AN ABSTRACT OF THE THESIS

OMAR MOHAMED SABRY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACY PRESENTED ON FEBRUARY 5, 2004. TITLE: NEW BIOACTIVE NATURAL PRODUCTS FROM MARINE ALGAE AND CYANOBACTERIA

Abstract approved: Redacted for privacy

William H. Gerwick

This thesis is an account of investigation on the natural products deriving from various marine algae and has resulted in the discovery of eleven novel bioactive metabolites. Isolation and characterization of these new molecules were carried out using different chromatographic techniques and by analyses of different spectroscopic data, respectively.

Using bioassay guided fractionation (brine shrimp toxicity assay), I isolated and identified five new, biologically active compounds [2β,3α-epitaondiol, flabellinol, flabellinone, stypoaldehyde and stypohydroperoxide], together with five known compounds [2-geranylgeranyl-6-methyl-1, 4-benzoquinone, (-) epistypodiol, (-) stypoldione, fucoxanthin and iditol] from the marine brown alga *Stypopodium flabelliforme,* collected from Papua New Guinea. All of the new compounds were found to have cytotoxic activity (EC_{50} ranges from 0.8 – 10 µg/ml) in human lung cancer (NCI-H460). 2β,3α-epitaondiol and flabellinol exhibited strong sodium channel blocking activity (EC_{50} = 0.3 and 0.9 µg/ml, respectively).

As a result of efforts to identify bioactive agents from marine algae, I have isolated and identified one new halogenated monoterpene [(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene] in addition to another three known halogenated monoterpene compounds from the red alga *Plocamium cartilagineum* collected from the eastern coast of South Africa. [(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene] was found to be active as a

cytotoxic agent in human lung cancer (NCI-H460) and mouse neuro-2a cell lines (EC_{50} 4 μg/ml).

As part of continued search for bioactive secondary metabolites from marine sources using a bioassay guided fractionation approach (anti-trypanosome activity), I examined the organic extract of a Papua New Guinean collection of the green alga *Udotea orientalis* growing on a coral wall and collected in September 1998. Successive HPLC separations resulted in the isolation of three new compounds; (+) curcuepoxide A , (+) curcuepoxide B and (+)-10α-hydroxycurcudiol. In addition I isolated four known compounds; (+)-10β-hydroxycurcudiol, (+) curcuphenol , (+) curcudiol and (+) curcudiol-10-one.

A bioassay guided investigation approach (anti-Sirt2) of a *Lyngbya majuscula* collection from Key West Florida, led to the discovery of two novel bioactive natural products [(+)-malyngamide X and one cyclic depsipeptide, (+)-floridamide]. The new cyclic depsipeptide, (+)-floridamide contains four amino acids units beside the unique unit, 2,2-dimethyl-3-hydroxyoctanoic acid (Dhoaa).

NEW BIOACTIVE NATURAL PRODUCTS FROM MARINE ALGAE AND CYANOBACTERIA

by

Omar Mohamed Sabry

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CONTRIBUTION OF AUTHORS

Cytotoxicity bioassays against human lung cancer and mouse neuro-2a were carried out by Dr. Douglas Goeger in our laboratory. X-ray crystallography of 2β,3α-epitaondiol was carried out by Dr. Alexandre Yokochi, Department of Chemistry, OSU. Modulations of Ca^{++} concentration in rat neurons assays were carried out by Dr. Keith T. LePage in the laboratory of Dr.Thomas Murray, Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia. Cytotoxicity bioassays against human colon cancer and human leukemia cell lines were carried out by Fred Valeriote, Josphine Ford Cancer Research Center, Detriot, MI.

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LIST OF ABBREVIATIONS

Ala	Alanine
Ara-A	Adenine arabinoside
Ara-C	Cytosine arabinoside
br	Broad
CI	Chemical Ionization
COSY	^{1}H-^{1}H Chemical Shift Correlation Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement Polarization Transfer.
Dhoaa	2,2-Dimethyl-3-hydroxy-octanoic acid
DMSO	Dimethylsulfoxide
EIMS	Electron-Impact Mass Spectrometry
EtOAc	Ethyl Acetate
FAB	Fast Atom Bombardment
GC	Gas Chromatography
Gly	Glycine
HMBC	Heteronuclear Multiple-Bond Coherence Spectroscopy
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
IC	Inhibitory Concentration
IR	Infrared Spectroscopy
LD	Lethal Dose
m	Multiplet
Me	Methyl
MeOH	Methanol
MS	Mass Spectroscopy
MTPA-Cl	2-Methoxy-2-(trifluoromethyl)phenylacetyl chloride
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
NOESY	Nuclear Overhauser Exchange Spectroscopy
Phe	Phenylalanine

LIST OF ABBREVIATIONS (*continued*)

Pro	Proline
q	Quartet
ROESY	Rotating Overhauser Exchange Spectroscopy
RT	Room Temperature
s	Singlet
SCUBA	Self-Contained Underwater Breathing Apparatus
t	Triplet
TOCSY	Total Correlation Spectroscopy
t_R	Retention Time
UV	Ultraviolet Spectroscopy
Val	Valine
VLC	Vacuum liquid chromatog

NEW BIOACTIVE NATURAL PRODUCTS FROM MARINE ALGAE AND CYANOBACTERIA

CHAPTER ONE

GENERAL INTRODUCTION

As disease resistance to drugs continues to grow at an alarming rate, natural products chemists are increasingly turning to new sources in the search for biologically active compounds. Approximately 45% of all current drugs are either a natural product, natural product derivative, or inspired by natural products chemistry, while 37% of all pharmaceutical sales are from natural product derived medicines.[1] An estimated 57% of the 150 most prescribed drugs are either natural product derivatives or are synthetic complements provided by natural product lead structures. In the specific field of cancer treatment, approximately 60% of the agents in clinical trials owe their origin to natural products.[2]

Oceans comprise over 70% of our world's surface and harbor a tremendous variety of flora and fauna, even though the majority of reported natural products originate from terrestrial sources.[3-6] Although still quite young by many standards, the field of marine natural products has already proven to be a productive source for biologically active natural products. The marine environment is characterized by new, exciting, and potentially useful chemistry. The development of SCUBA in the 1960's, and more recently submersible vehicles, has allowed relatively easy access to both shallow and deep-water marine organisms for studies by natural products chemists.

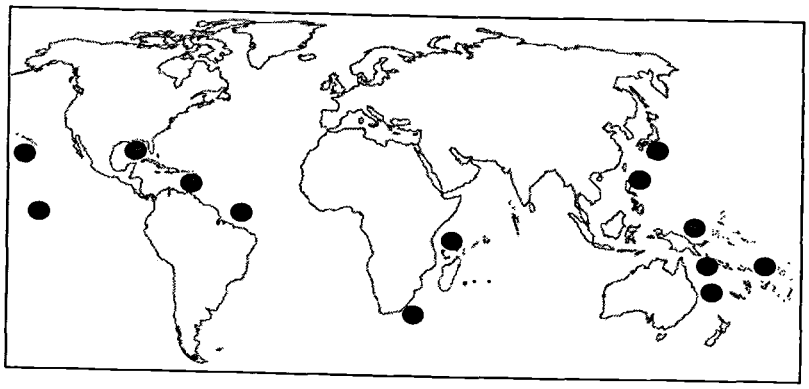


Figure I. 1. Primary collection sites for marine algae in Gerwick's laboratory.

Many marine derived secondary metabolites are structurally complex with unique functionalities, in contrast to their terrestrial counterparts. This is due in part to differences in the physicochemical nature of the two environments. Higher pressures, lower temperatures, lack of light as well as high ionic concentrations present in the sea may account for the biosynthesis of highly functionalized and unusual molecules in marine organisms. Production of natural products from living organisms increases as a result of the presence of competition environments. It is because of these structural novelties that natural products chemists hope to uncover new classes of therapeutic agents with unique pharmacophores, which may lead to the definition of novel sites in proteins and enzymes that are involved in human illnesses.

MARINE NATURAL PRODUCTS

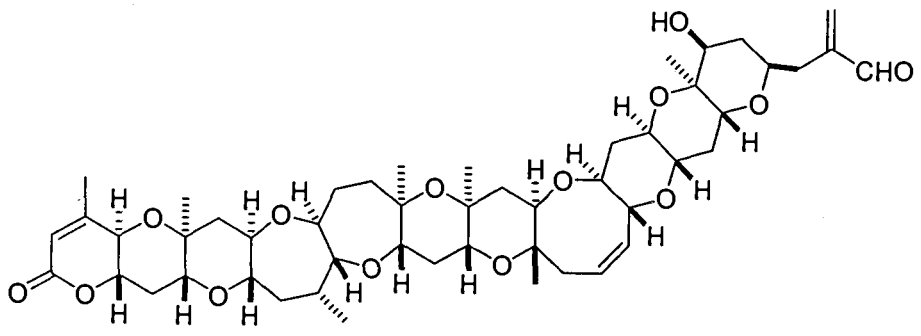
A number of promising compounds have been identified from marine sources that are already at advanced stages of clinical trials (mostly for the treatment of cancer) or have been selected as promising candidates for extended preclinical evaluation.[7] Interestingly, the majority of marine natural products currently in clinical trials or under preclinical evaluation are produced by invertebrates such as sponges, soft corals, sea squirts, and bryozoans. This differs greatly from the situation present in terrestrial natural products where plants by far exceed animals with regard to the production of bioactive metabolites.[8] The types of compounds characterized from marine organisms are diverse and represent many different structural classes, including polyethers, terpenoids, acetogenins, alkaloids, macrolides, polypeptides and polyphenolics.[7] In addition, these metabolites possess a wide spectrum of bioactivities, including anticancer activity, anti-inflammatory activity, antimalarial activity, antimicrobial activity, immunosuppressant activity, antifeedant activity, molluscicidal activity and ichthyotoxic activity.[7]

MARINE NATURAL PRODUCTS CHEMISTRY

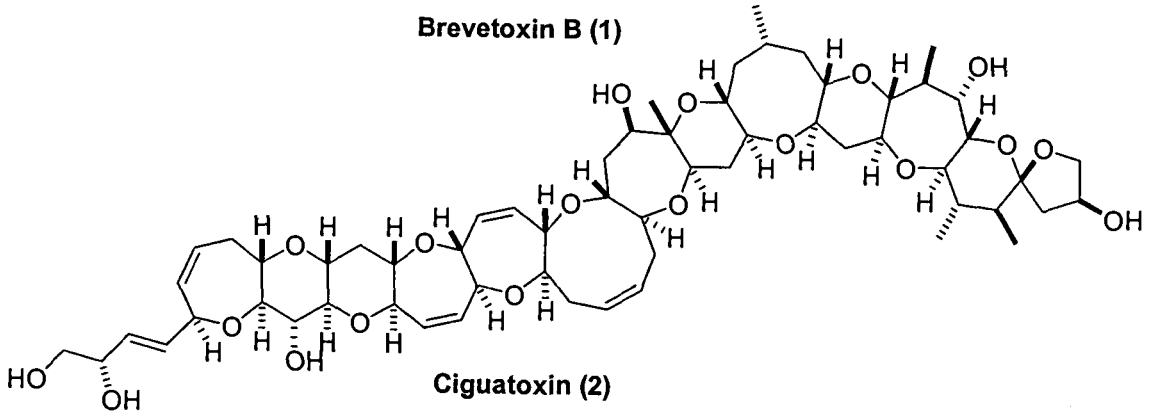
Marine Toxins

An important class of marine macromolecules, belonging mainly to the polyether structural class, forms a large group of marine toxins. Some of the well known marine polyether toxins are: brevetoxin B (**1**), isolated from the dinoflagellate *Gymnodinium*

breve;[9] ciguatoxin (**2**), a toxic constituent implicated in ciguateric seafood poisoning;[10] maitotoxin (**3**), isolated from *Gambierdiscus toxicus*;[11,12] and okadaic acid (**4**), a causative agent of diarrhetic shellfish poisoning, found from two species of dinoflagellates, *Prorocentrum lima* and *Dinophysis* species;[13,14] palytoxin (**5**), is a complex polyol isolated from the zoanthid, *Palythoa toxicus*.[15]



Brevetoxin B (1)

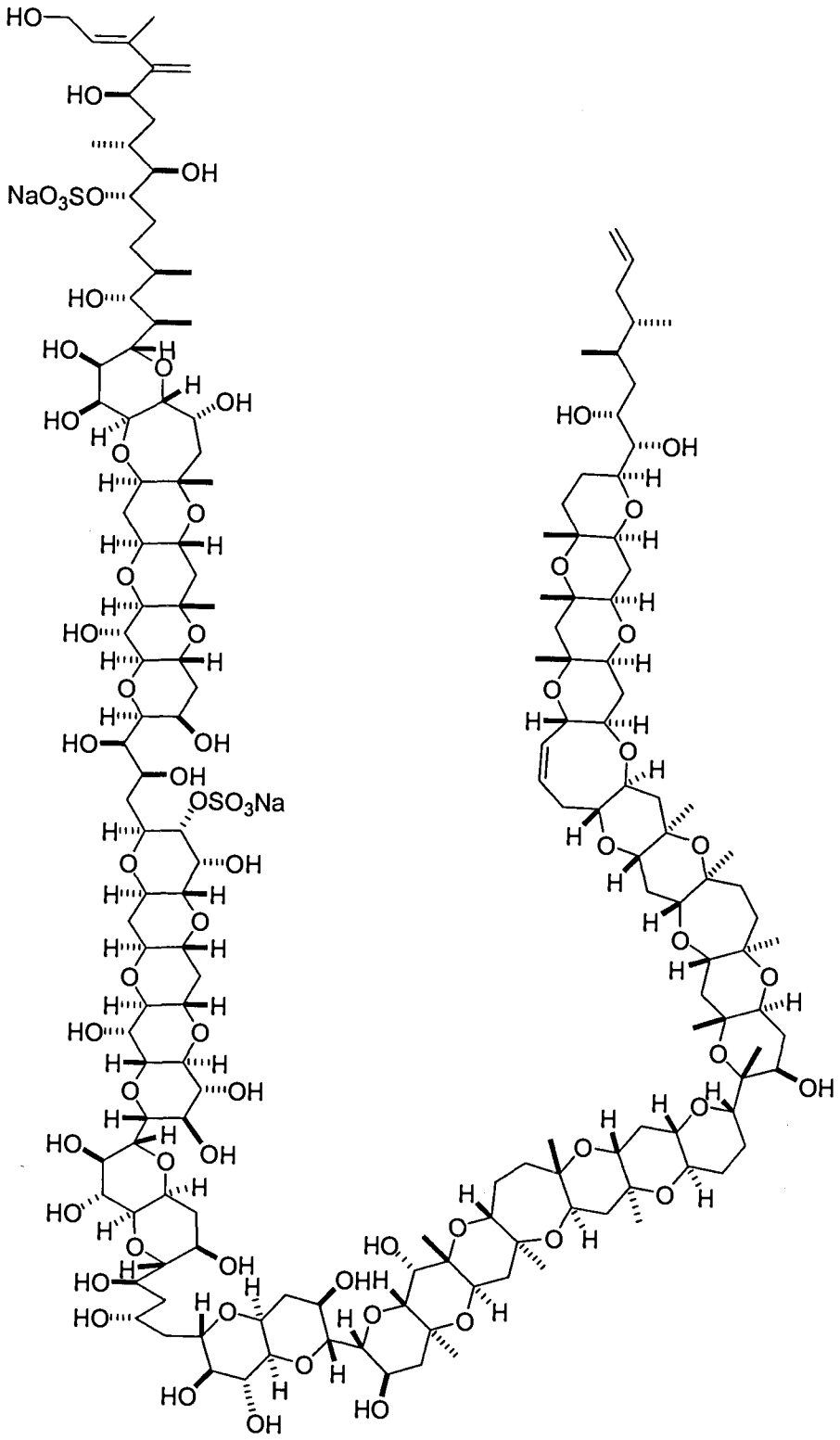


Ciguatoxin (2)

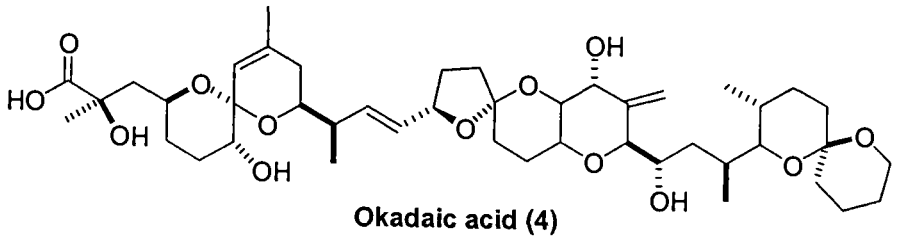
Marine Biomedicinals

The main aim of most marine natural products programs is to discover secondary metabolites of biomedicinal utility. This is especially true in the area of cancer research where numerous marine natural products are being screened for anticancer properties.

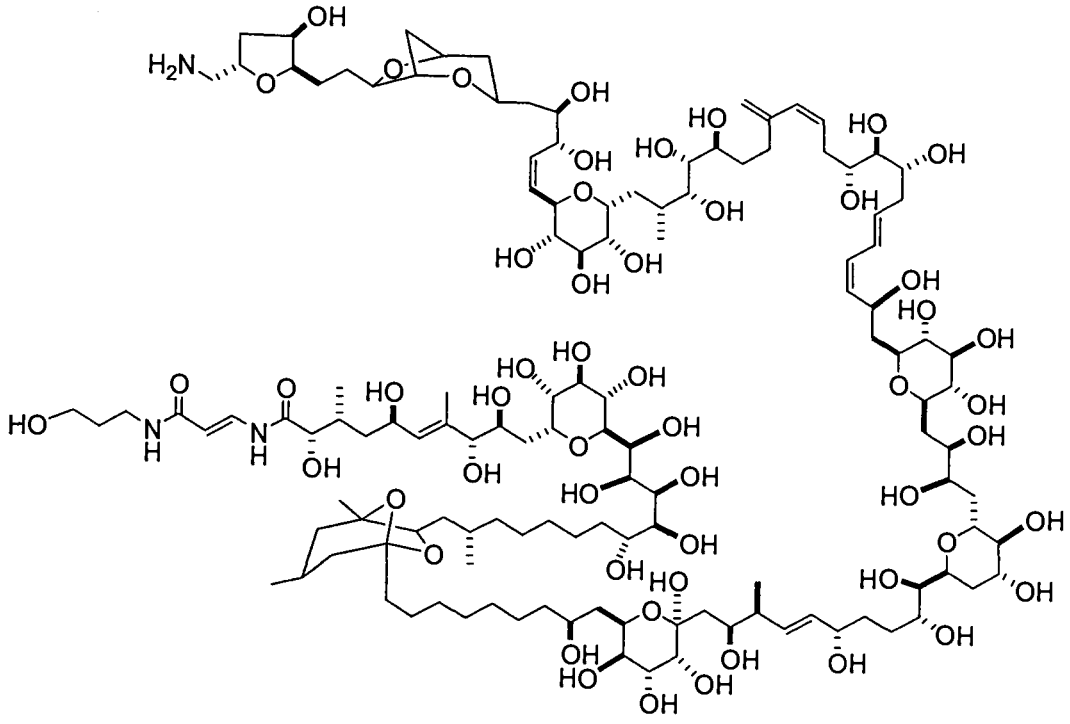
The first steps toward obtaining therapeutic agents from the marine environment were made in the 1950's by Bergmann and Feeney through their discovery of the nucleosides spongouridine (**6**) and spongothymidine (**7**) from the sponge *Tethya crypta*.[16] Subsequent development of synthetic analogues has provided the clinically relevant agents arabinosyl adenine (Ara-A, **8**), an anti-viral agent,[17] and arabinosyl cytosine (Ara-C, **9**), an anti-cancer agent for the treatment of acute myelocytic leukemia and non-Hodgkin's lymphoma.[18]



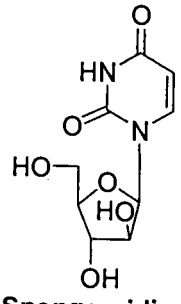
Maitotoxin (3)



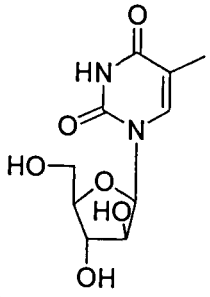
Okadaic acid (4)



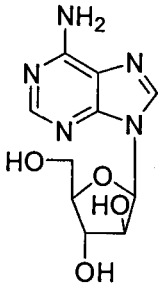
Palytoxin (5)



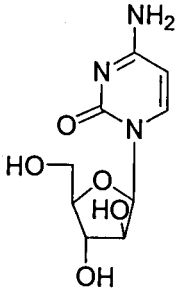
Spongouridine (6)



Spongothymidine (7)



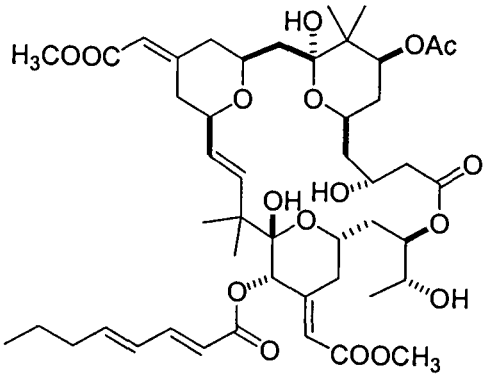
Ara-A (8)



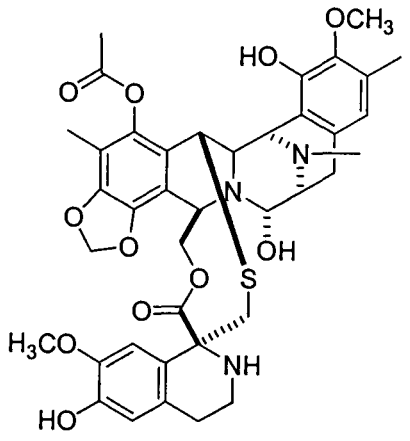
Ara-C (9)

Among the more promising antitumor natural products is bryostatin 1 (**10**), a polyketide first isolated in minute quantity from the bryozoan, *Bugula neritin*a in the 1970's.[19] Bryostatin 1 (**10**) is currently in phase II clinical trials for the treatment of various leukemias, lymphomas, melanoma, and solid tumors.[20] Recent research has found that this cytotoxic macrolide shows potential in the treatment of ovarian and breast cancer and it also enhances lymphocyte survival in patients undergoing radiation treatment.[21]

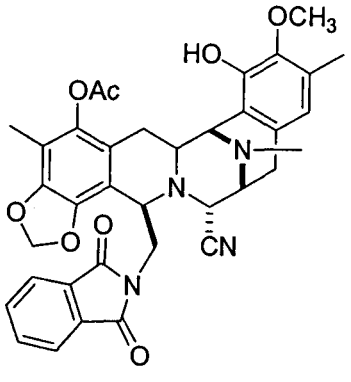
Another promising anticancer agent that is currently in phase III clinical trial is ecteinascidin 743 (**11**),[22] isolated from the Caribbean ascidian, *Ecteinascidia turbinata* and currently being investigated for potential use in the treatment of small cell lung cancer and skin cancer.[23,24]The structural complexity of ecteinascidin 743 has prompted the design of less complicated analogues of equal potency and greater stability, such as phthalascidin (**12**).[25]



Bryostatin 1 (10)



Ecteinascidin 743 (11)

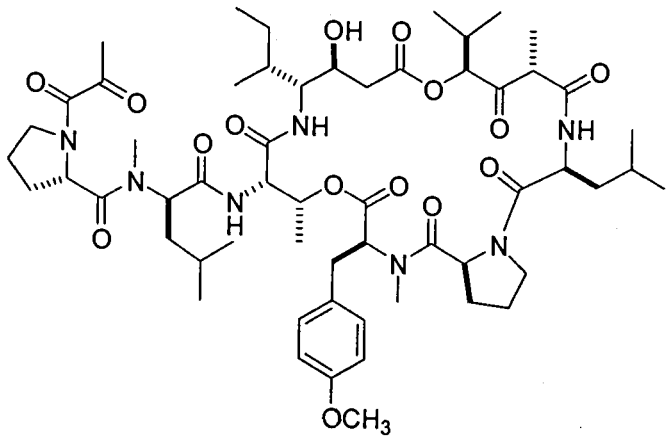


Phthalascidin (12)

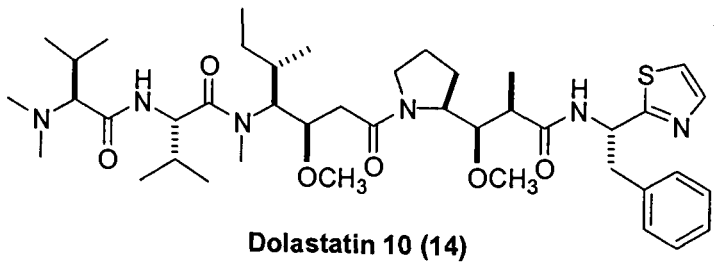
Two other marine-derived compounds that are in clinical trial (phase I) are aplidine (dehydrodidemnin B) (**13**) from the tunicate, *Aplidium albicans*[26] and dolastatin 10 (**14**), first isolated from the herbivorous seahare, *Dolabella auricularia* collected from the Indian Ocean.[27] Aplidine is ten times more effective in killing cancer cells than didemnin B, and is less toxic to normal cells.[28] Subsequent re-isolation of large amounts of dolastatin 10 (**14**) from the marine cyanobacterium *Symploca sp.* has unequivocally demonstrated the true origin of this bioactive metabolite.[29]

Other noteworthy marine-derived metabolites undergoing intense investigation as anticancer agents are: halichondrin B (**15**)[30] and isohomohalichondrin B (16)[31] from the sponge *Axinella sp.*; aplyronine A (**17**) from the seahare, *Aplysia kurodai*;[32] discodermolide (**18**) from the sponge, *Discodermia dissolute*;[33] eleutherobin (**19**) from the soft coral, *Eleutherobia albifora*;[34] sarcodictyin A (**20**) from the coral, *Sarcodictyon roseum*;[35] curacin A (**21**) from the Curaçao cyanobacterium, *Lyngbya majuscula*;[36] kahalalide F (**22**) from the Hawaiian mollusk, *Elysia rufescens*[37] and the bioactive peptide cryptophycin A (**23**), originally isolated from a cyanobacterium *Nostoc sp.* for its antifungal activity, but now well known for its potential application in the treatment of cancer.[38]

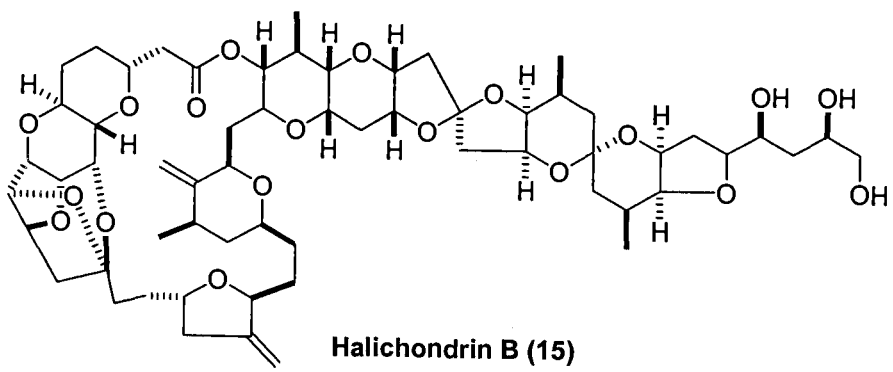
Marine organisms have also yielded a number of important anti-inflammatory agents. Most of these compounds act as phospholipase A_2 inhibitors, preventing the release of arachidonic acid from the lipids of cell membranes.[39] A recent bioassay screening of marine natural products having such inhibitory activities includes: pseudopterosins A (**24**) and E (**25**), from the Caribbean gorgonian, *Pseudopterogorgia elisabethae*;[40,41] topsentin (**26**) from the sponge, *Spongosorites ruetzleri*;[42] scytonemin (**27**), a pigment found in the extracellular sheaths of blue-green algae,[43] manoalide (**28**) from the sponge, *Luffariella variabilis,*[44] cyclomarin A (**29**)[45] from a marine *Streptomyces sp.* and the pain-killing marine natural product, ω-conotoxin. The latter compound has successfully completed phase III clinical trials for two therapeutic applications: to alleviate pain associated with malignant diseases and as an analgesic for nonmalignant neuropathic pain.[46] It was found to consist of 25-amino-acid linear peptide found along with other similar peptides in the venom of the predatory Indo-Pacific marine mollusc *Conus magus*, which is a fish-hunting mollusc that uses its venom to paralyse prey and proved to be 1,000 times more active than morphine in animal models.[46]



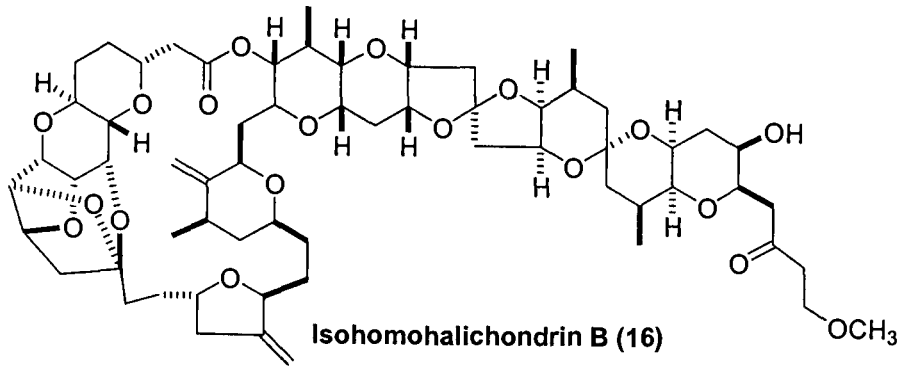
Aplidine (13)



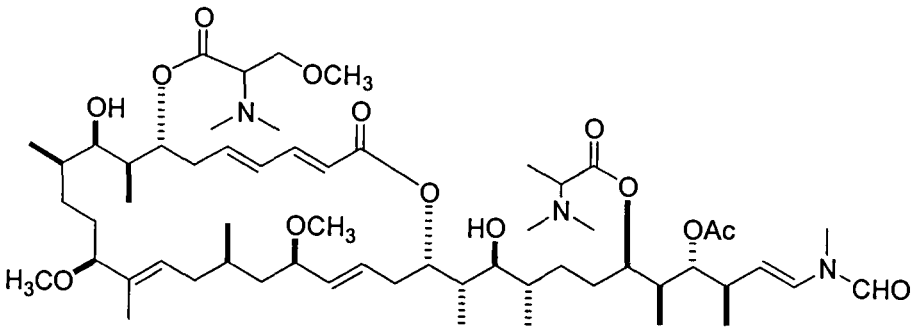
Dolastatin 10 (14)



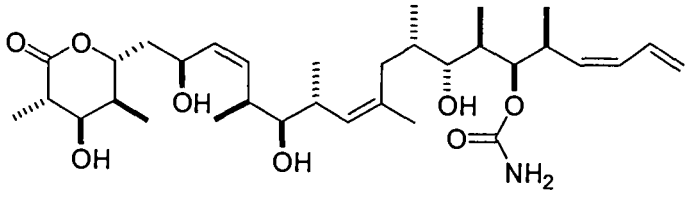
Halichondrin B (15)



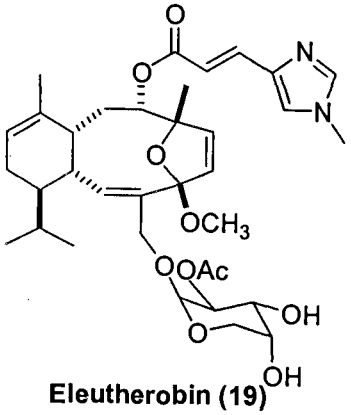
Isohomohalichondrin B (16)



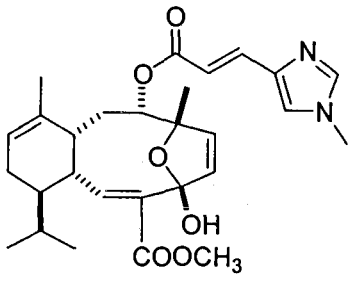
Aplyronine A (17)



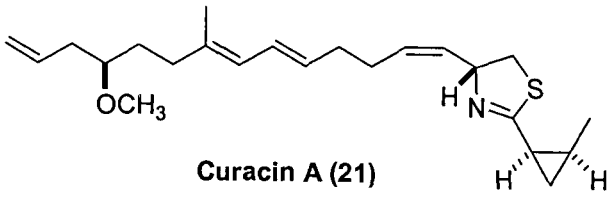
Discodermolide (18)



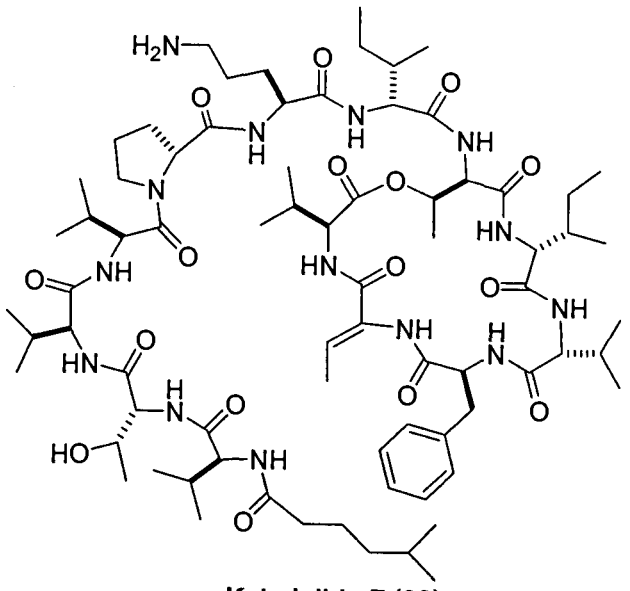
Eleutherobin (19)



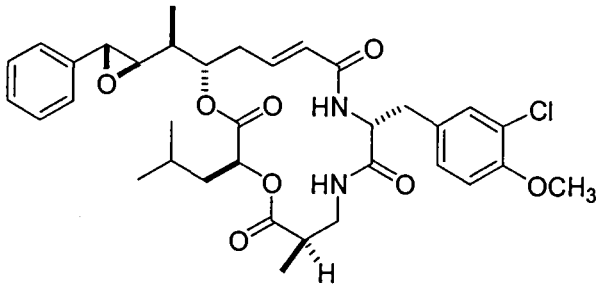
Sarcodictyin A (20)



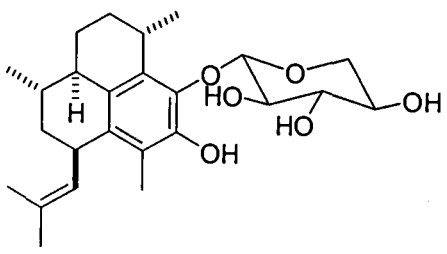
Curacin A (21)



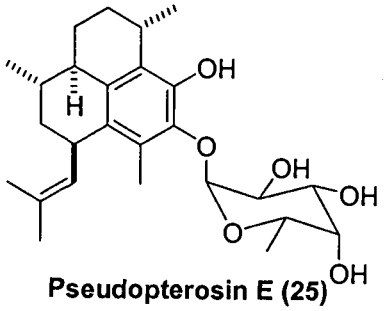
Kahalalide F (22)



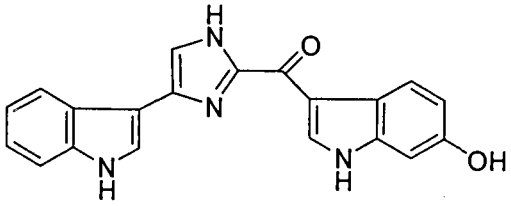
Cryptophycin A (23)



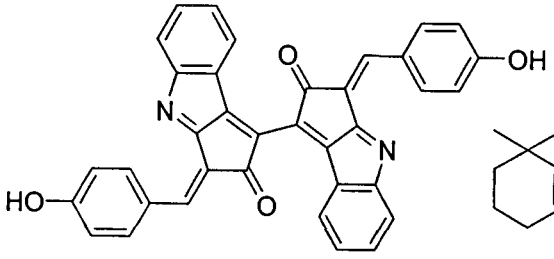
Pseudopterosin A (24)



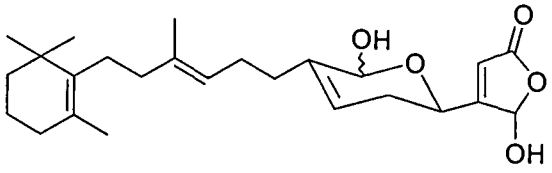
Pseudopterosin E (25)



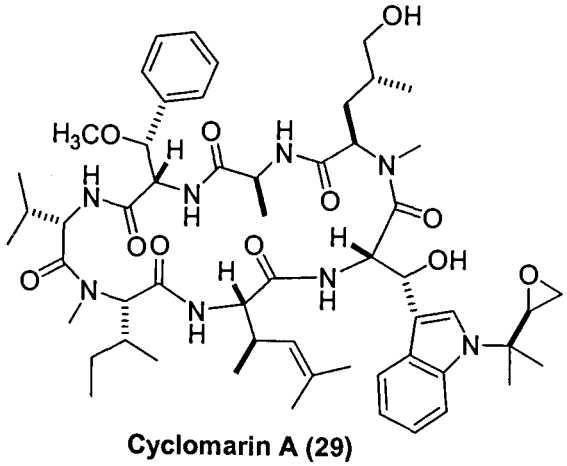
Topsentin (26)



Scytonemin (27)

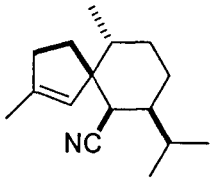


Manoalide (28)

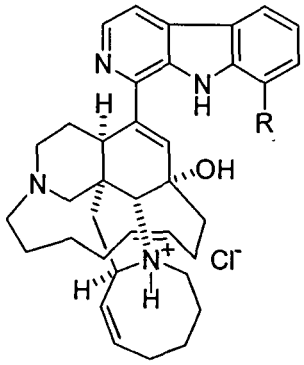


Cyclomarin A (29)

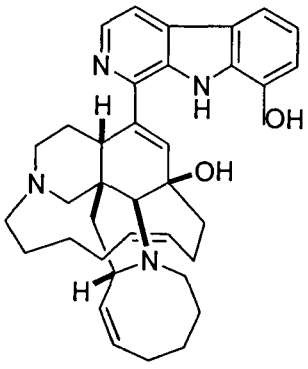
In an initial screening of extracts from marine sponges for antimalarial properties, *Acanthella klethra* yielded the active component, axisonitrile 3 (**30**), a bicyclic spiro-sesquiterpene with an isonitrile group, which possesses potent *in vitro* antimalarial activity with no detectable cytotoxicity.[47] The manzamines belong to a new class of alkaloids from sponges and are gaining attention as antimalarial agents. Four members of this series, manzamine A (**31**), 8-hydroxymanzamine A (**32**), its enantiomer (**33**), and a manzamine dimer, *neo*-kauluamine (**34**) gave encouraging results in preliminary *in vivo* tests.[48]



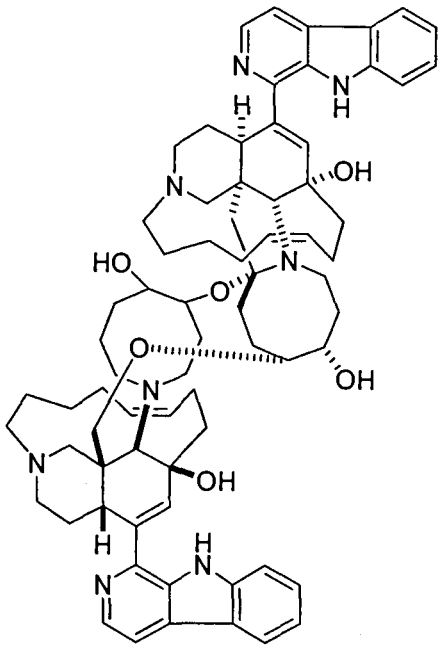
Axisonitrile 3 (30)



Manzamine A (31) R = H
8-Hydroxymanzamine A (32) R = OH



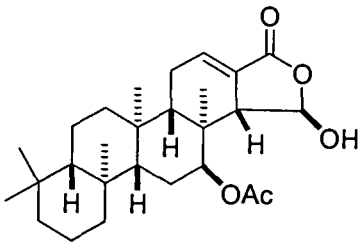
***ent*-8-Hydroxymanzamine A (33)**



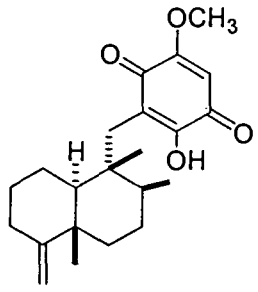
***neo*-Kauluamine (34)**

In the process of discovering new antituberculosis agents, three new leads were generated from marine sources. These were the C-19 hydroxy steroids, scalarin (**35**),[49] and tetrabromospirocyclohexadienylisoxazolines.[50]

In the area of antiviral research, especially anti-HIV (human immunodeficiency virus), two main leads have been found from marine sources. The marine natural product ilimaquinone (**36**) is currently being investigated as a chemotherapeutic in HIV infection.[51] This compound acts by targeting the RNase function of the reverse transcriptase enzyme encoded by HIV.



scalarin (35)



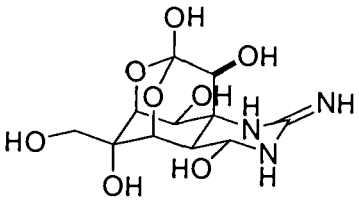
Ilimaquinone (36)

The marine environment has also provided a number of compounds useful as molecular probes for understanding cellular processes. Molecules such as tetrodotoxin (**37**), saxitoxin (**38**), and the conotoxins are instrumental in detailing the functions and structures of the membrane channels involved in nerve transmission. Other marine secondary metabolites that are becoming important as molecular tools are latrunculin A (**39**),[52] swinholide A (**40**),[53] and jaspamide (=Jaspakinolide) (**41**),[54,55] which bind specifically to the intracellular actin network; ilimaquinone (**36**),[56] which affects vesiculation of the Golgi apparatus; and more recently a unique sponge compound, adociasulfate 2 (**42**),[57] which is an inhibitor of the intracellular motor protein kinesin.

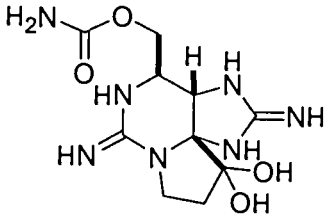
Marine Chemical Ecology

Many sessile marine organisms produce chemicals to inhibit settling by fouling organisms. This has led chemists to search for nontoxic natural marine antifoulants that could be incorporated in paints to be applied on ships hulls as well as offshore platforms. Over 90 marine antifoulants have now been characterized and they exhibit a high degree of structural diversity. Some examples are the renillafoulins (**43** to **45**) from an octocoral,[58] ambiol A (**46**)[59] and furospongolide (**47**)[60] from sponges, and halogenated

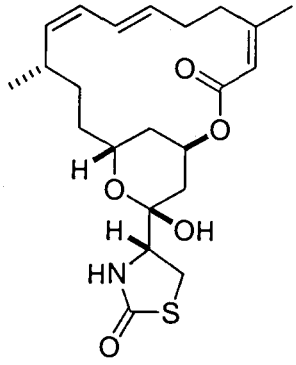
furanones (e.g. compound **48**) from the marine agla *Delisea pulchra*.[61] Compound such as stypoldione (**49**)[62] from the brown alga *Stypopodium zonale* was discovered on the basis of its ichthyotoxicity and was later shown to be cytotoxic.[63]



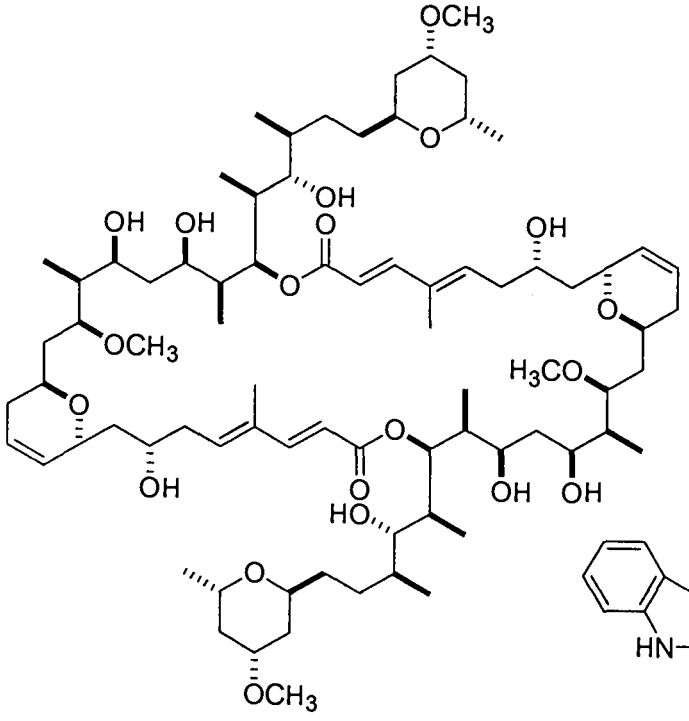
Tetrodotoxin (37)



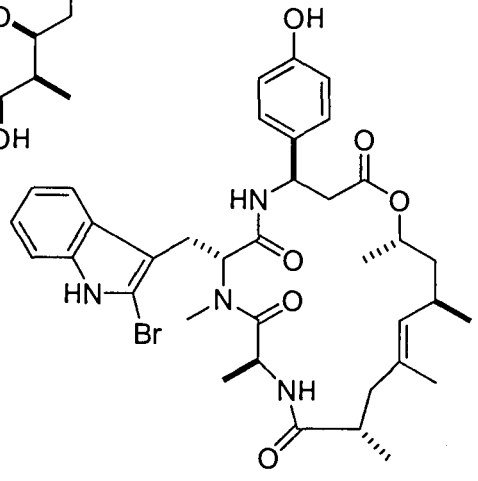
Saxitoxin (38)



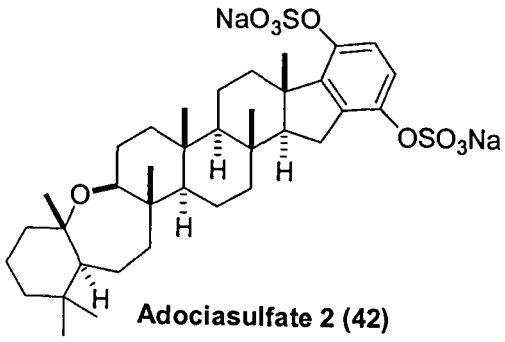
Latrunculin (39)



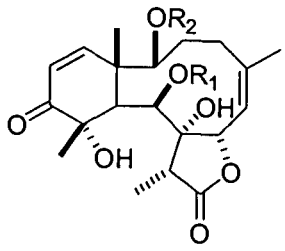
Swinholide A (40)



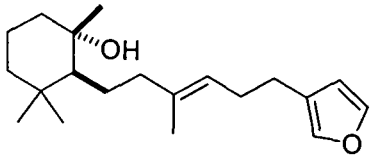
Jaspamide (= Jaspakinolide) (41)



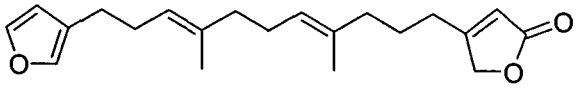
Adociasulfate 2 (42)



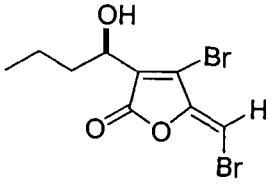
Renillafoulin A (43) $R_1 = R_2 = Ac$
Renillafoulin B (44) $R_1 = Ac$, $R_2 = C_2H_5CO^-$
Renillafoulin C (45) $R_1 = Ac$, $R_2 = n\text{-}C_3H_7CO^-$



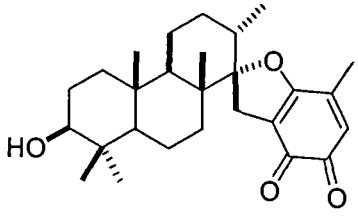
Ambiol A (46)



Furospongolide (47)



Compound (48)



Stypoldione (49)

ALGAL TAXONOMY

Algae are photosynthetic organisms that occur in most habitats, ranging from marine and freshwater to desert sands. They vary from small, single-celled forms to complex multicellular forms, such as the giant kelps of the eastern Pacific that grow to more than 60 meters in length and form dense marine forests. Algae may contribute to mass mortality of other organisms, in cases of algal blooms. According to the type of chlorophyll, accessory pigments, storage proteins, and cell wall composition, algae are typically divided into the following four main divisions:[64-66]

1. Chlorophyta (green algae)

Chlorophytes range in size from microscopic to quite large. The typical color of plants in the Chlorophyta, resulting from the dominant chlorophyll pigments, is some shade of apple or grass green, although certain species may appear yellowish green or

blackish green due to the presence of carotenoid pigments or high concentrations of chlorophyll. Green algae have chlorophylls a and b and store starch as a food reserve inside their plastids. The cell wall is made mainly of cellulose and pectin.

2. Cyanophyta (blue-green algae)

Blue-greens contain chlorophyll a, as in eucaryotic phototrophs. They produce free oxygen as a byproduct of photosynthesis. The variety of striking colors exhibited by Cyanobacteria are a result of their major light-gathering pigments, the phycoerythrin and phycocyanin, that are bound to protein granules, (phycobilisomes), that are attached to the photosynthetic membranes. Large blooms of freshwater Cyanobacteria may produce toxins that can kill livestock. They store glycogen as a food reserve. The cell wall is made mainly of peptidoglycan. Cyanophyta, while capable of photosynthesis, lack organized organelles(thus being prokaryotes) and apparently utilize some of the biosynthetic machinery extant in bacteria.

3. Phaeophyta (brown algae)

The colors of brown algae (predominantly due to the brown accessory pigment fucoxanthin) cover a spectrum from pale beige to yellow-brown to almost black. In tropical seas, they range in size from microscopic filaments to several meters in length. Brown algae have chlorophylls a and c and store laminarin as a food reserve inside their plastids. The cell wall is made mainly of cellulose.

4. Rhodophyta (red algae)

Rhodophytes are usually multicellular and grow attached to rocks or other algae, but there are some unicellular or colonial forms. Rhodophytes contain chlorophyll a and d which is masked by phycoerythrin, phycocyanin and zeaxanthin pigments bound to proteins. Rhodophytes store starch as a food reserve. The cell wall is made mainly of cellulose.

FUNCTIONS OF BIOACTIVE METABOLITES IN THE PRODUCING ORGANISMS

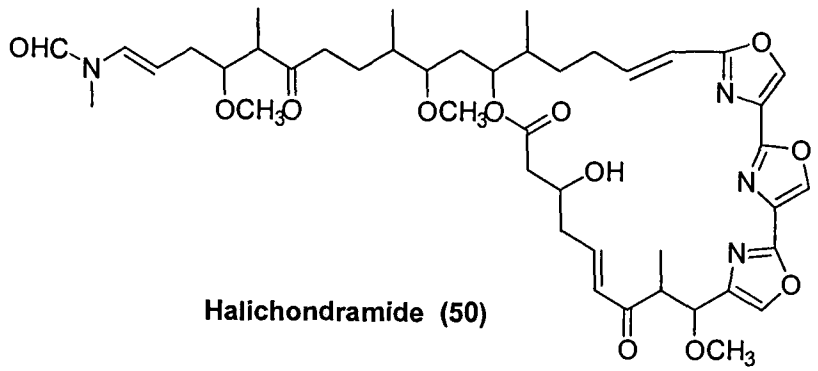
Secondary metabolites are naturally produced compounds that are not essential for the survival of the producing organism, in contrast to primary metabolites. The function of secondary metabolites has been a subject of debate since the first isolation and characterization of these often times structurally complex chemical compounds. Screening and bioassay of these compounds have revealed a wide range of biological

activities, such as cytotoxicity, ichthyotoxicity, antimicrobial and antifeedant activity, antifoulant and anticancer activities.[7]

This apparent paradox has generated two conflicting views on the origin and rationale for the production of secondary metabolites. The first hypothesis posits that secondary metabolites are waste products that no longer play a role in enhancing the fitness of an organism.[67] The second view postulates that they have definite biological activities that increase the fitness and survival of the producer.[68]

Logically, the energy expended for producing and processing this dazzling variety of chemical structures and motifs is too great for organisms to be merely maintaining unnecessary biosynthetic pathways. Chemical compound biosyntheses are costly because they utilize resources that could be allocated to growth and reproduction.[69] Thus, secondary metabolism must serve a purpose in the general economy of the producing organism. The most obvious use for these compounds is in providing defense against predators or competing species.

In the field of marine chemical ecology, it has been shown repeatedly that chemical defense through accumulation of toxic or distasteful natural products is an effective strategy to fight off potential predators, such as fish and sea urchins, or to suppress competitors, pathogens and fouling organisms.[70] The opisthobranch sea hare *Stylocheilus longicauda* is a reef grazer that feeds specifically on *Lyngbya majuscula* and also sequesters bioactive cyanobacterial metabolites, presumably for defense. Another example is represented by the nudibranch mollusc *Hexabranchus sanguineus* which is protected from predation by reef fish or hermit crabs due to macrocyclic oxazole alkaloids, such as halichondramide (**50**), which act as feeding deterrents.[71] These anti-feedant alkaloids are concentrated in the most vulnerable parts of the mollusc, the dorsal mantle and the egg ribbons. The origin of the halichondramide was determined as dietary and ultimately traced to sponges of the genus *Halichondria*.



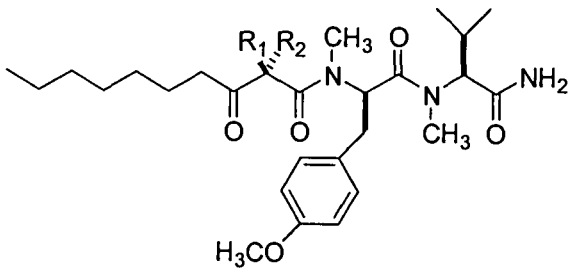
Halichondramide (50)

Marine algae are another group of organisms rich in secondary metabolites proven to serve multiple roles through field and laboratory bioassays: defense against consumers, antifouling, antimicrobial and allelopathic effects.[72] The majority of natural products isolated from marine macroalgae are terpenoids, polyketides or aromatic compounds.[72] Nitrogenous compounds, while frequently encountered in marine invertebrates, are very rare in macroalgae. The component of bioactive metabolites in macrophytes is known to vary widely, depending on geographic location, environmental conditions and the induced or basal state of the alga.[71] Studies aimed at investigating the presence of inducible production of metabolites in a manner similar to terrestrial plants have been very scarce. One study reported that grazing by the amphipod *Ampithoe longimana* induced an increased accumulation of dictyol-type terpenes in the marine brown alga *Dictyota menstrualis*.[73] These terpenes also reduced the palatability of *D. menstrualis* to the amphipod. Another experiment showed that water-borne cues from actively feeding herbivorous gastropods can serve as external signals to induce production of defense chemicals (phlorotannins) in unharmed individuals of the brown seaweed *Ascophyllum nodosum*. Furthermore, the increased levels of defense chemicals deterred further feeding by *Littorina obtusata*.[74]

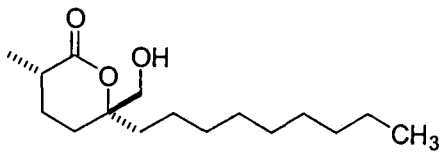
Marine cyanobacteria are known as very rich producers of toxic and bioactive secondary metabolites with practical applications, even though the biological and ecological roles of these metabolites are still relatively uninvestigated. Most of the studies on the ecological significance of cyanobacterial metabolites have been performed in the past ten years, after recognizing the importance of cyanobacteria as producer organisms for many compounds that were initially isolated from other sources.

Through studies on their activity as anti-feedants, it is well-established that toxic secondary metabolites in marine cyanobacteria have chemical defense roles. For example, a recent report found that the organic extracts of either *Lyngbya majuscula*, or mixed assemblages of *Lyngbya* sp., *Schizothrix calcicola* and *Microcoleus* sp. all deterred feeding by parrotfish (*Scarus schlegeli*), but to varying extents. Further chemical characterization of the crude extracts revealed the existence of distinct 'chemotypes', with characteristic secondary metabolite profile, even between monoculture samples of the same species.[75] Purified secondary metabolites isolated from *L. majuscula*,[76] *H. enteromorphoides*[77] and mixed assemblages[78] were tested in similar experiments and proved to be effective feeding deterrents to juvenile rabbitfish

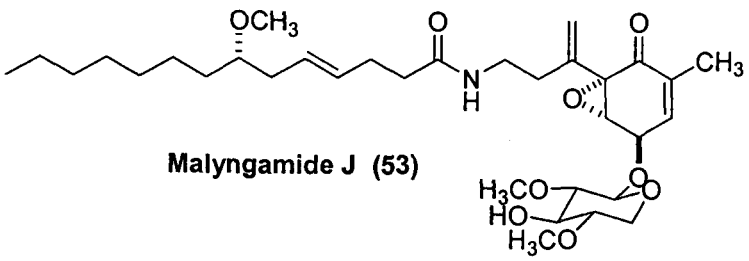
and parrotfish. Among the most active were: majusculamides A and B (**51**), malyngolide (**52**), malyngamide J (**53**) and laxaphycin (**54**).



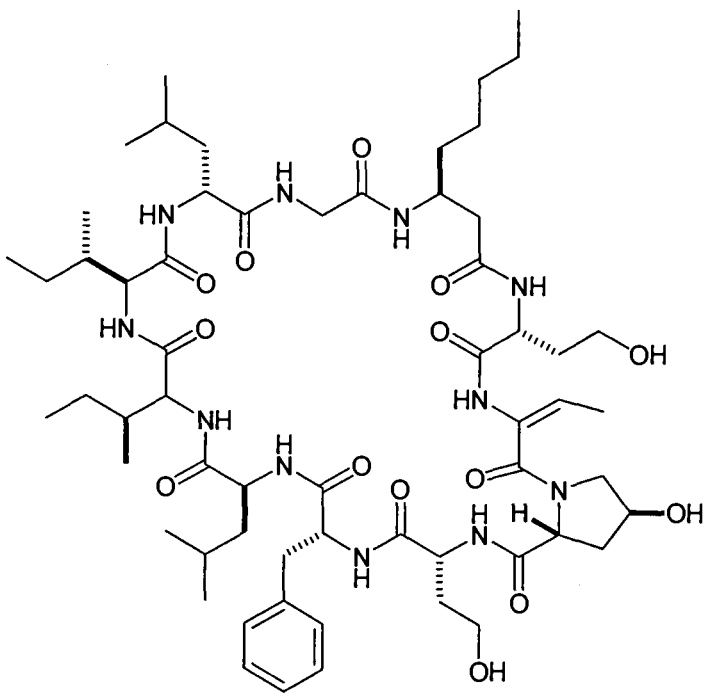
Majusculamides A $R_1 = CH_3$, $R_2 = H$
(51) **B** $R_1 = H$, $R_2 = CH_3$



Malyngolide (52)

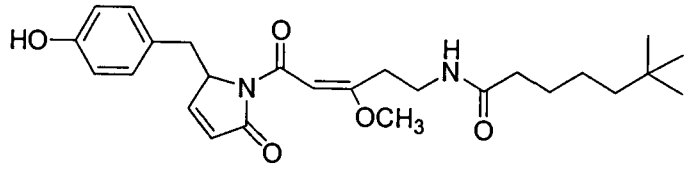


Malyngamide J (53)



Laxaphycin A (54)

The lipopeptide ypaoamide (**55**) which acts as a feeding deterrent to herbivorous reef fish and sea urchins, is produced by *L. majuscula* mixed with *S. calcicola*.[79]



Ypaoamide (55)

Another rationale for studying the marine natural products is the biology and ecology of marine secondary metabolites, especially in many cases where toxins produced by marine cyanobacteria and dinoflagellates have dangerous effects on human health and the fishing industry through increasingly frequent harmful algal blooms. Knowledge accumulated in this field could serve in the prediction, monitoring and/or prevention of future outbreaks.

General Thesis Contents

The search for new and useful natural products from marine algae has been the main focus of our laboratory. Our extensive collections of marine algae are the results of continual expeditions to different parts of the world, including the Caribbean, Indonesia, Japan, Papua New Guinea, Fiji, South Africa, and Madagascar. As such, the various chapters outlined in this thesis have unifying themes, which entail isolation, structure elucidation (both planar and stereochemical determinations), and biological characterization of novel secondary metabolites from marine algae collected from these different localities.

This thesis begins with the first chapter which is a general introduction to marine natural products, the biomedical utility of some recently discovered marine natural products, and a discussion of the roles they might play in the producing organisms.

Chapter two discusses the investigation of the brown alga *Stypopodium flabelliforme*. Using bioassay guided fractionation, I isolated and identified five new, biologically active compounds [2β,3α-epitaondiol, flabellinol, flabellinone, stypoaldehyde and stypohydroperoxide], together with five known compounds (2-geranylgeranyl-6-methyl-1, 4-benzoquinone, (-) epistypodiol, (-) stypoldione, fucoxanthin and iditol) from the marine brown alga *Stypopodium flabelliforme,* collected from Papua New Guinea. All of the new compounds were found to have cytotoxic activity (EC_{50} ranges from 0.3 – 10 μg/ml) in human lung cancer (NCI-H460) and mouse neuro-2a cell lines. 3α-epitaondiol and flabellinol exhibited strong sodium channel blocking activity (EC_{50} = 0.3 and 0.9 μg/ml, respectively). Stypoaldehyde, as well as the previously known compound, stypoldione, effected a change in the calcium concentrations in rat neurons. The acetate derivatives of 3α-epitaondiol and flabellinol, were significally less active than the natural products. The molecular structures of the isolated compounds were determined by extensive analysis of their spectroscopic data (1D and 2D NMR, LRMS, HRMS, IR and UV). The relative stereochemistries of the compounds 3α-epitaondiol, and flabellinol were determined by 1D and 2D NOE experiments. X-ray crystallography confirmed the relative stereochemistry of 2β,3α-epitaondiol. Absolute stereochemistry of the latter compound was detemined bythe modified Mosher's ester method.

Chapter three presents the isolation and identification of one new cytotoxic halogenated monoterpene metabolite [(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-

methyl-1,5,7-octatriene] in addition to three known compounds from the red alga *Plocamium cartilagineum* collected from the eastern coast of South Africa. (-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene was found to be active as a cytotoxic agent in human lung cancer (NCI-H460) and mouse neuro-2a cell lines (EC_{50} 4 µg/ml). Two of the known compounds were found to have promising activity as cytotoxins in other cell line assays, especially to human leukemia and human colon cancers and are now in the *in-vivo* evaluation stage. However, none of these metabolites were active as sodium channel blockers or activators.

A total of three new compounds; (+) curcuepoxide A, (+) curcuepoxide B and (+)-10α- hydroxycurcudiol were isolated and identified. In addition I isolated four known compounds; (+)-10β- hydroxycurcudiol, (+) curcuphenol, (+) curcudiol and (+) curcudiol-10-one. Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed for construction of their planar structures. Curcudiol was found to be active in an *in-vitro* assay of anti-trypanosome activity (EC_{50}10 µg/ml). The isolated compounds were found to have variable cytotoxic activity in human lung cancer cell lines. The most active compounds in the latter assays were compounds(+)-10β- hydroxycurcudiol and (+) curcudiol-10-one (EC_{50} = 2 and 4 ug/ml, respectively). However, they both displayed sodium channel blocking activity at this dose but not at the lower dose of 1 µg/ml.

In chapter five, a bioassay guided investigation (Anti-Sirt2) of a *Lyngbya majuscula* collection from Key West Florida in 1995, led to the discovery of two novel bioactive natural products [(+)-malyngamide X and another cyclic depsipeptide, (+)-floridamide]. Their structures were deduced through extensive analysis of 1D and 2D NMR spectroscopic data and supported by HRFAB mass spectrometry. The new cyclic depsipeptide contains four amino acids units, including *N*-methyl phenylalanine (*N*-MePhe), proline (Pro), valine (Val) and alanine (Ala), beside the unique unit, 2,2-dimethyl -3-hydroxy-octanoic acid (Dhoaa). In addition to the discovery of these two new compounds, two previously reported metabolites were also isolated and identified from this cyanobacterial collection, the (-) C-12 lyngbic acid and the antibacterial agent (-) malyngolide.

The thesis will end with a concluding chapter, summarizing the results presented in the preceding chapters as well as comments on structural trends that are emerging in marine algal and cyanobacterial secondary metabolites.

REFERENCES

1. Frormann, S.; Jas, G. Business Briefing: *Future Drug Discover.* **2002**, 84-90.

2. Cragg, G. M.; Newman, D. J. *Expert Opinion on Investigational Drugs* **2000**, *9*, 2783-2797.

3. Davies, D. G.; Parsek, M. R.; Pearson, J. P.; Iglewski, B. H.; Costerton, J. W.; Greenberg, E. P. *Science* **1998**, *280*, 295-298.

4. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. *Microbiology* **1999**, *145*, 283-29.

5. De Nys, R.; Wright, A. D.; Konig, G. M.; Sticher, O. *Tetrahedron* **1993**, *49*, 11213-11220.

6. Rasmussen, T. B.; Manefield, M.; Andersen, J. B.; Eberl, L.; Anthoni, U.; Christophersen, C.; Steinberg, P.; Kjelleberg, S.; Givskov, M. *Microbiology* **2000**, *146*, 3237-3244.

7. Faulkner, D. J. *Nat. Prod. Rep.* **2000**, *17*, 1-6.

8. Proksch, P.; Edrada, R. A.; Ebel, R. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 125-134

9. Lin, Y.–Y.; Risk, M.; Ray, S. M.; Van Engen, D.; Clardy, J.; Golik, J.; James, J. C.; Nakanishi, K. *J. Am. Chem. Soc.* **1981**, *103*, 6773–6775.

10. Murata, M.; Legrand, A.–M.; Ishibashi, Y.; Yasumoto, T. *J. Am. Chem. Soc.* **1989**, *111*, 8927–8931.

11. Murata, M.; Nakoi, H.; Iwashita, T.; Matsunaga, S.; Sasaki, M.; Yokoyama, A.; Yasumoto, T. *J. Am. Chem. Soc.* **1993**, *115*, 2060–2062.

12. Norimura, T.; Sasaki, M.; Matsumori, N.; Miata, M.; Tachibana, K.; Yasumoto, T. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1675–1678.

13. Yasumoto, T.; Oshima, Y.; Sugawara, W.; Fukuyo, Y.; Oguri, H.; Igarashi, T.; Fujita, N. *Nippon Suisan Gakkaishi* **1980**, *46*, 1405–1411.

14. Murakami, Y.; Oshima, Y.; Yasumoto, T. *Nippon Suisan Gakkaishi* **1982**, *48*, 69–72.

15. Moore, R. E.; Scheuer, P. J. *Science* **1971**, *172*, 495–498.

16. Bergmann, W.; Feeney, R. J. *J. Org. Chem.* **1951**, *16*, 981–987

17. Lopez, C.; Giner-Sorolla, A. *Ann. N. Y. Acad. Sci.* **1977**, *284*, 351-357

18. Bodey, G. P.; Freirich, E. J.; Monto, R. W.; Hewlett, J. S. *Cancer*

Chemother. **1969**, *53*, 59–66

19. Pettit, G. R.; Herald, C. L.; Doubek, D. L.; Herald, D. L.; Arnold, E.; Clardy, J. *J. Am. Chem. Soc.* **1982**, *104*, 6846–6848.

20. Propper, D. J.; Macaulay, V.; O'Byrne, K. J.; Braybrooke, J. P.; Wilner, S. M.; Ganesan, T. S.; Talbot, D. C.; Harris, A. L. *British J. Canc.* **1998**, 78, 1337-41.

21. Kraft, A. S. *J. Natl. Cancer Inst.* **1993**, *85*, 1790–1792.

22. Aune, G. J.; Furuta, T.; Pommier, Y. *Anti-Cancer Drugs* **2002**, 13, 545-555.

23. Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55*, 4508–4511.

24. Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Kiefer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512–4515.

