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Novel approaches to the control of infectious diseases

Damaris Agathe Meujo Foping
University of Mississippi

NOVEL APPROACHES TO THE CONTROL OF INFECTIOUS DISEASES

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Damaris Agathe Meujo Foping

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ABSTRACT

As part of our ongoing efforts to find new drug leads against infectious diseases, several terrestrial and marine macro- and microorganisms were investigated. Several bioactive molecules, presented below, were isolated from these natural products.

The mass-screening of dozens of marine *Actinomycetes* was completed and several *Streptomyces* with interesting biological activity profiles identified. A few examples are the *Streptomyces* code H747 and the *Streptomyces* code H668, a bacterium from which a new (**5**) and two known polyethers, the antimalarial agent K41-A (**6**) and its C-29 analog (**7**) were isolated.

Five new six-membered ring cyclic peroxides: plakinastreloic acid A (**8**), methyl plakinastreloate A **(9**), the C-12 epimers of methyl 13, 14-epoxyplakinastreloate (**10** & **11**), and plakinastreloic acid B (**12**) were isolated from a marine sponge of the genus *Plakinastrella*. Compounds **8** and **9** exhibited antifungal activities against *Candida albicans* (IC_{50} = 6.5 µg/mL and 3.5 µg/mL, respectively), *Aspergillus fumigatus* (IC_{50} = 4.0 µg/mL and 9.0 µg/mL, respectively) and *Cryptococcus neoformans* (IC_{50} = 4.0 µg/mL and 9.0 µg/mL, respectively). A moderate antimalarial activity against CQ-resistant and CQ-sensitive strains of *Plasmodium falciparum* was observed, as well. It was also established that **8** possesses anti-HCV (Hepatitis C Virus) activity.

Two other compounds were isolated from HCV active methanol extracts of *Inga fagifiolia* (twigs) and *Diplostephium rhodendroides* (leaves). These compounds were identified as 2,3,4,5,6-pentahydroxy-2-(hydroxymethyl)hexanamide (**14**), the amide of a known molecule and 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic acid (**15**). The bioassay-guided approach used for their isolation and structure elucidation is reported here.

The potential of $scCO_2$ to inactivate bacteria trapped in the gut of oysters was studied. It was established that exposing oysters to CO_2 at 100 bar and 37 °C for 30 minutes and at 172 bar and 60 °C for 60 minutes induced 2-log and 3-log reductions in the APC loads, respectively. The decrease in the microbial load as a result of treatment with $scCO_2$ was found to be significant ($P = 0.002$). A blind study allowing sensory analysis of treated vs. untreated oysters was also completed; no significant difference in the physical appearance, smell, or texture was recorded.

DEDICATION

This work is dedicated to my God, whose faithfulness and love has brought me this far. It is also dedicated to my family, especially my parents Mr. & Mrs. Foping, my beloved grand-mother Suzanne, my sister Dolores and my brothers Colince, Jean-Louis, Nicanor and Franclin.

LIST OF ABBREVIATIONS AND SYMBOLS

ABC: ATP-Binding Cassette

ACI: AntiCoccidial Index

ADV: Angara Disease Virus

AIDS: Acquired Immunodeficiency Syndrome

APC: Aerobic Plate Count

ASP: Amnesic Shellfish Poisoning

AZP: AZaspiracid Shellfish Poisoning

CD_3OD: Deuterated Methanol

CDC: Centers for Disease Control and Prevention

CFP: Ciguatera Fish Poisoning

CFU: Colonies Forming Units

CNS: Central Nervous System

COSY: COrrelation SpectroscopY

CQ: ChloroQuine

CSPI: Center for Science for Public Interest

CVM: Center for Veterinary Medicine

DDE: p,p'-DichloroDiphenyldichloroEthylene

DDT: DichloroDiphenylTrichloroethane

DEPT: Distortionless Enhancement by PolarizationTransfer

DHA: DocosaHexAenoic

DMSO: DiMethyl SulfOxide

DSP: Diarrheic Shellfish Poisoning

EC: European Commission

EC_{50}: Effective Concentration, 50%

ED_{50}: Effective Dose, 50%

EEC: European Economic Community

EIA: Enzyme ImmunoAssay

ELISA: Enzyme-Linked ImmunoSorbent Assay

EO: Electrolyzed Oxidizing

EPA: Environmental Protection Agency

EPA: EicosaPentaenoic Acid

EPA: Environmental Protection Agency

EtOAc: Ethyl acetate

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

GC: Gas Chromatography

gp120: envelope glycoprotein 120

HAB: Harmful Algae Blooms

HACCP: Hazard Analysis & Critical Control Points

HAV: Hepatitis A Virus

HCP: Heat-Cool Pasteurization

HCV: Hepatitis C Virus

hERG: human Ether-à-go-go Related Gene

HHP: High Hydrostatic Pressure

HIV: Human Immunodeficiency Virus

HMBC: Heteronuclear Multiple Bond Coherence

HMQC: Heteronuclear Multiple Quantum Coherence

HPB: Histamine-Producing Bacteria

HPLC: High Pressure Liquid Chromatography

HRMS: High Resolution Mass Spectrometry

HSQC: Heteronuclear Single Quantum Coherence

i.v.: intravenous

i.p.: Intraperitoneal

IC_{50}: Inhibitory Concentration, 50%

ICH: International Conference on Harmonisation

IQF: Individual Quick Freezing

ISO: International Organization for Standardization

KmTxs: Karlotoxins

LACF: Low-Acid Canned Food

LC: Liquid Chromatography

LC-MS: Liquid Chromatography–Mass Spectrometry

LC–QqQ-MS: Liquid Chromatography coupled with triple-Quadrupole Mass-Spectrometry

LD_{50}: Lethal Dose, 50%

LDH: Lactate DeHydrogenase

mCPC: Cellobiose Polymyxin Colistin

MDR: MultiDrug-Resistant

MIC: Minimum Inhibitory Concentration

MOA: Mechanisms Of Action

MPN: Most Probable Number

MRL: Maximum Residue Limit

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MS: Mass Spectrometry

NDV: Newcastle Disease Virus

NMR: Nuclear Magnetic Resonance

NOE: Nuclear Overhauser Effect

NOESY: Nuclear Overhauser Effect SpectroscopY

NSP: Neurologic Shellfish Poisoning

OA: Okadaic Acid

p.o: Oral

PCBs: PolyChlorinated Biphenyls

PHP: Post-Harvest Processing

PrepLC: Preparative Liquid Chromatography

PSP: Paralytic Shellfish Poisoning

PUFAs: n-3 PolyUnsaturated Fatty Acids

QF: Quick Frozen

ROESY: Rotating frame Overhause Effect SpectroscopY

RT-PCR: Real-Time Polymerase Chain Reaction

$scCO_2$: supercritical CO_2

SCF: Supercritical Fluid

SCID: Severe Combined Immune Deficient

SI: Selectivity Index

TAPC: Total Aerobic Plate Count

TB: Tuberculosis

TDE: 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane

TDH: Thermostable Direct Hemolysin

TLC: Thin-Layer Chromatography

TOF: Time-Of-Flight

TRH: TDH-Related Hemolysin

UV: Ultra Violet

VLC: Vacuum Liquid Chromatography

VRE: Vancomycin-Resistant Enterococci

WHO: World Health Organization

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INTRODUCTION

Each year, millions of lives are claimed by diseases of all sorts. Nine of the top ten threats to human life are disease related; the deadliest being coronary heart disease, stroke and other cerebrovascular diseases, lower respiratory infections, chronic obstructive pulmonary disease, diarrheal diseases, AIDS, tuberculosis and lung cancer. Several of the diseases that burden the world population presently are extremely old. There are for instance reports of malaria and tuberculosis going back to antiquity [1, 2]. Yet after centuries of fight to see to their eradication, many are still major public health issues.

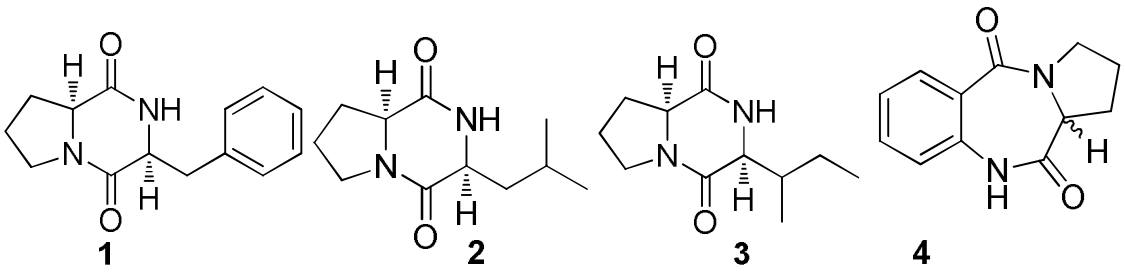
Malaria, for instance, is a leading cause of death and morbidity in third world countries; this disease claims the life of a child every 30 seconds. One of the major hindrances towards taking full control of this disease is the emergence and rapid spread of strains of the parasite that are resistant to major antimalarial drugs currently marketed. Tuberculosis (TB), Multi Resistant *Staphylococcus aureus* (MRSA) infections and AIDS are other major infectious illnesses that burden our world. Tuberculosis (TB) is a pandemic and the leading cause of death among people suffering from AIDS [3]. It is caused by *Mycobacterium tuberculosis,* which presently infects about one third of the world population. One of the major barriers to fully control the disease is the emergence of MDR strains; about 450,000 new cases of MDR-TB are reported worldwide annually [3]. Like tuberculosis, MRSA which can cause fatal

invasive infections constitutes a serious public health issue faced by countries all over the world. MRSA infections are of particular concern in hospital environments. The emergence of vancomycin resistant strains is cause for greater concern, as vancomycin is currently the drug of choice in the fight against MRSA related infections. The incidence of several of these diseases is on the rise. These menaces put an unprecedented amount of pressure on the necessity to discover new drugs/drug leads.

Multidrug resistance is not the only concern that threatens public health today; pharmaceutical drug companies facing major problems with their pipelines is another matter. As research and development (R&D) spending has grown exponentially over the years, a steady decline in the number of NCE approved has been noted instead. It is important to point out that combinatorial chemistry had failed to solve this issue. It has been speculated that increase in attrition, longer R&D timelines, and increased costs for some key R&D components (novel technologies for instance) were, among others, the factors that drive R&D costs [4-6]. Because the decline in the number of approved NCE had paralleled the reduced interest of pharmaceutical industries in natural products related research, several experts believe that there is a correlation between these two events [4].

Because of the exceptional contribution made, nature stands out in the drug discovery arena. According to a recent report [7], of the 974 small molecules new chemical entities reported between 1981 - 2006, 63% can be traced to nature, either as a natural or a synthetic compound inspired from a naturally occurring molecule (Figure 0-1). An impressive number of currently marketed antibiotics were derived from *Actinomycetes*.

The first project was to screen a set of marine *Actinomycetes* previously isolated by one of our collaborators, namely Dr. Russell Hill - University of Maryland, Center of Marine Biotechnology (COMB). Several bacteria with interesting biological activity profiles were identified. One of these bacteria was a *streptomyces* code H747 which crude extract had outstanding anti-MRSA and anti-HIV activities. Four known bioactive molecules, three diketopiperazines (**1**, **2** and **3**) and a pyrrolobenzodiazepine (**4**) were isolated from the crude extract of this bacterium.



Another *Streptomyces* with outstanding bioactivity against the malaria parasite *Plasmodium falciparum* and MRSA was identified during a different screening program. The bioassay-guided approaches used for the isolation of anti-infective metabolites from this bacterium and several other marine and terrestrial organisms are presented below.

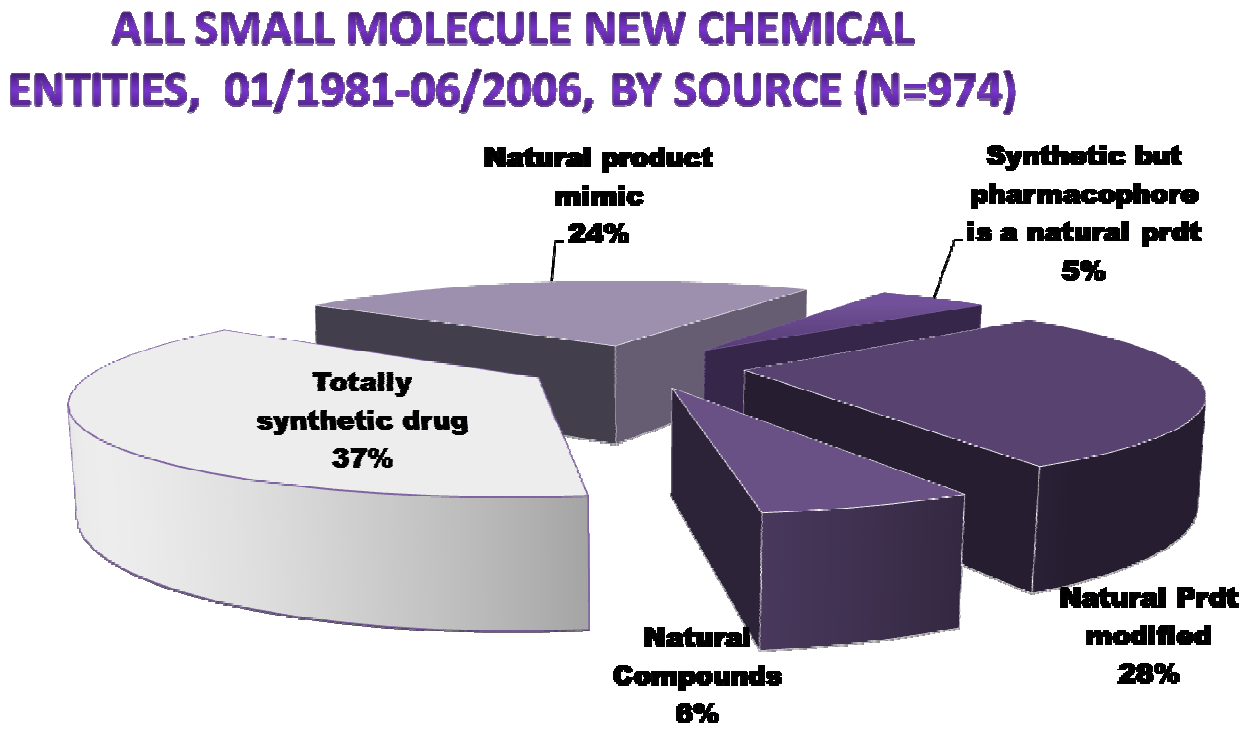


Figure 0-1. All small molecule new chemical entities, 01/1981-06/2006, by sources (N974)

Chapter 1 : POLYETHER IONOPHORES: BROAD-SPECTRUM AND PROMISING BIOLOGICALLY ACTIVE MOLECULES FOR THE CONTROL OF DRUG-RESISTANT BACTERIA AND PARASITES

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Authors: Kevin Il DA, Meujo DA, and Hamann MT

Background: As multidrug-resistant (MDR) pathogens continue to emerge, there is a substantial amount of pressure to identify new drug candidates. Carboxylic polyethers, also referred to as polyether antibiotics, are a unique class of compounds with outstanding potency against a variety of critical infectious disease targets including protozoa, bacteria and viruses. The characteristics of these molecules that are of key interest are their selectivity and high potency against several MDR etiological agents.

Objective: Although many studies have been published about carboxylic polyether antibiotics, there are no recent reviews of this class of drugs. The purpose of this review is to provide the reader with an overview of the spectrum of activity of polyether antibiotics, their mechanism of action, toxicity and potential as drug candidates to combat drug-resistant infectious diseases.

Conclusion: Polyether ionophores show a high degree of promise for the potential control of drug-resistant bacterial and parasitic infections. Despite the long history of use of this class of drugs, limited medicinal chemistry and drug optimization studies have been reported, thus leaving the door open to these opportunities in the future.

Scifinder and PubMed were the main search engines used to locate articles relevant to the topic presented in this review. Keywords used in our search were specific names of each of the compounds presented in the review, as well as more general terms such as polyethers, ionophores, carboxylic polyethers and polyether antibiotics.

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1. Introduction

Polyether antibiotics or carboxylic ionophores [8, 9] represent a unique class of compounds reported as potent antibiotics. They belong to the larger family of naturally occurring ionophores. First used in 1967, the term ionophore refers to the molecule's ability to bind a metal ion and facilitate its transport across cellular membranes. This chemo-physiological property has made polyether ionophores a useful tool in the study of cation transport mechanisms and has been the rationalization for their biological activities [9, 10].

Polyether antibiotics are believed to affect their targeted cells by modification of the permeability of cellular membranes to cationic metal species. Polyether ionophores

possess distinctive structural features that are crucial to their ability to interact with metal species. These features include an exterior alkyl backbone that confers their lipophilic characteristics, and an oxygen-rich internal cavity that can bind metal ions [8, 11, 12].

Naturally occurring ionophores are grouped into three categories with regard to the mechanism through which membrane permeability is altered. The first group is the mobile carriers that bind the metal species in an internal polar cavity, thus shielding their charge. The second group is the quasi-ionophores. These molecules form trans-membrane hydrophilic channels that permit the flow of metal ions. The last group consists of the neutral ionophores that facilitate the diffusion of cations across membranes according to the membrane potential [8, 13].

Naturally occurring carboxylic ionophores are relatively high in molecular weight, 500 – 1,000 amu, and are lipid soluble compounds that display a high affinity for cations such as K^+, Na^+, Ca^{2+}, thus yielding mobile cation complexes that can freely travel across biological membranes [9]. With the exception of lasalocid, (**16)** all naturally occurring carboxylic ionophores possess an affinity for Na^+ and K^+. Lasalocid (**16**) forms dimers and divalent complexes with Ca^{2+} and Mg^{2+}. This metal complex formation results in a paracyclic complex as a result of the head-to-tail hydrogen bonding.

The normal physiologic steady state of most living cells is dependent on the establishment of intracellular and extracellular concentration gradients of Na^+ and K^+ [14]. These ion concentration gradients are essential for cell function. Carboxylic ionophores can easily penetrate cellular membranes owing to their lipophilic properties

[14-16] and perturb the intracellular cation balance. Carboxylic ionophores thus present significant biological interests not only for their therapeutic utility but also for the possibility of using these chemo-physiological dynamics in combination with traditional or current therapeutics [10, 16, 17].

A total of 53 bacteria of the *Streptomycetaceae* family are reported to produce carboxylic ionophores. These microorganisms belong to three genera, *Streptomyces*, *Actinomadura* and *Dactylosporangium*. *Streptomyces* sp. are the main producers of these types of compounds and ~ 50% of the known carboxylic ionophores are derived from two *Streptomyces* species (*Streptomyces hygrocropicus* and *Streptomyces albus*) [9]. Industrially, these highly active molecules are produced via fermentation by microorganisms; the cost of production is generally low [10, 18, 19].

2. Biological activity

Polyether antibiotics show a broad spectrum of bioactivity including antibacterial, antifungal, antiparasitic, antiviral and tumor cell cytotoxicity. In addition to the previously mentioned biological activities, several studies have investigated the herbicidal [20] and anti-inflammatory activities [21] of these molecules, as well as their effect on the CNS and immunoregulatory systems (Table 1-1) [22, 23].

2.1 Antibacterial

The antibacterial potential of carboxylic ionophores is widely documented; reports on the discovery of these compounds typically mention their activity against some fungi and Gram-positive and Gram-negative bacteria. From these reports, it can

be gathered that carboxylic polyethers are molecules of outstanding potency against numerous pathogenic bacteria (drug-sensitive and drug-resistant). The fact that their potency against serious threats such as MRSA and VRE rivals that of clinically used drugs such as vancomycin and oxacillin, adds to their potential utility. At this point, it is difficult to fully assess the true value of these molecules as potential antibacterial therapeutic agents simply because none of these molecules has been tested on animal models.

2.1.1 Activities against drug-sensitive bacterial strains

A similar inhibitory pattern, namely, a strong selectivity towards Gram-positive bacteria, emerged from reports on the antibacterial potential of carboxylic polyethers [9, 11, 15, 16, 19, 24-28]. Monensin (**17**), narasin (**18**), salinomycin (**19**), nigericin (**20**), lenoremycin (**21**), septamycin (**22**), carriomycin (**23**), X-14766A (**24**), noboritomycin A (**25**), alborixin (**26**), ionomycin (**27**), A23187 (**28**), X-14547A (**29**), lysocellin (**30**) and lasalocid (**16**) were all evaluated against eight Gram-positive bacteria, seven Gram-negative bacteria, and four fungal species. All but ionomycin (**27**) showed potent activity against Gram-positive bacteria (MIC varying in the range 0.006 – 12.5 μg/mL). In contrast, not one of these compounds displayed activity against the tested Gram-negative bacteria (MIC > 100 μg/mL). Several other carboxylic ionophores are reported as having a similar biological activity profile against Gram-negative and Gram-positive bacteria. These are grisorixin (**31**) [18], ionomycin (**32**) [27], octacyclomycin (**33**) [25], X-14931A (**34**) [28], dianemycin (**35**) [16], ionomycin B (**36**) [19] and C (**37**) [19], antibiotic 6016 (**38**) [26], inostamycin (**39**) [29], inostamycin B (**40**) [29] and leuseramycin (**41**) [30].

Not all carboxylic polyethers show this selectivity towards Gram-positive bacteria. Compounds such as septamycin (**22**), noboritomycin A (**25**) and B (**42**) stand out for their ability to target not only Gram-positive bacteria but also Gram-negative bacteria. The reported MIC of septamycin (**22**) against *Escherichia coli* was 0.1 µg/mL [31]. Noboritomycin A (**25**) and B (**42**) inhibited the growth of *Neisseria pharyngi* with an MIC of 0.01 µg/mL [32]. The MIC of mutalomycin (**43**) against the same pathogen was 0.3 µg/mL [33]. Not all carboxylic ionophores possess significant activity against Gram-positive bacteria. For example, laidlomycin (**44**) was reported as inactive against *Staphylococcus aureus* [34]. Although in general anaerobic bacteria are more resistant to carboxylic polyethers, a few *Clostridium* sp, *Eubacterium* sp, *Propionibacterium* sp and *Peptococcus* sp are reported as extremely sensitive to compounds such as nigericin (**20**), monensin (**17**), dianemycin (**35**), lysocellin (**30**), lasalocid A (**16**) and A23187 (**28**) with MIC values as low as 0.049 µg/mL in many cases [9]. Carboxylic ionophores have a broad spectrum of activity against Gram-positive but not Gram-negative bacteria. Guyot *et al.* [35] attributed this overall differential selectivity to the presence of an outer membrane in Gram-negative bacteria. The outer membrane is believed to be impermeable to hydrophobic compounds. These authors came to this conclusion while using autoradiography; they could show that only *Bacillus cereus,* not *E. coli,* could incorporate the radiolabeled calcimycin (**28**) [36].

2.1.2 Activities against multidrug-resistant strains of bacteria

Carboxylic polyethers were reported as potent agents against a variety of MDR strains of pathogenic bacteria. Lycocellin (**30**), for instance, was reported as

active against a streptomycin-, erythromycin-, chloramphenicol and penicillin-resistant strain of *S. aureus* , MIC 4 µg/mL [15]. The MIC for the Na^+ salt of the antibiotic No 6016 (**38**) against a similar strain was reported to be 1.56 µg/mL [26]. Noboritomycin A (**25**) and B (**42**) were also reported as being active against several resistant strains: penicillin-resistance strains of *S. aureus*, MIC 0.01 µg/mL, tetracycline-resistance strains of *Micrococcus* sp, MIC 0.01 µg/mL, aminoglycoside-resistant strains of *Streptococcus faecalis*, MIC 0.01 µg/mL and macrolide-resistant strains of *Sarcina lutea* , MIC 0.01 µg/mL [32].

Table 1-1. Overview of reported bioactive naturally occurring polyethers (from 1967-1994*), sources, and their reported in vivo and in vitro testings

Name (acronyms), molecular formula, molecular weight	Organism from which it was isolated	In vitro activities	Tested in vivo	Availability of crystal structure	Discovery (year, journal, authors)
Lasalocid A (**16**), $C_{34}H_{54}O_8$ 590.8	*Streptomyces lasaliensis NRRL 3382*	Antiparasitic [37-39], antiviral [40], antibacterial [9, 41], antifungal [9, 42]	Antimicrobial and/or antiparasitic effect [43-47]	[48]	[48]
Monensin A (**17**), $C_{36}H_{62}O_{11}$ 670.9	*Streptomyces cinnamonensis ATCC 15413*	Antiparasitic [37, 49-53], antiviral [40, 54], antibacterial [55, 56], antifungal [9], antiproliferative/apoptotic [55, 57]	Antimicrobial and/or antiparasitic effect [37, 44, 58], immunoregulatory effect [59], cardiovascular effect [60, 61], antiviral [62]	[63]	[63]
Narasin A (**18**), $C_{43}H_{72}O_{11}$ 765.0	*Streptomyces aureofaciens NRRL 5758*	Antibacterial [9, 56, 64], antifungal [9] , antiparasitic [37, 51, 52]	Antiviral [62], antiparasitic [64]		[64]
Salinomycin (**19**), $C_{42}H_{70}O_{11}$ 751.0	*Streptomyces lasaliensis*	Antiparasitic [38, 51, 52], antiviral [40], antibacterial [9, 56], antifungal [9]	Antimicrobial and/or antiparasitic effects ,immunoregulatory effect [40, 44, 47, 58, 65-68]		[69]

Nigericin (**20**), $C_{40}H_{68}O_{11}$ 725.0	*Streptomyces hygrocopicus*	Antiparasitic [37, 53, 70], antiviral [40, 71, 72], antibacterial [9], antifungal [9], antiproliferative/apoptotic [57, 73]	Antimicrobial and/or antiparasitic effect [37, 74-76], effect on CNS tissues [23, 77], antitumor [73], antiherbicidal/insecticidal [20], Antiviral [62]	[78]	[78]
Lenoremycin (**21**), $C_{47}H_{77}O_{13}Na$, 873.1		Antibacterial, antifungal [9, 39]		[79]	[79]
Septamycin (**22**), $C_{48}H_{82}O_{16}$ 915.2	*Streptomyces hygroscopicus NRRL 5678*	Antibacterial, antifungal [9, 31], antiproliferative/apoptotic [31]	Antiparasitic[16, 31], antiviral [31]		[31]
Carriomycin (**23**), $C_{47}H_{80}O_{15}$ 885.2	*Streptomyces hygroscopicus*	antiproliferative/apoptotic [57], antibacterial, antifungal [80]	Antimicrobial and/or antiparasitic effect [72, 81]	[82]	[82]
Antibiotic X-14766A (**24**), $C_{43}H_{62}ClO_{14}Na$ 861.4 .	*Streptomyces malachitofuscus* subsp. downeyi	Antibacterial antifungal [83]		[84]	[84]
Noboritomycin A (**25**), $C_{43}H_{63}O_{14}Na$ 827.0	*Streptomyces Noboritoensis NRRL 8123*	Antibacterial [32]	Antibacterial [32]		[32]

Alborixin (**26**), $C_{48}H_{84}O_{14}$ 885.9	*Streptomyces albus*	Antibacterial [9], antiviral [85], antiparasitic [51, 52, 86], anticocicidal [50]	Antimicrobial and/or antiparasitic effect [37, 39, 87], cardiovascular effect [18]	[88]	[88]
Ionomycin (**27**), $C_{41}H_{70}CaO_9$ 747.1	*Streptomyces conglobatus ATCC 31005*	Antiparasitic [51], antibacterial, antifungal [9, 89], antiproliferative/apoptotic [90, 91]	Immunoregulatory effect [92-96], effect on CNS tissues [22]		[89]
Calcimycin (A23187) (**28**), $C_{29}H_{37}N_3O_6$ 523.6	*Streptomyces Chartreusensis*	Antiparasitic [51], antibacterial, antifungal [9, 36]		[97]	[97]
X14547 A (**29**)		Antiparasitic [51]			[98]
Lysocellin (**30**), $C_{34}H_{59}O_{10}$ Na.1/2H_2O 659.8	*Streptomyces cacaoi*	antiproliferative/apoptotic [57], antibacterial [15]	Antimicrobial and/or antiparasitic effect [81]	[99]	[99]
Grisorixin (**31**), $C_{40}H_{68}O_{10}$ 709.0	*Streptomyces griseus*	Antiparasitic [51], antibacterial [18]	Antimicrobial and/or antiparasitic effect [52], cardiovascular effect [100-102]	[35]	[35]
Lonomycin A (**32**), $C_{44}H_{76}O_{14}$ 829.1	*Streptomyces hygrocopicus*	Antibacterial [27], antiproliferative/apoptotic [57], antiparasitic [37, 51]	Antimicrobial and/or antiparasitic effect [103], cardiovascular effect [104]		[27]

Octacyclomycin (**33**), $C_{52}H_{88}O_{19}$ 1,016	*Streptomyces* sp.	Antibacterial, antifungal, antiproliferative/apoptotic [25]			[25]
X-14931A (**34**), $C_{40}H_{66}O_{11}$ 723.0		Antibacterial [28, 105], antifungal [28]	Antiparasitic [28]	[28]	[28]
Dianemycin (**35**), $C_{47}H_{78}O_{14}$ 867.1		Antiviral [40], antibacterial [16], antiproliferative/apoptotic [57]	Anti-inflammatory [21]	[106]	[106]
Lonomycin B (**36**), $C_{44}H_{75}O_{14}Na$ 851.1	*Streptomyces ribosidificus TM-481*	Antibacterial and antifungal [19]			[19]
Lonomycin C (**37**), $C_{43}H_{73}O_{14}Na$ 837.0	*Streptomyces ribosidificus TM-481*	antiproliferative/apoptotic [57], antibacterial [19]			[19]
Antibiotic 6016 (**38**), $C_{46}H_{78}O_{16}$ 887.1	*Streptomyces albus*	Antibacterial, antiparasitic [26]		[107]	[107]
Inostamycin (**39**), $C_{38}H_{68}O_{11}$, 700.9	*Streptomyces* sp. *MH816-AF15*	antiproliferative/apoptotic [24], antibacterial [29]		[108]	[108]
Inostamycin B (**40**), $C_{37}H_{66}O_{11}$ 686 M^+	*Streptomyces* sp. *MH816-AF15*	Antibacterial, antifungal [29]			[29]

Leuseramycin (**41**), $C_{47}H_{78}O_{13}$ 851.1	*Streptomyces hygroscopicus TM-531*	Antibacterial, antifungal [30]			[30]
Noboritomycin B (**42**), $C_{44}H_{65}O_{14}Na$ 841.0	*Streptomyces Noboritoensis NRRL 8123*	Antibacterial [32]	Antiparasitic [32]		[32]
Mutalomycin (**43**) S-11743 C 41 H 69 O 12 Na, 755.0	*Streptomyces mutabilis*	Antiparasitic [33]			[33]
Laidlomycin (**44**) AB-78 $C_{37}H_{62}O_{12}$ 698.9	*Streptoverticillum eurocidium*	Antiproliferative/apoptotic [34, 57] antibacterial [34, 41, 56], antiviral [85]			[34]
X-14885 (**45**), $C_{27}H_{31}N_2O_7$ Na·H_2O 536.57	*Streptomyces* sp. X-14885	Antibacterial, antifungal [109]		[109]	[109]
Cationomycin A (**46**), $C_{45}H_{70}O_{15}$ 850.5	*Actinomadura azurea* sp *Nov*	Antiparasitic [51], antibacterial [9, 110]	Antiparasitic [110]		[110]
AntibioticX-206 (**47**), $C_{47}H_{82}O_{14}$ 870	*Streptomyces* sp	Antiparasitic [111]	Antimicrobial and/or antiparasitic effect [111-113] cardiovascular effect [114]	[115]	[115]

K-41-A (**48**), $C_{48}H_{82}O_{18}$ 947.2	*Streptomyces gypseum NRRL 11168*	Antiparasitic [116], antibacterial [117]	Antiherbicidal/insecticidal [118]	[119]	[117]
Hawaiimycin I (**49**), $C_{36}H_{64}O_{12}Na$ 711.4	*Streptomyces* sp.	Antiparasitic [120]			[120]
Maduramicin (**50**) $C_{47}H_{80}O_{17}$ 917.1	*Actinomadura rubra*	Antibacterial [121], antiparasitic [52, 122]	Antimicrobial and/or antiparasitic effect [44, 87, 123]		[121]
CP-82,009 (**51**), $C_{49}H_{84}O_{17}$ 945.2	*Actinomadura* sp. (ATCC 53676)	Antibacterial [124]	Antiparasitic [124]	[124]	[124]
CP-84,657 (**52**), $C_{45}H_{78}O_{14}$ 843.1	*Actinomadura* sp.	Antibacterial [93]	Antiparasitic [93]	[93]	[93]
CP-80,219 (**53**), $C_{47}H_{78}O_{14}$ 867.1	*Streptomyces hygroscopicus*	Antibacterial [125]	Antiparasitic [125]	[125]	[125]
Kijimicin (**54**), $C_{37}H_{64}O_{11}$ 684.9	*Actinomadura* sp *MI215-NF3*	Antibacterial [126], antiviral[40, 127, 128], antiparasitic [129]	Antiparasitic [126]	[126]	[126]
Endusamycin (**55**), $C_{47}H_{77}O_{14}Na$ 889.1	*Streptomyces endus* subsp. aureus (ATCC39574)	Antibacterial [130]	Antiparasitic [130]	[130]	[130]
X-14868A (**56**),	*Nocardia* X-14868	Antibacterial,	Antiparasitic [131]		[131]

$C_{47}H_{80}O_{17}$ 917.13		antifungal [131]			
X-14868B (**57**), $C_{48}H_{82}O_{17}$ 931.2	*Nocardia* X-14868	Antibacterial, antifungal [131]	Antiparasitic [131]		[131]
SF 2361 (**58**), $C_{48}H_{82}O_{16}$ 915.2	*Actinomadura* sp. *SF-2361*	Antiviral [40], antiproliferative/apoptotic [57]			[132]
SF2324 (**59**), $C_{52}H_{88}O_{19}$ 1017.2		Antiviral [40, 43], antiproliferative/apoptotic [57]			[133]
SF 2487 (**60**), $C_{42}H_{63}O_{12}Na$ 805	*Actinomadura* sp *SF2487*	Antibacterial [134] antiviral [40, 134], antiproliferative/apoptotic [57], antiparasitic [129]		[134]	[134]
Monensin B (**61**) $C_{35}H_{60}O_{11}$ 656	*Streptomyces cinnamonensis ATCC 15413*	Antiviral [9]			[112]
A 28695B (**62**) $C_{48}H_{82}O_{17}$ Na 953.5	*Streptomyces albus NRRL 3883*		Antiparasitic [16], Antiviral [62]		[16]
A-204 (**63**) $C_{49}H_{84}O_{17}$ 945.2	*Streptomyces albus*	Antiviral [9]		[135]	[135]
Deoxy-(0-8)-salinomycin (**64**),	*Streptomyces albus ATCC 21838*	Antibacterial, antifungal [114]		[114]	[114]

$C_{42}H_{70}O_{10}$ 734					
CP-91,243 (**65**), $C_{50}H_{83}O_{18}Na$ 995.1	*Actinomadurra roseorufa*	Antibacterial, antiparasitic, antifungal [136]			[136]
CP-120,509 (**66**), $C_{45}H_{76}O_{17}$ 889.1	*Actinomadura roseorufa*	Antibacterial, antifungal [137]	Antiparasitic [137]	[137]	[137]
Iso-lasalocid (**67**), $C_{34}H_{54}O_8$ 590.8	*Streptomyces lasaliensis*	Antibacterial [138]		[138]	[138]
20-Deoxynarasin (**68**), $C_{43}H_{72}O_{10}$ 748	*Streptomyces aureofaciens* NRRL 11181	Antibacterial, antiviral [82]	Antiparasitic [82]		[82]
20-deoxy-epi-17-narasin (**69**) $C_{42}H_{70}O_{10}$ 735.0	*Streptomyces aureofaciens* NRRL 11181	Antibacterial, antiviral [82]	Antiparasitic [82]		[82]
Moyukamycin (**70**), $C_{47}H_{75}O_{13}Na$ 871	*Streptomyces hygroscopicus* TM-581 (FERM-BP 274)	Antibacterial, antifungal [139]			[139]
(Deoxy-(*O*-8)3)-epi-l7-salinomycin (**71**), $C_{42}H_{70}O_{10}$	*Streptomyces albus ATCC 21838*	Antibacterial, antifungal [114]	Hypertention [140]	[114]	[114]

735.0					
CP-54,883 (**72**), $C_{41}H_{61}O_{12}Cl_2$ 838	*Actinomadura routienii Huang* sp.	Antibacterial, antiparasitic [141]			[141]
X-14868C (**73**), $C_{46}H_{78}O_{17}$ 903.1	*Nocardia* X-14868	Antibacterial, antifungal [131]	Antiparasitic [131]		[131]
X-14868D (**74**), $C_{47}H_{80}O_{17}$ 917.1	*Nocardia* X-14868	Antibacterial, antifungal [131]			[131]
Abierixin (**75**), $C_{40}H_{68}O_{11}$ 725.0	*Streptomyces albus NRRL B-1865*	Antibacterial, antifungal [142]	Antiparasitic, [142]		[142]
Martinomycin (**76**), $C_{49}H_{84}O_{17}$ 967.5	*Streptomyces salvialis*	Antibacterial [143]	Antiherbicidal/insecticidal [143]		[143]
Portmicin (**77**), $C_{44}H_{76}O_{14}$ 828	*Nocardiopsis* sp *No. 6270*	Antibacterial, antifungal [144]	Antiparasitic [144]		[144]

X-14667 A (**78**), C_{44} $H_{88}NO_{12}Na$ 826.0	*Streptomyces cinnamonensis*	Antibacterial, antifungal [145]			[145]
X-14667 B (**79**), $C_{45}H_{70}NO_{12}Na$ 840.0	*Streptomyces cinnamonensis*	Antibacterial, antifungal [145]			[145]
CP-96,797 (**80**), $C_{47}H_{80}O_{17}$ 917.1	*Streptomyces* sp	Antibacterial, antifungal, [146]	Antiparasitic [146]	[146]	[146]
UK-58, 852 (**81**) $C_{52}H_{88}O_{18}$ 1000	*Actinomadura roseorufa* Huang sp. nov., ATCC 39697		Antiparasitic, antibacterial [147]		[147]

The sodium salt of septamycin (**22**) was also reported as effective against several MDR bacterial strains. These included a penicillin-resistant strain of *Staphylococcus aureus* (MIC 0.31 µg/mL), a tetracycline-resistant strain of *Micrococcus* sp. (MIC 0.31 µg/mL), an aminoglycoside-resistant strain of *Streptococcus faecalis* (MIC 0.1 µg/mL) and finally, a macrolide-resistant strain of *Sarcina lutea* (MIC 0.31 µg/mL) [31]. In 2007, Yoo *et al.* [56] published a report on the potential of several carboxylic polyethers, namely, laidomycin (**44**), monensin (**17**), salinomycin (**19**) and narasin (**18**), to inhibit VRE and MRSA [56]. In the case of MRSA, the MIC of these compounds varied from 0.5 to 4 µg/mL; in the case of VRE, it varied from 8 to 16 µg/mL against. Narasin (**18**) was the most potent compound. All these compounds were more potent than vancomycin and oxacillin. The MIC of vancomycin against VRE was 64 µg/mL and that of oxacillin against MRSA was > 32 µg/mL [56].

2.2. Antifungal

In general, fungi are more resistant to carboxylic ionophores. Several authors have reported MIC values greater than the highest tested concentration [9, 15, 18, 25, 27]. There are some exceptions: several species of fungi, namely, *Paecilomyces variotii, Candida albicans, Saccharomyces cerevisiae* and *Penicillium digitatum* were reported as moderately sensitive to some carboxylic polyethers. These included for example, grisorixin (**31**) [18], monensin (**17**) [9], narasin (**18**) [9], salinomycin (**19**) [9], lenoremycin (**21**) [9], carriomycin (**23**) [9], alborixin (**26**) [9], A23187 (**28**) [9], dianemycin (**35**) [11] , lysocellin (**30**) [9], X-14931A (**34**) [105] and leuseramycin (**41**) [30] , with MIC values varying in the range of 20 – 100 µg/mL. *P. variotii, C. albicans, S. cerevisiae* and

P. digitatum were quite sensitive to several other carboxylic polyethers (lysocellin (**30**) [9], A23187 (**28**) [9], lenoremycin (**21**) [9] and nigericin (**20**) [9]). The MIC values for these compounds were < 2 µg/mL. More recent studies about the antifungal capacities of carboxylic ionophores have focused on opportunistic infections caused by *Pneumocystis carinii* [46]. Lasalocid (**16**) and nigericin (**20**), two common carboxylic ionophores, were found to possess a significant inhibitory effect on the growth of *P. carinii* in vitro [42, 46].

2.3 Antiparasitic

2.3.1 Plasmodium sp

The emergence and spread of resistant strains of the malaria parasites to several of the antimalarial drugs marketed at present, especially CQ, constitute a serious public health threat [148-151]. To address this issue, combination therapy, i.e., the simultaneous use of more than one drug known to have different mechanisms of action (MOAs) is important. Such an approach slows the development of resistance to individual drugs, thus limiting the spread of resistant strains [152]. The success of combination therapy is dependent on the availability of molecules with unique MOA. In recent years, a great deal of effort has been dedicated to the discovery of new antimalarial agents. Among the potential candidates reported thus far are carboxylic ionophores. From the reports we gathered, it emerges that these molecules stand out as potential antimalarial drug candidates for several reasons.

First, carboxylic ionophores are potent in vitro antimalarial agents, with IC_{50} values in the nanomolar and picomolar range [51, 111]. Carboxylic ionophores are

typically organized into three subclasses. The first category includes compounds specific to monovalent cations such as alborixin (**26**), lonomycin A (**32**), nigericin (**20**), grisorixin (**31**), narasin A (**18**), salinomycin (**19**), cationomycin (**46**) and monensin A (**17**). The second group includes molecules that are specific to divalent cations; few examples are calcimycin (**28**), X14885 A (**45**) and X14547A (**29**) (Figure **I-1**). The third category includes molecules that show no particular specificity; among these are lysocellin (**30**), lasalocid A (**16**) and ionomycin (**27**). Of these three subgroups, carboxylic ionophores specific to monovalent cations are reported as the most potent with IC_{50} values in the range of 0.6 – 1.5 ng/mL. Cationomycin (**46**), IC_{50} = 35 ng/mL, is an exception.

Second, carboxylic ionophores are also reported to have potent activity in vivo. The in vivo antimalarial potential of alborixin (**26**), ionomycin A (**32**), nigericin (**20**), narasin (**18**) and monensin A (**17**) was recently evaluated using rats infected with *Plasmodium chabaudi* and *Plasmodium vinckei petteri* [37] . Alborixin (**26**), ionomycin A (**32**), nigericin (**20**), narasin (**18**) and monensin A (**17**) showed significant in vivo antimalarial activity, decreasing the parasitemia by ≥ 70% by day 5 in *P. chabaudi* and *P. vinckei petteri* infected mice. Overall, the ED_{50} varied between 0.4 and 4.1 mg/kg. Carboxylic ionophores that are specific to monovalent ions including alborixin (**26**), lonomycin A (**32**), nigericin (**20**), narasin (**18**) and monensin A (**17**) seemed to be the most potent. The therapeutic index using the intraperitoneal route of administration for lonomycin A (**32**), nigericin (**20**), narasin A (**18**), monensin A (**17**) and lasalocid A (**16**) ranged from 2 to 6 [37]. A similar study was completed by Adovelande and Schrevel who assessed the in vivo antimalarial activity of monensin (**17**) and nigericin (**20**) on P.

vincke petteri infected mice. The ED_{50} and ED_{90} values for monensin (**17**) were 1.1 and 3.5 mg/kg, respectively. The ED_{50} and ED_{90} for nigericin (**20**) were 1.8 and 4.6 mg/kg, respectively. Monensin (**17**) seemed more effective at curing mice than nigericin (**5**); 100% of mice that received 10 mg/kg of monensin (**17**) were cured. Only one-third of mice that received a similar dose of nigericin (**20**) were cured [74].

Third, according to several studies, these molecules act in a selective manner. The threshold of toxicity varies from one subclass of carboxylic ionophores to the next. Gumila *et al.* [51] reported the in vitro differential ionophore activity between *P. falciparum* and normal mammalian cells (Human Jurkat lymphoblasts and U937 macrophage cell lines). It appeared that mammalian cells were only affected by compounds such as nigericin (**20**), alborixin (**26**), lonomycin (**32**), narasin (**18**) and monensin (**17**) at concentrations that are significantly (i.e., at least 35-fold) higher than the IC_{50} against the malarial parasite [51]. While the IC_{50} of narasin (**3**), monensin A (**17**) and nigericin (**20**) against the malaria parasite varied in the range 0.6 – 1.5 ng/mL, the LD_{50} against against U937 macrophage, varied between 23.5 and 305 ng/mL. The LV_{50}, LD_{50} against Jurkat lymphoblast, varied between 45 and 500 ng/mL [51]. Gumilla *et al.* [37] assessed the acute and subacute toxicity of these compounds using rats infected with *P. chabaudi* and *P. vinckei petteri* and determined that alborixin (**26**), LD_{50} = 1 mg/kg, was the most toxic. Overall, carboxylic ionophores such as alborixin (**26**), lonomycin (**32**), nigericin (**20**), narasin (**18**) and monensin A (**17**) that are specific to monovalent ions appeared to be more toxic; the reported LD_{50} values for those molecules were in the range 4 - 30 mg/kg. The LD_{50} values for polyethers specific to divalent metal ions on the other hand varied in the range of 30 – 80 mg/kg [37].

Fourth, two carboxylic ionophores were reported to be significantly more potent than CQ. Adovelande and Schrevel assessed the antimalarial activity of monensin (**17**) and nigericin (**20**) alongside that of CQ and showed that in vitro, these were 25- and 30,000-fold more potent, respectively [74].

Fifth, several carboxylic ionophores were recently reported as having outstanding activity against chloroquine-resistant strains of *P. falciparum*. Otoguro *et al.* [111, 153] published two reports in 2001 and 2002 in which the effects of several carboxylic ionophores were evaluated against chloroquine-resistant and sensitive strains of *P. falciparum* alongside several drugs marketed at present. These antimalarial agents were artesunate, artemisin, CQ, pyrimethamanine, amodiaquine, quinine and trimethoprim. These studies revealed that carboxylic ionophores such as X-206 (**47**), lonomycin A (**32**), nigericin (**20**), narasin (**18**), salinomycin (**19**), dianemycin (**35**), monensin (**17**) and lysocellin (**30**) were extremely potent against CQ-resistant strains of *P. falciparum* with IC_{50} values varying in the range 0.15 – 6.4 nM (most potent). These molecules seemed to be more potent than the standard drugs mentioned earlier. The IC_{50} values for these drugs varied from 7.6 to values > 100,000 nM. Artemether (IC_{50} = 7.6 µg/mL) was the most potent of these standard compounds. The cytotoxicity against mammalian cells, a human diploid embryonic cell line, was also assessed. Lasalocid A (**16**), octocyclomycin (**33**) and X-206 (**47**) displayed high selectivity indexes. The highest selectivity index was that of X-206 (**47**), that is, 3,673 against the CQ-resistant strain and 1,080 against the CQ-sensitive strain. These compounds showed higher potency and selectivity towards CQ-resistant strains when compared to CQ-sensitive strain [111]. Lastly, these authors performed a comparative assessment of the in vivo

effect of X-206 (**47**), artesunate and artemether in *Plasmodium berghei* infected mice. X-206 (**47**) showed a narrow therapeutic window and had an ED_{50} = 0.53 mg/kg but this compound was toxic at 3 mg/kg. In 2002, these authors reported another compound, K-41 (**48**), as active in vitro against a CQ-resistant strain of *P. falciparum.* K-41 (**48**) (IC_{50} = 8.5 nM) showed an activity similar to that of artemether and artesunate and was more potent than artemisinin, CQ and pyrimethamine. K-41 (**48**) had a moderate selectivity index in vivo*;* it was active orally against the CQ-resistant strain used; the ED_{50} (7.0 mg/kg) was close to that of artemether, CQ and artesunate. As far as acute toxicity of K-41 (**48**) is concerned, an LD_{50} > 100 mg/kg was reported [153].

Finally, carboxylic ionophores are believed to affect the malaria parasite by means of a mechanism that is distinct from that of drugs available at present. It is important to point out that there is some conjecture over this subject. Adovelande and Schrevel believe that the mechanism of action of carboxylic ionophores is similar to that of CQ [74]. The latter acts by increasing the internal pH of the food vacuole and by binding to ferriprotoporphyrin, thus preventing its crystallization into hemozoin. Hemozoin will then accumulate to a toxic level inside the vacuole [74, 154, 155]. It is generally accepted that carboxylic ionophores act via anti-transport of cation with H^+ after inserting themselves in cellular membranes, acting as ion channels. According to Adovelande and Schrevel, this anti-transport of cations with H^+ will result in the alkalination of the food vacuole of the parasite in a similar manner as CQ [74]. Gumilla *et al.* [51] have a different point of view. According to these authors, the anti-transport of cations with H^+ cannot justify the outstanding potency of these compounds, for the

simple reason that changes that occur in the plasma membrane of infected erythrocytes reduce the efficiency of ion shuttling across the membrane. These authors believe that carboxylic ionophores are completely internalized [51, 156]. Once inside the cell and in close proximity with the parasite, they can inhibit its development either by altering its ion content or by disturbing the pre-established ionic gradient between the host and parasite cytosol [51]. The parasite is reportedly more sensitive during the shizont stage [37].

A polyether isolated from the marine *Streptomyces* sp. H668 and named hawaiimycin-I (**49**) [120] reinforced the critical role of certain key structural features including the terminal carboxylic group on the bioactivity of carboxyl ionophores. Hawaiimycin-I (**49**) is close structurally to a set of carboxylic ionophores reported as potent antimalarial [37, 153], that is, monensin A (**17**), K-41 (**48**), nigericin (**20**), lonomycin (**32**) and grisorixin (**31**). These molecules share a unique skeleton: five interconnected tetrahydrofuran and pyran rings. The key difference between hawaiimycin-I (**49**), established to have only a weak antimalarial activity (IC_{50} = 100 – 200 ng/mL against *P. falciparum)* [120], and these molecules is the absence the terminal carboxylic group. Crystal structures of this type of compounds reveal that the terminal carboxylic moieties can either be implicated in ion coordination or be involved in hydrogen bonding with distant OH groups [8, 11].

Toxicity concerns remain the primary hindrance in the development of carboxylic polyethers into antimalarial drugs. Apart from this issue, it can be gathered from the literature that carboxylic polyethers posses a great potential as antimalarial drug candidates and leads for further optimization. Carboxylic polyethers owe their

outstanding potency, in vitro and in vivo, to their unique MOA. Studies published by Gumilla *et al.* [37] illustrate that it is possible through semisynthetic modification of these molecules to generate better drug candidates. These authors reported that the therapeutic index could be improved in the case of monensin (**17**) and lasalocid A (**16**); the therapeutic index of monensin A (**17**) is 5 p.o. but that of its synthetic analog, monensin A methyl ether, is 12 i.p. The therapeutic index of lasalocid A (**16**) is 3.3 i.p. but that of its analog, 5-bromo lasalocid A, is 22 i.p. [37, 65, 123, 157].

2.3.2 Eimeria sp

Eimeria sp are apicomplexan protozoan parasites and etiological agents of coccidiosis, a disease affecting cattle and poultry. In an attempt to control these diseases, anticoccidial drugs are generally added to the feed of these animals. Several carboxylic polyethers are among the drugs that are now approved for use as control therapeutics for coccidiosis. The first approved application of veterinary carboxylic ionophores was in the prophylactic and therapeutic treatment of coccidiosis in poultry [65, 123, 157]. Carboxylic ionophores are reported to also have a positive impact on the growth of these animals [59, 92]. Maduramicin (**50**), salinomycin (**19**), narasin (**18**), lasalocid (**16**) and monensin (**17**) are the most prevalent of the feed additives marketed at present [9, 59, 92, 93]. Several other carboxylic ionophores are also reported to have anticoccidial utility in chickens and turkeys. Although these are the most common additives used now, there are several other carboxylic ionophores reported to be more effective than additives marketed at present [65, 131].

Dirlam *et al.* [124] performed a comparative study of the anticoccidial activity of CP-82,009 (**51**), salinomycin (**19**) and maduramicin (**50**) in chickens infected with several pathogenic strains of *Eimeria: E. tenella, E. necatrix, E. acervulina, E. maxima* and *E. brunette.* CP-82,009 (**51**) at doses of 5 or 10 mg/kg of feed showed a broader spectrum and tolerance [124]. These same authors reported in 1990 that another carboxylic polyether, CP-84,657 (**52**) had outstanding anticoccidial potential. This agent at dosage ≤ 5 mg/kg cured chickens infected with different types of *Eimeria* sp. CP-84,657(**37**) was more potent than several marketed molecules, such as salinomycin (**19**), narasin (**18**), lasalocid (**16**) and monensin (**17**) [93]. CP-80,219 (**53**) was reported to have an anticoccidial effects against *E. tenella* in chickens at dosages of 30 and 120 mg/kg of feed [125].

Kijimicin (**54**), tested along with monensin (**17**) and salinomycin (**19**) in chicken infected with *E. tenella,* was more effective than several marketed drugs. Its anticoccidial index (ACI) was 156.8 versus 144.1 for monensin (**17**) sodium salt and 127.1 for salinomycin (**19**) sodium salt [126]. Endusamycin (**55**) showed a protective effect on chicken infected with *E. tenella* and *E. acervulina* at doses of 10 – 40 mg/kg of feed [130]. The toxicity of this compound in rats was analyzed; when administered to rats orally, the LD 50 was 7.5 mg/kg [130].

Liu *et al.* [131] reported that X-14868A (**56)** and X-14868B (**57**) were potent anticoccidial agents for chickens infected with *E. tenella* . Doses of 7 and 15 mg/kg of X-14868A (**56)** and X-14868B (**57**), respectively, were estimated to be protective quantities against coccidiosis. These agents are more potent than well-known coccidiostats agents; required doses for monensin (**17**), lasalocid (**16**) and salinomycin

(**19**) are 98 – 121 mg/kg, 75 – 125 mg/kg and 60 – 100 mg/kg, respectively [131]. Smith and Strout suggested an MOA for the coccidiocidal effect of carboxylic ionophores [157]. They suggested that anticoccidial agents act, either by inhibiting the development of intracellular parasites or by inducing destruction of intracellular sporoizoids [157].

2.3.3 Cryptosporidium parvum

Cryptosporidium parvum infections in immunocompetent individuals are generally self-limiting. In immunocompromised patients, particularly in AIDS patients, these infections are severe and are becoming increasingly prevalent [158-160]. Although many drugs are used for the clinical treatment of *C. parvum,* effective treatments for infected AIDS patients are scare [158, 159]. The screening of several anticoccidial molecules has led to the in vitro and in vivo examination of several carboxylic ionophores [38, 39, 44, 53, 58, 161]. Monensin (**17**), maduramicin (**50**), salinomycin (**19**), alborixin (**26**), and lasalocid (**16**) were all evaluated for their respective activity against *C. parvum* [38, 39, 44, 53, 58, 87, 121, 161]. When compared with control anticoccidial agents, monensin (**17**) reduced the development of *C. parvum* by > 90% [53]. The administration of maduramicin (**35**) and Alborixin (**26**) to severe combined immune deficient (SCID) mice inoculated with 10^6 oocysts (bovine) oral gavage, beginning 4 weeks post infection at 3 mg/kg/day for 3 weeks, has resulted in a 96% reduction in fecal parasite load ($p < 0.003$) (case of maduramicin (**50**)) [87]; this correlated with significant reductions in parasite loads in cross-sections of small intestine tissue ($p < 0.000002$) and colon ($p < 0.000006$). Alborixin (**26**) showed less effective results with oocyst reduction at 71% after 3 weeks [87]; some toxicity was observed [87].

2.3.4 Toxoplasma gondii

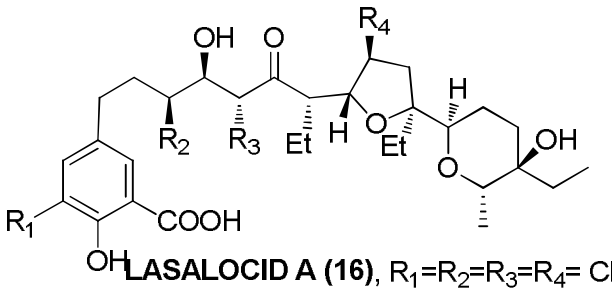
T. gondii is best known for its neurological effects and its particularly hearty cystic stage; it infects a wide variety of organisms [75, 162-164]. *T. gondii's* most deleterious activity is its induction of toxoplasmic encephalitis, thus causing fatal CNS lesions. *T. gondii* is an opportunistic pathogen in AIDS patients [49, 70, 162, 164, 165]. Treatment for toxoplasmic encephalitis is typically a combination therapy involving pyrimethamine and sulfadiazine. However, this type of chemotherapy is not effective against the cystic stage of *T. gondii*, thus, reactivation of the bradyzoites is chronic, especially in the CNS [23, 76].

Previous data has demonstrated that several polyether antibiotics are highly active against the tachyzoite stage of *T. gondii* [49, 73, 77, 78, 164]. In vitro and in vivo studies on the bradyzoites stage revealed under immunofluoresence and electron microscopy that very low dosages, 0.0001 µg/mL, of monensin (**17**) significantly altered the cytological physiology, that is, swollen cysts and large numbers of vacuoles, of *T. gondii* [49]. Temporal, in vivo mouse brain *T. gondii* bradyzoites, evaluations of a 6- and 48-h exposure yielded significant antiparasitic activity [124, 129]. Six-hour single dose treatment of 0.1 µg/mL of monensin (**17**) effectively eliminated the viability or lysed the cells completely. In a similar 48-h trial with concentrations of as small as 0.0001 µg/mL, the bradyzoites were swollen or lysed, and at a concentration of 0.01 µg/mL all parasites seemed permanently altered beyond viability under electron microscopic evaluation [49]. Apart from carboxylic ionophores antibiotics only two other drugs have shown activity against the bradyzoites stage, atovaquone [166] and 2′,3′-dideoxyinosine (ddi) [167]. In vitro and in vivo tests have shown that these other molecules take a

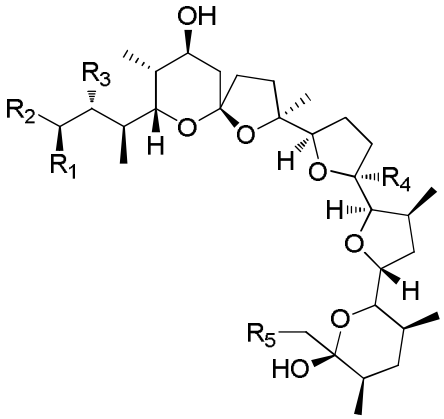
significantly longer time to have the same activity as monensin (**17**) [78, 129, 137, 164].

2.3.5 Neospora canium

The coccidian parasite *N. canium* is often misdiagnosed as *T. gondii.* The antimicrobial potential of several compounds, including, a few carboxylic polyethers against this pathogen was recently investigated. To examine the inhibition of tachyzoite multiplication, a 2-day treatment monoclonal antibody-based enzyme immunoassay (EIA), and a 5-day treatment cell culture flask lesion-based assay were developed. In this study 43 chemotherapeutics, sulfonamides, dihydrofolate reductase/thymidylate synthase inhibitors, macrolides, lincosamides, pentamidine analogues, eight miscellaneous antiprotozoal and six carboxylic polyethers were evaluated for use in treating *Neospora canium* infections. This assay determined that five out of the six ionophores evaluated, that is, lasalocid (**16**), maduramicin (**50**), narasin (**18**), monensin (**17**) and salinomycin (**19**), caused 100% reduction in tachyzoite induced lesions at concentrations of 0.000001 µg/mL in the CCF assay. Alborixin (**26**) was toxic to host cells at these concentrations [52].

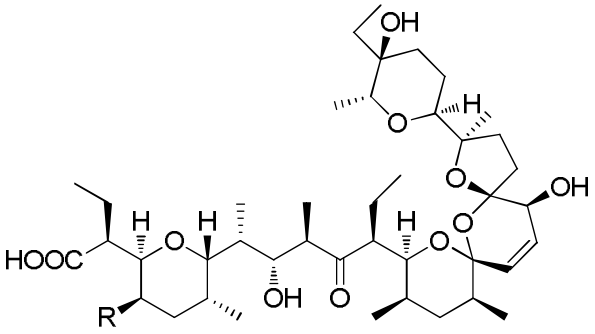


LASALOCID A (16), $R_1=R_2=R_3=R_4= CH_3$
LASALOCID B (82), $R_1=Et$, $R_2=R_3=R_4= CH_3$
LASALOCID C (83), $R_2=Et$, $R_1=R_3=R_4=CH_3$
LASALOCID D (84), $R_1=R_2=R_4=CH_3$, $R_3=Et$,
LASALOCID E (85), $R_1=R_2=R_3=CH_3$, $R_4=Et$,

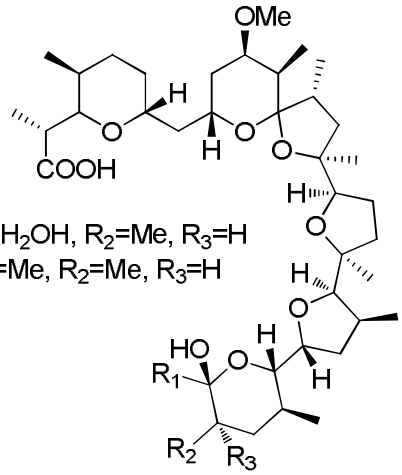


MONENSIN A (17), $R_1=CH_3$,$R_2=COOH$, $R_3=OMe$, $R_4=Et$, $R_5=OH$
MONENSIN B (61), $R_1=CH_3$,$R_2=COOH$, $R_3=OMe$, $R_4=Me$, $R_5=OH$
LAIDLOMYCIN (44), $R_1=CH_3$,$R_2=COOH$, $R_3=COOEt$, $R_4=Me$, $R_5=OH$
MONENSIN C(86), $R_1=H$,$R_2=CH_2CH_2COOH$, $R_3=OMe$, $R_4=Et$, $R_5=OH$
26-DEOXY-LAIDLOMYCIN (87), $R_1=CH_3$,$R_2=COOH$, $R_3=COOEt$, $R_4=Me$, $R_5=H$

Figure 1-1. Structures of carboxylic polyethers

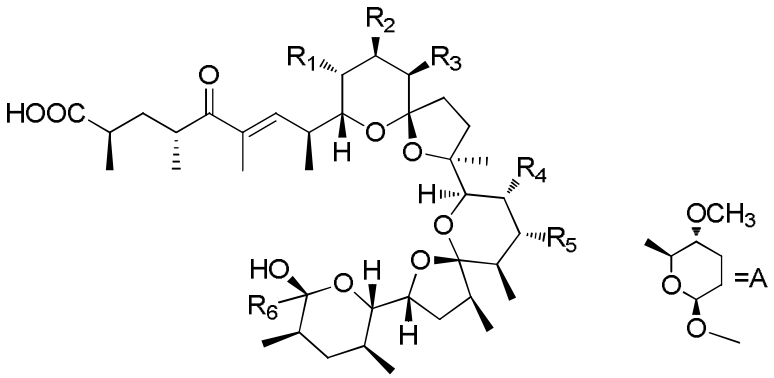


NARASIN (18), R= Me
SALINOMYCIN (19), R=H



NIGERICIN(20), R_1=CH_2OH, R_2=Me, R_3=H
GRISORIXIN (31), R_1=Me, R_2=Me, R_3=H

Figure 1.1. (cont.) Structures of carboxylic polyethers



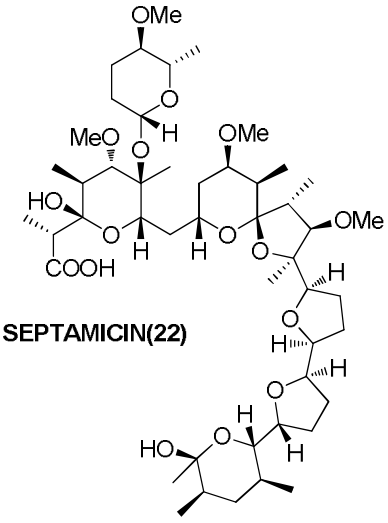
LENOREMYCIN (21), R_1=H,R_2=A, R_3=CH_3,R_4=R_5=H, R_6=CH_2OH

X14931A(34), R_1=CH_3, R_2=OH, R_3=R_4=R_5=H, R_6=CH_2OH

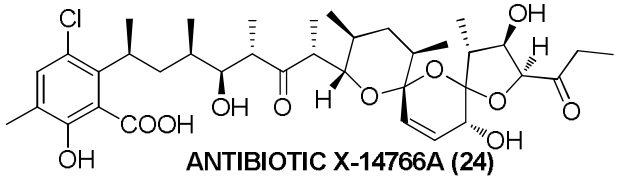
DIANEMYCIN (35), R_1=Me, R_2=OH, R_3=R_4= H, R_5=A, R_6=CH_2OH

LEUSERAMYCIN (41), R_1=Me, R_2=OH, R_3=R_4= H, R_5=A, R_6=Me

ISO-DIANEMYCIN (88), R_1=CH3, R_2=R_3=R_4= H, R_5=A, R_6=CH_2OH

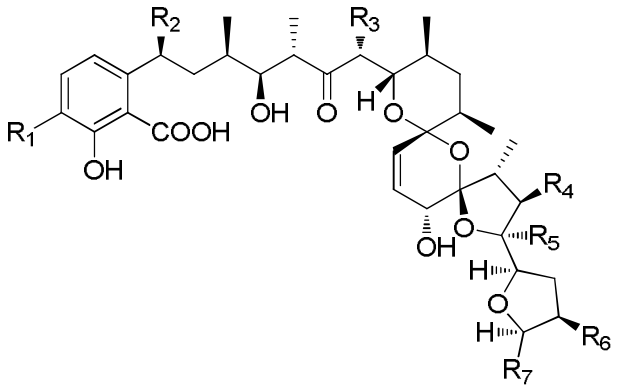


SEPTAMICIN(22)

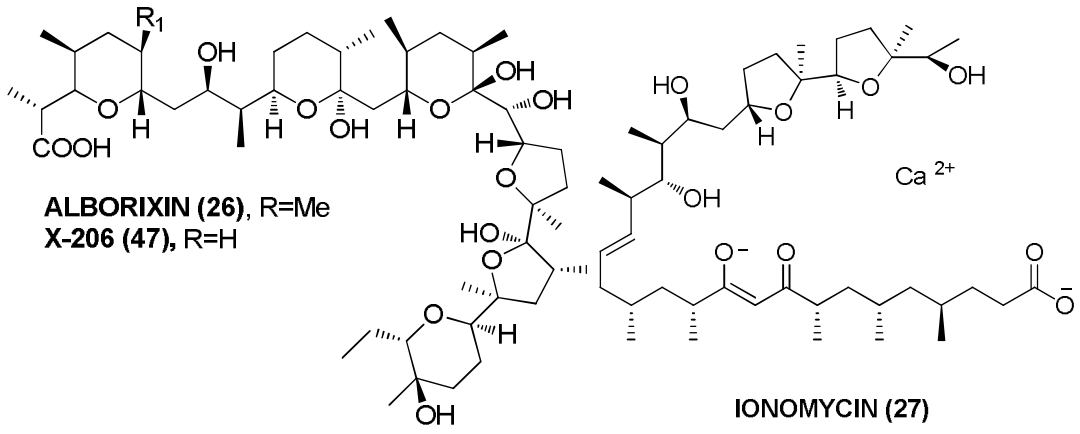


ANTIBIOTIC X-14766A (24)

Figure 1.1. (cont.) Structures of carboxylic polyethers



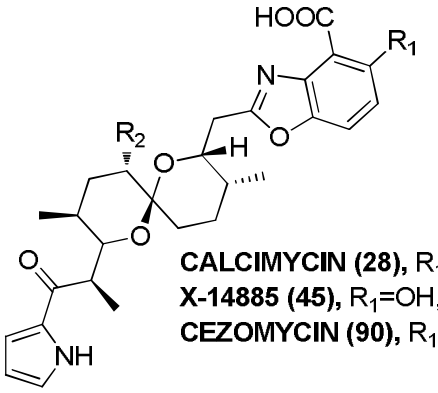
NOBORITOMYCIN A (25), $R_1=R_2=R_3=R_5=Me, R_6=OH$, $R_4=OMe, R_7=COOCH_2CH_3$
NOBORITOMYCIN B (42), $R_1=Et$, $R_2=R_3=R_5=Me, R_6=OH$, $R_4=OMe, R_7=COOCH_2CH_3$
CP44161 (89) $R_1=CH_3$, $R_2=R_4=R_6=H$, $R_3=R_5=Et$, $R_7=CHOHCH_3$



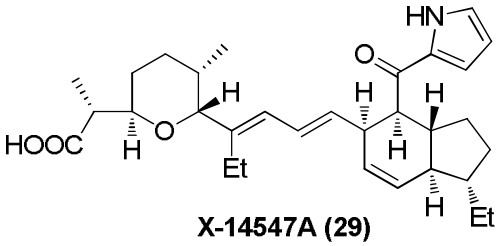
ALBORIXIN (26), R=Me
X-206 (47), R=H

IONOMYCIN (27)

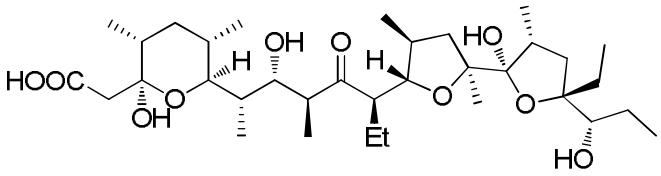
Figure 1.1. (cont.) Structures of carboxylic polyethers



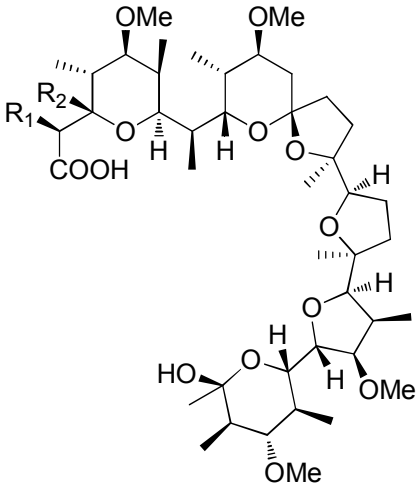
CALCIMYCIN (28), R_1=$NHCH_3$, R_2=Me
X-14885 (45), R_1=OH, R_2=H
CEZOMYCIN (90), R_1=H, R_2=Me



X-14547A (29)

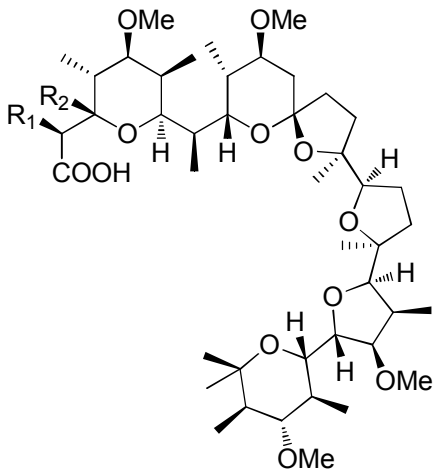


LYSOCELLIN (30)

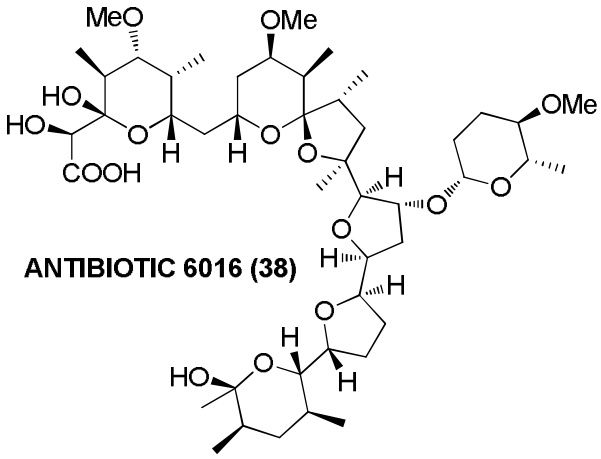


LONOMYCIN A (32), R1= αMe, R2=αOH
LONOMYCIN B (36), R1= αMe, R2=βOH
LONOMYCIN C (37), R1= H, R2=αOH

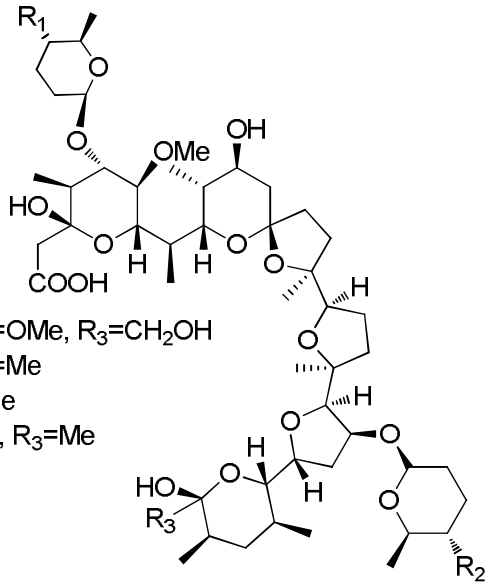
Figure 1.1. (cont.) Structures of carboxylic polyethers



LONOMYCIN A (32) R_1=αMe, R_2=αOH
LONOMYCIN B (36) R_1=αMe, R_2=βOH
LONOMYCIN C (37) R_1=H, R_2=αOH

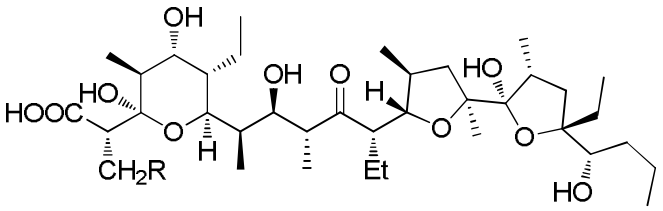


ANTIBIOTIC 6016 (38)

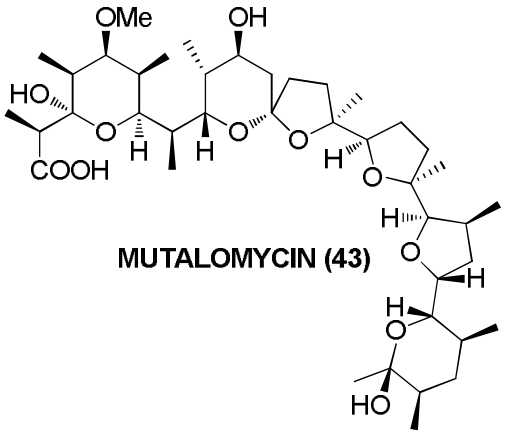


OCTACYCLOMYCIN (33), R_1=R_2=OMe, R_3=CH_2OH
UK-58,852 (81), R_1=R_2=OMe, R_3=Me
CP-91, 243(65), R_1=R_2=OH, R_3=Me
CP-91, 244 (91), R_1=OH, R_2=OMe, R_3=Me

