DITERPENE BIOSYNTHESIS IN EARLY ONTOGENETIC STAGES OF PSEUDOPTEROGORGIA ELISABETHAE AND PSEUDOPEROGORGIA BIPINNATA

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Diterpene Biosynthesis in Early Ontogenetic Stages of *Pseudopterogorgia elisabethae* and *Pseudoperogorgia bipinnata*

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Russell G. Kerr, Department of Chemistry and Biochemistry, and has been approved by the members of her supervisory committee. It was submitted to the faculty of The Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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There is growing evidence that secondary metabolites isolated from some marine invertebrates may actually be produced by a symbiont. The capacity of early ontogenetic stages of *Pseudopterogorgia elisabethae* and *Pseudopterogorgia bipinnata* to biosynthesize biologically active and pharmaceutically important diterpenes was examined. These early life stages lack the algal symbionts found in adult colonies of the species, thus removing one level of complexity. The larvae and polyps of these two species produced high concentrations of the diterpenoid secondary metabolites, one of which proved to significantly deter fish-feeding in *in situ* feeding assays. Additionally, a novel cembrenoid diterpene was isolated from *P. bipinnata*. The structure of this compound, bipinnatolide L (**40**) was solved using 1D and 2D NMR experiments.

To Dr. Kalinath Mukherjee, we were but stones, your light made us stars

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Introduction and Background

1.1-Natural products

Mankind has 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).

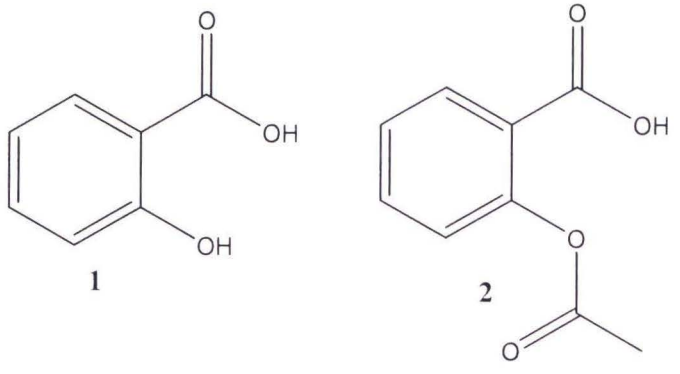
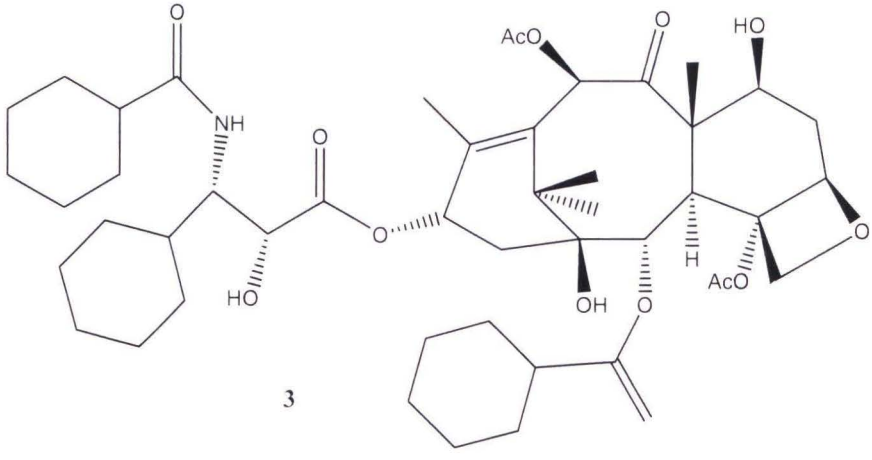


Figure 1. Structure of salicylic acid (**1**) and acetylsalicylic acid (**2**).

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®, **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.



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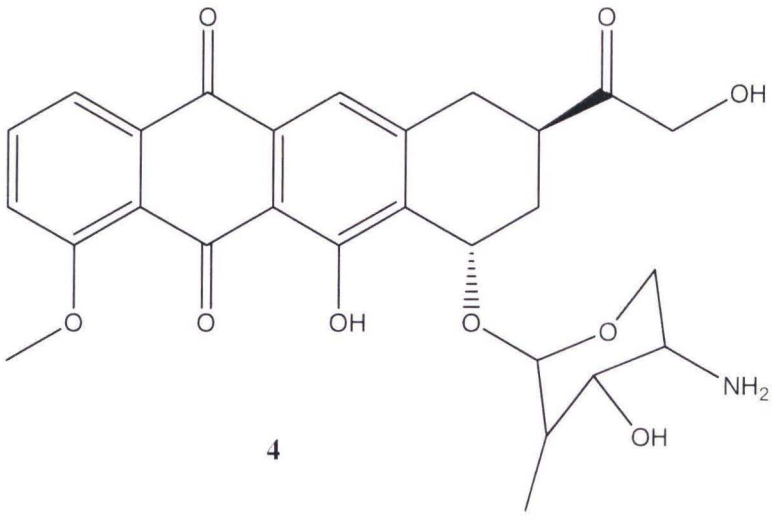


Figure 2. Structures of paclitaxel (**3**) and doxorubicin (**4**).

1.2-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 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®, **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).

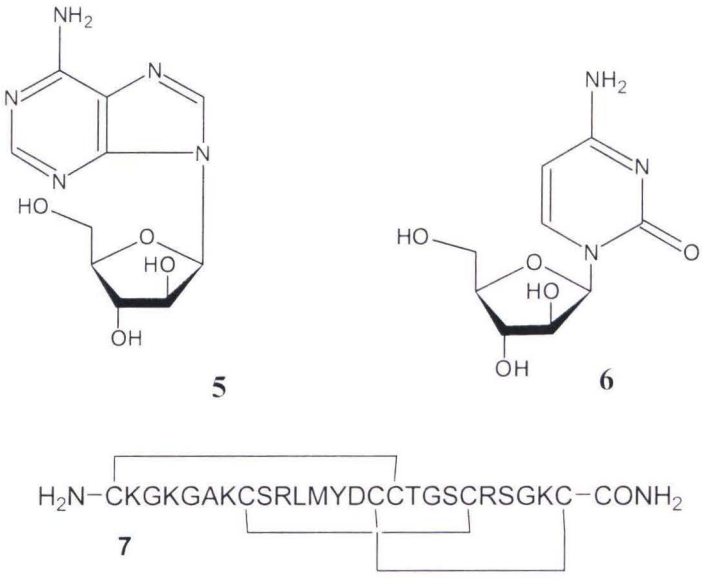


Figure 3. structures of ara-A (**5**), ara-C (**6**) and Prialt® (**7**)

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®). 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).

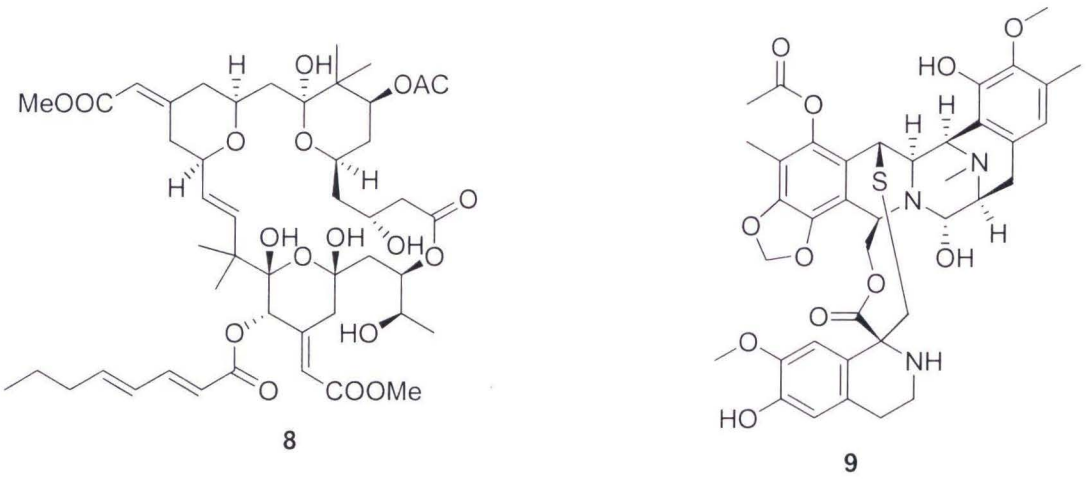


Figure 4. Structures of bryostatin 1 (**8**) and ecteinascidin-743 (**9**).

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 anti-inflammatory 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 slow-moving marine invertebrates that lack morphological defense structures such as spines or a protective shell is no coincidence but reflects the ecological importance of these constituents 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^2 per day (Carpenter, 1986). With this intense selective pressure only those organisms will survive that can rely 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 inter-species 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 for protection (Faulkner, 2000).

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; Van Alstyne and Paul, 1992; 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 (Burkholder and Burkholder, 1958; 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 Carribbean 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 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_{50} values 0.9, 3.2, and 1.5 μg/mL, respectively. Additionally, bipinnatin B was found to inhibit [^{125}I]-α-toxin binding to intact cells and block α-toxin binding to detergent-extracted receptor. Bipinnatin B also inhibits [^{125}I]-α-toxin binding to receptor-rich

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

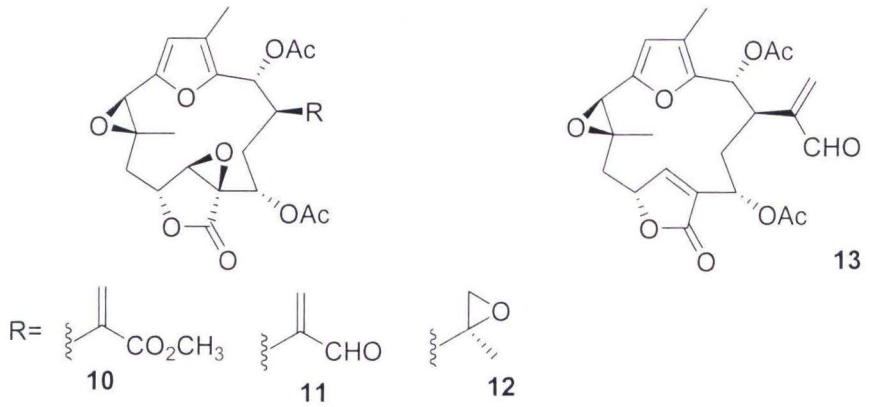


Figure 5. Structures of bipinnatans A-D (**10-13**).

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).

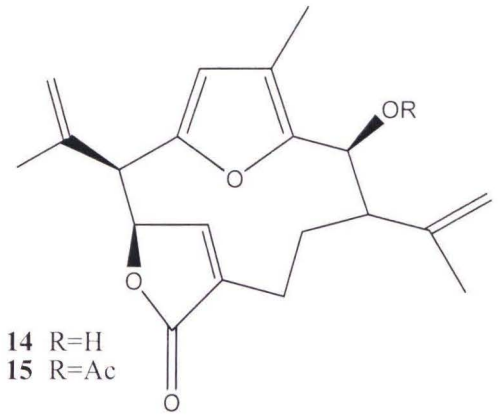
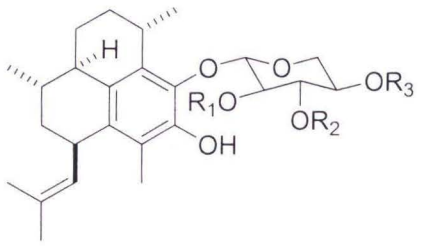


Figure 6. Stuctures of kallolide A (**14**) and kallolide A acetate (**15**).

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 isolated 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 possess 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).



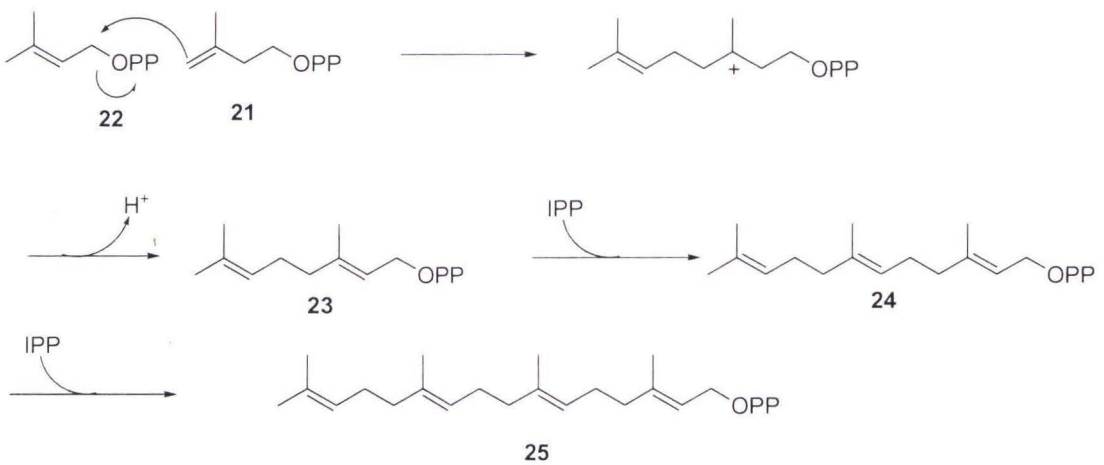
17 R_1= R_2= R_3=H
18 R_1= Ac, R_2= R_3=H
19 R_2= Ac, R_1= R_3=H
20 R_3= Ac, R_1= R_2=H

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

1.3-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 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}).



Scheme 1. 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).

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. ^{3}H or ^{14}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 [^{3}H-C_1]-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.

1. 4-Localization of biosynthesis

There is growing evidence that secondary metabolites isolated from some marine invertebrates may actually be produced by a symbiont. Structurally similar or identical

compounds have been isolated from marine invertebrates and terrestrial microorganisms (Anthoni et al. 1990; Kobayashi and Ishibashi 1993). Production of some metabolites has been localized to symbiont via the physical separation of the symbiont from host cells (e.g., Faulkner et al., 1999) and microorganisms cultured from host material have produced compounds identical to those isolated from the host (Stierle et al., 1988; Elyakov et al., 1991).