25. Martinez, E. J.; Corey, E. J.; Owa, T. *Chem. Biol.* **2001**, *8*, 1151-60

26. Sakai, R.; Rinehart, K. L.; Kishore, V.; Kundu, B.; Faircloth, G.; Gloer, J. B.; Carney, J. R.; Namikoshi, M.; Sun, F.; Hughes, R. G.; Garcia Gravalos, D.; de Quesada, T. G.; Wilson, G. R.; Heid, R. M. *J. Med. Chem.* **1996**, *39*, 2819–2834.

27. Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinamn, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B.; Bontems, R. *J. Am. Chem. Soc.* **1987**, *109*, 6883–6885.

28. Geldof, A. A.; Mastbergen, S. C.; Henrar, R. E. C.; Faircloth, G. T. *Cancer Chemother. Pharmacol.* **1999**, *44*, 312-318

29. Luesch, H.; Moore, R. E.; Paul, V. J.; Mooberry, S. L.; Corbett, T. H. *J. Nat. Prod.* **2001**, *64*, 907-910

30. Hirata, Y.; Uemura, D. *Pure Appl. Chem.* **1986**, *58*, 701–710.

31. Litaudon, M.; Hart, J. B.; Blunt, J. W.; Lake, R. J.; Munro, M. H. G. *Tetrahedron Lett.* **1994**, *35*, 9435–9438.

32. Yamada, K.; Ojika, M.; Ishigaki, T.; Yoshida, Y.; Ekimoto, H.; Arakawa, M. *J. Am. Chem. Soc.* **1993**, *115*, 11020–11021.

33. Gunasekera, S. P.; Gunasekera, M.; Longley, R. E.; Schulte, G. K. *J. Org. Chem.* **1990**, *55*, 4912–4915.

34. Lindel, T.; Jensen, P. R.; Fenical, W.; Long, B. H.; Casazza, A. M.; Carboni, J.; Fairchild, C. R. *J. Am. Chem. Soc.* **1997**, *119*, 8744–8745.

35. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta.* **1987**, *70*, 2019–

2027.

36. Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. L. *J. Org. Chem.* **1994**, *59*, 1243–1245.

37. Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825–5826.

38. Smith, C. D.; Zhang, X.; Mooberry, S. L.; Patterson, G. M. L.; Moore, R. E. *Cancer Res.* **1994**, *54*, 3779-3784

39. Wylie, B. L.; Ernst, N. B.; Grace, K. J. S.; Jacobs, R. S. *Prog. Surg.* **1997**, *24*, 146–152.

40. Look, S. A.; Fenical, W.; Matsumoto, G. K.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5140–5145.

41. Roussis, V.; Wu, Z, Fenical, W.; Strobel, S. A.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1990**, *55*, 4916–4922.

42. Bartik, K.; Braekman, J. C.; Daloze, D.; Stoller, C.; Huysecom, J.; Vandevyver, G.; Ottinger, R. *Can. J. Chem.* **1987**, *65*, 2118–2121.

43. Proteau, P.; Gerwick, W. H.; Garcia–Pichel, F.; Castenholz, R. *Experientia* **1993**, *49*, 825–829.

44. De Silva, E. D.; Scheuer, P. J. *Tetrahedron Lett.* **1980**, *21*, 1611–1614.

45. Renner, M. K., Shen, Y.-C., Cheng, X.-C., Jensen, P. R., Frankmoelle, W., Kauffman, C. A., Fenical, W., Lobkovsky, E. and Clardy, J. *J. Am. Chem. Soc.* **1999**, *121*, 11273-11276

46. Olivera, B. M. In: Fusetani, N. (ed), *Drugs from the sea.* Karger **2000**, 74-85

47. Wright, A. D.; Konig, G. M. *J. Nat. Prod.* **1996**, *59*, 710–716.

48. Baldwin J. E.; Whitehead, R. C., *Tetrahedron Lett.* **1992**, 33, 2059.

49. Fattorusso, E.; Magno, S.; Santacroce, C.; Sica, D. *Tetrahedron* **1972**, 28, 5993-7.

50. El Sayed, K. A.; Bartyzel, P.; Shen, X.; Perry, T. L.; Zjawiony, J. K.; Hamann, M. T. *Tetrahedron* **2000**, *56*, 949–953.

51. De Clercq, E. *Med. Res. Rev.* **2000**, *20*, 323–349.

52. Spector, I.; Shochet, N. R.; Kashman, Y.; Groweiss, A. *Science* **1983**, *219*, 493–495.

53. Kitagawa, I.; Kobayashi, M.; Katori, T.; Yamashita, M.; Tanaka, J.; Doi, M.; Ishida, T. *J. Am. Chem. Soc.* **1990**, *112*, 3710–3712.

54. Zabriskie, T. M.; Klocke, J. A.; Ireland, C. M.; Marcus, A. H.; Molinski, T. F.; Faulkner, D. J.; Xu, C.; Clardy, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3123–3124.

55. Crews, P.; Manes, L. V.; Boehler, M. *Tetrahed. Lett.* **1986**, *27*, 2797–2800.

56. Luibrand, R. T.; Erdman, T. R.; Vollmer, J. J.; Scheuer, P. J.; Finer, J.; Clardy, J. *Tetrahedron* **1979**, *35*, 609–612.

57. Blackburn, C. L.; Hopmann, C.; Sakowicz, R.; Berdelis, M. S.; Goldstein, L. S. B.; Faulkner, D. J. *J. Org. Chem.* **1999**, *64*, 5565–5570.

58. Keifer, P. A.; Rinehart, K. L.; Hooper, I. R. *J. Org. Chem.* **1986**, *51*, 4450–4454.

59. Walker R. P.; Faulkner, D. J. *J. Org. Chem.* **1981**, *46*, 1098–1102.

60. Kashman, Y.; Zviely, M. *Experientia* **1980**, *36*, 1279–1279.

61. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. *Microbiology* **1999**, *145*, 283–291.

62. Gerwick, W.; Fenical, W.; Fritsch, N.; Clardy, J. *Tetrahed. Lett.* **1979**, 145.

63. O'Brien, E. Timothy; W., Steven; J., Robert S.; Boder, G. B.; Wilson, L. *Hydrobiologia* **1984**, 116-117.

64. Abbott, I. A. and Dawson, E. Y. *In How to Know the Seaweeds*, 2nd Ed., WCB McGraw-Hill: Boston, **1987**, pp. 1-19.

65. O'Clair, R. M.; Lindstorm, S.C. *In North Pacific Seaweeds*, Plant Press: Alaska, **2000**, pp. 1-8.

66. Sze, P. *A Biology of the Algae*, 3rd Ed., WCB McGraw-Hill: Boston, **1998**.

67. Davies, J. *Mol. Microbiol.* **1990**, *4*, 1227-1232.

68. Stone, M. J.; Williams, D. H. *Mol. Microbiol.* **1992**, *6*, 29-34.

69. Herms, D. A.; Mattson, W. J. *Quart. Rev. Biol.* **1992**, *67*, 283-335.

70. Proksch, P. *Toxicon* **1994**, *32*, 639-655.

71. Pawlik, J. R.; Kernan, M. R.; Molinski, T. F.; Harper, M. K.; Faulkner, D. J. *J. Exp. Mar. Biol. Ecol.* **1988**, *119*, 99-109.

72. Hay, M. E. *J. Exp. Mar. Biol. Ecol.* **1996**, *200*, 103-134.

73. Paul, V. J. *Seaweed chemical defenses on coral reefs, in Ecological roles for*

marine natural products (ed. Paul, V. J.), Comstock Publishing Associates, Ithaca, **1992**, 24-50.

74. Cronin, G.; Hay, M. E. *Ecology* **1996**, *77*, 2287-2301.

75. Toth, G. B.; Pavia, H. *Proc. Nat. Acad. Sci. USA* **2000**, 97,14418-14420.

76. Nagle, D. G.; Paul, V. J. *J. Phycol.* **1999**, *35*, 1412-1421.

77. Thacker, R. W.; Nagle, D. G.; Paul, V. J. *Mar. Ecol. Prog. Ser.* **1997**, *167*, 21-29.

78. Pennings, S. C.; Pablo, S. R.; Paul, V. J. *Limnol. Oceanog.* **1997**, *42*, 911-917.

79. Nagle, D. G.; Paul, V. J. *J. Exp. Mar. Biol. Ecol.* **1998**, *225*, 29-38.

CHAPTER TWO

NEW BIOLOGICALLY ACTIVE COMPOUNDS FROM THE MARINE BROWN ALGA *STYPOPODIUM FLABELLIFORME*

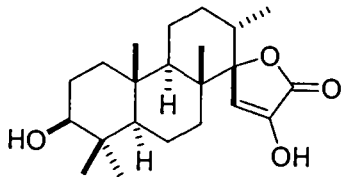
ABSTRACT

Using bioassay guided fractionation (brine shrimp toxicity assay), I isolated and identified five new, biologically active compounds [2β,3α-epitaondiol (**12**), flabellinol (**13**), flabellinone (**14**), stypoaldehyde (**15**) and stypohydroperoxide (**16**)], together with five known compounds (2-geranylgeranyl-6-methyl-1, 4-benzoquinone, (-) epistypodiol, (-) stypoldione, fucoxanthin and iditol) from the marine brown alga *Stypopodium flabelliforme,* collected from Papua New Guinea. All of the new compounds were found to have cytotoxic activity (EC_{50} ranges from 0.3 – 10 μg/ml) in human lung cancer (NCI-H460) and mouse neuro-2a cell lines. 2β,3α-epitaondiol and flabellinol exhibited strong sodium channel blocking activity (EC_{50} = 0.3 and 0.9 μg/ml, respectively). Stypoaldehyde, as well as the previously known compound, stypoldione, effected a change in the calcium concentrations in rat neurons. The acetate derivatives of 3α-epitaondiol and flabellinol, were significally less active than the natural products. The molecular structures of the isolated compounds were determined by extensive analysis of their spectroscopic data (1D and 2D NMR, LRMS, HRMS, IR and UV). The relative stereochemistries of the compounds 2β,3α-epitaondiol, flabellinol, flabellinone, stypoaldehyde and stypohydroperoxide were determined by 1D and 2D NOE experiments. X-ray crystallography confirmed the relative stereochemistry of 2β,3α-epitaondiol. Absolute stereochemistry of the latter compound was detemined by the modified Mosher's ester method.

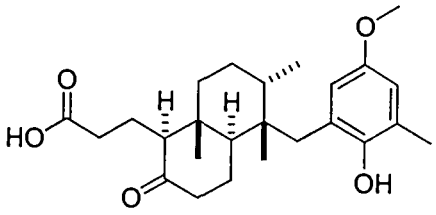
INTRODUCTION

Brown algae possess the ability to produce a great variety of secondary metabolites with different skeletal types and functionalities.[1] Among this group are the brown algae *Stypopodium flabelliforme* and *S. zonale* which are well known for their high yield of a unique series of cyclic terpenes of mixed biogenesis.[1-3] These components vary for a given species depending on collection, location and season.[3-5] Recent research on this genus has yielded a number of metabolites that display diverse bioactivities. Stypolactone (**1**)[5] and atomaric acid (**2**)[2] have cytotoxicity against human lung and colon carcinoma while 14-keto-stypodiol diacetate (**3**) from *S. flabelliforme* showed inhibition of microtubule assembly.[6] Stypodiol (**4**), epistypodiol (**5**), stypotriol (**6**) and taondiol (**7**) have ichthyotoxic activity.[2] Diacetylepitaondiol (**8**), a diterpenoid from the alga, has a negative inotropic effect on the isolated rat atrium.[7] Stypoquinonic acid (**9**) and atomaric acid (**2**) showed inhibition of tyrosine kinase.[8] Stypoldione (**10**) showed many biological activities such as inhibition of cell division activity,[9] inhibition of the division of sea urchin embryo activity[10] and ichthyotoxic activity.[1] Isoepitaondiol (**11**) is reported to have an insecticidal activity.[11]

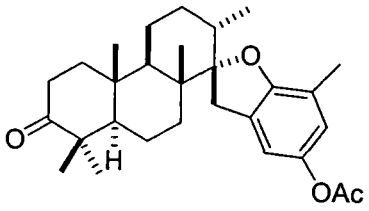
As a result, I have targeted this organism for isolation of additional biologically active compounds. A bioassay-guided examination of the lipid extract from the marine brown alga *S. flabelliforme* collected from Papua New Guinea (December 1999) led to the discovery of five novel diterpenoids (**12-16**). This work describes the structural characterization and biological activities of these five novel *Stypopodium* metabolites.



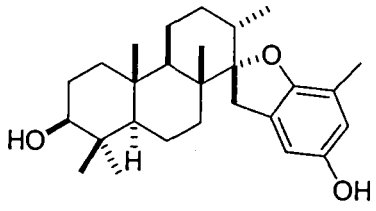
Stypolactone (1)



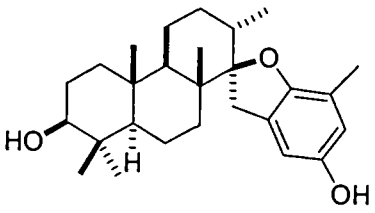
Atomaric acid (2)



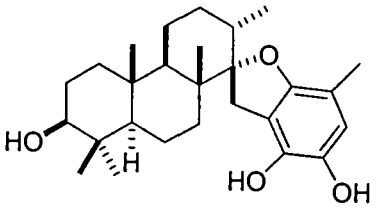
14-Keto-stypoldiol acetate (3)



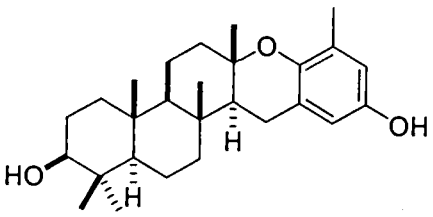
Stypoldiol (4)



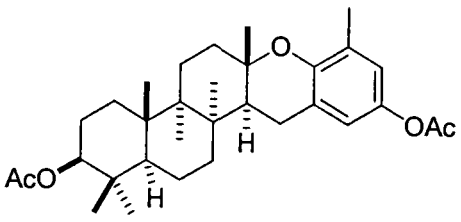
Epistypoldiol (5)



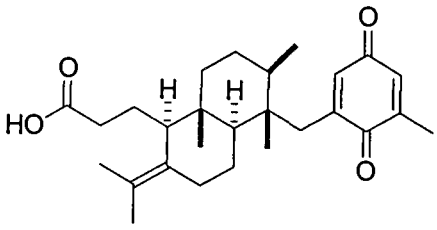
Stypotriol (6)



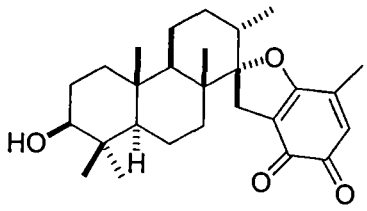
Taondiol (7)



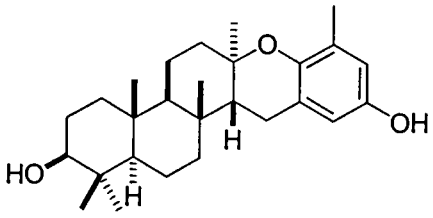
Diacetylpitaondiol (8)



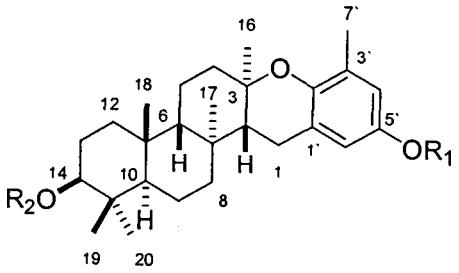
Stypoquinonic acid (9)



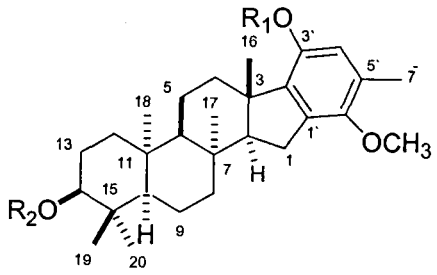
Stypoldione (10)



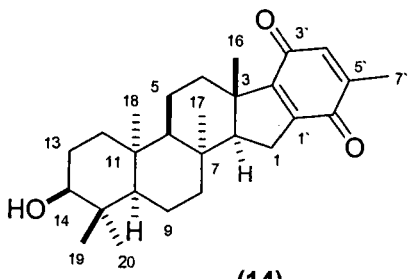
Isoepitaondiol (11)



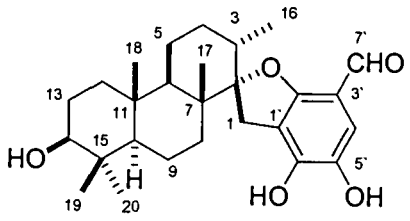
(12) R1, R2 = H
(17) R1 = H R2 = OAc
(18) R1 = OAc R2 = OAc



(13) R1, R2 = H
(19) R1, R2 = OAc



(14)



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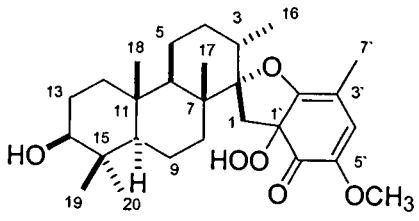

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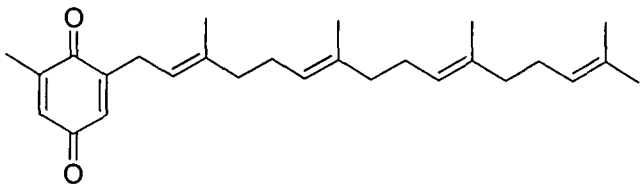
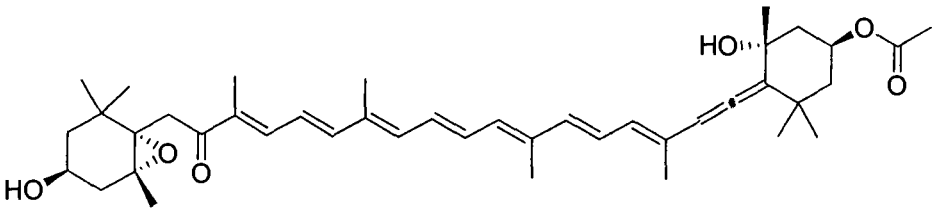
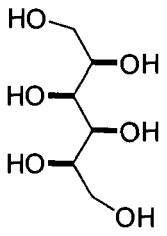


2-geranylgeranyl-6-methyl-1, 4-benzoquinone (20)



Fucoxanthin (21)



Iditol (22)

RESULTS AND DISCUSSION

In my continuing efforts using bioassay guided fractionation to characterize the prolific natural products from marine algae, I investigated the crude organic extract of *Stypopodium flabelliforme* from South Pacific collections, which have never been studied previously. *S. flabelliforme* was collected by hand using SCUBA (45-55 feet) of water near the Papua New Guinea coast (December 1999). After extraction of the alcohol-preserved tissue with CH_2Cl_2/MeOH (2:1), initial fractionation was accomplished by VLC (EtOAc/hexanes gradient) over silica gel. Successive normal phase HPLC, resulted in the isolation of five new compounds (**12-16**), in addition to five known compounds [2-geranylgeranyl-6-methyl-1, 4-benzoquinone (**20**); (-) epistypodiol (**5**); (-) stypoldione (**10**); fucoxanthin **(21)** and iditol **(22)**[12]].

Spectral analysis of the isolated compounds (UV, IR, LRMS, HRMS, 1D and 2D NMR) allowed for construction of their planar structures while 1D and 2D NOE experiments were used to determine relative stereochemistries. X-ray crystallography was performed on compound **12** to confirm the relative stereochemistry. The absolute stereochemistry of the latter compound was determined using the modified Mosher method. Mono and diacetate derivatives of compound **12** (**17-18**) and a diacetate derivative of compound **13** (**19**) were prepared for biological activity testing.

Compound **12** showed a HRFABMS $[M+H]^+$ ion at *m/z* 413.2977 for a molecular formula of $C_{27}H_{40}O_3$, and therefore possessed eight degrees of unsaturation. ^1H NMR experiments, were carried out in CD_3OD, C_6D_5N and $CDCl_3$ to provide good chemical shift resolution. ^1H NMR signals were well dispersed in C_6D_5N. The data (Table II.1) collected in this solvent indicated the presence of two free aromatic protons meta to each other at δ_H 7.0 and 6.95, one phenolic hydroxy group (δ_H 5.8), and one alcoholic hydroxy group (δ_H 3.66), and six singlet methyls located on quaternary carbons (δ_H 2.34, 1.26, 1.21, 1.11, 1.07 and 0.99). Analysis of ^{13}C NMR (Table II.1), DEPT135 and DEPT90 spectra in C_6D_5N revealed the presence of eight quaternary carbons (δ_C 149.0, 146.9, 129.4, 138.2, 136.1, 39.6, 38.9, 45.8 and 38.5), six methines (δ_C 117.1, 114.6, 78.9, 46.5, 55.2, 54.5), seven methylenes (δ_C 42.0, 39.6, 34.0,30.4, 23.8, 20.8, 18.1), and six methyl groups (δ_C 30.1, 22.8, 21.4, 20.9, 17.3, 17.1). After assigning all proton resonances to their directly bonded carbon atoms, aided by HSQC data, it was possible to deduce four molecular fragments using ^1H-^1H COSY spectra (Figure II.6). From the ^1H-^1H COSY spectrum of **12** four ^1H-^1H spin systems could be deduced. Coupling was observed between H_3-7` and H-4`, H-4` further coupled to H-6` and also H-6 coupled to

H-1. Furthermore, couplings were observed between H-2 and H_2-1 (fragment **a**, figure II.6). Another spin system was shown by the coupling between H_a-4 and H_a-5, and H-6` further coupled to H_b-5 (fragment **b**, Figure II.6). Moreover, couplings were observed between H_a-8 and H_a-9, H_2-9 and H-10 to give fragment **c** (Figure II.6). Couplings between H_2-12 and H_2-13, between H_2-12 and H-14 and between the proton of the hydroxy group at position 14 and H-14 afforded fragment **d**. This information in conjunction with the interpretation of the HMBC spectrum finally led to the planar structure of **12** (Figure II.7). Thus, starting with fragment **d**, cross-peaks in the HMBC spectrum were seen between H_2-12/C-10, C-11 and C-13. Further, HMBC cross-peaks between H-14 and C-13, C-14, C-15, C-19 and C-20, H_3-19 and C-10, C-13, C-14, C-15 and C-20, in addition cross-peaks between H_3-20 and C-10, C-13, C-14, C-15 and C-19 extended fragment **d**. Heteronuclear multiple bond couplings were also seen from H-18 to C-11, C-12, C-13, C-10, C-9 and C-6. H-6 correlation with C-11, C-17 and C-5 was observed. Another correlations were observed between H_3-17 and C-7, C-8 and C-2. Fragments **d**, **c** and **b** as deduced from ^{1}H-^{1}H COSY and HMBC could now be connected to extended fragment **a** between C-12, C-11and C-6 and also connected C-14, C-15, C-10, C-9, C-8, C-7 and C-2 to give the bicyclic ring. Also, as deduced from ^{1}H-^{1}H COSY and HMBC, fragment **b** could now be connected to extended fragment **d** Further cross-peaks in the HMBC spectrum were shown between H_3-16/C-3, C-4 and C-2, the latter showing coupling with H_2-1, enabled fragment **a** to be placed with the extended fragment **d** to give the planar structure of compound **12** (Figure II.7).

The planar structure of **12** was found to be the same as that of taondiol (**7**)[13], epitaondiol (**23**)[14], isotaondiol (**24**)[15] and isoepitaondiol (**11**)[11]. By comparing ^{1}H NMR and ^{13}C NMR data of the isolated compound and those of the above mentioned compounds, I noticed significant differences in the NMR data. I concluded that this compound is a new isomer of the previously reported compounds. Determination of the relative stereochemistry of **12** was carried out by full investigation of 1D and 2D NOE experiments. The relative stereochemistry was confirmed by carrying out an X-ray crystallographic experiment on this compound [I acknowledge Dr. Alex. Yokochi for carrying out the X-ray crystallography] (Figure II. 8).

The absolute stereochemistry of compound **12** was determined using the modified Mosher method.[16-18] Acylation of **12**, in separate experiments, with *R*-(-)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride yielded the expected product from *R*(-)-MTPA chloride, the C-14, C-5` *bis S* ester and from (*S*)-MTPA chloride, the C-14, C-5`

bis R ester.The products were purified by normal-phase HPLC in each case, and the chemical shifts of the two diesters were analyzed separately by ^{1}H NMR spectroscopy. The *bis* C-14 *S*, C-5`*S* and C-14 *R*, C-5`*R* esters were examined for their ^{1}H NMR shifts. The esters showed negative values $\Delta(\delta_H S\text{-}\delta_H R)$ for H-12, H-13 and H-18 and positive values for H-9, H-10, H-19 and H-20 (Table II. 2) and (Figure II.10a, 10b and 10c). Interestingly, the same trend was observed during examination of the ^{13}C NMR shifts of both of the two Mosher ester derivatives, in fact, bigger overall $\Delta\delta$ values were observed in the ^{13}C NMR spectra as versus the ^{1}H NMR spectra (Figure II.10d). The esters showed negative values $\Delta(\delta_C S\text{-}\delta_C R)$ for C-12 and C-13 and positive values for C-6, C-9, C-10, C-19 and C-20. Measuring $\Delta(\delta_C S\text{-}\delta_C R)$ may be useful in the future in addition to measuring $\Delta(\delta_H S\text{-}\delta_H R)$ to confirm the absolute stereochemistry of different compounds, especially with the great advances in NMR probe technology such as microprobes and microcoils. This is the first time that absolute stereochemistry has been determined for taondiol or one of it's isomers. However, total synthesis of stypoldione (**10**) has led to the same absolute stereochemistry in this structure class.[19] By the aid of X-ray crystallography[20] and modified Mosher's ester data, the chiral centers at C-2, C-3, C-6, C-7, C-10, C-11 and C-14 were then assigned the following absolute streochemistry *R, R, S, S, R,* and *S,* respectively (Figure II. 10a and 10b).

Table II.1. ^{1}H- and ^{13}C-NMR Data for (**12**) in C_6D_5N[a]

Position	δ_H mult. *J* (Hz)	δ_C	HMBC[b]
1	2.58 dd (12, 7)	23.8 CH_2	C-1', C-2
	2.56 dd (12, 5)		C-1', C-2
2	1.55 m	54.5 CH	C-3, C-7
3	----------	76.6 C	
4	2.09 m	42.0 CH2	C-3, C-5
	1.67 m		C-3, C-7
5	1.54 m	20.8 CH2	C-4, C-6
	1.30 m		
6	1.20 dd (12, 6)	55.2 CH	C-5, C-7, C-11
7	----------	37.5 C	
8	1.50 d (14.2)	39.6 CH2	C-7, C-9
	1.38 d (14.2)		
9	1.60 m	18.1 CH2	C-8, C-10
	1.36 m		
10	1.64 dd (11.7, 5)	46.5 CH	C-9, C-11
11	----------	36.6 C	
12	1.95 2H, m	30.4 CH2	C-10, C-11, C-13
13	1.4 2H, m	34.0 CH2	C-12, C-14
14	3.48 dd (17,5)	78.9 CH	C-13, C-15, C-19, C-20
15	----------	40.2 C	
16	1.21 (3H, s)	21.4 CH3	C-2, C-3, C-4
17	1.11 (3H, s)	20.9 CH3	C-2, C-6, C-7, C-8
18	0.99 (3H, s)	22.8 CH3	C-6, C-9, C-10, C-12, C-13
19	1.26 (3H, s)	30.1 CH3	C-10, C-13, C-14, C-15, C-16
20	1.07 (3H, s)	17.3 CH3	C-10, C-13, C-14, C-15, C-17
1'	----------	123.6 C	
2'	----------	145.3 C	
3'	----------	127.1 C	
4'	7.00 d (2.75)	117.1 CH	
5'	----------	151.9 C	
6'	6.95 d (2.75)	114.6 CH	C-5', C-2
7'	2.34 (3H, s)	17.1 CH3	C-2', C-3', C-4'
OH (C-5`)	5.8		
OH (C-14)	3.66		

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

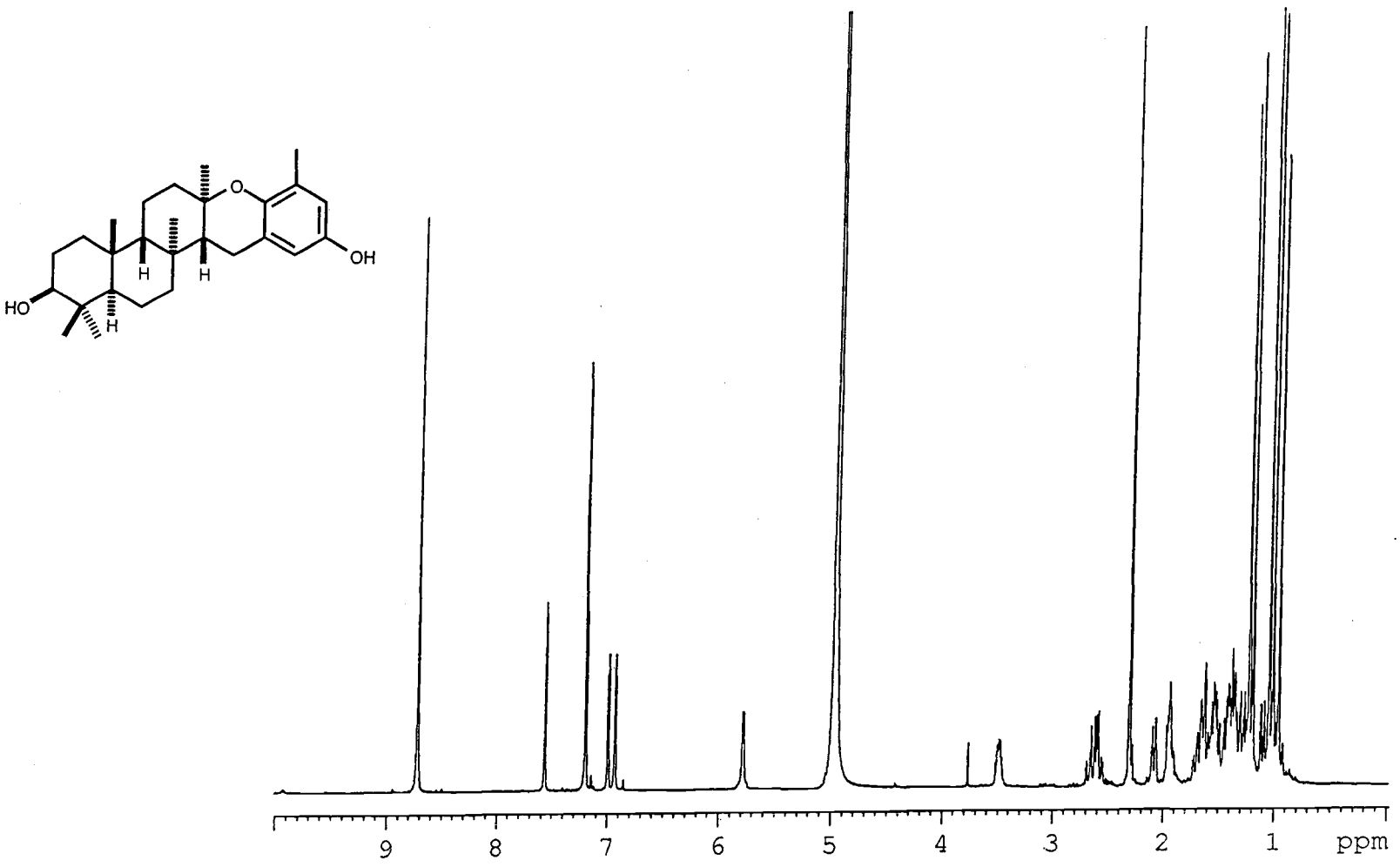


Figure II.1. ^{1}H NMR spectrum of (**12**) in C_6D_5N [400 MHz]

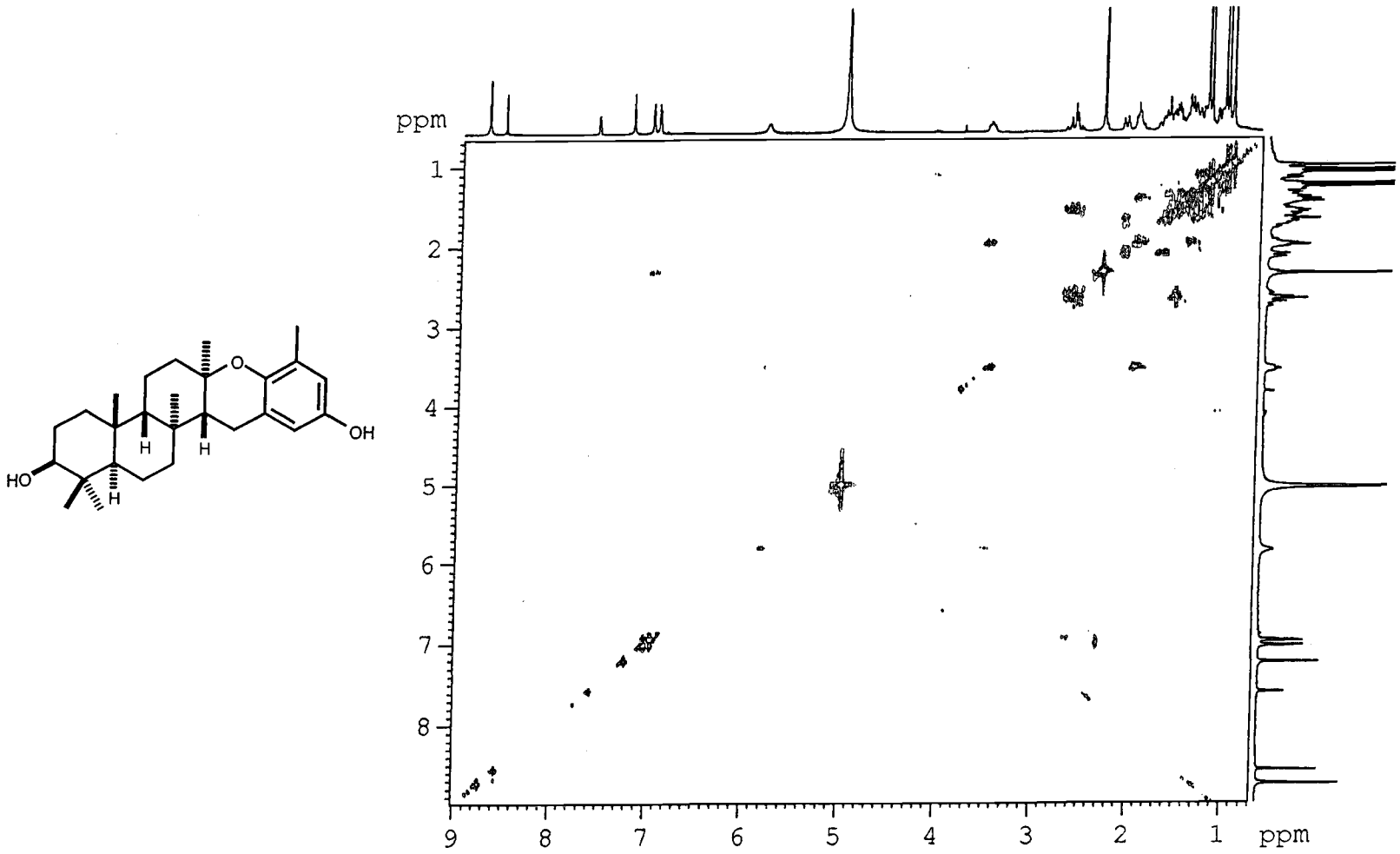


Figure II.2. ^{1}H-^{1}H COSY spectrum of (**12**) in C_6D_5N

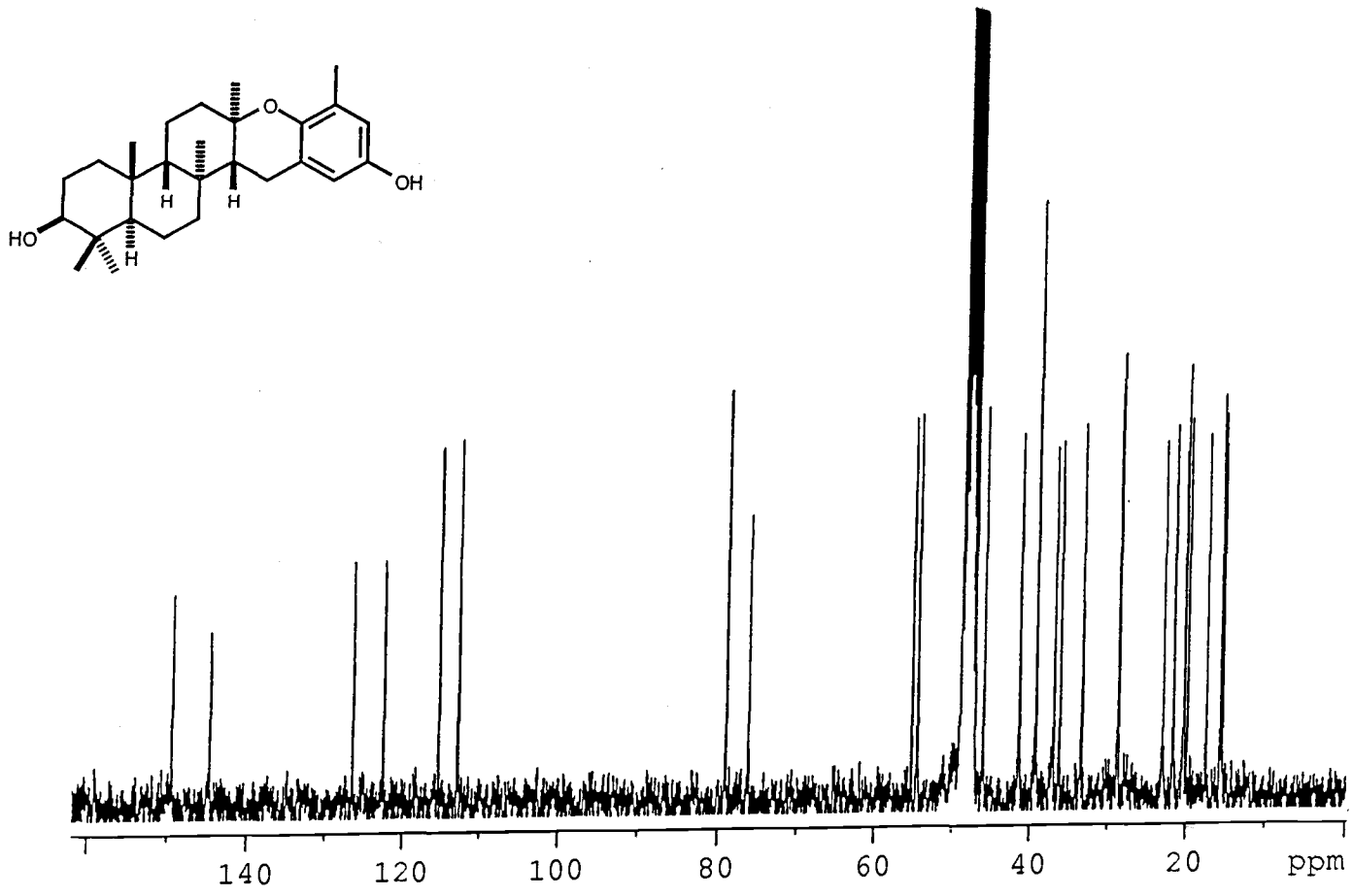


Figure II.3. ^{13}C NMR spectrum of (**12**) in CD_3OD

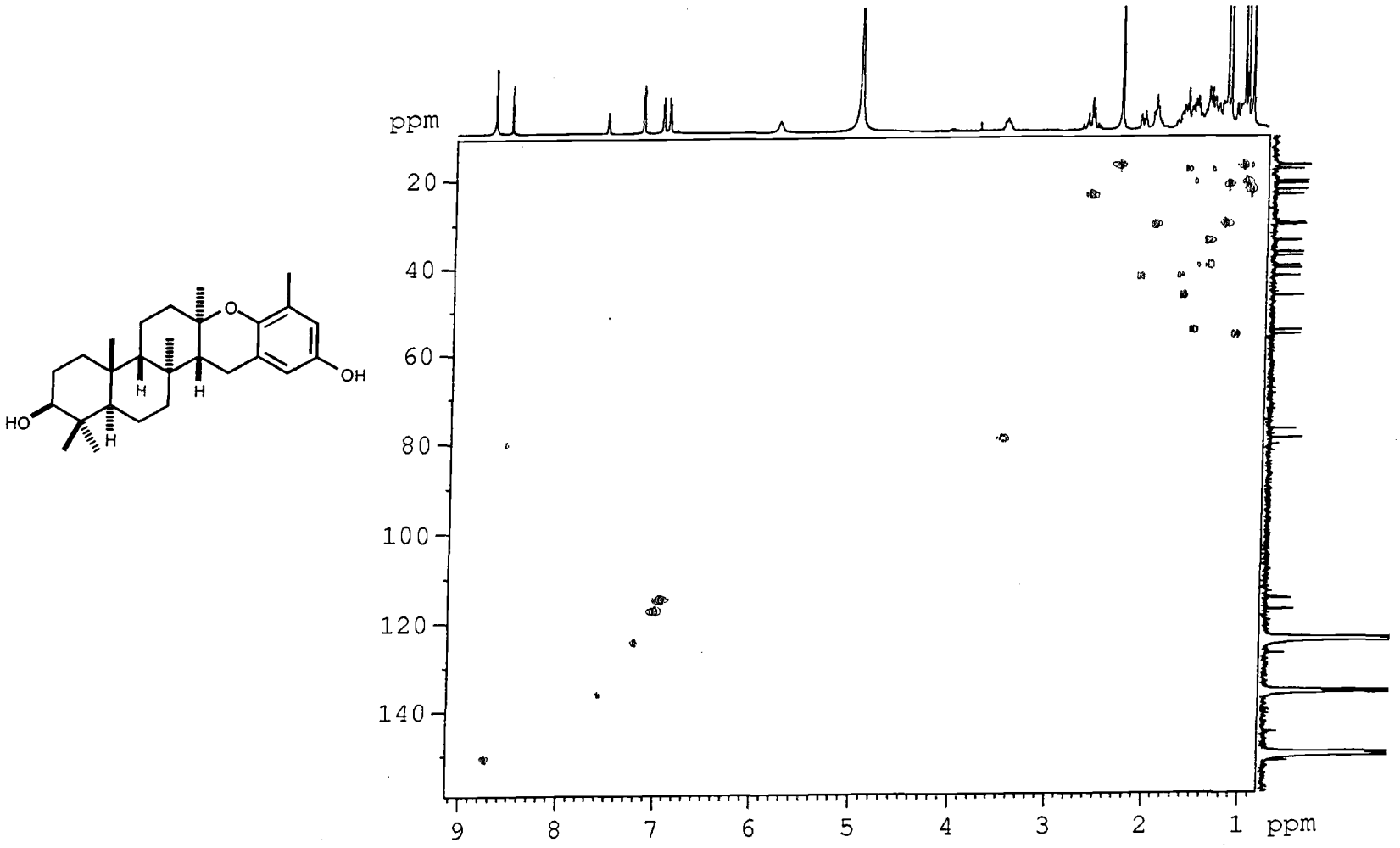


Figure II.4. HSQC spectrum of (**12**) in C_6D_5N

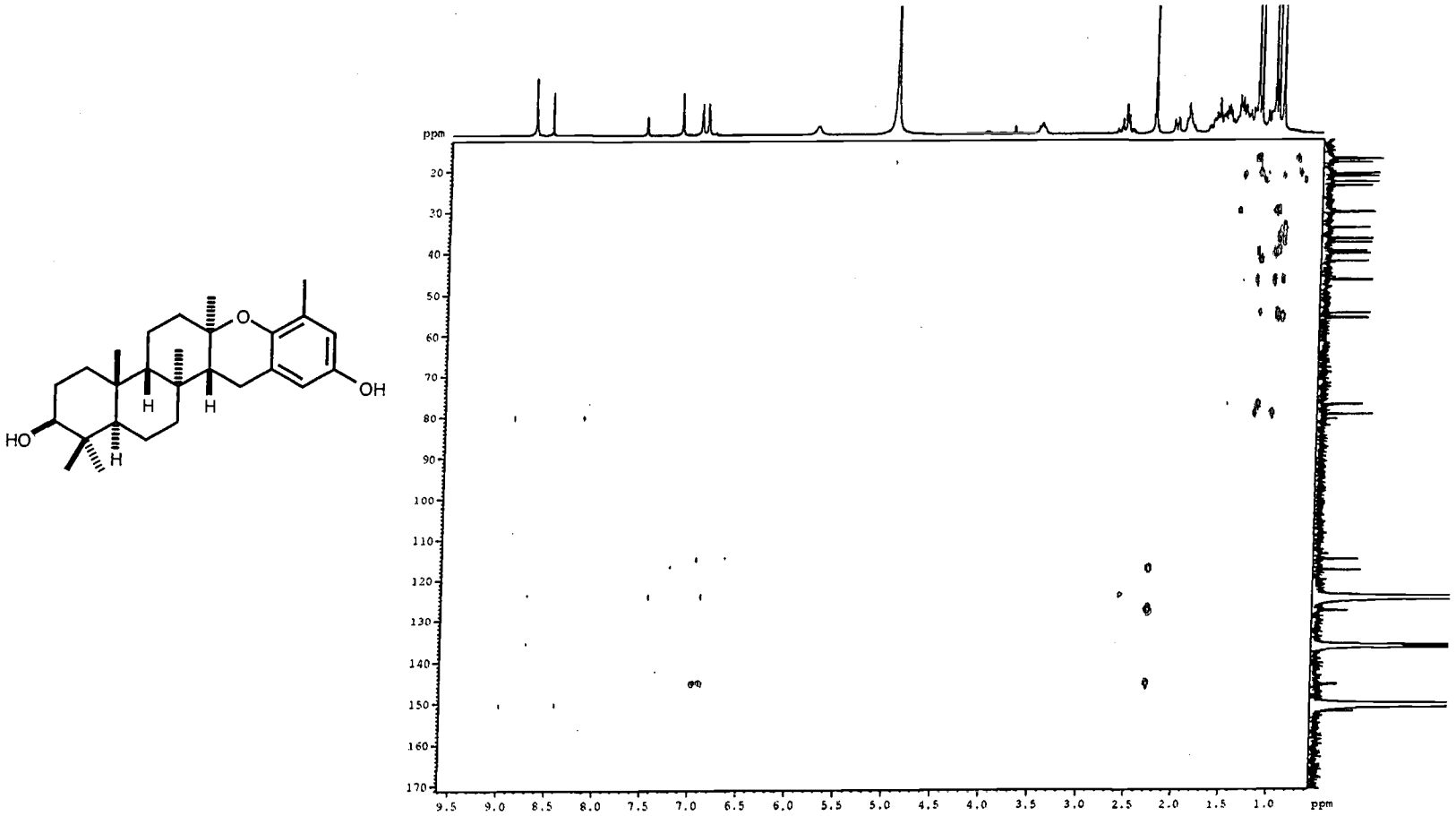


Figure II.5. HMBC spectrum of (**12**) in C_6D_5N

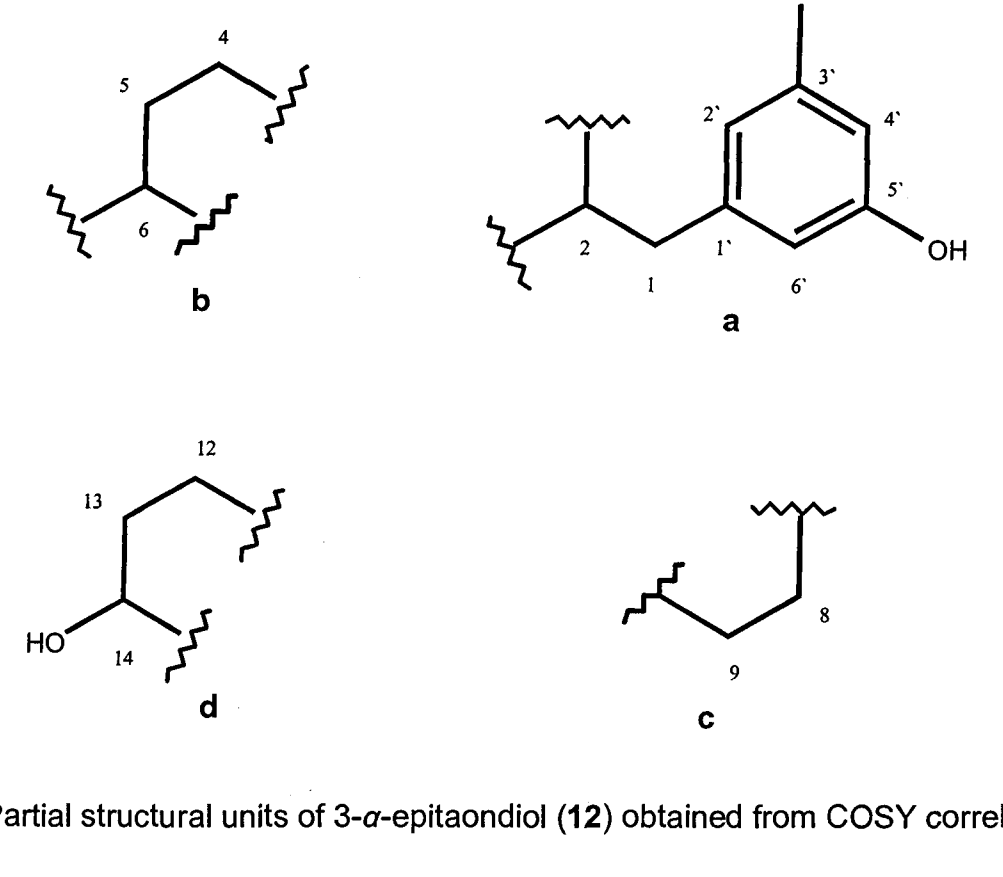


Figure II.6. Partial structural units of 3-*α*-epitaondiol (**12**) obtained from COSY correlations

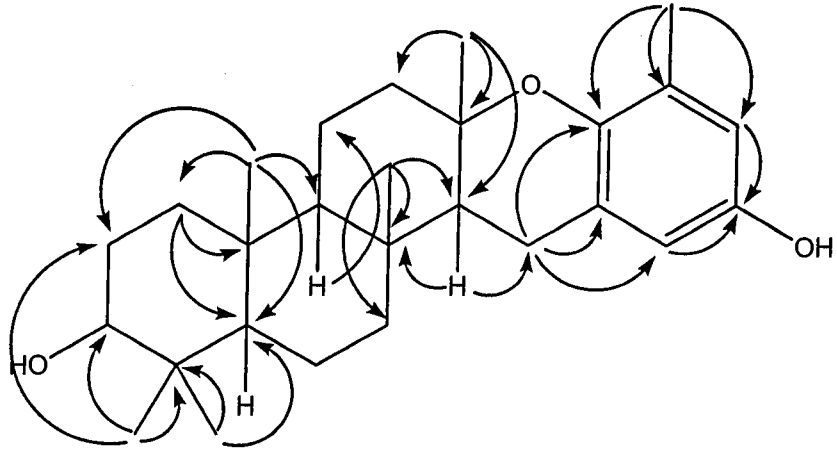
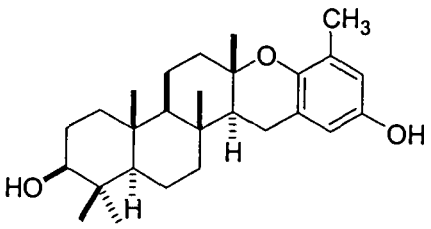
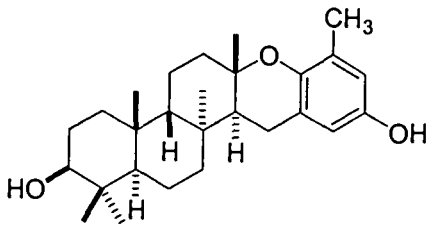


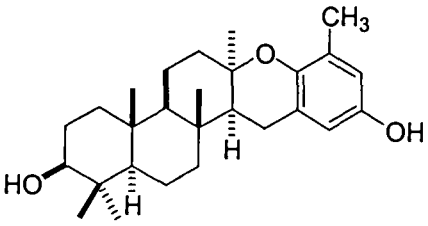
Figure II. 7. HMBC correlations of 2β,3α-epitaondiol (**12**)



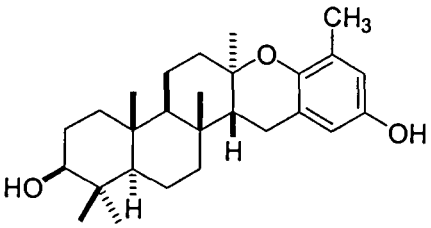
Taondiol (7)



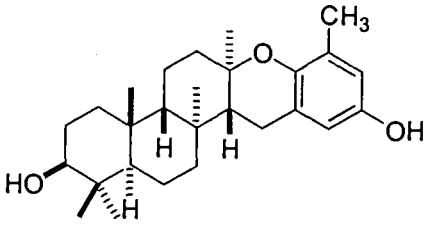
Epitaondiol (23)
(Revised structure)[14]



Isotaondiol (24)



Isoepitaondiol (11)



2β,3α-epitaondiol (12)

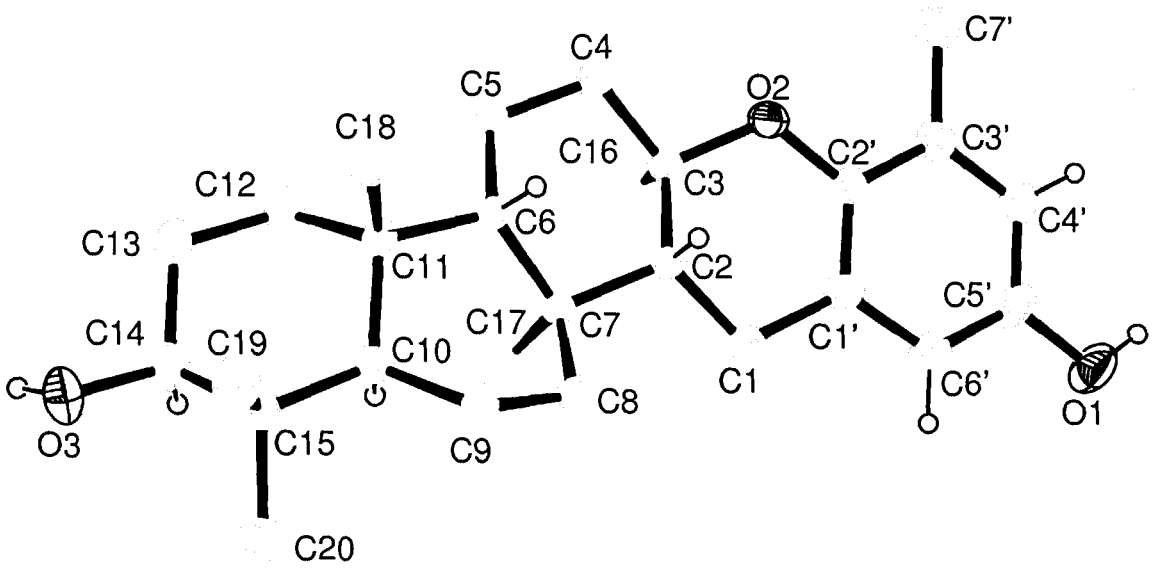


Figure II. 8. ORTEP of compound **12** with 30% displacement ellipsoids.

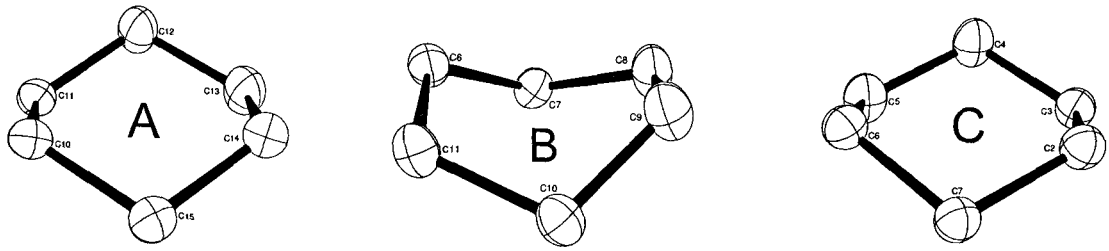


Figure II. 9. Diagrams depicting the conformations of rings A, B and C in compound **12**.

Table II.2. Selected ^1H NMR Chemical Shifts and $\Delta\delta$ Values for Mosher Ester

δ			
Proton #	**(S) Mosher's ester**	**(R) Mosher's ester**	**Δδ (δS- δR)**
H-6	1.20	1.18	+ 0.02
H-10	1.68	1.66	0.02
H-14	4.73	4.76	- 0.03
H-12	1.53	1.56	- 0.03
H-13b	1.8	1.6	-0.2
H_3-18	0.96	0.99	- 0.03
H_3-19	0.815	0.80	0.01
H_3-20	0.91	0.84	0.07

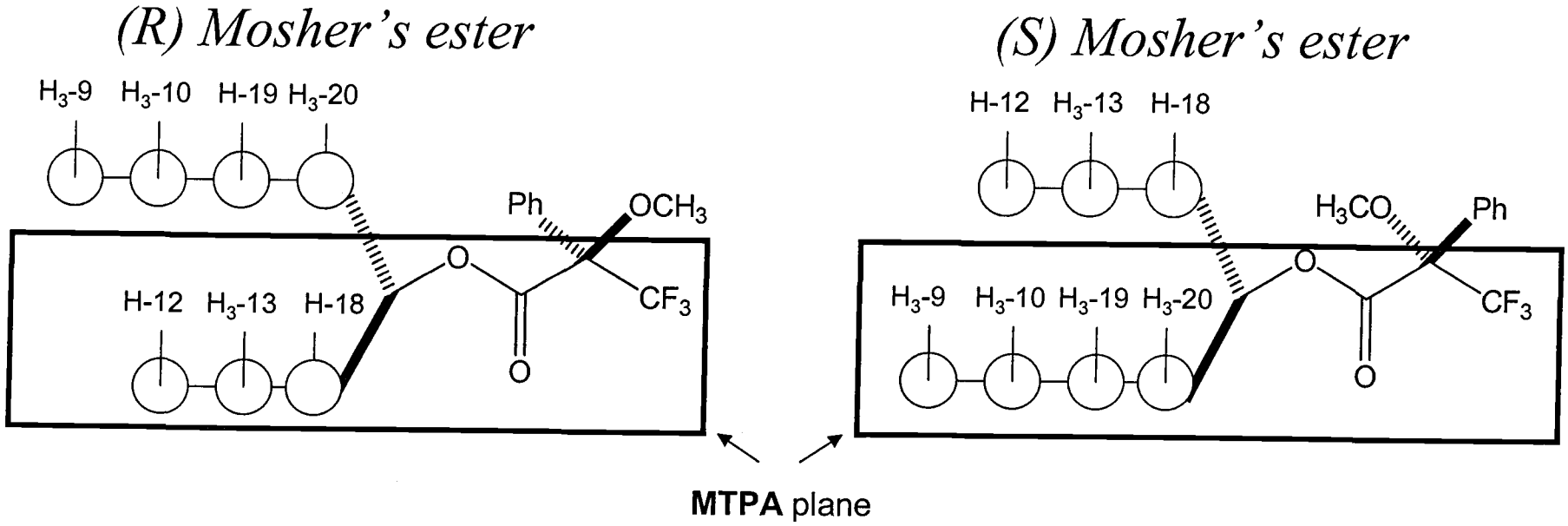


Figure II.10a. Idealized conformation of the MTPA Ester and the MTPA plane

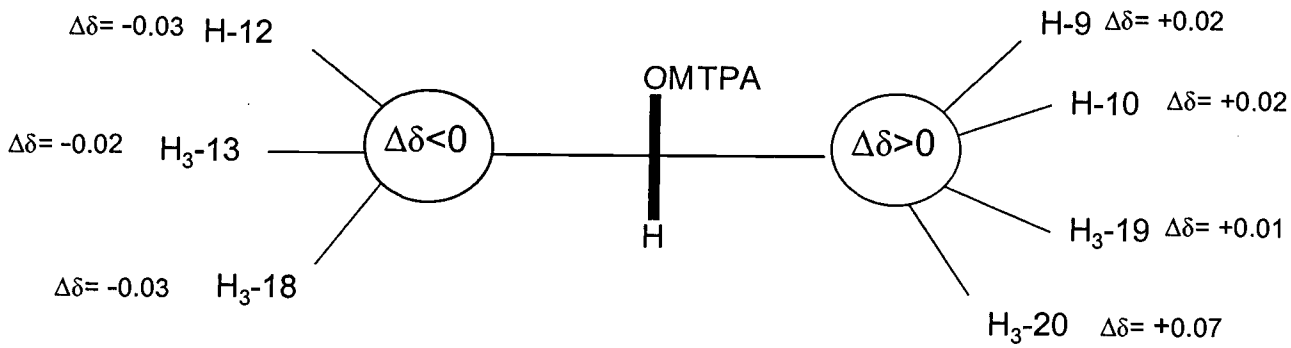
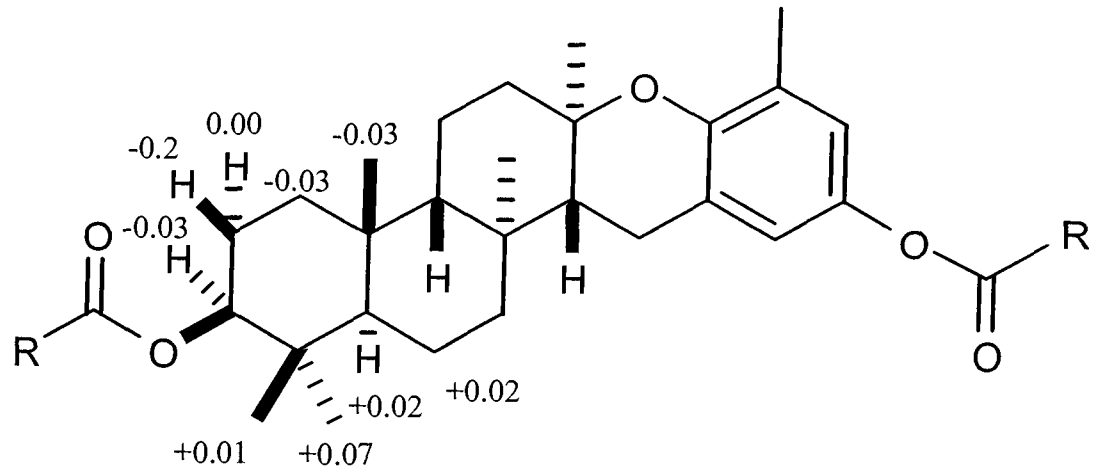


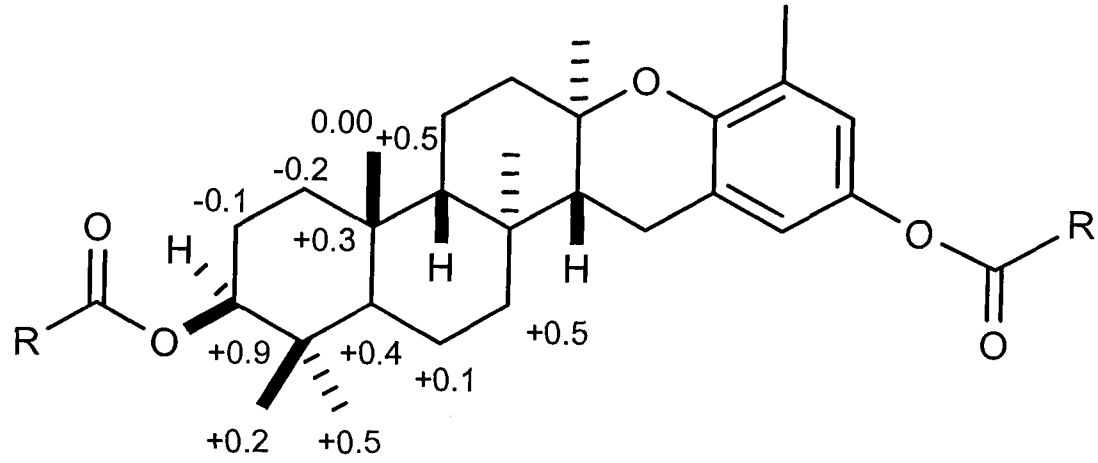
Figure II.10b. Model for determination of the absolute stereochemistry of secondary alcohols

$\Delta\delta_H$ (δS- δR)



Figurell.10c. Selected $\Delta\delta_H$ Values for Mosher ester derivative of compound **12**

$\Delta\delta_C$ (δS- δR)



Figurell.10d. Selected $\Delta\delta_C$ Values for Mosher ester derivative of compound **12**

Compound **13** showed a HRFABMS $[M+H]^+$ ion at *m/z* 427.3126 for a molecular formula of $C_{28}H_{42}O_3$, and therefore possessed eight degrees of unsaturation. The IR spectrum showed absorption bands at 3602, 3375 cm^{-1}, indicating the presence of two hydroxy group functionalities. Both ^1H NMR and ^{13}C NMR signals were well dispersed in $CDCl_3$ (see table II. 3, 4). These NMR data indicated the presence of one free aromatic proton at δ_H 6.39 (3H, s), one alcoholic hydroxy group (δ_H 4.37) and six singlet methyl groups located on quaternary carbons (δ_H 2.2, 1.25, 1.22, 1.19, 1.02 and 0.82). Analysis of ^{13}C NMR (Table II.4), DEPT135 and DEPT90 spectra revealed the presence of eight quaternary carbons (δ_C 149.3, 146.9, 129.4, 138.2, 136.1, 39.6, 38.9, 45.8 and 38.5), six methines (δ_C 117.1, 114.6, 78.9, 46.5, 55.2, 54.5), seven methylenes (δ_C 42.0, 39.6, 34.0,30.4, 23.8, 20.8, 18.1), and six methyl groups (δ_C 30.1, 22.8, 21.4, 20.9, 17.3, 17.1).

Two-dimensional NMR experiments, including HSQC and ^1H-^1H COSY were mainly used for assigning the partial structures. The partial structures were connected from HMBC data (Figure II.15). Determination of the relative stereochemistry of **13** was carried out by 1D and 2D NOE experiments. From the 1D NOE experiments (Table II.4) on protons at δ2.59 and δ2.88, it is evident that irradiation of each of these protons causes enhancement of CH_3-16 and CH_3-17, respectively. This is only possible if the CH_3-16 and CH_3-17 are on opposite faces of the molecule. The proton at δ1.80 (H-2) and the methyl at C16 must also be *trans* in order for irradiation of the proton at δ2.88 (H-1) to cause enhancement both at resonance δ1.80 (H-2) and resonance δ1.25 (H-17). The correlation network between the protons at δ1.22 (H_3-18), δ3.22 (H-14), δ1.13 (H-10), δ1.10 (H-6), δ 0.82 (H_3-19) and δ1.25 (H_3-17) suggest that these protons are on the same face. A correlation in the 2D NOE spectrum between H-6 and H-2 suggests that these two protons are also on the same face (Figure II.16, 17).

Table II.3. ^{1}H NMR Data for **13-16** [400 MHz].