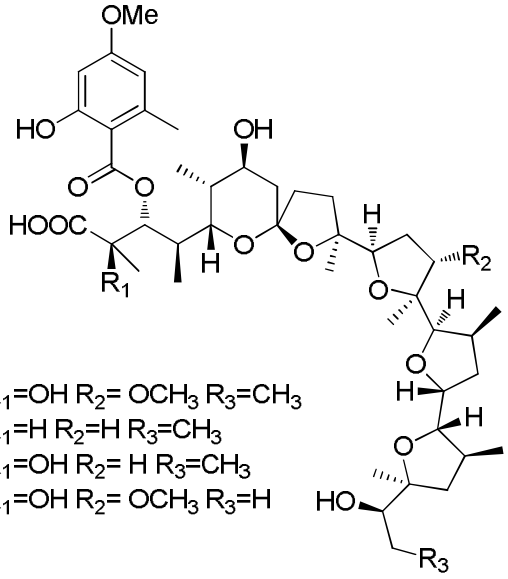
Figure 1.1. (cont.) Structures of carboxylic polyethers



INOSTAMYCIN (39), R=Me
INOSTAMYCINB (40), R=H

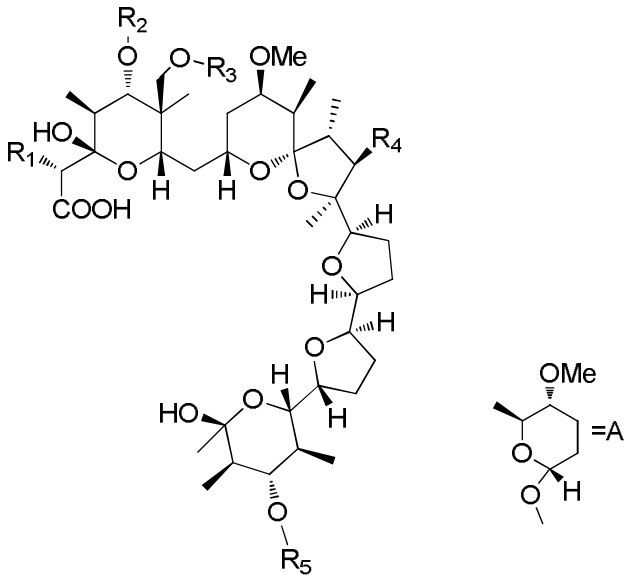


MUTALOMYCIN (43)



CATIONOMYCIN A (46), R_1=OH R_2= OCH_3 R_3=CH_3
CATIONOMYCIN B (92), R_1=H R_2=H R_3=CH_3
CATIONOMYCIN C (93), R_1=OH R_2= H R_3=CH_3
CATIONOMYCIN D (94), R_1=OH R_2= OCH_3 R_3=H

Figure 1.1. (cont.) Structures of carboxylic polyethers



K-41 A (48), R_1=OH, R_2=R_3=Me, R_4=OMe, R_5=A

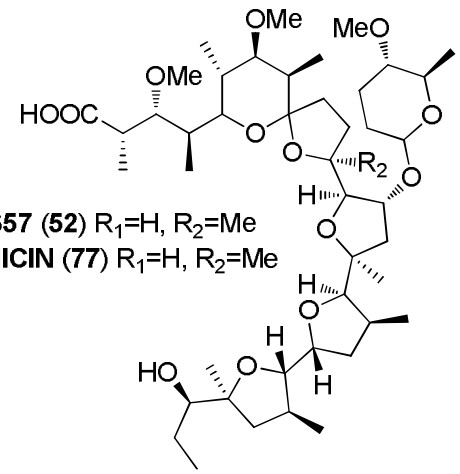
CP-82,009 (51), R_1=R_2=Me R_3=A, R_4= R_5=Me

MARTINOMYCIN (76) R_1=R_2= R_3=Me, R_4=OMe, R_5=A

CP-96,797(80), R_1=R_2=Me R_3=A, R_4= R_5=Me

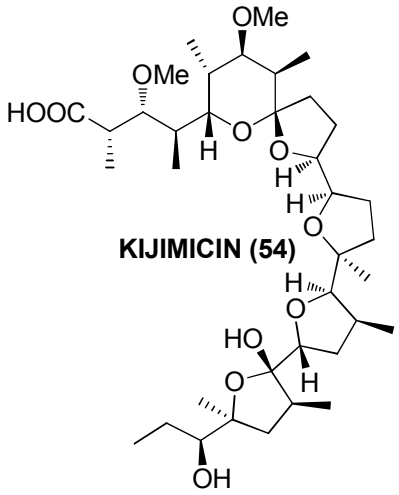
K-41 B (95), R_1=OH, R_2=R_3=Me, R_4=R_5=A

A-204 (96) R_1=Me, R_2=A, R_3=Me, R_4=OMe R_5=Me



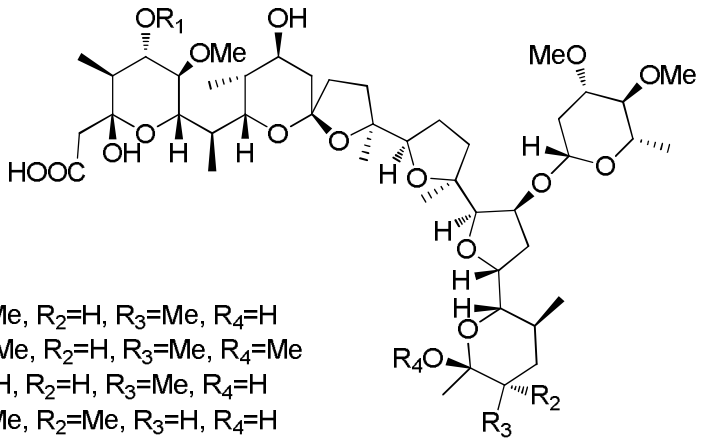
CP-84,657 (**52**) R_1=H, R_2=Me

PORTMICIN (**77**) R_1=H, R_2=Me

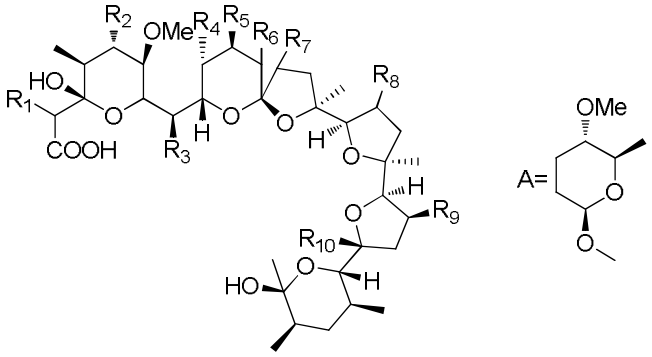


KIJIMICIN (54)

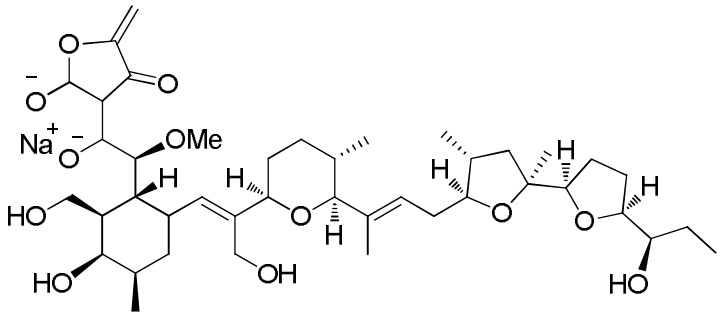
Figure 1.1. (cont.) Structures of carboxylic polyethers



X-14868A (56) R_1=Me, R_2=H, R_3=Me, R_4=H
X-14868B (57) R_1=Me, R_2=H, R_3=Me, R_4=Me
X-14868C (73) R_1=H, R_2=H, R_3=Me, R_4=H
X-14868D (74) R_1=Me, R_2=Me, R_3=H, R_4=H

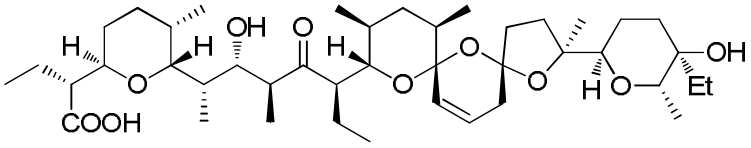


SF2324 (59), R_1=R_{10}=R_6=R_7=R_8=H, R_5=OH, R_3=R_4=CH_3,R_9=R_2=A
SF2361 (58), R_2=R_5=OCH_3, R_1=R_6=R_7=CH_3, R_3=R_4=R_5=R_9= R_{10}=H,R_8=A
*configuration for SF2324

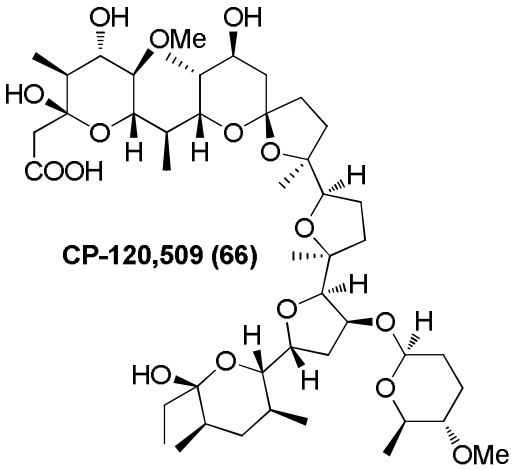


SF2487(60)

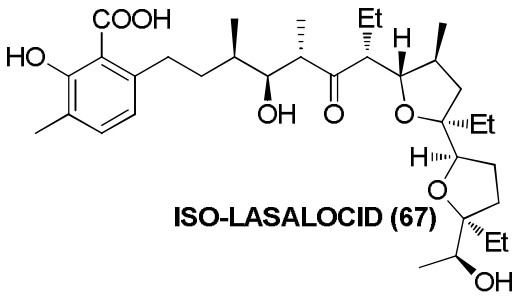
Figure 1.1. (cont.) Structures of carboxylic polyethers



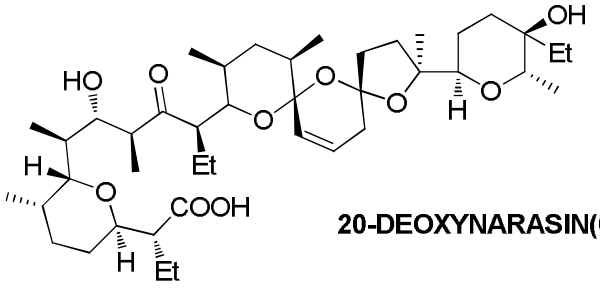
DEOXY-(O-8)-SALINOMYCIN (64)



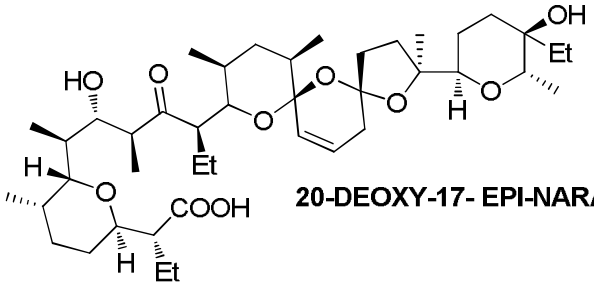
CP-120,509 (66)



ISO-LASALOCID (67)

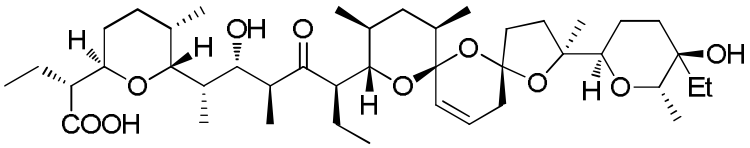


20-DEOXYNARASIN(68)

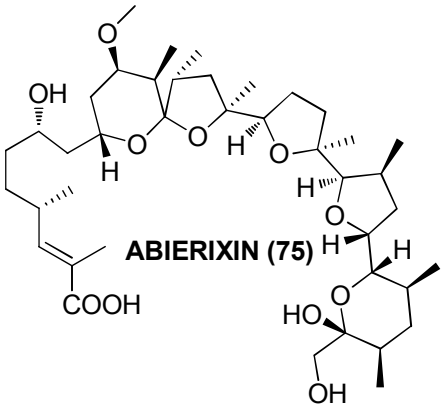


20-DEOXY-17- EPI-NARASIN(69)

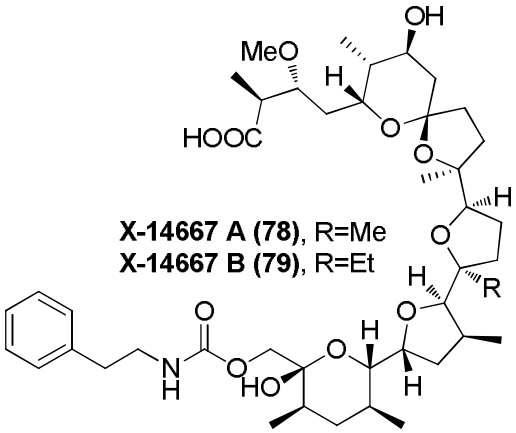
Figure 1.1. (cont.) Structures of carboxylic polyethers



DEOXY-(O-8)-EPI-17-SALINOMYCIN (71)

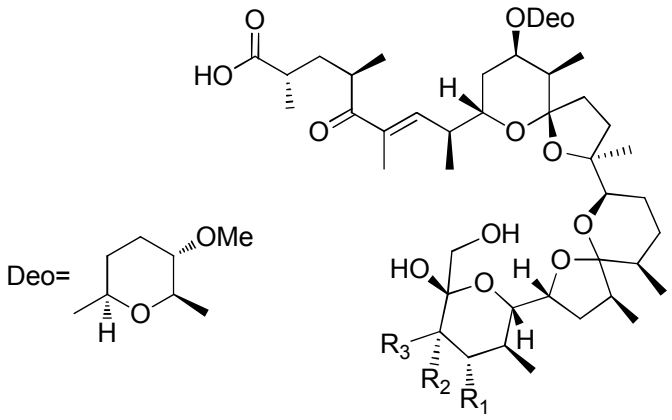


ABIERIXIN (75)

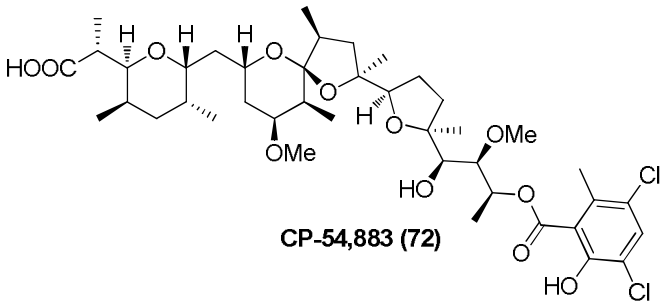


X-14667 A (78), R=Me
X-14667 B (79), R=Et

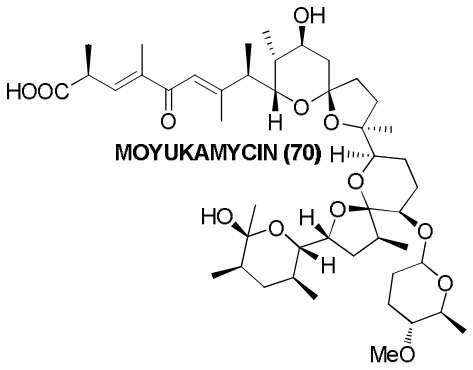
Figure 1.1. (cont.) Structures of carboxylic polyethers



A-130A (97)R_1= H ,R_2= H, R_3= Me
A-130B(98) R_1= ODeo,R_2= H, R_3= Me
A-130C (99)R_1= H ,R_2= Me, R_3= H



CP-54,883 (72)



MOYUKAMYCIN (70)

Figure 1.1. (cont.) Structures of carboxylic polyethers

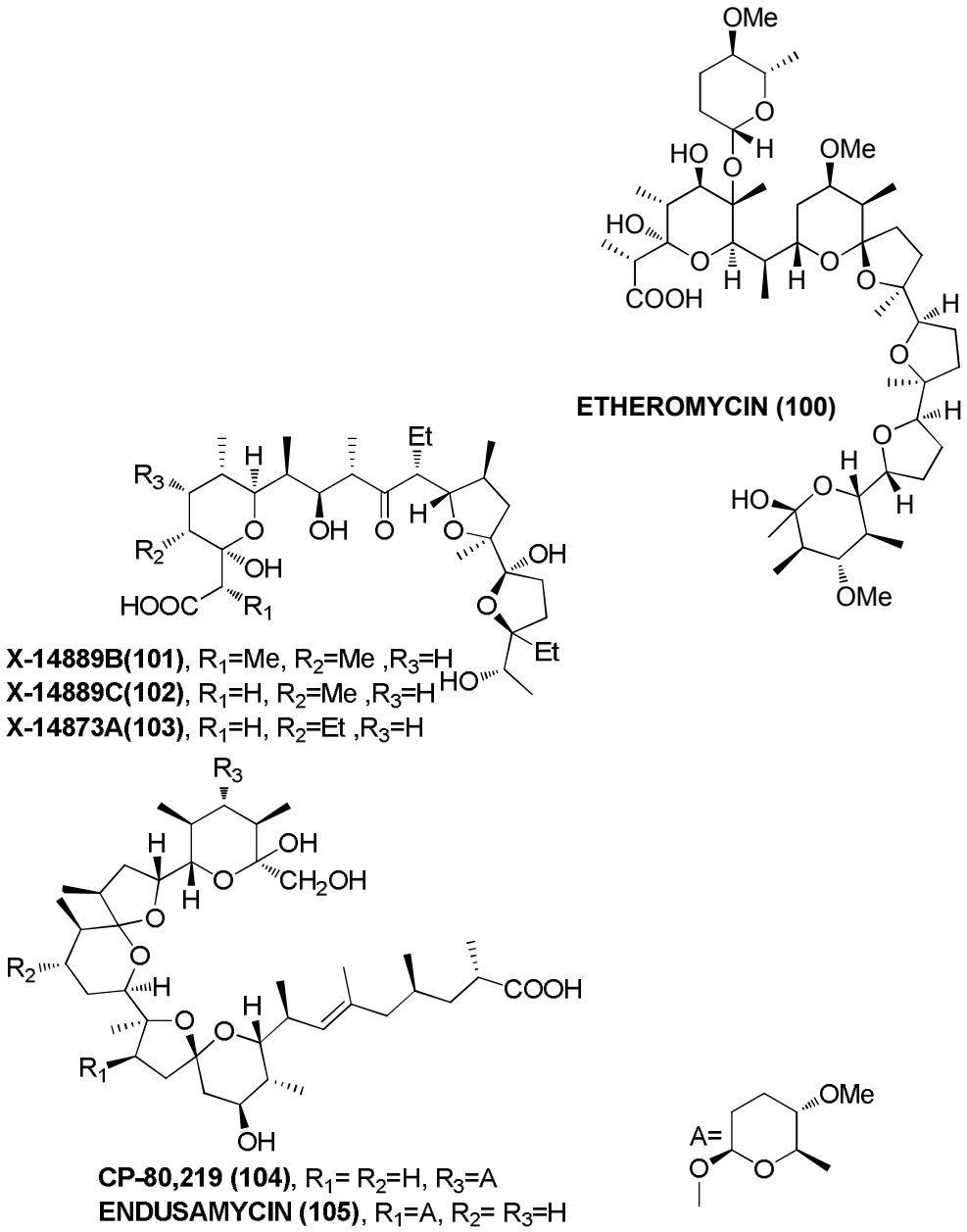
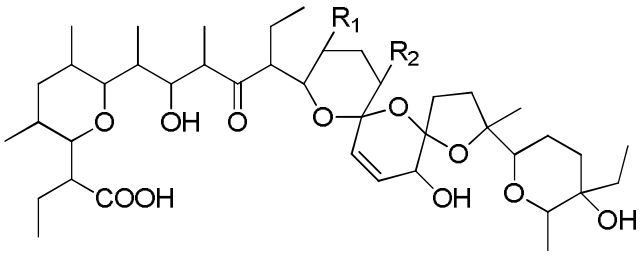
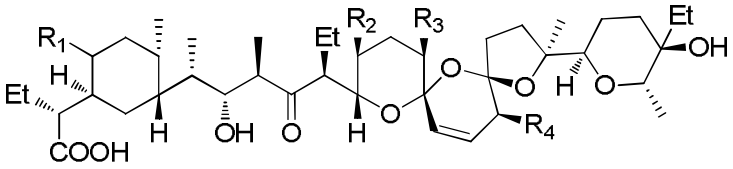


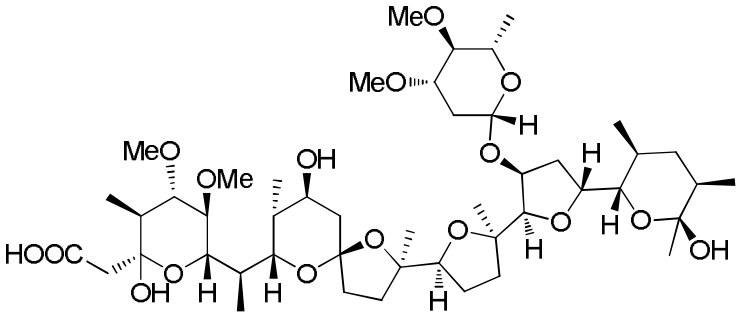
Figure 1.1. (cont.) Structures of carboxylic polyethers



NARASIN B (106) $R_1=R_2=CH_3$, $R_3=$ =O
NARASIN D (107) $R_1=CH_3$ or Et, $R_2 = CH_3$ or Et, R_3= OH



ANTIBIOTIC SY-1 (108), R_1=H, $R_2= R_3=CH_3$,R_4=H
A-28086D(109) $R_1=CH_3$, $R_2=CH_3$, R_3=Et,R_4=OH
A-28086D (110) $R_1=CH_3$, $R_3=CH_3$, R_2=Et,R_4=OH



MADURAMYCIN (111)

Figure 1.1. (cont.) Structures of carboxylic polyethers

2.4 Antiviral activity

From the literature reviewed, it can be gathered that carboxylic polyethers possess several features that could qualify them as promising anti-HIV-1 leads. Although none of the reports reviewed assessed the potency of these molecules alongside clinically used drugs the reported IC_{50} values against HIV-1 targets are significant. These molecules are also reported to have the potential to inhibit the replication of the virus in key cells such as CD4-expressing lymphoid cells and

mononuclear phagocytes. In addition, carboxylic polyethers have proven to be effective against acute, as well as chronic infections in vitro. The effective dose and toxicity level varied from molecule to molecule but a few molecules have proven effective at nontoxic concentrations. As a group, carboxylic polyethers do not belong to a specific class of known anti-HIV drugs. Some carboxylic polyethers such as monensin (**17**) and kijimicin (**54**) were evaluated as protease inhibitors, with respect to their interference with posttranslational modifications of HIV-1 proteins. It is well established that HIV-1, which mutation rate is extremely high, readily develops resistance to protease inhibitors by altering the active site of the targeted protease enzyme. Whether carboxylic polyethers that interfere with posttranslational modifications of HIV-1 proteins will share this fate is not well established at this time due to the limited understanding of the exact sequence of events through which compounds such as monensin (**17**) and kijimicin (**54**) impair posttranslational modifications. None of the studies reviewed investigated the effectiveness of these carboxylic polyethers in an animal model.

2.4.1 Activity against HIV-I-AIDS

Several reports describe the potential of carboxylic ionophores: monensin (**17**), salinomycin (**19**), lasalocid (**16**), nigericin (**19**), dianemycin (**35**), alborixin (**26**), kijimicin (**54**), SF2361 (**58**), SF2324 (**59**), SF2487 (**60**) and Laidomycin (**44**) as anti-HIV agents [86-91] [40, 54, 127, 128, 168, 169].

Nakamura *et al.* [40] assessed the effect of 10 carboxylic ionophores, monensin (**17**), salinomycin (**19**), lasalocid (**16**), nigericin (**19**), dianemycin (**35**), alborixin (**26**), kijimicin (**54**), SF2361 (**58**), SF2324 (**59**) and SF2487 (**60**), on the replication of HIV-1.

All but SF2324 (**59)** showed a dose-dependent inhibition of the virus replication. Apart from SF2324 (**59**), the concentration that inhibited reverse transcription activity, viral replication, by 50%, EC_{50}, varied in the range 0.40 - 0.040 to 7.12 - 4.4 µg/mL. The IC_{50} and EC_{50} of SF2324 (**59**) in H9 cells (primary infection) was determined to be > 100 µg/mL, the highest tested concentration. The IC_{50} of the remaining compounds varied in the range 6.98 ± 2.9 to 78.40 ± 4.7 µg/mL (Table 1-2). For these compounds, an inhibitory effect at nontoxic doses was observed. The ratio IC_{50} /EC_{50} varied in the range 3.18 – 76.72 (Table 1-2). The chronically infected cells, U937, were sensitive to all but SF2487, but a higher cytotoxicity was observed as shown by the low IC_{50} /EC_{50} ratio, which varied here from 1.18 to 8.32 (Table 1-2) [40, 127].

It is believed that all carboxylic ionophores do not share a common MOA against HIV. These compounds target different stages of the HIV infective cycle, that is, the pre- and post-absorption steps [40, 54, 127, 128, 169]. Nakamura *et al*. [40] using a time of addition design partitioned these agents in two main groups on the basis of their MOA. These authors exposed H9 cells to these molecules before, during and after viral absorption. The first group, believed to inhibit viral absorption was made of lasalocid (**16**), dianemycin (**35**), SF2361 (**58**), SF2324 (**59**), SF2487 (**60**) and alborixin (**26**). The second group, believed to interfere with post-viral adsorption events, was made of monensin (**17**), salinomycin (**19**), lasalocid (**16**), nigericin (**20**), Dianemycin (**35**), alborixin (**26**), kijimicin (**54**), SF2361 (**58**), SF2324 (**59**) and SF2487 (**60**) [40]. Yamauchi *et al.* [128] working specifically with kijimicin (**54**), tested at concentrations of 0.08 – 10.0 µg/mL confirmed its lack of ability at interfering with the absorption step, as well as early stage of the replication, such as integration. It was proposed that kijimicin (**54**) acted by

decreasing the infectivity of the virus, which was possibly accomplished by means of incomplete glycosylation of gp120 [128].

A dose-dependent study of the effect of monensin (**17**) on chronically infected MOLT-3/HTLV-IIIB revealed a marked inhibitory effect on the proteolytic processing of gpl60 to gpl20, after 7 h of exposure at 10 μM [54]. This effect was specific as no change on the protein level of gag proteins Pr53 gag and p24 was noted. No effect on the expression of tat, vif and nef gene products was observed, as well. Monensin (**17**) also showed a marked reduction in the formation of syncytia at concentration > 3 nM when MOLT-3/HTLV-IIIB cells were co-cultured with CEM cell. Both effects were reversible upon removal of monensin (**17**) [54]. All these findings were later confirmed by a second group [169]. Laidlomycin (**44**) was also reported to induce a dose-dependent inhibition of HIV replication. The MOA of laidlomycin (**44**) was proposed to be via inhibition of gp120 expression. Laidlomycin (**44**) also induced an inhibitory effect on syncithium formation at concentrations as low as 1 μg/mL [85].

2.4.2 Activity against other types of viruses

Many carboxylic ionophores are reported to be active against several other DNA and RNA viruses: monensin (**17**), monensin B (**61**), nigericin (**20**), narasin (**18**), lasalocid (**16**), X-206 (**47**), septamycin (**22**), A 28695B (**62**) and A204 (**63**) were tested against transmissible gastroenteritis coronavirus, Newcastle disease virus (NDV), infectious canine hepatitis virus and infectious bovine rhinotracheitis virus [9]. All these agents showed a significant activity against transmissible gastroenteritis coronavirus, MIC ranging from 0.005 to 0.25 μg/mL. Nigericin (**20**), narasin (**18**) and A28695B (**62**)

were effective against NDV with MIC ranging from 0.02 to 2.0 µg/ml. Only septamycin (**7**) was active against infectious canine hepatitis virus (MIC = 0.32 µg/mL). Monensin B (**61**), nigericin (**20**), narasin (**18**), X-206 (**47**), septamycin (**22**) and A204 (**63**) were effective against the infectious bovine rhinotracheitis virus, MIC ranging from 0.005 to 0.08 µg/mL [9].

2.5 Cytotoxicity and anticancer

Resistance to chemotherapy is a common clinical problem in patients with cancer. During treatment, tumor cells are often found to be refractory to a variety of drugs with different structures or modes of actions. These cancers are referred to as MDR forms. Most multidrug resistances are caused by *trans*-membrane xenobiotic transport proteins belonging to the superfamily of ATP-binding cassette (ABC) transporter efflux pumps [170, 171]. Carboxylic polyethers are interesting antineoplastic drug candidates for several reasons; they possess potent anticancer activity in vitro and in vivo. Some carboxylic polyethers such as nigericin (**5**) have proven to be selectively cytotoxic, inhibiting DNA replication of tumor cells in vivo. Another feature that makes carboxylic polyethers even more appealing as antineoplastic drug candidates is their ability to reverse multidrug resistance in human carcinoma. Carboxylic polyethers are reported as chemosensitizing agents. These molecules selectively increase the sensitivity of cancerous cells, but not normal cells, to several cytotoxic agents of which the clinically used anticancer drug paclitaxel [172].

Kawada *et al.* [57] evaluated the effects of several carboxylic ionophores on colchicine resistance in human carcinoma MDR KB-C410 [57]; these compounds were

inostamycin (**39**), lysocellin (**30**), laidlomycin (**44**), monensin (**17**), dianemycin (**35**), leuseramycin (**41**), nigericin (**20**), lonomycin A&C (**32** , **37**), carriomycin (**23**), desoxy-salinomycin, antibiotic 6016 (**38**), SF2324 (**59**), SF2361 (**58**) and SF2487 (**60**). It was discovered that the cancer cells responded better to colchicine when some polyethers were added simultaneously. Laidlomycin (**44**), monensin (**17**), dianemycin (**35**) and leuseramycin (**41**) led to > 100-fold potentiation of colchicine cytotoxicity. The most potent compound was laidlomycin (**44**); at 0.3 and 1 µg/mL, it potentiated the cytotoxicity of KB-C4 cells by ~ 725-fold [57]. Compared to tumor cells, the potentiation of normal cells was negligible; the ratio of IC_{50} in the absence of polyether versus the IC_{50} in the presence of polyether only varied in the range 0.4 – 2.4 µg/mL. Inostamycin (**39**) was also reported to have a chemosensitizing effect on paclitaxel in Ms-1 cell, small cell lung carcinoma.

Table 1-2 HIV-1 infectivity and cytotoxicity

	Acute infection (H9)			Chronic (U937)		
	EC_{90} (µg/mL)	IC_{50} (µg/mL)	IC_{50}/EC_{90}	EC_{90} (µg/mL)	IC_{50} (µg/mL)	IC_{50}/EC_{90}
Compounds						
Monensin (**17**)	4.22 ± 2.6	13.44 ± 2.8	3.18	0.5 ± 0.25	0.59 ± 0.19	1.18
Salinomycin (**19**)	0.4 ± 0.04	6.98 ± 2.9	17.63	0.09 ± 0.02	0.28 ± 0.05	3.14
Lasalocid (**16**)	2.37 ± 2.9	48.80 ± 4.7	20.63	0.2 ± 0.2	1.35 ± 0.35	6.92
Nigericin (**20**)	0.68 ± 0.46	14.46 ± 8.0	21.26	0.05 ± 0.33	0.4 ± 0.007	8.32
Dianemycin (**35**)	7.12 ± 4.4	78.40 ± 4.7	11.01	1.1 ± 0.14	1.1 ± 0	1.00
SF2361 (**58**)	0.64 ± 0.36	49.10 ± 4.6	76.72	1.35 ± 0.49	2.1 ± 0.14	1.56
SF2324 (**59**)	> 100	> 100	> 1.00	3.7 ± 0.71	10.1 ± 0.14	2.73
SF2487 (**60**)	0.49 ± 0.18	7.8 ± 1.3	15.92	0.03 ± 0.15	1.85 ± 0.28	64.91
Alborixin (**26**)	2.5 ± 1.1	13.50 ± 3.1	5.4	0.12 ± 0.04	0.80 ± 0.28	6.53
Kijimicin (**54**)	1.63 ± 1.9	18.62 ± 11	11.41	0.1 ± 0.007	0.55 ± 0.07	5.79
Dextran Sulfate	< 1	> 100	> 100	NT		

EC_{50}: Concentration that inhibits reverse transcription activity by 50% (viral replication); IC_{50}: Concentration that decreases cell viability by 50%. From Nakamura *et al.*, 1992 [40].

This effect was specific to these types of cells and to paclitaxel, as no potentiation was noted with the other tested agents, driamycin, vinblastine, methotrexate, cisplatin, etoposide and camptothecin [172]. Inostamycin (**39**) was also reported to reverse multidrug resistance in these cell lines. Using radiolabeled vinblastine, Kawada *et al*. [57] established that MDR cells treated with inostamycin (**39**) (0.5 – 2 µg/mL) accumulated the drug. Inostamycin (**39**) (1 µg/mL) acted by inhibiting drug efflux [57].

Baibakov *et al.* [73] and Margolis *et al.* [173] studied the antineoplastic MOA of nigericin (**20**) and established that nigericin (**20**) was a unique type of cytostatic agent. The compound (**20**) acts not by interfering with spindle microtubules or transcription/translation processes directly, but rather by altering intracellular pH [73, 173]. Nigericin (**20**) showed anticancer potential (cytostatic agent) in Ehrlich ascites carcinoma cells. The proposed MOA related to its ability to acidify the intracellular environment, a consequence of its H^+ /K^+ anti-transport ability [173]. It is believed that by decreasing the pH, DNA synthesis is halted. Nearly 100% inhibition of the DNA synthesis was achieved at 0.5 µM nigericin (**20**), with the pH of the incubation medium being 7.0 [173]. An in vivo method, using tridimensional histocultures of human lung tissues, with normal cells present in the same histoculture, determined that this antineoplastic effect of nigericin was selective. At 1µM of nigericin (**20**) neither the histology of normal cells nor their DNA synthesis was negatively affected. In cancerous tissues however, the same concentration of nigericin (**5**) halted the DNA synthesis in 67% of the cases, induced pyknotic nuclei and dystropic alteration in 27% of tumors exposed to nigericin (**20**) and necrosis in 13% of cases. Another carboxylic polyether,

ionomycin (**27**), is also reported to possess antineoplastic properties. It induced inhibition of the growth of a human bladder cancer cell line, HT1367, in vitro in a dose (0.1 – 100 µg/mL), and time dependent manner. The MOA here is reported to be the induction of apoptosis by means of the downregulation of Bcl-2 and upregulation of Bax. Ionomycin (**27**) was also effective in vivo, as it inhibited HT1367 tumor's growth [90].

2.6 Cardiovascular effects

Several carboxylic polyethers were reported to show ionotropic, chronotropic and hemodynamic properties. Because of the critical role of Ca^{2+} in cardiovascular contractile systems, this was predictable at least for those carboxylic polyethers that are selective for this ion, such as lasalocid (**16**) and A23187 (**28**). It is well established that lasalocid (**16**), also known as X-537A, is a positive ionotropic agent. In canine studies, lasalocid (**16**) was able to increase the contractility index of the heart by approximately threefold at 2 mg/kg [10, 13]. X-537A is also reported as a positive ionotropic and chronotropic agent in dogs. In barbiturated dogs, at dosage 2 mg/kg, it caused a threefold increase in the contractility, as well as a modest rise in aortic pressure and heart rate. It also induced a drop in total peripheral resistance followed by a drastic increase in blood flow through the coronary arteries of the left ventricle [10]. The exact MOA through which these carboxylic polyethers induce contractility of cardiac muscle is still unclear. Initially, it was believed that this could be the result of their ability to shuttle Ca^{2+} and catecholamine across membranes. This was refuted after the discovery that carboxylic polyethers specific to monovalent cations were able to induce, even more effectively, this type of response. Nonetheless, it is believed that catecholamine is partially involved because the effect of these molecules on the cardiac muscle is

suppressed by α adrenergic inhibitors.

Several other carboxylic ionophores were reported to possess a cardiovascular effect. Monensin (**17**) reportedly induced an increase of arterial blood pressure, myocardial contractility and total peripheral resistance when administered intravenously to anesthetized cats at a dose of 0.075 – 0.375 mg/kg [60]. A similar effect was observed when 0.125 – 2.00 mg/kg were administered via the same route to anesthetized dogs [60]. Monensin (**17**) was also effective on isolated organs; it showed a vasoconstrictor effect on isolated rabbit aortic strips at concentration ≤ 7 µg/mL and a myocardial stimulation effect in isolated rabbit hearts [60].

Three other carboxylic ionophores, grisorixin (**31**), alborixin (**26**), Ionomycin A (**32**), are reported to have similar effects. Grisorixin (**31**) induced a coronary vasodilator effect in dogs at doses of 60 µg/mL; this was associated with a marked increase in the coronary blood flow, which reached a maximum at this concentration. At doses of 125 – 500 g/kg, further inotropic and hypertensive effects were observed [174]. Grisorixin (**31**) was also reported to have a cardiotonic effect on isolated perfused rat hearts [100]. In guinea pigs, grisorixin (**31**) and alborixin (**26**) 4 mg/kg/min i.v. are reported to increase blood pressure. The MOA is believed to be linked to their intrinsic ionophorous potential: the ability of the molecules to alter the blood concentration of cations such as K^+ and Na^+ [101]. Moins *et al.* [175] working with anesthetized dogs reported similar findings, as 2 mg/kg of grisorixin (**31**) and 1 mg/kg of alborixin (**26**) increased the ventricular contractile force and systolic and diastolic arterial pressures. This was associated with an increase in the plasma concentration of K^+ followed by a decrease in the concentration of Na^+ [175]. Because it has been reported that compounds such as

grisorixin (**31)** are able to induce the release of catecholamine, the physiological response associated with the release of catecholamine was also proposed as a possible justification of the cardiovascular effects of these compounds[174]. Lonomycin A (**32**), another carboxylic polyether, was also reported to induce a coronary vasodilatation effects in dogs. Its MOA was proposed to be the stimulation of the Na^+, K+-ATPase activity [105].

As stressed by Pressman, and despite their toxicity, carboxylic polyethers are important drug leads for control of various cardiovascular conditions. Their value lies in their outstanding ionotropic, chronotropic and hemodynamic potentials. These molecules could prove life-saving tools in controlling conditions such as failure from myocardial infarction and other similar forms of shock. Carboxylic polyethers such as lasalocid (**16**) (1 mg/kg) were proven effective at restoring dogs in cardiogenic shock. Similar data were reported for salinomycin (**19**) (0.15 mg/kg). According to Pressman, the narrower the transport spectrum of a molecule, the fewer the number of associated undesirable effects [10].

2.7 Other biological activities of carboxylic polyethers: immunoregulatory, herbicidal and anti-inflammatory

2.7.1 Immunoregulatory activity

Two measures are usually adopted to protect poultry against a variety of etiological agents such as Angara disease virus (ADV) and Newcastle disease virus **(**NDV), and to promote growth. These include vaccination and addition of substances such as antibiotics, coccidiostat and vitamins to feed. Among additives currently

approved for use are several carboxylic polyethers (lasaloicid (**16**), monensin (**17**), salinomycin (**19**) and manduramycin (**50**)). There have been some recent concerns over the possible implication of additives in vaccine failure in poultry. A few reports have actually proven that therapeutic agents such as cyclophosphamide and corticosteroids possess immunomodulatory properties. Munir *et al.* in 1994 and 2007 studied the immunomodulatory effects of salinomycin (**19**) and monensin (**17**); specifically, the effects of these molecules on the protective immune response in NDV and ADV vaccinated chickens [59, 176]. These authors established that unlike cyclophosphamide, salinomycin (**19**), at a dosage of 0.1 g/kg, did not adversely affect the bursal, splenic, thymic and liver weight gain [176]. It was also established that NDV vaccinated chickens that received salinomycin (**19**), 60 mg/kg of feed, had higher antibodies titers at days 14, 21, 28, 35, 42 compared to unmedicated chickens [59, 176] or vaccinated chickens that were medicated with monensin (**17**) 12 mg/kg instead [59]. These salinomycin medicated chickens also gained significantly more weight, $p < 0.05$, compared to all the other groups evaluated. These chickens were challenged with both ADV and NDV after vaccination. No post-ADV or post-NDV challenge mortality was observed in vaccinated chickens that received salinomycin (**19**) and no clinical sign of the disease was observed [59, 176].

From these studies, it seemed that far from causing vaccine failure, carboxylic polyether such as salinomycin (**19**) actually seem to have a boosting effect on the anti-NDV and anti-ADV immune response [59, 176]. Another carboxylic polyether reported as having immunoregulatory potential was ionomycin (**27**), a calcium-specific ionophore. Ionomycin (**27**) interferes with physiological processes by increasing the intracellular

level of calcium, an effect that could have several implications on key cells of the immune system such as neutrophils and macrophages. They exert their action by production of microbicidal species such as superoxide anions via an NADPH-dependent process known as 'respiratory burst' in response to *in situ* triggers such as inflammatory agents. Ca^{2+} is documented as an enhancer of NADPH oxidase activity. Finkel *et al.* [177] reported that ionomycin (**27**) at a concentration of 1 – 10 nM can prime neutrophils to release approximately sevenfold more superoxide anions during stimulation. Ionomycin (**27**) was also reported to induce T cell activation [177] .

2.7.2 Herbicidal activity

It has been reported that nigericin (**20**) also possess herbicidal properties. A concentration-dependent study of the effect of nigericin (**20**) on the growth of the radicle of garden cress seed showed a dose-dependent inhibitory effect. A 50% reduction in the elongation was achieved at 1.3 – 2.0 µg/mL; the maximum inhibition was reported at ~ 3.33 µg/mL. Nigericin (**5**) did not induce browning or necrosis of tissues in the process [20].

2.7.3 Anti-inflammatory activity

Dianemycin (**35**) was recently reported as having topical anti-inflammatory potential on several animal models for cutaneous inflammation. In a croton-oil induction of ear edema, dianemycin (**35**) had a comparable activity to prednisolone, a potent steroidal anti-inflammatory. In the UV-induced erythema test or in the delayed type sensitivity test, no anti-inflammatory activity was observed. Additionally, no acute toxicity was observed with topical application ≤ 10 mg/ear [21].

3. Toxicity

Acute toxicity of the naturally occurring polyether ionophores is relatively specific to the affected organism. It is clear that minimal exposure to these very active compounds initiates some type of physiological response [13]. In the case of higher organisms and specifically mammals and birds, these effects are almost equally diverse [9]. For example, a common poisoning scenario is a horse consuming a small quantity of feed prepared with a ruminant's polyether antibiotic and dying rather quickly, or wild turkeys consuming anticoccidial chicken feed and dying. Although the ionophoric specificity of molecules can be predicted, respective acute toxicity values are diverse and unpredictable. For example, LD_{50} ± SE values for an acute oral dose of monensin (**17**) were: mouse (m) 70.0 ± 9.0 mg/kg, (f) 96.0 ± 12.0 mg/kg; rat (m) 40.1 ± 3.0 mg/kg, (f) 28.6 ± 3.8 mg/kg; dog (m) > 20.0 mg/kg, (f) > 10.0 mg/kg; rabbit 41.7 ± 3.6 mg/kg; monkey > 160.0 mg/kg; chicken 200.0 mg/kg; cattle 26.4 mg/kg; sheep 11.9 ± 1.2 mg/kg; goat 26.4 ± 4.0 mg/kg; swine 16.7 ± 3.57; horse 2 – 3 mg/kg; trout > 1000.0 mg/kg [9, 13].

Dogs given a daily oral dose of 0, 1.25, 2.5, 5, or 7.5 mg/kg of monensin (**17**) for 1 year survived with no evidence of toxicity in the two lowest dose groups. Four generations of rats were continuously maintained on diets containing 0, 33, 50, or 80 ppm of monensin (**17**)… except for a decrease in body weight gain, there were no compound-related effects on reproduction or observed teratogenic effects" [77]. These same dosing conditions were evaluated for a 2-year period, with respect to the skeletal and cardiac muscles, with no increase in chronic lesions or neoplasms being detected [77]. Despite these maverick chemo-physical qualities of the ionophores, considerable

numbers of toxicology studies have attempted to illustrate/translate the specific toxicology and pharmacology of the most common polyether ionophores to human health utilization [13].

4. Cardiac toxicity

Whether a monovalent or divalent ionophore is used, these molecules will disrupt the Ca^{2+} concentrations, either forming Ca^{2+} complexes or depolarizing the membrane potential, changing the H^{+} concentrations in the mitochondria [12]. It is clear that Ca^{2+} is an essential ion in the physiological activities of composite functionality in the entire human body's daily functions. Cardiac function is especially sensitive to the roles of Ca^{2+} owing to the intrinsic functions of the ion and its role in muscular contractions; so, expectedly, potent effects of polyether antibiotics have been observed in mammalian cardiac studies [10, 73, 78, 106, 126, 129]. Canine studies are accepted as the translative animal model for human cardiac response and for the effects of carboxylic polyethers. These studies have shown that as little as 2 μg/kg i.v. can induce coronary dilation [86, 106]. The concern for clinical applications of these ionophores is that people with coronary heart disease who have coronary arterial dilation, as part of the hearts own auto regulatory response to occlusion of coronary arterial vessels, will dilate the affected vessels further in the presence of an indiscriminate vasodilator. The unaffected vesicles would dilate further and the already maximally dilated obstructed vessels would divert the blood supply away from the ischemic regions of the myocardium. This phenomenon is called 'coronary steal' and has been observed in the administration of the drug dipyridamole, a thrombus inhibitor and chronic vasodilator [88, 101]. As a high percentage of the population is affected by coronary heart disease

these concerns have validity, although this phenomena was experimentally evaluated and the coronary steal was not observed [88]. Only recently have the in vitro tools been available for cardiac toxicity evaluations with respect to interaction on the *hERG* (human Ether-a-go-go Related Gene) gene and the associated Kv 11.1 potassium ion channel that depolarizes the IKr current in the cardiac action potentials. The polyether antibiotics have not yet been critically evaluated in hERG assays. The drug interactions related to hERG ion channel were not mandated by ICH guidelines until November 2005 (mandate #CHMP/ICH/423/02). Consequently, the effects of carboxylic polyether ionophores on the QTc, with respect to the interaction with hERG have not yet been evaluated.

5. Conclusion

Carboxylic polyethers are broad spectrum antibiotics. They are active against a wide range of biological targets: bacterial, fungal, protozoan, viral, neoplastic and cardiovascular. A few authors have also reported carboxylic polyethers as immunomodulating agents. In vitro, these molecules have proven to be effective at very low concentrations. The malaria parasite (*P. falciparum*) is especially sensitive to several of these compounds with IC_{50} values in the nanomolar range. Based on the reports in this review it can be argued that carboxylic polyethers are highly selective. Unlike Gram negative bacteria or fungi for instance, Gram positive bacteria tend to be extremely sensitive to these antibiotics. Moreover, the IC_{50} values of several of these molecules against mammalian cells tend to be significantly higher, when compared to the IC_{50} against targeted etiological agents.

Despite these interesting features, the only current application for carboxylic polyethers is in veterinary medicine, where they are used as controls for coccidiosis and feed absorption efficiency. The main obstacle for the use of carboxylic polyethers as drugs to control human diseases seems to be the issue of toxicity. Carboxylic polyethers possess some interesting qualities against serious threats such as MRSA and VRE and have proven to be more effective (in vitro) than drugs that are currently used clinically, such as vancomycin and oxacillin. They have also shown to be effective against several other resistant bacterial strains including penicillin resistant strains of *S. aureus,* tetracycline resistant strain of *Micrococcus* sp, aminoglycoside resistant strain of *Streptococcus faecalis*, and macrolides resistant strain of *Sarcina lutea*. Carboxylic polyethers have also proven effective at nanomolar range against CQ resistant strains of *P. falciparum* and are more potent against these resistant strains than several currently marketed antimalarial drugs. Their mechanism of action against the malaria parasite appears uniquely different from that of the currently utilized drugs. This makes them possible candidates for use in combination therapy.

6. Expert opinion

On the basis of our assessment of the literature, polyether ionophores show a high degree of promise for the potential control of drug-resistant bacterial and parasitic infections. Many of these infectious diseases fall under the umbrella of neglected disease with limited options for therapeutic intervention. Furthermore, recent decades have emphasized the importance of combination therapies for the long-term control of disease and mitigation of resistance. The long history of the utilization of polyether ionophores as growth promoting antibiotics by the agricultural industry validates the

potential cost-effective use of these antimicrobial agents for other applications. The polyether antibiotics seem to provide reasonable oral availability, half-life, metabolic and general stability, absence of a UV sensitizing chromophore combined with significant potency. Despite the long history of use of the class very limited medicinal chemistry and drug optimization studies have been reported leaving the door open to these opportunities in the future. In many instances, the potency and in vivo efficacy for the control of bacterial and parasitic infections is truly remarkable.

However, concerns about toxicity need to be addressed with further optimization and preclinical evaluation. On the basis of the available literature, there is still insufficient data to clearly classify these molecules as either too toxic or safe as a class. Several carboxylic polyethers have indeed been shown to be too toxic in animal models for malaria, but the results cannot be generalized to the entire class. Using an in vivo model for cancer, nigericin (**20**) was recently reported to be highly selective in targeting cancerous cells, broadening the scope of possible utility for this class [80]. As a result, an organized and carefully orchestrated identification and optimization of leads using in vitro models followed by an in vivo assessment of toxicity and efficacy would seem to yield viable drug candidates for the control of drug-resistant infectious diseases and cancer with a reasonable understanding of pharmacokinetics, stability and cost of goods. The reported toxicity for monensin (**17**) of > 160.0 mg/kg (LD_{50}) in primates and frequent utilization of this drug in agriculture makes the polyether class an attractive group of molecules for optimization and development of human health applications for the control of drug-resistant diseases such as malaria and MRSA.

Chapter 2 : ANTIMALARIAL, ANTI-MTB AND ANTI-MRSA METABOLITES FROM A MARINE *STREPTOMYCES*

PART 1 - A NEW ANTIMALARIAL POLYETHER FROM A MARINE *STREPTOMYCES* SP. H668

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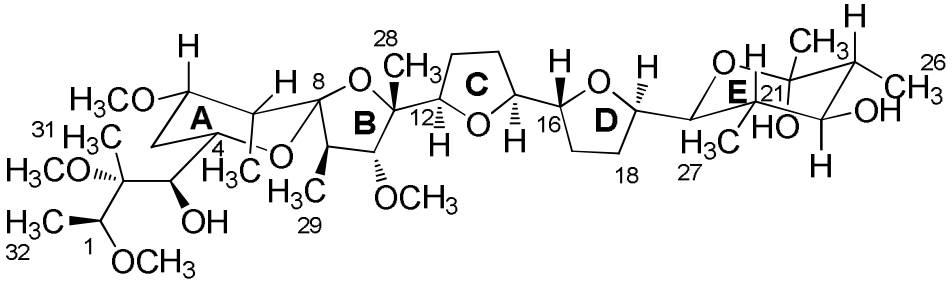
Authors: Na M, Meujo DA, Kevin D, Hamann MT, Anderson M, Hill RT

ABSTRACT - The antimalarial-guided fractionation of the crude extract of the marine *Streptomyces* sp. strain H668 led to the isolation of a new polyether (**5**). This new metabolite showed in vitro antimalarial activity against the CQ-susceptible (D6) and resistant (W2) clones of *Plasmodium falciparum*, without cytotoxicity to normal cells (Vero) making it a promising drug lead.



5

Malaria is a tropical infection caused by four different species of protozoa of the genus *Plasmodium* transmitted to humans by the Anopheles mosquito. During past decades, CQ and other aminoquinolines have been utilized as frontline antimalarial agents. However, an increase in drug resistance in *Plasmodium falciparum* has made it essential to develop new chemotherapeutic agents in addition to combination therapies utilizing available antimalarial drugs with different modes of action [178]. Recently, some specific polyether antibiotics have been reported to have potent antimalarial activity [37, 51, 111, 153]. K41-A, a carboxylic acid containing polyether antibiotic produced by *Streptomyces hygroscopicus* has been reported to exhibit nanomolar range in vitro antimalarial activity against the *P. falciparum* strains K1 (drug resistant) and FCR3 (drug sensitive) [153]. Furthermore, it showed high selectivity in vivo against *P. berghei* strain N and *P. yoelii* strain NS-infected mice, when administered orally [153]. Several other polyethers from this class (ionomycin A, nigericin, and monensin, among others) have been identified as potent antimalarial agents, as well [37, 51].These compounds are classified as ionophores due to their potential to interact with ions (cations); this is believed to be their putative mechanism of action [37, 51]. There are two main classes of ionophores: mobile carriers or true ionophores which bind cations into an oxygen rich pocket in order to facilitate their transport across the cell membrane and quasi-ionophores which, instead, form trans-membrane channels through which cations flow. Compounds of the latter group possess a polycyclic alkyl backbone which confers their lipophilic character. They also possess a terminal carboxylic group which plays an important role in the formation of the oxygen rich internal cavity which interacts with metal ions [37, 51, 179].

Bacteria, especially *Streptomyces* sp. isolated from soil samples are reported as the primary producers of this type of compounds, with at least 21 producer strains reported thus far [180]. Reports concerning the isolation of these compounds from marine *Streptomyces* are scarce; a few examples include aplasmomycin [181], arenaric acid, and oxolonomycin [182, 183].

H668 was isolated from Hawaiian marine sediments and cultured in ISP2 medium. It was established that its crude extract possessed potent in vitro activity against *P. falciparum* and no significant cytotoxicity to Vero cells. H668 was identified as a *Streptomyces* sp. by 16S rRNA analysis. A bioassay-guided fractionation of the EtOAc-soluble fraction of the H668 culture led to the isolation of a new polyether metabolite. In this report, we describe the isolation and structure elucidation of this metabolite.

The antimalarial active fraction eluting with 70% MeOH in H_2O using reversed phase C_{18} VLC was further fractionated by preparative HPLC [Phenomenex C_8 column (21.2 × 250 mm); flow rate 5 mL/min] using a gradient from 50% to 100% MeOH in H_2O over 80 min. The active fractions were further purified by preparative HPLC [Phenomenex C_{18} column (21.2 × 250 mm) at 5 mL/min] eluting with a gradient from 70% to 100% MeOH in H_2O, to afford a semipure metabolite. Purification of this semipure compound by HPLC [5 micron C_{18} (10 × 250 mm); flow rate 3 mL/min] using an isocratic solvent of 75% MeOH in H_2O gave 3.0 mg of the pure metabolite **5**.

The ^{1}H NMR spectrum of **5** indicated the presence of eleven oxymethine hydrogens (3.1 – 4.3 ppm), four methoxy groups (3.2 – 3.6 ppm), eight methyls (0.9 – 1.3 ppm), and five methylene (1.2 – 2.2 ppm). This was supported by the ^{13}C and DEPT NMR spectroscopic data. In addition, two oxygenated quaternary carbon signals at δ_C 82.8 and 78.4, as well as two ketal (or hemiketal) signals at δ_C 100.9 and 106.2 were observed. Four independent spin systems were identified by a COSY experiment, in which a sequential correlation from H-12 to H-23 and two methyl signals at C-26 and C-27, were observed, along with correlations for H-9 and H-10, H-3 through H-7, and H-1 and one methyl group at C-1. Several ambiguous correlations in the methylene area of the COSY data were identified based on the analysis of HSQC data. From the HSQC data, each protonated carbon for the partial structures could be assigned as shown in Table 1-1. The HMBC data of **5** revealed correlations from H-12 (δ_H 3.89) to C-14 (δ_C 28.2) and C-15 (δ_C 81.7) and from H-15 (δ_H 3.74) to C-12 (δ_C 81.9), suggesting a structure of a tetrahydrofuran ring (ring C). In a similar fashion, HMBC correlations assisted in identifying ring D. Ring E was deduced from the HMBC analysis, where both the methyl protons at δ_H 0.97 (3H, d, J = 6.4 Hz, H-27) and 1.01 (3H, d, J = 6.4 Hz, H-26) showed correlations with C-22 (δ_C 74.6), and H-22 (1H, t, J = 10.0 Hz) exhibited 2- and 3-bond correlation with C-20 (δ_C 75.3), C-21 (δ_C 40.9), C-23 (δ_C 47.8), and C-24 (δ_C 100.9), along with a correlation from the methyl signal at δ_H 1.30 (3H, s, H-25) to C-24. Moreover, the HMBC correlations from H-12 to C-11 (δ_C 82.8) and C-10 (δ_C 94.8), as well as from neighboring protons (H-10, H-9, H-7, and H-6) to the ketal carbon at δ_C 106.2 facilitated the construction of a spiroketal analog linked with ring C. These results suggest that compound **5** is a polyether type metabolite. Comparison of the NMR data

of **5** with those of polyether compounds previously reported supported the NMR assignments for the rings A – D [146, 182, 184, 185]. However, the residual part was quite different from reported polyethers. The biggest difference was the lack of a carboxylic group in **5**. COSY data confirmed the linkage of H-3 (δ_H 4.29, 1H, d, *J* = 10.0 Hz) and H-4 (δ_H 4.10, 1H, br s), and of H-1 (δ_H 3.45, 1H, m) and H-32 (δ_H 1.02, 3H, d, *J* = 6.4 Hz). The HMBC correlations from H-3 to C-2 (δ_C 78.4), as well as to the methyl carbon at δ_C 11.6 revealed the connection from C-1 to C-3 bearing two methyl groups (C-31 and C-32). In addition, two methoxy groups at δ_H 3.54 and 3.39 were identified and attached at C-1 and C-2, respectively, by the HMBC data analysis. Taken together, the structure of **5** was determined as shown in Figure 2-1, and all the ^{1}H and ^{13}C NMR data were assigned as in Table 2-1. The relative configuration of **5** was determined on the basis of ROESY NMR data. NOE correlations were observed as described in Table 2-1, which indicated that **5** has the same relative configuration in rings A-E as those of K41-A (Figure 2-2).

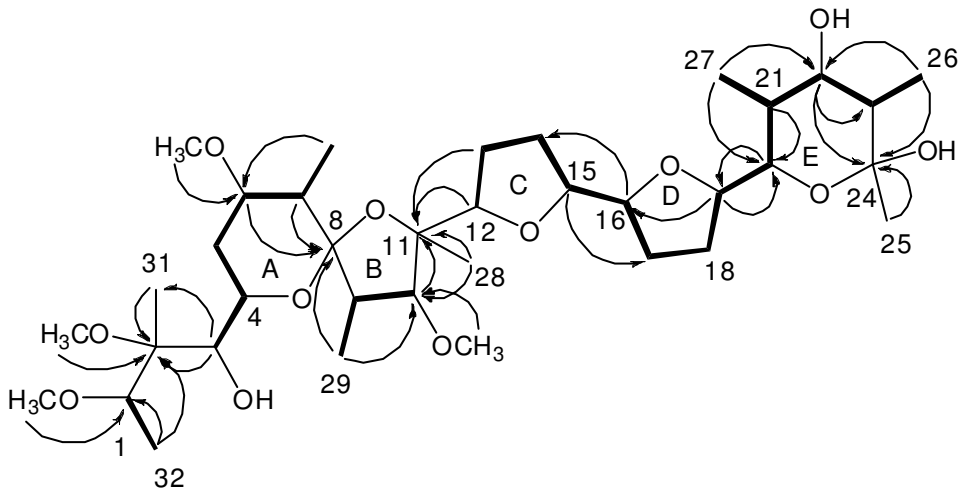


Figure 2-1. Spin system ^{1}H-^{1}H COSY data (—) and HMBC correlations of 5

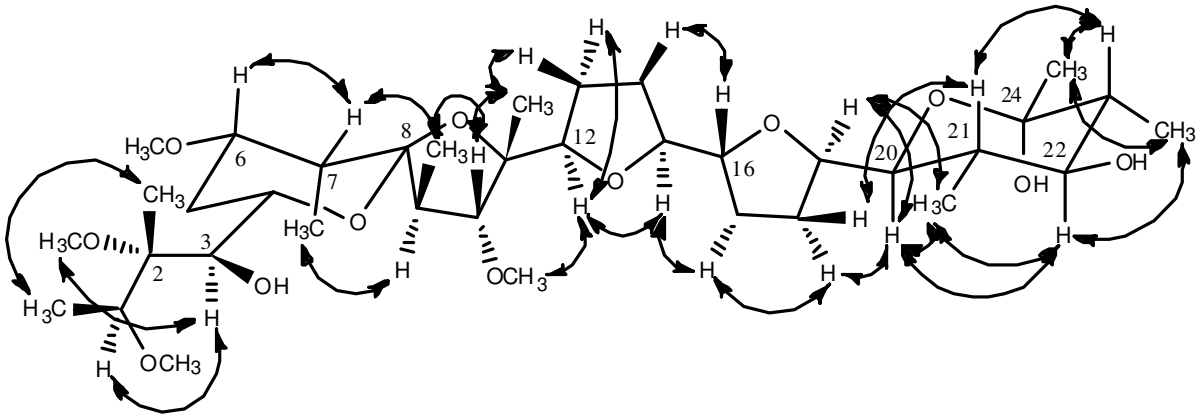


Figure 2-2. Key NOE correlations of 5

The antimalarial activity of the new metabolite (5) was evaluated against both the CQ-susceptible (D6) and -resistant (W2) clones of *P. falciparum*, and their toxicity was tested against Vero cells. Compound **5** showed antiprotozoal activity against both the D6 and W2 clones, with IC_{50} values ranging from 100 – 200 ng/mL. Although the in vitro antimalarial activity of **5** was significantly less than the original extract, no cytotoxicity was observed at 4.75 µg/mL, the highest concentration tested. The high index of selectivity ($SI = IC_{50\ Vero}/IC_{50\ \textit{P. falciparum}}$) of **5** indicates that the polyether metabolite **5** is highly specific to the parasite. As discussed earlier, the mechanism of action for ionophores (quasi-ionophore as mobile carier) is via alteration of normal membrane permeability to cationic species [37, 51, 186]. Ionophores (mobile carriers) possess unique structural features which are crucial to their ability to interact with metal species such as Na^+, K^+, and Ca^{2+} [37, 51, 56]; these include (1) an alkyl backbone (2) an internal oxygen rich pocket and (3) a terminal carboxylic group [179]. A few examples of

mobile cariers are: monensins A, B, C, nigericin, laidlomycin, grisorixin, mutalomycin, lonomycins A, B, X-206, alborixin, lenoremycin, dianemycin, carriomycin, septamycin, etheromycin, A-204A, K41-A, K41-B, and A-601612. All these compounds possess a terminal carboxylic moiety [186].

The polyether **5** isolated from the marine *Streptomyces* sp. H668 clearly belongs to a unique class of compounds. Although it is closely related to several members of the mobile carriers group, the fact that it lacks the carboxylic functionality, suggests that the mode of action against the parasite might be unique; this warrants additional investigation.

Table 2-1 NMR assignment of 5 in CD_3OD

	δ_C[a]	δ_H mult. (*J* in Hz)[b]	HMBC	ROESY
1	81.8[c]	3.45, m[c]	C-2, 1-OMe	H-3
2	78.4	/	/	/
3	66.6	4.29, d (10.0)	C-2, C-31	H-1, 2-OMe
4	61.4	4.10, br s	/	/
5	32.2	1.40, m[c]	C-7	/
6	78.6	3.34, m	C-4, C-8	H-7
7	36.7	1.71, m	C-5, C-8, C-30	H-6, H-29, H-30
8	106.2	/	/	/
9	45.6	2.06, m	C-8, C-10, C-29	H-10, H-29, H-30
10	94.8	3.43, d (9.6)[c]	C-9, C-12, C-28, C-29, 10-OMe	H-9, H-28, H-29
11	82.8	/	/	/
12	81.9[c]	3.89, t (7.2)	C-10, C-11, C-14, C-15, C-28	H-13a, H-15, 10-OMe
13	26.0	1.99, m 1.83, m[c]	C-11, C-12, C-15	H-13a, H-14b, H-28 H-12, H-13b
14	28.2	1.71, m[c] 1.87, m[c]	C-12, C-15, C-16	H-13b, H-14a, H-16 H-14b, H-15
15	81.7	3.74, q (6.4)	C-13, C-12 (or C-16), C-17	H-12, H-14a, H-16, H-17a
16	81.9[c]	3.99, q (6.4)	C-14, C-19	H-14b, H-15, H-17b
17	28.5	2.06, m 1.83, m[c]	C-15, C-19	H-16, H-17a H-15, H-17b, H-18a
18	24.8	2.00, m 1.91, m[c]	C-16, C-19, C-20	H-18a, H-21 H-17a, H-18b, H-20
19	79.9	4.20, dt (6.4, 2.8)	C-20	H-20, H-27
20	75.3	3.40, m[c]	/	H-18a, H-19, H-22, H-27
21	40.9	1.25, m	C-19, C-20, C-22, C-27	H-18b, H-23, H-27
22	74.6	3.08, t (10.0)	C-20, C-21, C-23, C-24, C-26, C-27	H-20, H-26, H-27
23	47.8	1.40, m	C-22, C-24, C-25, C-26	H-21, H-25, H-26
24	100.9	/	/	/
25	21.2	1.30, s	C-23, C-24	H-23, H-26

26	11.5	1.01, d (6.8)[c]	C-22, C-23, C-24	H-22, H-23, H-25
27	12.3	0.97, d (6.4)	C-20, C-21, C-22	H-19, H-21, H-22
28	24.7	1.37, s	C-10, C-11	H-10, H-13b
29	10.5	0.97, d (6.4)	C-9, C-10	H-7, H-9, H-10
30	12.0	0.95, d (6.8)	C-6, C-7	H-7, H-9
31	11.6	1.10, s	C-2	H-32
32	11.2	1.02, d (6.8)[c]	C-1, C-2	H-31
1-OMe	60.0	3.54, s	C-1	/
2-OMe	49.1	3.39, s[c]	C-2	H-3
6-OMe	56.9	3.28, s	C-6	/
10-OMe	59.6	3.41, s	C-10	H-12

[a]Assignments based on DEPT, HMQC, and HMBC NMR data (100 MHz). [b]Assignments based on COSY and HMBC NMR data (400 MHz)

