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Natural Product Studies of Selected East Pacific Gorgonians

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NATURAL PRODUCT STUDIES OF SELECTED EAST PACIFIC GORGONIANS -

University of California, San Diego PH.D. 1982

UNIVERSITY OF CALIFORNIA

SAN DIEGO

Natural Product Studies of Selected East Pacific Gorgonians

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

by

Maury Melanie Bandurraga

Committee in charge:

Professor William H. Fenical, Co-Chair
Professor D. John Faulkner, Co-Chair
Professor Trevor C. McMorris
Professor William A. Newman
Professor George N. Somero

1982

The dissertation of Maury Melanie Bandurraga is approved, and it is acceptable in quality and form for publication on microfilm:

Co-Chair

Co-Chair

University of California, San Diego

1982

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VITA

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PUBLICATIONS

W. Fenical, R.K. Okuda, M.M. Bandurraga, P. Culver and R.S. Jacobs, "Lophotoxin: A Novel Neuromuscular Toxin from Pacific Sea Whips of the Genus Lophogorgia", Science 212, 1512 (1981).

R.R. Izac, M.M. Bandurraga, J.M. Wasylyk, F.W. Dunn and W. Fenical, "Germacrene Derivatives from Diverse Marine Soft-Corals (Octocorallia)", Tetrahedron 38(2), 301 (1982).

M.M. Bandurraga, B. McKittrick and W. Fenical, "Diketone Cembrenolides from the Pacific Gorgonian Lophogorgia alba", Tetrahedron 38(2), 305 (1982).

M.M. Bandurraga, W. Fenical, S.F. Donovan and J. Clardy, "Pseudopterolide: An Irregular Diterpene Lactone from a Caribbean Gorgonian", J. Am. Chem. Soc., in press (1982).

FIELDS OF STUDY

Major Field: Marine Natural Products Chemistry

Studies in Marine Natural Products Chemistry
Professor William H. Fenical
Professor D. John Faulkner

Studies in Natural Products Chemistry
Professor Trevor C. McMorris

Studies in Organic Synthesis and Mechanisms
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Studies in Marine Chemistry
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ABSTRACT OF THE DISSERTATION

Natural Product Studies of Selected East Pacific Gorgonians

by

Maury Melanie Bandurraga

Doctor of Philosophy in Oceanography

University of California, San Diego, 1982

Professor William H. Fenical, Co-Chair

Professor D. John Faulkner, Co-Chair

Gorgonian corals commonly occur in great numbers and diversity in the Caribbean, east Pacific and Indo-Pacific. Previous natural product investigations of Caribbean gorgonians have yielded a plethora of structurally and biologically unique compounds. These include diterpenes, sesquiterpenes, sterols and prostaglandins. Prior to this investigation, very little was known about the natural products composition of the east Pacific Gorgonacea. This dissertation reports the isolation and structure elucidation of twenty-seven natural products from fifteen species of east Pacific gorgonians. Twenty of the compounds described are new and seven were previously known from other marine and terrestrial sources. The gorgonians studied were collected off the coast of southern California and Pacific Mexico, and in the Gulf of California.

The structures of all of the compounds were elucidated by combined chemical and spectroscopic methods.

Examination of the extracts of four Lophogorgia species resulted in the isolation of a new neuromuscular toxin, lophotoxin, and six related furanocembrenolide compounds. Lophotoxin acts specifically and irreversibly at low concentrations to block indirect nerve stimulated muscle contraction. One of the Lophogorgia species studied, L. alba, also contained three new 1,4-diketone cembrenolides.

Eugorgia forreri, collected in the Gulf of California, possessed two cembrane-diterpenes which were previously isolated from a soft coral collected in Canton Atoll.

An investigation of the natural products composition of five Pacifigorgia species yielded seven sesquiterpene-derived metabolites. Six of the seven compounds possessed familiar germacrane, cadinane and guaiane ring systems. The remaining sesquiterpene contained a novel linear carbon skeleton.

Extracts of two Mexican Muricea species, M. fungifera and M. austera, contained three known germacrane derivatives. which had previously been isolated from a terrestrial plant.

A comparative natural products investigation of two local Muricea species yielded the known sterol, ergosterol peroxide, from both Muricea californica and Muricea fruticosa. In addition, only the less fouled Muricea fruticosa contained four new esterified aminosugar saponin derivatives. These compounds inhibit the growth of the marine

diatom, Phaeodactylum tricornutum, at concentrations comparable to those found in the gorgonian tissue. This result may indicate possible roles for these compounds in preferentially reducing fouling on the surfaces of Muricea fruticosa.

Chapter I

Introduction

Gorgonians are colonial, benthic invertebrates which belong to the phylum Cnidaria, class Anthozoa, subclass Octocorallia (Alcyonaria), order Gorgonacea (Figure 1).[1] Gorgonians are dominant members of shallow water communities in the tropical western Atlantic. They also exist in great numbers and diversity in tropical and subtropical regions of the east Pacific and Indo-Pacific. Each gorgonian colony consists of individual polyps connected by common tissue, the coenenchyme, surrounding a long, thin, axial endoskeleton (Figure 2).[2] The colonies grow in fan-, bush- or tree-like shapes. These shapes are the source of their common names, sea fans and sea whips. The body wall between individual polyps contains transport canals and calcareous spicules. The endoskeleton is comprised of either calcareous or wood-like collagenous material. Gorgonian reproduce by asexual budding as well as sexual reproduction. The latter includes the sequence polyp-egg-planula larva-polyp. Settlement of the larval stage is dependent upon finding an appropriate substrate--usually a hard surface on which to develop a holdfast. Gorgonians are commonly found in areas of strong currents or surge where the polyps have enhanced opportunities to filter feed.

The order Gorgonacea is comprised of two suborders: the Holaxonia and Scleraxonia (Figure 3). The Holaxonia endoskeleton consists of a woody, collagenous substance called gorgonin. Most of the common, shallow water species in the tropical western Atlantic and east Pacific

Figure 1. Taxonomy of the phylum Cnidaria[1,2]

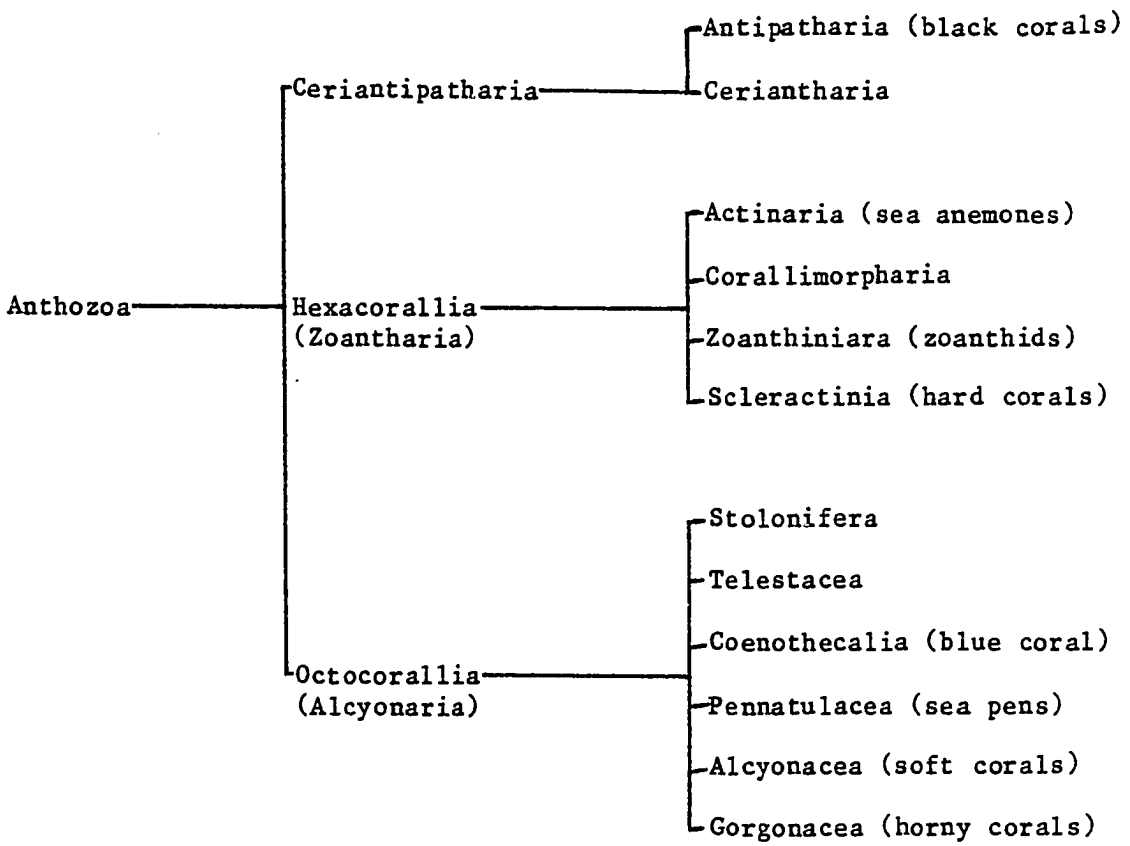
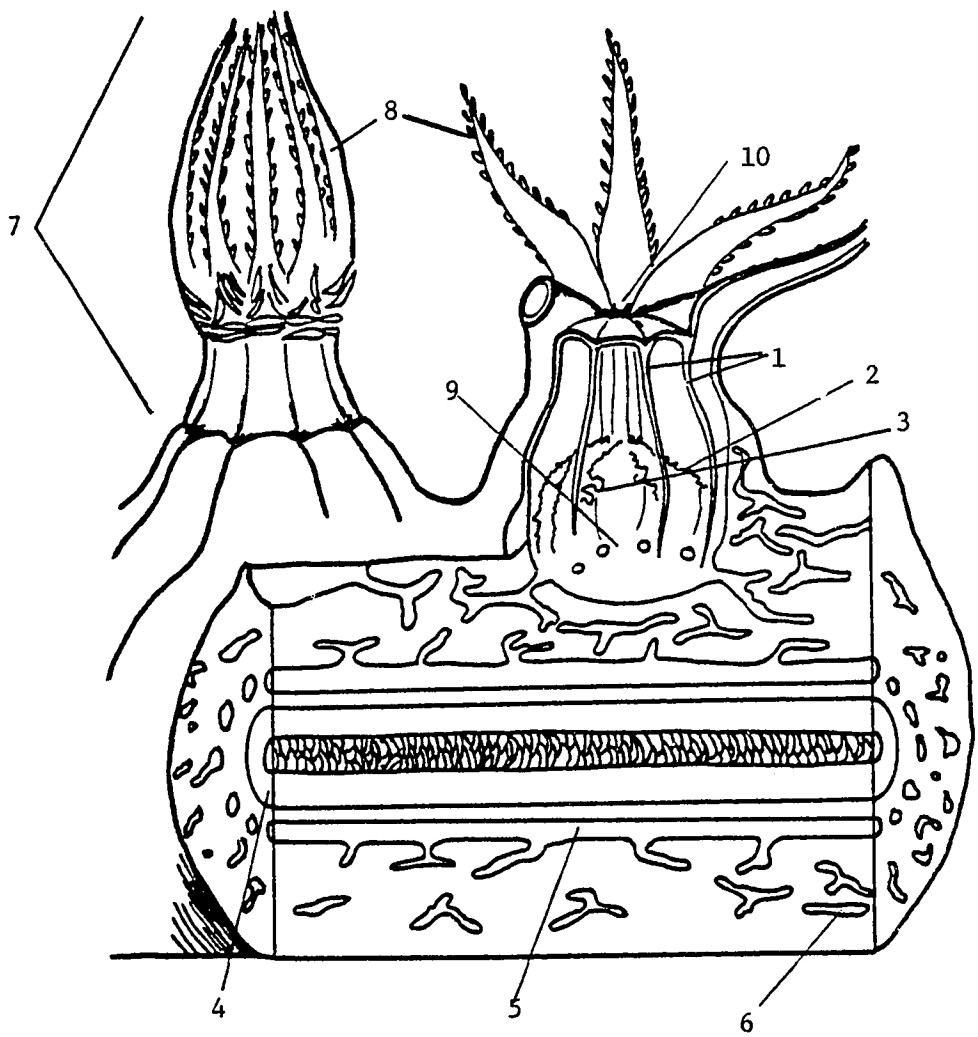


Figure 2. A generalized diagram of part of a gorgonian colony[3]

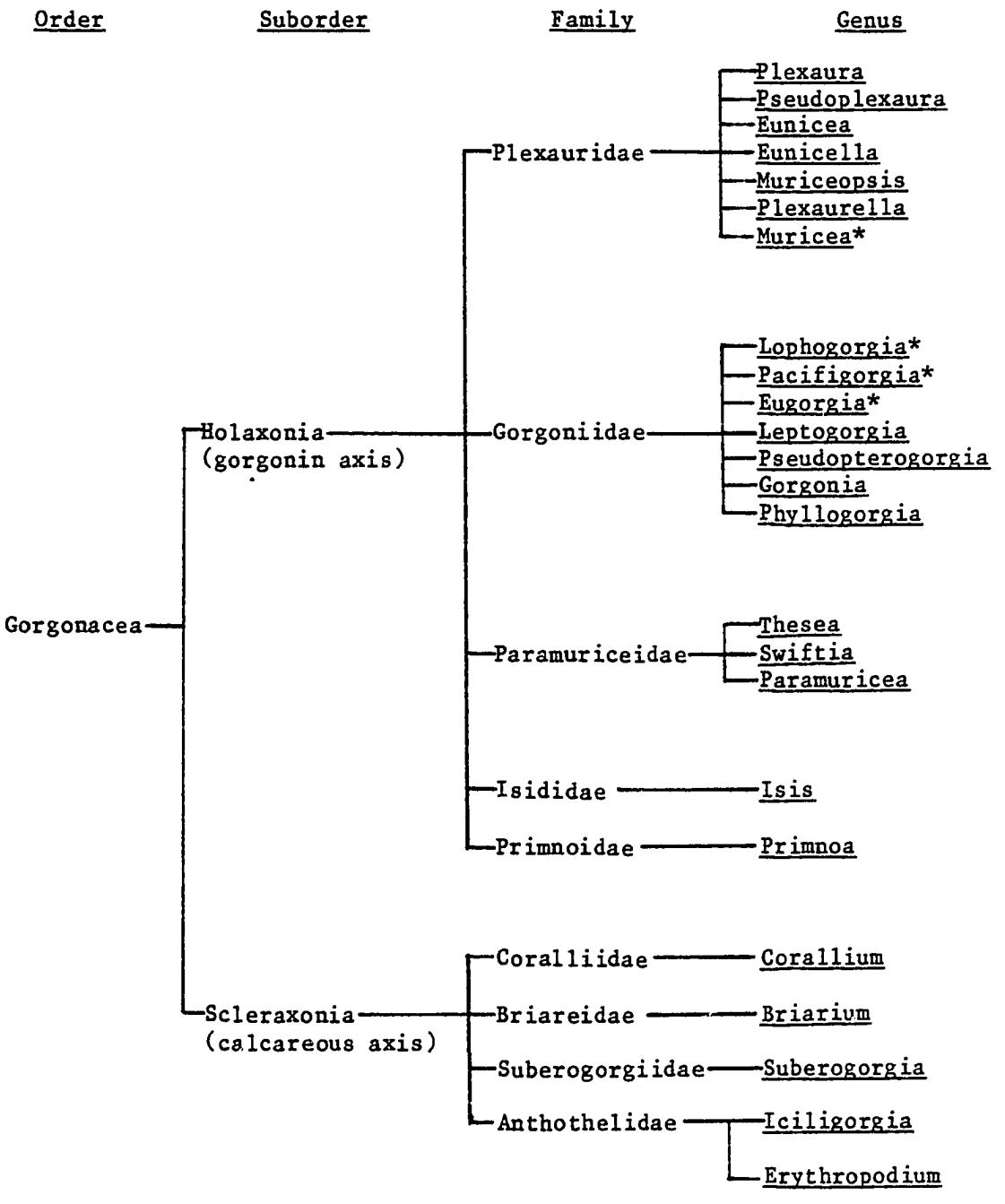


1, mesenteries; 2, mesenterial filament; 3, gonads; 4, axial skeleton; 5, stem canals; 6, solenia; 7, polyp; 8, tentacle; 9, coelenteron; 10, mouth

belong to the families Plexauridae and Gorgoniidae in the suborder Holaxonia. Gorgonians in the suborder Scleraxonia possess an endoskeleton made up of more calcareous, brittle material. This suborder includes many of the deep water and Indo-Pacific gorgonians, such as the deep water pink and red precious corals. Figure 3 summarizes current gorgonian taxonomy and indicates the gorgonian genera commonly found in the east Pacific.

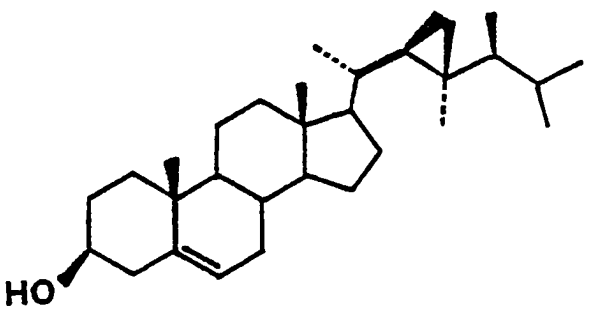
The natural products chemistry of gorgonian corals was first studied in 1896 by Dreschel, who isolated diiodotyrosine (iodogorgic acid) from Gorgonia carollino.[6] Two decades later, dibromotyrosine was isolated from another gorgonian, Primnoa laepadifera.[6] The isolation of gorgosterol (1) in 1943 from the Caribbean gorgonian Plexaura flexuosa[7], marks the beginning of a period of intensive study of the natural products chemistry of Caribbean gorgonians. The accessibility and abundance of shallow-water Caribbean gorgonians combined with the advent of modern tools of analytical organic chemistry have made this a productive area of research.[8,9] Natural product investigations of Caribbean gorgonians were stimulated by the discovery of many new compounds, some of which possess interesting biological activities (Figure 4). For example, the prostaglandins (2-3), isolated in the late 1960's from Plexaura homomalla,[10] are closely related to a number of pharmacolcgically important mammalian hormones. Caribbean gorgonians also produce cembrenoid diterpenes such as asperdiol (4)[11] and crassin acetate (5)[12] which possess antineoplastic activity. Pseudopterolide (6) typifies the kind of interesting chemical structures and biological activities possessed by Caribbean gorgonians. Pseudopterolide, isolated from Pseudopterogorgia

Figure 3. Taxonomy of the order Gorgonacea[1,5]

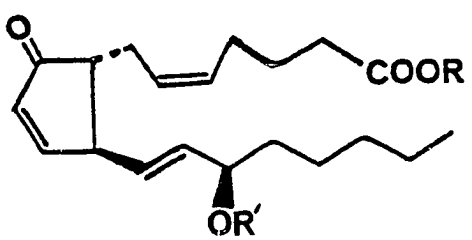


*Shallow water (>30 m) species commonly found in the east Pacific and studied during this research.

Figure 4. Examples of natural products isolated from Caribbean gorgonians[8,13]

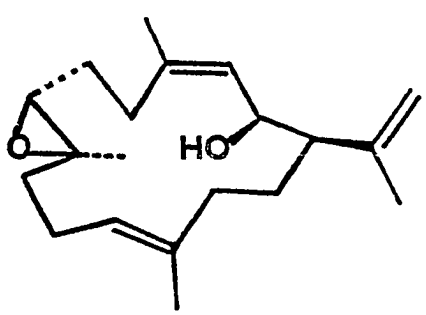


1 gorgosterol — Plexaura flexuosa[8]



2 R=R'= H

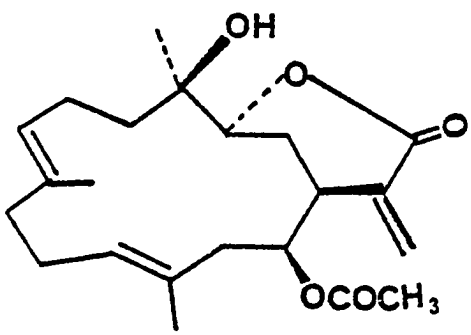
3 R= Me; R'= Ac — Plexaura homomalla[8]



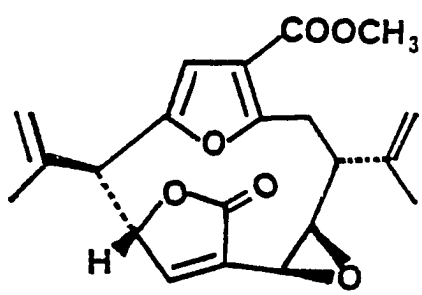
Eunicea tourneforti[13]

4 asperdiol — Eunicea asperula

Figure 4. continued



5 crassin acetate *Pseudoplexaura porosa* [13]



6 pseudopterolide *Pseudopterogorgia acerosa* [16]

acerosa, possesses a new, irregular diterpene ring system and inhibits the cell division of fertilized sea urchin eggs.[16]

Natural product studies of Caribbean gorgonians to date have yielded more than fifty new sesquiterpenoid and diterpenoid compounds.[13,15] This is in addition to a number of new sterols[14] and fatty-acid derived compounds.[8,9] Despite the plethora of interesting metabolites isolated from Caribbean gorgonians, investigations of gorgonians in other areas of the world have been almost nonexistent until very recently. Figures 6-11 illustrate compounds isolated from gorgonians outside of the Caribbean region. The majority of these studies were published after this work began.

Figures 5 and 6 summarize the sesquiterpene-derived compounds found recently in gorgonians from Turkey, Japan, the Red Sea and the East Pacific. Linderazulene (7)[17], guaiazulene (8),[18] and furanodiene (12),[20] have previously been isolated from terrestrial plants or fungi. Pacifigorgiol (13), an ichthyotoxin (LD_{100} = 1 ppm), possesses an irregular terpenoid skeleton.[21]

Figure 7 illustrates the diterpene metabolites isolated from the deep-water precious pink coral, Corallium sp., from Hawaii. These compounds are closely structurally related to xenicin from the soft coral Xenia elongata.[23]

Figure 8 lists fatty acid-derived compounds from related plexaurid species, and Figure 9 contains compounds of mixed biogenesis from the same source. Figure 10 relates some new, and Figure 11 shows some known sterols from several species of east Pacific gorgonians. These

Figure 5. Examples of sesquiterpenes isolated recently from non-Caribbean gorgonians

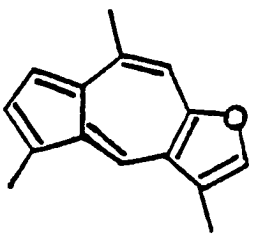
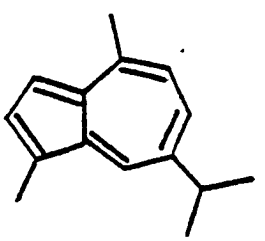
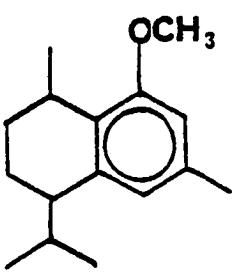
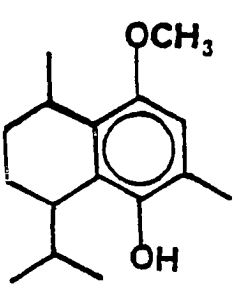
		Gorgonian	Collecting Site
			
7	linderazulene	Paramuricea chamaelon	Turkey[17]
			
8	guaiazulene	Euplexaura erecta	Japan[18]
			
9		Suberogorgia hicksoni	Red Sea[19]
			
10		Suberogorgia hicksoni	Red Sea[19]

Figure 6. Natural products previously isolated from east Pacific gorgonians

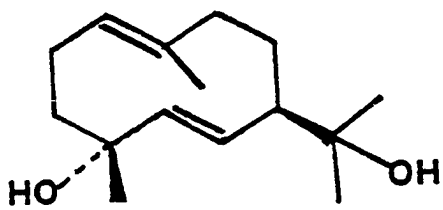
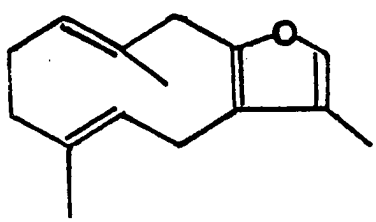
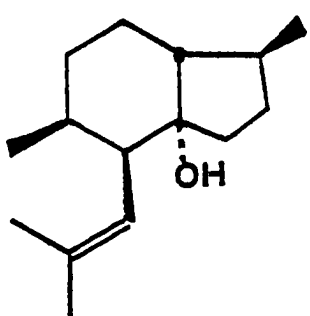
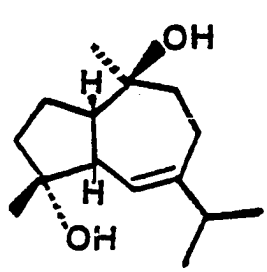
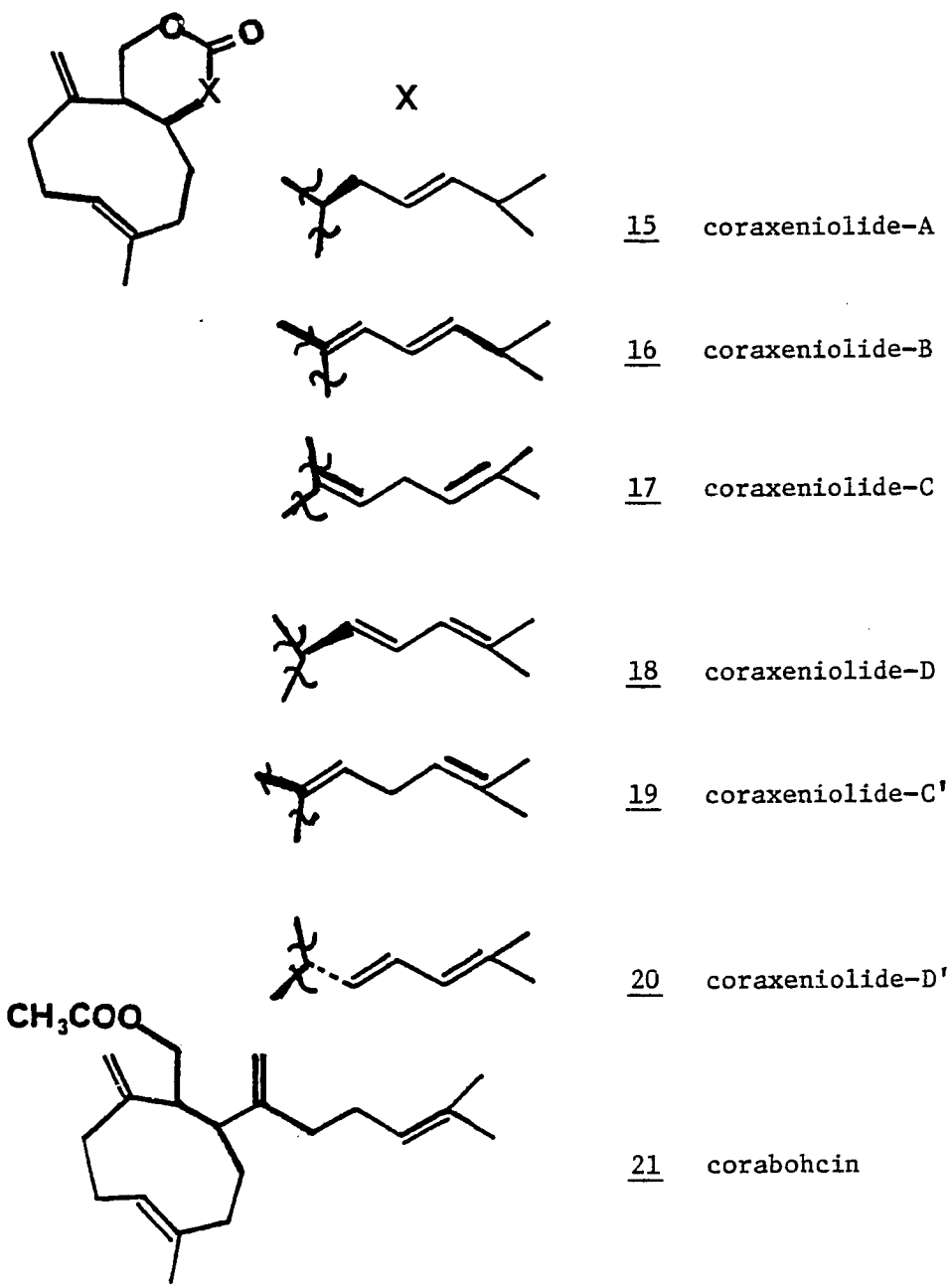
		Gorgonian	Collecting Site
			
11		Pacifigorgia media	Los Frailes[20]
			
12	furanodiene	P. media	Los Frailes[20]
			
13	pacifigorgiol	P. adamsii	Los Frailes[21]
			
14		P. eximia	La Paz[22]

Figure 7. Diterpenes isolated from a Hawaiian Corallium sp.[24]



15 coraxeniolide-A

16 coraxeniolide-B

17 coraxeniolide-C

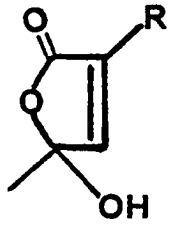
18 coraxeniolide-D

19 coraxeniolide-C'

20 coraxeniolide-D'

21 corabohcin

Figure 8. Fatty acid derived natural products from Pacific gorgonians



Gorgonian	Collecting Site
Euplexaura flava	Okinawa[25]

R

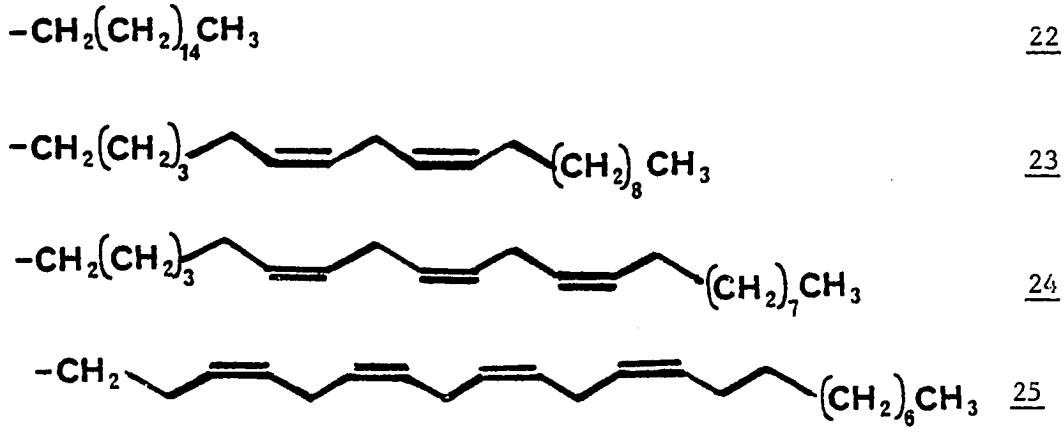
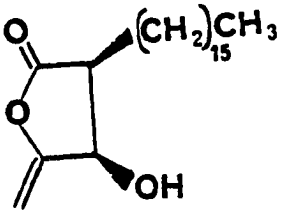


Figure 8. continued

Plexaura flava Australia[26]



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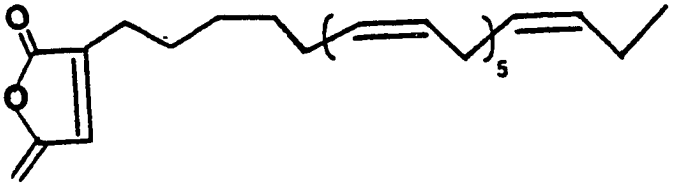

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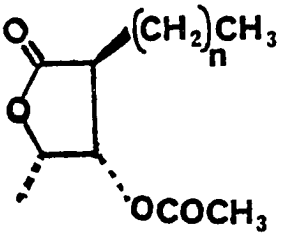
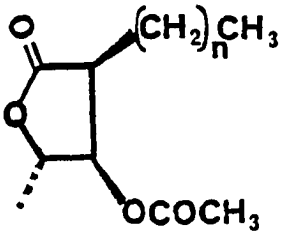


28 n= 15

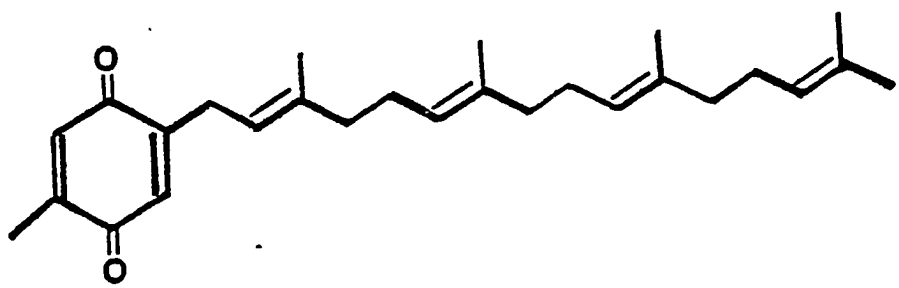
29 n= 13



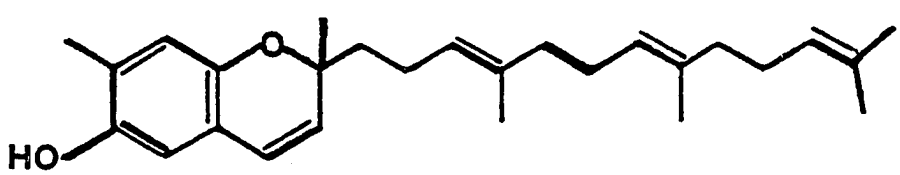
30 n= 15

31 n= 13

Figure 9. Natural products of mixed biogenesis from the Australian gorgonian Plexaura flava[26]

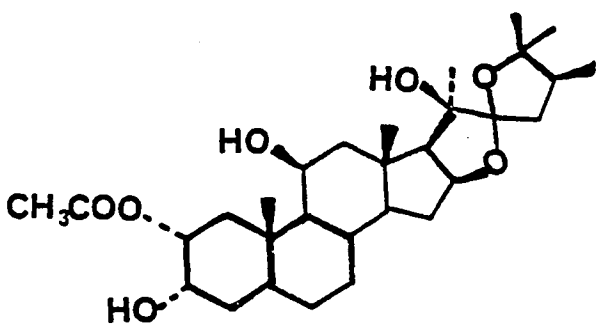


32



33

Figure 10. Sterols isolated from the Pacific gorgonian Isis hippuris[27]



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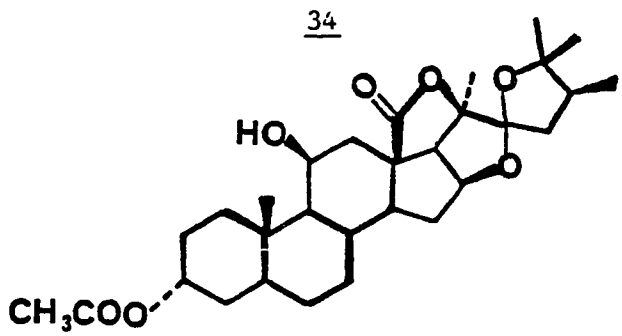

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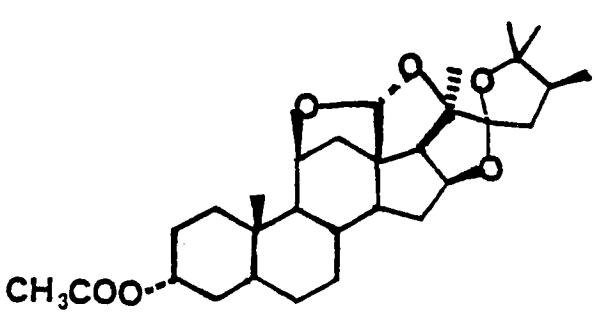

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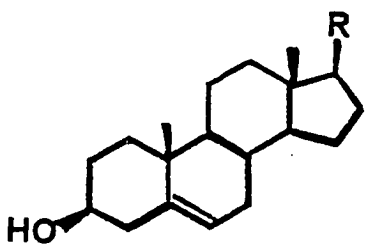
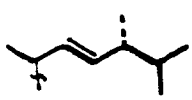
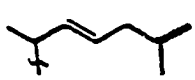
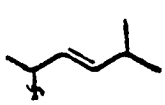
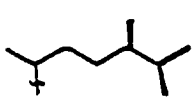
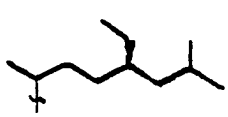
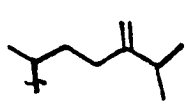
Figure 11. Sterols from two east Pacific gorgonians: Muricea californica (M)[28] and Eugorgia ampla (E)[29]



	Compound	Source
	37 cholesterol	M,E
	38 brassicasterol	M,E
	39 Δ^{22}- cholesterol	M,E
	40 Δ^{22}- norcholesterol	M,E
	41 24- methyl-cholesterol	M,E
	42 β-sitosterol	M,E
	43 24-methylene cholesterol	E
	44 pregna-5,20- diene- 3β- ol	M

known sterols are ubiquitous in marine organisms and their discussion is beyond the scope of this study. They have been the subject of excellent reviews by Goad (1978)[14] and Baker (1976)[9].

This thesis describes an investigation of the natural products chemistry of gorgonians collected in tropical and subtropical areas of the east Pacific. This region was chosen as a study site for a number of reasons. First and foremost was the opportunity to tap a previously unexplored resource for new and interesting natural products chemistry. The extent of this resource is reflected in Figure 12, which compares the number of endemic species in several families of gorgonians common to the east Pacific. Figure 13 also illustrates the abundance of species in several genera of east Pacific gorgonians.[5] Prior to this study very little was known about the natural products chemistry of any of the species listed in Figure 13. Only four bisabolane sesquiterpenes were known from the related Caribbean gorgonian Muricea elongata (Figure 14). The only other chemical studies of these east Pacific genera dealt with their pigments,[31,32] endoskeleton composition,[33,34] and lack of prostaglandin synthetase activity.[35] The precedent of chemically- and biologically-exciting chemistry from Caribbean gorgonians combined with the lack of previous exploration of the east Pacific Gorgonacea provided the impetus for this exploration.

Collections of east Pacific gorgonians were easily made by hand (using SCUBA) in and around San Diego and the Channel Islands. Collecting trips were also made annually to sites in the Gulf of California (Loreto, Los Frailes and Cabo San Lucas). Two cruises, on the RV Alpha Helix (in 1978) and the RV Fisherette (in 1980), provided the

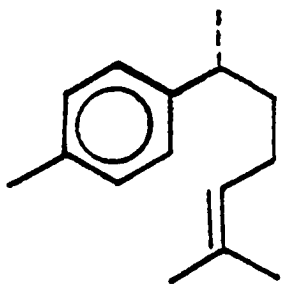
Figure 12. Geographical distributions of gorgonian species in the families Gorgoniidae and Plexauridae[1]

	Number of Species	
	Gorgoniidae	Plexauridae
Endemic Western Atlantic	34	35
Eastern Atlantic	9	5
Mediterranean	1	2
Indo-West Pacific	6	46
Endemic East Pacific	32	14

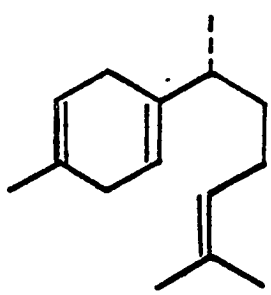
Figure 13. Comparison of the occurrence of some Holaxonian genera in the east Pacific and tropical western Atlantic[1,5]

	# Species Found in:	
Genera	East Pacific	Tropical Western Atlantic
Muricea	34	6
Pacifigorgia	19	1
Lophogorgia	30	9
Eugorgia	15	0

Figure 14. Sesquiterpenes from the Caribbean gorgonian *Muricea elongata*[9,13]



(-) α- curcumene



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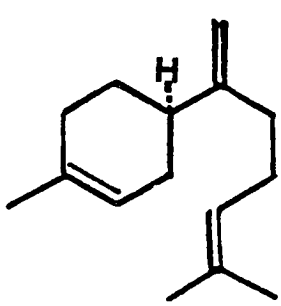
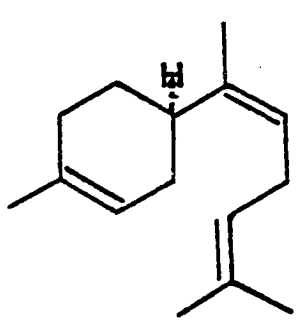


(+) β- bisabolene



(+) α- bisabolene

opportunity to sample the gorgonian fauna of the offshore and oceanic Mexican islands. More than fifty individual collections of many different species of gorgonians were made over a four year period (1978-1982) at the east Pacific collecting sites indicated in Figure 15.

This investigation has resulted in the isolation and structure elucidation of twenty seven compounds from fifteen species of east Pacific gorgonians. Twenty of these compounds are new and seven were previously isolated from other marine or terrestrial sources. Figure 16 lists the natural products discovered in the east Pacific Gorgonacea as a result of this work.

These gorgonian natural products were isolated by extraction of the whole animal with organic solvents, followed by separation of the extracts using a variety of chromatographic techniques. Each compound was purified by silica or reverse phase high performance liquid chromatography (hplc) or by recrystallization. The structures were elucidated using a combination of chemical and spectroscopic methods. These methods included Fourier transform ^{13}C and ^{1}H nuclear magnetic resonance (NMR), infrared (IR), and ultraviolet (UV) spectroscopy, in addition to low and high resolution mass spectrometry (HRMS). Synthetic derivatives of the natural products were made, when necessary, in order to gain information about specific functional groups in the molecule. The structural assignments were confirmed by either X-ray crystallography, comparison of the spectral data with that of known compounds, or synthetic interconversion with rigorously defined compounds.

Chapters two through seven describe the isolation and structure

Figure 15. East Pacific collecting sites

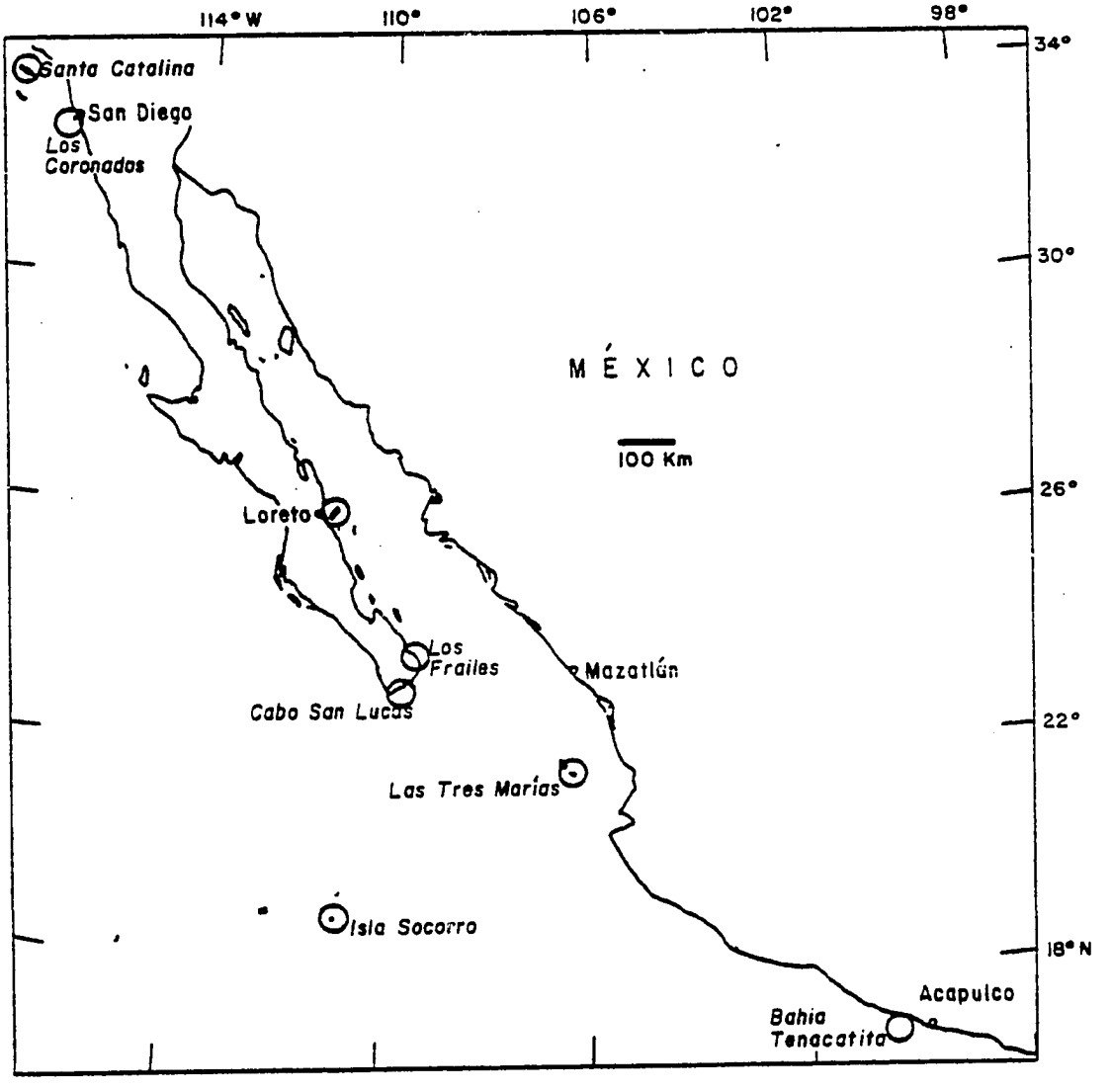
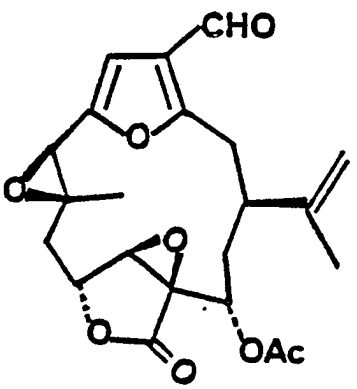


Figure 16. Natural products isolated from my investigation of east Pacific gorgonians



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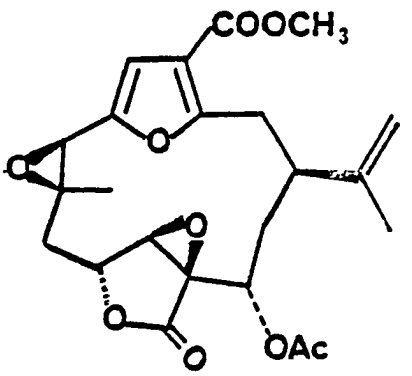

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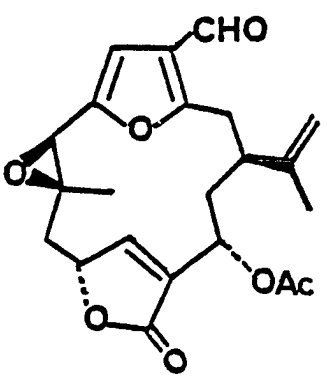

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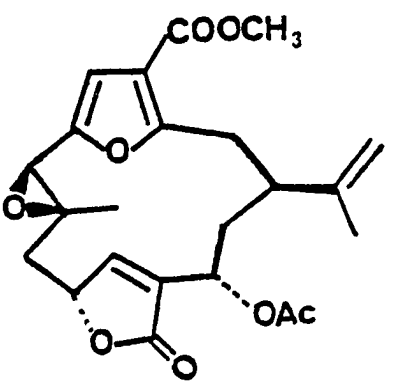

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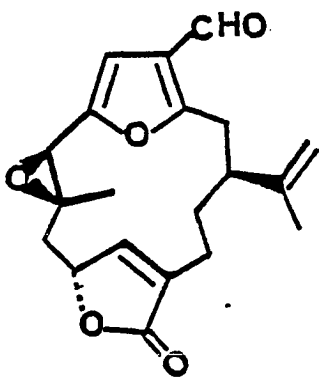

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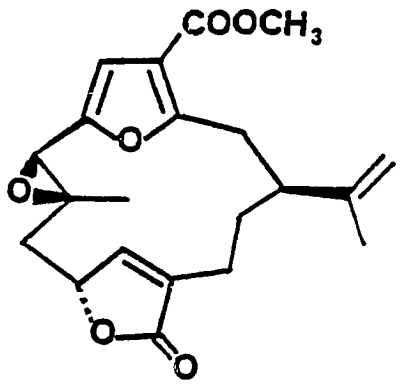

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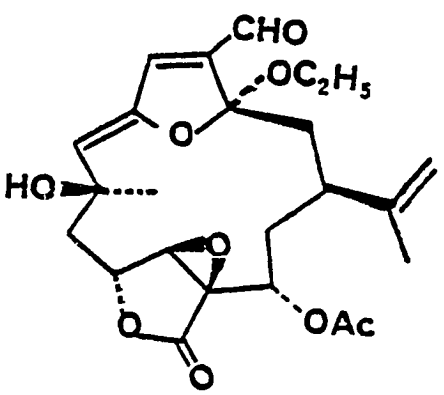
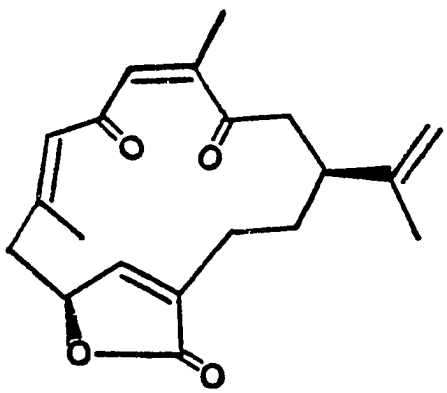


51

* previously isolated from another source

Figure 16. continued



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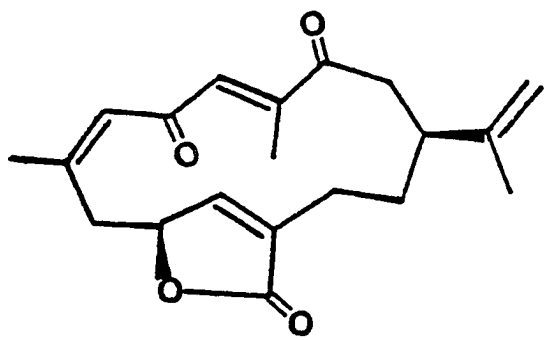

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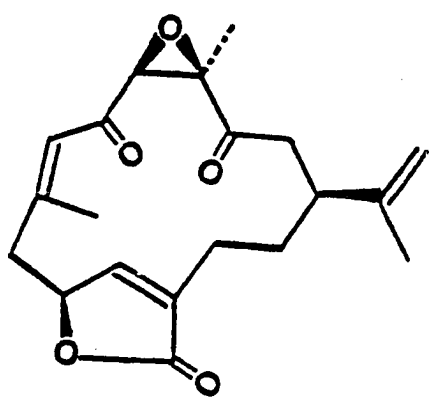

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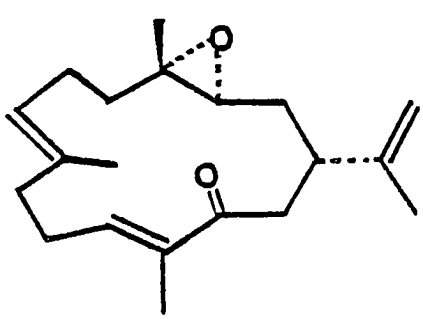

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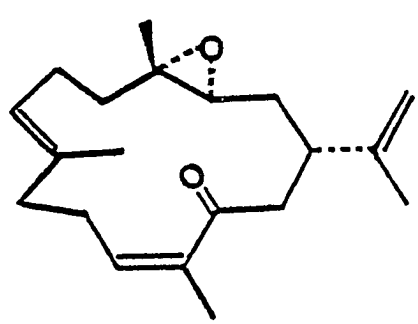

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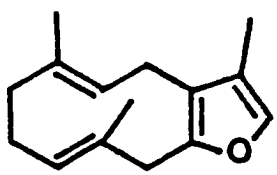

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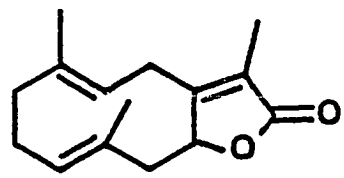

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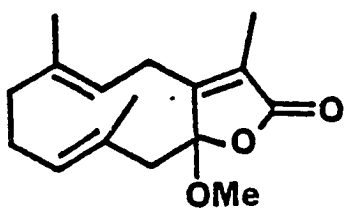

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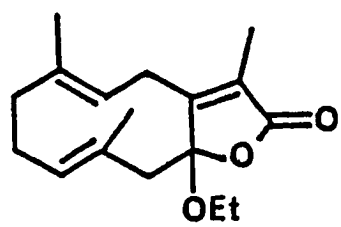

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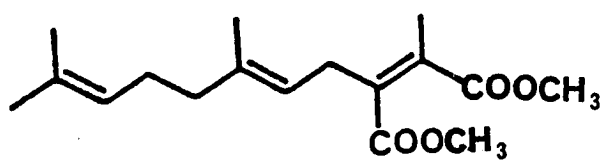

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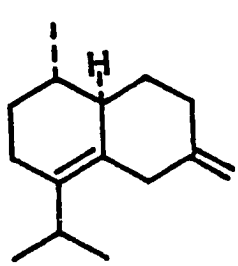

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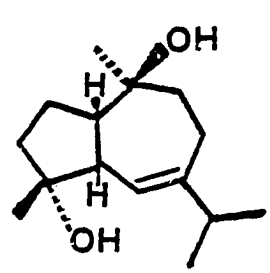

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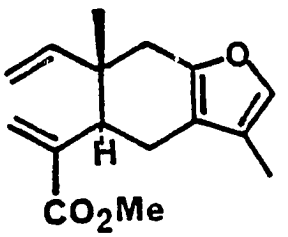

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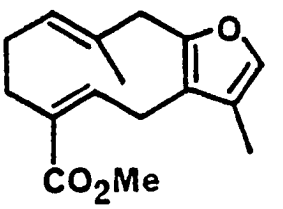

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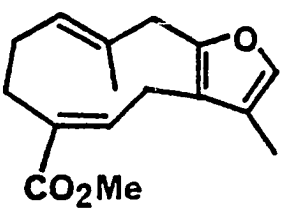

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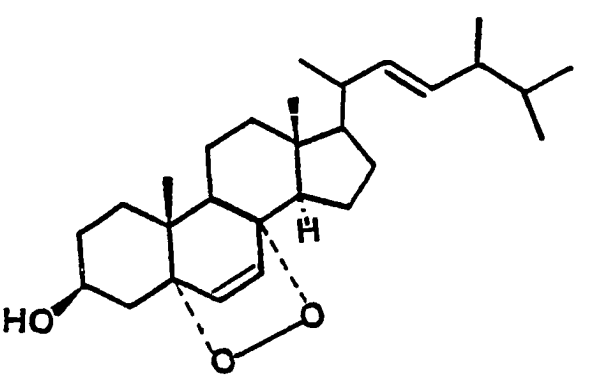

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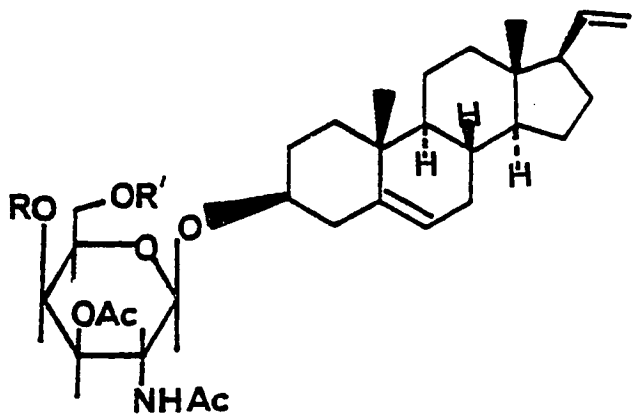

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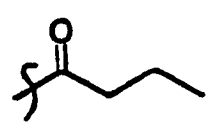
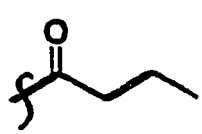
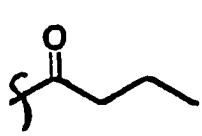
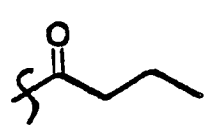
68 =	Ac	
69 =		Ac
70 =		

elucidation of natural products from east Pacific gorgonians using the techniques described above.

Chapter II describes the isolation and structure elucidation of seven furan-derived cembrenolides, 45-51, from four Lophogorgia species collected over a broad geographic range. One of these compounds, lophotoxin (45), is a new and potent neuromuscular toxin with unique pharmacological properties.

Chapter III reports the isolation of three new 1,4-diketone cembrenolides, 52-54, from one of the Lophogorgia species studied in Chapter II, L. alba.

Chapter IV reports the isolation of two cembrenoid diterpenes, 55 and 56, which were previously isolated from a Pacific soft coral.

An investigation of the natural products chemistry of six Pacifigorgia species resulted in the isolation of seven compounds with four different sesquiterpene ring systems. Chapter V describes the structure elucidation of four germacrenes (12 and 57-59), a rearranged linear sesquiterpene (60), a cadanane-derived compound (61), and a guaiane-diol (62).

Chapter VI reports the isolation of three additional sesquiterpene compounds, 63-65, from two Muricea species. These compounds have been previously isolated from a terrestrial plant.

Chapter VII gives the results of a comparative investigation of two local Muricea species: Muricea californica and M. fruticosa. This study resulted in the isolation of a known sterol, ergosterol peroxide

(66) from both gorgonians. Four new amino sugar saponins (67-70) were also isolated from only Muricea fruticosa, and this represents the first isolation of an amino sugar- or saponin-derived compound from a gorgonian. Therefore, this chemical investigation of the east Pacific Gorgonacea was extremely productive in terms of the numbers of compounds isolated and the different types of new chemistry discovered.

This chemical investigation has also provided an opportunity to explore several biological issues related to the east Pacific Gorgonacea. These issues include 1) the role of zooxanthellae in the biogenesis of terpenes in gorgonians, 2) the taxonomy of east Pacific gorgonians, and 3) the possible roles of gorgonian natural products in the marine environment.

1) Many gorgonians contain symbiotic algae within their tissues. These endosymbionts, called zooxanthellae, are also found in the tissues of the hard and soft corals. Zooxanthellae are unicellular algae (dinoflagellates) that exist in densities of approximately 30,000 cells/mm^3 in the gorgonian mesoglea and polyps. They contribute to the symbiosis with their host by utilizing gorgonian respiratory waste products and providing primary metabolites such as amino acids, sugars, sterols and fatty acids.[36] The role of zooxanthellae in the biogenesis of terpene-derived compounds in gorgonians and other soft corals was a subject for debate then study over the last several years.[13,15] All of the Caribbean shallow-water gorgonians examined to date for natural products chemistry have possessed zooxanthellae.

In sharp contrast to their Caribbean relatives, none of the east

Pacific gorgonians collected and studied in the course of this work contained zooxanthellae.[37] This study has shown that east Pacific gorgonians lacking zooxanthellae produce diverse and fascinating terpenoid compounds. Therefore gorgonians are capable of the de novo biosynthesis of compounds derived via the mevalonic acid pathway. This conclusion does not preclude the involvement of zooxanthellae in the production of natural products in gorgonians which contain the endosymbionts.

2) The taxonomy of many species of East Pacific gorgonians is currently confused and under review.[1,5,38,39] Most of the confusion is due to the presence of closely related morphological forms (color, size, and shape) which are difficult to distinguish by the traditional taxonomic criterion: comparison of the size and shape of the calcareous spicules embedded in the gorgonian tissue. The ability of a gorgonian species to produce unique natural products may provide a useful addition to the features which form the basis of gorgonian taxonomy. The results of my study support this proposal. Of the fifteen gorgonian species chosen for study, members in the genera Lophogorgia and Eugorgia were found to produce only diterpenes. In contrast, members of the genera Pacifigorgia and Muricea were found to produce sesquiterpenoids. These chemical relationships are summarized in Figure 17 along with the dates and locations of collection of each gorgonian species.

3) The last major biological issue pertinent to this study is that of the role of natural products in gorgonian adaptation. Secondary metabolites are energetically "expensive" to produce, and natural product chemists and ecologists have tried to examine how this cost can be outweighed by the benefic they yield to the producing organism. The

Figure 17. Natural products chemistry from east Pacific gorgonians[39]

I. Diterpenes

Gorgonian	Collection Site	Date	Compounds
Lophogorgia alba (Duchaissang and Michelotti)	Islas Tres Marias Bahia Tenacatita	6/8 6/78	45-54
L. chilensis (Verrill) (cf. L. panamensis (Duch & Mich)	Catalina La Jolla Los Coronados	2/79 6/79 6/80	45,47,51
L. cuspidata (Verrill)	Bahia Tenacatita Cabo San Lucas	6/78 12/78	45-51
L. rigida (Verrill)	Islas Tres Marias Los Frailes Los Frailes	6/78 12/78 12/79	45-51
Eugorgia forerri (Studer)	Los Frailes	12/79	55,56

II. Sesquiterpenes

Pacifigorgia pulchra exilis (Verrill)	Los Frailes	12/79	12,58-61
P. sp. B	Loreto	12/81	62
P. sp. A	Loreto	12/80	61
P. media (Verrill)	Los Frailes Islas Tres Marias	12/78 6/78	12 57,58,61
P. tenuis (Verrill)	Los Frailes	12/78	58
P. florae (Verrill)	Los Frailes	12/78	12
Muricea austera (Verrill)	Isla Socorro Bahia Tenacatita	6/78 6/78	63-65
M. fungifera (Valenciennes)	Los Frailes	12/79	63-65

III. Sterol derived chemistry

Muricea fruticosa (Verrill)	La Jolla	1/79	66-70
	Catalina	2/79	
	Catalina	4/79	
	Los Coronados	6/80	
Muricea californica (M. aurivillius)	La Jolla	1/79	66
	Catalina	2/79	
	Catalina	4/79	
	Los Coronados	6/80	

concept of chemical defense is well known in terrestrial plants. The presence of secondary metabolites helps the plants to respond to the pressure of enchroachment by pathogenic or herbivorous organisms.[40] These mechanisms are also known in insects and in marine molluscs. Some marine molluscs concentrate toxic sponge metabolites ingested as a result of their diet. This renders their tissues either unpalatable or toxic to fish.[41,42]

Secondary metabolites are also known to play a role in chemical communication within and between species. Interspecies interactions can be characterized by the metabolite giving an adaptive advantage to either the producing organism (allomone) or the receiving organism (kairomone).[43] For example, colonies of Millepora species (fire coral) are able to actively detect the presence of nearby gorgonians and redirect their growth to contact and overgrow them.[44] This is true only in the case of living gorgonians, and depends on the direction of water flow. Gorgonians may be producing kairomone type substances that the Millepora is able to detect. An example of an organism using allomones to provide a selective advantage is the already mentioned ability of nudibranchs to sequester dietary sponge metabolites to defend against predation.[41,42]

Pacific gorgonians are large, sessile organisms which live in a nutrient rich, highly competitive environment. This renders them susceptible to a variety of predators and fouling organisms. The noticeable lack of predation and fouling observed on most healthy gorgonians leads to the hypothesis that natural products produced by these organisms may be utilized in defensive strategies. Specific ecological

observations on the lack of predation and fouling by epibionts in a pair of local gorgonians, Muricea fruticosa and M. californica, stimulated my interest in the chemical study of these east Pacific gorgonians. Although the two gorgonians are morphologically very similar and grow side-by-side in the same habitat, ecologists noted that Muricea californica is fouled by a variety of epibionts much more frequently than M. fruticosa.[45] This leads to some specific questions which part of this thesis addresses:

(1) Is there a chemical difference between M. californica and M. fruticosa in terms of their natural products chemistry?

