# INTER- AND INTRA- SPECIES VARIATION IN SECONDARY METABOLITES FROM CARIBBEAN OCTOCORALS OF THE GENUS *PSEUDOPTEROGORGIA*: ANTIOXIDANT ACTIVITY AND A POTENTIAL ECOLOGICAL ROLE

by

#### MAIA STAPLETON MUKHERJEE

#### (Under the direction of William K. Fitt)

#### ABSTRACT

Trends of the past century suggest that coral bleaching events may become more frequent and severe as the climate continues to warm, exposing coral reefs to an increasingly hostile environment. The combination of higher than average temperatures (as small as 1°C–2°C) and intense light can lead to mass coral bleaching events, which result in apoptosis and/or the expulsion of the zooxanthellae from the host tissue. The decline of coral reefs is significant, because coral reefs maintain high levels of biodiversity, provide habitats for coastal fisheries, protect shorelines from erosion, and may contain potential pharmacological compounds.

A striking difference between Caribbean octocorals and other zooxanthellate cnidarians is their seemingly greater resistance of bleaching. While there have been numerous reports of scleractinian coral bleaching throughout the world's tropical oceans, there have been far fewer observations of bleaching among octocorals. If the increasing incidence of bleaching events continues, the relative insensitivity of most gorgonians to high temperatures may have important consequences for Caribbean reefs.

This research attempts to examine whether secondary metabolites from the genus *Pseudopterogorgia* act as antioxidants and thus mediate thermal and UV induced oxidative stress and thereby protect the coral-algal symbiosis. Chemical diversity in soft corals may have an adaptive value in mediating ecological interactions in reef environments. Extracts from *Pseudopterogorgia sps* have differing antioxidant potentials as seen using the FRAP assay. Diterpenes isolated from *P elisabethae* exhibit antioxidant potential in FRAP and pseudopterosins scavenge superoxide radicals generated in mouse neuronal cells deprived of NGF and prevent apoptosis. Most significantly, pseudopterosins (and especially the aglycone) reduce the loss of quantum photosynthetic yield in zooxanthellae exposed to excess heat and light, and reduced significantly the oxidation of the probe DCFH in the zooxanthellae/micro-plate based assay.

Index Words: Coral bleaching, Photoinhibition, Octocoral, Zooxanthellae, Antioxidant, *Pseudopterogorgia*, Pseudopterosin, Natural product, FRAP, DCFH-DA, Nerve Growth Factor, Diterpene

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**DOCTOR OF PHILOSOPHY** 

**ATHENS, GEORGIA** 

2008

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## **DEDICATION**

For my father and mother; we were but stones; your light made us stars.

For Kevin, who reminds me every day that the world is full of wondrous discoveries yet to be made.

## ACKNOWLEDGEMENTS

Thanks to my advisor, Dr. William Fitt, and the members of my committee, Dr. Jim Porter, Dr. Jim Franklin, Dr. Jay Shelton and Dr. Ron Carroll for all the support and advice.

Thanks to Dr. Gregory Schmidt and Dr. Bill Fitt for allowing me to use cultures of *Symbiodinium* spp and the PAM Fluorometer. Thanks to Clint Oakley for assisting me with the zooxanthellae based assays.

Thanks to Rebecca Kirkland and Jim Franklin for performing the assays with mouse sympathetic neurons.

Thanks to Dr. Prasoon Gupta and Dr. Lyndon West for the structural elucidation and characterization of pseudopterosins.

Additional thanks to Dr. West his support and for generously allowing me to use the facilities in his lab to conduct my research.

Thanks to Dr. Howard Lasker for supporting my field work, for generously sharing his expertise in octocoral biology and ecology, and all his advice and encouragement. Finally a very special thanks to the Mukherjees; mom, dad, Joia, Janam, Che, and Kevin-"If not for you, winter would have no spring, couldn't hear the robin sing, I just wouldn't have a clue, anyway it wouldn't ring true, if not for you."

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## **CHAPTER 1**

### **Introduction and Background**

#### 1.1-Coral reefs and coral bleaching

Corals and all of the associated biodiversity on reefs are being degraded at an alarming rate. Among the most degraded reefs are those of the Caribbean, which have suffered an 80% decline in coral coverage over the last thirty years (Gardner et al., 2003) and a consequent phase shift from hard coral to algal dominance (e.g. Hughes, 1994). Acropora palmata and Acropora cervicornis once spatially dominant and the primary framework builders of the Caribbean during the Pleistocene and Holocene (Carpenter et al., 2008) are now officially listed as threatened under the U.S. Endangered Species Act, while macroalgal cover has risen in the last thirty years from an average of  $\sim 5\%$  to 40% (Jackson, 2008). This transition has been the result of major disturbances to the coral reef ecosystem including altered food webs (Jackson 1997; Jackson et al. 2001), large scale bleaching events (Hoegh-Guldberg 1999; Wilkinson 2002), and the emergence of disease (Harvell et al. 1999; Harvell et al. 2002; Sutherland 2004). Several factors may have contributed to this process, including over-fishing, increasing CO<sub>2</sub> levels, changes in sea surface temperatures, eutrophication, sedimentation, and pollutants. However, the individual and synergisitic role of these stressors in causing reef decline is unclear.

Ecosystems are composed of biological communities and habitats. Biological communities are interacting populations of individual species. Coral reef ecosystems include the species residing in these habitat types, but they also include aquatic residents

of associated sand, macroalgae, seagrass, and mangrove habitats. Species have specialized roles or niches within habitats. Organisms within coral reef communities can be divided into four main groups. First, the epibenthos (sessile organisms, the living substrate) provides the complex structure of the reef itself. These are the coralline and fleshy algae, hard and soft corals, and sponges. Second, plankton (tiny floating plants and animals, most microscopic) provides food for the reef filter feeders. Third, the suprabenthos are the larger mobile animals that swim over and around the reef. These are the herbivores, corallivores, carnivores and detritivores. Fourth, the cryptofauna, which bores into the substrate and settles in holes and reef crevices. These bryozoans, sponges, tunicates, and polychaetes further increase reef habitat complexity.

Coral reefs form preferentially on elevated stable substrates and within a narrow range of physical parameters. They are restricted to depths of 0-50 m (within the photic zone), salinities between 32 and 38 ppt, temperatures that range between 22 and 29° C (mean annual values), light levels of 100-2000  $\mu$  E m<sup>-2</sup> s<sup>-1</sup>, waters with transmittance values < 90% and near 90% full saturation oxygen levels. As a result of these requirements, coral reefs are restricted to the tropics, primarily between 25° north and south latitude, and predominantly on the western boundaries of the world's oceans in the Caribbean and the Indo-Pacific (Porter and Tougas, 2001).

Corals are ancient animals that evolved into the modern reef-building forms over the last 250 million years. They initially appeared as solitary forms in fossils more than 400 million years ago (Romano and Palumbi, 1996; Romano and Cairns, 2000; Stanley, 2003). The primary building blocks of a reef are polyps of scleractinian corals. These polyps are sessile with a small cylindrical body and prey-capturing tentacles surrounding the opening or mouth. The polyps of scleractinian corals deposit a calcium carbonate skeletal cup around themselves. A coral reef is comprised of millions of these calcified polyps making up individual coral heads. Coral heads are often cemented together by coralline (calcareous) algae. Symbiotic photosynthetic, single-celled algae of the genus *Symbiodinium*, (collectively called zooxanthellae) live in the tissues of each coral polyp. Polyps depend on these microscopic plants for part of their nutrition, so are limited by their symbionts' requirements to a maximum depth light penetrates in clear, oceanic waters (around 150 ft). Corals also rely on intense feeding by herbivorous fishes and sea urchins to remove seaweeds that can overgrow and kill corals. Processes such as overfishing of herbivores, climate change, nutrient pollution, sedimentation, and diseases destabilize these mutualisms and degrade reefs. In 1983 and 1984, the dominant herbivore of most Caribbean reefs, the long-spined urchin *Diadema antillarum*, suffered a pathogen-induced mass mortality. Within a year, >90% of the population of this species had died and algal biomass increased rapidly throughout Caribbean reefs (Hughes, 1994). Reefs also rely on nearby ecosystems such as seagrass beds and mangrove forests as important sources of energy and nutrients and to provide nurseries for juvenile fishes that move to reefs as adults. These have suffered unprecedented losses due to coastal development and associated run-off.

Coral reef ecosystems have evolved in oligotrophic waters with a relative absence of dissolved nutrients (nitrogen and phosphorus). Yet reefs have rates of carbon fixation that rank them among the most productive ecosystems on Earth (Grigg et al., 1984). The coral reef is the most complex, species-rich, and productive marine ecosystem (Bryant et al., 1998; Stafford-Deitsch, 1993). It has been estimated that coral reefs have about 1 million

species, with only 10% percent described (Reaka-Kudla, 1996). The reefs are home to the greatest phyletic diversity on the planet, with 32 of the 34 describe phyla found in this ecosystem (Porter and Tougas, 2001). Reef-building corals form a complex topography and create a very stable substrate, both of which may persist even after the death of the coral. This topographic relief and spatial heterogeneity along with the cryptic refuges they provide facilitate the existence of an abundance of specialized interactions and an enormous diversity of ecological niches.

The endodermal tissue lining the gastrovascular cavity of scleractinian corals harbors dinoflagellate zooxanthellae (genus Symbiodinium). These endosymbiotic algae play a pivotal role in the survival of the coral host since they provide their host with energy from photosynthate, primarily in the form of glycerol, but also as glycerides, fatty acids, lipids, organic acids, sugars and amino acids (Muscatine et al., 1972; Muscatine and Porter, 1977; Patton et al., 1977; Tiltyanov and Tiltyanova, 2002). Zooxanthellae may translocate as much as 95% of their photosynthetic products to the host (Muscatine, 1990). In return, the host provides the zooxanthellae with by-product of respiration such as CO<sub>2</sub>, organic and inorganic nitrogen and phosphorus. Additionally they receive protection against herbivory. The most significant and apparently limiting nutrient in the picture is fixed nitrogen, a critical element in the construction of all proteins. The symbiotic arrangement allows the partners to avoid the loss of fixed N to the water, a process that normally occurs in both free floating algae and solo-living marine animal life forms. Symbiotic zooxanthellae provide their hosts with orders of magnitude more energy than normally available to heterotrophic organisms. This is accomplished through the production of a photosynthate consisting of carbohydrates and lipids and the

intracellular translocation of this photosynthate to the cells of the coral host. This energetic advantage is really what permits zooxanthellate corals to prosper in nutrient-limiting environments that otherwise could not support them (Stanley, 2003).

The critical coral-algal symbiotic relationship is highly sensitive to environmental changes, particularly during periods of elevated sea surface temperature. This plantanimal partnership comes at a metabolic cost since photosynthetic oxygen released by algal symbionts can greatly exceed the respiratory demand of the animal (Richier et el., 2005). This causes coral tissues to be hyperoxic (>250% air saturation) during daylight exposure which, in combination with high light intensities, can cause photooxidative toxicity to the symbiosis via the photodynamic production of cytotoxic ROS (Richier et el., 2005). Coral bleaching occurs when either there is an overall decline in the densities of zooxanthellae and/or the concentration of photosynthetic pigments within the zooxanthellae fall (Kleppel et al. 1989). Healthy reef-building corals contain around 0.5-5 x  $10^6$  zooxanthellae cm<sup>-2</sup> of live surface tissue and healthy zooxanthellae contain between 2-10 pg of chlorophyll. When corals bleach they commonly lose 60-90% of their zooxanthellae and each zooxanthella may lose 50-80% of its photosynthetic pigments (Glynn 1996). The combination of higher than average temperatures (as small as 1°C-2°C) and intense light can lead to mass coral bleaching events, which result in apoptosis and/or the expulsion of the zooxanthellae from the host tissue. Of great debate is the adaptive bleaching hypothesis, which views bleaching as having an adaptive value by allowing the host to rid itself of its resident phylotype of algae in order to increase the chances of becoming repopulated with a different phylotype of zooxanthellae that may be more tolerant of the new environmental regime (Buddemeier and Fautin, 1993).

In the early to mid 1980's coral reefs around the world began to experience large scale bleaching (Brown, 1997; Glynn, 1996; Goreau, 1992; Glynn, 1991; Hayes and Goreau, 1991; Goenaga and Canals, 1990; Goreau, 1990; Jokiel and Coles, 1990; Williams and Bunkley-Williams, 1990). Since then, bleaching events have occured almost every year, at one time or another impacting every reef region of the world, and across all depths of the tropical reef (Hoegh-Guldberg, 2005). The severity of these events has ranged from catastrophic, resulting in mass mortality throughout at least eighty percent of a reef system, to negligible in cases where only small patches of reef bleach and then recover (Wilkinson et al., 1999; Wilkinson, 1998). Factors that may influence the intensity and outcome of bleaching episodes include differences in genetically-based species- and population-specific responses (Buddemeier and Fautin, 1993), the location of the reef (e.g., depth), strength of the stressor(s) involved, and the duration of the stress (Wilkinson, 1998). Recent years have seen widespread and severe coral bleaching episodes around the world, with coral mortality reaching 70% in some regions. The decline of coral reefs is significant, because coral reefs maintain high levels of biodiversity, provide habitats for coastal fisheries, may contain potential pharmacological compounds, and protect shorelines from erosion. Trends of the past century suggest that coral bleaching events may become more frequent and severe as the climate continues to warm, exposing coral reefs to an increasingly hostile environment. This global threat to corals compounds the impacts of more localized anthropogenic factors that already place reefs at risk.

In response to a dramatic increase in the frequency and intensity of bleaching events over the past 15–20 years, research focused on the molecular events occurring

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during the dissociation of dinoflagellate-cnidarian symbioses have intensified. Bleaching is considered a symptom of stress and may be induced by a variety of physical and biological factors, including high and low sea surface temperatures (Jokiel and Coles, 1977; Fitt and Warner, 1995; Saxby et al., 2003; Hoegh-Guldberg and Fine, 2004), UV irradiation (Jokiel and Coles, 1977; Gleason and Wellington, 1993; Brown et al., 1994), bacterial infection (Kushmaro et al., 1996; Rosenberg and Falkovitz, 2004), lowered salinity, and pollution (Glynn, 1993). All of these factors have the ability to cause localized bleaching events; but it is increased SST and irradiance associated with periods of El Nino Southern Oscillation that causes mass bleaching and poses the greatest challenge to coral reefs worldwide (Hoegh-Guldberg and Smith, 1989; Porter et al., 1989; Glynn, 1991 and Glynn, 1993; Fitt and Warner, 1995; Hoegh-Guldberg, 1999 and Hoegh-Guldberg, 2004; Lough, 2000).

During conditions of high light and temperature, over-reduction of the electron transport chain downstream of the Photosystem II (PS II) reaction centre (Jones and Hoegh-Guldberg, 2001) contributes to an increased production of reactive oxygen species (ROS) within the dinoflagellates (Lesser,1997). Chloroplasts are hyperoxic, produce ROS, and are susceptible to oxidative stress. ROS in the chloroplast may damage PS II, primarily through oxidative degradation of the D1 protein (Warner et al., 1999), and also inhibit the repair of damage to PS II (Nishiyama et al, 2001). In addition to  ${}^{1}O_{2}$ ,  $O_{2}^{-}$  and HO' also are produced in the PS II reaction center.

Oxidative stress—the production and accumulation of reduced oxygen intermediates such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals—can damage lipids, proteins, and DNA. Many disease processes and

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the aging process involve oxidative stress in their underlying etiology. The production of reactive oxygen species is also prevalent in the world's oceans, and oxidative stress is an important component of the stress response in marine organisms exposed to a variety of insults as a result of changes in environmental conditions such as thermal stress, exposure to ultraviolet radiation, or exposure to pollution (Lesser, 2006).

Atmospheric  $O_2$  in its ground state is distinctive among the elements because it has two unpaired electrons (and thus is known as a biradical). This property significantly limits its ability to interact with organic molecules unless it is "activated." The univalent reduction of molecular oxygen produces reactive intermediates such as the superoxide radical ( $O_2^-$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical (HO<sup>•</sup>). Cumulatively the reduction products of oxygen are known as free radicals or reactive oxygen species (ROS) (Cadenas, 1989).

All photosynthetic and respiring cells produce ROS, including  $O_2^-$  via the univalent pathway;  $H_2O_2$  is formed by the continued reduction of  $O_2^-$ ; and eventually HO<sup>•</sup> is formed and then reduced to the hydroxyl ion and water. Oxidative stress can be defined as the production and accumulation of ROS beyond the capacity of an organism to quench these reactive species. It can result in damage to lipids, proteins, and DNA, but ROS can also act in signal transduction (Fridovich, 1998). The central purposes of antioxidant defenses in biological systems are to quench  ${}^{1}O_2$  at the site of production and to quench or reduce the flux of reduced oxygen intermediates such as  $O_2^-$  and  $H_2O_2$  to prevent the production of HO<sup>•</sup>, the most damaging of the ROS.

The reducing side of PS I can reduce  $O_2$  to  $O_2^-$  by the Mehler reaction and is the most significant site of  $O_2^-$  production in the chloroplast (Lesser, 2006). The production

of  $O_2^-$  increases under stressful conditions, such as exposure to xenobiotics or pollutants, high visible irradiances, exposure to ultraviolet radiation (UVR), and/or exposure to thermal stress. This elevated production can overwhelm antioxidant defenses and can result in damage to both PS II and the carbon fixation process.

The extent to which hosts lose their populations of symbiotic dinoflagellates varies among species (Hoegh-Guldberg and Salvat, 1995; Fabricius, 1999; Loya et al., 2001; Douglas, 2003), within species (Lasker et al., 1984; Porter et al., 1989; Glynn, 1990; Berkelmans and Oliver, 1999; Ulstrup et al., 2006; LaJeunesse et al., 2007) and sometimes between regions of a colony (Rowan and Knowlton, 1995; Rowan et al., 1997). This variability has been attributed to the thermal tolerance of the symbiont (Berkelmans and van Oppen, 2006; Ulstrup et al., 2006), differences in host tolerance (Loya et al., 2001; Brown et al., 2002b; Bhagooli and Hidaka, 2004; D'Croz and Maté, 2004), or particular host- symbiont combinations (Rowan, 2004; Goulet et al., 2005). In the Caribbean, octocorals are the most conspicuous reef macrofauna, with up to 40 species co-occurring in a single area (Sánchez et al., 2003). A striking difference between Caribbean octocorals and other zooxanthellate cnidarians is their seemingly greater resistance of bleaching. While there have been numerous reports of scleractinian coral bleaching throughout the world's tropical oceans, there have been far fewer observations of bleaching among octocorals (Lasker, 2003). If the increasing incidence of bleaching events continues, the relative insensitivity of most gorgonians to high temperatures may have important consequences for Caribbean reefs.

Octocorals or soft corals are important constituents of coral reef ecosystems (Goldberg, 1973; Kinzie, 1973; Kinzie, 1974; Lasker and Coffroth, 1983; Sánchez et al.,

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1997; Fabricius and Alderslade, 2001; Sánchez et al., 2003). Few studies, however, have examined how they and their symbionts differentially respond to environmental stress (Fabricius, 1999; Strychar et al., 2005). In a study done during the height of the 1998 mass bleaching event on the Great Barrier Reef, the octocoral families Briareidae, Clavularidae, Gorgonidae, and Nephtheidae exhibited low bleaching susceptibilities (Goulet et el., 2008). There was no indication that different *Symbiodinium* populations explained the occurrence of bleached or unbleached tissues. The authors of this study concluded that differential tolerance to stress among cnidarian species, irrespective of the Symbiodinium they host, may explain differences in bleaching. In the Caribbean, the vast majority of octocorals and other invertebrate hosts associate with Symbiodinium clade B, and more selectively, with a single lineage of this clade, Symbiodinium B1/B184 (Santos et al., 2004). Since specificity and stability in cnidarian-dinoflagellate symbioses are common (Rodriguez- Lanetty et al., 2003, 2004; LaJeunesse et al., 2004; Goulet, 2006; Thornhill et al., 2006), especially among octocorals (Goulet and Coffroth, 2003, 2004; Santos et al., 2003; Kirk et al., 2005; Goulet et al., 2008), rapid environmental changes may not lead to new symbiotic combinations in alcyonaceans. The physiological resiliency of host and symbiont under their current range of partner combinations may limit their potential for surviving acute environmental stress.

### **1.2-Natural products**

Humans have made use of natural products throughout most of its history. Plant extracts have been incorporated in the preparation of foodstuff, dyes, toxins and medicines by even the most ancient human civilizations. In the late eighteenth century, chemists moved this practice into the realm of modern science. They began to separate, purify and analyze active compounds made inside living cells. Aspirin or acetylsalicylic acid is a derivative of salicylic acid that is a mild, non-narcotic analgesic useful in the relief of headache and muscle and joint aches. The drug works by inhibiting the production of prostaglandins, metabolites that are necessary for blood clotting and which also sensitize nerve endings to pain.

Hippocrates, who lived sometime between 460 B.C and 377 B.C, is considered the father of modern medicinal science. Hippocrates has left historical records of pain relief treatments, including the use of a powder made from the bark and leaves of the willow tree to help heal headaches, pains and fevers. In the early 1800's, scientists discovered that it was the compound called salicin in willow plants that relieves pain. In 1828, Johann Buchner, professor of pharmacy at the University of Munich, isolated a tiny amount of bitter tasting yellow, needle-like crystals, which he called salicin. In 1838, Raffaele Piria a chemist at the Sorbonne in Paris, split salicin into a sugar and an aromatic component (salicylaldehyde) and converted the latter, by hydrolysis and oxidation, to a crystallized acid, which he named salicylic acid (1). The problem was that salicylic acid caused stomach irritation. In 1853, a French chemist Charles Frederic Gerhardt neutralized salicylic acid by buffering it with sodium (sodium salicylate) and acetyl chloride, creating acetylsalicylic acid (2). In 1899, a German chemist named Felix Hoffmann, who worked for a German company called Bayer, rediscovered Gerhardt's formula. Felix Hoffmann gave acetylsalicylic acid to his father who was suffering from the pain of arthritis. With good results, Felix Hoffmann then convinced Bayer to market the new wonder drug. Aspirin was patented on March 6, 1889. The scientists at Bayer came up with the name Aspirin, it comes from the 'A" in acetyl chloride, the "spir" in *Spiraea ulmaria* (the plant they derived the salicylic acid from) and the 'in' was a then familiar name ending for medicines (Andermann, 1996).



Figure 1. Structure of salicylic acid (1) and acetylsalicylic acid (2).

A natural product is generally defined as a secondary metabolite; one that is not necessary to sustain the life of the producing organism, but confers to it an adaptive advantage. Secondary metabolites produced by living organisms presumably have adaptive value that favors their evolution. Many plants and animals, both terrestrial and marine, produce compounds that help them survive by, for example, deterring potential predators, warding off pathogens, keeping their living space free from competitors, and reducing the impact of exposure to environmental stresses, such as high levels of UV radiation.

Nature has provided mankind with a broad array of structurally diverse and often pharmacologically active compounds that might be of use to combat disease or as lead structures for the development of novel synthetically derived drugs that approximate their structure and function in nature. Traditionally, higher plants and, since the discovery of the penicillins, terrestrial microorganisms have proven to be the richest sources of novel bioactive compounds. A well-known example of a plant-derived anti-cancer drug is paclitaxel (taxol<sup>®</sup>, **3**), isolated from the bark of the Pacific yew *Taxus brevifolia*. Examples of anti-cancer agents from bacterial sources include doxorubicin (adriamycin, **4**) and bleomycin from various *Streptomyces* strains.



Figure 2. Structures of paclitaxel (3) and doxorubicin (4).

## **1.3-Marine natural products**

The marine environment covers a wide thermal range with temperatures from below the freezing point in Antarctic waters to as high as 350°C in deep hydrothermal

vents. The ocean environment also exhibits a vast pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and varied light regimes (photic and non-photic zones). Due to this extensive variability the ocean is home to unmatched speciation at all phylogenetic levels, from microorganisms to mammals. Despite the fact that the biodiversity of higher taxa in the marine environment far exceeds that of the terrestrial environment, research of marine natural products is still in its infancy. The development of new diving techniques, manned submersibles and remotely operated vehicles and improved technologies for mapping has increased our capacity to collect marine samples.