Symbiotic bacterium on the embryos of the shrimp *Palaemon macrodactylus* and the lobster *Homarus americanus* produced compounds that prevent infection by the pathogenic marine fungus *Lagenidium callinectes* (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). There are two examples of symbiont producing an anti-predator chemical defense for its host. The first example is pederin, a complex polyketide isolated from rove beetles (*Paederus spp.*), which deters predation on *Paederus* eggs and larvae and is produced by a symbiotic bacterium (Kellner and Dettner, 1996; Kellner, 2001& 2002). Interestingly the structure of pederin is nearly identical to that of the mycalamides isolated from the marine sponge *Mycalie sp.* and onnamides isolated from the marine sponge *Theonella swinhoei*. The second example is the bryostatins, which are produced by a bacterial symbiont of the bryozoan *Bugula neritina* and have been shown to defend *B. neretina* larvae against predation (Lopanik et al., 2004; Haygood and Davidson, 1998; Haygood et al., 1999; Davidson et al., 2001).

The structural complexity and low natural concentration of many marine derived natural products frequently limit their commercial potential. It is not unusual that

bioactive natural compounds are discarded from clinical or preclinical trials due to one or both of these reasons. If symbionts are indeed responsible for the biosynthesis some of these pharmacologically promising secondary metabolites, then an effective solution to the supply problem may lie in the culture of these symbionts on a large scale.

1.5- Research objectives

The first objective of this project was to ascertain whether the azooxanthellate larvae of the sea whips *Pseudopterogorgia elisabethae* and *P. bipinnata* contain the diterpene glycosides associated with the zooxanthellate adults colonies collected in the same location, and the biosynthetic capacity of the larvae was examined via incubations with a radiolabeled precurser. The second objective was to test whether the pseudopterosins A-D and the pseudopterosin aglycone, diterpenes from *P. elisabethae* collected in the Bahamas, functioned as predator deterrents. Finally, characterization and structure elucidation was completed on a previously undescribed diterpene isolated from the CH_2Cl_2 fraction from *P. bipinnata*.

Chapter 2

Diterpene Biosynthesis in Azooxanthellate *Pseudopterogorgia elisabethae* and *Pseudopterogorgia bipinnata*

2. 1- Terpene biosynthesis in azooxanthellate gorgonian tissue

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; 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.

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.

2. 2- Biosynthetic studies of larvae from *P. elisabethae*

P. elisabethae larvae were collected by SCUBA from Little Bahamas Bank, Bahamas in November of 2003. Larvae were maintained in Millipore filtered seawater in a growth incubator, at a constant temperature of 26°C with a 12/12 light/dark cycle. After 72 hours larvae were subjected to chemical analysis to determine concentrations of pseudopterosins A-D (**17-20**). Larvae were extracted in MeOH/H_2O (9:1) (at room temperature for ~ 1hr) and partitioned with hexanes. The aqueous partitions were then

adjusted to MeOH/ H_2O (1:1), and partitioned a second time with CH_2Cl_2. HPLC conditions for isolation of pseudopterosins from *P. elisabethae* larval CH_2Cl_2 extract used a mobile phase gradient from hexanes/EtOAc (60:40) to 100% hexanes with detection at λ=283 nm.

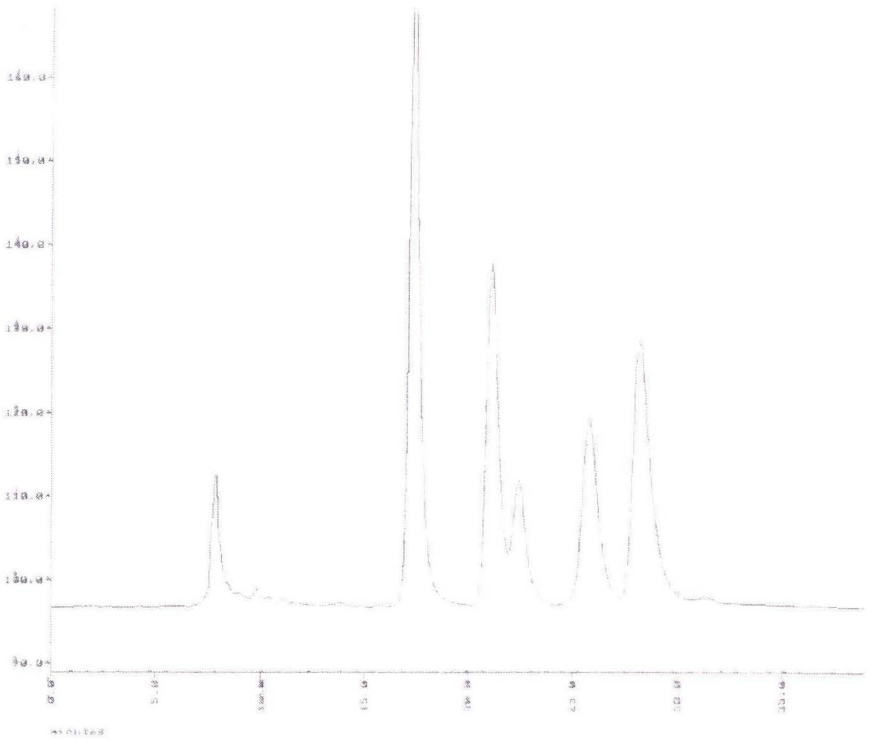


Figure 8. Normal phase HPLC of CH_2Cl_2 extract from *P. elisabethae* larvae. Peak at 17 min is pseudopterosin C, 21 min is pseudopterosin D, 26 min pseudopterosin B and 27 min pseudopterosin A (Mydlarz et al, 2003)

Concentrations of pseudopterosins in *P. elisabethae* adult holobiont and isolated *Symbiodinium* sp. cells collected in Sweetings Cay, Bahamas have been previously reported (5% of the lipid extract in holobiont, 11% of the algal lipid extract, Mydlarz et

al, 2003). Concentrations of pseudopterosins in the larvae were found to be markedly higher (table 1).

Larvae[1]	Zooxanthellae[2]	Holobiont[2]
30	11	5

1. based on an average of replicate experiments
2. as reported in the literature (Mydlarz et al, 2003)

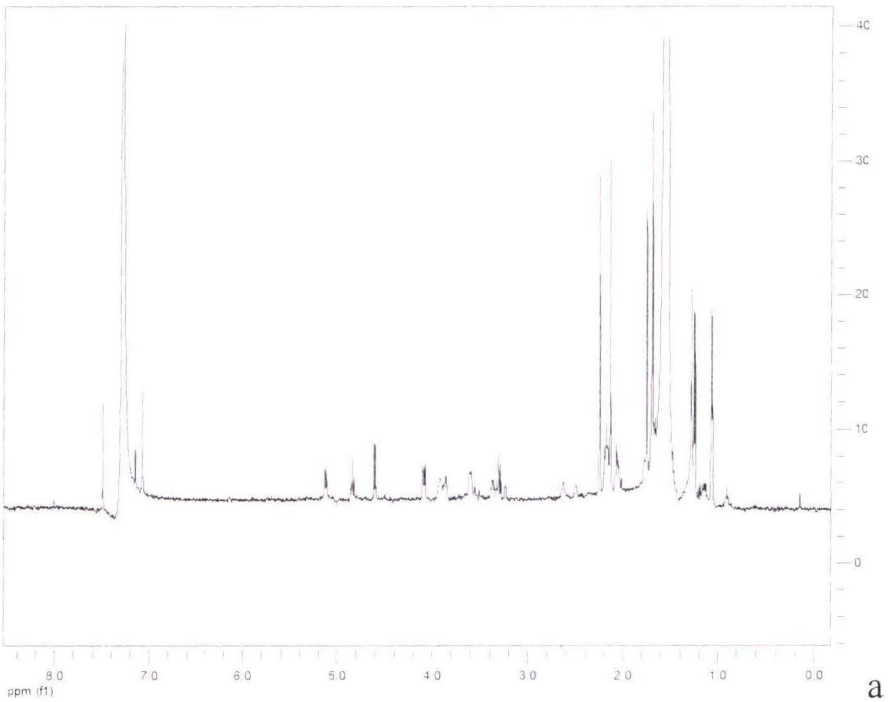
Table 1. Comparison of pseudopterosin content expressed as % organic extract.

To probe for biosynthetic capacity, the larvae were incubated with 4 μCi [^{3}H-C_1]-GGPP for 48 hr. Purification of the pseudopterosins by HPLC (as described above) and analysis by scintillation counting indicated that pseudopterosins A–D from the larvae were radioactive (table 2).

	mgs	% of organic	DPM	DPM/mmol
PsC	0.39	10.60%	14970	1.80E+07
PsD	0.24	6.45%	7660	1.50E+07
PsB	0.2	5.57%	7100	1.70E+07
PsA	0.29	7.77%	7520	1.10E+07

Table 2. Pseudopterosins A-D recovered from *P. elisabethae* extract.

Pseudopterosin C was further purified by RPHPLC using a gradient of acetonitrile/water (50-100%) and the ^{1}H-NMR (fig. 9) was compared with the literature to confirm the structure (Look et al, 1986).



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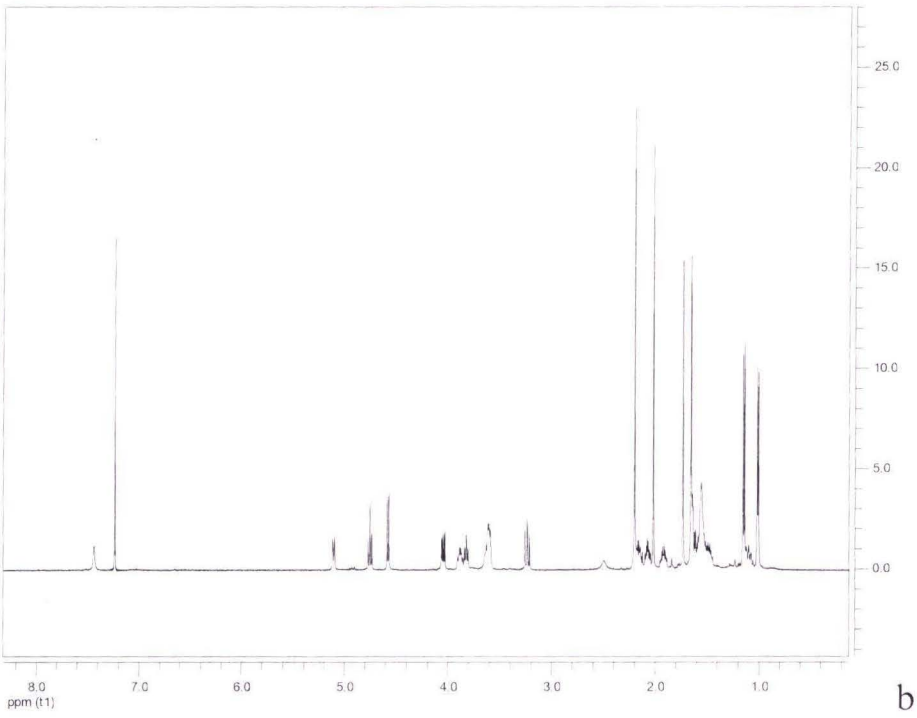

Figure 9. ^{1}H-NMR of pseudopterosin C from *P.elisabethae* larvae (a) and holobiont (b).

To ensure that radioactivity was due to the incorporation of the ^{3}H-GGPP rather than a coeluting impurity, larval pseudopterosin C was derivatized according to previously published methods (Mydlarz et al, 2003) and the derivative examined. A base hydrolysis converted pseudopterosin C to pseudopterosin A. The pseudopterosin A was then purified by normal and reversed phase HPLC (as described above) and radioactivity was assessed by scintillation counting. The specific activity remained in the same order of magnitude from product to reactant (6.05 x 10^{6} DPM/mmol for pseudopterosin C and

1.64 x 10^6 DPM/mmol for hydrolyzed product) confirming that the pseudopterosin C was radioactive.

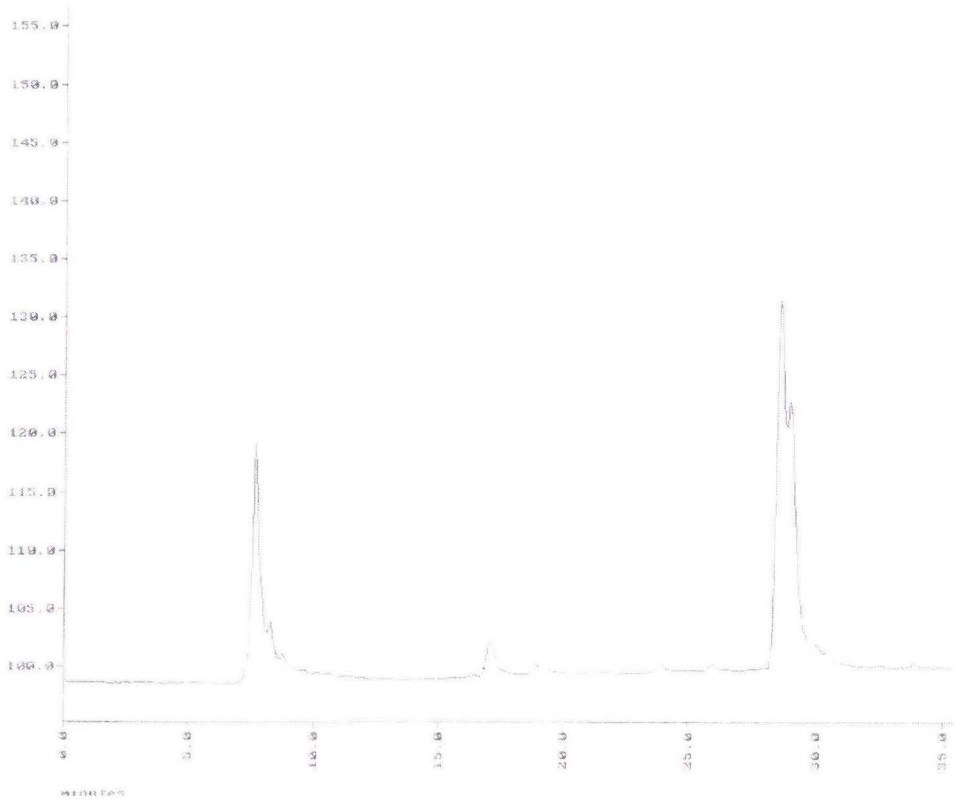

Figure 10. Normal phase HPLC purification of hydrolyzed pseudopterosin C. Peaks at ~27 min correspond to epimers of pseudopterosin A, the products of the hydrolysis reaction. Small peak at ~17 minutes is unreacted pseudopterosin C.

