AN ABSTRACT OF THE THESIS OF

Melodie A. Graber for the degree of Master of Science in Pharmacy presented on May 29, 1997. Title: Natural Products from Temperate and Tropical Marine Algae.

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William H. Gerwick

Marine algae are a rich source of bioactive and structurally novel compounds. Studies of algal metabolites have led to new medicinal agents, pharmacological probes for biochemical studies, as well as a greater understanding of our environment. The current need for new pharmaceutical agents has prompted our investigations of several genera of algae which displayed crude extract biological activity and/or unique thin-layer chromatographic characteristics. This research utilized modern chromatographic techniques coupled with spectroscopic analysis including IR, UV, MS, as well as 1-D and 2-D NMR.

Agardhilactone, a novel tricyclic oxylipin containing δ-lactone, cyclopentane and epoxide rings and a conjugated diene, was isolated from the marine red alga *Agardhiella subulata*. Its structure, including partial stereochemistry, was determined by NMR analysis of agardhilactone acetate and GC-MS analysis of menthoxycarbonyl derivatives. Agardhilactone represents a new oxylipin structure class, distinguished by the unprecedented position of the cyclopentyl ring, C6 to C10, within a 20-carbon chain framework.

Kalkipyrone, a novel α-methoxy-β,β'-dimethyl-γ-pyrone linked to an alkyl side chain, was isolated from an assemblage of the cyanobacteria *Lyngbya*

majuscula and *Tolypothrix sp.* Its structure, including stereochemistry, was determined by NMR, UV, and IR analysis and by GC-MS analysis of menthoxycarbonyl derivatives. Kalkipyrone displays toxicity to brine shrimp ($LD_{50} \approx 1$ μg/ml) and gold fish ($LD_{50} \approx 2$ μg/ml) and is structurally related to several *Streptomyces* metabolites, the actinopyrones.

Natural Products from Temperate and Tropical Marine Algae.

by

Melodie A. Graber

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Melodie A. Graber, Author

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LIST OF ABBREVIATIONS

COSY	^{1}H-^{1}H Chemical Shift Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
EAC	Epoxy Allylic Carbocation
EI	Electron Impact
EPA	Eicosapentaenoic Acid
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromotography Mass Spectrometry
HETE	Hydroxyeicosatetraenoic Acid
HHT	Hydroxyheptadecatrienoic Acid
HIV	Human Immunodeficiency Virus
HPETE	Hydroperoxyeicosatetraenoic Acid
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High-Performance Liquid Chromotography
HRCIMS	High Resolution Chemical Ionization Mass Spectrometry
HREIMS	High Resolution Electron Impact Mass Spectrometry
IC	Inhibitory Concentration
IR	Infrared or Infrared Spectroscopy
LC	Lethal Concentration
LD	Lethal Dose
LO	Lipoxygenase
LRMS	Low Resolution
MC	Menthoxylcarbonyl
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance

NP	Normal Phase
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Exchange Spectroscopy
PG	Prostaglandin
RT	Room Temperature
SCUBA	Self-contained Underwater Breathing Apparatus
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet Spectroscopy
VFDF	Very Fast Death Factor
XHCORR	Heteronuclear Chemical Shift Correlation Spectroscopy

This thesis is dedicated to the memory of Alicia Showalter Reynolds.

NATURAL PRODUCTS FROM TEMPERATE AND TROPICAL MARINE ALGAE

CHAPTER I

GENERAL INTRODUCTION

Natural products have played significant roles throughout the history of human kind. Numerous beneficial drugs such as penicillin, salicin, and streptomycin trace their origins to the natural environment. Potent toxins like batrachotoxin, a Na^+ channel blocking agent produced by the frog *Phyllobates,*[1] and tubocurarine (curare), a neuromuscular blocking agent from plant extracts of *Chondodendron* and *Strychnos* have been utilized in South American tribal warfare. The psychoactive constituent of coca leaves, cocaine, was used by the Incas for socio-religious ceremonies[2] and today is a chemical prototype for most local anesthetic drugs.[3]

The isolation and characterization of natural products continues to be both structurally and pharmacologically interesting. New discoveries have led to medicinal agents, probes for the study of biochemical functions, and a greater understanding of our environment. Although terrestrial plants and animals have been utilized as sources of natural products for centuries, marine organisms have been examined considerably less.

The advent of the self-contained underwater breathing apparatus (SCUBA) has permitted the study of the marine environment to grow within the last several decades. Advances in the fields of mass spectrometry, nuclear magnetic resonance spectroscopy and chromatography provided the foundation for a surge of isolations and characterizations of metabolites from marine organisms. Extensive literature reviews by D. J. Faulkner and P. J. Scheuer describe many of these compounds.[4-16] A significant number of these

natural products are bioactive and/or structurally novel, encouraging further investigations into the diverse underwater world.

Our research group specializes in the isolation and characterization of metabolites from marine organisms with a focus on marine algae, a rich source of bioactive and structurally novel compounds. When I began my studies at Oregon State University, it was my proposition that continued study of marine algae would produce additional bioactive and novel metabolites. Subsequently, the current need for new pharmaceutical agents prompted our investigations of several genera of algae which displayed crude extract biological activity or unique thin-layer chromatographic characteristics. This thesis describes some of our research in the area of algal oxylipins as well as the isolation a metabolite from cyanobacteria.

Marine Natural Products

Sponges have proved to be a rich source of bioactive compounds. The isolation and subsequent studies in the early 1950's of spongouridine (**1**, Figure I.1) and spongothymidine (**2**), two arabinosyl containing nucleosides from *Tethya crypta,*[17] initiated the search for antitumor compounds from the sea. Studies involving these pyrimidines eventually led to the synthesis of a new class of compounds, arabinosyl nucleoside analogs. One of the analogs, Ara-C (**3**), was shown to be an effective inhibitor of sarcoma, carcinoma, leukemia and lymphoma in mice.[18,19] Today it is used clinically in humans for the treatment of acute myelocytic leukemia and non-Hodgkins lymphoma.[20]

Another series of antitumor sponge metabolites, the halichondrins, was isolated from *Halichondria okadai.* Of these polyether macrolides, halichondrin B (**4**) exhibited the most potent *in vivo* activity[21] and was subsequently shown

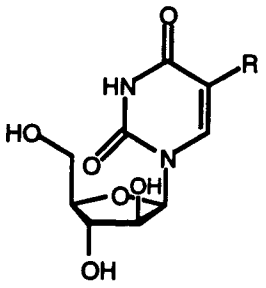
to be an antimitotic agent which binds in the *vinca* domain of tubulin.[22] A potent anti-inflammatory agent, manoalide (**5**) was isolated from the sponge *Luffariella variabilis*. This sesterterpenoid inhibits phospholipase A_2 as a covalent adduct and has been used as a tool to study the arachidonic acid cascade.[23,24]

The sea hare *Dolabella auricularia* yielded dolastatin 10 (**6**, Figure I.2), the most potent antineoplastic agent to date. This peptide is one of a series of potent cell growth inhibitors and antineoplastic agents[25,26] and was shown to be another antimitotic agent which binds to the *vinca* site.[27]

The didemnins are a series of cyclic depsipeptides isolated from the tunicate *Tridemnum solidum* and have exhibited a wide range of antitumor and antiviral activities.[28,29] Didemnin B (**7**) displays the most potent activities including cytotoxicity to murine leukemia, inhibition of protein synthesis and induction of apoptosis. This exciting activity profile led to testing (**7**) as the first marine natural metabolite to be evaluated in phase I and phase II clinical trials at the National Cancer Institute (NCI) as a potential anticancer agent.[30]

Marine algae are another source of bioactive compounds. The red alga *Laurencia* yielded tetracyclic polyethers deriving from squalene such as thyrsiferol (**8**, Figure I.3).[31] This metabolite as well as several derivatives have shown potent antiviral activities.[32] Of the numerous polyhalogenated monoterpenes isolated from the red alga, *Porteria*, halomon (**9**), was selected by the NCI Decision Network Committee for preclinical drug development due to its potent *in vitro* antitumor activities.[33] Preliminary *in vivo* screening has been promising in a xenograph model of a highly aggressive brain tumor line showing a high percentage (40 %) of "cures."[34]

Tetrodotoxin (**10**, Figure I.4) is one of the best known marine toxins. It is responsible for numerous cases of fatal food poisoning throughout the world. Although originally isolated from pufferfish, it's origin has been traced to a



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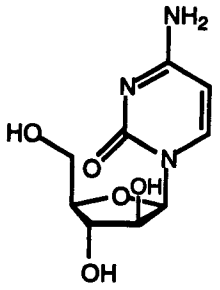
2 R = CH_3



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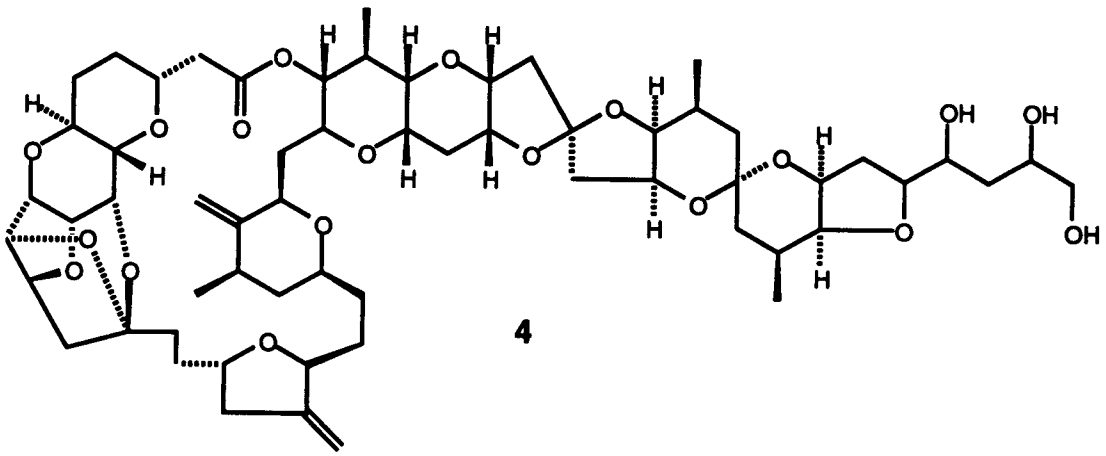

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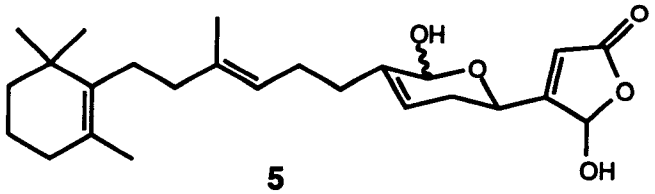

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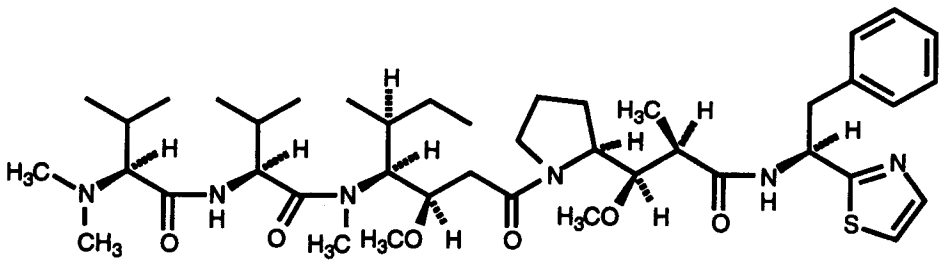
Figure I.1 Bioactive Sponge Metabolites



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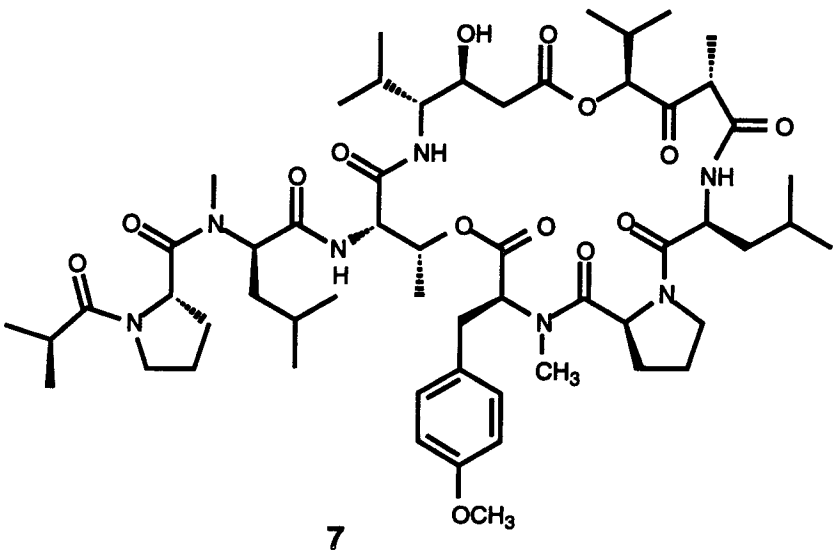

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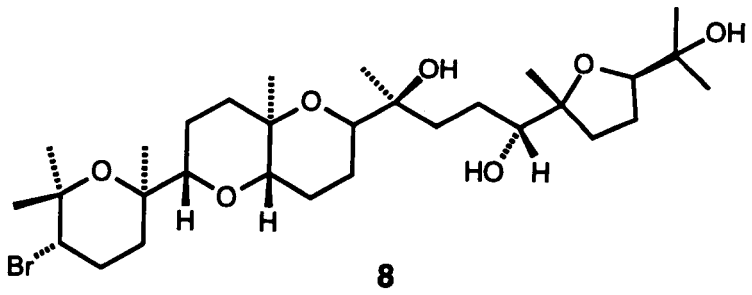
Figure I.2 Marine Metabolites with Biomedical Potential



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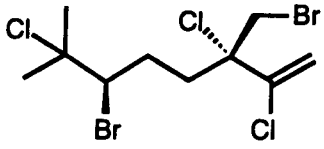

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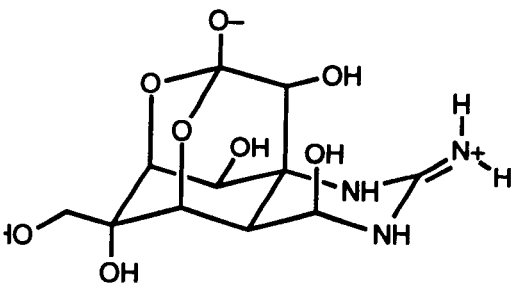
Figure I.3 Bioactive Algal Metabolites



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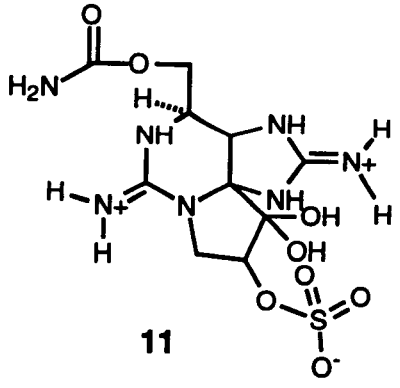

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Figure I.4 Marine Toxins

marine bacterium, *Shewanella alga*, that is ingested as part of the fish's food source. Subsequently, numerous other bacteria have been reported as producers of this deadly toxin.[35,36] Several genera of dinoflagelletes, as well as cyanobacteria, have been known to produce another fatal poison, saxitoxin (**11**). This tricyclic alkaloid was the first recognized toxic constituent of paralytic shellfish poisoning.[37] Subsequently, neosaxitoxin and a suite of derivatives have been described.[38] Saxitoxin and tetrodotoxin are both sodium channel blockers in excitable membranes. Studies of these metabolites have enabled pharmacologists to study the mechanisms of action of these critical channels.[39]

Oxylipins

One focus of our marine natural products laboratory is the discovery and characterization of marine algal oxylipins.[40,41] These oxygenated compounds arise from fatty acid metabolism with at least one step of mono- or dioxygenase-dependent oxidation. The term "eicosanoid" specifically refers to C20 derived fatty acids. However, numerous analogs with shorter or longer chain length exist in nature. The term "oxylipin" has been proposed as a more general term to encompass all chain lengths.[42]

Oxylipins play integral roles in mammalian systems such as regulation of the inflammation response, control of several reproductive functions, and regulation of the sleep/wake cycle.[43] Thromboxane A_2 induces platelet aggregation and leukotriene D_4 contracts and relaxes smooth muscles. Most prostaglandins (PG) of the E series are potent vasodilators.[44] Profound effects can occur even at extremely low concentrations.[43]

The release of arachidonic acid from membrane lipids by the action of phospholipase A_2[43] leads to three major mammalian biosynthetic pathways for

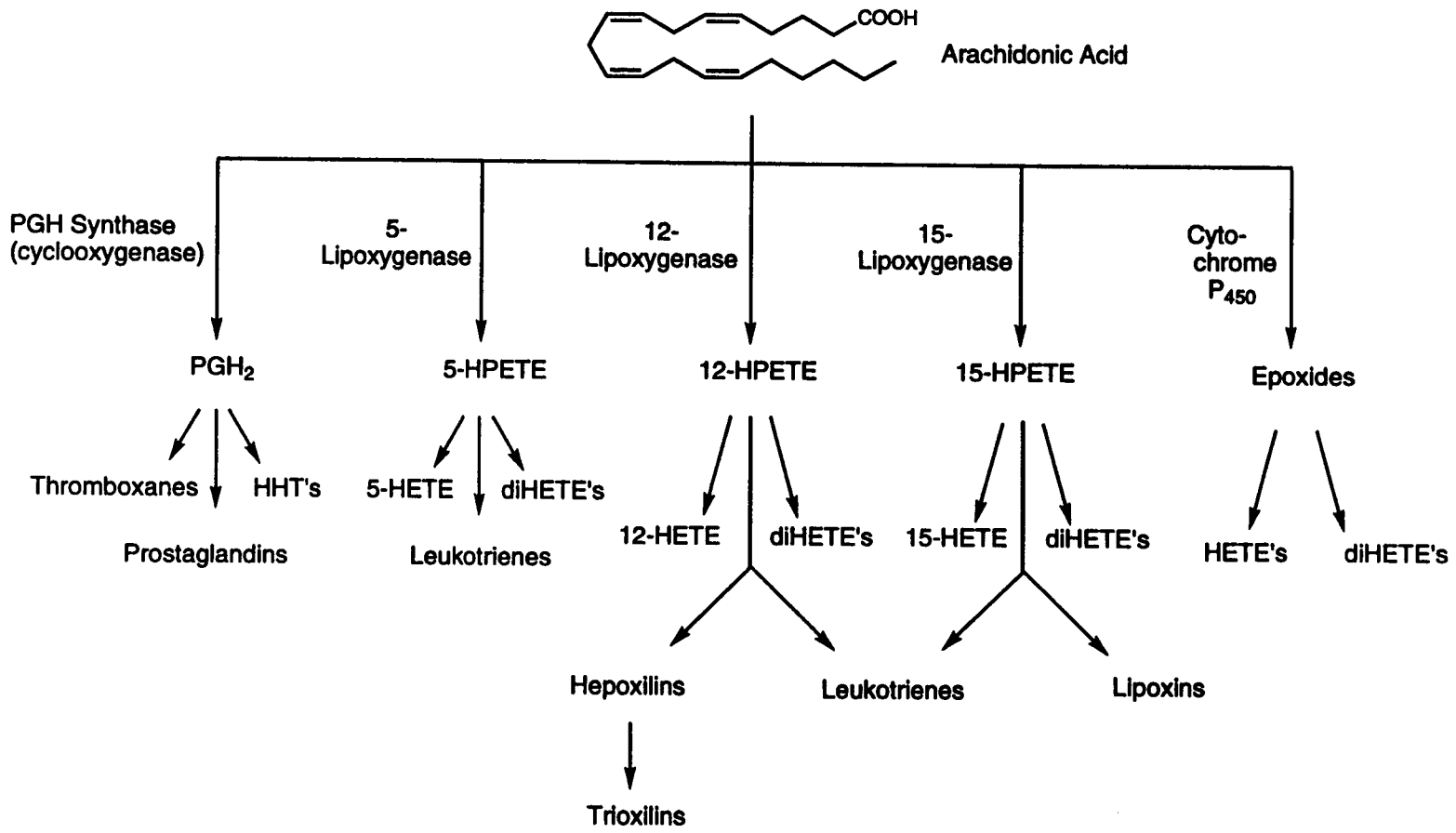


Figure I.5 Generalized Scheme for the Arachidonic Cascade in Mammals. PG, prostaglandins; HHT, hydroxy-heptadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid (adapted with modifications from ref. 41)

the production of oxylipins (Figure I.5). The prostaglandins and thromboxanes are produced by cyclooxygenase (PGH synthase). The lipoxygenases (LO) produce numerous oxygenated products such as leukotrienes, hydroperoxyeicosatetraenoic acids, hepoxilins, and lipoxins. The monooxygenase activity of cytochrome P450 yields some hydroxyeicosanoids.[44,45]

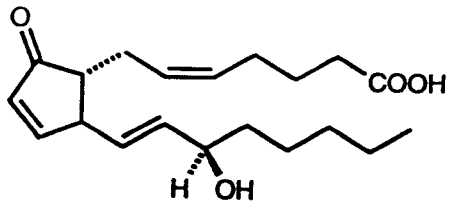
Since the first lipoxygenase enzyme was isolated from soybeans in 1947,[46] numerous LO metabolites and activities have been found throughout the classes of higher plants. Several literature reviews of plant LO have been published.[47,48] While most mammalian oxylipins derive from C20 precursors, plant oxylipins originate mainly from C18 fatty acids such as linoleic and α–linolenic acids.[47] Plant LO activity produces leukotrienes, lipoxins and other oxylipins. Allene oxides serve as intermediates in some pathways.[47] As one example, biosynthetic studies from several species have shown the production of 7-iso-jasmonic acid to proceed through an allene oxide intermediate. The pathway is initiated by LO catalyzed oxygenation of α-linolenic acid. After several steps, the allene oxide intermediate, 12,13 *(S)*-epoxy-9*(Z)*,11,15*(Z)*-octadecadienoic acid cyclizes to produce 12-oxophytodienoic which then proceeds through several intermediates to form 7-iso-jasmonic acid.[48-50] Jasmonic acid and its methyl ester are plant hormones which have been reportedly responsible for numerous activities such as growth inhibition, senescence, induction of leaf protein synthesis as well as abscission.[48]

Interest in oxylipins from the marine environment was initiated by the discovery in 1969 of 15*R*-PGA_2 (**12**, Figure I.6) and its diester from the soft coral *Plexaura homomalla*.[51] This prostaglandin is identical to one found in mammalian systems except it is epimeric at position 15. Both C-15 isomers were found to be effective antifeedent agents against predatory reef fish.[52]

Research evolving from this discovery has resulted in the isolation of numerous oxylipins from a broad range of organisms. Another soft coral *Telesto* yielded the punaglandins, halogenated prostanoids. Punaglandin 3 (**13**) displayed potent inhibition of leukemia cell proliferation.[53,54] Numerous biosynthetic studies involving soft corals and other marine invertebrates have been conducted to probe oxylipin production and the role they play in the marine environment.[42,45,55] Most notably, in comparison with mammalian systems was the discovery that *P. homomalla* produces prostanoids through non-cyclooxygenase pathways, most likely by the utilization of lipoxygenase activity to produce allene oxides as intermediates which cyclize to form the natural products.[42]

The structural diversity of marine oxylipins also is apparent in other invertebrates. An unusual oxylipin, aplydilactone (**14**), which exhibits phospholipase A_2 activating activity, was isolated from the sea hare, *Aplysia kurodai*.[56] Prostaglandin 1,15-lactones of the E and F series were characterized from the nudibranch *Tethys fimbria*. The E series lactones (PGE_2-1,15 lactone, **15**) play a role in autonomy, a behavioral defense mechanism. These lactones are synthesized from the corresponding prostaglandin and stored in the cerata, dorsal projections of the nudibranch. The cerata are autonomized to escape from predators and upon detachment of these appendages, the lactones are reconverted to the corresponding prostaglandin.[57-59] The prostaglandin lactones have been shown to be toxic to mosquito fish.[60] Cytotoxic 1, 2 dioxane metabolites, xestin A and B (**16**), were isolated from the Fijian sponge *Xestospongia* with xestin A exhibiting more potent activity against P388 leukemia cells *in vitro* (IC_{50} = 0.3 μg/ml).[61]

The marine algae also have proved to be a rich source of known and new oxylipins. The cyanobacterium *Lyngbya majuscula* produced the trioxilin,



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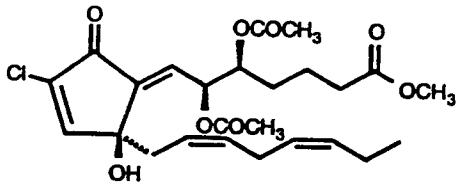

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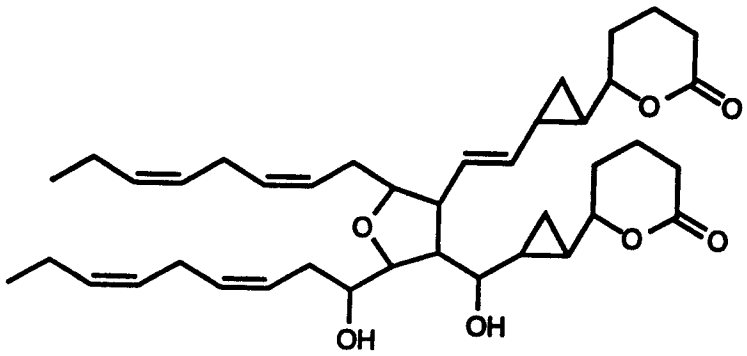

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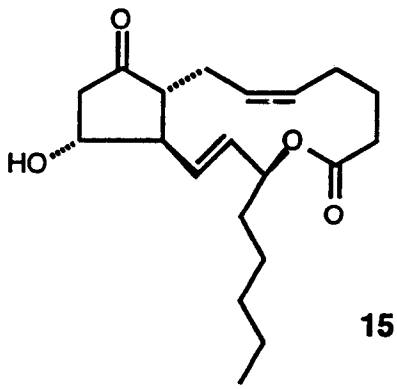

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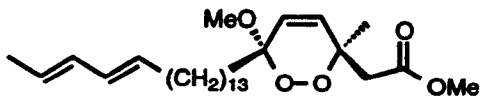

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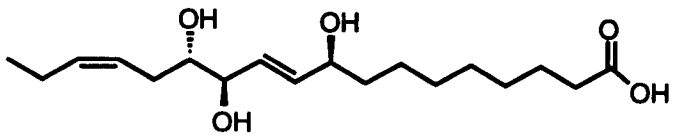
Figure I.6 Oxylipins from Marine Invertebrates

malyngic acid (**17**, Figure I.7)[62] as well as lyngbyalactone (**18**).[63] Green algae also have generated several oxylipin metabolites. *Acrosiphonia coalita* afforded an assortment of C18 derived metabolites such as (**19**) and coalital (**20**), a C10 metabolite which showed antimicrobial properties.[64] Recently, dictyosphaerin (**21**), a bicyclic lipid, was reported from an Australian *Dictyosphaeria sericea*.[65]

The Phaeophyceae have also demonstrated a capacity to produce oxylipins. The epoxycyclopentane derivatives ecklonialactones A and B (**22**, Figure I.8) as well as C-F were isolated from a Japanese kelp *Ecklonia stolonifera.* Ecklonialactone A and B displayed weak abalone antifeedent activity.[66,67] *Egregia menziesii* also afforded several ecklonialactones,[68] as well as the carbocyclic chlorinated metabolites, egregiachlorides A, B, and C (**23**).[69] Several collections of *Laminaria* from Oregon produced divinyl and hydroxy fatty acids.[70] Another Pacific Northwest brown, *Cymathere triplicata*, gave rise to the cymathere ethers A (**24**) and B, substituted oxanorbornanes containing cyclopentyl rings.[71]

The red algae represent another rich source of oxylipins, many identical to ones isolated from other organisms. From *Gracilaria lichenoides* prostaglandin $F_{2\alpha}$, a major uterine PG, and E_2 (**25**, Figure I.9) were isolated after investigation of the extract's antihypertensive properties. These oxylipins were the first reported prostaglandins found in plants.[72] Studies of human intoxication known as "Ogonori poisoning" after ingesting other *Gracilaria* species suggested PGE_2 as a causative agent.[73] Subsequently, the isolation and biological testing of polycavernosides A and B indicate these metabolites may be responsible for the activity.[74]

The mammalian insulin release factor hepoxilin B_3 (**26**) from *Platysiphonia miniata* and *Cottoniella filamentosa* [75] and 6(E)-leukotriene B_4



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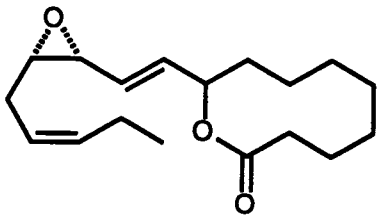

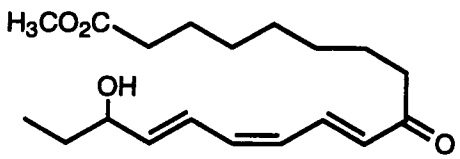

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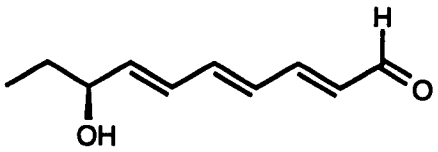

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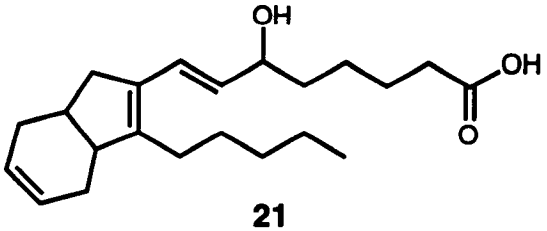

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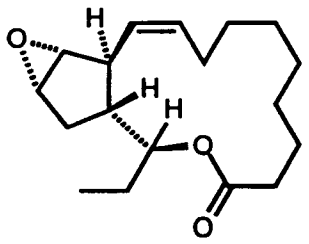
Figure I.7 Oxylipins from Cyanobacteria and Green Algae



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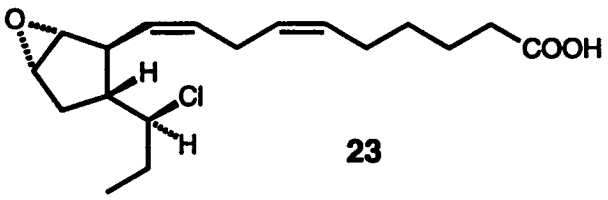

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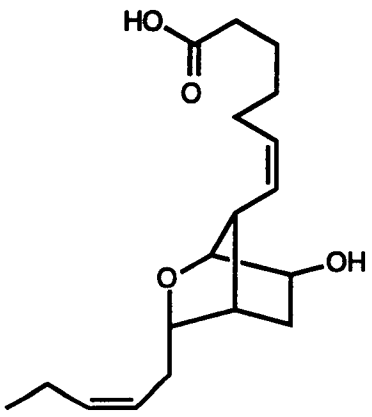

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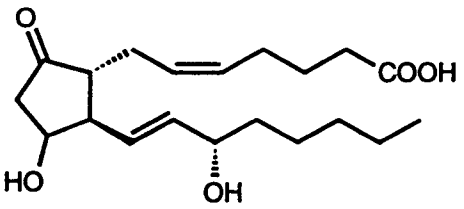
Figure I.8 Oxylipins from Phaeophyceae

from *Murrayella periclados*[76] represent metabolites identical to some involved in human metabolism. The plant hormone, jasmonic acid, was isolated from *Gelidium latifolium.*[77] *Farlowia mollis* produced several novel dihydroxyeicosanoids such as 12*R*, 13*S*-dihydroxyeicosapentaenoic acid. These oxylipins collectively inhibit activities such as dog kidney Na^+/K^+ ATPase and the metabolism of arachidonic acid by human neutrophils.[78] The epoxycyclopentane derivative hybridalactone (**27**) was characterized from *Laurencia hybrida.*[79] The constanolactones A and B (**28**) from *Constantinea simplex* possess cyclopropyl rings.[80] Several galactolipids (for example **29**) similar to those found in higher plants were characterized from *Gracilariopsis lemaneiformis.*[81] This alga has also been utilized in several studies in our laboratory involving oxylipin biosynthesis and lipoxygenase activity.[82,83]

Algal oxylipins appear to be the product of the lipoxygenase pathway and have been the subjects of several reviews.[40,41,84] A recent review discusses a general proposal in which epoxy allylic carbocations serve as conceptual intermediates for the biogenesis of numerous oxylipins from marine organisms.[63] A more detailed discussion of this idea is presented in chapter two of this thesis.