(2) If a difference exists, does it contribute to the lack of fouling in Muricea fruticosa by acting as an allomone?

In order to help answer these questions and to explore the role of these compounds in nature, all of the compounds isolated as a result of this thesis research were tested in a series of simple bioassays. Chapter eight reports the results of four biological assays which were selected to help indicate possible functions of gorgonian natural products in nature. All of the compounds were tested for inhibition of cell division of fertilized sea urchin eggs. Assays testing fish toxicity, inhibition of algal growth and antibacterial activity were also performed.

Chapter II

Furanocembrenolides with Neuromuscular Activity from Four Species of Lophogorgia

Caribbean gorgonians and Indo-Pacific soft corals have been the subject of intensive natural products investigations over the last twenty years. This effort has resulted in the isolation of many unique cembrenolides with potent biological activity.[16,46]

My studies of east Pacific gorgonians of the genus Lophogorgia have resulted in the isolation and structure elucidation of seven unique furanocembrenolide derivatives (45-51) which also possess potent biological activities. The major metabolite, lophotoxin (45), is an irreversible neuromuscular toxin comparable in activity and mode of action to the snake venom toxin, α-bungarotoxin.[47,48] The six other metabolites, 46-51, possess varying levels of neuromuscular activity. These compounds were isolated from four different Lophogorgia species collected in San Diego, the Gulf of California and Pacific Mexico. The collecting sites and distribution of secondary metabolites for each species studied are summarized in Figure 18.

The research described in this chapter was the result of a collaborative effort which involved several individuals.[47] An undergraduate at UCSD, Roy Okuda, under the supervision of Dr. William Fenical, performed the initial extraction and isolation of furanocembrenolides from Lophogorgia species collected in 1978 in Pacific Mexico. I isolated

Figure 18

Distribution of natural products from *Lophogorgia* spp.

Lophogorgia spp. Collection location and code	45 LTX	46 Lopholide	47 Deoxy LTX	48 Acetoxypukalide	49 Pukalide aldehyde	50 Pukalide	51 RAL	52 Lophodione	53 Isolophodione	54 Epoxylophodione
L. chilensis (L-2 = La Jolla)							X			
L. chilensis (L-6 = Los Coronados)	X		X							
L. rigida (V-5 = Los Frailes)	X	X	X	X	X					
L. rigida (AH60 = Tres Marias)	X	X	X	X	X	X	X			
L. alba (AH56 = Tres Marias)	X		X				X			
L. alba (AH74 = Bahia Tenacatita)	X						X	X	X	X
L. cuspidata (AH82 = Bahia Tenacatita)	X	X	X							
L. cuspidata (V-7 = Los Frailes)	X	X	X							

these same compounds from several collections of Lophogorgia species made locally and in the Gulf of California. I was responsible for the spectroscopic and stereochemical analyses and synthetic interconversions which resulted in the complete structure elucidation of all seven compounds. As a result of our participation in the Sea Grant Marine Pharmacology Program, Dr. Paul Culver and Professor Robert S. Jacobs at the University of California, Santa Barbara, provided studies of the neuromuscular activity of lophotoxin.[48]

This chapter describes the isolation and structure elucidation of lophotoxin and six other related compounds using spectroscopic methods and synthetic interconversion techniques. Spectral data for each of the natural products are summarized in tables and in the experimental sections located at the end of the chapter.

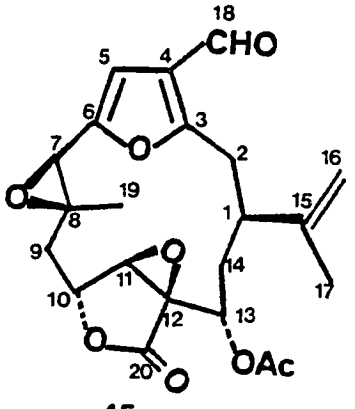
A. Isolation and partial structure elucidation of lophotoxin

Extracts of Lophogorgia rigida, L alba, L. cuspidata and L. chilensis were quickly chromatographed over silica gel and Florisil to give mixtures of seven new compounds in varying amounts (Figure 18). High performance liquid chromatography (hplc) of the mixtures using 60% ethyl acetate in isooctane on a μ-Porasil column yielded lophotoxin as the major component of the extracts. Lophotoxin (45) was isolated as white needles that crystallized from the hplc fractions, m.p. 164-166°C. Complete spectroscopic data for lophotoxin appear in Table 1.

A molecular formula of $C_{22}H_{24}O_8$ was established for lophotoxin by HRMS (M^+ obs. 416.1472, calc. 416.1471) This molecular formula indicated eleven degrees of unsaturation. Six unsaturations were

Table 1

Spectral Data for Lophotoxin (45)



45

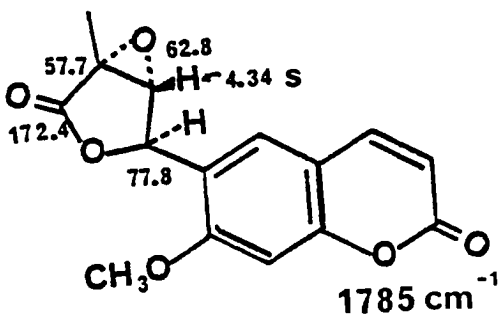
$C_{22}H_{24}O_8$, needles, m.p. 164-166°C; $[\alpha]_D^{27}$ = +14.2° (c = 1.7,$CHCl_3$); UV: λ_{max}^{MeOH} = 265 (1000), 208 (2000) nm; IR ($CHCl_3$) 3030, 2850, 1792, 1739, 1683, 1572, 1385, 1087, 976, 896 cm^{-1}

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	3.90 ddd (11,11,4)	36.5 (J_R = 16.4)
2	α 3.07 dd (-17,4), β 2.95 dd (-17,11)	32.9 (16.6)
3	--	161.8
4	--	123.0
5	6.57 bs	105.6 (31.6)
6	--	149.8+
7	4.09 bs	55.3 (26.1)
8	--	56.1
9	β 2.49 dd (-16,4), α 2.05 dd (-16,3)	39.0 (15.4)
10	4.81 dd (4,3)	76.7 (23.4)
11	4.17 s	64.2 (28.1)
12	--	61.3
13	4.99 d (7)	70.3 (22.2)
14	β 2.50 ddd (-14,11,7), α 1.70 d (-14)	31.7 (14.3)
15	--	148.8+
16	4.93 bs, 4.91 bs	110.9 (23.9)
17	1.90 bs	21.1 (14.4)
18	9.87 s	184.4 (38.0)
19	1.14 s	20.2 (12.4)
20	--	170.1
CH_3COO-	2.05s	168.1, 20.4 (14.9)

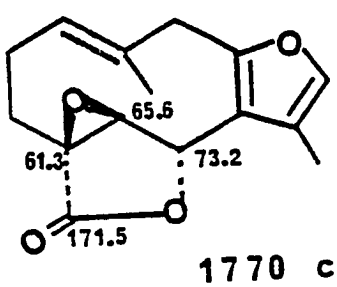
+may be interchanged

immediately accounted for by signals for three carbonyl and six olefinic carbons in the ^{13}C NMR spectrum, leaving five ring systems to be accounted for. Infrared absorptions at 2850 and 1689 cm^{-1}, coupled with ^{13}C NMR bands at 184.5 (d), 161.8 (s), 149.8 (s), 123.0 (s) and 105.6 (d) ppm, and a ^{1}H NMR signal at δ 6.57 (1 H, bs), allowed the formulation of an α,α-disubstituted furan-β-aldehyde group. An additional IR absorption at 1792 cm^{-1} initially suggested the presence of a saturated γ-lactone functionality; however, due to the presence of two trisubstituted epoxides in the ^{13}C NMR data (64.1 (d), 61.3 (s), 56.1 (s) and 55.3 (d) ppm), the γ-lactone was revised to an α,β-epoxy-γ-lactone group. Several epoxy-lactone model compounds such as micromelin (71)[51] and linderane (72)[52] (Figure 19) possessed similar carbonyl absorptions at 1785 and 1770 cm^{-1}. A further infrared absorption at 1735 cm^{-1}, along with a 3 proton singlet in the ^{1}H NMR at δ 2.05, confirmed the presence of an acetate ester. A normal diterpene ring system was suggested by the acetate ester and the presence of five methyl equivalents in the ^{1}H and ^{13}C NMR spectra. The two trisubstituted epoxides, γ-lactone, furan and diterpene ring made up the five remaining degrees of unsaturation calculated earlier from the molecular formula. The problem at this point consisted of the placement of these functional groups in a ring system and the structure proof of that assignment. This task was aided by the fortuitous isolation of pukalide (50) and five other derivatives of lophotoxin from several collections of *Lophogorgia*.

Figure 19. Model compounds containing an epoxy- lactone functionality



71 micromelin[51]



72 linderane[52]

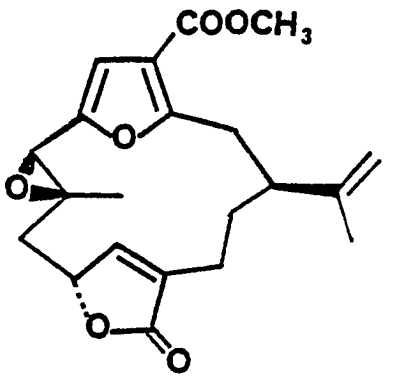
B. Isolation of pukalide and comparison with lophotoxin

Along with lophotoxin we were fortunate to isolate a related furanocembrenolide, pukalide. Pukalide (50) was previously isolated by Scheuer and coworkers from the Hawaiian soft coral Sinularia abrupta.[49] Pukalide has also been isolated recently by Fenical from an unknown Sinularia species (PO-226) from Ponape.[53] Pukalide was isolated in minor amounts from one collection of Lophogorgia rigida (from Islas Tres Marias) and knowledge of this structure was useful in providing a number of clues for the structure elucidation of lophotoxin. Table 2 gives the complete spectral data for pukalide. The optical rotation of 50 ($[\alpha]_D^{27}$ = +26.5° (c = 1.0, $CHCl_3$)), and all other spectral data, were comparable to those of the natural product previously reported ($[\alpha]_D$ = +44° (c = 1.1, $CHCl_3$)).[49] An unpublished x-ray study of pukalide (completed after publication) allowed us to completely assign its relative stereochemistry as shown (50).[54]

In analogy to pukalide, the spectral features of lophotoxin (Table 2) contained the elements of an isopropenyl group, a trisubstituted epoxide, an α,α-dialkyl-β-substituted furan and a γ-lactone group. Lophotoxin differed from pukalide in possessing an epoxy-lactone, a furanoaldehyde and an acetoxyl group. The gross structure of lophotoxin was assigned as 45 based on spectral comparisons between lophotoxin and pukalide (Tables 1 and 2), and on ^1H NMR decoupling experiments. The acetoxyl group in lophotoxin was placed adjacent to the lactone ring. This assignment was based on the downfield shift of the acetoxyl methine upon deoxygenation of the epoxy-lactone to an α,β-unsaturated-γ-lactone (δ 4.99 to 5.85). To confirm this structural assignment of lophotoxin

Table 2

Spectral Data for Pukalide (50)



50

$C_{21}H_{24}O_6$; needles, m.p. 203-204°C, $[\alpha]_D^{27}$ = +26.5° (c = 1.0, $CHCl_3$); UV: λ_{max}^{MeOH} = 248 (5200) nm; IR ($CHCl_3$) 2950,1754, 1709, 1567, 1437, 13777, 1274, 1235, 1058 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	3.55 m (11,3,.5)	40.7
2	β 3.00 dd (-18,11), α 2.90 dd (-18,3.5)	32.5
3	--	160.0
4	--	113.9
5	6.33 s	106.4
6	--	148.2+
7	4.04 s	55.0
8	--	57.0
9	β 2.50 dd (-15,3.5), α 2.20 dd (-15,2)	40.0
10	5.20 bm (3.5,2,1)	78.8
11	7.06 bs (1)	148.2
12	--	137.3
13	2.35 m	22.8
14	2.40 m, 1.78 m	32.5
15	--	145.8+
16	5.20 bs, 4.91 bs	112.9
17	1.75 bs	18.7
18	--	163.8
19	1.00 s	19.8
20	--	173.7
-OMe	3.75 s	51.2

+ may be interchanged

and determine the relative stereochemistry at the seven asymmetric centers, an X-ray crystallographic analysis was attempted. However, needles of lophotoxin had unfortunately incorporated solvent during recrystallization. The crystals cracked upon mounting, making them unsuitable for crystallographic study. Stereochemical analyses of lophotoxin using ^{1}H NMR decoupling data and synthetic interconversion with pukalide were attempted in order to unambiguously prove the structure of lophotoxin.

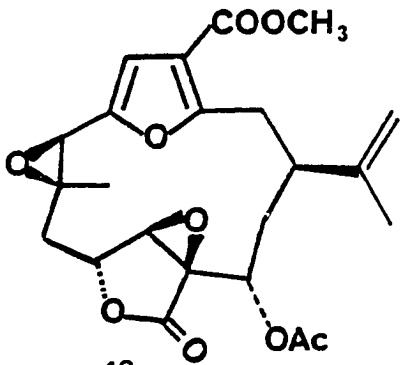
C. Isolation of lopholide and acetoxypukalide and interconversion of lophotoxin with pukalide

In addition to pukalide, several other related compounds were isolated from the four Lophogorgia species investigated. These compounds aided in the complete structure elucidation of lophotoxin by providing comparisons of their common spectral features. These compounds also provided common intermediates in the synthetic interconversion of lophotoxin to a derivative of pukalide.

One of these compounds, lopholide (46), was isolated from Lophogorgia rigida and L. cuspidata collected both in the Gulf of California and Pacific Mexico. Comparison of the ^{13}C and ^{1}H NMR spectral data of lophotoxin and lopholide showed that only one structural element differed between the two compounds (Table 3). Compound 46 possessed a molecular formula of $C_{23}H_{26}O_9$ (from HRMS), lophotoxin ($C_{22}H_{24}O_8$) plus $-CH_2O-$. A three proton singlet at δ 3.79 in the ^{1}H NMR spectrum, combined with the absence of an aldehyde proton, suggested that lopholide was the methyl ester derivative of lophotoxin. This conclusion was

Table 3

Spectral Data for Lopholide (46)



46

$C_{23}H_{26}O_9$; $[\alpha]_D^{23} = +1.2^\circ$ (c = 0.5, $CHCl_3$); UV: $\lambda_{max}^{MeOH} = 251$ (3000), 223 (3000) nm; IR ($CHCl_3$) 2899, 1786, 1736, 1715, 1582, 1441, 1374, 1073, 955 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, d_6 benzene)
1	3.84 m	36.7
2	3.00 m	34.5+
3	--	159.8
4	--	114.5
5	6.52 s	107.9
6	--	149.4++
7	4.07 bs	55.5
8	--	56.0
9	β 2.50 dd (-14,4), α 2.04 dd (-14,3)	38.9
10	4.79 dd (4,3)	76.7
11	4.16 s	64.2
12	--	61.6
13	4.98 d (7)	71.0
14	β 2.50 m (-15,7), α 1.70 d (-15)	32.1+
15	--	149.1++
16	4.90 bs, 4.88 bs	110.6
17	1.89 s	21.3
18	--	163.6
19	1.13 s	20.2
20	--	170.0
CH_3COO-	2.04 s	168.3, 20.2
-OMe	3.79 s	51.0

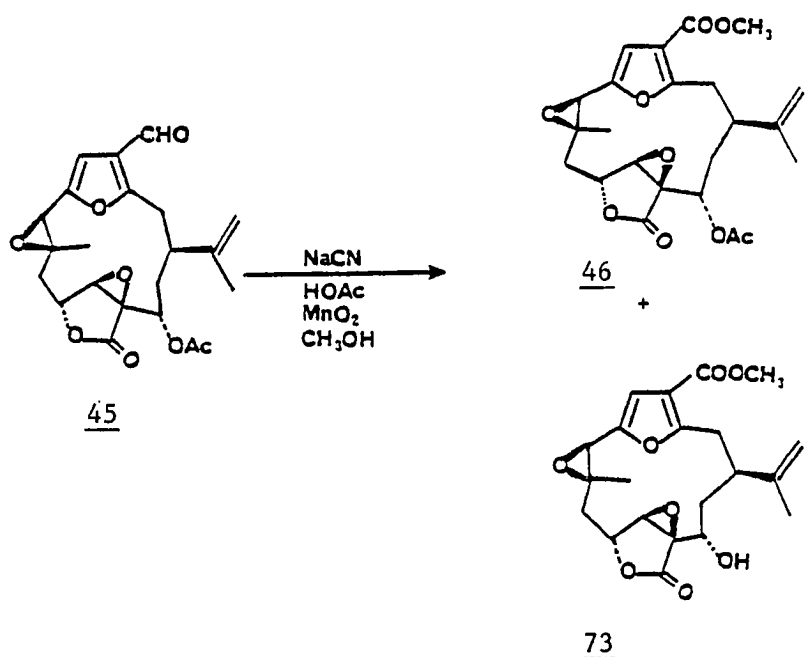
+,++ may be interchanged

supported by the shift in the IR absorption from 1689 to 1715 cm^{-1} for an unsaturated methyl ester. Treatment of lophotoxin with Corey's reagent (manganese dioxide, sodium cyanide and acetic acid in methanol) smoothly converted the furanoaldehyde to the corresponding methyl ester (Figure 20).[55] This synthetic methyl ester showed ^1H NMR features and an optical rotation comparable to the natural product, confirming the assignment of lopholide.

A hydroxy-lopholide derivative, 73, was also produced as a minor product from the MnO_2 oxidation of lophotoxin. Presumably this reaction occurred by acid hydrolysis of lophotoxin or lopholide in the presence of acetic acid. The structure of compound 73 was deduced by the molecular formula of $C_{21}H_{24}O_8$ (lopholide - C_2H_2O) from mass spectrometry, and by infrared absorptions at 3600 (for hydroxyl), 1786 (saturated γ-lactone) and 1712 cm^{-1} (unsaturated ester). Structure 73 was also clearly defined by the absence of the acetoxyl methyl singlet at ~δ 2.0 in the ^1H NMR spectrum, combined with the upfield shift of the C-13 methine proton from δ 4.99 to 4.10.

Lopholide was utilized as an intermediate in another reaction which confirmed the epoxy-lactone functionality in lophotoxin and lopholide. The two trisubstituted epoxides in lopholide were reduced using chromous chloride and acetic acid in acetone to give the corresponding diolefin product, deoxyacetoxypukalide (74) (Figure 21). Deoxyacetoxypukalide was readily identified by the disappearance in the ^1H NMR spectrum of the two epoxide methines at δ 4.07 and 4.16. These bands were replaced by two new olefinic absorptions at δ 7.0 and 6.1. Reduction of pukalide using the zinc-copper couple produced deoxypukalide (75).

Figure 20. MnO_2 oxidation of lophotoxin to lopholide



45 46 + 73

Mechanism of the MnO_2 oxidation:

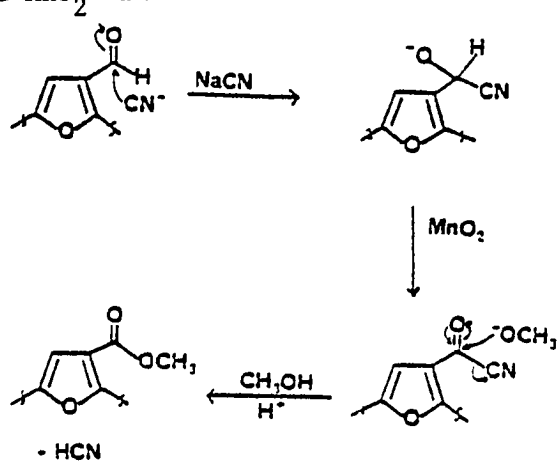
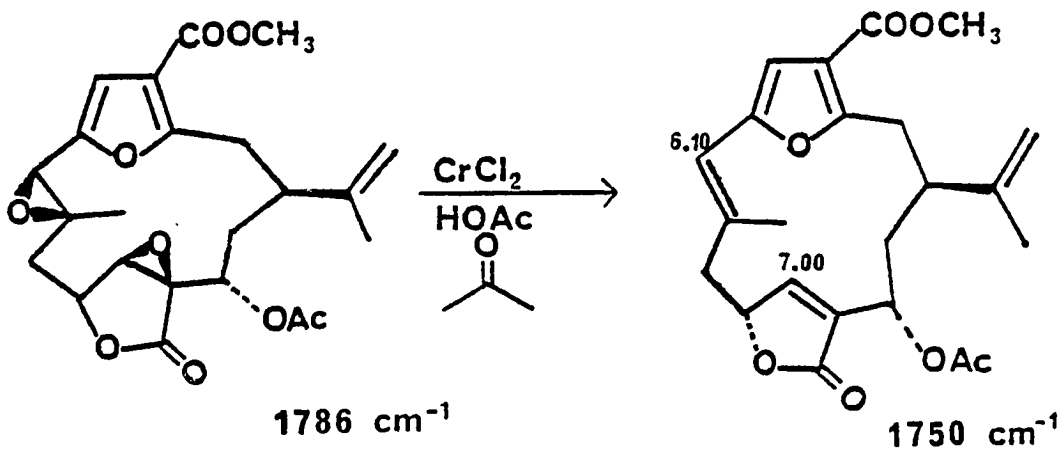
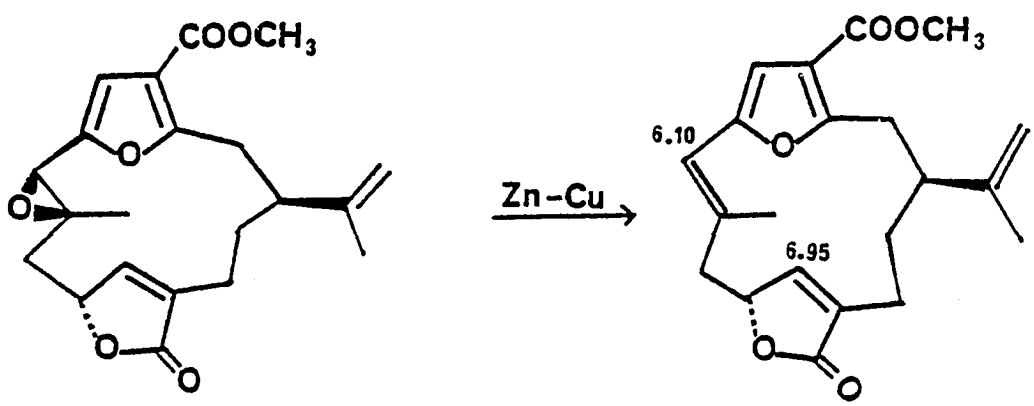


Figure 21. De-epoxidation of lopholide and pukalide



46 74



50 75

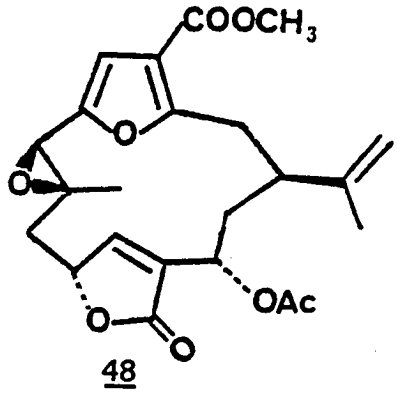
Compound 75 showed analogous signals in the ^1H NMR spectrum at δ 6.96 and 6.10 for the two olefinic protons at C-7 and C-11. The unsaturated lactone produced by the reduction of lopholide gave rise to an infrared absorption at 1750 cm^{-1}, reflecting the ~30 cm^{-1} shift also observed in the product from the de-epoxidation of linderane (72).[52]

The original plan to prove the structure of lophotoxin by interconversion with pukalide depended on the selective reduction of the C-7, C-8 epoxide in lopholide (46) to produce acetoxypukalide (48). However, reduction of lopholide (46) using chromous chloride did not yield 48 as had been reported previously,[47] but gave the diene 74 instead. Therefore, it was necessary to interconvert acetoxypukalide with the same diene 74.

The structure of acetoxypukalide was assigned as 48 based on HRMS and NMR spectral comparisons with lophotoxin, lopholide and pukalide (Tables 4-6). The IR absorptions at 1767, 1730 and 1715 cm^{-1}, combined with signals in the ^1H NMR spectrum at δ 7.28 (1 H, s), 5.80 (1 H, d), 3.78 (3 H, s) and 2.00 (3 H, s), revealed that acetoxypukalide was simply the acetoxyl derivative of pukalide at C-13. Close similarities of these data with the ^{13}C and ^1H NMR features of the model compound melampodin B (76) provided evidence for the β′-acetoxyl-α,β-unsaturated-γ-lactone functionality (Figure 22).[56] Reduction of acetoxypukalide using chromous chloride gave the same diolefin product, deoxyacetoxypukalide (74), that an analogous reduction of lopholide had earlier produced (Figure 22).[57] The reduction products were found to be identical on the basis of their superimposable ^1H NMR spectra and similar optical rotations. Deoxyacetoxypukalide from lopholide gave an

Table 4

Spectral Data for Acetoxypukalide (48)



48

$C_{23}H_{26}O_8$; $[\alpha]_D^{23}$ = +20.8° (c = 1.0, $CHCl_3$); UV: λ_{max}^{MeOH} = 245 (6500) nm; IR ($CHCl_3$) 3049, 2959, 1767, 1730, 1715, 1575, 1437, 1372, 1074, 1041 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	3.90 dd (12,11,4)	36.9
2	β 3.06 dd (-18,12), α 2.90 dd (-18,4)	34.2 (J_R = 17.6)
3	--	160.1
4	--	114.2
5	6.40 bs	106.9 (31.6)
6	--	149.0+
7	4.09 bs	55.0 (25.8)
8	--	56.8
9	β 2.50 dd (-14,4), α 2.20 dd (-14,3)	40.0 (15.2)
10	5.23 m (4,3,1)	77.7
11	7.28 bs (1)	151.0 (31.4)
12	--	135.0
13	5.80 d (10)	68.9 (24.4)
14	β 2.50 m (14,11,10), α 1.85 d (14)	36.0 (13.3)
15	--	148.3+
16	4.99 bs, 4.88 bs	110.9 (24.1)
17	1.88 bs	20.8 (13.3)
18	--	163.3
19	0.97 s	29.8 (12.7)
20	--	170.6
CH_3COO-	2.00 s	170.6, 20.6 (15.7)
-OMe	3.78 s	51.4 (20.7)

+ may be interchanged

Table 5

^{1}H NMR Data for Furanocembrenolides from Lophogorgia spp.

C		LTX 45	Lopholide 46	Deoxy LTX 47	Acetoxypukalide 48	Pukalide Aldehyde 49	Pukalide 50	RAL 51
1		3.90	3.84	3.90	3.90	3.62	3.55	2.10
2	α	3.07	3.00	3.01	3.06	2.97	3.00	2.39
	β	2.95	3.00	2.97	2.90	2.97	2.90	1.89
5		6.57	6.52	6.44	6.40	6.41	6.33	7.11
7		4.09	4.07	4.11	4.09	4.09	4.04	5.30
9	α	2.49	2.50	2.60	2.50	2.53	2.50	2.84
	β	2.05	2.04	2.25	2.20	2.30	2.20	1.99
10		4.81	4.79	5.20	5.23	5.18	5.20	4.84
11		4.17	4.16	7.28	7.28	7.10	7.06	3.88
13		4.99	4.98	5.85	5.80	2.41	2.35	4.90
14	α	2.50	2.50	2.50	2.50	1.83	2.40	2.65
	β	1.70	1.70	1.80	1.85	1.63	1.78	1.59
16		4.93	4.90	5.02	4.99	5.20	5.20	4.70
		4.91	4.88	4.92	4.88	4.96	4.91	4.70
17		1.90	1.89	1.90	1.88	1.79	1.75	1.54
18		9.87	--	9.85	--	9.83	--	9.72
19		1.14	1.13	0.98	0.97	1.01	1.00	1.57
CH_3COO-		2.05	2.04	2.01	2.00	--	--	2.01
-OMe		--	3.79	--	3.78	--	3.75	--
$-OCH_2$								3.27,3.18
CH_3								1.14

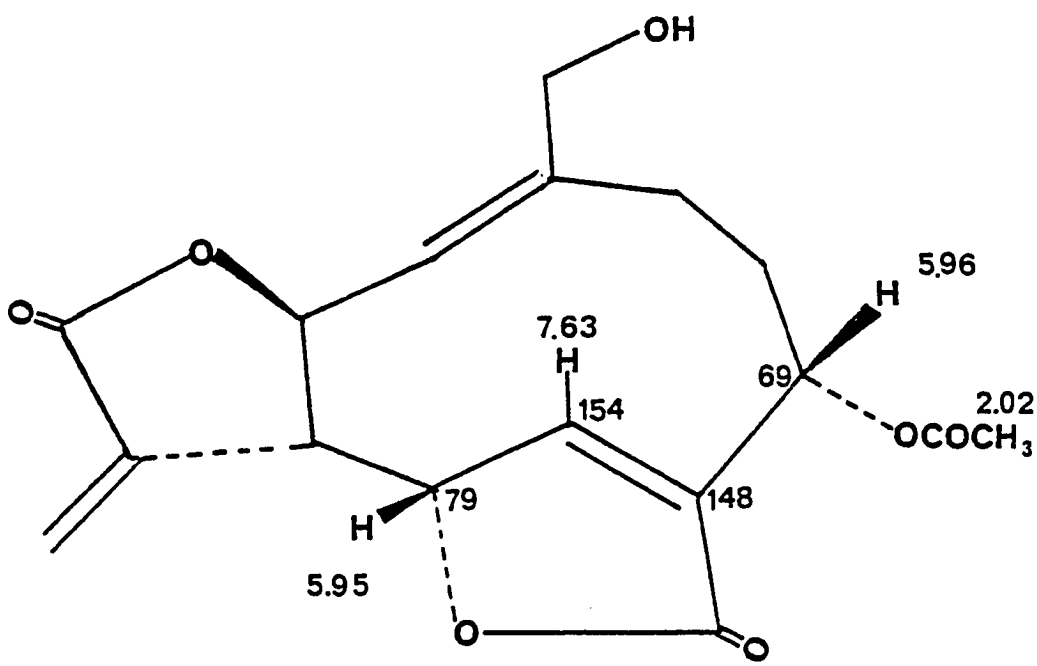
Table 6

^{13}C NMR Data for Furanocembrenolides from Lophogorgia*

	LTX	Lopholide**	Deoxy LTX	Acetoxypukalide	Pukalide aldehyde	Pukalide	RAL
C#	45	46	47	48	49	50	51
1	36.5	36.7	36.8	36.9	40.5	40.7	36.0
2	32.9	34.5‡	33.1	34.2	31.2	32.5	37.2+
3	161.8	159.8	162.3	160.1	162.7	160.0	115.8
4	123.0	114.5	123.2	114.2	123.3	113.9	138.4
5	105.6	107.9	104.4	10..9	104.2	106.4	143.1
6	149.8+	149.4+	149.8+	149.0+	149.9+	148.2+	150.4
7	55.3	55.5	54.8	55.0	54.8	55.0	120.3
8	56.1	56.0	56.8	56.8	57.7	57.0	71.4
9	39.0	38.9	39.3	40.0	39.9	40.0	42.7+
10	76.7	76.7	77.8	77.7	77.8	77.8	73.3
11	64.2	64.2	151.3	151.0	148.1	148.2	62.3
12	61.3	61.6	134.8	135.0	136.7	137.3	58.4
13	70.3	71.0	68.7	68.9	22.7	22.8	70.9
14	31.7	32.1‡	35.7	36.0	32.3	32.5	42.0+
15	148.8+	149.1+	148.5+	148.3+	145.2+	145.8+	147.4
16	110.9	110.6	111.3	110.9	113.5	112.9	112.7
17	21.1	21.3	19.7	20.8	18.8	18.7	18.4
18	184.4	163.6	184.4	163.3	184.5	163.8	185.1
19	20.2	20.2	20.7	19.8	19.9	19.8	29.1
20	170.1	170.0	170.4	170.6	170.0	173.7	170.5
-OMe	--	51.0	--	51.4	--	51.2	--
CH_3COO-	168.1	168.3	170.4	170.6	--	--	168.3
	20.4	20.2	20.5	20.6	--	--	20.5
$-OCH_2$	--	--	--	--	--	--	58.7
CH_3	--	--	--	--	--	--	15.2

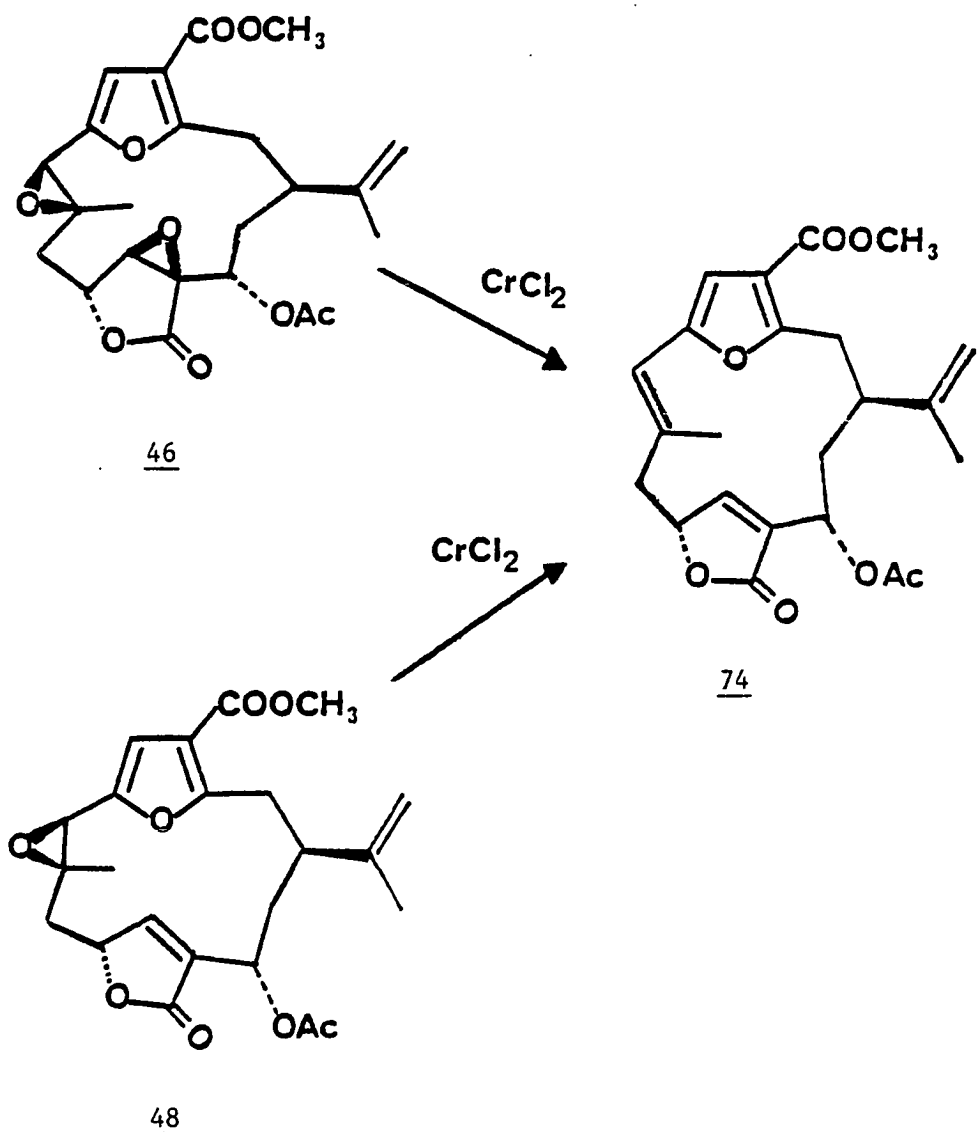
*assigned using J_R
**in d_6 benzene/TMS
+,++may be interchanged

Figure 22. Spectral data of the model compound melampodin B[56]



76

Figure 23. Deoxyacetoxypukalide from lopholide and acetoxypukalide



$[\alpha]_D^{25}$ +30.0° (c = 0.1, $CHCl_3$), while the same product from acetoxypukalide gave an $[\alpha]_D^{23}$ + 18.3° (c = 0.6, $CHCl_3$), ultimately interrelating lophotoxin and acetoxypukalide.

In order to remove the acetoxyl group and interconvert acetoxypukalide with pukalide, both compounds were treated with hydrogen and palladium over carbon in ethyl acetate. Hydrogenolysis of the acetoxyl functionality in acetoxypukalide gave hexahydropukalide (77), $[\alpha]_D^{27}$ = +34.0° (c = 0.3, $CHCl_3$), identical by all spectral features (IR, MS and ^1H NMR) to 77 produced from hydrogenation of pukalide $[\alpha]_D$ = + 33.5° (c = 0.9 $CHCl_3$) (Figure 24).[49]

The results of these reactions confirmed the gross structural assignments of lophotoxin, lopholide and acetoxypukalide by interconversion with pukalide. However, only two of the seven asymmetric centers (C-1 and C-10) remained intact throughout the complete interconversion scheme (Figure 25). Therefore, it was necessary to determine the relative stereochemistry of lophotoxin using other techniques.

D. Assignment of the relative stereochemistry of lophotoxin and related compounds

The relative stereochemistry of the remaining asymmetric centers in lophotoxin (45) and acetoxypukalide (48) were determined by ^1H NMR decoupling and nuclear Overhauser enhancement (nOe) experiments. ^1H NMR decoupling studies were used to identify neighboring sets of protons. The coupling constants measured are characteristic of the angles between adjacent protons.[58] Nuclear Overhauser enhancement experiments are based on proton spin lattice relaxation phenomena. NOe's are measured by the

Figure 24. Hexahydropukalide from acetoxypukalide and pukalide

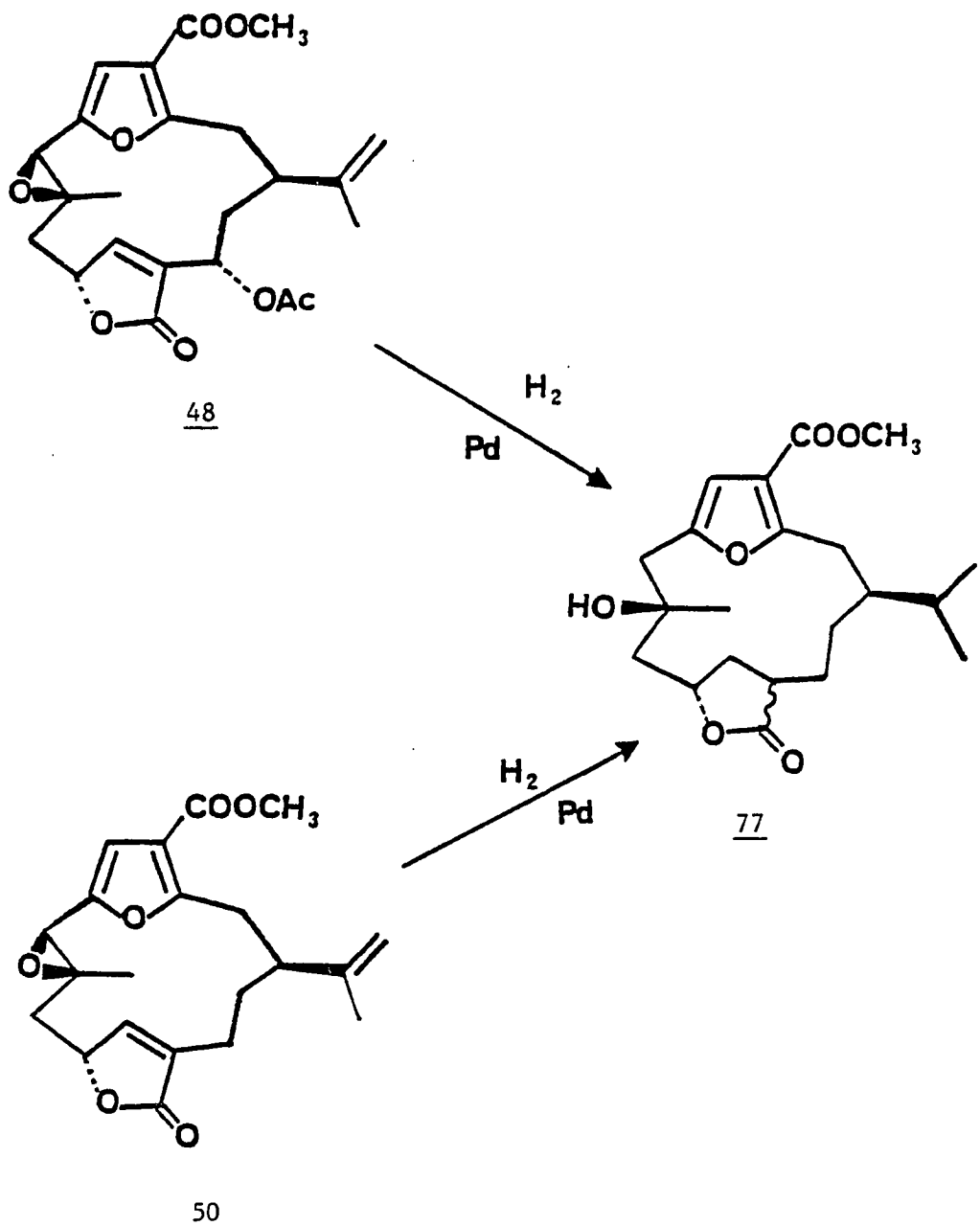
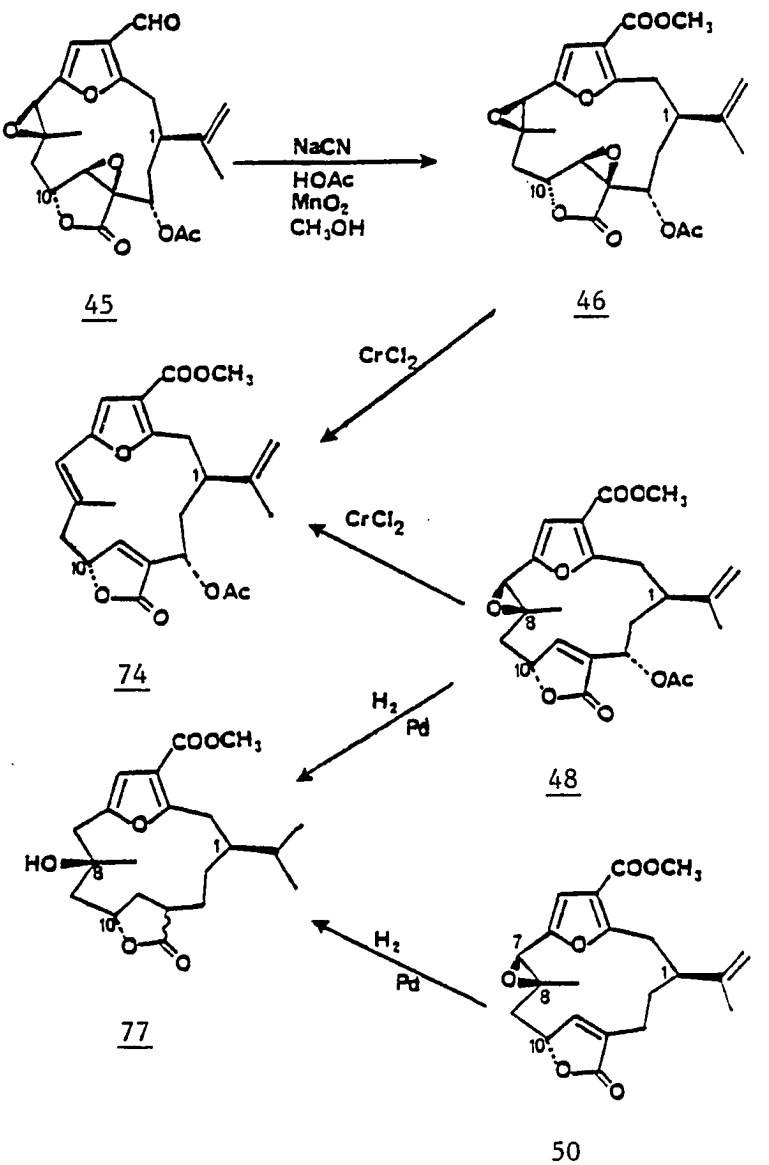


Figure 25. Interconversion of lophotoxin with pukalide



change in the integrated intensity of a proton after saturation of a neighboring proton. The quantitative increase in signal intensity observed depends, in part, on their spatial relationships. NOe experiments are useful in determining the proximity of protons less than 3.0 Å apart in space (using Dreiding molecular models where 1 cm = 0.4 Å).[59,60]

An nOe study of lophotoxin and acetoxypukalide resulted in the assignment of the stereochemistry at the C-7, C-8 epoxide in both compounds. The lack of nOe in the C-7 epoxide methine at δ 4.09 upon irradiation of the C-8 epoxide methyls in 45 and 48 resulted in the assignment of the C-7, C-8 epoxide as trans. Irradiation of the epoxide methyl groups resulted in an nOe enhancement in the C-11 methine and olefinic proton, at δ 4.17 and δ 7.28 respectively. Molecular models show that if the trans epoxide is placed in the "up" orientation, the C-11 methine and the epoxide methyl group both extend into the center of the cembrenolide ring and are in close proximity to one another. Due to the rigid ring system the "down" epoxide would result in severe steric crowding for the C-8 methyl. In addition, the proximity of the epoxide methyl group to the C-11 methine proton would be reduced. Based on molecular models and nOe results, the stereochemistry of the epoxide at C-7, C-8 in lophotoxin and acetoxypukalide must be placed "up" and trans, as shown in Figure 26. This is identical to the orientation of the epoxide in pukalide which was determined by nOe and X-ray studies.

The relationship of the protons at C-10 and C-11 in lophotoxin was defined on the basis of ^{1}H NMR decoupling studies. The C-10, C-11 coupling constant is less than 1 Hz due to their approximate 90°

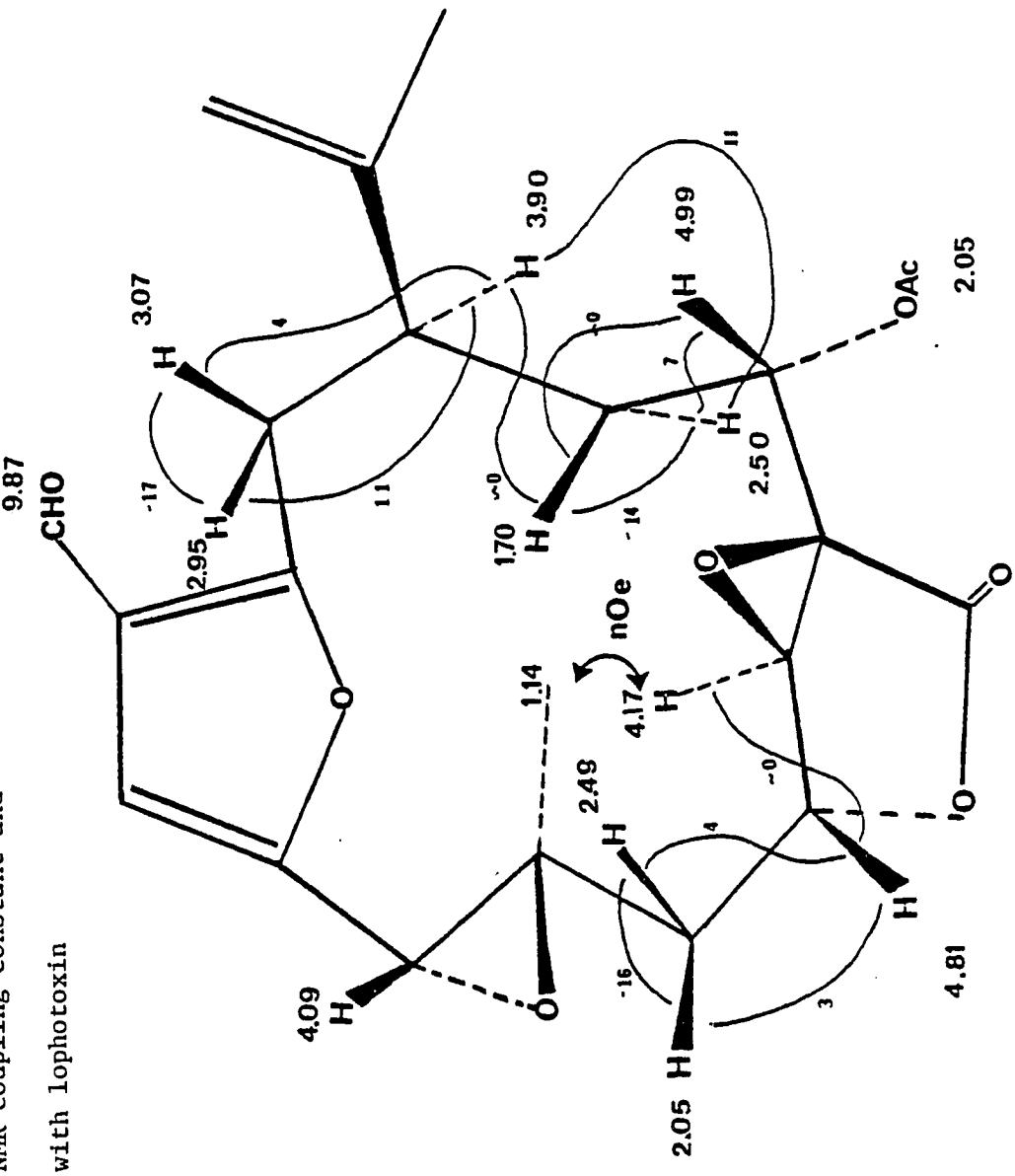
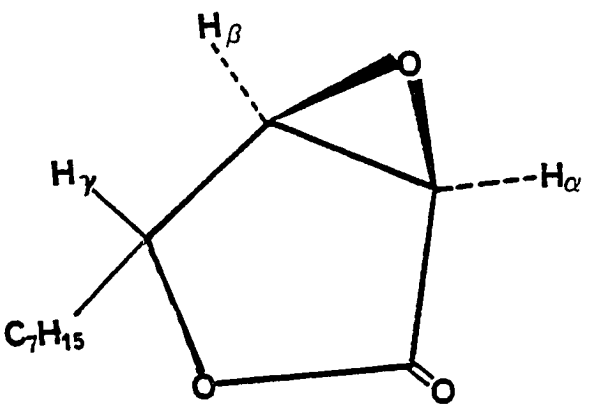


Figure 26. ^{1}H NMR coupling constant and nOe results with lophotoxin

dihedral angle. This is only possible if the protons are trans to one another. This result is well known in model compounds with cis and trans epoxy-γ-lactone functionalities. Figure 27 illustrates the difference in coupling constants between cis and trans epoxy-γ-lactones where trans $J_{\beta,\gamma} < 1$ Hz (78) and cis $J_{\beta,\gamma} \cong 2\text{-}3$ Hz (79).[61] The epoxide at C-11, C-12 was also placed "up" based on the nOe results, which indicated the proximity of the C-19 methyl and C-11 methine protons. The C-11, C-12 epoxide must be cis, based on the ring constraints of the γ-lactone.

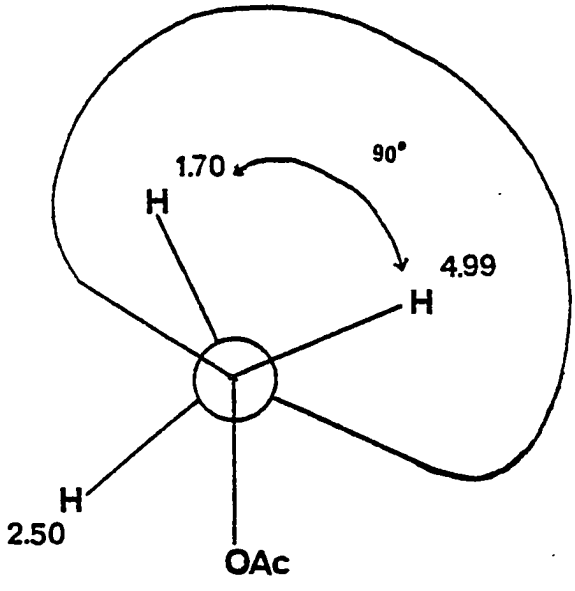
^{1}H NMR decoupling studies also helped to define the stereochemistry of the acetoxyl group at C-13 in lophotoxin. The readily resolved allylic methine proton at C-1, which is observed as an identical multiplet in all of the Lophogorgia furanocembrenolides, served as a cornerstone to establish the four carbon unit C-13, C-14, C-1 and C-2 in lophotoxin (see Figure 26). As in pukalide, the C-1 methine proton was only coupled to three of the four proximate protons. Both C-2 protons were coupled to C-1 (J = 11, 4 Hz), and only one of the C-14 methylene protons (δ 2.50) showed coupling to C-1 (J = 11 Hz). The C-14 methylene proton at δ 2.50 was also coupled (J = 11 Hz) to the acetoxyl bearing methine at C-13. The geminal coupling (J = -15 Hz) of the C-14 protons was easily established since their chemical shifts were widely separated (see Table 1). The lack of coupling between the C-14 methylene proton at δ 1.70 and both the C-1 isopropenyl and C-13 acetoxyl methine protons indicated dihedral angles for both sets of protons of near 90°. Using molecular models these dihedral angles can only be achieved, given the constraints of the ring system, if the acetoxyl group is placed pseudo-

Figure 27. Coupling constants in α,β- epoxy-γ- lactones

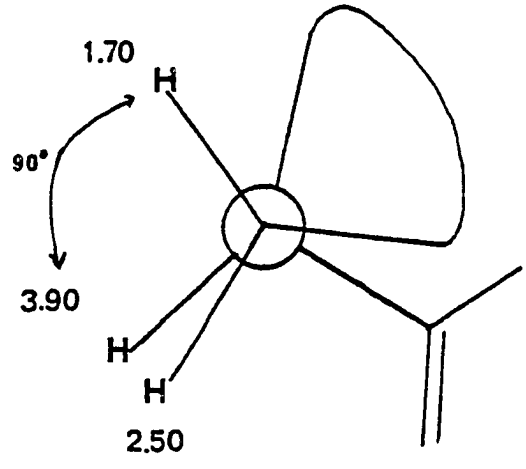


	H_β , H_γ	$J_{\beta,\gamma}$
78	trans (H_γ β or down)	< 1 Hz
79	cis (H_γ α or up)	≅ 2-3 Hz

Figure 28. Newman projections of the C-13, C-14 and C-1, C-14 bonds of lophotoxin



C-13, C-14



C-14, C-1

axial and down (Figures 26 and 28). This placement is also supported by the deshielding effect of the acetoxyl group on the isopropenyl methine proton due to their proximity. The C-1 methine proton shifts from δ 3.55 in pukalide (48) to δ 3.90 in lophotoxin (45). Therefore, on the basis of interconversion with pukalide and ^{1}H NMR studies, the relative stereochemistry of lophotoxin was defined as 45: C-1 (S*), C-7 (S*), C-8 (S*), C-10 (R*), C-11 (R*), C-12 (S*), C-13 (S*). Similar analyses for lopholide and acetoxypukalide allowed their structures to be defined as shown in 46 and 48.

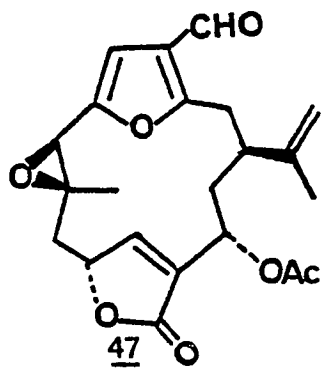
E. Structure elucidation of pukalide aldehyde (49) and deoxylophotoxin (47)

In addition to lophotoxin, lopholide, acetoxypukalide and pukalide, two closely related furanocembrenolides were also isolated from the four Lophogorgia species studied. The close similarity of their spectral features to those of the previously isolated compounds allowed the assignment of their structures (Tables 7 and 8). The structure of deoxylophotoxin (47) was assigned as the α,β-unsaturated-γ-lactone derivative of lophotoxin based on correlations of the spectral data with lophotoxin and acetoxypukalide. The infrared absorption at 1761 cm^{-1} and the broad singlet in the ^{1}H NMR at δ 7.28 were indicative of an α-substituted α,β-unsaturated-γ-lactone. Features of their ^{13}C and ^{1}H NMR spectra also correlated well with those of the β′-acetoxyl-α,β-unsaturated lactone-containing model compound melampodin B (76) shown in Figure 22 (Table 7).

The molecular formula of $C_{22}H_{24}O_7$, established for 47 by high

Table 7

Spectral Data for Deoxylophotoxin (47)



47

$C_{22}H_{24}O_7$; $[\alpha]_D^{23}$ = +46.1° (c = 1.8, $CHCl_3$); UV: λ_{max}^{MeOH} = 283 (4000) nm; IR ($CHCl_3$) 2933, 2849, 1761, 1727, 1672, 1374, 1218, 1087, 1046, 961 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	3.90 m	36.8 (J_R = 15.4)
2	α 3.01 m, β 2.97 m)	33.1 (16.4)
3	--	162.3
4	--	123.2
5	6.44 bs	104.4 (31.6)
6	--	149.8+
7	4.11 bs	54.8 (26.4)
8	--	56.8
9	2.60 dd (-15,2), 2.25 dd (-15,2)	39.3 (15.7)
10	5.20 bs (2,2)	77.8 (24.6)
11	7.28 bs	151.3 (32.4)
12	--	134.8
13	5.85 d (6)	68.7 (24.4)
14	β 2.50 m, α 1.80 d (-15)	35.7 (13.6)
15	--	148.5+
16	5.02 bs, 4.92 bs	111.3 (24.0)
17	1.90 bs	19.7 (13.0)
18	9.85 s	184.4 (38.1)
19	0.98 s	20.7 (12.4)
20	--	170.4
CH_3COO-	2.01 s	170.4, 20.5 (15.8)

+ = may be interchanged

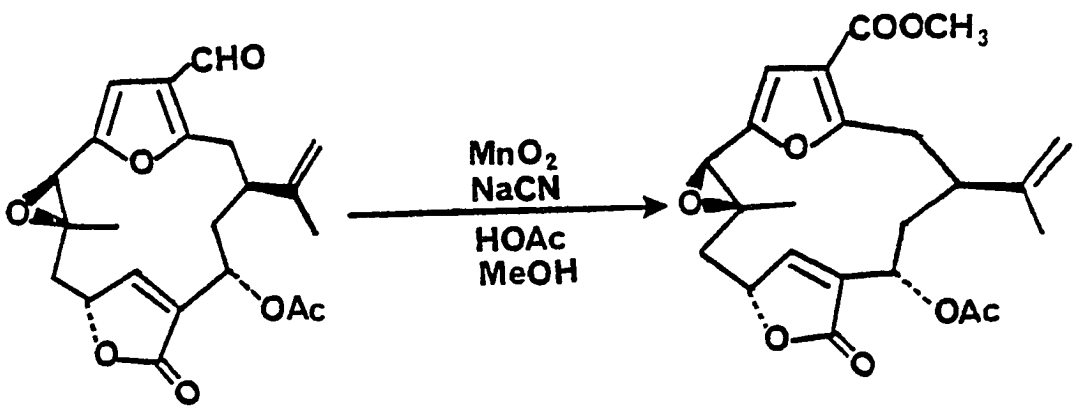
resolution mass spectrometry (lophotoxin minus one oxygen atom), supported this assignment. The structure of deoxylophotoxin (47) was confirmed by a smooth conversion to acetoxypukalide (48) using manganese dioxide oxidation of the aldehyde (Figure 29). synthetic acetoxypukalide, $[\alpha]_D^{23}$ = +33.0° (c = 0.4, $CHCl_3$), was comparable in all respects to the natural product, $[\alpha]_D^{23}$ = +20.8° (c = 1.0, $CHCl_3$).

Pukalide aldehyde (49) was shown to be the aldehyde derivative of pukalide based on similar reasoning (Table 8). The NMR spectra of pukalide and pukalide aldehyde were practically superimposable. The only difference was a new aldehyde proton singlet at δ 9.85 in a ^1H NMR spectrum and the doublet at 184.5 ppm in the ^{13}C NMR spectrum of 49. Smooth conversion of pukalide aldehyde to pukalide (50) using the MnO_2 oxidation reaction confirmed the structure of 49 (Figure 29). Synthetic pukalide, $[\alpha]_D^{26}$ = +30.3° (c = 0.6, $CHCl_3$), was identical in all respects to the natural product, $[\alpha]_D^{27}$ = +26.5° (c = 1.0, $CHCl_3$).

