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Title: Oxylipins from Temperate Marine Algae and a Photoprotective Sheath Pigment from Blue-Green Algae

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William H. Gerwick

Algae provide a diverse assortment of natural products. Although they have been studied over the past two decades, some classes of compounds produced by the algae, such as oxylipins, are just now receiving attention. The oxylipin chemistry of *Laminaria* spp., *Cymathere triplicata*, and *Sarcodiotheca gaudichaudii* has been explored. In addition, the structure of scytonemin, a sheath pigment common to many blue-green algae has been elucidated.

Our initial studies of brown algal oxylipins were with three species of *Laminaria*: *L. sinclairii*, *L. saccharina*, and *L. setchellii*. These algae were found to contain 15*S*-hydroxyeicosatetraenoic (15*S*-HETE), 15*S*-hydroxyeicosapentaenoic (15*S*-HEPE), 13*S*-hydroxyoctadecadienoic (13*S*-HODE), 13*S*-hydroxyoctadecatrienoic (13*S*-HOTE), and 13*S*-hydroxyoctadecatetraenoic acids (13*S*-HODTA). One of these, 13*S*-HODTA, had not previously been reported. We also discovered three new divinyl ether fatty acids in *L. sinclairii*. These metabolites all suggest that a lipoxygenase with ω6 specificity is present in these algae.

We next explored the chemistry of the kelp *Cymathere triplicata*. This alga was found to produce several hydroxy acids as well as the "cucumber" aldehydes 2*E*-nonenal and 2*E*, 6*Z*-nonadienal. More elaborate oxylipins containing cyclopentane epoxides, including four compounds that bear a cyclopropane ring, were also discovered. Finally, two 2-oxanorbornane containing compounds were characterized, revealing a structural element not previously encountered in natural products.

Investigations of the red alga *Sarcodiotheca gaudichaudii* revealed two new $C_{20}$ 11-membered ring lactones, both containing a *cis* epoxide. Two hydroxy fatty acids, 8*R*-HETE and 8*R*-HEPE, and the aldehyde, trideca-2*E*, 4*E*, 7*Z*, 10*Z*-tetraenal, were also

described. These metabolites suggest an 8-lipoxygenase in the alga, a specificity not previously reported for algae.

The blue-green algal sheath pigment, scytonemin, occurs in numerous genera under conditions of high UV light irradiance. Although its existence was known since the mid 1800's, the structure of this pigment had been unknown until now. The structure was determined by extensive NMR studies of the pigment , its reduced form, and an ozonolysis fragment of the reduced pigment. Scytonemin possesses a dimeric structure with each half containing indolic and phenolic subunits.

# Oxylipins from Temperate Marine Algae and a Photoprotective Sheath Pigment from Blue-Green Algae

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| CD | Circular dichroism |
| CIMS | Chemical ionization mass spectrometry |
| COLOC | Long-range $^{1}H$-$^{13}C$ correlation spectroscopy |
| COSY | $^{1}H$-$^{1}H$ chemical shift correlation spectroscopy |
| DEPT | Distortionless enhancement by polarization transfer |
| DMF | *N*,*N*-dimethylformamide |
| DMSO | Dimethylsulfoxide |
| EIMS | Electron-impact mass spectrometry |
| EtOAc | Ethyl acetate |
| E/H | Ethyl acetate in hexanes |
| FABMS | Fast atom bombardment mass spectrometry |
| FTIR | Fourier transform infrared spectroscopy |
| GC | Gas chromatography |
| GC/MS | Gas chromatography/mass spectrometry |
| HETE | Hydroxyeicosatetraenoic acid |
| HEPE | Hydroxyeicosapentaenoic acid |
| HMBC | Heteronuclear multiple-bond coherence spectroscopy |
| HODE | Hydroxyoctadecadienoic acid |
| HODTA | Hydroxyoctadecatetraenoic acid |
| HOTE | Hydroxyoctadecatrienoic acid |
| HPETE | Hydroperoxyeicosatrienoic acid |
| HPLC | High performance liquid chromatography |
| HR | High resolution |
| IR | Infrared spectroscopy |
| LAH | Lithium aluminum hydride |

| | |
|---|---|
| LR HETCOSY | Long-range $^{1}H$-$^{13}C$ correlation spectroscopy |
| $LTA_4$ | Leukotriene $A_4$ |
| $LTB_4$ | Leukotriene $B_4$ |
| MS | Mass spectrometry |
| NMR | Nuclear magnetic resonance spectroscopy |
| NOE | Nuclear Overhauser effect |
| NOEDS | Nuclear Overhauser effect difference spectroscopy |
| NOESY | Nuclear Overhauser exchange spectroscopy |
| PCC | Pyridinium chlorochromate |
| $PGE_2$ | Prostaglandin $E_2$ |
| $PGF_{2\alpha}$ | Prostaglandin $F_{2\alpha}$ |
| $PGI_2$ | Prostacyclin |
| THF | Tetrahydrofuran |
| TLC | Thin layer chromatography |
| TMS | Trimethylsilyl |
| $TXA_2$ | Thromboxane $A_2$ |
| UV | Ultraviolet spectroscopy |
| VC | Vacuum silica chromatography |
| XHCORR | Heteronuclear chemical shift correlation spectroscopy |

# OXYLIPINS FROM TEMPERATE MARINE ALGAE AND A PHOTOPROTECTIVE SHEATH PIGMENT FROM BLUE-GREEN ALGAE

## CHAPTER I

## GENERAL INTRODUCTION

The study of natural products has been a cornerstone of organic chemistry. Not only have natural products been of great use in medicine (morphine, atropine, digoxin) and industry (pyrethrins, lubricating oils), but they have also provided targets for innumerable synthetic organic efforts and have been key tools for biochemical studies. Central to the study of natural products are the isolation and structure elucidation of novel metabolites. Without these crucial first steps, further studies of the natural products would be greatly hampered.

Once a crude extract of an organism is obtained, the next stage in any further investigation is to determine which metabolite of thousands of compounds present is the one of interest. One of the most common approaches used to resolve this complex problem is bioassay guided fractionation. Much natural products research is driven by the desire to find compounds of novel structure types that can be used as drugs or drug leads. Simple tests can be utilized to pursue bioactivity, such as antimicrobial and brine shrimp toxicity assays, or more elaborate assays like the mechanism based screens provided by pharmaceutical companies and *in vitro* testing in the National Cancer Institute cancer cell lines.

Other natural products are investigated for their ecological importance. The hatching factor in barnacles,[1] trail pheromones in ants,[2] and feeding deterrent alkaloids in *Pitohui* birds[3] are just a few of many chemo-ecological phenomena. A third approach relies on professional intuition and does not follow rigid guidelines, but allows for the scientific knowledge of the individual researcher to lead the way to new compounds. This

ability to recognize novel components in a crude mixture develops after years of working with certain classes of compounds. Pattern recognition in NMR spectra is an example of a skill that is honed with time. After viewing hundreds of NMR spectra of crude fractions, the ability to recognize minor components that have unique chemical shifts grows (e.g. cyclopropyl protons between 0-1 ppm.). A familiarity with common compounds, such as sterols, pigments, fatty acids, and sugars, also develops, allowing for a visual "subtraction" of peaks corresponding to these generic compounds, greatly simplifying a complex crude spectrum. This experiential knowledge can lead to the discovery of novel structures that otherwise might have been overlooked.

Once a compound is obtained in pure form, structure elucidation can be accomplished. Spectroscopic techniques, including nuclear magnetic resonance (NMR), infrared (IR), ultraviolet (UV), and mass spectrometry (MS) are the mainstays of structure elucidation work, but degradative chemistry and knowledge of chemical manipulations remain vital research tools. The determination of a structure is not an endpoint, but rather opens doors for further research in areas such as biosynthesis, biochemistry, and synthetic organic chemistry.

This dissertation describes research focussed on these critical first stages of natural products chemistry, isolation and structure elucidation. Our research group specializes in marine natural products chemistry, with a focus on the marine algae. When I began my doctoral work my goal was to expand the investigation of a class of compounds, the oxylipins, in the marine algae, with a specific emphasis on studies of brown algae. We also had the opportunity to determine the structure of another algal metabolite, the blue-green algal sheath pigment scytonemin, in collaboration with biologists at the University of Oregon.

The following chapters will detail the results of research of four species of brown algae, one red algal species, and of a pigment present in numerous blue-green algal species. Chapter II describes the oxylipin chemistry of three species of temperate brown

algae of the genus *Laminaria*. These algae produce hydroxy and divinyl ether fatty acids which are indicative of a lipoxygenase with ω6 specificity. Chapter III provides an in-depth look at the products of oxylipin metabolism in the edible kelp *Cymathere triplicata*. This brown alga produces a series of prostanoid-like metabolites, in addition to simpler hydroxy acids. An investigation of a red alga is encountered in Chapter IV, where novel macrolactone oxylipins are described from *Sarcodiotheca gaudichaudii*. These metabolites suggest an 8-lipoxygenase activity which has not been previously reported for red algae.

A departure from oxylipin chemistry is described in Chapter V. Scytonemin is a blue-green algal sheath pigment that has been known for over 100 years. Its structure, however, has remained a mystery. Recently, the biological significance of this pigment as a photoprotective screen was determined. The structural study of this pigment is presented in Chapter V.

This introductory chapter will provide examples of the types of compounds encountered in marine natural products chemistry. These examples illustrate the novelty and complexity of secondary metabolites from the marine environment. A summary of oxylipin chemistry will then be presented to add perspective to our explorations in this field. And finally, a brief overview of metabolites from blue-green algae will be given.

## Marine Natural Products

Early natural products investigations utilized plants and animals, with bacterial sources gaining popularity in the mid 1900's. More recently, the natural products field has expanded to include the marine environment. The advances in marine natural products chemistry have been the subject of an on-going series of reviews by Faulkner[4-10] and Scheuer.[11,12] Their efforts help to keep track of the exciting new results in this branch of science.

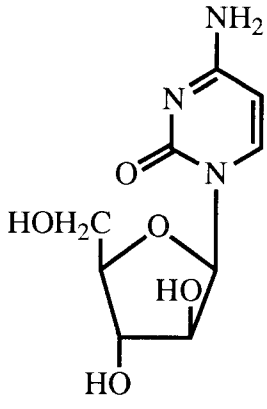
The search for novel and bioactive metabolites from the marine environment has produced several compounds that show promise as drugs or pharmacological tools. Ara-

C (**1**) was developed as an anticancer drug and ara-A (**2**) as an antiviral drug after the initial discoveries of spongothymidine (**3**) and spongouridine (**4**) from the sponge *Cryptotethia crypta* .[13,14] Manoalide (**5**), from a Palauan sponge,[15] has attracted attention for its antiinflammatory activity.[14,16] Okadaic acid (**6**), originally isolated from sponges[17] and subsequently from dinoflagellates,[18] is a tumor promoter and has found use as a phosphatase inhibitor in biochemical studies.[19,20] The tunicate metabolite eudistomin C (**7**) possesses activity against the herpes simplex virus.[21] Maitotoxin (**8**), a causative agent of ciguatera poisoning, was recently characterized structurally.[22] This highly toxic compound ($LD_{50}$ 50 ng/kg, mouse, ip) is perhaps the most extreme example of the complex and novel structures produced in the marine environment.

Although the above metabolites are from animals or microorganisms, the study of marine algae has also been a fruitful endeavor. Kainic acid (**9**) from *Digenea simplex* is the active principle of a crude drug used in Japan to combat intestinal parasites in humans.[14] It also has been used as a pharmacological tool in neurological studies.[23] A halogenated monoterpene (**10**) from *Portieria hornemannii* has shown exciting activity in NCI anticancer assays.[24] The recent report of a novel toxin, polycavernoside A (**11**), from the red alga *Polycavernosa tsudai* (formerly *Gracilaria edulis*) indicates that the marine algae still have much more to offer as far as new natural products.[25]

## Oxylipins

An area of marine natural products chemistry that has been of interest to our research group is the oxylipins. The term oxylipin was recently introduced to overcome the shortcomings of the term eicosanoids.[26,27] Eicosanoid implicitly describes $C_{20}$ derived fatty acid compounds. The type of metabolism that produces the eicosanoids, however, can also utilize alternative substrates of different chain lengths. Oxylipin, therefore, is an encompassing term for oxygenated compounds which are formed from fatty acids by reactions involving at least one step of mono- or dioxygenase-dependent



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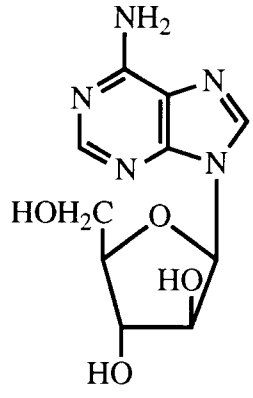

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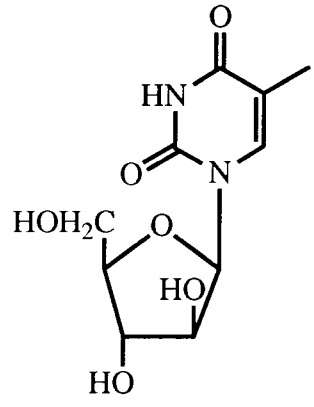

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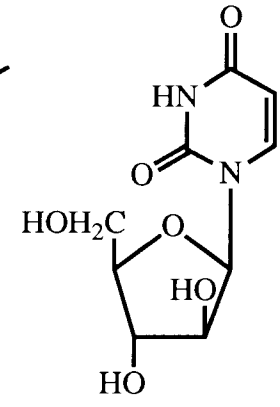

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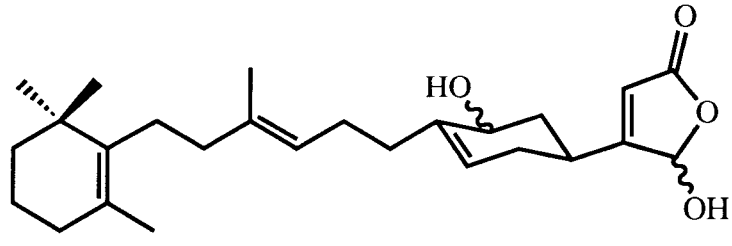

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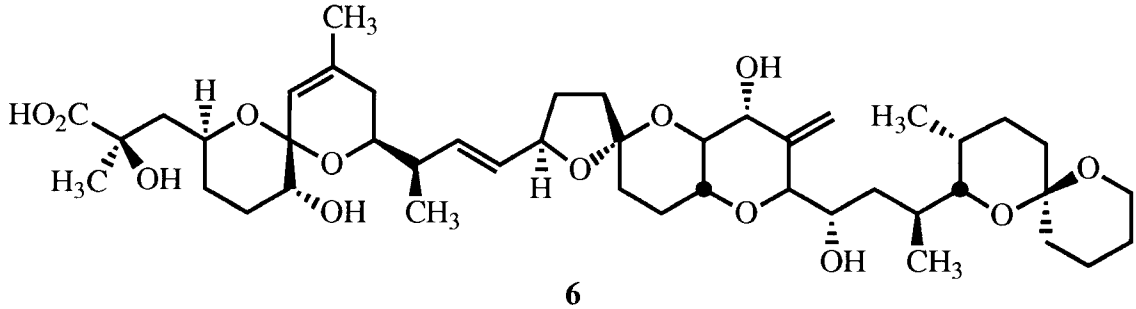

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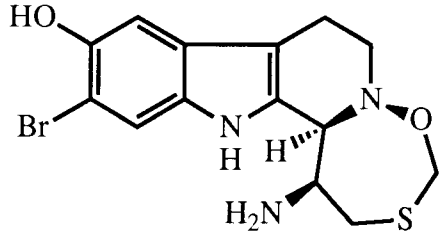

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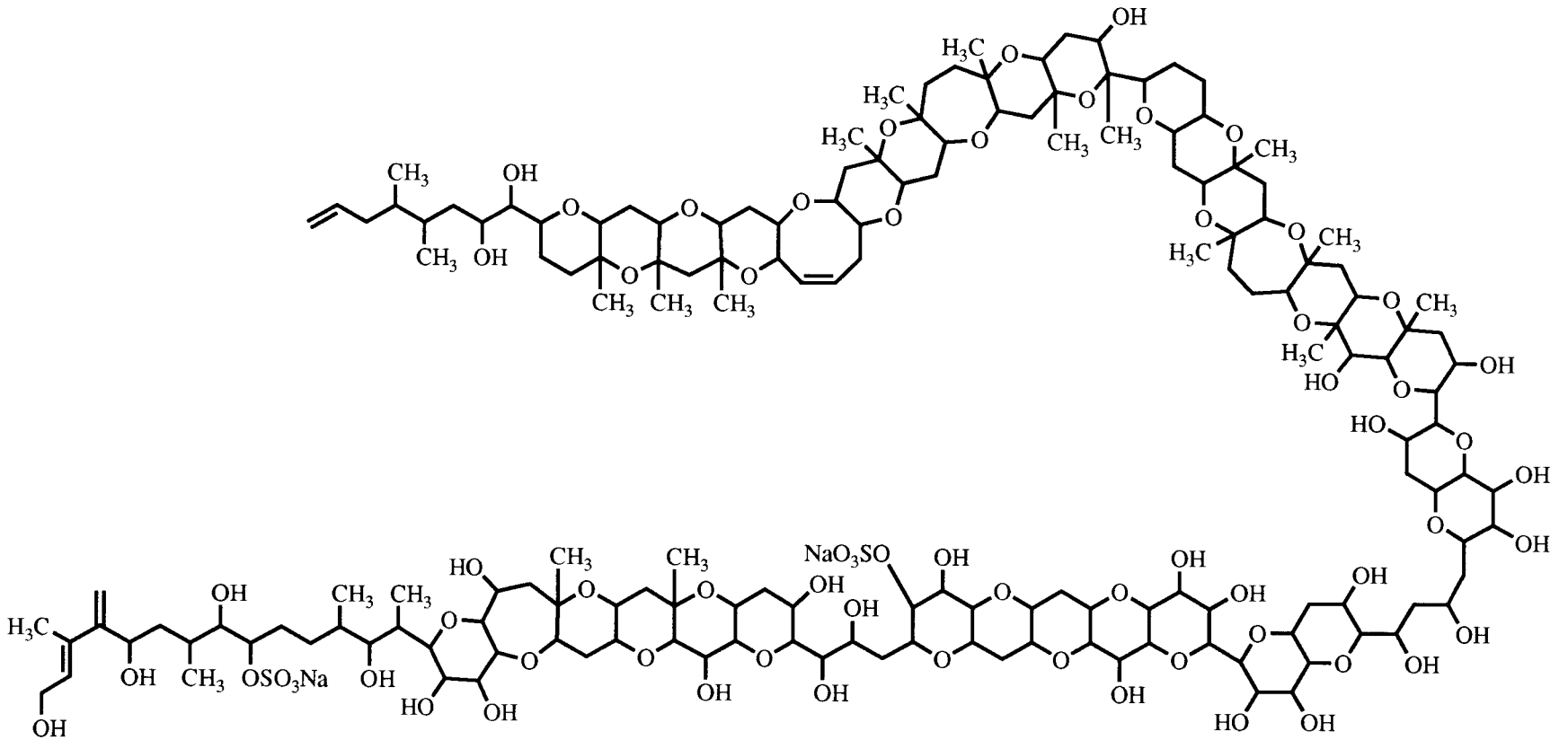
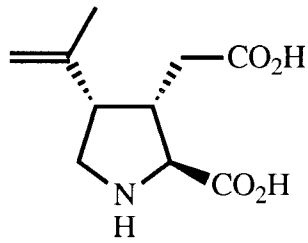


Maitotoxin (**8**)

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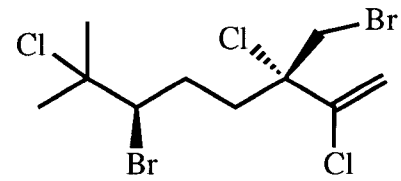


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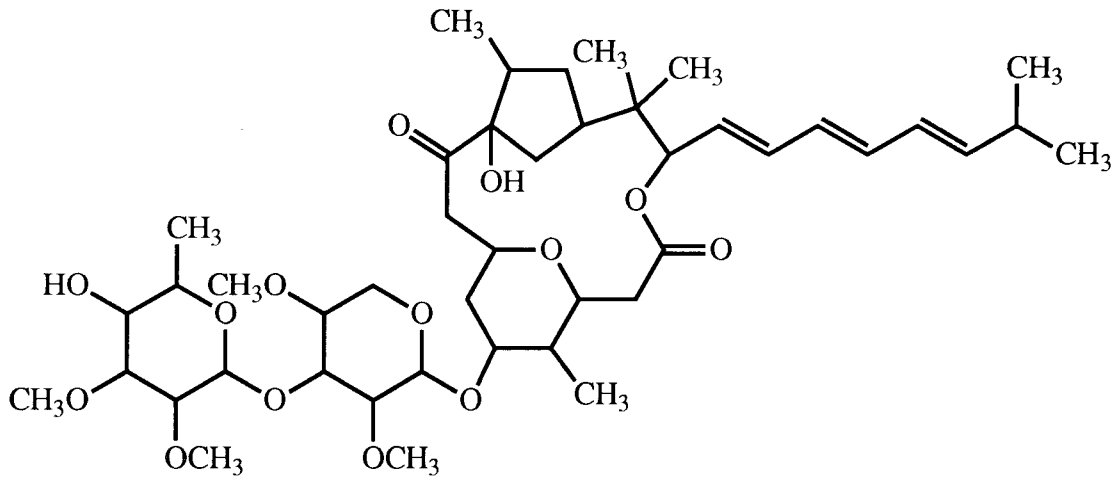


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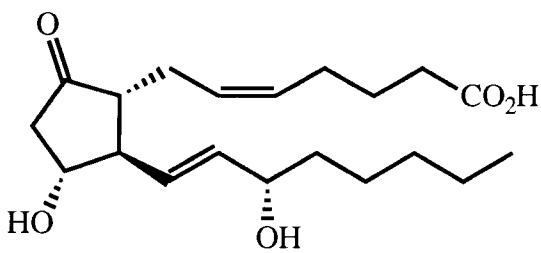


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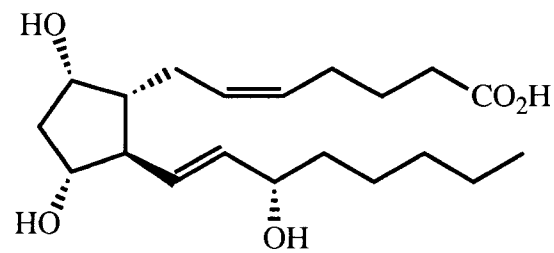


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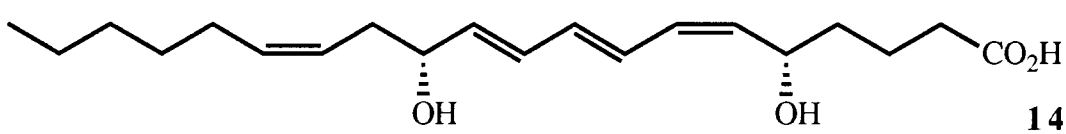


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oxidation. This definition now covers metabolites from a wide range of fatty acids, regardless of chain-length.

In mammalian metabolism, the oxylipins play a diversity of roles in a wide range of biological processes, including blood clotting, inflammation, and reproduction.[28] Prostaglandin $E_2$ ($PGE_2$ **12**) is involved in smooth muscle dilation and $Na^+$ excretion. $PGF_{2\alpha}$ (**13**) and thromboxane $A_2$ ($TXA_2$) are bronchoconstrictors and vasoconstrictors. On the other hand, prostacyclin ($PGI_2$) is a vasodilator. Leukotriene $B_4$ ($LTB_4$, **14**) is a chemotactic agent for various blood cells. Lipoxin $B_4$, a trihydroxy eicosanoid, inhibits natural killer cells. The hydroperoxy acid, 12-HPETE, is involved in migration of vascular smooth muscle cells and vasoconstriction. A final example, 14,15-*cis*-epoxy-5*Z*, 8*Z*, 11*Z*-eicosatrienoic acid, participates in glucagon secretion and can inhibit cyclooxygenase. Many of these activities have been shown to arise by participation of the oxylipins in intercellular signal transduction pathways.[28]

These diverse mammalian eicosanoids arise via three distinct pathways. The prostaglandins and thromboxanes arise via a cyclooxygenase pathway, whereas the lipoxins, leukotrienes and HPETEs arise via lipoxygenase initiated pathways (Figure I.1). A third pathway utilizes cytochrome $P_{450}$ enzymes to produce epoxy and hydroxy acids.[29]

The importance of oxylipins is not restricted to mammals. Research has shown that the insect immune response to bacterial infection is mediated by eicosanoids.[30] Further examples of oxylipin metabolism in marine invertebrates are given below.

Oxylipins in plant systems are also very important. A recent review covering plant lipoxygenases gives a detailed account of the enzymes involved in the formation of plant oxylipins.[31] While the first lipoxygenase enzyme was crystallized from soybeans in 1947, the crystal structure was not solved until this year.[32] The study of this enzyme and its products in plant systems preceded the discovery of a mammalian lipoxygenase by more than 25 years.[33] This is just one example of the relevance of studying oxylipins in less complex organisms.

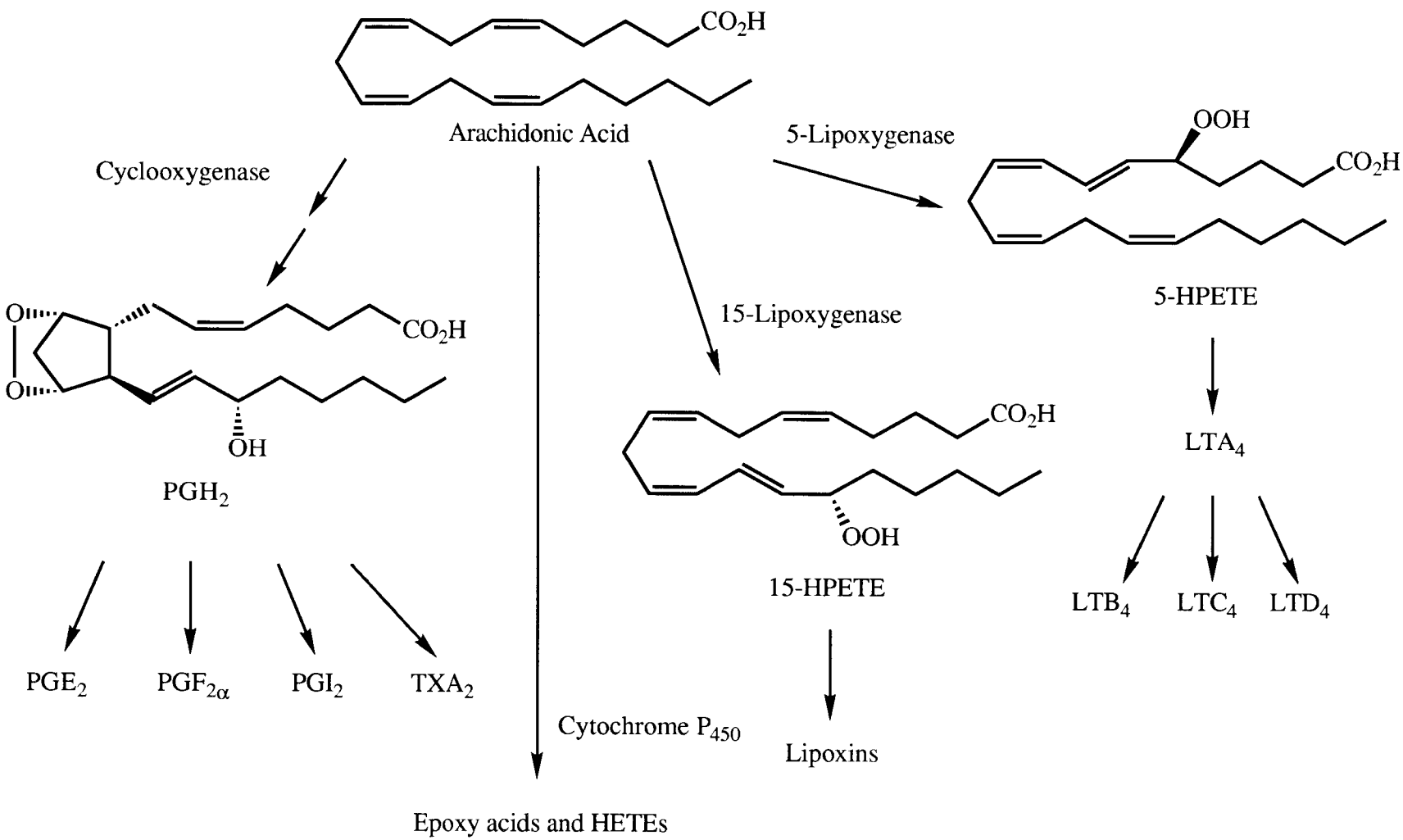



Figure I.1. Representative Examples of Cyclooxygenase, Lipoxygenase, and Cytochrome $P_{450}$ Products of Arachidonic Acid.

The plant hormones jasmonic acid, *iso*-jasmonic acid, and methyl jasmonate are important fatty acid derived metabolites. Their formation is initiated by a lipoxygenase with ω6 specificity which acts on α-linolenic acid to form 13*S*-hydroperoxyoctadecatrienoic acid, which is further metabolized by hydroperoxide dehydrase to an allene oxide.[34] Cyclization of the allene oxide by allene oxide cyclase then produces 12-oxophytodienoic acid.[34] Jasmonic acid derives from 12-oxophytodienoic acid via several β-oxidation steps (Figure I.2).[35] The jasmonates have been reportedly involved in growth inhibition, abscission, senescence, induction of leaf proteins, and induction of proteinase inhibitors.[31]

Other lipoxygenase products also play vital roles in plants. Rice plants are known to produce oxygenated fatty acids in response to infection by the rice blast fungus. The hydroxy acids, 9-hydroxyoctadecadienoic acid (9-HODE), 9-hydroxyoctadecatrienoic acid (9-HOTE), 13-HODE, 13-HOTE, and 16-HOTE are responsible for this antifungal activity.[36] Taro tubers produce the fatty acid 9, 12, 13-trihydroxy-10*E*-octadecenoic acid as a defensive substance against the black rot fungus *Ceratocytis fimbriata*.[37] Recent work has shown that the tomato increases its lipoxygenase activity in response to infection by powdery mildew.[38]

The marine environment is another source of both known and novel oxylipins. A recent comprehensive review of oxylipins from marine invertebrates covers the literature up to July of 1992.[27] A few examples of the diversity of this type of metabolism will be presented here. Initial interest in oxylipins from soft corals arose from the finding of $PGE_2$ and $PGF_{2\alpha}$ in *Plexaura homomalla*.[39] Although interest in the coral as a commercial source of prostaglandins died down, study of the biosynthetic pathways in this and related corals continued. These invertebrates apparently utilize a different pathway to the prostaglandins than do mammals. An allene oxide appears to be a key intermediate.[40,41] Punaglandin 1 (**15**), isolated from the octocoral *Telesto riisei*, is one of

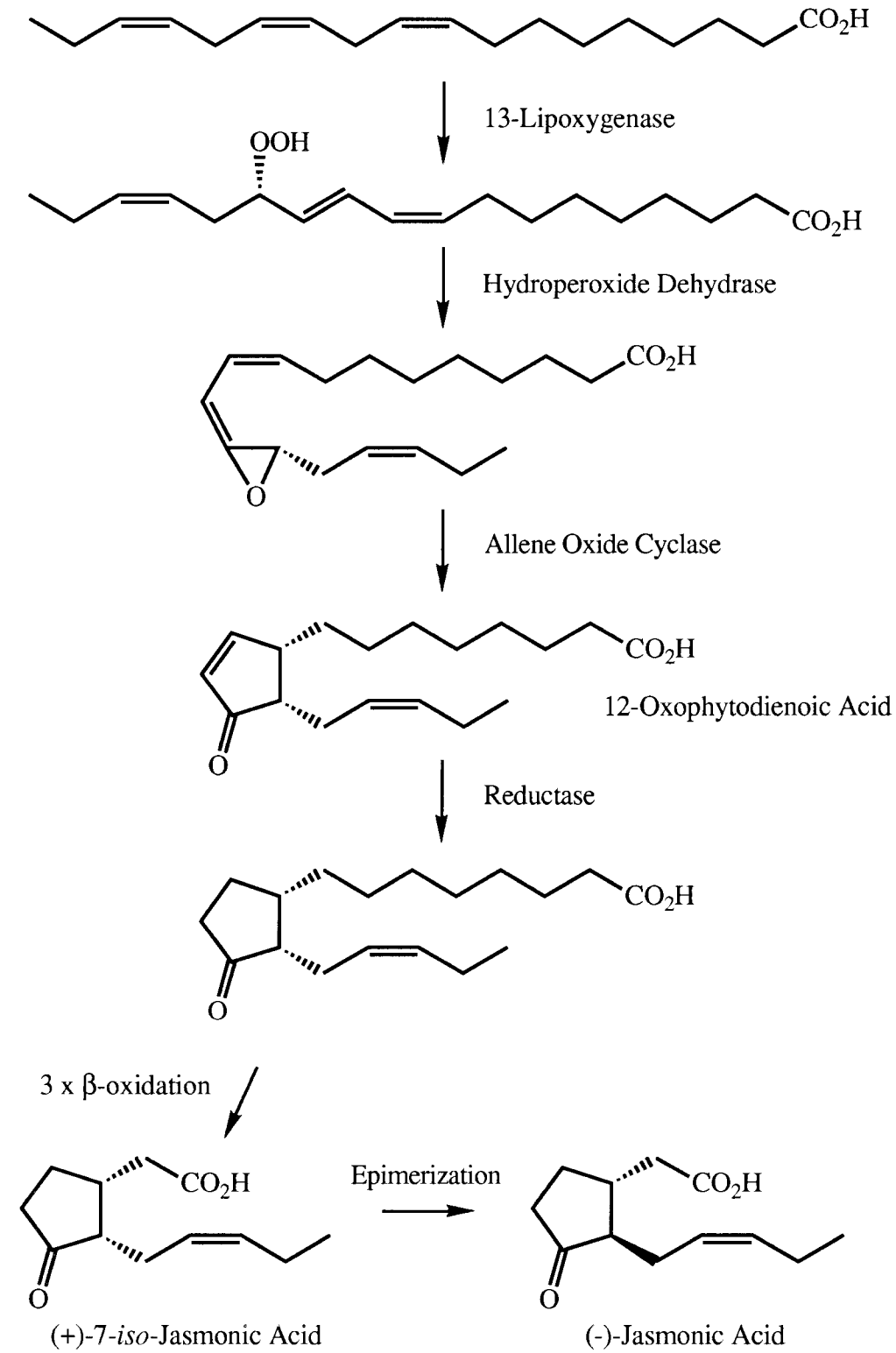



Figure I.2. Biosynthesis of Jasmonic Acid.[34,35]

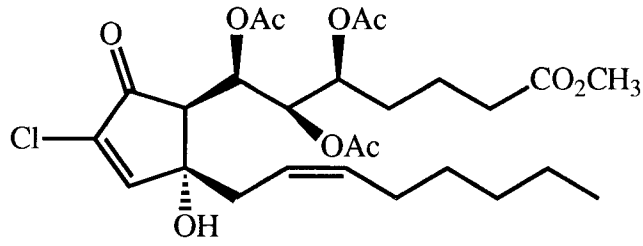
several compounds related to the prostaglandins that are unique to the marine environment.[42]

The ability to form allene oxides is also apparent in cell free studies with starfish.[43] The starfish have been shown to utilize 8*R*-hydroxyeicosatetraenoic acid (8*R*-HETE) as an oocyte maturation factor.[44]

Oxylipins have also been isolated from sponges, molluscs, and tunicates. Halicholactone (**16**) from the sponge *Halichondria okadai* represents a unique incorporation of a cyclopropane into an oxylipin.[45] The cyclopropyl containing metabolite aplydilactone (**17**), isolated from the sea hare *Aplysia kurodai*,[46] may arise by dimerization of constanolactone-like monomer units.[27] The macrolactone didemnilactone (**18**) from the tunicate *Didemnum mosleyi* provides a final example of the unique oxylipins that have been found from the marine invertebrates.[47]

Our research group has focussed on the oxylipins of marine algae. Several reviews of this topic have recently appeared.[48,49] The earliest report of oxylipins from marine algae was on the isolation of $PGE_2$ and $PGF_{2\alpha}$ from the red alga *Gracilaria lichenoides* in 1979 by an Australian group.[50] This work was followed later by the discovery of $PGE_2$ in *Gracilaria verrucosa* from Japan and its possible causative nature in "ogonori poisoning".[51] The recent isolation of polycavernosides A and B, however, suggests a re-evaluation of the role of the prostaglandins in this type of poisoning.[25] The early 1980's also witnessed the discovery of hybridalactone (**19**) from work with *Laurencia hybrida*.[52] This novel eicosanoid incorporates a cyclopentyl ring, a lactone, an epoxide, and a cyclopropyl ring in its structure.

Despite these scattered initial studies, no systematic research on the presence of oxylipins in marine algae appeared until our research group attacked the problem. Discovery of 12*S*-HETE (**20**) in the red alga *Platysiphonia miniata* was the first example of this eicosanoid in a non-mammalian system.[53] Since that initial report, 12*S*-HETE has been discovered in the red algae *Cottoniella filamentosa*,[54] *Constantinea simplex*,[55]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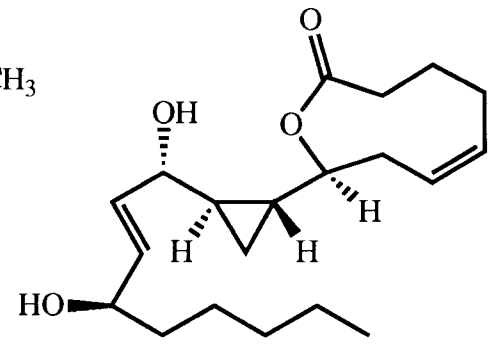
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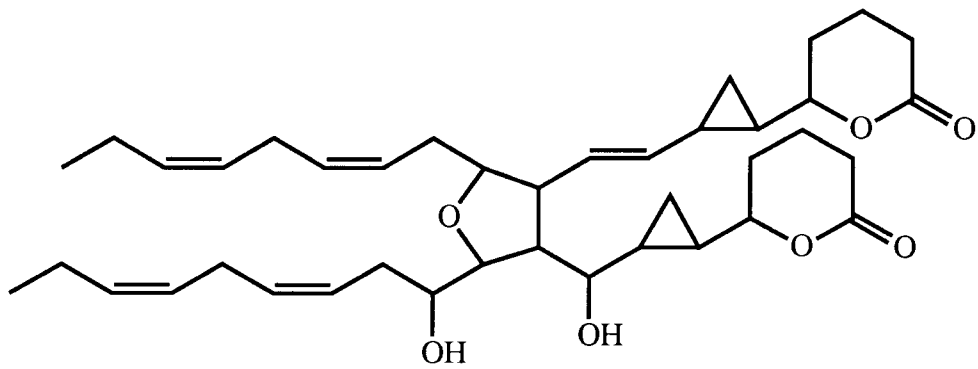
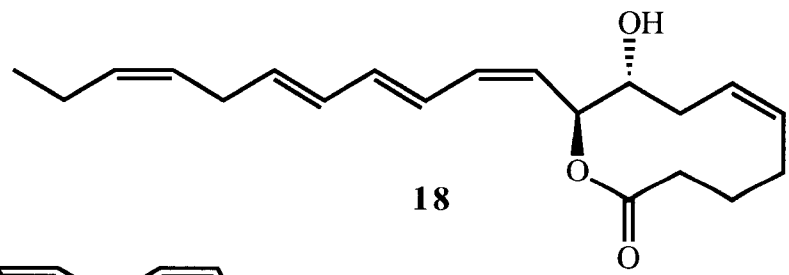
HO

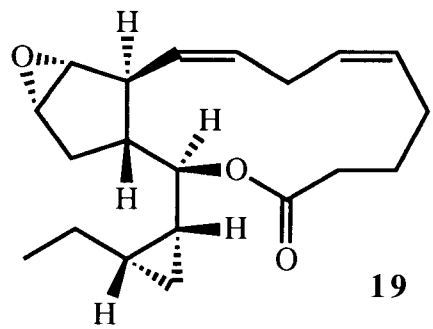
**16**


O
O
O
O
O
OH
OH

**17**


OH
O
O

**18**

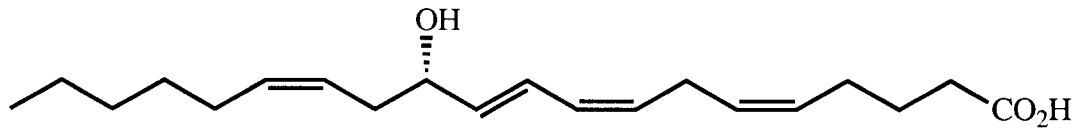

H
O
H
O
H
H
O
H

**19**

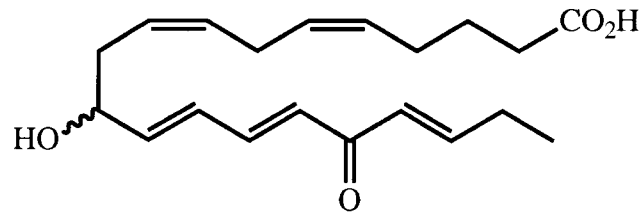
*Murrayella periclados*,[56] *Gracilariopsis lemaneiformis*,[57] and *Lithothamnion corallioides*/*L. calcareum*.[58] Other well-known mammalian eicosanoids, hepoxilin $B_3$, leukotriene $B_4$, 15-HETE, and 5-HETE have all been identified from red algae.[49] In addition to the known metabolites, new oxylipins have also been discovered. Ptilodene (**21**),[59] 12*R*,13*S*-diHETE (**22**),[60] and constanolactone A (**23**)[55] represent some of these novel metabolites. Up to this point in time, products of 5-, 9-, 11-, 12-, and 15-lipoxygenases (based on a $C_{20}$ substrate fatty acid) have been discovered in the red algae.[49]

The green algae also demonstrate a capacity for oxylipin metabolism. Freshwater green algae have shown lipoxygenase activity as determined by experiments with cell-free homogenates from *Chlorella pyrenoidosa*[61] and by the isolation of hydroxy acids from *Dunaliella acidophila*.[62] The marine green alga *Cladophora columbiana* also was shown to produce an oxylipin fragment proposed to arise via hydroperoxide lyase cleavage of an initial hydroperoxide.[49] A more extensive study of green algal metabolism was carried out with the Oregon chlorophyte *Acrosiphonia coalita*. This alga was shown to metabolize $C_{18}$ polyunsaturated fatty acids to hepoxilin-type compounds as well as chain-cleaved metabolites (**24**) and branched chain compounds (**25**).[63]

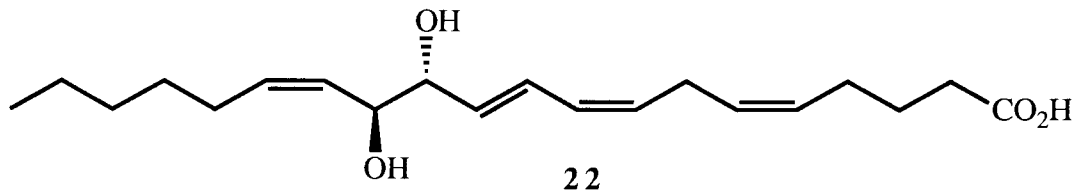
Upon initiating my Ph.D. studies, the brown algae were the least studied of the algae for oxylipin metabolism. The epoxy and tetrahydrofuran lipids (**26**-**28**) of *Notheia anomala*[64,65] and the ecklonialactones (**29**, **30**)[66] were the only examples. The *Notheia* lipids do not appear to derive via lipoxygenases, but rather by cytochrome $P_{450}$-type epoxidation and subsequent modifications. The ecklonialactones have putative origins in lipoxygenase metabolism.[49] A more recent article on further ecklonialactones offered an alternative biogenetic hypothesis involving bis-epoxide precursors.[67] The acylphloroglucinol **31** from the brown alga *Zonaria tournefortii* also is also a likely product of oxylipin metabolism.[68] A 15-lipoxygenase could potentially act on a free fatty acid prior to acylation or on a polyunsaturated acylphloroglucinol directly.



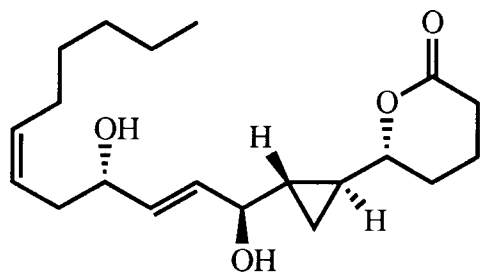

**20**




**21**




**22**

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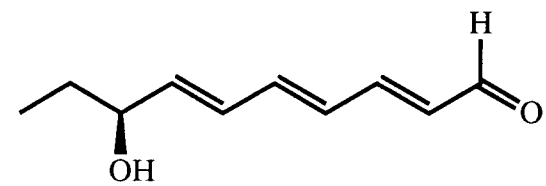


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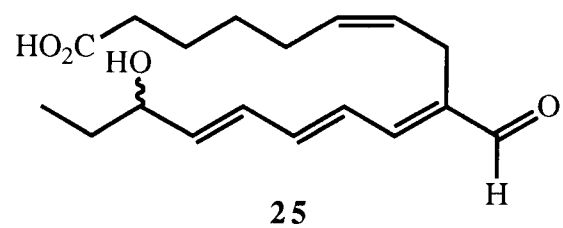


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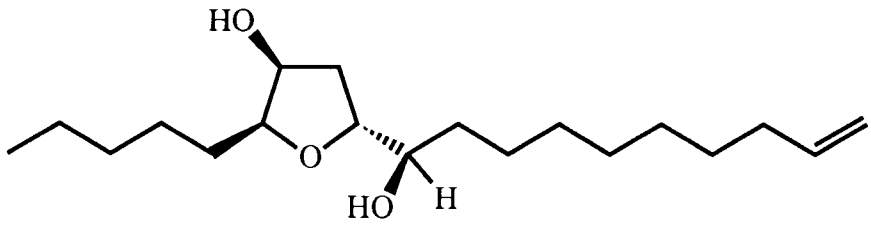
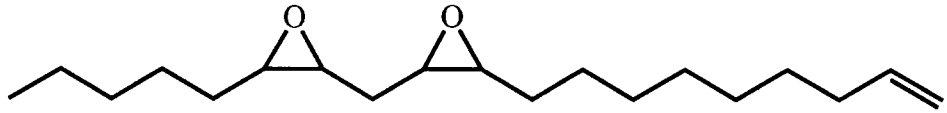



**26**

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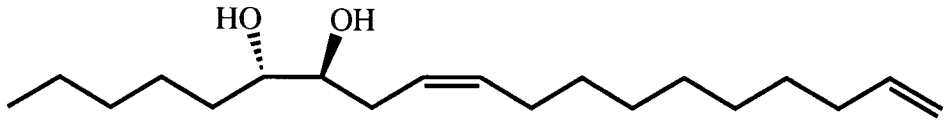


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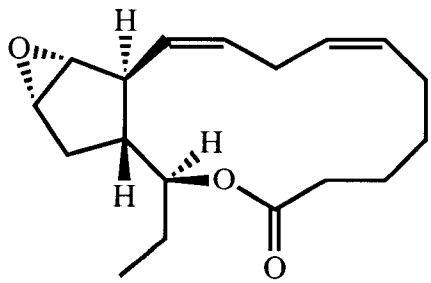
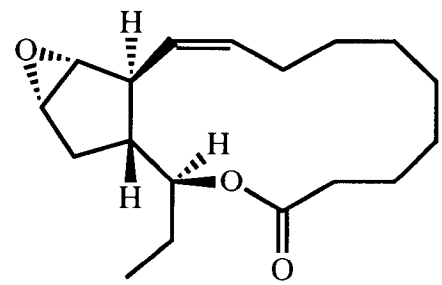
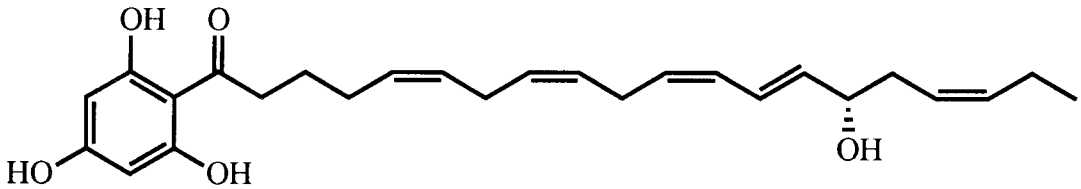



**29**




**30**



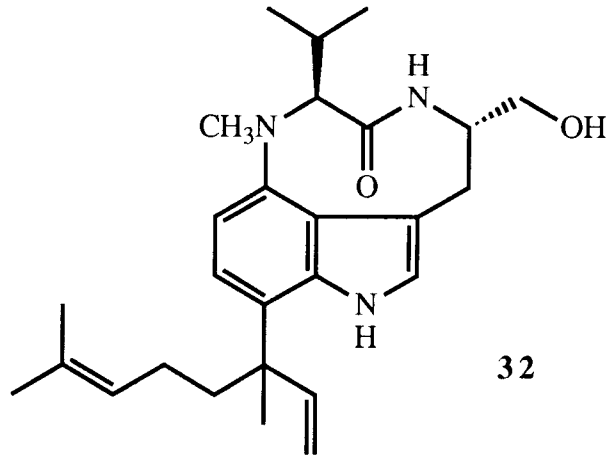

**31**

## Blue-green Algal Natural Products

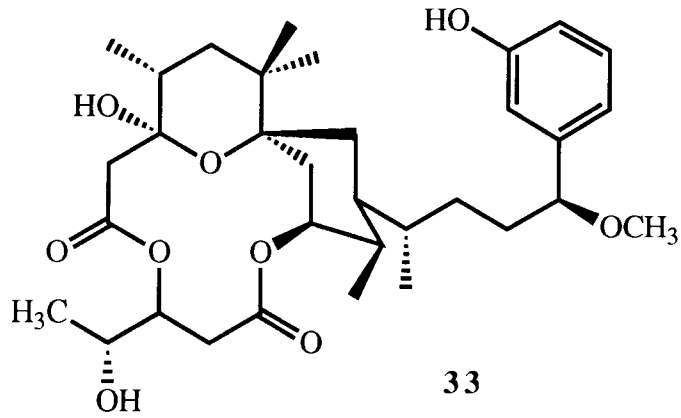
The blue-green algae have been a rich source of unique metabolites.[69,70] Although these photosynthetic organisms are called algae, they are taxonomically more closely related to bacteria. The blue-greens, or cyanobacteria, are prokaryotes, whereas true algae are eukaryotes. Among the novel metabolites of the blue-greens are a number of peptide toxins, polyketides, fatty acids, and indole derived compounds. Carmichael, in a recent review, divides the toxins from blue-green algae into two categories based on the type of bioassay used to identify the compounds.[70] Cytotoxins are compounds active in cultured mammalian cell lines, especially tumor cell lines. Biotoxins are compounds found by using mice or aquatic invertebrates as the assay vector.

The cytotoxins include lyngbyatoxin A (**32**), isolated from a Hawaiian *Lyngbya majuscula*,[71] and debromoaplysiatoxin (**33**), from a separate collection of *L. majuscula*.[72] Although structurally distinct, both are responsible for "swimmer's itch" which occurs occasionally in Hawaii. Nostocyclophane D (**34**) from *Nostoc linckia* and cylindrocyclophane A (**35**) from *Cylindrospermum licheniforme* were the first examples of paracyclophanes in nature.[73] A final example of a cytotoxin is the unique alkaloid, tantazole A (**36**), which was obtained from the extracts of *Scytonema mirabile*.[74]

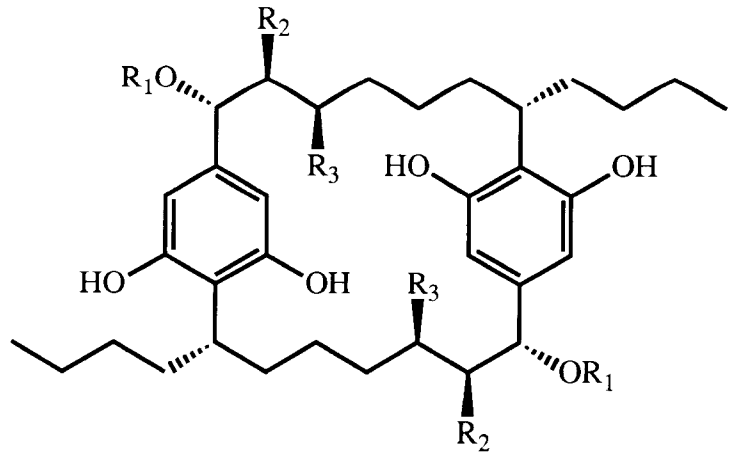
Anatoxin-a (**37**) from *Anabaena flos-aquae* was the first neurotoxin characterized from a cyanobacterium.[75,76] Another neurotoxin, anatoxin-a(s) (**38**), from *Anabaena* spp. was characterized more recently.[77] These biotoxins are responsible for numerous wildlife and livestock poisonings. The (s) in compound **38** stands for salivation, a condition induced in laboratory test animals selectively when **38** is present in samples rather than **37**.[70] Microcystin LR (**39**)[78,79] from *Microcystis aeruginosa* is representative of a number of cyclic peptide toxins that are present in numerous genera of cyanobacteria. These cyclic peptides are usually found to be hepatotoxins.