Exploration of the vast potential of marine organisms as sources of bioactive metabolites started in the late 1960s. The discovery of prostaglandins (important mediators involved in inflammatory diseases, fever and pain) in the gorgonian *Plexaura homomalla* by Weinheimer and Spraggins in 1969 is considered to be a critical starting point for the search for "drugs from the sea" (Weinheimer and Spraggins, 1969). From 1969–1999 approximately 300 patents on bioactive marine natural products were issued. The number of compounds isolated from various marine organisms now exceeds 10,000 (MarinLit, 2001). There are currently three FDA approved drugs of marine origin. Unusual nucleosides isolated from the Caribbean sponge *Cryptotethya crypta* (Bergmann and Feeney, 1951) served as lead structures for the development of the now commercially important anti-viral drug ara-A (**5**) and the anticancer drug for leukemia ara-C (**6**) (Arif et al., 2004). Ziconotide (Prialt<sup>®</sup>, 7), a 25 amino acid peptide isolated from the marine mollusk *Conus magnus*, was approved by the FDA in 2004 for use in alleviating chronic pain (Proksch et al., 2002; Associated Press, 2004; Newman et al., 2004).



Figure 3. structures of ara-A (5), ara-C (6) and Prialt<sup>®</sup> (7)

Many marine natural products are currently in clinical trials. Notable anti-cancer compounds from marine organisms include bryostatin 1 (8) and ecteinascidin-743 (9) (Yondelis<sup>®</sup>). Bryostatin 1 is polyketide isolated from the bryozoan *Bugula neritina* and is currently in Phase I/II clinical trials for treatment of various cancers (Newman et al., 2004). Currently there are four phase I and five phase II trials underway for the use of bryostatin 1 in combination with nucleotide derivatives or cytotoxic agents (Newman et al., 2004). Ecteinascidin-743 is a tetrahydroisoquinoline alkaloid from the tunicate *Ecteinascidia turbinata*. It is presently in phase III clinical trials and is effective against solid tumors such as sarcomas and breast cancer (Proksch et al., 2002; Arif et al., 2004).



Figure 4. Structures of bryostatin 1 (8) and ecteinascidin-743 (9).

Marine octocorals (Phylum Cnidaria) are a source of a wealth of biologically active and structurally unique compounds isolated and described by natural products chemists (Faulkner, 2000). These metabolites have been assumed to be important for the success of octocorals in the marine environment, and their real ecological functions have been experimentally tested (Cronin et al., 1995; Fenical and Pawlik, 1991; Harvell et al., 1988; Pawlik and Fenical, 1992; Pawlik et al., 1987; O'Neal and Pawlik, 2002). The defensive role against predation has been the most analyzed, in part due to the low predation rates found in these sessile and soft-bodied animals. Living in habitats characterized by high levels of predation and nutrient scarcity, octocorals seem to be free from predation with the exception of certain specialists such as the mollusks *Cyphoma gibbosum* (Harvell and Suchanek, 1987), and the butterfly fish *Chaetodon capistratus* (Lasker, 1985).

Caribbean gorgonians account for nearly 38% of known octocorals and over 195 species of octocorals have been recorded from this region (Fenical, 1987). Gorgonians

include organisms commonly referred to as sea plumes, sea rods, flat sea whips, and sea fans. Gorgonians have a central axis that is attached to a substrate by a holdfast. Radiating from the central axis are branchlets that contain polyps, which extend themselves through apertures called calyces for feeding. The shapes of these calyces are taxonomically distinct (Bayer, 1961). Different branching patterns and the three dimensional arrangement of these branching patterns also define different genera and species (Bayer, 1961).

Evidence of chemical defense among gorgonians is abundant. While detailed chemical analysis has only been conducted on about one-fifth of known Caribbean gorgonian species, a large number of novel metabolites have been discovered in the tissue of these organisms (reviewed in Rodriguez, 1995). Many natural products isolated from gorgonians are active in pharmacological assays, many exhibiting anti-cancer and antiinflammatory properties (Fenical, 1987; Rodriguez, 1995). Hypotheses regarding the ecological role of secondary metabolites have been tested in some cases. There is experimental evidence that the compounds may inhibit larval settlement, increase resistance to fungal pathogens, or prevent or inhibit overgrowth by other sessile organisms (Standing et al., 1984; Rodriguez, 1995; Kim et al., 2000). The most common hypothesis is that secondary metabolites are anti-predatory agents, and studies have consistently demonstrated antipredatory properties of gorgonian crude extracts and purified compounds (Pawlik et al. 1987; Fenical & Pawlik, 1991; Pawlik and Fenical, 1992; Paul and Van Alstyne, 1992; Harvell et al., 1993; Epifanio et al., 1999; Maia et al., 1999; Koh et al. 2000).

The wealth of bioactive metabolites isolated from soft-bodied, sessile or slowmoving marine invertebrates that lack morphological defense structures such as spines or a protective shell is no coincidence but reflects the ecological importance of these coumpounds for these organisms. It has been shown that chemical defense through accumulation of toxic or distasteful natural products is an effective strategy to fight off potential predators and/or organisms competing for space (Proksch et al., 2002; McClintock and Baker, 2001). Grazing pressure by predators such as fishes is higher on tropical and subtropical reefs than in any other ecosystem of the world. On tropical coral reefs fish have been estimated to bite the bottom in excess of 150,000 times per m<sup>2</sup> per day (Carpenter, 1986). With this intense selective pressures survival depends on effective means of chemical defense.

In many cases compounds that protect their invertebrate producers from predators or that help to fight off fouling organisms and those organisms competing for space have also attracted attention in pharmacological assays that are aimed at drug discovery. Thus, organisms that thrive in spite of pronounced biotic pressures can, to some degree, be expected to contain metabolites that are also of interest for drug discovery programs. While there is no direct correlation between defensive and biomedical potential of a compound, the fact that many cellular processes and structures such as enzymes and receptors are highly conserved implies that they must have been good targets for interspecies chemical warfare. For example, the production of a substance by one organism in order to affect a second organism in a detrimental manner- the broadest definition of antibiosis- is very common phenomenon in the marine ecosphere and may provide a convincing rationale for the production of antimicrobial and antiviral agents by marine invertebrates that are constantly in contact with marine bacteria, fungi and viruses. The rich diversity in chemical structures found in marine phyla may reflect the long period of time the organisms have had to perfect their chemical arsenal (Reviewed in Faulkner, 2000).

Gorgonian corals are found in all tropical and subtropical seas, but reach their greatest levels of diversity and abundance on Caribbean coral reefs (Bayer 1961, Kinzie 1973), where they attain densities as high as 25 colonies per m<sup>2</sup> (Goldberg 1973). Faunistically, extensions of the West Indian region reach into the Gulf of Mexico, all the Antilles, the Bahamas, the Florida Keys, the Bermudas, the Islands of the Caribbean, and south along the northeast coast of South America to the reefs of Brazil. All over this region, the families *Gorgoniidae* and *Plexauddae* flourish as they do nowhere else in the world. Gorgonian corals (order Gorgonacea, phylum Cnidaria) are conspicuous members of most tropical and subtropical marine habitats, being the most abundant octocorals found in the West Indies. With over 195 species documented from these two major families, gorgonian octocorals represent an estimated 38% of the known fauna.

Taxonomically, the Octocorallia include six orders the Alcyonacea (fleshy soft corals), Telestacea (common fouling organisms), Gorgonacea (horny or gorgonian soft corals), Stolonifera (matlike or stolonborne soft corals), Pennatulacea (sea pens), and Coenothecalea (blue-stony corals). Defensive chemistry has been reported or noted from all six orders (Coll, 1992). The Alcyonacea and Stolonifera are prominent in the Indo-Pacific, while the Gorgonacea and Pennatulacea dominate the Caribbean. There is little crossover of species between the two regions.

Unlike scleractinian corals, which have only a thin veneer of living tissue on an endoskeleton of rock-hard carbonate, gorgonians possess a relatively fleshy coenenchyme that usually surrounds a supportive rod or mesh of sclerotinized protein. Given the nutrient scarcity and high levels of predation reported for coral reef environments (Grigg et al., 1984), it might be expected that gorgonians would provide a source of food for a variety of reef predators, but this does not appear to be the case (Pawlik et al., 1987). Natural products chemists have isolated over 200 novel secondary metabolites from these animals (Faulkner, 2000; and previous reviews by the same author); some of these compounds occur at extraordinarily high concentrations within the coral tissues (Schneider et al., 1977). Although pharmacological effects of many of these compounds have been described (see references in Pawlik et al., 1987), only recently have the possible functions of these metabolites in gorgonians been addressed by employing ecologically relevant laboratory and field experiments (Gerhart, 1988; Pawlik et al., 1987; Harvell et al., 1989; Pawlik and Fenical, 1989; Pawlik and Fenical, 1989).

Gorgonians of the genus *Pseudopterogorgia* are commonly referred to as "sea plumes" based on their finely branch or plumose growth forms. *Pseudopterogorgia* species are among the most common of the Caribbean species. The first chemical analysis of a *Pseudopterogorgia* species began in 1968, with investigations of the sesquiterpene hydrocarbons from *Pseudopterogorgia americana* (Schmitz et al., 1969). The gorgonian corals, and octocorals in general, are a rich source of biologically active compounds which play a defensive role against predators (e.g. Pawlik et al. 1987; Harvell et al. 1988; Harvell and Fenical, 1989; Pawlik and Fenical, 1989; Fenical and Pawlik, 1991; Paul and Van Alstyne, 1992; Van Alstyne et al. 1992), competitors (Sammarco et al.,1983; La Barre et al., 1986), and microbes and fouling organisms (Targett et al., 1983; Bandurraga and Fenical, 1984; Gerhart et al., 1988; Ciereszko and Guillard, 1989; Kim, 1994; Slattery et al., 1995; Jensen et al., 1996).

Chemical defenses isolated from gorgonians and alcyonaceans are usually terpenoids (reviewed in Paul and Puglisi, 2004). Briarane and asbestinane diterpenes are found in adult colonies and the larvae of the gorgonian *Briareum asbestinum* and show anti-predator activity in fish feeding assays (Harvell et al., 1996). Similarly, pukalide and 11-acetoxypukalide were found in the eggs of the Pacific soft coral *Sinularia polydactyla* (Slattery et al., 1999). The concentration of pukalide was similar to that of the adults while the concentration of 11-acetoxypukalide was much lower. Recently, the sesquiterpene heterogorgiolide and a known eunicellane diterpenoid from *Heterogorgia uatumani* were reported to be unpalatable to natural assemblages of fishes in Brazil (Maia et al., 1999). A similar study of the gorgonian *Lophogorgia violacea* yielded a mixture of 5 furanocembranoid diterpenes that in combination also appear to deter fish predation (Epifanio et al., 2000).

Antimicrobial activities of crude extracts from many Caribbean gorgonian corals have been examined (reviewed in Paul and Puglisi, 2004). Bioassays carried out with the extracts of seven Caribbean gorgonians in the family *Plexauridae* and one gorgonian in the family *Gorgonidae* showed that non-polar fractions inhibited the growth of three species of marine bacteria and two non-marine species (Kim, 1998). Among cnidarians, gorgonians display some of the most potent antimicrobial activities (Jensen et al. 1996; Kim et al., 2000). The hydroquinones of *Pseudopterogorgia rigida* and *P. acerosa* have antiviral and antibacterial activity and deter predatory fish (Harvell et al., 1988). In a
broad survey of 39 Caribbean sea fans and sea whips, crude extracts were screened for antibacterial activity by the standard agar disc-diffusion method against a host of marine bacteria isolated from the surfaces of living colonies of *Briareum asbestinum* and decaying gorgonians and three bacterial species known to be pathogenic to marine invertebrates (Jensen et al., 1996). While only 15% of the extracts showed antimicrobial activity, extracts from *Pseudopterogorgia* spp. were quite active, inhibiting the growth of most bacterial strains tested.

Gorgonians from the Caribbean region are responsible for several diterpenoid carbon skeletal classes including cembranes, asbestinins, briareins, pseudopterosins, and pseudopteranes, along with other skeletons that do not belong to any major class. Most of these skeletal classes possess unique functionalities and substitution patterns. Cembranes represent the largest group of compounds isolated from gorgongians in the West Indian region (Rodríguez, 1995).

The gorgonian *Pseudopterogorgia bipinnata*, a widely distributed member of the genus *Pseudopterogorgia*, produces a large variety of cembrene derivatives somewhat dependent upon collection site. The characteristic of *Pseudopterogorgia* cembrenes is their high levels of oxygenation. Bipinnatins A-D (**10-13**) were first isolated from *P. bipinnata* collected off Jamaica Cay and Acklins Islands, Bahamas (Wright et al., 1989). Bipinnatins A, B, and D were active, *in vitro*, against P388 murine tumor cell lines with IC<sub>50</sub> values 0.9, 3.2, and 1.5 µg/mL, respectively. Additionally, bipinnatin B was found to inhibit [<sup>125</sup>I]- $\alpha$ -toxin binding to intact cells and block  $\alpha$ -toxin binding to receptor-rich

membrane fragments prepared from *Torpedo* electric organ (Culver et al., 1985, Abramson et al., 1991).



Figure 5. Structures of bipinnatans A-D (10-13).

Some *P. bipinnata* also contain diterpenes representative of the pseudopterane skeletal class including, kallolide A (14) and kallolide A acetate (15) (Look et al, 1985). Kallolide A has been shown to be a potent inhibitor of phorbol ester induced inflammation in the mouse ear assay at concentrations and efficacies equivalent to those of indomethacin (Marshall and Nelson, 1988).



Figure 6. Structures of kallolide A (14) and kallolide A acetate (15).

The pseudopterosins are a class of structurally diverse anti-inflammatory and analgesic metabolites isolated from the gorgonian *Pseudopterogorgia elisabethae* (Look et al., 1986). The pseudopterosins show anti-inflammatory activity equal to or great than the industry standard, indomethacin, and appear to possess a unique mechanism of action. There is also recent evidence suggesting that pseudopterosins may serve as anti-oxidants (Mydlarz and Jacobs, 2004). Currently pseudopterosins are used as cosmetic additives (Rouhi, 1995).

Pseudopterosins A-D (**17-20**) were originally from samples collected at Crooked Island, Bahamas (Look et al., 1986). The structure of pseudopterosin C was established by X-ray crystallography. *P. elisabethae* collected from different geographic locations posses different congeners of the pseudopterosins. So far, twenty-six pseudopterosin congeners have been described, all exhibiting the amphilectane skeleton and a glycoside linkage at either C-9 or C-10 (Rodriguez, 1995).



**17**  $R_1 = R_2 = R_3 = H$  **18**  $R_1 = Ac, R_2 = R_3 = H$  **19**  $R_2 = Ac, R_1 = R_3 = H$ **20**  $R_3 = Ac, R_1 = R_2 = H$ 

Figure 7. Structures of pseudopterosins A-D (17-20)

Due to their excellent antiinflammatory and analgesic activity, partially purified extracts containing pseudopterosins are currently incorporated into skin care preparations (Pomponi, 1999). The availability of pseudopterosins however, is limited by the actual supply of organic extracts of *Pseudopterogorgia elisabethae* which currently only comes from the Bahamas islands. The complex and expensive chemical synthesis of these compounds makes these animals an attractive study target in other areas of the Caribbean. One of the arguments often advanced for the conservation of coral reef ecosystems is the extraordinary diversity of these communities and the potentially useful bioactive compounds that reef species may produce (Bruckner, 2002). That potential creates a paradox, in that the discovery of compounds with potentially valuable properties generates the need to harvest that material. Even when laboratory syntheses of the compounds are possible it is often the case that the amounts of material needed for exploratory research will necessitate extensive collections. Bruckner (2002) for instance notes cases in which thousands of kilograms of tissue are needed to generate milligrams of the target compound.

To date, pseudopterosins from the Caribbean octocoral *Pseudopterogorgia elisabethae* are the only compounds from a reef species that have made their way to commercial use. Pseudopterosins exhibit anti-inflammatory and analgesic properties and are used by the cosmetics industry (Look et al., 1986). Over the last 12 years *P*. *elisabethae* has been harvested from the Little Bahama Bank in the Bahamas. The fishery is regulated through an export limit set by the Bahamas Department of Marine Resources. Annual harvest data are not publicly available, but Bruckner (2002) placed the value of the fishery at \$3–\$4 million and Puyana et al. (2004) notes that estimated demand for the pseudopterosins is 13–20 tons per year. The collection protocols in the fishery have been developed by the collectors. Colonies are collected by divers using either scuba or surface supplied air. The collectors clip the colony, removing most of the colony but with the goal of leaving at least one central branch, typically 5–15 cm in height, that contains 5–10 branchlets. The remaining tissue survives and regenerates new branches (Castanaro and Lasker, 2003). Colonies develop sufficient tissue for reharvesting in as little as 2 years (Goffredo and Lasker, 2008). Initially populations were allowed to recover for 2–3 years, which was the time generally required for new and recovering colonies to reach a size at which a harvest would be profitable. Additional input from more recent scientific studies (i.e., Lasker et al., 2003, Gutiérrez-Rodríguez and Lasker, 2004) has led the collectors to extend the recovery time to allow colonies to reach reproductive size and reproduce prior to a second harvest. Some of the sites have been harvested 3 times which is suggestive of sustainability, but the fishery has not been the subject of a quantitative analysis.

## 1.4 Reactive oxygen and antioxidants

Molecular oxygen is relatively unreactive due to its electron configuration. Activation of oxygen (i.e. the first univalent reduction step) is energy dependent and requires an electron donation. The subsequent one-electron reduction steps are not energy dependent and can occur spontaneously or require appropriate  $e^-/H^+$  donors. In biological systems transition metal ions (Fe<sup>2+</sup>, Cu<sup>+</sup>) and semiquinones can act as  $e^-$  donors. Fourelectron reduction of oxygen in the respiratory electron transport chain (ETC) is always accompanied with a partial one- to three-electron reduction, yielding the formation of ROS (Halliwell and Gutteridge, 1999). This term includes not only free radicals (superoxide radical, O<sub>2</sub><sup>--</sup>, and hydroxyl radical, OH), but also molecules such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), singlet oxygen ( $^{1}O_{2}$ ) and ozone (O<sub>3</sub>). Both O<sub>2</sub><sup>--</sup> and the hydroperoxyl radical HO<sub>2</sub><sup>--</sup> undergo spontaneous dismutation to produce H<sub>2</sub>O<sub>2</sub>. Although H<sub>2</sub>O<sub>2</sub> is less reactive than O<sub>2</sub><sup>--</sup>, in the presence of reduced transition metals such as Fe<sup>2+</sup> in a chelated form (which is the case in biological systems), the formation of OH<sup>-</sup> can occur in the Fenton reaction (Halliwell and Gutteridge, 1999).

A biological antioxidant has been defined as any substance that when present at low concentration compared with that of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1999). Since some free radical production in cells is inevitable and can be very damaging, a defense system against the deleterious action of free radicals has evolved (Cheeseman and Slater, 1993). These are known as antioxidant defenses and the two main categories are those whose role is to prevent the generation of reactive oxygen species (ROS), and those that intercept any radicals that are generated (Larson, 1997).

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. As such, free radicals can be formed in three ways: (i) by the hemolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons; (ii) by the loss of a single electron from a normal molecule; (iii) by the addition of a single electron to a normal molecule (Halliwell and Gutteridge, 1999). The latter, electron transfer, is a far more common process in biological systems than is homolytic fission, which generally requires high energy input from either high temperatures, UV light or ionizing radiation. Heterolytic fission, in which the electrons of the covalent bond are retained by only one of the fragments of the parent molecule, does not result in free radicals but in ions, which

are charged. Free radicals can be positively charged, negatively charged or electrically neutral. The unpaired electron and the radical nature of a species are conventionally indicated by writing it with a heavy superscript dot.

It can be a source of confusion that the electrons in one of the most important molecules in free radical biochemistry, oxygen, are distributed in such a way that two of the electrons are 'unpaired'. Thus, oxygen is sometimes considered a di-radical. While the di-radical nature of oxygen does enable it to react readily with many other free radicals, in general it reacts relatively slowly with non-radical species (Denisov and Afanas'ev, 2005). When considering its reactions in the context of free radical biochemistry, it is usually easiest to simply consider it as a normal molecule that can readily add to free radicals or accept a single electron from them, while not itself being a free radical (Halliwell and Gutteridge, 1999).

Arguably the most important free radicals in biological systems are radical derivatives of oxygen (Cheeseman and Slater, 1993). Reactive oxidizing species derived from oxygen are formed in cells by normal metabolism as well as by numerous types of environmental insults. Reduction of oxygen by the transfer to it of a single electron will produce the superoxide free radical anion ('superoxide').

 $O_2 + e^- > O_2^-$ 

A two-electron reduction of oxygen would yield hydrogen peroxide:

## $O_2 + 2e^{-} + 2H^{+}$

Hydrogen peroxide is often generated in biological systems *via* the production of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen.

 $2 O_2 \rightarrow 2H^+ \longrightarrow H_2O_2 + O_2$ 

Because the free radical reactants produce non-radical products this is known as a dismutation reaction. It can take place spontaneously (slow reaction kinetics) or can be catalyzed by the enzyme superoxide dismutase. Hydrogen peroxide is not a free radical but falls into the category of 'reactive oxygen species' (ROS) that includes not only oxygen free radicals but also non-radical oxygen derivatives that are involved in oxygen radical production (Denisov and Afanas'ev, 2005).

Hydrogen peroxide is an important compound in free radical biochemistry because it can rather easily break down, particularly in the presence of transition metal ions, to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical (OH):

$$H_2O_2 + Fe^{2+} \longrightarrow OH + OH^- + Fe^{3+}$$

The above reaction is referred to as the iron-catalysed Haber-Weiss reaction. The noncatalysed Haber-Weiss reaction is the reaction of superoxide directly with hydrogen peroxide:

 $O_2^{-+} H_2O_2^{+} \longrightarrow OH + OH^{-} + O_2$ 

All of the major classes of biomolecules may be attacked by free radicals but lipids are probably the most susceptible. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidizing radicals. The oxidative destruction of PUFAs, known as lipid peroxidation, is particularly damaging because it proceeds as a self-perpetuating chain-reaction. Lipid peroxidation has been implicated in a wide range of tissue injuries and diseases (Romero et al, 1998; Pansarasa et al., 1999; Beal, 2002). Free radical scavengers are preventive antioxidants. Antioxidants can act at different levels in an oxidative sequence. This may be illustrated by considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation (Denisov and Afanas'ev, 2005). Free radical chain reactions within a cell can be inhibited by adding chemicals that retard the formation of free radicals, by introducing substances that compete for the existing radicals and remove them from the reaction medium.

Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions causing the termination of the Fenton reaction (Rice-Evans et al., 1997). Phenols are intrinsically electron-rich compounds prone to enter into efficient electron-donating reactions with oxidizing agents. Typically, an electron is transferred from a phenol to the unfilled orbital of a one-electron oxidant such as a peroxyl radical, followed by rapid proton transfer:

 $Ph-OH + ROO \rightarrow Ph-O + ROOH$ 

The net result is the equivalent of a hydrogen atom transfer from the phenolic hydroxyl group to the free radical. The product, the phenoxyl radical, PhO, is stabilized by resonance delocalization of the unpaired electron to the *ortho* and *para* positions of the ring (Denisov and Afanas'ev, 2005).

In order to act as an effective antioxidant, the phenoxyl radical must not be reactive enough to initiate further free-radical reactions on its own. While the phenol itself is a rather ineffective antioxidant, if other radical-stabilizing features are accentuated, extremely potent antioxidants can result (Denisov and Afanas'ev, 2005). In addition to the intrinsic stability of the resulting radical due to resonance properties, various structural features such as the steric and inductive effects of ring substituents enhance this stability. For example, bulky or electron donating groups at the *ortho* position of a phenol is the signature feature of many synthetic and naturally occurring antioxidants (Denisov and Afanas'ev, 2005).

Diterpenes play diverse functional roles in vivo, acting as hormones (gibberellins), regulators of wound-induced responses (abietic acid), photosynthetic pigments (the phytyl chain of chlorophylls), and antioxidants (McGarvey and Croteau, 1995). Although tocopherols and carotenoids are, among lipid-soluble antioxidants, the best-characterized groups of compounds in their function of protecting the plant from oxidative stress, plants contain other compounds such as diterpenes displaying high antioxidant properties (Rice-Evans et al., 1997). Rosemary (*Rosmarinus officinalis*) leaf extracts show a very high antioxidant activity. The main compound responsible for the antioxidant activity is the diterpene, carnosic acid. (Aruoma et al., 1992). Its radical scavenging activity follows a mechanism analogous to that of other antioxidants such as  $\alpha$ -tocopherol and is caused by the presence of two hydroxyl groups in *ortho* position at C<sub>11</sub> and C<sub>12</sub>. (Richheimer et al., 1999). Subcellular localization studies show that carnosic acid protects chloroplasts from oxidative stress in vivo (Munné-Bosch and Alegre, 2001).