In November of 2004, *P. elisabethae* colonies were collected from Long Key, Florida by SCUBA at a depth of 25 m. These adult *P. elisabethae* colonies were transported to the Gumbo Limbo Environmental Center, in Boca Raton Florida, where

they were maintained in flowing seawater at ambient temperature and natural light. In the week following the new moon of November, these adult colonies spawned viable larvae. The larvae were carefully removed by pipette from the surface of the colonies and transported back to the lab in buckets of seawater. Larvae were maintained in MFSW in a growth incubator, at a constant temperature of 26°C with a 12/12 light/dark cycle. Larvae were subjected to chemical analysis (in the manner described above) to probe for the presence of pseudopterosin Y (**26**), the predominant congener in adult colonies collected in this location. The normal phase HPLC chromatogram of the analysis CH_2Cl_2 fraction was nearly identical to that of the adult standard (fig.11), with a major peak corresponding to pseudopterosins Y with the correct retention time.

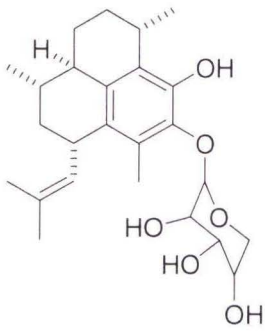

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Figure 11. Structure of pseudopterosin Y (**26**)

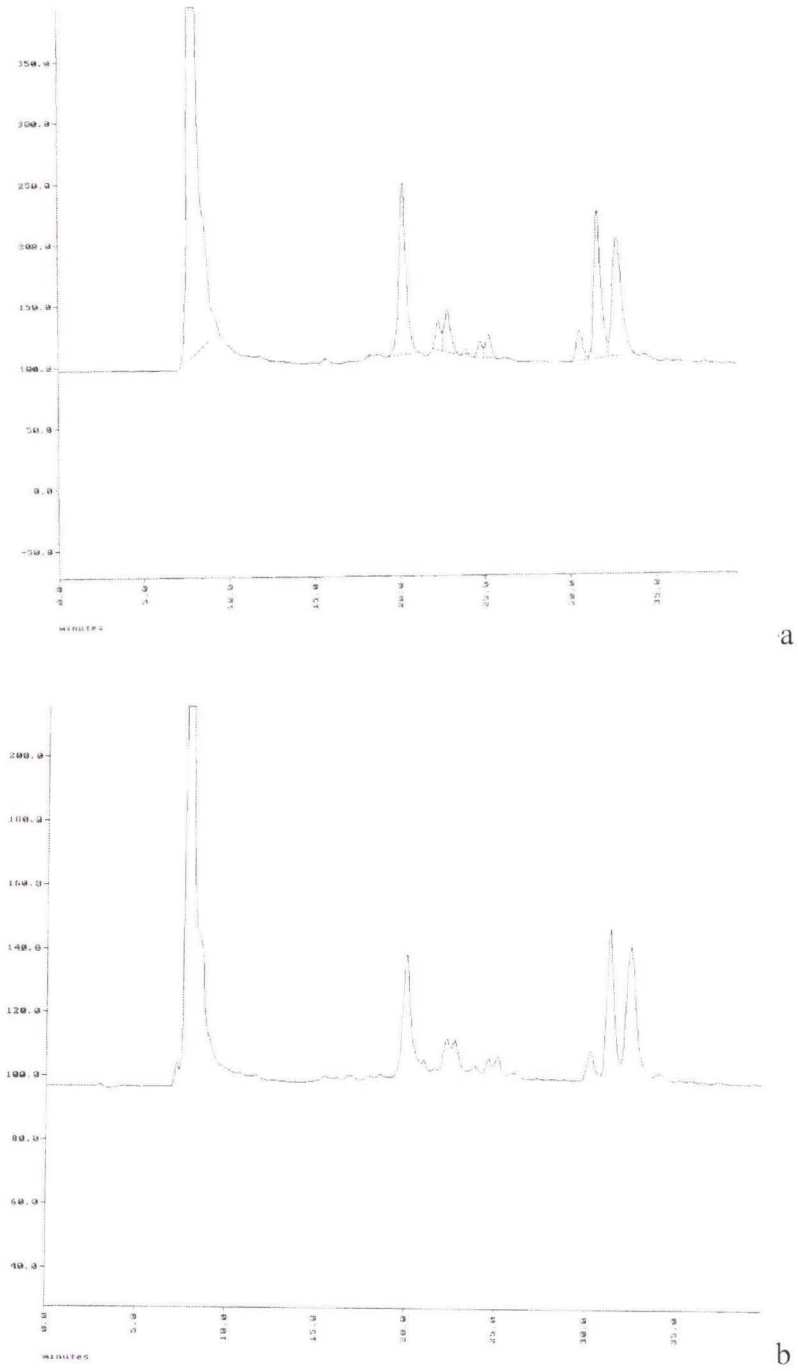


Figure 12. Normal phase HPLC of CH_2Cl_2 fraction from *P. elisabethae* larvae (a) and adult colony (b) collected in Long Key, Fl.

The biosynthetic capacity of these Keys larvae was tested in the same manner as the Bahamian larvae (described above). Purification of pseudopterosin Y by HPLC and analysis by scintillation counting indicated that it was radioactive. Pseudopterosin Y was further purified by RPHPLC but due to the low yield (0.13mg) it was not possible to obtain a ^{1}H-NMR of the compound. Specific activity of the isolated pseudopterosin Y was 1.17×10^{7} DPM/mmol.

The presence of two biosynthetic precursors in the hexanes partition of the larval extract (from both locations) elisabthaetriene and erogorgiaene (see scheme 2) was confirmed using the GC-MS (fig. 13). Retention times on the GC and mass spectra for these two compounds were compared to standards for confirmation.

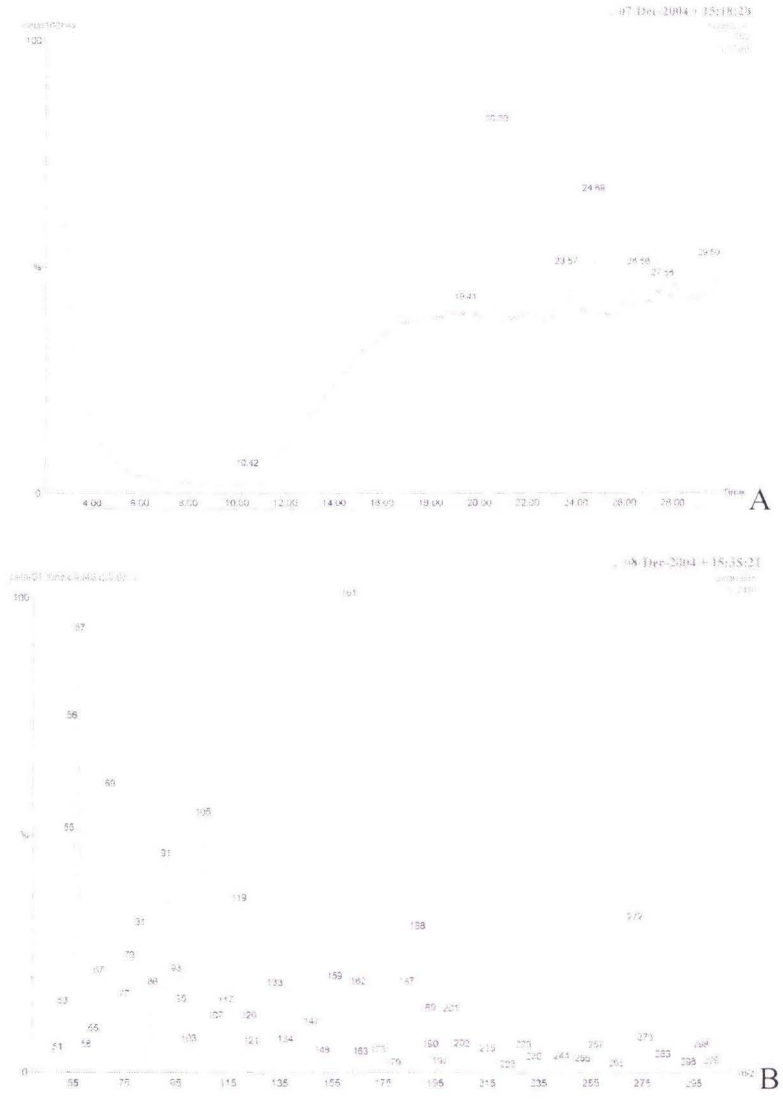


Figure 13. (A) Gas chromatogram of 100% hexanes fraction of *P. elisabethae* larvae from Little Bahamas Bank, (B) Mass spectrum of peak at ~24 min. MW=272

Based on the results of these experiments, we conclude that the larvae of *P. elisabethae* harbor the ability to biosynthesize pseudopterosins.

2. 3-Biosynthetic Studies of larvae from *P. bipinnata*

P. bipinnata larvae were collected from Long Key, Fl in December 2004. TLC analysis of adult colonies revealed that all spawning adults exhibited chemistry associated with chemotype A. Normal phase HPLC purification was performed on the *P. bipinnata* larval CH_2Cl_2 extract using a silica column and a mobile phase gradient from 100% hexanes to hexanes/EtOAc (50:50) with UV detection at λ=270nm.

Chemical analysis of the CH_2Cl_2 extract from the larvae yielded the known compounds kallolide A (**14**) and kallolide A acetate (**15**) based on normal phase HPLC analysis and previously established retention times.

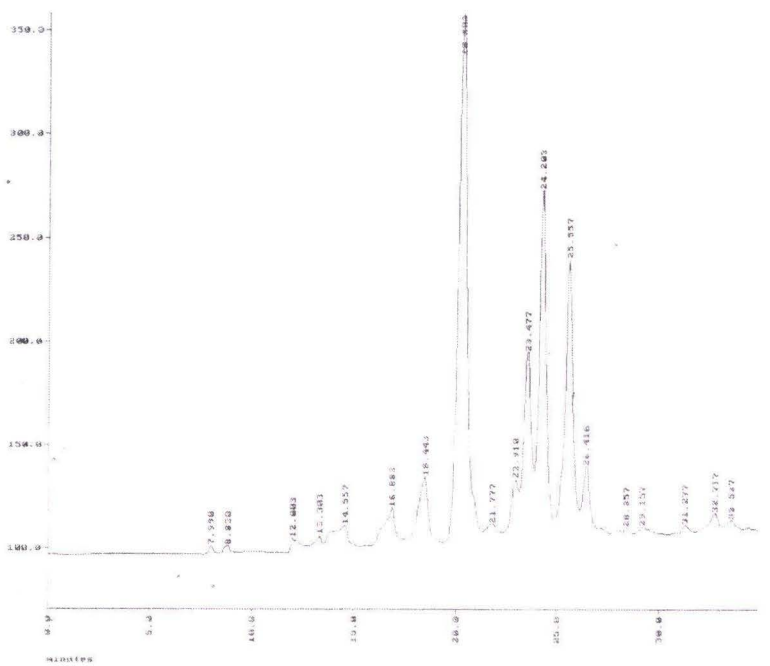


Figure 14. Normal phase HPLC of CH_2Cl_2 fraction from *P. bipinnata* larvae. Peak at ~24 min is kallolide A and peak at ~25min is kallolide A acetate.

Concentration of kallolide A acetate in the larval organic extract was found to be higher than extracts from either the holobiont or isolated zooxanthellae.

Larvae[1]	Polyps[1]	Zooxanthellae[2]	Holobiont[2]
23.5	18.7	7.3	4.3

1 based on average of replicate experiments
2 as reported in the literature (Boehnlein et al, 2005)

Table 3. Comparison of kallolide A Acetate content expressed as % organic extract.

Larvae were maintained in MFSW and incubated with 4 μCi ^{3}H-GGPP for 48 hr. Kallolide A acetate was purified from the CH_2Cl_2 fraction using normal phase HPLC. Kallolide A acetate comprised roughly 23.5% of the larvae organic extract. The presence of kallolide A acetate was confirmed by comparing ^{1}H-NMR spectra (fig. 14) to those reported in the literature (Look et al., 1985).

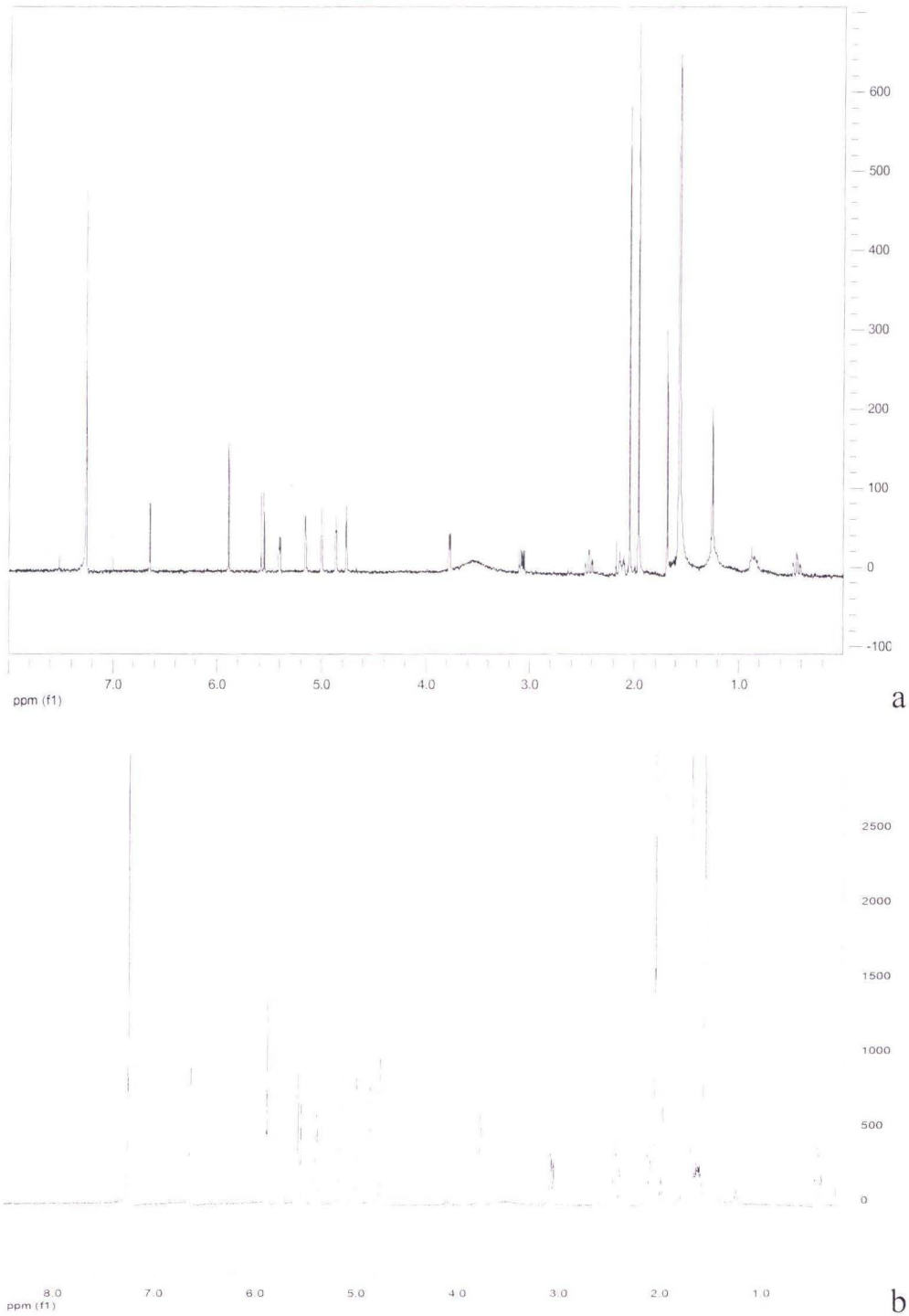


Figure 15. ^{1}H-NMR of kallolide A acetate from *P.bipinnata* larvae (a) and holobiont (b).