Cyanobacteria

Another area of focus in our laboratory involves the study of marine cyanobacteria. Marine microalgae constitute the majority of living species of organisms found in the oceans. Presently, more than 10,000 known species are separated into five major divisions: Chrysophyta (golden-brown, yellow algae and diatoms), Chlorophyta (green algae), Pyrrhophyta (dinoflagellates), Euglenophyta, and Cyanophyceae (blue-green algae).[38]



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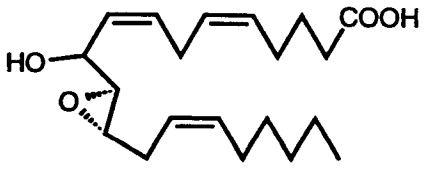

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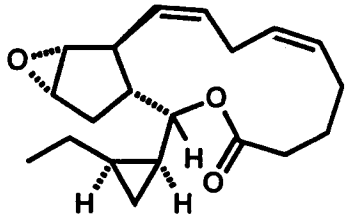

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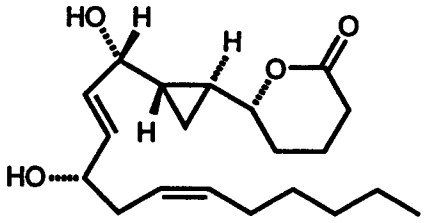

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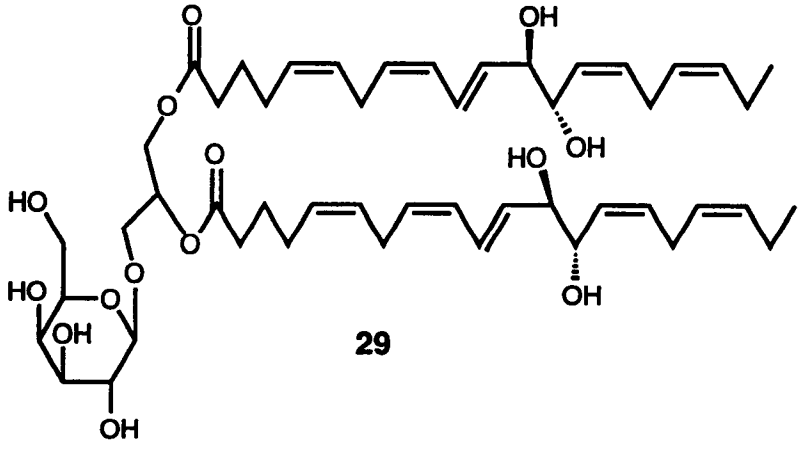

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Figure I.9 Oxylipins from Red Algae

Since the nomenclature and taxonomy of the microalgae have been a source of some disagreement among taxonomists, the classification of the cyanophyta has proven to be extremely difficult. Because they are procaryotic, some scientists place them in the bacteria (cyanobacteria) while others consider them algae (cyanophyta) due to their photosynthetic ability as well as the close similarities to their eucaryotic counterparts.[38] Others ascertain that the term "Cyanophyta" (Gr. n. Phyta plant), should not be used as it implies that the blue-greens are plants and rather the utilization of "Cyanophyceae" (Gr. n. Phykos algae) should be encouraged.[85]

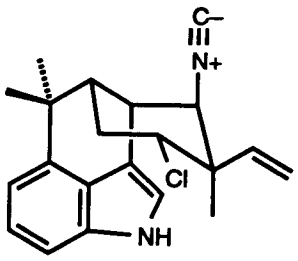
In 1932, the German phycologist Lothar Geitler produced a comprehensive botanical study of the cyanophyta. This work resulted in the recognition of about 1300 species, 145 genera, 20 families and 3 orders and serves as a basis for taxonomic identification along with several other complimentary treatises.[85,86] However, beginning in 1956, Drouet and Daily, in an attempt to simplify taxonomic classification of cyanobacteria proposed the "Drouet" system which eventually reduced over 2000 species in over 140 genera to 62 species in 24 genera.[85] Although most cyanobacterial taxonomists have rejected this system in favor of previous classifications, a few have utilized it. Thus the nomenclature of these life forms can be confusing.[85] The phylogenic positions of the cyanobacteria are important in considering the relationships of the metabolites produced and their biochemistry.[38]

The cyanobacteria are found in a diversity of habitats: freshwater, marine, damp soil, tree trunks, rocks, deserts, glacier, and hot springs.[87] They play important ecological roles as primary producers and nitrogen fixers.[88]

Numerous secondary metabolites have been isolated from terrestrial cyanobacterial forms. *Hapalosiphon fontinales* yielded hapalindole A (**30**, Figure I.10), a chlorine and isonitrile containing alkaloid with antialgal and

antimycotic activity.[89] It was the first of a series of 20 related metabolites from the same organism.[90] The scytophycins, (scytophycin B, **31**) were isolated from *Scytonema pseudohofmanni* and are closely related to the marine natural products, swinholide A and tolytoxin. These macrolides are potent cytotoxins and broad spectrum fungicides.[91] Another species of *Scytonema, S. mirabile,* yielded novel alkaloids, the tantazoles and related compounds. Tantazole B (**32**) exhibited potent murine solid tumor selective cytotoxicity.[92] A cyclic decapeptide containing chlorine, puwainaphycin C (**33**), was isolated from an *Anabaena sp.* collected from Punchbowl National Cemetery in Hawaii. This peptide proved to be a cardioactive agent with a strong, positive inotropic effect on isolated mouse atria.[93]

The freshwater forms of cyanobacteria are the most abundant[87]and are known to produce toxic algal blooms throughout the world.[87] Anatoxin-a (**34**, Figure I.11), initially isolated from *Anabaena sp.,* was the first toxin characterized from a freshwater source and is responsible for numerous deaths of livestock and waterfowl.[94] This alkaloidal neurotoxin serves as a potent postsynaptic cholinergic nicotinic agonist.[95] Since death occurs 2-5 minutes after ingestion by mice, it is called Very Fast Death Factor (VFDF).[94] Other cyanobacteria are reported to produce anatoxin-a.[96] Extracts of another *Anabaena* yielded anatoxin-a(s) (**35**), a phosphate ester of cyclic N-hydroxyguanine which produced marked salivation (designated s) in laboratory rats.[97] This neurotoxin was shown to be a potent anticholinesterase with the LD_{50} dose effective in mice at 10-30 minutes.[95,97] Microcystin-LR (**36**) from *Microcystis aeruginosa* is a member of a series of related metabolites which are responsible for toxic algal blooms.[95,98,99] These hepatotoxic heptapeptides have been isolated from numerous genera.[96]



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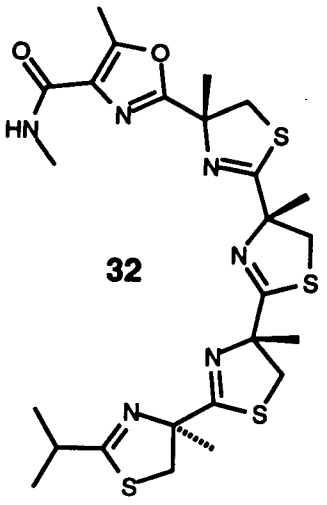

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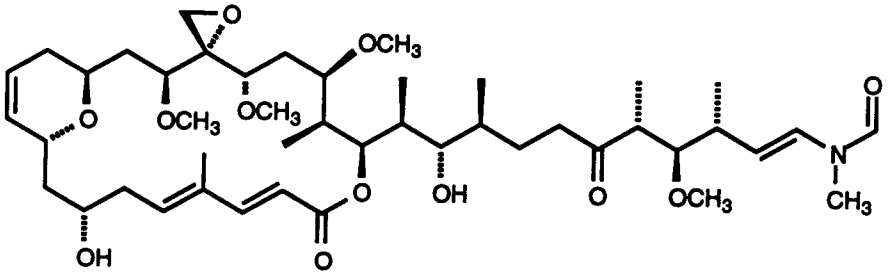

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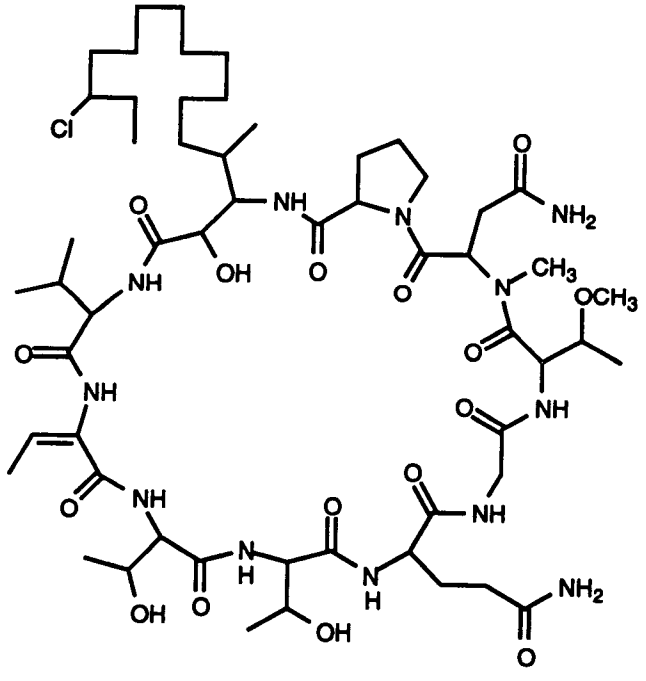

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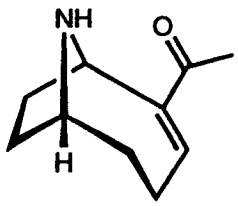
Figure I.10 Terrestrial Cyanobacterial Metabolites



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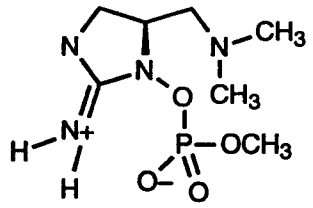

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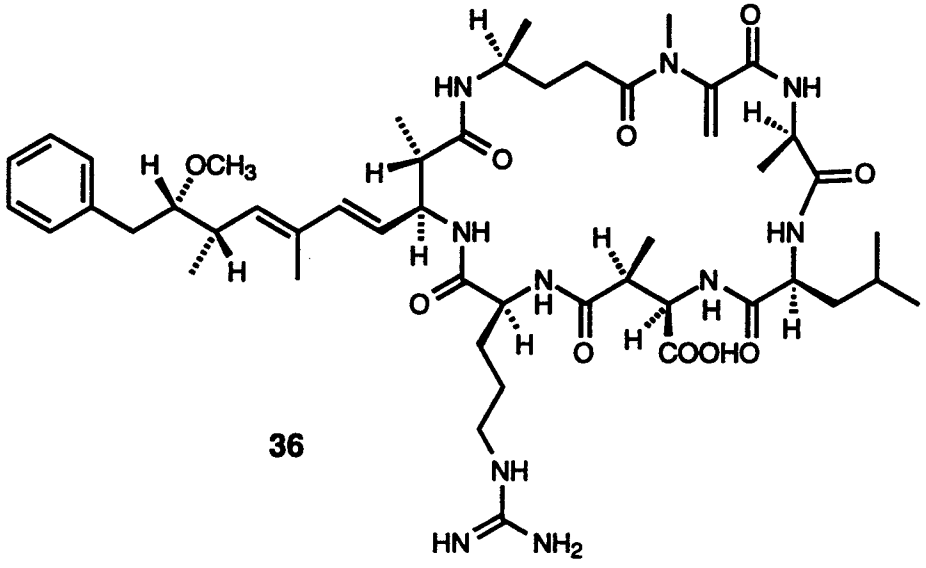

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Figure I.11 Freshwater Cyanobacterial Toxins

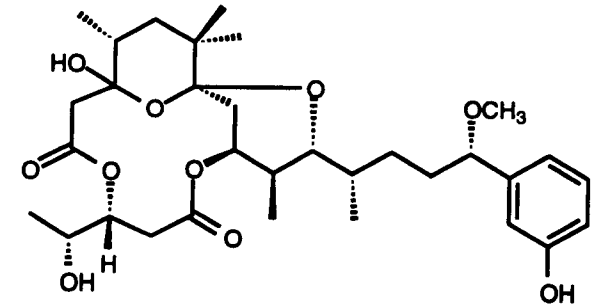
Although the freshwater forms have produced numerous toxins, the marine environment also has been a source of bioactive metabolites. A mixture of *Oscillatoria nigrovirdis* and *Schizothrix calcicola* yielded debromoaplysiatoxin (**37**, Figure I.12), an antitumor metabolite.[100] Hormothamnin A (**38**), a cyclic unadecapeptide with potent ichthyotoxicity, was isolated from a Caribbean form of *Hormothamnion enteromorphoides*.[101,102] A cyanobacterium, *Cylindrospermopsis raciborskii* from the tropical waters around Australia yielded cylindrospermopsin (**39**), a sulfated hepatotoxic alkaloid[103] and potent inhibitor of protein synthesis.[104] A strain of *Nostoc linckia* produced borophycin (**40**), a boron containing compound with potent cytotoxicity.[105] *Lyngbya majuscula* has produced numerous bioactive compounds, some of which will be discussed in chapter three.

General Thesis Contents

Our laboratory specializes in the isolation and characterization of novel and bioactive compounds from marine algae. This thesis describes some of our research in the area of algal oxylipins as well as the isolation of a bioactive metabolite from cyanobacteria.

This thesis contains four chapters and an appendix. After this general introduction, chapter two discusses the investigation of a carbocyclic oxylipin from the red alga *Agardhiella subulata*. Along with discussion of the isolation and characterization of this novel metabolite, agardhilactone, the biosynthetic pathway and biochemical significance are considered.

The discovery of kalkipyrone, a γ-pyrone natural product found in the extract of a cyanobacterial assemblage of *Lyngbya majuscula* and *Tolypothrix*, is described in chapter three. This chapter details the isolation,



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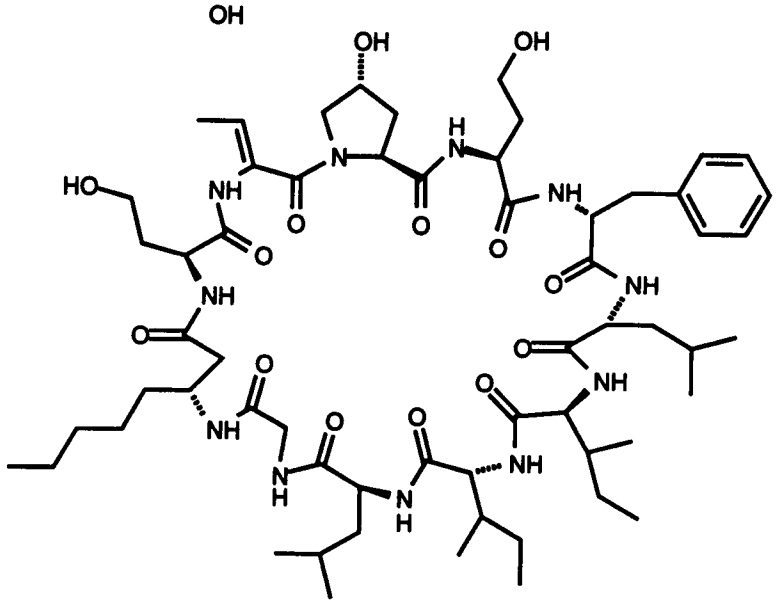

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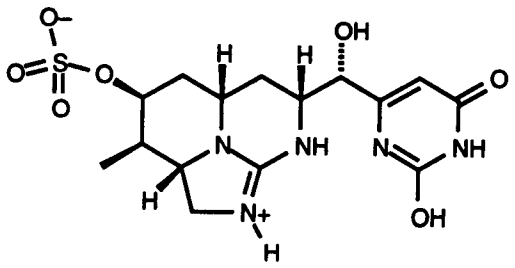

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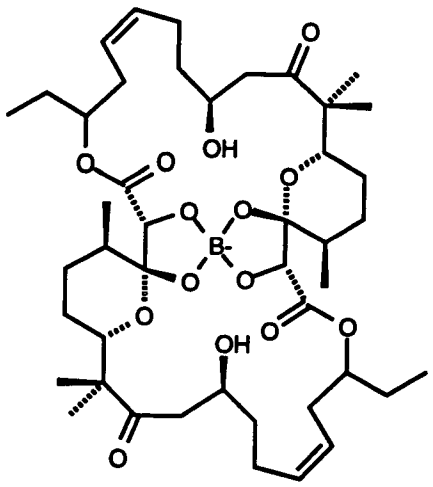

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Figure I.12 Marine Cyanobacterial Metabolites

characterization, biological activity as well as comparisons with other related natural products.

Chapter four contains conclusions regarding the discoveries of these metabolites from marine algae and their biological significance. The appendix consists of a description of the isolation of two known compounds, stypoldione from the brown alga *Stypopodium zonale* and malyngamide C acetate from *Lyngbya majuscula*. The modification of stypoldione to the triacetate of stypotriol is also discussed. These three compounds were utilized in biological testing.

CHAPTER II

A NOVEL OXYLIPIN FROM THE RED ALGA *AGARDHIELLA SUBULATA*

Abstract

Agardhilactone, a novel tricyclic oxylipin containing δ-lactone, cyclopentane and epoxide rings and a conjugated diene, was isolated from the marine red alga *Agardhiella subulata*. The structure, including partial stereochemistry, was determined by NMR and GC-MS analysis of menthoxycarbonyl derivatives. Agardhilactone represents a new oxylipin structure class, distinguished by the unprecedented position of the cyclopentyl ring, C6 to C10, within a 20-carbon chain framework.

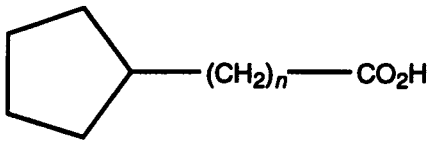
Introduction

Agardhiella subulata (Gigartinales, Solieriaceace) is a prominent species of red alga which grows along the coast of Atlantic North America from Massachusetts to Florida, throughout the Caribbean and Brazilian waters[106] and has recently been reported in the Mediterranean Sea.[107] The actual distribution of this species has been unclear due to misidentification and nomenclature changes that have been occurring since the alga was first reported in 1822 as *Sphaerococcus subulatus* and reclassified as *Agardhiella subulata* in 1985.[108] Recent previous taxons have included, *Neoagardhiella baileyi*, *Solieria tenera*, *Agardhiella baileyi* and the "northern entity" of *Agardhiella tenera.*[108]

Agardhiella is a valuable member of the intertidal flora as a producer of the commercially important phycolloids, carrageenan and agar. Both these sulfated polymers of galactose have a variety of uses. Carrageenan is used as a stabilizer for emulsions such as dairy products, cosmetics and paints.[109,110] Agar is utilized as a component of drug capsules, dental impression material, culture medium for microorganisms, and food preparation.[110] Due to the commercial importance of the alga, various studies have been undertaken to improve biomass production through tissue culture and whole plant cultivation.[109,111,112]

Although the nomenclature changes and commercial utility of *Agardhiella* has prompted detailed morphological [106,107] and production studies,[109,111,112] the natural products chemistry of this genus have not been extensively examined with few reported in the literature. Recently, a galactan sulfate isolated and partially purified from *A. tenera*, showed inhibition of the cytopathic effect of the human immunodeficiency virus type 1 and type 2

(HIV-1, HIV-2) as well as other enveloped viruses.[113] A study of sterol distribution in seven species of red algae from the waters of New York included *Agardhiella tenera* which was found to contain the highest total sterol content (117 mg/kg) with the majority as cholesterol (108 mg/kg). A sterol, cholest-7-en-3β-ol, previously unreported in Gigartinales, was also isolated.[114] Detailed fatty acid analysis of *Agardhiella tenera* along with several other members of the Solieriaceae, revealed ω-5 monounsaturated fatty acids. Several also contained terminal cyclopentyl rings such as dihydrohydnocarpic acid (**1**) and dihydrochaulmoogric (**2**, Figure II.1).[115]



1 $n = 10$ Dihydrohydnocarpic acid
2 $n = 12$ Dihydrochaulmoogric acid

Figure II.1 Cyclopentyl Fatty Acids from *Agardhiella*

Our interest in *Agardhiella subulata* was guided by previous TLC analysis of a crude extract. Several minor blue charring compounds were apparent suggesting the presence of oxylipins. In our continuing efforts to describe the organic chemistry of algal-derived oxylipins,[40,116] we extracted a large recollection of *Agardhiella subulata* and have now isolated a novel epoxycyclopentane derivative, agardhilactone (**3**). Agardhilactone is the first of a new oxylipin structure class, distinguished by the unprecedented position of the cyclopentyl ring, C6 to C10, within a 20-carbon chain framework.

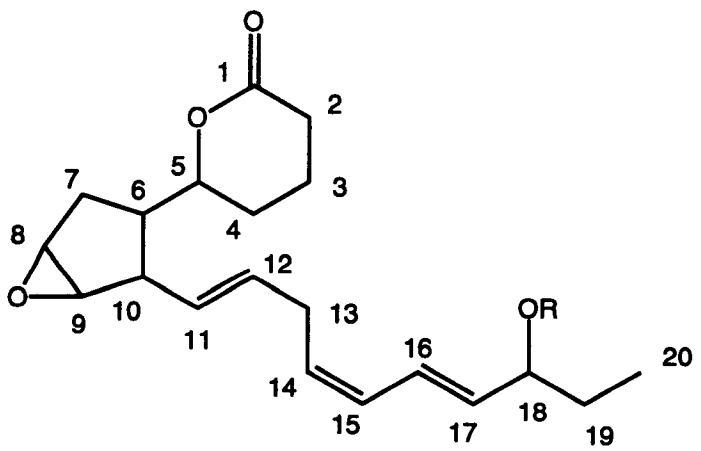
Results and Discussion

Agardhiella subulata was collected in the waters of the Massachusetts coast during September 1994, immediately frozen and sent to our laboratory for analysis. The defrosted alga was repeatedly extracted for its lipid metabolites with CH_2Cl_2/MeOH (2:1), to yield a dark oil (18.6 g). Vacuum silica chromatography of 8.6 g yielded several polar fractions containing blue-charring compounds (50% H_2SO_4) by TLC, indicative of oxylipins. These fractions were combined and further fractionated to provide a mixture of oxylipins. This mixture was acetylated (Ac_2O, pyridine) and subjected to successive fractionations to yield agardhilactone acetate (**4**) along with an intractable minor component (*ca.* 5%). Fractionation of an additional 10 g of extract gave agardhilactone (**3**) along with the same intractable minor isomer; this latter sample was used for stereochemical analysis (Figure II.2).

Agardhilactone acetate (**4**) yielded a molecular formula of $C_{22}H_{30}O_5$ by HRCIMS. Of the eight degrees of unsaturation inherent in this formula, five were defined by ^{13}C NMR (Table II.1) and IR data as two esters ($\upsilon_{C=O}$ 1732, 1738; δ170.1, δ171.8) and six olefinic carbons (δ127.7, 128.4, 129.1, 130.3, 131.2, 131.6) thereby indicating three additional rings. One of the rings was present as an epoxide (δ55.7, 60.2) and two of the olefinic bonds were in conjugation (UV λ_{max} = 236 nm, ε = ca. 39,000, MeOH).

Although overlap in the ^1H NMR (Table II.1) of the δ1.0-1.9 region (H3, H4a, H4b, H6, H7a, and H19) limited the utility of ^1H-^1H COSY, double quantum filter (DQF) ^1H-^1H COSY (Figure II.3) revealed one extended spin system, H2-H20 and an isolated acetate methyl group (H22). Correlations from a ^1H-^{13}C XHCORR showed the protons at δ4.24 (H5), δ5.22 (H18), δ3.46 (H8), and δ3.40

(H9) were attached to carbons bearing oxygen, the latter two as an epoxide. Complete analysis of the ^{1}H-^{13}C XHCORR in concert with DQF ^{1}H-^{1}H COSY defined a disubstituted cyclopentyl epoxide ring connected to a δ-lactone, fulfilling the remaining two degrees of ring unsaturation, and a 10-carbon chain which contained three double bonds and an acetylated hydroxyl group. The acetate ester was confidently placed at C18 by observing a δ1.11 upfield shift in H18 in the ^{1}H NMR spectrum of underivatized agardhilactone (**3**, Table II.1). The double bonds at C11-C12 (J=15.6 Hz) and C16-C17 (J=15.3 Hz) were defined as *trans* while the C14-C15 double bond was *cis* based on $^{3}J_{HH}$ (J=11.0 Hz) and NOESY correlations between H13 and H16 (Table II.1), completing the planar structure of agardhilactone acetate (**3**, Figure II.2). Although not completely characterized, the minor component was structurally similar from C1 to C10 but differed in the acyclic region, C11 to C20 by DQF ^{1}H-^{1}H COSY.



3 R = H
4 R = Acetate

Figure II.2 Planar Structure of Agardhilactone (**3**) and its Acetate Derivative (**4**)

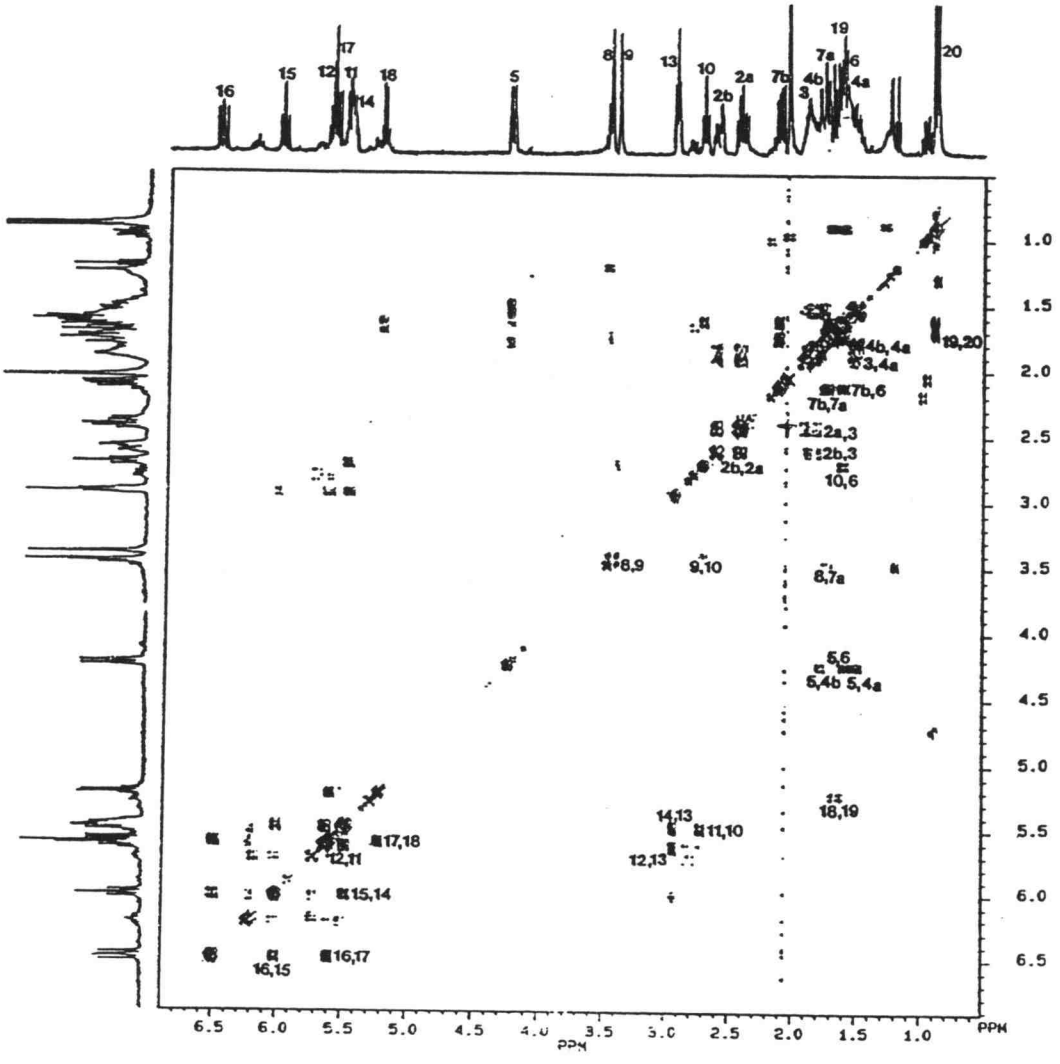
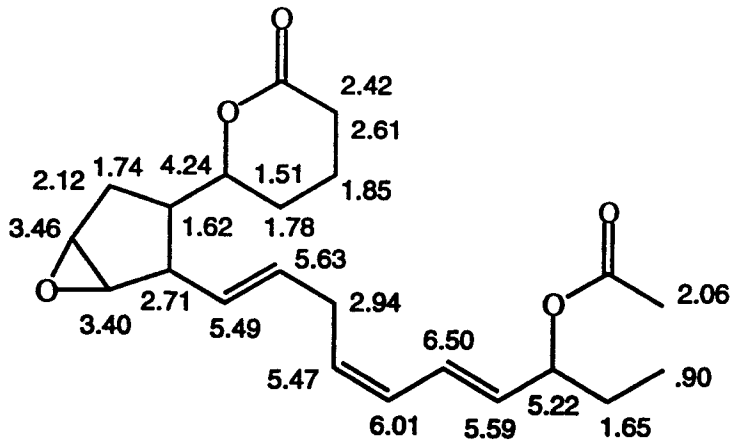
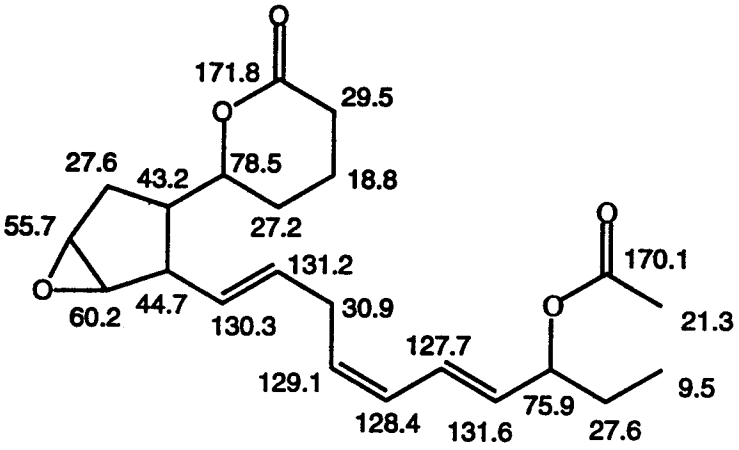


Figure II.3 Double Quantum Filter COSY of Agardhilactone Acetate (**4**) in $CDCl_3$ (400 MHz)



- ^{1}H NMR Chemical Shifts in $CDCl_3$ (400 MHz)



- ^{13}C NMR Chemical Shifts in $CDCl_3$ (100 MHz)

Figure II.4 ^{1}H and ^{13}C NMR Assignments of Agardhilactone Acetate (4)

Table II.1 ^{1}H and ^{13}C NMR Data for Agardhilactone (**3**) and its Acetate Derivative (**4**).[a]

	Compound 3[b]		Compound 4			
#C	^{13}C	^{1}H	^{13}C	^{1}H	NOESY Correlations	HMBC Correlations C ⇒ H
1	171.5	-	171.8	-	-	2a, 2b
2 a	29.5	2.42	29.5	2.42 dt 17.7, 7.5	2b	-
b		2.61		2.61 dt 17.7, 6.5	2a	
3	18.8	1.85	18.8	1.85 m	4b	2a, 2b
4 a	27.2	1.51	27.2	1.51 m	4b	2b
b		1.79		1.78 m	3, 4a	
5	78.5	4.24	78.5	4.24 ddd 11.7, 2.4, 2.4	-	4, 10
6	43.2	1.61	43.2	1.62 m	7b, 9	7, 8, 10
7 a	27.5	1.74	27.6	1.74 m	7b, 8	6, 8
b		2.12		2.12 dd 13.6, 7.5	6, 7a	
8	55.6	3.46	55.7	3.46 bd 7.5	6, 7a	7
9	60.2	3.40	60.2	3.40 dd 2.7, 1.4	6, 10	7
10	44.6	2.72	44.7	2.71 bdd 9.0, 9.0	9	7, 9, 10
11	129.3	5.49	130.3[c]	5.49 m	12	-
12	131.3	5.64	131.2	5.63 dt 15.6, 6.0	11	14, 18
13	30.9	2.95	30.9	2.94 dd 6.8, 6.8	14, 16	13
14	129.0	5.45	129.1[c]	5.47 m	13, 15	-
15	128.7	6.07	128.4	6.01 dd 11.0	14, 16	13, 17
16	125.5	6.50	127.7	6.50 dd 15.4, 11.0	13, 15, 17	15, 17, 18
17	136.4	5.71	131.6	5.59 dd 15.4, 6.8	16, 18	15, 19
18	74.0	4.11	75.9	5.22 dd 13.8, 6.8	17	19, 20
19	29.7	1.57	27.6	1.65 m	-	20
20	9.7	0.94	9.5	0.90 t 7.4	-	19
21	-	-	170.1	-	-	22
22	-	-	21.3	2.06 s	-	-

[a] All spectra recorded on a Bruker AM-400 spectrometer in $CDCl_3$ (^{1}H spectra referenced to TMS at 0.0 ppm; ^{13}C spectra referenced to the centerline of $CDCl_3$ at 77.0 ppm; data presented as δ, multiplicity in Hz).