# 1.5 The antioxidant potential of extracts and compounds from *Pseudopterogorgia* sps.

It has been shown that photosynthetic rates in zooxanthellae can be improved by the addition of exogenous antioxidants to coral exposed to artificially elevated light and temperature (Lesser, 1997). Antioxidants improve the rate of  $O_2$  evolution by scavenging free radicals within the dinoflagellate symbiont. Although dinoflagellates can ameliorate the oxidative burden through the use of endogenous antioxidant enzymes and photoprotective mechanisms, these systems can be overwhelmed, especially by the synergistic effects of thermal and irradiance stress.

Gorgonians from the Caribbean region are responsible for several diterpenoid carbon skeletal classes including cembranes, asbestinins, briareins, pseudopterosins, pseudopteranes, along with other skeletons that do not belong to any major class. Most of these skeletal classes possess unique functionalities and substitution patterns. Cembranes represent the largest group of compounds isolated from gorgonians in the West Indian region (Rodríguez, 1995).

Phenolic diterpenes have been shown to act as potent antioxidant in many pharmacological studies. Gorgonians of the genus *Pseudopterogorgia* biosynthesize many such diterpenes with substantial diversity in both the carbon skeleton and the location and type of substitutions to the skeleton. The antioxidant potential of these compounds was assessed using a number of pharmacologically and ecologically relevant assays. This research attempts to examine whether secondary metabolites from the genus *Pseudopterogorgia* act as antioxidants and thus mediate thermal and UV induced oxidative stress and thereby protect the coral-algal symbiosis. This was accomplished by screening semi-purified extracts as well as pure compounds from these organisms.

Chapter two provides an analysis of the variation in secondary metabolite chemistry of *Pseudopterogorgia elisabethae* across geographic and depth gradients. Secondary metabolites vary among species and the biosynthetic pathways producing the compounds are homologous traits which can be used for phylogenetic inference. Indeed some of the most intricate accounts of microevolution have been based on the evolution of secondary metabolites and chemical defense. Efforts to generate chemical fingerprints of samples of the genus Pseudopterogorgia have led to the development of HPLC and NMR libraries that can be used for the rapid identification of *Pseudopterogorgia spp*. collected from the northern Bahamas and the Florida Keys. Additionally we have evaluated the variation in secondary metabolites amongst *Pseudopterogorgia elisabethae* collected from different locations, different depths along the same reef tract and at different times of the year. Pseudopterogorgia elisabethae is currently commercially harvested for the pharmacologically active compounds the pseudopterosins and information about chemical variation across geographic, depth and seasonal gradients may prove useful for future management decisions regarding their harvest.

Chapter three includes results and analysis of assays which screen semi-purified extracts and pure compounds isolated from samples of the genus *Pseudopterogorgia* for antioxidant potential. First, extracts from seven species of the genus were partitioned into their polar (40% acetone water), intermediately polar (75% acetone-water), and non-polar (100% acetone) constituents using column chromatography. Then these fractions were then assayed using the ferric reducing ability of plasma (FRAP) assay. The FRAP assay is a measure of "antioxidant power" and offers a putative index of antioxidant, or

reducing, potential. In the assay ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Based on the results of these initial FRAP assays, it appears that the intermediately polar fractions of all seven species have antioxidant or reducing potential, with a significant interspecies variation. The species with the lowest overall FRAP values was *P. hystrix*, which according to Bayer (1961) is a deep-water specialist.

Extracts of *P. elisabethae* where subjected to HPLC and purified compounds were screened in the FRAP assay. These pure compounds had differing antioxidant potentials, and many of those isolated from *P elisabethae* had activity equal to or greater than Trolox, a water-soluble vitamin E derivative which is commonly used as a positive control in many antioxidant assays.

In Chapter four the *in vitro* antioxidant activity of the intermediately polar fractions of seven species of the genus *Pseudopterogorgia* was tested in a zooxanthellae based assays. This was accomplished using cultured zooxanthellae and the oxidative stress sensitive dye DCFH-DA in a micro-well plate. DCFH-DA diffuses into the cell where cellular esterases cleave the diacetate moiety to form the more polar DCFH, which is trapped within the cell. Oxygen radicals oxidize the intracellular DCFH to the fluorescent DCF. The level of fluorescence measured upon excitation is proportional to the level of oxidation. Antioxidants prevent oxidation of DCFH and reduce the formation of DCF. Extracts (40 µg each dissolved in methanol) were added to wells in triplicate, control wells had either methanol only or nothing added. For all seven species, the intermediately polar fraction significantly reduced fluorescence versus the control cells.

Four of the species caused reduction that did not differ significantly from the positive control (Trolox).

Compounds from *P. elisabethae* previously tested in the FRAP assay were screened using the DCFH-DA zooxanthellae assay. The pseudopterosins caused significant reduction in oxidative state in the cells as evidenced by a significant reduction in DCF fluorescence versus the control, with the pseudopterosin aglycone having the best overall activity (Tukey's HSD p<0.05).

The ability of secondary metabolites isolated from *Pseudopterogorgia elisabethae* to delay photoinhibition in stressed zooxanthellae was also tested. Samples from a clade B2 Symbiodinium culture from the temperate western Atlantic were subjected to combined irradiance (light=300 $\mu$ M) and thermal (temperature=34°C) stress. Pulse-amplitude modulated (PAM) fluorometry was used to characterize the maximum quantum efficiency of PSII (F<sub>V</sub>/F<sub>M</sub>). Results were compared to control samples to which no compounds were added and solvent-only controls. Pseudopterosins and especially the pseudopterosin aglycone seem to reduce the loss of quantum photosynthetic yield over the nine and a half hour period of the experiment.

In Chapter 5, the in vitro antioxidant activity of secondary metabolites isolated from samples of the genus *Pseudopterogorgia* was tested in a mouse sympathetic neuron cell model. Depriving sympathetic neurons of nerve growth factor (NGF) *in vitro* leads to neuronal apoptosis. Reactive oxygen species (ROS) appear a "trigger" in this apoptotic process- a burst of oxidative stress is detected 4 hr after NGF withdrawal, and superoxide dismutase (SOD) overexpression inhibits apoptosis. Apoptosis of cells induced by NGF withdrawal is inhibited by a number of antioxidants and scavengers of ROS.

The results presented here are evidence that octocorals may use secondary metabolites to mediate oxidative stress thus reducing the incidence of bleaching. First, extracts from *Pseudopterogorgia sps* have differing antioxidant potentials as seen using the FRAP assay. Second, diterpenes isolated from *P elisabethae* exhibit antioxidant activity in FRAP. Third, Pseudopterosins scavenge superoxide radicals generated in mouse neuronal cells deprived of NGF and prevent apoptosis. And finally and most significantly, pseudopterosins (and especially the aglycone) reduce the loss of quantum photosynthetic yield in zooxanthellae exposed to excess heat and light, and reduced significantly the oxidation of the probe DCFH in the zooxanthellae/ micro-plate based assay.

## CHAPTER 2

## Intra-specific chemical variability of Pseudopterogorgia elisabethae.

## 2.1 Caribbean octocorals

The octocoral fauna of the West Indies is unique in its profusion of gorgonian corals (also known as sea whips, sea fans, or sea plumes). Faunistically, extensions of the West Indian region reach into the Gulf of Mexico, all the Antilles, the Bahamas, the Florida Keys, the Bermudas, the Islands of the Caribbean, and south along the northeast coast of South America to the reefs of Brazil (Bayer, 1961). All over this region the families Gorgoniidae and Plexauridae flourish as they do nowhere in the world. Gorgonian corals (order Gorgonacea, phylum Cnidaria) are conspicuous members of most tropical and subtropical marine habitats, being the most abundant octocorals found in the West Indies. With over 195 species documented from these two major families, gorgonian octocorals represent an estimated 38% of the known fauna (Bayer, 1961). A diverse array of branching octocorals are present on reefs throughout the Atlantic and Indo-Pacific (e.g., Fabricius and Alderslade, 2001), and on many reefs in the Eastern Pacific and Western Atlantic/Caribbean the biomass and diversity of branching octocorals, and in particular Holaxonians, may be higher than that of scleractinian corals. Octocorals have always been identified as an important component of the reef benthos both in Florida and throughout the Caribbean (Opresko, 1973; Wheaton and Jaap, 1988; Kinzie, 1973; Lasker and Coffroth, 1983; Yoshioka and Yoshioka, 1989; Sanchez, 1998; Sanchez et al., 1999).

At many sites, octocorals now are the visually most abundant invertebrate due in large part to the dramatic loss of scleractinian cover (Porter and Meier, 1992; Hughes, 1994; Ostrander et al., 2000; Gardner et al., 2003; Cote et al., 2005). Octocorals play additional roles in benthic ecosystems such as generating sediments (Weinbauer and Velimirov, 1995) and providing habitat for fishes and other invertebrates. Branching octocorals have also been the subject of intense investigation by natural product chemists, and have proven to be rich in novel compounds with interesting ecological properties and in some cases properties of interest to biomedical researchers. Secondary metabolites from octocorals have been demonstrated to have anti-inflammatory, anti-carcinogenic, and anti-tumor activity (Pawlik et al., 1987; Rodriguez, 1995; Cobar et al., 1997; Mayer et al., 1998; Shi et al., 2002). While differences in natural product chemistry both between and within taxa have been noted, there has been only limited use of that variability in either the identification or systematics of octocorals. The only attempt to use the chemical data in a systematic context (Gerhart, 1983) identified some of the same ambiguities more recently identified in mtDNA sequence analyses (Sánchez et al., 2003).

#### 2.2 Terpene Biosynthesis

Terpenes, or isoprenoids, are ubiquitous metabolites found in all living organisms. Sterols, steroidal hormones, and carotenoids are all examples of essential isoprenoids which participate in many important metabolic pathways. Additionally, there are large numbers of terpenes or terpenoids for which physiological roles remain a mystery (Conolly and Hill, 1992). Terpenes represent the largest family of natural products with over 30,000 members (Buckingham, 1998). They are classified by the number of homologous isoprene units (5 carbon units) in their structure: monoterpenes  $C_{10}$  (2 isoprene units), sesquiterpenes  $C_{15}$  (3 isoprene units), diterpenes  $C_{20}$  (4 isoprene units), sesterterpenes  $C_{25}$  (5 isoprene units), and triterpenes  $C_{30}$  (6 isoprene units).

Terpenoid biosynthesis (Figure 8) involves the head to tail addition of isopentenyl diphosphate (21) (IPP) units (Ruzicka, 1953). The enzyme isopentenyl isomerase facilitates the isomerization of IPP to dimethylallyl diphosphate (22) (DMAP). Next, IPP and DMAP are condensed and the chain elongated by prenyltransferases to create geranyl diphosphate (23) (GPP-  $C_{10}$ ). The enzyme-bound geranyl diphosphate undergoes further condensation with additional IPP units to form larger prenyl diphosphates, namely, farnesyl diphosphate (24) (FPP-  $C_{15}$ ) and geranylgeranyl diphosphate (25) (GGPP-  $C_{20}$ ).



**Figure 8.** Biosynthesis of geranyl, farnesyl, and geranylgeranyl diphosphate (**23-25**) from isopentenyl diphosphate (**21**) and dimethylallyl diphosphate (**22**)

Cyclization, coupling and/or rearrangement reactions of these prenyl diphosphates forms parent carbon skeletons of monoterpenes, sesquiterpenes, and diterpenes (Singh et al., 1989; McGarvey and Croteau, 1995; Luthra et al., 1999). These carbon skeletons can then be subjected to structural modification through oxidation, reduction, isomerization, hydration, conjugation, halogenation, and/or other transformations to give rise to a variety of terpenoids (McGarvey and Croteau, 1995).

## 2.3-Terpenes from *Pseudopterogorgia* spp.

Caribbean gorgonian octocorals of the genus Pseudopterogorgia are abundant and chemically rich, responsible for the production of several classes of metabolites. Many of the compounds isolated from these marine invertebrates are of great interest because of their structural complexity and their pharmacological potential (Blunt et al., 2005). Two such families of *Pseudopterogorgia* metabolites are the pseudopterane and the cembrane diterpenoids. Several Caribbean species of Pseudopterogorgia (P. acerosa, P. kallos, and P. bipinnata) are known to biosynthesize diterpenoids based on the 12-membered carbocyclic pseudopterane skeleton (Tinto et al., 1990; Rodriguez, 1995,). Kallolide A (14), a major metabolite of P. bipinnata and P. kallos based on the 12-membered carbocyclic pseudopterane skeleton, possesses anti-inflammatory properties comparable to the potency of existing drugs such as indomethacin (Look et al., 1985; Fenical, 1987). While the pseudopteranes appear to be taxonomically restricted to these Pseudopterogorgia (Gorgonacea) species and one species of Gersemia (Alcyonacea), the cembranoids, on the other hand, are often found in many gorgonian and soft coral species. Diterpenoids of the cembrane class are common metabolites of several West

Indian gorgonian genera, especially Eunicea, Plexaura, and Leptogorgia (Rodriguez, 1995). On the other hand, Pseudopterogorgia species that produce cembranoid diterpenes do so in a much more restricted manner. Thus far, cembrane-based diterpenoids have been found in only two of the more than 15 species identified *P. acerosa* (Tintio et al., 1990, 1991 and 1995) and P. bipinnata (Fenical, 1987; Wright et al., 1989; Rodriguez and Shi, 1998). Of these, the major producer of cembrane derivatives is Pseudopterogorgia bipinnata Verrill (family Gorgoniidae), a widely distributed member of this genus. In 1987 Fenical reported that more than 15 cembrane derivatives were isolated from *P. bipinnata* collected in the Bahamas, (Fenical, 1987). In 1989, Wright et al. reported the isolation and structure elucidation of four cytotoxic furanocembranolides, denoted as bipinnatins A-D (10-13), from a specimen of P. bipinnata also collected in the Bahamas (Wright et al., 1989). In 1999, Rodriguez and co-workers reported the isolation and structure determination of nine previously unreported pseudopterane and cembrane metabolites from extracts of a Colombian specimen of P. bipinnata. These include bipinnapterolide A structurally related to the known metabolite kallolide A (14) and bipinnatins G-I are highly oxygenated cembranolides which are structurally similar to the known metabolites bipinnatins A-D (10-13). Additionally, Rodriguez et al (1999) isolated from five new related cembranolides bipinnatolides F-J.

Secondary metabolites isolated from gorgonians (sea whips and sea fans) are structurally diverse (Faulkner, 2001) and many exhibit biological activities (Paul, 1992). Since the polyps of the gorgonians lack physical defenses, the secondary metabolites from these animals have generally been hypothesized to serve as chemical defenses (Paul, 1992; Pawlik, 1993). During the past decade, laboratory and field experiments have shown that numerous gorgonians possess metabolites that are highly deterrent to generalist carnivorous fishes. Anti-predatory properties of the crude extracts from 32 species of Caribbean gorgonians were examined in laboratory assays with the generalist predator *Thalassoma bifasciatum* and 100% of these crude extracts proved to be deterrent (O'Neal and Pawlik, 2002). Included in these assays was crude extracts from *Pseudopterogorgia elisabethae,* which were among the most potent icthyodeterrent of the extracts tested. Of the gorgonians studied in detail, several metabolites responsible for icthyodeterrent activity have been isolated (O'Neal and Pawlik, 2002).

## 2.4 Pseudopterosins

Pseudopterosins are an interesting group of diterpene glycosides first discovered by Fenical and collaborators (Look et al., 1986) from specimens of the gorgonian coral *Pseudopterogorgia elisabethae*. So far, 26 pseudopterosins (PsA–PsZ) isolated from specimens collected in the Bahamas (Look et al., 1986), Bermuda (Roussis et al., 1990), the Florida Keys (Ata et al., 2003), and the Columbian Caribbean (Duque et al., 2004) have been reported. The structurally related secopseudopterosins A–D have also been identified in *Pseudopterogorgia kallos* collected near the Marquesas Keys in Florida (Look and Fenical, 1987).These pseudopterosins and secopseudopterosins exhibit more potent antiinflammatory and analgesic activities than the common market drug indomethacin (Mayer et al., 1998). It is suggested that the mechanism of action of the pseudopterosins may involve membrane stabilization, different from inhibition of eicosanoid release from inflammatory cells mediated by traditional non-steroidal drugs (Mayer et al., 1998). Experiments done to elucidate the biosynthetic pathway of pseudopterosins in *Pseudopterogorgia elisabethae* present evidence that the pseudopterosin aglycone (**26**) is the end product of pseudopterosin biosynthesis (Figure 9) and thus may be an active natural product *in vivo* (Ferns, 2005).



Figure 9. Pseudopterosin biosynthesis. Ferns et al., 2005

Perhaps then this represents an example of an activated defense system. Activated defense has been described as a rapid process in which innocuous metabolites that are stored in the tissue are converted to defensive metabolites in response to tissue damage

(reviewed in Paul, 1992). The advantage of activated defenses to organisms that possess them is not one of optimization (because the energy involved in production has already been expended), but rather the avoidance of autotoxicity. Activated defenses might be selected over constitutive defenses if the deterrent chemical is physiologically damaging to the producing organism (Baldwin and Callahan, 1993; Wolfe et al., 1997) or if the defensive chemical attracts predators (Carroll and Hoffman, 1980; Giamoustaris and Mithen, 1995).

The deterrence capacity of pseudopterosins A-D and the pseudopterosin aglycone was tested by incorporating these metabolites into artificial carrageenan-based diets (Mukherjee, 2005). Paired feeding experiments were conducted on a shallow patch reef (~3M) at Sweetings Cay, Bahamas. At natural volumetric concentrations (found in the adults colonies collected in Sweetings Cay, Bahamas) pseudopterosins A-D (0.6% dry weight) significantly inhibited feeding relative to controls with high statistical signifigance (Wilcoxon signed rank test 1 tail, p= 0.0062) while the aglycone (0.1% dry weight) was a significant feeding attractant (p= 0.0027). Based on these results it is hypothesized that the pseudopterosin aglycone has some other ecological role and that its activity as a feeding attractant may explain the low concentration of this metabolite found in undamaged *P. elisabethae* tissue (Mukherjee, 2005).

The activation of defenses has been widely documented in terrestrial plants (León et al., 2001). Cyanogenic plants contain glycosides composed of a cyanohydrin-type aglycone and a sugar moiety. Cyanogenic glycosides and their hydrolyzing enzymes are stored in different tissue compartments in intact plant tissue. Upon tissue damage, the glycosides are hydrolyzed, and HCN, which is toxic to non-specialist grazers, is released

(Vetter, 2000). Additional examples of activated defense in vascular plants include the conversion of phenolic glycosides to phenolics in quaking aspen Populus tremuloides (Clausen et al., 1989) and balsam poplar Populus balsamifera (Reichardt et al., 1990), and the hydrolysis of glucosinolates to form thiocyanates, isothiocyanates, or isonitriles in the Cruciferae and related plants (Van Etten and Tookey, 1979; Chew, 1988). There are examples of activated defense in marine organism as well. Van Alstyne and Paul (1992) found that most species of Halimeda on Guam immediately convert the lessdeterrent secondary metabolite halimedatetraacetate to the more potent feeding deterrent halimedatrial upon injury simulating fishes feeding on Halimeda plants. Extracts from injured plants contained higher amounts of halimedatrial and were more deterrent toward herbivorous fishes than extracts from control plants (Van Alstyne and Paul, 1992). Cetrulo and Hay (2000) investigated the frequency of activated chemical defenses in seaweeds using urchin and fish feeding assays of chemical extracts from 42 species of seaweeds that were damaged immediately before extraction in organic solvents (the potentially activated extract) versus extracts from undamaged tissue (the non-activated extract). Seven species exhibited changes in palatability consistent with activated defenses while 4 species became more, rather than less, palatable if they were damaged 30 s before extraction (Cetrulo and Hay, 2000).

Identifying the true biosynthetic origin of secondary metabolites isolated from marine organisms is confounded by the fact that most marine organisms live in tight symbiotic association with many marine microbes. Over the past decade, there has been a growing awareness that compounds whose biosynthesis had been attributed to marine invertebrates may actually be produced by a symbiont (Vacelet and Donadey, 1977;

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Anthoni et al., 1990; Kobayashi and Ishibashi, 1993). From both ecological and biomedical viewpoints, it is important to know whether the metabolites isolated from marine organisms are produced by symbiotic microorganisms (Faulkner, 1994). Most gorgonian species form obligate associations with dinoflagellates of the genus *Symbiodinium* in order to survive in the nutrient-poor tropical waters. Nutrition, in the form of translocated photosynthates, is the primary contribution of the dinoflagellate to the host. The dinoflagellate receives inorganic nutrients as well as protection from the intense predation of marine environment in return (Whitehead and Douglas, 2003). For corals which are endowed with symbiotic zooxanthellae, there is debate whether the coral itself, the zooxanthellae, or another microbial associate is the source of the secondary metabolites. Historically, terpenes have been viewed as plant metabolites; thus zooxanthellae were assumed to be the source of these compounds.

The diterpene crassin acetate was purified from the extract of zooxanthellae isolated from the gorgonian *Pseudoplexaura crassa* (Ciereszko, 1962), and diterpenes have been purified from the algal symbiont of *P. elisabethae* and *P. bipinnata* (Mydlarz et al, 2003; Boehnlein et al, 2005). Conversely, isolation of terpenoids from azooxanthellate *Lophogorgia alba* (Bandurraga et al., 1982) and azooxanthellate *Lobophytum compactum* (e.g. Michalek-Wagner et al, 2001) gives credence to terpene biosynthesis being under animal control. In the bryozoan *Bugula neritina*, biosynthesis of the bioactive bryostatins has been localized to bacterial symbionts (Davidson and Haygood, 1999). Research on the sponge *Dysidea herbacea* shows that a unique group of polychlorinated compounds isolated from the whole sponge tissue is limited to the

cyanobacterial filaments, whereas the accompanying sesquiterpenoids are found only in the sponge cells (Unson and Faulkner, 1993).

The larvae of the gorgonians *Pseudopterogorgia elisabethae* and *P. bipinnata* lack algal symbionts. Examining these for the presence of diterpenes, and assessing their biosynthetic capacity can therefore provide additional information about the true source of these ecologically and pharmacologically important secondary metabolites.

Hildebrand et al (2004) suggest the use of the following criteria when attempting to determine the source organism of a metabolite: 1) correlate presence of the symbiont with a function for the host, 2) remove the symbiont and show loss of function, 3) reintroduce the symbiont and show that function is regained, and 4) isolate the symbiont again. One difficulty lies in obtaining symbiont-free hosts, as many marine organisms are not viable without their symbiont. Also, isolating symbionts for reintroduction can be complicated, as obligate symbionts may not be culturable.

Since most organisms have many microbes associated with them, it is important to determine which of these are persistently associated with the host (Hildebrand et al 2004). When there are variations in natural products within a host species or related group of species, correlation of the presence of a particular microbe with a particular compound provides support for the microbial involvement in synthesis of that compound. If animals lacking a particular microbe also lack the metabolite, this suggests that the microbe might be a good candidate for further study. In some cases, symbionts can be reduced or eliminated by treatment with antibiotics or by other methods. Correlation of symbiont reduction or elimination with reduction or elimination of the metabolite can provide useful information.

Reproductive tissues in gametes and larvae are always important to examine for the presence of symbionts. Although symbionts can be recruited from the environment, in many cases, the host has evolved mechanisms to ensure vertical transmission. Microbes persistently associated with eggs and larvae are likely to have important roles in the life of the host, one of which could be synthesis of bioactive metabolites (Lopanik et al, 2004).

Biosynthetic experiments can be performed in different ways. One of these is feeding with an isotopically labelled precursor in order to prove whether this is incorporated into the final product or not. A radiolabeled (e.g. <sup>3</sup>H or <sup>14</sup>C) putative precursors can be used in *in vivo* or *in vitro* assays to monitor the transformation of a known precursor to the secondary metabolite of interest. In the case of terpene biosynthesis, the radiolabeled precursor [<sup>3</sup>H-C<sub>1</sub>]-GGPP can be used. Radiolabeled isotopes are extremely sensitive and easily detected by scintillation counting. This sensitivity is useful when the enzymatic turnover rate from precursor to secondary metabolite is low. When a radiolabeled precursor is being employed radiochemical purity for the isolated secondary metabolite must be assessed after isolation of the target compound. This can be done by derivatization of the compound followed by purification of the derivative and monitoring of specific activity. Finally, in order to conclude that a label was incorporated into a metabolite, radioactivity at least twice background (an inactive sample) should be obtained for the metabolite of interest.

Chemical analysis of the azooxanthellate larvae from the sea whips *Pseudopterogorgia elisabethae* and *Pseudopterogorgia bipinnata* revealed higher concentrations of diterpenes than are present in the intact holobiont.