Position	**13**[a] δ_H mult. *J* (Hz)	**14**[a] δ_H mult. *J* (Hz)	**15**[b] δ_H mult. *J* (Hz)	**16**[a] δ_H mult. *J* (Hz)
1	2.88 dd (15, 7)	2.67 dd (15, 7)	3.16 d (14.3)	3.01 d (14.3)
	2.59 dd (15, 7)	2.36 dd (15, 7)	2.70 d (14.3)	2.57 d (14.3)
2	1.80 m	1.70 m		
3			1.86 m	1.72 m
4	2.34 d (5)	2.33 d (5)	1.63 d (15.4)	1.63 d (15.4)
	1.72 d (5)	1.14 d (5)	1.58 *m*	1.56 m
5	1.86 2H, m	1.80 m	1.60 m	1.53 m
		1.60 m	1.50 m	1.37 m
6	1.10 d (4)	1.05 d (4)	1.57 t (12, 5)	1.36 t (11, 5)
7				
8	1.57 d (7)	1.54 2H d (7)	1.79 m	1.03 2H, t (12, 5)
	1.50 d (7)		1.07 m	
9	1.90 m	1.86 m	1.51 m	1.61 m
	1.48 m	1.42 m	1.04 m	1.44 m
10	1.13 m	1.08 (1H, m)	0.79 t (11, 5)	0.77 t (11, 5)
11				
12	1.74 d (7)	1.69 d (7)	1.05 t (12, 5)	1.53 t (12, 5)
	1.16 d (7)	1.16 d (7)	1.39 t (12, 5)	1.38 t (12, 5)
13	1.71 m	1.68 2H, m	1.56 m	1.59 m
	1.66 m		1.53 m	1.47 m
14	3.22 m	3.22 m	3.17 dd (11, 5)	3.20 dd (11, 4)
15				
16	1.19 3H, s	1.11 3H, s	0.73 3H, d (6.8)	0.91 3H, d (6.8)
17	1.25 3H, s	1.18 3H, s	1.05 3H, s	0.93 3H, s
18	1.22 3H, s	1.18 3H, s	0.92 3H, s	0.85 3H, s
19	1.02 3H, s	0.99 3H, s	0.94 3H, s	0.98 3H, s
20	0.82 3H, s	0.77 3H, s	0.77 3H, s	0.79 3H, s
1'				
2'				
3'				
4'	6.39 s	6.37 s	6.9 s	5.9 (1H, s)
5'				
6'				
7'	2.20 3H, s	2.0 3H, s	9.9 3H, s	2.12 3H, s
OCH_3	3.73 3H, s			3.73 (3H, s)
OH (C-5`)			6.76 s	
OH (C-14`)	4.37			

[a] Spectrum obtained in $CDCl_3$
[b] Spectrum obtained in CD_3OD

Table II.4. ^{13}C-NMR Data for **13-16** [400 MHz]

Position	**13**[a] δ_C	**14**[a] δ_C	**15**[b] δ_C	**16**[a] δ_C
1	27.1 CH_2	27.1 CH2	31.0 CH_2	34.4 CH_2
2	61.6 CH	59.8 CH	99.5 C	98.8 C
3	45.8 C	47.0 C	37.4 CH	37.3 CH
4	37.4 CH_2	35.5 CH2	27.0 CH_2	30.9 CH_2
5	23.0 CH_2	22.7 CH2	20.4 CH_2	26.6 CH_2
6	57.5 CH	57.5 CH	51.8 C	51.2 C
7	38.5 C	38.3 C	42.0 CH	42.8 CH
8	19.5 CH_2	19.4 CH2	38.7 CH_2	38.6 CH_2
9	30.7 CH_2	30.6 CH2	17.9 CH_2	18.2 CH_2
10	48.0 CH	48.9 CH	55.4 C	55.3 C
11	38.9 C	38.9 C	37.2 CH	37.4 CH
12	36.2 CH_2	36.1 CH2	33.2 CH_2	27.7 CH_2
13	28.1 CH_2	28.1 CH2	31.2 CH_2	32.8 CH_2
14	79.7 CH	79.6 CH	78.5 CH	79.2 CH
15	39.6 C	39.0 C	38.9 C	39.2 C
16	20.3 CH_3	19.0 CH3	14.9 CH_3	17.6 CH_3
17	30.8 CH_3	30.8 CH3	16.7 CH_3	15.8 CH_3
18	26.9 CH_3	26.9 CH3	15.1 CH_3	16.6 CH_3
19	28.6 CH_3	28.6 CH3	27.5 CH_3	28.4 CH_3
20	15.9 CH_3	15.9 CH3	15.8 CH_3	15.7 CH_3
1'	136.1 C	148.0 C	110.0 C	103.0 C
2'	138.2 C	156.3 C	161.5 CH	166.0 CH
3'	129.4 C	186.6 C	114.1 C	143.8 C
4'	116.6 CH	133.9 CH	108.9 CH	122.8 CH
5'	146.9 C	145.0 C	140.2 C	165.0 C
6'	149.3 C	187.5 C	150.7 C	169.0 C
7'	16.1 CH_3	15.9 CH3	187.2 CH	24.2 CH_3
OCH_3	60.6 CH_3			56.0 CH_3

[a] Spectrum obtained in $CDCl_3$
[b] Spectrum obtained in CD_3OD

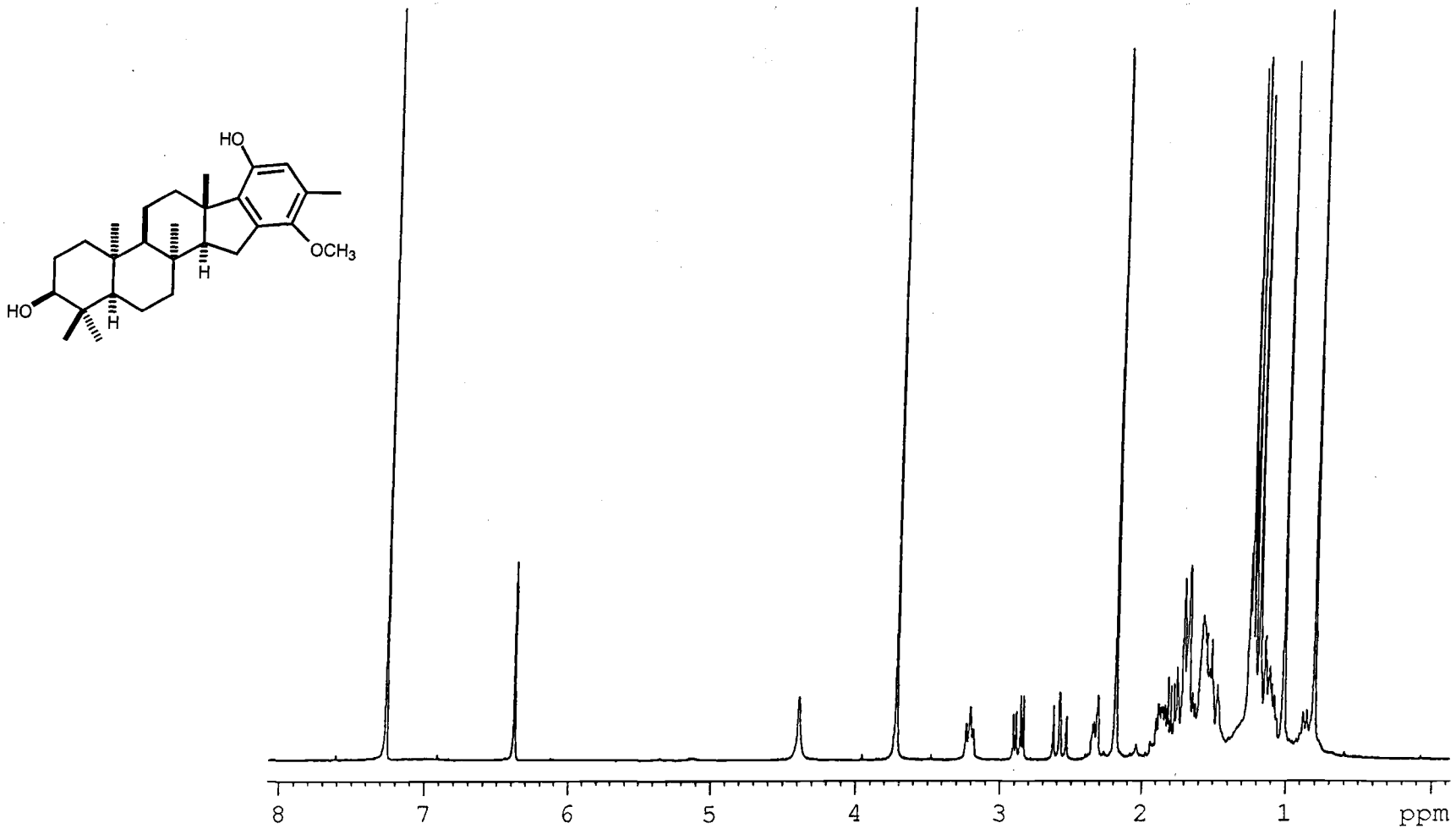


Figure II.11. ^{1}H NMR spectrum of (**13**) in $CDCl_3$ [400 MHz]

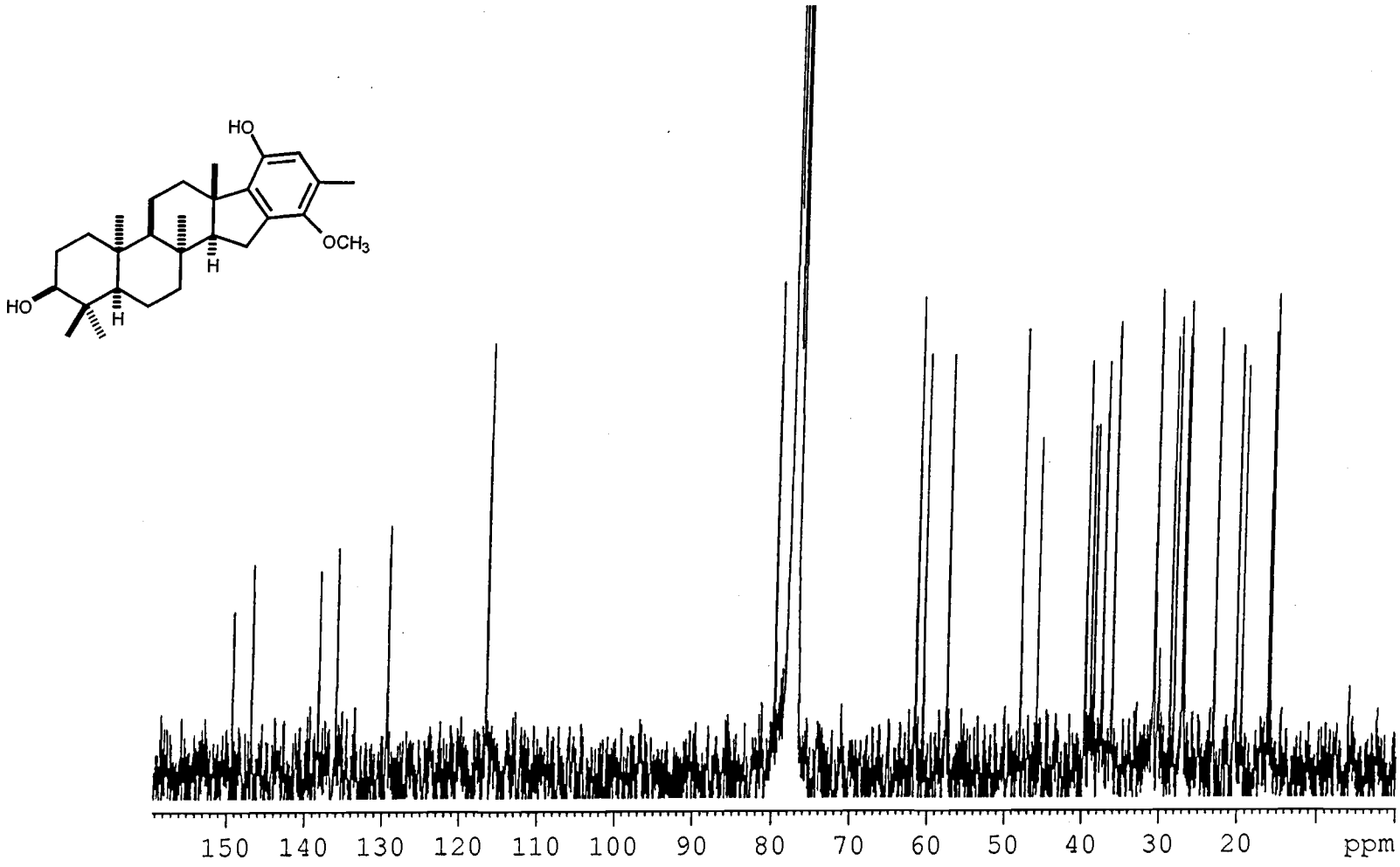


Figure II.12. ^{13}C NMR spectrum of (**13**) in $CDCl_3$

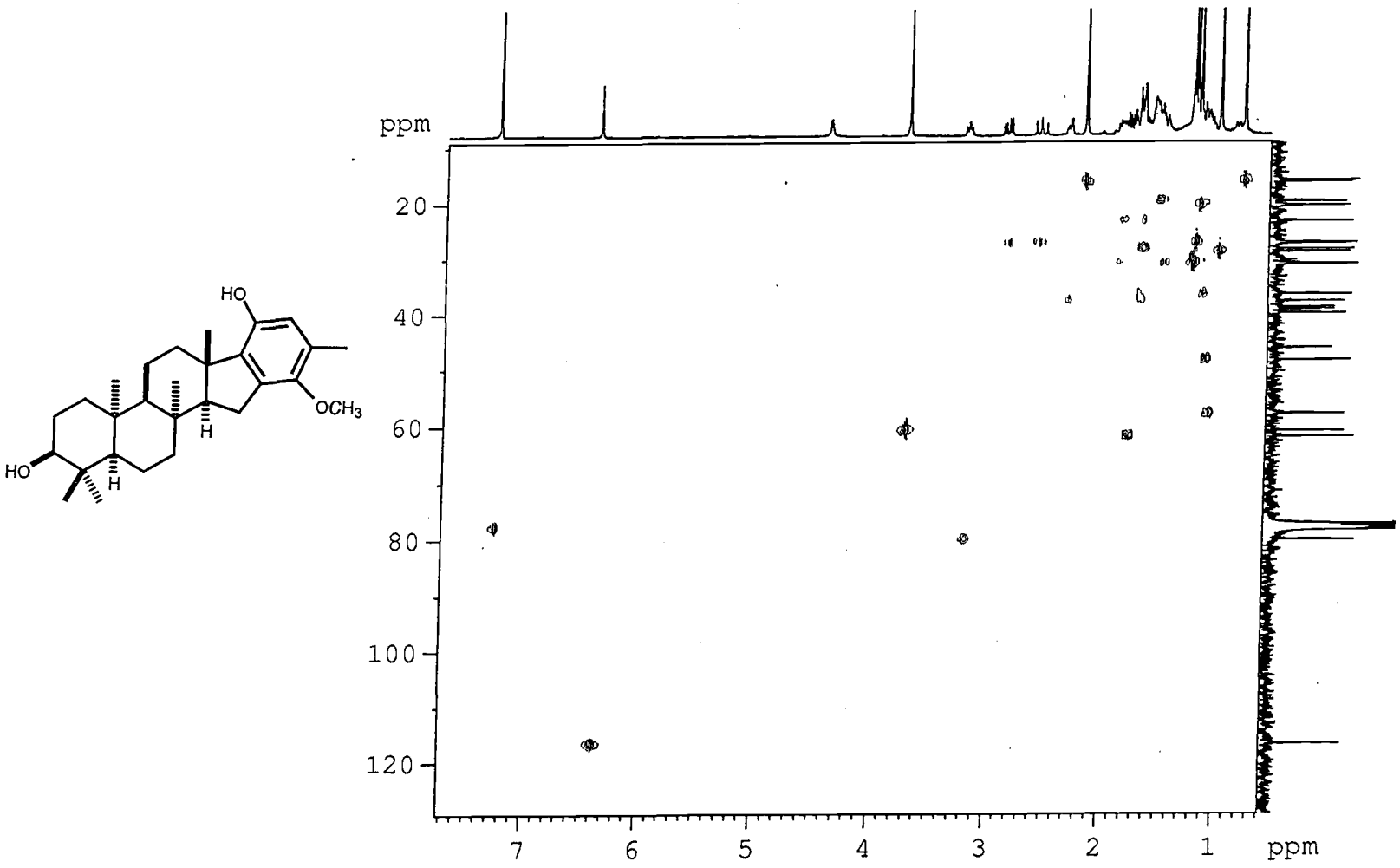


Figure II.13. HSQC spectrum of (**13**) in $CDCl_3$

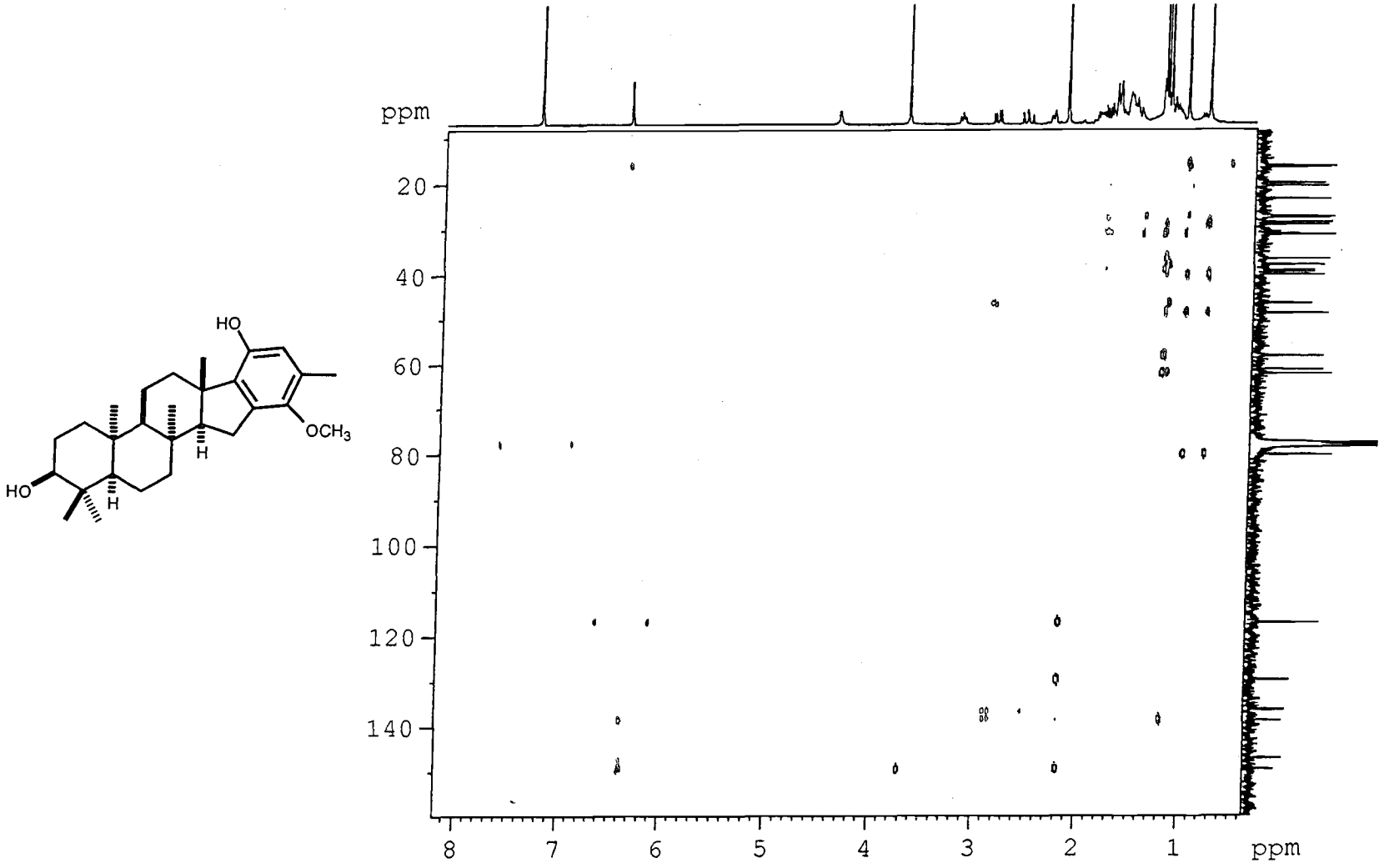


Figure II.14. HMBC spectrum of (**13**) in $CDCl_3$

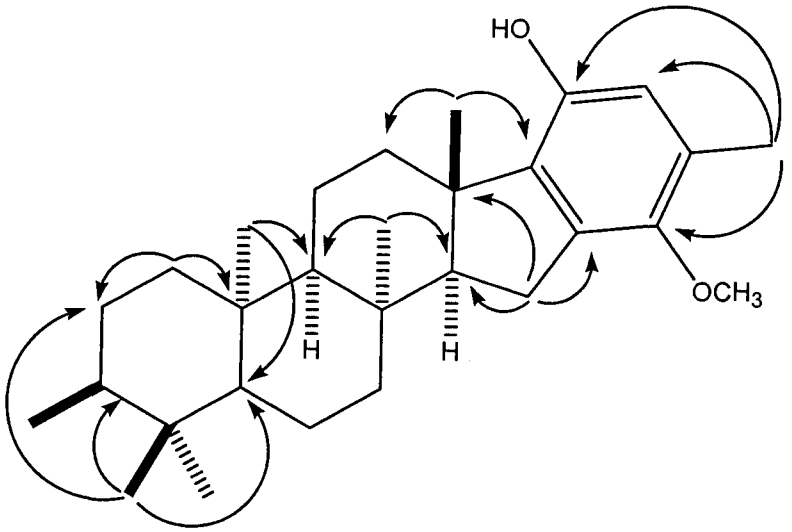


Figure II. 15. HMBC correlations of flabellinol (**13**)

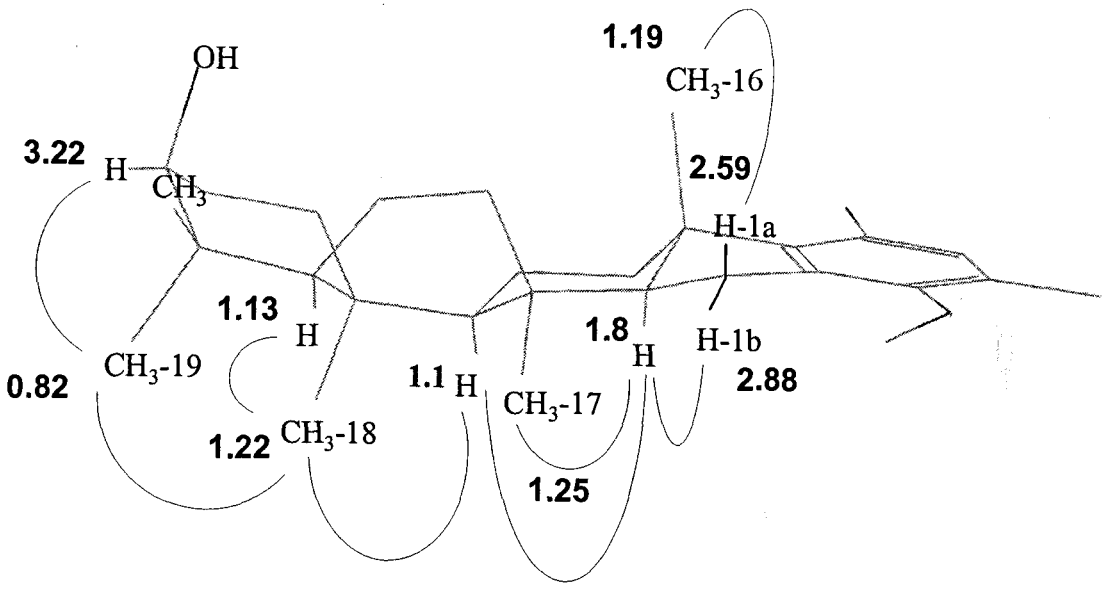
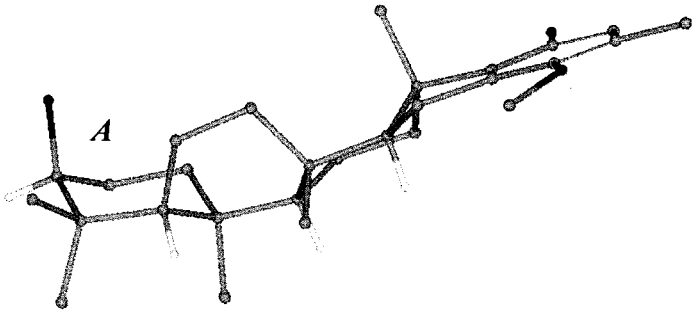


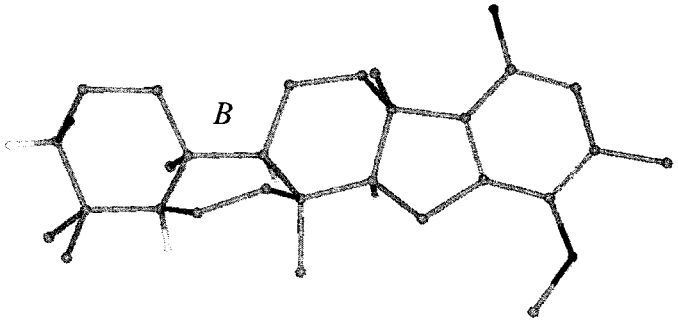
Figure II.16. Selected NOE correlations of flabellinol (**13**).

Signal Irradiated	Enhancements
3.22	0.82
0.82	3.22, 1.22
1.22	0.82, 1.13, 1.1
1.8	1.1, 1.25, 2.88
1.1	1.22, 1.8
1.19	2.59

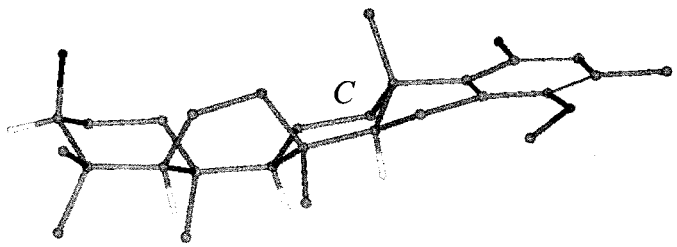
Table II.5. 1D NOE correlations of flabellinol (**13**) in $CDCl_3$ 400 MHz.



Ring A is chair



Ring B is boat



Ring C is chair

Figure II.17. Modeling of flabellinol (**13**) depicting the conformations of rings A, B and C.

Compound **14** showed a HRFABMS $[M+H]^+$ ion at *m/z* 411.2821 for a molecular formula of $C_{27}H_{38}O_3$, and therefore possessed eight degrees of unsaturation. The IR spectrum showed absorption bands at 1648 and 1598 cm^{-1}, indicating the presence of two carbonyl group functionalities. Both ^1H and ^{13}C NMR spectra in $CDCl_3$ (Table II.3, 4) indicated the presence of one deshielded olefinic proton and two carbonyl double bonds on a benzoquinone nucleus. The resonances for all of the methyl groups in the ^1H NMR spectrum were present as singlets, indicating that they were connected to six quaternary carbons. Analysis of ^{13}C NMR, DEPT135 and DEPT90 spectra revealed the presence of nine quaternary carbons, five methines, seven methylenes, and six methyl groups. Two-dimensional NMR experiments, including HSQC and ^1H-^1H COSY were mainly used for assigning the partial structures of **14.** The partial structures of **14** were connected from HMBC data (Figure II.22b) [Compound **14** was found to be a modified derivative of compound **13,** where two carbonyl groups replace the hydroxy and the methoxy groups in compound **13** in positions 3` and 6`].

Compound **15** showed a HRFABMS $[M+H]^+$ ion at *m/z* 443.2794 for a molecular formula of $C_{27}H_{38}O_5$, and therefore possessed nine degrees of unsaturation. The IR spectrum showed absorption bands at 3466 and 1611 cm^{-1}, indicating the presence of phenolic hydroxy group and one conjugated carbonyl group functionality. ^1H NMR was carried out in CD_3OD to provide good chemical shift resolution (Table II.3), and this indicated the presence of one free aromatic proton, two phenolic hydroxy groups, another alcoholic hydroxy group and an aldehyde group. The resonances for four methyl groups in the ^1H NMR spectrum were present as singlets, indicating that they were connected to four quaternary carbons. The resonance for the remaining methyl group in the ^1H NMR spectrum was present as a doublet, indicating that it was connected to a tertiary carbon. Analysis of the ^{13}C NMR spectrum in CD_3OD (Table II.4) revealed the presence of nine quaternary carbons, six methines, seven methylenes, and five methyl groups. Two-dimensional NMR experiments, including HSQC and ^1H-^1H COSY were mainly used for assigning the partial structures of **15.** The partial structures were then connected from HMBC data. Compound **15** is a modified derivative of stypotriol where the methyl group at position 7` is oxidized to an aldehyde. Determination of the relative stereochemistry was carried out by comparison of ^1H NMR and ^{13}C NMR data with that reported data for stypotriol.[1]

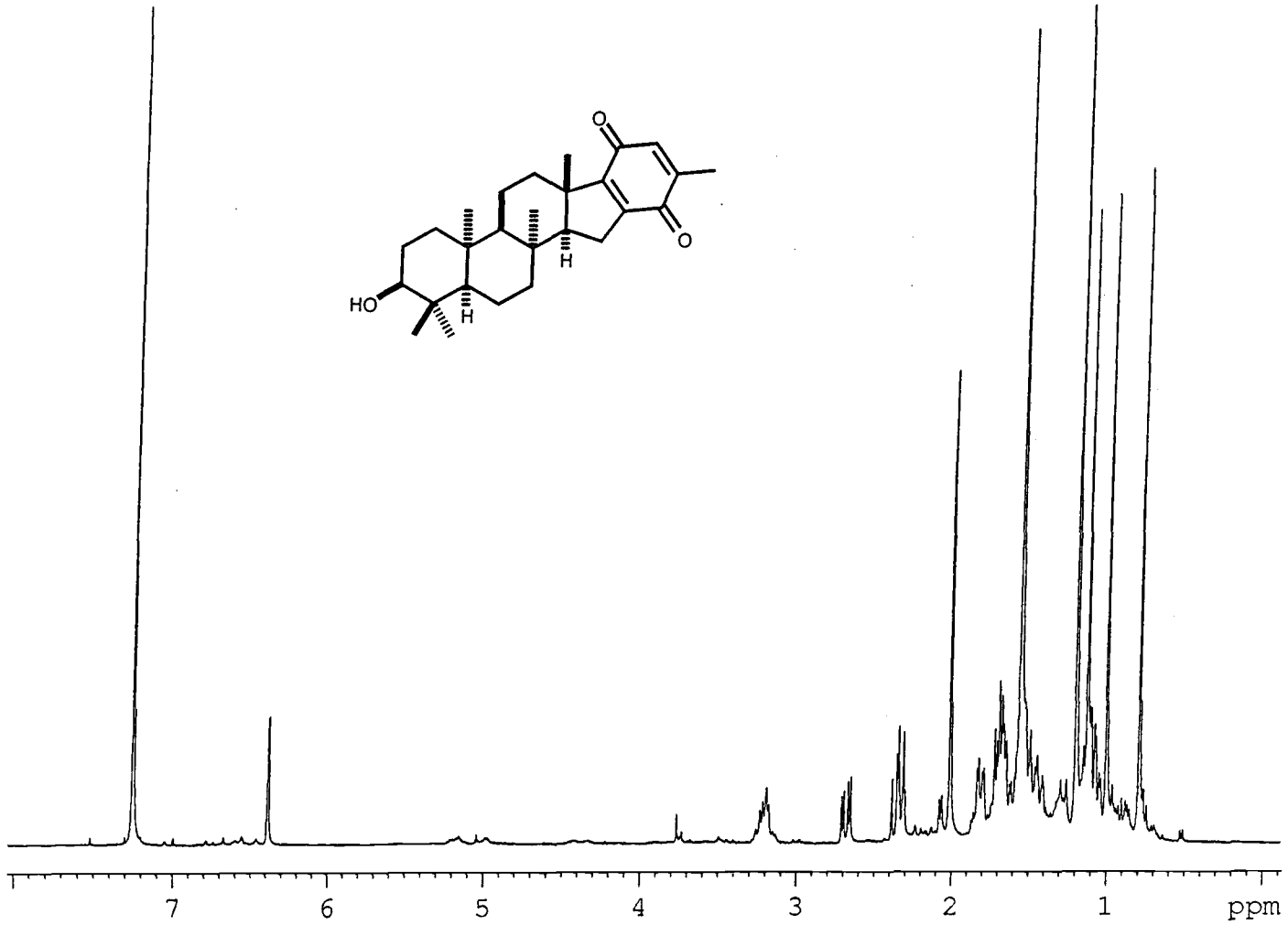


Figure II.15. ^{1}H NMR spectrum of (**14**) in $CDCl_3$ [400 MHz]

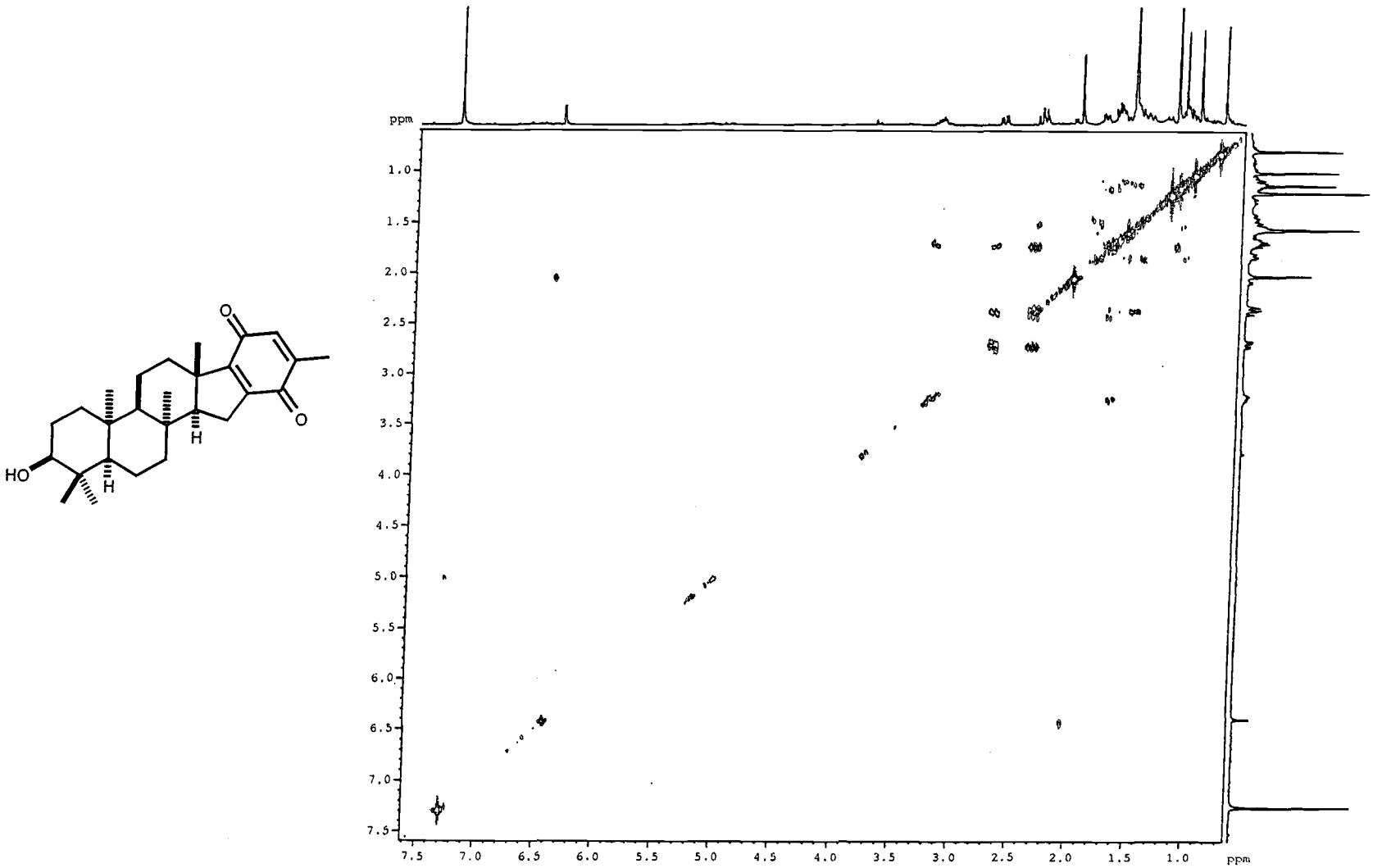


Figure II.19. ^{1}H-^{1}H COSY spectrum of (**14**) in $CDCl_3$

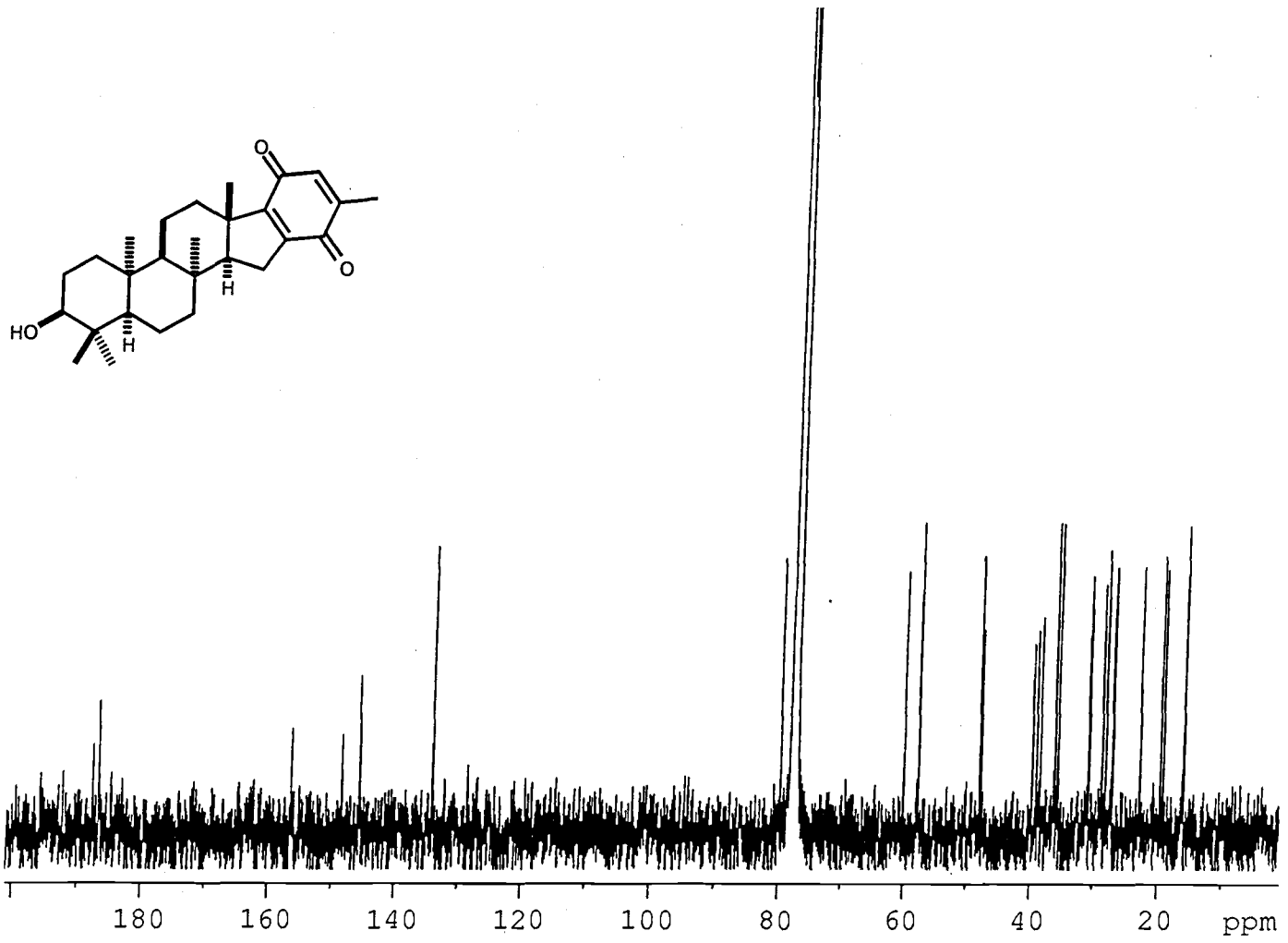


Figure II.20. ^{13}C NMR spectrum of (**14**) in $CDCl_3$

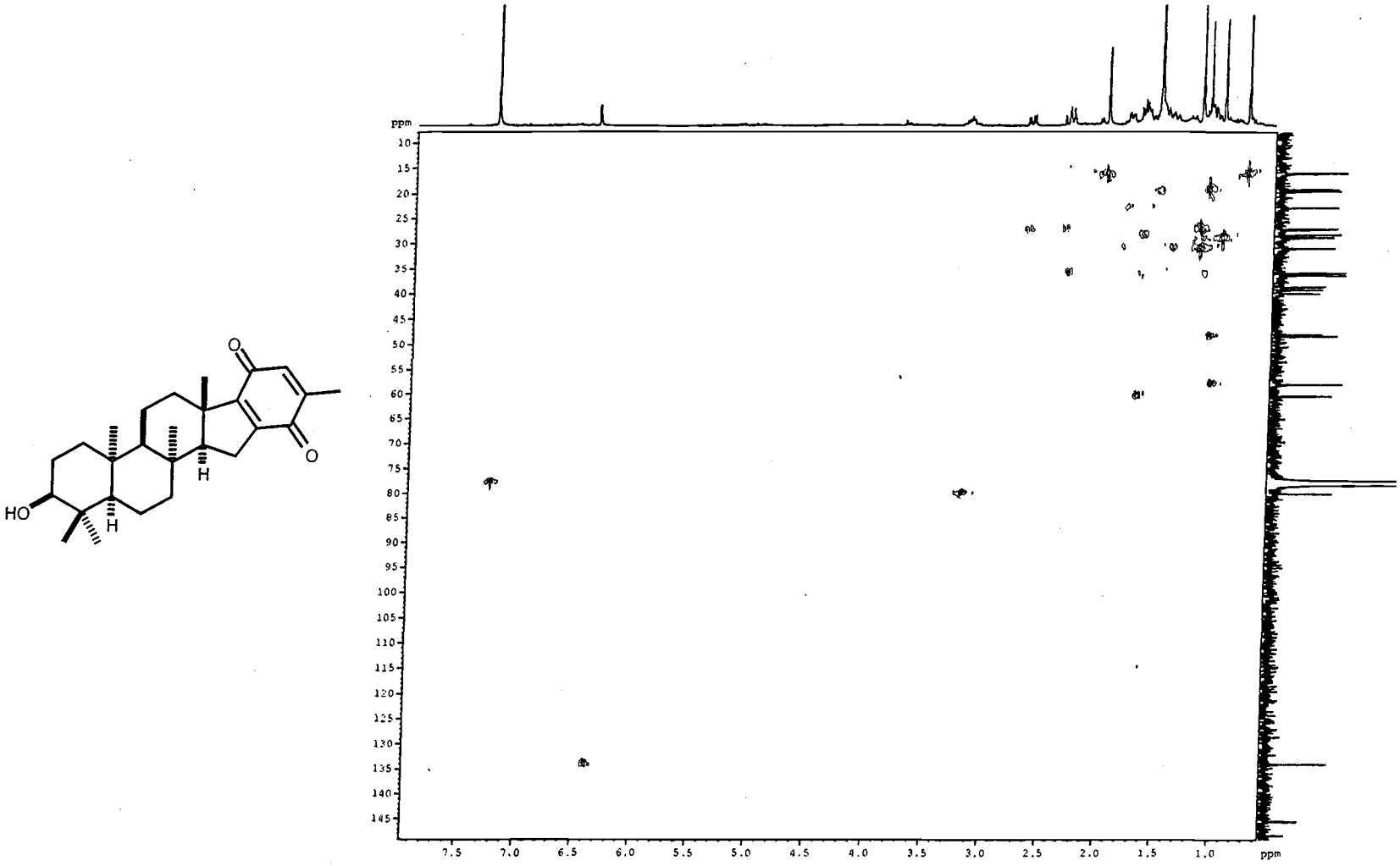
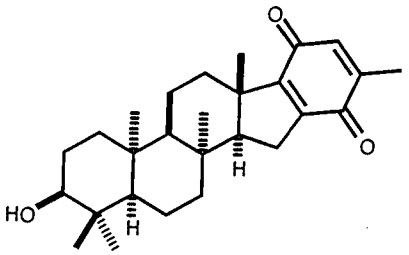


Figure II.21. HSQC spectrum of (**14**) in $CDCl_3$



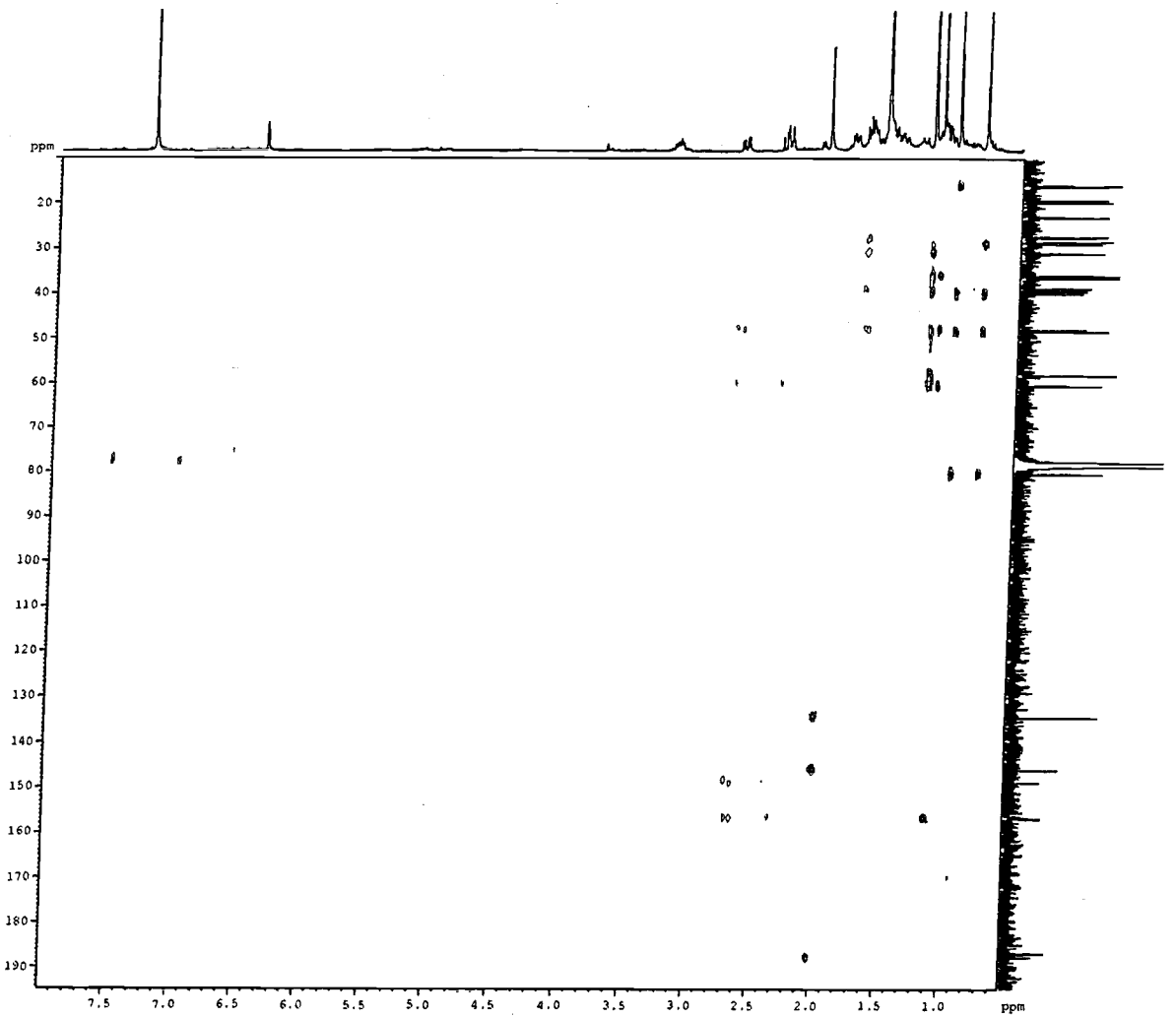


Figure II.22. HMBC spectrum of (**14**) in $CDCl_3$

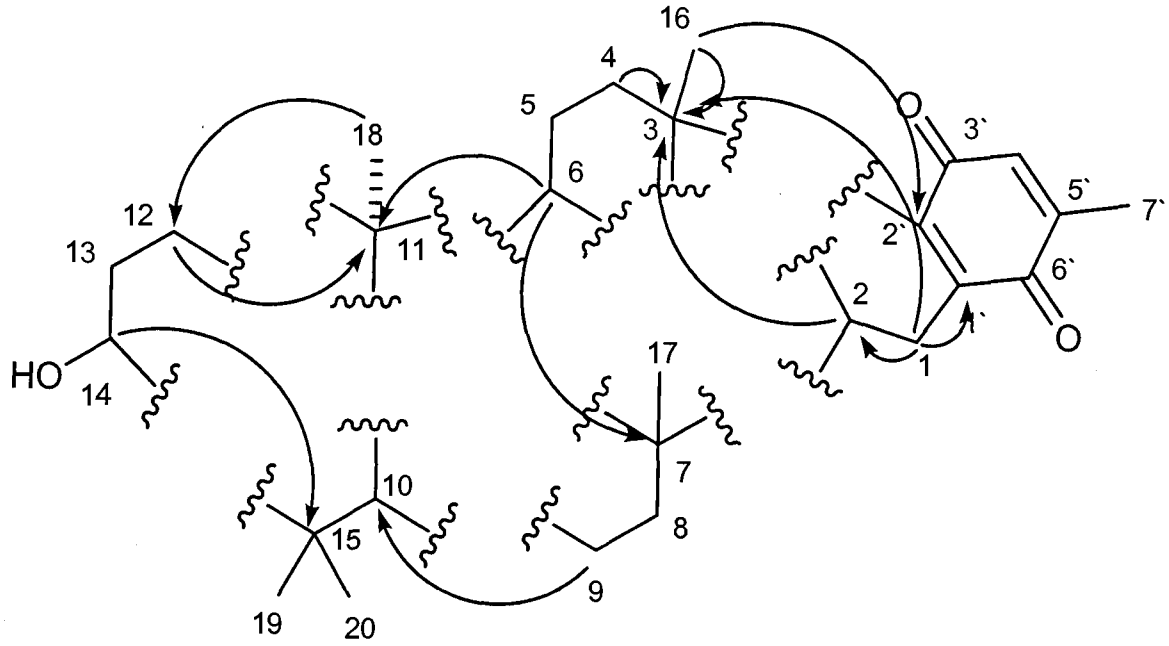


Figure II. 22b. Partial structures of **14** connected by HMBC correlations.

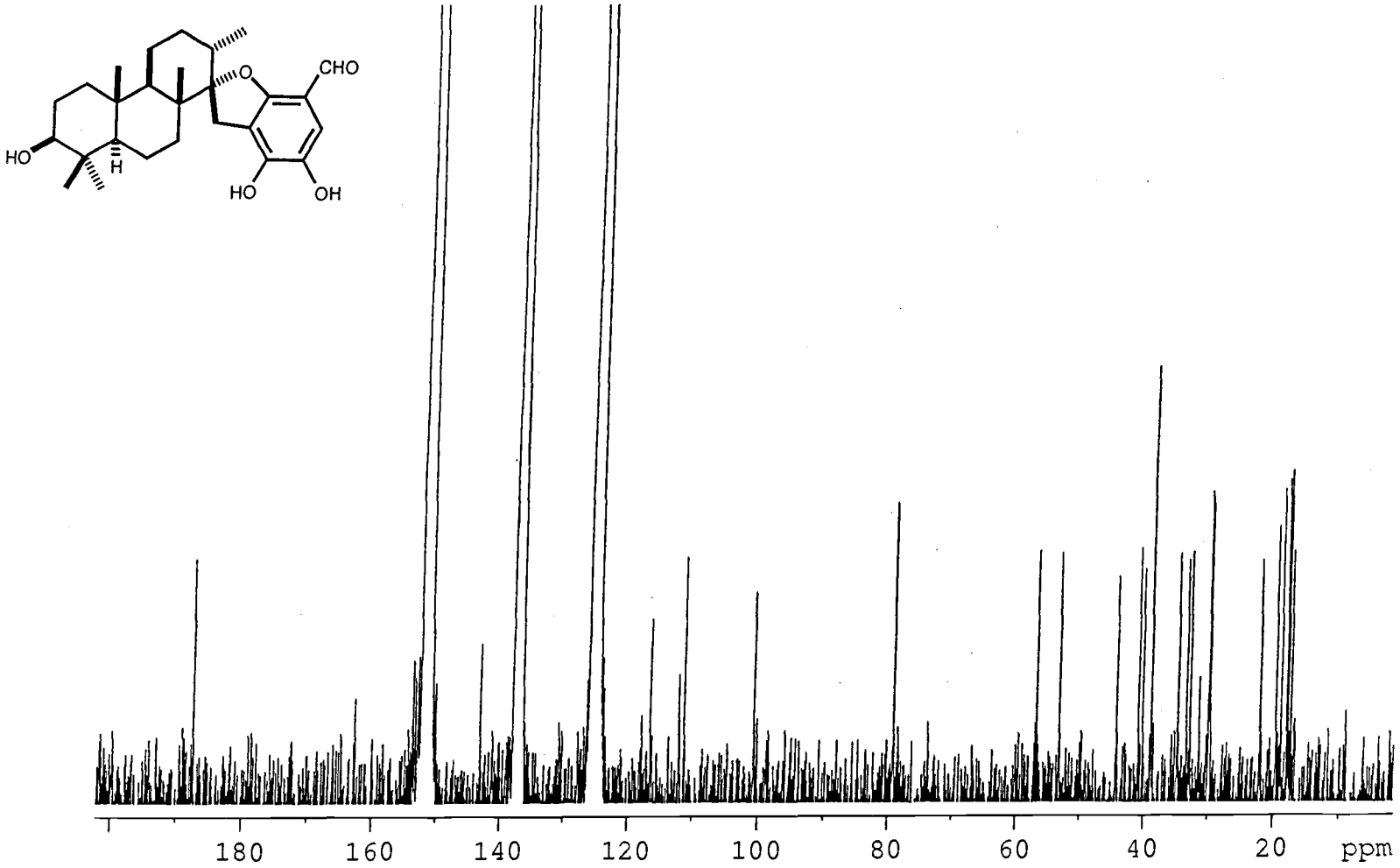


Figure II.23. ^{13}C NMR spectrum of (**15**) in C_6D_5N

Compound **16** showed a HRFABMS $[M+H]^+$ ion at *m/z* 475.2994 for a molecular formula of $C_{28}H_{42}O_6$, and therefore possessed eight degrees of unsaturation. The IR spectrum showed absorption bands at 1729 cm^{-1}, indicating the presence of one carbonyl group. Analysis of ^1H NMR and ^{13}C NMR spectra in $CDCl_3$ (Table II. 3, 4) indicated the presence of one free olefinic proton, one methoxy group, another alcoholic hydroxy group, nine quaternary carbons, four methines, seven methylenes, and six singlet and one doublet methyl groups. The singlet nature of the methyl groups indicated that they were connected to six quaternary carbons. Two-dimensional NMR experiments, including HSQC and ^1H-^1H COSY, were mainly used for assigning the partial structures. The partial structures were connected from HMBC data. The presence of the hydroperoxide group was confirmed from IR (absorption band at 1014 cm^{-1}), ^{13}C-NMR (shift of the carbon atom carrying the hydroperoxide group from the aromatic range to 103.0 ppm) and finally by chemical tests, such as potassium dichromate test and reduction with triphenyl phosphine to the corresponding alcohol, then confirmation of the identity of the product by MS (M.Wt. 458). Compound **16** is a modified derivative of stypotriol, where position 1` possesses a hydroperoxide group, position 5` contains a methoxy group and position 6` contains a carbonyl group. This compound was found to be strongly stable due to the formation of hydrogen bond between proton of the hydroperoxide group and the neighboring carbonyl group. Determination of the relative stereochemistry at most positions was carried out by comparison of the ^1H NMR and ^{13}C NMR data with that reported data for stypotriol.[1]

The isolated and semisynthetic compounds were evaluated for their biological activity in several systems. All of the new compounds were found to have cytotoxic activity (EC_{50} ranges from 0.8 – 10 µg/ml) in human lung cancer (NCI-H460) and mouse neuro-2a blastoma cell lines (I acknowledge Dr. Doug. Goeger, for carrying out these bioassays). Upon testing these compounds in a sodium channel assay, compounds (**12** and **13**) were found to have strong blocking activity with an EC_{50} of (0.3 and 1.0 µg/ml respectively) (Figure II. 37). This brings forth the possibility that these compounds may be used as lead compounds to develop a new class of sodium channel blockers, which are categorized as one of the subcategories of drugs used in epileptic seizures. Compound **12** was found to be the most active in the brine shrimp toxicity assay; at a concentration of 10 ppm, lethality was found to be 90%. Compound **14**, as well as one of the known compounds, stypoldione, caused a change in calcium concentrations in rat neurons (I acknowledge Dr. Thom. Murray's group for carrying out these bioassays).

The dose response curve analysis showed that compound **14** gives a biphasic response. This could be a result of receptor desensitization or cell death (w/o any rapid immediate cell lysis). The calculated EC_{50} for this compound **14** is approximately 9 μM whereas of stypoldione gave an EC_{50} of 35 μM (see table II. 6). Mono and diacetate derivatives of compound **12** (**17-18**) and a diacetate derivative of compound **13** (**19**) were prepared for biological activity testing. However, an obvious reduction in the bioactivities was seen upon comparison of the results of the biological assays of the new derivatives with those of the natural compounds.

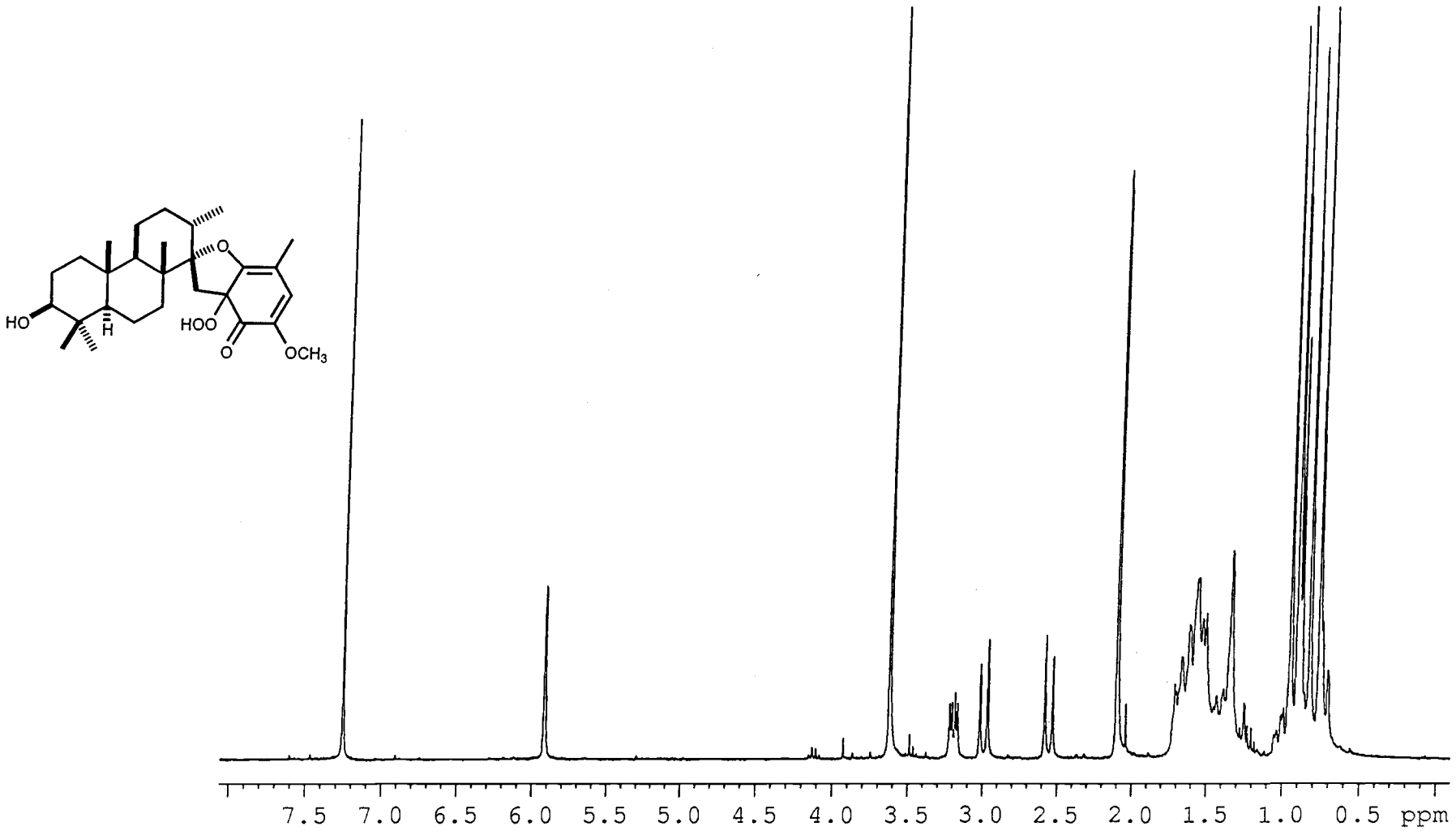
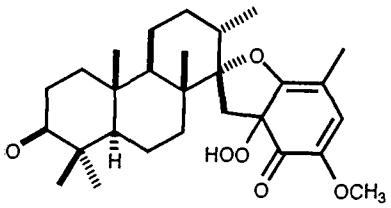


Figure II.24. ^{1}H NMR spectrum of (**16**) in $CDCl_3$ [400 MHz]



