STRUCTURE ELUCIDATION OF BIOACTIVE NATURAL PRODUCTS FROM MADAGASCAR MARINE ALGAE AND CYANOBACTERIA

Eric Hajaniriana Andrianasolo

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AN ABSTRACT OF THE THESIS OF

Eric Hajaniriana Andrianasolo for the degree of Doctor of Philosophy in Pharmacy presented on November 18, 2005.

Title: Structure Elucidation of Bioactive Natural Products from Madagascar Marine Algae and Cyanobacteria

Abstract approved:

William H. Gerwick

This thesis is an investigation of the natural products deriving from marine algae and cyanobacteria and has resulted in the discovery of eleven new secondary metabolites. The structure elucidations of these new molecules were performed using a variety of spectroscopic techniques.

Four new macrolides were isolated and characterized from the Madagascar marine cyanobacterium *Geitlerinema* sp. These ankaraholides are structurally similar to the potently cytotoxic swinholides and were found to have cytotoxicities ranging from 178 nM to 354 nM against human lung cancer (NCI-H460) and mouse neuro-2a cell lines. Since swinholide-type compounds were previously localized to the heterotrophic bacteria of sponges, these findings raise intriguing questions about their true metabolic source.

Geitlerinema sp. was found to be particularly rich in chemistry, and also produced the new linear lipopeptide mitsoamide with unusual structural features including an aminal moiety, a homolysine residue and a polyketide unit (3,7-dimethoxy-5-methyl- nonanedioic acid) (DMNA).

A collection of the red marine alga *Portieria hornemannii* from the south of Madagascar (Tolagniaro, Fort Dauphin), led to the isolation of the previously reported halogenated monoterpene, halomon, and the discovery of three new related metabolites. These molecules were found to inhibit DNA methyltransferase 1 (DNMT-1).

As a result of efforts to identify bioactive agents from the marine cyanobacterium *Lyngbya majuscula,* tanikolide dimer, a novel SIRT2 inhibitor (IC_{50} = 176 nM), and tanikolide seco-acid were isolated. The depside molecular structure of tanikolide dimer, which is likely a *meso* compound, was established by NMR, MS and chiral HPLC analyses. The structure of tanikolide dimer raises a number of intriguing configurational and biosynthetic questions for further study.

The bioassay guided fractionation of a collection of the brown marine alga *Dictyota* sp. from Netherland Antilles Playa Fort, led to the identification of a novel HDAC inhibitor with a dolastane carbon skeleton. The novel molecule was also found to possess antimalarial activity. Other known HDAC inhibitors with interesting antimalarial activity have been reported previously, and based on this efficacy against malaria, HDAC appears to be a viable target for the development of antiparasitic agents.

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by

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APPROVED:

Major Professor, representing Pharmacy

Dean of the College of Pharmacy

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Eric Hajaniriana Andrianasolo, Author

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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. Isolation and characterization of swinholide A from a Fijian cyanobacterium of the genus *Symploca* cf. was performed by Dr Harald Gross in our laboratory. Cyanobacterial Taxonomy was conducted by Mirjam Musafija-Girt in our laboratory. X-ray crystallography of Tanikolide seco-acid was carried out by Dr. Alexander Yokochi, Department of Chemistry, OSU. Microfilament disrupting assay and cytotoxicity using Sulforhodamine B (SRB) assays were carried out by Dr. Rachel M. Leal in the laboratory of Dr. Susan L. Mooberry, Southwest Foundation for Biomedical Research, San Antonio, Texas**.** DNMT-1, SIRT2 and HDAC inhibitory assays were carried out by Dennis France and Susan Cornell-Kennon, Novartis Institute for Biomedical Research, Summit, New Jersey.

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LIST OF ABBREVIATIONS

Ala	Alanine
Ara-A	Adenine arabinoside
Ara-C	Cytosine arabinoside
BME	Basal Medium Eagle
br	Broad
CARD-FISH	Catalysed Reporter Deposition-Fluorescence *In Situ* Hybridization
CI	Chemical Ionization
COSY	Correlated Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement Polarization Transfer
DMSO	Dimethylsulfoxide
DNMT-1	DNA Methyltransferase 1
EC	Effective Concentration
EDTA	Ethylenediaminetetraacetic acid
EI	Electron Impact
EtOAc	Ethyl Acetate
ESI	ElectroSpray Ionization
FAB	Fast Atom Bombardment
GC	Gas Chromatography
HDAC	Histone Deacetylases
HAT	Histone Acetyl Transferases
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HR	High Resolution
HSQC	Heteronuclear Single Quantum Correlation
HSQMBC	Heteronuclear Single Quantum Multiple Bond Correlation
IC	Inhibitory Concentration
Ile	Isoleucine
IR	Infrared Spectroscopy
LD	Lethal Dose
LR	Low Resolution

LIST OF ABBREVIATIONS (Continued)

m	Multiplet
MALDI-TOF	Matrix Assited Laser Desorption Ionization Time Of Flight
Me	Methyl
MeOH	Methanol
MS	Mass Spectrometry
MTT	Methylthiazoletetrazolium
NAD	Nicotinamide Adenine Dinucleotide
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Exchange
NP	Normal Phase
NRPS	Non-Ribosomal Peptide Synthetase
Phe	Phenylalanine
PKS	Polyketide Synthase
PMSF	Phenyl Methyl Sulfonyl Fluoride
PPA	Piperidin-2-amine
PBS	Phosphate Buffered Saline
q	Quartet
ROESY	Rotating Frame Overhauser Exchange Spectroscopy
RP	Reverse Phase
s	Singlet
SAHA	Suberoylanilide Hydroxamic Acid
SAM	*S*-adenosyl methionine
SIRT2	Sirtuin Type 2 (human)
SPE	Solid Phase Extraction
SRB	Sulforhodamine B
t	Triplet
TOCSY	Total Correlation Spectroscopy
TSA	Trichostatin A
t_R	Retention Time
UV	Ultraviolet Spectroscopy
Val	Valine
VLC	Vacuum Liquid Chromatography

STRUCTURE ELUCIDATION OF BIOACTIVE NATURAL PRODUCTS FROM MADAGASCAR MARINE ALGAE AND CYANOBACTERIA

CHAPTER ONE

GENERAL INTRODUCTION

HIGHLIGHTS OF MARINE NATURAL PRODUCTS

Introduction

Terrestrial plants have been used to treat human diseases since the ancient ages. Studies of the secondary metabolites of terrestrial plants were begun in the 1800's,[1] and have proven to be rich sources of natural drugs that are used for the treatment of fatal diseases such as cancer (taxol) and microbial infections (penicillin). However, due to the resurgence of pathogenic microorganisms and parasites that have developed resistance to traditional chemotherapies, natural products chemists are increasingly turning to new sources in the search for biologically active compounds, and specifically to marine realm.[2,3]

The oceans represent a huge unexplored resource and harbor a tremendous variety of flora and fauna. The marine environment offers a new frontier for research and attracts scientists from different disciplines, such as organic chemistry, bioorganic chemistry, pharmacology, biology and ecology. Although the field of marine natural products is a relatively new area of research, because of the difficulties involved in collecting samples, it has already proven to be a productive source for biologically active natural products. The development of SCUBA in the 1960s, and more recently submersible vehicles, have allowed easy access of both shallow and deep-water marine organisms for studies by natural products chemists.

Many marine derived secondary metabolites are structurally complex with unique functionalities and possess pronounced biological activity. This is due in

part to an extreme and harsh living environment, with high ionic concentrations, high pressure, variable temperatures, lack of light as well as low nutrient availability. Because conditions in the ocean are so markedly distinct, the chemistry produced by its inhabitants is also quite varied. While terrestrial sources of pharmaceuticals and biochemicals have been considerably explored, less than one percent of marine species have been examined for production of novel chemistry.[4-7] Thus, the oceans represent a rich and still largely untapped resource for biologically active compounds. In addition, completely unknown biochemical pathways in pathogens or disease may be discovered and targeted by such unique chemotypes, leading to the development of novel therapeutics.

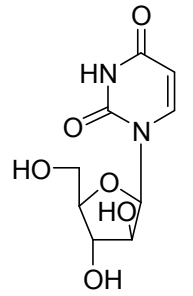
Marine natural products in general

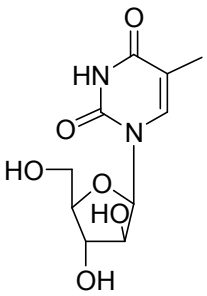
A number of promising compounds have been identified from marine sources that are already at advanced stages of clinical trials, mostly for the treatment of cancer, or have been selected as promising candidates for extended preclinical evaluation.[8] The majority of marine natural products currently in clinical trials or under preclinical evaluation are produced by invertebrates such as sponges, soft corals, sea squirts, and bryozoans. The types of compounds characterized from marine organisms are diverse and represent many different structural classes, including polyethers, terpenoids, alkaloids, macrolides, polypeptides. [8]

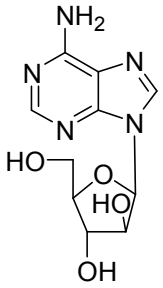
The first therapeutic agents from the marine environment were made in the 1950's by Bergmann and Feeney through their discovery of the nucleosides spongouridine (**1**) and spongothymidine (**2**) from the sponge *Tethya crypta*.[9] Subsequent development of synthetic analogues has provided the clinically relevant agents arabinosyl adenine (Ara-A, **3**), and arabinosyl cytosine (Ara-C, **4**), anti-cancer agents for the treatment of acute myelocytic leukemia and non-Hodgkin's lymphoma.[10]

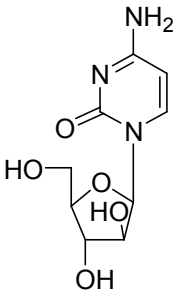
An important class of marine macromolecules, belonging mainly to the polyether structural class, forms a large group of marine toxins. Some of the well known marine polyether toxins are: brevetoxin B (**5**), isolated from the dinoflagellate *Gymnodinium breve*;[11] ciguatoxin (**6**), a toxic constituent implicated

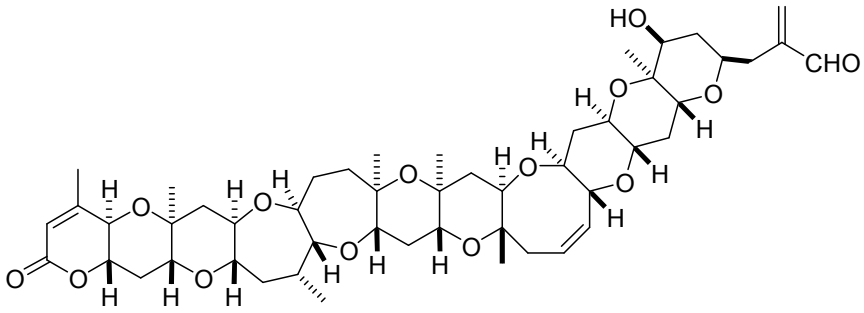
in ciguateric seafood poisoning;[12] maitotoxin (**7**), isolated from *Gambierdiscus toxicus*;[13,14] and okadaic acid (**8**), a causative agent of diarrhetic shellfish poisoning, found from two species of dinoflagellates, *Prorocentrum lima* and *Dinophysis* species;[14,15] palytoxin (**9**), is a complex polyol isolated from the zoanthid, *Palythoa toxicus*.[16]

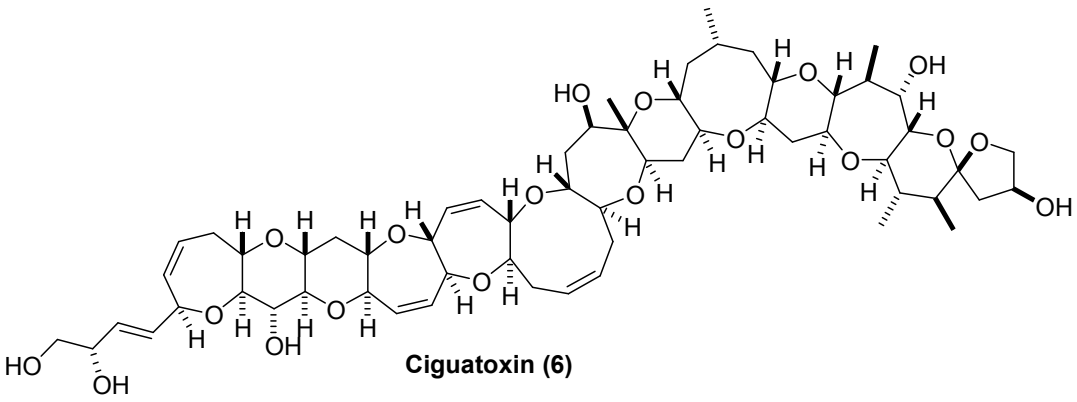

Spongouridine (**1**)

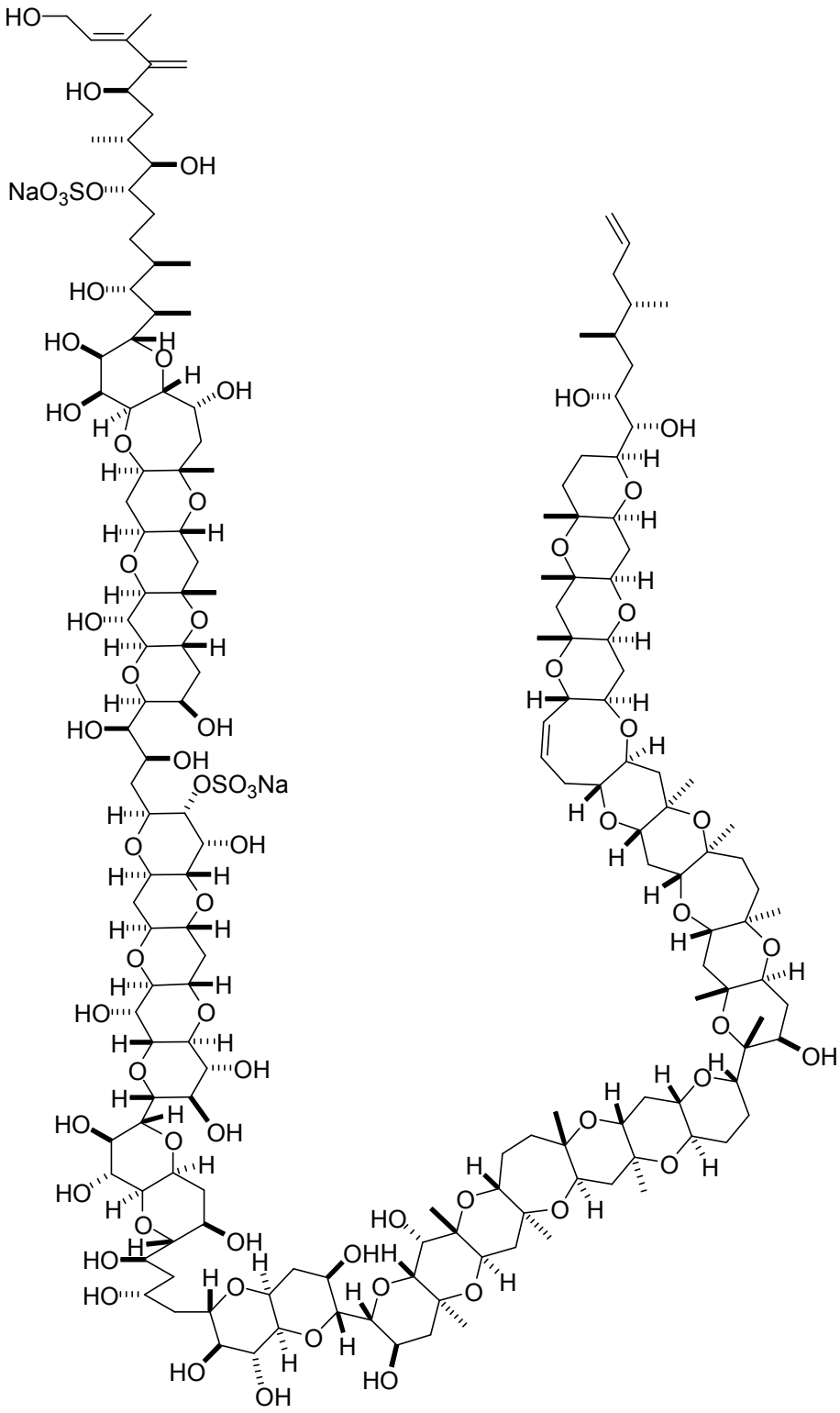

Spongothymidine (**2**)


Ara-A (**3**)

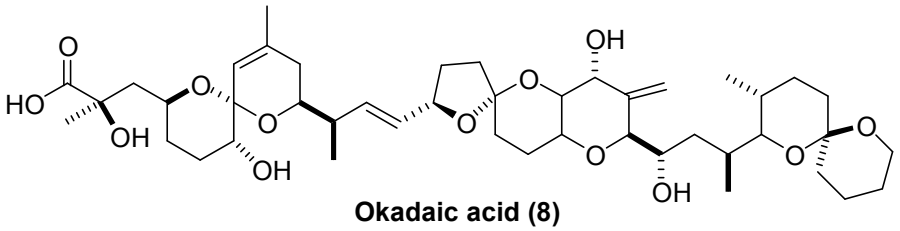

Ara-C (**4**)


Brevetoxin B (5)

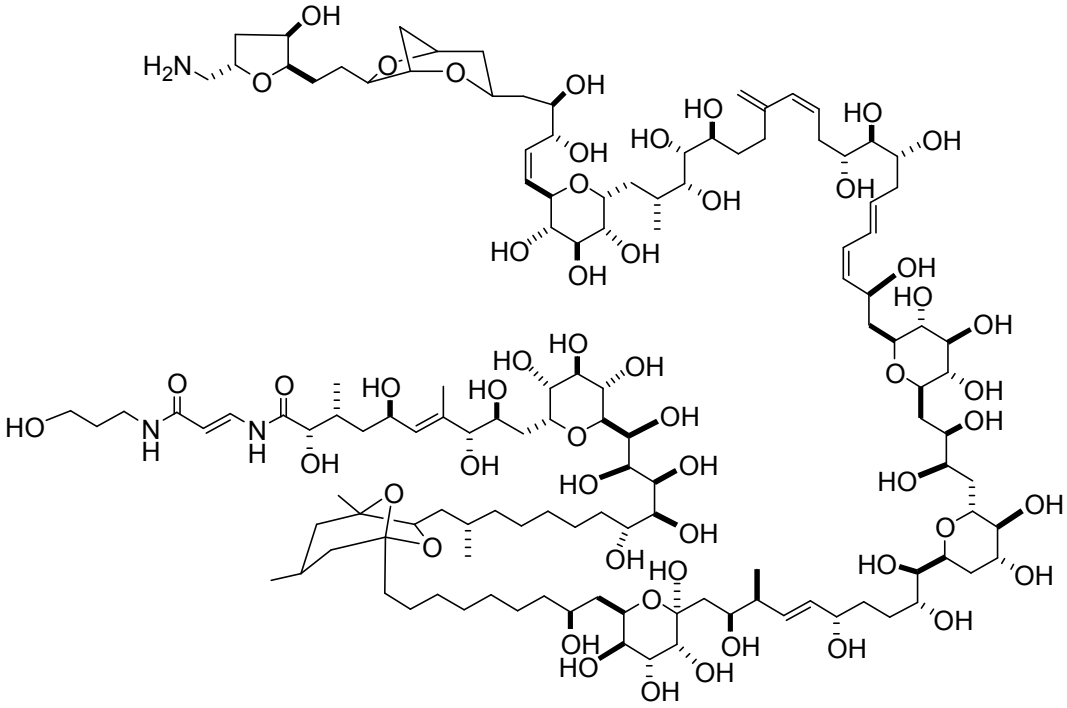

Ciguatoxin (6)



Maitotoxin (7)

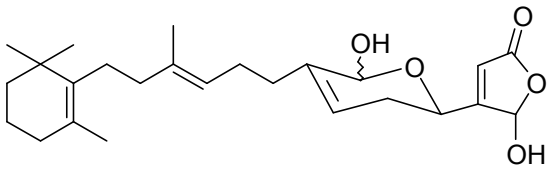


Okadaic acid (8)

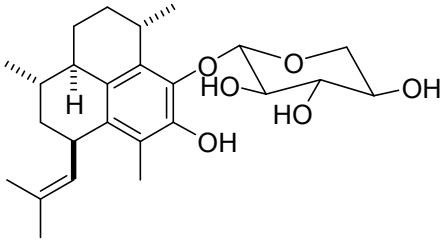


Palytoxin (9)

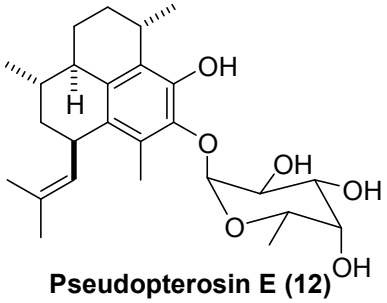
Another interesting structural class is the terpenoids, one of these compounds is the sponge metabolite manoalide (**10**). This compound was originally reported by Scheuer's group as an antibiotic isolated from the marine sponge *Luffariella variabilis*.[18] The groups of Jacobs and Dennis [19,20] independently established that this compound was a potent inhibitor of the enzyme phospholipase A_2. Moreover, two diterpene glycosides showing promising *in vitro* and *in vivo* activities as anti-inflammatory agents[21,22] are the pseudopterosins A (**11**) and E (**12**), compounds isolated from the Caribbean gorgonian, *Pseudopterogorgia elisabethae*.[23,24]



Manoalide (10)



Pseudopterosin A (11)



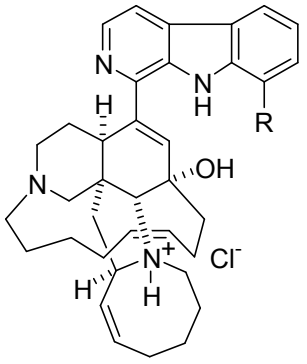
Pseudopterosin E (12)

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 (**13**), 8-hydroxymanzamine A (**14**), its enantiomer (**15**), and a manzamine dimer, *neo*-kauluamine (**16**) gave encouraging results in preliminary *in vivo* tests.[25]

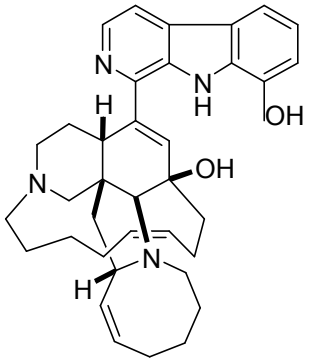
Other marine secondary metabolites that are becoming important as molecular tools are the macrolide swinholide A (**17**),[26] and its analogue misakinolide (**18**),[27] which bind specifically to the intracellular actin network and represent useful tools in investigations of actin organization, dynamics and function.

First reported in 1993, the kahalalides are collection of cytotoxic peptides deriving from a Hawaiian mollusc *Elysia rufescens*.[28] Interestingly, during collection of this herbivorous mollusc for chemical evaluation, *E. rufescens* was observed feeding on a green alga *Bryopsis* sp. Following collection of the alga and purification of its active constituents, the *Bryopsis* sp. yielded the identical chemistry, including kahalalide F, to that isolated from *E. rufescens*. Currently undergoing preclinical evaluation, kahalalide F (**19**) shows selectivity against various solid tumor cell lines.[29] Toxicity data also shows that high concentrations of kahalalide F are toxic to CNS neurons, suggesting a potential use for the

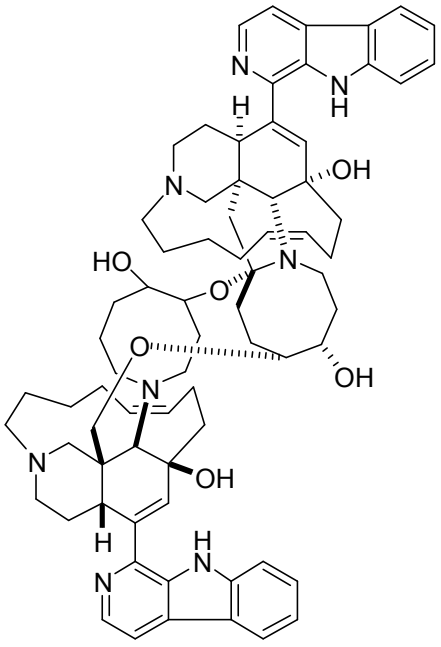
treatment of selected tumors, such as neuroblastoma. Phase I trials are expected to be initiated shortly.



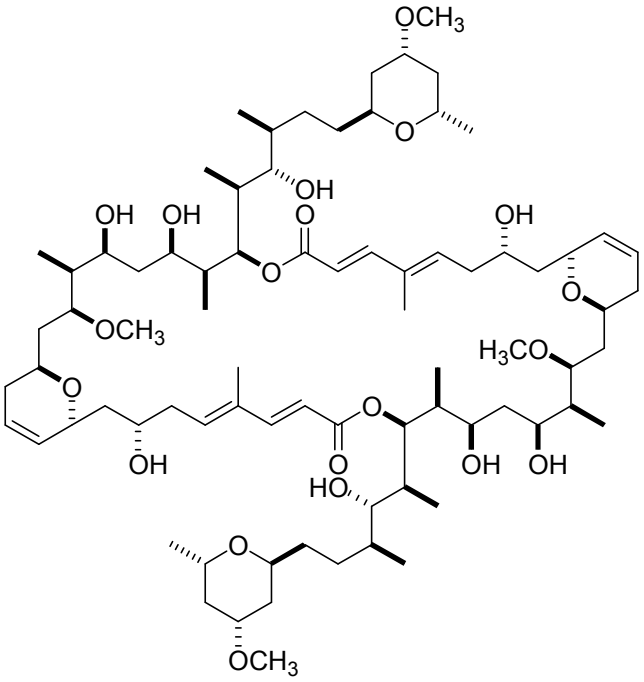
Manzamine A (13) R = H
8-Hydroxymanzamine A (14) R = OH



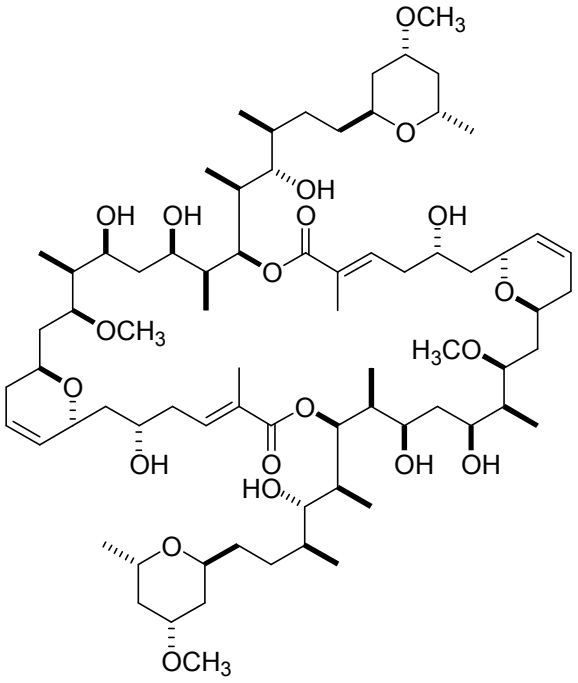
***ent*-8-Hydroxymanzamine A (15)**



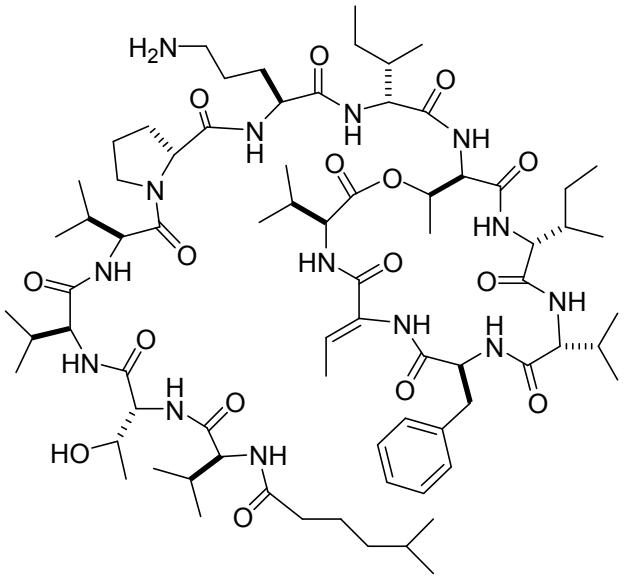
***neo*-Kauluamine (16)**



Swinholide A (17)



Misakinolide (18)



Kahalalide F (19)

Marine Natural Products as Anticancer Drugs

The main focus for marine natural products research has been the pursuit of new anticancer agents. Indeed a large number of metabolites deriving from marine organisms display antimitotic activity, with several candidates currently in clinical or preclinical trials. An illustrative example of the status of marine natural products in anticancer preclinical or clinical trials established by Newman *et al.* shows the diversity offered by marine life[30] (Table 1.1.).

a. Didemnin B from a Tunicate Harboring Diverse Cyanobacterial Symbionts[30]

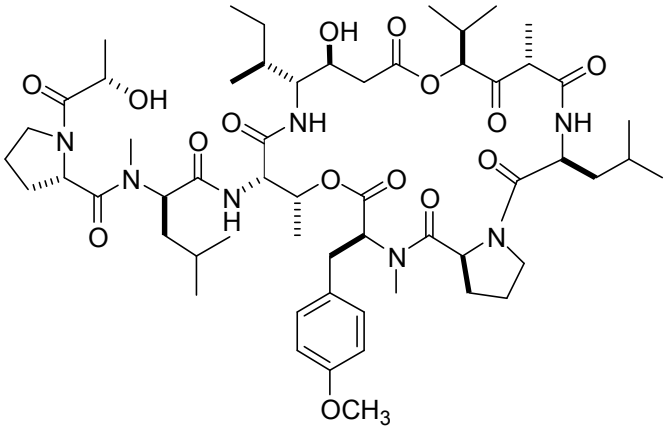
Didemnin B (**20**) ,a cyclic antiproliferative depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum*,[31] was the first marine natural product to enter clinical trial as an antitumor agent.[32] Based on a close structural resemblance of the didemnins to known cyanobacterial metabolites, Rinehart speculated that these potent cytotoxins likely derive from symbiotic cyanobacteria living in association with the tunicate.[33] Didemnin B demonstrated antitumor activity against a variety of models, and has been investigated in Phase II clinical trials for the treatment of breast, ovarian, cervical, myeloma, glioblastoma/astrocytoma, and lung cancers. Moreover, didemnin B displays several *in vitro* biological activities,

albeit with widely varying potencies (>5 orders of magnitude),[34] suggesting that the activities are mediated by different mechanisms. Didemnin B inhibits the synthesis of RNA, DNA and proteins,[35] and binds noncompetitively to palmitoyl protein thioesterase.[36] Moreover, rapamycin inhibits the didemnin-induced apoptosis of human HL60 cells, suggesting activation of the FK-506 apoptotic pathway. Didemnin B perhaps modulates the activity of FK-506 binding proteins as part of its immunomodulatory process and thus leads to cell death via apoptosis.[37]

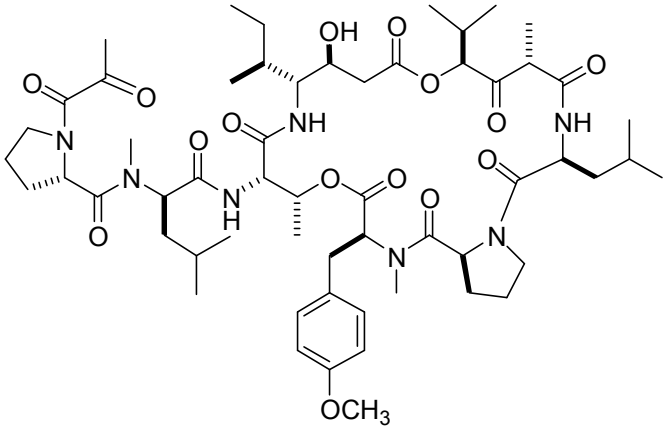
Despite a variety of treatment protocols and testing against many different cancer types, the compound was simply too toxic for use, which led to the termination of trials by the NCI in 1990. The experience gained from these trials led to the synthesis of closely related molecules, such as aplidine (**21**).[38] Similar to didemnin B, aplidine interferes with the synthesis of DNA and proteins and induces cell cycle arrest.[39] Moreover, aplidine possesses a unique and differential mechanism of cytotoxicity which involves the inhibition of ornithine decarboxylase, an enzyme critical in the process of tumor growth and angiogenesis. Furthermore, unlike didemnin B, aplidine blocks protein synthesis at the stage of polypeptide elongation.[40] This cytotoxicity is induced independently of MDR or p53 status and has demonstrated antiangiogenic effects by decreasing the secretion of Vascular Endothelial Growth Factor (VEGF) and reducing the expression of the VEGF-r1 receptor.[41,42] In preclinical studies, aplidine was more active than didemnin B and displayed substantial activity against a variety of solid tumor models, including tumors noted to be resistant to didemnin B. On the basis of its preclinical activity, aplidine entered phase I clinical trials in patients with solid tumors and lymphomas. The success of aplidine in phase I trials has led to its current evaluation in Phase II trials against solid tumors.[43,44]

Table 1. 1. Current status of marine natural products in anti-cancer preclinical or clinical trials.[30,101]

Compound	Source Organism	Chemical Class	Current Status
Ecteinascidin 743 (Yondelis®)	*Ecteinascidia turbinate* (tunicate) (possible bacterial source)	Tetrahydroisoquinolone alkaloid	Phase II
Dolastatin 10	*Dolabella auricularia / Symploca* sp. (mollusc/cyanobacterium)	Linear peptide	Phase II
Bryostatin 1	*Bugula neritina* (bryozoan)	Macrocyclic lactone	Phase II
Synthadotin (ILX651, Dolastatin 15 derivative)	*Dolabella auricularia/ Symploca* sp (synthetic analog)	Linear peptide	Phase II
Kahalalide F	*Elysia rufescens / Bryopsis* sp. (mollusc/green alga)	Cyclic depsipeptide	Phase II
Squalamine	*Squalus acanthias* (shark)	Aminosteroid	Phase II
Dehydrodidemnin B (Aplidine®)	*Trididemnum solidum* (tunicate, synthetic) (possible bacterial/cyanobacterial source)	Cyclic depsipeptide	Phase II
Didemnin B	*Trididemnum solidum* (tunicate)	Cyclic depsipeptide	Phase II (discontinued)
Cemadotin (LU103793, Dolastatin 15 derivative)	*Dolabella auricularia/ Symploca* sp (synthetic analog)	Linear peptide	Phase II (discontinued)
Soblidotin (TZT-1027, Dolastatin 10 derivative)	*Dolabella auricularia/ Symploca* sp (synthetic analog)	Linear peptide	Phase I
E7389 (Halichondrin B derivative)	*Halichondria okadai* (sponge, synthetic)	Macrocyclic polyether	Phase I
NVP-LAQ824 (Psammaplin derivative)	*Psammaplysilla* sp. (sponge, synthetic)	Indolic cinnamyl hydroxamate	Phase I
Discodermolide	*Discodermia dissolute* (sponge)	Lactone	Phase I
HTI-286 (Hemiasterlin derivative)	*Cymbastella* sp. (synthetic analog of sponge metabolite)	Linear peptide	Phase I
LAF-389 (Bengamide B derivative)	*Jaspis digonoxea* (sponge, synthetic)	ε-Lactam peptide derivative	Phase I
KRN-7000 (Agelasphin derivative)	*Agelas mauritianus* (sponge, synthetic)	α-galacosylceramide	Phase I
Curacin A	*Lyngbya majuscula* (cyanobacterium)	Thiazole lipid	Preclinical
DMMC	*Lyngbya majuscula* (cyanobacterium)	Cyclic depsipeptide	Preclinical
Salinosporamide A	*Salinospora* sp. (bacterium)	Bicyclic γ-lactam-β-lactone	Preclinical
Laulimalide	*Cacospongia mycofijiensis* (sponge)	Macrolide	Preclinical
Vitilevuamide	*Didemnin cucliferum / Polysyncration lithostrotum* (tunicates)	Cyclic peptide	Preclinical
Diazonamide	*Diazona angulata* (tunicate)	Cyclic peptide	Preclinical
Eleutherobin	*Eleutherobia* sp. */ Erythropodium caribaeorum* (soft corals)	Diterpene glycoside	Preclinical
Sarcodictyin	*Sarcodictyon roseum* (sponge)	Diterpene	Preclinical
Peloruside A	*Mycale hentscheli* (sponge)	Macrocyclic lactone	Preclinical
Salicylihalimides A and B	*Haliclona* sp. (sponge)	Polyketide	Preclinical
Thiocoraline	*Micromonospora marina* (bacterium)	Depsipeptide	Preclinical
Ascididemin	*Didemnum* sp. (sponge)	Aromatic alkaloid	Preclinical
Variolins	*Kirkpatrickia variolosa* (sponge)	Heterocyclic alkaloid	Preclinical
Lamellarin D	*Lamellaria* sp. (mollusc and various soft corals)	Pyrrole alkaloid	Preclinical
Dictyodendrins	*Dictyodendrilla verongiformis* (sponge)	Pyrrolocarbazole derivatives	Preclinical
ES-285 (Spisulosine)	*Mactromeris polynyma* (mollusc)	Alkylamino alcohol	Preclinical
Dolastatin 15	*Dolabella auricularia* (mollusc)	Linear peptide	Preclinical (discontinued)
Halichondrin B	*Halichondria okadai* (sponge)	Macrocyclic polyether	Preclinical (discontinued)



Didemnin B (20)



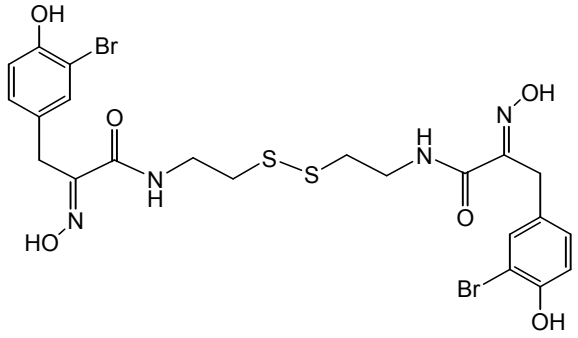
Aplidine (21)

b. Psammaplins from Verongid Sponges

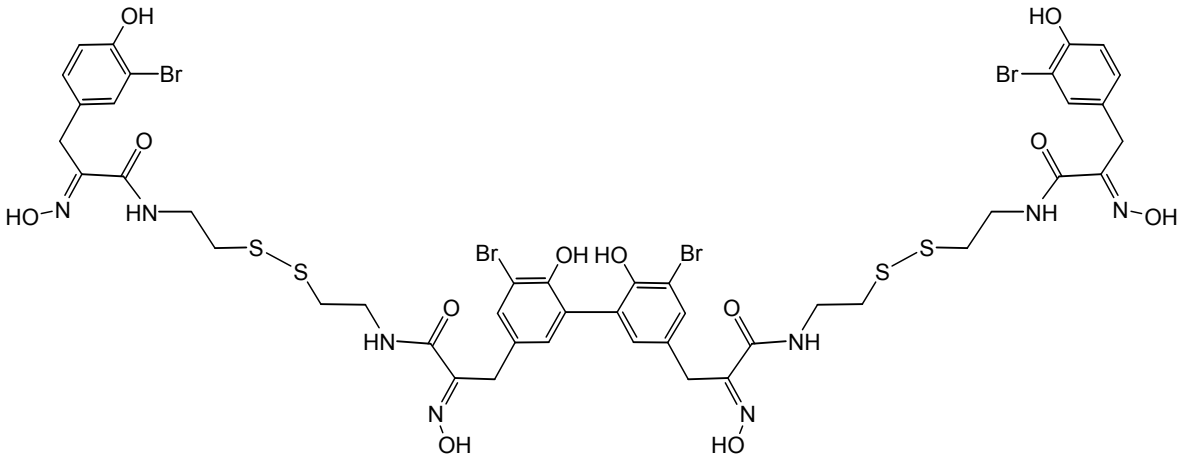
Initial isolations of the bromotyrosine metabolite psammaplin A (**22**) from various verongid sponges (e.g. *Psammaplysilla* sp.) were reported simultaneously by several research groups in 1987.[45,46] Psammaplin A, a symmetrical bromotyrosine disulfide possessing oxime moieties, was found to have potent cytotoxicity to P388 cells (IC_{50} of 0.3 μg/mL), and to co-occur with a dimeric metabolite, biprasin (**23**).[47] Additional psammaplin compounds have since been isolated, including various sulfated and salt derivatives (psammaplins B-L), and the degraded cysteine dimer, prepsammaplin A. Several of these were found to possess potent antibacterial activity. The fact that the psammaplins have been isolated from a diversity of sponge "sources", and that brominated aromatic amino

acid derivatives are common in marine bacteria, suggests that these metabolites may actually derive from biosynthetic pathways of microorganisms living in association with sponges.

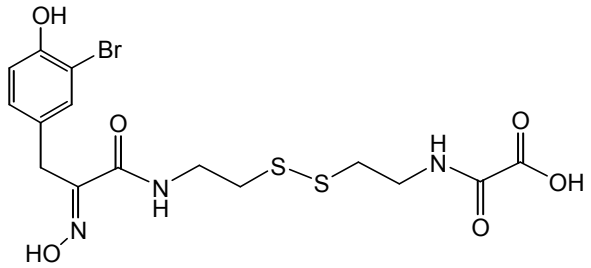
Recently, testing of known and new psammaplin metabolites as DNA methyl transferase (DNMT) and histone deacetylase (HDAC) inhibitors has been reported.[48] Remarkably, psammaplin A and biprasin proved to be dual inhibitors of the two enzymes tested, which is a significant finding in light of the potential relationship between DNMT and HDAC as epigenetic modifiers of tumor suppressor gene activity. In addition, psammaplin F (**24**) is a selective HDAC inhibitor while psammaplin G (**25**) is a selective DNMT inhibitor. Psammaplin A has also been reported to inhibit topoisomerase II,[49] and aminopeptidase N with *in vitro* angiogenesis suppression.[50] However, the physiological instability of the psammaplin class has so far precluded their direct clinical development.



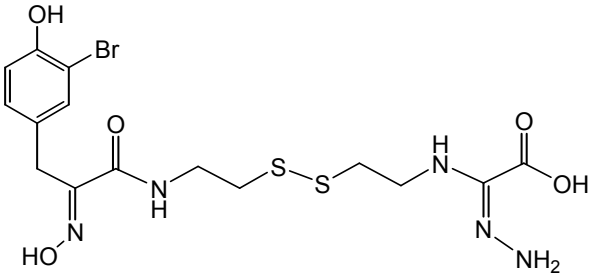
Psammaplin A (22)



Biprasin (23)



Psammaplin F (24)

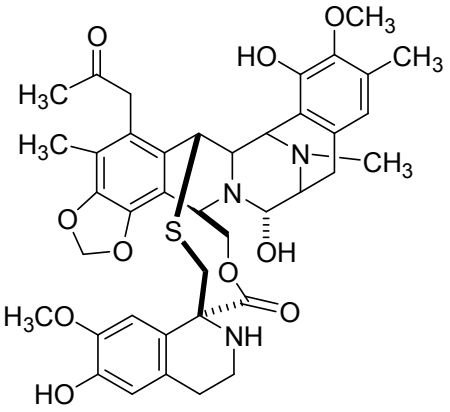


Psammaplin G (25)

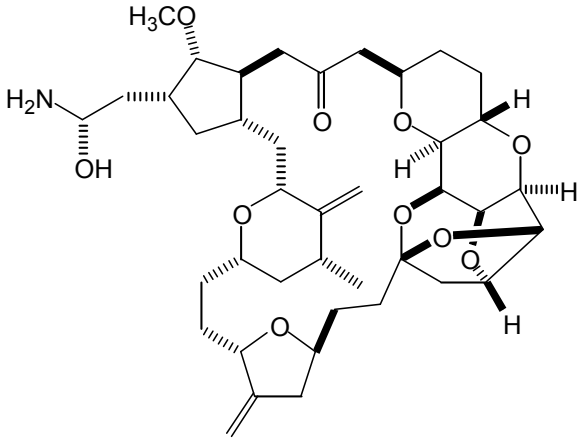
c. Ecteinascidin-743, an Alkaloid from Tunicates Rich in Symbionts

From early surveys of marine organisms for anticancer-type activity, the aqueous extracts of the Caribbean tunicate *Ecteinascidia turbinata* were known to contain potent substances. The molecular structures of the ecteinascidin alkaloids were first deduced as complex tetrahydroisoquinolones.[51,52] Ecteinascidin-743 (**26**) was the major metabolite, and although less potent *in vivo* than its N-demethyl analog (ET-729), its cytotoxicity (IC_{50} 0.5 ng/mL vs L1210 leukemia cells), stability and relatively high natural abundance made it most suitable for clinical development. However, mechanism of action and preclinical *in vivo* evaluation studies were hampered by a lack of material. Large-scale collections, aquaculture and synthetic efforts have all been employed,[53] and culminated in the development of a semisynthesis of ET-743 from cyanosafracin B, which was obtained in bulk through fermentation of the marine bacterium *Pseudomonas fluorescens*. Ecteinascidin's structure is consistent with a natural microbial origin (e.g. the saframycins). Indeed, there are two patents for bacterial symbionts of the tunicate *E. turbinata*. The first focuses on the isolation of the producing microbe,[54] while

the second uses 16S rDNA sequences to identify the endosymbiont as *Endoecteinascidia frumentensis*, the apparent producer of the ecteinascidins.[55]



Ecteinascidin 743 (26)



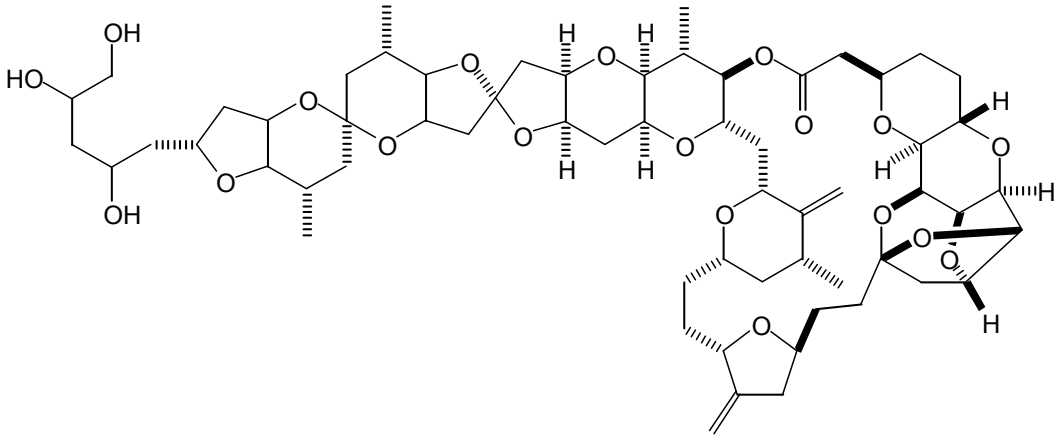
E7389 (27)

d. Halichondrin B, a Complex Polyether from Diverse Sponges

The halichondrins were first isolated from the Japanese sponge *Halichondria okadai* by Uemura and his colleagues and structures determined by X-ray crystallography.[56] Subsequently, halichondrin B (**28**) and several natural analogs were isolated from various unrelated sponges, including *Lissodendoryx* sp., *Phakellia carteri*, and *Axinella* sp., and thus strongly suggests that this skeletal type may be constructed by an associated microorganism. A number of studies subsequently examined their mechanism of cell toxicity, and it was discovered that the halichondrins are potent tubulin inhibitors, in this case non-competitively

binding to the *Vinca* binding site and causing a characteristic G_2-M cell cycle arrest with concomitant disruption of the mitotic spindle.[57,58]

Because of their phenomenal biological activity in killing cancer cells and great structural complexity, the halichondrins rapidly became targets for chemical synthesis. The first total synthesis was completed in 1990.[59] The Kishi group focused on the synthesis of structurally simplified halichondrin analogs which retained or had enhanced biological properties, and this eventually led to the discovery of the clinical candidate E7389 (**27**). Phase I clinical trials with E7389 have been initiated using an accelerated dose escalation schedule to evaluate MTD and pharmacokinetics. Dose limiting toxicity was reached in one patient at a single dose of 0.5 mg/m^2, and a 3-compartment model best described the plasma pharmacokinetics. Plasma levels of E7389 in excess of those required for cytotoxicity were observed in all patients for up to 72 hours, and patients with solid tumors are currently being recruited for additional Phase I trials.[60,61]



Halichondrin B (28)

e. Dolastatin 10 from Sea Hares and their Cyanobacterial Diets

In the early 1970's Pettit and co-workers discovered the extremely potent anti-cancer properties of extracts from the sea hare *Dolabella auricularia.* However, due to the vanishingly small abundance of the active principle (~ 1.0 mg/100 kg of collected organism), the structure elucidation of dolastatin 10 (**29**) took nearly fifteen years to complete. The low concentrations of dolastatin 10 in

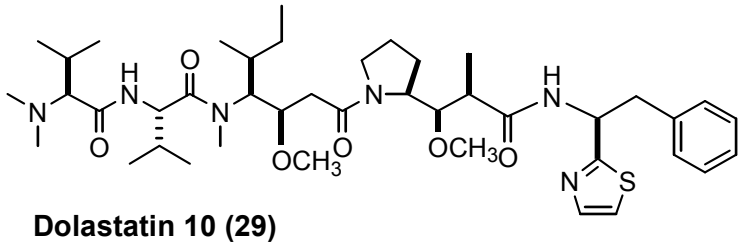
sea hares implicates a cyanobacterial diet as the origin of this bioactive secondary metabolite,[62] and this was subsequently confirmed by direct isolation of dolastatin 10 from field collections of the marine cyanobacterium *Symploca*.[63] Dolastatin 10 is a pentapeptide with four of the residues being structurally unique (dolavaline, dolaisoleucine, dolaproline, and dolaphenine, in addition to valine). Interestingly, at the time of its discovery, it was the most potent antiproliferative agent known with an ED_{50} = 4.6 x 10^{-5} μg/mL against murine PS leukemia cells.[64] Subsequently, dolastatin 10 was shown to be a potent noncompetitive inhibitor of *Vinca* alkaloid binding to tubulin (K_i 1.4 μM), and strongly affected microtubule assembly and tubulin-dependent GTP hydrolysis.[65] Further work revealed that dolastatin 10 binds to the rhizoxin/maytansine binding site[66] (adjacent to *Vinca* alkaloid site) as well as to the exchangeable GTP site on tubulin, causing cell cycle arrest in metaphase.[67]

Dolastatin 10 entered Phase I clinical trials in the 1990's through the National Cancer Institute and progressed to Phase II trials. Unfortunately, it was dropped from clinical trials, as a single agent, due to the development of moderate peripheral neuropathy in 40% of patients[68] and insignificant activity in patients with hormone-refractory metastatic adenocarcinoma[69] and recurrent platinum-sensitive ovarian carcinoma.[70] Nevertheless, dolastatin 10 offered a logical starting point for SAR studies and synthetic drug design, ultimately leading to the analog TZT-1027 (**30**).

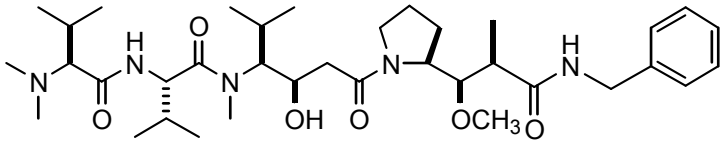
f. Dolastatin 15, another cyanobacterial peptide isolated from Dolabella auricularia

Dolastatin 15 (**31**) was also isolated from extracts of the Indian Ocean sea hare *Dolabella auricularia* in trace amounts (6.2 mgs from 1600 kg of wet sea hare 4 x 10^{-7} %), again strongly implicating a cyanobacterial source for this metabolite. Indeed, numerous dolastatin 15-related peptides have been isolated from diverse marine cyanobacteria.[71] Its linear depsipeptide sequence is composed of seven amino- or hydroxy-acid residues. In initial bioassays with the NCI's P388 lymphocytic leukemia cell line, dolastatin 15 displayed an ED_{50} = 2.4 x 10^{-3} μg/mL.[72] In contrast to dolastatin 10, dolastatin 15 binds directly to the *Vinca* domain of tubulin.[73] Obstacles to further clinical evaluation of dolastatin 15 include

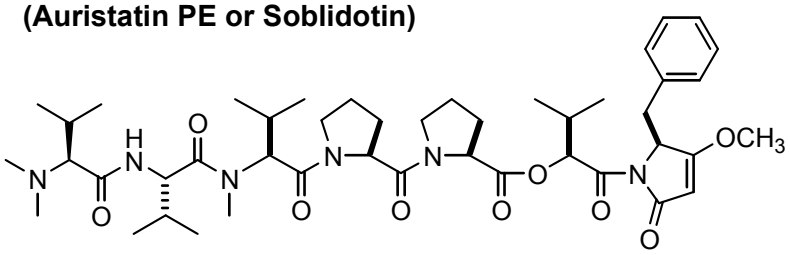
the complexity and low yield of its chemical synthesis, and its poor water solubility. However, these impediments have prompted the development of various synthetic analog compounds with enhanced chemical properties, including cemadotin (**32**) and synthadotin (**33**).



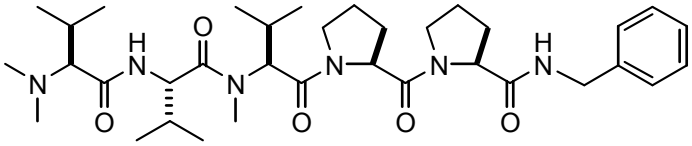
Dolastatin 10 (29)



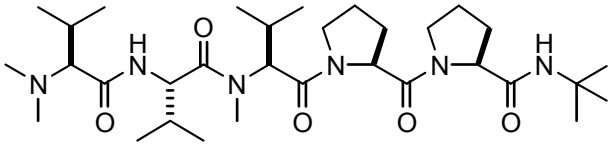
TZT-1027 (30)
(Auristatin PE or Soblidotin)



Dolastatin 15 (31)



LU 103793 (32)
(Cemadotin)



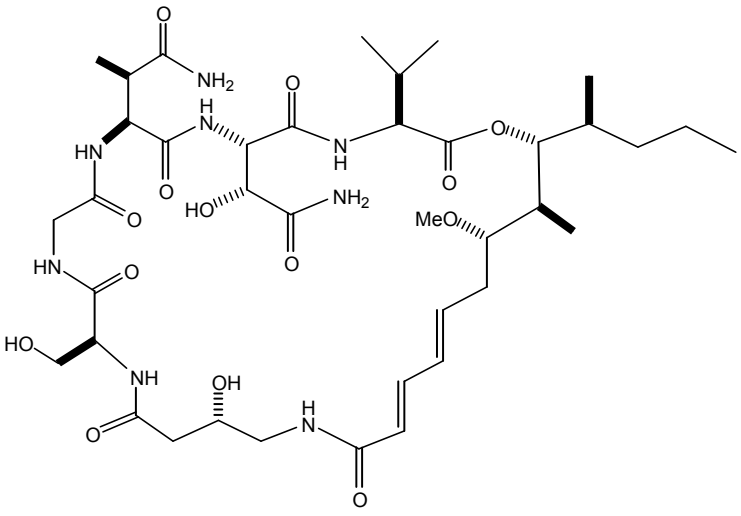
ILX651 (33)
(Synthadotin)

Bioactive Metabolites from Marine Invertebrates

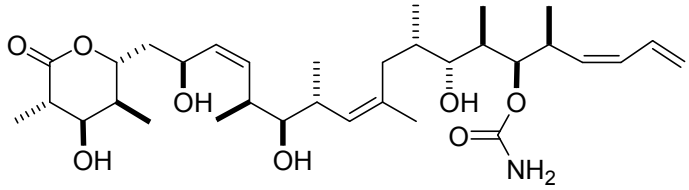
Because of their prevalence and ease of collection, sponges have been the dominant source of isolated marine natural products. Though they are the simplest of the multicellular animals, appearing on earth more than 500 million years ago, sponges produce some of the most intriguing and biologically active chemistry. One of them is the marine sponge of the genus *Thoenella* which is a rich source of unusual peptides possessing such biological activities as antibacterial, enzyme inhibitor, antifungal, and anti-HIV.[74,105]

Nagahamide A (**34**),an antibacterial compound isolated by Yumika *et al.* from the marine sponge *Theonella swinhoei*, has a seven-residue depsipeptide containing three unusual amino acids and a polyketide portion.[74]

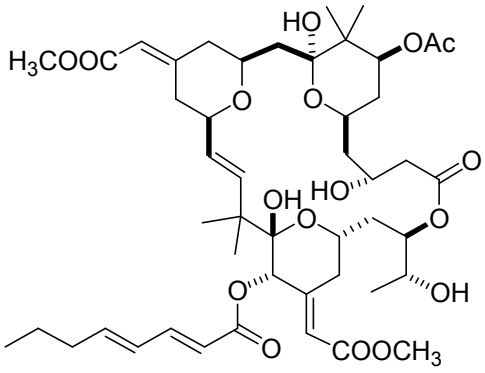
Another example of a novel metabolite includes the cytotoxin (+)-discodermolide (**35**) isolated from the sponge *Discodermia dissoluta* as a potent immunosuppressive agent and inducer of tubulin polymerization.[75] Discovered by researchers at Harbor Branch Oceanographic Institute, discodermolide was licensed to Novartis Pharmaceutical Corporation in 1998 for further development. Continued research focused on the preparation and evaluation of natural and synthetic analogs that may lead to more efficient syntheses and a better understanding of structure-activity relationships within this structurally novel class of antimitotics.[76]



Nagahamide A (34)



Discodermolide (35)



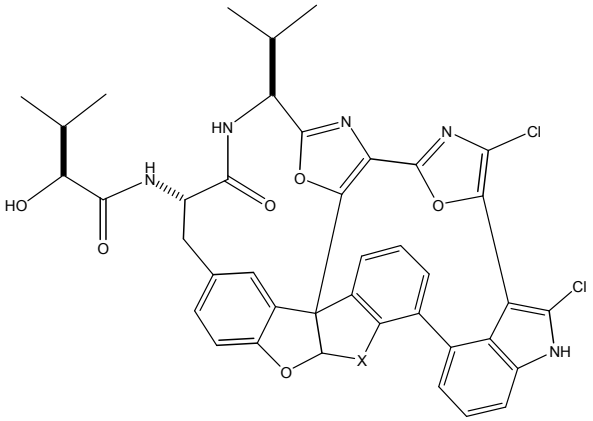
Bryostatin 1 (36)

Among the more promising bioactive natural products is bryostatin 1 (**36**), a polyketide first isolated in minute quantity from the bryozoan, *Bugula neritina,* in the 1970's.[77] Bryostatin 1 is currently in phase II clinical trials for the treatment of various leukemias, lymphomas, melanoma, and solid tumors.[78] 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.[79]

Ascidians, commonly referred to as tunicates or sea squirts, have been another tremendous source of novel bioactive natural products. Recent studies on the compound diazonamide A (**37**),[80] isolated from the marine ascidian *Diazona angulata*, revealed that it causes cells to arrest in mitosis. Both diazonamide A and the oxygen analog (**38**) are potent inhibitors of microtubule assembly, equivalent in activity to dolastatin 10 and far more potent than dolastatin 15.

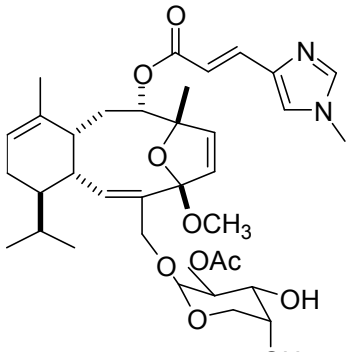
In 1993, off the northeastern coast of Australia, a new soft coral *Eleutherobia albicans* was identified. The bioactive natural product produced by this organism, eleutherobin (**39**), has proven to be one of the most potent cytotoxins discovered to date, displaying *in vitro* cancer cell inhibition with an IC_{50} range of 10-15 nM.[81] The related sarcodyctyins (sarcodyctin A, **40**) were detected

earlier in the Mediterranean Sea from a different coral species, *Sarcodictyon roseum*.[82]

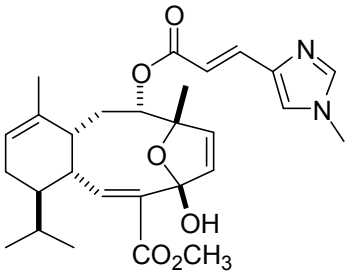


X = NH Diazonamide A (37)
X = O oxygen analog (38)

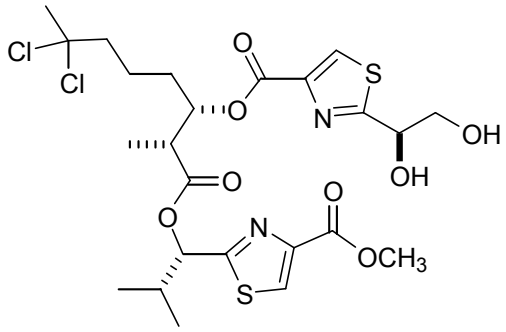
The sea hare, *Dolabella auricularia* has yielded several cytotoxic agents that share similarity with cyanobacterial metabolites, including the anticancer agent dolastatin 10 and the cytotoxic agent, dolabellin (**41**).[83]



Eleutherobin (39)



Sarcodictyin A (40)

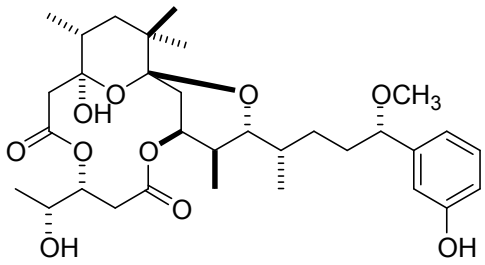


Dolabellin (41)

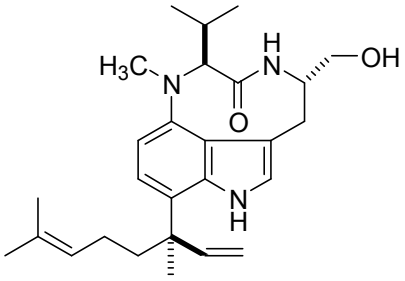
Uncommon chemistry of cyanobacterial metabolites

Cyanobacteria, the most ancient of the microalgae, are prokaryotic photosynthetic organisms which have been identified as a rich source of unusual bioactive secondary metabolites. The natural products chemistry of marine cyanobacteria is extraordinarily diverse, and directly reflects the exceptional biosynthetic capacities of these organisms. The major theme in cyanobacterial chemistry is the production of polypeptides modified with various lipid components to make diverse lipopeptides. Unusual functional groups, including heterocyclic rings, oxidations at unusual positions, and halogenations utilizing bromine, chlorine, and occasionally iodine, are introduced into these lipopeptide scaffolds by action of numerous tailoring reactions. The resulting metabolites show a diversity of potent biological activities and include a number with powerful anti-tubulin or anti-actin properties; hence, they have utility in the treatment of proliferative disorders such as cancer.

Collections of strain variants of *Lyngbya majuscula* from around the world have already yielded over 200 different compounds. Two highly inflammatory but structurally distinct metabolites, debromoaplysiatoxin (**42**)[84] and lyngbyatoxin A (**43**),[85] were isolated from a Hawaiian strain of *L. majuscula* as the agents responsible for causing severe contact dermatitis. Both classes of compounds were found to bind the phorbol ester receptor on protein kinase C (PKC) and have played an important role in the study of PKC signal transduction.

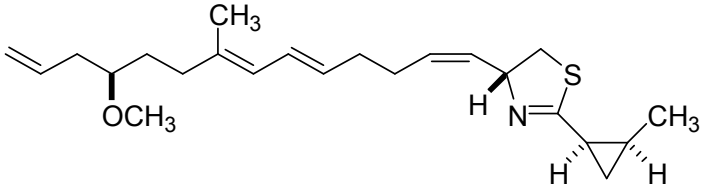


Debromoaplysiatoxin (42)



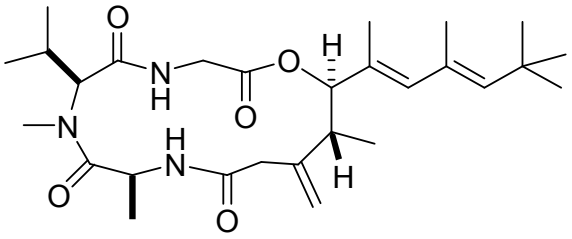
Lyngbyatoxin A (43)

Curacin A (**44**) is a novel thiazoline-containing lipid isolated from the marine cyanobacterium *Lyngbya majuscula*. Discovered originally through its toxicity to brine shrimp, curacin A and its structural analogues have shown in preclinical studies a potent microtubule polymerization inhibition through interaction at the colchicine binding site.[86] Recent efforts have mainly focused on development of synthetic curacins with greater stability and water solubility.[87] Because of its simplicity, therapeutic versions of an active curacin agent would be relatively inexpensive to produce.



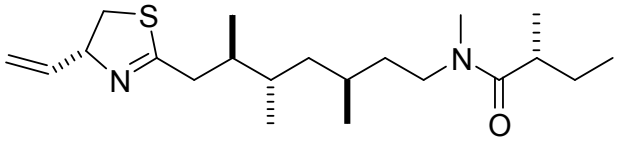
Curacin A (44)

A major metabolic theme in *L. majuscula* chemistry is the combination of polyketide synthase and non-ribosomal peptide synthetase biosynthetic pathways to produce a seemingly limitless number of possible secondary metabolites. One significant structural example is antillatoxin (**45**), a novel lipopeptide first reported for its potent ichthyotoxic activity[88] and later shown to depolarize membranes through activation of voltage sensitive sodium channels.[89,90] Antillatoxin appears to activate sodium channels through a unique binding site, adding to the array of probes available for sodium channel investigations.



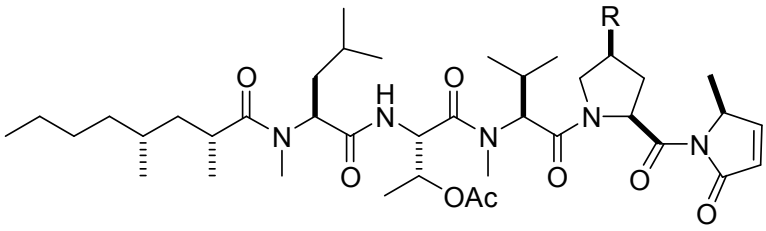
Antillatoxin (45)

The extract of a *L. majuscula* collection from Playa Kalki, Curaçao, displayed both ichthyotoxicity and brine shrimp toxicity. Bioassay-guided fractionation led to the discovery of kalkitoxin (**46**), as the active constituent. Subsequent screening against mouse neuro-2a neuroblastoma cells has established kalkitoxin as a powerful blocker of sodium channels. [90,91]



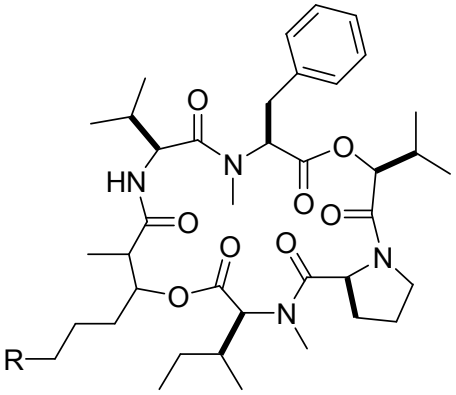
Kalkitoxin (46)

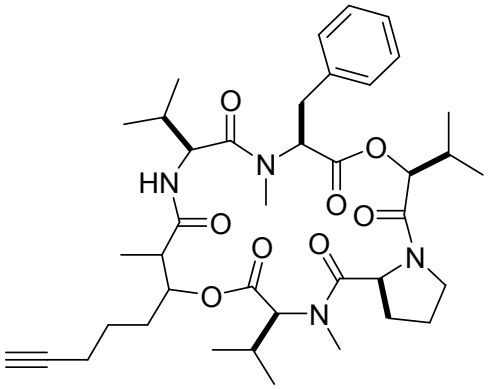
An alternation of PKS and NRPS modules is presumed to be involved in formation of the acyclic microcolins (**47**, **48**), bioactive peptides reported in 1992 from a Venezuelan *L. majuscula* collection.[92] *In vitro* results suggest that the antiproliferative and immunosuppressive properties of both compounds are possibly associated with apoptosis-inducing events, implying an additional potential for their development as antineoplastic agents.[93]



Microcolin A (47) R = OH
Microcolin B (48) R = H

Not only linear but also cyclic peptides are found in the chemistry of *L. majuscula.* Examination of a *Lyngbya majuscula* collection from Antany Mora, Madagascar,[94] led to the isolation of a series of depsipeptides, antanapeptins A-D (**49-52**) along with dolastatin 16 (**53**), a promising antineoplastic metabolite first reported from the marine mollusc *Dolabella auricularia.*



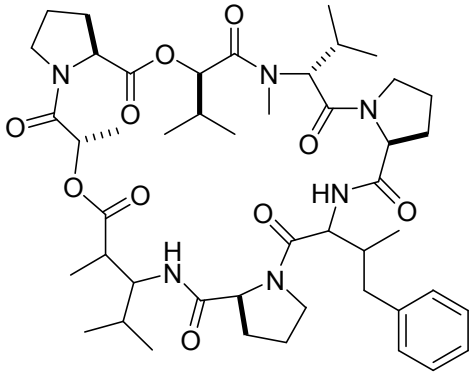


Antanapeptin A (49) **R =** 

Antanapeptin B (50) **R =** 

Antanapeptin C (51) **R =** 

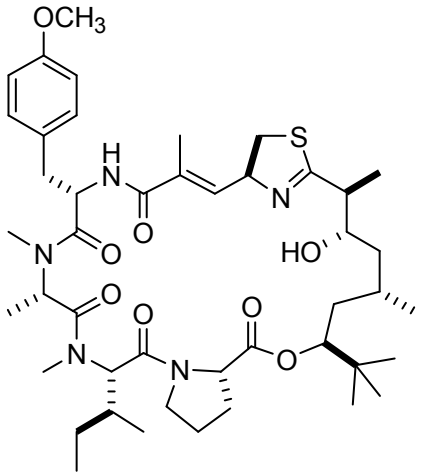
Antanapeptin D (52)



Dolastatin 16 (53)

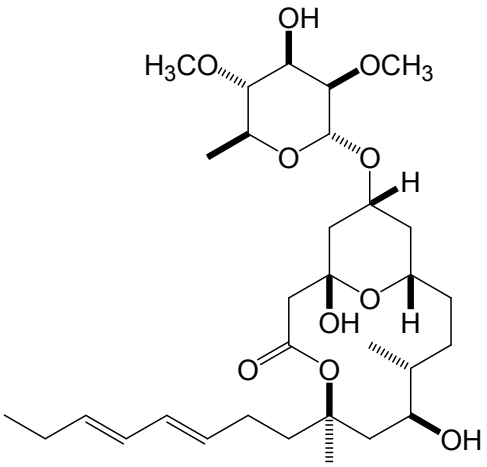
Apratoxin A (**54**), a potent cytotoxin with a novel skeleton, has been isolated from the marine cyanobacterium *Lyngbya majuscula.* This cyclodepsipeptide of mixed peptide-polyketide biogenesis bears a thiazoline ring flanked by polyketide portions, one of which possesses an unusual methylation pattern. Apratoxin A possesses IC_{50} values for in vitro cytotoxicity against human

tumor cell lines ranging from 0.36 to 0.52 nM; however, it was only marginally active in vivo against a colon tumor and ineffective against a mammary tumor.[95]



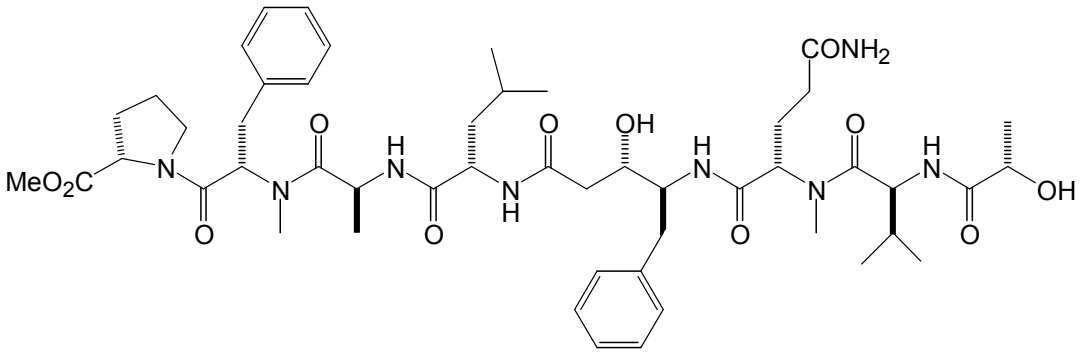
Apratoxin A (54)

Lyngbouilloside (**55**), a novel glycosidic macrolide isolated from the marine cyanobacterium *Lyngbya bouillonii* collected from Papua New Guinea, has close structural features to metabolites found in various marine invertebrates. This suggests that these “marine invertebrate-derived“substances actually derive from marine cyanobacteria, either through sequestration in the diet (e.g. mollusks) or through symbiotic associations (e.g. sponges).[96]



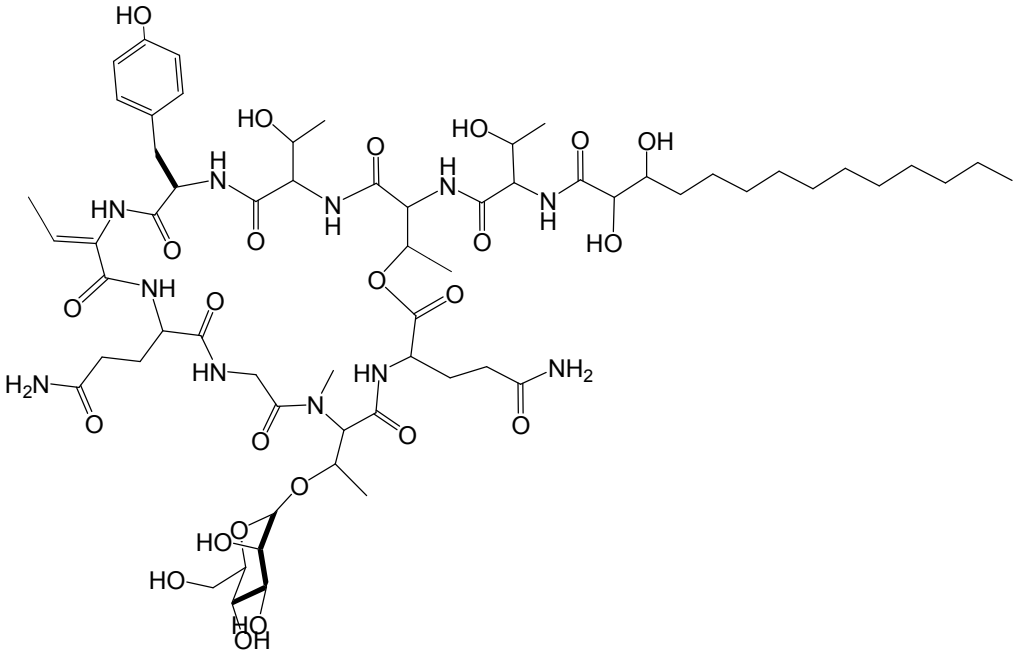
Lyngbouilloside (55)

Tasiamide B (**56**) a new cytotoxic peptide, which displayed an IC_{50} value of 0.8 μM against KB cells, has been isolated from the marine cyanobacterium *Symploca* sp.[97] The interesting structural feature of tasiamide B is the incorporation of an Ahppa (4-amino-3-hydroxy-5-phenylpentanoic acid) unit; this is a structural motif that has been known as a component of protease inhibitors from *Candida* and *Streptomyces* spp. and has also been widely utilized in the field of peptidomimetics. The incorporation of modified amino acids, formed by a combination of polyketide synthases and nonribosomal peptide synthetases, is one of the reasons investigations of these organisms remain so fruitful.