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	Larvae	Polyps	Zooxanthellae	Holobiont
Pseudopterosins	$30.39 \pm 1.48^{-1}$	N/A	$11^{2}$	$5^{2}$
A-D				
Kallolide A	$21.51 \pm 0.97^{-1}$	$18.75 \pm 2.11^{-1}$	7.3 <sup>2</sup>	4.27 <sup>2</sup>
acetate				

**Table 1.** Comparison of pseudopterosin A-D and kallolide A Acetate content from *P. elisabethae* and *P. bipinnate* expressed as % organic extract.

1 Mukherjee, 2005, Average of n = 3 experiments  $\pm$  st. dev.

2 Mydlarz et al., 2003

3 Boehnlien et al., 2005

Biosynthetic studies of the larvae with <sup>3</sup>H-labeled geranylgeranyl diphosphate indicated that they are capable of diterpene biosynthesis. Previous studies had suggested that pseudopterosin and kallolide biosynthesis proceeds in the absence of *P. elisabethae* (Mydlarz et al., 2003) and P.bipinnata (Boehnlien et al., 2005) cells. In these studies, zooxanthellae were separated using a gravimetric gradient of Percoll. To further investigate whether this is indeed the case, DNA from freshly isolated zooxanthellae, and DNA from the larvae (from each of these two host organisms) was subjected to PCR specific primers (Santos et al., reactions with zooxanthellae 2001) and Pseudopterogorgia specific primers (Gutierrez-Rodriguez et al., 2004). The reaction resulted in the amplification of a fragment of DNA corresponding to the presence of both zooxanthellae and coral DNA from the isolated zooxanthellae. The larvae DNA from both species clearly lacked algal DNA, as no amplicon was seen corresponding to the expected product of the zooxanthellae primers (Figure 10).

It is quite possible that results of previous studies, in which biosynthesis appeared to occur in the absence of coral cells, may have been confounded by the presence of coral cells in what was thought to be purified algal symbionts. Coral DNA was found in zooxanthellae isolates regardless of the number of times the purification procedure was repeated, up to three consecutive times. In an attempt to further localize the biosynthesis,



**Figure 10.** An example of an agarose gel showing amplification products from larvae, isolated zooxanthellae, and holobiont DNA using zooxanthellae and *Pseudopterogorgia* specific primers.

larvae from both species were treated over 48 hours with a combination of antimycotic and antibiotic agents, following a previously published procedure used successfully in with the larvae of the bryazoan *Bugula neritina* (Lopanik et al., 2004). Briefly, For each species, n=3 containers of 100 ml MFSW with ~100 larvae were treated with antibiotic/antimycotic twice over 48 hours with final concentrations as follows: 0.1 mg/ml kanamycin (a water-soluble, broad-spectrum antibiotic), 0.1 mg/ml streptomycin (active against both gram-positive and gram-negative bacteria, including species resistant to other antibiotics), 100 units/ml penicillin, 0.25  $\mu$ g/ml amphotericin B. At the end of the 48 hours the samples were incubated for 24 hrs with the tritiated diterpene precurser and then extracted and analysed as previously described. Treatment of the larvae from both species with antimicrobial agents did not reduce their capacity to synthesize these bioactive compounds (Table 2). Radiochemical purity of the pseudopterosin Y and kallolide A acetate was verified via derivitization reactions as previously described (Mydlarz et al., 2003 for pseudopterosins; Boehnlein et al., 2005 for kallolide A acetate; Mukherjee, 2005). In each case significant change in structure and physical properties between the parent compound and the derivative resulted in a significant retention time difference of over five minutes on both normal and reversed phase HPLC, ensuring that the associated radioactivity was due to the compound of interest and not the co-elution of a highly radioactive impurity. Structures of parent compounds and derivatives were confirmed by <sup>1</sup>H- NMR.

**Table 2.** Comparison specific activities of pseudopterosin and Kallolide A acetate from larvae treated with antibiotics and antimycotics and those untreated. Average of n = 3 experiments  $\pm$  st. dev.

Specific Activity (DPM/mmol)	P. elisabethae larvae	P. elisabethae larvae Ab/Am treated
pseudopterosin Y	2.4 ± 0.58 E7	$2.8 \pm 0.23 \text{ E7}$
Specific Activity (DPM/mmol)	<i>P. bipinnata</i> larvae	<i>P. bipinnata</i> larvae Ab/Am treated
Kallolide A Acetate	3.6 ± 0.89 E6	5.6 ± 1.22 E6

Based on these results, it seems likely that the terpenoid biosynthesis occurs within the coral cells. Further studies with these early ontegenetic tissues should include thorough examination of antibiotic and antimycotic treated larvae to confirm that they are free of microbial associates.

### 2.5 Variability of pseudopterosin content across a geographic gradient

Patterns of intraspecific variation in composition and concentration of secondary metabolites among geographic regions or habitats have been well documented for terrestrial plants (Gershenzon and Croteau, 1991). A few studies have shown variability in secondary metabolite content within or between individuals of a given species (e.g., Meyer and Paul, 1995; Maida et al., 1993; Harvell et al., 1993; Becerro et al., 1998; Amade and Lemee, 1998). Some of these reports have correlated the variation to spatial variables, such as depth or geography. For example, Maida et al. (1993) found significant differences in the major metabolites, flexibilide and sinulariolide, of the soft coral Sinularia flexibilis among sites at Lizard Island (Great Barrier Reef, Australia). In the Caribbean, Harvell et al. (1993) detected variation in composition and concentration of the major metabolites of the gorgonian Briareum asbestinum among depths and geographical sites. In a study done by Benavahu and co-workers (2000) intraspecific variation in composition of secondary metabolites of Parerythropodium fulvum, a common encrusting alcyonacean soft coral on Red Sea reefs as well as in concentrations of its major metabolites were correlated with different color morphs and varying depths.

The great degree of chemical variation among different specimens of *P*. *elisabethae* from various sites in the Caribbean region has been acknowledged by several authors. Different kinds of the closely related pseudopterosins have been isolated from Bahamian, Bermudan and Columbian specimens of *P. elisabethae* (Look et al., 1986; Roussis et al., 1990; Puyana et al., 2004). So far, it is not known if chemical variability and pseudopterosin production are genetically or environmentally determined.

Fragments of P. elisabethae colonies were collected by SCUBA at various sites and depth ranges around the islands of the Bahamas and Key Largo, Florida (for GPS coordinates see Table A1). Sample collection never involved removing whole colonies, only a terminal fragment of each individual colony was cut from the main gorgonian axis with sharp scissors. Gorgonian fragments were air-dried, labeled, and stored in the freezer. Samples were kept frozen until extraction. Colony fragments used for chemical analyses were collected at 10 sites around the Bahamas and 1 site off Key Largo between May of 2000 and September of 2007 (Figure 11). We collected on average 10 replicates The morphology of *P. elisabethae* colonies can be highly variable. per location. Therefore, to avoid confusion with other species, we corroborated the identity of each colony by comparison of the gross colony architecture and by morphology and dimensions of the three most common sclerite types (i.e., spindles, anthocodial rods, and scaphoids). To obtain sclerites, we clipped off a small fragment of the colony and digested it in household bleach. Once the organic tissue was digested, sclerites were repeatedly washed with distilled water and ethanol, and dried. Crude sclerite preparations were mounted on glass slides and analyzed under a microscope. For each colony, we measured 30 anthocodial rods, 30 spindles, and 30 scaphoids. For species designations, we followed Bayer (1961). Based on morphological analysis, all colonies collected were correctly identified in the field as *P. elisabethae*.



**Figure 11.** Collection sites of *Pseudopterogorgia elisabethae* in the Bahamas and Florida. Detail shows the sites clustered along the Little Bahamas Bank.

Each dried colony was cut in small pieces and weighed. 1 g of gorgonian tissue was extracted with methanol. Crude extracts were analyzed by thin layer chromatography (TLC) on silica plates with 40:60 hexanes:ethyl acetate as the developing solvent. Plates were visualized under  $UV_{254nm}$  light followed by charring with 10% H<sub>2</sub>SO<sub>4</sub> in ethanol. Characteristic TLC profiles were obtained for each location. The overall morphology of *P. elisabethae* can be variable, and chemical differences were not correlated to specific

morphs. We confirmed species identity of each colony by morphological and sclerite analysis and found no significant differences in sclerite dimensions among different colonies. Therefore, we ruled out the possibility that we were dealing with different species.

A portion of each crude extract was redissolved in methanol for additional purification. Methanol extracts of colony tissue are fractionated on the polymeric chromatographic support HP20. Dilation HP20 Poly(styrene-divinylbenzene) resin is used to create discrete fractions that can be examined using HPLC and NMR. The extract is pre-fractionated in three fractions, a polar, a medium-polar and a non-polar fraction. Most biologically active compounds need to be able to travel throughout a target organism's body in order to reach the intended sites of action. They therefore need to be soluble in both hydrophilic and hydrophobic environments (Zubay, 1998). Consequently a majority of biologically active compounds are neither extremely polar nor non-polar in character (possess intermediate polarity). We have found that by using the polymeric chromatographic resin Diaion HP-20 instead of traditional silica based supports (Silica gel, C18 etc.) medium-polar biologically active secondary metabolites such as terpenoids can be efficiently separated from water-soluble carbohydrates, salts, and non-polar fats and steroids that are found in high concentrations

We then employed HPLC analysis of the medium-polar fraction to obtain a characteristic chemical profile for each colony. HPLC profiles allowed us to confirm chemical differences among colonies of *P. elisabethae* collected from different sites. Chromatograms indicated five chemotypes (Figures 12, 13 and14), each with a distinct pattern of peaks with varying retention times. For simplicity we will refer to the

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chemotypes as follows, Burrows/San Salvador (BSS), Burrows/Little Bahama Bank Hybrid (BLH), Little Bahama Bank (LBB), Exuma Sound/Bimini (ESB) and Key Largo (KLP). Analysis of replicate samples from within each site showed consistent results, with the exception of the Burrows site, where we have identified two chemotypes. Although quantities of individual metabolites varied and some minor metabolites were absent in some cases, the general secondary metabolite profiles were consistent for each chemotype (Table 3). Quantification of pseudopterosins in crude extracts was performed by HPLC with UV detection, using a calibration curve generated with pure pseudopterosin A as a standard. All known pseudopterosins contain a similar chromophore facilitating their quantification by chromatographic methods using one standard.

The Bahamas Archipelago consists of vast carbonate banks (average depth  $\sim 3 \text{ m}$ ) with islands, deep channels, and deep-water basins. The collections sites were as follows. Cross Harbor, Long Rock, Gorda Cay, Sandy Point and AB102 are clustered along a  $\sim 20$  km stretch of the Little Bahamas Bank off the coast of Abaco Island at the northern end of the chain of Bahamian islands. Burrows is located at the northern end of Little Bahama Bank, separated from the other sites by  $\sim 20$  km. Little San Salvador, Cat Island and Hog Cay are in Exuma Sound. Exuma Sound and Little Bahamas Bank are separated by the Northeast Providence Channel. The Northeast Providence Channel  $\sim 4000 \text{ m}$  depth extends south to form the Tongue of the Ocean. The channel is about 40 km wide. The distance between the northern end of Cat Island and San Salvador is about 135 km. The open waters of the western North Atlantic Ocean lay in between. San Salvador Island lies





**Figure 12.** HPLC chromatograms of chemotypes of *P. elisabethae*-BSS chemotype: A = San Salvador, B = Burrows; BHY chemotype C= Burrows; PKL chemotype, L= Pickles Reef.








on a small, isolated bank at the eastern edge of the Bahamas. San Salvador represents both the eastern most and southern most site in our survey. Pickles Reef, off-shore of Key Largo, is separated from Bimini by the Straits of Florida channel, with a depth of about 800 m and a width of ~85 km. Bimini is on the northwest corner of Great Bahama Bank, and is separated from the sites along Little Bahama Bank by the Northwest Providence Channel.

The distribution of the five chemotypes across this geographic region does not lend it's self to an obvious interpretation. The sites clustered along the Little Bahama Bank display the same chemical profile (LBB), while Burrows, just ~20km north of the northern most site of this group has two chemotypes. BSS is distinctly different, and the BLH is essentially a hybrid of the BSS and LBB. It has previously been reported (Mukherjee, 2005; Kohl and Kerr, 2003) that *P. elisabethae* from Sweetings Cay, Bahamas are of the BSS chemotype. Sweetings Cay is ~ 10 km further north along the Little Bahama Bank from Burrows. San Salvador, the southern most site in our survey, is over 300 km from Burrows, yet colonies from these sites share the BSS chemotype. Colonies from the geographically disconnected sites in Exuma Sound and Bimini share the ESB chemotype. Additionally, *P elisabethae* from Pickles Reef off Key Largo, Florida produces the aglycone from which the pseudopterosins of the ESB chemotype are derived.

One of the central paradigms in the study of marine life history strategies is the relationship between the number of eggs/larvae produced, their size and mode of development (Thorson, 1950). A corollary to that relationship is that large lecithotrophic and brooded larvae do not disperse as widely as planktotrophs. That in turn, suggests

reduced connectivity and greater susceptibility to local extinctions among brooding species. Hughes et al. (1999) found differences in the patterns of spatial variance of brooding and broadcast spawning species, and Underwood and co-workers (2006) report that brooding by the coral *Seriatopora hystrix* has led to limited connectivity among Western Australia reefs. Sexual reproduction in *P. elisabethae* and *P. bipinnata* occurs via surface brooding. Fertilized (and possibly unfertilized) eggs are maintained in a mucus layer on the surface of the maternal colonies and develop there into competent planulae (Gutierrez-Rodriguez and Lasker, 2004). We might predict that *P. elisabethae* would have limited dispersal extending to zoogeographic barriers.



Figure 15. Map of the hydrography of the collections region.

The purified compounds were identified by UV, CD, MS, <sup>1</sup>H, and <sup>13</sup>C NMR spectroscopy (Appendix), and by comparison of our spectral data with those previously reported (Look et al., 1986; Look and Fenical, 1987; Roussis et al., 1990; Puyana et al., 2004). This research has yielded six previously undescribed pseudopterosin. All were

isolated from the samples collected at Cross Harbor, Long Rock, Gorda Cay, Sandy Point and AB102 (Table 1). Two novel variants of the previously reported pseudopterosin K were recovered. These differ from pseudopterosin K (**35**) only in the presence and location of an acetate group on the sugar moiety of the molecule. While the fucose of pseudopterosin K is not acetylated, the new ps K variants have an acetate group at carbon two (ps K 2' acetate; **36**) and carbon four (ps K 4' acetate **37**) of the sugar ring. Additionally, a series of novel pseudopterosins, the iso-pseudopterosins A-D were also isolated from samples collected at these sites. The iso-ps A-D series (**30-33**) are stereochemically identical to the previously described ps A-D (**17-20**) series (Look et al., 1986), but the glycoside linkage is at C-10 of the diterpene skeleton, not C-9 as on the ps A-D series (NMR data table A9).



Figure 16. Structures of pseudopterosins G-K, iso A-D and ps G-J aglycone.

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Distribution
1. Distribution

Chemical diversity in soft corals may have an adaptive value in mediating ecological interactions in reef environments (Sammarco and Coll, 1988). Several studies have demonstrated that interactions such as predation (Pawlik et al., 1987; Puglisi et al., 2000; Thornton and Kerr, 2002), competitive and allelopathic interactions (De Nys et al., 1991; Maida et al., 1993, 1995; Leone et al., 1995), and reproductive state (Coll et al., 1989, 1995) may influence the type and quantities of specific compounds produced by soft corals. When trying to understand chemical variability, the issue is complicated by the influence of ecological variables such as habitat characteristics and depth (Harvell et al., 1993; Roussis et al., 2000; Thornton and Kerr, 2002). At this point, we do not know the causes for the extreme chemical variability of *P. elisabethae* in the areas we surveyed. Thornton and Kerr (2002) found that predation by the ovulid gastropod Cyphoma gibbossum, or decreased levels of UV radiation had a significant effect on the concentration of pseudopterosins while simulated wounding or predation by the butterfly fish Chaetodon capistratus did not. Interestingly, the greatest increase of pseudopterosin concentration occurred in response to decreased UV radiation. The authors argue that pseudopterosin biosynthesis may involve, directly or indirectly, a photosynthetic organism (probably zooxanthellae). A great diversity of zooxanthellae has been recognized in hermatypic corals (Rowan and Knowlton, 1995; Toller et al., 2001). This diversity is closely related to environmental variables and can change in response to environmental stress. P. elisabethae harbors zooxanthellae (Rowan, 1988), and one would expect to find different symbionts on a wide geographical range (i.e., the Caribbean). A study done by Santos and co-workers (2003) used microsatellites to characterize the Symbiodinium sp. clade B populations hosted by the gorgonian *Pseudopterogorgia elisabethae* in the Bahamas. Many of the collection sites for their study are the same as those presented here. Twenty-three unique, two-locus genotypes were identified in association with these *P. elisabethae* colonies. Most colonies hosted only a single *Symbiodinium* sp. clade B genotype; however, in some instances (n=25), two genotypes were harbored simultaneously. For 10 of the 12 populations, 66–100% of the *P. elisabethae* colonies hosted the same symbiont genotype. Added to this, in 9 of the 12 populations, a *Symbiodinium* sp. clade B genotype was either unique to a population or found infrequently in other populations (Santos et al., 2003). These distinct symbiont genotypes do not correlate well with the chemotype data we have generated, and it seems highly unlikely that chemical variation is a result of symbiont variation.

Genetic analysis of the adult populations of *P. elisabethae* (Lasker, unpublished data) identified small but significant  $\theta$  and  $\rho$  values (estimators of  $F_{st}$  and  $R_{st}$ ) among the Little Bahama Bank (LBB) populations. According to this analysis, the data best fit a model of 4 populations, one being Little San Salvador, one Exuma Sound and Florida, and the other two distributed across the New Providence Channel from Bimini on the east to Southampton reef to the west. The New Providence Channel collections were made up of individuals whose genotypes are admixtures of 2 populations.

Interestingly, Lasker found that Hog Cay (HC) and Pickles Reef, Florida (FL) form a homogeneous population, and we found that the chemistry from these two geographically distant sites is also related, as the pseudopterosins from Exuma Sound and the pseudopterosin aglycone from Pickles reef are identical. Lasker concludes that that while the genetic analyses have identified clear structure between the New Providence Channel, San Salvador, Exuma Sound and Florida, the 5 loci currently in use do not have

the power to clearly assess differentiation among the Little Bahama Bank populations (Lasker, personal communication).

The intra-species chemical variation we have found in *P elisabethae* may likely represent phenotypic plasticity. Phenotypic plasticity describes the capacity of a genotype to exhibit a range of phenotypes in response to variation in the environment. Single genotypes can change their chemistry, physiology, development, morphology, or behavior or in response to environmental cues (Agrawal, 2001). Environmental variation encompasses both abiotic and biotic components of the environment, including interactions among organisms. The strength and outcome of many ecological interactions, ranging from antagonism to mutualism, are mediated through the phenotypically plastic responses of one or more players in the interaction (Fordyce, 2006). The conditions favouring phenotypic plasticity are certainly found on coral reefs, that is, sessile corals experience disruptive selection both in space and time (Bradshaw, 1965; Potts, 1984). The dispersal of larvae, with little motility and limited capacity for substrate selection, into the heterogeneous reef environment almost eliminates any chance of predicating what habitat offspring will settle in. Plasticity is important in the maintenance of natural populations, enhancing the survival and reproductive success by contributing to their ability to cope with environmental changes and to potentially adapt to new niches. Adaptive plasticity describes phenotypic responses to the environment that results in increased fitness for an organism, such as inducible defenses of plants in response to herbivore attack (Karban and Agrawal, 2002). Some phenotypic plasticity is clearly adaptive however, this is difficult to demonstrate empirically (Newman, 1992).

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Reciprocal transplant experiments of Todd and co-workers (2004) clearly demonstrated phenotypic plasticity in the morphology of two species of Indo-Pacific corals and there relation to environmental factors such as depth and sedimentation rates. If larval settlement in benthic habitats is random, the developing organism has the ability to generate the phenotype that conveys the greatest fitness for the local conditions. The phenotype is then acquired through development under the current environment and can be changed in the next generation, if conditions are modified (Todd et al., 2004).

In summary, the present study reveals a qualitative variation in the secondary metabolite content of *P. elisabethae* between collection sites. Although the potential adaptive nature of such variation is still unknown, it could well be in response to some yet undescribed and localized selection pressure. Higher resolution genetic data may lead to a closer correlation of chemotypes and population differences. *Pseudopterogorgia elisabethae* is currently commercially harvested for the pharmacologically active compounds the pseudopterosins, and information about chemical variation across geographic, depth and seasonal gradients may prove useful for future management decisions regarding their harvest. Future research in this area should examine between site variation in environmental parameters such as predation pressure, pathogen abundance, UV irradiance and temperature. Furthermore, one should take intraspecific variation into account when presenting a chemical fingerprint of a given species.

### **CHAPTER 3**

## Antioxidant potential of extracts and pure compounds from Pseudopterogorgia sps.

#### **3.1 Antioxidants and marine organisms**

One potential ecological role for the diverse and novel secondary compounds produced by gorgonians may be defense against oxidative stress. In a study done during the height of the 1998 mass bleaching event on the Great Barrier Reef, the octocoral families *Briareidae*, *Clavularidae*, *Gorgonidae*, and *Nephtheidae* exhibited low bleaching susceptibilities (Goulet et el., 2008). There was no indication that different *Symbiodinium* populations explained the occurrence of bleached or unbleached tissues. The authors of this study concluded that differential tolerance to stress among cnidarian species, irrespective of the *Symbiodinium* they host, may explain differences in bleaching.

In the Caribbean, octocorals are the most conspicuous reef macrofauna, with up to 40 species co-occurring in a single area (Sánchez et al., 2003). A striking difference between Caribbean octocorals and other zooxanthellate cnidarians is their seemingly greater resistance of bleaching. While there have been numerous reports of scleractinian coral bleaching throughout the world's tropical oceans, there have been far fewer observations of bleaching among octocorals (Lasker, 2003).

It has been shown that photosynthetic rates in zooxanthellae can be improved by the addition of exogenous antioxidants to coral exposed to artificially elevated light and temperature (Lesser, 1997). Antioxidants improve the rate of  $O_2$  evolution by scavenging free radicals within the dinoflagellate symbiont. Dinoflagellates can ameliorate this oxidative burden through the use of endogenous antioxidant enzymes and photoprotective mechanisms but these systems can be overwhelmed, especially by the synergistic effects of thermal and irradiance stress.

Seaweeds or their extracts have been studied as potential natural antioxidants during the last decade (Anggadiredia et al., 1997; Duval et al., 2000; Lim et al., 2002; Matsukawa et al., 1997; Ruberto et al., 2001; Ruperez et al., 2002; Tutour et al., 1998; Xue et al., 2001; Yan et al., 1998). Although most photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, they seldom suffer from any serious photodynamic damage in vivo. Relatively few attempts have been made to explore this vast resource of structurally unique chemistry for new antioxidant prototypes. Marine antioxidant research has largely focused on the antioxidant effects of crude extracts (Guzman et al., 2001). The previously characterized marine antioxidant substances are mainly chemicals that are structurally related to plant-derived antioxidants (Nakagawa et al., 1998; Son et al., 2002). These marine antioxidants include pigments such as chlorophylls (Yamamato et al., 1992; Wantanabe et al., 1993) and carotenoids (Schubert et al., 2006) and tocopherol derivatives such as vitamin E and related isoprenoids (Yamamato et al., 2001). Certain phenolic substances produced by marine algae (Fujimoto and Kaneda, 1984) have been shown to have antioxidant activity. UVabsorbing mycosporine- like amino acids found in marine microalgae and invertebrates have been shown to have antioxidant activity (reviewed in Klisch and Häder, 2008).

Griffin and Bhagooli (2004) showed that the ferric reducing antioxidant power (FRAP) assay could be a useful tool in determining antioxidant potential in corals. Their

results clearly demonstrated that when corals are exposed to thermal stress, an increase in enzymatic antioxidant concentrations can be measured using the FRAP assay. Other research on coral has shown an increase in individual enzymatic antioxidant concentrations (lipid peroxidase, MnSOD and Cu/ZnSOD) in corals after being subject to thermal stress (Dykens and Shick, 1982; Lesser, 1996; Downs et al., 2000 and Downs et al., 2002). The FRAP assay can provide information on total antioxidant potential in a relatively short time without having to run more lengthy tests for each individual antioxidant. It is demonstrated here how this assay could be a simple, reproducible and inexpensive tool for marine biologists involved in ecotoxicological studies.