[b] Assigned by comparison to **4** and model compounds.[117] Multiplicities and J_{HH} similar to those reported for **4**.

[c] Assignment may be interchanged.

NOESY analysis combined with ^{1}H-^{1}H coupling constants supported a relative stereochemistry around the five membered ring as 6*S**, 8*R**, 9*S**, 10*R**. NOe correlations between H9 and H6, and H8 and H6 indicated that the epoxide and C1-C5 chain were *cis* to each other (Figure II.5). The cyclopentyl epoxide must be *cis* due to steric constraints. According to the modified Karplus equations, the vicinal ^{1}H-^{1}H coupling constants are determined by the dihedral angles formed between the two protons. The coupling constants for *cis* vicinal protons in cyclopentanes are about 8 Hz, while *trans* approach 0 Hz, corresponding to angles of 0 and 90 degrees respectively.[118] The small coupling constant observed between H9 and H10 suggest they occupy a *trans* relationship. An energy minimized model (Chem 3D) of the cyclopentyl fragment showed the ring as slightly puckered. Analysis of a Newman projection of C9-C10 revealed a 98° dihedral angle between H9 and H10, thus supporting the *trans* stereochemical assignment (Figure II.6)

Some substituted cyclopentyl ring systems have shown the ranges of vicinal couplings as J_{cis} = 7.0-12.6, while J_{trans} = 4.0-9.6.[119] The lactone and C11-C20 substituents were defined as *trans* based on coupling constants (9.0 Hz) and ^{13}C NMR comparisons to the ecklonialactones, (ecklonialactone A, **6** and ecklonialactone E, **7**),[66,67] hybridalactone (**8**)[79] and cymathere lactone (**9**) [120] (Figure II.7). The ecklonialactones also contain a cyclopentyl ring in which the substituents relative stereochemistry is confirmed by crystal structure analysis[66] and through NOE difference experiments.[67] Although the ^{1}H-^{1}H coupling constants for the cyclopentyl bridge were considerably less in the model compounds, different conformations of the cyclopentyl ring could account for this dissimilarity. The cyclopentyl rings can be described in envelope conformations. In the model compounds, the distal carbon is cisoid to the epoxide, leading to a dihedral angle close to 90° and small coupling constants.

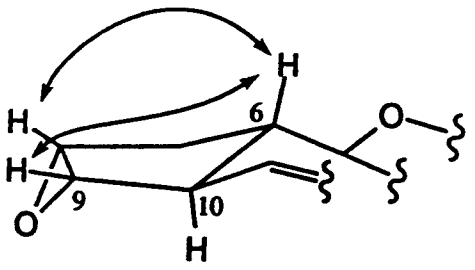


Figure II.5 Selected nOe Correlations for Agardhilactone Acetate Ring Fragment

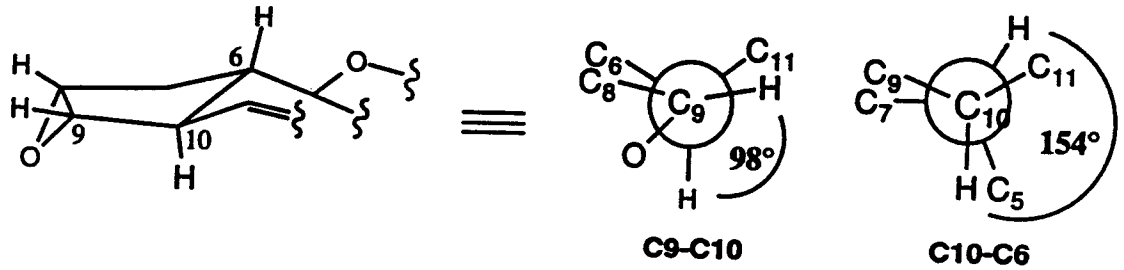
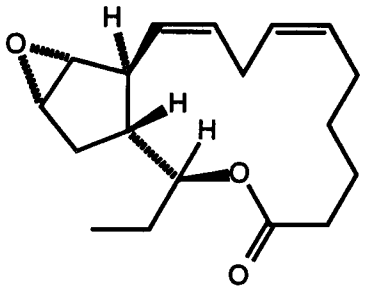


Figure II.6 Depiction of Selected H-C-C-H Dihedral Angles of Energy Minimized (Chem 3D) Agardhilactone Acetate Fragment



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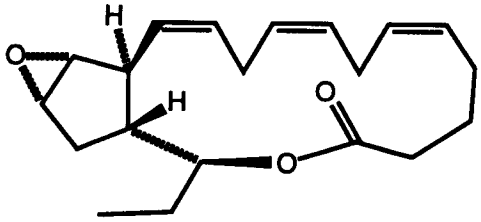

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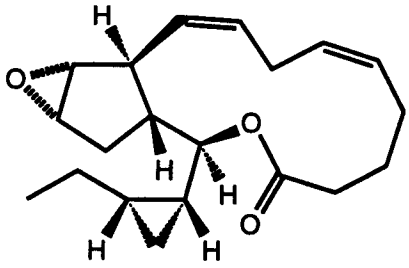

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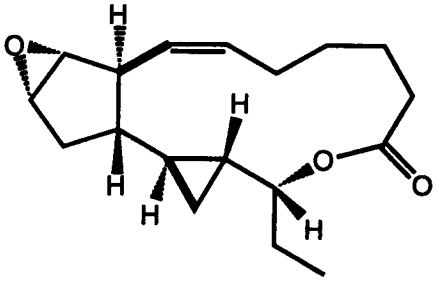

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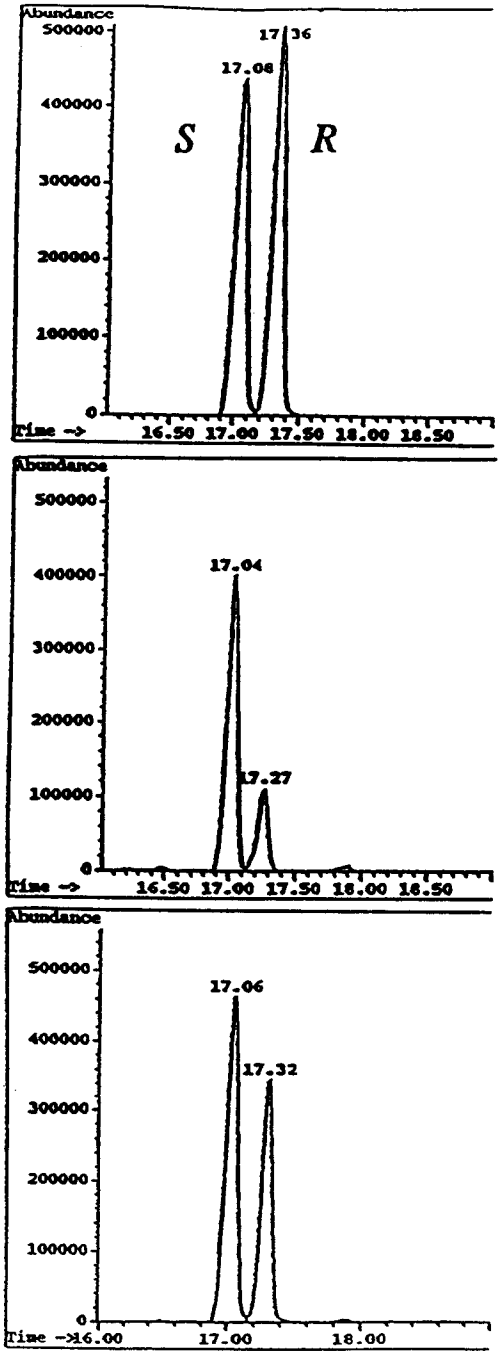
Figure II.7 Algal Metabolites with Cyclopentyl Rings

Alternatively, in agardhilactone, the distal carbon is transoid to the epoxide and a Newman projection of C10-C6 showed a 154° dihedral angle between H10 and H6, thus supporting the *trans* stereochemical assignment (Figure II.6). Although not likely, the 10S* stereochemistry in agardhilactone can not be eliminated due to the difference in bridgehead coupling constants in comparison to the model compounds. A Newman projection of C10-C6 showed a 154° dihedral angle between H10 and H6, thus supporting the *trans* stereochemical assignment (Figure II.6). Lack of nOe correlations between protons of the cyclopentane and lactone rings precluded stereochemical assignment at C5.

The absolute stereochemistry at C18 was determined by converting agardhilactone (**3**) (0.2 mg) to its corresponding (-)-menthoxycarbonyl (MC) derivative, oxidative ozonolysis to release the C17-C20 fragment and derivation of this fragment to the corresponding methyl ester.[121] GC standards for this same MC derivative gave baseline separation under optimized conditions (11.5 m of HP Ultra-1, 100-210°C at 3.0° C per min., then isothermal for 15 min.), with an elution time of 17.03 min. *(S)* and 17.27 min. *(R)*. The reaction product was coinjected with 2-hydroxy methyl butyrate as racemic standards and the observed peak enhancement clearly showed the agardhilactone MC derivative was partially racemic as 80% *S*, 20% *R*. (Figure II.8). Although the scalemic nature of C18 could be due to extraction or derivatization procedures, it should be noted that plant lipoxygenases are known to produce *S/R* mixtures exceeding the 80:20 ratio.[48]

A recent review has proposed an epoxy allylic carbocation (EAC) as a conceptual intermediate in the biogenesis of more than 50 marine oxylipin metabolites.[63] Initial lipoxygenase (LO) activity with an unsaturated fatty acid leads to hydroperoxide formation. Consistent with the mechanism of



(-)-Menthoxycarbonyl (MC) derivatives of racemic 2-hydroxy methyl butyrate as standards

Agardhilactone derived MC derivatives

Agardhilactone derived with added racemic 2-hydroxy methyl butyrate MC derivatives

Figure II.8 Elution Profile of (-)-Menthoxycarbonyl (MC) Derivatives by Gas Chromotography

cytochrome P-450 in higher plants, the hydroperoxide is homolytically cleaved by an iron-oxo complex. The resulting allylic alkoxyl radical cyclizes to an epoxide with an adjacent carbon-centered radical. Analogous to the mechanism of prostacyclin and thromboxane synthases, an iron-oxo complex proceeds to remove a single electron from the radical species to form the EAC (Figure II.9).

We propose the biogenesis of (**3**) to involve an 8-LO initiated oxidation of eicosapentaenoic acid (EPA) to hydroperoxyeicosatetraenoic acid (8*R*-HPETE) (Figure II.10). Reaction of the hydroperoxide with the 9,10-olefin with consequent loss of OH^- would form an EAC at C10. Electron flow from the 5, 6 olefin induces cyclopentyl ring formation as electrons from the carboxylate end generate the lactone ring. Completion of the agardhilactone structure could be accomplished by an ω-3 LO and the reduction of the resulting hydroperoxide. A related metabolite, sarcolactone A (**10**) is produced by a member of the same family (Solieriaceae, *Sarcodiotheca*) and has indicated precedence of an 8-LO.[120] Although isomerization of the 11,12-olefin from *cis* to *trans*, could occur at a late step in the biogenesis,[122] a proposed biogenetic scheme with isomerization at an earlier stage, envisions a common intermediate as producing sarcolactone A as well as agardhilactone (**3**, Figure II.10).[63] Several members of the same order (Gigartinales) contain high levels of the putative precursor EPA as well as arachidonic acid.[123] While ω-3 oxidation is relatively uncommon in algae, it was recently proposed for a series of green algal metabolites.[64]

The biochemical significance of this compound as a secondary metabolite from *Agardhiella* has not been determined. Due to the small yield of the isolated compound, this sample has not been tested for biological activity. Although red marine algae have been shown to be a rich source of novel

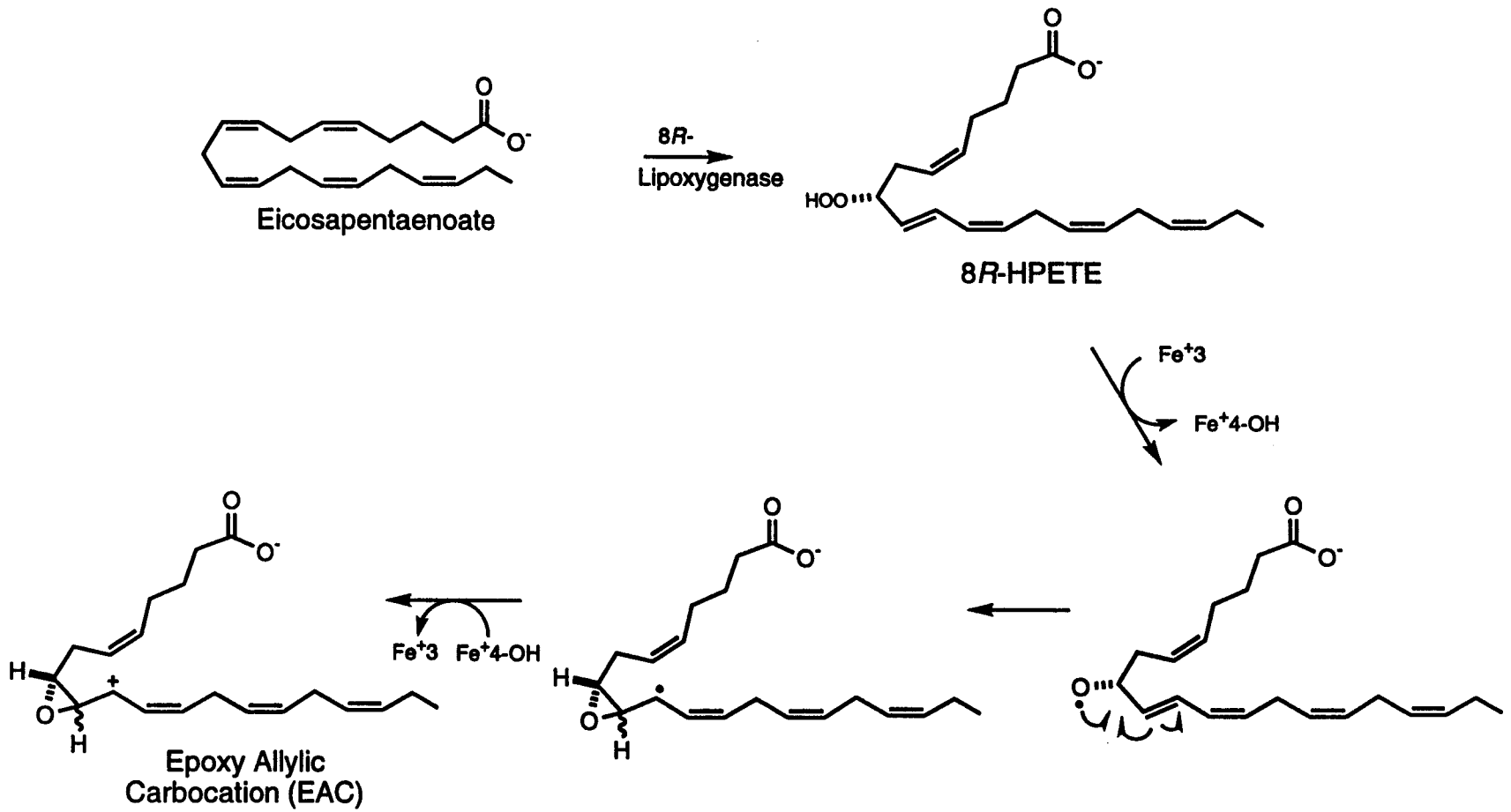


Figure II.9 Epoxy Allylic Carbocation Formation from Eicosapentaenoate (adapted with modification[63])

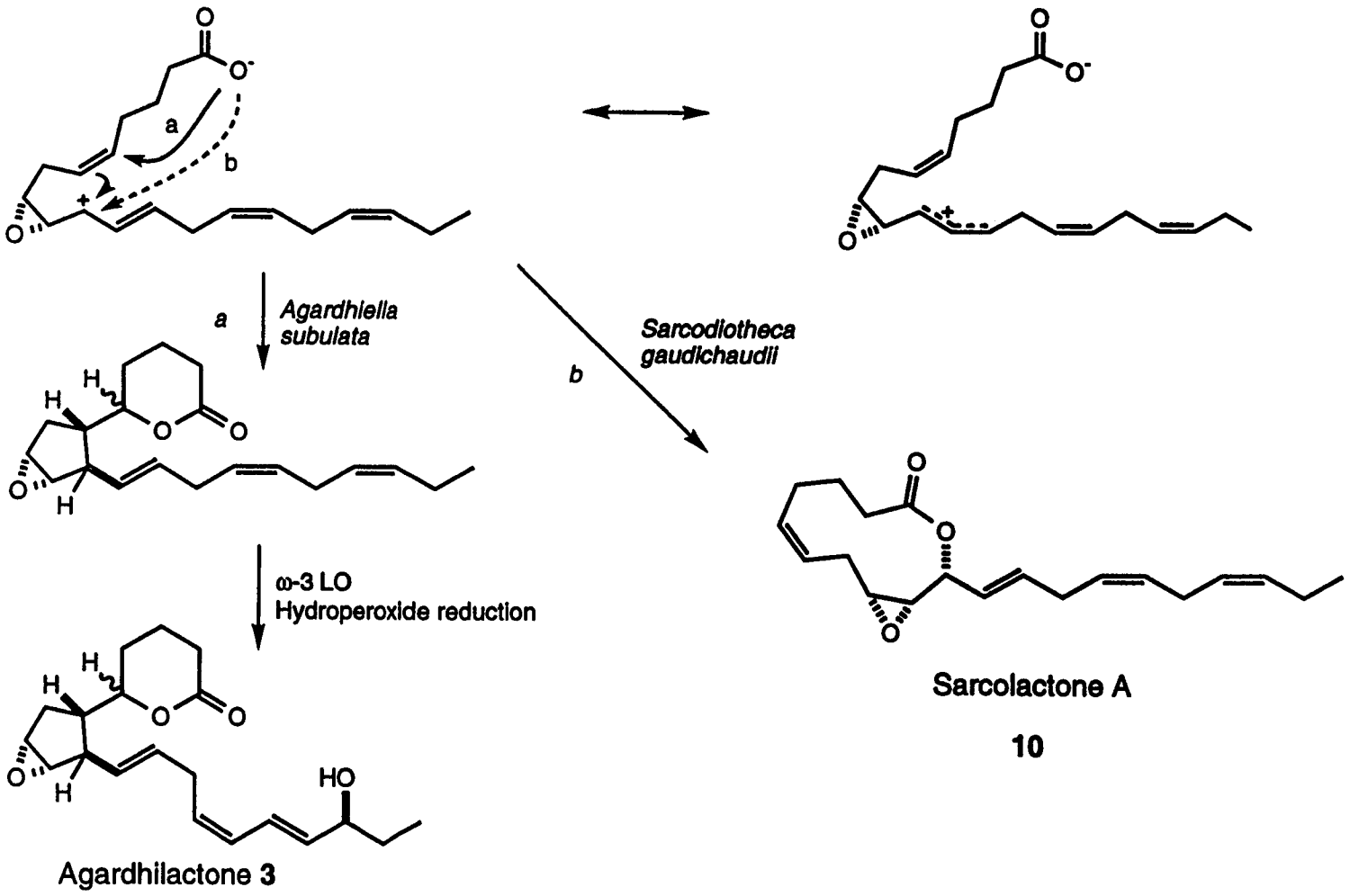


Figure II.10 Proposed Biogenesis of Sarcolactone A and Agardhilactone (adapted with modifications[63, 122])

oxylipins, the biological and ecological significance of a majority of these metabolites has not been described. It has been speculated that these red algal oxylipins may play roles in osmoregulation, tissue development, wound response, and reproductive biology of the algae.[84]

The significance of this class of compounds from red algae should be investigated further because many oxylipins isolated from other sources have been shown to be pharmacologically active substances.[44,45] As one example, the prostaglandin 1, 15-lactones from the nudibranch *Tethys fimbria* were briefly described in the introduction of this thesis. Low dosages of these compounds were toxic to mosquito fish, supporting the proposal that these metabolites serve as anti-predator allomones.[57-60] Perhaps agardhilactone, which is structurally related to the prostaglandins, is also utilized in chemical defense.

Other organisms may have adapted through evolution to exploit the production of oxylipins from *Agardhiella* for their own benefit. One hypothesis involves interspecies chemical signaling. While invertebrate metamorphic induction is not completely understood, some studies have indicated that environmental cues are involved.[124] As an example, free fatty acids have been shown to be inducers of larval settlement and metamorphosis in a marine polychaete *Phragmatopoma*. [125] Several macroalgae including *Agardhiella subulata* and the red alga *Gracilaria* are known to produce an exogenous factor that induces the onset of competence transition and metamorphosis in the marine gastropod *Aplysia californica*.[126] Since both algae produce oxylipin type chemistry which hypothetically derives from fatty acids, agardhilactone and prostaglandin E_2 and F_2 from *Gracilaria*,[127] perhaps these metabolites or closely related ones are involved in the chemical communications necessary for metamorphic transition in *Aplysia californica* and other species.

Further work possibly could identify the role of agardhilactone as well as the unidentified oxylipins apparent by TLC in the crude extract that are structurally related to agardhilactone.

Experimental

General. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AM-400 spectrometer in $CDCl_3$. Proton spectra were referenced to internal tetramethylsilane at 0.0 ppm. Carbon spectra were referenced to the centerline of $CDCl_3$ at 77.0 ppm. Ultraviolet (UV) spectra were obtained on a Hewlett Packard 8452A diode array spectrophotometer. Infrared (IR) spectra were run on a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, while high resolution mass spectra (HRMS) were recorded on a Kratos MS 50 TC. High-performance liquid chromatography was performed with a Waters M-6000 pump and either a Waters Lambda-Max 480 LC spectrophotometer or a R 401 differential refractometer spectrophotometer. Merck aluminum-backed thin layer chromatography (TLC) sheets (silica gel 60 F254) were utilized for TLC analysis with compounds detected by UV illumination and by heating the plates after spraying with 50% H_2SO_4 solution. Gas chromatography/mass spectrometry was performed on a Hewlett Packard 5890 Series II GC connected to a Hewlett Packard 5971 mass spectrometer. Reverse phase C-18 silica (Analytichem Bond Elut) was utilized for column chromatography. TLC-grade (10-40 μm) silica gel was used for vacuum chromatography. All solvents were glass distilled prior to use.

Collection, Extraction and Isolation. A collection of *Agardhiella subulata* (380 g dry weight) was made in waters of the Massachusetts coast during

September 1994. The alga was immediately frozen and stored at -20 °C. The defrosted alga was extracted in three portions for its lipid metabolites. Each portion was extracted once with warm CH_2Cl_2/MeOH (2:1) followed by six additional extractions with room temperature CH_2Cl_2/MeOH (2:1). The combined extracted yielded 18.6 g dark oil. Vacuum chromatography of 8.6 g (Hex/EtOAc/MeOH gradient) yielded several polar fractions (66.5 mg) containing blue-charring compounds (50% H_2SO_4) by TLC, indicative of oxylipins. These fractions were combined and further fractionated over reverse phase C-18 silica (H_2O/MeOH gradient), followed by normal phase prep TLC (10% MeOH/$CHCl_3$). After acetylation, 0.6 mg of agardhilactone acetate (**4**) was isolated by NP-HPLC (50% EtOAc/Hex), along with an intractable minor component (ca. 5%). Vacuum liquid chromatography, C-18 silica (H_2O/MeOH gradient), NP-HPLC (70% and 40% EtOAc/Hex), and TLC (5% MeOH/$CHCl_3$) of the additional 10 g of extract gave 0.6 mg of agardhilactone (**3**) along with the same intractable minor isomer; this latter sample was used for stereochemical analysis.

*Determinations of Absolute Stereochemistry at C18 of Agardhilactone (**3**).* An aliquot of agardhilactone (0.2 mg) was reacted with 20 µl of a 1 µmol/µl (-)-menthyl chloroformate solution (toluene), in 80 ml dry toluene and 10 ml dry pyridine for 30 min. at RT. Solvents were removed *in vacuo* and the residue resuspended in 100% hexanes. The reaction products were purified utilizing a 1.2 cm x 1 cm NP silica column with an EtOAc/Hex gradient. The (-)-menthyl chloroformate products eluting with 40% EtOAc/Hex were suspended in CH_2Cl_2 and ozonized for 2 minutes at -8 °C followed by 10 minutes RT. The reaction products then were treated with peracetic acid overnight at 50 °C. The reaction mixture was dried under nitrogen, resuspended in MeOH and the methyl ester generated with ethereal diazomethane. The resulting products were examined

by GC-EIMS (11.5 m of HP Ultra-1, 100-210°C at 3.0° C per min., then isothermal for 15 min), and compared with the corresponding standards; 17.03 min. *(S)* and 17.27 min. *(R)*.

Agardhilactone acetate (**4**). FTIR (film) υ 2962, 2930, 1738, 1732, 1716, 1652, 1372, 1240 cm^{-1}, UV (MeOH) λ_{max} = 236 nm, ε = ca. 39,000. HRCIMS *m/z* obs. $[M+H]^+$ 375.2170 (0.1 mmu dev.) $C_{22}H_{30}O_5$. For ^1H and ^{13}C NMR data see Table II.1.

Agardhilactone (**3**). For ^1H and ^{13}C NMR data see Table II.1.

CHAPTER III

KALKIPYRONE, A TOXIN FROM AN ASSEMBLAGE OF THE CYANOBACTERIA *LYNGBYA MAJUSCULA* AND *TOLYPOTHRIX SP.*

Abstract

Kalkipyrone, a novel α-methoxy-β,β'-dimethyl-γ-pyrone linked to an alkyl side chain, was isolated from an assemblage of the cyanobacteria *Lyngbya majuscula* and *Tolypothrix sp.* Its structure, including stereochemistry, was determined by NMR, UV, and IR analysis and by GC-MS analysis of menthoxycarbonyl derivatives. Kalkipyrone displays toxicity to brine shrimp ($LD_{50} \approx 1$ μg/ml) and gold fish ($LD_{50} \approx 2$ μg/ml) and is structurally related to several *Streptomyces* metabolites, the actinopyrones.

Introduction

Cyanobacteria are a recognized rich source of chemically and biologically interesting compounds. A major focus of our laboratory involves the isolation and characterization of cyanobacterial metabolites. The project discussed in this chapter began with the detection of a UV-active compound in the crude extract of a assemblage containing the cyanobacteria *Lyngbya majuscula* and *Tolypothrix sp.* This extract also displayed brine shrimp toxicity.