Tasiamide B (56)

Hassallidin A (**57**), a new antifungal glycosylated lipopeptide, was isolated from an epilithic cyanobacterium collected in Bellano, Italy, indentified as *Tolypothrix* (basionym *Hassallia*) species. This compound represents a new structural type due to the co-occurrence of cyclic peptide, fatty acid, and carbohydrate moieties and, thus, with respect to a recent evaluation of topology of cyanobacterial secondary metabolites, represents a new molecular archetype. Antifungal peptides known so far are linear or cyclic peptides and lipopeptides, but lack glycosyl residues.[98]

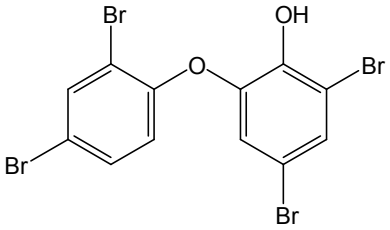


Hassallidin A (57)

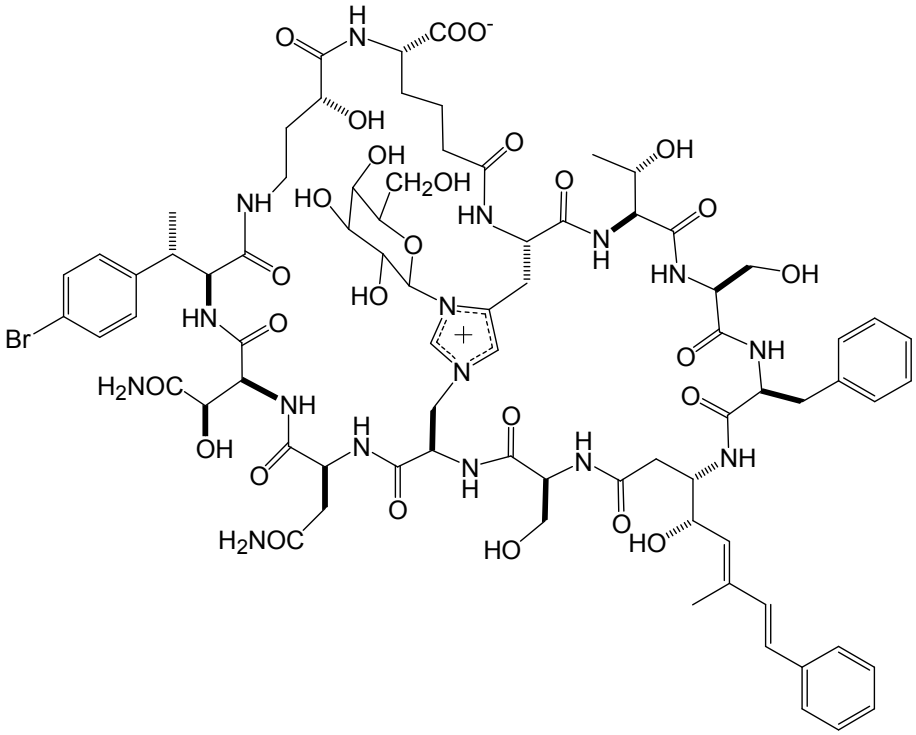
Bioactive metabolite symbioses

Many of the natural products isolated from marine invertebrates share structural homology with compounds of microbial origin, leading to the hypothesis that the marine compounds are actually produced by microorganisms (e.g. microalgae, cyanobacteria, and heterotrophic bacteria) living in association with the invertebrates (e.g. sponges, tunicates, and soft corals). Marine invertebrates are indeed largely sessile, filter-feeding organism that contain a complex assemblage of symbiotic microorganism. Thus, the real producer of biologically active compounds from marine invertebrates is always uncertain.

Detailed investigation of bioactive metabolite symbioses is a field that is still in its infancy. Every organism and every symbiotic system requires effort to understand the unique problems it presents. D. John Faulkner maintained an active interest in this research area, leading his group in many studies of symbiosis and marine natural products.[99] Under his guidance, brominated diphenyl ethers (**58**) were found in cyanobacterial symbionts of the sponge *Dysidea herbacea.* The potent polyketide cytotoxin swinholide A (**17**) was discovered in a mixed unicellular bacterial fraction of the marine sponge *Theonella swinhoei,* while



2',4,4',6-tetrabromo-2-phenoxyphenol (58)



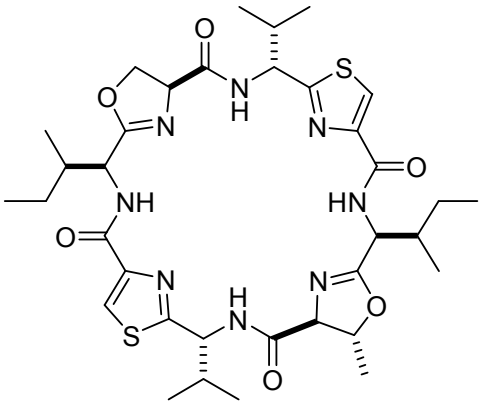
Theopalauamide (59)

a hybride peptide-polyketide, theopalauamide (**59**), was isolated from filamentous bacteria from the same sponge.

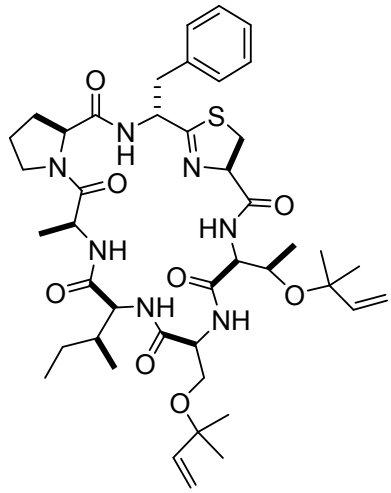
In some cases metabolites can be localized to either the symbiont or the host cells. Although it is logical to assume that the location of a compound reflects its site of origin, a compound may diffuse or be exported elsewhere, after synthesis. Free-living microbes often transport antibiotics that they synthesize out of their cells in order to protect themselves. Thus, the site of synthesis may not be where the metabolite is finally localized. The possibility that compounds are synthesized in another cell type and translocated to those storage cells cannot be ruled out.[100]

The most unequivocal evidence to demonstrate a symbiont origin of secondary metabolites is to culture the microbe and show production of the compound. Besides that, identification and isolation of the biosynthetic genes from the symbiont is a definitive proof.[101]

An illustrative example is the case of bioactive cyclic peptides, patellamide A (**60**) and trunkamide (**61**), from the ascidian *Lissoclinum patella.* Because these cyclic peptides resemble cyanobacterial metabolites and it is known that ascidians house cyanobacterial symbionts of the genus *Prochloron,* there is a high likelihood that *Prochloron* is the producer of these cyclic peptides. However, Salomon and Faulkner[102] demonstrated that the patellamides accumulate in the tunic tissue of the ascidian and are not associated with the cyanobacterial symbiont. In light of these contradictory results, it cannot be ruled out that the compounds are in fact produced and secreted by the cyanobacterial symbiont where they would then accumulate in the ascidian tissues. Indeed, Schmidt et al recently identified the patellamide A and C biosynthesis genes and confirmed their function, it was found to be a ribosomal peptide biosynthetic pathway (microcin-like pathway) in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinum patella.*[103,106] In addition, a related cluster was indentified in *Trichodesmium erythraeum* IMS101, an important bloom-forming cyanobacterium.[106]

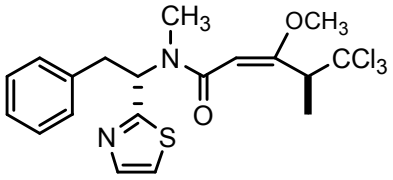


Patellamide A (60)

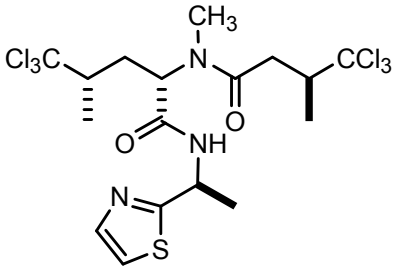


Trunkamide A (61)

Another example is the cloning of the gene cluster responsible for the biosynthesis of the chlorinated peptide barbamide (**62**) which has an identical trichloromethyl group as that of found in dysidenin (**63**), a metabolite isolated from the sponge *Dysidea herbacea*. The study of cell separation by flow cytometry technique has demonstrated that the chlorinated metabolites are associated with the cyanobacterial symbiont. Further more, isolation of the biosynthetic gene cluster from the sponge-symbiont biomass revealed that it contains similar gene as that of found to the barbamide biosynthetic gene cluster (*bar*). Localization of *bar*-like halogenase biosynthetic genes to the *O. spongellieae* symbiont were performed by Fluorescence in situ hybridization (FISH) techniques and demonstrated that the biosynthetic capacity to produce chlorinated peptides resided within the cyanobacterial cells.[104]



Barbamide (62)



Dysidenin (63)

GENERAL THESIS CONTENTS

The search for new and useful natural products from marine algae has been the main focus of our laboratory and of the research presented within this Ph.D. thesis. Our extensive collections of marine algae are the results of continual expeditions to different parts of the world. Madagascar was one of the collection sites which was revisited three times for sample collections and has been fruitful. 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 Madagascar marine algae and cyanobacteria.

Following the general introduction to marine natural products included in this first chapter, the second chapter describes the structure determination and biological activities of four novel macrolides, ankaraholides (A-D) isolated from a cyanobacterium *Geitlerinema* sp. collected in 2000 from Nosy be Island, Madagascar. 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). Ankaraholides were found to be potent cancer cell growth inhibitors with IC_{50} values ranging from 0.1 μM to 0.3 μM against human lung cancer (NCI-H460) and mouse neuro-2a cell lines and exert their cytotoxic effect by disruption of the actin cytoskeleton.

Chapter three presents the isolation and identification of new halogenated monoterpenes from the red marine alga *Portieria hornemannii* collected in 1997 from Tolagniaro, Madagascar. Three new halogenated monoterpenes along with the known compounds halomon and its two analogues were isolated and characterized using NMR spectroscopy in combination with mass spectral data analysis. These compounds were evaluated in a DNMT-1 enzyme inhibition assay.

The fourth chapter discusses the isolation and characterization of tanikolide dimer, a novel SIRT2 inhibitor, and tanikolide seco acid, isolated from the marine cyanobacterium *Lyngbya majuscula* collected in 1997 from Nosy be, Madagascar.

The molecular structure of tanikolide dimer was established by comprehensive NMR and MS spectroscopic analysis whereas the structure of tanikolide seco acid was elucidated by an X-ray experiment. Tanikolide dimer inhibited SIRT2 with an IC_{50} = 176 nM.

In chapter five, a bioassay guided investigation (HDAC inhibitor assay) of a brown marine alga *Dictyota* sp. led to the discovery of new tricyclic diterpene. The structure of this compound was deduced by NMR spectroscopy along with extensive masse spectroscopy analysis. This novel HDAC inhibitor was also found to possess antimalarial activity.

Chapter six details the isolation and structure determination of mitsoamide, a cytotoxic linear peptide with an unusual structure by virtue of a piperidine amine (aminal) moiety and the incorporation of a homolysine residue next to an uncommon polyketide portion. All partial structures were confirmed by the application of the 1D TOCSY pulse sequence. The planar structure of mitsoamide was established by an extensive analysis of 1D and 2D NMR spectroscopy data in addition to amino acid sequencing by MS/MS data.

The thesis ends with a concluding chapter that contains a brief summary of the projects described within the text as well as comments on the structural trends that are emerging in marine algal and cyanobacterial secondary metabolites.

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CHAPTER TWO

ANKARAHOLIDES, GLYCOSYLATED DERIVATIVES OF SWINHOLIDES FROM THE MADAGASCAR MARINE CYANOBACTERIUM *GEITLERINEMA* SP.

ABSTRACT

Chemical investigation of Madagascar marine cyanobacteria *Geitlerinema* sp. has led to the discovery of four new cytotoxic natural products, ankaraholides A (**1**), B (**2**), C(**3**) and D (**4**). The molecular structures of these compounds were established using NMR spectroscopy and extensive mass spectral analyses. These compounds are found to be glycosylated derivatives of the known compound swinholide A, which was recently isolated from a Fijian cyanobacterium of the genus *Symploca* cf. sp.[14] Since swinholide-type compounds were previously localized to the heterotrophic bacteria of sponges, these findings raise intriguing questions about their true metabolic source.

INTRODUCTION

In our ongoing search for new biologically active secondary metabolites from marine algae and cyanobacteria, bioassay-guided investigation of cyanobacterium, *Geitlerinema* sp. from a Madagascar field collection, resulted in the isolation of four new glycosylated swinholides, ankaraholides A (**1**), B (**2**), C(**3**) and D (**4**). Swinholide A was originally isolated by Kashman and Carmely from the marine sponge *Theonella swinhoei*.[1] The structure was first described as a monomer but subsequently recognized as a dimer by Kitagawa and co-workers.[2] Later, the absolute stereostructure of swinholide A was established,[3] and the related derivatives swinholide B-H, isoswinholide A, bistheonellides A[4] and B, and a monomeric seco acid of swinholide A were isolated from marine sponges belonging to the genera *Theonella*, *Lamellomorpha*, and *Tedania*.[5] Our report here is the first isolation of this compound class from field collections[14] of marine cyanobacteria and gives new insight into the metabolic source of these compounds in the various sponge-microorganism assemblages from which they have been reported.

RESULTS AND DISCUSSION

The cyanobacteria sample of *Geitlerinema* sp. were collected from Nosy Mitso-ankaraha Island Madagascar. Organic extracts CH_2Cl_2/MeOH (2:1) of this cyanobacteria was potently toxic to brine shrimp. Chromatographic separation using normal-phase silica VLC and C_{18} reverse-phase SPE and HPLC, all monitored using brine shrimp toxicity, yielded four new compounds, ankaraholides A (**1**), B (**2**), C(**3**) and D (**4**).

The molecular formula of **1**, $C_{90}H_{152}O_{28}$, was deduced by accurate high-resolution mass measurement. Comparison of its ^{1}H and ^{13}C NMR spectral data with those for swinholide A (see Table 2.1.) clearly showed that it was related to the swinholides. However, **1** showed only 10 upfield methyl resonances (two each at δ 0.88, 0.94, 1.01, 1.22, and 1.84), while swinholide A possesses 12 upfield methyl groups; thus, compound **1** was a bis-normethyl derivative of swinholide A. Extensive one- and two-dimensional NMR studies of **1** revealed that the C16/C-16' methyl groups of swinholide A were missing. Furthermore, compound **1** showed additional resonances in the ^{13}C NMR spectrum at δ 58.8 (CH_3), 60.0 (CH_3), 62.8 (CH_2), 73.0 (CH), 79.6 (CH), 84.0 (CH), and 103.0 (CH), which suggested the presence of a bis-methoxylated sugar moiety. On the basis of vicinal $^{3}J_{H,H}$ coupling constants and correlations in the ^{1}H-^{1}H COSY, HMBC, and ROESY spectral data, the sugar moiety was identified as 2,3-di-*O*-methyl-β-lyxopyranoside (Figure 2.8). The linkage between the aglycon portion and the sugar unit was determined by an HMBC correlation between H-32 and C-7, as well as several ROESY correlations. The relative stereochemistry of the aglycon portion was based on the close similarity of ^{1}H and ^{13}C NMR shifts and $J_{H,H}$ values between swinholide A and **1** and confirmed by ROESY correlations. The relative stereochemistry of the sugar and aglycon was determined by ROESY cross-peaks between H-32 and H-7 and between H-36α and H-8α, thereby establishing the relative stereostructure of ankaraholide A (**1**) as shown.

The high-resolution MALDI-TOF mass spectrum of compound **2** showed that the molecular formula ($C_{91}H_{154}O_{28}$) was 14 mass units higher than that of ankaraholide A (**1**). In addition, the ^{1}H NMR spectrum contained a new doublet methyl resonance at δ 0.86, which showed correlations with H-16' in the ^{1}H-^{1}H COSY spectrum and with C-15', C-16', and C-17' in the HMBC spectrum. Therefore, ankaraholide B (**2**) possessed an additional methyl group at the C-16' position relative to ankaraholide A (**1**).

The low and high-resolution MALDI-TOF mass spectra of compound **3** showed that **3** has the same molecular weight and formula as ankaraholide A (**1**). Comparison of the ^{1}H and ^{13}C NMR spectral data with those for ankaraholide A (**1**) revealed that one methyl group was shifted (δ 0.86)(data recorded in CD_3OD). A ^{1}H-^{1}H COSY correlation was seen between this methyl group (δ 0.86) and the proton at H-16 (δ 1.67). Long-range ^{13}C-^{1}H HMBC correlations were observed between the following carbons: C-15 (δ 78.1), C-16 (δ 41.71) and C-17 (δ 73.0) and the methyl group (δ 0.86). From these results it was deduced that ankaraholide C (**3**) was an isomer of ankaraholide A (**1**) with a methyl group attached to C-16/C-16' instead of C-20/C-20' as that of seen in **1**.

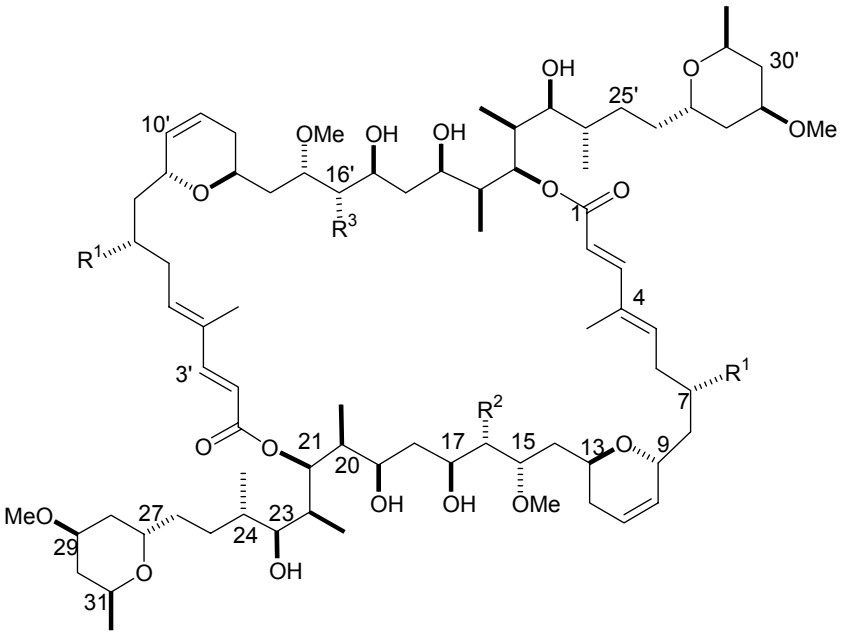
The molecular formula deduced by high-resolution MALDI-TOF mass spectrum of compound **4** was $C_{96}H_{160}O_{34}$. The difference between **4** and **1** or **3** was $C_6H_8O_6$. Comparison of its ^{1}H and ^{13}C NMR spectral data with those for ankaraholide A (**1**) and C (**3**) clearly showed that it was related to **3**. Two additional methylenes at δ 64.0 and δ 64.2, two methines at δ 72.8 and 73.25 were observed from ^{13}C NMR and multiplicity edited HSQC. Two carbonyls at δ 171.69 and 171.7 (after expansion of this region) were also seen in ^{13}C NMR spectrum. Another striking difference in proton NMR between **4** and **3** is the presence of signals at δ 4.25 and δ 4.75. ^{1}H-^{1}H COSY and Long-range ^{13}C-^{1}H HMBC correlation spectra revealed that δ 4.75 belongs to the sugar moiety and correlates to the carbonyl at δ 171.7. The proton at δ 4.25 displayed HMBC correlations to the methine carbon at δ 72.8 and the carbonyl group at δ 171.7. A ^{1}H-^{1}H COSY correlation between δ 4.25 and the methine protons (δ 3.75 and δ 3.85) was also seen. It was deduced from these results that the remaining portion $C_6H_8O_6$ of **4** was attached to the sugar moiety and likely derived from two glyceraldehyde units as shown.

Swinholide-based compounds are potent cancer cell growth inhibitors with IC_{50} values ranging from 0.37 nM to 1.0 μM against several cancer cell lines.[2,5] Swinholides exert their cytotoxic effect by disruption of the actin cytoskeleton.[6] One dimeric macrolide binds simultaneously to two molecules of G-actin, forming a tertiary complex with the two side chains of the macrolide (C-21/C-21' to C-27/C-27'), thus inhibiting polymerization by sequestration of G-actin.[7] In contrast to the bistheonellides, the swinholides additionally cause breakage of filamentous actin strands.[8] To evaluate the influence of the sugar moieties in ankaraholide A on the biological properties of these new swinholide derivatives, **1** was tested for cytotoxicity and microfilament inhibiting activity.

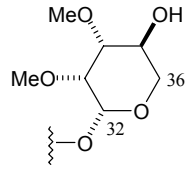
Ankaraholide A inhibited proliferation (IC_{50} values) in NCI-H460 (119 nM), Neuro-2a (262 nM), and MDA-MB-435 (8.9 nM) cell lines. Furthermore, in A-10 cells, **1** caused complete loss of the filamentous (F)-actin at 30 and 60 nM (Figure 2.30), coincident with dramatic changes in cell morphology. These normally fibroblastic cells became neuron-like with small core areas and multiple outwardly extending processes. The effects were specific for microfilaments, as there was no evidence of microtubule loss at these concentrations. Binuclear cells were present, consistent with inhibition of the actin-dependent process of cytokinesis. Hence, the mode of action as well as the biological activity of ankaraholide A (**1**) is comparable to that of swinholide A, and the additional sugar moieties in ankaraholide A do not affect its biological properties. Ankaraholide B (**2**) inhibited proliferation (IC_{50} values) in NCI-H460 (178 nM), Neuro-2a (354 nM), Ankaraholide C (**3**) NCI-H460 (236 nM), Neuro-2a (295 nM) and Ankaraholide D (**4**) NCI-H460 (215 nM), Neuro-2a (323 nM).

That the swinholides are produced by three taxonomically unrelated sponges suggests that symbiotic microorganisms may be the true producers of these metabolites. Due to the presence of cyanobacteria in these sponges and the close resemblance of swinholides to scytophycins (metabolites isolated from another cyanobacterium of the genus *Scytonema*[9]) it has been hypothesized that symbiotic cyanobacteria are the true metabolic origin of these compounds.[2,5,10] However, Bewley et al. showed that swinholide A was associated with a heterotrophic eubacterial fraction of the sponge *Theonella swinhoei*.[11] This latter approach presumes that the cellular location of a compound reflects its site of biosynthesis and neglects the possibility that substances may be excreted, diffuse within the sponge body, and absorb to cell types unrelated to its origin.

Our work reports the direct isolation of swinholide-type compounds from cyanobacteria,[14] thus unequivocally demonstrating that marine cyanobacteria possess the metabolic capacity to produce this skeletal class. In consequence, the true metabolic origin of the swinholides in sponges remains in question. However, it is conceivable that swinholide production occurs in multiple classes of bacteria due to gene transfer events.[12] Access to genetic methodologies and knowledge of the biosynthetic gene clusters encoding the biosynthesis of complex cyanobacterial natural products provides new techniques such as CARD-FISH analysis for probing such issues.[13]

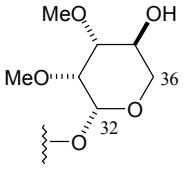


(**1**) R^1 =

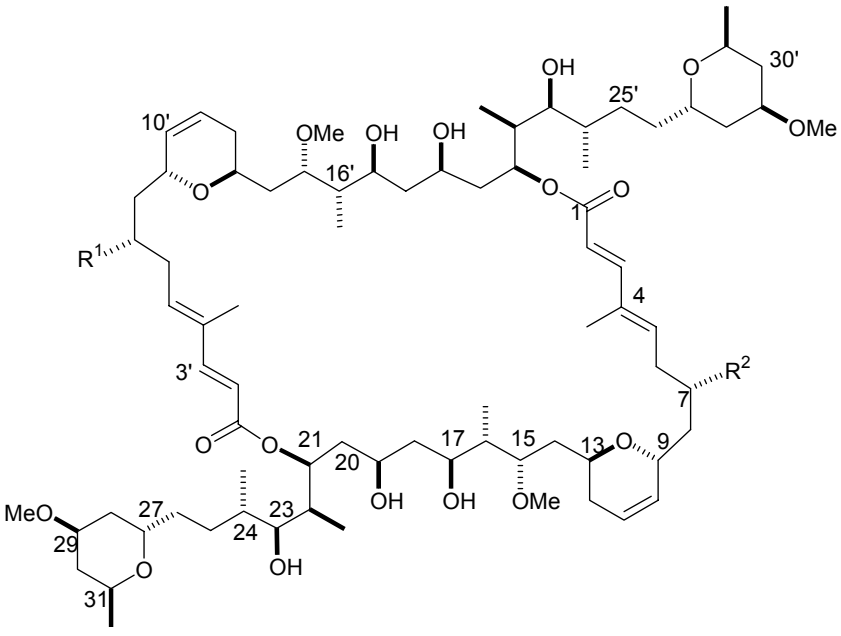


$R^2 = R^3 = H$

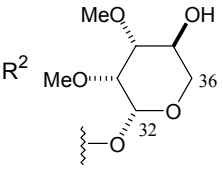
(**2**) R^1 =



R^2 = H, R^3 = Me



(**3**) $R^1 = R^2$



(**4**) R^2 =

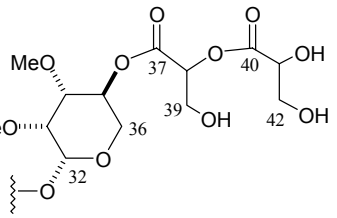


Table 2.1. ^{1}H and ^{13}C NMR Spectral Data for Compounds **1-2** in $CDCl_3$ (δ in ppm, *J* in Hz).

		Swinholide A[14]		**1**		**2**	
position	DEPT	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1/1’	qC	170.1		170.1		170.1	
2/2’	CH	113.3	5.79, d (15.8)	115.0	5.84, d (15.6)	115.0	5.84, d (15.3)
3/3’	CH	153.3	7.58, d (15.8)	151.9	7.51, d (15.6)	152.0	7.51, d (15.3)
4/4’	qC	134.3		134.6		134.6	
4/4’-Me	CH_3	12.3	1.81, s	12.7	1.84, s	12.7	1.84, s
5/5’	CH	142.3	6.08, dd (9.0, 5.1)	139.8	6.25, dd (6.3, 6.4)	140.2	6.26, m
6/6’	CH_2	37.4	2.17, br d (14.9) 2.46, ddd (19.9, 9.7, 9.7)	33.5	2.45, m; 2.69, m	33.5	2.45, m; 2.69, m
7/7’	CH	66.7	4.16, dd (7.2, 7.2)	76.7	4.07, m	76.6	4.09, m
8/8’	CH_2	41.1	1.60, m	40.1	1.35, m; 1.62, m	40.1	1.39, m; 1.62, m
9/9’	CH	65.9	4.52, br d (9.2)	69.9	4.38, m	69.9	4.38, m
10/10’	CH	129.9	5.69, d (10.2)	129.6	5.74, d (10.0)	129.9	5.78, brs
11/11’	CH	123.3	5.78, m	123.6	5.83, m	124.2	5.80, m
12/12’	CH_2	30.0	1.89, m 2.28, br d (17.2)	31.9	1.98, m; 2.00, m	31.9	1.98, m; 2.00, m
13/13’	CH	65.8	3.90, m	65.0	3.73, m	65.0	3.75, m
14/14’	CH_2	33.9	1.45, m; 2.15, m	39.7	1.63, m; 2.22, m	39.7 33.9*	1.60, m; 2.22, m 1.23, m*; 1.67, m*
15/15’	CH	75.1	4.01, m	74.7	3.75, m	74.7 ; 76.0*	3.76, m; 3.95, m*
15/15’-OMe	CH_3	57.5	3.35, s	57.1	3.37, s	57.1	3.37, s
16/16’	CH	41.1	1.68, m	43.6[a]	1.37, m; 1.65, m	43.6[a] 40.5*	1.40, m; 1.67, m 1.41, m*
16/16’-Me	CH_3	9.1	0.81, d (6.9)			9.7*	0.86, d (7.3)*
17/17’	CH	73.9	3.84, dd (9.5, 9.5)	69.4	4.11, m	69.3; 74.0*	4.12, m; 3.83, m*
18/18’	CH_2	38.5	1.58, m; 1.63, m	41.7	1.76, m	38.6; 35.4*	1.64, m; 1.63, m*
19/19’	CH	71.4	4.01, m	71.6	3.90, m	71.4	3.92, m
20/20’	CH	40.9	1.75, dq (9.7, 7.2)	40.7	1.69, m	41.8; 39.7*	1.70, m; 1.76, m*
20-Me	CH_3	9.4	0.98, d (6.9)	9.7	0.88, d (7.0)	9.7	0.88, d (4.8); 0.92, d (5.4)*
21/21’	CH	74.4	5.35, d (10.8)	74.8	5.38, d (10.1)	75.3	5.39, d (10.5)
22/22’	CH	37.7	1.93, m	37.2	1.92, m	37.2	1.92, m
22/22’-Me	CH_3	9.2	0.83, d (6.9)	9.8	0.94, d (6.7)	9.8	0.94, d (7.1)
23/23’	CH	76.0	3.13, d (9.7)	76.6	3.12, d (8.5)	76.6	3.12, m
24/24’	CH	33.3	1.66, m	33.6	1.69, m	33.3	1.72, m
24/24’-Me	CH_3	17.8	0.99, d (7.2)	17.9	1.01, d (6.3)	17.9	1.01, d (5.3)
25/25’	CH_2	24.0	1.26, m; 1.38, m	24.0	1.27, m; 1.39, m	24.0	1.23, m; 1.32, m
26/26’	CH_2	29.4	1.25, m; 1.87, m	29.7	1.27, m; 1.90, m	29.7	1.29, m; 1.92, m
27/27’	CH	71.4	4.01, m	71.4	4.02, m	71.5	4.04, m
28/28’	CH_2	34.9	1.59, m; 1.82, m	35.4	1.62, m; 1.84, m	35.4	1.62, m; 1.84, m
29/29’	CH	73.3	3.54, m	73.7	3.56, m	73.4	3.57, m
29/29’-OMe	CH_3	55.3	3.33, s	55.5	3.36, s	55.7	3.36, s
30/30’	CH_2	38.7	1.17, m; 1.97, m	39.0	1.19, m; 2.01, m	38.9	1.20, m; 2.04, m
31/31’	CH	64.6	3.70, m	64.4	3.66, m	64.3	3.67, m
31/31’-Me	CH_3	21.8	1.20, d (5.9)	22.3	1.22, d (6.3)	22.0	1.23, d (6.2)
32/32’	CH			103.0	4.58, d (5.5)	103.1	4.56, d (6.1); 4.54, d (6.2)
33/33’	CH			79.6	3.30, m	79.4	3.29, m
33/33’-OMe	CH_3			58.8	3.47, s	58.8	3.47, s
34/34’	CH			84.0	3.26, m	84.0	3.26, m
34/34’-OMe	CH_3			60.0	3.61, s	60.0	3.61, s
35/35’	CH			73.0	3.38, m	73.0	3.38, m
36/36’	CH_2			62.8	3.29, m; 4.03 m	62.8	3.29, m; 4.03, m

* indicates resonances at prime atom positions [a] CH_2

Table 2.2. ^{1}H and ^{13}C NMR spectral data for ankaraholide A (**1**) in CD_3OD.

Position	DEPT[a]	δ_H mult. (*J* in Hertz)[a]	δ_C[b]	COSY[a]	HMBC[c]	ROESY[a]
1/1'	qC		169.6			
2/2'	CH	5.90 d, (15.6)	115.6	3	1,3,4	4-Me
3/3'	CH	7.43 d, (15.6)	151.5	2	1,2,4,5,4-Me,5	5
4/4'	qC		135.0		2,4-Me,6	
4/4'-Me	CH_3	1.84 s	11.9		3,4,5	2
5/5'	CH	6.19 dd, (7.0,7.2)	139.2	6	3,4-Me,6,7	3,6
6/6'	CH_2	2.58 m, 2.60 m,	33.4	5,7	4,5,7	4,7,8,32
7/7'	CH	3.95 m,	76.0	6,8	6,8,32	6,8,9,32
8/8'	CH_2	1.50 m, 1.73 m,	39.2	7	7,9	6,7,9,32,36
9/9'	CH	4.37 m	69.6	10	8,7	6,8,10,14
10/10'	CH	5.80 d, (1)	129.6	11	11	8,9,14,32
11/11'	CH	5.82 m	123.6	10	12,13	14
12/12'	CH_2	1.96 m 2.04 m	31.1	11	11,13	13
13/13'	CH	3.74 m	64.3	12,14	14	12
14/14'	CH_2	1.73 m 2.01 m	39.4	13	15	9
15/15'	CH	3.63 m	75.07	14	15-OMe	17
15/15'-OMe	CH_3	3.31 s	55.6		15	
16/16'	CH_2	1.49 m, 1.76 m	42.5	15,17	17	19
17/17'	CH	3.92 m	67.2	16,18	16,18	15
18/18'	CH_2	1.56 m	42.1	17,19	19,17	20
19/19'	CH	3.81 m	68.8	18,20	18	16
20/20'	CH	1.87 m	39.7	19,20-Me,21	20-Me,21	18
20/20'-Me	CH_3	0.91 d, (2.9)	8.35	20	19,20,21	
21/21'	CH	5.43 d, (10.4)	75.0	20	1,19,20-Me,22-Me,23	19,23,24-Me
22/22'	CH	1.99 m	37.2	22-Me,23	22-Me,23	
22/22'-Me	CH_3	0.93 d, (3.1)	8.75	22	21,22,23	23
23/23'	CH	3.12 m	76.5	22	21,24-Me,25	22-Me,24-Me
24/24'	CH	1.72 m	33.6	24-Me	23,24-Me,26	
24/24'-Me	CH_3	0.98 d, (6.8)	16.9	24	23,24,25	21,23
25/25'	CH_2	1.25 m, 1.44 m	24.3	24,26	23,24-Me,26	27
26/26'	CH_2	1.21 m, 1.93 m	29.0	25,27	25,27	28
27/27'	CH	3.99 m	71.9	26	26	25
28/28'	CH_2	1.52 m, 1.87 m	35.0	27,29	29,30	26
29/29'	CH	3.62 m	73.3	28,30	29,29-OMe,30	31
29/29'-OMe	CH_3	3.34 s	54.4		29	27
30/30'	CH_2	1.12 m, 2.01m	38.7	29	28,29,21-Me	28
31/31'	CH	3.74 m	64.9	31-Me	31Me	29
31/31'-Me	CH_3	1.19 d, (6.1)	20.9	31	30,31	
32/32'	CH	4.38 d, (2.2)	102.9	33	7,33,34,36	5,6,7,8,10,34,36
33/33'	CH	3.25 dd, (3.6, 2.2)	79.8	32,34	32,34,33-OMe	
33/33'-OMe	CH_3	3.44 s	57.6		33	35
34/34'	CH	3.11 dd, (9.5, 3.6)	85.4	33,35	32,34-OMe,35	32,36
34/34'-OMe	CH_3	3.59 s	59.7		34	
35/35'	CH	3.23 dd, (9, 9.5)	73.5	34,36	33,36	33O-Me
36/36'	CH_2	3.20 d, (9), 4.01 m	63.0	35	32,33,34	8,32,34

[a]Recorded at 400 MHz. [b]Recorded at 100 MHz, [c]Recorded at 600 MHz, data reported for experiments optimized for either 4 Hz or 8 Hz.

Table 2.3 ^{1}H and ^{13}C NMR spectral data for ankaraholide B (**2**) in CD_3OD.

	DEPT[a]	δ_H mult. (*J* in Hz)[a]	$\delta_{H'}$ mult. (*J* in Hz)[a]	δ_C[b]	$\delta_{C'}$[b]	COSY[a]	HMBC[c]	ROESY[c]
1/1'	qC			169.6	169.6			
2/2'	CH	5.90 d, (15.4)	5.92 d, (15.4)	115.6	115.6	3	4	4-Me
3/3'	CH	7.43 d, (15.4)	7.41 d, (15.4)	151.5	151.5	2	1,4,4-Me	5
4/4'	qC			135.0	135.0			
4/4'-Me	CH_3	1.84 s	1.82 s	11.9	11.9		3,4,5	2
5/5'	CH	6.19 dd (6.6,7.6)	6.19 dd (6.6,7.6)	139.2	139.2	6	3,4	3,6
6/6'	CH_2	2.58 m, 2.60 m	2.58 m, 2.60 m	33.4	33.4	5,7		4,7,8,32
7/7'	CH	3.95 m	3.95 m	76.0	76.0	6,8	32	6,8,9,32
8/8'	CH_2	1.50 m, 1.73 m	1.50 m, 1.73 m	39.2	39.2	7		6,7,9,32,36
9/9'	CH	4.37 m	4.37	69.6	69.6	10		6,8,10,14
10/10'	CH	5.82 s	5.82 s	129.6	129.6	11		8,9,14,32
11/11'	CH	5.82 m	5.82 m	123.6	123.6	10		14
12/12'	CH_2	1.96 m, 2.04 m	1.96 m, 2.04 m	31.1	31.1	11		13
13/13'	CH	3.74 m	3.70 m	64.3	64.6	12,14		12
14/14'	CH_2	1.73 m, 2.01 m	1.54 m, 2.00m	39.4	33.4	13 13'		9
15/15'	CH	3.63 m	3.71 m	75.07	77.2	14 14'	15 15'	17
15/15'-OMe	CH_3	3.31 s	3.30 s	55.6	55.6			
16/16'	CH	1.56 m, 1.67m	1.63 m	42.5	42.3	15,17		19
16-Me	CH_3		0.86 d, (7.6)		9.42	16'	16',17',18'	15',16',17'
17/17'	CH	3.92 m	3.64m	67.28	72.1	16,18		15,15'
18/18'	CH_2	1.79 m	1.70 m, 1.72 m	35.8	38.2	17,19		20
19/19'	CH	3.81 m	3.88m	68.8	69.2	18,20		16
20/20'	CH	1.87 m	1.87m	39.7	39.7	19,20,21 19',20',21'		18
20/20'-Me	CH_3	0.91d, (2.9)	0.91 d, (2.9)	8.35	8.35	20 20'	19,20,21 19',20',21'	
21/21'	CH	5.43 d, (8.8)	5.41 d, (8.8)	75.0	75.0	20		19,23,24-Me
22/22'	CH	1.99 m	1.99 m	37.2	37.2	22, 23		
22/22'-Me	CH_3	0.93 d, (2.9)	0.93 d, (2.9)	8.75	8.75	22	21,22,23	23
23/23'	CH	3.12 m	3.12 m	76.5	76.5	22	25,24-Me	22-Me,24-Me
24/24'	CH	1.72 m	1.72 m	33.6	33.6	24-Me		
24/24'-Me	CH_3	0.98 d, (7.6)	0.98 d, (7.6)	16.9	16.9	24	23,24,25	21,23
25/25'	CH_2	1.25 m, 1.44 m	1.25 m, 1.44 m	24.3	24.3	24,26		27
26/26'	CH_2	1.21 m, 1.93 m	1.21 m, 1.93 m	29.0	29.0	25,27		28
27/27'	CH	3.99 m	3.99 m	71.9	71.9	26		25
28/28'	CH_2	1.52 m, 1.87 m	1.52 m, 1.87 m	35.0	35.0	27,29		26
29/29'	CH	3.62 m	3.62 m	73.3	73.3	28,30		31
29/29'-OMe	CH_3	3.34 s	3.34 s	54.4	54.4			27
30/30'	CH_2	1.12 m 2.01m	1.12 m 2.01m	38.7	38.7	29	28,29,31	28
31/31'	CH	3.74 m	3.74 m	64.9	64.9	31-Me		29
31/31'-Me	CH_3	1.19 d, (6.5)	1.19 d, (6.5)	20.9	20.9	31	30,31	
32/32'	CH	4.38 d, (2.2)	4.38 d, (2.2)	102.9	102.9	33	7	5,6,7,8,10,34,36
33/33'	CH	3.25 dd, (3.6, 2.2)	3.25 dd, (3.6, 2.2)	79.8	79.8	32,34	32,33-OMe	
33/33'-OMe	CH_3	3.44 s	3.44 s	57.6	57.6		33	35
34/34'	CH	3.11 dd, (9.4, 3.6)	3.11 dd, (9, 3.6)	85.4	85.4	33,35	34-OMe	32,36
34/34'-OMe	CH_3	3.59 s	3.59 s	59.7	59.7		34	
35/35'	CH	3.23 dd, (9.4, 9)	3.23 dd, (9, 9.5)	73.5	73.5	34,36		33-OMe
36/36'	CH_2	3.20 d, (9), 4.01 m	3.20 d, (9.5), 4.01 m	63.0	63.0	35	32,34	8,32,34

[a]Recorded at 400 MHz. [b]Recorded at 100 MHz, [c]Recorded at 600 MHz.

Table 2.4 ^{1}H and ^{13}C NMR Spectral Data for Compounds **3-4** in CD_3OD (δ in ppm, *J* in Hz).

		3		**4**			
Position	DEPT	δ_C	δ_H	δ_C	δ_H	$\delta_{C'}$	$\delta_{H'}$
1/1’	qC	169.6		169.6			
2/2’	CH	115.1	5.92, d (15.6)	115.6	5.93, d (15.5)		
3/3’	CH	150.9	7.47, d (15.6)	150.0	7.46, d (15.5)		
4/4’	qC	135.0		135.0			
4/4’-Me	CH_3	11.8	1.87, s	11.9	1.84, s		
5/5’	CH	138.9	6.20, dd (5.9, 6.2)	138.6	6.21, dd (5.9, 6.2)	138.6	6.07, dd (5.9,6.2)
6/6’	CH_2	33.3	2.56, m; 2.64, m	33.4	2.59, m; 2.61, m		
7/7’	CH	76.1	3.99, m	76.0	3.98, m		
8/8’	CH_2	39.0	2.01, m; 1.71, m	39.0	2.01, m; 1.71, m		
9/9’	CH	69.7	4.40, m	69.7	4.40, m		
10/10’	CH	129.3	5.84, d (10.2)	129.7	5.82, d (10.0)		
11/11’	CH	123.8	5.83, m	124.0	5.83, m		
12/12’	CH_2	31.1	1.96, m; 2.04, m	31.1	1.94, m; 2.04, m		
13/13’	CH	65.0	3.78, m	65.0	3.78, m		
14/14’	CH_2	36.0	1.57, m; 1.81, m	36.0	1.57, m; 1.81, m		
15/15’	CH	78.1	3.75, m	78.1	3.75, m		
15/15’-Ome	CH_3	56.5	3.35, s	56.5	3.35, s		
16/16’	CH	41.7	1.67, m	41.7	1.67, m		
16/16’-Me	CH_3	9.2	0.86, d (6.7)	9.2	0.86, d (6.8)		
17/17’	CH	73.0	3.74, m	73.0	3.76, m		
18/18’	CH_2	42.1	1.75, m;	42.1	1.75, m		
19/19’	CH	68.5	3.86, m	68.5	3.86, m		
20/20’	CH_2	41.4	1.55, m; 2.04, m	41.3	1.55, m; 2.04, m		
21/21’	CH	71.1	5.70, d (10.2)	71.2	5.70, d (10.3)		
22/22’	CH	41.1	1.81, m	41.0	1.81, m		
22/22’-Me	CH_3	9.9	0.98, d (6.5)	9.9	0.97, d (6.7)		
23/23’	CH	76.7	3.13, d (8.7)	76.5	3.12, d (8.6)		
24/24’	CH	34.0	1.72, m	34.0	1.72, m		
24/24’-Me	CH_3	17.1	0.98, d (6.5)	16.9	0.98, d (6.7)		
25/25’	CH_2	24.5	1.25, m; 1.44, m	24.3	1.25, m; 1.44, m		
26/26’	CH_2	29.2	1.31, m; 1.94, m	29.0	1.31, m; 1.93, m		
27/27’	CH	72.1	4.01, m	71.4	4.00, m		
28/28’	CH_2	35.1	1.52, m; 1.90, m	35.0	1.52, m; 1.87, m		
29/29’	CH	73.6	3.62, m	73.7	3.63, m		
29/29’-OMe	CH_3	54.6	3.36, s	54.6	3.36, s		
30/30’	CH_2	38.9	1.12, m; 2.01, m	38.7	1.12, m; 2.01, m		
31/31’	CH	65.9	3.70, m	65.9	3.70, m		
31/31’-Me	CH_3	21.1	1.20, d (6.2)	20.9	1.19, d (6.3)		
32/32’	CH	102.9	4.38, d (2.1)	102.9	4.38, d (2.1)	100.3	4.67, d (2.0)
33/33’	CH	79.9	3.26, dd (3.6, 2.1)	79.6	3.26,dd (3.6,2.0)	79.6	3.38,dd (3.7.2.2)
33/33’-Ome	CH_3	57.8	3.48, s	57.7	3.47, s	57.8	3.49,s
34/34’	CH	85.6	3.13, dd (9.4, 3.6)	85.6	3.13,dd (9.1,3.7)	83.0	3.38, dd (9,3.6)
34/34’-OMe	CH_3	60.0	3.60, s	59.9	3.63, s	59.9	3.63,s
35/35’	CH	73.6	3.24, dd (9.1, 9.4)	73.8	3.24,dd (9.1,9.3)	73.8	4.75, dd (9,9.1)
36/36’	CH_2	63.0	3.22, d (9.4) ; 4.03, m	63.0	3.22,d(9.3);4.03, m	63.0	3.29, d(9.1); 4.09, m
37						171.7	
38						72.8	4.25, dd (4.4,4.1)
39						64.2	3.75, 3.85
40						171.69	
41						73.2	3.76
42						64.0	3.75, 3.85

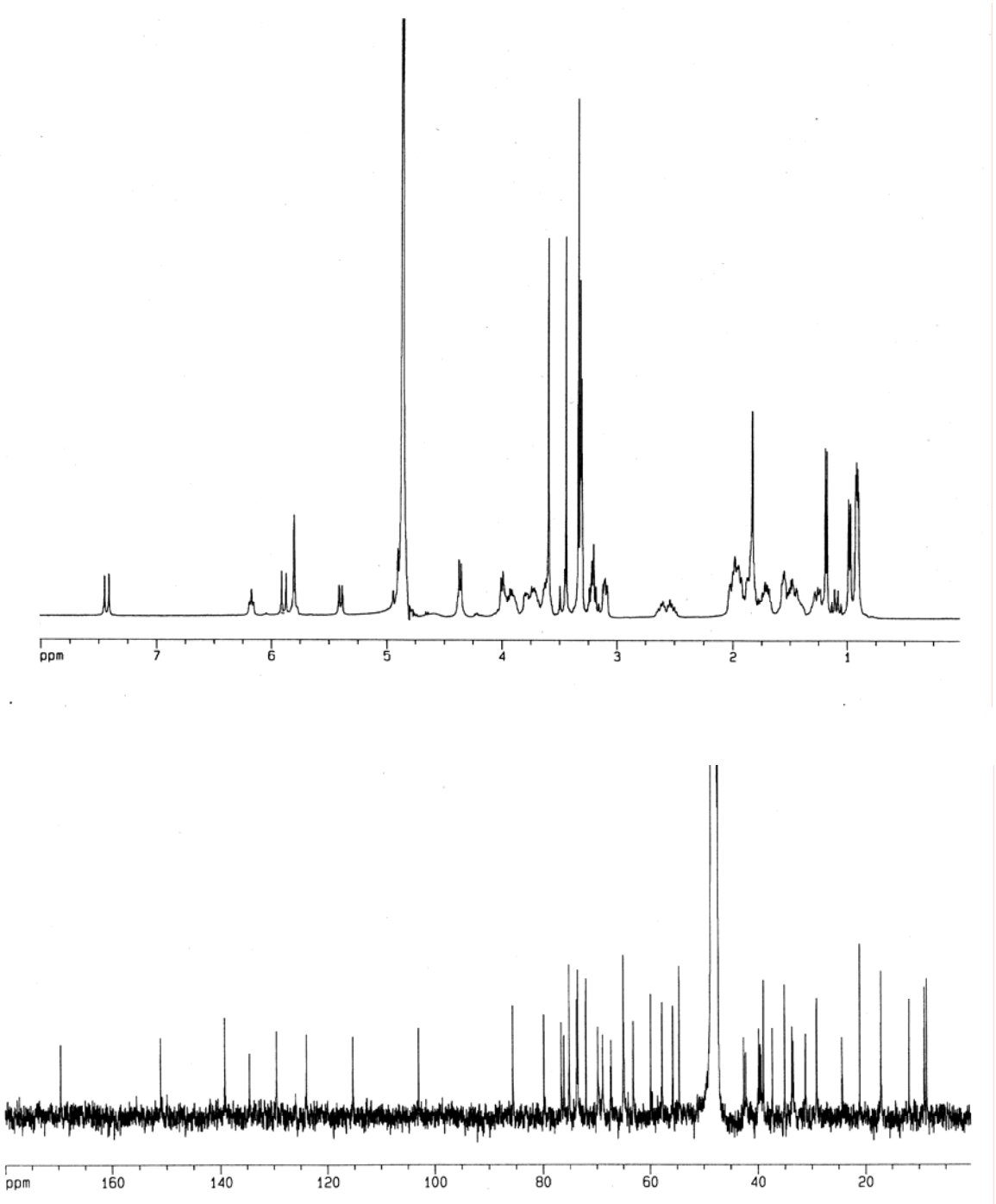


Figure 2.1. 400 MHz ^{1}H NMR spectrum and 100 MHz ^{13}C spectrum of ankaraholide A (**1**) in CD_3OD.

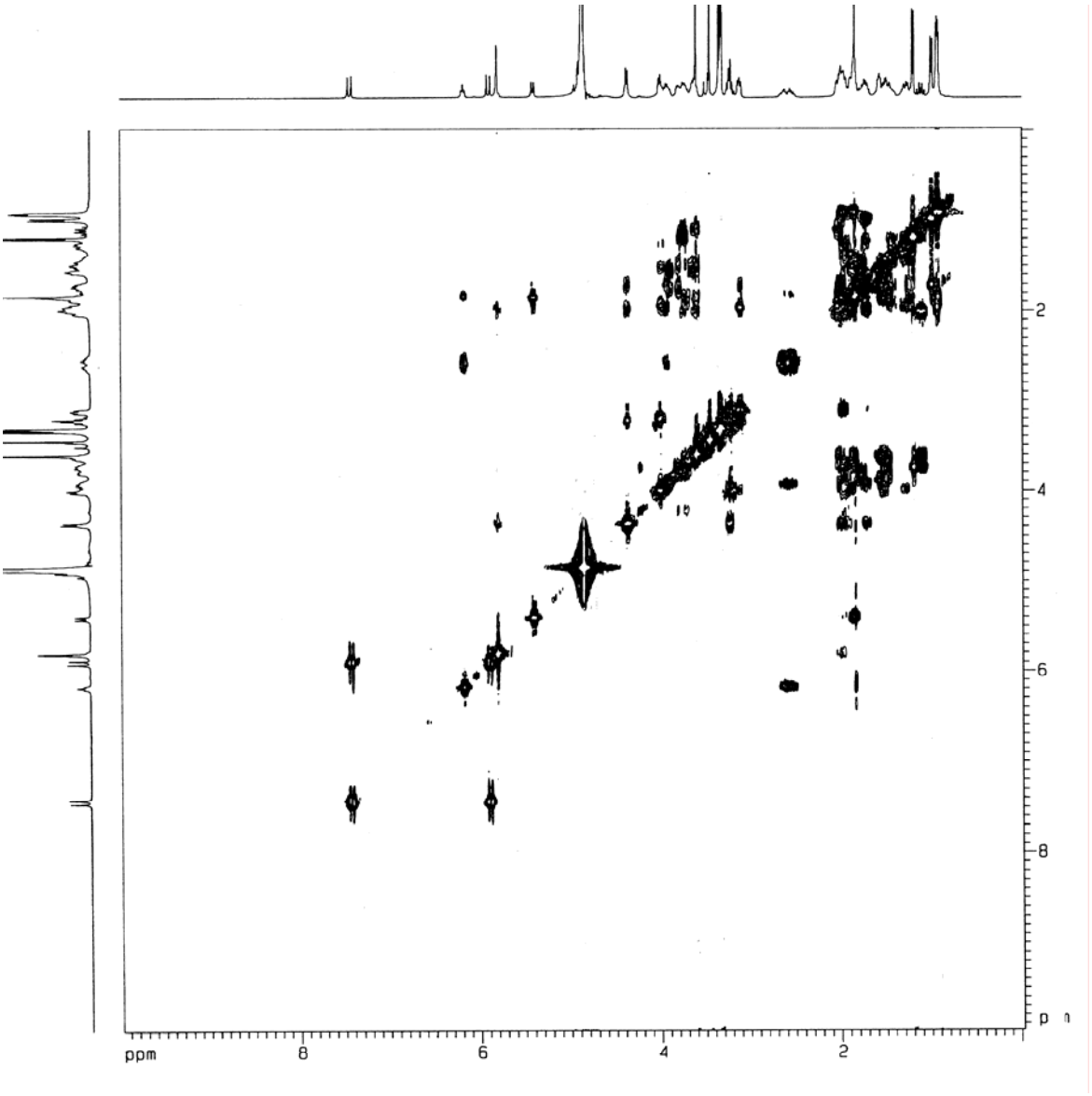


Figure 2.2. 400 MHz ^{1}H-^{1}H COSY spectrum of ankaraholide A (**1**) in CD_3OD.

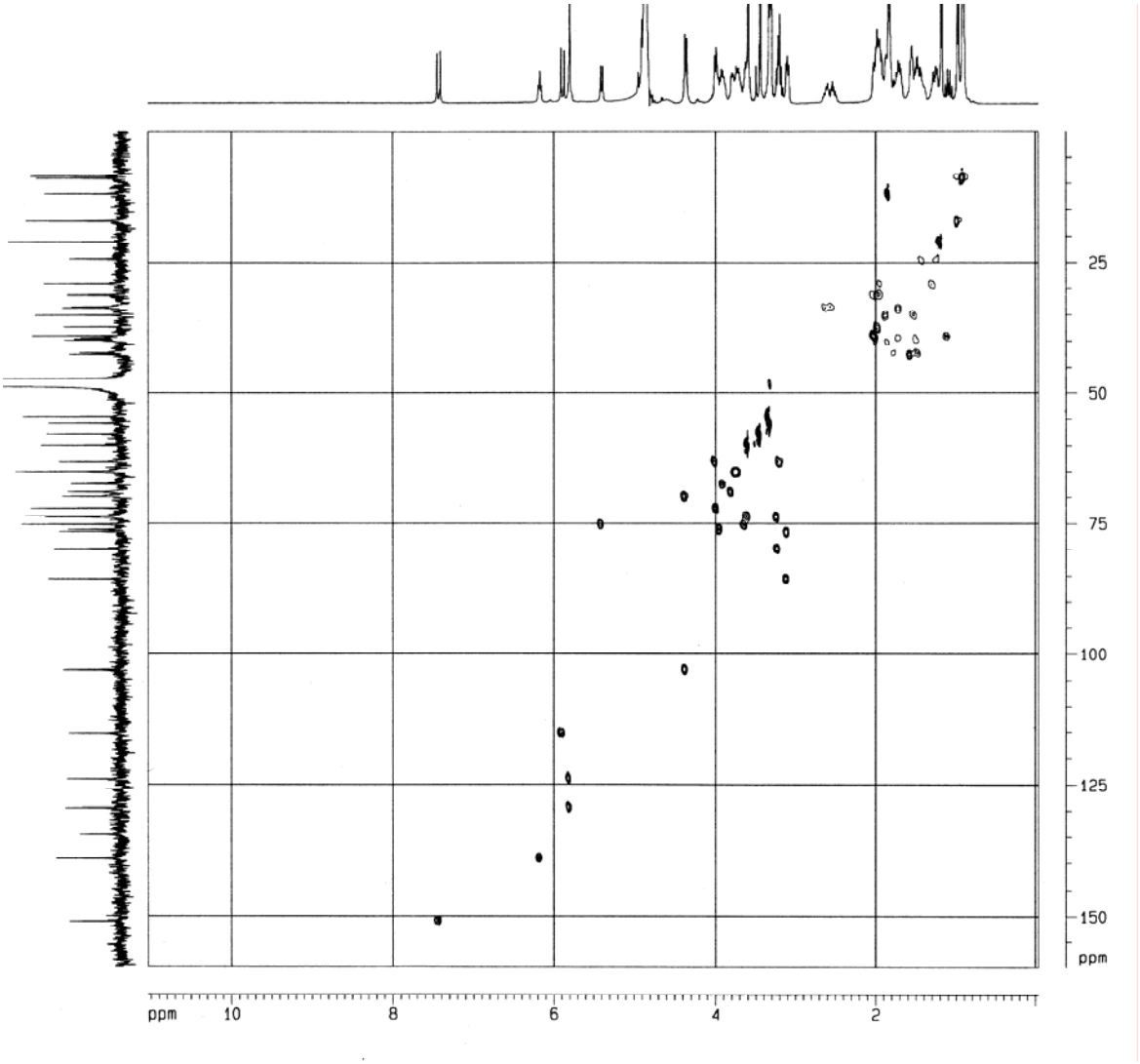


Figure 2.3. 400 MHz Multiplicity-edited HSQC spectrum of ankaraholide A (**1**) in CD_3OD.

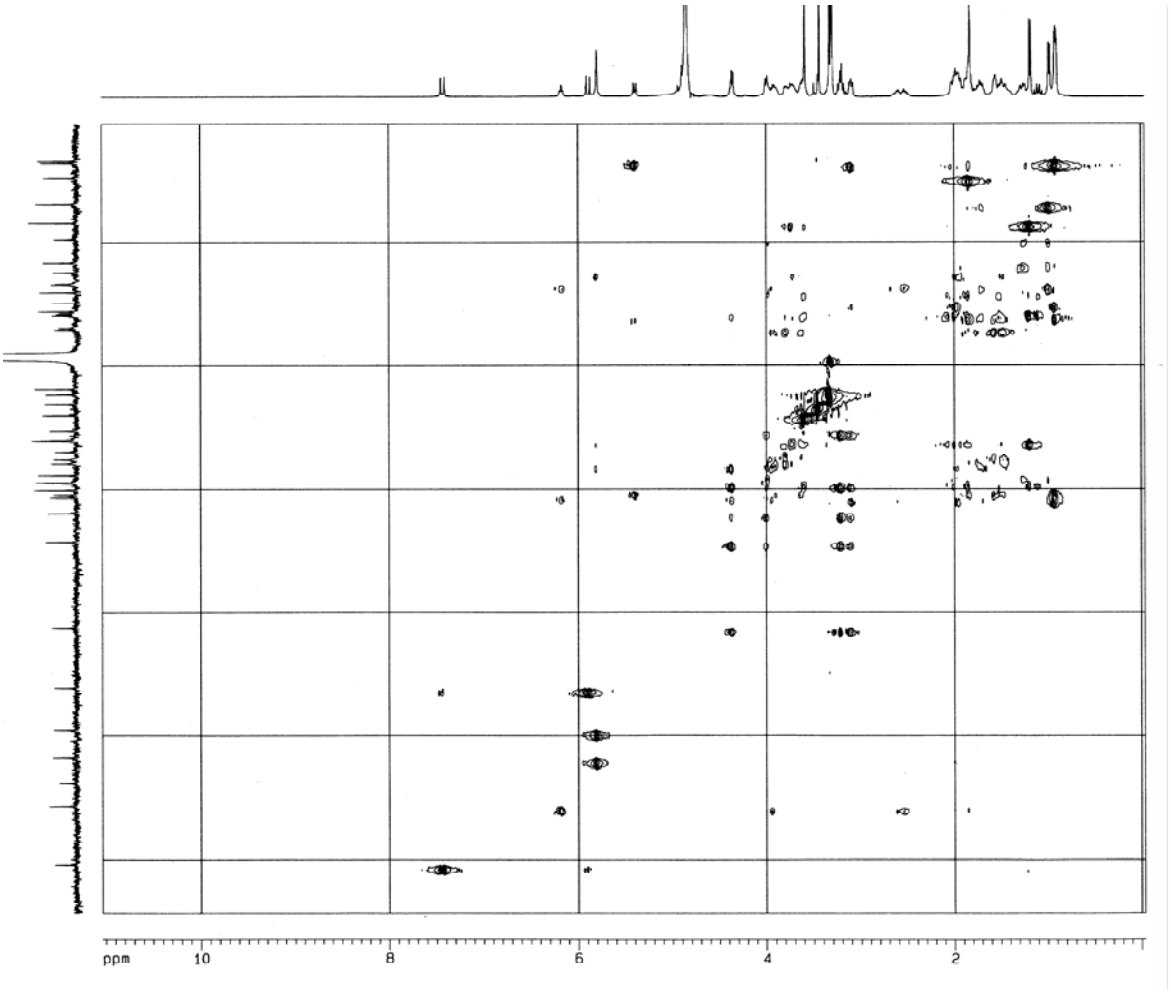


Figure 2.4. 400 MHz HSQC-TOCSY spectrum of ankaraholide A (**1**) in CD_3OD.

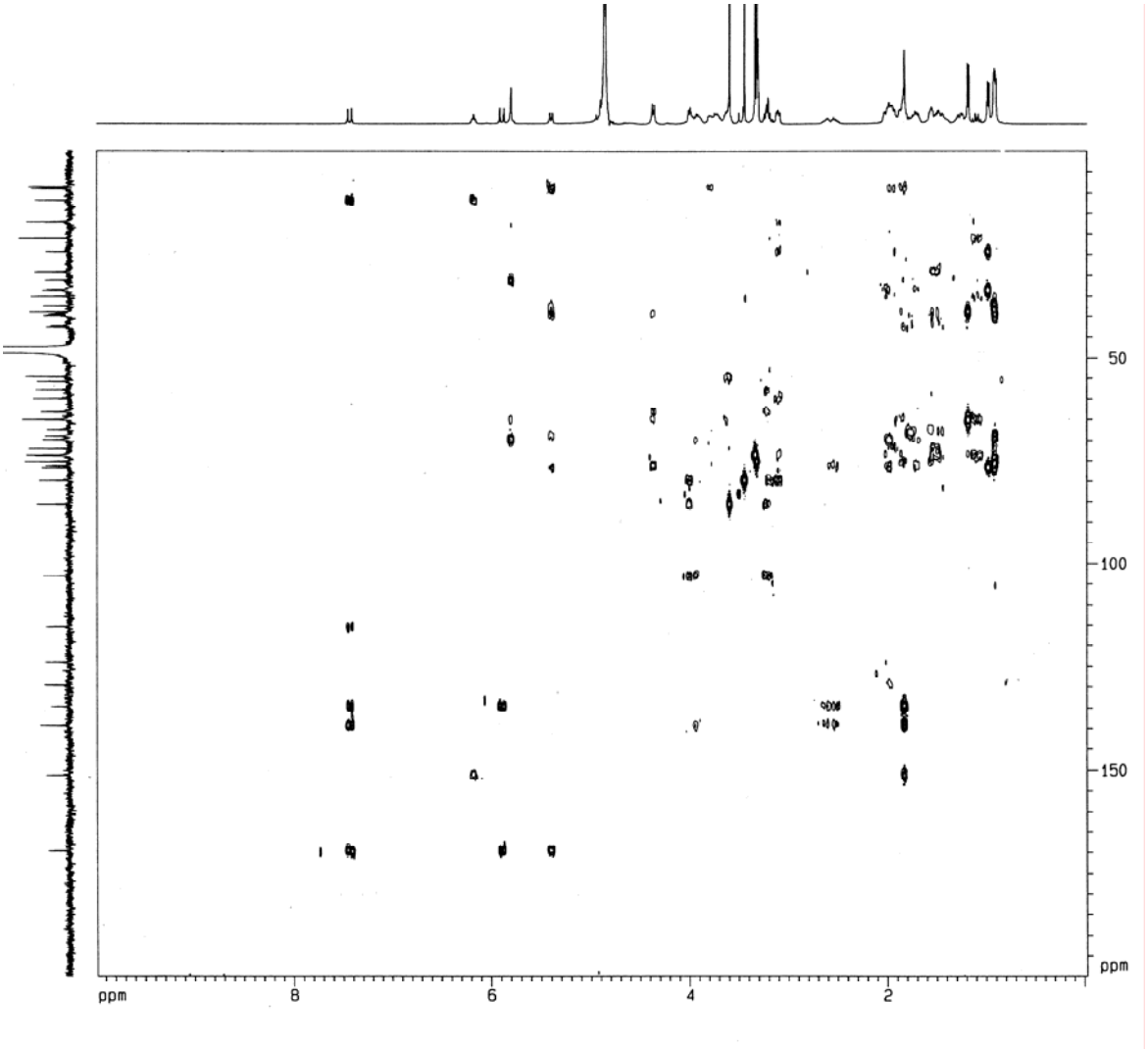


Figure 2.5. 600 MHz HMBC spectrum (optimized for *J* = 8Hz) of ankaraholide A (**1**) in CD_3OD.

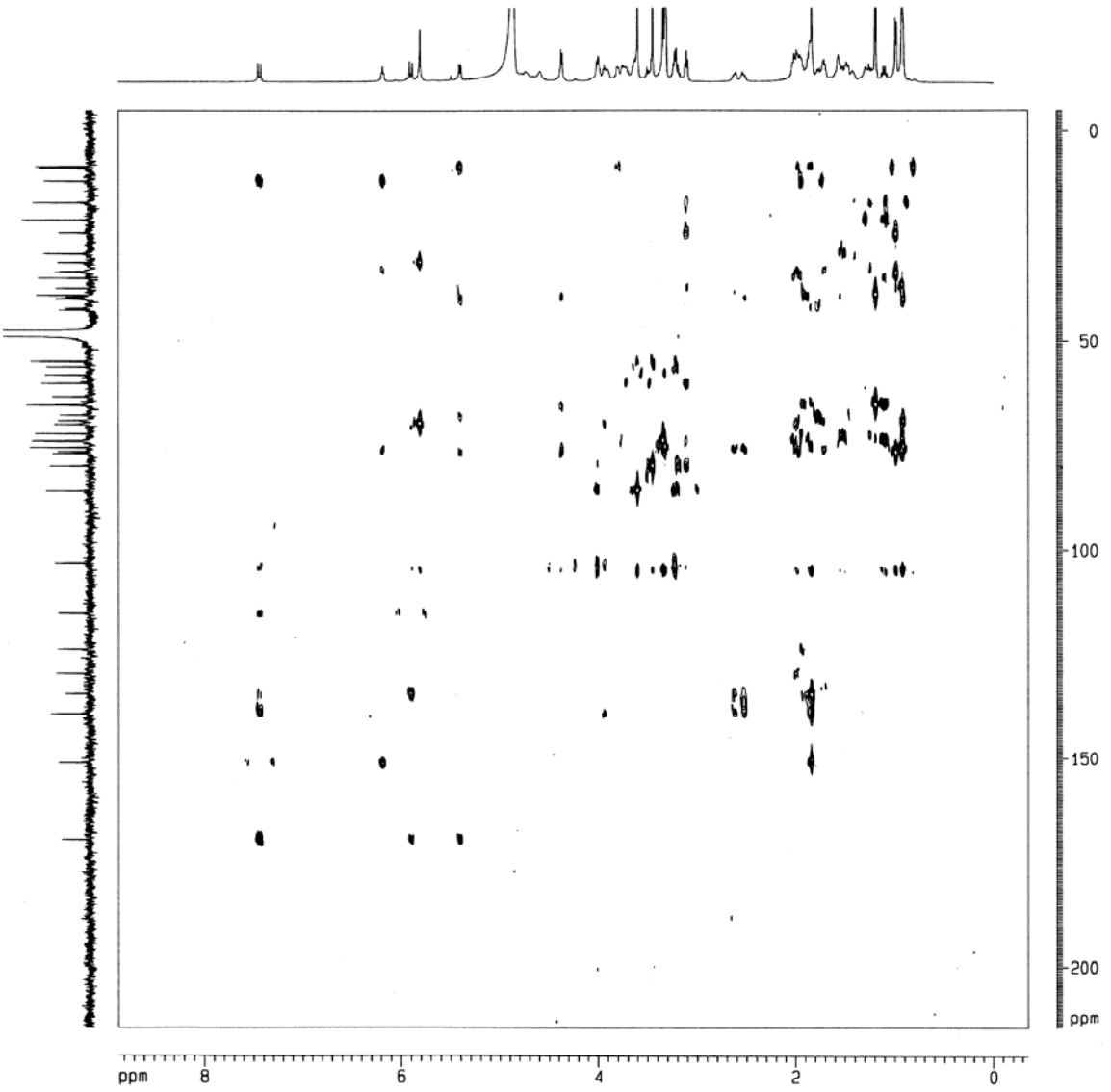


Figure 2.6. 600 MHz HMBC spectrum (optimized for *J* = 4Hz) of ankaraholide A (**1**) in CD_3OD.

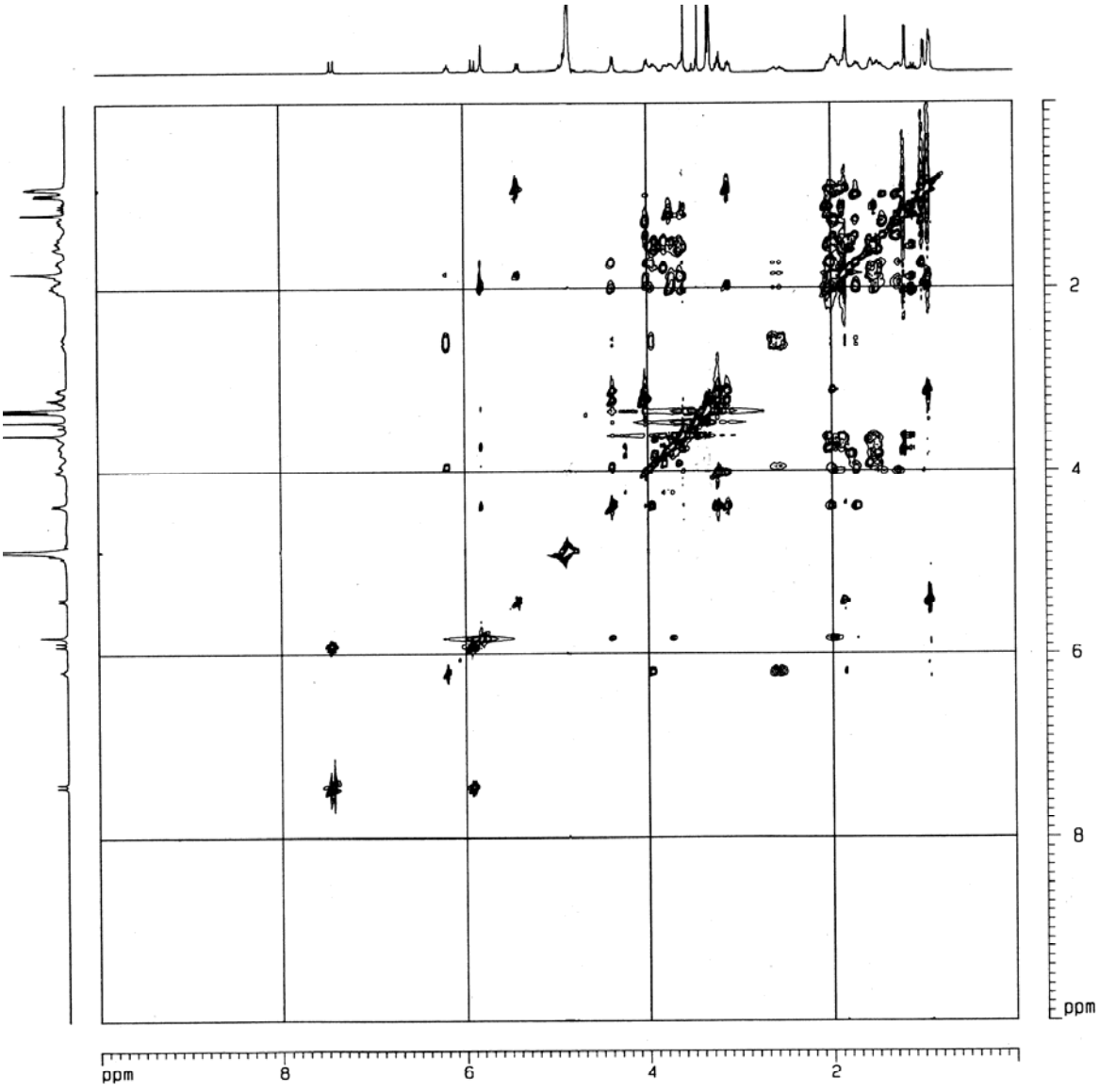


Figure 2.7. 400 MHz ROESY spectrum of ankaraholide A (**1**) in CD_3OD.