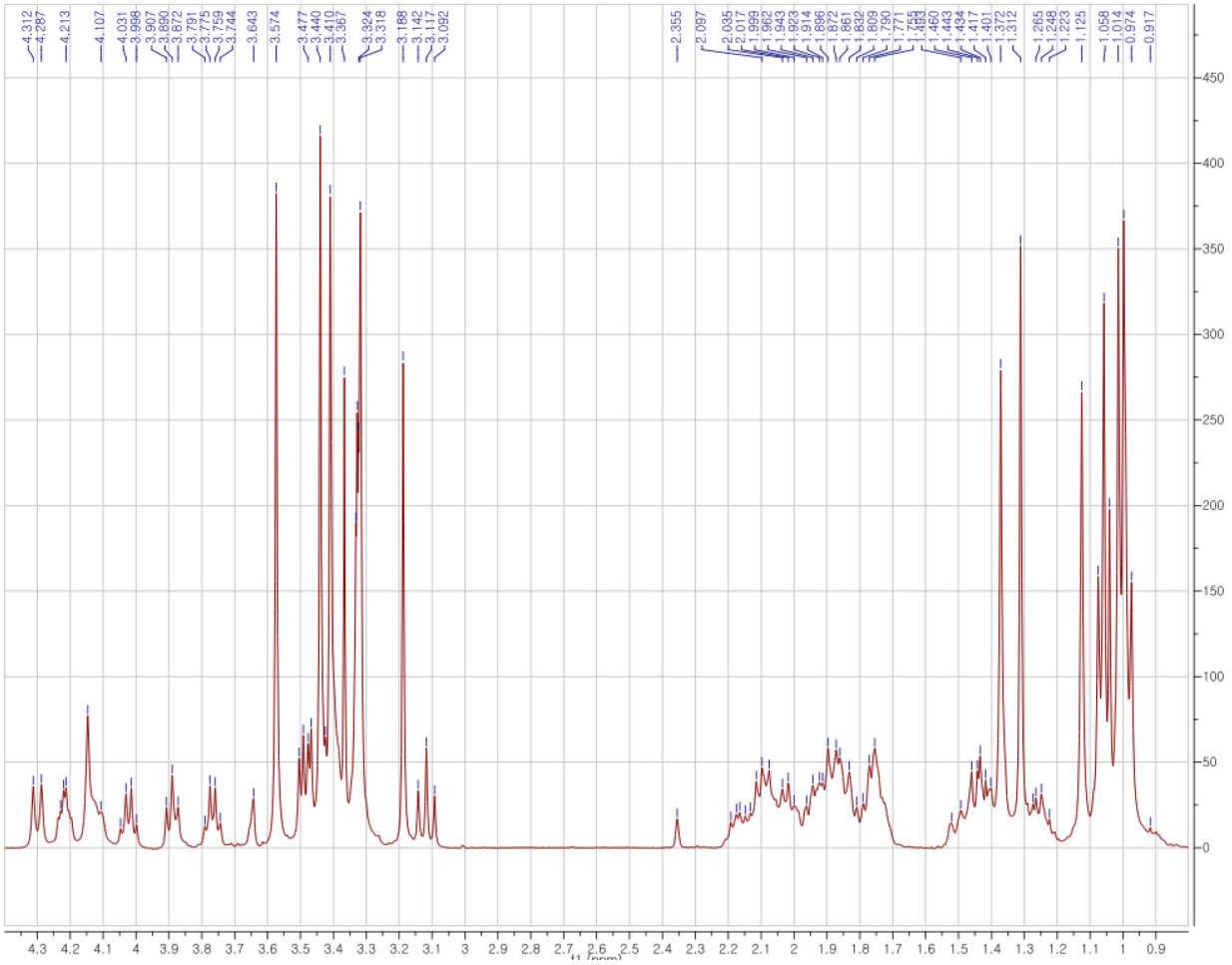


Figure 2-3. ^{1}H NMR spectrum of 5 in CD_3OD

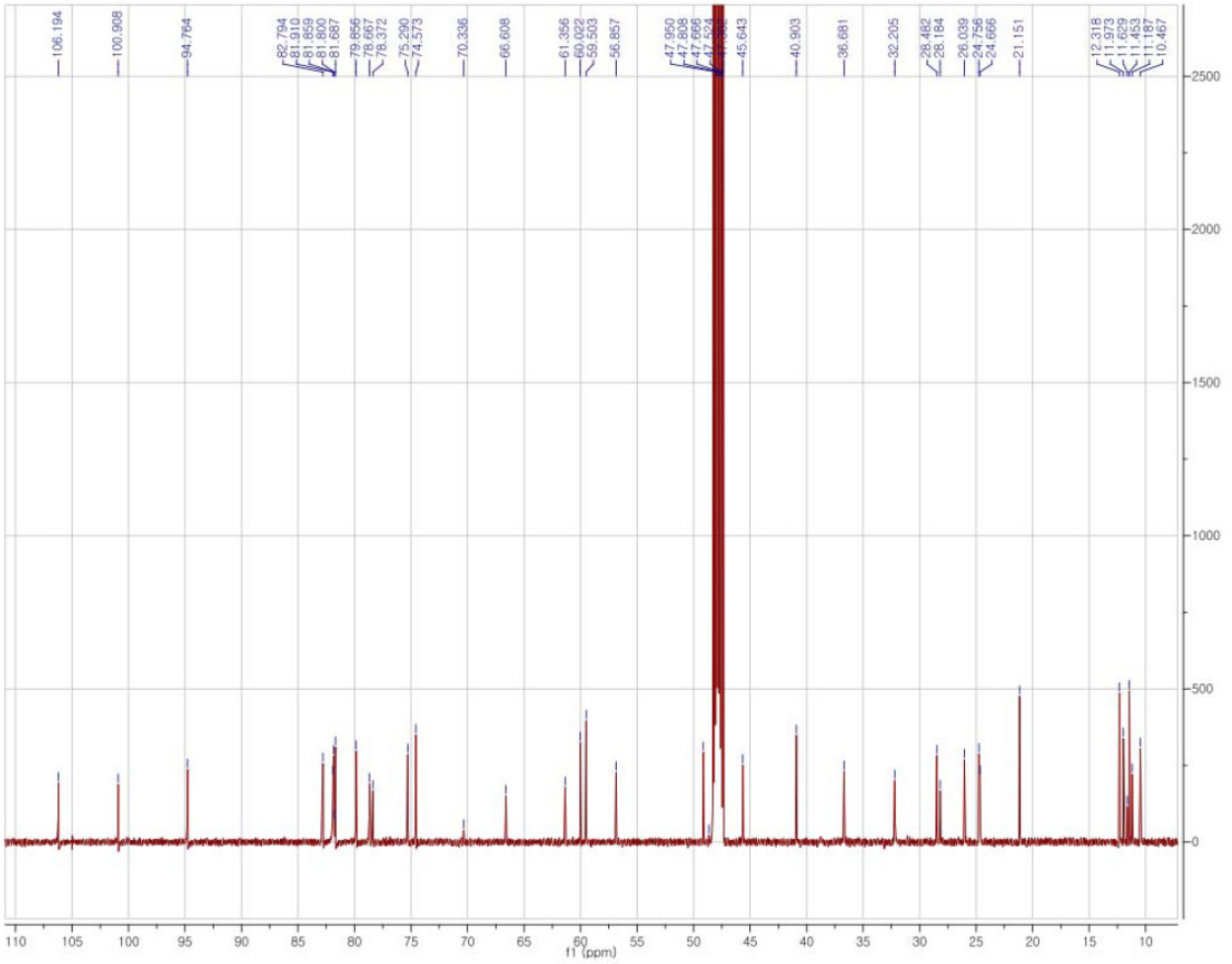


Figure 2-4. ^{13}C NMR spectrum of 5 in CD_3OD

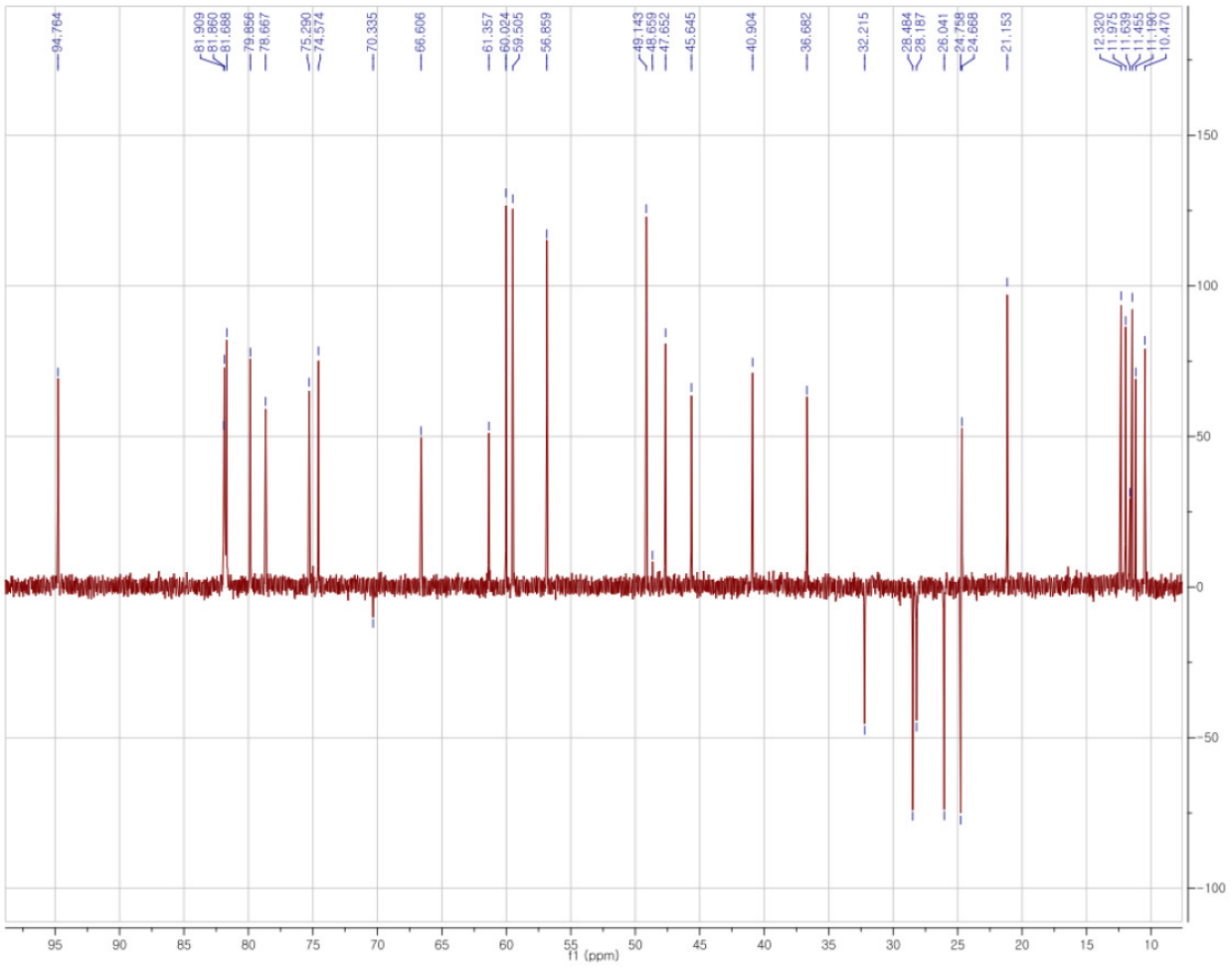


Figure 2-5. DEPT spectrum of 5 in CD_3OD

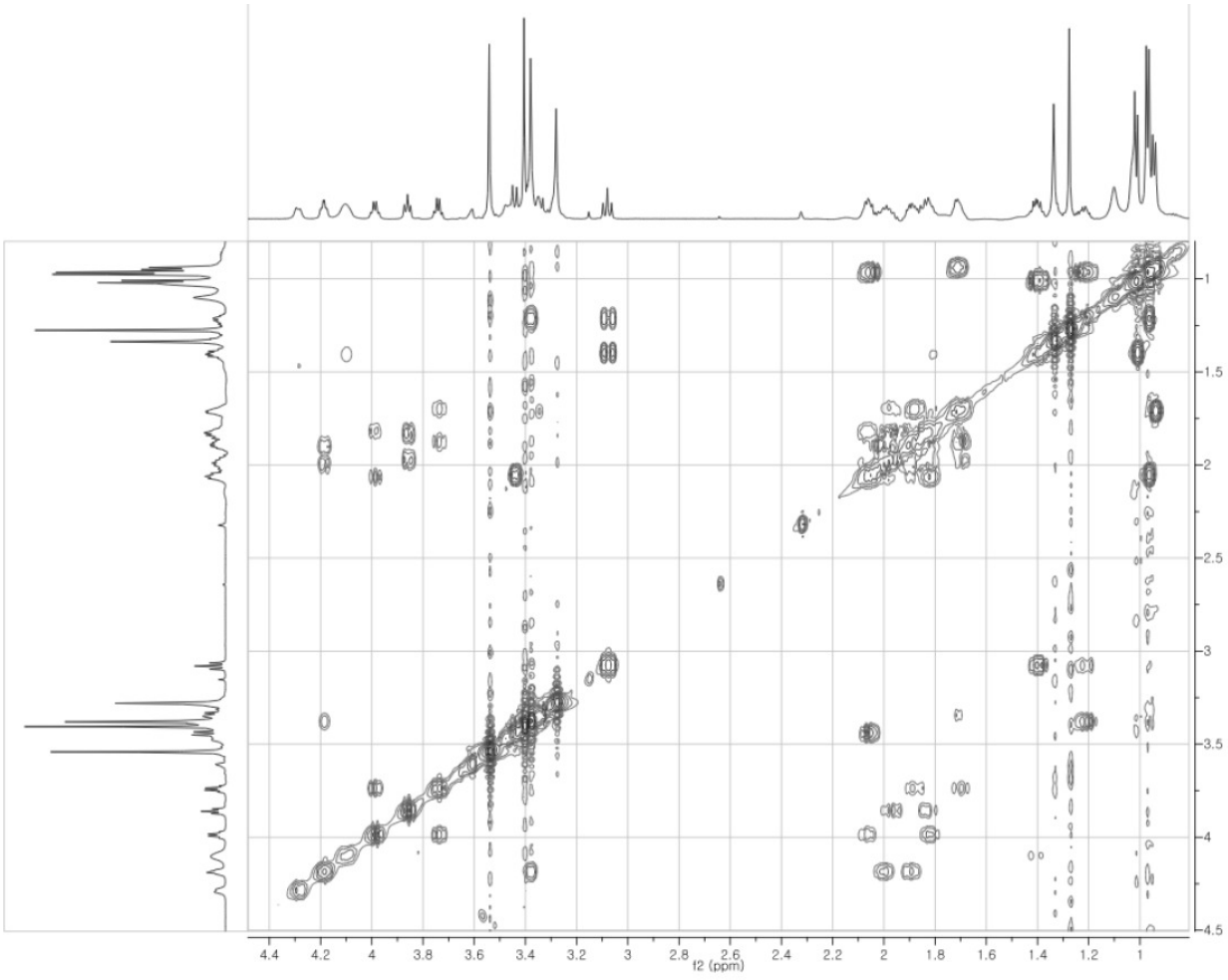


Figure 2-6. ^{1}H-^{1}H COSY spectrum of 5 in CD_3OD

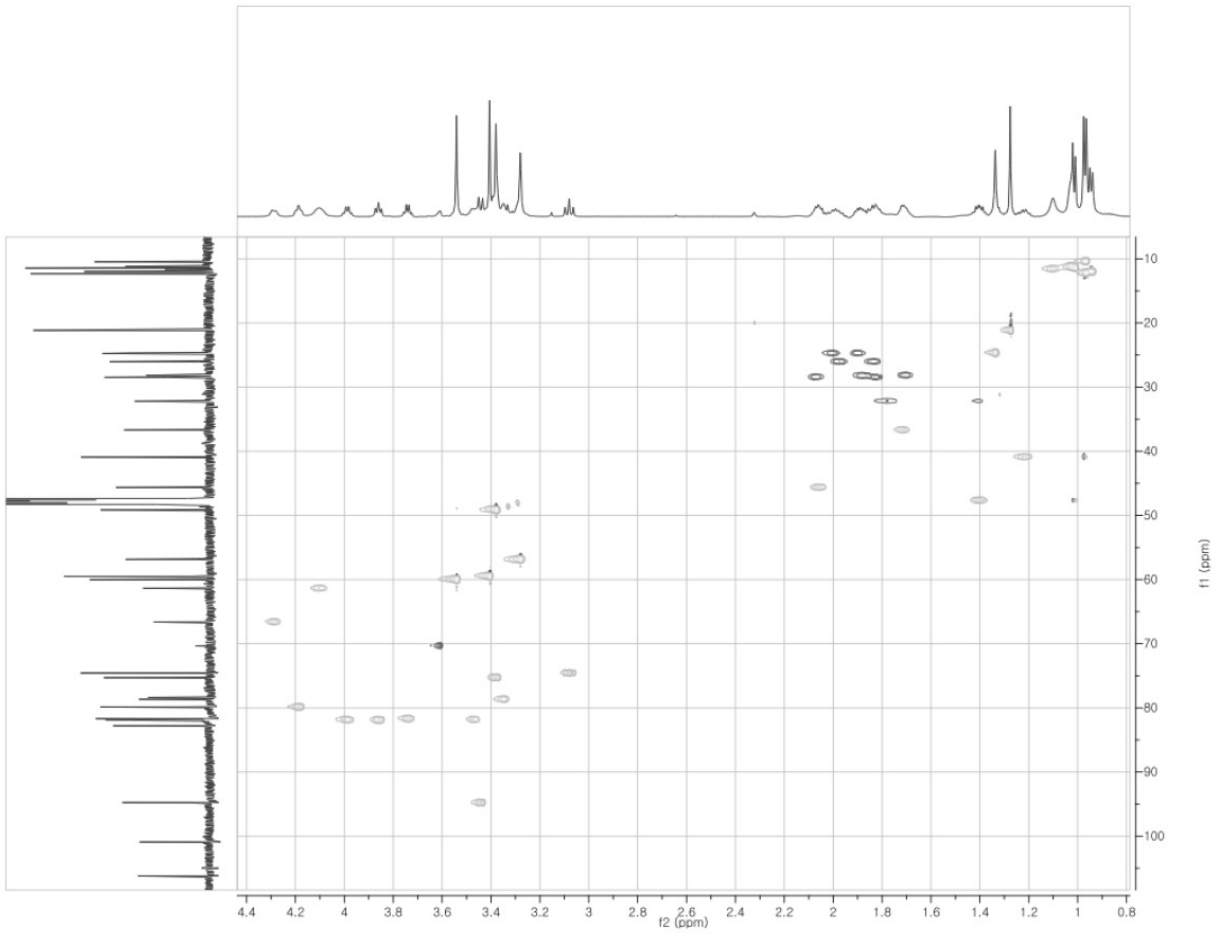


Figure 2-7. HSQC spectrum of 5 in CD_3OD

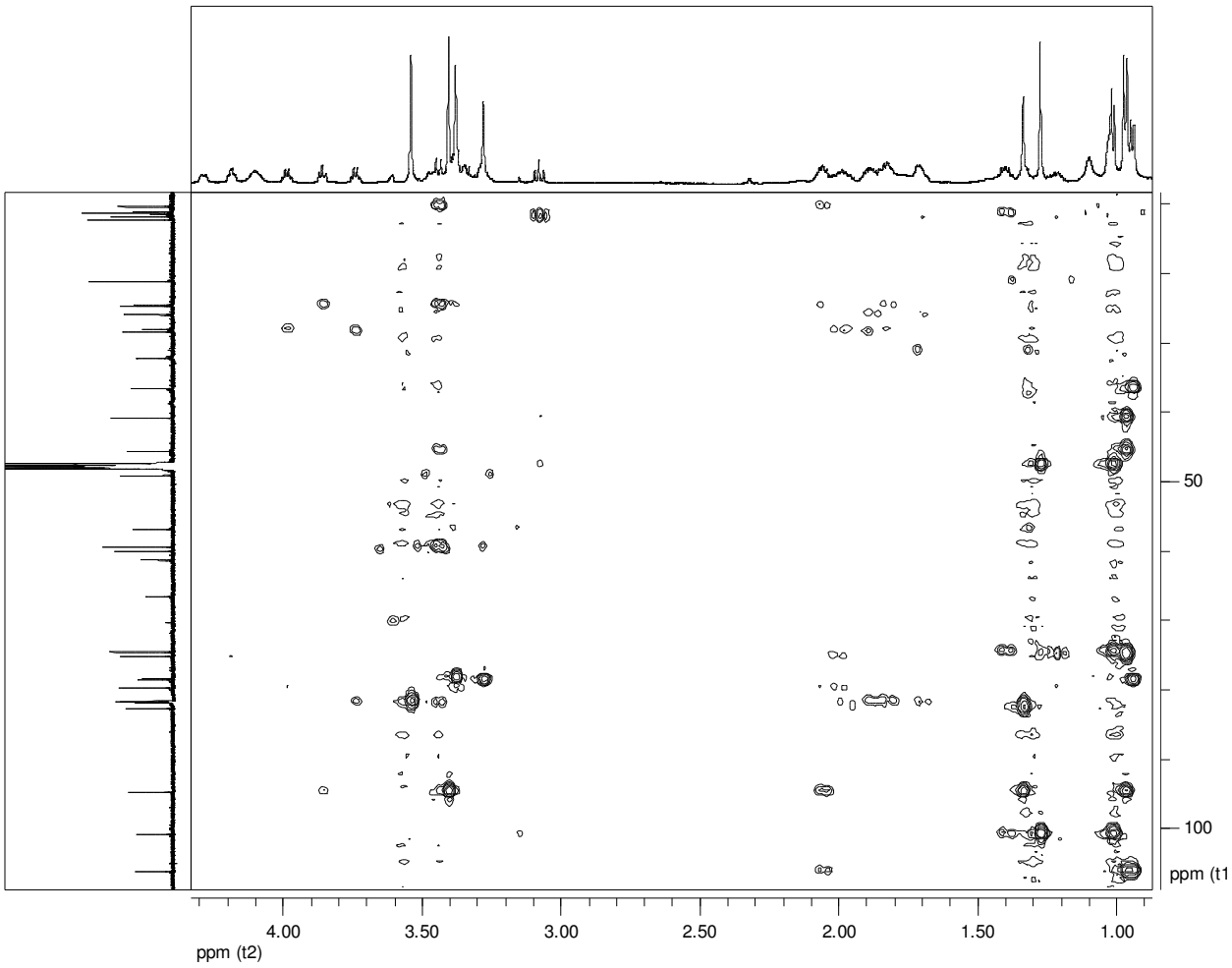
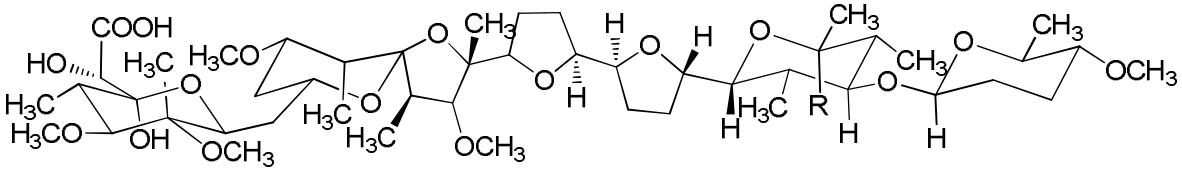


Figure 2-8. HMBC spectrum of 5 in CD_3OD

Chapter 2: ANTIMALARIAL, ANTI-MTB AND ANTI-MRSA METABOLITES FROM A MARINE *STREPTOMYCES*

PART 2 - EPIGENETIC STUDIES YIELDING POTENT ANTI-*MYCOBACTERIUM TUBERCULOSIS* AND ANTI-MRSA POLYETHERS IN A MARINE *STREPTOMYCES* SP

ABSTRACT—Two known polyethers (**6**, **7**) were isolated from a marine *Streptomyces* sp whose crude extract previouly showed potent antimalarial and anti-MRSA activities. It was established that metabolites responsible for these activities were the antibiotic K41-A (**6**) and its C-29 OMe (**7**) ether analog. It was also established that etherification at C-29 has a negative impact on the bioactivity of K41-A. In this chapter, we report the outstanding bioactivity of these molecules against two important pathogens: *Mycobacterium tuberculosis* and MRSA. Several other commercially available ionophores were assayed, as well.



K41-A: R= OH (**6**)
C-29 OMe K41-A: R= OCH_3 (**7**)

1. Introduction

Tuberculosis and methicillin-resistant *Staphylococcus aureus* (MRSA) infections are both serious conditions that can be transmitted from livestock and other animals to humans. According to the WHO, about 1/3 of the world's population is currently affected by TB [3]. In 2007, the CDC reported that an animal MRSA strain is currently responsible for >20% of MRSA cases reported in the Netherlands [187]. As the incidence of these two diseases continues to increase there is an unprecedented demand for the discovery of new drugs leads.

Nature has played a tremendous role in the drug discovery arena for decades with a wide range of bioactive metabolites reported thus far. Among these molecules are ionophores, a unique class of compounds with outstanding potency. Polyethers and peptides are often classified as ionophores; the peptide gramicidin D, has proven to be one of the most potent compound of this class. Due to limitations associated with cytotoxicity, none of these molecules is currently utilized beyond topical applications as a treatment to address human illnesses. It is however important to point out that ionophores are widely used in veterinary medicine. They have proven valuable tools for the control of coccidiosis, a serious problem in poultry and cattle [188].

In a continued effort to identify new drug leads, a bacterium (H668) was isolated from Hawaiian marine sediments. Based on the 16S rRNA analysis, it was established that H668 is a new species. It is related to *Streptomyces cacaoi* AB 184183 and *Streptomyces violaceoruber* AY999815. The ethyl acetate extract of the fermentation broth of this bacterium showed outstanding potency against MRSA, IC_{50} = 0.55 µg/mL.

Due to a frequent loss of bioactivity from batch to batch, a bioassay-guided epigenetic study was completed to explore which external stimuli had the most significant impact on active metabolite production. This was followed by a bioassay guided isolation of the active metabolites.

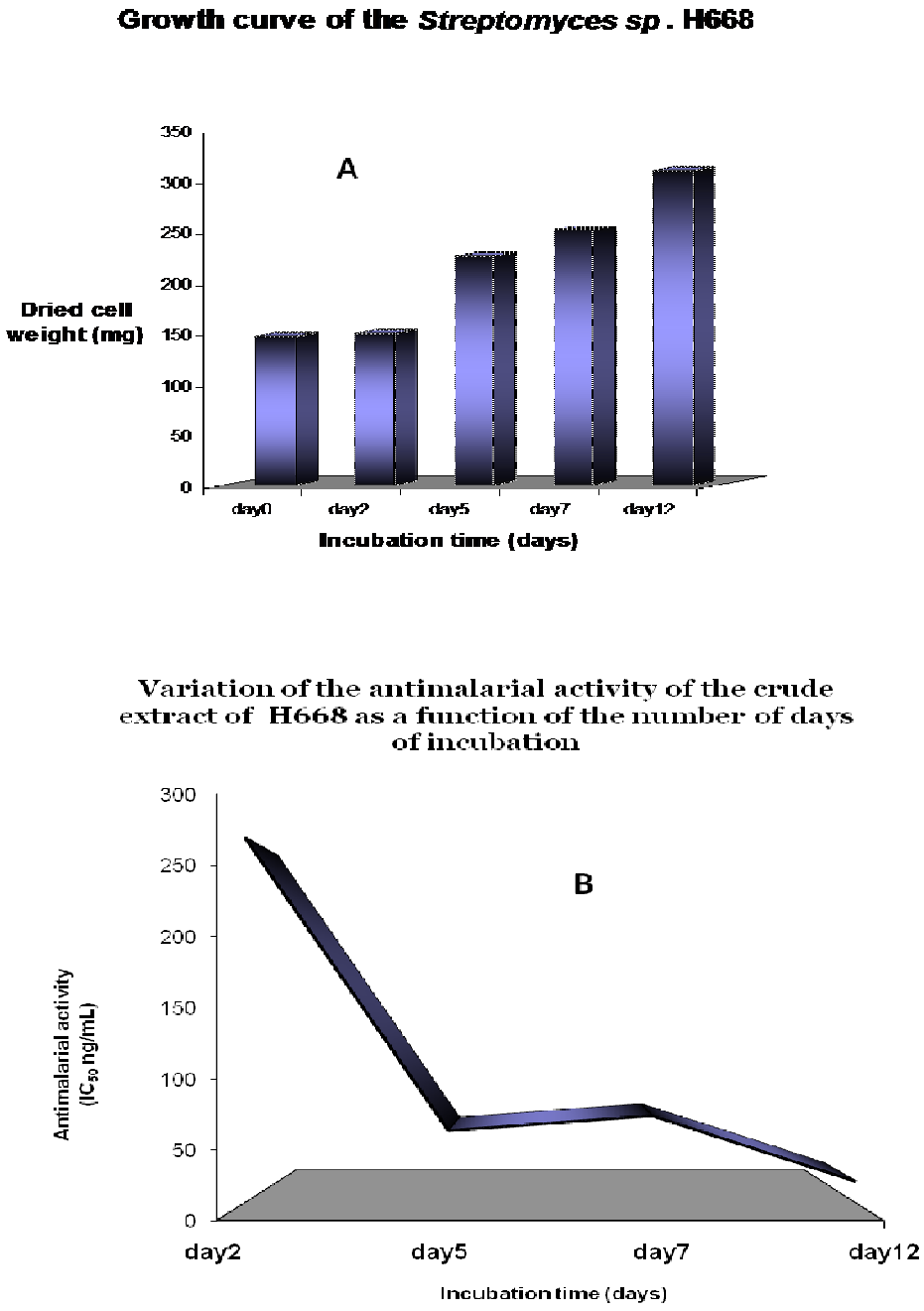


Figure 2-9. Growth pattern of H668 (A) and variation of the antimalarial activity of the crude extract as a function of the number of days of incubation (B)

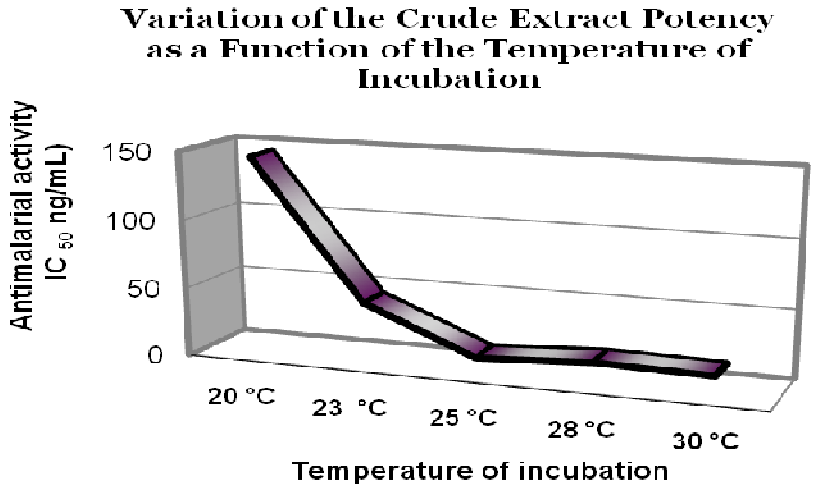
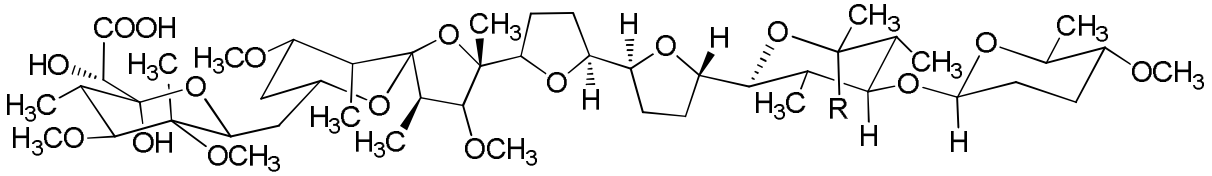


Figure 2-10. Variation of the antimalarial activity of crude extract of culture of H668 as a function of the temperature of incubation

Initial epigenetic studies involved the inoculation of 50 mL of ISP2 medium with an H668 spore suspension followed by incubation at different temperatures (20 - 30 ˚C) and for different periods of time (2, 5, 7, 12 days). It was determined that the best conditions for active metabolites production were long incubations (~12 days) and temperatures within the range 25 - 30 ˚C. These conditions were applied to generate enough crude extract for the isolation of bioactive secondary metabolites. The generated crude extract was first fractionated on C18 material (VLC) to generate a bioactive fraction (80% MeOH in H_2O) which was further purified by HPLC on a silica gel column. This bioassay-guided approach revealed that the bioactive metabolites were the polyether K41-A (**5**) [117] and its C-29 methyl ether (**6**) [189].



K41-A: R= OH (**6**)
C-29 OMe K41-A: R= OCH_3 (**7**)

Figure 2-11. Structure of K41-A (6) and its C-29 OMe Ether (7)

Though an OMe, instead of an OH, was the only difference between the flat structures of **6** and **7**, their NMR data varied considerably. Based on the NOESY data, the main difference between both compounds resided in their 3D structure, instead. In fact, in the case of **6**, long range correlations between atoms at opposite ends of the molecule were detected (Figure 2-12). This was not the case with **7** (Figure 2-13) and was interpretated as an indication of the chelation of a cation. It is important to point out that a new polyether was previously isolated from this same bacterium [120]

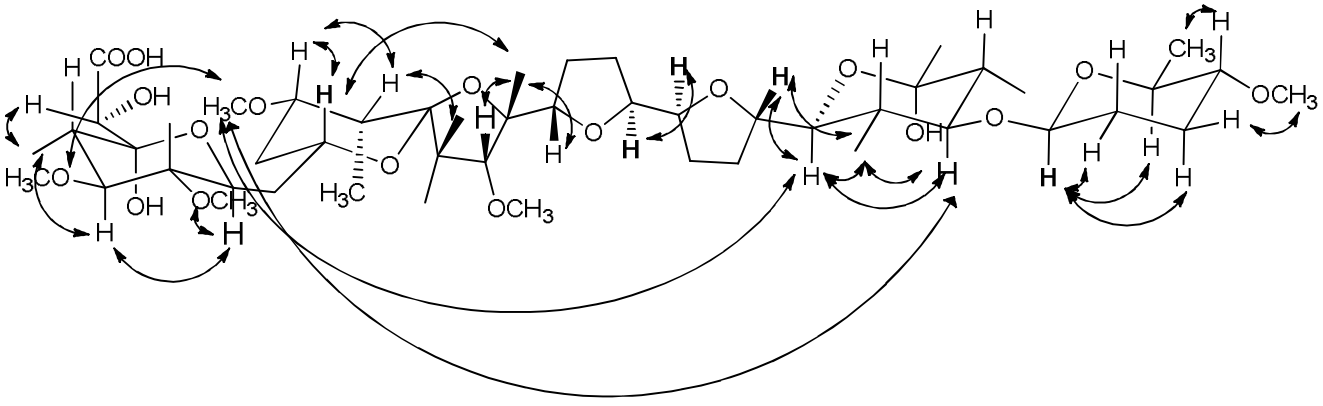


Figure 2-12. Key NOESY correlations of 6

The effects of these molecules along with several other standard ionophores including gramicidin D were evaluated against several disease targets. These included Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*), Gram positive bacteria *(Mycobacterium intracellulare, Mycobacterium tuberculosis* and Methicillin-resistant *Staphylococcus aureus*) and fungi (*Candida albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus*). K41-A and its methyl ether showed selectivity and outstanding potency against Gram-positive bacteria. The IC_{50}s (µg/mL) against MRSA were 0.065 and 0.55, respectively for K41-A and its methyl ether. Corresponding MICs (µg/mL) were 1.25 and 5.00, respectively, for K41-A and its methyl ether. The IC_{50} and MIC (µg/mL) values of K41-A against *M. intracellulare* were 0.15 and 0.31, respectively.

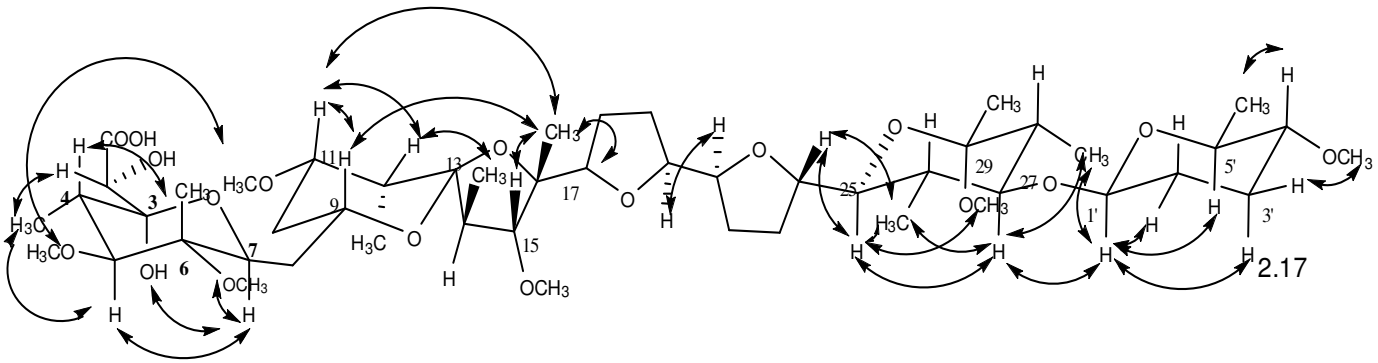


Figure 2-13. Key NOESY correlations of 7

K41-A, its methyl ether and several other ionophores (monensin, nigericin, calcimycin, gramicidin D, and valinomycin) were also tested again *M. tuberculosis* [190]. In the primary assay, the inhibition potential of all but valinomycin ranked between 99

and 100%. The MICs (µg/mL) of monensin, gramicidin D, K41-A and its ether were 0.88, 7.68, 3.70 and 5.96, respectively. Pending further investigations, these molecules are interesting drug leads for the control of zoonotic tuberculosis and MRSA.

Table 2-2 NMR assignment of 6 and 7 in CD_2Cl_2

	Hawaiimycin B (6)		Hawaiimycin C (7)	
	δ_C[a]	δ_H mult. (J in Hz)[b]	δ_C[a]	δ_H mult. (J in Hz)[b]
1.	174.9	/	178.7	/
2.	72.9	4.82 br s	71.6	3.79, m
3.	99.9		98.8	/
4.	38.8 [c]	2.90, m	38.6	2.04, m
5.	85.0	4.09 u[d]	85.0	3.29, br s
6.	78.3	/	78.1	/
7.	68.5	4.60, dd (1.8, 12.0)	66.5	3.80, m
8.	33.5	2.31, m	32.6	1.54, m
9.	61.7	4.70, m	61.3	3.93, m
10.	32.5 [c]	2.84 , m 1.89, m	31.1	1.14, m/2.07, m
11.	79.4	4.17, m	79.8	3.38[d]
12.	37.6[c]	2.60, m	36.8	1.81, m
13.	107.0		107.0	/
14.	48.3	2.30, m	46.1	2.12, m
15.	95.6	4.27, d (9.6)	94.6	3.53, m
16.	82.4	/	83.4	/
17.	84.8	4.43, dd (6.6, 9.6)	83.6	3.75, m
18.	25.9[c]	2.66, m 2.60, m	25.5	1.93m, /1.77m
19.	29.2[c]	2.76, m 2.38 , m	23.0	1.77, m
20.	81.4	4.35, m	79.5	3.94, m
21.	81.8	4.88, dd (6.6, 7.4)	79.4	4.43, m
22.	30.5[c]	2.92, m 2.47, m	29.2	1.44, m / 2.00, m
23.	25.4[c]	2.66, m	24.1	2.13, m / 1.84m
24.	80.2	4.99, dt (3.0, 7.2)	80.8	4.37, m
25.	76.6	4.19, m	74.2	3.89, dd (2.0, 10.0)
26.	40.1[c]	2.04m	39.1	1.23, m

27.	83.3	4.06, m	82.7	3.32[d]
28.	46.6[c]	2.80, m	47.0	1.42, m
29.	101.5	/	98.3	/
30.	22.2	2.07, s	26.7	1.26, s
31.	11.7[c]	1.75, m	12.3/12.5/13.0[c]	Interch[c]
32.	13.6[c]	1.79, m	12.3/12.5/13.0[c]	Interch[c]
33.	28.5	2.29, s	28.2	1.60, s
34.	13.6[c]	1.79, m	11.3	1.00, m
35.	12.9[c]	1.76, m	12.3/12.5/13.0[c]	Interch[c]
36.	11.4	1.87, s	10.9	1.09, s
37.	12.2	1.81, d (6.6)	11.8	1.03, d (3.0)
5-OMe	61.4	4.31, s	60.7	3.52, s
6-OMe	51.0	4.11, s	50.4	3.33, s
11-OMe	57.8	4.07, s	59.9	3.44, s
15-OMe	47.8	3.92, s	60.0	3.40, s
29-OMe	60.7	4.17, s	/	/
1'	103.1	5.19, dd (1.8, 9.6)	102.6	4.42, m
2'	31.1[c]	2.70, m	30.5	1.39, m / 1.93, m
3'	27.9[c]	2.95, m	27.3	2.19, m
4'	80.9	3.54, dt (4.2, 10.2)	80.3	2.78, m
5'	75.1	4.02, m	74.4	3.24, m
5'-Me	18.2	1.97, d (6)	18.1	1.20, d (6.0)
4'-OMe	57.1	4.09, s	56.4	3.31, s
5'-Me				

[a]Assignments based on DEPT, HMQC, and HMBC NMR data (100 MHz). [b]Assignments based on COSY and HMBC NMR data (400 MHz). [c]Signals partially overlapped.
[c] interchangeable methyl assignments, [d]berried signal

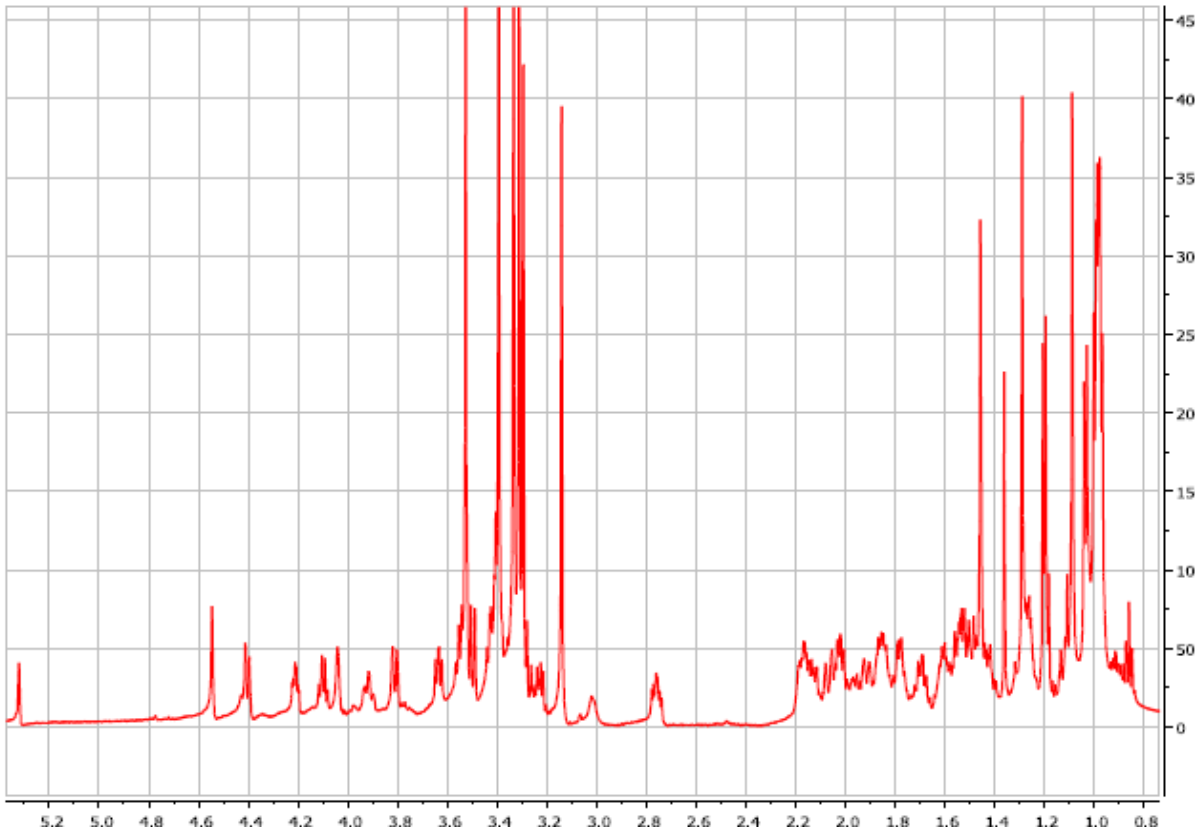


Figure 2-14. ^{1}H NMR spectrum of 6 in CD_2Cl_2

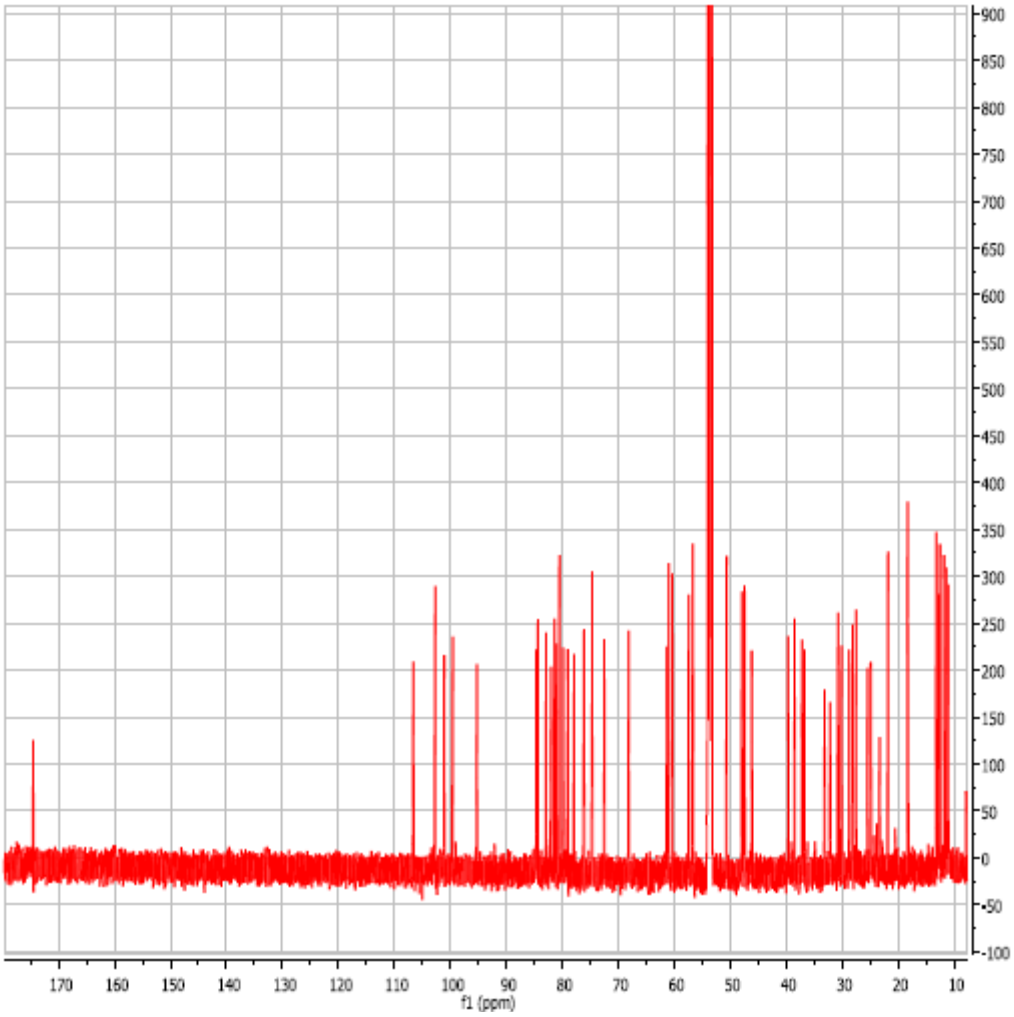


Figure 2-15. ^{13}C NMR spectrum of 6 in CD_2Cl_2

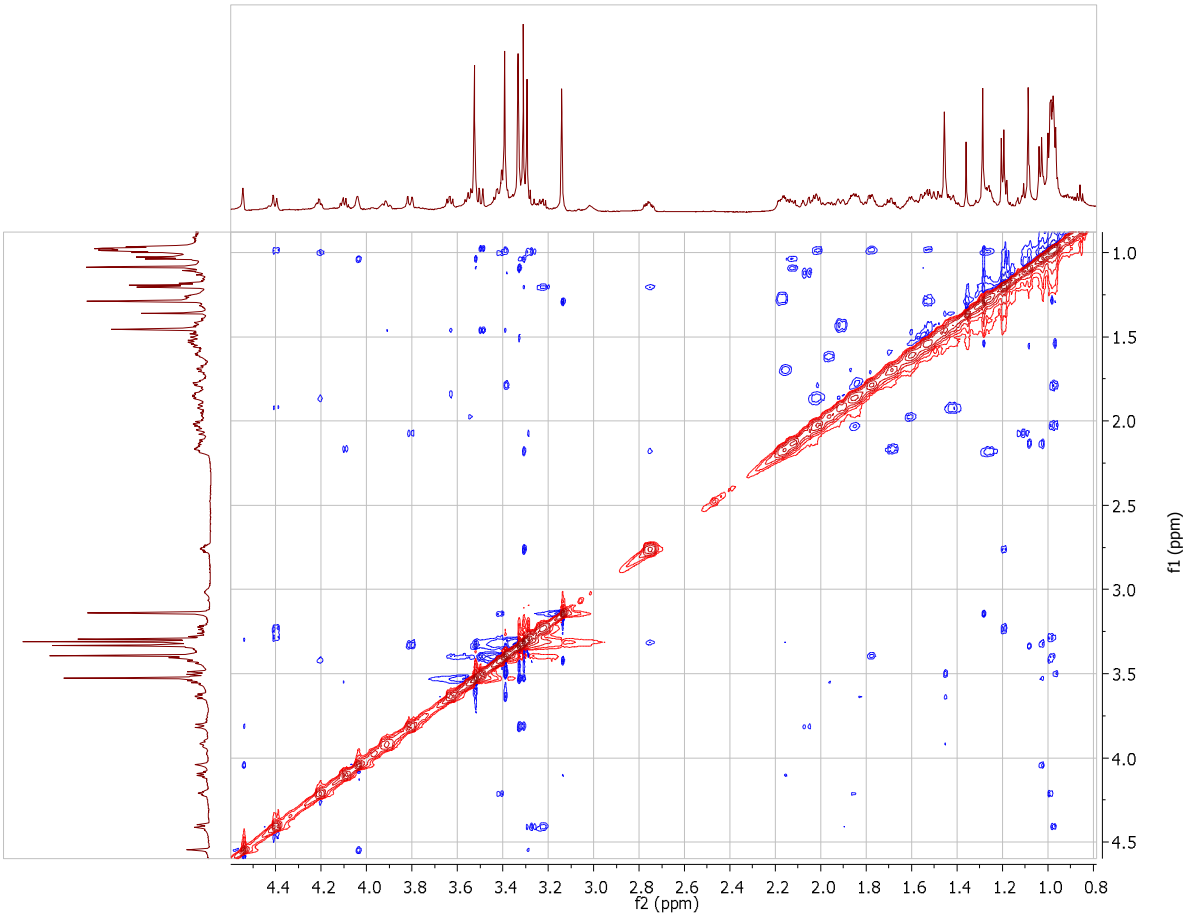


Figure 2-16. NOESY spectrum of 6 in CD_2Cl_2

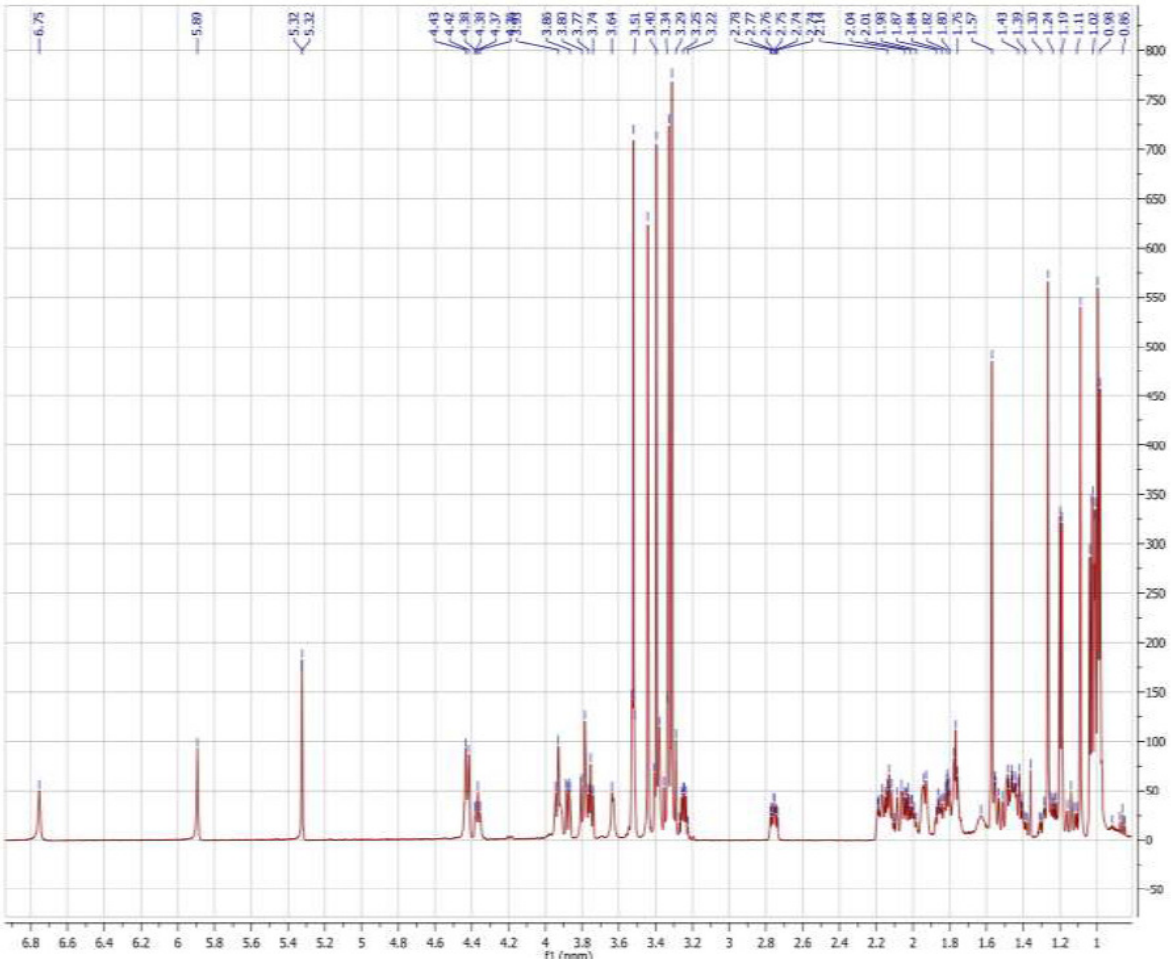


Figure 2-17. ^{1}H NMR spectrum of 7 in CD_2Cl_2

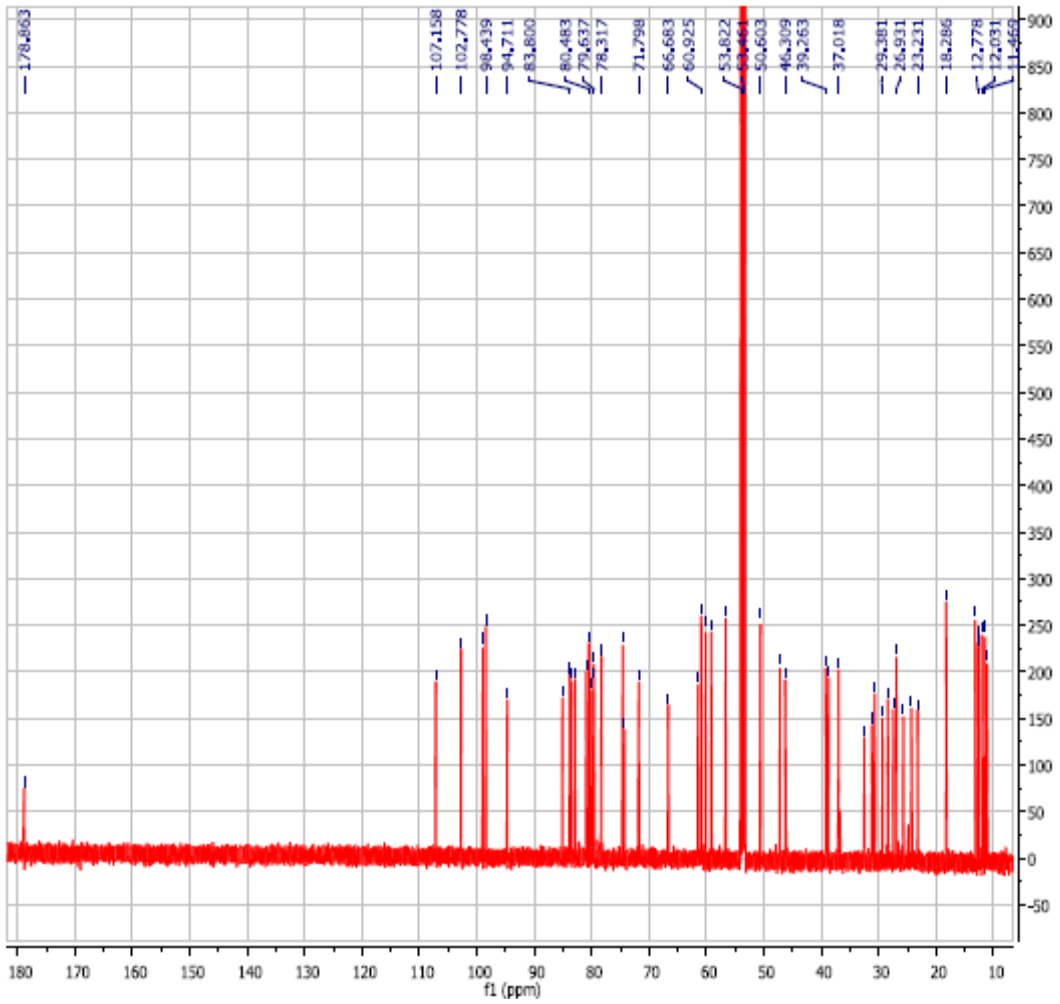


Figure 2-18. ^{13}C NMR spectrum of 7 in CD_2Cl_2

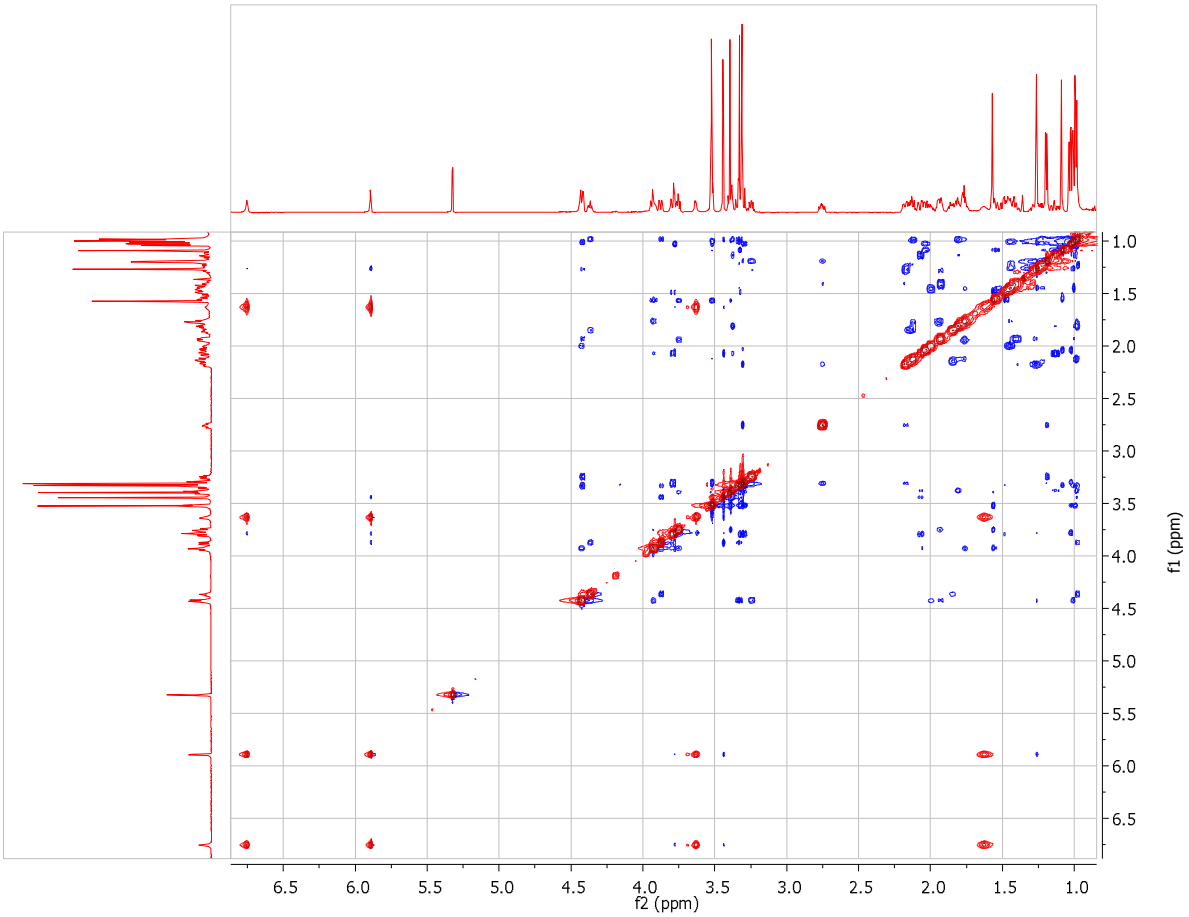


Figure 2-19. NOESY spectrum of 7 in CD_2Cl_2.

Chapter 3 : ANTIFUNGAL AND ANTI-HEPATITIS C VIRUS (HCV) CYCLIC PEROXIDES ISOLATED FROM A JAMAICAN SPONGE OF THE GENUS *PLAKINASTRELLA*

ABSTRACT— Five new six-membered ring cyclic peroxides, plakinastreloic acid A (**8**), methyl plakinastreloate A **(9**), the C-12 epimers of methyl 13, 14-epoxyplakinastreloate (**10** & **11**), and plakinastreloic acid B (**12**) were isolated from a marine sponge of the genus *Plakinastrella*. Their structures and relative configurations were determined. Compounds **8** and **9** exhibited antifungal activities against *Candida albicans* (IC_{50} = 6.5 µg/mL and 3.5 µg/mL, respectively), *Aspergillus fumigatus* (IC_{50} = 4.0 µg/mL and 9.0 µg/mL, respectively) and *Cryptococcus neoformans* (IC_{50} = 4.0 µg/mL and 9.0 µg/mL, respectively). A moderate antimalarial activity against CQ-resistant and CQ-sensitive strains of *P. falciparum* was observed, as well. It was also established that **8** possesses anti-HCV activity. The percentage of HCV's RNA inhibition at 10 µg/mL was estimated at 88%. Some cytotoxicity was observed, as well.

Marine sponges have proven to be an excellent source of secondary metabolites, several of which show interesting bioactivity against a variety of disease targets. Among these compounds are cyclic peroxides, a group of secondary metabolites isolated from

sponges of the genus *Plakortis* and *Plakinastrella* [191]. Examples include plakortide F [192], plakinic acid F [193], and epiplakinic acid F [193], plakortide Q and its analogues [194], ethyl plakortide Z, ethyl didehydroplakortide Z, and methyl didehydroplakortide Z, ethyl *seco*-plakortide Z, *epi*-ethyl *seco*-plakortide Z, and ethyl didehydro-*seco*-plakortide Z [195], andavadoic acid [196], nuapapuin A methyl ester [197], nuapapuin B [197]. Cyclic peroxides are bioactives molecules; they are reported to possess antifungal (primarily against *C. albicans* and *A. aspergillus*) [193, 198, 199], antimalarial [199, 200], antineoplastic [194-197, 201, 202] and antiviral (HSV) activities [200].

Figure 3-1. Jamaican sponge of the genus *Plakinastrella*

In a continued effort to identify new biologically active metabolites from the marine environment, several sponge samples were harvested from Jamaica (Rio Buenos Discovery Bay) using closed circuit rebreathers at a depth of - 40 m [203]. We previously reported the isolation of new peroxylactones from one of these samples

(*Plakinastrella onkodes*) [203]. Here, we report the isolation of five new six-membered ring cyclic peroxides from a liver sponge of the genus *Plakinastrella* (Figure 3-1) [203]. These include plakinastreloic acid A (**8**), methyl plakinastreloate A **(9**), the C-12 epimers of methyl 13, 14-epoxyplakinastreloate (**10** & **11**) and plakinastreloic acid B (**12**)**.**

Plakinastreloic acid A (**8**) was isolated as a colorless amorphous solid {$[\alpha]_D$ = + 38 (*c*.0.09, MeOH)}. The ^{13}C NMR spectrum suggested that **8** contained 25 carbons. Inspection of the ^{13}C and ^{1}H NMR spectra suggested that the molecular formula was $C_{25}H_{36}O_4$, indicating the presence of eight degrees of unsaturation. A molecular ion of 399.9 was obtained by LCMS analysis under negative ion mode. ^{13}C and ^{1}H NMR data are provided in Table 3-1 and

Table 3-2. Resonances attributable to five methyl groups were observed [δ_H 1.16 (3H, d, *J* = 6.6 Hz), δ_H 1.67 (3H, brs), δ_H 1.36 (3H, s), δ_H 0.91 (3H, d, *J* = 6.8 Hz), δ_H 0.93 (3H, d, *J* = 6.0 Hz)]. Compound **8** also contained 11 methine, four methylene, and four quaternary carbons. It was established based on the ^{1}H-^{1}H COSY spectrum analysis that **8** contained four independent spin systems; these spin systems are labeled A-D and shown in bold bonds in Figure 3-2; full tables of data are included.

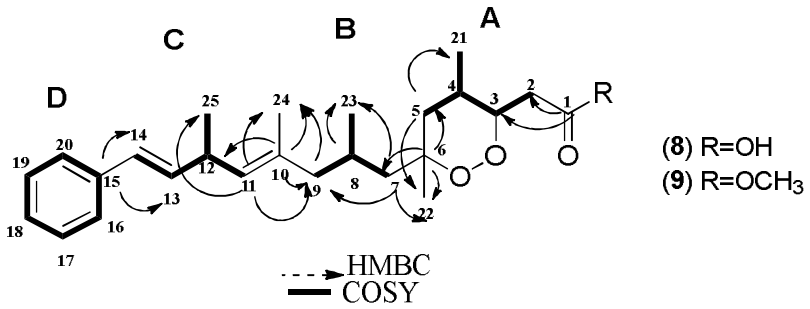
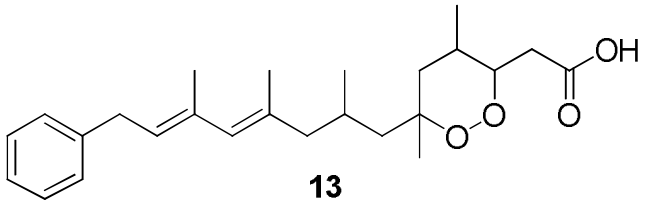


Figure 3-2. Key HMBC and ^{1}H-^{1}H COSY correlations for plakinastreloic acid A (8) and methyl plakinastreloate A (9)

These spin systems were connected based on HMBC correlations (Figure 3-2). First, several HMBC correlations were observed between the methylene protons H-2a (1H, δ_H 2.26, m), H-2b (1H, δ_H 2.68, dd, *J* = 2.4 Hz, 16.0 Hz) and C-1. Another HMBC correlation was observed between the methine proton H-3 (1H, δ_H 4.01, dt, *J* = 2.8 Hz, 9.6 Hz) and the same carbon (C-1). When taken in conjunction with the molecula formula and the chemical shift of C-1, this suggested that **8** was a free acid. The connection of the first and second spin system was made possible by HMBC correlations between H-7a (1H, δ_H 1.21, m), H-7b (1H, δ_H 1.50, dd, *J* = 2.8 Hz, 14.8 Hz) and C-6, on the one hand and between H-5a (1H, δ_H 1.21, m) and C-22 on the other. The existence of HMBC correlations between H-24 (3H, δ_H 1.67, brs) and C-9 on the one hand, and between H-24 and C-11 on the other, allowed the connection of the second and the third spin system (Figure 3-2). Finally, the HMBC correlation between H-14 (1H, δ_H 6.36, d, *J* = 16 Hz) and C-15 allowed the connection of the aromatic ring to the third spin system. The structure of **8** was determined to be as depicted in Figure 3-2.

A closely related molecule (**13**) and its methyl ester were reported in 1980 by Stierle and Faulkner [204]. These molecules were derived from a sponge of the genus *Plakortis.* Compounds **8** and **13** share the same molecula formula and skeleton; in both molecules, C-10 and C-11 are connected by a double bond. The only difference between **8** and **13** resides in the location of the second double bond. The second double bond of **8** connects C-13 and C-14 and in **13** C-12 and C-13, instead. Stierle and Faulkner reported **13** and its methyl ester as highly unstable compounds that

"decompose on standing" making data acquisition difficult. Several other reported peroxides did not share this feature [204].



13

Based on the NOESY spectrum analysis, it was established that the relative configuration of **8** is as depicted in Figure 3-3. A strong NOESY correlation was observed between H-3 (1H, δ_H 4.01, dt, J = 2.8 Hz, 9.6 Hz) and H-5a (1H, δ_H 1.21, m). It is important to note that no NOESY correlation was detected between H-3 and 5b (1H, δ_H 1.63, d, J = 4.4 Hz) and that the correlation between H-3 and H-2b (1H, δ_H 2.68, dd, J = 2.4 Hz, 16.0 Hz) was weak. Another important NOESY correlation was identified between H-22 (3H, δ_H 1.36, s) and H-4 (1H, δ_H 1.85, m).

The strong NOESY correlations between H-11 (1H, δ_H 5.07, d, J = 8.8 Hz), H-9a (1H, δ_H 1.87, m) and H-9b (1H, δ_H 1.96, m) suggested an E configuration for the C-10 - C-11 double bond. It is important to point out that no similar correlation with H-24 (3H, δ_H 1.67, br s) was observed.

Strong NOESY correlations were identified between H-13 (1H, δ_H 6.17, dd, J = 6.0 Hz, 16.0 Hz), H-14 (1H, δ_H 6.36, d, J = 16.0 Hz) and the aromatic protons H-16/H20. This is only possible if H-13 and H-14 are trans-oriented, suggesting an *E* configuration of the corresponding double bond; the large coupling constant between H-13 and H-14 (J = 16.0 Hz) confirms this assignment. No other proton had a NOESY correlation with

the aromatic protons. H-13 and H-14 both showed weak NOESY correlations to H-25, H-12 and H-11.

Methyl plakinastreloate A (**9**) was isolated as a colorless amorphous solid {$[\alpha]_D$= + 32 (*c*.0.03, MeOH)}. ^{13}C and ^{1}H NMR data for **9** are provided in Table 3-1 and 3-2. Analysis of its NMR spectrum revealed that it was similar to plakinastreloic acid A (**8**) in all but one point: the presence of an OMe signal (δ_H 3.67, δ_c 51.1); merged NMR spectra of **8** and **9** are included. This resonance showed an HMBC correlation to C-1 (δ_c 171.9), an indication that **9** was the methyl ester of plakinastreloic acid A (**8**). No difference was observed between the NOESY spectra of methyl plakinastreloate A (**9**) and that of plakinastreloic acid A (**8**), suggesting that these compounds had the same relative configuration (Figure 3-3).

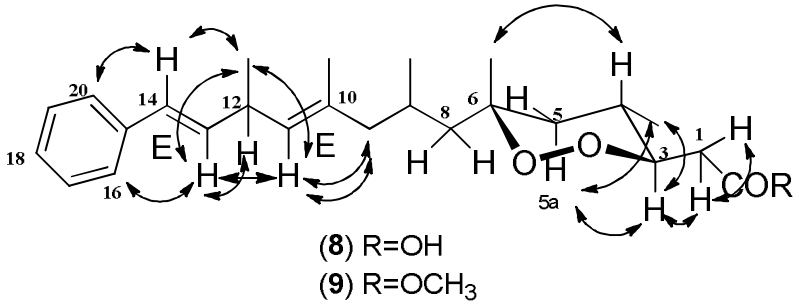
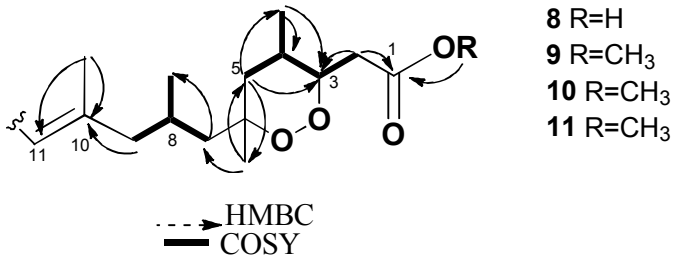


Figure 3-3. Relative configuration of plakinastreloic acid A (8) and methyl plakinastreloate A (9) based on NOESY correlations (↔)

Two additional new molecules, **10** and **11** were isolated after further purification of the HPLC fraction which eluted with 6:94 H_2O/CH_3CN [C_{18} column, 21.2 × 250 mm at 6 mL/min]. These two compounds eluted together, and because of a limited amount of material, their structures were solved as a mixture. The HRMS analysis (positive ion mode) of the mixture showed a single peak, an indication that **10** and **11** shared the

same molecular weight. ^{13}C and ^{1}H NMR data for **10** and **11** are provided in Table 3-1 and 3-2. Inspection of the ^{1}H and ^{13}C NMR spectra coupled with HRMS data suggested a formula of $C_{26}H_{38}O_5Na$ for **10** and **11** [(M+ Na) - *m/z* observed 453.2617], indicating the presence of eight degrees of unsaturation in each of these molecules.

A comparative analysis of the NMR spectra of **9** with that observed for the mixture of **10** and **11** suggested that these three compounds share a common moiety (C-1 through C-11) (Figure 3-4); full tables of data are included. The remainder of the structure of **10** and **11** was assembled through analysis of the ^{1}H-^{1}H COSY spectrum of **10** and **11** as follows**:** H-11 was coupled to the methine proton H-12 (δ_H 2.63 and δ_H 2.47, for **10** and **11**, respectively); this proton was in turn coupled to H-25 (δ_H 1.06 and δ_H 1.1, for **10** and **11**, respectively) and H-13 (δ_H 2.79 and δ_H 2.86, for **10** and **11** respectively). H-13 was in turn coupled to a methine proton H-14 (δ_H 3.69 and δ_H 3.7, for **10** and **11**, respectively).



8 R=H
9 R=CH_3
10 R=CH_3
11 R=CH_3

Figure 3-4. Structural moiety shared by 10, 11, 8 and 9

The chemical shifts of C-13 (δ_C 67.0 and 66.8, for **10** and **11**, respectively**)** and C-14 (δ_C 56.9 and 57.9, for **10** and **11** respectively) are consistent with oxygen substitution at these positions. When taken in conjunction with the molecular formula of these

molecules, this suggests the presence of an epoxide ring at C-13 – C-14. The strong HMBC correlation between H-14 (δ_H 3.66 and δ_H 3.77 for **10** and **11**, respectively) and aromatic carbons C-15 and C-16 allowed the incorporation of a phenyl substituent at C-14. The aromatic signal (δ_H 7.1-7.3) integrates for 10 instead of five protons. This further suggests that we are dealing with a mixture and that the aromatic rings of **10** and **11** are mono substituted. It was established based on the ^{1}H-^{1}H COSY spectrum analysis that **10** and **11** contained four independent spin systems; these are labeled A-D and shown in bold bonds in Figure 3-5; full tables of data are included. The planar structures of **10** and **11** are identical and depicted in Figure 3-5.

Table 3-1. ^{13}C NMR data for compounds 8 - 12 (600 MHz)

	8 (CD_3OD)	**9** (CD_3OD)	**10** (CD_3OD)[a]	**11** (CD_3OD)[a]	**12** (CD_2Cl_2)
1.	173.7	171.9	171.9	171.9	169.8
2.	36.0	35.8	35.7	35.7	69.6
3.	83.7	83.7	83.7	83.7	43.8
4.	30.3	30.4	30.3	30.4	45.6
5.	42.7	42.8	42.7	42.7	84.4
6.	81.5	81.8	81.7	81.7	51.6
7.	47.0	47.3	47.3	47.3	28.8
8.	26.0	26.2	26.1	26.1	39.0
9.	49.1	49.3	49.1	49.2	27.2
10.	133.2	133.3	135.6	135.8	29.6-30.0[u]
11.	130.4	130.6	126.8	127.6	29.6-30.0[u]
12.	35.6	35.7	34.5	35.5	29.6-30.0[u]
13.	134.7	134.8	67.0	66.8	29.6-30.0[u]
14.	127.5	127.6	56.9	57.9	29.6-30.0[u]
15.	137.9	138.1	138.0	138.0	29.6-30.0[u]
16.	125.6	128.3	125.3	125.4	29.6-30.0[u]
17.	128.1	125.8	128.3	128.3	29.6-30.0[u]
18.	126.5	126.6	127.8	127.7	32.1
19.	128.1	125.8	128.3	128.3	22.9
20.	125.6	128.3	125.3	125.4	14.1
21.	16.1	16.2	15.6	16.2	31.1
22.	19.9	20.0	20.0	20.0	28.1
23.	20.9	21.0	20.7	21.0	21.7

24.	15.1	15.2	15.4	15.6	/
25.	20.3	20.4	16.2	16.7	/
OMe	/	51.1	51.1	51.1	/

[u] interchangeable assignments (methylene carbons of the aliphatic chain). [a] the structures of compounds **10** and **11** were solved as a mixture; assigned signals are thus interchangeable for any given position

Table 3-2. ^{1}H NMR data of compounds 8-12 (600 MHz) δH,mult. (*J* in Hz)

	8 (CD_3OD)	**9** (CD_3OD)	**10** (CD_3OD)[a]	**11** (CD_3OD)[a]	**12** (CD_2Cl_2)
1.	/	/	/	/	/
2.	2.68, dd (2.4, 16.0)/ 2.26, m	2.72, dd (3, 16.2) 2.28, dd (9, 15.6)	2.71, m 2.26, m	2.71, m 2.26, m	/
3.	4.01, dt (2.8,9.6)	3.97, dt (3, 9.6)	3.95,m	3.96, m	2.56, dd (2.4,16.8) 2.39, d (16.8)
4.	1.85, m	1.85, m	1.81, m	1.81, m	1.93, dd (2.0, 14.0) 1.77, d (14.4)
5.	1.63, d (4.4) 1.21, m	1.63, d (6) 1.21, m	1.62, m 1.25, m	1.62, m 1.25, m	/
6.	/	/	/	/	1.62, m 1.46, dd (7.0, 15.2)
7.	1.50, dd (2.8, 14.8) 1.21, m	1.48, dd (2.4, 14.4) 1.17, m	1.24, m 1.57, m	1.24, m 1.57, m	1.62, m
8.	1.81, m	1.8, m	1.80, m	1.80, m	1.29, m 1.17, m
9.	1.87, m 1.96, m	1.82, m 1.9, m	1.82, m 1.91, m	1.82, m 1.91, m	1.27, m
10.	/	/	/	/	1.27, m
11.	5.07, d (8.8)	5.04, d (8.4)	5.00, m	5.00, m	1.27, m
12.	3.26, m	3.24, m	2.63, m	2.47, m	1.27, m
13.	6.17, dd (6.0, 16.0)	6.15, dd (6.2, 16.2)	2.79, dd (2.4, 6.0)	2.86, dd (2.4, 6.0)	1.27, m
14.	6.36, d (16.0)	6.33, d (16.2)	3.66, d (2.0)	3.77, d (2.0)	1.27, m
15.	/	/	/	/	1.27, m
16.	7.32, m	7.24, m	7.20, m	7.20, m	1.27, m
17.	7.27, m	7.30, m	7.30, m	7.30 , m	1.27, m

18.	7.17, m	7.14, m	7.23, m	7.23, m	1.27, m
19.	7.27, m	7.30, m	7.30, m	7.30, m	1.27, m
20.	7.32, m	7.24, m	7.20, m	7.20, m	0.88, t (7.2)
21.	0.91, d (6.8)	0.91, d (6.6)	0.86, m	0.86, m	1.34, s
22.	1.36, s	1.33, s	1.26s	1.29s	1.52, s
23.	0.93, d (6.0)	0.93, d (6)	0.81, d (7.0)	0.9, d (7.0)	0.97,d (6.6)
24.	1.67, brs*	1.64, s	1.64, brs	1.66, brs	/
25.	1.16, d (6.6)	1.12, d (6.6)	1.06, d (7.0)	1.1, d (6.0)	/
OMe	/	3.67, s	3.67, s	3.67, s	/

[u] interchangeable assignments (methylene carbons of the aliphatic chain). [a] the structures of compounds **3** and **4** were solved as a mixture; assigned signals are thus interchangeable for the same carbon position

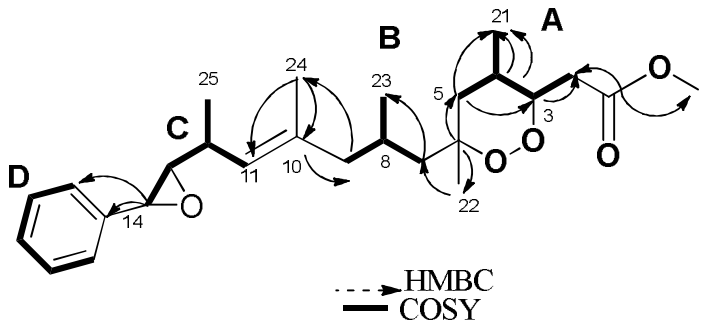


Figure 3-5. Key HMBC and ^{1}H-^{1}H COSY correlations for compounds 10 and 11

The next step was to attempt to determine what differentiated **10** from **11**. NOESY correlations appeared to be identical around the double bonds in **10** and **11**. The olefinic proton H-11 (δ_H 5.00 and δ_H 5.00, for **10** and **11,** respectively) did not show a correlation to the methyl signal at C-24 (δ_H 1.64 and δ_H 1.66, for **10** and **11,** respectively) but did show a correlation to H-25 (δ_H 1.06 and δ_H 1.1, for **10** and **11,** respectively) and H-9 (δ_H 1.82/1.91 and δ_H 1.82/1.91, for **10** and **11,** respectively)

leading to an E configuration of these double bonds in both compounds.

As for the configuration around the epoxide ring, H-13 (δ_H 2.79 and δ_H 2.86, for **10** and **11**, respectively), gave a NOESY correlation to the aromatic proton H-16 (δ_H 7.20 and δ_H 7.20, for **10** and **11**, respectively). This point added to the fact that there was no NOESY correlation between the aromatic protons and the olefinic signal H-11 was an indication that the aromatic ring had an α orientation while the C-11 – C-12 bond had a β orientation (Figure 3-6). The major difference between **10** and **11** is the chemical shifts of H-12 (δ_H 2.63 and δ_H 2.47, for **10** and **11**, respectively). This suggests that **10** and **11** differ in the relative configuration between C-11 and C-12. The epoxide ring present in **10** and **11** could have resulted from a nonselective epoxidation of the C-13 - C-14 double bond of **9**.

