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The Chemistry and Chemical Ecology of Indo-Pacific Gorgonians

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in

Oceanography

by

Tegan Matthew Eve

Committee in charge:

Professor William H. Fenical, Chair
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2001
The dissertation of Tegan M. Eve is approved, and it is acceptable in quality and form for publication on microfilm:

[Signatures]

University of California, San Diego

2001
To Mitchell Martin LaFontaine, an eternal inspiration in my life.
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FIELDS OF STUDY

Chemical Ecology and Natural Products Chemistry of Gorgonians
  Marine Natural Products Chemistry (isolation and structure elucidation)
  Chemical Ecology (fish feeding deterence and antimicrobial microdilution assays)
  Nuclear Magnetic Resonance Spectroscopy
  Analytical Organic Chemistry (LC-MS, HPLC, chromatography)
ABSTRACT OF THE DISSERTATION

The Chemistry and Chemical Ecology of Indo-Pacific Gorgonians

by

Tegan M. Eve

Doctor of Philosophy in Oceanography

University of California, San Diego, 2001

Professor William H. Fenical, Chair

Previous studies have demonstrated that gorgonians produce secondary metabolites that act as chemical defenses against predators and pathogens in the reef environment. While there have been investigations of the gorgonacea of the Western Atlantic which have shown that these organisms do contain defensive chemistry, the chemical ecology of Indo-Pacific gorgonians has not been investigated. The goal of this thesis research was to investigate the ecological role of Indo-Pacific gorgonian secondary metabolites in mediating interactions with potential predators and pathogens and to identify and describe the secondary metabolites responsible for observed activity.
This dissertation concerns Indo-Pacific gorgonian chemical defenses against predation by the generalist reef fish *Thalassoma lunare* and *Halichoeres melanurus*, and against the growth of fungi isolated from gorgonian tissues, including the known gorgonian pathogen *Aspergillus sydowii*. The identification and characterization of novel secondary metabolites from gorgonians is also presented in this dissertation.

The information gathered in these surveys led to projects involving gorgonian species of particular interest, with the aim of revealing the compound or compounds responsible for observed activities. Each project is described both biologically and chemically. The biological aspects described are the taxonomic identification of gorgonians and fungal strains, and the testing of extracts and pure compounds in assays. The chemical studies involve the isolation and structure elucidation of known and novel gorgonian secondary metabolites. The possible importance of specific compounds, or in some cases a class of compounds, in the chemical ecology of gorgonians is discussed.
Chapter I

Introduction

A. Summary of the Dissertation

Previous studies have demonstrated that gorgonians produce secondary metabolites that act as chemical defenses against predators and pathogens in the reef environment. This dissertation concerns Indo-Pacific gorgonian chemical defenses against predation by the generalist reef fish *Thalassoma lunare* and *Halichoeres melanurus*, and against the growth of fungi isolated from gorgonian tissues, including the known gorgonian pathogen *Aspergillus sydowii*. The identification and characterization of novel secondary metabolites from gorgonians is also presented in this dissertation.

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B. Gorgonian Biology and Systematics

Gorgonians are members of the subclass Octocorallia in the class Anthozoa, which is one of four classes included in the phylum Cnidaria.\textsuperscript{2} The phylum Cnidaria also includes the classes Hydrozoa, Cubozoa, and Scyphozoa. These four classes are represented by a diverse group of invertebrates including jellyfish, stony corals, sea anemones, hydroids, and sea fans (Figure 1).\textsuperscript{2,3} Invertebrates of the class Anthozoa are distinguished from other cnidarians by several morphological and life-history features, the most notably the complete lack of a medusoid stage.

The class Anthozoa is the largest of the cnidian classes, and contains over 6000 species. The Anthozoa is divided into two subclasses, the Hexacorallia (or Zooantharia) and the Octocorallia (or Alcyonaria). The Hexacorallia, represented by sea anemones and stony corals, possess twelve or more tentacles and septa. The Octocorallia always have eight tentacles, which are pinnate (possessing side branches, as does a feather, with the side branches having the same origin on the axis). There are also always eight complete septa, one each side of the tentacle base (Figure 2).\textsuperscript{2,4}

Octocorals have free or fused calcareous spicules imbedded in the mesenchyme giving octocorals an internal skeleton. As represented in Figure 2, the bodies of most gorgonians have a complex central axial rod composed of a tanned collagen called gorgonin, chitin, and histidine rich protein. This axial rod is commonly calcified. Surrounding the axis is a layer of coenenchyme that connects polyps embedded within it via gastrodermal tubes.\textsuperscript{2} Gorgonians are always covered with a thin layer of spicule-filled mesoglea, and these calcareous components vary widely in shape and
Figure 1. Systematic classification of the phylum Cnidaria.²

Phylum Cnidaria

   Class Hydrozoa (man-of-wars, hydroids, fire coral; ~2700 species)

   Class Scyphozoa (jellyfish; ~200 species)

   Class Cubozoa (jellyfish, velum present; ~15 species)

   Class Anthozoa (>6000 species)
      Subclass Hexacorallia (anemones and stony corals)
      Subclass Octocorallia (octocorals including gorgonians)
         Order Stolonifera (organ-pipe coral)
         Order Telestacea
         Order Helioporeacea (blue coral)
         Order Pennatulacea (sea pens, sea pansy)
         Order Alcyonacea (soft corals and gorgonians)
Figure 2. Structure of a branch of a gorgonian colony (drawing adapted from Bayer, et al. \(^4\) and Barnes\(^2\) by Sy Teisan).
color. Along with the presence of symbiotic zooxanthellae in many species, these spicules impart a variety of colors to gorgonians. Gorgonian morphology is widely varied, from the single cylindrical filaments of whip corals to gorgonians branched in only one plane to those which are highly branched and appear plant-like. In gorgonians that branch in only one plane the branches may not interconnect or the branches may anastomose, or be highly interconnected, to form a lattice. These sea fans usually orient themselves at a right angle to the water current to allow a large surface area for feeding.3,4

Gorgonian octocorals are found in all of the world’s tropical and sub-tropical seas. The diversity and abundance of these organisms reach their highest levels on Caribbean coral reefs, where densities may be as high as 25 colonies per square meter.5 However, even on coral reefs which are nutrient poor and where predation levels are high, gorgonians appear to have only a handful of predators and are generally free from fouling and microbial infection. The predators and microbial pathogens gorgonians do have often appear to be very particular in their choice of prey species,1,6 though some predators are not species specific.

While gorgonians enjoy high levels of diversity and abundance on Caribbean reefs, alcyonaceans (true soft corals) are scarce. This situation is reversed on Indo-Pacific coral reefs, where alcyonaceans dominate the Octocorallia and gorgonian diversity and abundance is low. One plausible explanation for this contrast in speciation of the Octocorallia between the two oceans is the difference in the geological evolution of the oceans. The carbonate fossils of hard corals are used as a proxy for octocoral speciation because the Octocorallia lack hard structures that
preserve in the fossil record. Fossils of hard corals show worldwide similarity in the pre-Miocene (25 mya) when the earth had a pan-Tethyan tropical sea. The situation would have been similar with respect to the Octocorallia. During the early Miocene, the closing of the Isthmus of Panama separated the tropical oceans, and subsequent events drastically affected coral diversities and distributions.

During the early Pleistocene (1 mya), the onset of the most recent major glaciation occurred. The Caribbean and Indo-Pacific ocean basins experienced this ice age very differently. First, the north-south orientations of the major mountain ranges in the North American landmass allowed glaciation to advance farther than it could in Asia, where the mountain ranges run predominantly east-west. In addition to this, the basin size of the Indo-Pacific associated with coral reef formation is approximately an order of magnitude greater than that of the Caribbean, giving it a significantly higher heat capacity. The combination of these factors resulted in a greater cooling of the Caribbean, which caused the extinction of many hard corals and true soft corals. Gorgonians, which appear better equipped to deal with colder climates, may have been pushed south but became the dominant coral after the ice age. Soft corals retained their dominance in the Indo-Pacific, with their early taxonomic radiation unfettered.

The separate evolutionary histories of Caribbean and Indo-Pacific octocorals may have led to differences in the evolution of chemical defenses. Gorgonians in the Indo-Pacific have experienced a long history of undisturbed competition and predation, while those in the Caribbean might have experienced a period when these factors were less important than fast growth and quick dispersal. Given the low diversity and abundance of gorgonians in the Indo-Pacific, it is reasonable to
hypothesize that those gorgonians that are found there are extremely well adapted to defend themselves from predation.

The identification and taxonomic classification of gorgonians at present is based on the morphology of the colony and the overall size and morphology of the spicules.\textsuperscript{9} Spicule morphology can be very distinct for a given species, as seen in Figure 3. The traits examined when assessing colony morphology include the overall size of the colony, the colony's shape and branching pattern, polyp distribution, axis structure, and color. Unfortunately, it has been observed that both colony morphology\textsuperscript{9,10} and to a much lesser degree spicule morphology\textsuperscript{11} can vary with environmental conditions such as current, depth, and other physical factors. While these issues do not rule out the use of spicule and colony morphology for taxonomic grouping, the variations possible must be taken into consideration and it must be realized that the classification of gorgonians at the species and even genus level is somewhat subjective.

Over the past half century Dr. Frederick Bayer has addressed the problems which continue to persist in gorgonian taxonomy in three published revisions of octocoral classification starting in 1951\textsuperscript{12}, with another in 1961\textsuperscript{9} and the latest in 1981.\textsuperscript{13} In his 1961 review Bayer stated that "it is not an exaggeration to state that we still do not know what an alcyonarian species is."\textsuperscript{9} Two decades later, Bayer, still concerned over the state of gorgonian classification, wrote that "it must be kept in mind that some common species have been repeatedly described under different names, and many others have been assigned to the wrong genera."\textsuperscript{13} As an example,
Figure 3. Examples of Gorgonian Spicules
Figure 3 (continued). Examples of Gorgonian Spicules.
Bayer noted that none of the species described by Nutting (1910) as *Heterogorgia* are still classified in that genus. Most of them have since been recognized as species of *Echinogorgia*, and Bayer further notes that most species then described as *Echinogorgia* actually belong in *Villogorgia* and *Menella*. In his 1981 review, Bayer also removes the Gorgonacea as an order of the Alcyonacea noting that such families as the Paragorgiidae and Briareidae challenge the taxonomic separation of the Gorgonacea. In studying the Briareidae, Bayer found a great deal of confusion in the literature between the genera *Briareum* and *Solenopodium*. Referring to a specimen as "one of those odd-ball *Solenopodium Briareum* things," Bayer suggested that "DNA (sequencing) might offer some useful information" in the resolution of the family taxonomy.\textsuperscript{14} Further complicating the taxonomy of the Briareidae is the suggestion by Williams that *Pachyclavularia*, which has traditionally been classified as a soft coral of the family Tubiporidae, is "more closely related to *Briareum*" and even "may be synonymous with *Briareum*."\textsuperscript{15} As further evidence of problems with the Gorgonacea-Alcyonacea complex, Bayer refers to the scleroproteinous axis of the gorgoncean suborder Holaxonia which seems to be an unequivocal character, but is compromised by a number of species placed in this suborder which combine this axial trait with the axial cortex composition typical of the suborder Scleraxonia.\textsuperscript{13}

While Bayer does place all gorgonian families into the order Alcyonacea in his removal of the order Gorgonacea, there is still an overall sense of certain traits which many taxonomists and certainly ecologists consider to characterize true gorgonians as distinct from soft corals (personal communication; Dr. Gary C. Williams of the California Academy of Sciences). In the field of chemical ecology, authors have
continued to classify organisms as either soft corals (Alcyonarians) or gorgonians in
the two decades following Bayer’s 1981 revision. The searchable database on the
literature of marine natural products, Marinlit, continues to classify gorgonians in the
order Gorgonacea in following with literature publication practices. For the purpose
of this dissertation, the chemical ecology of only those alcyonarians traditionally
considered to be gorgonians was studied. Discussions with Dr. Gary C. Williams
allowed the identification of eleven families of gorgonians that are represented in the
Indo-Pacific. The taxonomy of Indo-Pacific gorgonians to the family level, as revised
by Bayer in 1981 and compiled by Williams in 1996, is outlined in Figure 4.13
Figure 4. Taxonomy of gorgonians of the Indo-Pacific to the family level.¹³

Order Alcyonacea

[Suborder Alcyonacea]

Family Nidaliidae

[Suborder Scleraxonia]

Family Briareidae

Family Anthothelidae

Family Subergorgiidae

Family Melithaeidae

[Suborder Holaxonia]

Family Acanthogorgiidae

Family Plexauridae

Family Gorgoniidae

Family Ellisellidae

Family Ifalukellidae

Family Isididae
Dr. Gary C. Williams of the California Academy of Sciences generously helped me to work out the taxonomy of gorgonians used in the present studies for fish feeding and antifungal assays. Gorgonian voucher specimens and photographs showing gross colony size and morphology were deposited in the invertebrate zoology collection of the California Academy of Sciences. While it is desirable and ideal to have all studied specimens identified to the species level, the current state of gorgonian classification made this goal impossible. In many cases the gorgonians studied could only be identified to the genus level. Among the Plexaurids, eleven specimens could be identified only to the family level, with genus and species indeterminate. According to personal conversations with Dr. Williams, Dr. Bayer made an attempt during the last decade to make some sense of the Plexaurid family, and gave up in frustration. In his evaluation of one Plexaurid examined in this dissertation, Dr. Bayer wrote, "(I)t could be one of a number of inadequately described species lurking in the literature but running it down would be worse than searching for a needle in a haystack."16 As shown in Figure 5, sixty-six gorgonians were identified and studied in the course of this dissertation. This collection of gorgonians represents 49 distinct species from nine of the eleven families of gorgonians present in the Indo-Pacific.
Figure 5. Taxonomy of gorgonians studied as part of this dissertation, with collection numbers indicated.

ORDER ALCYONACEA (29 families, 221 genera) skeletal components: calcite and gorgonin

Family Nidaliidae:
Siphonogorgia sp. A (PNG-97-097)
Siphonogorgia sp. B (PNG-97-008)
Siphonogorgia godefferyi (PNG-97-019)

Family Briareidae:
Briareum sp. A (NCI-1868)
Briareum sp. B (95-121, Palau)
Briareum sp. C (PNG-96-038)
Briareum cf. stechei (PNG-97-064)
[“Pachyclavularia sp.”] (PNG-97-039)

Family Anthothelidae:
cf. Solenocaulon sp. (PNG-97-083)

Family Subergorgiidae:
Subergorgia reticulata (PNG-96-010)
Subergorgia mollis (PNG-97-091)

Family Melithaeidae:
Acabaria sp. (PNG-97-044)
Wrightella sp. (PNG-97-060)

Family Acanthogorgiidae:
Acalycygorgia sp. A (PNG-97-007, -034, -051)
Acalycygorgia sp. B (PNG-97-077)

Family Plexauridae: (36 genera)
Astrogorgia sp. (PNG-96-053)
Bebryce sp. (PNG-97-059)
Euplexaura sp. (PNG-97-089)
Villogorgia sp. A (PNG-96-054)
Villogorgia sp. B (PNG-97-071)
Echinomuricea sp. (PNG-97-062)
Echinogorgia (Menella) sp. A (PNG-97-038)
Echinogorgia (Menella) sp. B (PNG-97-082)
Echinogorgia (Menella) sp. C (PNG-97-076)
Figure 5. (continued)

Echinogorgia (Menella) sp. D (PNG-97-065)
Echinogorgia sp. A (PNG-97-090)
Echinogorgia sp. B (PNG-97-043)
Echinogorgia sp. C (PNG-97-053)
Echinogorgia sp. D (PNG-97-050)

Plexaurids, genus/species indeterminate:
A (PNG-96-53x)
C (PNG-97-080)
D (PNG-97-018)
E (PNG-97-031)
F (PNG-97-079)
G (PNG-97-072)
H (PNG-97-081)
I (PNG-97-058)
J (PNG-97-063)
K (PNG-97-094)
L (PNG-97-004)

Family Gorgoniidae:
Hicksonella cf. princeps (PNG-96-035)
Rumphella sp. A (PNG-96-027, PNG-97-033, -037)
Rumphella sp. B (PNG-97-009, -032)

Family Ellisellidae:
Junceella (Juncella) cf. fragilis (PNG-96-015, PNG-97-028, -046)
Ctenocella (Ellisella) sp. A (PNG-96-014, PNG-97-006, -095, -099)
Ctenocella (Ellisella) sp. B (PNG-97-001, -002, -005, -096)
Ctenocella (Ellisella) sp. C (PNG-97-003, -035)
Ctenocella (Umbracella) umbraculum (PNG-97-045, -066, -070)
Ctenocella (Umbracella) cf. granulata (PNG-97-036)
Ctenocella (Viminella) sp. (PNG-97-098)
C. Review of Gorgonian Chemical Ecology

Since the late 1960s, chemists have been studying unique and interesting metabolites present in marine organisms.\textsuperscript{17,18} These "secondary metabolites," so named because they are not involved in primary metabolic pathways, first attracted attention for their novel carbon skeletons and interesting chemical properties. Because these compounds often possess important physiological activities, they have historically been investigated for potential pharmaceutical and industrial use. The exact role or function of most secondary metabolites remains unclear. In the past, some researchers speculated that secondary metabolites were merely evolutionary mistakes or carbon and nitrogen storage products. Scientists began to observe, however, that soft-bodied and/or sessile marine organisms like gorgonians and acidians, which often lack physical or behavioral defenses against predation yet live in fiercely competitive habitats, are frequently rich in secondary chemistry. In contrast, organisms that are mobile and/or have hard, protective shells generally lack secondary metabolites. The hypothesis was proposed by some researchers, most notably Kitteridge in the early 1970s,\textsuperscript{18} that these compounds may impart some chemical defense against predation, fouling, or microbial infection to the organisms that produce them. Indeed studies conducted by Bakus three decades ago on the feeding habits of coral reef fishes led to the conclusion that the feeding habits "played an important role, by acting as agents of natural selection, in the evolution of protective mechanisms . . . (in) tropical shallow-water invertebrates."\textsuperscript{19} Bakus concluded that while protective and defensive mechanisms employed by these exposed invertebrates include rapid movement, structural defenses, and cryptic form, the organism's defense
was "probably most frequently noxious, toxic, and venomous defense mechanisms."19 Only recently, however, have ecologically rigorous experiments been undertaken to test this hypothesis.20 Gorgonians are one group of organisms which have been studied using such ecologically relevant experiments, and most of the experimental evidence collected to date has focused on defense against predation.

Gorgonians are relatively fleshy and conspicuous in the reef environment, and given the high levels of predation seen on coral reefs, predators might well be expected to feed on gorgonians. In fact, few animals appear to prey on gorgonians, and most of these are highly selective in their choice of gorgonian species.6 The butterfly fish Chaetodon capistratus has been observed to feed on the extended polyps of gorgonians,21 and the polychaete Hermodice carunculata is a predator of several gorgonian species.6 Ovulid molluscs are specialist predators of gorgonians, including Cyphoma gibbosum, which grazes specifically on gorgonian tissues,22-24 and Tritonia hamnerorum, which feeds on and has been observed at very high densities (as many as 1700 molluscs on a single colony) on the sea fan Gorgonia ventalina.25 These sea slugs of the subclass Opisthobranchia are shell-less and lack any physical means of defense, and many have been found to contain a large amount of secondary metabolites obtained from the organisms they prey upon. This observation led to the theory that loss of a protective shell occurred evolutionarily with the development of a dietary derived chemical defense mechanism.26 As specialist predators, the molluscs appear to derive protection from potential enemies by consuming and associating with
gorgonians (and other marine invertebrates) which contain defensive secondary metabolites.25

While gorgonians lack a protective shell, they do contain calcium carbonate sclerites which can comprise up to 80% of the dry weight in some species.27 A few studies have addressed the ability of these sclerites to act as a defensive mechanism against certain predators. Sclerites isolated by Harvell and coworkers from the Caribbean gorgonians Pseudopterogorgia acerosa and P. rigida were assayed in synthetic foods against an assemblage of natural predators on the reef.28 When sclerites were removed from gorgonian tissues by bleaching the tissue and then incorporated into carrageenan assay strips, the sclerites significantly deterred feeding at concentrations above 34% sclerites by volume. Lower concentrations of around 1% did not significantly deter feeding. In a subsequent study of the chemical and structural defenses of P. acerosa and P. rigida, the intracolony localization of each type of defense was studied.27 The authors found that sclerites comprise a large percent composition of the gorgonian axial tissue, but only a very small percentage of the branch tissue. The concentration of sclerites as a function of volume was negatively correlated with the concentration of defensive extract found in the gorgonians, with the deterrent metabolites found almost exclusively in the polyp tissue and only trace quantities in the axial tissue. Harvell concluded that this distribution of defenses may be a result of the fact that fish foraging on gorgonians consume only the polyps21 while predators such as the mollusc Cyphoma gibbosum preferentially attack the axial tissues.27 These findings are further supported by a study of the chemical
and structural defenses of *G. ventalina*. Addition of sclerites to an artificial diet reduced feeding by *C. gibbosum* by about 50% and reduced feeding by natural assemblages of tropical fishes by 95%. While these results may appear to indicate that sclerites defend gorgonians from fish predation, it must be remembered that fish eat only the sclerite-poor polyp tissue. In addition to the effect of localization of sclerites in gorgonian tissues, the incorporation of sclerites into artificial diets (such as imbedding them in molten carageenan) may not mimic the orientation and packing of sclerites in the gorgonian a predator might encounter. Thus, it remains unclear what effect sclerites have with respect to deterring fish predation, but it seems clear that the localization of chemical deterrents in polyp tissues is important.

The majority of studies of gorgonian chemical defense have focused on fish feeding deterrence and have been performed in the Caribbean Sea. A preliminary survey of chemical defense among gorgonians by Pawlik and coworkers studied the deterrence of 37 specimens of Caribbean gorgonians from at least 19 species and 11 genera. Aquarium assays were conducted using the common generalist reef fish, the blueheaded wrasse *Thalassoma bifasciatum*, in which food pellets were treated with the organic extracts of gorgonians and offered against control pellets. At the time, the assays were conducted on board ship, the extract concentrations in the test pellets were unknown. When these extract concentrations were later determined, they varied considerably relative to the concentrations in the gorgonian tissue. As tested, the extracts of 19 specimens (51%) of the gorgonians were highly unpalatable to the assay fish, with at most one of the five treated pellets consumed. Four of the extracts were found to be moderately palatable with two or three of the five pellets eaten, and the
remaining 14 types (38%) were palatable with four or five treated pellets eaten. While the concentrations of extract in the pellets and gorgonians did vary considerably, the concentration of extract in all gorgonians found to be palatable in the assay was below that of the treated pellet. The pellet concentrations were also high relative to that of the gorgonians in those types found to be moderately or highly unpalatable, raising questions about concentration effects in these samples. The authors did assay seven types at decreasing treatment concentrations in an effort to determine the threshold of deterrence and most were found to be deterrent near or below the concentrations observed in the gorgonians. In two of the gorgonians, Erythropodium caribaeorum and Pseudopterogorgia rigida, the extracts were found to be deterrent at 7 and 5 percent of gorgonian tissue concentrations respectively.

Pawlik's preliminary survey\textsuperscript{30} led to further work on a number of species found to be deterrent (or not). Crude extracts and purified compounds from the Caribbean gorgonians Pseudopterogorgia acerosa and P. rigida were assayed in both aquarium and field assays.\textsuperscript{28} As found in the survey, crude extracts of these gorgonians were active in aquarium assays. The crude extracts were also incorporated into carageenan food strips at concentrations that were found to be effective in aquarium assays. These treated food strips deterred feeding of a natural assemblage of reef fish when deployed on the reef and assayed against control food strips. The major terpenoid components of both gorgonians were purified and assayed. The sesquiterpenes curcuhydroquinone (1) and curcuquinone (2) from P. rigida were found to deter feeding in both aquarium and field assays at concentrations below that found in the gorgonian tissues, while a hydrocarbon mixture containing mostly curcumene (3) was not active (Figure 6). The
Figure 6. Secondary metabolites from gorgonians.

1. curcuhydroquinone

2. curcuquinone

3. curcumene

4. pseudopterolide

Pseudopterogorgia rigida

P. rigida

P. rigida

Pseudopterogorgia acerosa
diterpene pseudopterolide (4) was isolated as the major metabolite found in the extract of *P. acerosa*, and may function as a deterrent metabolite, though this was not experimentally determined.\textsuperscript{27} As discussed above, further work on these two gorgonians localized the vast majority of the defensive chemistry found in the gorgonian tissues in the polyps, which would be most vulnerable to fish predation.\textsuperscript{27}

Another gorgonian found to be highly active in Pawlik's preliminary survey,\textsuperscript{30} *Erythropodium caribaeorum*, was further assayed in the field.\textsuperscript{31} When incorporated into carageenan strips at the same volumetric concentration found in the gorgonian tissue, the crude extract of *E. caribaeorum* also deterred feeding by a natural population of reef fish in the field. Bioassay guided fractionation of the crude extract identified a fraction containing chlorinated diterpenes with the briarane class carbon skeleton as that possessing strong antifeedant activity. Again, the hydrocarbon fraction, which contained mainly the sesquiterpene erythrodiene (5), was not deterrent at natural concentrations. Three chlorinated diterpenes, erythroliodes A-C, were purified from the active fraction and assayed independently in the field at natural concentrations (Figure 7). Erythrolide B (6) and erythrolide D (7) were effective as antifeedants, while erythrolide A (8) was not, although it was present in the gorgonian tissue at concentrations similar to that of the two deterrent erythrolides. While the possible additive or synergistic effects of various combinations of the three erythrolides isolated was not examined, the authors state the strong activity of the erythrolide fraction (containing all three compounds, 6-8, and quite probably other minor analogs) suggests that such effects are likely.
Figure 7. Secondary metabolites from *Erythropodium caribaeorum*.

5. erythrodiene

6. erythrolide B

7. erythrolide D

8. erythrolide A

Figure 8. Secondary metabolites from *Pterogorgia anceps*.

9. ancepsenolide

10. unnamed ancepsenolide

11. unnamed ancepsenolide
*Pterogorgia anceps*, also found to be highly unpalatable in the Pawlik survey,\textsuperscript{30} was further examined for antipredator defenses in both aquarium and field assays.\textsuperscript{32} Bioassay guided fractionation was used to localize the deterrent activity of the crude extract, previously shown to deter predation in field assays. Aquarium assays of the fractions from the crude extract led to a fraction containing three acetogenins, ancepsenolide and two derivatives (9-11, see Figure 8). A mixture of these three compounds also deterred feeding in field assays. The diacetoxy derivative of ancepsenolide 11 was active in aquarium assays, but neither ancepsenolide (9) nor the monoacetoxy derivative 10 inhibited feeding. This study represents the first evidence of feeding deterrence by metabolites of the acetogenin class. Again, the authors noted that a less polar fraction containing unidentified hydrocarbons was not deterrent in aquarium assays.

In Pawlik's survey, most gorgonians of the genus *Plexaura* yielded palatable extracts,\textsuperscript{30} but a previous study of *Plexaura homomalla* had identified secondary metabolites which deterred feeding by the generalist wrasse *Halichoeres garnoti*.\textsuperscript{33} Food pellets treated with (15R)-prostaglandin A\textsubscript{2} (PGA\textsubscript{2}) (12) were observed to cause vomiting in fish assays. This inconsistency with the survey data led to further investigation of the prostaglandins found in the tissues of *P. homomalla*.\textsuperscript{34} In this subsequent investigation, only acetoxy methyl esters were found in the living tissues of the gorgonian. Investigators found that slow enzymatic hydrolysis leads to the formation of acetoxy acids, hydroxy methyl esters, and hydroxy acids after the gorgonian was collected. Field assays of the acetoxy methyl ester (13) found in the living gorgonian tissue at volumetrically equivalent concentrations to those in the
Figure 9. Secondary metabolites from *Plexaura homomalla*.

12. 15(R)-prostaglandin A$_2$

13. acetoxy methyl ester

14. hydroxy methyl ester

15. acetoxy acid

Figure 10. The furanocembranolide pukalide, from *Leptogorgia virgulata*.

16. pukalide
gorgonian showed no activity. The hydroxy acid (12) assayed in the original study by Gerhart, as well as the hydroxy methyl ester (14) and the acetoxy acid (15) were all found to be deterrent in field assays (Figure 9). However, the prostaglandin derivative naturally present in the living tissue of *P. homomalla*, (13), does not appear to afford the gorgonian protection from fish predation. These studies suggest that care must be taken in the handling and collection of gorgonian specimens that will be used to assess ecologically relevant activities.