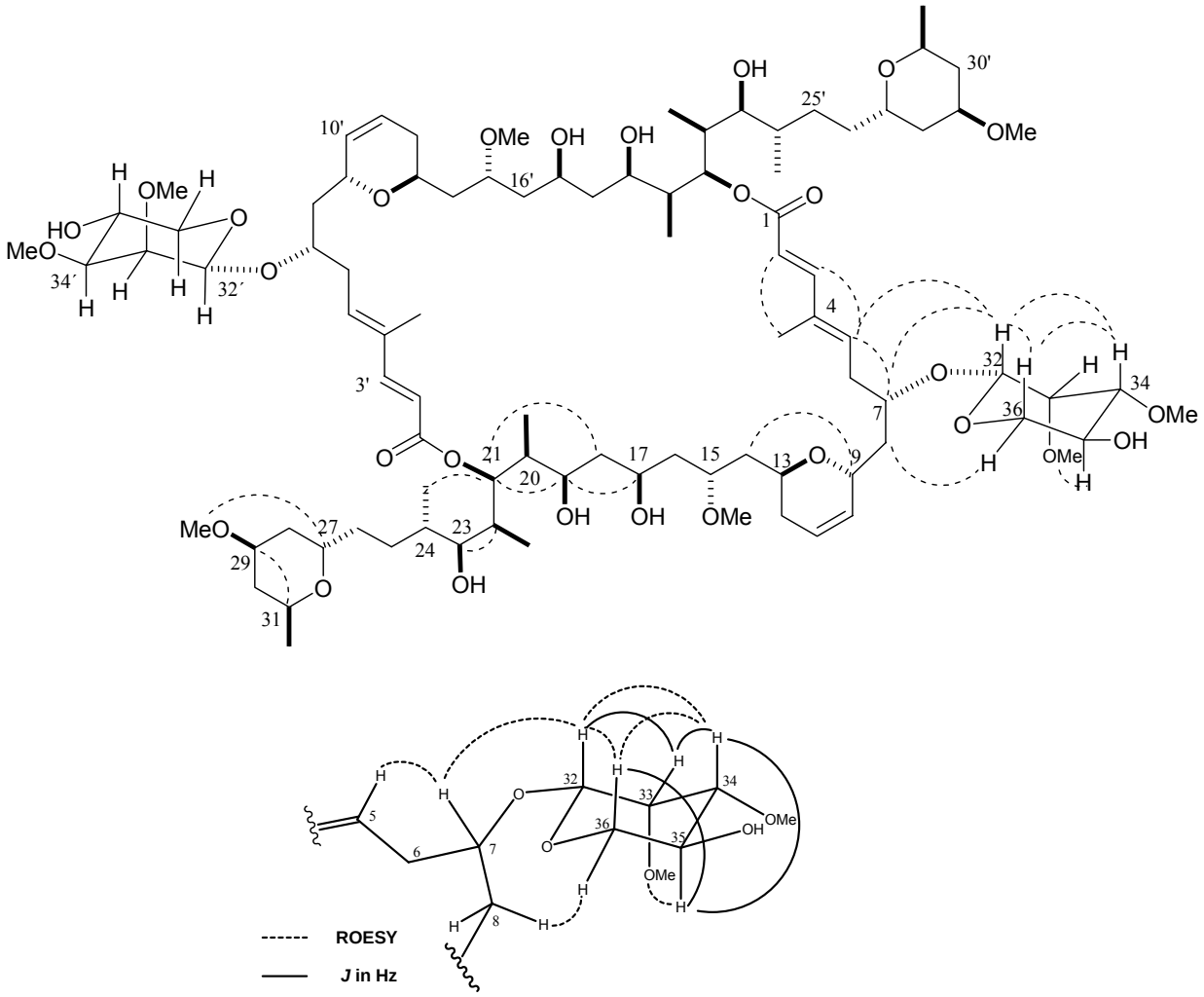


Figure 2.8. Coupling constant analysis and ROESY correlations of the 2,3-di-*O*-methyl-β-lyxopyranoside moiety ($^3J_{H,H}$ values were obtained in CD_3OD), ROESY correlations and relative stereochemistry of ankaraholide A (**1**).

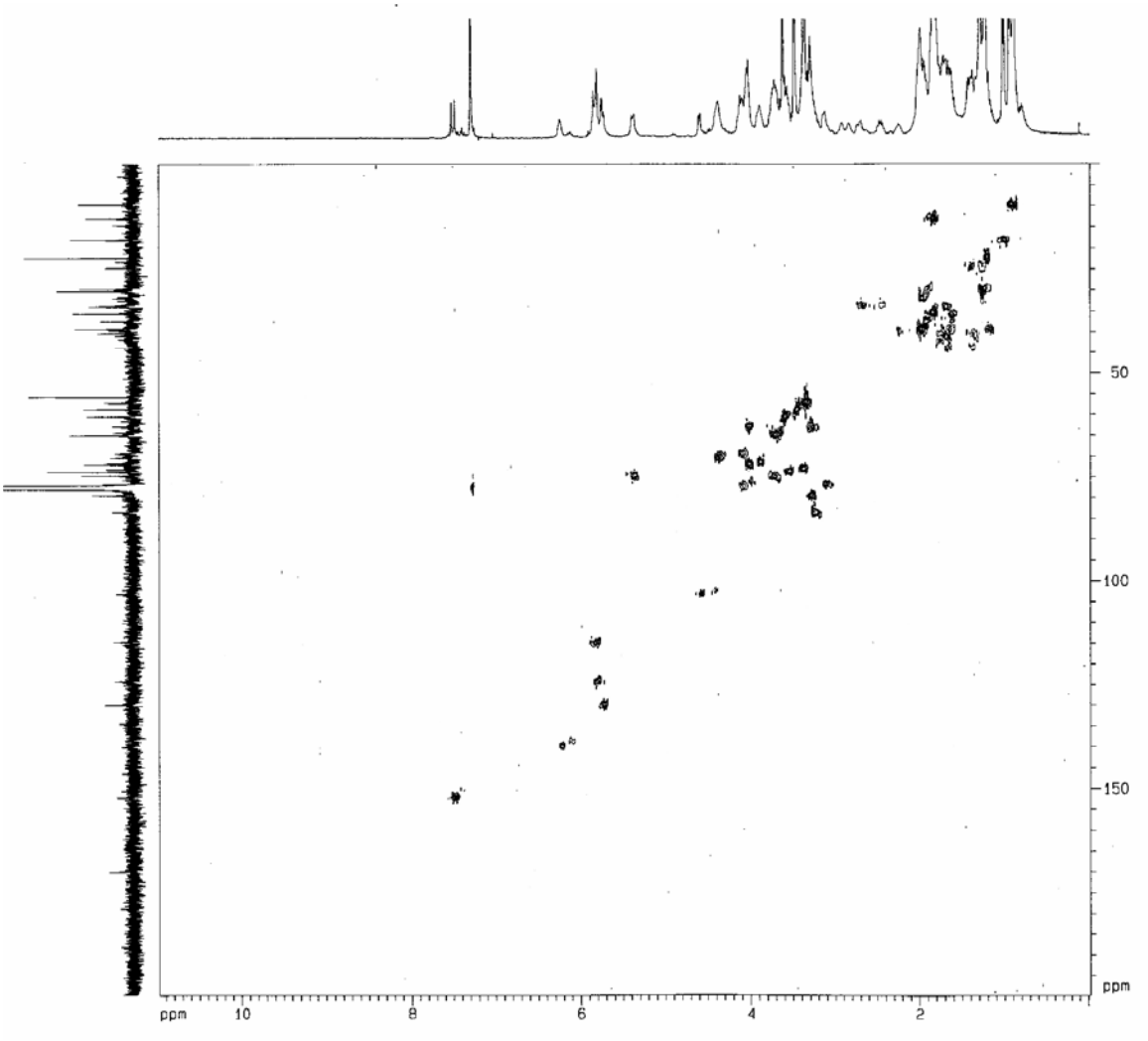


Figure 2.9. 400 MHz Multiplicity-edited HSQC spectrum of ankaraholide A (**1**) in $CDCl_3$

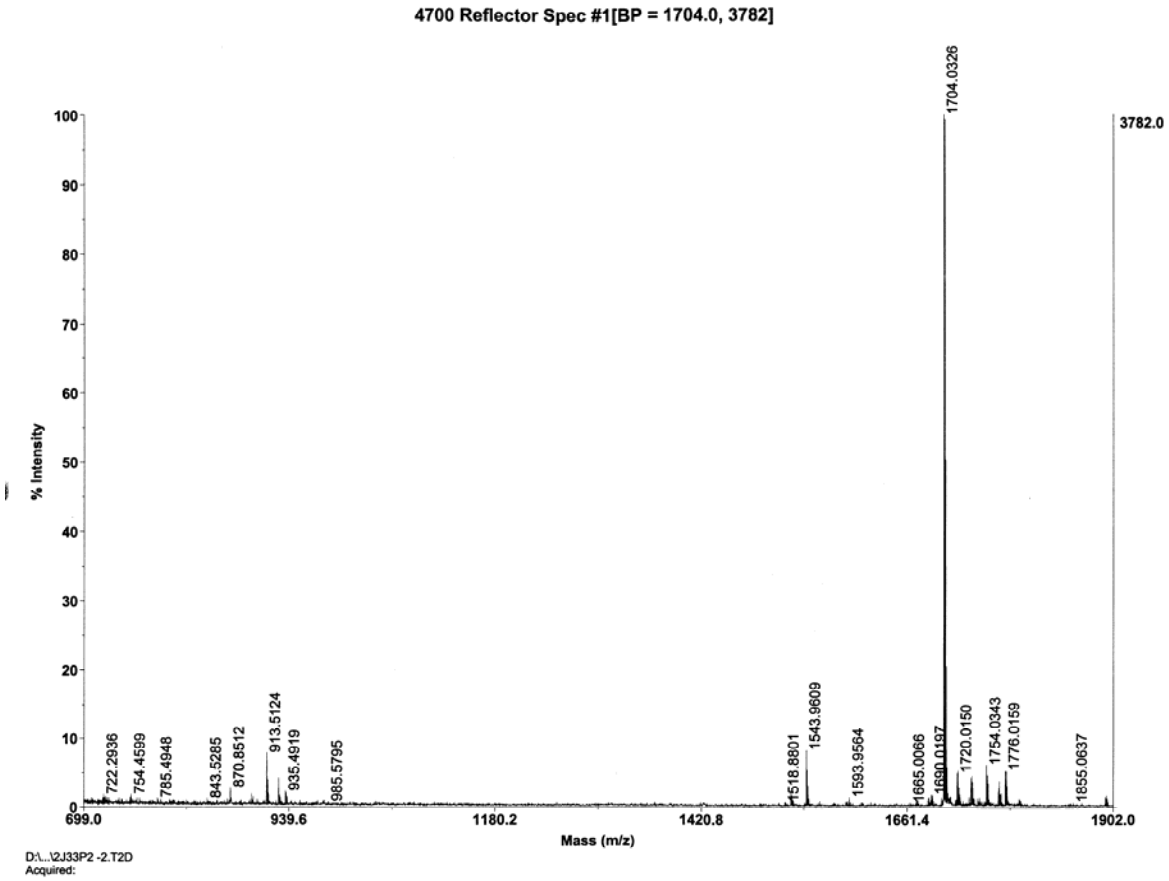


Figure 2.10. MALDI-TOF spectrum of ankaraholide A (**1**).

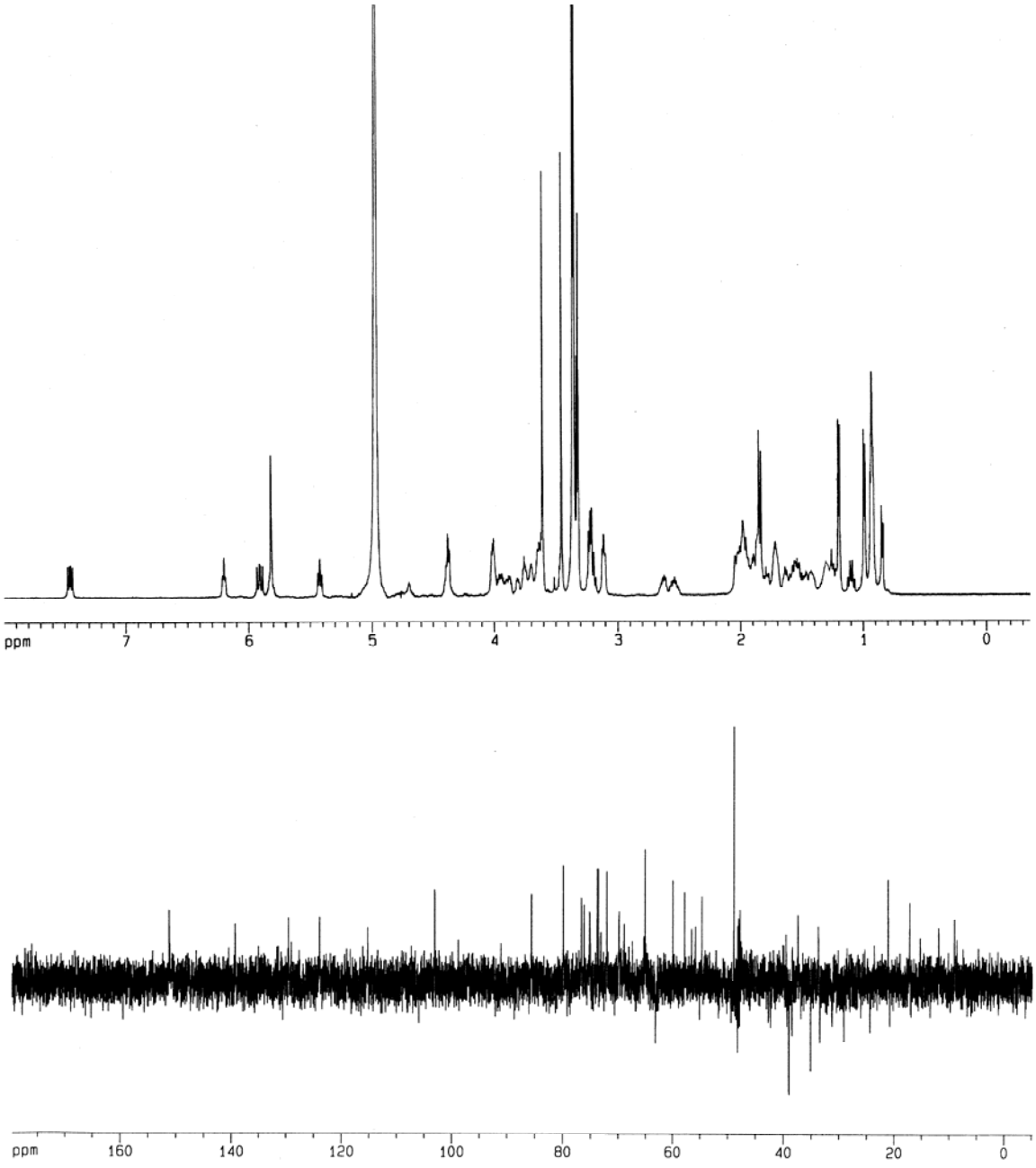


Figure 2.11. 400 MHz ^{1}H NMR spectrum and DEPT135 spectrum of ankaraholide B (**2**) in CD_3OD.

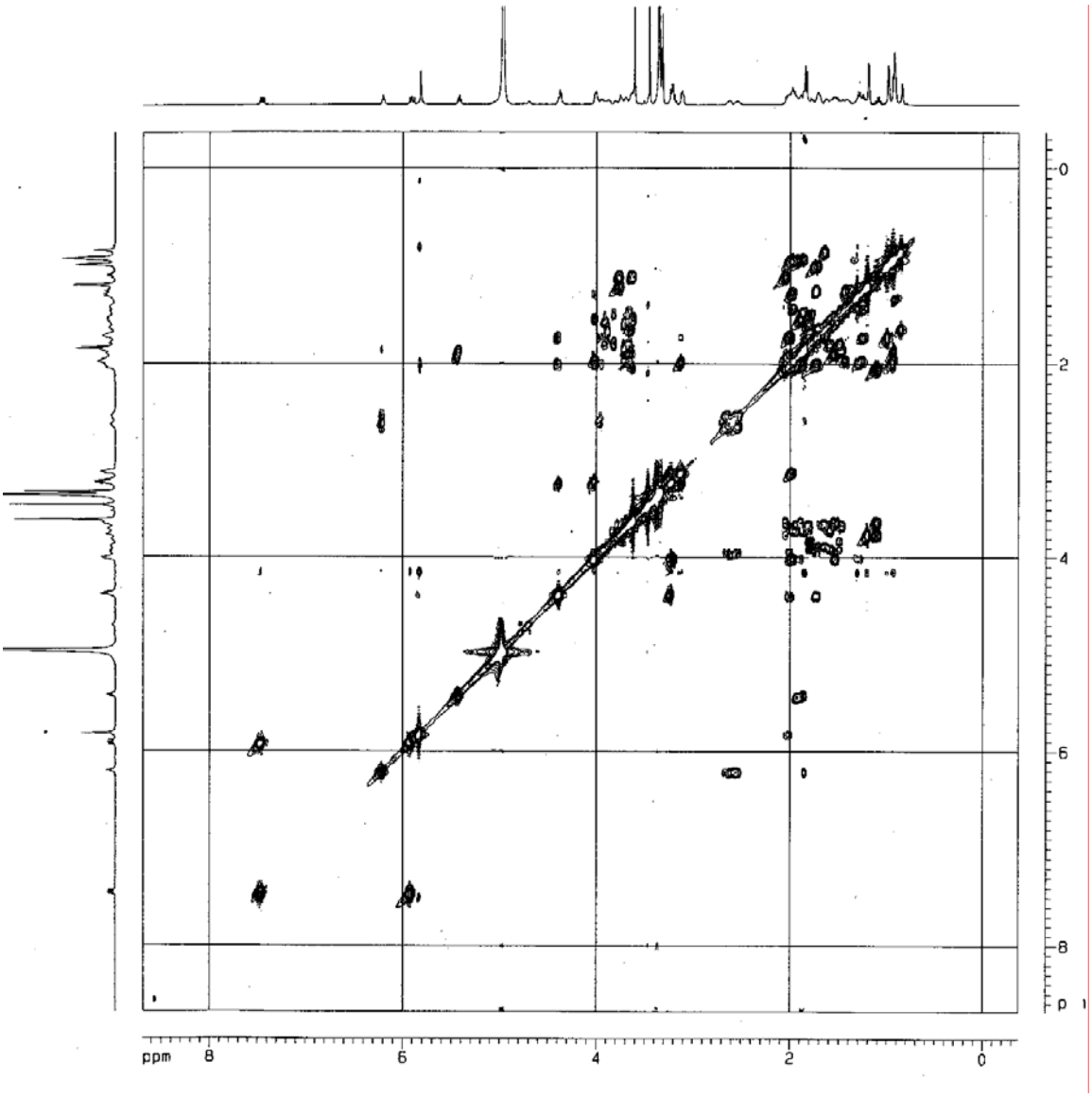


Figure 2.12. 400 MHz ^{1}H-^{1}H COSY spectrum of ankaraholide B (**2**) in CD_3OD

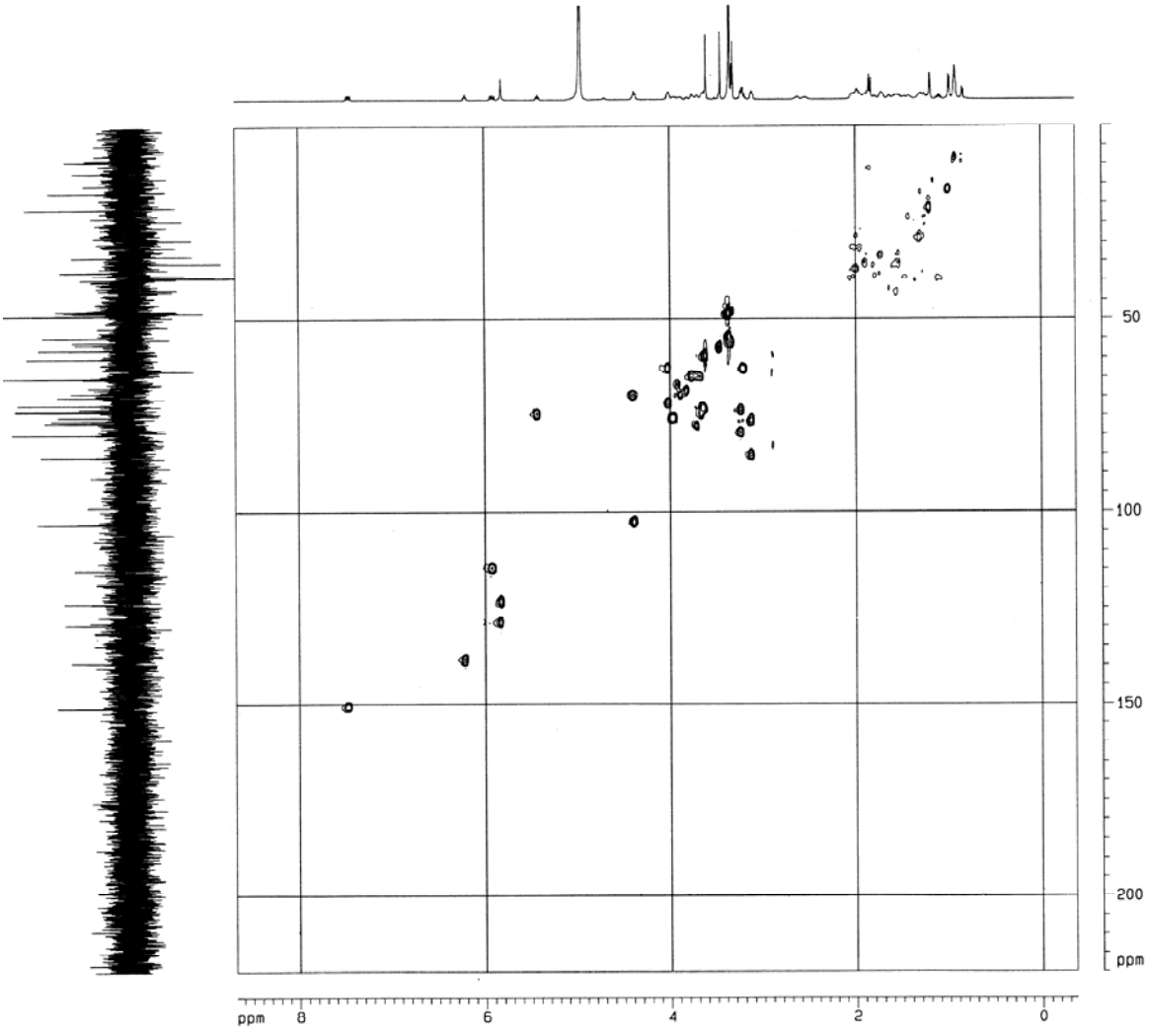


Figure 2.13. 600 MHz Multiplicity-edited HSQC spectrum of ankaraholide B (**2**) in CD_3OD.

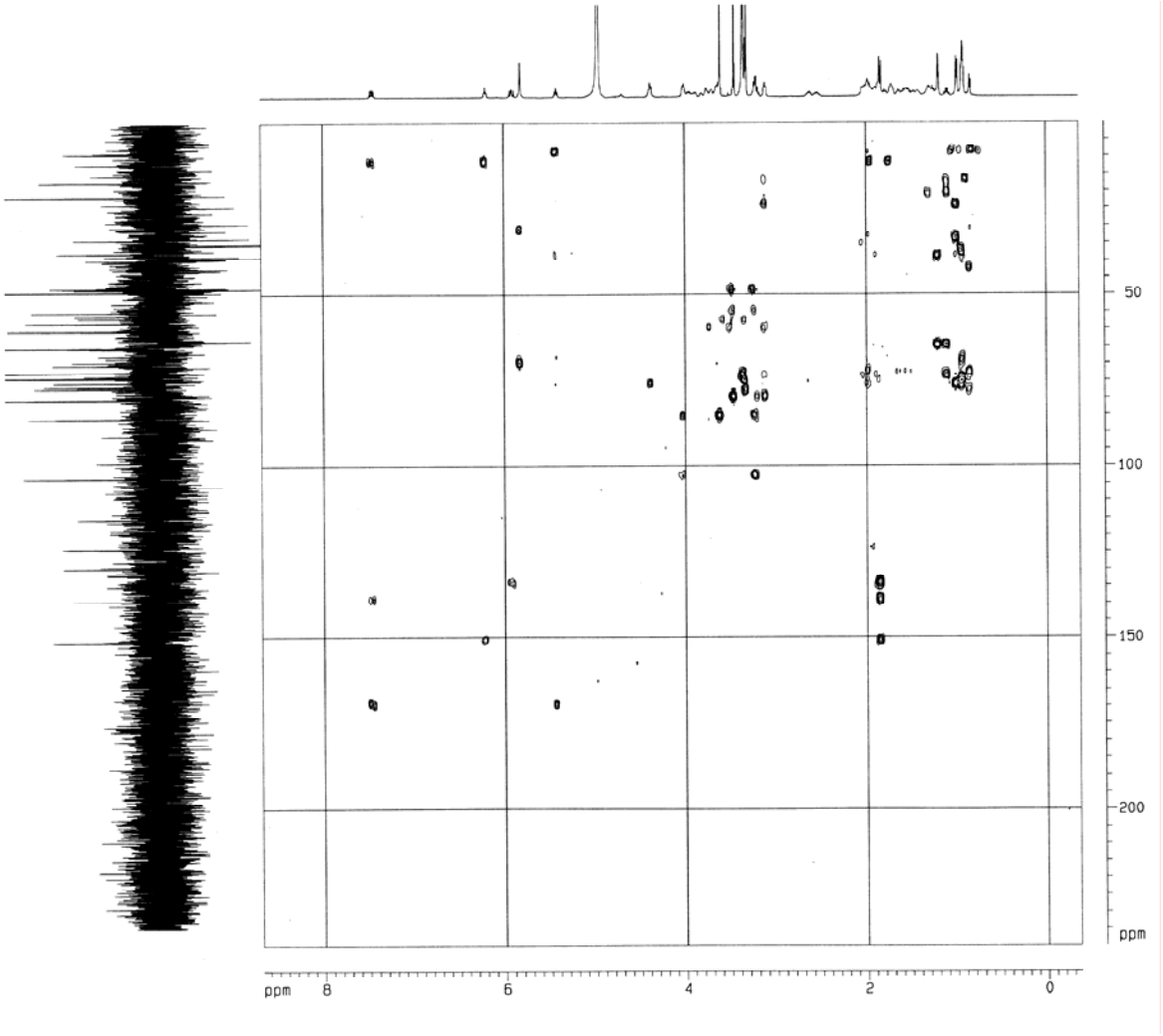


Figure 2.14. 600 MHz HMBC spectrum (optimized for *J* = 4Hz) of ankaraholide B (**2**) in CD_3OD.

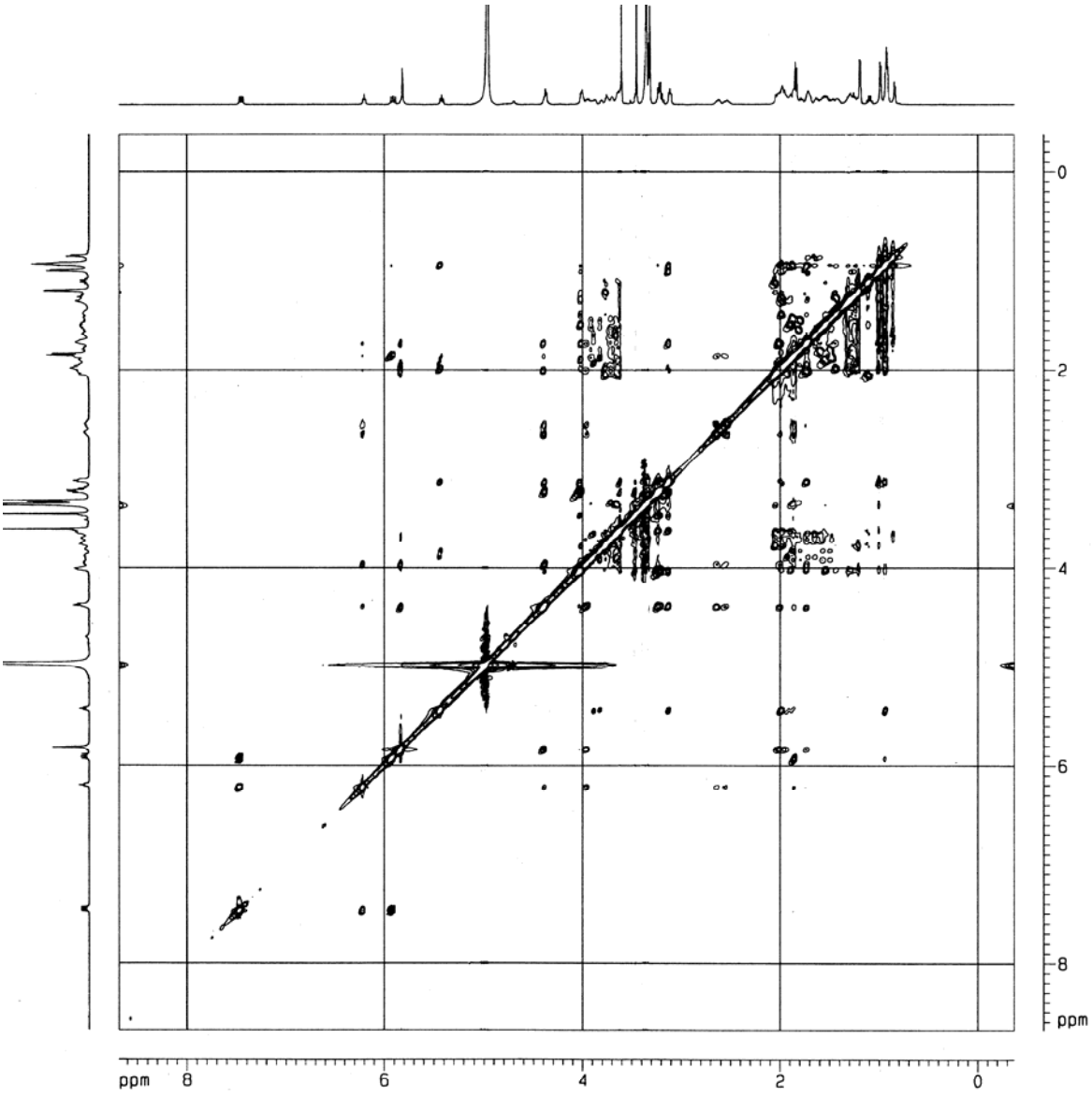


Figure 2.15. 600 MHz ROESY spectrum of ankaraholide B (**2**) in CD_3OD.

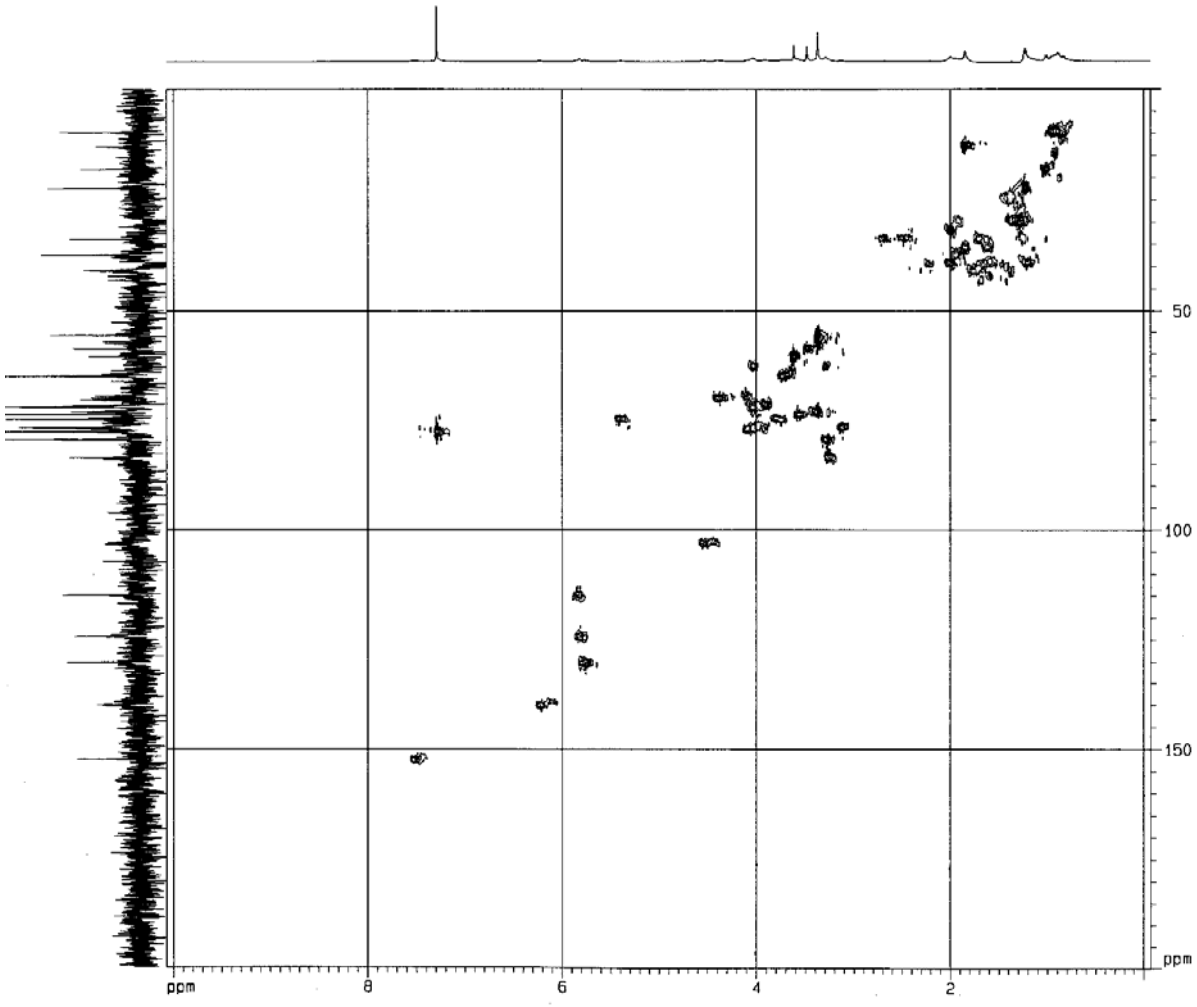


Figure 2.16. 400 MHz HSQC spectrum of ankaraholide B (**2**) in $CDCl_3$.

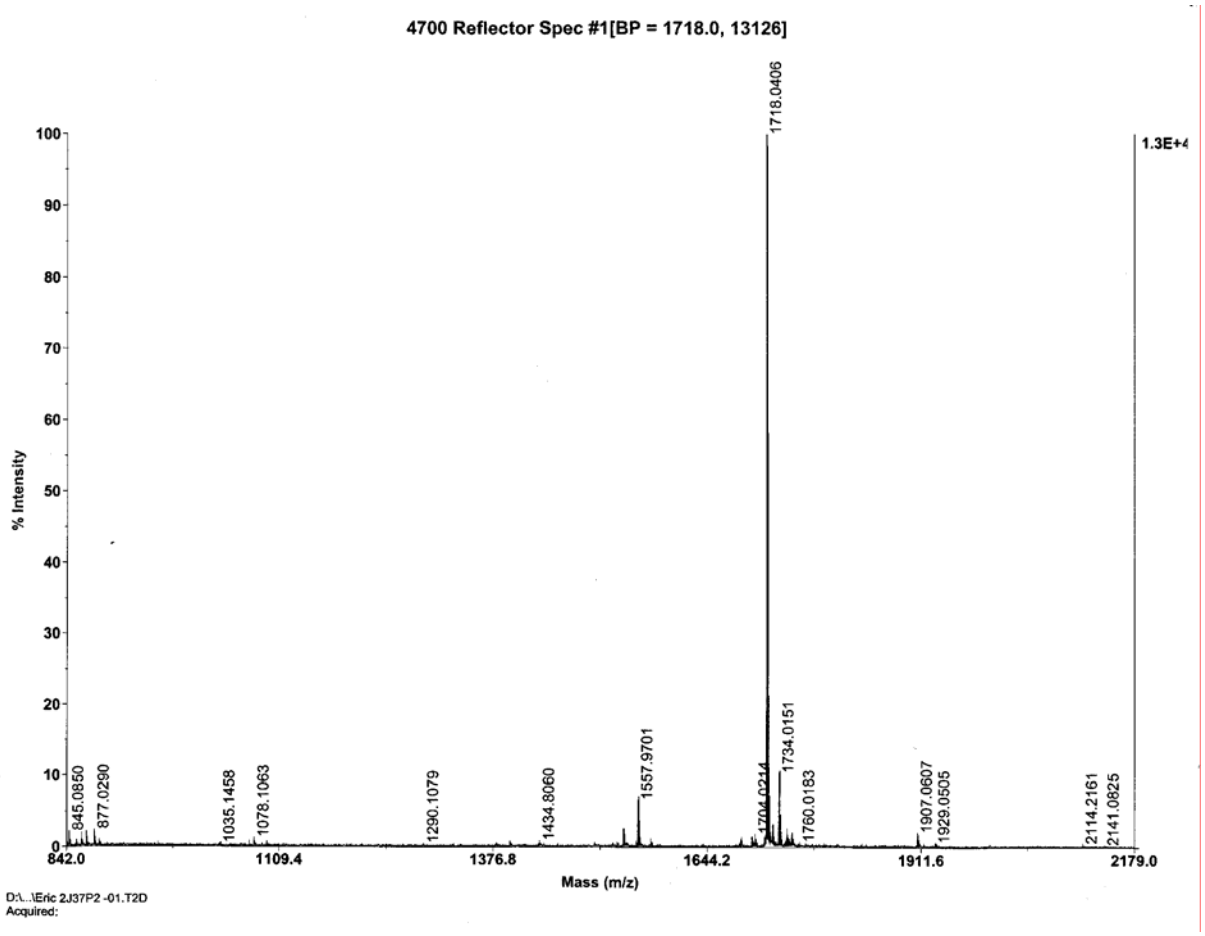


Figure 2.17. MALDI-TOF spectrum of ankaraholide B (**2**).

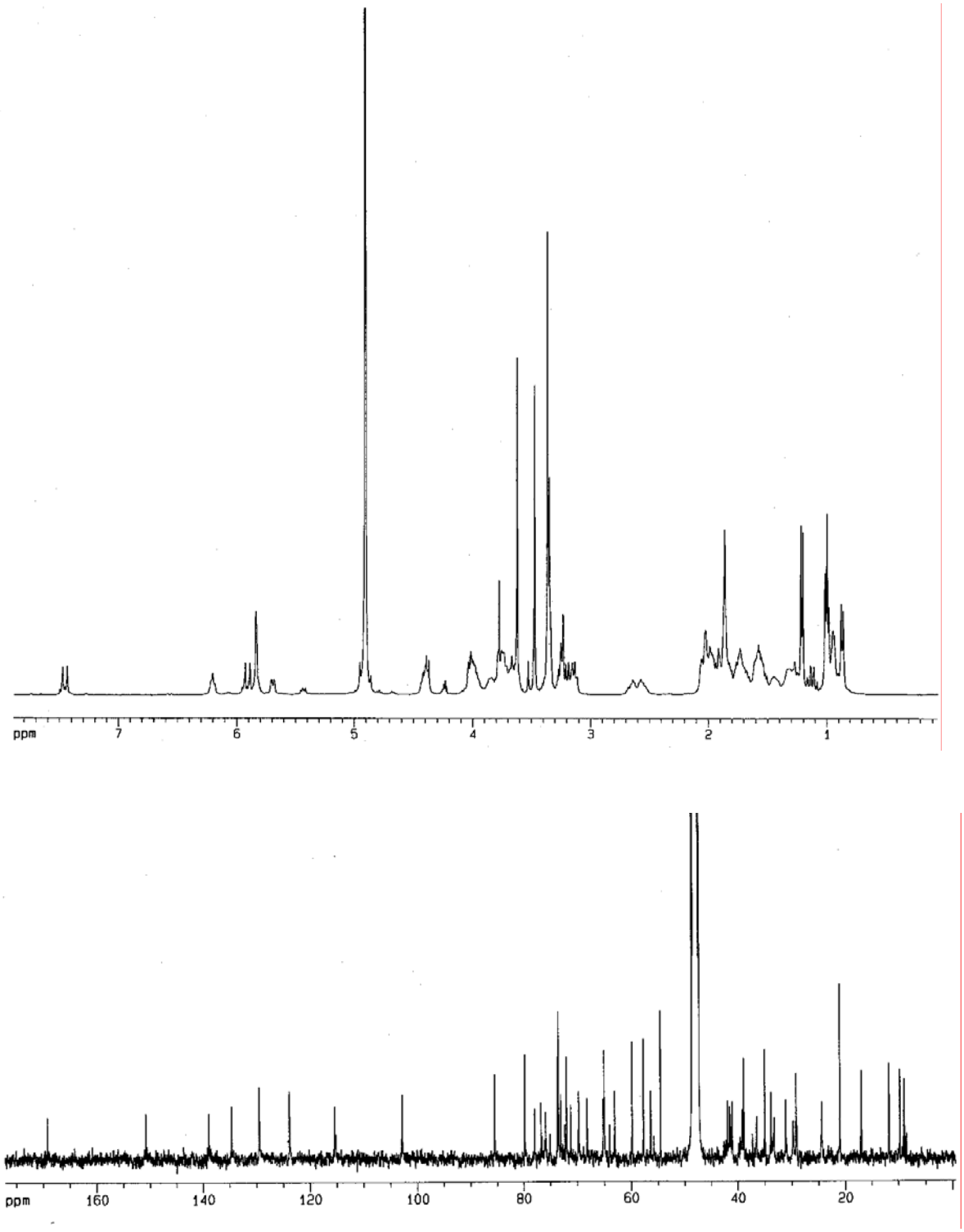


Figure 2.18. 400 MHz ^{1}H NMR spectrum and 100 MHz ^{13}C spectrum of ankaraholide C (**3**) in CD_3OD.

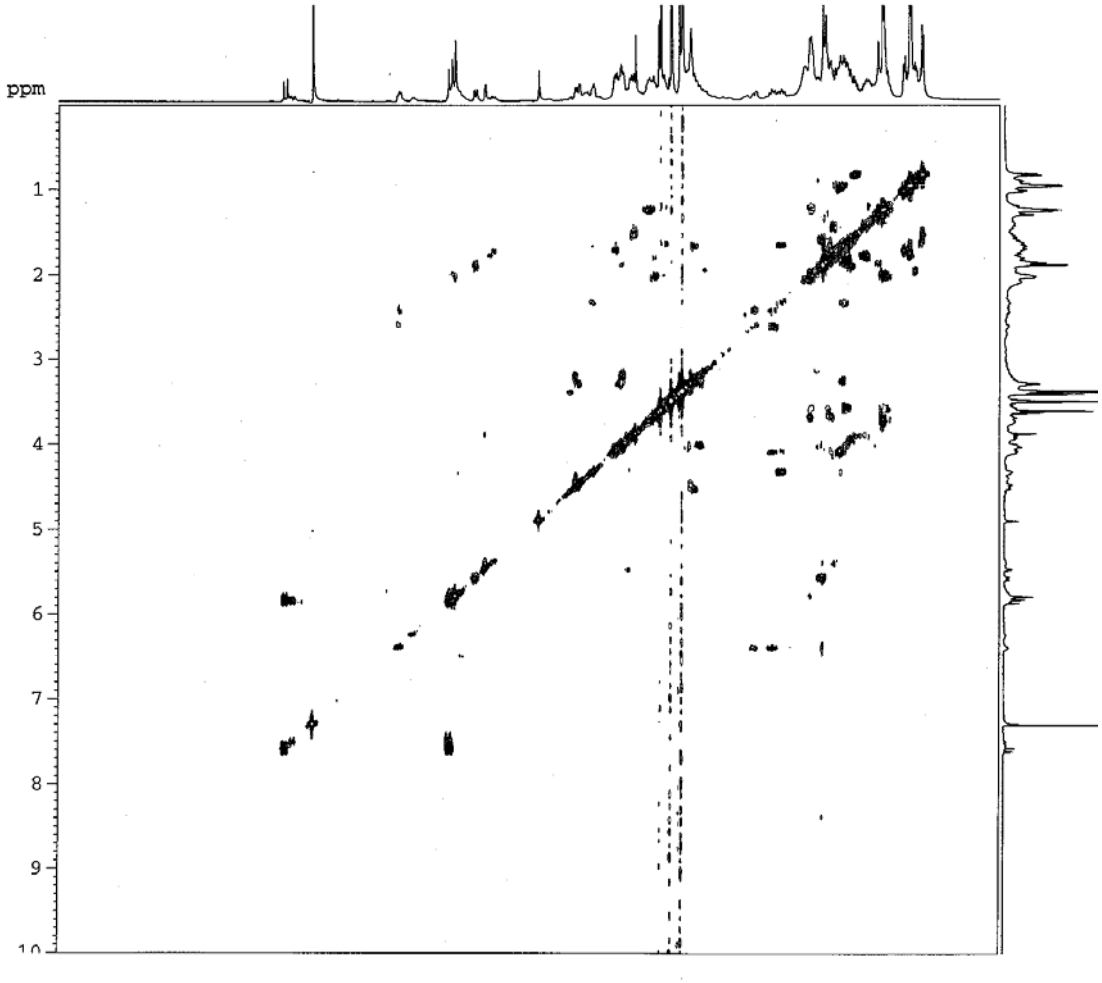


Figure 2.19. 400 MHz ^{1}H-^{1}H COSY spectrum of ankaraholide C (**3**) in CD_3OD.

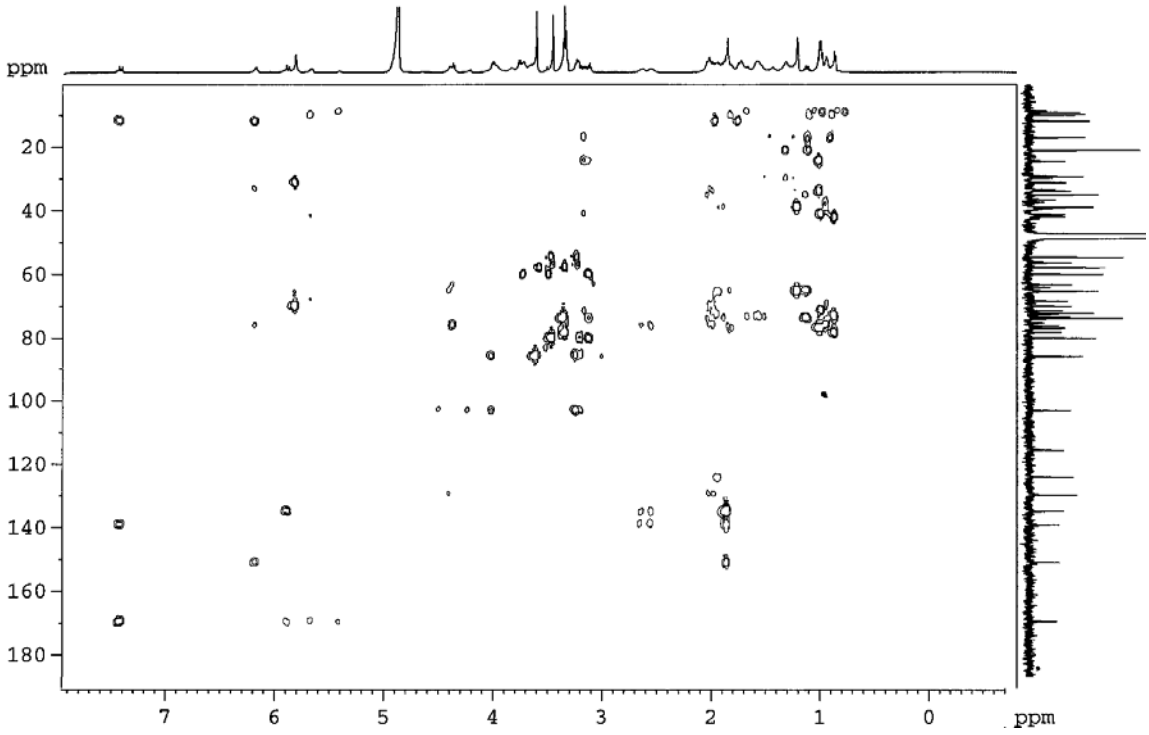


Figure 2.20. 400 MHz HMBC spectrum (optimized for *J* = 8Hz) of ankaraholide C (**3**) in CD_3OD.

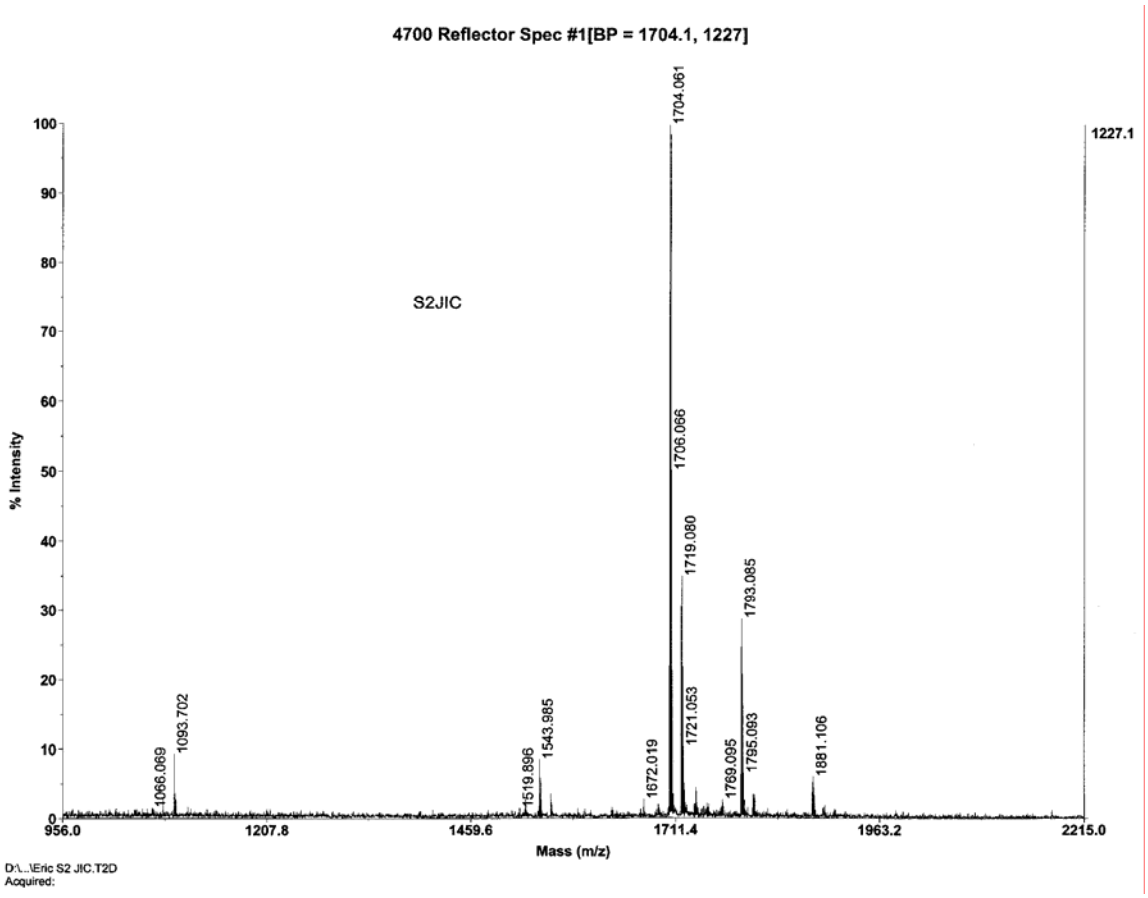


Figure 2.21. MALDI-TOF spectrum of ankaraholide C (**3**).

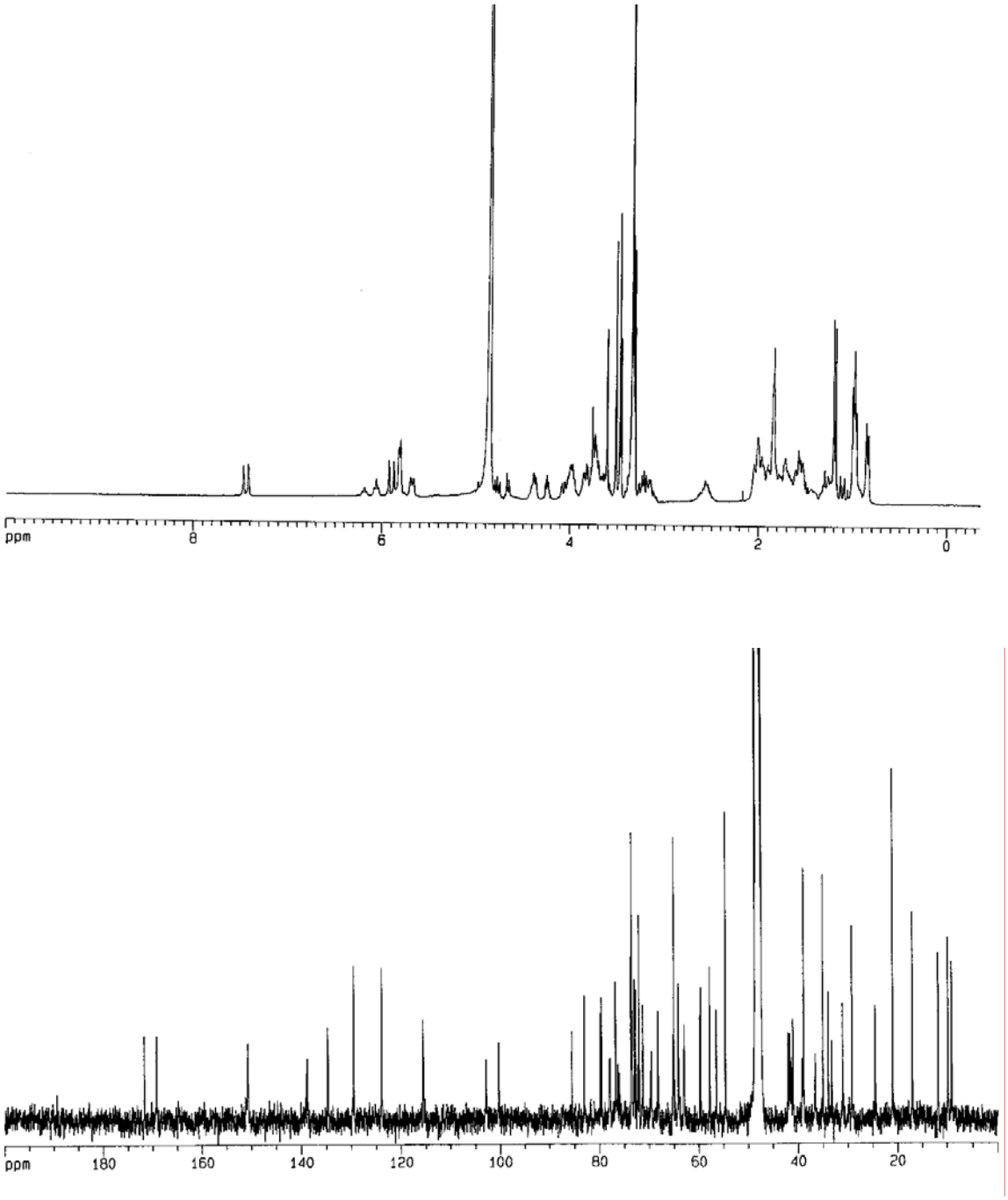


Figure 2.22. 400 MHz ^{1}H NMR spectrum and 100 MHz ^{13}C spectrum of ankaraholide D (**4**) in CD_3OD.

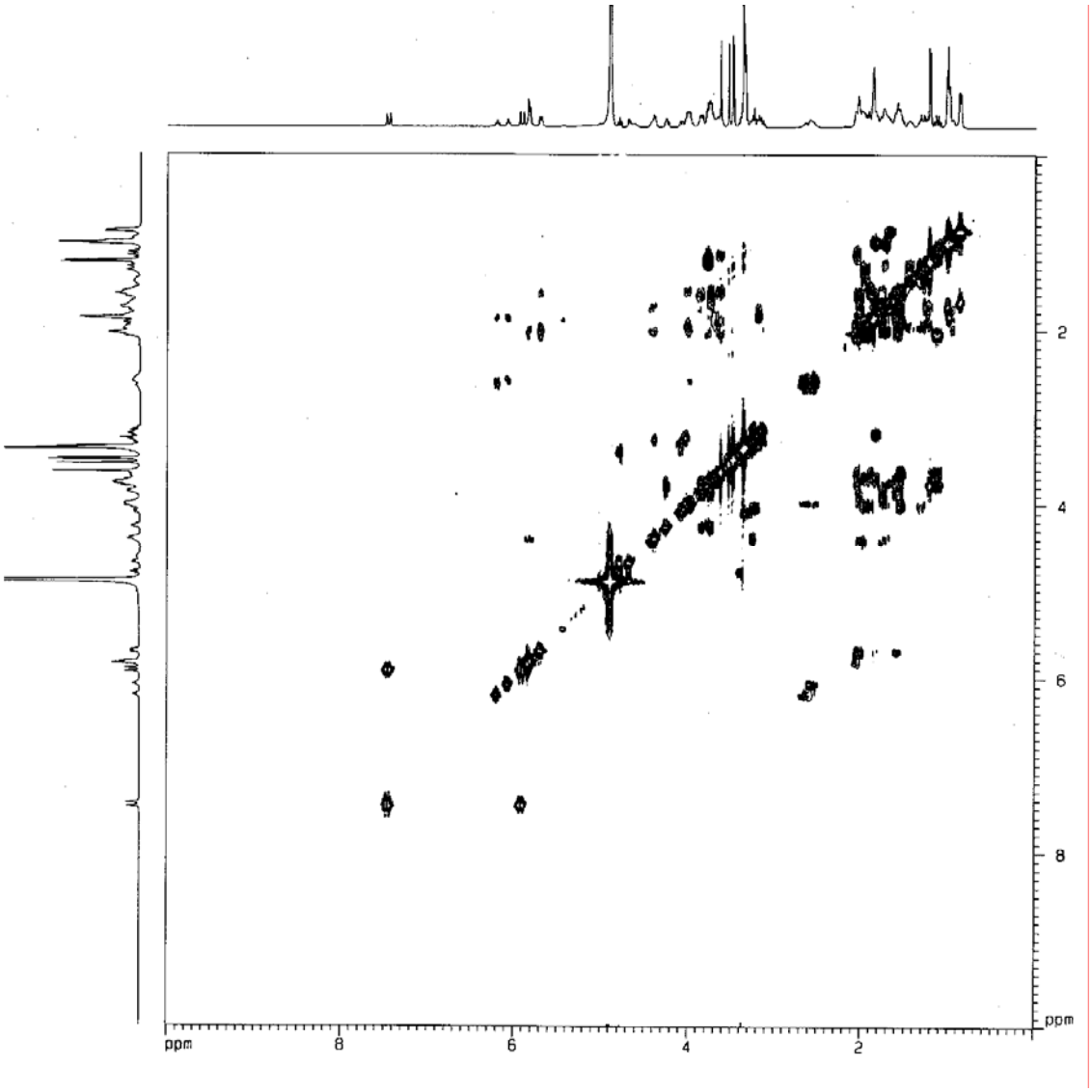


Figure 2.23. 400 MHz ^{1}H-^{1}H COSY spectrum of ankaraholide D (**4**) in CD_3OD

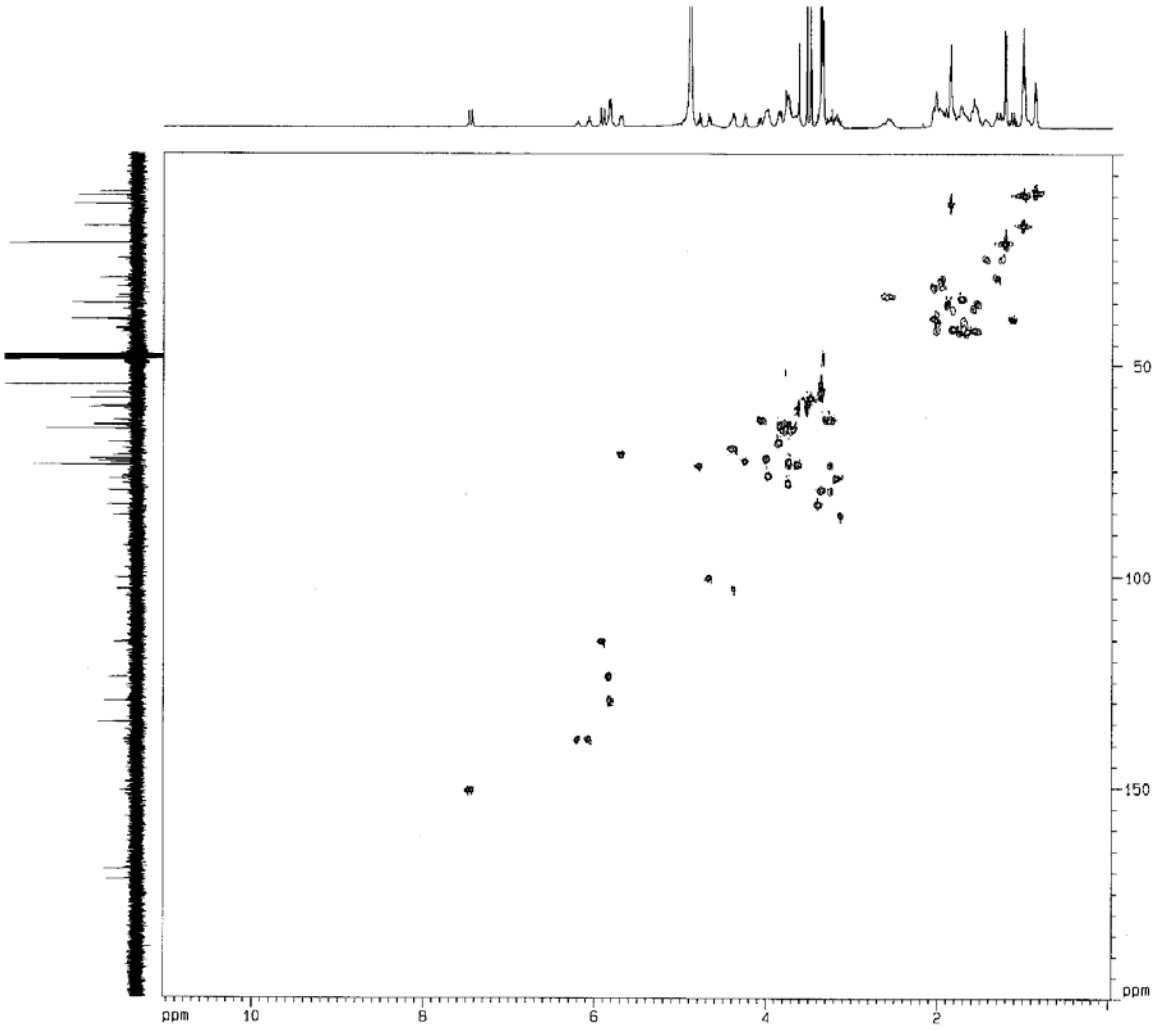


Figure 2.24. 400 MHz Multiplicity-edited HSQC spectrum of ankaraholide D (**4**) in CD_3OD

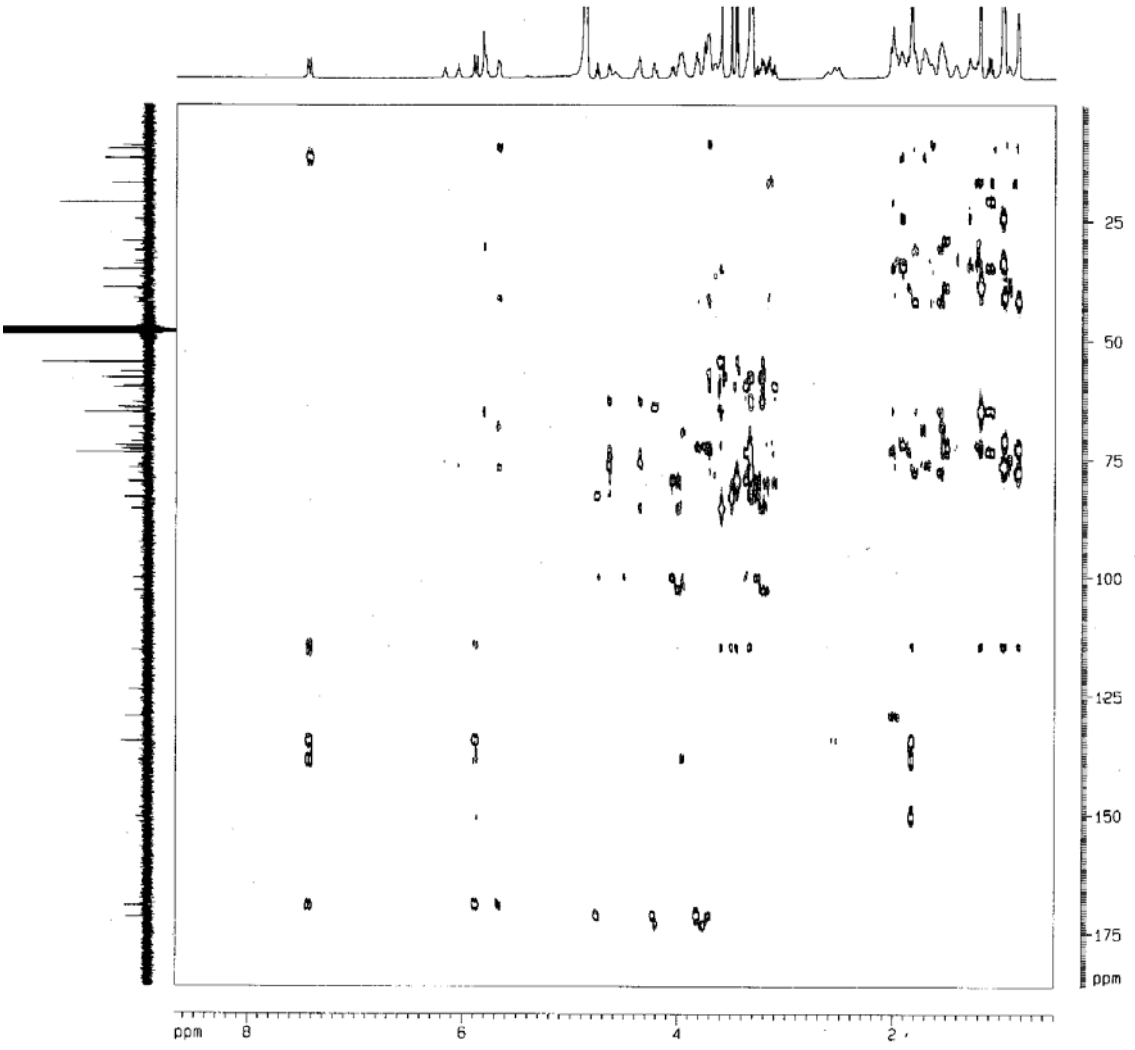


Figure 2.25. 600 MHz HMBC spectrum (optimized for *J* = 4Hz) of ankaraholide D (**4**) in CD_3OD.

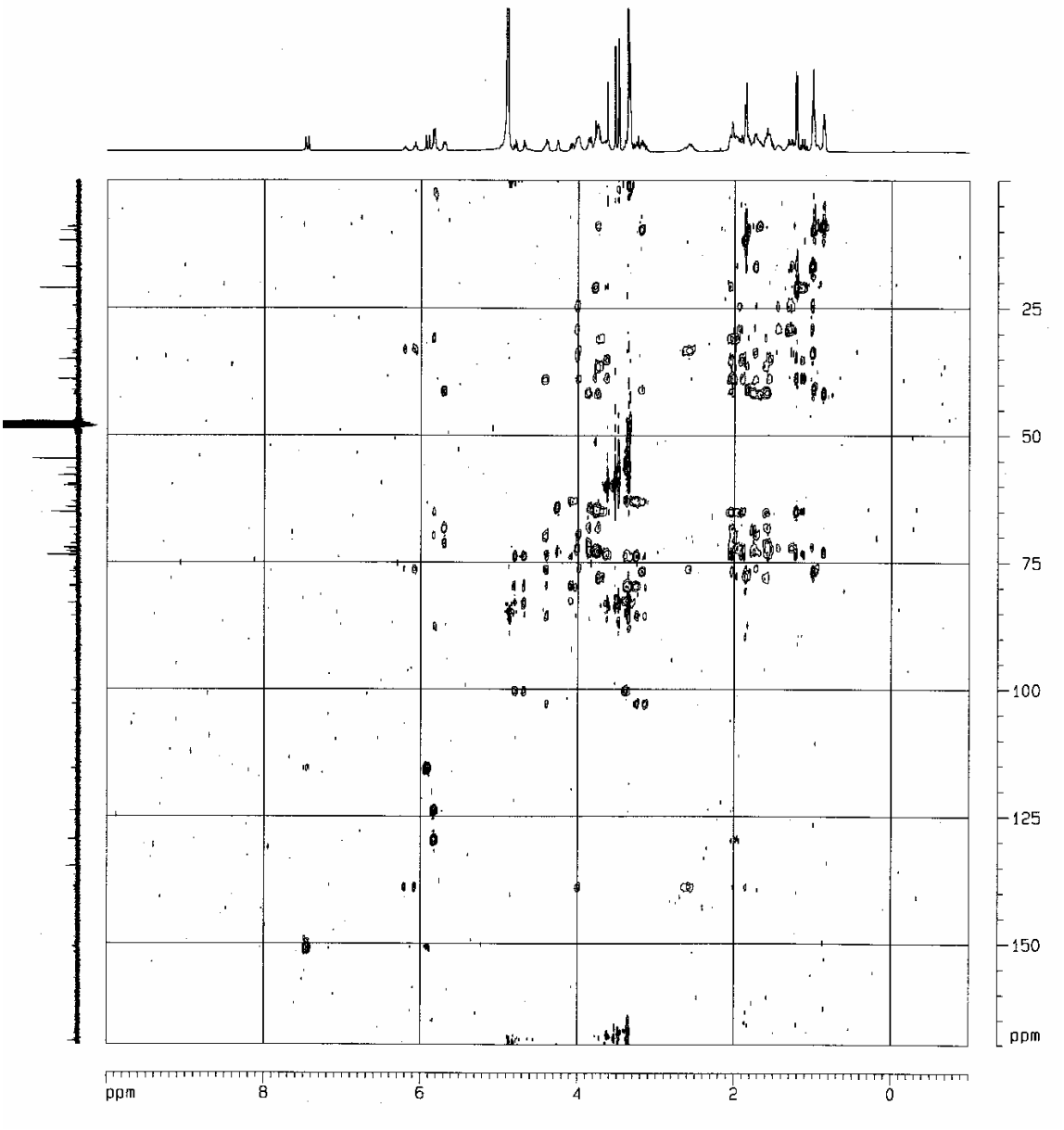


Figure 2.26. 400 MHz HSQC-TOCSY spectrum of ankaraholide D (**4**) in CD_3OD.

