IDENTIFICATION OF MARINE ANTIOXIDANTS

by

BRIGITTE E. TOWNSEND

(Under the Direction of Lyndon M. West)

ABSTRACT

Pharmaceutical science has benefited from the variety of bioactive lead compounds discovered in marine organisms over the past several decades. The remarkable structural diversity of marine secondary metabolites brings marine natural product chemistry to the forefront of drug discovery. In search of novel therapeutic interventions, marine natural products research has converged with antioxidant studies to form an exciting new research focus. Phenolic antioxidants and indole alkaloids are among the most bioactive classes of marine metabolite, but relatively little has been done to thoroughly investigate the biomedical potential of organisms containing these types of metabolites. In this study, several compounds representing both of these structural classes were identified in marine sponges collected from the Western Atlantic. Several of the compounds were found to have high antioxidant potential when screened with the ferric-reducing antioxidant power (FRAP) assay. Antioxidant activity of marine samples was detected primarily in shallow and intermediate-water sponges, but no correlation was found between activity and fraction polarity.

INDEX WORDS: Marine antioxidant, FRAP assay, chromazonorol, brominated aplysinopsin, *Smenospongia sp.*

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CHAPTER 1

History of Natural Products Chemistry

Long before chemistry became the refined discipline that it is today, early cultures experimented with chemical reactions using natural product reactants. Archeological evidence from the Mesopotamian era reveals the use of the mortar and pestle, crucibles, and double-rimmed earthenware vessels that could have been designed to percolate plant extracts from raw plant material. Naturally occurring pigments provided color for cosmetics and artwork. Organic hydrocarbons from animals and plants were used to color cloth. Dyed material was then treated with mineral salts as a mordant to bind dye to the cloth to prevent immediate fading. Egyptians and Assyrians combined sand with sodium carbonate, melting it to form glass that was then fashioned into ointment bottles or artwork. Reddish-colored glass artifacts indicate addition of gold, dissolved in a nitric- hydrochloric acid mixture, to the sand during the heating process. These early practices could only result from ongoing chemical inquiry and primitive experimentation.[1] Throughout history, human curiosity and innovation is evident from the creation of tools and the utilization of natural resources that increase labor effectiveness and improve the quality of life.

Medicinal history reveals many clues about the therapeutic uses of naturally occurring substances, whether from terrestrial or aquatic plants, microbes, or other living organisms. Historically—and still today—many of the substances relied upon for treating diseases and disorders are of plant origin. Ayurveda, traditional herbal medicine, can be traced to beginnings in India as far back as 3000 B.C. with use of herbs, plant extracts, and venoms for their curative

properties.[2] Sumerian innovations during the era from 3000-2400 B.C. constitute probably the earliest scientific records. An unidentified Sumerian physician left detailed records of ancient pharmaceutical practices, and many other clay tablets of information have been discovered as well. Prescriptions often required salt, minerals, nitrates, and soaps made from alkaline salts and fats. Other medical formulations included drugs such as henbane, poppy, and hemp. Figs, dates, roses, thyme, and pomegranate juice constituted several medicinal plant sources. Extracts of plants were also prescribed, with water soluble compounds easily extracted for medicines by steeping plant material in boiling water. Additionally, hydrophobic compounds were extracted using fermented juices, the alcohol content of which made a suitable solvent for water-insoluble plant components.[1, 3]

The Egyptians formed an extensive pharmaceutical library, even importing drugs from other empires. Prescriptions incorporated many herbs, plant compounds, and oils. Egyptians also experimented some with toxicities, becoming the leaders of the ancient world in cures and poisons.[3] Ancient Greek culture also relied on medicinal properties of natural products in the third and fourth centuries B.C. Comparable to the osteopathic and oriental medicine techniques of modern society, the Greek medical schools mainly focused on diet, exercise, and mental balance leading to health. As a culture immersed in philosophy, the Greeks were intent on acquiring descriptions and explanations. They acquired extensive knowledge of plants and herbs which, along with psychological treatment, comprised most of their remedies. Hippocrates' extensive studies and writings on observed properties and uses of plants are perhaps the most familiar historical association of natural products research. Many of these records are still available today and form the basis for what can be inferred about the role science played in early Greek history.

Preservation of scientific knowledge—from early civilizations and throughout history—imparts insight into key uses of natural products. Although much of indigenous methodology was based on mysticism and astrology, at least a basic grasp of healing remedies has been present for millennia. Currently, ethnobotany provides many researchers with a valuable starting point to investigate the medicinal properties of natural products.[2, 4]

Traditionally, terrestrial organisms supplied the main source of natural products. Gradually, the field has grown to include microorganisms and marine species as well. Marine natural products research was especially facilitated through development of SCUBA diving techniques in the 1940's, which extensively increased the scope of specimens available for collection. Emergence of new separation schemes and structural elucidation methods has also played a significant role in the expansion of natural product research.[5]

Compounds of interest are frequently present in such low concentrations within the sample biomass as to be undetectable except by highly sensitive analytical techniques. Although this is still a relevant challenge to the field, development of increasingly sensitive equipment including 600-800 Hz NMR has steadily improved the quality of research that can be performed. High- performance liquid chromatography (HPLC) is another method that has significantly advanced chromatographic separation efficiency by reducing the amount of sample necessary to obtain good product isolation.[5] Since the advent of HPLC, improvements in automation, probes, column design, and mass spectroscopy technology have impacted the versatility of column chromatography.[6]

Improving methods in natural products research also addresses the need to extend sustainability of resources. This is probably achieved most effectually with microorganisms, which can be cultured and maintained in abundant supply. Progress proceeds more slowly in the ability to replicate the numerous factors required to sustain marine organisms, but has already

met with some measure of success.[5] Further research in this area is necessary, because environmental stores of certain plants and marine organisms would be quickly depleted if collected on a wider scale than laboratory research, such as for pharmaceutical use. Tissue culture, genomic manipulation to optimize growth, and aquaculture are current means of preserving the ecology of natural product sources.[6-8]

Halichondrin B provides an example of an exciting natural product discovery that was challenged by low sample concentration and limited resources. Isolated first from the sponge *Halichondria okadai* off the coast of Japan (Figure 1.1), and later from sponges in the Western Pacific, Indian Ocean, and New Zealand coast, halichondrin B was purified and pinpointed as having activity that affected microtubule polymerization. However, after the initial screenings, not enough isolate was present to continue research. A collection initiative was launched by the National Cancer Institute, the National Institute for Water and Atmospheric Research, and the New Zealand government. The project resulted in not only an additional 300 mg of compound, but also in successful development of aquaculture for halichondrin-producing sponges.[5] Halichondrin B derivatives have since been synthesized and advanced to clinical trials as anti-cancer agents.

In addition to halichondrin B, a significant number of drugs that have entered the market in the past two decades are directly from natural products, semi-synthetic versions of natural products, or synthetic variations of a natural product lead compound. Examples include galantamine from the snow drop lily, a novel drug introduced in 2002 to treat Alzheimer's disease. Another potential Alzheimer's drug, huperzine from club moss, is currently undergoing FDA Phase II clinical trials for inhibition of acetylcholine esterase.[9] Natural products have also influenced the discovery of current anti-fungals, anti-virals, and neurological treatments. The anti-bacterial classes including β-lactams, tetracyclins, cephalosporins, streptogramins,

glycopeptides, rifamycins, lipopeptides, and chloramphenicol all were originally derived from natural product leads.[9]

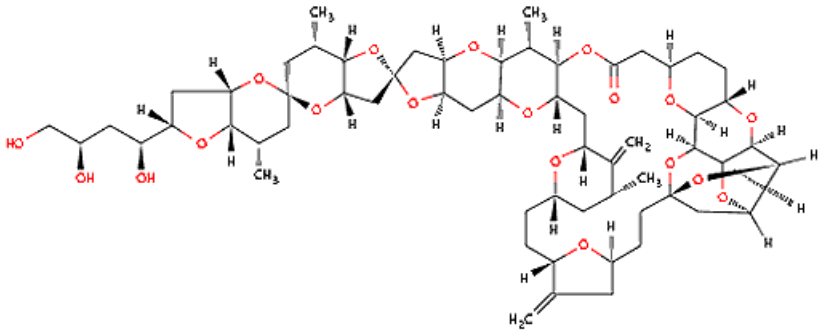


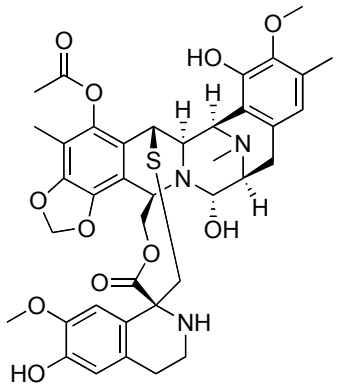
Figure 1.1. Marine sponge *Halichondria okadai* and its anti-cancer metabolite halichondrin B.[10]

Marine Natural Products in Current Drug Research

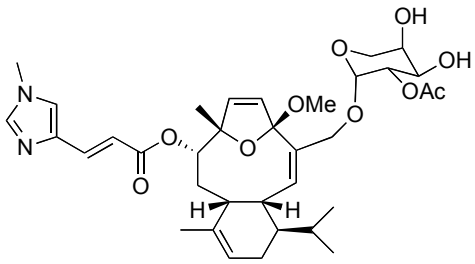
Marine organisms have become a prominent focus in drug discovery. Disease resistance and new emerging diseases demand novel lead compounds, and the marine environment is home to a wide spectrum of chemical structures that may have a significantly different effect on human and mammalian diseases than traditional drug structures. The vastly diverse array of specimens thriving in earth's oceans signals great discovery potential in the widely uncharted waters of marine natural product research.[11] Several prospective marine drugs are in clinical trials, most of

them having anti-cancer properties. The first marine natural product to be submitted to clinical trials was didemnin B from the tunicate *Tridemnum solidum*. However, didemnin B was removed during Phase II trials for toxicity reasons. More favorably, a series of bryostatin macrolides has been isolated from a bryozoan species *Bugula neritina.* Some of these structures show potential as anti-cancer, anti-depressant, anti-dementia agents. Currently in Phase II clinical trials, the effectiveness of bryostatin-1 is indicated only in combination with another natural product analogue, the anti-cancer drug Ara-C. Another anti-cancer drug, ecteinascidin 743, was isolated from sea squirts as early as 1969, but in insufficient quantities to bring the product to clinical trials. Successful development of a synthetic protocol ensued, and a synthetic version of this natural product is currently in Phase II clinical trials.[5, 12, 13] Other marine natural products are still in pre-clinical stages, such as the microtubule-binding agents peloruside A from the New Zealand sponge *Mycale hentscheli,* and eleutherobin from octocoral *Eleutherobia sp.,* and more recently from *Erythropodium caribaeorum* (Figure 1.2).[10]

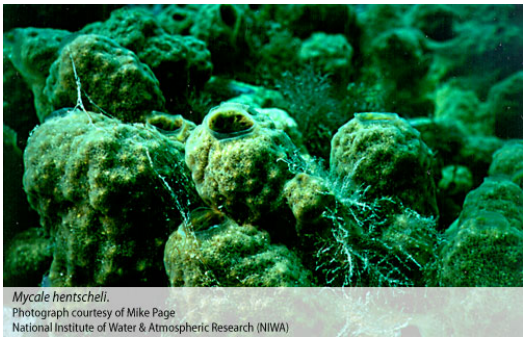
Like terrestrial plants, marine organisms produce primary metabolites consisting of essential proteins, lipids, and other complex molecules that form the building blocks for structural and functional maintenance of all living organisms. Viability is dependent on unimpaired primary metabolism. However, in most living things, additional energy is

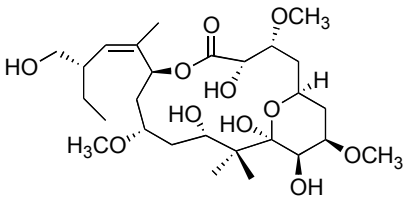


Ecteinascidin 743



Eleutherobin





Peloruside

Figure 1.2. Marine natural products provide a potential source of new anti-cancer agents.[10]

expended on secondary metabolites. Although not vital to an organism's survival, products of secondary metabolism enhance resilience through protective and defensive roles. Their

biosynthesis is therefore a significant aspect of an organism's biochemical makeup and long-term viability. It is these incredibly diverse molecules that constitute the focal point of natural products research.[2]

Because natural products often contain small molecules having neither an extremely polar or non-polar nature, they are ideal drug candidates. Natural products chemist Alan Harvey reports that "the use of natural products has been the single most successful strategy for the discovery of new medicines."[14] Soft-bodied, sessile marine invertebrates are of particular interest pharmaceutically. Lacking external functional defense mechanisms (such as scales or shells) and mobility to move away from danger or from a resource-depleted environment, these organisms rely on biochemical production of—often toxic—chemical compounds. Secondary metabolites in marine organisms serve several important ecological roles, whether to deter predators, aid food capture, or maintain environmental boundaries against invasion of space by overgrowth of neighboring organisms.[13] These same components hold significant appeal to pharmaceutical researchers. Therapeutic implications from the extensive variety of biologically active marine metabolites suggest potential drugs with anti-tumor, anti-viral, and anti-inflammatory properties.[13, 15] Some species of the *Hyrtios* class of marine sponges produces the related sesquiterpene compounds puuephenone and methoxypuupehenol, both of which display anti-fungal and anti-malarial activity.[16] The Caribbean sponge *Cryptotethia crypta* produces atypical nucleosides which have been synthesized and used as anti-cancer and anti-viral treatments since their discovery in the 1950's. Halogenated metabolites are also frequently identified in sponges, distinguishing marine metabolites from terrestrial counterparts. For centuries, Russians have been collecting freshwater sponges with iodine-containing compounds. The sponges, collectively called Badiaga, were ground into a powder for topical use. Polish physicians prescribed their

own therapeutic mixture of Badiaga powder, differing slightly from the Russian and Ukrainian versions based on the availability of sponge species.[15]