## 3.2 Ferric reducing antioxidant power (FRAP) assay.

Benzie and Strain (1996) considered the antioxidant as any species that reduces the oxidizing species that would otherwise damage the substrates. And the authors further treat the "total antioxidant power" as the "total reducing power". The antioxidant activity is then interpreted as the reducing capability. Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay. FRAP uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form which has an intense blue color can be monitored by measiring the change in absorbance at 593nm. This reaction is non specific, in that any half reaction has lower redox potential, under reaction conditons, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II ) ion formation. The change in absorbance is therefore directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture. The relevant chemical reaction of the FRAP method involves a single electron reaction between Fe  $(TPTZ)_2$  (III) and a single electron donor ArOH (Prior et al., 2005)

$$Fe(TPTZ)_2(III) + ArOH \rightarrow Fe(TPTZ)_2(II) + ArOH^+$$

The FRAP assay can be performed on a wide range of complex biological fluids, including plasma, serum, saliva, tears, urine, cerebrospinal fluid, exudates, transudates, and aqueous and ethanolic extracts of drugs, foods, and plants, as well as on simple and heterogeneous solutions of pure antioxidants (Benzie and Strain, 1996).

A major advantage of the FRAP method is its ability to quantify the amounts of total antioxidants or reductants in extracts. The FRAP assay is also the only one to directly measure antioxidants or reductants in a sample. Other commonly employed assays are more indirect because they measure the inhibition of reactive species (free radicals) generated in the reaction mixture. These results are strongly dependant on the type of reactive species used (Guo et al., 2003). The FRAP assay, in contrast, uses antioxidants as reductants in a redox-linked colorimetric reaction. Moreover, the other assays (but not FRAP) use a lag phase type of measurement. In previous experiments this has been difficult to standardize and has generated different results between different laboratories. In the FRAP assay, pretreatment is not required, stoichiometric factors are constant and linearity is maintained over a wide range (Benzie and Stezo, 1999).

In order to streamline the analysis of large collections we have developed a smallscale standardized solid phase extraction (SPE) and HPLC procedure to allow the rapid fractionation of multiple samples and generate HPLC data that can used to prioritize samples for scale-up isolation. The crude fractionation of the extract is achieved using a solid phase extraction (SPE) 12-port vacuum manifold to allow fractionation of multiple samples using HP-20. The extract is fractionated in three fractions, a polar, a medium-polar and a non-polar fraction. The objective of this step is to remove a majority of the very polar salts and carbohydrates and very non-polar fats and steroids in the crude extract. These pre-fractionated "enriched" materials can then be examined using NMR for the presence of novel compounds or screened for biological activity.

Initial separation of methanolic extracts of *Pseudopterogorgia sps* material were partitioned into three discrete fractions of decreasing polarity using the reverse-phase HP-20 chromatographic method described above. We have taken these three discrete fractions (40%, 75% and 100% Acetone:H<sub>2</sub>O) and examined them for antioxidant potential using the FRAP assay. Samples of each fraction (100  $\mu$ g) were loaded in triplicate onto 96-well microtitre plates with FRAP reagent. Activity was determined per µg of sample using a linear calibration curve generated from FeSO<sub>4</sub>·7H<sub>2</sub>O standards of known concentrations. Aqueous solutions of known Fe(II) concentration (FeSO<sub>4</sub> ·7H20) were used for calibration of the FRAP assay. Reaction of Fe(II) represents a one electron exchange reaction and is taken as unity, i.e., the blank corrected signal given by 100  $\mu$ M solution of Fe(II) is equivalent to a FRAP value of 100 µM. Typical Fe(II) standard concentrations used in these experiments are in the range of 50-1000  $\mu$ M (Figure 17). Results from these initial experiments correlate with our assumption that the intermediately polar fractions (75%) contain the majority of the biologically active compounds (Figure 18).



Figure 17. An example of a standard curve generated using concentrations of  $FeSO_4$ 



Antioxidant Potential of Pseudopterogorgia sps extracts

**Figure 18.** FRAP analysis of polar (40%), medium-polar (75%) and non-polar (100%) fractions of the crude extract of seven species of the genus *Pseudopterogorgia*.

Vitamin E is a generic name for all naturally occurring tocopherol and tocotrienol derivatives, among which  $\alpha$ -tocopherol exhibits the highest antioxidant activity (Fryer,

1993). It is well recognized that vitamin E ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol) is an outstandingly effective antioxidant and can scavenge active free radicals (Mukai et al., 1988). The study of Satoh et al. (1997) claims that antioxidative efficacy of Trolox (**38**) surpasses that of  $\alpha$ -tocopherol, the most effective of the vitamin E complex. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a hydrophilic vitamin E analog lacking the phytyl tail, with enhanced antioxidant capacity due to its increased cell permeability. Trolox is a phenolic antioxidant which was originally designed as a food preservative due to its free radical trapping capability (Davies et al., 1988).

The 75% fraction of extracts from seven species of *Pseudopterogorgia* were screened using the FRAP assay in an attempt to make a systematic comparison of the antioxidant potential of their biologically available secondary metabolites. The assay was standardized using Trolox, and results were expressed as  $\mu$ M Trolox equivalents per  $\mu$ g of extract. The standard curve was linear between 5 and 100  $\mu$ M Trolox (Figure 19). Values are reported as  $\mu$ M of Trolox per  $\mu$ g of extract.

The experimental results were expressed as mean  $\pm$  standard error of mean (SEM) of four replicates. The data were subjected to one way analysis of variance (ANOVA; Table A2) and differences between samples were determined by Tukey's HSD (P< 0.01) test using the Graphpad Prism program (Table 4; Figure 20). Intra-species variation was not statistically significant (p<0.01, Tukey's HSD) except for *Pseudopterogorgia rigida*, which has two different chemical profile, and the Key Largo sample of *elisabethae* with significantly greater activity than any of the other extracts screened (Table 4; Figure 18). It is possible that the differences seen in activity within the *P. rigida* samples may in fact be a result of misidentification of these. The two *rigida* samples with activity like

*elisabethae* did in fact differ morphologically from most of the other *rigida* samples collected. They had a strong acetic acid- like smell and turned the plastic storage bag



**Figure 19.** Example of a standard curve generate using concentrations of Trolox from  $5-100 \mu$ M in a FRAP assay.

yellow. While sclerite analysis did not differentiate them from other *rigida* specimen, it is quite possible that they are a different species. In his 1961 monograph, *The Shallow-water Octocorallia of the West Indian Region*, F.M. Bayer wrote, "It has been shown in the foregoing pages that the characters upon which the classification of octocorals depends – *i.e.*, the ramification and general form of the entire colony, the size, shape and color of the spicules, and the development of the anthocoidial armature in the polyps – are subject to a degree of variation and intergradation that imposes an uncomfortably large element of doubt upon the resultant system." We have found several populations in the Bahamas in which numerous individuals do not readily fit the *Pseudopterogorgia* spp. descriptions of Bayer (1961).

**Table 4.** FRAP assay of the intermediate polarity (75%) fraction of seven species of *Pseudopterogorgia*. Values given are  $\mu$ M of Trolox /  $\mu$ g extract, n=4 ± standard deviation. Different letters indicate significantly different means (*P* <0.01), Tukey's HSD.

		1
species	collection site	μM/μg equiv
acerosa <sup>a</sup>	Cross Harbor	$0.29 \pm 0.07$
acerosa <sup>a</sup>	Sandy Point	$0.12 \pm 0.03$
<i>bipinnata<sup>b</sup></i>	Cross Harbor (60ft)	$1.34 \pm 0.12$
bipinnata <sup>b,f</sup>	Cross Harbor (80 ft)	$0.93 \pm 0.04$
elisabethae <sup>c</sup>	Litlle San Salvador	$1.92 \pm 0.19$
elisabethae <sup>c</sup>	Bimini	$1.88 \pm 0.23$
elisabethae <sup>c</sup>	Cross Harbor	$1.90 \pm 0.27$
elisabethae <sup>c</sup>	Burrows (55 ft)	$1.95 \pm 0.23$
elisabethae <sup>c</sup>	Long Rock	$1.51 \pm 0.18$
elisabethae <sup>c</sup>	San Salvador	$1.62 \pm 0.15$
<i>elisabethae</i> <sup>c</sup>	Cat Island	$1.44 \pm 0.08$
<i>elisabethae</i> <sup>c</sup>	Hog Cay	$1.42 \pm 0.10$
elisabethae <sup>d</sup>	Pickles (KL)	$2.13 \pm 0.10$
elisabethae <sup>c</sup>	Cross Harbor	$1.89 \pm 0.14$
<i>hystrix</i> <sup>a</sup>	Cross Harbor	$0.22 \pm 0.05$
<i>hystrix</i> <sup>a</sup>	Cross Harbor	$0.23 \pm 0.05$
<i>hystrix</i> <sup>a</sup>	Cross Harbor	$0.21 \pm 0.01$
kallos <sup>a</sup>	AB102	$0.34 \pm 0.03$
kallos <sup>a,f</sup>	AB102	$0.49 \pm 0.05$
rigida <sup>c</sup>	Long Rock	$1.77 \pm 0.13$
rigida <sup>f</sup>	Long Rock	$0.61 \pm 0.07$
rigida <sup>f</sup>	Sandy Point	$0.61 \pm 0.09$
rigida <sup>c</sup>	Sandy Point	$1.61 \pm 0.08$
$sp^{a}$	Burrows (20 ft)	$0.23 \pm 0.03$



**Figure 20.** FRAP assay of 75% fractions of seven species of *Pseudopterogorgia*. Values represent  $\mu$ M Trolox/ $\mu$ g extract. Mean of n=4 ± standard deviation. Different letters indicate significantly different means (*P* < 0.01), Tukey's HSD.

There is a lot of inter-species variation. The least and the most potent extracts show a nearly twenty-fold difference in activity. *P. hystrix*, which had the lowest overall activity, is noted by Bayer (1961) to be a deep water specialist, and we have only observed this species at the deepest site in the region, a ridge along a steep wall that begins at approximately 28 m. Additionally, unlike the extracts of all the other species in genus, no terpenoid chemistry was apparent in the <sup>1</sup>H-NMR analysis of the 75% fraction of *P. hystrix*. Interestingly, extracts of *P. acerosa* and *P. kallos*, two of the most common species on the shallowest reefs in our survey (between 5 and 10 m), had activity that did not differ significantly from that of the *hystrix* extract.

To further investigate the antioxidant potential of these extracts, they were subjected to HPLC analysis. Briefly, 5 mg of each 75% fraction was subjected to semipreparative HPLC separation on a polymeric PRP-1 column. We are using an evaporative light scattering detector (ELSD) to estimate the quantity of compounds present in the fraction. In addition to HPLC data the medium polar fraction is also subjected to <sup>1</sup>H NMR and <sup>1</sup>H-<sup>1</sup>H COSY to allow the identification of compound classes present in the fraction. The HPLC is equipped with a fraction collector, which was program to collect everything eluted from the column into 96-well plates. These plates were then dried *in vacuo* and reconstituted with 200  $\mu$ L of 100% MeOH per well, 10% of which was transferred to 96-well microtitre plates with FRAP reagent and assayed for activity. In this fashion, activity can be correlated with chromatographic peaks, and active components can be rapidly identified for further study (Figure 21, 22). Activity for this assay is expressed as FeSO<sub>4</sub>  $\mu$ M equivalents per well.



**Figure 21.** comparison of chromatograms and graphical representations of FRAP analysis of 96-well plate collections of HPLC separation of 75% fraction.



**Figure 22.** FRAP assay of 96-well plate collection from HPLC of 75% fraction. For each of four of the chemotypes one chromatogram, corresponding FRAP plate and graph of FRAP values for each well. FRAP values expressed in  $\mu$ M equivalent FeSO<sub>4</sub>.

For each of the fractions analyzed in this way, FRAP activity was correlated to specific peaks on the chromatogram. In some cases, the identity of the active compounds could be assumed based on retention times, research previously done by this investigator (<sup>1</sup>H-NMR of 75% fraction) and previously published data. In the case of *P. rigida*, the active compound is the previously characterized sesquiterpene (-)-curcuphenol (McEnroe and Fenical, 1978). The active compounds from the 75% fraction of *P. bipinnata* are kallolide A and its acetate and (Figure 6; structures 14, 15) and the bipinnatins, (figure 5; structure 10-13). It is important to note Sanchez and co-workers have shown, using ITS1-5.8S-ITS2 phylogenetic analysis, evidence for considering the species P. kallos (Bielschowsky) as the shallow-most morphotype of P. bipinnata from exposed environments (Sánchez et al., 2007). Additionally the presence of the same metabolites within these organisms supports the theory that P. bipinnata and P. kallos belong to the same species. Yet, the activity of the 75% fractions from specimen of these two species have significantly different activity (p< 0.01, Tukey's HSD; Figure 16), and we have found that the concentration of active secondary metabolites is considerably greater in bipinnata (Data not shown).

The 75% fractions of *P. elisabethae* had significantly more antioxidant potential than extracts of the other species in the genus (with the exception of *P. rigida*) and since the activity in the fractions of *P. elisabethae* was localized to peaks corresponding to the pseudopterosins, these were further purified for additional screening.

Pseudopterosins were purified by chromatographic separation as follows. Ten to 30 mg of the 75% fraction was injected on a Beckman HPLC with a preparative scale C-18 column and eluted with a linear gradient of acetonitrile and water (70:30 ACN:  $H_2O$ 

to 100% ACN). The identity and purity of each compound was confirmed by UV, CD, MS, <sup>1</sup>H, and <sup>13</sup>C NMR spectroscopy (Appendix), and by comparison of our spectral data with those previously reported (Look et al., 1986; Look and Fenical, 1987; Roussis et al., 1990; Puyana et al., 2004).

All compounds were assayed in quadruplicate at a concentration of  $10\mu$ M. Values reported are  $\mu$ M Trolox per  $\mu$ M of pseudopterosin. The experimental results are expressed as mean  $\pm$  standard error of mean (SEM) of four replicates. The data were subjected to one way analysis of variance (ANOVA, table A3) and differences between samples were determined by Tukey's HSD (P< 0.01) test using GraphPad Prism (Table 5; Figure 23).

**Table 5.** FRAP assay of pure pseudopterosins and related molecules isolated from *Pseudopterogorgia elisabethae* Values given are  $\mu$ M of Trolox /  $\mu$ g extract, n=4 ± standard deviation. Different letters indicate significantly different means (*P* <0.01), Tukey's HSD.

pseudopterosin A <sup>a</sup>	$1.056 \pm 0.090$
pseudopterosin B <sup>a</sup>	$0.994 \pm 0.022$
pseudopterosin C <sup>a</sup>	$1.080 \pm 0.023$
pseudopterosin D <sup>a</sup>	$1.038 \pm 0.011$
ps A-D aglycone <sup>b</sup>	$0.698 \pm 0.046$
ps G <sup>a,d</sup>	$1.167 \pm 0.017$
ps I <sup>a,d</sup>	$1.190 \pm 0.044$
ps J <sup>a,d</sup>	$1.170 \pm 0.054$
ps K <sup>a</sup>	$1.123 \pm 0.046$
ps K 2' acetate <sup>a</sup>	$0.984 \pm 0.036$
ps K 4' acetate <sup>a</sup>	$1.065 \pm 0.028$
iso-A <sup>a</sup>	$0.941 \pm 0.044$
iso-B/iso-C <sup>a,d</sup>	$1.118 \pm 0.052$
iso-D <sup>b</sup>	$0.595 \pm 0.023$
ps G-J aglycone <sup>d</sup>	$1.311 \pm 0.058$



**Figure 23.** FRAP assay pseudopterosins and related molecules from *Pseudopterogorgia elisabethae* throughout the study region. Values represent  $\mu$ M Trolox/ $\mu$ M pseudopterosin. Mean of n=4 ± standard deviation. Different letters indicate significantly different means (*P* <0.01), Tukey's HSD.

Antioxidant activity did not vary widely among the suite of pseudopterosins tested. For twelve of the fifteen compounds tested, antioxidant potential appears to be equal to or greater than that of Trolox on a  $\mu$ M/ $\mu$ M basis. Slight, but statistically significant (Tukey's HSD, P < 0.01) differences were observed between the ps G-J series and the other pseudopterosins, and the aglycone of this series has the best activity. The ps A-D aglycone and iso-D displayed significantly less activity than the other compounds, but when the ps A-D aglycone was re-evaluted by <sup>1</sup>H-NMR, it was evident that the molecule had decomposed to an ~ 50:50 mix of the aglycone and a di-ketone derivative. We would expect that the conversion of the catechol to the di-ketone moiety would cause

the loss of antioxidant function. It is not clear why iso-D was significantly less active than the other compounds, though it is not unlikely that this difference may be the result of a quantitaion or experimental error.

Phenolics are diverse secondary metabolites abundant in plant tissues (reviewed in Grace and Logan, 2000). Polyphenols possess ideal structural chemistry for free radical scavenging activity and have been shown to be more effective antioxidants *in* vitro than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as Hydrogen or electron donors, and from the ability of the polyphenol-derived radical to perform the chain-breaking function of stabilizing and delocalizing the unpaired electron (Blokhina et al., 2003). Trolox (**38**; Figure 24) is a potent water-soluble antioxidant in biological fluids. It scavenges reactive oxygen and may, consequently, prevent oxidative damages to important biological macromolecules such as DNA, lipids and proteins (Satoh et al., 1997).



**Figure 24.** Structure of Trolox, a synthetic vitamin E analog with potent antioxidant activity.

Phenolic diterpenes from sage (*Salvia officinalis* L.) such as carnosic acid (**39**) and carnosol (**40**; Figure 25) have been shown to possess antioxidant and antiinflammatory activities. Carnosic acid is a diterpene that displays high antioxidant activity and which protects biological membranes from lipid peroxidation (Aruoma et al., 1992; Haraguchi et al., 1995). The antioxidant activity of carnosic acid is due to the two hydroxyl groups found in *ortho*-position at  $C_{11}$  and  $C_{12}$  of the molecule (Aruoma et al., 1992). The same mechanism of antioxidative protection by carnosic acid applies in rosemary and sage. Further more, it has been demonstrated that it is a complete set of biosynthetically related antioxidant molecules (and not a single mechanism) that is responsible for alleviating oxidative stress and reducing oxidative damage in species of the plant genera *Rosmarinus, Lepechinia,* and *Salvia* (Munné-Bosch and Alegre., 2001).



**Figure 25.** Structure of carnosic acid and carnosol, phenolic diterpenes from terrestrial plants with potent antioxidant activity

As a protective response against damage from excessive UV-B radiation, plants enhance the biosynthesis of UV-B absorbing and antioxidant phenolic compounds (Körner, 1999). The induction of enzymes involved in flavonoid biosynthesis with experimentally supplemented UV radiation has been demonstrated (Jaakola and Määttä-Riihinen, 2004). Previous studies on altitudinal variation of phenolic compounds in different wild and cultivated taxa from the Asteraceae suggested that factors related to altitude of the growing site are causing significant shifts in the quantity of secondary metabolites in flowering heads (Zidorn and Stuppner 2001; Zidorn et al. 2005; Spitaler et al. 2006). Spitaler and coworkers (2008) found an increase of caffeic acid derivatives in flowering heads of A. montana in response to environmental factors related to the altitude of the growing site. It is well established that enhanced UV-B radiation indirectly causes damage to plants by inducing the formation of free radicals, which subsequently target lipids, proteins, carbohydrates, and nucleic acids (Blokhina et al., 2003). It was also demonstrated that phenolic compounds play a vital role in the hydrogen peroxide scavenging system of plants, which besides phenolics comprises peroxidase, ascorbic acid, and glutathione (Takahama and Oniki, 1997).

Many studies have examined the pharmacological potential of the pseudopterosins. They display strong anti-inflammatory and analgesic activity. These diterpene glycosides had potencies superior to those of existing drugs such as indomethacin in mouse ear models (Look et al., 1986). These compounds appear to act by a novel mechanism of action; they are not active against PLA<sub>2</sub>, cyclooxygenase, and cytokine release, or as regulators of adhesion molecules. Evidence suggests the pseudopterosins block eicosanoid release rather than biosynthesis in murine macrophages. Purified pseudopterosins (pseudopterosin E) have been commercialized for use in cosmetics (Bruckner, 2002). They have also be show to exhibit antituberculosis activity. Typical values for growth inhibition of *Mycobacterium tuberculosis* range

between 20 and 60%. However, some of these marine diterpenoids show a very high growth inhibition rate (Rodríguez et al., 2000). In biological screenings pseudopterosins and related molecules show in vitro cancer cell cytotoxicity in the NCI's 60 cell-line tumor panel (Rodríguez et al., 1999; Rodríguez et al., 2000).

Considerably less is know about the ecological roles of these compounds. Thorton and Kerr (2002) found significantly higher concentrations of pseudopterosins in exposed to experimentally elevated levels of feeding by the mollusk Cyphoma gibbosum indicating the inducibility of pseudopterosin biosynthesis by predation. Similar results to C. gibbosum predation were obtained in response to decreased levels of UV/VIS radiation (Thorton and Kerr, 2002). It has been shown that pseudopterosins A-D (17-20) deter feeding by a natural assemblage of reef fishes, but that the pseudopterosin A-D aglycone (26) acts as a feeding attractant (Mukherjee, 2005). Ferns and co-workers (2005) have suggested that the aglycone may be the final product of the pseudopterosin biosynthetic pathway. The results presented here indicate that ps G-J aglycone has the highest anti-oxidant potential. It is quite possible that these molecules serve both as predator deterrence (in the glycosylated form), and as antioxidants (both glycosylated and the more active aglycone form). Optimization of defenses can lead to multiple roles for secondary metabolites. If costs of synthesizing and maintaining chemical defenses are high then utilizing a compound for multiple purposes should increase the overall fitness of an organism relative to producing several compounds for several purposes. The potent and varied biological activities of marine plant and animal extracts suggest that some compounds may fulfill several functions for their host organisms. However, demonstration that compounds are involved in multiple interactions requires that we know the identity of compounds, and that we test purified compounds rather than whole organisms or crude extracts. Therefore, rarely have discrete metabolites been tested for their capacity to fulfill multiple ecological roles.

In a study done by Thorton and Kerr (2002) he blocking of UV/visible radiation increased the pseudopterosin content by approximately 100% in the treatment groups. While the authors remark that ecological significance of this is not immediately clear, they imply that it suggests the involvement, either directly or indirectly, of a photosynthetic organism in pseudopterosin biosynthesis. If the pseudopterosins are being used as antioxidants in what would probably be a non-recycled fashion, we would not be surprised to find lower concentrations of these in the tissues of organisms exposed to higher light regimes.

The work presented in this chapter represents the first time secondary metabolites from octocorals have been examined for antioxidant potential. The results strongly suggest that secondary metabolites from organisms in the genus *Pseudopterogorgia* may serve as antioxidants. Goulet and co-workers (2008) assessed the relative sensitivities of zooxanthellate octocorals to thermal stress across numerous reefs in the central GBR system during the 1998 sea surface heating event. Video transects showed that across all taxa, on average 43% of inshore colonies and 21% of offshore colonies, were partially or totally bleached (Goulet et al., 2008). The percentage of bleached colonies differed substantially among taxa during the height of the bleaching event. For example at the height of the bleaching event <10% of colonies of *Briareum* spp., *Rhytisma fulvum*, *Lemnalia* spp., and *Paralemnalia* spp. were partially or totally bleached. Samples of bleached and unbleached tissues were collected for genetic analyses of resident *Symbiodinium* populations, and symbiont genetic identity was not found to be the cause of variability in bleaching response within and between soft coral species. The authors of the study conclude that differential tolerance to stress among octocoral species irrespective of the *Symbiodinium* they host, may explain differences in bleaching (Goulet et al., 2008). Soft corals of the genera *Lemnalia, Paralemnalia*, and *Rhytisma* are a rich source of sesquiterpenoids, and the genus *Briareum* is a prolific source of diterpenoids. Perhaps it is the isoprenoid chemistry that confers the organisms in these genera a reduce susceptibility to bleaching. Results presented here support this hypothesis as they provide a potential mechanism to effect this variability; differing secondary metabolite chemistry. If the increasing incidence of bleaching events continues, the relative insensitivity of octocorals to thermal stress may have important consequences for the future of reef ecosystems.

#### **CHAPTER 4**

# *In vivo* antioxidant activity of extracts and pure compounds from *Pseudopterogorgia* <u>sps.</u>

### 4.1 Photosynthesis and oxidative stress

Corals containing symbiotic dinoflagellates (zooxanthellae) of the genus Symbiodinium are dependent upon light energy to power photosynthesis. The photochemical apparatus within zooxanthellae is similar to those found in other oxygenevolving photosynthetic algae and plants. There are two photosystems involved, called Photosystem I (PS I) and Photosystem II (PS II). PS II is associated with the splitting of water molecules to form molecular oxygen. PS I involves transfer of energy to the Calvin Cycle, where inorganic carbon is converted to simple sugar. Photosynthesis begins with Photosystem II. If PS II fails, the electron flow from PS II to PS I stops, and the entire photosynthetic process grinds to a halt. Light-absorbing pigments called photopigments harvest light energy. Chlorophylls (chlorophyll a and chlorophyll  $c^2$ ) are the most recognized photopigments of zooxanthellae, but others are involved as well, and these are called accessory (or antennae) pigments. In corals, accessory pigments include peridinin and beta-carotene. Chlorophylls and accessory pigments can channel the energy they harvest to both photosystems, although it is possible for an accessory pigment (such as beta-carotene, peridinin, etc.) to transfer its collected energy almost exclusively to one photosystem.