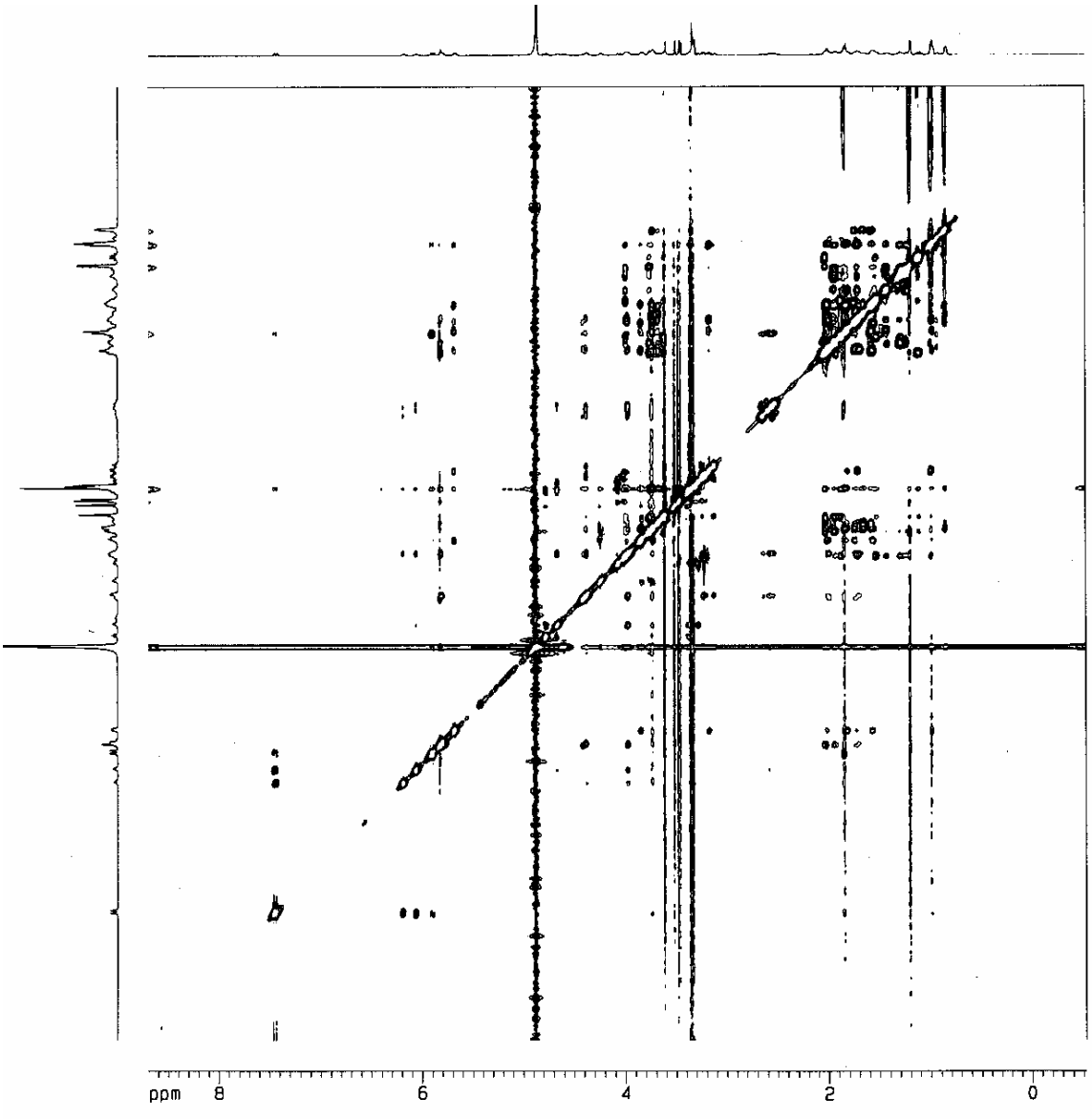


Figure 2.27. 600 MHz ROESY spectrum of ankaraholide D (**4**) in CD_3OD

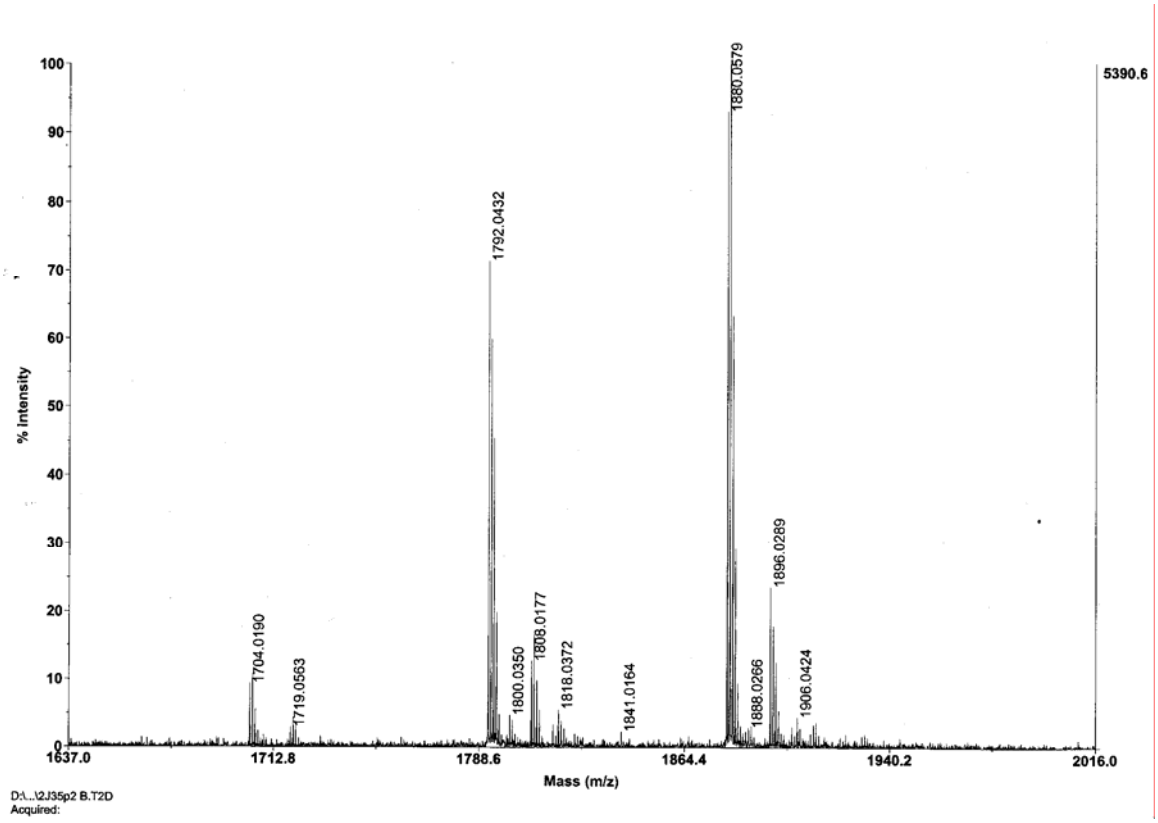


Figure 2.28. MALDI-TOF spectrum of ankaraholide D (**4**).

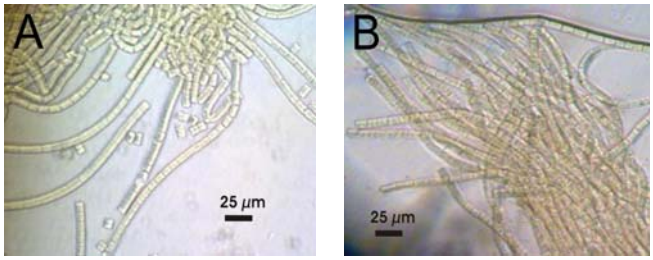


Figure 2.29 Photomicrographs of voucher samples of (A) *Symploca* cf sp. from Fiji[14] and (B) *Geitlerinema* cf sp. from Madagascar.

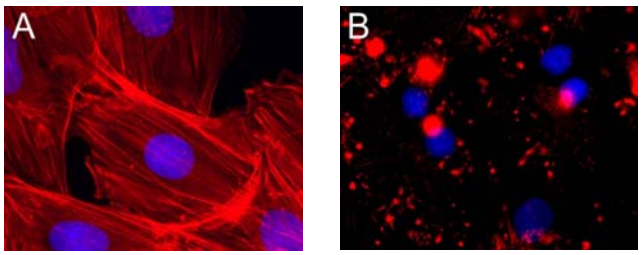


Figure 2.30 Effect of ankaraholide A (**1**) on the actin cytoskeleton of A-10 cells. After 24 h cells were processed and exposed to the microfilament staining reagent TRITC-phalloidin (visualized as red in the figure) and to the DNA-reactive compound DAPI (visualized as blue). (A) Control cells. (B) Treatment of the cells with ankaraholide A (**1**) at 60 nM, which caused complete loss of the cellular microfilament network and generated binucleated cells.

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on Perkin Elmer 141 and 243 polarimeters, respectively. UV and FT-IR spectra were obtained employing Hewlett Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on Bruker Avance DRX300, DPX400 and DRX600 spectrometers. Spectra were referenced to residual solvent signal with resonances at $\delta_{H/C}$ 7.26 / 77.1 ($CDCl_3$) and $\delta_{H/C}$ 3.31/49.15 (CD_3OD). MALDI-TOF and TOF-MS-ES data were recorded on Applied Biosystems 4700 TOF-TOF and Waters Micromass LCT Classic mass spectrometers, respectively. HPLC was carried out using a Waters system consisting of a Rheodyne 7725i injector, two 515 pumps, a pump control module and a 996 photodiode array detector. All solvents were purchased as HPLC grade.

Cyanobacterial Collection and Taxonomy

The marine cyanobacterium *Geitlerinema* cf sp. (voucher number MMA-18Apr00-02) was collected in April 2000 from Nosy Mitso-Ankaraha Island, NosyBe, Madagascar from a depth of 16 m by SCUBA. After collection, the microalga was stored in 2-propanol at -20 ^{o}C until workup. Trichomes of the Malagasy cyanobacterium sample were 5 to 6.25 µm wide. The cells were longer than broad or isodiametric. Cell length varied from 6.25 to 10 µm. No constrictions between the adjacent cells were observed and a sheath was absent. The live specimen was red colored and produced an external “slimy” gelatinous layer. Thus, dimensions and appearance of the sample are in accordance with the description of the form-genus *Geitlerinema* (Castenholz R. W.; Rippka R.; Herdman M. In *Bergey’s Manual of Systematic Bacteriology*; Boone D. R.; Castenholz R. W.; Garrity G. M., Eds; Springer-Verlag: New York, Berlin, Heidelberg, 2001; Vol. 1, p 544). Cyanobacterial strain was examined using a Zeiss light microscope (400 x magnification).

Extraction and Isolation procedures

A total of 164.0 g (dry wt) of the cyanobacterium *Geitlerinema* cf sp. was extracted repeatedly with CH_2Cl_2-MeOH (2:1, v/v) to produce 1.0 g of crude organic extract. The crude extract was found to be active at 10 ppm (90% mortality) in the brine shrimp toxicity assay. A portion of the crude extract (950 mg) was fractionated using

VLC on silica gel with a stepwise gradient of hexanes-EtOAc and EtOAc-MeOH to give 13 fractions. Fraction 10 showed a cytotoxic effect (67% toxicity 10 ppm to brine shrimp) and was further fractionated on a Waters C_{18} solid phase extraction cartridge (RP-18 SPE; 2 g) using a stepwise gradient from 50:50 MeOH/H_2O to 100% MeOH to give 11 subfractions. The fraction eluting in 90:10 MeOH/H_2O was subjected to reversed phase HPLC (column: Phenomenex Sphereclone ODS, 250 x 10.0 mm, 5µm) using a linear gradient from 85:15 MeOH/H_2O to 100% MeOH over 50 min at 2.5 mL·min^{-1}, yielded 2.1 mg of ankaraholide A (**1**), 1.1 mg of ankaraholide B (**2**) and 2 mg of ankaraholide D (**4**). Fraction 9 (eluting at 50:50 EtOAc/MeOH) showed a cytotoxic effect (63% toxicity 1 ppm to brine shrimp) and was further purified on analytical RP HPLC (Phenomenex Sphereclone ODS 5 µm, 250 x 10.0 mm, MeOH/H_2O (90:10)) to yield 1.7 mg of ankaraholide C (**3**).

Ankaraholide A (1): yellowish liquid; $[\alpha]^{25}_D$ - 47° (*c* 0.12, $CHCl_3$); UV ($CHCl_3$) λ_{max} 268 nm (ε 57000); IR (neat) ν_{max} 3440, 2925, 2855, 1685, 1618, 1463, 1384, 1316, 1185, 989, 758 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3COD), see Table 2.2; MALDI TOF MS *m/z* 1704.03260 (calcd for $C_{90}H_{152}O_{28}Na$ $[M+Na]^+$, 1704.03624).

Ankaraholide B (2): yellowish liquid; $[\alpha]^{25}_D$ - 37° (*c* 0.07, $CHCl_3$); UV ($CHCl_3$) λ_{max} 266 nm (ε 57000); IR (neat) ν_{max} 3442, 2926, 2861, 1694, 1626, 1463, 1389, 1316, 1186, 995, 757 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3COD), see Table 2.3; ESI-TOF-MS *m/z* $[M+Na]^+$ 1718.0591, MALDI TOF MS *m/z* 1718.0406 (calcd for $C_{91}H_{154}O_{28}Na$ $[M+Na]^+$, 1718.0524).

Ankaraholide C (3): yellowish liquid; $[\alpha]^{25}_D$ – 65° (*c* 0.19, $CHCl_3$); UV ($CHCl_3$) λ_{max} 268 nm (ε 57000); IR (neat) ν_{max} 3441, 2925, 2859, 1685, 1618, 1460, 1384, 1316, 1185, 989, 758 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3COD), see Table 2.4; MALDI TOF MS *m/z* 1704.03360 (calcd for $C_{90}H_{152}O_{28}Na$ $[M+Na]^+$, 1704.03624).

Ankaraholide D (4): yellowish liquid; $[\alpha]^{25}_D$ –55° (*c* 0.19, $CHCl_3$); UV ($CHCl_3$) λ_{max} 268 nm (ε 57000); IR (neat) ν_{max} 3441, 2926, 2861, 1694, 1623, 1463, 1388, 1315, 1186, 995, 759 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3COD), see Table 2.4; MALDI TOF MS *m/z* 1880.0579 (calcd for $C_{96}H_{160}O_{34}Na$ $[M+Na]^+$, 1880.0689).

Brine Shrimp Toxicity Bioassay.

Brine Shrimp *Artemia salina* toxicity was measured as previously described.[16] After a 24 h hatching period, aliquots of a 10 mg/mL stock solution of sample was added to test wells containing 5 mL of artificial seawater and brine shrimp to achieve a range of final concentrations. After 24 h the live and dead shrimp were tallied.

Cytotoxicity Assays with Neuro-2a and H460 cells.

Toxicity towards mouse neuro-2a cells and NCI-H460 lung tumor cells was measured using the method of Alley et al.[15] with cell viability being determined spectrophotometrically by MTT reduction as previously described.[17] Cells were seeded in 96-well plates at 6000 cells/well in 180 µL. After 24 h, pure compounds, dissolved in DMSO and diluted into medium without fetal bovine serum were added at 20µg/well. DMSO was less than 1% of the final concentration. After 48 h, the medium was removed and cell viability determined.

Sulforhodamine B (SRB) Assay.

Inhibition of MDA-MB-435 cell proliferation and cytotoxicity were measured using the SRB assay.[18] Cells were plated in 96-well plates and allowed to attach and grow for 24 h. The compounds or vehicle (ethanol) was added and incubated with the cells for 48 h. Following drug exposure, the cells were fixed with 10% trichloroacetic acid and then the cell layer was stained with 0.4% SRB. The absorbance of the SRB solution was measured at 560 nm. Dose-response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of the log dose-response curves.

Microfilament Disrupting Assay.

Ankaraholide A (**1**) was tested for microfilament-disrupting activity using rhodamine-phalloidin. A-10 cells were grown on glass coverslips in Basal Medium Eagle (BME) containing 10% fetal calf serum. The cells were incubated with the test compound for 24 h and then fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 2 min, and chemically reduced with sodium borohydride (1 mg/mL in PBS) three times for 5 min each. Following a 45 min incubation with 100 nM TRITC-phalloidin in phosphate buffered saline (to visualize the actin cytoskeleton), the coverslips were washed, stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize

DNA, mounted on microscope slides, and examined and photographed using a Nikon E800 Eclipse fluorescence microscope with a Photometrics Cool Snap FX3 camera. The images were colorized and overlayed using Metamorph® software.

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CHAPTER THREE

HALOGENATED MONOTERPENES FROM THE MADAGASCAR RED MARINE ALGA *PORTIERIA HORNEMANNII*

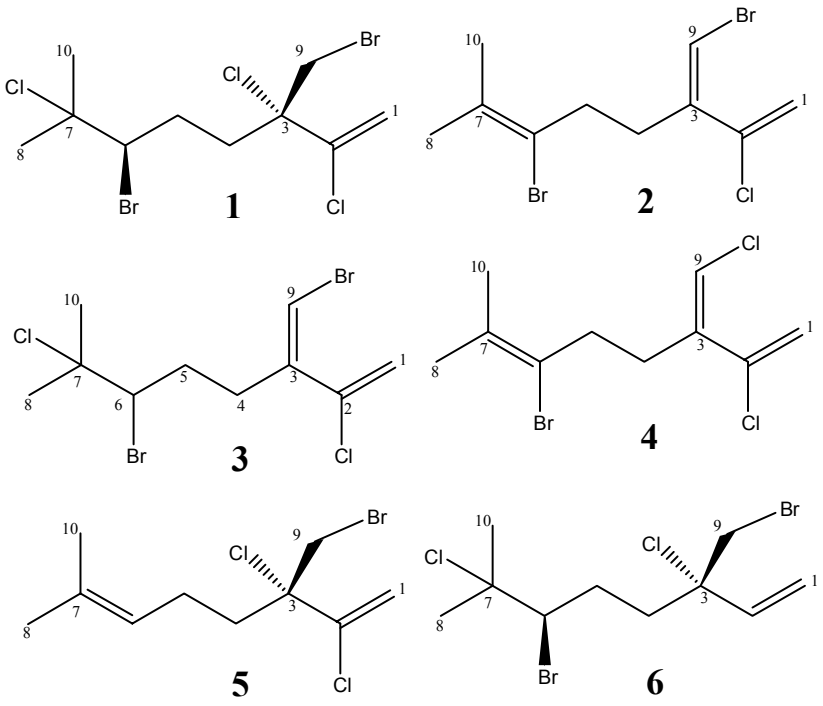
ABSTRACT

Three new halogenated monoterpenes, (**2**), (**3**) and (**4**), along with the known compounds halomon (**1**) and two analogues, (**5**) and (**6**), were isolated from the Madagascar red marine alga *Portieria hornemannii.* The molecular structures of all three new compounds were determined by NMR spectroscopy in combination with mass spectral data analysis. Compounds **1**, **2, 3** and **6** were evaluated in a DNMT-1 enzyme inhibition assay.

INTRODUCTION

Many species of red algae synthesize natural products that contain bromine, chlorine and occasionally iodine atoms.[1] Halomon [6(*R*)-bromo-3(*S*)-(bromomethyl)-7-methyl-2,3,7-trichloro-1-octene] was first isolated from the red alga *Portieria hornemannii* (Lynbye) collected in the Philippines in April 1992.[2] Halomon strongly exhibited differential cytotoxicity to brain, renal and colon derived cell lines in the National Cancer Institute's *in vitro* human tumor cell line screening panel.[2,3] On the basis of its unprecedented cytotoxicity profile, halomon was selected by the NCI for preclinical drug development.[2,4] However, research and development of halomon as an anticancer lead has been limited by the lack of a reliable natural source, and failure to show in vivo effects.[5] Attempts to re-isolate halomon from *Portieria hornemannii* collected from Batan Island in the Philippines, and other locations in the Pacific Ocean have been unsuccessful,[6] largely due to site-to-site and temporal variations in terpene content.[7] Chemical syntheses of halomon and analogues have been achieved although with some difficulties in regio and stereo-control.[8,9] Nevertheless, Thierry *et al.* reported a successful high yielding total synthesis of halomon (13% overall) and a variety of analogues.[10]

Screening of the organic extracts of marine algae and cyanobacteria for mechanism-based anticancer agents has been productive and led to the discovery of new chemotypes showing antiproliferative properties.[11,12,18] In our ongoing effort to discover and develop new marine natural product biomedicinals, we found that the organic extract of the Madagascar red alga, *Portieria hornemannii*, possessed a potent inhibitory activity to the DNA methyltransferase 1 (DNMT-1) isoform. DNMT-1 causes methylation of the cytosine phosphodiester linked guanine dinucleotide (CpG) by catalyzing the transfer of a methyl group from *S*-adenosylmethionine to the 5' position on cytosine residues residing at CpG sites.[12,18] In many cancers, promoters of tumor suppressor genes are silenced by hypermethylation at CpG sites, and thus, the inhibition of DNMT-1 could potentially reverse tumor growth.[12,18] Subsequently, the extract was subjected to bioassay-guided isolation and resulted in the isolation of three novel halogenated monoterpenes, (**2**), (**3**) and (**4**), along with the known compounds halomon (**1**) and its analogues (**5**) and (**6**). To our knowledge this is the first report of DNMT-1 enzyme inhibition by a halogenated monoterpene, including halomon.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RESULTS AND DISCUSSION

The algal sample was collected in the south of Madagascar (Tolagniaro, Fort Dauphin) in March 1997. It was found growing on shallow reef rocks, and taxonomically identified by its distinctive branching pattern and pungent odor when crushed.

The organic extract of *Portieria hornemannii* was initially fractionated by normal phase vacuum-liquid chromatography (NPVLC), and these fractions were tested for DNA methyltransferase-1 inhibitory activity. The least polar fraction (fraction A eluting with 100% hexanes) was active in this assay at 10 μg/mL. Preparative normal phase HPLC of fraction A yielded three sub-fractions and two were found to inhibit DNA methyltransferase (fraction A1 and A3 at 4 μg/mL and 8 μg/mL, respectively). Further fractionation of A3 by analytical NP-HPLC yielded halomon (**1)** and analog **6** as the major components. Continued purification of A1 using analytical normal phase HPLC led to seven fractions A1a to A1g, two of which were new compounds **2** and **3**. Further HPLC purification of fraction A1a and A1d led to the isolation of the new compound **4** and known compound **5**, respectively. The identity of halomon (**1**) was established by direct comparison of ^{1}H and ^{13}C NMR, HR-MS, and $[\alpha]^{20}_{D}$ with literature values.[1]

The LR CIMS of **2** displayed a number of complex fragment ion clusters, indicating that it had several halogen atoms. The fragment ion cluster at *m/z* 247/249/251 indicated the presence of one bromine and one chlorine atom whereas the fragment ion cluster at *m/z* 291/293/295 indicated the occurrence of two bromines. HR CIMS of the peak at *m/z* 246.99022 $[M\text{-}Br]^{+}$ analyzed for $C_{10}H_{13}ClBr$, and thus compound **2** had a molecular formula of $C_{10}H_{13}ClBr_2$, consistent with three degrees of unsaturation.

The ^{13}C NMR, DEPT and multiplicity-edited HSQC of **2** characterized 10 carbon atoms, and included six olefinic carbons (δ106.1, 118.9, 120.1, 132.2, 136.1, 142.3), two methyl groups (δ 20.9, 25.8) and two methylene carbons (δ 34.7, 36.2). The ^{1}H NMR of **2** showed the presence of two three-proton singlets (δ 1.80, 1.95) assigned as geminal olefinic methyl groups, a four-proton multiplet (δ 2.62) assigned as protons belonging to two methylene groups, and finally, three olefinic protons composed of one singlet (δ 6.25) and two doublets (δ 5.48, 5.68). A multiplicity-edited HSQC revealed that the two-doublet protons were attached to the same

carbon (δ 118.9) while the singlet proton was attached to a carbon at δ 106.1. Thus, the remaining olefinic carbons were fully substituted.

The arrangement of these 10 carbons was established using HMBC and ^{1}H-^{1}H COSY experiments. The two protons attached to the carbon at δ 118.9 showed HMBC correlations to the carbon at δ 136.1 establishing partial structure **a (**Figure 3.1.). The singlet olefinic proton at δ 6.25 displayed an HMBC correlation to the quaternary carbon at δ 142.3, indicating the connectivity of partial structure **b.** Strong HMBC correlations between the multiplet protons at δ 2.62 and carbons at δ 34.7 and δ 36.2 indicated that these two methylenes were adjacent (partial structure **c**). Finally, the methyl groups at δ 1.95 and δ 1.80 showed HMBC correlations to carbons at δ 132.2 and δ 120.1, thereby, establishing partial structure **d**.

The connection between partial structures **a** and **b** was established by HMBC correlations between the protons at δ 5.48 and 5.68 and the quaternary carbon at δ 142.3. Connectivities between partial structure **c** and **b** as well as **c** and **d** were established by HMBC correlations between protons at δ 2.62 and quaternary carbons at δ 142.3/106.1 and δ 120.1/132.2, respectively. By ^{1}H-^{1}H COSY an allylic coupling was observed between the protons at δ 2.62 and the methyl group at δ 1.95, confirming the connectivity of partial structure **c** and **d**.

Given the proton and carbon count from the molecular formula and their assigned positions as described above, all of the halogen atoms were required to be positioned on double bonds. Carbon-13 NMR spectroscopy played an important role in the assignment of the regiochemistry. While both bromine and chlorine markedly deshield the carbon to which they are attached, the effect of chlorine is generally larger.[15] For example, the chemical shift of the C-2 quaternary carbon (δ 136.1) indicated that chlorine was attached whereas the shift at the C-6 quaternary carbon (δ 120.1) indicated bromine was present. Similarly, the chemical shift at C-9 (δ106.1) indicated that bromine was attached at this position. To determine the geometry of the double bond between C-3 and C-9, a series of 1D NOE experiments were performed. Irradiation of H-9 (δ 6.25) showed NOE enhancement in H-5 (δ 2.62) but not H-1, thus indicating that the geometry was *Z*, and thus completing the structure of compound **2** (Figure 3.2.).

LR GCMS analysis of **3** showed major fragment peaks at *m/z* $[M\text{-}Br\text{-}HCl]^{+}$ 247/249/251, with relative intensities indicative of one chlorine and one bromine

atom, and *m/z* $[M-Br]^+$ 283/285/287, with relative intensities indicative of one bromine and two chlorine atoms, thus yielding a molecular formula of $C_{10}H_{14}Cl_2Br_2$; this was confirmed by HR CIMS (see experimental). The two degrees of unsaturation inherent to the molecular formula of **3** were assigned to two olefinic bonds (Table 3.2.). The ^{13}C NMR spectrum of compound **3** showed similarities to **2** at positions C-1 through C-4 and C-9, and similarities to compound **1** at positions C-5 through C-8 and C-10 (Table 3.2.). For example, the ^{13}C NMR resonances for C-6 and C-7 in **2** were missing in **3,** and new halogen-bearing carbons were present as for **1**. Further, the ^1H NMR showed an additional signal at δ 4.03 by comparison with compound **2**. Multiplicity-edited HSQC showed that this proton was attached to the halogen-bearing carbon at δ 64.5 and an HMBC correlation between this proton and the other halogen-bearing carbon at δ 72.4 revealed that these two carbons were arranged precisely as found in **1**.

The regiochemistry of compound **3** was established using ^{13}C NMR chemical shift analysis in comparison with **2** for C-2 and C-9 and with **1** for C-6 and C-7. Thus, bromine was attached to C-9 (δ106.1) whereas chlorine was attached to C-2 (δ135.7). Correspondingly, bromine was attached to C-6 (δ 64.5) and chlorine to C-7 (δ 72.4). The geometry of the C-3/C-9 double bond between was found to be *Z* by a 1D NOE experiment (irradiation of H-9 enhanced H-4).

Extensive GC-MS analyses showed that compound **4** also contained several halogen atoms. The fragment ion cluster at *m/z* $[M-Cl]^+$ 247/249/251 indicated the occurrence of one chlorine atom and one bromine atom, and thus, compound **4** had a molecular formula of $C_{10}H_{13}Cl_2Br$.

The ^1H NMR spectrum of compound **4** was quite similar to that of compound **2**. The primary difference was the proton chemical shift of H-9 which was shifted downfield to δ 6.97 in compound **4** from δ 6.25 in **2**. Coupled with the MS data and molecular formula, this chemical shift change was consistent with the replacement of the H-9 bromine atom with a chlorine atom. The chemical shift of C-9 (δ 112.6) confirmed this deduction. Thus, compound **4** was the C-9 chloro-analog of metabolite **2**.

Halomon (**1**) and compounds **2**, **3** and **6** were tested in a DNMT-1 enzyme inhibition assay. Halomon (**1**) and compound **2** were found to have comparable activities (1.25 and 1.65 μM, respectively) while compound **3** and **6** were only weakly

active (55 and 21.9 μM, respectively). Compounds **4** and **5** were not tested due to the small quantities isolated.

The biological halogenation of monoterpenes is believed to involve the formation of halonium ions and their subsequent Markovnikov addition to the olefinic bond of myrcene.[17] Vanadium bromoperoxidase (V-BrPO) catalyzes the oxidation of halides (Br^-,Cl^-) by the peroxo complex of V-BrPO.[13,14] The oxidized halogen can then react with an appropriate unsaturated organic substrate by an electrophilic mechanism (Br^+, Cl^+). The resulting carbocation intermediate can then either react with a nucleophilic halogen atom (Br^- or Cl^-) to form mainly saturated natural products like halomon, or can loose a proton to regenerate the unsaturated species. In this Malagasy specimen of *Portieria hornemannii,* both of these manifolds appear to be in operation.

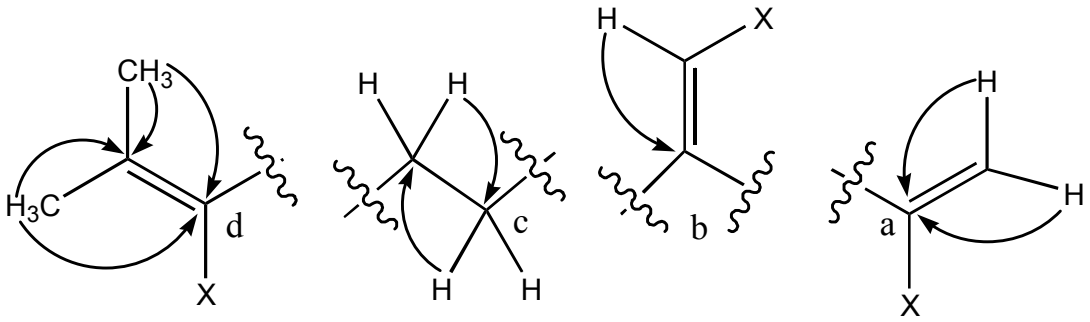

Figure 3.1. Partial structures **a**-**d** in compound **2** (arrows indicate HMBC correlations).

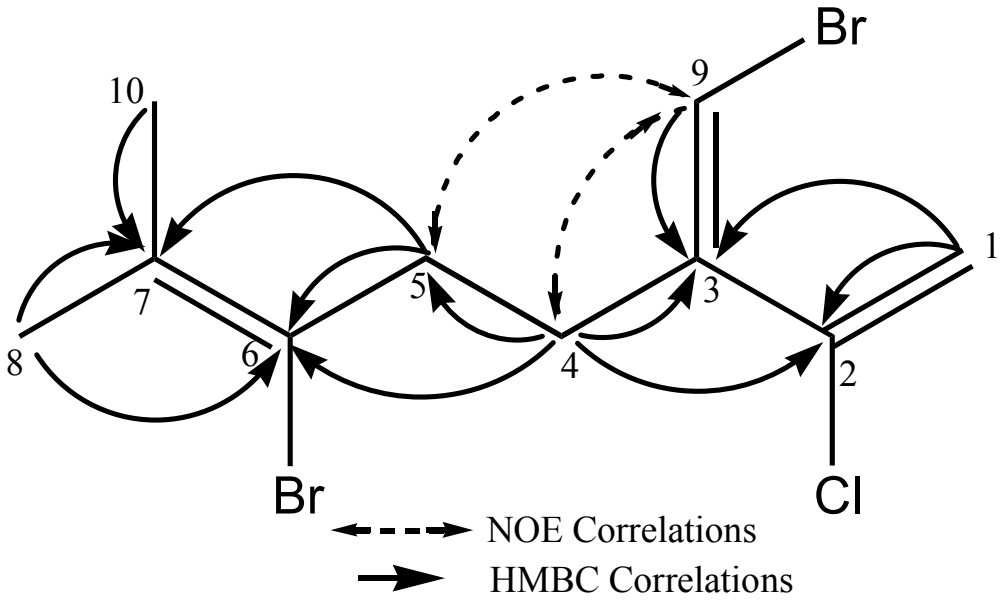

Figure 3.2. Key HMBC and selected NOE correlations for compound **2**.

Table 3.1. ^{1}H NMR assignments for compounds **1-4** (400 MHz, $CDCl_3$).

Position	**1**	**2**	**3**	**4**
1	5.68 (d, 2.3)	5.48 (d, 1.55)	5.49 (d, 1.65)	5.53 (d, 1.92)
	5.84 (d, 2.3)	5.68 (d, 1.55)	5.69 (d, 1.65)	5.70 (d, 1.92)
2				
3				
4	2.57 (ddd, 13, 9.3, 1.3)	2.62 (m)	2.80 (m)	2.70 (m)
	2.19 (t, 11.5)	2.62 (m)	2.50 (m)	2.75 (m)
5	2.57 (ddd, 13, 9.3, 1.3)	2.62 (m)	2.49 (m)	2.70 (m)
	2.02 (dddd, 9.3, 10.4, 11.5, 1.3)	2.62 (m)	1.89 (m)	2.75 (m)
6	4.08 (dd, 10.4, 1.3)		4.03 (dd, 11.3, 1)	
7				
8	1.71 (s)	1.80 (s)	1.69 (s)	1.84 (s)
9	3.89 (d, 11)	6.25 (s)	6.35 (s)	6.97 (s)
	3.83 (d, 11)			
10	1.85 (s)	1.95 (s)	1.81 (s)	1.90 (s)

Table 3.2. ^{13}C NMR assignments for Compounds **1-4** (100 MHz, $CDCl_3$).

Position	**1**	**2**	**3**	**4**
1	118.9	118.9	118.9	115.1
2	140.1	136.1	135.7	137.7
3	74	142.3	142.3	140.7
4	38.4	36.2	34.4	36.3
5	30.5	34.7	32.4	30.5
6	65.2	120.1	64.5	120.3
7	72.2	132.2	72.4	132.2
8	27.5	20.9	27.7	20.8
9	39.1	106.1	106.8	112.6
10	33.5	25.8	33.6	25.9

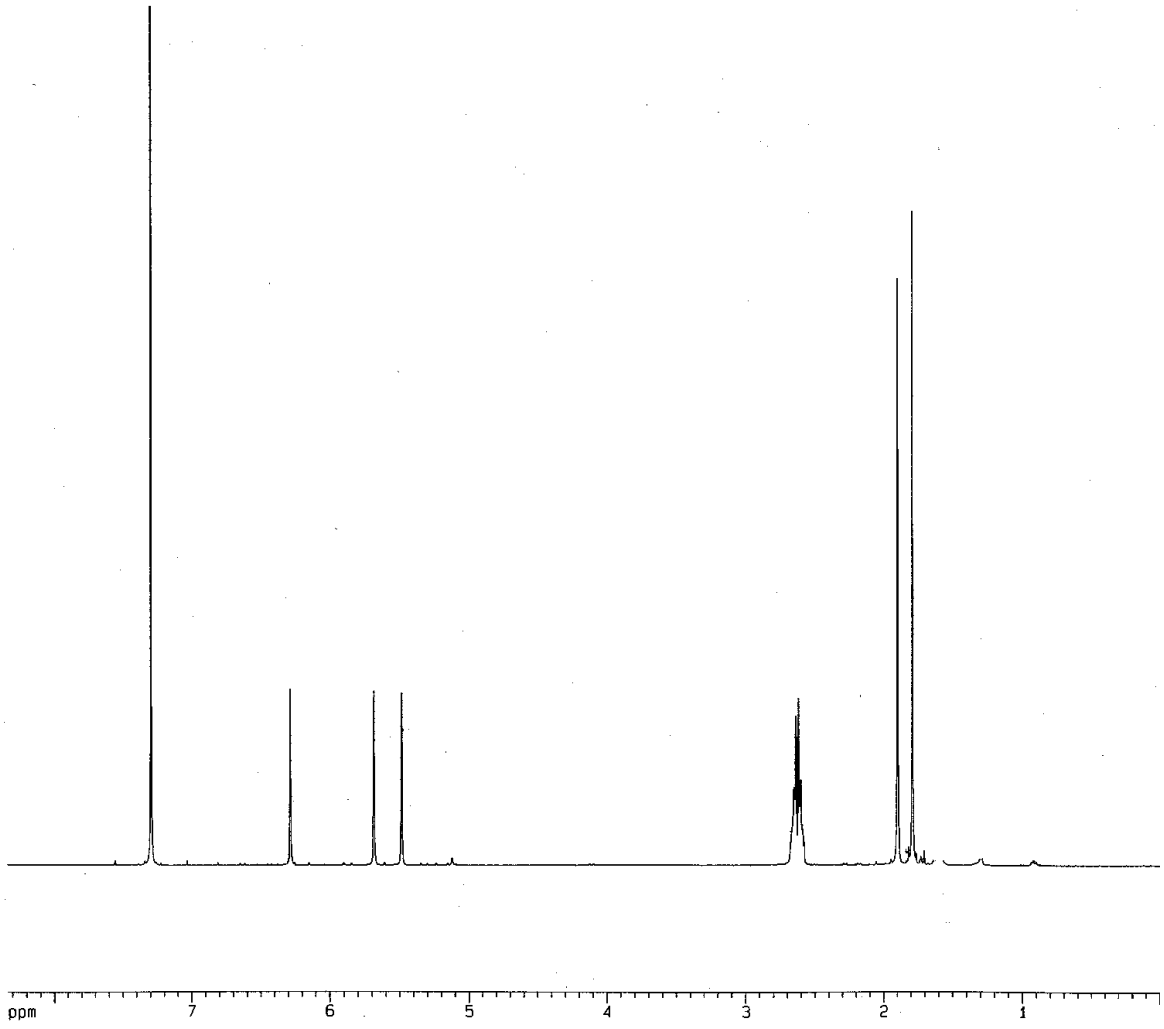

Figure 3.3. 400 MHz ^{1}H NMR spectrum of compound (**2**) in $CDCl_3$

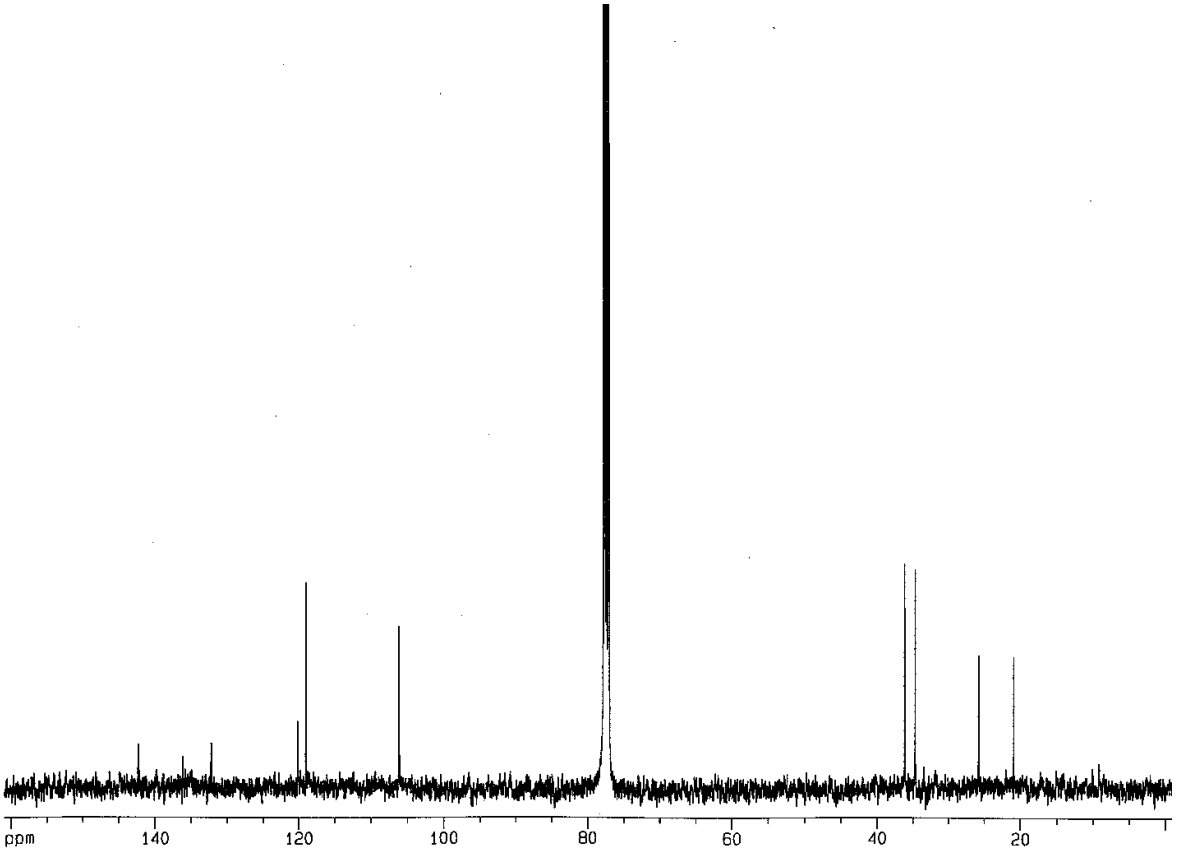

Figure 3.4. 100 MHz ^{13}C NMR spectrum of compound (**2**) in $CDCl_3$

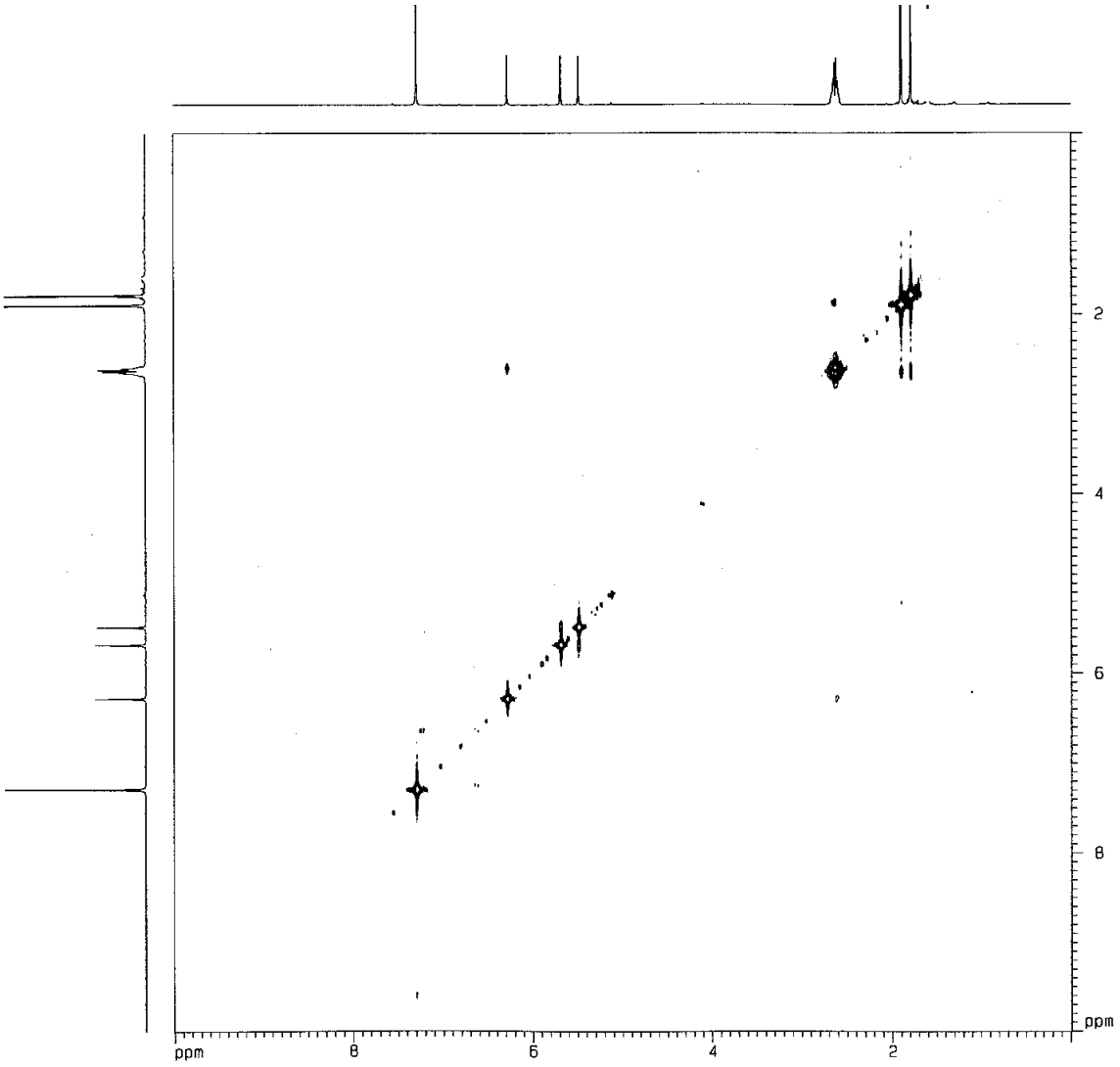

Figure 3.5. 400 MHz ^{1}H-^{1}H COSY spectrum of compound (**2**) in $CDCl_3$

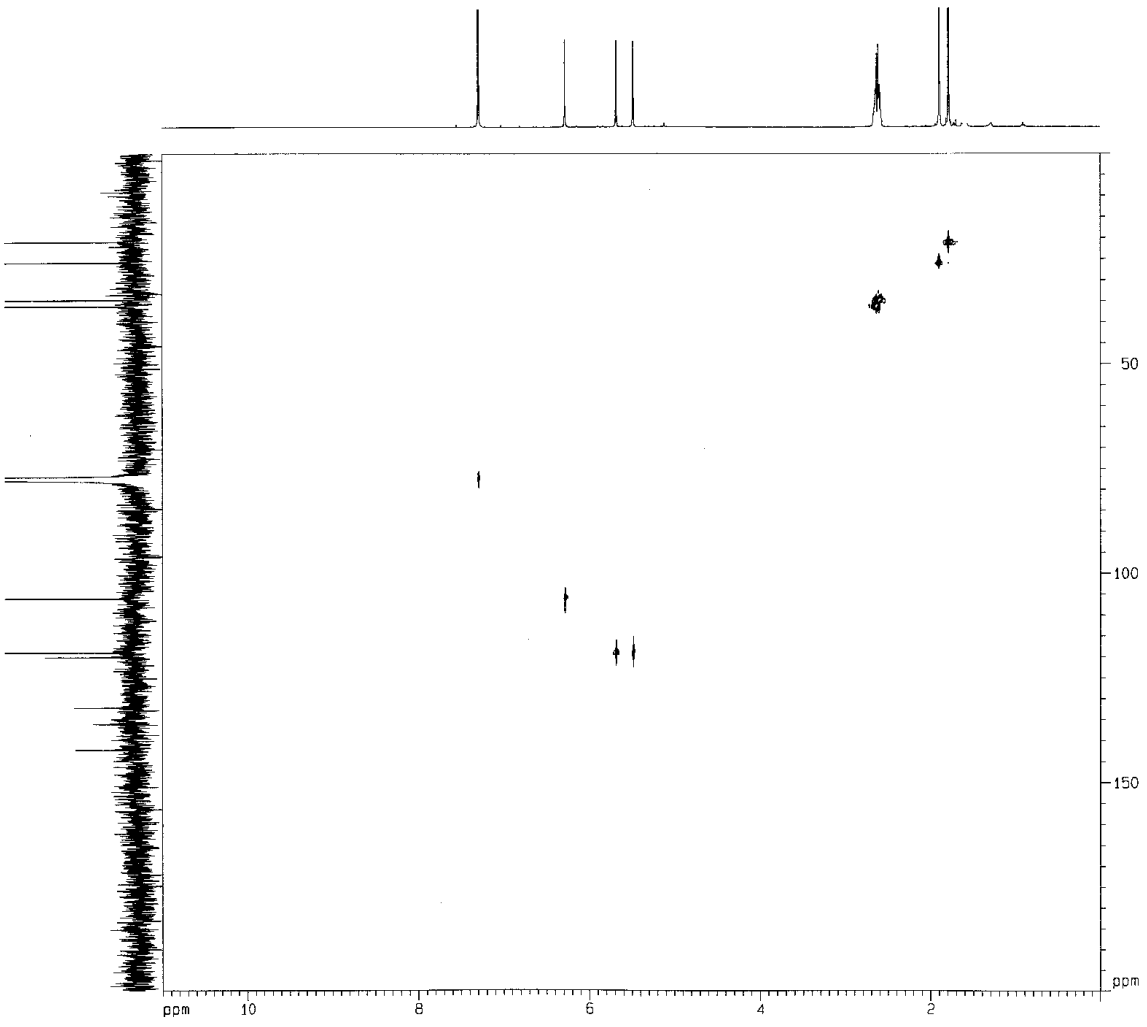

Figure 3.6. 400 MHz Multi-edited HSQC spectrum of compound (2) in $CDCl_3$

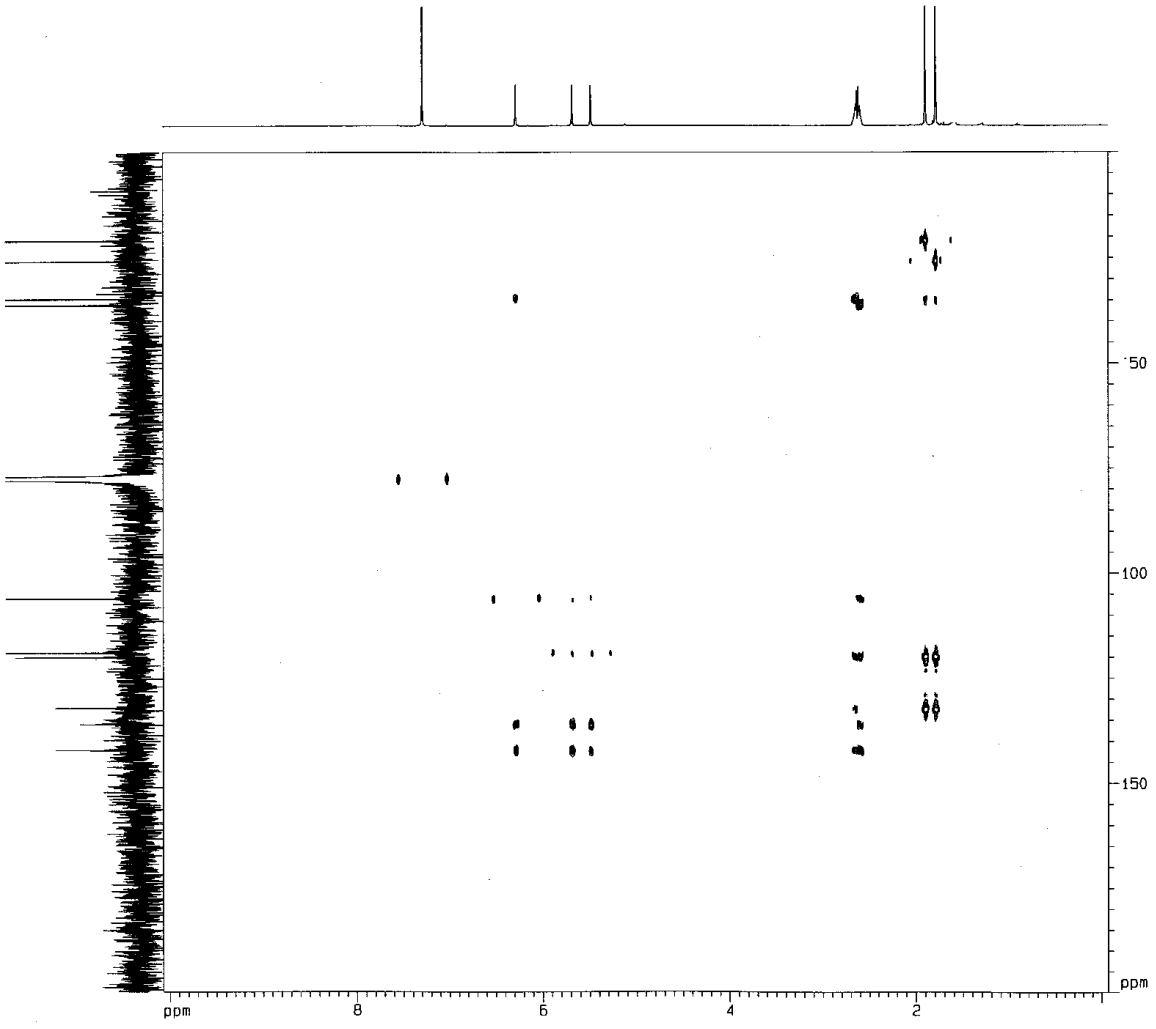

Figure 3.7. 400 MHz HMBC spectrum (optimized for *J* = 8Hz) of compound (**2**) in $CDCl_3$

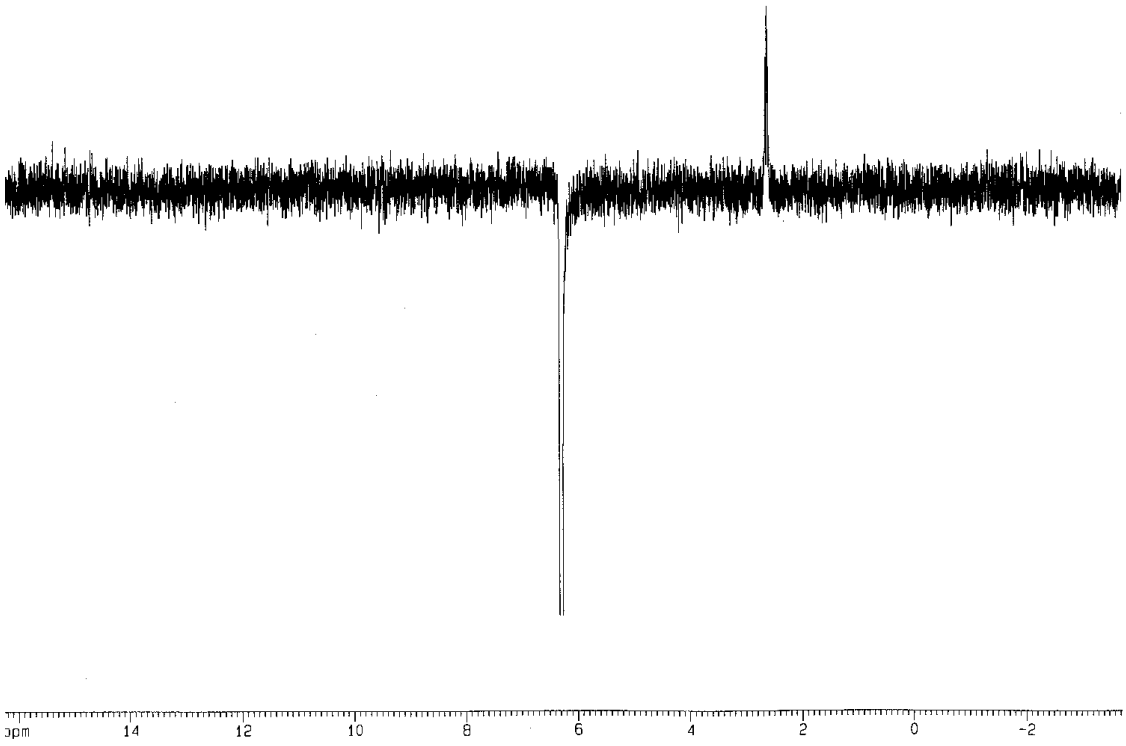

Figure 3.8. 400 MHz 1D NOE spectrum (irradiation of proton 6.25 ppm) of compound (**2**) in $CDCl_3$

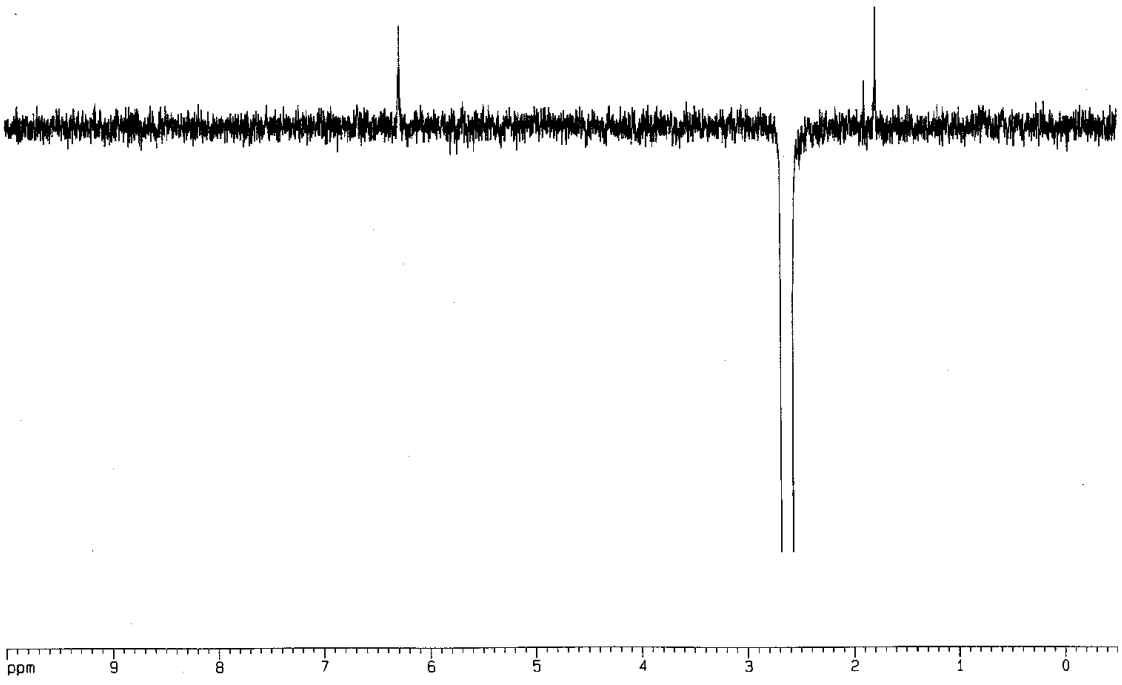

Figure 3.9. 400 MHz 1D NOE spectrum (irradiation of proton 2.62 ppm) of compound (**2**) in $CDCl_3$

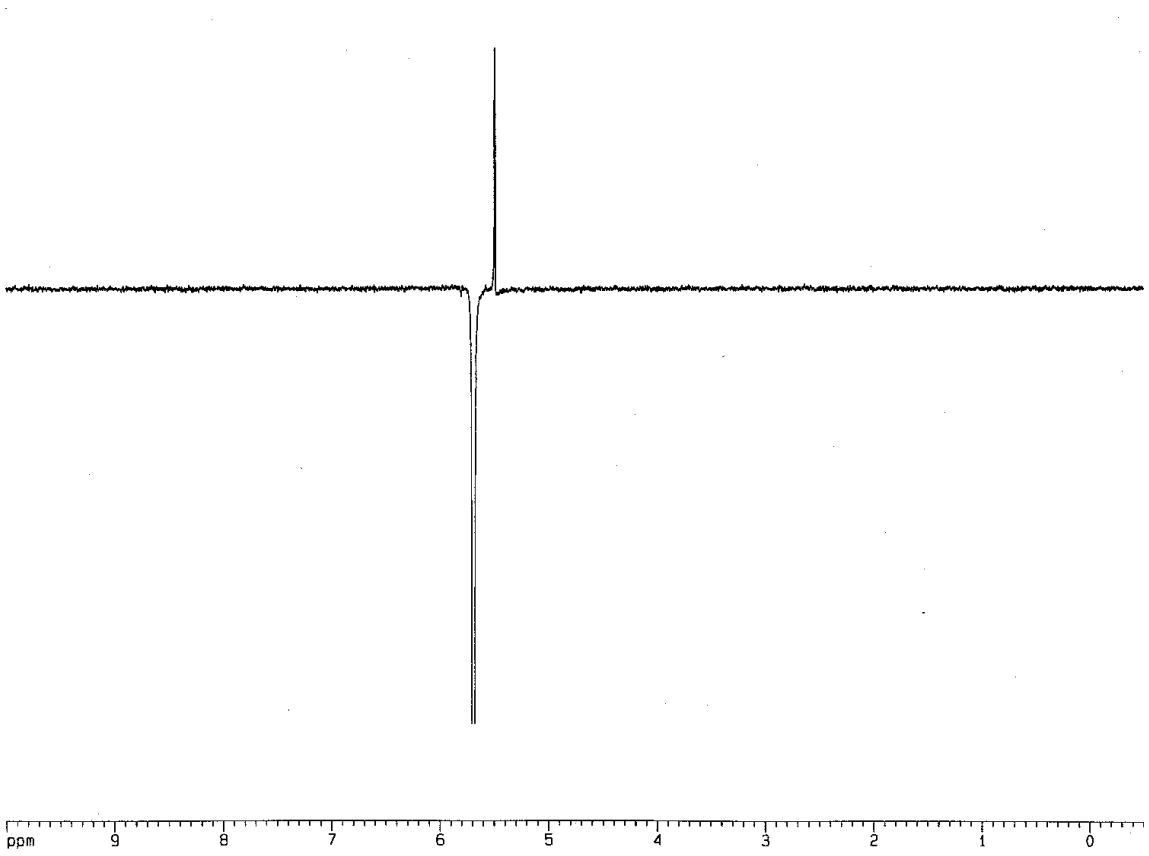

Figure 3.10. 400 MHz 1D NOE spectrum (irradiation of proton 5.68 ppm) of compound (**2**) in $CDCl_3$

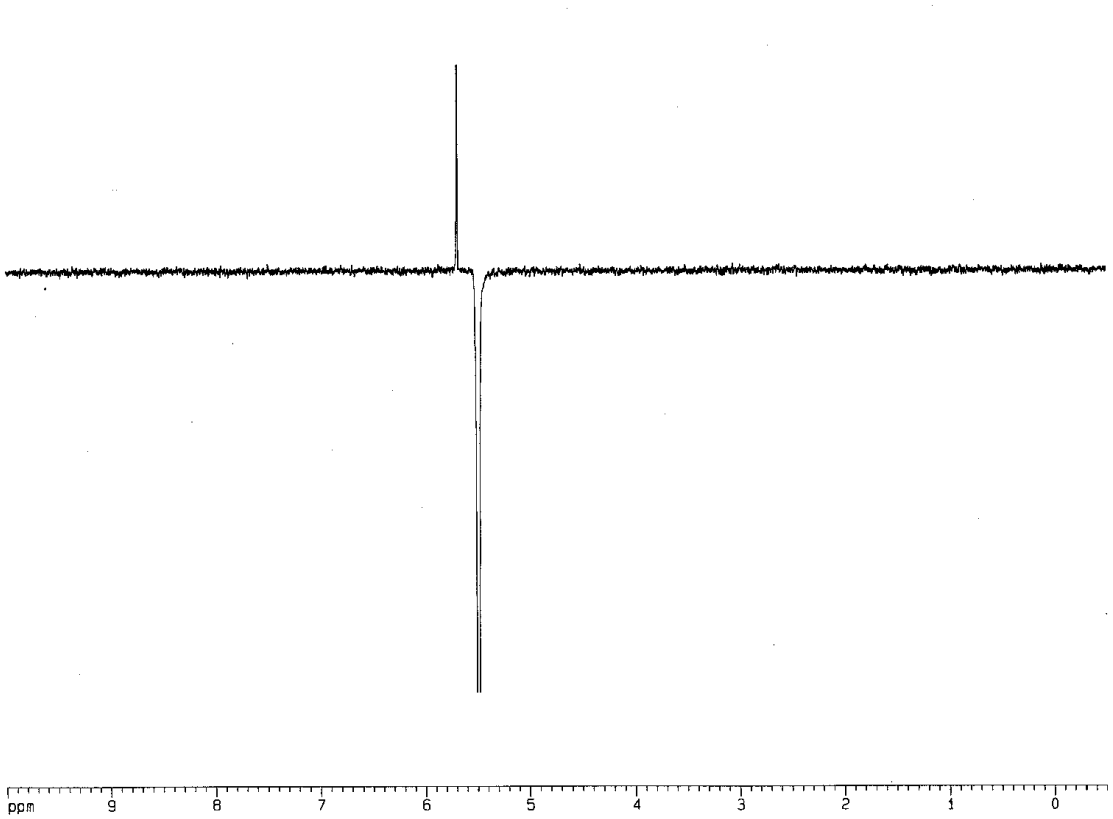

Figure 3.11. 400 MHz 1D NOE spectrum (irradiation of proton 5.48 ppm) of compound (**2**) in $CDCl_3$

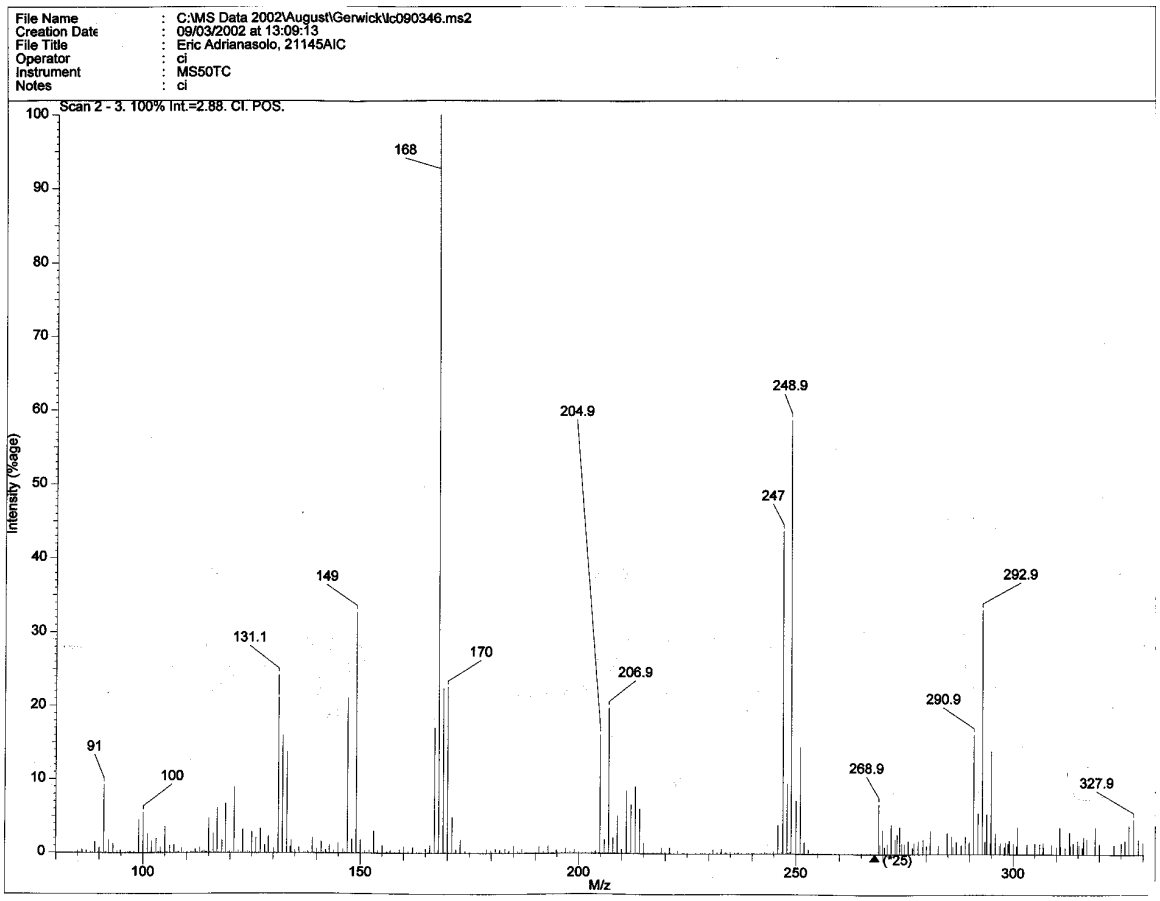

Figure 3.12. LRCI MS spectrum of compound (**2**)

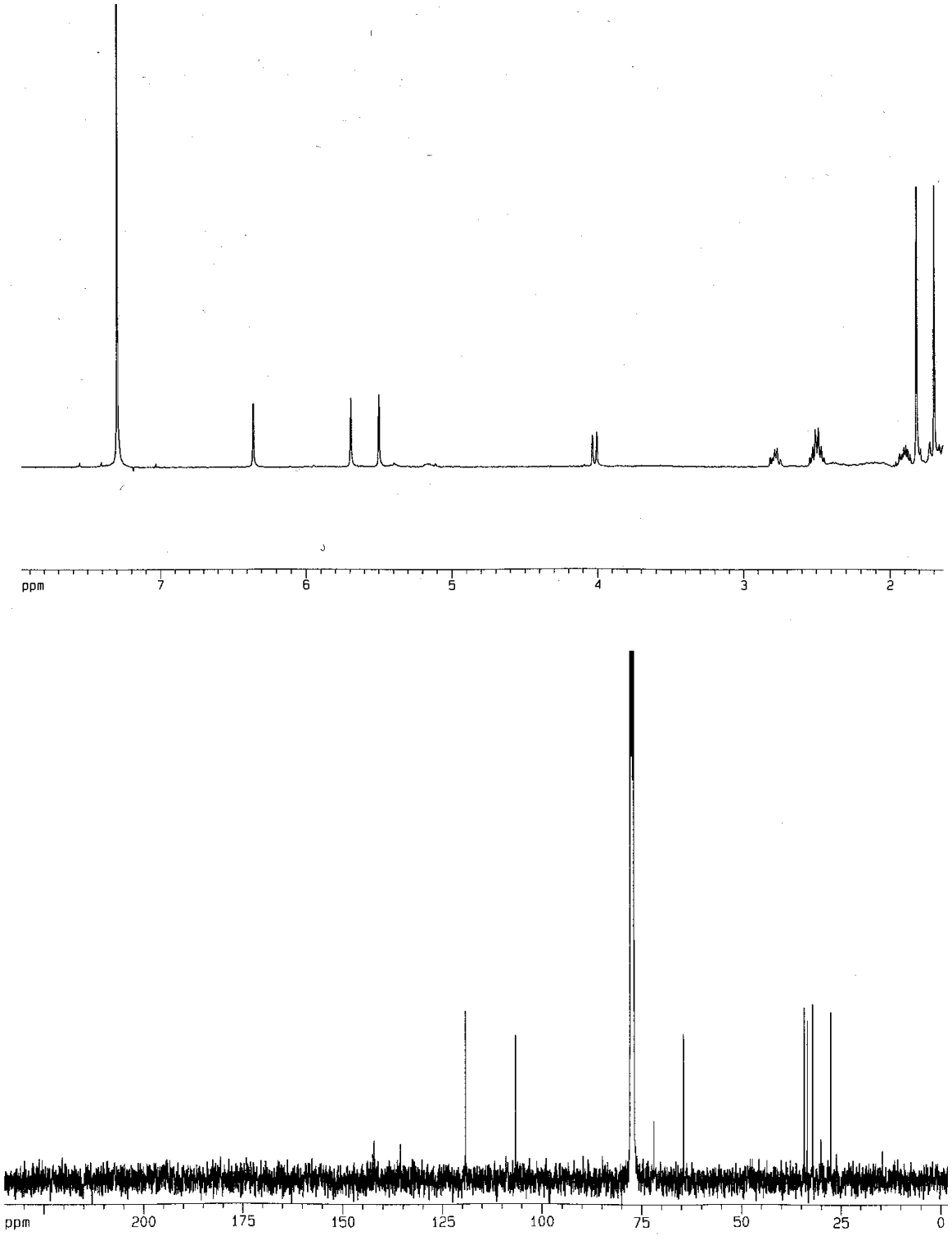

Figure 3.13. 400 MHz ^{1}H NMR spectrum and 100 MHz ^{13}C spectrum of compound (**3**) in $CDCl_3$