Interestingly, the phylum Porifera is distinguished as the most biosynthetically active class of marine invertebrates. Like other marine organisms, many of the metabolites emitted are toxic, and production varies with changing environmental conditions, light exposure, and growth rates. While the exact function of these compounds is hard to determine, it can be reasonably proposed that they exist to keep the sponge free of barnacles, biofilms, and parasites, while also maintaining a zone clear of ecological competitors.[15] Over half of marine natural products thus far identified are sponge-derived.[17]

Marine Natural Product Interest in the Western Atlantic Region

Home to the third largest reef system in the world and extensive underwater visibility, the Caribbean waters of the Bahamas (Figure 1.3) are a wealthy resource for marine research.[18] The clarity of the water allows sufficient sunlight to support incredible underwater ecosystems of flora and fauna. A combination of coral reefs, sessile marine invertebrates, microalgae, and autotrophic zoozanthellae all contribute to the uniqueness of the marine environment. Productivity is enhanced by symbiotic relationships between some marine organisms, contributing to the diversity of nutrients and metabolites that are produced.[19]

While sunlight is essential to energy metabolism of phototrophic marine organisms, excess sunlight or lack of protective mechanisms can be harmful to a reef system. Antioxidant defenses in reef ecology are just beginning to be studied in their protective role against deleterious effects of UV radiation. The field of marine ecology is expanding to address oxidative stress issues through investigation of the unique relationships among corals, marine microbes, and sponges.[19-21]

During the second half of the 19th century, explorers and fisherman began commercially harvesting sponges from the waters around the Bahamas and the Florida Keys. By the 1930's, overfishing and disease had taken a visible toll on the status of the Caribbean reef system.[22] Since that time, the reefs have undergone phases both of recovery and of damage from hurricanes and coral bleaching.[23]

Figure 1.3. Biodiversity in the marine environment of the Bahamas' reef system provides resources for potential pharmaceuticals.[18]

Sponges (Phylum Porifera)

The phylum *Porifera*, including thousands of varieties of sponges, is one of the first classes of marine invertebrates to be studied by natural products chemists. Sponges are multicellular, undifferentiated organisms equipped with an adept water-filtration system. Although technically considered animals, physiologically sponges are among the simplest Metazoans. Possessing no differentiated tissue to form digestive, nervous, and circulatory systems, they are composed primarily of a porous network of channels and chambers through which water moves. Flagellated cells (choanocytes) and phagocytotic cells (archaeocytes) lining

these channels propel water in the appropriate direction, and help capture nutrients that are swept along by the water current. In the space between water channels, a gelatinous connective matrix called the mesohyl houses skeletal materials. Following phagocytosis through apertures at the inhalant surface (ostia), food particles are filtered through progressively smaller sieve-like channels into the mesohyl for metabolism.[7, 24] The sieves are designed because indiscrete intake of water and particles by non-selective filter feeders results in ingestion of many particles which are of no biochemical value to the sponge. In order to retain sufficient nutrients for metabolism while simultaneously keeping waste levels from overwhelming the filtering system, the sponge must constantly excrete particles from its aqueous environment. Materials that cannot be used for energy are eliminated through apertures (oscules) in the exhalant surface. The entire sponge biomass is involved primarily in maintaining its low-pressure water pumping system.[24]

Taxonomists have long been frustrated by a lack of consistent defining characteristics of sponge morphology. Amorphous shapes, color variability within a species, and both flexibility in size and pigment intensity depending on the degree of light exposure create classification challenges. Unique skeletal characteristics of spicule formation provide the primary departure point from species ambiguity, and have served as the fundamental basis of classification. Skeletal polymorphism results from the differences in components of skeletal materials. Depending on the Poriferan class, skeletal elements can be composed of collagenous, siliceous, or calcareous fibers, arranged in patterns which lead to the diverse spicule arrangement. Still, proper classification is a tedious and uncertain process. Advancements in biochemical and histological tests aid classification of collected sponges, and other researchers have begun exploring the interesting technique of chemotaxonomy—identification by differential metabolite production. Chemotaxonomy has been used to distinguish sponges down to the genera, but cannot be used to determine classification at the species level.[4]

Three classes of sponge are commonly recognized. The most prominent class, Demospongiae, includes 95% of identified species and spans a wide range of habitats from intertidal depths to marine trenches in fresh or brackish water. Spicules are intracellularly produced from silica in most species, while other species are devoid of spicules. The deep-water class, Hexactinellida, is characterized by six-membered hexactine siliceous spicules. This rather unusual class lacks a mesohyl matrix, and is morphologically the most distinct from other Poriferan classes. Finally, the third class Calcarea is composed of calcium carbonate-based skeletal materials. Crystalline calciferous spicules may grow individually or as one mass.[24]

Despite constant engagement in the water filtration process, sponges are also proliferate metabolite producers. Osinga *et al.* report that –remarkably—"the structural diversity of sponge secondary metabolites is larger than that of any other marine phylum."[7] Although sponges were the primary target for historical marine invertebrate research, publications presenting biochemical information on their secondary metabolites were not available sixty years ago.[24] Current research has yet to definitively link each metabolite to its role, but the functional nature of many metabolites can be credibly theorized: toxins are most likely involved in spatial competition, while antibacterial compounds prevent ingested bacteria from dominating sponge metabolism. Sponge ecology become an important consideration in uncovering environmental or biochemical factors that would suggest a need for certain types of metabolite production.[7]

Identification and Isolation of Bioactive Compounds

Marine organisms contain a plethora of metabolites, some of which will display bioactivity, while many others will not. From a pharmaceutical perspective, it becomes necessary to screen out the non-active compounds early in the research. Investigators are therefore

challenged to use time-effective separation schemes that will remove unwanted materials from an extract with minimal loss of bioactive compounds.

Traditional approaches to natural products research generally focused on the chemistry of novel compounds, without intense interest in biological activity until total isolation and characterization was complete. Thus the process from sample collection to biological screening often involved extensive timeframes.[2, 4] Current natural product chemistry is primarily bioassay-guided, due largely to molecular-targeted high through-put screening (HTS) which allows rapid screening of multiple compounds at one time. The development of HTS increased the demand for new natural products and natural product derivatives so dramatically that analytical chemists could not supply purified compounds fast enough.[25] In order to meet these new dynamics of the field, natural products chemists had to reorganize their approach to separation schemes. Now, as well as a continued search for novel therapeutic compounds, current research projects for natural product scientists include assay and method development aimed at reporting quicker, more effective methods of collection and extraction.[6]

Historical methods of preliminary screening involved recognizing patterns of previously identified thin layer chromatography signatures. In contrast, modern *in vitro* cellular assays screen compounds with precise molecular targets. A wide variety of assays are currently available, aiding the search for anti-inflammatory, anti-cancer, anti-bacterial, and many other biomedically relevant activities. This chemical-biological interface has been a significant change in the structure of natural products approaches to chemistry. For instance, although chemical assays have traditionally been used to characterize antioxidant activity of marine organisms, there is a growing demand for cell-based antioxidant data. Laboratories may use a variety of screens to speed up isolation of useful compounds.[26] Organisms displaying a certain threshold of biological activity will be selected for further research. Isolation of bioactive compounds is

continued in a step-wise, assay-guided manner, with each new fraction generated being subjected to biological testing. This approach ensures that at each subsequent step in the separation scheme, the fraction or compound pursued for purification is bioactive.

Certain disadvantages are often unavoidable when using bio-screening to identify compounds of interest. Results processing is typically the rate-limiting step in bioassay guided fractionation, and may impede timely investigation. Also, depending on the type of assay, procedures can be expensive. Repeated testing at each stage of separation depletes often-miniscule quantities of available sample material. *In vivo* assays in particular are expensive and time-consuming; however, the medical field prefers *in vivo* results which reveal multi-target capabilities rather than activity of an isolated point within a biochemical network.

Another advance in natural product research is the development and expansion of databases through which characteristics of unknown compounds can be compared with previously identified compounds to avoid overlapping research and discovery. Before the existence of these databases, it was difficult to determine whether or not a seemingly novel compound had already been identified in a lab across the country or in another part of the world. Investigators needed a way to access natural product compound libraries so as to avoid spending tedious time elucidating structures of already-known compounds. Structural dereplication— comparison of similar structural data through the compound libraries— usually follows preliminary screening to extracts.

Semi-purification followed by preliminary screening for activity reduces the time spent on isolation and purification because potential hit compounds are identified in the early stages of compound separation. Crude extracts are separated into semi-pure fractions, at which point compounds of identical or similar fractions are grouped, profiled, and prioritized based on interesting characteristics. Thorough natural product databases are essential to employing

information from existing compound profiles into a separation scheme.[6] Isolation and identification procedures have also benefited from increased automation and the availability of simultaneous fractionation and screening methods via hyphenated techniques such as MS-NMR and reversed-phase HPLC-MS. Renewed interest in natural products chemistry is largely due to the combination of expanded natural product libraries and improved methodology, both of which effectively streamlined the dereplication process.[27]

Drugs typically require an amphiphilic nature in order to cross both aqueous and lipid barriers presented by a cellular environment. In a crude extract, therefore, molecules of intermediate polarity are the compounds most likely to be compatible with physiological considerations of adsorption, distribution, and metabolism. Unfortunately, drug-like compounds of intermediate polarity are generally isolated in the lowest concentration from natural product biomass, or are left undetected by traditional separation schemes. In response to this problem, a method has been developed to achieve initial separation of fractions of intermediate polarity from the more prominent polar and non-polar fractions.[2] The low-mass, intermediately polar fraction obtained by this method allows basic structural analysis prior to complete purification. Compound assessment for novel or clearly recognized classes of molecules in the intermediate polarity range is not possible by analysis of crude extract. In relation to the low amounts of amphiphilic compounds, higher concentrations of lipids, salts, and carbohydrates will obscure the smaller signals. In the cyclic loading method, traditional silica-based chromatography (silica gel or C-18 stationary phase) supplies substrate to which polar compounds may become irreversibly bound. In contrast, the macroporous polymeric resin Diaion HP20 (polystyrene-divinylbenzene) lacks polar sites, effectively preventing irreversible binding of polar compounds to the stationary column. Intermediately polar compounds will adhere to the HP20 substrate while the majority of unwanted compounds are washed away. After the removal of water-

soluble salts and carbohydrates, using a 40% Me_2CO/H_2O eluant, intermediately polar compounds are eluted with 75% Me_2CO/H_2O, and non-polar fats and steroids removed with Me_2CO. In order to decrease the often-tedious amount of time spent concentrating to dryness the Me_2CO/H_2O–containing fractions, the fraction of interest is back-loaded onto a smaller HP20 column and metabolites eluted with Me_2CO. This chromatographic loading method has been used for metabolite extraction of numerous marine sponges, and provides an effective way of reducing purification time of drug-like compounds.[2]

Emerging Methods in Natural Products Chemistry

Although traditional medicine has always recognized the significant role of natural products, the past two decades marked a decline in pharmaceutically-funded natural products research. Seeking to avoid the tedious—and often labor-intensive— methods characteristic of natural product discovery, companies anticipated a surge in combinatorial chemistry libraries that, disappointingly, has not surfaced. However, a significant change in screening methods has emerged with the development of high throughput screening. HTS can screen more compounds than was ever previously possible, by using robotic systems and multi-well assay plates.[6] Pure compounds are needed for screening to avoid the ambiguity of false positive and negative results. Unfortunately, natural products chemistry does not lend itself to the HTS regime. Natural product compounds involve more complexity than synthetic substances, and often the size and diversity of structures makes it difficult to determine the physical and molecular properties of those compounds. Unidentified chemical characteristics may interfere with the screening process, challenging the accuracy of assay results.[6]

Specific Aims

In this study, a sampling of marine sponge extracts from various locations in the Western Atlantic and collection depths will be separated into hydrophilic, hydrophobic, and amphiphilic fractions. Each fraction will be screened for antioxidant activity using the FRAP assay. Assay-guided isolation and purification of hit compounds will direct elucidation of potential marine antioxidants. Additionally, in order to test the ability of the potential antioxidants to cross membranes, a fluorescent, cell-based assay will be carried out on purified compounds using zoozanthellae cells.