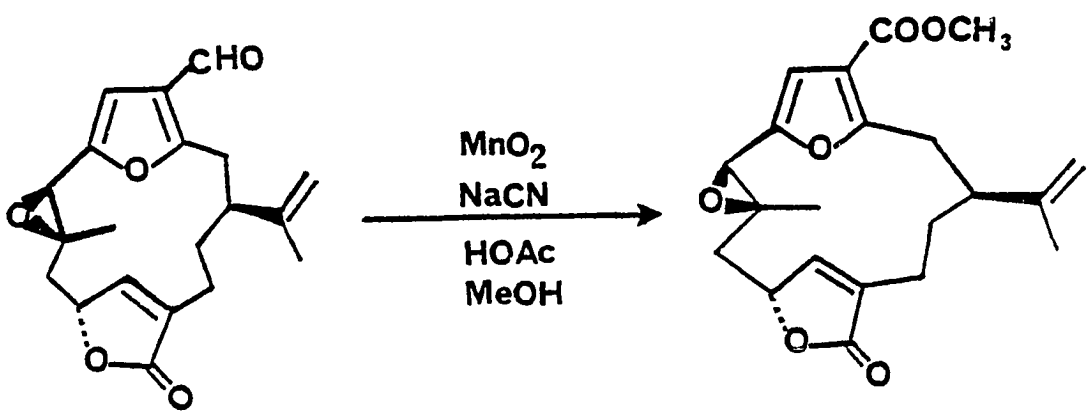
F. Isolation and structure elucidation of the rearranged aldehyde

An interesting related compound was isolated from L. rigida, L. alba, and L. chilensis, in addition to the furanocembrenolide compounds 45-50. The new compound appeared similar to lophotoxin by ^1H and ^{13}C NMR. However, it lacked absorptions in the ^1H NMR spectrum for the furan proton at ~δ 6.5 and the epoxide methine at δ 4.1. Absorptions for two new olefinic protons appeared as broad singlets at δ 7.11 and 5.30. Low resolution mass spectrometry gave a molecular ion of 462, for $C_{24}H_{30}O_9$. This molecular formula calculated for ten degrees of unsaturation and represented lophotoxin ($C_{22}H_{24}O_8$) plus C_2H_6O. Comparison

Figure 29. MnO_2 oxidation of deoxylophotoxin and pukalide aldehyde



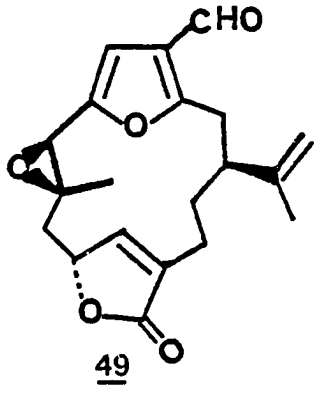
47 48



49 50

Table 8

Spectral Data for Pukalide Aldehyde (49)



49

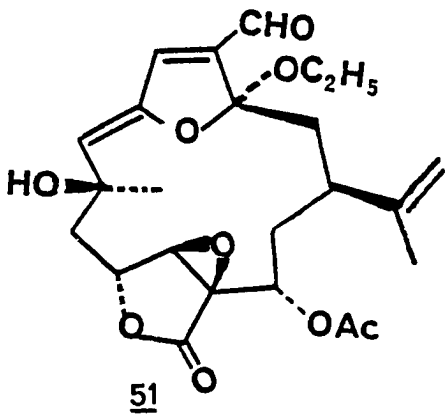
$C_{20}H_{22}O_5$; $[\alpha]_D$ = + 43.5° (c = 0.2, $CHCl_3$); UV: λ_{max}^{MeOH} = 267 (3000) nm; IR ($CHCl_3$) 2941, 2755, 1754, 1678, 1555, 1242, 1081, 1047 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	3.62 m	40.5 d (J_R = 14.6)
2	2.97 m	31.2 t (16.6)
3	--	162.7 s
4	--	123.3 s
5	6.41 s	104.2 d (31.7)
6	--	149.9 s+
7	4.09 s	54.8 d (26.4)
8	--	57.7 s
9	2.53 dd (-16,3), 2.30 (-16,3)	39.9 t (15.7)
10	5.18 bs	77.8 d (27.5)
11	7.10 s	148.1 d (31.3)
12	--	136.7 s
13	2.41 m	22.7 t (14.0)
14	1.83 m, 1.63 m	32.3 t (11.4)
15	--	145.2 s+
16	5.20 bs, 4.96 bs	113.5 t (24.5)
17	1.79 s	18.8 q (14.5)
18	9.85 s	184.5 d (38.3)
19	1.01 s	19.9 q (13.0)
20	--	170.0 s

+ may be interchanged

Table 9

Spectral Data for the Rearranged Aldehyde (RAL) (51)



51

$C_{24}H_{30}O_9$; needles, m.p. 281-219°C; $[\alpha]_D$ = +133° (c = 1.2, $CHCl_3$); UV: λ_{max}^{MeOH} = 315 (13,000) nm; IR ($CHCl_3$) 3571, 2941, 1792, 1739, 1689, 1597, 1374, 1244, 1144, 916 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	2.10 m (12,5,4)	36.0 (J_R = 13.2)
2	2.39 dd (-15,5), 1.89 dd (-15,4)	37.2+ (15.4)
3	--	115.8
4	--	138.4
5	7.11 s	143.1 (33.3)
6	--	150.4
7	5.30 s	120.3 (26.8)
8	--	71.4
9	2.84 dd (-14,6), 1.99 m	42.7+ (15.9)
10	4.84 m (6)	73.3 (24.2)
11	3.88 s	62.3 (27.9)
12	--	58.4
13	4.90 bs (6.5,1)	70.9 (22.1)
14	2.64 m (-15,12,6.5), 1.59 m (-15)	42.0+ (12.9)
15	--	147.4
16	4.70 bs	112.7 (24.4)
17	1.54 s	18.4 (14.6)
18	9.72 s	185.1 (39.1)
19	1.57 s	29.1 (14.7)
20	--	170.5
CH_3COO-	2.01 s	168.3, 20.5 (15.5)
-CH_2O-	3.27 dq (9,7), 3.18 dq (9,7)	58.7 (18.3)
CH_3	1.14 t (7)	15.2 (12.6)

+ may be interchanged

of the spectral features of lophotoxin and the "rearranged aldehyde" (Tables 1 and 9) revealed that the new compound was most likely an addition product of ethanol to the furan ring of lophotoxin. ^{1}H NMR signals at δ 3.27 (1 H, dq) and 3.18 (1 H, dq), geminally coupled, and 1.14 (3H, t) supported this reasoning. In the ^{13}C NMR spectrum, absorptions at 58.7 (t) and 15.2 (q) also indicated an ethoxy group. Examination of the mode of workup revealed that the rearranged aldehyde was isolated only from Lophogorgia extracts previously stored in or extracted with ethanol (see Figure 30). The compound was not isolated from extracts with no ethanol contact.

Information gained from low and high resolution mass spectrometry provided useful clues to the structure of the rearranged aldehyde. The molecular ion was not present in the high resolution mass spectrum, but ions which calculated for $M^{+}-H_2O$ (444) and $M^{+}-$ ethoxy (417) were observed. The $M^{+}-H_2O$ ion and the weak, broad infrared absorption at 3470 cm^{-1} indicated the presence of an alcohol. This assignment was supported by a new singlet in the ^{13}C NMR spectrum at 71.4 ppm. The shift in the ^{1}H NMR spectrum of the methyl epoxide group from δ 1.00 to 1.57 was characteristic of a methyl group attached to a carbon bearing a tertiary alcohol. The tertiary nature of the alcohol was also confirmed by its inability to acetylate under a number of rigorous conditions. From these data, it appeared that the epoxide at C-7, C-8 had opened to produce a tertiary alcohol at C-8. The formation of the tertiary alcohol did not appear to be the result of simple nucleophilic attack on the epoxide. This structure was ruled out due to changes in the spectral data of 51 indicating loss of the furan

Figure 30. Occurrence of the rearranged aldehyde (51) and method of workup

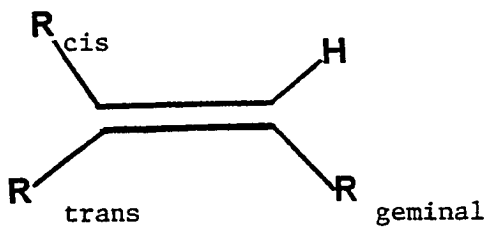
Lophogorgia spp.	Collection Site	Mode of Storage	Extraction of RAL	Occurrence
L. chilensis	La Jolla (L-2)	ethanol	70% $CHCl_3$/MeOH	Yes
	Los Coronados (L-6)	isopropyl alcohol	70% $CHCl_3$/MeOH	No
L. rigida	Los Frailes (V-5)	methanol	70% $CHCl_3$/MeOH	No
	Tres Marias (AH 60)	frozen	ethanol, followed by 70% $CHCl_3$/MeOH	Yes
L. alba	Tres Marias (AH 56)	frozen	ethanol, followed by 70% $CHCl_3$/MeOH	Yes
	Bahia Tenacatita (AH 74)	frozen	ethanol and 70% $CHCl_3$/MeOH	Yes
L. cuspidata	Bahia Tenacatita (AH 82)	frozen	ethanol and 70% $CHCl_3$/MeOH	No
	Los Frailes (V-7)	isopropyl alcohol	70% $CHCl_3$/MeOH	No

functionality. The new signal at δ 7.11 in the ^{1}H NMR spectrum was indicative of a β proton in an α,β-unsaturated carbonyl group. The lack of coupling between the two new olefins at δ 7.11 and 5.30 combined with the addition of ethoxy led to the proposal of 51 as the structure of the rearranged aldehyde. This structral assignment was supported by chemical shift calculations for the olefinic protons at C-5 and C-7. The calculations gave δ 6.93 and 5.02, respectively (observed δ 7.11 and 5.30), using the formula for substituted ethylenes (Figure 31).[63] This structure was also indicated by the characteristic singlet in the ^{13}C NMR spectrum at 115.8 for C-3, similar to the corresponding ketal carbon in the model compound 80 (Figure 32).[62] The ultraviolet absorption at 315 nm (13,000), indicating extended conjugation, confirmed the α,β,γ,δ-unsaturated aldehyde functionality (calculations for the ultraviolet absorption of this aldehyde gave 304 nm).[63]

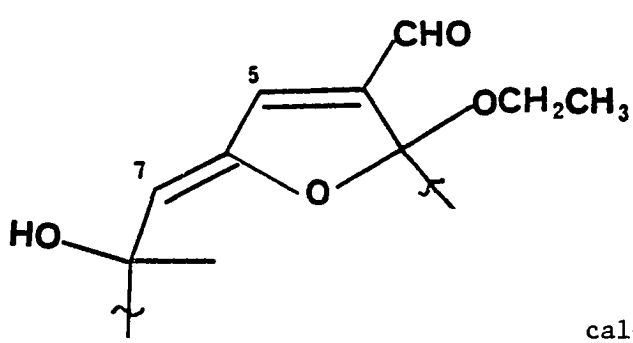
Ethanol addition to the furan seems reasonable under the acidic conditions of solvent storage and workup. This addition may occur by nucleophilic attack of ethoxide on the unsaturated aldehyde, followed by rearrangement and displacement of the protonated epoxide (Figure 33). A number of attempts were made to repeat this reaction *in situ* using lophotoxin in ethanol combined with several different acids (acetic, sulfuric and p-toluenesulfonic acid). These reactions were not successful. Attempts to induce nucleophilic attack on the furan ring using NaOMe also failed.

Analysis of ^{1}H NMR decoupling data with molecular models was used to determine the relative stereochemistry of the rearranged aldehyde. All of the protons in the rearranged aldehyde were clearly

Figure 31. Calculations of the chemical shift of the olefin protons at C-5 and C-7 in the rearranged aldehyde (51)[63]

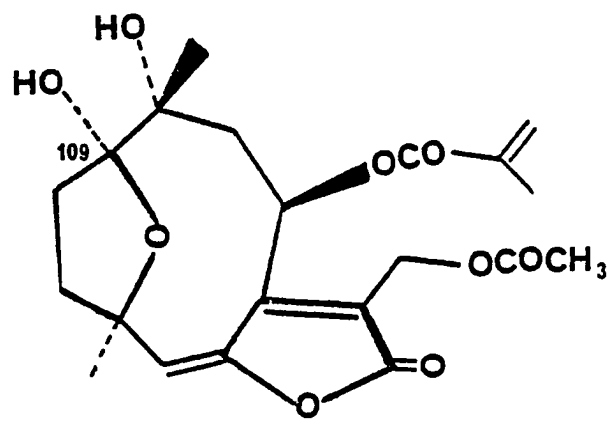


Substituent	Z_i		
R	geminal	cis	trans
alkyl- ring	0.71	-0.33	-0.30
-C=C-	0.98	-0.04	-0.21
-C=C-, conjugated	1.26	0.08	-0.01
-CHO	1.03	0.97	1.21
-OR, R conjugated	1.14	-0.65	-1.05



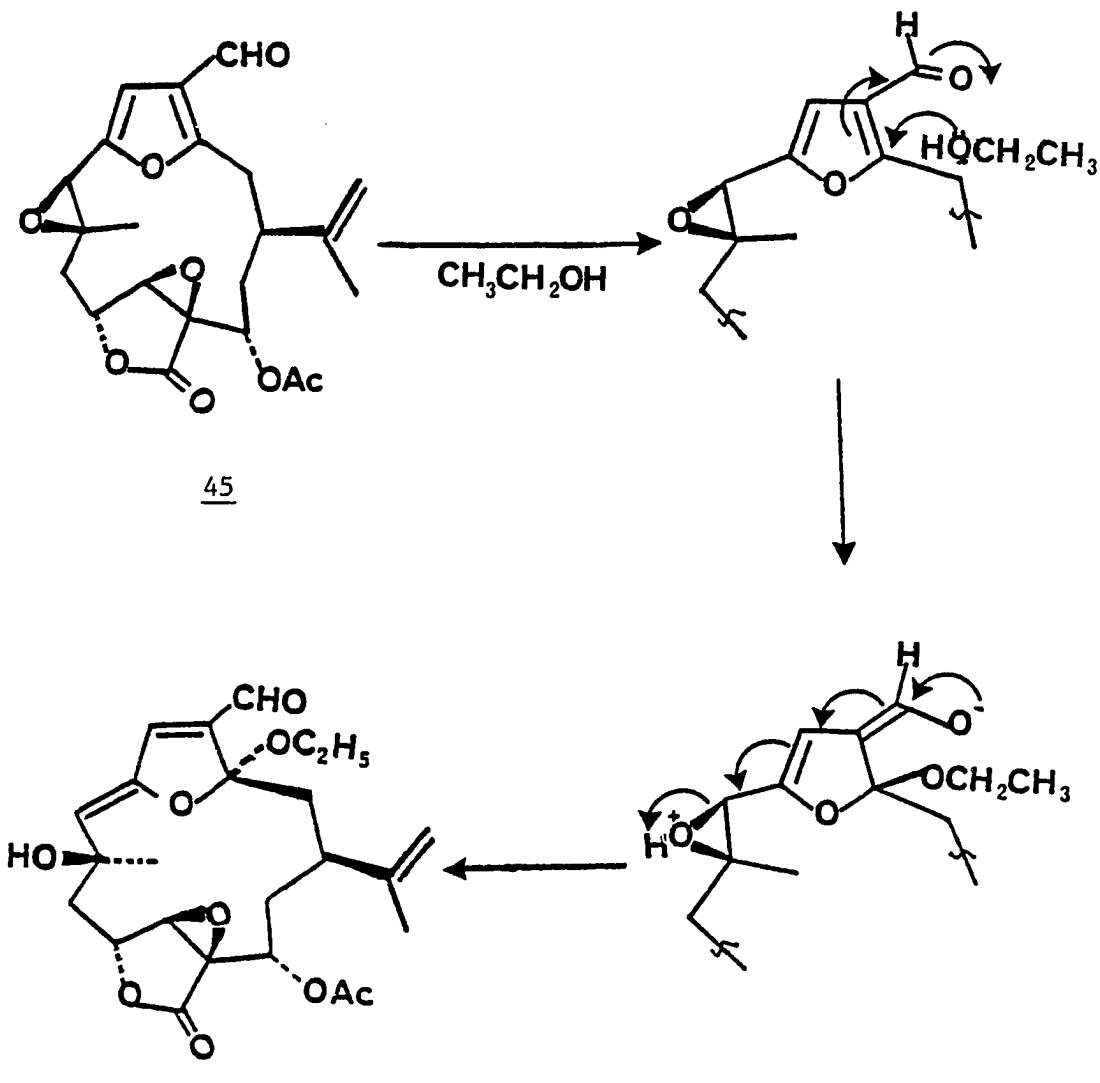
		Chemical Shift	
		calculated	observed
C-5:	5.28 + 0.98 + 0.97 - 0.30 =	6.93	7.11
C-7:	5.28 + 0.71 + 0.08 - 1.05 =	5.02	5.30

Figure 32. Piptocarphin A[62]



80

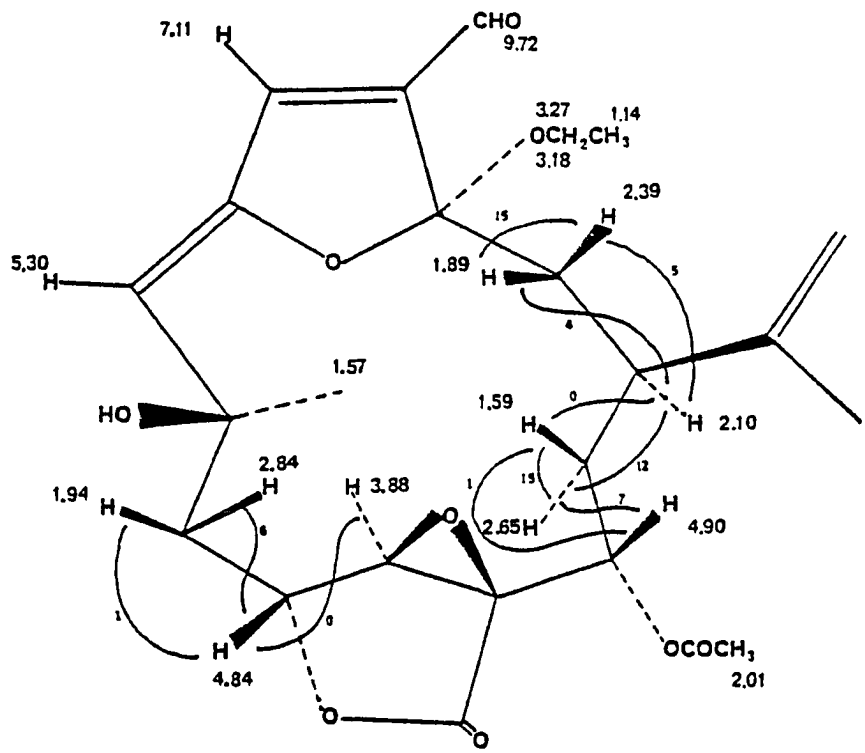
Figure 33. Possible mechanism for the formation of the rearranged aldehyde 51



45

51

Figure 34. ^{1}H NMR chemical shifts and coupling constants depicting the stereochemistry of the rearranged aldehyde (51)



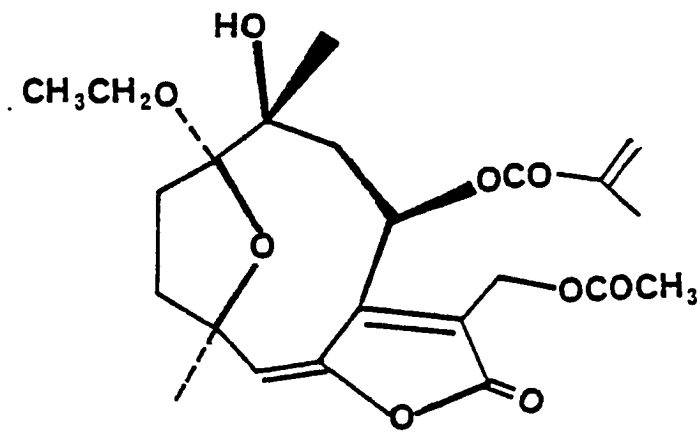
51

visible in the ^1H NMR spectrum. Their coupling constants revealed spatial relationships which turned out to be identical to those in lophotoxin. Figure 34 illustrates these features. If lophotoxin is presumed to be the precursor of the rearranged aldehyde, then the relative stereochemistry of the tertiary alcohol would be as shown in 51. I have assigned the relative stereochemistry of the ethoxy group as down, by assuming nucleophilic attack from the less hindered, outside face of the cembrenolide ring. The magnetic nonequivalence of the ethoxy methylene protons (δ 3.27 dq, 3.18 dq) in the ^1H NMR spectrum reflects the sterically hindered quaternary center and the stereospecificity of attack. This magnetic nonequivalence is observed in two other sterically hindered ethoxy derivatives - ethoxypacifigorgiolide (59) (reported in Chapter 5) and piptocarphin E (81)[62], where the chemical shift of the ethoxy protons are also widely separated (Figure 35).

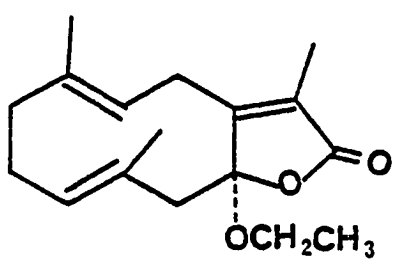
G. The pharmacological activity of lophotoxin and related furanocembrenolides

The pharmacological activity of lophotoxin and its furanocembrenolide analogues has been intensively studied by Drs. Paul Culver and Robert Jacobs at the University of California, Santa Barbara, as part of the Sea Grant Program in Marine Pharmacology and Dr. Culver's dissertation research. Lophotoxin is only moderately toxic in mice as compared to some other well known marine toxins. Lophotoxin has an LD_{50} in mice of 8.9 mg/kg compared to LD_{50}'s of 10 µg/kg for saxitoxin[64] (from a red tide dinoflagellate), 0.3 mg/kg for lyngbyatoxin[65] (from a blue green algae) and 0.025 µg/kg for palytoxin[66] (from a zooanthid species). Subcutaneous injection of lophotoxin caused death in mice after thirty

Figure 35. Natural products containing an ethoxy ketal functionality



81 piptocarphin E[62]



59

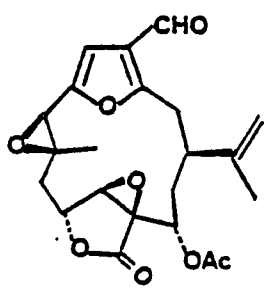
minutes. Death was preceded by ataxia, paralysis and severe respiratory depression: indications of neuromuscular blockage. Lophotoxin is a very potent inhibitor of nerve transmission, however, when measured in the *in vitro* systems. Experiments in the rat phrenic nerve hemidiaphragm preparation demonstrated that lophotoxin irreversibly inhibits indirect nerve stimulated muscle contraction without affecting direct electrical stimulation of the muscle. Neuromuscular blockage was observed with bath concentrations as low as 8×10^{-8}M. Both the kinetics and irreversibility of lophotoxin resemble those of α-bungarotoxin, a paralytic component of the venom of the Southeast Asian snake *Bungarus multicinctus*, characterized by an increase in the latency period of onset with decreasing concentration of compound.[47,48]

The activity of the five furanocembrenolides related to lophotoxin was compared with lophotoxin by measuring their relative potency. Similar activity to lophotoxin was observed, in each of the compounds but with far less potency. We hoped that the close structural relationship of the six cembrenolides would lead to an understanding of which functional groups were responsible for lophotoxin's biological activity. However, as Figure 36 indicates, there is no straightforward correlation between the level of neuromuscular activity and type of functional group present. Other factors such as lipid solubility, differential rates of binding, or adsorption may be important to understanding the differences observed in activity.[67]

This comparative study does reveal the importance of both the furanoaldehyde and epoxy-lactone functionalities to the activity of lophotoxin. Acetoxypukalide (48) has only weak to moderate toxicity and

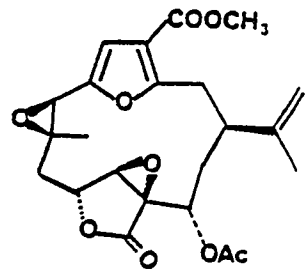
Figure 36. Neuromuscular activity relative to lophotoxin[67]

1000



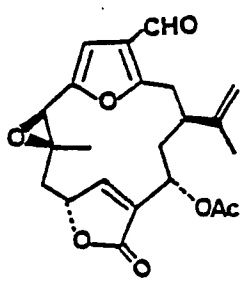
45 lophotoxin

90



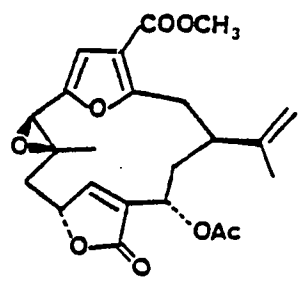
46 lopholide

90



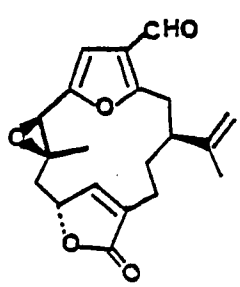
47 deoxylophotoxin

250



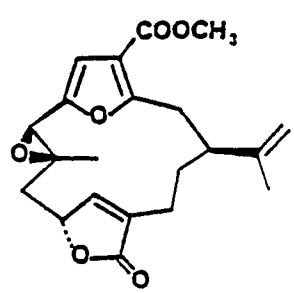
48 acetoxypukalide

20



49 pukalide aldehyde

2



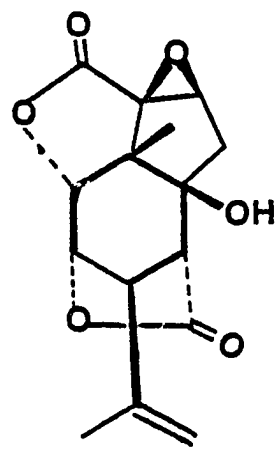
50 pukalide

pukalide (50) shows almost no activity. The epoxy-lactone and furanoaldehyde functionalities are found separately in only a few other natural products. These groups have not previously been observed in marine natural products. Other α,β-epoxy-γ-lactone containing natural products possess important biological activities. Micromelin (71), isolated from the Asian evergreens Micromelum integenium and M. minutum, for example, demonstrates significant activity against P-388 lymphocytic leukemia and Lewis lung cancer. The corresponding synthetic α,β-unsaturated-γ-lactone derivative of micromelin showed no such activity.[51] Picrotoxinin (82), an ichthyotoxin from the poisonous plant genus Menispermum, also possesses the epoxy-lactone functionality, and apparently acts by blocking γ-aminobutyric acid to produce central nervous system stimulation and convulsions.[68]

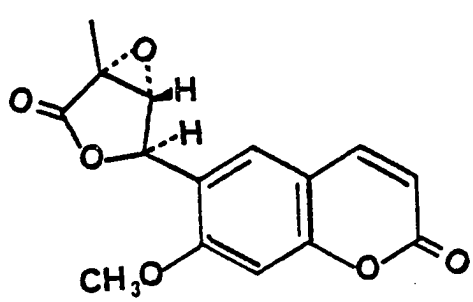
The inference that the epoxy-lactone and furanoaldehyde groups may be responsible for the potent biological properties of lophotoxin leads to speculation concerning their respective modes of action. It is clear that both functionalities could react with biological nucleophiles (such as the sulfhydryl groups in enzymes) providing a site for highly selective alkylation. If the rearranged aldehyde is an artifact of the extraction/isolation procedure, then the high reactivity of the epoxy furanoaldehyde moiety towards the weak nucleophile ethanol may provide a model for the reactivity of lophotoxin with biological nucleophiles.

Lophotoxin's most unique feature, however, in terms of its potent in vitro neuromuscular activity is the fact that it does not possess cationic ammonium functional groups. This is in sharp contrast to all the other established neurotoxins. This has led to intensive

Figure 37. Examples of epoxy-lactone-containing compounds which possess significant biological activity



82 picrotoxinin[68]



71 micromelin[51]

research by several research groups around the country who are currently using lophotoxin as a pharmacological tool to study the mode of action of neuromuscular toxicity.

H. Experimental - Chapter II

General. Infrared and ultraviolet spectra were recorded on Perkin-Elmer Model 137 and Perkin-Elmer Model 124 double beam spectrophotometers, respectively. Some ultraviolet spectra were also recorded on a Beckman MVl spectrophotometer. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter using a 10 cm microcell. ^{1}H NMR spectra were recorded on Varian HR-220, 360 and T-60 spectrometers and ^{13}C NMR spectra were recorded on a Varian CFT-20 or Nicolet 50 MHz multinuclear wide-bore spectrometer (all chemical shifts are reported relative to tetramethyl silane (TMS)). NMR chemical shifts are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, bs = broad singlet, m = multiplet, + = interchangeable assignment. Low resolution electron impact mass spectra recorded at Scripps were obtained using a Hewlett-Packard 5930-A mass spectrometer. High resolution electron impact and low resolution field desorption mass spectra were obtained from the Bio-organic, Biomedical Mass Spectrometry Resource (A.L. Burlingame, Director) supported by NIH Research Grant #RR00719 from the Division of Research Resources. Several high resolution electron impact spectra were also obtained from the Department of Chemistry, University of California, Los Angeles and the Department of Chemistry, Colorado State University. Melting points were determined on a Fisher-Johns melting point apparatus and are reported uncorrected. All solvents used were distilled from glass prior to use. The equipment listed above was used in the chemical projects detailed in this chapter as well as in subsequent chapters (3-7).

Collection, extraction and chromatography. Gorgonians were collected by hand using SCUBA in a number of locations outlined in Figure 17 at 30 to 80'. Samples were frozen or stored in alcohol to preserve them upon collection. Repeated extraction of the ground animal with warm 70% chloroform/methanol was followed by removal of the solvents under vacuum. The aqueous residue obtained was partitioned between $CHCl_3$ and water, and the organic layer was concentrated and dried over $MgSO_4$ to give a crude extract (usually 3-4% of the dry weight of the animal). A mixture of furanocembrenolides was eluted from a silica gel column separation of the crude extract using mixtures of 50-100% EtOAc/CH_2Cl_2. This mixture was further purified using Florisil chromatography and silica hplc (5μ silica column, 60-70% EtOAc in isooctane) to give pure compounds in varying amounts from each gorgonian (0.2-3% crude extract and 0.01-0.08% dry weight). For example: Lophogorgia chilensis (L-2) collected in San Diego (1979) and stored in ethanol, yielded 0.2 g (51) (0.7% crude extract, 0.02% dry weight). L. chilensis (L-6) collected in Los Coronados (1980) and stored in isopropyl alcohol, yielded 0.4 g each lophotoxin (45) and deoxylophotoxin (47) (2% crude extract, 0.04% dry weight). L. rigida (V-5), collected in Los Frailes (1979) and stored in methanol, yielded 1.6 g lophotoxin (45) (2.7% crude, 0.08% dry weight), 1.0 g lopholide (46) (1.7% crude, 0.05% dry weight) and approximately 0.1 g each deoxylophotoxin (47), acetoxypukalide (48) and pukalide aldehyde (49) (0.2% crude, 0.01% dry weight). All other Lophogorgia spp. were stored frozen and extracted initially with ethanol, except for L. cuspidata (V-7) from Los Frailes which was stored in methanol. The distribution of compounds from all these sources is indicated in Figure 18. The compounds were eluted in the

following order of increasing polarity: pukalide, lopholide, pukalide aldehyde, lophotoxin, acetoxypukalide, deoxylophotoxin and the rearranged aldehyde.

Lophotoxin (45, Table 1). ^{1}H NMR (1:1 $CDCl_3$, d_6-benzene) δ 9.76 (1H, s) 6.54 (1H, s), 4.99 (1H, bs), 4.97 (1H, d (8.3)), 4.93 (1H, bs), 4.43 (1H, dd (4,3)), 4.05 (1H, bs), 3.96 (1H, ddd (12, 10, 5)), 3.70 (1H, s), 2.96 (1H, dd (-18, 5)), 2.87 (1H, dd (-18, 12)), 2.47 (1 H, ddd (-15, 10, 8.3)), 2.11 (1H, dd (-16, 3)), 2.00 (3H, s), 1.92 (3H, s), 1.83 (1H, dd (-16, 4)), 1.58 (1H, d (-15)), 0.96 (3 H, s); HRMS: M^+ obs. 416.1472 (23.1) (calc. 416.1471 for $C_{22}H_{24}O_8$), 356.1251 (22.5) for M^+-HOAc, 149.0599 (72.8), 121.0650 (100). LRMS (70 eV): M^+ 416, 401, 356, 284, 256.

Lopholide (46, Table 3). HRMS: M^+ obs. 446.1568 (23.0), (calc. 446.1577 for $C_{23}H_{26}O_9$), 386.1370 (12.3) for M^+ -HOAc, 354.1087 (190), 168.0426 (100.0), 121.0658 (74.3); LRMS (70 eV): M^+ 446, 430, 409, 386, 354, 205, 149.

Deoxylophotoxin (47, Table 7). HRMS: M^+ obs. 400.1521 (19.1) (calc. 400.1522 for $C_{22}H_{24}O_7$), 340.1313 (35.0) for M^+ -HOAc, 283.0963 (32.5), 231.1028 (55.0), 178.0634 (75.9), 138.0318 (100.0), 136.0524 (86.9), LRMS (70 eV) M^+ 400, 340, 283, 282.

Acetoxypukalide (48, Table 4). ^{13}C NMR (d_6-benzene, 20 MHz) 170.0 (s), 170.0 (s), 163.6 (s), 160.2 (s), 150.9 (d), 149.7 (s), 149.0 (s), 134.8 (s), 114.7 (s), 111.0 (t), 107.0 (d), 77.4 (d), 69.2 (d), 56.5 (s), 55.0 (d), 50.9 (q), 39.8 (t), 37.1 (d), 36.2 (t), 34.7 (t), 20.9 (q), 20.3 (q), 19.6 (q); HRMS: M^+ obs. 430.1631 (22.7) (calc.

430.1628 for $C_{23}H_{26}O_8$), 370.1415 (23.0) for M^+ -HOAc, 261.1126 (39.9), 208.0739 (100.0), 168.0422 (90.2), 165.0560 (83.2); LRMS (70 eV): M^+ 430, 368, 342, 285.

Pukalide aldehyde (49, Table 8). HRMS: M^+ obs. 342.1470 (68.4) (calc. 342.1467 for $C_{20}H_{22}O_5$), 188.0840 (65.0), 178.0637 (76.2), 165.0560 (16.4), 138.0320 (100.0); LRMS (70 eV): M^+ 342, 285.

Pukalide (50, Table 2). HRMS: M^+ obs. 372.1577 (49.6) (calc. 372.1573 for $C_{21}H_{24}O_6$), 340.1300 (31.0), 208.0736 (96.9), 165.0566 (100.0).

Rearranged aldehyde (51, Table 9). HRMS: $M^+ - H_2O$ obs. 444.1778 (12.2) (calc. 444.1784 for $C_{24}H_{28}O_8$: $M^+ = C_{24}H_{30}O_9$ not seen), M^+ -OEt obs. 417.1584 (4.2) (calc. 417.1549 for $C_{22}H_{25}O_8$), 196.0741 (62.5), 168.0424 (76.7), 165.0556 (100.0), 121.0652 (71.7); LRMS (70 eV) M^+ 462, 447, 444 ($M^+ - H_2O$), 433, 402 (M^+ -HOAc), 387, 373.

Lopholide (46) from MnO_2/NaCN oxidation of lophotoxin (45). Activated MnO_2 was prepared using the following procedure.[69] A solution of 30.2 gm $MnSO_4.4H_2O$ in 40 ml H_2O and 31.8 ml of 40% NaOH (aqueous) solution was added to a hot solution of 26.1 g $KMnO_4$ (oil bath, 80-90°C) over a period of one hour, resulting in the precipitation of $MnO_2.H_2O$ as a fine brown solid. After addition was completed, the mixture was stirred for one hour, centrifuged, and washed with water several times. The solid was rinsed with water, filtered, and dried under a vacuum for five to twelve hours. MnO_2 was stored in cyclohexane or benzene. The MnO_2 powder was activated by azeotroping with benzene or toluene several times and oven dried, resulting in 25 g MnO_2. Activated MnO_2, 372 mg,

15 µl glacial HOAc, and 66 mg NaCN were added to 58.7 mg (1.4×10^{-4} moles) lophotoxin in 8 ml MeOH.[55] The reaction mixture was stirred at room temperature for 21 hours. The product, 45.7 mg, was recovered after filtration of the solid material and partitioning between CH_2Cl_2 and H_2O. Hplc separation of the reaction mixture in 60% EtOAc/TMP gave 12.4 mg (2.8×10^{-5} M) pure lopholide (20% yield) $[\alpha]_D^{25} = +0.4^o$ (c 1.0, $CHCl_3$) (identical to the natural product by ^1H NMR).

Hydroxylopholide (73) from MnO_2/NaCN oxidation of lophotoxin (45). From the same reaction mixture of lophotoxin, MnO_2, HOAc, and NaCN in MeOH as described above, 12.3 mg (3×10^{-5} moles) (21% yield) of a new compound, hydroxylopholide (73) was isolated by hplc separation of the reaction mixture in 60% EtOAc/TMP. ^1H NMR ($CDCl_3$) δ 6.5 (1H, s), 5.3 (1H, bs), 5.0 (1H, bs), 4.9 (1H, bm), 4.1 (1H, d), 4.1 (1H, s), 4.0 (1H, bs), 3.9 (1H, m), 3.8 (3H, s), 3.3 (1H, m), 3.1 (1H, d), 2.9 (1H, m), 2.5 (1H, m), 2.1 (1H, m), 1.9 (3H, s), 1.7 (1H, m), 1.1 (3H, s); IR ($CHCl_3$) 3600, 2950, 1786, 1712, 1570, 1430, 1070, 920 cm^{-1}; LRMS (70 eV) M^+ 404 for $C_{21}H_{24}O_8$, 389, 372.

Deoxyacetoxypukalide (74) from $CrCl_2$ reduction of lopholide (46). A $CrCl_2$ solution was prepared as follows:[52] 1.22 g $CrCl_3$ was dissolved in 4 ml H_2O, and flushed with Ar while stirring in ice bath. Next, 2.08 g Zn dust was added slowly to the solution. The reaction mixture was stirred, flushing with Ar, for 20 min, after which 2.8 ml conc. HCl was added. The reaction was stirred for 2 more hours. The clear blue $CrCl_2$ solution was decanted, removing the ZnCl and $CrCl_3$ precipitates. Lopholide, 74 mg (1.7×10^{-4} moles), was dissolved in 2 ml acetone and 0.7 ml HOAc and 1.2 ml $CrCl_2$ solution was added slowly,

while continuously flushing with Ar, over a period of 20 minutes. The reaction mixture was stirred, under Ar, at room temperature for 3 hours. The reaction was quenched by adding H_2O, and then extracted 2 x with CH_2Cl_2, 2 x with $NaHCO_3$ and dried over $MgSO_4$, to give 11.5 mg of a mixture of products. This mixture was separated by hplc in 50% EtOAc/TMP to give 1 mg (2.4×10^{-6} moles) (1.4% yield) of deoxyacetoxypukalide (74): $[\alpha]_D^{25} = +30.0^\circ$ (c = 0.1, $CHCl_3$), UV: λ_{max}^{MeOH} 237 (1000), 218 (2000) nm; IR ($CHCl_3$) 2899, 1750, 1724, 1710, 1440 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.0 (1H, bs), 6.5 (1H, s), 6.1 (1H, bs), 5.5 (1H, m), 5.0 (1H, m), 4.8 (2H, bs), 3.9 (3H, s), 2.1 (3H, s), 2.0 (3H, s), 1.8 (3H, bs). HRMS did not give clear fragmentation.

Deoxyacetoxypukalide (74) from $CrCl_2$ reduction of acetoxypukalide (48). A $CrCl_2$ solution was prepared as previously described for the reduction of lopholide. 1.0 ml of the $CrCl_2$ solution was added to 35 mg (8.1×10^{-5} moles) acetoxypukalide and 0.7 ml HOAc in 2 ml acetone. The reaction mixture was stirred, under Ar, at room temperature for 3 hours and the reaction mixture was treated as before to give 17.1 mg of a crude product. Separation of the crude product with hplc in 50% EtOAC/TMP gave 6 mg (1.4×10^{-5} moles) (17% yield) deoxyacetoxypukalide (74) $[\alpha]_D^{23} = +18.3^\circ$ (c = 0.6, $CHCl_3$), identical by ^1H NMR analysis to the same compound produced earlier by the reduction of lopholide.

Deoxypukalide (75) from Zn-Cu couple of pukalide (50). The Zn-Cu couple was prepared as described in Fieser and Fieser.[70] Pukalide, 20 mg (5.4×10^{-5} moles), was warmed in 50 ml EtOH with ~50 mg Zn-Cu couple to give 6 mg (1.7×10^{-5} moles) (31% yield) deoxypukalide (75). ^1H NMR

($CDCl_3$): δ 6.95 (1H, s), 6.43 (1H, s), 6.10 (1H, s), 5.00 (1H, m), 4.92 (1H, bs), 4.89 (1H, bs), 3.82 (3H, s), 3.50 (1H, dd (17, 13)), 3.14 (1H, t (12)), 2.75 (1H, dd (12,4)), 2.67 (1H, dd (17,4)), 2.43 (2H, m), 2.10 (1H, m), 2.00 (3H, s), 1.80 (3H, s).

Difference nOe study of lophotoxin (45). Lophotoxin, 4.3 mg (0.01 M) in 1 ml 0.5% TMS/$CDCl_3$, was prepared for a nOe experiment by carefully degassing with Ar for 30 minutes. ^1H NMR decoupling experiments identified the C-7 epoxide methine at δ 4.09, the C-19 epoxide methyl at δ 1.14 and the C-11 lactone epoxide methine at δ 4.17. The decoupler power was then decreased until the irradiated peaks were barely nulled. The decoupler was gated for on delay only with a 90° pulse angle, and the delay time between irradiations was increased from 2 to 15 sec. Difference nOe techniques[60] were employed which revealed no nOe effect at δ 4.09 by irradiating the methyl groups at δ 1.14, thus indicating a trans epoxide at C-7, C-8. Irradiation at δ 1.14 resulted in a significant enhancement of the C-11 methine product at δ 4.17, indicating their proximity in space.

Difference nOe study of acetoxypukalide (48). Acetoxypukalide, 3.0 mg (7×10^{-3} M) in 1 ml 0.5% TMS/$CDCl_3$, was prepared for a nOe experiment by degassing with Ar for 30 min. ^1H NMR decoupling experiments identified the C-7 epoxide methine at δ 4.09 and the C-19 epoxide methyl at δ 0.97, and the C-11 lactone olefinic proton at δ 7.29. The decoupler was gated and the delay (D_5) increased to 15 seconds, as before. Difference nOe spectra revealed no enhancement between the epoxide methyl and methine protons, indicating the same trans relationship as in lophotoxin and pukalide. Irradiation of the epoxide methyl

resulted in an enhancement of the lactone olefinic proton, and vice versa, indicating their proximity in the center of the ring.

Hexahydropukalide (77) from catalytic hydrogenolysis of acetoxypukalide (48). Acetoxypukalide, 25 mg (5.8×10^{-5} moles) in 20 ml EtOAc, was treated with H_2 over Pd/C at room temperature for 16 hours. The solution was filtered through a Florisil pipette column to recover 18 mg of a mixture of products. The components of the mixture were purified by hplc using 75% EtOAc/TMP to give 12 mg (3.2×10^{-5} moles) (55% yield) hexahydropukalide and 3 mg (6.8×10^{-6} moles) (12% yield) of hexahydroacetoxypukalide (acetoxyl group still present). Hexahydropukalide (77) was identical by all spectral feature to that synthesized by hydrogenation of pukalide.[49] $[\alpha]_D^{27}$ = +34.0° (c = 0.3, $CHCl_3$); IR ($CHCl_3$): 3550, 2960, 1751, 1704, 1603, 1560, 910 cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.5 (1H, s), 4.7 (1H, bm), 3.8 (3H, s), 3.3 (1H, dd (-15, 2)), 3.0 (1H, d (-15)), 2.8 (1H, d (15)), 2.5 (3H, m), 2.2 (1H, d (-15)), 1.40 (3H, s), 1.0 (3H, d), 0.9 (3H, d). HRMS: M^+ obs. 378.2043 (5.5) (calc. 378.2042 for $C_{21}H_{30}O_6$), 346.1777 (56.5) (M^+-MeOH), 303.1237 (100.0). LRMS (70 eV): M^+ 378, 346, 303, 221.

Hexahydropukalide (77) from catalytic hydrogenation of pukalide (50). Pukalide, 98 mg (2.6×10^{-4} moles) in 25 ml EtOAc, was hydrogenated over Pd/C at room temperature for ten hours. The solution was filtered and separated by HPLC in 75% EtOAc/TMP to give 42.8 mg (1.1×10^{-4} moles) (42% yield) hexahydropukalide, identical by ^1H NMR and optical rotation to that produced by Scheuer previously[49] and by hydrogenolysis of acetoxypukalide. $[\alpha]_D^{27}$ = +33.5° (c = 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 6.54 (1H, s), 4.71 (1H, m), 3.81 (3H, s), 3.27 (1H, dd (-

15, 3)), 3.03 (1H, d (-15)), 2.78 (1H, d (-15)), 2.50 (3H, m), 2.21 (1H, m), 1.37 (3H, s), 0.96 (3H, d (7)), 0.93 (3H, d (7)).

Acetoxypukalide (48) from MnO_2/NaCN oxidation of deoxylophotoxin (47). Activated MnO_2, 0.5 g, prepared as described previously, was added to a solution containing 200 µl HOAc, 90 mg NaCN and 48 mg (1.2×10^{-4} moles) deoxylophotoxin in 3 ml CH_2Cl_2 and 6 ml MeOH. The reaction mixture was stirred at room temperature for fifteen hours and treated as before to give 3.9 mgs (9.1×10^{-6} moles) (8% yield) acetoxypukalide and 6.0 mgs (1.5×10^{-5} moles) (13% yield) starting material. The synthetic acetoxypukalide had an $[\alpha]_D^{23} = +33^o$ (c = 0.4, $CHCl_3$) and was identical to the natural product $[\alpha]_D^{23} = +20.8^o$ (c = 1.0, $CHCl_3$) by comparison of ^1H NMR data: δ ($CDCl_3$): 7.29 (1H, s), 6.40 (1H, s), 5.84 (1H, d (6)), 5.24 (1H, bm), 4.99 (1H, bs), 4.88 (1H, bs), 4.09 (1H, s), 3.95 (1H, m), 3.79 (3H, s), 3.04 (1H, dd (-18, 12)), 2.88 (1H, dd (-18, 3)), 2.52 (2H, m), 2.19 (1H, dd (-15, 3)), 2.00 (3H, s), 1.88 (3H, bs), 0.97 (3H, s).

Pukalide (50) from MnO_2/NaCN oxidation of pukalide aldehyde (49). Activated MnO_2, 0.3 g, was added to a solution containing 10 µl HOAc, 30 mg NaCN and 30 mg (8.8×10^{-5} moles) pukalide aldehyde in 3 ml MeOH. The mixture was stirred at room temperature for fifteen hours and next treated by filtration followed by extraction with CH_2Cl_2 to give 8.2 mg (2.2×10^{-5} moles) (25% yield) pukalide, $[\alpha]_D^{26} = +30.3^o$ (c = 0.6, $CHCl_3$), which was identical with the natural product by ^1H NMR comparison. δ ($CDCl_3$) 7.1 (1H, bs), 6.3 (1H, s), 5.20 (2 H, bs), 4.9 (1H, bs), 4.0 (1H, s), 3.8 (3H, s), 1.8 (3H, s), 1.0 (3H, s).

Chapter III

Diketone Cembrenolides from the Pacific Gorgonian *Lophogorgia alba*

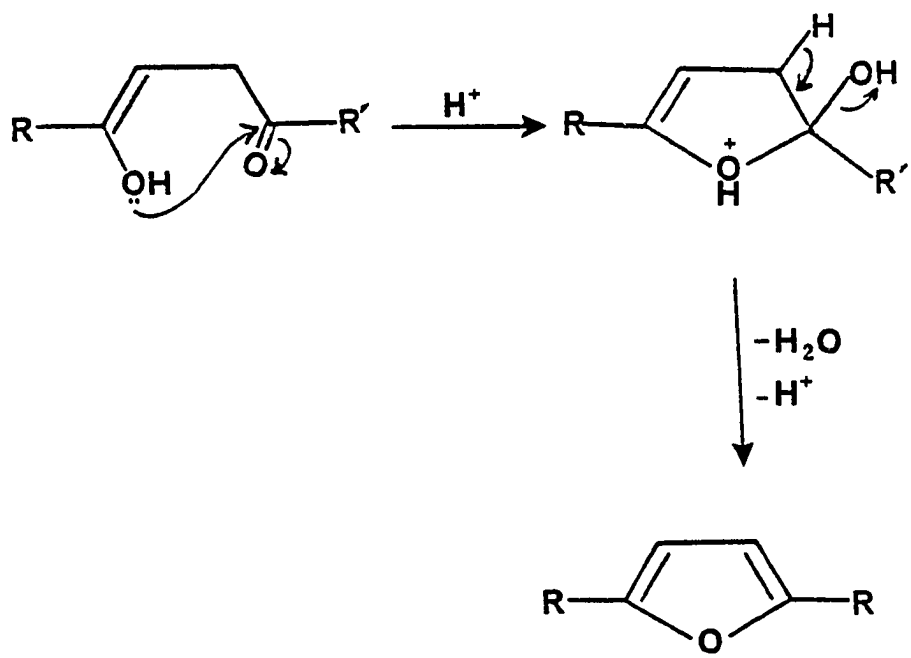
During the search for novel metabolites from *Lophogorgia* species, seven furan-derived cembrenolides **45**-**51** were isolated from four different *Lophogorgia* species. Three additional cembrenolides were isolated from one of these, *Lophogorgia alba*, collected in Bahia Tenacatita (near Acapulco in Pacific Mexico). The work described in this chapter has been published previously elsewhere. This is the first report of natural products isolated from a gorgonian that lacks the symbiotic algae, zooxanthellae.[71] Although a number of cembrenolides and other terpenoid compounds have previously been isolated from Caribbean gorgonians,[16,46] their biosynthetic origin has been questioned due to the presence of symbiotic algae in their tissues. The isolation of these cembrenolides from a gorgonian which does not possess zooxanthellae indicates that the algae are not essential to the production of secondary metabolites in some gorgonians. In fact, none of the Pacific gorgonians examined during this thesis work possess zooxanthellae. Therefore the animals must be capable of *de novo* terpene biosynthesis. Although many higher plants and some marine organisms (e.g., algae, gorgonians with zooxanthellae, sponges and molluscs) produce terpenes, very few terrestrial animals are capable of *de novo* terpene biosynthesis. An exception to this exists in some families of insects (particularly the termites). Isolation of new terpenes from this animal source represents

an important contribution to the exploration of terpene biosynthesis in nonphotosynthetic organisms.

The three 1,4-diketone cembrenolides described in this chapter are interesting from a biosynthetic viewpoint because of their simultaneous coisolation with the furan-containing cembrenolides described in chapter II. 1,4-Diketones are known to give furans when treated with acid. This occurs by intramolecular addition of an enol alcohol to a ketone to form a hemiacetal (or hemiketal), which eliminates water to form a furan (Figure 38).[73] Therefore, the 1,4-diketone cembrenolides isolated from Lophogorgia alba may represent possible precursors to this group of furan-containing cembrenolides. It is interesting to note that the 1,4-diketone compounds were isolated from only one collection of Lophogorgia alba, whereas eight collections of four species of Lophogorgia contained varying amounts of the furanocembrenolides. If the diketone cembrenolides are precursors to the furanocembrenolides, one would expect to find them present in other collections of Lophogorgia spp.

The chemical variation observed in two collections of L. alba, is interesting from a taxonomic point of view. Taxonomic assignment of both AH-56 and AH-74 as Lophogorgia alba was made reluctantly, and with some difficulty, by a gorgonian expert. This difficulty was due to minor morphological differences between both of the voucher samples as compared with the museum type specimen.[74] The fact that only one collection of L. alba, AH-74 from Bahia Tenacatita, contains the diketone compounds suggests that these are, in reality, different species. This conclusion is based on their chemical and morphological differences. Alternatively, the same species of gorgonians may be producing different

Figure 38. Formation of furans from 1,4 diketones[73]



chemistry at different locations.

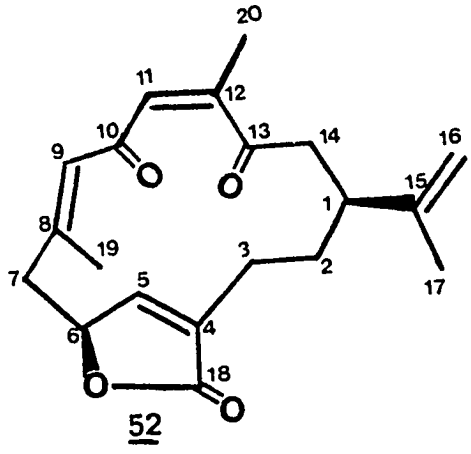
The isomeric diketones 52 and 53 were isolated by repeated column and high performance liquid chromatography (hplc) of the chloroform/methanol extract of the pink sea whip Lophogorgia alba (Duch. & Mich.), collected in Pacific Mexico. Both compounds were sensitive to silica gel chromatography; therefore Florisil and rapid elution chromatography techniques were employed. Isolophodione (53) was found as the major terpenoid component (0.51% of the extract). The corresponding isomer, lophodione (52), was less concentrated (0.36% extract). An epoxide isomer, epoxylophodione (54) was also a minor component. The structure of lophodione (52) was assigned by X-ray crystallography. The structures of isolophodione (53) and epoxylophodione (54) have been assigned based upon interconversion with lophodione and by proton difference decoupling and nOe experiments.

A. Structure elucidation of lophodione (52)

Lophodione (52) crystallized from one hplc fraction, m.p. = 172-174°, and a molecular formula of $C_{20}H_{24}O_4$ was assigned by a combination of low resolution mass and ^{13}C NMR spectrometry (Table 10). An IR absorption at 1751 cm^{-1}, similar to that in pukalide[49], suggested an α,β-unsaturated-γ-lactone. This was confirmed by ^1H NMR bands at δ 6.97 (1H, bs) and 5.31 (1H, m) and ^{13}C NMR bands at 173.1 (s), 148.4 (d), 134.1 (s) and 80.1 (d) ppm. Additional IR absorptions at 1669 and 1616 cm^{-1} indicated the presence of an α,β-unsaturated ketone with possible further conjugation. This functionality was supported by the two carbonyl bands in the ^{13}C NMR spectrum at 190.7 (s) and 205.4 (s) ppm.

Table 10

Spectral Data for Lophodione (52)



52

$C_{20}H_{24}O_4$, needles, m.p. 172-174°C; $[\alpha]_D^{23}$ = -275° (c = 0.8,$CHCl_3$); UV: λ_{max}^{MeOH} 267 nm (8000); IR ($CHCl_3$) 2950, 1751, 1669, 1616, 1433, 1202, 1115 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	41.3
2	--	30.3 (J_R = 16.1)
3	--	25.7 (16.7)
4	--	134.1
5	6.97 bs	148.4 (37.3)
6	5.31 m	80.1 (28.9)
7	3.04 dd (-13.3,4.6), 2.61 bd (-13.3)	43.5 (18.9)
8	--	156.3
9	6.12 bs	125.9 (31.2)
10	--	190.7
11	6.42 bs	133.4 (32.5)
12	--	144.8
13	--	205.4
14	2.64 bd (-14.0), 2.40 m (-14.0)	45.7 (17.2)
15	--	145.5
16	4.97 bs, 4.72 bs	115.9 (27.7)
17	1.60 bs	17.0 (16.0)
18	--	173.1
19	2.19 bs	22.6 (18.5)
20	1.84 bs	21.5 (16.7)

Evidence for the presence of an enone with extended conjugation also came from the UV absorption at 267 nm (ϵ = 8000).

The α,β-unsaturated-γ-lactone, two carbonyl carbons and three other olefins indicated by ^{13}C NMR, provided eight of the nine degrees of unsaturation required by the molecular formula. Therefore lophodione was monocarbocyclic. The presence of four methyl groups or vestiges in the ^{13}C and ^{1}H NMR spectra (the lactone carbonyl plus three olefinic methyl groups) suggested that lophodione contained a cembrenolide ring system with an isopropenyl group and two trisubstituted olefins.

^{1}H NMR decoupling and nuclear Overhauser enhancement (nOe) experiments[59] allowed the stereochemistry of the two trisubstituted olefins to be determined.[75] Proton decoupling showed that the methyl group at δ 1.84 was coupled to the olefinic proton at δ 6.42, and the methyl group at δ 2.19 was coupled to the olefinic proton at δ 6.12. Irradiation of the methyl group at δ 1.84, under nOe conditions, resulted in an enhancement of 16% in the integrated intensity of the proton at δ 6.42. This indicated a Z configuration for the olefin. Irradiation of the methyl group at δ 2.19 under the same conditons did not result in significant enhancement in the proton at δ 6.12. The two groups are therefore assigned as trans to one another. This resulted in an E configuration for the second trisubstituted olefin.

Due to the predominant lack of distinguishable coupling in the ^{1}H NMR spectrum, we were not able to place the functional groups in the cembrenolide ring system. Therefore, suitable crystals of lophodione (52) were submitted for X-ray diffraction analysis to Dr. Jon Clardy of

Cornell University.[71] The X-ray experiment defined the relative stereochemistry of both C(1) and C(6) as (S*). The C(8)-C(9) double bond was assigned the E configuration and the C(11)-C(12) configuration was assigned as Z. The double bonds hold the 14-membered ring in a fairly open conformation as illustrated in Figure 35. The enone-containing fragment C(8)-C(9)-C(10)-O(23) is completely conjugated as judged by a dihedral angle of 180°. The C(13)-O(24) carbonyl shows little p-orbital overlap with the C(11)-C(12) double bond as recognized by the dihedral angle of 49°. The C(10)-O(23) carbonyl is also isolated from the C(11)-C(12) olefin with a dihedral angle of 42°. Figure 40 is a stereodrawing with ^{1}H NMR assignments which illustrates the proximity of the olefinic protons in 52 which showed positive nOe results.

In addition to lophodione, a very closely related crystalline compound, isolophodione, was also isolated and its structure determined by similar methods.

B. Structure elucidation of isolophodione (53)

Isolophodione (53) crystallized from one hplc fraction, m.p. = 172-175°, and possessed the same molecular formula as lophodione. The major structural features of lophodione (α,β-unsaturated-γ-lactone, two trisubstituted olefins, isopropenyl group and two ketones) were present in isolophodione by examination of the IR, UV, ^{1}H and ^{13}C NMR spectra. The spectra, however, showed very slight differences in chemical shifts (Tables 10 and 11). The strong correlation of all spectral data with 52 suggested the two compounds were geometrical isomers of one another at the trisubstituted olefins. Isomerization of lophodione to

Figure 39. A computer generated drawing of the X-ray structure of lophodione (52)

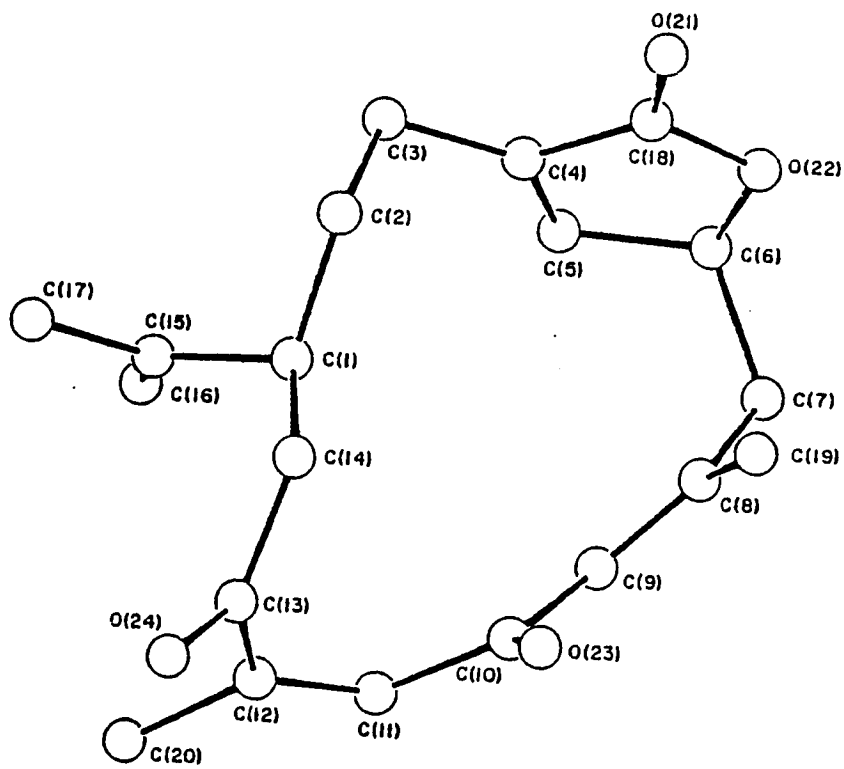
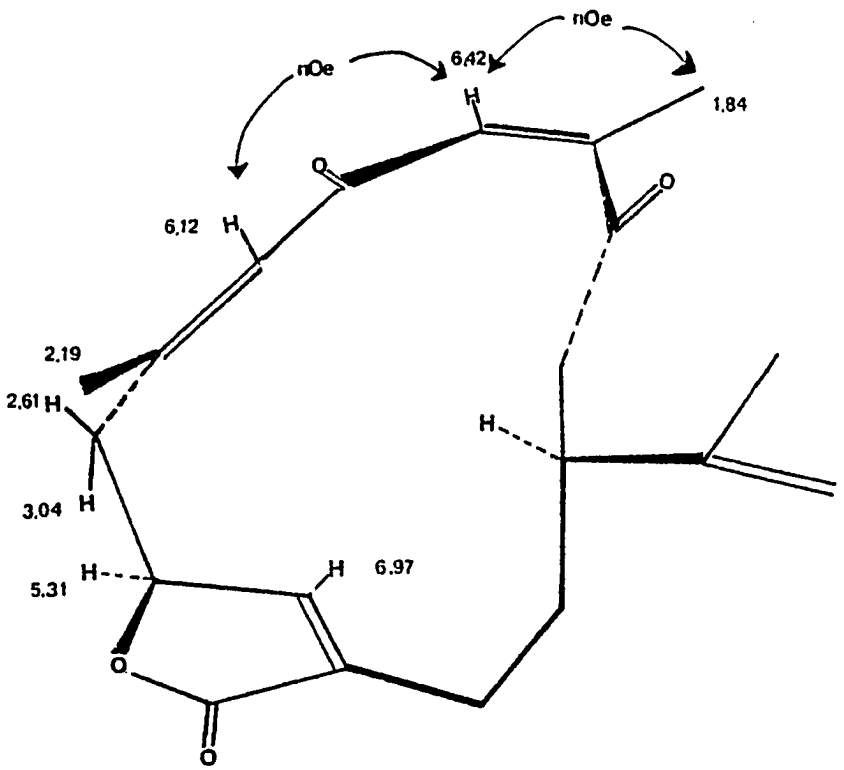


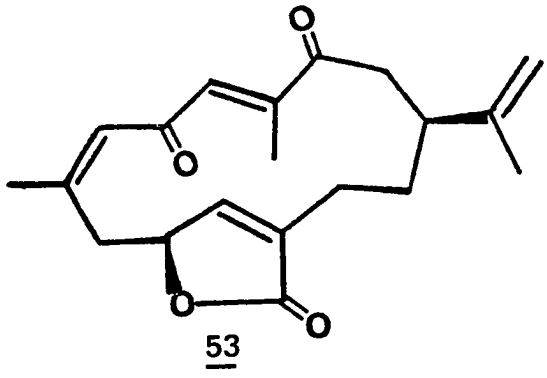
Figure 40. Chemical shifts and nOe results for selected protons in lophodione (52)



52

Table 11

Spectral Data for Isolophodione (53)



53

$C_{20}H_{24}O_4$, needles, m.p. 172-175°C; $[\alpha]_D^{18}$ = -232° (c = 1.0, $CHCl_3$); UV: λ_{max}^{MeOH} = 261 nm (10,000); IR ($CHCl_3$): 2941, 1754, 1675, 1618, 1439, 1208, 1115 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	40.5
2	--	31.2
3	--	22.2
4	--	133.1
5	7.00 bs	148.6 (J_R = 37.5)
6	4.99 bm	77.9 (25.2)
7	2.67 m, 2.41 m	37.3
8	--	146.4
9	6.26 bs	128.0 (33.6)
10	--	189.1
11	6.15 bs	126.5 (31.0)
12	--	154.1
13	--	208.3
14	--	44.8
15	--	146.4
16	4.90 bs, 4.70 bs	114.0 (26.8)
17	1.68 bs	19.1 (15.4)
18	--	173.1
19	2.04 bs	26.9 (17.8)
20	1.89 bs	22.2 (16.0)

isolophodione, and vice versa, was accomplished using iodine in benzene (Figure 41). Since the C(8) E and C(11) Z olefins in lophodione could potentially isomerize to either the corresponding E,E; Z,Z or Z,E olefins, this left the geometry of the two trisubstituted olefins in isolophodione still in question.

Irradiation at δ 2.04 in the ^{1}H NMR spectrum of 53 under ^{1}H NMR nOe conditions, caused considerable enhancement of the proton at δ 6.26. This result illustrated that one olefin was in the Z configuration. Irradiation of the methyl group at δd 1.89 yielded no enhancement in the olefin proton at δ 6.15. Clearly the other trisubstituted olefin was in the E configuration. Both olefins in 53 had therefore isomerized (E,Z to Z,E). Irradiation of the two olefinic protons under nOe conditions resulted in enhancements which demonstrated their proximity in space.