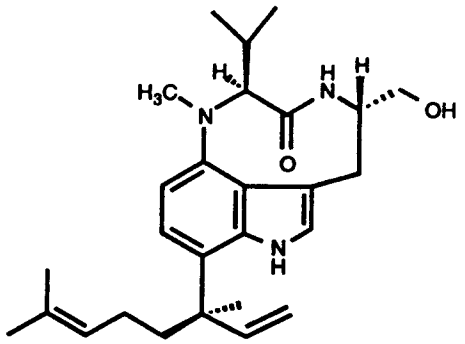
Lyngbya Agardh 1824 (Nostocales, Oscillatoriaceae) was named after a Danish botanist H. C. Lyngbye.[85] The German phycologist Lothar Geitler described almost 100 *Lyngbya* species in the 1930's.[86] More recently this microalgal genus has been used as a generic term for members of the "LPP" group (= *Lyngbya, Phormidium,* and *Plectonema).* This group is characterized as filamentous with a distinct mucoid sheath. According to some taxonomists, the uniqueness of *Lyngbya* as a taxonomic unit is uncertain.[85] *Lyngbya* is found frequently in brackish waters, but also in marine and freshwater habitats.[87]

Lyngbya majuscula is a cosmopolitan species commonly found in tropical marine environments.[86] This genus is taxonomically identified through analysis of cell and trichome morphology. Sheath and filament width can be extremely variable with the latter ranging from 16-60 μm, although mostly 20-40 μm.[86,128] Since this range overlaps many of the smaller species of *Lyngbya*, one taxonomist, T. V. Desikachary states that it is "doubtful whether one can really separate subordinate taxa with any degree of certainty."[128] The "Drouet" system, as described in chapter one, furthered the confusion by revising *Lyngbya majuscula* to *Microcoleus lyngbyaceus.* Most taxonomists do not accept this nomenclature although it still may appear in the literature.[129]

The cyanobacterium *Lyngbya majuscula* is a rich source of novel natural products which have been isolated during the last several decades. Populations from many locations around the world have shown a variety of compound classes, many with potent biological activity. The indole alkaloid, lyngbyatoxin A (**1**, Figure III.1) was isolated from a Hawaiian collection and is one causative agent of a severe contact dermatitis known as "swimmer's itch."[130] This inflammatory agent was found to be identical to the *Streptomyces mediocidicus* metabolite teleocidin A-1[131] which stimulates the phosphorylation of threonine and serine residues in proteins.[132] This same Hawaiian collection also yielded several pyrrolic compounds, known as the pukeleimides (pukeleimide A, **2**), [133,134] as well as (+)-(S)-α-butryramido-γ-butyrolactone.[135]

Several amides of (-)-*trans*-7*(S)*-methoxytetradec-4-enoic acid, named malyngamides A, B, and C (**3**), were isolated from another Hawaiian collection.[136] They represent the first members of an extended series of malyngamides. Another causative agent of "swimmer's itch," debromoaplysiatoxin (**4**), was briefly discussed in chapter one. This macrocycle was isolated originally from the marine gastropod *Stylocheilus sp.* [137] but was reisolated from a deep water variety of *Lyngbya sp.* in the Marshall Islands. *Lyngbya* seems to be the preferred food source of *Stylocheilus sp.* Debromoaplysiatoxin has shown activity against P-388 lymphocytic leukemia in mice.[100] Another variety of *L. majuscula* produced majusculamide C (**5**). This antifungal depsipeptide is related structurally to dolastatin 11[138,139] and is strongly cytotoxic, exhibiting similar activity to the mitosis blocker, cytochalasin B.[132]

A Guam collection of *Lyngbya majuscula* yielded ypaoamide (**6**, Figure III.2), an herbivore antifeedent compound.[140] A *Lyngbya* species from Australia produced a brominated fatty acid (**7**)[141] which is related structurally to



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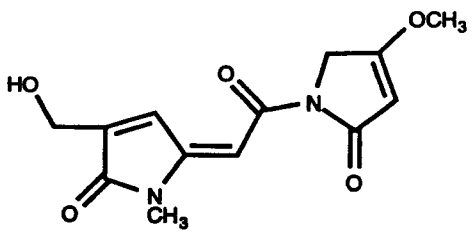

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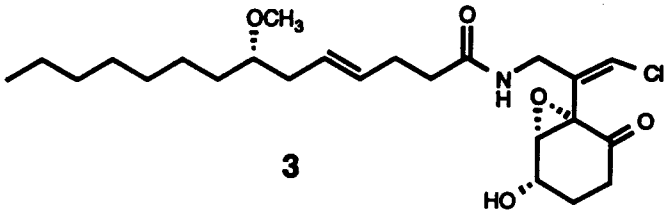

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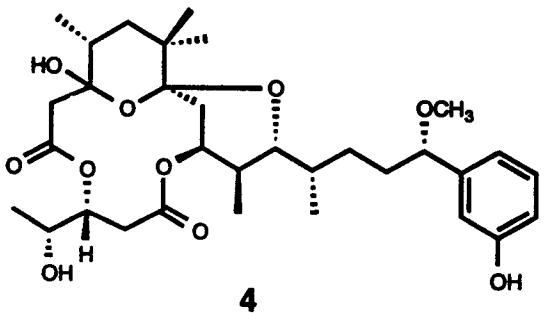

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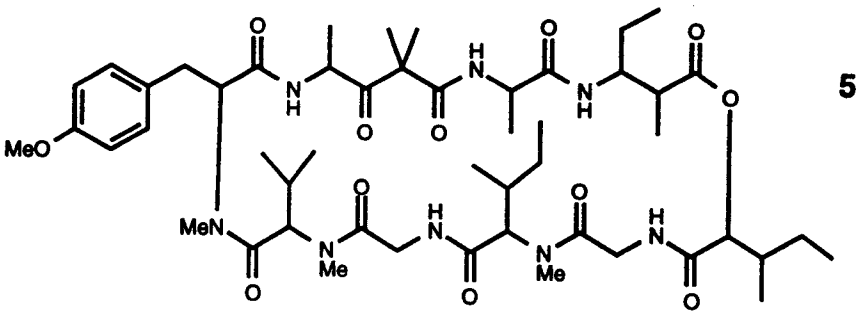

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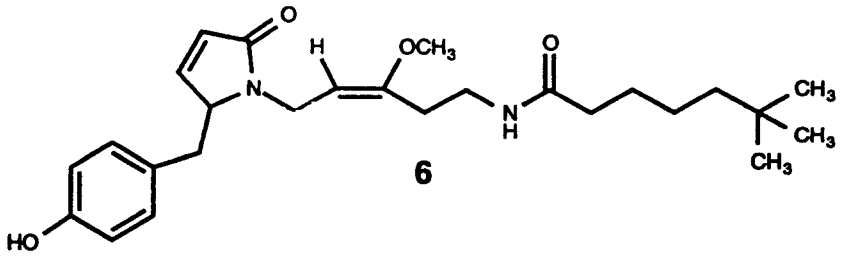
Figure III.1 Metabolites from Pacific *Lyngbya majuscula*

grenadadiene (**8**), a metabolite of a *L. majuscula* from Grenada.[142] A Venezuela *L. majuscula* yielded microcolins A (**9**) and B (**10**). These linear peptides are potent inhibitors of murine P-388 leukemia and the murine mixed lymphocyte response.[143]

An in depth study by our laboratory of *Lyngbya majuscula* from the Caribbean, and in particular Curaçao, has led to the isolation of a suite of novel bioactive compounds. Initially, curacin A (**11**, Figure III.3) was obtained and characterized as a thiazoline containing lipopeptide. The isolation was guided by brine shrimp toxicity bioassays. Subsequently, curacin A was shown to be a potent antimitotic and antiproliferative to cancer cells grown *in vitro.*[144] Another collection containing highly ichthyotoxic fractions yielded antillatoxin (**12**), a cyclic lipopeptide.[145] This same extract also produced a molluscicidal agent, barbamide (**13**), a chlorinated compound containing a thiazole ring.[146] Another collection yielded kalkitoxin (**14**), a thiazoline containing lipid with potent ichthyotoxicity (LC_{50} = 5 ng/ml).[147]

The other major constituent of the cyanobacterial assemblage utilized in this project was a *Tolypothrix sp.* The genus *Tolypothrix* (Nostocales, Scytonemataceace) was first identified in the 1840's and Geitler has described almost 50 species.[86] These sheathed cyanobacteria are filamentous and contain heterocysts utilized in nitrogen fixation. They are found in marine, fresh water and terrestrial environments and often form macroscopic tufts or entangled masses with other algal genera.[85] One species, *T. lophopodellophila,* is known to be associated with freshwater polyzoa,[128] some of which are symbionts in the intestines of various species of termites.[148] Another species, *T. tenius,* has been utilized for numerous axenic culture studies of chromatic adaptation.[85]



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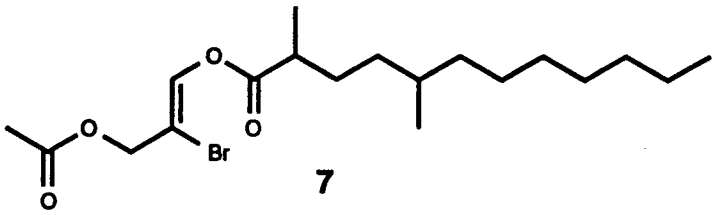

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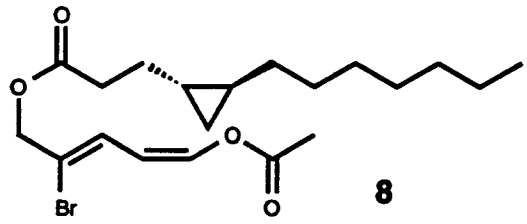

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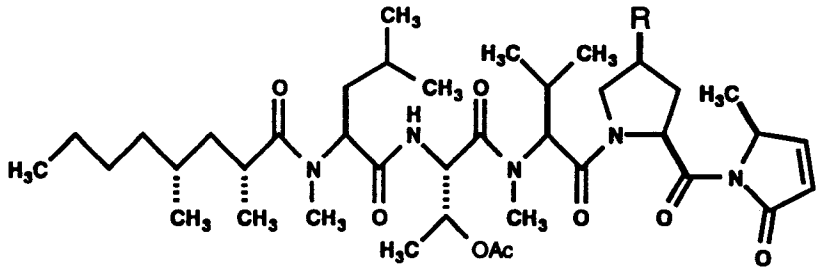
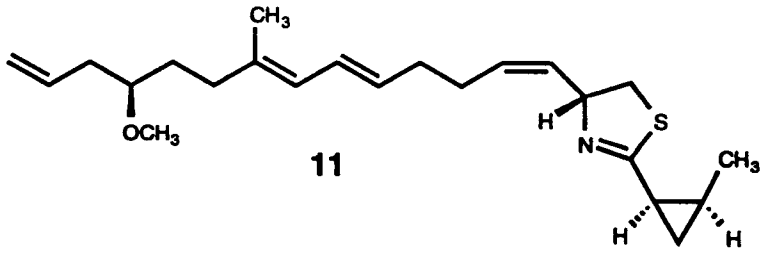


9 R = OH
10 R = H

Figure III.2 Several Metabolites of *Lyngbya majuscula*



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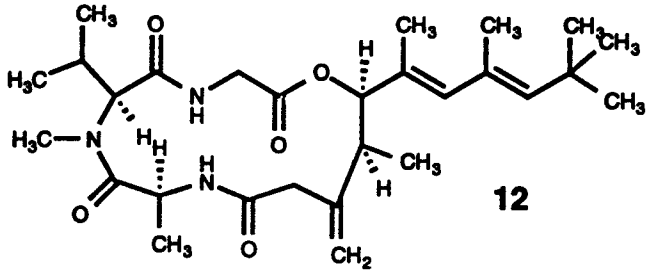

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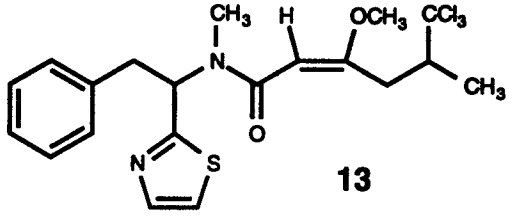

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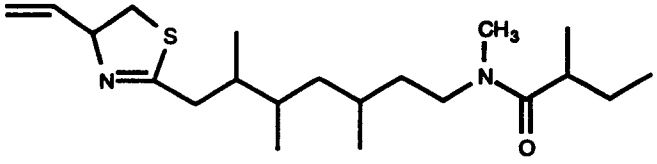

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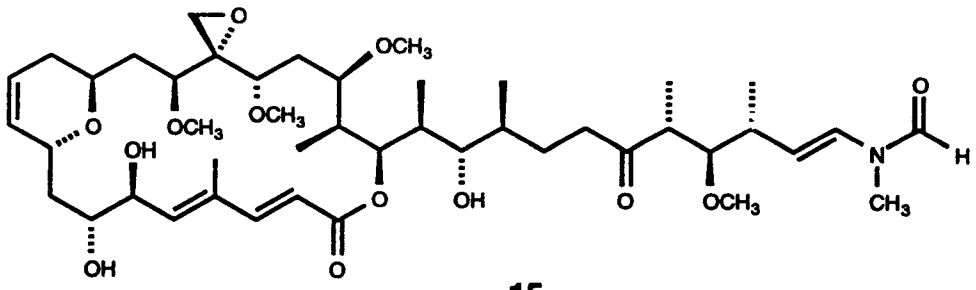
Figure III.3 Metabolites of *Lyngbya majuscula* from Curaçao

Although the secondary metabolites of *Tolypothrix* have not been extensively examined, most of the reported compounds are bioactive. The macrolactone tolytoxin (**15**, Figure III.4) was isolated initially from a collection of terrestrial *T. conglutinata* var. *colorata*.[129] Subsequently, tolytoxin, as well as a family of related compounds, have been isolated from *Scytonema*, a related genus. Collectively these metabolites are known as the scytophycins.[149] Tolytoxin exhibits antifungal activity along with cytotoxicity as a potent microfilament-depolymerizing agent.[150,151] During the isolation of tolytoxin, a series of non-toxic isotactic polymethoxy-1-alkenes (for example **16**) was obtained from the same extract.[152]

An extract of a collection of *T. byssoidea* displayed potent bioactivity in a variety of assays. Initially, tubercidin (**17**, Figure III.5), an antineoplastic nucleoside originally isolated from *Streptomyces tubercidicus* was obtained.[153] Further investigation of the extract afforded tolypophycins A and B which displayed positive inotropic effects in isolated mouse atria and fungicidal activity. These metabolites have not been fully characterized. A mixture of several *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O,O*-dimethyl-α-cyclodextrins (for example **18**) was isolated and blocked the fungicidal but not the cardiotonic activity of the tolypophycins.[154]

Tubercidin, its cyanoderivative toyocamycin (**19**), and the corresponding 5'-α-D-glucopyranose derivatives were isolated from *Tolypothrix* during a comparison study of the Scytonemataceae. Toyocamycin was originally obtained from *Streptomyces toyocaenis*. All of these metabolites are cytotoxic and antimycotic.[155]

A soil sample from Florida yielded *T. tjipanasensis* which produced the tjipanazoles, fifteen *N*-glycosides of indolo[2,3] carbazoles. Tjipanazole A1 (**20**) and A2 exhibited antifungal activity.[156] The lipophilic extract of *T. nodosa*



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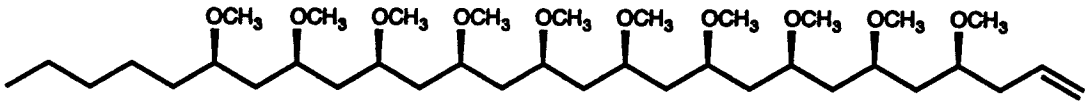

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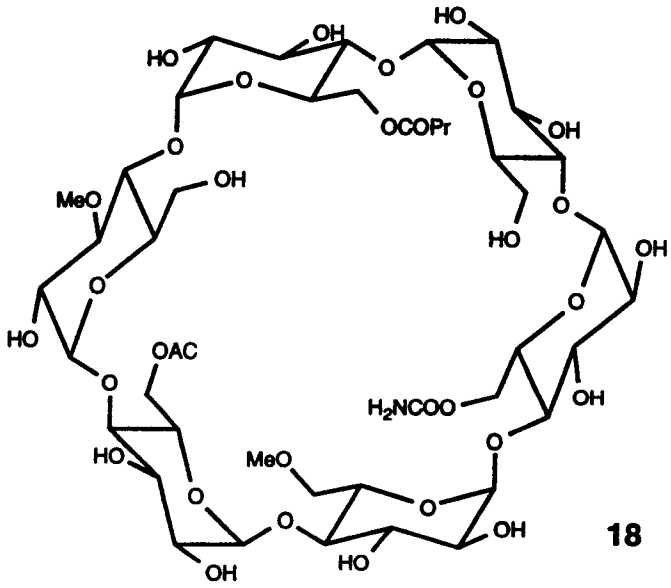

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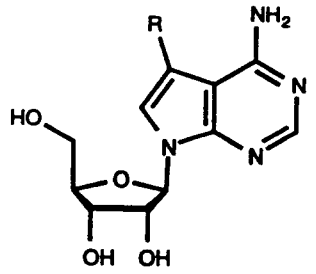
Figure III.4 Metabolites from *Tolypothrix*

afforded the tolyporphins, a series of porphyrin derivatives. Tolyporphin A (**21**) reversed multidrug resistance in a vinblastine-resistance subline of human ovarian adenocarcinoma.[157,158] Recently, an antiinflammatory diterpenoid tolypodiol (**22**) has been reported from this same collection of *Tolypothrix*. This metabolite represents the first reported meroditerpenoid compound from cyanobacteria.[159]

Our interest in cyanobacteria has been encouraged by the discovery of extensive number of metabolites which display bioactivity. Furthermore, a literature summary of the *Lyngbya majuscula* and *Tolypothrix* reveals numerous bioactive compounds. Clearly studies of these genera could provide new and exciting natural products. In our continuing efforts to describe the organic chemistry of the cyanobacteria, we extracted a collection of an assemblage of *Lyngbya majuscula* and *Tolypothrix sp.* and have now isolated a novel γ-pyrone derivative, kalkipyrone (**23**). Kalkipyrone is the first γ-pyrone moiety isolated from either genera and displays potent brine shrimp ($LD_{50} \approx 1$ μg/ml) and gold fish ($LD_{50} \approx 2$ μg/ml) toxicity.



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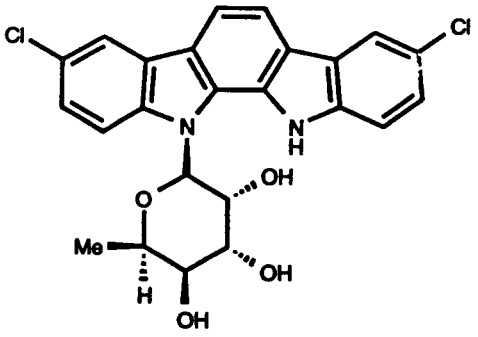
19 R = CN



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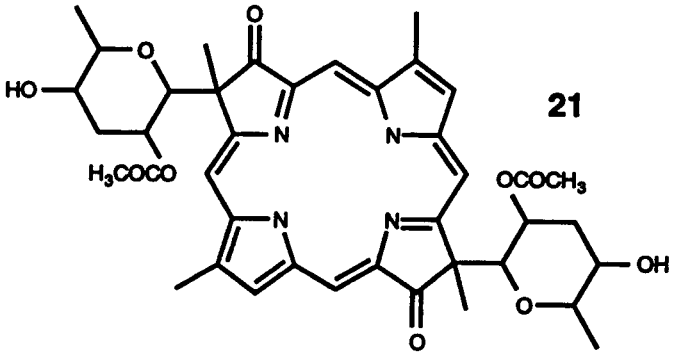

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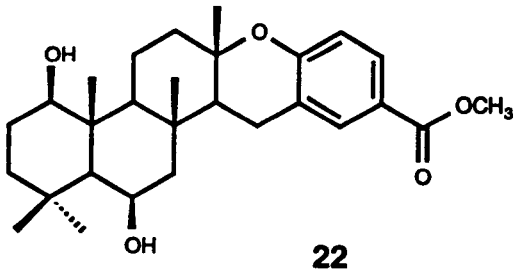

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Figure III.5 Additional Metabolites from *Tolypothrix*

Results and Discussion

A mixture of *Lyngbya majuscula* and *Tolypothrix sp.* was collected from Playa Kalki, Curaçao in May 1996. This sample was preserved in isopropyl alcohol and stored at -20 °C. The defrosted algae were repeatedly extracted for their lipid metabolites with CH_2Cl_2/MeOH (2:1) and the resulting solution partitioned between H_2O and CH_2Cl_2. The organic layer was concentrated *in vacuo* to produce a dark oil (550 mg).

Initial two-dimensional TLC analysis indicated an interesting UV-active compound. Vacuum silica chromatography (Hex/EtOAc/MeOH gradient) followed by reverse phase C-18 silica chromatography (MeOH/H_2O, 40%-100%) yielded 7.3 mg of kalkipyrone (**23**) as a colorless oil which was utilized for initial characterization. An additional 8.5 mg of **23** was isolated and used for biological evaluation and further NMR studies.

The structure elucidation of kalkipyrone was based on interpretation of spectral and physical properties. The molecular formula was determined by high resolution EI mass spectrometry as $C_{20}H_{28}O_4$, indicating 7 degrees of unsaturation. Initial examination by ^{13}C NMR in $CDCl_3$ showed only 18 carbons, implying overlapping resonances. Subsequent NMR experiments in C_6D_6 revealed 20 carbons, which included one carbonyl (δ180.7) and 10 olefinic carbons (δ 100.1, 118.8, 119.2, 126.8, 133.8, 136.4, 137.2, 137.9, 156.8, 162.3), accounting for 6 degrees of unsaturation; thereby, the compound had one ring. The IR spectrum contained hydroxyl (υ 3374, br) and dienone (υ 1665) absorption bands while UV indicated the presence of a conjugated olefinic system (λ_{max} = 238 nm, ε = ca. 33,000).

The partial structures, **a**, **b**, and **c** (Figure III.6) were deduced from ^1H-^1H COSY analysis. The C15-C13 portion (partial structure **a**) was defined based

on the proton of an oxygenated methine showing connectivity to a methyl group and an olefinic proton. The structural subunit **b** was characterized by a *trans* double bond (J = 15.6 Hz) contiguous to a methylene at C9. The partial structure **c** was defined by an olefinic proton connected to a methylene at C6. Additionally, the ^{1}H NMR spectrum indicated five singlet methyl groups, four as vinylic (δ 1.47-2.10) and one as a methoxy (δ 3.20). The chemical shifts of the protonated carbons were determined by 2D ^{1}H-^{13}C correlation experiment (HMQC).

Long-range heteronuclear ^{13}C-^{1}H coupling (HMBC) together with a NOESY experiment enabled the spin systems and methyl groups to be put together (Figure III.7, Table III.1). The coupling between the olefinic proton H13 (δ 5.44) and the vinylic methyl group C16 (δ 13.1) as well as between H11 (δ 6.05) and C16, connected partial fragments **a** and **b** and placed C16. The ^{13}C shift of C12 (δ 133.8) was deduced by the connection between H16 (δ 1.60) and C12 along with the correlation between H10 (δ 5.53) and C12. Coupling between the fully substituted olefinic carbon C8 and both sets of methylene protons H6 (δ 2.85) and H9 (δ 2.66) allowed the assignment of C8 (δ 137.9) and connected partial fragments **b** and **c**. The vinylic methyl group C17 (δ 16.7) was confidently placed at C8 by observing a H17 (δ 1.47) correlation to C8. The configuration of the trisubstituted olefinic bonds was determined as *E* by the values of the carbon signals assigned to C16 (δ 13.1) and C17 (δ 16.7). Both resonate at less than 20 ppm indicating *E* stereochemistry as a result of a γ-shielding effect.[160-162] NOE correlations between H16 and H10 as well as between H17 and H6 further confirmed the structure of the alkyl chain.

While the connectivity of the alkyl chain was easily determined through HMBC experiments, the lack of protons in the ring portion did not provide sufficient C-H connectivities to resolve this substructure with certainty prior to

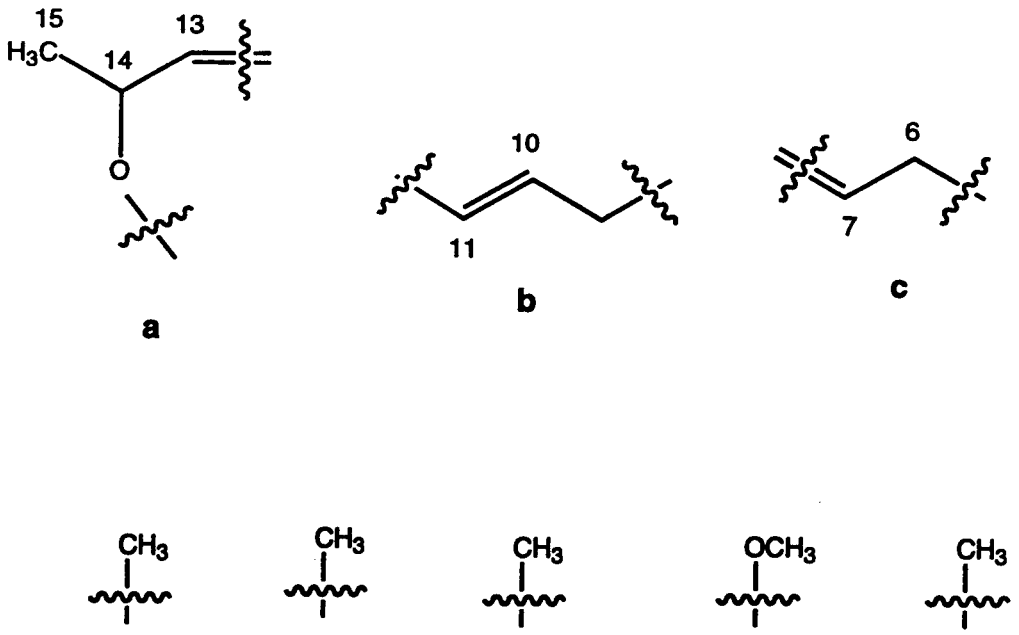


Figure III.6 Partial Structures of Kalkipyrone (**23**)

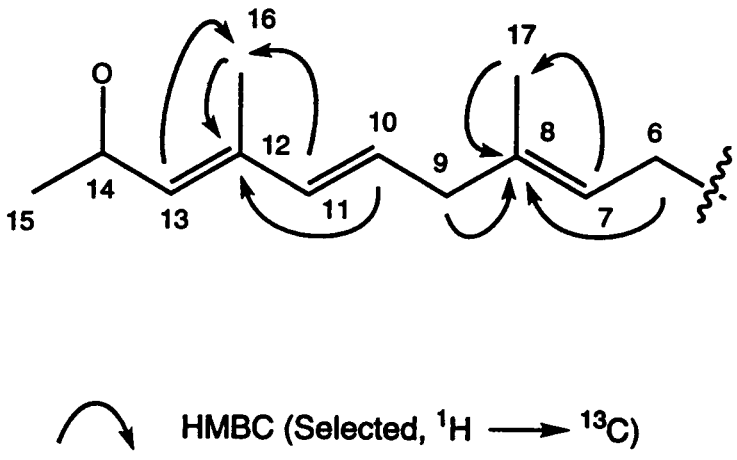


Figure III.7 Alkyl Tail of Kalkipyrone (**23**) Connected by HMBC

Table III.1 ^{1}H, ^{13}C, HMBC and NOESY Data of Kalkipyrone (23)

#C	^{13}C $CDCl_3$[a*] δ	^{1}H $CDCl_3$ δ mult, J in Hz	^{13}C C_6D_6[b] δ	^{1}H C_6D_6 δ	NOESY Correlations	HMBC Correlations H ⇒ C
1	162.2 s	-	162.3	-	-	-
2	99.4 s	-	100.1	-	-	-
3	181.1 s	-	180.7	-	-	-
4	118.1 s	-	118.1	-	-	-
5	157.0 s	-	156.8	-	-	-
6	30.0 t	3.30 d 7.2	30.3	2.85	7, 17, 18	5, 7, 8
7	118.1 d	5.25 bt 7.2	119.2	5.12	6	6, 9, 17
8	137.8 s	-	137.9	-	-	-
9	42.8 t	2.80 d 7.2	43.5	2.66	10, 11	7, 8, 10
10	127.0 d	5.61 dd 7.2, 15.6	126.8	5.53	9, 11, 16	8, 9, 11, 12
11	136.0 d	6.04 d 15.6	137.2	6.05	9, 10	9, 12, 13, 16
12	134.3 s	-	133.8	-	-	-
13	134.3 d	5.40 d 8.4	136.4	5.44	14	11, 15, 16
14	64.6 d	4.69 dt 6.4, 8.4	64.8	4.47	13, 15	12, 13, 15
15	23.5 q	1.27 d 6.4	24.2	1.15	14	13, 14
16	12.7 q	1.78 s	13.1	1.60	10	12, 13
17	16.5 q	1.72 s	16.7	1.47	6	7, 8, 9
18	9.8 q	1.95 s	10.5	1.98	6	3, 4, 5
19	6.8 q	1.83 s	7.7	2.10	-	1, 2, 3
20	55.2 q	3.91 s	55.0	3.20	-	1

[a] Spectra in $CDCl_3$ recorded on a Bruker AM-400 spectrometer (^{1}H spectra referenced to TMS at 0.0 ppm; ^{13}C spectra referenced to the centerline of $CDCl_3$ at 77.0 ppm).

[b] Spectra in C_6D_6 recorded on a Bruker DRX-600 spectrometer (^{1}H spectra referenced to C_6D_6 at 7.16 ppm; ^{13}C spectra referenced to the centerline of C_6D_6 at 128.39 ppm).

* ^{13}C NMR multiplicity assigned by a DEPT 135 experiment.

literature consultation. Two ring substructures were possible from IR, HMBC, ^{13}C and ^{1}H NMR data. The carbonyl C3 (δ 180.7) correlated to a singlet methyl H19 (δ 2.10) which was also connected to two olefinic carbons C1 (δ 162.3) and C2 (δ 100.1). One olefinic carbon C1 was also coupled to a methoxy group H20 (δ 3.20). This fragment was defined as a ketone conjugated with a fully substituted polarized olefinic bond. The ring could be completed as a γ–pyrone (**A**) or as a 5 membered ring with an exocyclic double bond (**B**). One olefinic carbon C4 (δ 118.1) was coupled to a vinyl methyl group H18 (δ 1.98) while another olefinic carbon C5 (δ 156.8) correlated to a methylene H6 (δ 2.84) and H18. A three bond coupling from C3 to H18 gave the pyrone system (**A**) while a possible four bond correlation indicated the five membered ring (**B**, Figure III.8).