CHAPTER 2

Antioxidant Activity of Marine Organisms from the Western Atlantic

Antioxidants in the Marine Environment

Reactive oxygen species (ROS) are encountered by all aerobic organisms, with oxidative proclivity being especially high at electron rich areas such as metabolic or photosynthetic sites. Marine organisms are exposed to particularly high levels of ROS through a combination of photosynthesis, symbiont oxygen production, and intense sunlight intensities leading to UV-induced free radical production. The conjecture that organisms highly exposed to ROS will have effective antioxidant mechanisms has certainly not disappointed natural product chemists. Many species contain powerful plant-like—or completely novel— antioxidant compounds.[28] Research progress in the marine antioxidant field has led to antioxidant screening of pure compounds and semi-pure fractions rather than crude extracts as was typical in earlier antioxidant research. Takamatsu *et al.* investigated antioxidant activity of over one hundred purified marine natural product compounds using the chemically-based DPPH (2,2-diphenyl-1-picrylhydrazyl radical) TCL method as well as the cell-based DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) assay. Demonstrating that marine products are active in living cells advances the compounds of interest one step closer to pharmaceutical applicability. Importantly, the side-by-side comparison approach of chemical-based and cell-based assays used by Takamatsu *et al.* allows researchers to distinguish chemical reductants from biological antioxidants.[26]

While marine antioxidants are a relatively new focus area in natural products chemistry, emerging interest has led to several unique discoveries. A compound from brown algae exhibited

radical scavenging activity comparable to the common antioxidant food additive BHT. Certain species of marine *Streptomyces* bacteria produce a class of superoxide scavenging molecules called aburatubolactams.[13]Another research group characterized an antioxidant, anti-apoptotic isolate from the sponge *Hymeniacidon helophila.* Although the protective functions of sponge metabolites are often difficult to determine, this amino acid derivative, L-5-hydroxytryptophan, presumably protects the intertidal *Hymeniacidon* sponges from oxidative stress caused by intense sunlight exposure.[29]

Bioassays for Antioxidant Activity

Physiological disorders, including neurodegenerative diseases, atherosclerosis, and cancer have been linked to oxidative stress in humans, and are a prominent target for novel therapeutic interventions. Normally, aerobic challenges can be overcome by enzymes, chelating agents, and non-enzymatic antioxidants which are present in aerobic organisms to protect DNA, proteins, and lipids from the detriments of oxidation. Disease conditions may arise from excessive oxidative stress, such as exposure to radiation.[30, 31] Compounds that can be administered pharmacologically to counteract ROS damage may be effective both in prevention and treatment of neurodegenerative disorders.[26, 32-36]

At the core of oxygen utilization in biological systems are reduction-oxidation, or "redox" reactions which involve transfer of electrons from one substrate to another. The substrate receiving the electrons is reduced, while the substrate providing the electrons is oxidized. Oxidation and reduction reactions must always exist in balance: one cannot occur without the other. Redox substrates are typically classified as reductants and oxidants in chemistry, but the terms antioxidant and pro-oxidant are more frequently encountered in biological systems. Specifically, a pro-oxidant, (reactive oxygen species or ROS), is a

pathological oxidant. ROS are implicated in numerous disorders including cancer, Parkinson's, and Alzheimer's disease.[37] When free radicals—molecules having become charged through the loss of an electron— are formed in consequence of aerobic metabolism, photosynthesis, radiation, or ROS-generating conditions, they will react with the first available oxidizable substrate. Oxidation of substrates such as DNA, proteins, or membranes can create considerable damage inside a cell. When antioxidants are present in a cell, they replace other oxidizable substrates as the reductant component of redox reactions. As long as the antioxidant capacity is not overwhelmed, this mechanism protects vulnerable cellular components. Specifically, an antioxidant is classically defined as a "substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate."[38] The products of this reaction will have little to no toxicity, as opposed to the highly detrimental reactions of ROS with non-antioxidant substrates.[37, 39]

Antioxidants are classified as preventative when they prevent formation of free radicals. Others are classified as radical scavengers, functioning to halt further propagation of chain reactions. The final class of antioxidants consists of repair molecules which ameliorate oxidative damage to a cell. Phenolic antioxidants often found in plant materials—and more recently in numerous marine organisms—are thought to play a preventative role in radical formation. Relatively little has been done to identify the mechanisms responsible for antioxidant action of phenolic marine metabolites, however. This class of compounds, predominantly the phenolic terpenes, forms the most bioactive category of marine structures. Utkina *et al.* studied a number of marine sesquiterpenequinones to determine both antiradical and antioxidant activity.[31] Antiradical activity was defined as the ability to prevent oxidation by DPPH radicals, while antioxidant activity was tested based on the ability of the compounds to prevent, delay, or stabilize lipid peroxidation of linseed oil. In the latter test, antioxidant capacity was quantified

for auto-oxidation and for lipid peroxidation induced by lipid hydroperoxides or Fe^{2+} molecules. Phenolic sponge metabolites puupehenone and its analogue 15-methoxypuuepenol are potent antiradicals in the DPPH assay, but although they slowed the process of lipid peroxidation, neither could actually stop it. Thus both compounds qualify as preventative antioxidants, but do not possess effective chain-breaking antioxidant potential. Both these compounds were compared to the α-tocopherol, which is the major antioxidant component of lipid-soluble vitamin E. Because it is known to be able to prevent as well as halt oxidative chain reactions, it is often used as a control in antioxidant assays.[31]

Methods to quantify antioxidant activity have been an important component of biomedical research. Initially created by Benzie and Strain[40] to test total antioxidant power of biological fluids, the ferric reducing antioxidant potential (FRAP) assay has since seen extended use for plant compounds, food and nutrition chemistry, and marine natural product research. Differing slightly in principle from other assays which quantify resistance to free radicals, the FRAP assay evaluates the strength of an antioxidant compound as a reductant. The assay procedure can be completed fairly quickly and lends itself to gathering preliminary data on a large number of samples. Once the appropriate samples have been added to the FRAP reagent, a colorimetric change to deep blue signals reduction of the ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to ferrous TPTZ (Fe^{2+}-TPTZ). Molar concentration of the reductant can be plotted as a function of color intensity as measured on a spectrophotometer.[35, 40, 41]

One of the disadvantages of the FRAP assay is that its scope is limited to measuring chemically-defined reductant capacity, which may not be identical to antioxidant capacity. In this assay, a reductant which reduces Fe^{3+} to Fe^{2+} is not necessarily an antioxidant because Fe^{3+} is not a pathological oxidant. Fe^{2+} is actually a pro-oxidant which forms free radicals from peroxide. Therefore the FRAP assay provides reflective antioxidant capacity, but not true antioxidant

power. Prior points out that antioxidants which reduce both Fe^{3+} and Fe^{2+} will be detected in the assay, while other effective antioxidants which lack the ability to reduce Fe^{3+} will be undetectable.[37]

Often it is necessary to obtain a variety of information about antioxidant potential other than what can be concluded from the FRAP assay alone. Cell-based assays in particular can be used to gain appreciable evidence of the potential *in vivo* antioxidant effectiveness of a compound. One such method, the dichlorofluorescein assay, provides a straightforward approach to measuring ROS sequestration by an antioxidant. The assay can also be used to quantify ROS levels induced by mechanical or chemical oxidative stress factors. This cell-based method can also be applied to measurement of antioxidant activity within an oxidative environment. Cells readily absorb the non-polar 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe. As the probe crosses membranous cellular boundaries, it becomes trapped internally via hydrolyzation to the non-fluorescent polar compound 2',7'-dichlorodihydrofluorescein (DCF). Inside the cell, DCF fluoresces when oxidized by peroxyl radicals are formed by treatment with a radical generator. Measuring fluorescent intensity of oxidized DCF reveals overall oxidative stress in the cell. If antioxidants are present, fluorescent intensity is limited to the extent that free radical scavenging counteracts the peroxyl radicals.[30, 42]

Antioxidant Activity in Sponges

Sponges grow at a range of depths; accordingly, not all are exposed to identical UV intensity. Under the oxidative pressures of the marine environment, survival is influenced by the measure of balance maintained between ROS generated within the sponge and its defensive antioxidant mechanisms. This correlation between antioxidant activity and ecological factors leads to the collection of sponges that appear to thriving in highly-oxidative environments. For

example, the metabolic products of an intertidal sponge investigated by Lysek *et al.* was expected to include a significant concentration of antioxidant compounds due to its shallow water, high-light exposure surroundings. The main constituent of the sponge *Hymeniacidon heliophila,* L-5-hydroxytryptophan, exhibited anti-apoptotic activity by sequestering UV radiation-induced ROS.[29] In another investigation of sponge-derived antioxidants, Regoli *et al.* examined oxidative stress levels in the sponge *Petrosia ficiformis* in both symbiotic and aposymbiotic relationships. This particular sponge can be found in diverse habitats ranging from light-exposed water to dark caves. Investigation of oxygen production in *P. ficiformis* revealed a correlation between heightened antioxidant defenses and increased O_2 due to symbiotic relationships. Interestingly, exposure of symbiotic *P. ficiformis* to more intense radiation did not necessarily result in increased antioxidant protection, with outer layers being especially vulnerable to radiation stress.[20] Older studies investigating the UV exposure of corals surviving at different reef depths report that shallow water corals have heightened ability to absorb UV radiation compared to those found in deeper water.[19, 43]

Specific Aims

The aims of this study were to provide evidence of antioxidant capacity in several sponge specimens native to the Western Atlantic. Specifically, we were interested in possible correlations between 1) antioxidant bioactivity and collection depth, and 2) fraction polarity and concentration of antioxidant compounds. Based on previous publications involving antioxidant assays of marine product extracts, we hypothesized that the samples collected in shallow water or at intermediate depths would display the highest FRAP readings.

Results and Discussion

Lyophilized specimens of marine sponges collected from the Western Atlantic from a range of depths were selected for comparison of antioxidant activity. Of the nine specimens chosen for the assay, three were collected in deeper water off the coast of Panama City, Florida, while the others had been previously collected from the Bahamas. During initial separation, methanolic extracts of sponge material were partitioned into three discrete fractions of decreasing polarity using the reverse-phase HP20 chromatographic method described in Chapter 1. Samples of each fraction were loaded in triplicate onto 96-well plates for preliminary antioxidant screening based on the FRAP assay first described by Benzie and Strain.[40] This assay, used in order to determine general distribution of antioxidant compounds within the sponges, showed ferric-reductant capability across the range of hydrophilic to hydrophobic compounds. Marine sponge fractions were initially assayed using ~100 μg/mL of each sample dissolved in methanol. Absorbance was measured at 600 nm and recorded 12-15 min following addition of samples to FRAP reagent. Equivalence values for each sample were then determined based on a linear calibration curve using known concentrations of $Fe_2SO_4 \cdot 7H_2O$. The fractions of semi-purified sponge extracts exhibited variable antioxidant potential, with some of the intermediate and shallow water specimens exhibiting activity equaling or surpassing the activity of the control antioxidant, 100 μM α-Tocopherol, in all three fractions (Figure 2.1). While FRAP activity was found in sponges from intermediate or shallow collection depths (Figure 2.2), the deeper water Floridian sponges displayed little or no activity (Figure 2.3). This agrees with previous reports of an inverse relationship between antioxidant concentration and collection depth.[44]

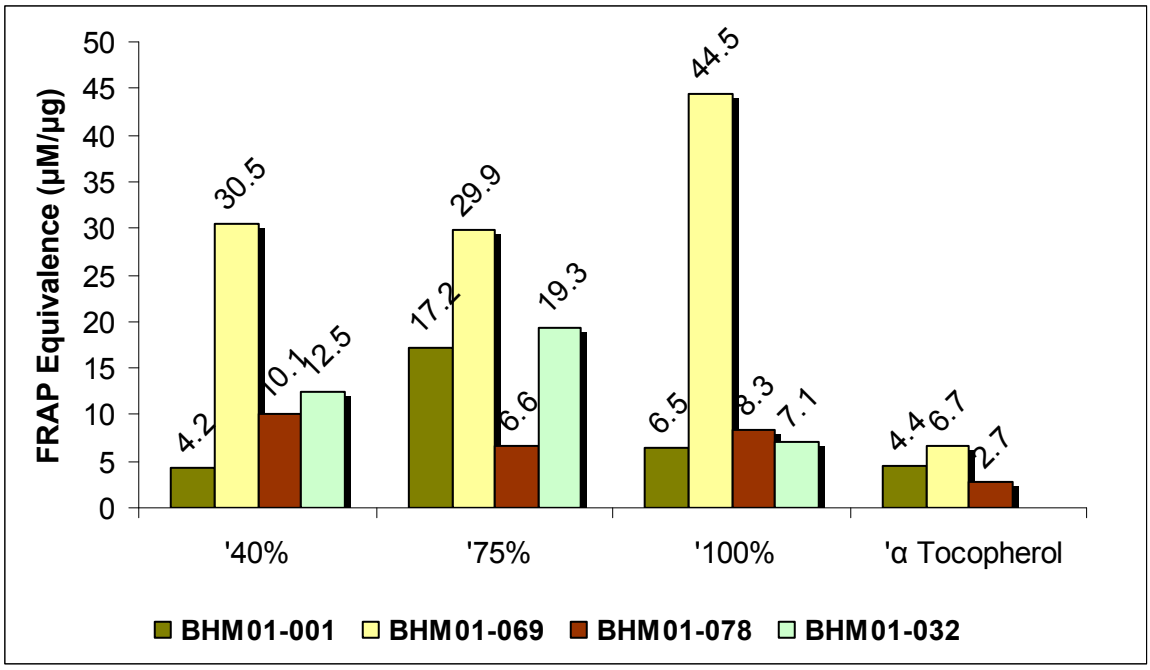


Figure 2.1. Bioactive fractions of marine sponges from the Western Atlantic.