Studies of the gorgonian *Leptogorgia virgulata* revealed that tissue and extractable metabolites from this gorgonian cause vomiting in fish and inhibit settling by marine fouling organisms. Bioassay guided purification of the anti-fouling activity led to the isolation of pukalide (16), a furanocembranolide diterpene found in a variety of octocorals (Figure 10). The authors investigated the ability of ecologically relevant doses of orally delivered pukalide to induce vomiting in the sympatric fish *Fundulus heteroclitus*. At concentrations of pukalide in treated pellets that approximated levels found in *L. virgulata* coenenchyme, 53% of fish ingesting treated pellets vomited. The effect of pukalide was found to be dose-dependant, with fish receiving higher doses of pukalide vomiting significantly sooner than those receiving the threshold dose of 0.05 mg/g body weight. However, all fish that ingested pukalide showed signs of distress including gulping movements with the mouth and rapid, erratic swimming. The authors acknowledge that pukalide (16) appears to lack the ability to reduce palatability in an ecologically significant manner, but suggest that if pukalide does indeed act as a defensive toxin it may be through learned aversion in fish due to nausea and distress. Though few data were
presented, the authors also state that pukalide is a highly effective inhibitor of settlement by some marine invertebrate larvae, and suggest a possible dual role for pukalide in defense from both predation and biofouling.

Recently, the feeding deterrent properties of a several Brazilian gorgonians have been studied by Epifanio and coworkers. In one study, the crude extracts of Plexaura regia and Phyllogorgia dilatata were tested at natural concentrations in field studies conducted in the habitat of gorgonian origin. The crude extract of P. regia had no apparent feeding deterrent properties; in fact the authors report that the extract seemed to stimulate feeding. The crude extract of P. dilatata did reduce feeding by reef fish relative to controls, and bioassay guided fractionation of the crude extract led to a medium polarity partition which also had feeding deterrent properties. A less polar fraction containing a mixture of hydrocarbons was noted to be inactive in field assays. Chromatography of the deterrent fraction led to the isolation of two compounds, 11β, 12β-epoxypukalide (17) and a novel germacrane sesquiterpene 18. Field assays with the two compounds revealed that the pukalide derivative 17 was highly deterrent while the sesquiterpene 18 was inactive. In contrast with feeding studies of pukalide (16), which induced vomiting but did not reveal feeding deterrence properties directly in aquarium assays, the epoxy product of oxidation at the α,β-unsaturation site (17) exhibited high levels of feeding deterrence in field assays (Figure 11).

A subsequent report by Epifanio and coworkers describes the feeding deterrent properties of the known compound lophotoxin (19) from the Brazilian gorgonian
Figure 11. Secondary metabolites from Brazilian gorgonians.

17. 11\beta, 12\beta-epoxypukalide

18. germacrane sesquiterpene

19. lophotoxin

20. (6E)-2\alpha,9\alpha-epoxyeunicella-6,11(12)-dien-3\beta-ol

21. heterogorgiolide
*Lophogorgia violacea* (Figure 11).\textsuperscript{41} Bioassay guided fractionation of the deterrent crude extract of *L. violacea* led to **19** and four minor derivatives, which are not feeding deterrent at individual natural concentrations but appear to contribute, in an additive manner, to the deterrency of **19**. Lophotoxin (**19**) is a furanocembranolide diterpene closely related to pukalide (**16**) and its epoxy derivative **17**, giving further evidence that compounds of this class provide chemical protection from predation.

Another Brazilian gorgonian, *Heterogorgia natumani*, was found to contain two feeding deterrent compounds (Figure 11), a previously reported eunicellane diterpenoid **20** and the novel sesquiterpenoid, heterogorgiolide (**21**).\textsuperscript{40} While both of these compounds were found to inhibit fish feeding in field assays when tested at natural concentrations, it is noted that the crude extract was never assayed and the activity of the compounds was not discovered through bioassay guided fractionation. It is notable that both a feeding deterrent sesqui- and diterpene were derived from the same gorgonian. Feeding deterrent activity can be ascribed to the individual compounds, but caution must be taken when extrapolating this deterrency to the gorgonian, whose crude chemical extract could possibly contain feeding stimulants.

Though individual compounds responsible for the observed deterrency were not isolated, the crude extract and flash column chromatography fractions of *Gorgonia ventalina* were found to significantly reduce feeding by both natural assemblages of fish in field assays and the specialist predator of *G. ventalina*, the mollusc *Cyphoma gibboum*.\textsuperscript{29} Crude extracts reduced feeding by *C. gibboum* by about 50%; even though the mollusc is a specialist predator it is not immune to the chemical defenses of *G. ventalina*. Further experiments revealed that the mollusc preferentially attacks
areas of the gorgonian colonies in which a lower concentration of extractable material is present. Fish feeding on artificial food treated with the crude extract was reduced by 87% compared to controls. All but two of seven flash column fractions of the *G. ventalina* extract were significantly deterrent at natural concentrations, including the least polar fraction eluted with pure hexane. While this is the first report of very non-polar extracts being active in feeding assays, the authors note that the proton NMR spectra of all flash fractions showed that oxygenated sesquiterpenes were present.\textsuperscript{29}

Another study showed that the specialist nudibranch *Tritonia hamnerorum* preferentially sequesters secondary metabolites from the gorgonian *Gorgonia ventalina*.\textsuperscript{25} Extracts from *T. hamnerorum*, which contained the same secondary metabolites as *G. ventalina* extracts, deterred feeding in a number of fish, including wrasses, grunts, and damsels. After the deterrent activity was concentrated in a particular flash column fraction of the DCM soluble extract from *T. hamnerorum*, seven individual compounds were isolated and tested for activity against the wrasse *T. bifasciatum*. The same compounds were also isolated directly from the gorgonian and assayed; in both cases only one compound significantly deterred feeding by the wrasse, the furano-germacrene julieannafuran (\textsuperscript{22}, see Figure 12).

Extracts of the Caribbean gorgonian *Briareum asbestinum* were found to be highly unpalatable in Pawlik’s preliminary survey,\textsuperscript{30} and though no publications have to date identified the specific compound(s) responsible for the observed deterreny, *B. asbestinum* is known to contain relatively large quantities of briarane and asbestinane class compounds. Unpublished data,\textsuperscript{42} the above mentioned study of *E. caribaeorum*,\textsuperscript{31} and experimental evidence included in this dissertation indicate that
these types of compounds are highly feeding deterrent, often at volumetric concentrations an order of magnitude lower than that found in the gorgonians. A study of the chemical defenses of the embryos and larvae of the gorgonian *B. asbestinum* revealed that they were rejected when offered to the generalist fish *Thalassoma bifasciatum* against control foods. The study also found that the concentrations of the four major asbestinanes of the gorgonian did not vary significantly in the embryos or larvae except during transitions in development, suggesting that the compounds were not simply sequestered in the embryos and larvae but actually produced by the larvae. The marked decline of asbestinane concentrations during ontogeny may be due to a transition in the use of metabolic energy during the rapid cell division that occurs during metamorphosis, or could be due to the increased susceptibility of developing tissues to autotoxic effects of the metabolites. While no specific compounds were identified through bioassay guided fractionation, the author states that unpublished data from two sources indicate that most asbestinane compounds tested from *B. asbestinum* were deterrent to fish.

The feeding deterrent activity of gorgonian extracts and metabolites has been the most investigated and reported aspect of gorgonian chemical ecology, but other ecological functions have been ascribed to gorgonian extracts and metabolites. Targett and coworkers found that the water-soluble extracts of the gorgonians *Leptogorgia virgulata* and *L. setacea* inhibited the growth of the potential fouling diatom *Navicula salinicola*. The water-soluble extract contained homarine (23), which also inhibited fouling at concentrations found in the gorgonians. Homarine has been isolated from numerous marine organisms and cnidarians (Figure 12).
Figure 12. Secondary metabolites from gorgonians.

22. juleannafuran from *Gorgonia ventalina*

23. homarine from *Leptogorgia virgulata*

Muricins 1-4 from *Muricea fruticosa*.

24. muricin 1

25. muricin 2

26. muricin 3

27. muricin 4
While Gerhart's studies of the defensive chemistry of *L. virgulata* focused on fish feeding deterrence$^{35}$ (see above), both punkalide (16) isolated in that study and homarine (23) have been ascribed possible anti-fouling activity.

In a comparative study of two closely related Pacific gorgonians, *Muricea californica* and *M. fruticosa*, it was shown that the much less fouled *M. fruticosa* contains four novel esterified aminogalactose saponins.$^{46}$ These compounds, assigned as muricins-1 through -4 (24-27), were found to inhibit growth of the marine diatom *Phaeodactylum tricornutum* when assayed at approximately natural concentrations (Figure 12). While no ecologically rigorous experiments were conducted to determine the ichthyodeterrence of the muricins, it is noted that similar aminoglucose saponins had previously been described as the active components in the defensive secretion of the sole *Paradachirus pavoninus*.$^{47}$ The authors suggest that the ability of the muricins to inhibit growth of *P. tricornutum* may play a significant role in the reduced fouling observed on *M. fruticosa*.

Many of the unusual secondary metabolites isolated from gorgonians have been found to have biomedically relevant antimicrobial activity. However, few studies have explored the role of these metabolites in defending gorgonians against ecologically relevant microbial pathogens. Antimicrobial chemical defenses are well documented in the terrestrial environment,$^{48}$ and microbial pathogens have been shown to be important selective forces in the evolution of secondary metabolites. Marine invertebrates are certainly susceptible to microbial pathogens,$^{49,50}$ but, with a noted exception,$^{1}$ gorgonians generally appear to be free of microbial infection.
Because only a few studies have shown evidence of antimicrobial chemical defense in gorgonians, a correlation between the biomedically relevant activities seen in gorgonian metabolites and microbial chemical defense cannot be established. However, it seems logical that the high level of antimicrobial compounds found in gorgonians has some evolutionary significance.51

A first study, in 1958,52 showed gorgonians may contain metabolites that inhibit microbial growth, and led to the conclusion that this antimicrobial activity may be responsible for a noted lack of surface fouling on gorgonians.53 Gorgonian tissue fragments and extracts were tested for their ability to inhibit the growth of marine and pathogenic bacteria that had been inoculated on agar plates. 52 A limited number of species was tested, and no further work to identify active metabolites was reported, but "striking" antimicrobial activity was observed in some species.52 Aside from the above mentioned studies involving the ability of gorgonian metabolites to prevent fouling by diatoms,43,46 no further investigations of ecologically relevant antimicrobial activity were published until 1994 by Kim.54 This study reported that the non-polar extracts of eight gorgonian species exhibited varying levels of bacterial inhibition in all but one test out of 40 (8 gorgonians x 5 bacteria) when tested in agar disc diffusion assays against three strains of marine bacteria and two strains of non-marine bacteria.54 While these results seem to indicate that the gorgonians all possessed antimicrobial activity, it must be noted that the study scored even a thin halo of less than 0.5 mm as indicative of antimicrobial activity, and more than half of the observed inhibition zones were 0.5 mm or less. *Plexaura homomalla* displayed
significant activity against all five bacterial strains, while *Pseudoplexaura flagellosa*
significantly inhibited only the two non-marine bacteria. The activities of the six other
gorgonians showed no activity in tests. The author concludes that “all gorgonians
tested possessed antimicrobial properties,” and therefore the antimicrobial activity “is
an important ecological characteristic of these corals.” However, the selection of
bacteria, including those of marine origin, was based on ease of culturing and ability
to form mats on simple media, and the inhibition zones observed in most cases were
very small, raising questions about the ecological significance of this work. The study
seems to confirm only that which was already known; many gorgonian extracts and
metabolites exhibit antimicrobial activity, but the ecological significance has not been
rigorously tested.

Antimicrobial activity of gorgonian extracts was subsequently surveyed for a
taxonomically comprehensive group of abundant gorgonian species from the
Caribbean. Jensen and coworkers used ecologically relevant bacteria to determine if
gorgonians produce broad-spectrum antibiotics as a chemical defense. The study
assessed the activities of 39 gorgonian extracts against 15 strains of marine bacteria.
For each gorgonian species or chemotype three replicate collections were made and
tested independently. The bacterial strains consisted of three known invertebrate
pathogens, six strains isolated from healthy *Briareum asbestinum* tissue, and six
strains isolated from decayed gorgonian tissue. The authors acknowledge that the
distribution of compounds with respect to surface colonization in the producing
gorgonian is unknown, and it is difficult to test extracts at the concentrations
experienced by microorganisms in nature. They proposed that testing extracts at
concentrations equal to those in the gorgonian tissue provides a rational approach for assaying activities if caution is exercised in interpreting the results of tests performed. Testing extracts at such concentrations probably exposes the bacteria to much higher concentrations of metabolites than would be experienced in nature because the metabolites undoubtedly diffuse in agar assays at greater rates than they diffuse away from animal tissues. The results of the assays were interpreted conservatively compared to Kim’s much more limited survey, with assays being scored as active only if the extracts generated inhibition zones of more than 5 mm in diameter. Jensen suggests that this conservative approach is necessary because low levels of activity may be an artifact of the extraction and testing procedures and because the variability inherent to zones of inhibition is estimated to be ±1.0 mm. For these reasons, small and non-reproducible zones of inhibition were not considered indicative of antimicrobial chemical defense.

The survey found that of the 544 gorgonian/bacteria assays scored, only 15% resulted in antibacterial activity. In addition, one third of the 39 gorgonian extracts inhibited only one bacterial strain, and 9 of these extracts showed no activity at all. The authors conclude that these results suggest that most gorgonians “do not possess potent, broad-spectrum antibacterial activity inhibitory to the growth of opportunistic marine pathogens and bacteria associated with healthy and decayed gorgonian surfaces.” The alternative conclusions from this survey as compared to Kim’s survey are explained by the difference in criteria used to evaluate the assays. Had Jensen’s criteria been applied to Kim’s assay results, only one of the eight extracts would have been considered active. Jensen’s findings suggest that in contrast to
terrestrial plants, bacteria may not have been important agents in the evolution of gorgonian secondary metabolites.

While gorgonians overall did not possess broad-spectrum antibiotic activity, the extracts of four *Pseudopterogorgia* species showed high levels of activity, inhibiting 43 to 86% of the bacterial strains. These four species include *P. acerosa* and *P. rigida*, both known to possess feeding deterrent metabolites as well. Gorgonian extracts of the genus *Gorgonia* were also found to have higher levels of antibacterial activity and are ichthyodeterring, although other gorgonians (*Briareum asbestinum*, *Erythropodium caribaeorum*, *Eunicea*, and *Pterogorgia*) which possess feeding deterrent properties showed no antibacterial activity.

Fungal infections in gorgonians have recently been demonstrated to be an ongoing problem across Caribbean. The ascomycete *Aspergillus sydowii* was responsible for mass disease and mortalities among Caribbean gorgonians of the genus *Gorgonia*. While fungi are known pathogens of other coelenterates, this is the first report of a pathogenic fungus infecting gorgonians. The hyphae of the fungus were embedded in the receding edge of diseased coenenchyme tissue, the outer organic rich tissue containing the living polyps. There were no hyphae in healthy rind tissue, and a secondary infection by a cyanobacterium similar to that responsible for black-band disease in hard corals was extensive on some gorgonians. The extensive damage caused by *A. sydowii* infection of these gorgonians has prompted studies of the ability of *Gorgonia* spp. to resist infection and the consequences of this pathogenesis are being examined.
An investigation of the ability of the crude extract of *Gorgonia ventalina* and *G. flabellum* to resist infection by *A. sydowii* showed that concentrations of extract similar to those estimated in living tissue of the gorgonian inhibited fungal spore germination. The concentration of extracts was higher in healthy gorgonians surveyed, suggesting a role in reducing susceptibility to fungal infection. Antifungal activity of the extracts increased near infection sites, suggesting an inducible defensive response to infection. The antibacterial activity of the extracts, previously determined to be among the highest observed in a survey of gorgonians, had a strong positive correlation with antifungal activity patterns seen in *Gorgonia* spp. While the specific compound or compounds responsible for the observed antifungal and antibacterial activity were not isolated or quantified, the study concluded that sea fans of the genus *Gorgonia* possess chemical resistance to microbial pathogens. It is notable, however, that while gorgonians of the genus *Gorgonia* seem to possess strong antimicrobial activity, they are the gorgonians most often infected by *Aspergillus sydowii*. It is unclear why other gorgonian genera are not susceptible to this pathogen, though a recent report by Kim and coworkers examined the ability of 20 common Caribbean gorgonian extracts to inhibit the growth of *A. sydowii*. Several of the crude extracts had inhibitory activity, though in many cases the extracts were not present in sufficient concentrations in living gorgonian tissue to be effective against the fungal pathogen. This report will be discussed in further detail in Chapter III of this dissertation due to its relevance to the research discussed there.

A further investigation of the consequences of fungal pathogenesis of *Gorgonia ventalina* found significant decreases in the furano-germacrene (22)
metabolite previously determined to be responsible for the observed feeding
deterrency of the gorgonian. Grazing by the specialist mollusc Cyphoma gibbosum
increased on infected sea fans, and feeding assays determined that the feeding
deterrent compound 22 was deterrent to fish only at concentrations found in
uninfected sea fans. The observed lowering of antifeedant defenses occurring while
antifungal defenses are being built up during a pathogenic infection might result from
a shift in the metabolic energies the gorgonian spends to defend itself or other
requirements such as repair. In any case, it is clear from this study that fungal
infection has both direct and indirect consequences on gorgonian health.

The studies of gorgonian chemical ecology discussed above have begun to shed
light on the secondary metabolites responsible for ecological activities. In a review of
the state of marine chemical ecology, Hay notes that field and laboratory tests suggest
that secondary metabolites commonly function as a defense against potential
predators, but have also been shown to reduce fouling, inhibit microbial pathogens and
competitors, and in some organisms act as gamete attractants. These other
potentially important activities have received less attention as evidenced by the reports
above; indeed, the ability of gorgonian metabolites to inhibit microbial pathogens has
only received serious attention since 1994, and little work has sought to reveal the
actual compound or compounds responsible for the observed activity. Hay also
remarked that although work on the defensive metabolites found in gorgonians has
progressed, very little is known about how the consumers of these compounds
perceive them or how quantities of compounds consumed affect physiology or overall
fitness. Though a defensive or ecologically active metabolite may be isolated from an
organism, there is usually little knowledge of the variation in these chemical defenses over geographic regions, habitats, and within individuals in a habitat.\textsuperscript{20}

Hay states that as a science, marine chemical ecology has benefited by maturing from a time when chemists isolated unusual metabolites from marine organisms and assumed an ecological function, or biologists assumed an underlying chemical influence for observed ecological interactions, to a present state where ecologically realistic and relevant studies simultaneously investigate both chemical and biological aspects.\textsuperscript{20} Many outstanding areas of investigation for marine chemical ecology remain, including how metabolites act on consumers when eaten, the variance of metabolites in spatial and temporal patterns, how compounds act in conjunction with all aspects of the organism and its environment, when and how ontogenetic shifts in types and concentrations of metabolites occur, and what role microbes may play in the ecology of marine organisms. For such studies to succeed, Hay points out that “more novel, rigorous, and ecologically relevant methodologies need to be developed and applied to investigations” of the possible functions of marine secondary metabolites.\textsuperscript{20}

As shown in the following section which reviews the numerous, varied, and unusual compounds that have been isolated from gorgonians, only a few have been tested for or ascribed any ecological function. The study of gorgonian chemical ecology, though decades old, is only at the beginning of understanding the ecological significance of this plethora of metabolites, and it would seem that limitless possibilities exist in investigating and understanding gorgonian chemical ecology.
D. Review of Gorgonian Natural Products Chemistry

Studies of the interesting chemistry of gorgonian corals have been continuing at an ever-increasing pace since Bergmann's description of gorgosterol in 1943\textsuperscript{58} and Burkholder's finding of gorgonian antimicrobial activity in 1958.\textsuperscript{52} Starting in the early 1970's, the rate at which novel compounds and new carbon skeletons were being reported from gorgonians increased dramatically. The increase in reports of gorgonian chemistry reflects an increased interest in marine natural products and advances in spectroscopic techniques, such as proton NMR and single crystal X-ray diffraction, which aid greatly in structure elucidation.

The numerous reports of gorgonian secondary metabolites have been reviewed and summarized by specialists.\textsuperscript{17,59-67} While these combined reviews comprehensively detail the metabolites isolated from gorgonians (and in one case a limited taxonomic look at the chemistry isolated specifically from West Indian gorgonians), no review to date has surveyed the complete literature on gorgonian chemistry by taxonomic family. The intent of the present section is twofold: first, to provide an overall picture of the types of metabolites which have been isolated from gorgonians by family, and second, to provide a list of gorgonian chemistry references which is as comprehensive as possible. For each gorgonian family, structures of examples of the types of metabolites discovered and the references of subsequent reports of similar compounds will be presented. Collection sites for the gorgonians will be provided when available. In compiling the references cited in this section, Faulkner's reports in \textit{Marine Natural Products}\textsuperscript{17} were used heavily and were extremely helpful. In addition to Faulkner's reviews, a survey by Rodriguez\textsuperscript{67} was
consulted, Chemical Abstracts (CA Selects) were reviewed, and computer literature searches of MarinLit were carried out. Every effort has been made to include all references for reports of novel gorgonian chemistry. These references are comprehensive through 1999 and should be comprehensive through 2000 as well, though some papers published in journals which less commonly report marine natural products may have been omitted. As previous reviews have detailed synthetic work on gorgonian metabolites as well as interesting biological and pharmacological properties of gorgonian metabolites, that information will not be repeated here.

The chemistry of gorgonians is presented here by taxonomic family, with the intent of providing an overview of the types of metabolites that have been isolated from each family. However, it should be noted that many classes of compound are known from more than one family, and some gorgonian families have had no chemistry described at all. In 1995, Rodriguez reviewed the literature on West Indian gorgonian chemistry and stated that less than 20% of the species from that region had been chemically described.\(^\text{67}\) It must be noted, however, that researchers do not report on species for which no interesting chemistry is found, such as the common and conspicuous Indo-Pacific gorgonian *Siphonogorgia* sp. Of those gorgonians for which novel chemistry has been reported, researchers have found acetogenins, sesquiterpenoids, diterpenoids, prostanoids, and highly functionalized steroids. Many of these compounds are of chemical classes previously unknown from terrestrial organisms.

Five families of gorgonians have had no secondary metabolites reported from them. The families Pseudogorgiidae and Sibogagorgiidae both have only one genus,
the family Ifalukellidae has two genera, the Keroeididae has five, and the Chrysogorgiidae twelve genera. None of the twenty-one genera represented by these five families are very common, and the lack of reports of chemistry from them may simply be due to the fact that only biologists have encountered them to date. The thirteen families for which chemistry has been reported are described below in alphabetical order.

1. **Family Acanthogorgiidae**

The family Acanthogorgiidae comprises seven genera, though chemistry has only been reported from two of them, *Acalycigorgia* and *Muricella*. Three compounds having the guaiazulene carbon skeleton were reported from *Acalycigorgia* sp. in 1987.\(^{68}\) The novel compound 2,3-dihydrolinderazulene (28) and the two known compounds linderazulene (29) and guaiazaulene (30) were found (Figure 13), and are responsible for the blue color of gorgonians containing guaiazulene class compounds. Further guaiazulenoid pigments reported from this family include gorgiabisazulene (31) and gorgiagallyazulene (32) from a Japanese gorgonian.\(^{69}\) A number of xenicanes have been reported from *Acalycigorgia* sp., starting with acalycixeniolides A (33) and B (34) from *A. inermis* in 1987 (Figure 13).\(^{70}\) The same authors reported the related xenicane, ginamallene (35) from four Japanese specimens of *Acalycigorgia* sp. the following year,\(^{71}\) with the related minor compounds acalycixeniolides B' and C reported in 1989.\(^{72}\) Further reports of xenicane diterpenes from Japanese gorgonians of the family Acanthogorgiidae include acalycigorgins A, B, and C in
Figure 13. Secondary metabolites from the family Acanthogorgiidae.

33 $R = -(\text{CH}_2)_3\text{CH}=\text{CH}_2$
34 $R = -(\text{CH}_2)_2\text{CH}=\text{C}=\text{CH}_2$
35
36 $R = \text{H}$
37 $R = \text{OAc}$
38
39
1993,73 with D and E reported the following year,74 and acalyxeniolides D through G from a Korean specimen of *A. inermis* reported and corrected in 2000.75,76

Ophirin (36), a diterpene of the cladiellane class, was first reported from *Muricella* sp. collected in the Red Sea in 1980,77 with the revised structure and the related compound 37 described in 1997.78 Three aromatic seco steroids, calicoferols C (38), D (39), and the related E were reported from *Muricella* sp. collected in Korea in 1995,79 with the report of calicoferols F-I following in 1998 from another Korean gorgonian.80

2. **Family Anthothelidae**

Though the Anthothelidae comprises 12 genera, the chemistry reported from this family is dominated by diterpenes from the species *Erythropodium caribaeorum*. A 1984 report described two interesting diterpenes of the briarane class, erythrolides A (40) and B (41).81 The structure of erythrolide A was determined by X-ray analysis (Figure 14). The structures of erythrolides C-I from a Caribbean gorgonian were subsequently reported in 1991,82 with erythrolides E (42), F (43), and I (44) containing the first example of a C-2,8 ether. The known erythrolides were extended with the 1993 description of erythrolide J from a Tobago collection,83 and the 1998 report of the unusual erythrolide K (45) which was linked to erythrolide B by a series of interconversions.84 The erythrolide derivatives erythrolide E 3-acetate, erythrolide F 3-acetate, and erythrolide H 16-acetate were reported from a Jamaican collection in 1999.85 The briaranes are very interesting secondary metabolites whose carbon
Figure 14. Secondary metabolites from the family Anthothelidae.

\[ \text{40} \]

\[ \text{41} \]

\[ \text{42} \quad R = \text{Ac} \]

\[ \text{43} \quad R = \text{COCH}_2\text{OAc} \]

\[ \text{44} \quad R = \text{COCH}_2\text{OH} \]

\[ \text{45} \]

\[ \text{46} \]

\[ \text{47} \]

\[ \text{48} \]
skeleton had not previously been observed from terrestrial resources, but have been
isolated from organisms throughout all orders of the Alcyonaceans. The three ring
system of the briaranes, and especially the ten-membered ring, make it nearly
impossible to accurately depict the relative stereochemistry of briarane compounds in
a two dimensional drawing. A more in depth analysis of briaranes and their
uniqueness will be presented in a later chapter.

In a very interesting 2000 report, *E. caribaeorum* from the southern Caribbean
was found to contain eleutherobin (46) and six related diterpenes (Figure 14).
Eleutherobin was isolated as the major metabolite, with desmethylleletherobin,
desacetylleletherobin, isoeletherobin A, Z-eletherobin, caribaeoside, and
caribaeolin all isolated as minor derivatives. Eleutherobin was first described from
the soft coral *Eleutheroobia* sp. in 1997 and drew interest due to its pharmacological
activity in mimicking paclitaxel (Taxol) by stabilizing microtubules. This is the only
report of the discovery of the eleutherobin carbon skeleton in gorgonians, but the
occurrence of this metabolite in both gorgonians and alcyonarian soft corals highlights
the close taxonomic relationship of these organisms.

Two sesquiterpenoids have been reported from this family, including the
spirobicyclic sesquiterpene (-)-erythrodiene (47) described from *E. caribaeorum*
collected in the Caribbean in 1993. The only other species of the family
Anthothelidae for which secondary chemistry has been described is *Alertigorgia* sp.
The sesquiterpene dimer alertenone (48) was isolated from an Australian gorgonian
and is a dimer of the previously reported suberosenone from *Subergorgia suberosa* of
the family Subergorgiidae.
3. **Family Briareidae**

The family Briareidae has only one genus, yet the chemistry described from that genus is extensive. The Briareidae produce a number of diterpenes, including briaranes, asbestinins, eunicellins, and cembranoids. The family has been condensed into one genus, as *Briareum* has been found to be synonymous with *Solenopodium* and probably *Pachyclavularia*. However, the chemistry will be presented here as reported in the literature for clarity, with the reports from each species grouped and presented alphabetically by species. In addition, structures will be presented as drawn in original publications to illustrate the difficulty faced in trying to represent the relative configuration of these compounds, as previously disscussed. The original publication should be referenced when attempting to decipher the relative stereochemistry of these compounds.

**Briareum asbestinum**

The structure of briarein A (49) from *Briareum asbestinum* was announced at a conference in 1975 and the X-ray structure was subsequently published in 1977. The discovery of this metabolite was very exciting, as briarein A had a novel carbon skeleton, was highly oxygenated, and chlorinated (Figure 15). Briarein A became the first of many briaranes to be reported due to the interest in its unusual structure and the fact that latter investigations found that gorgonians of the *Briareum* genus typically contained a relatively large quantity of often a dozen or more similar metabolites.

Subsequent reports from a Belize collection of *B. asbestinum* described asbestinins 1-5 (50-54) and asbetinin epoxide (55) and asbestinin-5 acetate (56). The structures were assigned based on the X-ray structure of the diol (57) to which all
Figure 15. Secondary metabolites of the family Briareidae.