Radioactivity was assessed by scintillation counting, with the average specific activity from kallolide A acetate samples counted equal to 2.3 x 10^6 DPM/mmol (table 4).

	mgs	% of organic	DPM	DPM/mmol
Kal A ac	0.8	18.9	5250	2.33E+06
Kal A	0.9	21.2	5460	1.99E+06

Table 4. Kallolide A and kallolide A acetate recovered from *P. bipinnata* larval extracts.

To assess radiochemical purity of kallolide A acetate (**15**), a derivatization reaction was performed. Following previously established methodology (Rodriguez et al, 2000), we performed catalytic hydrogenation and hydrogenolysis of the radiolabelled kallolide A acetate (fig. 15) and obtained the hexahydro product (**27**) (confirmed by ^{1}H-NMR).

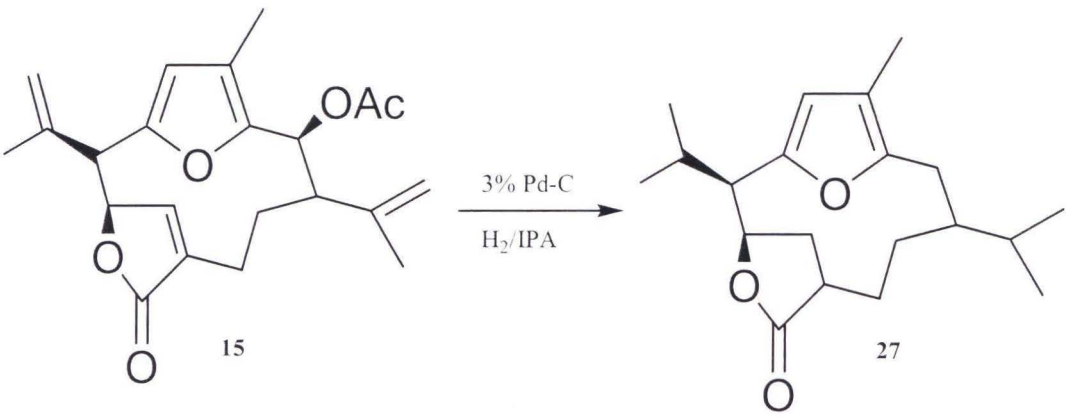


Figure 16. Hydrogenation/hydrogenolysis reaction converts kallolide A acetate (**15**) to the derivative **27**.

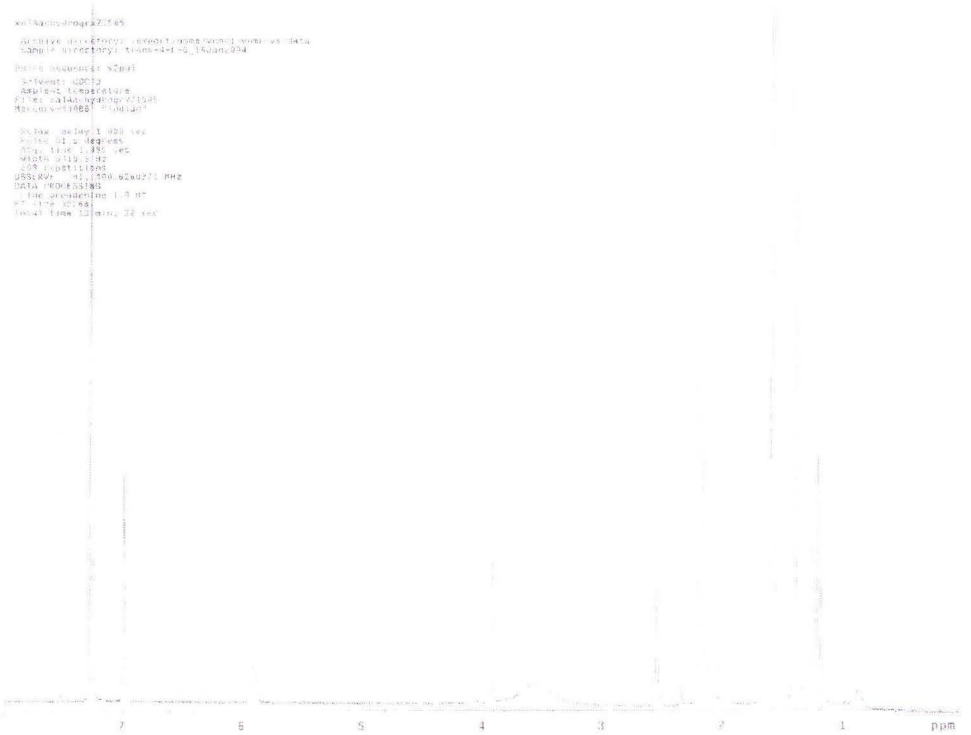


Figure 17. ^{1}H-NMR of hydrogenation/hydrogenolysis reaction product.

The specific activity of the derivative was determined to be 2.12 x 10^6 DPM/mmol.

Larvae were maintained in MFSW. These settled and metamorphosed after 11 days. Five polyps were extracted in the same fashion as the larvae. HPLC chromatograms of the polyp CH_2Cl_2 fraction peaks characteristic of those found in the HPLC chromatograms of the adult *P. bipinnata* extracts.

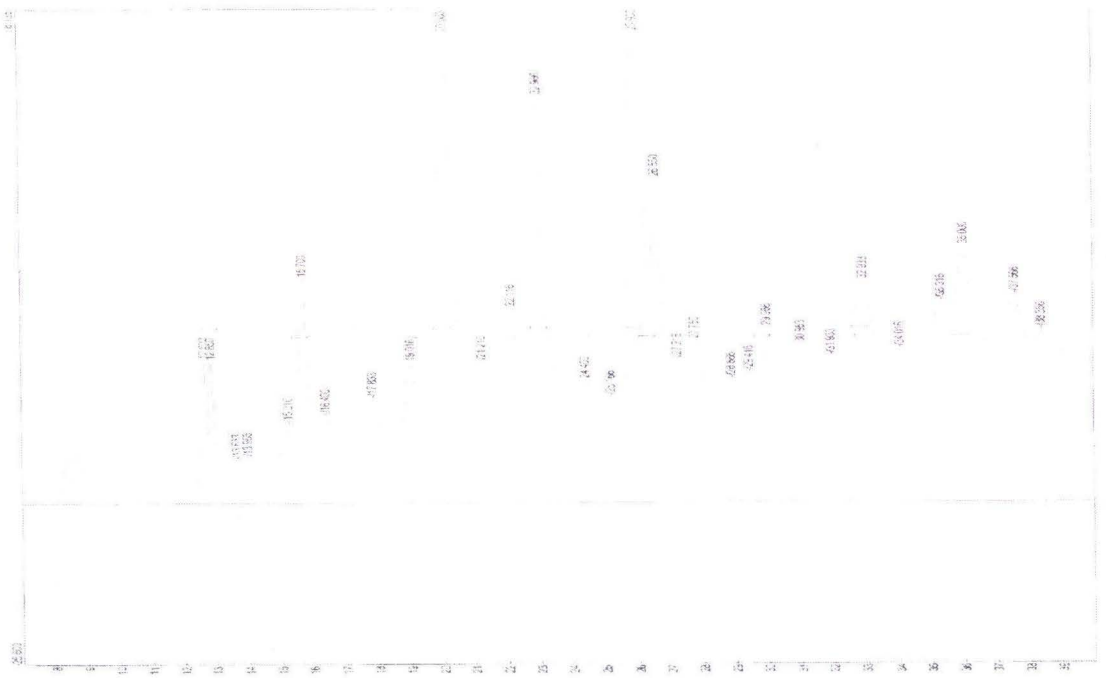


Figure 18. Normal phase HPLC of CH_2Cl_2 fraction from *P. bipinnata* polyps. Peak at ~25 min is kallolide A and peak at ~26min is kallolide A acetate.

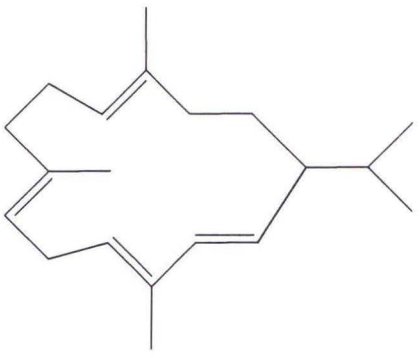
To confirm the biosynthesis of these diterpenes in the polyps, 10 polyps were incubated with 4 μCi ^{3}H-GGPP for 48 hr, and extracted and analyzed in the same manner as the larvae. Radioactivity of the kallolide A acetate was assessed by scintillation

counting. Specific activity of the kallolide A and kallolide A acetate isolated from the polyps was the same order of magnitude as that from the larvae (table 5).

Specific Activity (DPM/mmol)	*P.b.*larvae	*P.b.*polyps
Kallolide A Acetate	2.3 E6	3.7 E6
Kallolide A	1.99 E6	3.2 E6

Table 5. Specific activity data for kallolide A and A acetate from *P. bipinnata* larvae and polyp extracts

Additionally, cembrene (**28**) and neocembrene (**29**), diterpene hydrocarbons assumed to be the first committed step in the biosynthesis of kallolide diterpenes, are present in the hexanes partition of the larvae and polyp extracts. This was confirmed by GC-MS.



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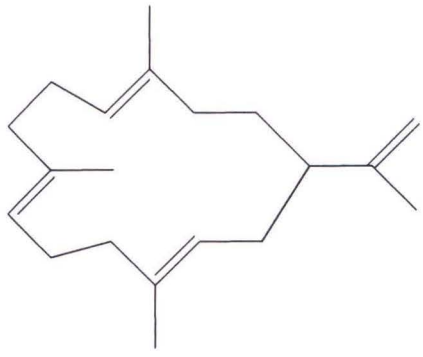

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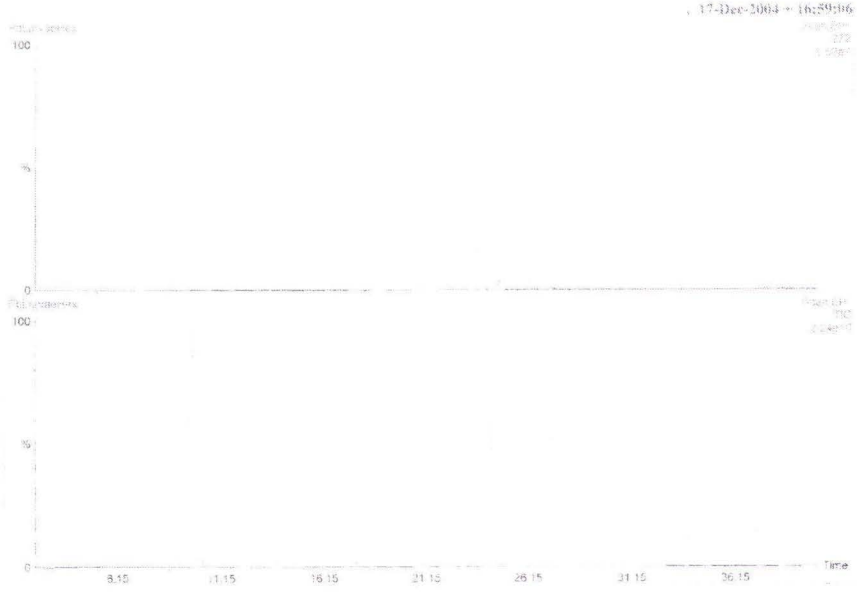
Figure 19. Structures of cembrene (**28**) and neocembrene (**29**)



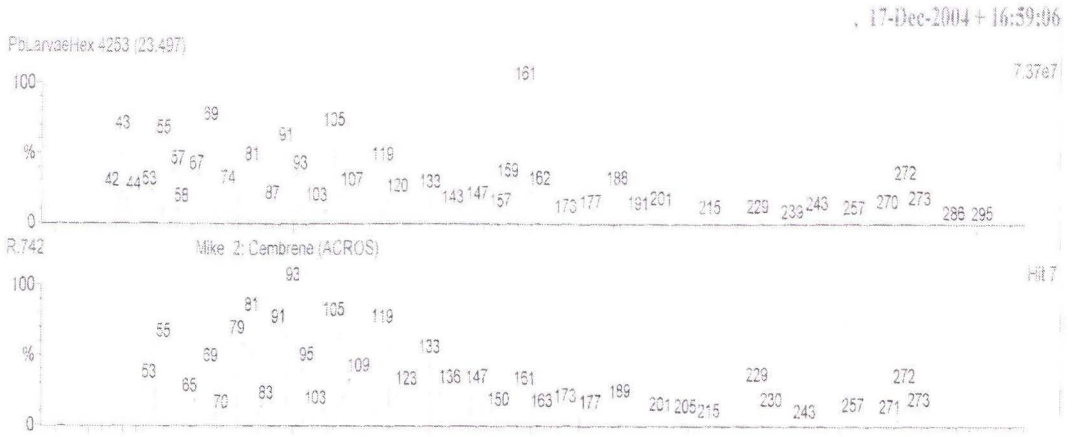


Figure 20. Gas chromatogram of the 100% hexanes fraction of *P. bipinnata* larvae and mass spectrum of the diterpene hydrocarbon cembrene

Neocembrene was purified by HPLC and RPHPLC from the larvae hexanes partition, and its radioactivity assessed by scintillation counting. The specific activity of the neocembrene was found to be 2.1×10^6 DPM/mmol. Neocembrene was present as 2.3% of the larvae hexanes partition.