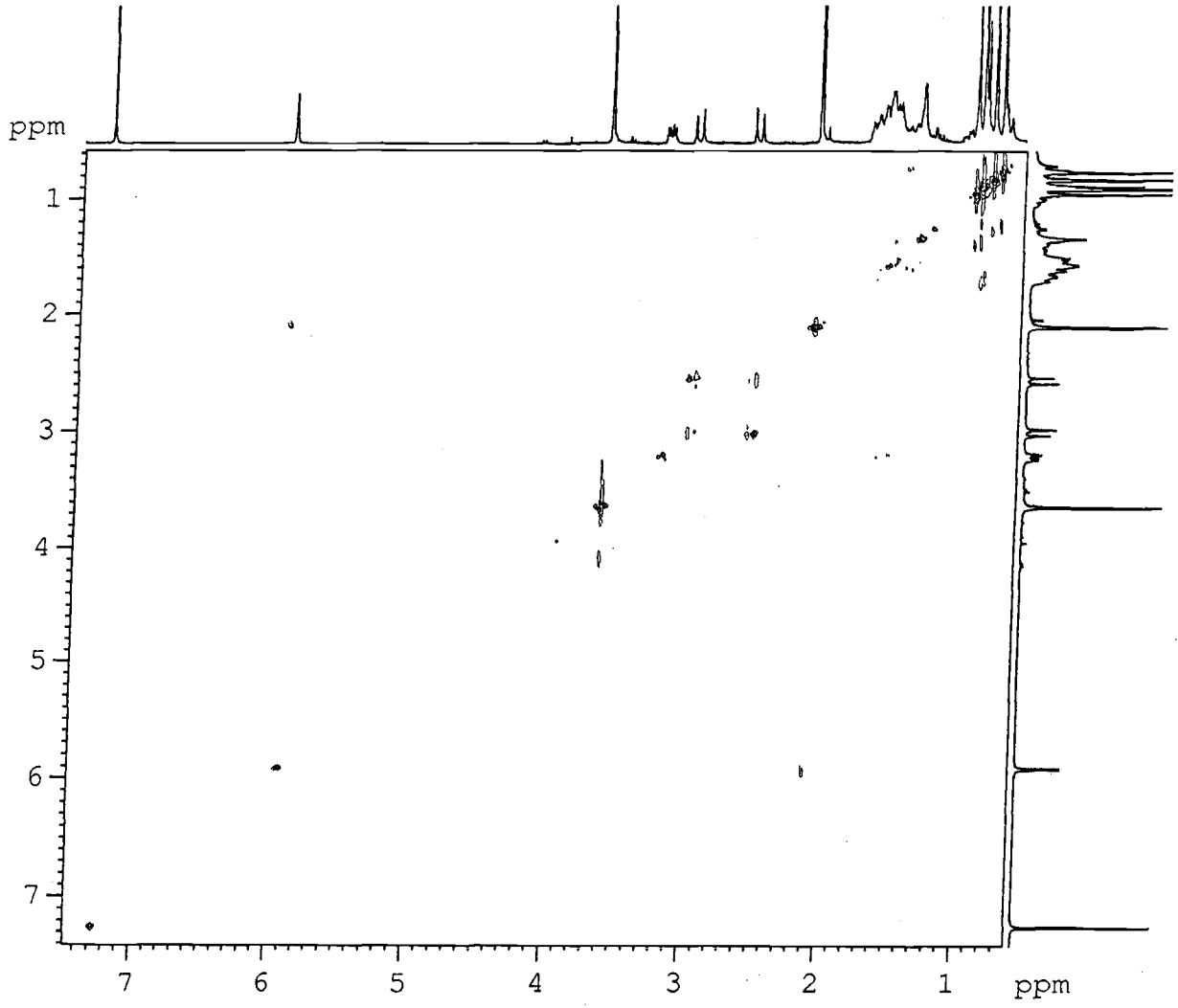


Figure II.25. ^{1}H-^{1}H COSY spectrum of (**16**) in $CDCl_3$

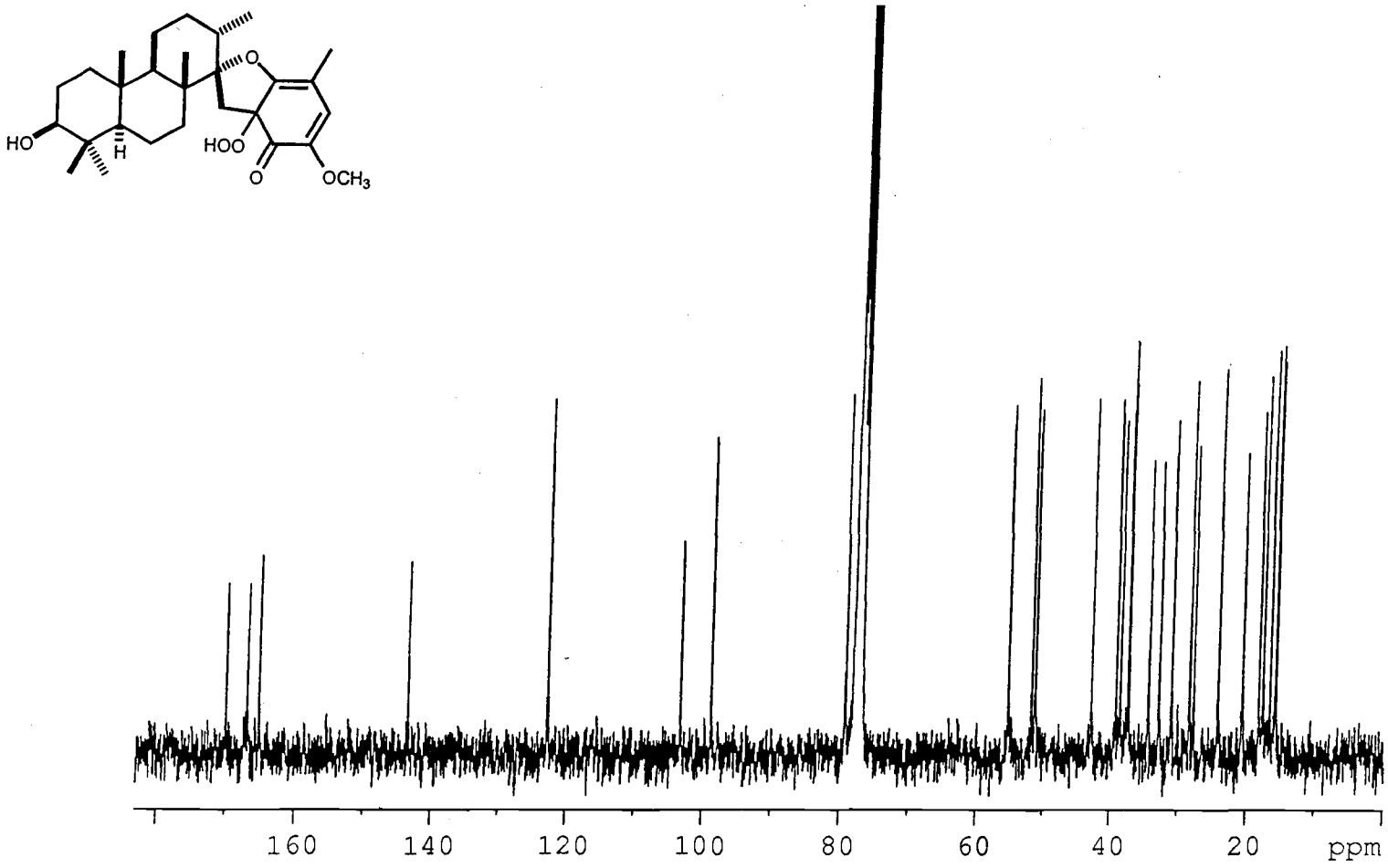


Figure II.26. ^{13}C NMR spectrum of (**16**) in $CDCl_3$

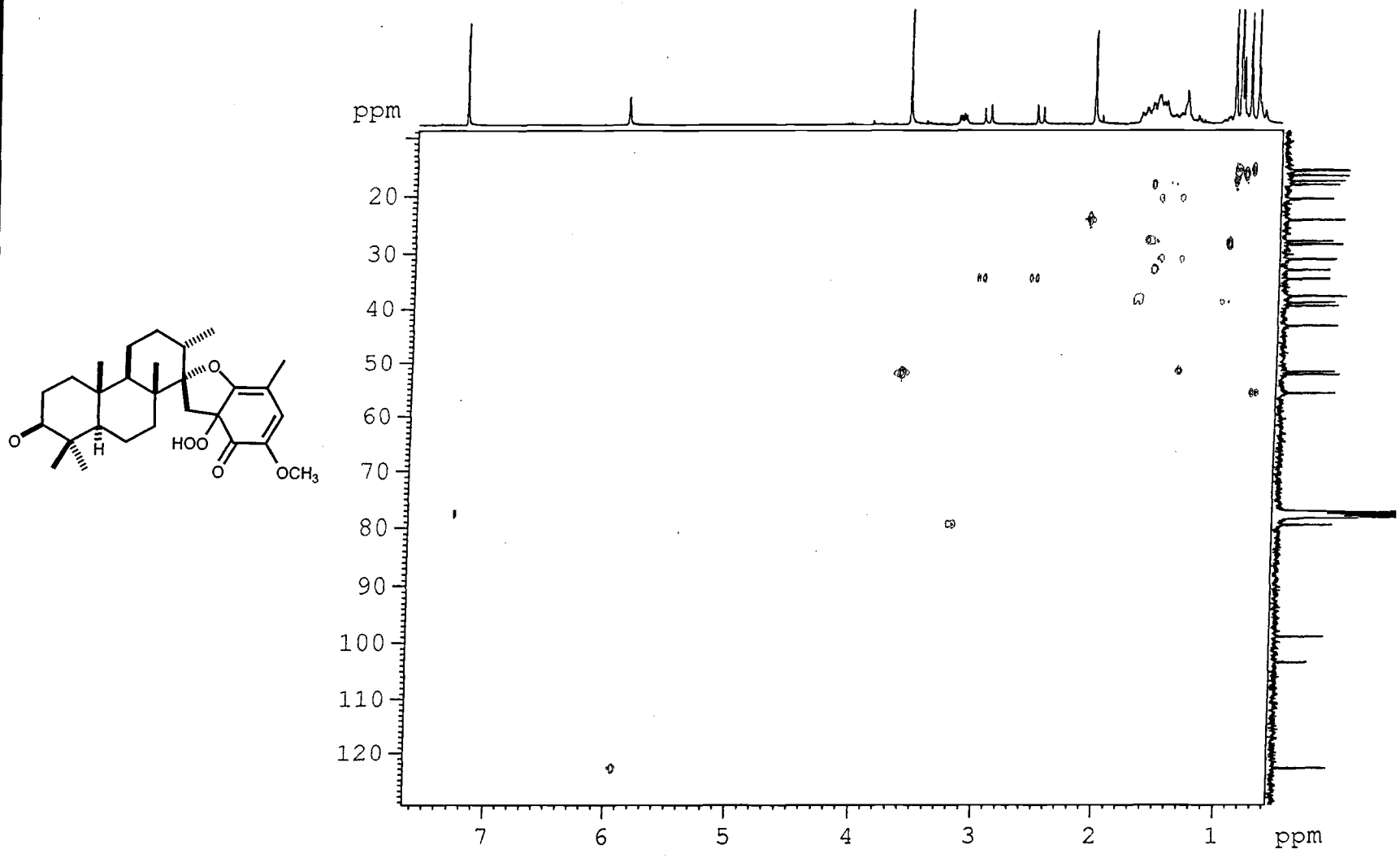


Figure II.27. HSQC spectrum of (**16**) in $CDCl_3$

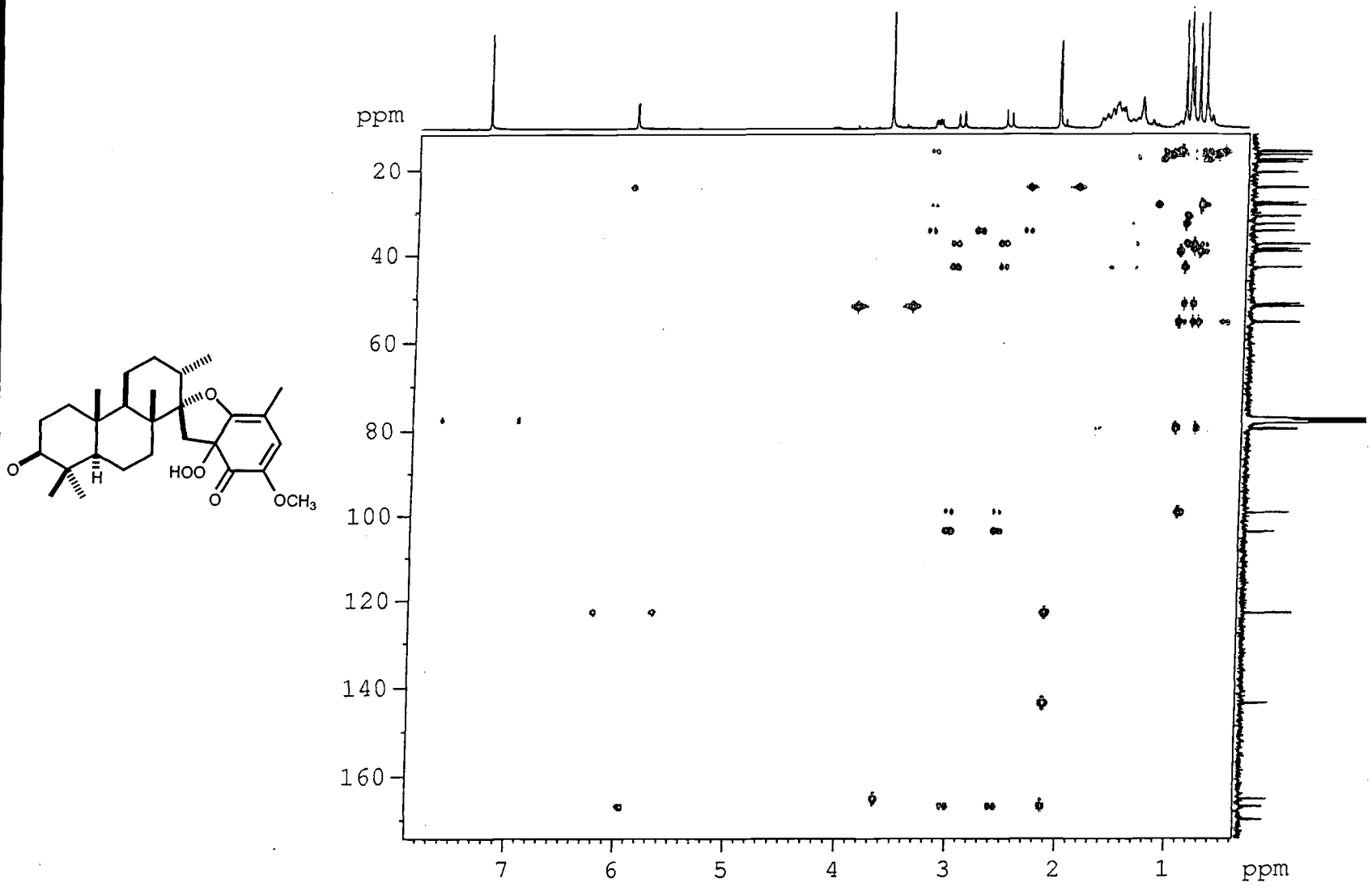


Figure II.28. HMBC spectrum of (**16**) in $CDCl_3$

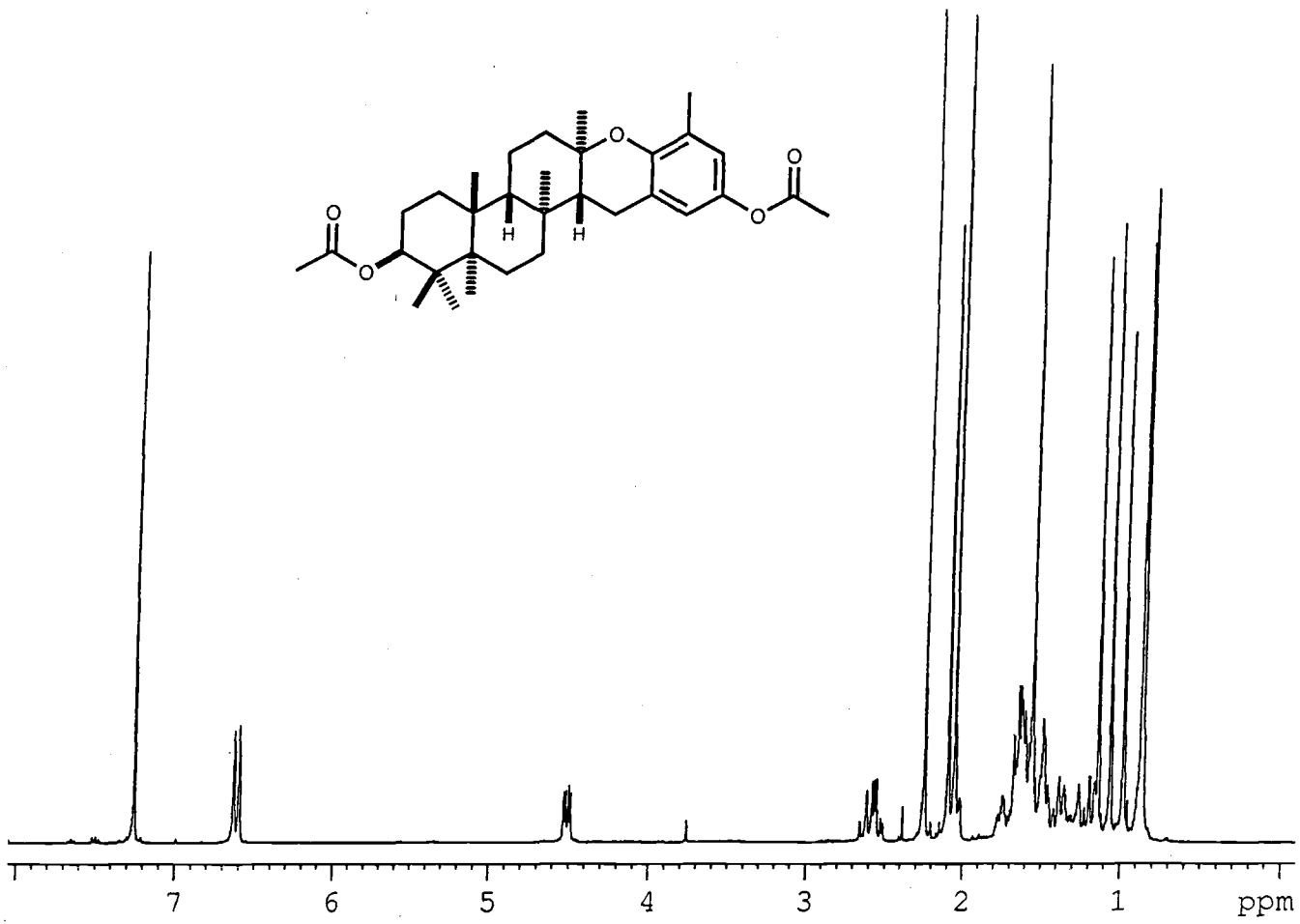


Figure II.29. ^{1}H NMR spectrum of (**18**) in $CDCl_3$ [400 MHz]

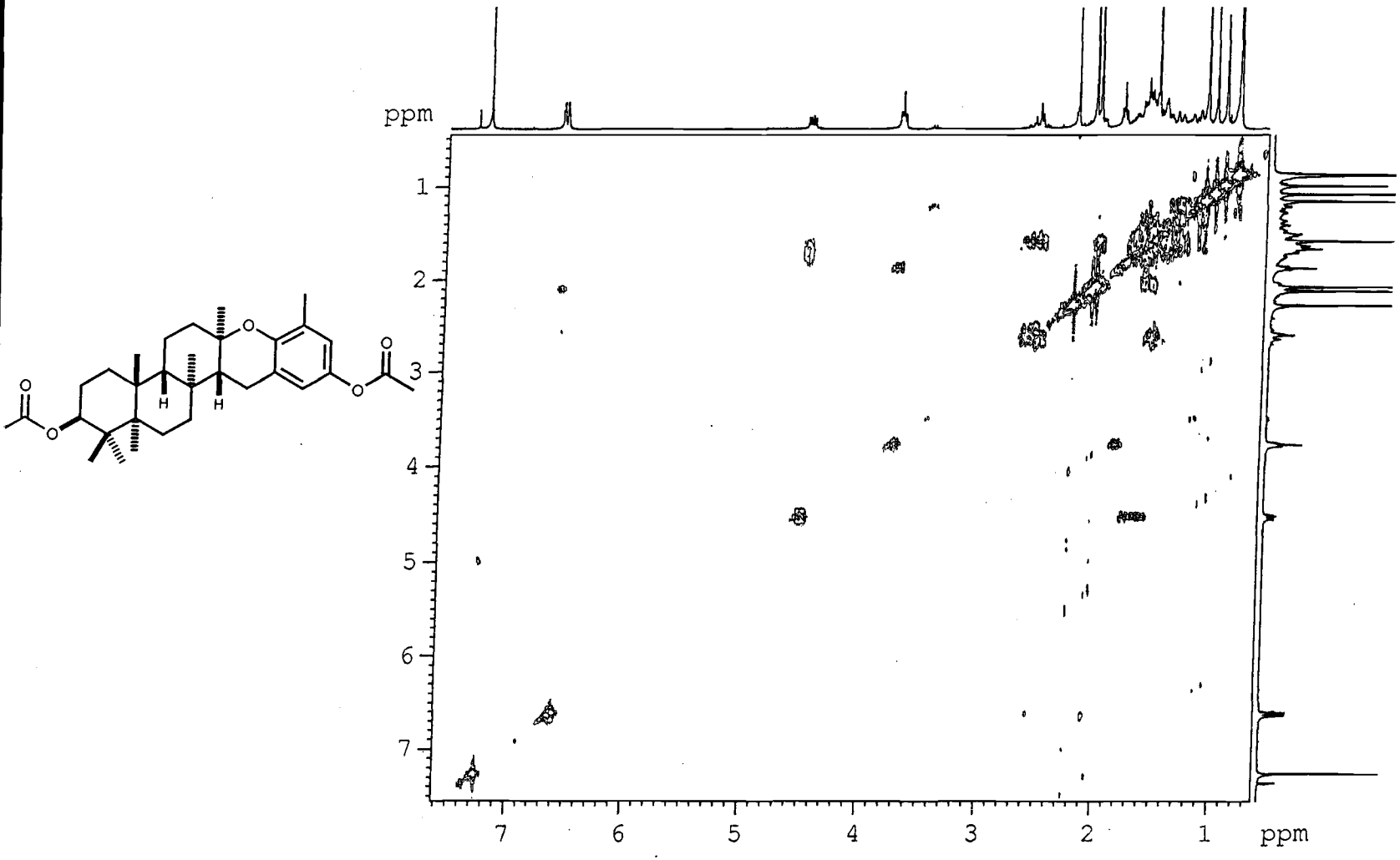


Figure II.30. 1H-1H COSY spectrum of (**18**) in $CDCl_3$

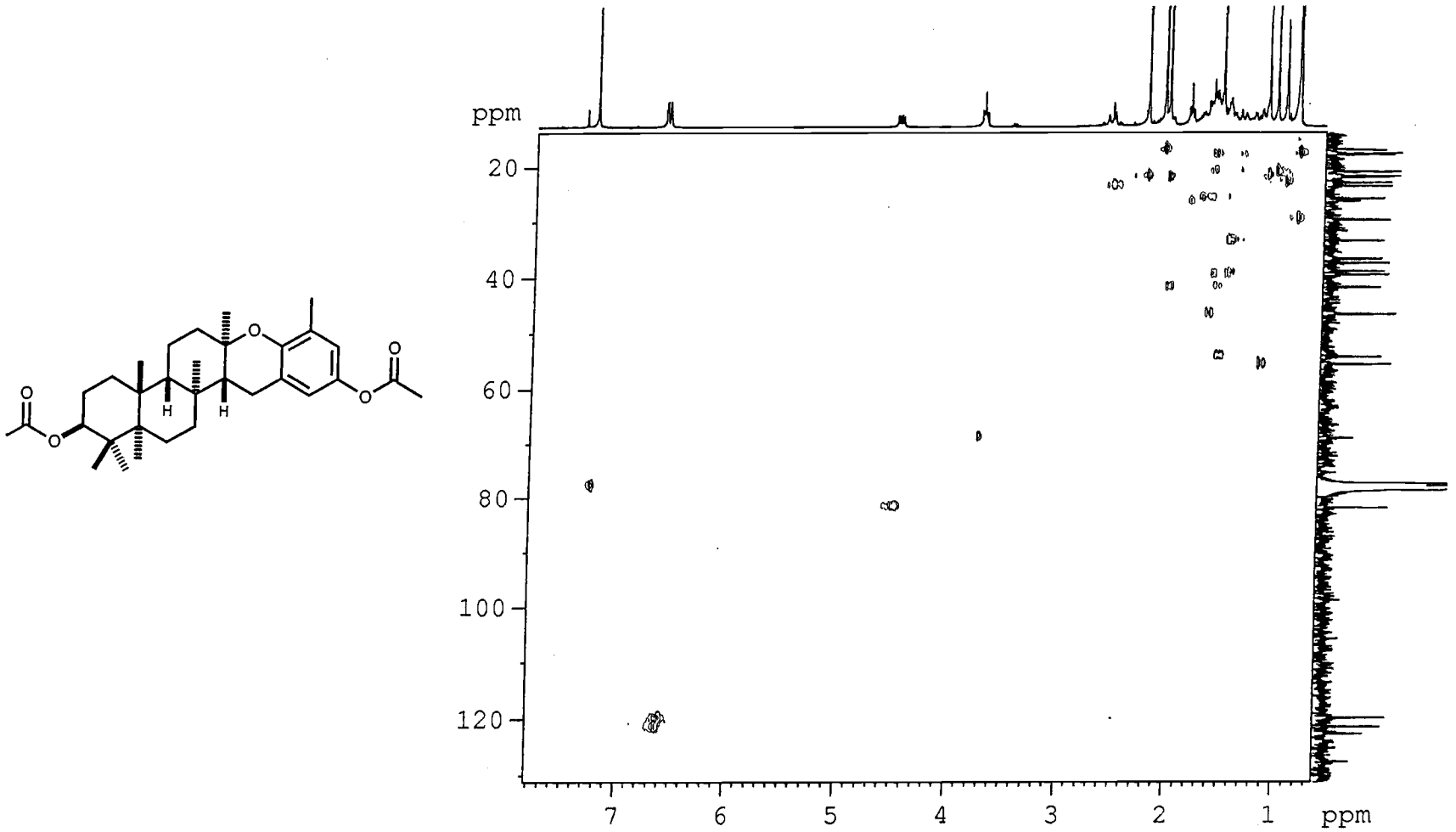


Figure II.31. HSQC spectrum of (**18**) in $CDCl_3$

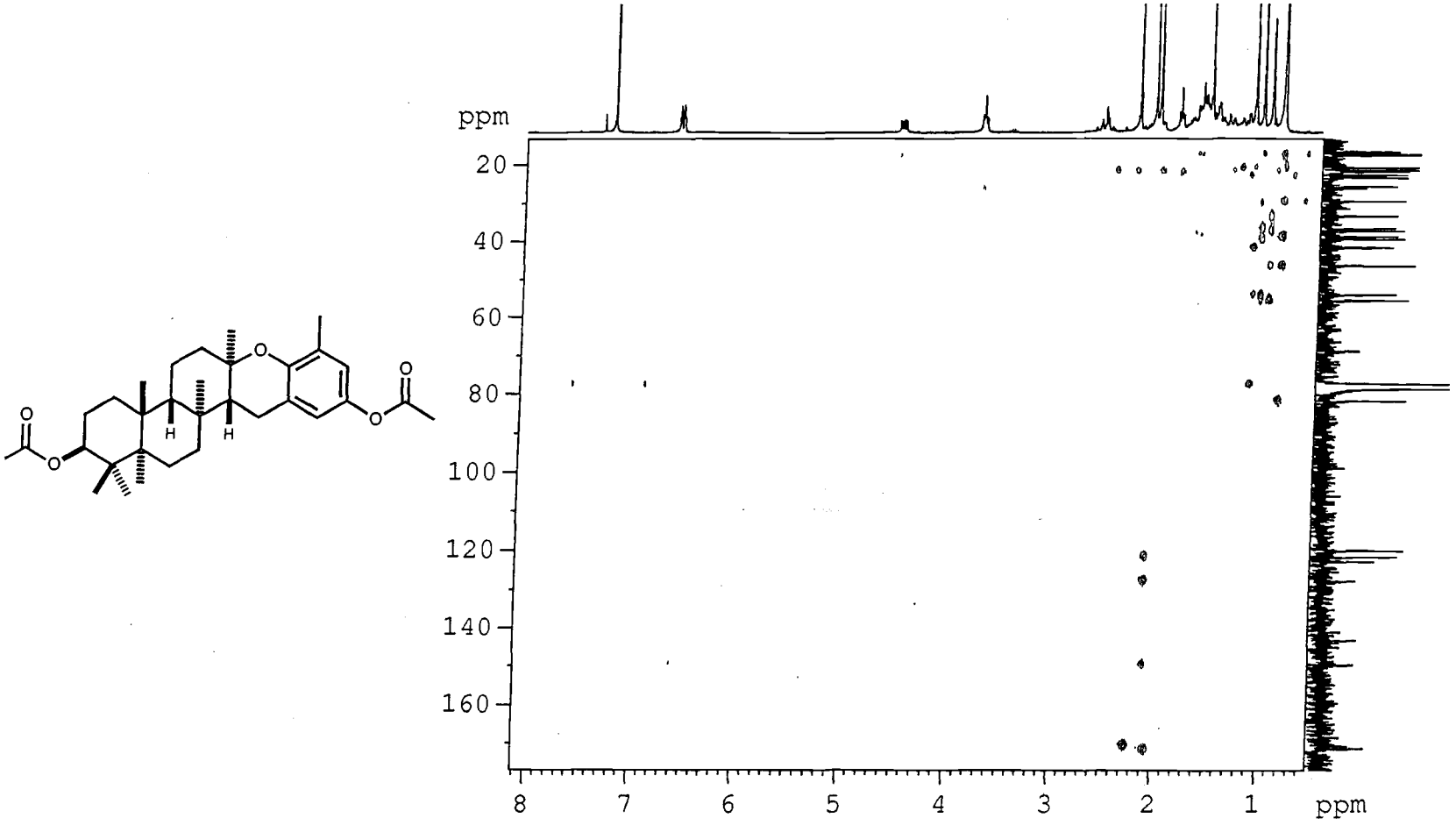


Figure II.32. HMBC spectrum of (**18**) in $CDCl_3$

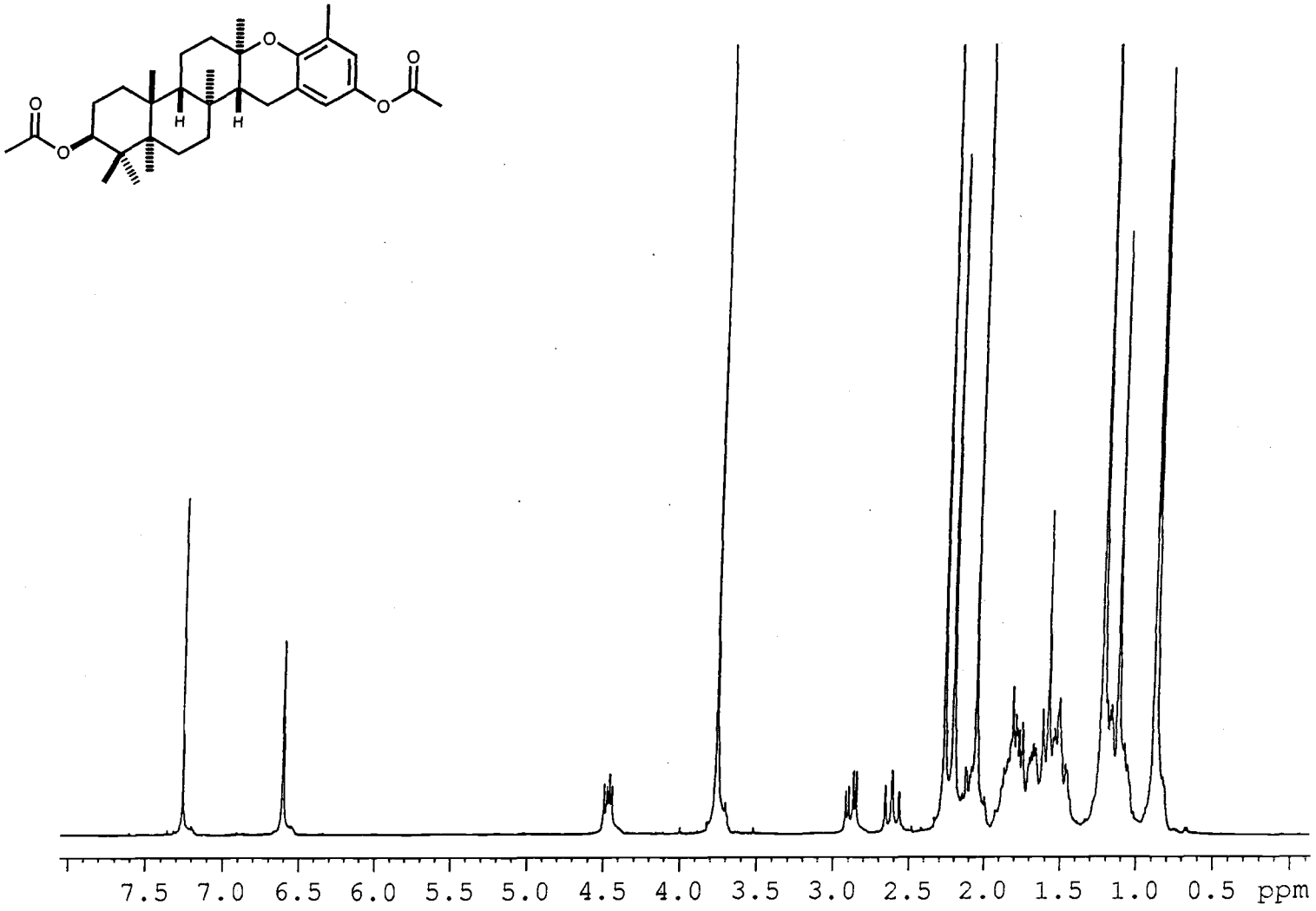


Figure II.33. ^{1}H NMR spectrum of (**19**) in $CDCl_3$ [400 MHz]

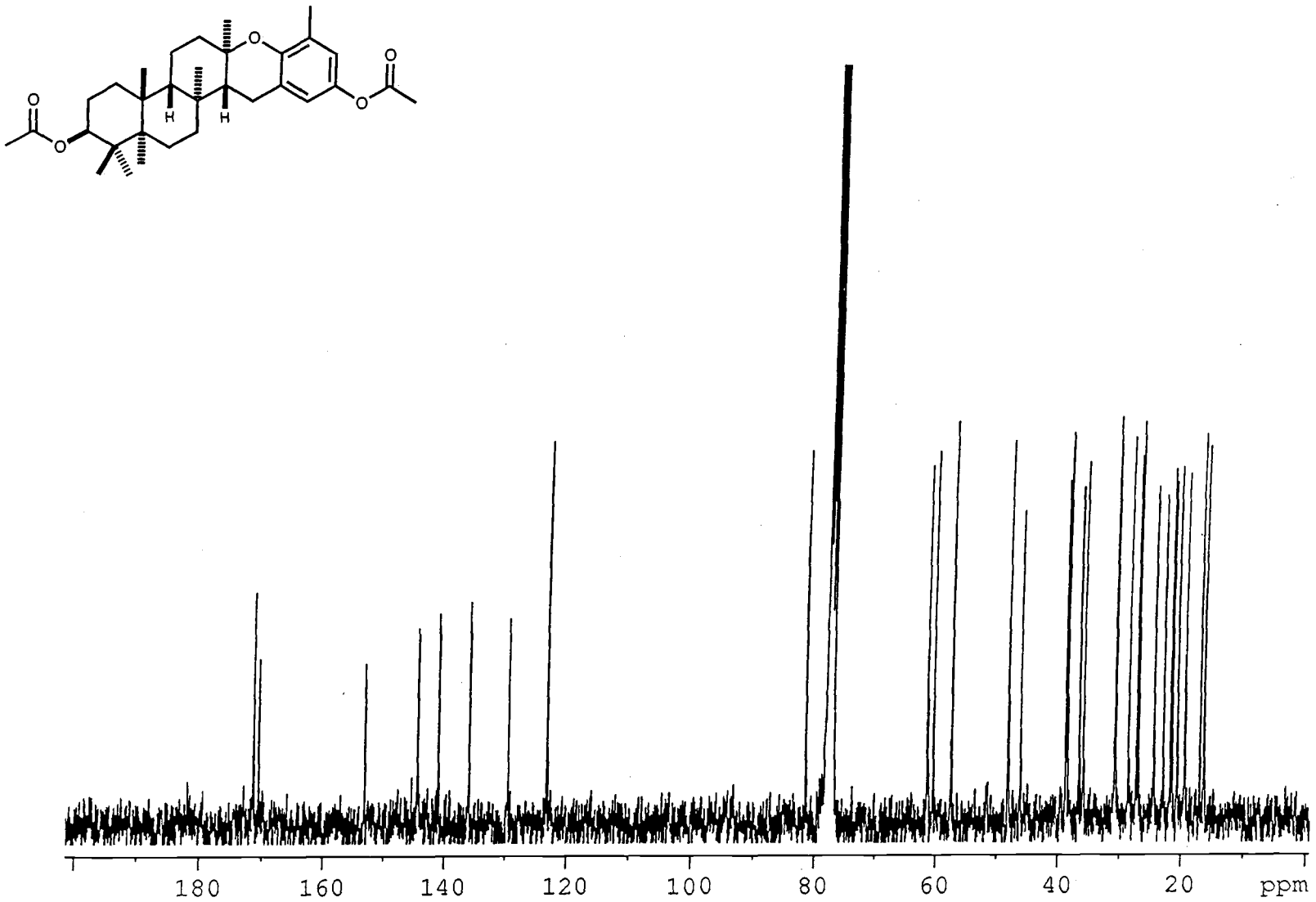


Figure II.34. ^{13}C NMR spectrum of (**19**) in $CDCl_3$

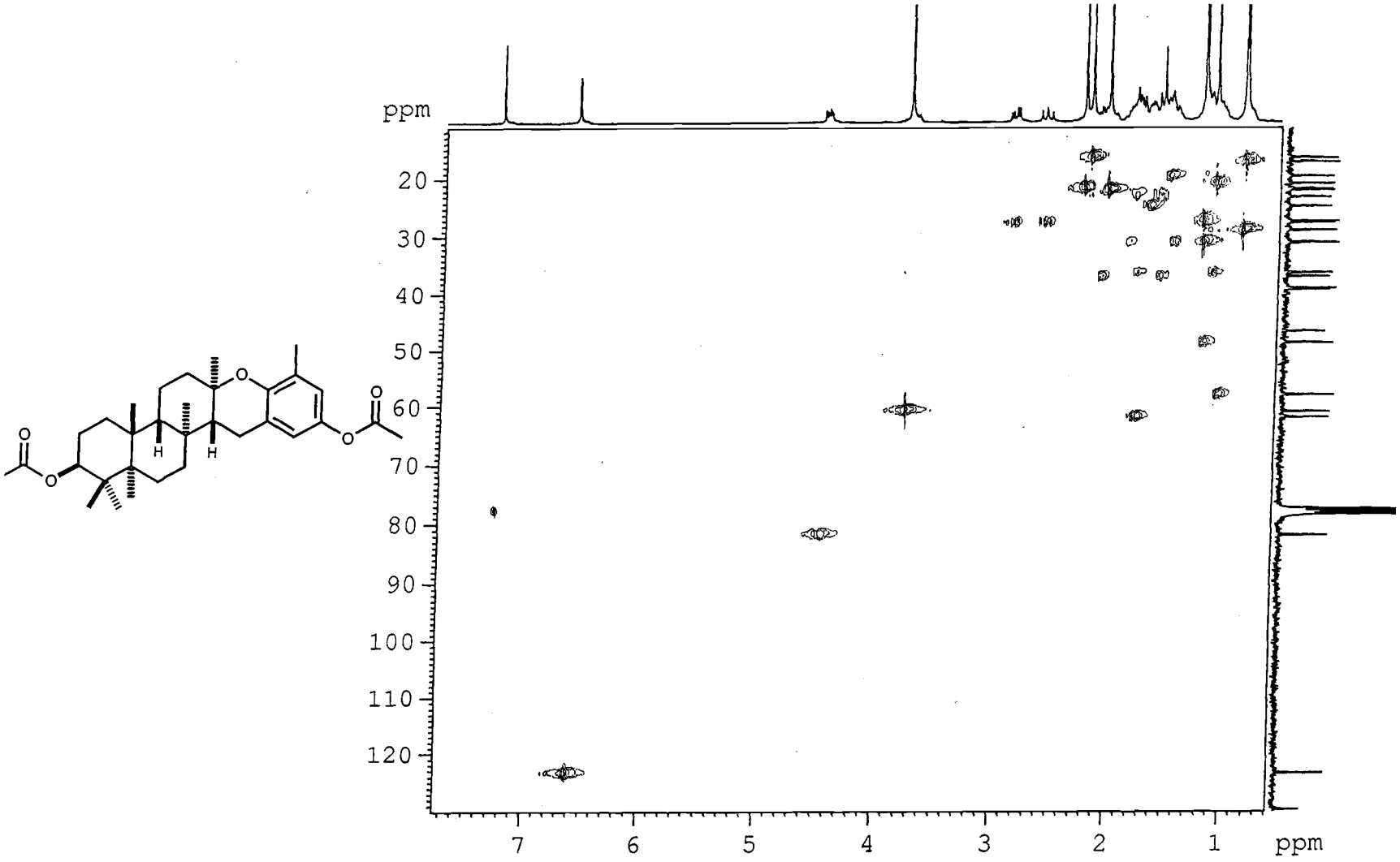


Figure II.35. HSQC spectrum of (**19**) in $CDCl_3$

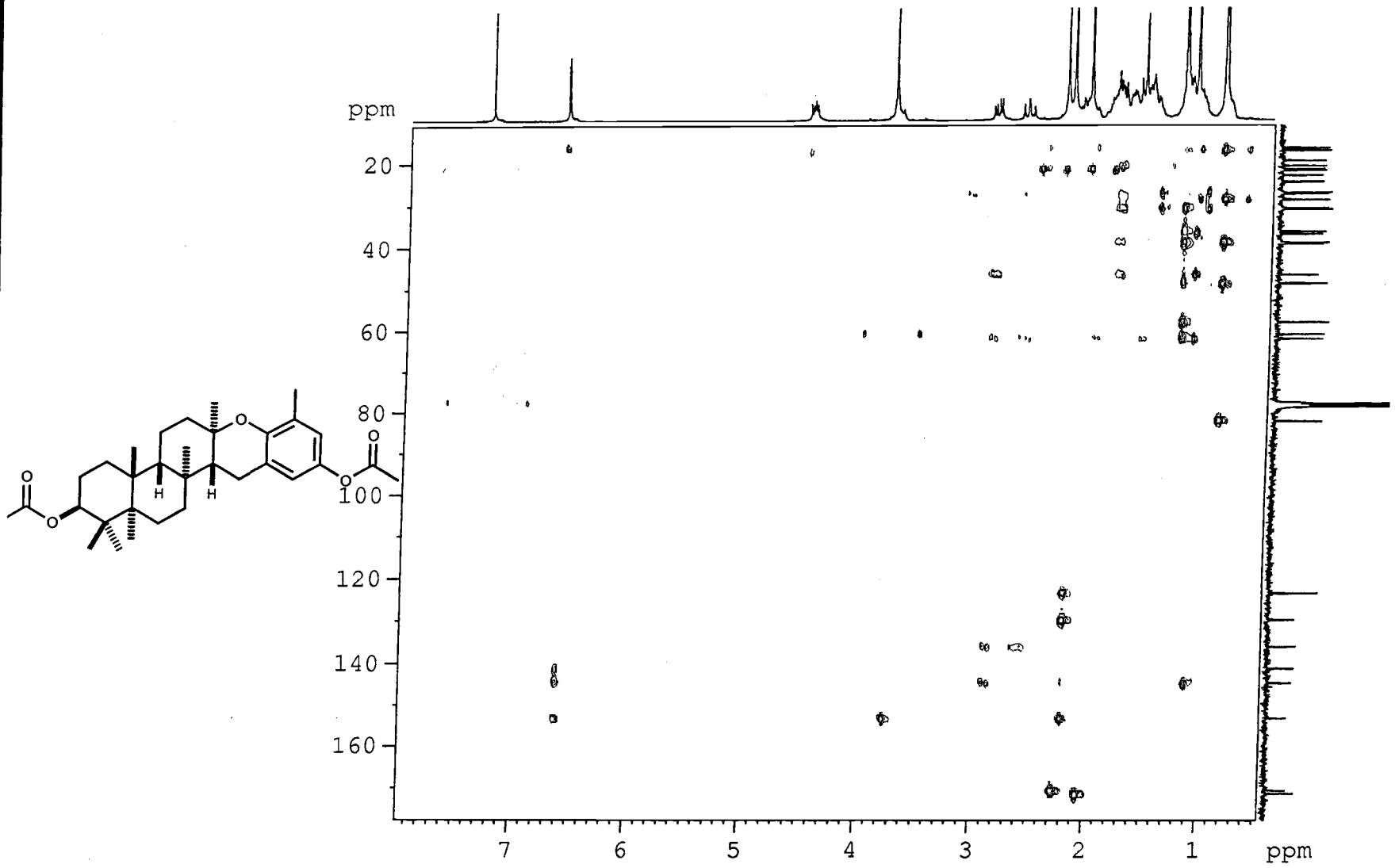


Figure II.36. HMBC spectrum of (**19**) in $CDCl_3$

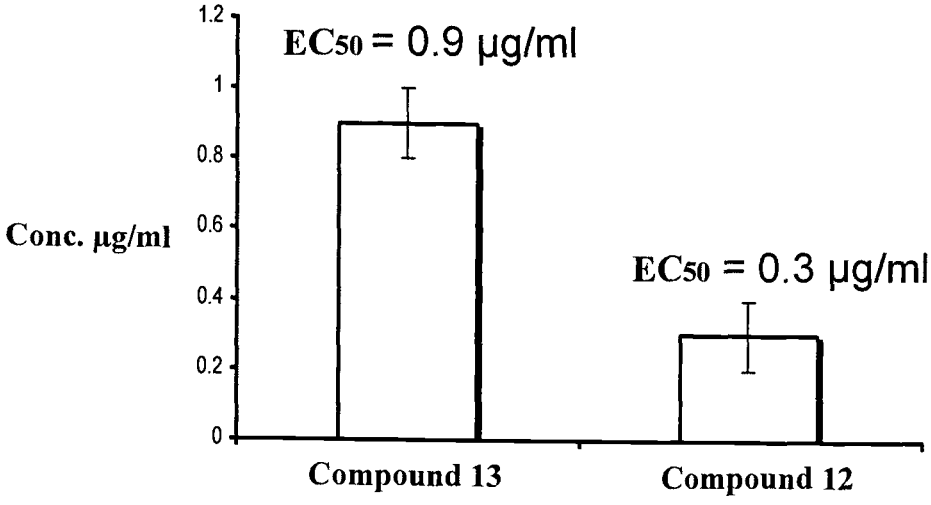


Figure II.37. Results of sodium channel blocking activity of **12** and **13**.

Compound Test	(12)	(13)	(14)	(15)	(16)	(18)	(19)	(5)	(10)	(20)
Brine shrimp toxicity 10 ppm	92%	50%	NT	NT	NT	NT	NT	NT	90%	NT
Cytotoxicity human lung cancer (NCI-H460) (EC_{50})	0.8 ug/ml	4.0 ug/ml	6.0 ug/ml	1.0 ug/ml	5.0 ug/ml	>10.0 ug/ml	6.0 ug/ml	5.0 ug/ml	>10.0 ug/ml	0.8 ug/ml
Cytotoxicity mouse neuro-2a (EC_{50})	4.0 ug/ml	5.0 ug/ml	2.0 ug/ml	1.0 ug/ml	>10.0 ug/ml	>10.0 ug/ml	>10.0 ug/ml	2.5 ug/ml	7.0 ug/ml	0.7 ug/ml
Sodium channel blocking activity (EC_{50})	0.3 ug/ml	1.0 ug/ml	NA	NA	NA	NA	NA	NA	NA	NA
Calcium concentrations modulation in rat neurons (EC_{50})	NA	NA	NA	9 uM	NA	NA	NA	NA	35UM	NA

NT: Not tested (because of the isolation of these compounds from weakly active fractions)

NA: Not active (not effective at 100 μM)

Table II.6. Biological activities of different *Stypopodium* metabolites

Possible biosynthetic origin of Compounds 12, 13 and 16.

The biosynthesis of **12** may occur by the cyclization of 2-(2,3-epoxy-geranylgeranyl)-5-methyl-1, 4-benzohydroquinone.[14] The initiating step is most probably the breaking of the epoxide to form the secondary alcohol with the adjacent tertiary carbocation. In all likelihood, this cascade is initiated by an enzyme similar to the cyclase enzyme that acts on 2,3-oxidosqualene. The cyclization culminates in the attack by the phenyl hydroxyl alcohol on the carbocation left on C-3, forming an ether ring (Figure II.38).

The biosynthesis of compound **13** can be initiated in the same way, but the termination of cyclization must be different in order to form a five-membered carbocyclic ring and maintain two oxygens in a *para* relationship. It is uncertain exactly when in the biosynthetic process the aromatic methyl ether is formed. The proposed scheme (Figure II.39) suggests that this methylation occurs after cyclization which seems logical because of the isolation of compound **14** which has no methoxy group. Compound **13** is not an artifact, because I couldn't isolate any form of this compound with a free phenolic group at position 6`.

The biosynthesis of **16** can be initiated in the same way, but the termination of cyclization must be different in order to form the five-membered spiro nucleus in the stypotriol compound. The proposed scheme for biosynthesis of compound **16** (Figure II.40) suggests the use of singlet oxygen reaction. Singlet oxygen reacts with many organic compounds including olefins, dienes, sulphides, aromatics, hetero-aromatics, terpenes, steroids, fatty acids, flavones, tetracyclines, vitamins and synthetic polymers Most of the reactions fall into three general classes. I predict that the methylation step (the aromatic methyl ether) occurs after cyclization and hydroxylation, as I didn't isolate any methylated derivative of 2-geranylgeranyl-6-methyl-1, 4-benzoquinone (**20**).

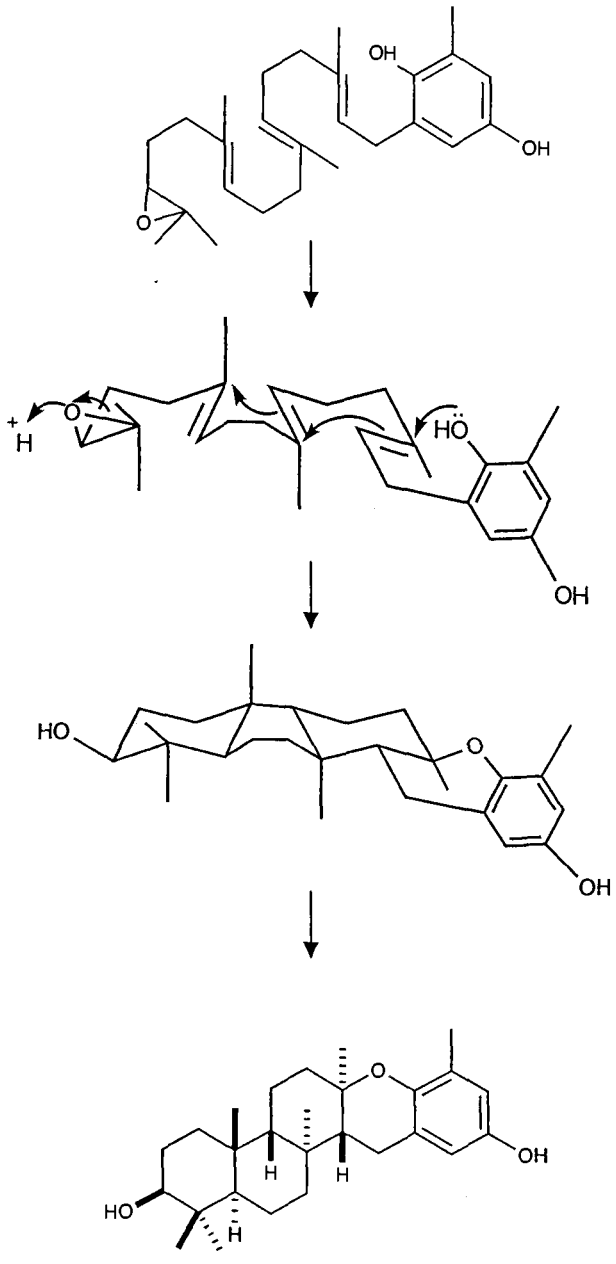


Figure II.38. Proposed biogenetic pathway of 2β,3α-epitaondiol (**12**)

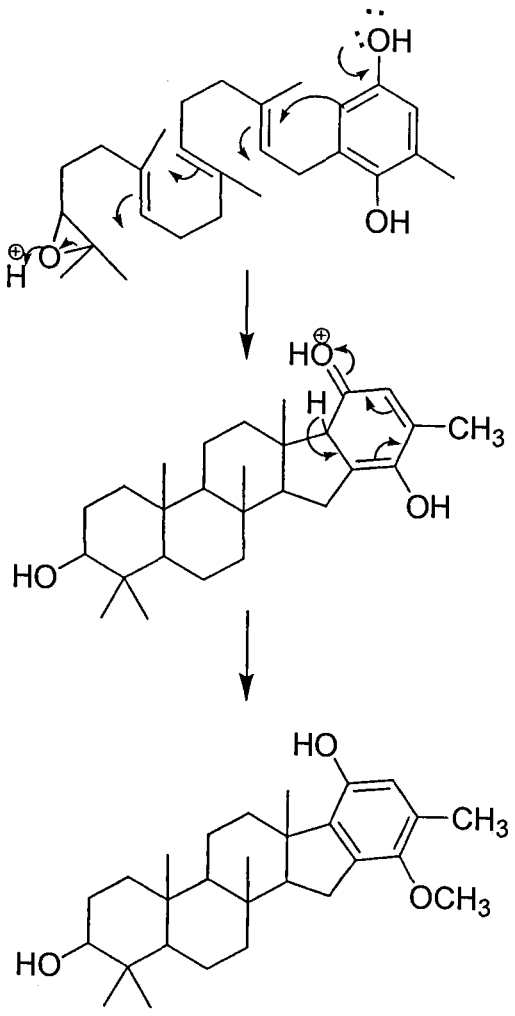


Figure II.39. Proposed biogenetic pathway for flabellinol (**13**).

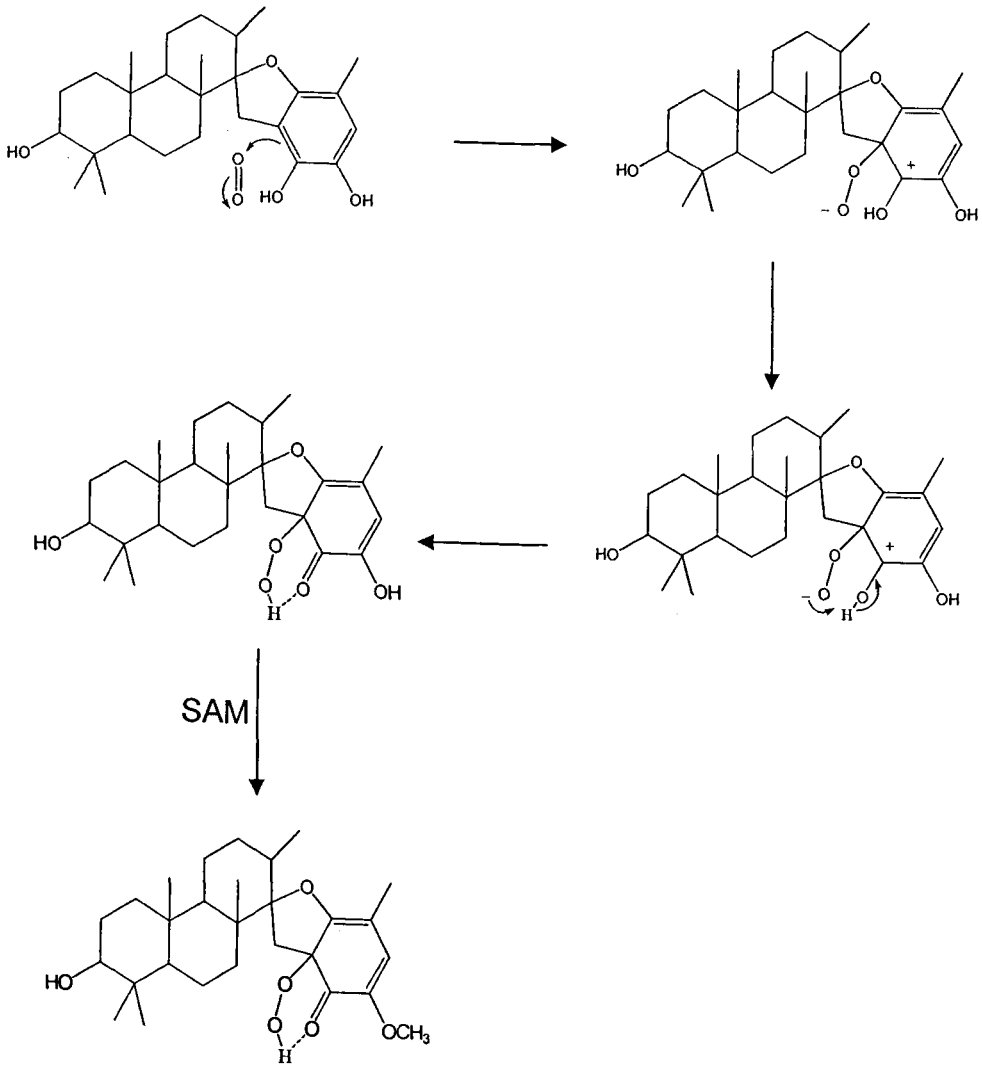


Figure II.40. Possible biogenesis of stypohydroperoxide (**16**)

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. NMR spectra were recorded on a Bruker DPX400 spectrometer, with the solvent ($CDCl_3$ at δ_C 77.2, δ_H 7.26) used as an internal standard. Mass spectra were recorded on a Kratos MS50TC mass spectrometer, and HPLC isolations were performed using Waters Millipore model 515 pumps and a Waters 969 diode array detector.

Algal Collection. The marine brown alga *Stypopodium flabelliforme* (voucher specimen available from WHG as collection number PNGP7-9 December 99-1). The sample was collected by hand using SCUBA from (45-55 feet) of water near Papua New Guinea coast (December 1999). The material was stored in 2-propanol at -20 ^{0}C until extraction.

Extraction and Isolation. Approximately 69 g (dry wt.) of *Stypopodium flabelliforme* was extracted repeatedly with 2:1 CH_2Cl_2/MeOH to produce 17 g of crude organic extract. A portion of the extract (4 g) was subjected to normal phase vacuum liquid chromatography (VLC, hexanes/EtOAc/MeOH) to produce nine chemically distinct fractions. The fraction eluting with 40% EtOAc in hexanes was purified on a silica solid phase extraction (SPE) cartridge (7:3 hexanes/ EtOAc) then by normal phase HPLC (15-25% gradient EtOAc/ hexanes; dual silica, Phenomenex Luna 10u Silica 100A 250×4.6mm column) to yield 2 mg of compound **4**. The VLC fractions eluting with 60 and 80% EtOAc were subjected to C_{18} SPE (60-100% MeOH/H_2O). Fractions from 70% and 80% MeOH/H_2O were subjected to normal phase HPLC (20-40% gradient EtOAc /hexanes) on dual silica, Phenomenex Luna 10u Silica 100A 250×4.6mm. to yield compounds **12** (6.1 mg), **13** (8 mg), **3** (1.2 mg) and **4** (2.6 mg) in addition to the know compounds, 2-geranylgeranyl-6-methyl-1,4-benzoquinone **20** (6 mg), epistypodiol **5** (5.4 mg), stypoldione **10** (3.2 mg), fucoxanthin **21** (4 mg) and iditol **22** (70 mg).

Acetylation of 12 and **13**.[2] All acetylations were conducted in a similar manner. Separately, the natural products **12** and **13** (1 mg) were combined with excess pyridine (ca. 2 ml) and acetic anhydride (ca. 2 ml) and stirred at room temperature. After 24 h, ice and then water were added and the mixture was extracted with diethyl ether (3 x 25mL). The combined ether extracts were washed with 5% HCl (3 x 25 mL) and followed by saturated $NaHCO_3$ solution and dried over anhydrous $MgSO_4$. The concentrated product

was purified using HPLC (5-10% gradient EtOAc /hexanes; dual silica, Phenomenex Luna 10u Silica 100A 250×4.6mm column) to obtain the acetylated compounds **17-19.**

Preparation of *S*- and *R*-MTPA Ester Derivatives of compound 12.[16-18] To a solution of compound **12** (1.5 mg in 1 mL of $CHCl_3$) were added sequentially *N,N*-diisopropylethylamine (10 μL), (*S*)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (3.0 μL), and catalytic amounts of pyridine and 4-(dimethylamino)pyridine (DMAP). In a separate experiment, compound **12** (1.5 mg) was treated with the (*R*)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride as described above. Each mixture was heated at 50 ^{0}C for 24 h under N_2. The solutions were diluted with water and extracted with ethyl acetate. Compound **12** treated with (*R*)-MTPA-Cl furnished the *S*-ester, while treatment of **12** with (*S*)-MTPA-Cl furnished the *R*-ester derivatives. The crude ester mixtures were purified by normal Phase SPE and then normal phase HPLC eluting with 5% ethyl acetate/hexanes (dual silica, Phenomenex Luna 10u Silica 100A 250×4.6mm column) to give *S*-ester (2.8 mg, approximately 90% yield of the theoretical maximum) and *R*-ester (2.3 mg, approximately 74% yield of the theoretical maximum), respectively.

X-ray Crystallography.[20] Compound **12** (4 mg) was recrystallized from neat acetonitrile. Determination of the crystallographic parameters, data collection and structure solution and refinement was done as described elsewhere[16], with the following details:

A well shaped crystal of dimensions 0.20 x 0.20 x 0.10 mm^3 was selected and mounted on the tip of a thin glass fiber using epoxy glue. An automated routine was used to find and center reflections with $3° < \theta < 12.5°$, with which the crystal was indexed. The reflection list was then expanded to include 87 reflections with $3.42° < \theta < 24.91°$, and the lattice parameters refined against this list. All unique data, including a small set of redundant reflections, were collected (-7 – 7, -14 – 14, -35 – 35). Monitoring of three strong reflections as intensity standards during data collection showed no decay. Correction for the effects of absorption anisotropy was carried out by means of psi-scans using the program XEMP v4.3.

The structure was solved using direct methods as programmed in SHELXS-90 and refined using the program SHELXL-97, followed by Fourier synthesis. Though all hydrogen atoms could be clearly identified from the Fourier map, in order to preserve a favorable data-to-parameter ratio the hydrogen atoms were placed in geometrically idealized positions. The hydrogen atoms were given a displacement parameter equal to 1.5 times (methyl group) or 1.2 times (all other hydrogens) the equivalent isotropic

displacement parameter of the atom it is attached to. During the final cycle of least squares refinement, all non-hydrogen atoms were refined with anisotropic displacement parameters. Though the refined value of the absolute structure parameter (Flack parameter) of -0.2 (2) suggests the model obtained accurately depicts the absolute structure of the molecule, the large uncertainty associated with this parameter indicates that this cannot be stated with absolute certainty. An ORTEP of the final model is given in Figure 1, with displacement ellipsoids drawn at the 30% probability level. Separate diagrams illustrating the conformations of rings A, B and C are given in Figure II.7.

Reaction of stypohydroperoxide with Triphenylphosphine.[21] To a stirred solution of 0.5 mg. (1 μM) of stypohydroperoxide in 1 ml of ether was added 1 mg of triphenylphosphine in 0.5 ml of ether. After stirring at room temperature for two hours, the ether was removed under vacuum and then subjected to normal phase SPE with 50% ethyl acetate /hexanes. The sample eluting from the SPE was subjected to NP HPLC (ethyl acetate /hexanes gradient) to get the purified reduction product (0.3 mg).

Compound 12: Colorless radiating needles; $[\alpha]^{25}_{D}$ +70 (c 0.2, $CHCl_3$); UV (MeOH) $_{max}$ 224 nm (ε 1210), 294 nm (ε 1301); IR (neat) 3342, 2933, 2865, 1620, 1469, 1376 cm^{-1}; ^1H and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables 1 and 2 respectively; HRFABMS *m/z* $[M+H]^+$ 413.2985 (calculated for $C_{27}H_{40}O_3$, 412.2977).

Compound 13: Colorless oil; $[\alpha]^{25}_{D}$ +18 (c 0.12, $CHCl_3$); UV (CD_3Cl) $_{max}$ 284 nm (ε 960), 244 nm (ε 745); IR (neat) 3602, 3375.23, 2937.30, 2861.50, 1450, 1416 cm^{-1}; ^1H and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables 1 and 2 respectively; HRFABMS *m/z* $[M+H]^+$ 427.3126 (calculated for $C_{28}H_{42}O_3$, 427.3134).

Compound 14: Yellowish oil; $[\alpha]^{25}_{D}$ +25 (c 0.12, $CHCl_3$); UV (CD_3Cl) $_{max}$ 254 nm (ε 1240), 276 nm (ε 1039), 354 nm (ε 123); IR (neat) 3480, 2933, 2861, 1648, 1598, 1460 cm^{-1}; ^1H and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables 1 and 2 respectively; HRFABMS *m/z* $[M+H]^+$ 411.29685 (calcd. for $C_{27}H_{40}O_3$, 411.2977)

Compound 15: Yellow oil; $[\alpha]^{25}_{D}$ +22 (c 0.12, $CHCl_3$); UV (MeOH) $_{max}$ 216 nm (ε 7910), 288 nm (ε 2560), 348 nm (ε 1190); IR (neat) 3466, 2927, 1714, 1655, 1611, 1454 cm^{-1}; ^1H and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables 1,2; HRFABMS *m/z* $[M]^+$ 443.2794 (calcd for $C_{27}H_{39}O_5$, 443.2797).

Compound 16: Colorless oil; $[\alpha]^{25}_{D}$ -21 (c 0.12, $CHCl_3$); UV (CD_3Cl) $_{max}$ 266 nm (ε 658), 290 nm (ε 675), 345 nm (ε 109); IR (neat) 3417, 2941, 2875, 1729, 1667, 1616,

1000 cm^{-1}; ^1H and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables 1 and 2 respectively; HRFABMS *m/z* $[M]^+$ 475.2994 (calcd for $C_{28}H_{42}O_6$, 474.2982).

Compound 17: Colorless oil; UV ($CDCl_3$) $_{max}$ 296 nm; IR (neat) 3342, 2933, 2861, 1620, 1469, 1376 cm^{-1}; ^1H NMR δ 6.44 (1H, d, *J* = 2.0 Hz), 6.37 (1H, d, *J* = 2.0 Hz), 4.51 (1H, m), 4.2 (1H, s), 2.56 (1H, dd, *J* = 12.0, 5.1 Hz), 2.51 (1H, dd, *J* = 12.0, 5.1 Hz), 2.08 (3H, s), 2.05 (3H, s), 1.98 (1H, m), 1.70 (1H, m), 1.69 (1H, m), 1.16 (1H, m), 1.66 (1H, m), 1.66 (1H, m), 1.65 (1H, m), 1.64 (1H, m), 1.58 (1H, m), 1.54 (1H, m), 1.52 (1H, m), 1.51 (1H, m), 1.45 (1H, m), 1.40 (1H, m), 1.20 (1H, m), 1.12 (3H, s), 1.06 (3H, s), 1.00 (3H, s), 0.98 (3H, s), 0.87 (3H, s), 0.86 (3H, s); LRFABMS *m/z* $[M+H]^+$ 455.0 (calculated for $C_{29}H_{42}O_4$).

Compound 18: Colorless oil; $[\alpha]^{22}_D$ +83 (*c* 0.18, $CHCl_3$); UV ($CDCl_3$) $_{max}$ 242 nm (ε 1686), 288 nm (ε 570), 250 nm (ε 446); IR (neat) 2937, 2867, 1758, 1732, 1475, 1371 cm^{-1}; ^1H NMR δ 6.62 (1H, d, *J* = 2.0 Hz), 6.58 (1H, d, *J* = 2.0 Hz), 4.51 (1H, m), 2.56 (1H, dd, *J* = 12.0, 5.1 Hz), 2.49 (1H, dd, *J* = 12.0, 5.1 Hz), 2.26 (3H, s), 2.11 (3H, s), 2.04 (3H, s), 2.0 (1H, m), 1.74 (1H, m), 1.66 (1H, m), 1.64 (1H, m), 1.63 (1H, m), 1.61 (1H, m), 1.60 (1H, m), 1.56 (1H, m), 1.47 (2H, m), 1.40 (1H, m), 1.36 (1H, m), 1.35 (1H, m), 1.16 (1H, m), 1.14 (3H, s), 1.06 (3H, s), 0.98 (3H, s), 0.87 (3H, s), 0.87 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 171.5 q, 171.0 q, 149.0 s, 143.0 s, 127.1 s, 122.5 s, 121.3 d, 119.7 d, 81.3 d, 68.4 s, 55.1 d, 53.7 d, 46.1 d, 41.4 t, 39.1 s, 38.5 t, 37.1 s, 36.4 s, 33.2 t, 29.4 q, 25.6 t, 23.3 t, 22.6 q, 22.17 q, 21.7 q, 21.4 q, 20.7 q, 20.7 t, 17.5 t, 17.3 q, 16.6 q; HRFABMS *m/z* $[M]^+$ 497.6200 (calculated for $C_{31}H_{44}O_5$, 497.6211)

Compound 19: colorless oil; $[\alpha]^{25}_D$ +25 (c 0.36, $CHCl_3$); UV (CD_3Cl) $_{max}$ 240 nm, 280 nm; IR (neat) 2934, 2961, 2860, 1768, 1731, 1476 cm^{-1}; ^1H NMR 6.64 (1H, s), 4.5 (1H, dd, *J* = 12, 4 Hz), 2.56 (1H, d, *J* = 5.1 Hz), 2.51 (1H, d, *J* = 5.1 Hz), 1.54 (1H, m), 1.98 (1H, m), 1.58 (1H, m), 1.70 (1H, m), 1.65 (1H, d, *J* = 15.3 Hz), 1.64 (1H, d, *J* = 12.2 Hz), 1.66 (1H, d, *J* = 14.2 Hz), 1.52 (1H, d, *J* = 14.2 Hz), 1.66 (1H, m), 1.40 (1H, m), 1.2 (1H, d, *J* = 11.7 Hz), 1.69 (1H, m), 0.98 (1H, m), 1.51 (1H, m), 1.45 (1H, m), 4.51 (1H, dd, *J* = 17, 5 Hz), 0.87 (3H, s), 0.86 (3H, s), 1.00 (3H, s), 1.06 (3H, s), 1.12 (3H, s), 6.44 (1H, d, *J* = 2.5 Hz), 6.37 (1H, d, *J* = 2.75 Hz), 2.05 (3H, s), 4.2 (1H, s), 2.08 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 16.3 q, 17.0 q, 19.4 t, 20.6 q, 21.5 q, 21.7 q, 22.8 t, 24.4 t, 26.9 q, 27.2 t, 28.5 q, 30.6 t, 30.7 q, 35.8 t, 36.5 t, 38.5 s, 38.6 s, 38.8 s, 46.1 s, 48.1 d, 57.4 d, 60.4 q, 61.4 d, 81.4 d, 123.2 d, 129.5 s, 136.0 s, 141 s, 144.5 s, 153.2 s, 170 s, 171.4 s; HRFABMS *m/z* $[M]^+$ 511.3352 (calculated for $C_{32}H_{46}O_5$, 511.3368).

Cytotoxicity against NCI-H460 human lung cancer and mouse neuro-2a blastoma cell lines.[22] Cytotoxicity was measured to lung tumor cells (NCI-H460) and mouse neuro-2a blastoma cells using the method of Alley et. al.[17] with cell viability being determined by MTT reduction. Cells were seeded in 96-well plates at 5000 and 8000 cells/well in 180 µl for H460 and neuro-2a cells, respectively. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µg/well. DMSO was less than 1% final concentration. After 48 hr, the medium was removed and cell viability determined.

Sodium channel modulation.[23] Isolated compounds were evaluated for their capacity to either activate or block sodium channels using the following modifications to the cell-based bioassay of Manger et. al. [12] Twenty-four hr prior to chemical testing, mouse neuroblastoma (neuro-2a) cells were seeded in 96-well plates at 8 X 10^4 cells/well in a volume of 200 µl. Test chemicals dissolved in DMSO were serially diluted in medium without fetal bovine serum and added at 10 µl/well. DMSO was less than 1% final concentration. Plates to evaluate sodium channel activating activity received 20 µl/well of either a mixture of 3 mM ouabain and 0.3 mM veratridine (Sigma Chemical Co.) in 5 mM HCl in addition to the test chemical. Plates were incubated for 18 hr and results compared to similarly treated solvent controls with 10 µl medium added in lieu of the test chemical. The sodium channel activator brevetoxin PbTx-1 (Calbiochem) was used as the positive control and added at 10 ng/well in 10 µl medium. Sodium channel blocking activity was assessed in a similar manner except that ouabain and veratridine were 5.0 and 0.5 mM, respectively, and the sodium channel blocker saxitoxin (Calbiochem) was used as the positive control. Plates were incubated for approximately 22 hr.

Brine Shrimp Toxicity Assay.[24] Screening of the crude extract, fractions, and pure compounds (for brine shrimp toxicity) was performed by a slight modification of the original method.[15] About 15 hatched brine shrimp (*Artemia salina*) in ca. 0.5 ml seawater were added to each well containing different concentrations of sample in 50 µL of EtOH and 4.5 ml of artificial seawater to make a total volume of ca. 5 ml. Samples and controls were run in duplicate. After 24 h at 28 C, the number of alive and dead brine shrimp were counted.

Cerebellar granule cell culture.[25] Primary cultures of cerebellar granule neurons (CGN) were obtained as described.[25] Cerebella were removed from 8-day-old Sprague-Dawley rats and dissected from their meninges. The isolated cerebella were

then minced by mild trituration with a Pasteur pipette and treated with trypsin (2200 U/mL) in Kreb's buffer containing 3 mg/ml BSA. The cell suspension was then shaken mildly for 15 min at 37°C. The digestion was terminated by the addition of 166 µg/mL soybean trypsin inhibitor (SBTI), 26 µg/mL DNAse and 1.7 mM $MgSO_4$. Following centrifugation at 228 x g for 1 min the cells were resuspended in Kreb's/BSA containing 500 µg/mL SBTI, 80 µg/mL DNAse and 2.8 mM $MgSO_4$, and triturated with a Pasteur pipette. The suspension was allowed to settle and the resultant supernatant was centrifuged at 228 x g for 5 min. The cell pellet was resuspended in Basal Eagle's medium (BME) containing 25 mM KCL, 2 mM glutamine and 100 µg/mL gentamycin to a final concentration of 6.4 x 10^5 cells/mL. The neurons were plated at a density of 9.6 x 10^4 cells/well on to 96-well (9 mm) clear-bottomed black well culture plates (Costar) coated with poly-L-lysine (M.W. = 393,000) and incubated at 37°C in a 5% CO_2, 95% humidity atmosphere. Cytosine arabinoside (10 µM final concentration) was added to the cultures after 18 – 24 h to inhibit the growth of non-neuronal cells. After 7 – 8 days in culture the neurons were fed by the addition of 10 µL of a 25 mg/mL dextrose solution.