The ring conformation of isolophodione is similar to lophodione based upon analysis molecular models. In both compounds it is possible for only one of the trisubstituted olefins to be conjugated with one of the ketones. Shifts in the ^{13}C NMR spectrum of isolophodione at 189.1 (s) and 208.3 (s) ppm, reflecting both a conjugated and a nonconjugated ketone,[76] substantiated this. The ^{13}C NMR data in Figure 42 also support this conformation by demonstrating the changes in polarization of the enone olefins in the conversion from E to Z.[77]

C. Structure elucidation of epoxylophodione (54)

In the course of repeated isolation of lophodione and isolophodione from extracts of Lophogorgia alba, one of the related minor constituents found appeared to be very closely related to lophodione. The

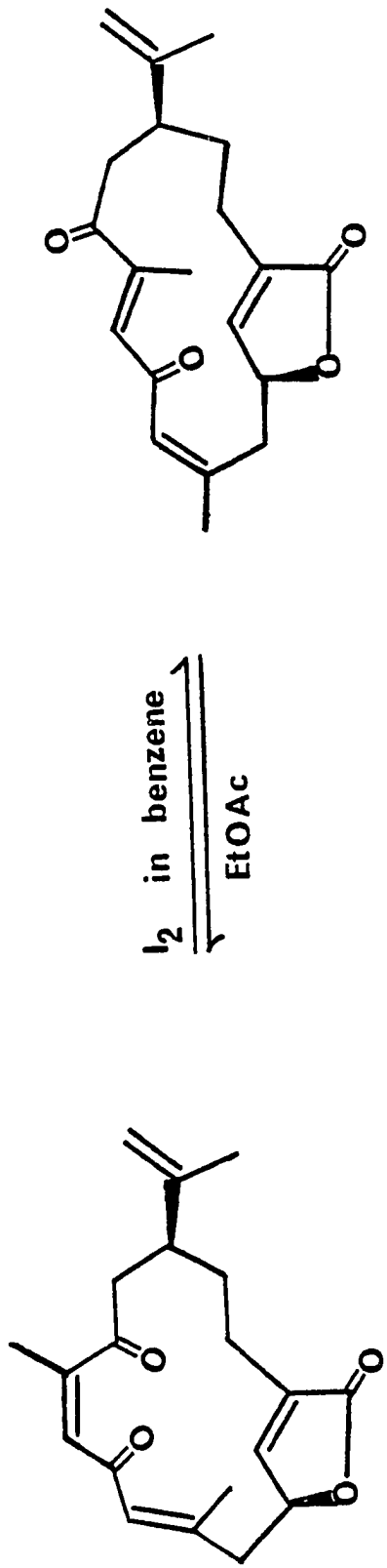


Figure 41. Interconversion of lophodione and isolophodione

Figure 42. ^{13}C NMR comparison of the E and Z olefins in 52-54

Olefin	Stereochemistry	52	53	54
C(8)	E	156.1		157.9
C(9)		125.9		123.2
C(11)	E		154.1	
C(12)			126.5	
C(8)	Z		146.4	
C(9)			128.0	
C(11)	Z	133.4		
C(12)		145.5		

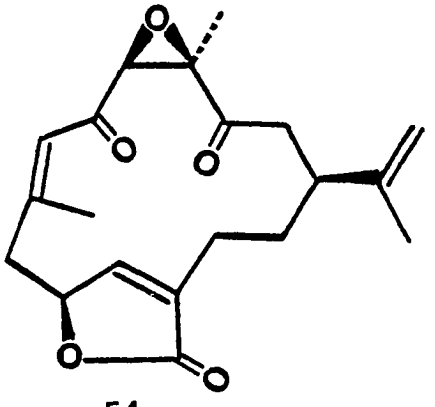
molecular formula of epoxylophodione (54) was determined as $C_{20}H_{24}O_5$, based on high resolution mass and ^{13}C NMR spectrometry (Table 12). The ^1H NMR spectrum of epoxylophodione was almost identical to that of lophodione. The major difference was the absence of one olefinic proton and the appearance of a new epoxide proton at δ 3.47 (Tables 10 and 12). In addition, one of the single olefinic methyl groups in lophodione was shifted upfield from δ 1.89 to 1.58 in epoxylophodione. The absence of any alcoholic absorption in the IR spectrum of 54 indicated that epoxylophodione was an epoxide derivative of one of the trisubstituted olefins in lophodione. ^{13}C NMR absorptions at 65.8 (d) and 66.8 (s) ppm supported this proposal, along with the high degree of correlation of the remaining ^{13}C NMR bands with those of lophodione (Table 13).

^1H NMR decoupling studies of expoxylophodione allowed the clear delineation of every proton in the molecule, placing the position of the epoxide at C(11)-C(12). The C(8)-C(9) olefin was determined to be intact on the basis of the strong similarities in the chemical shifts and coupling constants of the protons at C(6), C(7) and C(19) to those in lophodione (Table 14). This correlation was lacking in the NMR characteristics of isolophodione. An IR absorption at 1736 cm^{-1}, and the ^{13}C NMR absorption at 205.2 ppm, indicated the presence of an isolated ketone. This functionality could only be accounted for by structure 54 for epoxylophodione.

Difference ^1H NMR nOe studies were employed to define the stereochemistry of the epoxide and the remaining enone olefin of epoxylophodione. Irradiation of the methyl group at δ 1.58 resulted in a enhancement of the epoxide proton at δ 3.47, indicating a cis epoxide.

Table 12

Spectral Data for Epoxylophodione (54)



54

$C_{20}H_{24}O_5$); $[\alpha]_D^{23} = -114^\circ$ (c = 1.1, $CHCl_3$); UV: λ_{max}^{MeOH} 250 nm (13,000); IR ($CHCl_3$): 3021, 1757, 1736, 1678, 1613, 1443, 1241 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)	^{13}C NMR (50 MHz, $CDCl_3$)
1	2.71 m	42.8
2	1.82 m, 1.42 m	31.0
3	2.43 m, 2.24 m	22.9
4	--	135.7
5	7.22 bs	147.6
6	5.24 m (4.6,3.2,1)	79.5
7	2.99 dd (-13.4,4.6), 2.67 dd (-13.4,3.2)	42.1
8	--	157.9
9	6.23 bs	123.2
10	--	192.7
11	3.47 s	65.6
12	--	66.8
13	--	205.2
14	2.50 dd (-16.1,6.8), 2.32 (-16.1,7.3)	43.8
15	--	146.4
16	4.79 bs, 4.70 bs	111.9
17	1.68 bs	19.1
18	--	173.1
19	2.15 bs	22.8
20	1.58 bs	19.4

Table 13

^{13}C NMR Data for Compounds 52-54

	Lophodione (52)[a]		Isolophodione (53)[a]		Epoxylophodione (54)[b]
C	^{13}C ppm	J_R	^{13}C ppm	J_R	^{13}C ppm
1	4.13		40.5		42.8
2	30.3	16.1	31.2		31.0
3	25.7	16.7	22.2		22.9
4	134.1		133.1		135.7
5	148.4	37.3	148.6	37.5	147.6
6	80.1	28.9	77.9	25.2	79.5
7	43.5	18.9	37.3		42.1
8	156.3		146.4		157.9
9	125.9	31.2	128.0	33.6	123.2
10	190.7		189.0		192.7
11	133.4	32.5	126.5	31.0	65.6
12	144.8[c]		154.1		66.8
13	205.4		208.3		205.2
14	45.7	17.2	44.8		43.8
15	145.5[c]		146.4		146.4
16	115.9	27.7	114.0	26.8	111.9
17	17.0	16.0	19.1	154.0	19.1
18	173.1		173.1		173.1
19	22.6	18.5	26.9	17.8	22.8
20	21.5	16.7	22.2	16.0	19.4

[a]Assignments are based upon multiplicities, chemical shifts and residual coupling constants as determined by single frequency off-resonance and broad band decoupling techniques. Spectra were recorded on a Varian CFT-20 spectrometer in $CDCl_3$ with internal TMS. Measurement of the residual coupling constants for isolophodione were recorded in 75% $CDCl_3$/benzene-d_6 solution.

[b]Assignments are based upon chemical shifts and proton substitution as determined by INEPT (insensitive nucleus enhancement phase transfer) methods, and two level broad band decoupling experiments. Spectra were recorded on a Nicolet 50 MHz ^{13}C multinuclear wide-bore spectrometer.

[c]Assignments may be reversed.

Table 14

^{1}H NMR Data for Compounds 52-54

	Lophodione (52)		Isolophodione (53)		Epoxylophodione (54)	
C	δ	Mult. J	δ	Mult.	δ	Mult. J
1					2.71	m
2					1.82	m
					1.42	m
3					2.43	m
					2.24	m
5	6.97	bs	7.00	bs	7.22	bs
6	5.31	m	4.99	m	5.24	m
	2.61	bd -13.3	2.41	m	2.67	dd -13.4, 3.2
9	6.12	bs	6.26	bs	6.23	bs
11	6.42	bs	6.15	bs	3.47	s
14	2.64	bd -14.0			2.50	dd -16.1, 6.8
	2.40	m			2.32	dd -16.1, 7.3
16	4.97	bs	4.90	bs	4.79	bs
	4.72	bs	4.70	bs	4.70	bs
17	1.60	bs	1.68	bs	1.68	bs
19	2.19	bs	2.04	bs	2.15	bs
20	1.84	bs	1.89	bs	1.58	bs

[a]Assignments are based on chemical shift, decoupling and nOe experiments. ^{1}H NMR spectra were recorded on a Varian HR 220 or 360 MHz spectrometers using $CDCl_3$ with TMS.

Lack of similar enhancement of the olefinic proton at δ 6.23, after irradiation of the methyl group at δ 2.15, enabled us to assign the olefin at C(8)-C(9) as E. Irradiation of the olefinic proton at δ 6.23, under nOe conditions, also produced an enhancement in the epoxide proton at δ 3.47 ppm. This indicated their conformational proximity, in complete analogy to 52 and 53. The demonstrated proximity of these two protons suggested the conformation of epoxylophodione was identical to lophodione. Attempts to chemically interrelate the two compounds by selective epoxidation of 52 were unsuccessful, however. There was insufficient material to attempt the deoxygenation of 54 to produce 52.

D Experimental - Chapter III

General. IR spectra were recorded on a Perkin-Elmer Model 137 spectrophotometer and UV spectra were recorded on a Beckman MVI instrument. Low resolution mass spectra were obtained on a Hewlett-Packard Model 5930A mass spectrometer and high resolution mass spectra were obtained from the Department of Chemistry, Colorado State University on an AEI MS-902 instrument. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter with a one decimeter microcell. All solvents were distilled prior to use.

Collection, extraction and isolation. Lophogorgia alba (Duch. & Mich.), Voucher no. AH-74, was collected by hand using SCUBA in, June 1978, at Bahia Tenacatita during a cruise of the R.V. Alpha Helix to Pacific Mexico. Repeated extraction of the ground animal (6.5 kg dry weight) with 70% chloroform/methanol was followed by removal of the solvents under vacuum. The aqueous residue obtained was partitioned between $CHCl_3$ and water. The organic layer was dried over $MgSO_4$ and concentrated to give 230 gm of crude extract. A mixture of lophodione, isolophodione and epoxylophodione was eluted from a silica gel column using 60-100% $CHCl_3$/petroleum ether, and the mixture was further purified using Florisil chromatography.

Lophodione (52, Table 10).

The 8(E), 11(Z) isomer was eluted from a Florisil column using 80-100% $CHCl_3$/petroleum ether and purified by silica hplc (5μ silica column, 50% ethyl acetate in isooctane). Recrystallization from

EtOAc/isooctane yielded 0.142 g of 52, m.p. = 172-174°, $[\alpha]_D^{23}$ = -275° (c 0.8, $CHCl_3$), (0.36% extract). Lophodione exhibited the following spectral features: UV: λ_{max}^{MeOH} = 267 nm (8000); IR ($CHCl_3$): 2950, 1751, 1669, 1616, 1433, 1202, 1115 cm^{-1}; MS: M^+ m/z 328 for $C_{20}H_{24}O_4$ (low resolution), 232, 178, 151.

Isolophodione (53, Table 11).

The 8(Z), 11(E) isomer was eluted from a Florisil column with 60-80% $CHCl_3$/petroleum ether and purified by silica hplc (5μ silica column, 45% ethyl acetate in isooctane) to give white crystals $[\alpha]_D^{18}$ = -232° (c 1.0, $CHCl_3$), m.p. = 172-175° (0.51% extract). Isolophodione exhibited the following spectral characteristics: UV: λ_{max}^{MeOH} = 261 nm (10,000); IR ($CHCl_3$): 2941, 1754, 1675, 1618, 1439, 1208, 1115 cm^{-1}; MS: M^+ obs. 328.1679, calc. 328.1675, for $C_{20}H_{24}O_4$, $M^+-C_5H_4O_2$ obs. 232.1464, calc. 232.1463, $M^+ -C_9H_{10}O_2$ obs. 178.1002, calc. 178.0994, $M^+ -C_{11}H_{13}O_2$ obs. 151.0764, calc. 151.0759.

Epoxylophodione (54, Table 12).

The 8(E), 11(Z) epoxide was separated from a mixture of 52 and 53 on Florisil using 60% $CHCl_3$/petroleum ether and purified by silica hplc (5μ silica column, 50% EtOAc in isooctane) to produce 0.013 g of a noncrystalline white solid $[\alpha]_D^{23}$ = -114° (c 1.1, $CHCl_3$). Epoxylophodione exhibited the following spectral features: UV: λ_{max}^{MeOH} = 250 nm (13,000); IR ($CHCl_3$): 3021, 1757, 1736, 1678, 1613, 1443, 1241 cm^{-1}; MS: M^+ obs. 344.1605, calc. 344.1624 for $C_{20}H_{24}O_5$.

Isomerization of lophodione to isolophodione. Five drops of a solution containing one small crystal of I_2 dissolved in 10 ml benzene were added to 10 mg of lophodione in 2 ml EtOAc. Partial conversion to isolophodione was seen after 12 hr by tlc examination. The reaction was quenched using 5 ml of an aqueous sodium dithionite solution and extracted using EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to yield a 3:1 mixture of lophodione and isolophiodione as analyzed by 220 MHz ^1H NMR.

Isomerization of isolophodione to lophodione. Five drops of a solution of I_2 in benzene (see preceding) was added to 5 mg of isolophodione in 2 ml EtOAc. After fifteen minutes, conversion to lophodione was noted by tlc, and the reaction was quenched and the product isolated as above. ^1H NMR analysis of the purified products showed an equimolar mixture of lophodione and isolophodione to have been produced.

Nuclear Overhauser enhancement study of lophodione (52). Lophodione, 3 mg (0.009 M), in 1 ml of 0.5% TMS/$CDCl_3$ was carefully degassed by bubbling Ar through the solution for 60 min. ^1H NMR decoupling experiments identified the two pairs of vicinal olefinic methyl and vinyl proton constituents. The olefin proton at δ 6.42 was allylically coupled to the methyl group at δ 1.84. The protons at δ 6.12 and 2.19 were mutually coupled. The decoupler power was decreased until the irradiated peaks were barely nulled. The decoupler was gated for on delay only and the delay time between irradiations was increased from 2 to 20 sec. A sequence of 30 irradiations was performed alternating off resonance irradiations with irradiatons of each of the four resonances under consideration. A total of 3 replicates were run for each olefinic

methyl or vinyl proton irradiation. Enhancements were calculated by comparing the intensity of a olefinic absorption during each irradiation with its intensity under off resonance conditions set to 100%. An enhancement greater than 10% was considered a positive result, indicating proximity of the two groups involved of 3.0 A or less (1 cm = 0.4 A in molecular models). Summary of the nOe experiment results: irradiation at δ 6.12 resulted in an increase of 11% in the peak at δ 6.42. Irradiation of the methyl group at δ 1.84 produced an increase of 16% in the olefin proton at δ 6.42. Therefore, the protons at δ 6.42 and 1.84 are cis, indicating a Z olefin. These data place these bands at C(11) and C(20), using the X-ray model.

Difference nOe study of isolophodione (53). Isolophodione, 4 mg (0.012 M), in 1 ml of 0.5% TMS/$CDCl_3$ was prepared for a nOe experiment as above. A ^1H NMR decoupling study revealed that the olefin proton at δ 6.26 was coupled to the methyl group at δ 2.04, and the protons at δ 6.15 and δ 1.89 were mutually coupled. The nOe experiment was executed and interpreted in the same manner as before. Difference nOe techniques[60] were also employed. Irradiation of the proton at δ 6.26 produced a nOe enhancement in the proton at δ 6.15 indicating their proximity. Irradiation of the methyl group at δ 2.04 resulted in a positive enhancement of 29% in the olefin proton at δ 6.26, indicating their cis relationship. No other significant enhancements were observed. This placed the Z olefin at C(8) and C(9).

Difference nOe study of epoxylophodione (54). Epoxylophodione, 4 mg (0.012 M), in 1 ml 0.5% TMS/$CDCl_3$ was prepared for an nOe experiment as previously discussed. A ^1H NMR decoupling study showed that the

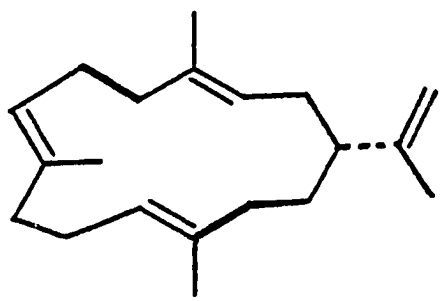
olefinic proton at δ 6.23 was allylically coupled to the methyl group at δ 2.15, and the that protons at δ 3.47 and δ 1.58 were mutually coupled. Irradiation of the olefinic proton at δ 6.23 resulted in a positive enhancement of the epoxide proton at δ 3.47 of 11%. Irradiation in the reverse direction produced a smaller enhancement of 5%. Irradiation of the epoxide methyl group at δ 1.58 caused an enhancement of 10% in the epoxide proton at δ 3.47, indicating their cis relationship. No other significant nOe enhancements were observed. These results indicated that the epoxide at C(11)-C(12) was Z, and that epoxylophodione possessed a very similar conformation to that of lophodione.

Chapter IV

Cembranoid Diterpenes from *Eugorgia forreri*

Cembrenolides, cembrane-derived diterpenoids with α,β-unsaturated lactone functionalities, are characteristic constituents of many Caribbean and east Pacific gorgonians. This fact is well illustrated by the 1,4-diketone and furan-containing cembranolides discussed in the previous two chapters. Cembranoid diterpenes are characteristic of many members of the Alcyonacea (soft corals), and some examples of these compounds are listed in Figure 43. This chapter reports the isolation of two additional cembrane derivatives from the Pacific gorgonian, *Eugorgia forreri*. The investigation of *E. forreri* represents the first isolation of natural products from a *Eugorgia* species. This is particularly interesting in view of the fact that the genus *Eugogia* is endemic to the east Pacific region, and has never been investigated as a source of natural products chemistry. Previous chemical investigations of *Eugorgia* species have been extremely limited. They concerned the study of gorgonian pigments (*Eugorgia ampla*)[32] and lack of prostaglandin-endoperoxide synthetase activity (*Eugorgia rubens*).[35] Over the past four years I examined several *Eugorgia* species for interesting natural products chemistry. The gorgonians were screened by thin-layer chromatography and by ^{1}H NMR analysis of both the crude extract and silica gel column chromatography fractions. Nothing of interest was found using these screening techniques in either *Eugorgia rubens*, a local San Diego gorgonian, or several other *Eugorgia* species collected in the Gulf

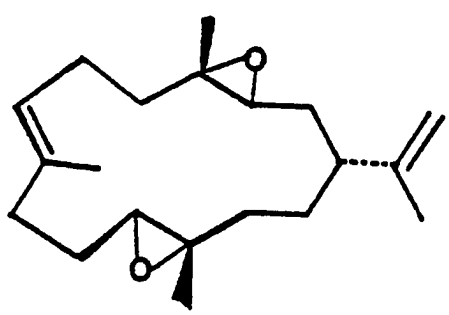
Figure 43. Examples of cembranoid diterpenes isolated from soft corals[13]



R-(-)-cembrene A

Sinularia flexibilis

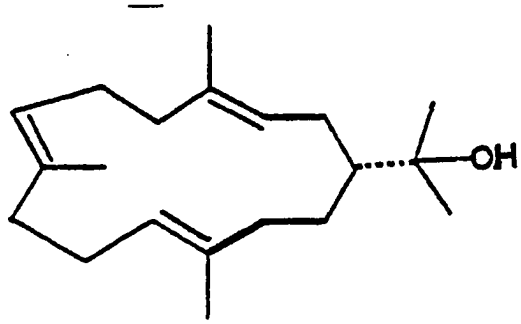
83



3,4,11,12-diepoxycembrene A

Sinularia flexibilis

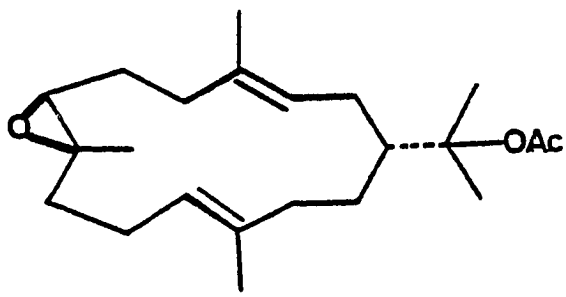
84



nephthenol

Nephthea sp.

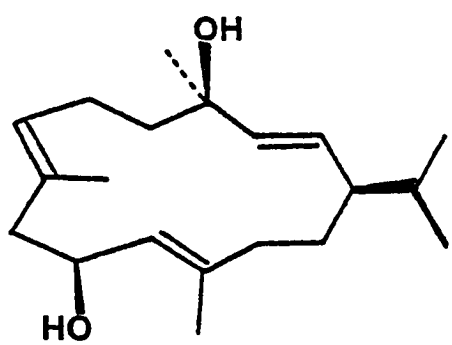
85



epoxynephthenol acetate

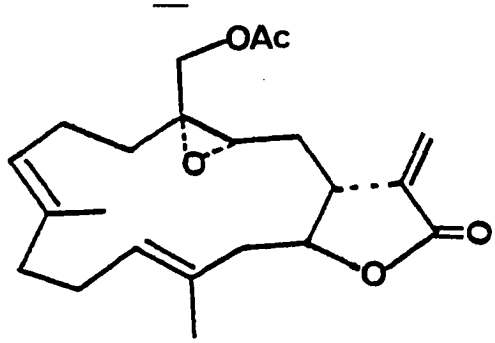
Nephthea sp.

86



Sarcophyton glaucum

87



lobolide

Lobophytum crassum

88

of California, with the exception of E. forreri.

A. Isolation of 55 and 56 from Eugorgia forreri

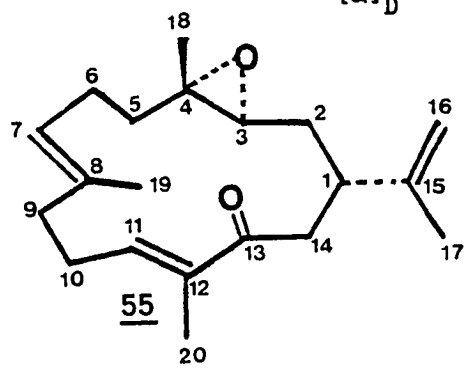
The red pinnately-branched sea whip, Eugorgia forreri, (V-21) was collected by hand using SCUBA from Bahia Los Frailes Canyon, Baja California, from 24 m in January, 1980. The gorgonian was preserved in ethanol and the ground animal was extracted in warm 70% chloroform/methanol to give 34 g of an organic extract from 1 kg dry weight of the animal (3.4% dry weight). The extract was fractionated on a silica gel column, using mixtures of isooctane, dichloromethane and ethyl acetate. A fraction containing two compounds was eluted in 50% ethyl acetate/dichloromethane and appeared interesting by tlc examination. Both compounds showed UV activity and stained dark red after spraying with sulfuric acid. The compounds were rechromatographed with an open silica gel column and by high performance liquid chromatography (in 20% ethyl acetate-isooctane) to give 200 mg of compound A (55) (rf = 0.42 in 100% diethyl ether) (0.6% crude extract, 0.02% dry weight); and ~2.0 g of compound B (56) (rf = 0.38 in 100% diethyl ether, 6% crude extract, 0.2% dry weight).

Examination of the ^{13}C NMR spectrum of the less polar metabolite (55) revealed it to possess a molecular formula of $C_{20}H_{30}O_2$. Absorptions defining four of the six degrees of unsaturation calculated from the molecular formula were present in the ^{13}C NMR spectrum: six olefinic carbons and one carbonyl group. ^{13}C NMR absorptions at 60.5 (doublet) and 59.6 (singlet) were characteristic of a trisubstituted epoxide, which left one carbocyclic ring (presumably cembranoid).

Table 15

Spectral Data for 55

$[\alpha]_D^{27} = -0.03^\circ$ (c = 0.8, $CHCl_3$)



C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	2.92 m	38.3 (J_R = 17.0)
2	1.76 m	38.2 (14.5)
3	2. 64 t (7)	60.5 (72.6)
4	--	59.6
5	2.10 m	39.0 (15.1)
6	2.10 m	23.6 (15.2)
7	5.07 bt (6.4,4)	124.0 (26.0)
8	--	137.4+
9	2.25 m	31.0 (15.6)
10	2.33 m	29.3 (16.0)
11	5.67 bt (7.4,4)	134.5 (27.8)
12	--	135.5+
13	--	185.5
14	2.78 m	44.9 (16.7)
15	--	147.3
16	4.85 bs, 4.73 bs	110.6 (26.8)
17	1.81 bs	20.6 (15.8)
18	1.22 bs	16.7 (14.0)
19	1.62 bs	16.8 (14.5)
20	1.86 bs	21.8 (16.2)

+ may be interchanged

Comparison of the ^{13}C and ^{1}H NMR spectral data of 55 and 56 (Tables 15, 16 and 17) suggested that the two compounds were isomeric at one of the olefins. The major difference between the two compounds was the ^{1}H NMR shift of one of the olefinic protons from δ 5.67 in 55 to δ 6.60 in 56.

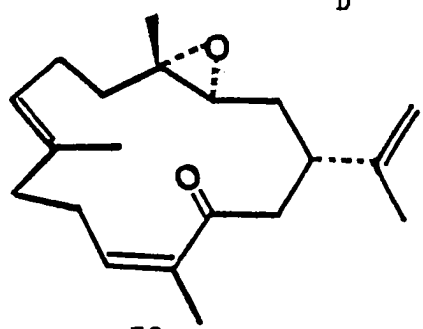
Comparison of these spectral data to those of model cembranoid compounds isolated from soft corals revealed that 55 and 56 were identical to two compounds previously isolated by Ravi and Faulkner from an unknown Sarcophyton sp. (soft coral) collected in Canton.[77] The optical rotation for 56, $[\alpha]_D^{27}$ = +20.6° (c = 0.9 $CHCl_3$), from Eugorgia forreri, agreed quite well with that of the compound from the soft coral, $[\alpha]_D^{20}$ = +12.8°. However, the optical rotation for the gorgonian-derived 55, $[\alpha]_D^{27}$ = -0.03° (c = 0.8, $CHCl_3$), deviated from that of the compound originally isolated from the soft coral, $[\alpha]_D^{20}$ = +8.8°. This is perhaps due to the presence of differing ratios of enantiomers or impurities, although both compounds were purified by hplc several times before rotations were taken. Therefore, 55 was assigned as (1S*, 3S*, 4S*, 7E, 11Z)-3,4-epoxy-13-oxo-7,11,15-cembratriene and 56 was assigned as (1S*, 4S*, 7E, 11E)-3,4-epoxy-13-oxo-7,11,15 cembratriene. The structures of the soft coral-derived metabolites were proven by ^{1}H NMR analysis of both the natural products and their synthetic derivatives.[77]

The isolation of common metabolites from both a soft coral and a gorgonian is reminiscent of the isolation of pukalide from Lophogorgia rigida[47] and Sinularia abrupta (a soft coral from Hawaii).[49] It is interesting to note that identical compounds result from the biosynthetic pathways of gorgonians and soft corals. Soft corals possess the

Table 16

Spectral Data for 56

$[\alpha]_D^{27}$ = +20.6° (c = 0.9, $CHCl_3$).



56

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	2.63 m	43.7 (J_R = 15.6)
2	1.76 (-13,7) 1.44 ddd (13,5,2)	32.4 (14.5)
3	2.77 dd (7,5)	61.9 (21.7)
4	--	59.6
5	2.20 m	38.1 (15.6)
6	2.20 m	24.0 (15.8)
7	5.16 bt (6,1)	125.4 (26.2)
8	--	137.2
9	2.34 m	38.5 (16.3)
10	2.34 m	25.4 (16.1)
11	6.60 bt (6,1)	143.3 (30.5)
12	--	134.0
13	--	191.1
14	3.29 dd (-18,8), 2.20 m	40.7 (20.3)
15	--	147.3
16	4.72 bs, 4.62 bs	110.7 (26.1)
17	1.72 bs	15.6 (15.4)
18	1.23 bs	16.5 (14.0)
19	1.64 bs	19.3 (14.5)
20	1.75 bs	11.3 (15.4)

Table 17

Comparison of the ^{13}C NMR Data for the Cembrenes 55 and 56.[a]

C	55	56
1	38.3 (d)	43.7
2	38.2 (t)	32.4
3	60.5 (d)	61.9
4	59.6 (s)	59.6
5	39.0 (t)	38.1
6	23.6 (t)	24.0
7	124.0 (d)	125.4
8	137.4 (s)	137.2
9	31.0 (t)	38.5
10	29.3 (t)	25.4
11	134.5 (d)	143.3
12	135.5 (s)	134.0
13	185.5 (s)	191.1
14	44.9 (t)	40.7
15	147.3 (s)	147.3
16	110.6 (t)	110.7
17	20.6 (q)	15.6
18	16.7 (q)	16.5
19	16.8 (q)	19.3
20	21.8 (q)	11.3

[a] ^{13}C NMR spectra were run on a Varian CFT-20 NMR spectrometer using $CDCl_3$ solutions with Me_4Si as the internal standard. Assignments were made using residual coupling constants, multiplicities, and by consideration of model compounds.

endosymbiotic zooxanthellae, whereas neither of the gorgonians investigated possess zooxanthellae. This difference provides one way to investigate the role of zooxanthellae in the biosynthesis of terpenes in these alcyonarians. Currently, pukalide is being examined for differences in $^{13}C/^{12}C$ isotope ratios between samples isolated from gorgonians and soft corals. The comparison of $^{13}C/^{12}C$ ratios is being used to determine if the biosynthetic pathways of terpene-derived compounds differ in animals with and without zooxanthellae.[72] These ratios reflect a discrimination against ^{13}C in photosynthetic organisms. Compounds 55 and 56 from Eugorgia forreri would also be good candidates for this type of study because they have previously been isolated from soft corals.

Chapter V

Sesquiterpene-Derived Metabolites from Gorgonians of the Genus Pacifigorgia

Gorgonians produce a number of interesting sesquiterpene-derived metabolites as well as the cembrenolide and cembranoid diterpenes discussed in Chapters 2-4. While many of these sesquiterpenes belong to common ring systems and have been isolated from other sources, others have novel ring systems and unique biological properties. For example, pacifigorgiol (13), isolated from the east Pacific sea fan Pacifigorgia adamsii, possesses an unprecedented and irregular terpenoid skeleton as well as moderate ichthyotoxic activity.[21] Examples of the great variety of ring systems in sesquiterpene-derived metabolites previously isolated from gorgonians are illustrated in Figures 44-50 along with their sources.

Figure 44 illustrates the five known sesquiterpene hydrocarbons possessing the bisabolane ring system. All of the bisabolanes were isolated from Caribbean gorgonians, and with the exception of (+) α-bisabolene (90), each have been previously isolated from terrestrial sources.[13]

Figure 45 reports gorgonian sesquiterpenes belonging to the germacrene ring system. Compounds 11 and 12 were isolated from east Pacific gorgonians, and compounds 94 and 95 were found in Caribbean gorgonians. (-) Germacrene-A (94) has also been found in an unidentified

Figure 44. Bisabolenes isolated from gorgonians[9,13]

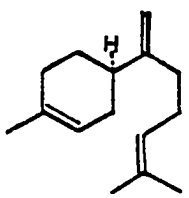
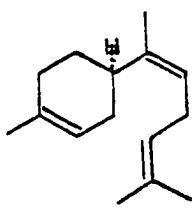
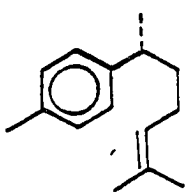
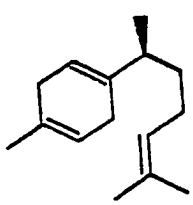
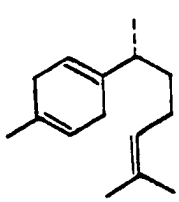
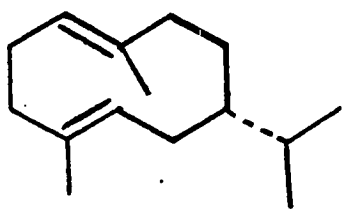
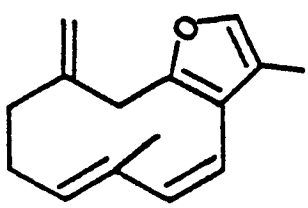
	Compound	Source
 89	(+) β- bisabolene	Plexaura dichotoma P. fusifera P. grisea P. nutans Muricea elongata
 90	(+) α- bisabolene	Plexaura dichotoma P. fusifera P. grisea P. nutans Muricea elongata
 91	(-) α- curcumene	Plexaura dichotoma P. fusifera P. grisea P. nutans Muricea elongata
 92	(+) β- curcumene	Plexaura dichotoma P. fusifera P. grisea
 93	(-) β- curcumene	Plexaura nutans Muricea elongata

Figure 45. Germacrenes isolated from gorgonians



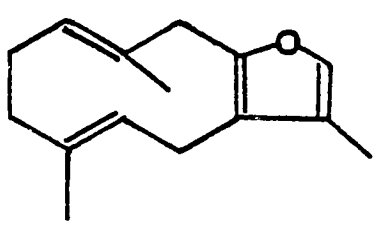
(-)-germacrene- A — Eunicea mammosa[13]

94



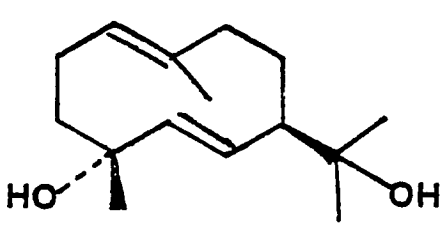
Pseudopterogorgia americana[20]

95



furanodiene — Pacifigorgia media[20]

12



Pacifigorgia media[20]

11

soft coral, Lobophytum sp.[79], and furanodiene (12) was originally isolated from a fungus, Curcuma zeodaria[80]. Furanodiene has been isolated subsequently from several Indo-Pacific soft corals, including the organ pipe coral, Tubipora musica[20], and two unidentified soft corals from the family Xeniidae.[81]

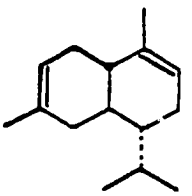
Cadinane derived metabolites from gorgonians are listed in Figure 46. Compounds 96-98 were isolated from Caribbean gorgonians[13]. Compounds 9 and 10 were isolated from gorgonians collected in the Red Sea.[19] Closely related members of the cadinane family are well known from one genus of the brown algae Dictyopteris, and from terrestrial sources.[9]

Figure 47 illustrates numerous sesquiterpenes isolated from Caribbean gorgonians with the copaene and muurolane ring systems.[13]. (+) β-Copaene (100) has also been found in the soft coral Sinularia mayi.[82] (+) α-Muurolene (101) and several closely related derivatives have been found in the Red Sea soft coral Heteroxenia fuscescens.[83]

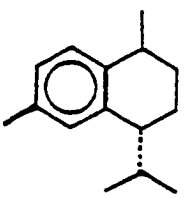
Figure 48 lists the maaliane-derived and aristolane sesquiterpenes, 102-105, isolated from the Caribbean gorgonian Pseudopterogorgia americana.[13].

In contrast to the ten-membered germacrene and bicyclo[4.4.0]-sesquiterpene ring systems (cadinanes and selinanes), gorgonians sesquiterpenes with bicyclo[5.3.0] ring systems, aromadendranes and guaianes, are reported in Figure 49. Alloaromadendrene (108) has been found in an Indo-Pacific soft coral as well as in several Caribbean gorgonians.[13] Linderazulene (7), isolated from a gorgonian collected in Turkey, has

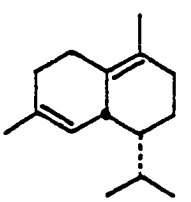
Figure 46. Cadanane - derived compounds from gorgonians



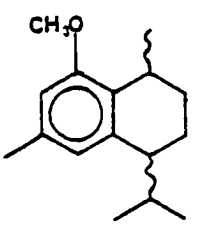
96 (+) cadinene Plexaura crassa[13]



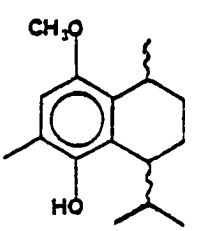
97 (+) calamenene Pseudoplexaura porosa[13]



98 (-) δ- cadinene P. porosa
P. wagenaari
P. flagellosa[13]

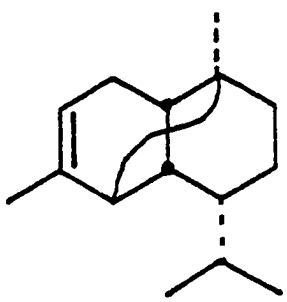


9 Suberogorgia hicksoni[19]



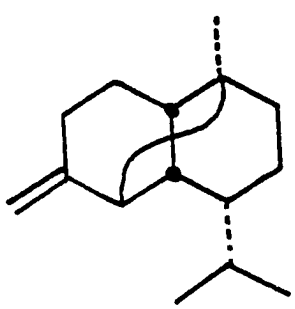
10 Suberogorgia hicksoni[19]

Figure 47. Copaane- and muurolane - derived compounds from gorgonians[13]



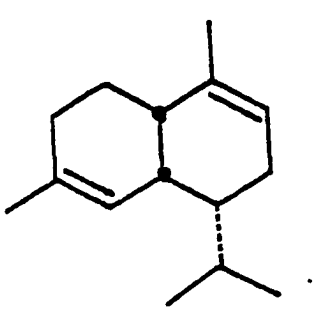
99

(+) α- copaene — Pseudoplexaura porosa; P. wagenaari; P. flagellosa



100

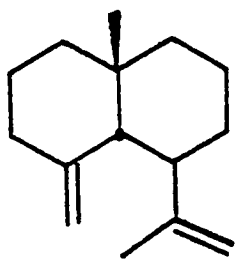
(-) β- copaene — P. porosa; P. wagenaari; P. flagellosa; Eunicea palmeri



101

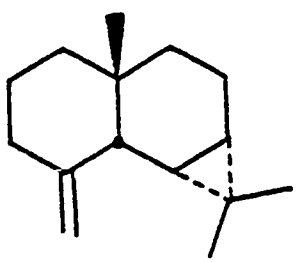
(+) α- muurolene — Eunicea mammosa; E. palmeri; Pseudoplexaura porosa; P. wagenaari; P. flagellosa; Plexaurella grisea; P. fusifera; P. dichotoma

Figure 48. Maaliane- and aristolane-derived compounds from the Caribbean gorgonian Pseudopterogorgia americana[13]



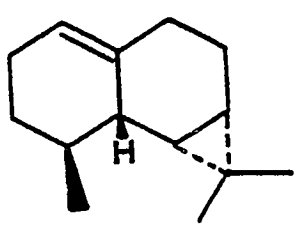
(+) β- gorgonene

102



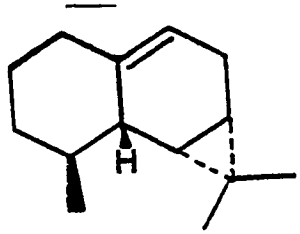
(+) γ- maaliene

103



(-) 1(10)- aristolene

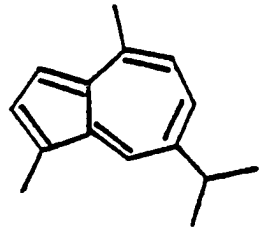
104



(+) 9- aristolene

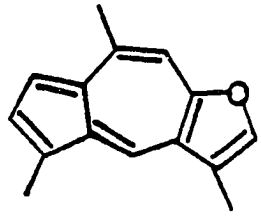
105

Figure 49. Guaiane- and aromadendrane - derived compounds from gorgonians



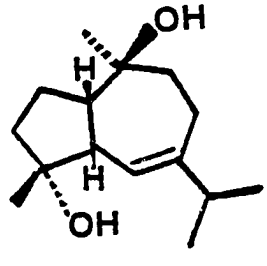
guaiazulene — Euplexaura erecta[18]

8



linderazulene — Paramuricea chamaelon[17]

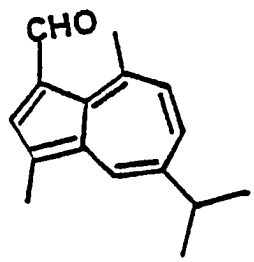
7



Pacifigorgia eximia[22]

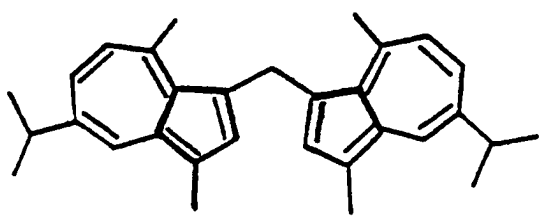
14

Figure 49. continued



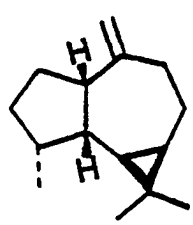
106

unidentified deep-sea Hawaiian gorgonian[85]



107

unidentified deep-sea Hawaiian gorgonian[85]



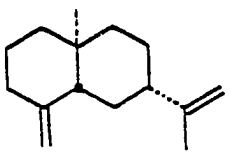
108

Pseudoplexaura porosa

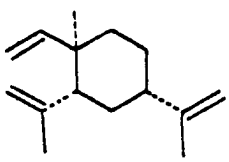
P. wagenaari

P. flagellosa[13]

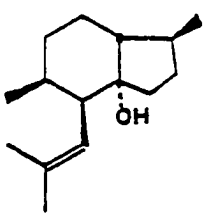
Figure 50. Miscellaneous sesquiterpenoid -derived compounds representing additional ring systems from gorgonians



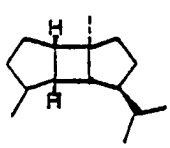
109 (−) - selinene *Eunicea mammosa*



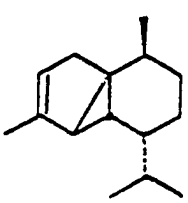
110 (+) - elemene *E. mammosa*[13]



13 pacifigorgiol *Pacifigorgia adamsii*[21]



111 (+) - bourbenene *Pseudoplexaura flagellosa*
P. porosa
P. wagenaari[13]



112 (+) - cubebene

also been isolated from an unidentified deep sea gorgonian collected in Hawaii. This Hawaiian gorgonian also produces several related compounds with an additional carbon atom, 106 and 105.[84] Guaiazulene (8), isolated from a gorgonian from Japan, has previously been found in both terrestrial plants and red algae.[18] The guaiane diol 14, from an east Pacific gorgonian, has also been isolated from the Indo-Pacific soft coral Lemnalia africana.[22]

Other miscellaneous gorgonian-derived sesquiterpenes are listed in Figure 50. With the exception of pacifigorgiol (13) mentioned earlier, all of these compounds were isolated from Caribbean gorgonians. (+) β-elemene (110) has also been isolated from the brown algae, Dictyopteris divaricata[9], and an unidentified soft coral from the genera Lobophytum.[79]

The compounds in Figures 44-50 illustrate the diversity of sesquiterpene ring systems previously found from gorgonians. This diversity is also a striking feature of the chemistry of east Pacific gorgonians of the genus Pacifigorgia. My investigation of six Pacifigorgia species resulted in the isolation of five new and two previously known sesquiterpene-derived compounds which possess four different ring skeletons. Four of the compounds are germacrene-derived (12 and 57-59). Compound 61 belongs to the cadinane class and compound 62 belongs to the guaiane ring system. The remaining compound, 60, has an unprecedented rearranged linear sesquiterpene skeleton. The structures of all of the compounds were elucidated by chemical and spectroscopic means. The structure of pacifigorgiolide (57) was confirmed by an X-ray study provided by Dr. Jon Clardy.[85]

All of the gorgonians were collected by hand, using SCUBA at 3-24 m, at several locations in Pacific Mexico and the Gulf of California. Pacifigorgia pulchra exilis, collected at Bahia Los Frailes, and P. media, collected at Islas Tres Marias, contained the highest number of compounds, with the germacrane and cadanane ring systems in common. Pacifigorgia tenuis and P. florae, from Cabo San Lucas, both possessed germacrene-derived metabolites. Two unidentified Pacifigorgia spp. (A and B), collected in Loreto, contained the cadanane compound, 61. The guaiane diol 62 was isolated from only Pacifigorgia sp. B. Figure 51 summarizes the distribution of natural products found in these and several related Pacifigorgia species which were investigated prior to this work.

A. Isolation of furanodiene

Extraction of Pacifigorgia pulchra exilis, from Bahia Los Frailes, and P. florae, from Cabo San Lucas, yielded large quantities (~0.4% dry wt.) of a nonpolar compound. This compound was readily identified as furanodiene (12) on the basis of comparison of its ^{1}H NMR spectral features with previously published data.[80] ^{13}C NMR data for furanodiene, which showed 5 singlets and 3 doublets in the low field region and 4 triplets and 3 quartets in the high field region, also supported this assignment. ^{1}H NMR decoupling studies allowed the complete assignment of all of the ^{1}H NMR signals. ^{13}C bands were assigned using residual coupling constants (J_R) and by comparison with model compounds (Table 18). Furanodiene was previously isolated from the terrestrial fungus Curcuma zeodaria[80], several soft corals[20,81] and one other Pacifigorgia species, P. media[20], also from Bahia Los Frailes.

Figure 51. Summary of the distribution of natural products from *Pacifigorgia* species

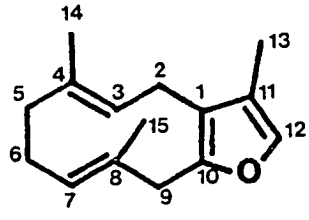
Pacifigorgia spp. and Collection Location	12	57	58	59	60	61	62	11	13
* *P. pulchra exilis* (V-25 = Los Frailes)[20]	x		x	x	x	x			
* *P. media* (AH-57 = Tres Marias)		x		x		x			
* *P. sp. A* (V-42 = Loreto)						x			
* *P. sp. B* (V-54 = Loreto)						x	x		
* *P. tenuis* (V-8 = Cabo San Lucas)				x					
* *P. florae* (V-13 = Cabo San Lucas)	x								
P. media (V-2 = Los Frailes)[20]	x							x	
P. adamsii (V-1 = Los Frailes)[21]									x
P. eximia (La Paz)[22]							x		

*results from my chemical studies

Table 18

Spectral Data for Furanodiene (12)

$C_{15}H_{20}O$



12

C	^{1}H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	121.8
2	3.06 d (7.2)	39.5 (J_R = 15.6)
3	4.73 bt (7.2)	127.6 (24.0)
4	--	128.8+
5	1.85 m, 1.72 m	24.4 (17.6)
6	2.24 m, 2.10 m	26.8 (13.1)
7	4.94 bt (7.8)	129.0 (24.7)
8	--	134.4+
9	3.47 AB d (16)	40.9 (18.7)
10	--	149.7
11	--	118.9
12	7.07 bs	136.0 (40.5)
13	1.92 s	8.8 (14.7)
14	1.60 bs	16.4 (14.0)
15	1.27 bs	16.2 (13.1)

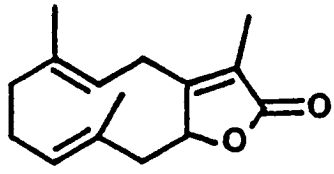
+ may be interchanged

B. Structure elucidation of pacifigorgiolide (57)

Along with furanodiene, several other related germacrene derivatives were isolated from the Pacifigorgia species studied. Pacifigorgia media, collected at Islas Tres Marias in 1978, was extracted sequentially with ethanol and 70% chloroform/methanol. The extract was fractionated using silica gel chromatography to yield furanodiene and 80 mg (0.01% dry wt.) of a more polar new metabolite, pacifigorgiolide. Pacifigorgiolide (57) was eluted with 80% dichloromethane-petroleum ether and purified by hplc with 30% ethyl acetate-isooctane to give colorless needles, m.p. 132-134°C (Table 19). High resolution mass spectrometry gave a molecular formula of $C_{15}H_{20}O_2$, which calculated for six degrees of unsaturation. ^{13}C NMR bands at 173.7 (s), 125.9 (s), 162.8 (s), and 82.8 (d), combined with an infrared absorption at 1740 cm^{-1}, indicated a tetrasubstituted α,β-unsaturated-γ-lactone to be present. These assignments accounted for the two oxygen atoms and three of the six degrees of unsaturation inherent in the molecular formula. ^{13}C NMR bands at 132.8 (s), 130.6 (s), 130.6 (d) and 123.7 (d), which were assigned to two trisubstituted olefins, left one degree of unsaturation, presumably in the form of a sesquiterpene macrocyclic ring. ^{1}H NMR decoupling studies revealed three sets of mutually coupled protons which resulted in the assignments of partial structures a-c (Figure 52). The homoallylic coupling observed between the olefinic methyl group at δ 1.87 and the lactone methine proton at δ 4.97 allowed the methyl group to be placed at the α position of the α,β-unsaturated-γ-lactone. The high field chemical shift of this methyl (8.9 ppm) in the ^{13}C NMR spectrum also supported this assignment. These partial structures accounted for all the

Table 19

Spectral Data for Pacifigorgiolide (57).

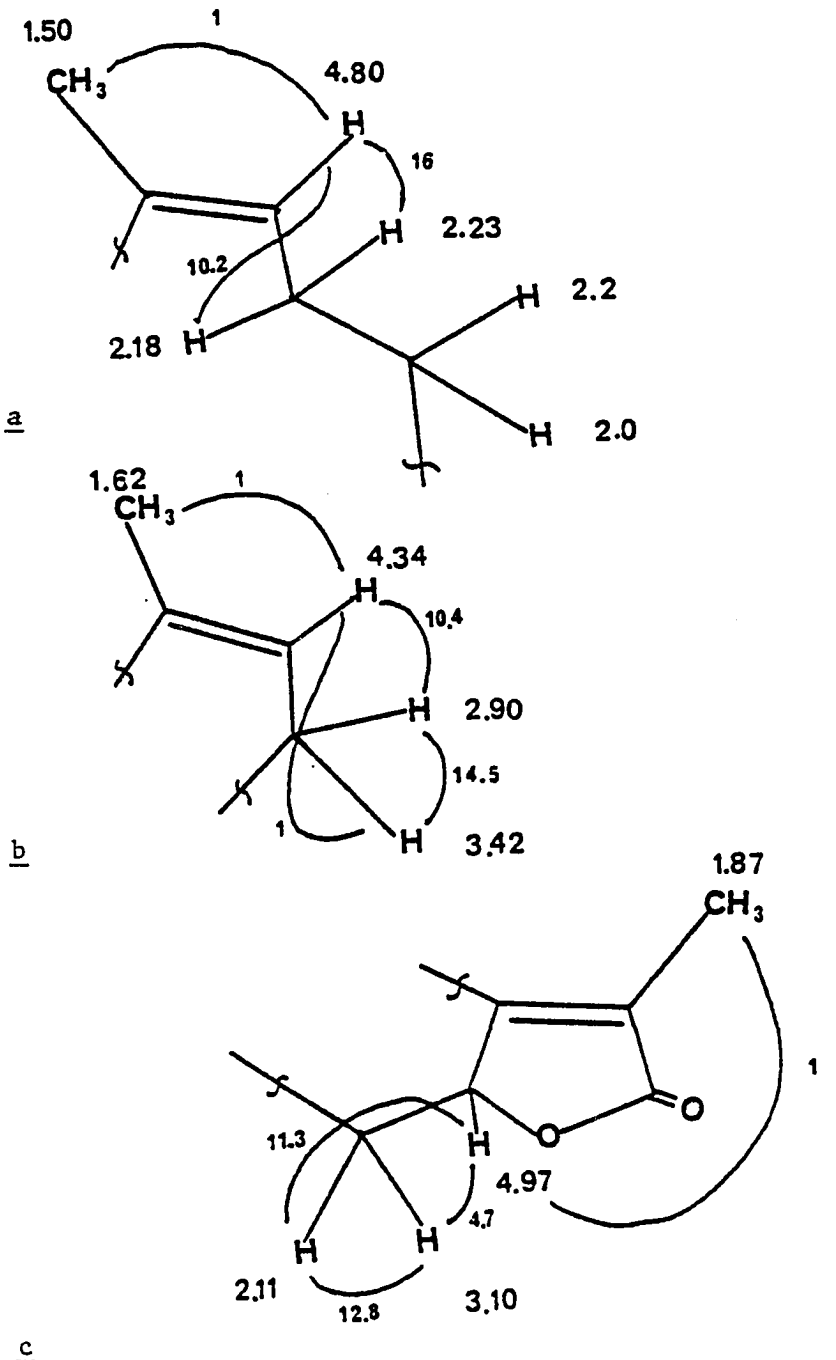


$C_{15}H_{20}O_2$, racemic white needles, m.p. 132-134°C; UV: λ_{max}^{MeOH} = 212 nm (14,000); IR ($CHCl_3$): 2925, 1740, 1445, 1380, 1110 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)	^{13}C NMR (50 MHz, $CDCl_3$)
1	--	162.8
2	3.42 bd (-14.5), 2.90 dd (-14.5, 10.0)	38.4 (J_B = 27.9)
3	4.34 bd (10.9, 1)	123.7 (47.0)
4	--	130.6+
5	2.2 m	27.5++ (33.5)
6	2.2 m	25.6++ (29.4)
7	4.86 bdd (16.0, 10.2)	130.6 (47.9)
8	--	132.8+
9	3.10 bd (-12.8, 4.7), 2.11 dd (-12.8, 11.3)	47.1 (31.7)
10	4.97 bdd (11.3, 4.7, ~1)	82.8 (47.9)
11	--	125.9
12	--	173.7
13	1.87 s	8.9 (28.4)
14	1.62 bs	16.8 (26.7)
15	1.50 bs	16.4 (26.5)

+, ++ may be interchanged

Figure 52. Partial structures of pacifigorgiolide (57) as determined by ^{1}H NMR decoupling experiments

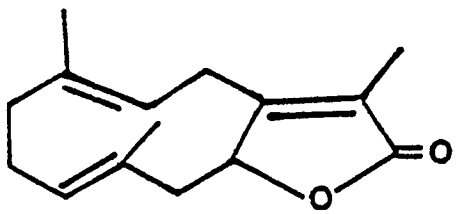


pieces of the molecule.

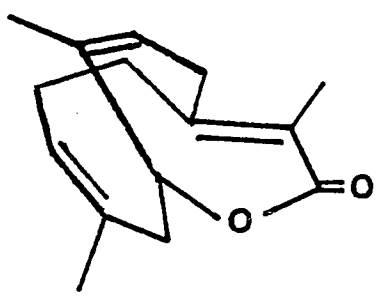
The low field chemical shifts of the methylene protons in pacifigorgiolide and their lack of further coupling illustrated that all of the methylene groups were either allylic (δ 2.1-3.1) or bis-allylic (δ 2.9-3.4). The only way these partial structures could be arranged in a regular isoprenoid fashion in a monocarbocyclic ring, was in the germacrenoid structure 57. This structure is closely related to that of furanodiene (12). ^{1}H NMR nuclear Overhauser enhancement differences studies (nOeds) revealed that both trisubstituted olefins had the E configuration. This was deduced by the lack of nOe observed between the respective olefinic methyl and olefin protons. This fact, combined with other spectral evidence, eliminated possible structures such as 57′ and 57". Both structures would have required E olefins (Figure 53).

The conformation of the germacrenoid ring of pacifigorgiolide was determined by using ^{1}H NMR decoupling and nOeds results. The lack of coupling observed between the olefin proton at δ 4.34 and the methylene proton at δ 3.42 indicated an approximate 90° dihedral angle. Nuclear Overhauser enhancements were observed between the protons at δ 4.97 and δ 1.50, and and between those at δ 1.87 and δ 4.34. The proximity of these groups was observed in molecular models if both of the olefinic methyls at C-4 and C-8 have the same relative orientation. Figure 54 illustrates the proposed conformation of pacifigorgiolide, defined by coupling constant analysis and by nOeds results. The extremely high field chemical shift of the olefinic proton at δ 4.34 can be explained by this conformation due to the shielding effect of the overlapping C-7, C-8 olefin π orbitals.

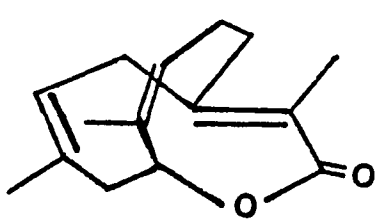
Figure 53. Possible structural proposals for pacifigorgiolide (57)



57



57'



57''

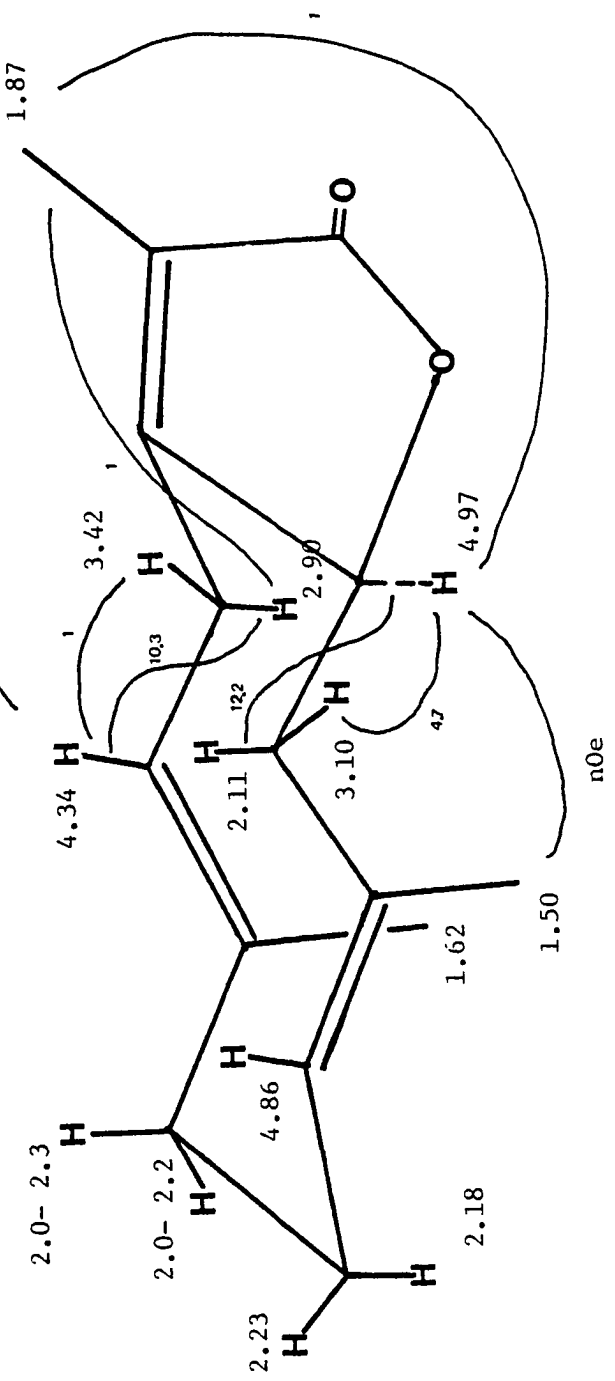


Figure 54. The results of ^{1}H NMR decoupling and nuclear Overhauser enhancement studies on pacifigorgiolide (57)

An X-ray crystallographic analysis of pacifigorgiolide was provided by Dr. Jon Clardy of Cornell University to confirm this structural proposal.[85] The proposed structure 57 was confirmed to possess the same conformation proposed by the ^1H NMR decoupling and nOed experiments (Figure 55). Needles of 57, recrystallized in ethyl acetate-isooctane, were determined to be racemic. The achirality of this molecule suggests that it may be an artifact. However, other natural products, such as the guaiane diol 63, are also found only in racemic mixtures. This makes it difficult to draw any definite conclusions about the source of pacifigorgiolide, especially since butenolides are well known in many natural products. In addition to pacifigorgiolide, several related compounds were also isolated from several Pacifigorgia species.