Based on the results of these experiments, we conclude that the larvae and polyps of *Pseudopterogorgia bipinnata* harbor the ability to biosynthesize diterpenes of the kallolide family.

2.4- DISCUSSION

Analysis of CH_2Cl_2 extracts from *P. elisabethae* larvae and *P. bipinnata* larvae and polyps revealed that these contain diterpenes previously reported from mature organisms of these species. Biosynthetic experiments were carried out with [^{3}H-C_1] - GGDP, the ubiquitous diterpene precursor. The recovered diterpenes (pseudopterosin C from Bahamian *P. elisabethae* and kallolide A acetate from *P. bipinnata*) were radioactive, and derivatization and monitoring of specific activity of these metabolites demonstrated their radiochemical purity. 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. Structure of parent compounds and derivatives were confirmed by ^{1}H- NMR.

Recent research has shown that diterpene biosynthesis occurs within the algal symbionts of these two species of *Pseudopterogorgia* (Mydlarz et al, 2003 and Boehnlein et al., 2005). Research presented here shows that diterpene biosynthesis occurs in the azooxanthellate larvae of both of these species. Together this evidence suggests that perhaps these diterpenes are produced by microbes symbiotic to or persistently associated with both the coral and the algae.

Another possible explanation is that there has been some form of horizontal gene transfer to the genomes of both the coral species and the algal symbionts. Horizontal gene transfer

involves the transmittal of genetic material between cells or genomes belonging to unrelated species, by processes other than normal reproduction. Bacteria have been known to exchange genes across species barriers in nature. There are three ways in which this is accomplished. In conjugation, genetic material is passed between cells in contact; in transduction, genetic material is carried from one cell to another by infectious viruses; and in transformation, the genetic material is taken up directly by the cell from its environment (Lorenz and Wackernagel, 1994). For horizontal gene transfer to be successful, the foreign genetic material must become integrated into the cell's genome, or become stably maintained in the recipient cell in some other form. In most cases, foreign genetic material that enters a cell by accident, especially if it is from another species, will be broken down before it can incorporate into the genome. Under certain ecological conditions which are still poorly understood, foreign genetic material escapes being broken down and becomes incorporated in the genome.

While horizontal gene transfer is well-known among bacteria, it is only within the past 10 years that its occurrence has become recognized among higher plants and animals (Ho et al., 1998). The scope for horizontal gene transfer is essentially the entire biosphere, with bacteria and viruses serving both as intermediaries for gene trafficking and as reservoirs for gene multiplication and recombination. There are many potential routes for horizontal gene transfer to plants and animals. Transduction is expected to be a main route as there are many viruses which infect plants and animals (Ho et al., 1998).

Further research in this area should include experiments designed to further localize the biosynthesis of these pharmacologically promising diterpenes to the source organism using such techniques as antimicrobial knockout experiments like those employed in the studies of pederin biosynthesis (Kellner, 2002) and bryostatin byosynthesis (Lopanik et al., 2004), molecular techniques such a DGGE to identify associated microbes (Haygood et al., 2001), and the physical separation of the symbiont from host cells (e.g. Faulkner et al., 1999) and culturing of microorganisms from host material to probe for compounds isolated from the holobiont (Stierle et al., 1988; Elyakov et al., 1991).

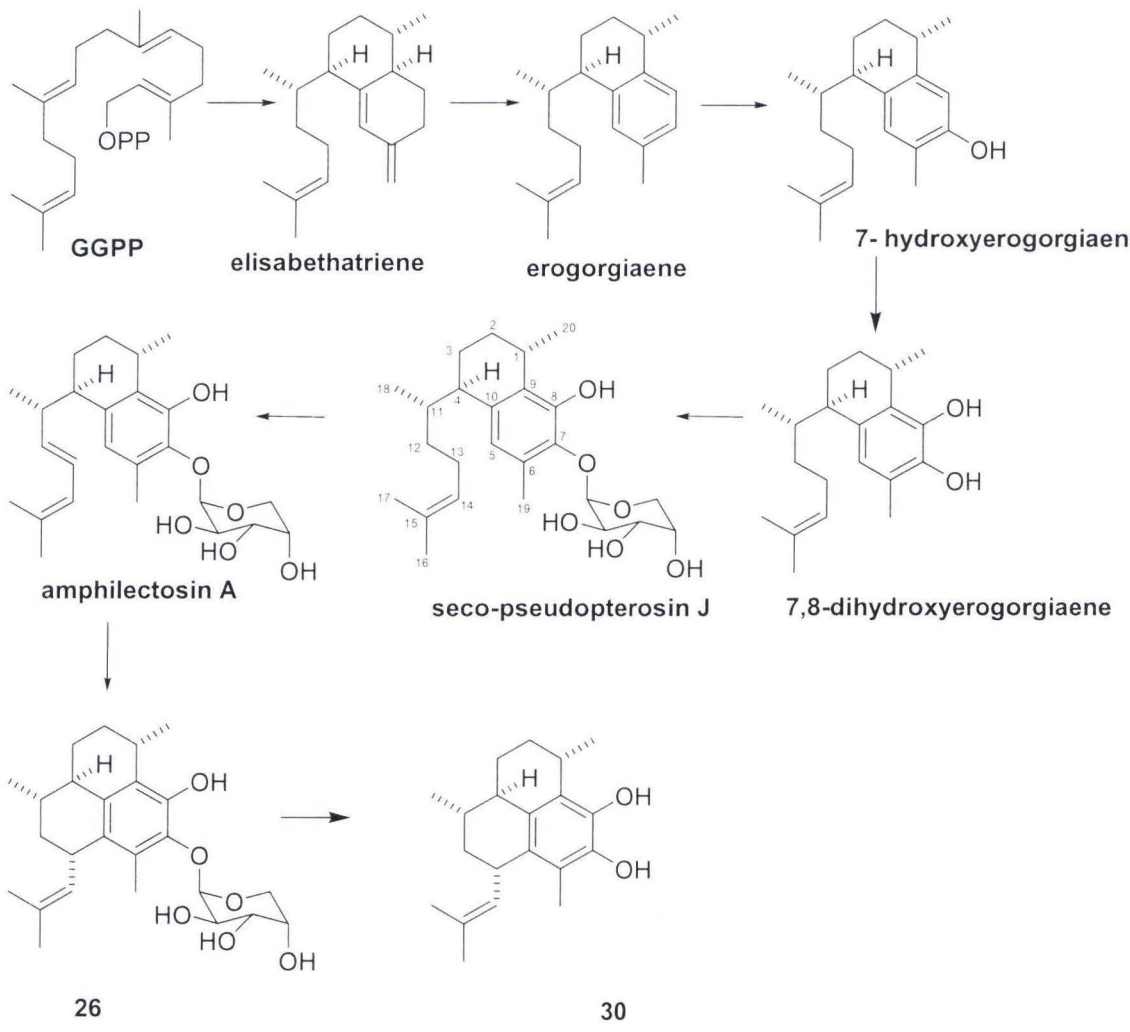
Chapter 3

Icthyodeterrent activity of diterpenes isolated from *Pseudopterogorgia elisabethae*

3. 1- Ecological roles of natural products

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 were 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).

Experiments done to elucidate the biosynthetic pathway of pseudopterosins in *Pseudopterogorgia elisabethae* present evidence that the pseudopterosin aglycone (**30**) is the end product of pseudopterosin biosynthesis (scheme 2) and thus may be an active natural product *in vivo* (Ferns, 2005).



Scheme 2. Pseudopterosin biosynthesis.

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 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 organisms as well. Van Alstyne and Paul (1992) found that most species of *Halimeda* on Guam immediately convert the less-deterrent 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).

3.2-Icthyodeterrence of pseudopterosins

In order to test whether the pseudopterosins or the pseudopterosin aglycone (**30**), or both were active as feeding deterrent metabolites, *in situ* feeding assays were conducted. Pseudopterosins A – D (**17**-20) were purified from *P. elisabethae* collected in Sweetings Cay, Bahamas by normal phase HPLC. Due to the difficulty in isolating sufficient quantities of aglycone, the pseudopterosin aglycone was synthesized by acid hydrolysis.

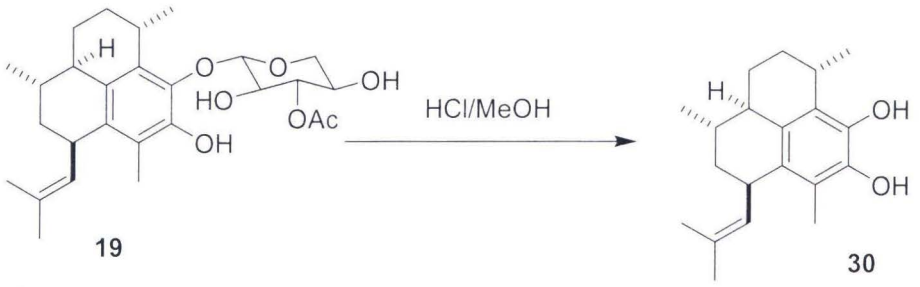
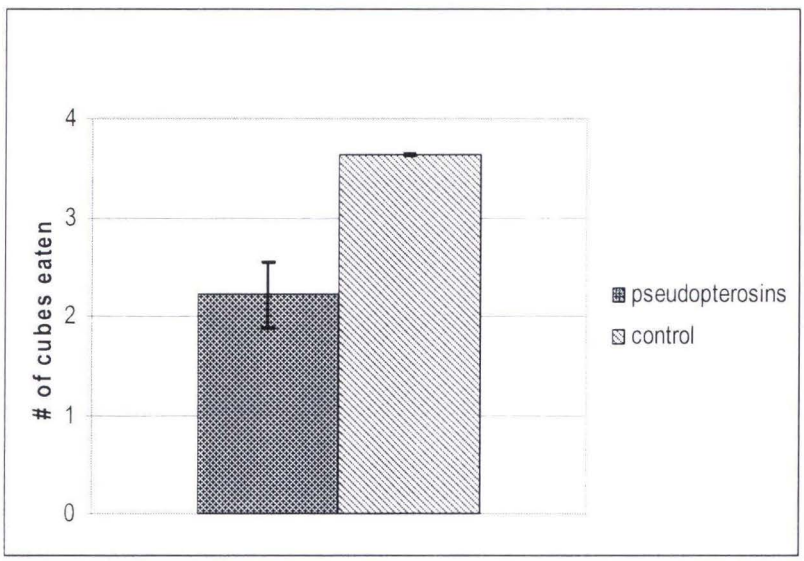


Figure 21. Acid hydrolyzed conversion of pseudopterosin C (**19**) to pseudopterosin aglycone (**30**).

The deterrence capacity of pseudopterosins A-D and the pseudopterosin aglycone was tested by incorporating these metabolites into artificial carrageenan-based diets. Harvell et al. (1988) described similar assays conducted in the Caribbean on extracts from the gorgonian *B.asbestinum*. Paired feeding experiments were conducted on a shallow patch reef (~3 m) at Sweetings Cay, Bahamas. Pseudopterosins A-D and the pseudopterosin aglycone were incorporated into an artificial diet that consisted of carrageenan agar, and water. The mixture was combined then heated in a microwave oven on the highest setting for 75 s. After the agar/carrageenan mixture was heated, pulverized fish food pellets and test compounds were added. Compounds dissolved in organic solvents or solvents only (for control diets) were stirred into the carrageenan mixture as it cooled. To incorporate the compounds into the diet, compounds were dissolved in a minimal amount of 95% ethanol (1 mL). After the metabolites were added,

the carrageenan diet was poured into molds and allowed to gel creating replicate 1 cm^3 pieces.

The cubes were presented to fishes on 50 cm long pieces of 3 strand, 1/4" yellow polypropylene rope in which four 3 cm long safety pins were attached equidistantly along the top 30-40 cm of the rope. The ropes were placed into the field in pairs to allow for comparisons of feeding on control versus treated cubes. Differences in consumption rates of artificial diet on the pairs of ropes were counted after at least half of all of the cubes on the two ropes had been consumed. Consumption rates were compared with a Wilcoxon Signed Ranks test. 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 significance (Wilcoxon signed rank test 1 tail, $p= 0.0062$) while the aglycone (0.1% dry weight) was a significant feeding attractant ($p= 0.0027$).



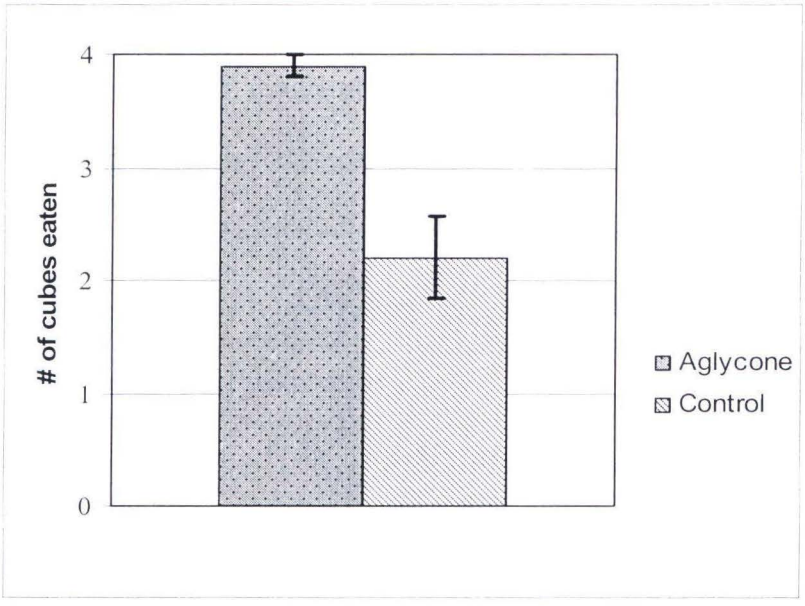


Figure 22. Mean values (error bars represent +/- 1 SE) of cubes eaten for pseudopterosins A-D (0.6% dry weight) N=14 and aglycone (0.1% dry weight) N=10 versus solvent only control.