Intracellular Ca^{2+} monitoring.[26] CGN cultures were used at 10 – 13 days in culture for experimental determination of toxin-induced Ca^{2+} influx. The growth media was removed and replaced with dye loading media containing 4 µM fluo-3 AM and 0.04% pluronic acid in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 8.6 mM HEPES, 5.6 mM glucose and 0.1 mM glycine, pH 7.4). The acetoxymethyl ester of fluo-3 is taken up by the cells and entrapped intracellularly following hydrolysis to fluo-3 by neuronal esterases. Dye loading was found to be optimal after 1 h at 37° C. After the 1 h incubation cells were washed four times in fresh Locke's buffer using an automated cell washer (Labsystems, Helsinki, Finland) and transferred to the incubation chamber of a fluorescent laser imaging plate reader (FLIPR®, Molecular Devices, Sunnyvale, CA.). The final volume of Locke's buffer in each well was 200 µL. The FLIPR operates by illuminating the bottom of a 96-well plate with an argon laser tuned to 488 nm. Ca^{2+} - bound fluo-3 has a fluorescence emission in the range of 500 – 560 nm and a CCD camera recorded the signal with the shutter speed set at 0.4 s. An automated 96-well pipettor can be programmed to deliver precise quantities of solutions from a source plate to all 96 cultures simultaneously. Prior to each experiment, average baseline fluorescence was set to between 5,000 and 10,000 relative fluorescent units by adjusting the power output of the laser. Measurements were taken every 3 sec for 1 min before

the addition of the treatments to establish the baseline and at the same interval for 2 min more to observe any rapid response to the toxins. After the 3 min time period subsequent readings were taken every 6 sec with a total exposure time of 24 min.

Quantification of results.[26] Data were analyzed and graphs generated using the GraphPad 3.0 analysis package (GraphPad Software, San Diego, California, USA). Area under the curve (AUC) values were calculated using the trapezoid rule to integrate the fluorescence vs. time curves. The EC_{50} value of toxin-induced increases in fluo-3 fluorescence and LDH was determined by non-linear least squares fitting of a logistic equation to the toxin concentration versus AUC or LDH units per plate.

REFERENCES

1. Faulkner, D. J. *Nat. Prod. Rep.* **2002**, *19*, 1-48, and references therein.

2. Gerwick, W. H.; Fenical, W. *J. Org. Chem.* **1981**, *46*, 22-27.

3. Gerwick, W. H.; Fenical, W.; Norris, J. N. *Phytochemistry* **1985**, 24, 1279-83.

4. Dorta, E.; Diaz-Marrero, A. R.; Cueto, M.; Darias, J. *Tetrahedron* **2003**, 59, 2059- 2062.

5. Dorta, E.; Cueto, M.; Diaz-Marrero, A. R.; Darias, J. *Tetrahedron Letters* **2002**, 43, 9043-9046.

6. Depix, M. S.; Martinez, J.; Santibanez, F.; Rovirosa, J.; San Martin, A; Maccioni, R. B. *Molecular and Cellular Biochemistry* **1998**, 187, 191-199.

7. Martinez, J. L.; Sepulveda, S. P.; Rovirosa, J.; San Martin, A. *Anales de la Asociacion Quimica Argentina* **1997**, 85, 69-75.

8. Wessels, M.; Koning, G. M.; Wright, A. D. *J. Nat. Prod.* **1999**, *62*, 927-930

9. O'Brien, E. Timothy; W., Steven; J., Robert S.; Boder, G. B.; Wilson, L. *Hydrobiologia* **1984**, 116-117.

10. White S. J; Jacobs R. S. *Mol. Pharmacol.* **1983**, 24, 500-8.

11. Rovirosa, J.; Sepulveda, M.; Quezada, E.; San-Martin, A. *Phytochemistry* **1992**, 31, 2679-81.

12. Sampli, P.; Tsitsimpikou, C.; Vagias, C.; Harvala, C.; Roussis, V. *Nat. Prod. Lett.* **2000**, 14, 365-372.

13. Gonzalez, A. G.; Darias, J.; Martin, J. D. *Tetrahedron Lett.* **1971**, 29, 2729-32.

14. Sanchez-Ferrando, Francisco; San-Martin, Aurelio. *J. Org. Chem.* **1995**, 60, 1475-8.

15. Gonzalez, Antonio G.; Alvarez, Miquel A.; Darias, Jose; Martin, Julio D. *J. Chem. Soc., Perkin.* **1973**, 22, 2637-2642.

16. Cahn, R. S.; Ingold, C. K.; Prelog, V. *Experientia* **1956**, *12*, 81-124.

17. Cahn, R. S.; Ingold, C. K.; Prelog, V. *Angew. Chem., Int. Ed.* **1966**, *5* (4), 385-415.

18. Hazzard, James T.; Moench, Susan J.; Erman, James E.; Satterlee, James D.; Tollin, Gordon. *Biochemistry*, **1988**, 27, 2002-2008.

19. Mori, K.; Koga, Y. *Bioorg. Med. Chem. Lett.* **1992**, 2, 391-394.

20. Blakemore, P. R., Kim, S. K., Schulze, V. K., White, J. D., Yokochi, A. F. T. *J. Chem. Soc. Perkin Trans. 1*, In Press.

21. Denney, Donald B.; Goodyear, William F.; Goldstein, Bernard. *J. Am. Chem. Soc.* **1960**, 82, 1393-1395.

22. Alley, M. C.; Scudiero, D. A. *Cancer Research* **1988, 48**, 589-601.

23. Manger, R. L.; Leja, L. S. *Journal of AOAC Int.* **1995, 78**(2), 521- 527.

24. (a) Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31–34.

(b) Gerwick, W. H.; Proteau, P.J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. *J. Org. Chem.* **1994**, *59*, 1243–1245.

25. Berman, F.W.; Murray, T.F. *J. Neurochem.* **2000**, 74, 1443-1451.

26. Koh, J.Y.; Choi, D.W. *J. Neurosci. Methods.* **1987**, 20, 83-90.

CHAPTER THREE

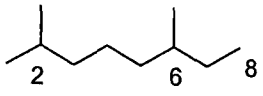
BIOLOGICALLY ACTIVE HALOGENATED MONOTERPENES FROM THE MARINE RED ALGA *PLOCAMIUM CARTILAGINEUM*

ABSTRACT

As a result of my efforts to identify bioactive agents from marine algae, I have isolated and identified one new halogenated monoterpene **28** [(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene] in addition to three known compounds (**1**, **2** and **10**) from the red alga *Plocamium cartilagineum* collected from the eastern coast of South Africa. Compound **28** was found to be active as a cytotoxic agent in human lung cancer (NCI-H460) and mouse neuro-2a cell lines (EC_{50} 4 µg/ml). Two of these compounds (**1** and **2**) were found to have promising activity as cytotoxins in other cell line assays, especially to human leukemia and human colon cancers and are now in the *in-vivo* evaluation stage. However, none of these metabolites were active as sodium channel blockers or activators. All structures were determined by spectroscopic methods (UV, IR, LRMS, HRMS, 1D NMR and 2D NMR). 1D and 2D NOE experiments were carried out on compounds **1**, **2** and **28** to confirm the geometry of the double bonds.

INTRODUCTION

As a result of their predominant growth in temperate and tropical locations, red algae are among the most frequently investigated sources for marine natural products. They include more than 4000 species distributed in different localities around the world.[1] *Plocamium cartilagineum* is a species of red algae (family Plocamiaceae, order Gigartinales). This species is characterized by its interesting secondary metabolites, being a rich source of diverse polyhalogenated monoterpenes, with a surprising degree of halogen incorporation.[2-5] Polyhalogenated monoterpenes vary for the given species depending on collection, location and season.[6] Recent research on this genus has yielded a number of halogenated metabolites that display considerable biological activity such as cytotoxic activity,[7-9] anti-feedant activity [10] anti-fungal activity, molluscicidal activity and insecticidal activity.[11] These secondary metabolites can be categorized into two predominant skeletal types, the 2,6-dimethyloctanes (**1-14**) and cyclohexanes (**15-27**).[5,12-23] The latter group is actually composed of three subtypes as shown in figure III.1.



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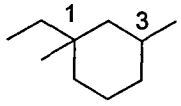

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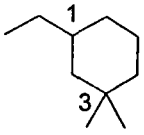

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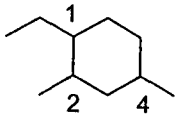
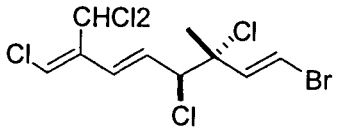


1-Ethyl-2,4-dimethylcyclohexane

Figure III.1. The four predominant skeletal types isolated from the genus *Plocamium*.



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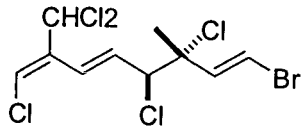

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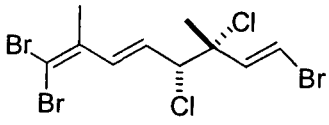

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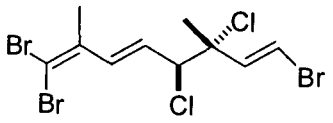

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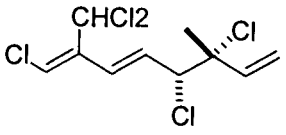

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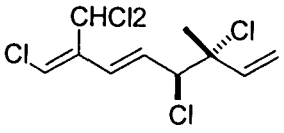

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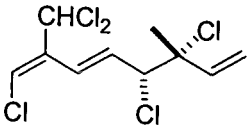

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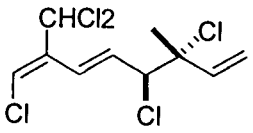

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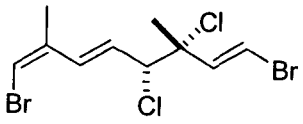

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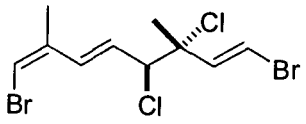

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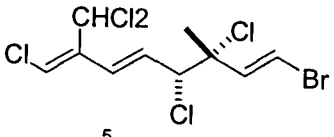

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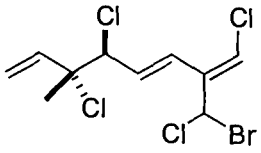

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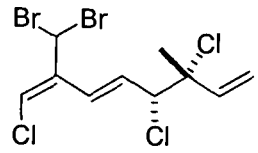

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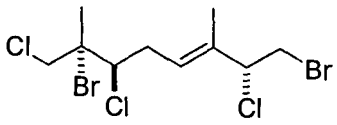

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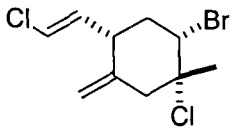

Plocamadiene A

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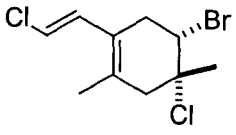

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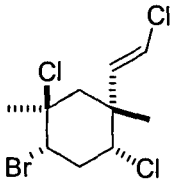

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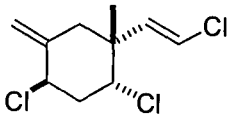
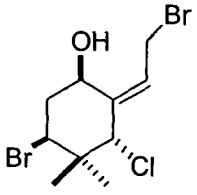
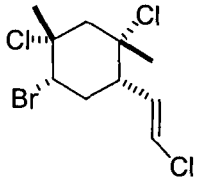


Violacene

(18)[18]



(19)[18]



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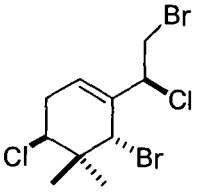

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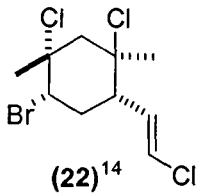

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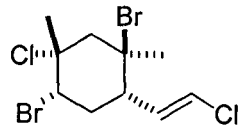

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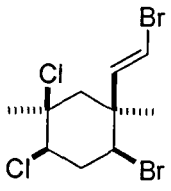

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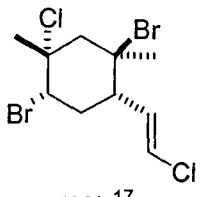

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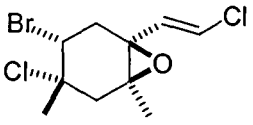

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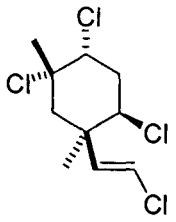
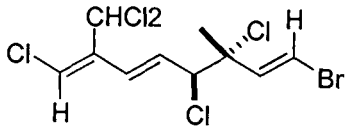


(27) [23]

RESULTS AND DISCUSSION

In our continuing efforts using bioassay guided fractionation to characterize the prolific natural products from marine algae, a detailed examination of the crude organic extract of *Plocamium cartilagineum* from a South African collection was carried out. After extraction of the alcohol-preserved tissue with CH_2Cl_2/MeOH (2:1), initial fractionation was accomplished by VLC (EtOAc/hexanes gradient) over silica gel. Successive normal phase HPLC fractionations and purifications resulted in the isolation of one new compound (**28**), in addition to three previously known compounds (**1**, **2** and **10**).[5,24]



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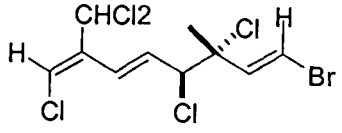

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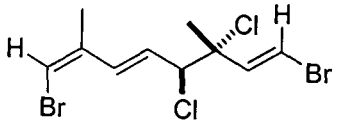

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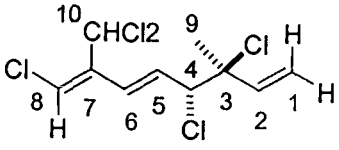


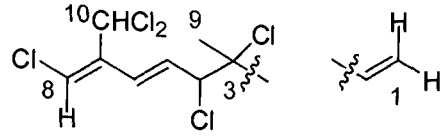
28

Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed construction of the in planar structures. HMBC and MS fragmentation were used to confirm these statements. ^{1}H-^{1}H coupling constants, ^{13}C NMR, 1D and 2D NOE were used to confirm the double bond geometry.

Compound **28** showed in the LRCIMS the presence of fragment ion clusters $[M\text{-}Cl]^+$ (*m/z* 271, 273, 275, 277), $[M\text{-}Cl\text{-}HCl]^+$ (*m/z* 235, 237, 239, 241) and $[M\text{-}Cl\text{-}2HCl]^+$ (*m/z* 199, 201, 203). HRCIMS showed an ion at *m/z* 305.93079 $[M]^+$ for a molecular formula of $C_{10}H_{11}Cl_5$, and therefore possessed three degrees of unsaturation. The IR spectrum of **28** showed absorption bands at 2923 cm^{-1}, indicating the presence of an olefinic group functionality. The ^{1}H NMR spectrum in $CDCl_3$ of **28** (Table III.1) showed signals corresponding to one dihalomethylene group at δ_H 6.96 (1H, s) and six olefinic protons at δ_H 6.3 (1H, s), 6.35 (1H, dd, *J* = 17, 7 Hz), 6.34 (1H, dd, *J* = 17, 2 Hz), 5.41 (1H, d, *J* = 17 Hz), 5.29 (1H, d, *J* = 11 Hz) and 6.07 (1H, dd, *J* = 17, 11 Hz). Additionally there was a doublet of doublet at δ_H 4.55 (1H, dd, *J* = 7, 2 Hz), attributed to a mono halomethylene group proton and one methyl group geminal to a halogen atom (δ_H 1.77, 3H, s). The ^{13}C NMR spectrum of **28** in $CDCl_3$ (Table III.1) showed signals for 10 carbons. The number of attached hydrogen atoms were determined from the HSQC and HMBC spectra: one methyl at δ 25.1, one methylene, at δ 116.0, six methines (four olefinic at δ140.0, δ 131.0, δ 127.0, δ 119.7 two bearing halogen at δ 69.2 and 66.0), and two nonprotonated carbons at δ138.1 and 72.0 were observed. This was in keeping with the three degrees of unsaturation required by the molecular formula.

Chemical shift arguments and ^{1}H-^{1}H COSY correlations supported by MS data and HMBC allowed the assignment of fragments **"a"** and **"b"** (Figure III.2). From the ^{1}H-^{1}H COSY NMR spectrum of **28**, it was possible to differentiate two discrete spin systems. The coupling between signals corresponding to the olefinic protons at δ 5.41, 5.29 and 6.07 established the connectivity of the H-1-H-2 fragment in **a**. The coupling between one of the protons bearing halogen (δ 4.55) and the methine protons at δ 6.35 and 6.34 established the connectivity of the H-4 to H6 in fragment **b**. HMBC data were used to confirm remaining of fragment **b** from the correlations between H-4 and C-2, C-3, C-5, C-6. The C-5/C-6/C-7 constellation was determined by the correlation between H-6 and C-5, C-7, and also by the correlations of H-8 with C-10, C-7, and C-10. The C-8/C-7/C-10 linkage was confirmed by the correlation of H-8 with C-7 and C-10. The linkage C-2/C-3 was secured by the correlations between H-1 and C-2, C-3 and suggested the

overall planar structure **28**. The *5E,7Z* stereochemistry of the double bonds were deduced from the ^{13}C NMR chemical shift, ^{1}H-^{1}H coupling constants, 1D NOE and 2D NOE (Table III.1). Many trials were carried out to crystallize compound **28** from different solvents at low temperature, but all failed. In order to investigate the stereochemistry of compound **28**, we applied the empirical rules of Faulkner[25] and Crews[26]. We noticed that the C-3 and C-4 chiral centers of compound **28** must be assigned (3*R**, 4*S**) according to the previous rules, as C-3 was found to possess δ_C = 25.1 and δ_H = 1.77 ppm and this fits the chemical shift range reported in the previous rules for the (3*R**, 4*S**) stereochemistry of these compounds. The suggested structure of compound **28** was found be similar to the previously reported compound **6** (3*R**, 4*S**) and not similar to **5** (3*R**, 4*R**) (Table III.2 and Table III.3). However, the differences in ^{1}H chemical shift between the two compounds, and the strong negative sign of the optical rotation of compound **28** ($[\alpha]^{25}_D$ = -92.0^0) and the positive sign of compound **6** ($[\alpha]^{25}_D$ = + 5.1^0), strongly supported the suggestion that compound **28** is an enantiomer to that of the previously reported compound **6** at C-3 and C-4. The 3*S**, 4*R** relationship at C-3 and C-4 was reinforced by 1D and 2D NOE experiments [No NOE effects between H-4 and H_3-9 were observed, confirming a relative (*R**)-configuration for the C-4 chiral center]. Compound **28**, which had a negative optical rotation, $[\alpha]^{25}_D$ – 92.0 (*c* 0.07, $CHCl_3$), was thus shown to be a 3,4-*erythro* compound. However, we can conclude that ^{1}H shifts are not very discriminating as shown by comparing the data of compounds **5**, **6** and **28**. Hence, the ^{13}C NMR values are more useful for establishing the threo (28 ppm) versus erythro (25 ppm) relationship of substituents at C-3 and C-4.[24]



b **a**

Figure III.2a. Partial structures of compound **28**.

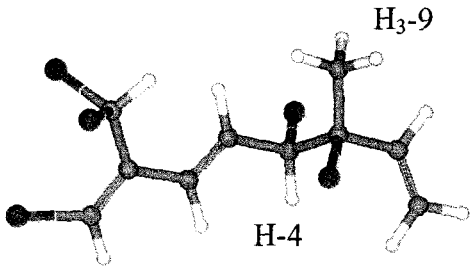


Figure III. 2b. Molecular modeling of compound **28**

Table III.1. ^{1}H- and ^{13}C-NMR Data for **28** in $CDCl_3$[a]

Position	^{1}H mult. *J* (Hz)	^{13}C	HMBC	NOE
1a	5.41 (d, 17)	116.0 CH_2	C-2, C-3	
1b	5.29 (d, 11)			H-2
2	6.07 (dd, 17, 11)	140.0 CH		H-1a
3		72.0 C		
4	4.55 (dd, 7, 2.1)	69.2 CH	C-2, C-3, C-5, C-6	H-5, H-6
5	6.35 (dd, 17, 7)	127.0 CH		H-4
6	6.34 (dd, 17, 2.1)	131.0 CH	C-4, C-8	H-4
7		138.1 C		
8	6.30 (s)	119.7 CH	C-9	
9	1.77 (s)	25.1 CH_3	C-3, C-4	
10	6.96 (s)	66.0 CH		

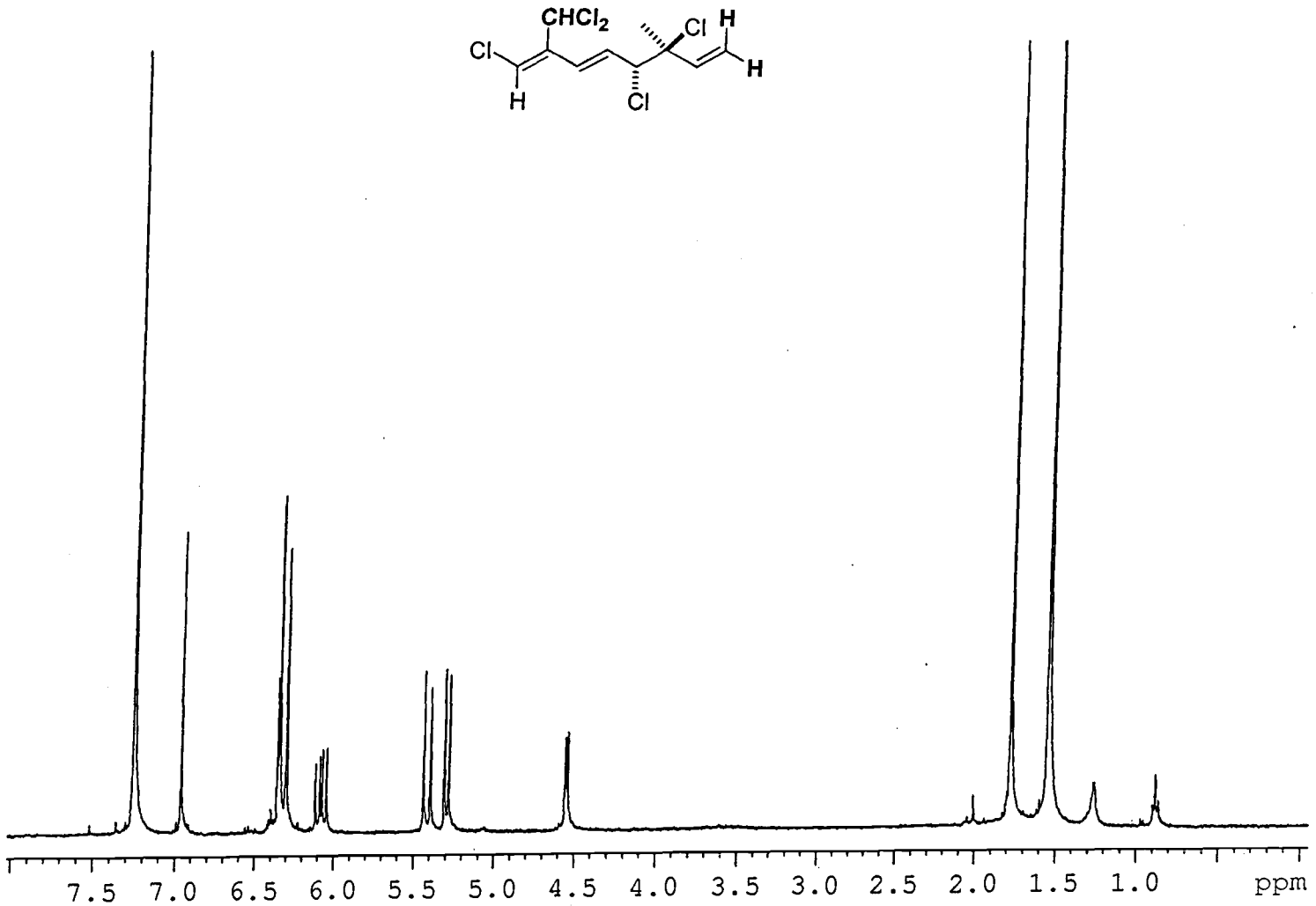


Figure III.3. ^{1}H NMR spectrum of compound **28** in $CDCl_3$

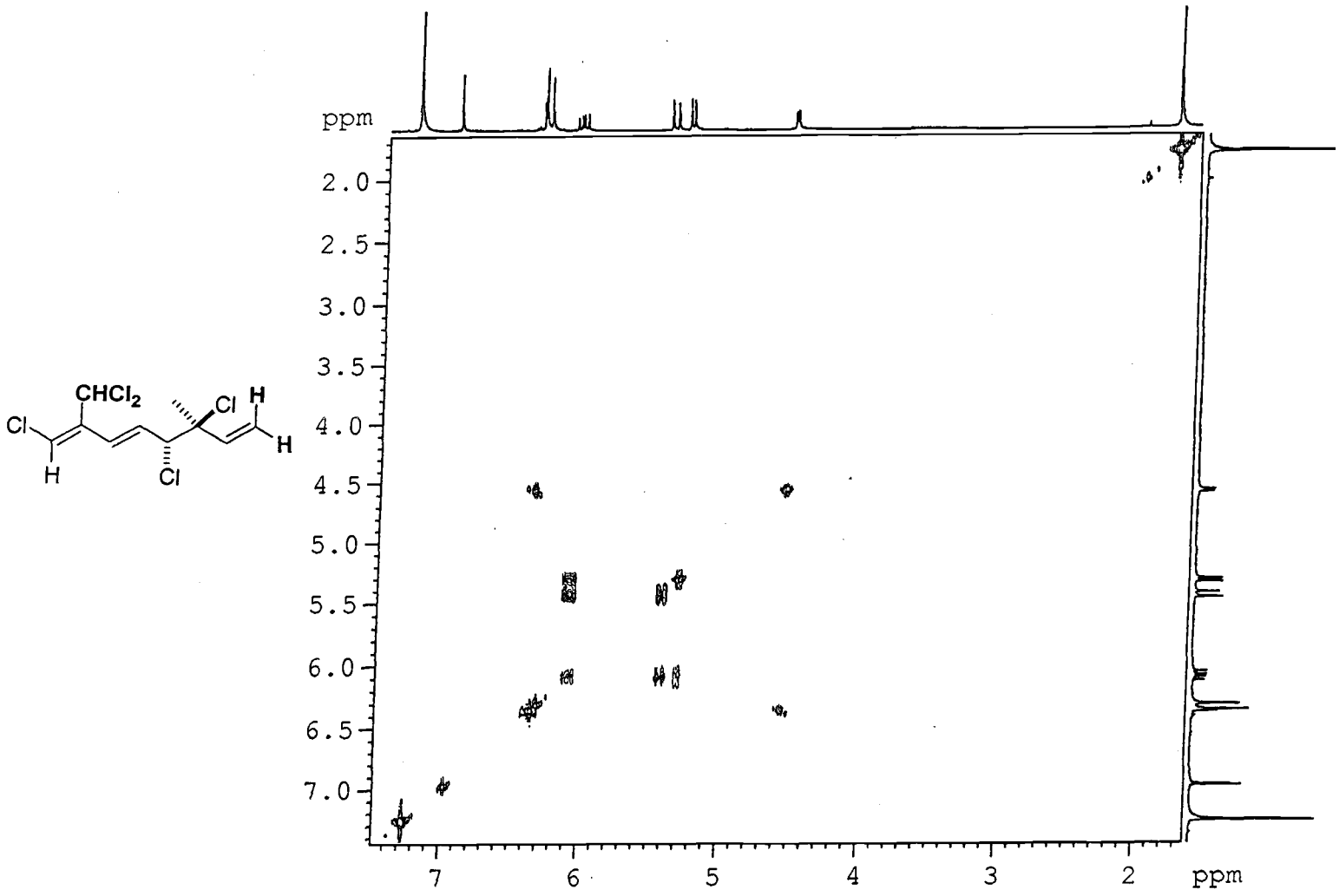


Figure III.4. COSY spectrum of compound **28** in $CDCl_3$

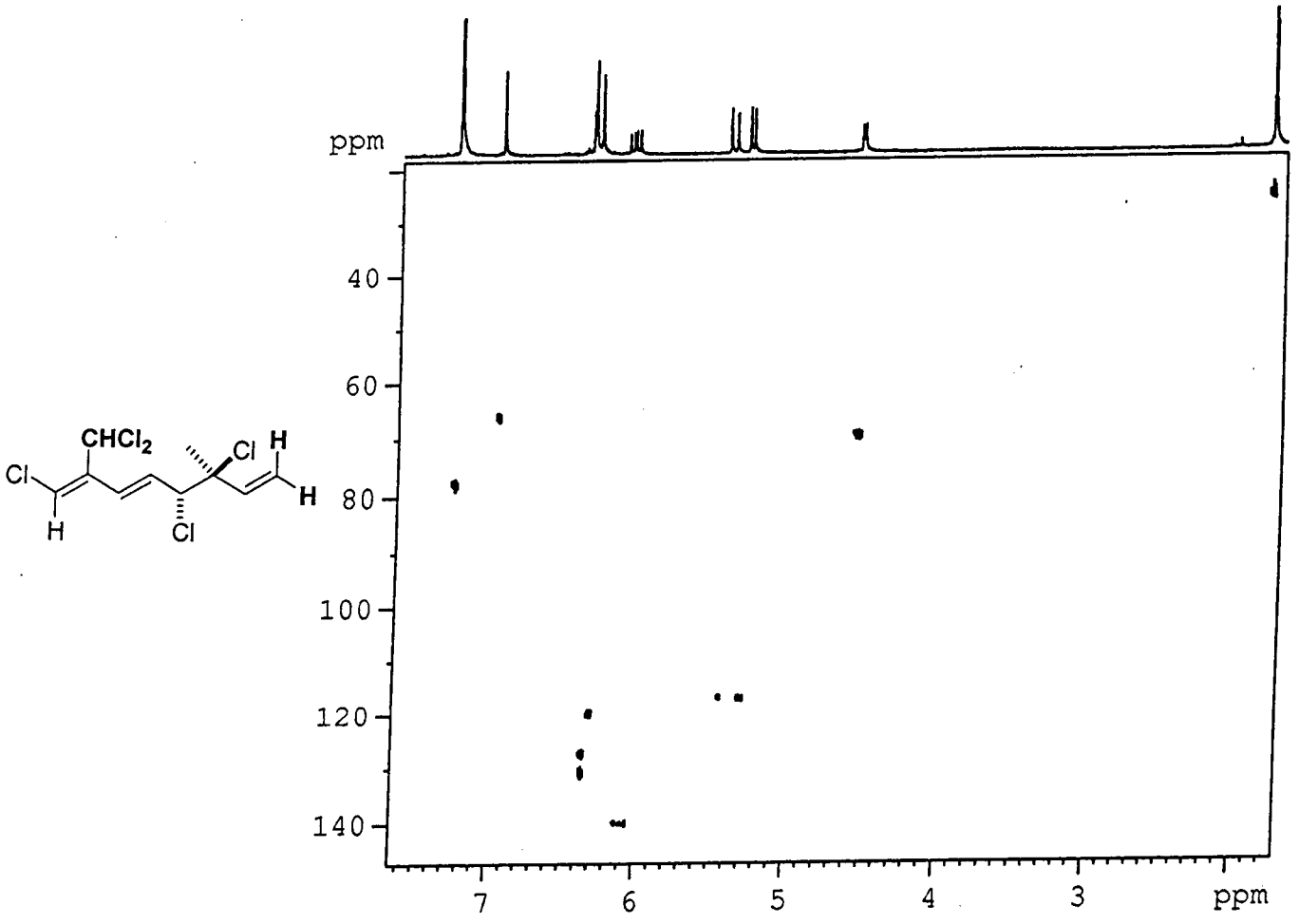


Figure III.5. HSQC spectrum of compound **28** in $CDCl_3$

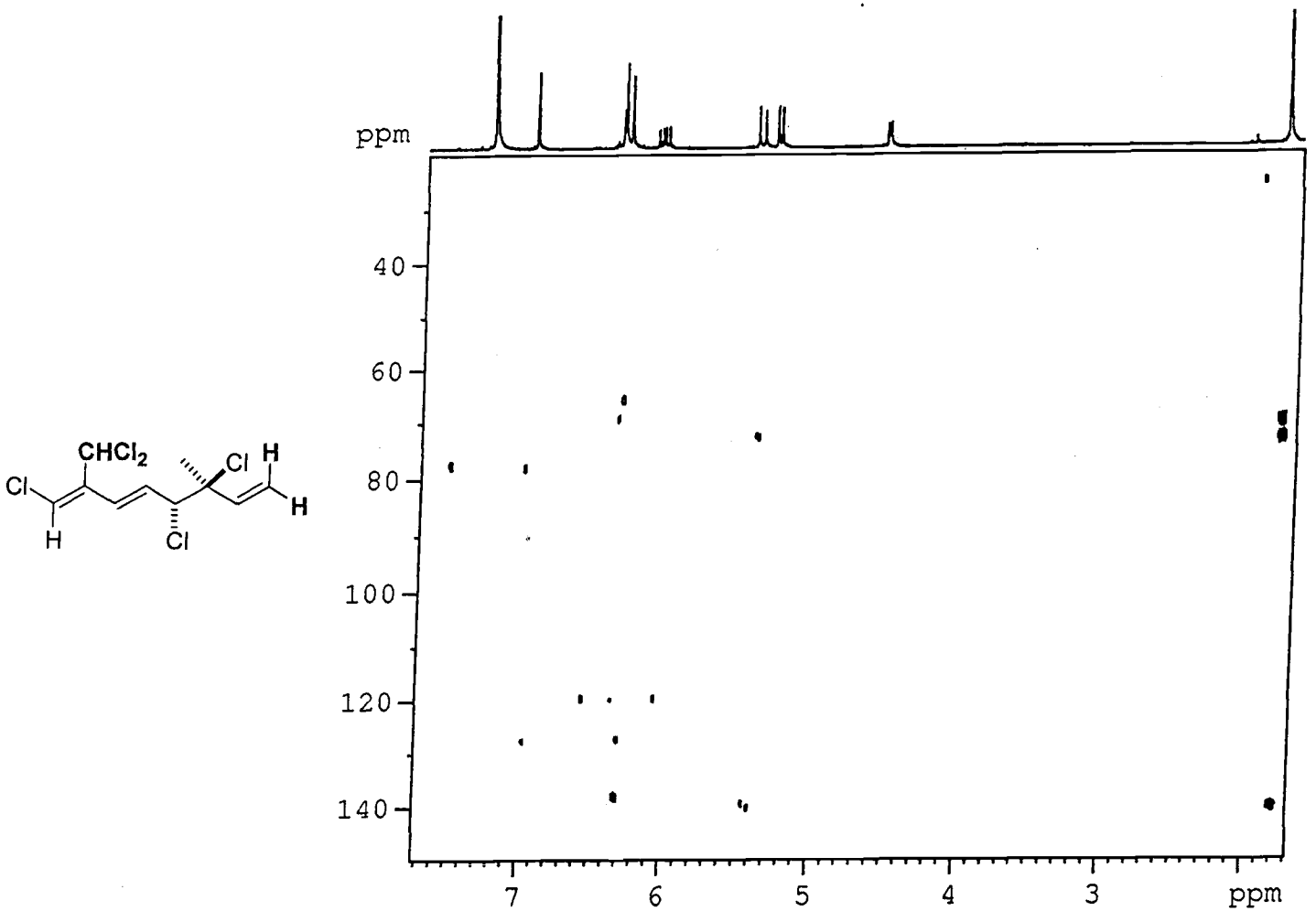


Figure III.6. HMBC spectrum of compound **28** in $CDCl_3$

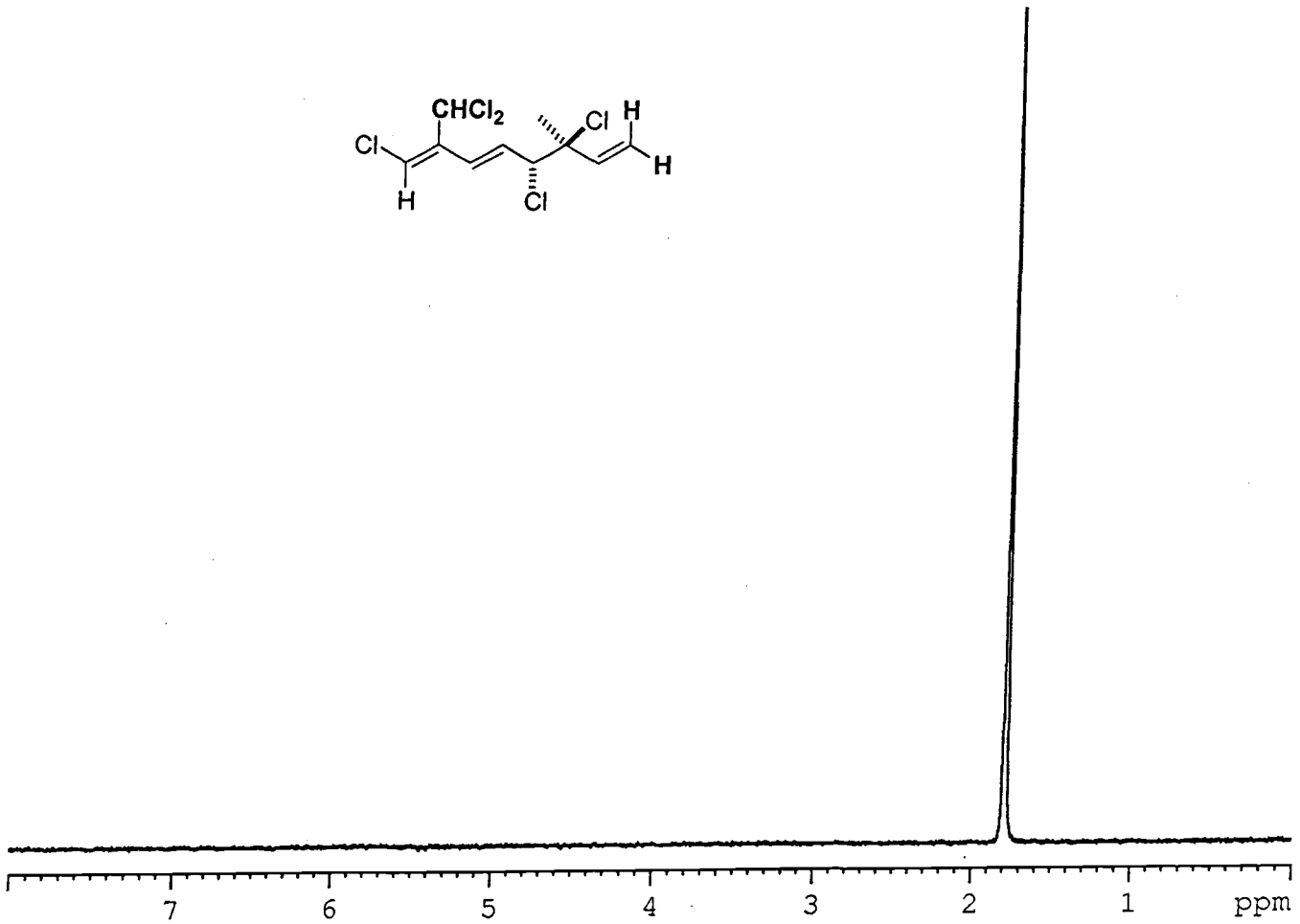


Figure III.7. 1D NOE spectrum of compound **28** in $CDCl_3$ irradiated at 1.77 ppm

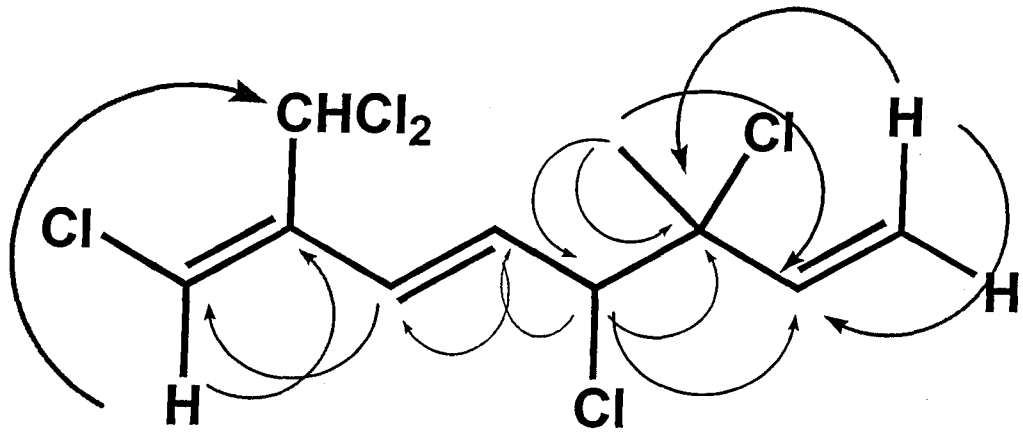
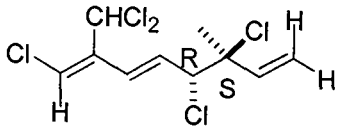
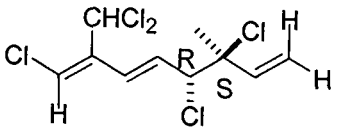


Figure III.8. HMBC correlations of compound **28**

Table III.2. ^{1}H NMR Data for comparison of compounds **28** and **5** in $CDCl_3$[a]



Compound 5 (threo)[5]
$[\alpha]^{25}_{D}$ + 34.6

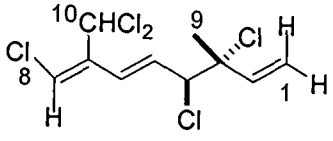


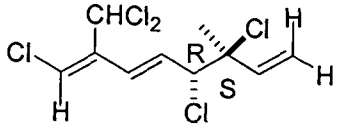
Compound 28 (erythro)
$[\alpha]^{25}_{D}$ – 92.0

Position	^{1}H mult. *J* (Hz)[13]	^{1}H mult. *J* (Hz)	$\Delta\ \delta_H$
1	5.43 (d, 16.5)	5.41 (d, 17.0)	-0.02
	5.29 (d, 11.0)	5.29 (d, 11.0)	
2	6.04 (dd, 16.5, 11)	6.07 (dd, 17, 11)	0.03
3			
4	4.49 (d, 7)	4.55 (dd, 7, 2)	0.06
5	6.24 (dd, 17, 7)	6.35 (dd, 17, 7)	0.11
6	6.28 (d, 17)	6.34 (dd, 17, 2)	0.06
7			
8	6.23 (s)	6.30 (s)	0.07
9	1.77 (s)	1.77 (s)	0.00
10	6.88 (s)	6.99 (s)	0.11

[a] Spectral data reported in ppm.

Table III.3. ^{1}H NMR Data for comparison of compounds **28** and **6** in $CDCl_3$[a]





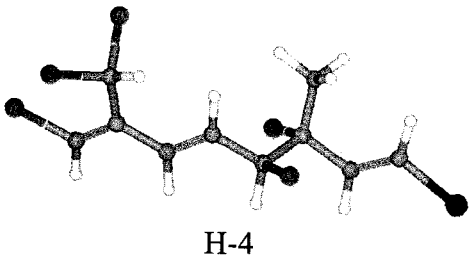
Compound 6 (erythro)[5] $[\alpha]^{25}_{D}$ + 5.1

Compound 28 (erythro) $[\alpha]^{25}_{D}$ – 92.0

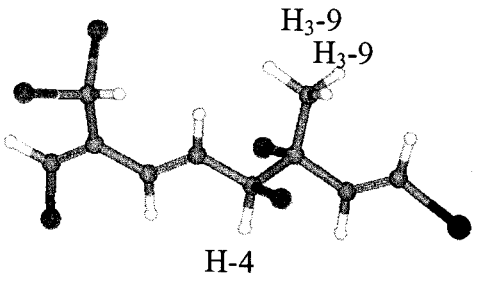
Position	^{1}H mult. *J* (Hz)[13]	^{1}H mult. *J* (Hz)	$\Delta\ \delta_H$
1	5.38 (d, 17.0)	5.41 (d, 17.0)	0.03
	5.25 (d, 10.5)	5.29 (d, 11.0)	0.04
2	6.03 (dd, 17.0, 10.5)	6.07 (dd, 17, 11)	0.04
3			
4	4.48 (d, 7.5)	4.55 (dd, 7, 2)	0.07
5	6.31 (dd, 16.0, 7.5)	6.35 (dd, 17, 7)	0.04
6	6.30 (d, 16.0)	6.34 (dd, 17, 2)	0.04
7			
8	6.27 (s)	6.30 (s)	0.03
9	1.73 (s)	1.77 (s)	0.04
10	6.90 (s)	6.99 (s)	0.09

[a] Spectral data reported in ppm

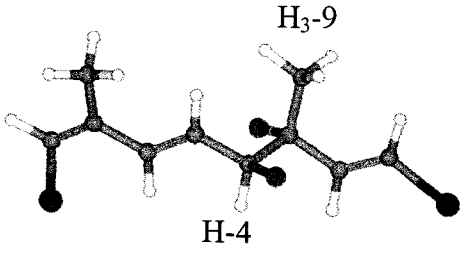
I also isolated from this algal species the previously reported metabolites **1, 2** and **10**.[5,24] I carried out a full investigation of the spectroscopic data of these compounds in the course of this work. My isolation of compounds "**1**", "**2**" and "**10**" were found to possess the same physical and spectroscopic data (Tables III.4, III.5, III.6, III.7 and III.8) as reported in the literature.[5,24] 1D and 2D NOE experiments on compounds **1, 2** and **10** involving the protons of the methyl group (H-9) at 1.77, 1.78 and 1.75 gave weak enhancements or even no enhancements of the protons at δ 4.53 (H-4), δ 4.58 (H-4) and δ 4.59 (H-4), respectively. This suggested that this methyl group and the proton attached to the chloromethine group at C-4 are directed to the opposite face of the molecule (see figure III.9). Our data were found to obey the empirical rules of Crews[26], but to some extent contradicts Mynderse and Faulkner rules[25] as they mainly depended only on the ^{1}H NMR chemical shifts of H_3-9 beside the sign of the optical rotation to determine the stereochemistry of the chiral centers at C-3 and C-4 of the halogenated monoterpene compounds. For example, of two compounds with H_3-9 in the same chemical shift range and with the same sign of optical rotation, one was assigned 3R*, 4S* and the other assigned 3R*, 4R* by Mynderse and Faulkner.[27] The ^{13}C NMR of compound **10** was at δ 25.6, consistent with an erythro relationship of substituents at C-3 and C-4, and consistent with previous isolates of the compound.[24] Because there were no ^{13}C NMR data published in the previous literature[5] for compound **1**, I recorded and assigned those data here as part of this study (see tables III.4 and III.5). Also during my investigations, I noticed that over time, compound **1** in $CHCl_3$ and ethyl acetate (acidic medium) was partially converted to the more stable compound **2**. The mechanism of this isomerization is proposed in figure III.10.



Compound **1** (erythro).



Compound **2** (erythro).



Compound **10** (*erythro*).

Figure III.9. Molecular modeling of compounds **1**, **2** and **10**. All of these compounds would not be expected to show strong NOE correlations between H-4 and H_3-9

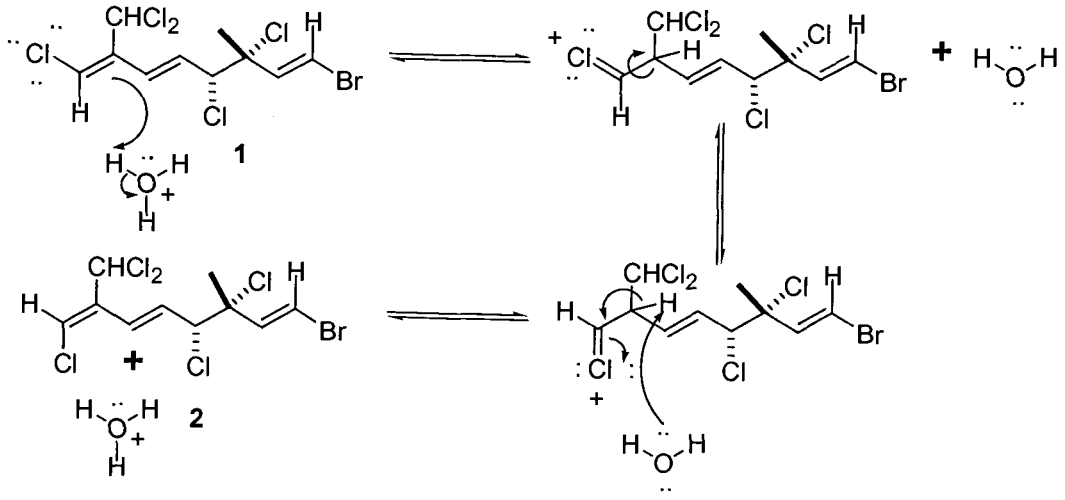


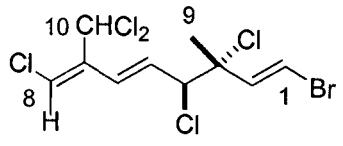
Figure III.10. Proposed mechanism of the isomerization of **1** to **2**.

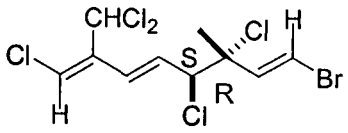
Table III.4. ^{1}H- and ^{13}C-NMR Data for our isolation of **1** in $CDCl_3$[a]

Position	^{1}H mult. *J* (Hz)	^{13}C
1	6.58 (d, 13.5)	110.7 CH
2	6.44 (d, 13.5)	138.9 CH
3		72.2 C
4	4.53 (d, 6.8)	66.0 CH
5	6.30 (dd, 14.5, 6.8)	130.0 CH
6	6.35 (d, 14.5)	127.8 CH
7		137.9 C
8	6.32 (s)	120.1 CH
9	1.77 (s)	25.8 CH_3
10	6.97 (s)	68.7 CH_2

[a] Spectral data reported in ppm.

Table III.5. ^{1}H NMR Data for comparison of compound **1** and our isolation of **1** in $CDCl_3$[a]





	Compound 1 (erythro)[5] $[\alpha]^{25}_D$ – 21.9	**our isolation of 1 (erythro)** $[\alpha]^{25}_D$ – 26.0	
Position	**^{1}H mult. *J* (Hz)**[13]	**^{1}H mult. *J* (Hz)**	**$\Delta\ \delta_H$**
1	6.52 (d, 14.0)	6.58 (d, 13.5)	0.06
2	6.38 (d, 14.0)	6.44 (d, 13.5)	0.06
3			
4	4.49 (d, 7.4)	4.53 (d, 6.8)	0.04
5	6.29 (dd, 15.6, 7.4)	6.30 (dd, 14.5, 6.8)	0.01
6	6.32 (d, 15.6)	6.35 (d, 14.5)	0.03
7			
8	6.28 (s)	6.32 (s)	0.04
9	6.90 (s)	6.97 (s)	0.07
10	1.74 (s)	1.77 (s)	0.03

[a] Spectral data reported in ppm.

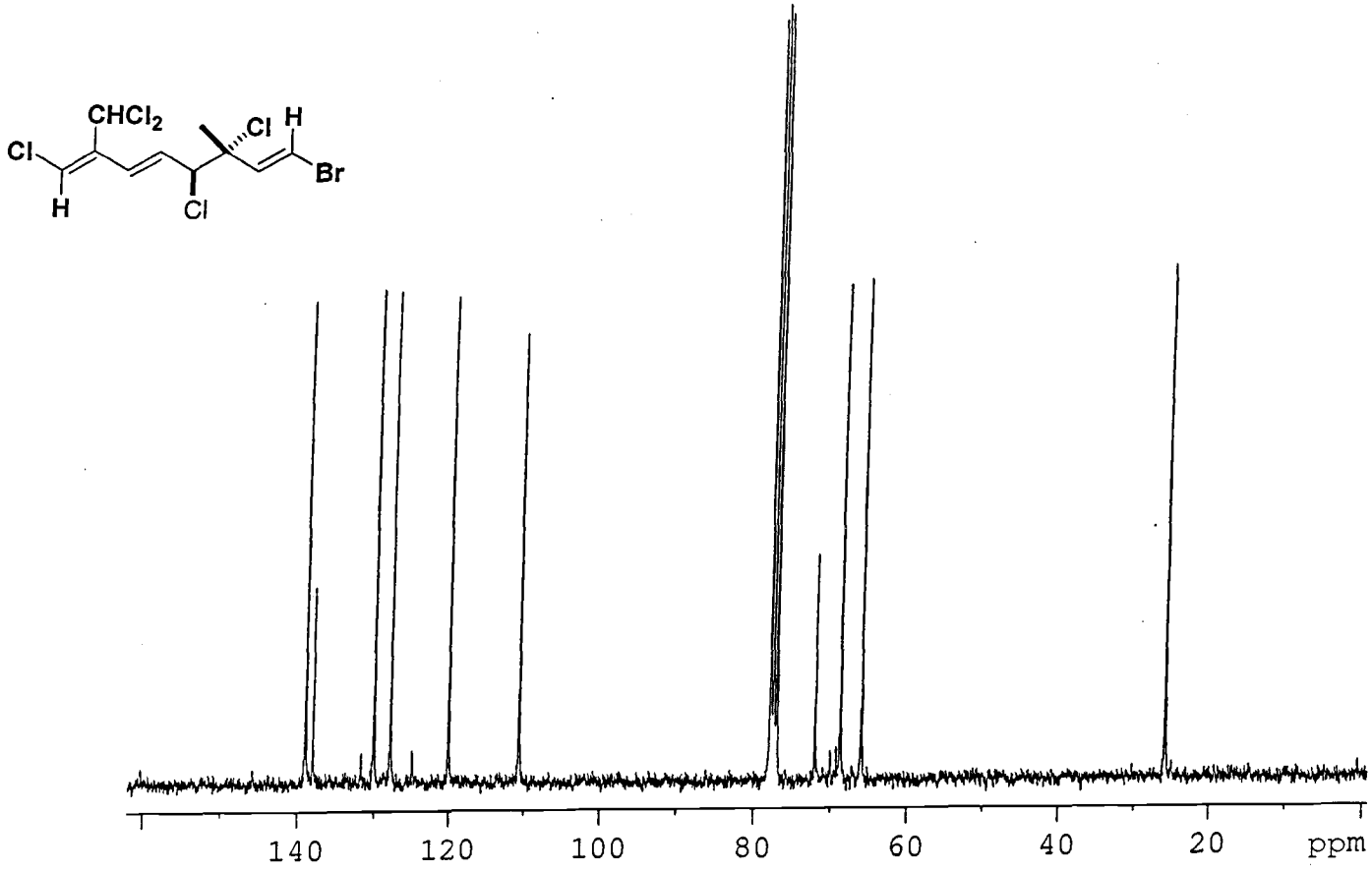


Figure III.11. ^{13}C NMR spectrum of our isolation of compound **1** in $CDCl_3$

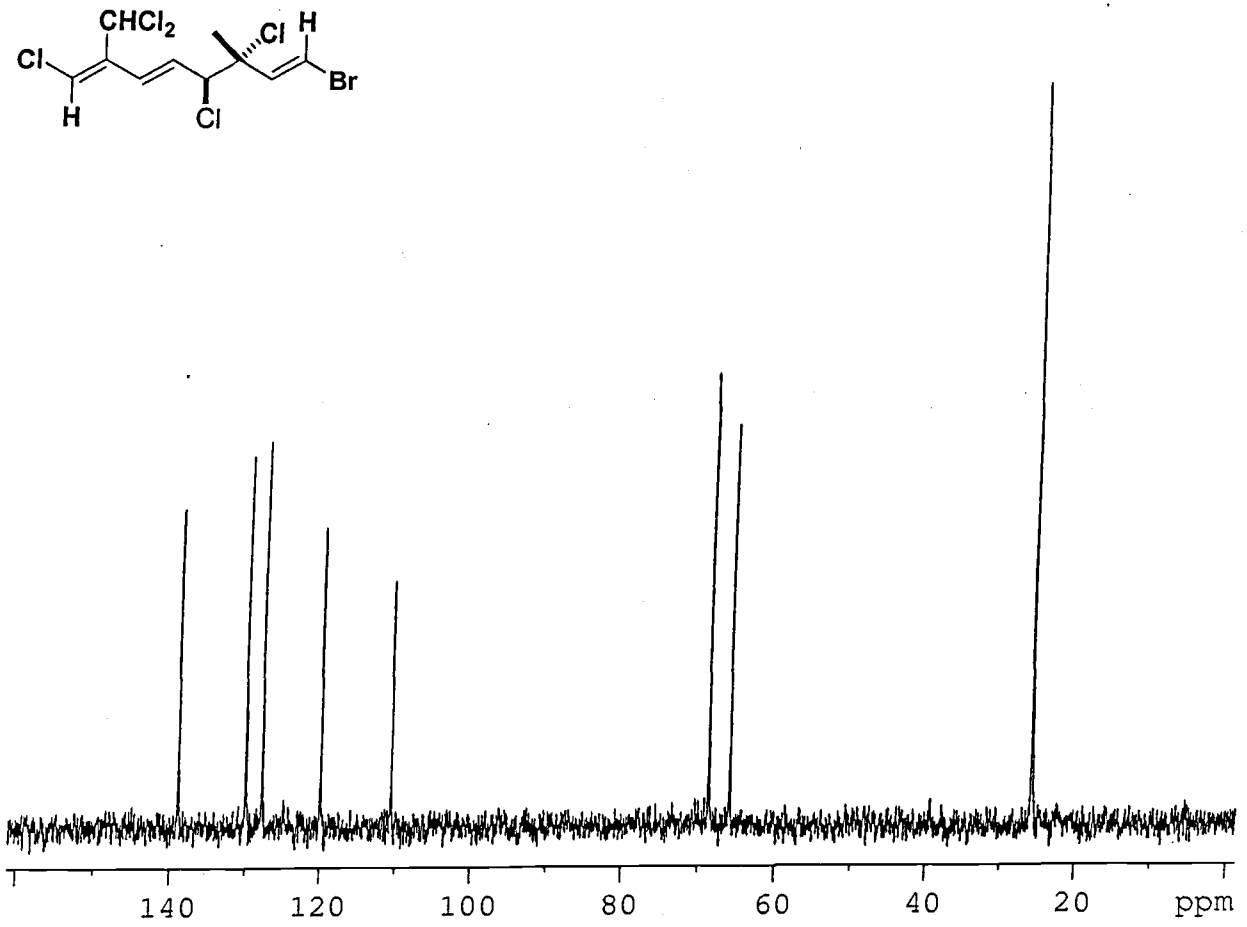


Figure III.12. DEPT90` spectrum of our isolation of compound **1** in $CDCl_3$

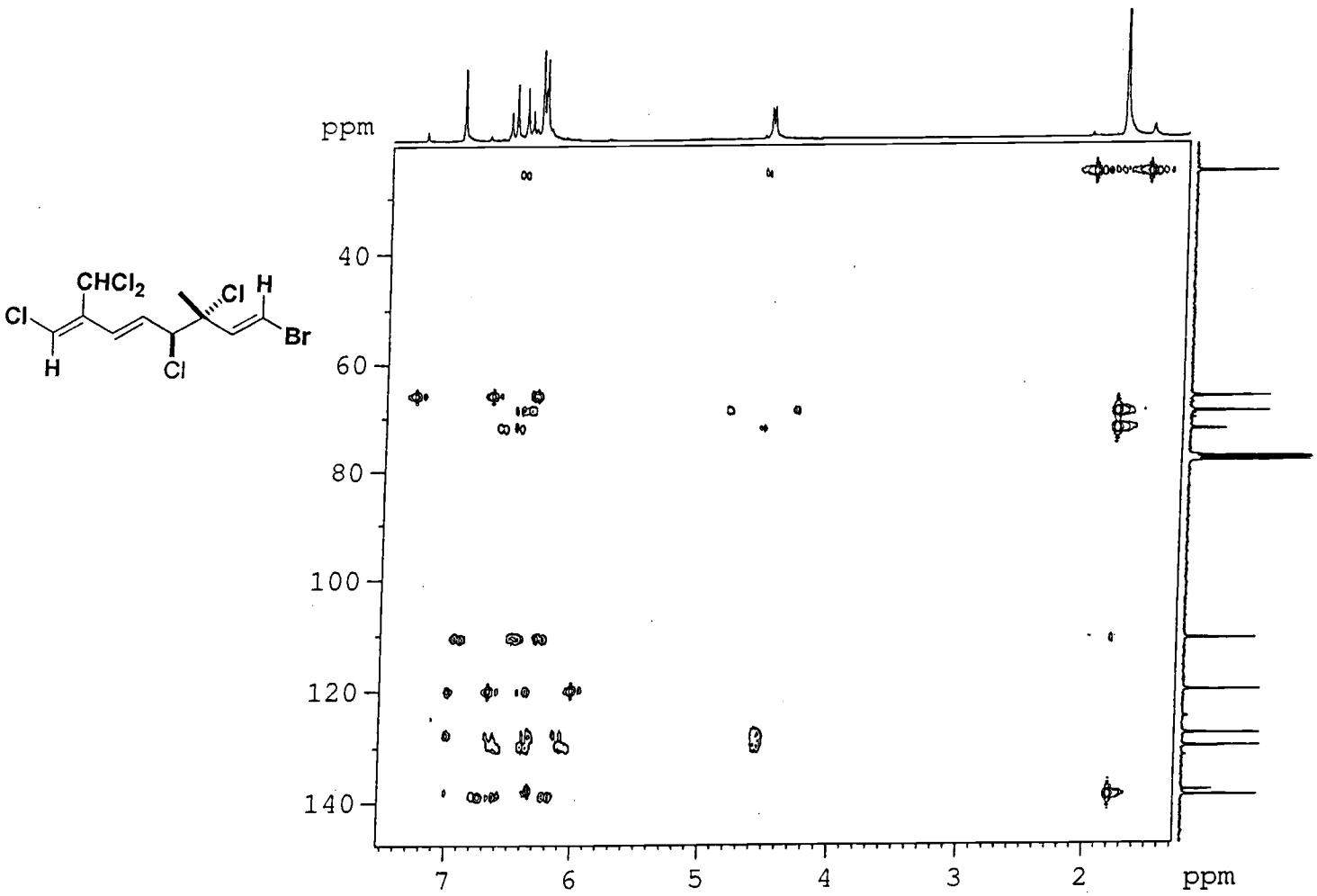


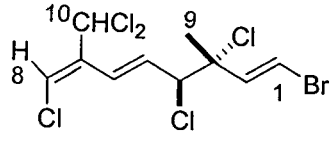
Figure III.13. HMBC spectrum of our isolation of compound **1** in $CDCl_3$

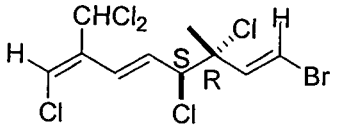
Table III.6. ^{1}H- and ^{13}C-NMR Data for our isolation of **2** in $CDCl_3$[a]

Position	^{1}H mult. *J* (Hz)	^{13}C	HMBC	NOE
1	6.58 (d, 14.0)	110.7 CH	C-2, C-3	
2	6.43 (d, 15)	138.9 CH	C-1, C-3	
3		71.9 C		
4	4.58 (d, 8.0)	69.1 CH	C-2, C-3, C-5, C-6 C-10	H-5, H-6
5	6.48 (dd, 14.5, 8.0)	131.6 CH		H-4
6	6.56 (d, 14.0)	124.8 CH	C-4, C-5, C-8	H-4
7		136.3 C		
8	6.39 (s)	124.9 CH	C-7, C-10	H-10
9	1.78 (s)	25.7 CH_3	C-1, C-2, C-3, C-4	
10	6.76 (s)	69.9 CH_2	C-3, C-4	H-4

[a] Spectral data reported in ppm.

Table III.7. ^{1}H NMR Data for comparison of compound **2** and our isolation of **2** in $CDCl_3$[a]





	Compound 2 (erythro)[5] $[\alpha]^{25}_{D}$ – 4.4	**our isolation of 2 (erythro)** $[\alpha]^{25}_{D}$ – 11.0	
Position	**^{1}H mult. *J* (Hz)**[13]	**^{1}H mult. *J* (Hz)**	**$\Delta\ \delta_H$**
1	6.52 (d, 14.0)	6.58 (d, 14.0)	0.06
2	6.40 (d, 14.0)	6.43 (d, 14.0)	0.03
3			
4	4.52 (d, 7.4)	4.58 (d, 8.0)	0.06
5	6.43 (dd, 15.6, 7.4)	6.48 (dd, 14.5, 8.0)	0.05
6	6.58 (d, 15.6)	6.56 (d, 14.5)	- 0.02
7			
8	6.33 (s)	6.39 (s)	0.06
9	6.74 (s)	6.76 (s)	0.02
10	1.74 (s)	1.78 (s)	0.04

[a] Spectral data reported in ppm.