49

50 $R^1 = \text{Ac}, R^2 = \text{COC}_3\text{H}_7$

52 $R^1 = \text{H}, R^2 = \text{COC}_3\text{H}_7$

57 $R^1 = R^2 = \text{H}$

51

53 $R^1 = R^2 = \text{O}$

54 $R^1 = \text{H}, R^2 = \text{OH}$

56 $R^1 = \text{H}, R^2 = \text{OAc}$

55

58 $\text{AcO}, \text{OCOPr}$

COOMe
Figure 15. Secondary metabolites of the family Briareidae (continued).

60 $R^1 = \text{Ac}, R^2 = H, R^3 = \text{OAc}$
61 $R^1 = \text{Ac}, R^2 = H, R^3 = \text{OCOC}_7\text{H}_{15}$
62 $R^1 = \text{Ac}, R^2 = H, R^3 = \text{Cl}$
63 $R^1 = \text{OCOC}_7\text{H}_{15}, R^2 = H, R^3 = \text{OCOC}_3\text{H}_7$
64 $R^1 = \text{OCOC}_3\text{H}_7, R^2 = R^3 = \text{OAc}$
Figure 15. Secondary metabolites of the family Briareidae (continued).

73

74

75

76 R = H
77 R = Ac

78

79 R = OCOPr

80 $R^1 = \text{COPr}, R^2 = R^3 = H$
81 $R^1 = \text{COPr}, R^2 = \text{Ac}, R^3 = H$
82 $R^1 = \text{COEt}, R^2 = R^3 = H$
83 $R^1 = \text{COPr}, R^2 = \text{Ac}, R^3 = H$
84 $R^1 = \text{Ac}, R^2 = R^3 = H$
85 $R^1 = R^2 = \text{Ac}, R^3 = H$
86 $R^1 = \text{COPr}, R^2 = \text{Ac}, R^3 = \text{OAc}$
87 $R^1 = \text{COEt}, R^2 = \text{Ac}, R^3 = \text{OAc}$
88 $R^1 = R^2 = \text{Ac}, R^3 = \text{OAc}$

89/91 R = OAc
90 R = H

92

93
of the compounds were interrelated. Examination of the asbestinin structures (50-56) reveals a trend common among asbestinins and briaranes. The compounds vary in sites of oxidation, saturation/unsaturation/epoxidation, and the functional groups observed, such as hydroxyl, acetoxy, or n-butyryl groups. These variations allow for an almost infinite number of different metabolites based on the same carbon skeleton, which accounts for the numerous and ever increasing reports of metabolites from these organisms.

Methyl briareolate (58) is the first reported briaran that does not possess a γ-lactone ring and was isolated 1992 from a Caribbean gorgonian (Figure 15). Another specimen from Tobago contained three compounds related to methyl briareolate (58) and two non-chlorinated briaranes, including 59 which has an unusual ether bridge (Figure 15). Briareins C-L were reported in 1996 from a Puerto Rican species, and briareins H-L (60-64) display the variability in substitution seen in the briarane class compounds. Briareolate esters have also been observed from B. asbestinum from Tobago, as represented by the report of briareolate esters D-I, which were isolated along with briareolides J and K. The three briareolate esters D-F (65-67) are shown in Figure 15. Other reports of briaranes from B. asbestinum include brianthein V and briarolides A-I.

An asbestinin metabolite containing an oxetane ring (68) was isolated in conjunction with briaranes (Figure 15), and the another specimen revealed the first reported seco-asbestinin (69). A 1995 report described ten new asbestinins from a Puerto Rican gorgonian named briarellins A-I, which contain a lactone as shown by
briarellins B (70) and C (71), and secobriarellin (72). Other reports of asbestinins from *B. asbestinum* include the four new asbestinins 4-deoxyasbestinin A, 11-acetoxy-4-deoxyasbestinin B, 4-deoxyasbestinin C, and 11-acetoxy-4-deoxyasbestinin D, asbestinins 6-10, and sixteen minor asbestinins from various collections made around Puerto Rico along with a revision of the structures of asbestinins 6 and 7.

*Briareum excavatum*

*B. excavatum* was reported as *Solenopodium excavatum* in the chemical literature prior to 1998. In 1998, the briarane diterpenes excavatolides A-E were reported, with the stereostructure of excavatolide B confirmed by X-ray analysis. In 1999, four reports described an additional 30 briaranes from this species: excavatolides F-M, N-T, U-Z, and having exhausted the alphabet briaexcavatolides A-J. Excavatolides A-J, F-M and U-Z were from Taiwanese gorgonians, while N-T were obtained from a Western Australian specimen. All of these compounds vary by simple group substitutions as described above.

*Briareum polyanthes*

Cardellina and coworkers published all of the reports of chemistry from *B. polyanthes*. Three chlorinated briaranes isolated from a Bermuda collection of *B. polyanthes* in 1983 were designated briantheins X-Z. Interestingly, brianthein W (73) was published the following year, complete with X-ray structure drawn in an unconventional manner. The group later published the structure of bissetone (74), which is a pyrone that appears to be unrelated to any other coelenterate metabolites.
In 1995 *B. polyanthes* continued to provide surprising chemistry with the report of emmottene (75), the carbon skeleton of which had not been previously observed in marine organisms and is the first report of a naturally occurring trans-bicyclo[5.1.0]octane.113

**Briareum steckeii**

Only four briaranes have been reported from *B. steckeii*, and are unnamed and vary only in the functional group substitutions of carbons 2, 3, and 4.114

**Briareum sp.**

An Australian specimen of *B. sp.* yielded nine novel briarane derivatives along with two new cladiellin-based diterpenes (76 and 77), a known cembranoid, and a novel tricyclic diterpene (78).115 A second specimen collected at Darwin, N. T. and described from the same group contained two new briaranes.116 The structure of brianolide from a Japanese specimen was determined by X-ray crystallography.117 The briaranes 2β-acetoxy-2-(debutyrlyloxy)stecholide E, 9-deacetylstylatulide lactone, and 4β-acetoxy-9-deacetylstylatulide lactone were reported from a Korean specimen.118 An Indonesian specimen contained 2,9-diacetyl-2-debutyryl-stecholide H, 13-dehydroxystecholide J, and 2β-acetoxy-2-(debutyrlyloxy)stecholide E acetate as well as several known briaranes.119 Another Japanese gorgonian yielded two briarane derivatives.120 Violides C-I,121 violides J-M,122 and N-P123 were reported from a Japanese species, with a subsequent correction.124
Solenopodium excavatum

There is only one report of chemistry from a New Guinea species of S. excavatum, describing stecholides I-N and 16-hydroxystecholide C. The X-ray structure of stecholide I (79) revealed an unexpected conformation of the ring juncture.

Solenopodium stechei

The single report of chemistry from a specimen of S. stechei collected off the Great Barrier Reef yielded 25 diterpenes representing three carbon skeletons. The stecholides, each of which is individually named in the paper, are briaranes. Nine of the twenty stecholides, 80-88, are shown in Figure 15 for comparison to the other metabolites isolated and to again reveal the variability of functional group substitution in these compounds. In addition to the stecholides, the eunicellin-type diterpenoids solenopodins A-D (89-92) and one cembranoid (93) were isolated.

Solenopodium sp.

An Indo-Pacific gorgonian provided six novel briaranes, solenolides A-F, which were assigned by spectral analysis and chemical modifications.

4. Family Coelogorgiidae

The family Coelogorgiidae has only one genus, Coelogorgia, and there is only one report of chemistry to date. In 1990, palmosalides A-C (94-96) were reported from an Indian Ocean collection of Coelogorgia palmosa (Figure 16). Palmosalide
C possess a bakkane skeleton and its structure was determined by X-ray crystallography and later by total synthesis.\textsuperscript{129}

5. **Family Coralliidae**

The family Coralliidae has two genera, and there is only one report of chemistry from this family to date. In 1981, Five xenicane class diterpenes were reported, corazenolides A-C (97-99), C' (100), and corabohcin (101).\textsuperscript{130} The Coralliidae are valued for their use in jewelry.

6. **Family Ellisellidae**

The Ellisellidae comprise 7 genera, and chemistry has been reported from three of these, especially *Junceella*. The *Junceella* are sea whips, often found as single strands of a meter in length, and typically grow in groups, as reproduction can occur by a tip of the whip breaking off, falling to the bottom, and giving rise to another whip. While studies of these gorgonians have typically involved collection of a number of individual whips and examination of the extract of the combined collection, research presented in following chapters will show that each individual whip may possess a distinct chemical profiles, and that chemical phenotypes can be found across geographic collection sites.

The compounds reported from *Junceella* have all been of the briarane class. A 1989 report from a South China Sea collection of *J. fragilis* described four new briaranes, juncellolides A-D\textsuperscript{131} together with the known compounds praelolide\textsuperscript{132} and juncellin\textsuperscript{133}, previously reported in Chinese journals. Junceellolide A (102) has
Figure 16. Secondary metabolites from the family Coelogorgiidae.

Figure 17. Secondary metabolites from the family Coralliidae.
an unusual C-4,8 ether bridge while junceellolide B (103) is hydroxylated at C-8 (Figure 18). The other four compounds are similar derivatives of these two compounds. Five further junceellolides, (-)-4-deacetyljunceellolide D, (+)-11α,20α-epoxyjunceellolide D (104), (-)-11α,20α-epoxy-4-deacetyljunceellolide D (105), 11α,20α-epoxy-4-deacetoxyjunceellolide D (106), and (+)-junceellolide A were reported from an Indonesian specimen of *J. fragilis.*

A West Pacific Ocean collection of *J. fragilis* yielded the three additional junceellolides E-G. A Great Barrier Reef collection of *J. gemmacea* revealed three new briaranes, including 107 (Figure 18). *J. gemmacea* continued to provide novel briarane diterpenes with the report of gemmacolides A-F from a Pohnpei collection. The structures of gemmacolides D (108) and E (109) are shown in Figure 18. Juncins A-F (110-115) are six new chlorinated briaranes isolated from a Red Sea collection of *J. juncea.* The locations of the isovalerate and isobutyrate esters in 114 and 115 were not determined (Figure 18). The juncins were extended in 1997 with the report of juncins G and H from an Indian Ocean collection of *J. juncea.* An unidentified gorgonian from Pohnpei contained known compounds previously isolated from *Junceella* sp., as well as four novel briaranes, nuiinoalides A-D.

Three polyhydroxylate sterol hemiacetals, pectinoacetals A (116), B, and C, were isolated from *Ctenocella pectinata* as the corresponding 18-acetate derivatives in 1993. A later report found a mixture of isomeric hemiacetals related to the pectinoacetals that were identified by spectroscopic analysis of the 18β-acetate.
Figure 18. Secondary metabolites from the family Ellisellidae.

102

103

104 R = OAc
105 R = OH
106 R = H

107

108 R = Ac
109 R = H

110 R₁ = R² = R³ = Ac, R⁴ = R⁵ = H
111 R₁ = R² = R³ = Ac, R⁴ = OAc, R⁵ = H
112 R₁ = R² = R³ = Ac, R⁴ = R⁵ = OAc
113 R₁ = R² = R³ = Ac, R⁴ = R⁵ = H (11,20-deoxy)
114 R² = Ac, R¹,R³,OR⁴ = 2Ac + isovalerate, R⁵ = H
115 R¹⁻³,OR⁴ = 3Ac+isobutyrate, R⁵ = H(3,4-dihydro)

116

117

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The Indian Ocean gorgonian *Gorgonella umbraculum* has been found to contain both briarane class diterpenes and a polyoxygenated steroid. Junceellin and praelolide, previously isolated from *Junceella* sp., were found along with a new briarane in a 1998 report. The same researchers later published further briaranes designated umbraculolides B-D from the same gorgonian and designated the previously unassigned compound umbraculolide A. The same group later found that the more polar fractions contained a novel polyoxygenated steroid (117).

7. **Family Gorgoniidae**

The Gorgoniidae comprise 13 genera, and account for a large portion of the gorgonian chemistry reported to date, second only to the Plexaurids. Because the number of chemistry reports from the Gorgoniidae is so large, and similar chemistry is found in different genera, the chemistry of the Gorgoniidae will be reported by structure type. Examples of compounds of each structural type will be given and subsequent publications of similar compounds will be detailed.

A number of sesquiterpenes have been isolated from members of the family Gorgoniidae, though many are remarkable only in that they are enantiomers of terpenes previously isolated from terrestrial sources. A 1968 report found β-gorgonene (118) from a specimen of *Pseudopterogorgia americana* (Figure 19). Three antimicrobial sesquiterpenes were isolated from *P. rigida* and identified as (-)-curcuphenol (119), (-)-curcuquinone (120), and (-)-curcuhydroquinone (121), and another report from a Caribbean collection described the unusual sesquiterpene...
derivative, mochiquinone. In 1995 the known curcuquinone sesquiterpenoids from gorgonians were extended with the isolation of curcuhydroquinone 1-monoacetate from *Pseudopterogorgia americana* collected in Barbados, and another quinone, rigidone, was isolated from *P. rigida* collected in the Bahamas in 1997. Germacrene derivatives isolated from the family include the diol 122 from *Pacifigorgia media*, the unusual furanogermacrene furanotriene (123, structure revised in 1990), and the related isofuranotriene from *Pseudopterogorgia americana*, and the known furanodiene 124, which was isolated from both *Pacifigorgia pulchra* and *P. media*. Pacifigorgiol (125) is another unusual sesquiterpenoid isolated from *P. cf. adamsii*. 12-hydroxy-(E)-γ-bisabolene (126) was isolated from a gorgonian of the genus *Pseudopterogorgia*. Another *Pseudopterogorgia* species contained elemanolide (127). The structure of (+)-lepidozene (128) from *Lophogorgia ruberrima* was determined by analysis of spectroscopic data and X-ray analysis of the bis-epoxide derivative. The nardosinane sesquiterpene 11,12-epoxynardosin-1(10)-ene (129) was isolated from the Brazilian gorgonian *Phyllogorgia dilatata*, and a later report found 12-hydroxynardosin-1(10),11(13)-diene from the same species. Also reported from *P. dilatata* is (E)-germacra-1(10),4(15),7(11)-trien-5-ol-8-one together with known compounds.

Though reports of sesquiterpenes from gorgonians declined in the 1990's, a few new compounds were reported. For example, a single 1991 paper reported a new furan (130) and its corresponding γ-hydroxybutenolide, piccolamine (131), and piccolamine N-oxide (132) from the Senegalese gorgonian *Leptogorgia piccola*.159
Figure 19. Secondary metabolites from the family Gorgoniidae.
Figure 19. Secondary metabolites from the family Gorgoniidae (continued).
Figure 19. Secondary metabolites from the family Gorgoniidae (continued).
Figure 19. Secondary metabolites from the family Gorgoniidae (continued).
In 1996, five highly oxygenated guanolides named americanolides A-C and two methyl esters (believed to be artifacts) were isolated from *Pseudopterogorgia americana*. Americanolides A (133), C (134), and the methyl ester of A (135) are shown in Figure 19. The americanolides were later extended to include americanolides D-F and methoxyamericanolides E and G from a Puerto Rican specimen of *P. americana*. Another Puerto Rican specimen of the same species gave 10-epimethoxyamericanolide A, 10-epiamericanolide C, 8-epimethoxyamericanolide A, 8-epiamericanolide C, methoxyamericanolide H, and methoxyamericanolide I. In 1999, a single report of a gorgonian sesquiterpene gave 5,10-epoxymuurolane (136) from a Bahamas collection of *Gorgonia ventalina*.

There have been numerous reports of diterpenes of a number of classes from the Gorgoniidae. The report of eunicellin (137) from *Eunicella stricta* established the carbon skeleton for the eunicellin class of compounds, which are seen in other families as well. Five further eunicellin class compounds, palmonines A-E, were reported from a Spanish specimen of *Eunicella verrucosa*. The five palmonines and the subsequently reported palmonine F comprise various derivatives of palmonines A (138) and C (139). Another eunicellin-type compound was isolated from the Mediterranean gorgonian *Eunicella cavolini*, followed by a report of massileunicellins A-C, which have an unusual second ether bridge as shown by massileunicellin B (140), from the same species. Five new eunicellins, labiatamides A and B and labiatins A-C, were isolated from the Senegalese gorgonian...
*Eunicella labiata*,\(^{169}\) from which labiatins D and E were later reported.\(^{170}\) A Spanish specimen of *E. labiata* contained \((1S^*,2Z,6E,10R^*,11S^*,12S^*,13S^*,14R^*)-12,13\)-diacetoxycladiella-\(2,6\)-dien-\(11\)-ol (141)\(^{171}\), which has the eunicellin carbon skeleton but lacks oxygenation at C-9, and might represent a biosynthetic precursor to both eunicellin and cladiellane diterpenes.

There have been many reports of compounds of the pseudopterane class from gorgonians in family Gorgoniidae. Lophotoxin (142) has been isolated from four species of *Lophogorgia*, including *L. alba*, *L. chilensis*, *L. cuspidata*, and *L. rigida*.\(^{172}\) The structure of lophotoxin was elucidated by interconversion with pukalide (16), which is a minor metabolite of these gorgonians and other alcyonarian soft corals. Pukalide was first isolated from the soft coral *Sinularia abrupta* by Scheuer and coworkers in 1975,\(^{173}\) and has since been isolated along with a number of related compounds from a number of gorgonians and soft corals, again illustrating the close chemotaxonomic relationship of many alcyonarians. The three related diketones lophodione, isolophodione (143), and epoxylphodione were also isolated from *L. alba*.\(^{174}\) Pseudopterolide (144) is another pseudopterane isolated from *Pseudopterogorgia acerosa*.\(^{175}\) Kallolides A-C and kallolide A acetate are four further pseudopteranes obtained from the Caribbean gorgonian *P. kallos*,\(^{176}\) and bipinnatins A-D were obtained from the Caribbean gorgonian *P. bipinnata*.\(^{177}\) A new pseudopterane containing nitrogen, tobagolidie (145), was isolated from a Tobago specimen of *P. acerosa*.\(^{178}\) Further collections of *P. acerosa* from Tobago gave a mixture of acids which were esterified with diazomethane to yield acerosolide,
deoxypseudopterolide, and tobagolide, the pseudopteranes
diepoxygorgiaacerone, pseudopterolide-methanol adduct, gorgiaceron,
gorgiacerodiol, methoxygorgiacerol, isogorgiacerodiol, bis(gorgiacerol)amine, and
11-goriacerol. Also from P. acerosa, aceropterine (146) is an unusual
dimethylamino pseudopterane, with the lactone ring transposed from the usual
position. An additional five pseudopteranes, pseudopteradiene, pseudopteradienoic
acid, 11-pseudopteranol, pseudopteranoic acid, and diepoxygorgiacerodiol, and the
unusual norditerpene alanolide (147) were all obtained from the same
specimen. A Puerto Rican specimen of P. acerosa yielded a bis-epoxide
pseudopterane and gorgiacerolide. Isolated from the Caribbean gorgonian P.
bipinnata were pinnatins A-C (148-150) and D-E, which contain a cyclopropane
ring, and bipinnatin J (151), photolysis of which gives kallolide A. A
Columbian specimen of P. bipinnata yielded bipinnapterolide A, bipinnatins G-I, and
bipinnatolides F-J, along with the diterpenoid dimer bisgersolanolide (152). A
novel seco-furanocembranolide was isolated along with bipinnatolide K from a West
Indian collection of P. bipinnata. The hexane extracts of a sample of P. bipinnata
collected in San Andrés Island, Colombia, led to the isolation of an unprecedented
heptacyclic C30 bis-diterpenoid, bisgersolanolide. 11β,12β-epoxyypukalide was
isolated from Leptogorgia setacea, and its structure was elucidated by comparison of
its spectral data with those of related compounds.

Other diterpenes isolated from the Gorgoniidae include the pseudopterosins and
seco-pseudopterosins. Pseudopterosins A-D (153-156) are anti-inflammatory
compounds that were isolated from the Caribbean gorgonian *Pseudopterogorgia elisabethae*. A new species of *Pseudopterogorgia* contained seco-pseudopterosins A-D (157-160), which also possess anti-inflammatory properties. A subsequent study of a Caribbean species of *Pseudopterogorgia* yielded three new pseudopterosins and one new seco-pseudopterosin. Reinvestigation of two collections of *P. elisabethae* from the Caribbean yielded pseudopterosins E-L and the methylated aglycone of pseudopterosin E, and pseudopterosins E-J were also found in a Bermudian collection. A Columbian collection of *P. elisabethae* contained pseudopteroxazole (161) and secopseudopteroxazole (162), and a second collection from the same location yielded elisabatins A (163) and B (164).

Other diterpenes isolated from Colombian collections of *P. elisabethae* include elisabethins A-C (165-167) and elisabanolide (168), the nor-diterpenes sandresolides A (169) and B (170), colombiasin A, which has an unusual tetracyclic carbon skeleton, and three unnamed novel diterpenes along with a norditerpene and an unusual tetrisnorditerpene which represent new classes of C$_{19}$ and C$_{16}$ rearranged terpene carbon skeletons. A West Indian collection expanded the elisabethins to include elsiabethin D and elisabethin D acetate, and also yielded 3-epi-elisabanolide and elisaporterosins A and B. The two novel lactones elisabetholide and amphilectolide were isolated from the hexane extracts of *P. elisabethae*, two novel serrulatane diterpenes, erogorgiaene and 7-hydroxyerogorgiaene, and an unusual C$_{40}$ bisditerpene were isolated from a West Indian collection of *P. elisabethae*, and another West Indian collection gave two diterpenes, cumbiasins A and B, having the
novel cumibane carbon skeleton, in addition to cumbiasin C, a ring cleavage product of cumbiasin B that possesses an unusual carbocyclic framework named seco-cumbiane.\(^{206}\)

A number of sterols have also been isolated from the Gorgoniidae. Four polyoxygenated cholestenone derivatives were isolated from the Mediterranean gorgonian *Leptogorgia sarmentosa*, as represented by the enolized \(\alpha\)-diketone 171, which is the major product.\(^{207}\) Another specimen of *L. sarmentosa* contains three new steroids, (20S)-20-hydroxycholestan-3,16-dione, (16S, 20S)-16,20-dihydroxycholestan-3-one, and (20S)-20-hydroxycholest-1-ene-3,16-dione together with a known related compound.\(^{208}\) Prena-4,20-dien-11\(\alpha\)-ol-3-one acetate was isolated from *Eunicella cavolini*,\(^{209}\) and the pregnane glycoside verrucoside was found from *E. verrucosa* collected in Spain.\(^{210}\) A polyhydroxylated 4\(\alpha\)-methylsterol, acerosterol (172), was isolated from *Pseudopterogorgia acerosa*.\(^{211}\) Three novel 9,11-secosterols were isolated from *Pseudopterogorgia* sp. collected in Florida.\(^{212}\) One of these secosterols, 173, was later reported from a Barbados collection of *P. americana*,\(^{213}\) and a similar secosterol, (22R,23R,24R)-22,23-methylene-23,24-dimethyl-9,11-secocolesterol-5en-9one-3\(\beta\),11-diol, was reported from a Caribbean collection of *P. hummelinkii*.\(^{214}\) The unusual 17-hydroxy sterol punnicin was isolated from the Brazilian gorgonian *Lophogorgia punicea*.\(^{215}\) A Puerto Rican collection of *Pseudopterogorgia americana* yielded two polyhydroxylated dinostane sterols,\(^{216}\) and an Indonesian *Lophogorgia* species contained 3\(\beta\),7\(\beta\),11-trihydroxy-5\(\alpha\),6\(\alpha\)-epoxy-9,11-secogorgostan-9-one.\(^{217}\)
A variety of bisbutenolides have been isolated from the Gorgoniidae, including antcepsenolide (174), which has been isolated from both *Pterogorgia aniceps* and *P. guadalupensis*.\(^{218}\) Seven further butenolides were isolated together with antcepsolide (174) from the Caribbean gorgonian *Pterogorgia citrina*,\(^{219}\) and three butenolides (175-177) also related to antcepsolide were obtained from *P. aniceps*.\(^{220}\)

8. **Family Isididae**

The family Isididae is comprised of 38 genera, though to date secondary metabolites have only been reported from gorgonians of the genus *Isis*. Polyoxygenated sterols called hippurins have been isolated from *Isis hippuris*.\(^{221}\) The structure of hippurin-1 (178) was determined by X-ray analysis of its 3-monoacetate derivative, but the stereochemistry at C-22 was later shown to have been inverted during the acetylation reaction (Figure 20).\(^{222}\) New hippurins were reported with the revision of hippurin-1 (178), including 22-*epi*-hippurin-1 (179), hippuristanol (180), 22-*epi*-hippuristanol (181), and 2α-hydroxyhippuristanol (182). A later report described the C-18 lactone (183) and the C-18 acetal (184) of hippuristanol 3-acetate.\(^{223}\) Another report detailed five new hippurins from a specimen of *I. hippuris* collected in India,\(^{224}\) and hippuristerone A was reported from another specimen.\(^{225}\) A novel gorgosterol derivative, 185, has also been reported from *I. hippuris*.\(^{226}\)
Figure 20. Secondary metabolites from the family Isididae.

178 $R^1 = \text{OAc}$, $R^2 = \text{H}$
180 $R^1 = R^2 = \text{H}$
182 $R^1 = \text{OH}$, $R^2 = \text{H}$
179 $R^1 = \text{OAc}$, $R^2 = \text{H}$
181 $R^1 = R^2 = \text{H}$
183
184
185
186
187 $R^1 = \text{OH}$, $R^2 = \text{H}$
188 $R^1 = \text{H}$, $R^2 = \text{OH}$
190 $R^1 = \text{OAc}$, $R^2 = \text{H}$
191 $R^1 = \text{H}$, $R^2 = \text{OAc}$
189
A novel ceramide, isisamide (186) was reported from a Chinese specimen of *Isla*
sp. Also, five novel suberosane sesquiterpenes, suberosenol A (187), B (188),
suberosanone (189), suberosenol A acetate (190), and suberosenol B acetate (191)
have been reported from *I. hippuris*.228

9. **Family Melithaeidae**

The Melithaeidae comprises five genera, and there have been only three
chemistry reports from two of those five. Four 3β,7α-dihydroxy-5α,6α-epoxy-Δ8-
steroids named melithasterols A-D (192-195) were isolated from the Okinawan
gorgonian *Melithaea ocracea* (Figure 21).229 Three related 3β,5α,6α-trihydroxy-
7α,8α-epoxy-sterols and a fourth sterol isolated as its 3,6-diacetate derivative were
isolated from the Korean gorgonian *Acabaria undulata*.230 Also isolated from *A.
undulata* were the four ceramides 196-199, and their absolute stereochemistry was
determined by synthesis.231

10. **Family Nidaliidae**

The Nidaliidae include 8 genera with the possibility that two more genera should
be placed in this family, yet there is only a single report of chemistry from this family.
The six novel polyhydroxylated pregnadienes, pregna-5,20-diene-3α,7α-diol 3α-
acetate (200), pregna-5,20-diene-3β,7α-diol 7α-acetate (201), pregna-5,20-diene-
3α,7α,11α-triol 3α-acetate (202), pregna-5,20-diene-3α,7α,11α-triol 3α,7α-diacetate
(203), pregna-5,20-diene-3α,7α,19-triol 3α,19-diacetate (204), pregna-5,20-diene-
3α,7α,11α,19-tetrol 3α,7α,19-triacetate (205) were isolated from a South African
collection of Pieterfaurea unilobata (Figure 22).232

11. Family Paragorgiidae

The family Paragorgiidae has only one genus, Paragorgia, and two report of
chemistry from this genus, both of xenicane class compounds, has been published.
Arboxeniolide-1 (206) was isolated together with the previously reported
coraxeniolides B (98) and C (99) from the deep water gorgonian P. arborea (Figure
23).233 One further xenicane, 207, was reported from a Kurile Island collection of P.
arborea.234

12. Family Plexauridae

The Plexaurids are the second largest gorgonian family with 36 genera, and
account for a large portion of the reports of chemistry from gorgonians to date. As
with the Gorgoniidae, the chemistry of the Plexaurids will be reported here by
structural type, with examples of structures given and subsequent reports of similar
chemistry listed.

A number of sesquiterpenes have been isolated from the Plexaurids, including
linear hydrocarbons such as 208 and 209 from Plexauraella grisea,235 guaiazulene
(210) from the gorgonian Euplexaura erecta,236 and the related linderazulene (211)
from Paramuricea chamaeleon,237 the later two gorgonians having brilliant blue
polyps due to the compounds described. A related keto-lactone of linderazulene was
Figure 21. Secondary metabolites from the family Melithaeidae.

192 $R = \text{[structure]}$
193 $R = \text{[structure]}$
194 $R = \text{[structure]}$
195 $R = \text{[structure]}$
196 $R = \text{CH}=\text{C}H\text{C}_9\text{H}_{19} \ (E)$
197 $R = \text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{C}_7\text{H}_{15} \ (E,E)$
198 $R = \text{CH}=\text{CH}(\text{CH}_2)_7\text{CH}(\text{CH}_3)_2 \ (E)$
199 $R = \text{C}_{11}\text{H}_{23}$

Figure 22. Secondary metabolites from the family Nidaliidae.