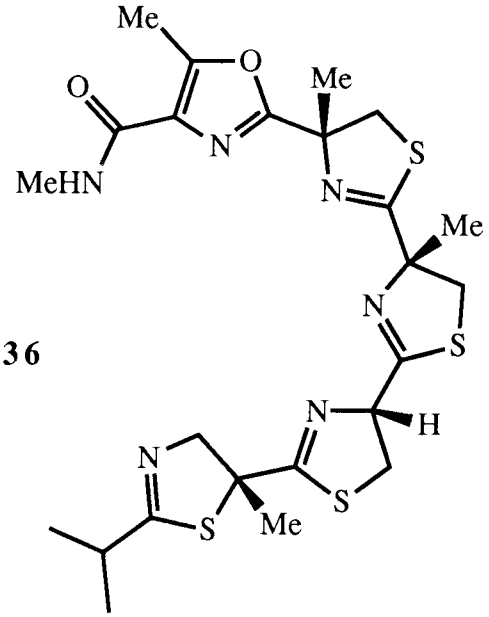

**32**



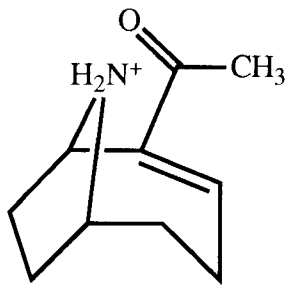

**33**



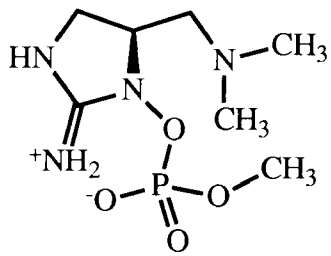

$R_1$ = Me, $R_2$ = H, $R_3$ = Cl **34**
$R_1$ = H, $R_2$ = Me, $R_3$ = H **35**


Me
O
Me
O
N
S
MeHN
N
Me
N
S
N
H
N
S
Me

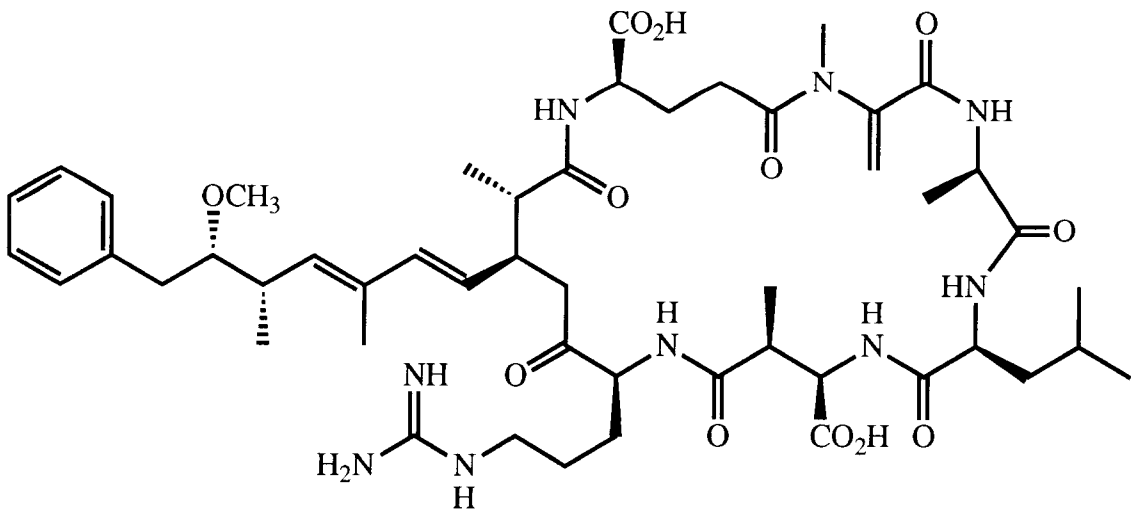
**36**


O
$H_2N^+$
$CH_3$

**37**


HN
N
$CH_3$
N
$CH_3$
O
$^+NH_2$
$CH_3$
$^-O$
P
O
O

**38**


$CO_2H$
HN
N
O
NH
O
$OCH_3$
O
O
HN
H
N
H
N
NH
O
$H_2N$
N
H
O
$CO_2H$
O

**39**

# CHAPTER II

## DIVINYL ETHERS AND HYDROXY FATTY ACIDS FROM THREE SPECIES OF *LAMINARIA* (BROWN ALGAE)


### Abstract

Three species of brown algae, *Laminaria sinclairii*, *L. saccharina*, and *L. setchellii*, have been investigated for the presence of oxylipins. From one, *L. sinclairii*, three new divinyl ether fatty acids have been characterized as methyl ester derivatives (methyl 12-[1'*Z*, 3'*Z*-hexadienyloxy]-6*Z*, 9*Z*, 11*E*-dodecatrienoate, methyl 12-[1'*Z*, 3'*Z*-hexadienyloxy]-9*Z*, 11*E*-dodecadienoate, and methyl 14-[1'*Z*, 3'*Z*-hexadienyloxy]-5*Z*, 8*Z*, 11*Z*, 13*E*-tetradecatetraenoate) by a variety of spectroscopic methods. In addition, one new (13*S*-hydroxy-6*Z*, 9*Z*, 11*E*, 15*Z*-octadecatetraenoic acid) and four known monohydroxy polyunsaturated fatty acids have been isolated from all three species as their methyl ester derivatives. The occurrence of these compounds in brown algae strongly suggests that these organisms possess an active lipoxygenase(s) with ω6 specificity.

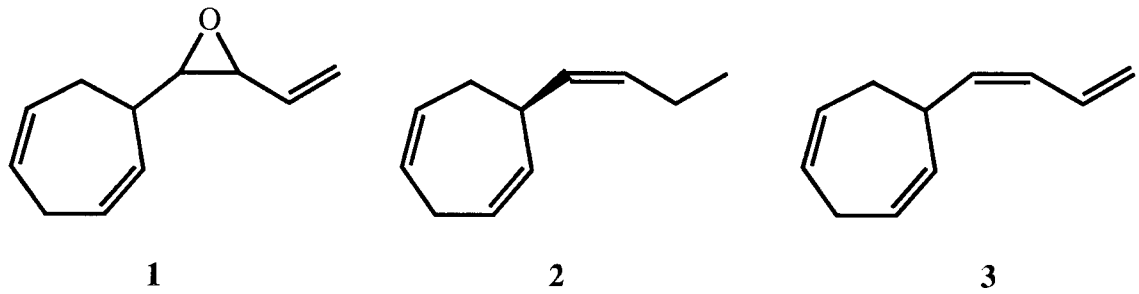
## Introduction

The brown algal genus *Laminaria* has approximately 30 recognized species distributed worldwide in temperate waters.[80] Along the northeast Pacific coast alone, ten species are known.[81] Algae of this genus are of importance both economically and in folk medicine. Several species in Japan are commonly eaten and are called kombu. A harvest of 160,000 tons of kombu in Japan in 1982/83 was worth $600 million.[82] *Laminaria digitata* in France and *L. hyperborea* in the UK and Norway are important sources of alginic acid, a phycocolloid that is used as a thickener and emulsifier in food preparations.[83] Alginic acid also has a variety of uses in the pharmaceutical industry. A polysaccharide from *Laminaria* species called laminarin has anticoagulant properties similar to heparin in a highly sulfated form and antilipemic (lipid lowering) properties in a lower-sulfated form.[84] Laminine dioxalate, an amino acid derivative from *Laminaria*, has purported antihypertensive properties.[84]

Additional studies have examined the hydrocarbons[85] and fatty acids of *Laminaria* species.[86] One study provides evidence that the antimicrobial activity of crude *Laminaria* extracts is due to a high concentration of free fatty acids.[87] Despite extensive study of these algae for nutritional value, alginate composition, and medical utility, the detailed examination of secondary metabolite production of these algae has been almost non-existent. A reason for this may be the commonality of these algae. Many species of *Laminaria* are locally abundant and are generally considered to be sources of polysaccharides or are used as food. Since many studies of secondary metabolites are driven by the search for new biomedicinals, common organisms can often be overlooked, especially ones that are used for human nutrition. The polysaccharide content of these algae may also be a deterrent to further study. If these algae are extracted using a standard protocol that involves maceration of the plant material, extensive emulsions will inevitably form. The practical end of manipulating the emulsions could discourage further

investigations of these organims. In practice, extracting the whole plant provides a satisfactory lipid extract with an acceptably low level of polysaccharides.

One class of secondary metabolites that has been studied in *Laminaria* is pheromones. The female gametes of members of the Laminariaceae secrete a volatile compound called lamoxirene (**1**).[88] This pheromone induces spermatozoid-release and -chemotaxis. In addition to lamoxirene, the $C_{11}$ hydrocarbons ectocarpene (**2**) and desmarestene (**3**) were identified as minor volatile components (Figure II.1). Although these minor hydrocarbons did not have pheromonal activity in algae of the Laminariaceae, they are pheromones in other brown algal families.[88] Recent studies on the biosynthesis of ectocarpene in several brown algae have shown that it derives from eicosapentaenoic acid.[89] These authors speculate that a 9-lipoxygenase may be involved in the biosynthetic pathway.




**1** **2** **3**

Figure II.1. Pheromones in the Brown Algae.

Our initial interest in the *Laminaria* species was guided by the presence of oxylipins in the related alga *Ecklonia stolonifera* from Japan[66] and from TLC observations in our laboratory of crude extracts of *Laminaria sinclairii*. Gray char reactions suggested the possibility of oxlipins. Another reason to study the secondary metabolites of *Laminaria* spp. is to further characterize the constituents of potential food items. Since the

oxylipins have numerous pharmacological activities, it would be important to know if they were present in foods.

Three species of *Laminaria*, *L. sinclairii*, *L. saccharina*, and *L. setchellii*, were examined in this initial foray into brown algal oxylipin chemistry. All three of these algae were found to contain hydroxy fatty acids. In addition, three divinyl ether fatty acids were found in *L. sinclairii*. These metabolites suggest that a lipoxygenase with ω6 specificity is present in these algae.[90] No signs of 9-lipoxygenase products were detected.

## Results and Discussion

*Laminaria sinclairii* was collected on the Oregon coast and immediately frozen with dry ice. Extraction of the defrosted alga with warm $CHCl_3$/MeOH provided a viscous dark brown oil which was fractionated by vacuum silica gel chromatography. Several non-polar fractions showed UV active, brown-charring spots on TLC. These were combined, methylated with $CH_2N_2$, and further fractionated to provide material rich in divinyl ethers and monohydroxy fatty acid methyl esters. The divinyl ethers were finally purified by normal phase HPLC to yield a 4:1 mixture of compounds **5** and **7** (determined by GC integration of hydrogenated **5** and **7**) and pure compound **9**. Attempts to isolate **5** and **7** in pure form were unsuccessful as the compounds degraded with each step of purification.

Since compounds **5** and **7** were not separable without extensive decomposition, data were collected for the mixture. The $^1H$ NMR spectrum showed minor shoulders on several of the olefinic peaks and distortions in upfield patterns indicating that **5** and **7** were closely related. The $^{13}C$ NMR data (Table II.1) and HR EIMS provided a molecular formula of $C_{19}H_{28}O_3$ for the major component. An analysis of the NMR data revealed five olefins and one ester carbonyl. Despite the presence of compound **7**, two isolated spin systems [H(2)-H(12) and H(13)-H(18)] were obtained from the $^1H$-$^1H$ chemical shift correlation spectroscopy (COSY) spectrum (Figure II.2). Since two of the oxygens in the formula are contained in the ester function, the remaining oxygen logically would connect the two spin systems. The resulting divinyl ether structure explains the polarization of the olefinic carbons at the termini of these spin systems (C-12, δ147.91, C-11, δ106.48; C-13, δ141.79, C-14, δ105.70). The magnitudes of the coupling constants for the olefins adjacent to the ether oxygen are also consistent with a divinyl ether structure. Enol ethers show distinctive coupling constants with $J_{trans}$=12.0-12.6 Hz and $J_{cis}$= 6.2-6.7 Hz.[91] Thus, the C(11)-C(12) double bond was *trans* (11.9 Hz) while the C(13)-C(14) olefin

Table II.1. NMR Data for Methyl Ester Derivatives of Oxylipins from *Laminaria sinclairii*[a] [δ (ppm), multiplicity (*J*, Hz)]

| | Methyl Divinyl Ether **5** | | Methyl Divinyl Ether **9** | Methyl 13-HODTA (**14**) | |
|---|---|---|---|---|---|
| C# | $^{13}C$ | $^{1}H$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 174.12 | --- | --- | 174.22 | --- |
| 2 | 33.93 | 2.32, t (7.5) | 2.30, t (7.5) | 33.93 | 2.32, t (7.6) |
| 3 | 24.53 | 1.65, tt (7.8, 7.5) | 1.62, bt (7.3) | 24.52 | 1.65, tt (7.6, 7.6) |
| 4 | 29.03 | 1.40, tt (7.8, 7.4) | 1.4-1.2, m | 28.99 | 1.39, tt (7.6, 7.6) |
| 5 | 26.83 | 2.09, bdt (7.5, 7.4) | 1.4-1.2, m | 26.87 | 2.08, m |
| 6 | 129.83 | 5.38, m | 1.4-1.2, m | 129.99 | 5.38, m |
| 7 | 127.83 | 5.38, m | 1.4-1.2, m | 127.61 | 5.38, m |
| 8 | 26.00 | 2.86, bdd (7.5, 7.2) | 2.10, m | 26.12 | 2.93, bddd (6.2,5.8,1.5) |
| 9 | 127.30 | 5.26, dt (10.5, 7.5) | 5.30, dt (10.8, 7.6) | 130.41 | 5.38, m |
| 10 | 123.32 | 5.86, bdd (11.1, 10.5) | 5.85, bdd (11.3, 10.8) | 127.81 | 5.99, bdd (11.1, 10.9) |
| 11 | 106.48 | 6.07, dd (11.9, 11.1) | 6.05, bdd (11.9, 11.3) | 125.39 | 6.56, ddt (15.2,11.1,1.2) |
| 12 | 147.91 | 6.61, d (11.9) | 6.59, d (11.9) | 135.73 | 5.73, dd (15.2, 6.3) |
| 13 | 141.79 | 6.21, d (6.2) | 6.20, d (6.2) | 72.02 | 4.23, m |
| 14 | 105.70 | 5.51, ddd (11.5,6.2,0.9) | 5.50, ddd (11.5, 6.2, 1.5) | 35.23 | 2.33, m |
| 15 | 119.54 | 6.32, bdd (11.5, 11.3) | 6.32, bdd (11.5, 11.1) | 123.76 | 5.38, m |
| 16 | 133.09 | 5.43, m | 5.42, bdt (11.1, 7.5) | 135.18 | 5.57, dtt (10.2, 7.2, 1.4) |
| 17 | 20.98 | 2.16, dqd (7.5, 7.5, 1.4) | 2.16, dqd (7.5, 7.5, 1.5) | 20.73 | 2.08, m |
| 18 | 14.11 | 1.00, t (7.5) | 1.00, t (7.5) | 14.19 | 0.97, t (7.5) |
| OMe | 51.46 | 3.67, s | 3.67, s | 51.51 | 3.67, s |
| OH | --- | --- | | --- | 1.91, d (3.9) |

a) All spectra recorded on a Bruker AC300 spectrometer ($^{1}H$ spectra referenced to TMS at 0.00 ppm; $^{13}C$ spectra referenced to the center line of $CDCl_3$ at 77.00 ppm). Assignments based on $^{1}H$-$^{1}H$ COSY and $^{1}H$-$^{13}C$ XHCORR spectra and on model compounds.[58]

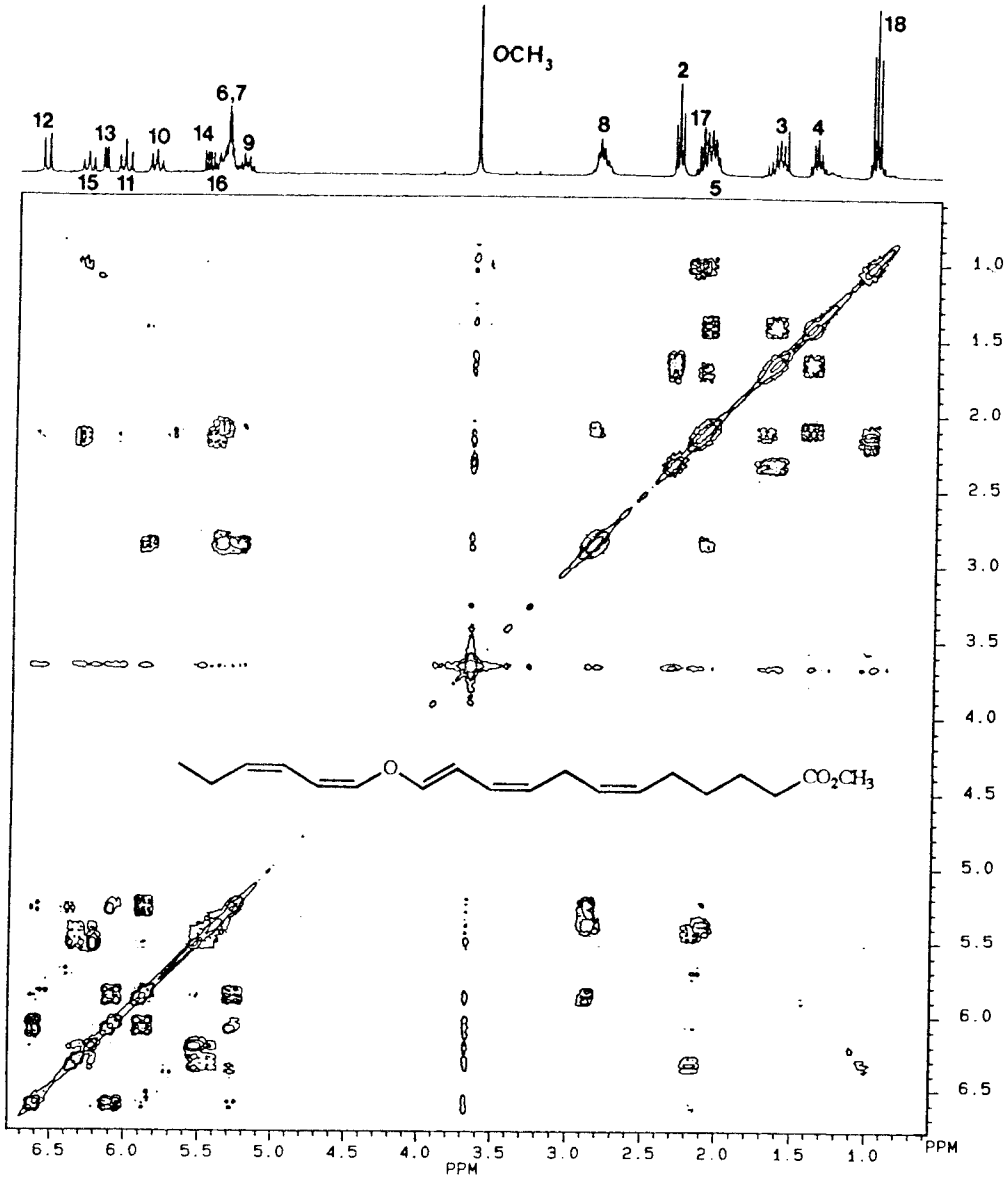


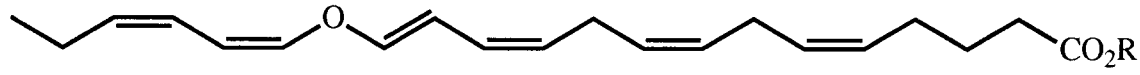

Figure II.2. COSY Spectrum of a Mixture of Methyl Divinyl Ethers **5** (major) and **7** (minor).

was *cis* (6.2 Hz). The C(9)-C(10) (10.5 Hz) and C(15)-C(16) (11.3 Hz) protons showed coupling constants of more typical *cis* olefins. The C(5)-C(6) olefin was also *cis* based on the $^{13}C$ shifts of the adjacent methylene carbons[92] (H-6 and H-7 were a degenerate multiplet). Hence, structure **5** was defined as methyl 12-(1'Z, 3'Z-hexadienyloxy)-6Z, 9Z, 11*E*-dodecatrienoate (Figure II.3).

The $^1H$-$^1H$ COSY spectrum (Figure II.2) of the mixture of **5** and **7** suggested that compound **7** was a $C_{20}$ analog based on an extra correlation from δ2.10 (H-4) to δ1.71 (H-3). The H-3 protons of Δ5 $C_{20}$ polyunsaturated fatty acids and derivatives typically occur at δ1.71. Also, the bisallylic methylene at δ2.86 in **5** appeared to have small, broad, doublets of doublets obscured to both the downfield and upfield sides of the main pattern, indicating the presence of two bisallylic methylenes in compound **7**. Analysis by GC/MS supports this conclusion. Although divinyl ethers **5** and **7** thermally isomerize to olefin isomers under GC/MS conditions, a peak that eluted after the major enol ether **5** showed a peak at *m/z* 330 which corresponds to the $M^+$ for a $C_{20}$ species with six olefins. A fragment peak at *m/z* 233 indicated an ether oxygen between C-14 and C-15. The mixture of divinyl ethers **5** and **7** was hydrogenated and the derivatized minor component had a $M^+$ 342 peak which implied six olefins in **7** and a major fragment peak at *m/z* 257, consistent with the loss of a $C_6H_{13}$ unit to the distal side of the oxygen. Since the $^1H$ NMR patterns of **5** and **7** essentially overlap and the above GC/MS evidence supports a $C_{20}$ analog with six double bonds, we propose structure **7**, methyl 14-(1'Z, 3'Z-hexadienyloxy)-5Z, 8Z, 11Z, 13*E*-tetradecatetraenoate, for the minor component. The olefin geometries shown are consistent with the functionality of **5** and the likely $C_{20}$ fatty acid precursor, 5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoic acid.

Compound **9**, the least polar divinyl ether from *L. sinclairii*, contained the same chromophore as in **5** ($\lambda_{max}$ 268 nm). The $^1H$ NMR spectrum of **9** was highly comparable to that of **5**, except for two less olefinic protons and four more protons in the high field region of the spectrum (Table II.1). This suggested that **9** was the C(6)-C(7) dihydro

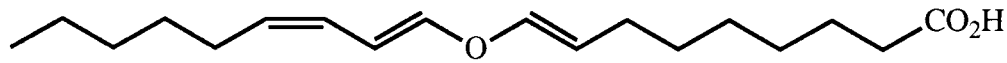

**4** R = H
**5** R = $CH_3$



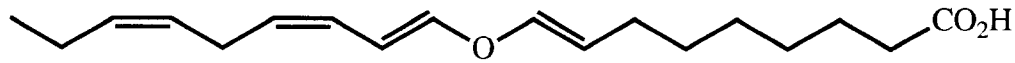

**6** R = H
**7** R = $CH_3$


**8** R = H
**9** R = $CH_3$

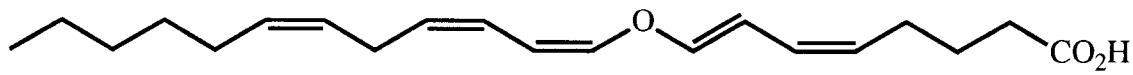
Figure II.3. Divinyl Ether Fatty Acids from *L. sinclairii*.




**10**




**11**




**12**

Figure II.4. Colneleic (**10**), Colnelenic (**11**), and Polyneuric (**12**) Acids.

analog of **5** which was further supported by $^1$H-$^1$H COSY analysis. Confirmation of this structure was obtained from mass spectral analysis. A significant $M^+$ 306 peak was present (49%) and the base peak at *m/z* 81 is consistent with cleavage to the distal side of the ether oxygen. Hydrogenation of compounds **5** and **9** and subsequent GC/MS analysis showed that these two hydrogenated products were identical.

The only previously known examples of divinyl ether fatty acids in nature are colneleic (**10**) and colnelenic (**11**) acids from potato tubers (Figure II.4)[93] and polyneuric acid (**12**) from the marine red alga *Polyneura latissima*.[94] Compounds **4** and **8** differ from colneleic and colnelenic acids in the position of oxygenation and in the double bond stereochemistry about the oxygen linkage. Colneleic and colnelenic acids have a *trans* olefin on either side of the ether oxygen, while compounds **4** and **8** have a *cis* double bond to one side of the oxygen and a *trans* olefin on the other side. The potato tuber fatty acids arise from a pathway initiated by a 9-lipoxygenase acting on linoleic or α-linolenic acid.[95] Assuming an analogous pathway to the new compounds, a 13-lipoxygenase is indicated in the formation of **4** and **8** from $C_{18}$ precursors (Figure II.5)[96] and a 15-lipoxygenase in the formation of **6** from a $C_{20}$ precursor (ω6 in each case). Divinyl ether **4** would derive from stearidonic acid and compound **8** would arise from α-linolenic acid.

The complex mixture of monohydroxy polyunsaturated fatty acid methyl esters from *L. sinclairii* was purified by a combination of normal and reverse phase HPLC to yield five major components: **14**, **16**, **18**, **20**, and **22**. Four of these were known compounds (Figure II.6): methyl 15*S*-hydroxy-5*Z*, 8*Z*, 11*Z*, 13*E*, 17*Z*-eicosapentaenoate (methyl 15*S*-HEPE, **16**), methyl 13*S*-hydroxy-9*Z*, 11*E*, 15*Z*-octadecatrienoate (methyl 13*S*-HOTE, **18**), methyl 15*S*-hydroxy-5*Z*, 8*Z*, 11*Z*, 13*E*-eicosatetraenoate (methyl 15*S*-HETE, **20**), and methyl 13*S*-hydroxy-9*Z*, 11*E*-octadecadienoate (methyl 13*S*-HODE, **22**). Methyl 15*S*-HETE, methyl 15*S*-HEPE, and methyl 13*S*-HOTE were identified by $^1$H NMR, $^{13}$C NMR, $^1$H-$^1$H COSY, optical rotations, and GC/MS analysis of TMS derivatives and comparison to literature data.[58,97-101] Methyl 13*S*-HODE was

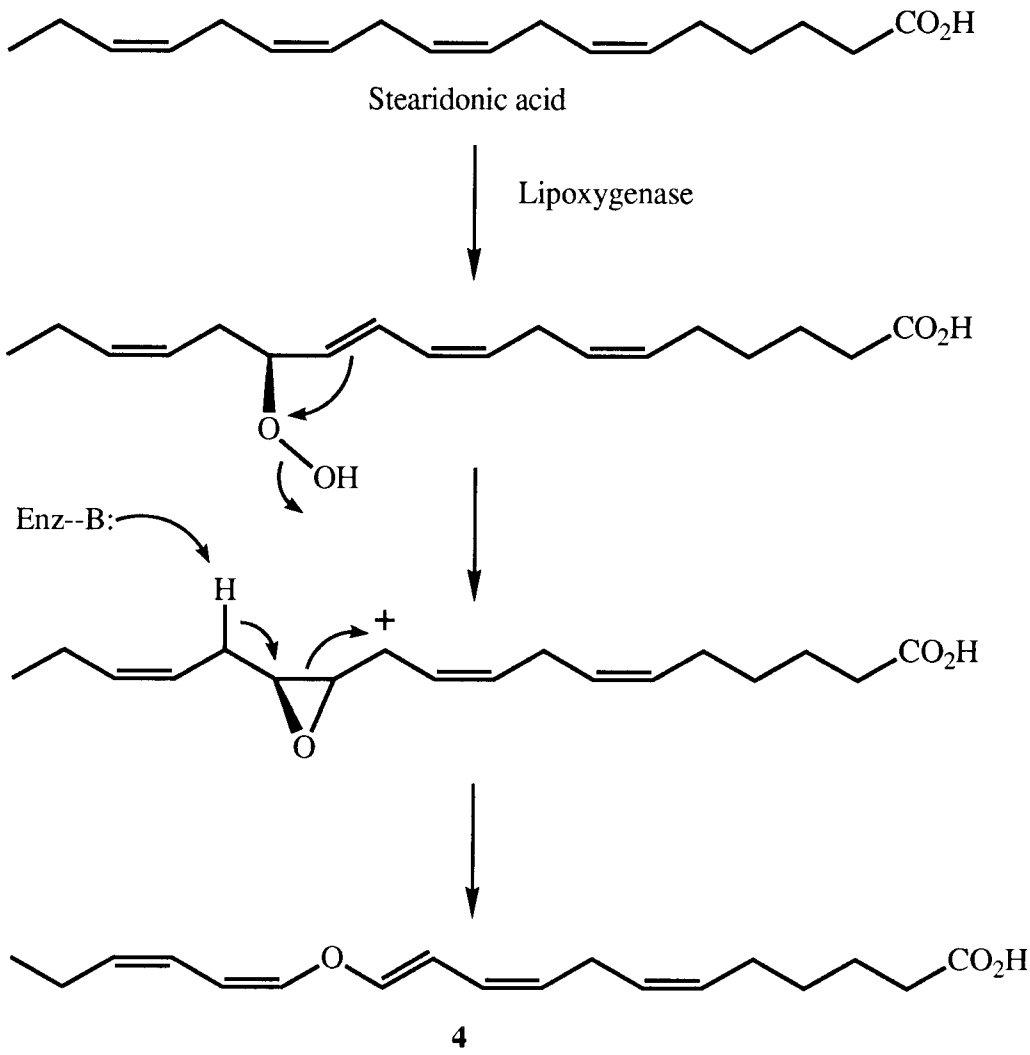



Figure II.5. Proposed Biogenesis of Major Divinyl Ether (**4**) from *L.sinclairii*. (Based on the Biosynthesis of Colneleic Acid.[96])

characterized by $^1$H NMR, optical rotation,[102] and GC/MS analysis of the TMS derivative.[103] The fifth compound, **14**, was closely related to the above hydroxy acid methyl esters by NMR and analyzed for $C_{18}H_{30}O_3$ by HR EIMS. The H(2)-H(6) (**A**), H(7)-H(15) (**B**), and H(16)-H(18) (**C**) spin systems (Figure II.7) could be assembled based on $^1$H-$^1$H COSY data (Figure II.8), but due to overlap in the olefinic and allylic methylene regions, connection of these fragments was not possible. The two possibilities for combining these fragments gave rise to either a 9-hydroxy or a 13-hydroxy compound. Biogenetic arguments would favor the 13-hydroxy compound based on the co-occurrence of methyl 13-HOTE and methyl 13-HODE. Low resolution EIMS supported this by showing a significant fragment at *m/z* 237, corresponding to cleavage distal to C-13. Further proof for oxidation at C-13 in **14** was obtained by separately hydrogenating small amounts of methyl 13-HODE, methyl 13-HOTE, and compound **14** and analyzing the products as TMS ethers by GC/MS. All three samples analyzed for the TMS ether of methyl 13-hydroxystearate.[104] Carbon NMR shifts in **14** (Table II.1) were assigned based on a $^1$H-$^{13}$C XHCORR experiment and model compounds.[58] The positive optical rotation obtained for this sample indicated *S* stereochemistry at position 13, based on the known positive rotations of methyl 13*S*-HOTE[101] and methyl 13*S*-HODE.[102] This stereochemical assignment was verified by analyzing the ozonolysis product of the (-)-menthoxycarbonyl derivative[105] by GC/MS and comparison to known standards.[33] The malate-derived product significantly enriched the peak corresponding to the L-malate derivative when co-injected into the GC/MS with a mixture of D- and L-standards. These data allowed the assignment of the new structure as methyl 13*S*-hydroxy-6*Z*, 9*Z*, 11*E*, 15*Z*-octadecatetraenoate (methyl 13*S*-HODTA, **14**). All five of these hydroxy acid derivatives were also obtained in 0.15-0.4% yield from extracts of *L. setchellii* and *L. saccharina*.[106]

The occurrence of these hydroxy acids **13**, **15**, **17**, **19**, and **21** in several species of *Laminaria* suggests that algae of this genus possess a lipoxygenase with predominant

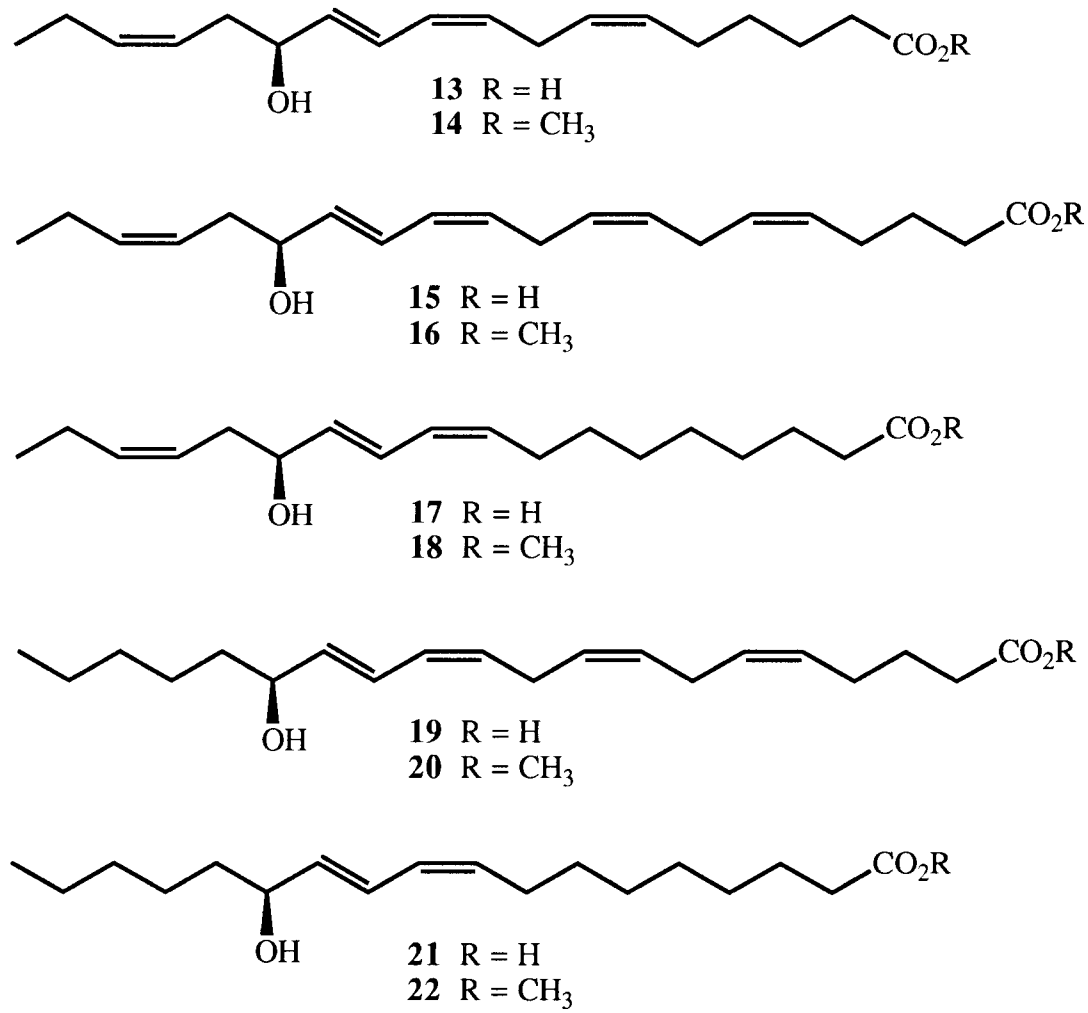



Figure II.6. Hydroxy Fatty Acids from *Laminaria* spp.

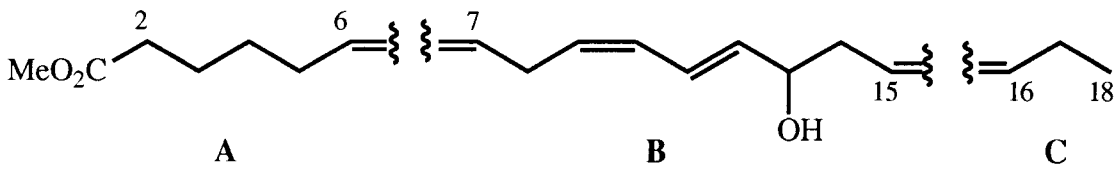



Figure II.7. Partial Structures for Compound **14**.

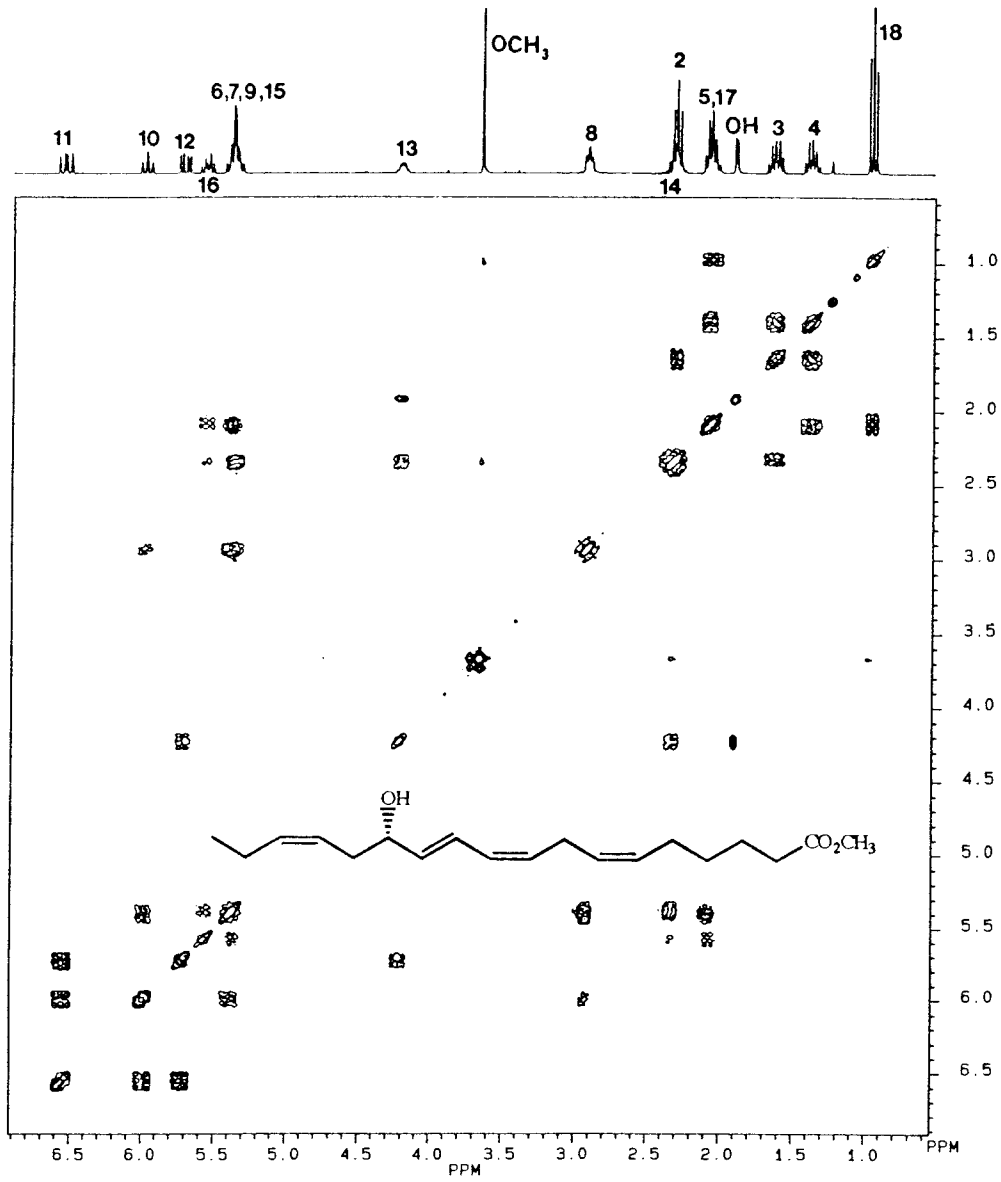



Figure II.8. COSY Spectrum of Methyl 13-HODTA (**14**).

positional specificity for C-13 in $C_{18}$ substrates and C-15 in $C_{20}$ substrates (ω6 in both structure classes). The presence of oxylipins in *Ecklonia stolonifera*,[66,67] *Cymathere triplicata*,[107] and now in several species of *Laminaria* suggests that other brown algae of the Laminariaceae offer a potential source of these novel fatty acid derivatives. The physiological relevance of these compounds in the algae is unknown, but related compounds appear to act as endogenous elicitors of phytoalexin production in rice plants which have been challenged with a fungal infection.[108] The role that the divinyl ethers play is also unknown, although colneleic acid has shown *in vitro* activity as a 5-lipoxygenase inhibitor.[109]

## Experimental

*General.* Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC300 instrument operating at 300.13 MHz for $^{1}H$ NMR and at 75.46 MHz for $^{13}C$ NMR. Proton spectra were referenced to internal tetramethylsilane at 0.00 ppm. Carbon spectra were referenced to $CDCl_3$ at 77.00 ppm. Infrared (IR) spectra were obtained using a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were run on a Hewlett-Packard 8452a spectrophotometer. High-resolution electron-impact mass spectra (HR EIMS) were recorded on a Varian MAT 311 spectrometer. High-performance liquid chromatography (HPLC) utilized a Waters M6000A pump, Rheodyne 7125 injector, and a Waters Lambda-Max 480 LC spectrophotometer. Optical rotations were obtained on a Perkin-Elmer 141 polarimeter. Merck aluminum-backed thin-layer chromatography (TLC) sheets (silica gel 60 $F_{254}$) were used for TLC. Compounds were detected by UV illumination or by heating plates sprayed with a 50% $H_2SO_4$ solution. Gas chromatography/mass spectrometry (GC/MS) was done utilizing a Hewlett-Packard 5890 Series II GC connected to a Hewlett-Packard 5971 mass spectrometer. Trimethylsilyl (TMS) ethers were prepared using TriSil® reagent (Pierce Chemicals). Hydrogenation reactions were carried out with 0.1-0.2 mg samples in ethanol using hydrogen gas and 5% palladium on activated carbon catalyst (Aldrich Chemical Co.).

*Collection, extraction, and isolation. Laminaria sinclairii* (Harvey *ex* Hooker f. *et* Harvey) Farlow, Anderson *et* Eaton was collected from the low intertidal at Strawberry Hill on the Oregon coast in May, 1990. The algal material was immediately frozen with dry ice and stored at -20° C until extraction. Extraction of the defrosted alga (640 g dry wt) with warm $CHCl_3$/MeOH (2:1, vol/vol) provided 7 g of a viscous dark brown oil which was fractionated by vacuum chromatography (7.5 cm x 5 cm, Merck Silica Gel G for TLC) using increasingly polar mixtures of hexanes and ethyl acetate. Fractions eluting with 40-50% EtOAc showed UV-active, brown charring reactions on TLC. These

were combined (497 mg) and methylated with diazomethane. The methylated material was fractionated by flash chromatography (Merck Kieselgel 60, 230-400 mesh) using a stepped gradient from 5% to 20% EtOAc in hexanes. Fractions eluting with 5-10% EtOAc in hexanes were enriched with divinyl ether compounds, while those eluting with 20% EtOAc contained monohydroxy fatty acid methyl esters (236.6 mg). The divinyl ethers were further purified by HPLC (Maxsil Silica 10 μ, 500 mm x 10 mm; 2.5% EtOAc/hexanes) to give ~40 mg of methyl 12-(1'*Z*, 3'*Z*-hexadienyloxy)-6*Z*, 9*Z*, 11*E*-dodecatrienoate (**5**) plus methyl 14-(1'*Z*, 3'*Z*-hexadienyloxy)-5*Z*, 8*Z*, 11*Z*, 13*E*-tetradecatetraenoate (**7**) and 3.4 mg of methyl 12-(1'*Z*, 3'*Z*-hexadienyloxy)-9*Z*, 11*E*-dodecadienoate (**9**). Compounds **5** and **7** were unstable and attempts to separate these compounds led to their degradation. The monohydroxy compounds (50.7 mg portion) were fractionated initially by normal phase HPLC (Maxsil Silica 10 μ, 500 mm x 10 mm, 15% EtOAc/hexanes) and then by reverse phase HPLC (Merck Lichrosorb RP-18 7 μ, 250 mm x 10 mm, 85% MeOH/$H_2O$) to yield: a) a mixture of methyl 13*S*-hydroxy-6*Z*, 9*Z*, 11*E*, 15*Z*-octadecatetraenoate (**14**) and minor related species, b) methyl 15*S*-hydroxy-5*Z*, 8*Z*, 11*Z*, 13*E*, 17*Z*-eicosapentaenoate (**16**), methyl 13*S*-hydroxy-9*Z*, 11*E*, 15*Z*-octadecatrienoate (**18**), and methyl 12-hydroxy-9,13,15-octadecatrienoate (**23**), and c) methyl 15*S*-hydroxy-5*Z*, 8*Z*, 11*Z*, 13*E*-eicosatetraenoate (**20**) plus methyl 13*S*-hydroxy-9*Z*, 11*E*-octadecadienoate (**22**). Final purification of compounds **14** (5.9 mg), **20** (4.7 mg), and **22** (2.4 mg) was accomplished by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 30 cm; 10% EtOAc/hexanes). Separation of compound **16** (6.4 mg) and a 4:1 mixture of **18** and **23** (4.8 mg; ratio determined by integration of GC peaks) was also achieved by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 30 cm, 0.75% isopropanol/hexanes). All pure compounds were colorless oils.

*L. setchellii* Silva was collected at Boiler Bay on the Oregon coast in April, 1991 and *L. saccharina* (L.) Lamouroux was obtained from Puget Sound, Washington in July, 1990. Both of these algae were processed in a manner similar to the above procedure to

obtain compounds **14**, **16**, **18**, **20**, and **22**. Neither of these algae showed any evidence for the divinyl ether fatty acids.

*Methyl 12-(1'Z, 3'Z-Hexadienyloxy)-6Z, 9Z, 11E-dodecatrienoate* (**5**). A colorless oil. FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3013, 2963, 2934, 1739, 1646, 1595, 1434, 1261, 1228, 1163 $cm^{-1}$. UV $\lambda_{max}^{MeOH}$ nm: 268 ($\varepsilon$ = 30,000) For $^{1}H$ and $^{13}C$ NMR data, see Table II.1. Low resolution electron-impact mass spectrum (LR EIMS) *m/z* (rel. intensity) 304 ($M^+$, 12), 275 (4), 175 (10), 105 (38), 91 (82), 79 (100). HR EIMS *m/z* obs. $M^+$ 304.204 ($C_{19}H_{28}O_3$, 0 mmu dev.). LR EIMS *m/z* (rel. intensity) of hydrogenated **5**: 283 ($M^+$-$OCH_3$, 3), 229 (47), 214 (36), 197 (100), 181 (20), 171 (27), 163 (24), 143 (60), 129 (22), 97 (46), 87 (87), 74 (85), 69 (51), 55 (63).

*Methyl 14-(1'Z, 3'Z-Hexadienyloxy)-5Z, 8Z, 11Z, 13E-tetradecatetraenoate* (**7**). LR EIMS *m/z* (rel. intensity) of **7** or a geometrical isomer: 330 ($M^+$, 1), 250 (10), 233 (8), 201 (20), 183 (33), 159 (16), 150 (23), 131 (27), 121 (100), 117 (46), 105 (40), 91 (85), 79 (77), 67 (51). LR EIMS *m/z* (rel. intensity) of hydrogenated **7**: 342 ($M^+$, 0.5), 311 ($M^+$-$OCH_3$, 2), 257 (29), 242 (31), 225 (42), 199 (23), 143 (44), 111 (25), 97 (44), 87 (83), 74 (100), 69 (59), 55 (62).

*Methyl 12-(1'Z, 3'Z-Hexadienyloxy)-9Z, 11E-dodecadienoate* (**9**). A colorless oil. FTIR (neat) 2929, 2855, 1740, 1647, 1595, 1434, 1250, 1228, 1164 $cm^{-1}$. UV $\lambda_{max}^{EtOH}$ nm: 268 ($\varepsilon$ = 30,000) For $^{1}H$ NMR data, see Table II.1. LR EIMS *m/z* (rel. intensity) 306 ($M^+$, 49), 275 (5), 245 (6), 149 (35), 131 (40), 107 (34), 96 (48), 81 (100), 67 (86), 55 (96). HR EIMS *m/z* obs. $M^+$ 306.220 ($C_{19}H_{30}O_3$, +1 mmu dev.).

*Methyl 13S-Hydroxy-6Z, 9Z, 11E, 15Z-octadecatetraenoate* (**14**). A colorless oil. FTIR $\lambda_{max}^{film}$ $cm^{-1}$: 3438 (br. OH), 3011, 2960, 2934, 1740, 1437, 1212, 1202, 1175, 985. $[\alpha]_D^{27}$ +12° (*c* 0.66, acetone), UV $\lambda_{max}^{MeOH}$ nm: 238 ($\varepsilon$ = 27,000). For $^{1}H$ and $^{13}C$ NMR data, see Table II.1. LR EIMS *m/z* (rel. intensity) 306 ($M^+$, 0.5), 288 ($M^+$ - $H_2O$, 7), 275 (2), 237 (35), 219 (22), 205 (41), 187 (40), 159 (50), 107 (78), 81 (100), 69

(57). HR EIMS *m/z* obs. $M^+$ 306.219 ($C_{19}H_{30}O_3$, 0 mmu dev.). LR EIMS *m/z* (rel. intensity) of TMS derivative: 363 ($M^+$ - $CH_3$, 0.7), 347 ($M^+$ - $OCH_3$, 0.7), 309 (100), 243 (10), 219 (6), 187 (10), 145 (19), 129 (16), 103 (21), 91 (24), 73 (89).

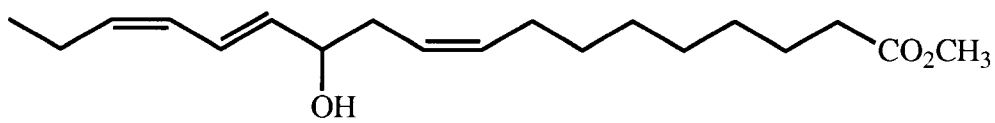
*Methyl 15S-Hydroxy-5Z, 8Z, 11Z, 13E, 17Z-eicosapentaenoate* (**16**). A colorless oil. $[\alpha]_D^{29.5}$ +9.9° (*c* 0.71, acetone). Based on the known positive optical rotation for ethyl 15-*S*-HETE,[58] this positive rotation also indicates*S* stereochemistry at position 15. The predominantly *S* stereochemistry was confirmed by oxidative ozonolysis of the (-)-menthoxycarbonyl derivative[105] and subsequent comparison by GC/MS of the malate derivative with standards prepared from **D**- and **L**-malate.[33] The experimentally obtained malate fragment significantly enriched the peak corresponding to the **L**-malate derivative when co-injected into the GC/MS with a mixture of **D**- and **L**-standards (due to incomplete resolution of the **D**- and **L**-malate-derived standards under our GC/MS conditions, quantitative determination of the *R* and *S* enantiomers was not possible). $^1H$ NMR (300 MHz, $CDCl_3$): δ6.56 (1H, *dddd*, *J*=15.2, 11.1, 1.1, 1.1 Hz, H-13), 6.00 (1H, *dd*, *J*=11.1, 10.8 Hz, H-12), 5.73 (1H, *dd*, *J*=15.2, 6.3 Hz, H-14), 5.58 (1H, *bdt*, *J*=10.8, 7.2 Hz, H-18), 5.39 (6H, *m*, H-5, H-6, H-8, H-9, H-11, H-17), 4.23 (1H, *m*, H-15), 3.67 (3H, *s*, $OCH_3$), 2.96 (2H, *bdd*, *J*=6.3, 6.0 Hz, H-10), 2.81 (2H, *bdd*, *J*=5.4, 5.3 Hz, H-7), 2.34 (2H, *m*, H-16), 2.33 (2H, *t*, *J*=7.4 Hz, H-2), 2.10 (2H, *m*, H-4), 2.09 (2H, *m*, H-19), 1.84 (1H, *d*, *J*=4.0 Hz, OH), 1.71 (2H, *tt*, *J*=7.4, 7.4 Hz, H-3), 0.97 (3H, *t*, *J*=7.5 Hz, H-20). $^{13}C$ NMR (75 MHz, $CDCl_3$; assignments based on model compounds (compound **14** and ref. 58): δ174.14 (C-1), 135.77 (C-14), 135.27 (C-18), 130.21 (C-11), 128.74 (C-5), 128.98 (C-6), 128.59 (C-8), 127.97 (C-9), 127.56 (C-12), 125.34 (C-13), 123.70 (C-17), 71.98 (C-15), 51.53 ($OCH_3$), 35.26 (C-16), 33.40 (C-2), 26.52 (C-4), 26.10 (C-10), 25.62 (C-7), 24.72 (C-3), 20.73 (C-19), 14.20 (C-20).