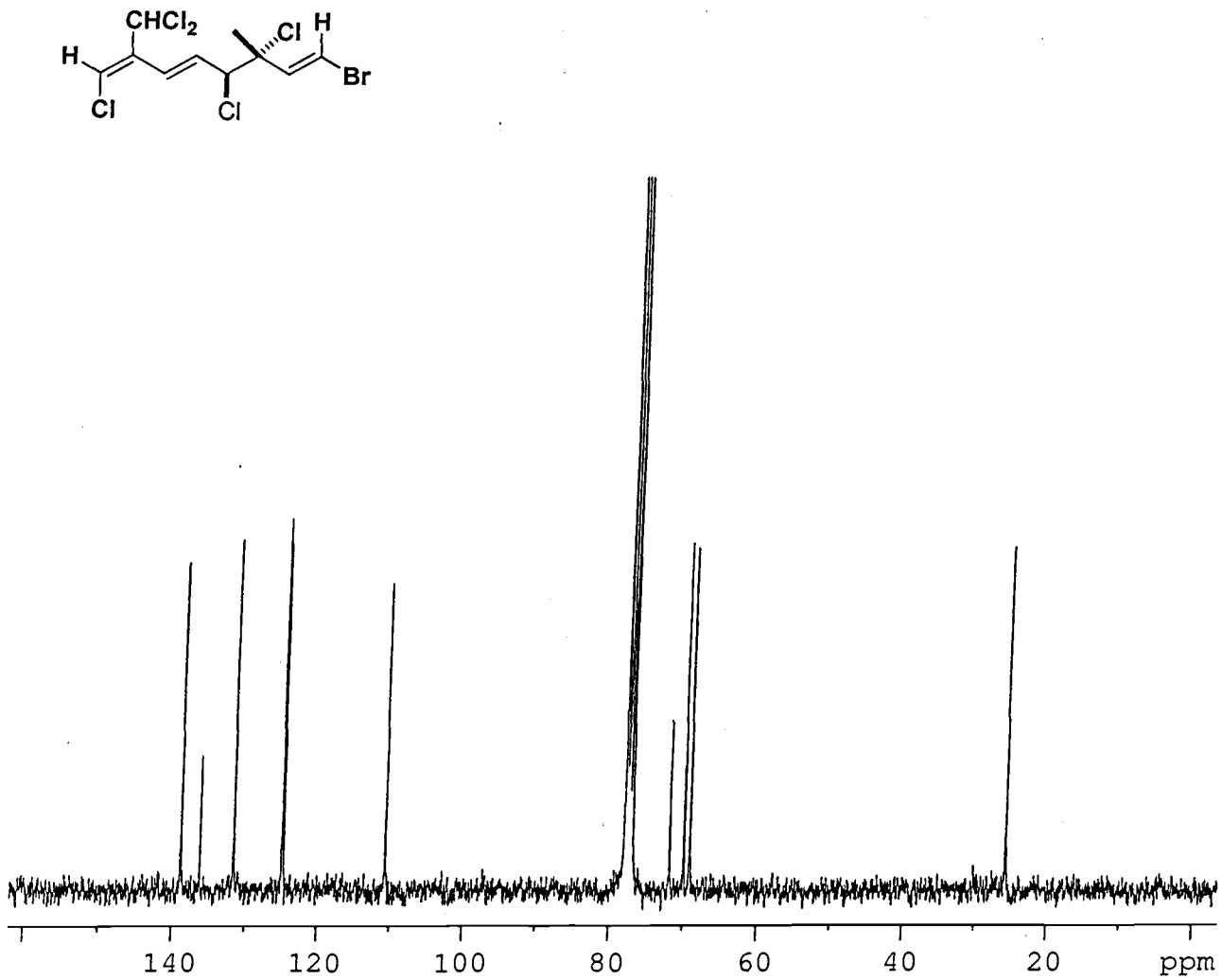


Figure III.14. ^{13}C NMR spectrum of our isolation of compound **2** in $CDCl_3$

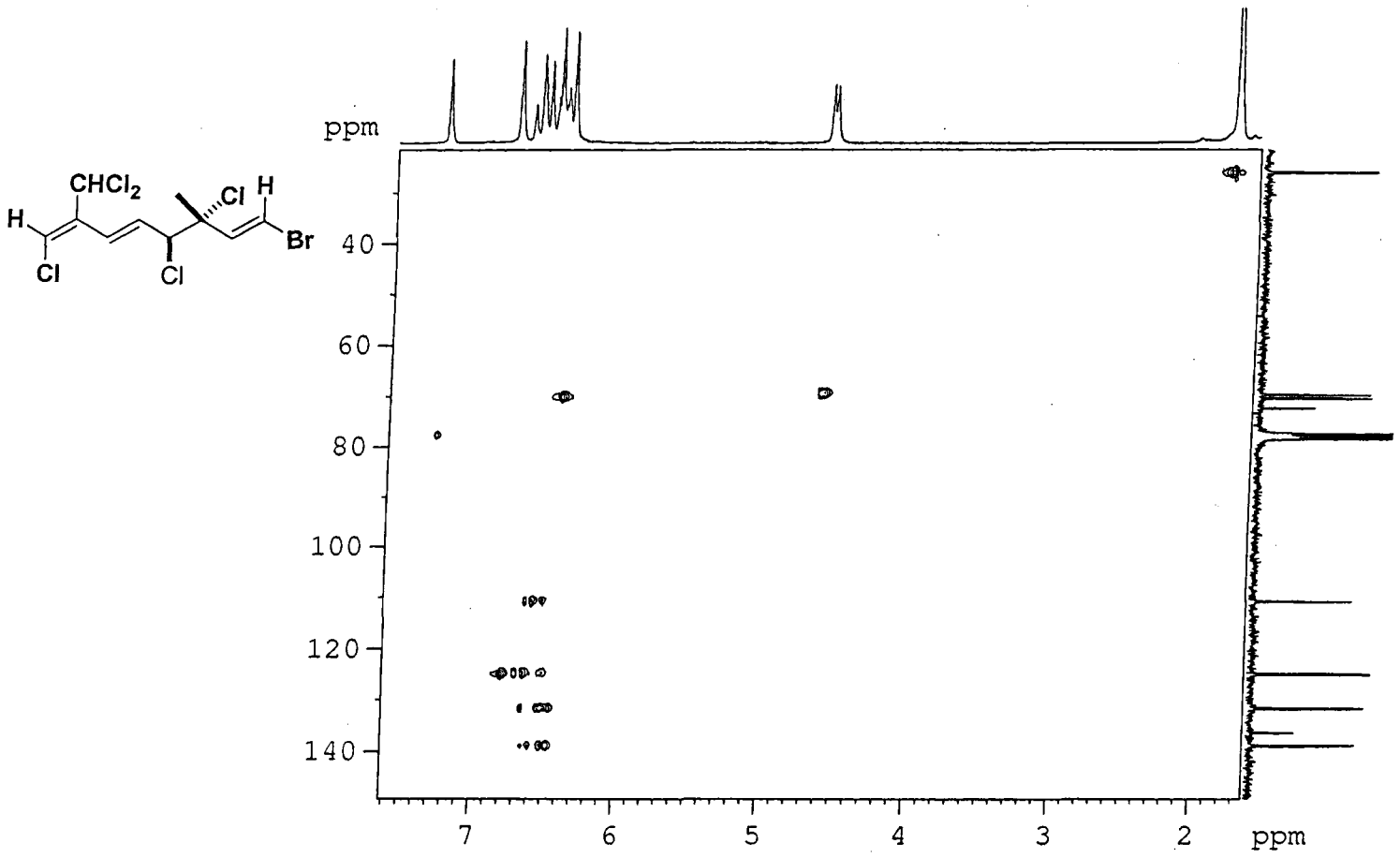
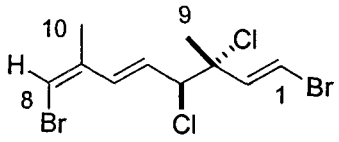
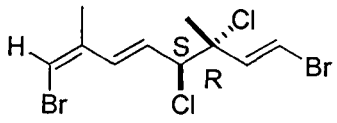


Figure III.15. HSQC spectrum of our isolation of compound **2** in $CDCl_3$

Table III.8. ^{1}H NMR Data for comparison of compound **10** and our isolation of **10** in $CDCl_3$[a]





	Compound 10 (erythro)[5, 24] $[\alpha]^{25}_{D}$ – 46.0	**our isolation of 10 (erythro)** $[\alpha]^{25}_{D}$ – 37.7	
Position	**^{1}H mult. *J* (Hz)**[13]	**^{1}H mult. *J* (Hz)**	**$\Delta\ \delta_H$**
1	6.51 (d, 14.0)	6.56 (d, 14.0)	0.05
2	6.40 (d, 14.0)	6.46 (d, 14.0)	0.06
3			
4	4.53 (d, 9.0)	4.59 (d, 9)	0.06
5	5.87 (dd, 15.5, 9)	5.91 (dd, 15, 9)	0.04
6	6.8 (d, 15.5)	6.86 (d, 15)	0.06
7			
8	6.17 (s)	6.2 (s)	0.03
9	1.73 (s)	1.75 (s)	0.02
10	1.91 (s)	1.91 (s)	0.00

[a] Spectral data reported in ppm.

The isolated compounds were evaluated for their biological activity in several systems. Compound **28** was found to have cytotoxic activity (EC_{50} = 4 µg/ml) to a human lung cancer cell line (NCI-H460) and the mouse neuro-2a neuroblastoma cell line. Upon testing these compounds in a sodium channel modulation assay, none of the compounds were found to have blocking or activating activity. Compounds **1** and **2** were found to have a promising cytotoxic activity in human colon cancer (CFU) cell lines and are now in the *in-vivo* evaluation stage.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. NMR spectra were recorded on a Bruker DPX400 spectrometer, with the solvent ($CDCl_3$ at δ_C 77.2, δ_H 7.26) used as an internal standard. Mass spectra were recorded on a Kratos MS50TC mass spectrometer, and HPLC isolations were performed using Waters Millipore model 515 pumps and a Waters 969 diode array detector.

Algal Collection. The marine red alga *Plocamium cartilagineum* (voucher specimen available from WHG as collection number ZAT-26 March 97-03) was collected intertidally by hand from South Africa eastern coast (March 1997). The material was stored in 2-propanol at -20 ^{0}C until extraction.

Extraction and Isolation. Approximately 47 g (dry wt.) of the *Plocamium cartilagineum* was extracted repeatedly with 2:1 CH_2Cl_2/MeOH to produce 2.8 g of crude organic extract. The crude extract (1.5) g was subjected to Si vacuum liquid chromatography (VLC, hexanes/EtOAc/MeOH) to produce 9 chemically distinct fractions. The fraction eluting with 100% hexanes (1.073 gm) was subjected to normal phase HPLC (0-30% ethyl acetate/hexanes) dual silica, Phenomenex Luna 10u Silica 250×4.6mm to yield 540 mg of compound **1** and 60 mg of compound **2**, 2 mg of compound **10** and 4 mg of compound **28**.

Compound 1: $[\alpha]^{25}_D$ – 11 (c 0.1, $CHCl_3$) (literature - 4.4)[5]; ^{1}H and ^{13}C NMR data see Table III.3; with remaining physical and spectroscopic properties identical to those previously reported.[5]

Compound 2: Colorless oil; $[\alpha]^{25}_D$ – 26 (c 0.1, $CHCl_3$) (literature – 22.9)[5]; UV ($CHCl_3$) $_{max}$ 248 (ε 1973) nm; IR (neat) 3086, 2990, 2931, 1617, 1577, 1448, 1379, 1207, 1052, 964, 936, 854, 721cm^{-1}; ^{1}H NMR and ^{13}C NMR (400 MHz, $CDCl_3$) see Tables III.3 and III.5; HRCIMS *m/z* $[M]^+$ 383.8398 (calculated for $C_{10}H_{11}BrCl_5$).

Compound 10: Colorless oil; $[\alpha]^{25}_{D}$ – 38 (c 0.07, $CHCl_3$) (literature – 46.0)[5]; with remaining physical and spectroscopic properties identical to those previously reported.[5]

Compound 28: Colorless oil; $[\alpha]^{25}_{D}$ – 92 (c 0.07, $CHCl_3$); UV ($CHCl_3$) $_{max}$ 242 (ε 3623) nm; IR (neat) 2923, 2853, 1459, 1375, 1215, 961, 932, 819, 749, 720 cm^{-1}; ^1H NMR and ^{13}C NMR (400 MHz, $CDCl_3$) see Table III.1; HRCIMS *m/z* $[M\text{-}Cl]^+$ 271.236 (calculated for $C_{10}H_{11}Cl_5$).

Cytotoxicity against NCI-H460 human lung cancer and neuro-2a neuroblastoma cell line.[27] Cytotoxicity was measured to NCI-H460 human lung tumor cells and mouse neuro-2a blastoma cells using the method of Alley et. al.[27] with cell viability being determined by MTT reduction. Cells were seeded in 96-well plates at 5000 and 8000 cells/well in 180 µl for H460 and neuro-2a cells, respectively. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µg/well. DMSO was less than 1% final concentration. After 48 hours, the medium was removed and cell viability determined.

Sodium channel modulation.[28] Isolated compounds were evaluated for their capacity to either activate or block sodium channels using the following modifications to the cell-based bioassay of Manger et. al.[28] Twenty-four hours prior to chemical testing, mouse neuro-2a blastoma cells were seeded in 96-well plates at 8 X 10^4 cells/well in a volume of 200 µl. Test chemicals dissolved in DMSO were serially diluted in medium without fetal bovine serum and added at 10 µl/well. DMSO was less than 1% final concentration. Plates to evaluate sodium channel activating activity received 20 µl/well of either a mixture of 3 mM ouabain and 0.3 mM veratridine (Sigma Chemical Co.) in 5 mM HCl in addition to the test chemical. Plates were incubated for 18 hr and results compared to similarly treated solvent controls with 10 µl medium added in lieu of the test chemical. The sodium channel activator brevetoxin PbTx-1 (Calbiochem) was used as the positive control and added at 10 ng/well in 10 µl medium. Sodium channel blocking activity was assessed in a similar manner except that ouabain and veratridine were 5.0 and 0.5 mM, respectively, and the sodium channel blocker saxitoxin (Calbiochem) was used as the positive control. Plates were incubated for approximately 22 hour.

REFERENCES

1. Gerwick, W. H.; Tan, L. T.; Sitachitta, N. *Alkaloids*, **2001,** 57, 75-184.

2. Norton, R. S.; Warren, R. G.; Wells, R. J.; *Tetrahedron Lett.* **1977**, 3905-3908.

3. Capon, R. J.; Engelhardt, L. M.; Ghisalberti, E. L.; Jefferies, P. R.; Patrick, V. A.; White, A. H. *Aust. J. Chem.* **1984**, *37*, 537-544.

4. Wright, A. D.; Coll, J. C.; Price, I. R. *J. Nat. Prod.* **1990**, *53*, 845-861.

5. Mynderse, J. S.; Faulkner, D. J. *Tetrahedron.* **1975**, 31, 1963-7.

6. Fuller, R. W.; Cardellina, J. H., II; Kato, Y.; Brinen, L. S.; Clardy, J., Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1992**, *35*, 3007-3011.

7. Naylor, S.; Hanke, F. J.; Manes, L. V; Crews, P. *Prog. Chem. Org. Nat. Prod.* **1983,** *44*, 189-241.

8. Ortega, M. J.; Zubia, E.; Salva, J. *J. Nat. Prod.* **1997**, *60*, 482-484.

9. Wessels, Matthias; Koenig, Gabriele M.; Wright, Anthony D. *J. Nat. Prod.* **2000**, 63, 920-928.

10. Argandona, V. H.; Rovirosa, J.; San-Martin, A.; Riquelme, A.; Diaz-Marrero, A. R.; Cueto, M.; Darias, J.; Santana, O.; Guadano, A.; Gonzalez-Coloma, A. *J. Agr. Food Chem.* **2002**, 50, 7029-7033.

11. Watanabe, K.; Miyakado, M.; Ohno, N.; Okada, A.; Yanagi, K.; Moriguchi, K. *Phytochemisty* **1989**, 28, 77-78.

12. Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R. *J. Org. Chem.***1984**, 49, 1371-1377.

13. Crews, P.; Myers, B. L.; Naylor, S.; Clason, E. L.; Jacobs, R. S.; Staal, G. B. *Phytochemisty* **1984**, 23, 1449-51.

14. Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R. *J. Org. Chem.***1984**, 49, 1371-7.

15. Blunt, J. W.; Bowman, N. J.; Munro, M. H. G.; Parsons, M. J.; Wright, G. J.; Kon, Y. K. *Aust. J. Chem.* **1985**, 38, 519-25.

16. Thomas S. G.; Beveridge A. A. *Clin. Exp. Pharm. Phys.* **1993**, 20, 223-9.

17. Abreu, Pedro M.; Galindro, Jose M. *J. Nat. Prod.* **1996**, 59, 1159-1162.

18. Mynderse, J. S.; Faulkner, D. J. *J. Am. Chem. Soc.* **1974**, 96, 6771-6772.

19. Burreson, B. J.; Woolard, F. X.; Moore, R. E. *Chemistry Lett.* **1975**, 11, 1111-14.

20. Higgs, M. D.; Vanderah, D. J.; Faulkner, D. J. *Tetrahedron* **1977**, 33, 2775-80.

21. Gonzalez, A. G.; Arteaga, J. M.; Martin, J. D.; Rodriguez, M. L.; Fayos, J.; Martinez-Ripolls, M. *Phytochemistry* **1978**, 17, 947-8.

22. Abreu, P. M.; Galindro, J. M. *Ind. J. Chem.* **1998**, 37B, 610-611.

23. San-Martin, A.; Rovirosa, J. *Biochem. System. Ecol.* **1986**, 14, 459-61.

24. Crews, Phillip. *J. Org. Chem.* **1977**, 42, 2634-6.

25. Mynderse, J. S.; Faulkner, D. J. *Tetrahedron* **1975**, *31*, 1963-1967.

26. Crews, P.; Kho-Wiseman, E. *J. Org. Chem.* **1974**, *39*, 3303-3304.

27. Alley, M. C.; Scudiero,D. A. *Cancer Research* **1988**, 48, 589-601.

28. Manger, R. L.; Leja. L. S. *Journal of AOAC International* **1995,** 78, 521-527.

CHAPTER FOUR

CURCUEPOXIDES AND HYDROXY CURCUDIOL: NEW PHENOLIC SESQUITERPENES FROM A PAPUA NEW GUINEAN COLLECTION OF THE GREEN ALGA *UDOTEA ORIENTALIS* GROWING ON THE SURFACE OF UNKNOWN CORAL

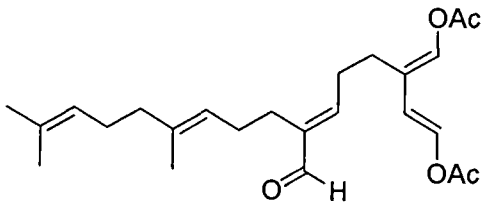
ABSTRACT

As part of my continued search for bioactive secondary metabolites from marine sources using a bioassay guided fractionation approach (Anti-trypanosome activity), I examined the organic extract of a Papua New Guinean collection of the green alga *Udotea orientalis* growing on a coral wall and collected in September 1998. Successive HPLC separations resulted in the isolation of three new compounds; (+) curcuepoxide A **5**, (+) curcuepoxide B **6** and (+)-10α- hydroxycurcudiol **7**. In addition I isolated four known compounds; (+)-10β- hydroxycurcudiol **8**, (+) curcuphenol **9**, (+) curcudiol **10** and (+) curcudiol-10-one **11**. Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed for construction of their planar structures. Curcudiol was found to be active in an *in-vitro* assay of anti-trypanosome activity (EC_{50}10 µg/ml). The isolated compounds were found to have variable cytotoxic activity in human lung cancer cell lines. The most active compounds in the latter assays were compounds **8** and **11** (EC_{50} = 2 and 4 µg/ml, respectively). However, they both displayed sodium channel blocking activity at this dose but not at the lower dose of 1 µg/ml. This activity was 72 and 82% inhibition of blocking, respectively, compared to 87% for saxitoxin at 0.05 µg/ml.

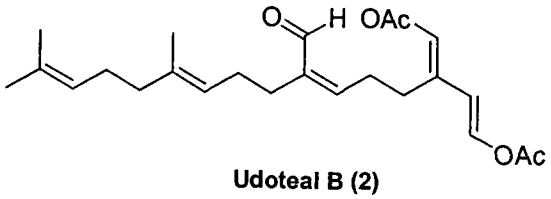
INTRODUCTION

The *Udotea* species grow in the shape of small calcareous fans, and contain calcium carbonate. The calcium carbonate content within *Udotea* gives them a hard body with varying textured plates, and the plates often have lines of calcium carbonate deposits centered around the middle. *Udotea* usually anchors into sandy or muddy silt bottoms or even the walls of coral reefs with a holdfast stemming from or around a single stalk. They are quite common species in tropical near shore marine environments. Their fan plates grow in various shapes, such as in a cup form like that of *U. cyathiformis*, or in clusters. These species grow from shallow sea grass flats to deeper depths of 50 feet or more, reaching three to eight inches in height.

Investigations of the different species of the marine green alga *Udotea sp.* have yielded several biologically active diterpene compounds such as udoteal (**1**) (induced feeding avoidance in the herbivorous fish *Eupomacentrus leucostictus*),[1-3] udoteal B (**2**), a new linear diterpenoid (antibiotic activity, inhibition of cell division in the fertilized sea urchin egg, and toxicity toward herbivorous damselfish),[4] lectins (antiviral, antibacterial, antifungal, ichthyotoxic, cytotoxic, antitumor, anticoagulant),[5-6] petiodial **3** (feeding deterrent)[7] and udoteafuran **4** (antimicrobial).[8] However upon searching the literature I didn't find any research work about investigation of bioactive secondary metabolites from this species, *U. orientalis*. Therefore, I investigated this organism for the presence of new bioactive natural products.



Udoteal (1)



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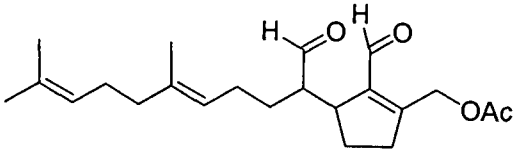
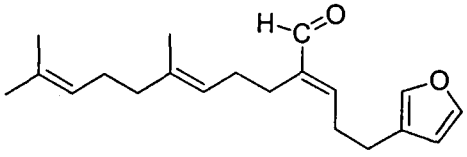
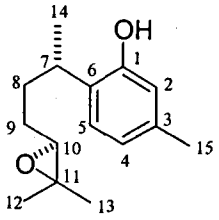


Petiodal (3)



Udoteafuran (4)

As part of my ongoing search for structurally and pharmacologically interesting substances from marine algae, a detailed exploration of a Papua New Guinean collection of the green alga *Udotea orientalis* growing on the wall of a coral reef and collected in September 1998 was undertaken. During this study, several metabolites of known identity but unusual to isolate from *Udotea* species were obtained including (+)-10β- hydroxycurcudiol **8**, (+) curcuphenol (**9**),[9-11] (+) curcudiol (**10**)[11,12] and (+) curcudiol-10-one (**11**)[10]. In addition, three new compounds, (+) curcuepoxide A (**5**), (+) curcuepoxide B (**6**) and (+)-10α- hydroxycurcudiol **7** were discovered in the same organic extract. This chapter describes the chromatographic isolation and structure elucidation of this new series of *U. orientalis* bioactive compounds.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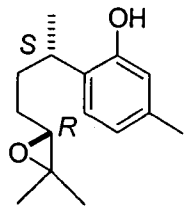
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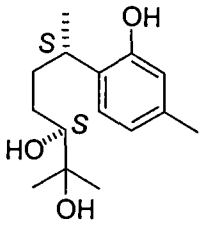
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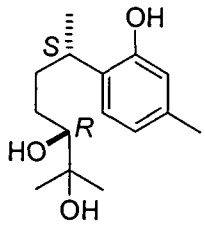
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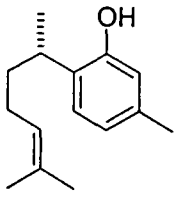
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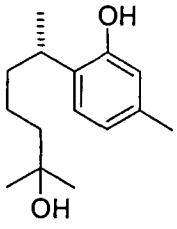
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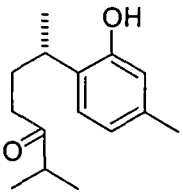
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RESULTS AND DISCUSSION

I have examined the organic extract of this organism, which was collected by hand using SCUBA from Papua New Guinea coast in September 1998. After extraction of the alcohol-preserved tissue with CH_2Cl_2/MeOH (2:1), initial fractionation was accomplished by VLC (EtOAc/hexanes gradient) over silica gel. Successive normal phase HPLC resulted in the isolation of three new compounds (**5-7**) in addition to four previously known compounds [(+)-10β- hydroxycurcudiol **8**,curcuphenol **9**, curcudiol **10**, and (+)-curcudiol-10-one **11**]. Curcuphenol and related compounds have previously been reported as natural products of the sponge *Didiscus oxeata* [9-11] and the coral *Pseudopterogorgia rigida* [12].

Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed for construction of their planar structures. HMBC and MS fragmentation were used to confirm their structures.

Compound **5** was isolated as a colorless oil. It showed a HRFABMS $[M+1]^+$ ion at *m/z* 235.1698 for a molecular formula of $C_{15}H_{23}O_2$, and therefore possessed five degrees of unsaturation. The IR spectrum displayed bands characteristic for a hydroxyl group (3420 cm^{-1}) as well as the presence of an aromatic system (2976 cm^{-1}). The ^1H NMR spectrum of **5** in C_6D_6 (Table IV.1.) displayed a sharp doublet at δ7.01 (1H, d *J*= 7.7 Hz), which was consistent with an aromatic proton ortho to another one that was present as a sharp doublet of doublets at δ 6.76 (1H, dd *J*= 7.7, 0.8 Hz). Another sharp doublet at δ 6.81 (1H, d *J*= 0.8 Hz), which was consistent with an aromatic proton meta to the latter proton, and a sharp singlet at δ 6.9 (1H, s), which was consistent with a phenolic hydroxy proton were detected. I recognized a sharp doublet of doublets at δ 2.42 with *J* values of 12, 2.8 Hz, which was consistent with a proton attached to a carbon atom attached to an epoxy group. Another two sharp singlets at δ 0.97 and 0.94, assignable to two geminal olefinic dimethyl protons, were also observed. In addition a sharp doublet at δ 1.18, which was consistent with an aliphatic methyl group attached to a methine group, was shown. In addition, the NMR spectrum of **5** showed a multiplet at δ 3.32 (methine proton), four methylene signals (δ 1.60, 1.28, 1.25 and 1.94), and an olefinic methyl at δ 2.13. The ^{13}C NMR spectrum of **5** (Table IV.1) showed signals for all 15 carbons.

The number of attached hydrogen atoms were determined from the HSQC spectra in C_6H_6. These data showed that **5** was a sesquiterpene composed of a trisubstituted benzene ring, one epoxy ethylene, one methine, two methylenes, and four methyl groups. Since the aromatic ring and the epoxy group accounted for the five

degrees of unsaturation, compound **5** was inferred to be bicyclic. The complete NMR assignments were established by the combined analysis of ^{1}H -^{1}H COSY, HSQC and HMBC (Table IV.1. 1) data.

The ^{1}H -^{1}H COSY spectrum of **5** contained only two spin systems. The benzylic methine proton (H-7, δ 3.32), which shared a 6.8 Hz coupling with the secondary methyl group (H-14, δ 1.18). Strong couplings were detected between H-7/H-8, H-8/H-9 and also H-9 and H-10. This left C-11 without two bonding partners. The appearance of two methyl groups (H-12 and H-13) as singlets in the ^{1}H NMR spectrum, as well as the HMBC cross peaks observed from both methyls to C-11 (δ 58.7), C-10 (δ 66.2) and also with each other, proved that they were bonded to C-11. This completed fragment **a** of the structure, as shown in Figure 1. In the aromatic region, H-5 (δ 7.01 d, J = 7.7 Hz) *ortho* coupled to H-4 (δ 6.76 d, J = 7.7 Hz), which in turn showed a weak *meta* coupling with H-2 (δ 6.81, d, J = 0.8 Hz), thus completing the second spin system. The substitution pattern of the trisubstituted aromatic ring was revealed by a HMBC experiment (J = 8.0 Hz). Correlations were observed from the aromatic methyl group (H-15) to C-2, C-3 and C-4, indicating that H-15 was attached to C-3. Also the residence of the aromatic hydroxy function at C-1 (δ 155.0) was evident from HMBC cross peaks observed between H of the hydroxy group with C-1, C-2 and C-6 as well as H-5/C-1, giving the second structural fragment, **b**. Fragments **a** and **b** could be bisabolane type sesquiterpene. Partial structures of **5** were connected through the HMBC correlations between H-7/C-6 and H-14/C-6, H-14/C-7 and H-14/C-8. Thus, the gross structure of **5** was determined to be curcuepoxide. The unambigious assignment of the relative stereochemistry of the two chiral centers (C-7 and C-10) within **5** was not possible by analysis of the 1D NOE experiment. However, the co-occurrence in the same extract of (+) curcuphenol (9) and (+) curcudiol (**10**) suggested that **5** possessed *S* configuration at C-7 as well. The *S* configuration at C-7 of (+) curcuphenol and (+) curcudiol had been previously confirmed by chemical synthesis.[13]

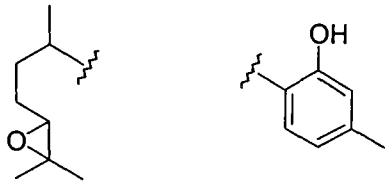
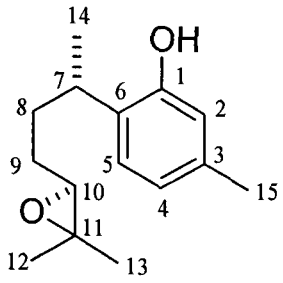


Figure 1. Partial structures of **5**



5

Table IV.1. ^{1}H- and ^{13}C-NMR Data for (**5**) in C_6D_6[a]

Position	^{1}H mult. *J* (Hz)	^{13}C	HMBC[b]
1	------	155.0 C	
2	6.8, s	118.5 CH	
3	------	137.3 C	
4	6.76, d (7.7)	122.0 CH	
5	7.01, d (7.7)	127.0 CH	C1-C3
6	------	130.0 C	
7	3.32, m	30.6 CH	
8	1.60 m	37.3 CH_2	
	1.28 m		
9	1.25 m	25.8 CH_2	
	1.05 m		
10	2.42 dd (7.5, 2.8)	66.2 CH	
11	------	58.7 C	
12	0.97 3H, s	24.7 CH_3	C-10, C-11, C-13
13	0.94 3H, s	18.9 CH_3	C-10, C-11, C-12
14	1.18 d (6.8)	22.8 CH_3	C-6, C-7, C-8
15	2.15, s	21.0 CH_3	C-2, C-3, C-4

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

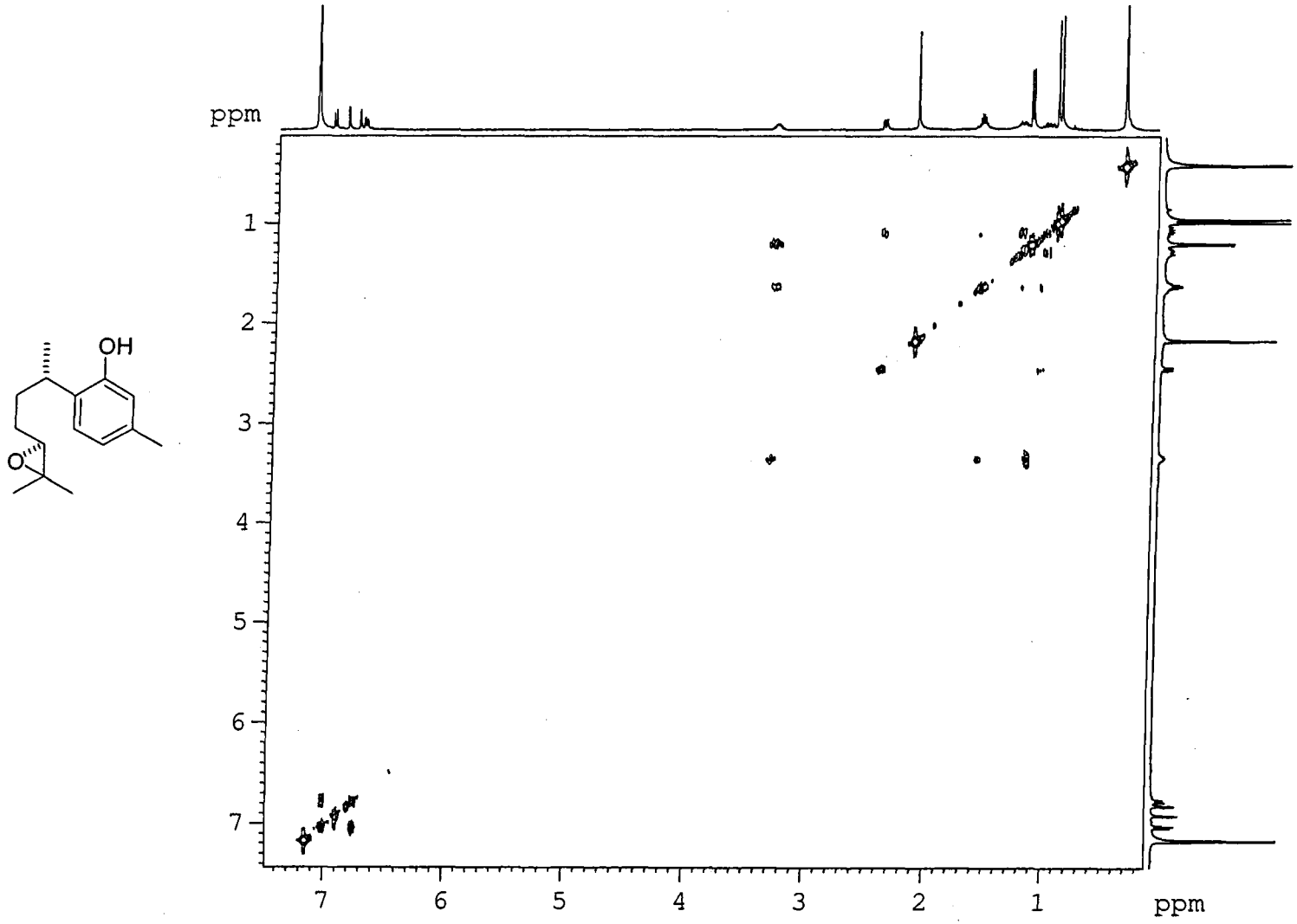


Figure IV. 2. COSY spectrum of compound **5** in C_6D_6

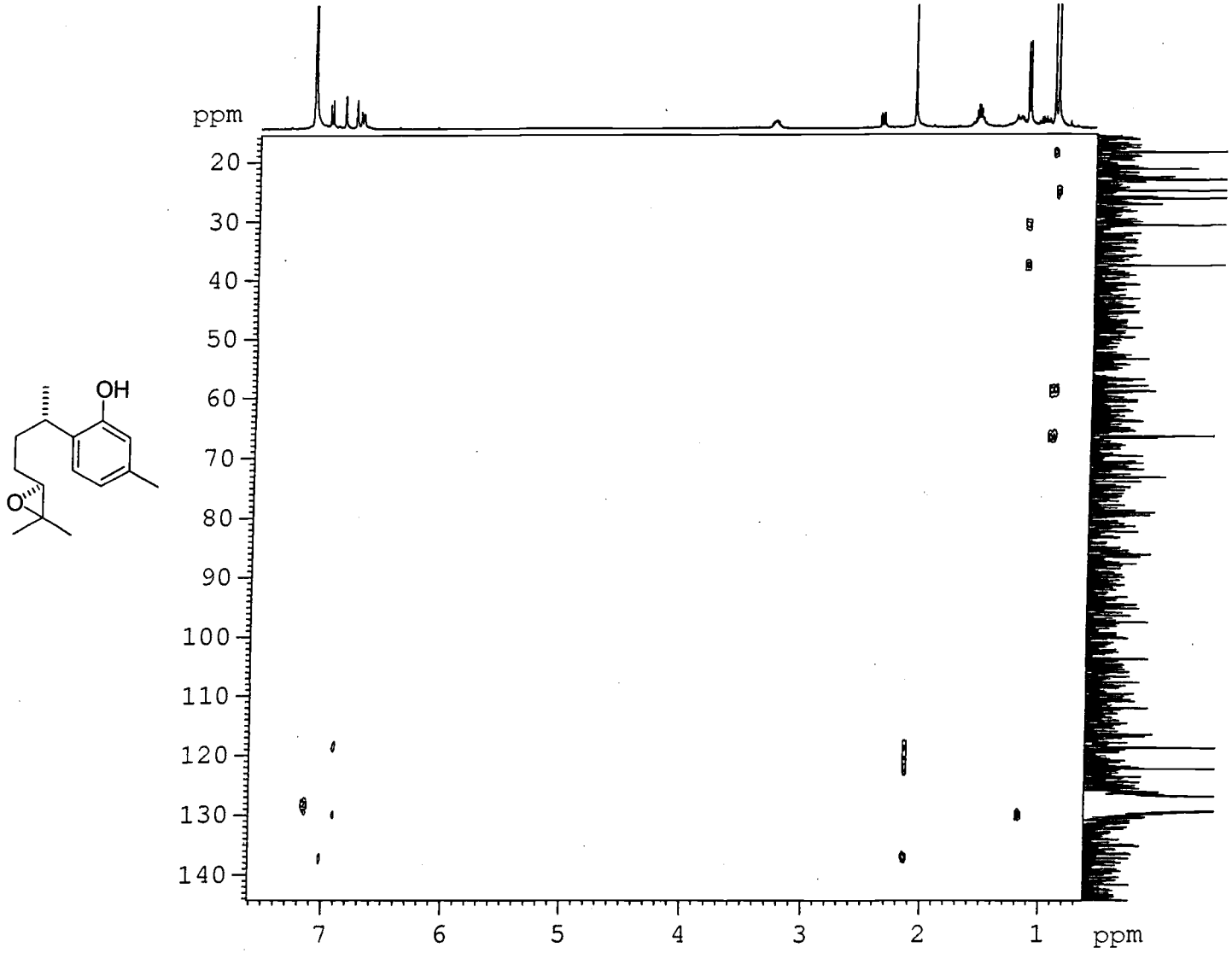
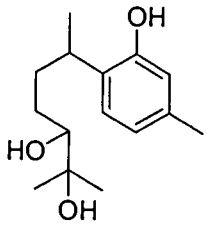


Figure IV. 3. HSQC spectrum of compound **5** in C_6D_6

Compound **6** was isolated as a colorless oil. It showed a HRCIMS $[M]^+$ ion at *m/z* 234.1616 for a molecular formula of $C_{15}H_{23}O_2$, and therefore possessed five degrees of unsaturation. The IR spectrum displayed bands characteristic for a hydroxyl group (3392 cm^{-1}) as well as the presence of an aromatic system (2958 cm^{-1}). ^1H NMR experiments, were carried out in CD_3OD and C_6D_6 to provide good chemical shift resolution. ^1H NMR signals were well dispersed in CD_3OD. The ^1H NMR spectrum of **6** in CD_3OD (Table IV.2. 2) displayed a sharp doublet at δ 6.95 (1H, d J= 7.6 Hz), which was consistent with an aromatic proton ortho to another sharp doublet at δ 6.58 (1H, d J= 7.6 Hz), a sharp singlet at δ 6.56 (1H, s). Another sharp triplet was found at δ 2.75 with J values of 13, 6.4 Hz, which was consistent with a proton attached to a carbon atom attached to an epoxy group. Another two sharp singlets at δ 1.22 and 1.14, assignable to two geminal olefinic dimethyl protons were also observed. Additionally a sharp doublet at δ 1.18, which was consistent with an aliphatic methyl group attached to a methine group, was observed. In addition, a multiplet at δ 3.16 (methine proton), four methylene signals (δ 1.79, 1.64, 1.45 and 1.42) and an olefinic methyl at δ 2.21 were detected. 2D NMR techniques (^1H-^1H COSY, HSQC, and HMBC) were used to establish the connectivities. An HSQC experiment showed the direct ^1H-^{13}C correlations involving all protonated carbons, while an HMBC experiment allowed the assignment of the nonprotonated carbons.

Compound **7** was isolated as a colorless oil. It showed a LRCIMS $[M+NH_4]^+$ ion at *m/z* 270 for a molecular formula of $C_{15}H_{24}O_3$, and therefore possessed four degrees of unsaturation. The IR spectrum displayed bands characteristic for a hydroxyl group (3340 cm^{-1}) as well as the presence of an aromatic system (2960 cm^{-1}). The ^1H NMR spectrum in CD_3OD of **7** (Table IV.2. 2) displayed a sharp doublet at δ 6.95 (1H, d J= 7.5 Hz), which was consistent with an aromatic proton coupled to another proton in the ortho position. Another sharp doublet at δ 6.85 (1H, d J= 7.5 Hz), consistent with an aromatic proton coupled to the previous proton, was recognized. A sharp singlet at δ 6.57 (1H, s), a sharp doublet of doublet at δ 3.36 with *J* values of 10.5, 1.5 Hz, which was consistent with a proton attached to a carbon atom attached to hydroxy group were detected. Two sharp singlets at δ 1.45 and 1.47, assignable to two geminal olefinic dimethyl protons attached to quaternary carbon were also displayed. Another sharp doublet at δ 1.19 with *J* value of 7.2 HZ, which was consistent with methyl aliphatic protons attached to a methine group was observed. In addition, a multiplet at δ 3.09 (methine proton), four

signals for protons attached to methylene groups (δ 1.82, 1.63, 1.60 and 1.36) and an olefinic methyl at δ 2.21 attached to an aromatic nucleus were obevious. 2D NMR techniques (^{1}H-^{1}H COSY, HSQC, and HMBC) were used to establish the connectivities and finally to the planar structure of **7**.



7

Table IV.2. ^{1}H- and ^{13}C-NMR Data for (**6**) in CD_3OD and $CDCl_3$ respectively.

Position	^{1}H mult. *J* (Hz)	^{13}C	HMBC[b]
1	------	153.0 C	
2	6.56, s	116.0 CH	C-6, C-4
3	------	137.5 C	
4	6.58, d (7.6)	122.0 CH	C-2, C-6
5	6.95, d (7.6)	127.0 CH	C1-C3
6	------	130.0 C	
7	3.16, m	32.1 CH	
8	1.79 m 1.64 m	34.0 CH_2	
9	1.45 m 1.42 m	27.0 CH_2	C-10, C-8
10	2.75 t (13, 6.4)	65.0 CH	
11	------	59.1 C	
12	1.25 3H, s	25.0 CH_3	C-10, C-11, C-13
13	1.14 3H, s	19.6 CH_3	C-10, C-11, C-12
14	1.18 d (7)	21.0 CH_3	C-6, C-7, C-8
15	2.21, s	21.3 CH_3	C-2, C-3, C-4

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

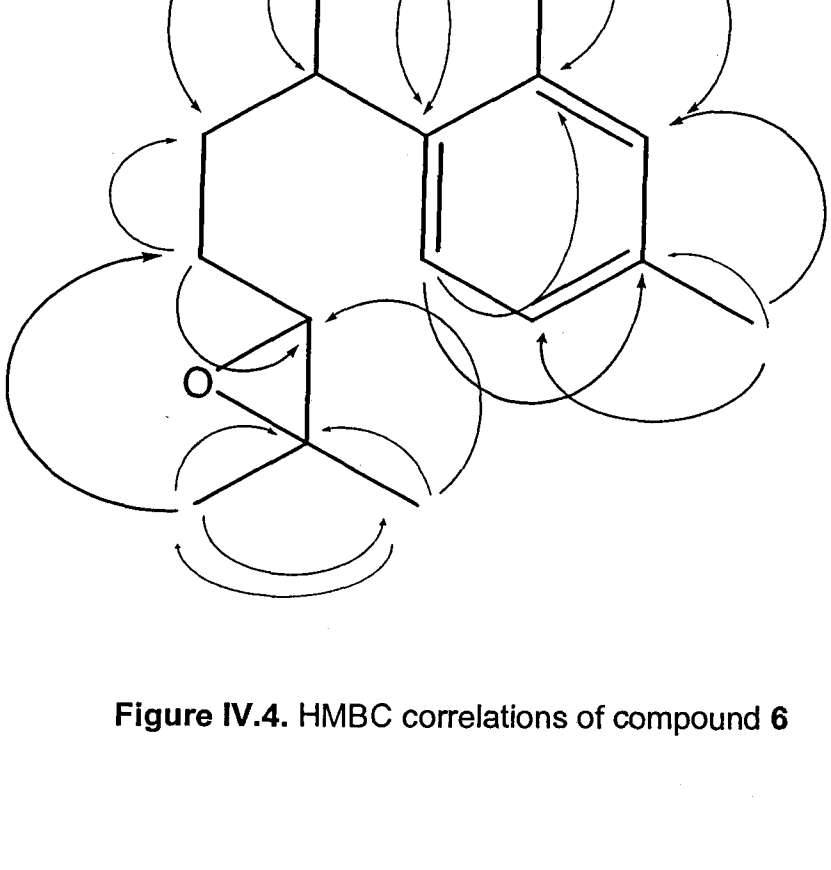


Figure IV.4. HMBC correlations of compound **6**

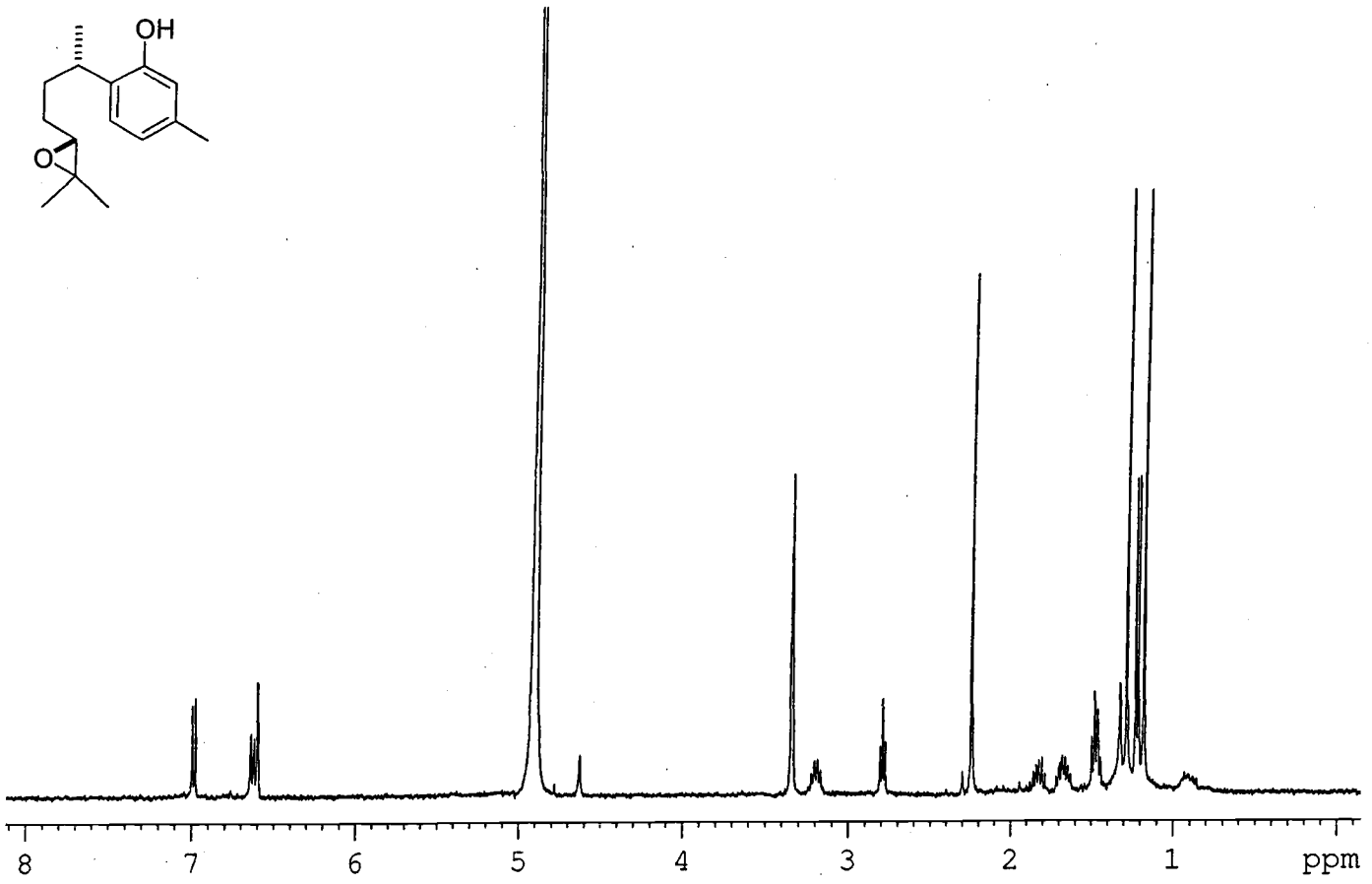


Figure IV.5. ^{1}H NMR spectrum of compound **6** in CD_3OD

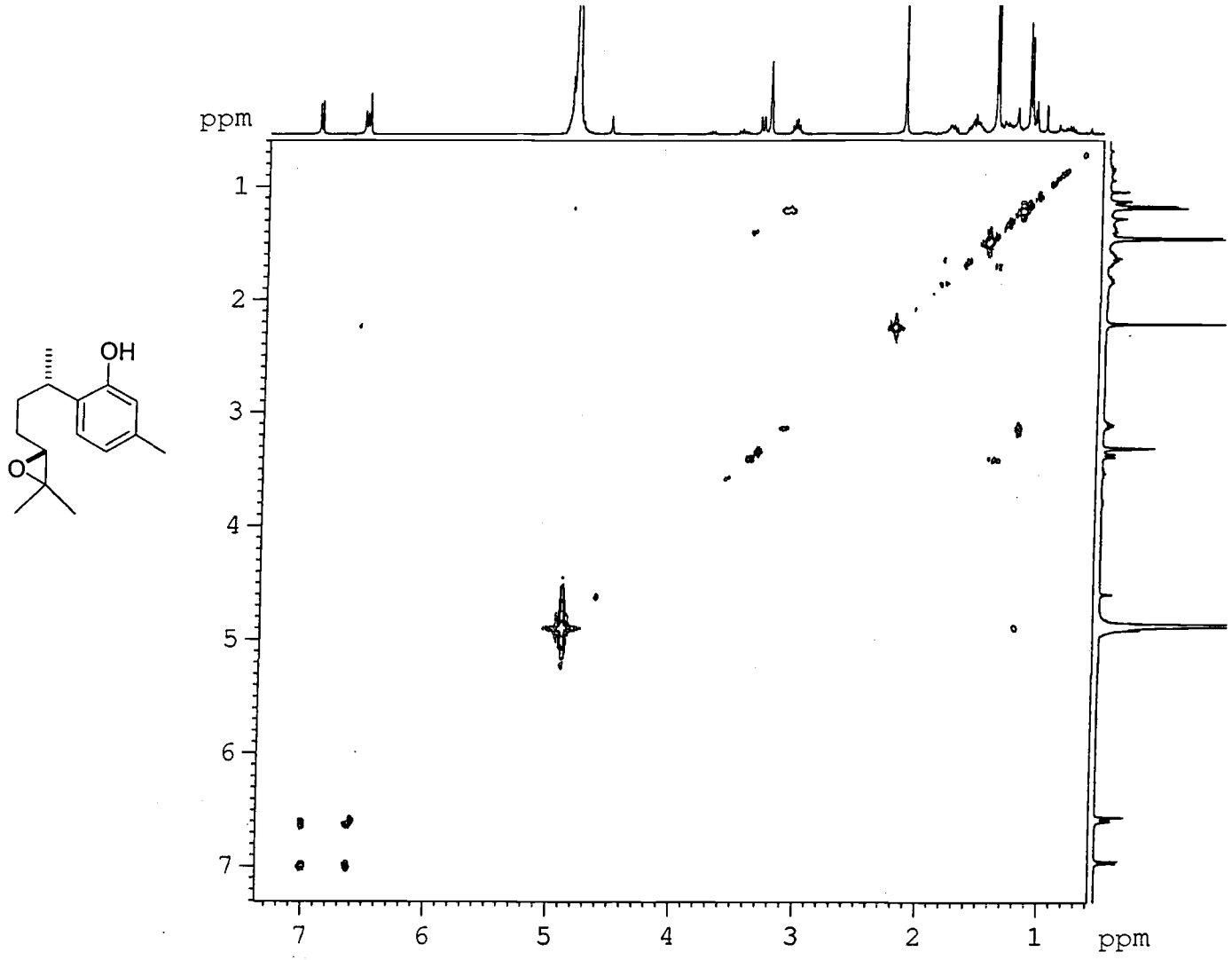


Figure IV.6. COSY spectrum of compound **6** in CD_3OD

Table IV.3. ^{1}H- and ^{13}C-NMR Data for (7) in CD_3OD

Position	^{1}H mult. *J* (Hz)	^{13}C	HMBC[b]
1	------	154.0 C	
2	6.57, s	115.7 CH	C-6, C-4
3	------	136.0 C	
4	6.85, d (7.5)	120.0 CH	C-2, C-6
5	6.95, d (7.5)	126.0 CH	C1-C3
6	------	130.7 C	
7	3.09, m	32.0 CH	
8	1.82 m 1.6 m	34.7 CH_2	
9	1.63 m 1.36 m	29.9 CH_2	
10	3.36 dd (10.5, 1.5)	73.5 CH	
11	------	79.0 C	
12	1.47 3H, s	28.3 CH_3	C-10, C-11, C-13
13	1.45 3H, s	27.4 CH_3	C-10, C-11, C-12
14	1.19 d (7.0)	20.46 CH_3	C-6, C-7, C-8
15	2.21, s	20.0 CH_3	C-2, C-3, C-4

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

(*S*)-(+)-Curcuphenol (**9**) $[\alpha]^{25}_{D}$ = +29.7 ^{0} provided the starting point for the assignment of the unknown stereochemistry at C-10 of metabolites **5**, **6** and **7**. The presence of two freely rotating methylene groups between the C-7 stereocenter and the distant additional stereogenic carbon at C-10 of **5**, **6** and **7** minimizes any direct chiroptical perturbation between stereocenters and allows for application of van't Hoff's principle (asymmetric centers separated by several single bonds make simple additive contributions to the molar rotation angle) of optical superposition.[10] The molar rotations ($[M]_D$) of curcuphenol and metabolites **5**, **6** and **7** were determined by experimental procedure. (*S*)-(-)-3-Ethyl-2,2-dimethyl-oxirane,[13] (*R*)-(+)-3-ethyl-2,2-dimethyl-oxirane[13] and (*S*)-(-)-2-methylpentane-2,3-diol[10] were used as references in the analysis of the configuration at C-10 for **5**, **6** and **7**, respectively, because the substitution pattern around the stereocenter in these compounds is closely related to the compounds isolated from *U. orientalis*. Since the *S*-configuration at C-7 in curcuphenol and in **5**, **6** and **7** is constant, the expected molar rotations for all possible diastereomers of **5**, **6** and **7** were readily derived (Table IV.4). Stereochemical assignments were possible due to the considerable differences in the observed and calculated molar rotations between the different stereoisomers. The (7*S*,10*S*) and (7*S*,10*R*) assignments for **5** and **6** respectively, provided the best agreement between the observed data and the values obtained by additional calculations for the two stereocenters (Table IV.4). However, the observed molar rotation of compound **7** (7*S*,10*S*) deviated to some extent from the calculated value, however it may be considered within the range.[10] I didn't have sufficient quantities of the previous compounds to determine the absolute stereochemistry using Mosher's ester analysis.

I also isolated from this algal species the previously reported metabolites **8-11**.[9-12] I carried out a full investigation of the spectroscopic data of these compounds in the course of this work. Compounds **8-11** were found to possess the same physical and spectroscopic data as reported in the literature.[9-11] Stereochemistry assignment of compound **8** was found to be the same as that reported in the literature[10] by comparing the ^{1}H, ^{13}C NMR data and specific angle of rotation, and finally compared with calculated values for the molar rotation (Table IV.5).

Isolation of these three new metabolites from the organic extract of a Papua New Guinean collection of the green alga *Udotea orientalis* growing on coral wall in addition

to the four known compounds from this single collection, demonstrates the capability of marine echology to produce a remarkable variety of structures in the same organism.

The isolated compounds were evaluated for their biological activity in several systems. Compounds **8** and **11** were found to have strong cytotoxic activities (EC_{50} = 2, 4 µg/ml), respectively in human lung cancer (NCI-H460) cell lines. In addition, they both displayed sodium channel blocking activity at this dose but not at the lower dose of 1 µg/ml. This activity was 72 and 82%, respectively, compared to 87% for saxitoxin at 0.05 µg/ml.

Table IV.4. Calculated Molar Rotations ($[M]_D$) of Different Curcuphenol Substituents at positions 7 and 10.

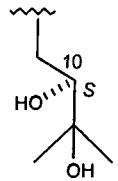
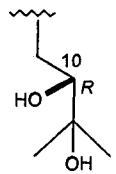
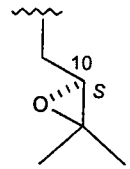
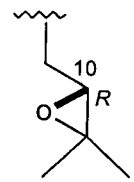
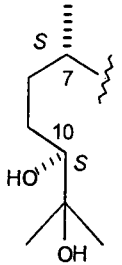
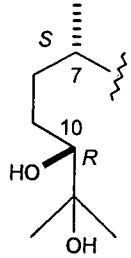
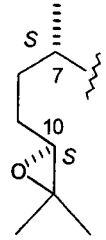
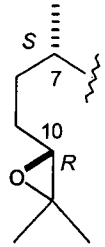
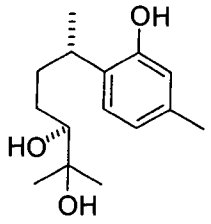
Curcuphenol	**Diols**		**Epoxides**	
	10***S***  (-37.2)	10***R***  (+37.2)	10***S***  *Levo* *<[0]*	10***R***  *Dextro* *>[0]*
7***S***  (+29.7)	7***S*** 10***S***  -7.5	7***S*** 10***R***  +66.9	7***S*** 10***S***  <[+29.7]	7***S*** 10***R***  >[+29.7]

Table IV.5. Molar Rotations ($[M]_D$) for Metabolites **5-8** and Differences between Observed and Calculated Values.

Stereochemistry	Diols		Epoxides	
	7***S*** 10***S***	7***S*** 10***R***	7***S*** 10***S***	7***S*** 10***R***
Calculated molar rotations	-7.5	+66.9	<[+29.7]	>[+29.7]
Compound **5** 7***S*** 10***S***			+2.0	
Compound **6** 7***S*** 10***R***				+80
Compound **7** 7***S*** 10***S***	+3.0			
Compound **8** 7***S*** 10***R***		+62		



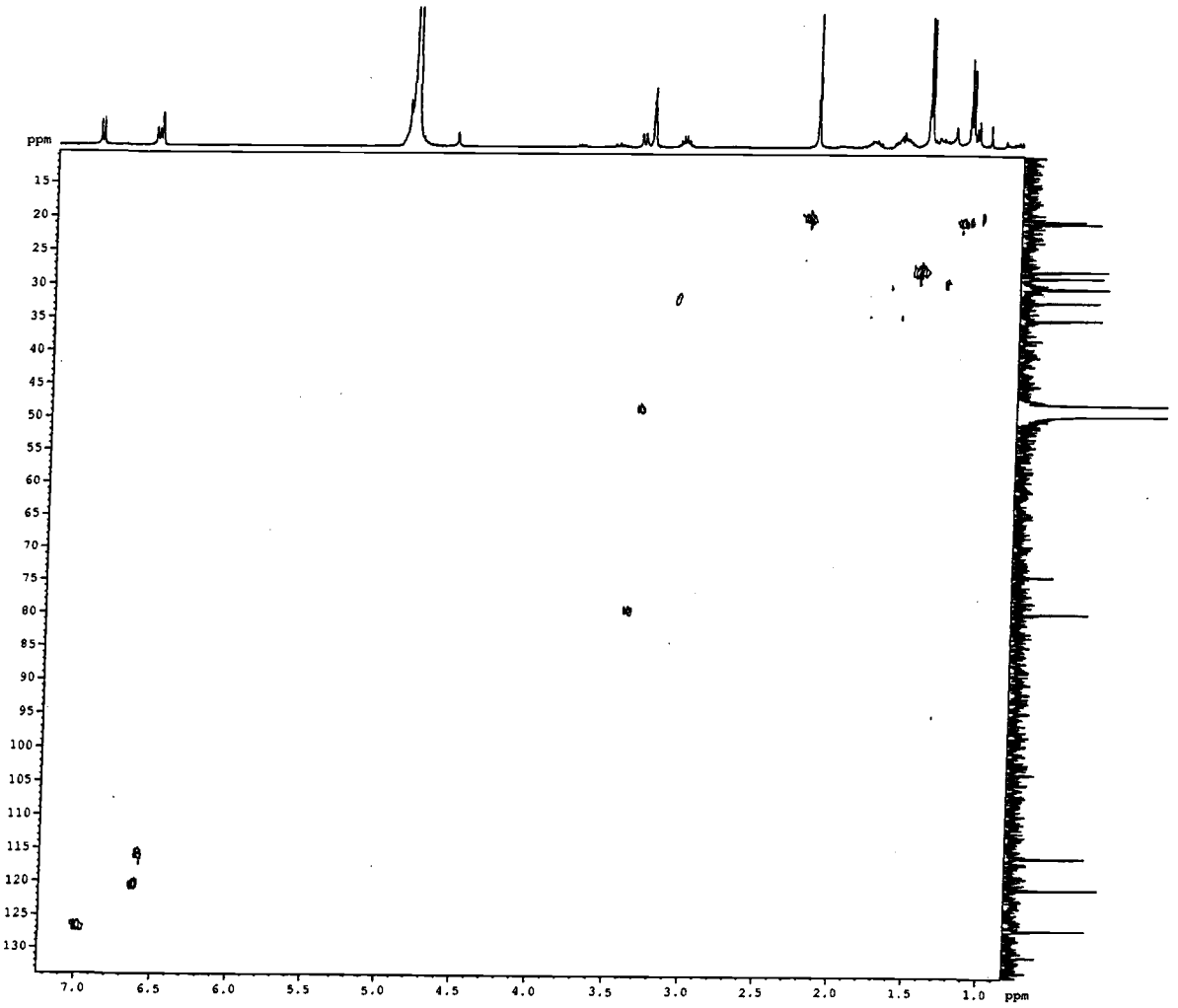


Figure IV.7. HSQC spectrum of compound **7** in CD_3OD

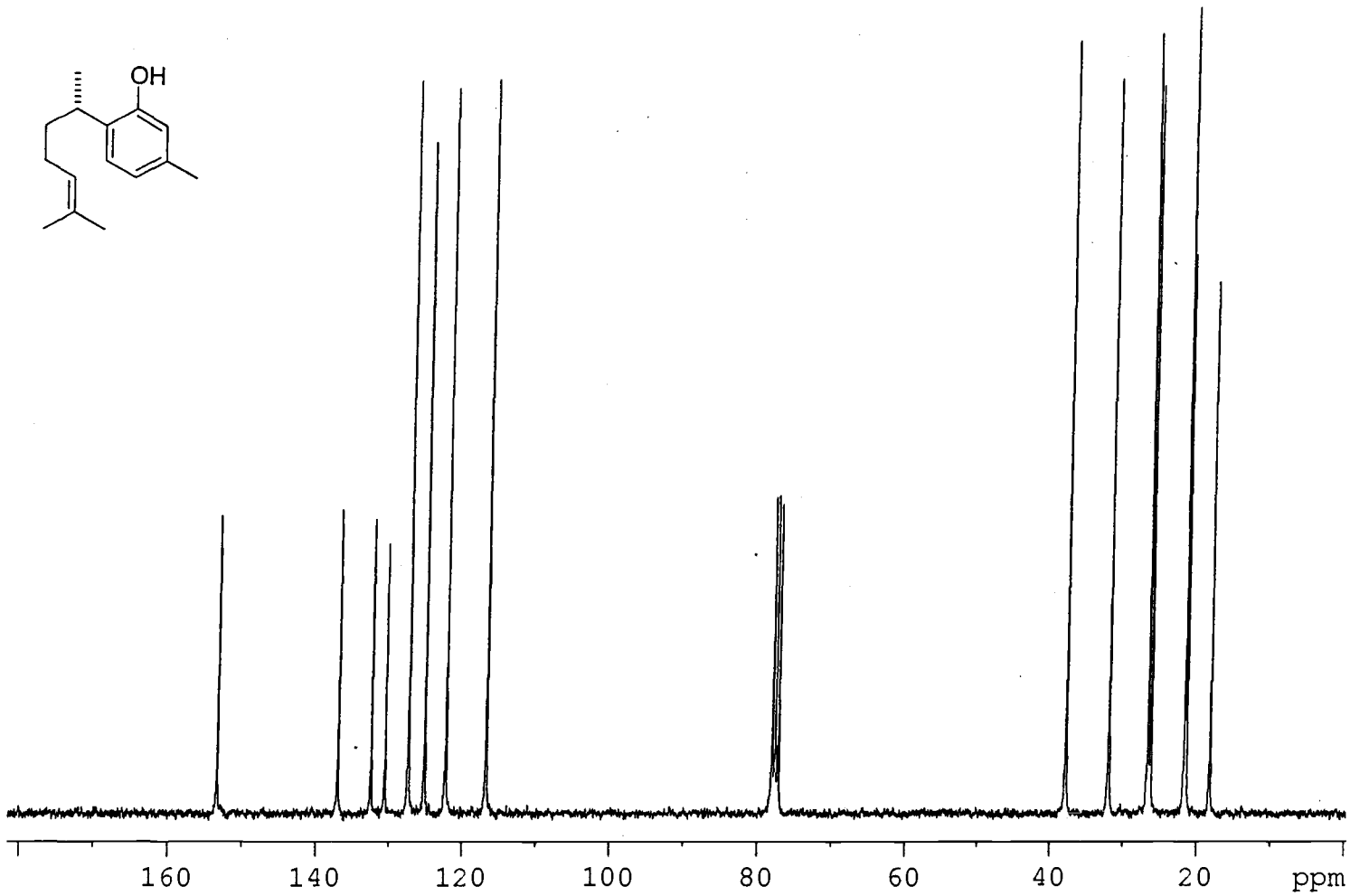


Figure IV.8. ^{13}C NMR spectrum of compound **9** in $CDCl_3$

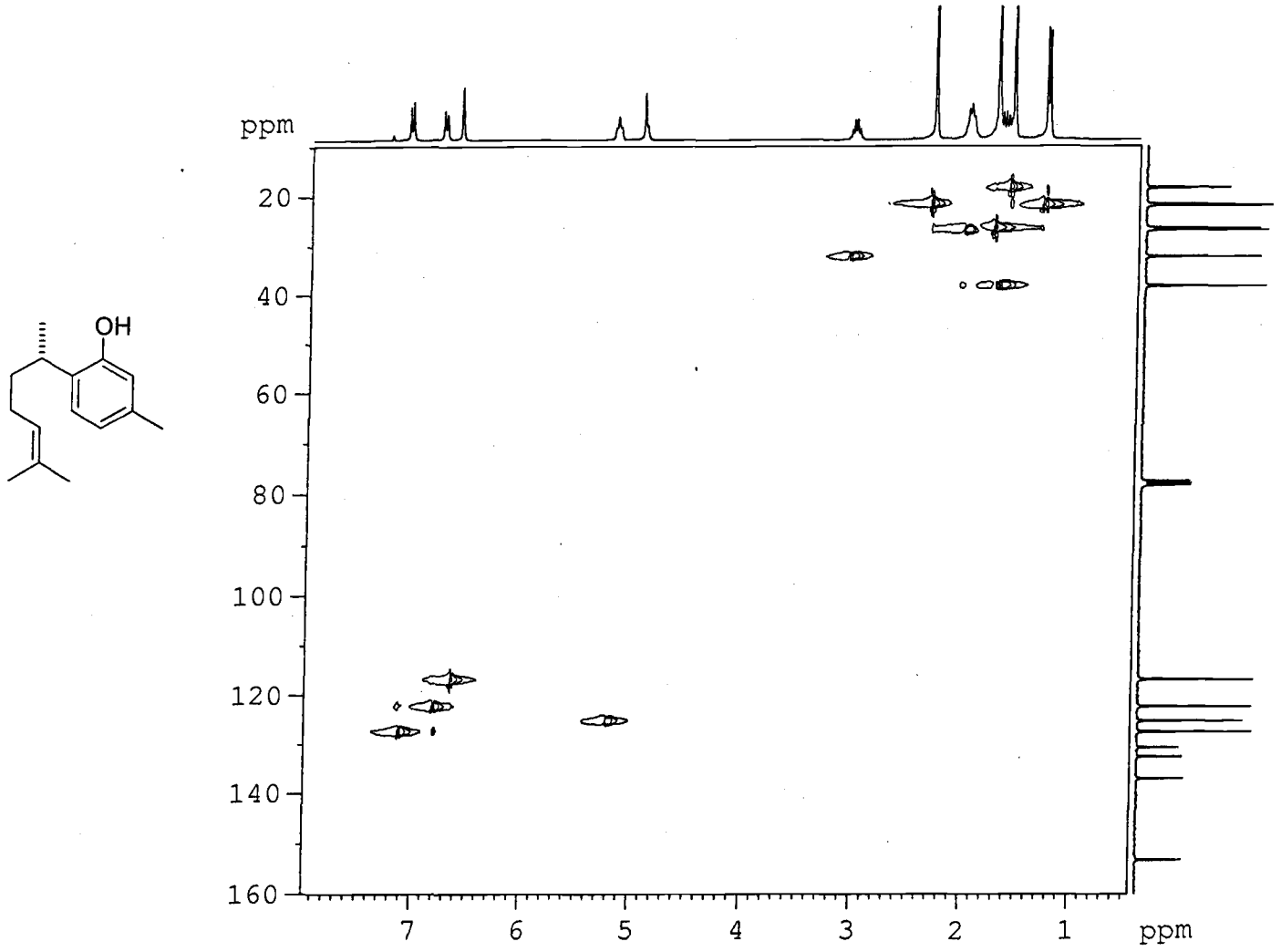


Figure IV.9. HSQC spectrum of compound **9** in $CDCl_3$

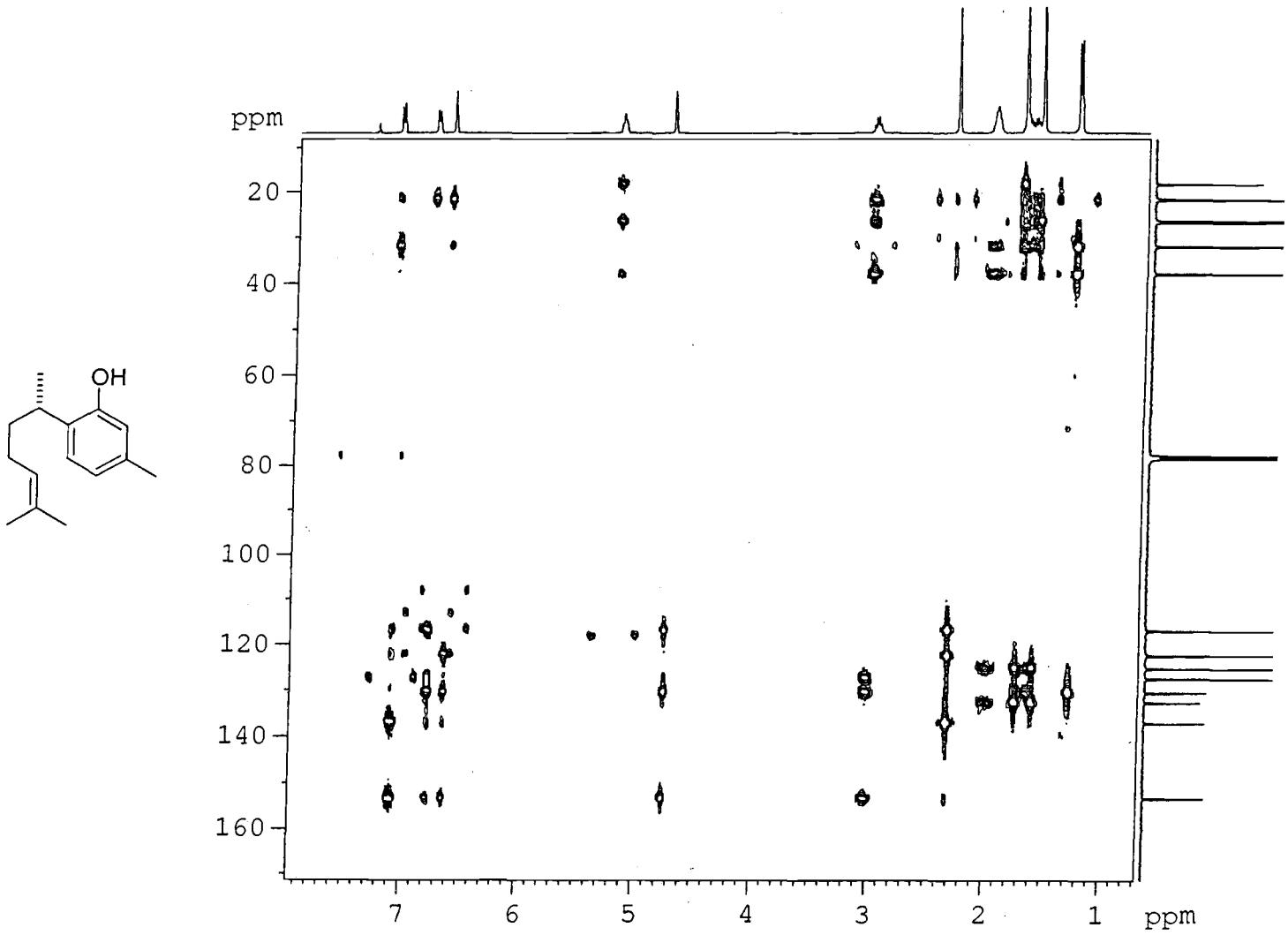
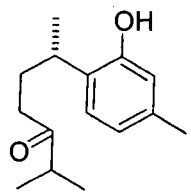