200 $\text{[structure]}$
201 $R^1 = \text{OH}; R^2 = R^4 = R^5 = \text{H}; R^3 = \text{OAc}$
202 $R^1 = R^5 = \text{H}; R^2 = \text{OAc}; R^3 = R^4 = \text{OH}$
203 $R^1 = R^5 = \text{H}; R^2 = R^3 = \text{OAc}; R^4 = \text{OH}$
204 $R^1 = R^4 = \text{H}; R^2 = R^5 = \text{OAc}; R^3 = \text{OH}$
205 $R^1 = \text{H}; R^2 = R^3 = R^5 = \text{OAc}; R^4 = \text{OH}$

Figure 23. Secondary metabolites from the family Paragorgiidae.

206 $\text{[structure]}$
207 $\text{[structure]}$
reported from a gorgonian of the genus *Placogorgia*.\textsuperscript{238} Other guaiazulenes reported from this family included 3-chloroguaiazulene (212), 3-bromoguaiazulene (213), ehuazulene (214),\textsuperscript{239} and *N,N*-dimethylamino(guaiazulen-3-yl)methane (215) from a second communication.\textsuperscript{238} Another guaiainoid, echinofuran, was isolated from the Okinawan gorgonian *Echinogorgia praelonga*.\textsuperscript{240} 2,2'-bisguaiazulenyl was isolated together with other known guaiainoid pigments from the Korean gorgonian *Calicogorgia granulosa*,\textsuperscript{241} and bebryazulene is a guaiane furan from a Comoros Islands collection of *Bebryce grandicalyx*.\textsuperscript{242} The known germacrene derivatives sericenine (216), neosericenine, and isosercenine were isolated from both *Muricea austera* and *M. fungifera*.\textsuperscript{153} Heterogorgiolide (20) was isolated from the Brazilian gorgonian *Heterogorgia uatumani* during studies of fish feeding deterrents.\textsuperscript{40}

There have been numerous reports of diterpenes from diverse classes from the Plexaurids. Crassin acetate (217) is a cembranolide diterpene obtained from several species of *Pseudoplexaura* including *P. crassa*, from which crystals can be obtained by squeezing the juice from the fresh gorgonian.\textsuperscript{243} Eunicin (218) was reported from a Bimini collection of *Eunicea mammosa*,\textsuperscript{147} while a Jamaican collection of the same species gave the related compound jeunicin as the major metabolite.\textsuperscript{243} Another cembranolide, eupalmerin acetate, was isolated from *E. palmeri*.\textsuperscript{243} Cembranoids isolated from gorgonians also include asperdiol (219) from both *Eunicea asperula* and *E. tournefortii*,\textsuperscript{244} another cembranolide, peunicin, from *E. succinea*,\textsuperscript{245} and 12,13-di-epi-eupalmerin which may be a biosynthetic precursor to eunicin (218) and jeunicin.\textsuperscript{246} Reports of cembranoids continued with the report of ceunicin found from
E. mammosa collected near Curacao,\textsuperscript{247} report of the three novel compounds dihydroplexaurolone, dehydroplexaurolone, and its epimer from a Caribbean species of *Plexaura*,\textsuperscript{248} euniolide from the Caribbean gorgonians *Eunicea succinea* and *E. mammosa*,\textsuperscript{249} (-)-sarcophytol A and (+)-marasol from the Caribbean gorgonian *Plexaura flexuosa*,\textsuperscript{250} eupalmerin as a minor constituent of a Caribbean collection of *Eunicea mammosa*,\textsuperscript{251} (1E,3E,11E)-1,3,11-cembratrien-6-one, (1Z,3Z,11E)-1,3,11-cembratrien-6-one, and (1E,3Z,11E)-1,3,11-cembratrien-6-one from *E. calyculata*,\textsuperscript{252} five cembranoids from an undescribed gorgonian, *Eunicea* sp. collected in the Caribbean,\textsuperscript{253} the cembranolides 12,13-bisepieupalmerin acetate, 12-epi-eupalmerin, and succinolide from *E. succinea*,\textsuperscript{254} and the novel compounds euplamerone, pseudoplexauric acid methyl ester, (1S,3S,4R,7E,11E)-3,4:15,17-diepoxycebra-7,11-diene, (-)-eunicenone, a diketone, and a chlorohydrin from *E. mammosa*.\textsuperscript{255} The unusual 14-deoxycrassin (220) and pseudoplexaurol were isolated from the Caribbean gorgonian *Pseudoplexaura porosa*.\textsuperscript{256} Eighteen cembranolides were isolated from another Caribbean collection of *Eunicea mammosa*, including uproloide A acetate (221), 8-epi-uproloide A acetate, uproloide B, 8-epi-uproloide B, uproloide C, 7-epi-uproloide C acetate, uproloide D (222), uproloide E acetate, uproloide F diacetate, uproloide G acetate, and various acetate derivatives including 223,\textsuperscript{257,258} *Eunicea succinea* from Mona Island, Puerto Rico yielded four new cembranoids and the precursor 224, a geranylgeraniol derivative,\textsuperscript{259} and seven minor cembranoids were later reported from the same species collected in the same location.\textsuperscript{260}
Figure 24. Secondary metabolites from the family Plexauridae.

208

209

210

211

212 \( R^1 = \text{Cl}, R^2 = \text{H} \)

213 \( R^1 = \text{Br}, R^2 = \text{H} \)

214 \( R^1 = \text{Cl}, R^2 = \text{Br} \)

215 \( R^1 = \text{CH}_2\text{NMe}_2, R^2 = \text{H} \)

216

217

218

219

220

221

222 \( R = \text{H} \)

223 \( R = \text{Ac} \)
**Figure 24.** Secondary metabolites from the family Plexauridae (continued).

224

225

226

227

228

229 $R = H$

230 $R = Me$

231

232 $R_1 = CHO, R_2 = H$

233 $R_1 = H, R_2 = CHO$

234 $R_1 = CHO, R_2 = H$

235 $R_1 = H, R_2 = CHO$
Figure 24. Secondary metabolites from the family Plexauridae (continued).

236 10α
237 10β

238 $R^1 = \text{Me}$, $R^2 = \text{H}$
239 $R^1 = \text{H}$, $R^2 = \text{Ac}$

240 $R^1 = \text{Ac}$, $R^2 = \text{OH}$, $R^3 = \text{H}$
241 $R^1 = R^2 = \text{H}$, $R^3 = \text{OAc}$
242 $R^1 = R^3 = \text{H}$, $R^2 = \text{OAc}$

244

245

246

247

248
Figure 24. Secondary metabolites from the family Plexauridae (continued).

249

250 \( R = C_{16}H_{33} \)
251 \( R = C_{14}H_{29} \)

252 \( R^1 = R^2 = \text{Ac} \)
253 \( R^1 = \text{COPr}, R^2 = \text{Ac} \)
254 \( R^1 = \text{Ac}, R^2 = \text{COPr} \)
255 \( R^1 = R^2 = \text{COPr} \)

256 \( R^1 = \text{OH}, R^2 = \text{H} \)
257 \( R^1 = \text{H}, R^2 = \text{Ac} \)

259

260
Figure 24. Secondary metabolites from the family Plexauridae (continued).
The diterpene fuscol (225) was isolated from *Eunicea fusca*, and the ketone 226 was isolated from the same animal.\textsuperscript{261} Related diterpene glycosides fuscosides A (227), B, C (228), and D were also isolated from *E. fusca*.\textsuperscript{262} Asperketals A-F are dilophol class diterpenes isolated from *Eunicea asperula*,\textsuperscript{263} and asperketals A (229), B (230), and C (231) are shown in Figure 24. The trisnorditerpenes norasperenals A-D (232-235) were isolated from an undescribed species of *Eunicea* from the Caribbean.\textsuperscript{264} Calyculones D (236), E (237), F, and G are diterpenes isolated from *E. calyculata* along with some cembranoids.\textsuperscript{252} The diterpenoid cyclohexanes eunicones A (238) and B (239) were isolated from a new species of *Eunicea* collected from the Tobago Cays.\textsuperscript{265} Three dilophol-type diterpene glycosides, calyculaglycosides A-C (240-242), were isolated from a Columbian gorgonian of the genus *Eunicea*.\textsuperscript{266} Three compounds with the cubitane carbon skeleton not previously seen from marine resources, calyculones A (243), B, and C, were isolated from *E. calyculata*.\textsuperscript{267} The known diterpene ophirin (36) and a new cladiellin, astrogorgin (244) were isolated from the Japanese gorgonian *Astrogorgia* sp.\textsuperscript{268} Similar cladiellins, calicophrins A and B, have been reported from a species of *Calicogorgia*.\textsuperscript{269} The cyclic ether praelolide (245) was isolated from *Plexaureides praelonga*.\textsuperscript{270}

Metabolites with the dolabellane carbon skeleton have also been isolated from gorgonians. Two such compounds, including 246, were isolated from *Eunicea calyculata*.\textsuperscript{271} The dolabellane palominol (247) was first reported from *E. calyculata* and *E. laciniata* in 1990,\textsuperscript{272} but was revised in 1991,\textsuperscript{273} along with the report of five further dolabellanes later isolated from a Caribbean collection of *E. laciniata*.\textsuperscript{274}
Another investigation of *E. laciniata* gave five further dolabellanes, edunone, edunone, edudione, edunol, and isoedunol.\textsuperscript{274} The related gorgonian *E. tourneforti* contained four additional dolabellanes and the unusual diterpene \textsuperscript{248}.\textsuperscript{275}

A few butenolides have been isolated from gorgonians of the Plexauridae. Six butenolides were reported from *Plexaura flava*,\textsuperscript{276} and are represented by \textsuperscript{249}, \textsuperscript{250}, and \textsuperscript{251}. Four similar hydroxylated butenolides were isolated from *Euplexaura flava*.\textsuperscript{277}

A number of sterols and secosterols have been reported from the Plexaurids. The saponins muricins 1-4 (\textsuperscript{252}-\textsuperscript{255}) were isolated from *Muricea fruticosa*.\textsuperscript{46} Two steroidal glycosides, dimorphosides A (\textsuperscript{256}) and B (\textsuperscript{257}), were reported from *Anthroplexaura dimorpha*.\textsuperscript{278} A novel pregnene glycoside similar to the muricins was isolated from *Pseudoplexaura wagenaari*,\textsuperscript{279} and another from a *Eunicea* species collected from Columbia.\textsuperscript{280} A novel gorgosterol was isolated together with known gorgosterol derivatives from the Caribbean gorgonian *E. laciniata*.\textsuperscript{281} The Korean gorgonian *Euplexaura anastomosans* contained the hemiacetals anastomosacetals A-D, represented by anastomosacetal A (\textsuperscript{258}) in Figure 24.\textsuperscript{282} A secosterol, astrogorgiadiol (\textsuperscript{259}), was reported from a Japanese species of *Astrogorgia*,\textsuperscript{268} and related calicoferols A and B were isolated from another Japanese gorgonian of the genus *Calicogorgia*.\textsuperscript{283}

A unique farnesylhydroquinone glycoside, moritoside (\textsuperscript{260}), was found from a Japanese species of *Euplexaura*,\textsuperscript{284} and the related eplexides A-E were later reported from the Korean gorgonian *Euplexaura anastomosans*.\textsuperscript{285}
Prostanoids and their precursors have been observed in the Plexaurids. Preclavulone-A (261) was observed from a Florida collection of *Pseudoplexaura porosa*, and is proposed to be a key intermediate in marine prostanoid biosynthesis. *Plexaura homomalla* contains 15-epi-PGA₂ acetate methyl ester (13), PGF₂-9-O-acetate methyl ester (262), and an incubation of arachidonic acid with the acetone powder preparation of *P. homomalla* produced two eicosanoids, the allene oxide (263) and the cyclopropyl alcohol (264). (11R)-Hydroxyeicosatetraenoic acid (265) was isolated from *P. dichotoma*, and is a proposed intermediate to prostanoids in coelenterates.

Other metabolites isolated from Plexaurids include the calciogorgins A, B (266), and C (267) which are sphinganine derivatives from a Japanese species of *Calicogorgia*, the indole alkaloids villagorgins A (268) and B (269) from a New Caledonian specimen of *Villogorgia rubra*, and (5Z,9Z)-14-methylpentadeca-5,9-dienoic acid (270) from *Eunicea succinea*.

13. Family Subergorgiidae

There have been a few reports of interesting secondary chemistry from the single genus, *Subergorgia*, in the family Subergorgiidae. Two sesquiterpenes, 8-methoxycalamenene (271) and 5-hydroxy-8-methoxycalamenene (272), were reported from *S. hicksoni*. The sesquiterpenoid subergorgic acid (273) was isolated from *S. suberosa* and was later synthesized in a stereocontrolled manner. Subsequently, four subergorgic acid analogues, 274-277, were obtained from the same
species collected in the Indian Ocean. Suberosenone (278) is another
sesquiterpenoid isolated from the same species, and is of the quadrone class of
compounds.

A few seco steroids have also been isolated from S. suberosa, including 3,9-
dioxo-9,11-secocholesta-5,7-dien-11-al (279) from a specimen collected from the
Mandapam Coast of India. Another Indian collection yielded 3β,6α,11-trihydroxy-
9,11-seco-5α-cholest-7-ene-9-one (280), and both 24S- and 24R-methyl-3β,6α,11-
trihydroxy-9,11-seco-5α-cholest-7,22E-diene-9-ones (281 and 282).

E. General Techniques Used

The study of gorgonians for the production of natural products requires careful
collection, identification, and storage of specimens. The investigation of the
secondary chemistry expressed in these organisms requires exhaustive extraction,
extensive chemical separations of the crude extract, and careful analysis of
spectroscopic data. The general techniques used in this study of gorgonian secondary
metabolites will be briefly described here.

Gorgonian specimens were collected by hand with the aid of SCUBA at depths
ranging from 5 to 120 feet. The location, collection depth and date were recorded and
a taxonomic voucher specimen was taken for each collected gorgonian. The portion to
be extracted was either immediately immersed in solvent, or was placed in plastic bags
and frozen at -20°C for future investigation.
Figure 25. Secondary metabolites from the family Subergorgiidae.

271 R = H
272 R = OH

274 R¹ = Me, R² = H
275 R¹ = Me, R² = Ac
276 R¹ = R² = H

278

279

280 R =
281 R =
282 R =
Gorgonian tissue volumes were determined by displacement of seawater, after which the gorgonians were broken into small pieces and freeze dried. The freeze-dried tissue was then extracted twice with 1:1 dichloromethane:methanol (1:1 DCM/MeOH) and a final time with 100% methanol. The extracts were combined, filtered through celite to remove emulsion-causing particulates, and concentrated under reduced pressure to yield a crude solvent extract. Chemical separation and isolation of metabolites was accomplished by a number of methods including solvent partitioning, thin layer chromatography using preparative plates, flash or vacuum chromatography, and high performance liquid chromatography. Chromatography media included silica and reversed phase (RP) C-18 supports. In many separations, after initial solvent partitioning, medium polarity partitions were subjected to silica flash chromatography initially with non-polar solvents such as isoctane (TMP) to remove large amounts of fats and chlorophyll (present in gorgonians containing symbiotic zooxanthellae) which tend to complicate separation schemes. Other less common separation techniques such as high-speed counter-current chromatography (HSCCC) and size exclusion chromatography (SEC) were used in special cases.

Structures were elucidated by a host of techniques, including \(^1\text{H}\) and \(^{13}\text{C}\) nuclear magnetic resonance (NMR), ultraviolet (UV), and infrared spectroscopy, as well as mass spectrometry (MS). The elucidation of many structures relied heavily on the use of one- and two- dimensional NMR spectroscopy (Figure 26). Two-dimensional experiments used during the course of work completed as part of this dissertation include Coupled Spectroscopy (COSY), Nuclear Overhauser Enhancement Spectroscopy (NOESY), Heteronuclear Multiple Quantum Coherence (HMQC) and
Heteronuclear Multiple Bond Coherence (HMBC). The use of an inverse detection probe and especially gradient NMR sequences during the later part of this dissertation research allowed for faster acquisition of higher quality data sets on smaller quantities of natural products. Liquid chromatography/mass spectrometry (LC/MS) was a very powerful tool in rapidly identifying and quantifying metabolites from multiple samples, and allowed efficient assessment of the variability of compound concentrations in various gorgonian populations.

The dereplication of known gorgonian secondary metabolites was accomplished by using chemical structure databases and literature searches. Most important of the computer based search programs in this respect was Marinlit, a database of marine

![Diagram](image)

**Figure 26.** Two-dimensional proton detected NMR experiments.
natural products literature which was developed, and is maintained by, the Marine Chemistry Group at the University of Canterbury, New Zealand. The strategies used in searching for known compounds with this database included structure, substructure, molecular formula, biological source, keywords, compound classification, and geographic area of origin. The ongoing series of marine natural products literature reviews authored by Professor D. John Faulkner and published in *Natural Product Reviews* was extremely helpful in directing literature searches. The Chemical Abstracts Service (CAS) on-line database was crucial to the confirmation that a metabolite was novel.

Specimens of fungi isolated from gorgonian tissues for studies of antifungal gorgonian metabolites were collected *in situ* with the aid of SCUBA and plated out on media containing antibiotics to retard bacterial growth. All but two of the eight cultures collected were filamentous fungi, and all cultures were grown on marine media as listed in Table 1. Both YPG and YPM media are nutrient rich and were useful in the growth of all fungal cultures used in this research.

<table>
<thead>
<tr>
<th></th>
<th>YPG</th>
<th>YPM</th>
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<tbody>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
<td>Yeast Extract</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 g</td>
<td>Peptone</td>
</tr>
<tr>
<td>Agar</td>
<td>17 g</td>
<td>Agar</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 g</td>
<td>Mannitol</td>
</tr>
<tr>
<td>Seawater</td>
<td>1 L</td>
<td>Seawater</td>
</tr>
</tbody>
</table>

*For Isolation Media Add Penicillin 50 mg/L and Streptomycin 50 mg/L*
Isolated cultures were stored at -80°C in 10% glycerol in the usual culture media in 2 mL cryovials. Before use, cryovials were thawed for 15 minutes and the contents plated onto media. Once growth of the pure strain was evident, a small block of the fungal hyphae on the agar media was transferred to 10 mL of the appropriate liquid culture media in a 50 mL culture tube and grown for 48 hours with constant agitation.
References


14. Bayer, F. M. Personal communication with Dr. William Fenical. 1996.

15. Williams, G. C., Octocoral Classification. Adapted by Williams from Bayer's 1981 revision and used in the determination of taxonomic classification for this dissertation.


Chapter II

Feeding Deterrent Properties and Chemistry of Indo-Pacific Gorgonians

A. Introduction

As discussed in the review of gorgonian chemical ecology (Chapter I), while hundreds of secondary metabolites have been identified from gorgonian chemical extracts, only a handful have been identified as being responsible for feeding deterrent activity. In addition, investigations of the ability of gorgonian extracts to deter feeding by co-occurring generalist predators have also been limited to gorgonians found in the Atlantic Ocean, predominantly from the Caribbean Sea and Brazil. These studies, including Pawlik's preliminary survey\(^1\) and several investigations of specific gorgonians, have revealed that many gorgonians do contain chemical extracts and secondary metabolites which significantly deter feeding by generalist predators in both field and laboratory assays.

While the great diversity and abundance of gorgonians in the tropical Atlantic, where they often dominate the invertebrate benthic fauna\(^2,3\) make these areas attractive sites for studies of gorgonian antifeedant activity, there has been relatively little effort to establish a similar ecologically relevant role for Indo-Pacific gorgonian extracts and metabolites. As detailed in Chapter I, the differences in evolutionary history and environment experienced by Indo-Pacific gorgonians may have led to differences in the evolution of chemical defenses.\(^4\) Gorgonians in the Indo-Pacific have experienced a long history of undisturbed competition and predation compared to Caribbean gorgonians, which may have experienced periods when fast growth and quick dispersal were key factors.
Given these evolutionary considerations and the low diversity and abundance of gorgonians in the Indo-Pacific, it is reasonable to hypothesize that gorgonians which are found there are extremely well adapted to defend themselves from predation. The survey of antifeedant activity from Indo-Pacific gorgonians presented in this chapter represents the first investigation of the level of deterrency found in these gorgonians, and allows for comparison to levels of deterrency observed in Caribbean gorgonians.

B. Fish Feeding Assay Design

Most of the gorgonians were collected during a field expedition to Madang Lagoon in April of 1997 by SCUBA from reefs throughout and outside the lagoon. Those gorgonians not collected during this expedition (those which do not have a PNG-97-# collection number) were collected in Palau, the Philippines, or sites other than Madang in Papua New Guinea, were immediately frozen and returned to Scripps Institution of Oceanography (SIO), and remained frozen until use. A scheme illustrating the extraction, identification, and quantification procedures used is presented in Figure 27. The gorgonian tissue extracted for use in each assay was taken from one individual colony, though multiple colonies of some readily identifiable species were grouped under one collection number. After removing tissue for extraction and a voucher sample for later identification, the remaining tissue was labeled and frozen for return to SIO. Instant film photography with a size indicator was used for those gorgonians having distinct colony morphology that was not preserved during bagging of the colonies for freezing. Gorgonians were identified at
Figure 27. Extraction and assay scheme used in the survey of gorgonian chemical defense.

Single Gorgonian Colony

3-10 mL of fresh tissue chopped and extracted 2 x with 40 mL MeOH:DCM

Remaining sample labelled and frozen, shipped to SIO

Extract reduced to minimal volume

Extract taken up in equal tissue volume of feeding matrix

Assay vs. Controls
the California Academy of Sciences (CAS) with generous help from Dr. Gary C. Williams on the basis of spicule preparations, photographs of whole colony morphology, and visual examination of voucher specimens which were deposited in the invertebrate collection at CAS.

The tissue removed for extraction was separated from the axial skeleton when possible, cut into small pieces and placed in a graduated 50 mL centrifuge tube. Forty mL of a 1:1 mixture of methanol and dichloromethane was added and the gorgonian tissue volume was determined by subtracting 40 mL from the total displacement in the centrifuge tube. The capped tubes were inverted and agitated repeatedly during a 24 hour extraction period, after which the mother liquor was decanted and filtered and another 40 mL of 1:1 MeOH:DCM was added for an additional 12 hour extraction period. The mother liquor from the second extraction was decanted and filtered, and combined with the first, and reduced to dryness by removing the DCM and MeOH on a rotary evaporator at ambient temperature followed by removal of trace amounts of water on a Savant Speed-Vac vacuum concentrator.

Early experiments assessing the defensive role of secondary metabolites in preventing predation on marine invertebrates involved ecologically questionable techniques. In some assays, fresh-water fish species were immersed in the aqueous extracts of test invertebrates and the toxic effects were observed.\textsuperscript{5,6} This type of testing raises a number of concerns about the ecological significance of experimental results, such as the test organism and the assay fish being from completely different environments and the extracts being experienced by the assay fish in a manner not consistent with feeding by that organism. Over the past two decades, studies have
become more refined to be as ecologically relevant as possible. Assays used in the survey presented in this chapter and for antifeeding experiments described in the rest of this dissertation were modeled after those developed by Pawlik and coworkers.\textsuperscript{1,7,8} These assays provided a number of experimental advantages, as they can test fresh extracts (those from gorgonians collected in Madang) which minimizes the extent of decomposition or air oxidation of potentially unstable metabolites. Further, the extracts are tested in an assay matrix which simulates the protein content of the extracted gorgonians and were tested at natural concentrations, and the assay organisms were generalist predators collected from the same environment as the gorgonians being investigated.

This assay method used an artificial food matrix into which extracts or compounds were be homogenized at controlled concentrations for treatment pellets and without additives for control pellets. The matrix was formed by adding 5 g of ground, freeze-dried squid mantle and 3 g of sodium alginate as a powder to 100 mL of distilled water, and mixing vigorously to homogenize the matrix resulting in a thick paste. Extracts from known volumes of gorgonian tissue were placed in vials in minimal amount of carrier solvent and the appropriate volume of assay mixture was added from a graduated syringe, followed by vigorous mixing to achieve homogeneity and break up any lumps. Pellets were formed from the assay mixture by loading the paste into a 10 mL syringe and extruding the paste into a 0.25 M solution of calcium chloride, forming a long spaghetti-like strand. The hardened strand was removed, rinsed in seawater, and cut into 4 mm pieces with a razor blade. Control pellets were made the same way, but without the addition of extract. Control and treated pellets
were offered in replicate experiments to ten different fish held in separate aquarium cells. Fish in each cell are offered either a treated or control pellet followed by the other. If the second pellet offered was the treated and was rejected, another control pellet was offered to ensure that rejection is not due to the fish having ceased eating. Pellets were considered to be rejected if not eaten after three attempts to consume the pellet by taking it into the mouth cavity, or if the pellet was ignored after one such attempt. For a single assay of 10 replicates, an extract was significantly deterrent if four or more pellets are rejected when analyzed by the Fisher exact test, which evaluates the significance of differential consumption of treated vs. control pellets (p≤0.043, 1-tailed test).

The assay utilized the co-occurring generalist wrasse *Halichoeres melanurus*, which is abundant on reefs throughout the Indo-Pacific. This generalist predatory fish is known to sample a wide variety of benthic invertebrates, and was selected for this assay because it was abundant on the reefs from which gorgonians were being collected. A generalist was desired as antipredator defenses would be directed against generalist predators, and generalists are less likely to have evolved means to circumvent gorgonian chemical defenses. The fish were caught by hand nets on the same reefs from which gorgonians were being collected and were quickly placed in groups of 2 to 4 into aquarium cells supplied with flowing seawater at ambient temperatures. The fish acclimated to the aquarium within hours and began accepting control food pellets, and the same group of fish remained healthy and was used for all assays in this survey.
C. Survey of Indo-Pacific Gorgonian Antifeedant Defenses

The crude extracts of 65 Indo-Pacific gorgonians representing nine families and more than 40 species (identified and unidentified) were tested as part of this survey of chemical defense against predation in gorgonians. All PNG gorgonian extracts tested were found to be highly unpalatable in laboratory assays against *Halichoeres melanurus*, with the highest number of treated pellets eaten for any one gorgonian being three. All control pellets were eaten by fish in every assay. The results of the fish feeding assays are presented in Table 2.

*Halichoeres melanurus*, the generalist predatory wrasse used in this assay, was abundant on the reefs from which gorgonians were being collected, and was easily acclimated to aquarium conditions. Competition was observed in the aquarium cells for offered food pellets, whether treated or not, and the fish consumed control pellets without satiation during the entire course of feeding assays. Treated pellets were obviously rejected in almost all cases (see Table 2, overall for the entire survey, only 37 out of 650 (6%) treated pellets were scored as consumed). It is notable, as well, that the competition in the aquarium cells was high, and treated pellets were often sampled multiple times by multiple fish and continually rejected until mechanical wear on the pellets by fish sampling and rejection caused them to break down. It is possible that the observed competition was a contributing factor in the consumption of some of the treated pellets scored as eaten.

As discussed in Chapter I, the classification and identification of the Gorgonacea is often problematic, which gave rise to certain issues when surveying antifeedant properties of gorgonian extracts. In 29 cases identification was only
Table 2. Survey of the antifeedant properties of Indo-Pacific gorgonian extracts. Score for number eaten is out of 10 pellets offered.