C. Structure elucidation of methoxypacifigorgiolide

Pacifigorgia pulchra exilis was collected at Bahia Los Frailes Canyon in 1979. The gorgonian was preserved in ethanol and extracted with 70% chloroform-methanol. The extract was separated by silica gel chromatography using mixtures of ethyl acetate-isooctane. 360 MHz ^1H NMR analysis of hplc fractions (20% ethyl acetate-isooctane) showed an interesting compound to be present, which appeared to be closely related to pacifigorgiolide. Close similarities in their spectral features (Tables 19 and 20) revealed that the new compound (58) differed from pacifigorgiolide only in the addition of a methoxy group at the lactone γ-carbon. This conclusion was evident from the absence of the lactone methine proton at δ 4.97 and the presence of a new three proton singlet at δ 3.17 in the ^1H NMR spectrum of 58. In addition, the C-9 methylene protons in methoxypacifigorgiolide were coupled only to each other with

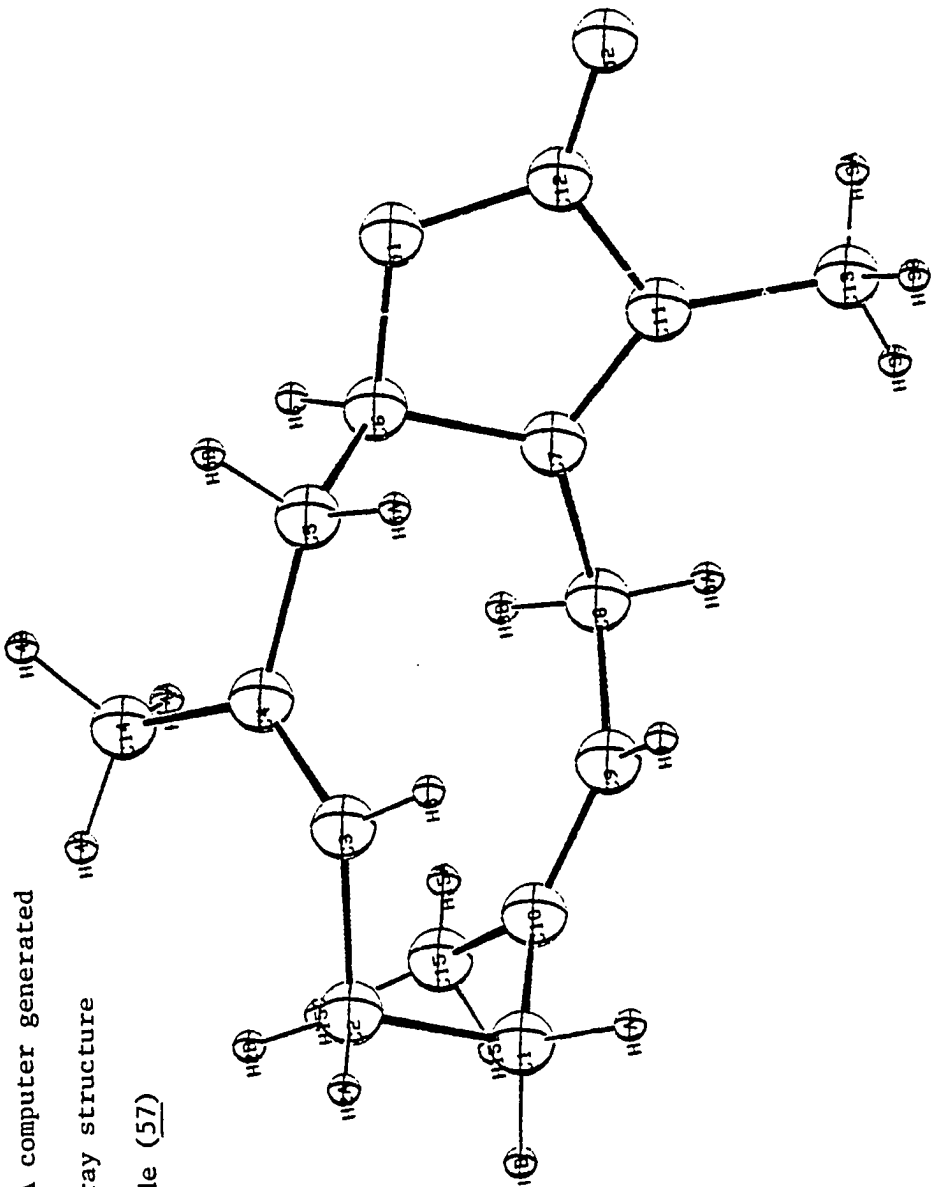
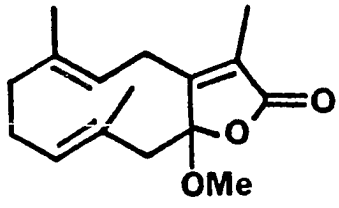


Figure 55. A computer generated drawing of the X-ray structure of pacifigorgiolide (57)

Table 20

Spectral Data for Methoxypacifigorgiolide (58)



58

$C_{16}H_{22}O_3$, racemic; UV: λ_{max}^{MeOH} = 254 nm (2000), 210 nm (16,000); IR ($CHCl_3$): 2970, 1750, 1450, 1300, 1150,950 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)	^{13}C NMR (50 MHz, $CDCl_3$)
1	--	160.5
2	3.11 bd (-13.6, 1), 2.86 dd (-13.6, 10.5)	36.8 (14.9)
3	4.26 bd (10.5, 1, 4)	123.3 (21.9)
4	--	133.2$^+$
5	2.11 m, 1.99 m	25.5^{++} (15.5)
6	2.25 m, 2.20 m	25.6^{++} (15.5)
7	4.78 bdd (11.3, 4.1, <1)	131.8 (22.1)
8	--	134.5$^+$
9	3.21 d (13.8), 2.30 d (13.8)	50.9 (16.9)
10	--	112.5
11	--	129.7
12	--	170.9
13	1.91 s	8.7 (15.0)
14	1.58 bs	17.2 (14.3)
15	1.62 bs	16.9 (14.3)
-OMe	3.17 s	50.5 (19.2)

+, ++ may be interchanged

a geminal coupling of -13.8 Hz. This is in contrast to the additional vicinal coupling found in pacifigorgiolide. Comparison of the ^{13}C NMR data of the two compounds also supported this modification, as the two spectra were practically superimposable except for one difference. The lactone methine carbon at δ 82.8 (d) in pacifigorgiolide was replaced by a singlet at 112.5 ppm (typical for an allylic carbon bearing two oxygens) in the new compound. All other spectral data, including the infrared absorption at 1750 cm^{-1} for the α,β-unsaturated-γ-lactone, indicated that no other changes had occurred in the molecule. The HRMS of 58, which gave a molecular formula of $C_{16}H_{22}O_3$ (pacifigorgiolide + $-CH_2O$), confirmed this structural proposal.

^1H NMR decoupling studies of 58 in $CDCl_3$, d_6-acetone and d_6-benzene allowed the complete assignment of every proton in the ^1H NMR spectrum (Table 21). These decoupling data, combined with the results of ^1H NMR nuclear Overhauser difference studies (nOeds), allowed the assignment of the conformation of methoxypacifigorgiolide. As in pacifigorgiolide, the lack of coupling between the C-2 methylene proton at δ 3.11 and the C-3 olefin proton at δ 4.26 indicated an approximately 90° dihedral angle. Irradiation of the C-13 olefinic methyl at δ 1.91 produced an nOe in the C-3 olefinic proton at δ 4.26 and the C-9 axial methylene proton at δ 2.30. Irradiation of the methoxy group at δ 3.17 resulted in enhancements for the C-15 vinyl methyl at δ 1.62, the C-2 axial methylene proton at δ 2.86, and the C-9 methylene protons at δ 3.21 and 2.30. Irradiation of δ 2.86, in turn, resulted in enhancements of the methoxy group at δ 3.17 and the C-15 methyl group at δ 1.62. This result indicated both groups possessed axial orientations on the

Table 21

360 MHz ^{1}H NMR Data for Methoxypacifigorgiolide (58)

C	$CDCl_3$/TMS	d_6-acetone/TMS	d_6-benzene/TMS
1	--	--	--
2	3.11 bd, 2.86 bd	3.28, 2.93	2.75, 2.52
3	4.26 bd	4.37	4.03
4	--	--	--
5	2.11 m, 1.99 m	2.25-2.00	1.83, 1.70
6	2.25 m, 2.20	2.00	2.00
7	4.78 bdd	4.86	4.44
8	--	--	--
9	3.21 d, 2.30 d	2.99, 2.33	3.16, 2.17
10	--	--	--
11	--	--	--
12	--	--	--
13	1.91 s	1.86	1.64
14	1.58 bs	1.58	1.37
15	1.62 bs	1.62	1.50
-OMe	3.17 s	3.14	2.79

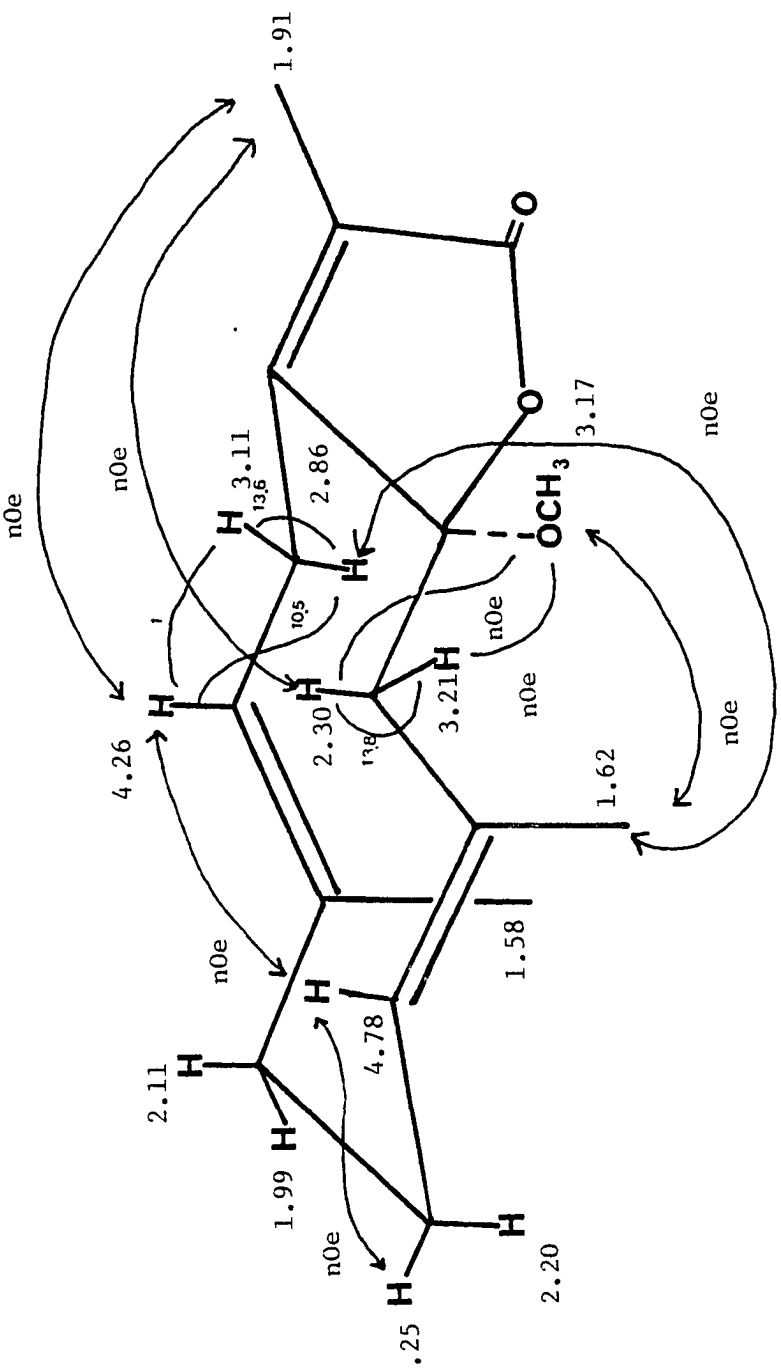
same face of the ring. Irradiation of the olefinic protons at δ 4.78 and 4.26 resulted in mutual enhancement, confirming their proximity and orientation on the side of the ring opposite to the methoxy group. Figure 56 summarizes the nOe experimental results and illustrates the predicted conformation of 58.

Methoxypacifigorgiolide is also apparently racemic from optical rotation measurements. The optical rotation at the sodium D line, as well as at four mercury absorptions, was near zero. This again raises the question of the source of this compound. Methoxypacifigorgiolide was additionally, and fortuitously, isolated from chloroform-methanol extracts of an Indo-Pacific gorgonian, Nicaule crucifera (Pg 134) collected in Palau in 1979. Compound 58, isolated from both sources, was identical by ^{1}H and ^{13}C NMR, IR, MS and optical rotation analysis. Nicaule crucifera, like Pacifigorgia pulchra exilis, also contained large quantities of furanodiene (12). The coisolation of methoxypacifigorgiolide and furanodiene from these two sources is suspicious. The discovery of a corresponding ethoxy derivative from ethanol extracts of several Pacifigorgia species suggests those products are derived from the photooxidation of furanodiene in alcoholic extracts. This issue will be more fully addressed in Section E.

D. Structure elucidation of ethoxypacifigorgiolide (59)

Extracts of Pacifigorgia pulchra exilis (from Bahia Los Frailes), P. media (from Islas Tres Marias) and P. tenuis (from Cabo San Lucas) were all either stored in or extracted with ethanol. After silica gel chromatography of the crude extracts, analytical hplc in 15%

Figure 56. The results of ^{1}H NMR decoupling and difference nuclear Overhauser enhancement experiments on 58



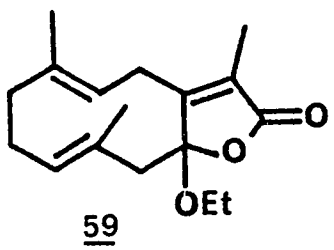
ethyl acetate-isooctane gave the least polar pacifigorgiolide compound, ethoxypacifigorgiolide (59). Compound 59 was also isolated in a racemic mixture. Both low and high resolution mass spectrometry gave a molecular formula of $C_{17}H_{24}O_3$: pacifigorgiolide + C_2H_4O. The major difference in the ^1H NMR between pacifigorgiolide (57) and 59 was the absence of the lactone methine proton at δ 4.97 and the presence of absorptions which were assigned to an ethoxy group: δ 3.48 (1 H, dq, (-9.0, 7.0)), 3.22 (1H, dq (-9.0, 7.0)) and 1.22 (3H, t (7.0)). In the ^{13}C NMR spectrum, a singlet at 112.2 ppm, analogous to that in the spectrum of methoxypacifigorgiolide, replaced the C-10 lactone methine doublet of pacifigorgiolide at 82.8 ppm. All other spectral features of ethoxypacifigorgiolide were comparable to pacifigorgiolide and methoxypacifigorgiolide (Table 22). Tables 23 and 24 contain the assignments and comparisons of the ^1H and ^{13}C NMR spectral data of the three Pacifigorgia butenolide compounds (57-59) with furanodiene (12). Based on similarities of chemical shifts and coupling constants in the ^1H NMR spectrum of 59 in relation to that of methoxypacifigorgiolide, 59 has been assigned the same conformation.

Similarities in the fragmentation pattern of 57-59 substantiated their related structures. The HRMS of pacifigorgiolide (57) showed fragments for C_9H_{13} and C_8H_{11}. The HRMS of methoxypacifigorgiolide (58) showed fragments for C_9H_{14} and C_8H_{11}. Both 58 and 59 showed fragments for M^+ -ROH (R = Me or Et) and M^+ $-C_9H_{14}$ (their respective base peaks). Figure 57 outlines these fragmentation reactions.

In order to definitively relate 58 and 59, both compounds were treated with lithium aluminum hydride in anhydrous diethyl ether at 0^o

Table 22

Spectral Data for Ethoxypacifigorgiolide (59)



59

$C_{17}H_{24}O_3$, racemic; UV: λ_{max}^{MeOH} = 211 nm (11,000); IR ($CHCl_3$): 2950, 1750, 1670, 1440, 1260, 990 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)	^{13}C NMR (50 MHz, $CDCl_3$)
1	--	161.0
2	3.20 bd (-18.8), 2.85 dd (-18.3, 10.8)	38.4 (J_B = 26.5)
3	4.27 bd (10.8, 1.0)	123.2 (43.0)
4	--	133.4+
5	2.25 m, 1.92 m	25.5 (30.9)
6	2.25 m, 2.10 m	25.5 (30.9)
7	4.78 bdd (11.4, 4.3, 1.0)	131.6 (44.5)
8	--	134.4+
9	3.13 bd (-13.8), 2.28 d (-13.8)	51.2 (30.0)
10	--	112.2
11	--	129.3
12	--	170.7
13	1.90 s	8.7 (26.4)
14	1.62 bs	17.2 (25.3)
15	1.60 bs	16.9 (24.8)
$-OCH_2-$	3.48 dq (-9.0, 7.0), 3.22 dq (-9.0, 7.0)	58.8 (35.8)
CH_3	1.22 t (7)	15.2 (23.3)

+ may be interchanged

Table 23

Comparison of ^1H NMR Data for Furanodiene (12) and the Pacifigorgiolides (57-59)+

C	12	57	58	59
2	3.06	3.42	3.11	3.20
	--	2.90	2.86	2.85
3	4.73	4.34	4.26	4.27
5	1.85	2.2	2.11	2.25
	1.72	--	1.99	1.92
6	2.24	2.2	2.25	2.25
	2.10	--	2.20	2.10
7	4.94	4.86	4.78	4.78
9	3.47	3.10	3.21	3.13
	--	2.11	2.30	2.28
10	--	4.97	--	--
12	7.07	--	--	--
13	1.92	1.87	1.91	1.90
14	1.60	1.62	1.58	1.62
15	1.27	1.50	1.62	1.60
-OMe	--	--	3.17	--
$-OCH_2-$	--	--	--	3.48, 3.22
$-CH_3$	--	--	--	1.22

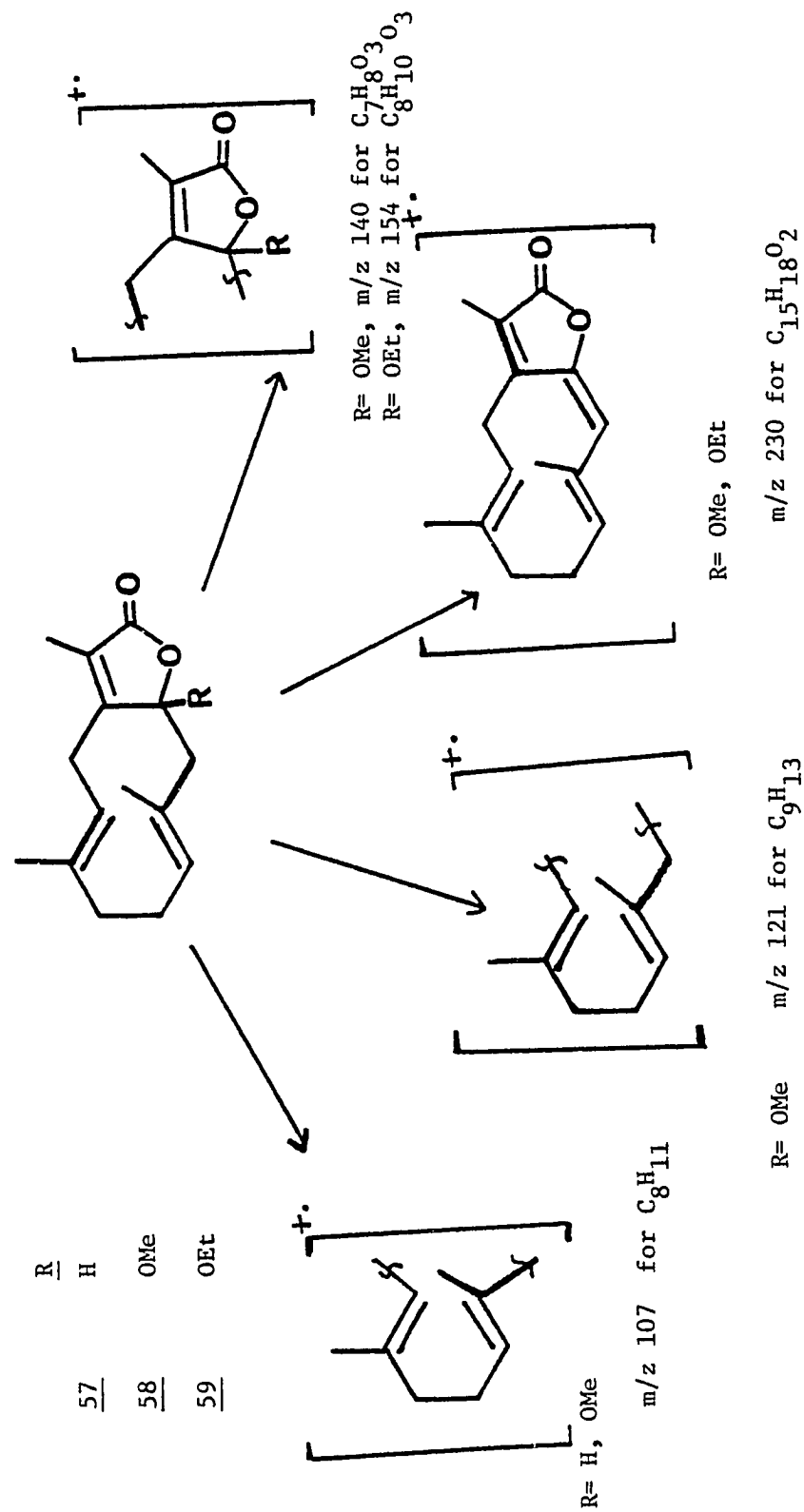
+ 360 MHz, taken in $CDCl_3$/TMS.

Table 24

Comparison of ^{13}C NMR Data for Furanodiene (12) and the Pacifigorgiolides (57-59).[+]

C	(12)	(57)	(58)	(59)
1	121.8	162.8	160.5	161.0
2	39.5	38.4	36.8	38.4
3	127.6	123.7	123.3	123.2
4	128.8[+]	130.6[+]	133.2[+]	133.4[+]
5	24.4	27.5[++]	25.5[++]	25.5
6	26.8	25.6[++]	25.6[++]	25.5
7	129.0	130.6	131.8	131.6
8	134.4[+]	132.8[+]	134.5[+]	134.4[+]
9	40.9	47.1	50.9	51.2
10	149.7	82.8	112.5	112.2
11	118.9	125.9	129.7	129.3
12	136.0	173.7	170.9	170.7
13	8.8	8.9	8.7	8.7
14	16.4	16.8	17.2	17.2
15	16.2	16.4	16.9	16.9
-OMe	--	--	50.5	--
$-OCH_2-$	--	--		58.8
$-CH_3$	--	--		15.2

+,++ may be interchanged



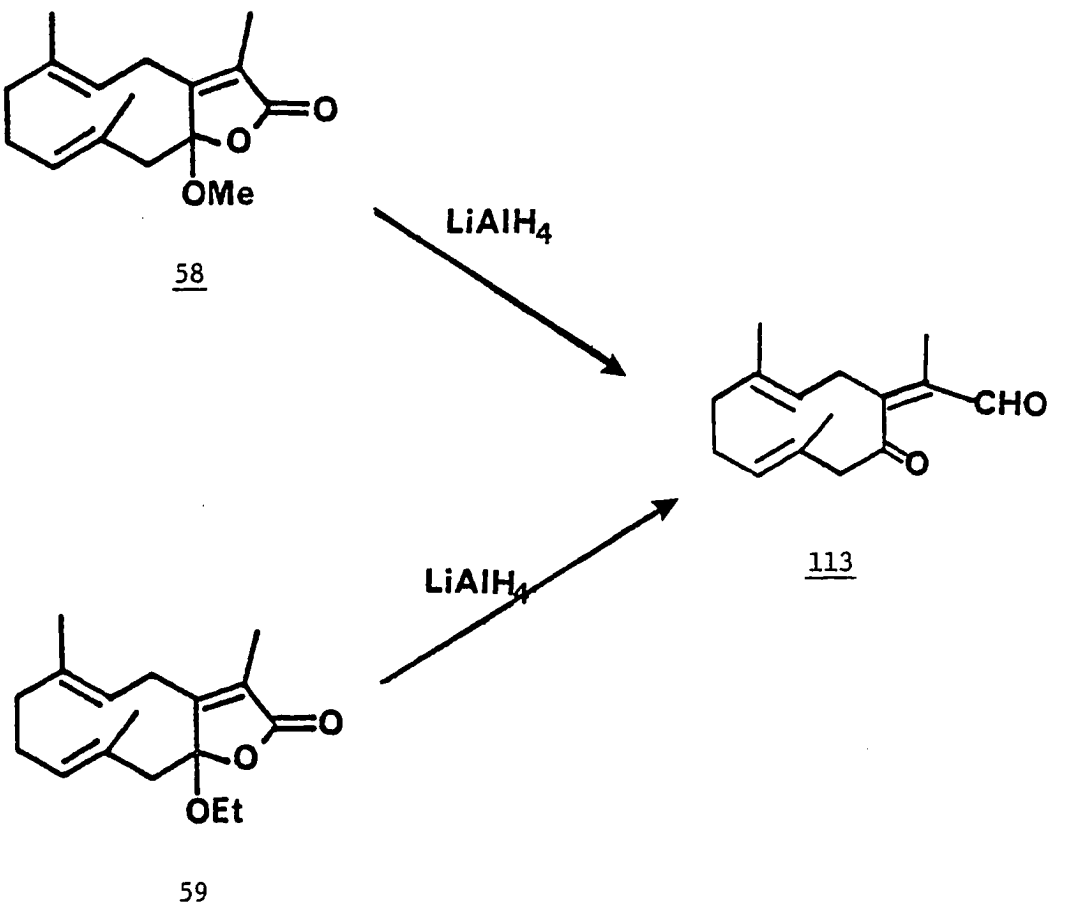
	R
57	H
58	OMe
59	OEt

Figure 57. Mass spectral fragmentation of 57 - 59

for 15-20 minutes. Both reactants yielded the same α,β-unsaturated keto-aldehyde 113, which was identical by ^1H NMR comparison (Figure 58) from each source. HRMS of the keto-aldehyde gave the molecular formula $C_{15}H_{20}O_2$ which is isomeric with pacifigorgiolide. The keto-aldehyde was readily identified by the aldehyde singlet in the ^1H NMR spectrum at δ 9.79, and by absorptions in the ^{13}C NMR spectrum at 203.7 (s) and 189.5 (d) ppm. ^1H NMR decoupling studies resulted in the assignment of all of the ^1H NMR bands as shown in Figure 59 (Table 23). The large downfield shift of the C-9 methylene protons from $H_{equatorial}$ = δ 3.1-3.2 and H_{axial} = δ 2.1-2.3 in 57-59 to δ 3.51 and 3.13 in 113 reflects their new position α to a carbonyl group.

Exposure to air, silica gel chromatography (upon hplc) or acidic conditions (unpurified $CHCl_3$ or CH_2Cl_2), resulted in the decomposition of 113 yielding a mixture of unknown products. One of the major components of this mixture appeared to be the oxidation product of 113, hydroxypacifigorgiolide (114). This assignment was supported by infrared absorptions at 1790 and 1770 cm^{-1}, typical of γ-hydroxybutenolides.[86] HRMS of the decomposition product gave a molecular ion of 248.1425 for $C_{14}H_{20}O_3$ (calc. 248.1412) for the hydroxy-lactone. ^{13}C NMR analysis of the keto-aldehyde (113) revealed a singlet at 99.0 ppm attributable to the hydroxybutenolide impurity. The mechanism for the reduction of 58 and 59 with $LiAlH_4$ to the keto-aldehyde 113, and subsequent formation of the γ-hydroxybutenolide 114, is outlined in Figure 60. An analogous reaction occurred via the Jones' oxidation of the γ-keto-enol derivative of nakafuran-8 to the γ-hydroxybutenolide (Figure 61).[87]

Figure 58. $LiAlH_4$ reduction of 58 and 59 to the keto-aldehyde 113

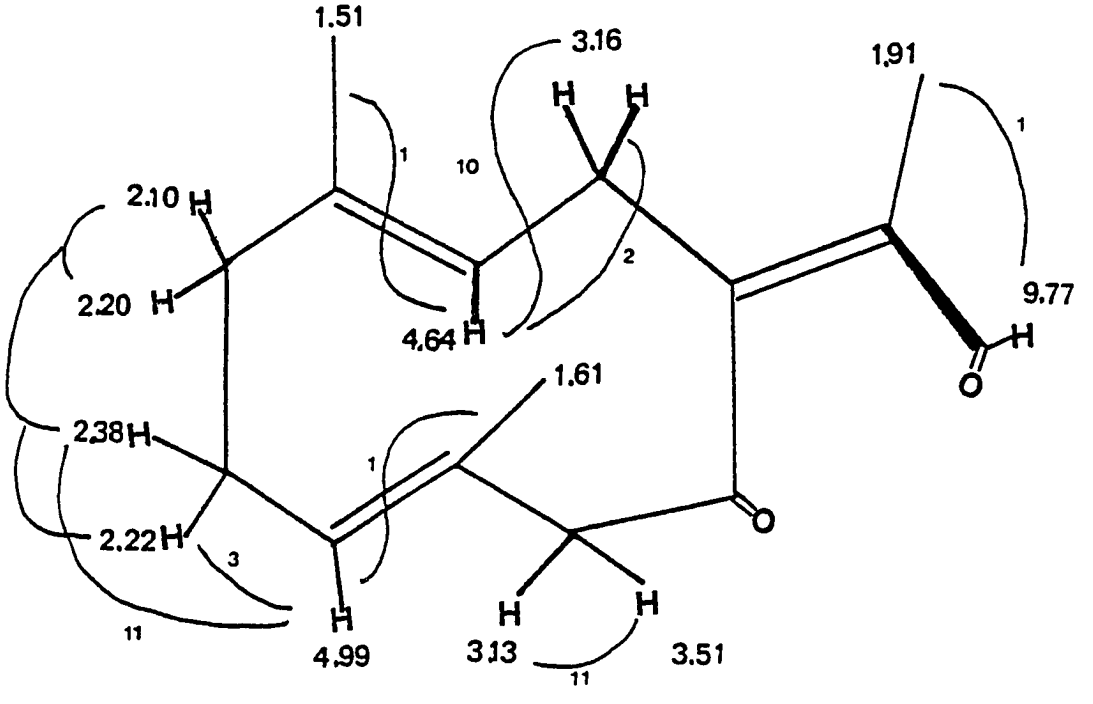


58

113

59

Figure 59. ^{1}H NMR decoupling studies of the keto-aldehyde 113

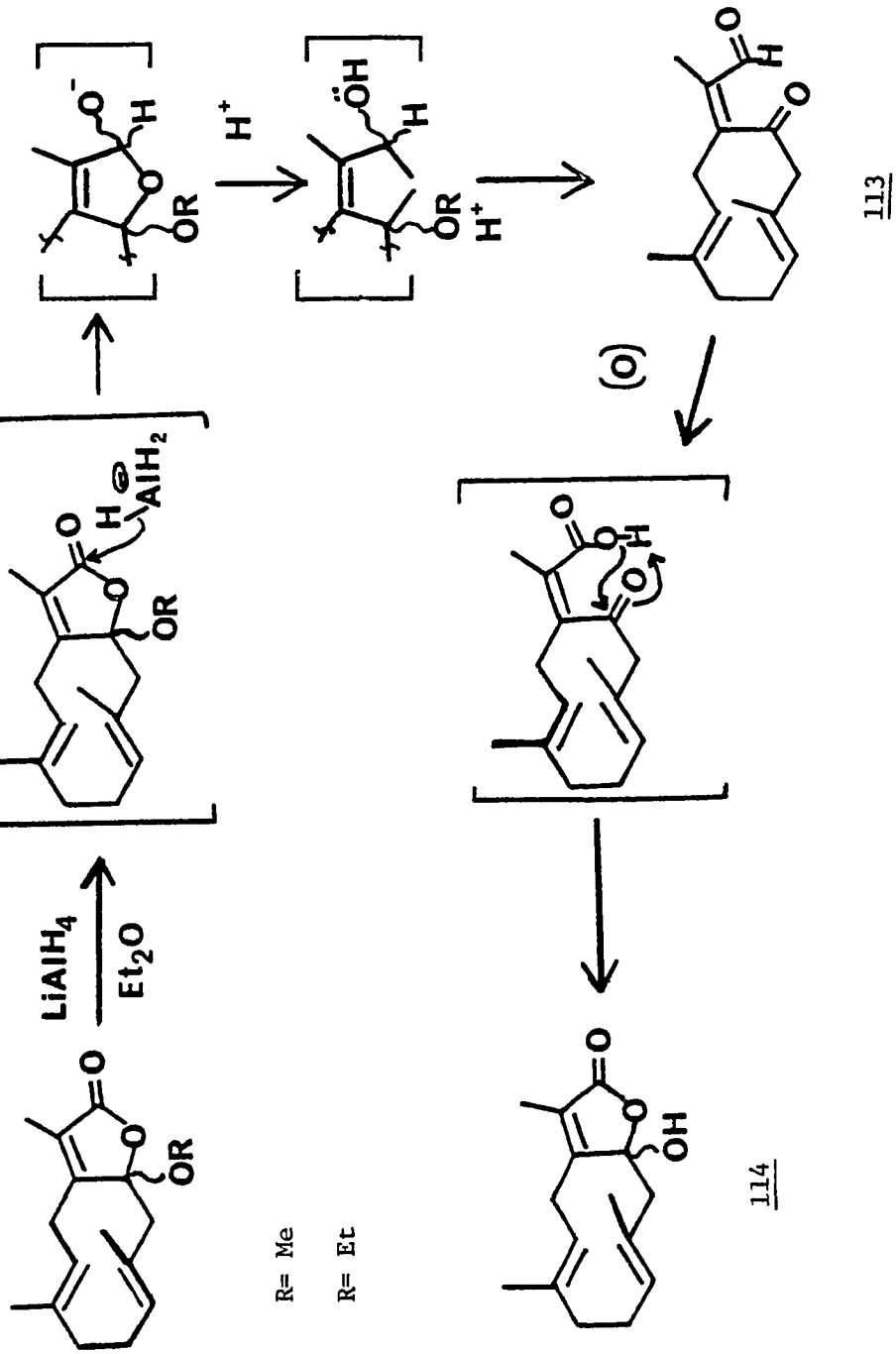


113

E. The role of furanodiene in the formation of methoxy- and ethoxy-pacifigorgiolide

Several features of the isolation of the alcohol derivatives of pacifigorgiolide point to the possibility that they are not natural products. Instead, they may be solvolysis products due to photooxidation of furanodiene in the alcohol extracts of the gorgonians. Several arguments in favor of this proposal can be found. First, furanodiene is present in the methanol extracts of Pacifigorgia pulchra exilis and Nicaule crucifera, from which methoxypacifigorgiolide was isolated. The occurrence of furanodiene in the ethanol extracts of P. media, from Islas Tres Marias, and P. tenuis, from Cabo San Lucas, is not known. It is highly likely, however, and needs to be ascertained. Second, the methoxy-derivative was found only in extracts that were in contact with methanol during the extraction procedure. Similarly, the ethoxy derivative was found only where ethanol was used as a storage or extraction solvent. Third, the racemic nature of the alcohol derivatives of pacifigorgiolide is indicative of a nonenzymatic synthetic pathway. Most biosynthetic pathways involve stereospecific enzymatic processes which result in chiral products. The racemic nature of these compounds points to their probable formation by photooxidation of furanodiene in the methanol or ethanol extract.

In order to test this assumption, a singlet oxygen photosensitized oxygenation reaction of furanodiene was attempted. Furanodiene was irradiated in a solution of 1:1 methanol-dichloromethane using Rose Bengal as the sensitizer. The expected methoxybutenolide product was not formed by this reaction, however a methoxyhydroperoxide derivative



58 R= Me

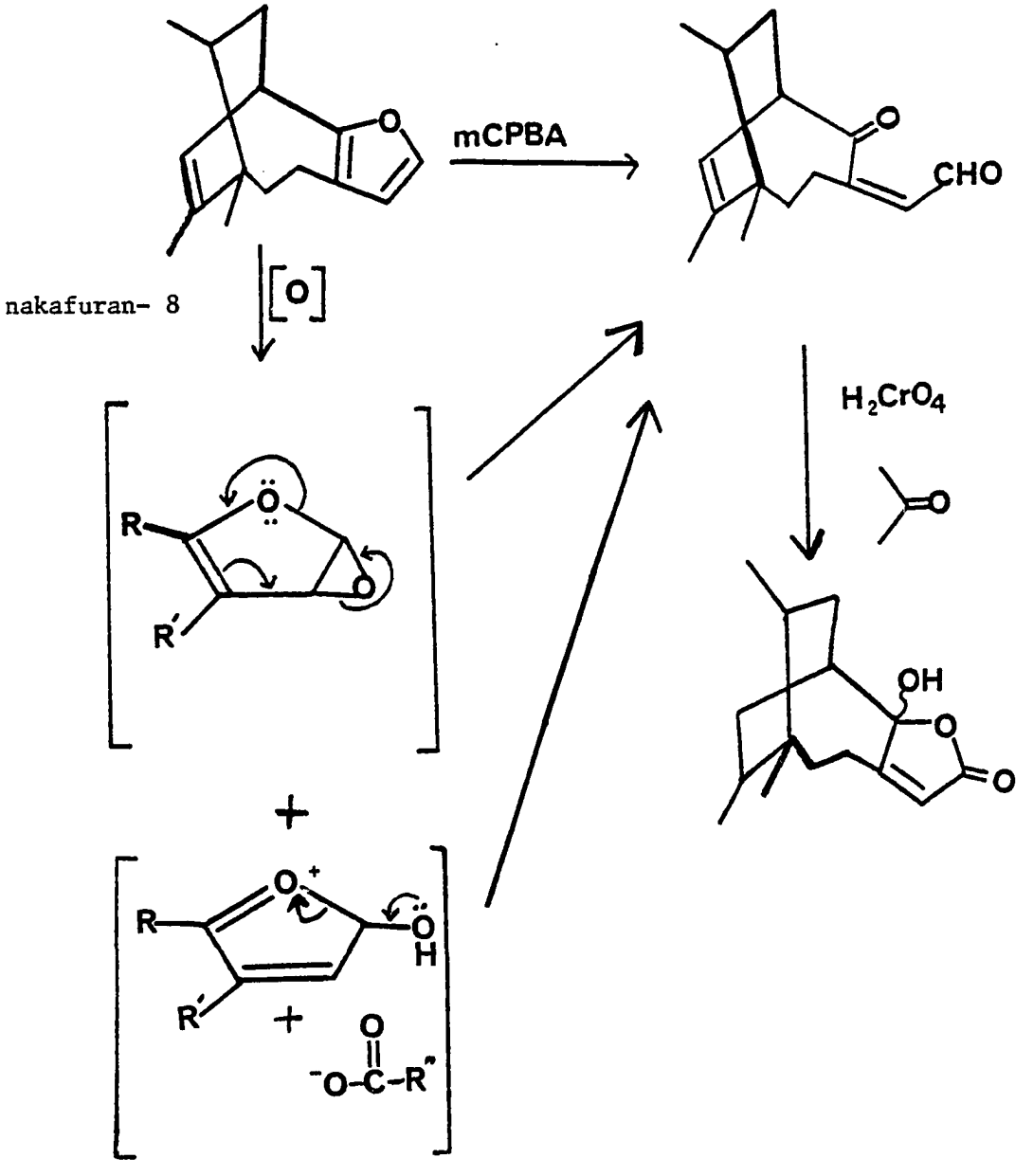
59 R= Et

113

114

Figure 60. Mechanism of the keto-aldehyde formation from 58 and 59; decomposition of 113 to the hydroxybutenolide 114

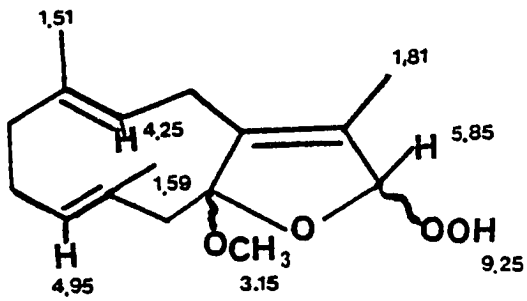
Figure 61. Formation of a γ- hydroxybutenolide from the γ-ketoenal derivative of nakafuran- 8[87]



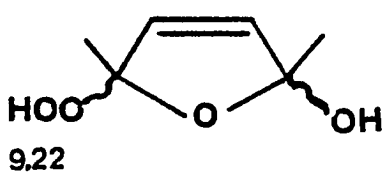
was recovered. The methoxyhydroperoxide derivative, 115, was identified on the basis of ^1H NMR comparison to furanodiene, methoxypacifigorgiolide and model hydroperoxide compounds. Figure 62 contains the ^1H NMR assignments of 115 which are based partly on analysis of the spectral features of the several model compounds shown. The hydroperoxide proton appeared as a one proton singlet at δ 9.25, which compared closely with those of model compounds 116 and 117. Compounds 116 and 117 were both produced from their respective furan precursors. In addition, a three proton methyl singlet appeared at δ 3.15 for the methoxyl group, along with a new band at δ 5.85 (1H, s) for the acetal proton. By ^1H NMR analysis, the olefinic methyl at δ 1.81 remained intact as did the two trisubstituted olefins (δ 4.95 (1H, dd), 4.25 (1H, bd), 1.59 (3H, bs) and 1.51 (3H, bs). Although the hydroperoxide was isolated, it possessed fleeting stability and decomposed to unidentified products after several days in chloroform at room temperature.

Methoxyhydroperoxides have previously been isolated from endoperoxide adducts of highly substituted furans by singlet oxygen oxidation in methanol. Pyrolysis of the methoxyperoxide 117 gave the corresponding methoxybutenolide (Figure 63).[88] Treatment of the peroxide 117 with acid or base, or upon air oxidation after several weeks, gave the corresponding hydroxybutenolide (118). Compound 118 could be converted to the methoxybutenolide derivative with dilute HCl in methanol.[88] It is possible that the hydroperoxide derivative of furanodiene is an intermediate to the methoxy and ethoxybutenolide compounds via acid catalyzed solvolysis of the hemiacetal intermediate during workup. However, less substituted furans are known to react with

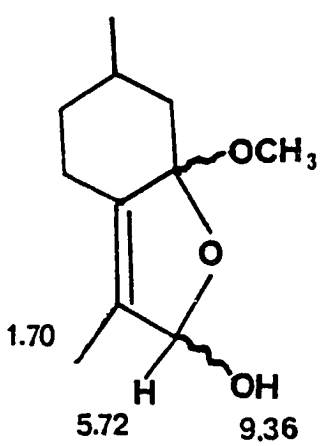
Figure 62. ^{1}H NMR assignments of the methoxy hydroperoxide derivative of furanodiene and several model compounds[88]



115

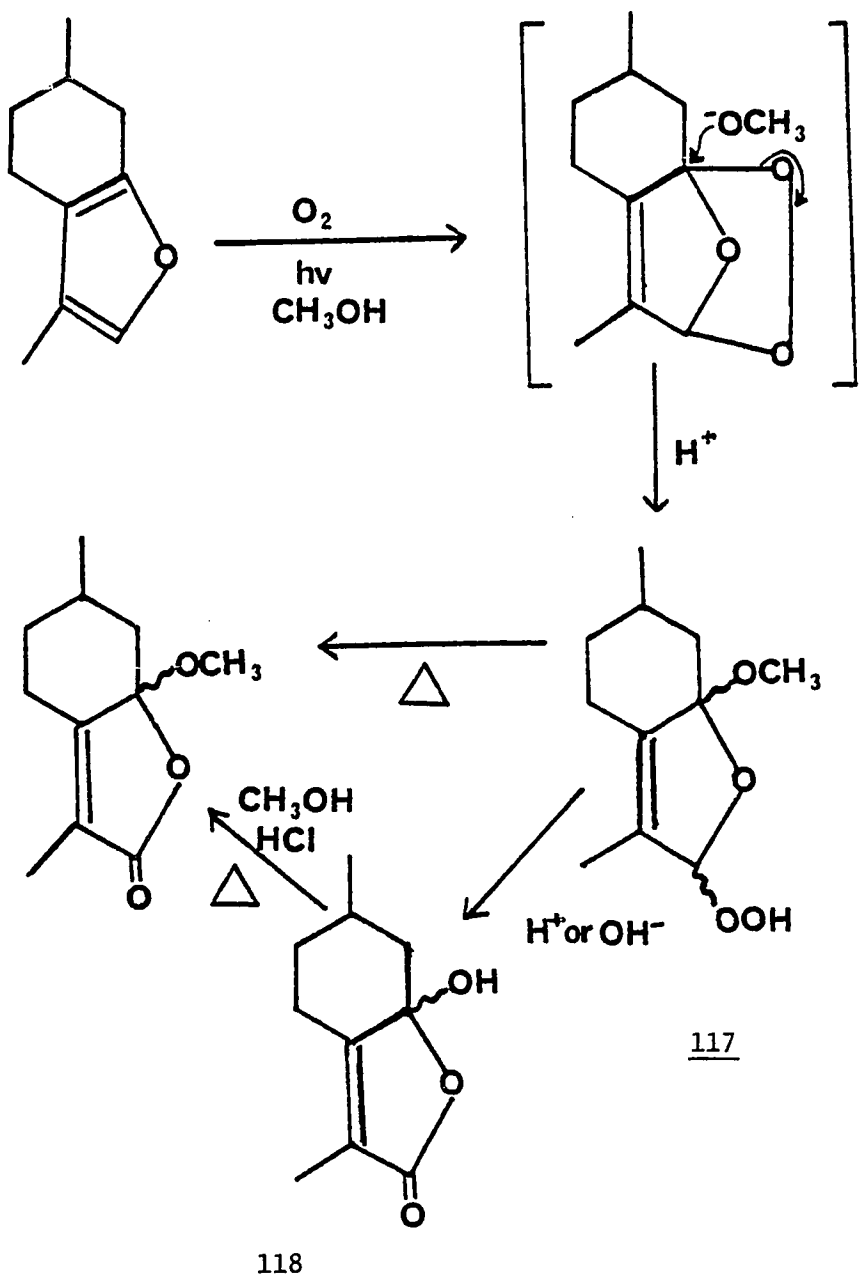


116



117

Figure 63. Photooxidation of menthofuran[88]



singlet oxygens to form β,γ-epoxy-γ-lactone intermediates. The epoxides can open by methanol or ethanol addition under the acidic conditions of workup to give the corresponding alkoxybutenolides.[89] These two possible schemes for the formation of 58 and 59 from furanodiene are both outlined in Figure 64. In support of pathway B, very minor amounts (4 mg) of a hydroxybutenolide derivative of pacifigorgiolide (118) were isolated from Pacifigorgia media collected in Islas Tres Marias. This compound was identified by the infrared absorption at 1765 cm^{-1} and ^1H NMR comparison with 57-59. However, the extremely minor amounts involved and the isolation of this pseudoacid from just one collection of gorgonians makes it a very weak argument. The true solvolysis intermediates, under actual extraction and storage conditions, may have an extremely limited life span and thus be essentially undetectable.

Other examples of methoxybutenolides have been found in nature, isolated from methanol extracts with their corresponding furans. Figure 65 lists several examples of this coisolation relationship found in methanol extracts of marine sponges and nudibranchs.[90-93] All of these examples make a stronger case for the autooxidation of furans to alkoxybutenolide derivatives. In contrast, the formation of a butenolide such as pacifigorgiolide from the autooxidation of furanodiene is highly unlikely since reduction of the hydroxybutenolide is necessary to form the butenolide.

F. Structure elucidation of the rearranged dimethyl ester 60

In addition to pacifigorgiolide, two other new natural products were also isolated from extracts of Pacifigorgia pulchra exilis

Figure 64. Possible formation of 58 and 59 from furanodiene[89, 90]

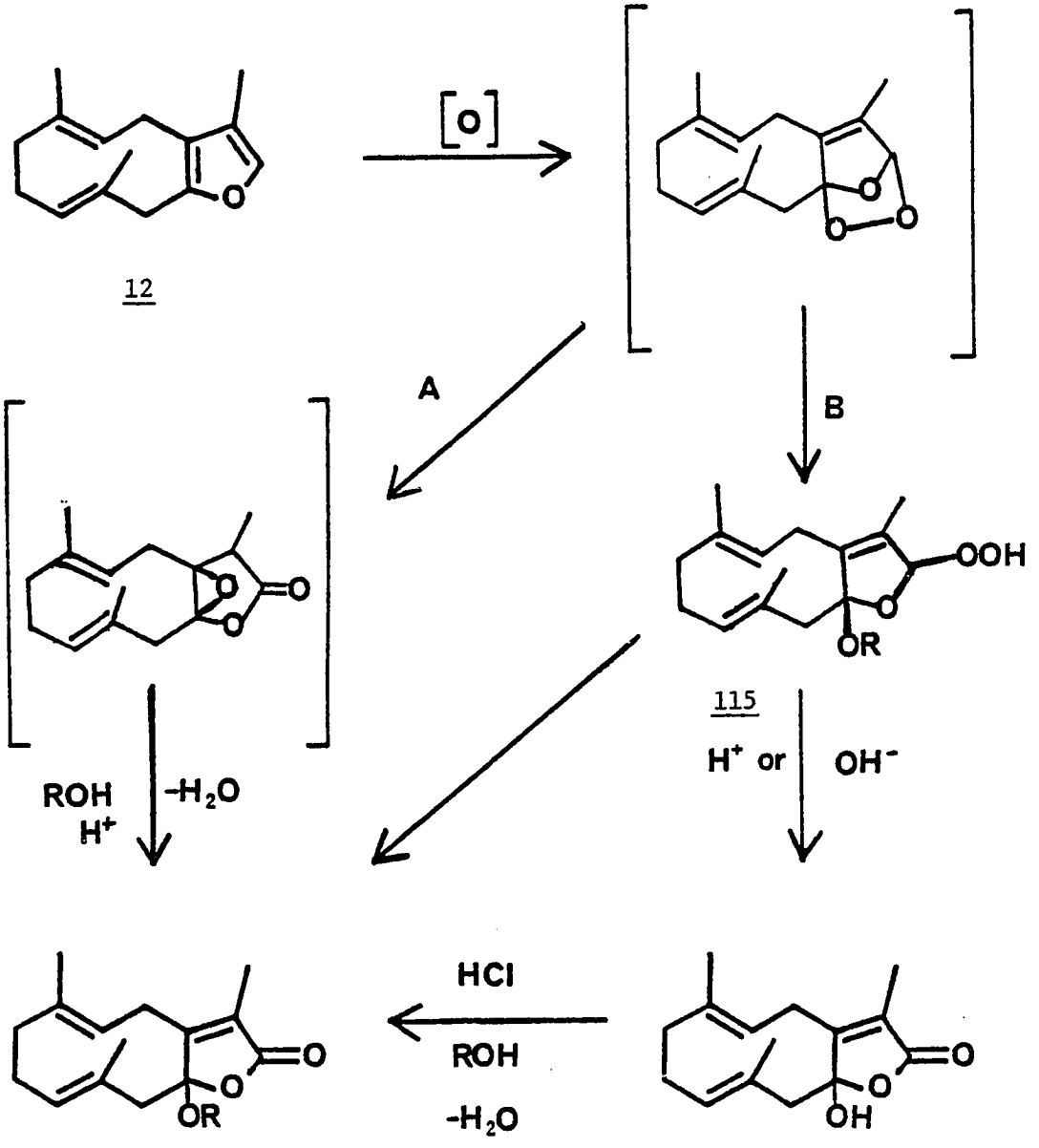
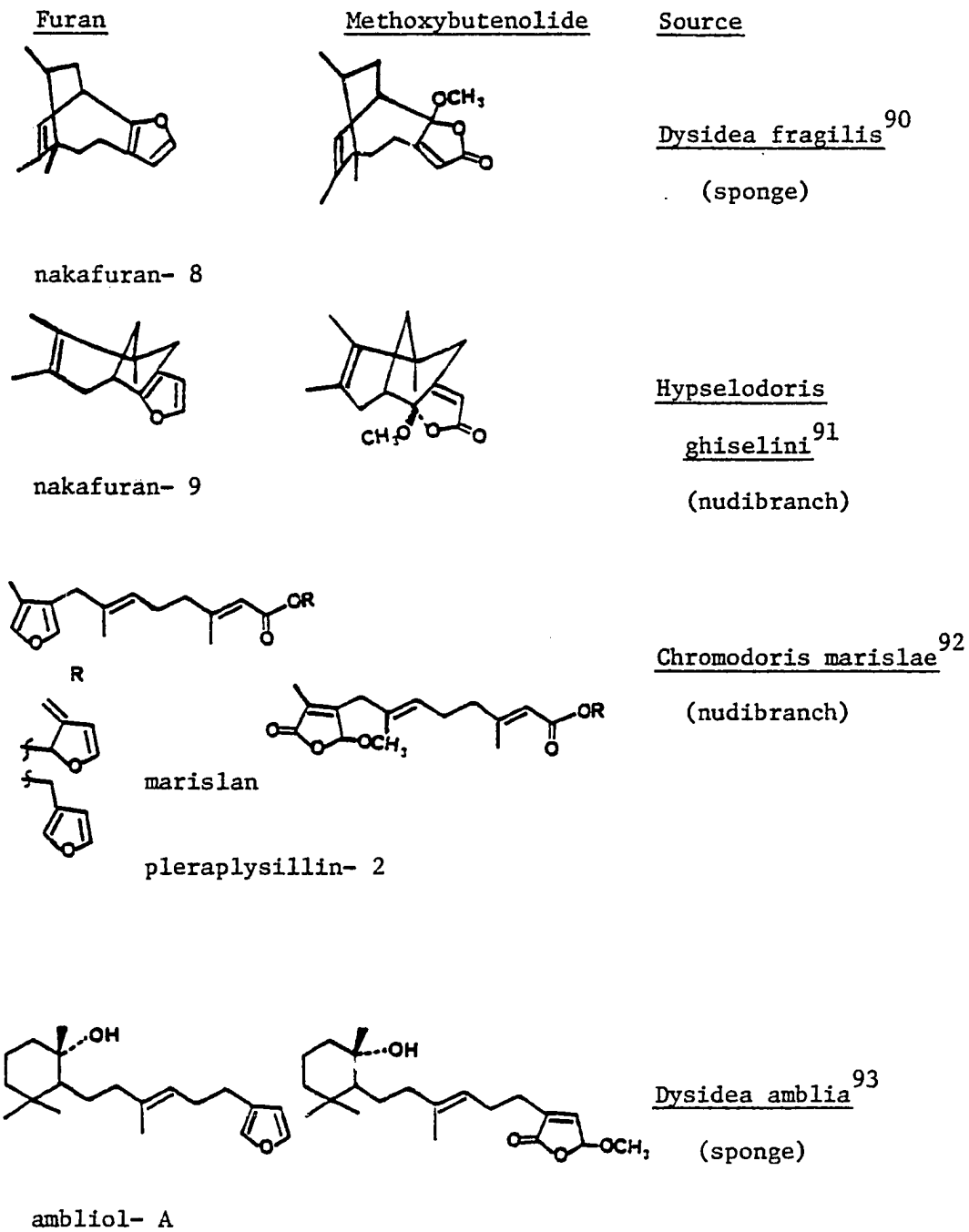


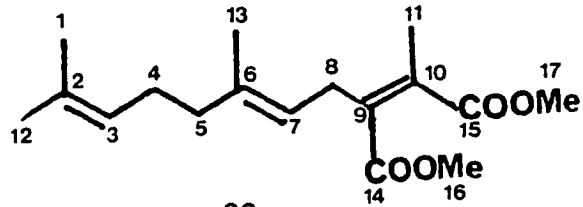
Figure 65. Examples of the coisolation of methoxybutenolides with furan "precursors" in marine organisms



(collected at Bahia Los Frailes). Hplc separation of silica gel chromatography column fractions containing a mixture of 58 and 59 yielded a more polar constituent, 60. Compound 60 was purified by silica hplc using 25% ethyl acetate-isooctane. Compound 60 appeared to be quite different from the pacifigorgiolide compounds by ^{1}H and ^{13}C NMR analysis. Analysis of ^{13}C NMR data for 60, combined with high resolution mass measurement, resulted in the assignment of a molecular formula of $C_{17}H_{26}O_4$, which contained five degrees of unsaturation. Several features of the molecule were readily identified by their characteristic absorptions in the ^{1}H and ^{13}C NMR spectra (Table 25). Two methyl singlets in the ^{1}H NMR spectrum at δ 3.45 and 3.40 were characteristic of two methyl esters, and this conclusion was further supported by ^{13}C NMR signals for four such carbons at 169.3 (s) and 52.1 (q) ppm. Three olefinic methyl groups and two olefinic protons were also present, as illustrated by ^{1}H NMR signals at δ 1.65 (3H, s), 1.54 (3H, s), 1.52 (3H, s), 5.15 (1H, bt (7)) and 5.18 (1H, bt (7)). The ^{13}C NMR spectrum contained absorptions for six olefin carbons at 138.1 (s), 132.1 (s), 131.6 (s), 131.5 (s), 124.0 (d) and 118.4 (d). This accounted for the three remaining degrees of unsaturation. Therefore, 60 was assigned a linear structure, in contrast to the germacrene ring system found previously in Pacifigorgia spp. ^{1}H NMR decoupling experiments were useful in defining the backbone of this linear compound. The olefinic proton at δ 5.15 was found to be coupled to the two proton multiplet at 2.08 (J = 7 Hz), which was in turn coupled to the two proton multiplet at 1.96 (J = 7 Hz). Allylic coupling (<1 Hz) between the olefinic proton at δ 5.15 and the two olefinic methyls at δ 1.65 and 1.54 was also observed. The olefinic proton at δ 5.18 was directly coupled to a vicinal methylene group

Table 25

Spectral Data for the Dimethyl Ester 60



60

$C_{17}H_{26}O_4$; UV: λ_{max}^{MeOH} = 208 nm (27,000); IR ($CHCl_3$): 2980, 2940, 1750-1730, 1650, 1445, 1390, 1280, 1180, 1110, 920 cm^{-1}.

C	^{1}H NMR (360 MHz, d_6-benzene)	^{13}C NMR (50 MHz, $CDCl_3$)$^+$
1	1.54 bs	17.7 (23.9)
2	--	131.5
3	5.15 bt (7)	124.0 (J_R = 44.0)
4	2.08 m (7)	26.6 (24.7)
5	1.99 m (7)	39.6 (24.6)
6	--	138.1
7	5.18 bt (7)	118.4 (45.3)
8	2.96 d (7)	28.9 (29.8)
9	--	132.1
10	--	131.6
11	1.73 s	15.1 (25.7)
12	1.65 bs	25.7 (23.2)
13	1.52 bs	16.2 (25.2)
14	--	169.3
15	--	169.3
16-OMe	3.45 s	52.1 (37.1)
17-OMe	3.40 s	52.1 (37.1)

$^+$Assigned based on ^{1}H NMR decoupling and nOe experiments, residual coupling constants and comparison with model compounds.

at 2.96 (d, J = 7 Hz) and allylically coupled to both the methyl group at δ 1.52 and the multiplet at 1.96. The methylene group at δ 2.96, which appeared to be bisallylic by the nature of its low field chemical shift, also showed a small homoallylic coupling to the olefinic methyl group at δ 1.73. These ^{1}H NMR decoupling results, combined with the ^{13}C NMR data, allowed the partial structure a to be drawn, as shown in Figure 66, leaving only the placement of the two methyl esters and the stereochemistry of the olefins to be defined.

Several other features, in addition to the ^{1}H NMR decoupling data, placed the two esters on the terminal olefin in an irregular isoprenoid fashion. First, the presence of six methyl groups or equivalents in the molecule (four olefinic methyls plus the two carbonyl groups) was indicative of a rearranged isoprenoid skeleton. A regular linear sesquiterpene compound would have only five methyl equivalents. Second, the infrared absorption at 1730-1750 cm^{-1} and the high value of the UV absorption extinction coefficient (λ_{max}^{MeOH} = 208 nm (27,000)) were characteristic of an unsaturated ester functionality with extended conjugation. Figure 67 gives examples of the UV and IR absorptions of model compounds which demonstrate this point. Third, even without the decoupling evidence, the two methyl esters must be placed on the tetrasubstituted olefin based on chemical shift arguments alone. As the model compounds in Figure 67 demonstrate, the chemical shift of olefinic protons in a diester containing di- or trisubstituted olefin ranges from ~δ 5.8-7.0 in the ^{1}H NMR spectrum. As both of the olefinic protons in 60 absorb at ~δ 5.1-5.2, this rules out the possibility of their being α or β to an ester. Therefore, the two methyl esters must be placed on

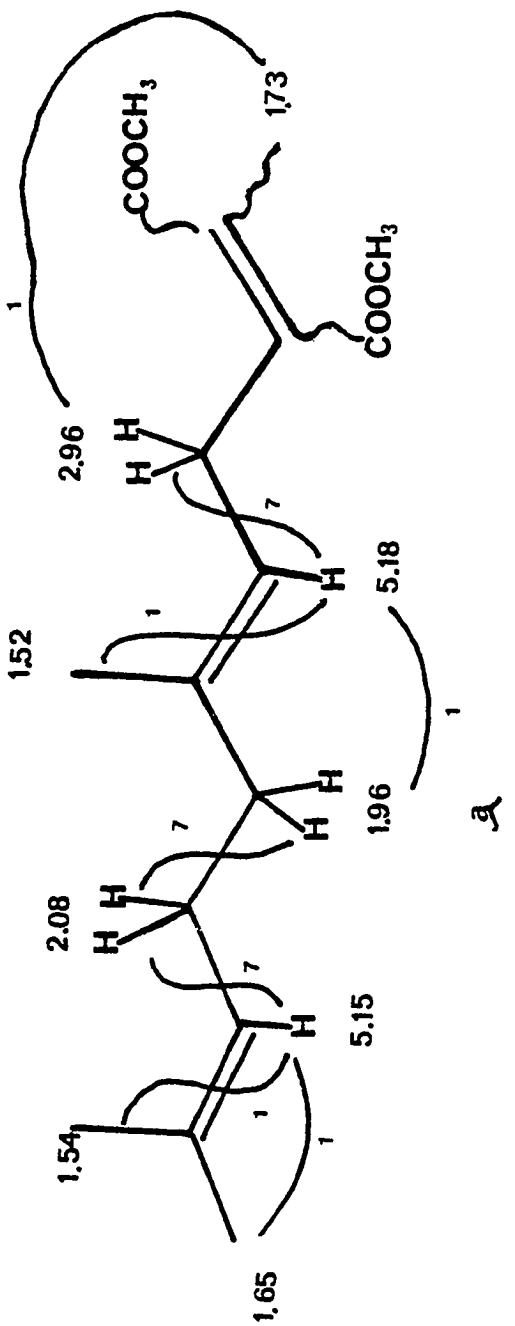
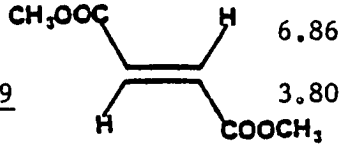
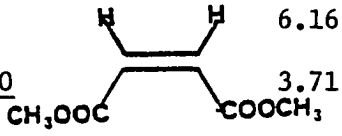
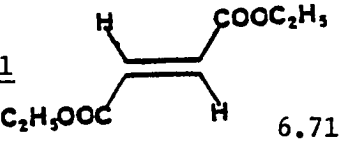
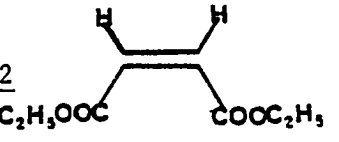
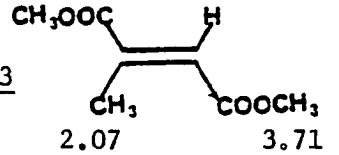
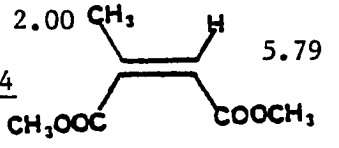
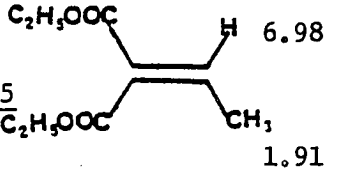


Figure 66. Results of ^1H NMR decoupling experiments on 60 (d_6-benzene)

Figure 67. Spectral data of model compounds for comparison with 60[94]

	Proton NMR (ppm)	Infrared (cm^{-1})	Ultraviolet (nm)
119 	6.86 3.80	1721 1440	215 (16,000)
120 	6.16 3.71	1730 1640 1435	
121 	6.71	1721 1642	213 (30,000)
122 		1721 1640	
123 	3.81 2.07 3.71	1725 (br) 1650 1440	
124 	2.00 5.79 3.60 3.70		
125 	6.98 1.91	1725 (br) 1645 1440	207 (900)

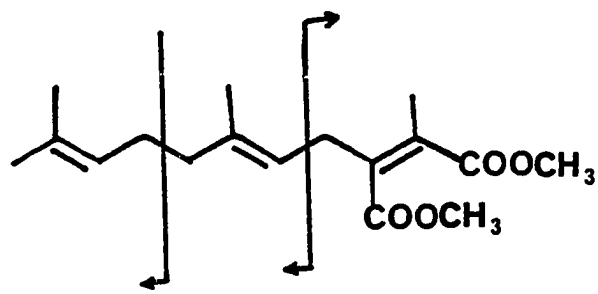
the tetrasubstituted olefin.

The high resolution mass spectrum of 60 provided additional evidence for the placement of the two methyl esters at the C-9, C-10 olefin position. The high resolution mass spectrum showed fragments reflecting the expected loss of $-OCH_3$ and $-CH_3OH$ from the methyl esters. However, the fragments observed for $M^+ - C_9H_{15}$ and $M^+ - C_9H_{14}$, which represent allylic cleavage at the C-7, C-8 bond, are only possible for a structure such as a with both esters placed at the terminal olefin. These conclusions were further supported by the base peak at 193.0860 for $C_{11}H_{13}O_3$ ($M^+ - CH_3OH - C_5H_9$) and the fragment at 225.1136 for $M^+ - C_5H_9$. Figure 68 depicts these fragmentation patterns.