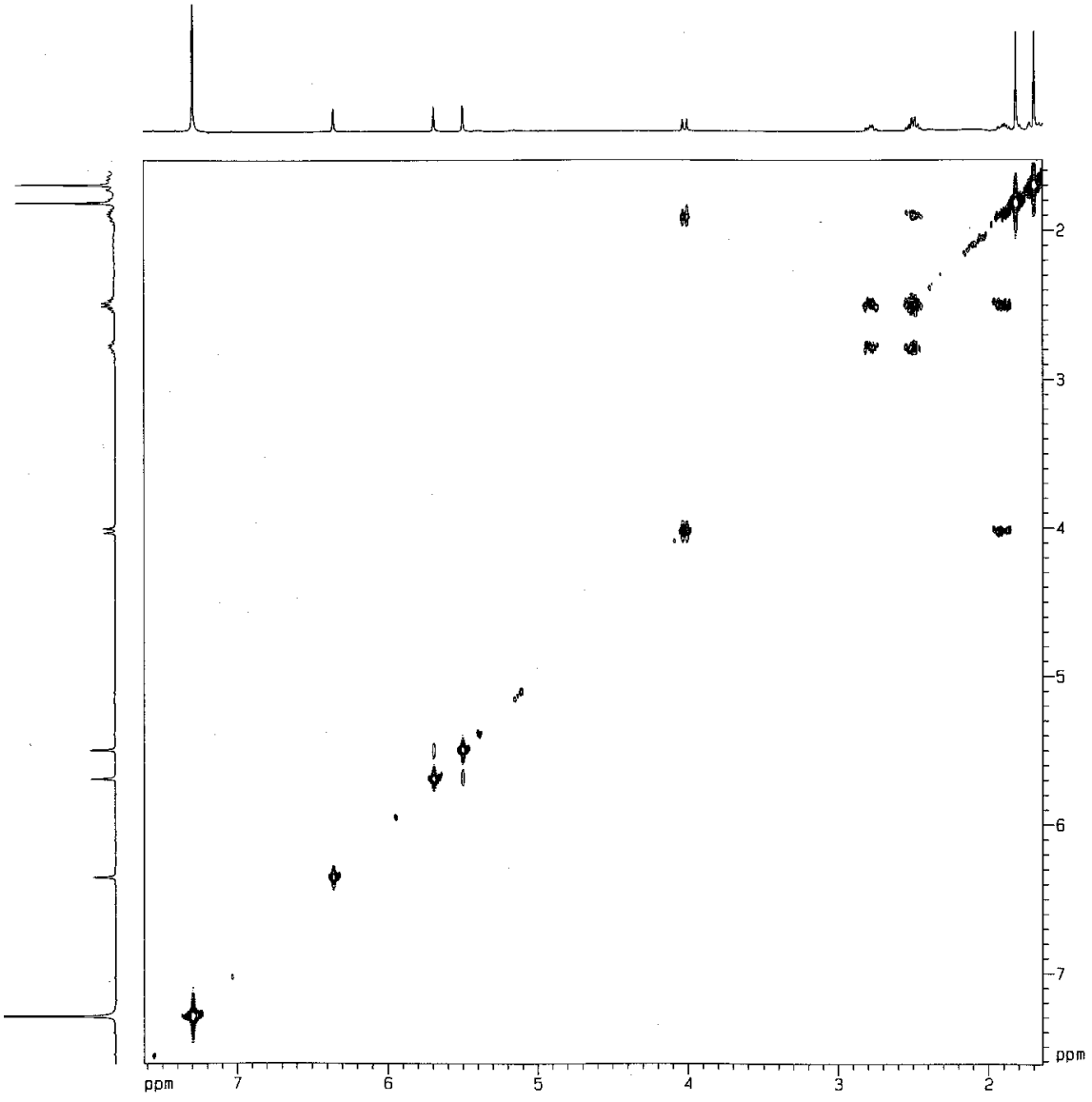

Figure 3.14. 400 MHz ^{1}H-^{1}H COSY spectrum of compound (**3**) in $CDCl_3$

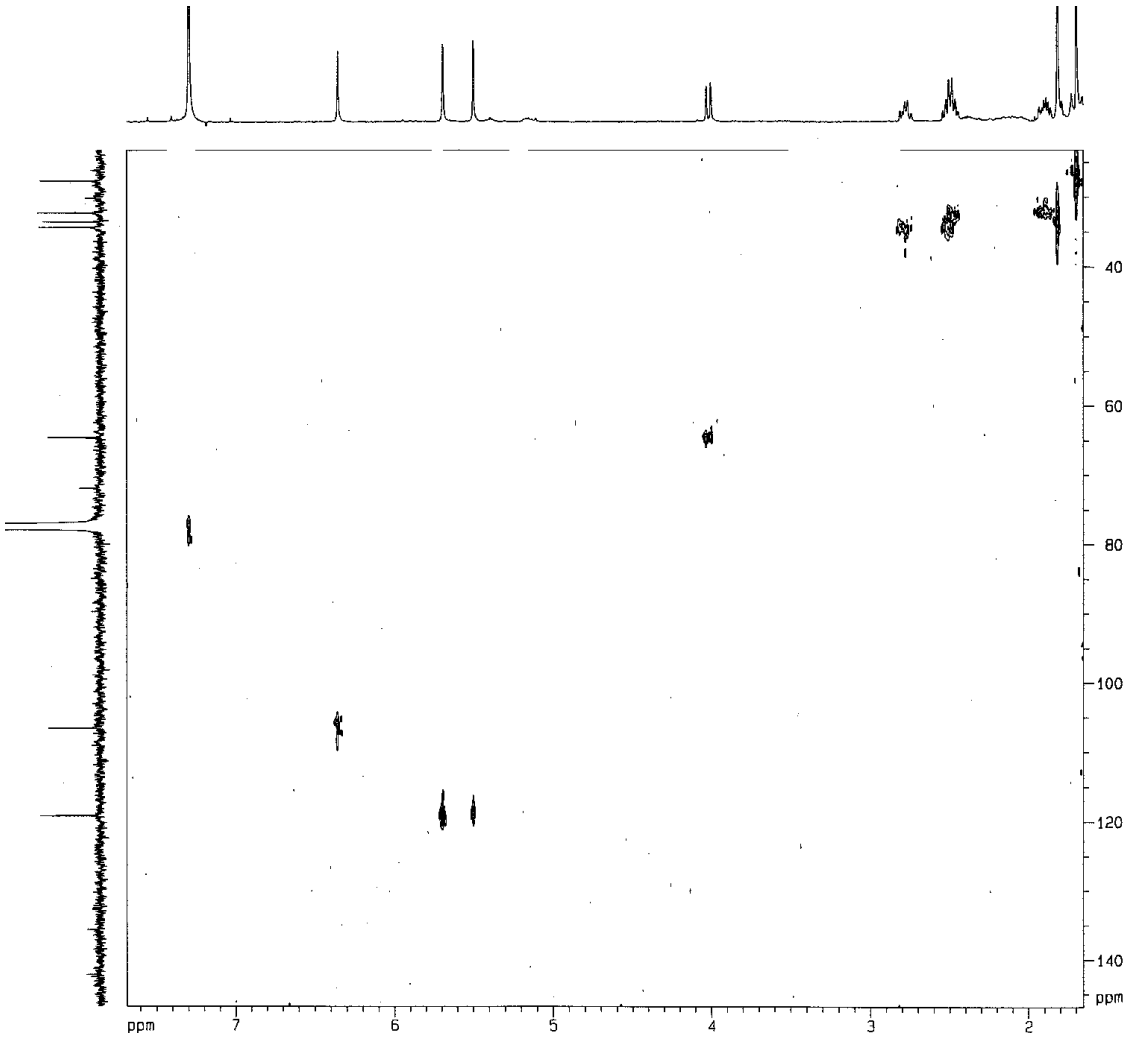

Figure 3.15. 400 MHz Multi-edited HSQC spectrum of compound (**3**) in $CDCl_3$

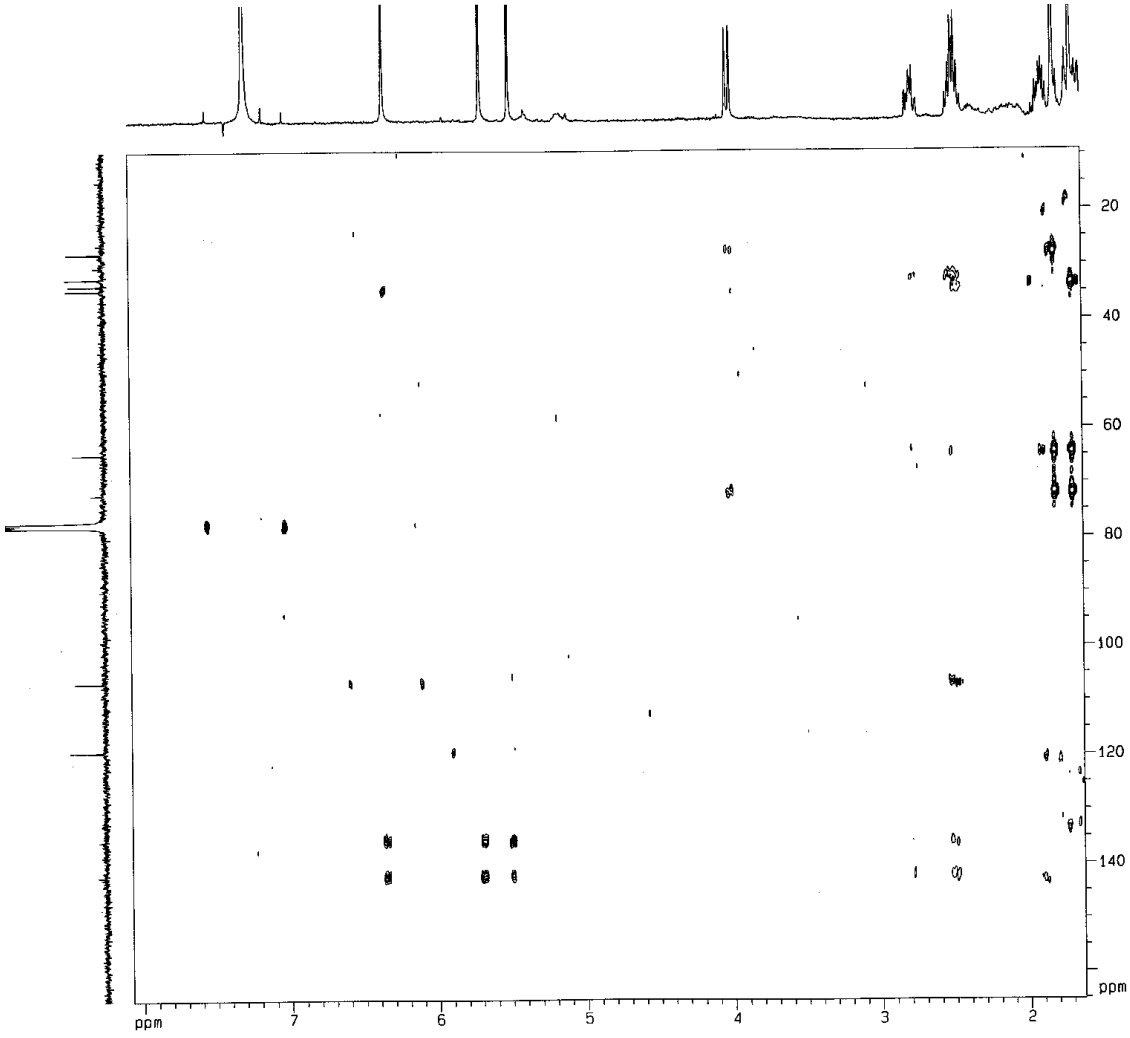

Figure 3.16. 400 MHz HMBC spectrum (optimized for J = 8Hz) of compound (**3**) in $CDCl_3$

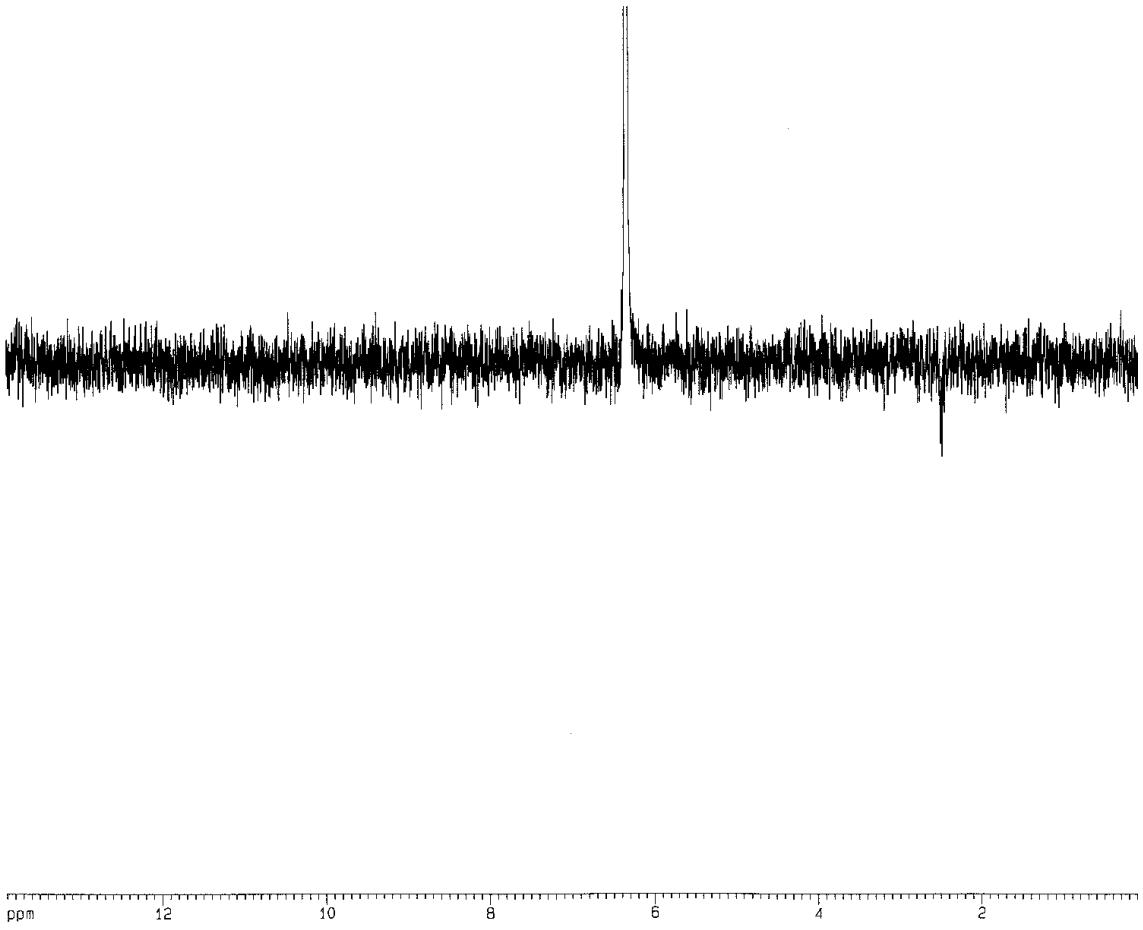

Figure 3.17. 400 MHz 1D NOE spectrum (irradiation of proton 6.35 ppm) of compound (**3**) in $CDCl_3$

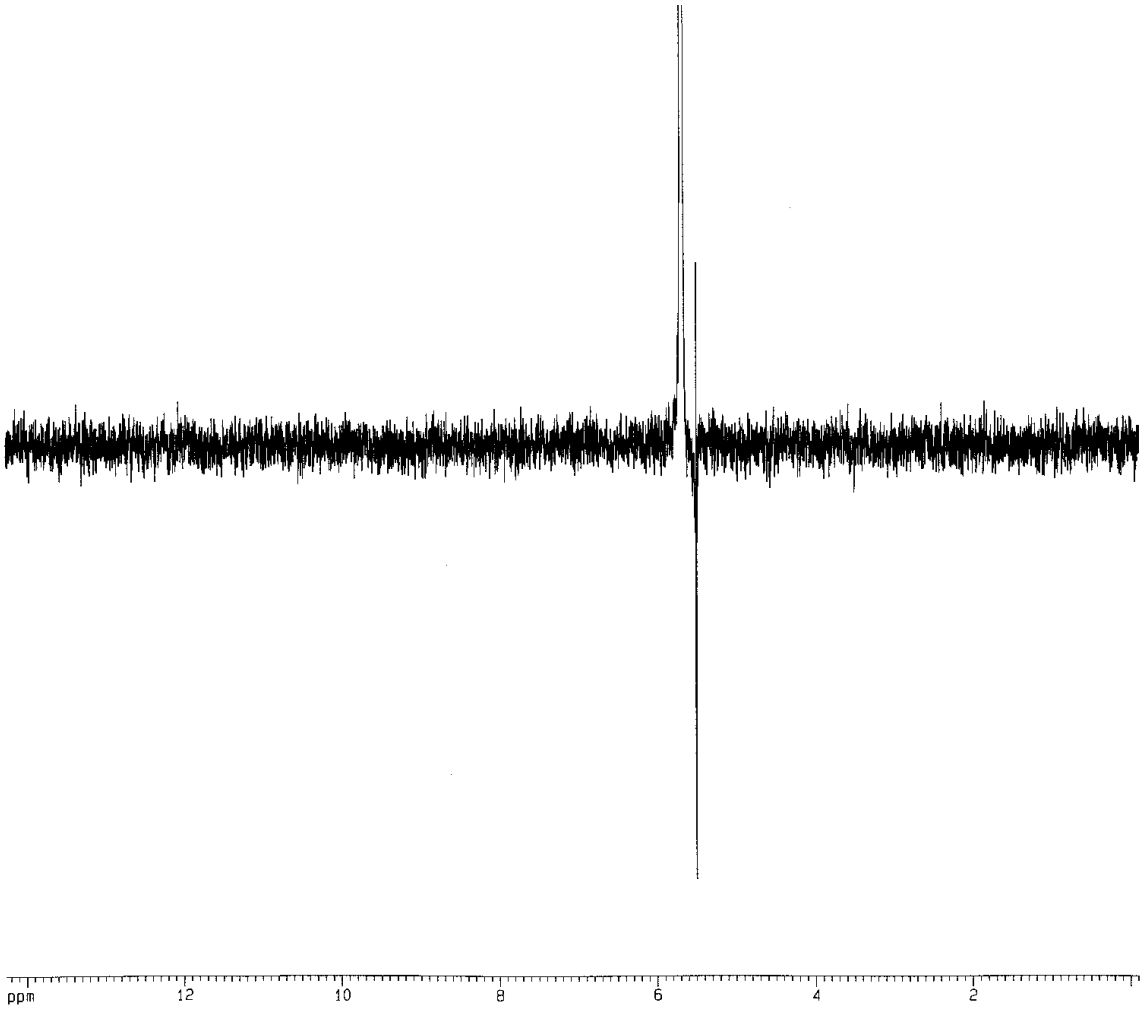

Figure 3.18. 400 MHz 1D NOE spectrum (irradiation of proton 5.69 ppm) of compound (**3**) in $CDCl_3$

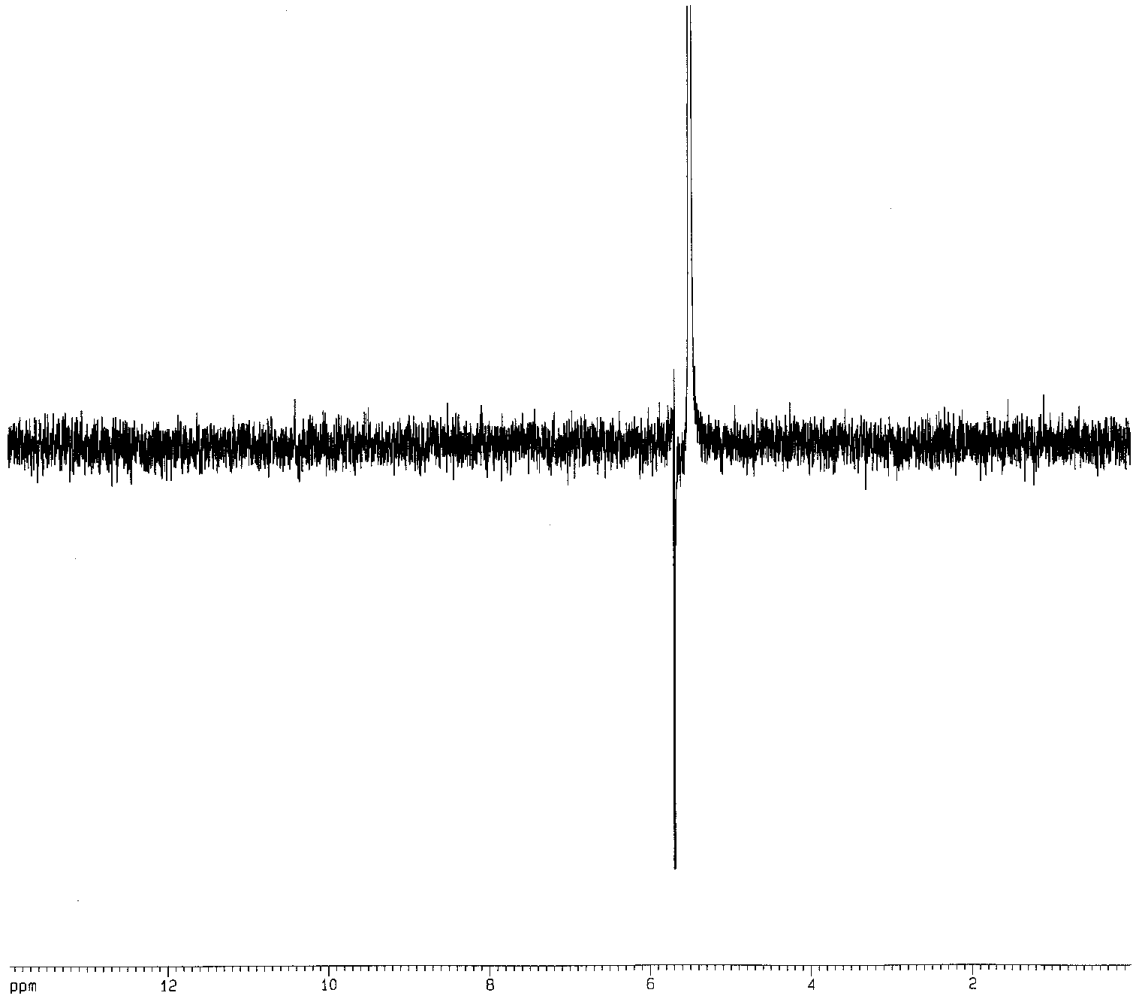

Figure 3.19. 400 MHz 1D NOE spectrum (irradiation of proton 5.49 ppm) of compound (**3**) in $CDCl_3$

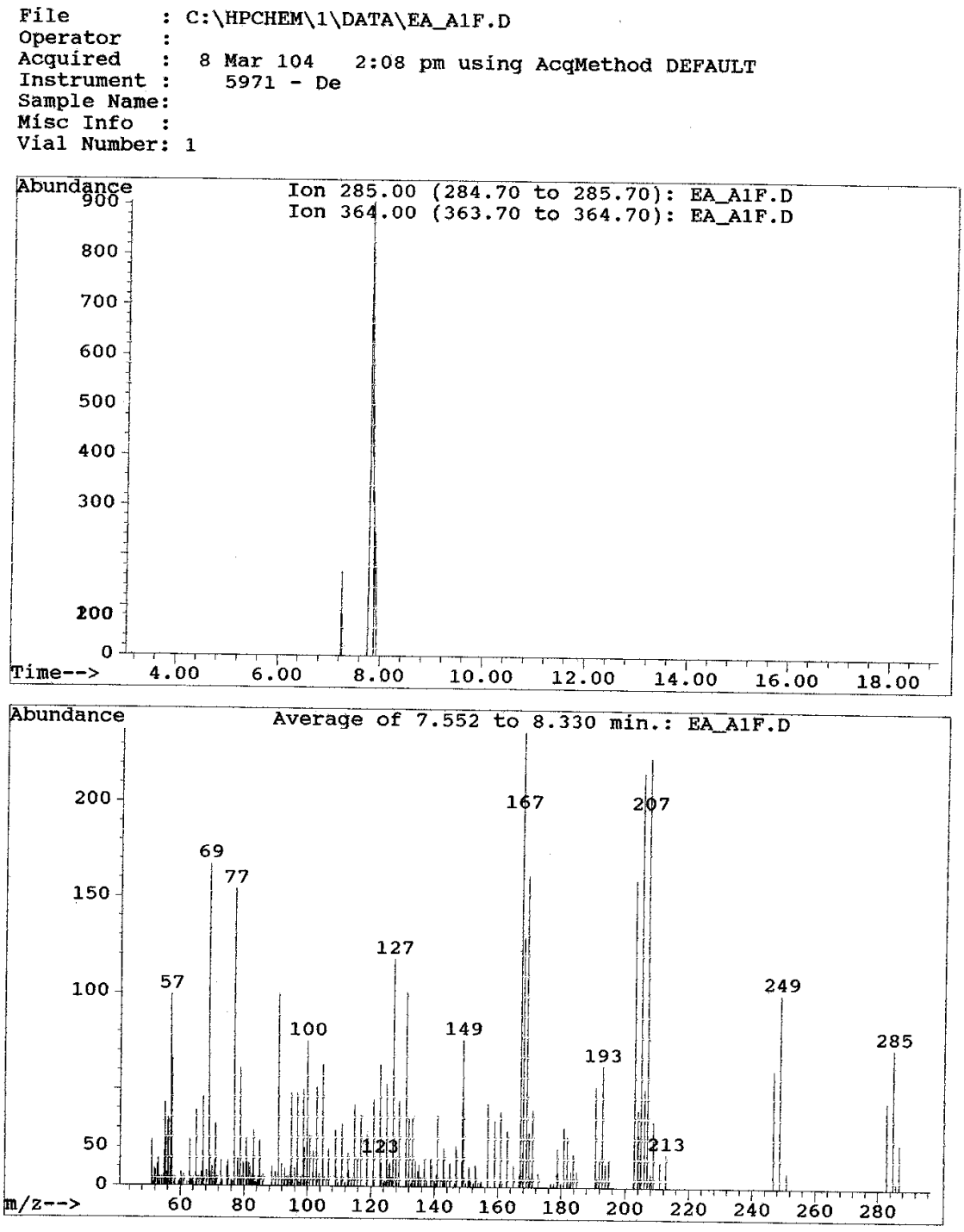

Figure 3.20.GC/MS spectrum of compound (**3**)

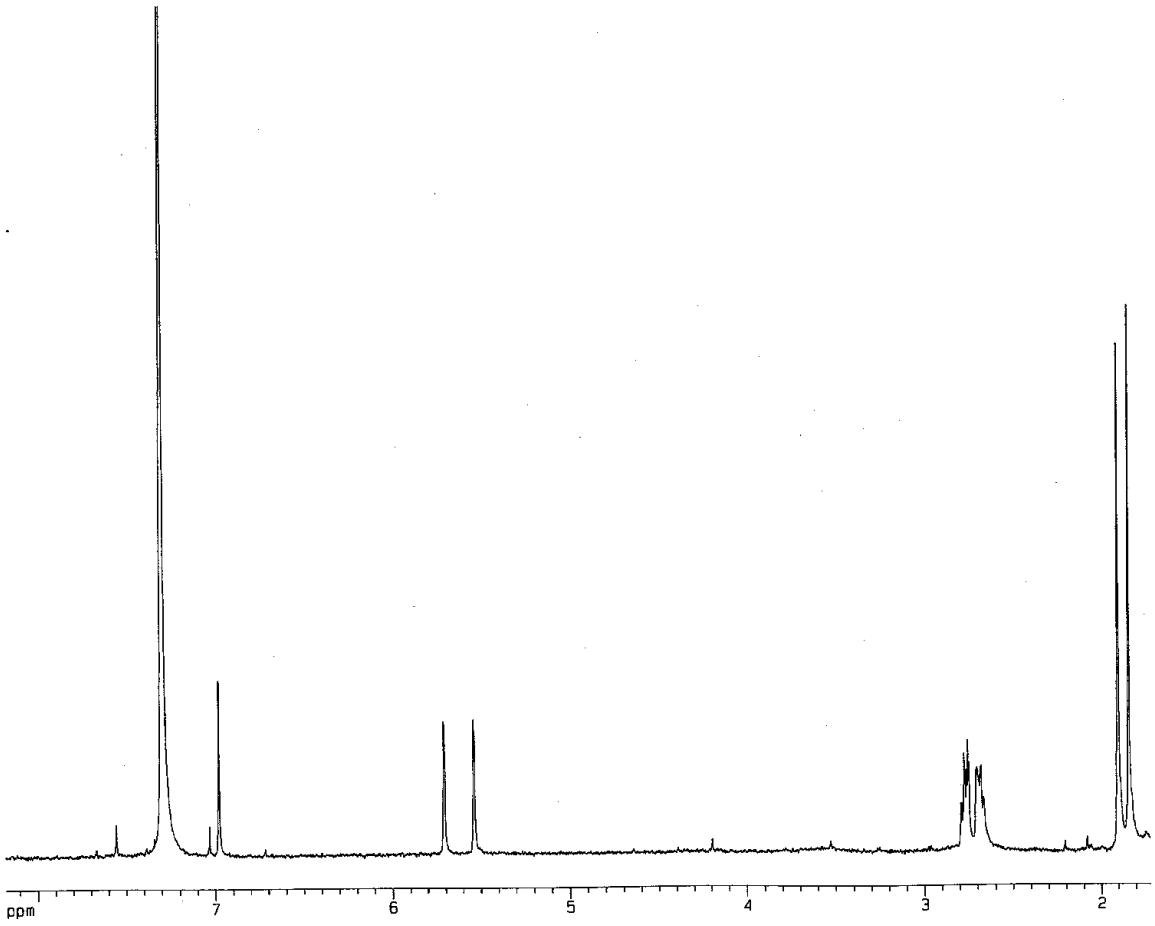

Figure 3.21. 400 MHz ^{1}H NMR spectrum of compound (**4**) in $CDCl_3$

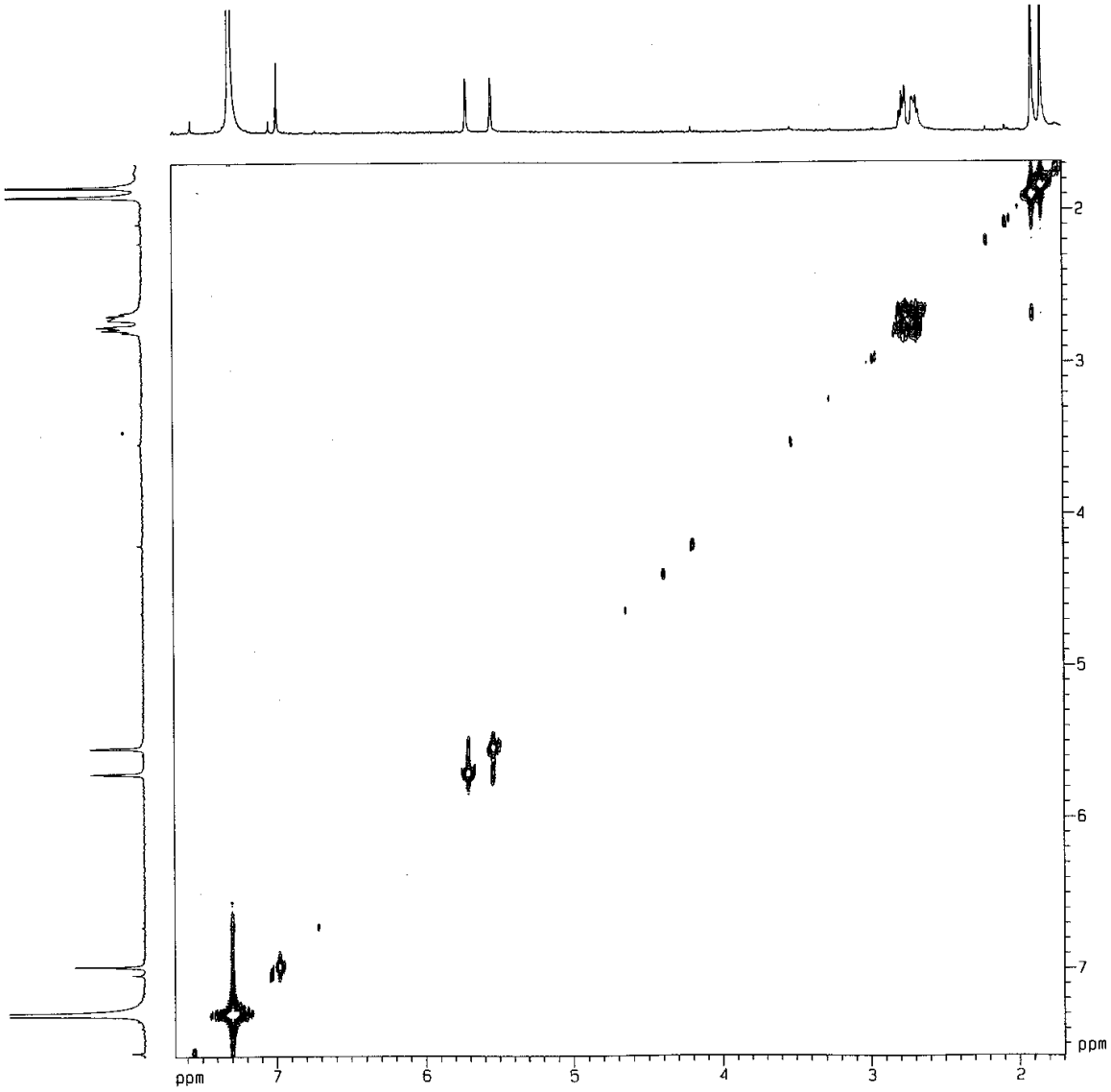

Figure 3.22. 400 MHz ^{1}H-^{1}H COSY spectrum of compound (**4**) in $CDCl_3$

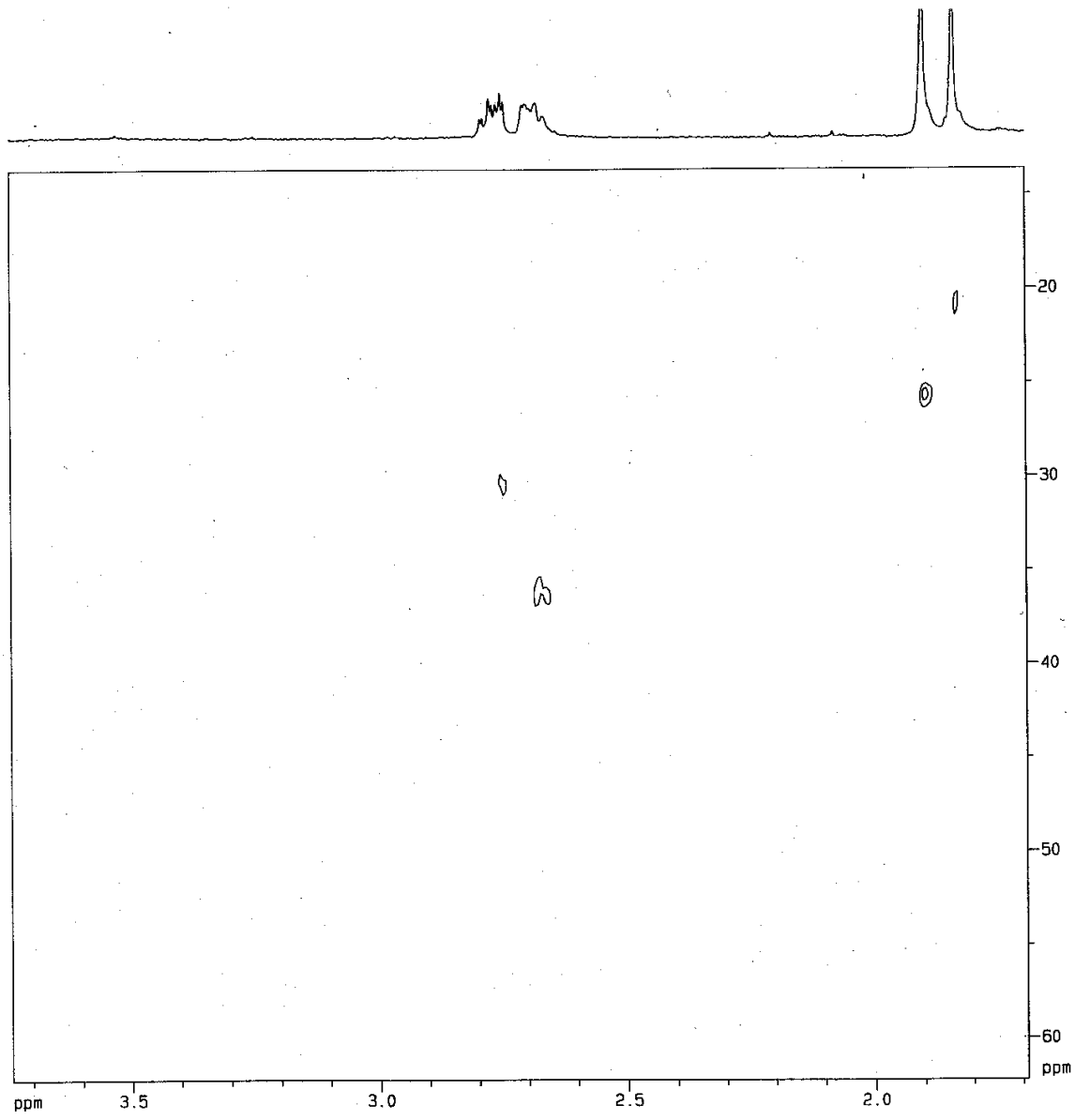

Figure 3.23. 400 MHz HSQC spectrum of compound (**4**) in $CDCl_3$ (partial view of the signal belonging to the two methyl groups)

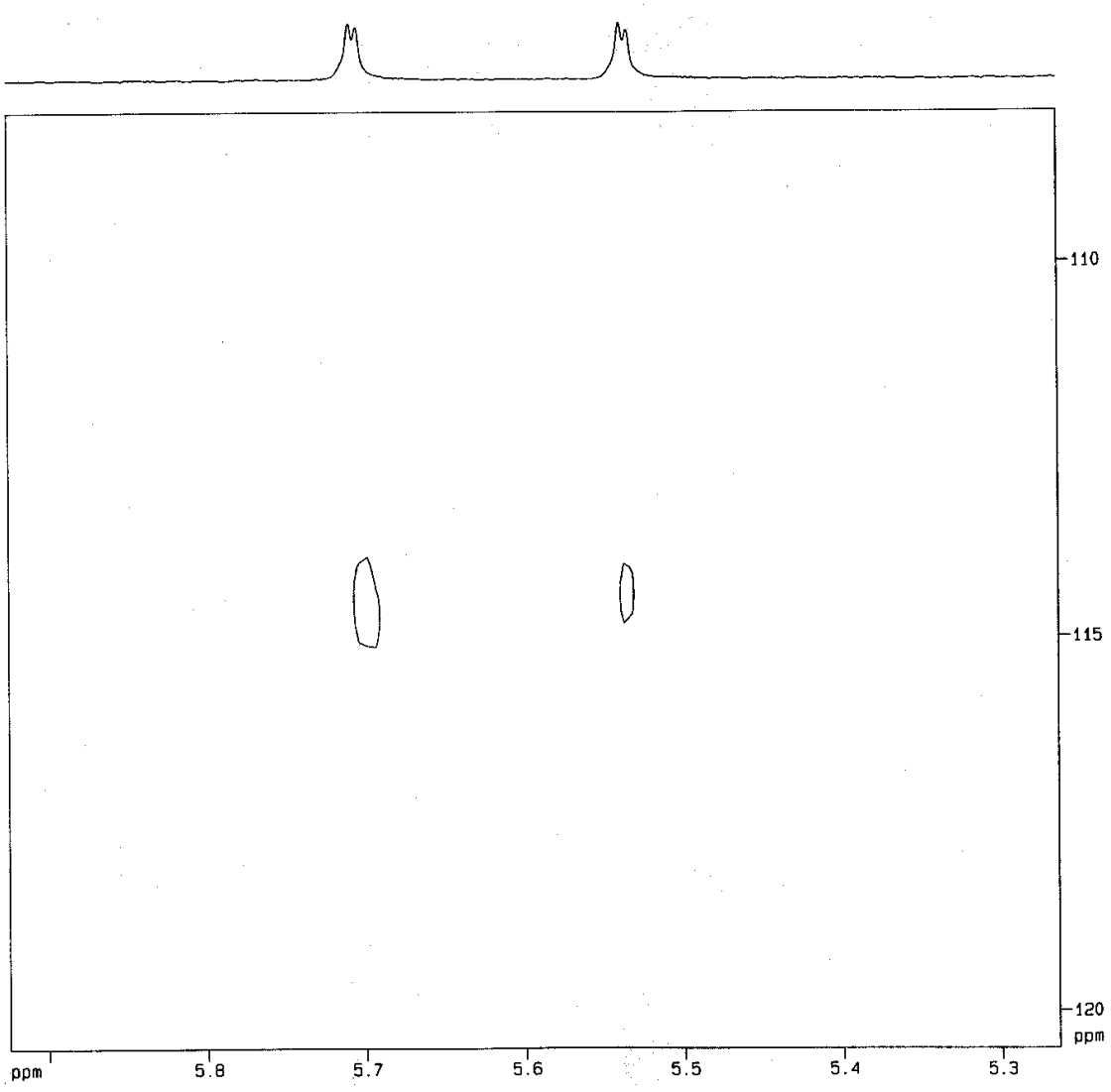

Figure 3.24. 400 MHz HSQC spectrum of compound (**4**) in $CDCl_3$ (partial view of the signal belonging to the proton doublets at 5.53 and 5.70 ppm)

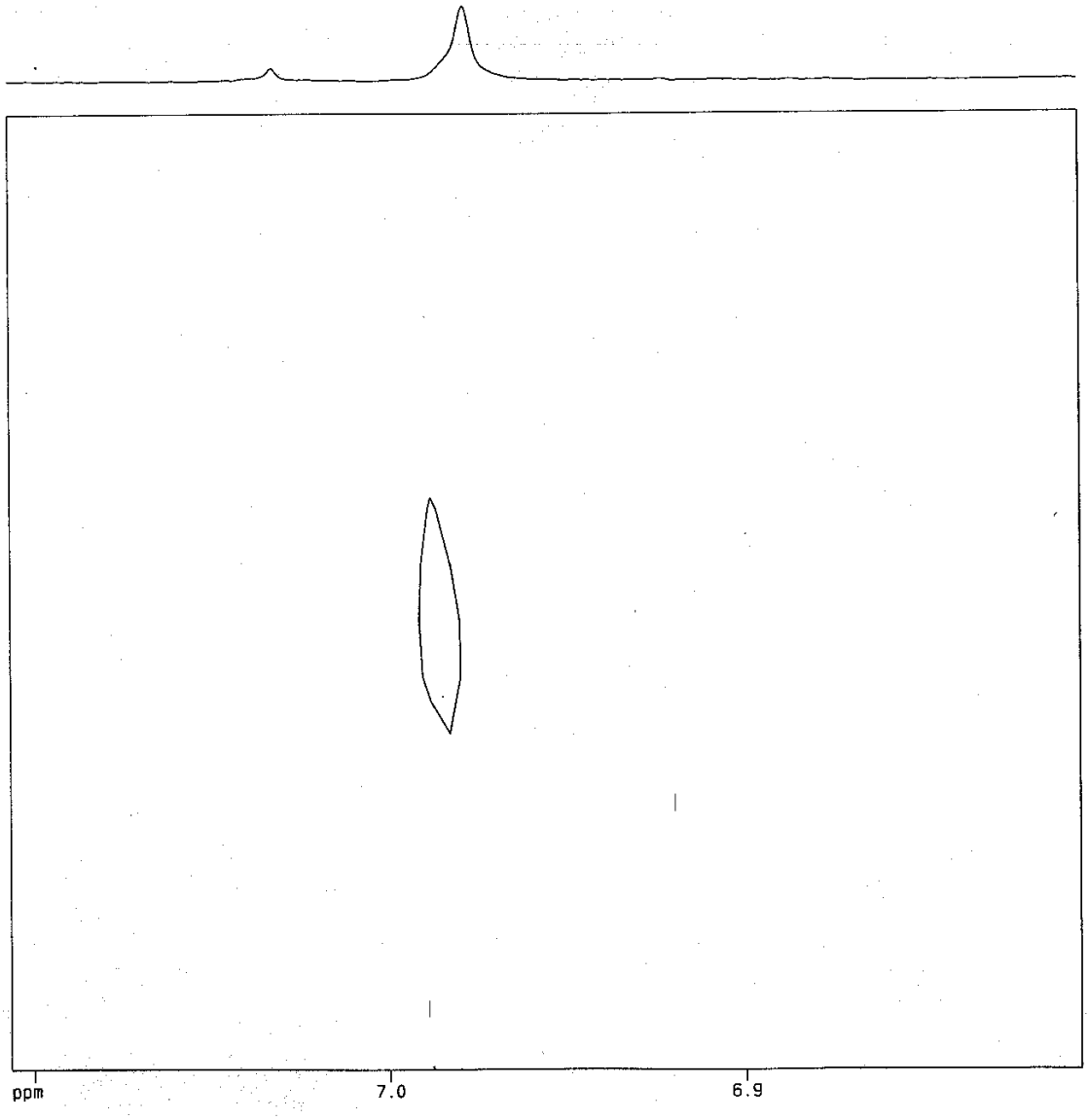

Figure 3.25. 400 MHz HSQC spectrum of compound (**4**) in $CDCl_3$ (partial view of the signal belonging to the proton at 6.97 ppm)

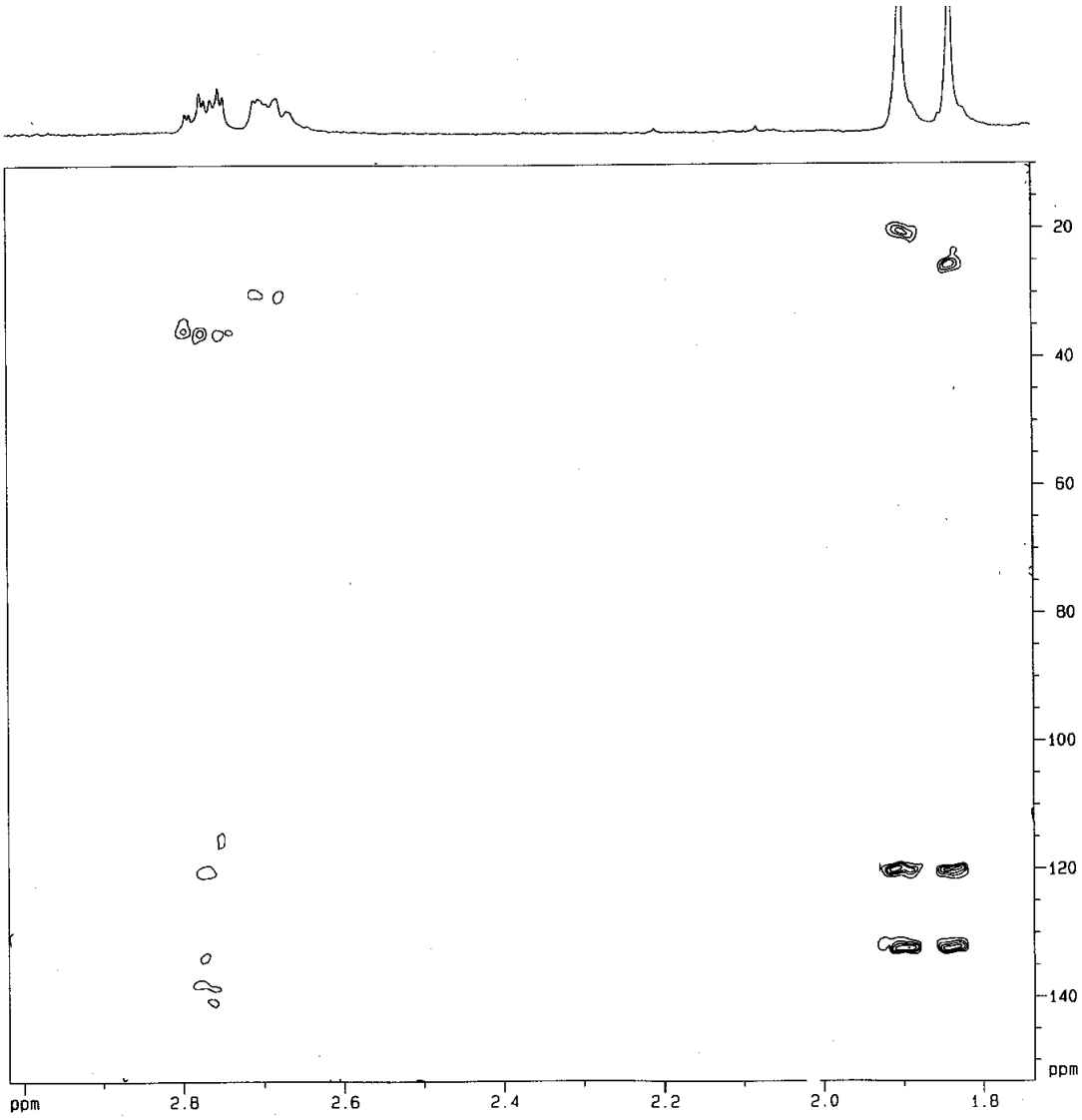

Figure 3.26. 400 MHz HMBC spectrum of compound (**4**) in $CDCl_3$ (observed signal from the two methyl groups)