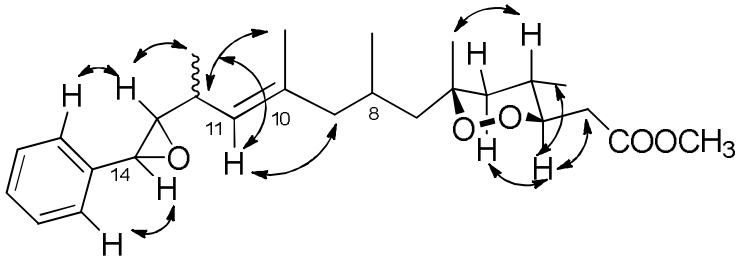


Figure 3-6. Key identical NOESY correlations for 10 and 11

A fifth compound, plakinastreloic acid B (**12**), was isolated as a colorless amorphous solid {$[\alpha]_D$= + 60 (*c*.0.06, MeOH)}. The inspection of the ^{13}C and ^{1}H NMR spectra coupled with the HRMS data suggested a formula of $C_{23}H_{45}O_4$ [$(M+H^+)$ *m/z* observed 385.4380]**,** indicating the presence of two degrees of unsaturation**.** The ^{1}H NMR spectrum indicated the presence of four methyl groups [δ_H 0.88 (3H, t, *J* = 7.2 Hz),

δ_H 0.97 (3H, d, *J* = 6.6 Hz), δ_H 1.34 (3H, s) and δ_H 1.52 (3H, s)]. Complex signals were present between δ_H 1.33 - 1.20 (23 H, m), suggesting the presence of an aliphatic chain. Based on the HSQC spectrum, these signals could be correlated to complex carbon signals in the δ_C 29.6 -30.0 region. This was further confirmed by the DEPT spectrum.

Based on the HSQC spectrum, the methylene protons could be assigned as follows: 2H attached to C-9, 2H attached to C-19, 1H attached to the methylene carbon C-8, and finally, 2H attached to C-18. By simple subtraction, it was established that the remaining 16 hydrogens (eight methylene) belonged to the aliphatic chain. Based on the HSQC, three additional methylene groups were identified, including C-3, C-4 and C-6. It was established that the molecule contained three quaternary carbons as well as a methine signal. All these carbons add up to a 23 carbon molecule.

Analysis of the ^{1}H-^{1}H COSY spectrum showed evidence for the existence of three independent spin systems; these are labeled A-C and shown as bold bonds in Figure 3-7; full tables of data are included.

The peroxide ring depicted in Figure 3-7 was assembled based on the following HMBC correlations: first, the correlations between the methyl protons H-21 (3H, δ_H 1.34, s) and C-1, C-2, C-3, C-4 and then the correlations between the methyl protons H-22 (3H, δ_H 1.52, s) and the carbons C-5, C-4 and C-6. Because of the existence of an HMBC correlation between H-6a (1H, δ_H 1.46, dd, *J* = 7.0, 15.0), H-6b (1H, δ_H 1.62, m) and C-5, it was determined that the spin system that extended from C-6 trough C-8 was connected to the peroxide ring. Another HMBC correlation was identified between H-8a (1H, δ_H 1.17, m), H-8b (1H, δ_H 1.29, m) and a carbon at δ_C 27.2 which is part of the

aliphatic chain that extended from C-8 to C-20. H-20 (3H, δ_H 0.88, t, J = 7.2) gave an HMBC correlation to C-19 and C-18. Based on these correlations, the structure of plaskinatrealoic acid B (**12**) was determined to be as depicted in Figure 3-7.

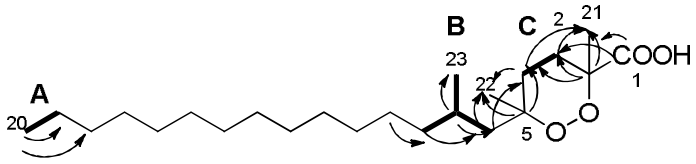


Figure 3-7. Key HMBC and ^{1}H-^{1}H COSY correlations of 12

Based on the NOESY spectrum, it was established that the relative configuration around the peroxide ring of 12 is as depicted in Figure 3-8. A NOESY correlation was observed between H-22 (3H, δ_H 1.52, s) and H-3b; no similar correlation to H-3a was observed. A weak correlation was also detected between H-23 (3H, δ_H 0.97, d, J = 6.6) and H-22. Another NOESY correlation was observed between H-6a (1H, δ_H 1.46, dd, J = 7.0 Hz, 15.2 Hz) and H-4a; no correlation between H-6a and H-4b was observed. Finally, there were NOESY correlations between H-21 (3H, δ_H 1.34, s) and both protons of the methylene C-3 and C-4, suggesting spin diffusion.

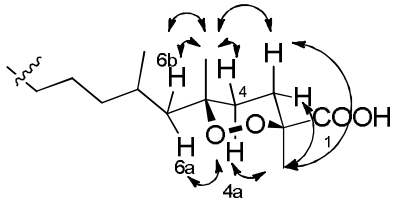


Figure 3-8. Relative configuration of 12 based on NOESY correlations (↔)

Plakinastreloic acid A (**8**) and methyl plakinastreloate A (**9**) showed an interesting bioactivity profile. The IC_{50} values for plakinastreloic acid A (**8**) and methyl plakinastreloate A (**9**) against *Candida albicans* were 6.5 and 3.5 µg/mL, respectively; against *Aspergillus fumigatus*, the IC_{50} values were 4.0 and 9.0 µg/mL, respectively. Finally, against *Cryptococcus neoformans*, the IC_{50} values were 4.0 and 9.0 µg/mL respectively. A moderate antimalarial activity against the CQ-resistant (strain W2) and sensitive strains of *P. falciparum* (strain D6) was also observed for **8** and **9**. For plakinastreloic acid A (**8**), the IC_{50} values were 3.8 and 2.7 µg/mL, respectively against D6 and W2. The selectivity indexes were higher than 1.3 and 1.8, respectively for these 2 strains. For **9**, the IC_{50} values against the malaria parasite were 2.3 µg/mL and 1.8 µg/mL, respectively, for the CQ-sensitive (D6) and the CQ-resistant strains (W2) of *P. falciparum*. The selectivity indexes were higher than 2.1 and 2.6, respectively for these two strains. No cytotoxicity against Vero cells was observed up to 4.7 µg/mL, the highest concentration tested. Plakinastreloic acid A (**8**) also exhibited antiviral activity against the Hepatitis C Virus (HCV) in a dose dependent manner. At the highest concentration (10 µg/mL), the percentage of HCV's RNA inhibition was estimated at 88%. It is important to note that a significant reduction in the level of rRNA replicon was noted as well (Figure 3-9) suggesting that plakinastrella A (**8**) acts in a non-selective manner (cytotoxic). The EC_{50} was 2.2 µg/mL and the $EC_{90} > 10$ µg/mL.

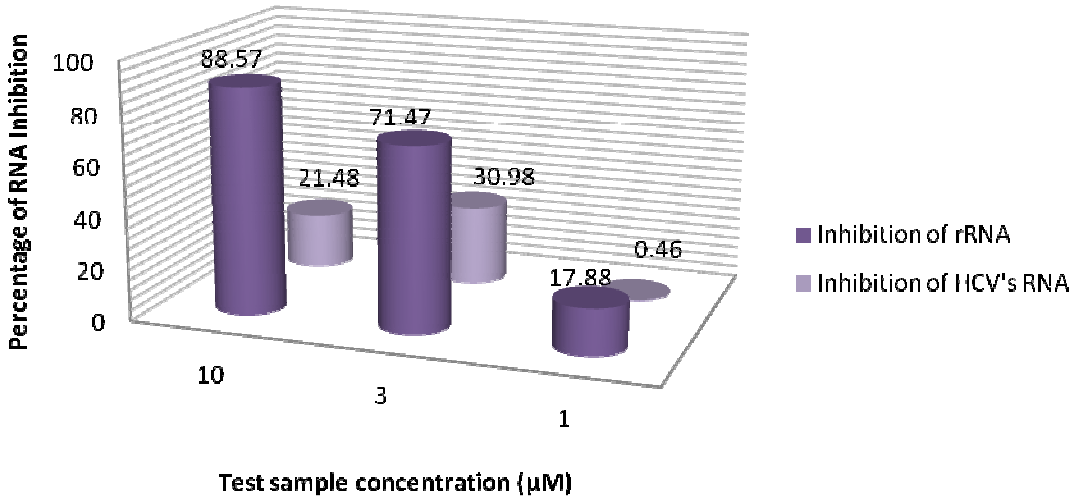


Figure 3-9. Dose-dependent anti-HCV activity of plakinastreloic acid A (8)

2. Experimental

2.1. General experimental procedures

1D and 2D NMR spectra were recorded on a 600 MHz NMR spectrometer (Varian; 3mm inverse probe with variable temperature control (vtc). Chemical shifts are expressed in ppm with the residual solvent signal used as standard.

Semi-preparative and preparative HPLC were carried out using a Waters PrepLC system; Reverse (C_{18}, C_8) and normal phase (silica gel) HPLC columns were used subsequently. The MW of **8** was determined by LCMS analysis under negative (Bruker Daltonics MicroTOF LCMS). The high resolution mass spectra (HRMS) for **10**, **11**, and **12** were recorded on a Waters Micromass Q-TOF® Micro mass™ spectrometer equipped with a NanoLockSpray™ source.

2.1.1 Extraction and isolation

A bioassay-guided approach was used for the isolation of all compounds mentioned in this report. Targeted biological assays included tests against *P. falciparum*, HCV and a variety of microbial pathogens (Gram positive and negative bacteria, as well as, a set of fungi). The initial fractionation of the crude extract was completed by VLC on silica gel using a *n*-hexane/acetone gradient. The most active fraction was further purified by HPLC. The first HPLC was completed on a 100 × 250 mm C_8 column. The flow rate was 25 mL/min and the solvent system varied from 100:0 H_2O/CH_3CN → 40:60 H_2O/CH_3CN over 60 minutes and then from 40:60 H_2O/CH_3CN → 0:100 H_2O/CH_3CN over an additional 240 minutes. The fraction collection was UV–guided (254 nm) and 62 fractions were collected. Two of these fractions that eluted with 100% CH_3CN, namely the fraction 44, from which **8**, **9**, **10, 11** were isolated and the fraction 58 from which **12** was isolated, were selected for further purification. The HPLC of fraction 44 using the same column (C_8, 100 × 250 mm) but a different solvent system led to poor separation; solvents were set to vary from 25:75 H_2O/CH_3CN → 20:80 H_2O/CH_3CN over 40 minutes and then from 20:80 H_2O/CH_3CN→ 0:100 H_2O/CH_3CN over an additional 80 minutes. Nonetheless, the further purification of a fraction that eluted with 100% CH_3CN by prep-TLC led to the isolation of **9.** The solvent system for the prep-TLC was 80:20 *n*-hexane-EtOAc. A better separation was achieved by switching the column to Phenomenex C_{18} column (21.2 × 250 mm). The solvent system varied from 25:75 H_2O/CH_3CN → 20:80 H_2O/CH_3CN (40 minutes) and from 20:80 H_2O/CH_3CN →0:100 H_2O/CH_3CN for an additional 80 minutes; the flow rate was 6 mL/min; using this second approach, **8** eluted with 14:86 H_2O/CH_3CN. The fraction that

eluted with 6:94 H_2O/CH_3CN was further purified using a Phenomenex C_8 column (10 × 250 mm). In this particular case, the solvent system varied from 15:85 H_2O/CH_3CN → 0:100 H_2O/CH_3CN (25 minutes) and **10** and **11** eluted with 0:100 H_2O/CH_3CN. The amount of plakinastreloic acid A (**8**) derived from 30 mg of fraction 44 was 3.5 mg and the combined weight of **10** and **11,** epimers of 13, 14 –epoxyplakinatreloate, was 1.6 mg; the structure of **10** and **11** was solved as a mixture. The fifth compound (plaskinastreloic acid B) was isolated from the fraction 58. The amount of plakinastreloic acid C (**12**) derived from 40 mg of fraction 58 was 3.5 mg. The purification of plaskinastreloic acid B (**12**) was completed using a Luna 10µ silica HPLC column (21.2 × 250 mm). The solvent system varied from 0:100 2-propanol/*n*-hexane → 10:90 2-propanol/*n*-hexane over 45 minutes and then from 10:90 2-propanol/*n*-hexane → 40:60 2-propanol/*n*-hexane (25 minutes). All these isolations were monitored by UV (254 nm) and **12** eluted with 14:86 2-propanol/*n*-hexane.

Plakinastreloic acid A [**8**]. Colorless amorphous solid; $[\alpha]_D$ = + 38 (*c*.0.09, MeOH); MS negative mode m/z 399.9 (very weak signal) [M-H] (calcd for $C_{25}H_{35}O_4$, 399.2535); ^{1}H NMR (CD_3OD)

Table 3-2 and ^{13}C NMR (CD_3OD) Table 3-1

Methyl plakinastreloate [**9**]. Colorless, amorphous solid; $[\alpha]_D$= + 32 (*c*.0.03, MeOH); $C_{26}H_{38}O_4$, calcd = 414.2770; ^{1}H NMR (CD_3OD);

Table 3-2 and ^{13}C NMR (CD_3OD) Table 3-1

Epimer of Methyl 13, 14-epoxyplakinastreloate [**10** and **11**] Colorless amorphous solid. The HRMS m/z 453.2617 [M + Na^+] (calcd for $C_{26}H_{38}O_5Na$, 453.2617); ^{1}H NMR (CD_3OD);

Table 3-2 and ^{13}C NMR (CD_3OD) Table 3-1

Plakinastreloic acid B [**12**]. Colorless, amorphous solid $[\alpha]_D$= + 60 (*c*.0.06, MeOH); HRMS m/z 385.3318 [M + H^+] (calcd for $C_{23}H_{45}O_4$, 385.6033); ^{1}H NMR (CD_2Cl_2);

Table 3-2 and ^{13}C NMR (CD_2Cl_2) Table 3-1

2.2. Material and methods

2.2.1. Antifungal and antibacterial assay

Several strains: *Candida albicans* ATCC 90028*, Cryptococcus neoformans* ATCC 90113*, Aspergillus fumigatus* ATCC 90906, Methicillin resistant *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellular* ATCC 23068) were used in these assays. Positive controls were amphotericin B (for *C. albicans, C. neoformans and A. fumigatus)* and ciprofloxacin for the rest the remaining bacteria. This assay was completed in a 96 well plate format [205].

2.2.2. Antiviral assay

The anti-HCV activity was determined in the HCV replicon (Clone B) system (Huh 7 clone B cells containing HCV RNA). Cells were seeded onto 96-well tissue culture plates (3,000 cells/well); the compounds were tested in a dose response at concentrations of 10, 3, 1 uM (triplicate). After addition of test compounds the plates were incubated for five days (37 ℃, 5%, CO_2). Afterwards the total cellular RNA was extracted using the manual Perfect Pure RNA 96 cell Vacki from 5 Prime. The replicon RNA and the internal control (TaqMan rRNA control reagent, applied Biosystems) were amplified by RT-PCR. The antiviral potency of the tested molecule was determined by

subtracting the threshold RT-PCR cycle of the tests compounds from the threshold RT-PCR of the negative control (no drug): #CtHCV (a 3.3 value being considered a 1 log reduction in replicon RNA level (in other words 90% reduction). The cytotoxicity was calculated using #Ct rRNA values and RS-446 (2-Me-C) was used as control to determine the EC_{50} and EC_{90} values [206, 207].

2.2.3. Antimalarial assay

The test was based on the determination of plasmodial LDH activity. Suspensions of red blood cells infected with D6 or W2 strains of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL amikacin) was added to the wells of a 96-well plate containing 10 µL of test samples diluted in medium at various concentrations. The plate was placed in a modular incubation chamber (Billups-Rothenberg, CA) flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 and incubated at 37 ºC, for 72 h. Parasitic LDH activity was determined according to the procedure of Makler and Hinrichs (1993). 20 µL of the incubation mixture was mixed with 100 µL of the Malstat™ reagent (Flow Inc., Portland, OR) and incubated at room temperature for 30 minutes. 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was added and the plate is further incubated in the dark for 1 h. The reaction was stopped by the addition of 100 µL of a 5% HOAc solution. The plate was read at 650 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). IC_{50} values were computed from the dose response curves. Artemisinin and CQ were included in each assay as the drug controls. DMSO (0.25%) was used as a vehicle control [208].

Table 3-3. Complete 1D and 2D data of 8 (CD_3OD)

	^{13}C	δ H mult. (*J* in Hz)	COSY	HMBC	NOESY
1.	/	/	/	H-2a, H-2b, H-3	/
2.	36.0	2.68, dd (2.4, 16.0)/ 2.26, m	H-3, H-2a, H2b	/	/
3.	83.7	4.01, dt (2.8,9.6)	H-2a, H-2b, H-4	H-21, H-2a, H-2b, H-5a, H-5b, H-4	H-21, H-5a, H-2a, H-2b
4.	30.3	1.85, m	H-3, H-21, H-5a, H-5b	H-21, H-5	H-5a, H-22
5.	42.7	1.63, d (4.4) 1.21, m	H-4	H-22, H-21	H-4, H-23
6.	81.5	-		H-7a, H-7b, H-5a, H-5b, H-22	
7.	47.0	1.50, dd (2.8, 14.8) 1.21, m	H-8	H-23, H-22, H-9a, H-9b	H-9a, H-9b
8.	26.0	1.81, m	H-7a, H-7b, H-23, H-9ab^{m}	H-23, H-7a, H-7b	
9.	49.1	1.87, m 1.96, m	H-8^{m}	H-24, H-23, H-7a, H-7b	H-7a, H-7b, H-23
10.	133.2	/	/	H-12, H-9, H-24	/
11.	130.4	5.07, d (8.8)	H-12	H-25, H24, H-9a, H-9b, H-12, H-14	H-13, H14, H-25
12.	35.6	3.26, m	H-11, H-25, H-13	H-25	H-13, H-14, H-25
13.	134.7	6.17, dd (6.0, 16.0)	H-12, H-14	H-25, H12	H-11,H-12, H-16/H-20, H-25, H-14

14.	127.5	6.36, d (16.0)	H-13	H-13, H-12	H-11,H-12, H-16/H-20, H-25, H-13
15.	137.9	/	/	H-14, H-13, H-17	
16.	125.6	7.32, m	H-17/H-19[m]	H-20, H-14, H-18	H-13, H-14
17.	128.1	7.27, m	H-18, H20/H16[m]	H-19, H-15	/
18.	126.5	7.17, m	H-17		/
19.	128.1	7.27, m	H-18, H20/H16[m]	H-17, H-15	/
20.	125.6	7.32, m	H-17/H-19[m]	H-16	H-13, H-14
21.	16.1	0.91, d (6.8)	H-4	H-5a, H-5b	H-3, H-2b, H-4
22.	19.9	1.36, s	/	H-7a, H-7b, H-5a	/
23.	20.9	0.93, d (6.0)	/	H-7a, H-7b, H-9a, H-9b	H-5b, H-9a, H-9b[W], H-7a, H-22, H-8
24.	15.1	1.67, br s[*]	/	H-9a, H-9b, H-11	H-12
25.	20.3	1.16, d (6.6)	H-12	H-11, H-12, H-13, H-14	H-12, H-11, H-13

*assigned as a broad singlet, slightly split at the top but has no COSY correlation,
[m] merged signals
[w] weak correlations

Table 3-4. Complete 1D and 2D data of 10 and 11 (CD_3OD)

	^{13}C 10	^{13}C 11	δ_H mult. (*J* in Hz) 10	δ_H mult. (*J* in Hz) 11	COSY	HMBC	NOESY
1.	171.9	171.9	/	/	/	OMe, H-2a, H-2b,	/
2.	35.7	35.7	2.71, m 2.26, m	2.71, m 2.26, m	H-2a, H2b,H-3	/	/
3.	83.7	83.7	3.95,m	3.96, m	H-2a, H-2b, H-4	H-21, H-2a, H-2b, H-5b	H-21, H-5a, H-2a, H-2b
4.	30.3	30.4	1.81, m	1.81, m	H-3, H-21, H-5a, H-5b	H-21, H-5a, H5b	/
5.	42.7	42.7	1.62, m 1.25, m	1.62, m 1.25, m	H-4	H-4, H-3, H-21, H-22	/
6.	81.7	81.7	/	/	/	H-5a, H-22	/
7.	47.3	47.3	1.24, m 1.57, m	1.24, m 1.57, m	H-8	H-22, H-23^{w}, H-9a, H-9b	/
8.	26.1	26.1	1.80, m	1.80, m	H-7a, H-7b, H-9ab^{m}	H-23, H-7a, H-7b	/
9.	49.1	49.2	1.82, m 1.91, m	1.82, m 1.91, m	H-8^{m}	H-23, H-24, H-7a, H-7b	/
10.	135.6	135.8	/	/	/	H-24, H-9a, H-9b, H-12	/
11.	126.8	127.6	5.00, m	5.00, m	H-12	H-9, H-24, H-25, H-12	H-25, H9a, H9b, H13, H14
12.	34.5	35.5	2.63, m	2.47, m	H-25, H-11, H-13	H-25, H13	H-14, H-24, H-25
13.	67.0	66.8	2.79, dd (2.4, 6.0)	2.86, dd (2.4, 6.0)	H-14, H-12	H-25, H-12	H-16/H-20

14.	56.9	57.9	3.66, d (2.0)	3.77, d (2.0)	H-13	H-15, H16	H-16/H-20
15.	138.0	138.0	/	/	/	H-14, H-16	/
16.	125.3	125.4	7.20, m	7.20, m	m	H-14, H-20, H-18	H-14, H-13
17.	128.3	128.3	7.30, m	7.30 , m	m	H-19, H-15	/
18.	127.8	127.7	7.23, m	7.23, m	m	/	/
19.	128.3	128.3	7.30, m	7.30, m	-m	/	/
20.	125.3	125.4	7.20, m	7.20, m	-m	/	/
21.	15.6	16.2	0.86, m	0.86, m	/	H-5	H-2b, H-3, H-4
22.	20.0	20.0	1.26s	1.29s	/	H-7a, H-7b, H-5a	H-42b, H-4
23.	20.7	21.0	0.81, d (7.0)	0.9, d (7.0)	/	H-9a, H-9b, H-7b	
24.	15.4	15.6	1.64, brs	1.66, brs	/	H-11, H-9a, H-9b	H-12
25.	16.2	16.7	1.06, d (7.0)	1.1, d (6.0)	/	/	H-12, H-11
OMe	51.1	51.1	3.67, s	3.67, s	/	/	/

[w] weak signal, [m] merged

Table 3-5. Complete 1D and 2D data of 12 (CD_2Cl_2)

	^{13}C (CD_2Cl_2)	δ_H mult. (*J* in Hz) (CD_2Cl_2)	COSY	HMBC	NOESY
1.	169.8	/	/	H-3a, H-3b, H21	/
2.	69.6	/	/	H-21, H-3a, H-3b, H-4a, H-4b,	/
3.	43.8	2.56, dd (2.4,16.8) 2.39, d (16.8)	H4b, H-3a, H-3b	H-21, H-4a[w], H-4b,	H-21, H-4a
4.	45.6	1.93, dd (2.0, 14.0) 1.77, d (14.4)	H3b, H-4a, H-4b	H-21, H22, H-3a, H-3b, H-6a[w], H-6b[w],	H-3a, H-21, H-6a[4]
5.	84.4	/	/	H-22, H-4a, H-6a, H-6b	/
6.	51.6	1.62, m 1.46, dd (7.0, 15.2)	H-6a, H-6b	H-22, H-23, H-4a, H-4b[w],	H-23, H-4a[6]
7.	28.8	1.62, m	H-23, H-8a, H-8b	H-23, H-6a, H-6b,	/
8.	39.0	1.29, m 1.17, m	/	H-23, H-6a, H-6b,	/
9.	27.2	1.27, m	-[m]	-[m]	-[m]
10.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
11.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
12.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
13.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
14.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
15.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
16.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
17.	29.6-30.0[u]	1.27, m	-[m]	-[m]	-[m]
18.	32.1	1.27, m	-[m]	H-20	-[m]
19.	22.9	1.27, m	-[m]	H-20	-[m]
20.	14.1	0.88, t (7.2)	-	H-19-H-9[m]	-

21.	31.1	1.34, s	-	H-3a, H-3b, H-4a, H-4b,	H-3a, H-3b, H-4a, H-4b,
22.	28.1	1.52, s	-	-	H-4b
23.	21.7	0.97, d (6.6)	-	H-6a, H-6b,	H-6a, H-7/H6b, H-22

[m] merged
[u] interchangeable
*stronger signal
[6]Correlation to H-6a
[4]Correlation to H-4a

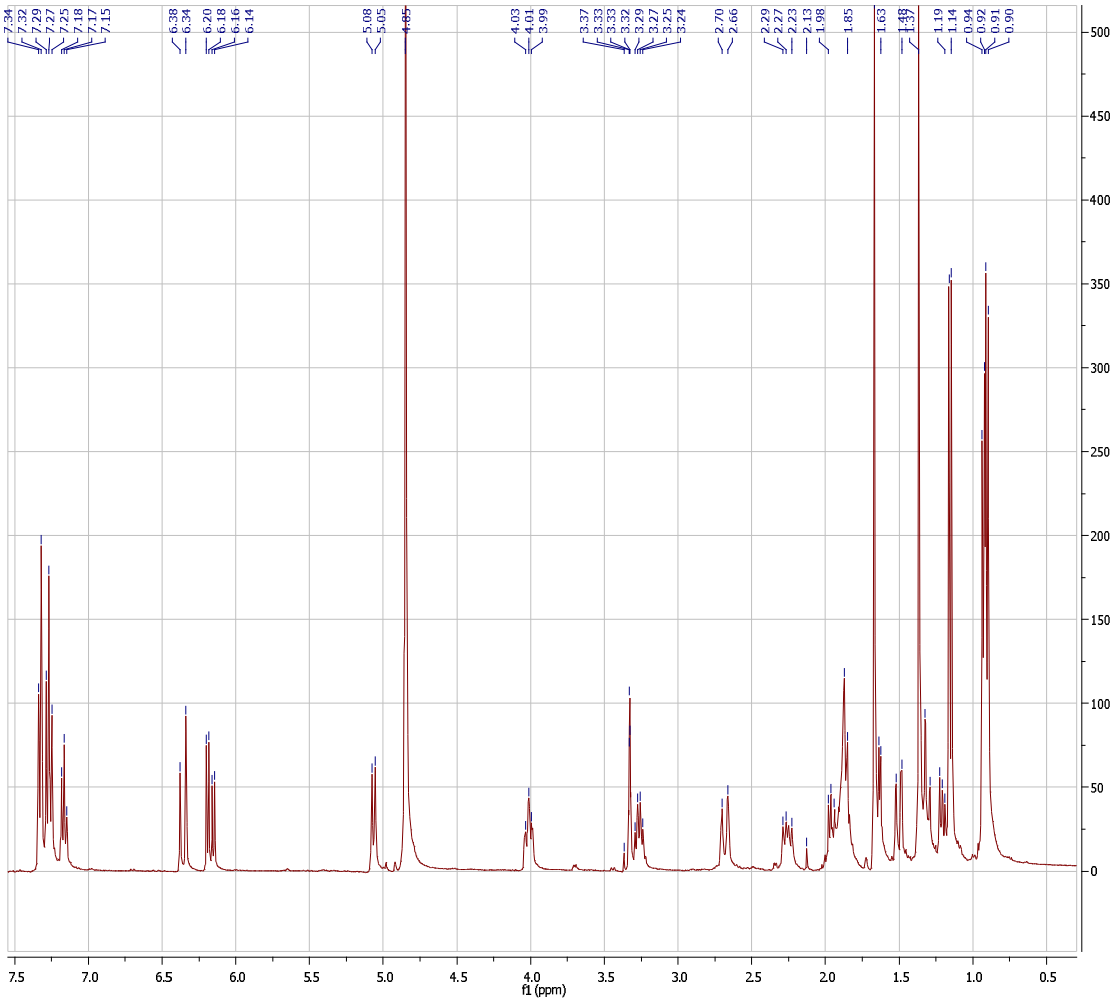


Figure 3-10. ^{1}H NMR spectrum of the plakinastreloic acid A (8) (CD_3OD)

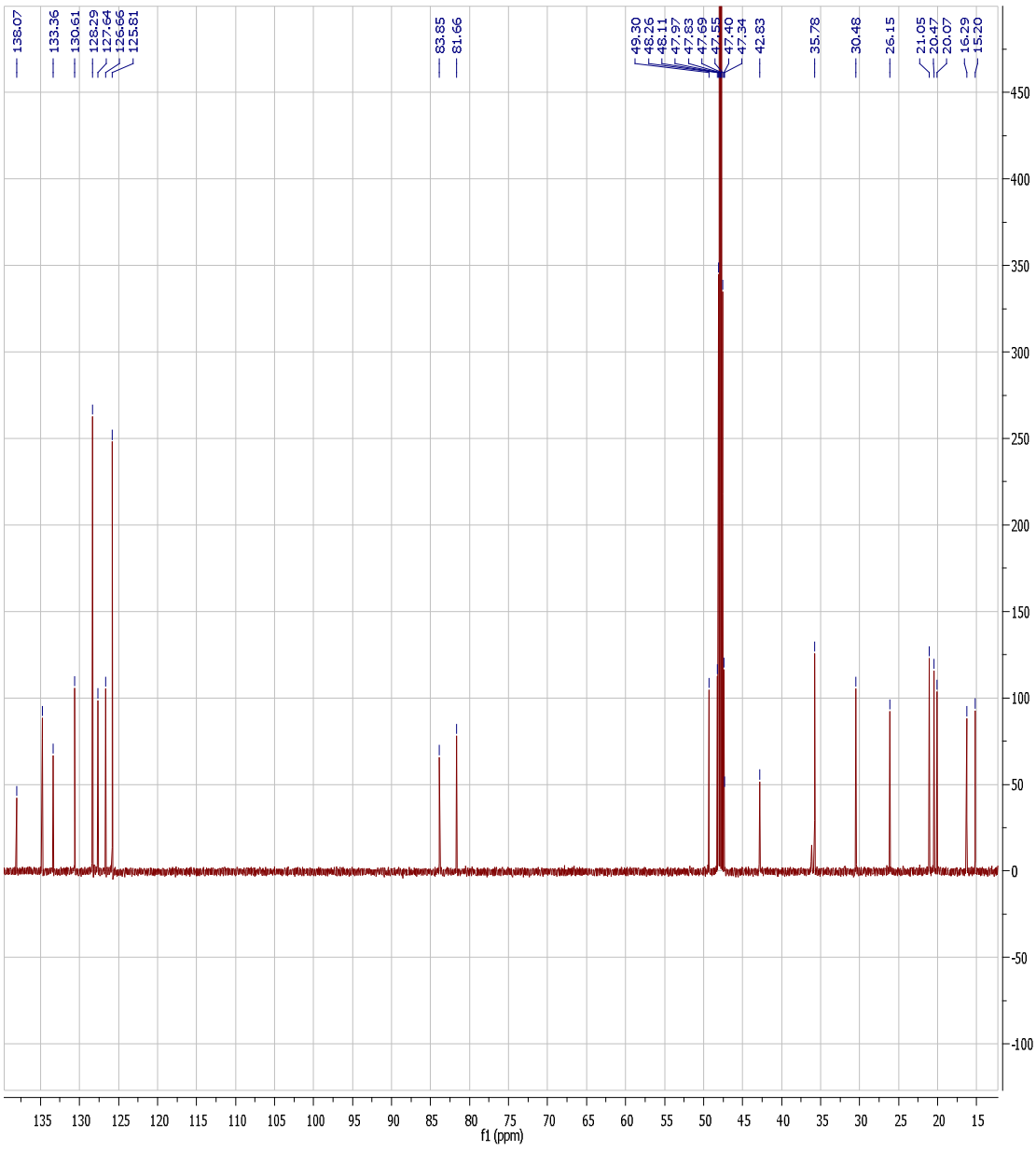


Figure 3-11. ^{13}C NMR spectrum of the plakinastreloic acid A (8) (CD_3OD)

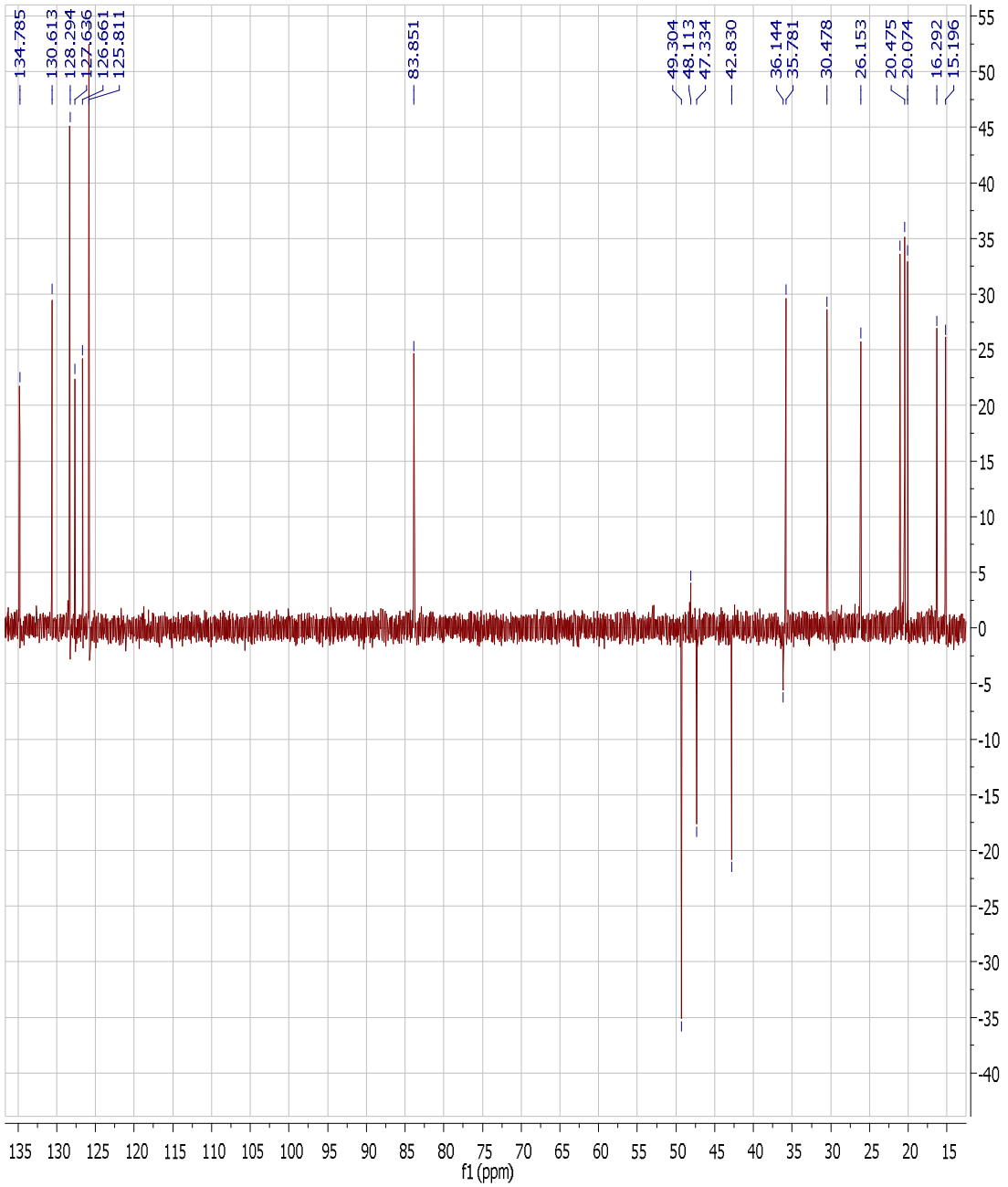


Figure 3-12. DEPT spectrum of the plakinastreloic acid A (8) (CD_3OD)

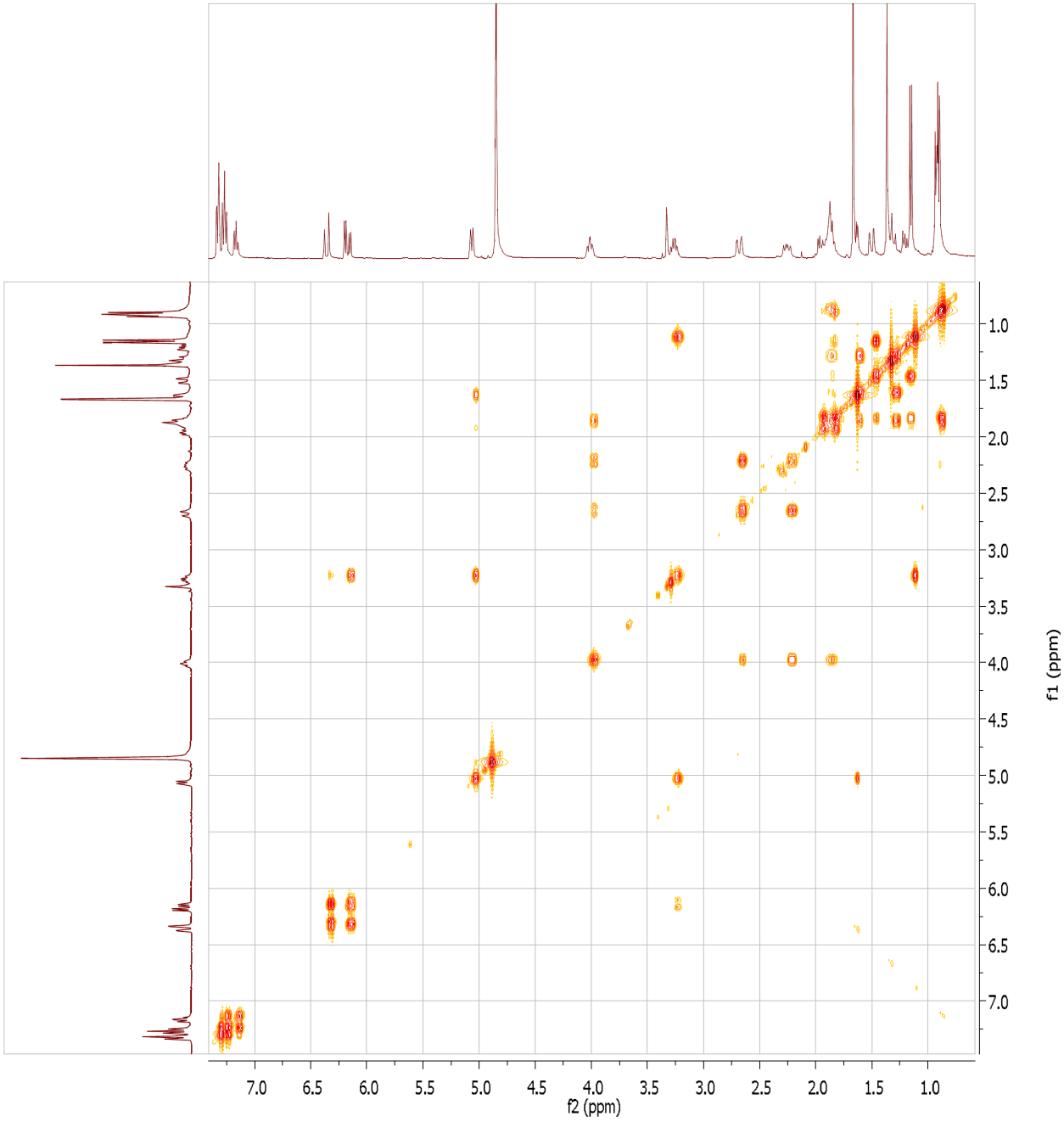


Figure 3-13. ^{1}H-^{1}H COSY spectrum of the plakinastreloic acid A (8) (CD_3OD)

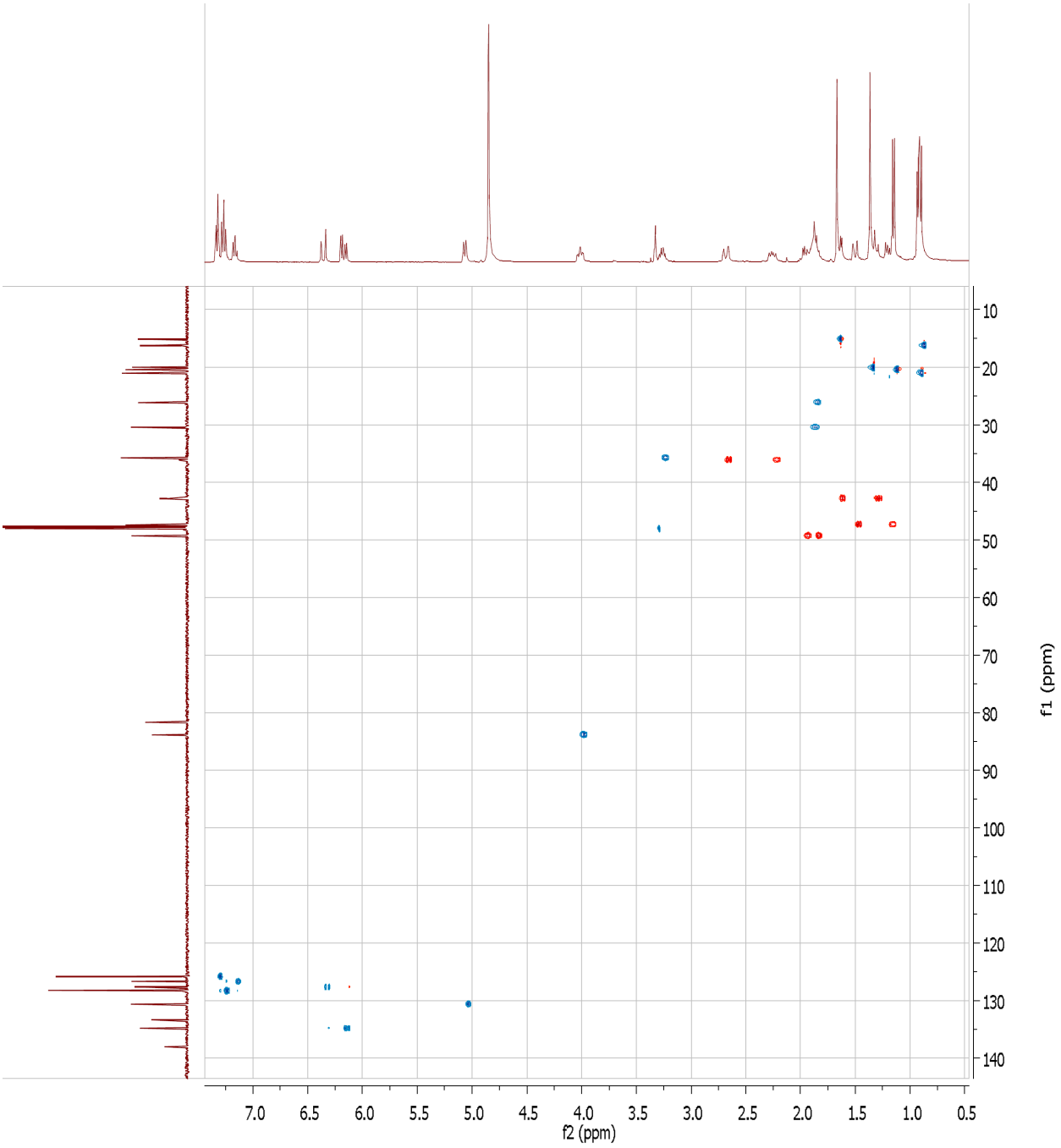


Figure 3-14. HSQC spectrum of the plakinastreloic acid A (8) (CD_3OD)

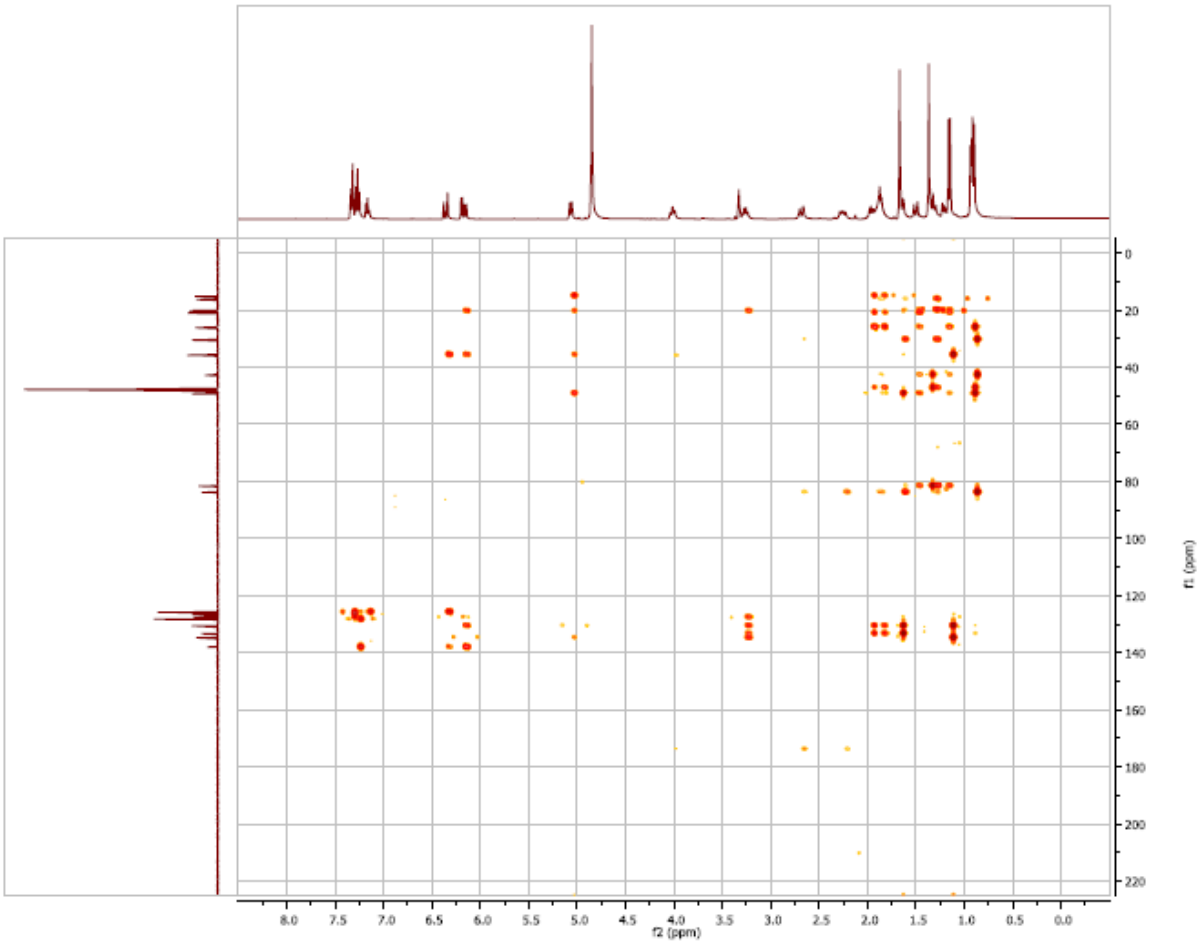


Figure 3-15. HMBC spectrum of the plakinastreloic acid A (8) (CD_3OD)

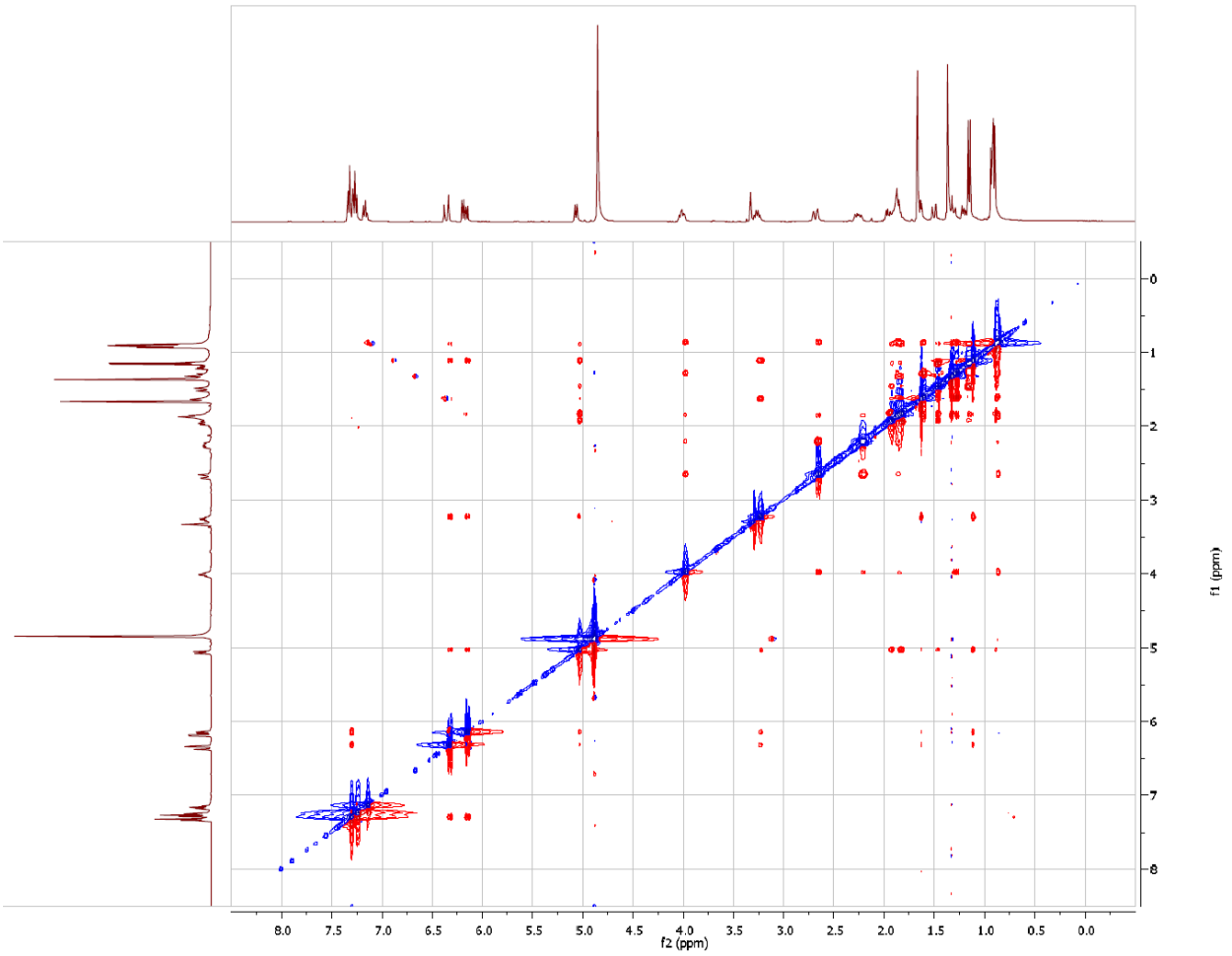


Figure 3-16. NOESY spectrum of the plakinastreloic acid A (8) (CD_3OD)

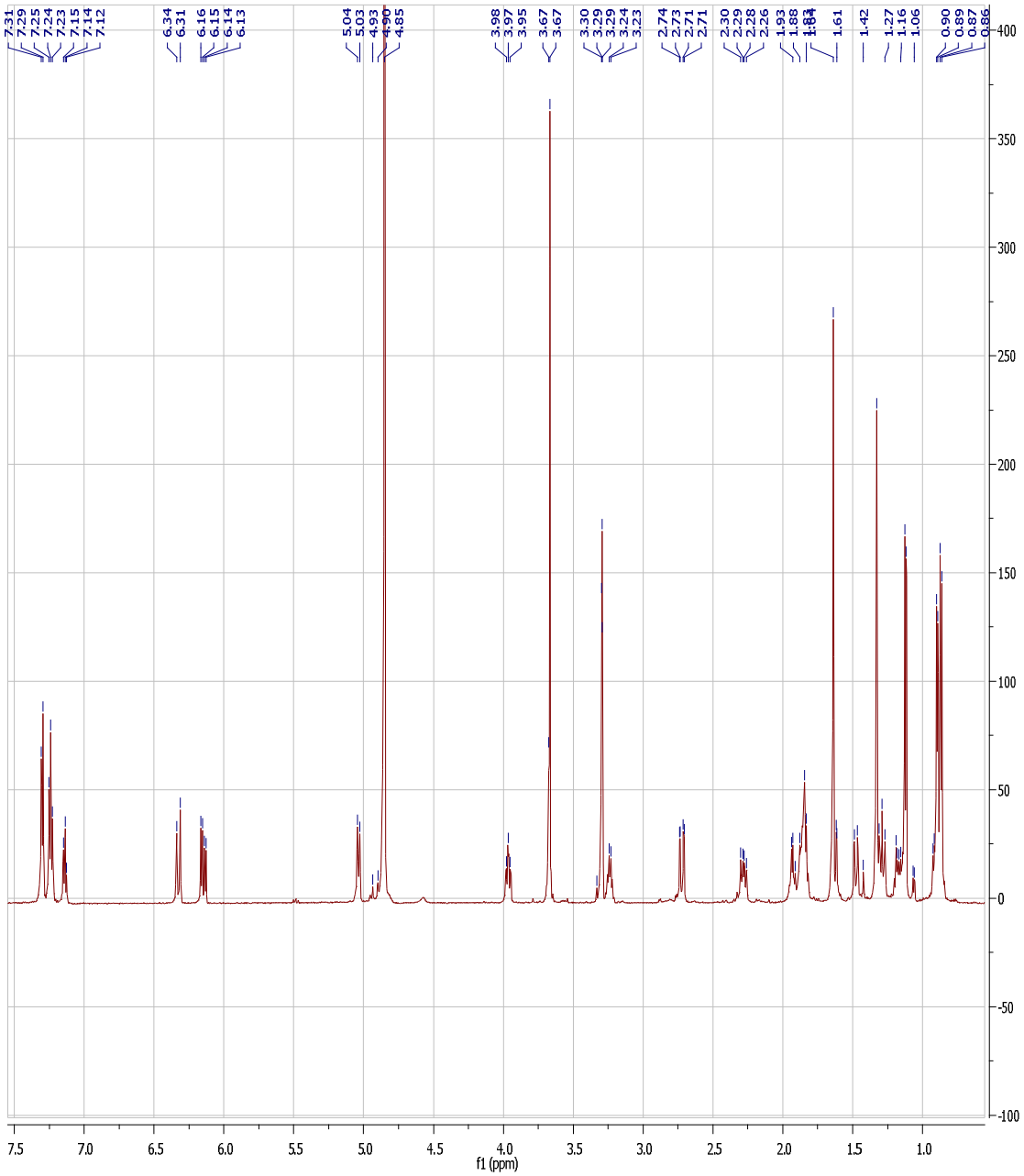


Figure 3-17. ^{1}H NMR spectrum of the methyl plakinastreloate (9) (CD_3OD)

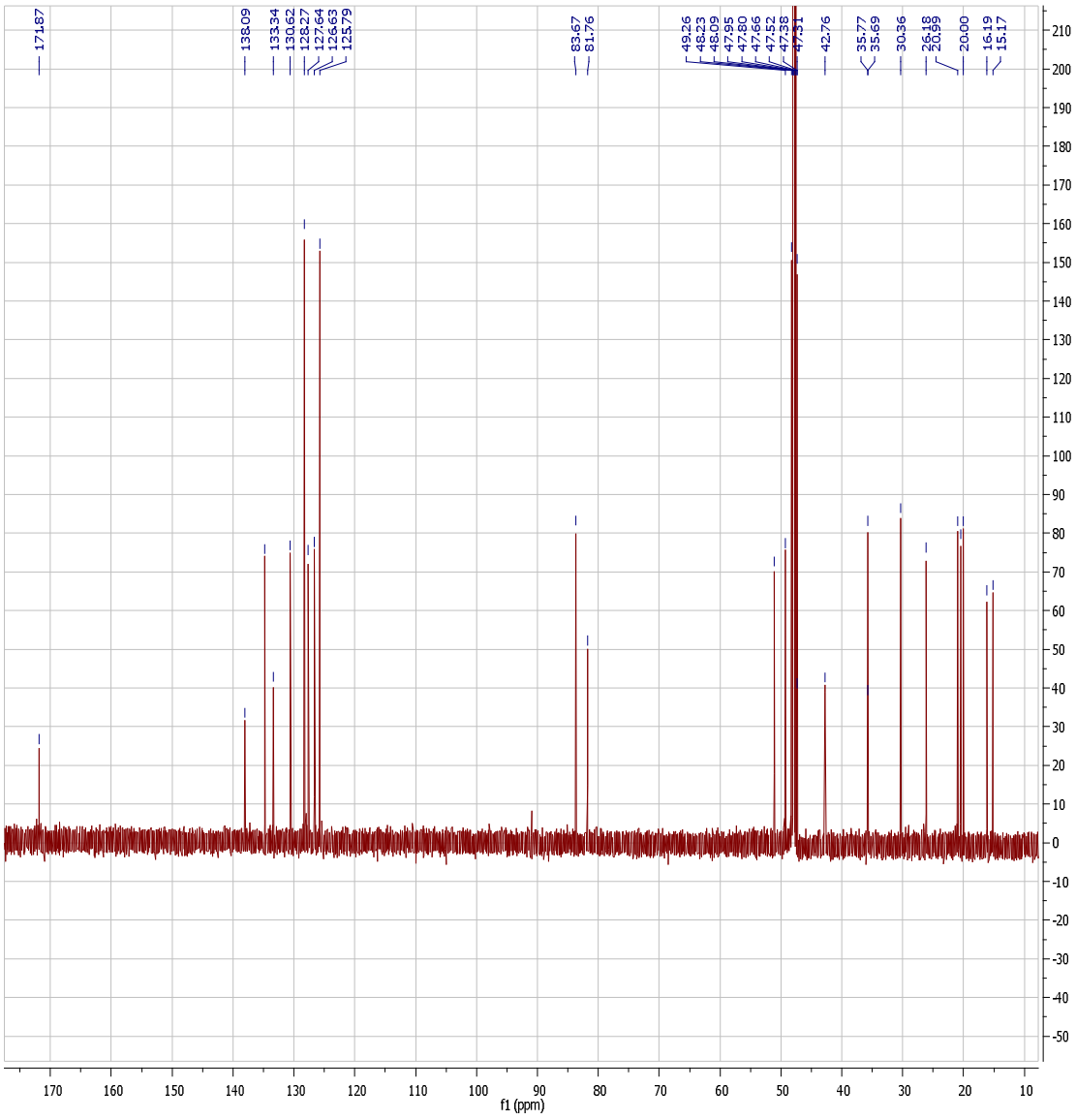


Figure 3-18. ^{13}C NMR spectrum of the methyl plakinastreloate (9) (CD_3OD)

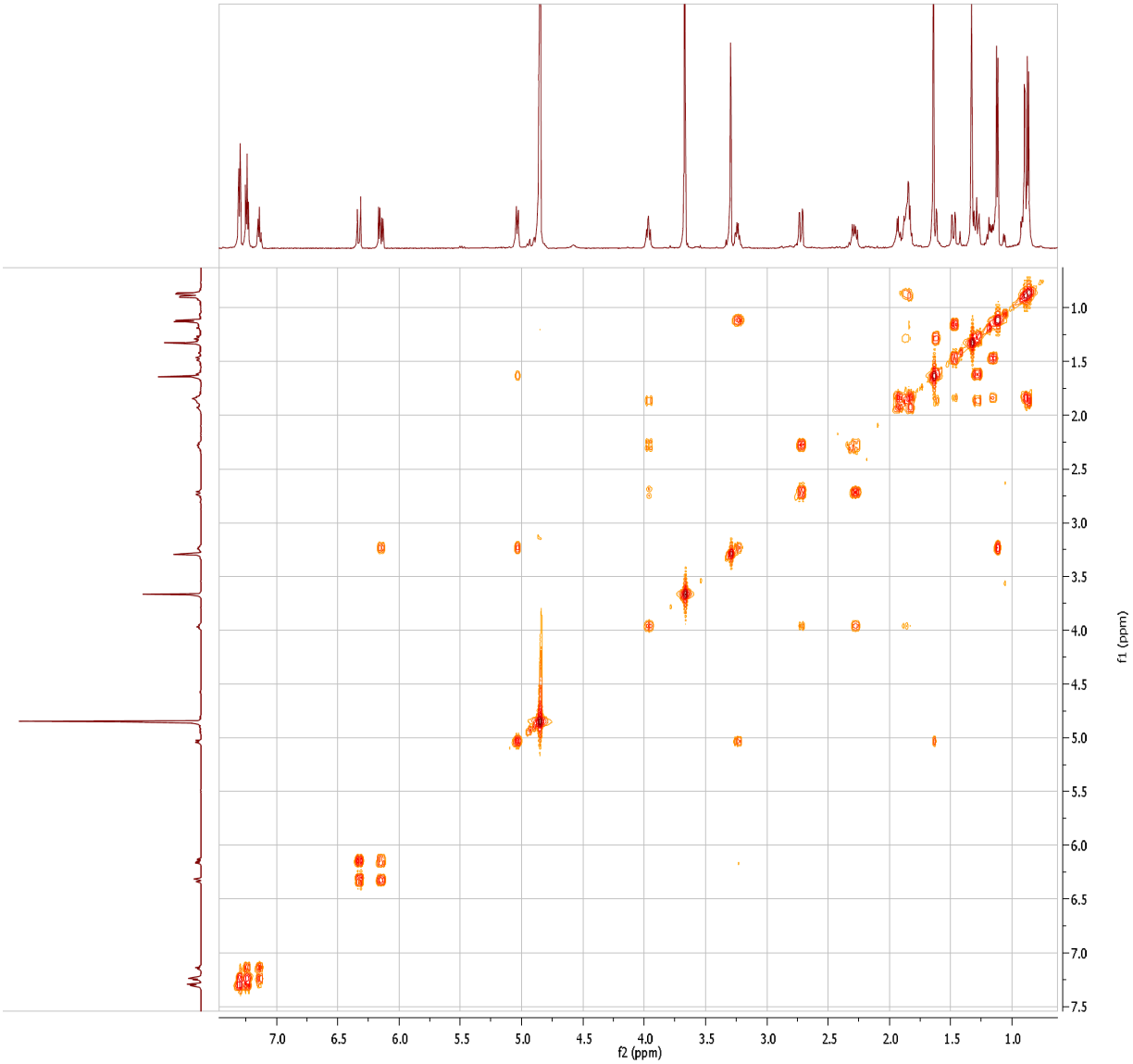


Figure 3-19. ^{1}H-^{1}H COSY spectrum of the methyl plakinastreloate (9) (CD_3OD)

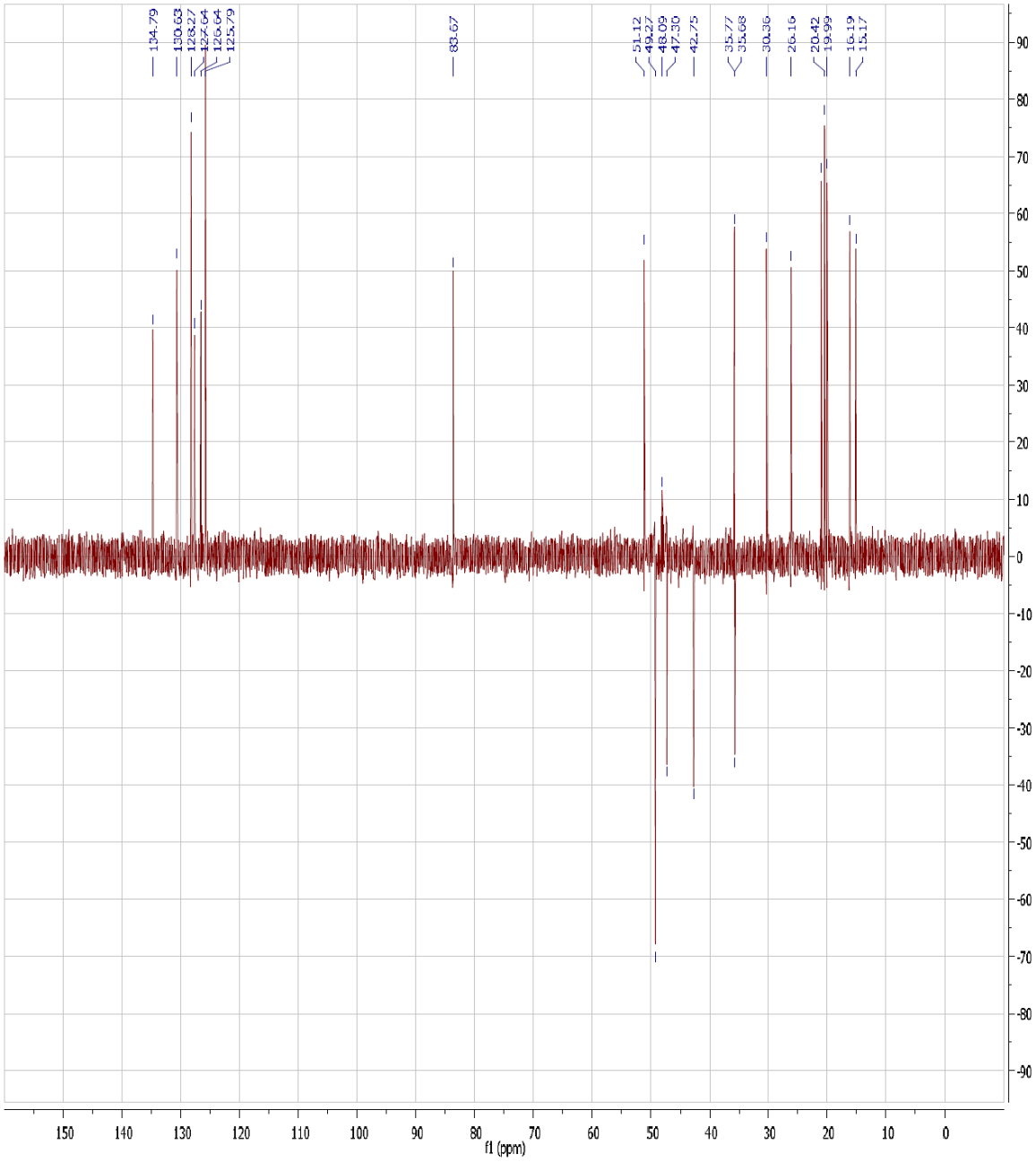


Figure 3-20. DEPT spectrum of the methyl plakinastreloate (9) (CD_3OD)

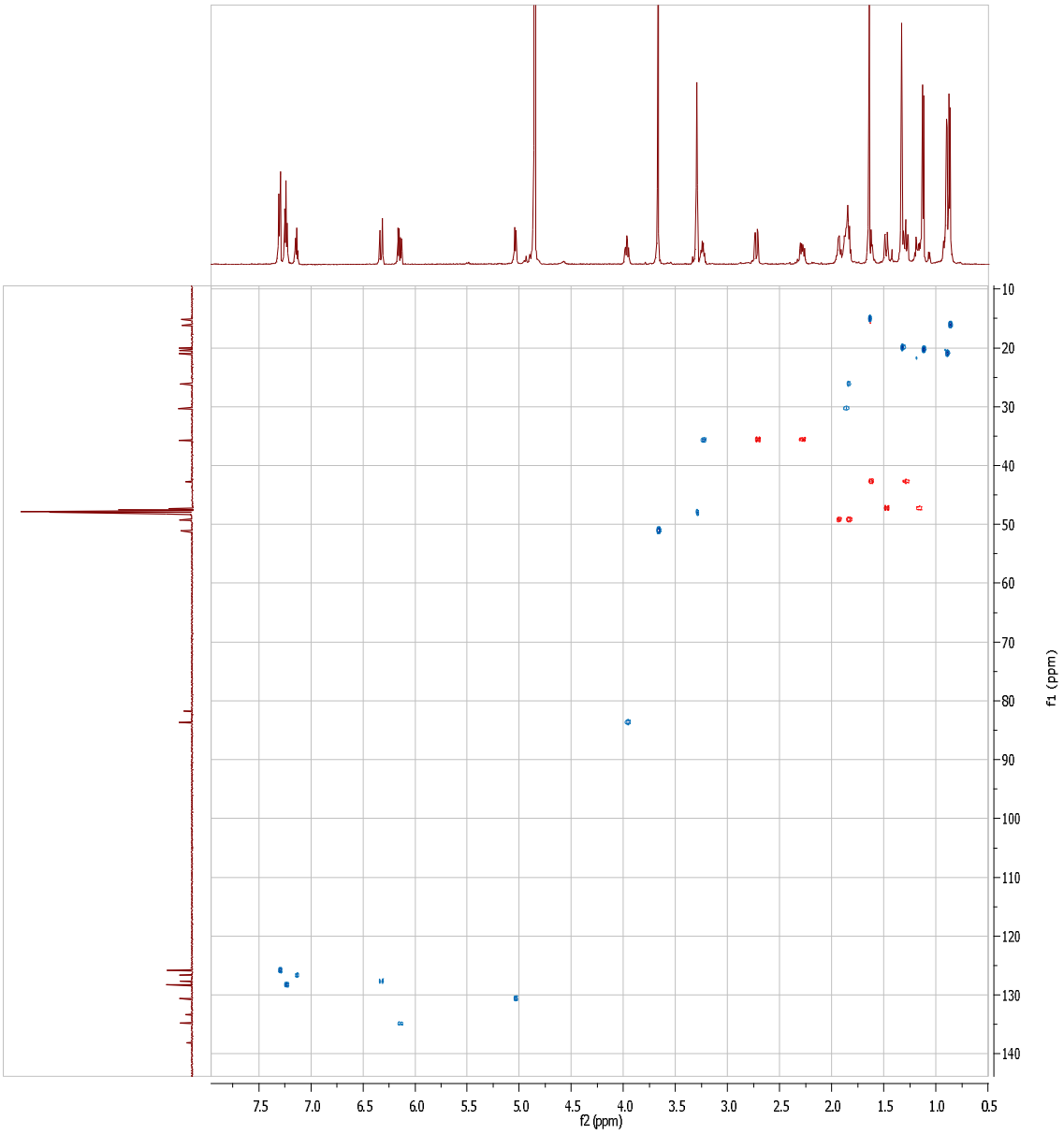


Figure 3-21. HSQC spectrum of the methyl plakinastreloate (9) (CD_3OD)

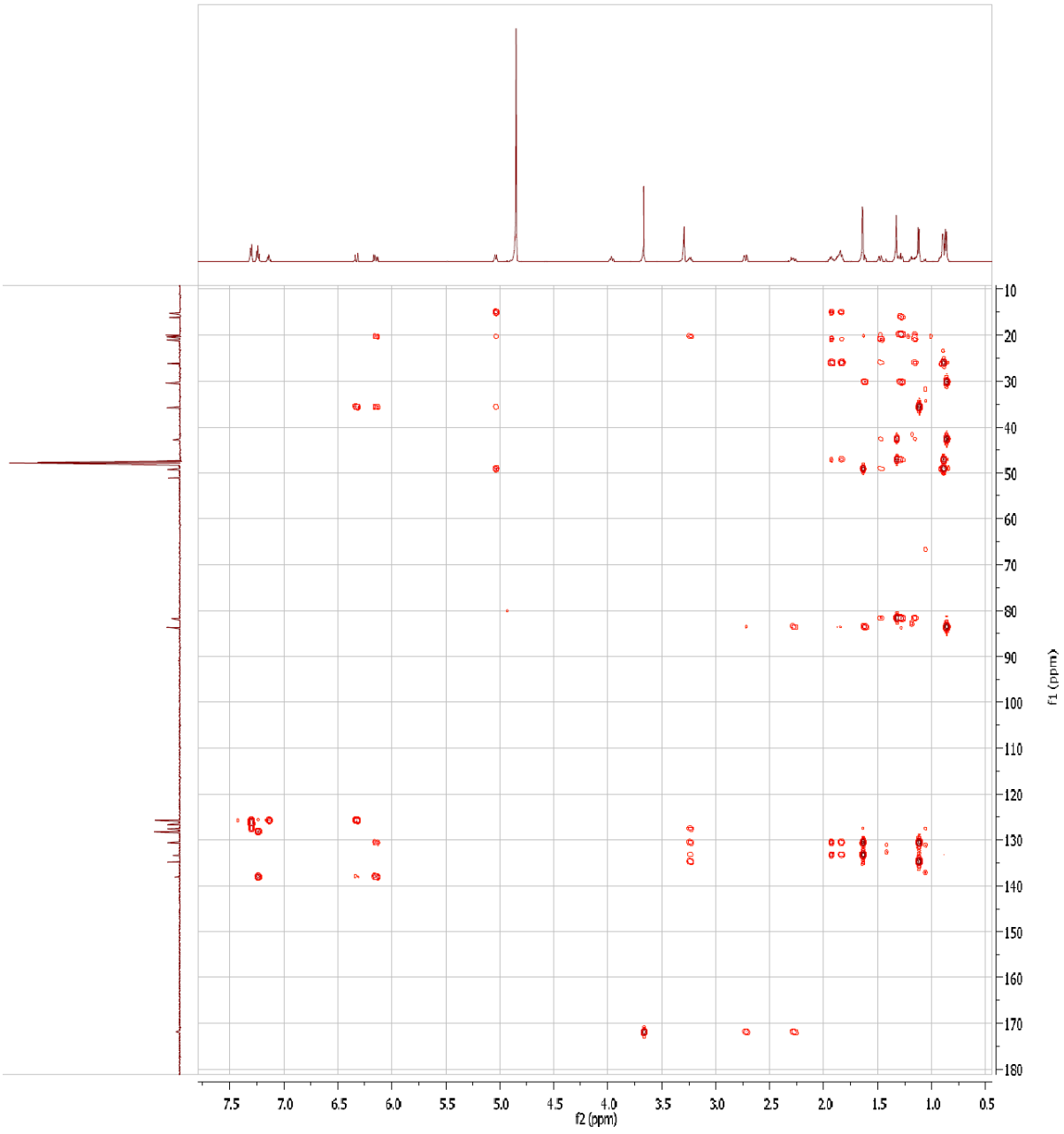


Figure 3-22. HMBC spectrum of the methyl plakinastreloate (9) (CD_3OD)

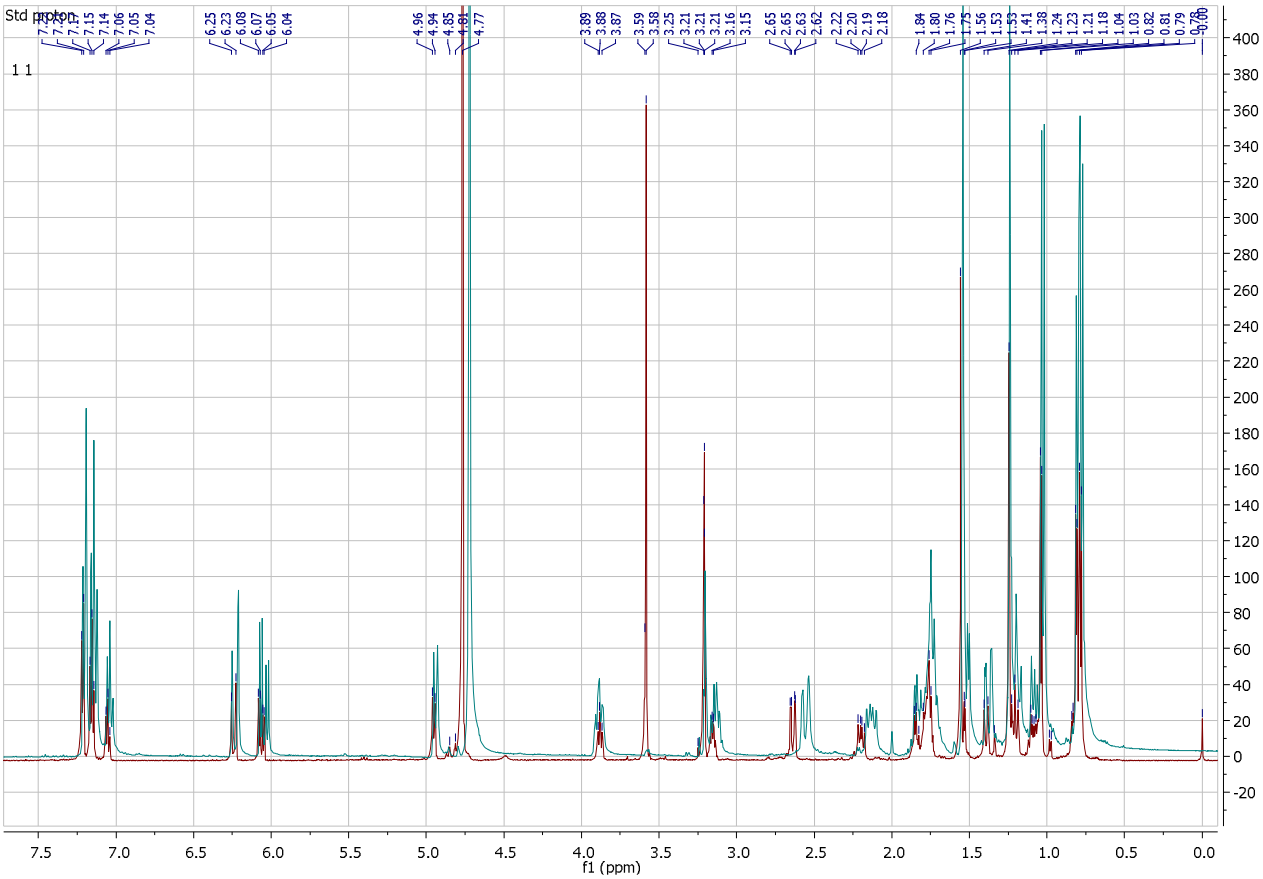


Figure 3-23. Merged ^{1}H NMR spectra of 8 (green) and 9 (red)