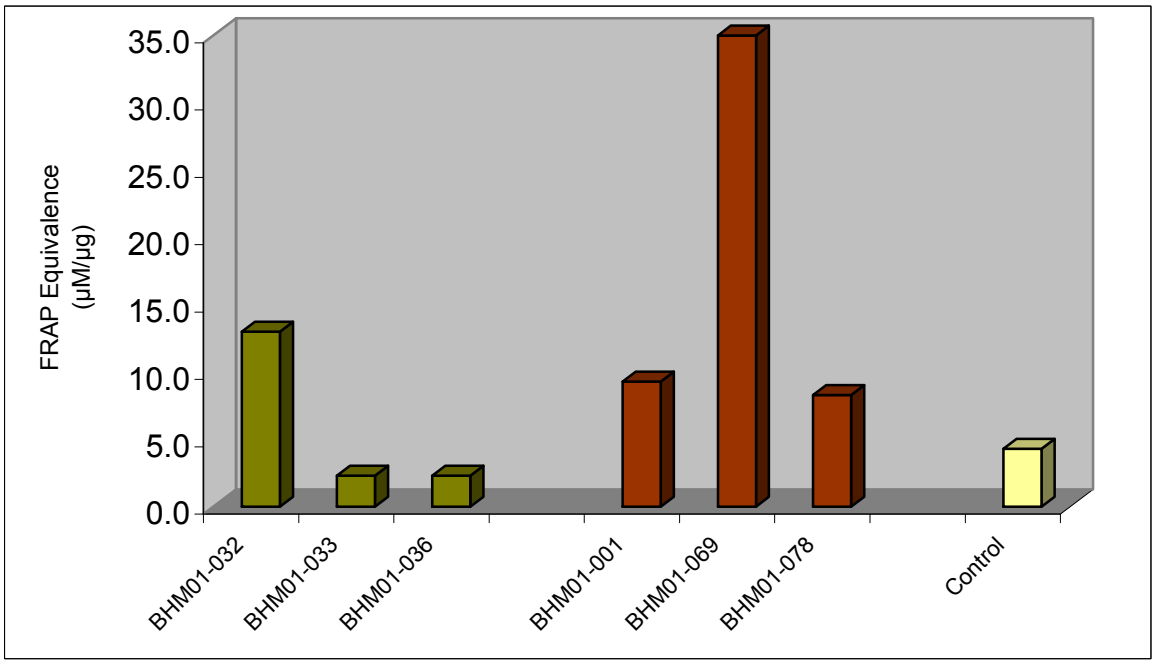


Figure 2.2. Average FRAP equivalence values of Bahamian sponge samples from various collection depths.

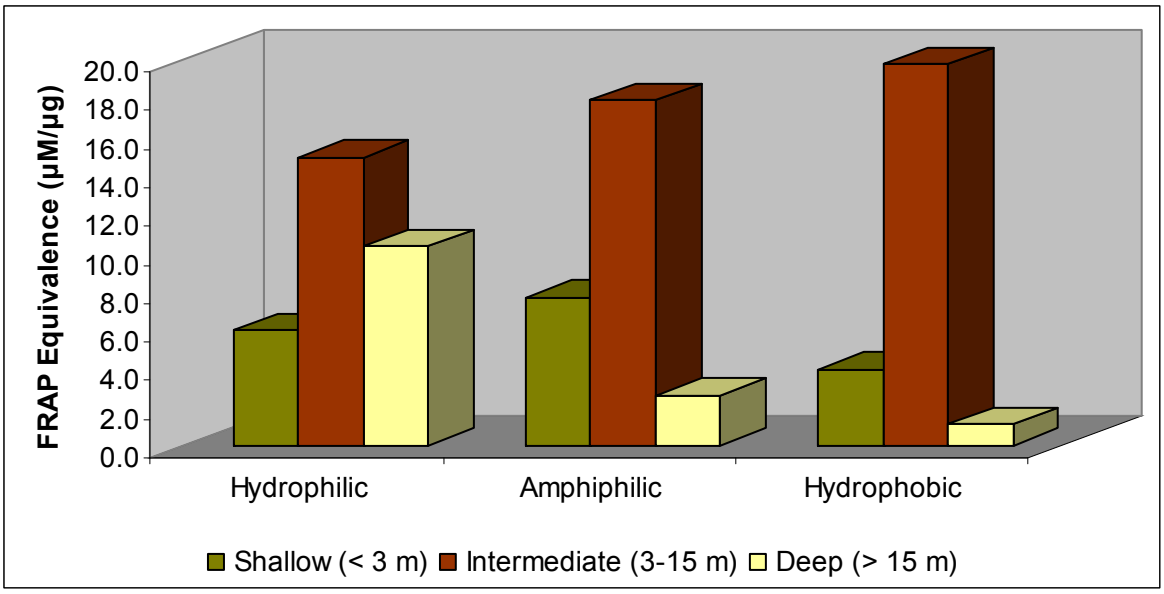


Figure 2.3. Average antioxidant activity distribution by polarity.

The results of this assay revealed comparable FRAP equivalence values among the three fractions screened in the majority of sponges used in this study (Figure 2.3). Although the intermediately polar fraction contained the majority of compounds with antioxidant activity, the most active component was found in the non-polar fraction of Bahamian sponge, collection ID BHM01-069 (Figure 2.4). This compound **(1)** was later isolated and identified as a chroman-sesquiterpene.

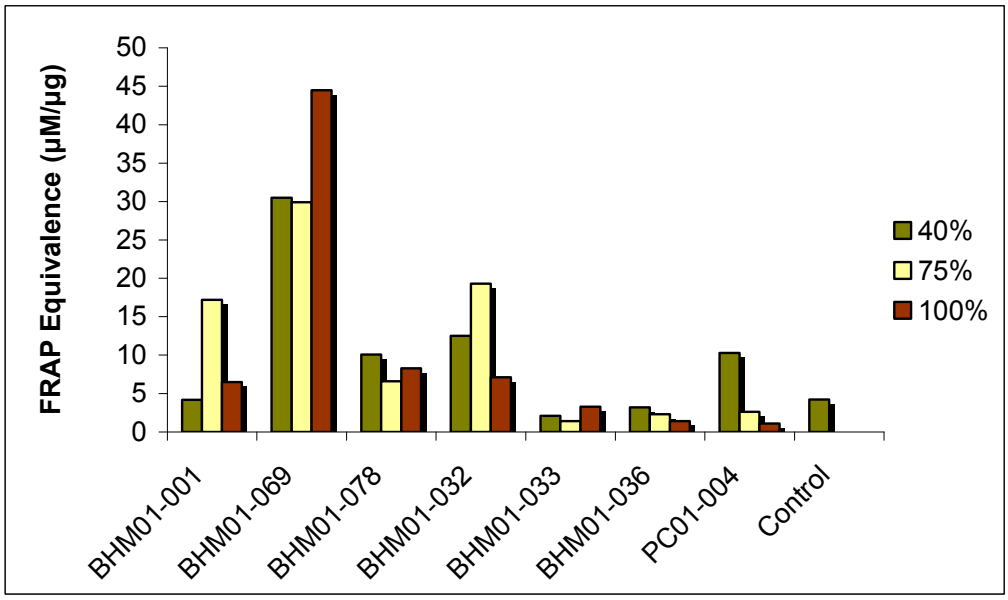


Figure 2.4. Antioxidant potential of individual marine sponge extracts varies with the polarity of the fraction assayed.

Although the sample size of collected sponges available for screening in this study was insufficient to determine a trend in the nature and distribution of antioxidant properties, the majority of antioxidant compounds in these sponges were contained in the amphiphilic fraction. Additionally, specimens collected from the 3 m to 15 m range were active, while sponges collected at depths greater than 15 m displayed little to no FRAP activity. It would be interesting to continue the study with a broader sample size in order to investigate statistical correlations between distribution of antioxidant compounds and molecular polarity.

CHAPTER 3

Bioactive Compounds from the Bahamian Sponge *Smenospongia sp.*

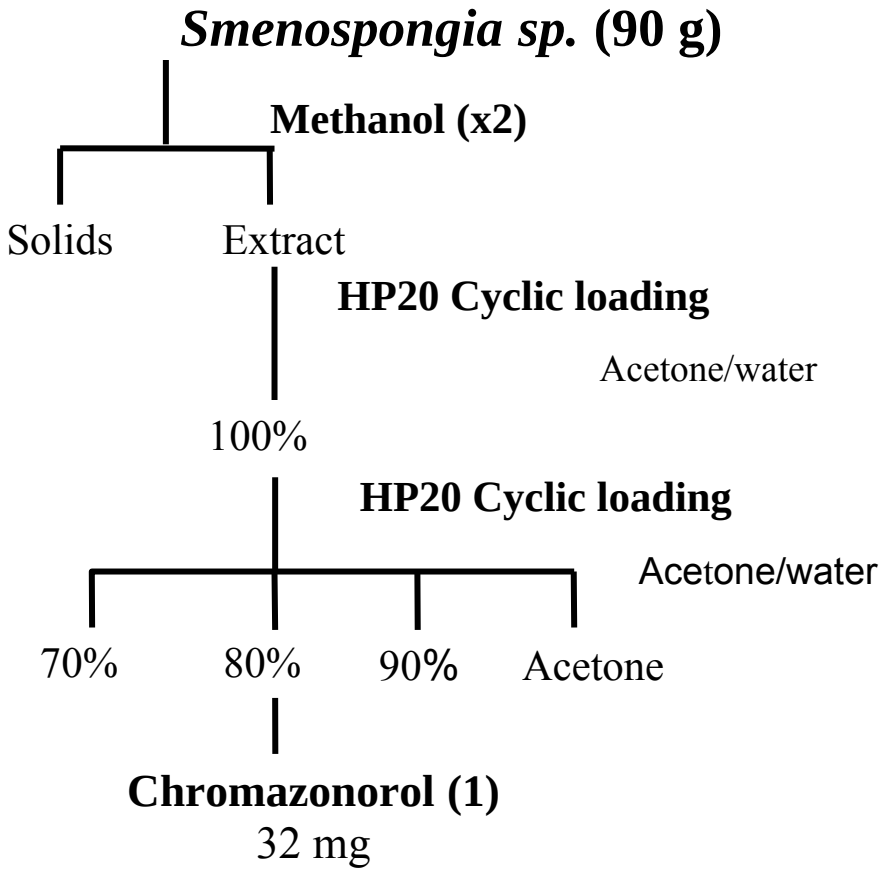
Smenospongia sp. (Figure 3.1) was collected from the Bahamas at a depth of 10 meters. Similar species from the genus *Smenospongia* have been collected from Korea and from Papua New Guinea.[45, 46]

Figure 3.1. Marine sponge *Smenospongia sp.*

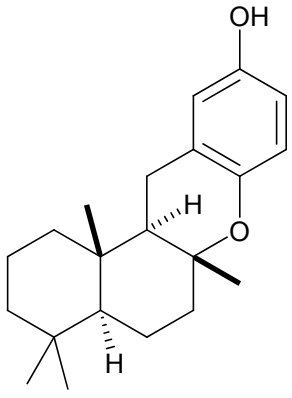
Investigation of antioxidant constituents from the marine sponge *Smenospongia sp.* (collection number BHM01-069) yielded the previously described compounds chromazonorol **(1)**, 6-bromo-2'-de-*N*-methylaplysinopsin **(2)**, and 6-bromoaplysinopsin **(3)**.