<table>
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<tr>
<th>Voucher number</th>
<th>Family</th>
<th>Species identification</th>
<th># eaten</th>
</tr>
</thead>
<tbody>
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<td>PNG-97-097</td>
<td>Nidaliidae</td>
<td>Siphonogorgia sp. A</td>
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<tr>
<td>PNG-97-008</td>
<td>Siphonogorgia sp. B</td>
<td></td>
<td>0</td>
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<tr>
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<td>Siphonogorgia godefferyi</td>
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<td>NCI-1868</td>
<td>Briareidae</td>
<td>Briareum sp. A</td>
<td>0*</td>
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<tr>
<td>95-121</td>
<td>Briareum sp. B</td>
<td></td>
<td>0</td>
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<tr>
<td>PNG-96-038</td>
<td>Briareum</td>
<td>Briareum sp. C</td>
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</tr>
<tr>
<td>PNG-97-064</td>
<td>Briareum c.f. stechei</td>
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<td>Subergorgiidae</td>
<td>Subergorgia reticulata</td>
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<td>F</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-072</td>
<td>G</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-081</td>
<td>H</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Plexaurids, genus/species indt:
Table 2. Survey of the antifeedant properties of Indo-Pacific gorgonian extracts. Score for number eaten is out of 10 pellets offered (continued).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Family</th>
<th>Species</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNG-97-058</td>
<td></td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>PNG-97-063</td>
<td></td>
<td>J</td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-094</td>
<td></td>
<td>K</td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-004</td>
<td></td>
<td>L</td>
<td>0</td>
</tr>
<tr>
<td>PNG-96-035</td>
<td>Gorgoniidae</td>
<td>Hicksonella cf. princeps</td>
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<tr>
<td>PNG-96-027</td>
<td></td>
<td>Rumphella sp. A</td>
<td>3</td>
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<tr>
<td>PNG-97-033</td>
<td></td>
<td>Rumphella sp. A²</td>
<td>2</td>
</tr>
<tr>
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<td>Rumphella sp. B²</td>
<td>1</td>
</tr>
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<td>PNG-96-015</td>
<td>Ellisellidae</td>
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<td>Ctenocella (Ellisella) sp. A²</td>
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<td></td>
<td>Ctenocella (Ellisella) sp. A²</td>
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</tr>
<tr>
<td>PNG-97-099</td>
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<td>Ctenocella (Ellisella) sp. A²</td>
<td>1</td>
</tr>
<tr>
<td>PNG-97-001</td>
<td></td>
<td>Ctenocella (Ellisella) sp. B</td>
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</tr>
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<td>Ctenocella (Ellisella) sp. B²</td>
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</tr>
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<td>Ctenocella (Ellisella) sp. B²</td>
<td>0</td>
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<tr>
<td>PNG-97-096</td>
<td></td>
<td>Ctenocella (Ellisella) sp. B²</td>
<td>2</td>
</tr>
<tr>
<td>PNG-97-003</td>
<td></td>
<td>Ctenocella (Ellisella) sp. C</td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-035</td>
<td></td>
<td>Ctenocella (Ellisella) sp. C²</td>
<td>1</td>
</tr>
<tr>
<td>PNG-97-045</td>
<td></td>
<td>Ctenocella (Umbracella) umbraculum</td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-066</td>
<td></td>
<td>Ctenocella (Umbracella) umbraculum²</td>
<td>0</td>
</tr>
<tr>
<td>PNG-97-070</td>
<td></td>
<td>Ctenocella (Umbracella) umbraculum²</td>
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</tr>
<tr>
<td>PNG-97-036</td>
<td></td>
<td>Ctenocella (Umbracella) cf. granulata</td>
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</tr>
<tr>
<td>PNG-97-098</td>
<td></td>
<td>Ctenocella (Viriminella) sp.</td>
<td>0</td>
</tr>
</tbody>
</table>

² Score for a purified compound from NCI-1868 at natural concentration.

Replicated assay.
possible to the genus level, and eleven Plexaurids were keyed as genus and species indeterminate. Despite the difficulties faced in gorgonian identification, this survey examined a broad range of Indo-Pacific gorgonians, with 9 of 11 families represented and at least 40 distinct species assayed.

This survey indicated that as a group, Indo-Pacific gorgonians are well defended against predation from generalist predatory fish such as *Halichoeres melanurus*. While it is not known what levels of tolerance to gorgonian chemistry other generalist predators may have, the high levels of deterrence seen in these assays suggest that gorgonian secondary chemistry is capable of and directed toward defending the gorgonians from these predators.

As previously discussed, in a survey of the patterns of chemical defense among Caribbean gorgonians, Pawlik and coworkers found that while over half of those gorgonians surveyed had unpalatable chemical extracts, 38% of tested extracts were palatable to assay fish.¹ These assays were performed by a standardized method that tested the extracts at an unknown percent dry weight of extract in the assay pellet which was subsequently determined and compared to the percent dry weight of extract in the gorgonian. Palatable extracts of Caribbean gorgonians were always tested at higher concentrations in assay pellets than they occurred in gorgonian tissues. The high levels of deterrence seen in all tested Indo-Pacific gorgonians relative to Pawlik’s Caribbean survey may be accounted for by the differences in evolutionary histories of the two groups and the different environmental stresses they experience.

Studies of the natural products found in Indo-Pacific gorgonians have been motivated by the search for novel classes of compounds or pharmacologically active
metabolites. This survey indicates that research is warranted for the study of the chemical ecology of these organisms. A number of studies of Western Atlantic gorgonians have shown that the specific compound or compounds responsible for the observed deterrence of those organisms can be isolated and identified through the use of bioassay directed separations.8,10-17 Research that follows in this dissertation was directed toward the identification, isolation, and characterization of gorgonian secondary metabolites which provide Indo-Pacific gorgonians with a chemical defense against predation.

The unique secondary metabolites produced by Caribbean gorgonians are often responsible for the observed deterrence 1,8,13,18. In these experiments, co-occurring generalist predators were presented with a food matrix in which chemical fractions or compounds from the gorgonians were homogenized at natural (or lower) concentrations. Through such bioassay-guided fractionation, chemists can perform chemical separations of the components of gorgonian extracts and follow the deterrent activity to the compound(s) responsible for this activity. In this manner, ecologists can garner valuable information about why these unusual compounds are present in these organisms and what purpose these compounds serve in their environment.

D. Further Study of the Antifeedant Activity of Gorgonian Extracts

While the feeding deterrent survey presented in this chapter gives an initial look at the levels of deterrence in Indo-Pacific gorgonian extracts, one of the overall goals of this dissertation was to identify the component(s) responsible for that activity. During the survey of gorgonian chemical deterrence in Madang, I made large
collections of many gorgonians tested in the assay. These gorgonians were frozen and returned to SIO, where they and previous collections of Indo-Pacific gorgonians awaited chemical investigation. The Hubbs Hall experimental aquarium at SIO has warm flowing seawater facilities in which tropical fish can be kept (Ron Mc Connaughey, personal communication), and the Indo-Pacific wrasse Thalassoma lunare, which is prevalent throughout the Indo-Pacific, has been housed at SIO in the past for use in chemical ecology studies. After obtaining the necessary approval from the UCSD Animal Subjects Program (Protocol# S98003), I obtained assay fish collected in the Solomon Islands from tropical fish wholesalers in Los Angeles.

Holding cells for Thalassoma lunare were made by dividing 10 gallon aquaria with an opaque divider, and providing aeration and a drain to allow a constant flow of seawater heated to 78-81°F. Fish obtained for the assays acclimated well to the aquaria and always began feeding within a day. Intermittent problems were experienced during periods of upwelling when the seawater intakes on the SIO pier pumped unusually cold seawater. Due to the necessary heating of the seawater, the water would become supersaturated with oxygen, which caused embolisms in some assay fish as evidenced by subcutaneous bubbles forming in the fin areas or protrusion of the eyeballs. Affected fish were isolated and not used in assays until they had recovered.

To examine the possibility that using another fish might affect assay results, a small number of Halichoeres melanurus were obtained during the initial incorporation of assays at SIO, and a set of nine gorgonian extracts were tested at natural concentrations against both species. Due to availability from tropical fish wholesalers
and cost, only six tests were run against *H. melanurus* for each extract. The results of those assays are given in Table 3. The high levels of deterrenacy seen in laboratory assays in Madang were also observed for both species in assays at SIO, which suggests that both organisms are suitable assay species. A purified briarane class compound (described later in this dissertation) isolated from *Briareum* sp. A, collection number NCI-1868, was found to be deterrent in assays in Madang at 0.75 mg/mL, which is less than 5% of the natural concentration of the metabolite in gorgonian tissues. The same compound was found to be deterrent at 1.5 mg/mL when tested at SIO against *T. lunare*, further suggesting that these two assay organisms have similar responses to gorgonian extracts and metabolites.

Table 3. Comparison of SIO laboratory feeding assays for *Thalassoma lunare* and *Halichoeres melanurus*.

<table>
<thead>
<tr>
<th>Voucher number</th>
<th>Species identification</th>
<th>Pellets eaten*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>T. lunare</em></td>
<td><em>H. melanurus</em></td>
<td></td>
</tr>
<tr>
<td>PNG-96-010</td>
<td><em>Subergorgia reticulata</em></td>
<td>2/10</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-014</td>
<td><em>Ctenocellia (Ellisella) sp. A</em></td>
<td>1/10</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-015</td>
<td><em>Junceella (Junceella) cf. fragilis</em></td>
<td>0/10</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-035</td>
<td><em>Hicksonella cf. princeps</em></td>
<td>2/10</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-038</td>
<td><em>Briareum</em> sp. C</td>
<td>1/10</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-053</td>
<td><em>Astrogorgia</em> sp.</td>
<td>0/10</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-53x</td>
<td>Plexaurid A</td>
<td>0/10</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>PNG-96-054</td>
<td><em>Villogorgia</em> sp. A</td>
<td>3/10</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>95-121</td>
<td><em>Briareum</em> sp. B</td>
<td>0/10</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

* Treated pellets eaten/control pellets eaten.
To isolate and identify the component(s) of an antifeedant gorgonian extract, the crude extracts of three gorgonians from Table 3 were partitioned between hexanes and methanol to give an initial separation of compounds based on their polarity. In all three cases, the antifeedant activity was found in only one of the solvent partitions when tested at natural concentration, as seen in Figure 28. The two solvent partitions of each gorgonian were also examined by TLC analysis and proton NMR spectroscopy, and in all three cases the partition observed to have significant deterrence (Fisher’s exact test, p < 0.05) was qualitatively found to contain secondary metabolites by both methods of analysis.

The activity of the hexane solvent partition seen for Plexaurid A (PNG-96-53x) was somewhat surprising, because no other gorgonian metabolite with feeding deterrent activity is non-polar, as discussed in Chapter I. The feeding deterrent

![Diagram](image)

**Figure 28.** Feeding deterrent activity of gorgonian extract solvent partitions.
properties of this gorgonian were examined in detail as described in Chapter IV of this dissertation. The continued study of Plexaurid A and other gorgonians described later in this dissertation was directed by assaying the feeding deterrent activity of extract partitions and fractions created via chemical separations towards the discovery of the metabolite(s) responsible for the deterrence.
References


Chapter III

Antifungal Properties and Chemistry of Indo-Pacific Gorgonians

A. Introduction

As discussed in the review of gorgonian chemical ecology above, gorgonian metabolites are well known to have biomedically relevant antimicrobial activities, but relatively little is known about how these metabolites defend gorgonians from ecologically relevant microbial pathogens.\(^1\) However, there has been increasing interest in the antimicrobial properties of gorgonians due to the increasing frequency of reports of disease and mass mortality in these and other marine organisms.\(^2\) Though incidents of black band disease\(^3-5\) and cyanobacterial infection\(^6\) have been reported in gorgonians, to date research on the antimicrobial activity of gorgonian extracts has focused on antibacterial activity\(^1,7-9\) and, more recently, antifungal activity.\(^10,11\)

The recent studies of the chemical resistance of gorgonians against fungal infections were stimulated by a 1996 report of a putative pathogen associated with diseased tissues of the Caribbean gorgonians *Gorgonia ventalina* and *G. flabellum*.\(^12\) Smith and coworkers observed distinct recession of the coenenchyme exposing the axial skeleton in diseased specimens, with fungal hyphae imbedded in the receding edge of the coenenchyme but not in healthy tissue. After reciprocal plating, it was found that one type of fungal pathogen was common to all the diseased gorgonians, which was identified as *Aspergillus* sp. by morphological, physiological, and nucleotide-sequence data.\(^12\) The fungal pathogen was later confirmed to be
Aspergillus sydowii, a soil-borne ascomycete known to be an opportunistic pathogen of terrestrial organisms. The widespread disease of sea fans throughout the Caribbean by this pathogen has continued to be observed and tracked with some interest. Though this pathogen had been initially observed only in conjunction with infected gorgonians of the genus Gorgonia, recent reports suggest that the disease may be spreading to other gorgonians, including Pseudopterogorgia americana and Briareum sp. Kock’s postulates have not been fulfilled to establish A. sydowii as the causative agent in these cases. Kock’s postulates require the following to unequivocally identify a pathogenic microorganism: the pathogen must always be found in association with the disease, the pathogen must be isolated from the disease state and grown in pure culture under laboratory conditions, the pure culture must produce the disease when inoculated onto a healthy animal, and the pathogen must be reisolated from the newly diseased animal and identified as the same pathogen. It is also notable that A. sydowii has been isolated from several other species in the Caribbean basin were Gorgonia sp. are currently affected by this pathogen. If aspergillosis is not restricted to gorgonians of the genus Gorgonia, disease from this pathogen may have far greater impact on gorgonians worldwide. In examining the host range of A. sydowii, Kim and coworkers suggest that the pathogen may not be host specific, and that other affected species may not have been identified yet, as disease symptoms may vary among infected species. While lesions indicating infection in Gorgonia species are readily identifiable and well documented, this may be due to the network of inter-connected branches of these gorgonians that
remain as evidence of infection for an extended period of time. Branches on arborescent colonies may weaken at infection sites and shed from the diseased colony, making detection of disease more difficult.

Despite this observed susceptibility of at least some gorgonians to fungal pathogenesis, the role gorgonian secondary metabolites play in antifungal chemical defense is largely unknown. It is known that *Gorgonian ventalina* and *G. flabellum*, the two gorgonians most affected by *A. sydowii*, do contain antifungal properties. Crude extracts from both of these gorgonians inhibited *A. sydowii* spore germination at levels estimated to be within the range of concentrations in living tissue,11 and diseased gorgonians had higher levels of antifungal activity relative to non-diseased sea fans, suggesting an inducible defense. It must be noted, however, that while the gorgonian extracts did inhibit spore germination at ecologically significant concentrations, the aspergillosis observed in diseased gorgonians has been found to be due to hyphal invasion of gorgonian tissue, not sporulation.12

The only evidence of antifungal chemical defense in gorgonians comes from gorgonians known to be susceptible to fungal infection. This is surprising given the abundance of gorgonian secondary metabolites (see review of gorgonian natural products chemistry, Chapter I), many of which have been shown to possess antimicrobial and antifungal activity against human pathogens.19-27 If the activities against human pathogens are mirrored by an antifungal chemical defense, it may help explain the lack of observed infection in other gorgonian genera. At present, there is no known correlation between biomedically relevant antifungal activity and antifungal
chemical defense, as there have been no studies to determine if gorgonians possess antifungal properties against ecologically relevant fungi.

Although much attention has been given to the recent outbreaks of disease and mass mortality observed in *Gorgonia* spp. in the Caribbean, there are only limited reports of fungal pathogenesis in other gorgonians of the Caribbean and no reports of mass disease in Indo-Pacific gorgonians. The objective of the research presented in this chapter was to survey Indo-Pacific gorgonian extracts for their ability to inhibit the growth of ecologically relevant fungi, including the known fungal pathogen *A. sydowii*. The gorgonians studied here represent a taxonomically diverse and comprehensive group of gorgonians found in the Indo-Pacific. The antifungal activities of these gorgonian extracts were tested either with microdilution methods adapted for fungal strains which produce either hyphae or spores in culture, or by standard agar disc-diffusion methods for spore-producing fungi. The extracts were tested against the *A. sydowii* pathogen and other fungi cultured from both healthy and decaying Indo-Pacific gorgonian tissue. If gorgonians do indeed produce broad-spectrum antifungal metabolites as a chemical defense, I would expect these bioassays to detect such substances.

B. Antifungal Assay Design

During the April 1997 field expedition to Madang Lagoon, a number of co-occurring fungi were isolated from dead and live gorgonian tissues while collections of gorgonians were made. While these fungi were readily isolated from the gorgonian tissues, no gorgonians visually examined appeared to be suffering from fungal
infections. The fungi were isolated by air-drying pieces of gorgonian tissue collected in sterile bags in a laminar flow hood, and then placing the tissue on agar plates treated with antibiotics to retard bacterial growth. All but one of the fungi produced only filamentous growth when grown up on specific nutrient agar media. While assays employing spore or conidia forming fungi are common (almost universally developed to assay fungal pathogens of humans), I found no mention of assays developed to allow testing of strictly filamentous fungi. Techniques from Espinel-Ingroff et al., which were developed for antifungal assays using conidial suspensions of medically important filamentous fungi, were modified to allow testing in microdilution assays with strictly filamentous fungi.28 The techniques for microdilution antifungal assays employed from Espinel-Ingroff et al. gave high degrees of interlaboratory reproducibility (90-97%) using amphotericin B as a standard antifungal agent against 25 pathogenic fungal isolates from five species when tested by six independent labs, which indicated that these assays are precise and reproducible.

Antimicrobial chemical defenses in gorgonians may affect pathogenic microorganisms by toxicity (by cell death or the inhibition of cell growth), by causing a negative chemotactic response of the pathogen, and possibly by the competitive exclusion of potential pathogens through the attraction of favorable epibionts.1 Investigation of the toxicity of gorgonian crude extracts and compounds should be possible using standard agar disc-diffusion antimicrobial assays and microdilution assays if certain criteria are met. As discussed in the study of the antibacterial properties of gorgonian extracts by Jensen and coworkers, the microbes employed in an assay should be ecologically relevant, the agents responsible for antibiotic activity
must be extracted efficiently from the gorgonians, and the concentrations at which the microbes are exposed to the extracts should reflect those concentrations the microbes would experience in nature. Meeting these criteria can be problematic in many ways. First, with the exception of the known fungal pathogen *Aspergillus sydowii*, neither the potential pathogens of the gorgonians nor the potential targets of a gorgonian's chemical defense are known. In addition, it is unknown how microbes in the gorgonian's natural environment experience the chemical substances in gorgonians, because there is no reliable information on the distribution of antifungal compounds in gorgonian tissues. For the *Aspergillus* gorgonian pathogen, it appears that the concentrations of antimicrobial substances within cells would be important, because the fungus apparently attacks the gorgonian through hyphal penetration.\textsuperscript{12} In contrast, for other potential pathogens, the surface concentrations of antimicrobial compounds in gorgonians may be key.

Given these limitations, it should be reasonable to begin assessing the antifungal properties of gorgonians using known pathogens of gorgonians, and other diverse fungi known to be experienced by the gorgonian in its environment. In testing gorgonian extracts, care must be taken to insure that microbes are not exposed to unreasonably high concentrations of gorgonian compounds when assessing the ability of those compounds to inhibit microbial growth. In the analysis of their own data and in reviewing the results of Kim's\textsuperscript{9} gorgonian antibacterial study, Jensen and coworkers note that the results of tests performed even at natural gorgonian extract concentrations must be interpreted with due caution as "extract diffusion-rates in agar undoubtedly exceed the rates at which metabolites are released from animal tissues in
nature." In microdilution assays, similar problems in interpreting assay results are faced as it is unknown if microbes experience gorgonian chemistry at natural concentrations in the environment. In addition, testing at natural concentrations may expose assay microorganisms to unduly high levels of degradation chemistry from the degradative oxidation of compounds such as fatty acids and chlorophyll. Extracted chlorophyll degrade to chlorophyllides and fatty acids air oxidize to hydroperoxides, both of which possess antibiotic properties.

While the studies of gorgonian antibiotic activity reported to date have focused on determining whether gorgonian extracts possess antibiotic properties at natural concentrations, they have varied both in the methods used to perform assays (standard agar disc diffusion assays and microdilution assays) and the way assay results have been interpreted (the definition of an active response and the comparison of MICs to natural concentrations). For the purposes of this study, a much more conservative approach was adopted with respect to the concentration at which gorgonian extracts were tested. Extraction of gorgonian tissue yields 5 to 25 mg of extract per mL of "living" gorgonian tissue, but as previously discussed, the distribution of the chemical components making up this extract in the gorgonian tissue is unknown. To avoid this problem and the possibility of exposing assay microorganisms to larger amounts of degradation products, the gorgonian extracts examined as part of this initial survey were tested at approximately an order of magnitude lower in concentration than they were found, on average, in gorgonian tissues. By using 10 μL of a 25 mg/mL solution of gorgonian crude extract and diluting in 190 μL of assay matrix, all samples were tested at an actual crude
concentration of 1.25 mg/mL. The conservative extract concentrations the fungal strains were exposed to in the assay allowed more definitive interpretations of observed activity in the assay results.

Following the approach of Espinel-Ingroff,\textsuperscript{28} I used spectrophotometric techniques were employed to develop a standardized suspension of the fungal inoculate. The optical density (OD) of the inoculum suspension solution was determined at 530 nm to eliminate light absorption by organic molecules and to give a true indication of the light scattering caused by suspended hyphae. To determine the optimal OD for the fungal inoculum for each of the four fungal isolates used in this survey, serial dilutions of the suspensions produced by fungal spores or homogenized hyphal tissue were made. The suspensions of each fungal isolate were diluted to four OD's, (0.05, 0.15, 0.25, 0.35) and the resulting suspension was diluted 1:100 with sterile seawater and 100 \( \mu \text{L} \) of this suspension was plated on an agar plate and spread with a glass hockey stick. Three replicates of this procedure gave an OD window of 0.15-0.25 for approximately 100-200 colony forming units (CFUs), read by randomly dividing the plates into quarters, counting CFUs in one quarter of the plate, and multiplying the result by four. For a spore suspension, a CFU would be a viable spore that germinates when plated on agar. With the homogenized filamentous suspension, a "CFU" would be a viable hyphal fragment that gives an individual point of growth. A CFU density of 100-200 points of growth per 100 \( \mu \text{L} \) of inoculum gave an overall inoculum concentration of 1 to 2 \( \times 10^3 \) CFU/mL.

Fungi were grown on agar plates until spore production, and the spores were harvested by washing the plate with 5 mL of sterile seawater. Gentle agitation with a
sterile rod assisted in loosening spores. The spore suspension was then diluted with sterile seawater to the predetermined OD window (0.15-0.25) which gave a suspension resulting in an appropriate fungal CFU concentration. The inoculum was then diluted 1:1 with double strength RPMI 1640 media, and one percent by volume of 100x alamar Blue™ indicator was added. The suspension was delivered into 96 well plates at 190 μL per well giving approximately 10,000 to 20,000 CFUs per well, and 10 μL of a 25 mg/mL methanol solution of gorgonian crude extract was added with mixing. Positive growth controls were run both with and without methanol, and negative controls were run with a series dilution of amphotericin B, a broad-spectrum antifungal compound. Results for microdilution assays run with spore producing fungi were read after 1 to 2 days. Standard agar disc diffusion assays with spore producing fungi were performed by inoculating the fungal strain onto the surface of a YPG agar plate and spreading evenly with a sterile glass hockey stick. Gorgonian extracts were tested by pipeting 16 μL of a 2.5 mg/mL solution of the extract onto standard circular paper discs with a volume of 33 μL to give a final concentration of approximately 1.25 mg/mL, allowing the solvent to evaporate, and placing the discs onto the surface of the inoculated plates. Assays were run until a confluent film had grown over the agar surface, usually from 2 to 4 days. Controls were run on each test plate with standardized paper discs laced with 10 μg of amphotericin B, paper discs wetted with 16 mL of methanol and then air dried, and blank paper discs.

For inoculum preparation, fungal filaments from an agar plate were grown in 10 mL liquid media with shaking for 5-7 days. The filaments were then separated on a sterile filter and placed in 5 mL of sterile seawater, and homogenized with a tissue
homogenizer (Tissue Tearor™, model 985-370, Biospec Products, Inc.) for at least 60 seconds until a homogenous suspension was obtained. As described above for spore producing fungi, the suspension was then diluted to an OD of 0.15-0.25 to give a fungal inoculum suspension resulting in the desired concentration of "CFU" producing hyphal fragments. The homogenate was then treated as above for spore forming fungi.

C. Survey of Indo-Pacific Gorgonian Chemical Defenses Against Fungal Strains

The crude extracts of 65 Indo-Pacific gorgonians representing nine families and more than 40 species were obtained from gorgonians collected and identified as described (Chapters I and II). These crude extracts were tested for antifungal activity against four strains of fungi, all isolated from gorgonian surfaces. The four strains used in this survey were:

- CNL 536: Aspergillus sydowii, a pathogen of gorgonians isolated from a Caribbean gorgonian
- CNL 672: a spore producing fungus isolated from a PNG gorgonian
- CNL 687: a filamentous fungus isolated from a PNG gorgonian
- CNL 759: a filamentous fungus isolated from a PNG gorgonian.

Fatty acid analysis by Microbial ID, Inc. of the fungal strains isolated from PNG gorgonians gave the following strain identifications: CNL 672 was confirmed to be Aspergillus niger by microscopy and colony appearance, CNL 687 gave a high similarity index to Botrytis spp., and CNL 759 a high similarity index to an unknown species of the genus Pithomyces. Gorgonian extracts were tested against the two spore producing Aspergillus species using both microdilution methods and disc diffusion methods. Microdilution methods were used to test for activity using the two filamentous fungi.
Activity for microdilution assays was scored visually against controls as per Espinel-Ingroff et al.,\textsuperscript{28} using alamar Blue\textsuperscript{TM}, which can be used as a quantitative measure of cellular respiration in animal cell lines as well as in bacteria and fungi.

The assay incorporated an oxidation-reduction (REDOX) indicator that gives a visually scorable colorimetric response based on the detection of metabolic activity in which the oxidized blue indicator is reduced to a red form.\textsuperscript{30} The growth, as detected by the alamar Blue\textsuperscript{TM} indicator, was scored for each well as compared to growth of controls and ranked in the following manner:

- **na**: no activity, no reduction in growth
- **+**: some minimal inhibition, minor reduction in growth
- **++**: noticeable growth inhibition
- **+++**: strong antifungal activity (fungistatic or fungicidal), no growth.

Inhibition of fungal growth for the agar disc diffusion assays was seen as clear halos (zones) surrounding disc. The zone diameters were measured in millimeters, the diameter of the paper disc was subtracted, and activity is given as a number representing the zone of inhibition in millimeters, with zones of inhibition $>1$ mm being considered active. Table 4 gives the results of antifungal testing for each specimen versus all four strains of fungi, with the zone of inhibition for the disc diffusion assay given in mm after the microdilution assay score for CNL 672. Table 5 gives the percentage of species tested against each strain displaying a certain level of activity in the microdilution assays.

None of the agar disc diffusion assays of the extracts tested against CNL 536, the *Aspergillus sydowii* gorgonian pathogen, produced a zone of inhibition greater than 1 mm, so no data were reported in Table 4 for that assay. This lack of activity
Table 4. Gorgonian antifungal activity against fungal test strains.

<table>
<thead>
<tr>
<th>Gorgonian</th>
<th>PNG #</th>
<th>CNL Isolate #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>536</td>
<td>672</td>
</tr>
<tr>
<td>Family Nidaliidae:</td>
<td></td>
<td></td>
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<tr>
<td><em>Siphonogorgia</em> sp. A</td>
<td>PNG-97-097</td>
<td>+</td>
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<tr>
<td><em>Siphonogorgia</em> sp. B</td>
<td>PNG-97-008</td>
<td></td>
</tr>
<tr>
<td><em>Siphonogorgia godefferyi</em></td>
<td>PNG-97-019</td>
<td>l</td>
</tr>
<tr>
<td>Family Briareidae:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Briareum</em> sp. A</td>
<td>NCI-1868</td>
<td>nt</td>
</tr>
<tr>
<td><em>Briareum</em> sp. B</td>
<td>95-121</td>
<td>++</td>
</tr>
<tr>
<td><em>Briareum</em> sp. C</td>
<td>PNG-96-038</td>
<td>+</td>
</tr>
<tr>
<td><em>Briareum</em> cf. stechei</td>
<td>PNG-97-064</td>
<td>+</td>
</tr>
<tr>
<td>[Pachyclavularia sp.]</td>
<td>PNG-97-039</td>
<td>+</td>
</tr>
<tr>
<td>Family Anthothelidae:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cf. <em>Solenocaulon</em> sp.</td>
<td>PNG-97-083</td>
<td>+</td>
</tr>
<tr>
<td>Family Subergorgiidae:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Subergorgia reticulata</em></td>
<td>PNG-96-010</td>
<td>+</td>
</tr>
<tr>
<td><em>Subergorgia mollis</em></td>
<td>PNG-97-091</td>
<td>++</td>
</tr>
<tr>
<td>Family Melithaeidae:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acabaria</em> sp.</td>
<td>PNG-97-044</td>
<td>+</td>
</tr>
<tr>
<td><em>Wirghtella</em> sp.</td>
<td>PNG-97-060</td>
<td>++</td>
</tr>
<tr>
<td>Family Acanthogorgiidae:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acalycygoria</em> sp. A</td>
<td>PNG-97-007</td>
<td>-034</td>
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<td></td>
<td></td>
<td>-051</td>
</tr>
<tr>
<td><em>Acalycygoria</em> sp. B</td>
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<td></td>
</tr>
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<td>Family Plexauridae:</td>
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<td></td>
</tr>
<tr>
<td><em>Astrogorgia</em> sp.</td>
<td>PNG-96-053</td>
<td>+</td>
</tr>
<tr>
<td><em>Bebryce</em> sp.</td>
<td>PNG-97-059</td>
<td>++,2</td>
</tr>
<tr>
<td><em>Euplexaura</em> sp.</td>
<td>PNG-97-089</td>
<td>+</td>
</tr>
<tr>
<td><em>Villogorgia</em> sp. A</td>
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<td>+</td>
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<td>PNG-97-062</td>
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<td>+</td>
</tr>
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<td>PNG-97-082</td>
<td>+</td>
</tr>
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<td><em>Echinogorgia (Menella)</em> sp. C</td>
<td>PNG-97-076</td>
<td>++</td>
</tr>
<tr>
<td><em>Echinogorgia (Menella)</em> sp. D</td>
<td>PNG-97-065</td>
<td>l</td>
</tr>
<tr>
<td><em>Echinogorgia</em> sp. A</td>
<td>PNG-97-090</td>
<td>+</td>
</tr>
<tr>
<td><em>Echinogorgia</em> sp. B</td>
<td>PNG-97-043</td>
<td>++,l</td>
</tr>
<tr>
<td><em>Echinogorgia</em> sp. C</td>
<td>PNG-97-053</td>
<td>+</td>
</tr>
<tr>
<td><em>Echinogorgia</em> sp. D</td>
<td>PNG-97-050</td>
<td>++</td>
</tr>
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<td>Family</td>
<td>Plexauridae (cont.)</td>
<td>CNL Isolate #</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Family</td>
<td>Plexaurids, genus/species indt.</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>PNG-96-53x</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>PNG-97-080</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>PNG-97-018</td>
<td>++,2</td>
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<tr>
<td>E</td>
<td>PNG-97-031</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>PNG-97-079</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>PNG-97-072</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>PNG-97-081</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>PNG-97-058</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>PNG-97-063</td>
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<tr>
<td>K</td>
<td>PNG-97-094</td>
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<td>Family</td>
<td>Gorgonidae</td>
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</tr>
<tr>
<td>Hicksonella</td>
<td>cf. princeps</td>
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<td>PNG-96-027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PNG-97-033</td>
<td></td>
</tr>
<tr>
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<td>-037</td>
<td></td>
</tr>
<tr>
<td>Rumphella sp. B</td>
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<td></td>
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<tr>
<td>Family</td>
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<td>Junceella</td>
<td>(Junceella) cf.</td>
<td>PNG-96-015</td>
</tr>
<tr>
<td>fragilis</td>
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<td></td>
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</tr>
<tr>
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<td>-046</td>
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</tr>
<tr>
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<td>(Ellisella) sp. A</td>
<td>PNG-96-014</td>
</tr>
<tr>
<td></td>
<td>PNG-97-006</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>-099</td>
<td></td>
</tr>
<tr>
<td>Ctenocella</td>
<td>(Ellisella) sp. B</td>
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<tr>
<td></td>
<td>-002</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>-096</td>
<td></td>
</tr>
<tr>
<td>Ctenocella</td>
<td>(Ellisella) sp. C</td>
<td>PNG-97-003</td>
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<tr>
<td></td>
<td>-035</td>
<td></td>
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<tr>
<td>C. (Umbracella) umbraculum</td>
<td>PNG-97-045</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-066</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-070</td>
<td></td>
</tr>
<tr>
<td>C. (Umbracella) cf. granulata</td>
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<td>4</td>
</tr>
<tr>
<td>Ctenocella</td>
<td>(Viminella) sp.</td>
<td>PNG-97-098</td>
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Table 5. Percentage of Species Tested Displaying Activity.