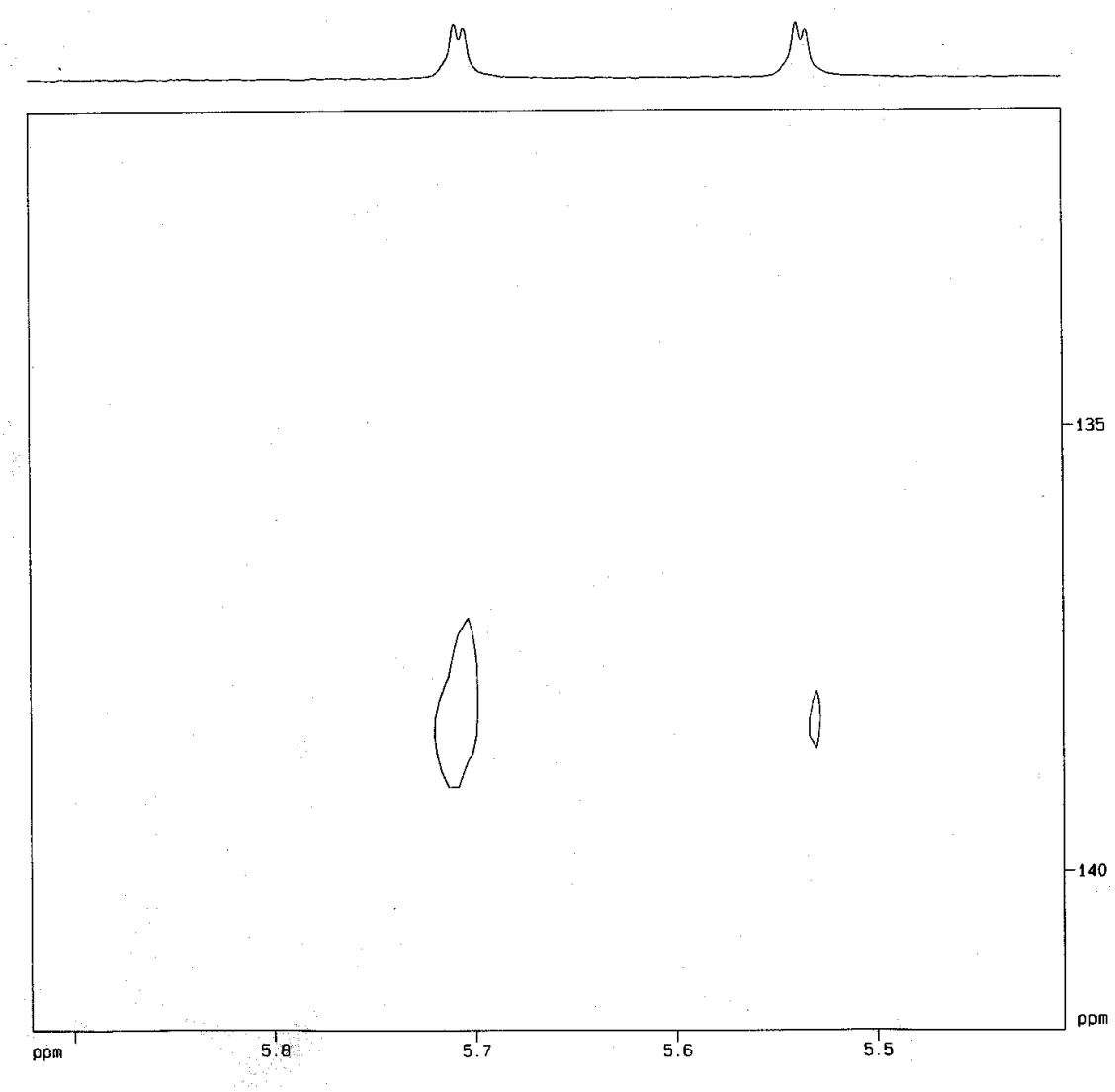

Figure 3.27. 400 MHz HMBC spectrum of compound (**4**) in $CDCl_3$ (observed signal from the proton doublets at 5.53 and 5.70 ppm)

```
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Acquired   :  8 Mar 104  10:00 am using AcqMethod DEFAULT
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Sample Name: 21141ala4
Misc Info  :
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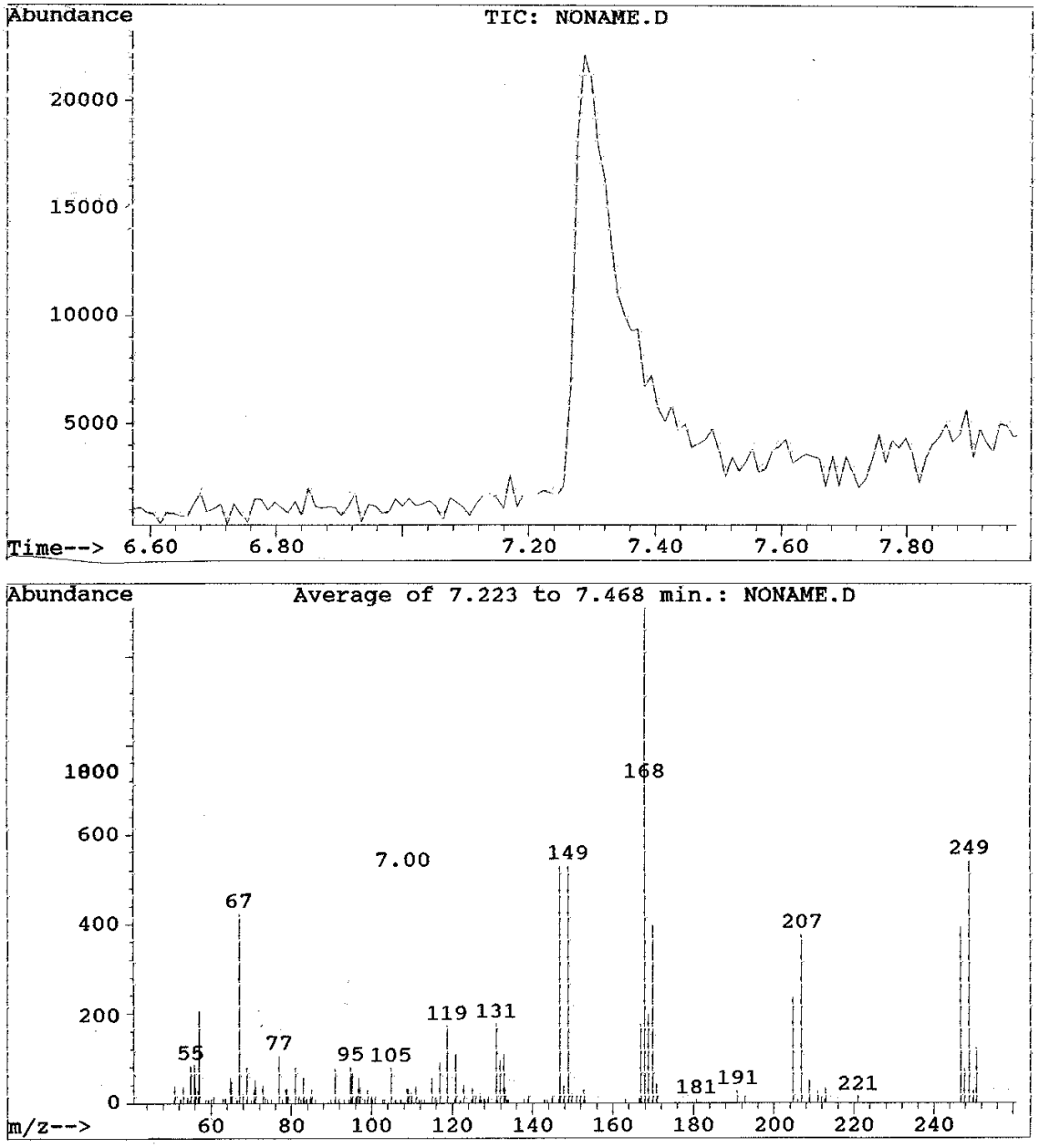

Figure 3.28. GC/MS spectrum of compound (**4**)

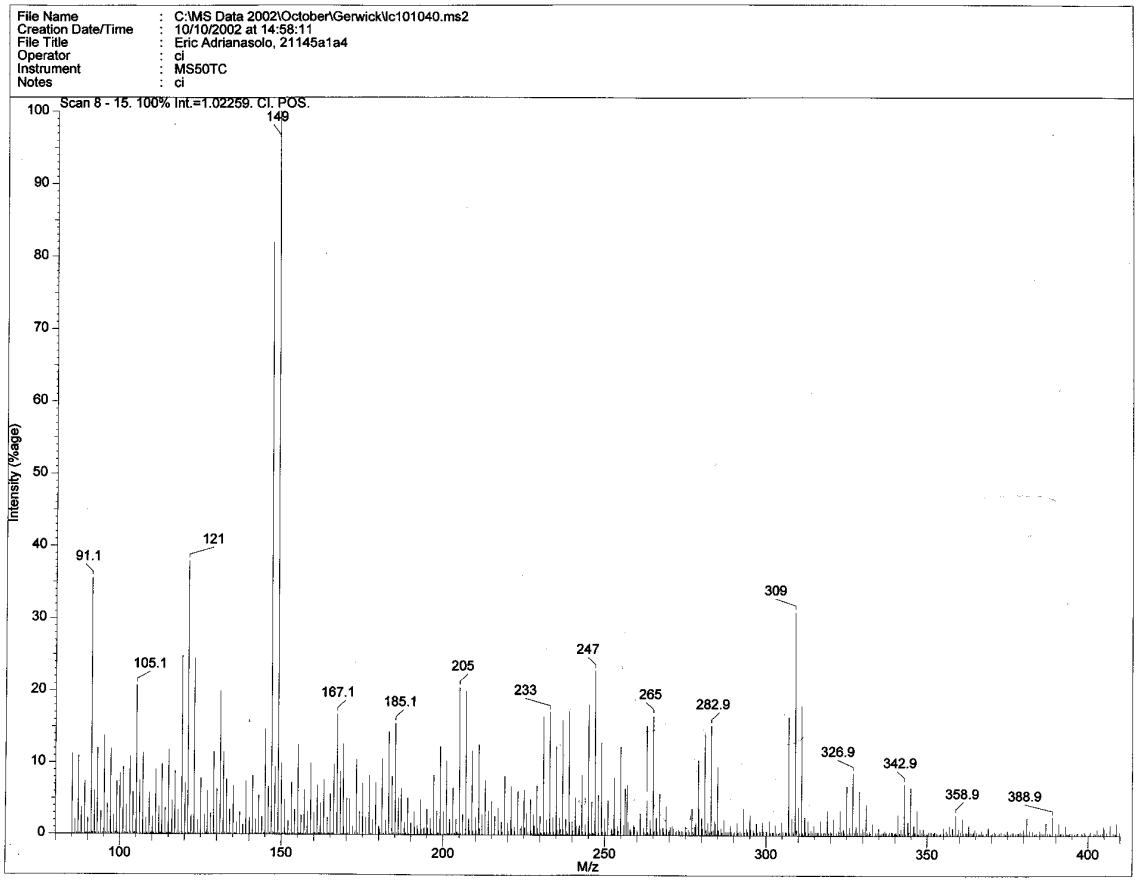

Figure 3.29. LRCI MS spectrum of compound (**4**)

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on a JASCO P 1010 polarimeter. UV and FT-IR spectra were obtained employing Hewlett Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on a Bruker Avance DPX400 spectrometer using residual $CHCl_3$ as reference (δ_H 7.27; δ_C 77.0). HPLC separations were performed using Waters 515 HPLC pumps, a Rheodyne 7725i injector, and Waters 996 photodiode array detector. LR and HR CI mass spectra were acquired on a Kratos MS50TC instrument and GC/MS data were acquired on Hewlett Packard 5890 series II chromatograph with Hewlett Packard 5971 series mass selective detector.

Collection, Extraction, and Isolation.

Portieria hornemannii was collected by hand using SCUBA in March 1997 from southern Madagascar (Bay of Fort Dauphin) (voucher specimen available from W.H.G. as collection number MFM-31/Mar/97-03). The alga was stored at -20ºC in 70% EtOH until workup. The alga (78.5 g dry wt) was extracted four times with CH_2Cl_2-MeOH (2:1) to give a crude organic extract (4.65 g). A portion of the extract (4.50 g) was fractionated on silica gel by NPVLC to give nine fractions using a stepwise gradient of hexanes-EtOAc and EtOAc-MeOH. Fraction A, eluting with 100% hexanes, was further chromatographed on preparative normal phase HPLC (Phenomenex Maxsil 10 Silica 10 µm, 500 x 10.0 mm, 100% hexanes) and yielded three fractions A1 to A3. Fraction A1 was subjected to analytical normal phase HPLC (Phenomenex Luna silica 10 µm, 250 x 4.60 mm, 100% hexanes) to yield successively 4.1 mg of **2**, 1.8 mg of **3** and mixtures A1a and A1d. Fraction A1a was further purified on analytical RP HPLC (Phenomenex Sphereclone ODS 5 µm, 250 x 10.0 mm, 100% acetonitrile) to yield 1 mg of **4**. Fraction A1d was further purified on analytical NP HPLC (Phenomenex Luna silica 10 µm, 250 x 4.60 mm, 100% hexanes) to yield 4.7 mg of **5** as the major component. Fraction A3 was subjected to analytical NP HPLC (Phenomenex Luna silica 10 µm, 250 x 4.60 mm, 100% hexanes) to yield 2 mg of **1** and 2 mg of **6**.

Enzyme Bioassay. The DNMT-1 assay was carried out as a homogeneous scintillation proximity assay (SPA) in the following manner. Using a 50 mM Tris pH 8.0 buffer in a 96-well Isoplate (Wallac, 1450-51), preparations of 25 nM DNMT-1 were combined with test compounds, 200 nM biotinylated hemi-methylated DNA substrate, and the methyl donor S-adenosyl-L-[methyl-3H]methionine (3H-SAM at 250 nM) and mixed for 2 hr at 37 ° C. Following addition of streptavidin-coated SPA beads, bead-associated methylated substrate was quantitated in a microplate scintillation counter.[19,20]

Compound 2: colorless oil; UV ($CHCl_3$) λ_{max} 252 nm (ε 2000); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables III.1 and III.2.; HMBC correlations, H-1/C-2,C-3,C-9; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-3,C-4,C-6,C-7; H-8/C-5,C-6,C-7,C-10; H-9/C-2,C-3,C-4; H-10/C-5,C-6,C-7,C-8; LR CIMS fragment ions at *m/z* $[M\text{-}C_5H_5BrCl]^+$ 147(25)/149(30), *m/z* $[M\text{-}2Br]^+$ 168(95)/ 170(25), *m/z* $[M\text{-}Br]^+$ 247(45)/249(60)/251(15), $[M\text{-}Cl]^+$ 290.9(19)/292.9(34)/ 294.9(17), *m/z* $[M]^+$ 325.9(1)/327.9(3)/329.9(1) and *m/z* $[M\text{-}Cl]^+$ 290.9(19)/ 292.9(34)/294.9(17); HR CIMS *m/z* $[M\text{-}Br]^+$ 246.9902 (calcd for $C_{10}H_{13}{}^{35}Cl^{79}Br$, 246.9889).

Compound 3: colorless oil; $[\alpha]^{25}{}_D$ +1° (*c* 0.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} 249 nm (ε 2000); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables III.1. and III.2.; HMBC correlations, H-1/C-2,C-3; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-3, C-4,C-6; H-6/C-5,C-7; H-8/C-5,C-6,C-7,C-10; H-9/C-2,C-3,C-4; H-10/C-5,C-6,C-7,C-8; LR GCMS fragment ion at *m/z* $[M\text{-}2HBr\text{-}Cl]^+$ 167(100)/169(30), *m/z* $[M\text{-}HBr\text{-}Br]^+$ 203(30)/205(18)/207(3), *m/z* $[M\text{-}2Cl\text{-}Br]^+$ 213(5)/215(5), *m/z* $[M\text{-}Br\text{-}HCl]^+$ 247(7)/249(10)/251(2) and *m/z* $[M\text{-}Br]^+$ 283(6)/285(10)/287(5). HR CIMS *m/z* $[M\text{-}Br]^+$ 282.96557 (calcd for $C_{10}H_{14}{}^{35}Cl_2{}^{79}Br$, 282.96559).

Compound 4: colorless oil; UV ($CHCl_3$) λ_{max} 251 nm (ε 2000); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables III.1. and III.2.; HMBC correlations, H-1/C-2; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-4; H-8/C-6,C-7,C-10; H-9/C-3; H-10/C-6,C-7,C-8; LR CIMS at *m/z* $[M+Na+2H]^+$ 307(17)/309(30)/311(19); LR GC/MS fragment ions at *m/z* $[M\text{-}C_5H_5Cl_2]^+$ 147(30)/149(30), *m/z* $[M\text{-}Cl\text{-}Br]^+$ 168(100)/170(35) *m/z* $[M\text{-}Cl]^+$ 247(45)/249(60)/251(14). HR CIMS *m/z* $[M\text{-}Cl]^+$ 246.9901 (calcd for $C_{10}H_{13}{}^{35}Cl^{79}Br$, 246.9889).

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CHAPTER FOUR

TANIKOLIDE DIMER AND TANIKOLIDE SECO ACID FROM THE MADAGASCAR MARINE CYANOBACTERIUM *LYNGBYA MAJUSCULA*: INTRIGUING STEREOCHEMICAL INSIGHTS AND IMPLICATIONS

ABSTRACT

Tanikolide dimer (**1**), a novel SIRT2 inhibitor, and tanikolide seco acid (**2**), were isolated from the Madagascar marine cyanobacterium *Lyngbya majuscula*. The depside molecular structure of **1**, most likely a *meso* compound, was established by NMR, MS and chiral HPLC analyses whereas the structure of **2**, isolated as the pure *S** enantiomer, was elucidated by an X-ray experiment. Tanikolide dimer inhibited SIRT2 with an IC_{50} = 176 nM. The co-occurrence of a *meso* dimer and an enantiomerically pure monomer is perplexing and has a number of intriguing implications for the biosynthetic assembly of these metabolites.

INTRODUCTION

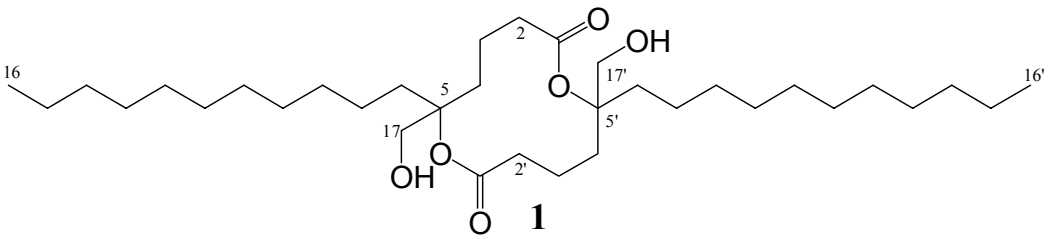
Tanikolide (**3**) is a biologically active δ-lactone isolated from the lipid extract of the Malagasy marine cyanobacterium *Lyngbya majuscula*.[23] Tanikolide possesses a single chiral center, a quaternary carbon with hydroxyl, hydroxymethyl group and two multicarbon chains, and exhibits potent antifungal activity against *Candida albicans* (13-mm diameter zone inhibition using 100 μg/disk). Additionally, it shows strong toxicity in the brine shrimp *Artemia salina* and the snail *Biomphalaria glabrata* (LC_{50} = 3.6 μg/mL and 9.0 μg/mL, respectively) biological assays.

Tanikolide has been the target of several enantioselective syntheses.[1] Optically active tertiary alcohols and related compounds are widely distributed in biologically active compounds, including many drugs.[2] Chiral syntheses of tertiary alcohol derivatives have been prepared by various biological and chemical methods, including enantioselective alkylation of ketones, kinetic resolution of racemates and desymmetrization of prochiral tertiary alcohols.[2,3] However, high levels of chiral induction (>95% ee) have rarely been attained by these methods. In contrast, a recent report described the synthesis with high purity of > 95% ee (*R*)-(+)-tanikolide through the stereospecific C-H insertion of dichlorocarbene into an optically active secondary alcohol.[4] The most recent synthesis of (+)-tanikolide by Tadaaki et *al.*[5] utilized a bromoalkene intermediate conveniently prepared by regioselective elimination from a vicinal dibromoalkane.

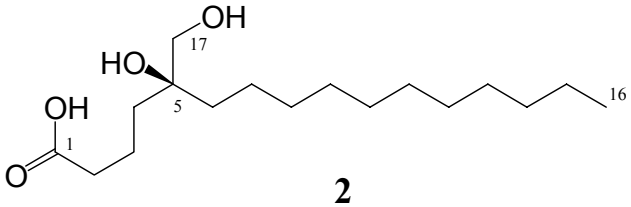
SIRT2 is a NAD^+-dependent cytoplasmic protein that is co-localized with HDAC6 on microtubules. SIRT2 has been shown to deacetylate α-tubulin and to control mitotic exit from the cell cycle.[6,7] Human SIRT2 proteins are closely related in structure to the NAD-dependent deacetylases of other species.[8] The natural substrate for SIRT2 is believed to be p53.[9] Binding of p53 to DNA is activated by its acetylation in the C-terminal domain,[10] thus, deacetylation of p53 by SIRT2 could be important in the regulation of cellular responses to DNA-damaging agents.[11] Because SIRT2 functions to silence apoptotic responses mediated by p53,[12] inhibitors of these proteins are of interest as potential anticancer drugs.

As part of our ongoing search for structurally and pharmacologically interesting substances from cyanobacteria,[20-22] a detailed exploration of a Malagasy *L. majuscula* collection was undertaken. Using a SIRT2 bioassay guided approach, we

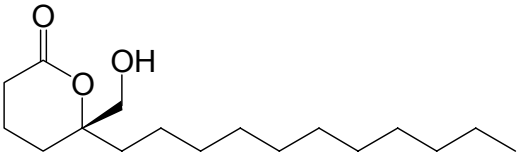
report here the isolation and structural elucidation of the naturally occurring dimer of tanikolide (**1**) and its seco acid derivative (**2**). Tanikolide dimer (**1**), a naturally occurring depside, was found to be a potent inhibitor of SIRT2 as well as an active sodium channel blocking agent. Conversely, the seco-acid **2** exhibited only moderate cytotoxicity against the H-460 cancer cell line and was inactive in either the SIRT2 or sodium channel blocking assays.



1



2



3

RESULTS AND DISCUSSION

L. majuscula was collected from near Tanikely Island, Madagascar, extracted with CH_2Cl_2/MeOH (2:1) and fractionated by Si VLC. The resulting fractions were tested for SIRT2 inhibitory activity, and revealed that the 40% EtOAc/hexanes fraction was strongly active (IC_{50} = 2.5 µg/mL). This material was subjected to further fractionation by solid-phase extraction (SPE), and two fractions (D3 and D4) were found active (81% and 75% inhibition at 10 µg/mL, respectively). Analytical reversed-phase HPLC purification of D3 and D4 led to the isolation of pure tanikolide dimer (**1**) which showed an IC_{50}=176 nM to SIRT2. Compound **2** was isolated as a crystalline substance from a VLC fraction eluting with 25% MeOH in EtOAc, and was inactive in the SIRT2 assay.

The parent molecular ion for **1** was unambiguously deduced by various modes of ionization, including FABMS in various matrices, ESIMS and EIMS. LR FABMS displayed three peaks at *m/z* 591.4 $[M+Na]^+$, *m/z* 569.3 $[M+H]^+$, and *m/z* 551.2 $[M-OH]^+$. HR FABMS of the *m/z* 569.47804 $[M + H]^+$ peak showed a molecular formula of $C_{34}H_{65}O_6$ whereas HR EIMS of the *m/z* 551.4691 $[M-OH]^+$ peak showed a molecular formula of $C_{34}H_{63}O_5$. However, the ^{13}C NMR and DEPT spectra for **1** indicated the presence of only 17 carbons and 31 carbon-bound hydrogens. These data combined with the MS information indicated that only half of the signals were appearing in the NMR spectra, and suggested that **1** was a symmetrical dimer. The three degrees of unsaturation implied by the formula were accounted for by the presence of two carbonyls (δ172.1) and one ring.

^{13}C NMR showed the presence of an ester carbonyl (δ172.1; IR 1716 cm^{-1}), a methylene carbon attached to oxygen (δ67.9), a quaternary carbon attached to oxygen (δ86.9), a methyl group (δ14.5), and 13 highfield methylene carbons. ^1H NMR of **1** showed the presence of a mutually coupled pair of doublets (δ3.68 and 3.59) for an isolated methylene attached to oxygen, and a two-proton multiplet (δ2.51) assigned to a methylene adjacent to a carbonyl group. Five methylene groups appeared as multiplets in the range δ1.29 to δ1.95, and a broad singlet (δ1.27) accounted for an additional 14 degenerate protons. A three-proton triplet at δ 0.86 was assigned to a terminal methyl group.

Analyses of ^1H-^1H COSY and HMBC experiments of **1** led to the deduction that **1** was closely related to tanikolide **3**, Figure 4.1.).[23] For example, the

oxymethylene protons at δ 3.68/3.59 (H_2-17) showed HMBC correlations with carbons at δ 86.9 (C-5), 27.0 (C-4), and 37.1 (C-6). Additionally, a pair of methylene protons at δ 2.51 (H_2-2) showed HMBC correlations with carbons at δ 172.1 (C-1), 17.1 (C-3), and 27.0 (C-4). ^{1}H-^{1}H COSY delineated a connected spin system for the methylenes at C-2, C-3 and C-4. A methyl group signal at δ 0.86 (H_3-16) showed HMBC correlations with two methylene carbons at δ 23.1 (C-15) and 32.3 (C-14), a sequence confirmed by ^{1}H-^{1}H COSY analysis, and hence, was at the terminus of a long lipid tail. Based on the close concordance in chemical shifts and coupling patterns between **1** and **3**, and the MS analysis described above, compound **1** was deduced to be a symmetrical dimer of tanikolide (**3**).

Because tanikolide had been isolated as an optically active natural product, and by modified Mosher ester methods, the lone stereocenter was *R*, it was surprising that the dimer **1** gave no rotation at the sodium D-line. Hence, an optical rotatory dispersion (ORD) study was conducted, but this also showed a lack of optical rotation. This perplexing result was further examined by hydrolysis of **1** and analysis by chiral HPLC, first with diode array detection (DAD) and then with ESI LCMS. In both cases, a 1:1 ratio of two peaks were obtained which both analyzed for the seco-acid, although the first was *S** and the second *R** as determined by comparison with the optically pure seco-acid **2** (see below). Hence, these results suggested that **1** was a *meso* compound with one monomer deriving from the *R*-acid and one from the *S*-acid. However, upon further consideration, we realized that two other structural possibilities existed for this material. The results above (absence of rotation, equal quantities of R- and S-acid produced from hydrolysis of dimer) were also consistent with 1) an equal mixture of R,R-dimer and S,S-dimer, or 2) a 1:2:1 ratio of R,R-, S,R-/R,S-, and S,S-dimer. The latter scenario, which would be biosynthetically plausible if the monomer precursor were present as a racemate and then assembled by a condensation reaction in which there was no chiral discrimination, predicts the presence of both enantiomeric as well as diastereomeric material in the isolated “tanikolide-dimer substance”. Because chiral HPLC of tanikolide dimer gave a single sharp peak, and all NMR bands were clean and without evidence of multiple molecular entities, this possibility seems unlikely. However, at the present time we are unable to distinguish whether **1** exists as a true *meso* compound or as an equal mixture of RR- and SS- dimers, and represents a challenging analytical problem.

HR FABMS analyses of compound **2** gave an $[M + Na]^+$ ion at *m/z* 325.2366 for a formula of $C_{17}H_{34}O_4Na$ (one less DBE than tanikolide). ^{13}C NMR revealed the presence of a carbonyl group, and based on its chemical shift, likely that of a carboxylic acid (δ176.8). The C-5 quaternary carbon shift (δ74.4) was present in **2,** indicating that hydroxyl, hydroxyl methyl and two alkyl groups were attached at this position. Indeed, by the data presented above and COSY, HSQC and HMBC, four distinct sections of the molecule were formulated; a hydroxy group, a hydroxymethyl group, a butanoic acid chain and an undecanyl chain, all of which were connected to the C-5 quaternary carbon. Because X-ray quality crystals of **2** were deposited from a fraction eluting with 25% MeOH in EtOAc, these structural features were confirmed via a diffraction study. Figure 4.2. shows the result of the X-ray analysis, establishing the length of alkyl chain and its overall structure.

Most intriguing, the seco acid **2** was found to have significant optical activity, and only a single enantiomer was observed in the X-ray study. By its negative rotation, it likely is the opposite *S*-configuation of that of found in tanikolide at C-5. When a sample of **2** was analyzed by chiral phase HPLC, only a single entaniomer was observed. Hence, while the dimer is comprised of an equal molar ratio of *R*- and *S*- monomers, the free seco acid is essentially the pure *S*-enantiomer. To our knowledge, this is the first such case in nature, although is reminiscent of nonactin and its biosynthesis.[26,27]

Pure compounds **1** and **2** were tested in SIRT2 inhibitory assay as well as a mammalian cell sodium channel blocking assay.[24] Compound **1** was found to have an IC_{50} value of 176 nM against SIRT2 whereas **2** was inactive at the highest concentration tested (50 μM). Comparing these data with those reported for sirtinol and 8,9-dihydroxybenzo-[4,5]furo[3,2-c]chromen-6-one (A3) (IC_{50} values of 38 and 45 μM respectively)[13] indicates that **1** is 200-300 fold more active than either of these agents. In the sodium channel blocking assay, compound **1** induced a 54% inhibition at 5.2 μM while **2** was again inactive at the highest concentration tested (10 μM). However, cytotoxicity assays revealed that **2** was moderately toxic at 9.9 μM to the human lung H460 cell line while **1** did not have any activity up to 10 μM.

A recent study on novel inhibitors of SIRT2 reported that a phenol or hydroxyl group is important for inhibitory activity based on the structure of putative SIRT2 active site.[14] These polar functional groups together with a hydrophobic moiety and hydrogen-bonding features are suggested to form an active SIRT2 pharmacophore.

In addition, it is believed that SIRT2 inhibitors must sterically block the opening of a narrow channel near the putative active site, adjacent to Ile169 and Asp170, and continuing throughout the length of the enzyme.[14] Interestingly, the functional groups and overall dimeric structure of **1** meets these requirements. However, that the seco acid **2** was inactive despite its containing two hydroxyl groups suggests that either the free carboxyl functionality interferes with binding of the drug, or that the entire dimeric structure is sterically required for effective inhibition.

It seems likely that the biosynthetic pathway of tanikolide (**3**), its seco acid (**2**), and its dimer (**1**) proceeds *via* a PKS biosynthetic pathway. However, the occurrence of a branching carbon atom at C-5(5') in these metabolites, a site on the polyketide backbone logically deriving from C-1 of acetate, suggests the involvement of an HMGCoA synthase as has recently been deduced in curacin A and jamaicamide A biosynthesis.[28,29] However, because tanikolide dimer is most probably a *meso* compound, this suggests that the HMGCoA synthase mediated acetate addition to the β-carbonyl intermediate occurs in both configurations, and it is both intriguing and puzzling as to how this is organized within a PKS, or possibly, a PKS dimer which simultaneously builds each monomer for subsequent tandem cleavage to form the depside. Hence, while structurally quite simple, the structure of tanikolide dimer (**1**) raises a number of intriguing configurational and biosynthetic questions for further study. Given tanikolide dimer's potent biological effects to SIRT2, developing a keener understanding of its structural subtleties and mechanism of formation is important to developing its lead compound and biotechnology potential.

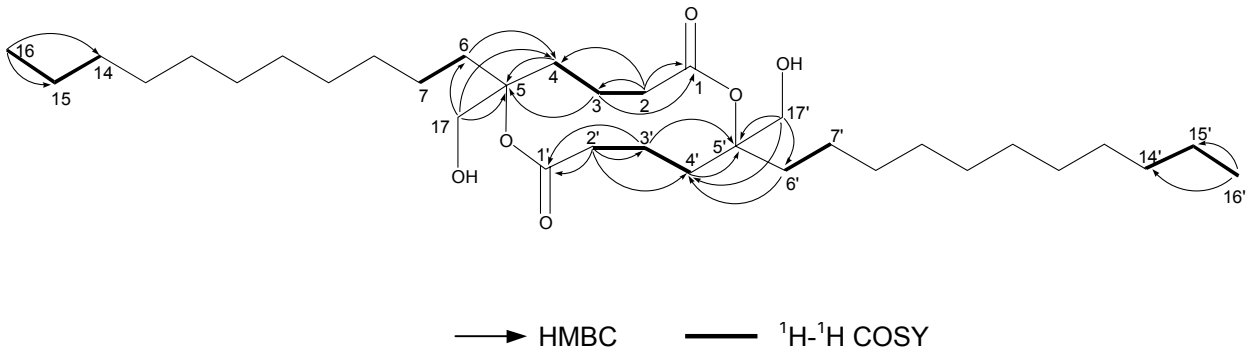


Figure 4.1. Selected HMBC and ^{1}H-^{1}H COSY correlations for tanikolide dimer (**1**).

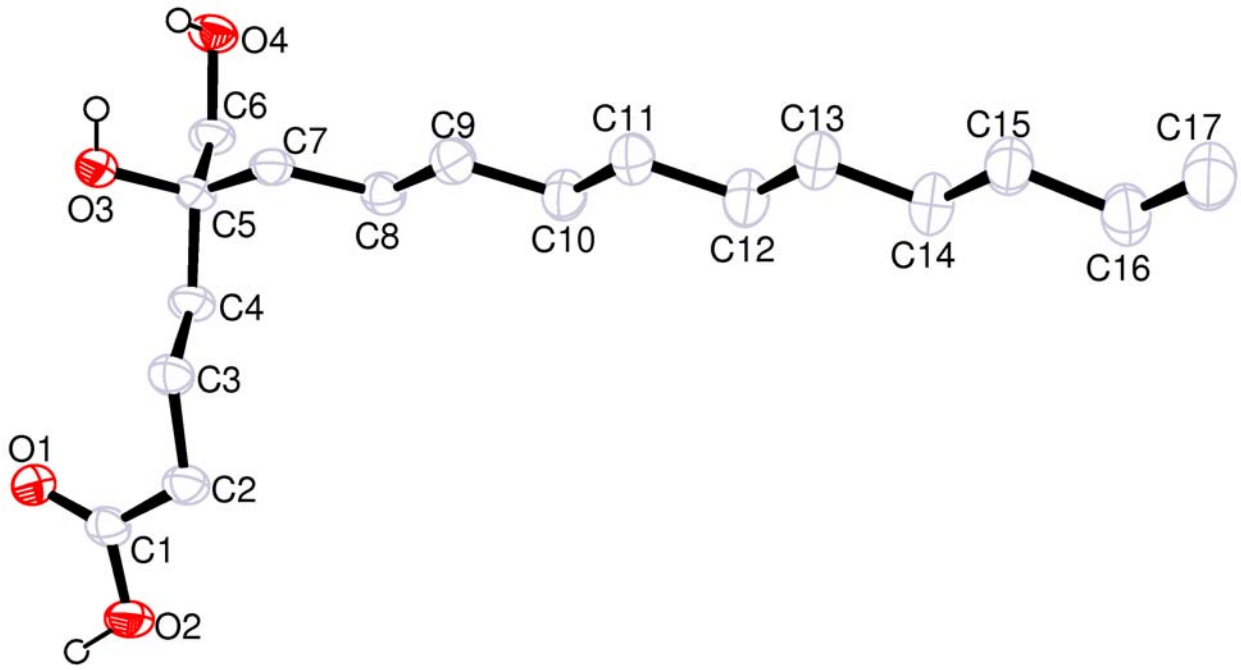


Figure 4.2. ORTEP of tanikolide seco acid (**2**) with 50% displacement ellipsoids.

Table 4.1. ^{1}H and ^{13}C NMR assignments for tanikolide dimer (**1**).[a]

Position	δ_C	δ_H	^{1}H-^{1}H COSY	HMBC
1/1'	172.1			
2/2'	30.2	2.51 (m)	3	1,3,4
3/3'	17.1	1.92 (m)	2,4	1,5
4/4'	27.0	1.78 (m) 1.95 (m)	3	3,5
5/5'	86.9			
6/6'	37.1	1.66 (m) 1.75 (m)	7	4,5,17
7/7'	23.9	1.29 (m) 1.32 (m)	6	
8/8'	30.4	1.27 (bs)		
9/9'	30.2	1.27 (bs)		
10/10'	30	1.27 (bs)		
11/11'	30	1.27 (bs)		
12/12'	29.9	1.27 (bs)		
13/13'	29.7	1.27 (bs)		
14/14'	32.3	1.27 (bs)	15	
15/15'	23.1	1.30 (m)	14,16	
16/16'	14.1	0.86 (t, *J*=7.2 Hz)	15	15,14
17/17'	67.9	3.59 (d, *J*=11.3 Hz) 3.68 (d, *J*=11.3 Hz)		4,5,6

[a] 400 MHz and 100MHz, $CDCl_3$

Table 4.2. ^{1}H and ^{13}C NMR assignments for tanikolide seco acid (**2**).[a]

Position	δ_C	δ_H
1	176.8	
2	34.6	2.29 (t *J*= 6.7 Hz)
3	19.1	1.67 (m)
4	35.5	1.49 (m)
5	74.4	
6	36.2	1.49 (m)
7	22.7	1.29 (bs)
8	29.9	1.29 (bs)
9	29.6	1.29 (bs)
10	29.6	1.29 (bs)
11	29.6	1.29 (bs)
12	29.6	1.29 (bs)
13	29.6	1.29 (bs)
14	32.3	1.29 (bs)
15	23.1	1.29 (m)
16	13.4	0.91 (t *J*= 6.9 Hz)
17	67.1	3.59 (d, *J*=14.1 Hz) 3.68 (d, *J*=14.1 Hz)

[a] 400 MHz and 100MHz, CD_3OD

Crystal structure data

Table 4.3. Crystal data and structure refinement for WG112003.

Identification code	WG112003	
Empirical formula	$C_{17}H_{34}O_4$	
Formula weight	302.44	
Temperature	100(2) K	
Wavelength	1.54180 Å	
Crystal system	Monoclinic	
Space group	$P2_1$	
Unit cell dimensions	a = 5.524(4) Å	α = 90°
	b = 8.048(5) Å	β= 97.180(14)°
	c = 20.295(12) Å	γ = 90°
Volume	895.2(10) $Å^3$	
Z	2	
Density (calculated)	1.122 Mg/m^3	
Absorption coefficient	0.618 mm^{-1}	
F(000)	336	
Crystal size	0.30 x 0.10 x 0.02 mm^3	
Theta range for data collection	4.39 to 71.19°.	
Index ranges	0<=h<=6, -9<=k<=9, -24<=l<=24	
Reflections collected	8951	
Independent reflections	2294 [R(int) = 0.1640]	
Completeness to theta = 71.19°	85.5 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.0000 and 0.1337	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	2294 / 1 / 196	
Goodness-of-fit on F^2	1.071	
Final R indices [I>2sigma(I)]	R1 = 0.0882, wR2 = 0.2253	
R indices (all data)	R1 = 0.0975, wR2 = 0.2358	
Absolute structure parameter	0.5(7)	
Largest diff. peak and hole	0.274 and -0.339 $e.Å^{-3}$	

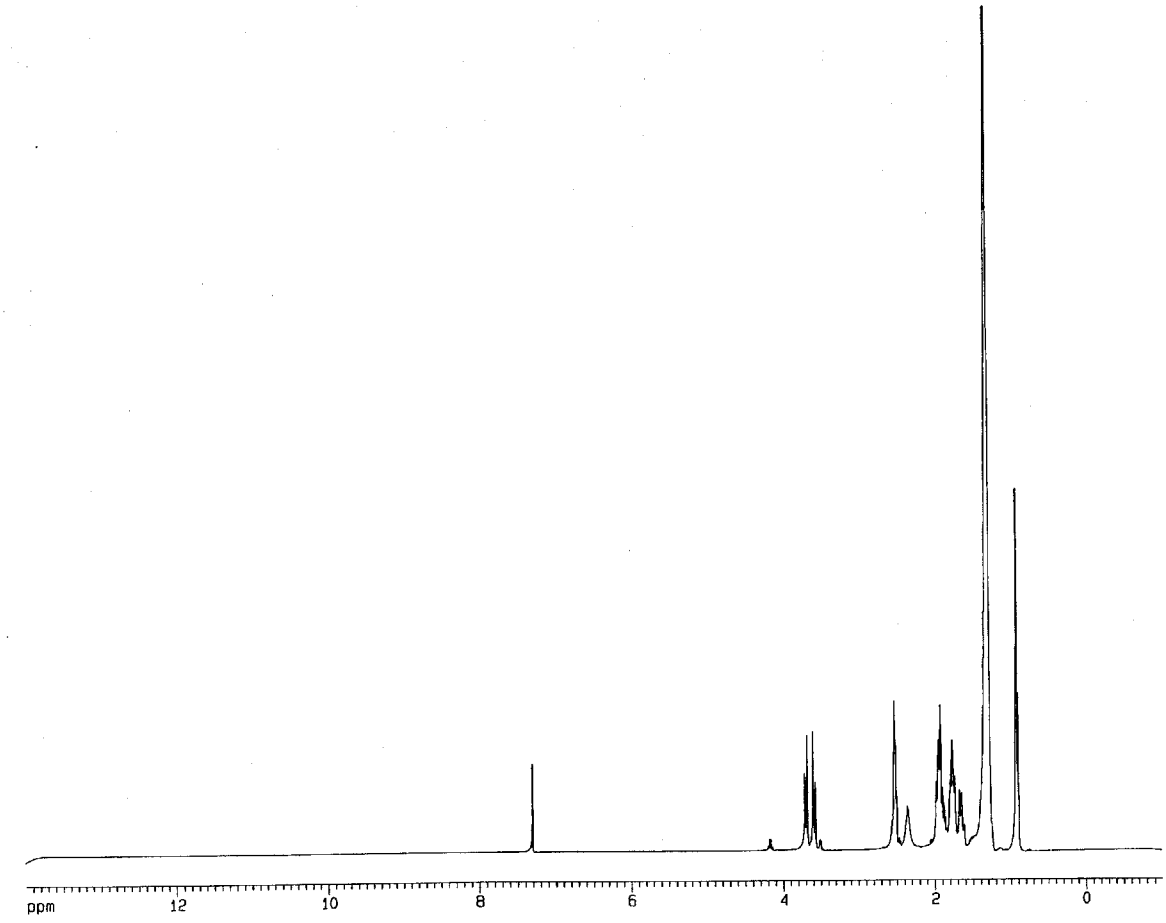


Figure 4.3. 400 MHz ^{1}H NMR spectrum of compound (**1**) in $CDCl_3$

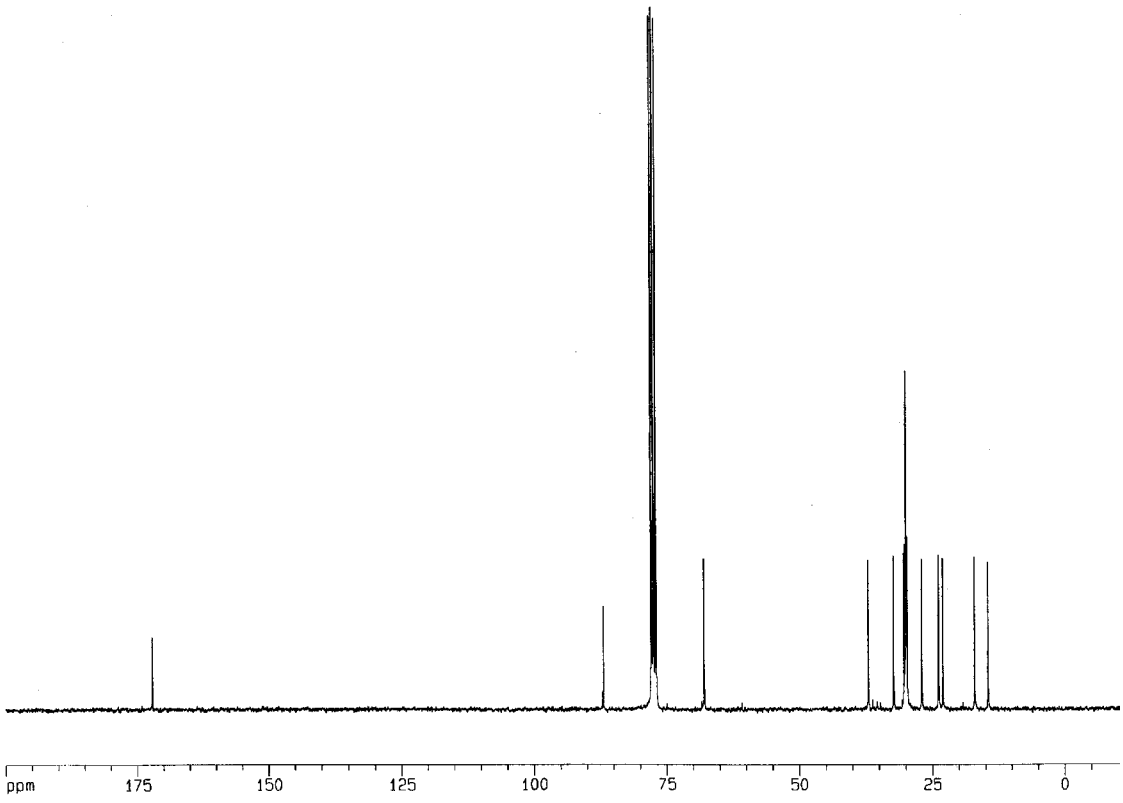


Figure 4.4. 100 MHz ^{13}C NMR spectrum of compound (**1**) in $CDCl_3$

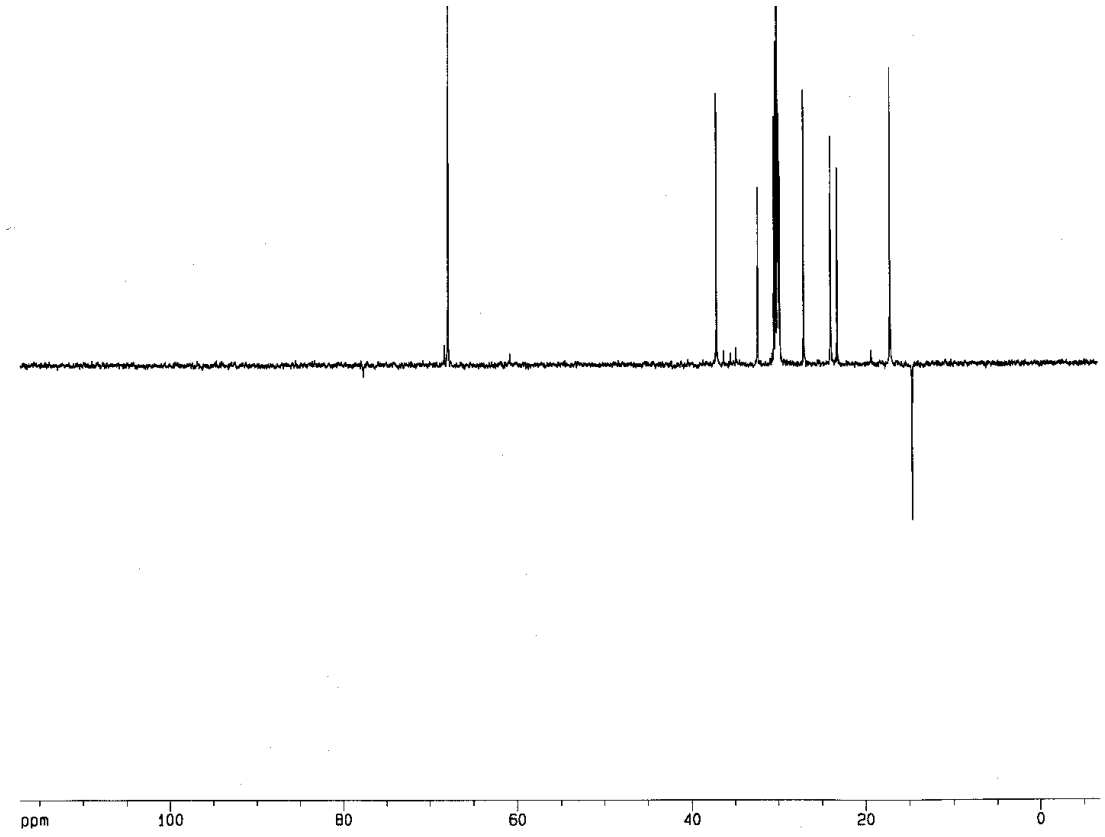


Figure 4.5. 100 MHz DEPT 135 spectrum of compound (**1**) in $CDCl_3$

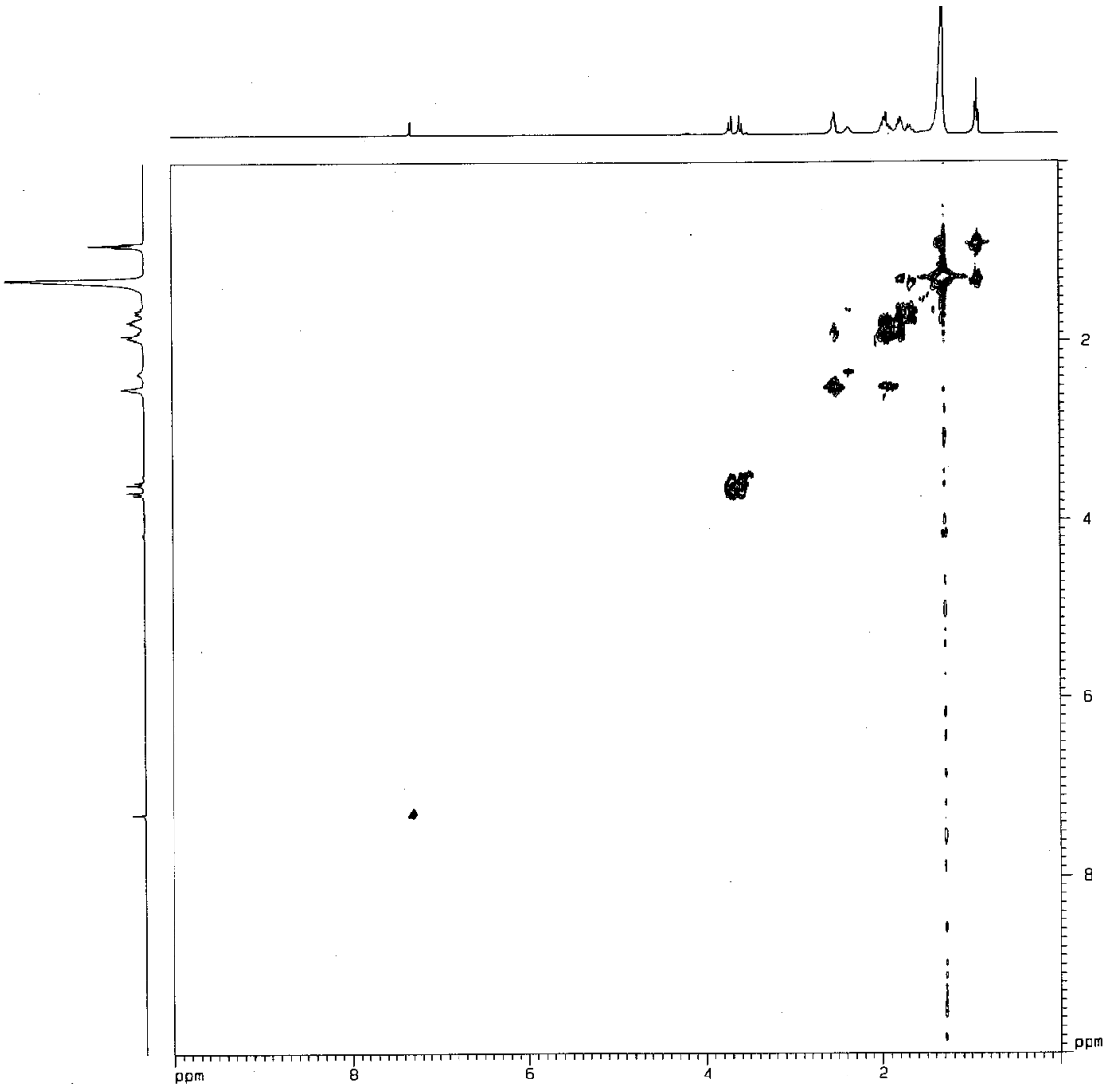


Figure 4.6. 400 MHz ^{1}H-^{1}H COSY spectrum of compound (**1**) in $CDCl_3$

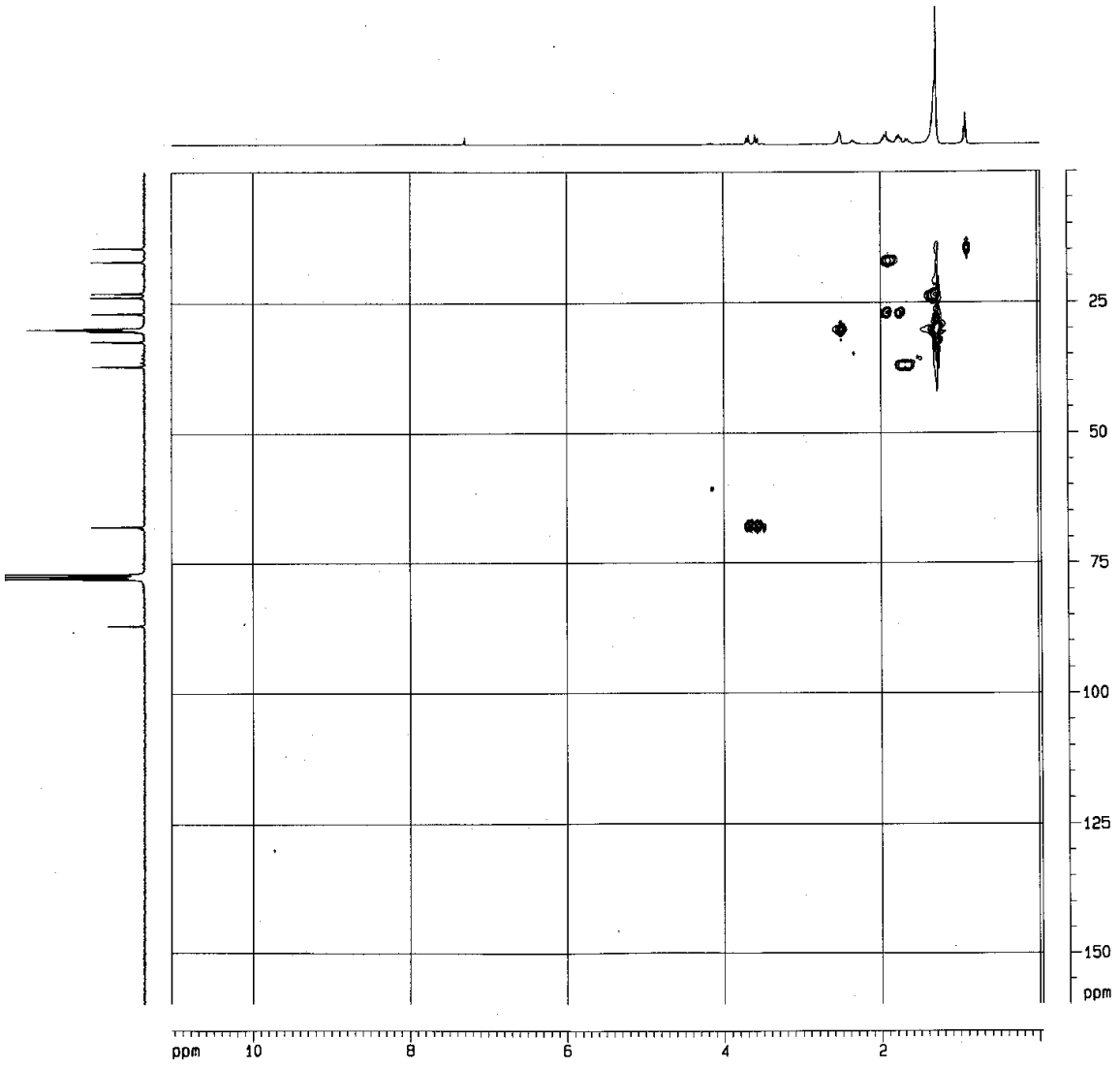


Figure 4.7. 400 MHz Multi-edited HSQC spectrum of compound (**1**) in $CDCl_3$

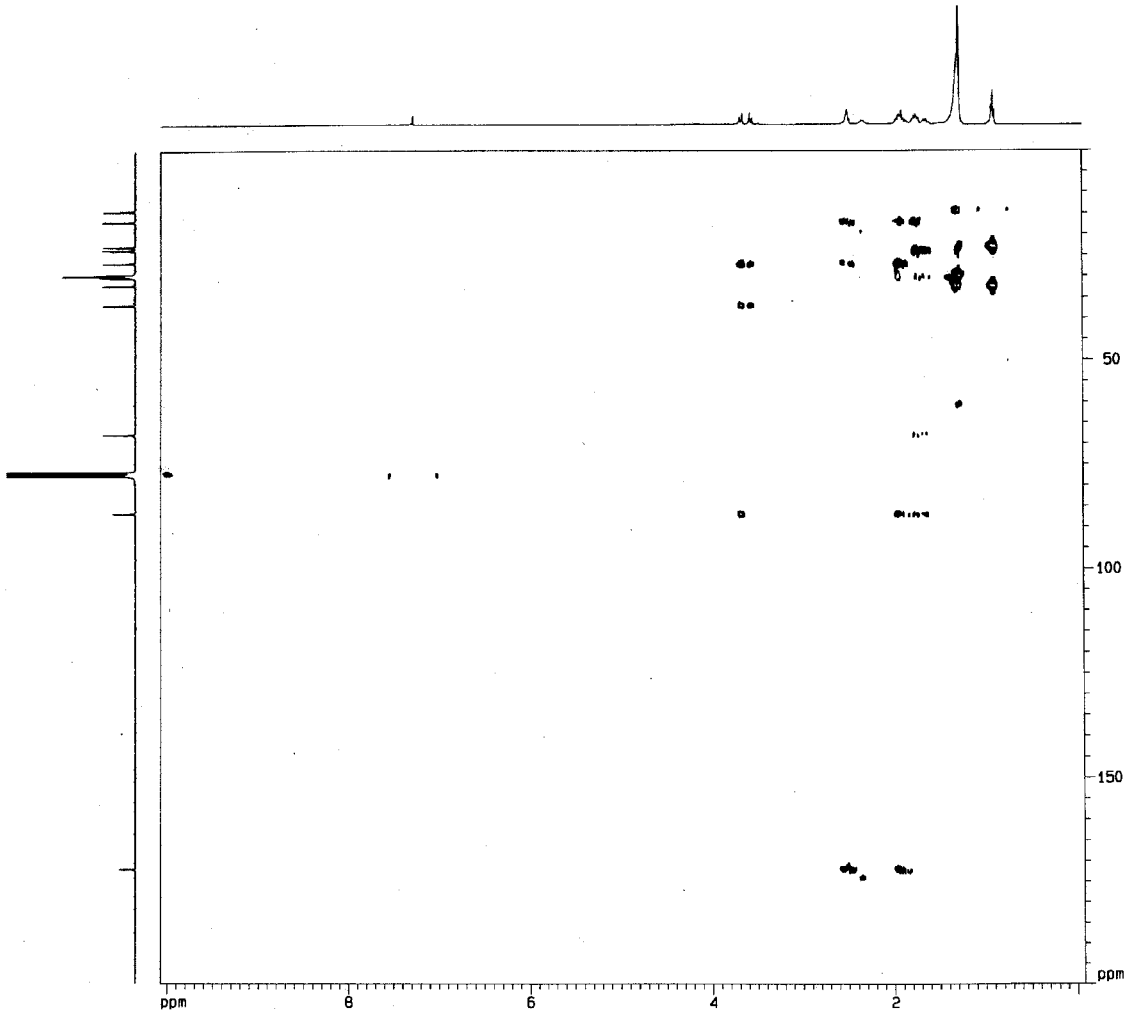


Figure 4.8. 400 MHz HMBC spectrum (optimized for *J* = 8Hz) of compound (**1**) in $CDCl_3$

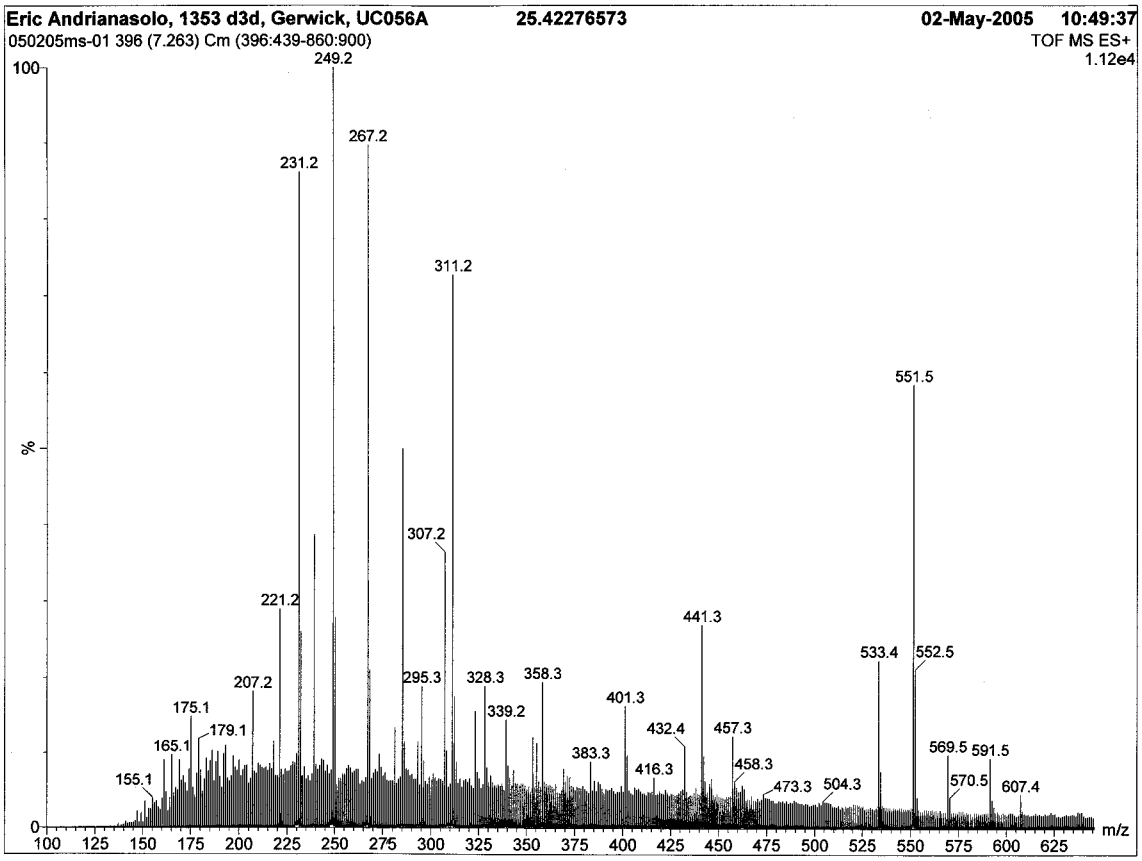


Figure 4.9. LR TOF MS ES+ spectrum of compound (**1**)

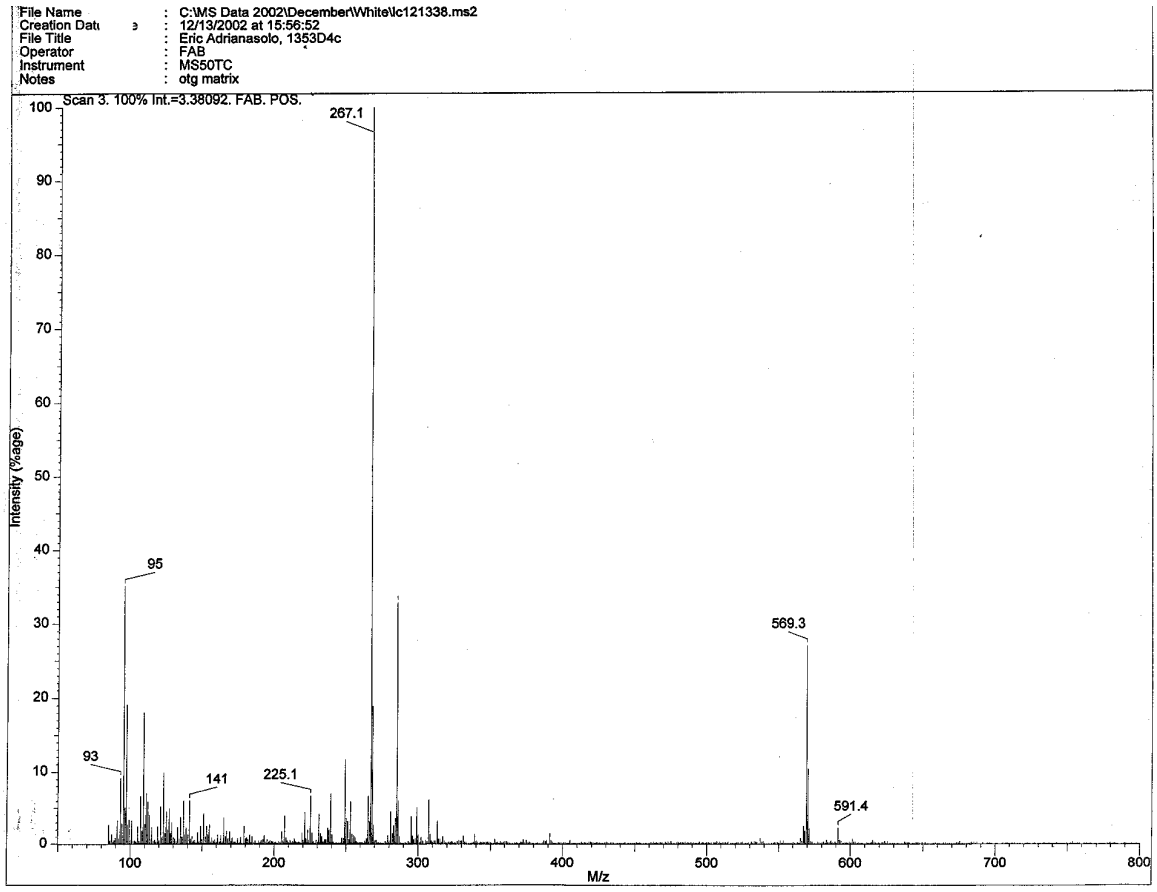


Figure 4.10. LR FAB (otg matrix) spectrum of compound (**1**)

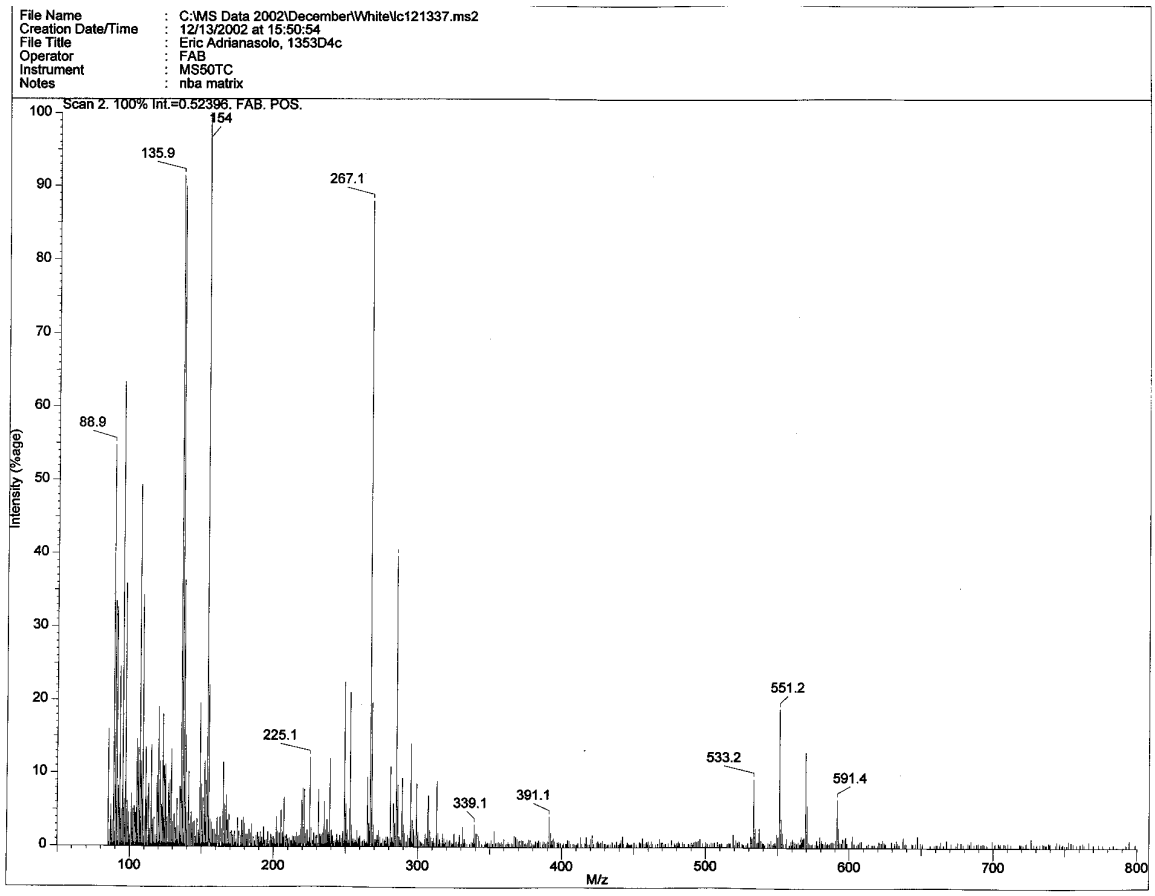


Figure 4.11. LR FAB (nba matrix) spectrum of compound (**1**)

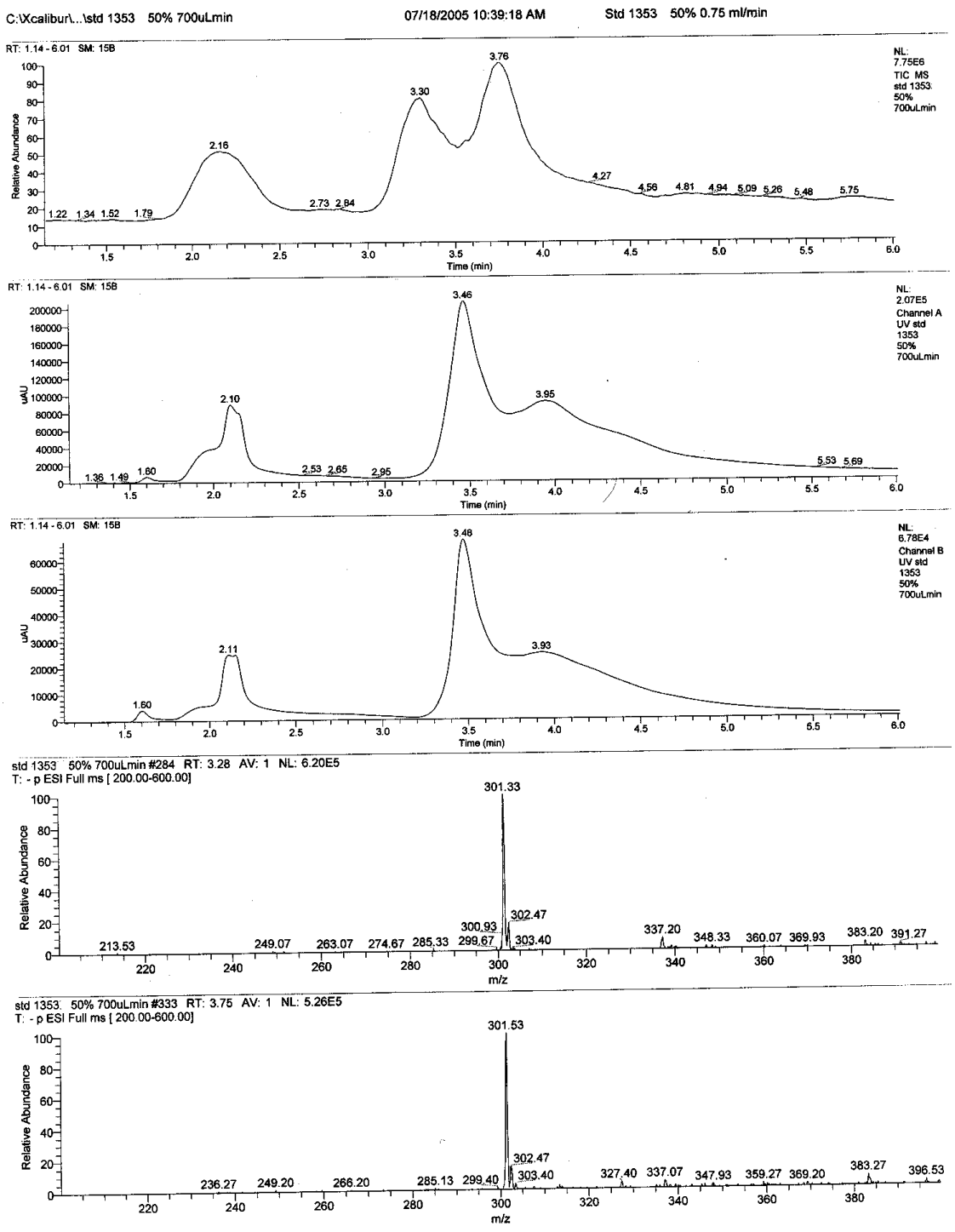


Figure 4.12. Hydrolyzed compound **1** ESI LCMS negative ionization

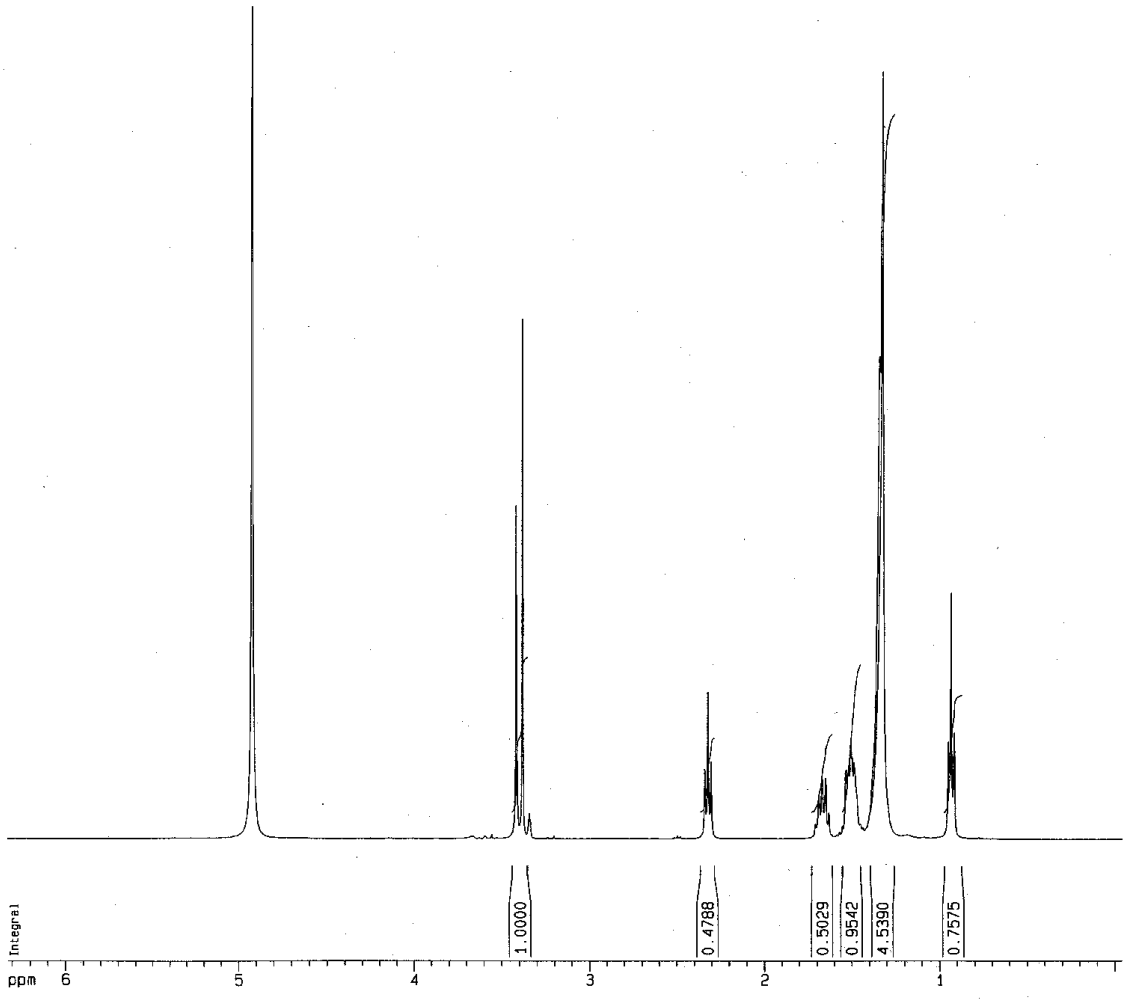


Figure 4.13. 400 MHz ^{1}H NMR spectrum of compound (**2**) in CD_3OD

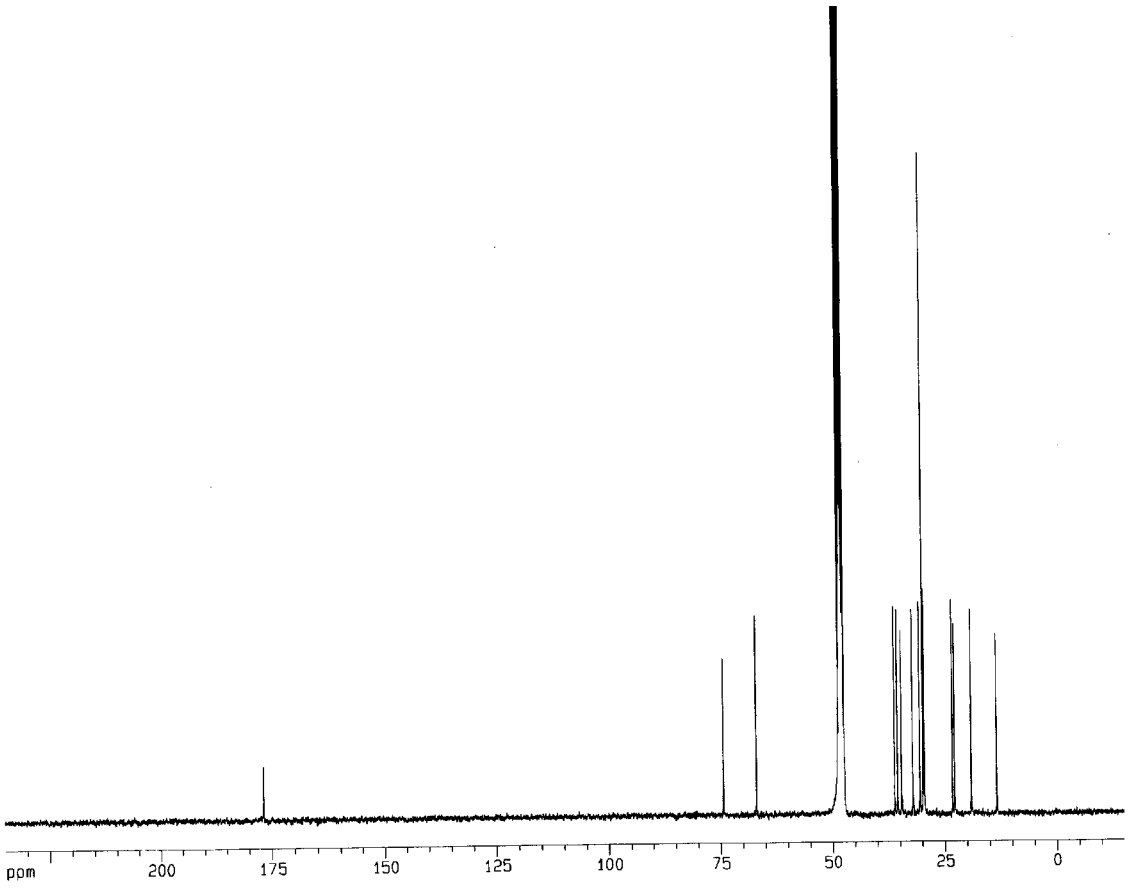


Figure 4.14. 100 MHz ^{13}C NMR spectrum of compound (**2**) in CD_3OD

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on JASCO P 1010 and Perkin-Elmer model 141 polarimeters. UV and FT-IR spectra were obtained employing Hewlett Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on a Bruker Avance DPX400 spectrometer. Spectra were referenced to residual solvent signal with resonances at $\delta_{H/C}$ 7.26 / 77.1 ($CDCl_3$) and $\delta_{H/C}$ 3.31/49.15 (CD_3OD). X-ray diffraction data were collected on a Siemens P-4 X-ray diffractometer with HiStar area dectector (Cu Kα radiation). Structure solution and refinement was carried out using SHELXS and SHELXL, respectively. HPLC separations were performed using Waters 515 HPLC pumps, a Rheodyne 7725i injector, and Waters 996 photodiode array detector. Analytical chiral HPLC analyses were performed on CHIRIOBIOTIC T (Teicoplanin) - 10µm (size 4.6 x 100 mm). LCMS analyses were performed on Agilent 1100 LC/MSD instrument. LR and HR FAB as well as HR EI mass spectra were acquired on a Kratos MS50TC instrument. LR TOF-ESI data were acquired on a Water's Micromass LCT Classic mass spectrometer.

Collection, Extraction, and Isolation Procedures

Lyngbya majuscula was collected by hand using SCUBA in April 2000 near Tanikely Island, Nosy-Be Madagascar (voucher specimen available from W.H.G. as collection number MNT-26/Apr/00-02). The alga was stored at -20ºC in 70% EtOH until workup. A total of 944 g (dry wt) of the alga was extracted three times with CH_2Cl_2-MeOH (2:1) to yield 8.73 g of crude extract. A portion of this (1.5 g) was fractionated using vacuum liquid chromatography (VLC) on Si gel by a stepwise gradient of hexanes-EtOAc and EtOAc-MeOH to give 9 fractions. Fraction D (96.7 mg, 40% EtOAc in hexanes) showed inhibitory activity against SIRT2 and was subjected to further fractionation by solid phase extraction (NP SPE) to yield five fractions (D1-D5). Fraction D3 and D4 gave 80% and 75% inhibition at 10 µg/ mL, respectively, in the SIRT2 assay. Analytical reversed-phase HPLC (Phenomenex Sphereclone ODS, 250 x 10.0 mm, 5 µm, 2.5 mL/min, RI detection, MeOH/H_2O, 95:5) purification of D3 and D4 led to the isolation of 15 mg of **1** (t_R = 8 min) as a colorless oil. Chiral LCMS analyses of **1** with a Chirobiotic T (Teicoplanin) column yielded a single peak (t_R = 3 min) using a gradient elution starting with 70%