However, the problem of the exact placement of these methyl esters on the C-9, C-10 olefin remained. Three possibilities exist, as depicted in Figure 69: with the methyl ester cis to one another (a), trans (b) or geminal (c). Partial structure c was ruled out on the basis of comparisons of the spectral data of 60 with that of model compounds shown in Figure 67. The geminal diester model compound, ethylidene diethyl malonate (125)[94] possesses an extremely small extinction coefficient for the UV absorption at 207 nm (ϵ = 900). This is very much lower than extinction coefficient of 61 (27,000). The small extinction coefficient in 126 is representative of steric hindrance preventing maximum orbital overlap, a case which obviously does not exist for 60. Perhaps more importantly, structure c may be ruled out on the basis of ^{13}C NMR calculations for tetrasubstituted olefins. Calculations for the case where the methyl esters are geminal give the α and β carbons as 119.5 and 158.5 ppm, reflecting the polarizability of the

Figure 68. High resolution mass fragmentation of 60

m/z 123 for C_9H_{15}



m/z 225 for $C_{12}H_{17}O_4$

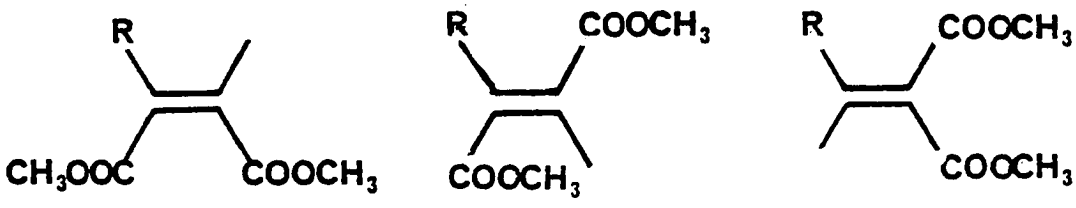
$= M^+ - C_5H_9$

m/z 172 for $C_8H_{12}O_4$

m/z 193 for $C_{11}H_{13}O_3$

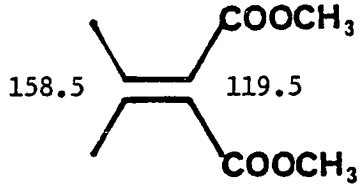
$= M^+ - C_5H_9 - CH_3OH$

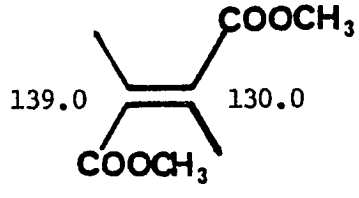
Figure 69. Possible stereochemistry of the tetrasubstituted olefin in 60

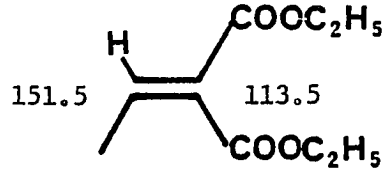


a b c

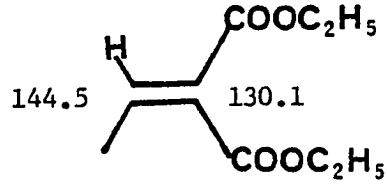
Figure 70. ^{13}C NMR calculations for olefinic carbons in tri- and tetrasubstituted diester containing olefins[76]







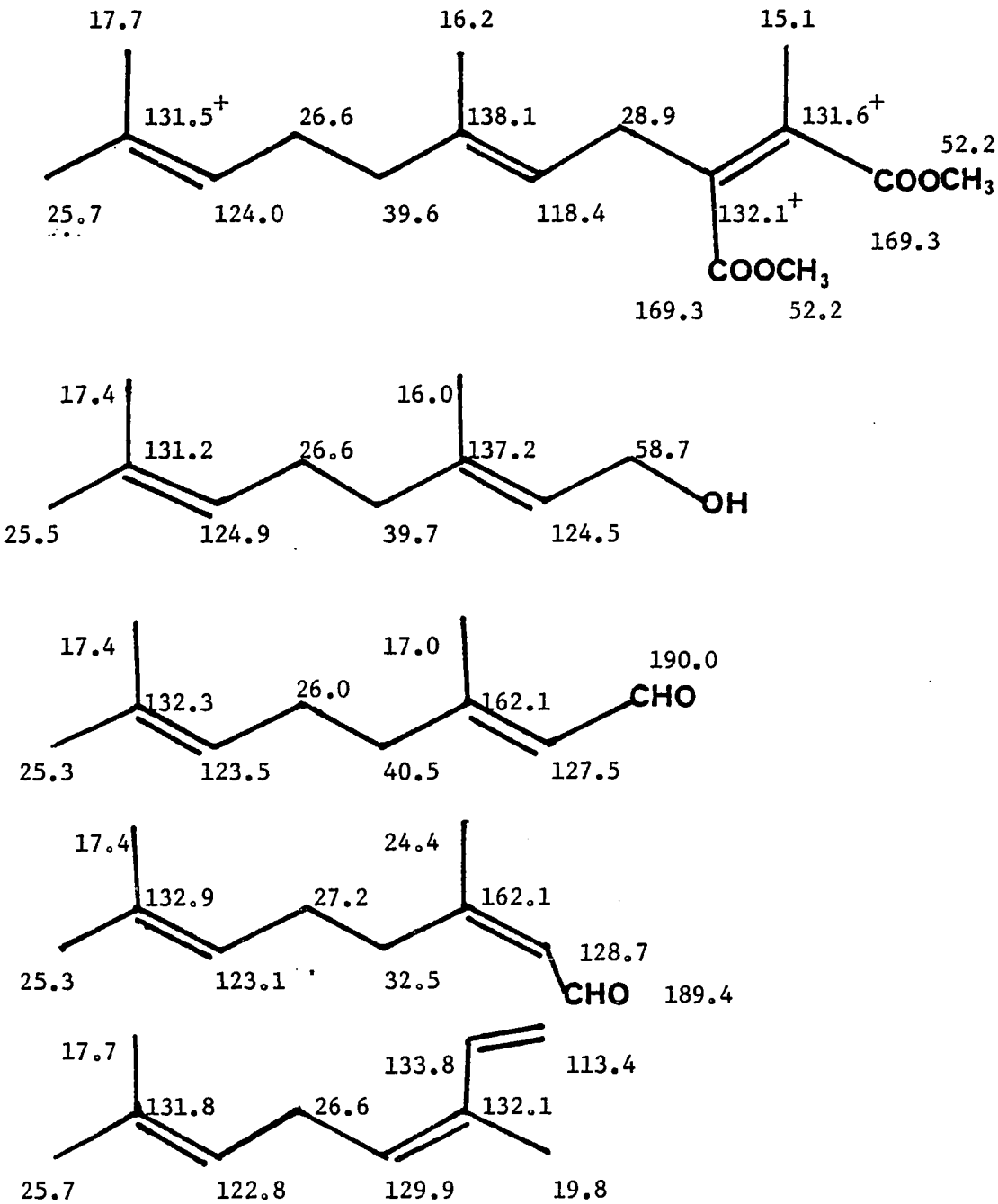
Observed[94]



olefin. In the isomeric olefin where the esters are vicinal, the calculations for both carbons are identical, 139.0 ppm (Figure 70).[76] The actual ^{13}C NMR bands observed for model compound 126 (with geminal esters) reflect this difference. The α carbon absorbs at 130.1 and the β carbon at 144.5 ppm,[94] and the calculated values are 113.5 and 151.5 ppm, respectively.[76] The polarization effect of unsaturated geminal esters is not observed in the ^{13}C NMR bands for the C-9, C-10 olefinic carbons of 60, ruling out structure c. The ^{13}C NMR data for the isoprenoid portion of 60 compared very closely with geraniol 126 and other monoterpene model compounds, 127-129 (Figure 71),[95] leaving the singlet bands at 132.1 and 131.6 assigned to the terminal olefinic bond bearing the methyl esters.

Now the problem of determining the stereochemistry at the C-9, C-10 olefin and the C-6, C-7 olefin remained. These problems were resolved using comparisons of the ^{13}C NMR assignments with model compounds and by ^{1}H NMR nuclear Overhauser enhancement difference studies (nOeds).[60] The C-6, C-7 olefin was assigned as E based on the high field ^{13}C NMR chemical shift of the olefinic methyl group (16.2 ppm). It is well known that the methyl groups of E olefins usually absorb at 8-10 ppm higher field than those for corresponding Z olefins (Figure 71).[77,95] The nOeds results confirmed this assignment, as no enhancement was observed in the olefinic methine at δ 5.18 upon irradiation of the methyl at δ 1.52 or vice versa. If the two esters at the C-9, C-10 olefin were oriented trans to one another, one would expect irradiation of the olefinic methyl at δ 1.73 to enhance both of the methyl esters as shown in a in Figure 72. However, irradiation of the olefinic methyl

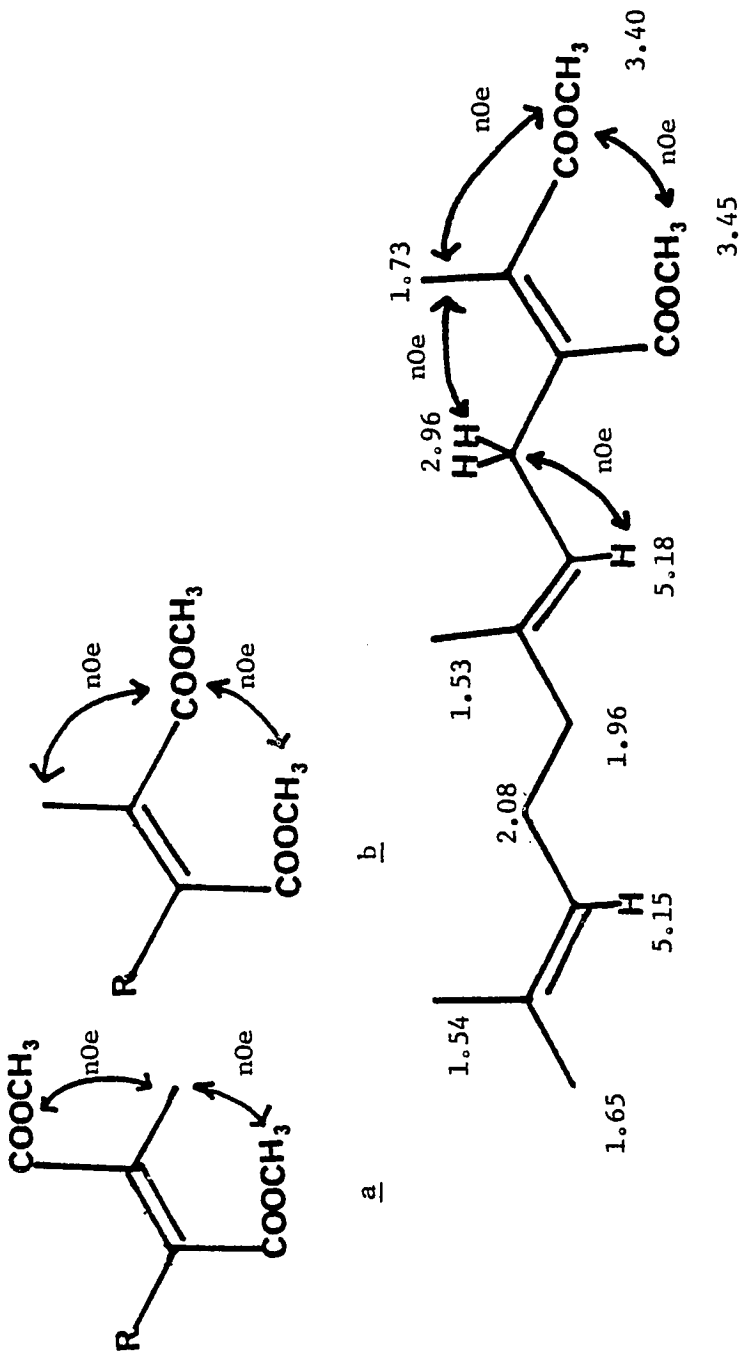
Figure 71. ^{13}C NMR spectral data for 60 and related model compounds[95]



resulted in only one enhancement of the methyl group at δ 3.40, ruling out structure a. Irradiation at δ 3.40 resulted in enhancements in both signals at δ 1.73 and δ 3.45, indicating that the methyl esters existed in a cis relationship. Irradiation of the methylene group at δ 2.96 resulted in a enhancement of the olefinic methyl at δ 1.73, indicating their cis relationship and confirming the structure of 60 as shown in Figure 72.

Several proposals for the biogenesis of the unusual irregular isoprenoid skeleton of 60 can be made. A 1,3 methyl transfer reaction in a farnesol precursor seems likely, followed by oxidation to the methyl esters. An alternative scheme, based on similarities between structures of 58 and 60 is also depicted in Figure 73. This scheme presumes nucleophilic attack by methoxy anion on the lactone carbonyl of 58, followed by formation of a diester, with resultant displacement of an alkyl group. This scheme does not seem likely, however, based on the poor leaving group abilities of an alkyl group compared with methoxy. Nevertheless, it does result in the sterospecific cis diester found in 60. It is also a mechanism for forming the irregular sesquiterpene skeleton from a related compound present in the extract. If 58 is simply a photooxidation product of furanodiene in methanol, then it seems unlikely that 60 would be formed from 58 in the extract. If 58 is, in fact, a natural product, then enzymatic mechanisms could be invoked for the formation of 60 from 58.

Figure 72. Results of ^{1}H NMR difference nOe studies of 60



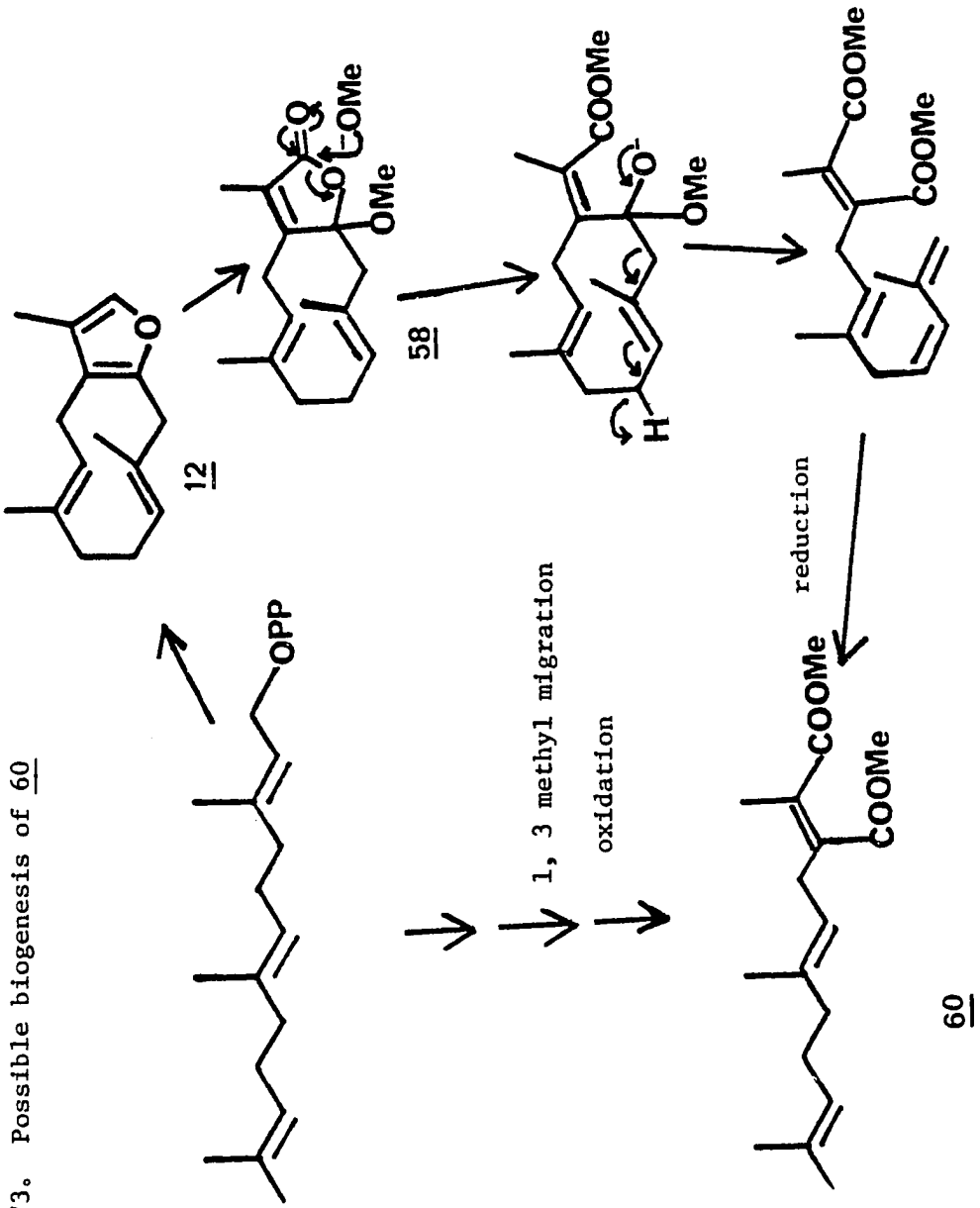


Figure 73. Possible biogenesis of 60

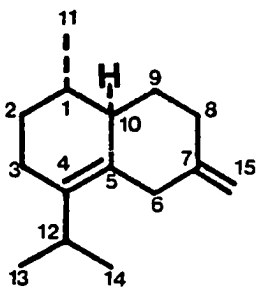
G. Structure elucidation of isoepizonarene (61).

The concept of diversity in the sesquiterpene-derived compounds found in gorgonians (as discussed in the introduction to this chapter) was reinforced by the isolation of a new cadanane hydrocarbon, 61, from four species of Pacifigorgia. Nonpolar fractions of P. pulchra exilis from Bahia Los Frailes, P. media from Islas Tres Marias, and two unidentified sea fans from Loreto (P. spp. A and B) yielded large quantities of mixtures of hydrocarbons. This mixtures were separated by hplc in 100% isooctane or hexane to give one major component by ^{1}H NMR. Compound 61 was isolated as a colorless oil which showed $[\alpha]_D^{26} = -24.4^\circ$ (c = 0.9, $CHCl_3$). High resolution mass measurement gave a molecular formula of $C_{15}H_{24}$ for 61, indicating a sesquiterpene hydrocarbon with four degrees of unsaturation. One exomethylene and one tetrasubstituted olefin were present by interpretation of ^{13}C NMR bands at 149.6 (s), 133.7 (s), 130.2 (s) and 106.1 (t), leaving two carbocyclic rings to account for (Table 26).

^{1}H NMR decoupling studies with 61 provided clues to several partial structures of the molecule. Irradiation of the exomethylene protons at δ 4.62 and 4.58 (1H each, bs) resulted in sharpening of signals for allylic methylene protons at δ 2.0-2.2. Irradiation of the doublet at δ 3.39 (1H, J = -14 Hz) resulted in the collapse of the broad doublet at δ 2.35 (1H, J = -14 Hz), indicating their geminal relationship, and a sharpening of the broad singlets of the exomethylene protons at δ 4.62 and 4.58. Irradiation of the methylene proton at δ 2.35, in turn, resulted in the collapse of the doublet at δ 3.39 to a sharp singlet. The simultaneous sharpening of the bands for the exomethylene protons at

Table 26

Spectral Data for Isoepizonarene (61)



61

$C_{15}H_{24}$; $[\alpha]_D^{26} = -24.4^\circ$ (c - 0.9, $CHCl_3$); UV: $\lambda_{max}^{Hex} = 240$ nm (3600); IR ($CHCl_3$): 2950, 1631, 1440, 1370, 890 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDcl_3$)
1	2.10 m	35.0
2	1.2-1.8 m	38.3^+
3	1.8-2.1 m	35.4^+
4		130.2^{++}
5		133.7^{++}
6	3.39 d (-14), 2.35 d (-14)	38.3^+
7		149.6
8	2.20 bdd, 2.0 m	23.1
9	1.2-1.8 m	30.7^+
10	2.35 m	46.0
11	0.98 d (7)	28.7
12	2.93 m (7)	29.6
13	0.92^+ d (7)	20.8^{+++}
14	0.94^+ d (7)	20.6^{+++}
15	4.62 bs, 4.58 bs	106.1

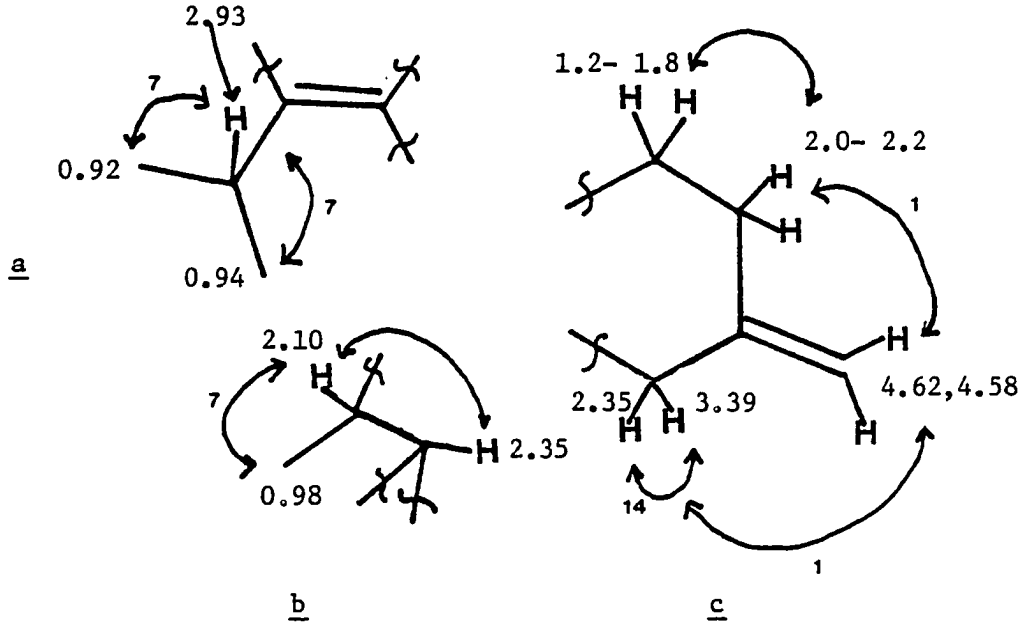
+,++,+++ may be interchanged

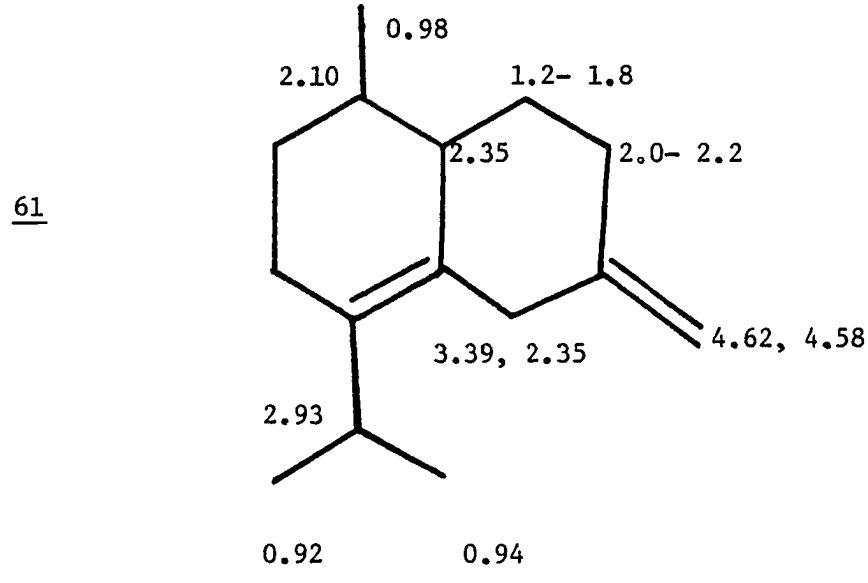
δ 4.62 and 4.58 also demonstrated their allylic relationship. Irradiation of the low field multiplet at δ 2.93 (1H, J = 7 Hz) resulted in collapse of the two methyl doublets at δ 0.94 and 0.92 (3H each, J = 7 Hz) to two singlets. Irradiation of these two methyl groups, in turn, converted the singlet at δ 2.93 band to a broad singlet. Irradiation of the third methyl doublet at δ 0.98 (3H, J = 7Hz) resulted in a major change in the multiplet centered around δ 2.10. This information allowed the proposal of partial structures a-c as shown in Figure 74. The methine proton at δ 2.93 was assigned to an allylic position of the tetrasubstituted olefin based on chemical shift considerations and the lack of further coupling.

These partial structures accounted for both olefins, all three methyls, two methylenes and two methine groups present in the ^{13}C NMR spectrum. This left one methine and three methylene groups to account for. Due to the blocked position of the geminal methylene protons at δ 3.39 and δ 2.35, and their low field chemical shifts, these geminal protons were placed next to the tetrasubstituted olefin. Assuming the compound was a regular sesquiterpene, and using the ^{13}C NMR spectral data (Table 26), the remaining methine in the ^{13}C NMR spectrum was assigned to a bridgehead carbon. Therefore the gross structure of the compound was formulated as 61, possessing a cadanane ring system.

In order to confirm the gross structure of this cadanane derivative, 61 was aromatized by dehydrogenation using palladium over carbon in refluxing xylene for 20 hours. Distillation of the product from xylene followed by silica hplc purification (100% isooctane) gave cadalene (130) (Figure 75). Compound 130 was identical in all respects

Figure 74. Partial structures of 61 from ^{1}H NMR decoupling results





to the natural product previously isolated from the brown alga Dictyopteris divaricata, and from other terrestrial sources.[94,96]

Dehydrogenation of 61 to cadalene provided conclusive confirmation that 61 possessed the cadanane ring system, but this left the stereochemistry of the two asymmetric centers at C-1 and C-10 to be defined. In order to determine the stereochemistry at these two centers, 61 was converted to the endocyclic olefin cadanane derivative, 131, by acid catalyzed rearrangement of the exocyclic olefin. The structure of 131 was confirmed from the ^{1}H NMR spectrum of the product. The exomethylene protons at δ 4.62 and 4.58, and the methylene protons δ 3.39 and 2.35, were replaced by two new signals at δ 1.77 (3H, bs) and δ 6.22 (1H, bs) for the olefinic methyl and olefinic proton. The three secondary methyl groups remained at δ 0.99, 0.96 and 0.95, and the isopropyl methine at δ 2.93 shifted to δ 3.03 ppm (Figure 75).

Figure 76 lists the four known isomers of 131 with their spectral data.[97] Three of the four isomers are known natural products, previously isolated from the brown alga, (Dictyopteris),[98] and from other terrestrial sources.[97] A racemic mixture containing the fourth compound, (+) zonarene (134), has been synthesized from farnesol using BF_3-etherate. Comparison of the spectral data of 131 with these compounds resulted in the assignment of the structure of 131 as epizonarene, based on similarities in the ^{1}H NMR data and their optical rotations. In both enantiomers of zonarene (133 and 134), the chemical shift of the C-1 methyl comes at much higher field (~δ 0.80) than the dobulet methyl in 131 (δ 0.99). This methyl shift is identical to the chemical shift of the C-1 methyl in the enantiomers of epizonarene (131 and 132).

Figure 75. Aromatization and isomerization reactions of isoepizonarene (61)

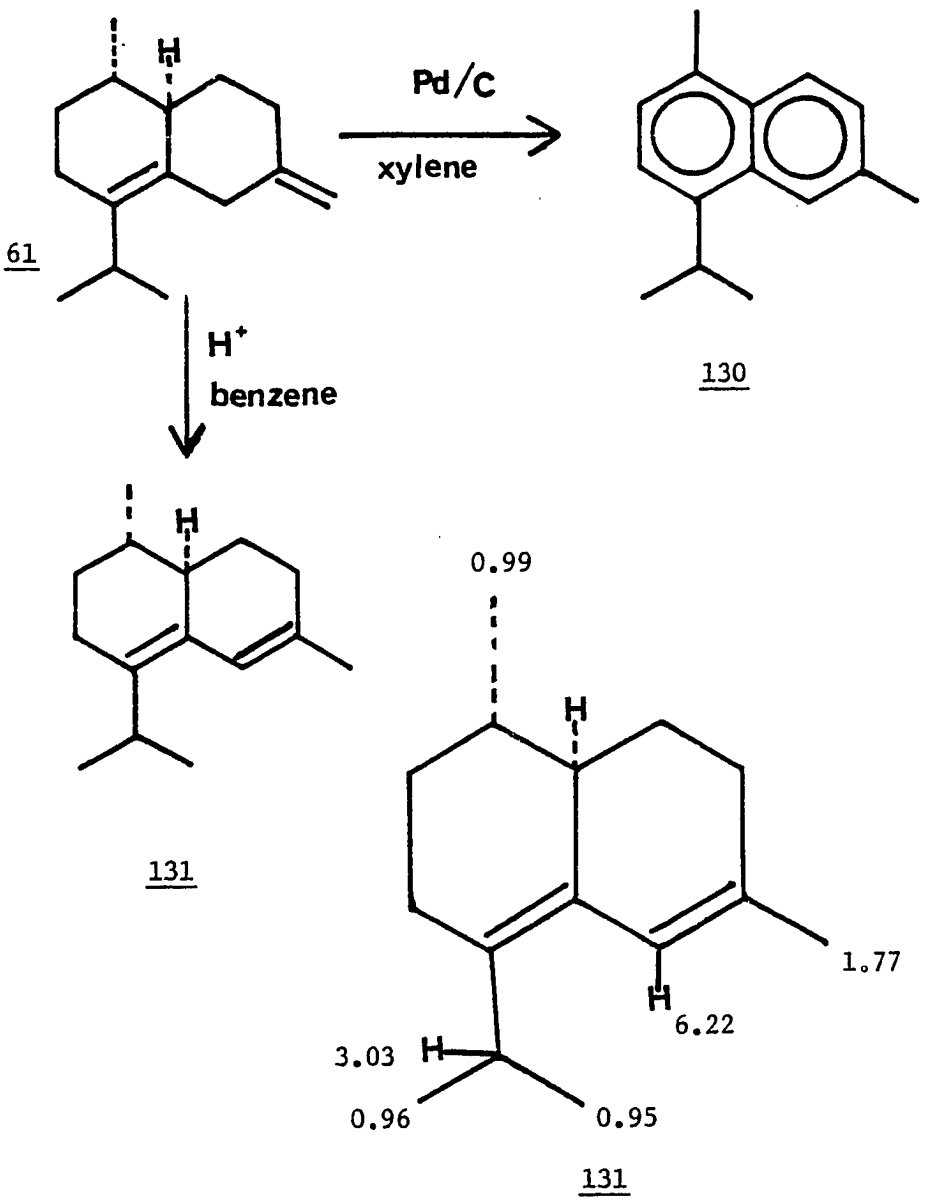
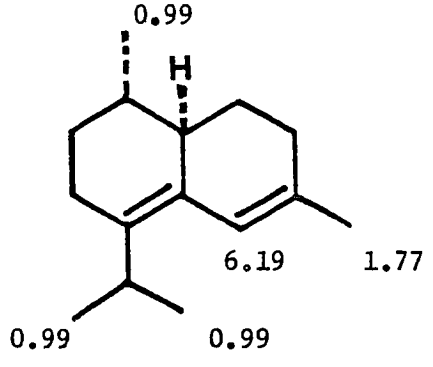
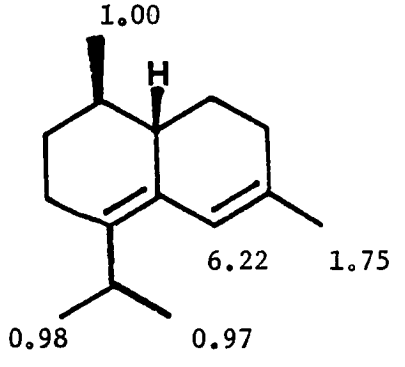


Figure 76. Spectral data for the stereoisomers of zonarene[97]



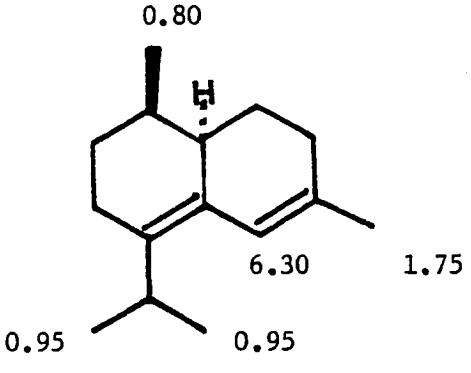
131 epizonarene

$[\alpha]_D$ = - 175



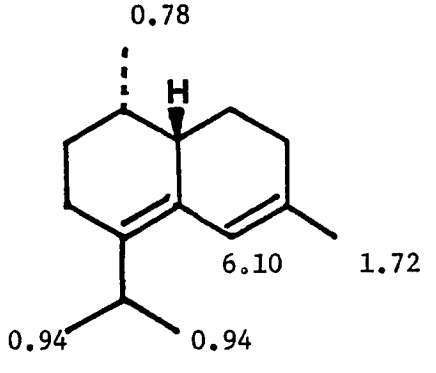
132 entepizonarene

$[\alpha]_D$ = +95



133 (-) zonarene

$[\alpha]_D$ = - 218



134 (+) zonarene

Therefore the C-1 methyl and C-10 proton must be cis to one another based on ^1H NMR chemical shifts of the C-1 methyl group. Based on the negative value of the optical rotation of the rearrangement product 131, its structure must be assigned as epizonarene. Therefore it follows that 61 is a new cadanane-based compound, isoepizonarene, as shown in Figure 75. The low value of the $[\alpha]_D$ of 131 compared with epizonarene may be due to the presence of either impurities or a partial racemic mixture in the sample on which the rotation was taken.

Several other cadanane-based compounds have previously been isolated from Caribbean and Red Sea gorgonians (Figure 45). Cadanane-derived compounds are also well known from several species of the brown algae Dictyopteris. It is interesting that isoepizonarene appears to be such a ubiquitous constituent of different species of Pacific gorgonians of the genus Pacifigorgia, collected over a broad geographic range.

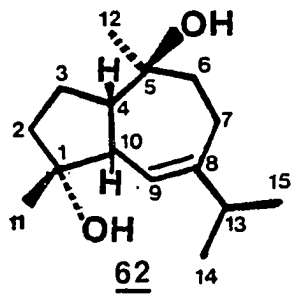
H. Isolation of the guaiane diol 62.

Examination of the polar fractions of an extract of an unknown Pacifigorgia species (P. sp. B collected in Loreto in 1981), revealed an interesting red staining compound (H_2SO_4) with no UV absorption. Repeated hplc purification of these fractions using 60-65% ethyl acetate-isooctane, followed by recrystallization from diethylether-hexane, yielded crystals of 62; $[\alpha]_D^{25} = -2.3^o$ (c = 0.3, $CHCl_3$); m.p. 141-142°C. High resolution mass measurement gave the molecular formula of $C_{15}H_{24}O$ for M^+-H_2O ($M^+ = C_{15}H_{27}O_2$ using ^{13}C NMR data), with three degrees of unsaturation. The infrared spectrum showed a broad hydroxyl absorption at 3500 cm^{-1}, with no carbonyl absorptions. The ^{13}C NMR

spectrum indicated two singlets at 80.0 and 75.6 ppm, for two tertiary alcohols. Absorptions for one trisubstituted olefin (140.0 (s) and 121.2 (d)) in the ^{13}C NMR spectrum left two carbocyclic rings to account for. Comparison of the ^{1}H and ^{13}C NMR data, and all other spectral data (Table 27), resulted in the assignment of the guaiane diol as 62, identical to the natural product previously isolated by Walker and Faulkner from the east Pacific gorgonian Pacifigorgia eximia (collected near La Paz).[22] The guaiane diol 62 has more recently also been isolated from extracts of the Australian soft coral, Lemnalia africana.[22] Related guaiane-derived aromatic compounds have also been isolated from gorgonians in Japan, Hawaii and Turkey (Figure 49).

Table 27

Spectral Data for the Guaiane-Diol (62)



62

$C_{15}H_{26}O_2$, m.p. 141-142°C; $[\alpha]_D^{25}$ = -2.3° (c = 0.3, $CHCl_3$) IR ($CHCl_3$): 3500, 2960, 1460, 1380, 1110, 935 cm^{-1}.

C	^1H NMR (360 MHz, $CDCl_3$)*	^{13}C NMR (50 MHz, d_6-acetone)
1		79.8^{+}
2		25.6^{++}
3		41.1^{+++}
4	2.15 m	51.2^{++++}
5		74.3^{+}
6		22.3^{++}
7		43.6^{+++}
8		148.9
9	5.50 bm (3)	123.4 (45.8)
10	2.15 m	50.8^{++++}
11	1.22 s	22.7 (21.9)
12	1.27 s	21.7 (22.1)
13	2.22 m (7)	37.9 (25.3)
14	0.99 d $(7)^{+}$	21.8 (21.1)
15	0.98 d $(7)^{+}$	21.5 (20.7)

*Assigned based on ^1H NMR decoupling experiments and the results of a ^1H NMR nOe experiment. Irradiation of δ 5.50 under nOe conditions produced enhancements at δ 1.22 and 2.22, resulting in their assignment at C-11 and C-13.

+,++,+++,++++ may be interchanged

I. Experimental - Chapter V

General. IR spectra were recorded on a Perkin-Elmer Model 137 spectrophotometer and UV spectra were obtained in methanol or hexane on a Beckman Acta XIV instrument. Proton NMR spectra were recorded in 0.5% TMS/$CDCl_3$ solution (unless otherwise noted) on a Varian HR-220-FT or 360 MHz Nicolet spectrometer, and ^{13}C NMR spectra were obtained on either a Varian CFT-20 or Nicolet 50 MHz multinuclear instrument. Low resolution mass spectra were recorded at 70 eV on a Hewlett-Packard Model 5930A instrument, and high resolution spectral measurements were obtained through the Bio-Organic, Biomedical Mass Spectrometry Resource (A.L. Burlingame, Director), supported by NIH Research Grant #RR00719 from the Division of Research Resources. All solvents were dried and distilled from glass prior to use.

Collection, extraction and chromatography. Gorgonians were collected by hand using SCUBA in locations described in Figure 51 at -20-80′. Samples were frozen or stored in alcohol for preservation. Repeated extraction of the whole animal with 70% chloroform in methanol was followed by removal of the solvents under vacuum. The resulting aqueous residue was partitioned several times between dichloromethane and water and the organic layer was concentrated and dried over $MgSO_4$ to give a crude extract (usually 2-3% of the dry weight of the animal). The various sesquiterpene-derived metabolites were purified by elution from silica gel columns and then rechromatographed on preparative and analytical silica hplc using mixtures of ethyl acetate and isooctane. All of the gorgonian vouchers except AH-57 were identified by Dr. David Harden. AH-57 (Pacifigorgia media) was identified by Dr. Frederick

Bayer. The extraction and isolation procedure for each of the individual collections of gorgonians are summarized below.

Pacifigorgia pulchra exilis (V-25), an orange-yellow sea fan with white polyps, was collected from Los Frailes Canyon at -70-80′ in January, 1980 and preserved in ethanol. Extraction with 70% chloroform-methanol gave 16.8 g crude extract from 533 g dry weight of the gorgonian (3.2% yield). Silica gel chromatography using mixtures of ethyl acetate-isooctane gave 0.3 g of 61 (0.05% dry wt) from 100% isooctane, and ~2 g furanodiene (12) (12% extract, 0.4% dry wt) in 20% ethyl acetate-isooctane. A mixture of ethoxy-(59) and methoxypacifigorgiolide (58) was eluted in 30% ethyl acetate-isooctane (0.3 g 59 and 0.5 g 58; 0.05 and 0.09% dry wt respectively). The diester 60 was also eluted with 30% EtOAC-isooctane to give 0.05 g (0.01% dry wt).

Pacifigorgia media (AH-57), a purple sea fan, was collected from Islas Tres Marias in Pacific Mexico in May of 1978. The frozen animal was extracted initially with ethanol followed by 70% chloroform/methanol to give 40.0 g extract from ~1.5 kg dry weight (2.7% yield). Silica gel column chromatography in mixtures of pet ether, dichloromethane and ethyl acetate yielded 57, 59, and 61. Pacifigorgiolide (57), 80 mg, was eluted with 80% dichloromethane-pet ether (0.2% extract, 0.01% dry wt).

Pacifigorgia sp. A (V-42), a dark orange sea fan, was collected in Loreto, Baja California, in December 1980, from -30-40′, and stored in ethanol. The crude extract, 12.7 g, was obtained by the chloroform-methanol extraction of 657 g dry weight of the animal (1.9% yield). Rapid filtration chromatography using tlc grade silica gel in a

scintered glass funnel and the house vacuum gave 200 mg of 61, eluted in 100% isooctane (0.03% dry wt).

Pacifigorgia sp. B (V-54), a purple sea fan with white polyps, was collected from Puerto Escondido, Baja California, at -20-30′. The frozen gorgonian was extracted with chloroform/methanol to give 8.0 g of extract from 745 g of animals (dry weight 1% yield). Both the cadanene-based compound 61 and the guaiane diol 62 were isolated from V-54 after rapid silica gel chromatography in ethyl acetate-isooctane. The diol was eluted in 60% ethyl acetate-dichloromethane to give a total of 100 mg (0.01% dr wt).

Pacifigorgia tenuis (V-8), a red sea fan with white polyps, and P. florae (V-13), a red sea fan with gold polyps, were both collected in Cabo San Lucas by snorkeling in -10-20′. The freshly collected gorgonians were stored in ethanol. Analysis of the ^{1}H NMR spectra of thick layer silica gel fractions of the crude extract (rf = 0.4-0.6 in 100% Et_2O) revealed that V-8, P. tenuis, contained ethoxypacifigorgiolide (59) and P. florae, V-13, contained furanodiene (12).

Isolation of the germacrene butenolides from other marine sources

As part of my investigation of the chemistry of Pacific gorgonians, Nicaule crucifera (Pg 134), a dark brown sea whip, was collected in Palau (Micronesia) in September 1979 at -60-70′ and frozen. Extraction of the frozen animal with 70% chloroform-methanol gave 12 g crude extract from 500 g dry wt (2.4% yield). A silica gel column using pet-ether, dichloromethane and ethyl acetate eluted 1 g furanodiene (12)

(0.2% dry wt) with 10% CH_2Cl_2/pet ether and 0.25 g methoxypacifigorgiolide (58) (0.05% dry wt) in 100% CH_2Cl_2. These latter ompounds were identical by all spectral features to the natural products isolated from east Pacific gorgonians.

Pacifigorgiolide (57, Table 19). Compound 57 was isolated from an extract of Pacifigorgia media, AH-57, after hplc purification of column fractions using 30% EtOAc-isooctane. $[\alpha]_D^{28} = +0.11^\circ$ (c = 0.9, $CHCl_3$); $[\alpha]_{578} = +0.22^\circ$, $[\alpha]_{546} = +0.07^\circ$, $[\alpha]_{436} = +0.07^\circ$, $[\alpha]_{365} = 0^\circ$ (c = 0.9, $CHCl_3$, 28°C). HRMS: M^+ obs 232.1468 (14.0) (calc. 232.1463 for $C_{15}H_{20}O_2$), 217.1227 (11.3) ($M^+ - CH_3$), 121.1018 (100) (calc. 121.1017 for C_9H_{13}), 107.0862 (71.9).

Nuclear Overhauser enhancement (nOe) difference study of pacifigorgiolide (57). Compound 57 1.9 mg (8.2×10^{-3} M), in 1 ml of 0.5% TMS/$CDCl_3$ was prepared for a nOe experiment by carefully degassing the solution with bubbling Ar for 30 minutes. The decoupler was gated for on delay only with a 90° pulse angle and the time between irradiations (D_5) was increased to 20 seconds. The decoupler power was decreased and difference nOe techniques were employed.[60] Irradiation of the C-15 methyl at δ 1.50 resulted in a enhancement at the lactone methine proton at δ 4.97, indicating their proximity. Irradiation of the lactone olefinic methyl (C-13) at δ 1.87 resulted in enhancement of the C-3 olefinic proton at δ 4.34.

Methoxypacifigorgiolide (58, Tables 20 and 21). Compound 58 was purified by hplc using 20% ethyl acetate-isooctane. $[\alpha]_D^{28} = +0.05^\circ$ (c = 0.7,$CHCl_3$); $[\alpha]_{578} = -0.05^\circ$, $[\alpha]_{546} = -0.05^\circ$, $[\alpha]_{436} = -0.1^\circ$, $[\alpha]_{365}$ =

0^{o} (c = 0.7,$CHCl_3$, 28°C). HRMS: M^+ 262.1558 (1.2) (calc. 262.1569 for $C_{16}H_{22}O_3$), 247.1347 (0.9) ($M^+ -CH_3$), 230.1312 (20.4), (M^+ - MeOH) (calc. 230.1307), 140.0471 (100.0) (calc. 140.0471 for $C_7H_8O_3$, $M^+ -C_9H_{14}$), 122.1091 (5.4) (C_9H_{14}), 107.0863 (41.0) (C_8H_{11}). LRMS (70 eV): M^+ 262, 248, 230, 140.

Nuclear Overhauser enhancement difference study (nOeds) of methoxypacifigorgiolide (58). Compound 58, 2.5 mgs (9.5 x 10^{-3} M), in 1 ml 0.5% TMS/$CDCl_3$ was prepared for a nOe experiment by degassing with bubbling Argon. The decoupler was gated, the delay increased to 20 sec., and line broadening increased to 2 Hz. Difference nOe[60] experiments gave the following results:

Irradiation	nOe enhancement observed
δ 4.78 (C-7)	4.26 (C-3) 2.25 (C-6)
4.26 (C-3)	4.78 (C-7) 1.91 (C-13)
3.17 (-OMe)	3.21 (C-9) 2.86 (C-2) 2.30 (C-9) 1.62 (C-15)
2.86 (C-2)	3.17 (-OMe) 1.62 (C-15)
1.91 (C-13)	4.26 (C-3) 2.30 (C-9)

Ethoxypacifigorgiolide (59, Table 22). Compound 59 was purified by hplc in 15% ethyl acetate-isooctane. $[\alpha]_D^{28}$ = -0.28° (c = 0.9, $CHCl_3$); $[\alpha]_{578}$ = 0°, $[\alpha]_{546}$ = 0°, $[\alpha]_{436}$ = 0°, $[\alpha]_{365}$ = 0° (c = 0.9,

$CHCl_3$, 28°C). ^1H NMR (d_6-benzene): δ 4.46 (C-7, 1H, dd), 4.07 (C-3, 1H, bd), 3.22 (C-9, 1H, m), 3.21 ($-OCH_2$, 1H, m), 2.83 (C-2, 1H, m), 2.81 ($-OHC_2$, 1H, m), 2.57 (C-2, 1H, dd), 2.17 (C-9, 1H, d), 2.01 (C-5, C-6, 2H, m), 1.92 (C-5, C-6, 2H, m), 1.68 (C-13, 3H, s), 1.59 (C-15, 3H, bs), 1.39 (c-14, 3H, bs), 0.99 (-OEt, 3H, t); ^{13}C NMR (d_6-benzene) 170.0 (s), 160.0 (s), 133.8 (s),133.6 (s), 131.7 (d), 129.7 (s), 123.4 (d), 111.7 (s), 58.6 (t), 51.6 (t), 38.7 (t), 25.8 (t), 25.5 (t), 17.4 (q), 16.7 (q), 15.3 (q), 8.6 (q) ppm; HRMS: M^+ 276.1719 (3.5) (calc. 276.1725 for $C_{17}H_{24}O_3$), 230.1300 (18.7) (M^+ -EtOH), 154.0644 (100.0) (calc. 154.0627 for $C_8H_{10}O_3$; M^+ $-C_9H_{14}$), 126.0314 (83.5). LRMS (70 eV): M^+ 276, 248, 230, 154, 126.

Lithium aluminum hydride reduction of (58). $LiAlH_4$ (100 mg) was added to 52 mg (2.0×10^{-4} moles) 58 in 3 ml Et_2O at 0°C. The reaction was quenched after 20 minutes with ethyl acetate and ice, then filtered and partitioned between 50% Et_2EtOAc and H_2O. The aqueous layer was re-extracted several times with CH_2Cl_2. The organic layers were combined and dried over $MgSO_4$ and concentrated to give 42.1 mg of recovered product. Hplc purification of the reaction product yielded only 5.1 mg (2.2×10^{-5} moles) (11% yield) of the keto-aldehyde 113: ^1H NMR ($CDCl_3$) δ 9.77 (C-12, 1H, s), 4.99 (C-7, 1H, bdd (11, 3, 1)), 4.64 (C-3, 1H, bdd (10, 2, 4)), 3.51 (C-9, 1H, d (-11)), 3.16 (C-2, 2H, AB q), 3.13 (C-9, 1H, d (-11)), 2.38 (C-6, 1H, m), 2.22 (C-6, 1H, m), 2.10 (C-5, 2H, m), 1.91 (C-13, 3H, s), 1.61 (C-15, 3H, bs), 1.51 (C-14, 3H, bs). Compound 113 was identical (by ^1H NMR) to the LAH reduction product of ethoxypacifigorgiolide.

$LiAlH_4$ reduction product of (59). $LiAlH_4$ (65 mg) was added to 48 mg (1.7×10^{-4} moles) 59 in 3 ml anhydrous Et_2O at 0^oC. The reaction was quenched after fifteen minutes with ethyl acetate and ice, filtered and partitioned several times between 50% diethyl ether-ethyl acetate and dichloromethane and water. The organic layers were combined and dried over $MgSO_4$ to give 25.7 mg of product which was purified by hplc using 20% EtOAc/isooctane to give 9.2 mg (4×10^{-5} moles) (24% yield) of the α,β-unsaturated keto-aldehyde 113, previously identified as the LAH reduction product of methoxypacifigorgiolide. ^1H NMR ($CDCl_3$): δ 9.79 (C-12, 1H, s), 5.00 (C-7, 1H, bdd (12, 3,1)), 4.65 (C-3, 1H, bdd (10, 2, 1)), 3.50 (C-9,1 H, d (-14)), 3.16 (C-2, 2H, m), 3.14 (C-9, 1H, d (-14)), 2.38 (C-6, 1H, m), 2.17 (C-6, C-5, 3H, m), 1.91 (C-13, 3H, s), 1.61 (C-15, 3H, bs), 1.51 (C-14, 3H, bs); ^{13}C NMR (d_6-benzene): 203.7 (s) (C-10), 189.5 (d) (C-12), 137.2 (s), 135.0 (s), 132.9 (d), 121.2 (d) ppm. HRMS: M^+ 232.1470 (15.8) (calc. 232.1463 for $C_{15}H_{20}O_2$), 230.1303 (2.4), 121.1018 (33.9) (calc. 121.1017 for C_9H_{13}).

Air oxidation of the keto-aldehyde 113. Exposure of 113 to air, silica gel (upon hplc), or to $CHCl_3$ or $CDCl_3$ solvents resulted in its decomposition to unidentified products. One of these products appeared to be a hydroxylactone derivative of pacifigorgiolide, 114, as deduced by IR absorption at 1790 and 1765 cm^{-1}, and a singlet in the ^{13}C NMR spectrum of the γ-ketoenal at 99.0 ppm. HRMS: M^+ 248.1425 (2.9) (calc. 248.1412 for $C_{15}H_{20}O_3$).

Singlet oxygen oxidation of furanodiene (12). A reaction mixture containing 33 mg furanodiene (1.5×10^{-4} moles) and 1 mg Rose Bengal in 30 ml 50% MeOH/CH_2Cl_2 in an ehrlenmeyer flask was flushed with O_2

and sealed. Enough O_2 was added to inflate a rubber pipette bulb attached to the side arm of the flask. The mixture was cooled to -78°C and a 200 watt light bulb irradiated the reaction flask for one hour, after which the light was removed and the reaction was stirred at room temperature for half an hour. The resulting product was concentrated and redissolved in CH_2Cl_2 and filtered through a Florisil pipette column to remove the Rose Bengal dye. A mixture of products (34 mg) was recovered. A ^1H NMR spectrum of the mixture showed a 40% yield of the methoxy hydroperoxide 115, isolated by silica hplc (10% ethyl acetate-isooctane). ^1H NMR ($CDCl_3$): δ 9.25 (1H, bs), 5.85 (1H, bs), 4.75 (1H, bdd), 4.25 (1H, bd), 3.15 (3H, s), 1.81 (3H, s), 1.59 (3H, bs), 1.51 (3H, bs). After three days at room temperature, the product was completely decomposed. The compound was also sensitive to silica gel (upon hplc). Only 2.4 mg (8.5×10^{-6} moles) (6% yield) of 115 were recovered after separation of the crude reaction mixture.

Hydroxypacifigorgiolide (114). A polar compound, 4 mg, assigned as 114 was isolated by hplc in 30% ethyl acetate-isooctane from P. media, from Tres Marias. IR($CHCl_3$): 3450 (weak), 3070, 2960, 1765, 1520, 1430 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.99 (1H, bdd), 4.25 (1H, bd), 3.13 (2H, ABq), 3.05 (1H, bd (-14)), 2.38 (1H, d (-14)), 1.85 (3H, s), 1.59 (6H, s).

The rearranged sesquiterpene dimethyl ester (60) (Table 25). Compound 60 was isolated from extracts of P. pulchra exilis, (V-25) after hplc purification of a column fraction containing a mixture of 58, 59 and 60 using 25% ethyl acetate-isooctane. ^1H NMR ($CDCl_3$): δ 5.05 (C-3, C-7, 2H, m (7)), 3.75 (-OMe, 3H, s), 3.74 (-OMe, 3H, s), 3.06 (C-

8, 2H, d (7)), 2.02-2.04 (C-4, C-5, 4H, m), 1.96 (C-11, 3H, s), 1.65 (3H, bs), 1.62 (3H, bs), 1.59 (3H, bs). ^{1}H NMR (d_6-acetone): δ 5.08 (1H, m), 5.06 (1H, m), 3.68 (3H, s), 3.67 (3H, s), 3.07 (2H, d (7)), 2.06-2.01 (4H, m), 1.95 (3H, s), 1.67 (3H, bs), 1.65 (3H, bs), 1.58 (3H, bs). HRMS: M^+ 294.1846 (1.4) (calc. 294.1831 for $C_{17}H_{26}O_4$), 279.1576 (0.6) (calc. 279.1597 for $M^+ -CH_3$), 263.1633 (25.6) (calc. 263.1648 for $M^+ -OCH_3$), 262.1572 (20.0) (calc. 262.1570 for M^+ -MeOH), 234.1610 (12.0) (calc. 234.1620 for M^+ -COOMe), 225.1136 (1.1) (calc. 225.1127 for $M^+ -C_5H_9$), 193.0860 (100.0) (calc. 193.0865 for $C_{11}H_{13}O_3$), 172.0737 (30.2) (calc. 172.0736 for $C_8H_{12}O_4$), 166.0992 (20.5) (calc. 166.0994 for $C_{10}H_{14}O_2$), 140.0473 (80.5) (calc. 140.0474 for $C_7H_8O_3$), 123.1174 (48.1) (calc. 123.1174 for C_9H_{15}); 83.0860 (9.6) (calc. 83.0861 for C_6H_{11}). LRMS (70 eV): M^+ 294, 263, 234, 193, 140, 91.

Difference nOe study on the dimethyl ester 60. Compound 60, 2 mg (6.8×10^{-3} M), was dissolved in 1 ml of 0.5% TMS/d_6-benzene. The solution was degassed by bubbling Argon through it for half an hour to prepare for a nOe difference experiment[60] Difference decoupling experiments revealed that the signal at δ 5.18 was coupled to the broadened C-13 methyl singlet at δ 1.52 and the doublet at δ 2.96. Irradiation of the C-13 methyl at δ 1.52 under nOe difference conditions resulted in no enhancement for the olefinic proton at δ 5.18. Irradiation of the methylene group at δ 2.96 resulted in a distinct enhancement of the olefin proton at δ 5.18 and the C-11 olefinic methyl group at δ 1.73, indicating their proximity. Irradiation of the C-11 methyl at δ 1.73, in turn, resulted in an enhancement in only one of the unsaturated methyl ester signals, at δ 3.40. Irradiation of δ 3.40 resulted in

enhancements of both δ 1.73 and 3.45, resulting in the assignment of the C-9, C-10 olefin as Z.

Isoepizonarene (61, Table 26). Compound 61 was purified by silica hplc in 100% isooctane or hexane. HRMS: M^+ 204.1879 (27.0) (calc. 204.1878 for $C_{15}H_{24}$), 189.1648 (17.8) (calc. 189.1643 for M^+-CH_3), 161.1326 (86.8) (calc. 161.1330 for $M^+-C_3H_7$), 147.1169 (36.3), 119.0864 (59.0), 81.0702 (100.0). LRMS (70 eV): M^+ 204, 189, 161.

Dehydration of isoepizonarene (61). Compound 61 480 mg (2.4×10^{-3} moles), were refluxed in 4 ml xylene with 100 mg of 10% palladium over carbon. After 20 hours, xylene was removed by distillation (b.p. xylene 137-140°C). The reaction mixture was filtered over a tlc grade silica gel pipette column using methylene chloride. 250 mg of the filtrate was collected and separated by hplc with 100% isooctane to give 32 mg (1.6×10^{-4} moles) (7% yield) of 130, identical in all respects to cadalene by comparison of their spectral features. UV (nm): λ_{max}^{Hex} 325 (600), 290 (3700), 280 (3700), 230 (32,200); IR ($CHCl_3$): 2985, 1600, 1460, 1383, 913, 837 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.91 (2H, m), 7.30 (1H, bdd), 7.24 (2H, m), 3.71 (1H, m (7)), 2.64 (3H, bs), 2.55 (3H, bs), 1.38 (6H, d (7)).

Isomerization of isoepizonarene (61) to epizonarene. Paratoluenesulfonic acid, 25 mg, was added to 480 mg (2.4×10^{-3} moles) 61 in 4 ml benzene. The reaction mixture was stirred at room temperature for three hours, and next partitioned between diethyl ether and water. The organic layer was sequentially washed with sodium bicarbonate, dried over $MgSO_4$, filtered, and concentrated to give 244 mg recovered product.

Hplc purification of the product (100% isooctane) gave 102 mg (5 x 10^{-4} moles) (21% yield) of a product identified as epizonarene (131) by $[\alpha]_D$, IR and ^1H NMR comparison. $[\alpha]_D^{26}$ = -16.0° (c = 0.4, $CHCl_3$); UV: λ_{max}^{Hex} = 238 nm (20,000); IR ($CHCl_3$): 2924, 1613, 1440, 1366 cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.22 (C-6, 1H, bs), 3.03 (C-12, 1H, m (7)), 1.77 (C-15, 3H, bs), 0.99 (C-11, 3H, d (6)), 0.96 (C-13 or C-14, 3H, d (7)), 0.95 (C-14 or c-13, 3H, d (7)).

Isolation of the guaiane-diol 62 (Table 27). Compound 62 was isolated from an extract of Pacifigorgia sp. B after repeated silica purification hplc of column fractions using 65% ethyl acetate-isooctane. ^1H NMR (d_6-benzene): δ 5.59 (1H, bs (2.6)), 2.18 (1H, m (7)), 2.06 (2H, m), 1.62-1.80 (5H, m), 1.40-1.50 (4H, m), 1.35 (1H, dd (12.7, 10.6)), 1.06 (3H, s), 1.05 (3H, s), 0.99 (3H, d (7)), 0.98 (3H, d (7)), ^1H NMR (d_6-acetone): δ 5.59 (1H, bs), 2.21 (1H, m (7)), 1.20 (3H, s), 1.13 (3H, s), 0.98 (3H, d (7)), 0.97 (3H, d (7)). ^{13}C NMR (d_6-acetone): 148.9 (s), 123.4 (d), 79.8 (s),74.3 (s), 51.2 (d), 50.8 (d), 43.6 (t), 41.1 (t), 37.9 (d), 25.6 (t), 22.7 (q), 22.3 (t), 21.8 (q), 21.7 (q), 21.5 (q) ppm; HRMS: M^+-H_2O 220.1825 (23.3) (calc. 220.1827 for $C_{15}H_{24}O$), 202.1719 (11.3) (calc. 202.1722 for $M^+ -2H_2O$), 187.1486 (16.4) (calc. 187.1487 for $M^+ -CH_3-2H_2O$), 177.1287 (19.6) (calc. 177.1279 for $M^+ C_3H_7-H_2O$), 162.1408 (100.0) (calc. 162.1409 for $M^+ -C_3H_8O_2$), 159.1179 (49.9) (calc. 159.1174 for $M^+ -C_3H_7 -2H_2O$).

Chapter VI

Germacrene Derivatives from *Muricea austera* and *M. fungifera*

Gorgonians of the genus *Muricea* are common members of shallow water communities in both the east Pacific and tropical western Atlantic region (Figure 13).[1,5] Despite their abundance, the natural products chemistry of only one *Muricea* species has been described previous to this study. Four sesquiterpenes of the bisabolene-type were isolated from the Caribbean gorgonian *Muricea elongata* (Figure 14).[9,13]

My investigation of two east Pacific *Muricea* species has resulted in the isolation of three germacrene-derived sesquiterpenes, **63**-**65**, related structurally to furanodiene (**12**). *Muricea austera* was collected at Isla Socorro in May, 1978, and *M. fungifera* was collected at Bahia Los Frailes in January, 1980. Tlc examination of the organic extracts of these gorgonians revealed several UV active compounds at r_f = 0.6 in 1:1 Et_2O-benzene. The compounds stained dark purple after treatment with acid and heat. Chloroform-methanol extracts from both collections of gorgonians were chromatographed over silica gel in isooctane and dichloromethane. A total of 1.3 g of a mixture of compounds was eluted in 80% dichloromethane-isooctane from 18.8 g of *M. austera* extract. Hplc fractionation of this mixture yielded 0.4 g **63** (0.02% dry wt.), 0.6 g **64** (0.03% dry wt.) and 0.1 g **65** (<0.01% dry wt.), in order of elution. Fractionation of 5.8 g crude *M. fungifera* extract (from 283 g dry weight of the gorgonian) gave similar results.

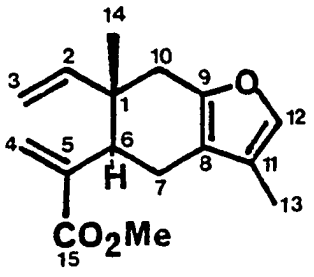
The least polar compound, 63, possessed a molecular formula of $C_{16}H_{20}O_3$ based upon interpretation of a combination of low resolution mass spectrometry and ^{13}C NMR spectral data (Table 28). A three proton singlet at δ 3.73 in the ^{1}H NMR spectrum indicated a methyl ester functionality. This was supported by absorptions in the ^{13}C NMR spectrum at 168.7 (s) and 52.0 (q) ppm. The presence of this functionality implied that 63 possessed a sesquiterpene ring system from the molecular formula. The ^{13}C and ^{1}H NMR data of 63 indicated a total of five methyl groups or methyl equivalents. These included two terminal vinyl groups, the ester carbon and two methyl groups. This indicated a rearranged sesquiterpene ring system.

Absorptions in the ^{13}C NMR spectrum for 8 olefinic carbons and one carbonyl carbon accounted for five of the seven degrees of unsaturation implicit in the molecular formula. The ^{13}C NMR data also indicated the presence of a trisubstituted furan functionality based on comparison with model compounds. This assignment was supported by the characteristic signal in the ^{1}H NMR spectrum at δ 7.06 (1H, s) for a β furan proton.