A small proportion of excitation energy is dissipated by the emission of fluorescence, stemming almost exclusively from chlorophyll a of photosystem II. Fluorescence emission competes with two other de-excitation processes that deactivate the excited chlorophyll states. These processes reduce (or quench) the amount of fluorescence, and are referred to as photochemical quenching  $(q_P)$  and nonphotochemical quenching  $(q_N)$ . Photochemical quenching reflects useful photochemistry (i.e. assimilatory or non-assimilatory electron flow), and depends upon the presence of oxidized  $Q_a$  (a quinone-type primary electron acceptor in photosystem II). When  $Q_a$  is oxidized, it can accept electrons from the photosystem II reaction centre, and pass these along the photosynthetic electron transport chain. The result is the oxidation of water, oxygen evolution, the reduction of NADP<sup>+</sup> to NADPH, membrane proton transport, ATP synthesis and eventually to the reduction of CO<sub>2</sub> to carbohydrate in the dark reactions of photosynthesis (Calvin cycle). The other means by which excited chlorophyll states can be deactivated is non-photochemical quenching, which reflects photoprotective dissipation of excess absorbed energy as heat in the light-harvesting antennae (Demmig-Adams, 1990; Horton and Ruban, 1994).

Active oxygen species are produced by a variety of metabolic processes; however the major source of active oxygen species in plant tissues is the photosynthetic electron transport system (Asada 1994). In photosynthesis, molecular oxygen is produced from the oxidation of water by the photosynthetic electron transport chain (Foyer *et al.* 1994). Oxygen can also be used as an electron acceptor, a reaction that results in the formation of singlet oxygen ( $^{1}O_{2}$ ) and superoxide radicals ( $O^{2-}$ ) (Fridovich 1986; Foyer *et al.* 1994). Singlet oxygen is highly destructive as it reacts with most biological molecules (Knox and Dodge 1985). Superoxide is also highly toxic with several cellular targets, however it's primary impact is an indirect one, as it gives rise to more powerful oxidants such as hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl ion (HO) (Fridovich, 1986). The principal sites of damage from these toxic oxygen species include the primary carboxylating enzyme, Rubisco, and Photosystem II, in particular, the D1 protein of PSII, which appears to have very little protection against oxidative damage (Asada and Takahashi 1987; Richter et al., 1990; Tschiersch and Ohmann, 1993). Damage to the D1 protein caused by reactive oxygen is irreversible, and can be corrected only by the enhanced synthesis and replacement of the protein (Long et al., 1994).

Symbiotic cnidarians and their zooxanthellae do produce hydroxyl radicals upon illumination (Dykens et al. 1992). Increased activities of antioxidant enzymes in the *Symbiodinium* sp. appears to be insufficient to scavenge the increase in the cellular flux of superoxide radicals and hydrogen peroxide produced during exposure to UV radiation and elevated temperatures (Lesser, 1996). Additionally, Lesser (1997) has shown that antioxidants prevent the coral from bleaching and affect the mechanism of symbiont loss from the coral host.

### 4.2 Detection of intracellular ROS using 2',7'-dichlorodihydrofluorescein diacetate

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 light 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 (Dunlap and Walter, 2003). Researchers have recently begun to look at the antioxidant effects of natural products in living systems. In a recent study, Takamatsu and coworkers (2003) investigated antioxidant activity of over one hundred purified marine natural product compounds using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) cell-based method to directly examine the ability of natural products to penetrate living human carcinoma cells (HL-60) and inhibit reactive oxygen species (ROS)-catalyzed oxidation. Their results clearly demonstrate the potential of marine natural products to act as potent antioxidants.

Screening of extracts and pure compounds of seven species of the genus *Pseudopterogorgia* using the FRAP assay (as described in Chapter 3) indicate that these have promising antioxidant potential. 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. To examine the potential ecological significance of these results, two assays were developed employing cultured *Symbiodinium* spp cells.

The *in vivo* antioxidant activity of the intermediately polar fractions of seven species of the genus *Pseudopterogorgia* was tested in a zooxanthellae based assays. This was accomplished using cultured zooxanthellae and the oxidative stress sensitive dye
DCFH-DA in a micro-well plate. DCFH-DA diffuses into the cell where cellular esterases cleave the diacetate moiety to form the more polar DCFH, which is trapped within the cell. Oxygen radicals oxidize the intracellular DCFH to the fluorescent DCF. The level of fluorescence measured upon excitation is proportional to the level of oxidation. Antioxidants prevent oxidation of DCFH and reduce the formation of DCF.

Fluorescent technology has made it possible to evaluate antioxidants in living cells using specific probes such as 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This cell-based fluorescent method is useful to directly examine the ability of natural products to penetrate cell membranes and inhibit ROS. Due to the indiscriminate nature of DCFH, which can be oxidized by various ROS and not just  $H_2O_2$ , the increase of intracellular DCF fluorescence does not necessarily reflect the levels of ROS directly, but rather an overall oxidative stress index (OS) in cells (Wang and Joseph, 1999). Quantifying cellular OS by the DCF assay using a fluorescent microplate reader is an easy and efficient method with low variability which can be used to quantify the potency of pro-oxidants or can be adapted to evaluate the efficacy of antioxidants against ROS in various cell lines (Wang and Joseph, 1999).

The formation of the fluorescent DCF from DCFH involves a complex process that occurs in close interaction with the superoxide radical and hydrogen peroxide. These two species react to form hydroxyl radicals in the Fe-catalysed Fenton (Haber-Weiss) reaction. DCFH reacts with hydroxyl radicals to for the DCF radical that reacts with oxygen and produces the green-fluorescent DCF (Zu et al., 1994). Glutathione suppresses DCF formation as it reconstitues the DCF radical into DCFH (Zu et al., 1996), and antioxidants such as mannitol scavenge active oxygen species in competition with DCFH (Shen et al., 1997). Trolox works in a similar fashion as mannitol.

An experiment was designed for the assessment of active oxygen production and the evaluation of the antioxidant activity of the extracts and pure compounds from Pseudopterogorgia sps. Clade C2 Symbiodinium culture from the lab of Gregory Schmidt, Department of Plant Biology, University of Georgia were used in this assay. We chose this particular culture because results of a previous study suggest that they are relatively more sensitive to the synergistic effects of thermal and irradiance stress than other clades (Berkelmans and van Oppen, 2006). Zooxanthellae cells were incubated in 10 µM DCFA-DA probe for 30 min with constant agitation. Excess probe was than washed away and the cells were resuspended in seawater. A total of 190 µL cell suspension was added to each well of black-bottom 96-well microtiter plates. Extracts (50 µg each dissolved in methanol) were added to wells in quadruplicate, control wells had either methanol only or cells incubated with the probe only. The plates were maintain in a water bath at 32°C and exposed to 180 µM of light. A control plate with the same volume of DCFH-DA treated cells per well as the experimental plates was maintained at room temperature in ambient light. Fluorescent intensity was recorded using a monochromatic spectrophotometer set at excitation wavelength 485 and emission wavelength 538. The level of fluorescence measured upon excitation is proportional to the level of oxidation. Antioxidants prevent oxidation of DCFH and reduce the formation of DCF. Readings were taken once an hour for four hours, and plates were dark acclimated for twenty minutes prior to each reading. Fluorescence in control cells that were exposed to the experimental thermal and irradiance stress was 6.63 times higher on average than those that were maintained at  $26^{\circ}$  C in ambient light ( $60\mu$ M). This serves to validate that the method employed to induce oxidative stress in the zooxanthellae was successful.

Raw data was converted to percent inhibition versus the solvent only control cells using the following equation:

 $[(F_{c} - F_{t}) / F_{c}] \ge 100$ 

Were  $F_c$  is the average of the solvent only control wells and  $F_t$  is the average for a given treatment (extract or compound). It is important to note that the addition of the solvent (MeOH) did cause a slight but not statistically significant reduction in fluorescence, thus the solvent only controls have been used to calculate the percent reduction.

**Table 6.** DCFH-DA assay of the intermediate polarity (75%) fraction of seven species of *Pseudopterogorgia*. Values given are % reduction in DCF fluorescence versus MeOH control,  $n=4 \pm$  standard deviation. Different letters indicate significantly different means (*P* <0.01), Tukey's HSD.

<i>acerosa</i> Bu <sup>a</sup>	5.28 ± 3.93	
acerosa SP <sup>a</sup>	4.41 ± 3.57	
<i>bipinnata</i> CH <sup>b</sup>	21.26 ± 2.96	
<i>bipinnata</i> CH <sup>b</sup>	19.99 ± 3.51	
<i>elisabethae</i> Bi <sup>b,c</sup>	31.51 ± 1.48	
<i>elisabethae</i> Bu <sup>b,c</sup>	28.36 ± 2.08	
<i>elisabethae</i> CH <sup>b,c</sup>	25.69 ± 3.33	
<i>elisabethae</i> KL <sup>c</sup>	34.15 ± 1.70	
<i>elisabethae</i> LR <sup>b,c</sup>	26.24 ± 4.43	
elisabethae SP <sup>b,c</sup>	27.02 ± 2.88	
<i>elisabethae</i> SS <sup>b,c</sup>	28.62 ± 2.12	
hystrix CH <sup>a</sup>	5.73 ± 1.55	
kallos SP <sup>a,d</sup>	11.56 ± 1.87	
<i>rigida</i> CH <sup>a,d</sup>	15.55 ± 2.77	
<i>rigida</i> LR <sup>c</sup>	30.94 ± 2.86	
sp CH <sup>a</sup>	7.15 ± 2.08	

#### **DCFH-DA zooxanthellae assay**



**Figure 26.** Results of the zooxanthellae-based DCFH-DA assay given as percent fluorescence inhibition versus control cells (MeOH only).  $n=4 \pm$  standard deviation. Different letters indicate significantly different means (P < 0.01), Tukey's HSD.

The experimental results were expressed as mean  $\pm$  standard error of mean (SEM) of four replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Tukey's HSD (P< 0.01) test using the Graphpad Prism program (Table 6; Figure 26). Means were significantly different (ANOVA P< 0.05; see Appendix). Intra-species variation was not statistically significant (p<0.01, Tukey's HSD) except for *Pseudopterogorgia rigida*, which has two different chemical profiles, one of which was as active as the *P elisabethae* fractions. The overall activity in this assay correlates well with the results of the FRAP assay presented in

Chapter 3. We can conclude that extracts not only exhibit antioxidant potential in a solution-based assay but can also be taken up by living cells and maintain their activity.

Pure pseudopterosins were screened in this assay following the same protocol as detailed above, except each of the compounds were added to the wells so that the final concentration of each was 20  $\mu$ M. Trolox was used as a positive control, also at a 20 $\mu$ M final concentration. The data were subjected to one way analysis of variance (ANOVA, means significantly different, p<0.05; see Appendix) and differences between samples were determined by Tukey's HSD (P< 0.01) test using GraphPad Prism (Table 7; Figure 27). Antioxidant activity did not vary widely among the suite of pseudopterosins tested. For ten of the fifteen compounds tested, activity appears to be equal to that of Trolox at the same concentration. Ps A-D aglycone displayed significantly less activity than the

**Table 7.** FRAP assay of pure pseudopterosins and related molecules isolated from *Pseudopterogorgia elisabethae*, given as percent fluorescence inhibition versus control cells (MeOH only)  $n=4 \pm$  standard deviation. Different letters indicate significantly different means (P < 0.01), Tukey's HSD.

pseudopterosin A <sup>a,b</sup>	44.951 ± 2.284
pseudopterosin B <sup>a,c</sup>	47.454 ± 0.916
pseudopterosin C <sup>a,c</sup>	47.793 ± 0.682
pseudopterosin D <sup>a,b</sup>	44.538 ± 1.626
ps A-D aglycone <sup>b</sup>	38.340 ± 3.495
pseudopterosin G <sup>a,c</sup>	46.460 ± 1.400
pseudopterosin I <sup>a,b</sup>	44.801 ± 0.791
pseudopterosin J <sup>a,b</sup>	45.026 ± 3.262
pseudopterosin K <sup>a,c</sup>	45.928 ± 1.218
pseudopterosin K 2' acetate <sup>a,c</sup>	48.082 ± 1.673
pseudopterosin K 4' acetate <sup>a,c</sup>	48.136 ± 3.130
iso-A <sup>a,c</sup>	45.460 ± 2.030
iso-B/iso-C <sup>a,c</sup>	45.891 ± 2.095
iso D <sup>a,c</sup>	46.878 ± 0.972
ps g-J aglycone <sup>a,c</sup>	51.451 ± 0.614
	52.686 ± 2.287

positive control and ten of the fourteen other compounds, but when the ps A-D aglycone was re-evaluated by <sup>1</sup>H-NMR, it was evident that the molecule had decomposed to an  $\sim$  50:50 mix of the aglycone and a di-ketone derivative. We would expect that the conversion of the catechol to the di-ketone moiety would cause the loss of antioxidant function. It appears evident from the results of this experiment that at least some portion of the bioactive components from these intermediately polar fractions is able to penetrate the *Symbiodinium* sp cells. Additionally, these fractions of the extracts from the seven species of *Pseudopterogorgia* and pure pseudopterosins reduce zooxanthellar intracellular OS as evidence by the reduction in DCF fluorescence. The pseudopterosins appear to be equally as efficient in reducing OS in zooxanthellae as Trolox at the 20 $\mu$ M concentration.



**Figure 27.** Results of the zooxanthellae-based DCFH-DA assay given as percent fluorescence inhibition versus control cells (MeOH only). N=3  $\pm$  standard deviation. Different letters indicate significantly different means (P < 0.01), Tukey's HSD.

Oxidative stress within the host tissues can occur as a consequence of oxidative stress in the algal symbiont because hydrogen peroxide can diffuse through biological membranes into host tissues (Asada and Takahashi 1987). It is known that Trolox exerts its antioxidant effect through its capacity to scavenge free radicals, but it is not possible to conclude that this is the nature of the activity of the pseudopterosins *in vivo*. In any case, it is clear that the antioxidant activity of the secondary metabolites of species from the genus *Pseudopterogorgia* would have adaptive value, particularly given the increasing incidence of El Niño/Southern Oscillation events and associated increase in prevalence and severity of coral bleaching.

# 4.3 Detection of photoinhibition using PAM fluorometry

Photoinhibition has been defined as light-dependent inhibition of photosynthesis (Powles, 1984; Greer and Laing, 1991). It can be described as the decreased capacity of a photosystem to capture and process photons (Long et al., 1994; Osmond, 1994), and is characterized by the accumulation of photochemically inactive PSII reaction centers (Krause, 1994). This typically results in a decrease in the overall photosynthetic rate (Richter et al., 1990). Photoinhibition is one of the daily challenges faced by most photosynthetic organisms (Hoegh-Guldberg and Jones, 1999). Photoinhibition occurs when the utilization of energy by the fixation of  $CO_2$  is exceeded by the amount of incoming light energy (Greer and Laing, 1991). Reduction in photosynthetic electron transport combined with continued high absorption of excitation energy leads to inactivation of, or damage to, PSII from the production of toxic oxygen species (Osmond, 1994; Lesser, 1996). It has been proposed that the reduction of PSII efficiency may act as

a protective mechanism to prevent damage to the photosynthetic apparatus, rather than indicating the degradation of the D1 protein of PSII. Therefore it is important to discriminate between the inactivation of PSII as a reversible downregulation of photosynthesis, and the irreversible effects of photodamage (Critchley and Russell, 1994).

The source of chlorophyll fluorescence in algae and plants is the antennae pigments of photosystem II. The initial fluorescence,  $F_0$ , of the samples is a measure of fluorescence when the reaction centers of photosystem II are fully oxidized. A saturating pulse of white light causes reduction of the photosystem II reaction centers and fluorescence increases to a maximal value  $(F_m)$ . The change in fluorescence from  $F_0$  to  $F_{\rm m}$  ( $\Delta F$ ) denotes the variable fluorescence. The ratio of variable to maximal fluorescence in a dark-adapted sample  $(F_v/F_m)$  can be correlated to the quantum yield of photosynthesis and serves as a convenient measure of the maximum potential quantum yield (Björkman and Demmig, 1987; Schreiber and Neubauer, 1990). Chlorophyll fluorescence measured in this fashion provides valuable information on the efficiency of photosynthesis, and fluorescence measurements are now widely used in studies on the effects of environmental stress. Knowledge of the variation in fluorescence characteristics among plants grown under non-stress conditions, and of the relationship between these characteristics and the photon yield in stressed plants is therefore of considerable importance. Kitajima and Butler (1975) have proposed that the ratio of variable to maximum fluorescence  $(F_v/F_m)$  emitted from photosystem II (PSII) may serve as a quantitative indicator of the efficiency of the photochemistry of PSII.

The endosymbiotic dinoflagellate microalgae (Symbiodinium spp.) located intracellularly within the tissues of scleractinian corals are very sensitive to elevated water temperature (Coles and Jokiel, 1978; Glynn and D.Croz, 1990; Iglesias- Prieto et al., 1992). Several studies have shown that at temperatures of 32°C photosynthesis is impaired (Iglesias Prieto et al., 1992; Fitt and Warner 1995; Warner et al., 1996; Iglesias-Prieto, 1997; Lesser, 1997; Jones et al., 1998). This thermal sensitivity of Symbiodinium spp. Is believed to be the underlying cause of the phenomenon of coral bleaching (Iglesias-Prieto et al., 1992), and has been investigated by photo-respirometry and chlorophyll fluorescence techniques. The chlorophyll fluorescence techniques have been particularly useful because they are rapid and non-invasive, and they allow diagnostic analyses of the effects of heat stress (see for example Warner et al., 1996; Jones et al., 1998). Temperature and light stress in corals is readily detectable as a change in the photosynthetic fitness of the zooxanthellae using non-invasive pulse amplitude modulation (PAM) fluorometry. The fluorometer uses a range of flashing (pulsed) lights to measure the photosynthesis. These pulses of light are modulated, and the height (or amplitude) of the pulse of light governs its intensity.

The ratio of variable fluorescence of variable  $(F_v)$  to maximal  $(F_m)$  fluorescence  $(F_v/F_m)$  can be quantitately related to the efficiency of photosynthesis. When all reaction centers are open, the probability of excitation escape is low and thus fluorescence is minimal  $(F_0)$ . When the reaction centers are become closed, absorbed excitation energy cannot be directed to photochemistry and fluorescence emitted rises to the maximum value,  $F_m$ . The ratio of quantum yields for fully open and fully closed reaction centers, normalized to  $F_m$  is a measure of quantum yield of photochemistry in PS II, and reflects

the probability that PS II reaction centers will use available excitation energy for photochemistry.

Coral bleaching is related to the general phenomenon of photoinhibition. The use of pulse amplitude modulated (PAM) fluorometry to study heat-stressed a coral has initiated the identification of the component of the photosynthetic metabolism that fails when zooxanthellae are exposed to heat stress (Iglesias-Prieto et al., 1992; Fitt and Warner, 1995; Warner et al., 1996). PAM measures variable fluorescence which is a relative measure of the rate at which PS II can use light to process electrons flowing from the water-splitting reactions of photosynthesis. Bleached corals have low maximum quantum yield (i.e. photochemical efficiency) of the photosystem II (PSII) as measured by the ratio of variable  $(F_v)$  to maximal  $(F_m)$  fluorescence  $(F_v/F_m)$  (Fitt and Warner, 1995; Warner et al., 1996, 1999; Brown et al., 2000; Jones et al., 2000; Bhagooli and Hidaka, 2002) and display a decline in the D1 reaction center protein (Warner et al., 1999). Recent studies showed that photoinhibitory damage of zooxanthellae as measured by long-term reduction in photochemical efficiency leads to bleaching (Brown et al., 2000; Jones and Hoegh- Guldberg, 2001). Corals often bleach first on their upper sunlight-exposed surfaces (Williams and Bunkley- Williams, 1990). This pattern is consistent with manipulative experiments that show that impairment of photosynthesis in corals during heat stress is dependent on both the quality (Fitt and Warner, 1995) and quantity (Jones et al., 1998) of light.

A decrease in  $F_v/F_m$  of the symbiotic dinoflagellates in the corals *Agaricia lamarckii*, *A. agaricites*, *Montastrea annularis*, *Siderastrea radians* (Fitt and Warner, 1995; Warner et al., 1996) and *Stylophora pistillata* (Jones et al., 1998) has been recorded in laboratory experiments during heat stress. In these studies, the decrease in Fv /Fm preceded loss of dinoflagellates from the tissues and bleaching. The temperature at which photosynthetic dysfunction occurs during manipulative studies of heat stress in corals is similar to those observed during natural coral bleaching events.

To cope with oxidative stress, plant cells have developed many highly-efficient defense systems, with both enzyme and non-enzyme constituents. The non-enzymatic antioxidants are generally small molecules, such as ascorbate and glutathione, that occur in the aqueous phase (intracellular fluid), and lipophilic antioxidants (tocopherols and carotenoids) which are active in cell membranes (Noctor and Foyer, 1998; Munne-Bosch and Alegre, 2002. In a study done to investigate the response of an ascorbate-deficient Arabidopsis thaliana, mutant vtcl plants (deficient in ascorbate due to a mutation) were exposed to short-term increased UV-B. Gao and Zhang (2008) found  $F_v/F_m$  decreased much more in the vtc1 mutant than in the wild type, validating the importance of the nonenzymatic antioxidant defense. The research of Havaux et al. (2005) shows that vitamin E protects Arabidopsis thaliana against photoinhibition and photooxidative stress. Additionally, herbicide-induced blocking of the tocopherol synthesis in the green alga Chlamydomonas reinhardtii exposed to high light was associated with a loss of photosystem II (PSII) activity (Trebst et al., 2002). Based on this observation, it was suggested that  $\alpha$ -tocopherol could be specifically involved in the maintenance of the PSII function by scavenging  ${}^{1}O_{2}$  produced at the PSII reaction center. In a study by Lesser (1997) the Caribbean coral Agaricia tenuifolia, was exposed to temperature induced stress and exposure to full solar radiation. They exhibited an overall decrease in photosynthetic performance which was followed by bleaching. Exposure of corals to the exogenous antioxidants, Vitamin C ( $125\mu$ M) and mannitol (10mM), known to scavenge reactive oxygen species during temperature-induced stress, improved maximum photosynthetic capacity to rates indistinguishable from corals measured at the ambient temperature of their site of collection and prevented bleaching.

The ability of secondary metabolites isolated from *Pseudopterogorgia elisabethae* to delay photoinhibition in stressed zooxanthellae was tested as follows. Samples from a clade B2 Symbiodinium culture from the temperate western Atlantic were subjected to combined irradiance (light=300 $\mu$ M) and thermal (temperature=34°C) stress. Maintaining constant temperature was achieved by placing the sample vial in a jacket with water circulating though a water bath (Figure 28). Pulse-amplitude modulated (PAM) fluorometry was used to characterize the maximum quantum efficiency of PSII (F<sub>V</sub>/F<sub>M</sub>). Samples were dark acclimated for 20 minutes before initial PAM reading. Each sample was then subjected to 14 automated cycles consisting of 35 minutes of light followed by 5 minutes after which a PAM measurement was recorded using a Diving-PAM (Walz, Germany).





**Figure 28.** The photograph shows a) the apparatus used for the photoinhibition assay, and b) the Diving PAM fluorometer.



**Figure 29.**  $n=3 \pm$  standard deviation. Different letters indicate significantly different means (*P* <0.05), Tukey's HSD.

Tukey's Multiple Comparison Test	q	Significant? P < 0.05?
CONTROL vs DMSO	6.20	Yes
CONTROL vs Ps G-J Aglycone 100 µM	20.59	Yes
CONTROL vs Ps K 75 µM	3.80	No
CONTROL vs K 2' ac 50µM	2.01	No
CONTROL vs K 2' ac 75 µM	2.74	No
DMSO vs Ps G-J Aglycone 100 µM	26.79	Yes
DMSO vs Ps K 75 µM	10.00	Yes
DMSO vs K 2' ac 50µM	8.21	Yes
DMSO vs K 2' ac 75 µM	8.94	Yes
Ps G-J Aglycone 100 µM vs Ps K 75 µM	16.79	Yes
Ps G-J Aglycone 100 µM vs K 2' ac 50µM	18.58	Yes
Ps G-J Aglycone 100 µM vs K 2' ac 75 µM	17.85	Yes
Ps K 75 μM vs K 2' ac 50μM	1.79	No
Ps K 75 μM vs K 2' ac 75 μM	1.06	No
K 2' ac 50μM vs K 2' ac 75 μM	0.73	No

Table 8. Pairwise comparison of treatments; Tukey's HSD, p<0.05.