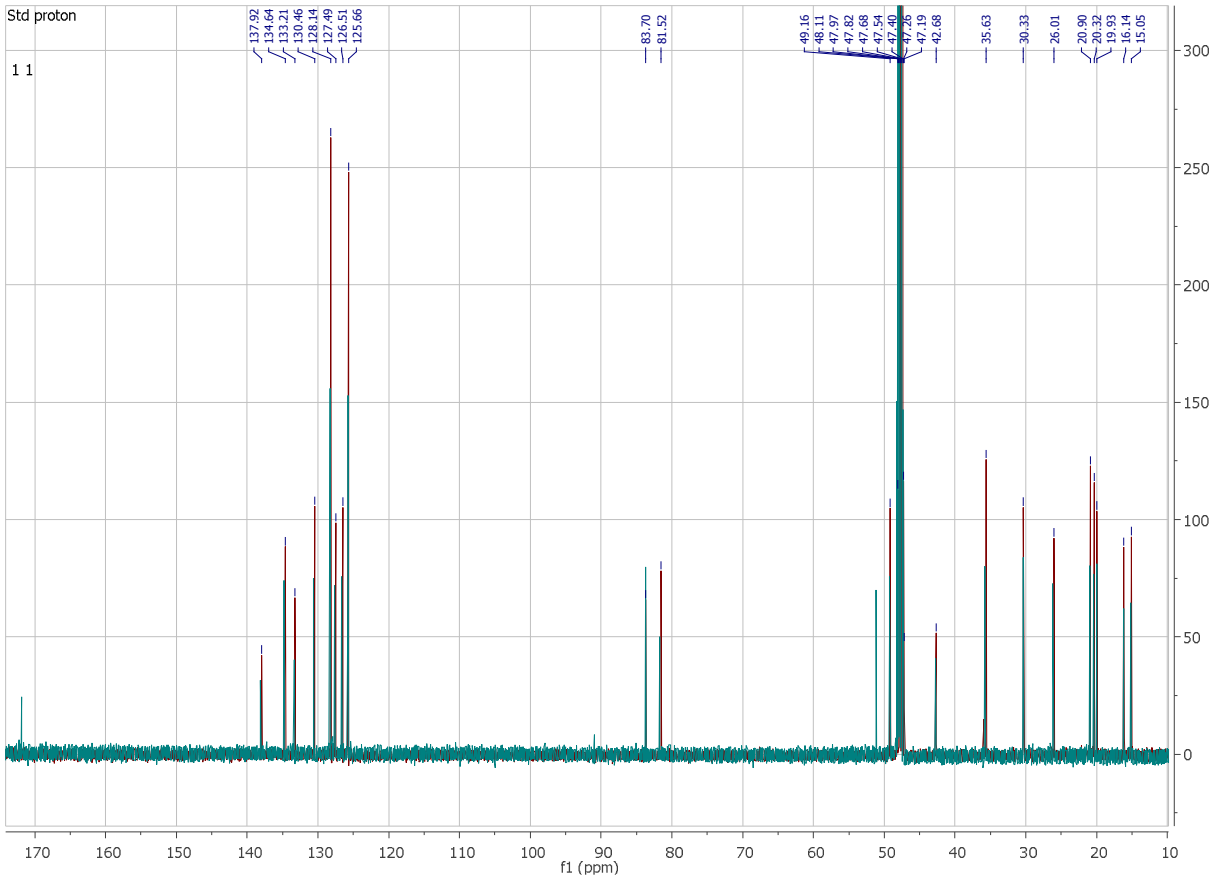


Figure 3-24. Merged ^{13}C NMR spectra of 8 (green) and 9 (red)

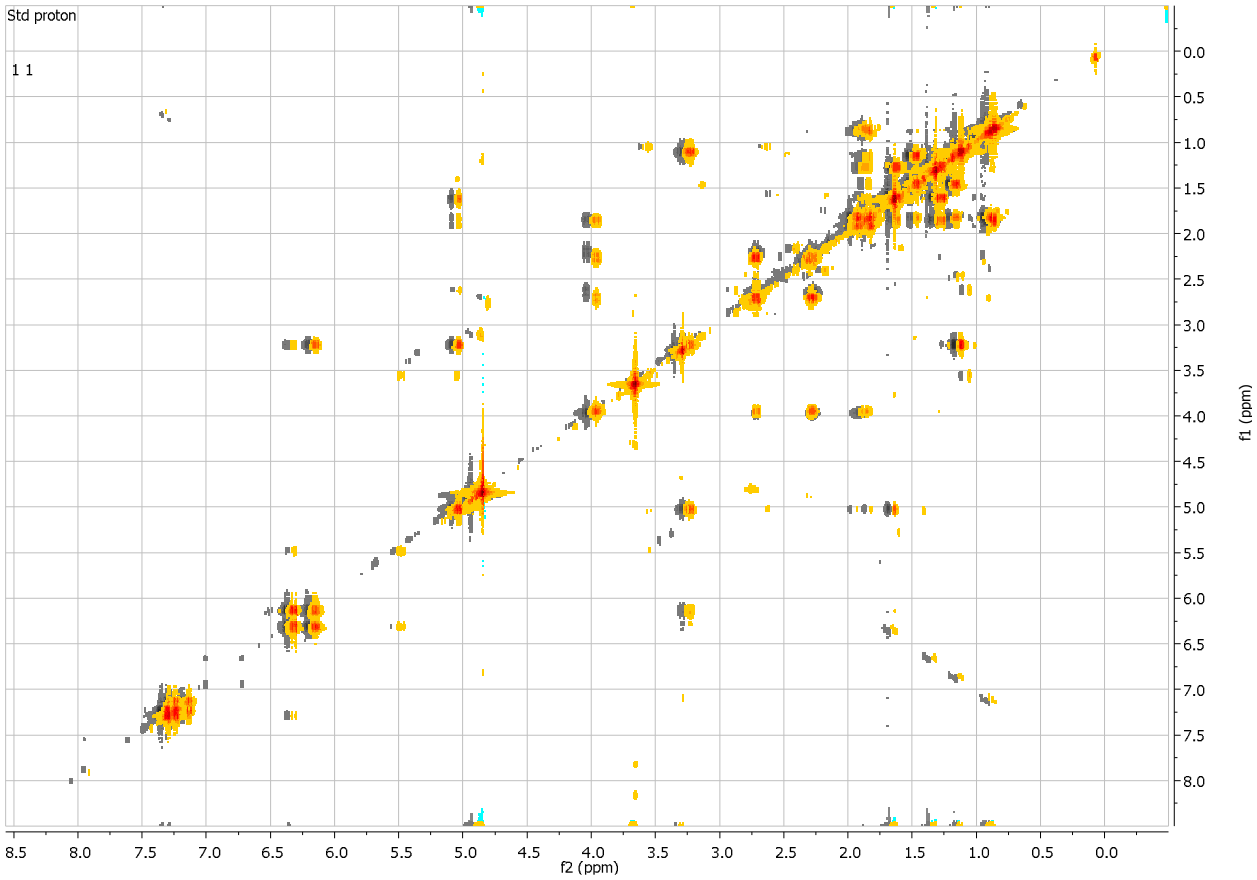


Figure 3-25. Merged ^{1}H-^{1}H COSY spectra of 8 (gray) and 9 (orange)

Figure 3-26. Merged HMBC spectra of **8** (**red**) and **9 (gray)**

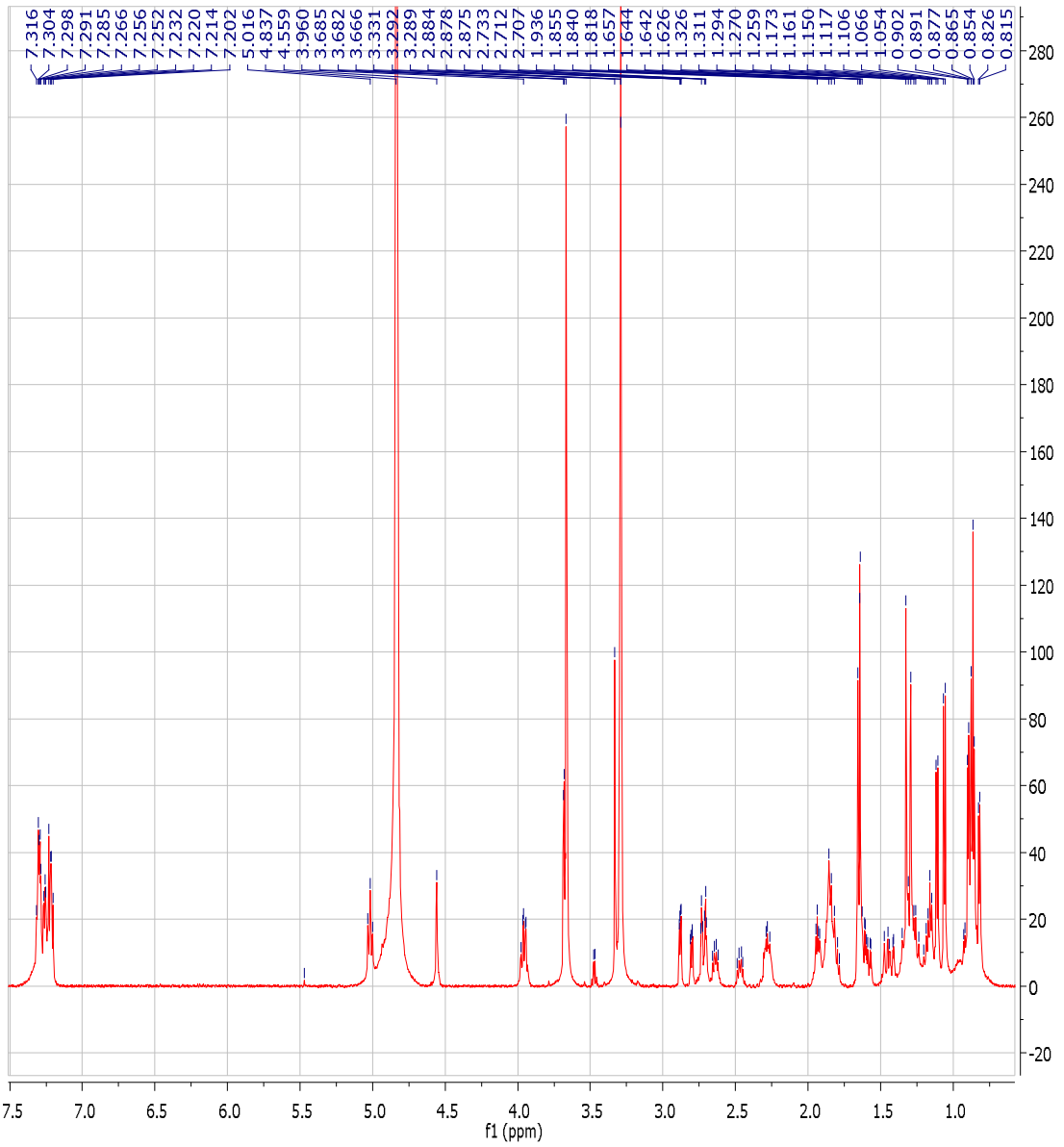


Figure 3-27. ^{1}H NMR of the methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

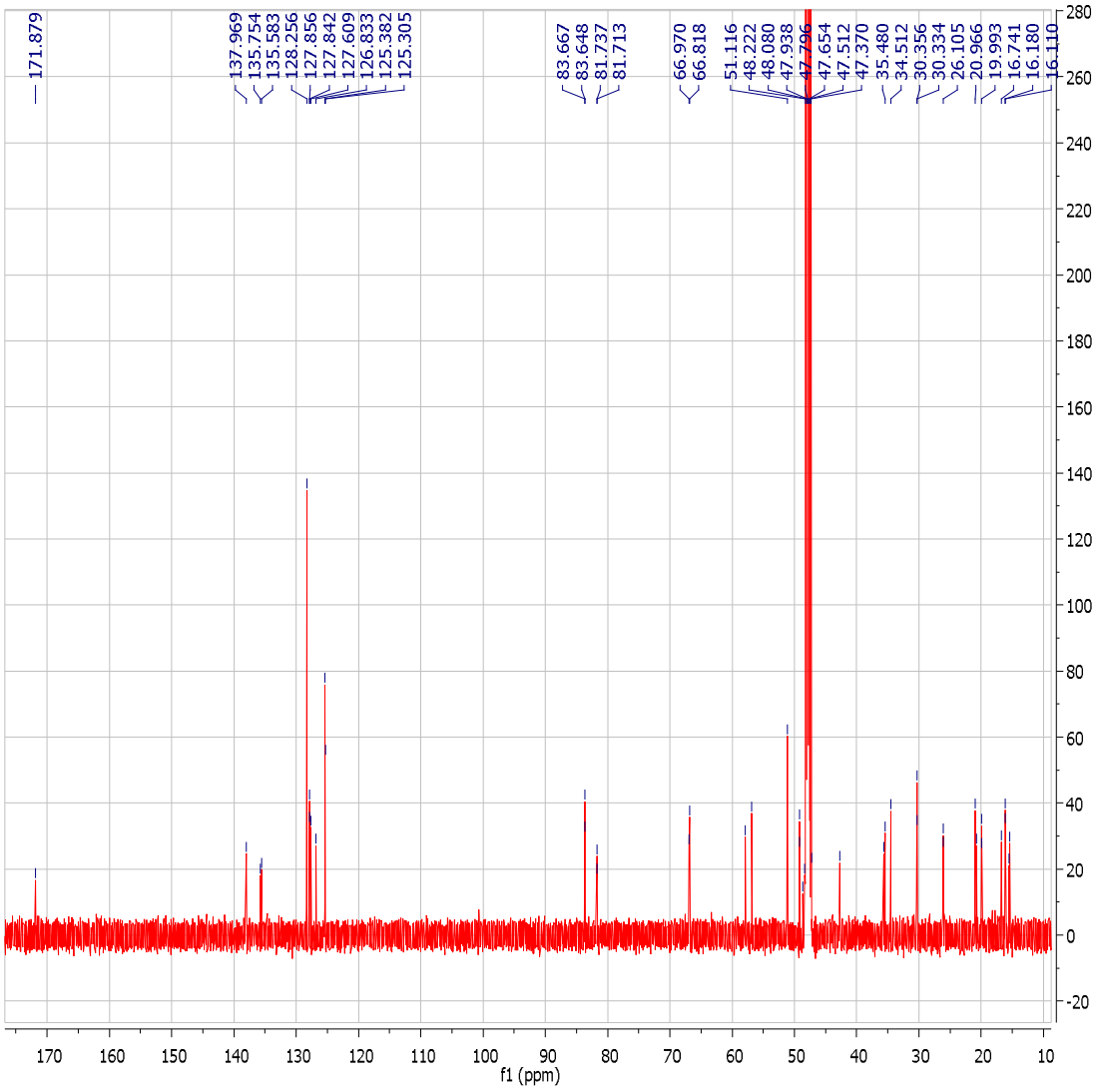


Figure 3-28. ^{13}C NMR spectrum of the methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

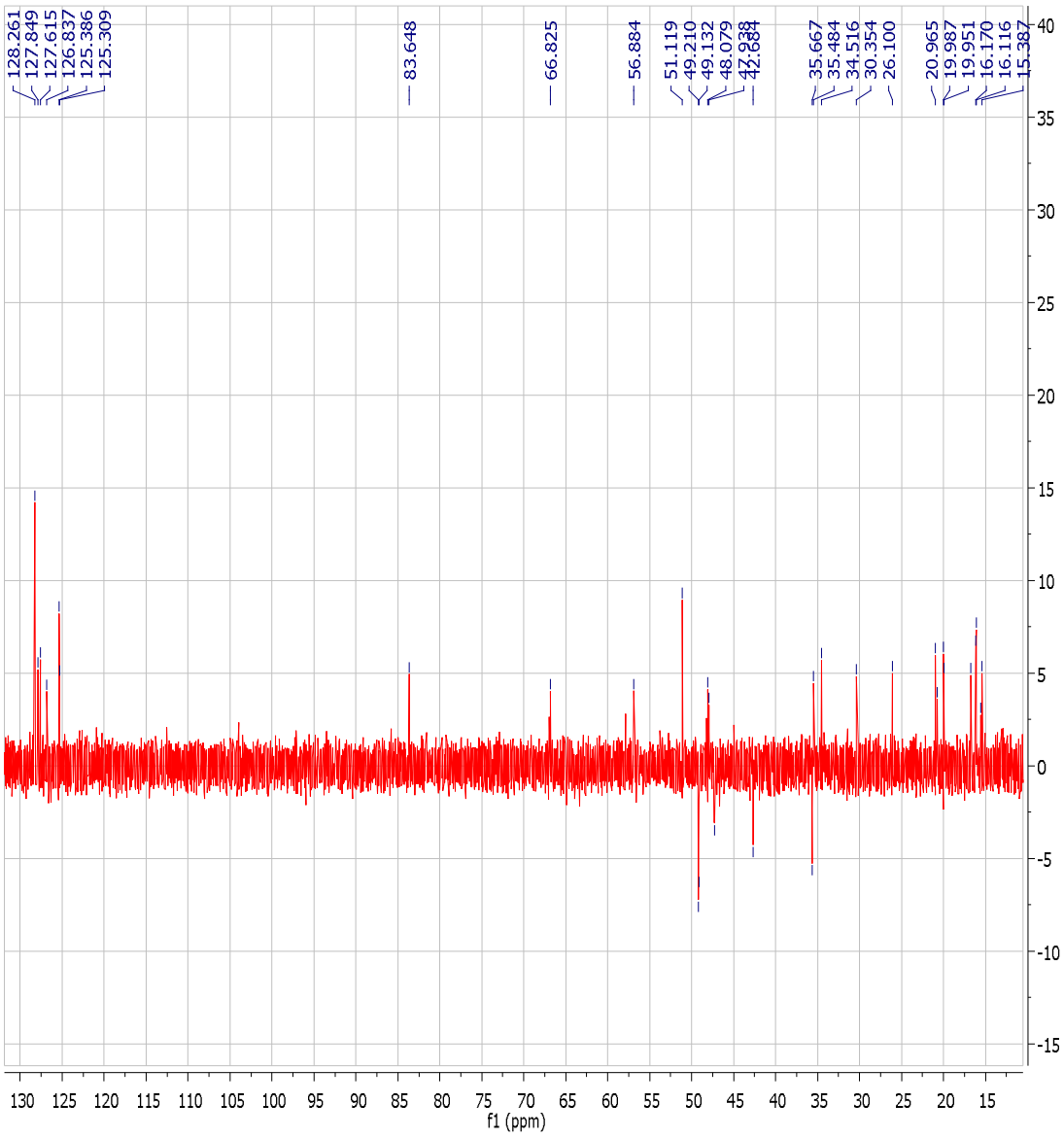


Figure 3-29. DEPT spectrum of the methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

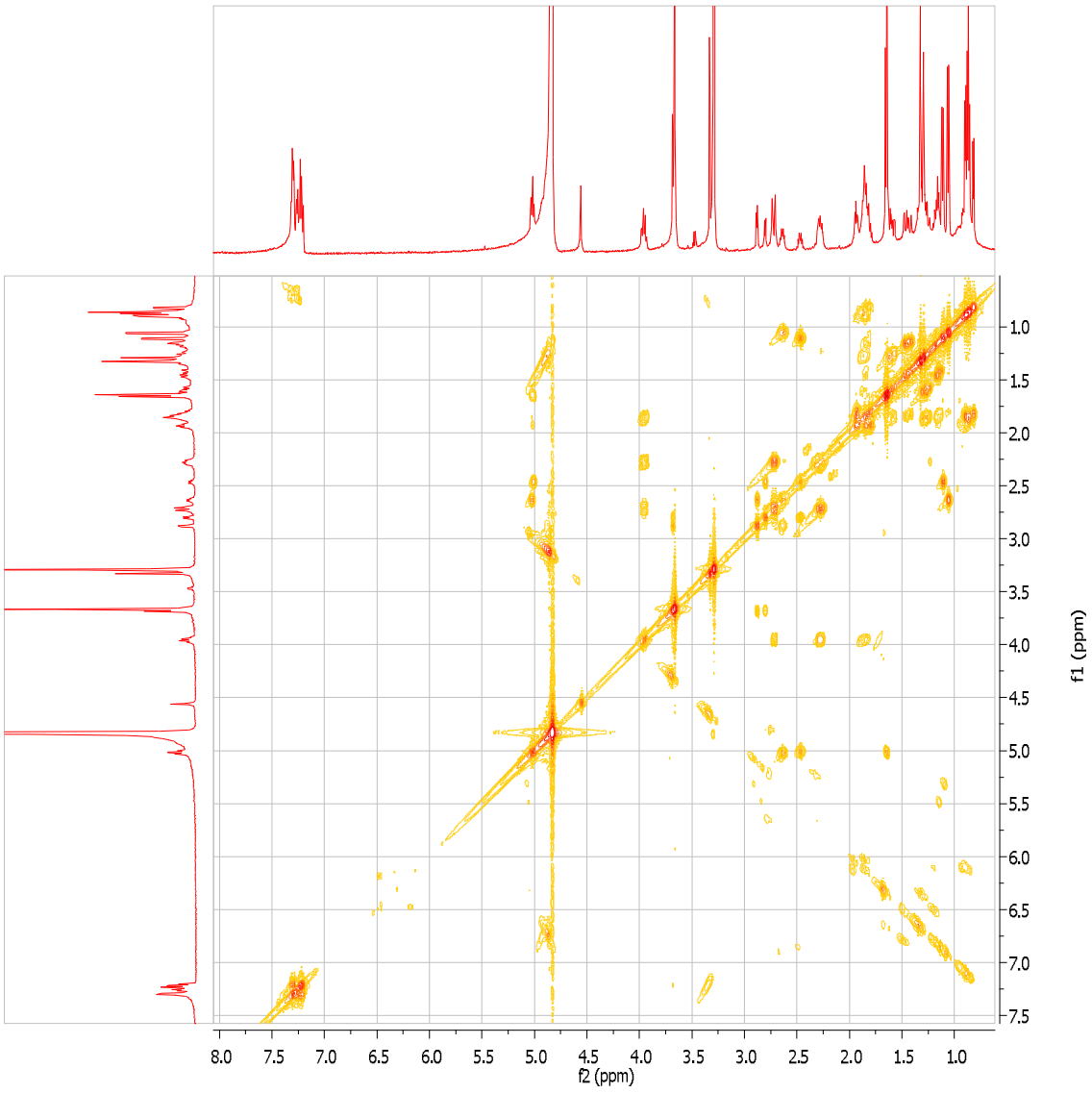


Figure 3-30. ^{1}H-^{1}H COSY spectrum of methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

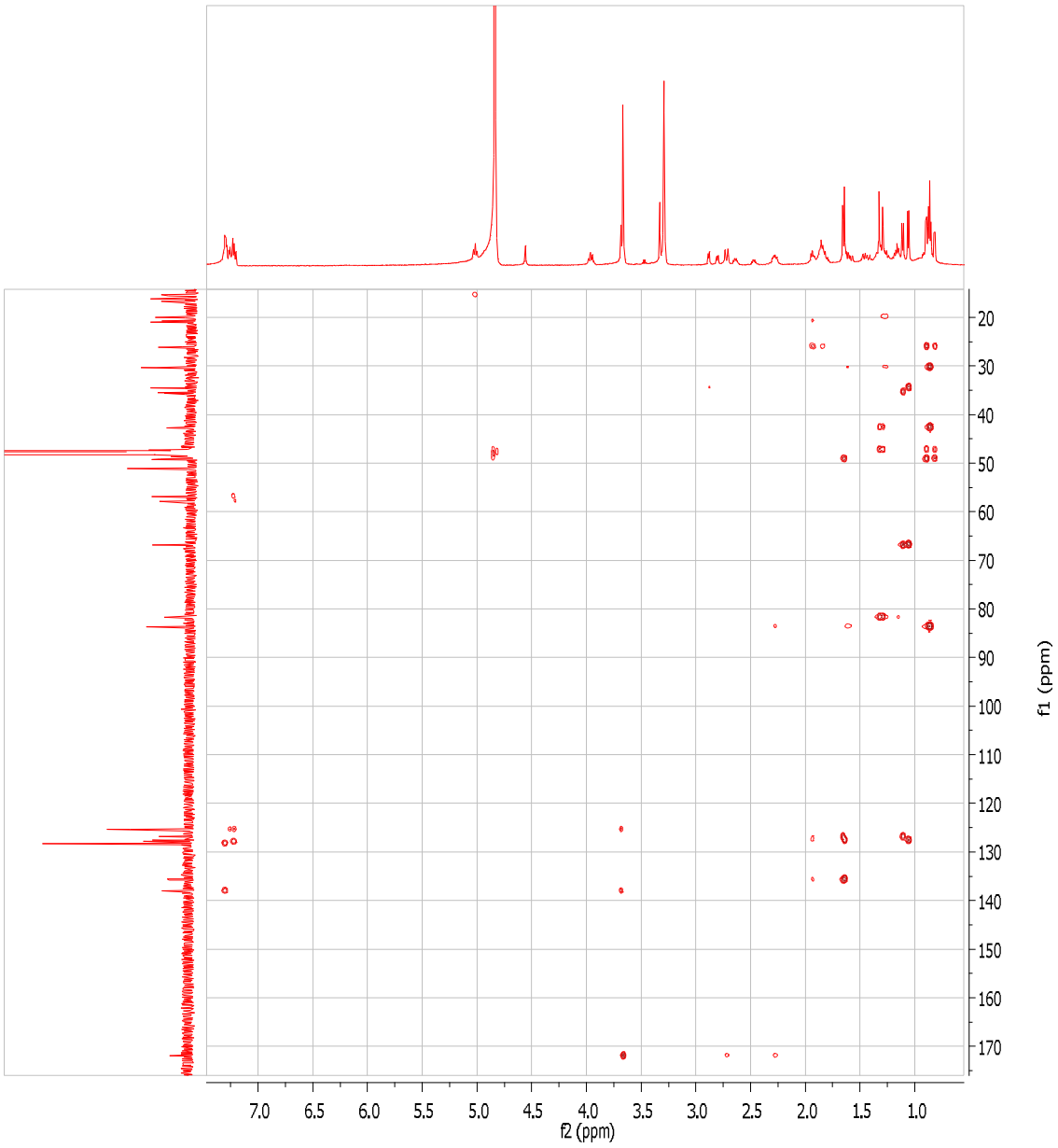


Figure 3-31. HMBC spectrum of methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

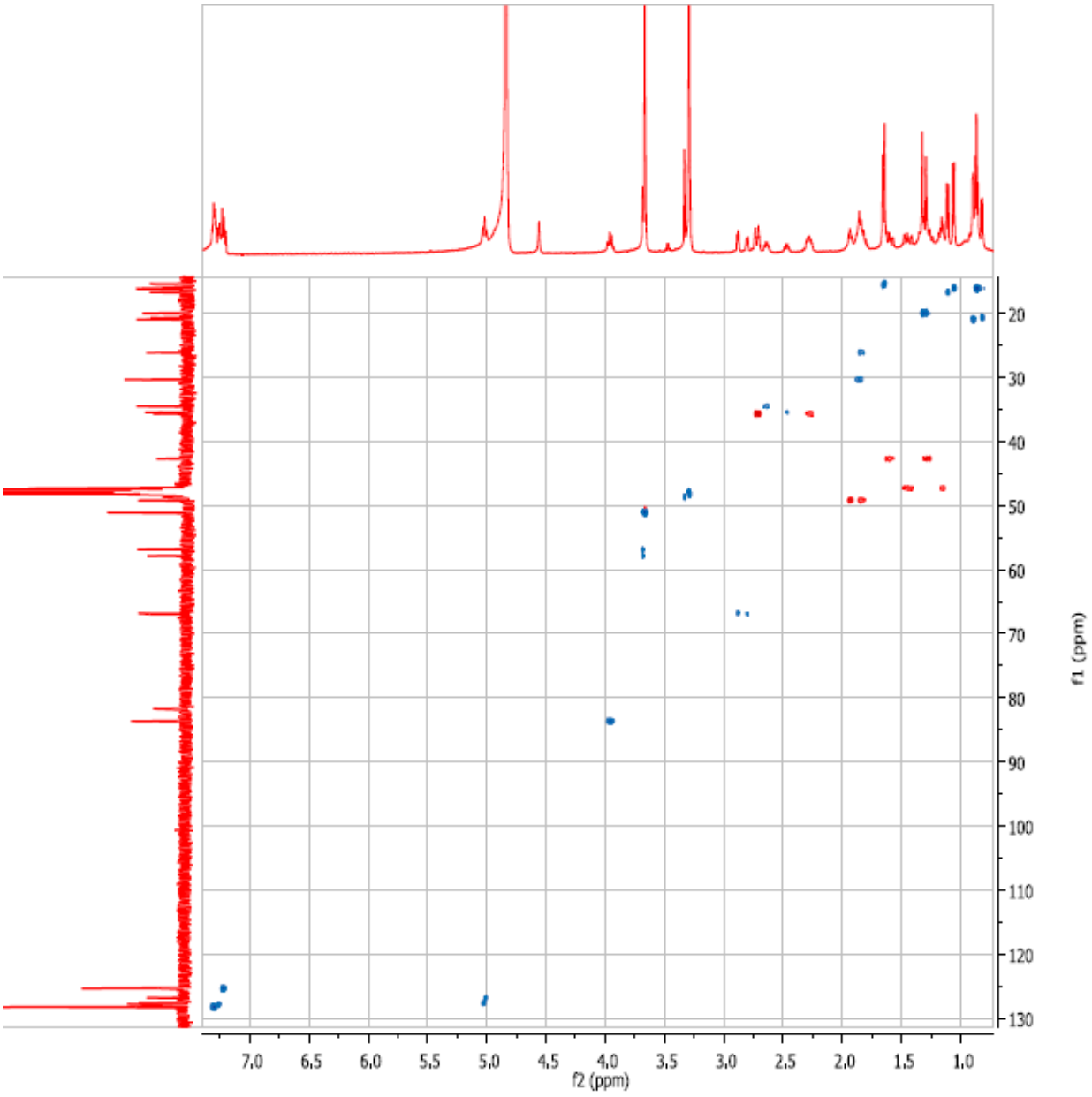


Figure 3-32. HSQC spectrum of methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

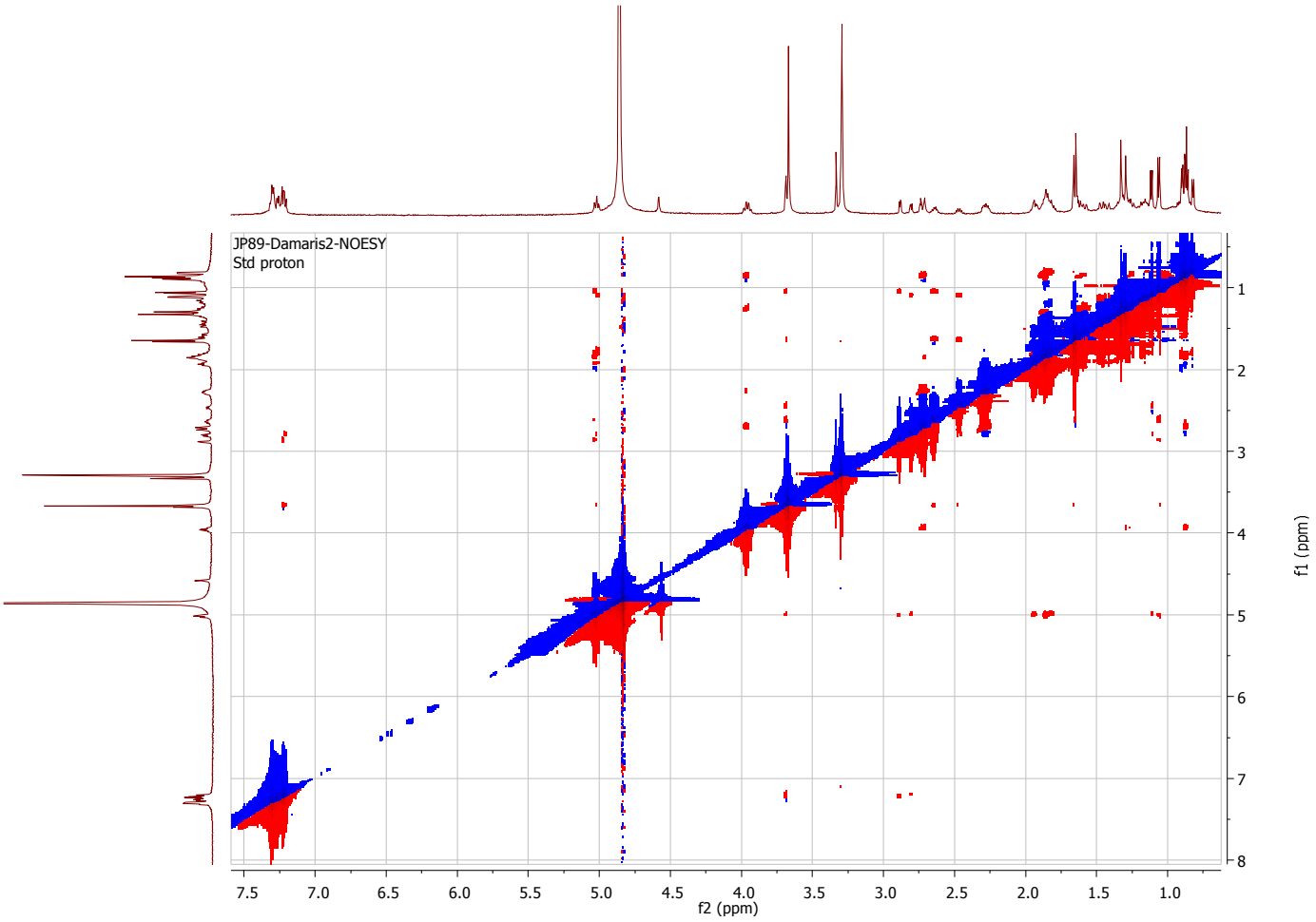


Figure 3-33. NOESY spectrum of methyl 13, 14-epoxyplakinastreloate (10 and 11) (CD_3OD)

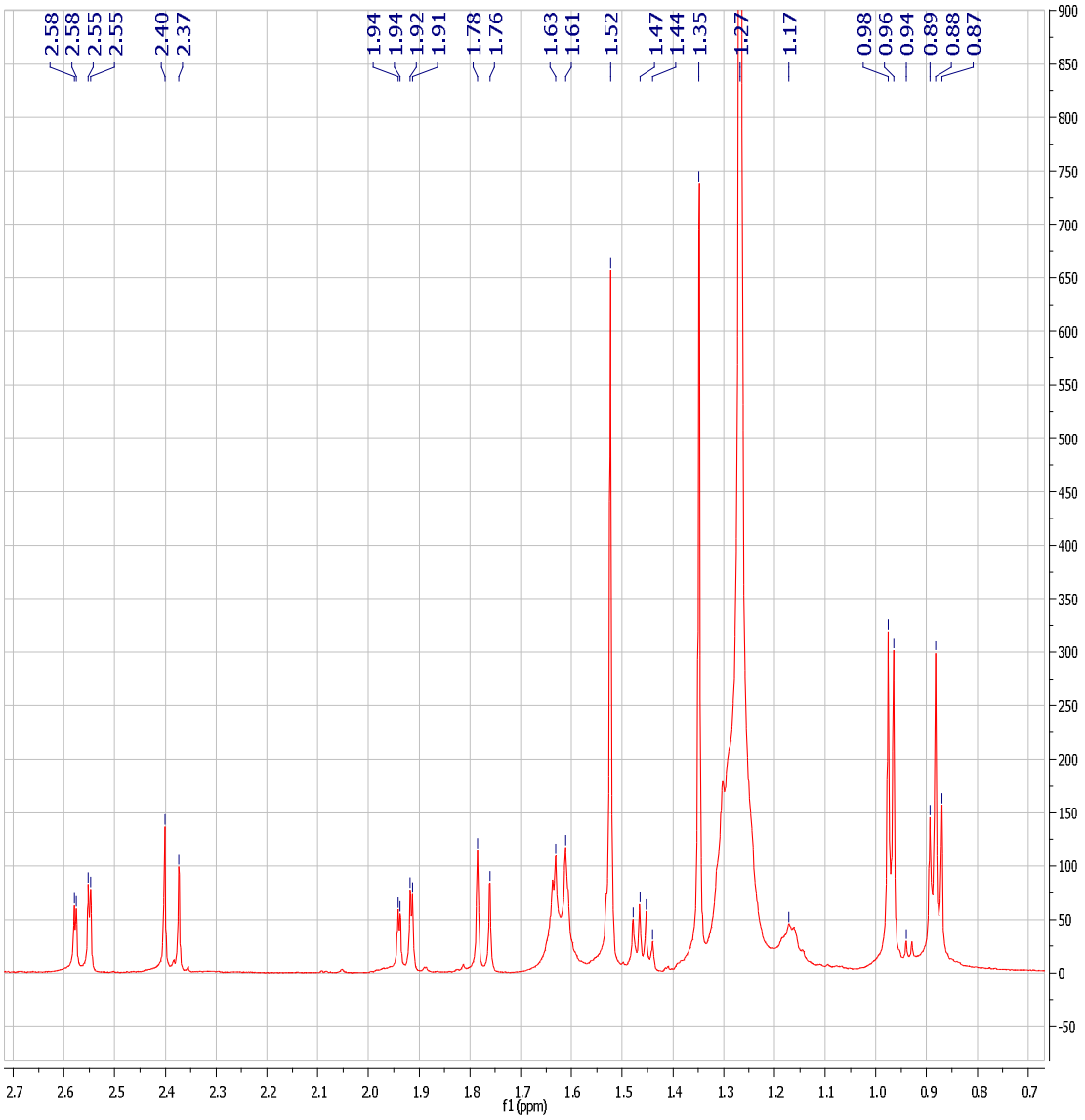


Figure 3-34. ^{1}H NMR spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

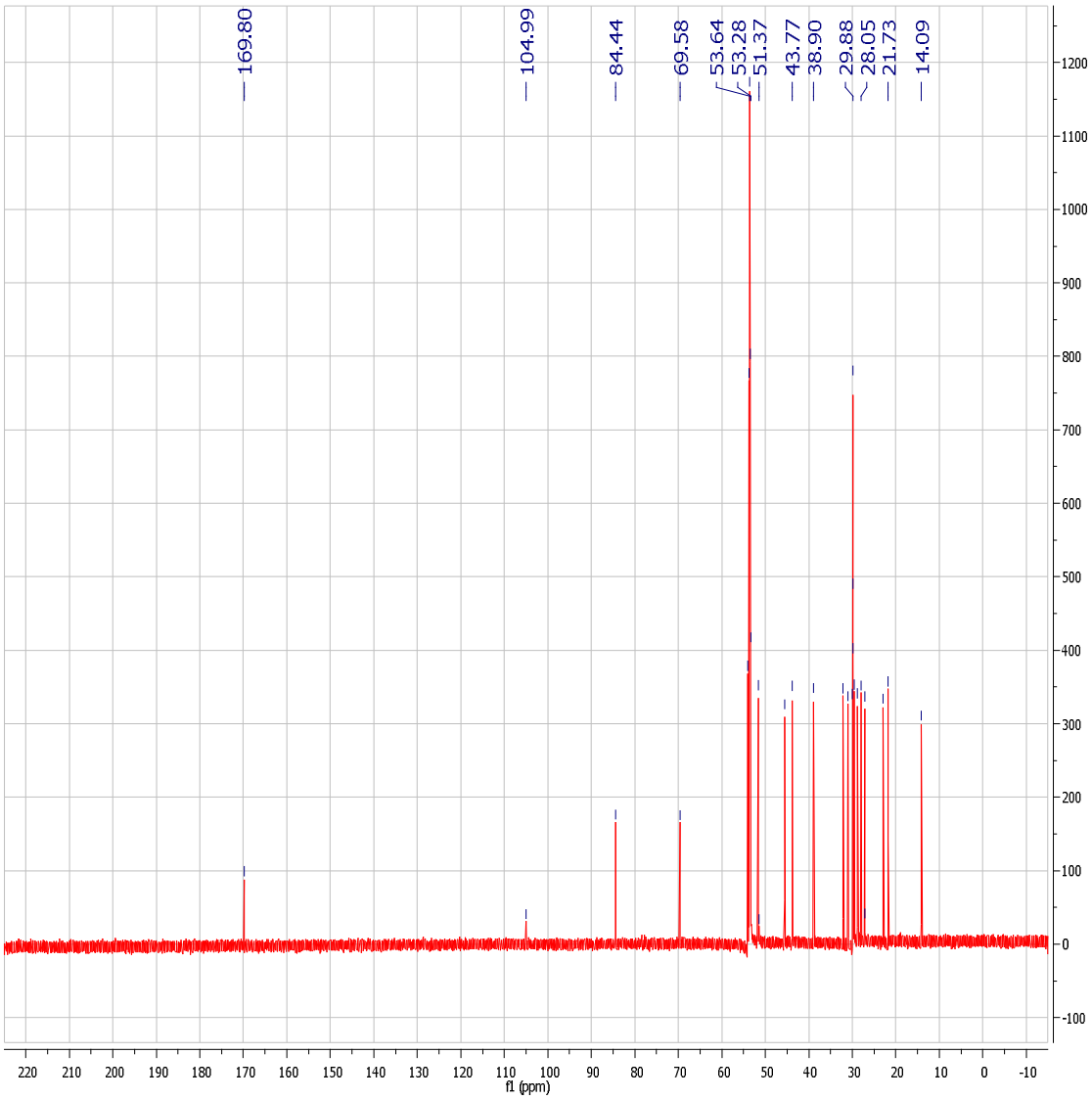


Figure 3-35. ^{13}C NMR spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

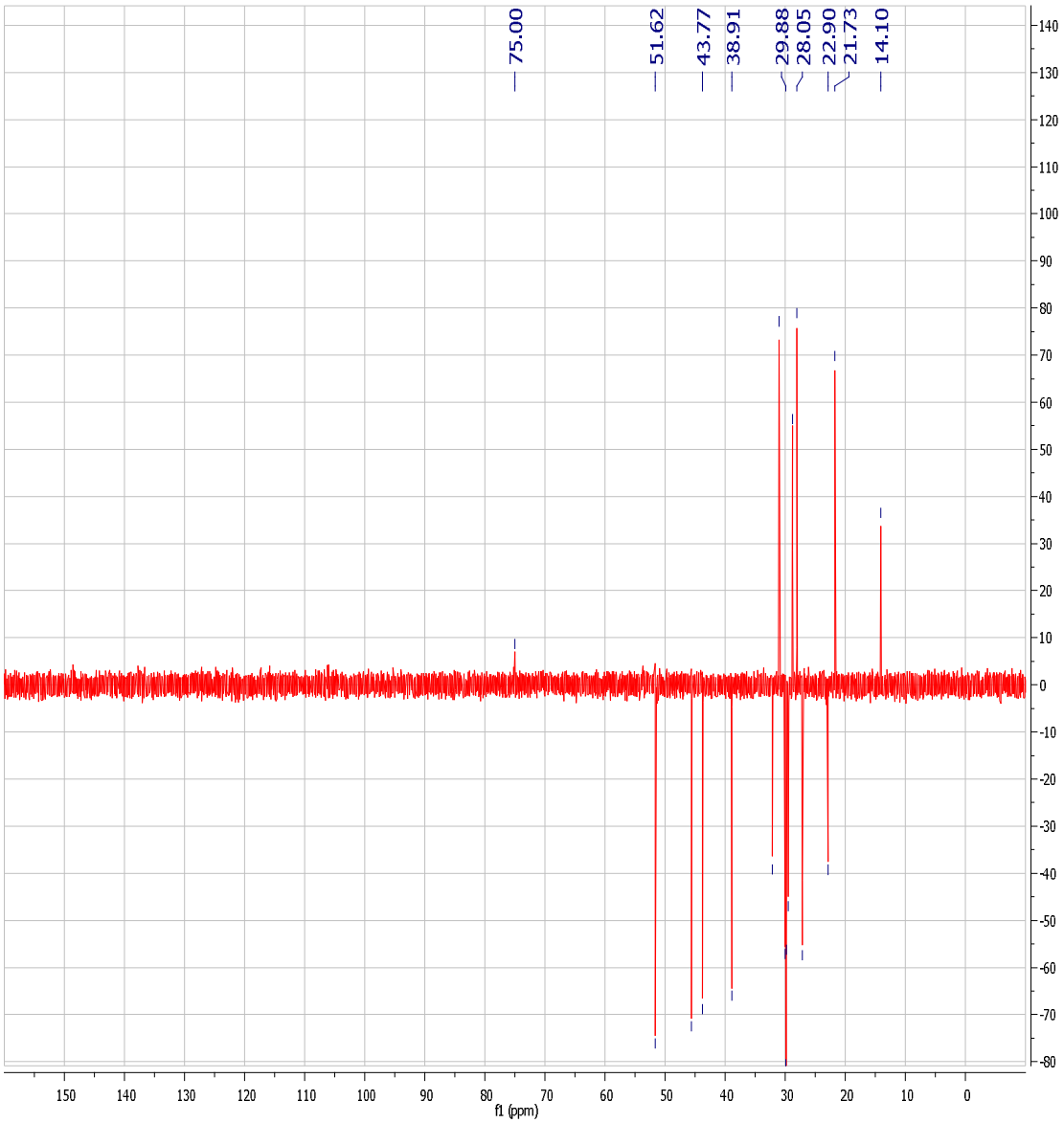


Figure 3-36. DEPT spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

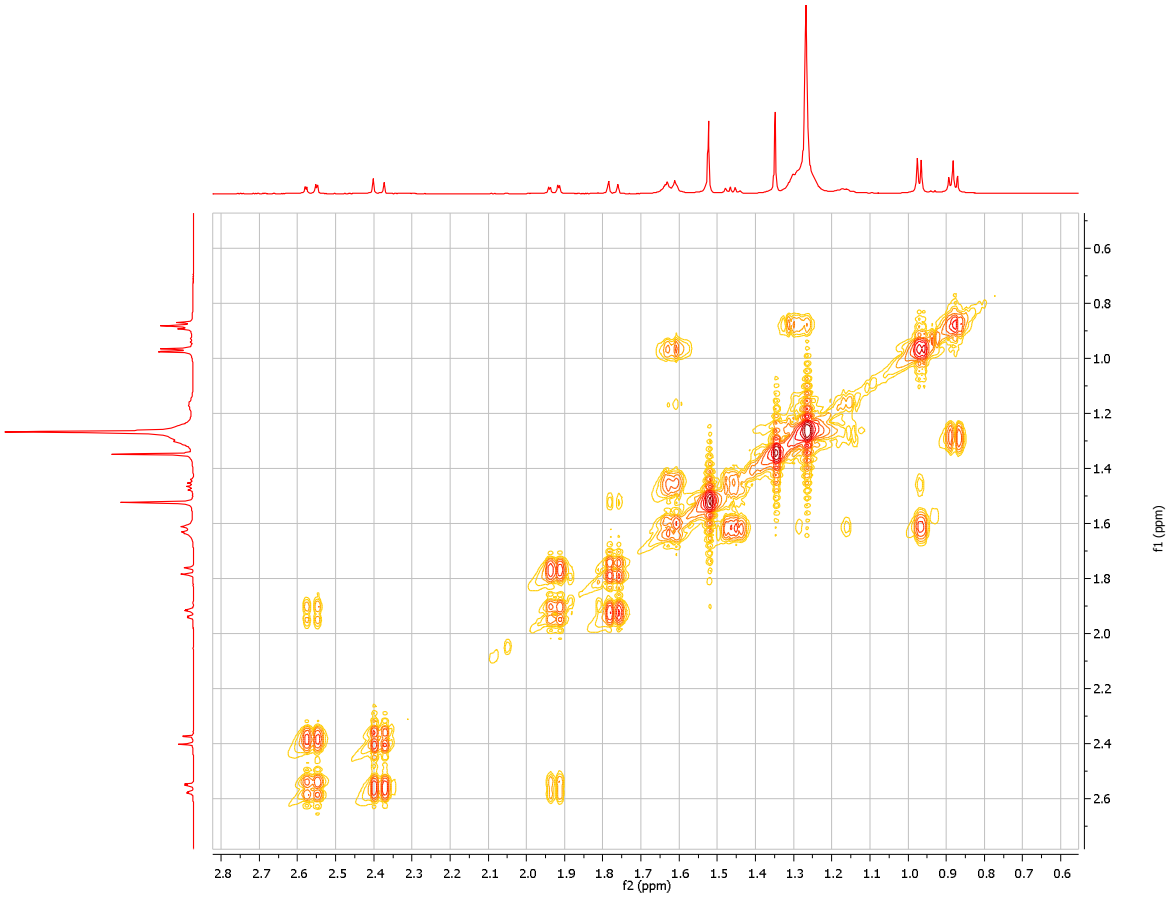


Figure 3-37. ^{1}H-^{1}H COSY spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

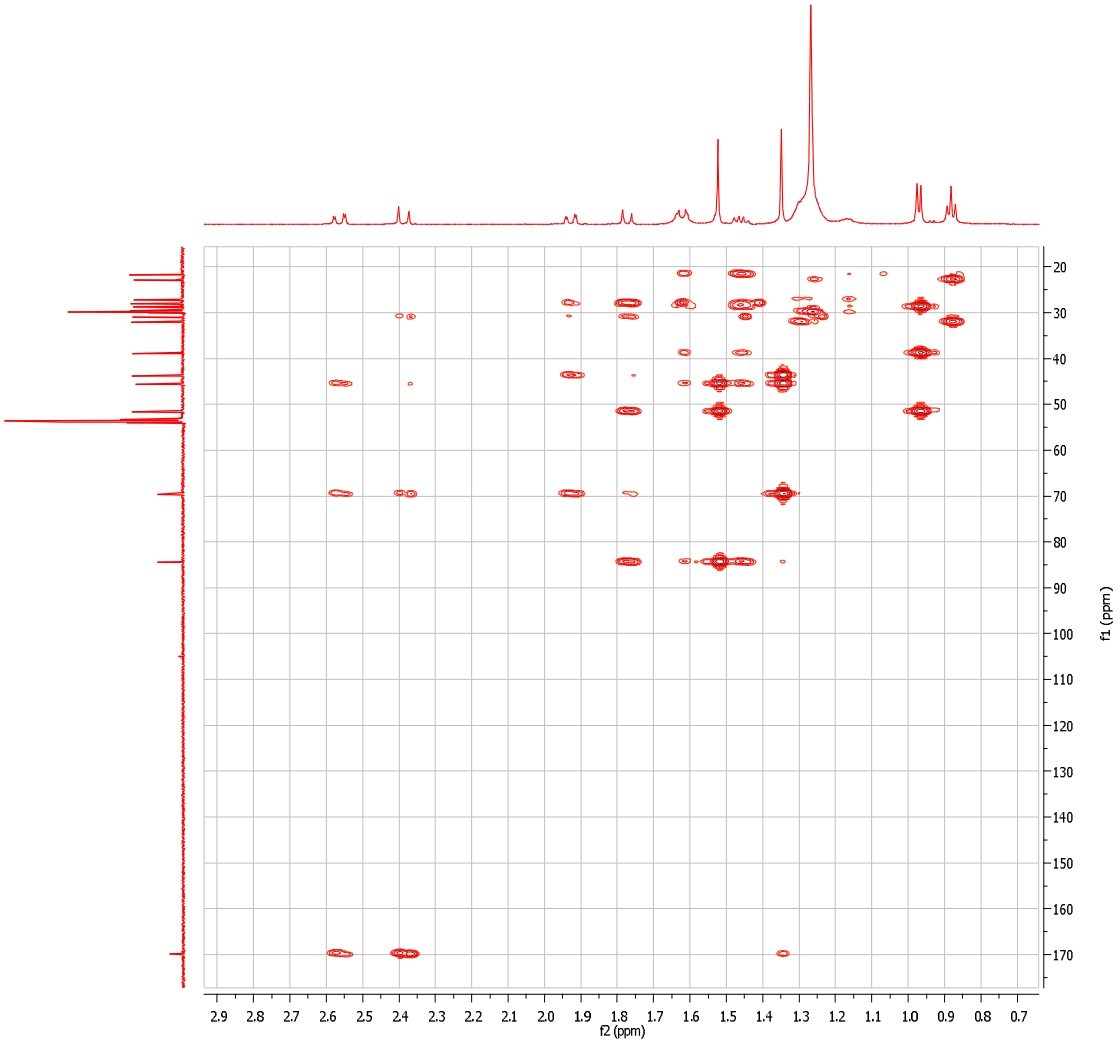


Figure 3-38. HMBC spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

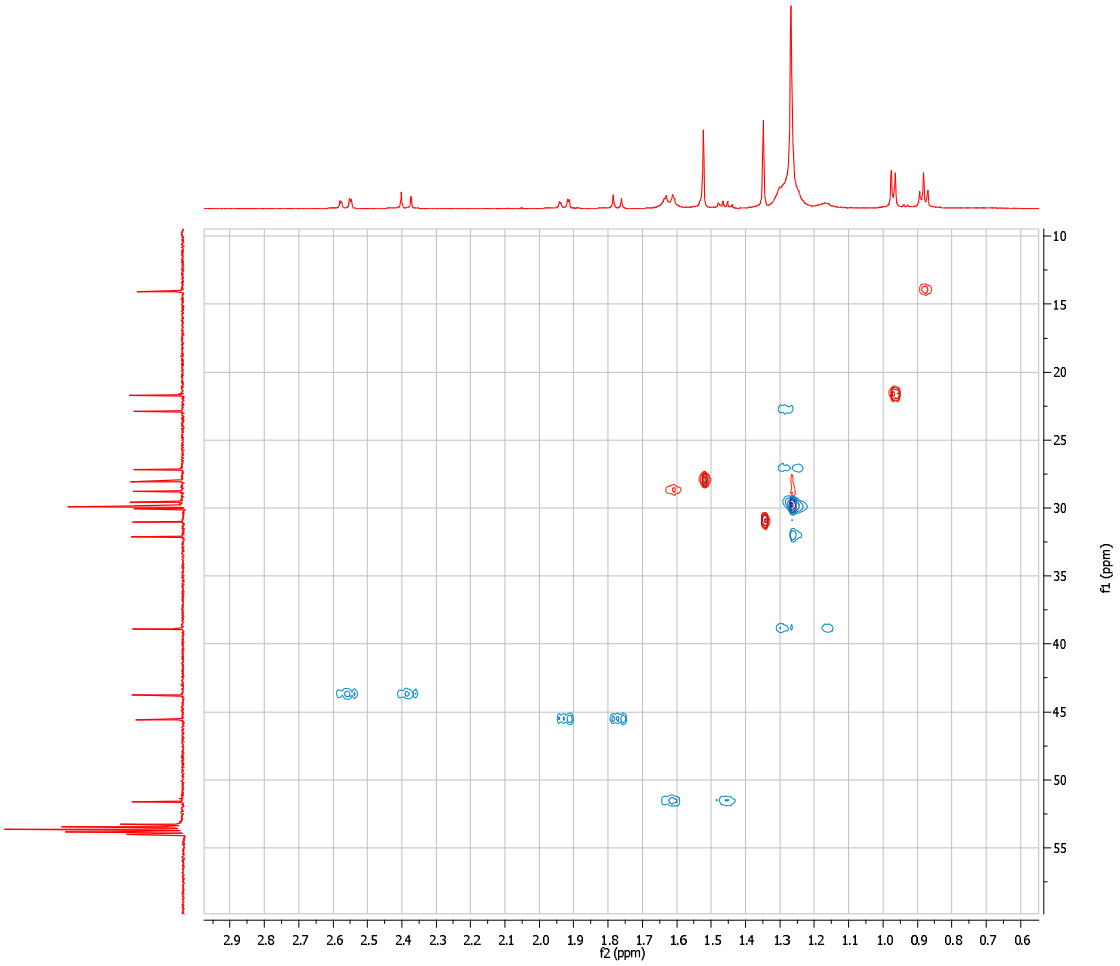


Figure 3-39. HSQC spectrum of plakinastrelloic acid B (12) (CD_2Cl_2)

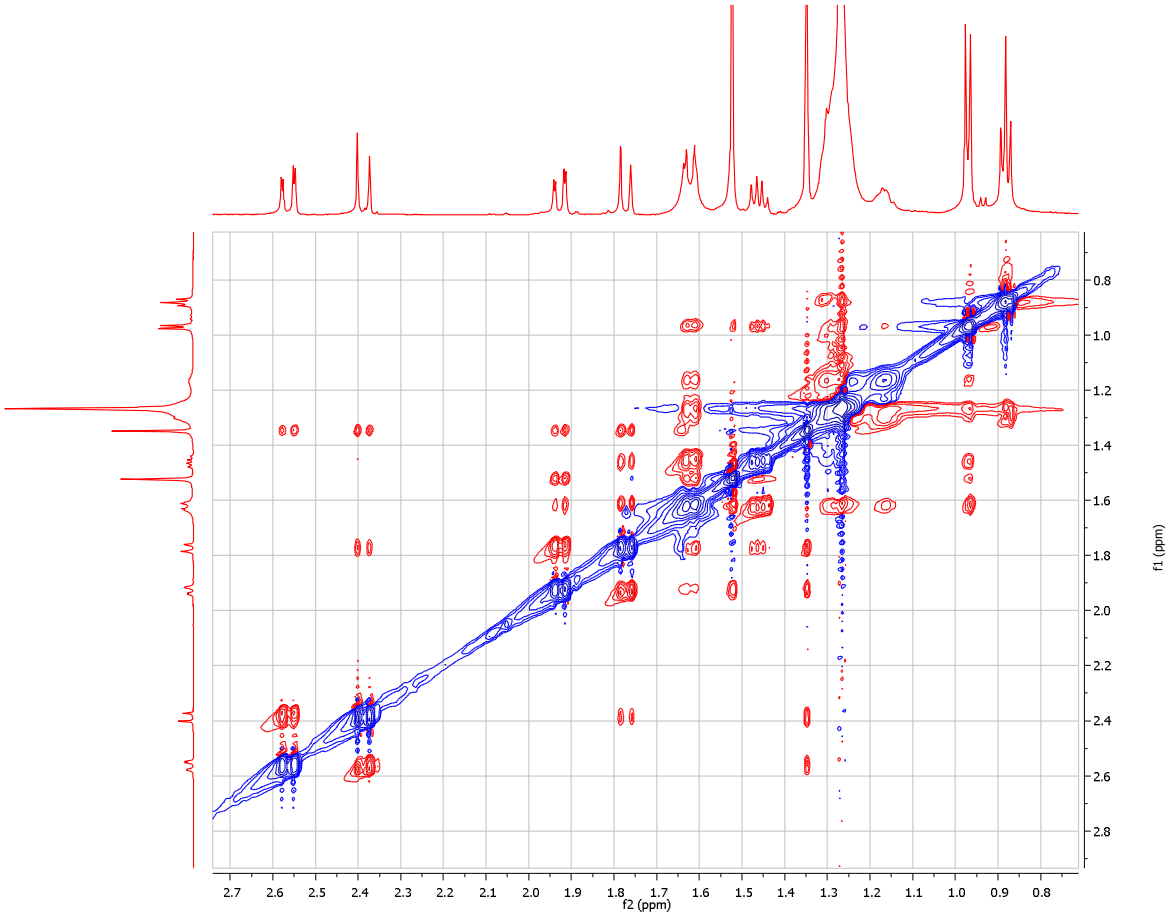


Figure 3-40. NOESY spectrum of plakinastreloic acid B (12) (CD_2Cl_2)

Chapter 4 : BIOASSAY-GUIDED ISOLATION OF HCV ACTIVE METABOLITES FROM *INGA FAGIFOLIA* AND *DIPLOSTEPHIUM RHODENDROIDES*

ABSTRACT - Two new molecules were isolated from Hepatitis C Virus (HCV) active methanol extracts of *Inga fagifiolia (*twigs) and *Diplostephium rhodendroides* (leaves). These compounds were identified as 2,3,4,5,6-pentahydroxy-2-(hydroxymethyl) hexanamide (**14**) and 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy]pentanoic acid (**15**). The bioassay-guided approach used for their isolation and structure elucidation is reported here.

1. Introduction

Hepatitis C, caused by HCV is a serious condition which, according to the CDC, currently affects about 3.2 million Americans. HCV targets the liver causing inflammation. HCV is a highly mutable virus; this property has made it difficult to address this disease effectively. Consequently, there is an urgent need for new anti-HCV drug leads.

In a continuous effort to find new drug leads against infectious diseases, we initiated a screening campaign of plant and marine extracts from the National Cancer Institute repository of natural products. These extracts were screened for their ability to inhibit HCV. Two extracts with outstanding activity were the crude extracts of the twigs of *I. fagifiolia* and the leaves of *D. rhodendroides.*

I. fagifiolia (Figure 4-0-1), also known as *Mimosa fagifolia L., I. tetraphylla (Vell.) Mart, M. laurina Sw., M. tetraphylla Vell, Feuilleea fagifolia (L.) Kuntze or F. laurina (Sw.) Kuntze* is a well known tree belonging to the order *Fabales,* family *Fabaceae* and sub-family *Mimosoideae. I. fagifolia* is a non climbing perennial tree and not an endangered species. The reported use of this tree is environmental, being used as a shade tree. The geographical distribution of this plant is wide. *I. fagifolia* has been reported in many countries around the world. These include the Caribbean regions, Central America, Indian Ocean regions, and South American regions. It possesses a variety of vernacular names; these include Pois Doux Blanc, Arbre a Miel, Caspiro, Cujincuil, Gina, Jina, Nacaspiro, Palal, Paternillo, Paternillo and Spanish Oak [209, 210]. Reports concerning the isolation of secondary metabolites from *I fagifolia*, especially those of biological importance are scarce. A few molecules have been isolated from this plant in recent years. These include a protein inhibitor of trypsin isolated from seeds [209], and three new galloyl depsides of tyrosine [211] that are abundantly produced in young leaves of *I. Fagifolia.* The primary structure of the protein was EVVVDSDGEMLRNGGKYY LSPANPIGGGAIISAAIRHGDHLCSLAVVS. The structures of these new galloyl depsides are displayed below [209, 211].

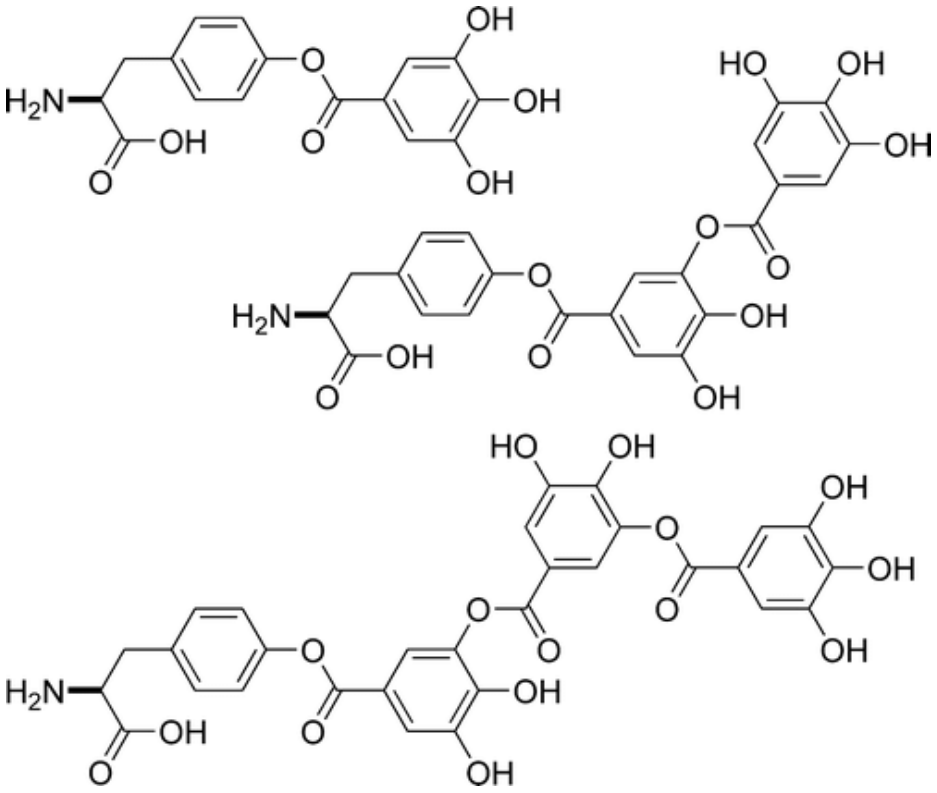


Figure 4-0-1. *Inga fagifolia*

D. rhodendroides, on the other hand, is a flowering plant that belongs to a subgroup of *Diplostephium* with characteristic disk flowers. The latter are more or less clavate and merely bifid; corresponding branches are very short and ovate. *D. rhodendroides* has been reported in Ecuador and Columbia. Plants of the genus *Diplostephium* are believed to have evolved from *Aster*, another genus of the familly *Asteraceae,* by adapting to extreme paramos' conditions. Among other evolutionary variations was the development of tomentum under leaves and on branches. About 110 species (trees and shrubs) have been reported to belong to the genus *Diplostephium.* Plants of this genus are mainly distributed in Columbia. Several species have been

reported in several other nearby countries, such as Ecuador, Peru, Chile, Coasta Rica, Venezuela, and Bolivia [212, 213].

2. Results and Discussion

The crude extract of twigs of *I. fagifolia* was received from the National Cancer Institute and fractionated using VLC on silica gel. The percentage of HCV RNA inhibition for the MeOH fraction was estimated at 99 but this fraction was also cytotoxic. HCV active and cytotoxic constituents of the methanol fraction were successfully separated by HPLC using a Phenomenex NH2 column (21.2 × 250 mm). The fraction that eluted from 100 -107 minutes was active against HCV and showed no cytotoxicity. This fraction was further separated on a smaller scale using a Phenomenex NH2 column (10 × 250 mm) to yield two well resolved peaks.

The fraction corresponding to the second peak was active against HCV and showed no toxicity (percentage of HCV RNA inhibition = 96, percentage of rRNA inhibition = - 54); this fraction contained compound **14**. The molecular formula was determined to be $C_7H_{15}NO_7$ based on a pseudo-molecular ion peak at *m/z* 226.9440 $[M + H]^+$ obtained by HRMS. The odd molecular weight of **14** was interpreted as an indication that the molecule contains an odd number of nitrogen atoms (nitrogen rule). It was determined that the molecule contained two quaternary carbons, two methylene, and three methine (Table 4-0-1). A ^1H-^1H COSY correlation was observed between the diastereotopic methylene protons H-7a (1H, δ_H 3.62/3.47, m) and H-7b (1H, δ_H 3.62 /3.47, m). Another COSY correlation was observed between the methylene protons H-6 (2H, δ_H 3.62, m) and H-5 (1H, δ_H 3.9, m). An HMBC correlation was observed between

H-3 (1H, δ_H 3.7, d, *J* = 10 Hz) and C-5. Another HMBC correlation was detected between the methylene signals H-7a, H-7b and the quaternary carbon C-2 (Figure 4-0-3). The fact that H-4 was a doublet of doublet, suggested that it was positioned between the only two other methines of this compound namely C-3 and C-5. The presence of a terminal amide group in **14** was determined based on the chemical shift of C-2, the degree of unsaturation and the molecular weight of the compound. All these points suggested that the structure of **14** is as depicted in Figure 4-0-2.

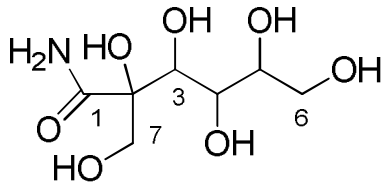


Figure 4-0-2. Structure assignment of 14 [2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl) hexanamide]

This molecule was identified as 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl) hexanamide (**14**). The free acid analog of **14**, 2-hydroxymethyl-D-arabinohexanoic acid, was reported recently [214-216]. In fact, the potential of various salts of the free acid of **14** to boost the immune response after immunization has been broadly studied. These studies, which also involved several other similar molecules, have resulted in several patents [214-216]. These compounds were investigated for use as components of vaccine adjuvants, a safer, less toxic alternative to cholera toxin [214-216].

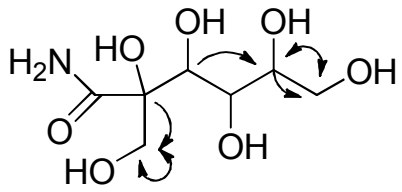


Figure 4-0-3. COSY (↔) and HMBC (→) correlations of 14 [2,3,4,5,6-pentahydroxy-2-(hydroxymethyl)hexanamide]

The crude extract of *Diplostephium rhodendroides* was also received from the National Cancer Institute and fractionated using VLC on silica gel; solvents included *n*-hexane, DCM, EtOAc and MeOH. The methanol fraction showed promising HCV activity and was further purified using a 250 × 21.2 mm NH2 column. The resulting ELSD chromatogram indicated that this fraction was constituted of a large number of minor metabolites that elute early during the HPLC. The major constituents of this methanol fraction were simple sugar molecules, none of which possessed anti-HCV activity. Early fractions that eluted from 10-12 minutes and from 13.9-15.9 minutes were active against HCV and non toxic. It was determined that the 10-12 minutes fraction was constituted of a mixture of trace amounts of HCV active molecules predominantly distributed in the stem and twigs of this plant. The fraction that eluted from 13.9-15.9 minutes was further purified using a Phenomenex NH2 column (10 × 250 mm). The ELSD chromatogram of this fraction indicated that, aside from very minor metabolites, it also contained **15;** about 1.5 mg of **15** were isolated from 400 mg of the methanol fraction; compound **15** eluted at around 38 minutes. Based on a pseudo-molecular ion peak at *m/z* 295.0491 $[M - H]^-$ (HRMS, negative mode), its molecular formula was determined to be $C_{11}H_{20}O_9$, corresponding to two degree of unsaturation. The 11 carbons included a quaternary

carbon, a methyl group, a ketal moiety, six other methines, and two methylenes (Table 4-0-2). It was established based on the ^{1}H-^{1}H COSY spectrum analysis that **15** contained two independent spin systems labeled A and B and shown in bold bonds in Figure 4-0-5. The first involved protons from H-6 (3H, δ_H 1.52, d, *J* = 6 Hz) to H-2 (1H, δ_H 4.46, m) and the second involved protons from H-2'a (1H, δ_H 2.73, dd, *J* = 3 Hz, 18 Hz) and H-2'b (1H, δ_H 3.02, dd, *J* = 5 Hz, 18 Hz) to H-5'a (1H, δ_H 3.67, dd, *J* = 4 Hz, 11 Hz) and H-5'b (1H, δ_H 4.12, m).

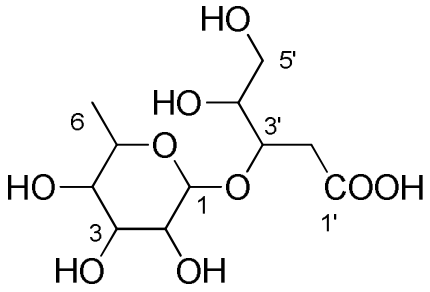


Figure 4-0-4. Structure of 15 [4, 5-dihydroxy-3-{(3, 4, 5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl} oxy) pentanoic acid]

The HMBC correlation between H-1 (1H, δ_H 5.24) and C-5 indicate a six membered ring involving carbons C-1 through C-5. Similarly, the HMBC correlation between H-1 and C-3' allowed the connection of this ring to the rest of the molecule. Based on these analyses, it was determined that the structure of **15** is as depicted in Figure 4-0-4.

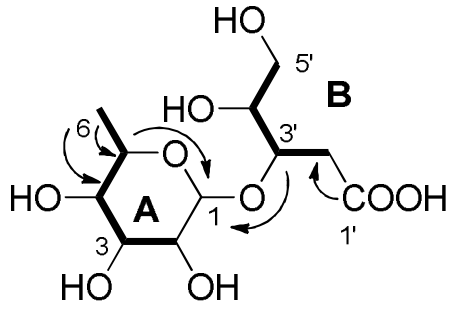


Figure 4-0-5. COSY (—) and HMBC (→) correlations of 15 [4,5-dihydroxy-3-{(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy}pentanoic acid]

Based on the NOESY spectrum of **15**, it was established that its relative configuration is as depicted in Figure. 4-0-6. NOESY correlations were detected between H-5a' and H-1, H-3' and H-4'. Another correlation was detected between the methyl protons H-6 and H-3; no such correlation was detected between H-6 and H-4. There was also a correlation between H-4' and H-5'b. Finally a correlation was detected between H-2 and H-3.