MeOH/30% H_2O. Chiral LCMS analyses on the same column of the hydrolysate of **1** (6N HCl at 105 °C for 16 hr) yielded two peaks (t_R = 4.73 min and 5.22 min) using an isocratic elution (40% H_2O/60% EtOH).

X-ray Crystallography

The crystalline sample of **2** obtained from the fraction eluting with 25% MeOH/75% EtOAc was used without further preparation. Determination of the crystallographic parameters, data collection and structure solution and refinement was done as described elsewhere,[15] with the following details.

A well shaped crystal of dimensions 0.30x 0.10 x 0.02 mm[17] was selected and mounted on the tip of a thin glass fiber using a dab of Paratone. Crystal quality evaluation and preliminary indexing were performed from four images of 10 degrees rotation about omega, each separated from the others by 50 degrees. Proving to be a satisfactory crystal, a full set of 160 frames of 5 degrees rotation about omega was collected. The frames were integrated using the determined unit cell, using the program TwinSolve as included in Rigaku/MSC's software package CrystalClear to yield a redundant data set of 8951 reflections. Correction for the effects of absorption anisotropy was carried out by means of multiscans[16] as programmed in TwinSolve. Finally, a data set consisting of 2294 unique reflections in the range (0 – 6,-9 – 9,-24 – 24) was generated with an R(merge) of 0.1640. The reported unit cell was refined using all 2048 reflections with intensities greater than 10 times their e.s.d.s in the range 2.25° < θ < 64.64°.

The structure was solved using direct methods as programmed in SHELXS-90[17] and refined using the program SHELXL-97.[18] Although 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 to which it was attached. During the final cycle of least squares refinement, all non-hydrogen atoms were refined with anisotropic displacement parameters. The refined value of the absolute structure parameter (Flack parameter)[19] of 0.5(7) indicates that no clear indication of the absolute structure of the molecule can be derived from the diffraction experiment alone. An ORTEP[20] of the final model is given in Figure 4.2, with displacement ellipsoids drawn

at the 50% probability level.

SIRT2 inhibitory assay

The assay was run in 96-well format in a final volume of 50 µl. All reaction components were prepared in Buffer A. Blank wells contained no NAD. The reaction was run for 2 h at 37^{o} and stopped with 50 µl of Stop Buffer. Development was allowed to proceed for 20 minutes, and then the plate was read with a fluorescence plate reader (excitation at 360 nm, emission at 460 nM).

Final assay concentrations contained 50 µM fluor de Lys substrate, 270 nM SIRT2 (12.5 µg/50 µl), 1 mM NAD, ± inhibitor, and adjusted to a 50 µL volume with Buffer A. Buffer A was composed of 25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$. Stop Buffer was composed of 2.7 mg/mL trypsin in Buffer A.

Sodium channel blocking assay

Chemicals 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*.[24] Twenty-four hr prior to chemical testing, mouse neuroblastoma (neuro-2a) cells were seeded in 96-well plates at 6.0 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 than0.5% 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 or 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-3 (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.

Cytotoxicity assay

Cytotoxicity was measured in NCI-H460 lung tumor cells and neuro-2a cells using the method of Alley *et. al*[25] with cell viability being determined by MTT

reduction.[24] Cells were seeded in 96-well plates at 6000 cells/well in 180 µl. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µl/well DMSO was less than 0.5% final concentration. After 48 hr, the medium was removed and cell viability determined.

Tanikolide dimer (1): $[\alpha]^{25}_{D}$ 0° (*c* 1, $CHCl_3$); UV ($CHCl_3$) λ_{max} 202 nm (ε 100); IR ν_{max} (neat) 3420, 3402, 2853, 2360, 1716, 1248, 1049, 940 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 4.1.; LR FABMS (otg matrix) obs *m*/*z* (rel int) 591.4 (5) $[M+Na]^+$, 569.3 (35) $[M+H]^+$, 267.1 (100); LR FABMS (nba matrix) obs *m*/*z* (rel int) 591.4 (7) $[M+Na]^+$, 569.3 (14) $[M+H]^+$, 551.2 (20) $[M-OH]^+$, 533.2 (10) $[M-2OH-H]^+$, 267.1 (100); LR TOF MS ES+ obs *m*/*z* (rel int) 607.4 (5) $[M+K]^+$, 591.5 (5) $[M+Na]^+$, *m*/*z* 569.5 (10) $[M+H]^+$, *m*/*z* 551.4 (60) $[M-OH]^+$, and *m*/*z* 533.4 (20) $[M-2OH-H]^+$; HR FABMS obs $[M + H]^+$ *m*/*z* 569.47804 for $C_{34}H_{65}O_6$ (0.1 mmu); HR EI MS *m/z* 551.4691 $[M-OH]^+$ and 533.4567 $[M-2OH-H]^+$ for $C_{34}H_{63}O_5$ (-0.5 mmu) and $C_{34}H_{61}O_4$ (2.8 mmu), respectively.

Compound 2: $[\alpha]^{25}_{D}$ -10° (*c* 0.87, $CHCl_3$); no UV; IR ν_{max} (neat) 3425, 3410, 2850, 2360, 1715, 1250, 1050, 939 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 4.2.; LR FABMS (nba) obs *m*/*z* (rel int) 325.3 (100) $[M+Na]^+$; HR FABMS obs $[M + Na]^+$ *m*/*z* 325.23668 for $C_{17}H_{34}O_4Na$ (-1.2 mmu).

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CHAPTER FIVE

TRICYCLIC DITERPENES FROM BROWN MARINE ALGA *DICTYOTA* SP.

ABSTRACT

A novel HDAC inhibitor belonging to the dolastane carbon skeleton has been isolated from the brown marine alga *Dictyota* sp. The molecular structure of this compound was determined by NMR spectroscopy in combination with mass spectral data analysis. The relative stereochemistry was deduced by 1 D NOE and ROESY experiments along with chemical conversion to the known compound amijiol. The novel HDAC inhibitor was also found to possess an antimalarial activity.

INTRODUCTION

The reversible modification of histones by acetylation, carried out by histone acetyl transferases (HATs) and histone deacetylases (HDACs) enzymes, is involved in chromatin remodeling and plays an important role in gene expression.[1] When HDACs are inhibited, histone hyperacetylation occurs. The disruption of the chromatin structure by histone hyperacetylation leads to the transcriptional activation of a number of genes.[2] One important outcome of the activation is induction of the cyclin-dependent kinase inhibitory protein $p21^{WAF1/CIP1}$, which causes cell cycle arrest.[3] HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) have been reported to inhibit cell growth, induce terminal differentiation in tumor cells,[4] and prevent the formation of malignant tumors in mice.[5] Therefore, HDACs have emerged as attractive targets in anticancer drug development, and HDAC inhibitors have also been viewed as useful tools to study the function of these enzymes.

Many groups have ongoing research programs to find nonpeptide small-molecule inhibitors of HDACs, and these efforts have led to the identification of several classes of inhibitors.[6] HDAC inhibitors belonging to hydroxamic acid derivatives such as TSA and SAHA are thought to chelate the zinc ion in the active site in a bidentate fashion through its CO and OH groups.[7] However, hydroxamic acids occasionally have been associated with problems such as poor pharmacokinetics and severe toxicity.[8] Thus, it has become increasingly desirable to find replacements that possess strong inhibitory action against HDACs. In addition, in terms of biological research, the discovery of novel zinc-binding groups (ZBGs) may lead to a new type of HDAC isozyme-selective inhibitors which are useful as tools for probing the biology of the enzyme.[9] Thus far, *o*-aminoanilide,[9,10] electrophilic ketones,[11] and *N*-formyl hydroxylamine[12] have been reported as ZBGs in small-molecule HDAC inhibitors. However, most of them have reduced potency as compared to hydroxamic acid, and unfortunately, HDAC inhibitors bearing electrophilic ketones[11] have a metabolic disadvantage in that they are readily reduced to inactive alcohols in vivo, even within cells.

As part of our ongoing search for structurally and pharmacologically interesting substances from marine organisms,[13-15] a detailed exploration of a brown marine alga *Dictyota* sp. was undertaken. Using a HDAC bioassay guided isolation approach we report here the isolation and structural elucidation of novel

HDAC inhibitor with an IC_{50}= 2.9 μM, the inhibitor is a tricyclic diterpene that belongs to the dolastane skeleton and bears an acetate group at C-4. The novel inhibitor was also found to possess an antimalarial activity at 7 μg/mL.

RESULTS AND DISCUSSION

Brown marine alga *Dictyota sp* was collected from Netherland Antilles Playa Fort, extracted with CH_2Cl_2/MeOH (2:1) and fractionated by Si VLC. The resulting fractions were tested for HDAC inhibitory activity, and revealed that the 10% EtOAc in hexanes fraction was strongly active (IC_{50} = 4.6 μM). This material was subjected to further fractionation. Analytical normal-phase HPLC purification of this material led to the isolation of pure compound (**1**) which showed an IC_{50}= 2.9 μM in HDAC inhibitory assay.

LR GCMS analysis of **1** showed peak at *m/z* $[M]^+$ 346 which was also seen in the LR CIMS. The HR CIMS analysis of the peak at m/z 346.25074 $[M]^+$ showed a molecular formula of $C_{22}H_{34}O_3$ (-0.2 mmu) consistent with six degrees of unsaturation. Extensive observation and analysis of the ^{1}H NMR, ^{13}C NMR and the multiplicity edited HSQC data of **1** displayed the presence of five methyls (δ 18.0, 20.7, 21.7, 21.8, 27.9), seven methylenes (δ 22.4, 27.2, 27.4, 27.7, 28.1, 43.3, 46.3), one methine (δ 26.9), one oxygen-bearing methine (δ 82.7), together with three fully substituted carbon atoms (δ 44.7, 51.3, 79.8), one tetrasubstituted double bond (δ 138.7, 139.8), one double bond with an exocyclic methylene (δ 109.8, 151.8), and one carbonyl (δ 169.9). The comparison of these carbon chemical shifts to the known compound amijiol[18] belonging to the dolastane skeleton revealed that **1** is the acetate derivative at C-4 of amijiol. This was confirmed by HMBC correlation between the methine proton at C-4 (δ 4.80) and the carbonyl (δ 169.9). Furthermore, irradiation of the carbonyl (δ 169.9) by the application of 1D HMBC experiment displayed couplings to the methine proton (δ 4.80) and the methyl group (δ 2.18) confirming the attachment of OAc group to C-4
(Figure 5.1.). The planar structure of **1** was then deduced and confirmed using additional ^{1}H-^{1}H COSY and HMBC correlations (Figure 5.2.).

The structure of **1** displays four stereocenters at C-4, C-5, C-12 and C-14. Fenical *et al*. and P. Crews *et al.* within different publications reported the structure of amijiol by X-ray crystallography[16,17] with a aim of stereochemistry determination of other related structures. However, only relative stereochemistry could be established. The relative stereochemistry of **1** could be implied by converting the acetate portion into its corresponding alcohol. The cleavage of the

acetate unit was performed by base hydrolysis using LiOH. Chemical shift analyses between the corresponding alcohol and the known compound amijiol[18] were performed. The optical rotation between these two compounds was also compared. Interestingly, ^{1}H NMR and ^{13}C NMR data as well as the optical rotation of the corresponding alcohol of **1** was similar to that of amijiol. These analyses led to the conclusion that the relative stereochemistry of **1** was similar to that of amijiol. Furthermore, 1D NOE experiment showed that irradiation of the methine proton at C-4 (δ 4.80) enhances the methyl signal at C-16 (δ 0.84) (Figure 5.10.). ROESY cross peak between these protons was also seen. These results implied that the relative stereochemistry at C-4 and C-5 were similar to that of found to amijiol. The unusual deshielded shift of the Methyl at C-20 (δ 1.38) indicated that the OH attached to C-14 and this methyl at C-20 are coplanar or a syn 1,3 Me-OH orientation, hence the relative stereochemistry at C-14 and C-12 were similar to that of found to amijiol (Figure 5.3.).

Compound **1** was tested in HDAC inhibitory assay as well as in antimalaria assay.[19] Compound **1** was found to have IC_{50} value of 2.9 μM against HDAC1 and an antimalaria activity of 20 μM. Study on apicidin,[22] a novel antiprotozoal agent that inhibits parasite histone deacetylase, revealed that this natural product, apicidin [cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)], has broad spectrum activity against the Apicomplexan parasites and kills parasites (malaria *Plasmodium falciparum*) by inhibiting histone deacetylase (HDAC), a key nuclear enzyme involved in transcriptional control.

Other known HDAC inhibitors such as sodium butyrate, trapoxin A, and HC-toxin were also evaluated and found to possess antiparasitic[22] activity including the malaria parasite *Plasmodium falciparum*, suggesting that HDAC is an attractive target for the development of novel antiparasitic agents. However, the issues of selectivity of inhibitors for parasite versus host enzyme will need to be addressed, but based on the efficacy against malaria, HDAC appears to be a viable target for the development of antiparasitic agents.

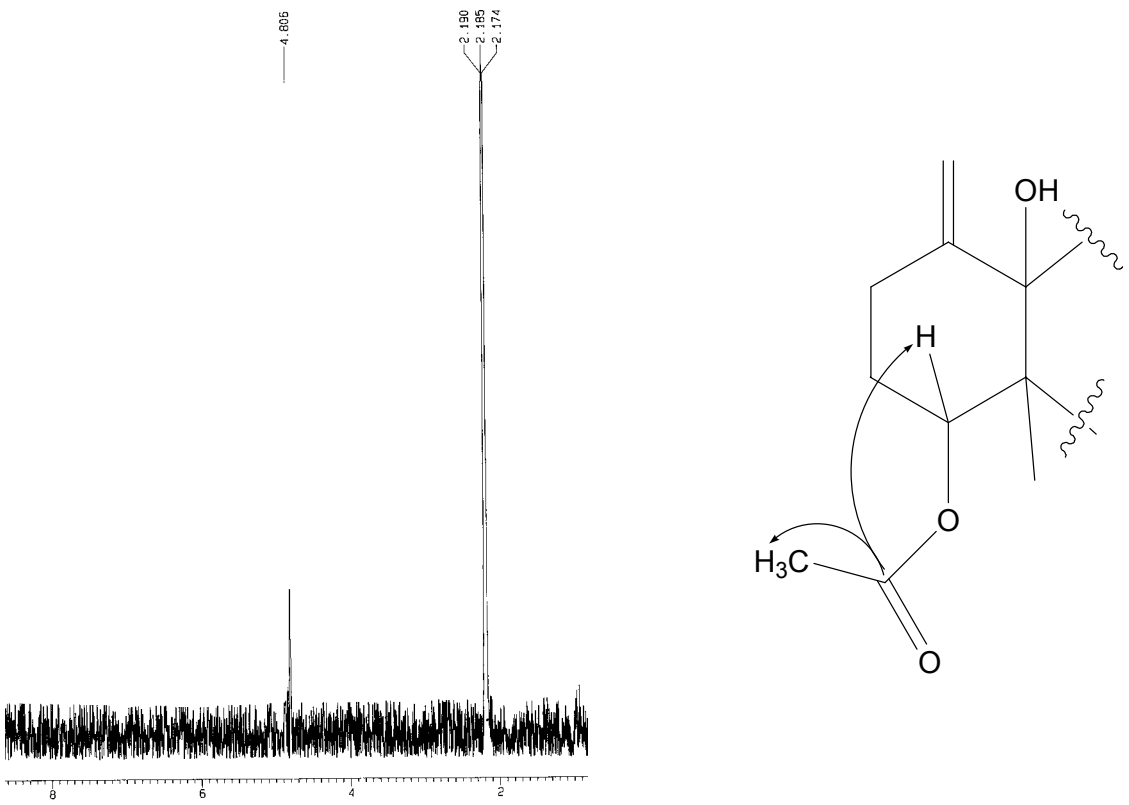


Figure 5.1. 1 D HMBC of **1**. Irradiation of carbonyl (δ 169.9) displayed couplings to methyl (δ 2.18) and H_4 (δ 4.80).

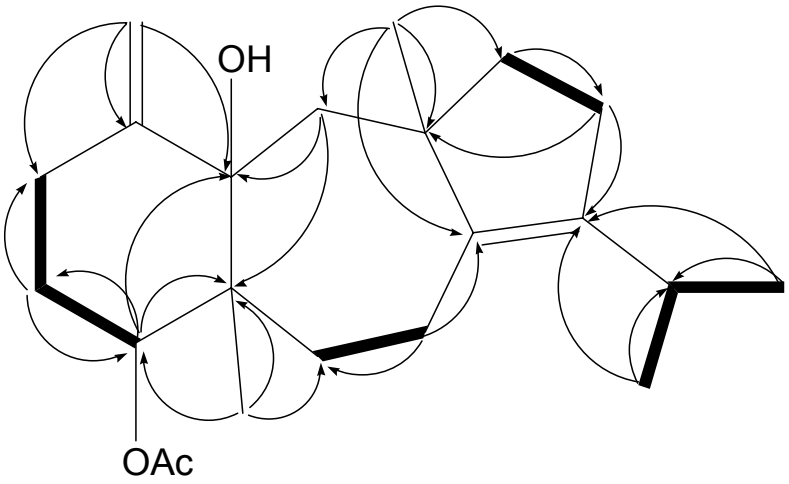
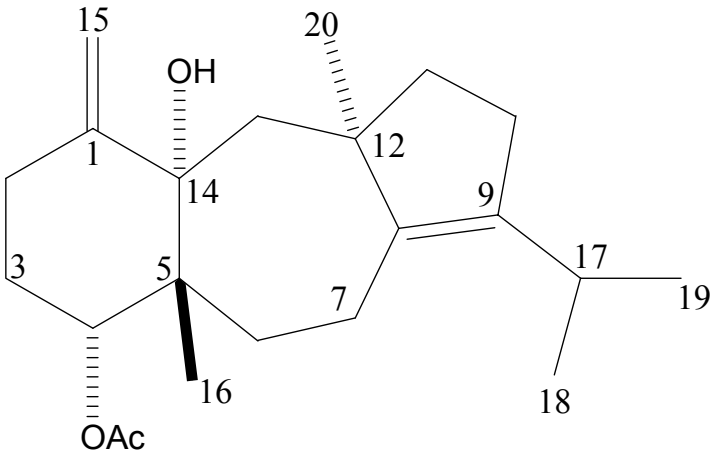
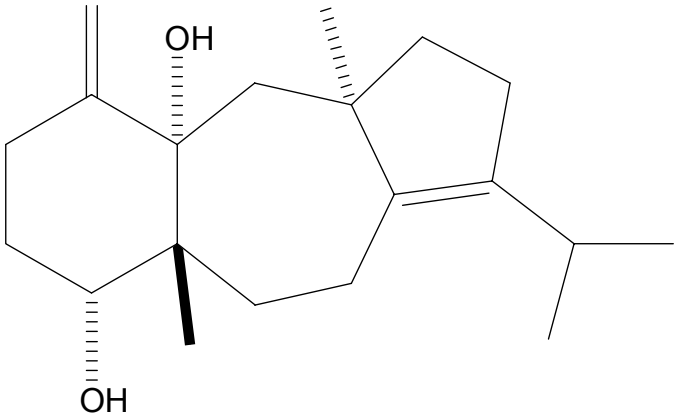


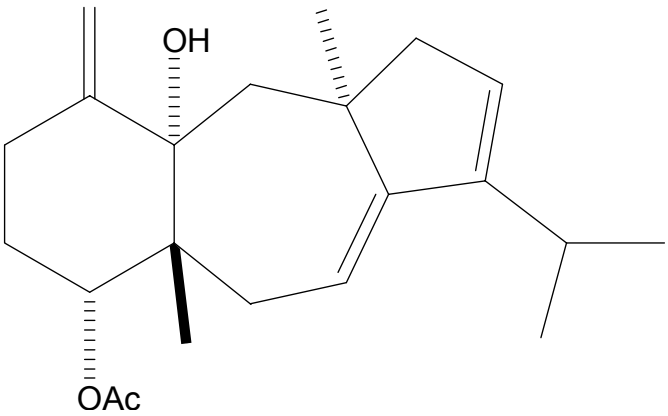
Figure 5.2. Selected HMBC and ^{1}H-^{1}H COSY correlations for compound (**1**).



Compound (**1**)



Amijiol



Compound (**2**)

Figure 5.3. Structure of **1** and related structures in the dolastane skeleton.

Table 5.1. ^{1}H and ^{13}C NMR assignments for compound **1**.[a]

Position	δC	δH		^{1}H-^{1}H COSY	HMBC
1	151.8				
2	27.4	2.20 (m)	2.80 (m)	3	3
3	27.2	1.70 (m)	1.80 (m)	2,4	1,2,4,5
4	82.7	4.80 (bs)		3	3,5,14,16
5	44.7				
6	28.1	1.37 (m)	2.70 (m)	7	5,7,8
7	22.4	2.22 (m)	2.51 (m)	6	5,6,8,12
8	139.8				
9	138.7				
10	27.7	2.20 (m)		11	8,9,12
11	43.3	1.57 (m)	1.67 (m)	10	9,10,12
12	51.3				
13	46.3	1.72 (d, *J*=13 Hz) (d, *J*=13 Hz)	1.9C		1,5,12,14
14	79.8				
15	109.8	4.90 (s) 4.93 (s)			1,2,14
16-Me	18.0	0.84 (s)			4,5,6,14
17	26.9	2.78 (m)		18,19	8,9,18,19
18-Me	20.7	0.91 (d, *J*=6.8 Hz)		17	9,17,19
19-Me	21.8	0.99 (d, *J*=6.8 Hz)		17	9,17,18
20-Me	27.9	1.38 (s)			8,11,12,13
21	169.9				
22-Me (Ac)	21.7	2.2 (s)			4,21

[a] 400 MHz and 100MHz, $CDCl_3$

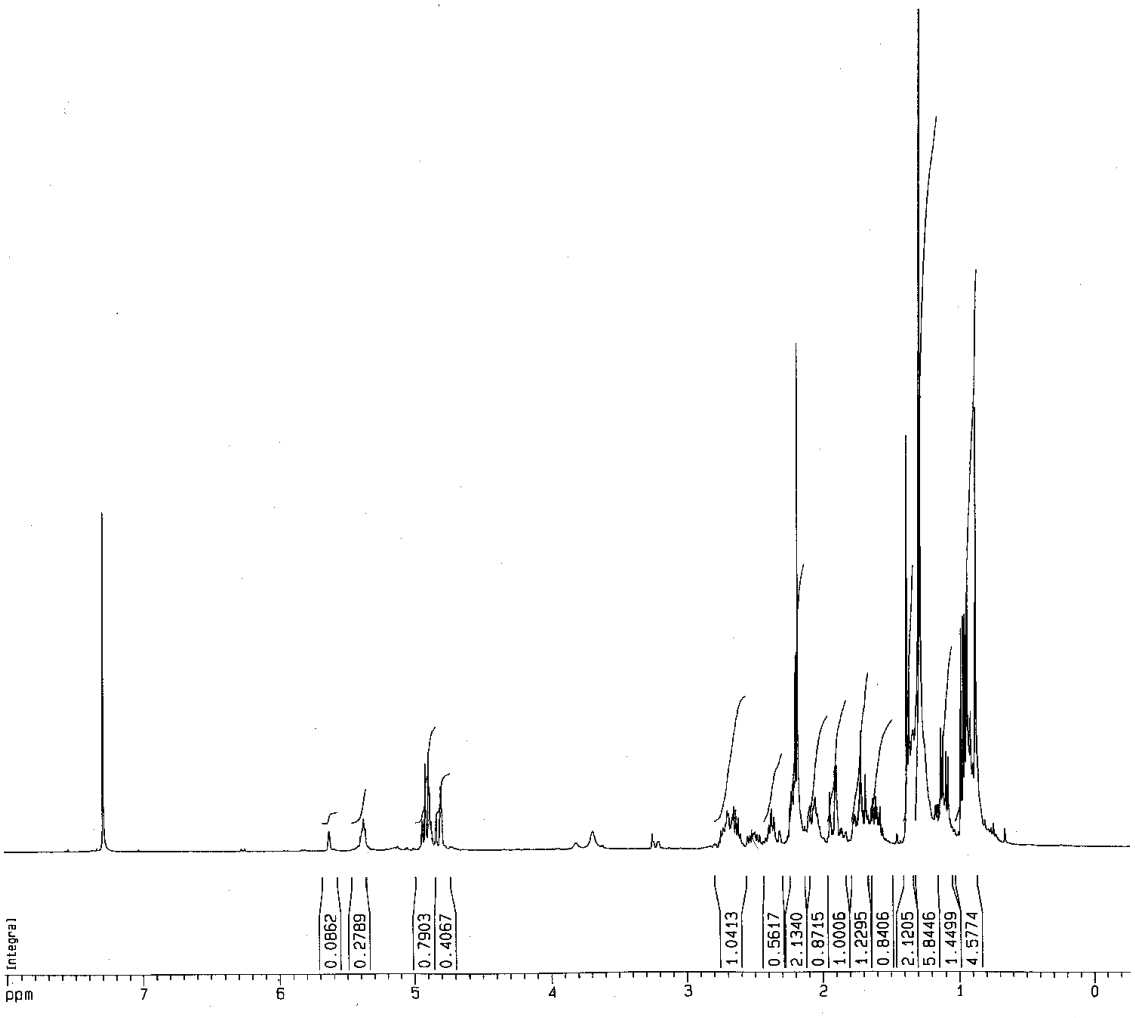


Figure 5.4. 400 MHz ^{1}H NMR spectrum of compound (**1**) in $CDCl_3$

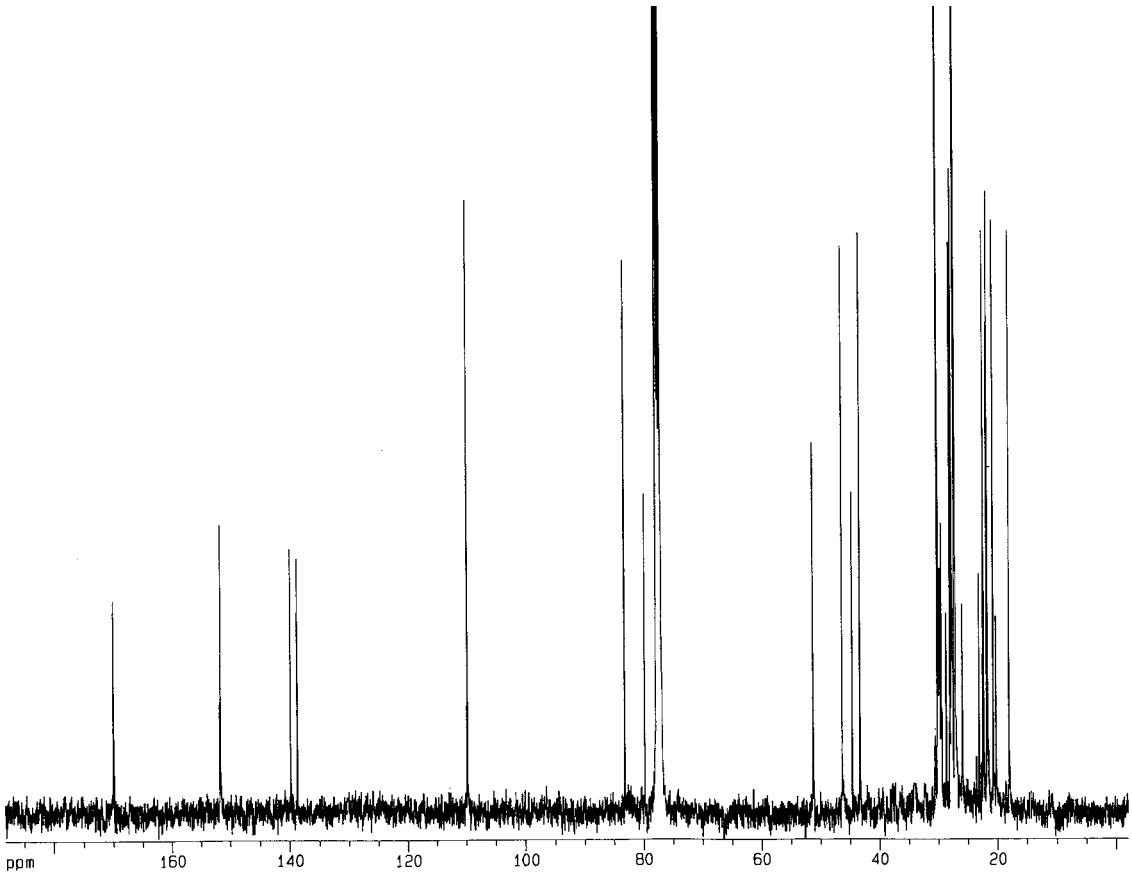


Figure 5.5. 100 MHz ^{13}C NMR spectrum of compound (**1**) in $CDCl_3$

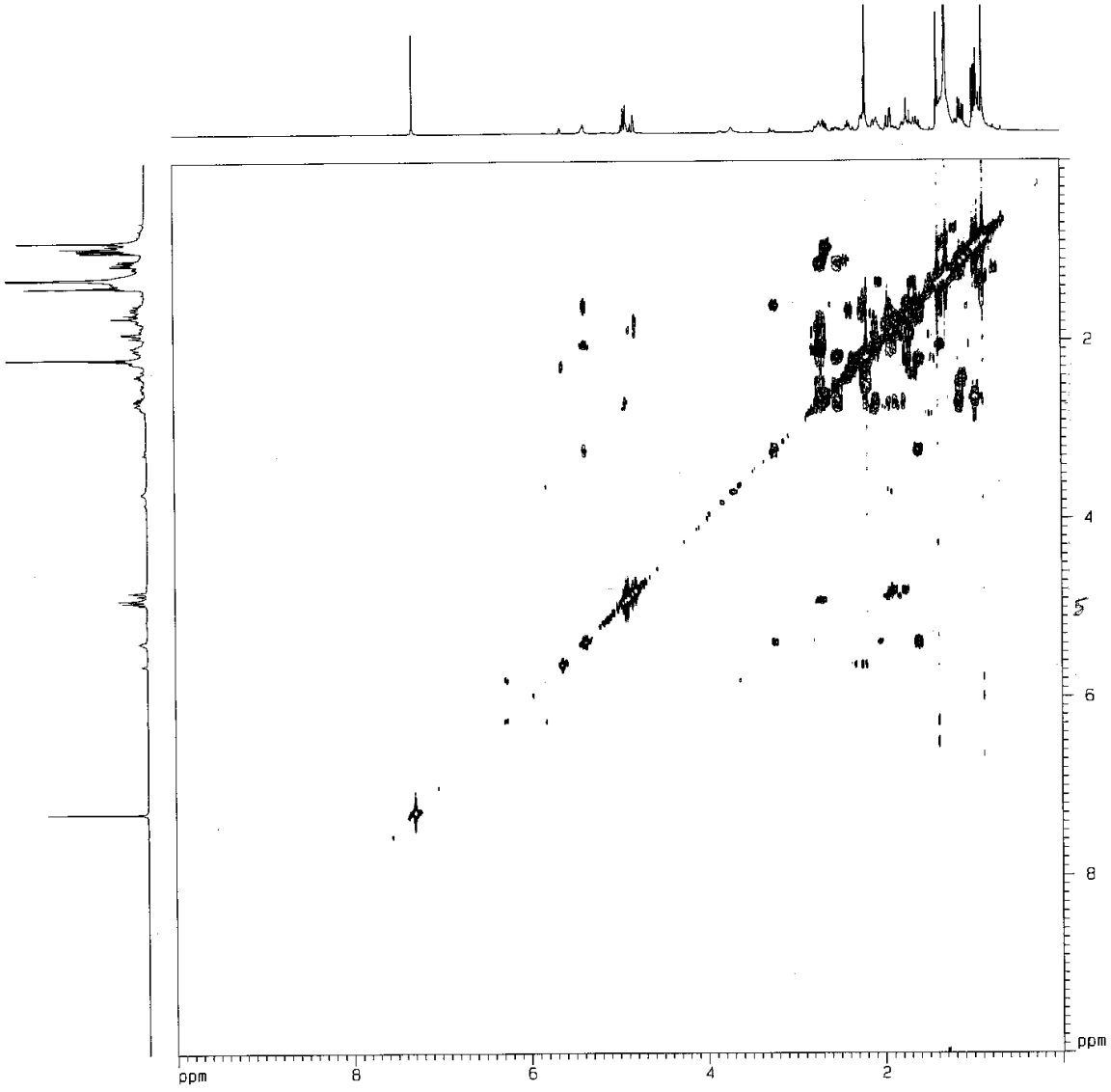


Figure 5.6. 400 MHz ^{1}H-^{1}H COSY spectrum of compound (**1**) in $CDCl_3$

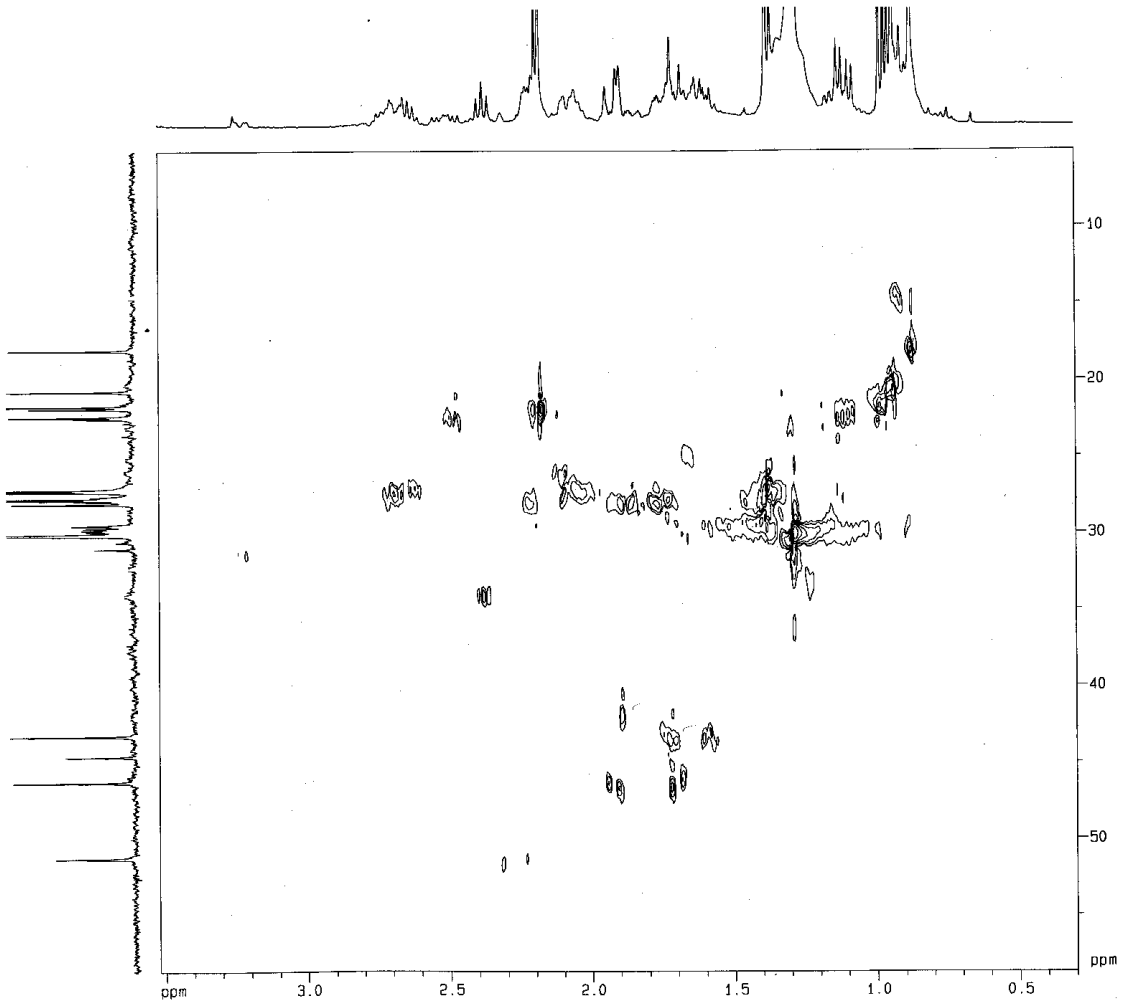


Figure 5.7. 400 MHz HSQC spectrum of compound (**1**) in $CDCl_3$ (Partial view).

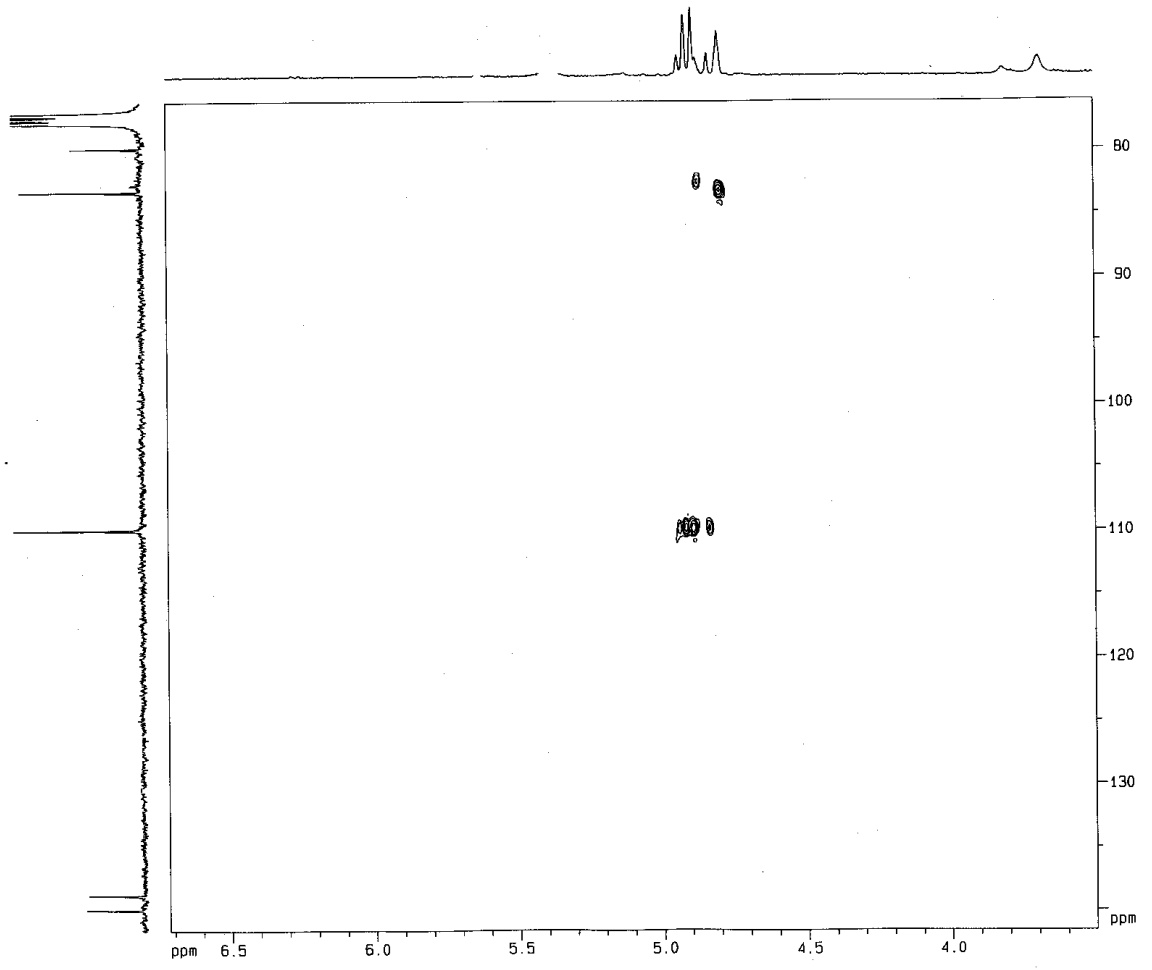


Figure 5.8. 400 MHz HSQC spectrum of compound (**1**) in $CDCl_3$ (Partial view).

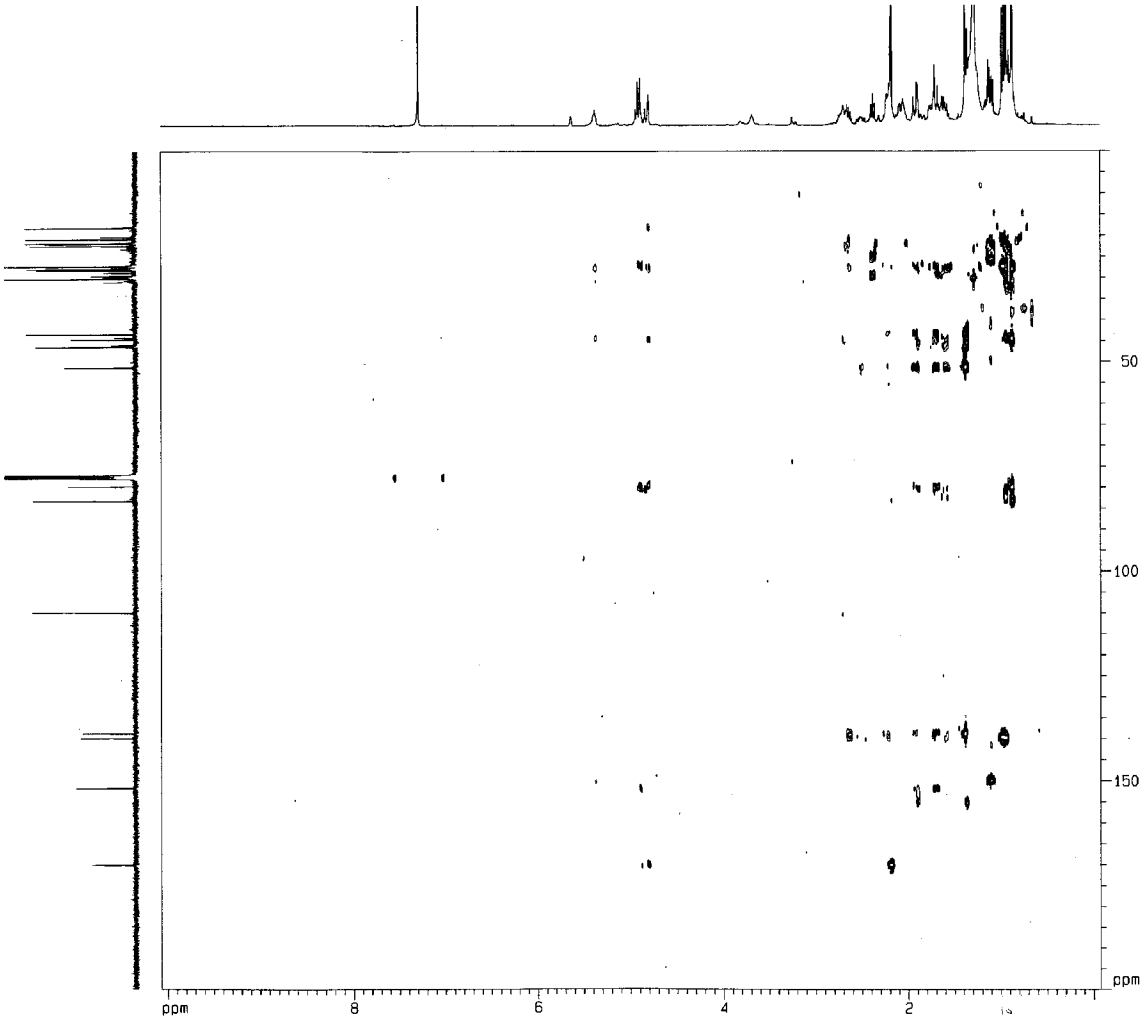


Figure 5.9. 400 MHz HMBC spectrum (optimized for *J* = 8Hz) of compound (**1**) in $CDCl_3$

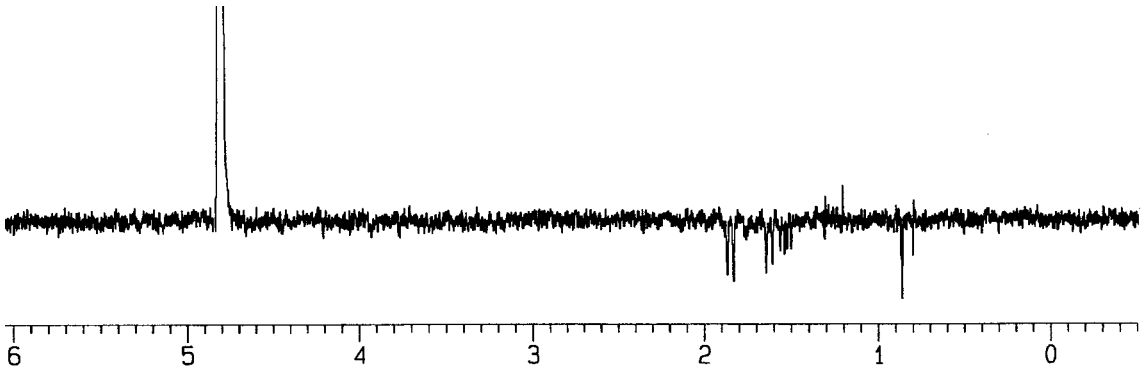


Figure 5.10. 400 MHz 1D NOE of compound (**1**) irradiation of the methine proton at C-4 (δ 4.80) enhances the methyl signal at C-16 (δ 0.84).

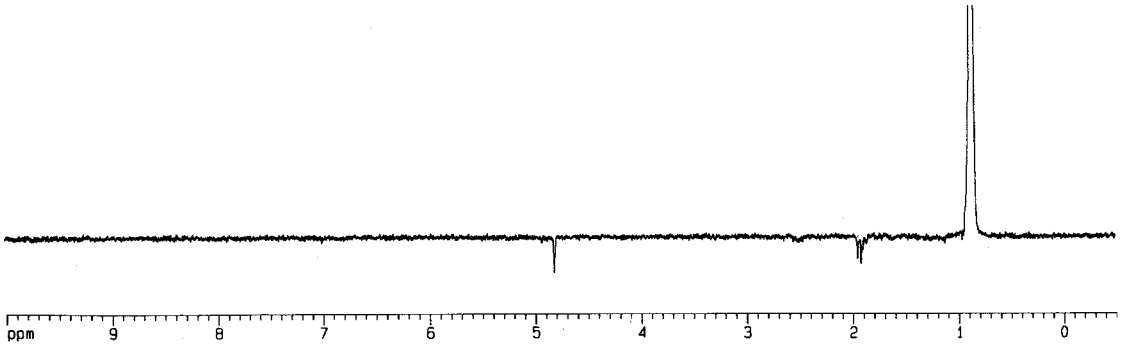


Figure 5.11. 400 MHz 1D NOE of compound (**1**) irradiation of the methyl at C-16 (δ 0.84) enhances the methine proton signal at C-4 (δ 4.80).

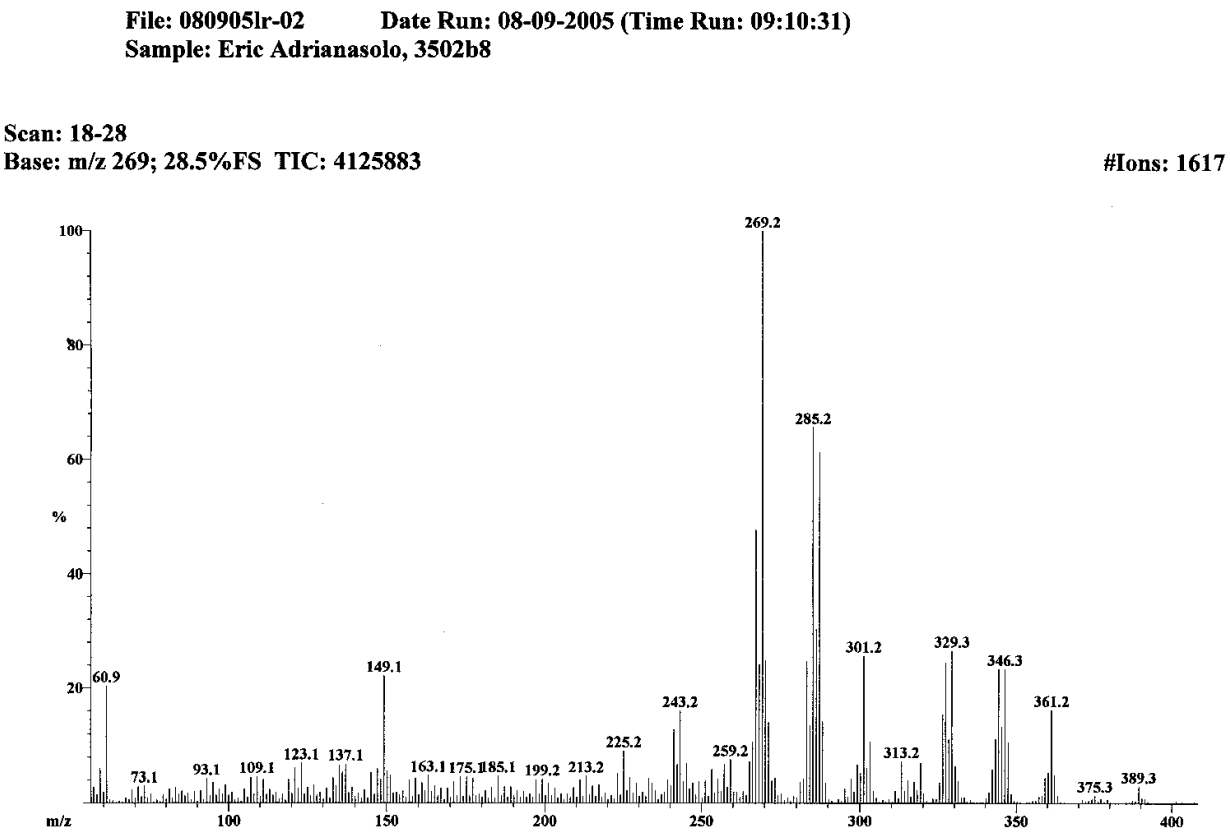
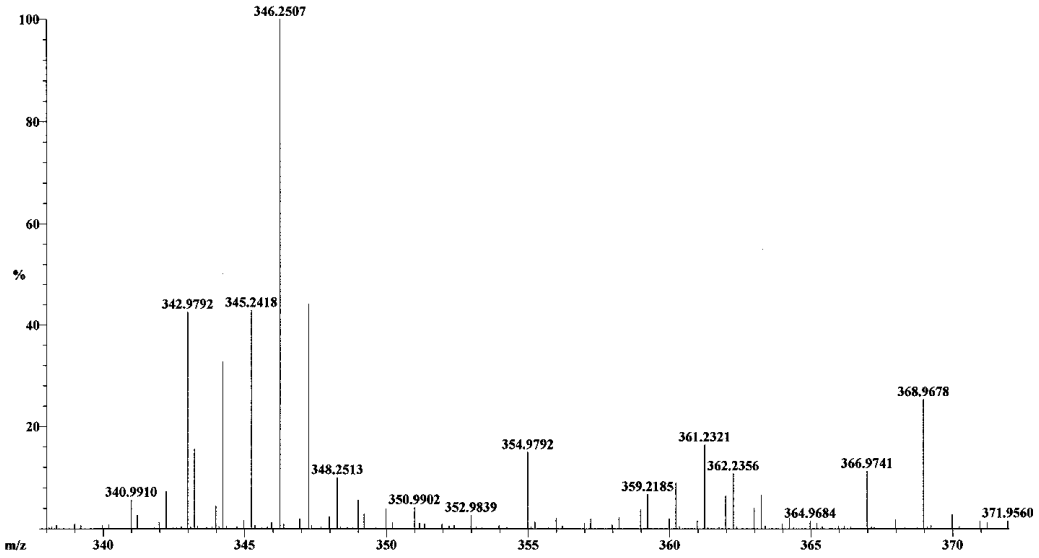


Figure 5.12. LR CIMS spectrum of compound (**1**)

File: 081005hr-01 Date Run: 08-10-2005 (Time Run: 08:06:19)
Sample: Eria Andrianasolo, 3502b8

Scan: 39-45
Base: m/z 346; 19.3%FS TIC: 1050060 #Ions: 196



Selected Isotopes : $H_{0-50}C_{0-25}N_{0-1}O_{0-6}$ Error Limit : 5 ppm

Measured Mass	*% Base*	*Formula*	*Calculated Mass*	*Error*
346.25074	100.0%	$C_{22}H_{34}O_3$	346.25080	-0.2

Figure 5.13. HR CIMS spectrum of compound (**1**)

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on a JASCO P 1010 polarimeter. UV and FT-IR spectra were obtained employing Hewlett Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on a Bruker Avance DPX400 spectrometer. HPLC separations were performed using Waters 515 HPLC pumps, a Rheodyne 7725i injector, and Waters 996 photodiode array detector. LR and HR CI mass spectra were acquired on a Kratos MS50TC instrument and GC/MS data were acquired on Hewlett Packard 5890 series II chromatograph with Hewlett Packard 5971 series mass selective detector.

Collection, Extraction, and Isolation.

Brown alga *Dictyota* sp. was collected by snorkeling in August 1994 from Netherland Antilles Playa Fort (voucher specimen available from W.H.G. as collection number NAF-11/Aug/94-1). The alga was stored at -20ºC in 70% EtOH until workup. The alga (70.5 g dry wt) was extracted four times with CH_2Cl_2-MeOH (2:1) to give a crude organic extract (4.35 g). A portion of the extract (4.30 g) was fractionated on silica gel by NPVLC to give nine fractions using a stepwise gradient of hexanes-EtOAc and EtOAc-MeOH. Fraction B, eluting with 90% hexanes, was further chromatographed on preparative normal phase HPLC (Phenomenex Maxsil 10 Silica 10 µm, 500 x 10.0 mm, using gradient system as mobile phase starting with 100% hexanes to 90% hexanes) and yielded ten fractions B1 to B10. Fraction B8 was subjected to analytical normal phase HPLC purification (Phenomenex Luna silica 10 µm, 250 x 4.60 mm, using gradient system as mobile phase starting with 100% hexanes to 90% hexanes) to yield 4 mg of **1** and 0.3 mg of the known compound **2**.

HDAC Enzyme Assay. HDAC is partially purified from H1299, human non-small cell lung carcinoma cells. Cells were grown to 70-80% confluence in RPMI media in the presence of 10% fetal calf serum, harvested and lysed using sonication. The lysate was centrifuged at 23,420xg for 10-15 min, the supernatant applied to Hiload 26/10 High Performance Q-sepharose column (Amersham Pharmacia Biotech), previously equilibrated with a Buffer A (20mM Tris pH 8, 0.1 mM EDTA, 10mM NH_4Cl, 1mM β-mercaptoethanol, 5% glycerol, 2 µg/mL leupeptin, and 400 mM phenyl methyl sulfonyl fluoride (PMSF)) and eluted with a linear gradient of 0-500 mM NaCl in Buffer A at a flow rate of 2.5 mL/min.

4 mL fractions were collected and each was titrated for HDAC activity using a modification of the published procedure[23] to determine the optimal amount needed to obtain a signal to noise ratio of at least 5:1. The substrate used is a peptide of amino acid sequence SGRGKGGKGLGKGGAKRHRKVLRD, corresponding to the twenty-four *N*-terminal amino acids of human histone H4, biotinylated at the N-terminus and peracetylated with ^{3}H-acetate at each lysine residue. The substrate is diluted in 10 µL of Buffer B (100 nM Tris pH 8.0, 2 mM EDTA), added to the enzyme mixture and incubated at 37 ºC for 1.5 h. The reaction is stopped by addition of 20 µL of 0.5N HCl/0.08 M HOAc, extracted with TBME and an aliquot of the organic layer is added to Opti-Phase Supermix liquid scintillation cocktail (Wallac). The mixture was read on a 1450 Microbeta trilux liquid scintillation and luminescence counter (Wallac) with a color/chemical quench and dpm correction and the data corrected for background luminescence.

Protozoal inhibition bioassays.

The anti-plasmodial activity was evaluated using a fluorometric method[11] based on the detection of parasite DNA with the fluorochrome PicoGreen® using a chloroquine-resistant strain (Indochina W2) of *Plasmodium falciparum*.[12] The parasites were maintained *in vitro* by a modification of the method of Trager and Jensen.[13]

Compound 1: colorless oil; $[\alpha]^{25}_{D}$ -87° (*c* 2, $CHCl_3$); UV ($CHCl_3$) λ_{max} 208 nm (ε 6000); IR ν_{max} (neat) 3500, 2900, 1731, 1640, 1370, 1240 and 911 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Table 5.1.; LR CIMS fragment ions at *m/z* 346.3 $[M]^+$, 329.3 $[M\text{-}OH]^+$, 285.2 $[M\text{-}Ac\text{-}H_2O]^+$, 269.2 $[M\text{-}OAc\text{-}H_2O]$; HR CIMS *m/z* $[M]^+$ 346.25074 (calcd for $C_{22}H_{34}O_3$, 346.25080).

Deacetylation of 1 to amijiol. 3.5 mg of **1** in solution with $THF/H_2O/CH_3OH$ (3/1/1, 1 mL) was treated with 1 N aqueous LiOH (1 mL) and stirred at 25 º C for 72 h. Extracted with $CHCl_3$, dried down on rotavapor.

$[\alpha]^{25}_{D}$ -106° (*c* 0.4, $CHCl_3$).

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CHAPTER SIX

MITSOAMIDE, A CYTOTOXIC LINEAR LIPOPEPTIDE FROM THE MADAGASCAR MARINE GYANOBACTERIUM *GEITLERINEMA* SP.

ABSTRACT

A new cytotoxic linear peptide (IC_{50} 0.46 μM to NCI-H460 human lung tumor cells) was isolated from the marine cyanobacterium *Geitlerinema* sp. The planar structure of mitsoamide (**1**) was deduced by 1D and 2D NMR experiments in combination with MS analyses. The structure of **1** contains an unusual polyketide unit (3,7-dimethoxy-5-methyl-nonanedioic acid) (DMNA), incorporates a homolysine residue and possesses a highly unusual aminal moiety. The configurations of the relatively common amino acids present in **1** (Ala, Ile, *N,O*-Me-Ile, Phe, Val) were determined by chiral HPLC analysis of the acid hydrolysate.

INTRODUCTION

A number of the most therapeutically interesting natural products are mixed polyketide/nonribosomal peptides, such as bleomycin, the epothilones, FK506 and rapamycin.[1-3] The biosynthesis of these clinically important natural products is achieved through the action of two related enzyme families: the nonribosomal peptide synthetases (NRPSs)[1] and polyketide synthases (PKSs).[1,2] These large, multimodular proteins are subdivided into discrete domains, each possessing a distinct catalytic function that contributes to the biosynthetic assembly line process.

Cyanobacteria are prolific producers of secondary metabolites of a peptidic nature that are commonly modified by the incorporation of polyketide portions to produce mixed peptide-polyketide hybrids.[4] These arise from the interdigitated functioning of type I nonribosomal peptide synthetases (NRPS)[5] and type I polyketide synthases (PKS)[6] as revealed by the growing number of sequenced biosynthetic gene clusters from this biological source which includes curacin A,[7] barbamide,[8] jamaicamide,[9] lyngbyatoxin,[10] microcystin[11] and cryptophycin.[12] In general, the metabolites of cyanobacteria constitute a highly diverse set of structures in which many novel biosynthetic transformations are revealed, including unusual halogenations, unsaturations, cyclizations and addition of pendant carbon atoms to polyketide chains. As a result, many cyanobacterial natural products have unique and potent biological properties, including cancer cell toxicity through interaction with tubulin or actin,[13,14] neurotoxicity via interference with normal ion channel function,[15] and antifungal or other antimicrobial properties.[16,17]

As part of our effort to discovery and characterize structurally-novel and biologically-interesting substances from marine cyanobacteria,[18-20] we initiated a detailed examination of a Malagasy marine cyanobacterium, *Geitlerinema* sp., which showed potent brine shrimp toxicity in its crude organic extract (LC_{50} 0.01 µg/mL). Using this assay to guide the isolation of bioactive constituents, we obtained an amazing array of bioactive molecules, including a small quantity of curacin A,[21] a curacin A related substance under continuing investigation, several new swinholide related metabolites including ankarharolide A,[14] and from quite a

polar fraction, a new linear lipopeptide which we have named mitsoamide (**1**). This paper describes our isolation and structure elucidation of this latter metabolite which possesses several novel structural features including a homolysine residue, a 3,7-dimethoxy-5-methyl-nonadioic acid residue, and a piperidine aminal, and was strongly cytotoxic to the H-460 human lung cancer cell line (IC_{50} 460 nM).

RESULTS AND DISCUSSION

The marine cyanobacterium *Geitlerinema* sp. was collected from Mitso-Ankaraha Island near NosyBe, Madagascar, extracted with CH_2Cl_2/MeOH (2:1) and fractionated by Si VLC. The resulting fractions were tested for brine shrimp toxicity assay and revealed that the 50:50 EtOAc/MeOH eluting material was strongly active (63% toxicity 1 ppm). This material was further purified on analytical RP HPLC to give a small quantity of mitsoamide (**1**, 3 mg, 0.3% of extract; LC_{50} 450 nM to the brine shrimp).

Mitsoamide (**1**) was isolated as yellow oil with a molecular mass of 1093.6835 by HR TOF MS ES+ that indicated a formula of $C_{55}H_{96}ClN_9O_{11}$. This was consistent with fragment ion clusters at *m/z* 1093.7/1095.7 observed in both FABMS and MALDI TOF MS experiments which confirmed the presence of one chlorine atom. 1D-NMR spectra exhibited resonances typical for a peptide natural product, including several exchangeable NH signals between 6 - 8 ppm, a number of resonances in the α-proton region, and a packet of carbonyl carbons around δ170 (Table 6.1.). Expanding outward from these NH and α-protons, standard analysis by ^1H-^1H COSY, multiplicity edited HSQC and HMBC NMR, in combination with literature values, quickly revealed the side chains corresponding to alanine, valine, two isoleucine residues, and phenylalanine. However, one of the isoleucine residues showed no α-proton to NH correlation, and by HMBC, was shown to be *N*-methyl substituted (δ_H 2.99, δ_C 31.2). Interestingly, this latter residue was also shown by HMBC to possess a methyl ester (δ_H 3.64, δ_C 52.07), and thus *N*,*O*-dimethyl isoleucine formed the carboxy terminus of mitsoamide (**1**).