Isolation and Identification of Chromazonorol

Separation of potential antioxidants from *Smenospongia sp.* was structured around high levels of FRAP activity detected in crude fractions during an initial screening (Scheme 1) Initially, semi-preparative separation of crude methanolic extract of dried *Smenospongia sp.* sample was performed using the reverse-phase HP20 cyclic loading method as described in Chapter 1. Potential antioxidant compounds were detected by FRAP screening of each of the three fractions eluted, and all fractions showed substantial activity. A smaller HP20 column was used to achieve further fractionation of both the 75% Me_2CO/H_2O and 100% Me_2CO fractions using incremental, increasing concentrations of Me_2CO/H_2O. The 80% Me_2CO/H_2O fraction yielded a phenolic compound **(1)** that displayed significant FRAP activity. The molecular formula of compound **1**, $C_{21}H_{30}O_2$, was determined from the HRESI of the $[M+H]^+$ ion at *m/z* 315.2338, requiring seven degrees of unsaturation. Initial analysis of the ^{13}C NMR spectrum (Figure 3.2 and 3.3) revealed 6 carbons in the aromatic/olefinic region, 14 aliphatic carbons, and an oxygenated carbon. These data suggested that compound **1** consisted of an aromatic ring connected to a sesquiterpene skeleton. Analysis of the NMR data (^1H, ^{13}C, HMBC, and HSQC) allowed identification of the compound as chromazonorol, previously isolated by Cimino *et al.* in 1975 from the sponge *Disidea pallescens*.[47] Chromazonorol shares structural similarities with previously identified phenolic sesquiterpenes puupehenone and aureol, both bioactive marine secondary metabolites. Puupehenone exhibits anti-fungal and anti-microbial properties against *Staphylococcus aureus* ,and *Clostridium tropicalis,* as well as anti-malarial properties when tested with three different strains of *Plasmodium falciparum.*[16]Additionally, puupehenone is active as an antioxidant in both chemical DPPH and cell-based DCFH-DA assays.[26]



Scheme 1. Separation scheme of chromazonorol from the Bahamian sponge *Smenospongia sp.*



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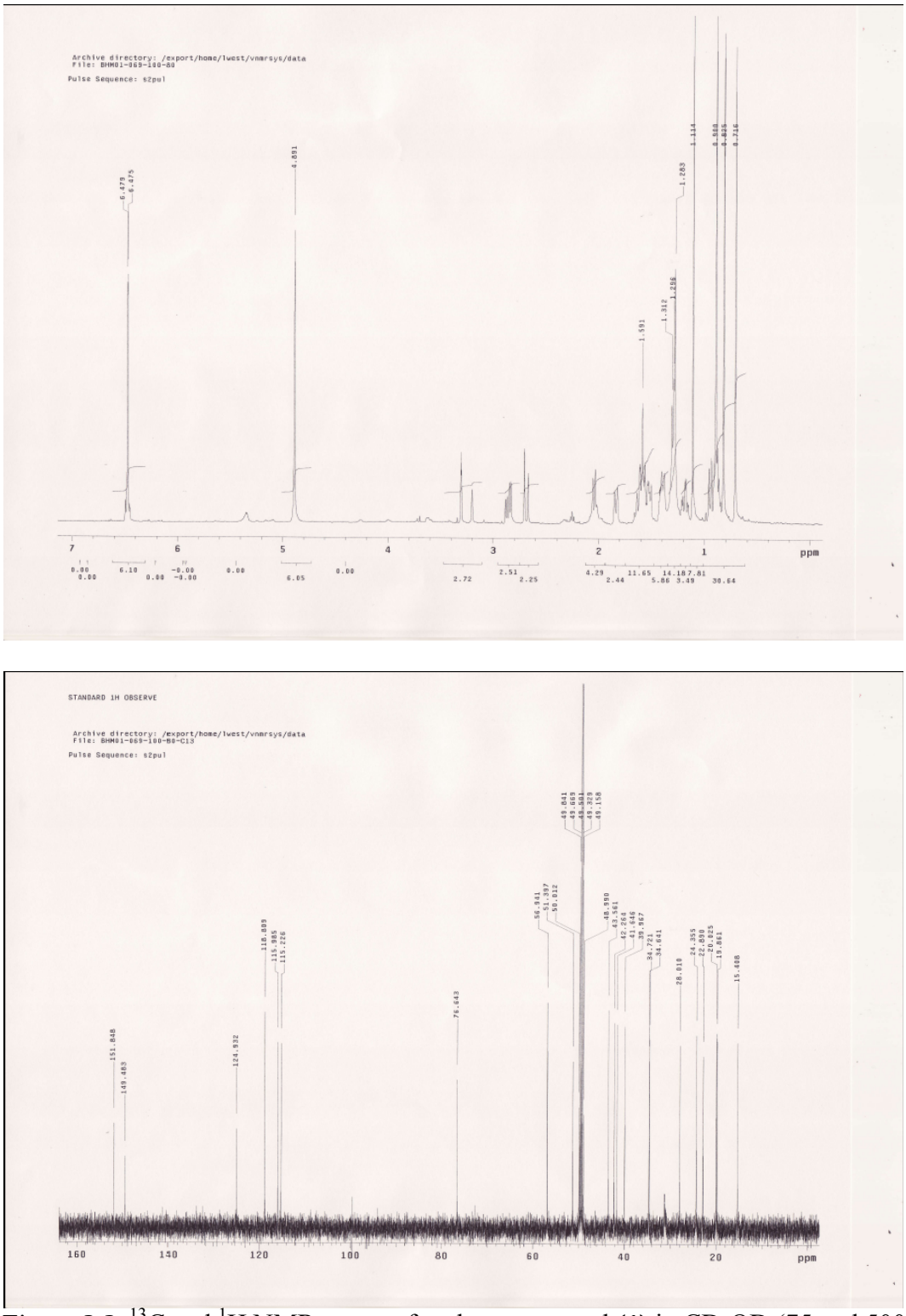


Figure 3.2. ^{13}C and ^{1}H NMR spectra for chromazonorol **(1)** in CD_3OD (75 and 500 MHz).

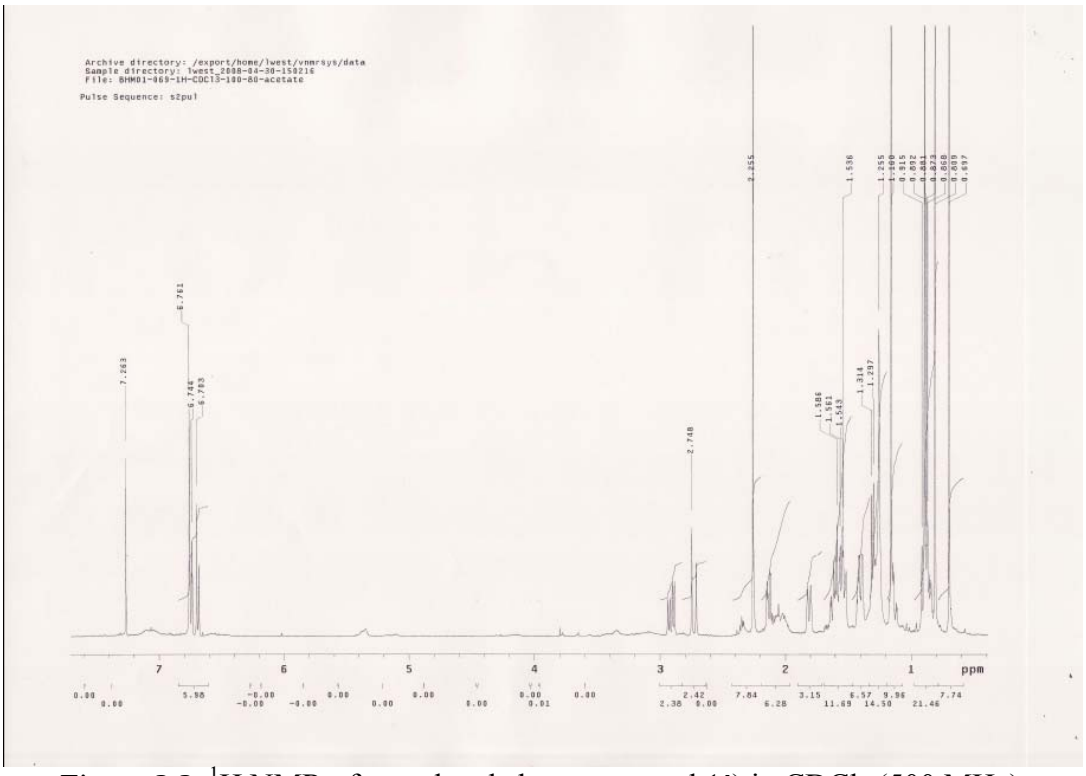


Figure 3.3. ^{1}H NMR of acetylated chromazonorol **(4)** in $CDCl_3$ (500 MHz).

FRAP Activity of Chromazonorol (1)

Several well-described, naturally occurring antioxidants were used as a comparison for antioxidant capacity of marine compounds. Bioactivity of purified chromazonorol exhibited more intense ferric-reductant potential than positive controls resveratrol, catechin, quercetin, ellagic acid, and α-tocopherol. Absorbance readings of $FeSO_4 \cdot 7H_2O$ standards of known concentration were used to generate a linear calibration curve from which equivalent concentrations of antioxidant sample were obtained. Purified sponge compounds were assayed for FRAP activity using 10 μg sample per well, while antioxidant controls were tested at a concentration of 20 μM. The FRAP values obtained were calculated as μM $FeSO_4 \cdot H_2O$ per μM antioxidant sample, and results are shown as mean FRAP equivalence from three independent values (Figure 3.4).

In order to identify the source of antioxidant activity in chromazonorol, compound **1** was acetylated using acetic anhydride and pyridine to give the acetylated chromazonorol **(4)**. Compound **4** was assayed for antioxidant activity and found to have significantly reduced activity (Figure 3.4). The antioxidant activity of chromazonorol and significantly reduced activity of acetylated chromazonorol indicated that the phenolic hydroxyl group is essential to the antioxidant activity of chromazonorol.

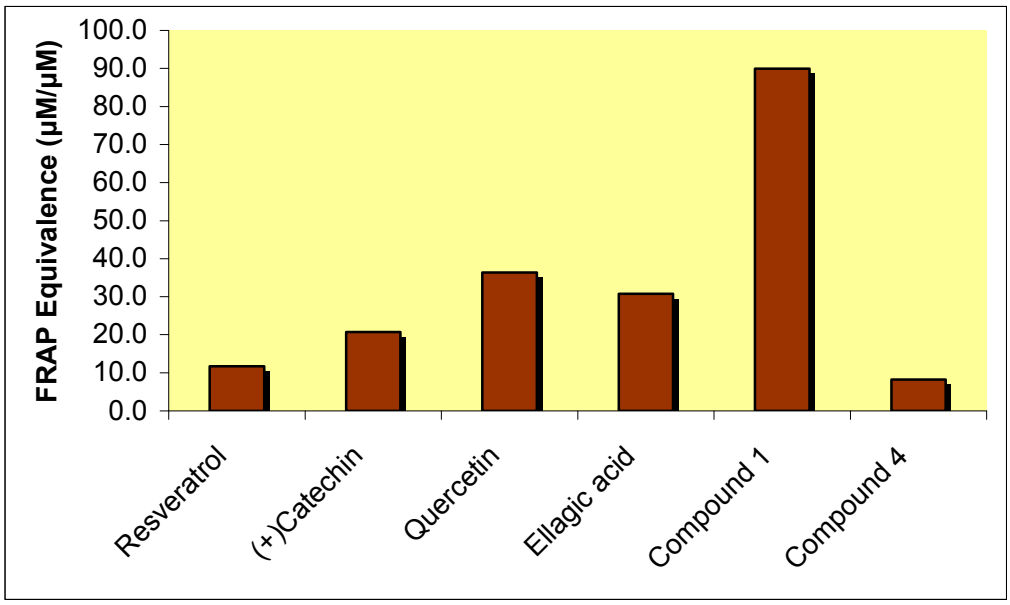
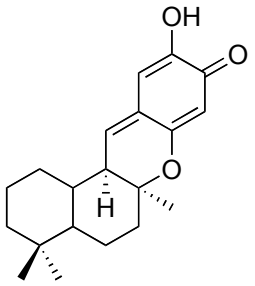


Figure 3.4. Average FRAP equivalence of antioxidants, chromazonorol **(1)**, and acetylated chromazonorol **(4)**.

Puupehenone has also been found to exhibit antioxidant activity. Similar in structure to chromazonorol, puupehenone is a phenolic sesquiterpene isolated from the sponge *Hyrtios sp.* The two hydroxyl functions contribute to its strong antioxidant activity.[16] As with other phenolics, the presence of at least one hydroxyl moiety in members of this class of molecules provides a ready substrate for free radicals. Phenolic antioxidant activity generally increases with an increasing number of hydroxyl groups attached to the aromatic ring. This was demonstrated by Kim *et al*.[48-50] using the vitamin C equivalent antioxidant capacity assay to test nutritional

polyphenolic compounds. The increase in activity is linked to the relative ease by which the hydroxyl proton is donated to neutralize free radicals. Electron donation is affected by stereochemistry and bond dissociation energy, and is impeded by the presence of electron withdrawing groups in proximity to the –OH function. Thus the position—as well as the number— of hydroxyl groups plays a significant role in conveying antioxidant activity.[48]