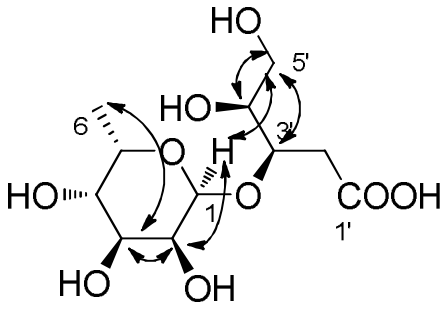


Figure. 4-0-6. Relative configuration of 15

3. Supporting information

Compound **14**. White solid, $[\alpha]_D$ = -12 (*c*.0.03, MeOH); HRMS *m/z* 226.9440 [M + H+] (calcd for $C_7H_{15}NO_7$, 226.0927)**;** ^{13}C NMR (400 MHz, D_2O) Table 4-0-1; ^{1}H NMR

(400 MHz, D_2O)

Compound **15**. Clear solid; $[\alpha]_D$ = -40 (*c*.0.02, MeOH); HRMS *m/z* 295.0491 [M - H]$^-$ (calcd for $C_{11}H_{19}O_9$, calcd 295.1029); ^{13}C NMR (400 MHz, pyridine) Table 4-0-2; ^1H NMR (400 MHz, pyridine) Table 4-0-2

3.1. Experimental Section

3.1.1. *Inga fagifolia*

The crude extract of twigs of *I. fagifolia* was initially fractionated using VLC on silica gel and the methanol fraction showed promising activity. Next, HCV active and cytotoxicity constituents were separated by HPLC using a Phenomenex NH2 column (21.2 × 250 mm). Fractions collection was guided by an ELSD detector. The solvent system was 100/0 DCM/MeOH → 75/25 DCM/MeOH (90 minutes), followed by 75/25 DCM/MeOH → 0/100 DCM/MeOH (30 minutes). The fraction that eluted between 100 - 107 minutes was active against HCV and showed no cytotoxicity; this fraction was further purified on a smaller scale using a Phenomenex NH2 column (10 × 250 mm). The solvent system was 70/30 DCM/MeOH → 30/70 DCM/MeOH (60 minutes), followed by 30/70 DCM/MeOH → 0/100 DCM/MeOH (5 minutes) and the flow rate was 6 mL/min. The ELSD chromatogram showed two well resolved peaks; corresponding fractions were collected at 22 and 28 minutes, respectively. The fraction collected at 28 minutes showed promising HCV activity and displayed no cytotoxicity. The percentage of inhibitions against HCV RNA and rRNA were estimated at 96 and - 54, respectively; this fraction contained compound **14;** 3 mg were derived from 300 mg of the methanol fraction.

3.1.2. *Diplostephium rhodendroides*

The crude extract of *D. rhodendroides* was fractionated using VLC on silica gel. Next, the MeOH fraction was purified using a Phenomenex NH2 column (21.2 × 250 mm). The ELSD chromatogram of this fraction indicated that it was comprised of a large number of minor metabolites that eluted early during the HPLC. None of the major metabolites was bioactive. Using the solvent system 50:50 DCM/MeOH (10 minutes) → 0/100 DCM/MeOH (18 minutes) followed by 0:100 DCM/MeOH (5 minutes), minor metabolites were combined in a few fractions and sent for assay. The fractions that eluted from 10-12 minutes and from 13.9-15.9 minutes were active against HCV and non toxic. The 10-12 minutes fraction was constituted of trace amounts of HCV active molecules predominantly distributed in stem and twigs. About 2 mg of a mixture of these molecules were derived from 400 mg of the methanol fraction. The fraction that eluted from 13.9-15.9 minutes was further purified using a Phenomenex NH2 column (10 × 250mm). The solvent system used was 100:0 DCM/MeOH (10 minutes), followed by 100:0 DCM/MeOH → 75:25 DCM/MeOH (40 minutes) and 75:25 DCM/MeOH→ 0:100 DCM/MeOH (40 minutes). The ELSD chromatogram indicated that, aside from very minor metabolites, this fraction also contained a metabolite (**15**); about 1.5 mg were isolated from 400 mg of the methanol fraction; **15** eluted at around 38 minutes.

3.2. Anti-HCV assay

Antiviral assay

The anti-HCV activity was determined in the HCV replicon (Clone B) system (Huh 7 clone B cells containing HCV RNA). Cells were seeded onto 96-well tissue

culture plates (3,000 cells/well). The compounds were tested in a dose response at concentration 10, 3, 1 uM (triplicate). After addition of test compounds the plates were incubated for five days (37 °C, 5%, CO_2). The total cellular RNA was extracted using the manual Perfect Pure RNA 96 cell Vacki from 5 Prime. The replicon RNA and the internal control (TaqMan rRNA control reagent, applied Biosystems) were amplified by RT-PCR. The antiviral potency of the tested molecule was determined by subtracting the threshold RT-PCR cycle of the tests compounds from the threshold RT-PCR of the negative control (no drug): #CtHCV (a 3.3 value being considered a 1 log reduction in replicon RNA level (in other words 90% reduction). The cytotoxicity was calculated using #Ct rRNA values and RS-446 (2-Me-C) was used as control to determine the EC_{50} and EC_{90} values [206, 207].

Table 4-0-1. 1D and 2D data of 14

	^{13}C	δ H mult. (*J* in Hz)	COSY	HMBC
1.	/	/	/	/
2.	98.0	/	/	H-7a, H-7b
3.	67.9	3.7, d (10.0)	/	/
4.	70.1	3.81, dd (3.0, 10.0)	/	/
5.	69.5	3.9, m	H-6	H-6, H-3
6.	63.7 or 64.1[u]	3.62, m	H-5	/
7.	63.7 or 64.1[u]	3.62/3.47, m	H-7a, H-7b	/

Table 4-0-2. 1D and 2D data of 15

	^{13}C	δ H mult. (*J* in Hz)	COSY	HMBC	NOESY
1	103.1	5.24 *	/	/	H-5'[a]
2	73.2	4.46, m	/	/	H-3
3	74.0	4.35, m	/	/	H-6, H-2
4	74.9	4.24, m	/	H-6	
5	71.4	4.13, m	H-6	H-6, H-1	
6	19.8	1.52 d (6.0)	H-5	/	H-3
1'	178.0	/	/	H-2'a, H-2'b	
2'	40.1	2.73, dd (3.0, 18.0) /3.02, dd (5.0, 18.0)	H-3'	/	
3'	70.3	4.74, m	H-2'	H-1	H-5'[a]
4'	88.6	4.9, m	H-5'		H-5'[ab]
5'	68.6	3.67, dd (4.0, 11.0) /4.12, m	H-4'		H-1[a], H-3'[a], H-4'[ab]

*buried signal, presence inferred based on the HSQC
[a] correlation to H-5a
[b] correlation to H-5b

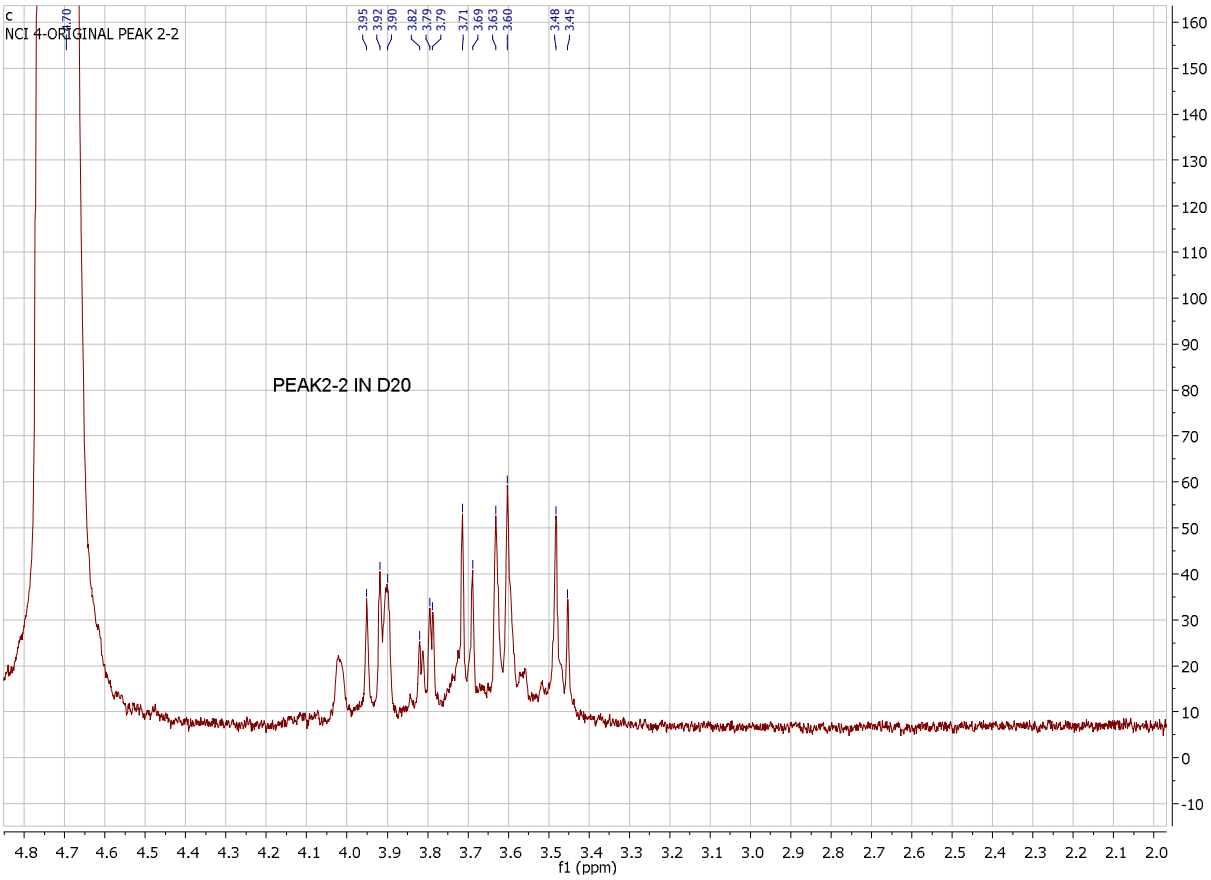


Figure 4-0-7. ^{1}HNMR spectrum of 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl)hexanamide (14) (D_2O)

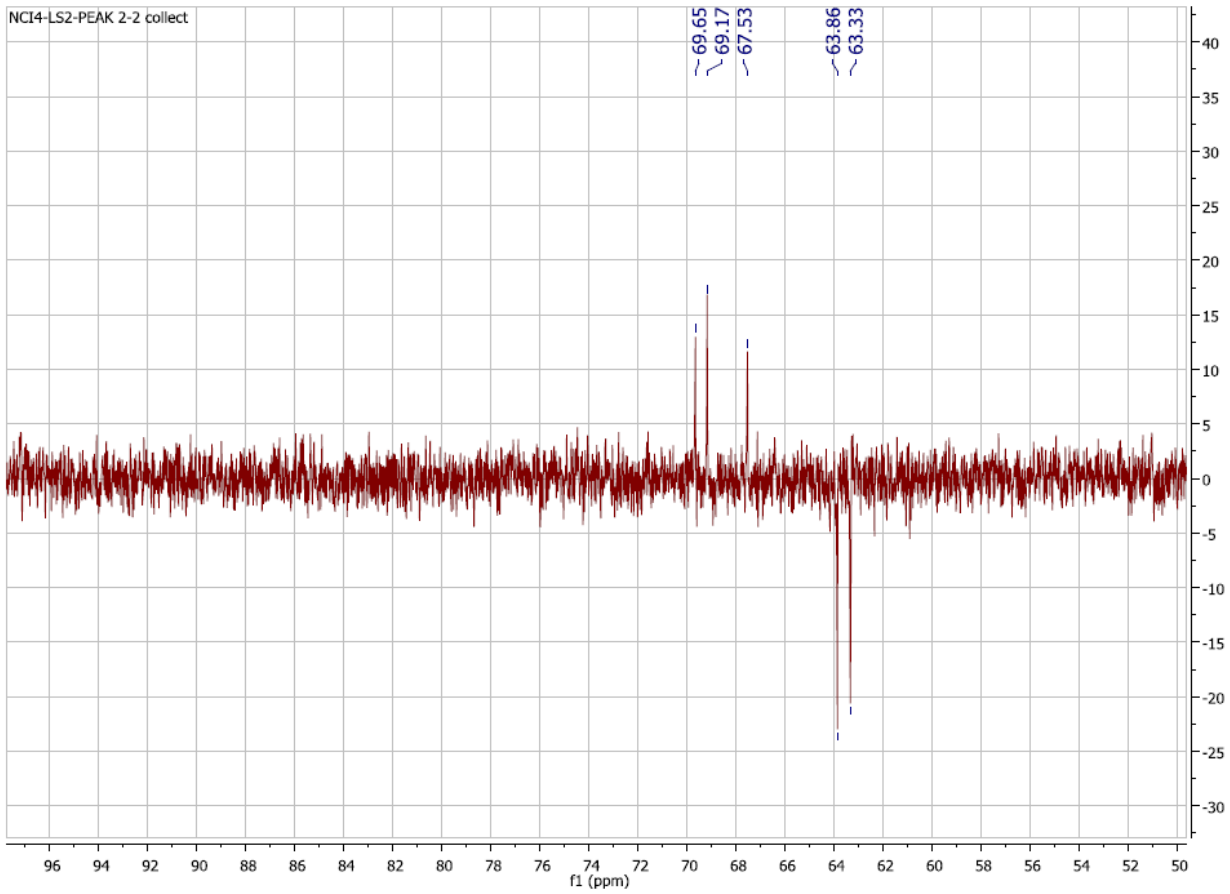


Figure 4-0-8. DEPT spectrum of 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl)hexanamide (14) (D_2O)

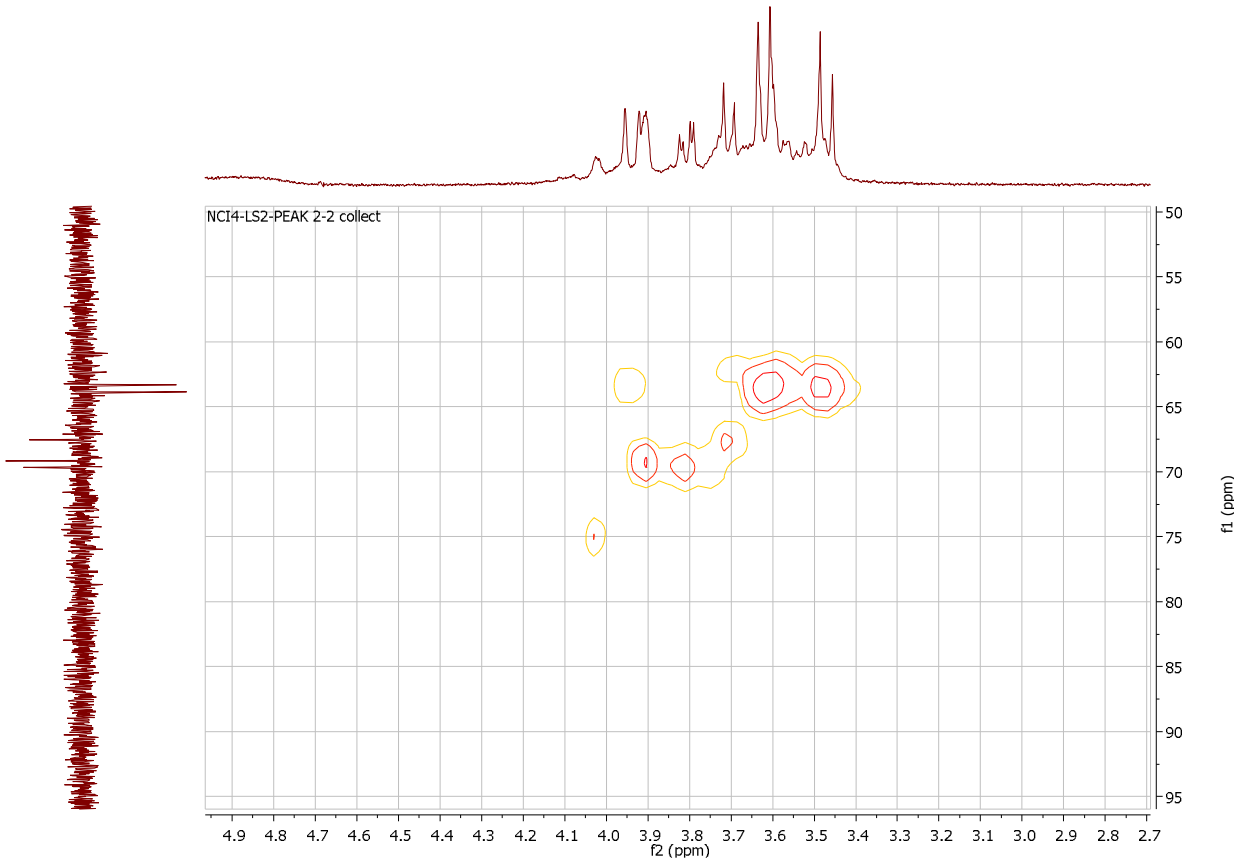


Figure 4-0-9. HSQC spectrum of 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl)hexanamide (14) (D_2O)

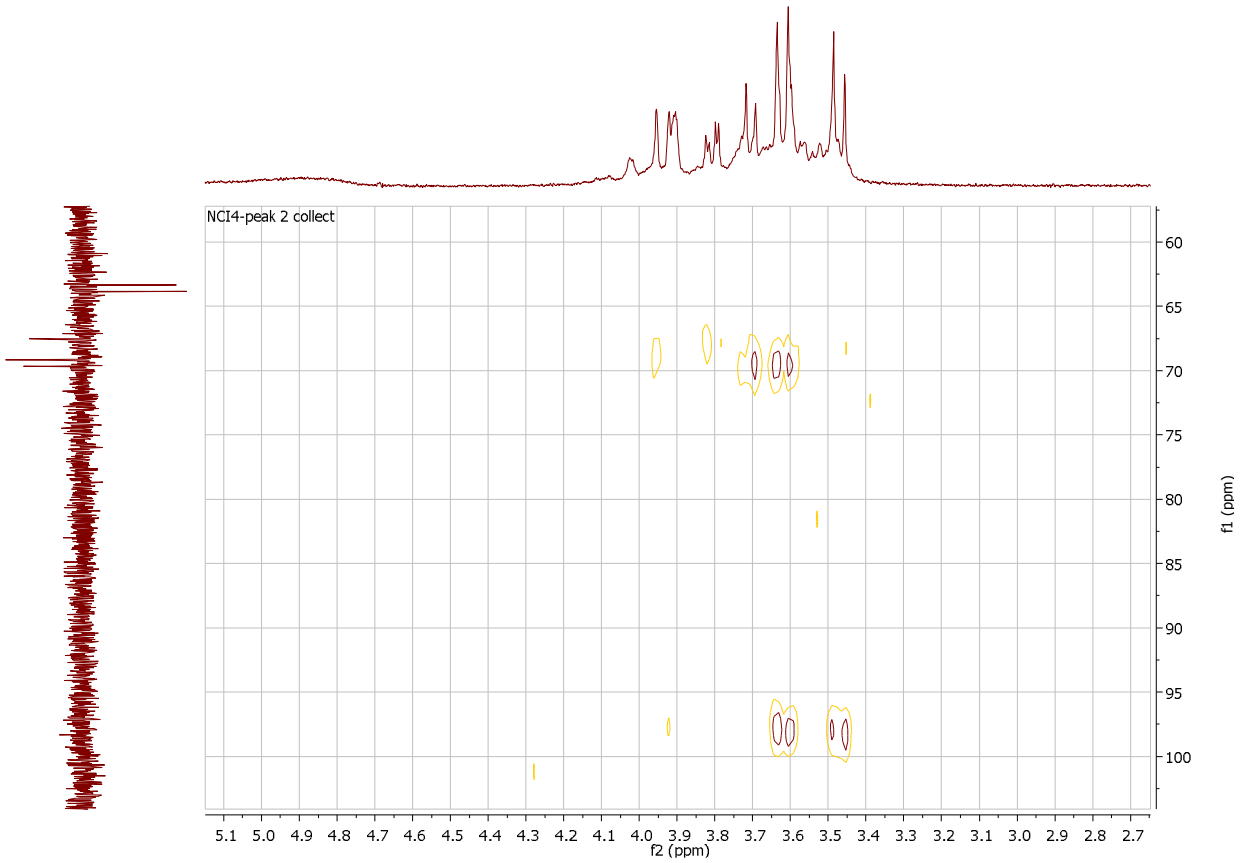


Figure 4-0-10. HMBC of 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl)hexanamide (14) (D_2O)

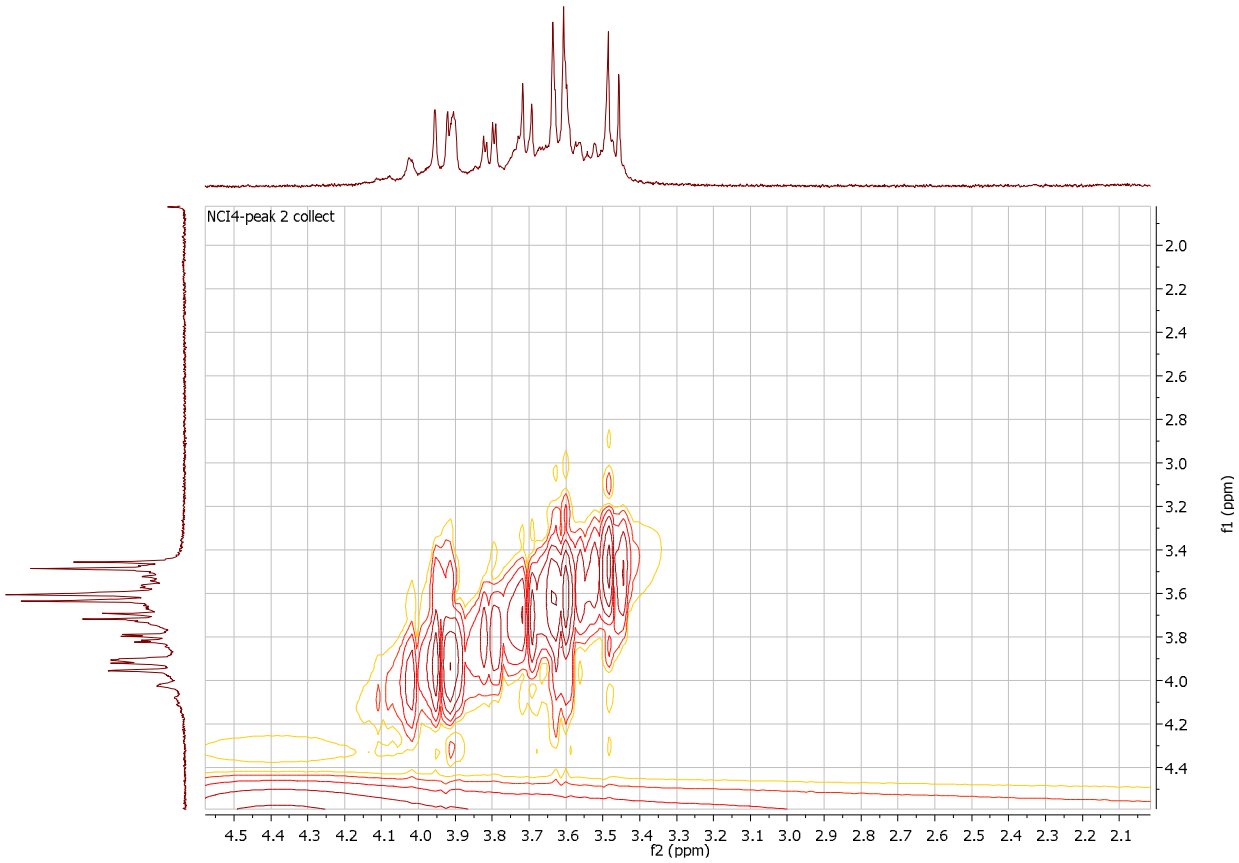


Figure 4-0-11. COSY of 2, 3, 4, 5, 6-pentahydroxy-2-(hydroxymethyl)hexanamide (14) (D_2O)

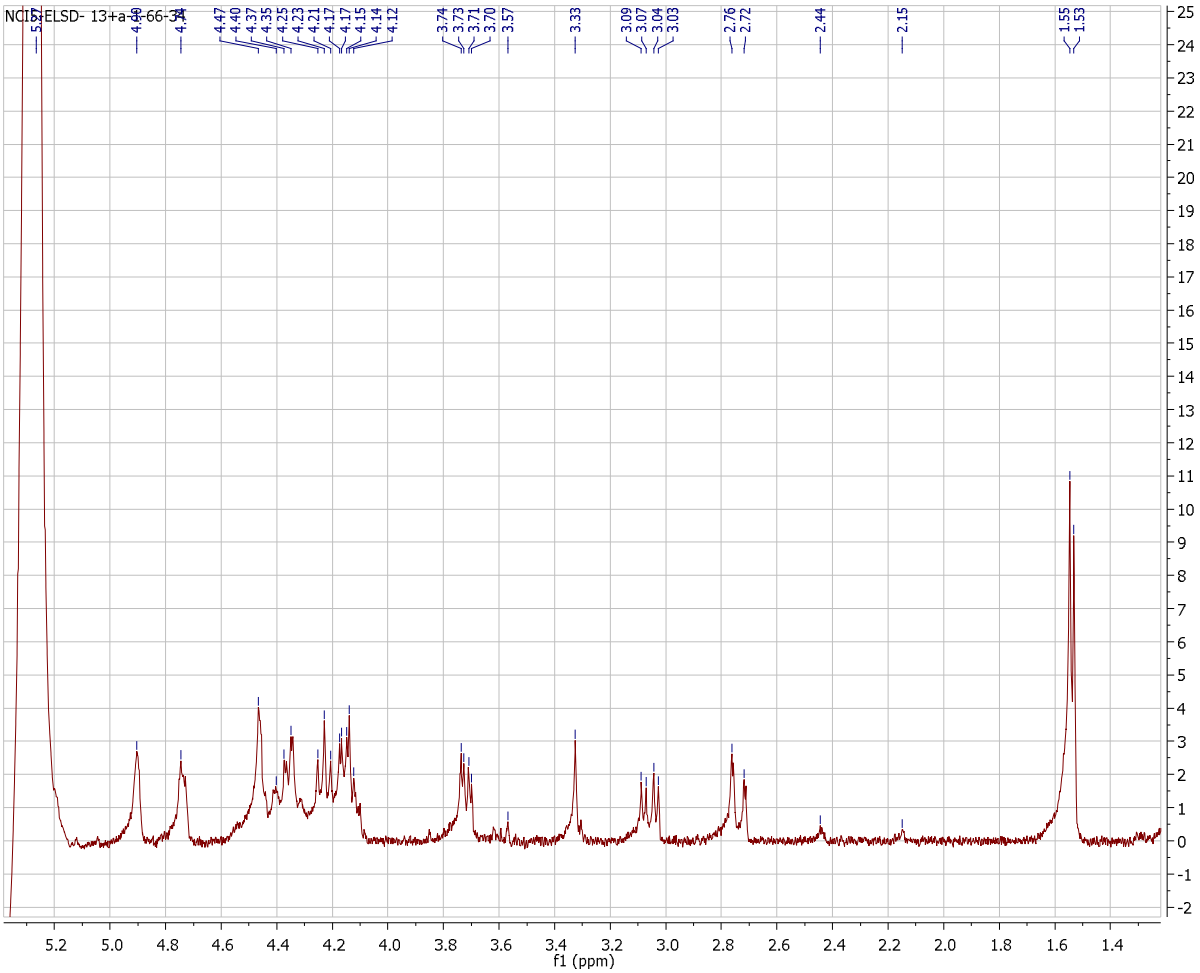


Figure 4-0-12. ^{1}H NMR of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic (15) (pyridine)

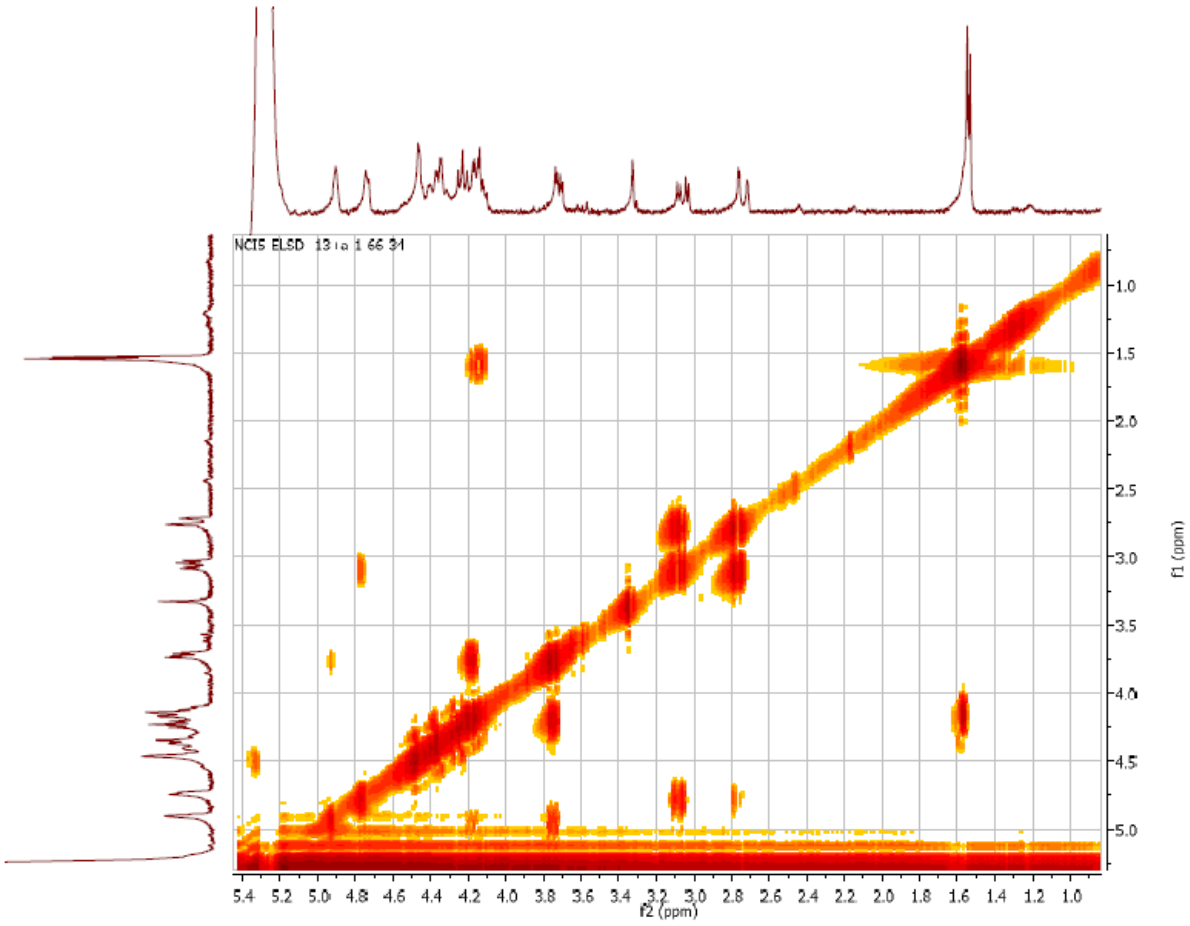


Figure 4-0-13. ^{1}H-^{1}H COSY of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic (15) (Pyridine)

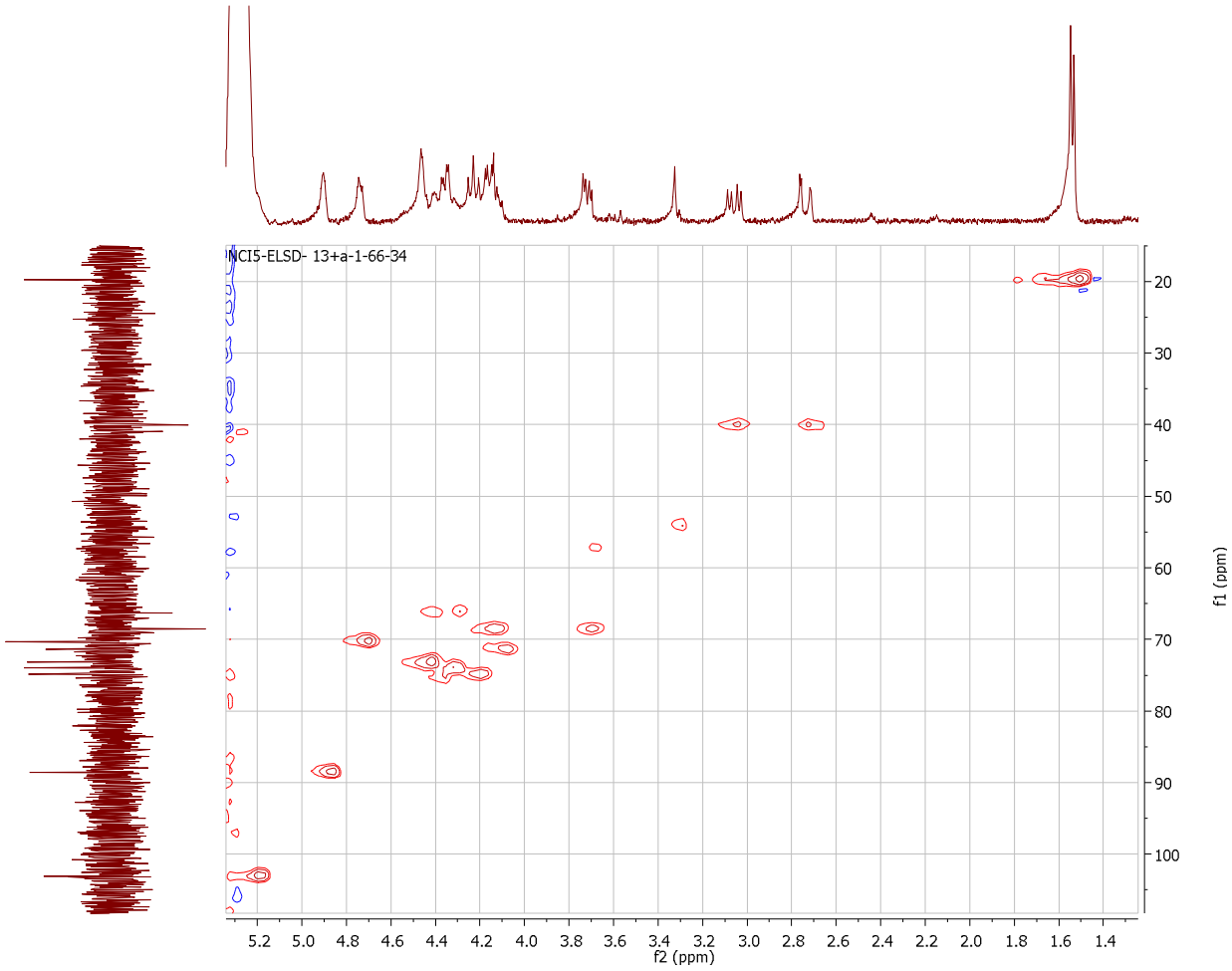


Figure 4-0-14. HSQC of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic (15) (Pyridine)

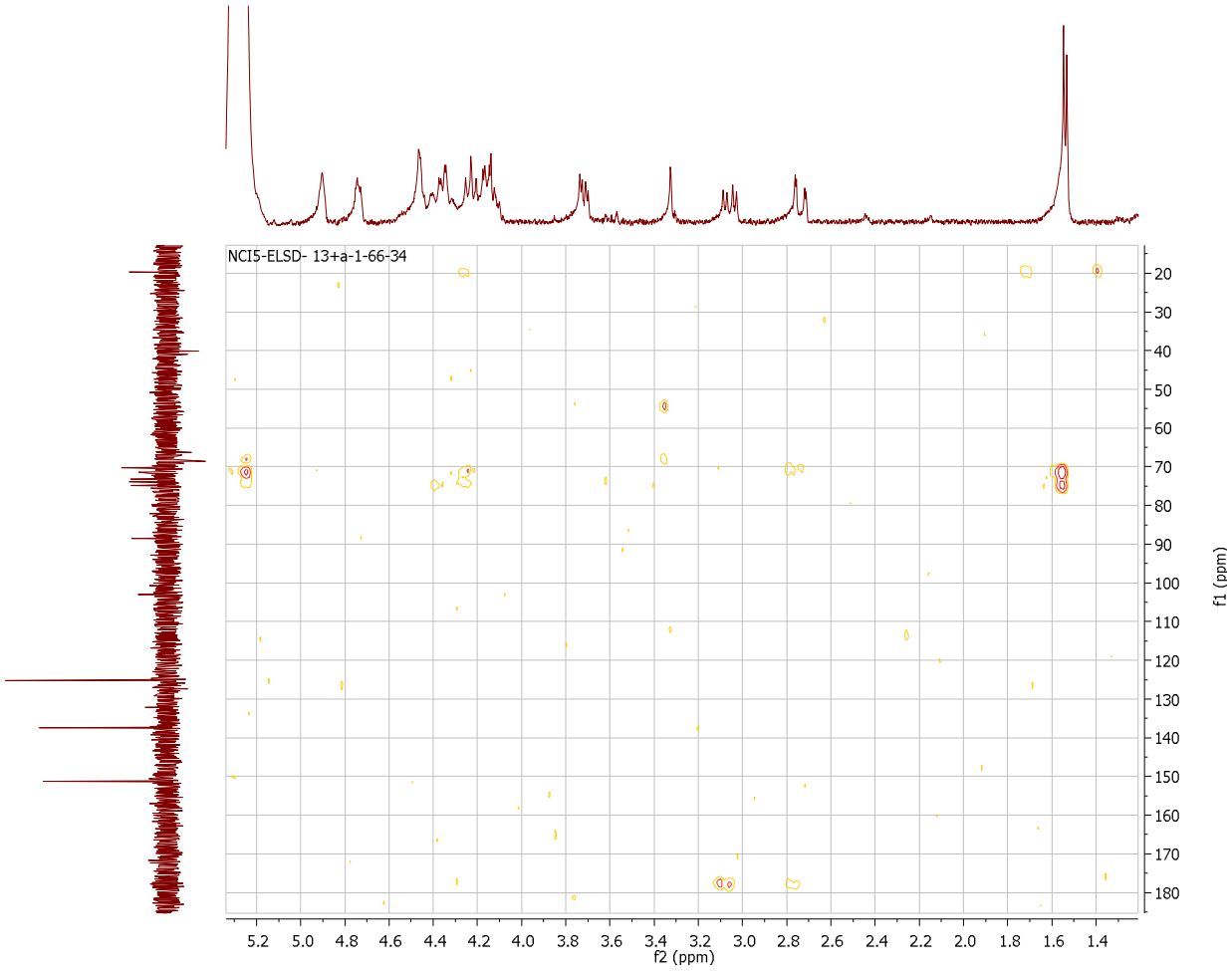


Figure 4-0-15. HMBC of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic (15) (Pyridine)

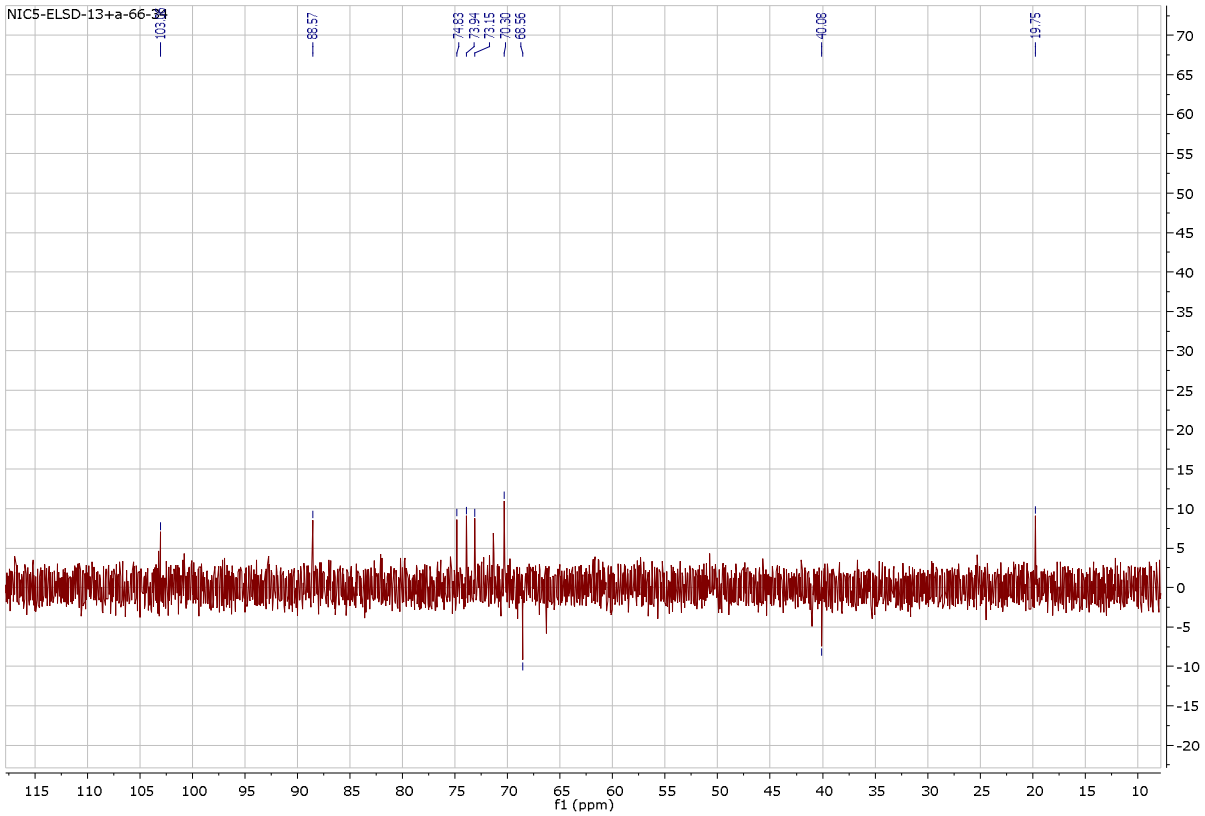


Figure 4-0-16. DEPT of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-*2H*-pyran-2-yl)oxy] pentanoic (15) (Pyridine)

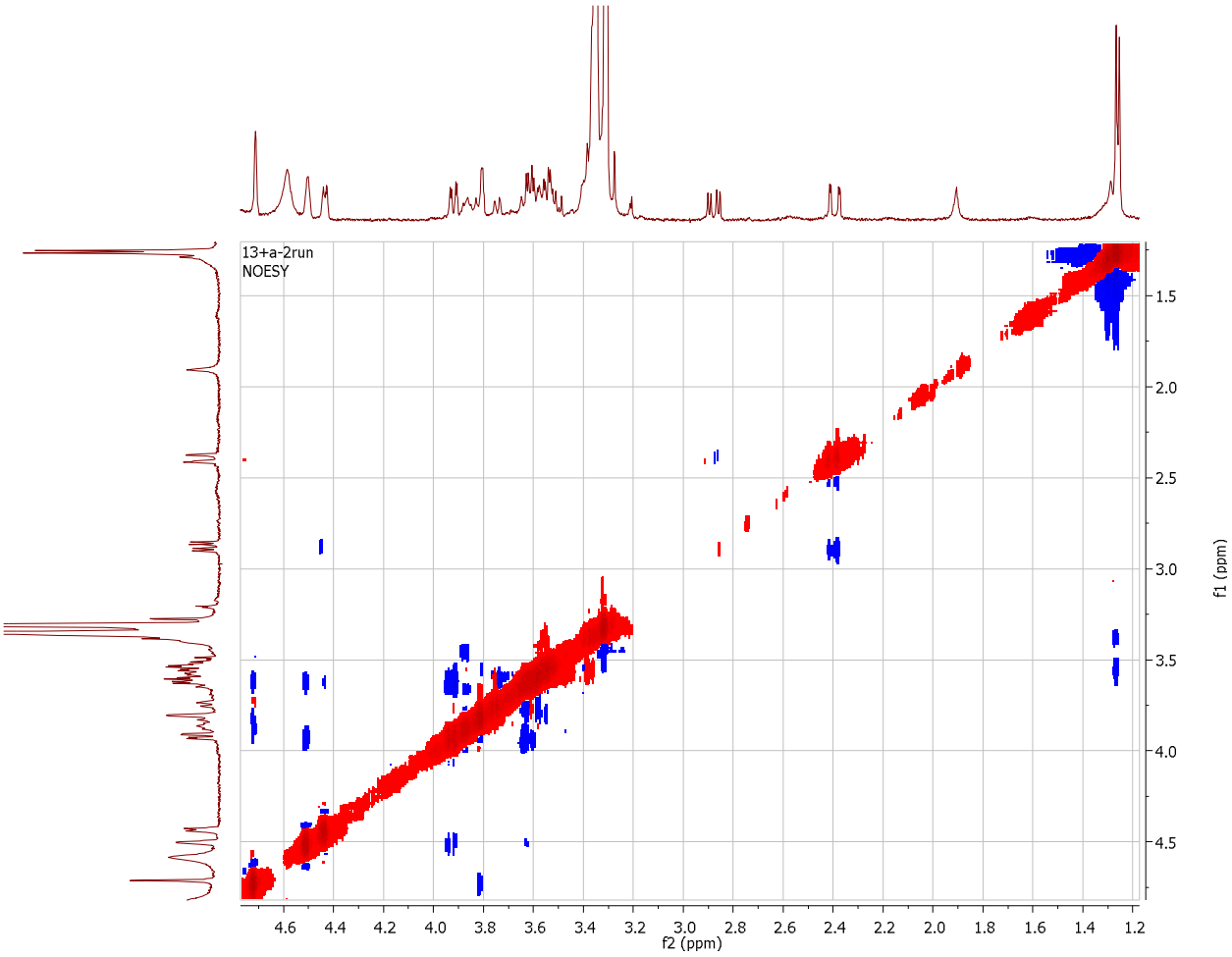


Figure 4-0-17. NOESY of 4,5-dihydroxy-3-[(3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy] pentanoic (15) (CD_3OD)

Chapter 5 : ENVIRONMENTAL TOXICOLOGY – ENCYCLOPEDIA OF SUSTAINABILITY SCIENCE AND TECHNOLOGY "SCIENCE, POLICY, AND RISK MANAGEMENT: THE CASE OF SEAFOOD SAFETY"

(Book Chapter – Elsevier, in press)

Authors: Meujo DA and Hamann MT

1. **Glossary** (Definitions of keywords)

"An outbreak involves two or more ill people" – CSPI.

Action levels: "Action levels and tolerances represent limits at or above which the FDA will take legal action to remove products from the market" - FDA.

Biological contaminants: in the context of this document, these are pathogenic microorganisms (bacteria, viruses and parasites) found in seafood.

Chemical contaminants: in the context of this chapter, are regrouped under this denomination, all non-biological contaminants (deleterious chemicals) traceable to seafood.

Environmental pollutants: seafood-associated deleterious substances traceable to the environment such as heavy metals and persistent organic pollutants.

Etiological agent: a microorganism responsible for a given disease.

Food safety hazards: according to the Seafood HACCP Regulation, a "food safety hazard" is "any biological, chemical, or physical property that may cause food to be

unsafe for human consumption."

Seafood-associated toxins: harmful chemical substances produced either by seafood-associated bacterial contaminants, cyanobacteria or toxic microscopic algae (dinoflagellates and diatoms) on which seafood feed.

Seafood: edible marine plants and animals (fish and shellfish) are usually grouped under the denomination of seafood; in some contexts, these are referred to as “fish and fishery products” [217] This same term is often given a broader meaning: all edible aquatic plants and animals.

Tolerance threshold: maximum allowable amount of ubiquitous deleterious substance in seafood.

2. Definition of the subject.

In order to function properly, the human body needs a wide range of essential nutrients, which it gets from food that is ingested on a daily basis. Unfortunately, food also represents a vector for harmful creatures (bacterial, viral, protozoan pathogens) and chemical substances (organic toxins as well as toxic metals and various environmental contaminants). According to the most recent surveys of the Center for Science for Public Interest (CSPI), for more than a decade now, seafood has ranked first as the most likely source of foodborne disease outbreaks of established origin [218, 219]. Based on these surveys, seafood-associated hazards that have caused the largest number of outbreaks are toxins (especially scombrotoxin and ciguatera), followed by bacteria, the most problematic of which are *Vibrio* spp, and finally viruses, especially norovirus. Though food safety is primarily the responsibility of regulatory

agencies, several other groups are involved. These include industries, consumers and the scientific community upon which rests the responsibility of developing cutting edge technologies capable of eliminating seafood-associated biological and chemical contaminants. The international community also relies on science for the development of revolutionary technologies for a faster, cheaper, easier and more accurate detection of seafood-associated health hazards; tools without which enforcing laws and regulations set forth by regulatory agencies is virtually impossible. In this chapter, we review different categories of seafood-associated health hazards as well as a few relevant regulatory and scientific efforts dedicated to reduce the incidence of seafood-borne illnesses.

3. Introduction.

Seafood constitutes a significant portion of the world's food supply and is renowned for its delightful taste. It is a critical component of the human diet because of its unique nutritional properties. Fish, for instance, is a good source of protein as its major components are proteins and lipids. All essential amino acids can be derived from fish consumption. Approximately 40% of the lipids found in fish are comprised of highly unsaturated long-chain fatty acids. Other outstanding nutritional qualities are reduced saturated fats and carbohydrates and plentiful essential nutrients. Some fish species are a valuable source of important nutrients such as vitamins A and D, phosphorus, iron, calcium, magnesium, selenium and iodine [220, 221].

There are numerous reports of health benefits associated with the consumption of seafood. Several of these health benefits have been attributed to seafood's high content of vital nutrients, such as n-3 polyunsaturated fatty acids (PUFAs), specifically

eicosapentaenoic acid (EPA), and docosahexaenoic (DHA). These health benefits include a reduced risk of developing serious diseases such as depression [222], myocardial infarction, Alzheimer's [223], dementia [224] and weight loss [225]. A number of reports have associated seafood consumption with a reduced risk of mortality among individuals suffering from coronary heart disease [226] and a reduced risk of developing diseases such as ischemic and thrombotic strokes, colon and intestinal cancers as well as others [68, 225, 227, 228]. These positive effects are counted among the factors that have driven current market trends. There has been a steady increase in the world's per capita fish and fishery products consumption for several decades now [229]. According to the December 2009 Food Outlook Report of the Food and Agriculture Organization (FAO), the annual per capita fish consumption in the world during the years 2007 - 2009 was estimated at ~ 17.1 kg. It is important to note that in the 1970s, 1980s and 1990s, these values were 11.5, 12.8 and 16.4 kg per capita respectively [230].

As indicated by recent estimates, there has been a net increase in the demand for seafood in countries around the world [231]. In the United Kingdom for instance, the seafood retail market has experienced a considerable increase between the years 2003 and 2007, increasing from £2.4bn (retail price) to an estimated £3.25bn in 2007 [229]. A significant increase in seafood demand in developing countries has been observed, as well [231]. Millions of tons of seafood are caught each year worldwide to sustain the current demand. There has been a steady increase in the total world fish production since the 1950's, from 19.3 million tons to about 134 million tons in 2002 [232]. According to a 2009 FAO report, the current world production (capture fisheries plus

aquaculture) is estimated at 144.1 million tons, divided into 98.8 million from capture fisheries and 54.3 million from aquaculture [230].

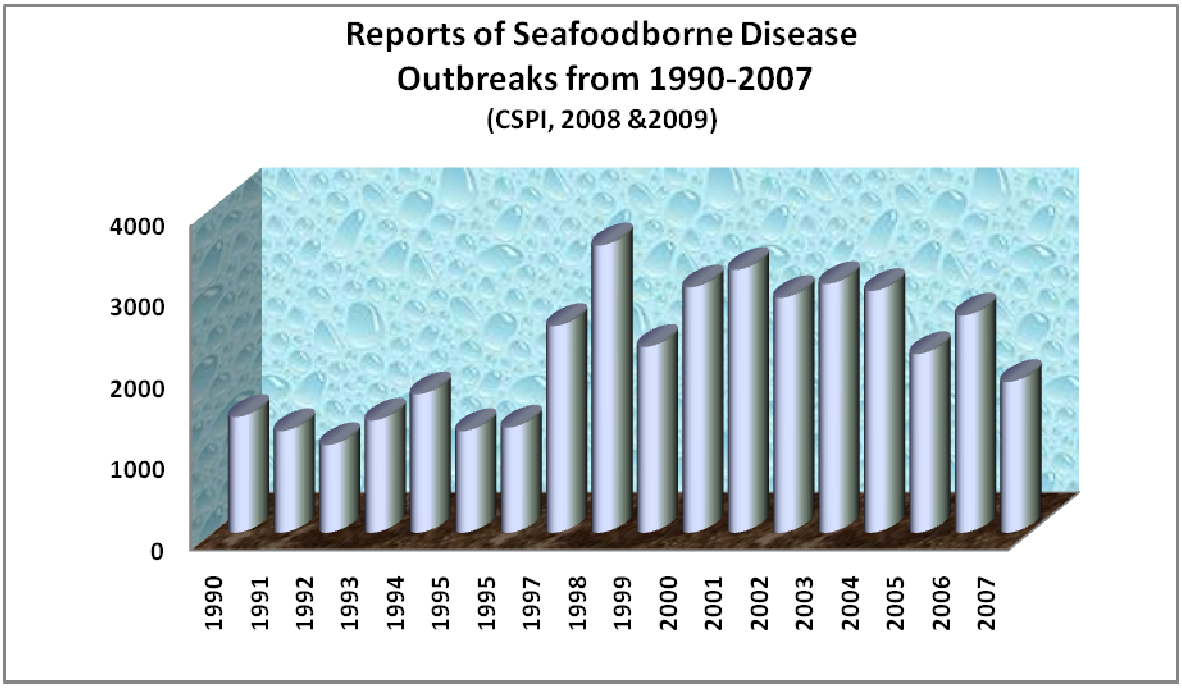


Figure 5-1. Variation in the number of reported seafood-borne disease outbreaks since the 1990's in the U.S.A.

Unfortunately, seafood consumption is not without risks and food is an important vector of a wide range of health hazards (Figure 5-1). Foodborne illnesses are a serious public health concern and according to the Centers for Disease Control and Prevention (CDC) roughly 76 million foodborne illnesses corresponding to about 325,000 hospitalizations and 5,000 deaths are recorded in the U.S. each year [233]. A recent survey conducted by the Center for Science for Public Interest (CSPI) [218], revealed that the food categories that were associated with the largest number of outbreaks in

the United States during the period 1990-2006 were seafood, produce, poultry, beef and eggs. Seafood was responsible for 1,140 out of 5,778 outbreaks and therefore, was the most problematic food (Figure 5-2). Also reported was an increase in the number of seafood related outbreaks compared to the early 1990s (Figure 5-1). It is important to mention that the number of reported cases of seafood-borne illness has remained constant over the years. Though it ranked second as far as number of outbreaks, produce caused the largest number of cases of illness during that same time frame [218].

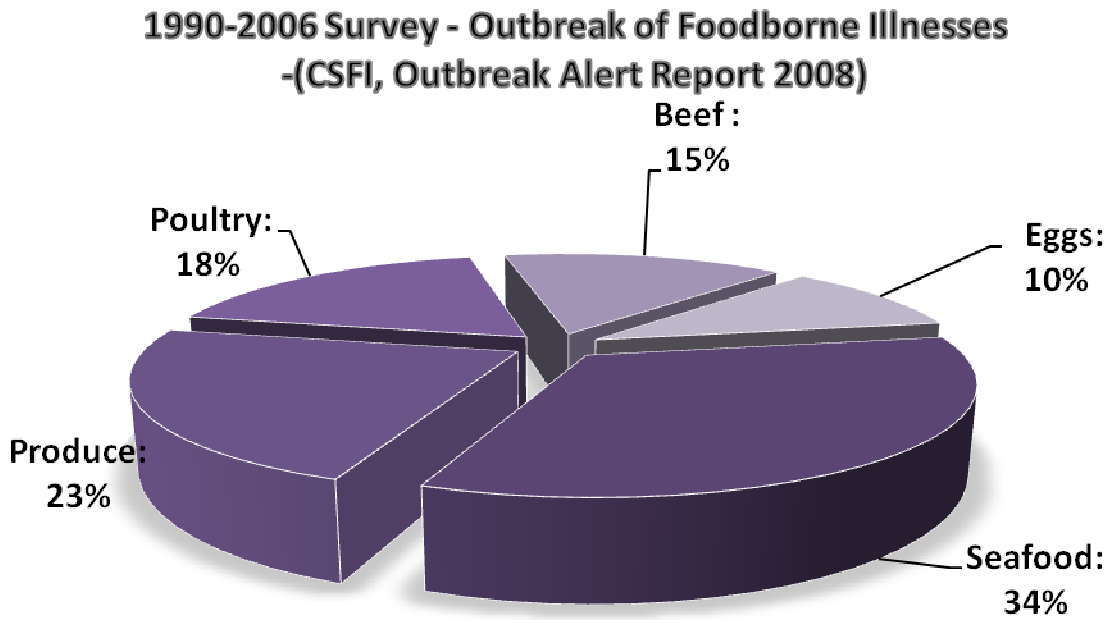


Figure 5-2. Foodborne outbreaks reported during the years 1998 - 2007 by category of food in the U.S.A.

4. "Food safety hazards" associated with seafood

Seafood-associated health hazards can be classified into two main categories: (1) biological contaminants (includes a long list of bacteria, viruses, parasites) and (2) chemical contaminants such as environmental pollutants (pesticides, heavy metals, approved or unapproved drug substances) and finally natural toxins from a variety of structural classes. According to the CSPI's 2008 report, the latter category was associated with the largest number of seafood-borne outbreaks from 1990 through 2006 (Figure 5-3) [218]. Currently known health hazards associated with seafood are either naturally occurring or from various anthropogenic activities. Seafood becomes contaminated either as a result of feeding on poisonous phytoplankton species or in sewage contaminated marine environments. Seafood contamination can also arise from inappropriate storage or accidental exposures during handling. Certain types of seafood are more likely vehicles of dangerous substances and pathogenic microorganisms than others.

Categories of seafood that have been associated with greater public safety risks are considered a high priority in sampling and surveillance efforts by the Food and Drug Administration (FDA) [234]. At the top of the FDA's seafood watch list are products such as molluscan shellfish from uncertified sources, refrigerated reduced oxygen packaged products, ready-to-eat seafood, seafood mixes containing cooked, raw or partially-cooked seafood components, as well as, scombrotoxin (histamine)-forming fish, aquaculture derived seafood and finally salt-cured or dried uneviscerated finfish [234]. According to the 2008 CSPI report, based on the number of reported outbreaks, finfish (such as tuna and grouper) was the most dangerous type of seafood between the

years 1990 through 2006. Finfish was responsible for 61% of all reported seafood-borne outbreaks during this period, followed by molluscan shellfish (15%) (Figure 5-4). The largest number of cases of seafood-borne illness was attributable to molluscan shellfish (Figure 5-4) [218].

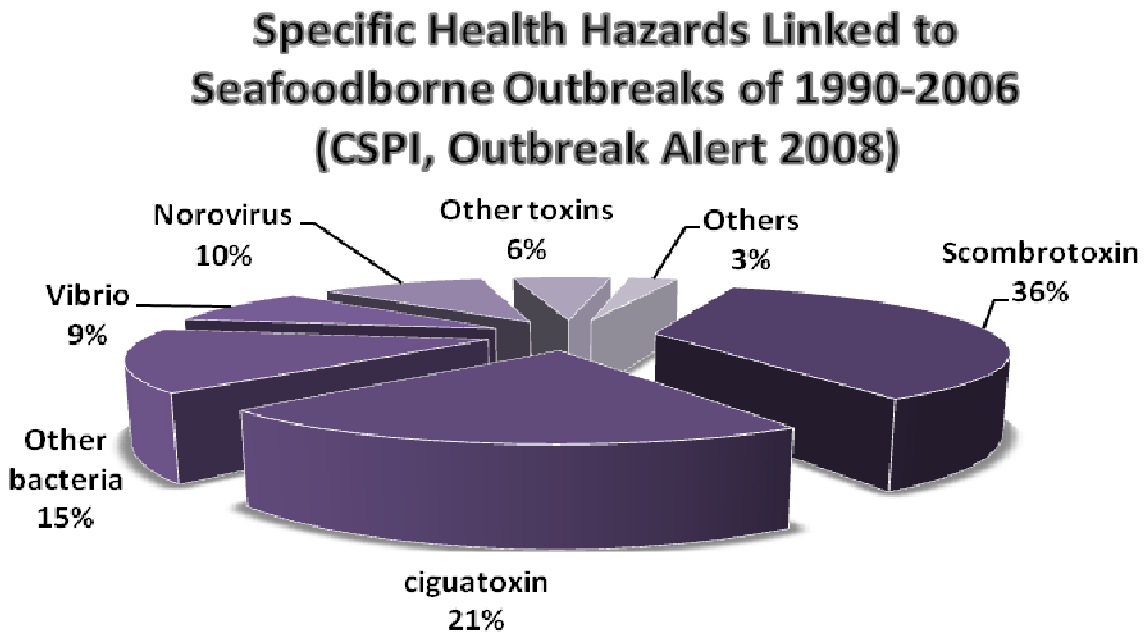


Figure 5-3. Specific health hazards that caused seafood-borne outbreaks reported during the years 1990 through 2006 in the U.S.A.

4.1. Seafood associated toxins

Seafood-associated toxins, especially scombrotoxin and ciguatoxin, have been linked to the majority of seafood-borne outbreaks that occurred during the last decade and as such, could be viewed as the most dangerous seafood-associated "food safety hazard" [218, 219]. Scombrotoxin and ciguatoxin alone were responsible for 57% of all seafood-related outbreaks in the United States reported from 1990 through 2006 [218].

Seafood-associated toxins have been linked to a wide variety of intoxications. These include, poisoning associated with shellfish consumption, namely Diarrheic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), Neurologic Shellfish Poisoning (NSP), AZaspiracid Shellfish Poisoning (AZP), and, poisonings associated with fish consumption. The latter include Ciguatera Fish Poisoning (CFP) and puffer fish poisoning [235]. It is necessary to point out that some seafood-associated biotoxins, namely ciguatoxin and toxins responsible for PSP, NSP and ASP can be lethal [236]. Seafood-associated toxins are generated either by bacterial contaminants that freely proliferate when seafood is improperly stored or by cyanobacteria and toxic microscopic algae (dinoflagellates and diatoms) on which the seafood feed. The blooms of these latter organisms, which occur from season to season, forming red tides (Harmful Algae Blooms (HAB) are a subject of public health and environmental concerns, affecting the tourism and fishing industries [237].

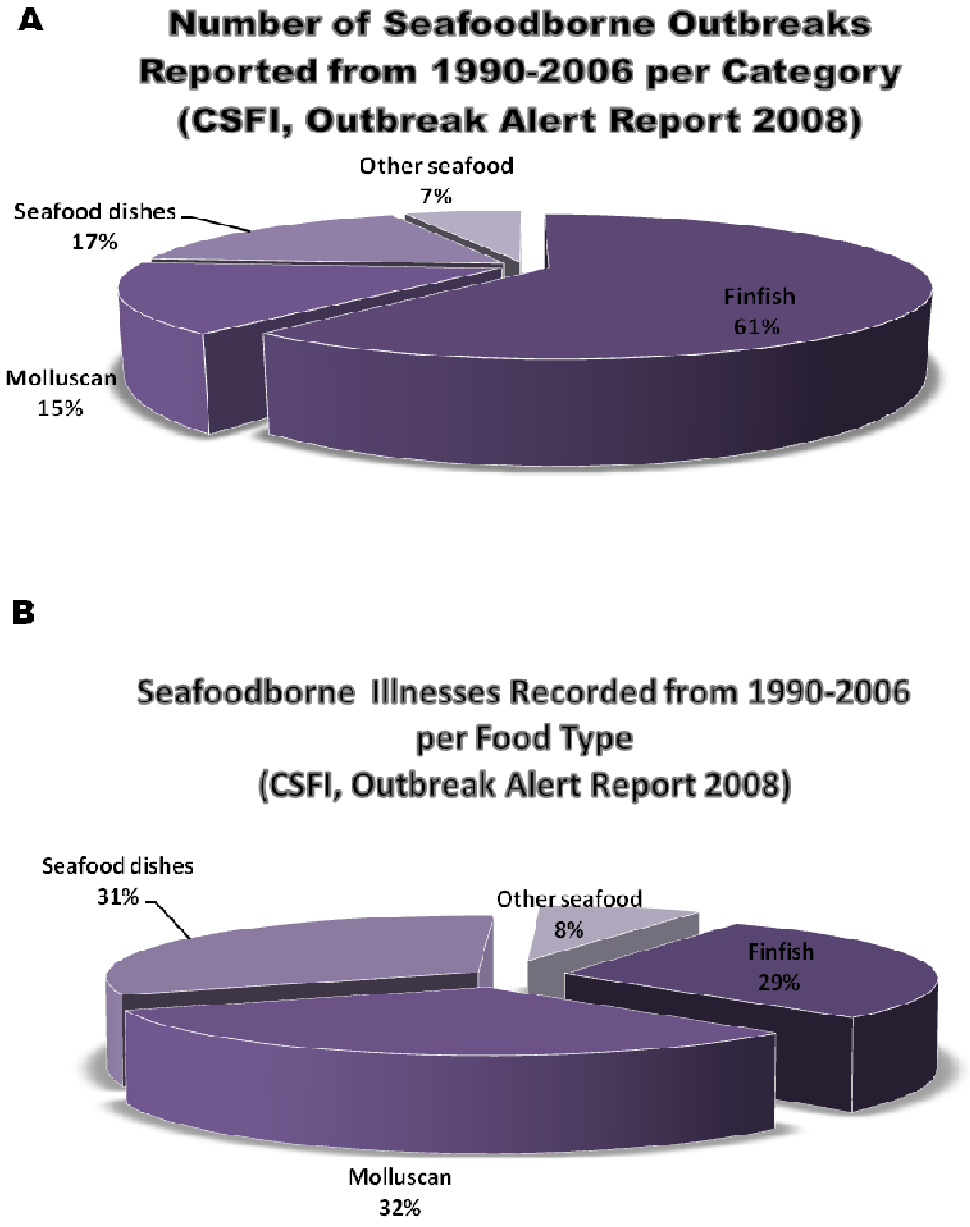


Figure 5-4. Seafood-borne, outbreaks (A) and cases of illnesses, (B) reported in the U.S.A. during the years 1990 through 2006 (by category of seafood)

4.1.1. Ciguatera fish poisoning

Ciguatera fish poisoning has been reported in countries around the world (Europe, Africa, America, Asia, and Oceania). This form of poisoning is frequent

between the months of April through August and has been linked to the consumption of certain fish. Some examples are shark, barracuda, snapper, hogfish**,** horse-eye jack, red grouper, gray triggerfish, Spanish mackerel, narrowhead gray mullet, chinamanfish, swordfish and amberjack. Ciguatera fish poisoning is caused by a set of heat resistant polyether toxins known as ciguatoxins (Figure 5-5), which are the product of *in situ* gambiertoxin biotransformation [237, 238]. Ciguatoxin, maitotoxin, palytoxin, and scaritoxin are members of this group. These toxins are produced by a variety of organisms; these include *Gambierdiscus toxicus*, *Gymnodinium sangieneum, G. polyedra, Ostreopsis lenticularis, Prorocentrum concavum, P. mexicanum,* and *P. rhathytum* among others [235, 239]. Symptoms of ciguatera fish poisoning are mainly gastrointestinal and neurological. A few therapeutic approaches in case of poisoning include, but are not limited to, antihistamines, antiemetics (droperidol, prochlorperazin, metoclopramide), atropine, as well as, intravenous hydration [237].

4.1.2. Scombrotoxic fish poisoning.

Scombroid fish poisoning differs from other types of toxin mediated seafood poisonings because the responsible toxin is not produced by a microalgae. Instead, it is generated under improper storage conditions (temperature >20 ˚C). This toxin is the result of a catalytic reaction involving the conversion of *in situ* histidine into histamine (). The enzyme responsible for this conversion, histidine decarboxylase, can be produced by several types of bacteria. These include various *Vibrio* sp. *Clostridium, Enterobacteriaceae* (such as *Morganella morganii* and *Klebsiella pneumoniae* and *Hafnia alvei*) and *Lactobacillus* sp. [240]. Scombrotoxin is stable to both heat and cold conditions. Scombroid fish (fish containing a high level of free histidine) and non-

scombroid fish have been implicated in this form of poisoning. These include fish such as amberjack, abalone, tunas, sardines, mackerel, bonito and bluefish, just to name a few [239]; associated symptoms rank from mild and self-limiting to severe. Groups at risk for developing the severe form of this disease are people with respiratory and cardiac conditions or those on medication such as isoniazid and doxycycline that slow histamine degradation [237, 239, 241]. Symptoms of scombrotoxic fish poisoning include headache, abdominal cramps, nausea, diarrhea and palpitations among others. As far as pathophysiology, bioamines other than histamine are believed to play a critical role; a few examples are spermine, cadaverine, agmatine and putrescine [239, 242]. There are several therapeutic approaches for this type of food poisoning. These include administration of activated charcoal, diphenhydramine, cimetidine and famodine [241].

4.1.3. Other major seafoodborne poisonings.

Paralytic shellfish poisoning *(PSP)* can be caused by a wide range of tetrahydropurine type toxins (carbamate, N-sulfo-carbamoyl, decarbamoyl and deoxydecarbamoyl) [238] collectively called saxitoxins (Figure 5-5). These are neurotoxins that act by blocking sodium channels, causing symptoms like numbness, paralysis and disorientation. Saxitoxins are produced by dinoflagellates and blue-green algae. Dinoflagellates that have been linked to this form of poisoning include *Pyrodinium bahamense, Gymnodinium catenatum,* as well as several organisms belonging to the genus *Alexandrium* [235, 238, 243]. Various types of seafood can serve as vectors; these include clam, crabs, cockles, oysters, salmon, mackerels, scallops, and whales to name a few [235, 237]. PSP has been reported on all continents. Regrettably, there is no antidote for PSP, and therapeutic approaches are mostly supportive and include

respiratory support in a life-threatening situation, gastric emptying, dialysis and enhancing renal clearance [237].

Diarrheic shellfish poisoning *(DSP)* has also been reported worldwide. Diarrhea, nausea, cramps and vomiting are common signs of DSP. It is usually associated with consumption of contaminated clams, mussels, oysters and scallops. This syndrome is caused by a group of acidic (okadaic acid and related dinophysistoxins) and neutral toxins (pectenotoxin). Yessotoxins have also been reported to cause this form of poisoning (Figure 5-5) [238, 244]. These toxins are produced by a variety of marine dinoflagellates including *Dinophysis* spp (*D. acuta, D. acuminate, D. caudate, D. mitra, D. norvegica*) as well as *Protoceratium* spp., *Prorocentrum* spp*. Gonyaulax* spp*.* and *Phalacroma* spp [235]*.*

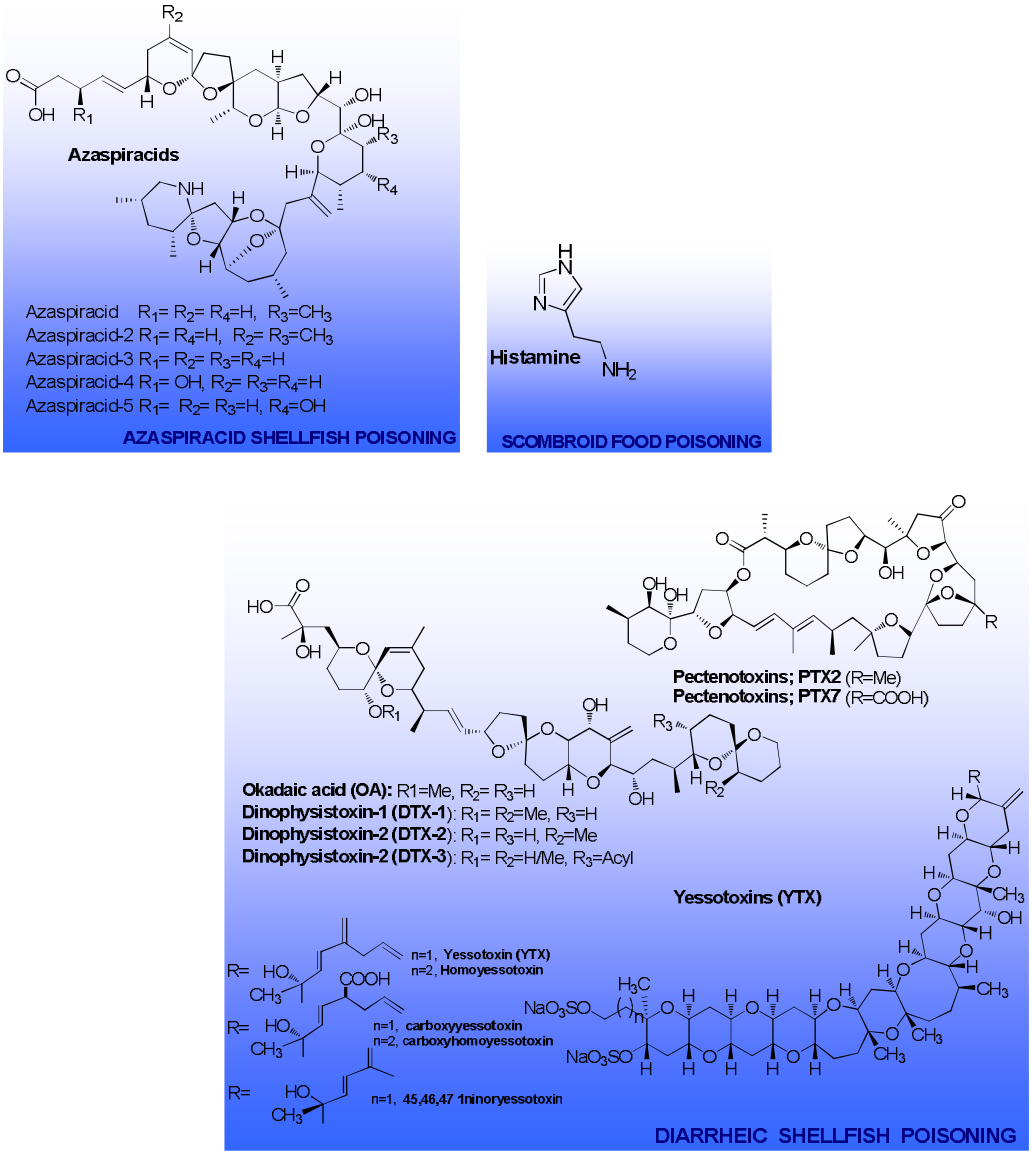


Figure 5-5. Toxins involved in seafoodborne intoxications

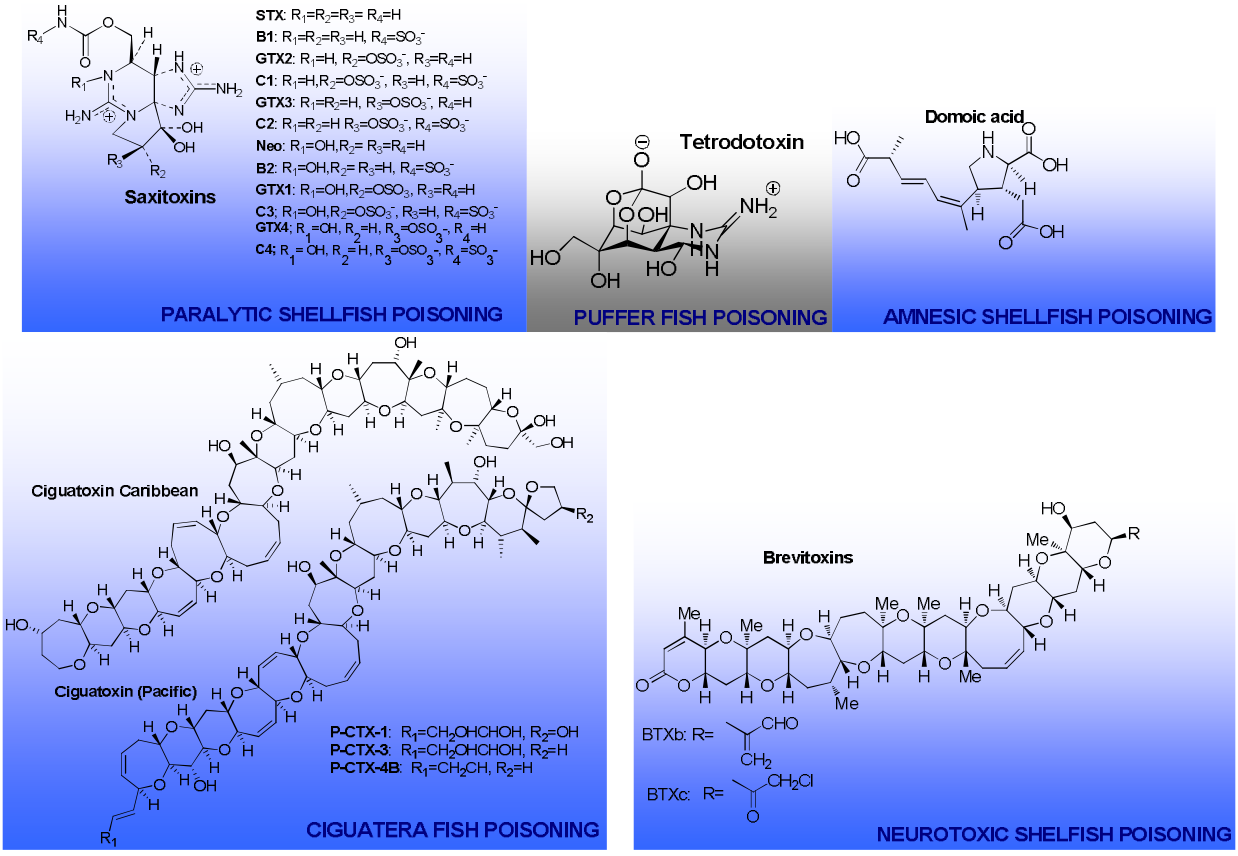


Figure 5-5. (cont) Toxins involved in seafoodborne intoxications

Amnesic shellfish poisoning **(ASP)** has been reported in various areas around the world including Europe, North, Central and South America, Asia and Oceania. Several types of seafood, including anchovies, clams, crabs, oysters, mussels, mackerels, lobsters, scallops and gastropods are potential vectors. ASP is caused by a marine biotoxin called domoic acid (Figure 5-5). This toxin is produced by a red-brown marine diatom called *Pseudo-nitzschia pungens*. Diarrhea, nausea and abdominal pain are examples of symptoms that indicate amnesic shellfish poisoning [235].