3.3- Discussion

The results of the study done by Centrulo and Hay (2000) indicate that secondary chemistry is the primary means of defense for Caribbean gorgonians against fish predators. In that study, crude organic extracts from the species *P. elisabethae* were among the most effective of the extracts tested. Here we have examined the ichthyodeterrent capacity of the isolated metabolites pseudopterosins A-D and the pseudopterosin aglycone at natural volumetric concentrations. Our results indicate that pseudopterosins A-D are highly deterrent against a natural assemblage of reef fish and the pseudopterosin aglycone is a significant feeding attractant at the concentrations

tested. We may hypothesize 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. If aglycone represents an activated defensive compound, then perhaps it may function as an inhibitor of microbial infection. Recent work by Ferns and Jett (unpublished) suggests that the aglycone is an effective antimicrobial, based on disc-diffusion assays.

If the ecological role of the pseudopterosins is ichtyodeterrence, it is not surprising the concentrations of these compounds were found to be much higher in the early life stages versus adult colonies of *P. elisabethae*. Predation is generally considered the most important source of pre-settlement larval mortality in marine invertebrates. Many larvae can survive attack and rejection by predators suggesting strong selection pressures for defenses that cause larvae to be ignored or quickly released by predators (Lindquist and Hay, 1996). Chemical defenses may be essential in allowing the release of large, well-provisioned larvae during daylight periods when the larvae could use photic cues to find suitable habitat and are most likely to be consumed by visual predators (Lindquist et al., 1992). If the "problem" of predation has largely been solved by the production of chemical defenses, then the larval stage should be free to evolve traits that reduce losses due to other sources of mortality, even at subsequent life stages. As newly settled juveniles, the survival of benthic invertebrates is enhanced by increased size (Lindquist, 2002). Selection for larger size may occur with chemically defended larvae because their conspicuousness should not increase the risk of mortality giving them an

adaptive advantage over species with undefended larvae. *P. elisabethae's* relative success in the highly competitive environment of the Caribbean reefs may in large part be due to the effectiveness of its chemical arsenal.

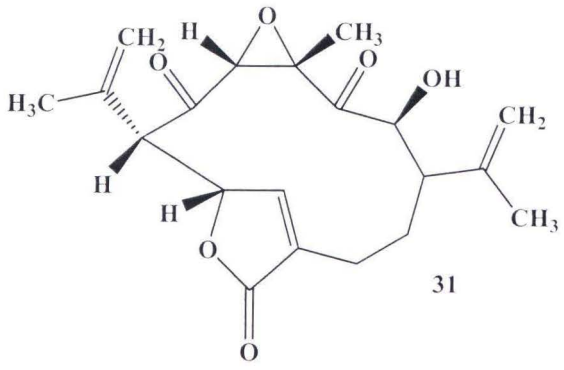
Chapter 4

Bipinnatolide L (40), a novel cembrenoid diterpene isolated from *Pseudopterogorgia bipinnata*

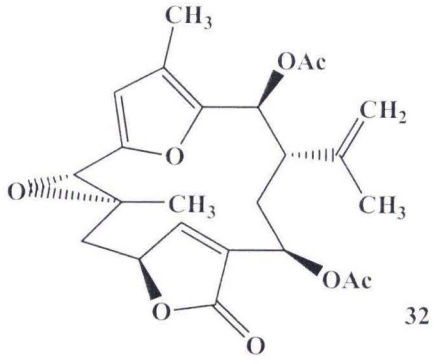
4.1-Terpenes from *Pseudopterogorgia* spp.

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.

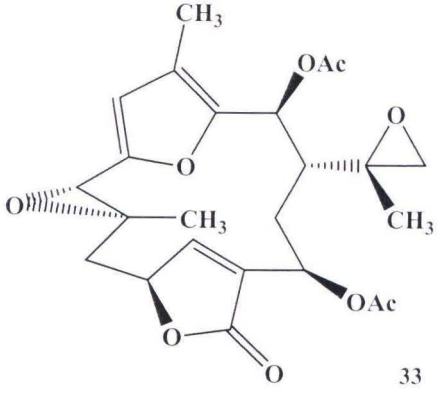
Thus far, cembrane-based diterpenoids have been found in only two of the more than 15 species identified *P. acerosa* (Tintio et al., 1990, 1991 and 1995) and *P. bipinnata* (Fenical, 1987; Wright et al., 1989; Rodriguez and Shi, 1998). Of these, the major producer of cembrane derivatives is *Pseudopterogorgia 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 (**31**) structurally related to the known metabolite kallolide A (**14**) and bipinnatins G-I (**32-34**) highly oxygenated cembranolides, structurally similar to the known metabolites bipinnatins A-D (**10-13**). Additionally, Rodriguez et al (1999) isolated five new related cembranoid bipinnatolides F-J **(35-39).**


CH2
H
O
CH3
H3C
O
O
OH
CH2
H
H
O
CH3
O
O

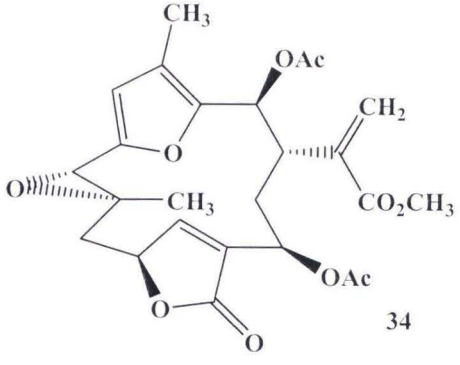
31


CH3
OAc
CH2
O
O
CH3
CH3
OAc
O
O

32


CH3
OAc
O
O
O
CH3
CH3
OAc
O
O

33


CH3
OAc
CH2
O
O
CH3
CO2CH3
OAc
O
O

34

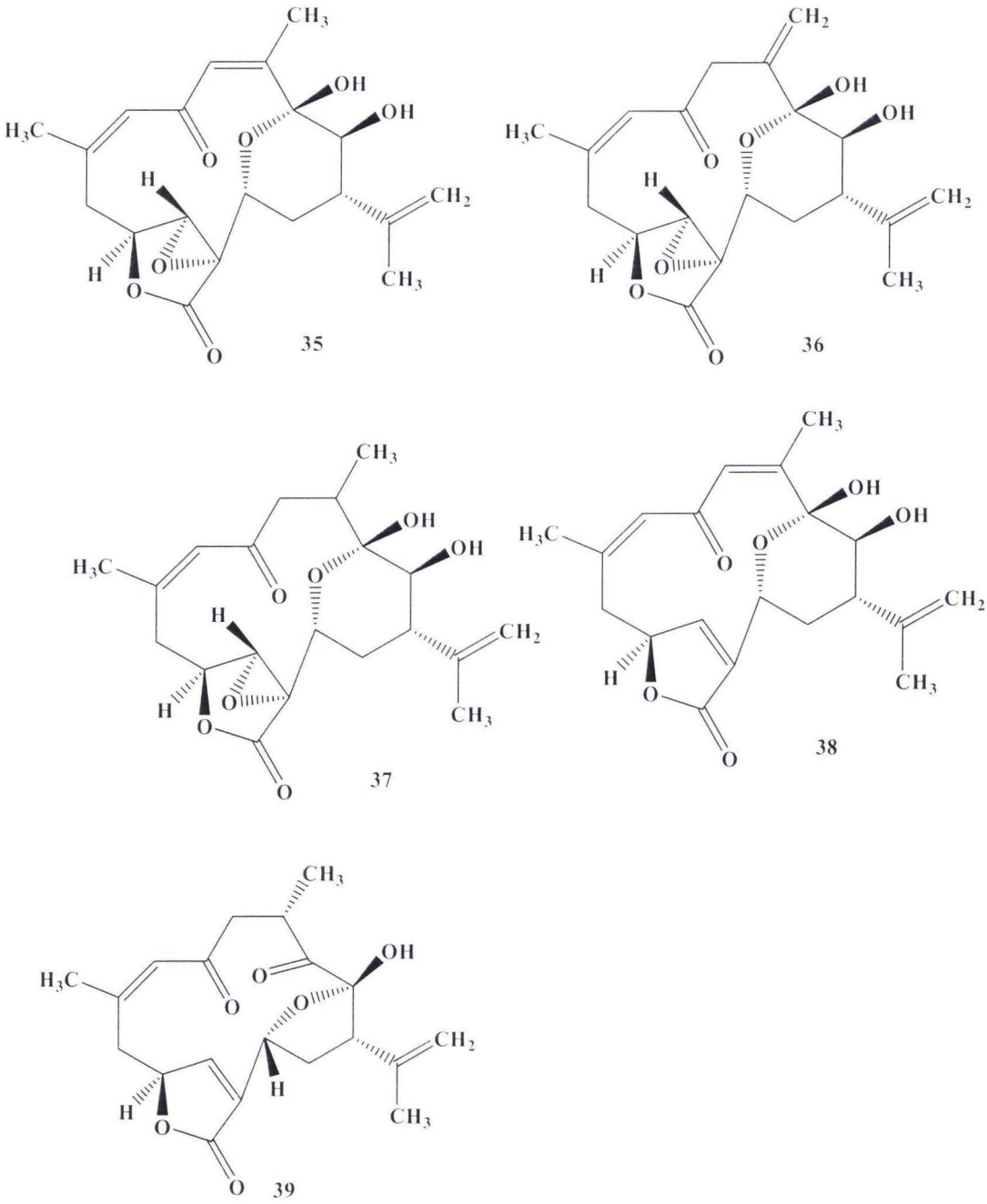


Figure 23. Structures of bipinnapterolide A (**31**), bipinnatins G-I (**32-34**) and bipinnatolides F-J (**35-39**)

4.2-Isloation and characterization of a novel diterpene from *Pseudopterogorgia bipinnata*

Normal phase HPLC analysis of the CH_2Cl_2 fraction of *P. bipinnata* larvae incubated with 4 μCi ^{3}H-GGPP (as described in chapter two) revealed a peak with a retention time of ~44 minutes (~ 20 min after kallolide A acetate) that had radioactivity upon scintillation counting that was comparable to that of kallolide A and kallolide A acetate. In order to collect enough of this compound for spectral analysis, a bulk extraction of dried adult *P. bipinnata* colonies was performed, yielding 6.45 mg of the peak at ~44 minutes.

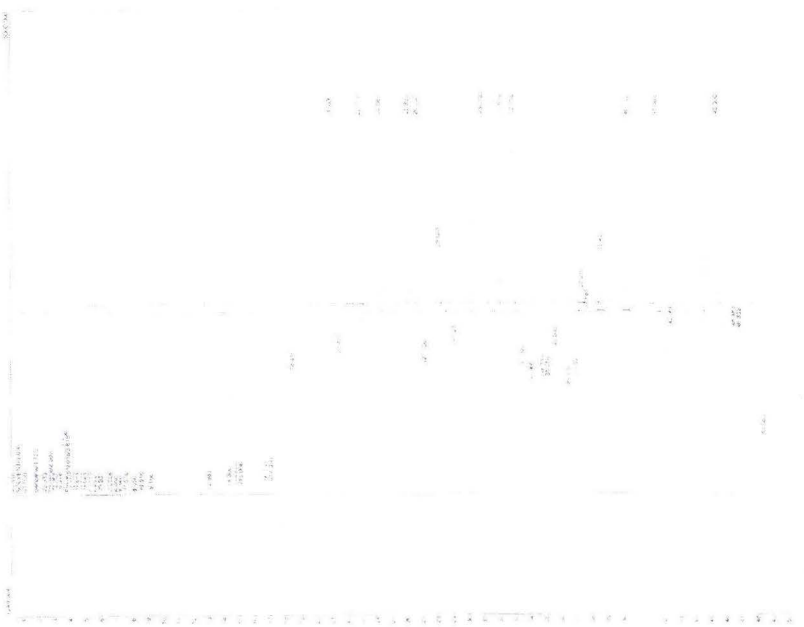
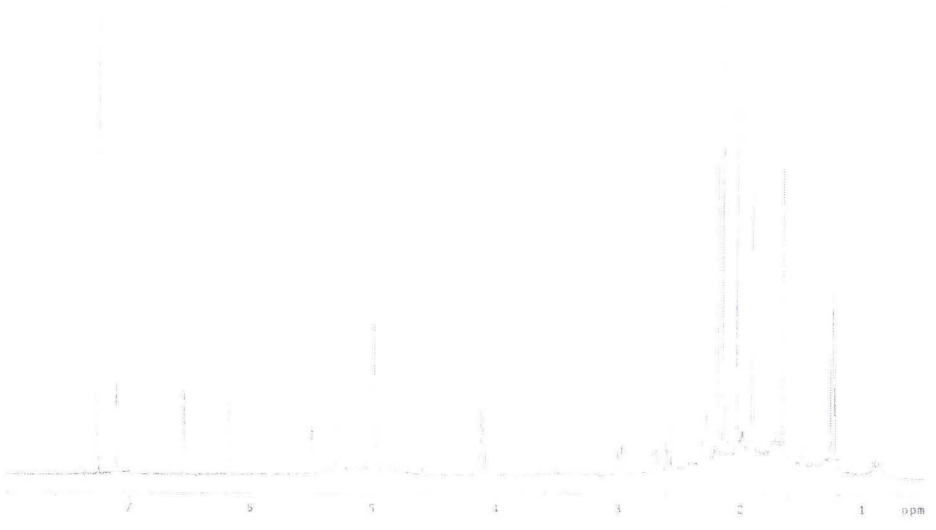
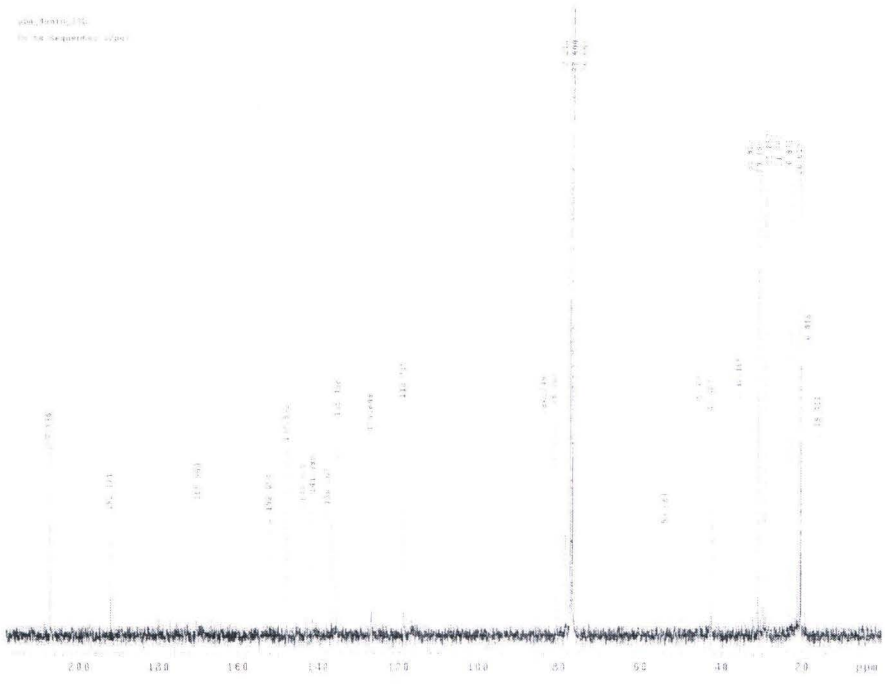


Figure 24. HPLC chromatogram of CH_2Cl_2 fraction of *P. bipinnata* peak with retention time of 45.300 min corresponds to bipinnatolide L (**40**)

An initial analysis of the ^{1}H and ^{13}C NMR spectra and comparison of spectral data to compounds previously isolated from *P. bipinnata* revealed structural similarities to the bipinnatins.