Figure IV.10. HMBC spectrum of compound **9** in $CDCl_3$



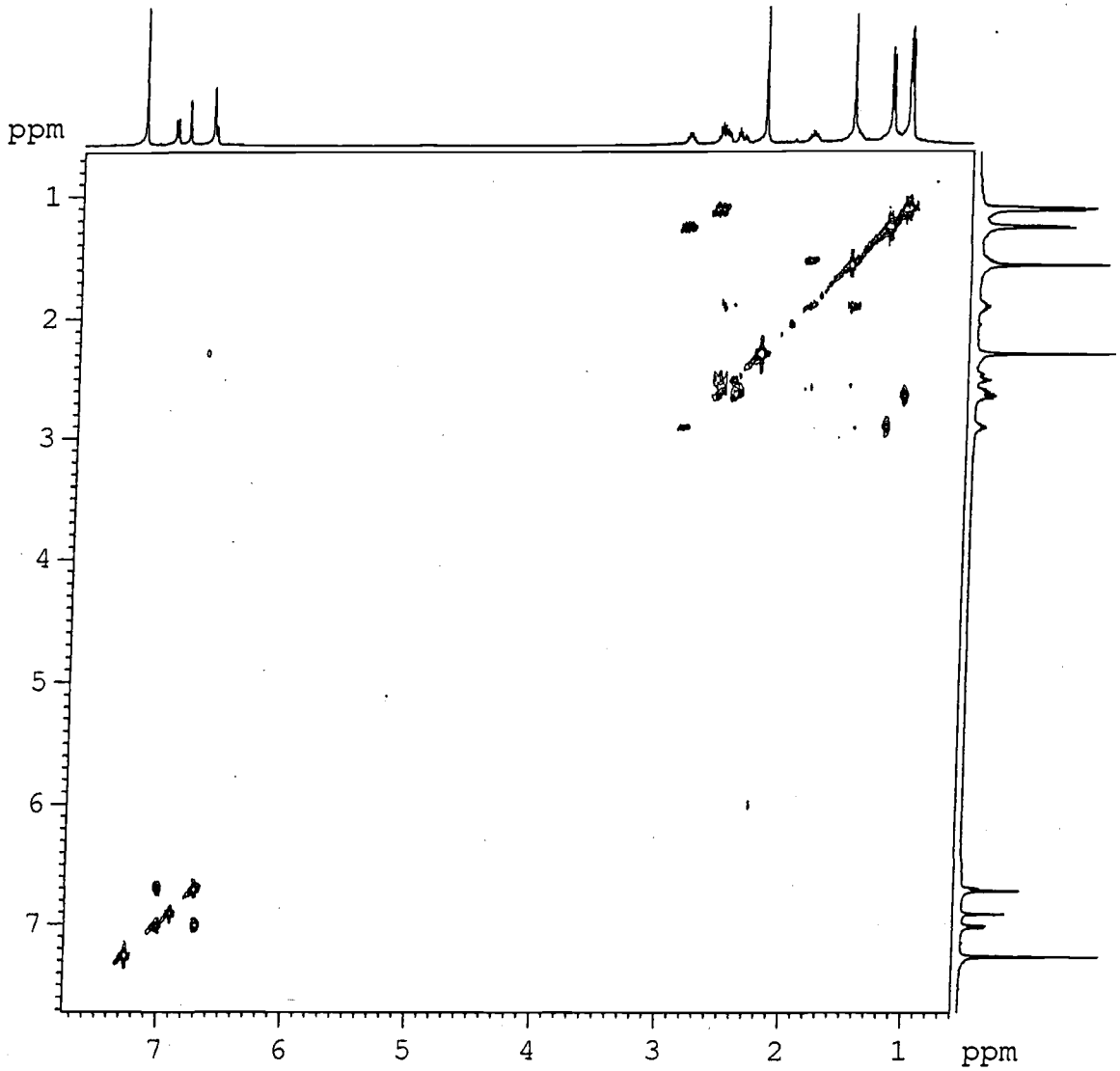


Figure IV.11. COSY spectrum of compound **11** in CD_3OD

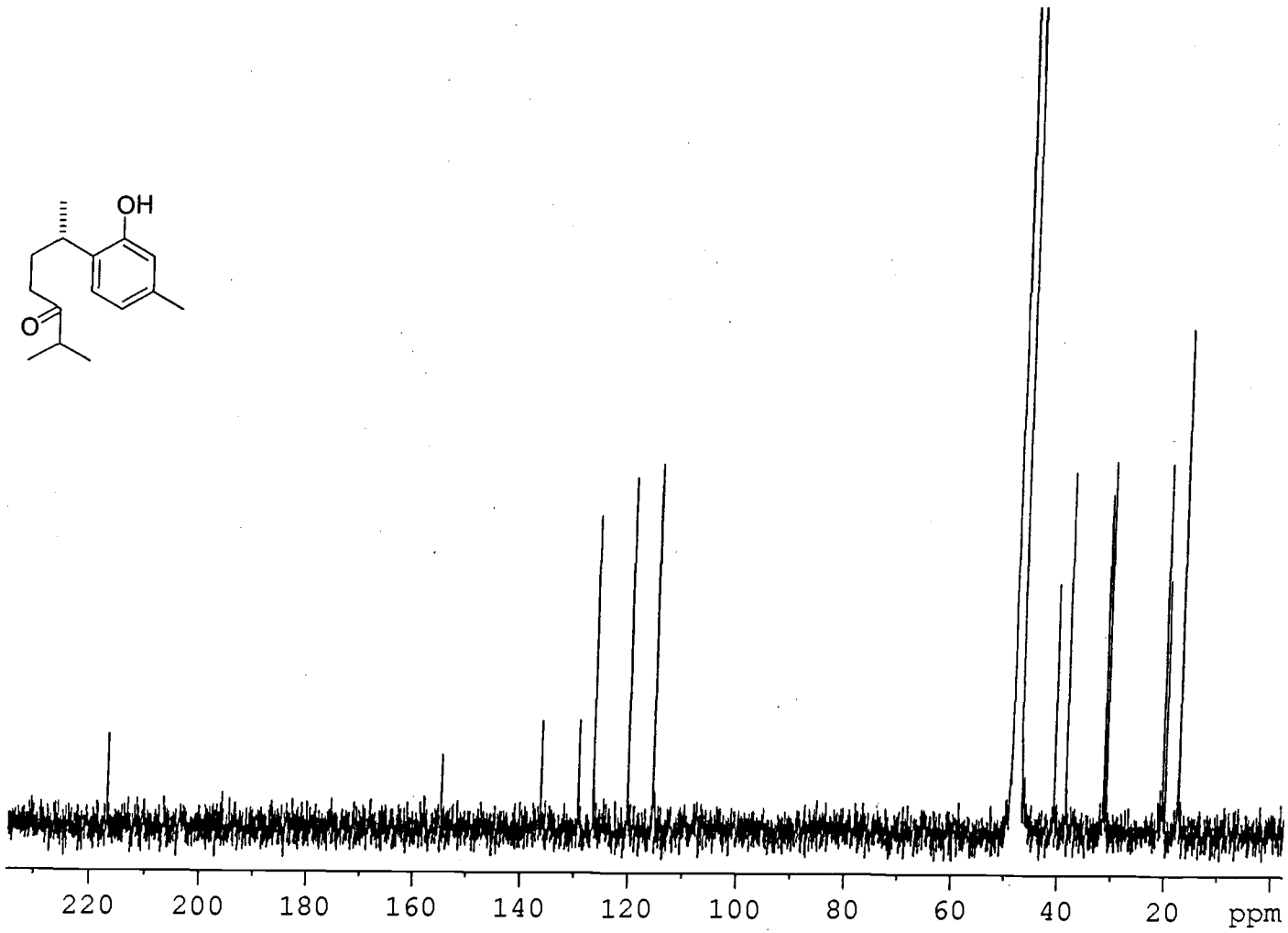


Figure IV.12. ^{13}C NMR spectrum of compound **11** in CD_3OD

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. NMR spectra were recorded on a Bruker DPX400 spectrometer, with the solvent ($CDCl_3$ at δ_C 77.3, δ_H 7.26 or C_6H_6 at δ_C 128.39, δ_H 7.16 CD_3OD at δ_C 49.15, δ_H 3.31 ppm) used as an internal standard. Mass spectra were recorded on a Kratos MS50TC mass spectrometer, and HPLC isolations were performed using Waters Millipore model 515 pumps and a Waters 969 diode array detector.

Plant material. The Papua New Guinean collection of the green alga *Udotea orientalis* growing on the surface of a coral (voucher specimen available from WHG as collection number PNHL-9 September 98-3) was collected by hand using SCUBA from 40 feet of water near Papua New Guinea coast (September 1998). The material was stored in 2-propanol at -20 ^{0}C until extraction.

Extraction and Isolation. Approximately 14 g (dry wt.) of the green alga *Udotea orientalis* was extracted repeatedly with 2:1 CH_2Cl_2/MeOH to produce 0.9 g of crude organic extract. The extract (0.9 g) was subjected to Si vacuum liquid chromatography (VLC, hexanes/EtOAc/MeOH) to produce 9 chemically distinct fractions. The fraction eluting with 10% EtOAc in hexanes was purified by a silica solid phase extraction (SPE) cartridge (7:3 hexanes/ EtOAc) then normal phase HPLC (3% EtOAc/ hexanes) dual silica, Phenomenex Luna 10u Silica 250×4.6mm to yield 0.7 mg of compound **5**, 0.8 mg of compound **6**, 1.0 mg of compound **7**, 0.9 mg of compound **8**, 240 mg of compound **9**, and 3.0 mg of compound **11**. The VLC fractions eluting with 40% EtOAc were subjected to normal phase HPLC (20-40% gradient EtOAc /hexanes) dual silica, Phenomenex Luna 10u Silica 250×4.6mm to yield 2 mg of compound **10**.

Compound 5 : Colorless oil; $[\alpha]^{25}_D$ +2 ^{0} (c 0.07, $CHCl_3$); UV ($CHCl_3$) $_{max}$ 245 nm (ε 771), 276 nm (ε 1079), 278 nm (ε 1085); IR (neat) 3400, 2967, 2921, 2870, 1466; ^{1}H NMR and ^{13}C NMR (400 MHz, $CDCl_3$) see Table IV.1. 1; HRFABMS $[M+1]^+$ *m/z* 235.1698 (calcd for $C_{15}H_{23}O_2$).

Compound 6 : Colorless oil; $[\alpha]^{25}_D$ +80 ^{0} (c 0.07, $CHCl_3$); UV ($CHCl_3$) $_{max}$ 244 nm (ε 791), 276 nm (ε 1085), 278 nm (ε 1080); IR (neat) 3392, 2958, 2924, 2858, 1455, 1422 cm^{-1}; ^1H NMR and ^{13}C NMR (400 MHz, $CDCl_3$) see Table IV.2. 2; HRCIMS *m/z* $[M]^+$ 235.1616 (calcd for $C_{15}H_{23}O_2$).

Compound 7 : Colorless oil; $[\alpha]^{25}_D$ +3 ^{0} (c 0.8, $CHCl_3$); UV ($CHCl_3$) $_{max}$ 244 nm (ε 792), 274 nm (ε 925), 282 nm (ε 1096); IR (neat) 3400, 2967, 2921, 2870, 1517, 1466, 1378, 1266, 1291, 807 cm^{-1}; ^1H NMR and ^{13}C NMR (400 MHz, $CDCl_3$) see Table IV.3. 3; LRCIMS $[M+NH_4]^+$ ion at *m/z* 270 (calcd for $C_{15}H_{24}O_3$)

Compound 8 : Colorless oil; $[\alpha]^{25}_D$ +52 ^{0} (c 0.07, $CHCl_3$); reported +61.8° ;[10] UV ($CHCl_3$) $_{max}$ 246 nm (ε 238), 274 nm (ε 461), 282 nm (ε 515); IR, ^1H, ^{13}C NMR, and MS data were identical to previously reported data.[10]

Curcuphenol (9): colorless oil, $[\alpha]^{25}_D$ +29 ^{0} (c 1.00, $CHCl_3$), reported +24.6 ± 2° ;[1] UV, IR, ^1H, ^{13}C NMR, and MS data were identical to previously reported data.[1,2]

Curcudiol (10): colorless oil, $[\alpha]^{25}_D$ +8 ^{0} (c 0.10, $CHCl_3$), literature value +9° ;[1] UV, IR, ^1H, ^{13}C NMR, and MS data were similar to literature.[1,2]

Curcudiol-10-one (11): colorless oil, $[\alpha]^{25}_D$ +38 ^{0} (c 0.10, $CHCl_3$); reported +40.5° ;[10] UV, IR, ^1H, ^{13}C NMR, and MS data were identical to previously reported data.[1,2]

Cytotoxicity against NCI-H460 human lung cancer and mouse neuro-2a neuroblastoma cell line.[14] Cytotoxicity was measured to NCI-H460 human lung tumor cells and neuro-2a blastoma cells using the method of Alley et. al. with cell viability being determined by MTT reduction. Cells were seeded in 96-well plates at 5000 and 8000 cells/well in 180 µl for H460 and neuro-2a cells, respectively. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µg/well. DMSO was less than 1% final concentration. After 48 hours, the medium was removed and cell viability determined.

Sodium channel modulation.[15] Isolated compounds were evaluated for their capacity to either activate or block sodium channels using the following modifications to the cell-based bioassay of Manger et. al. Twenty-four hours prior to chemical testing,

mouse neuro-2a blastoma cells were seeded in 96-well plates at 8 X 10^4 cells/well in a volume of 200 µl. Test chemicals dissolved in DMSO were serially diluted in medium without fetal bovine serum and added at 10 µl/well. DMSO was less than 1% final concentration. Plates to evaluate sodium channel activating activity received 20 µl/well of either a mixture of 3 mM ouabain and 0.3 mM veratridine (Sigma Chemical Co.) in 5 mM HCl in addition to the test chemical. Plates were incubated for 18 hr and results compared to similarly treated solvent controls with 10 µl medium added in lieu of the test chemical. The sodium channel activator brevetoxin PbTx-1 (Calbiochem) was used as the positive control and added at 10 ng/well in 10 µl medium. Sodium channel blocking activity was assessed in a similar manner except that ouabain and veratridine were 5.0 and 0.5 mM, respectively, and the sodium channel blocker saxitoxin (Calbiochem) was used as the positive control. Plates were incubated for approximately 22 hours.

REFERENCES

1. Paul, V. J.; Sun, H. H.; Fenical, W. *Phytochemistry* **1982**, 21, 468-9.

2. Paul, V. J.; Fenical, W. *Marine Ecology* **1986**, 34, 157-69.

3. Paul, V. J.; Nelson, S. G.; Sanger, H. R. *Marine Ecology* **1990**, 60, 23-34.

4. Iliopoulou, D; Vagias, C; Harvala, C; Roussis, V. *Nat. Prod. Lett.* **2000**, 14, 373-378.

5. Siddhanta, A. K.; Shanmugam, M. *J. Ind. Chem. Soc.* **1999**, 76, 323-334.

6. Uhlenbruck, G.; Hanisch, F. G.; Kljajic, Z.; Poznanovic, S.; Schroeder, H. C.; Mueller, W. E. G. *Behring Institute Mitteilungen* **1992**, 91, 67-77.

7. Fattorusso, E.; Magno, S.; Mayol, L.; Novellino, E. *Experientia* **1983**, 39, 1275-6.

8. Nakatsu, Tetsuo; Ravi, B. N.; Faulkner, D. John. *J. Org. Chem.* **1981**, 46, 2435-8.

9. Fusetani N; Sugano M; Matsunaga S; Hashimoto K. *Experientia* **1987**, 43, 1234-5.

10. El Sayed K. A; Yousaf M.; Hamann M. T; Avery M. A; Kelly M.; Wipf P. *J. Nat. Prod.* **2002**, 65, 1547-53.

11. Tasdemir, D.; Bugni, T. S.; Mangalindan, G. C.; Concepcion, G. P.; Harper, M. K.; Ireland, C. M. *Turkish J. Chem.* **2003**, 27, 273-279.

12. McEnroe, F. J.; Fenical, W. Tetrahedron **1978**, 34, 1661-4.

13. Davis, F. A.; Harakal, M. E.; Awad, S. B. *J. Am. Chem. Soc.* **1983**, 105, 3123-6.

14. Alley, M. C.; Scudiero,D. A. *Cancer Research* **1988**, 48, 589-601.

15. Manger, R. L.; Leja. L. S. *J. AOAC Int.* **1995**, 78, 521- 527.

CHAPTER FIVE

A BIOLOGICALLY ACTIVE NEW MALYNGAMIDE AND CYCLIC DEPSIPEPTIDE FROM A KEY WEST, FLORIDA COLLECTION OF *LYNGBYA MAJUSCULA*

ABSTRACT

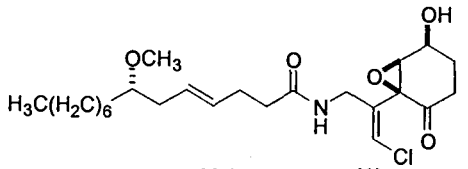
A bioassay guided investigation (Anti-Sirt2) of a *Lyngbya majuscula* collection from Key West Florida in 1995, led to the discovery of two novel bioactive natural products [(+)-malyngamide X **30** and another cyclic depsipeptide, (+)-floridamide **31**]. Their structures were deduced through extensive analysis of 1D and 2D NMR spectroscopic data and supported by HRFAB mass spectrometry. The new cyclic depsipeptide **31** contains four amino acids units, including *N*-methyl phenylalanine (*N*-MePhe), proline (Pro), valine (Val) and alanine (Ala), beside the unique unit, 2,2-dimethyl -3-hydroxy-octanoic acid (Dhoaa). In addition to the discovery of these two new compounds, two previously reported metabolites were also isolated and identified from this cyanobacterial collection, the (-) C-12 lyngbic acid **20** and the antibacterial agent (-) malyngolide **29**.

INTRODUCTION

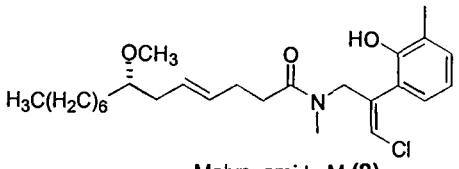
Many collections of the marine cyanobacterium *Lyngbya majuscula* produce a class of lipopeptide metabolites known as the malyngamides.[1-6] To date, more than thirty members belonging to this growing class of cyanobacterial metabolites have been discovered. Representative examples of these natural products (**1-18**) are shown in Figure V.1. There are two distinct portions present in the malyngamides; a methoxy fatty acid (known trivially as "lyngbic acid") and a variety of functionalized amines, linked through an amide bond. The different lyngbic acids (**19-23**) present in the malyngamides are shown in Figure V.2. These lyngbic acids have varying chain lengths, ranging from C-12 to C-20, with a methoxy group at C-7 as well as a *trans* double bond at C-4. Most of the reported malyngamides contain the C-14 lyngbic acid. The C-16 analog is present in malyngamides D (**7**) and E (**8**), while the C-12 analog is present in malyngamide G (**14**). The C-12 and C-14 lyngbic acids have been detected in free form from marine cyanobacteria. The absolute stereochemistry of these lyngbic acids has been confirmed by total chemical syntheses.[7-9]

The marine cyanobacterium *Lyngbya majuscula* is also considered an established source of unique and bioactive peptides (Figure V.3). Examples include the antimicrobial and actin polymerization-inhibiting lyngbyabellins A (**24**) and B (**25**), the cytotoxic lyngbyastatin 2 (**26**) and the pro-inflammatory lyngbyatoxins A (**27**) and B (**28**).[10-12]

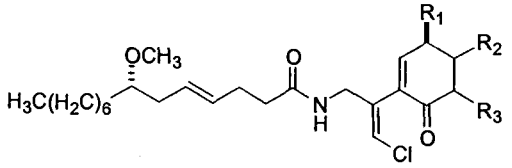
As part of my ongoing search for structurally and pharmacologically interesting substances from *Lyngbya majuscula*, a detailed exploration of a Key West Florida *L. majuscula* collection was undertaken. During this study, two metabolites of known identity were isolated, including the anti-microbial (-)-malyngolide (**29**) and the (-)-C-12 lyngbic acid (**30**). In addition, two new compounds, (+)-malyngamide X (**31**) and (+)-floridamide (**32**), were discovered in the same organic extract. This chapter describes the chromatographic isolation and structure elucidation of these two new metabolites of *L. majuscula*.



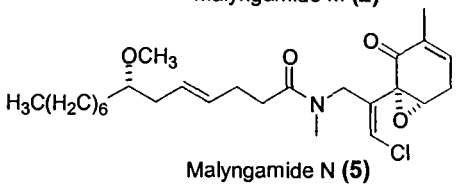
Malyngamide C **(1)**



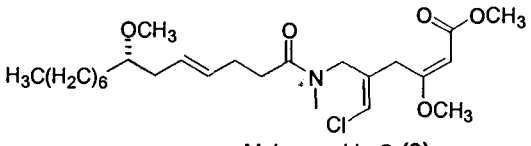
Malyngamide M **(2)**



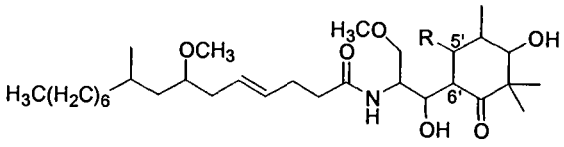
Malyngamide K $R_{1,2,3}$ = H **(3)**
Malyngamide L R_1 = H, R_2 = OH, R_3 = CH_3 **(4)**



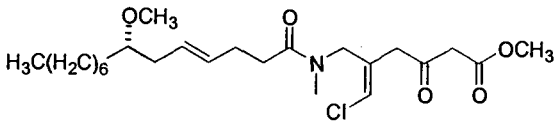
Malyngamide N **(5)**



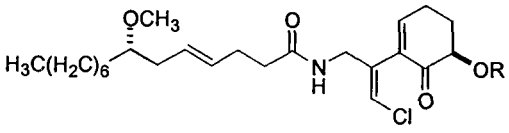
Malyngamide O **(9)**



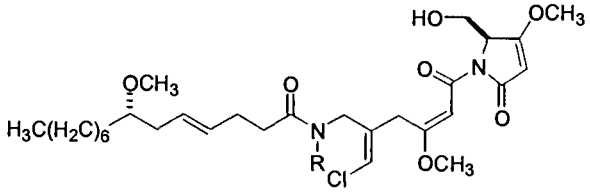
Malyngamide D R = OH **(7)**
Malyngamide E R = H, $\Delta^{5',6'}$ **(8)**



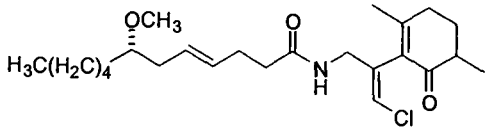
Malyngamide P **(10)**



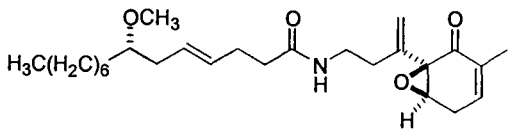
Malyngamide F R = H **(11)**



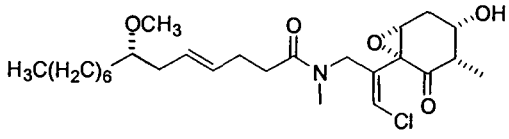
Malyngamide Q R = H **(12)**
Malyngamide R R = CH_3 **(13)**



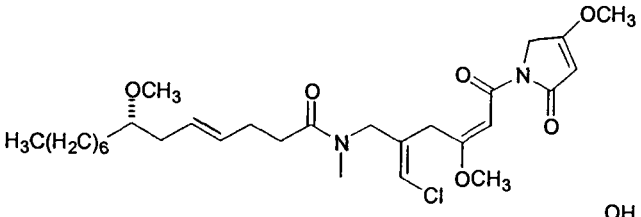
Malyngamide G **(14)**



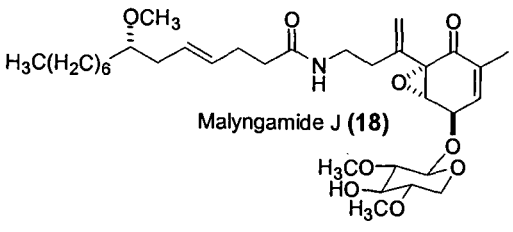
Malyngamide H **(15)**



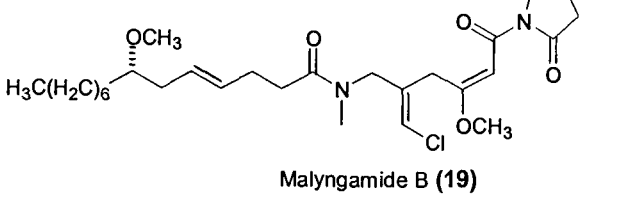
Malyngamide I **(16)**



Malyngamide A **(17)**



Malyngamide J **(18)**



Malyngamide B **(19)**

Figure V.1. Representative examples of the known malyngamides

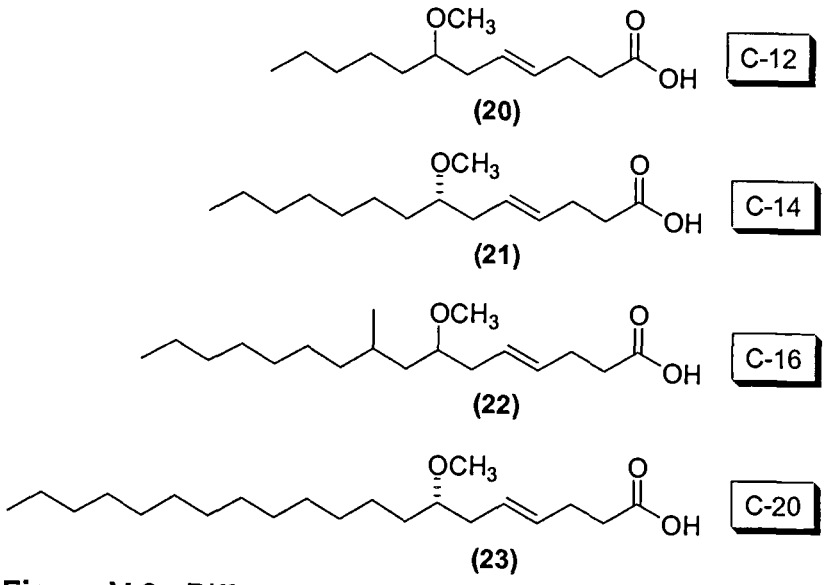


Figure V.2. Different lyngbic acids present in malyngamides

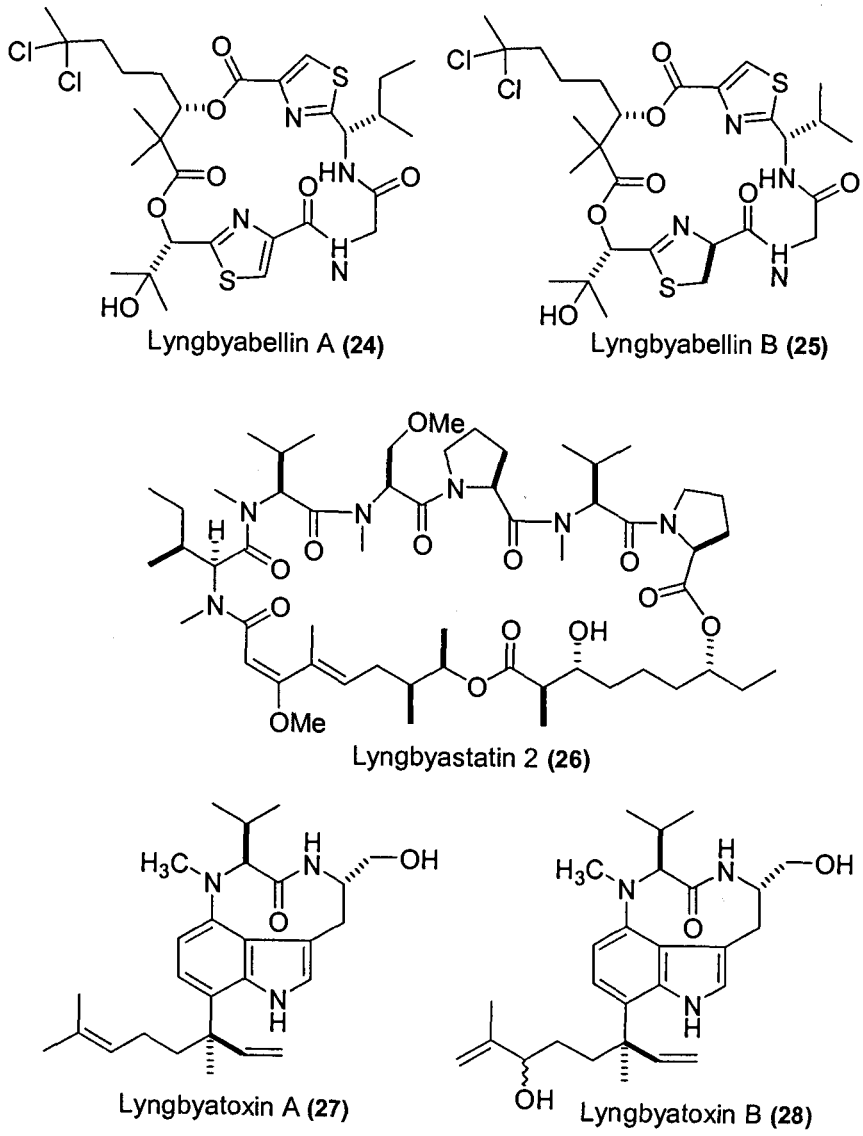
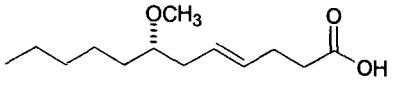
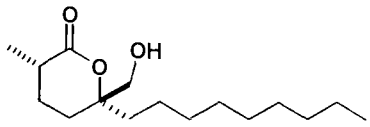


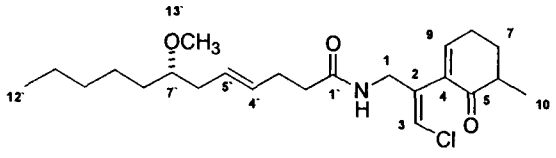
Figure V.3. Bioactive peptides from *Lyngbya majuscula*



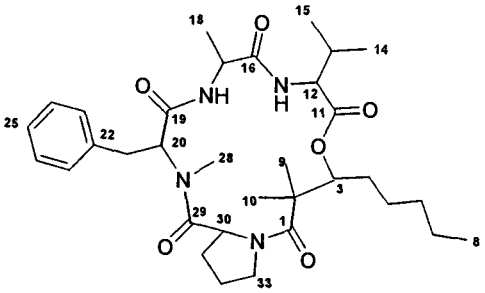
(-)-C-12 Lyngbic acid (**20**)



(-)-Malyngolide (**29**)



Malyngamide X (**30**)



Floridamide (**31**)

RESULTS AND DISCUSSION

A collection of *L. majuscula* (active in anti-sirt2 assay) was obtained from Key West, Florida in November 1995, was extracted with 2:1 CH_2Cl_2/MeOH and fractionated by silica vacuum liquid chromatography. Initial fractionation was accomplished by VLC (EtOAc/hexanes gradient) over silica gel. Successive reversed phase SPE and HPLC fractionations and purifications resulted in the isolation of two new compounds (**30**) and (**31**), in addition to two previously known compounds (**20** and **29**).

Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed construction of their planar structures. HMBC and MS fragmentation were used to confirm these.

Malyngamide X (**30**) was isolated as a colorless oil from the bioactive organic extract of *L. majuscula* (anti-sirt2 assay). The isotope pattern observed for the molecular ion (FAB) indicated the presence of one chlorine atom, while HRFABMS established a molecular formula of $C_{23}H_{37}ClNO_3$, and therefore possessed six degrees of unsaturation. The IR spectrum of **30** showed absorption bands at 3301 and 1675 cm^{-1}, indicating the presence of an amide group functionality. Inspection of the ^1H NMR spectrum indicated the presence of an olefinic proton triplet signal at δ 6.87 (H-9). Another sharp singlet at δ 6.18, indicative to an olefinic proton attached to an olefinic carbon with a chlorine atom (H-3), was detected. Additionally a broad triplet at δ 6.02 for an amide proton coupled with methylene group protons, was observed. Another two doublet of doublets at δ 4.2 and 4.06 ($H1_a$ and $H1_b$) were seen. A sharp singlet at 3.31 was recognized, indicating the presence of one methoxy group (C-13`). Moreover a sharp triplet appeared at δ 3.14, equivalent to a proton attached to a carbon carrying oxygen (H-7`). A sharp doublet was observed at δ 1.16, indicating the presence of a methyl group attached to another methine group. Finally, a terminal methyl triplet (δ 0.89) was observed (H_3-12). The ^{13}C NMR spectrum in $CDCl_3$ confirmed the presence of twenty-three carbon atoms. Analysis of ^{13}C NMR, DEPT135 and DEPT90 data, revealed the presence of one ketone resonance at δ 202.4 (C-5), an amide resonance at δ 172.4 (C-1`), six olefinc carbons (δ 149.0, 138.7, 138.4, 131, 127 and 120.1), ten methylene groups (δ 39.4, 31.0, 36.9, 36.8, 33.74, 32.4, 29.1, 26.12, 25.37, 23.9), two aliphatic methine groups (81.1 and 42.5) and also three methyl groups (56.9, 15.48 and 14.5). This was in keeping with five

degrees of the six degrees of unsaturation required by the molecular formula and comfirmed the need for one ring to fit the six degrees of unsaturations.

Chemical shift arguments, ^{1}H-^{1}H COSY and TOCSY correlations supported by MS data and HMBC allowed the assignment of the planar structure of **30** (Figure V.2). From the ^{1}H-^{1}H COSY NMR spectrum of **30**, it was possible to differentiate three discrete spin systems. A continuous spin system was evident in which a broad amide proton triplet (δ 6.02) was coupled to two mutually coupled midfield methylene resonances (δ 4.2 and 4.0 H-1a and H-1b), which in turn, showed couplings to an olefinic ^{1}H resonance (δ 6.1, H-3). The proton signal at δ 6.8 (H-9) was coupled to the proton at δ 2.4 (H-7) which by itself coupled to the methylene proton at δ 2.0 (H-7). The latter proton was found to couple with the methine proton at δ 2.4 (H-6). This completed the second spin system.

The third spin system was the C-12 chain of lyngbic acid. This spin system starts from the terminal methyl protons at δ 0.88 (H-12`) which were found to couple with the methylene group at δ 1.23 (H-11`). The latter proton signal was coupled to the proton at δ 1.22 (H-10`), which by itself, coupled to the methylene proton at δ 1.35 (H-9`). The H-9` proton was found to couple with the methine proton at δ 1.6 (H-8`), and H-8` coupled to a proton at δ 3.12 attached to a methine carbon carrying the methoxy group (H-7`). The latter proton signal was coupled to the proton at δ 2.15 (H-6`). The proton at δ 2.15 (H-6`) was found to couple with the olefinic proton at δ 5.4 (H-5`), and (H-5`) was found to couple with (H-4`) and (H-3`), and this completed the third spin system. TOCSY was carried out to confirm these connections.

HMBC correlations from δ 4.20 (H-1) to C-2, C-3, and C-4 combined with HMBC correlations from the methyl singlet at δ 1.12 (H-10) to C-5, C-5, and C-7 established the C-3/C-4/C-5/C-9 fragment of **30**. HMBC correlations from H-1 at δ 4.20 to C-1`, established the C-1/N-H/C-1` second fragment of **30.** Final securing of the cyclohexene ring structure was made by placement of the carbonyl functional group at C-5 due to the observation of HMBC correlations from the methyl group resonance at δ 1.12 to C-5, C-7, and C-8. The C-2 (δ 138.7)/C-3 (δ 120.1) double bond was determined as being terminal with the chlorine at C-3 by HMBC correlations from H-4 to C-1 (δ 39.4), C-2, and C-3. ^{13}C NMR and 2D-NMR established the fatty acid as a 12-carbon chain with unsaturation at the 4'-position. Placement of the methoxy group (δ_H 3.29 and δ_C 56.9) was made by virtue of an HMBC correlation from the methoxy proton singlet to C-7' (δ 81.1). HMBC correlations from H-3 to C-1, C-2, and C-9 confirmed the connectivity

deduced from COSY data. H-3 also showed an HMBC correlation to C-4, thereby completing the cyclohexene ring. Similarly, HMBC correlations from CH_3-10 to C-5, C-6, and C-7 positioned the methyl group (δ 1.12) at C-6. Strong HMBC correlations from δ 6.1 (H-3) to C-1 confirmed the stereochemistry of the vinyl chloride group to be *E.*

Table V.1. ^{1}H and ^{13}C NMR Data of malyngamide X (**30**) in $CDCl_3$[a]

Position	^{1}H mult *J* (Hz)	^{13}C	COSY	HMBC[b]
1_a	4.2 (dd, 4.9, 6.0)	39.4 CH_2	H-3, N-H	C-2, C-3, C-4, C-1`
1_b	4.0 (dd, 4.9, 6.0)		H-3, N-H	
2		138.7 C		
3	6.1 (s)	120.1 CH	H-1	C-1, C-2, C-4
4		138.4 C		
5		202.4 C		
6	2.4 (m)	42.5 CH	H-7	C-7, C-5
7a	2.0	31.0 CH_2	H-6, H-8	C-6, C-8
7_b	1.7		H-6, H-8	
8_a	2.4 (m)	26.1 CH_2	H-7, H-9	C-6, C-7, C-9, C-10
8_b	1.8 (m)		H-7, H-9	
9	6.8 (m)	14.9 CH	H-8	C-5, C-8, C-4
10	1.12	14.6 CH_3		C-5, C-6, C-7, C-8
N-H	6.02 (brt, 6.0)			
1'		172.4 C		
2'	1.6 (m)	25.4 CH_2		C-1`
3'	2.2 (m)	29.1 CH_2	H-4`	
4'	5.4 (m)	127.0 CH	H-3`, H-5`	C-5', C-3'
5'	5.4 (m)	131.0 CH	H-4`, H-6`	C-6`, C-4`
6'	2.15 (m)	36.9 CH_2	H-5`, H-7`	C-5`, C-7`
7'	3.12 (m)	81.1 CH	H-6`, H-8`	C-6`, C-8`, C-12`
8'	1.60 (m)	36.8 CH_2	H-7`	C-9`, C-10
9'	1.35 (m)	33.7 CH_2		C-8, C-10
10'	1.23 (m)	32.4 CH_2		C-9
11'	1.22 (m)	23.9 CH_2	H-12`	C-9, C12
12'	0.88 (t, 6.8)	14.5 CH_3	H-11`	C-11
13'	3.29 (s)	56.9 CH_3		C-7`

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

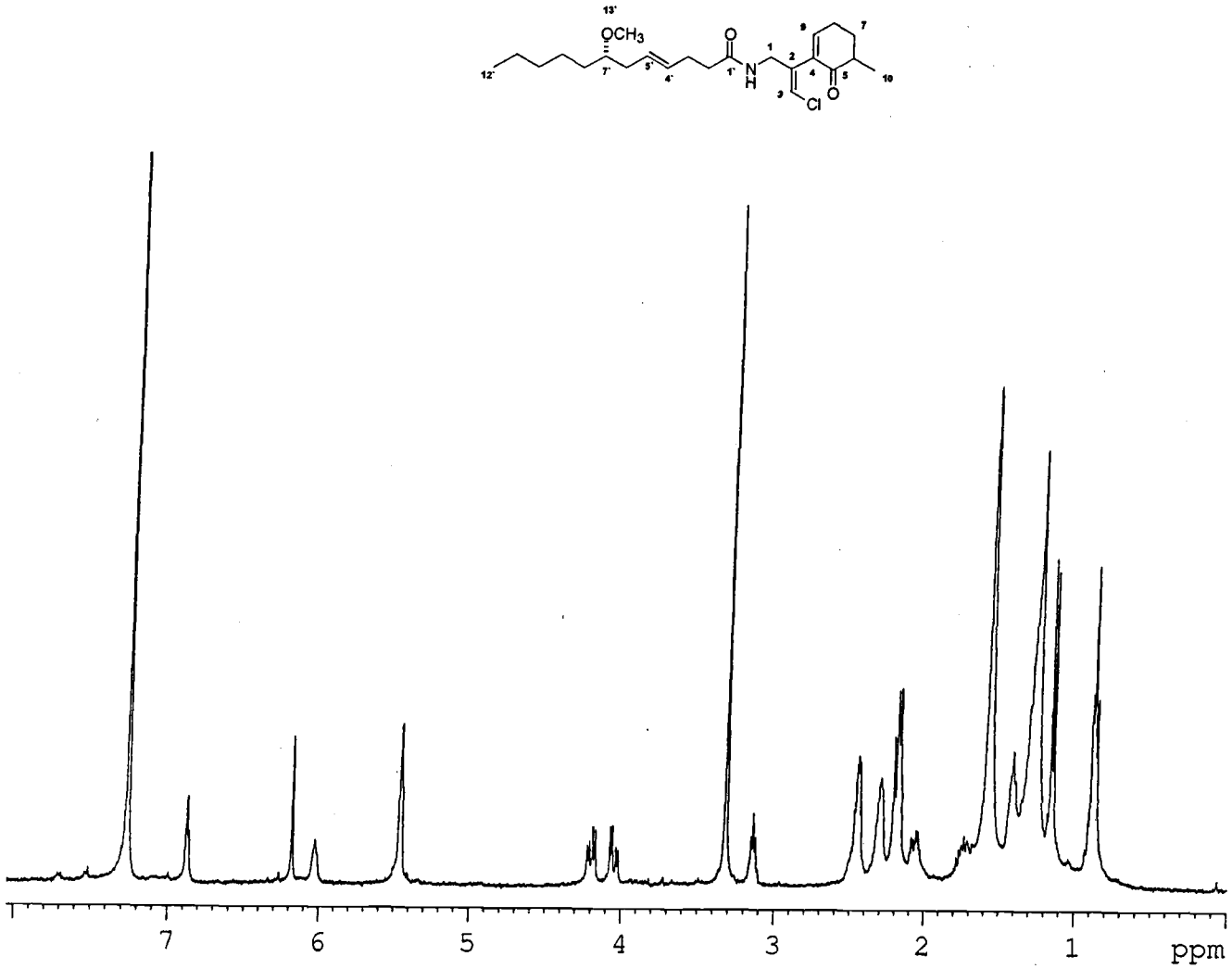


Figure V.4. ^{1}H NMR spectrum of compound **30** in $CDCl_3$

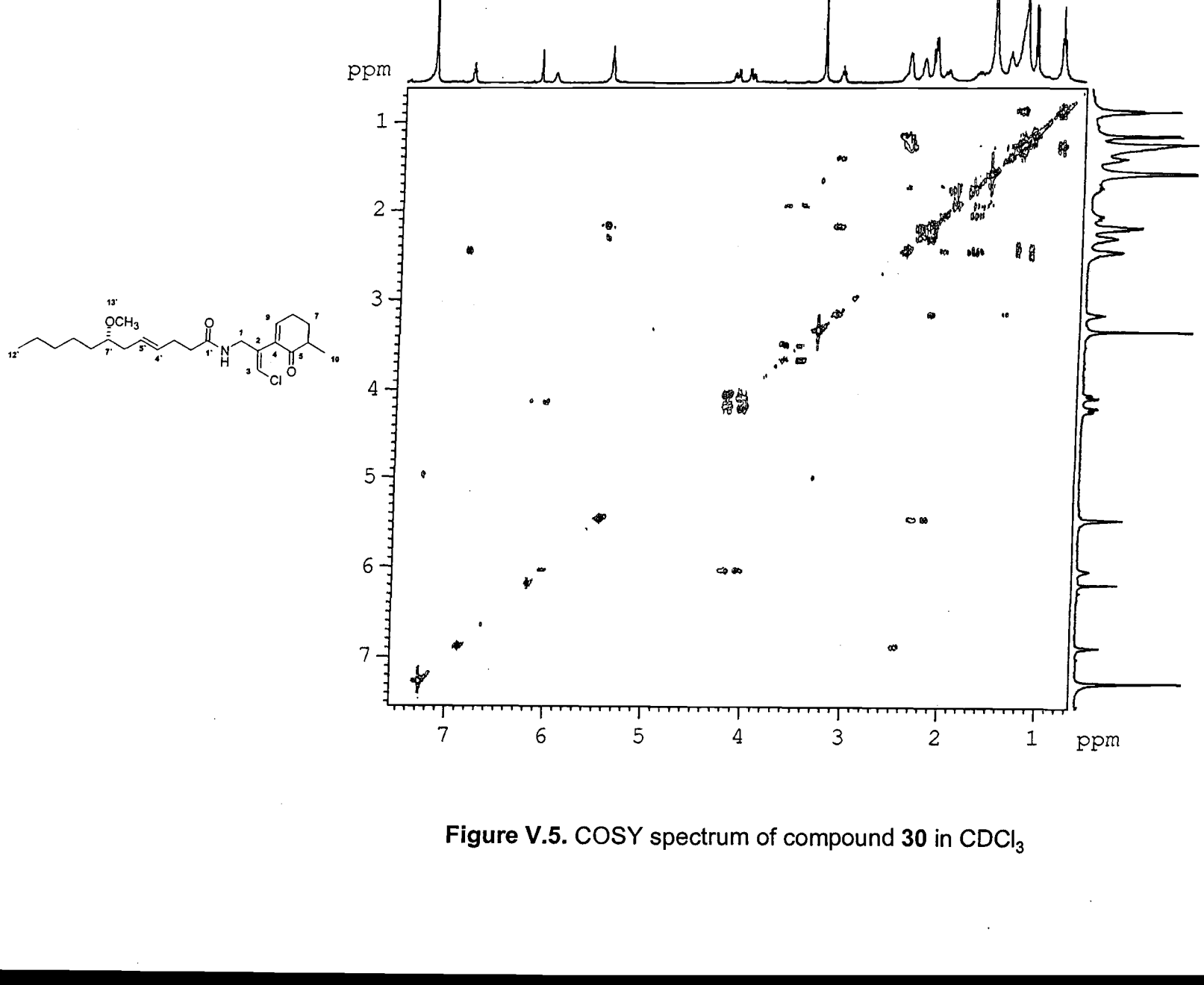


Figure V.5. COSY spectrum of compound **30** in $CDCl_3$

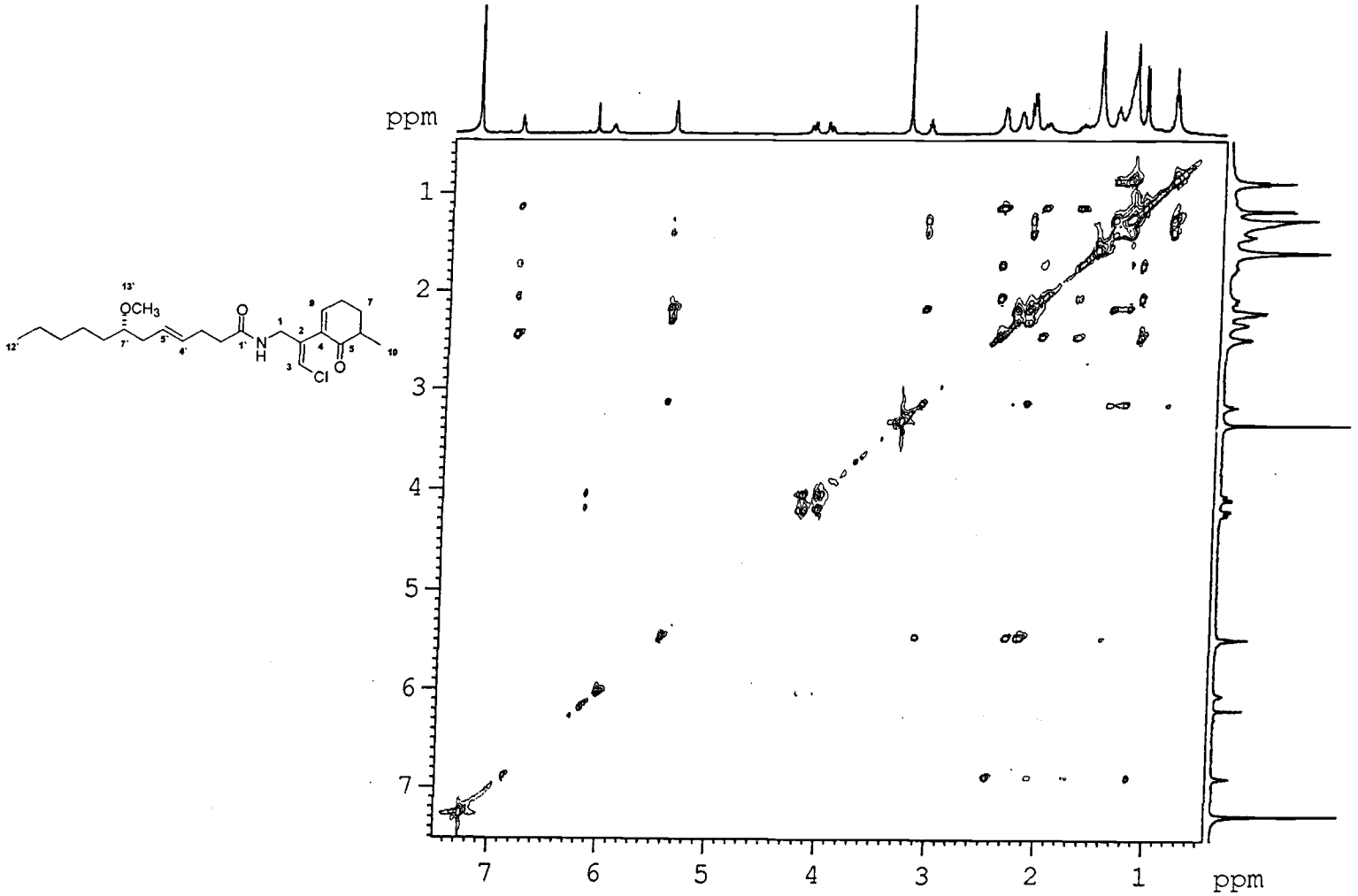


Figure V.6. TOCSY spectrum of compound **30** in $CDCl_3$

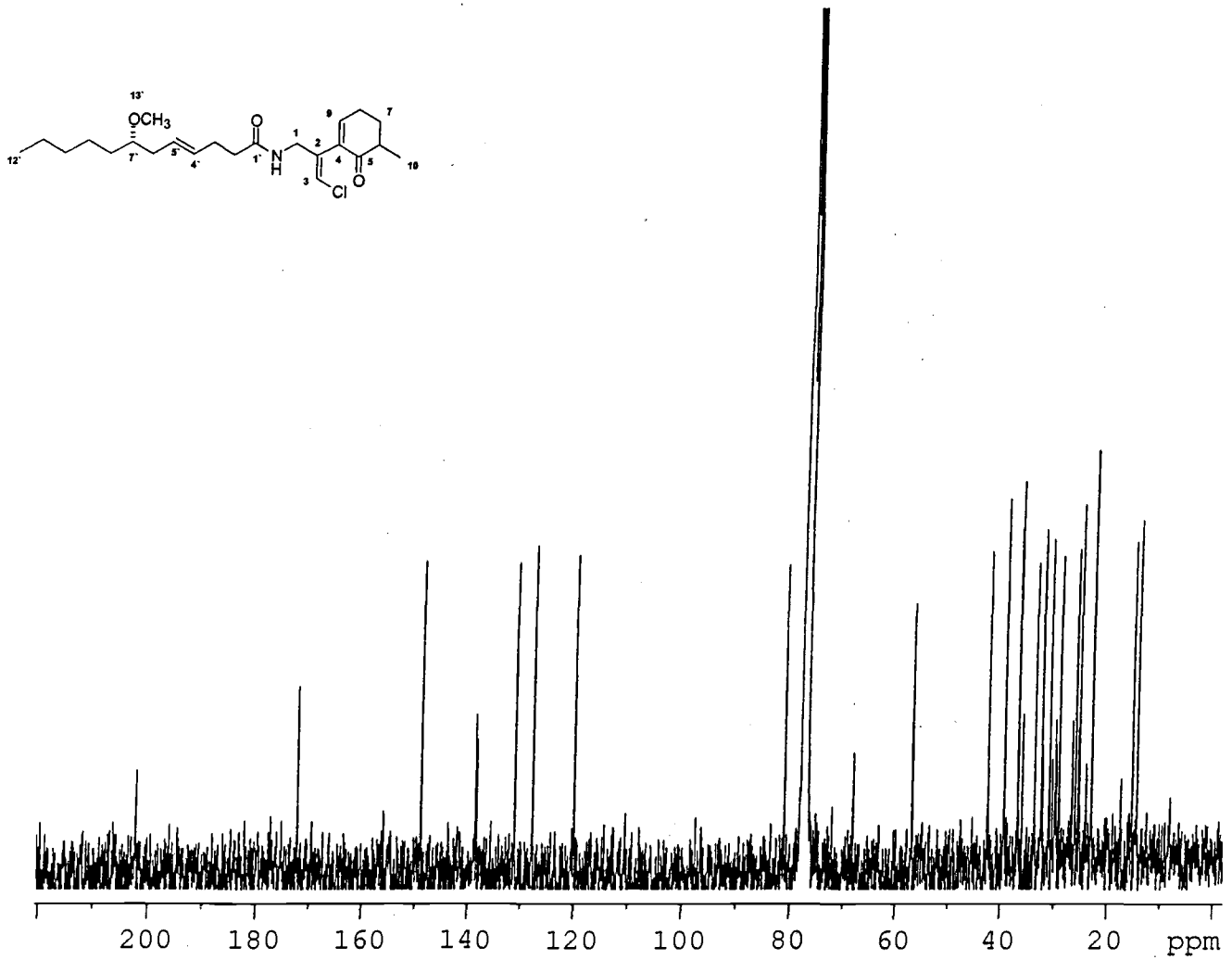


Figure V.7. ^{13}C NMR spectrum of compound **30** in $CDCl_3$

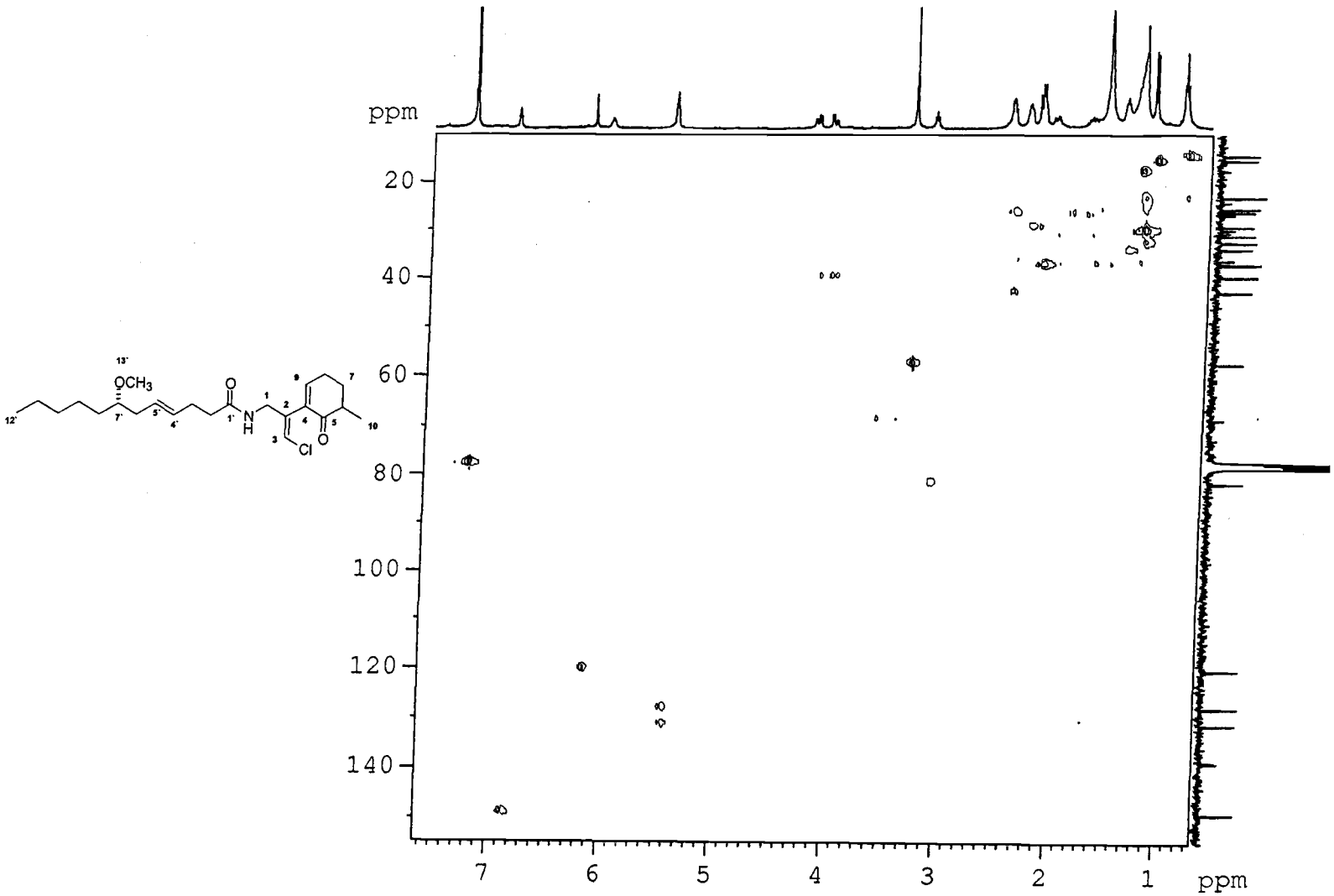


Figure V.8. HSQC spectrum of compound **30** in $CDCl_3$

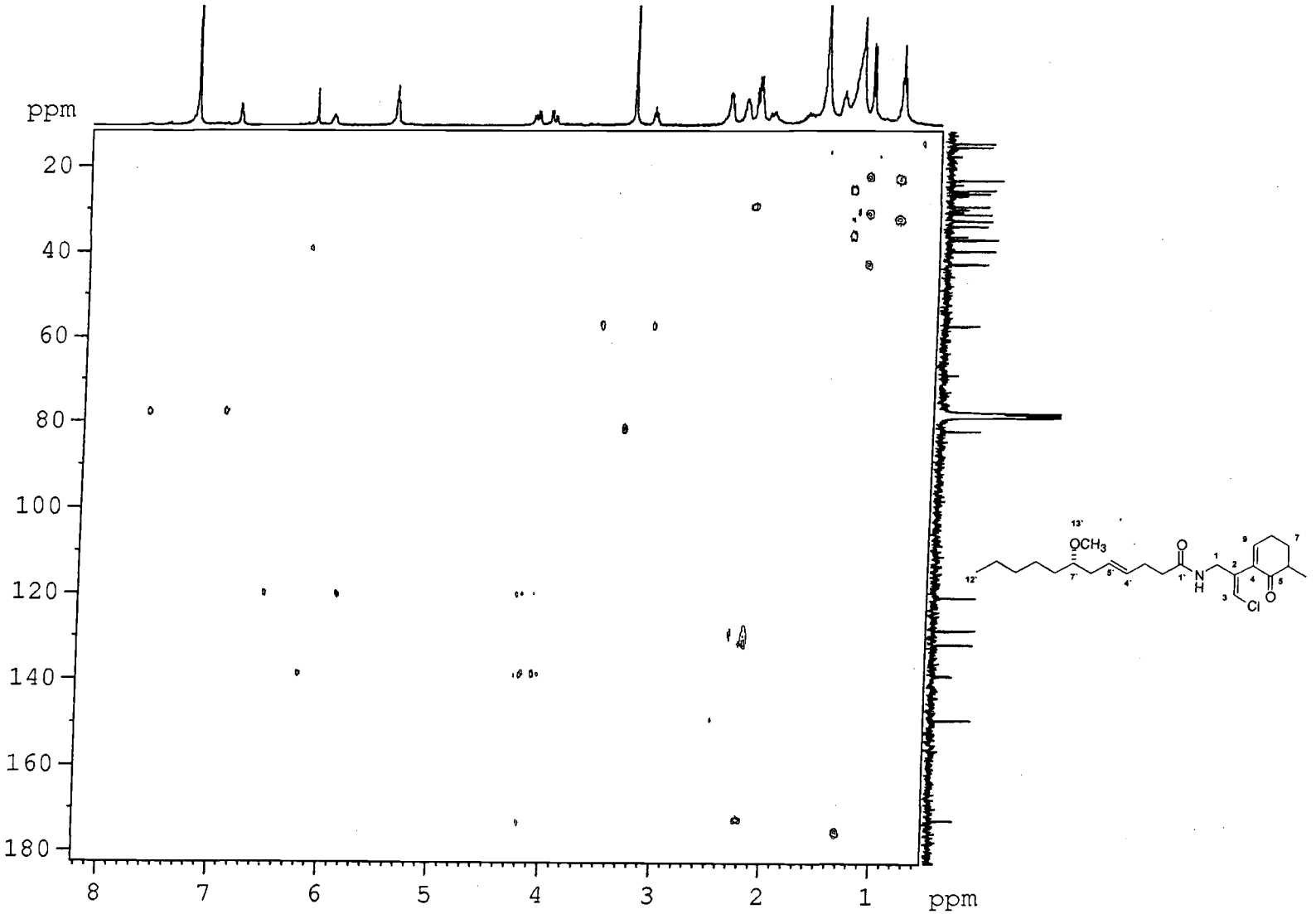


Figure V.9. HMBC spectrum of compound **30** in $CDCl_3$

Floridamide (**31**) was isolated as a colorless oil with a molecular formula of $C_{33}H_{50}N_4O_6$ as determined by ESIMS (observed $[M+Na]^+$ at *m/z* 621.9). The IR spectrum of floridamide (**31**) gave characteristic absorption bands at 3293, 1734, 1650, 1625 cm^{-1}, indicative of ester/amide carbonyl functionalities. Of the 11 degrees of unsaturation inherent to the molecular formula, four could be accounted for by a phenyl group as suggested in the ^1H NMR spectrum. In addition, the peptidic nature of **31** was indicated by exchangeable *N*H protons resonating at δ 8.6 and δ 6.7. One distinct *N*CH_3 proton singlet was also observed in the ^1H NMR data at δ 2.95. Two other high field CH_3 proton singlets were also observed at δ 0.92 and δ 1.29. Thirty-three carbon signals were observed in the ^{13}C NMR data of floridamide (**31**), which include characteristic low field aromatic carbon signals suggesting a mono-substituted phenyl group as well as five signals belonging to amide/ester carbonyls in the 169-173 ppm range. One oxygenated sp^3 carbon resonating at δ 77.9 was also detected in the HSQC spectrum.

From 1D and 2D NMR data, including HMBC and TOCSY, I noticed the presence of two conformers of this polypeptide in a ratio of 2:1 was noted. In this work I focused on the data of the major conformer. Five substructures were assembled for floridamide (**31**), including four amino acids (*N*-MePhe, Pro, Val and Ala) and one hydroxy acid, 2,2-dimethyl-3-hydroxy-octanoic acid (Dhoaa). The latter hydroxy acid, Dhoaa, is a unique unit previously reported from cyanobacterial depsipeptides and was deduced solely from HMBC and TOCSY data. The sequence of these five moieties in floridamide (**31**) was established mainly from CIMS (Figure V. 14) and HMBC correlations (Figure V. 15). Sequential HMBC correlations in CD_2Cl_2 were observed between H-20, H-28, H-30/C-29; H-17, H-20, *N*H (δ 6.7)/C-19; H-12, H-3/C-11; H-3, H-9, H-10, H-30/C-1 and H-17, H-12, *N*H (δ 8.6)/C-16 which gave rise to the Pro/*N*-MePhe/Ala/Val/Dhoaa sequence.

Absolute stereochemistry of *floridamide* (**31**) is not determined yet because of the limited time. This absolute stereochemistry could be carried out by ozonolysis followed by acid hydrolysis and analysis by Marfey's method[127] as well as chiral GC-MS. Acid hydrolysis and derivatization with Marfey's reagent could be followed by comparative HPLC analysis with derivatized standard *D*- and *L*-amino acids.