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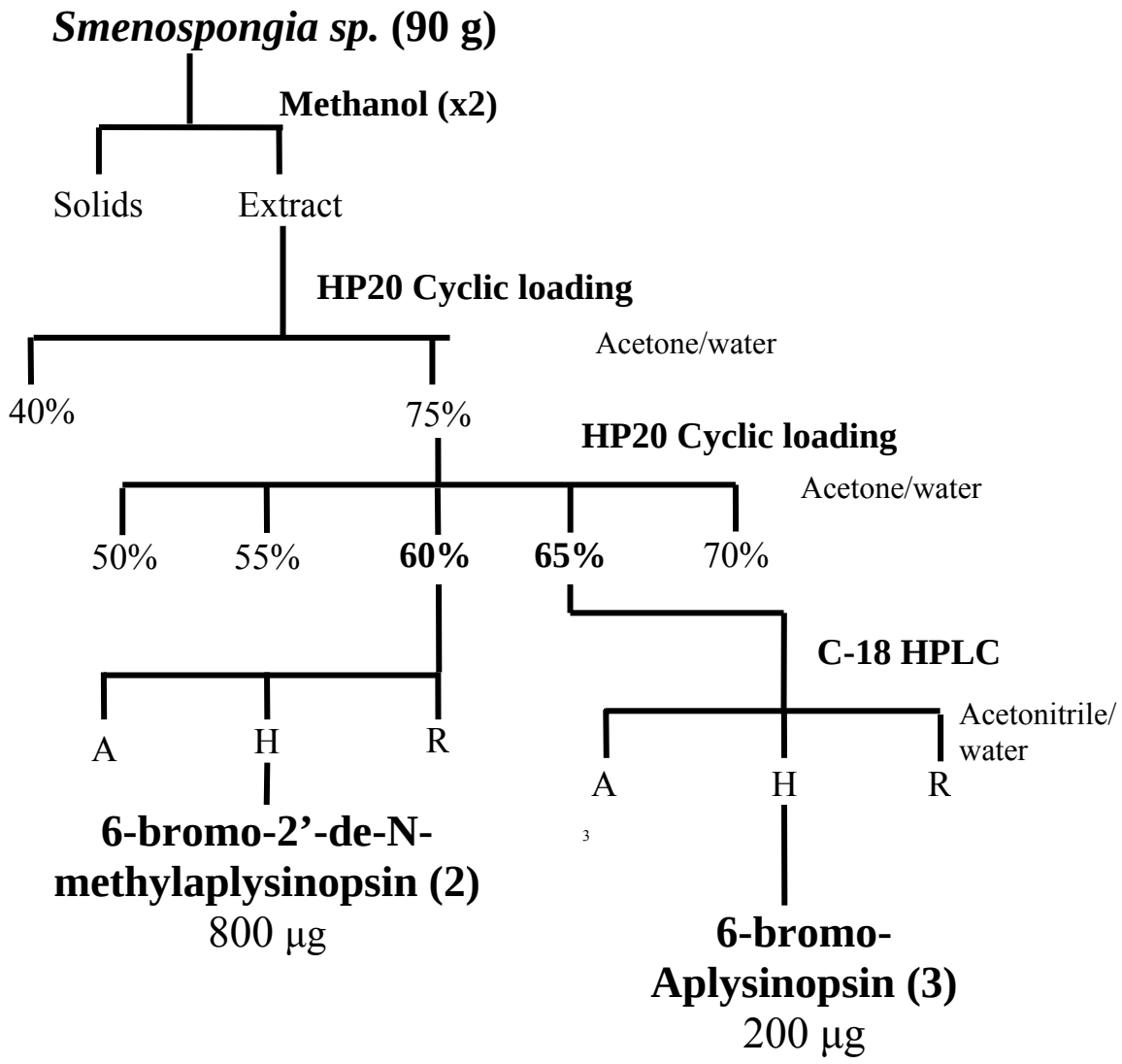
In addition to the placement and frequency of hydroxyl groups as determinants for antioxidant capacity of phenolics, effective antioxidant compounds must also possess molecular properties allowing efficient uptake by cells. Sometimes antioxidant activity conveyed by a compound in a solution-based assay is lost during cell-based or *in vivo* screening. This may be due to inability of the compound to cross cell membranes, or result from other properties incompatible with the internal cellular nature. However, the reverse is also true—non-active acetylated compounds are activated within the cell by loss of the acetyl group.[26, 49] In this study, chromazonorol **(1)** and acetylated chromazonorol **(4)** were tested for antioxidant activity in zoozanthellae cells using the fluorescent DCFH-DA assay (data not shown). Compound **1** was unable to prevent DCF oxidation at a concentration of 20 µg, but cells treated with compound **4** inhibited DCF oxidation. The activity of **4** in this assay may be attributed to its increased hydrophobicity conveyed by the acetate group, facilitating cellular uptake. Similar results were obtained by Takamatsu *et al.* in their investigation of marine antioxidants. Algal metabolites

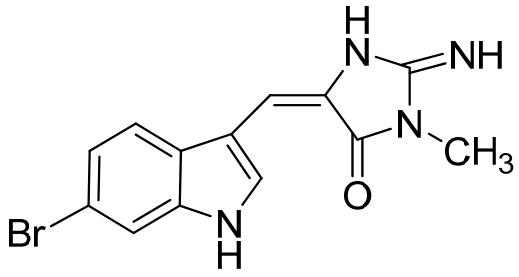
cymopol and 7-hydroxycymopol were screened for antioxidant activity using the DPPH radical trapping assay, along with the fluorescent DCFH-DA assay.[26] The compounds were found to be active in both assays, but the hydroxyl-containing compound displayed lower antioxidant activity in the cell-based assay. The authors concluded that the diminished activity was a result of increasing polarity of the molecule.

Isolation and Identification of Aplysinopsin Derivatives from Smenospongia sp.

Various indole alkaloids have been isolated from marine organisms with reported anti-microbial or anti-inflammatory, and anti-cancer activity, representing a promising class of compounds for pharmaceutical research. The indole alkaloid class comprises a quarter of known marine metabolites and the diversity found within this class is accentuated by the addition of atoms such as bromine which are not typically found in plant compounds.[51] Following the identification of chromazonorol from the 100% Me_2CO fraction of *Smenospongia sp.* extract, the 75% Me_2CO/H_2O fraction from the initial HP20 column, also found to contain antioxidant activity, was chromatographed further into five additional sub-fractions (Scheme 2). Using NMR data, two of these sub-fractions were selected for purification by reverse-phase C-18 HPLC using an acetonitrile/water gradient method to achieve clear separation of compounds (Figure 3.5). Fractions were collected at 30 sec intervals and combined by peak. The fraction library was transferred to a 96-well plate and assayed for FRAP activity to identify active compounds. The major compound of the 60% Me_2CO/H_2O fraction **(2)** was found to be active, while the major component of the 65% Me_2CO/H_2O fraction **(3)** displayed no activity. An additional quantity of the 75% Me_2CO/H_2O fraction was then re-chromatographed by HPLC and the original active peaks subjected to NMR analysis.



Scheme 2. Separation scheme of 6-bromo-2'-de-*N*-methylaplysinopsin **(2)** and 6-bromoaplysinopsin **(3)** from Bahamian sponge *Smenospongia sp.*



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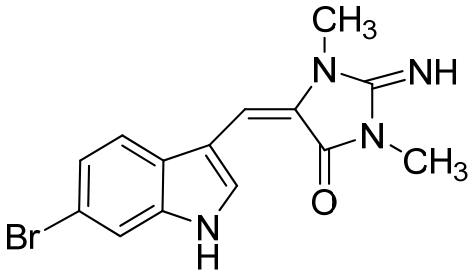

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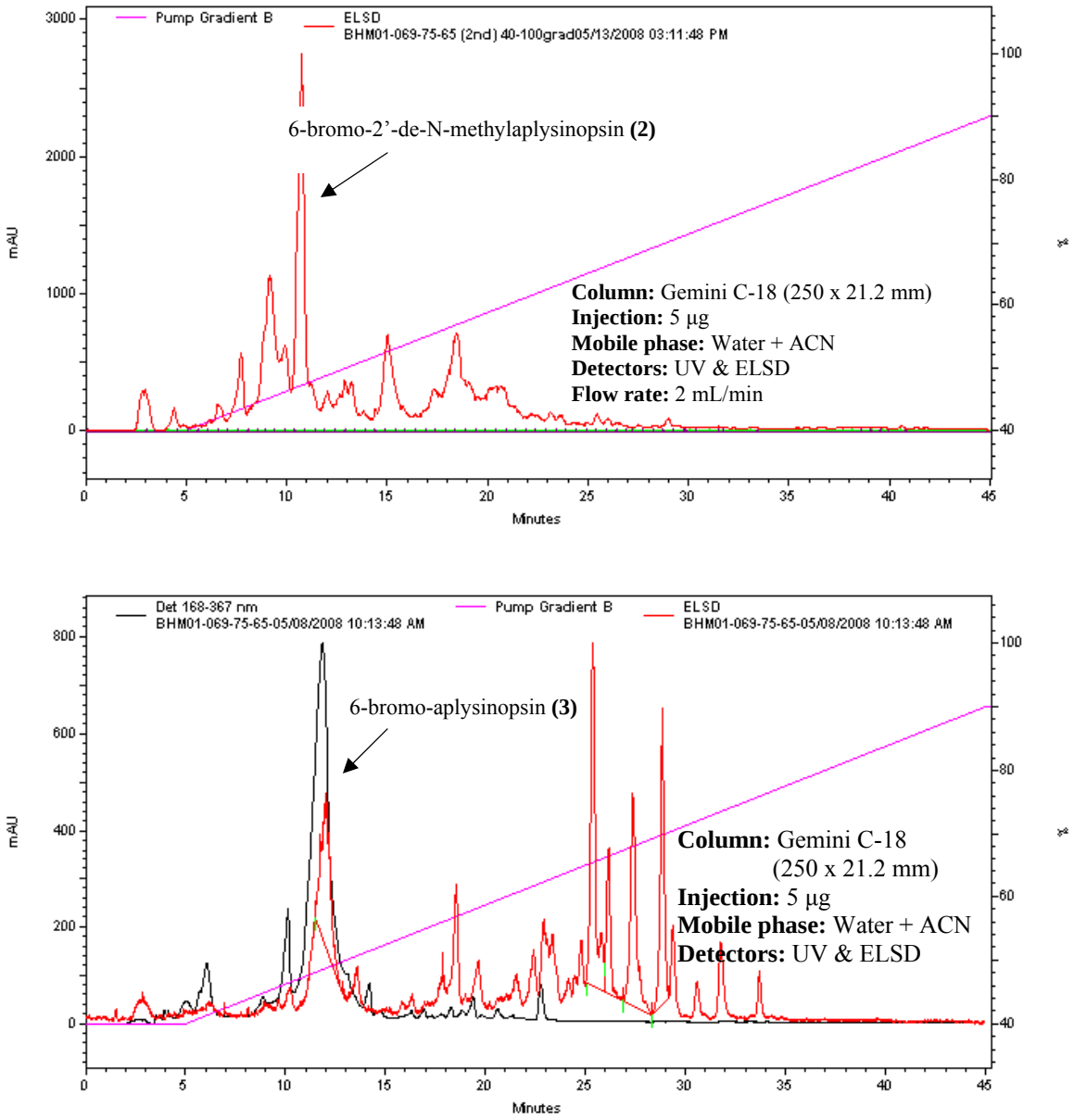

Figure 3.5. HPLC trace of the 60% Me_2CO/H_2O fraction with compound **2** (top) and the 65% Me_2CO/H_2O fraction with compound **3**.

The ESI of compound **2** showed $[M+H]^+$ ion peaks at *m/z* 318 and 320 with nearly equal intensity, and indicated the presence of one bromine atom. Analysis of ^1H and HMBC NMR data revealed deshielded aromatic protons of an indole moiety connected to a glycocyamidine side chain (Figure 3.6). A search of the literature revealed a large number of indoles and bis(indole) alkaloids have been reported from the marine environment. A comparison of the spectral data of **2** to a number of these compounds allowed it to be identified as 6-bromo-2'-de-*N*-

methylaplysinopsin. The ESI of the compound **3** was 14 mass units different than compound **2** and showed $[M+H]^+$ ions at *m/z* 332 and 334, also indicating the presence of one bromine atom. The ^1H NMR data was similar to compound **2**, except that it contained an additional *N*-methyl group. This suggested the proton at the R_1 position of the glycoamidine side chain was replaced with a methyl group. A comparison of the spectral of **3** allowed the compound to be identified as 6-bromoaplysinopsin. Isolation of the sesquiterpene phenols and brominated aplysinopsin metabolites in the genus *Smenospongia* has been reported in the literature.[45, 52] Thus, it is not surprising to find both chromazonorol and the bromoaplysinopsin derivatives in a Bahamian species of the sponge.