A



B

Figure 25. ^{1}H-NMR (A) and ^{13}C-NMR (B) of bipinnatolide L (**40**)

Characterization and structure elucidation of bipinnatolide L was done with the assistance of Dr Lyndon West. The molecular formula of bipinnatolide L (**40**) was established as $C_{22}H_{26}O_6$ from a HRESMS measurement of the molecular $[M + Na]^+$ ion peak *m/z* 409.1632 (Δ 2.4 ppm), and revealed ten double bond equivalents. The ^{13}C NMR spectrum contained 22 signals, consistent with the molecular formula. An analysis of the ^{13}C NMR and the DEPT revealed four carbonyl groups (δ_C 199.0, 192.2, 174.0 and 174.0), and four C-C double bonds (δ_C 152.0, 147.8, 143.0, 141.4, 136.7, 135.5, 126.8, and 118.7). These data accounted for eight of the ten double bond equivalents, and indicated that bipinnatolide L (**40**) was bicyclic.

A detailed analysis of the ^{1}H, ^{13}C, COSY, HSQC and HMBC data (Table **6**) led to the establishment of five substructures A-E as shown in Figure **26**. An α,β

unsaturated-γ-lactone (substructure A) was indicated from a one proton signal δ 7.08 in the ^{1}H NMR spectrum and carbon resonances at δ 174.0 (s), 147.8 (d), 136.7 (s), and 78.4 (d). A broad proton signal at δ 4.97 (2H) with allylic coupling to a broad methyl singlet at δ 1.65 and the carbon resonances at δ_C 141.4 (s), 118.7 (t), 19.4 (q) could be assigned to an isopropylene group (substructure B). The presence of two ketones conjugated with double bonds (α,β-unsaturated ketones) were indicated from carbonyl carbons with chemical shifts of δ_C 199.0 and 192.2, and the highly polarized nature of the C-C double bond carbons [δ_C 143.0 (C-4), 135.5 (C-5), and δ_C 126.8 (C-7), 152.0 (C-8)] (substructures C and D). The observation of absorption at λ=266 nm in the UV spectrum was consistent with this assignment. Methyl substitution of the double bonds at C-4 (δ_C 168.9) and C-8 (δ_C 152.0) was revealed in the COSY spectrum by the olefinic methines at H-5 (δ_H 6.52, δ_C 135.5) and H-7 (δ_H 6.17, δ_C 126.8) which both showed coupling to olefinic methyl singlet resonances at Me-18 (δ_H 1.89, δ_C 21.4) and Me-19 (δ_H 2.18, δ_C 22.8), respectively.

Finally, a linear substructure (substructure E) comprised of an oxygenated methine C-2 (δ_H 5.49, δ_C 80.7), methine C-1 (δ_H 2.69, δ_C 45.4), and two contiguous methylene groups C-14 (δ_H 2.00, 1.24 δ_C 32.2), and C-13 (δ_H 2.30, δ_C 20.4) was indicated from correlations in the COSY spectrum. A series of HMBC correlations, as illustrated in Figure **26** and detailed in Table **6,** confirmed the substructure assignments.

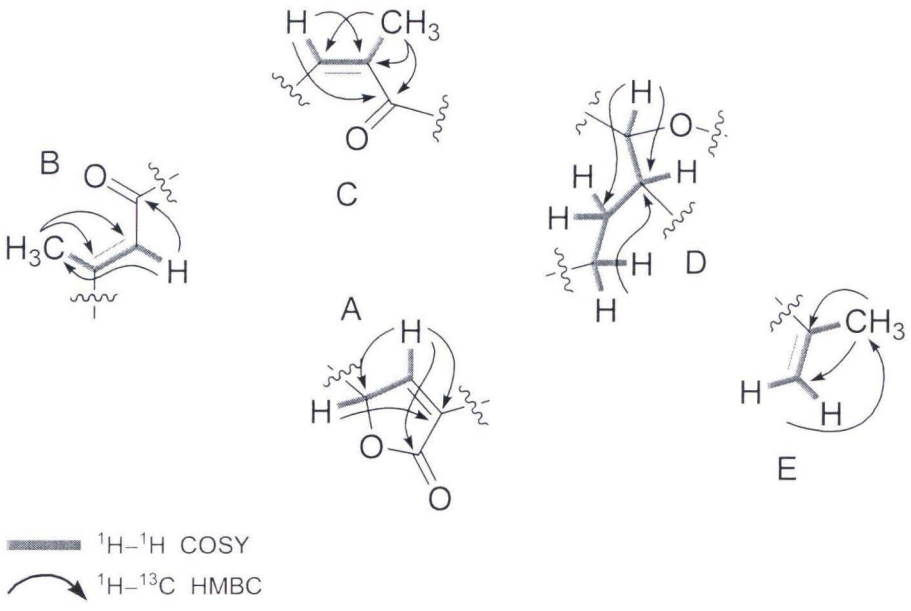


Figure 26. Substructures A–E bipinnatolide L (**40**).

Remaining to be assigned was a deshielded methylene group at δ_H 2.97, 2.56, δ_C 42.5, an acetate group (δ_H 2.10, δ_C 170.0, 20.6), and one double bond equivalent, indicating one more ring in the molecule. Assembly of the substructures with the remaining unassigned carbons was established from HMBC data as drawn in figure 27.

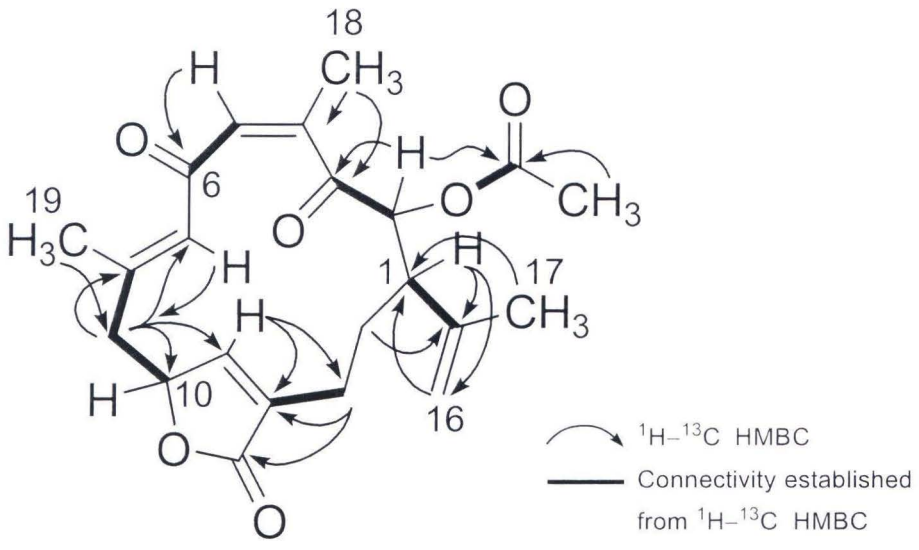


Figure 27. HMBC correlations used in the assembly substructures A - E of bipinnatolide L (**40**).

The relative configurations of C-1, C-2 and C-10 and the double bonds were determined from a combination of ^1H-^1H coupling constants, and ROESY correlations (Table 6). A small coupling of 4.0 Hz and NOE correlations observed between H-1 and H-2 together with correlations from both to H-14b established the *syn* relationship between these two protons. Allylic coupling between H-5 and Me-18 observed in the COSY spectrum and a ROESY correlation indicated the C4–C5 olefinic bond has a *Z* configuration. An NOE correlation observed between H-7 and H-9a together with no NOE correlation observed between Me-19 and H-7 is only consistent with the C7-C-8 double bond having the *E* configuration. NOE correlations observed between H-10 and H-11 to both Me-19 established the *syn* relationship of H-10 and Me-19. Due to the lack of transannular NOE's it is impossible to define the relationship between C1,

C2 and C10 therefore the stereochemistry of bipinnatolide L (**40**) was defined as (1*R**,2*R**,4*Z*,7*E*,10*R**) or (1*R**,2*R**,4*Z*,7*E*,10*S**)

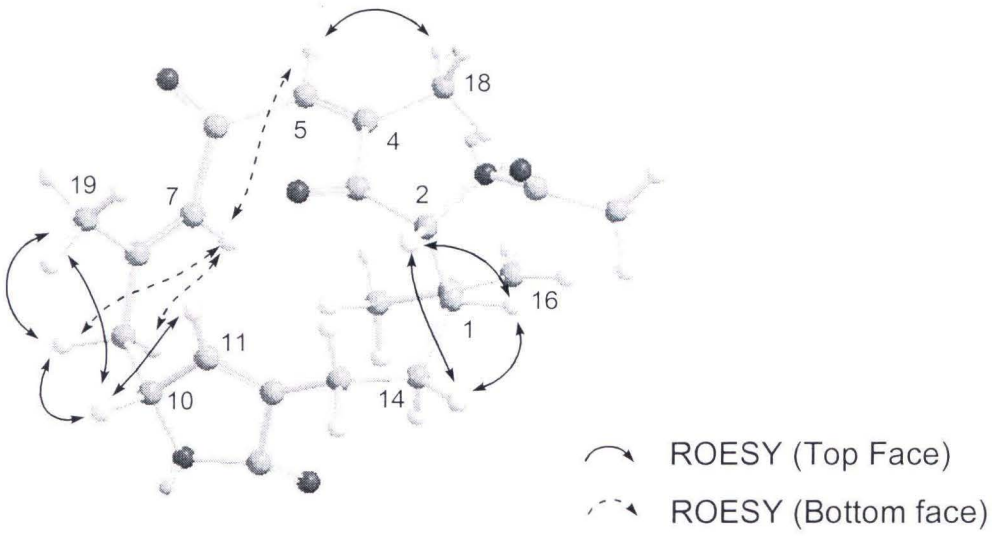
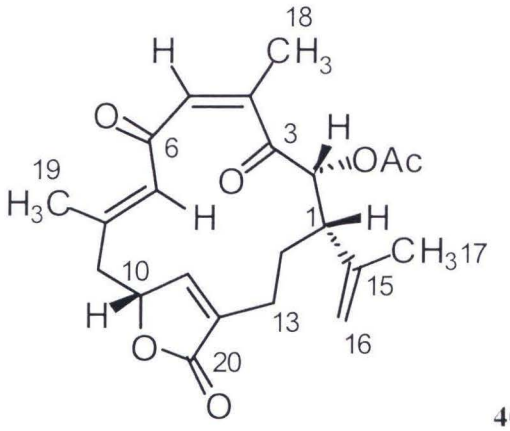


Figure 28. Selected ROESY correlations used to establish the relative stereochemistry of bipinnatolide L (**40**).