Results were compared to control samples to which no compounds were added and solvent-only controls. Pairwise comparison of treatments using Tukey's HSD indicate that pseudopterosins, and especially the pseudopterosin G-J Aglycone reduce the loss of quantum photosynthetic yield over the nine and a half hour period of the experiment versus the solvent only control (Figure 29, Table 8). Loss of quantum photosynthetic yield for the aglycone treated samples was also significantly less than the control samples without solvent. DMSO also significantly reduced quantum photosynthetic yield when compared to the cells only control. Dimethyl sulphoxide (DMSO) is commonly used as a solvent for water-insoluble substances applied to biological material. A very small volume was added to the samples, and thus it is unclear why these samples differed significantly from the control samples without solvent.

There is a strong suggestion that both coral reef organisms and communities exhibit acclimative and "adaptive" behaviors and considerable flexibility of response to environmental change, at time scales of years to decades (Buddemeier and Smith, 1999). Many compelling examples that confirm the prominence of protein metabolism as a central component of the acclimatization biology in tropical reef corals. For example, a suite of mycosporine-like amino acids (MAAs) screens UV radiation and protects corals from cellular and molecular damage (Jokiel and York, 1982; Gleason and Wellington, 1993). The ability of corals to increase and decrease expression levels of hsp 70, and effectively regulate the activity of antioxidant enzymes clearly reflects the intrinsic biological flexibility of reef corals with respect to their environment (Gates and Edmund, 1999). Reef corals regulate the activity of antioxidant enzymes to ameliorate the deleterious effects of oxygen radicals generated in their tissues in response to small environmental change (Lesser, 1997). Following exposure to elevated temperature and UV radiation, increased activities of superoxide dismutase (SOD) and catalase (CAT) have been directly measured in the coral *Pocillopora damicornis* exposed to differential flow regimes (Lesser et al., 1994), and in the soft-bodied symbiotic cnidarians *Anthopleura elegantissima* (Dykens and Shick, 1984) and *Palythoa caribaeorum* (Lesser et al., 1990). Different sides of *Goniastrea aspera* colonies that were either sun-exposed or shaded during emersion at low tide contained different levels of host heat shock proteins and the superoxidase dismutase (Brown et al., 2002).

Differential tolerance to stress among cnidarian species, irrespective of the *Symbiodinium* they host, may explain differences in bleaching (Gates and Edmunds, 1999; Loya et al., 2001; Bhagooli and Hidaka, 2004). While the studies referenced above have examined changes in protein metabolism in response to environmental stress, our study represents the first attempt to examine the relationship between secondary metabolite chemistry and oxidative stress in symbiotic cnidarians. It has been demonstrated previously that the addition of exogenous antioxidants to an illuminated culture of *Euglena gracilis* not only improved variable fluorescence and photosynthetic performance, but also decreased the rate of membrane-lipid peroxidation (Tschiersch and Ohmann, 1993). The addition of exogenous scavengers of oxygen radicals restores a significant portion of the photosynthetic capacity in cultured zooxanthellae exposed to UV radiation and elevated temperatures (Lesser, 1996). Lesser (1997) has also shown that in the coral *Agaricia tenufolia*, photosynthetic performance returned to pre-stress rates when the coral are treated with exogenous antioxidants.

Our results clearly demonstrate that the bioactive components of extracts from seven species in the genus *Pseudopterogorgia* are taken up by *Symbiodinium* cells. Furthermore, they reduce the overall oxidative stress index in the stressed cells as measured using the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Equivalent micromolar concentrations of ten of the fifteen pseudopterosins tested and the known antioxidant trolox exhibit the same activity. Thus, we conclude that a potential ecological role for the diverse and novel secondary compounds by species in the genus Pseudopterogorgia may be defense against oxidative stress. Previously it has been shown that the octocoral families Briareidae, Clavularidae, Gorgonidae, and Nephtheidae exhibited low bleaching susceptibilities (Goulet et el., 2008). Soft corals of the genera Lemnalia, Paralemnalia, and Rhytisma are a rich source of sesquiterpenoids, and the genus Briareum is a prolific source of diterpenoids. Perhaps it is this isoprenoid chemistry that confers the organisms in these genera and the Pseudopterogorgia their reduced susceptibility to bleaching. The experimental evidence that bleaching in corals exposed to elevated temperatures can be prevented by antioxidants (Lesser, 1997), along with the results presented here seem to support this hypothesis.

## **CHAPTER 5**

# A potential pharmacological role for the antioxidants from Pseudopterogorgia sps.

# 5.1 Oxidative stress and human diseases

All aerobic organisms are susceptible to oxidative stress simply because semireduced oxygen species, superoxide and hydrogen peroxide, are produced by mitochondria during respiration. However, during pathological conditions, the oxidant / antioxidant balance is altered. Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and aging (Dalle-Donne et al., 2006). Accumulation of free radical damage as the cause of aging is supported by studies that show increased oxidatively induced protein and lipid damage in pathological conditions of accelerated aging (Stadtman, 2004). A particularly important consequence of free radical damage in many cells, is the peroxidation of polyunsaturated fatty acids (PUFA), which results in the formation of lipid peroxides and aldehydes (Cheeseman and Slater, 1993), and carbonyl modifications of proteins which may affect a variety of cellular functions involving proteins: namely; receptors, signal transduction mechanisms, transport systems and enzymes.

Oxidative stress refers to the cytopathologic consequences of a mismatch between the production of free radicals and the ability of the cell to defend against them. Oxidative stress can thus occur when the production of free radicals increases, scavenging of free radicals or repair of oxidatively modified macromolecules decreases, or both. This imbalance results in a build-up of oxidatively modified molecules that can cause cellular dysfunction and, for postmitotic cells such as neurons, cell death. Mitochondrial oxidative metabolism, nitric oxide, phospholipid metabolism, and proteolytic pathways are potential sources of intracellular free radicals. Alterations in free radical defense systems may also contribute to oxidative stress. A net increase in reactive oxygen species can produce damage to lipids, proteins, and DNA and induce necrosis or apoptosis.

Free radicals are normal products of cellular aerobic metabolism (McCord, 1985; Halliwell and Gutteridge, 1985). Superoxide and hydroxyl species are the predominant cellular free radicals. Hydrogen peroxide and peroxynitrite although not themselves free radicals, contribute importantly to the cellular redox state. Together, these molecules are referred to as reactive oxygen species (ROS). An array of cellular defense systems exist to counterbalance ROS (McCord, 1985; Halliwell and Gutteridge, 1985). These include enzymatic and non-enzymatic antioxidants that lower the steady-state concentrations of free radical species and repair oxidative cellular damage. Because ROS can cause damage to virtually all cellular macromolecules, aerobic organisms have developed elaborate antioxidant defense systems for the prevention of ROS-mediated cellular damage. ROS can be reduced enzymatically by antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase or nonenzymatically by antioxidants such as vitamin E, vitamin C, and glutathione (Miquel, 2001). The antioxidant properties of green tea extract (Hong et al 2000), blueberry, spinach or strawberry (Joseph et al 1999), Allium sativum (Pedraza- Chaverri et al 1998) and Glycyrrhiza glabra (Vaya et al 1997) have already proven beneficial in reducing oxidative tissue injury. Previous studies

suggest that the antioxidant vitamin E may inhibit some aspects of the aging process by preventing lipid peroxidation. It is possible other antioxidants may have similar activity.

#### 5.2 Oxidative stress and neurodengerative diseases

For years researchers have known that free radicals can cause cell degeneration, especially in the brain. Growing data from experimental models and human brain studies suggest oxidative stress may play an important role in neuronal degeneration in diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. The brain is particularly vulnerable to oxidative processes because it has a high glucosedriven metabolic rate, has low levels of antioxidant defense enzymes, contains high concentrations of polyunsaturated fatty acids, which are potential substrates for lipid peroxidation and is rich in enzymatically active transition metals, which can potentially catalyze radical formation (Halliwell and Gutteridge, 1989). High-dose  $\alpha$ -tocopherol supplementation (5 g/kg) was associated with increased median and maximum life span and improved brain mitochondrial function in male mice from a short-lived strain (Navarro et al., 2005). Several studies have suggested that these increases in oxidative stress vulnerability and the resulting neuronal loss can be reduced through dietary supplementation of plant extracts that prevent brain atrophy as well as learning and memory impairments (Kanowski et al 1996; Moriguchi et al 1997; Nishiyama et al 1997). It has been demonstrated that certain phenolic antioxidants attenuate neuronal cell death induced by oxidative stress (Schroeter et al 2000; Youdim and Joseph 2001).

Oxidative stress has been associated with both necrosis and apoptosis. In necrosis, a selective loss of membrane permeability occurs, which results in swelling of organelles,

loss of membrane depolarization, and rupture of the plasma membrane. Apoptosis is a form of cell death that occurs during normal development and in pathological situations. Cells undergoing apoptosis exhibit certain characteristics, including cytoplasmic shrinkage, nuclear blebbing, and chromatin condensation (Kerr et al., 1972). Removal of NGF from sympathetic neurons in vitro triggers a classic apoptotic death (Chang et al., 2003) but progression along this cell death pathway can be aborted by readdition of NGF to an NGF-deprived sympathetic neuron before a cell has reached the commitment-to-die (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

Alzheimer's disease (AD) is the most common cause of dementia. This most common of the late-life dementias is rising in prevalence with the aging of populations in developed countries and may now affect 20 to 30 million people worldwide (Selkoe, 2005). AD effects the hippocampus, cortex and amaygdala and is characterised by loss memory loss and cognitive dysfunction. In people with Alzheimer's disease, deposits called amyloid plaques build up in the brain. These are composed, in part, of a protein called beta-amyloid, which is a fragment of the amyloid precursor protein (APP). A mutation in this gene in humans is believed to be responsible for 5 to 20 percent of all early onset familial Alzheimer's disease (Selkoe, 2005). Another neuropathological characteristic of Alzheimer's disease are abundant filamentous tau lesions, intracellular accumulations of abnormal filaments formed by the microtubule-associated protein tau. Both amyloid plaques and neurofibrillary tangles (NFT) are clearly visible by microscopy in brains of those afflicted by AD. Plaques are dense, mostly insoluble deposits of amyloid-beta protein and cellular material outside and around neurons. They continue to grow into insoluble twisted fibres within the nerve cell, often called tangles. Although

many older individuals develop some plaques and tangles as a consequence of aging, the brains of AD patients have a greater number of them in specific brain regions such as the temporal lobe. In vitro experiments support the observation that the neurotoxic effect of A $\beta$  is mediated by free radical mechanisms. A $\beta$  aggregation produces oxidative stress in neuronal cells and antioxidants such as vitamin E and melatonin attenuate this stress (Behl et al. 1992). Vitamin E clearly had a beneficial effect in an Alzheimer's disease clinical trial (Sano et al., 1997). The sympathetic nervous system is part of the autonomic nervous system, which is responsible for maintaining homeostasis. Postganglionic sympathetic neurons innervate endocrine and exocrine glands, cardiac muscle, and smooth muscle throughout the body carrying out vasomotor, pilomotor, secretomotor, sudomotor, and pupilodilator functions. The sympathetic nervous system regulates many physiological processes, including body temperature, blood pressure, respiration, cardiac output, blood glucose levels and gastrointestinal peristalsis (Glebova and Ginty, 2005). In response to an external or internal environmental change such as stress or a change in body posture, activation of the sympathetic nervous system affects multiple organ systems. Although an animal can survive without a sympathetic nervous system, it can do so only in conditions of constant ambient temperature and in the absence of stress. The importance of normal sympathetic function is underscored by the pathological states associated with sympathetic dysfunction, such as hypertension, congestive heart failure, dysautonomia, various neuropathies, and other disorders (De Quattro and Feng, 2002, Goldstein et al., 2002, Low, 2002). Sympathetic neurons in culture die by apoptosis when deprived of nerve growth factor (NGF). The neurotrophic factor hypothesis (Mattson, 1998) suggests that neurotrophic factor deprivation leads to formation of reactive oxygen

species (ROS) and a change in oxidant state (Dugan et al., 1997). The formation of free radicals acts directly to destroy phospholipids and as signaling molecules to upregulate cell death pathways leading to apoptosis or necrosis (Greenlund et al., 1995). The increase of mitochondrial-derived reactive oxygen species (ROS) that occurs in these NGF-deprived neurons has been demonstrated (Kirkland and Franklin, 2003). The prototypical model for investigating the cellular and molecular mechanisms underlying this death consists of rodent sympathetic neurons dissociated from late embryonic or early postnatal mice and grown in cell culture. Sympathetic neurons of this age require the neurotrophin nerve growth factor (NGF) to live.



**Figure 30.** Morphological changes during the apoptotic death of sympathetic neurons deprived of NGF.

Depriving these cells of NGF causes their apoptotic death both *in vivo* and *in vitro* (Kirkland et al., 2007; Figure 30). Thus, developing nerve growth factor (NGF)-dependent sympathetic neurons are one of the best-studied in vitro models of neuronal apoptosis and have been used to identify key components of the neuronal cell death pathway. During development of the rat, insufficient supply of NGF leads to the apoptotic death of approximately 40% of the sympathetic neurons in the superior cervical ganglion during the first few days after the animal is born (Wright et al., 1983). In a study done by Greenlund and co-workers (1995) microinjections of copper/ zinc SOD protein or a SOD expression vector delayed the apoptosis of sympathetic neurons while injection of an antisense SOD expression vector enhanced the death of neurons deprived of NGF. In addition, using a redox-sensitive dye to analyze the production of ROS after NGF deprivation, sympathetic neurons displayed a peak in fluorescence intensity 3 hr after deprivation. These data indicate that in sympathetic neurons ROS are important mediators of apoptosis induced by NGF deprivation (Greenlund et al., 1995).

# 5.3 Pseudopterosins promote survival in NGF deprived mouse sympathetic neurons

The pseudopterosins have displayed potent antioxidant activity in both solution based and cell based assays (as described in previous chapters). With these results in mind we have attempted to test whether pseudopterosins promote the survival of postganglionic sympathetic neurons by reducing the oxidative burden. The survival of these neurons was assessed in the absence of NGF, but in the presence of the compounds at varying concentrations. NGF was eliminated from the cultures with an antibody that neutralizes the activity of NGF. Cells that were not deprived of NGF serve as a positive



**Figure 31.** Treatment with 40µM pseudopterosin K, K 2'ac, and K 4'ac significantly enhanced the survival of NGF-deprived mouse sympathetic neuron, N=2 wells  $\pm$  standard deviation. Different letters indicate significantly different means (*P* <0.01), Tukey's HSD.

control. Cells deprived of NGF and without compound added serve as the negative control. Each treatment was applied to cells in two wells. The data presented represents the average of viable cells counted in the two wells  $\pm$  standard deviation. The means were significantly different across the treatments based on a one-way analysis of variance (p < 0.05; Appendix; Table 15). Based on the pairwise comparison of treatments by Tukey's HSD (p < 0.01) all three of the pseudopterosins tested at 40  $\mu$ M resulted in significantly higher survival of the NGF deprived sympathetic neurons (Figure 31). Additionally, the 40  $\mu$ M treatments of ps K 2' acetate and 4' acetate were not significantly different from the positive control.

#### 5.4 Pseudopterosins suppress ROS formation in NGF deprived sympathetic neurons

NGF withdrawal induces apoptosis in sympathetic neurons by causing the proapoptotic protein, Bax, to bind to the outer mitochondrial membrane where it induces release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm (Putcha et al., 1999). Increased levels of reactive oxygen species (ROS) occur in these neurons as a result. The intensity of the fluorescent redox-sensitive dye MitoSox Red increases in NGF-deprived sympathetic neurons indicating that it has become oxidized by the elevated levels of ROS in those cells (Kirkland et al., 2004). MitoSOX Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. MitoSOX Red reagent is livecell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits bright red fluorescence upon binding to nucleic acids (Haugland, 2005). MitoSOX can also be oxidized by ROS other than superoxide but is most sensitive to oxidation by superoxide (Kirkland et al., 2004). Pseudopterosins K and K4' acetate were screened in vitro for the ability to suppress ROS formation in sympathetic neurons deprived of NGF. Cultures were deprived of NGF and stained with the superoxide-sensitive dye MitoSOX 24 hr later. Deprived cultures were treated with various concentrations of the compounds at the time of deprivation.

Dye intensity was measured only in the cytoplasm. Nuclear staining occurred only in a small proportion of apoptotic cells that appeared to have compromised plasma membranes. Neurons in which cytoplasm could not be clearly differentiated from the nucleus were excluded from analysis. MitoSOX was excited with the 408 nm line of the



**Figure 32.** Quantification of ROS in NGF deprived mouse sympathetic neurons treated with pseudopterosins. Different letters indicate significantly different means (P < 0.01), Tukey's HSD.

confocal laser and the red photomultiplier channel of the confocal microscope was used for image acquisition. Generally, eight separate fields of view were scanned per plate. The measured intensity of each neuron was normalized to that of control neurons maintained in the standard culture medium containing NGF and receiving the same concentration of dye for the same period as did the experimental cells.

The pseudopterosins tested greatly suppressed the increase of MitoSOX intensity in NGF-deprived sympathetic neurons. A one-way analysis of variance confirmed that means differed significantly across treatments (p<0.05, Appendix; Table 16). All but the 10  $\mu$ M treatment of pseudopterosin K showed a significant reduction in ROS versus the NGF-deprived cells (Tukey's HSD p <0.01, Figure 32,33).



**Figure 33.** Confocal micrographs of sympathetic neurons exposed to the dye MitoSox Red **a**) NGF- (negative control) **b**) NGF + (positive control) **c**) ps K 40 $\mu$ M **d**) ps K 4' acetate 40 $\mu$ M

Many neurodegenerative disorders and syndromes are associated with an excessive generation of reactive oxygen species (ROS) and oxidative stress. Direct antioxidants such as chainbreaking free radical scavengers can prevent oxidative nerve cell death. Although there is ample experimental evidence demonstrating neuroprotective activities of direct antioxidants in vitro, the clinical evidence for antioxidant compounds to act as protective drugs is relatively scarce. Recent research efforts into the elucidation of the mechanisms of neurodegeneration in Alzheimer's disease (AD), Parkinson's disease (PD), stroke, and other neurodegenerative disorders has led to an increased understanding of the basic mechanisms of nerve cell death. In general, cell death can follow two basic pathways. Apoptosis (also called programmed cell death) is an active form of cell degeneration and is executed by enzymes (caspases). Necrosis, the other type of cell death, is frequently occuring during acute insults and is characterized by rapid cell lysis. Both processes of cellular degeneration are frequently accompanied by an overshooting generation of free radicals and other cellular events leading to oxidative stress (Behl and Moosmann, 2002). This final oxidative pathway of nerve cell death could be a basis for preventive and therapeutic avenues. With respect to neurodegenerative diseases and syndromes, multiple pathogenetic mechanisms may be involved upstream, ranging from inflammation to disturbed energy metabolism, many of which are accompanied by oxidative stress (Behl and Moosmann, 2002). Antioxidants in a pharmacological sense are compounds which counteract the adverse effects of oxidative stress. They can be differentiated with respect to their modes of action, which include the inhibition of free radical formation (indirect antioxidants), the direct chemical scavenging of generated free radicals (direct antioxidants), and/or the strengthening of the cellular capability to cope with high ROS loads via enzymatic processes which detoxify accumulating ROS, or to promote repair of the effected damage (Behl and Moosmann, 2002).

Based on the preliminary results presented here, pseudopterosins might be a promising drug candidate in the treatment of those neurodegenerative diseases in which oxidative stress plays an important role. Convincing evidence exist showing that different classes of antioxidants, mainly those that belong to the family of direct antioxidants, act as neuroprotectants *in vitro*. Vitamin E remains to be one of the most potent chainbreaking antioxidant structures. However, its passage through the blood brain barrier (BBB) is limited. Consequently, the identification of structures with much better BBB penetration and increased antioxidant activity may, ultimately, lead to the development of novel neuroprotective drugs.

# **CHAPTER 6**

#### Methods and materials

**Collection and identification of coral material.** Fragments of *Pseudopterogorgia* sps colonies were collected by SCUBA at a range depth of 5–30 m at various sites around the the Bahamas and Key Largo. Sample collection never implied removing whole colonies, only a terminal fragment of each individual colony was cut off the main gorgonian axis with sharp scissors. Gorgonian fragments were air-dried and stored in the freezer. Samples were kept frozen until the moment of extraction. Taxaonomic identification of each colony was made by comparison of the gross colony architecture and by morphology and dimensions of the three most common sclerite types (i.e., spindles, anthocodial rods, and scaphoids). To obtain sclerites, we clipped off a small fragment of the colony and digested it in household bleach. Once the organic tissue was digested, sclerites were repeatedly washed with distilled water and ethanol, and dried in an oven at 70±C for 24 hr. Crude sclerite preparations were mounted on glass slides and analyzed under a microscope. For each colony, we measured 30 anthocodial rods, 30 spindles, and 30 scaphoids. For species designations, we followed Bayer (1961).

**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**. HPLC separations were 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.

**Collection of larvae.** *Pseudopterogorgia elisabethae* were collected from spawning adult colonies in November 2004 at the marine aquaculture facility at the Gumbo Limbo Environmental Center in Boca Raton, Florida. These adult colonies had been collected by SCUBA at a depth of 15 M from Long Key, Florida in October, 2004. The larvae were then transferred to 1 L glass containers with Millepore (0.22µm) filtered seawater (~200 larvae/jar). Pseudopterogorgia bipinnata colonies were collected at Long Key, Fl by SCUBA at a depth of 25m in December of 2004. Spawning adult colonies were collected

and placed in individual sealed bags of seawater. Once transported to our lab in Boca Raton, larvae were separated from adult colonies by pipette, rinsed 3X in MFSW and placed in 1L glass containers of MFSW. All larval cultures were maintained in a growth incubator at 26° C with a 12/12 light/dark cycle and 40% of the water was changed once every second day while larvae were in culture.

Incubation of gorgonian larvae with  $[C_1-{}^3H]$  geranylgeranyl diphosphate. For each species, three replicates of 100 larvae each were transferred to 125 mL glass jars containing 100 mL of MFSW and were incubated with 4µCi of C<sub>1</sub>-  ${}^{3}H$ -GGDP (50-60 Ci/mmol) at 26°C and 200RPM on an environmental shaker for 24 h. Larvae were then transferred in a minimal amount of seawater (~1 ml) into 15 mL conical tubes. 9 mL of MeOH was added, and the tubes were centrifuged.

Extraction and purification of diterpenes from larvae. Larvae were extracted in MeOH/H<sub>2</sub>O (9:1) (at room temperature for ~ 1hr) and partitioned with hexanes. The aqueous partitions were then adjusted to MeOH/H<sub>2</sub>O (1:1), and partitioned a second time with CH<sub>2</sub>Cl<sub>2</sub>. Normal phase HPLC purification was performed using a Vydac semi preparative column. For isolation of diterpenes from *P.bipinnata* larval CH<sub>2</sub>Cl<sub>2</sub> extract, a mobile phase gradient from 100% hexanes to hexanes/EtOAc (50:50) with UV detection at 270nm. Reverse phase HPLC was performed using a semi preparative Phenomenex phenyl-hexyl column and a mobile phase gradient of MeOH/H<sub>2</sub>O (50:50) to MeOH (100%) with UV detection at 270nm. HPLC conditions for isolation of pseudopterosins from *P.elisabethae* larval CH<sub>2</sub>Cl<sub>2</sub> extract used a mobile phase gradient from

hexanes/EtOAc (60:40) to 100% ethyl acetate with detection at 283 nm. Pseudopterosin C was further purified by reversed phase HPLC using a gradient of acetonitrile/water (50-100%) as mobile phase and detection at 283nm. <sup>1</sup>H-NMR spectra were recorded at 400 MHz in CDCl<sub>3</sub> on a Varian Innova spectrometer.

Thin layer chromatography. TLC was carried out on pre-coated silica gel plates 60  $F_{254}$  or RP-18  $F_{254}$  plates with 0.5 or 1 mm film thickness (Whatman) with a mobile phase of Hexanes/EtOAc (60:40). Spots were visualized by UV light followed by spraying the plate with 10%  $H_2SO_4$  in MeOH and charring with a heat gun.