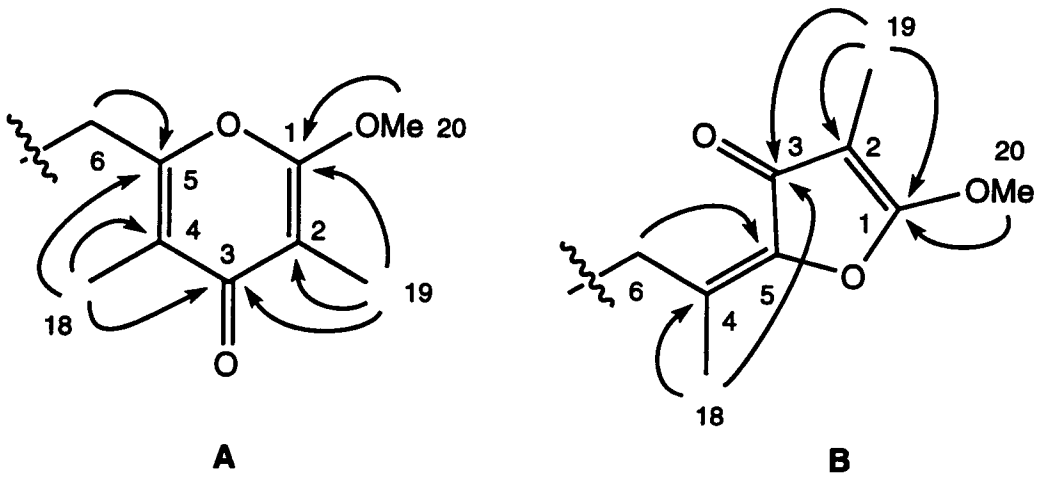


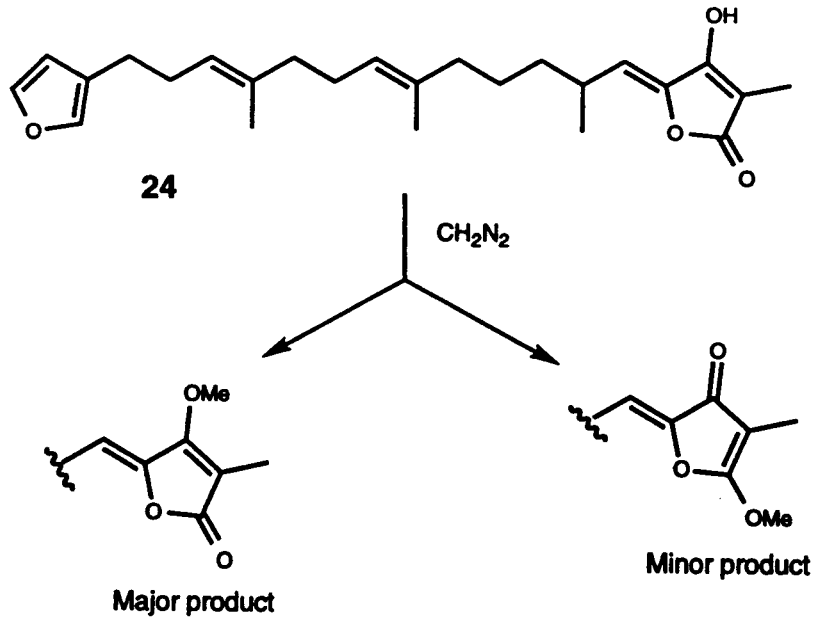
Figure III.8 Possible Ring Substructure of Kalkipyrone with HMBC Couplings

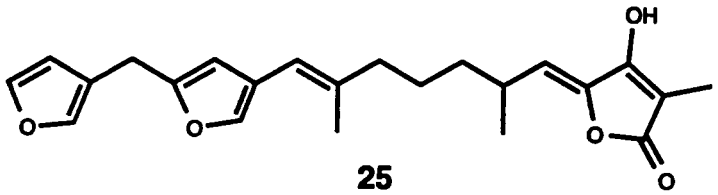
Since neither ring system has been reported in metabolites isolated from *Tolypothrix* or *Lyngbya majuscula*, other compounds within the field of natural products were used as structural models. The five membered ring substructure

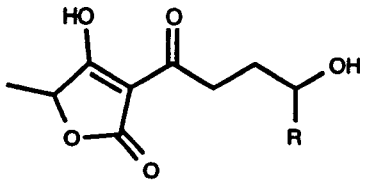
was reported as a minor product from the methylation with diazomethane of marine sponge metabolites which contain a tetronic acid moiety, including variabilin (**24**, Figure III.9)[163] and ircinins (ircinin 1, **25**) [164] from *Ircinia* species. Numerous fungi also produce tetronic acid metabolites, including carolic acid (**26**) and terrestic acid (**27**)[165] as well as serpenone (**28**).[166] If kalkipyrone contained a five membered ring, it could be derived biosynthetically from tetronic acid or a related compound. In addition, previous literature reported the difficulty of defining structures in the tetronate series.[167]

A red alga *Phacelocarpus* produces macrocyclic pyrones (for example **29**, Figure III.10).[168] Numerous marine molluscs (Opisthobranchia) biosynthesize secondary metabolites with pyrone moieties. These include the tridachiapyrones (tridachiapyrone 11, **30**)[169] from *Tridachia*, the peroniatriols (peroniatriol 1, **31**) from *Peronia*,[170] and the cyercenes (cyercene A, **32**) from *Cyerce*.[171] The origins of these metabolites are varied and some molluscs sequester chemicals from their algal diet possibly for chemical defense.[169] The pyrone system with an alkyl chain (**33**) also has been found in *Podolepis hieraciodes*, a higher plant from Australia.[172] Several strains of *Streptomyces* produce bioactive metabolites with the pyrone functionality such as spectinabilin (**34**, Figure III.11),[173] aureothin (**35**),[174] the actinopyrones A (**36**), B (**37**), and C (**38**)[175,176] and an analog (**39**).[177]

Comparisons with literature data, in particular the actinopyrones, clearly defined kalkipyrone as an α-methoxy-β,β'-dimethyl-γ-pyrone linked to an alkyl side chain. The IR bands as well as the ^{1}H and ^{13}C NMR data from the ring portion were comparable to those reported for the actinopyrones (Figure III.12). The pyrone ring of the actinopyrones was characterized by comparisons to other natural products, in particular, tridachione, which was confirmed by crystal structure anaylsis.[176,178] In addition, the structure of kalkipyrone (**23**, Figure







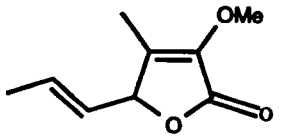
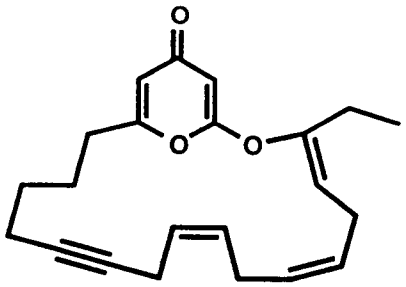


Figure III.9 Natural Products with a Tetronic Acid Moiety



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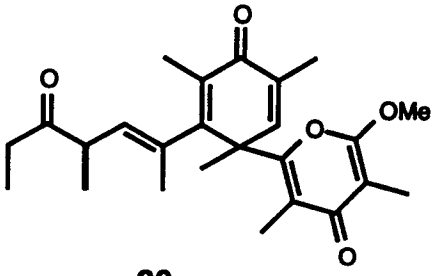

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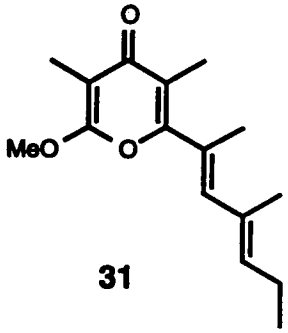

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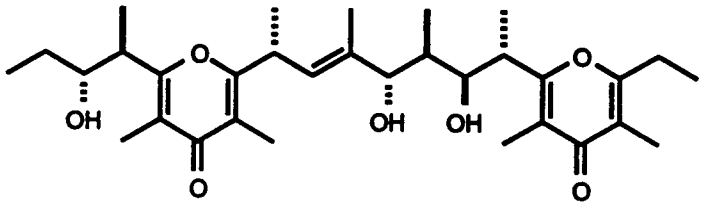

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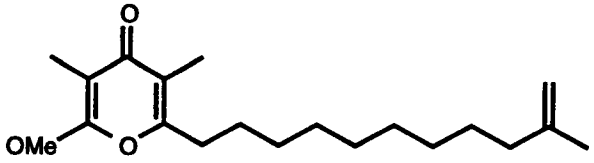

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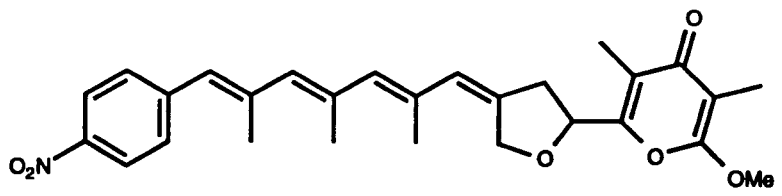
Figure III.10 γ-Pyrone Natural Products



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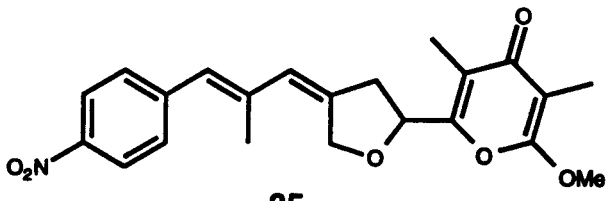

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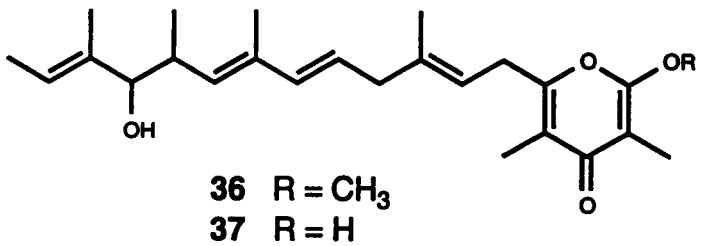

36 R = CH_3
37 R = H
38 R = CH_2CH_3

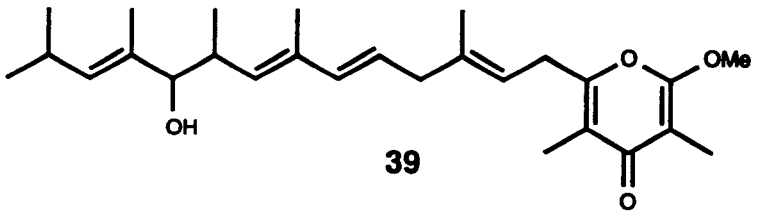

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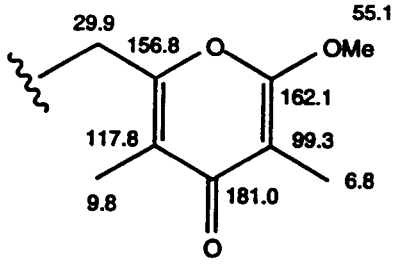
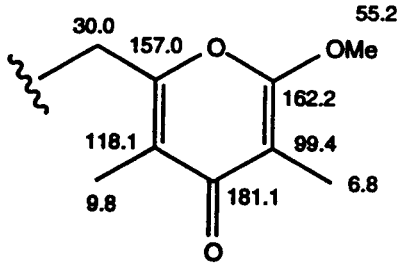
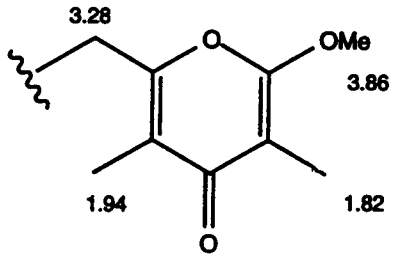
Figure III.11 γ-Pyrones from Streptomyces species



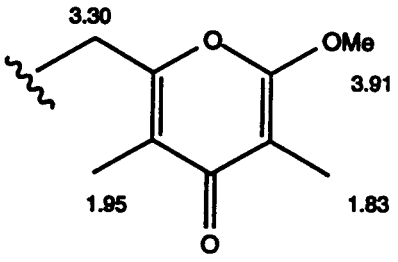
^{13}C NMR of actinopyrone A fragment in $CDCl_3$



^{13}C NMR of kalkipyrone fragment in $CDCl_3$



^{1}H NMR of actinopyrone A fragment in $CDCl_3$



^{1}H NMR of kalkipyrone fragment in $CDCl_3$

IR (film) υ_{max} 1660, 1590 cm^{-1} 1665, 1579 cm^{-1}

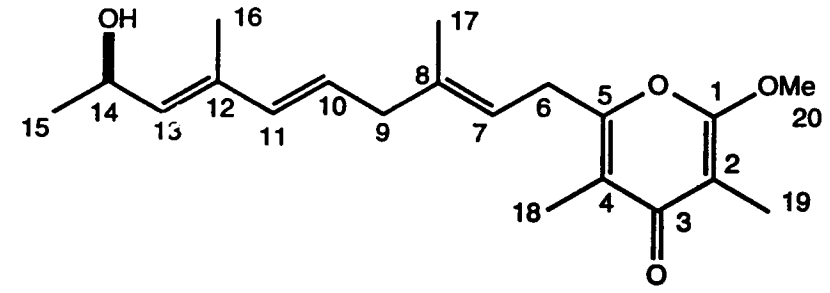
Figure III.12 Comparison of NMR (400 MHz) and IR data for Kalkipyrone (**23**) and Actinopyrone A (**36**)

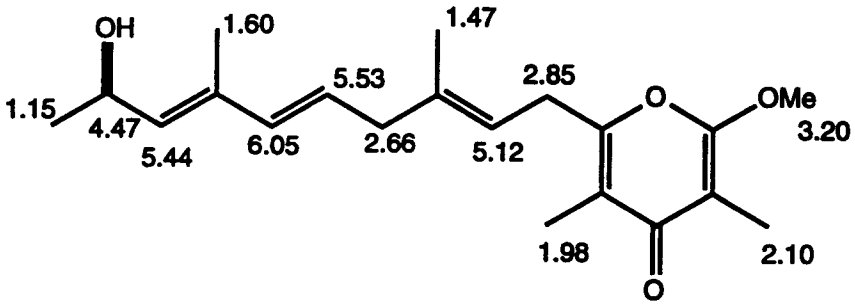
III.13) was completed by confidently placing the hydroxyl detected by IR at C14 since the ring substructure accounted for all of the remaining atoms in the molecular formula except for one hydrogen.

The absolute stereochemistry at C14 was determined by converting kalkipyrone (0.2 mg) to its corresponding (-)-menthoxycarbonyl (MC) derivative, followed by oxidative ozonolysis to release the C13-C15 fragment and derivation of this fragment to the corresponding methyl ester.[121] GC standards for this same MC derivative gave baseline separation under optimized conditions (11.5 m of HP Ultra-1, 100-210°C at 3.0° C per min., then isothermal for 5 min.), with an elution time of 14.81 min. *(S)* and 15.07 min. *(R)*. The reaction product was coinjected with lactate derived racemic standards and the observed peak enhancement clearly showed the kalkipyrone MC derivative was partially racemic as 84% *R*, 16% *S* (Figure III.14). Although the scalemic nature of C14 could be due to isolation or derivatization procedures, it should be noted that plant lipoxygenases are known to produce *S/R* mixtures which can exceed an 84:16 ratio.[48]

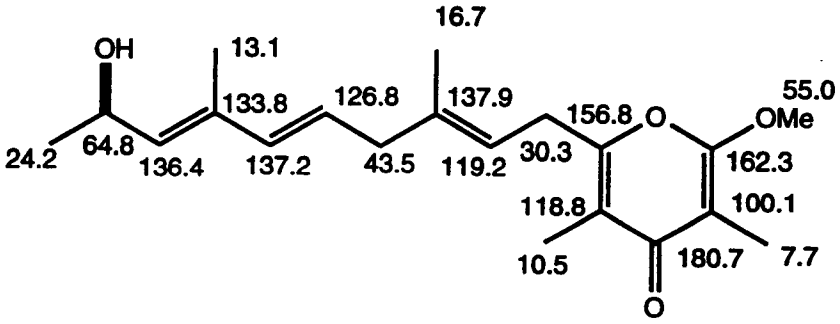
The initial structural analysis of kalkipyrone was hindered by decomposition of the metabolite during NMR experiments. Subsequently, kalkipyrone had to be reisolated from other fractions of the crude extract for biological evaluation and further experimentation. The chemical instability of this compound class is not unprecedented. For example, the actinopyrones are reported to be "sensitive to oxidation under air."[176]

Kalkipyrone was found to possess potent brine shrimp toxicity ($LD_{50} \approx 1.$ µg/ml) and ichthyotoxicity to goldfish ($LD_{50} \approx 2$ µg/ml). Since kalkipyrone represents 3% of the lipid soluble material from the assemblage, the producing organism clearly contains a greater percentage of the compound. This metabolite may function in nature to protect the alga from predation by



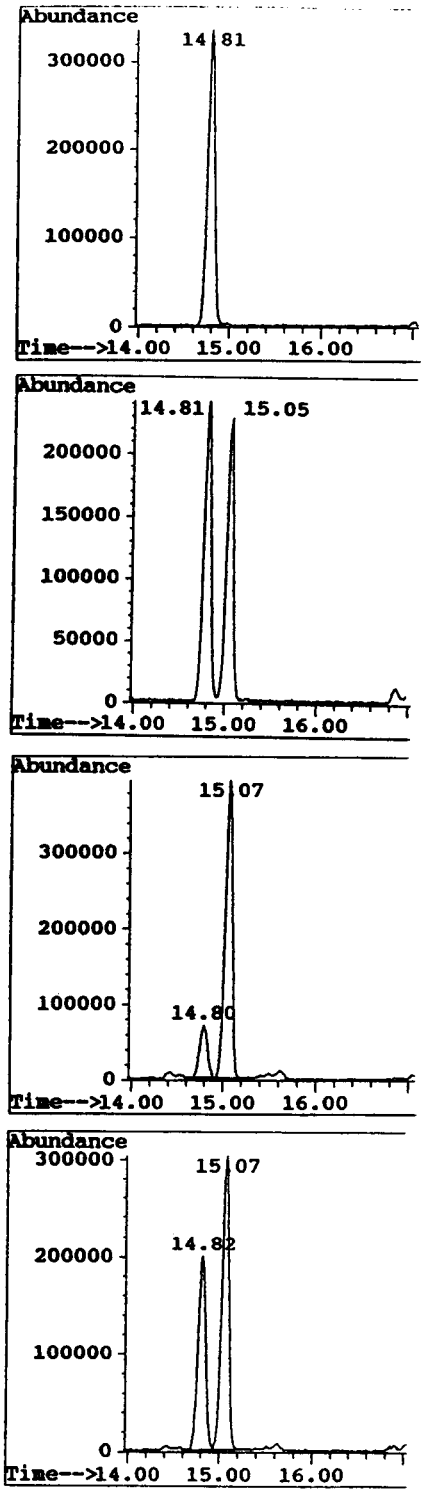


- ^{1}H NMR Chemical Shifts



- ^{13}C NMR Chemical Shifts

Figure III.13 ^{1}H and ^{13}C NMR Assignments of Kalkipyrone (**23**) in C_6D_6 (600 MHz)



(-)-Menthoxycarbonyl (MC) derivative of *S* methyl lactate as a standard

MC derivatives of racemic methyl lactate as standards

Kalkipyrone derived MC derivatives

Kalkipyrone derived with added racemic methyl lactate MC derivatives

Figure III.14 Elution Profile of (-)-Menthoxycarbonyl (MC) Derivatives by Gas Chromatography

Crustacea and herbivorous fish. These possible predator-prey interactions encourage additional investigations into the ecological relationships of these algae and the other organisms of the surrounding marine environment.

In addition, a precedent for ichthyotoxic plants to produce antifeedents, plant growth inhibitors, antitumor agents and insecticides was reported.[179] Subsequently, kalkipyrone was evaluated in the NCI's 60 human tumor cell line panel as a potential anticancer drug lead. The modest activity displayed in several cell lines of the renal and melanoma panels was not sufficient to warrant further investigations (Figure III.15 and 16). In comparison, the structurally related actinopyrones exhibited vasodilating and antimicrobial activity[175] while a close analog (**39**) was antiparasitic.[177]

Biogenetically, kalkipyrone may derive from polypropionate and acetate through the polyketide pathway. Structurally related compounds from marine molluscs have been shown to be produced biosynthetically *via* condensation of polypropionate units.[180] Although sea hares sequester chemicals from their algal diet,[169] biosynthetic studies of other polypropionate pyrone moieties from marine molluscs indicate the metabolites are of molluscan origin.[181]

Since the α-methoxy-γ-pyrone ring absorbs ultraviolet light, it has been suggested that in the molluscan metabolites they may serve a sunscreen protection role.[181] The mixture of cyanobacteria containing kalkipyrone was growing intertidally on a rock fully exposed to direct sunlight. Perhaps kalkipyrone also serves a UV protection role for its producer.

The structural similarity of kalkipyrone to the actinopyrones from *Streptomyces* is striking. Both *Tolypothrix* and *Lyngbya majuscula* have been reported to produce compounds identical to *Streptomyces* metabolites such as tubercidin (**17**), toyocamycin (**19**), and lyngbyatoxin A (**1**, teleocidin A-1). The

Figure III.15 NCI 60 Cell Line Tumor Growth Inhibition Dose Response Curves for Kalkipyrone (23)

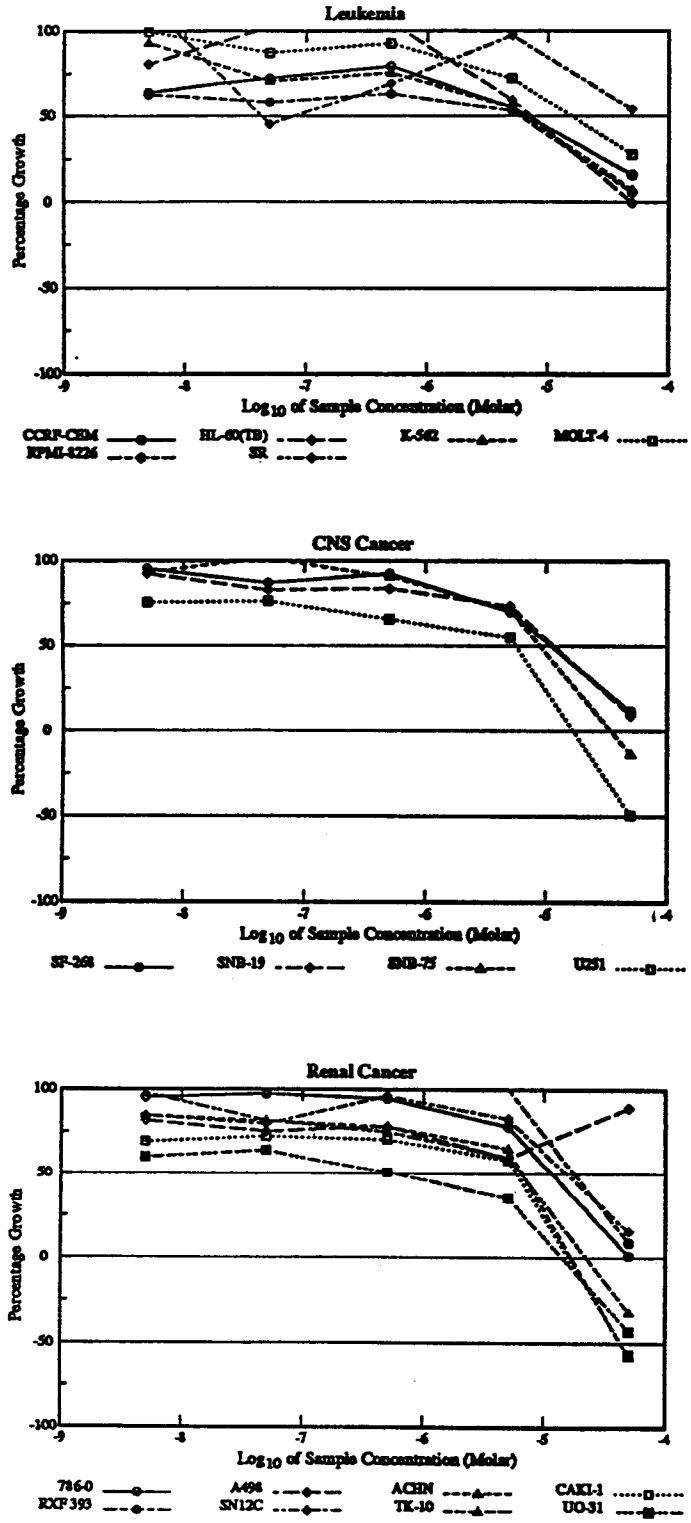


Figure III.15 Continued

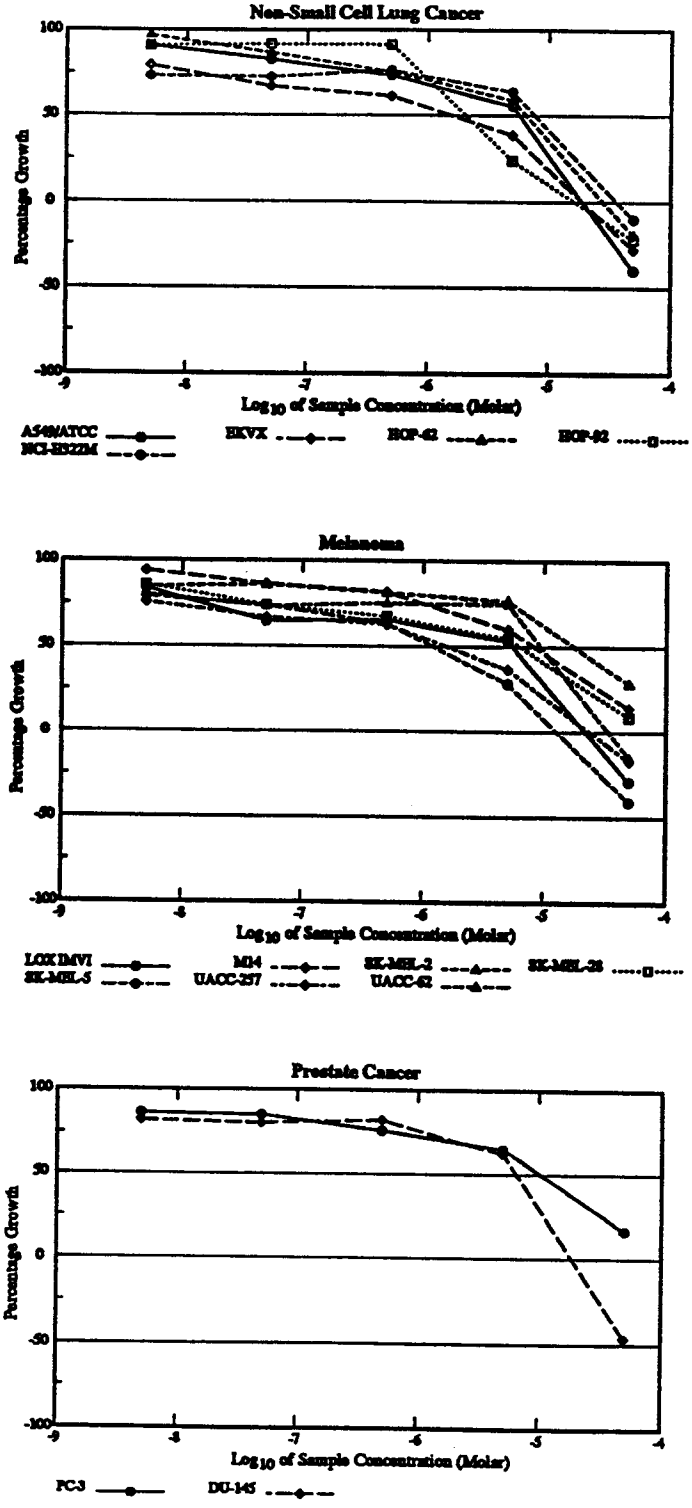


Figure III.15 Continued

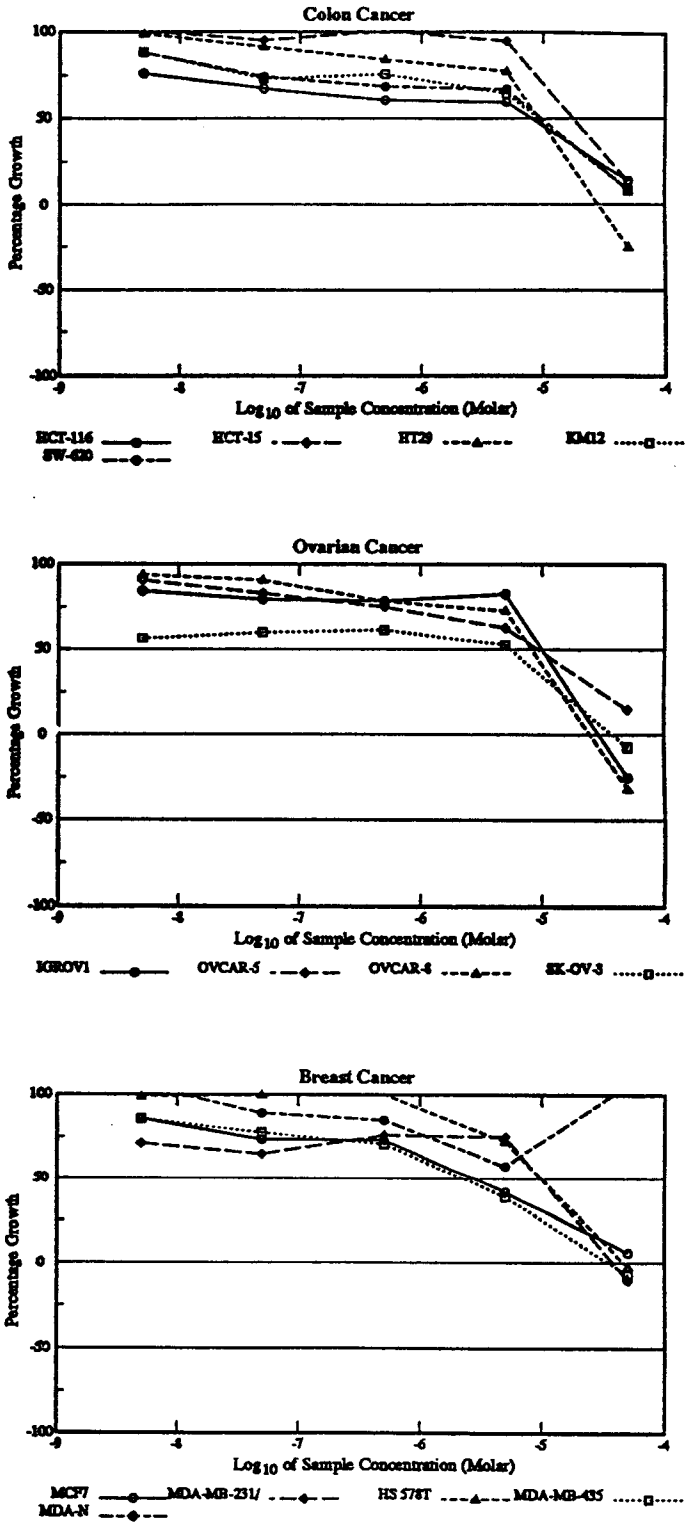


Figure III.15 Continued

Figure III.16 NCI 60 Cell Line Tumor Growth Inhibition Mean Graphs for Kalkipyrone (23) (GI, Growth Inhibition; TGI, Total Growth Inhibition)

Panel/Cell Line	Log_{10} GI50	GI50
Leukemia		
CCRF-CEM	-5.17	
HL-60(TB)	-5.14	
K-562	-5.20	
MOLT-4	-4.80	
RPMI-8226	-5.24	
SR		
Non-Small Cell Lung Cancer		
A549/ATCC	-5.24	
EKVX	-5.80	
HOP-62	-5.19	
HOP-92	-5.69	
NCI-H322M	-5.12	
Colon Cancer		
HCT-116	-5.09	
HCT-15	-4.75	
HT29	-5.03	
KM12	-5.04	
SW-620	-5.01	
CNS Cancer		
SF-268	-4.95	
SNB-19	-4.94	
SNB-75	-5.05	
U251	-5.25	
Melanoma		
LOX IMVI	-5.26	
M14	-5.08	
SK-MEL-2	-4.75	
SK-MEL-28	-5.20	
SK-MEL-5	-5.92	
UACC-257	-5.80	
UACC-62	-5.03	
Ovarian Cancer		
IGROV1	-5.00	
OVCAR-5	-5.03	
OVCAR-8	-5.08	
SK-OV-3	-5.26	
Renal Cancer		
786-0	-4.94	
A498	> -4.30	
ACHN	-5.18	
CAKI-1	-5.23	
RXF 393	-4.75	
SN12C	-4.81	
TK-10	-5.19	
UO-31	-6.26	
Prostate Cancer		
PC-3	-4.99	
DU-145	-5.19	
Breast Cancer		
MCF7	-5.56	
MDA-MB-231/ATCC	-5.01	
HS 578T	-5.00	
MDA-MB-435	-5.65	
MDA-N	> -4.30	
MG_MID	-5.14	
Delta	1.11	
Range	1.95	
		+3 +2 +1 0 -1 -2 -3

Figure III.16 Continued

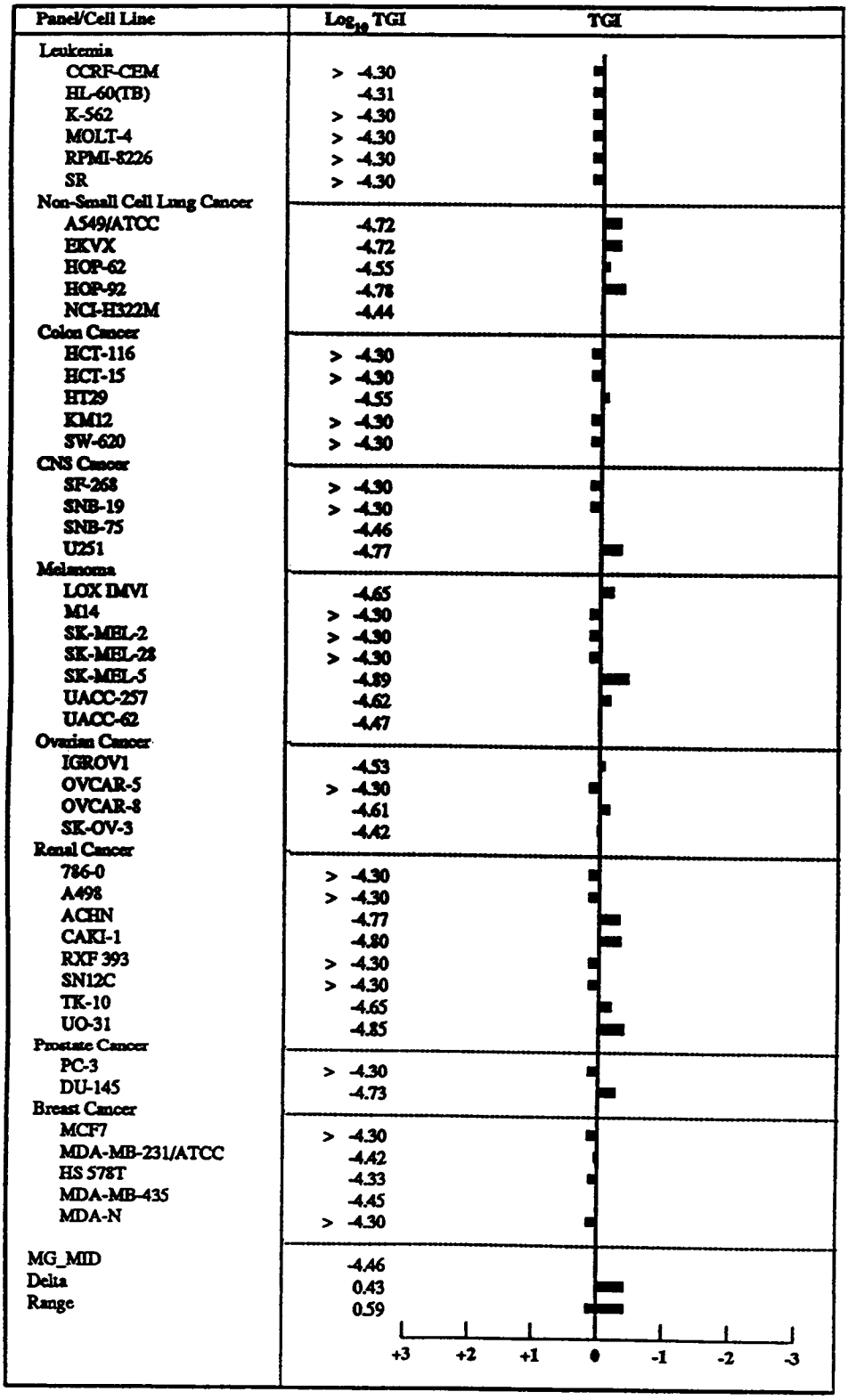


Figure III.16 Continued

occurrence of metabolites produced in common raises an interesting question regarding the relationship between cyanobacteria and *Streptomyces*.