Mitsoamide (**1**) incorporated several unique structural features not previously or rarely observed in marine cyanobacterial natural products. A carbon at δ 47.31 with protons at δ 3.17 and δ 3.65 was characteristic of a methylene group adjacent to nitrogen in a cyclic or asymmetric system. Long-range ^1H-^{13}C HMBC (various mixing times) and ^1H-^1H COSY experiments showed a sequential series of four methylene groups (see Table 6.1.), which then connected to a methine at δ 4.27 (δ 59.38). This latter signal was also coupled to an NH proton at δ 7.19. To unambiguously confirm this unusual spin system, a series of selective 1D TOCSY experiments were performed. Irradiation of the proton at H-2α (δ 3.65)

showed couplings to H-2β (δ 3.17), H-3α (δ1.90), H-3β (δ1.76), H-4α (δ1.20), H-4β (δ1.21), H-5α (δ1.86), H-5β (δ2.07), and H-6 (δ4.27). However, no coupling from the distal exchangeable NH proton was observed. Irradiation of the proton at H-6 (δ4.27) displayed similar couplings as seen above but with additional coupling to the NH proton at δ 7.19. Hence, this methine was attached to both the nitrogen between it and the CH_2-2 methylene and this latter amide-type NH, defining an aminal functionality (Figure 6.2.). Additional confirmation of this unusual structural feature was gained by comparing with various reference compounds as well with calculated ^{1}H and ^{13}C shifts for mitsoamide.[22]

A second uncommon structural feature in mitsoamide (**1**) was the presence of the relatively unusual amino acid homolysine. Initially, routine ^{1}H-^{13}C HMBC and ^{1}H-^{1}H COSY experiments were used to define a spin system that began with a deshielded methine at δ4.47 and extended through five sequential methylene groups. Again, application of selective 1D TOCSY was very powerful in confirming this portion of the molecule. Irradiation of α-proton H-8 (δ4.47) revealed couplings to an exchangeable NH proton (δ7.25), H-9α (δ1.87), H-9β (δ1.62), H_2-10 (δ1.36), H_2-11 (δ1.40), H-12α (δ1.46), H-12β (δ1.31), and H-13α (δ3.13). Irradiation of H-13α (δ3.13) displayed similar couplings as seen above but with the exclusion of the distal NH (δ7.25) and addition of the proximate NH (δ6.70). Irradiation of H-11(δ1.40), showed similar couplings as seen above with the inclusion of both exchangeable NH protons (δ7.25 and δ6.70). In complementary fashion, irradiation of H-12α (δ1.46) displayed all of the same couplings observed from irradiation of H-11 with the exception of the δ7.25 NH proton. This composite of results established a 2,7-diamino substitution and thus identified this residue as homolysine (Figure 6.3.). Homolysine, while rare in natural products, has been previously characterized in several cyanobacterial peptides, including microcystin LR and nodularin-Har [23,24]

A final unusual section of mitsoamide (**1**) was established by ^{1}H-^{1}H COSY, long-range ^{13}C-^{1}H correlation experiments (HMBC) with different mixing times and selective 1D TOCSY. By ^{13}C NMR, two nearly isochronous methoxy groups (δ 56.26, 56.38), two oxygen-bearing methine (δ 78.40, 78.41), four methylenes (δ 35.89, 36.18, 41.16, 41.44), one methyl (δ18.54) and one up field methine (δ 19.0) remained in **1**, and hence, was likely a polyketide fragment. HMBC

correlations revealed that the methyl group (δ18.54) was attached to the high field methine at δ19.0 which in turn was sequentially located between two methylenes (δ 35.89, 36.18) and the two methoxy methines. These mid-field methines were in turn adjacent to two similarly shifted methylenes, δ2.23/2.35 and δ2.33/2.43 with attached carbons at δ41.16 and δ41.44. From chemical shift reasoning, these latter methylenes were likely adjacent to carbonyl functionalities; HMBC correlations from δ2.23/2.35 to δ 171.70 and δ2.33/2.43 to δ171.42 confirmed this deduction. By 1D TOCSY, irradiation of the H-21α proton (δ2.33) resulted in couplings to protons at H-21B, H-20, H_2-19, H-18 and H_3-18-Me. Thus, the final remaining residue in mitsoamide A (**1**) was the essentially symmetric polyketide 3,7-dimethoxy-5-methyl-nonanedioic acid) (DMNA, Figure 6.1.).

The above spectroscopic reasoning defined eight residues in mitsoamide: alanine (Ala), valine (Val), isoleucine (Ile), phenylalanine (Phe), N,O-dimethylisoleucine (diMeILe), piperidin-2-amine (PPA), homolysine (HomoLys), and the poliketide (3,7-dimethoxy-5-methyl-nonanedioic acid) (DMNA). By comparing the carbon, proton, nitrogen, and oxygen count from these eight residues, $C_{55}H_{94}N_9O_{11}$, to the molecular formula established by HRMS, $C_{55}H_{96}ClN_9O_{11}$, the remaining unassigned atoms were two protons and one chlorine. Hence, on the basis of the observed chemical shifts for **1**, mitsoamide was isolated as the hydrochloride salt, likely present at the basic aminal nitrogen. Indeed, a similar hydrochloride salt was observed for the aminal portion of siastatin B.[22]

Long-range ^{1}H-^{13}C correlation experiments (HMBC) with different mixing times were used in combination with ESI-MS/MS fragmentation patterns to define the sequence of residues in mitsoamide (**1**). Connection between the PPA and homolysine residues was made by observing long range ^{1}H-^{13}C correlations between H-6, NH-6 and H-8 with the C-7 carbonyl at δ172.02. Similarly, H-15α, H-13α, and NH-13 all showed correlations to the C-14 carbonyl (δ171.70), thus connecting the homolysine and DMNA units. These connectivities were reinforced by ESI-MS/MS fragment ions at *m/z* 385 for HomoLys-DMNA-NH and *m/z* 484 for PPA-HomoLys-DMNA-NH. Additional fragment ion masses at *m/z* 942, 975, 724 and 611, resulting from consecutive Phe, Ala and Ile cleavages,

indicated an *N,O*-diMeIle-Phe-Ala-Ile sequence at the carboxy terminus. In turn, ions at *m/z* 212 $(Val\text{-}Ile)^+$, 298 $(Val\text{-}Ile\text{-}Ala\text{-}NH)^+$, and 459 $(Val\text{-}Ile\text{-}Ala\text{-}Phe\text{-}NMe)^+$ identified that Val was attached to the Ile residue to extend this sequence to *N,O*-diMeIle-Phe-Ala-Ile-Val. The Phe-Ala-Ile-Val sequence was confirmed by sequential HMBC correlations from each NH to the preceding carbonyl (Table 6.1.). Combination of these two sequences, PPA-HomoLys-DMNA plus Val-Ile-Ala-Phe-N,O-diMeIle, along with the hydrochloride salt feature of the PPA residue, completed the planar structure of mitsoamide **1**.

The absolute configurations of the ordinary amino acids in **1** were established by chiral HPLC in comparison with authentic standards following hydrolysis with 6 N HCl. All of the amino acids were shown to possess the L-configuration except phenylalanine which was found to have the D-configuration (Figure 6.25.). Assignment of the remaining five stereocenters in **1** (C-6, C8, C-16, C-18, C20) was precluded by decomposition of some of these constituents during acid hydrolysis of **1** (e.g. the PPA residue), lack of the appropriate reference standards (e.g. the DMNA unit), and the relatively small quantity of mitsoamide isolated.

Mitsoamide (**1**) was evaluated for cytotoxicity to the NCI-H460 human lung tumor cell line, and showed an LC_{50} of 460 nM, very similar to the potency observed in the brine shrimp toxicity assay. Biosynthetically, mitsoamide is consistent with other cyanobacterial natural products in that it shows a mixed NRPS and PKS origin; however, there are several unique structural features never or rarely seen previously. Mitsoamide biogenesis likely begins with lysine, which is decarboxylated and cyclized to the aminal at some stage in the process. Following amide linkage with the homolysine residue, it is the zeta amino function of this latter residue that forms an amide bond with an essentially symmetric polyketide which has the unusual feature of possessing carboxylic acids at both termini. While it is not clear how this occurs, the pattern of oxidation with methoxy groups at C-16 and C-20 suggests that C-18 is also derives from C-1 of acetate, and hence, the methyl group attached at this site likely derives from C-2 of acetate as described for curacin A[7] and jamaicamide A.[9] The mitsoamide pathway then reverts to an NRPS section which sequentially forms amide bonds with valine, isoleucine, alanine and phenylalanine. An epimerase domain is likely present in the latter module, converting L-Phe to D-Phe during the biosynthetic

assembly process. The pathway is predicted to finish with an NRPS module coding for a second isoleucine residue, this one likely containing an N-methyl transferase motif. Cleavage of mitsoamide from the NRPS-PKS enzyme cluster should involve a thioesterase domain, and methylation of the terminal carboxylate may involve either a post-assembly modification, or may occur as part of the thioesterase reaction and entail a biochemical equivalent of methanol.

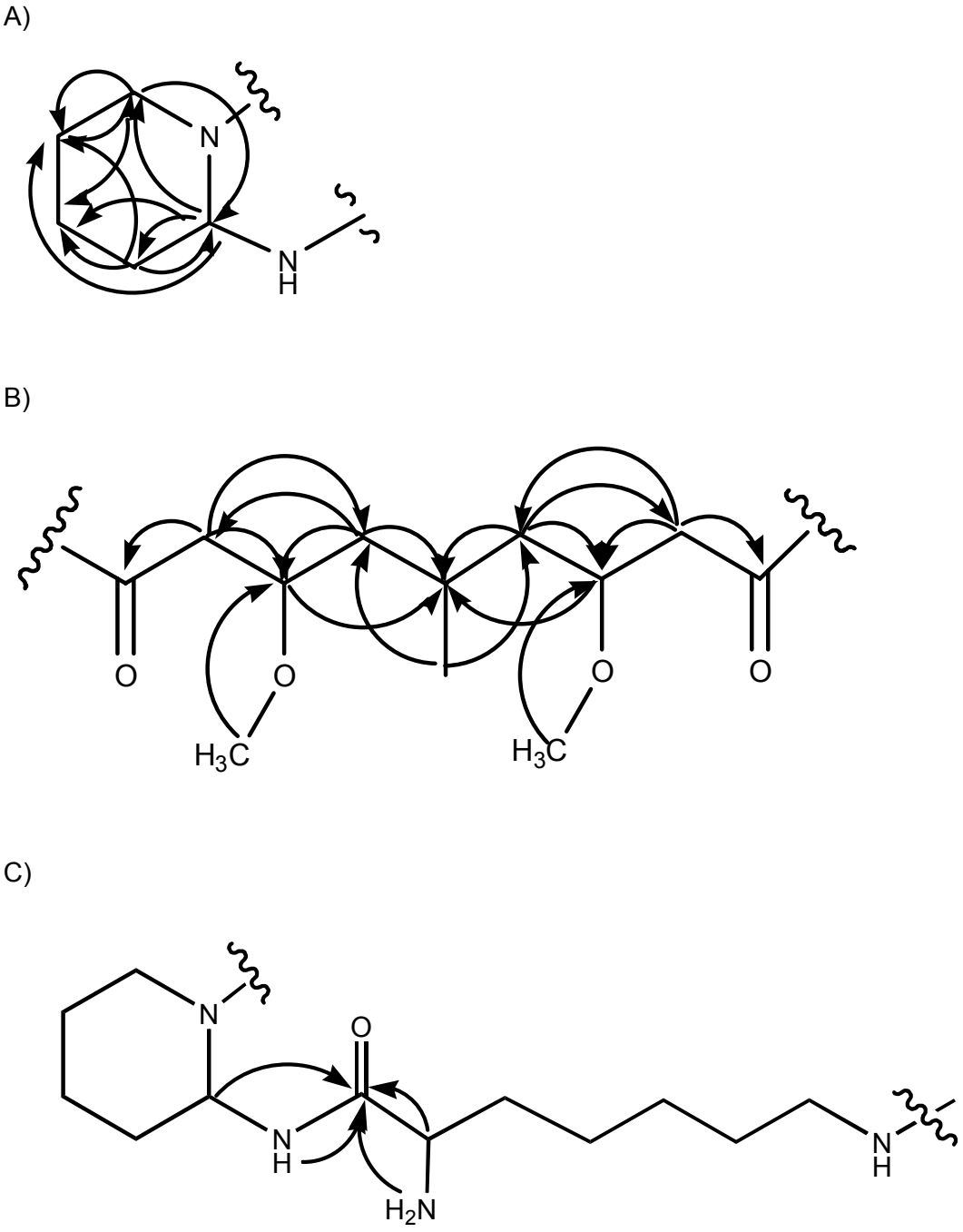
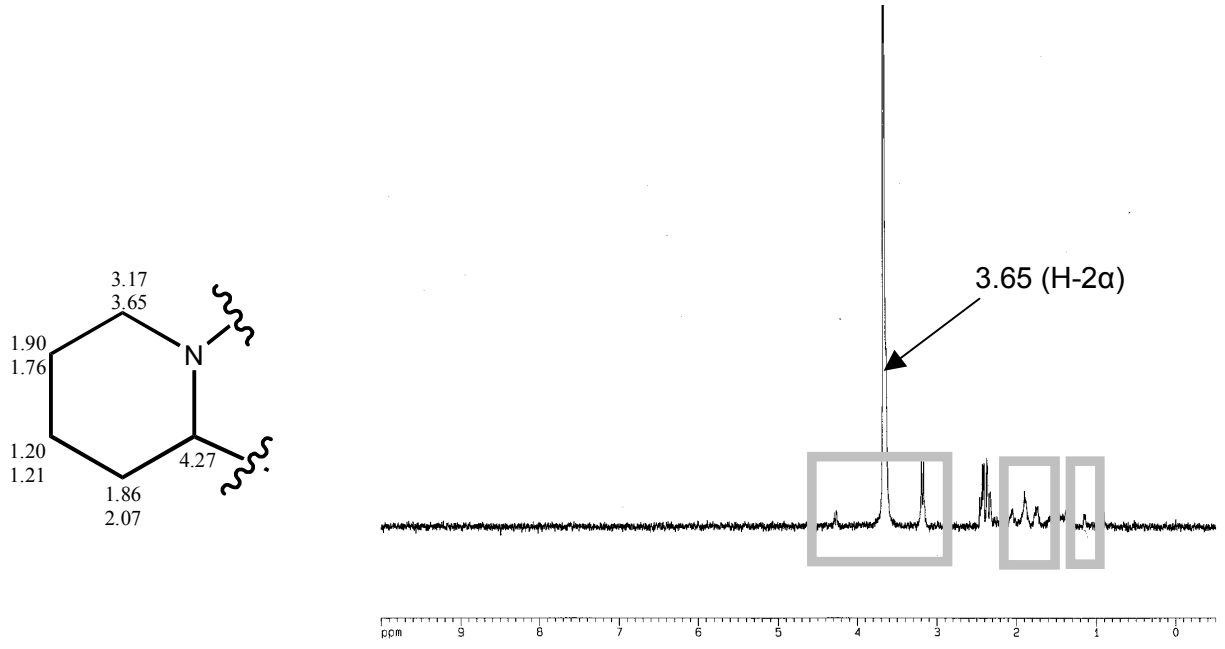
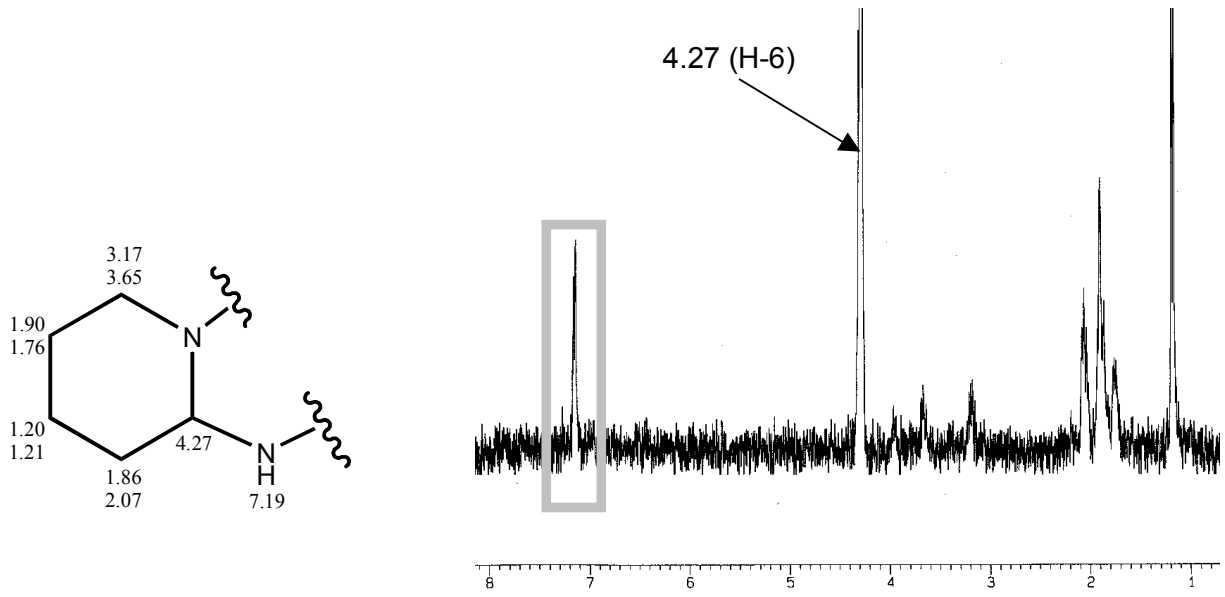


Figure 6.1. Selected HMBC correlations of mitsoamide (**1**); A) aminal (piperidin-2-amine, PPA); B) polyketide (3,7-dimethoxy-5-methyl-nonanedioic acid, DMNA); C) connection of PPA and homolysine residues in **1**

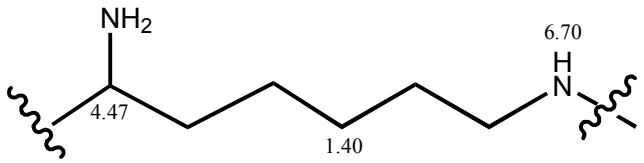


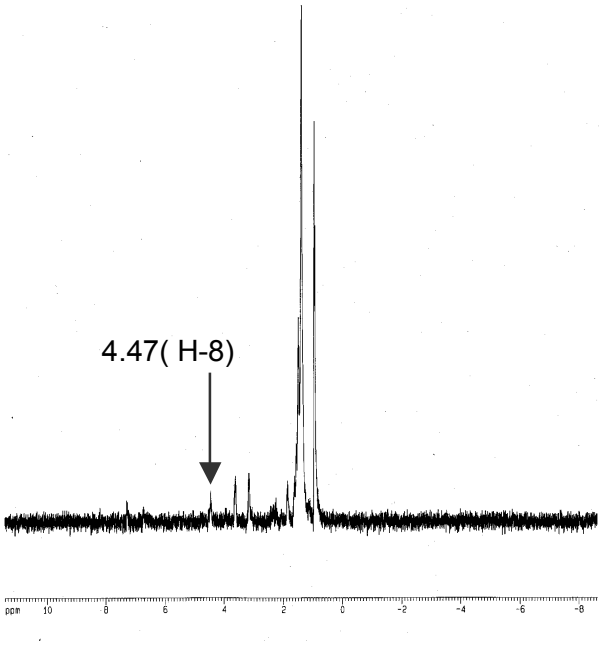
A) Irradiation of H-2α (δ 3.65)



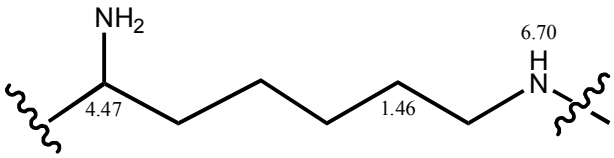
B) Irradiation of H-6 (δ4.27)

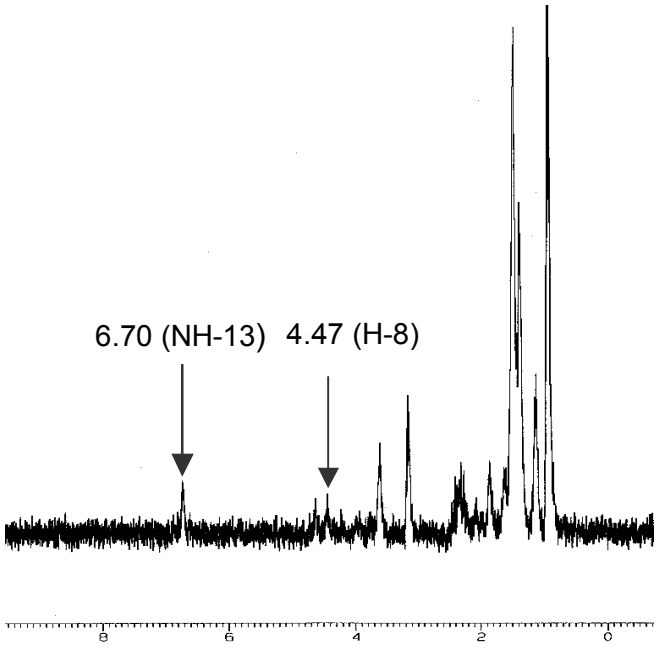
Figure 6.2. Application of 1D TOCSY pulse sequence on the aminal portion of **1**





A) Irradiation of H-11(δ1.40)





B)Irradiation of H-12α (δ1.46)

Figure 6.3. Application of 1D TOCSY pulse sequence on the homolysine residue of **1**

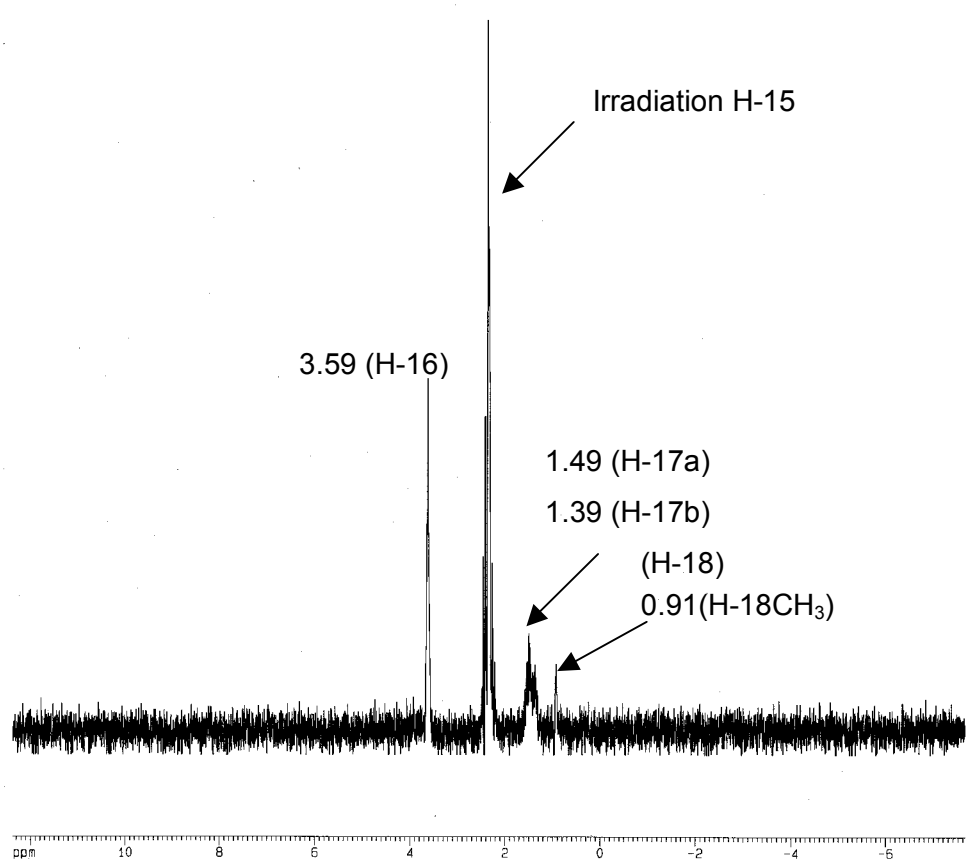


Figure 6.4. 400 MHz 1D TOCSY irradiation of δ 2.35 (H-15) of DMNA in mitsoamide (1) in CD_3CN.

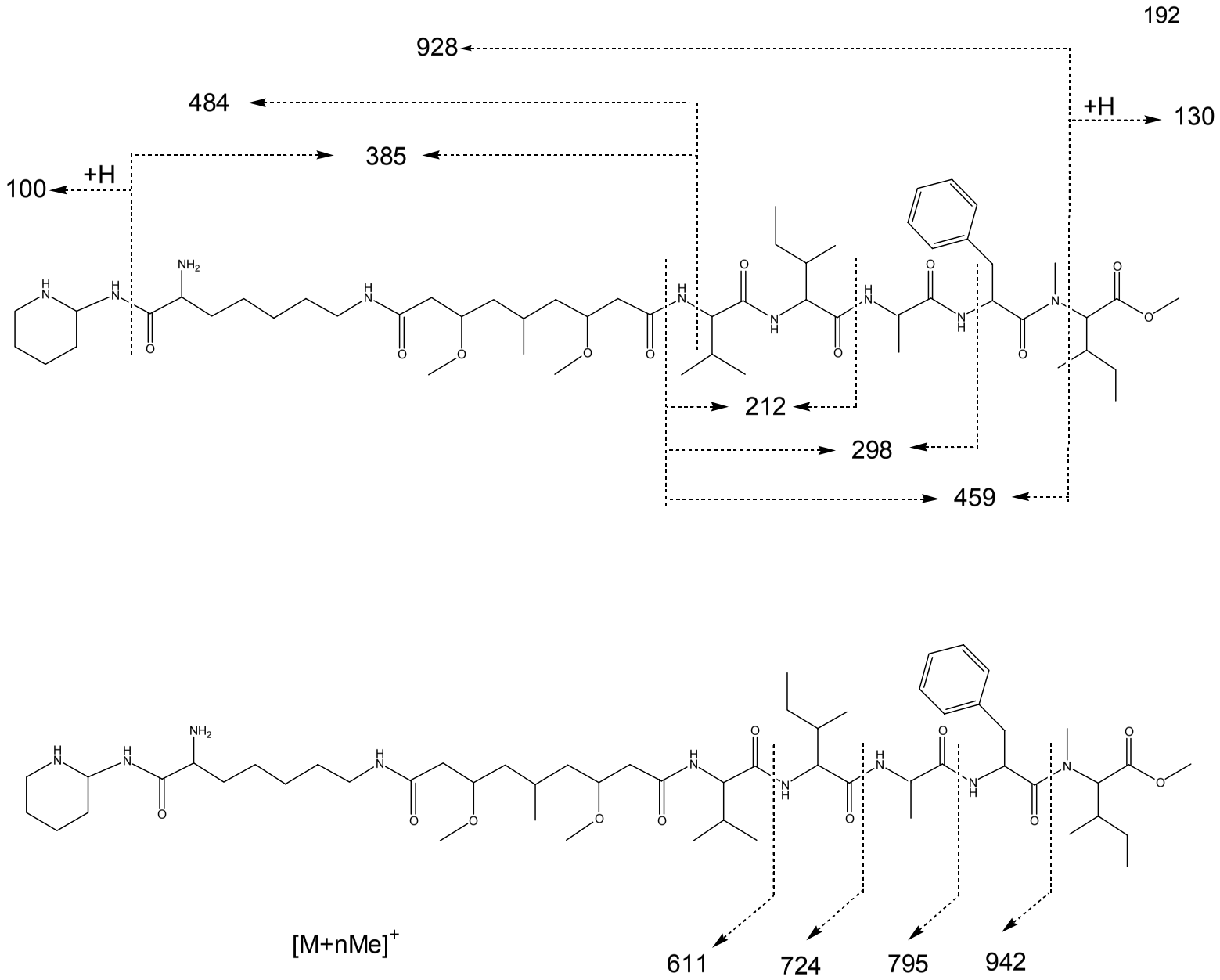


Figure 6.5. Key fragments from TOF MS/MS ES+ and FAB MS

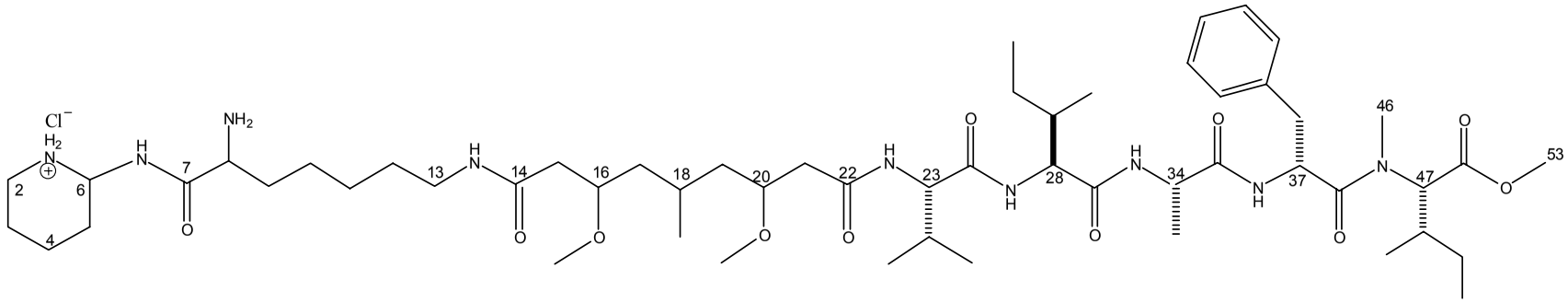


Figure 6.6. Structure of mitsoamide **1**

Table 6.1. NMR Spectral Data for Mitsoamide (1) in CD_3CN

Unit	Position	^{13}C	^{1}H (*J* in Hz)	HMBC[a]
Aminal	2	47.31	3.67, m	3,4,6
			3.15, m	
	3	24.96	1.90, m	2
			1.76, m	
	4	18.58	1.21, m	2
			1.20, m	
	5	29.41	2.07, m	3,4,6
			1.86, m	
	6	59.38	4.27, dd (4.1,3.7)	2,3,4,5
	6-NH		7.19, d (4.1)	
HomoLys	7	172.02		
	8	53.31	4.47, ddd (4.3,4.5,4.3)	7,9,10
	8-NH_2		7.25, d (7.4)	7
	9	32.10	1.87, m	7
			1.62, m	
	10	23.04	1.36, m	11
	11	18.60	1.40, m	12
	12	29.17	1.46, m	13
			1.31, m	
	13	38.96	3.13, m	12
			3.01, m	
	13-NH		6.70, dd (6.1,4.4)	14
DMNA	14	171.70		
	15	41.16	2.35, m	14,16,17
			2.23, m	
	16	78.40	3.59, m	18
	16-OMe	56.26	3.30, s	16
	17	35.89	1.49, m	15,16,18
			1.39, m	
	18	19.0	1.39, m	17,19
	18-Me	18.54	0.91, d (3.4)	17,18,19
	19	36.18	1.49, m	18,20,21
			1.39, m	
	20	78.41	3.59, m	18
	20-OMe	56.38	3.31, s	20
	21	41.44	2.43, m	19,20,22
			2.33, m	
	22	171.42		

[a] Proton showing HMBC correlation to indicated carbon.

Table 6.1. (Continued)

Unit	Position	^{13}C	^{1}H (*J* in Hz)	HMBC[a]
Val	22-NH		7.12, d (7.7)	22
	23	54.80	4.61, dd (8,7.7)	24,25,26,27
	24	32.04	2.07, m	23,25,26
	25	13.92	0.82, d (6.3)	23,24
	26	17.84	0.92, d (7.9)	23,24
	27	169.95		
Ile	27-NH		6.86, d (7.4)	27
	28	58.03	4.14, dd (7.4,7.1)	29,30,31,33
	29	36.87	1.86, m	28,30,31
	30	15.58	0.89, d (7.1)	28,29,31
	31	24.74	1.36	29,32
			1.06	
	32	10.8	0.94, d (3.4)	29,31
	33	171.38		
Ala	33-NH		7.14, d (6.8)	33
	34	49.42	4.29, d (6.8,6.2)	35,36
	35	18.26	1.16, d (6.2)	34,36
	36	172.16		
Phe	36-NH		7.44, d (7.7)	36
	37	52.5	4.90, dd (7.7,7.4)	38,39,45
	38	38.44	3.01	37,39,40/41
			2.92	
	39	137		
	40/41	130	7.25	38,39,42/43
	42/43	128.6	7.29	39,40/41,44
	44	127.1	7.22	42/43
	45	173.89		
N-Me-Ile-Me	46 (NMe)	31.20	2.99, s	47
	47	61.74	4.63, d (10.7)	48,52
	48	31.2	1.60, m	47,49,50
	49	14.72	0.93, d (8)	48
	50	26.46	1.50	48,51
			1.10	
	51	10.8	0.96, d (10)	48,50
	52	170.49		
	53 (OMe)	52.07	3.64, s	52

[a] Proton showing HMBC correlation to indicated carbon.

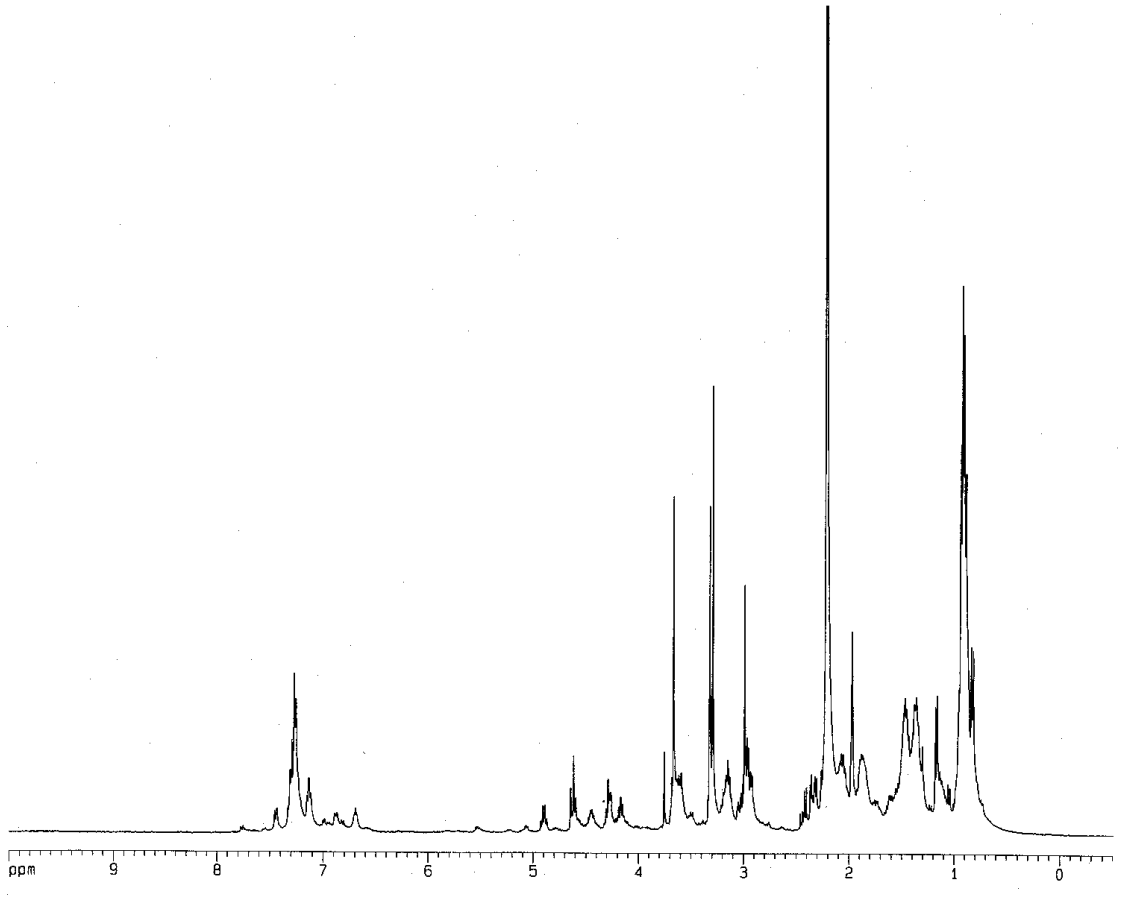


Figure 6.7. 400 MHz ^{1}H NMR spectrum of mitsoamide (**1**) in CD_3CN.

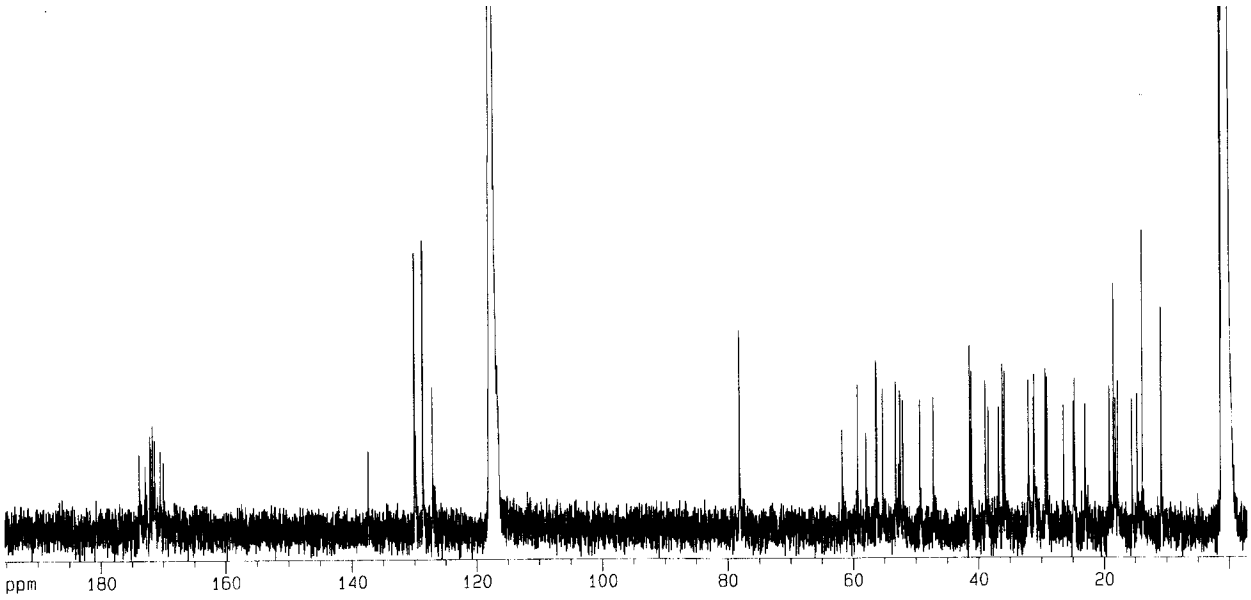


Figure 6.8. 100 MHz ^{13}C NMR spectrum of mitsoamide (**1**) in CD_3CN.

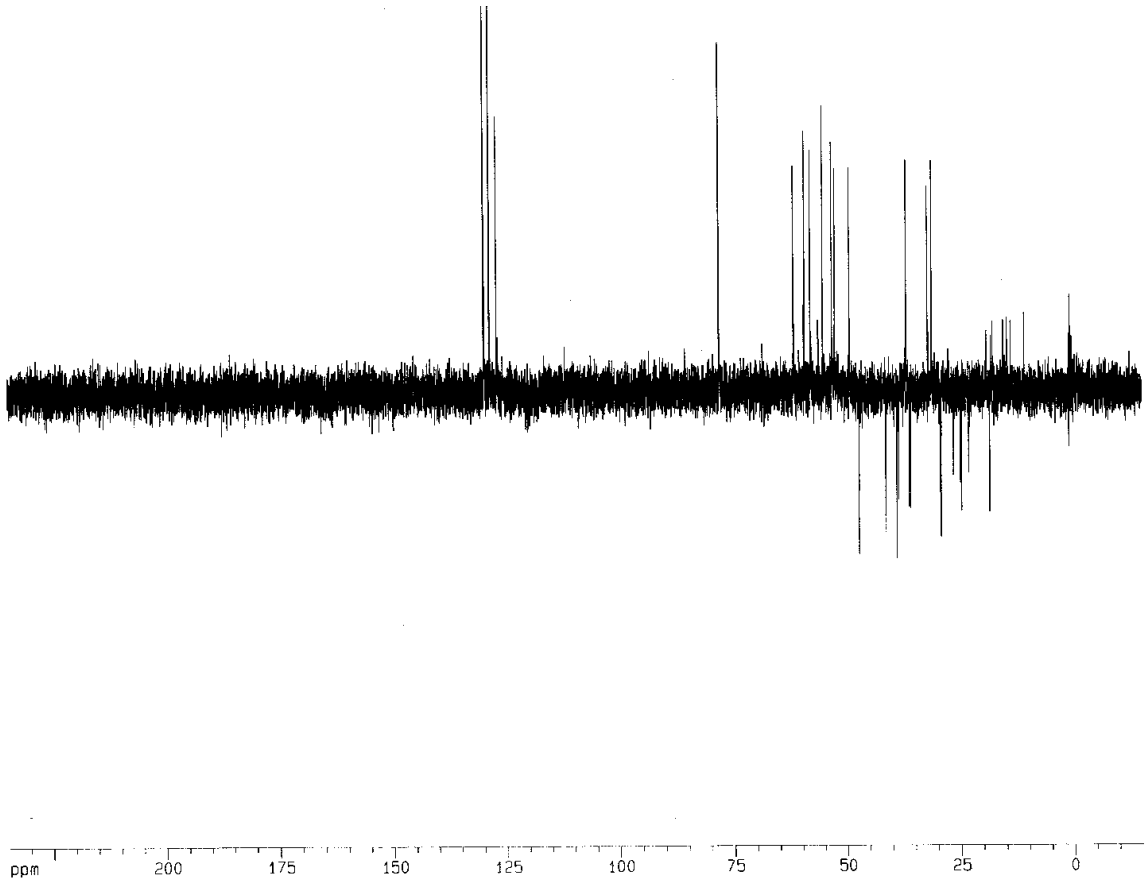


Figure 6.9. 100 MHz DEPT NMR spectrum of mitsoamide (**1**) in CD_3CN.

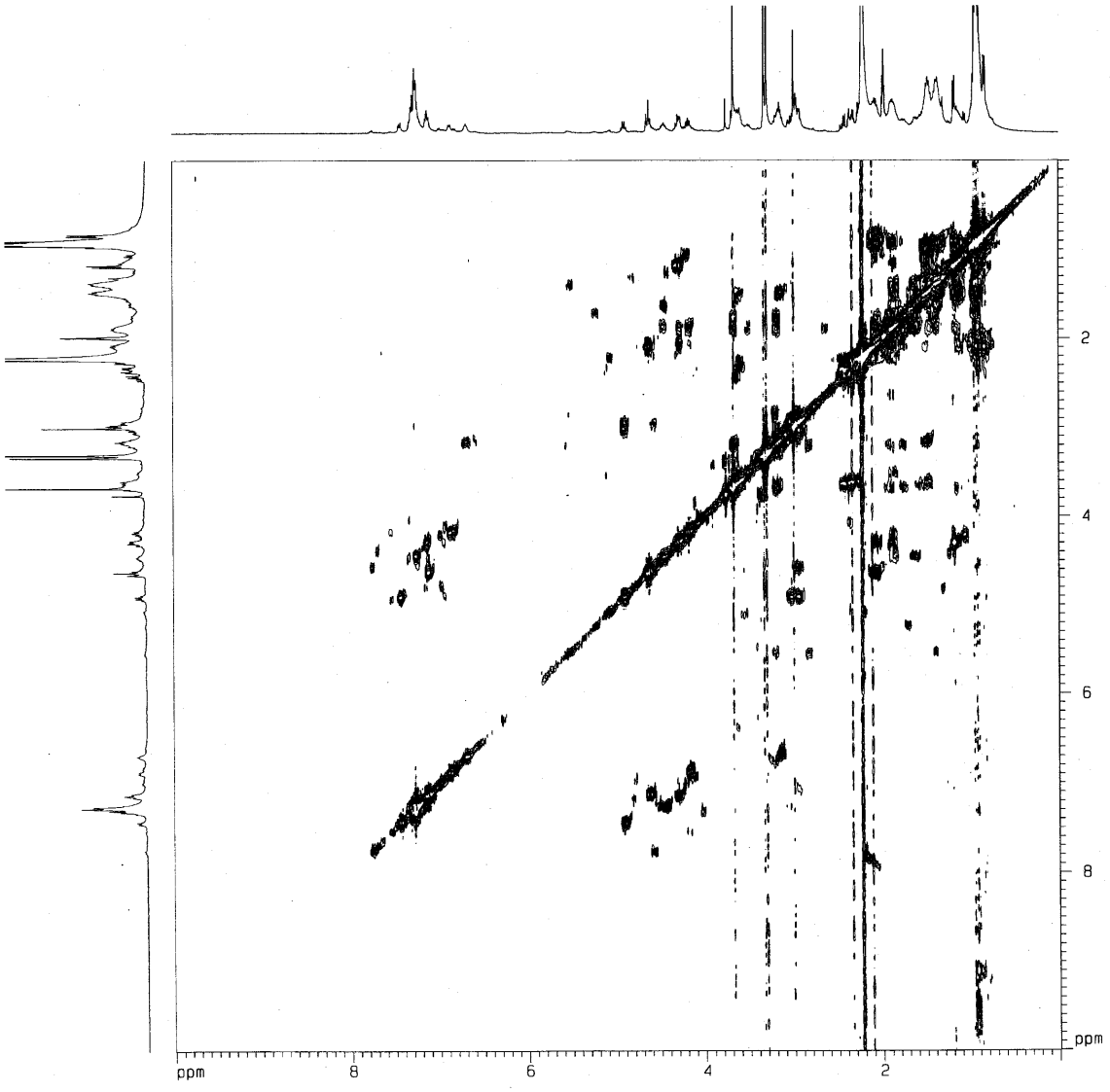


Figure 6.10. 400 MHz ^{1}H-^{1}H COSY spectrum of mitsoamide (**1**) in CD_3CN.

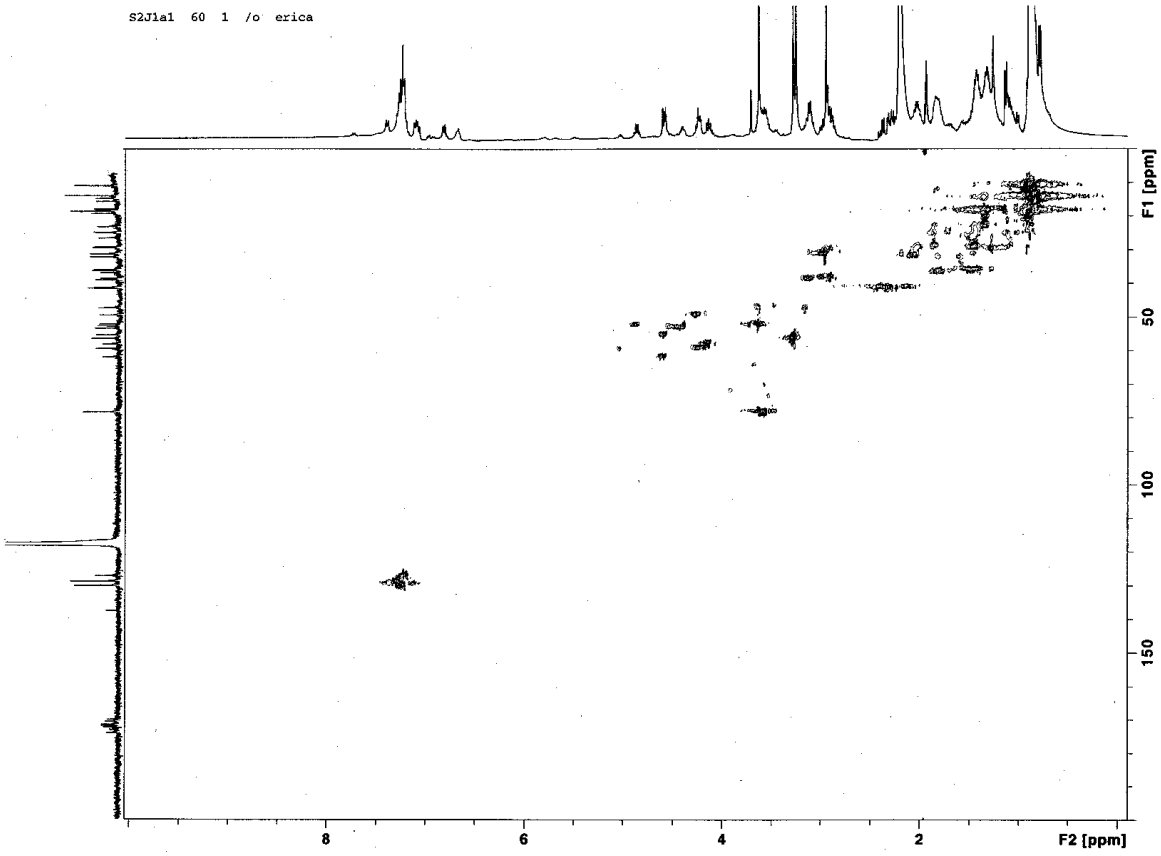


Figure 6.11. 400 MHz HSQC spectrum of mitsoamide (**1**) in CD_3CN.

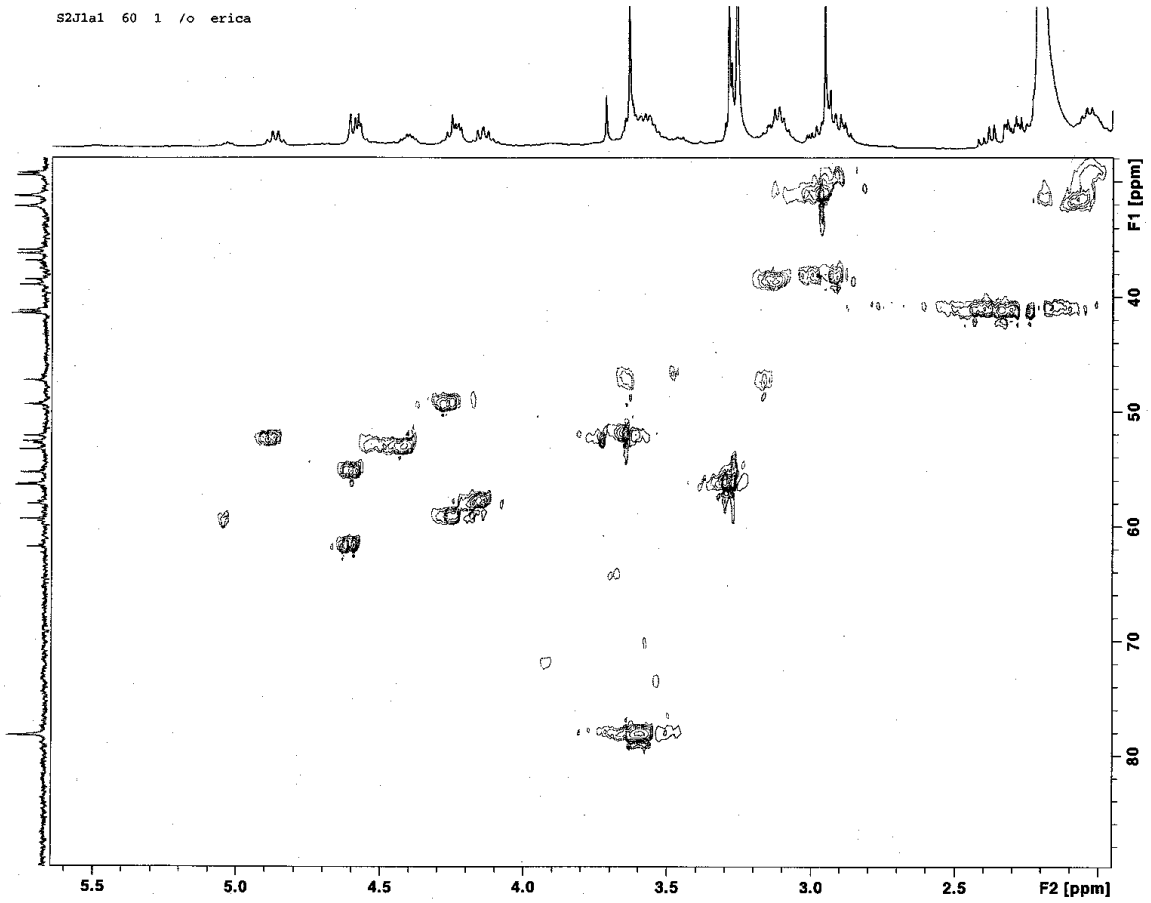


Figure 6.12. 400 MHz HSQC spectrum of mitsoamide (**1**) in CD_3CN (expansion plots ^{1}H δ2.8-5.5 ^{13}C δ30-80.)

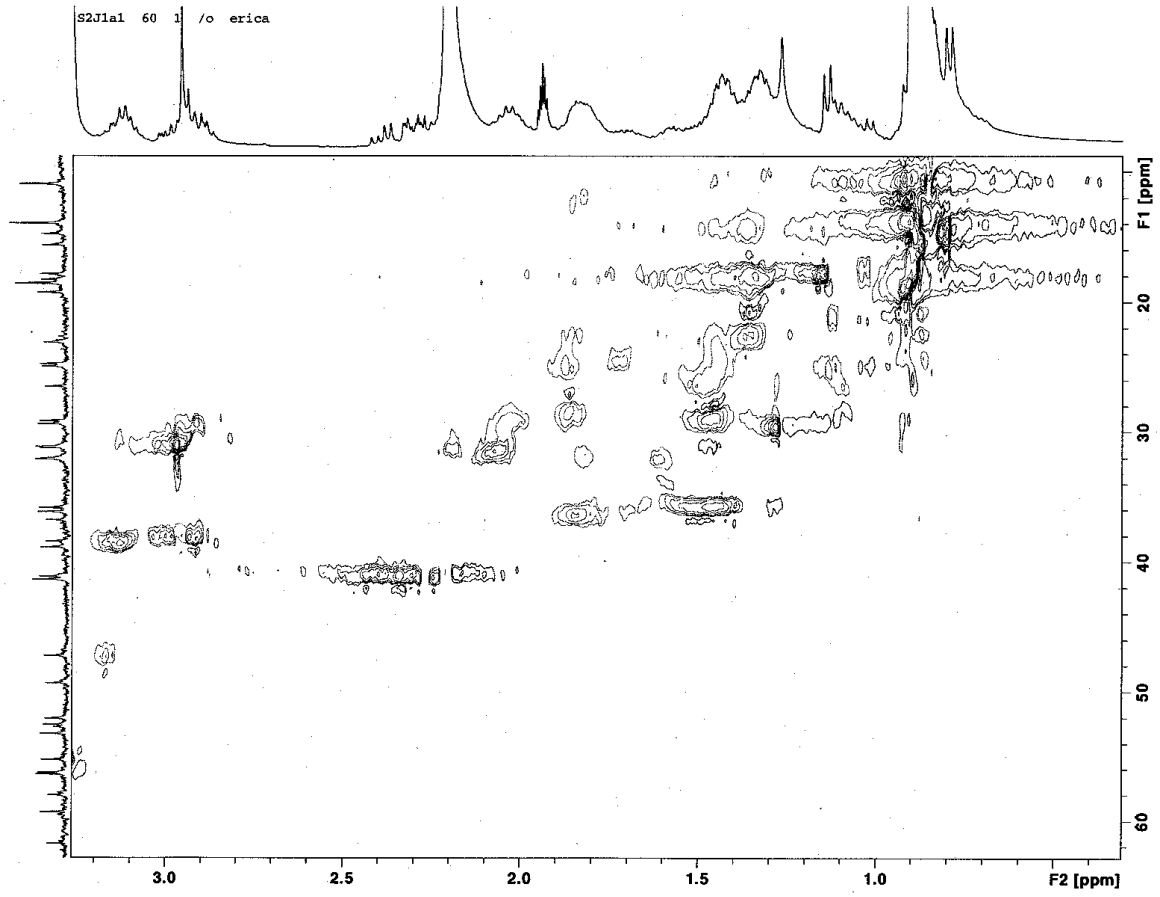


Figure 6.13. 400 MHz HSQC spectrum of mitsoamide (**1**) in CD_3CN (expansion plots ^{1}H δ 0.5-2.8 ^{13}C δ0-50.)

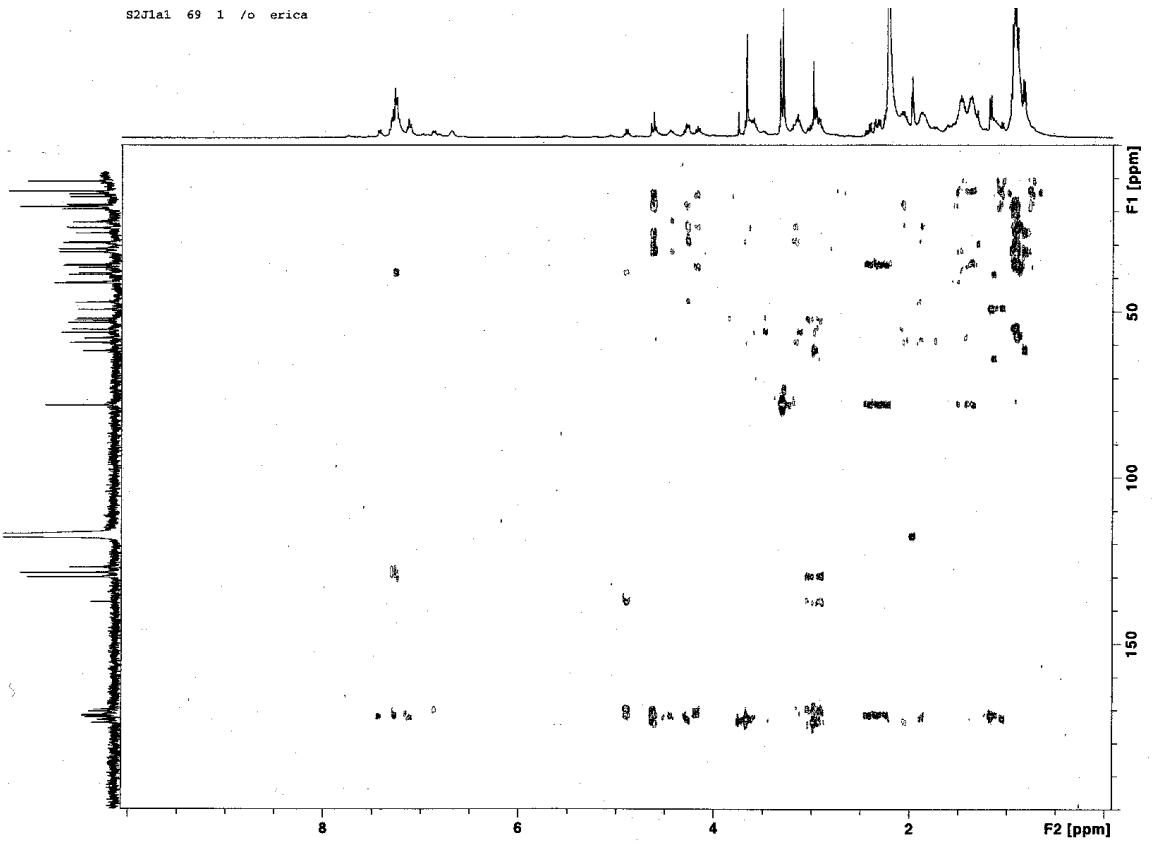


Figure 6.14. 400 MHz HMBC spectrum (optimized for *J* = 4Hz) of mitsoamide (**1**) in CD_3CN.

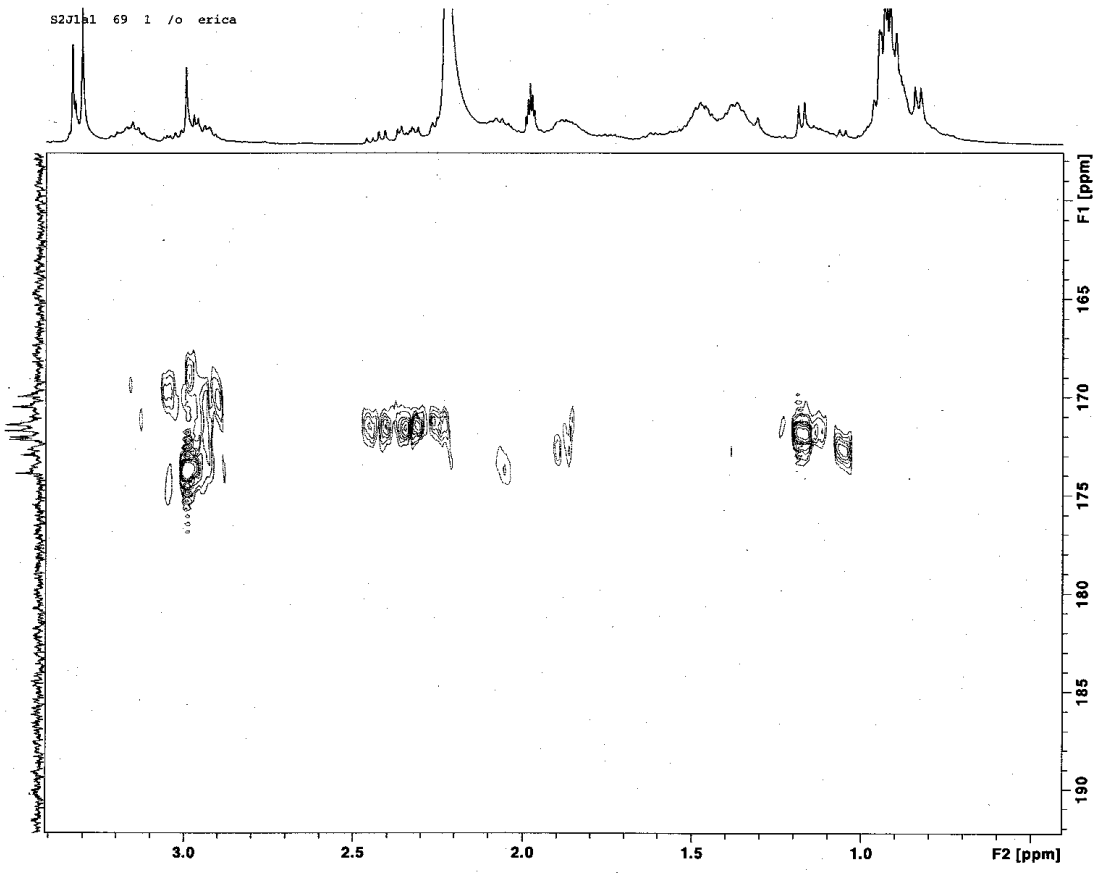


Figure 6.15. 400 MHz HMBC spectrum (optimized for *J* = 4Hz) of mitsoamide (**1**) in CD_3CN expansion plots ^{1}H δ 0-3.5 ^{13}C δ 160-175.

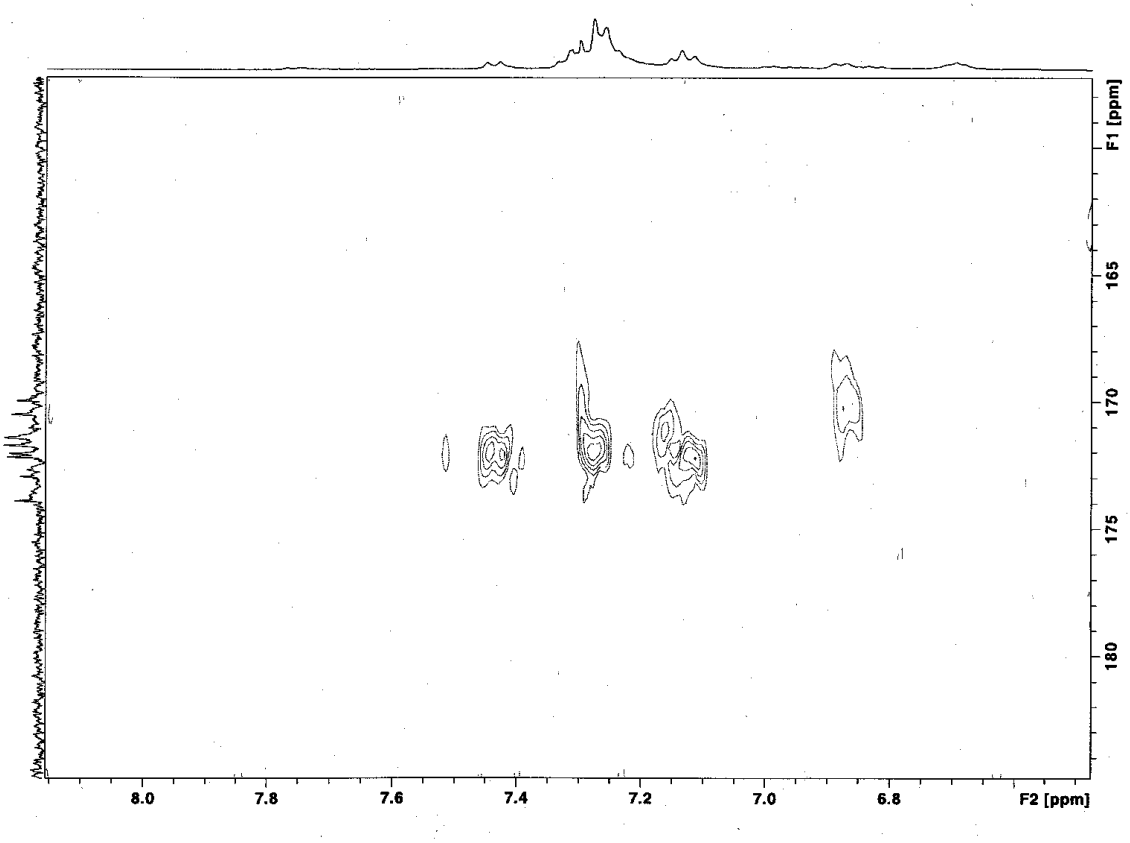


Figure 6.16. 400 MHz HMBC spectrum (optimized for *J* = 4Hz) of mitsoamide (**1**) in CD_3CN (expansion plots ^{1}H δ 6.6-8.0 ^{13}C δ 160-180).

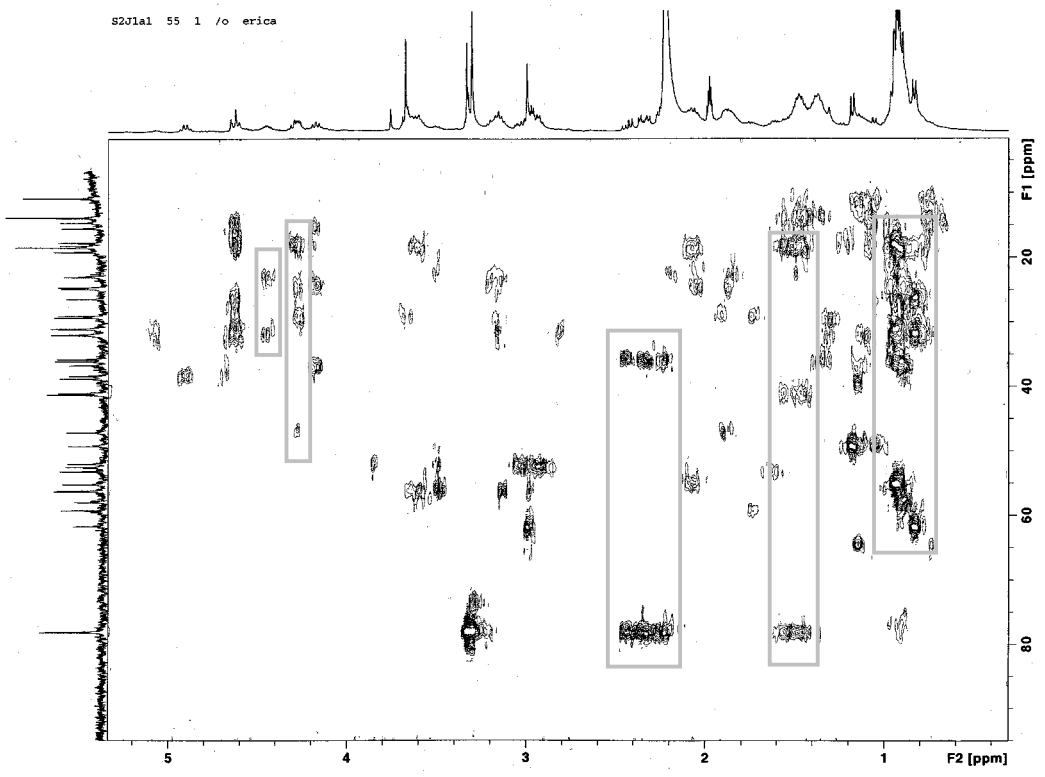


Figure 6.17. 400 MHz HMBC spectrum (optimized for *J* = 8Hz) of mitsoamide (**1**) in CD_3CN expansion plots ^{1}H δ 0-5 ^{13}C δ 10-85.

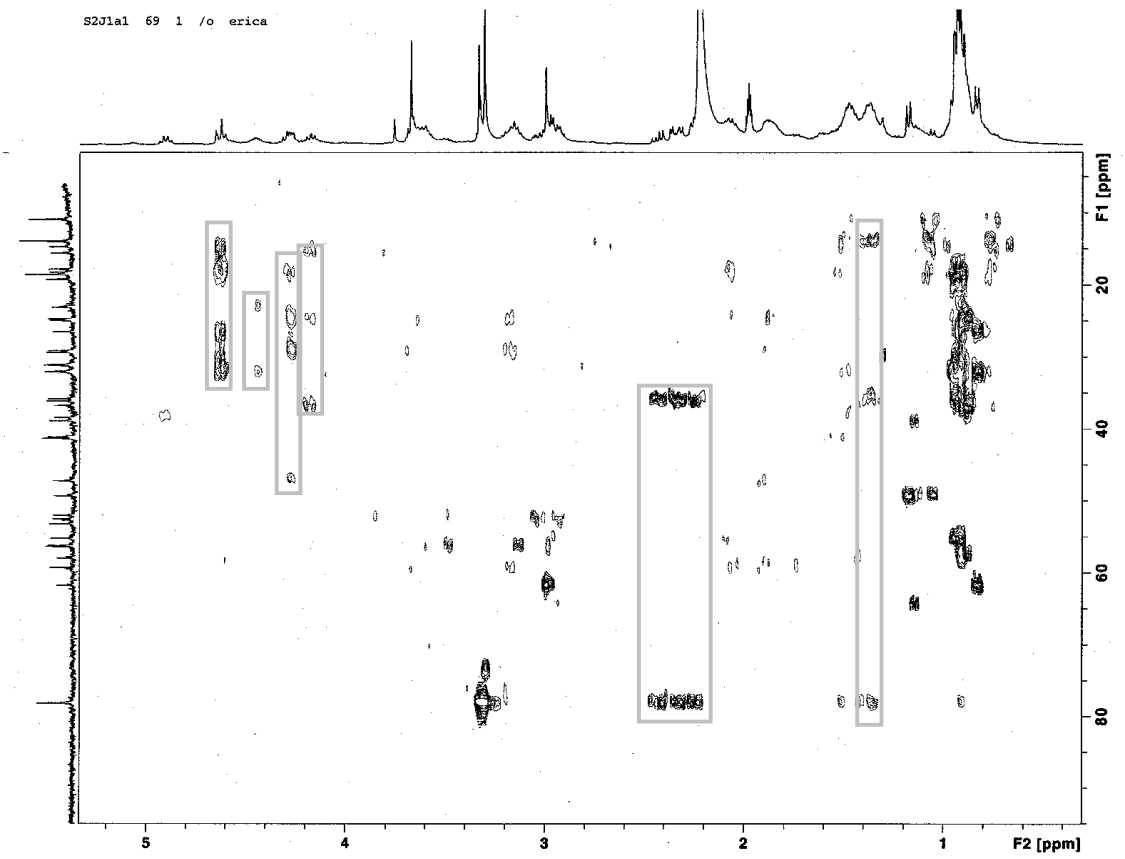


Figure 6.18. 400 MHz HMBC spectrum (optimized for *J* = 4Hz) of mitsoamide (**1**) in CD_3CN expansion plots ^{1}H δ 0-5 ^{13}C δ 10-85.

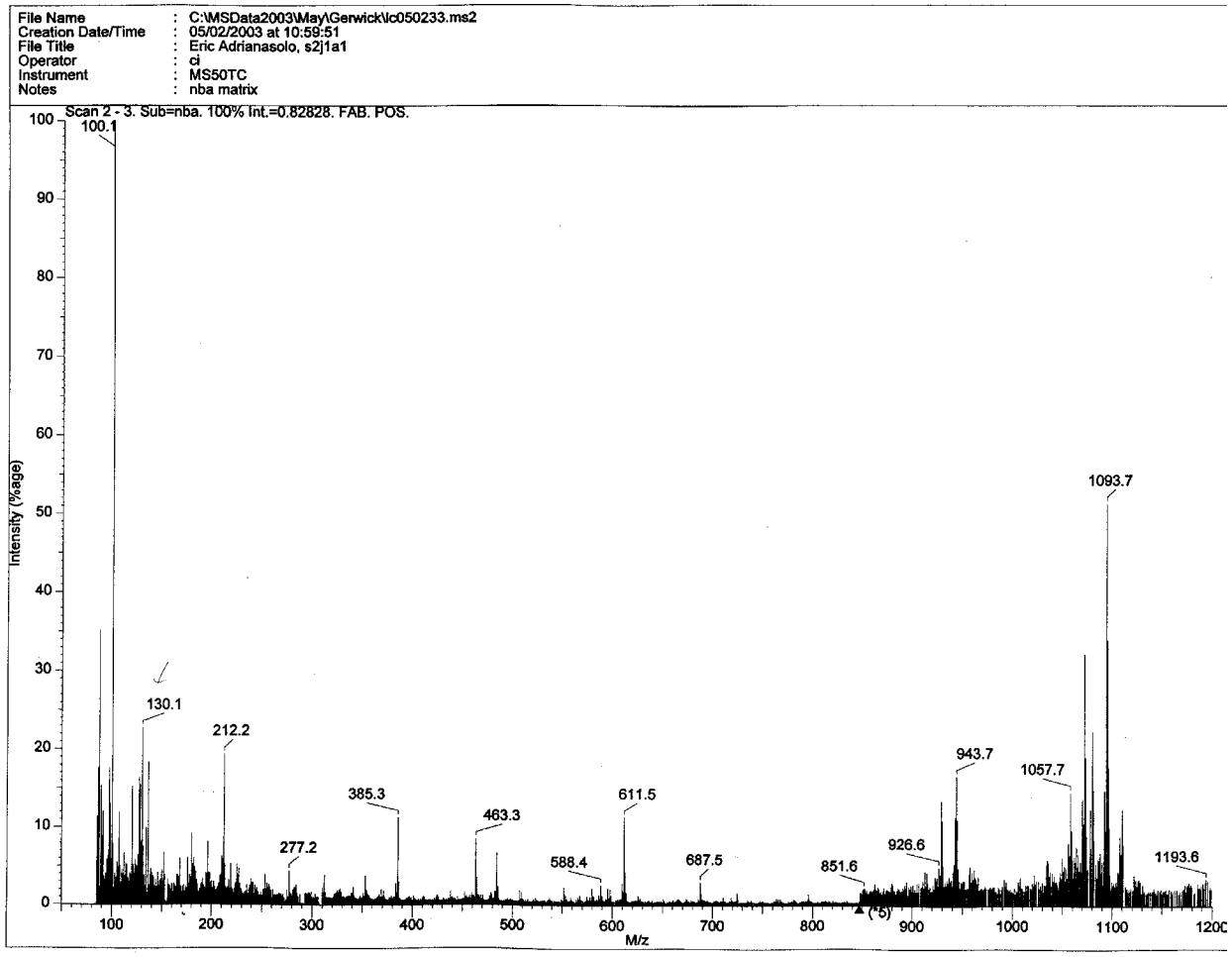


Figure 6.19. LR FAB spectrum of mitsoamide (**1**) (Kratos MS50TC instrument)

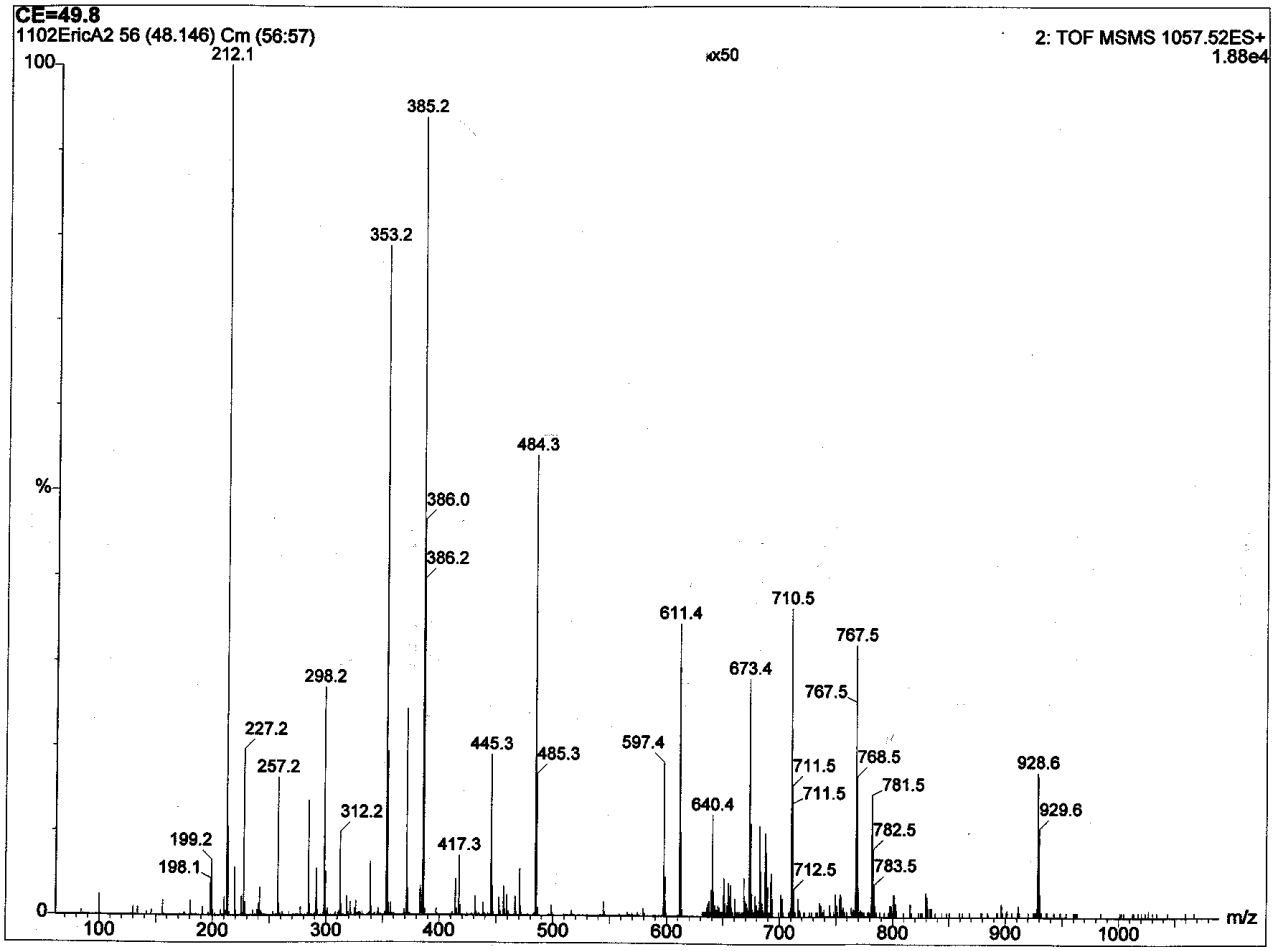


Figure 6.20. TOF MS/MS ES+ of m/z 1057.7 of mitsoamide (**1**).

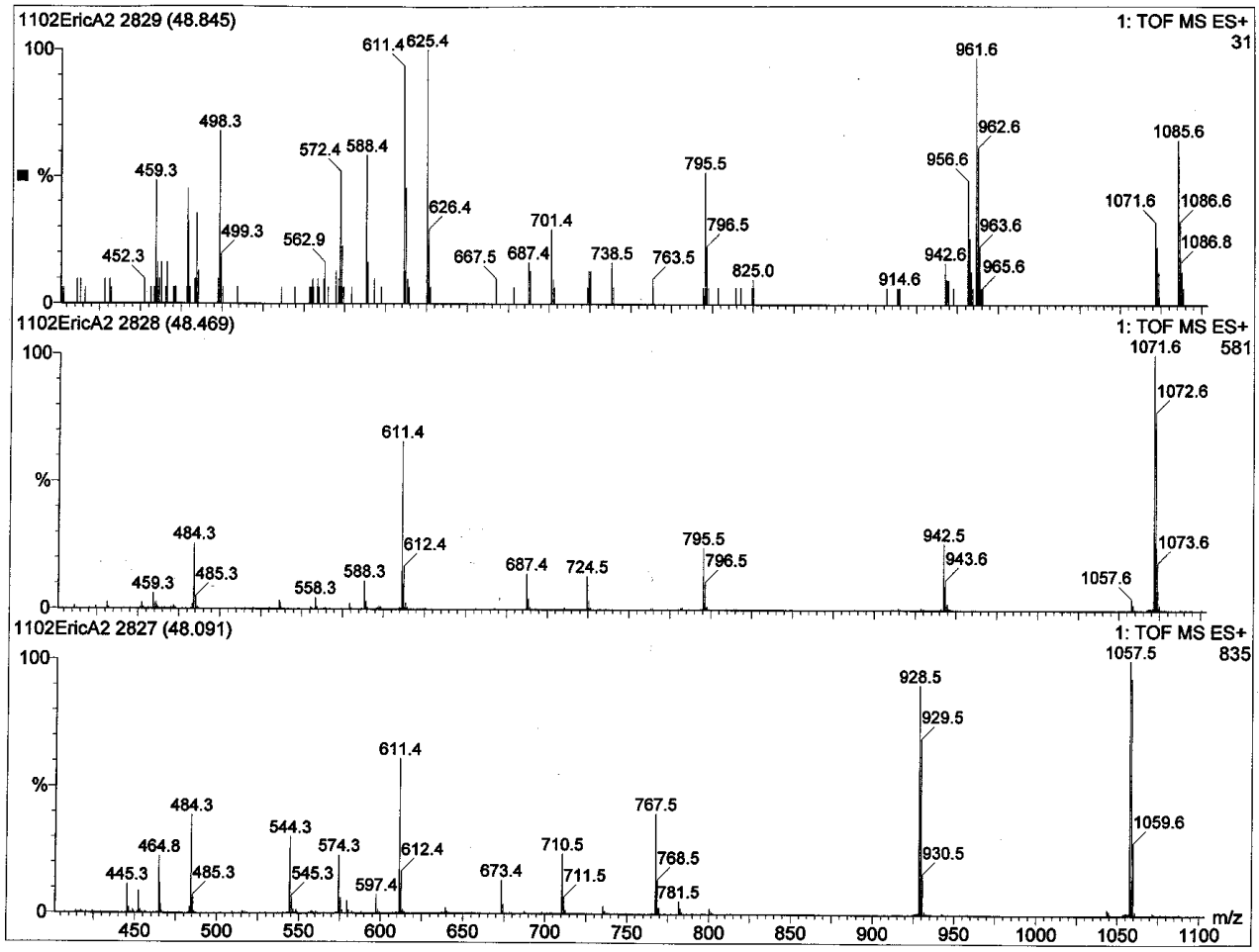


Figure 6.21. TOF MS ES+ of mitsoamide (**1**)

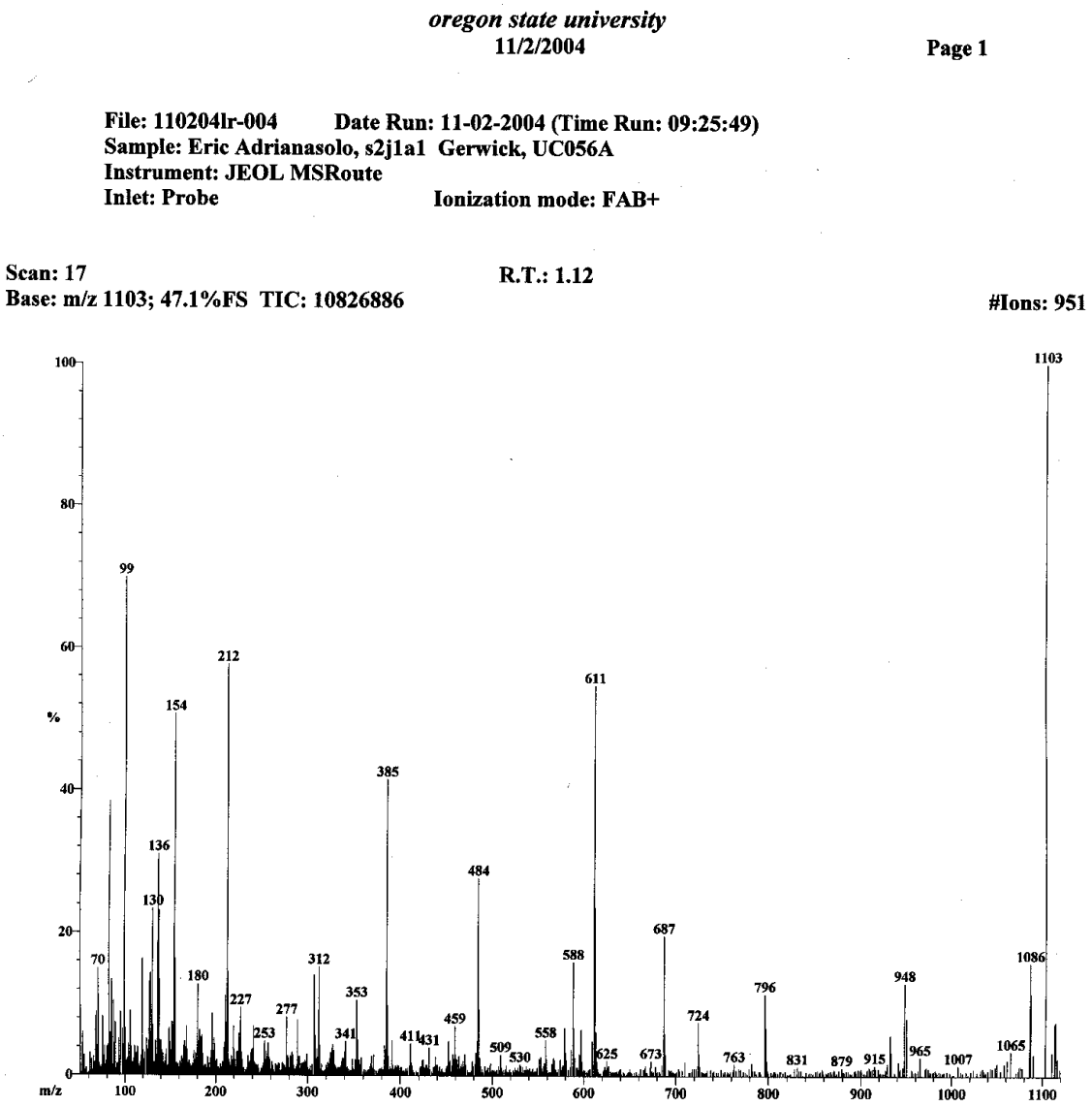


Figure 6.22. LR FAB spectrum of mitsoamide (**1**) (JOEL instrument, Inlet: probe)

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File: 110404lr-002 Date Run: 11-04-2004 (Time Run: 11:32:12)
Sample: Eric Andrianasolo, 1289J2B
Instrument: JEOL MSRoute
Inlet: My Inlet Ionization mode: FAB+

Scan: 7 R.T.: .23
Base: m/z 154; 100%FS TIC: 17108360 #Ions: 1332

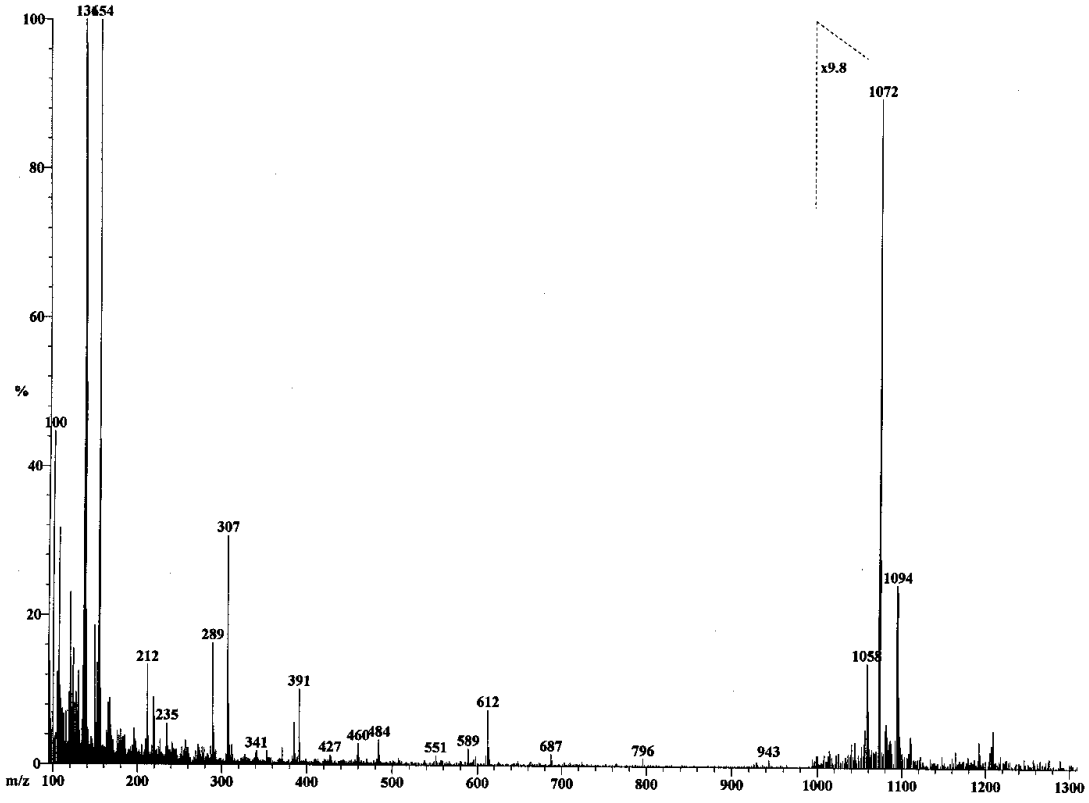
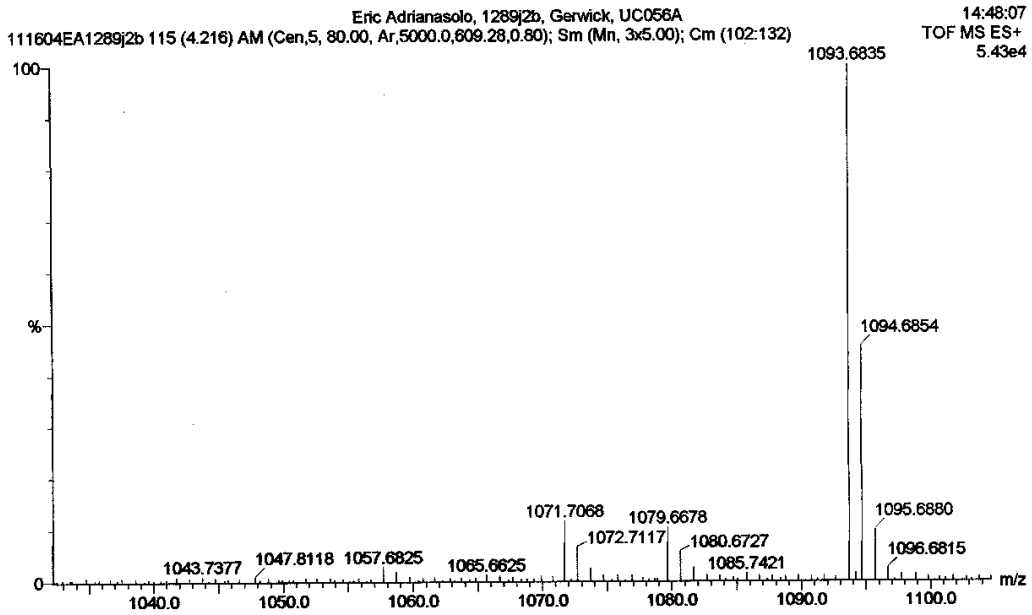


Figure 6.23. LR FAB spectrum of mitsoamide (**1**) (JOEL instrument, Inlet: My inlet)

Elemental Composition Report **Page 1**

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions



Minimum:				-1.5						
Maximum:		200.0	50.0	50.0						
Mass	Calc. Mass	mDa	PPM	DBE	Formula					
1093.6835	1093.6806	2.9	2.7	12.0	C56	H96	N7	O12	Cl	
	1093.6792	4.3	3.9	12.5	C54	H94	N10	O11	Cl	
	1093.6787	4.8	4.4	17.0	C57	H91	N9	O12		
	1093.6900	-6.5	-5.9	17.0	C56	H91	N11	O11		
	1093.6918	-8.3	-7.6	12.0	C55	H96	N9	O11	Cl	←
	1093.6680	15.5	14.2	12.5	C55	H94	N8	O12	Cl	
	1093.6666	16.9	15.4	13.0	C53	H92	N11	O11	Cl	
	1093.7025	-19.0	-17.4	16.5	C57	H93	N10	O11		
	1093.7030	-19.5	-17.8	12.0	C54	H96	N11	O10	Cl	
	1093.7044	-20.9	-19.1	11.5	C56	H98	N8	O11	Cl	

Figure 6.24. HR TOF MS ES+ spectrum of mitsoamide (**1**)

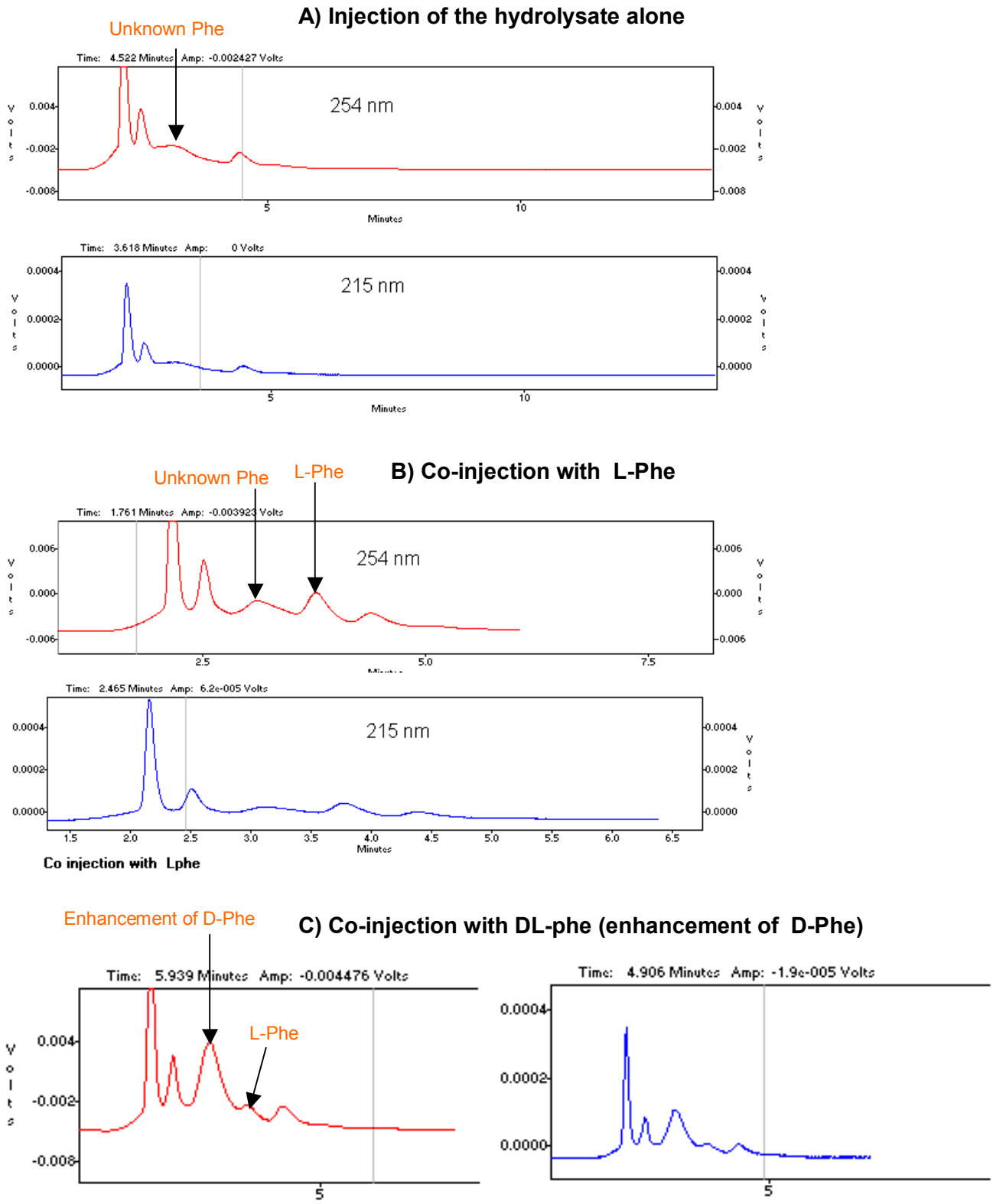


Figure 6.25. Amino acid analyses of mitsoamide **1** (D-Phe)

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on a Perkin Elmer 243 polarimeter and UV and FT-IR spectra were obtained employing Hewlett Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on Bruker Avance DRX300, DPX400 and DRX600 spectrometers with spectra referenced to residual solvent signal of CH_3CN ($\delta_{H/C}$ 1.94/118.69). LR FAB mass spectra were acquired on a Kratos MS50TC instrument. MALDI-TOF and TOF-MS-ES data were recorded on Applied Biosystems 4700 TOF-TOF and Waters Micromass LCT Classic mass spectrometers, respectively. HPLC was carried out using a Waters system consisting of a Rheodyne 7725i injector, two 515 pumps, a pump control module and a 996 photodiode array detector. All solvents were purchased as HPLC grade. Analytical chiral HPLC analyses were performed on CHIRIOBIOTIC T (Teicoplanin) - 10µm (size 4.6 x 100 mm).

Collection, Extraction, and Isolation Procedures

The marine cyanobacterium *Geitlerinema* cf sp. (voucher number MMA-18Apr00-02) was collected in April 2000 from Nosy Mitso-Ankaraha Island, NosyBe, Madagascar from a depth of 16 m by SCUBA. After collection, the microalga was stored in 2-propanol at -20 ^{o}C until workup. A total of 164.0 g (dry wt) of the cyanobacterium *Geitlerinema* sp. was extracted repeatedly with CH_2Cl_2-MeOH (2:1, v/v) to produce 1.0 g of crude organic extract. The crude extract was found to be active at 10 ppm (90% mortality) in the brine shrimp toxicity assay. A portion of the crude extract (950 mg) was fractionated using VLC on Si gel with a stepwise gradient of hexanes-EtOAc and EtOAc-MeOH to give 13 fractions. Fraction 9 (eluting at 50:50 EtOAc/MeOH) showed a cytotoxic effect (63% toxicity 1 ppm to brine shrimp) and was further purified on analytical RP HPLC [Phenomenex Sphereclone ODS 5 µm, 250 x 10.0 mm, MeOH/H_2O (90:10)] to yield 3 mg of 1.

Amino Acid Analysis of 1. Mitsoamide (**1**, 850 µg) was hydrolyzed in 6 N HCl at 105° C for 19 h, and dried under a stream of N_2. The residue was reconstituted with 510 µL of H_2O and 200 µL EtOH prior to chiral HPLC analysis [Chiral Chirobiotic T (Teicoplanin) column, 4.6 × 250 mm; UV detection at 215

and 254 nm], using mobile phase I, 70/30 H_2O/EtOH, flow rate 0.5 mL/min; mobile phase II, 50/50 H_2O/EtOH, flow rate 1 mL/min; mobile phase III, 90/10 EtOH/H_2O, flow rate 1 mL/min; mobile phase IV, 85/15 EtOH/H_2O, flow rate 1 mL/min; mobile phase V, 80/20 $H_2$0/EtOH. Flow rate 0.7 ml/min. Elution times of authentic standards in mobile phase I (t_R, min): L-Phe (3.84), D-Phe (3.15), mobile phase II (t_R, min): L-Ala (3.25), D-Ala (2.83), mobile phase III (t_R, min): L-Val (5.90), D-Val (8.50), mobile phase IV (t_R, min): L-Ile (5.58), D-Ile (10), mobile phase V (t_R, min): L-*N,O*-diMeIle (7.8), D-*N,O*-diMeIle Ile (6.8), The hydrolysate of **1** was chromatographed alone and co-injected with standards to confirm assignments of L-Val, L-Ile, L-Ala, and D-Phe. The presence of L-*N,O*-diMeIle was confirmed by methylation of standard D- and L-*N*-MeIle with diazomethane and compared to the hydrolysate. Because hypermethylation of the standards could occur, the hydrolysate of mitsoamide was also treated with diazomethane.

Cytotoxicity assay

Cytotoxicity was measured in NCI-H460 lung tumor cells using the method of Alley *et. al*[26] with cell viability being determined by MTT reduction.[25] Cells were seeded in 96-well plates at 6000 cells/well in 180 µl. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium without fetal bovine serum was added at 20 µl/well DMSO was less than 0.5% final concentration. After 48 hr, the medium was removed and cell viability determined.

Mitsoamide (1): $[\alpha]^{24}_D$ +17° (*c* 1, $CHCl_3$); UV ($CHCl_3$) λ_{max} 206 nm (log ε 3); IR ν_{max} (neat) 2925, 2861,1716,1694,1540, 1463,1370, 1186,1077, 995 cm^{-1}; ^1H NMR (CD_3CN, 400 MHz) and ^{13}C NMR (CD_3CN, 100 MHz), see Table 6.1.; LR FABMS (nba matrix) *m/z* $[M+HCl]^+$ 1093.7, *m/z* $[M]^+$ 1057.7;[27] MALDI-TOF *m/z* $[M+HCl]^+$ 1093.7;[27] HR TOF MS ESI+ *m/z* $[M+HCl]^+$ 1093.6835 (calc for $C_{55}H_{96}ClN_9O_{11}$,1093.6918).

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CHAPTER SEVEN

CONCLUSIONS

Investigation of marine natural products has been successful in the discovery of new chemistry with potential pharmaceutical, biochemical and agricultural applications. Cyanobacteria are one of the most productive sources of such novel and bioactive agents. Recent findings have proved that much of the unique chemistry isolated from marine invertebrates, such as sponges and tunicates, is also of cyanobacterial origins.

The focus of this research was the investigation of bioactive secondary metabolites of three Madagascar marine organisms using spectroscopic methods, primarily high field NMR and modern mass spectral techniques (MALDI-TOF, ESI and LCMS). Four novel macrolides, named ankaraholides A-D and one new linear peptide, mitsoamide, were isolated from the cyanobacterium *Geitlerinema* sp. collected in 2000 from Nosy be Island, Madagascar. The planar molecular structures were elucidated through two-dimensional NMR techniques in combination with low and high-resolution mass spectrometry.

The ankaraholides are new glycosylated derivatives of the known compound swinholide A, a potent cytotoxin which was originally isolated from the marine sponge *Theonella swinhoei*. Ankaraholide A showed biological activity comparable to that of swinholide A, and caused complete loss of the filamentous (F)-actin at 30 and 60 nM. Binuclear cells were present, consistent with inhibition of the actin-dependent process of cytokinesis. Ankaraholides B, C and D inhibited proliferation in NCI-H460 and Neuro-2a with IC_{50} values ranging from 178 nM to 354 nM. This work reports the isolation of swinholide-type metabolites directly from cyanobacteria, and unequivocally demonstrates that marine cyanobacteria possess the metabolic capacity to produce this skeletal class.

Mitsoamide is a new cytotoxic linear peptide, which displayed an IC_{50} value of 0.46 μM against NCI-H460 human lung tumor cells. The planar structure of

mitsoamide was established by 1D and 2D NMR experiments, including a 1D TOCSY pulse sequence, in combination with mass spectral data analyses. Mitsoamide displays uncommon structural features such as the unusual polyketide unit (3,7-dimethoxy-5-methyl- nonanedioic acid) (DMNA), the homolysine residue and the aminal moiety.

A red marine alga, *Portieria hornemannii*, collected from the south of Madagascar (Tolagniaro, Fort Dauphin) in March 1997 was discovered to produce the previously described halogenated monoterpene halomon. Extensive investigation using bioassay-guided isolation of several fractions of the same extract led to the isolation of three new halogenated monoterpenes, one of which displayed DNMT-1 inhibitory activity (1.65 μM). This is the first report of DNMT-1 enzyme inhibition by a halogenated monoterpene, including halomon (DNMT-1 inhibitory activity 1.25μM).

Tanikolide dimer, a novel SIRT2 inhibitor (IC_{50} = 176 nM), and tanikolide seco acid, were isolated from the marine cyanobacterium *Lyngbya majuscula* collected in April 2000 near Tanikely Island, Nosy-Be Madagascar. The depside molecular structure of tanikolide dimer, which is probably a *meso* compound, was established by NMR, MS and chiral HPLC analyses, whereas the structure of tanikolide seco acid, isolated as the pure *S** enantiomer, was elucidated by an X-ray experiment. The biogenesis of tanikolide, its seco acid, and its dimer proceeds *via* a PKS biosynthetic pathway. The co-occurrence of a *meso* dimer and an enantiomerically pure monomer is perplexing and has a number of intriguing implications for the biosynthetic assembly of these metabolites.

A novel HDAC inhibitor possessing a dolastane carbon skeleton has been isolated from the brown marine alga *Dictyota* sp., the only organism in this work that was not collected from Madagascar. The novel metabolite was also found to exhibit antimalarial activity at 7 μg/mL. Interestingly, other known HDAC inhibitors such as sodium butyrate, trapoxin A, and HC-toxin were also found to possess antiparasitic activity to the malaria parasite *Plasmodium falciparum*, making HDAC an attractive target for the development of novel antiparasitic agents.

This work has shown that marine algae and cyanobacteria are indeed an important source of new bioactive natural products. Marine cyanobacteria are capable of synthesizing compounds with potential for the treatment of a range of human diseases. Examining collections of marine algae and cyanobacteria from areas little studied for natural products, such as the coastal marine habitats of Madagascar, has been productive in the discovery of structurally new natural products with powerful biological properties. Several metabolites which target proliferation of cancer cells by inhibiting DNMT-1, SIRT2 and HDAC have been presented in this thesis. Isolation of the potent SIRT2 inhibitor, tanikolide dimer, also presents intriguing stereochemical insights. The cyanobacterial source of the ankaraholides provides strong evidence for the production of swinholide-type metabolites by cyanobacterial symbionts in sponges. It is then worthwhile to pursuit natural products research and investigation on these exceptional organisms.

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