*Methyl 13S-Hydroxy-9Z, 11E, 15Z-octadecatrienoate* (**18**). A colorless oil. $[\alpha]_D^{29}$ +5.1° (*c* 0.44, acetone) for a 4:1 mixture (by GC integration) of **18** and **23**.

*Methyl 15S-Hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoate* (**20**). A colorless oil. $[\alpha]_D^{32}$ +9.0° (*c* 0.52, acetone), $[\alpha]_D^{32}$ +4.4° (*c* 0.52, hexanes), $[\alpha]_D^{32}$ +8.8° (*c* 0.52, EtOH). $^1H$ and $^{13}C$ NMR data correlated well with previously reported values.[58]

*Methyl 13S-Hydroxy-9Z, 11E-octadecadienoate* (**22**). A colorless oil. $[\alpha]_D^{27}$ +8.9° (*c* 0.27, acetone).

*Methyl 12-Hydroxy-9,13,15-octadecatrienoate* (**23**) (This compound was previously characterized from a green alga.[62]) LR EIMS *m/z* (rel. intensity) 308 ($M^+$, 0.15), 290 ($M^+ - H_2O$, 3), 227 (2), 195 (3), 133 (3), 111 (100), 93 (12), 81 (11), 67 (12), 55 (14). A $C_{18}$ hydroxy acid methyl ester with three double bonds was indicated by the ion at *m/z* 308 ($M^+$). The base peak at *m/z* 111 suggested placement of the hydroxyl at C-12 with a diene unit oriented toward the methyl terminus. Assuming an α-linolenic acid precursor to this compound, the third olefin was positioned at C-9. The LR EIMS spectrum of the TMS-derivative of the hydrogenated species matched well with an authentic spectrum of methyl 12-(trimethylsilyl)oxyoctadecanoate (91% match with spectrum #45118 of the National Bureau of Standards mass spectral library #NBS54k included with the Hewlett-Packard 5971 MS operating software), confirming the placement of the hydroxyl at position 12. The stereochemistries of the olefins for compound **23** are proposed based on biogenetic arguments.




**23**

# CHAPTER III

## NOVEL OXYLIPINS FROM THE KELP *CYMATHERE TRIPLICATA*


### Abstract

Our investigation of the oxylipin chemistry of the temperate brown alga *Cymathere triplicata* has revealed several novel cyclopentane containing metabolites, and two new compounds that contain a 2-oxanorbornane skeleton. These compounds represent structural skeletons that have never been encountered before in natural products. One of the cyclopentane containing compounds is a macrocyclic lactone similar to the red algal metabolite, hybridalactone, but differing by having a cyclopropyl ring incorporated within the macrolactone, rather than external to it. In addition, several hydroxy acids (12*S*-HETE, 13*S*-HOTE, and 10*S*-HODTA) and hydroperoxide cleavage products have been identified. Two of the cleavage products, 2*E*-nonenal and 2*E*, 6*Z*-nonadienal, give the fresh alga its odor of cucumbers. All of the oxylipin metabolites in this alga are proposed to arise via a lipoxygenase-initiated event.

## Introduction

The success in finding oxylipins in the *Laminaria* spp. led us to investigate the natural products chemistry of an edible kelp from Washington, *Cymathere triplicata* (Postels et Ruprecht) J. Agardh, another member of the family Laminariaceae. This alga was collected during a general survey project looking for biomedicinals from marine algae of the Pacific Northwest. *C. triplicata* can be found from Puget Sound to the Bering Sea.[110] Several studies have been conducted to explore the potential use of this alga as a source of edible kelp products.[111,112]

A literature search indicated that this alga had been studied in the late 1940's by scientists from the colleges of pharmacy at the University of Washington and at Oregon State University. They found that antimicrobial components were present in extracts of this alga, but none of the components were characterized.[113] A general report of the hydrocarbon composition[85] and an analysis of the arsenic content[114] of *Cymathere* have also appeared.

Another report implies that *C. triplicata* produces the $C_{11}$ epoxide, lamoxirene, which is an important pheromone involved in sexual reproduction of brown algae of the order Laminariales.[88] It has recently been shown in several brown algae that $C_{11}$ hydrocarbon pheromones related to lamoxirene arise from $C_{20}$ fatty acids.[89] The authors suggested that a 9-lipoxygenase may initiate the pathway that leads to these pheromones.

An initial vacuum chromatography of the crude extract of *C. triplicata* filled the laboratory with the smell of cucumbers. At the time, the significance of the odor was not fully appreciated. After isolation of several monohydroxy fatty acids as their methyl esters, and researching the types of compounds responsible for the odor of cucumbers, the importance of these aldehydes as indicators of oxylipin metabolism was recognized. The odor of cucumber is due to 2*E*-nonenal and 2*E*, 6*Z*-nonadienal, both produced in cucumbers by hydroperoxide lyase action on 9-hydroperoxides of $C_{18}$ fatty acids.[31]

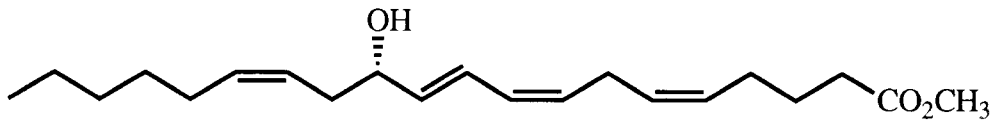
Upon re-collection of this alga, we realized that the odor was not just a laboratory phenomenon, but was readily detectable in the field. On one occasion, several schoolchildren were able to successfully identify the odor of cucumbers when they smelled a fresh blade of the alga. This odor can be quite strong under certain conditions. At one collecting site in Washington which we had not visited previously, we were even able to predict that *Cymathere* was present by smelling "cucumbers" when we exited the car, before even seeing the beach! Another field observation was that young plants seem to have a stronger odor than older plants.

The presence of these "simple" oxylipins was just a prelude to the isolation of the most complex set of oxylipins found in the algae to date. Several compounds were found that contain a cyclopropane, a cyclopentane, and an epoxide. One of these was a macrolactone similar to the red algal metabolite hybridalactone.[52] Two further compounds were isolated that contain a novel 2-oxanorbornane ring system, a previously unreported structural element. Also isolated were two cyclopentyl epoxides that are formally the open lactone forms of ecklonialactones A and B. In addition to these more elaborate oxylipins, hepoxilins $B_3$ and a $C_{11}$ aldehyde acid were also characterized.

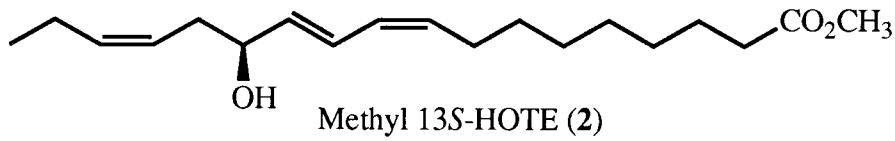
The presence of several of these oxylipins in *Cymathere* indicates that a lipoxygenase with predominant ω9 specificity is present. The opened ecklonialactone compounds and the 13*S*-HOTE suggest, however, that some metabolism is initiated by an ω6 lipoxygenase. Whether this occurs via the same lipoxygenase or a separate lipoxygenase is unknown. The major compounds isolated likely derive from stearidonic acid. The remaining cyclopentyl metabolites are proposed to arise from α-linolenic acid or eicosapentaenoic acid. All three of these putative fatty acid precursors have an ω3 double bond which seems to be a prerequisite for elaboration into the cyclopentyl metabolites. Arachidonic acid, which lacks this double bond, is metabolized by the lipoxygenase, as indicated by the isolation of 12*S*-HETE and 2*E*-nonenal, but does not appear to be converted into more complex structures.

## Results and Discussion

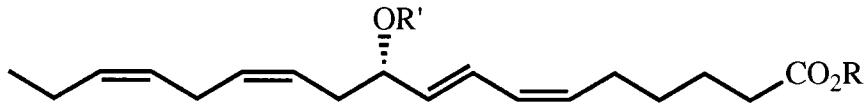
The first collection of *Cymathere triplicata* was made at Deception Pass in the state of Washington in 1990. Monohydroxy fatty acids were indicated by TLC analysis of the crude extract. After methylation and isolation, methyl 12*S*-HETE (**1**) and methyl 13*S*-HOTE (**2**) were identified from this initial collection of the alga. These compounds were characterized by $^{1}H$ NMR, $^{13}C$ NMR, GC/MS of TMS ethers, and optical rotation. Comparison of the data with that available in the literature secured the structures.[33,90,115] A third hydroxy acid (**3**), as its methyl ester (**4**), was identified from a second collection of the alga. Characterization of compound **4** followed from $^{1}H$ NMR, $^{13}C$ NMR, DEPT, $^{1}H$-$^{1}H$ COSY, XHCORR, UV, IR, and GC/MS analysis of the TMS ether. A $C_{18}$ hydroxy diene with two additional olefins was indicated. Analysis of the mass spectral fragmentation pattern of the TMS ether (*m/z* 269) and information from the COSY spectrum placed the hydroxyl at the 10 position of the chain. The positive optical rotation for the free alcohol **4** suggested an *S* configuration based on analogy to 12*S*-HETE and 13*S*-HOTE. The exciton chirality method was applied to a bromobenzoate derivative to determine the absolute stereochemistry. The 4-bromobenzoate derivative (**5**) was prepared using 4-bromobenzoyl chloride and the CD spectrum was recorded in EtOH. It showed a split Cotton curve with a positive first Cotton effect. This indicated that the alcohol at C-10 had the *S* configuration.[116,117] Compound **4** was defined as methyl 10*S*-hydroxyoctadeca-6*Z*, 8*E*, 12*Z*, 15*Z*-tetraenoate. As the acetate (**6**) of this compound was previously reported from the red alga *Gracilariopsis lemaneiformis*,[57] the acetate was prepared ($Ac_2O$, pyridine) from **4** and the $^{1}H$ NMR spectrum and mass spectral fragmentation patterns were compared, verifying that the structures were the same. The similarity of optical rotation values indicates that the product isolated from the red alga also has the 10*S* stereochemistry.



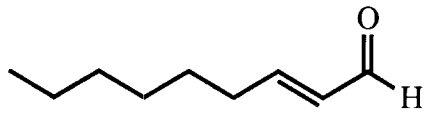

Methyl 12*S*-HETE (**1**)



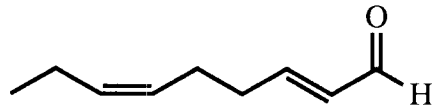

Methyl 13*S*-HOTE (**2**)



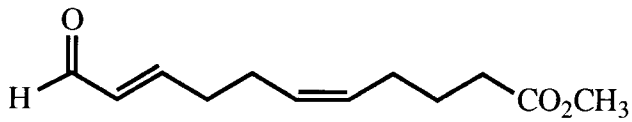

R = R' = H 10*S*-HODTA (**3**)
R = $CH_3$, R' = H Methyl 10*S*-HODTA (**4**)
R = $CH_3$, R' = 4-Bromobenzoyl (**5**)
R = $CH_3$, R' = $COCH_3$ (**6**)




2*E*-Nonenal (**7**)




2*E*, 6*Z*-Nonadienal (**8**)



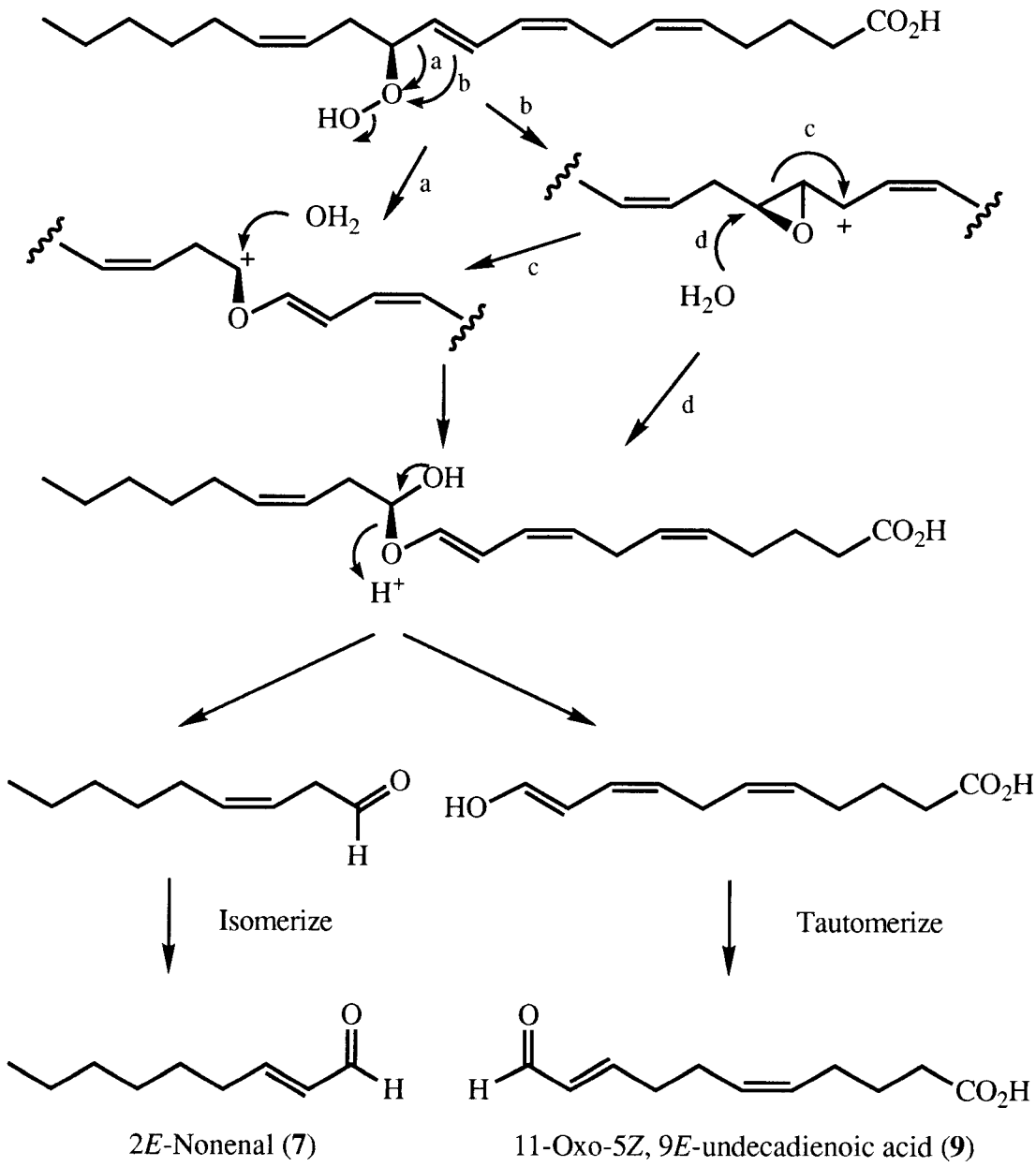

Methyl 11-Oxo-5*Z*, 9*E*-undecadienoate (**10**)

Figure III.1. Hydroxy Acids and Aldehydes from *C. triplicata*

Further evidence suggested that other types of oxylipin metabolism were occurring in this alga. One was the distinctive cucumber-odor associated with a non-polar vacuum chromatography fraction. Characterization of these *Cymathere* compounds using NMR and GC/MS supported their identities as 2*E*-nonenal (**7**) and 2*E*, 6*Z*-nonadienal (**8**), the same compounds responsible for the odor of cucumbers. The presence of these aldehydes is consistent with the activity of a fatty acid hydroperoxide lyase in *C. triplicata*. Compounds **7** and **8** have previously been identified as flavor compounds from several members of the Laminariaceae.[118]

If a lyase pathway does exist, the nonenals account for only half of the precursor hydroperoxide.[31] An aldehyde-acid fragment (**9**) would be the other expected cleavage product (Figure III.2). During the isolation of the hydroxy acid methyl esters, $^{1}H$ NMR evidence showed that aldehyde methyl ester compounds were also present in the alga. The major aldehyde compound **10** was isolated by HPLC. The $^{13}C$ NMR spectrum and the HR CIMS spectrum led to a formula of $C_{12}H_{18}O_3$. One of the carbons was the *O*-methyl of the methyl ester, so the chain length was eleven carbons. A conjugated enal function was apparent from the UV spectrum ($\lambda_{max}$ 222 nm), IR spectrum (1692 $cm^{-1}$) and the NMR data. The olefin in conjugation with the terminal aldehyde was *trans* as determined by a 15.6 Hz coupling constant and the other olefin was *cis* based on the $^{13}C$ chemical shifts of the methylenes adjacent to this double bond.[92] A final piece of supporting data was the formation of the methoxime derivatives. A 2.3:1 mixture of *E* and *Z* methoximes was produced and analyzed by GC/MS and $^{1}H$ NMR. The molecular ion at *m/z* 239 was expected based on the proposed structure. The final structure was methyl 11-oxoundeca-5*Z*,9*E*-dienoate (**10**), a previously unreported compound.

The free acid **9** can arise by hydroperoxide lyase cleavage of 12-HPETE or 12-HPEPE. Cleavage in a "heterolytic" mechanism would lead to this eleven carbon acid and a nine carbon aldehyde.[31] The two nonenals described earlier were logically the other cleavage products, after a facile isomerization. A twelve carbon aldehyde acid, 12-




Scheme III.2. Possible Mechanisms for Hydroperoxide Lyase.[31]

oxododeca-5*Z*, 8*E*, 10*E*-trienoic acid, had been described previously from three red algae[55,119,120] and was presumed to be the product of hydroperoxide lyase cleavage of 12-HPETE. If both the 11- and 12-carbon aldehyde acids can arise from the same 12-HPETE precursor, then the lyase enzymes involved must operate by different mechanisms. The red algal lyases apparently cleave the C(12)-C(13) bond of the hydroperoxide precursor. In contrast, the *Cymathere* lyase predominantly cleaves the C(11)-C(12) bond. The putative *Cymathere* lyase may not have complete specificity though, because the $^1$H NMR spectrum of an HPLC fraction containing impure minor aldehyde acid methyl esters suggested that methyl 12-oxododeca-5*Z*, 8*E*, 10*E*-trienoate was also present in small amounts.

During the purification of the nonenals, a compound that charred purple upon acid treatment of the TLC plate was also observed. Examination of this material by $^1$H NMR gave an exciting result. Several patterns upfield of 1 ppm were present, indicating a cyclopropyl group. Olefinic protons, a methyl triplet at $\delta$0.85, and the lack of any other methyl signals suggested that this compound might be a fatty acid metabolite. Due to our familiarity with this class of chemistry, the structure of hybridalactone was originally considered.[52] A comparison of the $^1$H NMR data (Table III.1) with that previously reported for hybridalactone (**11**) indicated similarities for the two, as well as several distinct differences. Examination of $^{13}$C NMR (Table III.1) and high resolution mass spectrometric data provided a formula of $C_{18}H_{26}O_3$. Six degrees of unsaturation were inherent in the molecular formula. An olefin, an ester carbonyl ($\delta$174.23), and the cyclopropyl ring accounted for three of the degrees of unsaturation. Extensive NMR experiments ($^1$H NMR in $CDCl_3$ and $C_6D_6$, $^{13}$C NMR, DEPT 135, $^1$H-$^1$H COSY (Figure III.3), and XHCORR) allowed us to construct the planar structure of this new oxylipin. An epoxide, a cyclopentane, and a lactone accounted for the final three degrees of unsaturation. With the aid of the COSY and XHCORR spectra, two distinct spin systems could be assembled (**A** and **B**; Figure III.4). In fragment **A**, the H-2 protons were

Table III.1. NMR Data for Cymathere Lactone **12** from *C. triplicata.*

| C # | $^{13}C$ | $^{1}H$ ($CDCl_3$) | $^{1}H$($C_6D_6$) |
|---|---|---|---|
| 1 | 174.23 | --- | --- |
| 2 | 32.86 | 2.52, m | 2.34, dd (14.3, 3.5) |
| | | 2.39, ddd (14.3, 5.7, 4.4) | 2.21, ddd (14.3, 5.1, 4.4) |
| 3 | 25.17 | 1.98, m | 2.00, m |
| | | 1.64, m | 1.37, m |
| 4 | 28.35 | 1.52, m | 1.33, m |
| | | 1.20, m | |
| 5 | 25.99 | 2.52, m | 2.41, m |
| | | 1.78, m | 1.60, m |
| 6 | 129.96 | 5.51, ddd (10.6, 9.7, 6.3) | 5.44, dddd (10.7, 9.7, 6.1, 0.6) |
| 7 | 128.49 | 5.13, dd (11.4, 10.6) | 5.02, bdd (11.3, 10.7) |
| 8 | 44.15 | 3.24, d (~11) | 3.31, d (11.3) |
| 9 | 61.37 | 3.26, d (~2.5) | 3.17, d (2.5) |
| 10 | 58.93 | 3.56, bd (2.3) | 3.25, bs |
| 11 | 32.42 | 1.95, d (4.9 (2nd order)) | 1.83, d (14.0) |
| | | | 1.60, m |
| 12 | 49.17 | 0.85, m | 0.63, dd (10.0, 9.8) |
| 13 | 22.46 | 1.17, m | 1.37, m |
| 14 | 8.44 | 0.38, ddd (8.7, 5.3, 5.3) | 0.18, ddd (8.8, 5.3, 5.2) |
| | | 0.23, ddd (9.3, 5.3, 5.3) | 0.02, ddd (9.3, 5.2, 5.2) |
| 15 | 23.55 | 0.87, m | 0.95, m |
| 16 | 75.56 | 5.19, ddd (10.8, 3.2, 3.1) | 5.35, m |
| 17 | 20.60 | 1.20, m | 1.06, m |
| | | 1.07, m | 0.71, m |
| 18 | 10.29 | 0.85, dd (7.3, 7.1) | 0.77, m (2nd order) |

$^{13}C$ NMR data recorded at 75.46 MHz; referenced to $CDCl_3$ at 77.00 ppm. $^{1}H$ NMR data recorded at 300.13 MHz; $CDCl_3$ spectrum referenced to TMS at 0.00 ppm, $C_6D_6$ spectrum referenced to the solvent line at 7.20 ppm. Coupling constants are in hertz.

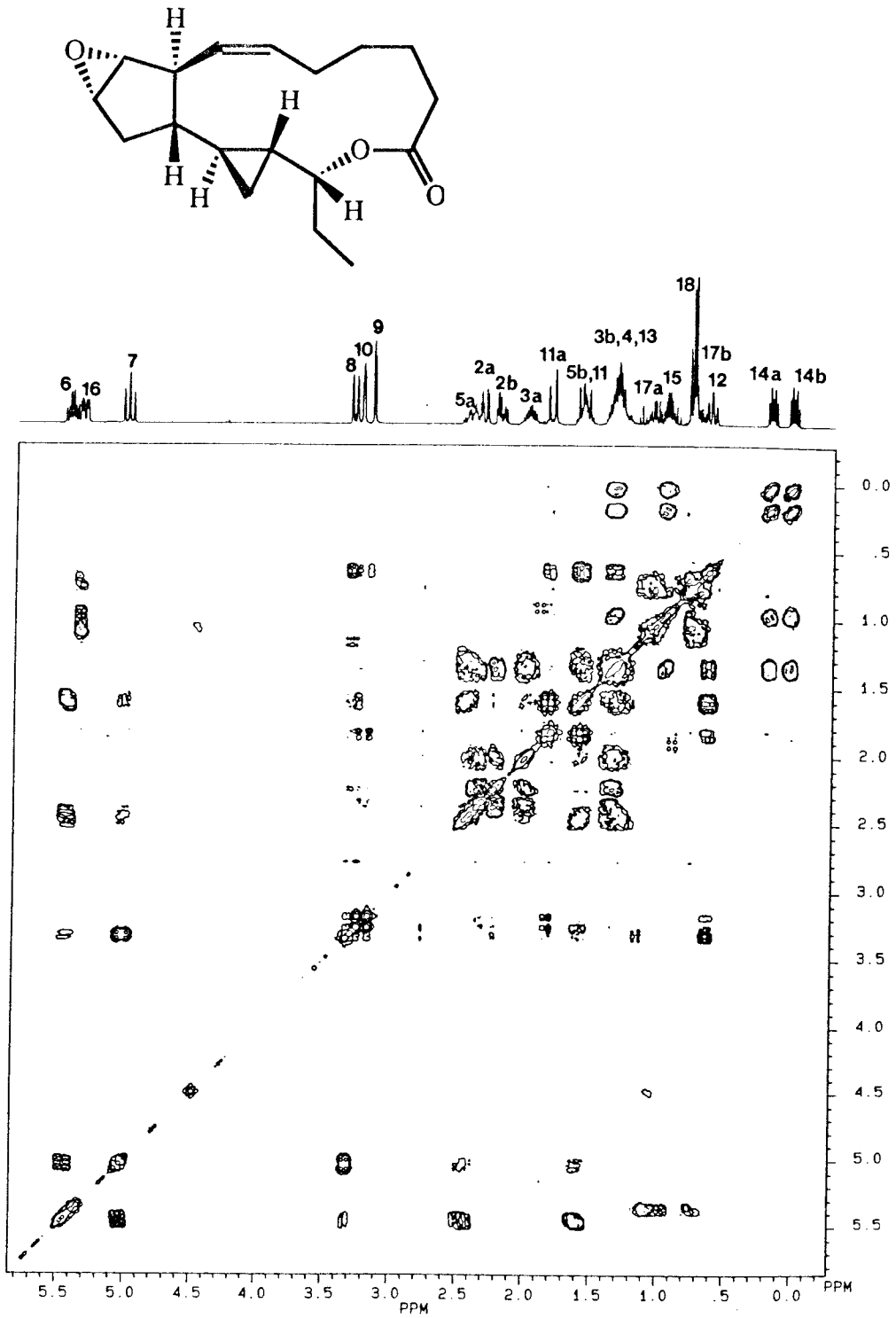
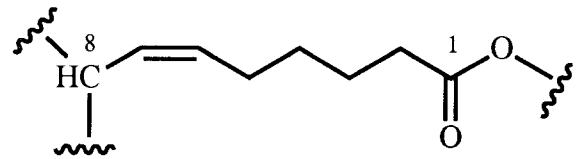
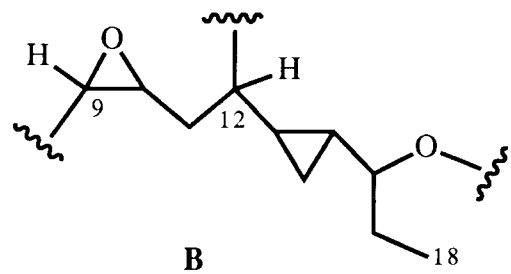



Figure III.3. COSY Spectrum of Cymathere Lactone (**12**).

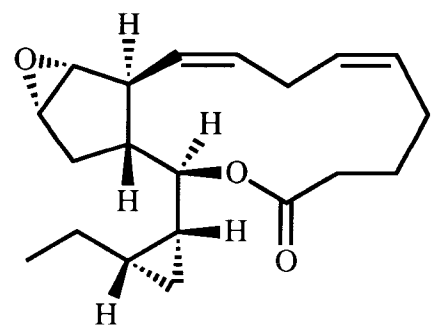



**A**




**B**

Figure III.4 Partial Structures for Cymathere Lactone.




Hybridalactone (**11**)

adjacent to an ester carbonyl based on chemical shifts. The epoxide in fragment **B** was defined by characteristic chemical shifts.[52,66,67,121] The H-8 proton at the terminus of fragment **A** showed weak COSY cross-peaks to both H-9 and H-12. Although the correlations were weak, possibly indicating long range coupling, direct connections from H-8 to H-9 and H-12 were made based on similarities to hybridalactone and the ecklonialactones.[52,66,67] Since all of the atoms required by the molecular formula were present in partial structures **A** and **B**, the final connection had to be from the oxygen at C-16 to the ester carbonyl at C-1, forming a 13-membered ring lactone. This new compound was termed cymathere lactone (**12**). Compound **12** differs from hybridalactone in chain length ($C_{18}$ vs $C_{20}$) and by having a *trans* cyclopropyl group within the macrolactone ring, rather than a *cis* cyclopropyl external to it. Further studies on the relative and absolute stereochemistry of cymathere lactone will be described below.

Several polar vacuum chromatography fractions also showed evidence for cyclopropyl groups by $^1$H NMR. Material in these fractions was methylated to improve their chromatographic properties. Repeated chromatographies using both normal and reverse-phase supports provided three polar cyclopropyl containing acids (**13-15**) as methyl esters (**16-18**). The major compound (**16**) was found to have a molecular formula of $C_{19}H_{30}O_4$. Inspection of the $^1$H NMR spectrum (Table III.2) indicated that one olefin, an epoxide, and a cyclopropyl group were present. A broad doublet at $\delta$3.06, similar to that of cymathere lactone, suggested that a cyclopentane ring was also present. A $^{13}$C NMR shift at $\delta$173.97 (Table III.3) and the IR stretch at 1739 cm$^{-1}$ indicated an ester carbonyl which accounted for the final degree of unsaturation. Analysis of the COSY led to a planar structure that was the formal equivalent of the methyl ester of the hydrolysis product of cymathere lactone. Verification of this structure was obtained by comparison to the base hydrolysis product of cymathere lactone (**12**). Compound **12** was treated with 5% KOH/MeOH and the resulting residue was methylated with diazomethane. After purification, the methylated hydrolysis product was examined by $^1$H NMR and was

Table III.2. $^1H$ NMR Data for Methyl Cymatherols A-C.[a]

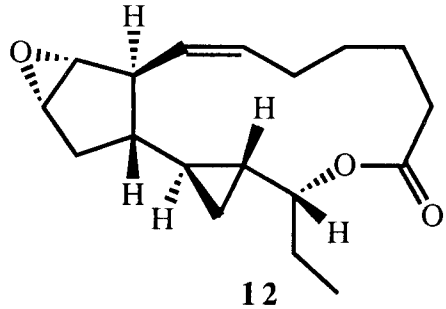
| Carbon #[b] | Compound **16** | Compound **17** | Compound **18** |
|---|---|---|---|
| (C2) | | | 2.34, t (7.5) |
| (C3) | | | 1.73, tt (7.5, 7.5) |
| C2 (C4) | 2.34, t (7.4) | 2.35, t (7.4) | 2.13, m |
| C3 (C5) | 1.67, m | 1.68, m | 5.40, m |
| C4 (C6) | 1.42, tt (8.1, 7.4) | 1.42, tt (7.7, 7.7) | 5.40, m |
| C5 (C7) | 2.16, tdd (7.4, 7.4, 1.5) | 2.18, tdd (7.7, 7.3, 1.5) | 2.87, bm |
| C6 (C8) | 5.40, dtd (10.8, 7.4, 1.0) | 5.40, dtd (10.7, 7.3, 1.0) | 5.37, dtd (10.7, 7.3, 0.9) |
| C7 (C9) | 5.10, ddt (10.8, 10.2, 1.5) | 5.11, ddt (10.7, 10.2, 1.5) | 5.12, ddt (10.7, 10.1, 1.6) |
| C8 (C10) | 3.06, d (10.2) | 3.10, d (10.2) | 3.09, d (10.1) |
| C9 (C11) | 3.25, d (2.6) | 3.25, d (2.6) | 3.26, d (2.6) |
| C10 (C12) | 3.54, d (2.6) | 3.54, bd (2.6) | 3.53, d (2.6) |
| C11 (C13) | 1.95, d (4.1) | 1.95, d (4.3) | 1.95, d (4.3) |
| C12 (C14) | 0.93, m | 0.96, m | 0.96, m |
| C13 (C15) | 0.93, m | 0.92, m | 0.93, m |
| C14a (C16) | 0.44, ddd (8.2, 4.8, 4.6) | 0.36, ddd (8.0, 4.8, 4.7) | 0.45, ddd (8.0, 4.7, 4.6) |
| C14b (C16) | 0.25, ddd (8.1, 4.6, 4.5) | 0.26, ddd (8.6, 4.7, 4.6) | 0.25, ddd (8.2, 4.6, 4.4) |
| C15 (C17) | 0.69, m | 0.65, m | 0.70, m |
| C16 (C18) | 2.79, bddd (8.8, 8.4, 3.7) | 2.81, m | 2.87, bm |
| C17a (C19) | 1.74, m | 1.56, m | 1.73, m |
| C17b (C19) | 1.58, m | | 1.58, m |
| C18 (C20) | 1.02, t (7.4) | 0.96, t (7.5) | 1.01, t (7.4) |
| $OCH_3$ | 3.68, s | 3.68, s | 3.68, s |

a) Spectra were recorded in $CDCl_3$; referenced to TMS at 0.00 ppm. Assignments were made by $^1H$-$^1H$ COSY spectra. Coupling constants are in hertz. Data for **16** and **17** collected at 300.13 MHz. Data for **18** collected at 400.13 MHz.
b) Carbon numbers in parentheses are for the $C_{20}$ compound **18**.

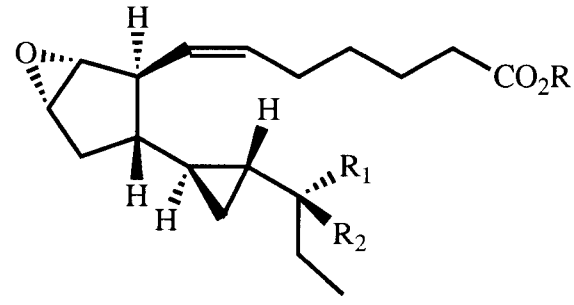
Table III.3. $^{13}C$ NMR Data for Methyl Cymatherols A-C.[a]

| Carbon #[b] | Compound **16**[c] | Compound **17**[d] | Compound **18**[d] |
|---|---|---|---|
| (C1) | | | 173.98 |
| (C2) | | | 33.44 |
| C1 (C3) | 173.97 | 174.16 | 24.74 |
| C2 (C4) | 33.92 | 33.89 | 26.65 |
| C3 (C5) | 24.62 | 24.51 | 129.53 |
| C4 (C6) | 29.08 | 28.99 | 128.27 |
| C5 (C7) | 27.32 | 27.22 | 25.93 |
| C6 (C8) | 130.48 | 130.61 | 129.18 |
| C7 (C9) | 128.83 | 128.88 | 128.83 |
| C8 (C10) | 44.16 | 44.51 | 44.24 |
| C9 (C11) | 61.16 | 61.21 | 61.11 |
| C10 (C12) | 58.08 | 58.21 | 58.09 |
| C11 (C13) | 32.26 | 32.34 | 32.28 |
| C12 (C14) | 48.60 | 48.42 | 48.62 |
| C13 (C15) | 24.94 | 24.78 | 24.89 |
| C14 (C16) | 10.38 | 10.21 | 10.33 |
| C15 (C17) | 26.42 | 26.06 | 26.40 |
| C16 (C18) | 77.30 | 77.11 | 77.20 |
| C17 (C19) | 29.99 | 29.71 | 29.97 |
| C18 (C20) | 10.29 | 10.16 | 10.26 |
| $OCH_3$ | 51.49 | 51.50 | 51.49 |

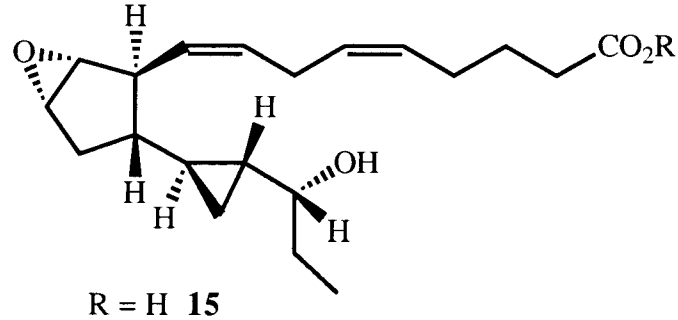
a) All spectra run in $CDCl_3$; referenced to $CDCl_3$ centerline at 77.00 ppm. $^{13}C$ NMR data for compounds **16** and **17** recorded at 75.46 MHz; data for **18** at 100.61 MHz.
b) Carbon numbers in parentheses are for the $C_{20}$ compound **18**.
c) Assignments made by analogy to related compounds.
d) Assignments based on an XHCORR experiment.



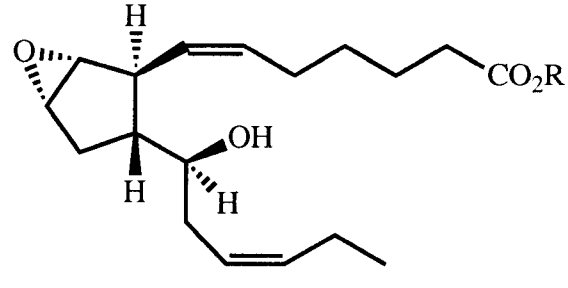
**12**



R = H, $R_1$ = OH, $R_2$ = H **13**
R = H, $R_1$ = H, $R_2$ = OH **14**
R = $CH_3$, $R_1$ = OH, $R_2$ = H **16**
R = $CH_3$, $R_1$ = H, $R_2$ = OH **17**



R = H **15**
R = $CH_3$ **18**



R = H **25**
R = $CH_3$ **28**

Figure III.5. *Cymathere triplicata* Cyclopentane Metabolites

shown to be identical to compound **16**. The optical rotations of the two compounds were almost identical, indicating that the lactone **12** and the opened form **13** are in the same stereochemical series. Compound **16** was trivially named methyl cymatherol A.

The second polar cyclopropyl-containing compound (**17**) had the same molecular formula as **16** ($C_{19}H_{30}O_4$) as determined by HR CIMS. This compound was determined by $^1H$, $^{13}C$, DEPT 135, $^1H$-$^1H$ COSY, and $^1H$-$^{13}C$ XHCORR NMR experiments to have the same planar structure as methyl cymatherol A **16**. The main differences in the NMR data (Tables III.2, III.3) were apparent in the region near the C-16 hydroxyl. A possible explanation for this would be that compound **17** was the C-16 epimer of the opened lactone **16**. Oxidation of these two compounds with pyridinium chlorochromate[122] to the ketone (**19**, Figure III.6) and comparison of the products by $^1H$ NMR, GC/MS, and optical rotation showed that the two oxidation products were identical, confirming that **16** and **17** were C-16 epimers. Compound **17** was named methyl cymatherol B.

The third polar cyclopropyl compound (**18**) was shown to be a $C_{20}$ compound by mass spectral data and $^{13}C$ NMR ($C_{21}H_{34}O_4$). Data from the COSY and XHCORR spectra allowed assembly of a structure that varied from methyl cymatherol A (**16**) only in the length of the alkyl chain leading to the carboxyl group. This side chain was two carbons longer than in **16** with an extra olefin present. Similarities of chemical shifts in the region near the alcohol center (Tables III.2, III.3) suggested that the $C_{20}$ compound had the same relative stereochemistry as compound **16**. Compound **18** was termed methyl cymatherol C.

Cymathere lactone contained seven chiral centers and one olefin. Several elements of stereochemistry were known. The olefin was *cis* based on a 10.7 Hz coupling constant and the cyclopentane epoxide must be *cis* due to steric constraints. The cyclopropyl group was *trans* based on coupling constants. The junction between the cyclopentane and macrolactone was also assumed to be *trans* based on coupling constants and similarities of the $^1H$ NMR spectrum with those of hybridalactone[52] and the ecklonialactones.[66,67] With

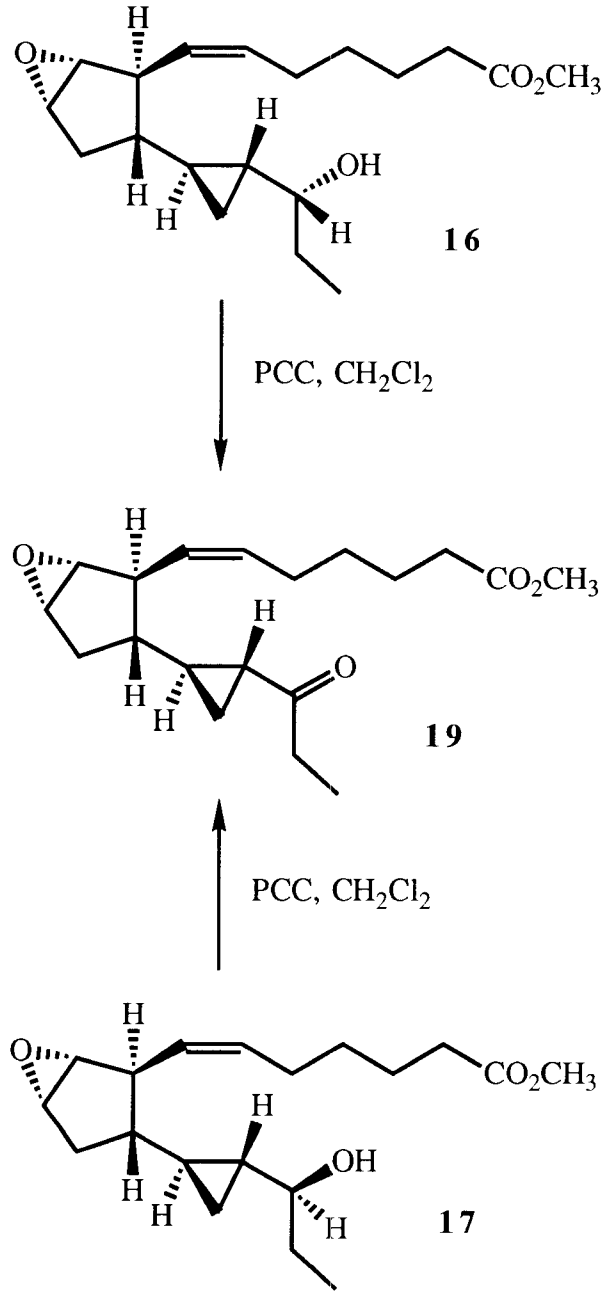
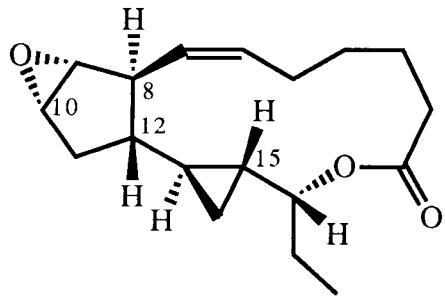


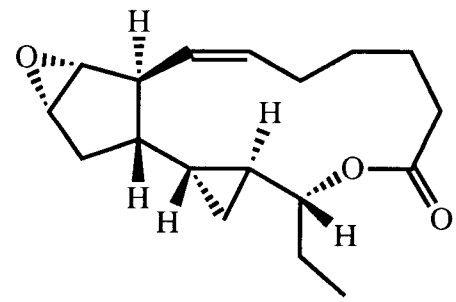

Figure III.6. Oxidation of Methyl Cymatherols A and B to a Common Ketone.

these stereochemical constraints, eight possible structures were constructed (in each enantiomeric series; Figure III.7). Energy minimization of these structures with CHEM 3D Plus™ provided models that were analyzed to see if the geometries obtained correlated to the coupling constant data for cymathere lactone. Although CHEM 3D Plus™ does not employ a global minimum search routine, useful results can be obtained by entering multiple starting conformations for each structure. At least ten starting conformations were used for each. Key coupling constants were H7-H8 11.4 Hz, H8-H12 <1 Hz (dihedral near 90°), H12-H13 ~10 Hz, and H15-H16 ~3 Hz. Five of the possible energy-minimized structures (**D**, **G**, **H**, **I**, and **J**) had H8-H12 dihedral angles >150° which would suggest a coupling constant of at least 8 Hz, ruling these structures out as possibilities. Two of the remaining structures (**E** and **F**) had H15-H16 dihedrals >160°, suggesting very large coupling constants. This was not consistent with the observed 3 Hz coupling. The final structure (**C**, lowest energy conformer) had the following dihedral angles: H8-H12 104° (0.4 Hz), H12-H13 170° (8.9 Hz), H15-H16 53° (2.7 Hz). The coupling constants were calculated using the Karplus-Conroy equation.[123] These angles were consistent with the experimental data. The relative stereochemistry of the epoxide and the cyclopentane side chains predicted by this model is the same as that found in hybridalactone and the ecklonialactones.

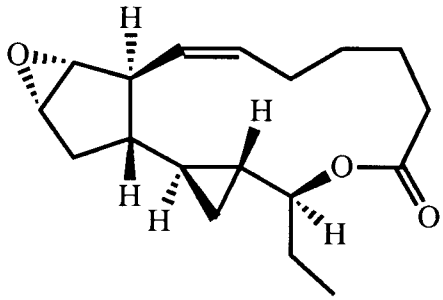
The predictive capabilities of the modeling studies were tested by experiments designed to determine the relative and absolute stereochemistry. A NOESY spectrum of cymathere lactone **12** in deuterobenzene showed correlations that eliminate several of the possible relative stereochemistry models (Figure III.8). Correlations between H-7 and H-9 and H-7 and H-12 indicate that these protons were on the same face of the cyclopentane ring. The H-7 to H-9 correlation indicated that the epoxide and the C1-C7 side chain were *trans* to each other. If the epoxide and the side chain were on the same face of the cyclopentane ring, the H-7 and H-9 protons would be too far apart to see an nOe. Models **G-J** were eliminated by these results.



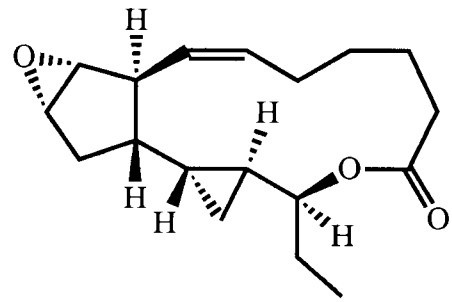

**C** (8*S*, 9*R*, 10*S*, 12*S*, 13*S*, 15*R*, 16*R*)



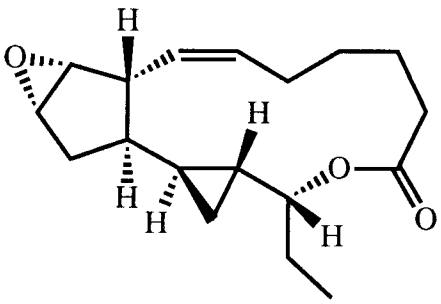

**D** (8*S*, 9*R*, 10*S*, 12*S*, 13*R*, 15*S*, 16*R*)



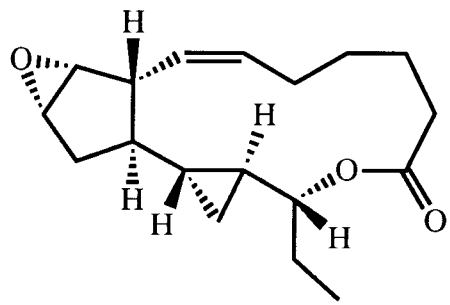

**E** (8*S*, 9*R*, 10*S*, 12*S*, 13*S*, 15*R*, 16*S*)



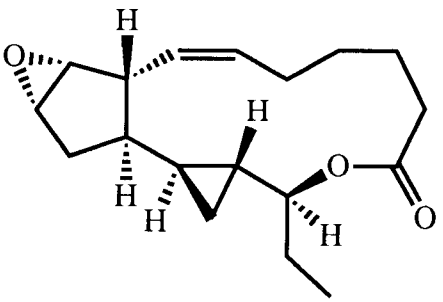

**F** (8*S*, 9*R*, 10*S*, 12*S*, 13*R*, 15*S*, 16*S*)



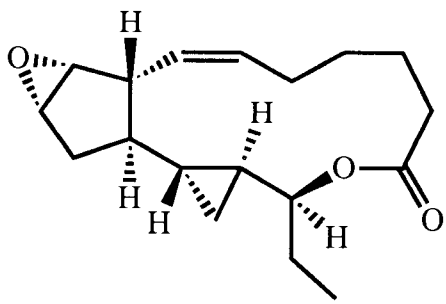

**G** (8*R*, 9*R*, 10*S*, 12*R*, 13*S*, 15*R*, 16*R*)




**H** (8*R*, 9*R*, 10*S*, 12*R*, 13*R*, 15*S*, 16*R*)

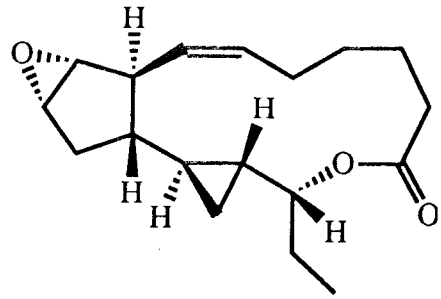



**I** (8*R*, 9*R*, 10*S*, 12*R*, 13*S*, 15*R*, 16*S*)




**J** (8*R*, 9*R*, 10*S*, 12*R*, 13*R*, 15*S*, 16*S*)

Figure III.7. Possible Relative Stereochemistries for Cymathere Lactone **12** (in one enantiomeric series).



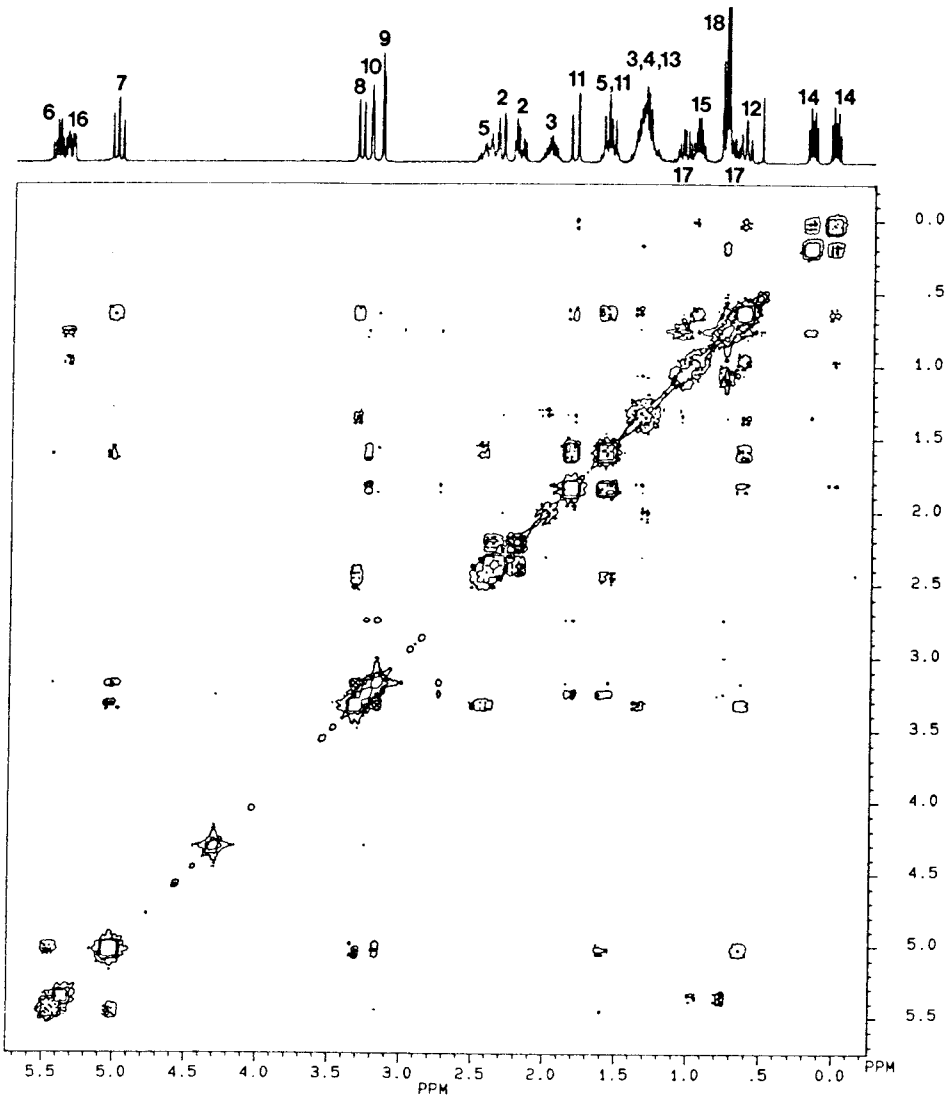
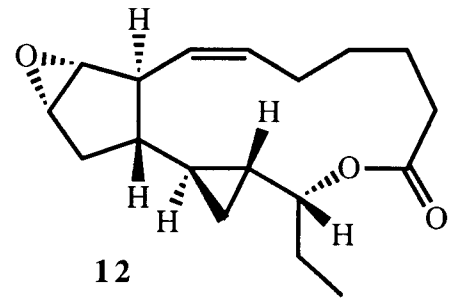




Figure III.8. NOESY Spectrum of Cymathere Lactone (**12**, $C_6D_6$).