Attempts to determine whether *L. majuscula* produced kalkipyrone, as versus *Tolypothrix*, were inconclusive. A sample of kalkipyrone as well as the crude extract were subjected to GC-EIMS analysis (11.5 m of HP Ultra-1, 70-250°C at 15.0 °C per min., then isothermal for 15 min.). Both contained several peaks (t_R = 12.29, 12.97 min.) which produced mass spectral fragments indicative of kalkipyrone. Olefinic isomerization in the alkyl chain can account for the occurrence of more than one peak during analysis. Two laboratory cultures of *L. majuscula* cells isolated from the mixture were extracted with CH_2Cl_2/MeOH (2:1, 3X), dried under nitrogen, resuspended in 10 % (v/v) EtOAc/hex, filtered, and analyzed by GC-EIMS. Kalkipyrone could not be detected in either sample.

Several possible circumstances could explain the inability to detect kalkipyrone. There may have been an insufficient amount of kalkipyrone in the cultured algal material for detection or an inefficient extraction procedure. Cultured *L. majuscula* may not generate this metabolite but rather the *Tolypothrix* species or another unidentified organism from the assemblage such as another bacterium. Furthermore, since many of the cyanobacteria reported to produce interesting metabolites have not been cultured under axenic conditions, little is known of their ability to produce compounds as single species.[38] Perhaps cultured *L. majuscula* could be induced to produce kalkipyrone.

Experimental

General. Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker AM-400 and DRX-600 spectrometers in $CDCl_3$ and C_6D_6. Proton spectra were referenced to C_6D_6 (7.16 ppm) or internal tetramethylsilane (0.0 ppm) in $CDCl_3$. Carbon spectra were referenced to the centerline of $CDCl_3$ (77.0 ppm) or C_6D_6 (128.39 ppm). Ultraviolet (UV) spectra were obtained using a Hewlett Packard 8452A diode array spectrophotometer. Infrared (IR) spectra were run on a Nicolet 510 Fourier transform IR (FTIR) spectrometer. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, while high resolution mass spectra (HRMS) were recorded on a Kratos MS 50 TC. Merck aluminium-backed thin layer chromatography (TLC) sheets (silica gel 60 F254) were utilized for TLC analysis with compounds detected by UV illumination and heating plates after spraying with a 50% H_2SO_4 solution. Gas chromatography/mass spectrometry was performed on a Hewlett Packard 5890 Series II GC connected to a Hewlett Packard 5971 mass spectrometer. Reverse phase C-18 silica (Analytichem Bond Elut) was utilized for column chromatography. TLC-grade (10-40 μm) silica gel was used for vacuum chromatography. All solvents were glass distilled prior to use.

Collection, Extraction and Isolation. A collection of an assemblage of *Tolypothrix sp.* and *Lyngbya majuscula* was obtained from Playa Kalki, Curaçao in May 1996. The algal material (80 g dry weight) was preserved in isopropyl alcohol and stored at -20 °C. The defrosted algae were homogenized in CH_2Cl_2/MeOH (2:1) and filtered. The algal residue was extracted with warm CH_2Cl_2/MeOH (2:1; 3X) and the extracts combined. After reduction on a rotary evaporator and partitioning with CH_2Cl_2 and H_2O, the organic extract yielded 550 mg dark oil. Vacuum silica chromatography of 540 mg (Hex/EtOAc/MeOH

gradient) yielded several fractions containing UV-active, charring compounds (50% H_2SO_4) by TLC. These fractions were combined (30.5 mg) and further fractionated over reverse phase C-18 silica (H_2O/MeOH gradient), to give 7.3 mg of kalkipyrone (**23**) as a colorless oil. Another 8.5 mg of (**23**) was isolated from other fractions of the crude extract and used for bioassays and further experimentation.

Determination of the Absolute Stereochemistry at C14 of Kalkipyrone (23) An aliquot of kalkipyrone (0.2 mg) was reacted with 20 µl of a 1 µmol/µl (-)-menthyl chloroformate solution (benzene), in 120 µl dry benzene and 20 µl dry pyridine for 2 hr at RT. The sample was dried under nitrogen and the residue resuspended in 100% hexanes. The reaction products were purified utilizing a 1.2 cm x 1 cm NP silica column with an EtOAc/Hex gradient. The (-)-menthyl chloroformate products eluting with 20-40% EtOAc/Hex were suspended in CH_2Cl_2 and ozonized for 2 minutes at -8 °C followed by 10 minutes at RT. The reaction products were then treated with peracetic acid overnight at 50 °C. The reaction mixture was dried under nitrogen, resuspended in MeOH and the methyl ester generated with ethereal diazomethane. The resulting products were examined by GC-EIMS (11.5 m of HP Ultra-1, 100-210°C at 3.0° C per min., then isothermal for 5 min.) and compared with the corresponding standards; 14.81 min. *(S)* and 15.07 min. *(R)*.

Kalkipyrone (**23**). FTIR (film) υ 3374 (br. OH), 2960, 2925,1665, 1579, 1327, 1165 cm^{-1}, UV (EtOH) λ_{max} = 204 nm, ε = ca. 20 000; λ_{max} = 238 nm, ε = *ca.* 33,000. HR EIMS *m/z* obs. (rel. int., mmu dev.) $[M]^+$ 332.1993 (12.5, 0.6) $C_{20}H_{28}O_4$, $[M-CH_3]^+$ 317.1762 (25.1, 1.0), $[M-H_2O]^+$ 314.1907 (52.5, 2.5), $[M-C_2H_3O]^+$ 289.1811 (55.1, 0.7), $[M-C_6H_9O]^+$ 235.1334 (22.6, 0.0), $[M-C_7H_{11}O]^+$ 221.1181 (74.2, 0.3), $[M-C_7H_{12}O_2]^+$ 204.1160 (41.0, 1.0), $[M-C_8H_{15}O_2]^+$ 189.0923 (37.0, 0.7), $[M-C_{11}H_{16}O]^+$ 168.0788 (100.0, 0.1), [M-

$C_9H_{15}O_4]^+$ 145.0992 (62.8, -2.5); Optical rotation $[\alpha]_D^{20}$ = +8.2° (*c* = 0.22, $CHCl_3$) For ^1H and ^{13}C NMR data see Table III.1.

Brine Shrimp Bioassay. Brine shrimp *(Artemia salina)* eggs were added to a shallow rectangular pan filled with artificial sea water (Instant Ocean®, Aquarium Systems, Inc.) and incubated at 28 °C for 24 hours. The hatching chamber contained a plastic divider with numerous 2 mm holes and eggs were added to the half of the chamber that was kept in the dark. After 24 hours, the phototrophic nauplii were gathered from the illuminated side of the chamber and used for assays. About 15 brine shrimp in *ca.* 0.5 ml sea water were added to each well containing different concentrations of sample in 50 µl EtOH and 4.5 ml artificial sea water to make a total volume of *ca.* 5 ml. Samples and controls were run in duplicate. After 24 hours at 28 °C, the brine shrimp were observed and counted utilizing a dissecting light microscope. The percentage of live shrimp versus total shrimp was used to estimate LD_{50} values.

Ichthyotoxicity Assay. Dilutions of samples in 40 µl EtOH were put into a 50 ml beaker containing 40 ml distilled water and mixed thoroughly. A single goldfish *(Carassius auratus)* was added and observed for one hour. Samples and controls were run in duplicate. End point was established as death and used for estimate LD values.

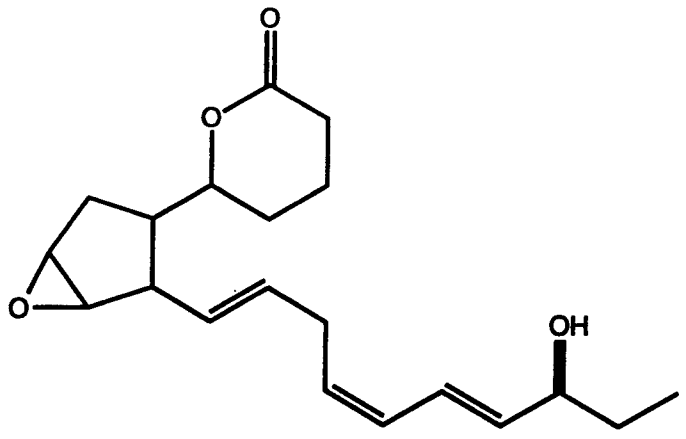
GC-EIMS Analysis of Cultured L. majuscula Cells. Two laboratory cultures of isolated cells of the *L. majuscula* were extracted with CH_2Cl_2/MeOH (2:1, 3X), dried under nitrogen, resuspended in 10 % (v/v) EtOAc/Hexanes, filtered, and analyzed by GC-EIMS(11.5 m of HP Ultra-1, 70-250°C at 15.0 °C per min., then isothermal for 15 min.). Peaks indicative of kalkipyrone (t_R = 12.29, 12.97 min.; *m/z* obs.; 332, 314, 189, 221, 168, 145) were not apparent in the cultured specimens.

CHAPTER IV
CONCLUSION

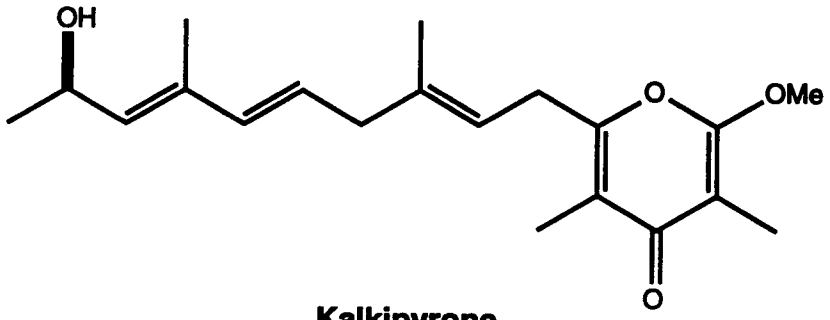
Intense grazing on marine algae by fish, sea urchins, gastropods, and Crustacea is a significant factor that determines abundance and distribution of the algae. Studies have shown that an annual average of 97% of the algal turf production is removed by herbivores in some locations.[182] Like other sessile organisms, the necessity for protective mechanisms is imperative for survial. A commonly employed strategy is chemical deterrence. Therefore, it is not surprising that marine algae are known as a rich source of structurally novel and bioactive compounds. My investigations of several genera of algae have led to the isolation of two metabolites, agardhilactone and kalkipyrone (Figure IV.1). Although these compounds may play a vital role in the survival of the organisms or species, their ecological significance has not been investigated in this thesis.

Agardhilactone, a novel tricyclic oxylipin isolated from the marine red alga *Agardhiella subulata*, represents a new structure class of oxylipins. The significance of this compound as a secondary metabolite has not been determined. Due to the small yield of isolated compound, this sample has not been tested for biological activity. Oxylipins play integral roles in mammalian systems as well as intraspecies interactions of plants and animals. Further investigations with agardhilactone could possibly identify its biochemical role and pharmacological significance in the marine environment.

Kalkipyrone, a γ-pyrone linked to an alkyl chain, was isolated from an assemblage of the cyanobacteria *Lyngbya majuscula* and *Tolypothrix sp.* Kalkipyrone displays toxicity to brine shrimp and gold fish. This metabolite may function in nature to protect the alga from predation by Crustacea and



Agardhilactone



Kalkipyrone

Figure IV.1 Metabolites from Temperate and Tropical Marine Algae

herbivorous fish. These possible predator-prey interactions should encourage additional investigations into the ecological relationships of these cyanobacteria and the other organisms of the surrounding marine environment.

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APPENDICES

APPENDIX A

THE ISOLATION OF STYPOLDIONE AND ITS DERIVATION TO STYPOTRIOL TRIACETATE FOR PHARMACEUTICAL EVALUATION

Abstract

The brown alga *Stypopodium zonale* is a source of numerous bioactive metabolites. The isolation of the previously reported compound, stypoldione and its derivation to stypotriol triacetate in our laboratory has lead to pharmaceutical evaluation of these two compounds in murine *in vivo* antitumor assays.

Introduction

The tropical brown alga *Stypopodium zonale* (Dictyotaceae, Phaeophyceae) produces a suite of structurally novel and bioactive metabolites. Previous work by Gerwick and Fenical led to the isolation of a majority of these compounds including stypoldione (**1**), stypotriol, stypodiol, epistypodiol, epitaondiol, 2-(geranylgeranyl)-5-methyl-1,4-benzohydroquinone, 2-(geranylgeranyl)-5-methyl-1,4-benzoquinone as well as the previously reported metabolites, taondiol and atomaric acid.[1,2] In particular, the diterpenoids, stypoldione and stypotriol, have shown potent ichthyotoxicity to reef dwelling fish. Initial hyperactivity of the fish is followed by narcosis and death. Subsequent testing of stypoldione indicated antitumoral activity as well as inhibition of synchronous cell division in a fertilized sea urchin egg assay (ED_{50} = 1.1 μg/ml), through inhibition of microtubule polymerization.[1] The isolation of stypoldione (**1**) and its derivation to stypotriol triacetate (**2**, Figure A.1) in our laboratory has lead to pharmaceutical evaluation of these two compounds in *in vivo* murine antitumor assays (Figure A.1)

Results and Discussion

The rapid air oxidation of the natural product stypotriol yielded the bright red ortho quinone, stypoldione, which was stable in the crude extract. Subsequently, the lipid extract of several collections of *Stypopodium zonale* were combined (57.4 g) and oxidized with AgO for 24 hr RT. Vacuum liquid chromatography (Hex/EtOAc/MeOH gradient) of an aliquot (30.7 g) of the resulting reaction mixture yielded several fractions of nearly

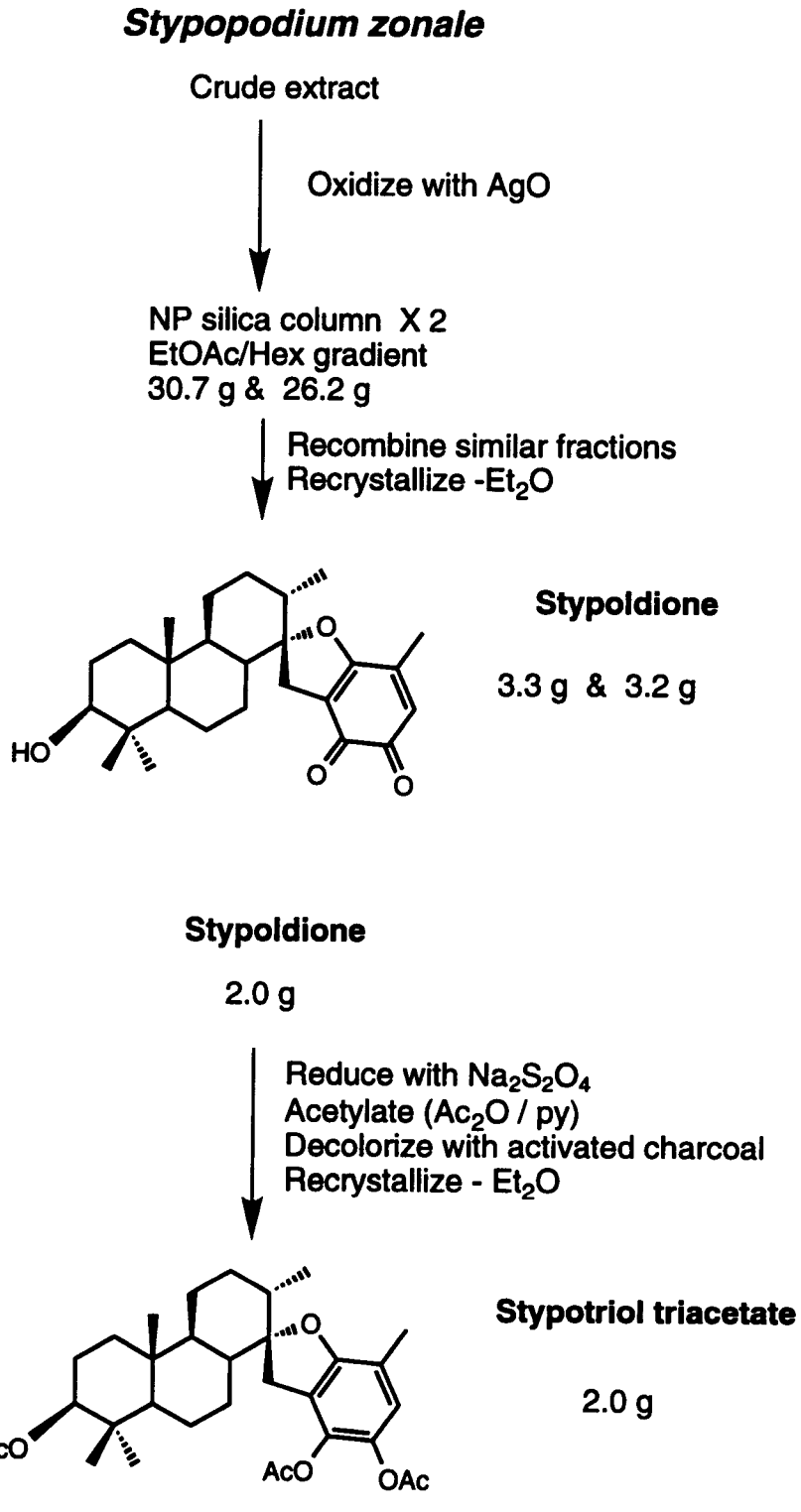

Figure A.1 Isolation Scheme of Stypoldione (**1**) and its Reduction and Acetylation to Stypotriol Triacetate (**2**)

pure stypoldione (**1**) which formed bright red crystals (3.4 g) upon recrystallization with diethyl ether. An additional 26.2 g of the oxidized crude extract afforded 3.2 g stypoldione. Crude stypotriol triacetate (**2**) was obtained from reduction of stypoldione (2.0 g) with $Na_2S_2O_4$ followed by acetylation (Ac_2O / py, RT,15 hr). The resulting reaction mixture was extracted with diethyl ether and the organic layer was decolorized with activated charcoal. Recrystallization from diethyl ether yielded stypotriol triacetate (2.0 g) as a white powder.

The structures of stypoldione and stypotriol triacetate were confirmed by ^1H NMR comparison of the pure compounds to the compounds previously reported.[1,2] Samples of stypoldione (2.0 g) and stypotriol triacetate (2.0 g) were submitted for murine *in vivo* antitumor assays.

Experimental

General. Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker AM-400 and AC-300 spectrometers in $CDCl_3$. Proton spectra were referenced to internal tetramethylsilane (0.0 ppm) in $CDCl_3$. Merck aluminum-backed thin layer chromatography (TLC) sheets (silica gel 60 F254) were utilized for TLC analysis with compounds detected by visual observation, UV illumination and spraying with 50% H_2SO_4 solution. TLC-grade (10-40 mm) silica gel was used for vacuum chromatography. All solvents were glass distilled prior to use.

Collection and Isolation The mixture of lipid extracts (93.5 g) of *Stypopodium zonale* included algal material collected from Vega Baja, Puerto Rico in March 1984, and Belize in 1985 as "Curlew Lab" and "South Water Cut." A portion of the crude extracts (57.4 g) was suspended in diethyl ether

and oxidized with AgO (7.4 g) for 24 hr RT. Vacuum liquid chromatography (Hex/EtOAc/MeOH gradient) of an aliquot (30.7 g) of the resulting reaction mixture yielded several fractions of nearly pure stypoldione which formed bright red crystals (3.3 g) upon recrystallization from diethyl ether. An additional 26.2 g of the oxidized crude extract afforded 3.2 g stypoldione.

Reduction of Stypoldione. An aqueous solution (50 ml) of $Na_2S_2O_4$ (2.4 g) was added to a stirred solution of stypoldione (2.0 g) in 150 ml EtOH. The red solution immediately turned milky white and after 2 min. at RT, 30 ml of 1 N HCl was added. The resulting reaction mixture was extracted with Et_2O (3 x 50 ml). The combined ether extracts were reduced to yield light green crystals of crude stypotriol.

Acetylation of Stypotriol. The crude stypotriol generated above was combined with 10 ml of pyridine and 10 ml acetic anhydride and stirred at RT. After 15 hr, the reaction was quenched with 25 ml ice followed by 50 ml of H_2O and extracted with Et_2O (3 x 300 ml). The combined Et_2O extracts were washed with 1 N HCl (3 x 15 ml), neutralized with a saturated $NaHCO_3$ solution (3 x 15 ml) and evaporated to dryness. The gummy residue was resuspended in Et_2O and hexanes and shaken with decolorizing activated charcoal. Filtration of the resulting mixture followed by recrystallization from Et_2O yielded stypotriol triacetate (2.0 g, 77%) as white crystals.

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2. Gerwick, W. H.; Fenical, W.; Fritsch, N.; Clardy, J. *Tetrahedron Lett.* **1979**, 145-148.

APPENDIX B

THE ISOLATION OF MALYNGAMIDE C ACETATE FROM THE CYANOBACTERIUM *LYNGBYA MAJUSCULA* FOR PHARMACEUTICAL EVALUATION

Abstract

The cyanobacterium *Lyngbya majuscula* has produced numerous bioactive metabolites. My isolation of the previously reported compound, malyngamide C acetate has lead to its submission to the National Cancer Institute 60 human tumor cell line panel as a potential anticancer drug lead.

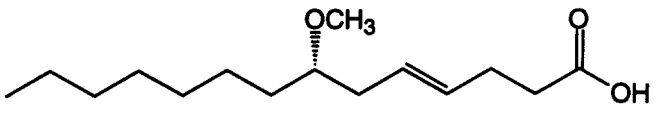
Introduction

The marine cyanobacteria *Lyngbya majuscula* (Nostocales, Oscillatoriaceae) has produced numerous structurally novel and bioactive metabolites, some of which were discussed in chapter three. In particular, several amides of (-)-*trans*-7*(S)*-methoxytetradec-4*(E)*-enoic acid (**1**) named malyngamides A (**2**) and B (**3**) were isolated in the 1970's (Figure B.1).[1,2] They represent the first members of an extended series of malyngamides. Malyngamide C (**4**) and its acetate derivative (**5**) were isolated by Moore and coworkers from a shallow water collection of *Lyngbya majuscula* from Fanning Island.[1,3] My isolation of malyngamide C acetate (**5**) has lead to pharmaceutical evaluation of this compound in the National Cancer Institute (NCI) 60 human tumor cell line panel as a potential anticancer drug lead.

Results and Discussion

A sample of *Lyngbya majuscula* was collected from Blue Inn, Grenada in July 1995. This sample was preserved in isopropyl alcohol and stored at -20 °C. The defrosted alga was repeatedly extracted for its lipid metabolites with CH_2Cl_2/MeOH (2:1) and the resulting solution partitioned between H_2O and CH_2Cl_2. The organic layer was concentrated *in vacuo* to produce a dark oil (434 mg).

Size exclusion chromatography (Sephadex LH-20, EtOAc/MeOH) of 330 mg of the crude extract followed by reverse phase C-18 silica chromatography (MeOH/H_2O) and NP silica column chromatography yielded 37.4 mg of malyngamide C acetate (**5**).



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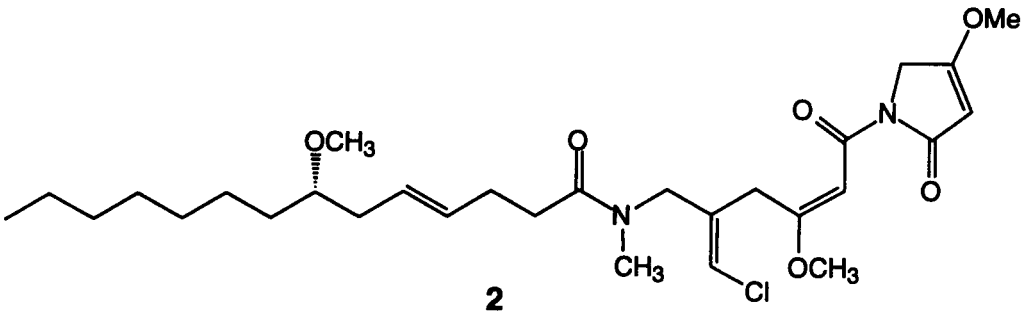

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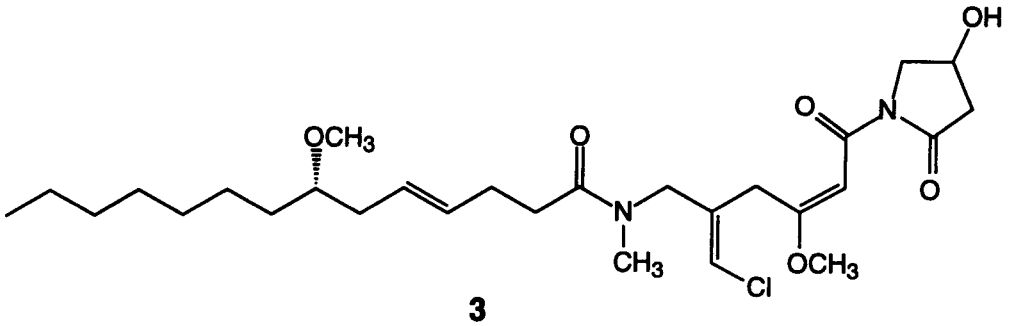

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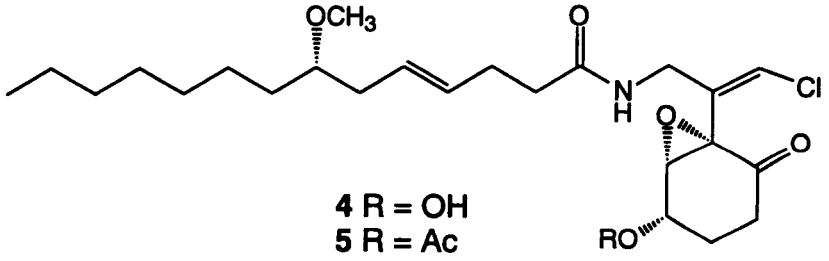

4 R = OH
5 R = Ac

Figure B.1 Several Malyngamides from *Lyngbya majuscula*

The structure of malyngamide C acetate was confirmed by ^{1}H and ^{13}C NMR comparison of the pure compound with previously reported data.[3] A sample of malyngamide C acetate (10 mg) was submitted for antitumor testing at NCI.

Experimental

General. Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker AM-400 and AC-300 spectrometers in $CDCl_3$. Proton spectra were referenced to internal tetramethylsilane (0.0 ppm) in $CDCl_3$. Carbon spectra were referenced to the centerline of $CDCl_3$ at 77.0 ppm. Merck aluminum-backed thin layer chromatography (TLC) sheets (silica gel 60 F254) were utilized for TLC analysis with compounds detected by UV illumination and heating plates after spraying with 50% H_2SO_4 solution. Sephadex LH-20 was utilized for size exclusion chromatography. Silica gel (60-200 mesh) and reverse phase C-18 silica (Analytichem Bond Elut) were utilized for column chromatography. All solvents were glass distilled prior to use.

Collection, Extraction and Isolation. A collection of *Lyngbya majuscula* was obtained from Blue Inn, Grenada in July 1995. The algal material (19 g dry weight) was preserved in isopropyl alcohol and stored at -20 °C. The defrosted algae were homogenized in CH_2Cl_2/MeOH (2:1) and filtered. The algal residue was extracted with warm CH_2Cl_2/MeOH (2:1; 3 x) and the extracts combined. After reduction on a rotary evaporator and partitioning with CH_2Cl_2 and H_2O, the organic extract yielded 434 mg dark oil. Size exclusion chromatography of 330 mg (Sephadex, EtOAc/MeOH) yielded several fractions containing UV-active, and/or charring compounds (50% H_2SO_4) by TLC. These fractions were combined (124.7 mg) and further fractionated over reverse

phase C-18 silica (H_2O/MeOH, 2-100%), and NP silica column chromatography (85% EtOAc/Hex) to yield 37.4 mg malyngamide C acetate (**5**).

References

1. Cardellina II, J. H.; Dalietos, D.; Marner, F.-J.; Mynderse, J. S.; Moore, R. E. *Phytochemistry* **1978**, *17*, 2091-2095.

2. Cardellina II, J. H.; Marner, F.-J.; Moore, R. E. *J. Am. Chem. Soc.* **1979**, *101*, 240-242.

3. Ainslie, R. D.; Barchi, J. J.; Kuniyoshi, M.; Moore, R. E.; Mynderse, J. S. *J. Org. Chem.* **1985**, *50*, 2859-2862.