<table>
<thead>
<tr>
<th></th>
<th>CNL536</th>
<th>CNL672</th>
<th>CNL687</th>
<th>CNL759</th>
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<tbody>
<tr>
<td>na</td>
<td>74%</td>
<td>53%</td>
<td>55%</td>
<td>86%</td>
</tr>
<tr>
<td>+</td>
<td>26%</td>
<td>18%</td>
<td>28%</td>
<td>14%</td>
</tr>
<tr>
<td>++</td>
<td>-</td>
<td>29%</td>
<td>8%</td>
<td>-</td>
</tr>
<tr>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>9%</td>
<td>-</td>
</tr>
</tbody>
</table>

was not surprising, as only low levels (+) of activity were seen in 17 of the 65 microdilution assays with *A. sydowii*. Of the 130 disc diffusion assays, only 21 (16%) resulted in zones of inhibition of at least 1 mm, and all of these activities were observed against CNL 672, identified as *Aspergillus niger*. As seen in Table 4, the observed antifungal activity was not specific to any particular family and variation was seen even among replicate assays of the same species (e.g. *Acalycygorgia* sp. A and *Junceella fragilis*, in which only one of three replicates of each species showed antifungal activity). Of the 21 extracts tested against CNL 672 in which at least a 1 mm zone of inhibition was observed, only seven also displayed antifungal activity in the microdilution assays with CNL 672. Zones of inhibition observed for control discs laced with 10 μg of amphotericin B gave consistent results for all test plates, with a range of 18-20 mm for CNL 536 inoculated plates and 14-17 mm for CNL 672 inoculated plates.

In the microdilution assays assessing gorgonian extract antifungal potency of four fungal strains, the extracts of gorgonians tested showed at least moderate levels of antifungal activity in 86 of the 230 tests analyzed (37%). Only 30 (13%) of the tests revealed extracts which either completely inhibited or gave more than minor reduction
(score of +++ or ++) in fungal growth, and these higher levels of inhibition were seen only against strains CNL 672 and 687. Observed activity against the gorgonian pathogen *Aspergillus sydowii* (CNL 536) and CNL 759 was minimal, with minor growth inhibition seen for 26% and 14% of the gorgonian extracts tested against the strains respectively. Only two gorgonians (of indeterminate genus and species of the family Plexauridae) had some activity against all four fungal strains, though a number of other Plexaurids (>50%) inhibited at least one fungal strain at high levels. Extracts from the four gorgonians from the family Briareidae gave inhibitory results in 9 of the 16 tests (56%), with none inhibiting strain CNL 759.

Extracts of the gorgonian species tested differed widely in their antifungal activity. As with the disc diffusion assays, observed antifungal activity was not specific to any particular family and variation was seen even among replicate assays of the same species (e.g. replicate assays of all three *Ctenocella* spp. and *Junceella fragilis* of the family Ellisellidae, see Table 4). Eighteen of the extracts tested showed no activity against any of the strains, and another 24 inhibited only one of the strains tested. The antifungal potency of the gorgonian extracts also differed widely between strains and within family and species groups.

This study's overall objective was to determine the extent to which Indo-Pacific gorgonians produce extractable organic compounds inhibitory to the growth of pathogenic and co-occurring fungi. The study was designed to allow unambiguous interpretation of any antifungal activity seen in assay results, and based on the examination of 65 gorgonian extracts representing over 40 species from nine gorgonian families, I conclude that the production of potent, broad-spectrum
antifungal compounds by Indo-Pacific gorgonians is not common. Disc diffusion assays against CNL 536 produced no antifungal activity, and in the tests against CNL 672 only 21 of 65 (32%) extracts produced antifungal activity. All but three of those results showed minimal activity, with zones of inhibition being only one or two mm. In microdilution assays with the four fungal strains, only 37% (86 out of 230) of the tests gave antifungal results. In both disc diffusion and microdilution assays antifungal potencies were generally low, with only moderate (<5 mm) zones of inhibition observed for disc diffusion assays, and just 13% (30/230) resulted in more than minor reduction in fungal growth in the microdilution assays.

While testing lower than natural concentrations in these assays may have missed some moderately antimicrobial extracts resulting in fewer positive results, it is not expected that the detection of a constitutive expression of potent, broad-spectrum antifungal chemistry by these gorgonians would be compromised by lower testing concentrations. It is highly unlikely that fungi on the surface of gorgonian tissues would contact gorgonian metabolites at concentrations approaching those in whole tissues. Indeed, lower concentrations allow more definitive interpretation of positive results as concern over false positive activity due to artifacts of the extraction and testing procedures, some of which are known to possess weak antibiotic activities, is thus avoided. I believe that, as represented by the results of the antifungal assays presented, Indo-Pacific gorgonians as a group do not possess potent, broad-spectrum antifungal activity, and that my inability to detect such activity in most gorgonian extracts accurately represents the absence of these activities in nature. There were, however, 13 extracts which displayed antifungal activity against at least three of the
four strains, and it remains possible that certain gorgonians do produce broad-spectrum antifungal metabolites which may function as an antimicrobial chemical defense. An example of this activity was seen in the family Briareidae, where three of the four extracts (75%) showed antifungal activity against CNL 536, 672, and 687, though it is noted that none of the Briareidae extracts were active against CNL 759.

Activity of extracts within families and even within replicates of individual species varied widely and little correlation was seen between the disc diffusion assay results and the microdilution assay results. In analyzing the data from CNL 672, only eight of the extracts showed activity in both assays, while 13 tests resulting in activity in the disc diffusion assay showed no activity in the microdilution assay. Similarly, 23 extracts for which antifungal activity was detected in the microdilution assay were not active in the disc diffusion assay. The differences in detecting antifungal activity between the two assays may be due to differences in the mechanisms by which the tested strains of fungi experience potentially antimicrobial gorgonian metabolites in the two media. In agar disc diffusion assays, it would be expected that compounds would need to diffuse into and through the agar to affect or be experienced by the fungi growing on the media, and in microdilution assays, compound solubility and solution structure may be important in determining antimicrobial efficacy.

Although the scope of the assays presented here was limited, the differences in activities of the gorgonian extracts in the two assays raise important questions about how fungi respond to potentially antifungal chemistry in different assays, and how that relates to the unknown mechanisms by which potential microbial pathogens and invertebrates interact in their natural environment. To my knowledge, this is the first
study that has examined the antifungal activity of gorgonian extracts in both disc
diffusion and microdilution assays, and only limited conclusions can be drawn from
the results presented here. More work is needed to determine the differences in ability
to detect antifungal activity in the two assays.

Subsequent to the completion of research presented in this chapter, a report
was published examining the chemical resistance of Caribbean gorgonians against the
fungal pathogen Aspergillus sydowii. The study by Kim and coworkers examined
the minimum inhibitory concentrations (MIC) of the extracts of 20 common gorgonian
species from the Florida Keys against the fungal pathogen. The authors tested a serial
dilution of the extracts, starting at 100 mg/mL, in a microdilution assay and concluded
that "(o)ur survey revealed considerable antifungal activity in this group of gorgonian
corals" after observing MICs of 8 to >15 mg/mL for the extracts, with more than
half the extracts tested having MICs equal to or greater than 15 mg/mL. The authors
then compared these MICs to the extract concentrations estimated in "living gorgonian
tissue." To determine these concentrations, the authors assumed that living tissue was
approximately 90% water, thus 1 g of dry tissue would equal about 10 mL of
gorgonian tissue volume. Dry tissue weight was determined by difference of the
whole coral weight minus skeletal weight and converted to living tissue volumes
based on the assumptions given above. In half of the gorgonians, extract
concentration in living tissue was found to fall below that of the MIC, giving a group
of "chemically susceptible" gorgonians and raising questions about the ability of these
extracts to inhibit fungal infection.
Although a number of the gorgonians did possess extract concentrations estimated to occur in their living tissue above the MICs detected in the assay, it is again unlikely that surface associated fungal pathogens are exposed to metabolite concentrations as high as those observed in whole gorgonian tissue. Indeed, the extract content estimated in the tissue of *Gorgonian ventalina* is higher than the MIC of the extract, yet this gorgonian is known to be highly susceptible to this pathogen. In addition, it is noteworthy that all of the gorgonian extracts were active at comparable and fairly high MICs, and that the component(s) responsible for the observed antifungal activity have not been determined. While the authors report that the "study indicates that gorgonian corals have antifungal chemicals in their crude extracts,"\(^{10}\) the effect of extraction and testing artifacts in these assays is unknown and cannot be discounted without further study. The authors cautiously suggest that the observed antifungal activity of the crude extracts of the gorgonians may indicate a possible ecological function for gorgonian secondary metabolites, and offer that a good deal of further study is needed to fully understand the role of chemistry in disease resistance. It must be noted, however, that without the isolation of the secondary metabolites responsible for observed activity, it is difficult to draw any meaningful conclusions.

The growing concern over outbreaks of disease in the marine environment is based upon the impact these diseases can have on marine systems.\(^2\) The attention given to the current gorgonian epidemic caused by *Aspergillus sydowii* in the Caribbean\(^2,10,11,16,17\) reflects the understanding that the host range is not known and the initial findings that this pathogen may be infecting other gorgonians in addition to
those of the genus *Gorgonia*. In a report investigating disease in the marine environment, Harvell and coworkers found that "most new diseases are not caused by new micro-organisms, but rather by known agents infecting new or previously unrecognized hosts." The host shift seen with *A. sydowii*, known to be a soil-borne opportunistic fungus, to the marine environment raises concern for gorgonian populations world-wide.

The research presented as part of this dissertation represents the first examination of Indo-Pacific gorgonian chemical defenses against a known gorgonian pathogen. Combined with the study of Caribbean gorgonian antifungal defense by Kim and coworkers, these studies represent a first look at the possibility that gorgonian secondary metabolites may function ecologically as a chemical defense. In my survey of Indo-Pacific gorgonians, the lack of activity in the disc diffusion assay and very low levels of activity seen for a limited number of the extracts tested in the microdilution assay suggest that this group of gorgonians as a whole does not possess a chemical defense against fungal infection. The study of Caribbean gorgonian chemical defense did detect antifungal activity in all gorgonians, but revealed that over half would be considered "chemically susceptible" by the authors' standards, and that even in those gorgonians that would appear to possess a chemical defense, infection on a large scale has been documented. These findings suggest to me that gorgonians have rarely evolved secondary chemistry as an antifungal defense, and that gorgonians world-wide may be susceptible to opportunistic pathogens like *A. sydowii*. Because of the importance of such disease-causing agents and the devastating impact they can
have on marine systems, additional studies addressing other possible gorgonian defenses against infection are warranted.

D. Further Study of Antifungal Activity Against CNL 687

While antimicrobial surveys such as the one presented in this chapter give an initial look at the levels of chemical defense that a group of organisms may have, one of the overall goals of this dissertation was to identify those gorgonian extracts which displayed antifungal activity and to identify the active components. To demonstrate which compounds in the mixture have antifungal activity, I tested fractions separated by polarity. I used 14 species with crude extracts that were moderately to highly fungistatic or fungicidal against the filamentous strain CNL 687. The crude extracts were partitioned between hexanes and methanol to give an initial separation of polar and nonpolar metabolites. These partitions were re-tested against CNL 687 employing a serial dilution scheme in which the extracts were tested at a variety of concentrations from 2.5 mg/mL to 0.08 mg/mL, allowing the minimum inhibitory concentration (MIC) of the partitioned extract to be determined, as reported in Table 6.

The results of these assays indicate that the antifungal properties of an extract can be isolated by chemical separations based on compound polarity. As indicated by bold MICs in Table 6, in a number of cases the antifungal activity was isolated completely in either the polar solvent partition or in one case (PNG-97-080) the nonpolar partition. When activity was preferentially distributed into one of the partitions but found in both or MICs were equal in both partitions, the component(s)
Table 6. MIC values of extracts tested against CNL 687*.

<table>
<thead>
<tr>
<th>Family</th>
<th>MIC (mg/mL)</th>
<th>hexane</th>
<th>MeOH</th>
</tr>
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<tbody>
<tr>
<td><strong>Briareidae:</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Briareum</strong> sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (95-121, Palau)</td>
<td>0.3</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>[&quot;Pachyclavularia sp.&quot;]</td>
<td></td>
<td>&lt;0.15</td>
<td>&lt;0.15</td>
</tr>
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<td><strong>Subergorgiidae:</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Subergorgia reticulata</td>
<td></td>
<td>na</td>
<td>0.3</td>
</tr>
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<td></td>
</tr>
<tr>
<td>Acalycygorgia sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (PNG-97-051)</td>
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<td></td>
<td>1.3</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Astrogorgia sp.</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(PNG-96-053)</td>
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<td>na</td>
<td></td>
</tr>
<tr>
<td>Echinogorgia (Menella) sp. C (PNG-97-076)</td>
<td>0.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Echinogorgia sp.</td>
<td></td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td>B (PNG-97-043)</td>
<td></td>
<td>na</td>
<td>0.3</td>
</tr>
<tr>
<td>Plexaurids, genus/species indeterminate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (PNG-96-53x)</td>
<td>&lt;0.15</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>C (PNG-97-080)</td>
<td>0.6</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>F (PNG-97-079)</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>G (PNG-97-072)</td>
<td>0.3</td>
<td>&lt;0.15</td>
<td>1.3</td>
</tr>
<tr>
<td>L (PNG-97-004)</td>
<td>0.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td><strong>Gorgoniidae:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hicksonella cf. princeps</td>
<td></td>
<td>na</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Ellisellidae:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctenocella (Elliselle) sp. A (PNG-96-014)</td>
<td>na</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

*Inoculum OD=0.22, MIC for AmpB was 0.012 mg/mL, assay read at 36hrs.

responsible for the observed activity may be of moderate polarity and therefore
distribute into both partitions, or there may be multiple components with different
polarities. Continued study of gorgonians described later in this dissertation was
directed by assaying the antifungal activity of extract partitions and fractions
accomplished via chemical separations towards the discovery of metabolites
responsible for observed antifungal activities.
References


30. alamar Blue™ literature sent with the product from AccuMed Internation, I., 29299 Clemens Road, Suite 1-K, Westlake, OH 44145-1051. 1996.
Chapter IV

The Chemical Ecology of Guaiazulene Producing Gorgonians

A. Introduction

Activity observed in the antifungal and feeding deterrence assays (Chapters II and III) directed an ecological study of azulene class compounds in two gorgonians from the family Plexauridae. This research represents the first ecological study of gorgonians that produce guaiazulene class compounds, which have been isolated from a number of gorgonians and other marine invertebrates. Guaiazulene and linderazulene were first reported from the gorgonians Euplexaura erecta and Paramuricea chamaeleon respectively in 1981.1,2 Prior to these reports naturally occurring azulenes had been found in the essential oils of terrestrial plants, liverworts, fungi, and marine algae, but these reports are the first isolation of azulenes from animals.1,2 Since 1981, guaiazulene and derivatives have been observed in a number of gorgonians from at least three families, as well as a number of soft corals, which are summarized in Table 7, which includes information on pharmacological activities. Azulenes have been reported to have a broad range of pharmacological activity and have been noted for giving gorgonian polyps an unusual and conspicuous brilliant blue coloring1, nothing has been reported concerning the chemical ecology of these compounds.

In the octocorallia, guaiazulene has been reported in eight species and has probably been observed in additional species, but not reported since it is a known compound. The wide distribution of azulenes in gorgonians suggests it plays an important ecological role or roles.
Table 7. Azulenes from gorgonians.

<table>
<thead>
<tr>
<th>Year</th>
<th>Species Identification</th>
<th>Compounds Isolated</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td><em>Euplexaura erecta</em></td>
<td>guaiazulene</td>
<td>fungi, bacteria</td>
</tr>
<tr>
<td>1981</td>
<td><em>Paramuricea chamaeleon</em></td>
<td>linderazulene</td>
<td></td>
</tr>
<tr>
<td>1982</td>
<td><em>Pseudothelia</em> sp.</td>
<td>guaiazulene, dimer</td>
<td></td>
</tr>
<tr>
<td>1983</td>
<td>(Alcyonium sp.)</td>
<td>guaiazulene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Cespitularia sp.)</td>
<td>azulene derivatives</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>family Paramuriceidae</td>
<td>halogenated guaiazulenes</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td><em>Placogorgia</em> sp.</td>
<td>guaiazulene, linderazulene, derivatives</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>family Paramuriceidae</td>
<td>guaiazulene, nitrogenous azulene derivatives</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>(Clavularia koellikeri)</td>
<td>guaianes</td>
<td></td>
</tr>
<tr>
<td>1987</td>
<td><em>Acalycigorgia</em> sp.</td>
<td>guaiazulene, linderazulene, derivatives</td>
<td>tumor, fungi, immunoreg.</td>
</tr>
<tr>
<td>1993</td>
<td><em>Acalycigorgia</em> sp.</td>
<td>guaiazulene, linderazulene, derivatives</td>
<td>brine shrimp, cell division</td>
</tr>
<tr>
<td>1996</td>
<td><em>Pseudoporterogorgia americana</em></td>
<td>guaianes</td>
<td></td>
</tr>
<tr>
<td>1996</td>
<td>(Sinularia gardineri)</td>
<td>guaianes</td>
<td>cytotoxicity</td>
</tr>
<tr>
<td>1996</td>
<td><em>Calicogorgia granulosa</em></td>
<td>guaiazulene, dimers</td>
<td>fungi, bacteria</td>
</tr>
<tr>
<td>1997</td>
<td><em>Pseudoporterogorgia americana</em></td>
<td>guaianes</td>
<td>cytotoxicity</td>
</tr>
<tr>
<td>1998</td>
<td><em>Bebryce grandicalyx</em></td>
<td>guaianes</td>
<td></td>
</tr>
</tbody>
</table>

a Parentheses indicate a soft coral species.
b Pharmacological activity observed in indicated assay.

B. Methods and Results

Two gorgonians, PNG-96-53x and PNG-97-079, were collected with SCUBA from two separate passes in the outer reef of Madang Lagoon and frozen immediately after removing voucher and assay samples. The gorgonians were later identified as Plexaurids A and F respectively; genus and species were indeterminate although the gorgonians represented two distinct species. The tissue volumes of the frozen
gorgonians were measured at SIO by displacement in chilled seawater in a large graduated cylinder. Plexaurid A gave 140 mL of tissue that was subsequently freeze-dried. During the freeze-drying process it was noticed that sublimation of a pigment had occurred as the ice that formed in the cold trap had a bright blue tinge. The gorgonian was immediately removed from the freeze-drier and extracted multiple times with dichloromethane and methanol. The combined fractions were reduced to an oil by rotary evaporation, and then partitioned between hexanes and methanol. TLC and proton NMR of the two solvent partitions indicated that the hexane partition was chemically rich while the methanol partition was not. Most of the hexane partition was reserved, and the equivalent of extract from 60 mL of gorgonian tissue (0.63 g) was fractionated on a silica gel column with a solvent gradient of iso-octane and ethyl acetate. Fractions were recombined on the basis of TLC analysis to give four fractions that yielded two blue pigments of the guaiazulene class, an unknown red pigment, and an unidentified cholesterol derivative. Based on the aquarium assay and the microdilution antifungal assays described earlier in this dissertation, the fish feeding deterrence and antifungal activity was isolated first in the hexane partition and subsequently in the two fractions containing the blue azulene pigments.

The first fraction yielded guaiazulene (283), the metabolite found to be responsible for the feeding deterrence and antifungal activity, which was isolated by normal phase silica HPLC. Guaiazulene was later isolated from Plexaurid F by analogous techniques. The second fraction contained a mixture of guaiazulene and small amounts of another known guaiazulene class compound, 2,2'-diguaiazulenylmethane (284). The third and fourth fractions contained an unidentified
red pigment and cholesterol derivative respectively, along with other minor metabolites, which were inactive in either assay.

\[
\begin{align*}
\text{283} & \quad \text{guaiazulene} \\
\text{284} & \quad 2,2'-\text{diguaiazulenylmethane}
\end{align*}
\]

Compound 283 was isolated as a deep blue oil, which had the molecular formula C_{15}H_{18} by $^{13}$C NMR, $^1$H NMR and low resolution EIMS (m/z 198, M$^+$). Compound 283 was readily identified as guaiazulene by comparison of spectral data with literature values.$^1$ The concentration of guaiazulene in the gorgonian could not be accurately determined during isolation, because a substantial amount of the compound had sublimed during the freeze-drying process and more material was probably lost due to rotary evaporation during isolation (hexane extraction of a portion of the melted blue-tinged ice which collected in the freeze-drier cold trap was shown to contain guaiazulene by TLC and proton NMR analysis). To accurately determine the concentration of guaiazulene in Plexaurids A and F, a standard curve was made from diode array detected HPLC of a serial dilution of the purified guaiazulene. Dilutions of 4, 2, and 1 mg/mL of guaiazulene were made and 20 µL of this solution was analyzed by a standard HPLC method. The area under the peak due to guaiazulene (4.3 minutes) was recorded at three $\lambda_{\text{max}}$ for the compound (210, 245, and
285 nm, see Figure 29) and plotted versus concentration to give standard curves by linear regression analysis (see Table 8 and Figure 30).

**Table 8.** Linear regression analysis of data for standard calibration curves for guaiazulene.

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>Area [mAU*sec] at Conc. (mg/mL)</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>210</td>
<td>4791</td>
<td>7371</td>
</tr>
<tr>
<td>245</td>
<td>9369</td>
<td>12494</td>
</tr>
<tr>
<td>285</td>
<td>13741</td>
<td>19463</td>
</tr>
</tbody>
</table>

Samples of the crude extract of Plexaurids A and F had been obtained from freshly collected wet gorgonian tissue which had been subjected to minimal vacuum during rotary evaporation and then immediately frozen during field assays in Madang. The crude extracts of Plexaurids A and F were diluted to natural concentration and 20 µL aliquots were analyzed by the same HPLC method used to create the standard curve. The concentrations of guaiazulene in the crude extracts were determined by plotting the area on the standard curve and correlating to the natural concentration of guaiazulene in the sample (see Table 9 and Figure 30).
Figure 29. UV spectra of guaiazulenes, showing $\lambda_{\text{max}}$ used in calibration curves.
Figure 30. Standard calibration curves for guaiazulene at selected $\lambda_{max}$ values.

Standard curve for guaiazulene at 210 nm.

$y = 3243.2x + 1282.5$

![Graph showing area vs. concentration for guaiazulene at 210 nm.]

Standard curve for guaiazulene at 245 nm.

$y = 5714.6x + 2618.5$

![Graph showing area vs. concentration for guaiazulene at 245 nm.]

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Figure 30. Standard calibration curves for guaiazulene at selected $\lambda_{\text{max}}$ values (cont.).

Standard curve for guaiazulene at 285 nm.

$$y = 9683.4x + 2473.0$$

Table 9. Mean concentration of guaiazulene in two Plexaurids.

<table>
<thead>
<tr>
<th>Gorgonian</th>
<th>Absorbance (Concentration)$^a$</th>
<th>Mean Conc. (Stnd. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>210 nm</td>
<td>245 nm</td>
</tr>
<tr>
<td>Plexaurid A</td>
<td>11251(3.07)</td>
<td>19784(3.00)</td>
</tr>
<tr>
<td>Plexaurid F</td>
<td>10283(2.77)</td>
<td>17170(2.54)</td>
</tr>
</tbody>
</table>

$^a$ Absorbance in mAU and concentrations in mg/mL.

$^b$ Not used in the calculation of mean concentration (see text).

Guaiazulene was present in Plexaurid A at a natural concentration of 3.04 ± 0.04 mg/mL, and in Plexaurid F at 2.66 ± 0.16 mg/mL. Data for Plexaurid F at 285 nm was not used to determine natural concentration as peak shape at that wavelength indicated that detector saturation had underrepresented the area calculated for that
peak. The determination of the natural concentrations was probably conservative as any loss of guaiazulene due to incomplete extraction, sublimation, or decomposition would result in underestimation of the concentration in the living gorgonian tissues.

Compound 284 was isolated as a blue gum, which had the molecular formula C₃₁H₆₅ by ¹³C NMR, ¹H NMR and low resolution EIMS (m/z 408, M⁺). Compound 284 was readily identified as 2,2'-diguaiazulenylmethane by comparison of spectral data with literature values.³¹³ 2,2'-Diguaiazulenylmethane was originally reported as a synthetic product, and was subsequently reported from the gorgonians Pseudothesia sp.³ and Calicogorgia granulosa.¹³ The approximate natural concentration of 2,2'-diguaiazulenylmethane was determined by methods analogous to those used for guaiazulene above, though the concentrations used in the dilution series were 2, 1, 0.5, and 0.25 mg/mL (see Table 10 and Figure 31).

Table 10. Linear regression analysis of data for standard calibration curves for 2,2'-diguaiazulenylmethane.

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>Area [mAU*sec] at Conc. (mg/mL)</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>210</td>
<td>4477</td>
<td>9652</td>
</tr>
<tr>
<td>245</td>
<td>7875</td>
<td>16495</td>
</tr>
<tr>
<td>285</td>
<td>14152</td>
<td>30415</td>
</tr>
</tbody>
</table>

² Data not used in standard curves due to detector saturation.

2,2'-Diguaiazulenylmethane was found to be present in Plexaurid A at a natural concentration of 0.22 ± 0.03 mg/mL, and in Plexaurid F at 0.14 ± 0.05 mg/mL. Data for the serial dilution of Plexaurid F at a concentration of 2 mg/mL detected at 285 nm were not used in linear regression analysis as peak shape at that wavelength indicated
that detector saturation had underrepresented the area due to that peak. The natural concentration of 2,2'-diguaiazulenylmethane determined for the two Plexaurids (0.22 and 0.14 mg/mL) fell below the range of values tested in the serial dilution analysis (0.25-2 mg/mL), and some caution must be exercised in interpreting these results. However, all r² values were very high (≥ 0.992) and the results are less than an order of magnitude below the lowest tested concentration in the serial dilution analysis, and it is expected that these values do accurately represent the values found in the gorgonian crude extract.

\[
\text{Table 11. Mean concentration of 2,2'-diguaiazulenylmethane in two Plexaurids.}
\]

<table>
<thead>
<tr>
<th>Gorgonian</th>
<th>Absorbance (Concentration)*</th>
<th>Mean Conc. (Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>210 nm</td>
<td>245 nm</td>
</tr>
<tr>
<td>Plexaurid A</td>
<td>3212(0.23)</td>
<td>5816(0.24)</td>
</tr>
<tr>
<td>Plexaurid F</td>
<td>1888(0.17)</td>
<td>3570(0.18)</td>
</tr>
</tbody>
</table>

*Absorbance in mAU and concentrations in mg/mL
Figure 31. Calibration curves for 2,2'-diguaiazulenylmethane at selected $\lambda_{\text{max}}$ values.