Aplysinopsin, the parent compound to the two indole molecules, is a yellow pigment that was first identified in the sponge *Aplysinopsis reticulata*.[53] Metabolites of marine sponges, including species of *Thorecta, Verongia, and Dercitus* also yield aplysinopsin, and analogues of this cytotoxic tryptophan derivative have been isolated from additional sponges.[54] Currently identified analogues include brominated and di-brominated versions found in *Hyrtios erecta, Smenospongia,* and *Dercitus,* and *Dentrophyllia.*[45, 46, 55] The analogue 6-bromoaplysinopsin was additionally identified outside the Poriferan phylum in an anthazoan species.[45, 56]

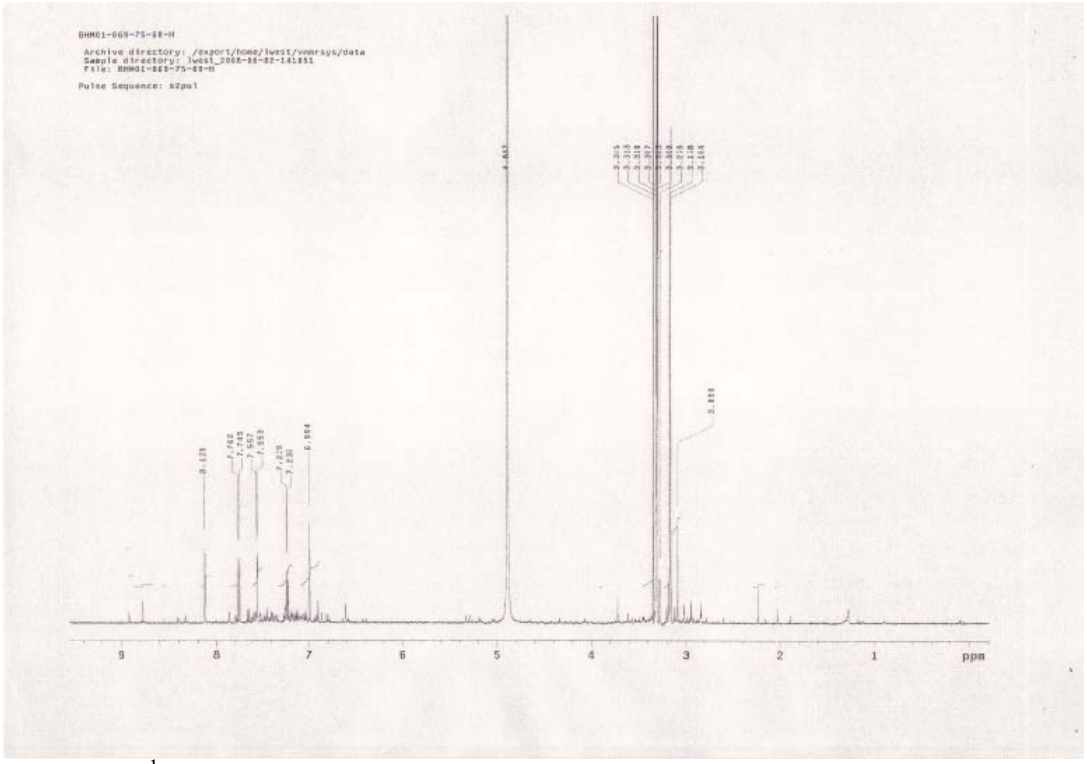

Figure 3.6. ^{1}H NMR of 6-bromo-2'-de-*N*-methylaplysinopsin (500 MHz) in CD_3OD.

Bioactivity of Brominated Aplysinopsin Derivatives

Compound **2** exhibited potential antioxidant capacity comparable to resveratrol, a natural product found in various plants that has been implicated as having anti-cancer and anti-inflammatory properties.[57] Interestingly, the methyl version exhibited high FRAP activity, while 6-bromoaplysinopsin **(3)**, having two methyl groups connected to the indole function, was inactive (Figure 3.8). Compounds **2-3** were also tested for activity in zoozanthellae cells using the DCFH-DA assay. Neither compound displayed activity when tested at a concentration of 20 μg (data not shown). The limited quantity of purified compound available prevented dose-response studies to determine if compounds **2-3** were active at higher concentrations. In a previous study by Hu *et al.*, 6-bromo-2'-de-*N*-methylaplysinopsin and 6-bromoaplysinopson were isolated from Jamaican *Smenospongia aurea* and tested for *in vitro* anti-malarial activity and cytotoxicity. The compound 6-bromo-2'-de-*N*-methylaplysinopsin showed anti-malarial

activity while 6-bromoaplysinopsin did not. Neither compound had cytotoxic effects at a concentration of 5 μg/mL. Both compounds were additionally tested, along with other aplysinopsin derivatives, for ability to displace antagonist drugs from several human serotonin receptors. Only the two brominated derivatives exhibited binding affinity strong enough to displace radio-labeled drug. The drug displaced from the 5-$HT2_C$ receptor was previously studied to treat Parkinson's disease, but had been removed from clinical trials due to toxicity.[58, 59]

Indole Alkaloid Antioxidants

The nitrogen of indoles plays an important role in the activity of indole alkaloids. Several familiar physiological metabolites, including tryptophan, serotonin, and melatonin are included in the class of indole alkaloids. The tryptamine melatonin is reported to be an effective ROS scavenger, while methoxy-substituted tryptamines exhibit both antioxidant and pro-oxidant activity, depending on the lipid content of their surroundings. Indole alkaloids from a variety of natural product sources display a wide rang of bioactivity including anti-cancer, anti-inflammatory, anti-bacterial, and other disease-countering activities. Compounds from marine sponges in particular have shown potential anti-cancer properties, and Aoki *et al*. reports selective nitric oxide synthase (NOS) inhibition by indole metabolites of the Okinawan sponge *Hyrtios erecta*.[32] These compounds represent a promising class from which to develop marine pharmaceuticals.

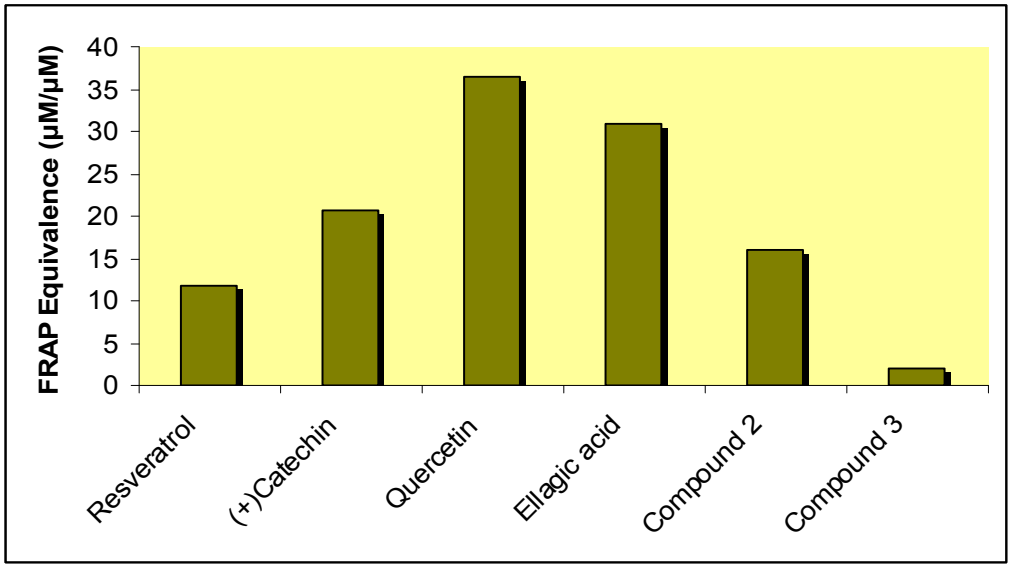

Figure 3.7. Average FRAP values of antioxidants and compounds **2-3**.

CHAPTER 4

Experimental Procedures

General Experimental Procedures

Optical rotation was measured on a Bellington Stanlay Ltd. ADP-220 digital polarimeter. UV spectra were obtained on a Perkin-Elmer λ-15 UV spectrophotometer. IR spectra were recorded on a Thermo Electronic Corporation Nicolet IR-100 spectrophotometer. All 1D and 2D NMR spectra were performed on a Varian Inova 500 MHz NMR spectrometer, and chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane as internal reference. HPLC purifications were performed on Beckman-Coulter 126P solvent module system using preparative HPLC column (Phenomenex Gemini 5μ, C18, 110A, 250 x 21.2 mm).

Solid phase Extractions (SPE) were performed on HP20 resin (3.0 g) in a 40-mL syringe-barrel column using a 12-port manifold (Altech Associates Inc). Semi-preparative HPLC separation were performed on a PRP-1 column (10 x 250 mm, 10 μm, Hamilton) using a Shimadzu HPLC system consisting of a Shimadzu DGU-20A online degasser, Shimadzu LC-20AT quaternary solvent delivery system, SPD-M20A Photodiode array detector, a Shimadzu LTII evaporative light scattering detector (ELSD) and a FRC-10A fraction collector (a *QuickSplit*™ Flow Splitter (ASI, El Sobrante, CA) was used to split the flow in 1:20 to the ELSD and fraction collector). The system was controlled with an SCL-10AVP system controller using EZStart chromatography software. Liquid handling is performed using a Precision XS microplate sample processor (BioTek Instruments, Inc, Winooksi, VT).

Collection and Preservation of Marine Biomaterial

Sponge specimens were collected using SCUBA at depths ranging from 3-90 feet in waters of the Western Atlantic. Samples collected in the Bahamas region were given identification numbers preceded by "BHM-01," while sponges from the coast of Panama City were labeled as "PC-01" samples. Biomaterial was lyophilized and stored at -20°C prior to extraction.

Solid-Phase Extraction

The organic extracts are fractionated using a solid phase Extraction (SPE) 12-port vacuum manifold. The organic extract (5 - 15 mL) is concentrated on to polymeric HP20 (3 g) using a savant vacuum centrifuge system. The HP20 is transferred into a 40-mL syringe-barrel SPE column, after washing of the column with water (20 mL) the column is eluted drop-wise with 15 mL fractions of; 1) 40% acetone/water 2) 75% acetone/water, and 3) 100% acetone. The eluent is collected in scintillation vials (20 mL) and is dried in a vacuum centrifuge.

Semi-preparative HPLC separation is performed on a Shimadzu HPLC system consisting of a Shimadzu LC-20AT quaternary solvent delivery system, SPD-M20A Photodiode array detector, evaporative light scattering detector (ELSD). We are using an evaporative light scattering detector to estimate the quantity of compounds present in the fraction. A sample of the 75% acetone/water fraction (5 mg) is subjected to semi-preparative HPLC separation on reversed phase. A post-column fixed flow splitter is used to split the flow in a ratio of 1:20 to the ELSD and the fraction collector, respectively. The elution protocol for the HPLC separation is isocratic elution with 20% acetonitrile in water for 2.5 min, followed by a linear gradient of acetonitrile from 20-90 % in 35 min, followed by isocratic elution with 90% for 10.0 min.

Extraction and Isolation

Crude extract of *Smenospongia sp.* (collection ID BHM01-069) was prepared by soaking ~90 g dried sponge material in 100% MeOH for 24 h. Extract was filtered, and the procedure repeated twice. The extract was passed through an HP20 column pre-equilibrated with methanol and separated into three discrete fractions of decreasing polarity based on the HP20 chromatographic method described previously. Polar compounds (40% Me_2CO/H_2O) were eluted first as the lowest-yielding fraction, giving only 84.2 mg compound. Intermediately polar fractions (75% Me_2CO/H_2O) yielded 346.1 mg compound. Non-polar fractions (100% Me_2CO) were eluted last, yielding 526.3 mg compound. Fractions were concentrated to dryness *in vacuo* and stored at 4°C until further use.

Assay-guided chromatographic fractionation led to selection of a especially active sample, collected from the Bahamas at a depth of 10 meters. This sponge, collection ID BHM01-069, was selected for additional research. Non-polar and intermediately polar fractions (75% Me_2CO/H_2O) and Me_2CO) were further fractionated by semi-preparative purification using increasing, 5% incremental concentrations of Me_2CO/H_2O. Isolation of the antioxidant components was directed by results from further FRAP reactions. Preliminary ^1H NMR data of the sub-fractions was also obtained to search for typical structural features of antioxidants (such as phenols). The 80% Me_2CO/H_2O was eluted as a pure compound, requiring no additional purification, and was subjected to complete structural analysis. The hydroquinone sesquiterpene was identified as chromazonorol **(1)**.

Compound **(2)** was purified from the 60% Me_2CO/H_2O fraction of the intermediately polar (75% Me_2CO/H_2O) extract. Structurally very different from the first antioxidant molecule, this compound was characterized as a brominated derivative of the indole alkaloid aplysinopsin.

Compound **3** was isolated additionally from the 65% Me_2CO/H_2O fraction as the 6-bromo analogue of methylaplysinopsin.

FRAP Assays

FRAP reagent was prepared before each assay with 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-triazine (TPTZ) solution, and 20 mM $FeCl_3$ in a 10:1:1 ratio. Reagent was heated to 37° prior to use. Assays were prepared in 96-well microtiter plates using 150 μL of warm FRAP reagent with 20 μL of sample. In the initial assay, each sample was diluted in MeOH to ~100 μg/mL. In subsequent assays, 5, 10, 18, or 20 μg of sample were added to the wells. Positive controls were tested at 10 and 20 μM concentrations. Resveratrol and catechin were dissolved in MeOH. Quercetin and ellagic acid were first dissolved in a minimal amount of 1 M NaOH, then further diluted to 10 and 20 μM with MeOH. A blank reading of the plate was taken before addition of samples. Following addition of the sample compounds, absorbance readings at 593 nm were recorded at 4, 12, and 30 minutes. The 12 min reading was selected for calculation of FRAP equivalence values.

DCFH-DA Assays

DCFH-DA (Sigma-Aldrich) was prepared as a 100 mM stock solution in MeOH and stored away from light at -20°C.

Cultured zoozanthellae (algal) cells were centrifuged briefly to remove media, and incubated in 10 μM DCFA-DA probe for 30 min with constant agitation. Following pre-loading with the probe, the probe was removed and cells resuspended in 36 mL seawater. A total of 190 μL cell suspension was added to each well of black-bottom 96-well microtiter plates. Samples were diluted to 1 μg/μL in MeOH and 5 or 20 μg added to each well. Cells were kept at 30°C and

subjected to radiation stress under a UV lamp for 3 h. Fluorescent intensity was recorded using a monochromatic spectrophotometer set at excitation wavelength 485 and emission wavelength 538. Fluorescent readings remained relatively unchanged after 24 h.