APPENDIX C

SPECTRAL DATA

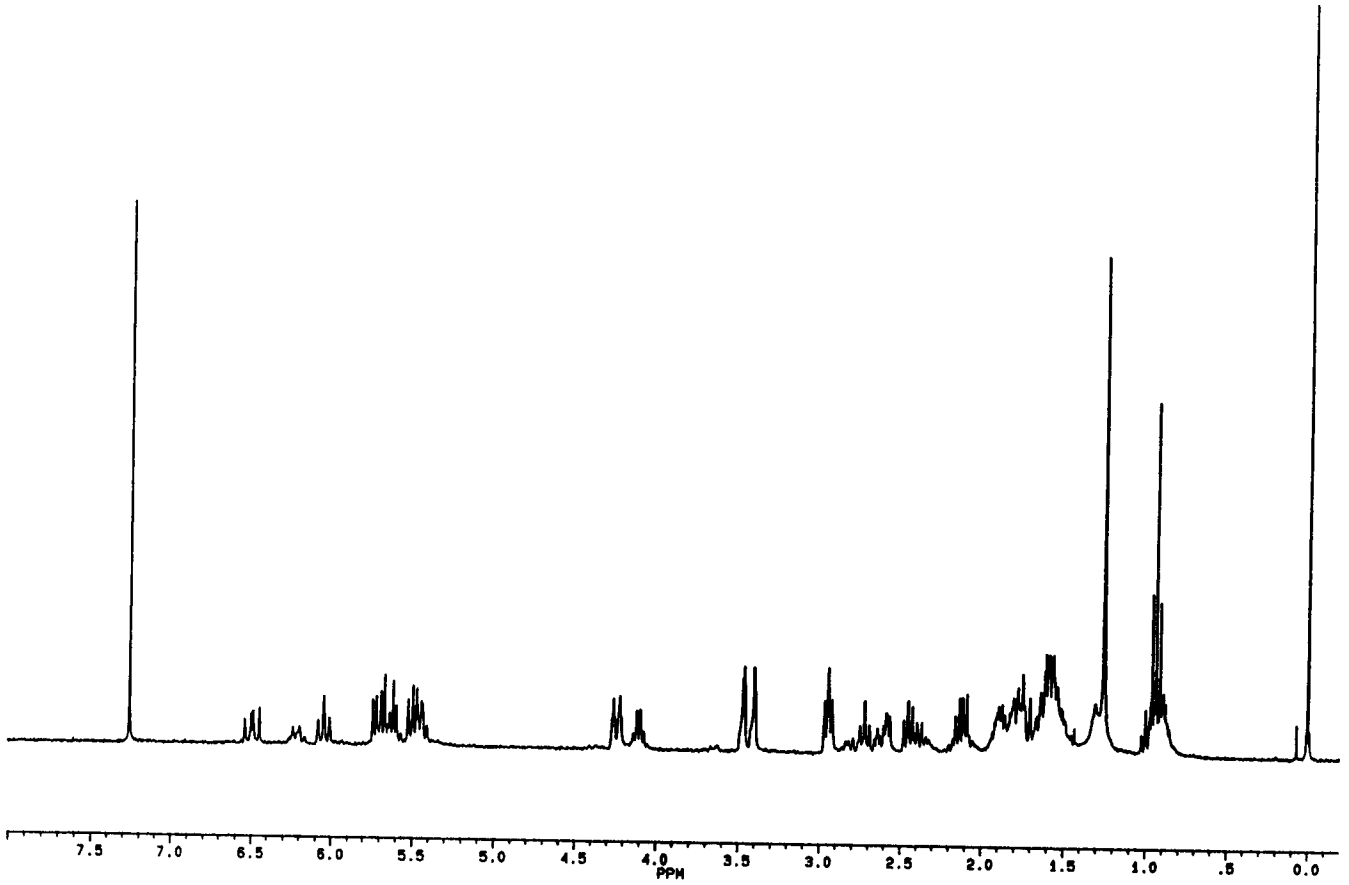

Figure C.1 ^{1}H NMR Spectrum of Agardhilactone (**3**) in $CDCl_3$ (300 MHz)

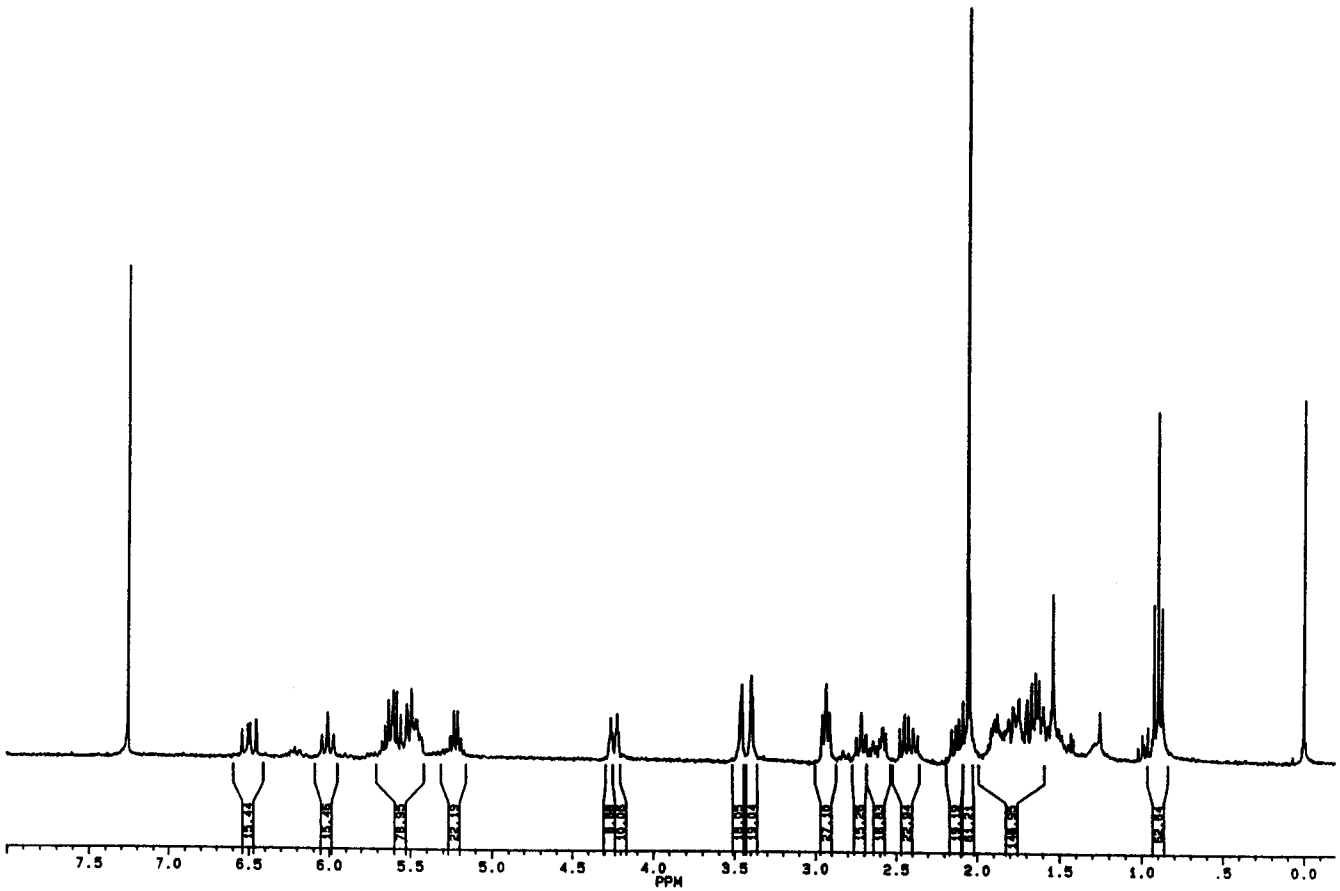

Figure C.2 ^{1}H NMR Spectrum of Agardhilactone Acetate (**4**) in $CDCl_3$ (300 MHz)

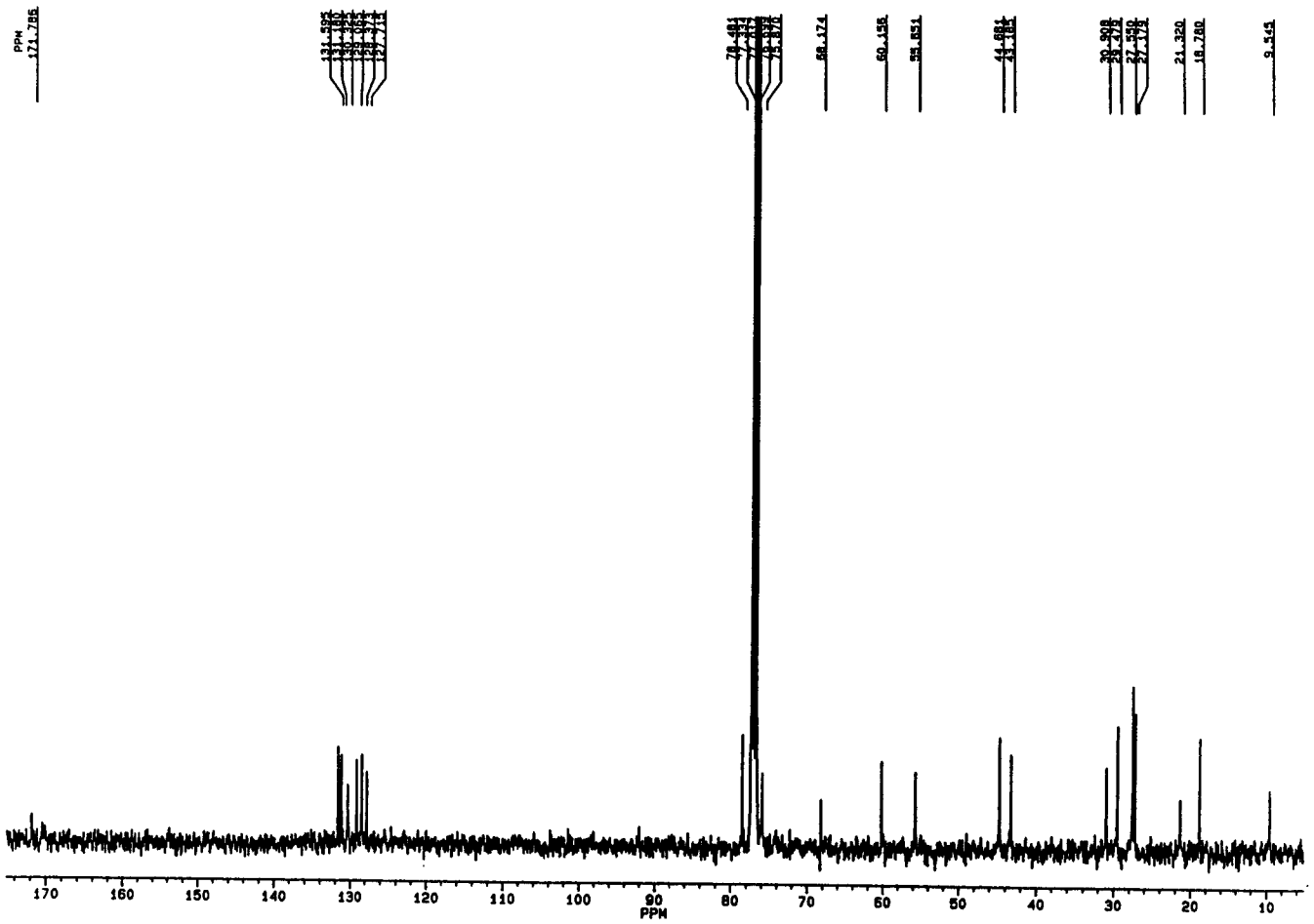

Figure C.3 ^{13}C NMR Spectrum of Agardhilactone Acetate (4) in $CDCl_3$ (100 MHz)

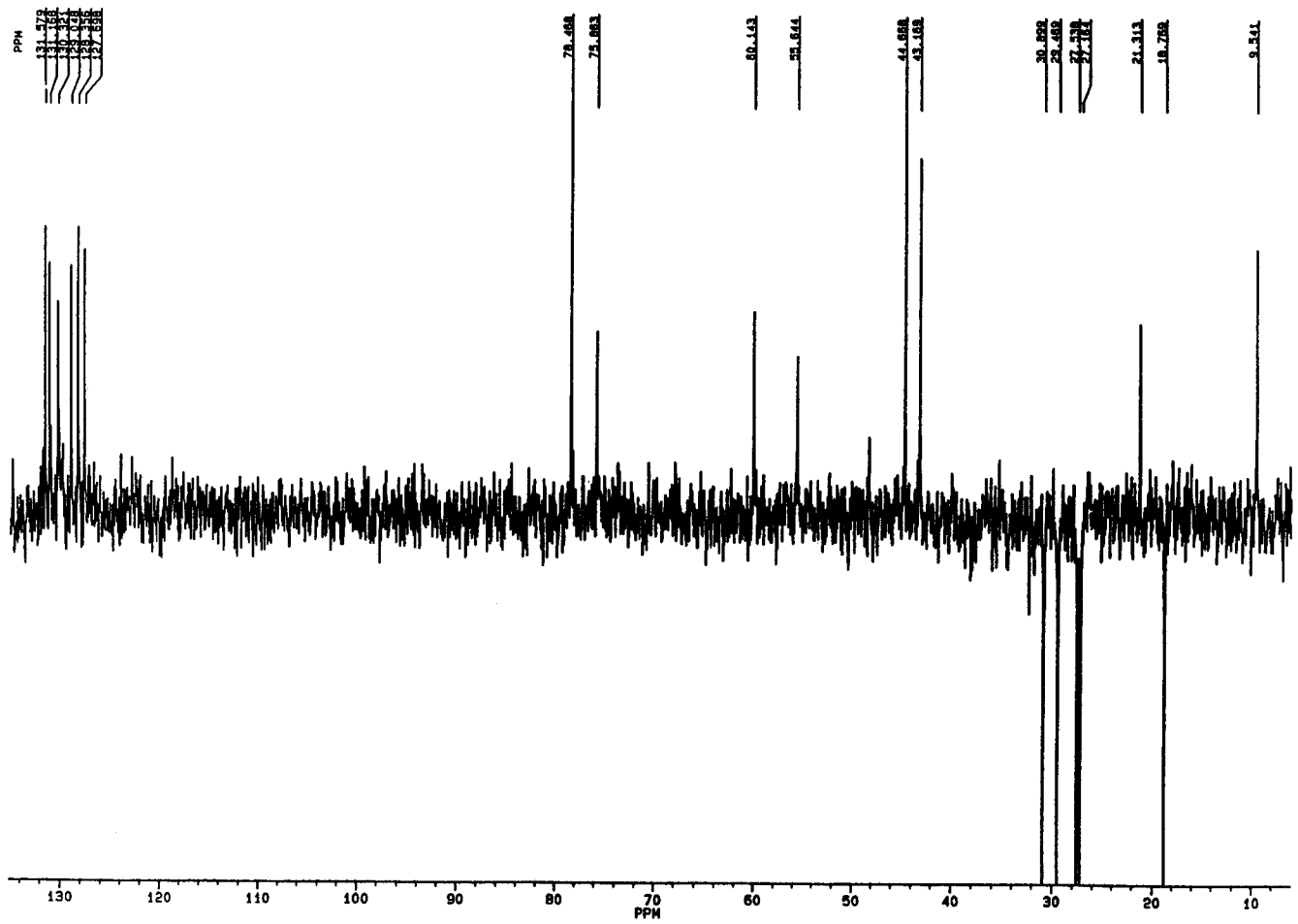

Figure C.4 DEPT 135 Spectrum of Agardhilactone Acetate (**4**) in $CDCl_3$ (100 MHz)

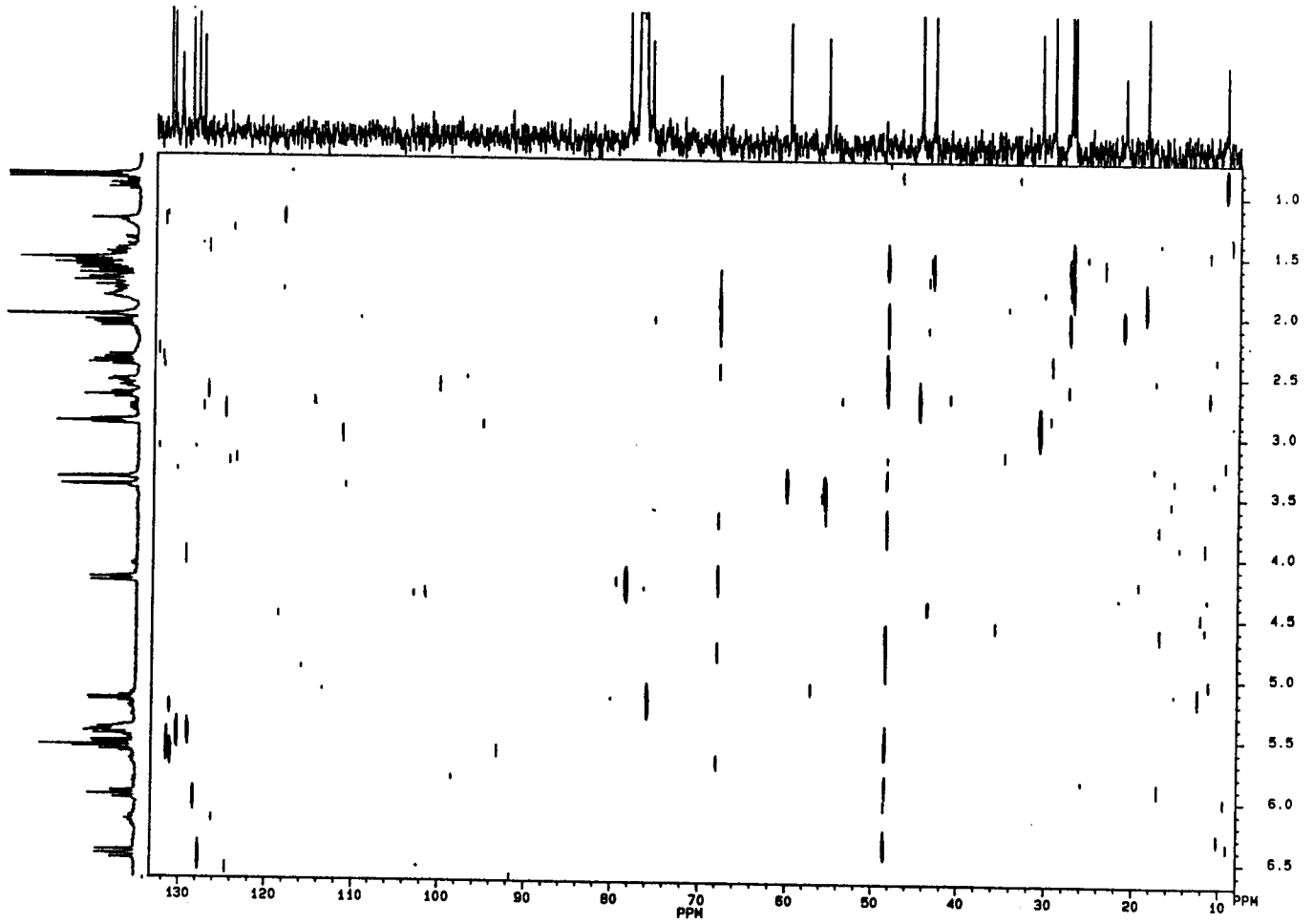

Figure C.5 XHCORR Spectrum of Agardhilactone Acetate (4) in $CDCl_3$ (100 MHz)

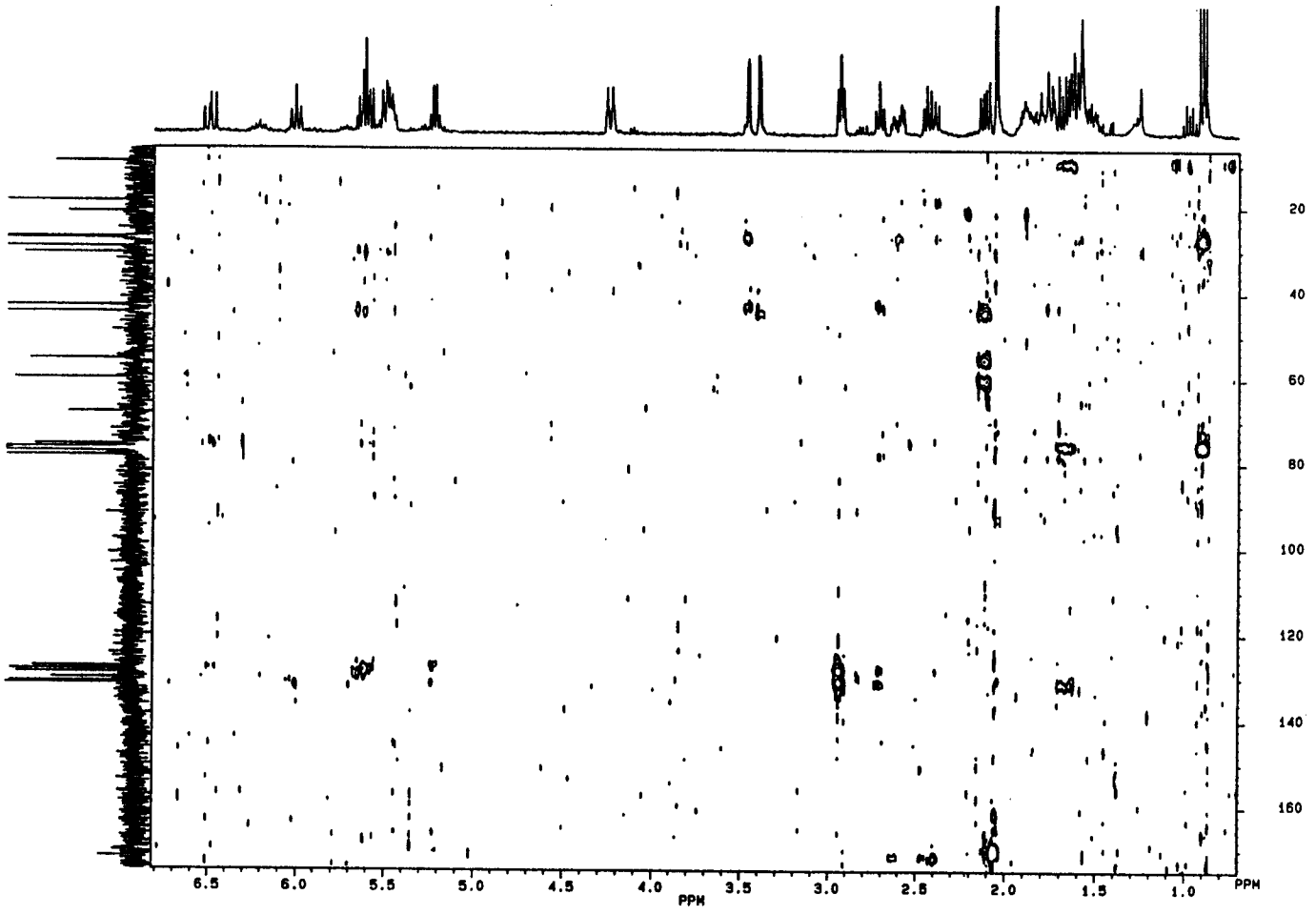

Figure C.6 HMBC Spectrum of Agardhilactone Acetate (4) in $CDCl_3$ (400 MHz)

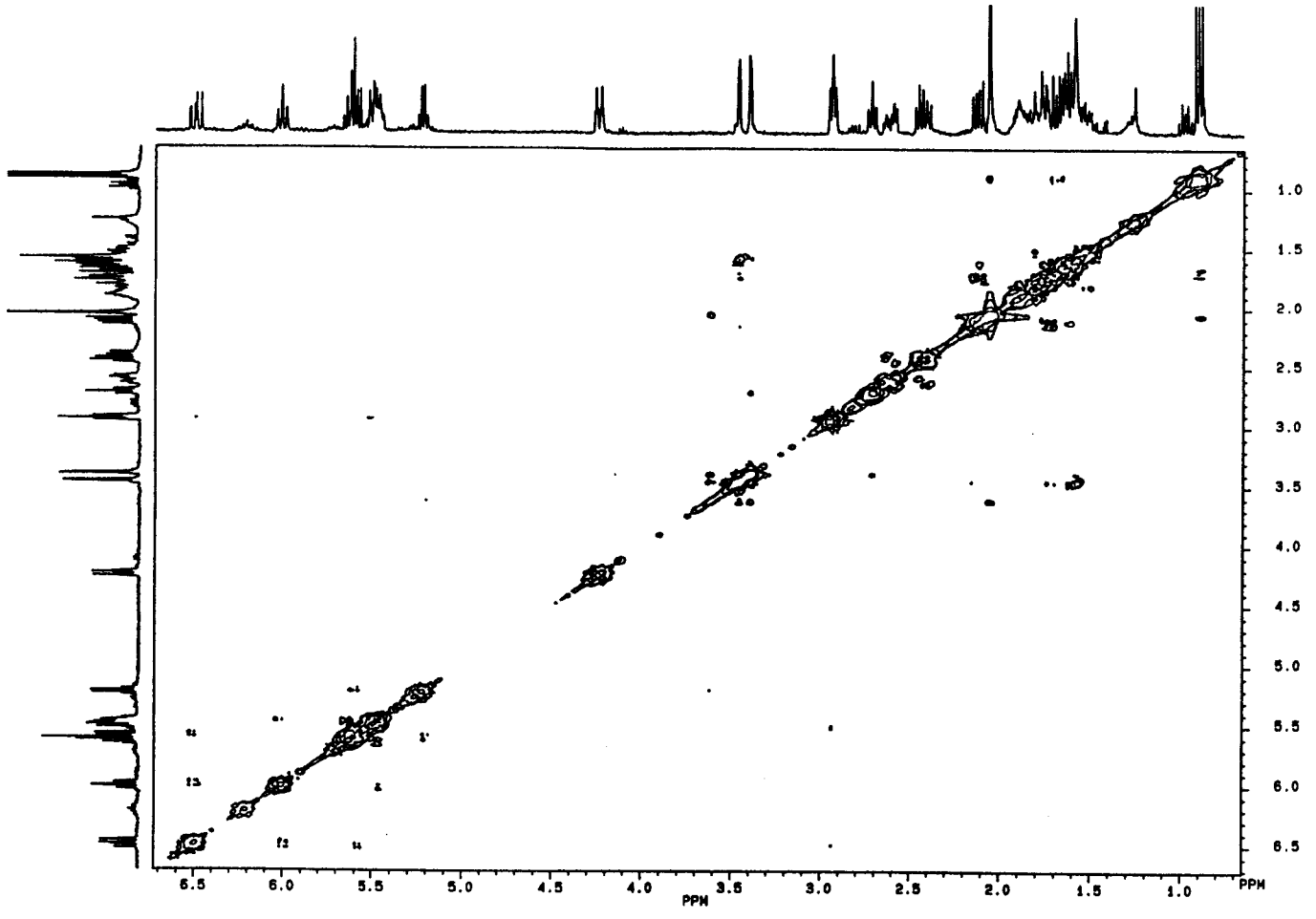

Figure C.7 NOESY Spectrum of Agardhilactone Acetate (4) in $CDCl_3$ (400 MHz)

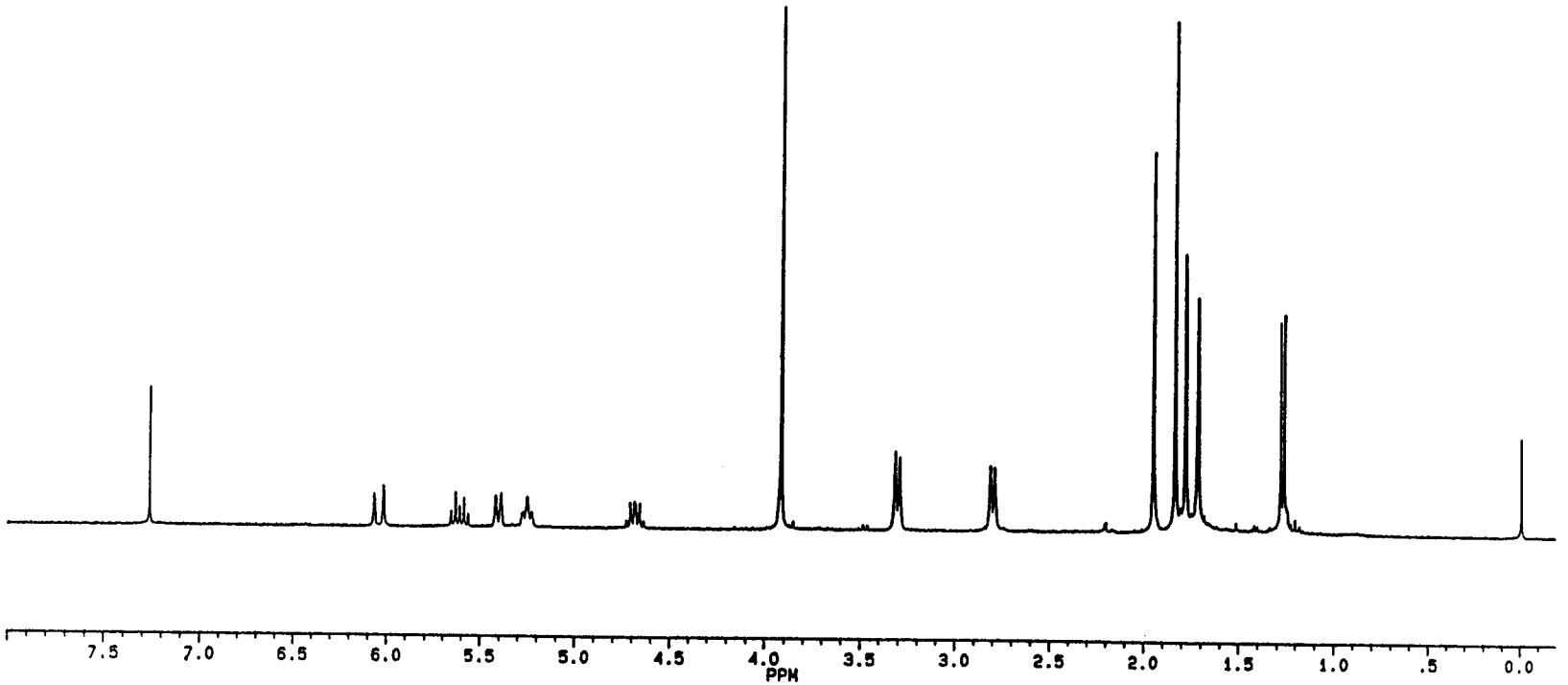

Figure C.8 ^{1}H NMR Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (300 MHz)

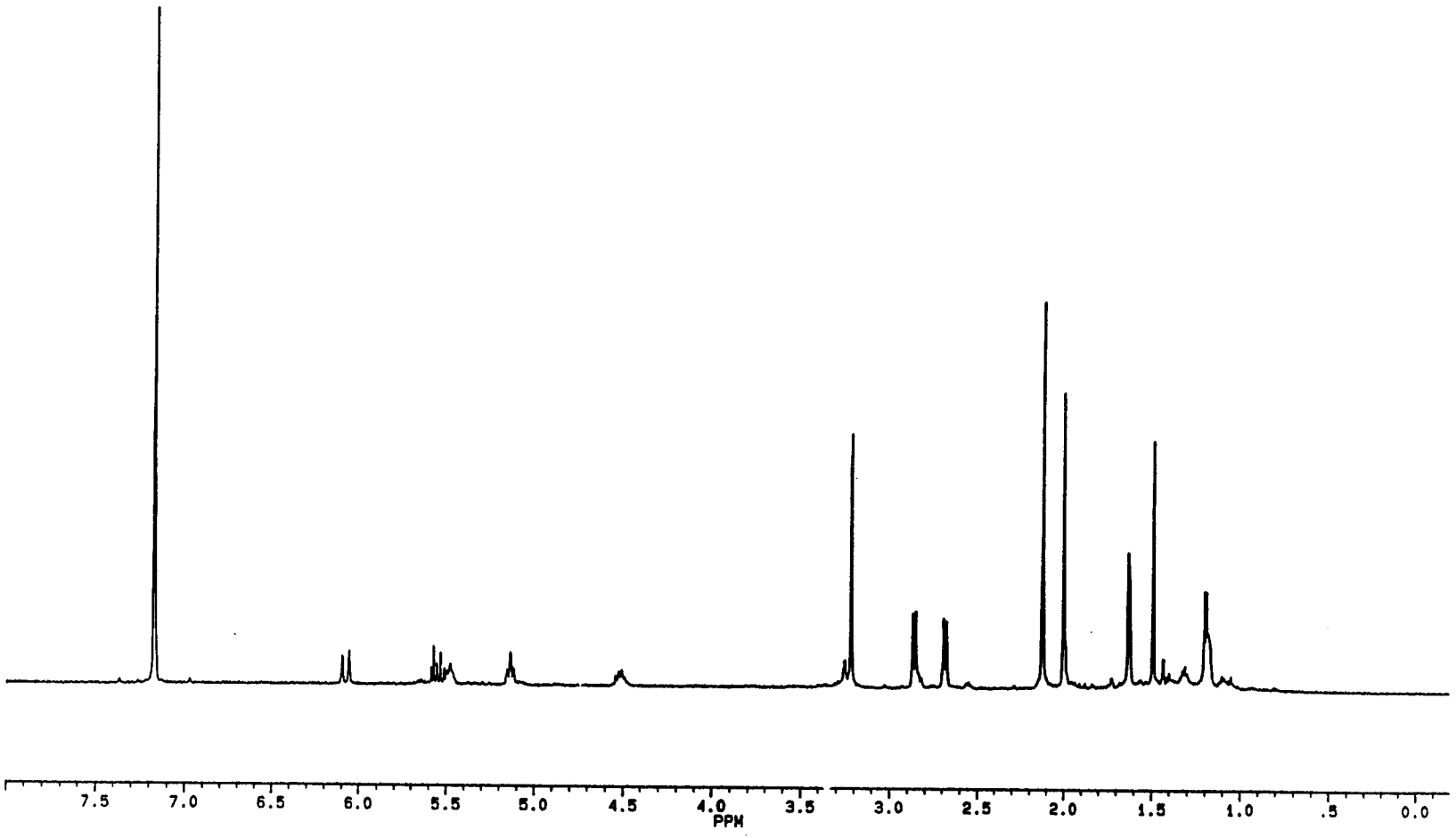

Figure C.9 ^{1}H NMR Spectrum of Kalkipyrone (**23**) in C_6D_6 (400 MHz)