Two approaches were used to determine the absolute stereochemistry. One was the exciton chirality method of Nakanishi and Harada.[124] The second was the modified Mosher ester method.[125,126] The 9, 10-dibromobenzoate derivative was envisioned as an appropriate derivative to apply the exciton chirality CD methodology.[127] The epoxide of cymathere lactone was opened to the diol using 1M $HClO_4$ in THF. Analysis of the trimethylsilyl ether derivative of this diol by GC/MS indicated a single major product for the reaction. Unfortunately, this diol product proved to be unstable when concentrated, so it was not fully characterized, but was taken directly on to the dibromobenzoate derivative. The diol was treated with 4-bromobenzoyl chloride to form the di-4-bromobenzoate of cymathere lactone (**20**). A NOESY spectrum of this derivative allowed assignment of the relative stereochemistry around the five-membered ring. A strong correlation was seen between the H-8 and H-10 protons, placing these protons on the same face of the cyclopentyl ring. Both of these protons also had weak correlations to the H-13 cyclopropyl proton. Other key correlations were between H-7 and H-9 and H-7 to H-12 (although H-4b and H-12 are overlapped in the $^1H$ NMR spectrum, the *cis* geometry of the olefin precludes the possibility of an nOe between H-7 and H-4b). Weak, but supporting, correlations were evident between H-11b and H-12 and H-11b and H-9. These three protons must be on the same face of the cyclopentane ring. The NOESY data clearly indicated that *trans* opening of the epoxide occurred at C-10 in the formation of this derivative. CD analysis of **20** showed a negative first Cotton effect with a maximum at 253 nm and a weak second positive Cotton effect at 236.5 nm. These results indicated a 9*R*, 10*R* absolute stereochemistry for the two alcoholic positions (Figure III.9). The NOESY spectrum allowed extension of the stereochemical descriptors to positions 8 and 12 (8*S*, 12*S*). The absolute configurations of the corresponding centers in cymathere lactone are: 8*S*, 9*R*, 10*S*, 12*S*.

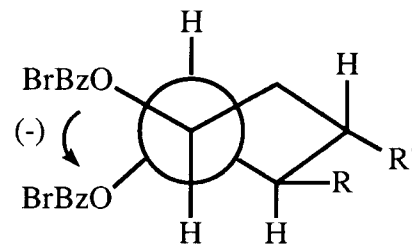
Application of the Mosher ester method required the preparation of both the *R* and *S* Mosher ester derivatives. Although cymathere lactone itself had no sites for derivation,

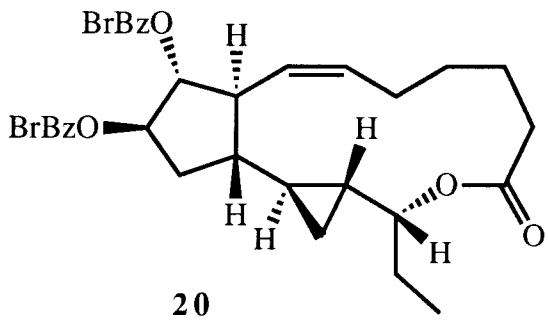



12

1) 1 M $HClO_4$, THF

2) 4-bromobenzoyl-Cl, $Et_3N$, DMAP, $CH_2Cl_2$




20

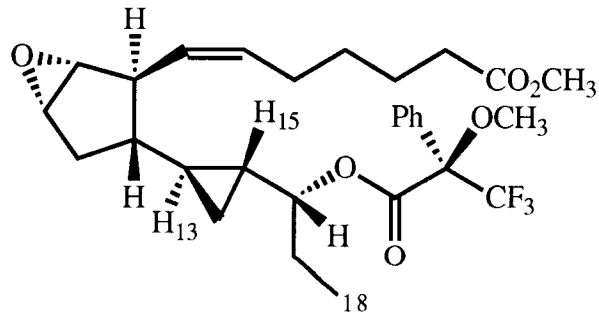
Figure III.9. Formation of Cymathere Lactone Dibromobenzoate and the Model Used to Determine Absolute Stereochemistry. (A negative first Cotton effect indicates a counterclockwise orientation of the bromobenzoate substituents)

methyl cymatherol A **16** was readily derivatized to the *R* (**21**) and *S* (**22**) Mosher esters. Using a modification of the Mosher ester method,[126] the chemical shifts of the protons adjacent to the Mosher ester group were tabulated and then the $\Delta\delta$ values were determined (Table III.4). The negative $\Delta\delta$ values represent protons that are to the left of the α-methoxy-α-trifluoromethylphenylacetate plane and the positive $\Delta\delta$ values represent protons to the right of the plane (Figure III.10). Application of standard Cahn-Ingold-Prelog priority rules lead to the 16*R* configuration for compound **16**. The absolute stereochemistry of this alcohol can be correlated back to the lactone C-16 center if we make the reasonable assumption that the hydrolysis proceeded via the Bac2 mechanism for base hydrolysis, in which the hydroxide ion attacks the ester carbonyl and stereochemistry at the alcoholic center is retained. The three other theoretical mechanisms for base hydrolysis of a carboxylic ester[128] do not seem to be viable choices under the conditions used. One of these, the Bac1 mechanism, can be eliminated because it has never been observed. The Bal1 mechanism requires the formation of an intermediate alkyl cation. Attack from both faces of the cation would give a mixture of products. Since only one alcoholic product was obtained from the reaction mixture, the Bal1 mechanism was ruled out. The Bal2 mechanism is very rare and requires $OH^-$ attack at an alkyl carbon. Because of steric congestion at C-16, this was not likely for the cymathere lactone. With five of the seven stereocenters known, only two possibilities remained for the absolute stereochemistry of cymathere lactone: 8*S*, 9*R*, 10*S*, 12*S*, 13*S*, 15*R*, 16*R* (**C**) and 8*S*, 9*R*, 10*S*, 12*S*, 13*R*, 15*S*, 16*R* (**D**). These structures represented the two possible orientations of the cyclopropyl ring at C-13 and C-15. Evaluation of the two energy minimized structures by coupling constant analysis and NOESY data, clearly indicated that the former (**C**; Figure III.11) represented the absolute stereochemistry of cymathere lactone. The lowest energy model **D** (Figure III.12) had an H-8 to H-12 dihedral angle of 156° which did not agree with the <1 Hz coupling constant between these two protons. The corresponding dihedral angle in **C** was 104°. The NOESY spectrum of cymathere

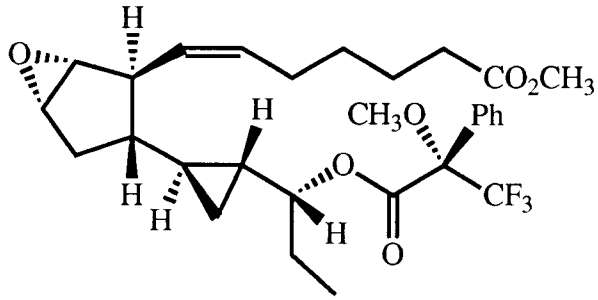
Table III.4. Selected $^1H$ NMR Chemical Shifts and $\Delta\delta$ Values for Mosher Ester Derivatives of Methyl Cymatherol A.

| | $\delta$ | | |
|---|---|---|---|
| H# | *S*-Mosher Ester **22** | *R*-Mosher Ester **21** | $\Delta\delta$ ($\delta S$ - $\delta R$) |
| H-12 | 0.93 | 0.97 | -0.04 |
| H-13 | 1.01 | 1.05 | -0.04 |
| H-14a | 0.62 | 0.66 | -0.04 |
| H-14b | 0.23 | 0.31 | -0.08 |
| H-15 | 0.76 | 0.87 | -0.11 |
| H-16 | 4.39 | 4.41 | -0.02 |
| H-17a | 1.89 | 1.85 | +0.04 |
| H-17b | 1.82 | 1.75 | +0.07 |
| H-18 | 1.00 | 0.86 | +0.14 |

Spectra were recorded in $CDCl_3$ at 400.13 MHz; referenced to TMS at 0.00 ppm. Chemical shifts are reported in ppm.




*R*-Mosher Ester of Cymatherol A (**21**)




*S*-Mosher Ester of Cymatherol A (**22**)

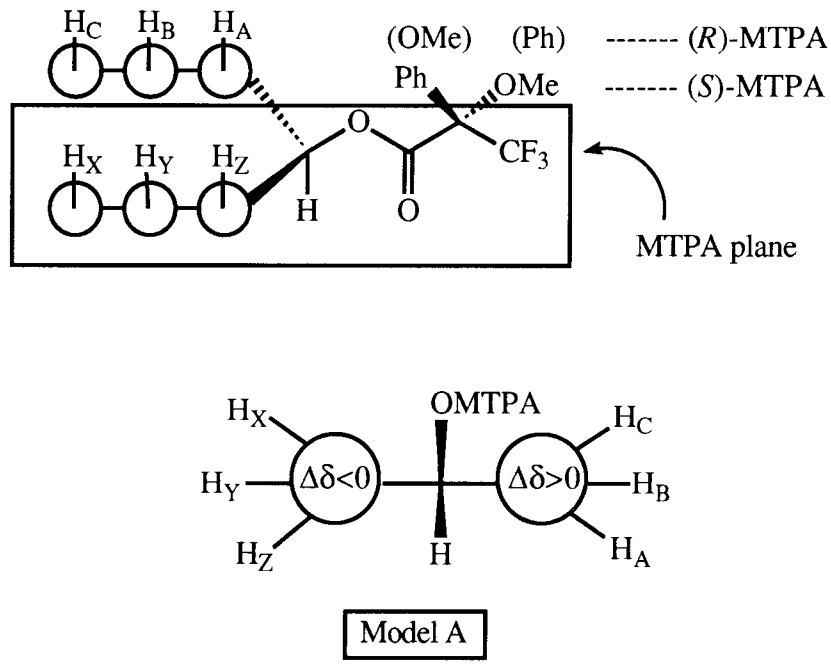



Figure III.10. (above) Idealized Conformation of the MTPA Ester and the MTPA Plane. (below) Model A for Determination of the Absolute Stereochemistry of Secondary Alcohols.[126]

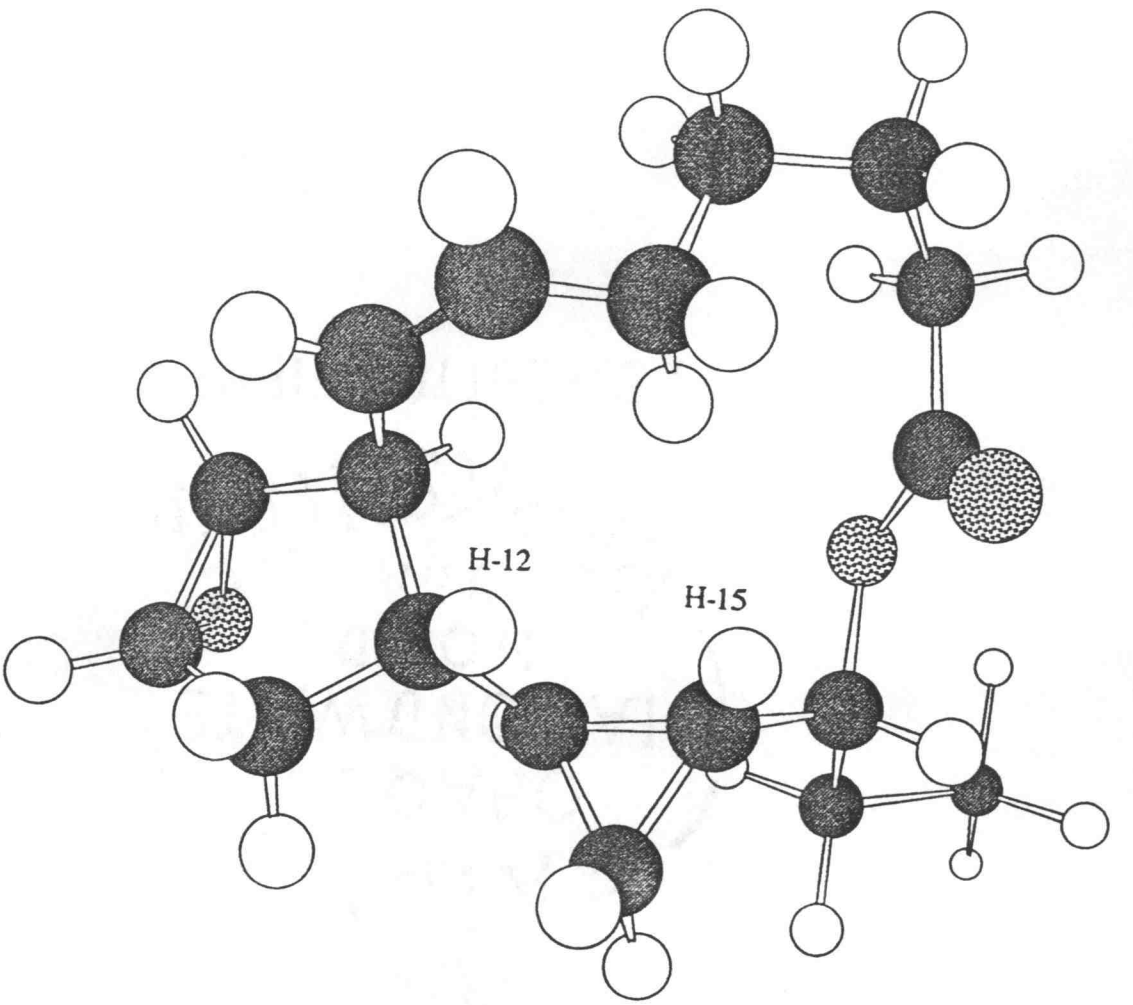



Figure III.11. Energy-minimized Structure of Cymathere Lactone Model C.

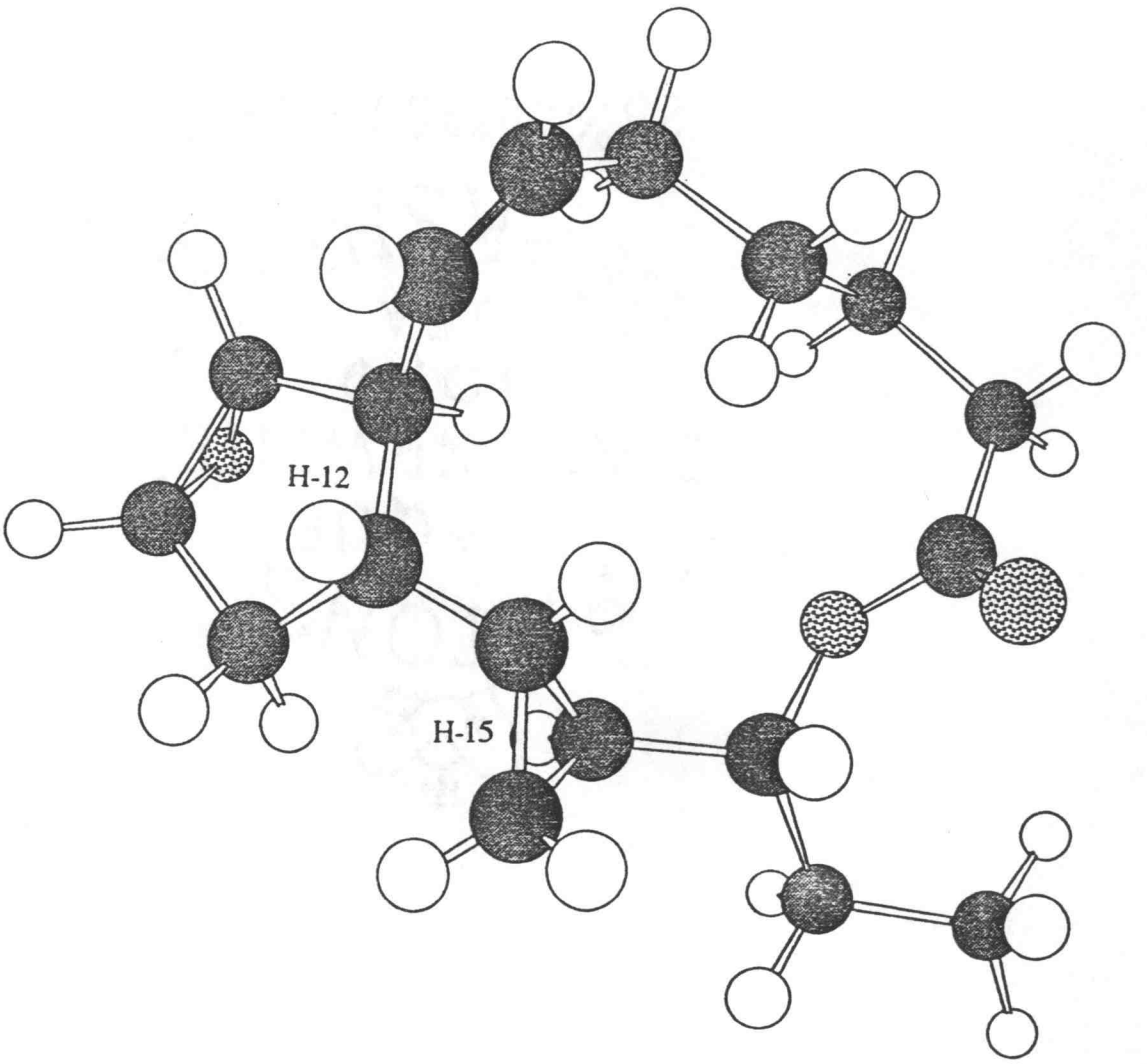



Figure III.12. Energy-minimized Structure of Cymathere Lactone Model **D**.

lactone (Figure III.8) showed a strong correlation between protons H-12 and H-15 which would not be possible for the 13*R*, 15*S* isomer (Figure III.12) according to modeling studies. As the final structure was the one predicted by the modeling studies, this indicates that the modeling programs, when used in conjunction with experimental data (coupling constants, nOe's), can be valuable tools for determing relative stereochemistry in complex molecules.

During the purification of the methyl cymatherols A-C, several other interesting new oxylipins (**23**-**25**) were isolated as methyl ester derivatives (**26**-**28**). Two of these compounds (**26**, **27**) gave dark red char reactions on TLC. Analysis of **26** by $^{13}C$ NMR and HREIMS provided a molecular formula of $C_{19}H_{30}O_4$. Examination of IR (1740 $cm^{-1}$) and NMR data (Table III.5) revealed one carbomethoxy ester and two olefins. The remaining two degrees of unsaturation must be satisfied by rings. A $^1H$-$^1H$ COSY spectrum (Figure III.13) revealed the H2-H9, H10-H11, and H13-H18 spin systems. The double bonds at C6-C7 (11.0 Hz) and C15-C16 (10.8 Hz) were *cis* based on $^3J_{HH}$. From a $^1H$-$^{13}C$ XHCORR spectrum, the protons at δ3.98 (H9), δ3.92 (H10) and δ3.82 (H13) were all attached to carbons bearing oxygen. A broad IR stretch at 3435 $cm^{-1}$ indicated that one of these carbons bore a hydroxyl. Since the NMR signal at H10 was the only one that altered upon exposure to $D_2O$, the alcohol was positioned at C10. Having accounted for three of the four oxygens, the last oxygen must connect C9 and C13 in an ether linkage. Overlap in the $^1H$ NMR spectrum in the 2.36-2.22 ppm region (H11b, H12, and H14a) prevented direct extension of these spin systems and use of alternative NMR solvent systems ($C_6D_6$ or acetone-$d_6$) did not resolve these signals. Combining the above spin systems with the remaining unassigned atoms in compound **2**, a methine, two reasonable bicyclic systems were assembled: **K**) a 2-oxabicyclo[2.2.1]heptane and **L**) a 6-oxabicyclo[3.2.0]heptane (Figure III.14). Several lines of evidence supported skeleton **K**. The orientation of the C10, C11 spin system in these structures incorporated the requirement for one intervening carbon atom between C11 and C13 to allow for the

Table III.5. $^{1}H$ and $^{13}C$ NMR Data for Methyl Cymathere Ethers A and B (**26**, **27**) in $CDCl_3$.[a] δ (ppm), multiplicity (*J*, Hz).

| C# | Methyl Cymathere Ether A (**26**) | | Methyl Cymathere Ether B (**27**) | |
|---|---|---|---|---|
| | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 174.07 | --- | 174.04 | --- |
| 2 | 33.89 | 2.33, t (7.3) | 33.41 | 2.33, t (7.5) |
| 3 | 24.55 | 1.66, tt (7.8, 7.3) | 24.70 | 1.71, tt (7.5, 7.3) |
| 4 | 28.84 | 1.42, tt (7.8, 7.4) | 26.60 | 2.12, m |
| 5 | 27.40 | 2.17, tdd (7.4, 7.2, 1.3) | 129.38 | 5.40, m |
| 6 | 133.21 | 5.51, dtd (11.0, 7.2, 1.5) | 128.24 | 5.40, m |
| 7 | 124.50 | 5.68, ddt (11.0, 7.9, 1.5) | 26.09 | 2.88, bdd (6.8, 5.8) |
| 8 | 47.47 | 3.03, dd (7.9, 1.2) | 131.61 | 5.49, dtd (10.9, 7.3, 1.5) |
| 9 | 82.14 | 3.98, bs | 124.50 | 5.70, ddt (10.9, 8.1, 1.6) |
| 10 | 73.80 | 3.90, bm | 47.52 | 3.06, bd (6.7) |
| 11 | 31.81 | a) 1.61, ddd (~14, 4.3, 3.5)<br>b) 2.26, m | 82.09 | 4.00, bs |
| 12 | 43.46 | 2.30, m | 73.79 | 3.91, bm |
| 13 | 79.85 | 3.82, bdd (7.5, 7.1) | 31.79 | a) 1.61, ddd (14.0, 3.8, 3.8)<br>b) 2.27, m |
| 14 | 29.66 | a) 2.30, m<br>b) 2.03, m | 43.50 | 2.31, m |
| 15 | 123.72 | 5.24, dtt (10.8, 7.3, 1.6) | 79.87 | 3.83, bdd (7.3, 7.0) |
| 16 | 133.92 | 5.47, dtt (10.8, 7.2, 1.5) | 29.66 | a) 2.31, m<br>b) 2.03, m |
| 17 | 20.76 | 2.06, qd (7.5, 7.2) | 123.71 | 5.24, dtt (10.9, 7.3, 1.5) |
| 18 | 14.18 | 0.97, t (7.5) | 133.94 | 5.47, m |
| 19 | --- | --- | 20.77 | 2.07, m |
| 20 | --- | --- | 14.18 | 0.97, t (7.5) |
| OMe | 51.49 | 3.67, s | 51.50 | 3.67, s |

a) All spectra recorded on a Bruker AC300 spectrometer ($^{1}H$ spectra referenced to TMS at 0.00 ppm; $^{13}C$ spectra referenced to the center line of $CDCl_3$ at 77.00 ppm). Assignments based on $^{1}H$-$^{1}H$ COSY and $^{1}H$-$^{13}C$ XHCORR spectra and on model compounds.[115]

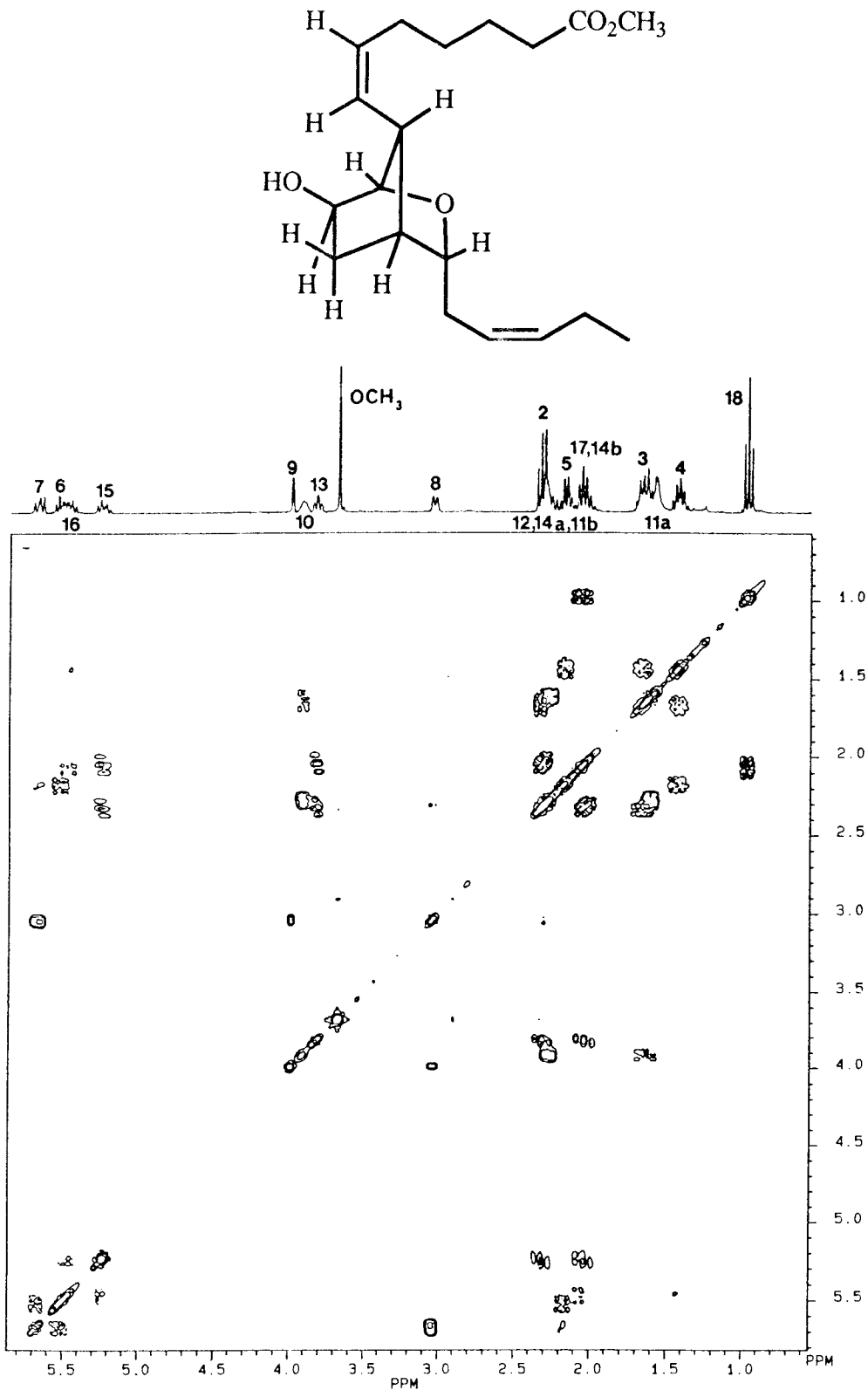
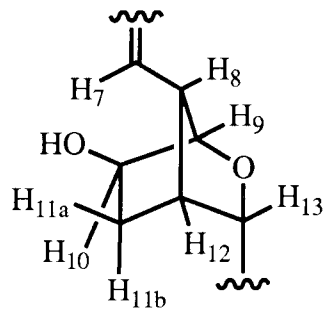
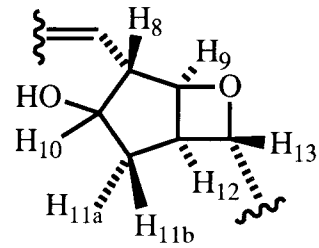



Figure III.13. COSY Spectrum of Methyl Cymathere Ether A (**26**).

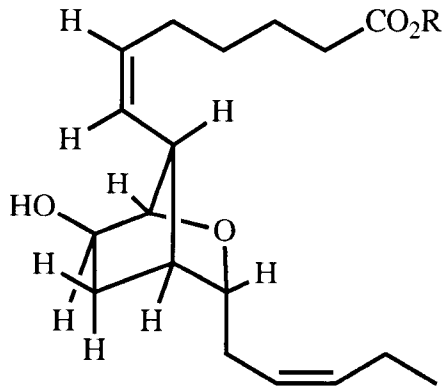



**K**

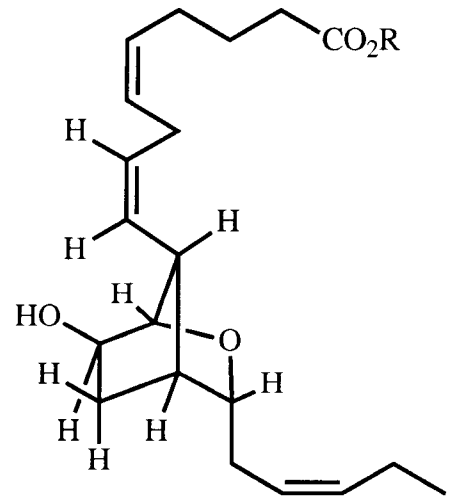



**L**

Figure III.14. Partial Structures for Methyl Cymathere Ether A.




R = H **23**
R = $CH_3$ **26**




R = H **24**
R = $CH_3$ **27**

observed W-coupling (strong correlation in the LRCOSY spectrum (Figure III.15)) between H11a and H13. In norbornane model systems[129] this type of coupling typically has a small value of 1-1.4 Hz. In structure **L**, a W-arrangement for H13 and either of the H11 protons was difficult to envision. A strong nOe (11%) in H13 when H8 was irradiated places these two protons in proximity. NOESY correlations also put H8 in the vicinity of H9 and H12 (Figure III.16). Structure **K** could accommodate these relationships. Due to the enforced *cis* ring junction in structure **L**, H8 and H12 would be on opposite faces of the bicyclic structure, preventing nOe. Using norbornane models, the observed couplings and nOe's were readily explained. The lack of coupling between H9 and H10 was consistent with bridgehead-*endo* coupling (0-2 Hz). That H10 was *endo* was supported by a $^{3}J_{HH}$ of ~4 Hz to H11a (*endo-exo* 2.5-5 Hz in norbornanes, *endo-endo* 6-7 Hz, *exo-exo* 9-10 Hz[129]). A 5% nOe when H7 was irradiated placed H11a in an *exo* position. The bridge proton H8 showed a correlation in the LRCOSY spectrum to H11b which could only be accommodated by a W-coupling, placing H11b in an *endo* position. The relative stereochemistry proposed is based on the above coupling constant data, NOEDS experiments, and a NOESY spectrum.

The 2-oxabicyclo[2.2.1]heptane ring system was further supported by $^{1}H$ NMR analysis of the hydrogenated derivative of **26**. The bridgehead proton H12 in this derivative was clearly resolved as a broad singlet that showed coupling in the $^{1}H$-$^{1}H$ COSY spectrum to both H11a and H13. Couplings to H8 and H11b were not observed, which was consistent with the small (0-3.5 Hz) couplings expected for this system. The H9 signal showed a minor coupling to both H10 and H12. Based on these data, structure **26** was established for methyl cymathere ether A.

Initially we proposed a 10*S* stereochemistry for cymathere ether A based on the co-occurrence of 10*S*-HODTA in the alga and a biogenetic hypothesis for formation of **23** initiated by a 10*S*-lipoxygenase (Figure III.17).[107] This biogenesis was based on a similar proposal by Corey for the formation of hybridalactone.[130] In the original scheme,

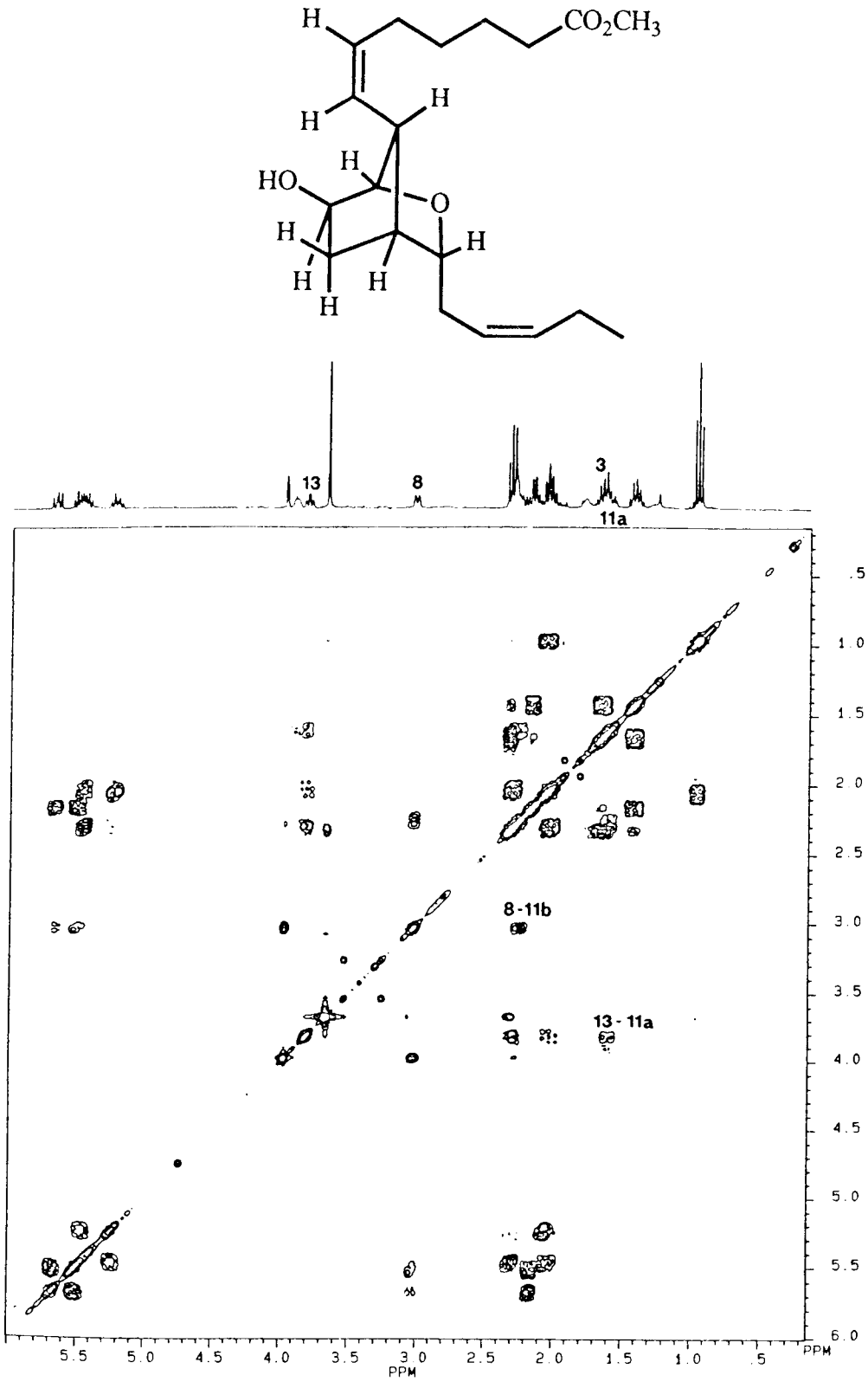
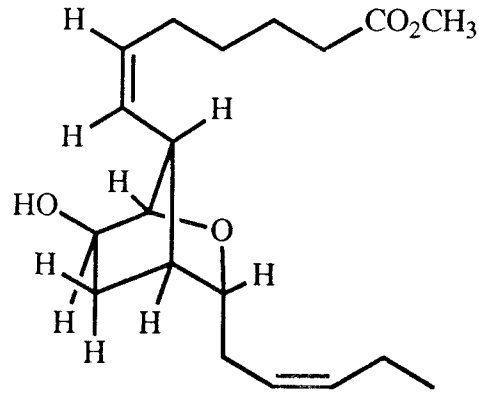



Figure III.15. Long-range COSY Spectrum of Methyl Cymathere Ether A (**26**).



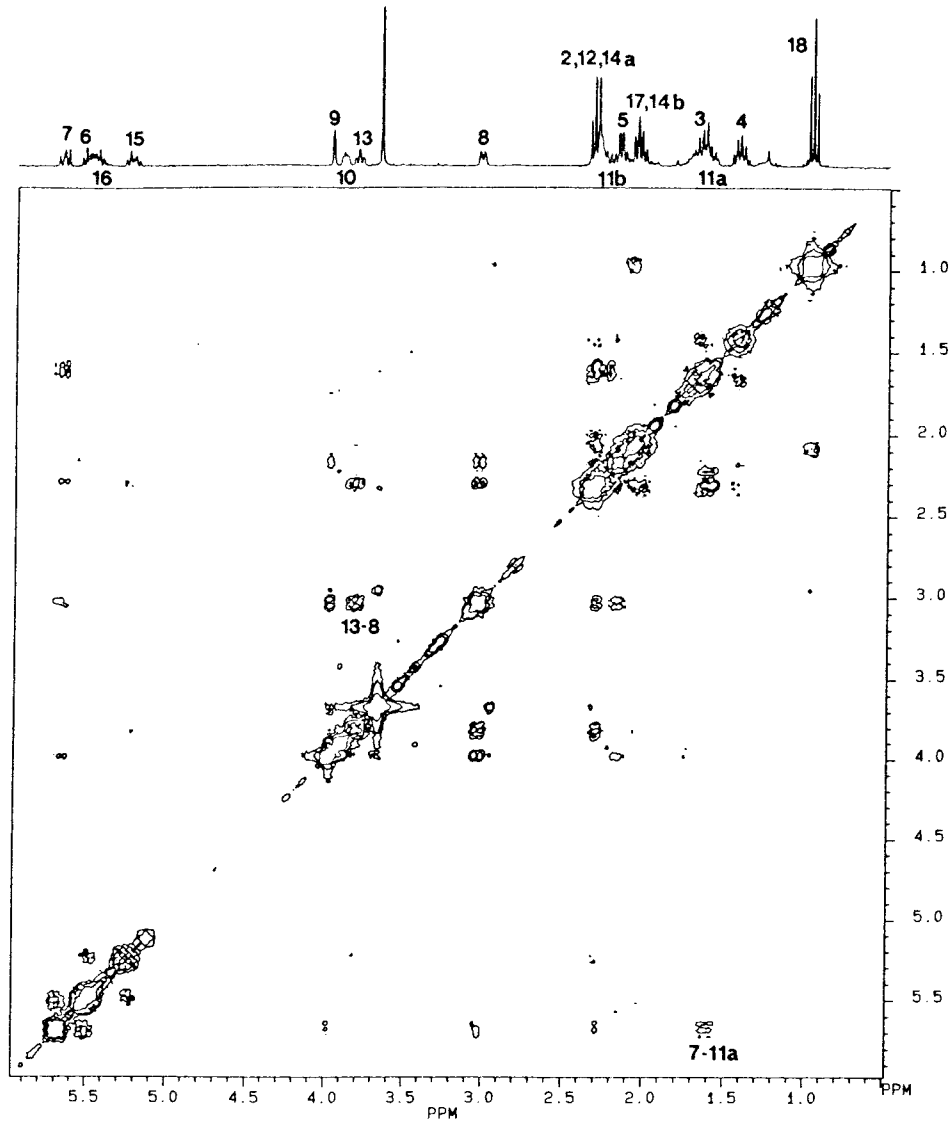




Figure III.16. NOESY Spectrum of Methyl Cymathere Ether A (**26**).

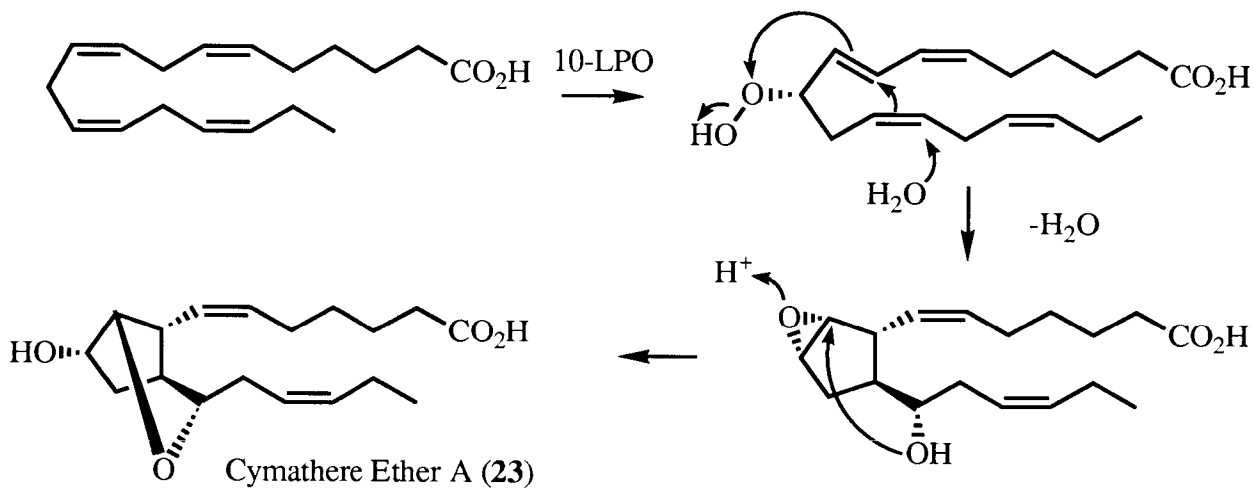



Figure III.17. Original Biogenetic Proposal for Formation of Cymathere Ether A (**23**).

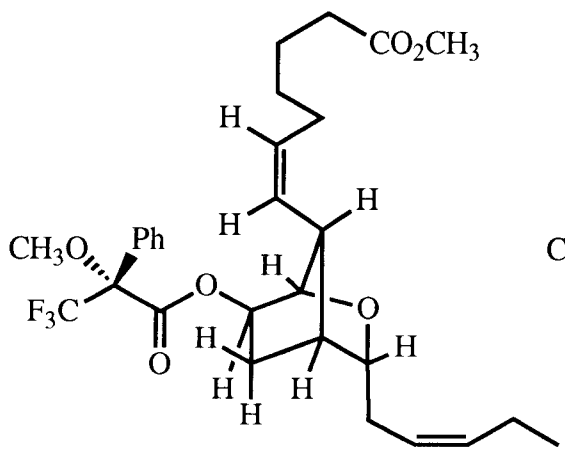
the key difference from Corey's proposal was the stereochemistry of the side-chains emerging from the cyclopentane ring. In hybridalactone, the carboxyl side chain is β and the terminal side chain is α. Our proposal depicted the carboxyl side chain α and the terminal side chain β based on the relative stereochemistry that we determined, assuming the 10*S*-stereochemistry. However, this biogenesis was proposed prior to the investigation of the relative stereochemistry of cymathere lactone. When the relative and absolute stereochemistry of cymathere lactone were determined, we were compelled to re-examine our initial biogenesis and prediction of absolute stereochemistry. Cymathere lactone and the cymatherols **13** and **14**, have the same cyclopentane relative stereochemistry as that in hydbridalactone and the ecklonialactones. If an interconnecting biogenesis is operative for the formation of the cymathere compounds, then cymathere ether A should also have the same relative configuration at the cyclopentane side-chains as cymathere lactone. With this constraint, and the known relative stereochemistry of **26**, the absolute stereochemistry at C-10 would have to be *R*. Since a secondary alcohol is available in **26**, we decided to apply the Mosher ester method to determine the configuration at C-10. Both *R* and *S* Mosher ester derivatives were prepared and the $^{1}H$ NMR spectra were recorded. Analysis of the differences in chemical shifts (Table III.6) and application to the model for Mosher ester derivatives yielded the *R* configuration at C-10 (see Figure III.10). The absolute stereochemistry for cymathere ether A is therefore 8*S*, 9*R*, 10*R*, 12*R*, 13*R*. A revised biogenetic scheme which is consistent with this new result is detailed below.

The second red char compound yielded a molecular formula of $C_{21}H_{32}O_4$ by HREIMS. Its $^{1}H$ spectrum (Table III.5) differed from that of **26** by having two extra olefinic protons, a bisallylic methylene at 2.83 ppm and the absence of a multiplet at 1.42 ppm. All other signals were essentially identical. The $^{13}C$ NMR spectrum (Table III.5) confirmed the presence of an extra olefin with two new signals at 129.38 and 128.24 ppm. A $^{1}H$-$^{1}H$ COSY spectrum positioned the extra two-carbon unit in the

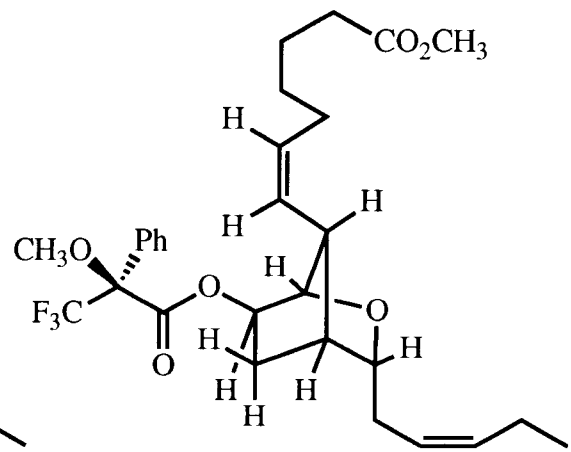
Table III.6. Selected $^1H$ NMR Chemical Shifts and $\Delta\delta$ Values for Mosher Ester Derivatives of Methyl Cymathere Ether A.

| | $\delta$ | | |
|---|---|---|---|
| H# | *S*-Mosher Ester **30** | *R*-Mosher Ester **29** | $\Delta\delta$ ($\delta S$ - $\delta R$) |
| H-7 | 5.27 | 5.17 | +0.10 |
| H-9 | 4.05 | 4.09 | -0.04 |
| H-10 | 4.92 | 4.90 | +0.02 |
| H-11a | 1.74 | 1.66 | +0.08 |
| H-11b | 2.385 | 2.391 | -0.006 |
| H-12 | 2.32 | 2.29 | +0.03 |

Spectra were recorded in $CDCl_3$ at 400.13 MHz; referenced to TMS at 0.00 ppm. Chemical shifts are reported in ppm.




**29**




**30**

carbomethoxy-containing side chain. The double bonds at C8-C9 (10.9 Hz) and C17-C18 (10.9 Hz) were *cis* based on proton coupling constants. Diagnostic $^{13}C$ shifts for C-4 (26.60 ppm) and C-7 (26.09 ppm) defined the stereochemistry of the C5-C6 olefin as *cis*.[92] The similarities in structure of **26** and **27** were confirmed by $^{13}C$-$^{1}H$ XHCORR, $^{1}H$-$^{1}H$ COSY, and NOEDS experiments. Hence, methyl cymathere ether B (**27**) was identified as a 20-carbon analog of **26**. The absolute stereochemistry, 10*S*, 11*R*, 12*R*, 14*R*, 15*R*, is proposed for **27** based on a similar positive optical rotation to **26** and on a related biogenetic origin.

Another cyclopentyl metabolite lacking a cyclopropyl ring was isolated in low yield. With a molecular formula of $C_{19}H_{30}O_4$, this compound was an isomer of the opened cymathere lactone. Compound **28** had two olefins, an epoxide, a cyclopentane ring, and a carbomethoxy group to account for the five degrees of unsaturation. One olefin was adjacent to the cyclopentane ring as in the other structures above. The second olefin was at the ω3 position in the methyl terminal side-chain. A hydroxyl was present in the structure as evidenced by a broad IR stretch at 3411 $cm^{-1}$ and by a $D_2O$ exchange experiment which eliminated the signal at δ3.78 in the $^{1}H$ NMR spectrum. The results from NMR experiments (COSY, LRCOSY, DEPT 135) placed this hydroxyl at C-13, next to the cyclopentane ring. The arrangement of the substituents around the cyclopentane ring was based on the similarities of the $^{1}H$ NMR patterns in the 3.2-3.8 ppm region to those of the opened ecklonialactone compounds (see below). Compound **28** was named methyl cymatherol D (Figure III.5).

An approach that we have taken when investigating the oxylipins of marine algae has been to identify a potentially unifying theme that ties together the occurrence of diverse fatty acid derived metabolites within a single alga. A biogenetic hypothesis that leads to several of the above metabolites starting with stearidonic acid is depicted in Figure III.18. Initial action of an ω9 specific lipoxygenase would produce the 10*S*-hydroperoxide. This hydroperoxide can be cleaved by a hydroperoxide lyase to yield 2*E*, 6*Z*-nonenal (**8**) or

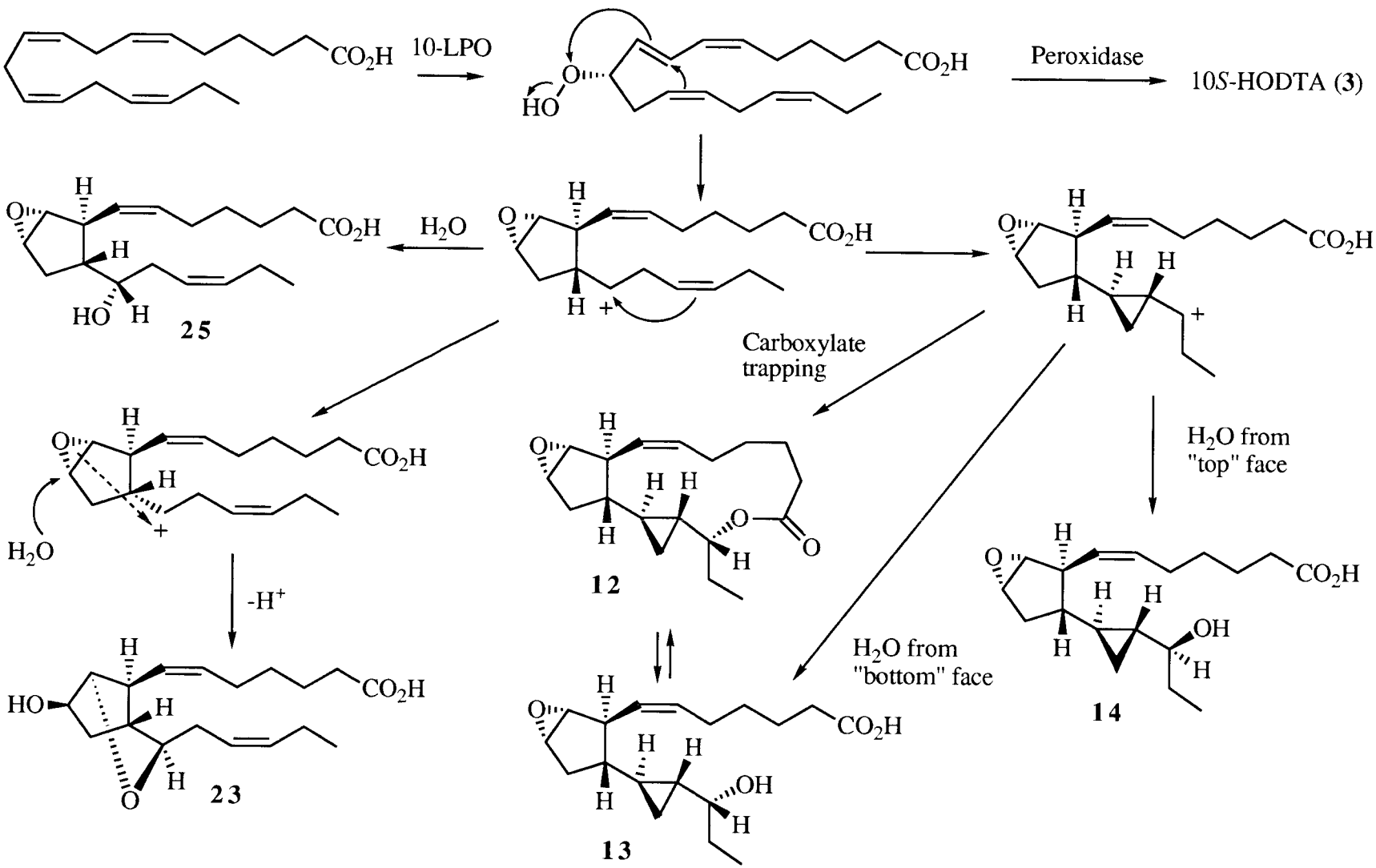
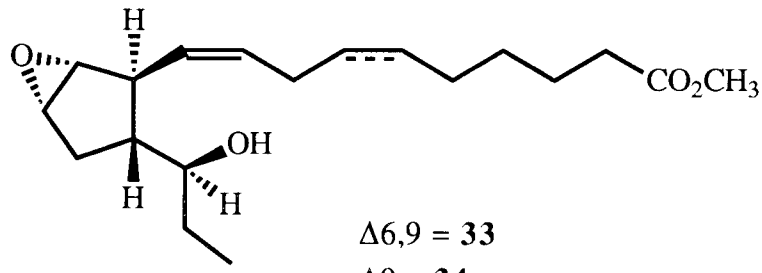



Figure III.18. Revised Biogenesis for *Cymathere* Oxylipins.

reduced by a peroxidase to 10*S*-HODTA (**3**). Alternatively, the hydroperoxide can undergo a cyclization reaction with formation of an epoxide and a C-13 cation, with subsequent formal loss of $OH^-$. This cyclopentyl cation is the common branch point. Trapping of the cation with water would produce compound **25**. Instead of direct water attack at the C-13 cation, water could attack the epoxide at the C-10 position and the resulting alkoxide (or alcohol) could internally trap the C-13 cation, forming cymathere ether A **23**, now with the correct 10*R* stereochemistry. A third option would be internal trapping of the cyclopentyl cation with the electrons from the distal olefin, producing a more stable cyclopropyl cation. This newly formed cation could be directly attacked by water to yield both **13** and **14**, or could be internally trapped by the terminal carboxylate to form cymathere lactone **12**. The role of the possible interconversion of **12** and **13** *in vivo* also must be considered. This revised biogenesis suggests an intriguing way to test this hypothesis would be to attempt a cell-free biosynthesis of the cymathere ethers under an atmosphere of $^{18}O_2$. If the latter biogenetic proposal is correct the ether oxygen would be labelled. If the former is operative, the C-10 alcohol would be labelled.