^{1}H NMR decoupling studies on 63 showed an ABX pattern in the protons at δ 5.82, 4.96 and 4.89, located on a monosubstituted olefin. A second terminal olefinic group was conjugated to the methyl ester functionality based on chemical shifts of the geminal vinylic protons at δ 6.27 and 5.56. Therefore, the remaining ring in 63 was assigned to a sesquiterpene elemane-type ring based on consideration of these and other spectral features. Comparison of their ^{1}H NMR spectral data led to the assignment of 63 as identical to isosericenine, an elemane

Table 28

Spectral Data for Isosericenine (63)



63

$C_{16}H_{20}O_3$, LRMS: M^+ 260, 160; $[\alpha]_D^{27}$ = -0.36° (c = 1.4, $CHCl_3$); UV: λ_{max}^{Hex} = 253 nm (5000); IR ($CHCl_3$): 2940, 1721, 1698, 1433, 1135 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	40.5
2	5.82 dd (17, 11)	146.0 (J_R = 38.0)
3	4.96 d (17), 4.89 d (11)	112.1 (27.5)
4	6.27 s, 5.56 s	124.7 (30.0)
5	--	142.6
6	3.21 dd (8, 6)	41.1 (18.4)
7	2.40 m	24.0 (16.4)
8	--	116.2
9	--	149.4
10	2.71 bd (-16), 2.38 bd (-16)	35.5 (18.0)
11	--	119.2
12	7.06 bs	137.4 (42.0)
13	1.92 s	8.1 (16.0)
14	0.96 s	18.0 (14.0)
15	--	168.7
-OMe	3.73 s	52.0 (22.7)

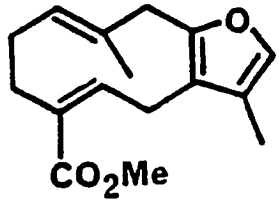
derivative previously isolated from the Japanese plant Neolitsea sericia.[99]

In contrast to the elemane ring system of 63, compounds 64 and 65 contained absorptions for only three olefinic methyl groups in their ^{1}H NMR spectra (Tables 29 and 30). This indicated a regular sesquiterpene ring system. The ^{13}C NMR spectra of both compounds appeared similar to that of furanodiene (12), with the addition of a methyl ester functionality. Comparison of their ^{1}H NMR data led to the assignment of 64 as neosericenine, with an E,Z germacradiene ring system.[100] Compound 65 was identified as sericenine, the E isomer of neosericenine at the methyl ester-bearing olefin.[101] Both germacrenoid compounds were also previously isolated from Neolitsea sericea and can be interconverted with isosericenine. Compound 63 is produced from neosericenine (64) via a thermally induced Cope rearrangement. Sericenine (65), isomerizes to neosericenine (64) under slightly more energetic conditions and ultimately results in the same product (Figure 77).[102]

The ^{13}C NMR assignments of 63-65 were based on chemical shift comparisons with model compounds, multiplicities, and residual coupling constants (J_R). These assignments, listed in Table 31, correlate well with those of model compounds 135 and 136. The ^{13}C NMR assignments of 135 and 136 were based on the results of lanthanide shift studies.[103]

Table 29

Spectral Data for Neosericenine (64)



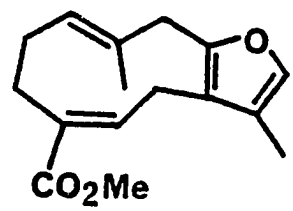
64

$C_{16}H_{20}O_3$; LRMS: M^+ = 260; UV: λ_{max}^{Hex} = 244 nm (10,000), 216 nm (30,000); IR ($CHCl_3$) 3000, 1717, 1626, 1433, 1140 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	134.4
2	5.09 dd (11, 6)	128.9 (J_R = 26)
3	2.20 m	28.0
4	2.93 ddd (-12, 4, 3), 1.67 ddd (-12, 6, 4)	35.5
5	--	123.4
6	5.54 dd (11, 3)	146.3 (28)
7	3.91 dd (-15, 11), 3.32 dd (-15, 3)	25.6 (20)
8	--	117.5
9	--	149.9
10	3.50 bs	40.5 (20)
11	--	121.5
12	7.08 bs	136.4 (42)
13	1.93 s	8.8 (16)
14	1.20 bs	16.4 (15)
15	--	169.6
-OMe	3.74 s	51.2 (23)

Table 30

Spectral Data for Sericenine (65).



65

$C_{16}H_{20}O_3$; UV: λ_{max}^{Hex} = 217 nm (10,000), 209 nm (10,000); IR ($CHCl_3$): 3120, 1706, 1550 cm^{-1}.

C	^1H NMR (220 MHz, $CDCl_3$)	^{13}C NMR (20 MHz, $CDCl_3$)
1	--	134.9
2	5.51 bm	127.3 (J_r = 26.1)
3	2.20	25.6+
4	2.69, 2.20	26.6+
5	--	127.8
6	6.65 bd	143.1 (31.8)
7	3.62 m, 2.83 m	24.0 (18.9)
8	--	116.3
9	--	150.8
10	3.59 d (-11), 3.15 d (-11)	39.6 (20.0)
11	--	121.5
12	7.07 bs	136.5 (42.2)
13	1.97 s	8.2 (16.0)
14	1.36 bs	16.8 (16.9)
15	--	169.5
-OMe	3.78 s	51.7 (23.0)

+ may be interchanged

Figure Interconversion of 64 and 65 with 63 via the Cope rearrangement[102]

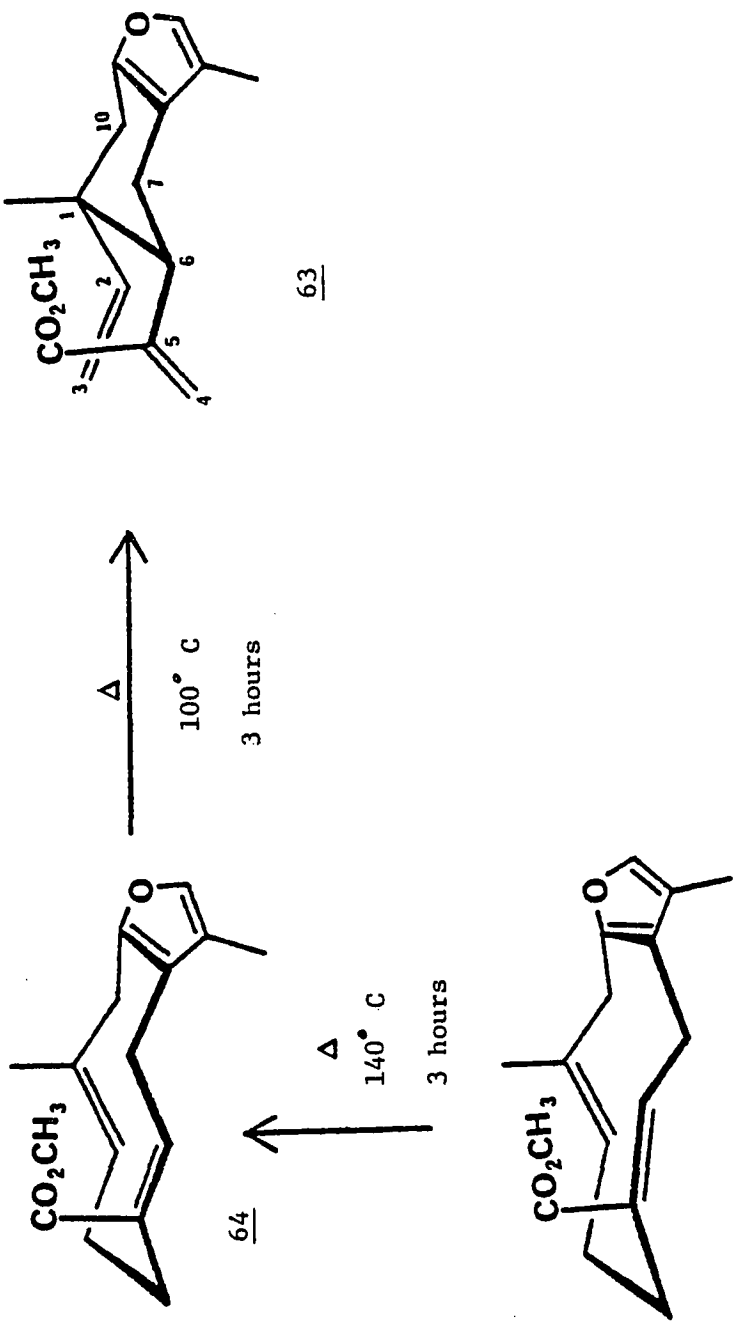
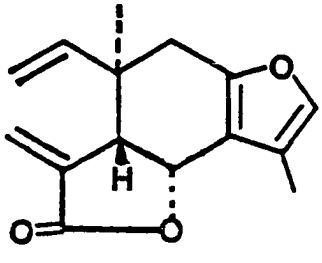


Table 31

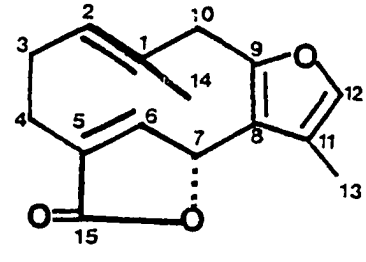
^{13}C NMR Assignments of 63-65
Based on Model Compounds 135 and 136[102,103+]

C	135	63	136	64	65
1	40.9	40.5	130.8	134.4	134.9
2	143.6	146.0	130.3	128.9	127.3
3	114.8	112.1	25.8	28.0	25.6
4	123.7	124.7	26.8	35.5	26.6
5	136.6	142.6	135.1	123.4	127.8
6	46.8	41.1	151.7	146.3	143.1
7	72.2	24.0	74.2	25.6	14.0
8	113.6	116.2	115.4	117.5	116.3
9	152.6	149.4	152.8	149.9	150.8
10	34.9	35.5	40.7	40.5	39.6
11	119.8	119.2	122.2	121.5	121.5
12	138.6	137.4	137.2	136.4	136.5
13	8.2	8.1	8.4	8.8	8.2
14	18.7	18.0	15.7	16.4	16.8
15	170.9	168.7	173.4	169.6	169.5
-OMe	--	52.0	--	51.2	51.7

[+]Recorded at 20 MHz on a Varian CFT-20 instrument in $CDCl_3$. Assignments are based upon off resonance decoupling experiments yielding J_R values, multiplicities and on comparisons with model compounds.



135



136

Chapter VII

A Comparative Natural Products Study of Two Local Gorgonians: *Muricea californica* and *Muricea fruticosa*

Muricea californica and *M. fruticosa* are two gorgonians which commonly occur in the same habitat in shallow water (9-24 m) off southern California and Baja California. *Muricea californica* is a red-brown sea whip with gold to cream colored polyps and *M. fruticosa* is a bushy, red sea whip with white polyps. *M. californica* is generally found in slightly more abundance than *M. fruticosa*. *M. californica* also tends to grow in more of a planar fashion than *M. fruticosa*, which usually appears smaller and bushier. Other than these very minor distinctions in morphology, tissue color and polyp color, similarities in the features of these two gorgonians make it very difficult to distinguish between them. In fact, these similarities, and their correspondence with other, closely related *Muricea* species have created uncertainties in the taxonomic assignment of these gorgonians. I have chosen to retain the names in common usage, for simplicity, although they are probably incorrect according to the current expert on their taxonomy, Dr. David Harden.[39]

The ecology and aspects of the life history of these two gorgonians have been closely examined by a number of local biologists.[45,104-110] During these studies, ecologists have noted major differences in the fouling susceptibility of the two gorgonians,

although they co-occur in the same environment and are closely related morphologically. This difference was first reported in 1960 by Cutress and Pequenat.[108] They described an encrusting zoanthid species, Parazoanthus lucificum, which grows only on Muricea californica. They found that up to 5% of the population of M. californica at their study site in Corona del Mar was affected by, while M. fruticosa was untouched by the zoanthid. Their specific comments follow:

> "It appears that once the zoanthid is established, the gorgonian has little or no defense against further encroachment. At the approach of the zoanthid's coenchyme the gorgonian's tissues soften and slough off, leaving exposed an area of bare skeleton which soon becomes occupied by a zoanthid polyp. Although two other genera of gorgonians are found off Corona Del Mar (Lophogorgia chilensis and Eugorgia rubens), Parazoanthus lucificum does not grow on them. P. lucificum has been found growing only on M. californica in spite of the fact that a second species--Muricea fruticosa--may in places grow side by side with the former."

The same authors also noted the encroachment on M. californica by another zoanthid, Epizoanthus induratum.

Several years later, Pequenat (1964)[109] made similar observations on the lack of fouling on Muricea fruticosa while a variety of fouling organisms, epizoic red algae, hydroids, ectoprocts and zoanthids, were found on M. californica. Ascidians, bryozoans, sponges, tube worms and barnacles were also found as epibionts on Muricea californica, but rarely, if ever, on M. fruticosa. These observations were reinforced by the results of a study comparing the population dynamics of the two Muricea species. Grigg (1977)[45] found that both species share similar patterns of life history and that detachment and abrasion, not predation, are the major sources of mortality. Despite these

similarities and their co-occurrence in the same habitat, he also reported differences in their fouling susceptibility. Approximately 1% of the local Muricea californica population he studied was overgrown by the zoanthids Parazoanthus lucificum and Epizoanthus induratum, while M. fruticosa appeared to be immune to these encrustations. He found a variety of fouling organisms on Muricea californica: coralline algae, sponges, bryozoans, ascidians, hydroids, algae, polychaetes, molluscs, pycnogonids, caprellids, isopods, decapods, cirripeds. Occasionally, he also observed several of these species on abraded areas of Muricea fruticosa. However, few of the gorgonians were completely overgrown, and the fouling organisms did not appear to spread significantly over a period of several years.

Personal observations of the two local Muricea gorgonians, made while collecting in Los Coronados, La Jolla and Catalina, have substantiated this difference in their fouling susceptibility. While M. fruticosa is rarely fouled, it is common to see whole colonies or portions of Muricea californica covered with a variety of fouling organisms.

The fact that these two species are so similar morphologically, and occur side-by-side, suggests that perhaps a chemical variation may be responsible for the observed differences in their fouling susceptibilities.

A chemical mechanism for reducing fouling has previously been implicated for several species of algae and gorgonians. An uneven distribution of fouling by epibionts and microorganisms was noted in several algae in the genera Ascophyllum,[111] Sargassum,[112] and

Laminaria.[113] Less fouled areas of the algae were found to contain higher concentrations of tannins than in the rest of the plant. Pyridine derivatives isolated from the unfouled Atlantic gorgonians Leptogorgia virgulata and L. setacea were found to inhibit the growth of a potential fouling diatom, Navicula salinicola.[114]

Partly because of these precedents, two questions were formulated.

1) Is there a chemical difference between the two local Muricea gorgonians in terms of their bioactive natural products?

2) If so, is this chemical variation responsible for the difference observed in their fouling susceptibilities?

In order to answer these questions, I embarked on a complete examination of the natural products chemistry of the two gorgonians.

A. Comparison of the natural products in the extracts of Muricea californica and M. fruticosa

Muricea californica and M. fruticosa were collected simultaneously at four separate locations between January, 1979, and June, 1980. Preliminary examination of the 70% chloroform-methanol extracts from each collection by thin layer chromatography (tlc) gave consistent results. This analysis of the crude extracts revealed that both gorgonians possessed the usual mixtures of lipids, pigments, fatty acids and sterols found in all gorgonians. In addition to these compounds, extracts of Muricea fruticosa contained several polar compounds at r_f =

0.1 in 1:1 diethyl ether-benzene. The extracts of both gorgonians were fractionated in an identical fashion, on tlc grade silica gel with a vacuum filtration column using mixtures of isooctane, dichloromethane, and ethyl acetate. Careful examination of each column fraction by tlc and 60 and 360 MHz ^{1}H NMR spectroscopy confirmed the preliminary results. However, in addition to the usual gorgonian dietary sterols, chromatography of both gorgonian extracts yielded ergosterol peroxide (66), eluted in 25% ethyl acetate-isooctane. Ergosterol peroxide was previously isolated from several sponges, fungi and lichens.[115] ^{1}H NMR spectra of Muricea fruticosa column fractions eluted with 50% ethyl acetate-dichloromethane showed a mixture of fatty acids and acetate-containing compounds with interesting peaks from δ 6.0-3.5. These mixtures were separated by hplc in 70-75% ethyl acetate-isooctane to give varying amounts of four new saponin derivatives, 67-70, in ~0.01% dry weight for each compound. The structures of these saponin derivatives were determined using a combination of chemical and spectroscopic techniques. Extracts of Muricea californica were examined intensively four separate times by the techniques described above. At no time were any of the saponin-derived compounds 67-70 encountered in any of the extracts of Muricea californica.

B. Isolation of ergosterol peroxide (66) from Muricea californica and M. fruticosa

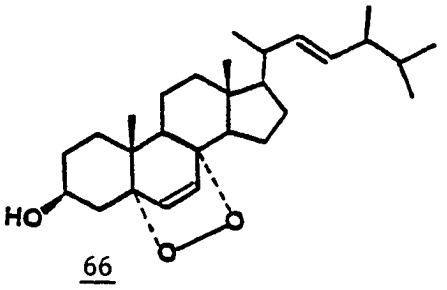
^{1}H NMR spectra of column fractions eluted in 25% ethyl acetate-dichloromethane, from extracts of both Muricea californica and M. fruticosa, revealed the presence of an interesting compound (66). The spectrum contained a pair of AB doublets, at δ 6.51 and 6.25 (J = 8.4

Hz), characteristic of the C-6 and C-7 olefinic protons in 5α, 8α-epidioxy sterols. The sterol was purified by hplc in 60% ethyl acetate-isooctane to give 66, in ~0.01% dry wt. from M. fruticosa, and 0.03% dry wt. from M. californica. The sterol was identified as 5α,8α-epidioxy-24-methyl cholesta-6,22-dien-3β-ol (ergosterol peroxide) (66) by comparison of its spectral data (Table 32) with that of the known sterol, previously isolated from several sponges, a tunicate, fungi and lichens.[115-117] The compound appeared to be racemic by the negligible value of its optical rotation compared with the known values of the 24R ($[\alpha]_D = -25^o$) and 24S ($[\alpha]_D = -6^o$) epimers.[115] The ^{13}C NMR spectrum of 66 was assigned by comparison with the ^{13}C NMR spectrum of ^{13}C enriched ergosterol labeled with [1-^{13}C] acetate.[118]

C. The isolation and structure elucidation of the four new saponin derivatives from Muricea fruticosa.

Silica gel chromatography of extracts of Muricea fruticosa resulted in the isolation of four interesting new saponin-derived compounds, 67-70. A mixture containing the compounds was eluted from a silica gel column using 50% ethyl acetate-dichloromethane. The compounds were separated by silica gel hplc in 70-75% ethyl acetate-isooctane and by reverse phase hplc in 90-95% methanol-water. The most polar compound, 67, appeared to contain four acetoxyl groups based upon the presence of four methyl singlets in the ^1H NMR spectrum at δ 2.14, 2.04, 2.00, and 1.97 (Table 33). A group of complex, mutually coupled protons observed from δ 3.8-5.4 were indicative of a sugar moiety. A doublet at 99.4 ppm in the ^{13}C NMR spectrum was characteristic of the anomeric carbon in a sugar and five additional ^{13}C NMR bands from 79.8

Table 32. Spectral Data for Ergosterol Peroxide 66



66

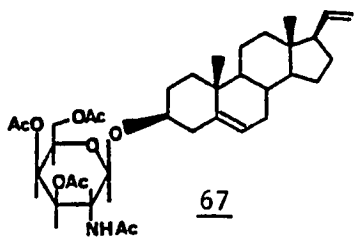
$C_{28}H_{44}O_3$; $[\alpha]_D^{26}$ = +0.8° (c = 0.5, $CHCl_3$); IR ($CHCl_3$): 2970, 1450, 1370, 1220, 1040, 1020, 910 cm^{-1}; LRMS: M^+ 428, 410, 396, 382.

^1H NMR (360 MHz, $CDCl_3$)

	δ	m	
1	--		37.0 (t)
2	--		30.1 (t)
3	3.98	m	66.3 (d)
4	--		37.0 (t)
5	--		82.2 (s)
6	6.51	d (8.4)	135.5 (d)
7	6.25	d (8.4)	130.8 (d)
8	--		79.5 (s)
9	--		51.2 (d)
10	--		39.5 (s)
11	--		23.4 (t)
12	--		37.0 (t)
13	--		44.6 (s)
14	--		51.8 (d)
15	--		23.4 (t)
16	--		30.1 (t)
17	--		56.2 (d)
18	0.88	s	12.9 (q)
19	1.25	s	12.9 (q)
20	--		39.5 (d)
21	0.99	d[+]	18.2 (q)
22	5.16	m	135.5 (d)
23	5.16	m	130.8 (d)
24	--		39.5 (d)
25	--		34.8 (d)
26	0.91	d[+]	20.7 (q)
27	0.83	d[+]	20.7 (q)
28	0.82	d[+]	18.2 (q)

[+] may be interchanged

Table 33. Spectral Data for the Saponin Derivative 67, 3-β-Pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,4′,6′-tri-O-acetyl-β-D-galactopyranoside



67

$C_{35}H_{51}NO_9$; $[\alpha]_D^{26} = -26.3^\circ$ (c = 1.6, $CHCl_3$); IR ($CHCl_3$): 3367-3546 (w), 2967, 1754 (s), 1686 (s), 1462, 1374, 1247, 1134, 1079, 1045, 1018 cm^{-1}.

^1H NMR (360 MHz, $CDCl_3$)			^{13}C NMR (50 MHz, $CDCl_3$)	
δ	H	m (J)	δ	J_R
5.76	1	ddd (16,11,8)	170.3 (s) x 4	--
5.64	1	d (9) (D_2O exchangeable)	140.3 (s)	--
5.41	1	dd (11,3)	139.7 (d)	46.9
5.36	1	bd (3)	122.0 (d)	48.0
5.36	1	bd (3)	114.5 (t)	45.6
4.98	1	d (11)	99.4 (d)	48.2
4.97	1	d (16)	79.8 (d)	36.5
4.89	1	d (8)	70.5 (d)	39.2
4.16	1	dd (-11,6)	69.6 (d)	46.5
4.12	1	dd (-11,7)	66.8 (d)	47.9
3.93	1	bt (7,3)	61.5 (t)	--
3.81	1	ddd (11,9,8)	55.9 (d)	17.6
3.51	1	m	55.3 (d)	27.9
2.14	3	w	52.4 (d)	38.0
2.04	3	s	50.4 (d)	19.4
2.00	3	s	43.4 (s)	
1.97	3	s	38.8 (t)	
0.99	3	s	37.3 (t)	
0.60	3	s	37.2 (d)	
			36.8 (s)	
			32.0 (t)	
			29.7 (t)	
			29.5 (t)	
			27.2 (t)	
			24.9 (t)	
			23.5 (q)	26.5
			20.7 (t)	
			20.7 (q) x 3	27.7
			19.4 (q)	23.1
			12.7 (q)	20.9

to 61.5 ppm supported this assignment. Two methyl singlets in the ^{1}H NMR spectrum at δ 0.99 and 0.60, for the C-19 and C-18 methyls, were indicative of a steroid nucleus. These data suggested 67 was a tetraacetate saponin derivative.

Field desorption mass spectrometry combined with the ^{13}C NMR data resulted in a molecular formula of $C_{35}H_{51}NO_9$ for 67, which calculated for eleven degrees of unsaturation. Six of the degrees of unsaturation could be accounted for by the four acetoxyl groups and two olefins present as deduced from the ^{1}H and ^{13}C NMR data. This left five rings: presumably one from the sugar moiety and four belonging to the steroid nucleus.

Due to the complexity of the spectral data and the large size of the molecule, it was difficult to assign the structure of 67 at this point. In order to reduce the complexity of the molecule, the saponin derivative 67 was cleaved into its component sugar and steroidal parts. Compound 67 was hydrolyzed using 3 N HCl at 40° for three hours. The aglycone, 137, was recovered in quantitative yield after repeated extraction of the hydrolysis reaction mixture with dichloromethane (Figure 78). ^{1}H NMR analysis of 137 revealed an ABX pattern in the olefin protons at δ 5.76 (1H, ddd, J = 16, 11, 8), 4.98 (1H, d, J_{cis} = 11 Hz), and 4.97 (1H, d, J_{trans} = 16 Hz). These bands were attributed to a terminal olefin, which was observed earlier in the saponin compound by comparison of both the ^{13}C and ^{1}H NMR data. The ^{1}H NMR spectrum of the aglycone also possessed the characteristic C-19 and C-18 sterol methyl singlets at δ 1.02 and 0.61, as well as an olefinic proton at δ 5.36 for the C-5, C-6 trisubstituted olefin. High resolution mass measurement of

Figure 78. The hydrolysis product from compound 67

67 —3 N HCl→ 137

$C_{35}H_{51}NO_9$	saponin	
- $C_{21}H_{31}O$	agiycone	pregna- 5, 20- dien- 3β- ol
$C_{14}H_{20}NO_8$	sugar + acetates	previously isolated from the
- $C_8H_{12}O_4$	- 4 acetates (4 x C_2H_3O)	sea raspberry Gersemia rubiformis [125]
$C_6H_8NO_4$	amino sugar	

137 gave a molecular ion of 300.2443 for $C_{21}H_{32}O$ (calc. 300.2453). A new absorption in the infrared spectrum at 3401 cm^{-1} was indicative of an alcohol functionality, and this conclusion was supported by the observation of a M^+-H_2O fragment in the HRMS. Analogies of these spectral data with those of known marine pregnane (C_{21} sterols) derivatives,[119-125] resulted in the assignment of 137 as pregna-5,20-dien-3β-ol. This assignment was confirmed by direct comparison of 137 with an authentic sample previously isolated from the Newfoundland sea raspberry, Gersemia rubiformis (Alcyonaceae).[124] The hydrolysis product, 137, showed $[\alpha]_D^{26} = -43.6^o$ (c = 0.6, $CHCl_3$), a value in good agreement with the natural product: $[\alpha]_D^{26} = -62.1^o$ (c = 0.7, $CHCl_3$). The structure and absolute stereochemistry of 137 was originally determined by synthesis from progesterone.[124,126] The C-3 alcohol was assigned as β on the basis of the large axial-axial coupling constants of the 3-α proton at δ 3.53.[119,124] It is interesting to note that pregna-5,20-dien-3-ol was previously isolated from Muricea californica during an examination of its sterol constituents.[28] Nevertheless, none of the saponin compounds 67-70 were ever isolated from Muricea californica.

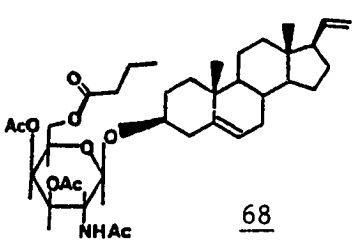
If the molecular formula of the aglycone 137 is subtracted from the saponin derivative 67 ($C_{35}H_{51}NO_9$- $C_{21}H_{31}O$), a formula of $C_{14}H_{20}NO_8$ results for the remaining sugar moiety. Subtraction of the four acetoxyl groups present in 67 from the sugar moiety gives $C_6H_8NO_4$ ($C_{14}H_{20}NO_8$-$C_8H_{12}O_4$) as the molecular formula.

The structure elucidation of the sugar component in compound 67 was aided by the co-isolation of three closely related saponin derivatives. The three other derivatives, 68-70, only differed from the

tetraacetate saponin derivative 67 in the number of acetoxyl groups they possessed. Compounds 68 and 69 were isomeric with one another, based on the molecular ion of 657 determined for both compounds by field desorption mass spectrometry. The molecular formula of $C_{37}H_{55}NO_9$ for both compounds was also supported by their ^{13}C NMR spectra (Tables 34 and 35). This molecular formula indicates an addition of C_2H_4 to the tetraacetate saponin derivative 67. Both compounds exhibited the absence of one acetoxyl methyl group in the ^1H NMR spectra compared with compound 67. Careful investigation of the ^1H NMR features illustrated that the acetoxyl group was replaced in both cases with an n-butyrate ester. The n-butyrate ester functionality was assigned on the basis of comparison of the ^1H and ^{13}C NMR data with model compounds (Figure 79) and the results of ^1H NMR decoupling experiments. For example, compound 68 showed a two proton multiplet at δ 1.62 mutually coupled by 7 Hz to both a two proton triplet at δ 2.27 and a three proton triplet at δ 0.93. These signals replaced the acetoxyl methyl group at δ 2.04 in 67. Similarly, an n-butyrate group, as evidenced by the protons in the ^1H NMR spectrum at δ 2.38 (2H, t (7)), 1.68 (2H, m (7)), and 0.97 (3H, t (7)), in 69 replaced the acetoxyl methyl group at δ 2.14 in 67. Other than these minor changes, the spectral data of the two compounds were practically superimposable. High resolution mass measurement of 69 did not result in a molecular ion (due to the large size, polarity and low volatility of the compound). HRMS of 69 did result in a fragment at 300.2433 for the aglycone (137), $C_{21}H_{32}O$, offering further support for the structural assignment of that portion of the molecule.

The remaining saponin derivative, 70, was most useful in terms

Table 34. Spectral Data for the Saponin Derivative 68, 3-β-Pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,4′-di-O-acetyl-6′-O-n-butyryl-β-D-galactopyranoside

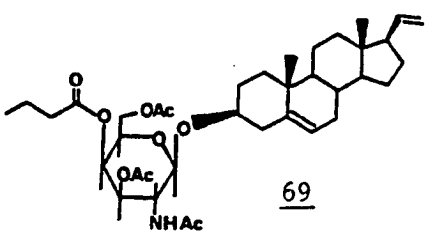


68

$C_{37}H_{55}NO_9$; $[\alpha]_D^{27} = -29.7°$; (c = 1.4, $CHCl_3$); IR ($CHCl_3$): 3340-3520 (w), 2960, 1745, 1680, 1370, 1240 1070 cm^{-1}.

^{1}H NMR (360 MHz, $CDCl_3$)			^{13}C NMR (50 MHz, $CDCl_3$)	
δ	H	m	δ	J_R
5.76	1	ddd (16,11,8)	173.0 (s)	
5.67	1	d (8)	170.3 (s)	
5.41	1	dd (11,3)	170.3 (s)	
5.36	1	bm (3)	170.2 (s)	
5.35	1	bm	140.3 (s)	
4.98	1	d (11)	139.7 (d)	46.1
4.97	1	d (16)	122.0 (d)	47.4
4.89	1	d (9)	114.5 (t)	45.4
4.17	1	dd (-11,7)	99.4 (d)	47.2
4.10	1	dd (-11,7)	79.8 (d)	36.2
3.93	1	bt (7,6)	70.5 (d)	39.6
3.82	1	ddd (11,9,8)	69.7 (d)	45.9
3.51	1	m (11,10,6,5)	66.8 (d)	47.5
2.27	2	t (7)	61.3 (t)	
2.14	3	s	55.9 (d)	18.4
2.00	3	s	55.3 (d)	27.0
1.97	3	s	52.4 (d)	37.7
1.62	2	m (7)	50.4 (d)	18.6
0.99	3	s	43.4 (s)	
0.93	3	t (7)	38.8 (t)	
0.60	3	s	37.3 (t)	
			37.2 (d)	
			36.88 (s)	
			35.9 (t)	26.6
			32.0 (t)	
			29.7 (t)	
			29.5 (t)	
			27.2 (t)	
			24.9 (t)	
			23.5 (q)	26.5
			20.7 (t)	
			20.7 (q) x 2	27.4
			19.4 (q)	22.0
			18.3 (t)	23.0
			13.6 (q)	21.1
			12.7 (q)	20.6

Table 35. Spectral Data for the Saponin Derivative 69, 3-β-Pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,6′-di-O-acetyl-4′-O-n-butyryl-β-D-galactopyranoside



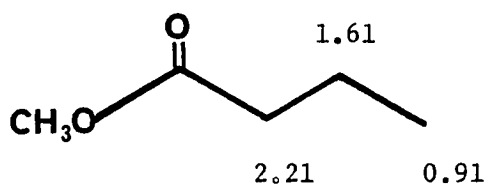
69

$C_{37}H_{55}NO_9$; $[\alpha]_D^{27} = -30.3^{\circ}$ (c = 1.2, $CHCl_3$); IR ($CHCl_3$): 3350, 2960, 1750, 1680, 1520, 1490, 1370, 1270, 1170, 1080, 1040 cm^{-1}.

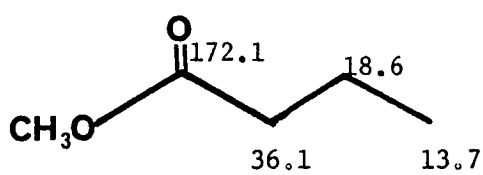
^1H NMR (360 MHz, $CDCl_3$)			^{13}C NMR (50 MHz, $CDCl_3$)	
δ	H	m (J)	δ	J_R
5.76	1	ddd (16,11,8)	172.9 (s)	
5.51	1	d (8)	170.3 (s) x 3	
5.41	1	dd (11,3)	140.3 (s)	
5.38	1	bd (3)	139.7 (d)	41.2
5.36	1	bd (5)	122.0 (d)	44.6
4.98	1	dd (11,2)	114.5 (t)	
4.97	1	dd (16,2)	99.4 (d)	44.8
4.89	1	d (8)	79.8 (d)	
4.17	1	dd (-11,7)	70.5 (d)	38.1
4.08	1	dd (-11,7)	69.7 (d)	42.0
3.93	1	bt (7)	66.5 (d)	45.2
3.79	1	ddd (11,9,8)	61.5 (t)	
3.51	1	m	55.9 (d)	
2.38	2	t (7)	55.3 (d)	
2.25	2	m	52.4 (d)	38.1
2.04	3	s	50.4 (d)	
1.99	3	s	43.4 (s)	
1.97	3	s	38.9 (t)	
1.00	3	s	37.3 (t)	
0.97	3	t (7)	37.3 (d)	
0.60	3	s	36.8 (s)	
			36.0 (t)	
			29.7 (t)	
			29.5 (t)	
			27.2 (t)	
			24.9 (t)	
			23.5 (q)	
			20.7 (q) x 2	
			20.7 (t)	
			19.4 (q)	
			18.6 (t)	
			13.6 (t)	
			12.7 (q)	

Figure 79。 Spectral data for n- butyrate esters[94]

^{1}H NMR



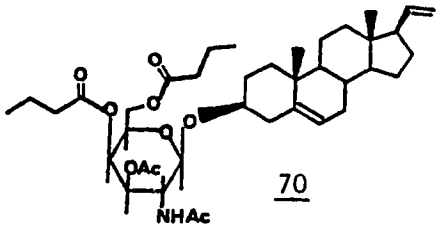
^{13}C NMR



of elucidating the total structures of these saponin derivatives. Compound 70 was the least polar and most abundant compound isolated. A molecular ion which measured 685.4152 was obtained for 70 by HRMS and this could be assigned to a molecular formula of $C_{39}H_{59}NO_9$. This molecular formula was supported by the ^{13}C NMR data (Table 36). The HRMS also showed a fragment for the aglycone (137) at m/z = 300.2455 for $C_{21}H_{32}O$, and a strong fragment for the aglycone $-H_2O$ at m/z 282.2350 for $C_{21}H_{30}$. The molecular formula reflects an addition of C_4H_8 to 67, and the ^1H NMR spectrum confirmed this by showing the addition of two n-butyrate ester groups. All other spectral features of 70 are comparable with the previous three compounds.

Hydrolysis of 70, with 10% H_2SO_4 at 35°C for two hours, yielded the aglycone 137. The aglycone was identical in all respects to the hydrolysis product from 67 and the natural product from Gersemia rubiformis. Reduction of 70 with $LiAlH_4$ in THF also yielded a small amount of the aglycone 137 as well as the free saponin, 138 (Figure 80). The free saponin, 138, was identified by the upfield shift of the protons attributable to the sugar moiety from δ 3.8-5.4 to δ 3.2-3.8, and the concomitant loss of three of the acetoxyl methyl groups at δ 2.14, 2.04 and 2.00. The rest of the molecule, including the aglycone, remained intact by examination of the ^1H NMR spectrum. The acetoxyl methyl not removed by $LiAlH_4$ reduction was assigned to an amide functionality, which was deduced to be present from the molecular formula of the sugar and from several other spectral features of 67-70. Infrared absorptions from 67-70, which centered around 3400 (weak) and 1685 cm^{-1} (strong), were characteristic of an amide functionality. This assignment was

Table 36. Spectral Data of the Saponin Derivative 70, 3-β-Pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′-O-acetyl-4′,6′-di-O-n-butyryl-β-D-galactopyranoside



70

$C_{39}H_{59}NO_9$, m.p. 119-121°C; $[\alpha]_D^{27}$ = 35.8° (c = 0.8, $CHCl_3$); IR ($CHCl_3$): 3500 (w), 2980, 1740, 1680, 1460, 1370, 1300, 1160, 1090, 1080 cm^{-1}.

^1H NMR (360 MHz, $CDCl_3$)			^{13}C NMR (50 MHz, $CDCl_3$)	
δ	H	m	δ	J_R
5.76	1	ddd (16,11,8)	172.9 (s) x 2	--
5.61	1	d (9) (D_2O exchangeable)	170.3 (s) x 2	--
5.40	1	dd (11,3.5)	140.8 (s)	--
5.38	1	bm	139.6 (d)	45.6
5.38	1	bd (3.5)	121.6 (d)	47.3
4.98	1	d (11)	114.5 (t)	45.9
4.97	1	d (16)	99.4 (d)	47.7
4.89	1	d (8)	79.7 (d)	35.9
4.18	1	dd (-11,7)	71.8 (d)	36.5
4.08	1	dd (-11,7)	69.8 (d)	44.5
3.81	1	ddd (11,9,8)	61.4 (t)	40.4
3.51	1	m	55.9 (d)	25.5
2.38	2	5 (i)	55.3 (d)	18.2
2.27	2	t (7)	52.3 (d)	37.3
1.99	3	s	50.4 (d)	18.8
1.96	3	s	43.4 (s)	--
1.68	2	m (8)	38.9 (t)	27.7
1.62	2	m (7)	37.3 (t)	21.9
1.55	1	m	37.2 (d)	--
1.00	3	s	26.8 (s)	--
0.98	3	t (8)	36.0 (t) x 2	27.3
0.94	3	5 (u)	32.0 (t)	--
0.60	3	s	30.1 (t)	21.7
			29.5 (t)	22.0
			27.2 (t)	--
			24.9 (t)	--
			23.5 (q)	26.7
			20.7 (t)	
			19.4 (q)	22.4
			18.6 (t)	23.4
			18.3 (t)	--
			13.6 (q) x 2	21.7
			12.7 (q)	20.6

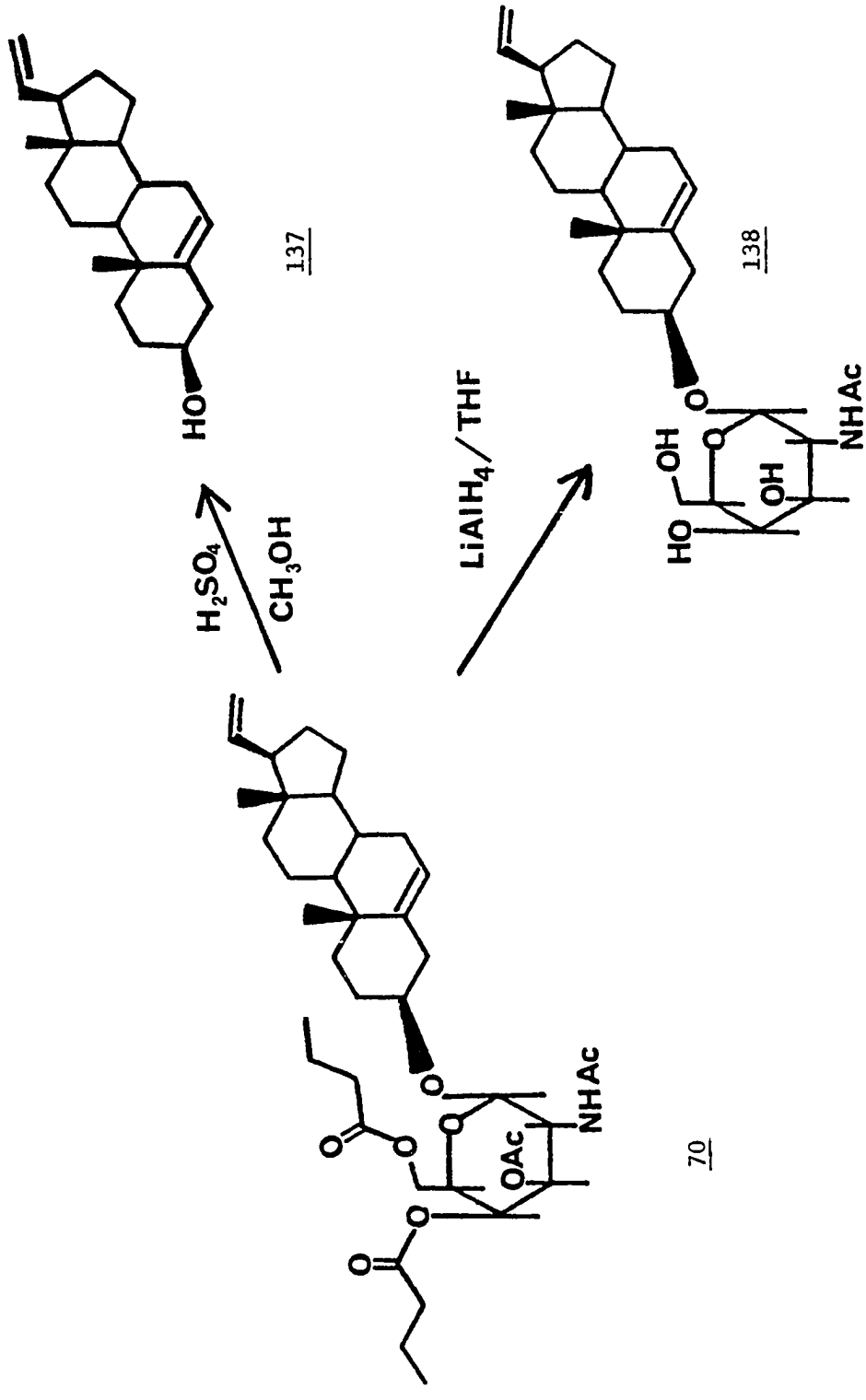
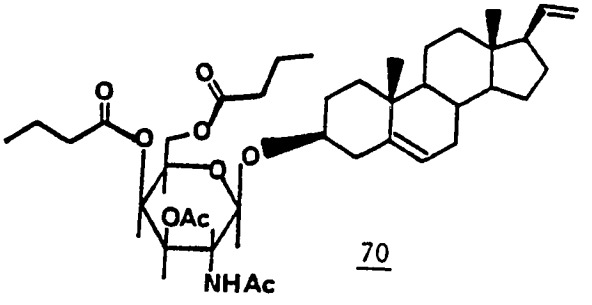


Figure 80. The hydrolysis and reduction products of compound 70

strongly supported by the presence of a doublet at ~δ 5.6 in the ^{1}H NMR spectra of 67-70 for the amide proton. Addition of D_2O caused this doublet to exchange slowly, over time, a characteristic property of amide protons.

^{1}H NMR spectra of 70, run in $CDCl_3$, d_6-acetone, and d_6-benzene (Table 37) clarified the sugar portion of the molecular by shifting out overlapping ester and sugar protons. ^{1}H NMR decoupling studies on 70 in $CDCl_3$ and d_6-acetone, combined with the ^{13}C NMR data of 67-70, established the structure and relative stereochemistry of the sugar. Subtraction of the aglycone portion of the molecule (assigned by comparison of the ^{13}C NMR data with that of the model compound sarcosterol 139[127] in Figure 81) from the ^{13}C NMR data of 67-70 left six absorptions for the sugar moiety. These ^{13}C NMR assignments are shown in Table 38. The doublet at 99.4 ppm was characteristic of the anomeric carbon in a sugar. The doublet at 52.3 ppm, with a large residual coupling constant, was typical of a carbon bearing an amide functionality. Three additional doublets, at 79.7, 69.8, and 66.5 ppm, and a triplet at 61.4 ppm indicated that the sugar moiety in 67-70 possessed a pyranose amino sugar skeleton. ^{1}H NMR decoupling experiments on 70 revealed the backbone of the sugar as shown in Figure 82. The C-1′ proton at δ 4.89 was coupled by 8 Hz only to the multiplet at δ 3.81, which was coupled in turn to both the amide proton at δ 5.61 (J = 9 Hz) and the doublet of doublets at δ 5.40 (J = 11 Hz), placing the amide functionality at C-2′. The C-3′ proton at δ 5.40 was coupled by 3.5 Hz to the C-4′ proton at δ 5.35. The C-4′ proton was coupled by the same amount to the broad triplet at δ 3.94 (C-5′). The C-5′ proton was coupled in turn by ~7 Hz to

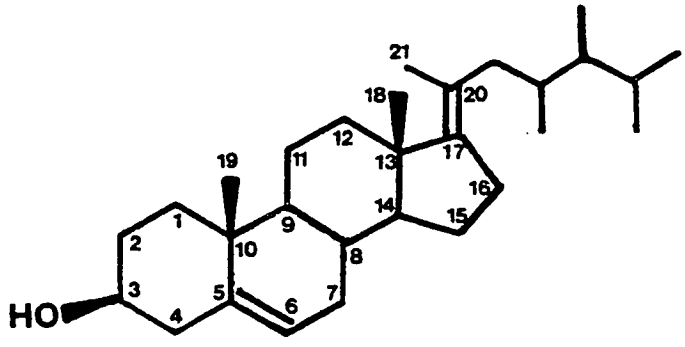
Table 37. 360 MHz ^{1}H NMR Comparison of the ^{1}H NMR Chemical Shifts of the Diacetate Saponin Derivative 70 in $CDCl_3$, d_6-Acetone, and d_6-Benzene.



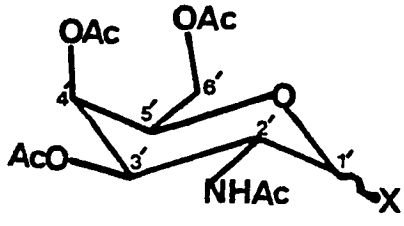
70

C	$CDCl_3$		d_6-Acetone			d_6-Benzene	
	δ	m	δ	H	m	δ	m
C-21	5.76	ddd	5.80	1	ddd (16,11,8)	5.85	ddd
-NH	5.61	d	7.10	1	d (9)	4.63	d
C-3′	5.40	dd	5.19	1	dd (11,3.5)	5.55	dd
C-4′	5.38	bd	5.35	1	bd (3.5)	5.50	bd
C-6	5.38	m	5.35	1	m	5.58	m
C-20	4.98	d	4.96	1	d (11)	5.08	d
C-20	4.97	d	4.95	1	d (16)	5.07	d
C-1′	4.89	d	4.89	1	d (8)	4.82	d
C-6′	4.18	dd	4.18	1	m	4.26	m
C-6′	4.08	dd	4.08	1	m	4.26	m
C-5′	3.94	bt	4.06	1	m	3.60	t
C-2′	3.81	ddd	4.02	1	ddd (11,9,8)	4.05	ddd
C-3	3.51	m	3.55	1	m	3.75	m
C-6"	2.38	t	2.40	2	t (9)	2.06	t
C-10"	2.27	t	2.26	2	t (7)	2.05	t
$-COCH_3$	1.99	s	1.90	3	s	1.80	s
$-NHCOCH_3$	1.96	s	1.85	3	s	1.60	s
C-7"	1.62	m	1.59	2	m (7)	1.57	m
C-11′	1.55	m	1.48	1	m	--	
C-19	1.00	s	1.08	3	s	0.95	s
C-8"	0.98	t	0.98	3	t (8)	0.80	t
C-12"	0.94	t	0.91	3	t (7)	0.77	t
C-18	0.60	s	0.64	3	s	0.57	s

Figure 81. Model compounds 139, 141 and 142 used to assign the ^{13}C NMR data for compounds 67-70



139 sarcosterol [127]



141: X = α-OAc

142: X = β-OAc

Table 38. Partial ^{13}C NMR Assignments of 67-70 Compared with Model Compounds+

C	67	68	69	70	137	139	141	142
1	37.3	37.3	37.3	37.3	37.3++	37.4++		
2	29.7++	29.7++	29.7++	30.1	29.7	31.8		
3	70.5	70.5	70.5	71.8	71.8	71.9		
4	38.8	38.8	38.9	38.9	42.3	42.4		
5	140.3	140.3	140.3	140.8	140.8	141.0		
6	122.0	122.0	122.0	121.6	121.6	121.8		
7	32.0	32.0	32.0	32.0	31.7	31.9		
8	37.2	37.2	37.3	37.2	32.0	31.5		
9	50.4	50.4	50.4	50.4	50.4	50.2		
10	36.8	36.8	36.8	36.8	36.6	36.7		
11	20.7	20.7	20.7	20.7	20.7	21.7		
12	39.5++	29.5++	29.5++	30.1	37.3++	37.8++		
13	43.4	43.4	43.4	43.4	42.3	44.5		
14	55.9	55.9	55.9	55.9	55.9	56.8		
15	24.9	24.9	24.9	24.9	24.9	24.7		
16	27.2	27.2	27.2	27.2	27.2	30.1		
17	55.3	55.3	55.3	55.3	55.3	144.4		
18	12.7	12.7	12.7	12.7	12.8q	16.5		
19	19.4	19.4	19.4	19.4	19.4q	19.6		
20	139.7	139.7	139.7	139.6	139.7d	124.2		
21	114.5	114.5	114.5	114.5	114.5t	17.8		
1′	99.4	99.4	99.4	99.4d			91.3	93.0
2′	52.4	52.4	52.4	52.3d			46.9	49.5
3′	69.6	69.7	69.7	69.8			67.8	70.3
4′	66.8	66.8	66.5	66.5			66.7	66.4
5′	79.8	79.8	79.8	79.7d			68.5	71.7
6′	61.5	61.3	61.5	61.4t			61.3	60.4

+ ^{13}C NMR spectra were obtained at 50 MHz in $CDCl_3$/TMS. Assignments were based on comparison with model compounds 139, 141 and 142.

++ may be interchanged

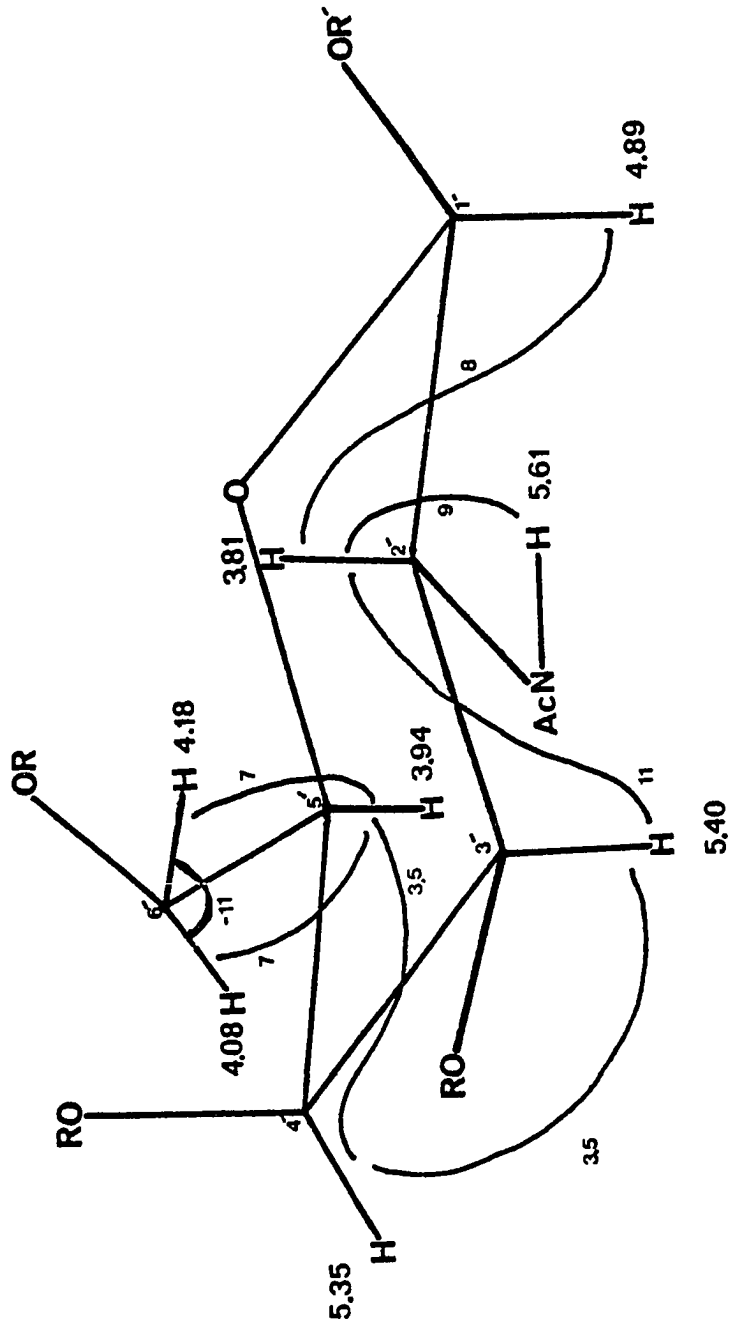


Figure 82. The results of ^{1}H NMR decoupling experiments on compound 70

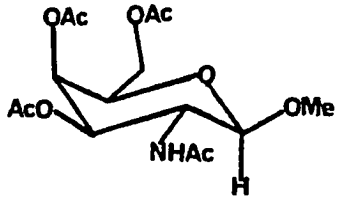
both C-6′ methylene protons at δ 4.18 and 4.08, which showed a geminal coupling constant of -11 Hz.

Coupling constants of protons in six membered rings are well known and reflect their dihedral angles. In pyranose sugars, the axial-axial coupling constant for vicinal diaxial protons ranges from 8-10 Hz. The coupling constant for axial-equatorial protons is 3-5 Hz while diequatorial protons show a coupling constant of 1-3 Hz.[128-131] Therefore, the protons at C-1′, C-2′ and C-3′ in 67-70 are all axial, while the proton at C-4′ must be equatorial, based on the observed coupling constants. The coupling constant of 3.5 Hz between the C-4′ and C-5′ protons also defines the C-5′ proton as axial.

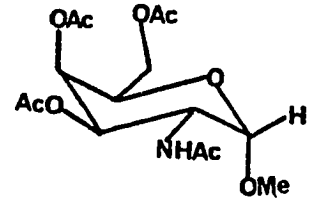
Comparison of the ^{1}H NMR chemical shifts and coupling constants with methyl-acetamido-2-3,4,6-triacetyl pyranose model compounds [132,133] resulted in the assignment of the sugar moiety in 67-70 as N-acetyl-β-galactosamine (Table 39). Comparison of the ^{13}C NMR data for the sugar moiety of 67-70 with model N-acetyl-2-amino sugar pyranose compounds yielded the same result.[134-137]

This assignment was supported by the isolation of N-acetyl-galactosamine derivatives from the hydrolysis reaction of 67. Acetylation of the water soluble fraction of the hydrolysis product resulted in a 10:1 mixture of α- and β-1′,3′,4′,6′ tetraacetyl-N-acetyl galactosamine. Compounds 141 and 142 were identified from the ^{1}H NMR spectrum of the mixture of products. The C-1′ equatorial proton in the α-anomer occurred at δ 6.22 ($J_{1',2'}$ = 3.6 Hz), while the C-1′ axial proton in the β-anomer was observed at higher field, δ 5.70 ($J_{1',2'}$ = 8.8 Hz).

Table 39. Comparison of the ^{1}H NMR Chemical Shifts of the Esterified N-Acetyl/Galactosamine Moiety of 67-70 with Methyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-and-α-D-galactopyranoside (143 and 144)+



143



144

C	67	68	69	70	β 143	α 144
-NH	5.64	5.67	5.56	5.76	5.95	5.89
C-1′	4.89	4.89	4.89	4.89	4.59	4.74
C-2′	3.81	3.82	3.82	3.82	4.05	4.52
C-3′	5.41	5.41	5.41	5.41	5.26	5.11
C-4′	5.36	5.35	5.35	5.35	5.33	5.33
C-5′	3.93	3.93	3.93	3.94	3.92	4.08
C-6′	4.12	4.10	4.08	4.08	4.12	4.08
	4.16	4.17	4.17	4.18	4.12	4.08
C-4′-OAc	2.14	2.14	--	--	2.15	2.14
C-6′-OAc	2.04	--	2.04	--	2.05	1.96
C-3′-OAc	2.00	2.00	1.99	1.99	2.00	2.03
C-2′-NHAc	1.97	1.97	1.96	1.96	1.97	1.96
C-4′-n-butyrate methyl	--	--	0.97	0.97	--	--
C-6′-n-butyrate methyl	--	0.93	--	0.93	--	--

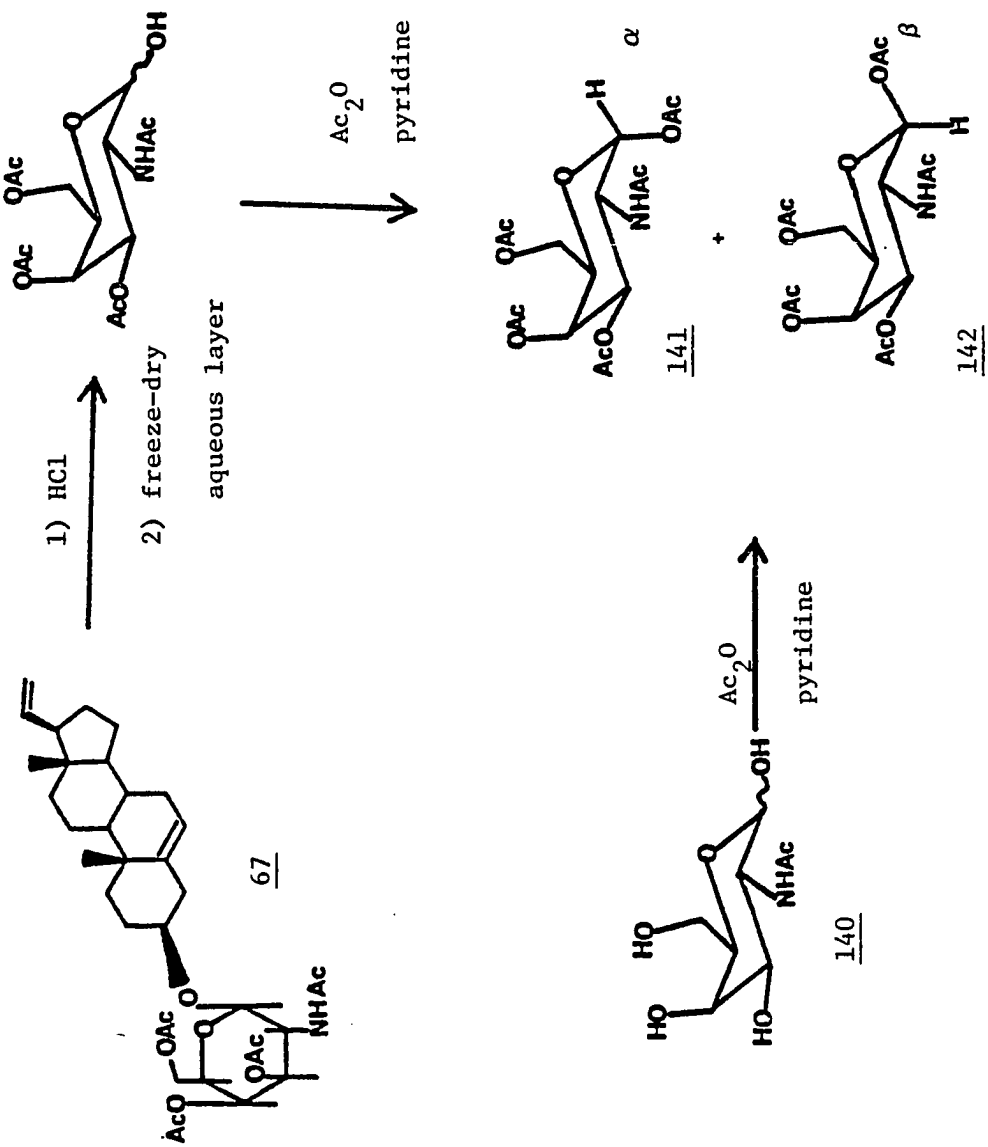
+The ^{1}H NMR spectra of all the compounds were run in $CDCl_3$/TMS. Assignments were based on ^{1}H NMR decoupling experiments and on comparison with model compounds. The acetate and n-butyrate ester methyl groups were assigned by using the results of lanthanide shift studies on 67, 68 and 70.

Acetylation of N-acetyl-D-galactosamine (140), obtained from Aldrich Chemical Company, under the same conditions, yielded the same ratio of products (Figure 83). This provided the final confirmation of the structural assignment of the sugar moiety of 67-70.

At this point, the absolute stereochemistry of the sugar moiety was still in question. The absolute stereochemistry of the sterol portion of the saponin had previously been defined by comparison of the optical rotation of the aglycone (137) with that of the natural product from Gersemia rubiformis. The N-acetyl-β-galactosamine could be either a D- or L-sugar. Only D-galactosamines have previously been found in nature.[138] This observation does not rule out the possibility of an L-amino sugar in 67-70. The amount of α- and β-1′,3′,4′,6′ tetraacetyl-2′-N-acetyl galactosamine isolated from the hydrolysis of 67 was too small and impure a sample on which to obtain an accurate optical rotation. Therefore the method of molecular rotation differences was employed to determine the absolute stereochemistry of the sugar in 67-70.

This method, alternatively called Hudson's rules of isorotation, assumes that the molecular rotation of a glycoside (sugar + terpene-derived compound) is made up of the sum of rotatory contributions from all the chiral centers in the molecule.[139-141] The molecular rotation [M] is defined as the molecular weight of the compound times the optical rotation divided by 100 ($[M]_D = MW[\alpha]_D/100$). Therefore, in the case of 67-70, the molecular rotation of each saponin compound should equal the sum of the molecular rotations of the sugar and sterol moieties ($[M]_{sap} = [M]_{sugar}+[M]_{sterol}$). Although this assumption is not theoretically

Figure 83. Isolation of the sugar from hydrolysis of compound 67



valid, the rules of isorotation have proven to be empirically useful.[140,141] This method is commonly used to determine the stereochemistry of pyranose sugars in glycosides and 2-deoxy-aminosugars have been found to obey the rules of isorotation quite well.[130,142,143]

The negative molecular rotation difference between the glycoside and its aglycone ($[M]_D$ -32 to -115 in chloroform) for 67-70 illustrated the β-D configuration of the sugar. The $[M]_D$ of methyl 2′-acetamido-3′,4′,6′-tri-O-acetyl-2′-deoxy-β-D-galactopyranose is -61 in $CHCl_3$, while the $[M]_D$ of the α-D-isomer is +325 in $CHCl_3$. The α- and β-L values for the $[M]_D$ of the isomers have not been measured, but would be opposite in sign to their D counterparts. Table 40 lists the values of the molecular rotations for 67-70, the aglycone, 137, and for the sugars 143 and 144, used in these calculations.