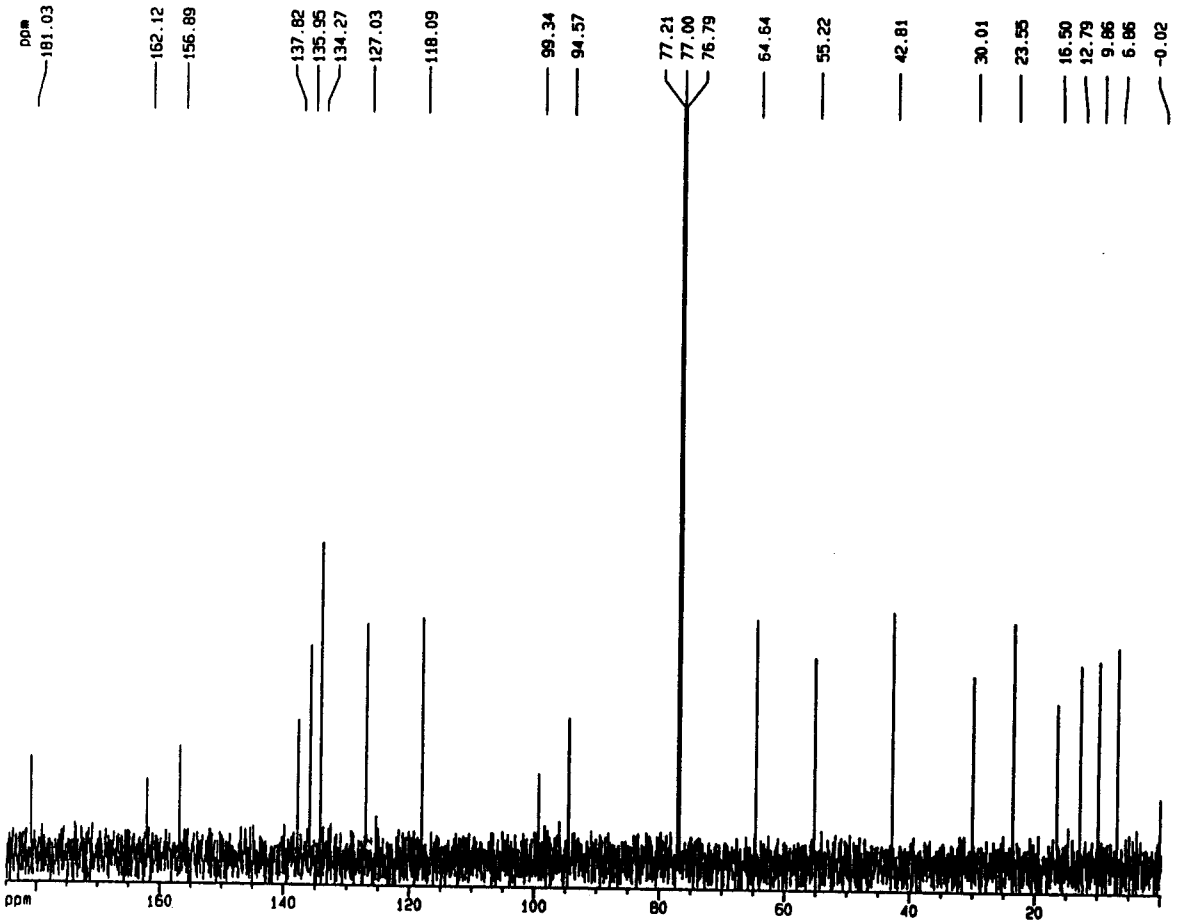

Figure C.10 ^{13}C NMR Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (75 MHz)

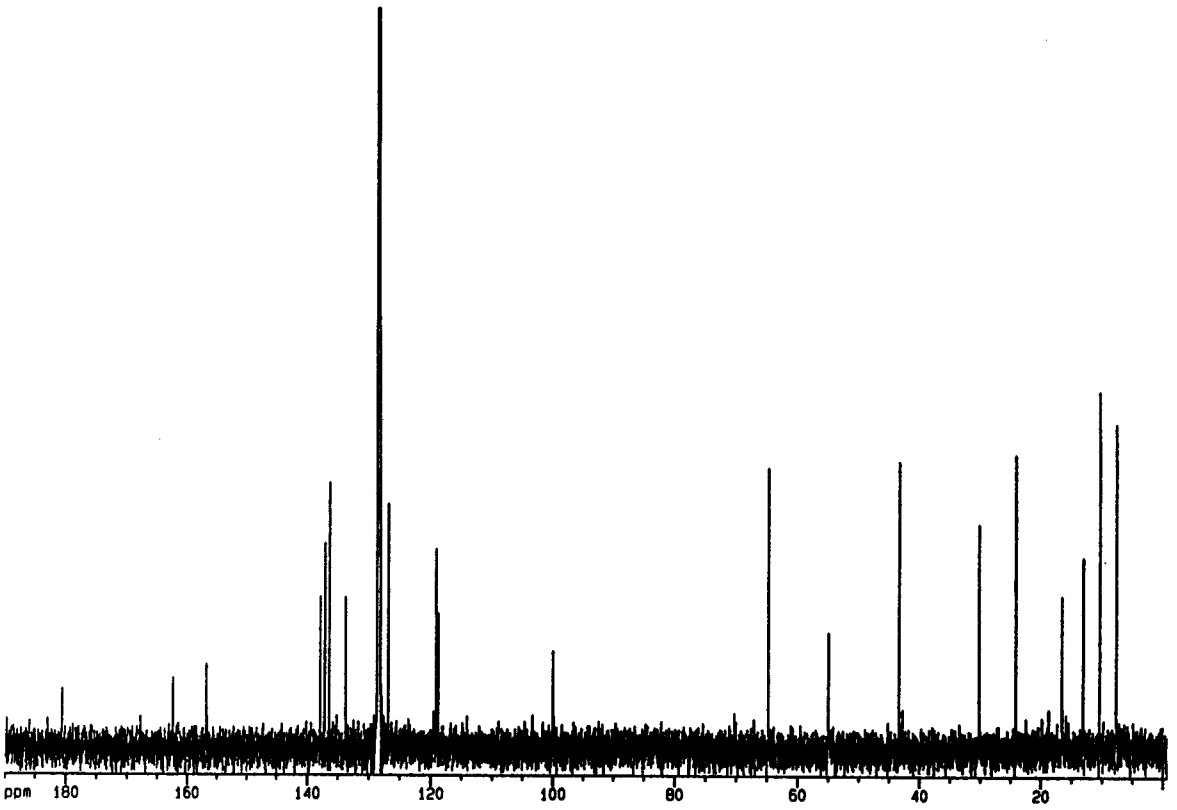

Figure C.11 ^{13}C NMR Spectrum of Kalkipyrone (**23**) in C_6D_6 (150 MHz)

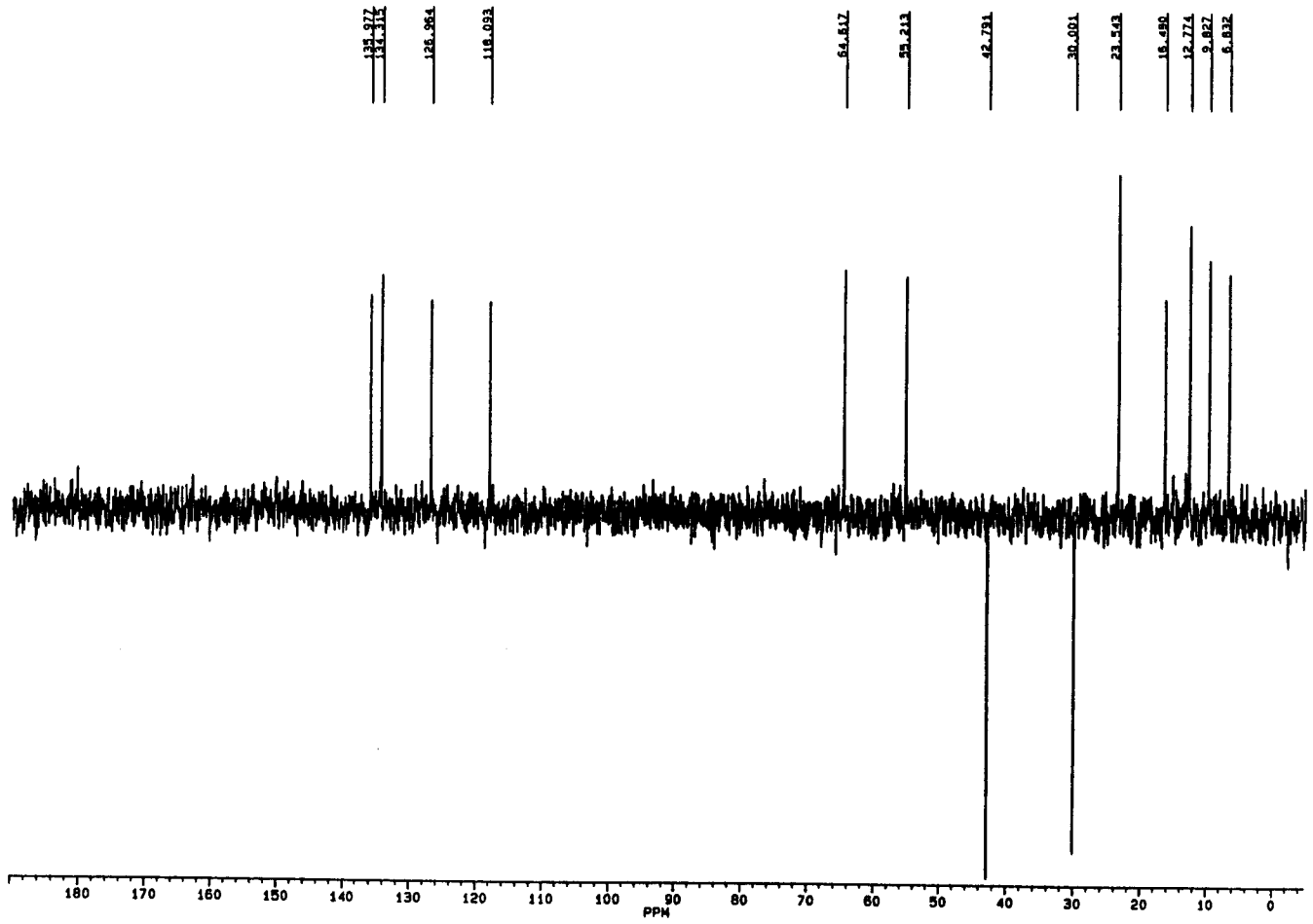

Figure C.12 DEPT 135 Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (100 MHz)

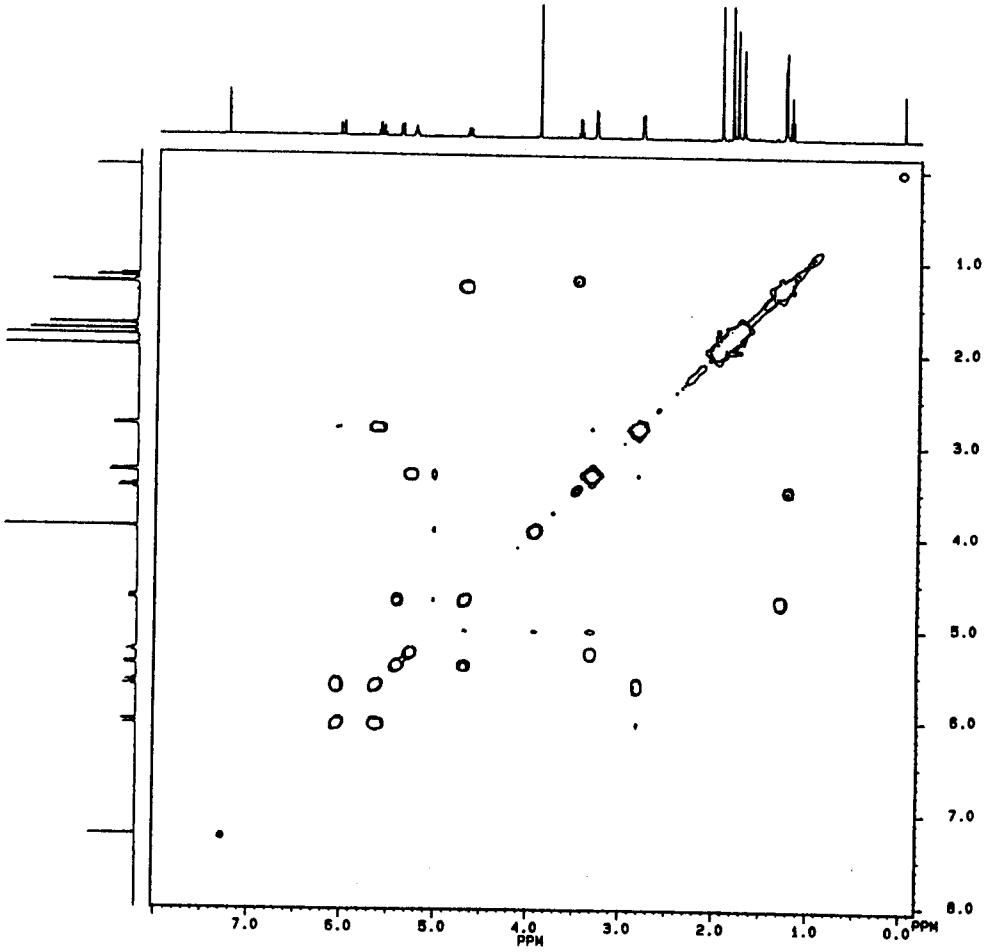

Figure C.13 COSY Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (400 MHz)

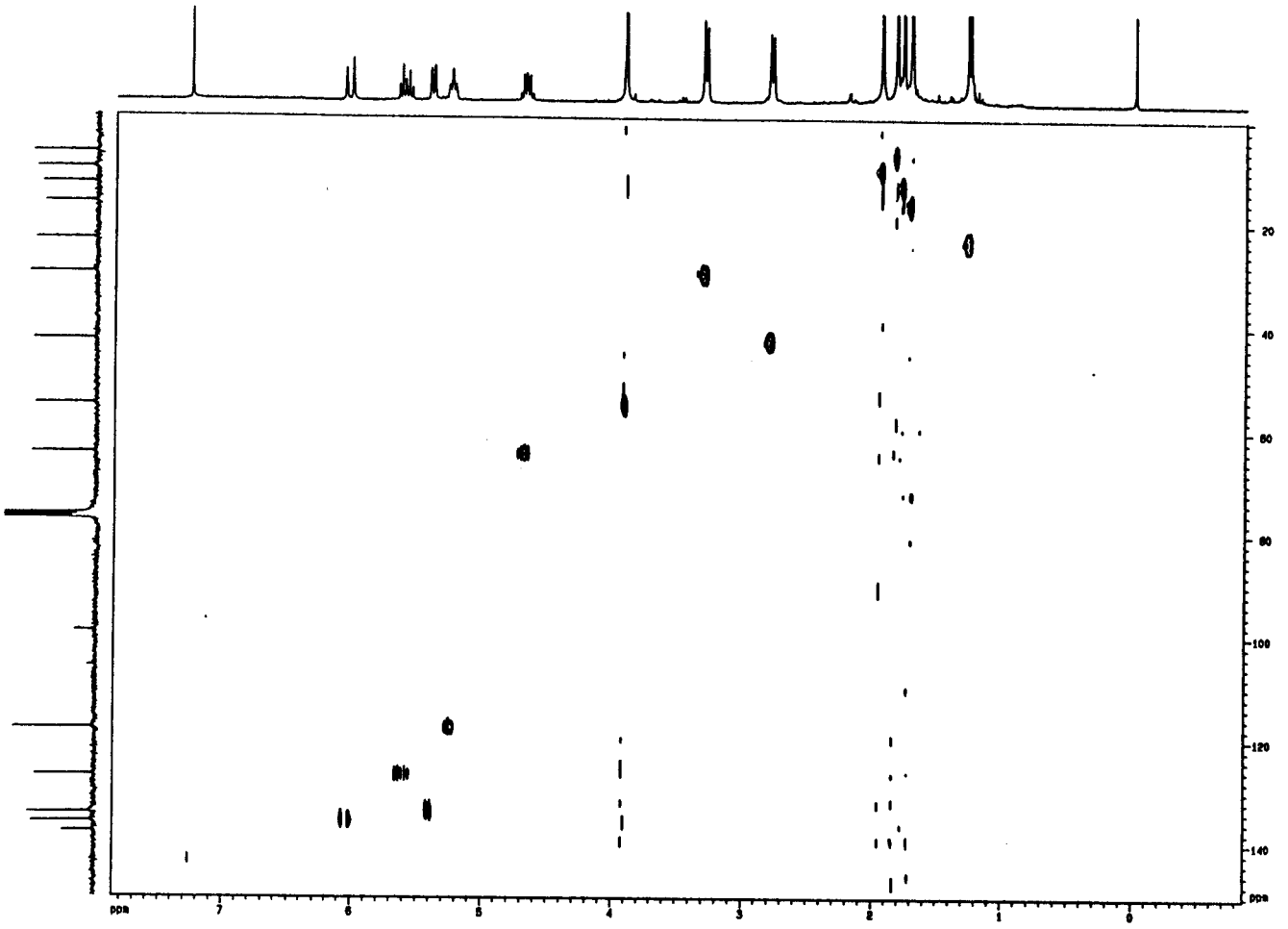

Figure C.14 HMQC Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (300 MHz)

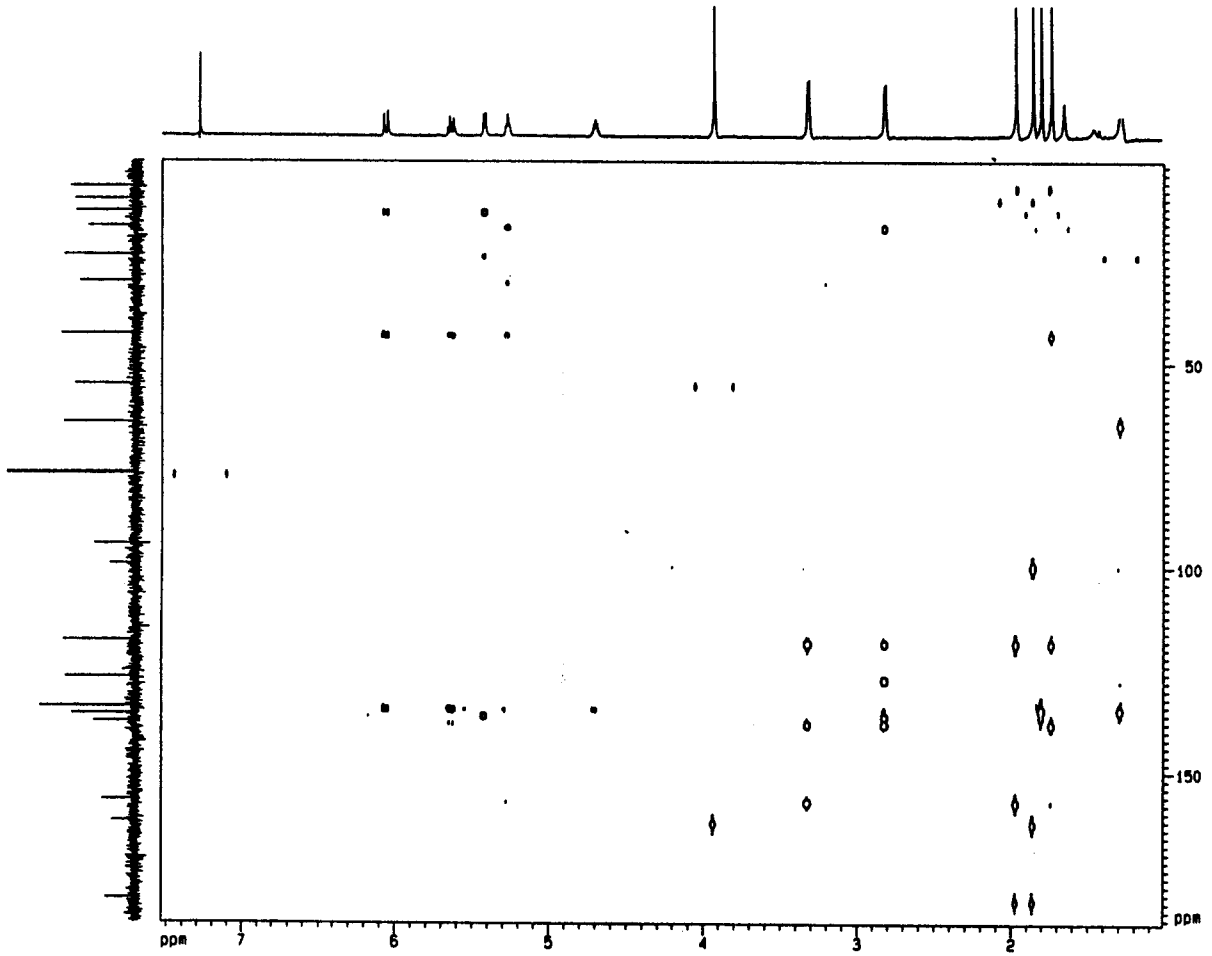

Figure C.15 HMBC Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (600 MHz)

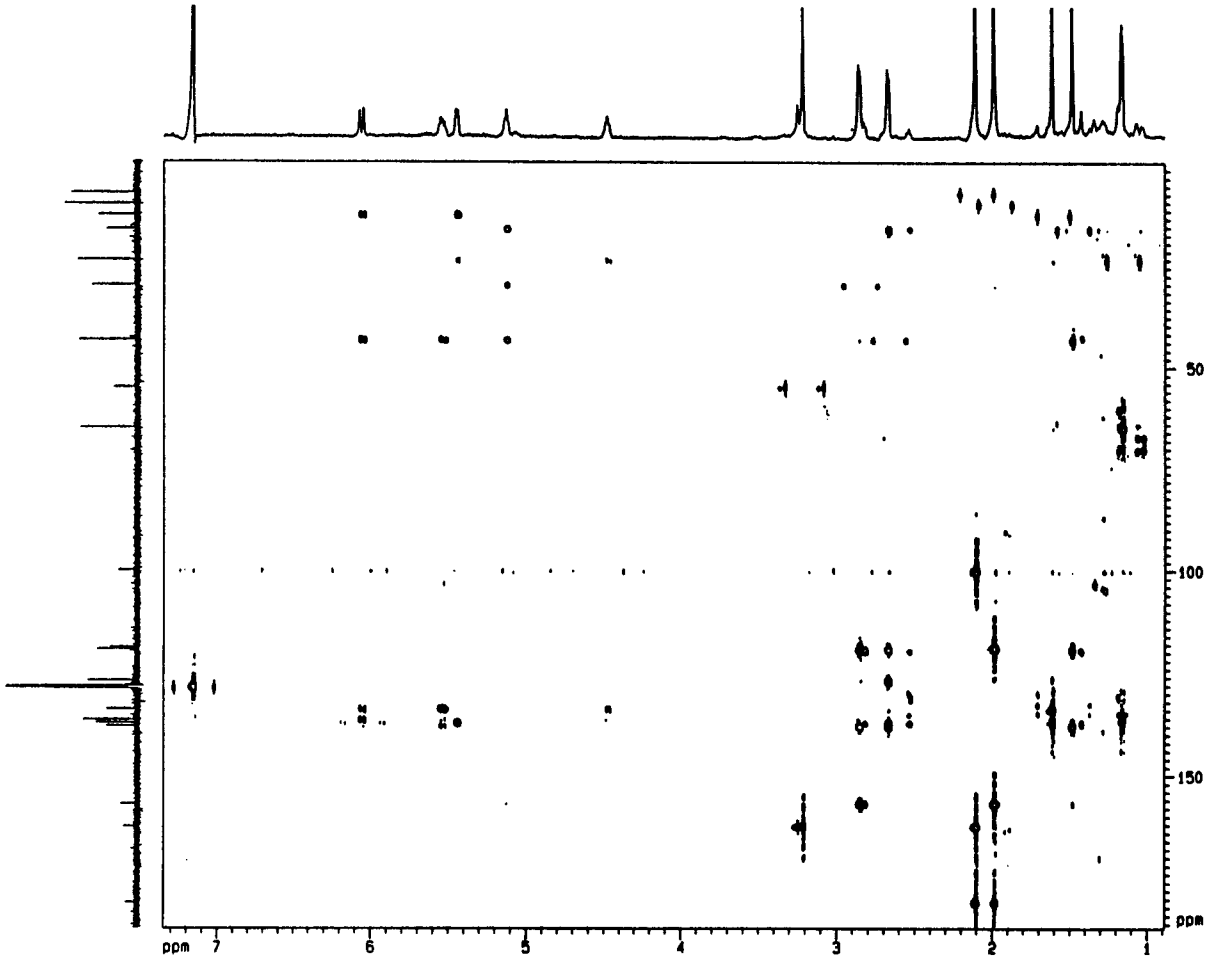

Figure C.16 HMBC Spectrum of Kalkipyrone (**23**) in C_6D_6 (600 MHz)

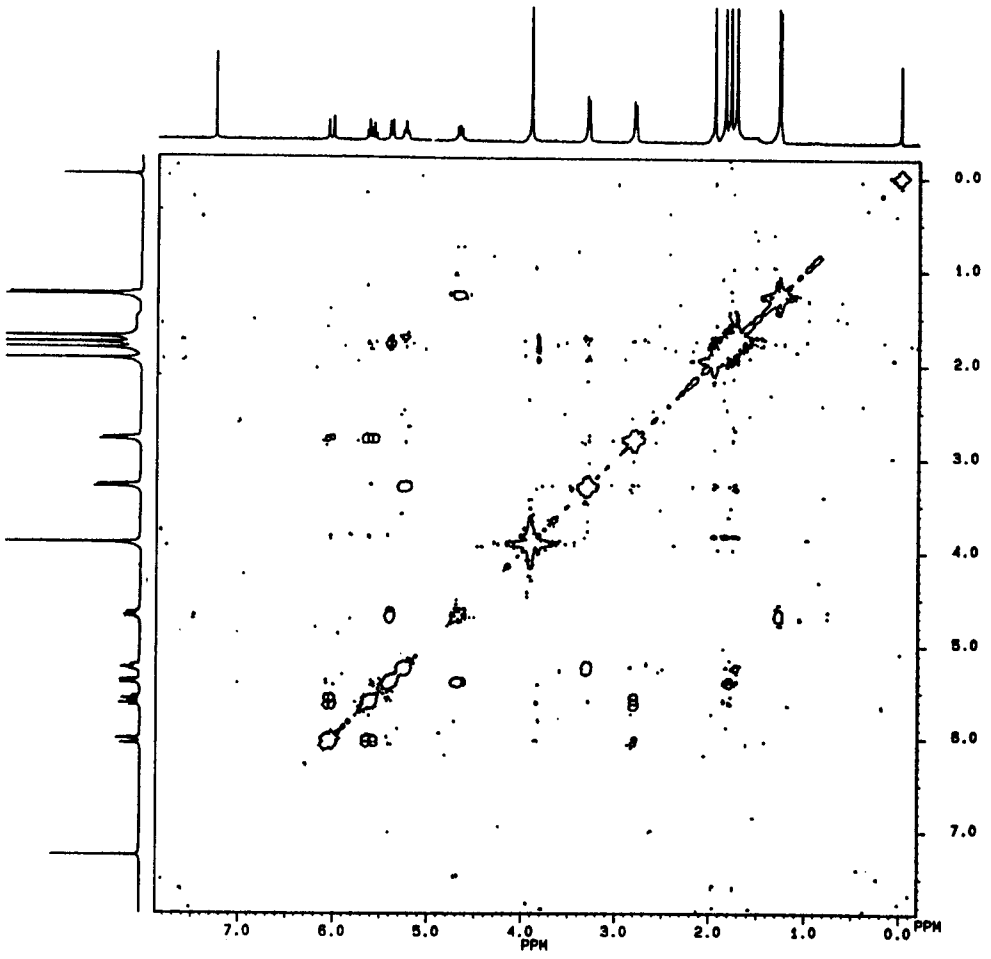

Figure C.17 NOESY Spectrum of Kalkipyrone (**23**) in $CDCl_3$ (300 MHz)

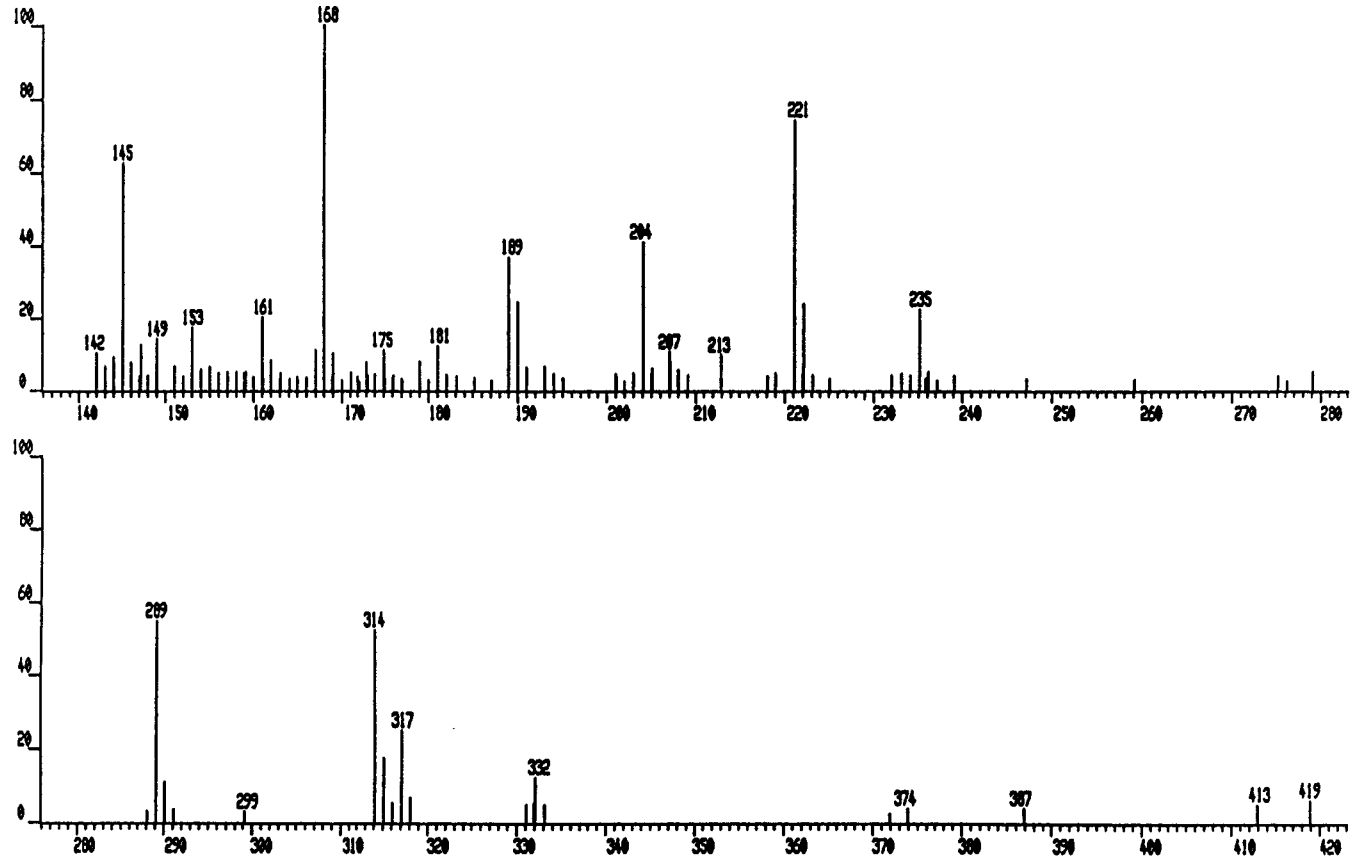

Figure C.18 LREI Mass Spectrum of Kalkipyrone (**23**)