Another pair of related cyclopentane containing compounds (**31**, **32**) that lack the cyclopropyl group were also characterized as the methyl ester derivatives (**33**, **34**). The first of these analyzed for $C_{19}H_{30}O_4$ by HR CIMS. The five degrees of unsaturation inherent in this formula were accommodated by two olefins, an ester carbonyl, an epoxide, and a cyclopentane ring. The two olefins were separated by a methylene group which had a characteristic chemical shift of 2.90 ppm. NMR analysis ($^1H$, $^{13}C$, DEPT, $^1H$-$^1H$ COSY, HMQC; Table III.7) led to the construction of a structure (**33**) that correlated in a planar sense to the methyl ester of the opened lactone form of the known brown algal metabolite, ecklonialactone A.[66] We were fortunate to have a supply of ecklonialactone A at our disposal from another project on-going in our laboratory with the brown alga *Egregia menziesii*. Dr. James S. Todd, a research associate in our laboratory, hydrolyzed a sample of ecklonialactone A with 5% KOH/MeOH and purified the products




Δ6,9 = **33**

Δ9 = **34**

Table III.7. NMR Data for Opened Ecklonialactone Methyl Esters from *C. triplicata.*

| | Opened Ecklonialactone A, Methyl Ester (**33**) | | Opened Ecklonialactone B Methyl Ester (**34**) | |
|---|---|---|---|---|
| C # | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 174.12 | --- | 174.25 | --- |
| 2 | 33.95 | 2.33, t (7.5) | 34.05 | 2.31, t (7.5) |
| 3 | 24.54 | 1.66, tt (7.7, 7.5) | 24.89 | 1.62, bt (7.5) |
| 4 | 29.01 | 1.41, m | 29.45 | 1.32, m |
| 5 | 26.92 | 2.10, m | 29.10 | 1.32, m |
| 6 | 130.27 | 5.42, m | 29.10 | 1.32, m |
| 7 | 127.38 | 5.35, bdt (10.7, 6.8) | 29.06 | 1.38, m |
| 8 | 25.91 | 2.90, bdd (6.8, 5.7) | 27.55 | 2.13, bdt (7.3, 7.0) |
| 9 | 129.74 | 5.42, m | 131.70 | 5.44, dtd (10.8, 7.3, 1.0) |
| 10 | 128.35 | 5.19, ddt (10.5, 10.2, 1.6) | 127.94 | 5.15, ddt (10.8, 10.5, 1.5) |
| 11 | 37.96 | 3.36, bd (10.2) | 37.83 | 3.33, bd (10.5) |
| 12 | 62.37 | 3.27, d (2.7) | 62.51 | 3.25, d (2.7) |
| 13 | 59.08 | 3.58, bdd (2.7, 1.6) | 59.08 | 3.57, bdd (2.7, 1.6) |
| 14 | 32.56 | a) 2.25, ddd (14.6, 10.3, 1.6)<br>b) 2.03, m | 32.67 | a) 2.25, ddd (14.6,10.3,1.6)<br>b) 2.00, dd (14.6, 1.8) |
| 15 | 47.90 | 2.03, m | 47.81 | 2.06, bd (10.3) |
| 16 | 75.45 | 3.36, m | 75.47 | 3.35, m |
| 17 | 29.59 | a) 1.50, m<br>b) 1.38, m | 29.58 | a) 1.48, m<br>b) 1.35, m |
| 18 | 10.42 | 0.90, t (7.4) | 10.43 | 0.89, t (7.4) |
| OMe | 51.48 | 3.67, s | 51.42 | 3.67, s |

Spectra were recorded in $CDCl_3$. $^{1}H$ NMR at 300.13 MHz; referenced to TMS at 0.00 ppm. $^{13}C$ NMR at 75.46 MHz; referenced to $CDCl_3$ centerline at 77.00 ppm. Coupling constants are in hertz. Assignments for C4-C7 of compound **34** are interchangeable.

by HPLC. One of the products had the same $^1$H NMR spectrum as this new compound from *Cymathere*. GC/MS comparison of the two products confirmed that they were the same. Not only does this confirm the proposed planar structure for the *Cymathere* compound, but since the relative stereochemistry of ecklonialactone A was known from an X-ray study, the relative stereochemistry of this opened lactone compound was also known. The absolute stereochemistry for **33** was proposed to be 11*S*, 12*R*, 13*S*, 15*R*, 16*S* based on the absolute stereochemistry of the co-occurring cymathere lactone compounds and 13*S*-HOTE. This absolute stereochemistry would be the same as that for the ecklonialactone A isolated from the related brown algae *Ecklonia stolonifera* (Laminariales, Laminariaceae)[66] and *Egregia menziesii* (Laminariales, Alariaceae).[127]

The second compound (**34**) was obviously related to this opened ecklonialactone A compound **33**. High-resolution CIMS revealed a molecular formula of $C_{19}H_{32}O_4$. The $^1$H and $^{13}$C NMR spectra (Table III.7) showed that this compound had one less olefin than **33**. The chemical shifts for the cyclopentane ring and the methyl terminal side-chain (assigned by $^1$H-$^1$H COSY (Figure III.19) and XHCORR experiments) agreed closely with the shifts for **33**. The only difference appeared to be in the carbomethoxy side-chain. With one less olefin in this side chain, this product is the equivalent of the methyl ester of the opened lactone form of ecklonialactone B. Hydrogenation of both compounds **33** and **34** and comparison by GC/MS showed the hydrogenation products to be identical. The relative and absolute stereochemistries are depicted in analogy to the opened ecklonialactone A. In contrast to *Ecklonia stolonifera* and *Egregia menziesii*, no sign of the ecklonialactones was evident.

The final oxylipins that were identified from the extract of *C. triplicata* were the methyl esters of the *erythro* and *threo* hepoxilins $B_3$ (**35**, **36**).[131] The $^1$H NMR spectrum of an HPLC fraction collected during the purification of methyl 10-HODTA showed two pairs of signals (δ4.68, *bdd*, *J*=~8, ~2 Hz; 3.05, *ddd*, *J*=5.4, 5.4, 2.2 Hz and δ4.33, *bdd*, *J*=~7, ~5 Hz; 2.97, *ddd*, *J*=5.4, 5.4, 2.2 Hz) that suggested the presence of

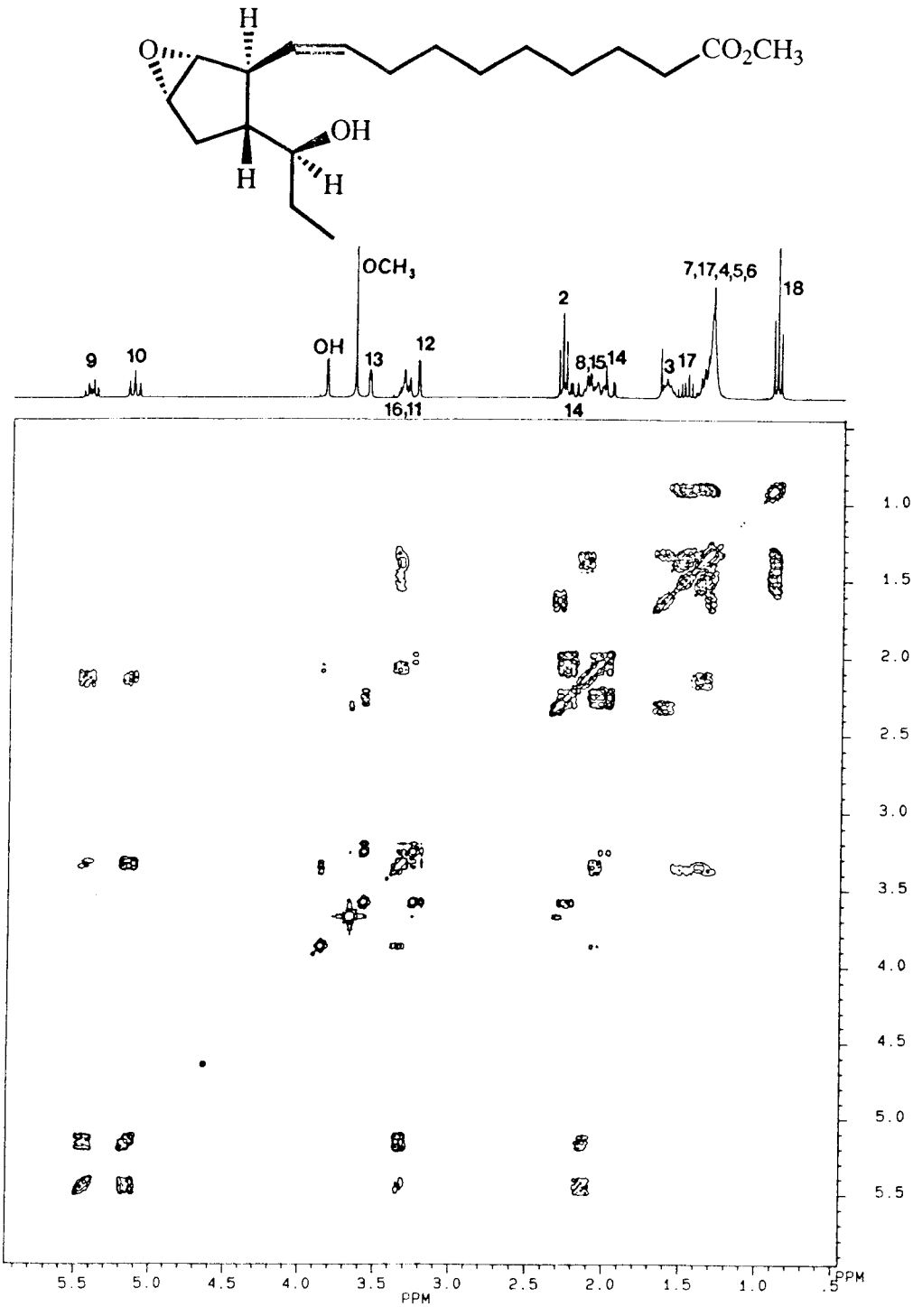
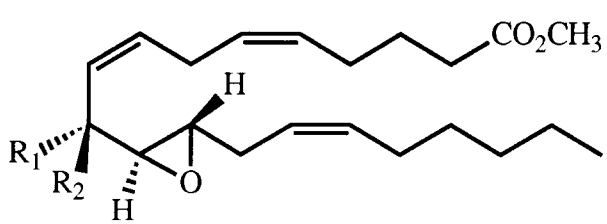



Figure III.19. COSY Spectrum of Opened Ecklonialactone B Methyl Ester (**34**).

hepoxilin-type compounds. Since several hepoxilin-type compounds had been previously isolated in our laboratory, a comparison by GC/MS of the TMS ether derivatives would be a rapid way to ascertain the identity of these compounds. A single major peak was present in the GC trace and the associated mass spectrum had a significant ion at *m/z* 269 and minor ions at *m/z* 391 and *m/z* 407. These latter ions are suggestive of [$M^+$ - $OCH_3$] and [$M^+$ - $CH_3$], respectively, for a $C_{20}$ fatty acid derived compound with three double bonds. The *m/z* 269 ion is characteristic for hepoxilin $B_3$ and comparison of the mass spectrum with one in the literature[132] showed a high degree of correlation. Both isomers of hepoxilin $B_3$ were available to us from earlier work in our laboratory with *Murrayella periclados*, a tropical red alga.[56] Co-injection of the mixture with each derivatized isomer gave a single GC peak in each case. Next the mixture and the standards were hydrogenated and derivatized to the TMS ethers. The *threo* and *erythro* isomers now separated and co-injections with the standards confirmed that both isomers were present in the alga. This is the first report of hepoxilins in a brown alga, although the red[54,56,94] and green algae[63] are known to produce these or closely related compounds.

The isolation and characterization of these novel oxylipins has unknown implications. It is now known that three species of brown algae produce elaborate cyclopentane containing compounds. The importance of these compounds to the algae has yet to be explored. From a biological activity standpoint, these compounds have yet to be fully tested. Cymathere lactone and methyl cymathere ether A were tested in the NCI 60 cell line assay for anticancer activity. Both were negative. Cymathere lactone and methyl cymathere ether B were tested in an *in vivo* assay in sheep by Dr. Fred Stormshak to see if they could re-introduce estrous. Neither compound was active.




$R_1$ = H, $R_2$ = OH **35**
$R_1$ = OH, $R_2$ = H **36**

## Experimental

*General.* Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC300 instrument operating at 300.13 MHz for $^{1}H$ NMR and at 75.46 MHz for $^{13}C$ NMR or on an AM400 instrument operating at 400.13 MHz and 100.61 MHz for $^{1}H$ and $^{13}C$ NMR, respectively. Proton spectra were referenced to internal tetramethylsilane at 0.00 ppm for $CDCl_3$ spectra and to residual solvent at 7.20 ppm for $C_6D_6$ spectra. Carbon spectra were referenced to $CDCl_3$ at 77.00 ppm. Infrared (IR) spectra were obtained as neat films with a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were run on a Hewlett-Packard 8452a spectrophotometer. Low-resolution electron-impact mass spectra (LR EIMS; probe) were recorded on a Finnigan 4023 spectrometer. High-resolution electron-impact mass spectra (HR EIMS) were recorded on a Varian MAT 311 spectrometer. High-resolution chemical ionization mass spectra (HR CIMS) were recorded on a Kratos MS 50 TC spectrometer. High-performance liquid chromatography (HPLC) utilized a Waters M6000A pump, Rheodyne 7125 injector, and a Waters Lambda-Max 480 LC spectrophotometer or a R 401 differential refractometer. Optical rotations were obtained on a Perkin-Elmer 141 polarimeter. Circular dichroism (CD) spectra were measured on Jasco J-41a or J-720 spectropolarimeters. Merck aluminum-backed thin-layer chromatography (TLC) sheets (silica gel 60 $F_{254}$) were used for TLC. Compounds were detected by UV illumination or by heating plates sprayed with a 50% $H_2SO_4$ solution. Gas chromatography/mass spectrometry (GC/MS) was done utilizing a Hewlett-Packard 5890 Series II GC connected to a Hewlett-Packard 5971 mass spectrometer. A methyl silicone column (HP Ultra-1, 11.5 m x 0.2 mm x 0.33 μm film thickness ) was used with helium as carrier gas. Trimethylsilyl (TMS) ethers were prepared using TriSil® reagent (Pierce Chemicals) or with pyridine, hexamethyldisilazane, and trimethylsilyl chloride. Pyridinium chlorochromate (PCC), 5% palladium on activated carbon, *R*-(-)-α-methoxy-α-

trifluoromethylphenylacetyl chloride, *S*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride, methoxylamine hydrochloride, and 4-bromobenzoyl chloride were obtained from Aldrich Chemical Co. Vacuum chromatography was performed with Merck Silica Gel G for TLC. Flash chromatography utilized Merck Kieselgel 60, 230-400 mesh. Chromatography solvents were distilled from glass prior to use.

*Collection, extraction, and isolation.* An initial collection of *Cymathere triplicata* (Postels et Ruprecht) J.G. Agardh was made at Deception Pass, Washington in July of 1990. A voucher sample has been deposited at the College of Pharmacy, Oregon State University, Corvallis, Oregon. The algae were frozen with dry ice for transport back to Oregon. Extraction of the algae (128 g dry wt) with two portions of 2:1 $CHCl_3$/MeOH yielded 1.2 g of a dark brown oily residue. The crude extract (1 g) was fractionated by vacuum silica chromatography (VC; 4.1 cm I.D. x 3.5 cm) using a gradient of EtOAc in hexanes. The fractions eluting with 40-50% EtOAc/hexanes (E/H) were combined and methylated. The methylated material was subjected to flash silica gel chromatography (1.6 cm O.D. x 11.5 cm), eluting first with 5% E/H, then 15% E/H. The fractions eluting with 15% E/H were then purified by NP HPLC (RSil Silica 10 μ, 500 mm x 10 mm, 15% E/H) then by RP HPLC (Merck Lichrosorb C18 Silica 7 μ, 25 cm x 1 cm, 85% MeOH/$H_2O$) to yield methyl 12-HETE (**1**, 4.8 mg, 0.5% yield) and methyl 13-HOTE (**2**, 1.7 mg, 0.2% yield), both as colorless oils.

Several vacuum chromatography fractions from this initial collection showed minor cyclopropyl peaks by $^1H$ NMR, suggesting that a re-collection was necessary. A second collection of *C. triplicata* was made at Deception Pass in May of 1991. The algae (207 g dry wt) were extracted three times with warm 2:1 $CHCl_3$/MeOH (the first extract is always weak due to the presence of large amounts of water) to yield 5.5 g of crude extract. The crude extract was fractionated by vacuum chromatography (8.5 cm I.D. x 5 cm) using a stepped gradient beginning with 5% E/H and increasing in the percentage of EtOAc up to

pure EtOAc. Final rinsing of the column was done first with 50% EtOAc/MeOH, then 100% MeOH.

*Non-polar metabolites.* The first two VC fractions (eluting with 5-10% E/H) were combined due to a high $R_f$ UV-active spot which coincided with a purple char. Two successive vacuum chromatographies using $CHCl_3$ as the eluent were used to remove pigments and the bulk of fatty acids present in the sample. The fatty acids can severely interfere with the separations because they tail on silica and act as an internal solvent, altering expected chromatographic behavior. Since the desired products are not fatty acids, methylation with $CH_2N_2$ and separation of the fatty acid methyl esters would be a better method of purification. Flash chromatography using 10% $Et_2O$/hexanes provided a fraction containing a 1:1 mix of cucumber aldehydes (**7** and **8**, 30 mg, 0.5% yield; exact yield is difficult to obtain due to volatility of these aldehydes) and another fraction enriched in a purple charring compound (80 mg). A portion (26.5 mg) of this material was purified by HPLC (Phenomenex Maxsil Silica 10 μ, 500 mm x 10 mm, 10% E/H) and the remainder was purified by treatment with activated charcoal to remove a yellow pigment and then high vacuum treatment to remove the remaining cucumber aldehydes (**12**, colorless oil, 60 mg, 1% yield).

*Polar metabolites.* The vacuum chromatography fractions eluting with 60% E/H to 50% EtOAc/MeOH were combined (554 mg) and methylated with $CH_2N_2$ to improve the chromatographic characteristics of the fatty acid components. The methylated material was vacuum chromatographed using 10% E/H, 30% E/H, then 100% EtOAc. The first fraction eluting with 30% E/H (192 mg) was fractionated by flash silica chromatography (5% IPA/hexanes) to yield a fraction enriched in dark blue and purple chars and UV-active spots. This material (157 mg) was then subjected to successive reverse-phase flash chromatography (95% MeOH/$H_2O$ first run, 85% MeOH/$H_2O$ second run). An early eluting fraction was purified by RP HPLC (Merck Lichrosorb C18 Silica 7 μ, 25 cm x 1 cm, 75% MeOH/$H_2O$) to yield aldehyde methyl ester **10** (22.1 mg, 0.4% yield) and a

mixed fraction containing the major aldehyde and a minor aldehyde tentatively identified as methyl 12-oxo-5*Z*, 8*E*, 10*E*-dodecatrienoate by comparison to an authentic $^1$H NMR spectrum. An HPLC run (Merck Lichrosorb C18 Silica 7 μ, 25 cm x 1 cm, 85% MeOH/$H_2O$) of a later eluting fraction from the RP flash chromatography gave four fractions: A) 12.9 mg; B) 19.8 mg; C) 31.5 mg; D) 7.9 mg. Fraction A was purified by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 30 cm, 15% EtOAc/hexanes) to yield methyl cymatherol D (**28**, 1.9 mg, 0.035% yield), opened ecklonialactone A methyl ester (**33**, 3.2 mg, 0.06%), and methyl cymatherol B (**17**, 5.4 mg, 0.1%). Fraction B was also purified by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 30 cm, 15% EtOAc/hexanes) to yield opened ecklonialactone B methyl ester (**34**, 10.1 mg, 0.2%). Fraction C was purified by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 30 cm, 20% EtOAc/hexanes) to yield methyl 10-HODTA (**4**, 19.0 mg, 0.35% yield) and several other uncharacterized hydroxy fatty acid methyl esters. Fraction D was examined by $^1$H NMR and was shown to contain a mixture of *erythro* (**35**) and *threo* (**36**) hepoxilin $B_3$.

The remainder of the material eluting with 30% E/H from the initial VC of the methylated material (80 mg) was de-pigmented by running through a short RP-silica plug using 90% MeOH/$H_2O$. This material was purified first by flash silica chromatography (5% IPA/hexanes), then by RP HPLC (Merck Lichrosorb C18 Silica 7 μ, 25 cm x 1 cm, 75% MeOH/$H_2O$) to yield the methyl esters of cymatherol A (**16**, 10.4 mg, 0.2%), cymatherol C (**18**, 4.6 mg, 0.08%), cymathere ether A (**26**, 13.5 mg, 0.25%), and cymathere ether B (**27**, 8.1 mg, 0.15%).

*Methyl 12S-Hydroxyeicosa-5Z, 8Z, 10E, 14Z-tetraenoate* (**1**). $[\alpha]_D^{27}$ +11° (*c* 0.53, acetone). $^1$H and $^{13}$C NMR data matched with literature values.[115] The TMS ethers were prepared by treating a small amount of compound **1** (<100 μg) with 2 drops each of pyridine, hexamethyldisilazane, and trimethylsilyl chloride. After 20 minutes at

room temperature, the solvents were removed *in vacuo* and the residue was triturated with hexanes. The hexanes solubles were transferred to a vial and analyzed by GC/MS.

*Methyl 13S-Hydroxyoctadeca-9Z, 11E, 14Z-trienoate* (**2**). $[\alpha]_D^{25}$ +8.9° (*c* 0.19, acetone). Compound **2** was characterized by $^1$H NMR, $^{13}$C NMR, $^1$H-$^1$H-COSY, and GC/MS of the TMS ether derivative. The $^1$H NMR spectrum was directly compared with an authentic spectrum.[90]

*Methyl 10S-Hydroxyoctadeca-6Z, 8E, 12Z, 15Z-tetraenoate* (**4**). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 3458 (br. OH), 3010, 2961, 2934, 1740, 1437, 1209, 1175, 987, 724. UV $\lambda_{max}^{MeOH}$ nm: 236 ($\varepsilon$ = 23,000). $[\alpha]_D^{25}$=+6.5° (*c* 0.49, acetone). $^1$H NMR ($CDCl_3$, 300 MHz): δ6.51 (1H, *dddd*, *J*=15.2, 11.1, 1.2, 1.2 Hz, H-8), 5.99 (1H, *bdd*, *J*=11.4, 11.1 Hz, H-7), 5.70 (1H, *dd*, *J*=15.2, 6.4 Hz, H-9), 5.55 (1H, *dtt*, *J*=10.8, 7.3, 1.4 Hz, H-13), 5.47-5.36 (3H, *m*, H-6, H-12, H-16), 4.23 (1H, *bdt*, *J*=6.4, 6.4 Hz, H-10), 3.67 (3H, *s*, $OCH_3$), 2.84 (2H, *bdd*, J=7.3, 7.1 Hz, H-14), 2.40-2.29 (2H, *m*, H-11), 2.32 (2H, *t*, *J*=7.4 Hz, H-2), 2.21 (2H, *dtd*, J=7.4, 7.4, 1.5 Hz, H-5), 2.07 (2H, *bqd*, J=7.5, 6.9 Hz, H-17), 1.75 (1H, *bs*, OH), 1.66 (2H, *tt*, *J*=7.8, 7.4 Hz, H-3), 1.42 (2H, *tt*, *J*=7.8, 7.4 Hz, H-4), 0.97 (3H, *t*, *J*=7.5 Hz, H-18). $^{13}$C NMR (75.46 MHz, $CDCl_3$): δ174.08 (C-1), 135.26 (C-9), 132.18 (C-16), 132.07 (C-6), 131.60 (C-13), 128.13 (C-7), 126.74 (C-15), 125.67 (C-8), 124.66 (C-12), 72.02 (C-10), 51.45 ($OCH_3$), 35.34 (C-11), 33.88 (C-2), 29.00 (C-4), 27.30 (C-5), 25.71 (C-14), 24.46 (C-3), 20.55 (C-17), 14.22 (C-18). The assignments for C-6 and C-16 may be interchanged. GC EIMS of TMS ether derivative 70 eV *m/z* (rel. int.): 378 $[M]^+$ (0.3), 363 $[M - CH_3]^+$ (0.5), 347 $[M - OCH_3]^+$ (0.5), 269 (100), 179 [M - TMSOH]$^+$ (4), 147 (24), 119 (36), 73 (75).

*Methyl 10S-(4-Bromobenzoyloxy)octadeca-6Z, 8E, 12Z, 15Z-tetraenoate* (**5**). The free alcohol (2.5 mg) was dissolved in 3 mL $CH_2Cl_2$ and several 4Å molecular sieves were added. Triethylamine ($Et_3N$; 30 ml, 26 equiv.), 4-bromobenzoyl chloride (12 mg, 6.7 equiv.), and a few crystals of 4-dimethylaminopyridine were then introduced. The reaction was monitored by TLC (20% E/H, $R_f$ = 0.43, UV-active, blue/gray acid char).

Several more portions of acid chloride were added during the course of the reaction (56.9 mg, 31.6 equiv. total) and $Et_3N$ (20 ml, 17 equiv). After 43 h, the reaction mixture was directly loaded onto a silica column (1.7 cm O.D. x 12.5 cm). Elution of the column with 10% E/H provided the desired compound contaminated with white crystals. The hexanes solubles were concentrated, then loaded onto a flash silica column (1.7 cm O.D. x 15 cm). Elution with 5% E/H gave a colorless oil (2.3 mg, 58% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3012, 2953, 2934, 1738, 1720, 1591, 1267, 1113, 1102, 757. $[\alpha]_D^{19}$ +84° (*c* 0.26, EtOH). UV $\lambda_{max}^{EtOH}$ nm: 240 (ε 36,000). CD (EtOH): Δε= -15.7, +19.1 ($\lambda_{max}$= 230, 250 nm). $^1H$ NMR ($CDCl_3$, 300 MHz): δ7.90 (2H, *d*, *J*=8.7 Hz, aromatic CH *ortho* to carbonyl), 7.58 (2H, *d*, *J*=8.7, aromatic CH *ortho* to Br), 6.59 (1H, *bdd*, *J*=15.1,11.1 Hz, H-8), 5.97 (1H, *dd*, *J*=11.1, 11.0 Hz, H-7), 5.71 (1H, *dd*, *J*=15.1, 7.3 Hz, H-9), 5.57 (1H, *ddd*, *J*=7.3, 6.7, 6.2 Hz, H-10), 5.54-5.43 (3H, *m*, H-6, H-12, H-13), 5.39 (1H, *dtt*, *J*=10.6, 7.1, 1.5 Hz, H-16), 5.27 (1H, *dtt*, *J*=10.6, 7.1, 1.5 Hz, H-15), 3.66 (3H, *s*, $OCH_3$), 2.80 (2H, *bdd*, *J*=7.1, 6.9 Hz, H-14), 2.62 (1H, *ddd*, *J*=14.7, 7.3, 6.7 Hz, H-11a), 2.51 (1H, *ddd*, *J*=14.7, 6.8, 6.2 Hz, H-11b), 2.30 (2H, *t*, *J*=7.4 Hz, H-2), 2.19 (2H, *dtd*, *J*=7.4, 7.4, 1.4 Hz, H-5), 2.05 (2H, *qd*, *J*=7.5, 7.1 Hz, H-17), 1.64 (2H, *tt*, *J*=7.8, 7.4 Hz, H-3), 1.41 (2H, *tt*, *J*=7.8, 7.4 Hz, H-4), 0.96 (3H, *t*, *J*=7.5 Hz, H-18). LR EIMS (probe) 70 eV *m/z* (rel. int.): 490 $[M(^{81}Br)]^+$ (0.05), 488 $[M(^{79}Br)]^+$ (0.04), 459 $[M(^{81}Br) - OCH_3]^+$ (0.17), 457 $[M(^{79}Br) - OCH_3]^+$ (0.13), 381 (2.4), 379 (2.4), 289 (12), 185 (91), 183 (100). LR CIMS (methane) *m/z* (rel. int.): 491 $[M(^{81}Br) + H]^+$ (1.6), 489 $[M(^{79}Br) + H]^+$ (2.0), 381 (2.5), 379 (2.7), 290 (100), 258 (26).

*Methyl 10S-Acetoxyoctadeca-6Z, 8E, 12Z, 15Z-tetraenoate* (**6**). The free alcohol (2.5 mg) was dissolved in 100 μL pyridine and 100 μL acetic anhydride. After stirring overnight, the reaction mixture was concentrated *in vacuo* to obtain the acetate (2.8 mg, quantitative). $[\alpha]_D^{23}$ -10° (c 0.31, MeOH). The $^1H$ NMR spectrum ($C_6D_6$) and EI mass spectrum matched those of methyl 10-HODTA acetate reported in the literature.[57] HR EIMS *m/z* obs. $[M - CH_3CO_2H]^+$ 288.20892 ($C_{19}H_{28}O_2$, 0.0 mmu dev.).

*2E-Nonenal* (**7**) *and 2E,6Z-Nonadienal* (**8**). NMR data were collected on the mixture of these two compounds. Assignments are based on a $^1$H-$^1$H COSY spectrum. $^1$H NMR (300 MHz, $CDCl_3$): (**7**) δ9.51 (1H, *d*, *J*=7.9 Hz, H-1), 6.85 (1H, *dt*, *J*=15.6, 6.6 Hz, H-3), 6.12 (1H, *ddt*, *J*=15.6, 7.9, 1.5 Hz, H-2), 2.34 (2H, *m*, H-4), 1.51 (2H, distorted *tt*, *J*=7.5, 7.1 Hz, H-5), 1.39-1.25 (6H, *m*, H-6, H-7, H-8), 0.89 (3H, distorted *t*, J=6.8 Hz); (**8**) δ9.51 (1H, *d*, *J*=7.9 Hz, H-1), 6.86 (1H, *dt*, *J*=15.6, 6.8 Hz, H-3), 6.14 (1H, *ddt*, *J*=15.6, 7.9, 1.5 Hz, H-2), 5.45 (1H, *dtt*, *J*=10.8, 7.2, 1.4 Hz, H-7), 5.31 (1H, *dtt*, *J*=10.8, 7.1, 1.5 Hz, H-6), 2.41 (2H, *m*, H-4), 2.26 (2H, *dt*, *J*=7.1, 7.1 Hz, H-5), 2.05 (2H, *qd*, *J*=7.5, 7.2 Hz, H-8), 0.97 (3H, *t*, *J*=7.5 Hz, H-9). $^{13}$C NMR (75.46 MHz, $CDCl_3$): δ194.10 (CH), 193.97 (CH), 159.05 (CH), 158.04 (CH), 133.23 (CH), 133.14 (CH), 132.87 (CH), 126.65 (CH), 32.66 ($CH_2$; 2C), 31.46 ($CH_2$), 28.73 ($CH_2$), 27.71 ($CH_2$), 25.34 ($CH_2$), 22.45 ($CH_2$), 20.51 ($CH_2$), 14.14 ($CH_3$), 13.97 ($CH_3$).

*Methyl 11-Oxo-5Z,9E-undecadienoate* (**10**). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 2950, 2738, 1737, 1692, 1437, 1173, 1157, 1127. UV $\lambda_{max}^{MeOH}$ nm: 222 (ε = 20,000). $^1$H NMR (300 MHz, $CDCl_3$): δ9.51 (1H, *d*, *J*=7.9 Hz, H-11), 6.84 (1H, *dt*, *J*=15.6, 6.6 Hz, H-9), 6.13 (1H, *ddt*, *J*=15.6, 7.9, 1.4 Hz, H-10), 5.41 (2H, *m*, H-5, H-6), 3.67 (3H, *s*, $OCH_3$), 2.40 (2H, *btd*, *J*=6.9, 6.6 Hz, H-8), 2.32 (2H, *t*, *J*=7.4 Hz, H-2), 2.26 (2H, *btd*, *J*=6.9, 6.8 Hz, H-7), 2.08 (2H, *td*, *J*=7.5, 6.4, H-4), 1.70 (2H, *tt*, *J*=7.5, 7.4, H-3). $^1$H NMR assignments based on $^1$H-$^1$H decoupling experiments. $^{13}$C NMR (75.46 MHz, $CDCl_3$): δ193.98 (C-11), 173.93 (C-1), 157.77 (C-9), 133.30 (C-10), 130.23 (C-5), 128.55 (C-6), 51.51 ($OCH_3$), 33.36 (C-2), 32.62 (C-8), 26.58 (C-4), 25.52 (C-7), 24.68 (C-3). Carbon assignments based on model compounds.[58] GC EIMS 70 eV *m/z* (rel. int.): 179 $[M - OCH_3]^+$ (0.4), 166 (11), 141 (100), 109 (51), 81 (90), 70 (68), 67 (52). LR CIMS (methane) *m/z* (rel. int.): 239 $[MH + C_2H_4]^+$ (11), 211 $[M+H]^+$ (100), 193 (17), 179 $[M - OCH_3]^+$ (64), 161 (48), 133 (23). HR CIMS *m/z* obs. $[M + H]^+$ 211.1334 ($C_{12}H_{19}O_3$, 0.0 mmu dev.).

*Methyl 11-Oxo-5Z,9E-undecadienoate, Methoxime Derivative.* The aldehyde **10** (1.0 mg) was dissolved in 100 μL of a 10 mg methoxylamine HCl/mL pyridine solution and allowed to sit at room temperature. After 16 hours, the solvent was evaporated under $N_2$ and the residue was dissolved in 5% EtOAc/hexanes and then passed through a short plug of silica. The concentrated oil was dissolved in hexanes and then analyzed by GC/MS. Two major peaks were detected in a 2.3:1 ratio. The mass spectra for these peaks were almost identical, consistent with the formation of the *E* and *Z* methoxime derivatives. (*E* isomer) $^1$H NMR (300 MHz, $CDCl_3$): δ7.67 (1H, d, J=9.4 Hz, H-11), 6.13 (1H, *bdd*, *J*=15.6, 9.4 Hz, H-10), 6.00 (1H, *dt*, *J*=15.6, 6.3 Hz, H-9), 5.38 (2H, *m*, H-5, H-6), 3.86 (3H, *s*, oxime $OCH_3$), 3.67 (3H, *s*, ester $OCH_3$), 2.31 (2H, *t*, *J*=7.5 Hz, H-2), 2.19 (4H, *m*, H-7, H-8), 2.07 (2H, *m*, H-4), 1.69 (2H, *tt*, *J*=7.5, 7.2 Hz, H-3). (*Z* isomer; partial) $^1$H NMR (300 MHz, $CDCl_3$): δ6.96 (1H, *d*, *J*=9.6 Hz, H-11), 6.64 (1H, *ddt*, *J*=15.8, 9.6, 1.5 Hz, H-10), 3.89 (3H, *s*, oxime $OCH_3$); the remaining signals were obscured or directly overlapped with patterns for the major *E* isomer. The $^1$H NMR spectrum was recorded on the 2.3/1 mixture. (*E* isomer; major) GC EIMS 70 eV *m/z* (rel. int.): 239 $[M]^+$ (3), 208 $[M - OCH_3]^+$ (7), 173 (34), 141 (13), 109 (43), 99 (100), 81 (87), 67 (74). (*Z* isomer; minor) GC EIMS 70 eV *m/z* (rel. int.): 239 $[M]^+$ (1.4), 208 $[M - OCH_3]^+$ (3), 173 (27), 141 (12), 109 (36), 99 (100), 81 (77), 67 (58).

*Cymathere Lactone* (**12**). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 3067 (w), 3004, 2967, 2930, 1727, 1223, 1151, 840. $[\alpha]_D^{26}$ +7.1° (*c* 0.34, MeOH). For $^1$H and $^{13}$C NMR data, see Table III.1. LR EIMS (probe) 70 eV *m/z* (rel. int.): 290 $[M]^+$ (6), 261 (4), 221 (31), 192 (57), 119 (35), 105 (36), 91 (59), 79 (100), 67 (92). HR EIMS *m/z* obs. $[M]^+$ 290.1880 ($C_{18}H_{26}O_3$, -0.2 mmu dev.).

*Methyl Cymatherol A* (**16**, natural product). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 3460 (br. OH), 3061 (w), 2931, 1739, 1438, 1210, 1174, 1156, 841. $[\alpha]_D^{26}$ -39° (*c* 1.0, acetone). See Tables III.2 for $^1$H NMR data and Table III.3 for $^{13}$C NMR data. GC EIMS 70 eV *m/z* (rel. int.): 304 $[M - H_2O]^+$ (0.1), 275 $[M - H_2O - CH_2CH_3]^+$ (1), 235 (6), 207 (40), 175

(43), 157 (44), 147 (37), 133 (72), 119 (39), 105 (49), 93 (78), 91 (93), 79 (100), 67 (53), 57 (50). LR CIMS (methane) *m/z* (rel. int.): 351 $[MH + C_2H_4]^+$ (2), 333 $[(MH + C_2H_4) - H_2O]^+$ (0.9), 323 $[M+H]^+$ (0.4), 305 $[(MH - H_2O]^+$ (10), 287 (18), 273 (7), 207 (100), 175 (9). HR CIMS *m/z* obs. $[M + H]^+$ 323.2222 ($C_{19}H_{31}O_4$, 0.0 mmu dev.).

*Methyl Cymatherol A* (**16**, from base hydrolysis of lactone). Cymathere lactone **12** (2.4 mg) was dissolved in 1 mL 5% KOH/MeOH solution. After 19 h, the reaction mixture was poured into a separatory funnel containing ether and 5% HCl solution. The aqueous layer was extracted with another portion of ether, and then the combined ether layers were washed with brine. Concentration of the dried organics ($Na_2SO_4$) provided a residue that was treated with $CH_2N_2$ in ether. The product was purified by flash chromatography (25% EtOAc/hexanes) to yield 1 mg (38% yield) of a colorless oil. $[\alpha]_D^{23}$ -36° (*c* 0.11, acetone). The product had an identical $^1H$ NMR spectrum to the natural product (300 MHz, $CDCl_3$) and gave a single spot on TLC (25% EtOAc/hexanes) when co-spotted with the natural product.

*Methyl Cymatherol A Acetate.* The free alcohol **16** (<100 μg) was dissolved in 3-4 drops each of pyridine and acetic anhydride. After sitting at room temperature overnight, the sample was concentrated and the residue was dissolved in hexanes. GC EIMS 70 eV *m/z* (rel. int.): 364 $[M]^+$ (0.1), 333 $[M - OCH_3]^+$ (0.1), 304 $[M - CH_3CO_2H]^+$ (2), 275 $[M - CH_3CO_2H - CH_2CH_3]^+$ (1), 235 (3), 207 (29), 206 (27), 175 (33), 168 (100), 157 (24), 147 (20), 133 (34), 105 (28), 93 (53), 91 (52), 79 (77), 67 (26).

*Methyl Cymatherol B* (**17**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3479 (br. OH), 3059 (w), 2935, 1739, 1438, 1207, 1175, 1155, 841. $[\alpha]_D^{29}$ -48° (*c* 0.60, acetone). See Tables III.2 for $^1H$ NMR data and Table III.3 for $^{13}C$ NMR data. GC EIMS 70 eV *m/z* (rel. int.): 235 (8), 207 (41), 175 (39), 157 (45), 147 (35), 133 (74), 119 (42), 105 (51), 93 (76), 91 (95), 79 (100), 67 (55), 57 (55). LR CIMS (methane) *m/z* (rel. int.): 351 $[MH + C_2H_4]^+$

(2), 333 [(MH + $C_2H_4$) - $H_2O$]$^+$ (1), 323 [M+H]$^+$ (0.2), 305 [(MH - $H_2O$]$^+$ (9), 287 (15), 273 (7), 207 (100), 175 (10). HR CIMS *m/z* obs. [M + H]$^+$ 323.2222 ($C_{19}H_{31}O_4$, 0.0 mmu dev.).

*Methyl Cymatherol B Acetate.* The free alcohol **17** (<100 μg) was dissolved in 3-4 drops each of pyridine and acetic anhydride. After sitting at room temperature overnight, the sample was concentrated and the residue was dissolved in hexanes. GC EIMS 70 eV *m/z* (rel. int.): 304 [M - $CH_3CO_2H$]$^+$ (3), 275 [M - $CH_3CO_2H$ - $CH_2CH_3$]$^+$ (2), 235 (5), 207 (53), 206 (48), 175 (54), 168 (44), 157 (41), 147 (30), 133 (61), 105 (41), 93 (71), 91 (80), 79 (100), 67 (40).

*Methyl Cymatherol C* (**18**). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 3466 (br. OH), 3062, 3010, 2956, 2927, 1737, 1438, 1246, 1224, 1201, 1156, 841. $[\alpha]_D^{26}$ -24° (*c* 0.50, acetone). See Tables III.2 for $^1$H NMR data and Table III.3 for $^{13}$C NMR data. GC EIMS 70 eV *m/z* (rel. int.): 261 (3), 243 (3), 233 (8), 229 (7), 201 (19), 183 (15), 161 (43), 131 (46), 117 (71), 105 (53), 91 (100), 79 (71), 67 (51), 57 (43). LR CIMS (methane) *m/z* (rel. int.): 377 [MH + $C_2H_4$]$^+$ (6), 359 [(MH + $C_2H_4$) - $H_2O$]$^+$ (3), 349 [M+H]$^+$ (5), 331 [(MH - $H_2O$]$^+$ (50), 289 (13), 313 (76), 233 (100), 201 (35). HR CIMS *m/z* obs. [M + H]$^+$ 349.2379 ($C_{21}H_{33}O_4$, 0.0 mmu dev.).

*Methyl Cymatherol C Acetate.* The free alcohol (<100 μg) was dissolved in 3-4 drops each of pyridine and acetic anhydride. After sitting at room temperature overnight, the sample was concentrated and the residue was dissolved in hexanes. GC EIMS 70 eV *m/z* (rel. int.): 330 [M - $CH_3CO_2H$]+ (0.6), 261 (5), 243 (5), 229 (11), 201 (18), 183 (14), 168 (100), 131 (42), 117 (74), 105 (50), 91 (92), 79 (98), 67 (46).

*Methyl Cymatherol A, C-16 Ketone* (**19**). Compound **16** (2.0 mg) was dissolved in 250 μL dry $CH_2Cl_2$. In another flask, pyridinium chlorochromate (PCC; 6 mg, 4.5 equiv.) and powdered sodium acetate (2.8 mg, 5.5 equiv.) were combined with 1 mL $CH_2Cl_2$. The sample solution was then added to the orange PCC solution via syringe. Rinses of the sample vial (3 x 250 μL dry $CH_2Cl_2$) were combined with the reaction

solution. The reaction mixture was stirred at room temperature for 1.5 h, then filtered through a short Florisil plug. Upon concentration, a colorless oil (1.9 mg, 95% yield) was obtained. FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3003, 2939, 1739, 1699, 1438, 1363, 1211, 1175, 1125, 842. $[\alpha]_D^{23}$ -140° (*c* 0.21, acetone). $^1H$ NMR ($CDCl_3$, 400 MHz) δ5.42 (1H, *dtd*, *J*=10.8, 7.3, 1.1 Hz, H-6), 5.11 (1H, *ddt*, *J*=10.8, 10.3, 1.5 Hz, H-7), 3.68 (3H, *s*, $OCH_3$), 3.52 (1H, *bd*, *J*=2.5 Hz, H-10), 3.22 (1H, *d*, *J*=2.5, H-9), 3.00 (1H, *d*, *J*=10.3 Hz, H-8), 2.51 (2H, *qd*, *J*=7.3, 1.2 Hz, H-17), 2.35 (2H, *t*, *J*=7.4 Hz, H-2), 2.17 (2H, *m*, H-5), 1.97 (2H, AB multiplet, H-11), 1.69 (1H, *m*, H-15), 1.68 (2H, *m*, H-3), 1.64 (1H, *m*, H-13), 1.45 (2H, *tt*, *J*=7.5, 7.5 Hz, H-4), 1.23 (1H, *ddd*, *J*=8.6, 4.6, 4.0 Hz, H-14a), 1.10 (1H, *m*, H-12), 1.08 (3H, *t*, *J*=7.3 Hz, H-18), 0.67 (1H, *ddd*, *J*=7.9, 6.4, 4.0 Hz, H-14b). GC EIMS 70 eV *m/z* (rel. int.): 304 (0.2), 289 $[M - OCH_3]^+$ (0.3), 273 (0.8), 263 (0.9), 245 (5), 235 (6), 206 (41), 175 (20), 157 (25), 133 (48), 119 (38), 105 (53), 96 (69), 91 (82), 79 (79), 67 (46), 57 (100).

*Methyl Cymatherol B, C-16 Ketone* (**19**). The C-16 epimer **17** (1.5 mg) was oxidized as above to yield 1.4 mg of the ketone **19** (93% yield). $[\alpha]_D^{25}$ -130° (c 0.16, acetone). The 400 MHz $^1H$ NMR spectrum was identical to the ketone derived from the open lactone. The GC EIMS spectrum was also identical. GC/MS co-injection of ketones from both open lactones under three different GC ramp conditions provided a single peak in all three cases (70-240°, 30°/min, $R_t$ = 7.56 min; 70-240°, 10°/min, $R_t$ = 16.83 min; 100-240°, 10°/min, $R_t$ = 13.84 min; all had an initial time of 30 seconds).

*Cymathere Lactone Dibromobenzoate* (**20**). Cymathere lactone **12** (11.9 mg) was dissolved in THF (0.4 mL) and 1M $HClO_4$ (0.2 mL). After stirring at room temperature for 16 h, the reaction mixture was diluted with 2 mL ether and then quenched with 0.5 mL saturated $NaHCO_3$ solution. The aqueous layer was extracted with three more portions of ether. The combined organics were washed twice with distilled water, then dried over $Na_2SO_4$, then over 4Å molecular sieves. The solution was filtered into a 25 mL flask and concentrated *in vacuo*. When the last visible signs of solvent were gone, the flask was

quickly removed from the rotary evaporator and briefly dried under a stream of $N_2$. The colorless oil was immediately dissolved in 3 mL dry $CH_2Cl_2$. (Two previous attempts to characterize the intermediate diol were unsuccessful. On the first try, the colorless oil darkened when $CHCl_3$ was added. On the second try, the oil turned gray after sitting on the rotary evaporator for several minutes after the last visible signs of solvent.) Several insoluble droplets were noticed on the walls of the flask. These were possibly water. Due to the potential instability of the diol when concentrated, the solution was directly submitted to the benzoate reaction conditions without further drying. Triethylamine (100 μL; 18 equiv.) and 4-bromobenzoyl chloride (90.7 mg; 10.6 equiv.) were added and the solution was stirred at room temperature. Within 10 minutes, a white precipitate had fallen out of solution. Another 1 mL of $CH_2Cl_2$ was added, but the white precipitate remained. TLC (30% E/H) monitoring of the reaction showed it to be sluggish, probably due to destruction of the acid chloride by the water present. After 27 h, more $CH_2Cl_2$ (2 mL), $Et_3N$ (100 μL), and 4-bromobenzoyl chloride (38.3 mg; 4.5 equiv.) were added. A few crystals of 4-dimethylaminopyridine catalyst were also introduced. Two more portions of acid chloride (37.6 mg; 4.4 equiv. total) were added before the reaction was quenched. After almost 3 days reaction time, the reaction mixture was diluted with hexanes (6 mL), which caused immediate formation of a white precipitate. The resulting mixture was directly loaded onto a short silica plug (2.1 cm I.D. x 4 cm), which was then eluted with 10% E/H. The fractions containing the desired compound (TLC 30% E/H, $R_f = 0.5$, UV-active, red-brown char with acid) were combined and concentrated. Final purification by HPLC (Phenomenex Maxsil 10 μ Silica, 500 mm x 10 mm, 10% E/H) provided a colorless oil (**20**, 17.3 mg, 66% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3073 (w), 2967, 2933, 1728, 1723, 1717, 1398, 1114, 1102, 1012, 756. $[\alpha]_D^{28}$ -85° (*c* 0.68, acetone). UV $\lambda_{max}^{EtOH}$ nm: 248 ($\varepsilon$ = 37,000). CD (EtOH): $\Delta\varepsilon$ = +5.6, -26.6 ($\lambda_{max}$= 236.5, 253 nm). $^1H$ NMR ($CDCl_3$, 400 MHz) δ7.90 (2H, *d*, *J*=8.5 Hz, aromatic CH *ortho* to carbonyl), 7.86 (2H, *d*, *J*=8.5 Hz, aromatic CH *ortho* to carbonyl), 7.59 (2H, *d*, *J*=8.5, aromatic CH *ortho* to

Br), 7.57 (2H, *d*, *J*=8.5, aromatic CH *ortho* to Br), 5.54 (1H, *ddd*, *J*=6.2, 6.2, 5.0 Hz, H-10), 5.48 (1H, *ddd*, *J*=10.4, 10.2, 4.5 Hz, H-6), 5.39 (1H, *dd*, *J*=10.4, 10.2 Hz, H-7), 5.30 (1H, *dd*, *J*=5.0, 5.0 Hz, H-9), 5.20 (1H, *ddd*, *J*=10.5, 4.8, 4.2 Hz, H-16), 3.12 (1H, *ddd*, *J*=10.2, 5.2, 5.2 Hz, H-8), 2.51 (1H, *m*, H-5a), 2.43 (2H, *m*, H-2), 2.26 (1H, *m*, H-11a), 2.17 (1H, *m*, H11b), 1.90 (1H, *m*, H-3a), 1.74 (1H, *m*, H5b), 1.63 (2H, *m*, H-3b, H-4a), 1.26 (2H, *m*, H-4b, H-12), 1.11 (3H, *m*, H-15, H-17a, H-17b), 1.05 (1H, *m*, H-13), 0.82 (3H, *t*, *J*=7.3 Hz, H-18), 0.56 (1H, *ddd*, *J*=8.4, 5.3, 5.3, H-14a), 0.32 (1H, *ddd*, *J*=9.1, 5.3, 5.2 Hz, H-14b). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ173.89 (C), 165.18 (C), 164.89 (C), 131.78 (CH; 4C), 131.18 (CH; 4C), 130.85 (CH), 130.62 (CH), 128.92 (C), 128.82 (C), 128.30 (C), 128.26 (C), 83.70 (CH), 78.60 (CH), 74.45 (CH), 47.91 (CH), 47.68 (CH), 35.93 ($CH_2$), 33.01 ($CH_2$), 28.19 ($CH_2$), 25.17 ($CH_2$), 24.28 ($CH_2$), 21.50 ($CH_2$), 20.75 (CH), 20.63 (CH), 10.26 ($CH_3$), 9.06 ($CH_2$). FABMS (3-nitrobenzyl alcohol) *m/z* (rel. intensity): 677 [M($^{81}$Br, $^{81}$Br) + H]$^+$ (2), 675 [M($^{81}$Br, $^{79}$Br) + H]$^+$ (4), 673 [M($^{79}$Br, $^{79}$Br) + H]$^+$ (2), 475 [MH - 4-bromobenzoic acid]$^+$ (6), 473 [MH - 4-bromobenzoic acid]$^+$ (6), 185 (99), 183 (100).

*Cymathere Lactone Diol, Bis-trimethylsilyl Ether.* The TMS ether derivative of the intermediate diol from the dibenzoate reaction was prepared as above for compound **1**. GC EIMS 70 eV *m/z* (rel. int.): 452 [M]$^+$ (1), 362 [M - TMSOH]$^+$ (4), 314 (47), 272 [M - 2 x TMSOH]$^+$ (3), 211 (32), 147 (45), 121 (22), 73 (100).