The structure of 67 was fully defined at this point as 3-β-pregna-5,21-dienyl-2′-acetamido-2′-deoxy-3′,4′,6′-tri-O-acetyl-β-D-galactopyranoside (Figure 84). The placement of the acetoxyl and n-butyrate ester functionalities had yet to be assigned in 68-70. The placement of these ester groups was determined by comparison of the ^1H NMR data with that of model compounds and by an ^1H NMR lanthanide induced shift (LIS) study of 67, 68 and 70. Table 39 compares the ^1H NMR chemical shift data for the acetoxyl and n-butyrate methyl groups in 67-70. It is well known that an axially-oriented acetoxyl methyl group occurs at a lower chemical shift than the corresponding equatorial acetoxyl methyl group. Axial acetoxyl groups are usually observed betweeen δ 2.15-2.18, while equatorial acetoxyl groups occur at ~δ 2.00-2.08.[129,130,144] Equatorial acetamide groups are generally found at

Table 40. Calculation of the Molecular Rotation Difference $(\Delta[M]_D)^+$ for Compounds 67-70.[139-142]

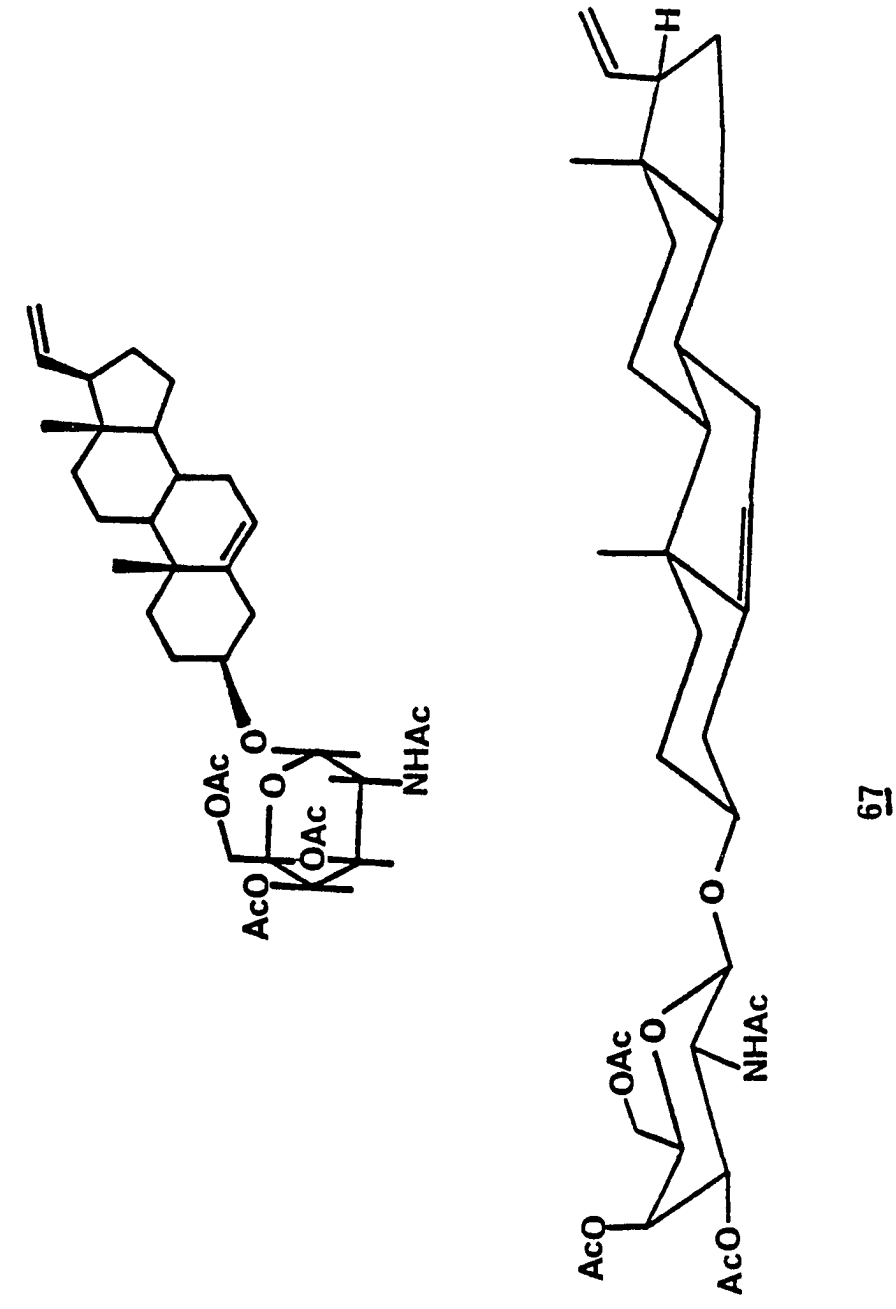
Compound	$[\delta]_D$ ($CHCl_3$)	MW	$[M]_D$	$\Delta[M]_D$
143++β	-17	361	-61	--
144++α	+90	361	+325	--
137 (aglycone)	-44	300	-132	--
67	-26	629	-164	-32
68	-30	657	-197	-65
69	-30	657	-197	-65
70	-36	685	-247	-115

$\Delta[M]_D = [M]_D$ (saponin)$-[M]_D$ (aglycone)$\cong[M]_D$ (sugar)

where $[M]_D$ (saponin)$=[M]_D$ (aglycone)$+[M]_D$ (sugar)

and $[M]_D=[\alpha]_D \times MW/100$

++Methyl-2′-acetamido-2′-deoxy--3′,4′,6′-tri-O-acetyl-D-galactopyranose[140]



67

Figure 84. The structure and stereochemistry of compound 67

δ 1.90-1.96.[58,129] This chemical shift difference for axial and equatorial acetoxyl methyl groups is clearly observed in the tetraacetate saponin derivative 67. The sugar moiety in 67-70 contains only one axial acetoxyl group, at C-4′. The chemical shifts of the acetoxyl methyl groups in 67 at δ 2.14, 2.04, 2.00, 1.97 reflect the presence of one axial and three equatorial acetoxyl methyl groups.

By comparison of the ^{1}H and ^{13}C NMR data with 67, both 68 and 69 possess only three acetoxyl groups with the addition of one n-butyrate ester, as previously discussed (Tables 39 and 41). The n-butyrate ester in 69 was easily placed at the C-4′ axial position, due to the absence of the axial acetoxyl methyl group at δ 2.14 in the ^{1}H NMR spectrum of 69. The n-butyrate ester in 68 was more difficult to place. The acetoxy methyl absorptions in the ^{1}H NMR spectrum of 68 occurred at δ 2.14, 2.00 and 1.97. This indicated that the n-butyrate ester could be placed at either the C-3′ equatorial or C-6′ position. This same problem arises when attempting to place the two n-butyrate ester groups in 70. The missing acetoxyl methyl absorption at δ 2.14 in the ^{1}H NMR spectrum of 70 places one of the n-butyrate groups at the C-4′ axial position. However, the second n-butyrate ester could occur at either the C-3′ or C-6′ position, as in 68. In order to resolve this problem, lanthanide induced shift (LIS) studies on the ^{1}H NMR spectra of 67, 68 and 70 were performed.

The LIS studies of 67, 68 and 70 were all performed in a similar manner, using low concentrations of $Eu(fod)_3$ to shift the acetoxyl methyl protons downfield in the ^{1}H NMR spectrum. The rate of the shift of each proton with increasing concentrations of $Eu(fod)_3$ (the induced

Table 41. Comparison of the ^{13}C NMR Data for the Ester Portions of 67-70 with the Acetates in 1,3,4,6-Tetraacetate-N-acetyl-α-D-galactosamine 141+

67	68	69	70	141
170.3 (s)	173.0 (s)	172.9 (s)	172.9 (s)	171.0 (s)
170.3 (s)	170.3 (s)	170.3 (s)	172.9 (s)	170.3 (s)
170.3 (s)	170.3 (s)	170.3 (s)	170.3 (s)	170.2 (s)
170.3 (s)	170.2 (s)	170.3 (s)	170.3 (s)	170.2 (s)
23.5 (q)	23.5 (q)	23.5 (q)	23.5 (q)	168.8 (s)
20.7 (q)	20.7 (q)	20.7 (q)	20.7 (q)	23.1 (q)
20.7 (q)	20.7 (q)	20.7 (q)	36.0 (t)	20.9 (q)
20.7 (q)	35.9 (t)	36.0 (t)	36.0 (t)	20.7 (q)
	18.3 (t)	18.6 (t)	18.6 (t)	20.7 (q)
	13.6 (q)	13.6 (q)	18.3 (t)	20.7 (q)
			13.6 (q)	
			13.6 (q)	

+ ^{13}C NMR spectra were run at 50 MHz in $CDCl_3$/TMS.

shift) depends on both the distance of the proton from the Europium metal and the angle between the two groups.[145,146] The induced shift ($\Delta\delta$) is determined by plotting the chemical shift of each proton signal against the molar quantity of $Eu(fod)_3$ added. The results of previous LIS studies on methyl-2′-acetamido-3′,4′,6′-tri-O-acetyl-2′-deoxy-D-pyranosides clearly showed that Europium complexes selectively with the amide carbonyl group,[132] as amides are stronger donors than esters toward lanthanide shift reagents.[147]

A qualitative analysis of the LIS study on 67 confirmed this result. The C-2′ proton at δ 3.80 showed an induced shift three times greater than the C-3′ and C-1′ protons at δ 5.40 and 4.89, and ten times greater than the C-5′ and C-6′ protons at δ 4.14 and δ 3.92. The induced shifts of the acetoxyl methyl groups in 67 gave similar results, which allowed their placement around the galactosamine ring. The acetamido methyl group at δ 1.97 moved three times faster than the C-3′ acetoxyl methyl at δ 2.00, six times faster than the C-4′ axial acetoxyl methyl at δ 2.14, and fifty times faster than the C-6′ acetoxyl methyl at δ 2.04. Shift studies on 68 and 70 gave corresponding results, resulting in the assignment of 68 with the n-butyrate ester at the C-6′ position. The second n-butyrate ester in 70 was also assigned to the C-6′ position. Therefore the structure and stereochemistry of all four compounds was determined as shown in Figure 85.

The aqueous extracts of both Muricea fruticosa and M. californica were examined for the presence of the free saponin analogue of 67-70. Acetylation of the freeze-dried aqueous fraction from extraction of both gorgonians failed to yield 67 by ^1H NMR examination. Therefore

Figure 85. Assignment of the ester functionalities in compounds 67 - 70

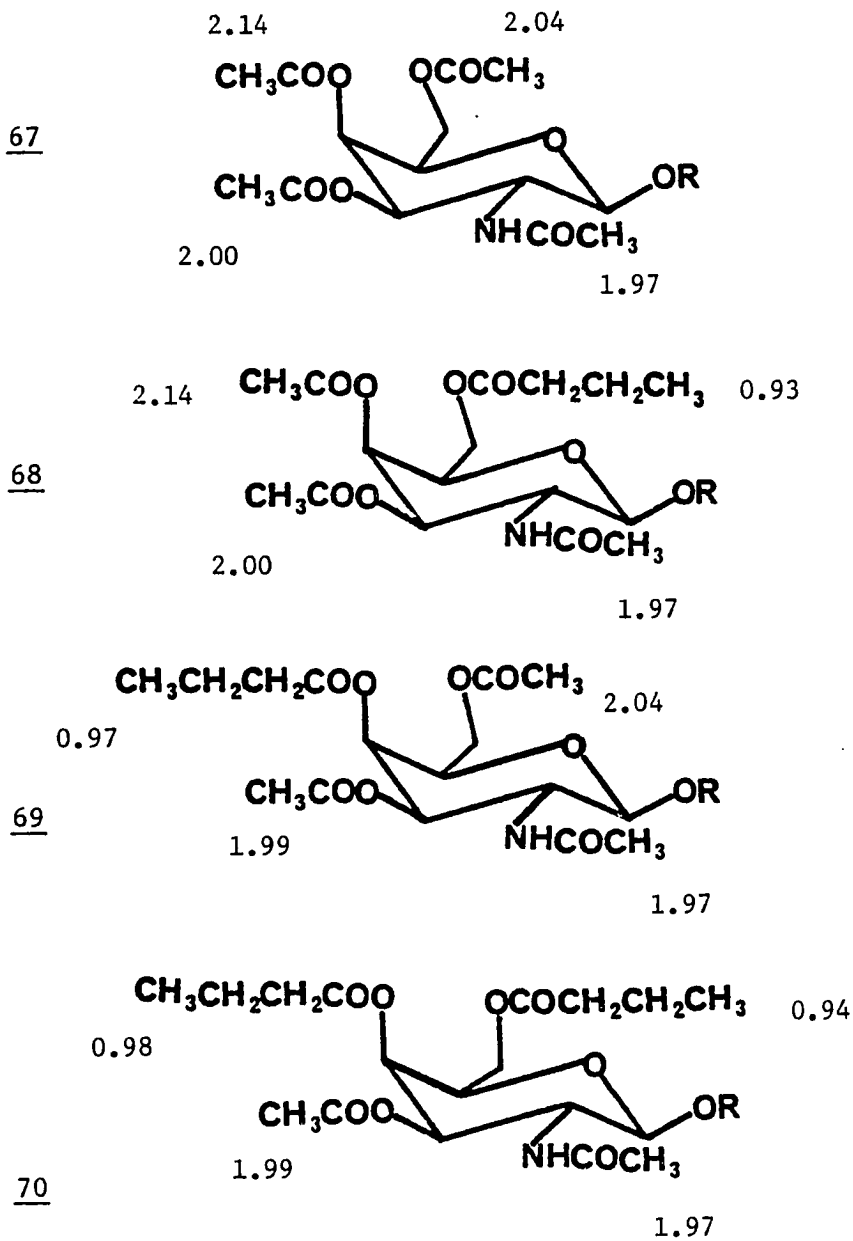
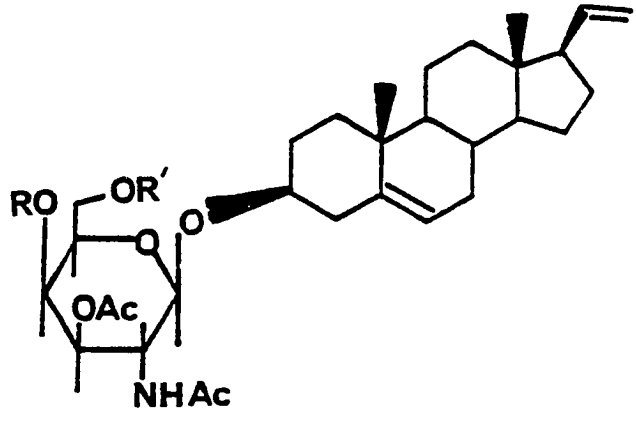
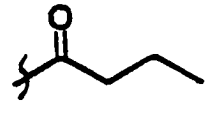
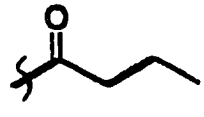
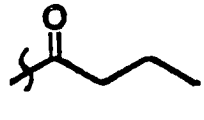
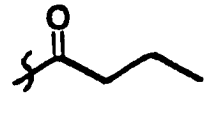


Figure 86. Saponin Derivatives Isolated from

Muricea fruticosa



	R	R′
67	Ac	Ac
68	Ac	
69		Ac
70		

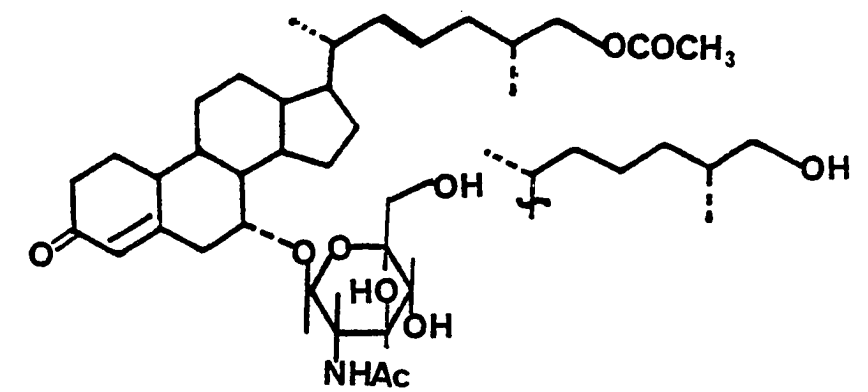
neither of the Muricea species possess the unesterified saponin in quantities detectable by this method.

The isolation of 67-70 from Muricea fruticosa represents the first isolation of a saponin-derived compound from the phylum Cnidaria and the first isolation of an aminosugar saponin from a marine organism. Marine saponins were previously known only in the phylum Echinoderma as common constituents of asteroids (starfish) and holothurians (sea cucumbers).[14,148] After this research was completed, a second set of aminosugar saponin-derivatives were isolated from the ichthyotoxic secretion of the Japanese fish Pardachinus pavonius. Four N-acetylglucosamine saponins, 144-147, were isolated from the defensive secretion of the sole, Pardachinus pavoninus (Figure 87).[149] Aminosugars, on the other hand, are found in many animal and bacterial polysaccharides and glycoproteins. D(+) Glucosamine is a major component of chitin, a structural polysaccharide which forms the exoskeleton of insects and crustaceans. D(+) glucosamine is also found in the cell walls of bacteria, in the form of muramic acid. D(+) Galactosamine occurs in the major polysaccharide of cartilage, chondroitin sulfate.[150]

D. The role of the acetylated aminosugar saponin derivatives (67-70) in reducing fouling in Muricea fruticosa.

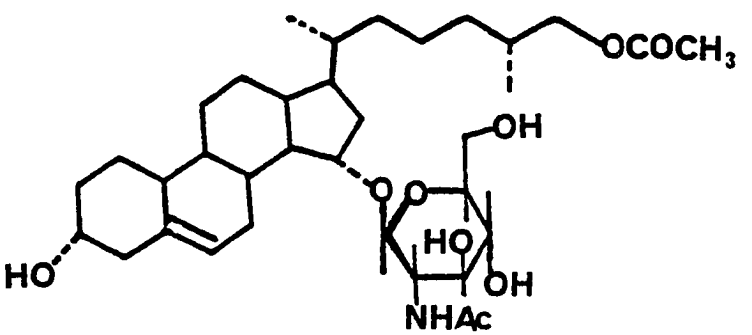
In order to examine the possible role of the saponins in reducing the amount of fouling on Muricea fruticosa, compounds 67-70 were tested in several simple but pertinent bioassays. The bioassays were designed to test the potential ability of these compounds to inhibit the growth of several primary foulers such as diatoms and marine bacteria.

Figure 87. Aminosugar saponins isolated from the Japanese sole *Pardachinus pavonius*[149]

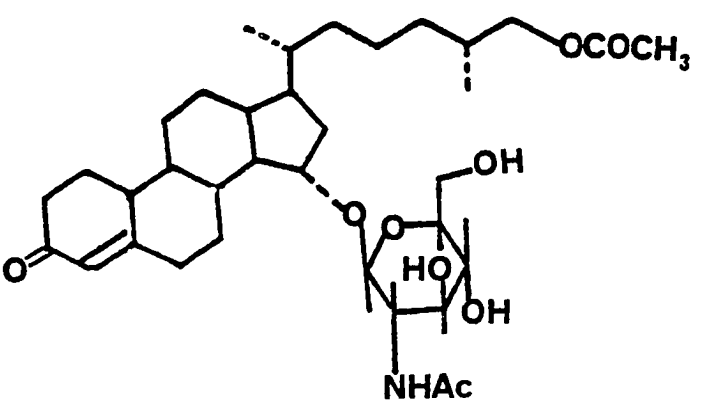


144

145



146



147

The compounds were also tested against the cell division of fertilized sea urchin eggs to examine their effect on invertebrate fouling organisms. The complete description and results of these assays are reported in Chapter eight. None of the compounds (67-70) were active in either the sea urchin egg or antibacterial assay. In contrast, all of the compounds inhibited the growth of the pennate marine diatom, Phaeodactylum tricornutum, at concentrations comparable to those found in the gorgonian tissue (100 ppm). This result does not prove a relationship between the lack of fouling observed in Muricea fruticosa and the occurrence of 67-70, but it does indicate a possible chemical defense mechanism for these compounds.

In support of this mechanism, it is well known that many saponins and aminosugar glycosides possess potent biological activities. Saponins from starfish and sea cucumbers possess cytotoxic, hemolytic, antiviral and antimicrobial activities.[14,148] Several saponins from starfish also induce an escape reaction in molluscs.[151] Holothurins, the saponins from sea cucumbers, possess both hemolytic and ichthyotoxic activities.[152] Saponins isolated from terrestrial plants also exhibit a broad range of biological activities.[153] The balanitin saponins were isolated from the methanol extract of an African root which is used medicinally in Africa. The compounds show insect antifeedant, antimicrobial and molluscidal activity.[154] Glycosides from the toxic Japanese ivory shell, Babylonia japonica inhibit nicotinic receptors in autonomic ganglia.[155] Aminosugar glycosides are also known for their potent biological properties. For example, the cytotoxic and antifungal agent, septacidin, contains the aminosugar 4-amino-4-deoxy-L-glucose.[156]

Compounds 67-70 are currently being tested for unusual pharmacological properties through the Sea Grant Marine Pharmacology Program at the University of California, Santa Barbara. So far, the compounds have not shown any cytotoxic or cardiac activity, although the tests are very incomplete at this time.

D. Experimental - Chapter VII

Muricea californica and M. fruticosa were collected simultaneously at four separate locations between January, 1979 and June, 1980 (Figure 17). After collection, samples were stored preserved in either ethanol or isopropyl alcohol. The alcohol was decanted and the whole animal was repeatedly extracted with 70% dichloromethane in methanol, followed by removal of the combined solvents under vacuum. The resulting aqueous residue was partitioned several times between dichloromethane and water. The resulting organic layer was concentrated and dried over $MgSO_4$ to give a crude extract (usually 2-3% of the dry weight of the animal). Extracts of Muricea californica and M. fruticosa were fractionated by rapid filtration chromatography using tlc grade silica gel in a scintered glass funnel. Fractions were eluted with mixtures of isooctane, dichloromethane and ethyl acetate.

Comparison of the organic extracts of Muricea californica and M. fruticosa.

Muricea californica, a dark reddish-brown sea whip with gold-colored polyps, was collected from La Jolla, Catalina and Los Coronados. A total of 5.7 g crude extract was obtained from 200 g dry weight of M. californica (collected in Catalina in June, 1980) (2.9% dry wt). The aqueous residue was set aside for further study. Separation of this extract, as described above, resulted in the isolation of ~50 mg of ergosterol peroxide (66) from a fraction eluted in 25% ethyl acetate-dichloromethane (0.9% extract, 0.03% dry wt).

Muricea fruticosa, a bushy, red sea whip with white polyps, was collected simultaneously with M. californica. A total of 3.0 g crude extract was obtained from 200 g dry wt. of M. fruticosa (collected in Catalina in June, 1980) (1.5% dry wt). The aqueous residue was set aside for further study. Separation of this extract gave ~30 mg 66 (eluted in 25% ethyl acetate-dichloromethane). ^{1}H NMR of the fraction eluted with 50% ethyl acetate-dichloromethane showed a mixture containing both fatty acids and compounds 67-70 (~0.5% extract, 0.01% dry wt for each compound). Collections of M. fruticosa from other locations yielded similar results with varying mixtures of 67-70. Compounds 67-70 were rechromatographed by hplc on silica in 60-75% ethyl acetate-isooctane. Reverse phase (C_{18}) hplc using 90-95% methanol-water was also employed to separate 67-70 from mixtures containing fats. The compounds were eluted from silica hplc in the following order of increasing polarity: 70, 69, 68 and 67.

Ergosterol peroxide (66, Table 32). ^{1}H NMR (360 MHz, d_6-benzene): δ 6.28 (1H, d (8.5)), 5.94 (1H, d (8.5)), 5.20 (2H, m), 3.95 (1H< m), 0.995 (3H, d (6.8)), 0.993 (3H, d (6.7)), 0.907 (3H, d (6.8)), 0.898 (3H, d (6.7)), 0.663 (3H, s), 0.608 (3H, s).

The saponin derivative 67, 3-β-pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,4′,6′-tri-O-acetyl-β-D-galactopyranoside (Table 33). Compound 67 was purified by silica hplc in 75% ethyl acetate-isooctane. Field desorption mass spectrometry (FDMS) gave: M+1 630 (100) (M^+ 629 for $C_{35}H_{51}NO_9$), 571 (15) (M^+-OAc), 338 (15), 332 (10) (M^+-$C_{21}H_{30}O$), 315 (10), 301 (10) ($C_{21}H_{33}O$), 259 (13).

The saponin derivative 68, 3-β-pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,4′-di-O-acetyl-6′-O-n-butyryl-β-D-galactopyranoside (Table 36). Compound 68 was purified from extracts of Muricea fruticosa by silica hplc in 70% ethyl acetate-isooctane. Field desorption mass spectral measurement (FDMS): M+1 658 (100) ($C_{37}H_{56}NO_9$), M^+ 657 (23) ($C_{37}H_{55}NO_9$), 367 (10) ($M^+-C_{21}H_{29}$), 283 (45) ($C_{21}H_{31}$), 205 (42), 171 (12), 153 (20).

The saponin derivative 69, 3-β-pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′,6′-di-O-acetyl-4′-O-n-butyryl-β-D-galactopyranoside (Table 37). Compound 69, isomeric with 68, was purified by silica hplc in 65% ethyl acetate-isooctane. FDMS: M+1 658 (20) ($C_{37}H_{56}NO_9$), M^+ 657 (24) ($C_{37}H_{55}NO_9$), 377 (3), 376 (42) ($M^+-C_{21}H_{29}$), 283 (37), 282 (100) ($C_{21}H_{30}$); HRMS: m/z 300.2433 (3.4) (calc. 300.2453 for $C_{21}H_{32}O$), 282.2344 (61.4) (calc. 282.2348 for $C_{21}H_{30}$), 267.2108 (26.4) (calc. 267.2113 for $C_{21}H_{27}$).

The derivative 70, 3-β-pregna-5,20-dienyl-2′-acetamido-2′-deoxy-3′-O-acetyl-4′,6′-di-O-n-butyryl-β-D-galactopyranoside (Table 34)). Compound 70 was purified by silica hplc in 70% ethyl acetate-isooctane. ^{13}C NMR (50 MHz, d_6-acetone); 173.1 (s), 172.9 (s), 170.2 (s) x 2, 141.5 (s), 140.3 (d, J_R = 44.7), 122.2 (d, 46.6), 115.0 (t), 100.7 (d, 45.8), 79.8 (d, 35.0), 71.4 (d, 44.1), 71.2 (d, 37.7), 67.5 (d, 46.9), 62.1 (t), 56.6 (d, 27), 56.1 (d, 17.6), 51.6 (d, 38.0), 51.4 (d), 44.0 (s), 39.6 (t), 38.1 (t), 37.5 (s), 36.3 (t), 32.7 (t), 27.8 (t), 25.4 (t), 32.1 (q, 26.0), 21.3 (t), 20.6 (q), 19.7 (q), 19.2 (t), 18.9 (t), 13.8 (q, 20.8), 13.0 (q, 20.9) ppm. HRMS: M^+ 685.4152 (0.1) (calc, 685.4190 for $C_{39}H_{59}NO_9$), 300.2455 (2.1) (calc. 300.2453 for

$C_{21}H_{32}O$), 285.2204 (1.0) (calc. 285.2218 for $C_{20}H_{29}O$), 282.2350 (48.7) (calc. 282.2348 for $C_{21}H_{30}$), 267.2105 (19.3) (calc. 267.2113 for $C_{20}H_{27}$), 231.1742 (1.8) (calc. 231.1749 for $C_{16}H_{23}O$), 229.1939 (3.5) (calc. 229.1956 for $C_{17}H_{25}$).

Hydrolysis of 67. Compound 67, 24.5 mg (3.9×10^{-5} moles), in 2 ml ethanol was treated with 1 ml of 10 N HCl and heated for three hours at 40°C. The reaction product was partitioned several times between dichloromethane and water and the organic layer was concentrated to give 11.8 mg (3.9×10^{-5} moles) (100% yield) of a single product, as deduced from the ^1H NMR spectrum. The product was identified as 137, identical to both the hydrolysis product of 70 and an authentic sample of 137, previously isolated from Gersemia rubiformis, by comparison of their ^1H NMR spectra. The aqueous fraction from this reaction was placed in a desiccator containing potassium hydride and placed under a high vacuum for 16 hours. A total of 5.5 mg of a white powder was recovered. Attempts to dissolved this power in a suitable ^1H NMR solvent ($CDCl_3$, d_6-acetone, d_4-methanol, deuterium oxide) were not successful. Acetylation of the aqueous hydrolysis product of 67. The powder, 5.5 mg recovered from the aqueous fraction resulting from the hydrolysis of 67, was dissolved in 6 ml pyridine and acetylated with 3 ml acetic anhydride for 20 hours at room temperature. The reaction was quenched by adding ice and water and the resulting solution was extracted several times with ethyl acetate. The organic layer was washed successively with 5% HCl and 5% bicarbonate solution and dried over $MgSO_4$. The recovered product, 4.0 mg of a yellow oil, was a mixture containing 1,3,4,6-tetraacetyl N-acetyl-α-D(+)-galactosamine (141) (10%) and 1,3,4,6-

tetraacetyl N acetyl β-D(+)-galactosamine (142) (1%) by ^1H NMR analysis. Comparison of the ^1H NMR spectrum with that of the synthetic products produced from acetylation of N-acetyl D-galactosamine (obtained from the Aldrich Chemical Company) showed the products were identical and to have been produced in the same relative proportion.

Acetylation of N-acetyl-D-galactosamine (140). N-acetyl-D-galactosamine, 125.5 mg (5.7×10^{-4} moles) in 8 ml pyridine, was acetylated with 4 ml acetic anhydride for 20 hours at room temperature. The reaction was quenched with ice and the solution was extracted several times with ethyl acetate. The organic layer was washed successively with 5% HCl and 5% $NaHCO_3$ and dried over $MgSO_4$. A white powder, 68.1 mg (1.8×10^{-4} moles, 32%), was recovered which appeared to be a mixture of 10:1 α- and β-1,3,4,6-tetraacetyl-N-acetyl-D-galactosamine (138 and 139) by comparison of ^{13}C and ^1H NMR spectral data. 1,3,4,6-tetraacetyl N acetyl-α-galactosamine (141). IR ($CHCl_3$): 3340-3510 (w), 1740, 1680, 1370, 1240, 1130, 1010, 910 cm^{-1}. ^1H NMR ($CDCl_3$/TMS): δ 6.22 (1H, d (3.6)), (C-1), 5.46 (1H, bd (9.1)) (-NH-), 5.42 (1H, d (2.2)) (C-4), 5.22 (1H, dd (11.6, 3.3)) (C-3), 4.73 (1H, m) (C-2′), 4.24 (1H, bt (7)) (C-5), 4.10 (2H, m) (C-6), 2.18 (6H, s), 2.03 (6H, s), 1.95 (3H, s). ^{13}C NMR ($CDCl_3$): 171.0 (s), 170.3 (s), 170.2 (s) x 2, 168.8 (s), 91.3 (d, J_R = 55.9)) (C-1), 68.5 (d (37.9)) (C-5), 67.8 (d (39.7)) (C-3), 66.7 (d (45.1)) (C-4), 61.3 (t (39.0)) (C-6), 46.9 (d (37.0)) (C-2), 21.1 (q (24.9)), 20.9 (q (25.3)), 20.7 (q (24.7)), 20.7 (q (25.0)) x 2 ppm. 1,3,4,6-tetraacetyl N acetyl-β-D-galactosamine (142). ^1H NMR ($CDCl_3$): δ 5.70 (1H, d (8.8)) (C-1), 5.50 (1H, bd) (NH), 5.38 (1H, d (3.1)) (C-4),, 5.19 (1H, dd (11.6, 3.3)) (c-3), 4.45 (1H, m) (C-

2), 4.17 (1H, bt) (C-5), 4.05 (2H, m), (C-6), 2.13 (3H, s), 2.05 (3H, s),2.02 (3H, s) x 2, 1.94 (3H, s).

Hydrolysis of the saponin derivative 70. Compound 70, 15 mg (2.2×10^{-5} moles) in 3 ml methanol, was treated with 3 ml of a solution containing 20% H_2SO_4. The reaction flask was warmed to 35°C for 2 hours and then stirred overnight at room temperature. The resulting mixture of products was partitioned several times between ethyl acetate and water. The organic layer was washed with bicarbonate, concentrated and dried over $MgSO_4$ to yield 6.2 mg (2.1×10^{-5} moles) of 137, $[\alpha]_D^{26}$ = 43.6° (c = 0.6, $CHCl_3$). IR ($CHCl_3$): 3401, 2950, 2865, 1450 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.76 (1H, ddd (16, 11, 8) (C-20), 5.36 (1H, m (5.2)) (C-6), 4.98 (1H, d (11)) (C-21), 4.96 (1H, d (16)) (C-21), 3.53 (1H, m) (C-3), 2.28 (2H, m) (C-4), 1.98 (1H, M) (C-17), 1.02 (3H, s) (C-19), 0.61 (3H, s) (C-18); ^{13}C NMR ($CDCl_3$): 140.8, 139.7, 121.6, 114.5, 71.8, 55.9, 55.3, 50.4, 42.3, 37.3 x 2, 36.6, 32.0 x 2, 31.7, 29.7, 27.2, 24.9, 20.7, 19.4, 12.8 ppm. HRMS: M^+ 300.2443 (&.3 (M^+ calc. 300.2453 for $C_{21}H_{32}O$), 282.2372 (16.2) (calc. 282.2348 for M^+-H_2O), 267.2105 (100) (calc. 267.2113 for $M^+-H_2O-CH_3$), 246.1994 (3.1) (calc. 246.1984 for $C_{17}H_{26}O$), 242.2050 (c.4) (calc. 242.2035 for $C_{18}H_{26}$), 213.1632 (21.8) (calc. 213.1643 for $C_{16}H_{21}$). Compound 137 was assigned as pregna-5,20-diene-3β-ol by comparison of the spectral data with an authentic sample of 137 isolated from Gersemia rubiformis.[125]

Pregna-5,20-dien-3β-ol (137) from Gersemia rubiformis. A crude mixture containing 60% of compound 137, isolated from the Newfoundland sea raspberry Gersemia rubiformis, was kindly supplied by Dr. John Kingston. Silica hplc of the mixture in 45% ethyl acetate-isooctane

yielded 8.2 mg of 137, $[\alpha]_D^{26}$ = -62.1° (c = 0.7, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.76 (1H, ddd (16.6, 11.5, 80) (C-20), 5.36 (1H, m (5.2)) (C-6), 4.98 (1H, d (11.5)) (C-21), 4.97 (1H, d (16.6)) (C-21), 3.53 (1H, bm) (C-3), 2.28 (2H, m) (C-4), 1.02 (3H, s) (C-19), 0.61 (3H, s) (C-18).

$LiAlH_4$ reduction of the saponin derivative 70. Compound 70, 40 mg (5.8×10^{-5} moles), in 3 ml THF, was treated with 100 mg of LAH at 0° for 1/2 hour. The ice bath was removed and the reaction was stirred at room temperature for two hours, at which time ethyl acetate and 0.5 ml H_2O were added to quench the excess reagent. The ensuing mixture was filtered over Celite, and the filtrate was concentrated and partitioned 2 x between ethyl acetate and water. The organic layer was dried over $MgSO_4$ and concentrated to give 2.6 mg (8.6×10^{-6} moles, 6% yield) of the aglycone 137, $[\alpha]_D^{26}$ = -19° (c = 0.1, $CHCl_3$) after silica hplc in 45% ethyl acetate-isooctane. The aglycone was identified by ^1H NMR spectral comparison with the authentic sample. The aqueous layer was lyophilized for four hours to give a small amount of a white powder. The methanol soluble portion of this powder was identified by ^1H NMR as the unesterified saponin 138 (1.0 mg (2.5×10^{-6} moles, 4% yield)). ^1H NMR d_6-acetone): δ 7.2 (1H, bd), 5.8 (1H, ddd), 5.4 (1H, bd), 5.0 (1H, bd), 4.95 (1H, d), 4.6 (1H, d) (C-1′), 3.5 (1H, m), 3.2-3.8 (6H, m), 1.9 (3H, s), 1.1 (3H, s), 0.6 (3H, s).

Acetylation of the aqueous extracts of Muricea californica and M. fruticosa. Aqueous residues from Muricea californica and M. fruticosa were examined for the presence of the free saponin, 141, produced by $LiAlH_4$ reduction of 70. The aqueous residues were taken from the extraction of the two Muricea species described at the beginning of the

Experimental Section. The aqueous fractions were lyopholized for twenty hours to yield 1.8 g of a yellow solid from M. fruticosa (0.9% dry wt) and 3.3 g from M. californica (1.7% dry wt). Both yellow solids were treated with 10 ml acetic anhydride in 20 ml pyridine for 24 hours at room temperature. The resulting mixtures were partitioned several times between ethyl acetate and water. The organic layers were washed sequentially with 5% HCl and 5% $NaHCO_3$ solutions, dried over $MgSO_4$ and concentrated. A total of 0.147 g of organic soluble material was isolated from acetylation of the aqueous residue of M. fruticosa. A total of 0.203 g of organic soluble material was isolated after similar treatment of M. californica. 360 ^1H NMR spectra of both of these fractions showed none of the expected tetraacetate saponin derivative, 67.

Lanthanide induced shift (LIS) study of the saponin derivative 67. Consecutive 0.05 molar aliquots of $Eu(fod)_3$, in $CDCl_3$, were added to an NMR tube containing 67 until a concentration of 0.25 molar equivalents was reached. The ^1H NMR spectrum of 67 was recorded after each addition of $Eu(fod)_3$ and double resonance (decoupling) experiments were performed to detect the downfield shift of each proton. The induced shifts ($\Delta\delta$) were determined by plotting the chemical shift of each proton signal against the molar quantity of $Eu(fod)_3$ added. The results of the shift study clearly placed the $Eu(fod)_3$ complexed selectively to the amide group, as reported previously in the literature[132] based on magnitude of the $\Delta\delta$ value of the protons at C-1′, C-2′, C-3′, and the C-2′ and C-3′ acetoxy groups compared with the rest of the protons in the molecule. LIS results: δ 5.76 (C-20, $\Delta\delta$ = 0), 5.46 (-NHAC, D_2O, exchanged), 5.42 (C-3′, $\Delta\delta$ = 2.1), 5.36 (C-4′, $\Delta\delta$ = 1.2),

5.36 (C-6, $\Delta\delta$ = 0), 4.98 and 4.97 (C-21, $\Delta\delta$ = 0), 4.89 (C-1′, $\Delta\delta$ = 2.2), 4.14 (C-6′, $\Delta\delta$ = 0.5), 3.92 (C-5′, $\Delta\delta$ = 0.6), 3.80 (C-2′, $\Delta\delta$ = 6.4), 3.50 (C-3, $\Delta\delta$ = 0.8), 2.137 (C-4′-OAc, $\Delta\delta$ = 0.3), 2.043 (C-6′-OAc, $\Delta\delta$ = 0.05), 2.004 (C-3′-OAc, $\Delta\delta$ = 0.6), 1.967 (C-2′-NHAc, $\Delta\delta$ = 1.9), 0.95 (C-19, $\Delta\delta$ = 0), 0.603 (C-18, $\Delta\delta$ = 0).

LIS study of the saponin derivative 68. Consecutive 0.05 molar aliquots of $Eu(fod)_3$, in $CDCl_3$, were added to an NMR tube containing 68 until a concentration of 0.15 molar equivalents was reached following the same procedure as described above. Close similarities in the $\Delta\delta$ values of 68 with those of 67, allowed the assignment of the n-butyrate ester in 68 at the C-6′ position. LIS results: δ 2.134 (C-4′-OAc, $\Delta\delta$ = 0.7), 2.002 (C-3′-OAC, $\Delta\delta$ = 1.3), 1.966 (C-2′-NHAc, $\Delta\delta$ = 4.2), 0.929 (C-6′n-butyrate, $\Delta\delta$ = 0).

LIS study of the saponin derivative 70. Consecutive 0.05 molar aliquots of $Eu(fod)_3$, in $CDCl_3$, were added to an NMR tube containing 70 until a concentration of 0.15 molar equivalents was reached following the established procedure described previously. Comparison of the $\Delta\delta$ values of 70 with those of 67 and 68 placed the n-butyrate groups at the C-4′ and C-6′ positions. LIS results: δ 1.991 (C-3′-OAc, $\Delta\delta$ = 3.0), 1.964 (C-2′-NHAC, $\Delta\delta$ = 8.3), 0.965 (C-4′-n-butyrate, $\Delta\delta$ = 0.8), 0.927 (C-6′-n-butyrate, $\Delta\delta$ = 0).

Chapter VIII

Biological Activity of Metabolites Isolated from East Pacific Gorgonians.

Gorgonians are large, sessile, benthic organisms which live in a nutrient rich, highly competitive environment. This makes them susceptible to a variety of predators and larval fouling organisms. However, field population studies have shown that the major causes of mortality in gorgonians are detachment and abrasion (by sand, coral, algae or gorgonian branches); very little predation has been observed.[45] An examination of the food sources of Caribbean reef fish by gut content analysis, also reveals an extremely low frequency of gorgonian ingestion. Gorgonian tissue was found in the stomachs of less than 5% of the fish investigated, and comprised only 2-10% of the total gut contents.[157]

The absence of fouling organisms on the surfaces of most gorgonians is also conspicuous. The large surface area that is exposed to currents in order for the polyps to efficiently filter feed, should make the gorgonian highly susceptible to larval settlement of encrusting organisms. The propagules of algae, anemones, ascidians, barnacles, bryozoans, hydroids, polychaetes, sponges and zoanthids, among others, have been observed to settle only on abraded or previously fouled areas of the gorgonian.[45,105] If these organisms spread, they are capable of smothering the gorgonian polyps, preventing feeding, and killing the underlying tissue.

The noticeable lack of predation and fouling on gorgonians leads to the hypothesis that the natural products they produce may be used in defensive strategies. In order to assess possible functions for the compounds isolated from east Pacific gorgonians, several simple bioassays were designed and performed.

A variety of natural products previously isolated from soft corals, algae and sponges have shown activity in assays for ichthyotoxicity, feeding inhibition, algal growth inhibition and antimicrobial activity.[6,13,112,113,158-161] Gorgonian extracts and natural products were active in a number of bioassays which include fish and copepod toxicity and growth inhibition of a ciliated protozoan, Tetrahymena pyriformis.[6,13,47,114,163] The protozoan was selected as an assay organism to study the effects of gorgonian compounds on ciliated larvae, which are typical fouling organisms. Extracts and natural products isolated from gorgonians have also been found to inhibit the growth of several marine and human pathogenic bacteria.[6,164]

The results of these bioassays do not prove specific functions for natural products in the marine environment, but they do indicate possible roles for the compounds isolated. Several bioassays, similar to those mentioned above, were performed on all of the natural products isolated from east Pacific gorgonians. The assays were designed to test for possible defensive functions against predation by fish and surface fouling by invertebrates, marine bacteria and diatoms. the concentrations tested (10-500 ppm) were in the range of those compounds found in gorgonian tissues (0.01-1%/dry weight).

A. Ichthyotoxicity

The toxicity of each compound isolated during this study was tested against two species of omnivorous Pacific damselfish. The black- and white-striped damselfish Dascyllus aruanas and the yellow-tailed blue damselfish, Pomacentrus coerulus were used interchangeably during the ichthyotoxicity assays. Both types of fish (~25 cm) yielded identical results. Compounds (2 mg dissolved in 100 µl ethanol) were added to 200 ml of fresh seawater, and the solutions were stirred until all of the compounds dissolved. Damselfish were exposed to the treated seawater for sixty minutes and their behaviors were observed. A seawater control and an ethanol control (100 µl) were run simultaneously with each experiment. No difference was observed between the two control fish.

Compounds were classified as toxic if a fish died as the result of exposure for 60 minutes. Most of the toxic compounds resulted in toxicity within 15-20 minutes after the start of the assay, but several showed a delayed effect. If the compound was judged toxic at a concentration of 10 ppm, and sufficient compound was available, the compounds were retested at 5 and 2.5 ppm. Toxic compounds were retested at 5 ppm in eight of ten cases and two compounds were retested at 2.5 ppm.

The results of the ichthyotoxicity assay are reported in Table 42. Ten out of the twenty-seven compounds tested were toxic at 10 ppm (37%). Eight of those ten (which were retested) were also ichthyotoxic at 5 ppm. Two of the toxic compounds tested (55 and 12) induced a violent escape response in the fish: the fish jumped out of the test

beaker several times during the test period. Compounds 57 and 58 caused the fish to regurgitate. Several compounds did not kill the fish, but resulted in severe impairment of their normal behavior (by comparison with the control fish). Fish treated with 45 (at 2.5 ppm) and 60 (at 10 ppm) exhibited severe "narcotic" effects. For example, the fish swam blindly into the sides of the beaker and aquaria, and appeared to have lowered rates of respiration. The fish also appeared to be unresponsive to stimuli such as a glass rod placed in the beaker. Some of the nontoxic compounds caused other signs of stress in the fish such as hyperventilation and tilting out of the vertical plane.

The severe reactions of damselfishes upon exposure to toxic and nontoxic compounds suggest that these natural products may play a role in preventing extensive gorgonian predation by fish. It is possible that the compounds may be perceived by predators as distasteful or harmful at concentrations found in or on gorgonian tissues. Results of feeding inhibition assays indicate that fish will mouth and then reject toxic tissues from marine organisms.[13,165] Observations by ecologists on the low frequency of sampling of gorgonian tissue by fish support these results.[45]

B. Inhibition of Cell Division in Fertilized Sea Urchin Eggs

The fertilized sea urchin egg cell division assay was used to examine the possible effects of gorgonian natural products on invertebrate fouling organisms. Several compounds previously isolated from Caribbean gorgonians and brown algae are potent inhibitors of sea urchin cell cleavage, and these compounds act by mechanisms similar to those of

Table 42. Ichthyotoxic Activity of Natural Products Isolated from East Pacific Gorgonians

Compound	Concentration		
	10 ppm	5 ppm	2.5 ppm
45	T	T	S
46	N-(1)	-	-
47	T	T	-
48	T	T	-
49	T	T	T
50	N	-	-
51	N-(1)	-	-
52	N	-	-
53	N	-	-
54	N	-	-
55	T-(2)	T	-
56	T	T	-
12	T-(2)	-	-
57	T-(3)	T-(3)	-
58	T	T-(3)	-
59	T	-	-
60	S	-	-
61	N	-	-
62	N	-	-
63	N	-	-
64	N	-	-
65	N	-	-
66	N	-	-
67	N	-	-
68	N	-	-
69	N	-	-
70	N	-	-

T = Toxic
S = Severe narcotic effect
N = Nontoxic
- = Not tested
1 = Fish hyperventilated and showed signs of severe stress
2 = Fish jumped out of test beaker
3 = Fish vomited

known cytotoxic agents such as cytochalasin-D[16] and colchicine.[159] Aspects of the pharmacology of this assay have recently been reported as a result of research done in the Sea Grant Marine Pharmacology Program.[162]

Three milliliters of a solution containing fertilized eggs from the local sea urchin, Lytechinus pictus, were added to plastic petri dishes containing 96 µg of each test compound in 3 ml of seawater. The gorgonian natural products were added to seawater in 25 µl of ethanol. Previous evaluations showed no effect on sea urchin cell cleavage at this concentration of ethanol.[163] Two controls for comparison of the cell division of treated and untreated cells were run during each experiment. One contained 25 µl ethanol and one was a seawater blank. The sea urchin eggs were left undisturbed for 2 hours at room temperature and then examined by light microscopy to determine the percentage of divided versus undivided cells. By the end of the test period most of the control cells had undergone 2-3 divisions (4-8 cells). Unfortunately, a condition called polyspermy made the results of the assay difficult to assess. Many of the eggs were fertilized by more than one sperm cell which made the cells difficult to count as divided or undivided because of the abnormal cell division which resulted.

By comparison of the division of test cells with the controls, only one compound, 61 (isoepizonarene), appeared to strongly inhibit cell division fertilized sea urchin eggs. The rest of the cells did not appear significantly different from the control cells. Therefore, if these compounds do inhibit invertebrate fouling organisms on gorgonian surfaces, they probably act via another mechanism. Other invertebrate

assay organisms, such as the acorn barnacle, Conopea galeata, which occurs only on previously abraded areas of local gorgonians, may be more suitable for future tests.[105]

C. Antimicrobial Assays

The development of microbial films on exposed surfaces in the marine environment results in a substrate for the attachment of larger fouling organisms such as algae and invertebrates.[168] Studies of fouling succession have shown that diatoms, algal spores and bacteria form a primary film on fouled surfaces. This film is quickly followed by algal settlement, and later by juvenile barnacles, hydrozoans, ascidians and bryozoans. This live primary film promotes secondary growth by stimulating attachment, and early growth of other fouling organisms, by providing food material or growth factors.[169]

Therefore, in order to test the possible role of gorgonian natural products in reducing fouling, two species of marine bacteria were used in an antibacterial assay. The standard agar plate-assay disc method on agar plates was used with the marine gram negative bacteria Vibrio anguillarum and Beneckea harveyi. At the same time, the compounds were tested against five human pathogenic bacteria and a yeast. Compounds (0.5 mg) were applied in acetone (10 µl) to 6.5 mm paper discs. After the solvent was evaporated, the discs were placed on agar surfaces freshly inoculated with a liquid microbial culture. The bacteria were incubated overnight (marine bacteria at 25°C and others at 37°C), at which time a dense bacterial lawn was observed. The plates were examined for zones of inhibition around the treated discs and these

Table 43. Bioactivity Data on the Inhibition of Cell Division in Fertilized Eggs of Lytechinus pictus

Compound	Percent Divided
Control 1[+]	70
Control 2[++]	70
45	70
46	70
47	70
48	70
49	70
50	70
51	70
52	70
53	70
54	70
55	70
56	70
12	70
57	70
58	70
59	70
60	70
61	20
62	70
63	70
64	70
65	70
66	70
67	70
68	70
69	70
70	70

[+] Control 1 = Seawater blank

[++] Control 2 = Blank containing 25 µl ethanol

zones were measured.

The results of these assays are reported in Table 44. Only two of the compounds (12 and 63) showed slight activity against the marine bacteria Vibrio anguillarum. Compound 12 (furanodiene) was also slightly active against the human pathogens Bacillus subtilis and Staphylococcus aureus, and the yeast Candida albicans. Compound 61 (isoepizonarene) was also slightly active against the same microorganisms. A number of compounds (10 out of 27) showed slight activity against the human pathogen Pseudomonas aeruginosa.

These results indicate that natural products isolated from east Pacific gorgonians (at the concentration tested) show only slight activity against marine bacteria.

It is interesting to note, in conjunction with this, that high levels ($\sim 10^6$ cells/cm^2) of bacteria have been found in the mucus of soft corals and gorgonians, although both organisms experience very little fouling.[170,171] This fact combined with other evidence suggests that mechanical sloughing of mucus may play an important role in removing fouling organisms from the surface of gorgonian tissue.[172] However, the mucus may also provide a dispersion mechanism for the organic compounds that the gorgonian produces. Experience handling freshly collected gorgonians reveals that high concentrations (as yet unmeasured) of volatile organic molecules are contained in the liquid gorgonian mucus. Natural products previously isolated from a soft coral have been identified in the soft coral exudates by thin layer chromatography.[173] Therefore the chemical and mechanical strategies to reduce fouling in gorgonians are

Table 44. Antimicrobial Activity of Natural Products Isolated from East Pacific Gorgonians[+]

	Marine Bacteria		Human Pathogens					Yeast
Compound	A	B	C	D	E	F	G	H
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	1	0
47	0	0	0	0	0	0	2	0
48	0	0	0	0	0	0	1	0
49	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0
54	0	0	0	0	0	0	1	0
55	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0
12	1	0	1	0	0	1	0	1
57	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	1	0
59	0	0	0	0	0	0	1	0
60	0	0	0	0	0	1	0	0
61	0	0	1	0	0	1	0	1
62	0	0	0	0	0	0	2	0
63	1	0	0	0	0	0	0	0
64	0	0	0	0	0	0	0	0
65	0	0	0	0	0	0	1	0
66	0	0	0	0	0	0	2	0
67	0	0	0	0	0	0	0	0
68	0	0	0	0	0	0	0	0
69	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	1	0

[+]Compounds were tested at 0.5 mg/disc
A = *Vibrio anguillarum*
B = *Beneckea harveyi*
C = *Bacillus subtilis*
D = *Escherichia coli*
E = *Enterobacter aerogenes*
F = *Staphylococcus aureus*
G = *Pseudomonas aeruginosa*
H = *Candida albicans*

0 = No activity
1 = Slight activity; zone of inhibition 7-10 mm
2 = Activity; zone of inhibition >10 mm

not mutually exclusive.

D. Inhibition of Algal Growth

Diatoms have been found, along with bacteria, in the surface fouling films of algae, gorgonians and other marine organisms.[109,114,170,111] Compounds previously isolated from Atlantic gorgonians reduced the growth of the benthic pennate diatom Navicula salinicola.[114]

In order to test the compounds isolated from east Pacific gorgonians in a diatom growth inhibition assay, I used the fast growing,hardy, pennate diatom Phaeodactylum tricornutum. This diatom was previously used to assay metabolites from brown algae with successful results.[167] An axenic culture of the diatom was kindly provided by Mr. James Lance. Pyrex culture tubes containing 1 ml GPM seawater medium and 100 µg of each compound were inoculated with 5 µl of the diatom culture. The compounds were introduced to the medium in 2.5 µl ethanol. The cells were grown for four days under continuous light at 24°C. The cell densities in each culture tube were counted at the end of the experiment under a microscope, using a haemocytometer. The experiment was repeated under similar conditions for five days at test concentrations of 200 µg/ml. The compounds isolated from Muricea fruticosa (66-70) were also tested for five days at 400 µg/ml. Controls containing ethanol and seawater only were run simultaneously with each experiment. Previous experiments determined that no effect on the growth of the diatom was observed at the concentrations of ethanol used.[167]

Table 45. Bioactivity Data on Inhibition of Growth in the Marine Diatom Phaeodactylum tricornutum

Compound	Cell Count[1]		
	100 ppm	200 ppm	400 ppm
Control #1[2]	19	29	29
Control #2[3]	18	22	11
45	16	2	--
46	14	3	--
47	6	2	--
48	4	2	--
49	11	1	--
50	11	14	--
51	16	5	--
52	7	5	--
53	10	5	--
54	10	4	--
55	15	1	--
56	3	1	--
12	0	4	--
57	1	2	--
58	1	1	--
59	5	1	--
60	1	1	--
61	14	2	--
62	5	1	--
63	3	5	--
64	3	3	--
65	18	4	--
66	24	8	8
67	3	11	4
68	5	23	2
69	7	2	3
70	6	10	3

1 = times 10^4
2 = seawater blank
3 = blank containing the amount of ethanol used to dissolve the compounds in each assay (2.5 µl for 100 ppm, 5.0 µl for 200 ppm, and 10.0µl for 400 ppm)

As Table 45 shows, more than half of the compounds tested inhibited the growth of the diatom by more than 60% at 100 ppm. Sixteen out of the 27 compounds tested resulted in diatom cell densities (p) of 7 x 10^4 cells/ml or less, compared with the controls which showed p = 20 x 10^4 cells/ml. Four additional compounds showed mild activity (densities between 8-10 x 10^4 cells/ml). At 200 ppm, all of the compounds except 68 and 50 showed less than 60% of the growth of the controls. At 400 ppm, all of the Muricea fruticosa saponin compounds 67-70 showed less than half the growth of the ethanol control. (At these higher concentrations, it appeared that the amount of ethanol used to dissolve the compounds did have an effect on the diatom growth. Therefore the activity of all of the compounds was compared to that of the ethanol control.)

These results support the hypothesis that natural products isolated from gorgonians may play a role in reducing fouling on gorgonians. As discussed previously, this role may not be exclusive of mechanical means of dealing with fouling such as the secretion of mucus. The mucus may provide a dispersive mode which enables the compounds to affect surface fouling organisms.

E. Summary of the Bioactivity Results

Table 46 summarizes the results of the biological assays performed on the twenty-seven compounds isolated from east Pacific gorgonians. Many of these compounds (37%) show ichthyotoxicity at 10 ppm and more than half (59%) inhibit the growth of a potential fouling diatom. Only one or two of the compounds showed slight activity in the assays

Table 46. Summary of Bioactivity Data

Compound[e]	Ichthyotoxicity[a]	Cell Division in Urchin Eggs[b]	Marine Bacterial Growth[c]	Diatom Growth[d]
45	T	0	0	0
46	N	0	0	0
47	T	0	0	2
48	T	0	0	2
49	T	0	0	1
50	N	0	0	1
51	N	0	0	0
52	N	0	0	2
53	N	0	0	1
54	N	0	0	1
55	T	0	0	0
56	T	0	0	2
12	T	0	1	2
57	T	0	0	2
58	T	0	0	2
59	T	0	0	2
60	S	0	0	2
61	N	1	0	0
62	N	0	0	2
63	N	0	1	2
64	N	0	0	2
65	N	0	0	0
66	N	0	0	0
67	N	0	0	2
68	N	0	0	2
69	N	0	0	2
70	N	0	0	2

a. Results of the ichthyotoxicity assay (tested at 10 ppm) see Table 42): T = toxic, S = severely narcotic, N = nontoxic.

b. Results of the fertilized sea urchin assay (tested (at 16 ppm) (see Table 43): 0 = no activity compared with the control, 1 = slight activity.

c. Results of the antibacterial assay using the marine bacteria Vibrio anguillarum, and Beneckea harveyi (at 0.5 mg/disc) (see Table 44): 0 = no activity, 1 = slight activity.

d. Results of the diatom growth inhbition assay (tested at 100 ppm) (see Table 45): 2 = activity ($\leq 7 \times 10^4$ cells/ml); 1 = mild activity ($8\text{-}10 \times 10^4$ cells/ml); 0 = no activity ($>11 \times 10^4$ cells/ml (control = 20×10^4 cells/ml)).

e. Trivial names for the compounds listed in Tables 42-46

Compound	Trivial Name
45	lophotoxin
46	lopholide
47	deoxylophotoxin
48	acetoxypukalide
49	pukalide aldehyde
50	pukalide
51	the rearranged aldehyde
52	lophodione
53	isolophodione
54	epoxylophodione
55	trans-keto-epoxide cembrene
56	cis-keto-epoxide cembrene
12	furanodiene
57	pacifigorgiolide
58	methoxypacifigorgiolide
59	ethoxypacifigorgiolide
60	the rearranged dimethyl ester
61	isoepizonarene
62	the guaiane diol
63	isosericenine
64	neosericenine
65	sericenine
66	ergosterol peroxide
67	tetraacetate saponin derivative
68	triacetate saponin derivative
69	triacetate saponin derivative
70	diacetate saponin derivative

against marine bacteria or the cell division of fertilized sea urchin eggs.

The results of these assays indicate possible roles for the natural products produced by east Pacific gorgonians in deterring predation by fish and surface fouling by primary foulers such as diatoms.

F. Experimental - Chapter VIII

Inhibition of cell replication in fertilized sea urchin eggs (Table 43). Local sea urchins, Lytechinus pictus, were provided by Dr. V. Vacquier of Scripps Institution of Oceanography. Injection of 0.1 ml of 0.5 M KCl into the body cavity of each sea urchin yielded yellow clumps of eggs or white streams of sperm. The eggs or sperm were collected separately from each individual urchin in a 50 ml beaker submerged in a 250 ml beaker containing seawater. The sea urchin eggs were rinsed with seawater (to remove feces) on a nylon mesh filter and the eggs were diluted with seawater to a volume of 100 ml. Sperm were added dropwise, while stirring the beaker containing the eggs, until fertilization was evident by microscopic examination (based on the formation of a second, inner cell membrane). Three milliliters of this solution was then added to 3 ml of seawater containing 96 µg of each compound in 60 mm plastic petri dishes (the compounds were added to seawater in 25 µl ethanol to give a test concentration of 16 ppm). Two blanks, one containing 25 µl ethanol and one without, were also run simultaneously with the experiment. The fertilized eggs were allowed to divide for two hours at room temperature, until the control embryos had undergone two or three divisions (4-8 cells). All of the assay dishes were then examined under a microscope to determine the percent of divided versus undivided cells.

Antimicrobial assays (Table 44). Antimicrobial bioassays were performed using the standardized agar plate-assay disc method. Each compound (0.5 mg) was applied to a 6.5 mm paper test disc (Difco #1599-33) in 10 µl acetone and the solvent was evaporated. The disc was then

placed on a freshly inoculated agar surface. The agar dishes were inoculated with microorganisms maintained in continuous culture by Ms. Katherine Steyn in the laboratory of Dr. D.J. Faulkner of Scripps Institution of Oceanography. Ms. Steyn and Dr. Faulkner provided all of the materials used in the bioassay and Ms. Steyn also assisted with the experiment.

The agar media used for the marine bacteria Vibrio anguillarum and Beneckea harveyi (B-392) consisted of:

5 g bacto-tryptone (Difco #0123-01)

3 g yeast extract (Difco #0127-02)

3 ml glycerol (analytical grade reagent)

17 g bacto-agar (Difco #0140-01)

250 ml deionized water

750 ml filtered seawater (8 μ millepore filter).

The five human pathogens (Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes and Staphylococcus aureus) and yeast (Candida albicans) tested were grown in the following agar media:

10 g bacto-tryptone

1 g yeast extract

8 g NaCl (analytical grade reagent)

1 g glucose

0.5 g Trizma-HCl (Sigma #T-3253)

17 g bacto-agar

1 l deionized water

Both agar media were sterilized by autoclaving for fifteen minutes at 15 psi and then poured into sterile 100 mm plastic petri dishes (Falcon #1001).

The human pathogens and yeast agar plates were incubated at 37° and the marine bacteria were incubated at 25° for 21 hours. The zone of inhibition for each compound was measured as the widest diameter around each disc of inhibited microbial growth.

Inhibition of diatom growth (Table 45). An axenic culture of the pennate marine diatom Phaeodactylum tricornutum Bohlin was obtained from Mr. James Lance in Dr. W. Fenical's laboratory at Scripps Institution of Oceanography. The diatoms were grown in a sterile standard GPM (Gonyaulex polyhedra media) medium. The instructions for preparing one liter of GPM medium are listed below. For 1 liter GPM medium, mix:

750 ml seawater

225 ml distilled water

2 ml 1 M KNO_3

0.2 ml 1 M K_2HPO_4 +

5 ml soil extract (soil:water, 1:1)

1 ml B_{12} (1 µg/ml)

1 ml thiamin HCl (1 mg/ml)

1 ml biotin (2 µg/ml)

+ Autoclave separately in 10 ml distilled water, cool, add to medium.

++PII Metal mix stock solution: Mix 6.0 g Na_2 EDTA, 0.29 g $FeCl_3.6\ H_2O$, 6.84 g H_3BO_4, 0.86 g $MnCl_2.4\ H_2O$, 0.06 g $ZnCl_2$ and 0.026 g $CaCl_2.6\ H_2O$ in distilled water to make 1.0 liter. Adjust the pH of the solution to 7.8-8.0 with NaOH (approx. 6-8 pellets).

One milliliter of GPM medium was pipetted into sterile 5 ml pyrex culture tubes. For the 100 ppm assay, each compound (100 µl) was dissolved in 2.5 µl ethanol and added to individual culture tubes with a micro-syringe. Two controls, one with 2.5 µl ethanol and one without, were run simultaneously with the experiment. One drop (~5 µl) of diatom culture inoculum ($\rho = 8 \times 10^4$ cells/ml) was added to each culture tube. The tubes were then covered and grown at 24°C under continuous light. After four days, the density of diatoms in each tube was measured by microscopic examination using a haemocytometer.

Similar procedures were followed for the diatom growth inhibition assay at 200 and 400 ppm concentrations. The compounds were again dissolved in ethanol (200 µg in 5 µl and 400 µg in 10 µl) and added to culture tubes containing 1 ml GPM medium. Twenty-five microliters of diatom inoculum ($\rho = 4.6 \times 10^5$ cells/ml) were added to each tube and ethanol controls for each concentration and a seawater blank were set up as before. After five days, the number of cells was counted using a haemocytometer.

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