Table V. 2. NMR spectral data for floridamide (**31**) at 400 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$.[a]

Position	^{1}H	mult	*J* (Hz)	^{13}C	HMBC[b]
			2,2-dimethyl-3-hydroxy-octanoic acid (Dhoaa)		
1				169.3 C	
2				44.7 C	
3	5.13	dd	7.0, 5.5	77.2 CH	1, 4, 11
4	2.13	m		29.0 CH_2	3, 5
5	1.77	m		27.0 CH_2	4, 6
6	1.38	m		23.4 CH_2	5, 7
7	1.00	m		22.9 CH_2	8
8	0.88	t	7.0	14.3 CH_3	7
9	0.92	s		16.4 CH_3	2, 3, 11
10	1.26	s		20.8 CH_3	2, 3, 11
			Val		
11				173.3 C	
12	4.14	d	11.0	52.5 CH	11, 13, 16
13	2.7	m		35.1 CH	12, 14, 15
14	1.26	d	6.7	14.5 CH_3	13
15	1.44	d	6.4	12.3 CH_3	13
(*N*-H)	8.60	s			12, 16
			Ala		
16				172.0 C	
17	4.76	m		51.8 CH	16, 18, 19
18	1.36	d	7.2	23.4 CH3	17
*N*H	6.7 d	brd	5.0		17, 19
			***N*-MePhe**		
19				172.9 C	
20	5.7	dd	12.1, 4.8	57.5 CH	19, 21, 29
21	3.45	dd	15.0, 5.0	33.8 CH_2	20, 22
	2.95	m			
22				137.0 CH	
23/27	7.15	m		128.6 CH	
24/26	7.21	m		129.0 CH	
25	7.16	m		126.7 CH	
28 (*N*-CH_3)	2.95	s		31.7 CH_3	20, 29
			Pro		
29				169.0 C	
30	4.40	m		58.5 CH	29, 31, 1
31	2.10	m		30.1 CH_2	30, 32
	1. 87	m			
32	1.20	m		22.9 CH_2	31, 33
	1.04	m			
33	3.60	m		47.0 CH_2	32, 1
	3.50	m			

[a] Spectral data reported in ppm.
[b] Optimized for 6 Hz.

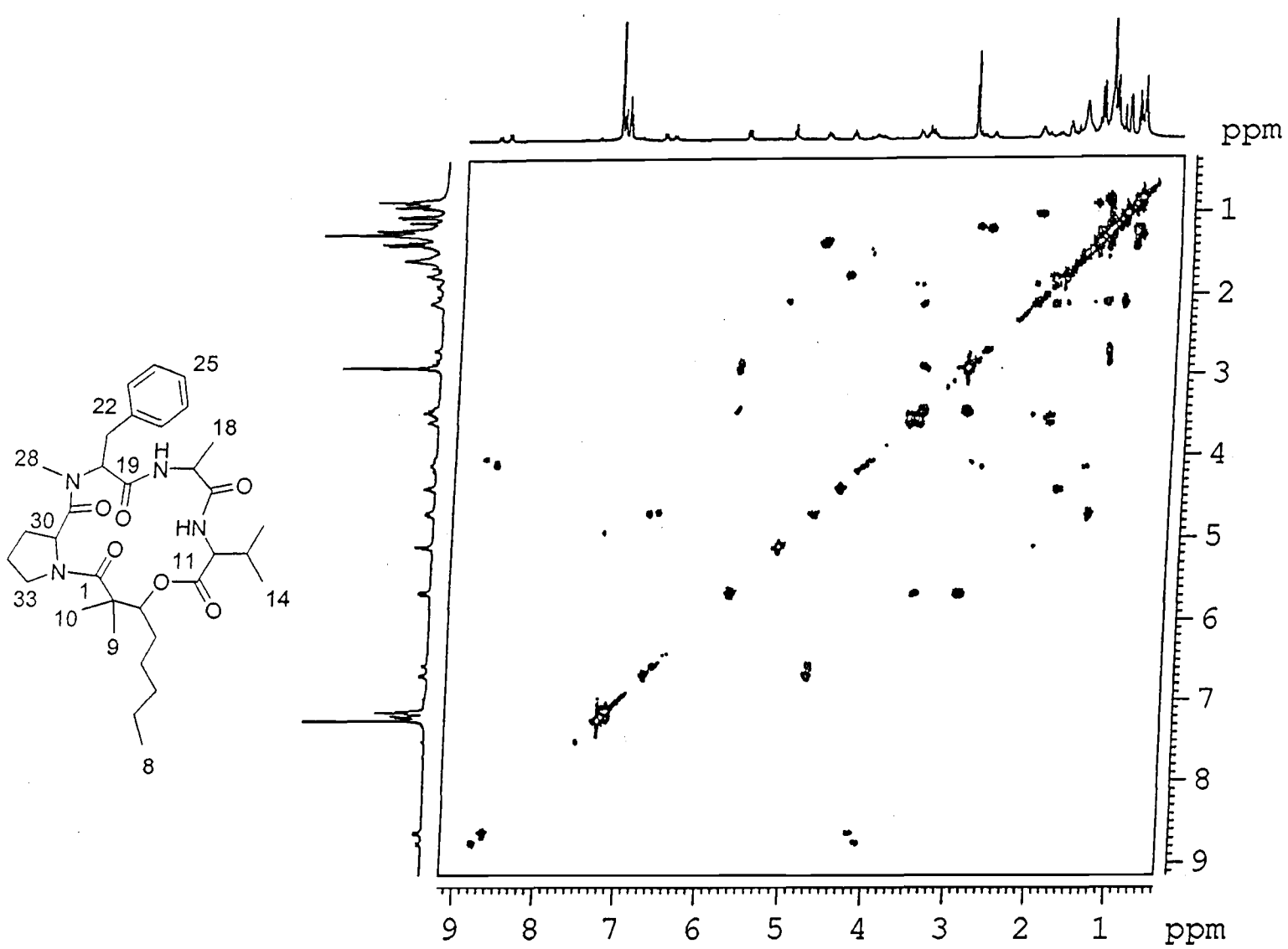
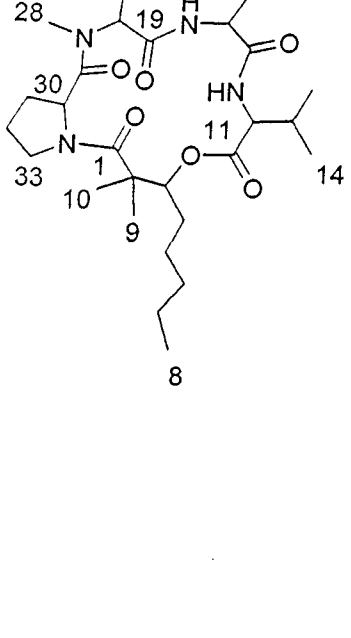


Figure V.10. COSY spectrum of compound **31** in $CDCl_3$



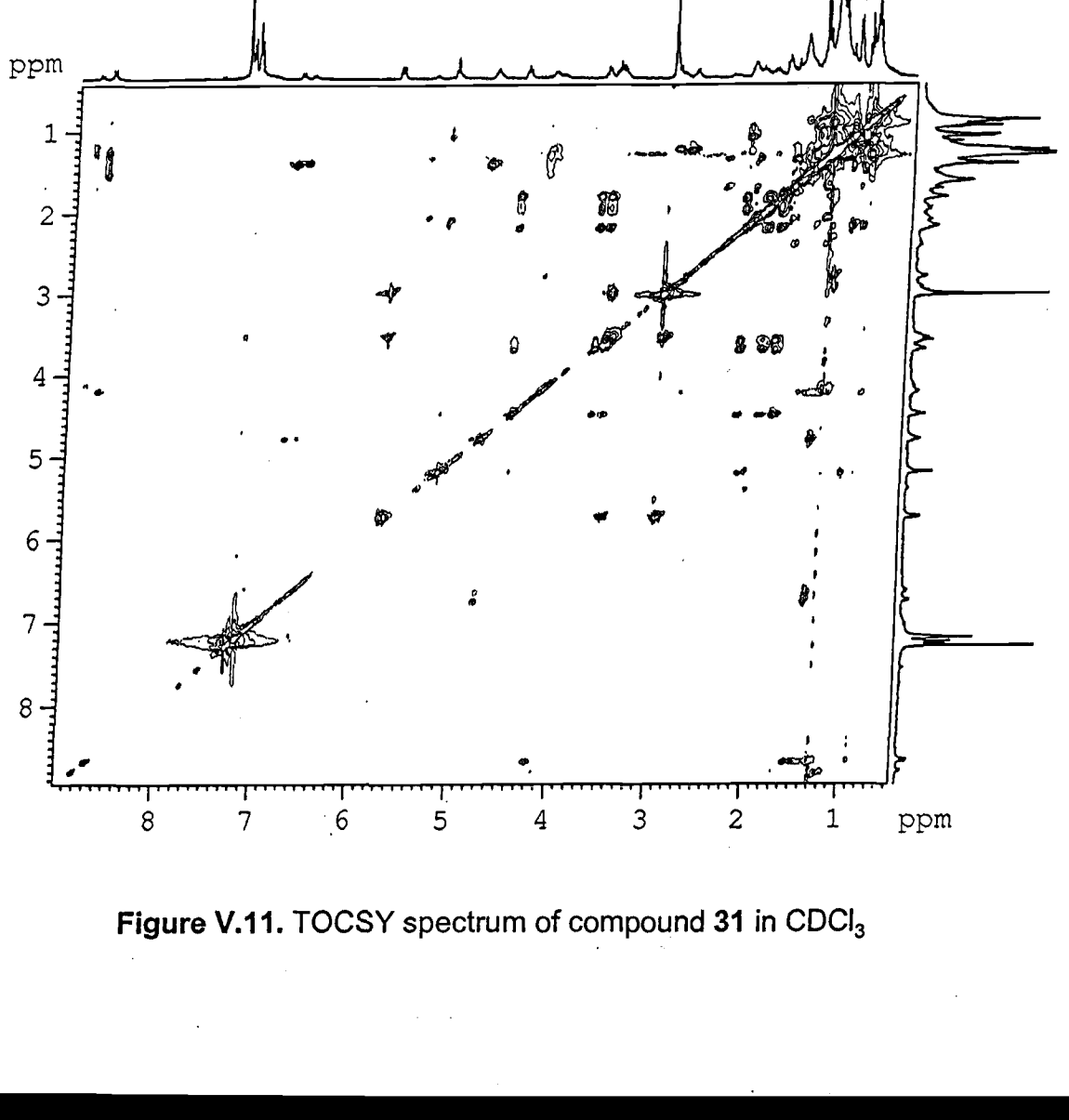


Figure V.11. TOCSY spectrum of compound **31** in $CDCl_3$

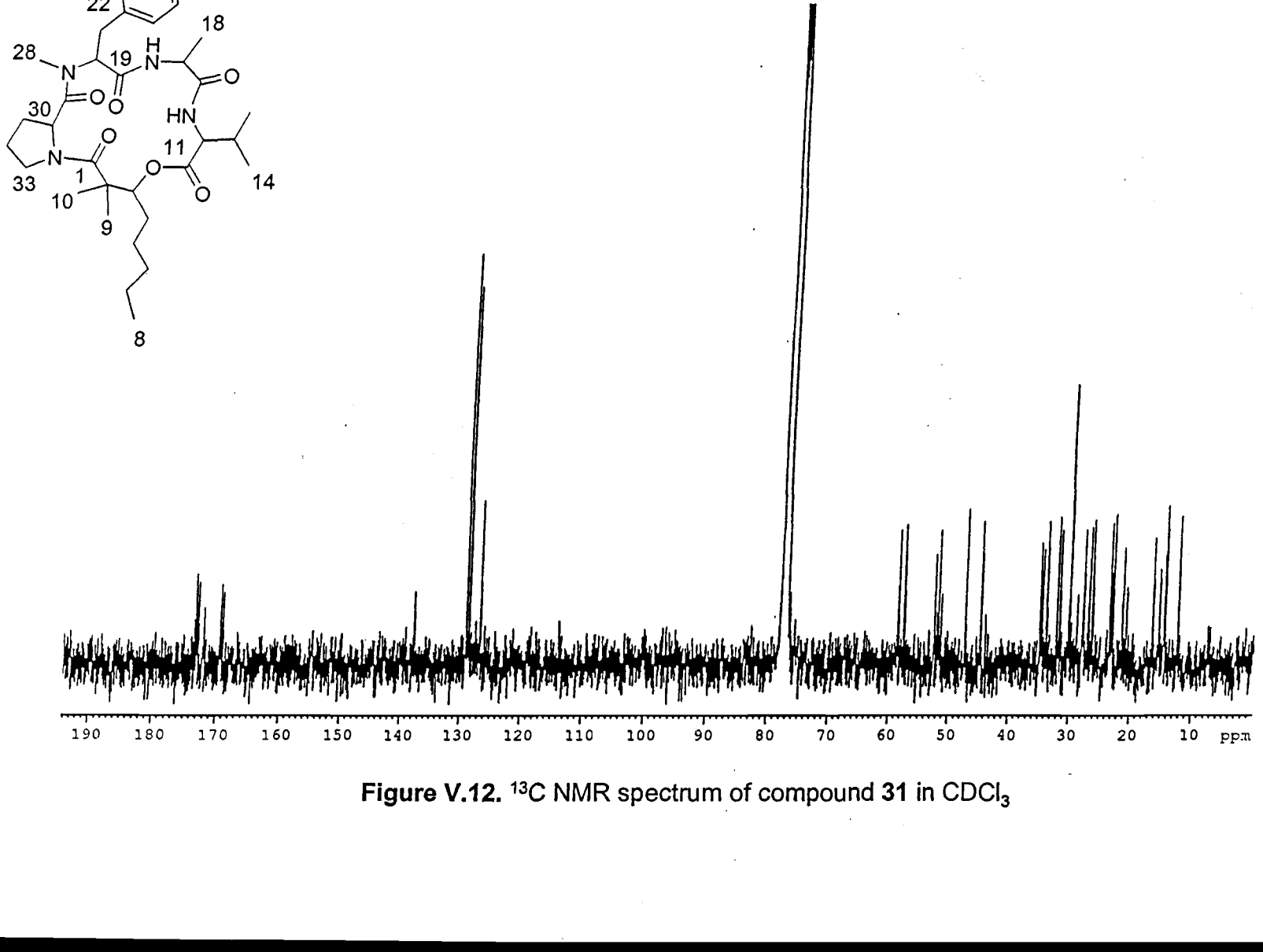


Figure V.12. ^{13}C NMR spectrum of compound **31** in $CDCl_3$

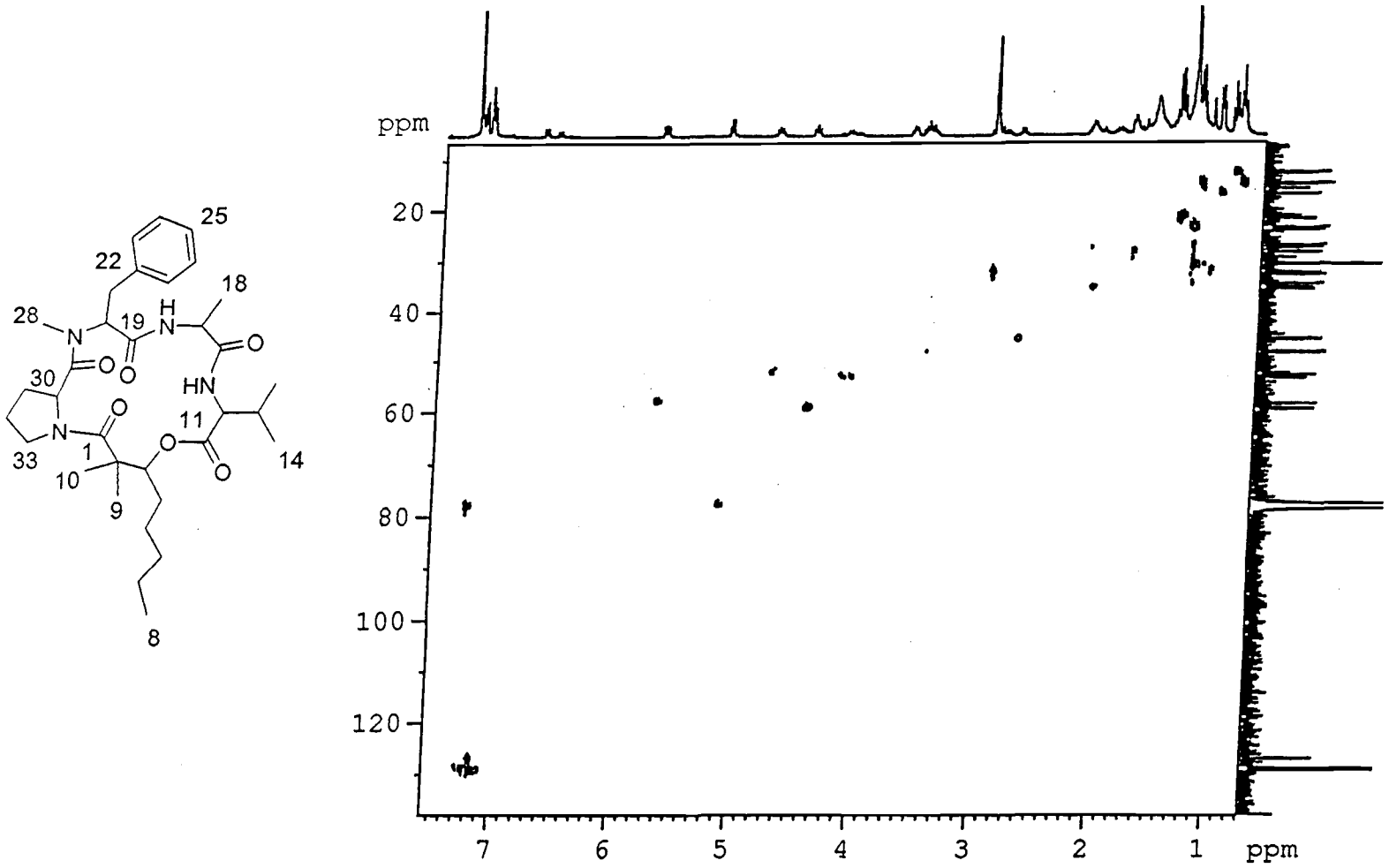


Figure V.13. HSQC spectrum of compound **31** in $CDCl_3$

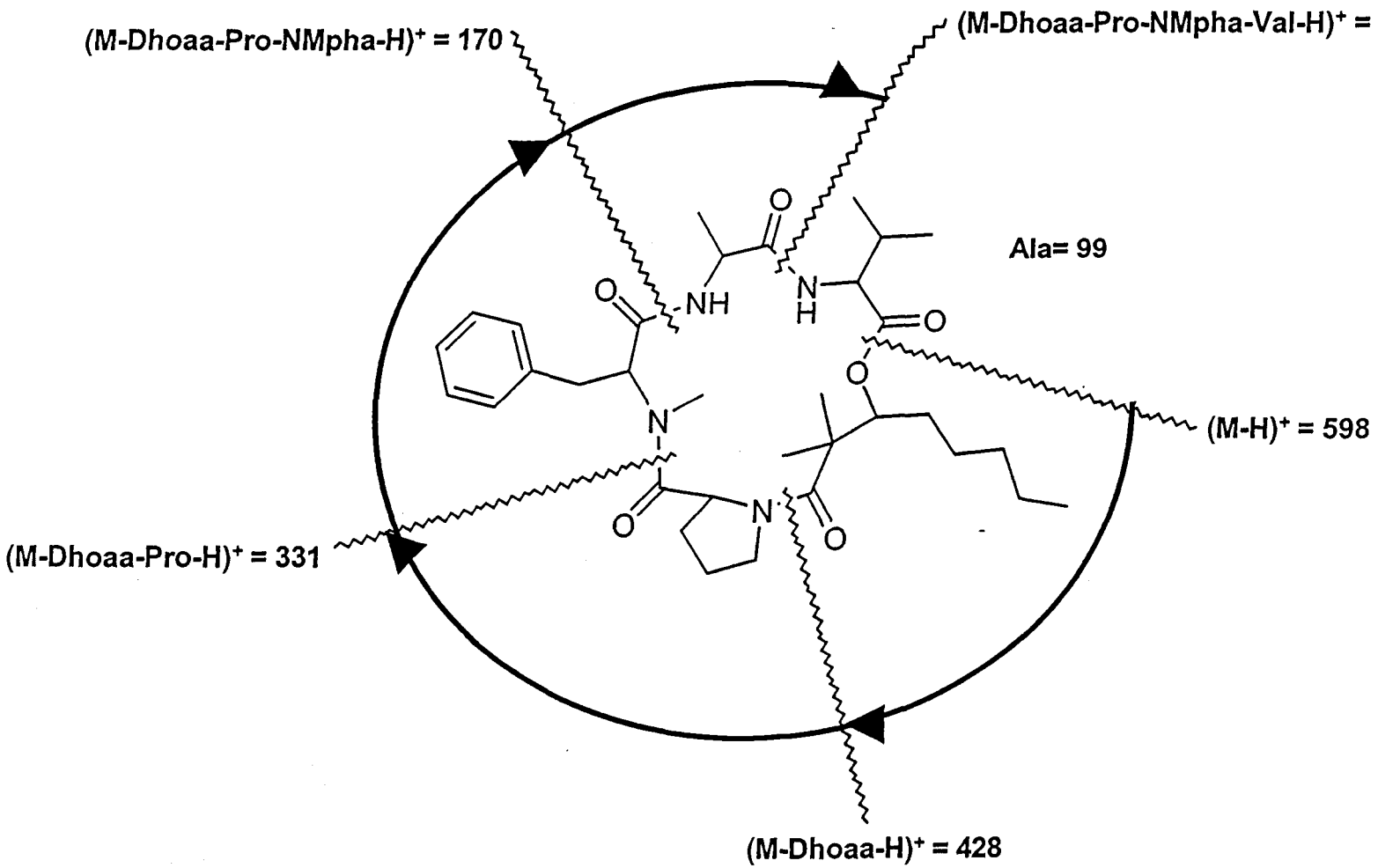


Figure V.14 CIMS fragmentation observed for floridamide (**31**).

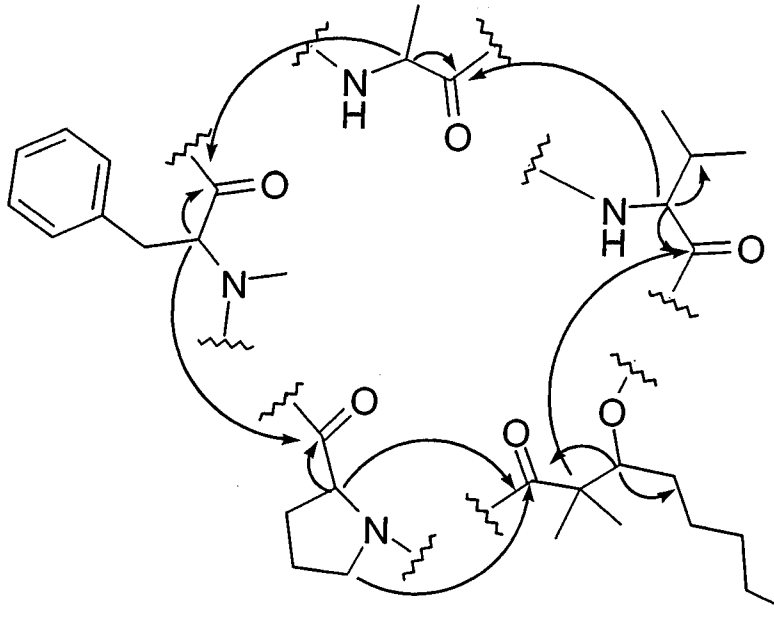


Figure V.15. Partial structures of **31** connected by HMBC correlations.

The isolated compounds were evaluated for their biological activity in several systems. Malyngamide X (**30**) was found to have cytotoxic activity (EC_{50} = 6 μg/ml) to a human lung cancer cell line (NCI-H460) and the mouse neuro-2a neuroblastoma cell line. However, floridamide (**31**) was found to have weak cytotoxic activity (EC_{50} = 10 μg/ml) in the latter assays. Upon testing these compounds in a sodium channel modulation assay, none of the compounds were found to have blocking or activating activity.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. NMR spectra were recorded on a Bruker DPX400 spectrometer, with the solvent ($CDCl_3$ at δ_C 77.2, δ_H 7.26) used as an internal standard. Mass spectra were recorded on a Kratos MS50TC mass spectrometer, and HPLC isolations were performed using Waters Millipore model 515 pumps and a Waters 969 diode array detector.

Cyanobacterial Collection. The marine cyanobacterium *Lyngbya majuscula* (voucher specimen available as collection number KWN-18/NOV/95-01) was collected by hand using SCUBA in Key West Florida, USA, on November 18, 1995. The material was stored in 2-propanol at -20 °C until extraction.

Extraction and Isolation. Approximately 40 g dry weight of the cyanobacterium was extracted repeatedly with 2:1 CH_2Cl_2/MeOH to produce 0.99 g of crude organic extract. A portion of the extract (0.97 g) was then fractionated by silica gel vacuum liquid chromatography. The fractions eluting with 60% EtOAc in hexanes was further purified with a C_{18} solid phase extraction (SPE) cartridge (8:2 MeOH/H_2O) and reversed-phase HPLC (9:1 CH_3OH/H_2O, Phenomenex Spheroclone 5 μ ODS) to yield 2.4 mg of C-12 lyngbic acid (**20**), 1.3 mg of Malyngamide X (**30**) and 1.0 mg of malyngolide (**29**). A second fraction eluting with 80% EtOAc in hexanes was further purified with a C_{18} solid phase extraction (SPE) cartridge (8:2 MeOH/H_2O) and reversed-phase HPLC (9:1 CH_3OH/H_2O, Phenomenex Sphereclone 5 μ ODS) to yield 2.0 mg of floridamide (**31**).

Malyngamide X (**30**): colorless oil; $[\delta]^{25}_D$ +14 ^{0} (c 0.09, $CHCl_3$); UV (MeOH) λ_{max} 250 (ε = 1729); IR ν_{max} (film) 3301, 2928, 2859, 1675, 1537, 1455, 1370, 1095 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, see Table 1; HRFABMS (3-NBA) obsd $[M + H]^+$ *m/z* 410.2467 (calcd for $C_{23}H_{37}ClNO_3$, 410.2462).

Floridamide (**31**): colorless oil; $[\delta]^{25}_D$ +56 ^{0} (c 0.1, $CHCl_3$); UV (MeOH) λ_{max} , 220 (log ε = 5.467), 278 (log ε = 4.453); IR ν_{max} (film) 3293, 2924, 2854, 2360, 2337, 1734, 1650, 1625, 1510, 1458, 1175 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, see Table 2; HRFABMS $[M + H]^+$ *m/z* 599.3815 (calcd for $C_{33}H_{51}N_4O_6$ 599.3808).

(-)-**Malyngolide (29:)** colorless oil, $[\delta]^{25}_{D}$ - 10 ° (c 0.10, $CHCl_3$), literature value - 12.0 °;[1] UV, IR, ^1H, ^{13}C NMR, and MS data were similar to literature.

(-)-**C-12 lyngbic acid (20)**: colorless oil; $[\delta]^{25}_{D}$ – 8 ^{0} (c 0.1, $CHCl_3$); (literature - 4.4); with remaining physical and spectroscopic properties identical to those previously reported.

Cytotoxicity against NCI-H460 human lung cancer and neuro-2a neuroblastoma cell line.[13] Cytotoxicity was measured to NCI-H460 human lung tumor cells and mouse neuro-2a blastoma cells using the method of Alley et. al.[27] with cell viability being determined by MTT reduction. Cells were seeded in 96-well plates at 5000 and 8000 cells/well in 180 µl for H460 and neuro-2a cells, respectively. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µg/well. DMSO was less than 1% final concentration. After 48 hours, the medium was removed and cell viability determined.

Sodium channel modulation.[14] Isolated compounds were evaluated for their capacity to either activate or block sodium channels using the following modifications to the cell-based bioassay of Manger et. al.[28] Twenty-four hours prior to chemical testing, mouse neuro-2a blastoma cells were seeded in 96-well plates at 8 X 10^4 cells/well in a volume of 200 µl. Test chemicals dissolved in DMSO were serially diluted in medium without fetal bovine serum and added at 10 µl/well. DMSO was less than 1% final concentration. Plates to evaluate sodium channel activating activity received 20 µl/well of either a mixture of 3 mM ouabain and 0.3 mM veratridine (Sigma Chemical Co.) in 5 mM HCl in addition to the test chemical. Plates were incubated for 18 hr and results compared to similarly treated solvent controls with 10 µl medium added in lieu of the test chemical. The sodium channel activator brevetoxin PbTx-1 (Calbiochem) was used as the positive control and added at 10 ng/well in 10 µl medium. Sodium channel blocking activity was assessed in a similar manner except that ouabain and veratridine were 5.0 and 0.5 mM, respectively, and the sodium channel blocker saxitoxin (Calbiochem) was used as the positive control. Plates were incubated for approximately 22 hour.

REFERENCES

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

2. Cardellina II, J. H.; Marner, F.; Moore, R. E. *J. Am. Chem. Soc.* **1978**, *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.

4. Wright, A. D.; Coll, J. C.; Price, I. R. *J. Nat. Prod.* **1990**, *53*, 845–861.

5. Mynderse, J. S.; Moore, R. E. *J. Org. Chem.* **1978**, *43*, 4359–4363.

6. Gerwick, W. H.; Reyes, S.; Alvarado, B. *Phytochemistry* **1987**, *26*, 1701–1704.

7. Praud, A.; Valls, R.; Piovetti, L.; Banaigs, B. *Tetrahedron Lett.* **1993**, *34*, 5437–5440.

8. Mesguiche, V.; Valls, R.; Piovetti, L.; Peiffer, G. *Tetrahedron Lett.* **1999**, 7473–7476.

9. Orjala, J.; Nagle, D.; Gerwick, W. H. *J. Nat. Prod.* **1995**, *58*, 764–768.

10. (a) Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611-615.
(b) Milligan, K. E.; Marquez, B. L.; Williamson, R. T.; Gerwick, W. H. *J. Nat. Prod.* **2000**, *63*, 1440-1443. (c) Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2000**, *63*, 1437-1439.

11. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 1702-1706.

12. (a) Cardellina, J. H. 2nd; Marner, F. J.; Moore, R. E. *Science* **1979**, *204*, 193-195.
(b) Fujiki, H.; Mori, M.; Nakayasu, M.; Terada, M.; Sugimura, T.; Moore, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3872-3876.
(c) Aimi, N.; Odaka, H.; Sakai, S.; Fujiki, H.; Suganuma, M.; Moore, R. E.; Patterson, G. M. L. *J. Nat. Prod.* **1990**, *53*, 1593-1596.

13. Alley, M. C.; Scudiero,D. A. *Cancer Research* **1988**, 48, 589-601.

14. Manger, R. L.; Leja. L. S. *J. AOAC Int.* **1995**, 78, 521- 27.

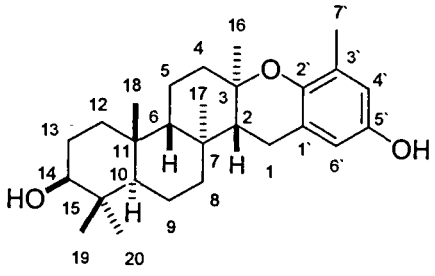
CHAPTER SIX

CONCLUSIONS

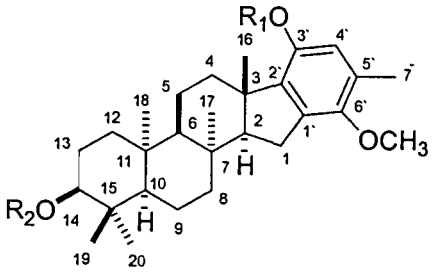
A total of four species of marine algae were examined for their bioactive chemical constituents in this thesis work. These algae are the brown alga, *Stypopodium flabelliforme* (from Papua New Guinea), the marine red alga *Plocamium cartilagineum* (collected from along the eastern coast of South Africa), the green alga *Udotea orientalis* (growing on the surface of unknown coral, from Papua New Guinea) and the marine cyanobacterium, *Lyngbya majuscula* (from Key West Florida). In addition to eleven new natural products, fourteen known compounds were identified from the organic extracts of these marine algae. The isolations and purifications of these molecules were achieved by using different chromatographic techniques, including VLC and HPLC. Structure determinations of these compounds were established by extensive 1D and 2D NMR experiments. Secondary metabolites isolated from the first three species are terpenoidal compounds. Compounds isolated from *Lyngbya* species were found to be nitrogen-containing with molecular sizes ranging from 410 Da to 599 Da. In addition to being new chemical structures, these molecules possessed various bioactivities including cytotoxicity, modulation of calcium concentration in rat neurons, sodium channel blocking activity and brine shrimp toxicty.

Using bioassay guided fractionation, I isolated and identified five new, biologically active compounds (2β,3α-epitaondiol, flabellinol, flabellinone, stypoaldehyde and stypohydroperoxide), together with five known compounds (2-geranylgeranyl-6-methyl-1, 4-benzoquinone, (-) epistypodiol, (-) stypoldione, fucoxanthin and iditol) from the marine brown alga *Stypopodium flabelliforme,* collected from Papua New Guinea. All of the new compounds were found to have cytotoxic activity (EC_{50} ranges from 0.3 – 10 μg/ml) in human lung cancer (NCI-H460) and mouse neuro-2a cell lines. Iso-isoepitaondiol and flabellinol exhibited strong sodium channel blocking activity (EC_{50} = 0.3 and 0.9 μg/ml, respectively). Stypoaldehyde, as well as the previously known compound, stypoldione, effected a change in the calcium concentrations in rat neurons. The acetate derivatives of iso-isoepitaondiol, flabellinol, were significally less active than the natural products. The molecular structures of the isolated compounds were determined by extensive analysis of their spectroscopic data (1D and 2D NMR, LRMS, HRMS, IR and UV). The

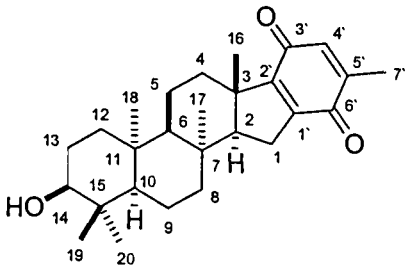
relative stereochemistries of the compounds iso-isoepitaondiol, and flabellinol were determined by 1D and 2D NOE experiments. X-ray crystallography confirmed the relative stereochemistry of 2β,3α-epitaondiol.



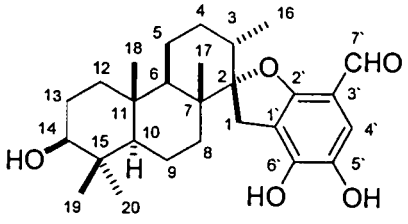
2β,3α-epitaondiol



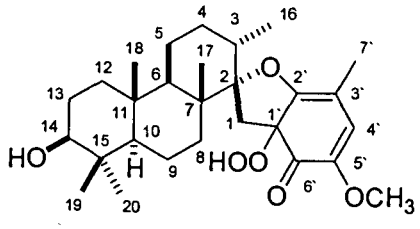
Flabellinol



Flabellinone



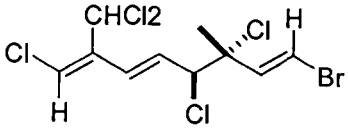
Stypoaldehyde

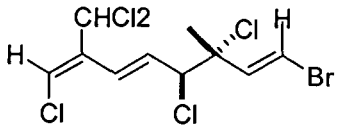


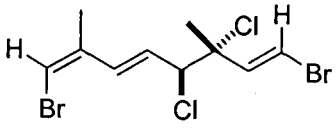
Stypohydroperoxide

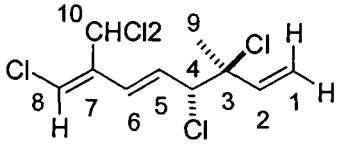
As a result of my efforts to identify bioactive agents from marine algae, I have isolated and identified one new halogenated monoterpene [(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene] in addition to another three known halogenated monoterpene compounds from the red alga *Plocamium cartilagineum* collected from the eastern coast of South Africa. [(-)-(5*E*,7*Z*)-3,4,8-Trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene] was found to be active as a cytotoxic agent in

human lung cancer (NCI-H460) and mouse neuro-2a cell lines (EC_{50} 4 µg/ml). Two of the known compounds were found to have promising activity as cytotoxins in other cell line assays, especially to human leukemia and human colon cancers and are now in the *in-vivo* evaluation stage. However, none of these metabolites were active as sodium channel blockers or activators. All structures were determined by spectroscopic methods (UV, IR, LRMS, HRMS, 1D NMR and 2D NMR). 1D and 2D NOE experiments were carried out on the four compounds to confirm the geometry of the double bonds.





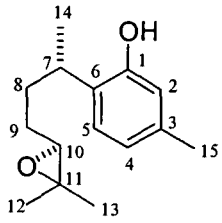




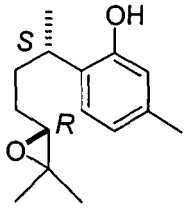
[(-)-(5*E*,7*Z*)-3,4,8-trichloro-7-dichloromethyl-3-methyl-1,5,7-octatriene]

As part of a continued search for bioactive secondary metabolites from marine sources using a bioassay guided fractionation approach (Anti-trypanosome activity), I examined the organic extract of a Papua New Guinean collection of the green alga *Udotea orientalis* growing on a coral wall and collected in September 1998. Successive HPLC separations resulted in the isolation of three new compounds; (+) curcuepoxide A, (+) curcuepoxide B and (+)-10α- hydroxycurcudiol. In addition I isolated four known compounds; (+)-10β- hydroxycurcudiol, (+) curcuphenol , (+) curcudiol and (+) curcudiol-10-one. Analysis of different spectroscopic data e.g. UV, IR, LRMS, HRMS, 1D NMR and 2D NMR of the isolated compounds allowed for construction of their planar structures. Curcudiol was found to be active in an *in-vitro* assay of anti-trypanosome activity (EC_{50}10 µg/ml). The isolated compounds were found to have variable cytotoxic activity in human lung cancer cell lines. The most active compounds in the latter assays were compounds (+)-10β- hydroxycurcudiol and (+) curcudiol-10-one (EC_{50} = 2 and 4 µg/ml, respectively). However, they both displayed sodium channel blocking activity at

this dose but not at the lower dose of 1 µg/ml. This activity was 72 and 82% inhibition of blocking, respectively, compared to 87% for saxitoxin at 0.05 µg/ml.



(+) curcuepoxide A



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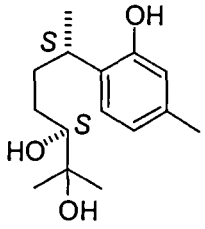
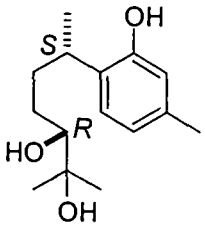
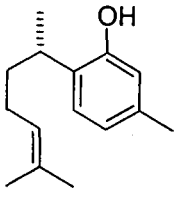


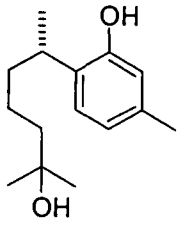
(+)-10α-hydroxycurcudiol



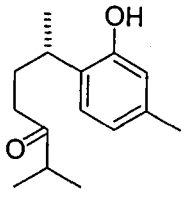
(+)-10β-hydroxycurcudiol



(+) curcuphenol

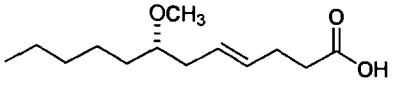


(+) curcudiol

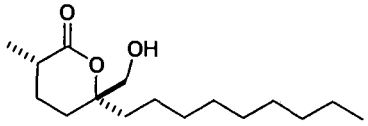


(+) curcudiol-10-one

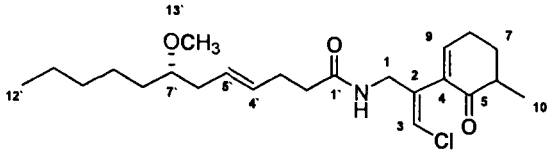
A bioassay guided investigation approach (Anti-Sirt2) of a *Lyngbya majuscula* collection from Key West Florida in 1995, led to the discovery of two novel bioactive natural products [(+)-malyngamide X and one cyclic depsipeptide, (+)-Floridamide]. Their structures were deduced through extensive analysis of 1D and 2D NMR spectroscopic data and supported by HRFAB mass spectrometry. The new cyclicdepsipeptide, (+)-floridamide contains four amino acids units beside the unique unit, 2,2-dimethyl-3-hydroxy-octanoic acid (Dhoaa). In addition to the discovery of these two new compounds, two previously reported metabolites were also isolated and identified from this cyanobacterial collection, including the antibacterial agent (-) malyngolide and (-) C-12 lyngbic acid.



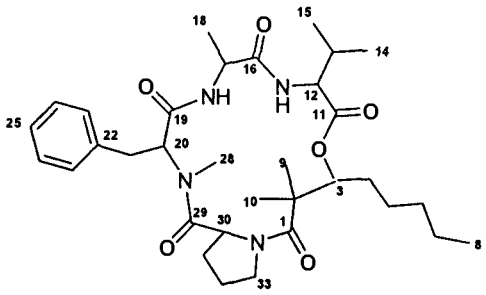
(-)-C-12 Lyngbic acid



(-)-Malyngolide



Malyngamide X



Floridamide

This thesis has shown that marine algae are indeed a prolific and important source of new and bioactive natural products. Some of these molecules have potential to be pharmaceuticals or as chemical leads in generating new pharmaceuticals. The most important findings of my thesis include the highly bioactive 2β,3α-epitaondiol, flabellinol, stypoaldehyde from the marine brown alga *Stypopodium flabelliforme*, two halogenated monoterpenes from the red alga *Plocamium cartilagineum* which show promising anticancer activity and floridamide, a *SIRT-2* active peptide. It is therefore a worthwhile pursuit to carry out natural products research on these phenomenal marine creatures.

BIBLIOGRAPHY

1. Abbott, I. A. and Dawson, E. Y. *In How to Know the Seaweeds*, 2nd Ed.,WCB McGraw Hill: Boston, **1987**, pp. 1-19.

2. Abreu, P. M.; Galindro, J. M. *Ind. J. Chem.* **1998**, 37B, 610-611.

3. Abreu, P. M.; Galindro, J. M. *J. Nat. Prod.* **1996**, 59, 1159-1162.

4. Aimi, N.; Odaka, H.; Sakai, S.; Fujiki, H.; Suganuma, M.; Moore, R. E.; Patterson, G. M. L. *J. Nat. Prod.* **1990**, *53*, 1593-1596.

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

6. Alley, M. C.; Scudiero, D. A. *Cancer Research* **1988**, **48**, 589-601.

7. Argandona, V. H.; Rovirosa, J.; San-Martin, A.; Riquelme, A.; Diaz-Marrero, A. R.; A. R.; Cueto, M.; Darias, J.; Santana, O.; Guadano, A.; Gonzalez-Coloma, A. *J. Agr. Food Chem.* **2002**, 50, 7029-7033.

8. Cueto, M.; Darias, J.; Santana, O.; Guadano, A.; Gonzalez-Coloma, A. *J. Agr. Food Chem.* **2002**, 50, 7029-7033.

9. Aune, G. J.; Furuta, T.; Pommier, Y. *Anti-Cancer Drugs* **2002**, 13, 545-555.

10. Baldwin J. E.; Whitehead, R. C., *Tetrahed. Lett.* **1992**, 33, 2059.

11. Bartik, K.; Braekman, J. C.; Daloze, D.; Stoller, C.; Huysecom, J.; Vandevyver, G.; Ottinger, R. *Can. J. Chem.* **1987**, *65*, 2118–2121.

12. Bergmann, W.; Feeney, R. J. *J. Org. Chem.* **1951**, *16*, 981–987

13. Berman, F.W.; Murray, T.F. *J. Neurochem.* **2000**, 74, 1443-1451.

14. Blackburn, C. L.; Hopmann, C.; Sakowicz, R.; Berdelis, M. S.; Goldstein, L. S. B.; Faulkner, D. J. *J. Org. Chem.* **1999**, *64*, 5565–5570.

15. Blakemore, P. R., Kim, S. K., Schulze, V. K., White, J. D., Yokochi, A. F. T. *J. Chem. Soc. Perkin Trans. 1*, In Press.

16. Blunt, J. W.; Bowman, N. J.; Munro, M. H. G.; Parsons, M. J.; Wright, G. J.; Kon, Y. K. *Aust. J. Chem.* **1985**, 38, 519-25.

17. Bodey, G. P.; Freirich, E. J.; Monto, R. W.; Hewlett, J. S. *Cancer Chemother.* **1969**, *53*, 59–66

18. Burreson, B. J.; Woolard, F. X.; Moore, R. E. *Chemistry Letters* **1975**, 11, 1111-14.

19. Cahn, R. S.; Ingold, C. K.; Prelog, V. *Angew. Chem., Int. Ed.* **1966**, *5* (4), 385-415.

20. Cahn, R. S.; Ingold, C. K.; Prelog, V. *Experientia* **1956**, *12*, 81-124.

21. Capon, R. J.; Engelhardt, L. M.; Ghisalberti, E. L.; Jefferies, P. R.; Patrick, V. A.; White, A. H. *Aust. J. Chem.* **1984**, *37*, 537-544.

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

23. Cardellina II, J. H.; Marner, F.; Moore, R. E. *J. Am. Chem. Soc.* **1978**, *101*, 240–242.

24. Cardellina, J. H. 2nd; Marner, F. J.; Moore, R. E. *Science* **1979**, *204*, 193-195.

25. Carter, D. C.; Moore, R. E.; Mynderse, J. S.; Niemczura, W. P.; Todd, J. S. *J. Org. Chem.* **1984**, *49*, 236-241.

26. Cragg, G. M.; Newman, D. J. *Expert Opinion on Investigational Drugs* **2000**, *9*, 2783 2797.

27. Crews, P.; Kho-Wiseman, E. *J. Org. Chem.* **1974**, *39*, 3303-3304.

28. Crews, P.; Manes, L. V.; Boehler, M. *Tetrahed. Lett.* **1986**, *27*, 2797–2800.

29. Crews, P.; Myers, B. L.; Naylor, S.; Clason, E. L.; Jacobs, R. S.; Staal, G. B. *Phytochem.* **1984**, 23, 1449-51.

30. Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R. *J. Org. Chem.***1984**, 49, 1371-1377.

31. Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R. *J. Org. Chem.***1984**, 49, 1371-7.

32. Crews, P. *J. Org. Chem.* **1977**, 42, 2634-6.

33. Cronin, G.; Hay, M. E. *Ecology* **1996**, *77*, 2287-2301.

34. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta.* **1987**, *70*, 2019–2027.

35. Davies, D. G.; Parsek, M. R.; Pearson, J. P.; Iglewski, B. H.; Costerton, J. W.; Greenberg, E. P. *Science* **1998**, *280*, 295-298.

36. Davies, J. *Mol. Microbiol.* **1990**, *4*, 1227-1232.

37. Davis, F. A.; Harakal, M. E.; Awad, S. B. *J. A. C. S.* **1983**, 105, 3123-6.

38. De Clercq, E. *Med. Res. Rev.* **2000**, *20*, 323–349.

39. De Nys, R.; Wright, A. D.; Konig, G. M.; Sticher, O. *Tetrahedron* **1993**, *49*, 11213-11220.

40. De Silva, E. D.; Scheuer, P. J. *Tetrahed. Lett.* **1980**, *21*, 1611–1614.

41. Depix, M. S.; Martinez, J.; Santibanez, F.; Rovirosa, J.; San Martin, A; Maccioni, R. B. *Molecular and Cellular Biochemistry* **1998**, 187, 191-199.

42. Dorta, E.; Cueto, M.; Diaz-Marrero, A. R.; Darias, J. *Tetrahedron Letters* **2002**, 43, 9043-9046.

43. Dorta, E.; Diaz-Marrero, A. R.; Cueto, M.; Darias, J. *Tetrahedron* **2003**, 59, 2059-2062.

44. El Sayed K. A; Yousaf M.; Hamann M. T; Avery M. A; Kelly M.; Wipf P. *J. Nat. Prod.* **2002**, 65, 1547-53.

45. El Sayed, K. A.; Bartyzel, P.; Shen, X.; Perry, T. L.; Zjawiony, J. K.; Hamann, M. T. *Tetrahedron* **2000**, *56*, 949–953.

46. Fattorusso, E.; Magno, S.; Mayol, L.; Novellino, E. *Experientia* **1983**, 39, 1275-6.

47. Fattorusso, E.; Magno, S.; Santacroce, C.; Sica, D. *Tetrahedron* **1972**, 28, 5993-7.

48. Faulkner, D. J. *Nat. Prod. Rep.* **2000**, *17*, 1-6.

49. Faulkner, D. J. *Nat. Prod. Rep.* **2002**, *19*, 1-48, and references therein.

50. Frormann, S.; Jas, G. Business Briefing: *Future Drug Discover.* **2002**, 84-90.

51. Fujiki, H.; Mori, M.; Nakayasu, M.; Terada, M.; Sugimura, T.; Moore, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3872-3876.

52. Fuller, R. W.; Cardellina, J. H., II; Kato, Y.; Brinen, L. S.; Clardy, J., Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1992**, *35*, 3007-3011.

53. Fusetani N; Sugano M; Matsunaga S; Hashimoto K. *Experientia* **1987**, 43, 1234-5.

54. G. M. L. *J. Nat. Prod.* **1990**, *53*, 1593-1596.

55. Geldof, A. A.; Mastbergen, S. C.; Henrar, R. E. C.; Faircloth, G. T. *Cancer Chemother. Pharmacol.* **1999**, *44*, 312-318.

56. Gerwick, W. H.; Fenical, W. *J. Org. Chem.* **1981**, *46*, 22-27.

57. Gerwick, W. H.; Fenical, W.; Norris, J. N. *Phytochemistry* **1985**, 24, 1279-83.

58. Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. L. *J. Org. Chem.* **1994**, *59*, 1243–1245.

59. Gerwick, W. H.; Proteau, P.J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. *J. Org. Chem.* **1994**, *59*, 1243–1245.

60. Gerwick, W. H.; Reyes, S.; Alvarado, B. *Phytochemistry* **1987**, *26*, 1701–1704.

61. Gerwick, W. H.; Tan, L. T.; Sitachitta, N. *Alkaloids*, **2001,** 57, 75-184.

62. Gerwick, W..; Fenical, W.; Fritsch, N.; Clardy, J. *Tetrahed. Lett.* **1979**, 145.

63. Gonzalez, A. G.; Arteaga, J. M.; Martin, J. D.; Rodriguez, M. L.; Fayos, J.; Martinez-Ripolls, M. *Phytochemistry* **1978**, 17, 947-8.

64. Gonzalez, A. G.; Darias, J.; Martin, J. D. *Tetrahed. Lett.* **1971**, 29, 2729-32.

65. Gonzalez, Antonio G.; Alvarez, Miquel A.; Darias, Jose; Martin, Julio D. *Journal of the Chemical Society, Perkin.* **1973**, 22, 2637-2642.

66. Gunasekera, S. P.; Gunasekera, M.; Longley, R. E.; Schulte, G. K. *J. Org. Chem.* **1990**, *55*, 4912–4915.

67. Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825–5826.

68. Hay, M. E. *J. Exp. Mar. Biol. Ecol.* **1996**, *200*, 103-134.

69. Hazzard, James T.; Moench, Susan J.; Erman, James E.; Satterlee, James D.; Tollin, Gordon. *Biochemistry*, **1988**, 27, 2002-2008.

70. Herms, D. A.; Mattson, W. J. *Quart. Rev. Biol.* **1992**, *67*, 283-335.

71. Higgs, M. D.; Vanderah, D. J.; Faulkner, D. J. *Tetrahedron* **1977**, 33, 2775-80.

72. Hirata, Y.; Uemura, D. *Pure Appl. Chem.* **1986**, *58*, 701–710.

73. Iliopoulou, D; Vagias, C; Harvala, C; Roussis, V. *Nat. Prod. Lett.* **2000**, 14, 373-378.

74. Kashman, Y.; Zviely, M. *Experientia* **1980**, *36*, 1279–1279.

75. Keifer, P. A.; Rinehart, K. L.; Hooper, I. R. *J. Org. Chem.* **1986**, *51*, 4450–4454.

76. Kitagawa, I.; Kobayashi, M.; Katori, T.; Yamashita, M.; Tanaka, J.; Doi, M.; Ishida, T. *J. Am. Chem. Soc.* **1990**, *112*, 3710–3712.

77. Koh, J.Y.; Choi, D.W. *J. Neurosci. Methods.* **1987**, 20, 83-90.

78. Kraft, A. S. *J. Natl. Cancer Inst.* **1993**, *85*, 1790–1792.

79. Lin, Y.–Y.; Risk, M.; Ray, S. M.; Van Engen, D.; Clardy, J.; Golik, J.; James, J. C.; Nakanishi, K. *J. Am. Chem. Soc.* **1981**, *103*, 6773–6775.

80. Lindel, T.; Jensen, P. R.; Fenical, W.; Long, B. H.; Casazza, A. M.; Carboni, J.; Fairchild, C. R. *J. Am. Chem. Soc.* **1997**, *119*, 8744–8745.

81. Litaudon, M.; Hart, J. B.; Blunt, J. W.; Lake, R. J.; Munro, M. H. G. *Tetrahed. Lett.* **1994**, *35*, 9435–9438.

82. Look, S. A.; Fenical, W.; Matsumoto, G. K.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5140–5145.

83. Lopez, C.; Giner-Sorolla, A. *Ann. N. Y. Acad. Sci.* **1977**, *284*, 351-357

84. Luesch, H.; Moore, R. E.; Paul, V. J.; Mooberry, S. L.; Corbett, T. H. *J. Nat. Prod.* **2001**, *64*, 907-910.

85. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611-615.

86. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 1702-1705.

87. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611-615.

88. Luibrand, R. T.; Erdman, T. R.; Vollmer, J. J.; Scheuer, P. J.; Finer, J.; Clardy, J. *Tetrahedron* **1979**, *35*, 609–612.

89. Mancini, I.; Guella, G.; Defant, A.; Candenas, M. Luz; A., Cristina P.; Depentori, D.; Pietra, F. *Helvetica Chimica Acta.* **1998**, 81, 1681-1691.

90. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. *Microbiology* **1999**, *145*, 283-29.

91. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. *Microbiology* **1999**, *145*, 283–291.

92. Manger, R. L.; Leja, L. S. *Journal of AOAC International* **1995,** 78, 521- 527.

93. Martinez, E. J.; Corey, E. J.; Owa, T. *Chem. Biol.* **2001**, *8*, 1151-60

94. Martinez, J. L.; Sepulveda, S. P.; Rovirosa, J.; San Martin, A. *Anales de la Asociacion Quimica Argentina* **1997**, 85, 69-75.

95. McEnroe, F. J.; Fenical, W. Tetrahedron **1978**, 34, 1661-4.

96. Mesguiche, V.; Valls, R.; Piovetti, L.; Peiffer, G. *Tetrahedron Lett.* **1999**, 7473–7476.

97. Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31–34.

98. Milligan, K. E.; Marquez, B. L.; Williamson, R. T.; Gerwick, W. H. *J. Nat. Prod.*

2000, *63*, 1440-1443.

99. Moore, R. E.; Scheuer, P. J. *Science* **1971**, *172*, 495–498.

100. Mori, K.; Koga, Y. *Bioorg. & Med. Chem. Lett.* **1992**, 2, 391-394.

101. Murakami, N.; Morimoto, T.; Imamura, H.; Ueda, T.; Nagai, S.; Sakakibara, J.; Yamada, N. *Chemical & Pharmaceutical Bulletin.* **1991**, 39, 2277-81.

102. Murakami, Y.; Oshima, Y.; Yasumoto, T. *Nippon Suisan Gakkaishi* **1982**, *48*, 69–72.

103. Murata, M.; Legrand, A.–M.; Ishibashi, Y.; Yasumoto, T. *J. Am. Chem. Soc.* **1989**, *111*, 8927–8931.

104. Murata, M.; Nakoi, H.; Iwashita, T.; Matsunaga, S.; Sasaki, M.; Yokoyama, A.; Yasumoto, T. *J. Am. Chem. Soc.* **1993**, *115*, 2060–2062.

105. Mynderse, J. S.; Faulkner, D. J. *J. A. C. S.* **1974**, 96, 6771-6772.

106. Mynderse, J. S.; Faulkner, D. J. *Tetrahedron* **1975**, *31*, 1963-1967.

107. Mynderse, J. S.; Moore, R. E. *J. Org. Chem.* **1978**, *43*, 4359–4363.

108. Nagle, D. G.; Paul, V. J. *J. Exp. Mar. Biol. Ecol.* **1998**, *225*, 29-38.

109. Nagle, D. G.; Paul, V. J. *J. Phycol.* **1999**, *35*, 1412-1421.

110. Nakatsu, Tetsuo; Ravi, B. N.; Faulkner, D. John. *J. Org. Chem.* **1981**, 46, 2435-8.

111. Naylor, S.; Hanke, F. J.; Manes, L. V; Crews, P. *Prog. Chem. Org. Nat. Prod.* **1983**, *44*, 189-241.

112. Norimura, T.; Sasaki, M.; Matsumori, N.; Miata, M.; Tachibana, K.; Yasumoto, T. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1675–1678.

113. Norton, R. S.; Warren, R. G.; Wells, R. J.; *Tetrahedron Lett.* **1977**, 3905-3908.

114. O'Clair, R. M.; Lindstorm, S.C. *In North Pacific Seaweeds*, Plant Press: Alaska, **2000**, pp. 1-8.

115. O'Brien, E. Timothy; W., Steven; J., Robert S.; Boder, G. B.; Wilson, L. *Hydrobiologia* **1984**, 116-117.

116. O'Brien, E. Timothy; W., Steven; J., Robert S.; Boder, G. B.; Wilson, L. *Hydrobiologia* **1984**, 116-117.

117. Olivera, B. M. In: Fusetani, N. (ed), *Drugs from the sea*. Karger **2000**, 74-85

118. Orjala, J.; Nagle, D.; Gerwick, W. H. *J. Nat. Prod.* **1995**, *58*, 764–768.

119. Ortega, M. J.; Zubia, E.; Salva, J. *J. Nat. Prod.* **1997**, *60*, 482-484.

120. Paul, V. J. *Seaweed chemical defenses on coral reefs, in Ecological roles for marine natural products* (ed. Paul, V. J.), Comstock Publishing Associates, Ithaca, **1992**, 24-50.

121. Paul, V. J.; Fenical, W. *Marine Ecology* **1986**, 34, 157-69.

122. Paul, V. J.; Nelson, S. G.; Sanger, H. R. *Marine Ecology* **1990**, 60, 23-34.

123. Paul, V. J.; Sun, H. H.; Fenical, W. *Phytochemistry* **1982**, 21, 468-9.

124. Pawlik, J. R.; Kernan, M. R.; Molinski, T. F.; Harper, M. K.; Faulkner, D. J. *J. Exp. Mar. Biol. Ecol.* **1988**, *119*, 99-109.

125. Pennings, S. C.; Pablo, S. R.; Paul, V. J. *Limnol. Oceanog.* **1997**, *42*, 911-

126. Pettit, G. R.; Herald, C. L.; Doubek, D. L.; Herald, D. L.; Arnold, E.; Clardy, J. *J. Am. Chem. Soc.* **1982**, *104*, 6846–6848.

127. Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinamn, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B.; Bontems, R. *J. Am. Chem. Soc.* **1987**, *109*, 6883–6885.

128. Praud, A.; Valls, R.; Piovetti, L.; Banaigs, B. *Tetrahedron Lett.* **1993**, *34*, 5437–5440.

129. Proksch, P. *Toxicon* **1994**, *32*, 639-655.

130. Proksch, P.; Edrada, R. A.; Ebel, R. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 125-134

131. Propper, D. J.; Macaulay, V.; O'Byrne, K. J.; Braybrooke, J. P.; Wilner, S. M.; Ganesan, T. S.; Talbot, D. C.; Harris, A. L. *British J. canc.* **1998**, 78, 1337-41.

132. Proteau, P.; Gerwick, W. H.; Garcia–Pichel, F.; Castenholz, R. *Experientia* **1993**, *49*, 825–829.

133. Rasmussen, T. B.; Manefield, M.; Andersen, J. B.; Eberl, L.; Anthoni, U.; Christophersen, C.; Steinberg, P.; Kjelleberg, S.; Givskov, M. *Microbiology* **2000**, *146*, 3237-3244.

134. Renner, M. K., Shen, Y.-C., Cheng, X.-C., Jensen, P. R., Frankmoelle, W., Kauffman, C. A., Fenical, W., Lobkovsky, E. and Clardy, J. *J. Am. Chem. Soc.* **1999**, *121*, 11273-11276.

135. Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Kiefer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512–4515.

136. Roussis, V.; Wu, Z, Fenical, W.; Strobel, S. A.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1990**, *55*, 4916–4922.

137. Rovirosa, J.; Sepulveda, M.; Quezada, E.; San-Martin, A. *Phytochemistry* **1992**, 31, 2679-81.

138. Sakai, R.; Rinehart, K. L.; Kishore, V.; Kundu, B.; Faircloth, G.; Gloer, J. B.; Carney, J. R.; Namikoshi, M.; Sun, F.; Hughes, R. G.; Garcia Gravalos, D.; de Quesada, T. G.; Wilson, G. R.; Heid, R. M. *J. Med. Chem.* **1996**, *39*, 2819–2834.

139. Sampli, P.; Tsitsimpikou, C.; Vagias, C.; Harvala, C.; Roussis, V. *Nat. Prod. Lett.* **2000**, 14, 365-372.

140. Sanchez-Ferrando, Francisco; San-Martin, Aurelio. *J. Org. Chem.* **1995**, 60, 1475-8.

141. San-Martin, A.; Rovirosa, J. *Biochem. System. Ecol.* **1986**, 14, 459-61.

142. Siddhanta, A. K.; Shanmugam, M. Journal of the Indian Chemical Society **1999**, 76, 323-334.

143. Smith, C. D.; Zhang, X.; Mooberry, S. L.; Patterson, G. M. L.; Moore, R. E. *Cancer Res.* **1994**, *54*, 3779-3784.

144. Spector, I.; Shochet, N. R.; Kashman, Y.; Groweiss, A. *Science* **1983**, *219*, 493–495.

145. Stone, M. J.; Williams, D. H. *Mol. Microbiol.* **1992**, *6*, 29-34.

146. Sze, P. *A Biology of the Algae*, 3rd Ed., WCB McGraw-Hill: Boston, **1998**.

147. Tasdemir, D.; Bugni, T. S.; Mangalindan, G. C.; Concepcion, G. P.; Harper, M. K.; Ireland, C. M. *Turkish J. Chem.* **2003**, 27, 273-279.

148. Thacker, R. W.; Nagle, D. G.; Paul, V. J. *Mar. Ecol. Prog. Ser.* **1997**, *167*, 21-29.

149. Thomas S. G.; Beveridge A. A. *Clin. Exp. Pharm. Phys.* **1993**, 20, 223-9.

150. Toth, G. B.; Pavia, H. *Proc. Nat. Acad. Sci.* **2000**, 97,14418-14420.

151. Uhlenbruck, G.; Hanisch, F. G.; Kljajic, Z.; Poznanovic, S.; Schroeder, H. C.; Mueller, W. E. G. *Behring Institute Mitteilungen* **1992**, 91, 67-77.

152. Walker R. P.; Faulkner, D. J. *J. Org. Chem.* **1981**, *46*, 1098–1102.

153. Watanabe, K.; Miyakado, M.; Ohno, N.; Okada, A.; Yanagi, K.; Moriguchi, K. *Phytochemisty* **1989**, 28, 77-78.

154. Wessels, M.; Koning, G. M.; Wright, A. D. *J. Nat. Prod.* **1999**, *62*, 927-930

155. Wessels, Matthias; Koenig, Gabriele M.; Wright, Anthony D. *J. Nat. Prod.* **2000**, 63, 920-928.

156. White S. J; Jacobs R. S. *Mol. Pharmacol.* **1983**, 24, 500-8.

157. Wright, A. D.; Coll, J. C.; Price, I. R. *J. Nat. Prod.* **1990**, *53*, 845-861.

158. Wright, A. D.; Coll, J. C.; Price, I. R. *J. Nat. Prod.* **1990**, *53*, 845–861.

159. Wright, A. D.; Konig, G. M. *J. Nat. Prod.* **1996**, *59*, 710–716.

160. Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55*, 4508–4511.

161. Wylie, B. L.; Ernst, N. B.; Grace, K. J. S.; Jacobs, R. S. *Prog. Surg.* **1997**, *24*, 146–152.

162. Yamada, K.; Ojika, M.; Ishigaki, T.; Yoshida, Y.; Ekimoto, H.; Arakawa, M. *J. Am. Chem. Soc.* **1993**, *115*, 11020–11021.

163. Yasumoto, T.; Oshima, Y.; Sugawara, W.; Fukuyo, Y.; Oguri, H.; Igarashi, T.; Fujita, N. *Nippon Suisan Gakkaishi* **1980**, *46*, 1405–1411.

164. Zabriskie, T. M.; Klocke, J. A.; Ireland, C. M.; Marcus, A. H.; Molinski, T. F.; Faulkner, D. J.; Xu, C.; Clardy, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3123–3124.

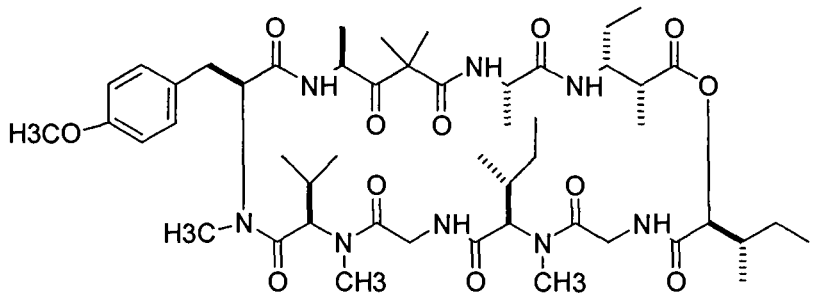
APPENDIX

APPENDIX A

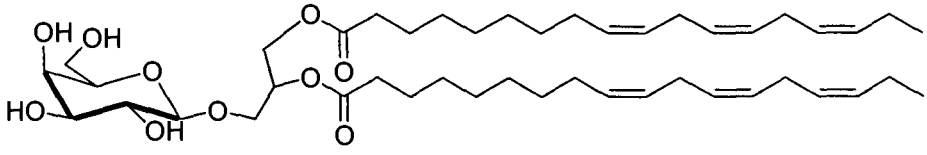
ISOLATION OF MAJUSCULAMIDE C AND GALACTOGLYCEROLIPID FROM A PAPUA NEW GUINEAN *LYNGBYA MAJUSCULA*

An organic extract of the marine cyanobacterium, *Lyngbya* majuscula, collected from Heina Lagoon, Papua New Guinea (PNHL 9 Sep 98-02) was found to be active in modulation of Ca^{++} concentration in rat neurons. Assay-guided fractionation of this extract led to the reisolation of the strongly cytotoxic cyclic depsipeptide, majusculamide C (**1**)[1] and the known anti-inflammatory galactoglycerolipid **2**[2-3]. Majusculamide C was originally reported in 1984 as an antifungal agent from a *L. majuscula* collection from Enewetak Atoll in the Marshall Islands.[1] The galactoglycerolipid **2** was originally isolated from an axenically cultured cyanobacterium, *Phormidium tenue,* and also from the green alga *Caulerpa taxifolia*.[2-3]

Fraction 8, eluted with 100% EtOAc from normal phase Si gel VLC of the organic extract, was subjected through a series of chromatographic techniques, including C_{18} SPE (8:2 MeOH/H_2O) followed by RP HPLC (Phenomenex Sphereclone 5μm ODS column, 17:3 MeOH/H_2O) to yield 3.2 mg of pure **1**. Compound **2** was isolated from the C_{18} SPE (9:1 MeOH/H_2O) followed by RP HPLC (Phenomenex Sphereclone 5μm ODS column, 18:2 MeOH/H_2O) to yield 6.2 mg of pure **2**. Comparison of spectroscopic and physical data, including 1D and 2D NMR, HRFABMS, IR and optical rotations showed these to be identical to the reported compounds.



1



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REFERENCES

1. Carter, D. C.; Moore, R. E.; Mynderse, J. S.; Niemczura, W. P.; Todd, J. S. *J. Org. Chem.* **1984**, *49*, 236-241.

2. Murakami, N.; Morimoto, T.; Imamura, H.; Ueda, T.; Nagai, S.; Sakakibara, J.; Yamada, N. *Chem. Pharm. Bullet.* **1991**, 39, 2277-81.

3. Mancini, I.; Guella, G.; Defant, A.; Candenas, M. Luz; A., Cristina P.; Depentori, D.; Pietra, F. *Helv. Chim. Acta.* **1998**, 81, 1681-1691.