*Methyl Cymatherol A, R-Mosher Ester Derivative* (**21**). Compound **16** (1.5 mg) was dissolved in dry pyridine and 10 μL of *S*-α-methoxy-α-trifluoromethylphenylacetyl chloride was added. After 80 minutes, the reaction was stopped by the addition of one drop of $H_2O$. The reaction mixture was concentrated under a stream of $N_2$. The product was purified by flash chromatography (1.3 cm O.D. x 15 cm) using 10% EtOAc/hexanes. The *R*-Mosher ester derivative was a colorless oil (2.0 mg, 80% yield). FTIR $\nu_{max}^{film}$ cm$^{-1}$: 3064 (w), 3004, 2948, 1740, 1453, 1438, 1265, 1168, 1124, 1019, 992, 842, 717. $[\alpha]_D^{24}$ +1.5° (*c* 0.22, acetone). $^1$H NMR ($CDCl_3$, 400 MHz) δ7.58 (2H, *m*, aromatic H),

7.38 (3H, *m*, aromatic H), 5.40 (1H, *ddd*, *J*=10.6, 7.6, 7.6 Hz, H-6), 5.08 (1H, *bdd*, J=10.6, 10.2 Hz, H-7), 4.41 (1H, *ddd*, *J*=9.4, 8.4, 4.0 Hz, H-16), 3.65 (3H, *s*, ester $OCH_3$), 3.59 (3H, *d*, J=1.2 Hz, ether $OCH_3$), 3.53 (1H, *d*, *J*=2.6 Hz, H-10), 3.24 (1H, *d*, *J*=2.6 Hz, H-9), 3.03 (1H, *d*, *J*=10.2 Hz, H-8), 2.31 (2H, *t*, *J*=7.4 Hz, H-2), 2.14 (2H, *m*, AB pattern, H-5), 1.95 (2H, *d*, *J*=4.8 Hz, H-11), 1.85 (1H, *m*, H-17a), 1.75 (1H, *m*, H-17b), 1.65 (2H, *tt*, *J*=7.8, 7.4 Hz, H-3), 1.42 (2H, *tt*, *J*=7.8, 7.6 Hz, H-4), 1.06 (1H, *m*, H-13), 0.97 (1H, *ddd*, *J*=10.4, 5.2, 5.2 Hz, H-12), 0.87 (1H, m, H-15), 0.86 (3H, *t*, *J*=7.4 Hz, H-18), 0.66 (1H, *ddd*, *J*=8.5, 4.8, 4.5 Hz, H-14a), 0.31 (1H, *ddd*, *J*=8.1, 5.0, 4.8 Hz, H-14b). LR CIMS (methane) *m/z* (rel. int.): 539 $[M+H]^+$ (3), 305 (17), 287 (21), 207 (100).

*Methyl Cymatherol A, S-Mosher Ester Derivative* (**22**). Compound **16** (1.8 mg) was dissolved in dry pyridine and 10 µL of *R*-α-methoxy-α-trifluoromethylphenylacetyl chloride was added. After 80 minutes, the reaction was stopped by the addition of one drop of $H_2O$. The reaction mixture was concentrated under a stream of $N_2$. The product was purified by flash chromatography using 10% EtOAc/hexanes. The *S*-Mosher ester derivative was a colorless oil (2.8 mg, 93% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3066 (w), 3004, 2947, 1740, 1452, 1440, 1263, 1169, 1124, 1018, 992, 842, 717. $[\alpha]_D^{24}$ -45° (*c* 0.31, acetone). $^1H$ NMR ($CDCl_3$, 400 MHz) δ7.55 (2H, *m*, aromatic H), 7.39 (3H, *m*, aromatic H), 5.39 (1H, *ddd*, *J*=10.8, 7.5, 7.2 Hz, H-6), 5.06 (1H, *bdd*, J=10.8, 10.2 Hz, H-7), 4.39 (1H, *ddd*, *J*=9.4, 7.8, 4.4 Hz, H-16), 3.65 (3H, *s*, ester $OCH_3$), 3.56 (3H, *d*, J=0.7 Hz, ether $OCH_3$), 3.53 (1H, *d*, *J*=2.4 Hz, H-10), 3.24 (1H, *d*, *J*=2.4 Hz, H-9), 3.02 (1H, *d*, *J*=10.2 Hz, H-8), 2.32 (2H, *t*, *J*=7.5 Hz, H-2), 2.13 (2H, *m*, AB pattern, H-5), 1.93 (2H, *d*, *J*=4.9 Hz, H-11), 1.89 (1H, *m*, H-17a), 1.82 (1H, *m*, H-17b), 1.65 (2H, *tt*, *J*=7.8, 7.5 Hz, H-3), 1.41 (2H, *tt*, *J*=7.8, 7.5 Hz, H-4), 1.01 (1H, *m*, H-13), 1.00 (3H, *t*, *J*=7.4 Hz, H-18), 0.93 (1H, *ddd*, *J*=10.7, 4.8, 4.8 Hz, H-12), 0.76 (1H, m, H-15), 0.62 (1H, *ddd*, *J*=8.4, 4.8, 4.5 Hz, H-14a), 0.23 (1H, *ddd*, *J*=8.2,

5.0, 4.8 Hz, H-14b). LR CIMS (methane) *m/z* (rel. int.): 539 $[M+H]^+$ (2), 305 (12), 287 (18), 207 (100).

*Methyl Cymathere Ether A* (**26**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3435 (br. OH), 3007, 2964, 2933, 1740, 1438, 1210, 1174, 1154, 1078, 1015. $[\alpha]_D^{15}$=+39° (*c* 0.41, acetone). For $^1H$ and $^{13}C$ NMR, see Table III.5. LR EIMS (probe) 70 eV *m/z* (rel. int.): 322 $[M]^+$ (12), 304 $[M - H_2O]^+$ (7), 293 $[M - CH_2CH_3]^+$ (4), 291 $[M - OCH_3]^+$ (4), 253 (68), 235 (28), 185 (51), 180 (57), 149 45), 133 (34), 119 (44), 107 (46), 91 (50), 79 (71), 67 (93), 55 (100). HR EIMS *m/z* obs. $[M]^+$ 322.2143 ($C_{19}H_{30}O_4$, -0.1 mmu dev.).

*Methyl Tetrahydrocymathere Ether A*. Methyl cymathere ether A (**26**) (1.8 mg) was dissolved in 1 mL EtOH and 5% Pd on activated carbon (1.4 mg) was added. A hydrogen balloon was attached to the reaction vial via a septum. After stirring for 40 minutes, the reaction solution was filtered through a Celite plug and concentrated to obtain the hydrogenated product as a colorless oil (orange-red char with acid on TLC). $^1H$ NMR (400 MHz, $CD_3OD$): 3.81 (1H, *bs*, H-9), 3.75 (1H, *m*, H-10), 3.70 (1H, *bdd*, *J*=5.8, 5.0, H-13), 3.64 (3H, *s*, $OCH_3$), 2.31 (2H, *t*, *J*=7.5, H-2), 2.20 (1H, *bs*, H-12), 2.10 (1H, *m*, H-11b), 2.07 (1H, *m*, H-8), 1.65-1.50 (4H, *m*, H-3, H-7), 1.51 (1H, *ddd*, *J*=13.7, 3.8, 3.7 Hz, H-11a), 1.41 (1H, *m*, H-14a), 1.40-1.27 (13H, *m*, H4-6, H14b, H15-H17), 0.90 (3H, *t*, *J*=6.8, H-18). GC EIMS 70 eV *m/z* (rel. int.): 326 [M]+ (8), 308 $[M - H_2O]$+ (12), 281 (25), 277 (13), 265 (18), 251 (34), 237 (30), 180 (30), 154 (34), 139 (82), 109 (41), 95 (71), 83 (100), 81 (89), 69 (63), 67 (63), 55 (91).

*Methyl Cymathere Ether B* (**27**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3429 (br. OH), 3009, 2966, 2934, 1739, 1437, 1246, 1219, 1201, 1157, 1078, 1016, 666. $[\alpha]_D^{19.5}$=+51° (c=0.77, acetone). For $^1H$ and $^{13}C$ NMR, see Table III.5. LR EIMS (probe) 70 eV *m/z* (rel. int.): 348 $[M]^+$ (31), 330 $[M - H_2O]^+$ (4), 319 $[M - CH_2CH_3]^+$ (2), 317 $[M - OCH_3]^+$ (1), 279 (51), 261 (23), 247 (12), 243 (13), 229 (21), 219 (17), 133 (40), 105 (51), 91 (76), 84 (67), 79 (87), 67 (100). HR EIMS *m/z* obs. $[M]^+$ 348.2298 ($C_{21}H_{32}O_4$, -0.2 mmu dev.).

*Methyl Cymatherol D* (**28**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3411 (br. OH), 2952, 2933, 1737, 1212, 1175, 839. $[\alpha]_D^{23}$ 0° (*c* 0.1, acetone). Since five chiral centers are present in this structure, the lack of an observed optical rotation probably indicates a weak rotation that is not detectable at low concentration. $^1H$ NMR (300 MHz, $CDCl_3$): δ5.46 (2H, *m*, H-6, H-16), 5.30 (1H, *dtt*, *J*=10.9, 7.3, 1.5 Hz, H-15), 5.16 (1H, *ddt*, *J*=10.6, 10.3, 1.5 Hz, H-7), 3.78 (1H, *bs*, OH), 3.67 (3H, *s*, $OCH_3$), 3.57 (1H, *bdd*, *J*=2.7, 1.5 Hz, H-10), 3.46 (1H, *m*, H-13), 3.38 (1H, *bd*, *J*=10.3 Hz, H-8), 3.26 (1H, *d*, *J*=2.7 Hz, H-9), 2.34 (2H, *t*, *J*=7.4 Hz, H-2), 2.21 (4H, *m*, H-5, H-11a, H-14a), 2.04 (5H, *m*, H-11b, H-12, H-14b, H-17), 1.68 (2H, *tt*, *J*=7.9, 7.4 Hz, H-3), 1.44 (2H, *tt*, *J*=7.9, 7.4 Hz, H-4), 0.94 (3H, *t*, *J*=7.5 Hz, H-18). $^{13}C$ NMR (75.46 MHz, $CDCl_3$): δ134.10 (C-16), 131.20 (C-6), 128.37 (C-7), 124.78 (C-15), 74.08 (C-13), 62.43 (C-9), 59.03 (C-10), 51.52 ($OCH_3$), 47.40 (C-12), 37.92 (C-8), 34.29 (C-14), 33.91 (C-2), 32.68 (C-11), 28.98 (C-4), 27.26 (C-5), 24.58 (C-3), 20.64 (C-17), 14.22 (C-18). Shifts and multiplicities were obtained from a DEPT 135 experiment. Quaternary carbons (C-1) were not observed in this experiment. Assignments were based on related metabolites in this alga. GC EIMS 70 eV *m/z* (rel. int.): 273 $[M - OCH_3 - H_2O]^+$ (0.3), 253 (0.7), 235 (17), 203 (14), 185 (100), 175 (36), 157 (46), 133 (52), 119 (36), 105 (32), 91 (63), 79 (62), 67 (31), 55 (32). LR CIMS (methane) *m/z* (rel. int.): 351 $[MH + C_2H_4]^+$ (7), 333 $[(MH + C_2H_4) - H_2O]^+$ (3), 323 $[M+H]^+$ (15), 305 $[(MH - H_2O]^+$ (35), 287 (49), 273 (24), 207 (100), 185 (26). HR CIMS *m/z* obs. $[M + H]^+$ 323.2222 ($C_{19}H_{31}O_4$, 0.0 mmu dev.).

*Methyl Cymathere Ether A, R-Mosher Ester derivative* (**29**). Compound **26** (1.3 mg) was dissolved in dry pyridine and 7 μL of *S*-α-methoxy-α-trifluoromethylphenyl-acetyl chloride was added. After 80 minutes, the reaction was stopped by the addition of one drop of $H_2O$. The reaction mixture was concentrated under a stream of $N_2$. The product was purified by flash chromatography (1.3 cm O.D. x 20 cm) using 10% EtOAc/hexanes. The *R*-Mosher ester derivative was a colorless oil (1.4 mg, 65% yield).

FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2953, 2874, 1744, 1452, 1438, 1254, 1170, 1123, 1037, 718. $[\alpha]_D^{26}$ +24° (*c* 0.16, acetone). $^1H$ NMR ($CDCl_3$, 400 MHz) δ7.48 (2H, *m*, aromatic H), 7.39 (3H, *m*, aromatic H), 5.48 (1H, *bdt*, *J*=11.0, 7.3 Hz, H-16), 5.23 (2H, *m*, H-6, H-15), 5.17 (1H, *m*, H-7), 4.90 (1H, *m*, H-10), 4.09 (1H, *bs*, H-9), 3.88 (1H, *bdd*, *J*=7.3, 7.1 Hz, H-13), 3.67 (3H, *s*, ester $OCH_3$), 3.52 (3H, *s*, ether $OCH_3$), 3.06 (1H, *bd*, *J*=8.1 Hz, H-8), 2.391 (1H, *m*, H-11b), 2.37 (1H, *m*, H14a), 2.30 (2H, *t*, *J*=7.5 Hz, H-2), 2.29 (1H, *m*, H-12), 2.07 (5H, *m*, H-5, H-14b, H-17), 1.66 (1H, obscured *m*, H-11a), 1.61 (2H, *tt*, *J*=7.8, 7.4 Hz, H-3), 1.34 (2H, *tt*, *J*=7.8, 7.4 Hz, H-4), 0.98 (3H, *t*, *J*=7.5 Hz, H-18). GC EIMS 70 eV *m/z* (rel. int.): 538 $[M]^+$ (0.5), 507 $[M - OCH_3]^+$ (0.6), 469 $[M - CF_3]^+$ (4), 304 (6), 189 (100), 185 (39), 119 (33), 105 (40), 91 (35).

*Methyl Cymathere Ether A, S-Mosher Ester derivative* (**30**). Compound **26** (1.4 mg) was dissolved in dry pyridine and 7 μL of *R*-α-methoxy-α-trifluoromethylphenyl-acetyl chloride was added. The remaining steps were as above. The *S*-Mosher ester derivative was a colorless oil (2.1 mg, 91% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2954, 2874, 1742, 1452, 1437, 1254, 1168, 1123, 1034, 717. $[\alpha]_D^{26}$ -20° (*c* 0.23, acetone). $^1H$ NMR ($CDCl_3$, 400 MHz) δ7.48 (2H, *m*, aromatic H), 7.39 (3H, *m*, aromatic H), 5.48 (1H, *bdt*, *J*=10.8, 7.3 Hz, H-16), 5.27 (2H, *m*, H-6, H-7), 5.25 (1H, *m*, H-15), 4.92 (1H, *m*, H-10), 4.05 (1H, *bs*, H-9), 3.88 (1H, *bdd*, *J*=7.9, 6.6 Hz, H-13), 3.67 (3H, *s*, ester $OCH_3$), 3.50 (3H, *s*, ether $OCH_3$), 3.04 (1H, *bd*, *J*=5.3 Hz, H-8), 2.385 (1H, *m*, H-11b), 2.37 (1H, *m*, H-14a), 2.32 (1H, *m*, H-12), 2.30 (2H, *t*, *J*=7.4 Hz, H-2), 2.07 (5H, *m*, H-5, H-14b, H-17), 1.74 (1H, *ddd*, *J*=14.2, 3.9, 3.5 Hz, H-11a), 1.61 (2H, *tt*, *J*=7.8, 7.4 Hz, H-3), 1.34 (2H, *tt*, *J*=7.8, 7.4 Hz, H-4), 0.98 (3H, *t*, *J*=7.5 Hz, H-18). GC EIMS 70 eV *m/z* (rel. int.): 538 $[M]^+$ (0.7), 507 $[M - OCH_3]^+$ (0.6), 469 $[M - CF_3]^+$ (5), 304 (6), 189 (100), 185 (39), 119 (32), 105 (39), 91 (34).

*Ecklonialactone A, seco-Acid Methyl Ester* (**33**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: $[\alpha]_D^{23}$ -19° (*c* 0.36, acetone). See Table III.7 for $^1H$ and $^{13}C$ NMR data. GC EIMS 70 eV *m/z* (rel. int.): 322 $[M]^+$ (0.7), 275 $[M - H_2O - CH_2CH_3]^+$ (1), 246 (7), 145 (31), 131 (75), 117

(100), 105 (66), 91 (94), 79 (70), 67 (43), 59 (34). Comparison by GC/MS with the product from base hydrolysis of ecklonialactone A showed the same retention times and mass spectral patterns. Co-injection provided a single peak on the GC trace. LR CIMS (methane) *m/z* (rel. int.): 351 $[MH + C_2H_4]^+$ (2), 333 $[(MH + C_2H_4) - H_2O]^+$ (3), 323 $[M+H]^+$ (12), 305 $[(MH - H_2O]^+$ (19), 287 (31), 273 (28), 247 (100), 215 (26). HR CIMS *m/z* obs. $[M + H]^+$ 323.2222 ($C_{19}H_{31}O_4$, 0.0 mmu dev.).

*Tetrahydroecklonialactone A, seco-Acid Methyl Ester.* A small amount (~100 μg) of compound **33** was dissolved in 200 μL of EtOH and a pinch of 5% Pd on activated carbon was added. A hydrogen balloon was attached to the reaction vial via a septum. After 15 minutes, the reaction mixture was filtered through Celite. The resulting solution was concentrated and the residue was dissolved in 500 μL hexanes for GC/MS analysis. GC EIMS 70 eV *m/z* (rel. int.): 308 $[M - H_2O]^+$ (0.8), 297 $[M - CH_2CH_3]^+$ (2), 279 $[M - H_2O - CH_2CH_3]^+$ (10), 277 $[M - H_2O - OCH_3]^+$ (6), 261 (10), 251 (12), 247 (30), 236 (16), 219 (8), 201 (9), 135 (28), 121 (38), 109 (33), 97 (74), 95 (72), 83 (91), 80 (100), 67 (62), 55 (58).

*Ecklonialactone B, seco-Acid Methyl Ester* (**34**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3428 (br. OH), 2929, 2856, 1740, 1462, 1438, 1247, 1198, 1172, 840. $[\alpha]_D^{29}$ -27° (*c* 1.1, acetone). See Table III.7 for $^1H$ and $^{13}C$ NMR data. GC EIMS 70 eV *m/z* (rel. int.): 306 $[M - H_2O]^+$ (0.7), 277 $[M - H_2O - CH_2CH_3]^+$ (3), 275 $[M - OCH_3]^+$ (8), 249 (22), 248 (21), 222 (23), 133 (38), 119 (78), 105 (69), 93 (100), 81 (76), 80 (78), 79 (79), 67 (55), 55 (53). LR CIMS (methane) *m/z* (rel. int.): 353 $[MH + C_2H_4]^+$ (0.7), 335 $[(MH + C_2H_4) - H_2O]^+$ (2), 325 $[M+H]^+$ (7), 307 $[(MH - H_2O]^+$ (10), 289 (13), 275 (29), 249 (100), 227 (10). HR CIMS *m/z* obs. $[M + H]^+$ 325.2378 ($C_{19}H_{33}O_4$, -0.1 mmu dev.).

*Dihydroecklonialactone B, seco-Acid Methyl Ester.* Prepared in the same manner as hydrogenated **33** above. GC/MS analysis yielded a peak with an identical mass spectrum as above. Co-injection of the two hydrogenated compounds yielded a single peak ($R_t$ = 14.67 min; 100°-240°, 30 sec initial delay, then 10°/min, 5 min final).

*Ecklonialactone B, seco-Acid Methyl Ester, C-16 Ketone.* The colorless oil **34** (<100 μg) was dissolved in 100 μL $CH_2Cl_2$. Small amounts of pyridinium chlorochromate and powdered NaOAc were dissolved in 200 μl $CH_2Cl_2$. The substrate solution was added to the orange PCC solution via syringe. After 1.5 h, the reaction mixture was filtered through a Florisil plug. The resulting solution was concentrated and dissolved in 500 μl hexanes for GC/MS analysis. GC EIMS 70 eV *m/z* (rel. int.): 322 [M]+ (0.2), 304 [M - $H_2O$]$^+$ (1.4), 293 [M - $CH_2CH_3$]$^+$ (0.7), 290 [M - $CH_3OH$]$^+$ (1.7), 247 (15), 233 (7), 165 (8), 147 (20), 133 (18), 119 (55), 105 (58), 92 (56), 80 (42), 57 (100).

*Erythro Hepoxilin $B_3$ Methyl Ester* (**35**) and *threo Hepoxilin $B_3$ Methyl Ester* (**36**). The mixture of these compounds was examined by $^1$H NMR ($CDCl_3$) and the TMS ethers were analyzed by GC/MS. Under the GC conditions used (100°-240°, 30 sec initial delay, then 10°/min, 5 min final), the TMS ethers of both diastereomers eluted at the same retention time (15.40 min). Co-injection with authentic samples of the *threo* and *erythro* isomers[56] confirmed that the hepoxilins were the dominant components of the mixture. The mixture and the standards were hydrogenated ($H_2$, 5% Pd/C, EtOH) and then the TMS ethers were formed. Under the same GC conditions used above, the hydrogenated TMS ether diastereomers separated cleanly, with the *threo* isomer eluting at 16.23 min and the *erythro* isomer eluting at 16.41 min. Co-injection with the hydrogenated standards confirmed that both isomers were present. With these hydrogenated compounds, a *threo/erythro* ratio of 3:2 was observed. This ratio roughly agrees with integration from the $^1$H NMR spectrum.

# CHAPTER IV

## SARCOLACTONES A AND B, NEW EICOSANOIDS FROM THE RED ALGA *SARCODIOTHECA GAUDICHAUDII*


### Abstract

The red alga *Sarcodiotheca gaudichaudii* has been examined for its oxylipin metabolism. From non-polar vacuum chromatography fractions, two new 11-membered ring lactone compounds have been isolated. These compounds incorporate a *cis* epoxide into the macrolactone ring and formally appear to be lactones of the known hydroxy epoxy hepoxilin-type compounds. In addition to the lactones, 8*R*-HETE, 8*R*-HEPE, and a previously unreported aldehyde, trideca-2*E*, 4*E*, 7*Z*, 10*Z*-tetraenal, have been identified from the crude extract. All of these metabolites suggest the presence of an 8-lipoxygenase in this alga, a specificity not observed in the algae to date.

## Introduction

*Sarcodiotheca gaudichaudii* (Montagne) Gabrielson (Gigartinales, Solieriaceae) is a red alga found subtidally from Baja California to southeastern Alaska, in the Galapagos Islands, and along the coast of Chile.[110] *S. gaudichaudii* was called *Neoagardhiella baileyi* and *N. gaudichaudii* in earlier taxonomic works.[133] It has not been investigated before for its secondary metabolite production.

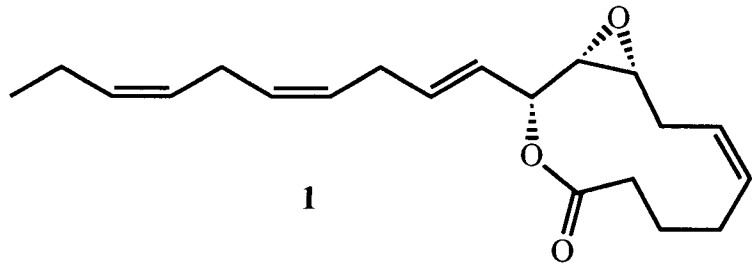
This alga was collected initially as part of a general survey of Pacific Northwest seaweeds. After preliminary positive testing results in protein kinase C and inosine monophosphate dehydrogenase assays at Syntex, the alga was recollected in larger quantity for pursuing the active chemistry.

Despite uninteresting levels of activity after fractionation of the crude extract, several oxylipins were discovered. Two new 11-membered ring epoxy lactones named sarcolactones A and B were isolated and characterized, along with 8*R*-HETE, 8*R*-HEPE, and a $C_{13}$ aldehyde. An 8-lipoxygenase is indicated by these metabolites. This positional specificity has not been observed before in the algae.[49]

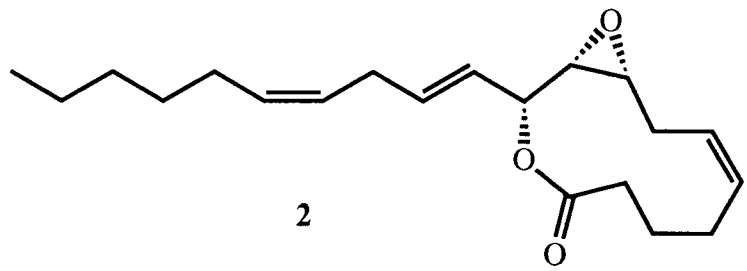
## Results and Discussion

*Sarcodiotheca gaudichaudii* was collected at Octopus Hole in the state of Washington in August, 1992. The crude 2:1 $CHCl_3$/MeOH extract was fractionated by vacuum chromatography using an increasing gradient of EtOAc in hexanes. A non-polar fraction (weakly active in the IMPDH assay) was found by TLC to have a blue char reaction upon treatment with 50% $H_2SO_4$, suggesting the presence of oxylipins. A crude $^1H$ NMR confirmed that fatty acid derived metabolites were present in this fraction and suggested that an epoxide might be present in the structure based on peaks at 3.07 and 3.25 ppm which had small coupling constants. Purification of this material utilized two successive vacuum silica chromatographies to remove the bulk of pigments and some fatty acids. Even at this stage, fatty acids were still interfering with the chromatography, so the enriched sample was methylated with diazomethane, and then loaded onto a Chromatotron. Elution with 5% EtOAc/hexanes provided several fractions enriched with both blue and purple charring substances on TLC. Normal phase silica HPLC using 5% EtOAc/hexanes provided pure sarcolactone A (**1**, blue char; 0.8% of crude extract) and sarcolactone B (**2**, purple char) contaminated with a small amount of triglycerides. The triglycerides were removed by Sephadex LH-20 chromatography to provide pure sarcolactone B (0.3%).

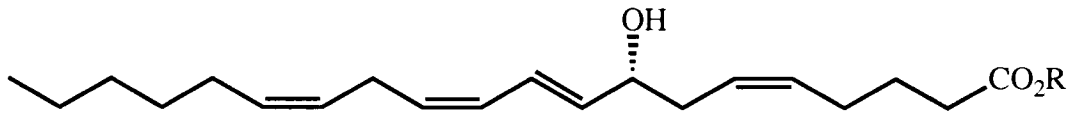
The structure of sarcolactone A was constructed using data from NMR experiments ($^1H$, $^{13}C$, DEPT 135, $^1H$-$^1H$ COSY, $^1H$-$^1H$ LRCOSY, $^1H$-$^{13}C$ XHCORR, NOESY; Table IV.1, Figure IV.2, IV.3, and IV.4). The $^{13}C$ NMR spectrum indicated that twenty carbon atoms were present and this was confirmed by HR CIMS ($C_{20}H_{28}O_3$). Four olefins and one ester carbonyl accounted for five of the seven degrees of unsaturation inherent in the molecular formula. The remaining two degrees of unsaturation were due to rings. The H2-H20 spin system was constructed directly from the COSY spectra. Small homoallylic couplings from H-4 to H-7 and H-16 to H-19 eliminate the problems caused



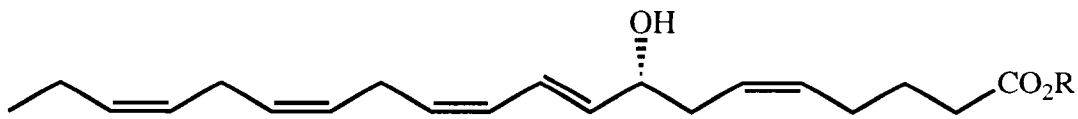
**1**



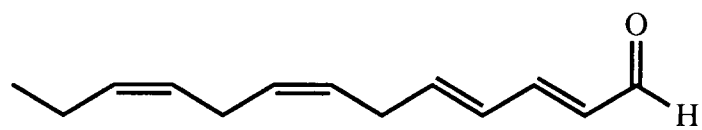
**2**



R = H **8**
R = $CH_3$ **10**



R = H **9**
R = $CH_3$ **11**



**12**

Figure IV.1. Oxylipins from *Sarcodiotheca gaudichaudii*.

Table IV.1. NMR Data for Sarcolactones A (**1**) and B (**2**).

| C# | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
|---|---|---|---|---|
| 1 | 172.82 | --- | 172.86 | --- |
| 2 | 35.21 | 2.40, ddd (13.3, 5.5, 3.8) | 35.23 | 2.39, ddd (13.5, 5.3, 4.0) |
| | | 2.25, m | | 2.23, m |
| 3 | 24.37 | 1.81, m | 24.39 | 1.81, m |
| 4 | 27.66 | 2.25, m | 27.68 | 2.23, m |
| | | 2.11, m | | 2.11, m |
| 5 | 129.80 | 5.43-5.33, m | 129.82 | 5.38, m |
| 6 | 124.54 | 5.43-5.33, m | 124.54 | 5.38, m |
| 7 | 27.21 | 2.69, m | 27.22 | 2.69, m |
| 8 | 56.43 | 3.24, ddd (8.6, 6.8, 4.3) | 56.44 | 3.25, ddd (8.6, 6.8, 4.3) |
| 9 | 57.34 | 3.06, dd (4.3, 1.6) | 57.38 | 3.06, dd (4.3, 1.6) |
| 10 | 68.62 | 5.70, dd (7.2, 1.6) | 68.68 | 5.70, dd (7.2, 1.6) |
| 11 | 125.80 | 5.60, ddt (14.7, 7.2, 1.5) | 125.60 | 5.63, ddt (15.0, 7.2, 1.5) |
| 12 | 133.53 | 5.86, dt (14.7, 6.1) | 133.91 | 5.85, dt (15.0, 6.1) |
| 13 | 29.94 | 2.85, dd (6.3, 6.1) | 29.99 | 2.81, bdd (6.8, 6.1) |
| 14 | 126.15 | 5.43-5.33, m | 125.87 | 5.36, dtt (10.8, 7.1, 1.3) |
| 15 | 129.76 | 5.45, m | 131.70 | 5.46, dtt (10.8, 7.1, 1.4) |
| 16 | 25.45 | 2.77, dd (6.5, 6.5) | 27.12 | 2.01, bdt (7.1, 6.5) |
| 17 | 126.81 | 5.29, dtt (10.6, 7.1, 1.4) | 29.21 | 1.28, m |
| 18 | 132.09 | 5.43-5.33, m | 31.46 | 1.28, m |
| 19 | 20.52 | 2.06, qd (7.5, 7.2) | 22.52 | 1.28, m |
| 20 | 14.22 | 0.97, t (7.5) | 14.04 | 0.88, distorted t (6.8) |

Spectra recorded in $CDCl_3$ at 300.13 MHz for $^{1}H$ and 75.46 MHz for $^{13}C$ NMR. Referenced to TMS at 0.00 ppm for $^{1}H$ and to $CDCl_3$ centerline at 77.00 ppm for $^{13}C$ spectra. Assignments based on XHCORR experments.

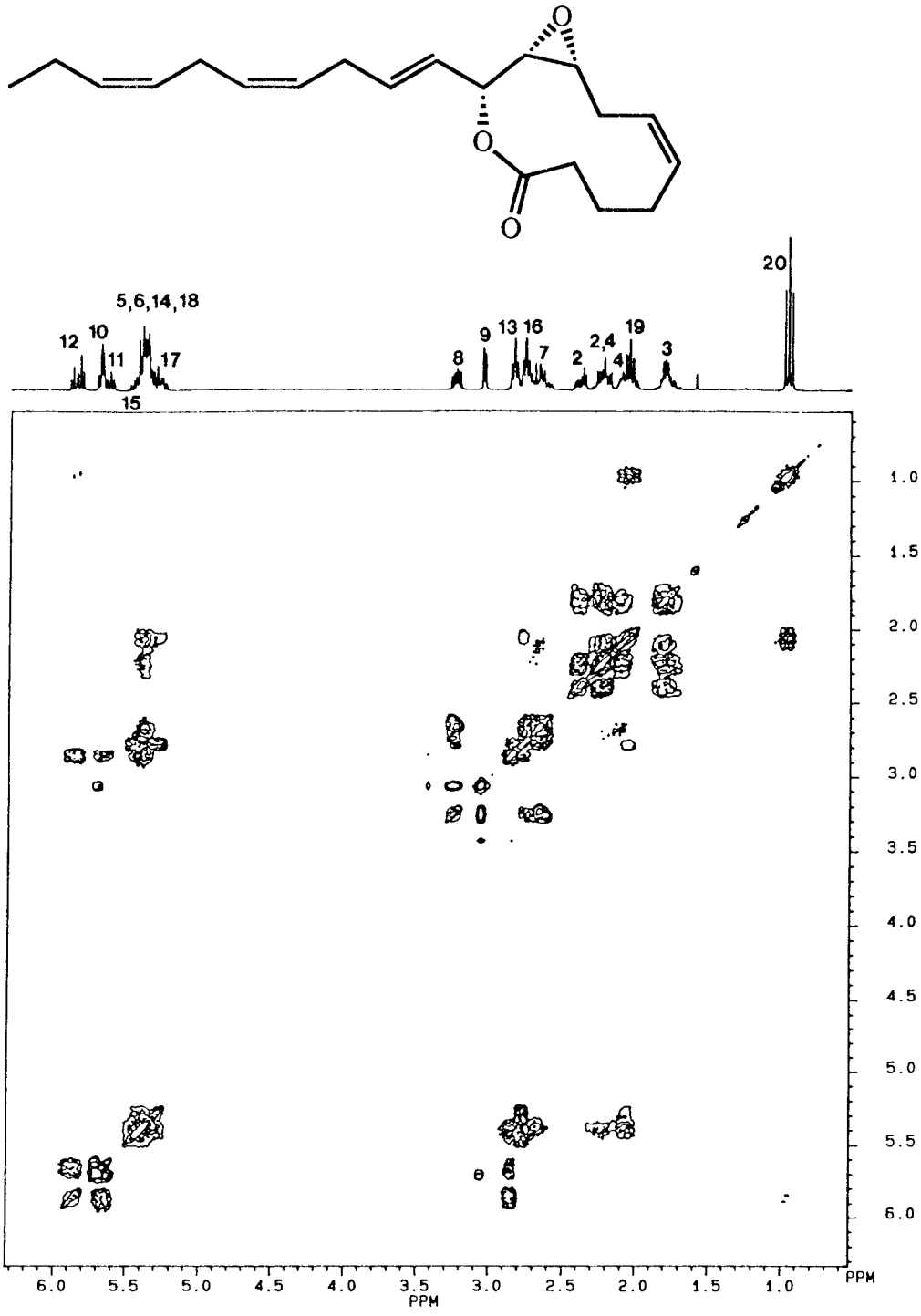



Figure IV.2. COSY Spectrum of Sarcolactone A (**1**).

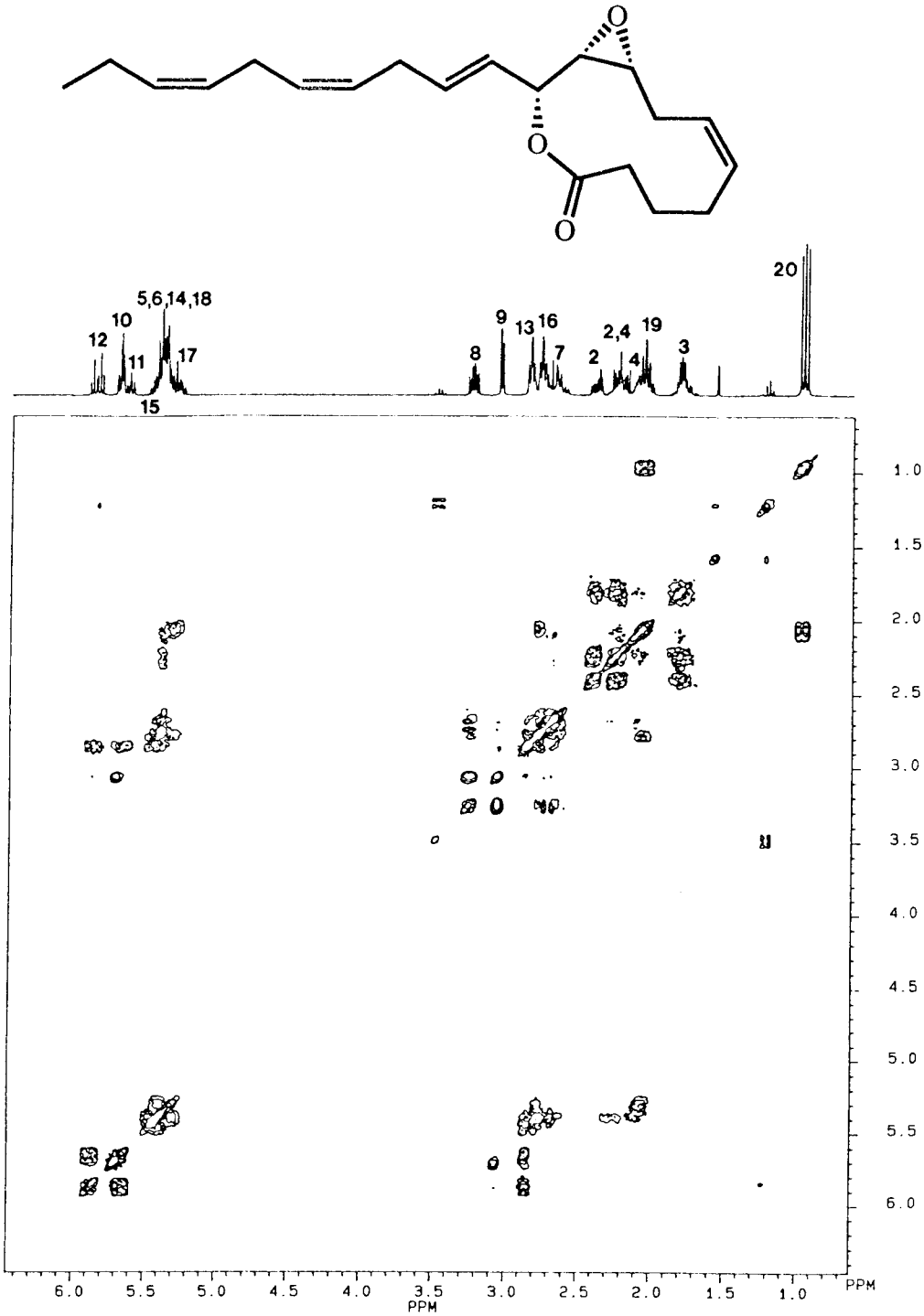



Figure IV.3. Long-range COSY Spectrum of Sarcolactone A (**1**).

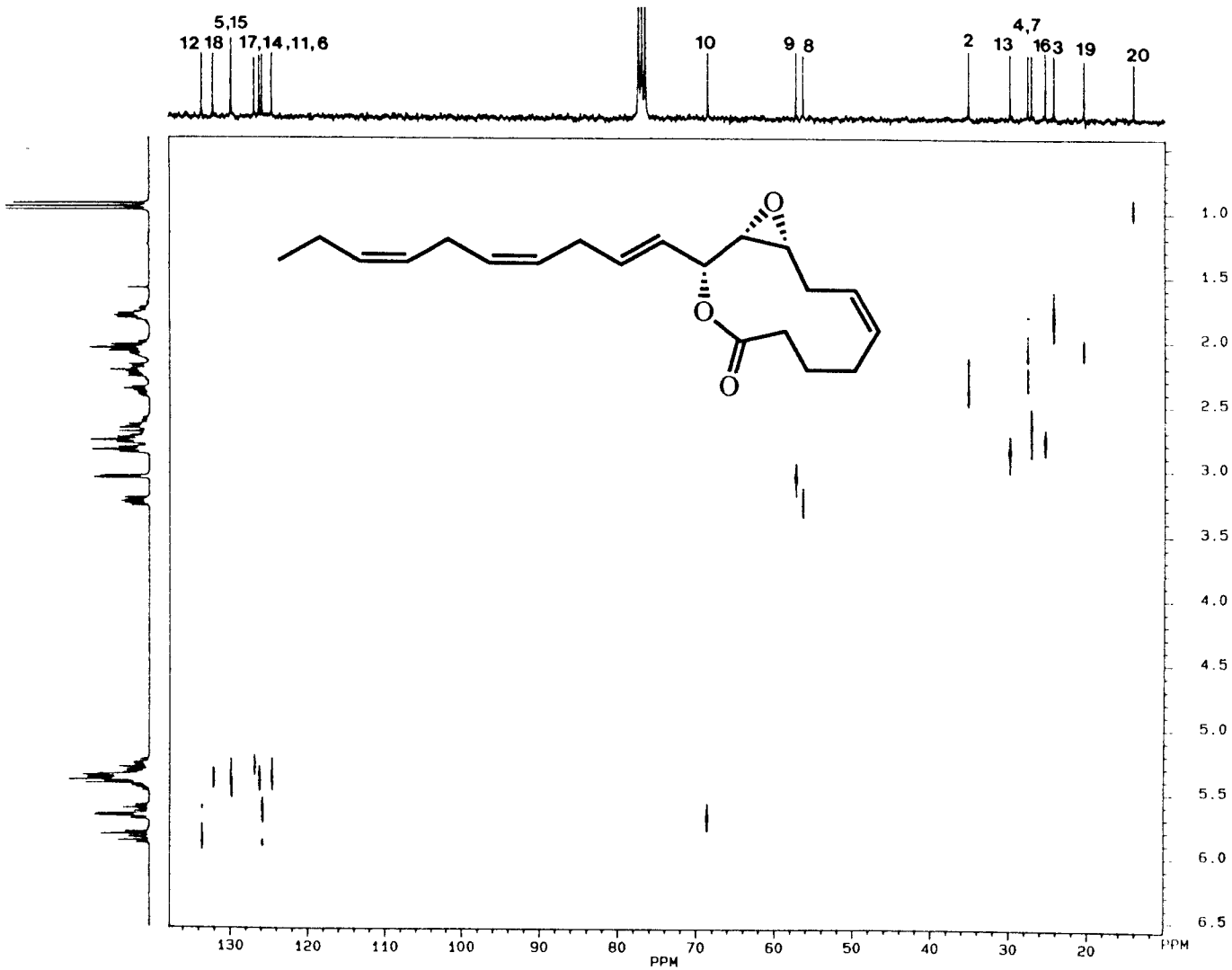



Figure IV.4. XHCORR Spectrum of Sarcolactone A (**1**).

by overlap in the olefinic region. The $^{1}H$ chemical shifts of the H-8, H-9, and H-10 protons (3.24, 3.06, and 5.70 ppm) indicated that the carbons bearing these protons were oxygenated. The carbon shifts for C-8 (56.43 ppm) and C-9 (57.34 ppm), combined with the $^{1}H$ NMR shifts, indicate an epoxide ring. The $^{1}H$ NMR shift (2.40 ppm) and the corresponding $^{13}C$ NMR shift (35.21 ppm) place the H-2 protons adjacent to an ester carbonyl. Since all of the protons and carbons required by the molecular formula have been accounted for, the last connection forms a lactone from C-1 to C-10. The geometries of three of the olefins can be determined by coupling constants (H11-12 *trans*, $J$=14.7 Hz; H14-15 *cis*, $J$=10.7 Hz; H17-18 *cis*, $J$=10.6 Hz). The remaining olefin was assigned the *cis* geometry based on the $^{13}C$ NMR shifts of the adjacent methylenes.[92] A 4.3 Hz coupling constant for the epoxide protons indicated a *cis* geometry.[134]

The remaining relative stereochemical element was the *threo* or *erythro* arrangement of the ester oxygen at C-10 and the epoxide oxygen at C-9. The coupling constant between H-9 and H-10 was 1.6 Hz. This small coupling constant suggested a dihedral angle between these two protons of near 60° or 115° according to the Karplus curve. Molecular modeling of the two possible structures, with truncated side chains, using CHEM3D Plus™ provided a low energy *threo* conformer that had a dihedral angle near 70°. The lowest energy *erythro* conformer obtained had a dihedral angle near 150°. The *threo* structure was more consistent with the coupling constant data.

The approach chosen to verify this modeling prediction took advantage of well determined models of 1,2- and 1,3-diol acetonides. The *threo* or *erythro* nature of the acetonides in either series of diol can be readily assessed by both $^{1}H$ and $^{13}C$ NMR data.[135-139] The chemical shifts of the methyl groups are used as indicators of the relative stereochemistry of the diols. In general, the methyl groups of the *threo* acetonides have the same chemical shift or very similar chemical shifts and the methyl groups of the *erythro* acetonides have distinct shifts, the difference being 1.5 to 3 ppm for 1,2-diol acetonides[136] and ~10 ppm for 1,3-diol acetonides in the $^{13}C$ NMR spectrum.[137-139] The

same trends are seen by $^1$H NMR, but the shift differences are of smaller magnitude (0.1-0.2 ppm).[135] The ability to use either the 1,2- or the 1,3-diol acetonide for this analysis lessens the requirement for regiospecific opening of the epoxide with hydride.

To implement this plan, sarcolactone A was first hydrogenated. GC/MS analysis indicated that two products were formed in approximately an 85:15 ratio. The major product was determined to be the desired perhydrosarcolactone A (**3**) based on $^1$H NMR patterns, decoupling experiments, and mass spectral data. Specifically, protons at 3.30 ppm and 2.92 ppm with a coupling constant of 4.1 Hz between them indicated that the epoxide was still present. The only other downfield peak, at 5.29 ppm, was coupled to the the 3.30 ppm signal. The absence of an OH stretch in the IR spectrum, a carbonyl stretch at 1733 cm$^{-1}$, and the chemical shift of the H-10 proton (5.29 ppm) confirmed that the lactone was intact. A significant $[M+H]^+$ 325 (35%) in the CI mass spectrum supported the addition of 8 hydrogens to sarcolactone A.

The minor component was identified as the saturated lactone with the epoxide hydrogenolytically cleaved to give the C-9 alcohol **4**. The presence of the alcohol was indicated by a broad OH stretch in the FTIR spectrum and by the ability of the compound to form a TMS ether derivative. Low resolution EI mass spectral analysis of the free alcohol showed a very weak molecular ion at *m/z* 326, an appropriate mass if the epoxide were reductively opened. Mass spectral data for the TMS ether gave the required molecular ion at *m/z* 398 in EI mode and $[M+H]^+$ 399 in CI mode. The $^1$H NMR spectrum of this derivative showed two downfield patterns, one at 4.82 ppm and the other at 3.95 ppm. Decoupling experiments in which both H-9 and H-10 were irradiated verified that these two proton signals were coupled to each other. If the C-8 alcohol had been formed, these downfield signals would not have been coupled.

The production of this opened epoxide species **4** had not been anticipated, but with the compound in hand, a new data point could be added to our molecular modeling approach to the relative stereochemistry. Again, the *threo* and *erythro* models, with the

side chain truncated to a butyl group, were energy minimized using the MM2 package of CHEM3D Plus™. The minimum energy *threo* conformer had a dihedral angle of 46°, while the *erythro* minimum energy structure had a 161° dihedral. The measured 3.1 Hz coupling constant again agrees better with the *threo* model.

The two hydrogenated species (**3** and **4**) were combined and reduced with lithium aluminum hydride (Figure IV.5). The crude product was subjected to conditions to form an acetonide (2,2-dimethoxypropane, *p*-toluene sulfonic acid). Analysis by GC/MS of the major product from this reaction showed a significant fragment ion at *m/z* 395. This was 25 mass units higher than the mass of the expected product! A possible explanation for this result was that the desired acetonide had formed, but a mixed acetonide also may have formed between the terminal alcohol and the reagent. A mixed acetonide of this nature would have a molecular ion at *m/z* 442. Loss of a methyl group and methanol from such a structure would provide the observed *m/z* 395. Based on the assumption that this was the product, this material was treated with ammonium chloride in aqueous acetone. This mild hydrolysis treatment provided a product **5** that gave a major ion at *m/z* 355 by GC/MS. Facile loss of a methyl group from the desired acetonide would produce this ion. High resolution mass spectral data confirmed that this product was consistent with the desired acetonide. The $^{1}H$ NMR data showed a single methyl peak at $\delta 1.38$[135] and the $^{13}C$ NMR spectrum also had a single methyl carbon line (determined by a DEPT experiment) at 27.34 ppm. These data support a *threo* 1,2-diol structure as determined by comparison to model compounds (Figure IV.6).[136] That the acetonide product **5** was indeed the 1,2-diol acetonide versus the 1,3-diol acetonide relied on $^{13}C$ NMR data. The shift for the ketal carbon for a 1,2-diol acetonide is ~107 ppm[136,140] and the shift for the 1,3-diol-derived ketal carbon is 98-101 ppm.[138-140] The product obtained had a $^{13}C$ NMR shift at 107.71 ppm, confirming the 5-membered ring structure. The relative stereochemistry of sarcolactone A was therefore established as *threo*.

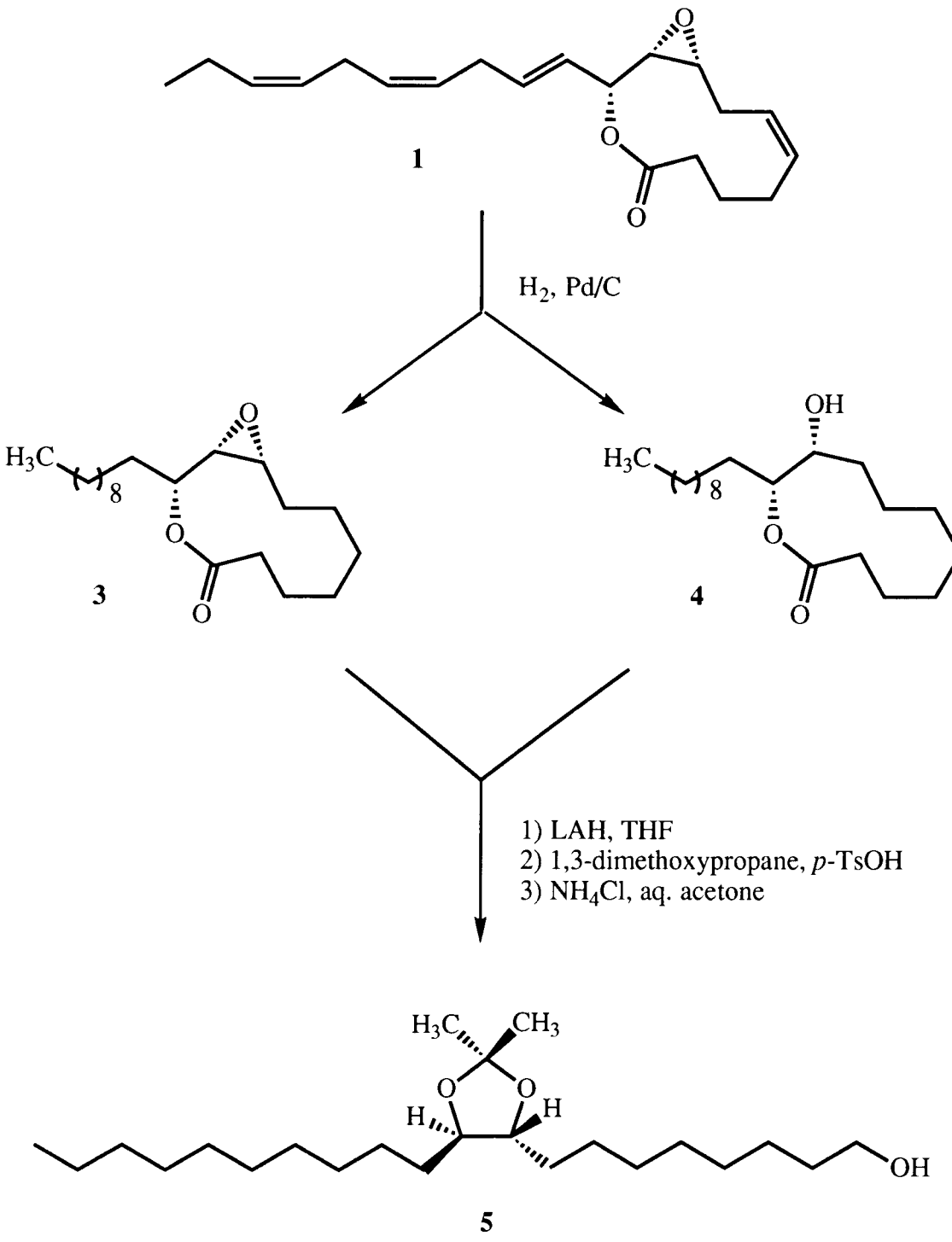



Figure IV.5. Formation of Sarcolactone A-Derived 1,2-Diol Acetonide.