Neurotoxic shellfish poisoning **(NSP)** is caused by brevetoxins and its analogs (Figure 5-5). Occurrences have been reported in countries around the world. This form

of poisoning has been associated with the consumption of contaminated clams, mullets, mussels, oysters, tunas and whelks. *Fibrocapsa japonica, Gymnodinium breve* (*Karenia brevis*), *Raphidophyceae* sp., and *Chattonella marina,* among others, are examples of organisms that produce these toxins [235, 238].

Puffer fish poisoning has been linked to the most potent and lethal marine neurotoxin: tedrodotoxin (Figure 5-5). It is produced by a variety of animals including the California newt, trumpet shell, the blue ringed octopus and puffer fish, especially a species known as fugu (present in the liver). Puffer fish is a delicacy in Japan and is the main vector for this form of poisoning. Once again, there is no antidote. The first case in Europe occurred in 2009 and involved an individual that had consumed trumpet shellfish (*Charonia sauliae*) harvested from the Atlantic Ocean in Southern Europe [245]. The main therapeutic approach upon poisoning is supportive and includes respiratory support (life-threatening circumstances). Activated charcoal, atropine, anticholinesterase agents, and alpha agonists, among others, are also recommended [246].

AZaspiracid shellfish Poisoning (AZP). Mussels and oysters are known vectors of toxins responsible for azaspiracid shellfish poisoning. AZP has been reported in countries around Europe, namely Norway, Portugal, the U.K. and Ireland. AZP is caused by marine toxins known as azaspiracids (Figure 5-5), which are produced by *Protoceratum crassipes and Protoperidinium.* Nausea, vomiting and diarrhea, are a few symptoms of azaspiracid shellfish poisoning [235, 238].

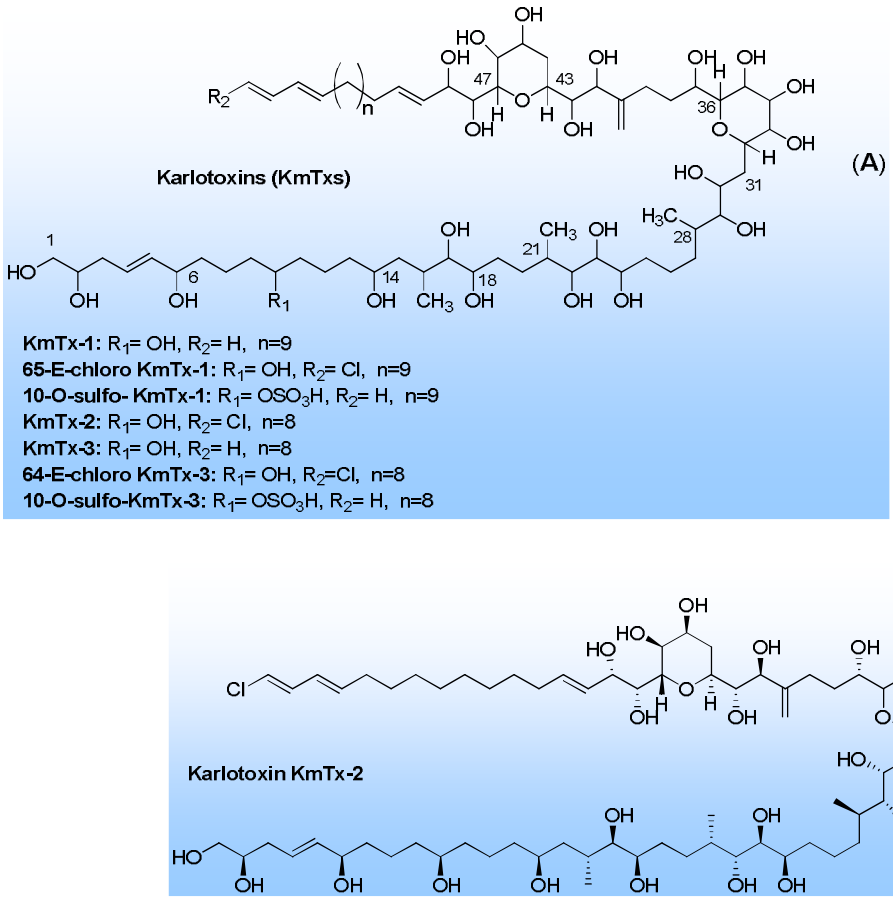


Figure 5-6. Structure of biotoxins produced by *Karlodinium veneficum*. (B) The absolute configuration of KmTx-2,

Several other marine biotoxins not listed above have been reported. A few examples able to impair human health are gymnodimine, neosurugatoxin, prosurugatoxin, polycavernoside and debromoaplysiatoxin [235]. It is also important to note that a number of biotoxin producers (dinoflagellates) has been associated with massive fish mortality and thus represent a major issue to the seafood and tourism industry worldwide. Examples in this case are *Pfiesteria piscicida* and *Karlodinium veneficum* however there is growing evidence that *P. piscicida* associated fish kills in

the past may have been indeed have been *K. veneficum* derived. Blooms of *K. veneficum* have been linked to various episodes of massive fish kill around the world. In this particular case, a set of toxins, believed to be the etiological agents, and regrouped under the denomination karlotoxins or KmTxs, has been isolated [247, 248]. Examples of such toxins include the karlotoxin-1 (KmTx-1), the 10-*O*-sulfo-KmTx-1, the KmTx-3, the 64-*E*-chloro-KmTx-3, the 10-*O*-sulfo-KmTx-3, the 65-*E*-chloro-KmTx-1, and finally, the KmTx-2 (Figure 5-6) [249], for which the relative and absolute configurations were assigned only recently [250]. Ongoing work in this area actually began with research by Abbott and Ballantine in the 1940s and '50s [251]. Karlotoxins kill fish by osmotic cell lysis, a result of the alteration of the ion transport system of the cell membrane. The fish dies as a result of damage to its vital gill epithelial tissues. These toxins are environmental pollutants and detectable in water during fish kill episodes. The ecological role of these toxins was investigated recently and because the toxins possesses an allelopathic inhibitory effect on competitors as well as a prey immobilization it was established that the organism produce these toxins in order to facilitate feeding and control of competition during a bloom [252, 253].

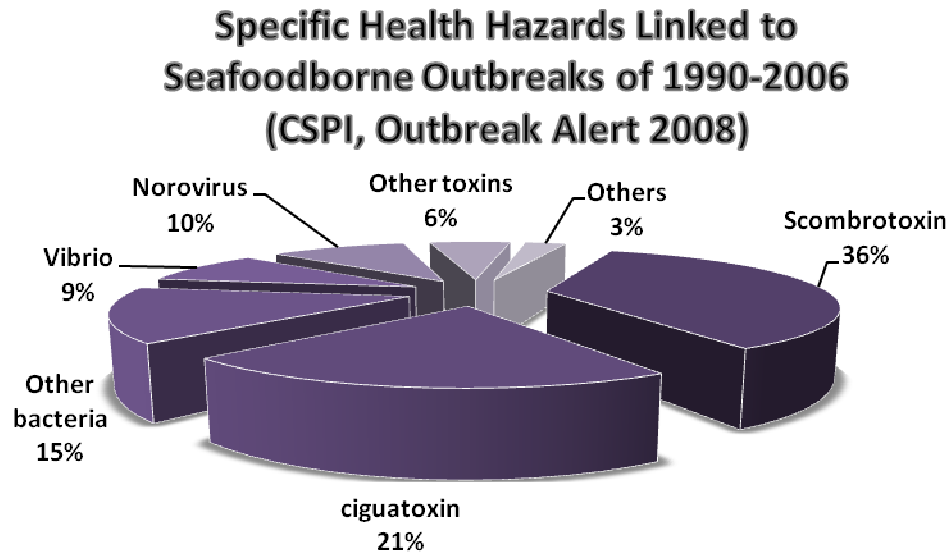


Figure 5-7. Specific health hazards that caused seafood-borne outbreaks reported during the years 1990 through 2006 in the U.S.A.

4.2. Microbial pathogens

Following chemical toxins, pathogenic microorganisms were the most likely cause of seafood-borne disease outbreaks throughout 1990-2006 (Figure 5-3). The CSPI's survey associated bacteria to 24% of reported outbreaks during this period, trailed by norovirus (10%) [218].

4.2.1. Pathogenic bacteria

Seafood's bacterial pathogens can be found either in their GI system (bivalve mollusks) or on their surface (crustaceans). These bacteria have been linked to various infections and intoxications. Pathogens accumulate in the digestive track of bivalve mollusks (cockles, mussels, oysters, clams) as a result of filter-feeding in heavily contaminated water. Seafood associated bacterial pathogens are either indigenous to the marine environment (case of *Vibrionaceae,* a source of greater concern) or

nonindigenous (resulting from fecal contamination). Members of the first category are pathogenic *Vibrio* spp. such as *V. cholerae, V. parahaemolyticus, V. vulnificus, Clostridium botulinum* (non-proteolytic types B, E, F), *Aeromonas hydrophila, Plesiomonas shigelloides,* and *Listeria monocytogenes*, just to name a few [236, 240, 254]. Diarrhea is a common symptom of infection caused by several of these microorganisms. *V. parahaemolyticus, V. vulnificus, V. hollisae,* and *V. cholera* non O-1 have been also associated with more serious conditions such as septicemia. These microorganisms are most prolific during summer months as the water temperature rises. The second category includes bacteria such as *Salmonella* (nontyphoidal), *Shigella, Campylobacter, Staphylococcus aureus,* and *Escherichia coli* [218]

Proper seafood storage is sufficient to protect against diseases caused by several of these bacteria. It has been established that their concentration in seafood is normally low (below the minimum infective dose) and will remain so providing that the seafood is stored in conditions that are not conducive to bacterial growth, multiplication or toxin production (refrigerated (4 ºC) and frozen (- 18 ºC)). It is important to note that this does not apply to mollusks and their predators [242, 255]. While, in several cases, a low concentration in seafood is tolerated, FDA regulations become more stringent when it comes to bacteria such as *L. monocytogenes, V. vulnificus, Salmonella, C. botulinum* and toxigenic 01 *V. cholera.* Current regulations require that these be undetectable in seafood requiring minimal cooking before consumption, for instance [217].

4.2.1.1. *Vibrio* spp.

Vibrio spp, especially *V. parahaemolyticus* and *V. vulnificus*, are a cause of significant concern in the U.S. and several Asian countries (Japan, Taiwan, India and China). *V. parahaemolyticus* (Vp), for instance, has been associated to the vast majority of seafood-borne gastroenteritis in the U.S. This bacterium has been associated with fewer outbreaks in Europe [254, 255]. The pathophysiology of Vp is centered on several virulence factors; a few examples in this case are the Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin (TRH), which are encoded by tdh and trh genes respectively [256]. Healthy individuals are also at risk of developing Vp associated infection [239]. The FDA can take legal action when seafood products are found to contain a Vp count ≥ 1 x 10^4/g [217]. Raw or improperly cooked fish and shellfish are potential vectors [239]. The minimal infective dose for this pathogen is > 10^6/g [240]. *V. vulnificus* (Vv) infections can also result from consumption of raw or undercooked seafood. In addition, transmission can occur via wound infection. Though Vv is not a major issue in healthy individuals, in certain groups, Vv can cause serious infections or death. People suffering from alcoholic cirrhosis, hemochromatosis/cirrhosis, chronic hepatitis, post-necrotic cirrhosis, as well as diabetics and alcoholics are at higher risk. *V. vulnificus* is the second leading cause of seafood-related fatality in the U.S. [257-259]. Vv infections that occur as a result of consumption of contaminated seafood (especially raw oysters) are primarily septicemia and gastroenteritis. One therapeutic approach is the use of antimicrobial agents (tetracycline and intravenous doxycycline with ceftazidime) [258].

2.2.1.2. Other seafood-associated bacteria

Clostridium botulinum – This bacterium is responsible for a condition known as botulism, the responsible agent being a toxin. Lightly preserved, semi-preserved, and fully preserved smoked, fermented, salted and pickled fish products are likely vectors. Cold-smoked and fermented fish products are of greater risk. Chilling, autoclaving, and salting are approaches used to prevent botulism [240, 242].

Listeria monocytogenes - Infections cause by this other bacterium can, at worst, result in septicemia spreading to several organs and even, in the case of pregnant women, to the fetus. This type of infection can be fatal. Groups most at risk are pregnant women, neonates, fetuses, and immuno-compromised patients. Shrimp is an important vector [240, 242, 260]. *S. aureus* has also been isolated from seafood. This bacterium is, as *C. botulinum,* a toxinogenic species. It produces toxins that are resistant both to enzymes degradation and heat. It is introduced in seafood as a result of environmental contamination or transferred from an infected worker involved in seafood handling [240, 242].

Enterobacteriaceae such as *Salmonella, Shigella* and *E. coli* have been also reported in seafood. *Enterobacteriaceae* usually occurs in seafood as a result of fecal contamination. *Salmonella* is responsible for salmonellosis and is especially problematic for the shrimp industry [260]. Compared to other food categories, seafood is a less likely vector of *Salmonella* [239, 240]. The minimum infective dose for *Salmonella* sp has been estimated to be in the range of $< 10^2$ - $>10^6$. Non-bloody diarrhea, fever, abdominal pain and nausea, just to name a few, are indicators of infection by this pathogen. Symptoms of a *Shigella* infection, on the other hand, are bloody stools,

severe abdominal cramps, fever and dehydration. The minimum infective dose in this case has been estimated at 10^{1-} 10^{2}. This is similar to what has been reported for *E. coli,* for which the minimal infective dose is 10^{1}- 10^{3} [240, 242].

Table 5-1. Safety levels set by FDA for several seafood associated bacteria

Hazards	FDA & EPA thresholds	Analytical approach	Targeted seafood	Higher risk populations
Salmonella sp.	Presence of organism [a]	Conventional culture methods	All fish	Severe in the elderly, infants, AIDS patients
E. coli	MPN of 230/100 grams [b] APC - 500,000/gram	Hemorrhagic colitis agar-direct plating method	Imported fresh and frozen clams and oysters	All people – most susceptible are young children and the elderly
S.aureus	Presence of staphylococcal enterotoxin, or a load ≥10^{4}/g (MPN) [c]	Specific precipitation with antiserum	All fish	All people
C. botulinum	Presence of viable spores or vegetative cells or toxin [c]	Mouse neutralization test	All fish	All people

Compliance policy/programs

[a] Sec 555.300 Compliance Policy Guide [261]

[b] Sec 560.600 Compliance Policy Guide [262]

[c] Compliance Program 7303.842 [263]

4.2.2. Seafood associated viruses

Seafood can also serve as a vector of viruses. Non A, non B enteral hepatitis viruses, Hepatitis A Virus (HAV), poliovirus, and norovirus, among others have been associated with seafood-borne outbreaks [239, 240, 264, 265]. Viruses end up in

seafood as a result of fecal contamination of the marine environment or when handled by an infected worker. Viruses are one of the most serious seafood-associated threats. So far, reports of seafood-borne infection outbreaks linked to viruses have emerged from countries around the world. HAV was associated with the largest seafood outbreak ever reported. This outbreak, which occurred in 1998, in the Chinese city of Shanghai, was linked to the consumption of contaminated clams and over 292,000 cases were reported [265, 266]. Another virus, namely norovirus, a single-stranded nonenveloped RNA virus, is currently responsible for roughly 50% of all foodborne outbreaks of gastroenteritis according to the CDC [267]. Norovirus was reported by CSPI as the most problematic seafood-associated virus during the period 1990 through 2006 (Figure 5-3) as it caused 10% of all reported seafood outbreaks during that time [218]. It is important to point out that of the five genogroups of norovirus, GII has been linked to the majority of infections. Filter feeding bivalve shellfish are important vectors [258]. Norovirus gastroenteritis-associated symptoms are vomiting, watery stools, non-bloody diarrhea with abdominal cramps, and nausea. It is a fairly resistant virus that can survive harsh conditions such as chlorine treatments (10 ppm), heating to 60 ℃ (4 hours), or freezing [267].

4.2.3. Seafood associated parasites

Seafood (raw or undercooked) can also serve as a vector of pathogenic parasites. These include nematodes, cestodes and trematodes. *Anisakis simplex, Pseudoterranova dicipiens, Gnathostoma* sp., *Capillaria* sp., and *Angiostrongylus* sp. are examples of seafood-associated nematodes. Examples of tape worms that can be isolated from seafood include *Diphyllobothrium latum* and *D. pacificum; Clonorchis* sp.,

Opisthorchis sp., *Metagonimus yokagawai, Heterophyes* sp., *Paragonimus* sp. and *Echinostoma* sp., on the other hand, are a few examples of seafood-associated trematodes [240].

Several parasite-infected seafood dishes such as sushi, crab, sashimi, herring roe and undercooked grilled fish have been associated with illnesses [217]. Compared to bacteria and viruses, however, parasites are of lesser concern. Trematodes (such as *Paragonimus westermani),* cestodes (such as *Diphyllobothrium latum, D. pacificum)* and nematodes (such as *Angiostrongylus cantonensis, Contraceacum osculatum)* have been associated with domestic fish and shellfish. Several other organisms have been associated with imported products instead. These include *Clonorchis sinensis, Heterophyes heterophyes, Metagonimus yokogawai, Opisthorchis felineus* and *Gnathostoma spinigerum*. Some nematodes, cestodes, and trematodes are of greatest concern as far as seafood safety. These include *Anisakis simplex*, *Pseudoterranova* spp*., Eustrongylides* spp*., Gnathostoma* spp*., Opisthorchis* spp*., Chlonorchis sinensis and Paragonimus* spp, just to name a few [217, 264].

4.3. Toxic heavy metals

Heavy metals are a threat to the environment and public health and are problematic in regard to their long-term persistence. A variety of heavy metal contaminants has been reported in seafood. These elements originate from natural occurrences (marine volcanism, geological and geothermal events) and anthropogenic activities. Several categories of anthropogenic activities threaten the marine environment. These include, activities that take place in tanneries, steel plants, battery

industries, thermal power plants and farms, especially those farms using heavy metal containing fertilizers and pesticides. Runoff from roadways has also been recently cited as an important source of contamination [220, 268, 269].

Heavy metals found in seafood include antimony, arsenic, cadmium, chromium, lead, mercury, nickel, copper, iron, manganese, selenium, zinc, aluminum, silver, strontium, thallium, and tin. Of these heavy metal contaminants, those of greatest concern are antimony, arsenic, cadmium, chromium, lead, mercury, and nickel. It is important to note that elements such as copper, selenium, iron and zinc (known essential micronutrients) are toxic only at high concentrations [220].

Arsenic can be present under a variety of forms: toxic (inorganic) and nontoxic (organic). Arsenic is an extremely potent poison in its trivalent form and can cause a wide range of acute and chronic illnesses. A few examples include cancer, nephritis, hepatomegaly, peripheral symmetrical neuropathy, and palmar hyperkeratosis, among others. In seafood, arsenic is mainly present in a nonpoisonous form known as arsenobetaine or arsenocholine [264]. A compilation of data (about 100,000 results) received from fifteen European countries revealed that seafood is among the food commodities with the highest arsenic levels [270].

Methyl mercury is a neurotoxic contaminant. Due to its potential effects on the fetus, it is one of the most regulated seafood-associated toxic metals. There are several related FDA recommendations to nursing/pregnant women, women of childbearing age, or children when it comes to seafood consumption. CH_3Hg^+ is present in nearly all seafood, but some types of fish such as shark**,** swordfish**,** king mackerel,

and tilefish are believed to have a higher content. The FDA and the U.S. Environmental Protection Agency (U.S.EPA) have recommended being very selective of the type of fish consumed, limiting uptake to ~ 12 ounces/week as a healthy approach for groups at risk [221]. This has been supported by scientific data [228, 271, 272]. Several reports have shown that moderate to high consumption of fish species containing only a low amount of CH_3Hg^+ during pregnancy, has a positive effect on fetal brain development. However, it is also important to note that several other studies, Myers et al. (2003) for instance, did not support the fact that exposure of pregnant women to methyl mercury through fish consumption could have deleterious effects on fetal development [228, 273].

Other toxic heavy metals include nickel, chromium, cadmium, selenium and lead. Cadmium tends to be bio-accumulated by crustacea and bivalves. Clinical signs of cadmium poisoning include osteoporotic and osteomalacic disease, as well as kidney damage. Lead poisoning, on the other hand, has been associated with anemia, convulsions, paralysis and proteinuria. This metal tends to accumulate in cortical and trabecular bone, kidney, lung, as well as the CNS. Edema, hepatitis, and hemorrhage are conditions that can result from selenium poisoning, which is also known, to result in congenital malformations and infertility. Arsenic (As), nickel (Ni) and chromium (Cr) are carcinogens [264].

4.4. Other chemical environmental contaminants

Organochlorine compounds. Until recently, organochlorine compounds were widely used. A wide range of polychlorinated substances can be found in seafood.

These include various insecticides, agrochemicals, industrial chemicals and byproducts. Examples of pesticides traceable to seafood include endrin, heptachlor, dieldrin, γ-1, 2, 3, 4, 5, 6-Hexachlorocyclohexane, chlordane, dichlorodiphenyltrichloroethane and lindane. Polychlorinated biphenyls and dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, are example of industrial chemicals that contaminate seafood. Prior to 1970, PCBs were widely used industrially [274]. Because they are non-biodegradable, toxic in nature and tend to bio-accumulate in seafood, organochlorine compounds now constitute a major environmental and public health concern. Several of these substances are known carcinogens (dioxins and polychlorinated biphenyls); but because seafood-associated health benefits outweigh potential risks, seafood consumption is recommended [228]. The level of these substances in fish is truly minimal. However, it is important to note that people whose diet is predominantly made of seafood are still at risk [274]. Reports on farm raised salmon (especially European farms) containing higher levels of these contaminants compared to wild type salmon is believed to have had a serious impact on the consumer acceptance of this type of seafood [274, 275].

Figure 5-8. Main causes of rejection or detention of seafood imports into Europe during the years 1999 – 2002.

Antimicrobial drug contaminants of seafood. This is a problem mainly associated with aquaculture, as farmers rely more and more on various drugs and antimicrobial agents to deal with specific seafood diseases (bacterial, fungal, viral), to improve the quality of water, and to manage pest-related issues.

There are quite a few reasons why there are concerns over drug residues being present in seafood. Several antimicrobial agents used in aquaculture have been linked to various adverse health effects, such as hypersensitivity reactions. In vivo studies, in animals, have established several others as carcinogens; these include nitrofuran, malachite green, gentian violet, and fluoroquinolones. Fluoroquinolones have been also

associated with antibiotic resistance. There are several drugs used to this end in the U.S. that have been approved by the Center for Veterinary Medicine (CVM) (Table 5-2). Substances such as ciprofloxacin, erythromycin, tetracyclines, chloramphenicol, nitrofurans, malachite green, gentian (crystal) violet, and fluoroquinolones have been frequently reported in seafood imported into the U.S. in recent years [234]. A short, recent survey by the FDA has shown that imports from China test positive for the presence of a variety of unapproved substances, such as gentian violet, malachite green, nitrofuran, and fluoroquinolones. Consequently, these products are the subject of great concern and the focus of regulatory surveillance [276].

Table 5-2. Aquaculture drugs approved in the U.S. and action levels

FDA-approved aquaculture drugs			
Drug name	**Tolerance level in the flesh**	**Type of seafood**	**Purpose**
Unapproved drugs	No trace tolerated [a]	All fish	-
Chorionic Gonadotropin[b]		Brood finfish	Reproductive
Formalin solution[c]		Salmon trout, catfish, largemouth bass, and bluegill	Antiparasitic and fungicidal/static
Tricaine methanesulfonate[d]		Catfish, salmon and trout, pike and perch	-
Oxytetracycline	2.0 ppm[e]	Salmonids, catfish, and lobster	Disease control
Sulfamerazine [f]	Undetectable [g]	Trout	-
Sulfadimethoxine/ Ormetoprim combination [h]	0.1 ppm [i]	Salmonids and catfish.	-

Compliance policy/programs

[a] Sec 615.200 Compliance Policy Guide [277]
[b] 21 CFR 522.1081 [278]
[c] 21 CFR 529.1030 [279]
[d] 21 CFR 529.2503 [280]
[e] 21 CFR 556.500 [281]
[f] 21 CFR 558.582 [282]
[g] 21 CFR 556.660 [283]
[h] 21 CFR 558.575 [284]
[i] 21 CFR 556.640 [285]

Similar issues have been reported in Europe. In fact, during the years 1999 through 2002, the presence of residues of antimicrobial drugs, mainly nitrofuran and chloramphenicol, was one of the main reasons for detention or rejection of seafood imports into Europe (Figure 5-8) [242]. A handful of aquaculture drugs approved in Europe are presented in Table 5-3.

Table 5-3. Aquaculture drugs approved in Europe and action levels

The Council of the European Communities approved aquaculture drugs		
Drug name	**Maximum Residue Limit (MRL) – muscle and skin in natural portion**	**Type of seafood**
Trimethoprim	50 µg/Kg *	Finfish
Flumequine	600 µg/Kg *	Finfish
Oxolinic acid	100 µg/Kg *	Finfish
Sarafloxacin	30 µg/kg *	Salmonidae
Tylosin A	100 µg/kg *	Finfish
Thiamphenicol	50 µg/kg *	Finfish
Colistin	150 µg/kg *	Finfish
Deltamethrin	10 µg/kg *	Finfish
Emamectin B1a	100 µg/kg *	Finfish
Oxolinic acid	300 µg/kg *	Finfish
Florfenicol	1 000 µg/kg *	Fish
Thiamphenicol	50 µg/kg *	Finfish
Deltamethrin	10 µg/kg *	Finfish
Compliance policy/programs * Council Regulation (EEC) No 2377/90 of 26 June 1990 [286]		

5. What are the approaches used to assure public safety?

Regulatory authorities and the scientific community are two groups with complementary goals that play a key role in ensuring public safety against seafood-associated health hazards.

5.1. **Regulatory agencies**

Seafood regulatory agencies, via promulgation and enforcement of various laws and regulations, guidance, and recommendations are usually at the heart of protecting the public from seafood-associated hazards. Numerous programs have been designed over the years to this end. In the U.S., the primary responsibility of assuring seafood safety falls to the FDA, which is in charge of setting the maximum safe levels of unavoidable toxic substances in seafood [276]. The FDA has the authority to detain and even refuse import entries into the U.S. A recent related event was the June 28, 2007, decision of the FDA to detain Chinese farm-raised catfish, basa, shrimp and dace until they were cleared from containing unapproved drug substances [276]. As for domestic seafood, the FDA can recommend legal sanctions, which include "warning letters, seizure of products, injunction against further non-compliant practices, or prosecution of an individual or establishment" [234]. There are a few other regulatory agencies; these include the EPA (Environmental Protection Agency) in charge of chemical contaminants such as pesticides and the National Marine Fisheries Service for instance. In Europe, the E.U. parliament is at the heart of food safety control. This organization promulgates food safety related laws, regulations and directives, which are mandatory in all states of the European Union. The E.U. parliament works in close collaboration with the European Food Safety Authority, which was established by regulation (EC) No.178/2002. This latter organization, more science oriented, is in charge of risk assessment.

There is a long list of guidance and regulations, mostly to seafood industries that protect the public from dangers associated with seafood consumption. Several of these

are preventive measures. A few examples in the U.S. are the National Shellfish Sanitation Program, the Salmon Control Plan, the Low-Acid Canned Food (LACF) Program, the Hazard Analysis & Critical Control Points (HACCP) Program and the Good Manufacturing Practice regulation. The latter is intended to assure that recommended processing conditions were used. The Salmon Control Program, on the other hand, was designed to assure the safety of salmon consumers and is a cooperative approach involving the FDA, industries, and various associations. As part of the Shellfish Sanitation Program in the U.S., the level of various pollutants in coastal water is to be monitored in order to classify each given area as suitable or unsuitable for shellfish harvest. As for the HACCP, it applies to domestic as well as imported seafood. HACCP requires both domestic and foreign processors of fish and fishery products to understand all concepts behind food safety hazards and through a system of precautionary control measures to prevent hazards from occurring [234, 276]. The E.U. parliaments, as well as regulatory agencies of countries around the world, have issued several similar regulations and directives that apply to domestic and imported seafood. A few examples of seafood-related regulations promulgated by the European Parliament include regulations (EC) No. 852/2004 and No. 853/2004, which established some key hygienic rules for food (including seafood) business operators and regulation (EC) No. 854/2004 in which key exigencies related to the organization of seafood official control programs are determined. Several of these regulations elaborate on the "tolerance threshold" for contaminants present in seafood. Examples of "tolerance threshold" for seafood-associated health hazards, set by regulatory agencies around the world are presented in Table 5-1 → Table 5-6.

Table 5-4. Seafood-associated marine biotoxins and action level set by regulatory agencies around the world

Hazards	Detection method (analytical methods for regulatory purposes)	Tolerated thresholds	Targeted seafood
Paralytic shellfish poison	***USA***- mouse bioassay	0.8 ppm (80µg/100g) saxitoxin equivalent [a] 800ug/Kg (live bivalve molluscs)	All fish
	EU - mouse bioassay [b]	80 mg STX eq/100 g of meat [c & d]	Bivalve molluscs
	Africa - mouse bioassay [b]	80 µg STX eq/100 g	molluscs
	Canada - mouse bioassay [b]	< 80 mg STX eq/100 g	Molluscs
	Asia - mouse bioassay [b]	400 MU/100 g	Shellfish
	Australia – mouse assay [b]	80 mg STX eq/100 g	Shellfish meat
Amnesic shellfish poison	***USA*** - LC method	20 ppm domoic acid (in general) [a] 30 ppm (in viscera of dungeness crab[a]	All fish
	Europe - LC method	20 mg/Kg of domoic acid [c]	Live bivalve mollusks
	Canada - LC method	20 mg DA/kg	Mussel
	New Zealand - LC method	20 mg DA/kg	Shellfish
Neurotoxic shellfish poison		0.8 ppm (20 mouse units/100 gram) (USA)	Clams, mussels and oysters, fresh, frozen or canned
Diarrheic shellfish poison	***EU*** - mouse bioassay [b]	160 ug of okadaic acid/Kg [c]	Molluscs, echinoderms, tunicates and marine gastropods

	Asia (Japan) mouse bioassay	5 MU/100 g whole meat	Shellfish
	Australia	16 to 20 µg OA eq/100 g	Shellfish
Azaspiracids	***USA*** - mouse or rat bioassay	160 ug azaspiracid equivalents/Kg [c]	Bivalve molluscs, echinoderms, tunicates and marine gastropods
	Europe - mouse or rat bioassay	160 µg/kg	Live bivalves
Ciguatoxin		Presence [c]	Fishery products
Histamine	***USA***- extraction coupled to fluorescence spectroscopy	50 ppm	Tuna, mahi mahi, and related fish
	Europe	< 200 ppm [e]	Scombridae, clupeidae, engraulidae and coryphaenidae

Compliance policy/programs

[a] Compliance Program 7303.842 or Sec 540.250 Compliance Policy Guide [263]

[b] FAO (2004) Marine Biotoxins Food and Agriculture Organization of the United Nations Rome, 2004 http://www.fao.org/docrep/007/y5486e/y5486e00.HTM available online, retrieved December 30, 2009 [238]

[c] Regulation (EC) No 853/2004 (European standards) [287]

[d] EU Directive 91/492/EEC[288]

[e] council directives 91/493/EEC [289]

Table 5-5. Seafood-associated toxic heavy metals and action level set by the FDA

Seafood Health Hazard	Tolerance threshold	Targeted seafood
Methyl mercury	1.0 ppm [a]	All fish
Arsenic	86 ppm (76 ppm for crustacea) [b]	Clams, oysters, and mussels
Cadmium	4 ppm (3 ppm for crustacean) [b]	Clams, oysters, and mussels
Chromium	13 ppm (12 ppm for crustacea) [b]	Clams, oysters, and mussels
Lead	1.7 ppm (1.5 ppm for crustacea) [b]	Clams, oysters, and mussels
Nickel	80 ppm (70 ppm for crustacean) [b]	Clams, oysters, and mussels

Compliance Policy/program
[a] Sec 540.600 Compliance Policy Guide [262]
[b] Appendix 5 - FDA & EPA Safety Levels in Regulations and Guidance [217]

Table 5-6. Seafood-associated environmental pollutants and action level set by the FDA

Seafood Health Hazard	Tolerance threshold	Targeted seafood
Polychlorinated Biphenyls (PCBs)	2.0 ppm (edible portion) [a]	All fish
DDT, TDE and DDE	5.0 ppm (edible portion) [b]	All fish
Chlordane -	0.3 ppm (edible portion) [b]	All fish
Chlordecone -	0.3 ppm (0.4 ppm in crabmeat)[b]	All fish
Mirex	0.1 ppm [b]	All fish
Diquat	0.1 ppm [c]	All fish
Heptachlor and heptachlor epoxide	0.3 ppm [b]	All fish
Glyphosate	0.25 ppm [d] 3.0 ppm (for Shellfish)	Fin fish
Fluridone	0.5 ppm [e]	Fin fish and crayfish
Simazine	12 ppm [f]	Fin fish
Aldrin and dieldrin -	0.3 ppm [b]	Fin fish and shellfish

Compliance Policy/program

[a] 21 CFR 109.30 [290]
[b] Sec 575.100 Compliance Policy Guide [291]
[c] 40 CFR 180.226 [292]
[d] 40 CFR 180.364 [293]
[e] 40 CFR 180.420 [294]
[f] 40 CFR 180.213 [295]

5.2. The Scientific community's contribution in ensuring seafood safety

Through the development of cutting edge technologies to solve problems at hand, science has also played a critical role in ensuring public safety against dangers associated with seafood. A simple literature search with a key word such as "seafood

safety" in ScienceDirect shows a significant and steady increase in seafood safety related research effort since 1991 (Figure 5-9). The international community relies on science, to develop cutting edge technologies and techniques that can rid seafood from associated biological and chemical contaminants in order to bring a safer product to the market. The development of cutting edge technologies for faster, cheaper, easier, and more accurate detection methods of seafood-associated health hazards is another way scientists have contributed in the protection of consumers against these dangers.

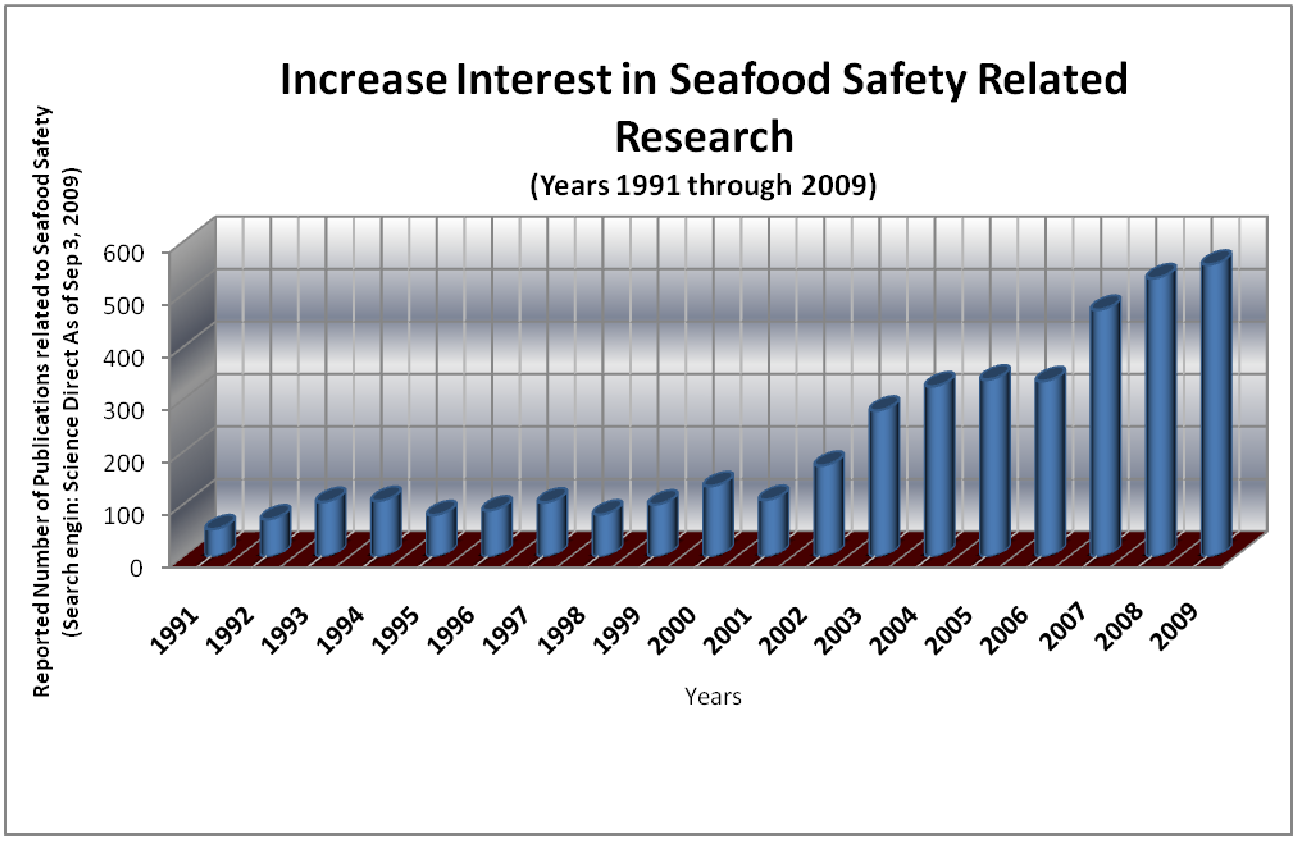


Figure 5-9. Increased interest in seafood safety related research.

5.2.1. Detection tools for seafood associated health hazards

Detection of biotoxins. Years of effort have yielded a wide range of approaches for toxin detection in seafood. These include various bioassays (in vivo and in vitro assays), biochemical techniques (immunoassays), and chemical techniques including fluorometric and colorimetric techniques, chromatographic techniques, electrophoretic techniques, mass spectrometry, and finally biosensor-based techniques (Table 5-7). In countries around the world, the mouse bioassay, despite its numerous shortcomings, and liquid chromatography are the two official methods recommended for biotoxin detection in seafood (Table 5-4).

Chromatographic techniques are at the heart of several effective analytical approaches to biotoxin separation and detection. Compared to animal assays, analytical techniques present the advantages of higher accuracy and sensitivity. While with animal bioassays, it is impossible to clearly determine the nature of contaminants, these techniques, coupled with the appropriate detection methods, will permit not only separation and accurate identification of incriminated toxins, but also, using a standard curve, their quantification. A variety of analytical approaches is currently available for the detection of marine toxins. These include Gas Chromatography (GC), Thin-Layer Chromatography (TLC), Liquid Chromatography (LC), Liquid Chromatography–Mass Spectrometry (LC-MS), and Capillary electrophoresis [235, 296]. In addition to advantages listed above, with chromatography-based detection methods, several toxins can be monitored simultaneously. A new liquid chromatography–tandem mass spectrometry method was developed recently [297]. With this method, up to 28 marine lipophilic toxins can be monitored at the same time. In this case, toxins are separated

using a gradient of acetonitrile/water at alkaline pH on a new type of C_{18} column proven stable under these conditions: a Waters X-Bridge C_{18} (150 mm × 3 mm, 5 µm) [297].

Biosensor-based detection methods have also been investigated for application in seafood safety programs. They are not only economical, straightforward and easy to use, but offer the advantages of high sensitivities/low limit of detection, plus, these technologies are portable [235]. An example of a recent development in this field is a new planar interdigital sensor-based sensing system developed by Syaifudin et al. (2009). This approach involves simple monitoring of variations of reactive impedance of the planar interdigital sensors. Using this approach, as little as 12.6 µg/g of domoic acid in mussel meat, for instance, can be successfully detected [298].

ELISA has also been investigated widely for application in seafood biotoxin detection. An example of a recent development in this field was made by Zhou et al. (2010), who reported a reliable ELISA-based approach to monitor brevitoxin in mollusks with reduce interference from the matrices. Oysters and cockles were used in this experiment. With such a method, the limit of detection of brevitoxin is improved. The main advantage of immunoassays, which are based on antibody-antigen interactions, is high specificity [299].

Table 5-7. Proposed methods for biotoxin detection in seafood [235, 238, 300]

	PSP	DSP	ASP	Ciguatera	NSP	SFP
Bioassays						
In vivo assays	Mouse bioassay	Mouse bioassay Suckling mouse assay Rat bioassay Daphnia magna assay Intestinal loop assays	Mouse bioassay	Mouse bioassay Chicken assay Mongoose and cat assay Brine shrimp assay Mosquito assay Diptera larvae assay	Mouse bioassay Fish bioassay	
In vitro assays	In vitro hippocampal slice assay Sodium channel blocking assay	Cytotoxicity assays (rat hepatocytes, KB cells, fibroblasts)	Receptor binding assays Hippocampal slice preparations	Sodium channel binding assays for ciguatoxins	Neuroblastoma cell assay Synaptosome binding assay Hippocampal slice assay	
Biochemical techniques	Immuno assay (ELISA)	Immunono assay (RIA or ELISA) Acid phosphatase assay	Immuno assay (ELISA)	Immuno assay (Radioimmunoassay Enzyme-linked immunosorbent assay (ELISA) Stick tests Immunoassays based on	Immuno assay (ELISA)	

				monoclonal antibodies Solid-phase immunobead assay)		
Chemical/analytical techniques	Fluorometric and colorimetric technique, MS Chromatographic and Electrophoretic techniques.	TLC,GC, LC MEKC, MS	Amino acid analysis TLC, LC, CE, MS	Chromatographic detection Nuclear magnetic resonance (NMR)/mass spectrometry (MS) Mass spectrometry Capillary zone electrophoresis	Micellar electrokinetic capillary chromatography detection Electrospray LC/MS Ionspray LC/MS LC/MS/MS	Chromatography coupled with fluorimetric detection or derivatization techniques (TLC, GC,CE, HPLC)
Biosensors based techniques	Sodium channels based biosensors	Immuno sensors Enzyme inhibition based biosensors	Optical immuno sensors Molecularly imprinted polymer based biosensors			

Drug residues in seafood. With the rise in demand of fish and shellfish, a large portion of seafood found in market places around the world comes from aquaculture, especially from China. As it is the case with any animal husbandry, veterinary drugs are often heavily used in aquaculture to control pests, infections, or to increase production. Unfortunately, drug residues, often molecules that have proven harmful to humans, are found in edible seafood tissues. As previously mentioned, lately in the U.S. as in Europe, there have been a significant number of alerts regarding seafood imports contaminated with unapproved drug residues (Figure 5-8). Over the years, through the dedication of the scientific community, better and more improved detection methods have been made available. An example in this case is liquid chromatography (LC) coupled with triple-quadrupole mass-spectrometry (LC–QqQ-MS). Until recently, in a single run, this sort of method could only analyze related molecular entities. An upgraded and more robust version, a multi-component quantitative HRLC–ToF-MS, was reported recently. This new approach has proven effective at simultaneously monitoring a wide range of unrelated drugs generally used in aquaculture or found in seafood tissues [301]. Smith et al. (2009) also developed an LC-ion trap mass spectrometry approach, effective at detecting several types of veterinary drugs in fish samples. In this case, the extraction of drug residues from seafood matrixes is completed in acetonitrile and hexane followed by HPLC separation on a phenyl column. In certain cases, imidazoles, macrolides, fluoroquinolones for instance, very low concentrations (0.01 ppm) could be detected using this technique. Other drugs involved in these studies included ionophores, macrolides, nitroimidazoles, benzimidazoles, anthelmintics, penicillines, quinolones, sulfonamides, tetracyclines, amphenicols, and

tranquilizers, among others [301, 302].

Detection of microbial pathogens in seafood. The best approaches for the detection of microbes in seafood are usually a combination of culture based and molecular based techniques; the latter being often used to assist in bacterial strain identification, while culture is required for enrichment purpose. The kind of medium used to this end is dictated by the type of microorganisms targeted. Over the years, a tremendous amount of effort has been dedicated to the conception of superior media. Recommended Standard Operating Protocols for use in microorganism detection, in seafood, are described in detail in the FDA's 1998 Bacteriological Analytical Manual (BAM) [303]. Targets of quality control programs are numerous. These include various pathogenic bacteria such as *Vibrio*, *Salmonella, S. aureus, L. monocytogenes* and *E. coli*) as well as indicators of fecal contamination [240].

Several molecular and culture-based methods have been developed to assist in the detection of *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv), which are major issues as far as seafood safety is concerned. Commonly used culture-based approaches, which present the main disadvantages of being time consuming, laborious, and inaccurate, are the MPN and the ISO cultural methods [254]. Quite a few *Vibrio*-specific media has been developed to assist in the isolation of these bacteria from seafood matrixes; these include TCBS agar (for *V. cholera* and *V. parahaemolyticus)* and modified cellobiose polymyxin colistin (mCPC) and CC agar for *V. vulnificus* [303]. The MPN method, coupled with various techniques to assist in the identification of suspect isolates, is recommended for detection of Vv and Vp in seafood. Examples of approaches used for these identifications are the establishment of a biochemical profile,

DNA probes or PCR. In the case of PCR for instance, DNA primers targeting tdh and *trh* genes are used to detect virulent strains of *V. parahaemolyticus.*

Until the advent of real-time PCR, this approach presented the main drawback of being limited to qualitative evaluation of food samples. Today, faster and quantitative assessment of *Vibrio*'s presence in food samples is possible [254]. More sophisticated methods have been introduced. In 2009 for instance, Espiñeira et al. introduced a sequential multiplex PCR system for the detection of *Vibrio* sp that have been involved in fish and shellfish poisoning, namely *V. cholerae*, *V. parahaemolyticus, V. vulnificus, V. alginoliticus* and *V. mimicus*. This method, which has been validated, is not only able to detect problematic *Vibrio* species, but it can also, using a fragment analysis, confirm the viable-dead status of these microorganisms and most importantly, probe for the presence of important serogroups and virulence factors [304]. Genetic markers, a precious tool for PCR, have been identified for several other seafood-borne pathogens. These include cytotoxin-hemolysin (*V. vulnificus), ctxAB* (*V. cholerae), oriC*, chromosomal origin of replication *(Salmonella* spp.), listeriolysin O (*hly*) and the 16S rRNA *(L. monocytogenes*), polymerase gene (Hepatitis A virus) and polymerase gene for norovirus, just to name a few [303].

5.2.2. Examples of recent technological breakthroughs in efforts to free seafood from associated contaminants

In recent years, outstanding breakthrough techniques and cutting edge technologies to aid in freeing seafood from associated contaminants have been developed. Molluscan shellfish and associated pathogens (especially *V. vulnificus* and

V. parahaemolyticus), scombrotoxin*,* fish safety (especially of cold-smoked salmon) and bio-preservation of seafood are a few examples of highly investigated topics.

Molluscan shellfish and associated pathogens. Nowadays, seafood regulatory authorities face major issues with molluscan shellfish. For years now, molluscan shellfish has been classified as “high risk” seafood by the FDA. They were responsible for the largest number of seafood-borne illnesses during 1990-2006 (Figure 5-4). Because of their filter-feeding habits, they tend to accumulate a wide range of etiological agents (pathogenic bacteria, parasites, and viruses) as well as biotoxins. The emergence of innovative FDA-approved Post-Harvest Processing (PHP) technologies such as Individual Quick Freezing (IQF), Heat-Cool Pasteurization (HCP), and High Hydrostatic Pressure (HHP), have revolutionized the industry of seafood, particularly in regard to oysters. These technologies, which are currently commercially available, have made it possible to bring raw and healthy products to consumers. An end product of high quality (fresh taste and superior appearance) is the major advantage of IQF, HCP and HHP. These processing techniques reduce the level of *Vibrio* bacteria to undetectable levels [304]. Though HHP is already approved by the FDA, several attempts to perfect this technology are currently on the way. In 2008 for instance, using a pressure resistant strain of Vp (MLT 403) to picture the worst-case scenario, Kural et al. proposed better pasteurizing conditions. Under such conditions, a 5-log reduction in the load of the pressure resistant Vp in live oysters could be achieved. These conditions were as follows: a two-minute treatment at pressure ≥ 350 MPa (1 °C to 35 °C) and a two-minute treatment at 40 °C (pressure ≥ 300 MPa) [305].

As far as seafood safety is concerned, HHP processing is an especially promising technology. Its inactivating effect on a variety of pathogenic agents isolable from seafood has been documented. These agents include viruses, parasites, and several other types of seafood-associated bacterial pathogens. The ability of HHP to inactivate oyster-associated viruses has been extensively studied [306-309]. A 5-min HHP treatment at pressure 400-MPa and 0 ℃ was established as an effective approach to bring murine norovirus-1 to undetectable levels in oysters [308]. HAV can also be effectively inactivated from oyster tissues using HHP. Calci et al. (2005) could achieve a PFU reduction >3 $\log_{10}$ with a one-minute treatment at 400 megapascals [306]. It is important to note that temperature, matrices' pH, and salinity have a great effect on the efficiency of pressure mediated HAV inactivation [307].

Another recent development in the field of molluscan shellfish safety is the application of super critical CO_2 ($scCO_2$), a known antibacterial substance widely used in the food industry to reduce the load of oyster-associated bacteria. Two conditions, 100 bar and 37 ℃ for 30 minutes and at 172 bar and 60 ℃ for 60 minutes, were reported as able to induce 2-log and 3-log reductions in the oysters' aerobic plate count respectively. No significant change in the physical appearance, smell, or texture was recorded (Figure 5-10) [310].

As previously stated, *V. vulnificus* and *V. parahaemolyticus* are major seafood-associated health concerns. In recent years, there has been a tremendous amount of effort to design approaches to reduce their load, especially in molluscan shellfish. One of the latest innovations in this field is the introduction of a "weak acidic electrolyzed water" based approach [311]. Quan et al. (2010) have demonstrated that weak acidic

electrolyzed water possesses outstanding antibacterial potency against Vv and Vp, especially when compared to sodium hypochlorite (NaClO), a commercial sanitizer [311].

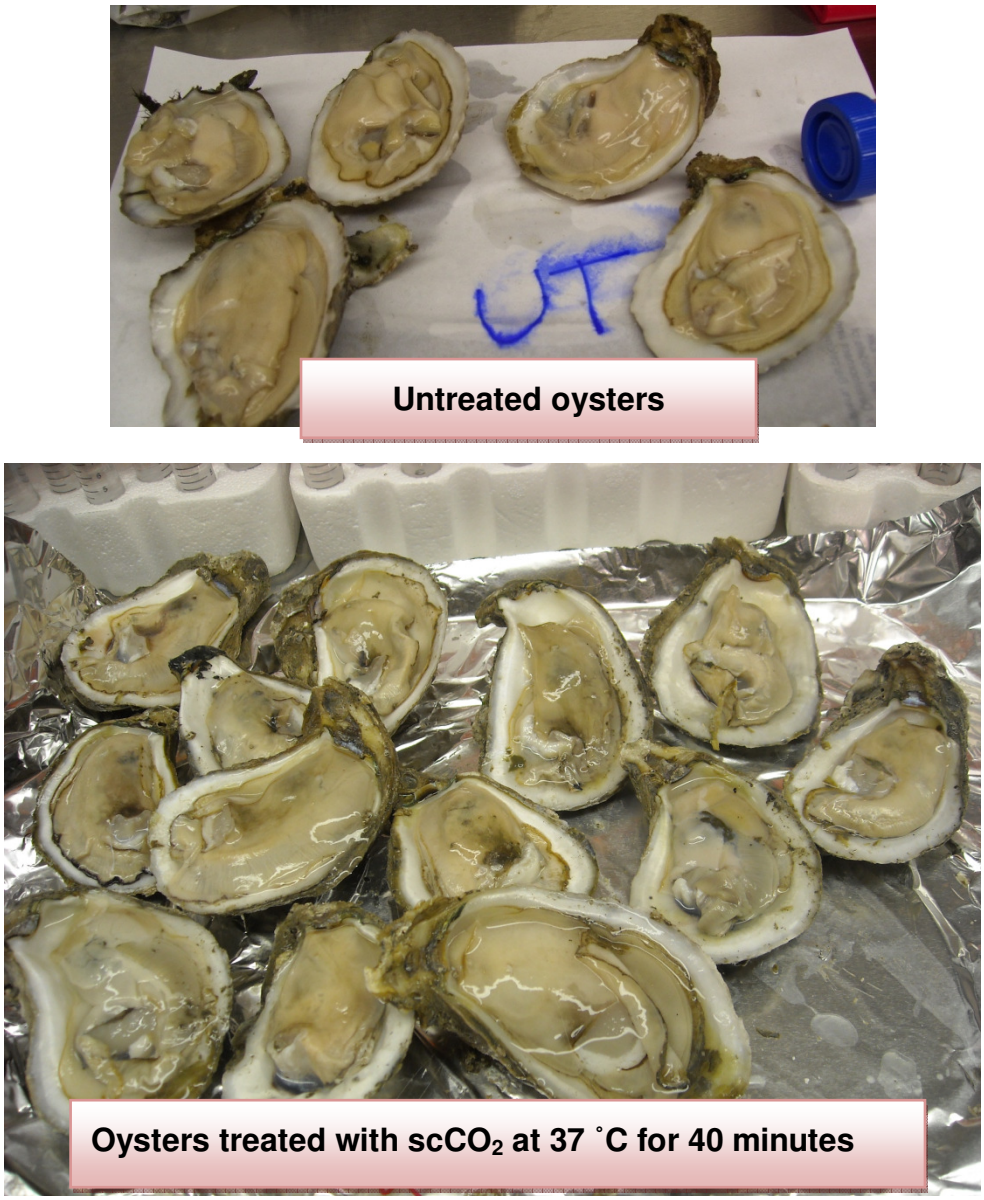


Figure 5-10. Appearance of oysters before and after a 40 minute exposure to $scCO_2$.

A chlorine dioxide (ClO_2) based approach was also recently introduced. According to Wang et al. (2010) a 6-hour treatment with 20 mg/L of ClO_2 is enough to disinfect oyster tissues contaminated with Vp. These authors particularly recommend this method because it is cost-effective; it also has the potential of increasing seafood's shelf life (~12 days) [312]. In addition, *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes*, other pathogens of importance regarding seafood safety, have proven sensitive to this type of treatment.

Scombrotoxin. Because of the large number of associated outbreaks, scombrotoxin can be viewed as the most dangerous seafood-associated health hazard [218, 219]. To store fish at low temperatures is generally considered sufficient to prevent the growth of causative bacteria. Unfortunately, such conditions cannot always be respected, especially during retail processes. Phuvasate and Su (2010) proposed an alternative to low temperature storage applicable under these circumstances, namely the use of Electrolyzed Oxidizing (EO) water and ice. According to these authors, the load of several histamine-producing bacteria on food surfaces and fish skin can be significantly reduced simply by using electrolyzed oxidizing water and ice. Conditions reported as effective using salmon and tuna's skin were, for EO water, 100 ppm chlorine for 120 minutes, and for EO ice, 100 ppm chlorine for 24 h [313]. Experts now agree, electrolyzed oxidizing water possesses a great potential as far as seafood safety. Its use is recommended not only because it is environmentally friendly, safe, and cost effective but also because its application is quite straightforward. Its antibacterial effects against several other seafood-associated bacteria have been reported. A few examples

are *E. coli* O157:H7, *L. monocytogenes,* as well as *Salmonella enteritidis*, *Campylobacter jejuni*, *Enterobacter aerogenes*, and *S. aureus* [314].

Bio-preservation of seafood. To add various chemicals to seafood in order to reduce its bacterial load or inhibit the growth of unwanted bacteria is an option that many have proposed as a solution to some seafood safety issues. Regrettably, consumer acceptance of these products is not always guaranteed. As chemical/preservative-free, ready-to-eat seafood products are gaining in popularity alternatives to the use of chemicals have emerged, and an example is bio-preservation. Recently there have been a few innovations in this field. An example in this case is the proposed use of *Carnobacterium divergens* M35 and divergicin M35 in an effort to rid seafood from one of its most persistent bacterial contaminants, namely *L. monocytogenes* [315]. Matamoros et al. (2009) characterized several strains of lactic acid bacteria that can be used to this end as well. These bacteria (*Lactobacillus fuchuensis, Leuconostoc gelidum, Lactococcus piscium,* and *Carnobacterium alterfunditum*) showed inhibitory potential against seafood spoiling and pathogenic bacteria [316]. Pinto et al (2009) also reported two bacteriocins produced by lactic acid bacteria *(Enterococcus faecium* and *Pediococcus pentosaceus)* that can be used to this end [317].

Irradiation and other recent breakthroughs. Several other cutting edge technologies designed to help deal with seafood-associated health issues have been introduced in recent years. A few examples are X-ray, gamma ray, and electron beam irradiation based technologies. Gamma irradiation (0.5, 1, 2, and 5 kGy) and electron beam irradiation have recently proven an effective non thermal approach to reduce the

load of *V. parahaemolyticus* as well as several other seafood-associated contaminants, namely *L. monocytogenes* and *S. aureus*, in a raw seafood dish (oyster *Jeotkal*). Organoleptic properties were not negatively affected by the irradiation. In this particular case, gamma irradiation appears a better alternative to electron beam irradiation [318].

The beneficial antibacterial effects of electron-beam irradiation applied to another seafood dish (salted and seasoned short-necked clam) were reported in 2009 by Kim et al. It is important to note that, in this case, no change in sensory qualities was observed. A significant microbial inactivation (coliform bacteria, aerobic bacteria, yeast and mold) was reported [319]. Similar results were achieved with cold-smoked salmon [320]. This technique appears to be a better alternative to HHP in regards to sanitization of cold-smoked salmon. In fact, while both approaches (Irradiation at 2 kGy and HPP: 450 MPa for 5 min) yielded a safer final product, the visual aspect of HHP-treated, not Irradiation-treated, salmon was negatively affected. The microbial load of both products did not exceed 6 $\log_{10}$ cfu/g after 35 days storage at 5 °C [320].

X-ray irradiation has also been investigated for use in improving seafood microbiological quality. Several recent publications have demonstrated its beneficial effects on *V. parahaemolyticus*, *V. vulnificus* (shrimp contaminants). It is important to note that several other shrimp-associated bacterial contaminants, namely *E. coli*, *Salmonella enterica*, and *Shigella flexneri*, were inactivated as well [321, 322].

High Hydrostatic Pressure (HHP) was also reported as an excellent alternative to reduce the level of *Listeria* in fish. According to Gudbjornsdottir et al. (2010), a 700–900MPa treatment of 10 s is sufficient to reduce the load of *L. innocua* to undetectable

levels. This experiment was completed with cold-smoked salmon. In this case, though there was no lipid oxidation, some variations in color and microstructure of the final product were noted [323]. The potential of HHP to rid mackerel from parasites, such as the nematode *Anisakis simplex*, was also recently reported. In this particular case, a complete inactivation of the larvae in the fish tissue was achieved at 300 MPa after a five-minute exposure [324].

Another recent report has proposed using CO_2 in packaging fish. Schirmer et al. (2009) proposed using CO_2 combined with various organic acids: citric acid (3% w/w, pH 5) and acetic acid (1% w/w, pH 5) in packaging fresh fish, as an effective way to improve its quality and shelf life. This combination has proven efficient at completely inhibiting bacterial growth in naturally contaminated salmon stored at 4 °C for 14 days. Monitored bacteria were *Enterobacteriaceae*, lactic acid bacteria, and sulphur reducing bacteria. Sensory analysis was not completed by these authors [325].

A better alternative to rid shrimp from *V. parahaemolyticus* was recently introduced. The use of chlorine is the current approach to reduce the level of shrimp-associated pathogenic bacteria. Unfortunately, health issues such as bronchitis and pulmonary edema in workers have been reported. Norhana et al. (2009) proposed an even simpler approach to deal with shrimp-associated bacterial pathogens. These authors show that washing shrimp with acidic fruit juice, namely bilimbi (*Averrhoa bilimbi*) or tamarind (*Tamarindus indica* L.) was also an effective way to significantly reduce its load of bacteria [326]. In 2007, Chaiyakosa et al. reported another safer alternative to reducing the load of *V. parahaemolyticus* in shrimp, namely the use of Chitosan [327]. Another author reported this same substance as an effective means of

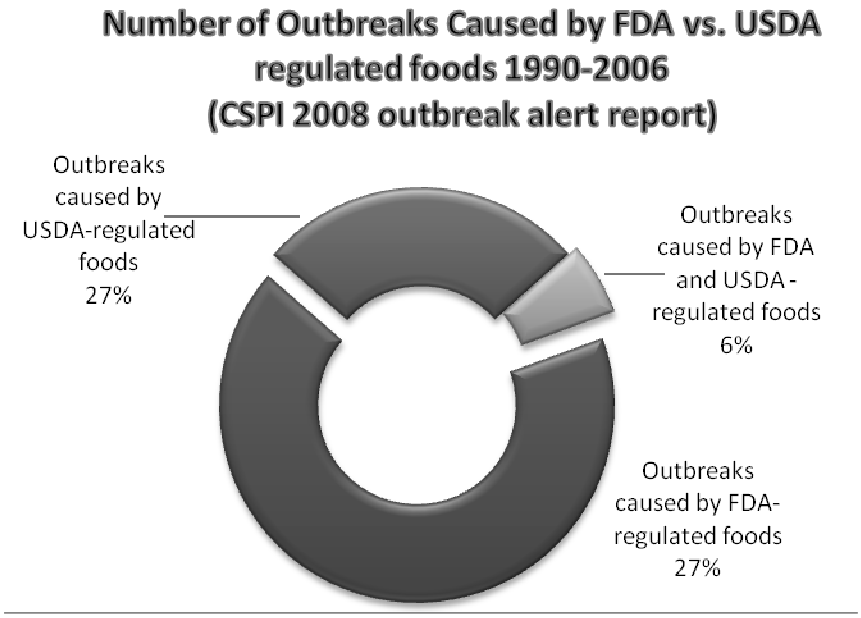
bringing safer salmon to consumers. Packing cold-smoked salmon in chitosan-coated plastic films containing 4.5 mg/cm^2 sodium lactate or either a combination of 4.5 mg/cm^2 sodium lactate plus 0.6 mg/cm^2 potassium sorbate or 2.3 mg/cm^2 sodium lactate plus 500 IU/cm^2 nisin was found to be beneficial. It was established that for seafood conditioned using this approach and stored at low temperature (~ 4 ºC), *L. monocytogenes'* growth was inhibited for at least six weeks [328].

6. Future directions and conclusions

From pathogenic bacteria, parasites, and viruses of all sorts, to life threatening biotoxins, the range of chemical and biological contaminants present in seafood is broad in scope and challenging to manage. Despite years of efforts from regulatory authorities and the scientific community, the public at large is still at risk of dangers associated with seafood consumption. For several years now, seafood has ranked foremost as the most significant source of food-borne disease outbreaks of known origin. Contributing factors are numerous and represent key points upon which urgent action, from regulatory authorities and the scientific community is required. Special attention should be paid to deleterious agents that have been associated with the largest number of outbreaks in the past years. In the U.S. for instance, major threats were scombrotoxin, responsible for 36% of all reported seafood-borne outbreaks from 1990 to 2006, followed by ciguatera (responsible for 21% of outbreaks), bacteria (especially *Vibrio* spp) responsible for 24% and finally noroviruses, which cause about 10% of all outbreaks reported during this same period [218]. It is important to note that these same agents pose serious problems in other parts of the world, as well. Scombroid fish poisoning, for instance, is also a serious problem in countries like Japan

and the U.K. [242]. *Vibrio* spp, especially *V. parahaemolyticus,* have been reported as a major problem in several Asian countries [254]. A recent study, a 2005 – 2008 survey of shellfish (mussels, clams and oysters), showed that norovirus is a problem in Italy, as well [329].

The task of ensuring consumers protection against the dangers of seafood is shared by several groups. These include (1) regulatory authorities, which depend heavily on the scientific community, and are in charge of the promulgation and enforcement of laws and regulations, (2) establishments involved in the harvest, processing, storage, and retail of seafood, and (3) consumers themselves. Failures and flaws at various levels of the current safety management system explain why seafood has been a persistent issue for the past few years. A certain number of defects in the seafood regulatory system of the U.S. for instance are presented in a 2008 report of the Center for Science in the Public Interest (CSPI). Mentioned shortcomings are the "voluntary recall" approach currently in place, added to financial issues. CSPI sees in the limited budget allocated to the FDA (Figure 5-11), a serious hindrance to its efficiency. This can be a serious hindrance, for instance, when it comes to law enforcement. CSPI reports an extremely low inspection rate of food processing companies by the FDA, a rate that is insignificant compared to the USDA's [218]. Currently, in the U.S., there are approximately 13,400 seafood-processing establishments. The FDA reports having inspected only 3,066, 2,830, and 2,456 during the fiscal years 2004, 2005 and 2006 respectively.



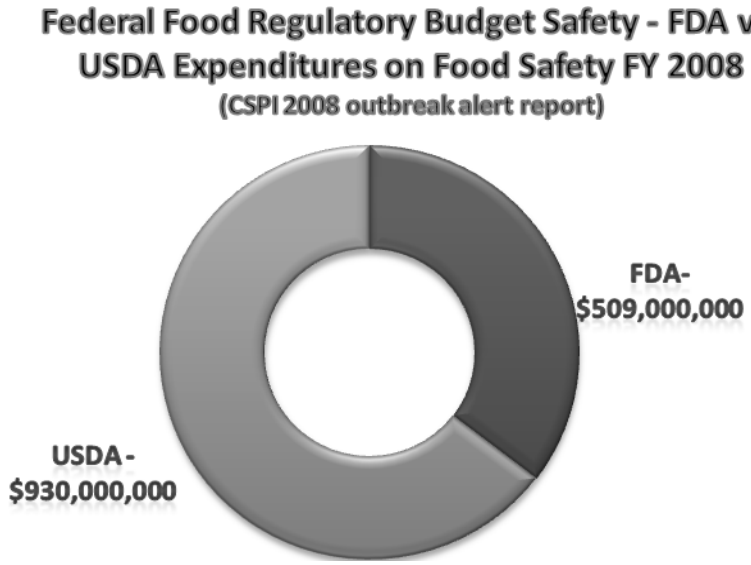


Figure 5-11. Graphical representation of some limitations in the current federal food safety regulatory system with signficant impact on seafood safety

Financial limitations are not the only obstacle to full efficiency of regulatory agencies. The unavailability of effective technological tools that could either help rid seafood from hazardous entities/substances, or assist regulatory authorities in effective risk assessment and management during various control programs is also critical. Without effectual detection methods, efficient law enforcement is nearly impossible. Though numerous approaches applicable to virtually all seafood-associated health hazards have been proposed so far, several gaps remain.

This is the case of biotoxins for instance, which alone were responsible for 63% of all reported seafood-borne diseases from 1990 to 2006. Because they are extremely resistant to various post-harvest processing techniques, as far as assuring seafood safety, preventive measures are a better option. More effective detection tools are thus critically needed in order to reduce the current incidence of biotoxin-linked seafood-borne illnesses. Presently, in this field, there is still a need for cost-effective, rapid, sensitive, specific, and straightforward methods that can be operated by untrained personnel on a routine basis.

Though several approaches to toxin detection in seafood have been proposed so far, and even significant progress made recently (Table 5-7)**,** animal-based assays and liquid chromatography, techniques that have their share of shortcomings, are still the official methods (Table 5-4)

Animal bioassays for instance, are fit to assess the overall toxicity of a sample but cannot give insight on the nature of the toxin(s) involved. Moreover, these assays are time consuming, of limited accuracy and controversial (ethics). As stated previously, liquid chromatography, on the other hand, when coupled with effectual detection methods, is valuable at accurately identifying the nature and concentration of the incriminated toxin(s). However, it is important to note that, chromatography-based methods are lengthy and not cost-effective; heavy equipments and trained specialists are needed. Better methods have been proposed, but, to our knowledge, none has been approved by regulatory authorities for routine use. Though these new methods present some clear advantages, it is important to point out that they also possess some weaknesses. Among these are difficulty of application on a routine basis, the necessity for heavy equipment, and the complexity of protocols and generated data, just to name a few.

There are flaws in current approaches to control scombroid fish poisoning and ciguatera. In the case of scombroid fish poisoning for instance, there is still a need for more effective alternatives to control Histamine-Producing Bacteria (HPB). Current recommendations of the FDA involve rapid cooling (≤ 40 ˚F) after visceration (for larger fish) upon death. Unfortunately, this approach possesses a few shortcomings. It is important to note that not all HPB are mesophiles. Several studies showed that histamine can be produced even at low temperature by physchrophilic HPB [242, 330]. Ciguatera was second to scombrotoxin as the most likely cause of seafood-borne disease outbreak from 1990 to 2006. Though ciguatera is such an issue in the U.S., “there are neither standards, nor an official method” that applies to Ciguatera Fish

Poisoning (CFP) in this country [238]. Innovations, regulation-wise, in this regard are obviously critical. Current efforts with respect to CFP prevention involve various toxin-monitoring programs, education, alongside with bans on the sale and capture of fish most likely to cause poisoning (Europe, Australia).

Bacteria were next to toxins as the most prevalent cause of seafood-borne illnesses during the past decade. Fortunately, in this case, there are currently several approved Post-Harvest-Processing (PHP) approaches aimed at reducing their load in seafood. These included IQF (Individually Quick Frozen), HCP (Heat Cold Pasteurization), and HHP (High Hydrostatic Pressure). Though these techniques have revolutionized the industry of oysters, for instance, there is still room for improvement, especially because oysters do not survive such processing [331]. In terms of shelf life, this can be an issue. To store, HHP-, IQF- and HCP-treated oysters at low temperatures is, unfortunately, not enough to solve the issue. Prapaiwong et al. (2009), studying variations in the bacterial load (total aerobic bacterial counts) of HHP-treated oysters stored at 4 °C for instance, determined that the bacterial count of processed products can rise quickly during storage and can even reach ~ 10^7 CFU/g in just 7 days [332]. Moreover, long-term storage presents the disadvantage of increasing the final production cost. Post-harvest-processing techniques non lethal to oysters would be, without the shadow of a doubt, a better alternative. Mahmoud and Burrage (2009c) reported X-ray irradiation as a better approach for reducing the load of oysters-associated *V. parahaemolyticus* because oysters are able to survive even extremely high X-ray doses [333]. Oysters have also been shown to be able to survive $scCO_2$ exposure, making it an attractive tool for further exploration [310].

Norovirus is another major seafood-associated health hazard. Major gaps in the current system are regulatory and scientific. Despite the clear threat posed by this virus, not much effort has apparently being exerted regulation-wise. As mentioned by Terio et al. (2010), “there is no virological standard for bivalve shellfish in European legislation” [329]. Though several approaches to the detection of norovirus in seafood have been proposed so far, much still needs to be done; an area in need of improvement is the development of more efficient methods of viral RNA extraction. RNA extraction is a critical step in several virus detection protocols. RNeasy Kit was recently presented by Husman et al. (2009) as a most useful alternative for viral RNA extraction after comparing five such approaches side by side. A modified paramagnetic silica-based guanidium extraction based technique was also developed recently [334, 335].

Other challenges faced in norovirus detection are related to the virus’ genetic variability and scarcity in samples. Interferences of seafood matrix, mainly, the presence of inhibitory substances, also represents a serious obstruction [335]. An effective TaqMan RT-PCR based approach for quantification of genogroups I and II norovirus, which presents the advantage of overcoming background inhibitory effects, was introduced recently by Gentry et al. (2009) [336]. An effective and more sensitive multiplex RT–PCR-based approach to norovirus and rotavirus detection in oysters was also introduced recently [337].

There is no doubt that the development of effective technologies able to surmount each and every one of these challenges will aid efforts to reduce outbreak incidents of seafood-borne infections attributable to viruses.

Chapter 6 : REDUCING OYSTER-ASSOCIATED BACTERIA LEVELS USING SUPERCRITICAL FLUID CO_2 AS AN AGENT OF WARM PASTEURIZATION

Running Title: SUPERCRITICAL CO_2 EFFECTS ON OYSTER'S BACTERIAL CONTAMINANTS

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Authors: Meujo DA, Kevin DA, Peng J, Bowling JJ, Liu J, Hamann MT

ABSTRACT - An innovative approach to Post-Harvest Processing (PHP) of oysters is introduced focusing on the effects of supercritical carbon dioxide ($scCO_2$) on bacterial contaminants trapped in the digestive system of oysters. Oysters were exposed to $scCO_2$ under two conditions: (1) 100 bar and 37 °C for 30 min and (2) 172 bar and 60 °C for 60 min. Using FDA standard guidelines for food analysis, variations in the Aerobic Plate Count (APC) were assessed. It was established that exposing oysters to CO_2 at 100 bar and 37 °C for 30 min and at 172 bar and 60 °C for 60 min induced 2-log and 3-log reductions in the APC respectively. The decrease in the microbial load as a result of treatment with $scCO_2$ was found to be significant (P=0.002). A release of adductor muscles from the shell was noted in oysters treated at 172 bar and 60 °C for 60 min; this was not the case for oysters treated at 100 bar and 37 °C for 30 min. A blind study

allowing sensory analysis of treated vs. untreated oysters was also completed and no significant change in the physical appearance, smell, or texture was recorded. In this paper, we also report the effect of $scCO_2$ on several bacterial isolates, including a referenced ATCC strain of a non-pathogenic *Vibrio* (*Vibrio fischeri*) as well as several other bacterial isolates cultured from oyster' tissues and found to share biochemical features common to pathogenic Vibrio strains. A complete inactivation (minimum 7-log reduction) was achieved with these latter bacterial isolates. A 6-log reduction was observed with *V. fischeri*.