**Zooxanthellae Isolation.** Coral tissue was homogenized in a Waring blender using deionized water. The resulting slurry was divided into 50mL Falcon tubes and centrifuged at 350 x g for 5 min. The supernatant was decanted and the pellet resuspended in deionized water. This was repeated until the supernatant was clear. The pellet was resuspended, layered over 100% Percoll, and centrifuged at 38 x g to pellet spicules. The zooxanthellae formed a brown band above the Percoll, which was transferred to another Falcon tube using a plastic pipet. This cell layer was diluted with deionized water and layered over a discontinuous gradient of 30, 70, and 100% Percoll and centrifuged at 350 x g for 30 minutes. The layer of cells was cleaned three additional times in the same manner until no further debris was observed under a light microscope.

**PCR analysis of DNA from isolated zooxanthellae and larvae.** All DNA was extracted using UltraClean<sup>™</sup> Soil DNA Kit (MoBio Laboratories, inc) according to the

manufacturer's protocol. The isolated DNA was quantified and assessed for purity by measuring the absorbance at 260 and 280 nm and calculating the A260:A280 ratio. The DNA was aliquoted at 50 ng µl-1 and stored at -80°C until used. The internal transcribed spacer region (ITS-rDNA) was PCR-amplified using the zooxanthellae- specific primers designed to amplify between the conserved regions of the 3' and 5' ends of the zooxanthellae 18S and 28S rDNA genes (Santos et al. 2001). Octocoral DNA was amplified using primers designed to target microsatellite loci in *P elisabethae* reported by Gutierrez-Rodriguez and Lasker (2004). In general, 1  $\mu$ l of DNA (50 ng  $\mu$ l-1) was used in a 50 µl PCR reaction containing 0.5 µl Taq (2.5 U; Qiagen), 5 µl 10× buffer, 3 µl 25 mM MgCl<sub>2</sub>, 1 µl 100 mM dNTPs, 1 µl of each primer (10mM concentration). PCR cycling conditions were an initial denaturing period of 2 min at 94°C followed by 30 cycles of 94°C for 40 s, 53°C for 1 min, and 72°C for 1 min. A final extension of 30 min at 72°C was added. 5 to 10 % of the reaction was loaded on a 1 % agarose gel (0.6% Metaphor agarose/0.6% Seakem agarose; Fisher Scientific), run at 90 V, and visualized by ethidium bromide.

Antibiotic/antimycotic treatment of larvae. 3 containers of 100 ml MFSW with ~100 larvae were treated with antibiotic/antimycotic twice over 48 hours with final concentrations as follows: 0.1 mg/ml kanamycin (a water-soluble, broad-spectrum antibiotic), 0.1 mg/ml streptomycin, 100 units/ml penicillin, 0.25  $\mu$ g/ml amphotericin B. At the end of the 48 hours the samples were incubated for 24 hrs with the tritiated diterpene precurser and then extracted and analysed as previously described.

Acid hydrolysis of pseudopterosins A-D to pseudopterosin aglycone. To a mixture of pseudopterosins A-D was added a 1N HCl methanolic solution (2 mL). The reaction vessel was agitated at 50°C for 3 hours and then the solution was cooled to 25°C, and water added (10 mL). The solution was extracted with methylene chloride (3 x 2 mL), the organic extracts combined, dried over anhydrous sodium sulfate, and the solvent allowed to evaporate under a nitrogen. The residue was purified by semi-preparative RP-C18 HPLC ( $\lambda = 283$  nm) using a linear gradient of acetonitrile:water (50:50 acetonitrile:water to 100% acetonitrile over 30 minutes, hold for 10 minutes) as mobile phase.

**Culture of rat sympathetic neurons.** Superior cervical ganglia were dissected from Sprague–Dawley rat fetuses (Harlan Bioproducts) on embryonic day 20 or 21. Neurons were enzymatically and mechanically dissociated from the ganglia and maintained in cell culture. Culture medium consisted of Eagle's minimum essential medium with Earle's salts (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µM fluorodeoxyuridine, 20 µM uridine, 1.4 mM L-glutamine, and 50 ng/ml 2.5S NGF. Cells used in survival assays were plated on a collagen substrate in 24-well Costar tissue culture dishes (Corning, Inc., Corning, NY, U.S.A.). Those used for fluorescent or confocal microscopy were plated on a collagen substrate coated on glass coverslips glued over holes cut in the bottoms of 35-mm Falcon tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). One-half to one ganglion was plated per culture in all experiments. NGF was withdrawn from cells by incubating cultures in the culture medium containing a NGF-

neutralizing antibody (Harlan Bioproducts) and lacking NGF. Cells had been in culture for 6–9 days at the beginning of experiments.

Quantification of ROS in sympathetic neurons. MitoSOX Red was purchased from Invitrogen (Eugene, OR). Cultures were incubated for 10 min at 35°C in the appropriate experimental medium containing MitoSOX (2  $\mu$ M). A Nikon (Melville, NY) C1 laserscanning confocal microscope mounted on a Nikon Eclipse TE 300 inverted microscope was used for all confocal microscopy. The confocal microscope was controlled by EZC1 software running on a Dell computer. Neurons, observed with a 60x plan oil immersion lens (numerical aperture, 1.4), were chosen at random and scanned by the confocal microscope. Laser power, confocal pinhole size, and photomultiplier gain were maintained at constant levels during an experiment. After incubation in MitoSOX, cultures were washed twice with L-15 medium and kept in the second wash for microscopy. MitoSOX was excited with the 408 nm line of the confocal laser and the red photomultiplier channel of the confocal microscope was used for image acquisition.

**FRAP assays.** FRAP reagent was prepared before each assay with 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution, and 20 mM FeCl<sub>3</sub> in a 10:1:1 ratio. Reagent was heated to  $37^{\circ}$  prior to use. Assays were prepared in 96-well microtiter plates using 150 µL of warm FRAP reagent with 20 µL of sample. 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 30, 90, and 120 minutes (SpectraMax
M2 microplate reader; Molecular Devices). The 90 min reading was selected for calculation of FRAP equivalence values. Standard curve were produced using 50, 125, 250, 500, and 1000  $\mu$ M of FeSO<sub>4</sub> and 5, 10, 20, 50 and 100  $\mu$ M of Trolox.

**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. Cells were centrifuged briefly to remove media, and incubated in 10  $\mu$ M 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  $\mu$ L cell suspension was added to each well of black-bottom 96-well microtiter plates. Cells were maintained in a water bath at 32°C and subjected to radiation stress under a UV lamp for 3 h. Fluorescent intensity was recorded using a monochromatic spectrophotometer (SpectraMax M2 microplate reader; Molecular Devices) set at excitation wavelength 485 and emission wavelength 538.

**Photoinhibition assay.** Cultured *Symbiodinium* sp (Clade B2) came from the lab of Dr G. Schmidt, Department of Plant Biology, University of Georgia. 1 ml of ~  $10^6$  cells/ml zooxanthellae were transferred to a flat bottom 1.2 ml vial. The samples was dark acclimated at rt for twenty minutes prior to initial PAM reading (PAM measurement was recorded using a Diving-PAM, Walz, Germany). The vial was maintained at 34 °C using a water bath and exposed to light at ~300  $\mu$ M for 35 minutes, followed by 5 minutes of dark and a PAM reading. This cycle was repeated 14 times. All compounds tested were dissolved in 10  $\mu$ L DMSO, and DMSO only controls contained 10  $\mu$ L DMSO.

**Statistical analysis.** In most cases, analysis of variance followed by nonparametric Tukey multiple comparison test was performed using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

**Purification and structure elucidation**. Optical rotation was measured on a Bellington Stanley Ltd. ADP-220 digital polarimeter. UV spectra were obtained on a Perkin-Elmer  $\lambda$ -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 ( $\delta$ ) relative to tetramethylsilane as internal reference. HPLC purifications were performed on Beckman Coulter 126P solvent module system using preparative HPLC column (Phenomenex Gemini 5 $\mu$ , C18, 110A, 250 x 21.2 mm). The structures of the new compounds were established using chemical and spectroscopic (ESIMS, <sup>1</sup>H NMR, <sup>13</sup>C NMR, COSY, HSQC, HMBC, ROESY) studies, whereas the known compounds were identified by comparison of their spectroscopic data (UV, MS and NMR) with published reports.

**Instrumentation.** 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*<sup>™</sup> 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).

**Pseudopterosin K 2' acetate:** amorphous solid;  $[\alpha]_D^{25} - 117.6$  (*c* 0.017, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{max}$  228 ( $\epsilon$  31232), 277 ( $\epsilon$  5477), 284 ( $\epsilon$  6075) nm; IR (film)  $\nu_{max}$  3344, 2927, 1745, 1456, 1432, 1374, 1242 cm<sup>-1</sup>; <sup>1</sup>H (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C (CDCl<sub>3</sub>, 125 MHz) NMR spectra, see Table 1; HRESIMS (positive-ion) *m/z* [M + Na]<sup>+</sup> 511.2655 (calcd for C<sub>28</sub>H<sub>40</sub>O<sub>7</sub>Na, 511.26717).

**Pseudopterosin K 4' acetate:** amorphous solid;  $[\alpha]_D^{25} - 130.4$  (*c* 0.046, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{max}$  228 ( $\epsilon$  36892), 277 ( $\epsilon$  6197), 284 ( $\epsilon$  6871) nm; IR (film)  $v_{max}$  3395, 2925, 1732, 1431, 1375, 1241 cm<sup>-1</sup>; <sup>1</sup>H (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C (CDCl<sub>3</sub>, 125 MHz) NMR spectra, see Table 1; ESIMS (positive-ion) *m/z* 489 [M + H]<sup>+</sup>; HRESIMS (positive-ion) *m/z* [M + H<sub>2</sub>O]<sup>+</sup> 506.3163 (calcd for C<sub>28</sub>H<sub>42</sub>O<sub>8</sub>, 506.2880).

**Pseudopterosin Iso-A:** amorphous solid;  $[\alpha]_D^{25} - 130.4$  (*c* 0.046, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\text{max}}$  228 ( $\epsilon$  15471), 277 ( $\epsilon$  3413), 284 ( $\epsilon$  3309) nm; IR (film)  $v_{\text{max}}$  3365, 2925, 1457, 1312, 1248 cm<sup>-1</sup>; <sup>1</sup>H (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C (CDCl<sub>3</sub>, 125 MHz) NMR spectra, see Table 2; HRESIMS (positive-ion) *m*/*z* [M + Na]<sup>+</sup> 455.2415 (calcd for C<sub>25</sub>H<sub>36</sub>O<sub>6</sub>Na, 455.2409).

**Pseudopterosin Iso-C:** amorphous solid;  $[α]_D^{25} - 130.4$  (*c* 0.05, CHCl<sub>3</sub>); UV (MeOH)  $λ_{max}$  228 (ε 16003), 277 (ε 4266), 284 (ε 3555) nm; IR (film)  $v_{max}$  3394, 2926, 1732, 1456, 1374, 1236 cm<sup>-1</sup>; <sup>1</sup>H (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C (CDCl<sub>3</sub>, 125 MHz) NMR spectra, see Table 2; ESIMS (positive-ion) *m/z* 492 [M + H<sub>2</sub>O]<sup>+</sup>; HRESIMS (positive-ion) *m/z* [M + H]<sup>+</sup> 475.2693 (calcd for C<sub>27</sub>H<sub>39</sub>O<sub>7</sub>, 475.2695).

**Pseudopterosin Iso-D**: amorphous solid;  $[α]_D^{25} - 133.3$  (*c* 0.015, CHCl<sub>3</sub>); UV (MeOH)  $λ_{max}$  228 (ε 15879), 277 (ε 3126), 284 (ε 3056) nm; IR (film)  $v_{max}$  3394, 2926, 1741, 1456, 1373, 1235 cm<sup>-1</sup>; <sup>1</sup>H (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C (CDCl<sub>3</sub>, 125 MHz) NMR spectra, see Table 2; HRESIMS (positive-ion) m/z [M + H]<sup>+</sup> 475.2693 (calcd for C<sub>27</sub>H<sub>39</sub>O<sub>7</sub>, 475.2695).

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## **APPENDICES**

Name	Latitude	Longitude
AB102	26 02.641N	77 28.014W
Burrows North	26 23.506N	77 46.028W
Burrows South	26 20.957N	77 45.034W
Cross Harbor Ridge	25 57.341N	77 20.821W
Gorda Rock	26 06.963N	77 33.684W
Long Rock	26 09.677N	77 34.973W
Sandy Point	26 00.170N	77 23.440W
Freeport	26 42.250N	79 00.200W
Marsh Harbor	26 33.000N	77 03.000W
Little San Salvador	24 35.000N	75 58.000W
Cat Island	29 09.000N	75 32.000W
San Salvador	24 04.000N	74 32.500W
Hog Cay	23 37.000N	75 21.000W
Bimini	25 44.119N	79 18.460W
Pickles	25 00.074N	80 22.400W

Table A1. GPS coordinates of *Pseudopterogorgia* sps. collection sites.

**Table A2.** One-way analysis of variance for FRAP assay of 75% fractions from seven species of the genus *Pseudopterogorgia* shows that means are significantly different amongst the treatments. (Graphpad Prism)

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? ( $P < 0.05$ )	Yes		
Number of groups	24		
F	130.7		
R squared	0.9766		
ANOVA Table	SS	df	MS
Treatment (between columns)	42.22	23	1.836
Residual (within columns)	1.011	72	0.01405
Total	43.23	95	

**Table A3.** One-way analysis of variance for FRAP assay of 15 pseudopterosins isolated from *Pseudopterogorgia elisabethae* shows that means are significantly different amongst the treatments. The water soluble vitamin E analog Trolox serves as the positive control. (Graphpad Prism)

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? $(P < 0.05)$	Yes		
Number of groups	15		
F	31.24		
R squared	0.9358		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.701	14	0.1215
Residual (within columns)	0.1167	30	0.003889
Total	1.818	44	

**Table A4.** One-way analysis of variance for DCFH-DA - zooxanthellae assay of 75% fractions from seven species of the genus *Pseudopterogorgia* shows that means are significantly different amongst the treatments. Cells with no treatment and cells with solvent only served as controls. (Graphpad Prism)

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? ( $P < 0.05$ )	Yes			
Number of groups	16			
F	7.6			
R squared	0.78			
ANOVA Table	SS	df	MS	
Treatment (between columns)	580	17	39	)
Residual (within columns)	160	32	5.1	1
Total	750	47		

**Table A5.** One-way analysis of variance for DCFH-DA - zooxanthellae assay of 15 pseudopterosins isolated from *Pseudopterogorgia elisabethae* shows that means are significantly different amongst the treatments. Cells with no treatment and cells with solvent only served as controls. (Graphpad Prism)

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? ( $P < 0.05$ )	Yes		
Number of groups	20		
F	52		
R squared	0.94		
ANOVA Table	SS	df	MS
Treatment (between columns)	3900	17	210
Residual (within columns)	240	60	4
Total	4100	79	

**Table A6.** Repeated measures analysis of variance for photoinhibition assay shows that means are significantly different amongst the treatments. Cells with no treatment and cells with solvent only served as controls. (Graphpad Prism)

Repeated Measures ANOVA			
P value	< 0.0001		
P value summary	***		
Are means signif. different? ( $P < 0.05$ )	Yes		
Number of groups	6		
F	80.12		
R squared	0.8513		
Was the pairing significantly effective?			
R squared	0.6744		
F	69.61		
P value	< 0.0001		
P value summary	***		
Is there significant matching? ( $P < 0.05$ )	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.3989	5	0.07978
Individual (between rows)	0.9705	14	0.06932
Residual (random)	0.06971	70	0.000996
Total	1.439	89	
**Table A7.** One-way analysis of variance for survival of mouse sympathetic neurons deprived of NGF and treated with three concentrations (10, 20 and 40  $\mu$ M) of pseudopterosin K, ps K 2'ac and ps K 4' ac shows that means are significantly different amongst the treatments.

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? $(P < 0.05)$	Yes		
Number of groups	11		
F	73		
R squared	0.99		
ANOVA Table	SS	df	MS
Treatment (between columns)	36000	10	3600
Residual (within columns)	540	11	49
Total	37000	21	

Table A8. One-way analysis of variance of ROS quantification using MitoSox Red dye.

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? ( $P < 0.05$ )	Yes		
Number of groups	8		
F	39		
R squared	0.83		
ANOVA Table	SS	df	MS
Treatment (between columns)	360000	7	51000
Residual (within columns)	74000	56	1300
Total	430000	63	

Pos.	Ps K 2' Ac		Ps K 4' Ac			
	$\delta_{\rm H}$ (mult., <i>J</i> in Hz)	$\delta_{ m C}$	$\delta_{\rm H}$ (mult., J in Hz)	$\delta_{ m C}$	HMBC	
1	3.62 (br m)	35.6	3.62 (m)	35.9	11, 13, 14	
2α	1.65 (m)	39.6	1.65 (m)	39.8	14	
$2\beta$	1.67 (m)		1.68 (m)			
3	1.55 (m)	30.2	1.57 (m)	30.3	1, 13	
4	2.02 (m)	42.7	2.00 (m)	43.1	13	
5α	1.12 (m)	28.0	1.11 (m)	28.4	4, 7	
$5\beta$	2.17 (m)		2.13 (m)			
6α	2.10 (m)	30.0	2.11 (m)	30.6	4, 8	
$6\beta$	1.44 (m)		1.45 (m)			
7	3.53	26.9	3.43	27.4	5, 8, 9, 13	
	(qdd, 7.55, 6.5, 5.3)		(qdd, 7.2, 6.6, 5.1)			
8	-	133.3	-	133.3	-	
9	-	142.1	-	142.6	-	
10	-	145.1	-	145.6	-	
11	-	121.2	-	121.6	-	
12	-	135.7	-	135.4	-	
13	-	129.9	-	128.9	-	
14	5.09 (d, 9.2)	130.2	5.11 (d, 9.3)	130.1	1, 12, 16, 17	
15	-	129.7	-	130.0	-	
16	1.67 (s)	25.7	1.68 (s)	26.0	14, 17	
17	1.75 (s)	17.8	1.75 (s)	18.0	14, 15, 16	
18	1.04 (d, 6.3)	21.1	1.03 (d, 6.0)	21.4	2, 4	
19	1.17 (d, 7.1)	24.0	1.17 (d, 6.7)	24.4	6, 8	
20	2.02 (s)	10.9	2.02 (s)	11.1	10, 12	
1′	5.07 (d, 4.0)	101.6	5.13 (d, 4.0)	103.2	9, 3', 5'	
2'	5.31 (dd, 10.4, 3.6)	72.5	4.04 (dd, 10.3, 4.0)	69.8	3'	
3'	4.25 (dd, 10.6, 3.6)	69.0	4.35 (dd, 10.4, 3.6)	69.3	2', 4'	
4′	3.98 (bd, 3.0)	72.9	5.29 (bd, 3.7)	73.8	3′, C=O	
5'	4.47 (q, 6.5)	67.6	4.48 (q, 7.0)	66.6	1', 4', 6'	
6′a	1.36 (d, 6.5)	16.3	1.26 (d, 7.0)	16.6	4', 5'	
OAc	2.28 (s)	21.2	2.19 (s)	21.3		
		171.3		172.4		
ОН	7.03 (s)		8.50 (s)		9, 11	

**Table A9.** NMR data for ps K 2' acetate and 4' acetate, ps iso- A, C and D. Recorded in CDCl<sub>3</sub> at 500 MHz (TMS as internal standard), chemical shifts, multiplicity and coupling constants (J, Hz) were assigned by means of <sup>1</sup>H, <sup>13</sup>C NMR and 2D NMR data.

Pos	Ps Iso-A Ps Iso-C			Ps iso-D		
1 05.	$\delta_{\rm H}$ (mult., <i>J</i> in Hz)	$\delta_{ m C}$	$\delta_{\rm H}({\rm mult.}, J {\rm in  Hz})$	$\delta_{ m C}$	$\delta_{\mathrm{H}}(\mathrm{mult.},J\mathrm{in}\mathrm{Hz})$	$\delta_{ m C}$
1	3.60 (br m)	35.3	3.51 (m)	35.7	3.62 (m)	35.6
2α	1.62 (m)	39.4	1.55 (m)	39.7	1.65 (m)	39.7
$2\beta$	1.68 (m)		1.59 (m)		1.66 (m)	
3	1.58 (m)	29.8	1.48 (m)	30.1	1.51 (m)	30.0
4	1.99 (m)	43.4	1.96 (m)	43.8	1.99 (m)	43.7
5α	1.06 (m)	28.2	1.05 (m)	28.4	1.05 (m)	28.3
5β	2.14 (m)		2.08 (m)		2.08 (m)	
6α	2.12 (m)	30.7	2.08 (m)	31.2	2.08 (m)	31.0
6β	1.48 (m)		1.39 (m)		1.39 (m)	
7	3.32 (m)	27.1	3.27 (m)	27.8	3.39 (bq, 6.5)	27.7
8	-	127.0	-	127.9	-	127.9
9	-	144.9	-	145.1	-	145.1
10	-	142.2	-	141.6	-	141.6
11	-	126.9	-	127.3	-	127.3
12	-	134.6	-	135.7	-	135.7
13	-	129.5	-	129.5	-	129.5
14	5.06 (d, 8.9)	130.2	5.02 (d, 9.0)	130.1	5.10 (d, 9.4)	130.1
15	-	128.7	-	129.9	-	129.9
16	1.68 (s)	24.6	1.60 (s)	26.0	1.67 (s)	26.3
17	1.75 (s)	16.5	1.65 (s)	17.9	1.73 (s)	17.9
18	1.04 (d, 6.7)	20.1	0.97 (d, 6.5)	21.4	1.04 (d, 6.1)	21.2
19	1.19 (d, 7.0)	22.3	1.15 (d, 7.0)	23.3	1.23 (d, 7.7)	231
20	2.10 (s)	10.9	2.04 (s)	11.9	2.17 (s)	11.8
1′	4.36 (d, 7.8)	106.9	4.51 (d, 8.0)	106.2	4.60 (d, 7.8)	105.5
2'	3.47 (dd, 9.1, 8.0)	74.1	3.77 (dd, 9.0, 8.0)	72.9	3.81 (dd, 9.5, 8.0)	74.5
3'	3.37 (t, 9.5)	76.6	4.74 (t, 9.5)	79.5	3.75 (t, 9.5)	71.4
4′	3.56 (dd, 10.0, 5.5)	69.6	3.82 (dd, 10.0, 5.5)	68.7	4.90 (dd, 10.0, 5.5)	81.6
5′a	3.14 (dd, 12.0, 11.0)	66.1	3.21 (dd, 12.0,	66.2	3.25 (dd, 11.0,	63.3
5′b	3.90 (dd, 11.8, 5.4)		4.00 (dd, 12.0, 5.5)		4.11 (dd, 11.8, 5.5)	
OAc	-	-	2.13 (s)	21.3	2.11 (s)	21.3
		-		173.3		171.1
ОН	-	-	7.08 (s)	-	6.98 (s)	-



Figure A1. In situ and ex situ photos of specimen of the genus Pseudopterogorgia. a) P bipinnata, b) P elisabethae; c) P hystrix, d) P rigida, e) P acerosa, f) P kallos, g) unidentified species



**Figure A2.** <sup>1</sup>H NMR spectrum of pseudopterosin A (17) in CD<sub>3</sub>OD (500 MHz)



Figure A3. <sup>1</sup>H NMR spectrum of pseudopterosin B (18) in CDCl<sub>3</sub> (500 MHz)



Figure A4. <sup>1</sup>H NMR spectrum of pseudopterosin C (19) in CDCl<sub>3</sub> (500 MHz)



Figure A5. <sup>1</sup>H NMR spectrum of pseudopterosin D (20) in CDCl<sub>3</sub> (500 MHz)





Figure A7. <sup>1</sup>H NMR spectrum of pseudopterosin I (28) in CDCl<sub>3</sub> (500 MHz)



**Figure A8.** <sup>1</sup>H NMR spectrum of pseudopterosin J (**29**) in CDCl<sub>3</sub> (500 MHz)



Figure A9. <sup>1</sup>H NMR spectrum of ps A-D aglycone (26) in CDCl<sub>3</sub> (500 MHz)



Figure A10. <sup>1</sup>H NMR spectrum of ps G-J aglycone (34) in CDCl<sub>3</sub> (500 MHz)







Figure A12. <sup>1</sup>H NMR spectrum of 2'Acetate ps K (36) in CDCl<sub>3</sub> (500 MHz)



Figure A13. <sup>1</sup>H NMR spectrum of 4'Acetate ps K (37) in CDCl<sub>3</sub> (500 MHz)



Figure A14. If third spectrum of iso-ps A (50) in CD<sub>3</sub>OD (500 MHz





Figure A16. <sup>1</sup>H NMR spectrum of *iso*-ps D (33) in CDCl<sub>3</sub> (500 MHz)