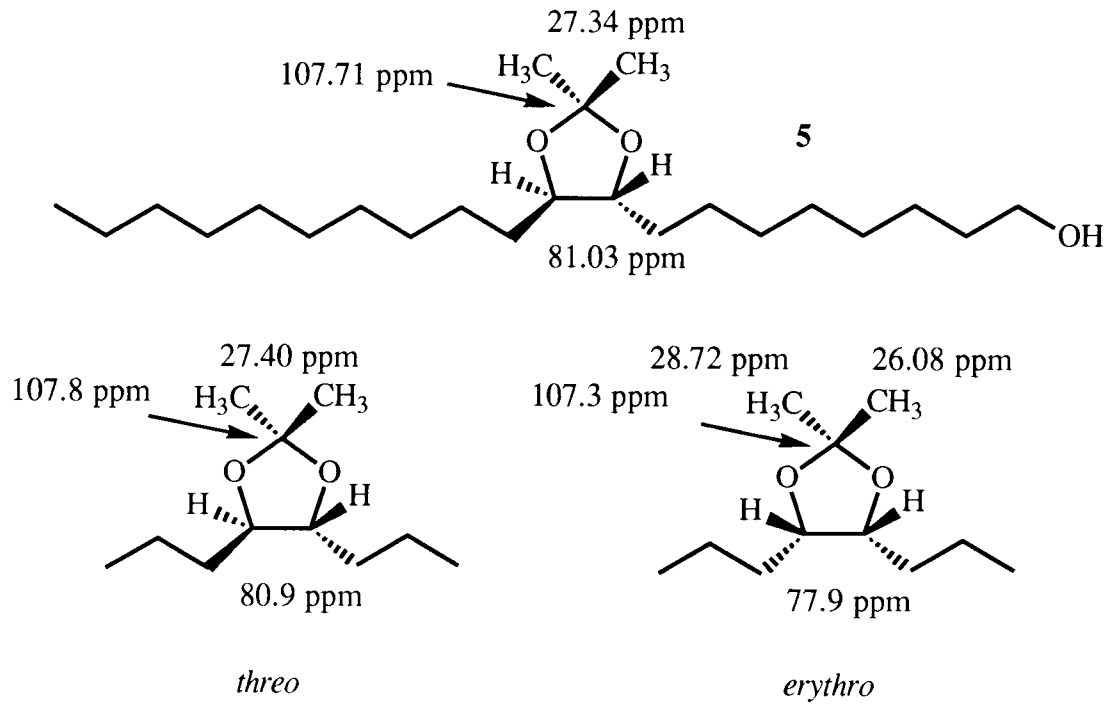



Figure IV.6. Sarcolactone A-Derived 1,2-Diol Acetonide and *threo* and *erythro* Model Compounds.[136]

The absolute stereochemistry was established by the exciton chirality method with a 4-bromobenzoate derivative. Sharpless and Nakanishi have shown that allylic alcohol absolute stereochemistry can be determined via this method.[116] Sarcolactone A was opened to the *seco* acid with 5% KOH in methanol. The acid was treated directly with diazomethane to make the methyl ester **6**. The alcohol at C-10 was then converted into the 4-bromobenzoate **7** with 4-bromobenzoyl chloride (Figure IV.7). Circular dichroic analysis of this product showed a positive first Cotton effect which indicates a 10*R* stereochemistry (Figure IV.8). The overall stereochemistry for sarcolactone A is therefore 8*R*, 9*R*, 10*R*.

Sarcolactone B (**2**) was shown to be the H17-H18 dihydro analog of sarcolactone A by NMR experiments ($^{1}H$, $^{13}C$, DEPT 135, $^{1}H$-$^{1}H$ COSY, $^{1}H$-$^{13}C$ XHCORR; Table IV.1). Sarcolactone B was also hydrogenated and compared with the hydrogenated sarcolactone A by GC/MS. The two hydrogenated products had identical retention times and mass spectral profiles. The same relative and absolute stereochemical assignments for sarcolactones A and B are based on similarities of NMR shift data (Table IV.1) and sign and magnitude of optical rotations. Both had rotations of -160°.

The sarcolactones superficially appear to be the lactone equivalents of known hydroxy epoxy hepoxilin-type compounds.[131] There are two important differences though. The epoxide in the sarcolactones is *cis* and the olefin adjacent to the alcoholic center is *trans*. This is just the opposite of that observed for the hepoxilins. These differences indicate that distinct enzymes are operative in this alga to produce these compounds.

The sarcolactones are not the only oxylipins produced by *Sarcodiotheca gaudichaudii*. Both 8*R*-hydroxyeicosa-5*Z*, 9*E*, 11*Z*, 14*Z* -tetraenoic (8*R*-HETE) and 8*R*-hydroxyeicosa-5*Z*, 9*E*, 11*Z*, 14*Z*, 17*Z*-pentaenoic acids (8*R*-HEPE) (**8**, **9**) were isolated as methyl ester derivatives (**10**, **11**). Their structures were characterized by $^{1}H$ NMR, $^{1}H$-$^{1}H$ COSY, UV, and GC/MS of the TMS ether derivatives. Once a preliminary

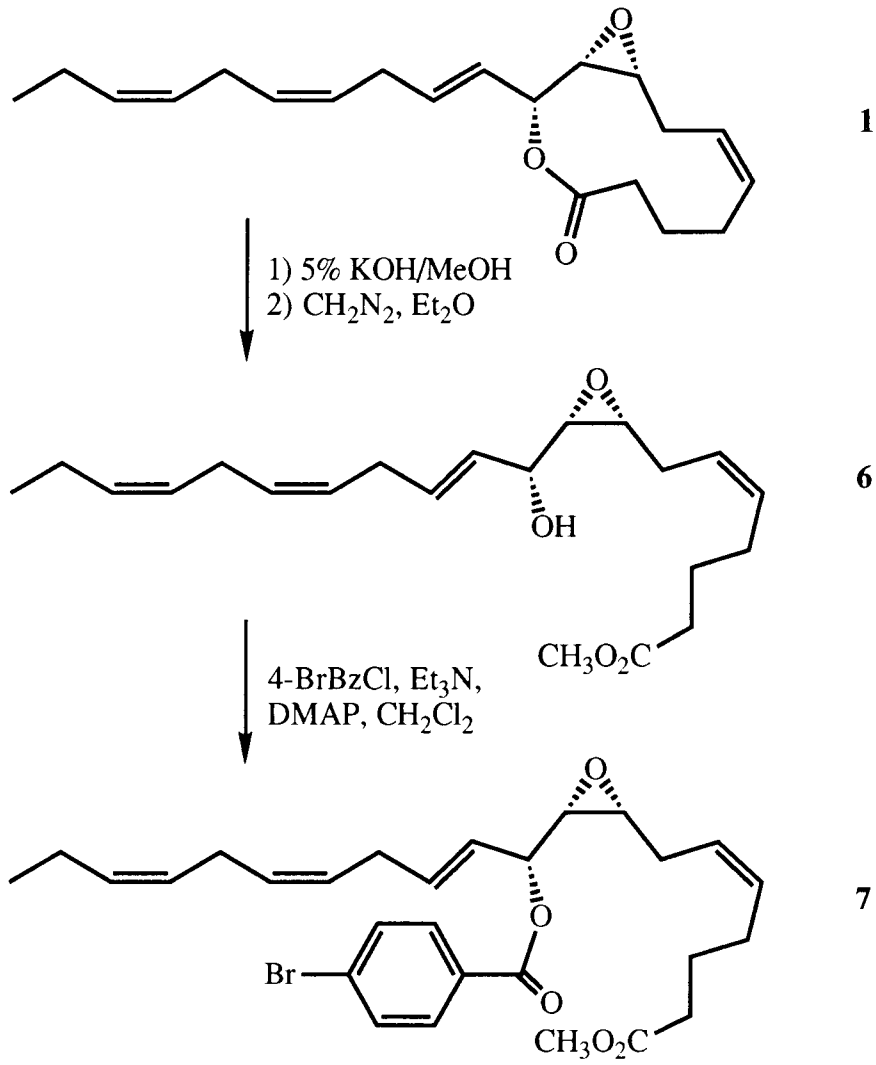



Figure IV.7. Preparation of the 4-Bromobenzoate Derivative of Sarcolactone A.

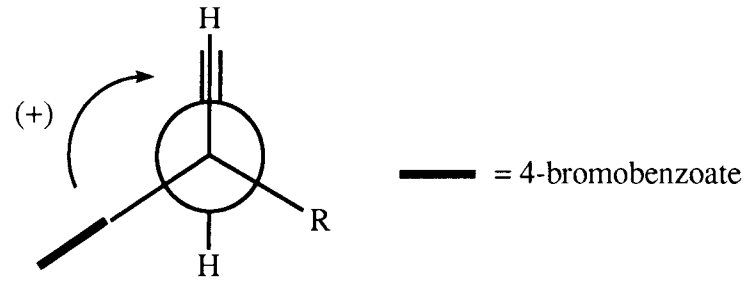



Figure IV.8. Model Showing Preferred Conformer and Arrangement of Benzoate and Olefin Required to Obtain a Positive Cotton Effect.[116]

identification of the structures was obtained, comparison of $^1$H NMR data and mass spectral fragmentation patterns of the TMS ether derivatives with literature values allowed confirmation of their identity.[141-143] The absolute stereochemistries were determined by preparing the menthoxycarbonate derivatives,[105] cleavage of the malate fragment using oxidative ozonolysis, and then GC analysis of the methyl esters versus malate standards.[33] Both hydroxy acids were found to be of the *R* configuration.

Another compound, trideca-2*E*, 4*E*, 7*Z*, 10*Z*-tetraenal (**12**), was also isolated from this alga. The linear structure was assembled in a straightforward manner from $^1$H-$^1$H COSY data. The conjugated dienal unit was supported by a UV $\lambda_{max}$ at 274 nm. The olefin geometries were determined by $^1$H-$^1$H coupling constants. Additional support for the structure was obtained by preparing the methoxime derivative and observing the expected molecular ion at *m/z* 219. The analogous 13-carbon aldehyde with the 10*Z* double bond saturated has been isolated from cell free incubations of starfish homogenates.[43]

The five oxylipins isolated from *Sarcodiotheca* can be correlated via a biogenetic hypothesis (Figure IV.9). Initial action of an 8-lipoxygenase on eicosapentaenoic acid would provide the hydroperoxy acid. This hydroperoxide can follow several possible pathways. Reduction would lead to 8-HEPE (**9**). Hydroperoxide lyase action on the hydroperoxide could produce the tridecatetraenal **12**. Rearrangement of the hydroperoxides to the C8-C9 epoxy cation, followed by trapping with the carboxylate would result in the formation of sarcolactones A (**1**). An analogous pathway starting with arachidonic acid would lead to 8-HETE (**8**) and sarcolactone B (**2**). The co-isolation of 8*R*-HETE and 8*R*-HEPE and the fact that the sarcolactones have 8*R* stereochemistry are strongly suggestive that these lactone compounds are initiated by an 8*R*-lipoxygenase.

These results expand the variations of the types of lipoxygenases that appear to be present in the algae. Examples of predominant 5-, 9-, 11-, 12-, and 15-lipoxygenase products are known.[49] This is the first reported occurence of an 8-lipoxygenase in the

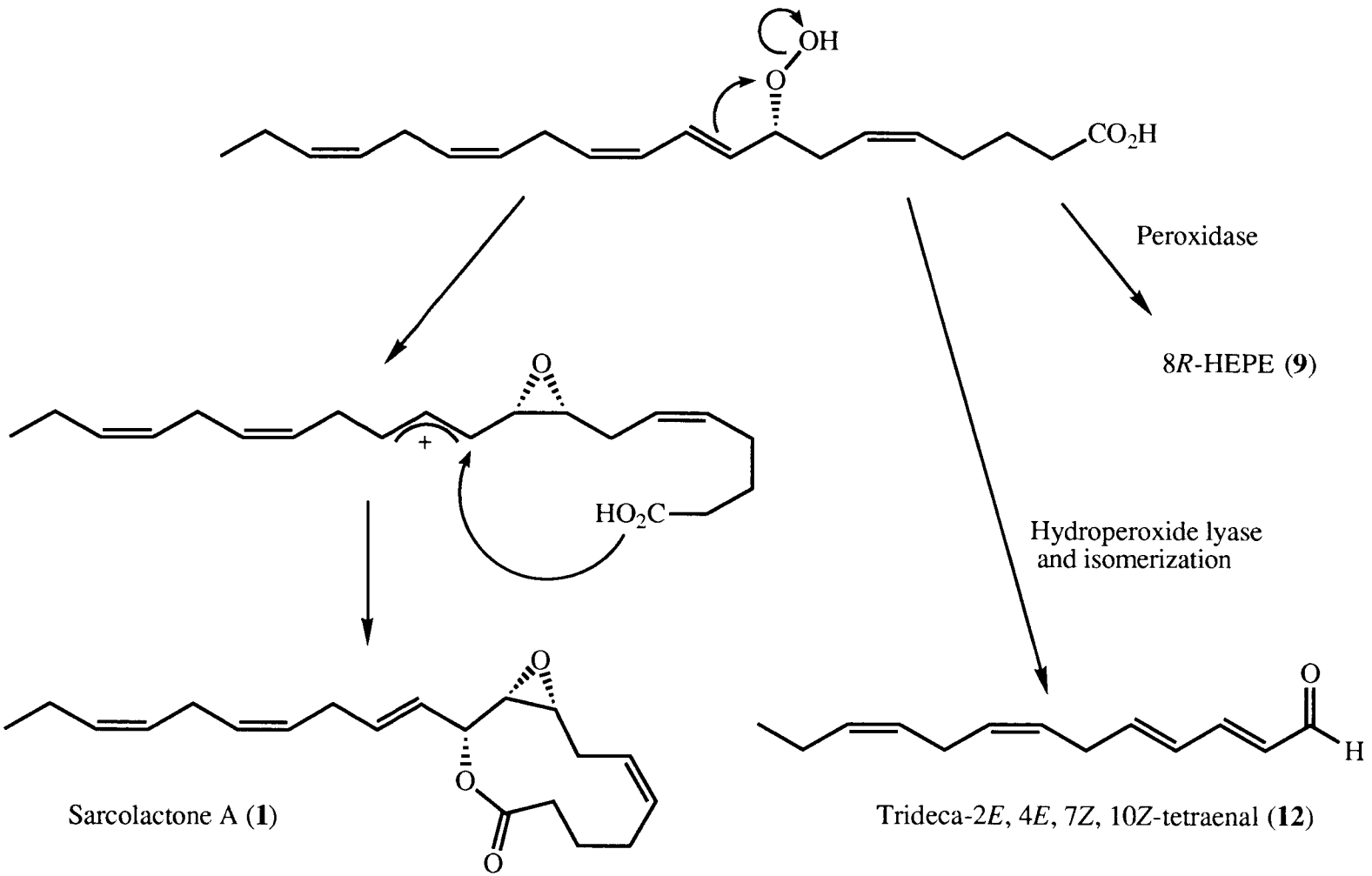



Figure IV.9. Proposed Biogenesis of *Sarcodiotheca* Oxylipins.

algae, but starfish and corals do possess lipoxygenases with this positional specificity.[117,143]

Although an 8-lipoxygenase seems to be predominant in this alga, GC/MS analysis of compounds with similar chromatographic properties to 8-HETE/8-HEPE indicated that other hydroxy acids are present in the alga. Analysis by GC/MS of mixtures of the free alcohol methyl esters and the trimethylsilyl ether, methyl esters suggested that 12-HETE, 12-HEPE, 15-HETE, 15-HEPE, 11-HETE, 14-HEPE, and other minor hydroxy acids and possibly keto acids were present. Further work on these minor components was not pursued.

These compounds have not been adequately tested for biological activities. Both 8*R*-HETE and 8*R*-HEPE, which were isolated while pursuing inosine monophosphate dehydrogenase inhibitory activity, failed to show activity when commercial samples were tested. Sarcolactone A was tested in an *in vivo* sheep bioassay for re-induction of estrous, but failed to show any positive results.

## Experimental

*General.* Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC300 instrument operating at 300.13 MHz for $^{1}H$ NMR and at 75.46 MHz for $^{13}C$ NMR or on an AM400 instrument operating at 400.13 MHz and 100.61 MHz for $^{1}H$ and $^{13}C$ NMR, respectively. Proton spectra were referenced to internal tetramethylsilane at 0.00 ppm. Carbon spectra were referenced to $CDCl_3$ at 77.00 ppm. Infrared (IR) spectra were obtained using a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were run on a Hewlett-Packard 8452a spectrophotometer. High-resolution electron-impact mass spectra (HR EIMS) were recorded on a Varian MAT 311 spectrometer. High-resolution chemical ionization mass spectra (HR CIMS) were recorded on a Kratos MS 50 TC spectrometer. High-performance liquid chromatography (HPLC) utilized a Waters M6000A pump, Rheodyne 7125 injector, and a Waters Lambda-Max 480 LC spectrophotometer or a R 401 differential refractometer. Optical rotations were obtained on a Perkin-Elmer 141 polarimeter. Circular dichroism (CD) spectra were measured on a Jasco J-41a spectropolarimeter. Merck aluminum-backed thin-layer chromatography (TLC) sheets (silica gel 60 $F_{254}$) were used for TLC. Compounds were detected by UV illumination or by heating plates sprayed with a 50% $H_2SO_4$ solution. Gas chromatography/mass spectrometry (GC/MS) was done utilizing a Hewlett-Packard 5890 Series II GC connected to a Hewlett-Packard 5971 mass spectrometer. A methyl silicone column (HP Ultra-1, 11.5 m x 0.2 mm x 0.33 μm film thickness) was used with helium as carrier gas. Trimethylsilyl (TMS) ethers were prepared using pyridine, 1,1,1,3,3,3-hexamethyldisilazane, and trimethylsilyl chloride. 5% palladium on activated carbon, methoxylamine hydrochloride, 4-bromobenzoyl chloride were obtained from Aldrich Chemical Co. Vacuum chromatography was performed with Merck Silica Gel G for TLC. Flash chromatography utilized Merck Kieselgel 60, 230-400 mesh. Chromatography solvents were distilled from glass prior to use.

*Collection, extraction, and chromatography. Sarcodiotheca gaudichaudii* (Montagne) Gabrielson was collected by SCUBA at a depth of 5 - 8 m in August of 1992 at Octopus Hole, Washington. The algae were transported back to Oregon on dry ice and was stored frozen prior to extraction. A voucher specimen is deposited at the College of Pharmacy, Oregon State University. The partially defrosted algae were extracted three times with warm 2:1 $CHCl_3$/MeOH, providing two grams of a viscous dark green oil after evaporation of solvents. The crude extract was initially fractionated by vacuum chromatography (Merck Silica Gel G for TLC, 60 ml sintered glass funnel, 3.7 cm bed height) using an increasing proportion of ethyl acetate in hexanes.

*Non-polar metabolites.* The first VC fraction, which eluted with 10% EtOAc/hexanes, contained a compound that charred blue as determined by 50% $H_2SO_4$ treatment during TLC analysis. Attempts to purify this component utilized two successive vacuum chromatographies using 3-10% EtOAc in hexanes. Although some undesired material was removed, significant amounts of fatty acids still contaminated the sample. These fatty acids interfere with chromatographic separations because they tail on the column and act as an internal solvent that lessens the efficiency of a separation. A TLC-scale methylation of an aliquot of the enriched material showed that the blue-charring material did not methylate, whereas the bulk of the tailing spot on TLC did methylate. The methylated fatty acids now have a higher $R_f$ value on TLC and do not have the tailing problem, so their removal should be much easier. The methylated fraction was loaded onto a Chromatotron (1 mm silica plate) and eluted with 5% EtOAc/hexanes. The fatty acid methyl esters eluted first, followed by triglycerides and an aldehyde, then a mixture of blue and purple charring substances and triglycerides. Purification by HPLC (Phenomenex Maxsil 10 m Silica, 500 mm x 10 mm, 5% EtOAc/hexanes) provided sarcolactone A (**1**, 16.2 mg, 0.8% yield of crude extract) and a mixture of sarcolactone B and triglycerides. The triglycerides were removed by Sephadex LH-20 column (1.7 cm

O.D. x 19 cm, 1:1 EtOAc/MeOH), providing pure sarcolactone B (**2**, 6.3 mg, 0.3% yield).

*Sarcolactone A* (**1**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3010, 2964, 2932, 1739, 1445, 1361, 1207, 1155, 1124, 1063. $[\alpha]_D^{27}$ -160° (*c* 0.56, MeOH). For $^1H$ and $^{13}C$ NMR data, see Table II.1. GC EIMS 70 eV *m/z* (rel. int.): 316 $[M]^+$ (0.2), 287 (0.5), 207 (5), 189 (8), 139 (26), 108 (30), 91 (55), 79 (100), 67 (67), 55 (63). LR CIMS (methane) *m/z* (rel. int.): 317 $[M+H]^+$ (100), 299 (73), 281 (14), 189 (39), 161 (65), 147 (50), 139 (92), 121 (37), 109 (32), 95 (45), 81 (39). HR CIMS *m/z* obs. $[M+H]^+$ 317.2116 ($C_{20}H_{29}O_3$, -0.1 mmu dev.).

*Sarcolactone B* (**2**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2958, 2929, 2857, 1740, 1445, 1207, 1155, 1121, 973. $[\alpha]_D^{27}$ -160° (*c* 0.64, MeOH). For $^1H$ and $^{13}C$ NMR data, see Table II.1. GC EIMS 70 eV *m/z* (rel. int.): 318 $[M]^+$ (1.6), 207 (16), 180 (21), 139 (89), 95 (75), 84 (67), 81 (82), 79 (80), 67 (80), 55 (100). HR EIMS *m/z* obs. $[M]^+$ 318.2194 ($C_{20}H_{30}O_3$, 0.0 mmu dev.).

*Perhydrosarcolactone A* (**3**). The colorless oil (4.1 mg) was dissolved in 1.5 mL EtOH and 5% Pd on activated carbon was added to the flask. Hydrogen was introduced into the flask via a balloon and septum apparatus. After 30 minutes, the reaction mixture was diluted with 1.5 mL 15% EtOAc/hexanes and then filtered through Celite. GC/MS analysis of the hexanes solubles showed an 85:15 mixture of products. Purification by flash chromatography (1.3 cm O.D. x 18 cm; 10% EtOAc/hexanes) yielded perhydrosarcolactone A (**3**, 3.0 mg, 71% yield) and 9-hydroxy-10-eicosanolide (**4**, 0.7 mg, 17% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2926, 2856, 1733, 1464, 1249, 1228, 1203, 1143, 998. $[\alpha]_D^{20}$ -1.2° (*c* 0.33, MeOH). $^1H$ NMR (300 MHz, $CDCl_3$): δ5.29 (1H, *ddd*, *J*=8.7, 5.6, 4.8 Hz, H-10), 3.30 (1H, *dd*, *J*=4.8, 4.1 Hz, H-9), 2.92 (1H, *ddd*, *J*=10.1, 4.1, 3.1 Hz, H-8), 2.33 (2H, *m*, H-2), 1.95 (1H, *m*, H-7a), 1.9-1.2 (27H), 0.88 (3H, distorted *t*, *J*=6.7 Hz, H-20). $^{13}C$ NMR (75.46 MHz, $CDCl_3$): δ174.47, 71.66, 58.98,

57.30, 35.27, 31.89, 31.55, 29.70, 29.58, 29.48, 29.39, 29.31, 26.46, 26.36, 26.24, 25.69, 23.85, 23.76, 22.67, 14.11. GC EIMS 70 eV *m/z* (rel. int.): 183 (2), 154 (3), 141 (100), 123 (4), 95 (65), 83 (9), 69 (16), 55 (19). GC CIMS (methane) *m/z* (rel. int.): 325 $[M+H]^+$ (35), 307 (8), 141 (96), 57 (100).

*Perhydrosarcolactone B*. An aliquot (<100μg) of sarcolactone B was dissolved in 300 μL absolute EtOH and a small amount of 5% Pd/activated carbon was added. A hydrogen balloon was then attached to the reaction vial via a septum. After 30 minutes, the reaction mixture was diluted with hexanes, then passed through a Celite plug. The concentrated residue was dissolved in hexanes and analyzed by GC/MS. The GC retention time and the mass spectral fragmentation pattern were identical with those of perhydrosarcolactone A. A co-injection of the two hydrogenated products gave a single GC peak.

*9R-Hydroxy-10R-eicosanolide* (**4**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3446 (br. OH), 2926, 2855, 1731, 1708, 1466, 1239, 1187, 1148. $^1H$ NMR (300 MHz, $CDCl_3$): δ4.82 (1H, *ddd*, *J*=7.9, 5.9, 3.1 Hz, H-10), 3.95 (1H, *ddd*, *J*=8.5,3.8, 3.1 Hz, H-9), 2.37 (2H, m, H-2), 1.9-1.2 (31H), 0.88 (3H, distorted *t*, *J*=6.7 Hz, H-20). GC EIMS 70 eV *m/z* (rel. int.): 326 [M]+ (0.1), 308 (0.5), 283 (3), 185 (7), 173 (14), 156 (79), 155 (100), 127 (53), 110 (34), 97 (30), 82 (32), 67 (29), 55 (47). GC EIMS of TMS ether derivative 70 eV *m/z* (rel. int.): 398 $[M]^+$ (4), 383 $[M-CH_3]^+$ (15), 308 (2), 243 (9), 211 (13), 185 (29), 157 (19), 136 (34), 129 (100), 73 (61). GC CIMS of TMS ether derivative (methane) *m/z* (rel. int.): 399 [M+H]+ (92), 383 (86), 365 (18), 309 (65), 291 (30), 245 (84), 229 (48), 73 (100).

*9R,10R-(Dimethylmethylenedioxy)-eicosan-1-ol* (**5**). The hydrogenated sarcolactone compounds (**3** + **4**, 3.7 mg) were dissolved in dry THF. The substrate solution was added to a lithium aluminum hydride (LAH, 2.8 mg; 6.7 equiv.) in THF (1 mL) solution via syringe. After 2 h, add 50 mL EtOAc and stir for 20 minutes to quench the remaining LAH. The reaction mixture was filtered through a short silica plug,

followed by rinses with 50% EtOAc/hexanes, ether, and EtOH. After concentration, the ether solubles were filtered through Celite to yield a crude residue which was treated with 2,2-dimethoxypropane (1 mL) and *p*-toluenesulfonic acid monohydrate (catalytic amount). After 2.5 h, $NaHCO_3$ (solid) was added to quench the reaction.[144] The reaction solution was diluted with an equal volume of hexanes and filtered through a silica plug. The colorless, mobile oil obtained after concentration was purified by flash chromatography (15% EtOAc/hexanes). The fraction with the bulk of the mass was analyzed by GC/MS and had two main peaks with a base peak in the mass spectrum of *m/z* 395. This was greater than expected, so this residue was initially dissolved in 300 μL acetone and 300 μL distilled water. Ammonium chloride (5 mg) was then added to the reaction vial. A milky layer was noticed at the top of the solution, so more acetone was added over the course of the reaction (1.8 mL total). After 20 h, the reaction solution was concentrated, then re-dissolved in 50% EtOAc/hexanes. Filtration through a glass frit removed the solid $NH_4Cl$. Concentration yielded a colorless oil (2.0 mg, 48% yield). GC/MS analysis indicated a 4.9/1 ratio of peaks that had *m/z* 355 as the base peak (probably the 1,2- and 1,3-diol acetonides, respectively). Data was collected without further purification. FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3429 (br. OH), 2927, 2855, 1465, 1378, 1368, 1240, 1225, 1057. $^1H$ NMR (300 MHz, $CDCl_3$): δ3.64 (2H, *bt*, *J*=6.2 Hz, H-1), 3.58 (2H, *bt*, *J*=3.1 Hz, H-9, H-10), 1.6-1.2 (32H), 1.38 (6H, s, acetonide $CH_3$s), 0.88 (3H, distorted *t*, *J*=6.7 Hz, H-20). $^{13}C$ NMR (75.46 MHz, $CDCl_3$): δ107.71, 81.03, 63.07, 33.04, 32.78, 31.91, 29.79, 29.71, 29.61, 29.52, 29.47, 29.34, 27.34, 26.16, 25.70, 22.68, 14.11. Only 17 carbon lines were observed. Signals at 81.03 and 27.34 ppm represent two carbons each. The cluster around 29.5 ppm probably represents more carbons than the six lines that resolved. GC EIMS 70 eV *m/z* (rel. int.): 355 $[M-CH_3]^+$ (100), 197 (7), 185 (6), 152 (7), 123 (11), 109 (22), 95 (42), 83 (34), 59 (60). LR CIMS (methane) *m/z* (rel. int.): 371 $[M+H]^+$ (16), 355 $[M - CH_3]^+$ (44), 341 (23), 313 (100), 311 (64), 295 (58). HR CIMS *m/z* obs. $[M+H]^+$ 371.3523 ($C_{23}H_{47}O_3$, -0.2 mmu dev.). GC EIMS of TMS ether

derivative 70 eV *m/z* (rel. int.): 427 $[M\text{-}CH_3]^+$ (100), 369 (25), 199 (13), 152 (8), 123 (23), 109 (25), 95 (43), 81 (40), 75 (54), 59 (41).

*Methyl 8R,9S-Epoxy-10R-hydroxyeicosa-5Z,11E,14Z,17Z-tetraenoate* (**6**). Sarcolactone A (3.7 mg) was dissolved in 1 mL 5% KOH/MeOH. After 75 minutes, the reaction solution was diluted with 5 mL ice water, transferred to a separatory funnel with 10 mL ether, and quenched with 1 mL of 5% HCl solution. The aqueous layer was extracted twice with ether and the combined organics were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated. The colorless oil was then treated with $CH_2N_2$ in ether. Concentration yielded a colorless oil (3.4 mg, 83% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3446 (br. OH), 3012, 2964, 2933, 1739, 1436, 1245, 1211, 1162, 974. $[\alpha]_D^{17}$ +71° (*c* 0.38, MeOH). $^1H$ NMR (300 MHz, $CDCl_3$): δ5.81 (1H, *dtd*, *J*=15.5, 6.2, 1.0 Hz, H-12), 5.56 (1H, *ddt*, *J*=15.5, 6.3, 1.5 Hz, H-11), 5.56-5.34 (5H, *m*), 5.30 (1H, *dtt*, *J*=10.7, 7.1, 1.4 Hz, H-17), 4.03 (1H, *bdd*, *J*=7.8, 6.3 Hz, H-10), 3.67 (3H, *s*, $OCH_3$), 3.05 (1H, *ddd*, *J*=6.5, 5.9, 4.3 Hz, H-8), 2.98 (1H, *dd*, *J*=7.8, 4.3 Hz, H-9), 2.86 (2H, *bdd*, *J*=6.1, 6.1 Hz, H-13), 2.78 (2H, *bdd*, *J*=7.1, 6.6 Hz, H-16), 2.44 (1H, *ddd*, *J*=14.6, 6.5, 6.5 Hz, H-7a), 2.33 (2H, *t*, *J*=7.4 Hz, H-2), 2.25 (1H, *ddd*, *J*=14.6, 5.9, 5.9 Hz, H-7b), 2.08 (4H, *m*, H-4, H-19), 1.71 (2H, *tt*, *J*=7.4, 7.4 Hz, H-3), 0.97 (3H, *t*, *J*=7.5 Hz, H-20). $^{13}C$ NMR (75.46 MHz, $CDCl_3$): δ132.13 (C-12), 132.08 (C-18), 131.64 (C-5), 129.71 (C-15), 127.91 (C-11), 126.82 (C-17), 126.34 (C-14), 124.75 (C-6), 70.83 (C-10), 60.05 (C-9), 57.04 (C-8), 51.52 ($OCH_3$), 33.32 (C-2), 30.06 (C-13), 26.72 (C-7), 26.59 (C-4), 25.47 (C-16), 24.61 (C-3), 20.55 (C-19), 14.25 (C-20); C-1 was not observed; assignments made by comparison to sarcolactone A. GC EIMS 70 eV *m/z* (rel. int.): 221 (2), 207 (3), 184 (3), 171 (5), 139 (13), 121 (19), 105 (25), 91 (44), 74 (48), 79 (100), 67 (81), 55 (49). GC EIMS of TMS ether derivative 70 eV *m/z* (rel. int.): 391 $[M\text{-}CH_2CH_3]^+$ (0.07), 389 $[M\text{-}OCH_3]^+$ (0.07), 311 (2), 279 (2), 237 (12), 209 (4), 181 (7), 171 (18), 142 (51), 129 (53), 73 (100). LR CIMS (methane) *m/z* (rel.

int.): 349 $[M+H]^+$ (4), 331 (82), 313 (60), 299 (36), 281 (21), 199 (40), 171 (100), 153 (33), 139 (68), 135 (45), 121 (34).

*Methyl 8R,9R-Epoxy-10R-(4-bromobenzoyloxy)eicosa-5Z,11E,14Z,17Z-tetraenoate* (**7**). Compound **6** (2.2 mg) was dissolved in 3 mL dry $CH_2Cl_2$ and then $Et_3N$ (300 μL, 341 equiv.), 4-bromobenzoyl chloride (21.4 mg, 15.5 equiv.), and a catalytic amount of 4-dimethylaminopyridine were added. After 21.5 h, the reaction mixture was diluted with hexanes (3 mL). A white residue precipitated immediately. After loading the reaction mixture directly onto a flash silica column (1.7 cm O.D. x 11 cm), the column was eluted with 5% E/H, then 60% E/H. The concentrated residue was further purified by Sephadex LH-20 chromatography (1.7 cm O.D. x 19 cm; 1:1 EtOAc/MeOH). Flash chromatography with 15% E/H (1.7 cm O.D. x 15.5 cm) provided the desired product as a colorless oil (2.4 mg, 68% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2962, 1733, 1724, 1591, 1267, 1100, 1012, 757. $[\alpha]_D^{26}$ +13° (*c* 0.27, MeOH). UV $\lambda_{max}^{MeOH}$ nm: 246 (ε = 19,000), 206 (ε = 25,000). CD (MeOH): $\Delta\varepsilon$= +2.47 ($\lambda_{max}$= 242.5 nm). $^1H$ NMR (300 MHz, $CDCl_3$): δ7.94 (2H, *d*, *J*=8.7 Hz, aromatic H *ortho* to carbonyl), 7.58 (2H, *d*, *J*=8.7 Hz, aromatic H *ortho* to Br), 5.91 (1H, *dtd*, *J*=15.6, 6.2, 0.9 Hz, H-12), 5.59 (1H, *ddt*, *J*=15.6, 6.7, 1.6, H-11), 5.53 (1H, *m*, H-5), 5.37 (2H, *m*, H-6, H-15), 5.38 (3H, *m*, H-10, H-14, H-18), 5.27 (1H, *dtt*, *J*=10.7, 7.0, 1.4 Hz, H-17), 3.67 (3H, *s*, $OCH_3$), 3.24 (1H, *dd*, *J*=8.6, 4.3 Hz, H9), 3.08 (1H, *ddd*, *J*=6.6, 6.1, 4.3 Hz, H-8), 2.86 (2H, *bdd*, *J*=6.6, 6.2 Hz, H-13), 2.76 (2H, *bdd*, *J*=7.0, 7.0 Hz, H-16), 2.47 (1H, *ddd*, *J*=14.6, 6.6, 6.6 Hz, H-7a), 2.33 (2H, *t*, *J*=7.5 Hz, H-2), 2.32 (1H, *m*, H-7b), 2.12 (2H, *btd*, *J*=7.3, 6.7 Hz, H-4), 2.04 (2H, *bqd*, *J*=7.5, 7.2 Hz, H-19), 1.72 (2H, *tt*, *J*=7.5, 7.3 Hz, H-3), 0.95 (3H, *t*, *J*=7.5 Hz, H-20). LR CIMS (methane) *m/z* (rel. int.): 533 $[M+H; {}^{81}Br]^+$ (3.1), 531 $[M+H; {}^{79}Br]^+$ (3.6), 391 (17), 331 (100), 313 (77), 299 (33), 201 (36).

*Trideca-2E,4E,7Z,10Z-tetraenal* (**12**). After the bulk of the fatty acid methyl esters had eluted from the Chromatotron, several fractions containing triglycerides and an aldehyde were collected (determined by GC/MS and $^1H$ NMR). These fractions were

combined, concentrated (10 mg), then loaded onto a Sephadex LH-20 column (1.7 cm O.D. x 19 cm) and eluted with 1:1 EtOAc/MeOH. The triglycerides eluted first, followed by the aldehyde and a yellow pigment. The bulk of the yellow pigment was removed using activated charcoal to give the aldehyde **12** as a pale yellow oil (1 mg; 0.05% yield). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 2962, 2929, 1686, 1638, 1156, 1117, 1012, 988. UV $\lambda_{max}^{MeOH}$ nm: 274 ($\varepsilon$ = 13,000). $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$9.54 (1H, *d*, *J*=7.9 Hz, H-1), 7.09 (1H, *dd*, *J*=15.4, 10.0 Hz, H-3), 6.35 (1H, *dd*, *J*=15.5, 10.0 Hz, H-4), 6.25 (1H, *dt*, *J*=15.5, 6.1 Hz, H-5), 6.09 (1H, *dd*, J=15.4, 7.9 Hz, H-2), 5.54 (1H, *dtt*, *J*=10.7, 7.1, 1.3 Hz, H-8), 5.42 (2H, *m*, H-7,11), 5.30 (1H, *dtt*, *J*=10.7, 7.1, 1.5 Hz, H-10), 3.00 (2H, *bdd*, *J*=6.6, 6.1 Hz, H-6), 2.80 (2H, *bdd*, *J*=7.1, 7.1 Hz, H-9), 2.06 (2H, *qd*, *J*=7.5, 7.2 Hz, H-12), 0.97 (3H, *t*, *J*=7.5 Hz, H-13). GC EIMS 70 eV *m/z* (rel. int.): 190 $[M]^+$ (1), 175 (1), 161 (6), 147 (7), 136 (25), 105 (28), 91 (64), 79 (100), 67 (37).

*Formation of Methoxime Derivative of* **12**. A small portion (~100 µg) of the aldehyde **12** was dissolved in 100 µL of a 10 mg methoxylamine HCl/mL pyridine solution and allowed to sit at room temperature. After 8 hours, the solvent was evaporated and the residue was dissolved in 5% EtOAc/hexanes and then passed through a short plug of silica. The concentrated oil was dissolved in hexanes and then analyzed by GC/MS. Two major peaks were detected in a 2.3:1 ratio. The mass spectra for these peaks were almost identical, consistent with the formation of the *E* and *Z* methoxime derivatives. Major isomer (probably *E*) GC EIMS 70 eV *m/z* (rel. int.): 219 $[M]^+$ (2), 204 (1), 188 (20), 118 (25), 110 (72), 108 (35), 91 (33), 79 (100). Minor isomer GC EIMS 70 eV *m/z* (rel. int.): 219 $[M]^+$ (1), 204 (1), 188 (17), 118 (24), 110 (57), 108 (37), 91 (36), 79 (100).

*Polar Metabolites*. The vacuum chromatography fractions eluting with 50% and 75% EtOAc/hexanes were combined (70 mg) and then fractionated by Sephadex LH-20 chromatography (1.7 cm O.D. x 19 cm, 1:1 EtOAc/MeOH). The fractions containing UV-active, gray-charring compounds (TLC in 10% MeOH/$CHCl_3$ and 60% EtOAc/hexanes)

were combined (36.4 mg). A $^1$H NMR spectrum of this crude material showed the presence of several hydroxy polyunsaturated fatty acids. This material was subjected to flash silica chromatography (7% isopropanol/hexanes plus 0.1% AcOH). The main hydroxy acid containing fractions were combined (11.3 mg) and separated by HPLC (Versapack Silica 10 μ, 2 x 4.1 mm x 25 mm, 2.5% isopropanol/hexanes plus 0.1% AcOH). One fraction from the HPLC run (3.3 mg) was mainly a 1:1 mix of ω6 and ω3 fatty acids as determined by the characteristic terminal methyl signals in the $^1$H NMR spectrum. For final purification, this fraction was methylated, then subjected to RP HPLC (Merck Lichrosorb C18 Silica 7 μ, 25 cm x 1 cm, 85% MeOH/$H_2O$) to yield methyl 8-HEPE (**11**, eluted first; 0.9 mg, 0.045% yield) and methyl 8-HETE (**10**, 0.9 mg, 0.045% yield). Several other fractions from the initial HPLC run were also shown to contain complex mixtures of hydroxy-diene fatty acids and ketodienes when the methyl esters and TMS ether methyl esters were examined by GC/MS.

*Methyl 8R-Hydroxyeicosa-5Z, 9E, 11Z, 14Z-tetraenoate* (**10**). Steric analysis: Methyl 8-HETE (0.1 mg) was dissolved in dry toluene (50 μL) plus pyridine (10 μL) and then 10 μL of a (-)-menthylchloroformate in toluene solution (1 μmol/μL) was added. After 30 minutes, the reaction mixture was diluted with 200 μL of hexanes. A white precipitate formed immediately. The solution was loaded directly onto a pipet column of silica, which was eluted with 10% E/H. The concentrated residue was dissolved in 1 mL $CH_2Cl_2$. After cooling the solution in a dry ice-ethylene glycol bath, a stream of ozone was blown over the surface of the solution for 2 minutes. The vial was capped and allowed to sit for 10 min at room temperature before concentration. The residue was dissolved in 250 μL of a peracetic acid solution (prepared from 1 mL AcOH and 250 μL of 30% $H_2O_2$ solution) and heated in a 50° sand bath overnight. The concentrated residue was dissolve 5 drops of MeOH, then methylated with $CH_2N_2$ in ether. The hexanes solubles from this procedure were analyzed by GC/MS. (HP Ultra-1 column, 11.5 m, 11 psi helium head pressure, 100-180° at 2°/min after an initial time of 30 seconds; L-

standard 35.35 min., **D**-standard 35.47 min). The retention time of the derived methyl menthoxymalate fragment correlated to the **D**-malate standard. Co-injection of the experimental sample with an ~50:50 mix of **D** and **L** standards showed a significant enrichment in the latter eluting **D** standard, verifying the *R* stereochemistry.

*Methyl 8R-Hydroxyeicosa-5Z, 9E, 11Z, 14Z, 17Z-pentaenoate* (**11**). Steric analysis was carried out in the same fashion as for 8-HETE. Again, co-injection of the product with a mix of standards showed significant enrichment of the **D**-malate derived peak indicating the *R* configuration at the alcoholic center.

# CHAPTER V

## SCYTONEMIN: A PHOTOPROTECTIVE PIGMENT FROM THE SHEATHS OF BLUE-GREEN ALGAE (CYANOBACTERIA)


### Abstract

The blue-green algae are the oldest type of organism known, having been in existence for several billion years. Throughout history, organisms have survived by adapting to the environments in which they live. An adaptation of the blue-green algae is their ability to produce a photoprotective sheath pigment under conditions of high irradiance which protects them from the damaging effects of high UV flux. Although this phenomenon has just recently been studied in depth, the sheath pigment has been known since the mid-1800's. Originally termed scytonemin, the structure of this pigment had remained unknown until this study. Presented here are the details of the structure elucidation of this pigment and a reduced form of the pigment, which is also known to occur in nature. The structures are novel dimers of subunits containing indolic and phenolic components.

## Introduction

Cyanobacteria or blue-green algae are representatives of the oldest known class of organisms, with fossils of cyanobacterial-like filaments dated as far back as 3.5 billion years.[145] The chemical constituents of these organisms have been studied since the early 1900's, with the majority of research occurring the last two decades. Initially, primary metabolites such as chlorophyll a, phycobiliproteins, carotenoids and lipids were characterized. More recently, blue-green algal toxins such as anatoxin-a and the microcystins have been described.[69,70] Most current natural products efforts are focussed on determining the structures of toxins[70] or finding pharmacologically active constituents.[146,147]

Another method of studying blue-greens takes an ecological approach. Since blue-greens inhabit a diversity of harsh environments, they have evolutionarily developed means to adapt to their surroundings. High temperatures, dessication, and high light intensity are some of the harsh conditions that these organisms encounter.

In animals, high light intensity can be dealt with in a variety of ways. One is mobility. Animals have the ability to move away from sunlight, finding shelter. A second way to deal with irradiation is chemical. Many organisms utilize melanins to act as a sunscreen.[148,149] This tyrosine-derived polymer[150,151] effectively absorbs most UV irradiation, protecting the particular organism from damage. Plants also have evolved compounds that appear to act as "sunscreens". The anthocyanins and flavone glycosides are examples of these photoprotective plant pigments.[152,153]

Biologists at the University of Oregon have recently shown that the cyanobacteria also have a pigment that is produced as a photoprotective compound.[154,155] During years of studying blue-greens, they had often encountered a yellow-green pigment that was present in the sheaths of the blue-greens. This sheath pigment was initially described in 1847[156] and in the 1870's, Nägeli named the pigment scytonemin, for the genus being

studied, *Scytonema.*[157] In the 1920's and 1930's the Swedish botanist Harald Kylin investigated the pigment in more detail and concluded that scytonemin was actually composed of two components: a green pigment that he called fuscochlorin and a red pigment he termed fuscorhodin.[158,159] Although he described extraction, solubility, and general acid-base phenomena, no structures were determined.

Despite knowledge of the existence of the sheath pigment, further information on its role in cyanobacteria was not available. The biologists at the Univeristy of Oregon, Garcia-Pichel and Castenholz, recently decided to examine the biology of pigment formation in more detail. Through culture studies they were able to document a direct correlation between pigment production and light intensity.[154,155] The blue-green algae were able to grow in the absence of the pigment, indicating that scytonemin was not involved in photosynthesis. Only under conditions of high irradiance was the pigment formed. Scytonemin therefore acts as a photoprotective pigment, rather than a photosynthetic pigment.

Although a role for the pigment was discovered, the structure was still unknown. Since the early work of Kylin, interest in scytonemin obviously eluded natural products chemists, because no further structural work appeared in the literature. This pigment was apparently "lost" to the chemical community, because in the early 1980's, the name "scytonemin A" was used to describe a peptide from a *Scytonema* sp.[160] The authors were apparently unaware of the previous usage of this term. To complement the biological studies, we undertook the structure elucidation of scytonemin and a related reduced pigment. Despite initial problems with solubility, we were able to determine the structure of this pigment with extensive 2D NMR experiments and by degradative chemistry. Scytonemin is a novel dimeric pigment in which the monomer unit has indolic and phenolic components.

Scytonemin is extremely stable and beyond its importance in the biology of the cyanobacteria, it may also be of importance as a biogeochemical marker. Because

scytonemin has been characterized from numerous genera of blue-green algae, it has been proposed that the ability to synthesize scytonemin was an early evolutionary characteristic. If blue-greens were producing scytonemin billions of years ago when the UV flux was higher than today, then perhaps this sheath pigment could have survived in certain geologic deposits and become a marker of ancient biological organisms.[161,162]

## Results and Discussion

Although scytonemin is present in numerous genera, the material used in our structural studies was obtained from three principle collections: 1) *Stigonema* sp. from the ultraoligotrophic, subalpine lake, Waldo Lake, in the Oregon Cascades, 2) a marine *Scytonema* sp. collected in the splash zone in Curaçao, Netherlands Antilles and 3) a marine *Lyngbya* sp. collected in the splash zone in Huahine, French Polynesia. A red reduced pigment was also isolated from these marine species. The solubility characteristics of the pigment allowed for a straightforward extraction and purification process. Scytonemin was soluble in DMF, pyridine, and THF, partially soluble in acetone and ethyl acetate, and poorly soluble/insoluble in $CHCl_3$, benzene, MeOH, hexanes, $H_2O$. Initial extraction of the alga with MeOH removed the bulk of unwanted material including carotenoids, sterols and other lipids, etc. Repeated extraction with EtOAc then provided an extract rich in the desired pigment. Concentration and then repetitive washings of the dark green brown residue with hexanes and MeOH provided scytonemin of 90-95% purity. The $^1H$ NMR spectrum showed the main impurities to be aliphatic residues which did not interfere with the signals due to the pigment itself.

The $^1H$ NMR spectrum of scytonemin (**1**) in pyridine-$d_5$ revealed seven separate signals that integrated for nine protons, all in the region downfield of 7 ppm (Table V.1). Four of these protons (two doublets at δ9.00 and δ7.34) represented a *para*-substituted benzene ring. Four of the remaining protons were part of an independent spin system representing a 1,2-disubstituted benzene ring. A broad singlet at δ8.00, representing a lone $sp^2$ methine, defined the remaining spin system. Recording the $^1H$ spectrum in acetone-$d_6$ uncovered another proton resonance at 9.49 ppm. The $^{13}C$ NMR spectrum in pyridine-$d_5$ showed 16 carbon lines, only seven of which were protonated based on a DEPT 135 experiment (Table V.1). The protonated carbons were all $sp^2$ methines. Analysis by HR FABMS provided a molecular formula of $C_{36}H_{22}N_2O_4$. This formula

Table V.1. NMR Data for Scytonemin, Reduced Scytonemin, and the Ozonolysis Fragment Derived from Reduced Scytonemin (chemical shifts in ppm, *J* in Hz).

| | Scytonemin[a] | | Reduced Scytonemin[b] | | Ozonolysis fragment[c] | |
|---|---|---|---|---|---|---|
| C# | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 129.83 | | 123.38 | | 177.01 | |
| 2 | 194.17 | | 196.41 | | 193.25 | |
| 3 | 118.67 | | 124.69 | | 119.41 | |
| 3a | 174.30 | | 145.76 | | 158.41 | |
| 4a | 163.94 | | 142.21 | | 140.63 | |
| 5 | 122.08 | 7.76 d (7.7) | 113.51 | 7.65 d (8.0) | 114.09 | 7.68 dd (8.0,0.9) |
| 6 | 135.14 | 7.49 ddd (7.7,7.6,1.1) | 124.93 | 7.31 ddd (8.0,7.2,1.2) | 126.38 | 7.42 ddd (8.0,7.3,1.1) |
| 7 | 126.64 | 7.22 dd (7.6,7.2) | 121.30 | 7.21 ddd (8.0,7.2,1.0) | 123.90 | 7.34 ddd (7.5,7.3,0.9) |
| 8 | 129.67 | 7.89 d (7.2) | 125.98 | 7.75 d (8.0) | 120.69 | 7.85 d (7.5) |
| 8a | 125.61 | | 124.37 | | 120.79 | |
| 8b | 158.63 | | 127.73 | | 123.60 | |
| 9 | 139.42 | 8.00 s | 128.22 | 7.42 s | 128.84 | 7.32 s |
| 10 | 126.36 | | 126.94 | | 124.44 | |
| 11 | 136.86 | 9.00 d (8.7) | 131.71 | 7.81 d (8.6) | 131.71 | 7.70 d (8.5) |
| 12 | 117.08 | 7.34 d (8.7) | 116.91 | 7.01 d (8.6) | 116.40 | 6.97 d (8.5) |
| 13 | 163.55 | | 160.22 | | 159.84 | |
| OH | | | | 10.34 bs | | 10.26 bs |
| NH | | | | 11.79 bs | | 12.21 bs |

a) Spectra recorded in pyridine-$d_5$. $^{1}H$ NMR at 300.13 MHz, $^{13}C$ NMR at 75.46 MHz. $^{1}H$ NMR spectrum referenced to TMS at 0.00 ppm; $^{13}C$ NMR referenced to pyridine centerline at 149.90 ppm. The centerline of the 7.22 ppm pattern in the $^{1}H$ NMR spectrum is obscured by the furthest upfield pyridine signal. Assignments based on $^{1}H$-$^{1}H$ COSY, XHCORR, and HMBC spectra.
b) Spectra recorded in DMF-$d_7$. $^{1}H$ NMR at 300.13 MHz, $^{13}C$ NMR at 75.46 MHz. $^{1}H$ NMR spectrum referenced to upfield methyl of DMF at 2.74 ppm; $^{13}C$ NMR referenced to DMF centerline at 162.70 ppm. Assignments based on $^{1}H$-$^{1}H$ COSY, XHCORR, LR HETCOSY, and COLOC spectra.
c) Spectra recorded in DMSO-$d_6$. $^{1}H$ NMR at 400.13 MHz, $^{13}C$ NMR at 100.61 MHz. $^{1}H$ NMR spectrum referenced to TMS at 0.00 ppm; $^{13}C$ NMR referenced to DMSO centerline at 39.50 ppm. Assignments based on XHCORR and HMBC spectra.

indicated that the pigment was a symmetrical dimer, based on the carbon count, but had two more protons than would be expected. The mystery of the extra protons was cleared up when a sample of reduced pigment (**2**; the pigment is readily reduced to a red compound with mild reductants such as ascorbic acid or dithiothreitol) showed the same molecular formula when analyzed by HR FABMS. Apparently a facile reduction took place in the mass spectometer.

Early research by Kylin suggested that crystals of scytonemin might be accessible.[158] Despite numerous attempts to obtain crystals suitable for X-ray crystallographic studies, only disordered clusters of needles were obtained. Attempts to form derivatives such as acetates and 4-bromobenzoates produced only residues that were poorly soluble and non-crystalline.