**Standard curve for 2,2'-diguaiazulenylmethane at 210 nm.**

\[ y = 21879x - 1823 \]

**Standard curve for 2,2'-diguaiazulenylmethane at 245 nm.**

\[ y = 38066x - 3290 \]
**Figure 31.** Calibration curves for 2,2'-diguaiazulenylmethane at selected λ<sub>max</sub> values.

The reef fishes *Thalassoma lunare* and *Halichoeres melanurus* were used in feeding assays as described in Chapter II of this dissertation. Bioassay guided fractionation identified guaiazulene (283) as the deterrent molecule. During the survey of feeding deterrence in gorgonian extracts in Madang, the crude extract of Plexaurid A strongly inhibited feeding by *H. melanurus*, with only one of ten assay pellets consumed. The results of feeding assays conducted in aquarium assays at SIO during fractionation and the isolation of the feeding deterrence in the fractions containing guaiazulene are shown in Figure 32. Guaiazulene isolated from both Plexaurid A and F was assayed at a range of concentrations, and was found to be
**Figure 32.** Bioassay-guided fractionation of Plexaurid A crude extract. Results of feeding deterrence assays are given for each separation. *Thalassoma lunare* assays were conducted using ten pellets for replicate assays, and six pellets were used for *Halichoeres melanurus*.

![Fractionation Diagram](image)

deterrent at levels of 2 mg/mL (see Figure 33). This concentration is below natural concentrations of guaiazulene found in Plexaurids A and F (> 2.5 mg/mL), indicating that these gorgonians are defended from predation by guaiazulene. 2,2'-

Diguaiazulenylmethane was also tested for feeding deterrence in aquarium assays, but did not retard feeding on its own, even at over twice natural concentration. 2,2'-
Diguaiazulenylmethane did show significant feeding inhibition (5 of 10 pellets eaten) at a concentration of 2 mg/mL, and may contribute to the overall detergency of the crude extract in an additive manner, though this hypothesis has not been tested.

**Figure 33.** Feeding deterrence of guaiazulene at various concentrations against *Thalassoma lunare.*

Bioassay-guided fractionation in antifungal assays also identified guaiazulene as the molecule responsible for observed activity. As discussed in Chapter III, Plexaurids A and F both showed some or total inhibition of the growth of strains CNL 536, 672, and 687 in the antifungal survey. Table 12 lists observed MICs for solvent partitions, fractions, and the two compounds from Plexaurid A against the three strains when tested in a serial dilution assay from 1.25 mg/mL to 0.01 mg/mL.

Guaiazulene was active against all three strains at MICs well below the observed natural concentrations of the compound in Plexaurids A and F. Guaiazulene was active against the gorgonian fungal pathogen *Aspergillus sydowii* at 0.16 mg/mL.
which is about 5% of the concentration found in Plexaurids A and F (3.04 and 2.66 mg/mL respectively). Guaiiazulene was active at even lower levels against the two other fungal strains tested, with MICs of 0.08 and 0.04 mg/mL against CNL 672 and 687 respectively. 2,2'-Diguaiazulenylmethane was active against CNL 687 at 0.08 mg/mL, which is below the natural concentrations found in Plexaurids A and F (0.22 and 0.14 mg/mL, respectively), although it was not active below natural concentrations against the other two strains, antifungal activity was observed at concentrations below 1 mg/mL.

<table>
<thead>
<tr>
<th>Table 12. Antifungal activity of Plexaurid A extract and compounds.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungal Isolate MIC (mg/mL)</strong></td>
</tr>
<tr>
<td><strong>Plexaurid A</strong></td>
</tr>
<tr>
<td>Solvent Partition:</td>
</tr>
<tr>
<td>methanol</td>
</tr>
<tr>
<td>hexanes</td>
</tr>
<tr>
<td>Hexane Fractions:</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>guaiiazulene</td>
</tr>
<tr>
<td>2,2'-diguaiazulenylmethane</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inoculum OD 0.17-0.20, MIC for Amphotericin B was 0.05-0.10 mg/mL.
<sup>b</sup> Inoculum OD 0.19-0.20, MIC for Amphotericin B was 0.02-0.10 mg/mL.
<sup>c</sup> Inoculum OD 0.19-0.22, MIC for Amphotericin B was 0.01-0.02 mg/mL.
C. Discussion

The identification of metabolites responsible for gorgonian chemical defenses gives insight into how these organisms reduce the incidence of predation and microbial infection in reef environments, into which chemical structures impart deterrent activity, and into how chemical defenses evolve. Compounds previously known to deter feeding by fish have had structures with polar attributes (see the review of gorgonian chemical ecology in Chapter I). In fact, in two cases non-polar sesquiterpene hydrocarbons from the extracts of gorgonians found to be deterrent in feeding assays were tested against assay fish and found to have no feeding deterrent properties.\textsuperscript{16,17} This study represents the first time that the deterrent activity of a gorgonian extract was found to be due to a hydrocarbon, broadening the range of chemical structures with ecologically relevant functions. In addition, the high levels of antifungal activity observed for guaiazulene suggest that secondary metabolites from gorgonians may play more than one ecologically relevant role. Other defensive roles secondary metabolites identified (or not) as having antifeedant properties may include antifouling activity, defense against other potential predators such as invertebrates, or protection from UV radiation.\textsuperscript{18} Very little work had been conducted to explore the possibility that gorgonian natural products are active in these, and many other, ecologically relevant roles.

In this study, guaiazulene had significant feeding deterrence against generalist predators in laboratory aquarium assays and showed strong antifungal activity against three fungal strains, including a known gorgonian pathogen, at MICs well below the natural concentrations observed in the gorgonians examined. The observance of
guaiiazulene and related compounds in a number of other octocorals, combined with the feeding deterrent and antifungal properties seen in this study, suggest that the production of these compounds may be a shared evolutionary defense mechanism of these organisms. The thresholds of tolerance for these compounds in other potential gorgonian predators remain unknown.
References


Chapter V

The Chemistry and Chemical Ecology of Briarane Producing Gorgonians

A. Introduction

Gorgonian corals are found in all tropical and subtropical seas, and are a rich source of structurally diverse novel secondary metabolites. These metabolites have been shown to possess both significant pharmacological properties and important ecological functions. As part of my continuing studies of the ecological importance of gorgonian secondary metabolites, I have studied the chemistry and chemical ecology of six briarane producing gorgonians. This study has led to the isolation of one novel briarane (285) together with the two known briaranes excavatolides B and C (286 and 287) from a Philippine collection of Briareum sp. A (NCI-1868), the identification of known briaranes in other collections, and a survey of the feeding deterrent properties of fractionated gorgonian extracts containing briaranes. Compounds 285-287 are closely related to other marine diterpenoids of the briarane skeletal class, first observed in briarein-A isolated from Briareum asbestinum. Related diterpenoids of the briarane skeletal class were later found in many other gorgonians and in organisms from other orders of the Alcyonarians such as true soft coral (Alcyonacea) of the genus Minabea, the sea pansy Renilla, and the sea pens (Pennatulacea) of the genera Ptilosarcus, Stylatula, Scytalium, Pteroides, and Cavernulina. Briaranes have long been known to possess potent pharmacological activities (cytotoxic, antiinflammatory, antiviral, and immuno-modulatory), yet the ecological functions of these compounds have been the subject of only two
reports. One report found that erythrolides B and D from *Erythropodium caribaeorum* act as fish feeding deterrents,21 and the other report found that the briaranes renillafoulins A-C from the sea pansy *Renilla reniformis* act as antifouling agents which inhibit the settlement of barnacle larvae.6 Other studies have detailed significant and interesting ecological properties of extracts known to contain briaranes, though the compound(s) responsible for the activity were not described. A survey of antifeedant properties in Caribbean gorgonian extracts by Pawlik and coworkers found significant levels of deterrence in extracts from three gorgonians known to produce briaranes.22 A study of the variation in the defensive chemistry of *Briareum asbestinum* by Harvell and colleagues found that the "basis of deterrence appears to reside in a diverse complement of 5 to 15 briarane and asbestinane diterpenoids,"23 and later reported that the embryos and larvae of this gorgonian appear to be defended from predation in the same manner.24 The presence of these compounds in many Alcyonarians and the initial ecological activities reported from briaranes suggest that they may be an important evolutionary defense. In the present study, the major metabolite, excavatolide B (286), deters feeding by fish in laboratory assays, as do the briarane containing fractions of other gorgonians. The structures and absolute stereochemistries of compounds 285-287 were determined by extensive spectral experiments (IR, MS, 1H and 13C NMR) and by comparison to known compounds.
B. Results of the Chemical Study of *Briareum* sp. A

Freshly collected specimens were stored frozen and subsequently extracted with dichloromethane and methanol. Flash silica gel chromatography of the lipid-soluble extract from *Briareum* sp. A, followed by high-performance liquid chromatography, yielded three briarane diterpenes. Excavatolide B (286) was by far the most abundant, representing more than 20% of the crude organic extract, while the two minor metabolites together comprised approximately 3% of the lipid-soluble material.

\[ \text{285 } R = \text{Ac} \]
\[ \text{286 } R^1 = \text{Ac}, R^2 = \text{n-PrCO} \]
\[ \text{287 } R^1, R^2 = \text{Ac} \]

4(R)-Butoxy-14(S)-hydroxy-excavatolide C (285) was purified by silica gel and ODS HPLC. Data from HR FAB MS and \(^{13}\text{C}\) NMR spectrometry of 285 established a molecular formula of C\(_{30}\)H\(_{42}\)O\(_{13}\) for the compound. The IR spectrum of 285 showed the presence of a hydroxyl group (3550-3500 cm\(^{-1}\)), a lactone (1780 cm\(^{-1}\)), and other ester carbonyl groups (1730 cm\(^{-1}\)). Carbonyl resonances in the \(^{13}\text{C}\) NMR spectrum of 285 (Table 13) at \(\delta\) 171.8, 172.2, 172.9, 173.6, and 174.4 confirmed the presence of a lactone and four other esters. Three of the esters were identified as
Table 13. $^1$H and $^{13}$C NMR Spectral Data of 4(R)-Butoxy-14(S)-hydroxy-excavatolide C (285).

<table>
<thead>
<tr>
<th>position</th>
<th>$^1$H$^a$</th>
<th>$^{13}$C$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.86, s, 1H</td>
<td>44.6 (C)$^b$</td>
</tr>
<tr>
<td>2</td>
<td>4.32, s, 1H</td>
<td>87.8 (CH)</td>
</tr>
<tr>
<td>3</td>
<td>6.27, s, 1H</td>
<td>74.6 (CH)</td>
</tr>
<tr>
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<td>5.24, d, 5, 1H</td>
<td>68.4 (CH)</td>
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<td>5.80, d, 5.5, 1H</td>
<td>141.5 (C)</td>
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<td>7</td>
<td>5.35, d, 8.5, 1H</td>
<td>124.7 (CH)</td>
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<td>2.60, dd, 8.5, 5, 1H</td>
<td>75.9 (CH)</td>
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<tr>
<td>9</td>
<td>2.44, m, 1H</td>
<td>72.0 (C)</td>
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<tr>
<td>10</td>
<td>3.93, ddd, 12, 4, 4, 1H</td>
<td>67.4 (CH)</td>
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<td>30.9 (CH2)</td>
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<td>12</td>
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<td>82.4 (CH)</td>
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<td>13$\alpha$</td>
<td>4.92, dd, 3, 3, 1H</td>
<td>18.5 (CH3)</td>
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<td>14</td>
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<td>17.1 (CH3)</td>
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<tr>
<td>16</td>
<td>1.58, s, 3H</td>
<td>10.5 (CH3)</td>
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<tr>
<td>17</td>
<td>1.08, d, 6.5, 3H</td>
<td>172.9 (C)</td>
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<td>acetates</td>
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<td></td>
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<tr>
<td>C-2</td>
<td>2.06, s, 3H</td>
<td>173.6 (C)</td>
</tr>
<tr>
<td>C-3</td>
<td>2.16, s, 3H</td>
<td>21.5 (CH3)</td>
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<td>C-9</td>
<td>2.32, s, 3H</td>
<td>171.8 (C)</td>
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<tr>
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<td>C-4</td>
<td>2.36, m, 2H</td>
<td>172.2 (CH)</td>
</tr>
<tr>
<td></td>
<td>1.65, m, 2H</td>
<td>21.3 (CH3)</td>
</tr>
<tr>
<td></td>
<td>0.95, t, 7.5, 3H</td>
<td>22.0 (CH3)</td>
</tr>
</tbody>
</table>

$^a$ Spectra recorded at 500 and 150 MHz in CD3OD at -20°C.

$^b$ Multiplicities deduced by DEPT. The δ values are in ppm and are referenced to the residual methanol signal (3.30 and 49.0 ppm).
acetate groups by the presence of methyl resonances in the $^1$H NMR spectrum (Table 13, Figure 34) at δ 2.32, 2.16, and 2.06. The other ester was determined to be a butyrate on the basis of $^1$H NMR studies including 2-D COSY experiments which showed the n-propyl group [δ 0.95 (t, J = 7.5, 3H), 1.65 (m, 2H), and 2.36 (m, 2H)]. Thus, ten of the 13 oxygen atoms in the molecular formula of compound 285 were accounted for by the γ-lactone and ester functionalities. A $^{13}$C DEPT experiment accounted for all but two of the hydrogens in the molecular formula, and these two hydrogens were assumed to belong to the hydroxyls suggested by the IR spectrum, though hydroxyl resonances were not seen in the $^1$H spectrum. The final oxygen was confirmed as an epoxide based on $^{13}$C NMR evidence [δ 63.4 (C) and 72.0 (C)]. Additional signals in the $^{13}$C NMR spectrum of 285 at δ 124.7 (CH) and 141.5 (C) suggested the presence of a trisubstituted olefin. The remaining $^{13}$C NMR signals confirmed the presence of one additional methylene [δ 30.9 (CH2)], a quaternary bridgehead carbon [δ 44.6 (C)], two methine carbons that did not bear heteroatoms [δ 38.1 (CH) and 42.1 (CH)], and four methyl groups [δ 9.5 (CH3), 10.5 (CH3), 17.1 (CH3), and 18.5 (CH3)].

Analysis of the spectral data obtained for 4(R)-butoxy-14(S)-hydroxy-excatavolide C suggested that the compound belonged to the briarane class of marine diterpenoids. A $^1$H-$^1$H COSY (Table 14) experiment allowed the determination of the spin systems which give the proton sequences H-2 to H-3 to H-4; H-6 to H-7 and H$_3$-16; and H-9 to H-10 to H-11 to H-12 to H-13$\beta$ to H-14. These data, combined with
Figure 34. 500 MHz Proton NMR spectrum of 4(R)-butoxy-14(S)-hydroxy-excavatolide C (285) in CD$_3$OD.
Table 14. Protons to Which Long-Range Correlations Were Observed in the HMBC, \(^1\)H-\(^1\)H COSY and NOESY spectra of 4(\(\dddot{R}\))-butoxy-14(\(S\))-hydroxy-excavatolide C (285).

<table>
<thead>
<tr>
<th>position</th>
<th>HMBC</th>
<th>(^1)H-(^1)H COSY</th>
<th>NOESY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-2, H-3, H-10, H-13(\beta), H(_3)-15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>H-4, H(_3)-15</td>
<td>H-3, H(_3)-15</td>
<td>H-3, H-14, H(_3)-15</td>
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<tr>
<td>3</td>
<td>H-2, H-4</td>
<td>H-2, H-4</td>
<td>H-2, H-10</td>
</tr>
<tr>
<td>4</td>
<td>H-2, H-6, H(_3)-16</td>
<td>H-3, H(_3)-16</td>
<td>H-7</td>
</tr>
<tr>
<td>5</td>
<td>H-3, H-4, H-6, H-7, H(_3)-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>H-4, H-7, H(_3)-16</td>
<td>H-7, H(_3)-16</td>
<td>H(_3)-16</td>
</tr>
<tr>
<td>7</td>
<td>H-6, H-9</td>
<td>H-6</td>
<td>H-4</td>
</tr>
<tr>
<td>8</td>
<td>H-9, H-10, H(_3)-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>H-10</td>
<td>H-10</td>
<td>H(_3)-15, H(_3)-18, H(_3)-20</td>
</tr>
<tr>
<td>11</td>
<td>H-9, H-10, H-13(\alpha)(\beta), H(_3)-20</td>
<td>H-12</td>
<td>H-10, H-12</td>
</tr>
<tr>
<td>12</td>
<td>H-13(\alpha), H-14, H(_3)-20</td>
<td>H-11, H-13(\alpha)(\beta)</td>
<td>H-10, H-11, H-13(\alpha)</td>
</tr>
<tr>
<td>13(\alpha)</td>
<td></td>
<td>H-12, H-13(\beta), H-14</td>
<td>H-12</td>
</tr>
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<td>(\beta)</td>
<td></td>
<td>H-12, H-13(\alpha), H-14</td>
<td>H-14</td>
</tr>
<tr>
<td>14</td>
<td>H-2, H-13(\beta), H(_3)-15</td>
<td>H-13(\alpha)(\beta)</td>
<td>H-2, H-13(\beta), H(_3)-15</td>
</tr>
<tr>
<td>15</td>
<td>H-10</td>
<td>H-2</td>
<td>H-2, H-9, H-14</td>
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<tr>
<td>16</td>
<td>H-4, H-6</td>
<td>H-6</td>
<td>H-6</td>
</tr>
<tr>
<td>17</td>
<td>H-9, H(_3)-18</td>
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<td>18</td>
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<td></td>
<td>H-9</td>
</tr>
<tr>
<td>19</td>
<td>H(_3)-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>H-10</td>
<td></td>
<td>H-9</td>
</tr>
</tbody>
</table>
correlations observed in an HMBC experiment and favorable NMR comparison to several known briarane diterpenoids, established the gross structure of 285. The butyrate methylene protons at δ 2.36 and the proton at δ 6.27 both correlated to the carbon signal at δ 174.4 in the HMBC spectrum which was assigned as the carbon atom of the butyrate carbonyl and placed it at C-4. The positions of the three acetate groups at C-2, C-3, and C-9 were confirmed in similar fashion, leaving the two hydroxyl groups at C-12 and C-14.

The major metabolite, excavatolide B (286), and the other minor metabolite, excavatolide C (287), were isolated and fully characterized as research undertaken as part of this dissertation, but prior to publication they were reported elsewhere.3 NMR data acquired for both of these compounds in multiple solvent systems showed slowly interconverting conformations in solution at both room and elevated temperatures (Figure 35). While Sheu, et al.,3 reported NMR data acquired in Me2CO-d6 at -70°C for 286 and 287, data were acquired for this study in CD3OD at -20°C (Figures 36 and 37). Careful analysis of spectroscopic data and comparison with published values confirm that 286 and 287 are identical to excavatolides B and C. Optical rotations found in this study, however, are of opposite sign and slightly different magnitude from those previously reported.3 While the literature values for the optical rotations of 286 and 287 are -23° and -13° respectively, the rotations were found to be +44° and +42° in this study. All other data confirm the gross structures of 286 and 287, and no explanation of this discrepancy is apparent. 4(R)-Butoxy-14(S)-hydroxy-excavatolide C is assumed to have the same relative configuration as 286 and 287 as it was isolated.
Figure 35. 500 MHz Proton NMR spectrum of excavatolide B (286) in CD$_3$OD at 25°C.
Figure 36. 500 MHz Proton NMR spectrum of excavatolide B (286) at -20°C in CD₃OD.
Figure 37. 150 MHz Carbon NMR spectrum of excavatolide B (286) at -20°C in CD3OD, with expansions shown.
from the same organism. Independent analysis of NOESY and coupling constant data confirm this assumption. Protons H-4 and H-7 were found to be within NOE proximity (Table 14), placing the butyrate group of 285 at C-4, which is not present in either 286 or 287, on the α face of the molecule.

![Chemical structure](image)

**Figure 38.** Selected NOE correlations of 4(\(R\))-butoxy-14(\(S\))-hydroxy-excavatolide C.

The absolute configuration of excavatolide B (286) was determined by modified Mosher's ester analysis\textsuperscript{25} by esterification at C-12. This method allows the measurement of the differential anisotropic shielding due to diamagnetic effect of the benzene ring of the (\(R\)) and (\(S\))-methoxytrifluoromethylphenylacetyl (MTPA) esters when oriented in the ideal configuration with the formation of the MTPA plane (Figure 39). Due to the diamagnetic effect of the aromatic ring, in the (\(R\))-MTPA ester the phenyl ring shields \(H_{A-C}\) and shifts their \(^1H\) NMR signals upfield relative to the (\(S\))-MTPA ester, and the reverse occurs for the \(^1H\) NMR signals of \(H_{X-Z}\). Because of these relative shifts in \(^1H\) NMR signals, when \(\Delta\delta = \delta_S - \delta_R\) positive values (where \(\Delta\delta > 0\)) are seen for \(H_{A-C}\) on the right hand side of the MTPA plane, and negative values are
seen for \( H_{x,z} \) on the left hand side of the plane, as indicated in Figure 39. Coupling this technique with high-field NMR and 2D NMR techniques that allow assignment of all protons in most molecules allows a large number of data points to be used in assigning the absolute stereochemistry of compounds.

![MTPA plane](image)

**Figure 39.** (A) Mosher ester, with the MTPA plane indicated with \( H_{A-C} \) on the right side of the plane and \( H_{x,z} \) on the left side. (B) View of (A) as seen by the outlined arrow, for absolute configuration determination using \( \Delta \delta \) values.

Excavatolide B (286) was selected for Mosher's analysis as it possessed a single secondary alcohol group at C-12 which molecular modeling indicated was not
Figure 40. (A) S-MTPA ester of 286. (B) R-MTPA ester of 286.
Figure 41. Analysis of the Mosher esters of 286. $^1$H $\Delta \delta$ (δS-δR) values expressed in Hz. The dashed line divides the compound along the MTPA plane.

overly sterically hindered. The (R) and (S)-MTPA esters of 286 were prepared and $^1$H NMR signals for the two compounds assigned (see Figure 40). The $\Delta \delta$ values were calculated and the protons with positive $\Delta \delta$ values were assigned to the right hand side of the molecule and those with negative $\Delta \delta$ values to the left hand side (Figure 40). Molecular models of the esters were constructed to confirm that all the protons with assigned positive and negative $\Delta \delta$ values were on the right and left sides of the MTPA plane, respectively. The absolute values observed for $\Delta \delta$ were also found to be proportional to the distance of the respective proton from the MTPA moiety, which is required as the diamagnetic effect of the benzene ring lessens with distance. The observed differential shielding pattern for the esters was clearly indicated the S configuration at C-12, placing the C-12 substituent in the β position. This analysis is in agreement with single crystal X-ray analysis of the closely related known diterpenoid brianolide.26 Brianolide was reported to have been previously isolated from the same gorgonian as excavatolide B.3 Thus, the overall absolute
stereochemistries of 286 and 287 were determined to be 1R, 2R, 3S, 7S, 8R, 9S, 10S, 11R, 12S, 14S, 17R. By analogy, the absolute configuration of 285 should be 1R, 2R, 3S, 4R, 7S, 8R, 9S, 10S, 11R, 12S, 14S, 17R.

Experimental Section

General Experimental Procedures: $^1$H NMR spectra were recorded at either 300 MHz or 500 MHz and $^{13}$C NMR spectra were recorded at either 50 MHz or 150 MHz, with chemical shifts referenced to residual methanol signal. Multiplicities of carbon signals were determined through DEPT experiments, and all carbon assignments were consistent with the DEPT results. HMBC and HMQC experiments were optimized for $n_j^{CH} = 8.0$ Hz and $j_j^{CH} = 150.0$ Hz, respectively. HPLC separations were accomplished using Rainin DYNAMAX-60Å SiO$_2$ or ODS column (250 x 10 mm) at a flow rate of 3 ml/min with refractive index detection. IR spectra were determined on a Hewlett-Packard 1600 FTIR spectrometer. Optical rotations were recorded on an Autopol 3.

Collection and Extraction: Briareum sp. (voucher number NCI-1868) was hand collected by SCUBA divers at 10 m in April 1993 in San Jose, Philippines, and stored frozen until use. Upon workup, the gorgonian was lyophilized and then twice extracted with a 1:1 mixture of methanol and chloroform followed by a final extraction in methanol. The extracts were then combined, filtered through celite, and evaporated under reduced pressure to give 36 g of crude extract (from 380 g, dry weight of animal). The crude extract was then triturated with methanol and dichloromethane to remove salts, giving 24.2 g of soluble extract after evaporating,
which was subsequently partitioned between hexanes and methanol. The methanol partition was evaporated to dryness and partitioned between ethyl acetate and water. The ethyl acetate partition was reduced to dryness and chromatographed on silica gel by vacuum flash chromatography. Fractions eluted with mixtures of isooctane and ethyl acetate. 4(R)-Butoxy-14(S)-hydroxy-excavatolide C (1) was the least polar of the three metabolites, eluting with 50% ethyl acetate. Next, excavatolides B (2) and C (3) eluted in 60% ethyl acetate.

**4(R)-Butoxy-14(S)-hydroxy-excavatolide C (285).** Purification by HPLC (70% ethyl acetate in isooctane) yielded 77 mg (0.32% of the triturated extract) of compound 285 as a white powder with mp 131-133°C; [α]$_D^{20}$ +134° (c 0.25, MeOH); IR (dry film) ν$_{max}$ 3500 (OH), 2960 (CH, aliphatic), 1780 (C=O, lactone), 1730 (C=O, ester), 1370, 1240, 1080, 1030 cm$^{-1}$; HRFAB MS m/z [M+Na]$^+$ 633.2527 (100%) (calcd for C$_{39}$H$_{42}$O$_{13}$Na$_4$, 633.2523).

**Excavatolide B (286).** Purification by HPLC (65% ethyl acetate in isooctane) followed by crystallization from ethanol/water yielded 6.23 g (25.7 % of the triturated extract) of compound 286 as needles with mp 220-222 °C; [α]$_D^{20}$ +44° (c 0.27, MeOH); IR (dry film) ν$_{max}$ 3510 (OH), 2965 (CH, aliphatic), 1785 (C=O, lactone), 1735 (C=O, ester), 1370, 1225, 1110, 1030 cm$^{-1}$; $^1$H NMR (500 MHz, CD$_3$OD at -20°C) 5.78 (d, 1H, J = 6.5 Hz, H-3), 5.53 (d, 1H, J = 7 Hz, H-7), 5.47 (d, 1H, J = 10 Hz, H-9), 5.38 (d, 1H, J = 7 Hz, H-6), 5.21 (br s, 1H, H-2), 4.76 (br s, 1H, H-14), 3.97 (dd, 1H, J = 16, 7.5 Hz, H-4β), 3.87 (ddd, 1H, J = 12, 4.5, 4 Hz, H-12), 3.05 (dd, 1H, J = 10, 4.5 Hz, H-10), 2.56 (m, 1H, H-11), 2.38 (s, 3H, 9-OAc), 2.20 (s, 3H, 14-OAc),
2.19 (m, 2H, 3-OCOPr), 2.18 (s, 3H, 2-OAc), 2.07 (d, 1H, J = 16 Hz, H-4α), 1.95 (s, 3H, H-16), 1.92 (m, 1H, H-13β), 1.79 (m, 1H, H-13α), 1.58 (m, 2H, 3-OCOPr), 1.53 (s, 3H, Me-18), 1.02 (d, 3H, J = 7 Hz, Me-20), 0.91 (t, 3H, J = 7.5 Hz, 3-OCOPr), 0.85 (s, 3H, Me-15); 13C NMR (150 MHz, CD3OD at -20°C) 172.6, 172.3, 172.3, 170.8, 170.6, 139.5, 122.5, 81.7, 81.6, 74.3, 73.9, 69.1, 66.3, 66.2, 60.6, 44.1, 40.3, 35.9, 35.5, 34.1, 29.8, 21.6, 21.4, 21.4, 20.6, 18.2, 17.7, 13.0, 9.3, and 8.6; HRFAB MS m/z [M+H]+ 595.2713 (100%) (calcd for C30H32O12, 595.2755).