Table 6. NMR Data for bipinnatolide L (**40**) in $CDCl_3$

C#	δ_C (DEPT)	δ_H mult (*J* in Hz)	HMBC (H – C)	ROESY
1	45.4 (CH)	2.69 ddd (11.5, 4.0, 1.5)	14, 15, 16	2, 14b
2	80.7 (CH)	5.49 d (4.0)	1, 3, 14, 2-OAc	1, 14b
3	199.0 (C)	-		
4	143.0 (C)	-		
5	135.5 (CH)	6.52 d (1.2)	3, 4, 5, 6	7, 18
6	192.2 (C)	-		
7	126.8 (CH)	6.17 s	6, 8, 9, 19	5, 9a, 9b
8	152.0 (C)	-		
9a	42.5 (CH_2)	2.97 dd (13.5, 3.5)	7, 8, 10, 11	9b, 7
9b		2.56 dd (14.5, 4.0)	7, 8, 10, 11	9a, 10, 7
10	78.4 (CH)	5.27 bs	12	9b, 11, 19
11	147.8 (CH)	7.08 s	10, 12, 13	10, 19
12	136.7 (C)	-		
13	20.4 (CH_2)	2.30 m	1, 12, 20	
14a	32.2 (CH_2)	2.00 m		
14b		1.24 m	15	2, 1
15	141.4 (C)	-		
16	118.7 (CH_2)	4.97 s	1, 15, 17	
17	19.4 (CH_3)	1.65 s	1, 15, 16	
18	21.4 (CH_3)	1.89 s	3, 4, 5	5
19	22.8 (CH_3)	2.18 s	7, 8, 9	10, 11
20	174.0 (C)	-		
2-OAc	170.0 (C)			
	20.6 (CH_3)	2.13	2-OAc	



40

Figure 29. Structure of bipinnatolide L (**40**)

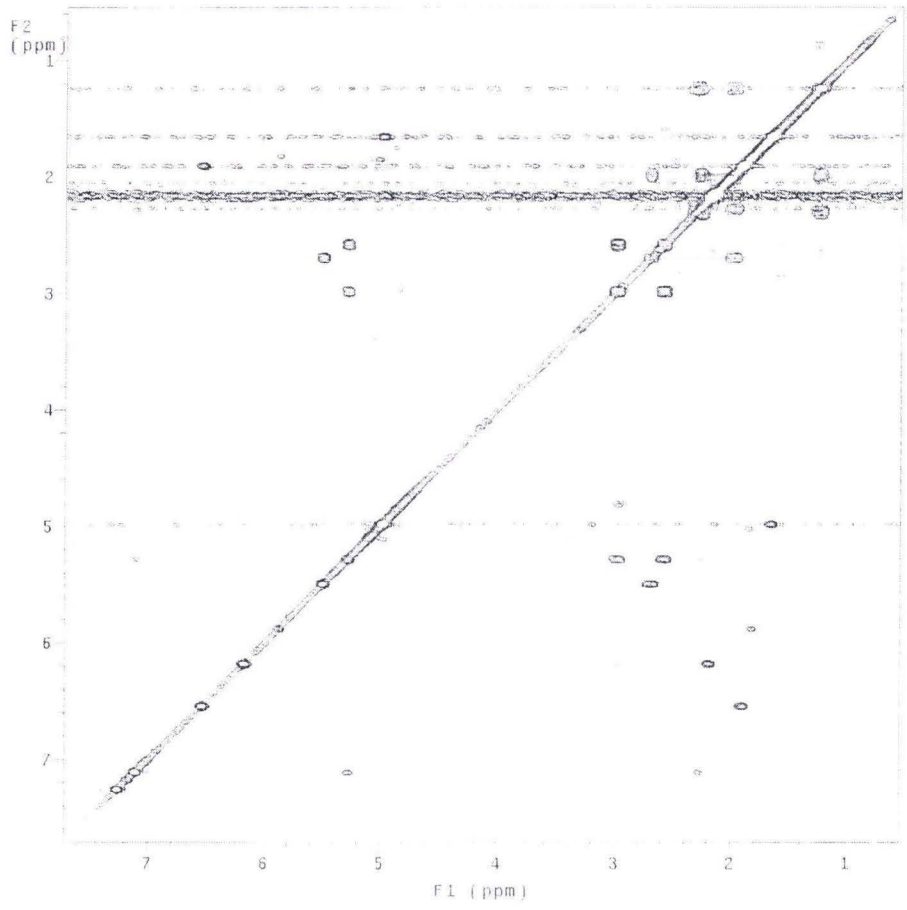


Figure 30. ^{1}**H -**^{1}**H** COSY of bipinnatolide L (**40**).

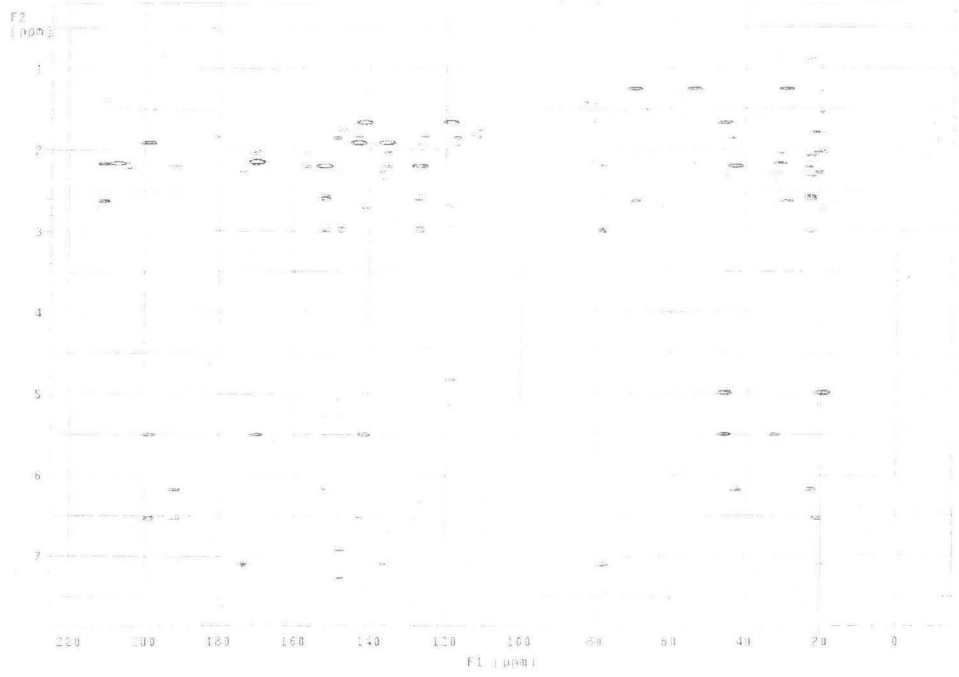


Figure 31. HMBC of bipinnatolide L (**40**).

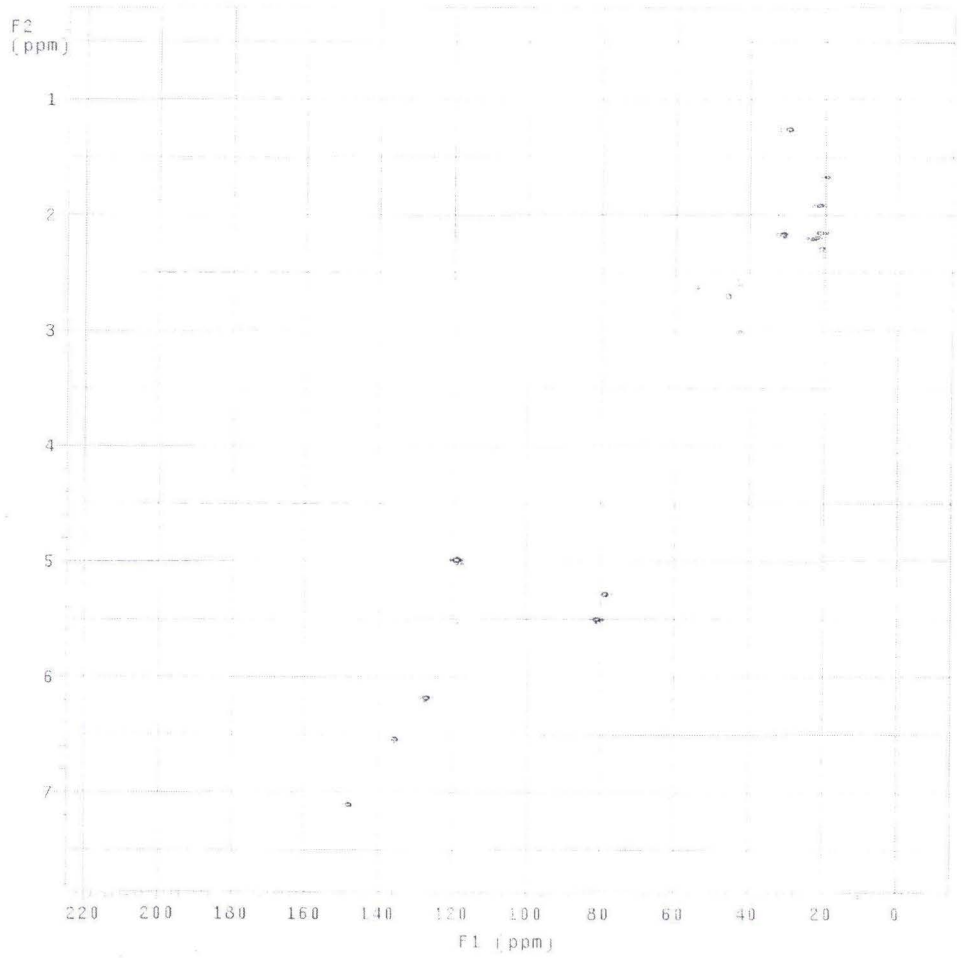


Figure 32. HSQC of bipinnatolide L (**40**).

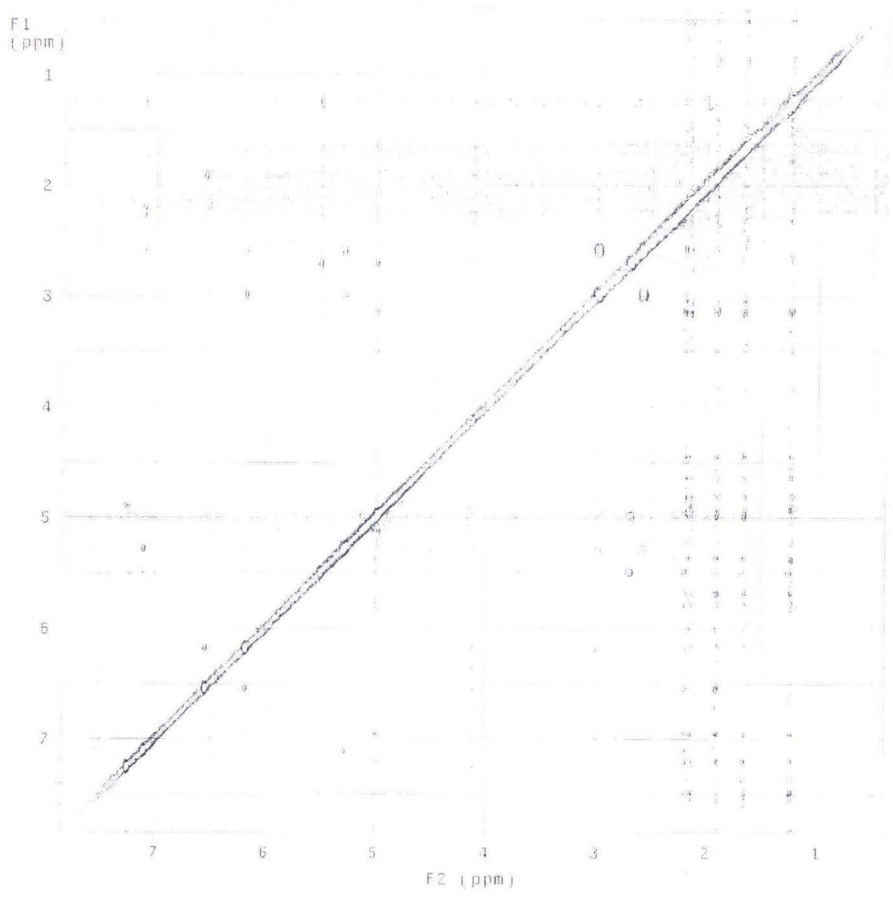


Figure 33. NOESY of bipinnatolide L (**40**)

Chapter 5

Materials and methods

Coral Material. *Pseudopterogorgia elisabethae* larvae, collected by SCUBA at a depth of 10 m off Little Bahamas Bank, Bahamas in November 2003, were generously donated to the lab by Dr. Howard Lasker. The larvae were then transferred to 1 L glass containers with Millipore (0.22μm) filtered seawater (~200 larvae/jar). *Pseudopterogorgia bipinnata* colonies were collected at Long Key, Fl by SCUBA at a depth of 25 m 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-^{3}H] 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_1- ^{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.

Larvae were extracted in MeOH/H_2O (9:1) (at room temperature for ~ 1hr) and partitioned with hexanes. The aqueous partitions were then adjusted to MeOH/ H_2O (1:1), and partitioned a second time with CH_2Cl_2.

General chromatographic procedures.

TLC analysis of adult *P.bipinnata* colonies was performed using precoated silica gel UV_{254} plates (Whatman) with a mobile phase of Hexanes/EtOAc (60:40). Spots were visualized by spraying the plate with 10% H_2SO_4 in MeOH and charring on a hot plate or in an oven. Davisil grade 633 type 60A 200-425 mesh (Fisher) silica gel was used with hexanes and EtOAc for normal phase column chromatography. Normal phase HPLC purification was performed using a Vydac semi preparative column. For isolation of diterpenes from *P.bipinnata* larval CH_2Cl_2 extract, a mobile phase gradient from 100% hexanes to hexanes/EtOAc (50:50) with UV detection at λ=270nm were used. Reverse phase HPLC was performed using a semi preparative Phenomenex phenyl-hexyl column and a mobile phase gradient of MeOH/H_2O (50:50) to MeOH (100%) with UV detection at λ=270nm. HPLC conditions for isolation of pseudopterosins from *P.elisabethae* larval CH_2Cl_2 extract used a mobile phase gradient from hexanes/EtOAc (60:40) to 100%

hexanes with detection at λ=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 λ=283nm. ^{1}H-NMR spectra were recorded at 400 MHz in $CDCl_3$ on a Varian Innova spectrometer. GC-MS analyses of hexanes partitions were performed on Perkin Elmer Gas Chromatograph with the following oven conditions- initial oven temperature 125°C hold 5 min, ramp 1-3°C/min to 175°C, ramp 2 10°C/min to 200°C hold 15 min.

Base hydrolysis of pseudopterosin C

Base hydrolysis of pseudopterosin C was accomplished by addition of 1 ml of 5% KOH / methanol, and the reaction mixture allowed to stir overnight. Ice was then added followed by 3 mL H_2O. The solution was acidified and the aqueous phase extracted with methylene chloride (3 x 15 mL). The combined methylene chloride layers were dried over anhydrous sodium sulfate, filtered, and and evaporated under N2 gas. The ^{1}H-NMR for the product was identical in all respects to the reported spectrum (Look et al. 1986).

Catalytic hydrogenation/hydrogenolysis of kallolide A.

Kallolide A was dissolved in 5 mL EtOAc and transferred to a flask containing 3% Pd activated charcoal and a stir bar. The flask was purged of air and filled with hydrogen three times. The reaction solution was allowed to stir at ambient temperature for 20 hrs, and then filtered through Celite. The reduced product was purified using reversed phase HPLC with 3% H_2O in MeOH as mobile phase.

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 (λ = 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. All spectral data collected were identical to that previously reported (Lazerwith et al. 2000).

Bipinnatolide L (40): clear oil; IR (KBr) ν_{max} 2956, 2930, 2870, 1752, 1702, 1619, 1442, 1377, 1233, 1102, 1038 cm^{-1}; UV (MeOH) λ_{max} 266 nm (ε 1068); ^{1}H and ^{13}C NMR ($CDCl_3$, 400 MHz) see Table 1; HRFABMS *m/z* 409.1632 $[M]^+$, calcd for $C_{22}H_{26}O_6$.

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