The reduced pigment proved to be a valuable derivative. Not only did it have better solubility characteristics than the original pigment, its proton spectrum was clearly resolved in both pyridine-$d_5$ and DMF-$d_7$. Since it had more favorable spectroscopic characteristics than scytonemin, the reduced pigment was used for structural studies. The $^1$H NMR spectrum in DMF-$d_7$ had two exchangeable protons in addition to seven separate carbon-bound protons signals (Table V.1). The doublets at $\delta$7.81 and $\delta$7.01 (representing 2 protons each) indicated that the *para*-substituted benzene ring was still intact. The *para*-substituted benzene ring was shown to be a vinyl substituted phenol by isolation of *p*-hydroxybenzaldehyde from the ozonolysis of both the oxidized and reduced forms of the pigment. The four coupled aromatic protons and the remaining carbon bound proton were also present in the reduced form. The extra hydrogen added during the reduction was an exchangeable hydrogen. Extensive 2D NMR analysis including $^1$H-$^1$H COSY, $^{13}$C-$^1$H XHCORR, LR HETCOSY[163] (Figure V.1), and COLOC (both 7 Hz and 3.5 Hz optimizations) experiments[164] (Table V.2) secured several partial structures. One of the exchangeable protons was associated with a phenolic ring. The other was attached to the nitrogen of an indole fragment.[165] Long-range $^1$H-$^{13}$C correlations (Table V.2) allowed

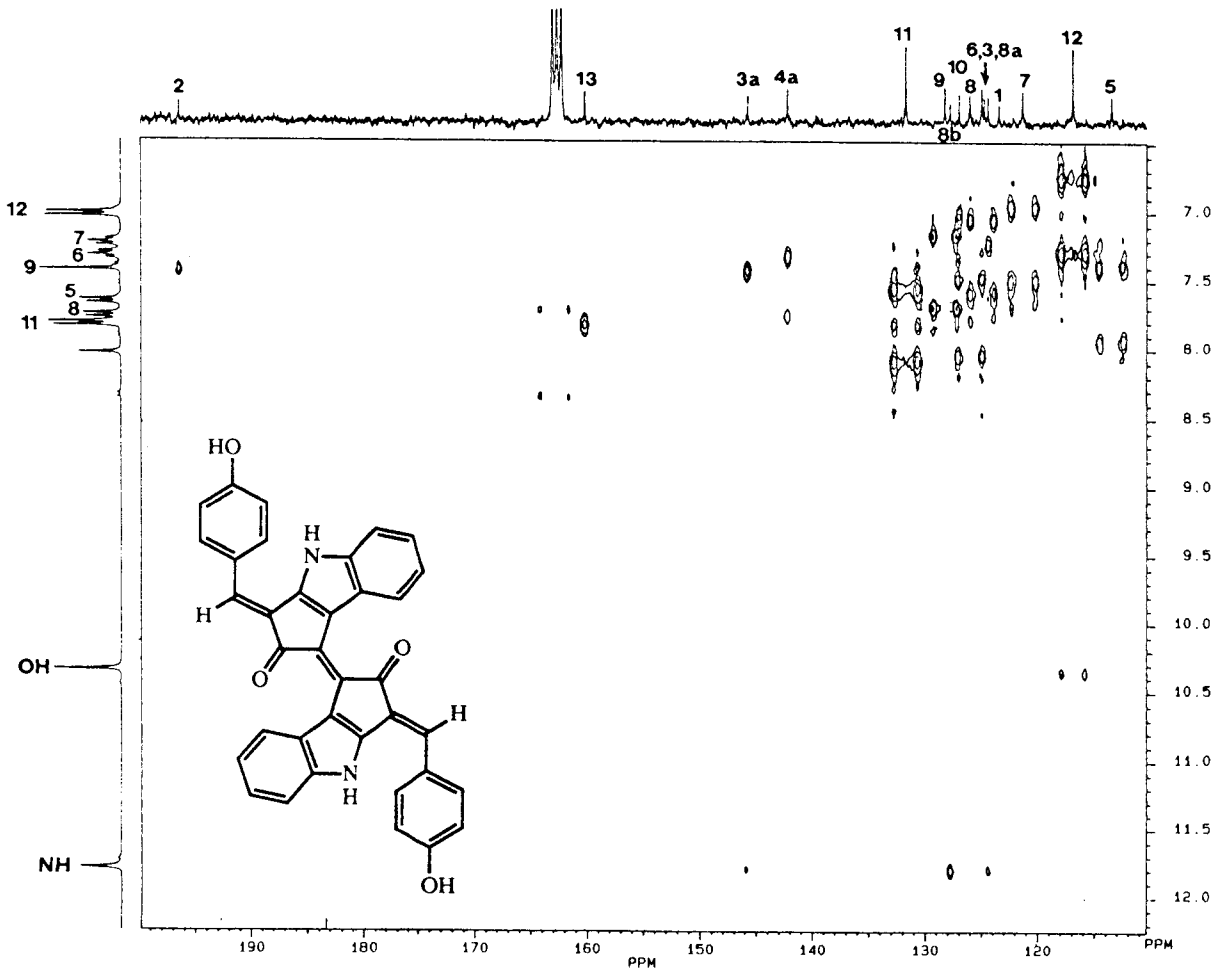



Figure V.1. LR HETCOSY Spectrum of Reduced Scytonemin (**2**).

Table V.2 Long-range $^{1}H$-$^{13}C$ Correlations for Reduced Scytonemin.

| Proton # | LR HETCOSY | COLOC (7 Hz) | COLOC (3.5 Hz) |
|---|---|---|---|
| NH | C-3a, C-8a, C-8b | C-3a, C-4a, C-8a, C-8b | C-3a, C-8b |
| OH | C-12(14) | C-12(14) | C-12(14) |
| H-5 | C-5 | C-7, C-8a | C-7, C-8a |
| H-6 | C-4a, C-6 | --- | C-4a |
| H-7 | C-7, C-8a | --- | --- |
| H-8 | C-4a, C-8 | C-4a | C-8b |
| H-9 | C-2, C-3a, C-9 | C-2, C-3, C-3a, C-9, C-11 | C-2, C-3, C-3a, C-9 |
| H-11, H-15 | C-11(15), C-13 | C-11(15), C-13 | C-9, C-13 |
| H-12, H-14 | C-10, C-12(14) | C-10, C-12(14) | C-10, C-13 |

Table V.3 HMBC Correlations for Reduced Scytonemin Ozonolysis Fragment.

| Proton # | HMBC Correlations |
|---|---|
| NH | C-3a, C-8a, C-8b |
| OH | C-12(14), C-13 |
| H-5 | C-7, C-8a |
| H-6 | C-4a, C-8 |
| H-7 | C-5, C-8a |
| H-8 | C-4a, C-6 |
| H-9 | C-2, C-3, C-3a, C-11(15) |
| H-11, H-15 | C-9, C-11(15), C-13 |
| H-12, H-14 | C-10, C-12(14), C-13 |

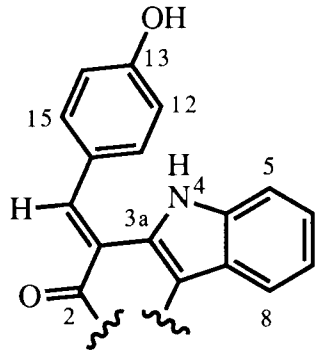
Table V.4. Long-range $^{1}H$-$^{13}C$ Correlations for Scytonemin.

| Proton # | HMBC Correlations | LR HETCOSY | COLOC Correlations |
|---|---|---|---|
| H-5 | C-7, C-8a | C-5 | |
| H-6 | C-4a, C-8 | C-6 | |
| H-7 | C-5, C-8a | C-7, C-8a | |
| H-8 | C-4a, C-6 | C-8 | |
| H-9 | C-2, C-3, C-3a, C-11 | C-2, C-3a, C-11 | C-2, C-3, C-3a, C-9, C-11 |
| H-11, H-15 | C-9, C-11(15), C-13 | C-11(15), C-13 | C-11(15) |
| H-12, H-14 | C-10, C-12(14), C-13 | C-10, C-12(14) | C-10, C-12(14) |

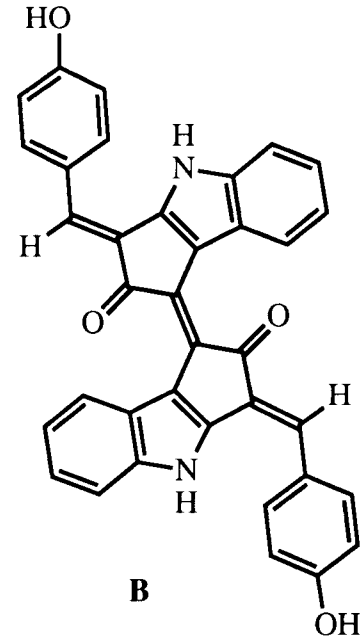
coupling of the indole unit with the phenol unit to yield substructure **A**. The geometry of the *exo* olefin was determined two ways. The $^{3}J_{CH}$s were measured for both C2-H9 (5.8 Hz) and C3a-H9 (10.3 Hz). The larger coupling constant for C3a-H9 indicated a *trans* relationship for these atoms, completely analogous to the *trans* > *cis* $^{1}H$-$^{1}H$ coupling constant relationship about an olefin.[166] Since C-3a and H-9 are *trans*, the olefin geometry was *E*. This olefin geometry was secured by observing an nOe between the indole proton and H-11(14).

Substructure A incorporates 17 of the 18 carbons required for half of the structure. The remaining quaternary carbon showed no correlations to any of the protons. Three possible structures were consistent with the NMR data (**B-D**; Figure V.2). The $^{13}C$ chemical shifts of cyclopentanones versus cyclohexanones are usually distinct, so analysis of the ketone shift (C-2) could potentially solve the problem. Several simple model compounds indicated shifts >190 ppm for fully unsaturated five-membered ring ketones[167,168] and shifts <190 ppm for unsaturated six-membered ring ketones.[169,170] The $^{13}C$ chemical shift of the ketone carbon (196.41 ppm) suggested one of the five-membered ring structures based on the simple model compounds. However, more complex models that incorporate the partial structures were unavailable, so a firm conclusion as to the structure was not reached.

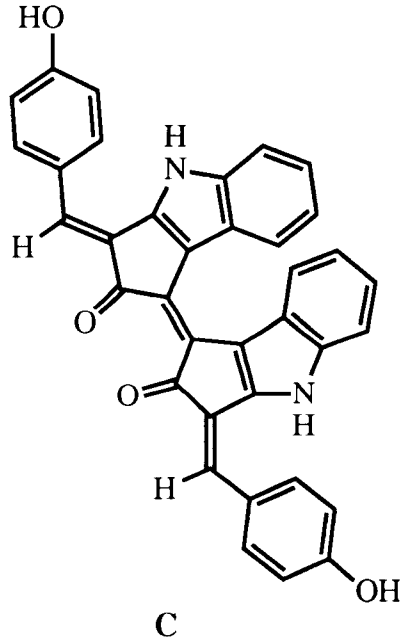
The solution to the structure of the reduced pigment came through ozonolysis studies. Ozonolysis of the reduced pigment in EtOAc at -78°C followed by work-up with dimethylsulfide provided an orange residue. Sephadex LH-20 chromatography was used to fractionate this residue. To our delight, orange crystals deposited out in several of the fractions. This material was combined and examined by $^{1}H$ NMR (Table V.1; Figure V.3). Signals for the indole, phenol, and vinyl proton were present, suggesting that this fragment could be the desired "half" of the structure. HR FABMS data verified that this fragment was half of the original dimer plus an extra oxygen from the ozonolysis reaction. NMR experiments (XHCORR, HMBC;[171] Table V.3) were used to connect the structural



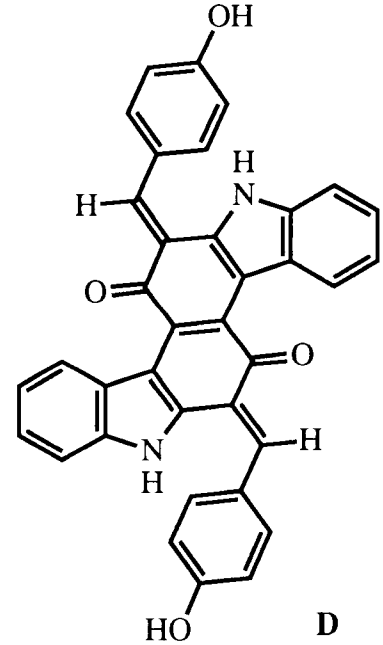

A




B




C




D

Figure V.2. Partial and Possible Structures for Reduced Scytonemin.

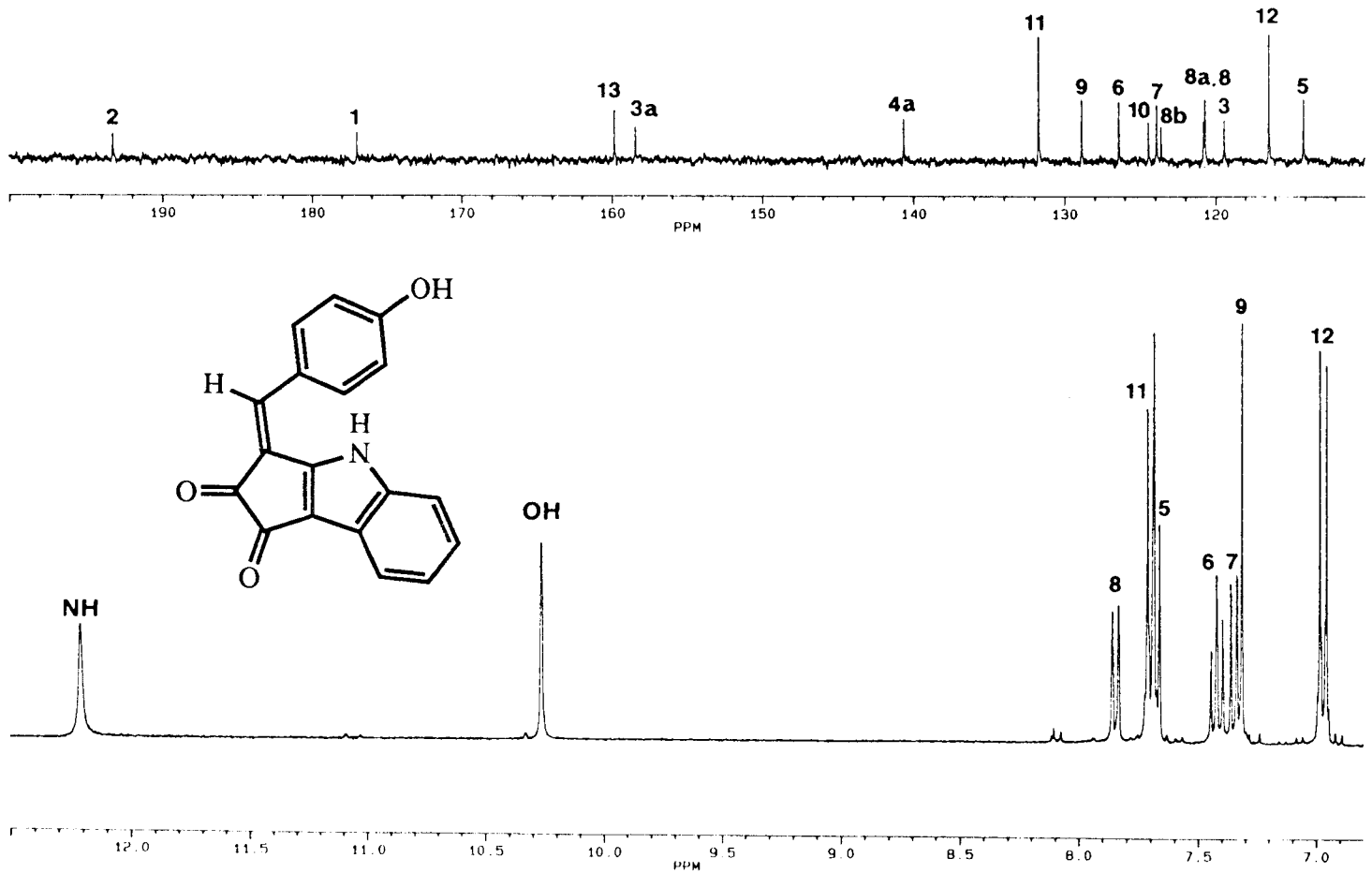
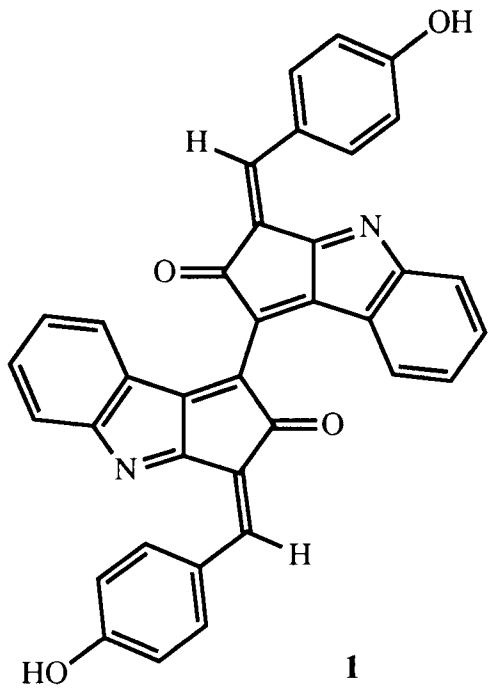



Figure V.3. $^{1}H$ and $^{13}C$ NMR Spectra of Reduced Scytonemin Ozonolysis Fragment (**3**) in DMSO-$d_6$.

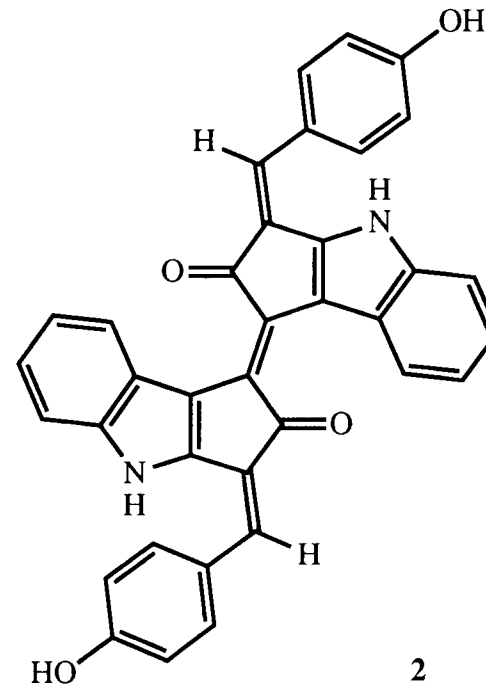
units, confirming that they were assembled as in the reduced pigment. A difference nOe experiment was used to show that the phenol ring and the indole nitrogen were adjacent. The reduced scytonemin ozonolysis fragment (**3**) has a fused 5,5,6 ring structure.

The structure determined for the ozonolysis fragment definitively ruled out the fused six-membered ring structure **D** for reduced scytonemin. An unanswered question for the five-ring linked structure was the configuration of the olefin connecting the two halves of the reduced pigment. Model building suggested that the configuration **B** would have less steric interactions, but twisting in the alternative structure **C** was not ruled out. Preliminary energy minimization with CHEM3D Plus showed that **B** was lower in energy in **C**, but only by about 0.5 kcal. More complete molecular mechanics analysis of these types of molecules with an extended pi system will require a more powerful computational program. A single energy minimization run for **B** using CHEM3D Plus took 2-3 days and found only a local minimum. Although these modeling results are prelimary, we propose the *E* geometry for the tetrasubstituted olefin connecting the two halves of the molecule of reduced scytonemin (**2**).

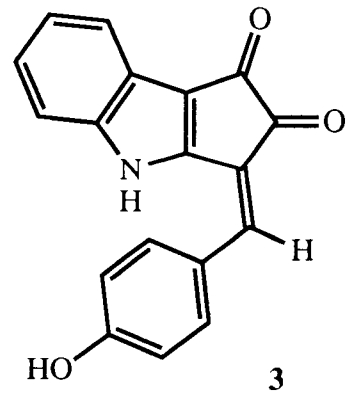
During the course of these studies, it was noticed by TLC that a red-brown pigment could also be detected in some solvent systems (50% THF/hexanes) directly below the reduced pigment. When "pure" reduced pigment was examined in these solvent systems, the red-brown substance was also noticed. This seemed to correlate with minor "contaminant" peaks that were always present in $^1$H NMR spectra. Two-dimensional TLC analysis showed that the brown component appeared to give rise to a dark red spot (reduced scytonemin) and a green spot (scytonemin) when eluted in the second dimension. This raised the possibility that the brown material could actually be the *Z* isomer of reduced scytonemin. An attempted HPLC purification of the red-brown pigment (50% THF/hexanes) revealed a minor peak just after the major red peak (reduced scytonemin). Concentration and subsequent $^1$H NMR analysis of the minor component in acetone-$d_6$ showed peaks characteristic for reduced scytonemin! Re-examination of this fraction by




1




2




3

Figure V.4. Scytonemin (**1**), Reduced Scytonemin (**2**), and the Reduced Scytonemin Ozonolysis Fragment (**3**).

TLC and HPLC showed three components: scytonemin, reduced scytonemin, and the brown component. The HPLC peak intensities showed that the reduced scytonemin and brown component were in approximately the same proportion as the original starting mixture. These results suggested that a photo-equilibrium was being established (Figure V.5). Molecular mechanics studies reveal that the *E* and *Z* isomers are only about 0.5 kcal different in steric energy, with the *E* isomer being of lower energy. Using the calculated energy difference of 0.5 kcal to calculate an approximate amount of each isomer at equilibrium yields an approximate 70:30 ratio.[172] The HPLC analysis suggested a slightly higher ratio, perhaps 80:20 or 85:15. Photoisomerization is a phenomenon that is known for N-substituted indigo pigments.[173]

The structure of scytonemin possessed two fewer hydrogens than reduced scytonemin. The key differences in the NMR spectra were the absence of the indole protons and a distinctive chemical shift at δ174.30 in the $^{13}C$ spectrum. The *p*-hydroxy phenyl group attached to the *exo* olefin was intact based on the isolation of *p*-hydroxybenzaldehyde from an ozonolysis reaction. The simple redox relationship between scytonemin and reduced scytonemin and the NMR data lead to structure **1** for scytonemin. This structure was supported by long range $^{1}H$-$^{13}C$ NMR experiments. Before we had the capability to run HMBC spectra, we performed LR HETCOSY and COLOC experiments on scytonemin. Even using overnight accumulations of data, only a partial set of correlations (Table V.4) was observed. More recently, an HMBC spectrum for scytonemin was recorded (Figure V.6). This experiment allowed the assignment of several more $^{13}C$ signals and confirmed the assignment of an imine-like carbon adjacent to the *exo*-olefin (Table V.4). An energy minimized 3-D representation of scytonemin in depicted in Figure V.7. The biphenyl-like arrangement about the bond connecting the two halves of the molecule suggested that chirality was possible if the barrier to rotation were sufficiently large. A CD spectrum was recorded to assess this possibility of chirality, but

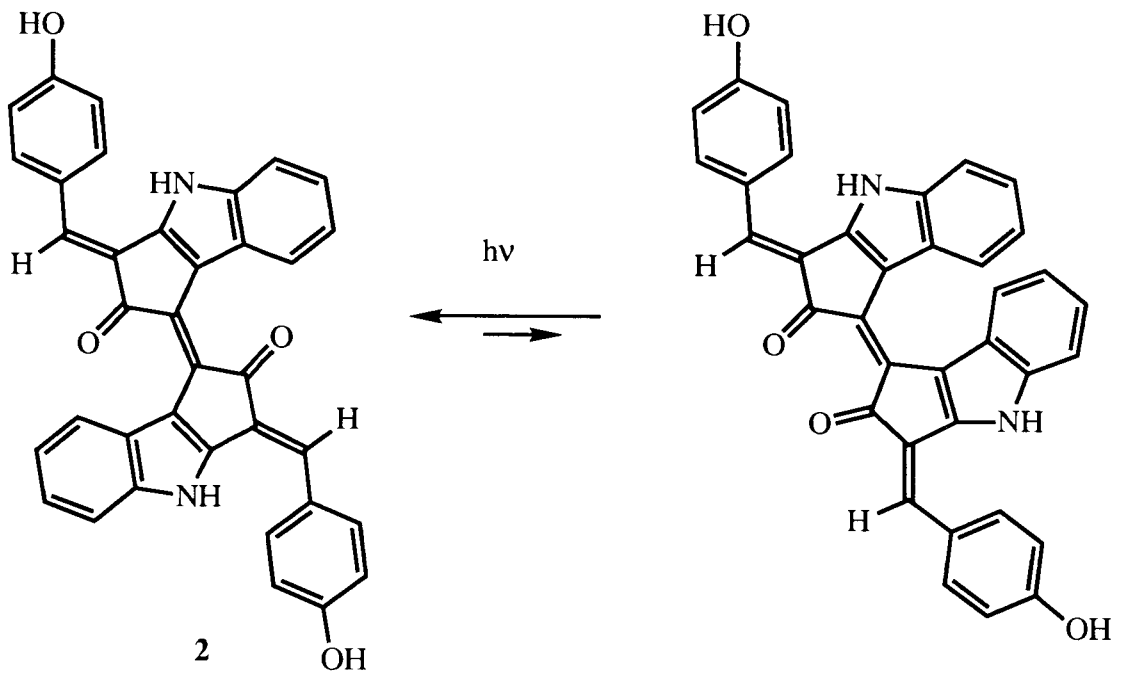



Figure V.5. Photoisomerization of Reduced Scytonemin?

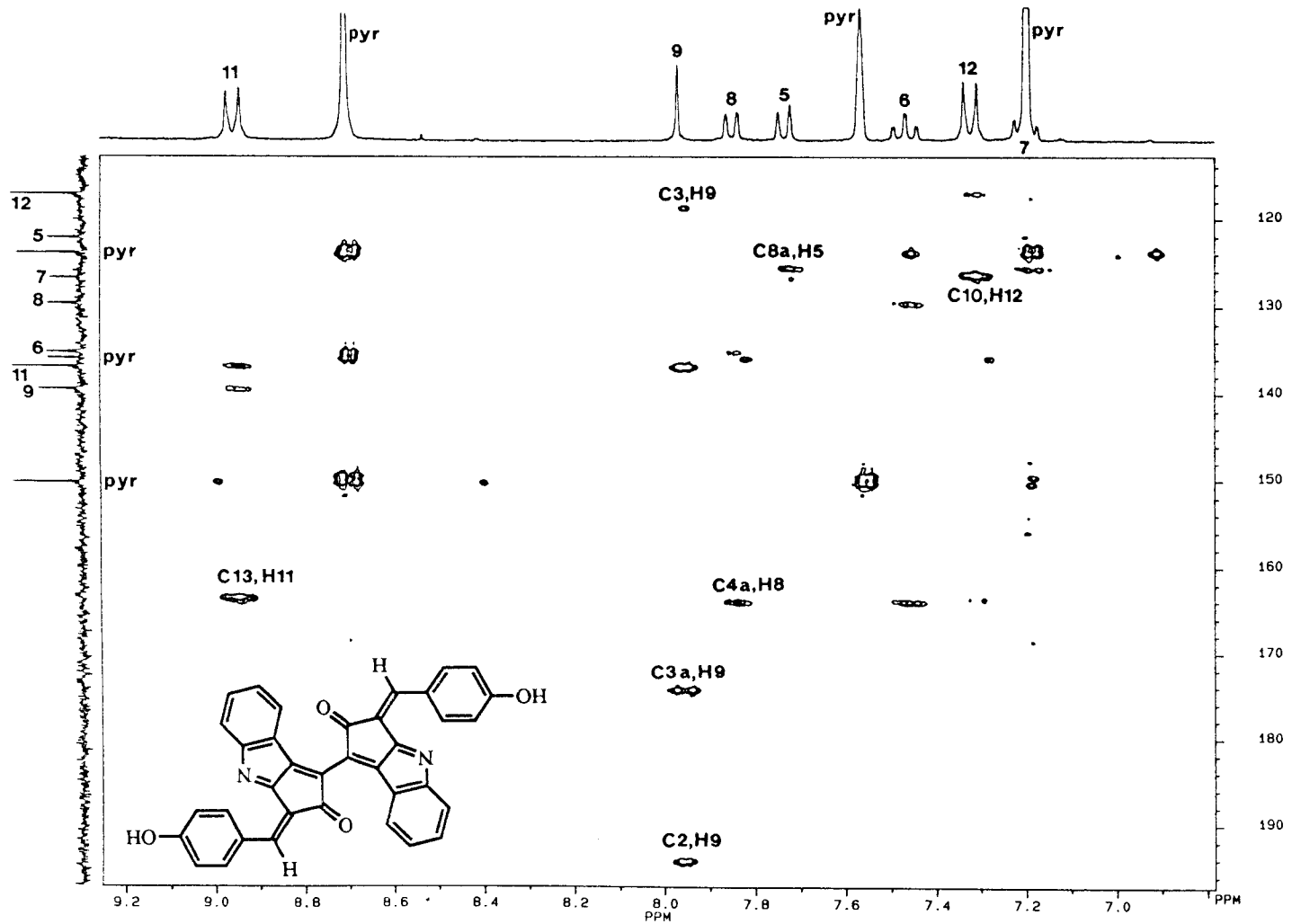



Figure V.6. HMBC Spectrum of Scytonemin (**1**) in Pyr-$d_5$.

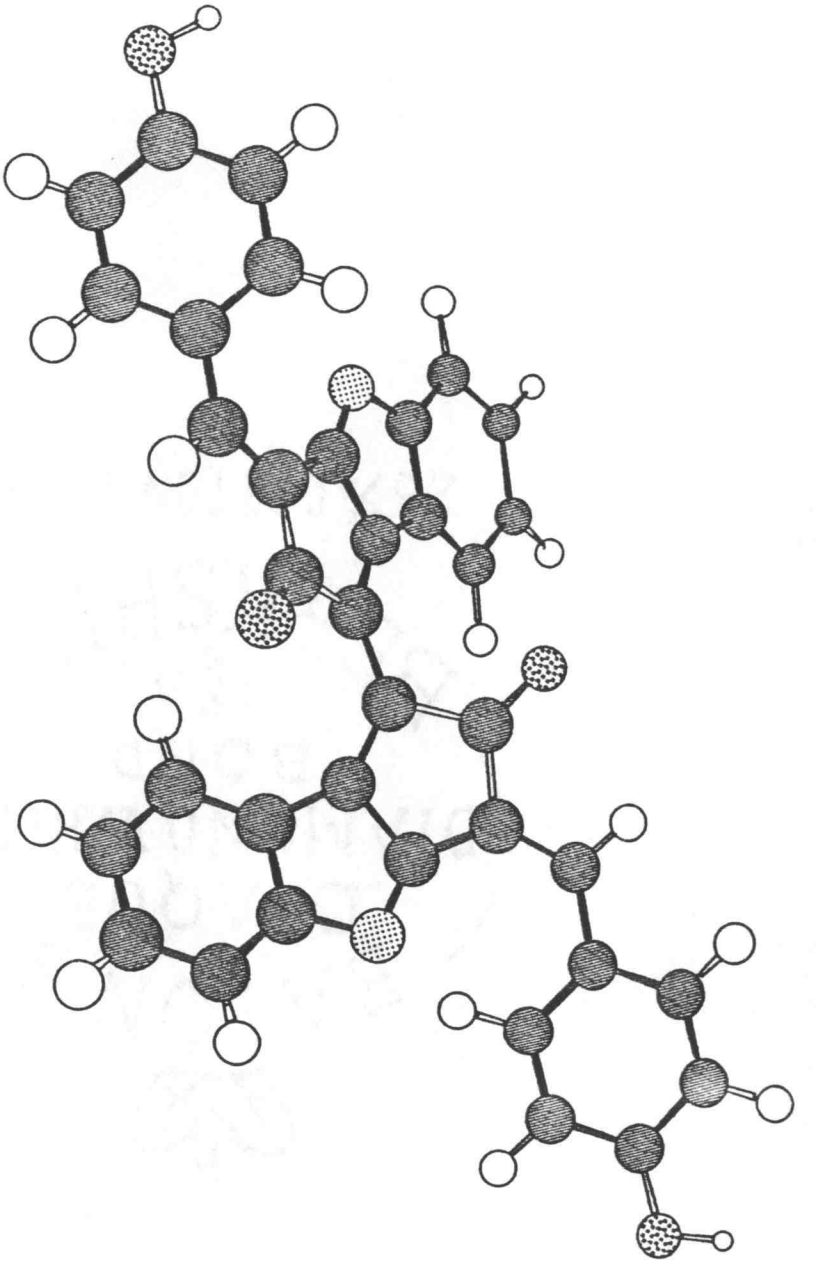


Figure V.7. Energy-minimized 3-D Model of Scytonemin (**1**).

no CD curve was obtained, indicating that the scytonemin sample examined was optically inactive.

The well-known pigment indigo provides an interesting parallel to reduced scytonemin. Both are indole derived dimers that have the possibility of *cis-trans* isomerism about the olefin joining the two monomeric fragments. Although indigo normally exists in the *trans* form, the *cis* form has been isolated.[174] The *cis* form rapidly reverts to the *trans* form on storage. A difference between reduced scytonemin and indigo is that in the *trans* form, indigo has the ability to hydrogen bond, which stabilizes this isomer. Isatin, an oxidative degradation product of indigo bears a striking resemblance to the reduced scytonemin ozone fragment (Figure V.8).[173] Indigo also has an oxidation product, dehydroindigo, which has obvious similarity to scytonemin.[175] Since indigo can be prepared by oxidative coupling of indoxyl,[176] it would be interesting to determine whether or not reduced scytonemin could be synthesized by oxidative coupling of an analogous monomer unit (Figure V.9).

Scytonemin, reduced scytonemin, and compound **3** have all been tested in the NCI anticancer assays. None showed positive results. All three were also negative in a tyrosine kinase inhibition assay performed at Syntex.

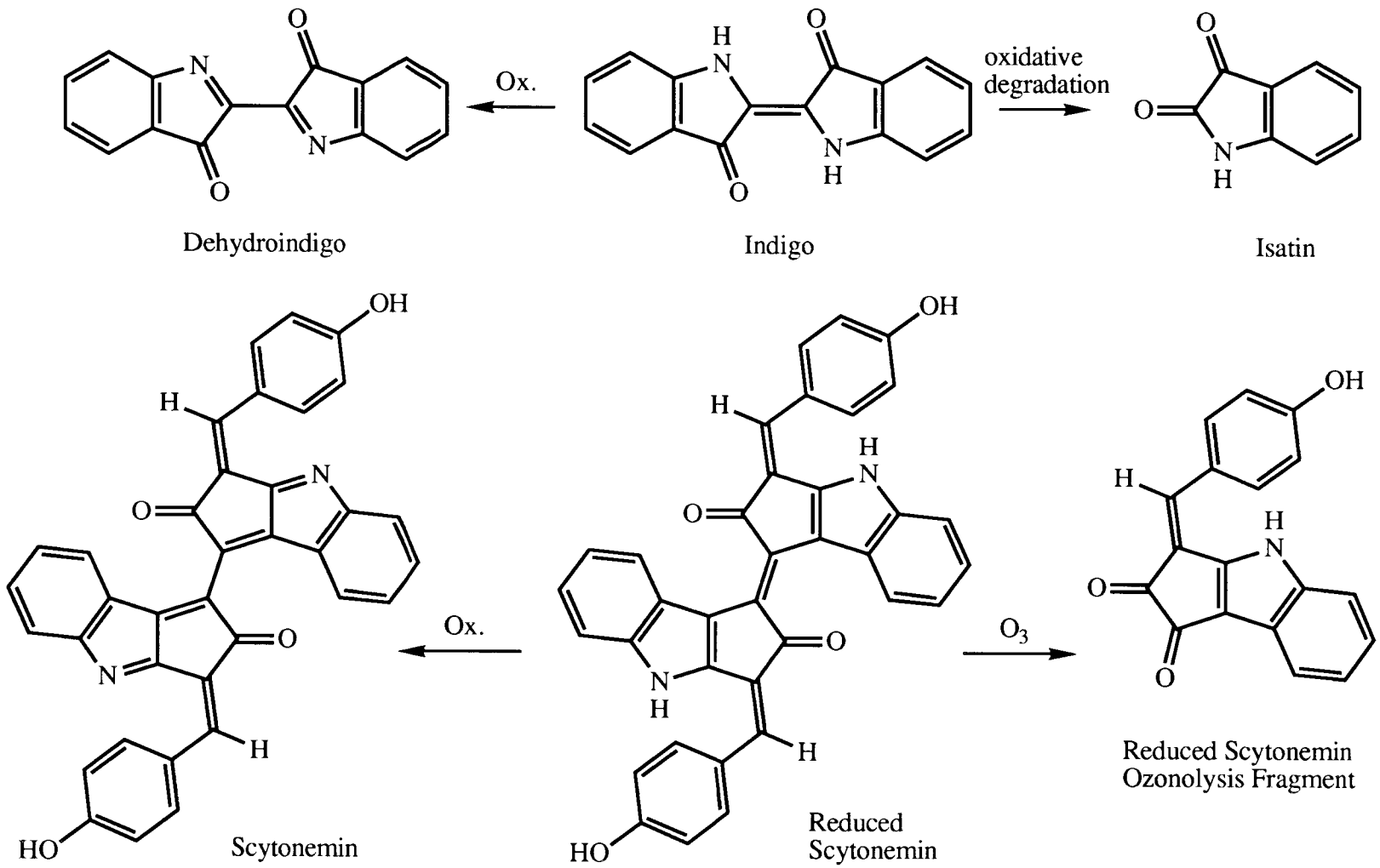



Figure V.8. A Comparison of Indigo and Reduced Scytonemin.

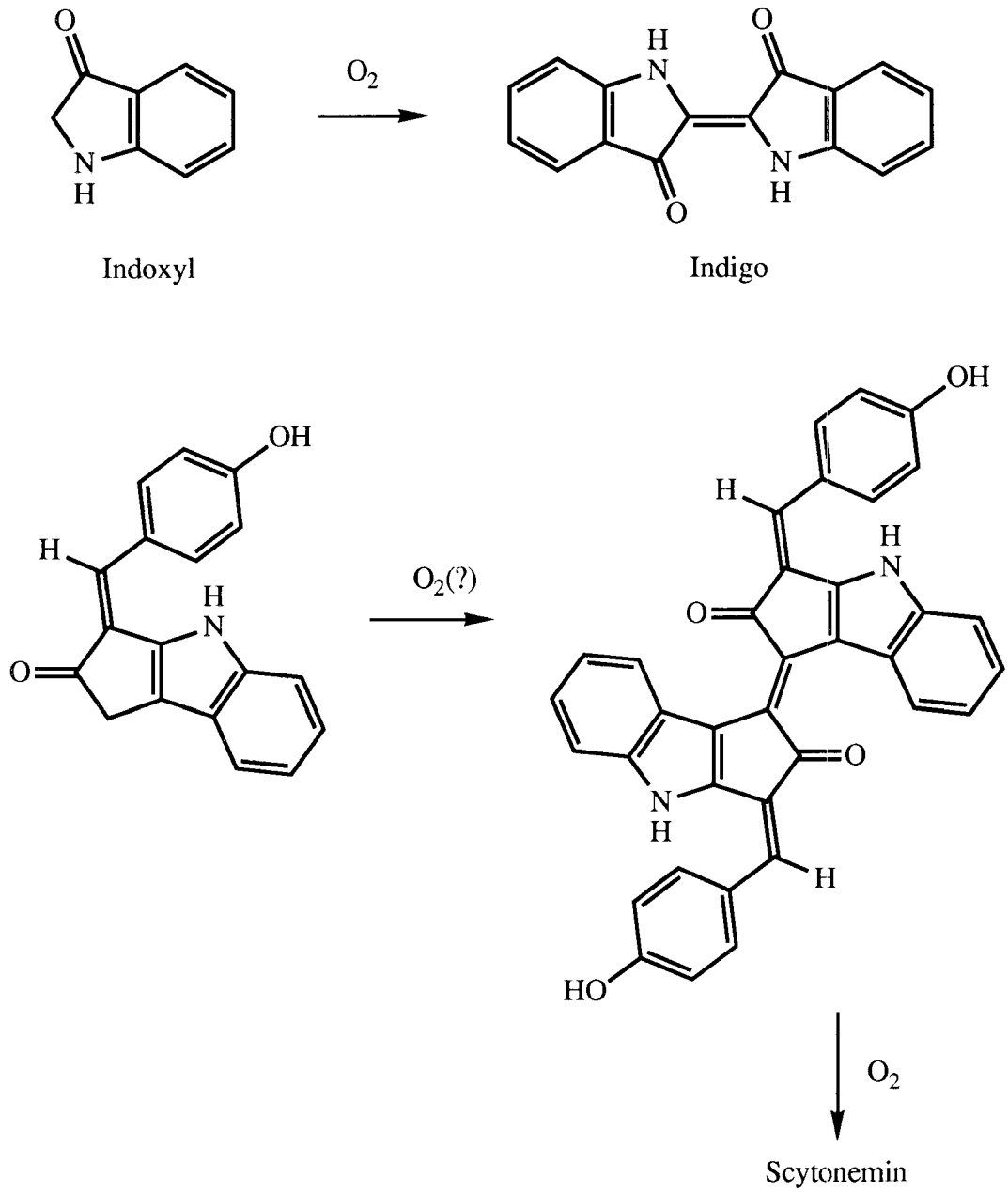



Figure V.9 Synthesis of Indigo and a Speculative Proposal for the Formation of Reduced Scytonemin.

## Experimental

*General.* Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC300 instrument operating at 300.13 MHz for $^{1}H$ NMR and at 75.46 MHz for $^{13}C$ NMR or on an AM400 instrument operating at 400.13 MHz and 100.61 MHz for $^{1}H$ and $^{13}C$ NMR, respectively. Proton spectra were referenced to internal tetramethylsilane at 0.00 ppm or to the residual solvent line. Carbon spectra were referenced to residual solvent. Infrared (IR) spectra were obtained using a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were run on a Hewlett-Packard 8452a spectrophotometer. Low and high-resolution fast atom bombardment mass spectra (FABMS) were recorded on a Kratos MS 50 TC spectrometer. High-performance liquid chromatography (HPLC) utilized a Waters M6000A pump, Rheodyne 7125 injector, and a Waters Lambda-Max 480 LC spectrophotometer. Merck aluminum-backed thin-layer chromatography (TLC) sheets (silica gel 60 $F_{254}$) were used for TLC. Compounds were detected visually or by UV illumination. Ozonolysis utilized an OREC Ozonator Model 03V10-0 with an oxygen gas supply.

*Collection and extraction.* A *Stigonema* sp. from Waldo Lake, Oregon was collected in September of 1991. A moss-like *Scytonema* sp. was collected at the Seaquarium in Curaçao, Netherlands Antilles in December, 1991. *Lyngbya* sp. from Huahine, French Polynesia, was collected in June, 1992. The extraction of the seaquarium *Scytonema* will be used as a representative example. The algae (178 g dry weight) were first extracted twice with MeOH. Most of the pigments and lipids are removed in these extracts. Next the algae were extracted over a one week period with eight portions of EtOAc. The residue from the EtOAc extracts was rinsed repeatedly with hexanes and then MeOH to provide scytonemin as a crusty green-brown solid (670 mg, 0.3% yield based on dry weight). TLC indicated that the reduced scytonemin was present in the MeOH rinse solution, along with other pigments. Pure reduced scytonemin is most

readily obtained by reduction of scytonemin. Yields of the sheath pigments are highly variable, since pigment production is directly correlated to amount of exposure to intense sunlight.

*Scytonemin* (**1**). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3308 (br. OH), 1716 (w), 1683 (w), 1597, 1578, 1558, 1512, 1286, 1172, 1142. UV $\lambda_{max}^{THF}$ nm: 252 ($\varepsilon$ = 42,000), 278 ($\varepsilon$ = 25,000), 300 ($\varepsilon$ = 24,000), 386 ($\varepsilon$ = 52,000), 560 (broad; $\varepsilon$ = 3,800). M.p.: >325°. Since the crystals did not melt, they were burned. A yellow flame was apparent initially, followed by an orange glow. The residue at this stage was examined under a microscope and was seen to be a black, charcoal-like mass. This residue burned completely with further heating. $^1$H NMR (300 MHz, acetone-$d_6$, TMS at 0.00 ppm): $\delta$9.49 (1H, *bs*), 8.78 (2H, *d*, *J*=8.7 Hz), 7.66 (1H, *s*), 7.64 (2H, *m*), 7.59 (1H, obscured *ddd*, *J*=7.6, 7.6, 1.2 Hz), 7.24 (1H, *ddd*, *J*=7.6, 7.2, 1.2 Hz), 7.07 (2H, *d*, *J*=8.7 Hz). $^1$H NMR (300 MHz, DMF-$d_7$, solvent line at 2.74 ppm): $\delta$8.78 (2H, *d*, *J*=8.7 Hz), 7.74 (1H, *bd*, *J*=7.2 Hz), 7.66 (1H, *s*), 7.66 (2H, *bm*), 7.27 (1H, *dd*, *J*=8.1, 7.1 Hz), 7.08 (2H, *d*, *J*=8.7 Hz). $^{13}$C NMR (75 MHz, DMF-$d_7$, solvent line at 162.70 ppm): $\delta$193.98, 138.98, 136.74, 126.46, 122.10, 116.68. These lines were the most intense under the conditions used. The signal to noise ratio was poor, so further assignments could not be made with confidence. HR (+)-FABMS (2% trifluoroacetic acid/3-nitrobenzyl alcohol) m/z obs. $[M + 2H]^+$ 546.1578 ($C_{36}H_{22}N_2O_4$, -0.2 mmu dev.)

*4,4'-Dihydroscytonemin* (**2**). The pigment **1** (107 mg) was dissolved in 5 mL DMF. This DMF solution was then dripped into a separatory funnel containing EtOAc (50 mL) and distilled $H_2O$ (75 mL). After removing the aqueous layer, the dark green organic layer was washed twice with $H_2O$ (75 mL each). The EtOAc solution was not dried, but was treated directly with L-ascorbic acid (522 mg, 2.96 mmol, 15 equiv). After 40 min, the bright red reaction mixture was washed with 3 x 50 mL portions of distilled $H_2O$, dried ($Na_2SO_4$), and concentrated. The reduced pigment appears as an almost black

residue when dry. (Note: Since scytonemin does not readily re-dissolve in ethyl acetate once concentrated to a solid residue, the DMF technique was devised to obtain concentrated EtOAc solutions. If scytonemin is not completely dissolved, the reduction step proceeds poorly) The reduced pigment can also be obtained by catalytic hydrogenation using 2:1 THF/EtOH as a solvent (1 h reaction time). FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3315 (br. OH), 1761 (w), 1683, 1601, 1498, 1451, 1363, 1232, 1127, 1097. UV $\lambda_{max}^{THF}$ nm: 246 (ε = 30,000), 276 (ε = 14,000), 314 (ε = 15,000), 378 (ε = 22,000), 474 (ε = 14,000), 572 (broad shoulder; ε = 7,600). $^1H$ NMR (300 MHz, pyr-$d_5$, TMS at 0.00 ppm): δ12.75 (1H, *bs*, NH), 8.41 (1H, *d*, *J*=7.8 Hz, H-8), 7.86 (2H, *d*, *J*=8.6 Hz, H-11(15)), 7.83 (1H, *s*, H-9), 7.65 (1H, *d*, *J*=7.8 Hz, H-5), 7.48 (1H, *ddd*, *J*=7.8, 7.2, 1 Hz, H-7), 7.41 (1H, *ddd*, *J*=7.8, 7.2, 1.2 Hz, H-6), 7.01 (2H, *d*, *J*=8.6 Hz, H-12(14)). $^{13}C$ NMR (75 MHz, pyr-$d_5$, solvent centerline at 149.90 ppm): δ196.73 (C-2), 160.53 (C-13), 146.43 (C-3a), 142.46 (C-4a), 131.54 (C-11(15)), 128.63 (C), 128.36 (C-9), 126.90 (C-10), 126.57 (C-8), 125.05 (C), 124.90 (C-6), 121.45 (C-7), 117.00 (C-12(14)), 113.08 (C-5). Assignments are based on XHCORR and LR HETCOSY experiments. Two quaternary carbons are not observed in pyridine-$d_5$ and are likely buried under a residual solvent line. Carbons assigned as (C) are quaternary carbons that do not have firm assignments. HR (+)-FABMS (2% trifluoroacetic acid/3-nitrobenzyl alcohol) m/z obs. $[M]^+$ 546.1578 ($C_{36}H_{22}N_2O_4$, -0.2 mmu dev.), HR (-)-FABMS (dithiothreitol/dithioerythritol) m/z obs. $[M - H]^-$ 545.1500 ($C_{36}H_{21}N_2O_4$, -0.1 mmu dev.).

Carbon-proton coupling constants were obtained using the Bruker GATEDEC experiment on a sample of the reduced pigment in DMF-$d_7$: δ196.41 (*d*, *J*=5.8 Hz), 160.22 (*btd*, *J*=8.9, 2.5 Hz), 145.76 (*dd*, *J*=10.3, 4.7 Hz), 142.21 (*btd*, *J*=9.5, 3.0 Hz), 131.71 (*ddd*, *J*=158, 6.7, 6.2 Hz), 128.22 (obscured *dt*, *J*=~160, 4.7 Hz), 127.73 (*dd*, *J*=5.7, 2.5), 126.94 (*t*, *J*=7.7 Hz), 125.98 (*ddd*, *J*=~161, 5.8, 2.2 Hz), 124.93 (*dd*, *J*=159, 8.8 Hz), 124.69 (*d*, *J*=2.7 Hz), 124.37 (*m*), 123.39 (*s*), 121.30 (*dd*, *J*=159, 7.8

Hz), 116.91 (*ddd*, *J*=160, 4.4, 4.4), 113.51 (*dd*, *J*=161, 7.3 Hz). Selective irradiation of the indole proton signal at 11.79 ppm resulted in the collapse of several signals: δ145.76 (*dd* → *d*, *J*=10.3 Hz), 142.21 (*btd* → *dd*, *J*=8.7, 8.4 Hz), 127.73 (*dd* → *d*, *J*=2.5 Hz), 124.37 (*m* → *dd*, *J*=~8.5, 5.5 Hz). Since the δ145.76 carbon was coupled two only two protons, the $^{3}J_{CH}$ for C3a-H9 must be 10.3 Hz.

*4,4'-Dihydroscytonemin Ozonolysis Fragment* (**3**). Dihydroscytonemin (53 mg in 70 mL EtOAc) was cooled to -78°, then $O_3$ was bubbled through the solution for 10 min. Dimethyl sulfide was added dropwise (1 mL) to the cold solution and then allowed to react at room temperature for 5 hr. The concentrated product was purified by first flash (5:95 MeOH/$CHCl_3$), then Sephadex LH-20 chromatography (1.7 cm O.D. x 19.5 cm; 1:1 EtOAc/MeOH) to give the ozonolysis product **3** (5.3 mg, 10% yield). An initial fraction from the flash chromatography provided *p*-hydroxybenzaldehyde. Compound **3** was recrystallized from 1:1 EtOAc/MeOH to yield orange needles. FTIR $\nu_{max}^{film}$ $cm^{-1}$: 3584 (sharp), 3228 (br. OH), 1733, 1664, 1599, 1584, 1508, 1452, 1282, 1235, 1162. M.p.: 130° darkening of crystals, 225° dec. UV $\lambda_{max}^{THF}$ nm: 244 (ε = 16,000), 276 (ε = 13,000), 296 (ε = 14,000), 372 (ε = 28,000). HR (+)-FABMS (0.1 M camphor sulfonic acid/3-nitrobenzyl alcohol) m/z obs. [M + H]+ 290.0816 ($C_{13}H_{12}NO_3$, -0.1 mmu dev.).

*Scytonemin Acetate*. This acetate derivative was only partially characterized. UV $\lambda_{max}^{CH_2Cl_2}$ nm: 248 (A=0.28), 282 (A=0.25), 300 (A=0.25), 348 (A=0.40), 450 (shoulder, A=0.1). The extinction coefficients were not determined, but the absorbance values are given to provide a sense of relative peak intensities. $^{1}H$ NMR (300 MHz, $CDCl_3$): δ8.71 (2H, *d*, *J*=8.7 Hz), 7.71 (1H, *s*), 7.66 (1H, *d*, *J*=7.6 Hz), 7.53 (1H, *ddd*, *J*=7.6, 7.6, 1.1 Hz), 7.47 (1H, *d*, *J*=7.5 Hz), 7.30 (2H, *d*, *J*=8.7 Hz), 7.20 (1H, *ddd*, *J*=7.6, 7.5, 0.8 Hz), 2.36 (3H, *s*). Decoupling at δ7.19 collapsed the patterns at δ7.53 and δ7.47. Decoupling at δ8.71 collapsed the doublet at δ7.30.

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