REFERENCES

(1) Cobb, C.; Goldwhite, H. In *Creations of Fire: Chemistry's Lively History from Alchemy to the Atomic Age;* Plenum Press: New York, **1995.**

(2) Houssen, W. E.; Jaspars, M. In *Natural Products Isolation, Second Edition;* Sarker, S. D., Latif, Z. and I., G. A., Eds.; Human Press Inc.: Totowa, NJ, **2006**; Vol. 20, pp 353-391.

(3) Mez-Magold, L. In *A History of Drugs;* Barnes & Noble: Totowa, N.J, **1986**.

(4) Verpoorte, R. Exploration of Nature's Chemodiversity: The Role of Secondary Metabolites as Leads in Drug Development. *Drug Discovery Today,* **1998**, *3*, 232-238.

(5) 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.

(6) Butler, M. S. The Role of Natural Products Chemistry in Drug Discovery. *J. Nat. Prod.* **2004**, *67*, 2141-2153.

(7) Osinga, R.; Tramper, J.; Wijffels, R. H. Cultivation of Marine Sponges for Metabolite Production: Applications for Biotechnology? *Trends in Biotechnology,* **1998**, *16*, 130-134.

(8) Osinga, R.; Tramper, J.; Wijffels, R. H. Cultivation of Marine Sponges. *Mar. Biotechnol. (NY)* **1999**, *1*, 509-532.

(9) Butler, M. S. Natural Products to Drugs: Natural Product Derived Compounds in Clinical Trials. *Nat. Prod. Rep.* **2005**, *22*, 162-195.

(10) Marinebiotech.org "Drugs from the Sea" Index. *http://www.marinebiotech.org/dfsindex_alpha.html* (accessed October 7, 2008).

(11) Folmer, F.; Jaspars, M.; Dicato, M.; Diederich, M. Marine Natural Products as Targeted Modulators of the Transcription Factor NF-κB. *Biochemical Pharmacology,* **2008**, *75*, 603-617.

(12) Sudek, S.; Lopanik, N. B.; Waggoner, L. E.; Hildebrand, M.; Anderson, C.; Liu, H.; Patel, A.' Sherman, D.H.; Haygood, M. G. 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.

(13) Minh Van Chau; Kiem Van Phan; Dang Hai Nguyen Marine Natural Products and their Potential Application in the Future. *ASEAN Journal on Science and Technology for Development; Vol 22, No 4 (2005)* **2007**.

(14) Harvey, A. Strategies for Discovering Drugs from Previously Unexplored Natural Products. *Drug Discovery Today*, **2000**, *5*, 294-300.

(15) Detmer Sipkema; Maurice, C. R. F.; Ronald Osinga; Johannes Tramper; René H. Wijffels Marine Sponges as Pharmacy. *Marine Biotechnology* **2005**, *7*, 142-162.

(16) Bourguet-Kondracki, M. L.; Lacombe, F.; Guyot, M. Methanol Adduct of Puupehenone, a Biologically Active Derivative from the Marine Sponge Hyrtios Species. *J. Nat. Prod.* **1999**, *62*, 1304-1305.

(17) McClintock, J. I.; Baker, B. J. In *Marine chemical ecology;* Boca Raton, Fla. : CRC Press, c2001.: 2001; , pp 610.

(18) Cline, W. Geography. *http://www.bahamasdiving.com/6729/with_flash/html/geography.html* (accessed 10/3/2008, 2008).

(19) Dunlap, W. C.; Shick, J. M. Ultraviolet Radiation-Absorbing Mycosporine-Like Amino Acids in Coral Reef Organisms: A Biochemical and Environmental Perspective. *J. Phycol.* **1998**, *34*, 418-430.

(20) Regoli, F.; Cerrano, C.; Chierici, E.; Bompadre, S.; Bavestrello, G. Susceptibility to Oxidative Stress of the Mediterranean Demosponge Petrosia Ficiformisâ€Š : Role of Endosymbionts and Solar Irradiance. *Mar. Biol.* **2000**, *137*, 453-461.

(21) Gorbi, S.; Regoli, F. Total Oxyradical Scavenging Capacity as an Index of Susceptibility to Oxidative Stress in Marine Organisms. *Comments on Toxicology* **2003**, *9*, 303.

(22) McClenachan, L.; Jackson, J. *Source Survey: The Historical Ecology of the Florida Keys Coral Reef Ecosystem*. **September, 2007**.

(23) Wilkinson, C.; Souter, D. In *Status of Caribbean coral reefs after bleaching and hurricanes in 2005.* Global Coral Reef Monitoring Network, and Reef and Rainforest Research Center: Townsville, 2008; pp 152.

(24) Bergquist, P. R. In *Sponges;* University of California Press: Berkeley, 1978; .

(25) 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.

(26) Takamatsu, S.; Hodges, T. W.; Rajbhandari, I.; Gerwick, W. H.; Hamann, M. T.; Nagle, D. G. Marine Natural Products as Novel Antioxidant Prototypes. *J. Nat. Prod.* **2003**, *66*, 605-608.

(27) Lam, K. S. New Aspects of Natural Products in Drug Discovery. *Trends in Microbiology* **2007**, *15*, 279-289.

(28) Dunlap, W.; Llewellyn, L.; Doyle, J.; Yamamoto, Y. A Microtiter Plate Assay for Screening Antioxidant Activity in Extracts of Marine Organisms. *Marine Biotechnology* **2003**, *5*, 294-301.

(29) Lysek, N.; Kinscherf, R.; Claus, R.; Lindel, T. L-5-Hydroxytryptophan: Antioxidant and Anti-Apoptotic Principle of the Intertidal Sponge Hymeniacidon Heliophila. *Z. Naturforsch.* **2003**, *58c*, 568-572.

(30) Wan, X. S.; Zhou, Z.; Kennedy, A. R. Adaptation of the Dichlorofluorescein Assay for Detection of Radiation-Induced Oxidative Stress in Cultured Cells. *Radiation Research* **2003**, *160*, 622-630.

(31) Utkina, N. K.; Makarchenko, A. E.; Shchelokova, O. V.; Virovaya, M. V. Antioxidant Activity of Phenolic Metabolites from Marine Sponges. *Chemistry of Natural Compounds* **2004**, *40*, 373-377.

(32) Aoki, S.; Ye, Y.; Higuchi, K.; Takashima, A.; Tanaka, Y.; Kitagawa, I.; Kobayashi, M. Novel Neuronal Nitric Oxide Synthase (nNOS) Selective Inhibitor, Aplysinopsin-Type Indole Alkaloid, from Marine Sponge *Hyrtios Erecta*. *Chem. Pharm. Bull.* **2001**, *49*, 1372-1374.

(33) LeBel, C. P.; Bondy, S. C. Sensitive and Rapid Quantitation of Oxygen Reactive Species Formation in Rat Synaptosomes. *Neurochemistry International* **1990**, *17*, 435-440.

(34) Kanski, J.; Aksenova, M.; Stoyanova, A.; Butterfield, D. A. Ferulic Acid Antioxidant Protection Against Hydroxyl and Peroxyl Radical Oxidation in Synaptosomal and Neuronal Cell Culture Systems in Vitro: Structure-Activity Studies. *The Journal of Nutritional Biochemistry* **2002**, *13*, 273-281.

(35) Tsao, R.; Deng, Z. Separation Procedures for Naturally Occurring Antioxidant Phytochemicals. *Journal of Chromatography B* **2004**, *812*, 85-99.

(36) Elisia, I.; Kitts, D. Anthocyanins Inhibit Peroxyl Radical-Induced Apoptosis in Caco-2 Cells. *Mol. Cell. Biochem.* **2008**, *312*, 139-145.

(37) Prior, R. L.; Cao, G. In Vivo Total Antioxidant Capacity: Comparison of Different Analytical methods1. *Free Radical Biology and Medicine*, **1999**, *27*, 1173-1181.

(38) Halliwell, B.; Gutteridge, J. M. C. In *Free Radicals in Biology and Medicine, 2nd ed.* Clarendon Press: Oxford, 1989.

(39) Wright, J. S.; Johnson, E. R.; DiLabio, G. A. Predicting the Activity of Phenolic Antioxidants: Theoretical Method, Analysis of Substituent Effects, and Application to Major Families of Antioxidants. *J. Am. Chem. Soc.* **2001**, *123*, 1173-1183.

(40) Benzie, I. F. F.; Strain, J. J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry,* **1996**, *239*, 70-76.

(41) Griffin, S. P.; Bhagooli, R. Measuring Antioxidant Potential in Corals using the FRAP Assay. *Journal of Experimental Marine Biology and Ecology,* **2004**, *302*, 201-211.

(42) Wang, H.; Joseph, J. A. Quantifying Cellular Oxidative Stress by Dichlorofluorescein Assay using Microplate Reader. *Free Radical Biology and Medicine* **1999**, *27*, 612-616.

(43) Dunlap, W. C.; Yamamoto, Y. Small-Molecule Antioxidants in Marine Organisms: Antioxidant Activity of Mycosporine-Glycine. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **1995**, *112*, 105-114.

(44) Shick, J. M.; Lesser, M. P.; Dunlap, W. C.; Stochaj, W. R.; Chalker, B. E.; Won, J. W. Depth-Dependent Responses to Solar Ultraviolet Radiation and Oxidative Stress in the Zooxanthellate Coral Acropora Microphthalma. *Mar. Biol.* **1995**, *122*, 41.

(45) Segraves, N. L.; Crews, P. Investigation of Brominated Tryptophan Alkaloids from Two Thorectidae Sponges: Thorectandra and Smenospongia. *J. Nat. Prod.* **2005**, *68*, 1484-1488.

(46) Rho, J. -.; Lee, H. -.; Shin, H. J.; Ahn, J. -.; Kim, J. -.; Sim, C. J.; Shin, J. New Sesterterpenes from the Sponge Smenospongia Sp. *J. Nat. Prod.* **2004**, *67*, 1748-1751.

(47) Cimino, G.; De Stefano, S.; Minale, L. Ent-Chromazonarol, a Chroman-Sesquiterpenoid from the sponge Disidea Pallescens. *Cellular and Molecular Life Sciences (CMLS)* **1975**, *31*, 1117-1118.

(48) Kim, D.; Lee, C. Y. Comprehensive Study on Vitamin C Equivalent Antioxidant Capacity (VCEAC) of various Polyphenolics in Scavenging a Free Radical and its Structural Relationship. *Critical Reviews in Food Science and Nutrition* **2004**, *44*, 253.

(49) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT - Food Science and Technology,* **1995**, *28*, 25-30.

(50) Rice-Evans, C.; Miller, N.; Paganga, G. Antioxidant Properties of Phenolic Compounds. *Trends in Plant Science,* **1997**, *2*, 152-159.

(51) Gul, W.; Hamann, M. T. Indole Alkaloid Marine Natural Products: An Established Source of Cancer Drug Leads with Considerable Promise for the Control of Parasitic, Neurological and Other Diseases. *Life Sciences* **2005**, *78*, 442-453.

(52) Tasdemir, D.; Bugni, T. S.; Mangalindan, G. C.; Concepcion, G. P.; Harper, M. K.; Ireland, C. M. Cytotoxic Bromoindole Derivatives and Terpenes from the Philippine Marine Sponge Smenospongia Sp. *Z. Naturforsch. [C].* **2002**, *57*, 914-922.

(53) Lovro Selič , Renata Jakscarone, Kristina Lampič , L. G.; , S. G.; - Grdadolnik, B. S., A Simple Stereoselective Synthesis of Aplysinopsin Analogs. *Helv. Chim. Acta* **2000**, *83*, 2802-2811.

(54) Stanovnik, B.; Svete, J. The Synthesis Aplysinopsins, Meridianines, and Related Compounds. *Mini-Reviews in Organic Chemistry* **2005**, *2*, 211-224.

(55) Djura, P., Faulkner, D.J. Metabolites of the Marine Sponge *Dercitus*. *J. Org. Chem.* **1980**, *45*, 735.

(56) Fattorusso, E.; Lanzotti, V.; Magno, S.; Novellino, E. Tryptophan Derivatives from a Mediterranean Anthozoan, *Astroides Calycularis*. *J. Nat. Prod.* **1985**, *48*, 924-927.

(57) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. *Science* **1997**, *275*, 218-220.

(58) Hu, J. -.; Schetz, J. A.; Kelly, M.; Peng, J. -.; Ang, K. K. H.; Flotow, H.; Leong, C. Y.; Ng, S. B.; Buss, A. D.; Wilkins, S. P.; Hamann, M. T. New Antiinfective and Human 5-HT2 Receptor Binding Natural and Semisynthetic Compounds from the Jamaican Sponge Smenospongia Aurea. *J. Nat. Prod.* **2002**, *65*, 476-480.

(59) Dupont, E.; Mikkelsen, B.; Jakobsen, J. Mesulergine in Early Parkinson's Disease: A Double Blind Controlled Trial. *J Neurol. Neurosurg. Psychiatry* **1986**, *49*, 390-390-395.