**Excavatolide C (287).** Purification by HPLC (70% ethyl acetate in isooctane) yielded 570 mg (2.4% of the triturated extract) of compound 287 as a white powder with mp 150-152 °C; [α]30° D +42° (c 0.23, MeOH); IR (dry film) νmax 3500 (OH), 2945 (CH, aliphatic), 1785 (C=O, lactone), 1740 (C=O, ester), 1370, 1225, 1115, 1085, 1030 cm−1; 1H NMR (500 mHz, CD3OD at -20°C) 5.76 (d, 1H, J = 7.5 Hz, H-3), 5.53 (d, 1H, J = 7 Hz, H-7), 5.47 (d, 1H, J = 10 Hz, H-9), 5.38 (d, 1H, J = 7 Hz, H-6), 5.21 (br s, 1H, H-2), 4.74 (br s, 1H, H-14), 3.96 (dd, 1H, J = 16, 7.5 Hz, H-4β), 3.87 (ddd, 1H, J = 12, 4.5, 4.5 Hz, H-12), 3.06 (dd, 1H, J = 10, 5 Hz, H-10), 2.56 (m, 1H, H-11), 2.39 (s, 3H, 9-OAc), 2.20 (s, 3H, 14-OAc), 2.18 (s, 3H, 2-OAc), 2.08 (d, 1H, J = 16 Hz, H-4α), 1.95 (s, 3H, H-16), 1.91 (s, 3H, 3-OAc), 1.82 (m, 1H, H-13β), 1.75 (m, 1H, H-13α), 1.53 (s, 3H, Me-18), 1.02 (d, 3H, J = 7 Hz, Me-20), 0.83 (s, 3H, Me-15); 13C NMR (150 MHz, CD3OD at -20°C) 173.8, 173.6, 171.9, 171.8, 171.3, 140.5, 123.6, 82.9, 82.4, 75.3, 75.1, 70.3, 67.3, 67.0, 61.5, 45.1, 41.2, 36.5, 34.8, 30.6, 22.6, 22.5, 22.3, 21.6, 20.9, 18.7, 10.2, and 9.5; HRFAB MS m/z [M+H]+ 567.2463 (100%) (calcd for C28H30O12, 567.2442).
**Preparation of Mosher's Ester (288).** Excavatolide B (5 mg, 0.0084 mmol) was dissolved in dichloromethane (400 µL). Added to the solution were DMAP (2 mg), pyridine (200 µL), and (S)-MTPA-Cl (7.5 µL, 0.041 mmol, 4.8 equiv.). The reaction was stirred at room temperature and followed by TLC until complete, 3 hours. The reaction mixture was then concentrated and quenched with 1M NH₄Cl (500 µL) for 25 minutes. The mixture was extracted with dichloromethane (3 x 1 mL), dried over MgSO₄, and concentrated to give a white solid (7.6 mg). Purification by HPLC (1:1 TMP:EtOAc) afforded ester 288 (2.3 mg, 34%). ^1H NMR (500 MHz, -20° C, CD₃OD) 5.82 (d, 1H, J = 7 Hz, H-3), 5.52 (d, 1H, J = 7 Hz, H-7), 5.45 (d, 1H, J = 7 Hz, H-6), 5.39 (d, 1H, J = 10 Hz, H-9), 5.25 (br s, 1H, H-2), 4.82 (br s, 1H, H-14), 3.97 (dd, 1H, J = 16, 4 Hz, H-4), 3.15 (dd, 1H, J = 10, 5 Hz, H-10), 2.60 (m, 1H, H-11), 2.38 (s, 3H, -OAc), 2.27 (s, 3H, -OAc), 2.19 (m, 2H, -OCOPr), 2.18 (s, 3H, -OAc), 2.15 (m, 1H), 2.07 (m, 1H), 2.03 (m, 1H), 1.99 (s, 3H, H-16), 1.57 (m, 2H, -OCOPr), 1.51 (s, 3H, H-18), 0.91 (t, 3H, J = 8 Hz, -OCOPr Me), 0.88 (d, 3H, J = 7 Hz, H-20), 0.86 (s, 3H, H-15).

**Preparation of Mosher's Ester (289).** Excavatolide B (5 mg, 0.0084 mmol) was dissolved in dichloromethane (400 µL). Added to the solution were DMAP (2 mg), pyridine (200 µL), and (R)-MTPA-Cl (7.5 µL, 0.041 mmol, 4.8 equiv.). The reaction was stirred at room temperature and followed by TLC until complete, 3 hours. The reaction mixture was then concentrated and quenched with 1M NH₄Cl (500 µL) for 25 minutes. The mixture was extracted with dichloromethane (3 x 1 mL), dried over MgSO₄, and concentrated to give a white solid (7.0 mg). Purification by HPLC (1:1
TCP: EtOAc) afforded ester 289 (3.2 mg, 47%). $^1$H NMR (500 MHz, -20° C, CD$_3$OD) 5.85 (d, 1H, J = 7 Hz, H-3), 5.54 (d, 1H, J = 7 Hz, H-7), 5.46 (d, 1H, J = 7 Hz, H-6), 5.43 (d, 1H, J = 10 Hz, H-9), 5.28 (m, 1H, H-12), 5.20 (br s, 1H, H-2), 4.78 (br s, 1H, H-14), 3.98 (dd, 1H, J = 16, 4 Hz, H-4), 3.21 (dd, 1H, J = 10, 5 Hz, H-10), 2.71 (m, 1H, H-11), 2.39 (s, 3H, -OAc), 2.27 (s, 3H, -OAc), 2.22 (m, 1H), 2.18 (m, 2H, -OCOPr), 2.18 (s, 3H, -OAc), 2.10 (m, 1H), 2.08 (m, 1H), 2.03 (m, 1H), 1.99 (s, 3H, H-16), 1.58 (m, 2H, -OCOPr), 1.54 (s, 3H, H-18), 1.04 (d, 3H, J = 7 Hz, H-20), 0.92 (t, 3H, J = 8 Hz, -OCOPr Me), 0.84 (s, 3H, H-15).

C. The Chemical Ecology of Briarane Containing Gorgonians

The effectiveness of excavatolide B (285), by far the major metabolite isolated from *Briareum* sp. A (NCI-1868), in suppressing feeding by common generalist predators was investigated along with fractions from other gorgonian extracts containing briaranes. Aquarium assays were performed (using methods described in Pawlik and Fenical$^{27}$) in wet labs located at the Christensen Research Institute (CRI) in Madang, Papua New Guinea and an experimental aquarium facility at Scripps Institution of Oceanography (SIO). Assays at CRI were run using the generalist predator wrasse *Halichoeres melanurus* that were collected by hand net from coral reefs in Madang Harbor. Assays performed at SIO were performed with specimens of *Thalassoma lunare*, another small generalist predatory wrasse found throughout the Indo-Pacific, that were purchased from Aquatic Depot in Long Beach, California. *Thalassoma lunare* from Aquatic Depot were collected in the Solomon Islands, delivered to SIO in healthy condition, and commenced feeding within a day of
arrival. Excavatolide B or extract fractions were dissolved in a minimal amount of methanol and incorporated at known concentrations into a palatable food matrix to create treatment pellets. Control pellets were made with only the addition of methanol to the food matrix. The treatment pellets were assayed and scored for palatability against the control pellets as described in Pawlik and Fenical.²⁷

Excavatolide B significantly deterred feeding (Fisher exact test; ²⁸ p ≤ 0.043, one tailed test) by *T. lunare* at concentrations as low as 1.5 mg/mL food matrix and by *H. melanurus* at concentrations down to 0.75 mg/mL. The natural weight per volume (w/v) concentration of excavatolide B was estimated by comparing the gorgonian’s wet weight before extraction to that of other samples of the gorgonian and then measuring the tissue volume of those samples. The concentration of excavatolide B in the extracted specimen was conservatively estimated to be at least 20 mg/mL tissue. Thus excavatolide B was found to be significantly deterrent to both of these generalist predators at concentrations of less than 10% w/v of those found in the gorgonian. These results are similar to the previously reported fish feeding deterrence of the extract of *Erythropodium caribaeorum* and two briaranes isolated from this gorgonian, erythrolides B and D.²¹²² The extract of *E. caribaeorum* was deterrent at less than 10% of the percentage dry weight the extract was found to have in the gorgonian when tested in aquarium assays against the generalist wrasse *Thalassoma bifasciatum*. Erythrolides B and D were later assayed in field studies with a natural assemblage of reef fish at the same concentrations as they occurred in gorgonian tissues, and inhibited feeding.
The extracts of five other gorgonians containing briaranes and deterring feeding in aquarium assays against both species of wrasse were further examined. Collections used were *Briareum* sp. B (95-121), *Briareum* sp. C (PNG-96-038), and three replicates of *Junceella* cf. *fragilis* (PNG-96-015, PNG-97-028, and -046). For each of those gorgonians, a portion of the crude extract equivalent to 20 mL of gorgonian tissue was partitioned first between hexanes and methanol to remove non-polar constituents. The resulting methanol partition was reduced by rotary evaporation to a gum that was partitioned between distilled water and ethyl acetate to remove polar constituents. The resulting ethyl acetate partition was divided in half to allow feeding deterrence assays with one 10 mL equivalent portion and chemical analysis of the other. $^1$H NMR, TLC, and LCMS confirmed the presence of briaranes for all five samples.

The extracts of the specimens studied here and other gorgonians containing briaranes have consistently been found to contain one or more briaranes present as major metabolites along with a number of minor metabolites which vary by oxygenation or substitution patterns (see Chapter I). For the five briarane containing ethyl acetate extracts studied here, the major metabolite(s) of the partition were identified. The ethyl acetate partition of *Briareum* sp. B (95-121) contained at least seven briaranes, with the most predominant compound being identified as 16-Acetoxyxystecholide C acetate (288) by comparison of spectral data to literature values.29 16-acetoxyxystecholide C acetate has been previously reported from the Australian gorgonian *Solenopodium stechei* as a minor metabolite. The ethyl acetate partition of *Briareum* sp. C (PNG-96-038) was very rich in compounds displaying
spectroscopic data which indicated that they were of the briarane carbon skeleton, and the previously reported briarane stecholide J (289) was the quantitatively predominant compound in the partition. Stecholide J was identified by comparison of spectroscopic data to literature values, and was first isolated as the most abundant briarane of a New Guinea collection of *Solenopodium excavatum*.\(^{30}\)

All chemical reports of compounds from gorgonians of the genus *Junceella* have described briarane class structures (see Chapter I), and at least 30 previously undescribed briaranes have been detailed from these gorgonians. The three *Junceella fragilis* ethyl acetate partitions were analyzed extensively by LCMS and \(^1\)H NMR, and though the levels at which the compounds occurred in the samples were not

**Figure 42.** Some briaranes from gorgonian extracts assayed.

![Chemical structures](image)

288 16-acetoxystecholide C acetate  
289 stecholide J  
290 juncellin  
291 praelolid  
292 juncellolide D
quantified, all three extracts from separate collections were found to contain the briaranes junceellin$^{31}$ (290), praelolide$^{32}$ (291), and junceellolide D$^{19}$ (292) in addition to a number of other briaranes. The two publications to date on the secondary metabolites from Junceella fragilis have both found these three compounds.$^{19,33}$

The ethyl acetate extracts of all five gorgonians were tested in aquarium assays at SIO against Thalassoma lunare at 1/1, 1/2, and 1/4 natural concentration. All five extracts were found to be highly deterrent to fish feeding at natural and 1/2 natural concentrations, and three of the five extracts were significantly deterrent when tested at 1/4 natural concentration (Figure 43).

The high levels of deterrence of briarane class compounds and extracts containing briaranes seen in this study and others described above suggest that this group of compounds may as a whole be an important chemical defense against predation in the gorgonians and other organisms containing them. The isolation of briaranes from many different Alcyonarians and their presence in complex mixtures further suggests that these defensive compounds may be synthesized by a pathway that has been preserved during the evolution of these organisms. While these compounds are highly deterrent against three generalist reef fish at concentrations well below those found in gorgonian tissues, the metabolic cost of producing the compounds would be expected constrain production of the compounds to minimally effective concentrations, especially considering the low nutrient reef environment these organisms exist in. However, thresholds of tolerance for these compounds in other potential gorgonian predators remains unknown, and it would be expected that specialist predators would be much more tolerant of the metabolites.
Figure 43. Feeding deterrence of gorgonian extracts containing briaranes at various concentrations.

The range of predators that gorgonians experience in their environment may also account for the often complex mixtures of briaranes present in many gorgonians. The mixture of briaranes may represent a "cocktail" of defensive chemistry that may have an additive or synergistic effect when sampled by various predators in the reef environment, though these theories have not been adequately tested.
References


Chapter VI

Natural Products Chemistry of Astrogorgia sp.

A. Introduction

Activity observed in the feeding deterrence assays (Chapter II) directed further investigation of the crude extract of Astrogorgia sp. (PNG-96-053). This study led to the isolation of seven secondary metabolites, including the known eunicellin diterpene ophirin (294), two novel eunicellin class compounds 295 and 296, three known 9,10-seco steroids 297-299, and the novel 9,10-seco steroid 300. Though partitions and fractions of the crude extract containing these and other compounds were active in feeding assays, the compound(s) responsible for the activity were not identified.

Compounds 294-296 are closely related to other marine diterpenoids of the eunicellin (297) skeletal class, first observed from the gorgonian Eunicella stricta in 1968.¹ Related diterpenoids of this type were later reported from the gorgonians Eunicella cavolini,²,³ E. labiata,⁴ E. verrucosa,⁵,⁶ Briareum asbestinum,⁷,⁸ Muricella sp.,⁹ and Alcyonarian soft corals of the genus Cladiella.¹⁰-¹³ Steroids and seco steriods are a major group of metabolites that have been isolated from gorgonians (Chapter I). The only publication of chemistry from Astrogorgia sp. reported the 9,10-seco steroid astrogorgiadiol (297) along with ophirin (294) and a related eunicellin, astrogorgin.¹⁴ The structures of compounds 294-300 were elucidated or confirmed by extensive spectral experiments (IR, MS, ¹H and ¹³C NMR) and by comparison to known compounds.
B. Results of the Chemical Study of *Astrogorgia* sp.

The freshly collected gorgonian was stored frozen and subsequently extracted with dichloromethane and methanol. Flash silica gel chromatography of the lipid-soluble extract from *Astrogorgia* sp., followed by high-performance liquid
chromatography (HPLC), yielded seven compounds. Astrocellins A (295) and B (296) were the most abundant metabolites, representing 1.2% of the lipid soluble material. All other metabolites accounted for less that 1% of the crude organic extract.

Compound 294 was purified by silica gel and ODS HPLC. Data from EIMS and $^{13}$C NMR spectrometry of 294 established a molecular formula of $C_{36}H_{38}O_7$ for the compound. The IR spectrum indicated the presence of ester carbonyl groups (1729 cm$^{-1}$). In the mass spectrum, consecutive loss of three 60 mass unit fragments from the molecular ion at $m/z$ 462 [402(11%), 342(8%), and 282(10%)] indicated the presence of three acetate groups. Carbonyl resonances in the $^{13}$C NMR spectrum of 294 (Table 15) at $\delta$ 170.6, 169.9, and 169.7, and three singlets in the $^1$H NMR spectrum at $\delta$ 1.99, 1.98, and 1.93 confirmed the presence of three acetate esters (Figure 44). Five additional singlets in the $^1$H NMR spectrum which integrated for 3 protons each ($\delta$ 1.80, 1.78, 1.76, 1.53, and 1.36) and the 20 carbons in the core of 294 suggested that the compound was a diterpene. With this information, a literature survey revealed that 294 was ophirin, an eunicellin previously isolated from the Red Sea gorgonian Muricella sp.$^9$ and a Japanese collection of Astrogorgia sp.$^{14}$ Comparison of the spectral data of ophirin showed very good correlation with published data for this compound.

Compounds 295 and 296 were isolated together by HPLC and were inseparable by the standard chromatographic techniques used. The compounds were purified together by ODS HPLC. Data from HRFAB MS, $^{13}$C NMR, and $^1$H NMR
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\(^a\) Spectra recorded at 400 MHz in CDCl\(_3\). \(^b\) Spectra recorded at 125 MHz in CDCl\(_3\).

The \(\delta\) values are in ppm and are referenced to the residual chloroform signal (7.24 and 77.0 ppm).

\(^c\) Number of attached protons deduced by DEPT.
spectrometry of the two compounds indicated that they were likely to be isomers.

Both $^{13}$C and $^1$H NMR spectra showed two related sets of resonances, and HRMS gave one parent ion ([M+Cs]$^+$ $m/z$ 553.1572) which established a molecular formula of $C_{32}H_{36}O_6$ for both compounds. The IR spectrum of the mixture showed the presence of ester carbonyl groups (1733 cm$^{-1}$), and carbonyl resonances in the $^{13}$C NMR spectrum (Table 15) at $\delta$ 170.3, 170.1, 169.5, and 169.4 suggested that each compound contained two esters. The esters were identified as acetate groups by the presence of four methyl resonances in the $^1$H NMR spectrum (Table 15 and Figure 45) at $\delta$ 2.05, 2.04, 1.88, and 1.86. A trisubstituted double bond was indicated for each compound [$\delta$ 126.7 (C), 126.5 (C), 130.6 (CH), and 128.4 (CH)] and six oxygen-bearing carbons (90.3, 90.1, 89.9, 86.8, 83.3, 82.9, 80.9, 78.4, 77.5, 74.1, 72.7, and 72.4) were revealed for each of the compounds. Of the six oxygen-bearing carbons, four from each compound were accounted for by two acetate groups each and the remaining two were proposed to be due to a pair of ether oxygens in each compound.

Spectral data suggested that these compounds were eunicellins closely related to ophirin (294), and careful analysis of 2D NMR spectra from HMBC, HMQC and COSY experiments revealed that the 10 membered rings of each compound were identical to that of ophirin. The ether and ester functionalities of the 10 membered rings left one acetate group and one ether bridge to be assigned in each isomer's six membered ring. Comparison of spectral data of astrocellin A (295) to known eunicellin compounds, and 2D NMR experiments, allowed assignment of the gross structure. A $^1$H-$^1$H COSY experiment allowed the determination of the spin system which gave the proton sequence H-2 to H-1 to H-14 to H-13$\alpha$ and H-13$\beta$ to H-12.
Figure 45. 400 MHz Proton NMR of the mixture of compounds A and B (295 and 296) in CDCl₃.
A HMBC experiment allowed the assignment of an oxygenated carbon at C-11 (δ 72.5), established ring connectivity with C-10 (δ 50.6), and the placement of the acetate (carbonyl at δ 170.3) at C-12 by correlations with H-12. HMBC correlations from the methyl protons at C-19 and C-20 placed an isopropyl group, connected by a quaternary carbon (δ 74.1) bearing an oxygen, at C-14. These data established an ether bridge from C-11 to C-18, creating a six-membered ring. Coupling constants could not be used to determine relative stereochemistry in the resultant bicyclic structure due to severe overlap of signals as seen in the ^1H NMR spectrum of astrocellins A and B (Figure 45). The relative stereochemistry of the methyl group at C-11 and the isopropyl group at C-14 of reported eunicellins has consistently been assigned as shown in 295 and 296. In the absence of direct evidence, the relative stereochemistry of 295 was tentatively assigned to match the known stereochemistry of the six membered ring of this class of compounds.

The gross structure of astrocellin B (296) was elucidated in the same manner as that of astrocellin A, and it was determined that the only difference was a switch in the placement of the ether bridge and the acetate group at C-11 and C-12 in the six-membered rings of 295 and 296. HMBC correlations from the methyl protons at C-19 and C-20 again placed an isopropyl group, containing a quaternary carbon (δ 83.3) bearing an oxygen, at C-14. HMBC correlations from H-12 established connectivity of the two rings at C-10, and a correlation was also seen to the quaternary carbon of the isopropyl group at C-14, establishing the ether bridge between C-12 and C-18. The acetate group was then assigned to the oxygen bearing carbon at C-11 (δ 83.0), resulting in the assignment of structure of 296. ^1H and ^13C NMR spectral data for
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<sup>a</sup>Spectra recorded at 500 and 150 MHz in CD$_3$OD at -20°C. The δ values are in ppm and are referenced to the residual methanol signal (3.30 and 49.0 ppm). <sup>b</sup>Multiplicities deduced by DEPT.
astrocellins A and B are given in Table 15. Due to the overlap of many signals in the
$^1$H NMR spectrum obtained for these compounds, the coupling constants and
integration values of some upfield resonances are not reported in Table 15, though
assignments were accomplished with the aid of COSY and HMQC experiments.

Compound 297 was purified by silica gel and ODS HPLC. Data from EIMS
and $^{13}$C NMR spectrometry experiments with 297 established a molecular formula of
C$_{27}$H$_{44}$O$_2$ for the compound. The 27 carbon signals observed in the $^{13}$C NMR spectrum
and five methyl protons in the $^1$H NMR spectrum suggested that 297 was a steroid
(Table 16). Six carbon NMR signals between δ 115-155 and three corresponding
proton NMR signals indicated that a trisubstituted benzene ring was present, and 2D
NMR methods confirmed the presence of a 3-hydroxy-6-methyl-benzyl group. With
this information, it was determined that 297 was astrogorgiadiol, a 9,10-secosterol
previously isolated in conjunction with ophirin (294) from Astrogorgia sp.$^{14}$

The molecular formula of calicoferol C (298) was determined as C$_{25}$H$_{44}$O$_2$ by
EIMS and $^{13}$C NMR spectrometry, and the spectral data of 298 were very similar to
those of 297. The sole difference in the $^{13}$C NMR data was the presence of an exo-
methylene group (see Table 16, C-24 and C-28). HMBC experiments placed the exo-
methylene group at C-24, confirming the structure of calicoferol C, a secosteroid
previously isolated in conjunction with astrogorgiadiol from Muricella sp.$^{15}$

The spectral data of calicoferol F (299) were also very similar to those of both
297 and 298. The compound analyzed for C$_{28}$H$_{44}$O$_2$ by EIMS and $^{13}$C NMR
spectrometry, and the differences in the $^{13}$C NMR spectrum compared to 297 indicated
the replacement of two upfield carbon signals with olefinic methines δ 135.9 (C-22),
Figure 47. 125 MHz Carbon NMR spectrum of calicogorgia J (300) in CDCl₃.

300 calicoferyl J

solvent
132.0 (C-23) and a new methyl group δ 18.0 (C-28) (Table 16). As with calicoferol C, analysis of the NMR data and combined 2D NMR experiments indicated that the difference occurred in the side chain of the molecule. $^1$H-$^1$H COSY and HMBC experiments placed the double bond between C-22 and C-23 and the methyl group at C-24, confirming the structure of calicoferol F (299), previously described in conjunction with astrogorgiadiol from *Muricella* sp.$^{16}$

The molecular formula of calicoferol J (300) was deduced as C$_{27}$H$_{42}$O$_2$ by HRMS and $^{13}$C NMR spectrometry, and the only significant difference in the $^{13}$C NMR spectrum compared to that of calicoferol F (299) indicated that the methyl group at C-24 was absent. Careful analysis of the $^1$H and $^{13}$C NMR spectra (Figures 46 and 47) combined with $^1$H-$^1$H COSY and HMBC experiments (Figure 48) confirmed the structure of calicoferol J (300), as the 23,23-didehydro derivative of astrogorgiadiol, thus extending the family of known calicoferols.

**Figure 48.** HMBC correlations establishing the structure of the side chain of 300.
Experimental Section

General Experimental Procedures: $^1$H NMR spectra were recorded at either 300 MHz or 400 MHz and $^{13}$C NMR spectra were recorded at 125 MHz, with chemical shifts referenced to the residual chloroform signal. Number of attached protons on carbon signals were determined through DEPT experiments, and all carbon assignments made were consistent with the DEPT results. HMBC and HMQC experiments were optimized for $^{n}J_{CH} = 8.0$ Hz and $^{1}J_{CH} = 150.0$ Hz, respectively. HPLC separations were accomplished using Rainin DYNAMAX-60Å SiO$_2$ or ODS column (250 x 10 mm) at a flow rate of 3 ml/min with refractive index detection. IR spectra were recorded on a Hewlett-Packard 1600 FTIR spectrometer.

Collection and Extraction: *Astrogorgia* sp. (voucher number PNG-96-053) was hand collected by SCUBA divers at 20 m in April 1996 in Madang Lagoon, Papua New Guinea, and stored frozen until use. Upon workup, the gorgonian was lyophilized and then twice extracted with a 1:1 mixture of methanol and chloroform followed by a final extraction in methanol. The extracts were then combined, filtered through Celite™, and evaporated under reduced pressure to give 13.3 g crude extract from 550 mL of gorgonian tissue. Because a large amount of inorganic salts were present in the crude extract, the extract was triturated with methanol to separate organic and inorganic components, and the remaining solids showed no organic characteristics by proton NMR. The resulting methanol soluble partition was evaporated under reduced pressure to give 9.8 g crude extract. This extract was
partitioned between hexanes and methanol, and the methanol partition was evaporated to dryness and subsequently partitioned between ethyl acetate and distilled water. The ethyl acetate partition was reduced to dryness and chromatographed on silica gel by vacuum flash chromatography. Fractions were eluted with a gradient of ethyl acetate (0 to 100%) in isooctane. Fractions eluted with 20-30% ethyl acetate contained a mixture of secosteroids (297-300), and those eluted with 40% ethyl acetate contained eunicellin class compounds (294-296).

**Astrocellins A (295) and B (296).** Purification by silica gel HPLC (35% ethyl acetate in isooctane) yielded 118 mg (1.2% of the triturated extract) of a mixture of the two compounds as an amorphous white powder with IR (dry film) ν\text{max} 2976 and 2930 (CH, aliphatic), 1733 (C=O, ester), 1464, 1369, 1256, 1068, 1027, 750 cm\(^{-1}\); HRFAB MS \text{m/z} [M+Cs]\(^+\) 553.1572 (100%) (calcd for C\(_{24}\)H\(_{36}\)O\(_6\)Cs, 553.1566), [M+Na]\(^+\) 443.2414 (100%) (calcd for C\(_{24}\)H\(_{36}\)O\(_6\)Na, 443.2410).

**Calicoferol J (300).** Purification by ODS HPLC (3% water in methanol) yielded 24 mg (0.2% of triturated extract) of a colorless oil with IR (film) ν\text{max} 3340 (OH), 2950 (CH, aliphatic), 1605, 1605, 1585, 1500, 1460, 1255, 965 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) 6.96 (d, 1H, J = 8.3 Hz, H-1), 6.63 (d, 1H, J = 2.5 Hz, H-4), 6.55 (dd, 1H, J = 8.3, 2.5 Hz, H-2), 5.25 (m, 1H, H-22), 5.19 (m, 1H, H-23), 4.03 (br s, 1H, H-9), 2.69 (ddd, 1H, J = 13, 11.5, 5 Hz, H-6), 2.40 (ddd, 1H, J = 13, 11, 5.5 Hz, H-6), 2.20 (s, 3H, Me-19), 2.02 (m, 1H, H-20), 1.81 (m, 1H, H-24), 1.79 (m, 1H, H-24), 1.75 (m, 1H, H-12), 1.73 (m, 2H, H-11), 1.63 (m, 1H, H-16), 1.55 (m, 1H, H-15), 1.52 (m, 1H, H-8), 1.51 (m, 3H, H-7 (x2), H-14), 1.48 (m, 1H, H-12), 1.21 (m, 1H, H-16), 1.19 (m, 1H, H-17), 1.08 (m, 1H, H-15), 0.99 (d, 3H, J = 7 Hz, Me-21), 0.84 (dd, 6H,
\[ J = 7, 1.2, \text{Me-26, Me-27}, 0.68 \text{ (s, 3H, Me-18); HRFAB MS } m/z [M]^+ 398.3188 \]
(40\%) (calcd for C\(_2\)H\(_4\)O\(_2\), 398.3185)

C. The Chemical Ecology of *Astrogorgia* sp.

In laboratory fish feeding assays conducted in Madang and at SIO, the crude extract of *Astrogorgia* sp. strongly inhibited fish feeding. None of the ten treated pellets tested against *Halichoeres melanurus* in Madang were consumed when the extract was tested at natural concentration, and only two of ten treated pellets were consumed when the extract was assayed at half natural concentration. Assays conducted at SIO with the methanol triturated extract were conducted with *Thalassoma lunare* and *H. melanurus*. Ten treated pellets were offered to *T. lunare* and six to *H. melanurus*, and none of the pellets were consumed.

The crude extract was partitioned between hexanes and methanol, and those extracts were assayed against *T. lunare* at natural concentrations. The hexane extract was not deterrent, with eight of ten treated pellets consumed, while the methanol extract was found to be deterrent, with two of ten treated pellets consumed. The methanol partition was further partitioned with ethyl acetate and distilled water, and both of these partitions were found to be deterrent at natural concentrations, with zero and two of ten treated pellets consumed for the respective extracts.

Further fractionation of both the ethyl acetate and water partitions resulted in a loss of activity. Although the fractions and isolated compounds were not recombined and tested, it is possible that some or all of the compounds isolated from this gorgonian (and other minor metabolites not isolated) act in an additive or synergistic
manner when tested together (Chapter I). No reports to date have detailed any
ecologically relevant activity of 9,10-secosterols, but one study of the eunicellin 6(E)-
2α, 9α-epoxyeunicella-6,11(12)-dien-3β-ol (20) from a Brazilian gorgonian reported
that the compound inhibited fish feeding in field assays when tested at natural
concentration (Chapter I).\textsuperscript{17}
References