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1. Introduction

As a direct consequence of filter-feeding in often heavily contaminated water, oysters can be loaded with pathogenic microorganisms. These microorganisms are absorbed concomitantly with nutrients and accumulate in the gastrointestinal (GI) system of oysters. Hepatitis viruses (A and E), *Salmonella*, *Shigella* spp., *Escherichia coli*, *Vibrio* spp., *Clostridium perfringens*, *C. botulinum* and *Yersinia enterocolitica* are some of the microbes that have been reported as part of this pathogenic microflora [265, 338, 339]. Since oysters are often consumed raw, these pathogens are a serious concern. These microorganisms have been associated with a wide range of mild to severe foodborne illnesses. Of pathogens that contaminate the GI system of oysters, *Vibrio* spp (especially *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv)) are of greater concern [338, 340, 341]. Among immunocompromised patients, especially those with chronic liver disease, *V. vulnificus* strains have been strongly associated with severe

and life-threatening conditions. *V. vulnificus* is the second leading cause of seafood-related fatality in the US [257, 259].

Pathogenic microorganisms are not the only concern associated with oyster consumption. Other risks include toxic metals, biotoxins and chemical environmental contaminants. These also accumulate in the GI system and tissues of oysters and have been associated with food poisoning outbreaks [235].

Until recently, all attempts to reduce the incidence of oyster-related foodborne illnesses were centered on consumer education. Other efforts to minimize consumer exposure to oyster-associated pathogens included natural depuration and harvesting restrictions. Despite these efforts, in 2002, Potasman et al. reported a worrisome increase in the number of outbreaks of bivalve-associated infections primarily from oysters, from 3 reports in the 1970s to 22 during the 1990s. The large majority of these outbreaks were due to raw oyster consumption [265].

The emergence of innovative FDA approved Post-Harvest Processing (PHP) technologies such as Individual Quick Freezing (IQF), introduced in 1989, Heat–Cool Pasteurization (HCP), introduced in 1995, and High Hydrostatic Pressure (HHP), introduced in 1999, have revolutionized the industry of seafood, particularly in regard to oysters. These technologies, which are currently commercially available, have made it possible to bring safer raw products to consumers. An end product of high quality (fresh taste and superior appearance) is the major advantage of Individual Quick Freezing (IQF), Heat–Cool Pasteurization (HCP) and High Hydrostatic Pressure (HHP) processing. These processing techniques also reduce the level of *V. vulnificus* to non-

detectable levels (i.e., < MPN/g). The level of spoilage bacteria is significantly reduced aswell. Unfortunately, oysters cannot survive any of the above PHP technologies [331].

In terms of shelf life, this can be an issue and thus it is mandatory to store HHP-, IQF- and HCP-treated oysters at low temperature; long term storage is expensive. Several other methods are under development including approaches using X-ray and Gamma radiation as cold pasteurizing agents.

In the present paper, we introduce an original and promising approach to post-harvest processing of oysters: warm pasteurization of oysters using $scCO_2$ (CO_2 at a temperature and pressure above its critical point). The antimicrobial potential of dense CO_2 was first reported in 1951 by Fraser while working with *E. coli* [342]. Extensive work has since been completed and currently, the bactericidal potential of $scCO_2$ is well-established over a wide range of pathogenic microorganisms. $ScCO_2$ has been documented to effectively inactivate viruses (HIV-1, Sindbis virus, polio Sabin type I virus, and *pseudorabies* virus [PRV]), yeast (*Saccharomyces cerevisiae*), as well as vegetative and sporal forms of various bacteria (*Salmonella*, *S. aureus*, *listeria*, *E. coli*, *Pseudomonas fluorescens*, etc.) [343-346]. So far, however, there has been no report on the effect of $scCO_2$ on *Vibrio* spp.

There are also several reports of studies related to the kinetics of inactivation of bacteria in food products (e.g., milk, fruit juice, herbs, ground beef, etc.) using $scCO_2$ [345-347]. However there is no reported attempt to assess whether treating oysters with $scCO_2$ could have a positive impact on the wide range of pathogenic bacteria which populate their GI system.

The present studies address several key questions in regards to oyster pasteurization by $scCO_2$. First, the effects of $scCO_2$ was evaluated in vitro on bacterial cultures of a non-pathogenic strain of *Vibrio* used here as a model for *Vibrio* spp (*Vibrio fischeri* ATCC® 7744™) and several bacterial isolates from an oyster homogenate. Second, the effect of $scCO_2$ on the bacterial community trapped in the oyster GI system was studied. Finally, using a panel of thirteen participants, a sensory analysis of fresh untreated oysters versus $scCO_2$-treated oysters was completed in a blind survey.

2. Material and Methods

2.1. Bacterial strains

Referenced bacterial strains used to assess the effect of $scCO_2$ on bacteria in vitro were *V. fischeri* (ATCC® 7744™) and *E. coli* (Migula) Castellani and Chalmers (ATCC® 25922™). *V. fischeri* was grown and maintained on either Luria Bertani Salt (LBS) medium (1% [wt./vol.] bacto tryptone [DB], 0.5% [wt./vol.] bacto yeast extract [BD, Franklin Lakes, NJ], 2% [wt./vol.] NaCl, 0.3% [vol./vol.] glycerol, 50 mM Tris–HCl [pH 7.5]) [348], or BOSS medium (3% [wt./vol.] NaCl [Fisher Scientific], 0.1% [wt./vol.] Glycerol [Sigma Aldrich], 1% [wt./vol.] bacto peptone [DB], 0.3% [wt./vol.] beef extract, [pH 7.3]; [349]. *E. coli* was grown and maintained in Bacto nutrient broth [Difco Laboratories, Detroit, MI], 23 g/L.

2.2. Oysters

Oysters were purchased from a seafood distributor in Memphis, TN, USA. These oysters were harvested on 04/13/09, properly maintained and delivered to our facility on

4/20/09. After arrival, the oysters were stored whole on ice overnight and our experiments were carried out the following day. Oysters were presumed alive unless the shell's adductor muscles were permanently released, in which case the oysters were discarded. Concerning morphology and size, the oysters were a mixture of “banana-shaped” and “good-quality shaped” oysters; “goodquality shaped” oysters constituted the majority. The morphology classification was performed according to procedures previously published by Lee et al., 2004 [350]. The length of the oysters varied from small (7.5 cm) to large (13 cm).

2.3. Supercritical unit and treatment of oysters using supercritical CO_2

The SFT-150 SFE system (Supercritical Fluid Technologies, Inc.) (Figure 6-1) was used for all $scCO_2$ treatments. The internal surfaces of the SFT-150's treatment chamber were sterilized with a 70% ethanol solution and allowed to dry at room temperature. The samples prepared for treatment were placed into the chamber which was then hermetically sealed. The liquid carbon dioxide source was opened filling the chamber and venting for 5 s to purge the chamber of any air. An external air source was utilized to supply the SCT-150's pneumatic compressor which increases the chamber's internal pressure. An external air pressure gauge was utilized to control the pneumatic compressor. The SCT-150's internal pressure was maintained and monitored via the fine-control valves and internal pressure displays located on the SCT-150 unit. Oysters were sampled in groups of 10–13 and placed in porous bags (Filter Specialists, Inc., [FSI] Michigan City, IN) which were placed into a 4 L capacity chamber. The oysters were exposed to $scCO_2$ under two different conditions: 100 bar for 30 min at 37 °C and 172 bar for 60 min at 60 °C. After treatment, the chamber was carefully vented, the

oysters removed, and their microbial load (APC) was assessed alongside untreated oysters.

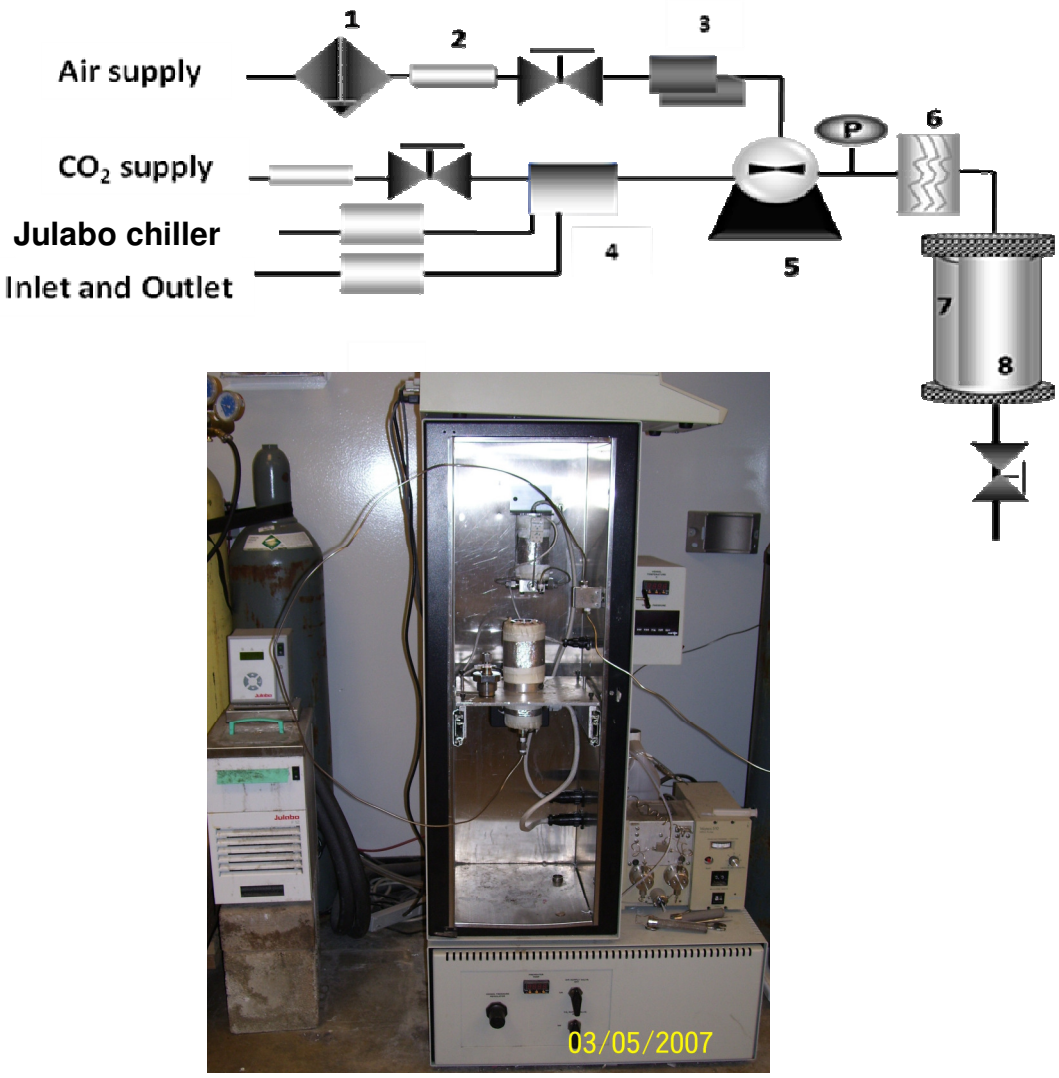

Figure 6-1. Standard bench-top SFT-150 (bottom) and schematic of supercritical sterilization apparatus (top).

(1) Filter. (2) Bulkhead. (3) Relieving Regulator. (4) CO_2 Pre-chiller. (5) Liquid CO_2 pump. (6) Preheater. (7) Vessel. (8) Sterilization unit.

2.4. Microbiological analysis of treated vs. untreated oyster samples

For microbiological analysis, each group of oysters was divided into three sub-samples which were analyzed independently. Each oyster's surface was carefully

cleaned with 70% ethanol solution and dried under a bacteriological hood using a paper towel. Oysters were opened aseptically in order to separate the flesh and interstitial fluid from the shell. The flesh and interstitial fluids were macerated in a sterilized blender for about a minute in an equal volume of Butterfield's phosphate-buffer to yield an oyster homogenate (dilution 1:2). A dilution of 1:10 was prepared by transferring 20 g of this homogenate into 80 mL of Butterfield's phosphate-buffered dilution water. Subsequent 10-fold dilutions were prepared. One mL of each dilution was pipetted into separate, duplicate, appropriately marked Petri dishes. About 12–15 mL plate count agar (cooled to about 45 ± 1 °C) was added to each plate and the agar was allowed to solidify. The plates were incubated promptly for 48 ± 2 h at 35 °C.

As a positive control a group of 12 oysters was split into three subgroups, placed in self-sealing sterilization pouches, and autoclaved (30 min at 121 °C and 18–20 psi). At the end of the sterilization process using the same procedure described above, the APC was assessed and confirmed to be zero.

2.5. Statistical analysis

All oysters' experiments were replicated twice and assessments of APC counts were run in triplicate for each replicate ($n = 2 \times 3$). Mean values ± standard deviations were used to plot the corresponding graph.

The test of the hypothesis that the bacterial load in treated vs. untreated oysters was significantly different was done by application of the Mann–Whitney U test to groups of log means of each set of two within sample replicates. The difference

between the set of treatment group log means and the set of untreated group log means was found to be significant when $P < 0.05$.

2.6. Biochemical characteristics of bacteria isolated from oyster tissue

Several bacterial colonies were isolated from the oysters' tissues (treated oysters); Gram staining, catalase tests, oxidase tests, mobility tests and fermentation profiles were completed for each isolate. The catalase reaction was completed with 3% H_2O_2. The ability of isolates to ferment specific carbohydrates was determined using phenol red broth (enzymatic digest of casein [1%], supplement sugar [0.5%–1%], sodium chloride [0.5%], phenol red [0.0018%], final pH: 7.4 ± 0.2 at 25 ℃) to which specific carbohydrates (glucose, sucrose, lactose or mannitol) were added. Oxidase reagent droppers [0.5 mL of a 1% aqueous solution of N, N, N′, N′-tetramethyl-*p* phenylenediamine dihydrochloride] were used for the oxidase test. Some isolates were also cultured on the *Vibrio* selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar (yeast extract [0.5%], proteose peptone No. 3 [0.1%], sodium citrate [1%], sodium thiosulfate [1%], oxgall [0.8%], saccharose [2%], sodium chloride [1%], ferric ammoniumcitrate [0.1%], bromothymol blue [0.004%], thymol blue [0.004%], and agar [1.5%]) (Table 6-2).

2.7. Effects of $scCO_2$ on bacteria isolated of oysters tissues

Four bacteria isolates possessing basic biochemical characteristics common to pathogenic *Vibrio* sp (i.e., Gram negative, catalase positive, oxidase positive and growth on TCBS agar) were cultured overnight at 35 ± 2 ℃ in ISP2. Ten milliliters of each culture was pipetted into 50 mL Sterile Corning® centrifuge tubes. A hole was made in

the cap of these tubes to allow the flow of $scCO_2$ into the tube. Dry sterilized filtered paper was used to cover the hole. Two such culture tubes were prepared for each of the four isolates. These were placed together in the 100 mL vessel of the unit of the Supercritical Fluid Technologies, Inc., SFT-150 SFE system and treated with $scCO_2$ at 100 bar and 37 ℃ for 30 min. Bacterial counts were estimated by plate spread technique using ISP2 or plate count agar; serial dilutions were completed in each case and these plates were seeded with 100 µL of treated or untreated culture

2.8. Kinetics of inactivation of two ATCC referenced Gram negative bacteria *V. fisheri* and *E. coli* by $scCO_2$

For each experiment, 2 mL of a freshly grown culture of *E. coli* and *V. fischeri* contained in a 15 mL Sterile Corning® Centrifuge tube (Corning Incorporated, Big Flats, NY), was introduced in the 100 mL vessel of the unit; a hole was also generated in the plug-sealed cap of the centrifuge tube to allow the flow of $scCO_2$ into the tube. The experiment was completed under three different pressures (100, 150, and 200 bar) at 37 ℃ with the purpose of evaluating the effects of pressure variation on the sterilization potential of CO_2; exposure times were 5, 10, 15 and 20 min. The initial load was estimated by plate count (*E. coli*) or by using a calibration curve (*V. fischeri*). At the end of each experiment, the number of surviving bacterial cells was counted by plate spread techniques [351]. Briefly, a fourfold serial dilution of each test culture was made in a 96 well-plate format. A few wells were selected for the bacterial count process via spreading 25 µL of the content of the given wells or dilution on the surface of a plate containing BOSS or nutrient agar; the bacterial count per well was repeated twice and

the average value was considered as the final count. The experiment was repeated to confirm the previously observed trend (n = 4).

2.9. Sensory assessment

The sensory analysis of oysters treated by $scCO_2$ was assessed by a panel of 13 people. The features evaluated included physical appearance, smell, and texture of oysters; the oysters were not tasted. Because they were not trained panelists, these panelists were presented with standards (Figure 6-4) that would assist them in the grading process. There were two such standards; the first was labeled as “fresh” and the second as “spoiled.” The first standard was a freshly shucked, untreated oyster while the second was an oyster that was left at room temperature for about two days (enough to allow it to develop the mild odor associated with spoiled oysters). Each panelist was asked to comparatively examine the physical appearance, smell, and texture of these standards after what they were led to the oyster stations where five groups of oysters were aligned side by side. One of these groups included untreated oysters while the rest included oysters treated under a variety of conditions ([20 min, 100 bar, 37 °C], [50 min, 100 bar, 37 °C], [50 min, 200 bar, 37 °C], and [20 min, 200 bar 37 °C]). These oysterswere labeled using simple codes: X1, X2, X3, X4 or X5 (blind study). The panelists were informed that one out of these five groups was made of standard/untreated oysters. The panelists were then asked to evaluate the physical appearance, smell, and texture of the oysters as closely resembling our standard for “fresh” or our standard for “spoiled”. A third option, “intermediate” was also offered. A score of two (2) was allocated for the adjective “fresh” (the most desirable). A score of zero (0) was allocated for the adjective “spoiled” (the least desirable). Finally, a score of

one (1) was allocated for “intermediate.” (Table 6-1) The overall “freshness score” for each given oyster was the sum of scores it received for its texture, physical appearance, and smell. This freshness score varied from 0 to 6. To be judged acceptable, an oyster must have obtained an overall score ≥ 3.

Data from all the 13 panelists were pooled for each group of oysters and an overall scorewas assigned per the group; this was the mean value of scores achieved by individual oyster of each particular group.

Table 6-1. Guide used in grading oysters (treated and untreated): blind study

Score	Physical appearance	Smell	Texture
2[a]	Fresh	Fresh	Fresh
1	Intermediate[b]	Intermediate[b]	Intermediate[b]
0[c]	Spoiled	Spoiled	Spoiled

[a] Extremely desirable.
[b] Not Fresh but definitely not spoiled.
[c] Extremely undesirable.

3. Results

3.1. Microbiological analysis of treated and untreated oyster samples

Oysters sampled in groups of 10–13 oysters were treated with $scCO_2$ under the following two conditions: 100 bar, 30 min, 37 ℃ and 60 min, 172 bar, 60 ℃. After exposure, the effect of $scCO_2$ on the aerobic microbial load (APC) that contaminates the GI system of these oysters was assessed using the FDA recommended approach to food analysis (n = 6). The microbial load (Aerobic Plate Count) present in the GI system

of untreated oyster samples was determined using this same approach and estimated to be 1.8×10^7 CFU/g of oyster meat (EAPC).

It was estimated that exposure of oysters to CO_2 (100 bar, 30 min, 37 °C) led to a significant decrease ($P = 0.002$) in the load of oysters' bacterial contaminants. This treatment induced a 97.6% reduction in the microbial load that contaminated their GI tract corresponding to a 2-log reduction. A similar result was observed in previous experiments with oysters exposed to $scCO_2$ at 100 bar, 40 min, and 37 °C (data not shown). The residual load of bacteria in these $scCO_2$-treated oysters was estimated to be about 4×10^5 CFU/g (EAPC) (Figure 6-2).

A greater bacteria inactivation level was observed with oysters exposed to 172 bar, 60 min and 60 °C. In this case the APC reduction was estimated to be 99.9%, which corresponds to a 3-log reduction. This translates into reducing the initial load of ~ 1.8×10^7 CFU/g (EAPC) of oyster meat to 3.3×10^4 CFU/g.

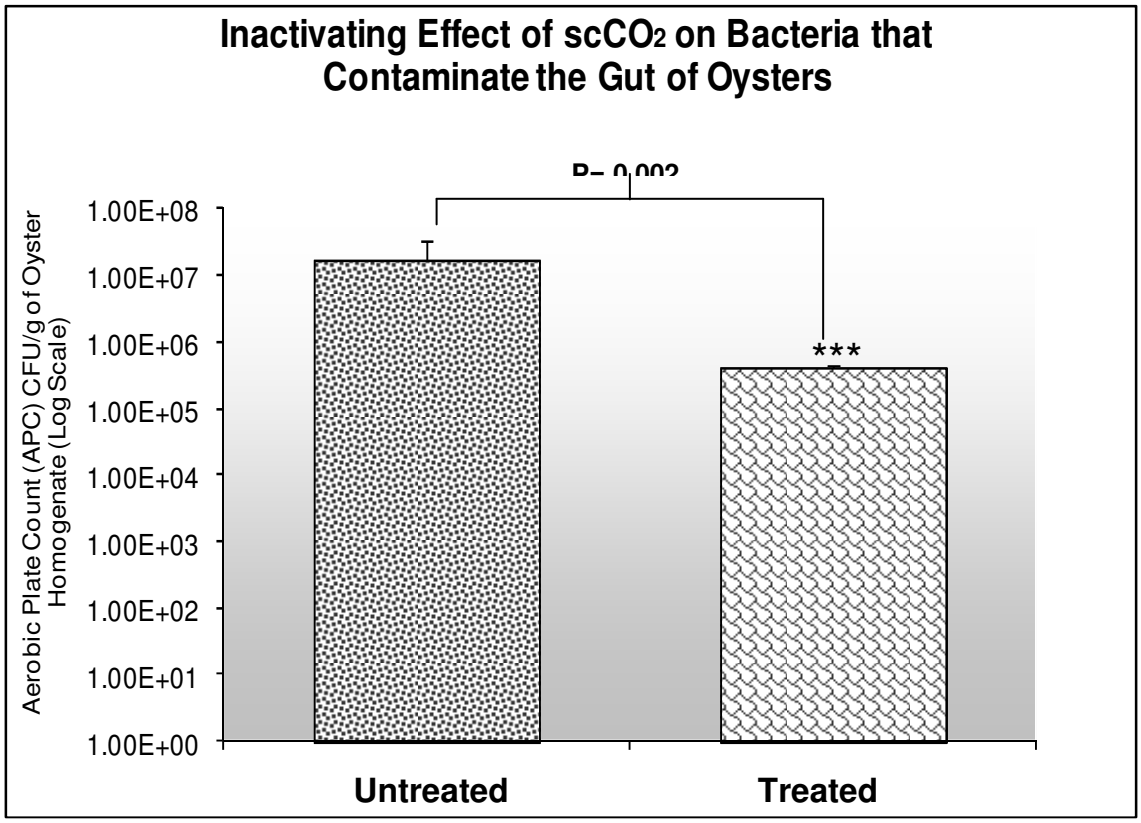

Figure 6-2. Inactivating effect of $scCO_2$ on bacterial contaminant of oyster.

Oysters were thoroughly cleaned and treated at 100 bars, 30 mins and 37 ˚C. The oysters were separate in 3 groups shucked in aseptic environment; the amount of APC was then estimated using FDA recommended approach to food analysis. This whole experiment was repeated twice for an "n = 6 (that is 2 * 3). Untreated Oysters were processed in a similar manner. The APC was estimated twice also for an n = 6 that is (2 * 3)

*** Statistically significant difference

3.2. Effects of $scCO_2$ on bacteria in vitro

As mentioned previously, *V. parahaemolyticus* and *V. vulnificus* are key players in quality control programs for raw oysters. We anticipate assessing the effect of $scCO_2$ on these microorganisms (in vitro and in vivo) in future studies. In the present document, we have evaluated the effect of $scCO_2$ on a non-pathogenic strain, namely *V. fischeri* which we used as a model for *Vibrio* sp. The kinetic of inactivation of V*. fischeri* by $scCO_2$ was studied under three different pressures conditions (100 bar, 150

bar, and 200 bar at 37 ℃); exposure times were 5, 10, 15 and 20 min. The effects of $scCO_2$ on oyster bacterial isolates possessing several key biochemical features common to pathogenic Vibrio spp (namely *V. parahaemolyticus* and *V. vulnificus*) was assessed as well.

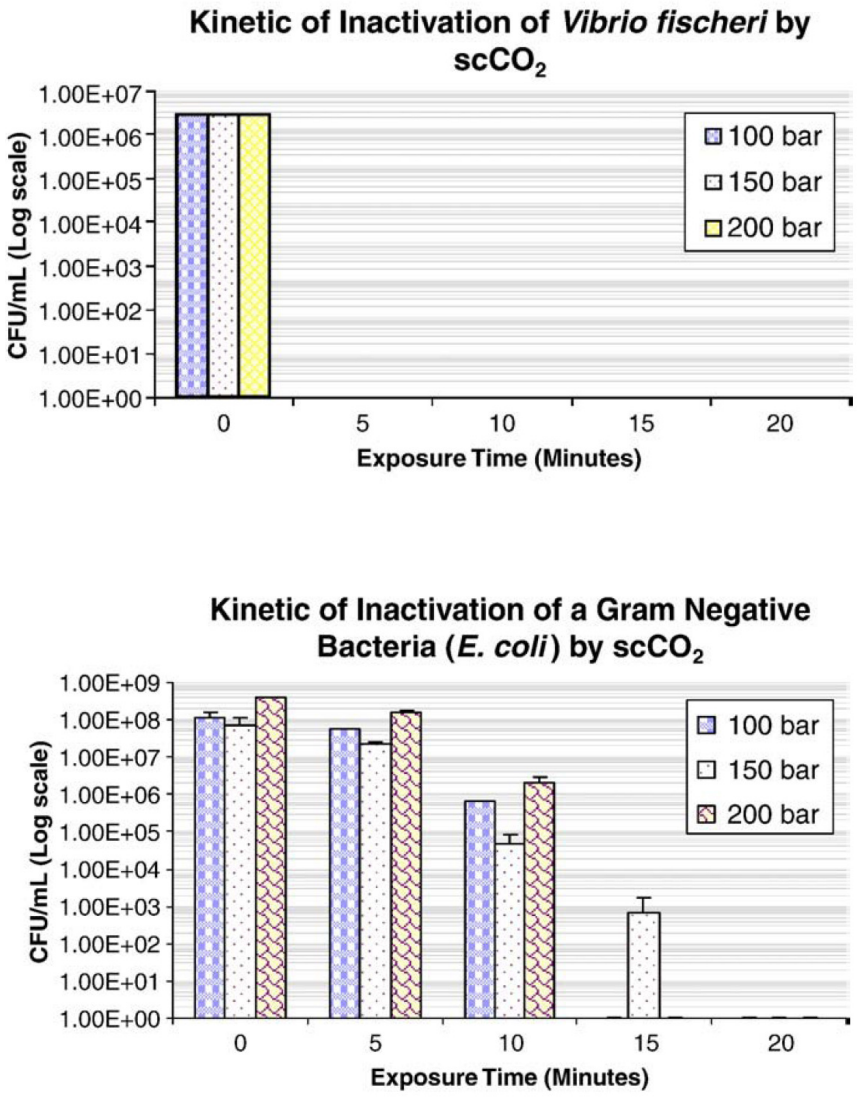

Figure 6-3. Comparative Effects of $scCO_2$ on *V. fisherii* and another Gram negative bacterium (*E. coli*) in vitro

The kinetic of inactivation of two Gram negative bacteria by $scCO_2$ was studied. Cultures of (A) *V. fisherii* (3×10^6 CFU/mL) and (B) *E. coli* (5×10^8 CFU/mL) were exposed to sc CO_2 at 100 bar, 150 bar and 200 bar for 5, 10, 15 and 20 minutes.

3.2.1. Effect of $scCO_2$ on bacterial isolates possessing some basic biochemical characteristics common to pathogenic *Vibrio* sp (in vitro).

Ten milliliters of overnight cultures of oysters' bacterial isolates (Table 6-2) was exposed to $scCO_2$ (100 bar, 37 °C) for 30 min. The initial microbial load was estimated to be higher than 2×10^7 CFU/mL of broth. A complete (100%) inactivation was obtained for all cultures. No growth was observed even in plates seeded with 100 µL of the undiluted sample (pipetted directly from the test tube) (Table 6-3).

This experiment was replicated and the number of survivals in each case was estimated in triplicate. This corresponds, at a minimum, to a 7-log reduction. Subsequent experiments helped to establish that a 30 minute-long exposure is not required to achieve this level of inactivation; similar results were achieved with cultures treated for only 10 min.

Table 6-2. A few biochemical features of bacterial isolates obtained from treated oyster tissues

Isolate code	Gram Type	Catalase reaction	Oxidase reaction	On TCBS			Mobility	
				Growth	Colonies	Colonies' Color conversion		
Isolate #13[*]	-	+	+	+	Green centered colonies	-	+	AF
Isolate #18[*]	-	+	+	+	Green	+[y]	+[*]	AF
Isolate #30[*]	-	+	+	+	Green	+[d]	+[*]	AF
Isolate #36[*]	-	+	+	+	Green	+[d]	+[*]	AF

[y]: conversion to yellowish color ; [d]: conversion to darker (blackish) colonies ; +: Positive ; -: Negative ; [*] did not ferment glucose, lactose or mannitol , +[*] poor growth, AF: anaerobie facultative

Table 6-3. Effect of $scCO_2$ on bacterial isolates possessing some basic biochemical characteristics common to pathogenic Vibrio sp (bacteria count 2E + 07 CFU/mL [a])

Bacteria	Condition of treatment	Level of inactivation
Isolate #13	100 bar, 37 °C, 30 min	Complete inactivation[b]
Isolate #18	100 bar, 37 °C, 30 min	Complete inactivation[b]
Isolate #30	100 bar, 37 °C, 30 min	Complete inactivation[b]
Isolate #36	100 bar, 37 °C, 30 min	Complete inactivation[b]

[a] Estimation according to ASTM Standard (Sutton, 2006).
[b] No growth from plating 100 µL of undiluted sample.

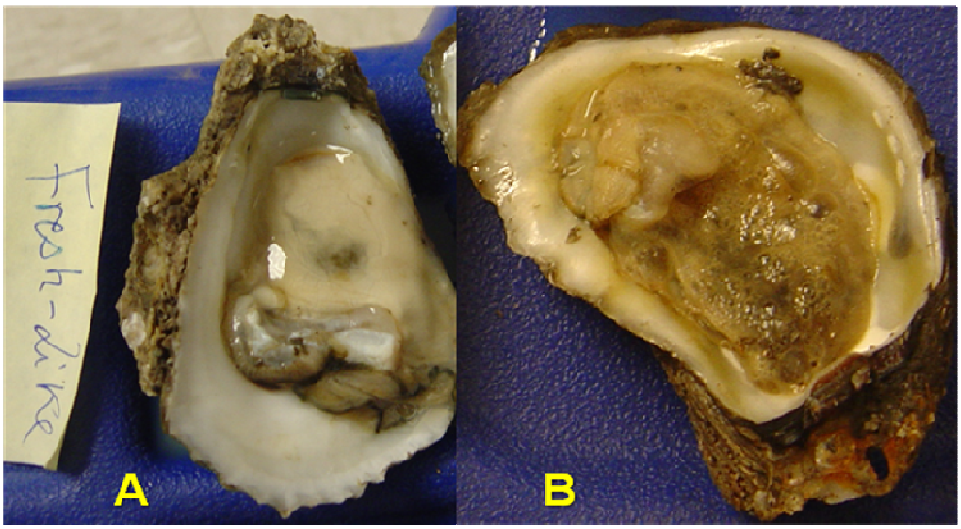

Figure 6-4. Standards used for sensory analysis of untreated vs. treated oysters

A sensory analysis of untreated vs. $scCO_2$ - treated oysters was completed. To grade the physical appearance, smell, and texture of oysters, a panel of 13 people used two standards; the first standard was that for "fresh" oysters and the second standard was that for "spoiled" oysters. (A) Standard for "fresh" (B) Standard for "spoiled"

3.2.2.. Comparative effects of $scCO_2$ on two ATCC referenced Gram-negative bacteria: *V. fisherii* and *E. coli* - kinetic of bacterial inactivation

V. fischeri was reduced to a non-detectable level (6-log reduction) after only 5 min of exposure to $scCO_2$; this occurred independently of pressure. *V. fischeri* appeared far more sensitive to the effect of $scCO_2$ when compared to *E. coli*, for instance. *E. coli* is considered one of the most sensitive bacteria to this treatment [352]. The complete

inactivation of *E. coli* (8-log reduction), under these same conditions, was achieved only after 20 min of exposure to $scCO_2$. Dillow et al. (1999) and also Smelt and Rijke (1992) reported similar results [353, 354]. Along with this decrease in bacterial viability came a decrease in the pH of the culture. This decrease in pH can suggest a possible mechanism for the bacterial inactivation.

3.3. Sensory assessment

Treated and untreated oysters were evaluated side by side by a panel of 13 volunteers in a blind study. A score was assigned to oysters individually and as a group. To be judged acceptable, an oyster or a group of oysters must have achieved an overall score ≥ 3. From the data collected, it was concluded that oysters remain acceptable, as far as their physical appearance, texture and smell, after exposure to CO_2 ([100 bar for 20 min at 37 °C], [100 bar for 50 min at 37 °C], [200 bar for 50 min at 37 °C] and [200 bar for 20 min at 37 °C]). The group of treated oysters that received the best rating were those treated at 100 bar for 20 min at 37 °C (score = 4.4). Ratings for the other groups were also ≥ 3. The group of untreated oysters achieved a score of 4.9.

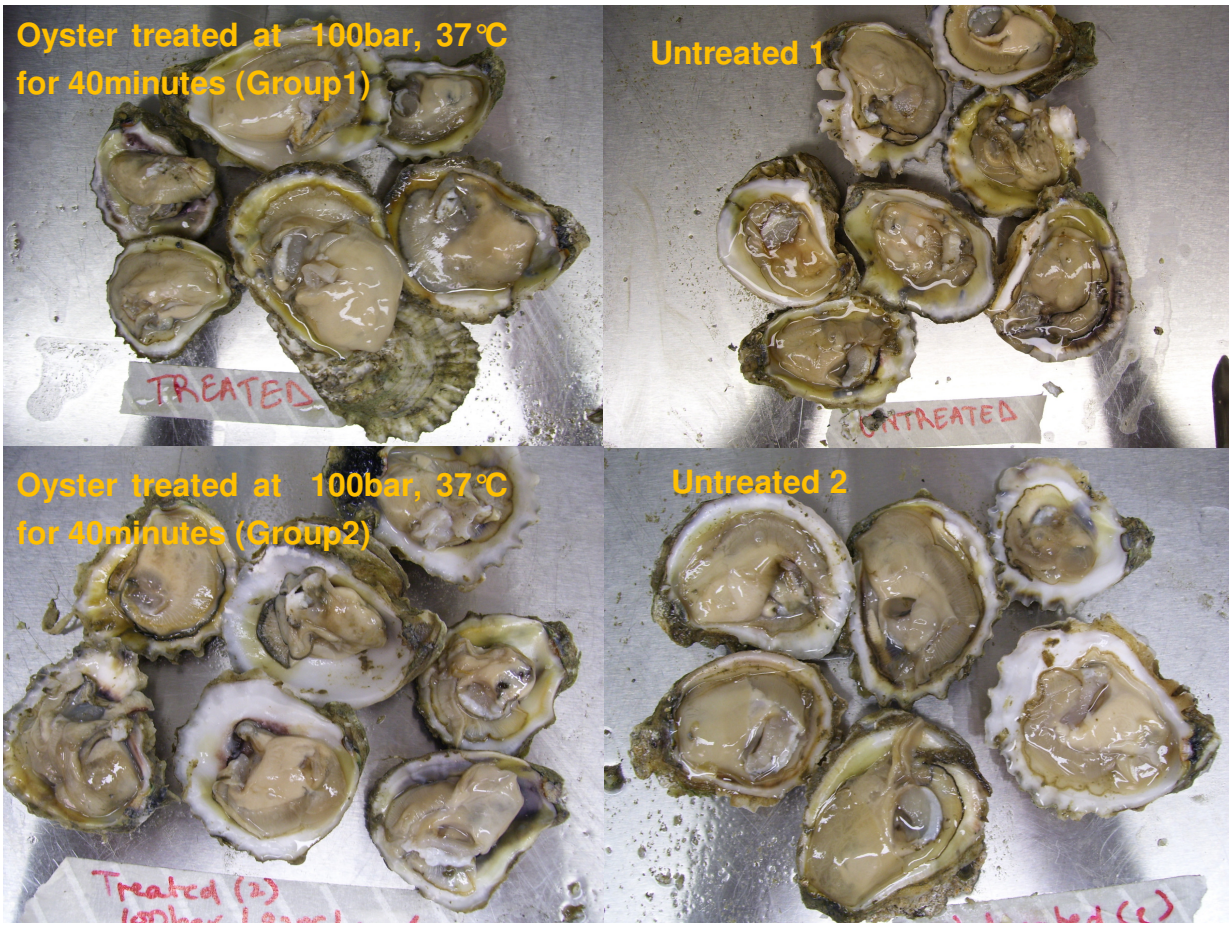

Figure 6-5. Appearance of oysters before and after a 40 minutes exposure to $scCO_2$.

The effect $scCO_2$ on the physical appearance of oysters is presented. Oysters were sampled in small groups and introduced in a supercritical unit (SFT-150 SFE system -Supercritical Fluid Technologies, Inc.) where they were exposed to $scCO_2$ for 40 minutes. They were then shucked and photographed. Temperature and pressure conditions were 37°C and 100 bar.

4. Discussion

Herein, we report that the level of bacterial inactivation (Aerobic Plate Count) achieved with $scCO_2$ (100 bar for 30 min at 37 °C or 172 bar for 60 min at 60 °C), is comparable to that achieved with several FDA approved PHP for oysters, namely, HHP (High Hydrostatic Pressure) and QF (Quick Frozen). In 2009, Prapaiwong et al. reported

a comparable residual amount of bacteria in HHP- and QF-treated oysters. Oysters included in their study were sampled from batches of treated oysters on their way to the market. These authors reported a Total Aerobic Plate Count (TAPC) of 1.4×10^4 CFU/g, 1.6×10^4 CFU/g, and 3.3×10^4 CFU/g in HHP-treated oysters harvested during summer, winter, and fall, respectively. They reported similar amounts of residual bacteria in QF-treated oysters. Untreated oysters harvested during these same periods reportedly contained 3.5×10^5, 2.8×10^5, and 1.4×10^9 CFU/g for oysters harvested during winter, fall, and summer, respectively [332]. Bacteria that survived these $scCO_2$ treatments were mostly Gram negative similar to what has been reported for HHP-treated and QF-treated oysters [332].

Twenty-four out of thirty isolates were identified as catalase positive, and eighteen as oxidase positive. Once in culture however, these isolates (a random selection of four) appeared to be extremely sensitive to the effect of $scCO_2$. According to Lin et al. (1994) [355], the complex physicochemical environment of food products especially a high fat content can increase the resistance of vegetative cells to the effect of $scCO_2$ by limiting the penetration of CO_2.

Each of the current three FDA approved PHP for oysters, namelyHHP, HCP, and IQF, has its advantages and disadvantages. One of the major selling points of High Hydrostatic Pressure (HHP), for instance, is the fact that it induces a release of adductor muscles from oysters' shells. Whether the release of these adductor muscles is truly a desirable effect is somewhat controversial. While it makes shucking oysters afterwards easier, it is important to note that the permanent release of such muscles is a clear indication that oysters did not survive the process which in terms of shelf life

could be an issue. Prapaiwong et al. (2009) [332] studying variations in the bacterial load of HHP-treated oysters stored at 4 °C, determined that the bacterial count can go from an initial 1.6×10^4– 3.3×10^4 CFU/g (post-treatment residual load) to about 10^7 CFU/g in just 7 days.

Based on the Cold Storage Chart of the FDA [356], the quality of such oysters can be maintained for a longer period of time (~ up to 3 – 6 months) provided they are stored at ~ –17 °C. We were able to verify that such long term storage does not affect the quality of oysters. In fact treated and untreated oysters used for our sensory study were stored at ~ –17 °C before analysis.

The $scCO_2$ approach stands out in this regard because the release of adductor muscles from oysters can be monitored. Oysters in this case were treated under two conditions, one that can be considered as mild (100 bar, 30 min and 37 °C) and another that can be considered harsher (172 bar, 60min and 60 °C). Contrary to what was observed at milder conditions (100 bar, 30 min and 37 °C), treating oysters at 172 bar for 60 min at 60 °C, caused a release of adductor muscles from the shell. Oysters exposed to the milder condition however did not release their adductor muscles and retained their fresh-like appearance closely resembling the untreated ones (Figure 6-5). On the other hand, the oysters treated at 172 bar, for 60 min at 60 °C though still watery appeared slightly cooked or steamed—certainly a direct consequence of the high temperature. This effect was most pronounced in another group of oysters treated at this same condition and left in the $scCO_2$ unit afterwards for about 45 min; this set of oysters was excluded from our analysis.

After processing, oysters that remain alive are undoubtedly a better alternative. This is, for instance, the major selling point of several emerging PHP techniques, namely those using X-ray and gamma irradiation as cold pasteurizing agents [357]. X-ray and gamma irradiation are believed to exert their bactericidal effect by damaging the bacterial DNA. Because of the high probability that oysters' DNA could sustain the same damage, concerns have been raised concerning the shelf life of X-ray and gamma irradiation-treated oysters. As mentioned previously, treating oysters at 100 bar for 30 min at 37 ℃ did not release the adductor muscles. We have good reasons to believe that oysters can survive mild $scCO_2$ treatments, such as 100 bar for 30 min at 37 ℃. During trial experiments, a few oysters treated under similar pressure and temperature conditions for 40 min (Figure 6-5) and afterwards put in a flask filled with water were actually able to open and close their shells (on contact with a hard object) for several days. This was a clear indication that they had survived the treatment. The ability of individual oyster to survive such high pressure and CO_2 concentration varies with individual health. The LD_{50} of $scCO_2$ on oysters will be addressed in future studies.

The sensory experiments revealed that oysters treated at milder conditions retain their acceptability. It is important to note that of all the groups of oysters included in this analysis, none received a perfect score of 6 (not even the group of untreated oysters). Two oysters from this group were graded as “spoiled.” Because of current facility limitations, the only feature used to decide whether any given oyster is alive or healthy (thus qualifying for inclusion into the study) is whether or not their shell is tightly closed. While it is true that this feature is shared by live oysters when brought out of the water, it is also true that some oysters, unhealthy or even dead, could share this feature. This

constitutes a source of bias and explains why two spoiled oysters were found in the group of untreated oysters. This justifies why, as stated above, at this point, it is difficult to clearly assess the LD_{50} of $scCO_2$ on oysters. Another difficulty and possibly a source of bias, especially when it comes to sensory analysis studies, is the way our $scCO_2$ unit is built; this instrument was designed for $scCO_2$ extraction; the lack of uniformity in the treatment chamber could result in some oysters being overexposed.

Although the exact mechanism of the bactericidal action of CO_2 is still unknown, it has been proposed that the bacterial inactivation might occur as a result of acidification of the medium or product being sterilized. This acidification occurs as a result of CO_2 interaction with water, an interaction which ultimately results in the generation of H_2CO_3. H_2CO_3 exists in equilibrium with HCO_3^- this equilibrium is responsible for the release of protons in the bacterial environment, thus decreasing pH.

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \longrightarrow \text{pH decrease}$$ ([353])

Low pH can then induce structural changes in the membrane of bacterial cells. These changes, in turn, will increase the permeability of the membrane to CO_2, which then can freely diffuse and accumulate in the bacterial cell, interfering with the normal metabolism of these living cells and ultimately resulting in the death of the bacteria [347, 353]. There have been several other proposed mechanisms of bacterial inactivation by $scCO_2$, such as removal of vital constituent from cell membrane, induction of intracellular electrolytes imbalance, and direct effect of CO_2 on bacterial metabolism, among others [345, 346].

In conclusion, the $scCO_2$ based approach to warm pasteurization of oysters is a promising PHP technique. In the present document, it is established that $scCO_2$ is as effective as several current FDA approved standard approaches to PHP of oysters (e.g., HHP and QF) aimed at reducing the microbial load (aerobic flora) present in the GI system of oysters. We believe that the $scCO_2$ approach will also be as effective in reducing the load of most problematic oysters-associated bacteria, namely, *V. parahaemolyticus* and *V. vulnificus*. The bacteria used as a model for *Vibrio* sp., namely *V. fischeri* showed an extremely high sensitivity to $scCO_2$ and bacterial isolates cultured from oysters' tissues and sharing common biochemical characteristics of *V. parahaemolyticus* and *V. vulnificus* showed an extremely high sensitivity to $scCO_2$, as well.

As a PHP approach, the $scCO_2$ based approach provides quite a number of advantages over other emerging oyster PHP technologies. Large scale $scCO_2$ based technologies for food processing are already available commercially and used industrially for processes such as coffee decaffeination. This represents a serious advantage over gamma and/or X-ray based PHP techniques. The use of $scCO_2$ in food industry is well-established and safe; it can thus be predicted that acceptance by the public will be easier. We have also completed a sensory analysis, and no significant change was noted as a result of oysters having been treated with $scCO_2$.

Finally, as mentioned in the introduction, microbial contaminants are not the only health hazard associated with oysters. Other risks include biotoxins, toxic metal, and chemical environmental contaminants. None of the currently proposed PHP treatments (e.g., HHP, IQF, HCP, X-ray based approach, and gamma-based approach) can reduce

the load of toxic metal, biotoxins, and chemical environmental contaminants in oysters. Though it is beyond the scope of this paper, it is important to note that the ability of $scCO_2$ to remove such contaminants from various biological substrates is well-established [358, 359]

BIBLIOGRAPHY

1. Cox F: **History of human parasitology.** *Clin Microbiol Rev* 2002, **15:**595-612.

2. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY-C, Gernaey AM, Galili E, Eshed V, Greenblatt CL, Lemma E, et al: **Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a neolithic settlement in the Eastern Mediterranean.** *PLoS One* 2008, **3:**1-6.

3. **Tuberculosis Facts** [www.who.int/tb/publications/2007/factsheet_2007.pdf]

4. Rouhi AM: **Rediscovering natural products cast aside for years, natural products drug discovery appears to be reclaiming attention and on the verge of a comeback.** In *The news magazine of the chemical world -Chemical & Engineering News*, vol. 81 American Chemical Society; 2003.

5. **Widening innovation-Productivity gap in the pharmaceutical industry - New challenges and future directions** [http://www.frost.com/prod/servlet/market-insight-print.pag?docid=128394740]

6. **The pursuit of high performance through research and development understanding pharmaceutical research and development cost drivers** [http://www.phrma.org/files/attachments/Accenture%20R&D%20Report-2007.pdf]

7. Newman DJ, Cragg GM: **Natural products as sources of new drugs over the last 25 years.** *J Nat Prod* 2007, **70:**461-477.

8. Pressman BC: **Biological applications of ionophores.** *Annu RevBiochem* 1976, **45:**501–530.

9. Westley JW: *Polyether antibiotics: naturally occurring acid ionophores.* New York: Marcel Dekker Inc.; 1982.

10. Pressman BC, deGuzman NT: **Biological applications and evolutionary origins of ionophores.** *Adv Exp Med Biol* 1977, **84:**285-300.

11. Dorkov P, Pantcheva IN, Sheldrick WS, Mayer-Figge H, Petrova R, Mitewa M: **Synthesis, structure and antimicrobial activity of manganese(II) and cobalt(II) complexes of the polyether ionophore antibiotic sodium monensin A.** *J Inorg Biochem* 2008, **102:**26-32.

12. Lutz WK, Winker FK, Dunitz. JD: **Crystal structure of the antibiotic monensin similarities and differences between free acid and metal complex.** *Helv Chim Acta* 1972, **54:**1103-1108.

13. Pressman BC, Fahim M: **Pharmacology and toxicology of the monovalent**

carboxylic ionophores. *Annu Rev Pharmacol Toxicol* 1982, **22:**465-490.

14. Hildebrandt J, Meingassner JG, Mieth H: **Mode of action of the anticoccidial agent septamycine.** *Zentralbl Veterinarmed B* 1978, **25:**186-193.

15. Ebata E, Kasahara H, Sekine K, Inoue Y: **Lysocellin, a new polyether antibiotic. I. Isolation, purification, physico-chemical and biological properties.** *J Antibiot (Tokyo)* 1975, **28:**118-121.

16. Hamill RL, Hoehn MM, Pittenger GE, Chamberlin J, Gorman M: **Dianemycin, an antibiotic of the group affecting ion transport.** *J Antibiot (Tokyo)* 1969, **22:**161-164.

17. Stern PH: **Ionophores chemistry, physiology and potential applications to bone biology.** *Clin Orthop Relat Res* 1977, **122:**273-298.

18. Gachon P, Kergomard A, Staron T, Esteve C: **Grisorixin, an ionophorous antibiotic of the nigericin group. I. Fermentation, isolation, biological properties and structure.** *J Antibiot (Tokyo)* 1975, **28:**345-350.

19. Mizutani T, Yamagishi M, Hara H, Omura Sea: **Lonomycins B and C, two new components of polyether antibiotics. Fermentation, isolation and characterization.** *J Antibiot (Tokyo)* 1980, **33:**1224-1230.

20. Heisey RM, Putnam AR: **Herbicidal effects of geldanamycin and nigericin, antibiotics from *Streptomyces hygroscopicus*.** *J Nat Prod* 1986, **49:**859-865.

21. Lee SJ, Kim HP, Park BK, Ahnm SC, Lee HS, Ahn JS: **Topical anti-inflammatory activity of dianemycin isolated from *Streptomyces* sp. MT 2705-4.** *Arch Pharmacal Res* 1997, **20:**372-374.

22. McCollum AT, Jafarifar F, Chan R, Guttmann RP: **Oxidative stress inhibits ionomycin-mediated cell death in cortical neurons.** *J Neurosci Res* 2004, **76:**104-109.

23. Parfenova H, Haffner J, Leffler CW: **Phosphorylation-dependent stimulation of prostanoid synthesis by nigericin in cerebral endothelial cells.** *Am J Physiol* 1999 **277:**C728-738.

24. Baba Y, Tsukuda M, Mochimatsu I, Furukawa S, Kagata H, Nagashima Y, Koshika S, Imoto M, Kato Y: **Cytostatic effect of inostamycin , an inhibitor of cytidine 5'-diphosphate 1,2-DIACYL-sn-GLYCEROL (CDP-DG): inositol transferase, on oral squamous cell carcinoma cell lines.** *Cell Biol Int* 2001, **25:**613-620.

25. Funayama S, Nozoe S, Tronquet C, Anraku Y, Komiyama K, Omura S: **Isolation and structure of a new polyether antibiotic, octacyclomycin.** *J Antibiot (Tokyo)* 1992, **45:**1686-1691.

26. Kusakabe Y, Mitsuoka S, Omuro Y, Seino A: **Antibiotic no. 6016, a polyether antibiotic.** *J Antibiot (tokyo)* 1980, **33:**1437-1442.

27. Omura S, Shibata M, Machida S, Sawada J: **Isolation of a new polyether antibiotic, Ionomycin.** *J Antibiot (Tokyo)* 1976, **29:**15-20.

28. Westley JW, Liu C, Sello LH, Troupe Nea: **Isolation and characterization of antibotic A-14931A, the naturally occurring 19-deoxyaglycone of dianemycin.** *J Antibiot (Tokyo)* 1984, **37:**813-815.

29. Odai H, Shindo K, Odagawa A, Mochizuki J, Hamada M, Takeuchi T: **Inostamycins B and C , new polyether antibiotics.** *J Antibiot (Tokyo)* 1994, **47:**939-941.

30. Mizutani T, Yamagishi M, Hara H, Kawashima A, Omura S, Ozeki M, Mizoue K, Seto H, Otake N: **Studies on the ionophorous antibiotics. XXIV. Leuseramycin , a new polyether antibiotic produced by *Streptomyces hygroscopicus*.** *J Antibiot (Tokyo)* 1980, **33:**137-143.

31. Keller-Juslen C, King HD, Kis ZL, Von Wartburg A: **Septamycin, a polyether antibiotic. Taxonomy, fermentation, isolation and characterization** *J Antibiot (Tokyo)* 1975 **28:**854-859.

32. Keller-Juslen C, King HD, Kuhn M, Loosli HR, von Wartburg A: **Noboritomycins A and B, new polyether antibiotics.** *J Antibiot (Tokyo)* 1978, **31:**820-828.

33. Fehr T, King HD, Kuhn M: **Mutalomycin, a new polyether antibiotic taxonomy, fermentation, isolation and characterization.** *J Antibiot (Tokyo)* 1977, **30:**903-907.

34. Kitame F, Utsushikawa K, Koama T, Saito T, Kikuchi M: **Laidlomycin, a new antimycoplasmal polyether antibiotic.** *J Antibiot (Tokyo)* 1974, **27:**884-888.

35. Alleaume M, Hickel D: **Crystal structure of the thallium salt of the antibiotic grisorixin.** *J Chem Soc, Chem Commun* 1972, **3:**175-176.

36. Guyot J, Jeminet G, Prudhomme M, Sancelme M, Meiniel R: **Interaction of the calcium ionophore A.23187 (calcimycin) with *Bacillus cereus* and *Escherichia coli*.** *Lett Appl Microbiol* 1993, **16:**192-195.

37. Gumila C, Ancelin ML, Delort AM, Jeminet G, Vial HJ: **Characterization of the potent in vitro and in vivo antimalarial activities of ionophore compounds.** *Antimicrob Agents Chemother* 1997, **41:**523-529.

38. Woods KM, Nesterenko MV, Upton SJ: **Efficacy of 101 antimicrobials and other agents on the development of *Cryptosporidium parvum* in vitro.** *Ann Trop Med Parasitol* 1996, **90:**603-615.

39. You XS, R.F.; Arrowood, M.J.; et al. : **In-vitro activities of paromomycin and lasalocid evaluated in combination against Cryptosporidium parvum** *J Antimicrob Chemother* 1998, **41:**293-296.

40. Nakamura M, Kunimoto S, Takahashi Y, Naganawa H, Sakaue M, Inoue Sea: **Inhibitory effects of polyethers on human immunodeficiency virus replication.** *Antimicrob Agents Chemother* 1992, **36:**492-494.

41. Edrington TS, Callaway TR, Varey PD, et al.: **Effects of the antibiotic ionophores monensin, lasalocid, laidlomycin propionate and bambermycin on *Salmonella* and *E. coli* O157:H7 in vitro** *J Appl Microbiol* 2003, **94:**207-213.

42. Cirioni O, Giacometti A, Barchiesi F, Scalise G: **In vitro activity of lytic peptides, inhibitors of ion transport systems and ionophorous antibiotics against *Pneumocystis carinii*.** *J Antimicrob Chemother* 1998, **42:**141-145.

43. Brasseur P, Lemeteil D, Ballet JJ: **Anti-cryptosporidial drug activity screened with an immunosuppressed rat model.** *J Protozool* 1991, **38:**230S-231S.

44. Folz SD, Lee BL, Nowakowski LH, Conder GA: **Anticoccidial evaluation of halofuginone, lasalocid, maduramicin, monensin and salinomycin.** *Vet Parasitol* 1988, **28:**1-9.

45. Lemeteil D, Roussel F, Favennec L, Ballet JJ, Brasseur P: **Assessment of candidate anticryptosporidial agents in an immunosuppressed rat model.** *J Infect Dis* 1993, **167:**766-768.

46. Oz HS, Hughes WT, Rehg JE: **Efficacy of lasalocid against murine *Pneumocystis carinii pneumonitis*.** *Antimicrob Agents Chemother* 1997, **41:**191-192.

47. Rehg JE: **Anticryptosporidial activity of lasalocid and other ionophorous antibiotics in immunosuppressed rats.** *J Infect Dis* 1993, **168:**1566-1569.

48. Johnson SM, Herrin J, Liu SJ, Paul IC: **The crystal and molecular structure of the barium salt of an antibiotic containing a high proportion of oxygen.** *J Am Chem Soc* 1970 **92:**4428-4435.

49. Couzinet S, Dubremetz JF, Buzoni-Gatel D, Jeminet G, Prensier G: **In vitro activity of the polyether ionophorous antibiotic monensin against the cyst form of *Toxoplasma gondii*.** *Parasitol* 2000, **12:**359-365.

50. Folz SD, Nowakowski LH, Lee BL, Conder GA, Rector DL, Brodasky TF: **Anticoccidial activity of alborixin.** *J Parasitol* 1987, **73:**29-35

51. Gumila C, Ancelin ML, Jeminet G, Delort AM, Miquel G, Vial HJ: **Differential in vitro activities of ionophore compounds against *Plasmodium falciparum* and mammalian cells.** *Antimicrob Agents Chemother* 1996, **40:**602-608.

52. Lindsay DS, Rippey NS, Cole RA, Parsons LC, Dubey JP, Tidwell RR, et al.: **Examination of the activities of 43 chemotherapeutic agents against *Neospora caninum* tachyzoites in cultured cells.** *Am J Vet Res* 1994, **55:**976-981.

53. McDonald V, Stables R, Warhurst DC, et al.: **In vitro cultivation of *Cryptosporidium parvum* and screening for anticryptosporidial drugs.** *Antimicrob Agents Chemother* 1990, **34:**1498-1500.

54. Pal R, Gallo RC, Sarngadharan MG: **Processing of the structural proteins of human immunodeficiency virus type 1 in the presence of monensin and cerulenin.** *Proc Natl Acad Sci USA* 1988, **85:**9283-9286.

55. Haney ME, Jr., Hoehn MM: **Monensin, a new biologically active compound.** *Antimicrob Agents Chemother* 1967**:**349-352.

56. Yoo JC, Kim JH, Ha JW, et al: **Production and biological activity of laidlomycin, anti-MRSA/VRE antibiotic from *Streptomyces* sp. CS684.** *J Microbiol* 2007, **45:**6-10.

57. Kawada M, Umezawa K: **Long-lasting accumulation of vinblastine in inostamycin -treated multidrug-resistant KB cells** *Jpn J Canc Res* 1991, **82:**1160-1164.

58. Tzipori SR, Campbell I, Angus KW: **The therapeutic effect of 16 antimicrobial agents on *Cryptosporidium* infection in mice.** *Aust J Exp Biol Med Sci* 1982, **60:**187-190.

59. Munir K, Muneer MA, Tiwari A, Chaudhry RM, Muruganandan S: **Effects of polyether ionophores on the protective immune responses of broiler chickens against Angara disease and Newcastle disease viruses.** *Vet Res Commun* 2007, **31:**909-929.

60. Atef M, Youssef SE-DAH, El-Sayed MGA, Shalaby MA: **Some cardiovascular effects of monensin.** *Tieraerztliche Wochenschrift* 1986, **93:**81-84.

61. Gurbanov KG, Kovalev GV, Paperno AA: **Experimental study of the cardiac and hemodynamic effects of the carboxylic ionophore monensin.** *Farmakologiya i Toksikologiya (Moscow)* 1990 **53:**17-19

62. Gale C, McDouglas LR, U.S. Patent 3,995,027, 1976.

63. Agtarap A, Chamberlin JW, Pinkerton M, Steinrauf L: **The structure of monensic acid, a new biologically active compound.** *J Am Chem Soc* 1967, **89:**5737-5739.

64. Berg DH, Hamill RL: **The isolation and characterization of narasin, a new polyether antibiotic.** *J Antibiot (Tokyo)* 1978 **31:**1-6.

65. Conway DP, Johnson JK, Guyonnet V, Long PL, Smothers CD: **Efficacy of semduramicin and salinomycin against different stages of *Eimeria tenella* and *E. acervulina* in the chicken.** *Vet Parasitol* 1993, **45:**215-229.

66. Johansen CH, Bjerrum L, Pedersen K: **Impact of salinomycin on the intestinal microflora of broiler chickens.** *Acta Vet Scand* 2007, **49:**30.

67. Kyriakis SC, Sarris K, Kritas SK, Saoulidis K, Tsinas AC, Tsiloyiannis VK: **The effect of salinomycin on the control of *Clostridium perfringens* type-A infection in growing pigs.** *Zentralbl Veterinarmed B* 1995, **42:**355-359.

68. Mozaffarian D, Longstreth WTJ, Lemaitre RN, Manolio TA, Kuller LH, Burke GL, Siscovick DS: **Fish consumption and stroke risk in elderly individuals: the cardiovascular health study.** *Arch Intern Med* 2005, **165:**200-206.

69. Kinashi ON, Yonehara H, Otake N, Yonehara H, Sato S, Saito T: **The structure of salinomycin, a new member of the polyether antibiotics.** *Tetrahedron Lett* 1973, **49:**4955-4958.

70. Couzinet S, Dubremetz JF, David L, Prensier G: ***Toxoplasma gondii*: activity of the polyether ionophorous antibiotic nigericin on tachyzoites in cell culture.** *Exp Parasitol* 1994, **78:**341-351.

71. Crance JM, Gratier D, Guimet J, Jouan A: **Inhibition of sandfly fever Sicilian virus (Phlebovirus) replication in vitro by antiviral compounds.** *Res Virol* 1997 **148:**353-365.

72. **Matsuno** THF, Yamazaki T: **Anticoccidial activity of a new polyether antibiotic, carriomycin , in battery trial.** *Takeda* 1982, **43:**192-193.

73. Baibakov BAF, George A, Margolis, Leonid B, Skulachev, Vladimir P: **Antitumor effect of potassium/hydrogen antiporter nigericin on human lung carcinoma grown in in vivo-like histocultures.** *Int J Oncol* 1993, **3:**1127-1129.

74. Adovelande J, Schrevel J: **Carboxylic ionophores in malaria chemotherapy: the effects of monensin and nigericin on *Plasmodium falciparum* in vitro and *Plasmodium vinckei petteri* in vivo.** *Life Sci* 1996, **59:**309-315.

75. Fruth IA, Arrizabalaga G: ***Toxoplasma gondii*: induction of egress by the potassium ionophore nigericin.** *Int J Parasitol* 2007, **37:**1559-1567.

76. Mahmoudi N, Garcia-Domenech R, Galvez J, Farhati K, Franetich JF, Sauerwein Rea: **New active drugs against liver stages of *Plasmodium* predicted by molecular topology.** *Antimicrob Agents Chemother* 2008, **52:**1215-1220.

77. Oh JH, O'Malley KL, Krajewski S, Reed JC, Oh YJ: **Bax accelerates staurosporine-induced but suppresses nigericin-induced neuronal cell death.** *Neuroreport* 1997, **8:**1851-1856.

78. Steinrauf LK, Pinkerton M, Chamberlin JW: **The structure of nigericin.** *Biochem Biophys Res Commun* 1968, **33:**29-31.

79. Blount JFE, Ralph H, Jr., Liu CM, Hermann T, Westley JW: **X-ray structure of Ro 21 - 6150 , a polyether antibiotic related to Dianemycin.** *J Chem Soc, Chem Commun* 1975, **20:**853-855.

80. Imada A, Nozaki Y, Hasegawa T, Mizuta E, Igarasi S, Yoneda M: **Carriomycin, a new polyether antibiotic produced by *Streptomyces hygroscopicus*.** *J Antibiot (Tokyo)* 1978, **31:**7-14.

81. Williams RD, Power C, E.P. Patent 19,870,305,000 1987.

82. Nakatsukasa WM, Marconi GG, Neuss N, Hamill RL, U.S. Patent 4,141,907, 1979.

83. Liu CM, Hermann TE, Prosser BL, Palleroni NJ, Westley JW, Miller PA: **X-14766A, a halogen containing polyether antibiotic produced by *Streptomyces malachitofuscus* subsp. downeyi ATCC 31547. Discovery, fermentation, biological properties and taxonomy of the producing culture.** *J Antibiot (Tokyo)* 1981, **34:**133-138.

84. Westley JW, Evans RHJ, Sello LH, Troupe N, Liu CM, Blount JF, Pitcher RG, Williams TH, Miller PA: **Isolation and characterization of the first halogen containing polyether antibiotic X-14766A, a product of *Streptomyces malachitofuscus* subsp. downeyi.** *J Antibiot (Tokyo)* 1981 **34:**139-147.

85. Nakamura M, Kunimoto S, Kawashima H, Takeuchi T, Ohno T: **Inhibitory effect of laidlomycin on human immunodeficiency virus replication.** *J Antibiot (Tokyo)* 2000, **53:**975-978.

86. Delhomme C, Kergomard A, Kergomard G, Staron T: **Alborixin, a new antibiotic ionophore: taxonomy, isolation and biological properties.** *J Antibiot (Tokyo)* 1976, **29:**692-695.

87. Mead JR, You X, Pharr JE, Belenkaya Yea: **Evaluation of maduramicin and alborixin in a SCID mouse model of chronic cryptosporidiosis.** *Antimicrob Agents Chemother* 1995, **39:**854-858.

88. Alleaume MBB, Farges C, Gachon P, Kergomard A, Staron T: **X-ray structure of alborixin, a new antibiotic ionophore.** *J Chem Soc, Chem Commun* 1975, **11:**4112.

89. Liu WC, Slusarchyk DS, Astle G, Trejo WH, Brown WE, Meyers E: **Ionomycin, a new polyether antibiotic S.** *J Antibiot (Tokyo)* 1978 **31:**815-819.

90. Miyake H, Hara I, Yamanaka K, Arakawa S, Kamidono S: **Calcium ionophore, ionomycin inhibits growth of human bladder cancer cells both in vitro and**

in vivo with alteration of Bcl-2 and Bax expression levels. *J Urol* 1999, **162:**916-921.

91. Park HJ, Makepeace CM, Lyons JC, Song CW: **Effect of intracellular acidity and ionomycin on apoptosis in HL-60 cells.** *Eur J Cancer* 1996, **32A:**540-546.

92. Dahlgren C, Karlsson A: **Ionomycin-induced neutrophil NADPH oxidase activity is selectively inhibited by the serine protease inhibitor diisopropyl fluorophosphate.** *Antioxid Redox Signal* 2002, **4:**17-25.

93. Dirlam JP, Belton AM, Bordner J, Cullen WP, Huang LH, Kojima Y, Maeda H, Nishida H, Nishiyama S, Oscarson JR, et al.: **CP-84,657, a potent polyether anticoccidial related to portmicin and produced by *Actinomadura* sp.** *J Antibiot (Tokyo)* 1990, **43:**668-679.

94. Holmes AG, Watt MJ, Carey AL, Febbraio MA: **Ionomycin, but not physiologic doses of epinephrine, stimulates skeletal muscle interleukin-6 mRNA expression and protein release.** *Metabolism* 2004, **53:**1492-1495.

95. Takeuchi TT, Tsuzaka K, Yoshimoto K, W.O. Patent 2,006,030,802, 2006.

96. Zhao JZY, Li H, Zeng X, Ji Y, He X: **Mechanisms underlying induction of IL-2 secretion by PDB plus ionomycin in CD4+CD25+ T cells from cord blood and adult peripheral blood.** *Zhongguo Bingli Shengli Zazhi* 2006, **22:**1133-1137.

97. Chaney MO, Demarco PV, Jones ND, Occolowitz JL: **Structure of A23187, a divalent cation ionophore.** *J Am Chem Soc* 1974, **96:**1932-1933.

98. Westley JW, Evan RH, Liu CM, Hermann T, Blount JF, : **Structure of antibiotic X- 14547 a carboxylic acid polyether produced by *Streptomyces antibiotics*.** *J Am Chem Soc* 1978, **100:** 6784-8786.

99. Otake N, Koenuma M, Kinashi H, Sato S, Saito Y: **The crystal and molecular structure of the silver salt of lysocellin, a new polyether antibiotic.** *J Chem Soc, Chem Commun* 1975**:**92 - 93.

100. Chollet-Debord F, Moins N, Renoux M, Gachon P: **Effects of the ionophore grisorixin on myocardial function and metabolism in isolated perfused working rat heart under normoxic and hypoxic conditions.** *Can J Physiol Pharmacol* 1986, **64:**631-640.

101. Gachon P, Moins N: **The cardiovascular effects of two monocarboxylic inophores, grisorixin and alborixin, in anesthetized guinea-pigs.** *Arzneimittelforschung* 1980, **30:**1502-1507.

102. Moins N, Gachon P, Maublant J: **Myocardial imaging in dogs treated with grisorixin: relationship between thallium-201 uptake and coronary blood**

flow. *J Nucl Med* 1982, **23:**330-336.

103. Miyagami T, Takei Y, Matsumoto Y, Otake N, Mizoue K, Mizutani T, et al.: **An in vitro study on the toxoplasmacidal activity of Ionomycin A in host cells.** *J Antibiot (Tokyo)* 1981, **34:**218-223.

104. Tsuchida K, Kaneko K, Aihara H, Chiba S: **Cardiovascular effects of an ionophorous antibiotic, Ionomycin A, in anesthetized dogs.** *Jpn J Pharmacol* 1985, **38:**109-112.

105. Westley JW, Liu CM, Sello LH, Troupe Nea: **Isolation and characterization of antibiotic X-14931A, the naturally occurring 19-deoxyaglycone of dianemycin.** *J Antibiot (Tokyo)* 1984, **37:**813-815.

106. Czerwinski EW, Steinrauf LK: **Structure of the antibiotic dianemycin.** *Biochem Biophys Res Commun* 1971, **45:**1284-1287.

107. Otake N, Ogita T, Nakayama H, Miyamae H, Sato S, Saito Y: **X-Ray crystal structure of the thallium salt of antibiotic-6016, a new polyether ionophore.** *J Chem Soc, Chem Comm* 1978**:**875- 876.

108. Imoto M, Umezawa K, Takahashi Y, Naganawa H, Iitaka Y, Nakamura H, Koizumi Y, Sasaki Y, Hamada M, Sawa T, Takeuchi T: **Isolation and structure determination of inostamycin, a novel inhibitor of phosphatidylinositol turnover.** *J Nat Prod* 1990 **53:**825-829.

109. Westley JW, Liu CM, Blount JF, Sello LH, Troupe N, Miller PA: **Isolation and characterization of a novel polyether antibiotic of the pyrrolether class, antibiotic X-14885A.** *J Antibiot (Tokyo)* 1983, **36:** 1275-1278.

110. Nakamura G, Kobayashi K, Sakurai T, Isono K: **Cationomycin, a new polyether ionophore antibiotic produced by *Actinomadura* Nov. sp.** *J Antibiot (Tokyo)* 1981, **34:**1513-1514.

111. Otoguro K, Kohana A, Manabe C, Ishiyama A, Ui H, Shiomi K, et al: **Potent antimalarial activities of polyether antibiotic, X-206.** *J Antibiot (Tokyo)* 2001 **54:**658-663.

112. Gorman MH, Robert L U.S. Patent 3,627,883, 1971.

113. Strout RG, Ouellette CA: ***Eimeria tenella*. Screening of chemotherapeutic compounds in cell cultures.** *Exp Parasitol* 1973, **33:**477-485.

114. Westley JW, Blount JF, Evans RHJ, Liu CM: **C-17 epimers of deoxy-(O-8)-salinomycin from *Streptomyces albus* (ATCC 21838).** *J Antibiot* 1977, **30:**610-612.

115. Blount JF, Westley JW: **X-ray crystal and molecular structure of the**

antibiotic X-206. *Chem Commun* 1975, **13:**533.

116. Omura SOK, Yamada H, J.P. Patent 2,003,335,667, 2003.

117. Tsuji N, Nagashima K, Kobayashi M, Wakisaka Y, Kawamura Y: **Two new antibiotics, A-218 and K-41. Isolation and characterization.** *J Antibiot (Tokyo)* 1976, **29**:10-4.

118. Ishiguro T, J.P. Patent 53,020,420, 1978.

119. Shiro M, Nakai H, Nagashima K, Tsuji N: **X-ray determination of the structure of the polyether antibiotic K - 41.** *J Chem Soc, Chem Commun* 1978, **16:**682-683.

120. Na M, Meujo DAF, Kevin D, Hamann MT, Anderson M, Hill RT: **A New Antimalarial Polyether from a Marine Streptomyces sp. H668.** *Tetrahedron Lett* 2008, **49:**6282-6285.

121. Fleck WF, Strauss DG, Meyer J, Porstendorfer G: **Fermentation, isolation, and biological activity of maduramycin: a new antibiotic from *Actinomadura rubra*.** *Z Allg Mikrobiol* 1978, **18** (6):389-98.

122. Caffarel-Mendez SD, C.; Jeminet, G. : **In vitro study of various ionophore antibiotics and some of their derivatives. II. Characterization of the ionophore properties of the compounds in a model system for Na^+ and K^+ ions.** *Reprod Nutr Dev* 1987, **27:** 921-928.

123. Cerruti SS, Leoni A, Agostini A, Castagnaro M: **Efficacy of maduramicin against turkey coccidiosis in battery: a clinical and pathological study** *Schweizer Archiv fur Tierheilkunde* 1996, **138:**201-206.

124. Dirlam JP, Belton AM, Bordner J, Cullen WP, Huang LH, Kojima Y, Maeda H, Nishiyama S, Oscarson JR, Ricketts AP, et al: **CP-82, 009, a potent polyether anticoccidial related to septamycin and produced by *Actinomadura*.** *J Antibiot (Tokyo)* 1992, **45:**331-340.

125. Dirlam JP, Presseau-Linabury L, Koss DA: **The structure of CP-80, 219, a new polyether antibiotic related to dianemycin** *J Antibiot (Tokyo)* 1990, **43:**727-730.

126. Takahashi Y, Nakamura H, Ogata R, Matsuda N, Hamada M, Naganawa Hea: **Kijimicin, a polyether antibiotic.** *J Antibiot (Tokyo)* 1990, **43:**441-443.

127. Nakamura M, Ohno T, Kunimoto S, Naganawa H, Takeuchi T: **Kijimicin: an inhibitor of human immunodeficiency virus in acutely and chronically infected cells.** *J Antibiot (Tokyo)* 1991, **44:**569-571.

128. Yamauchi T, Nakamura M, Honma H, Ikeda M, Kawashima K, Ohno T:

Mechanistic effects of kijimicin on inhibition of human immunodeficiency virus replication. *Mol Cell Biochem* 1993, **119:**35-41.

129. Tomio T, Arisuke W, Munekazu Iea, J.P. Patent 2,001,278,787**,** 2001.

130. Oscarson JR, Bordner J, Celmer WD, L.W.P. C, Huang H, Maeda H, Moshier PM, Nishiyama S, Presseau L, Shibakawa R: **Endusamycin, a novel polycyclic ether antibiotic produced by a strain of *Streptomyces endus* subsp. aureus.** *J Antibiot (Tokyo)* 1989, **42:**37-48.

131. Liu CM, Hermann TE, Downey A, Prosser BL, Schildknecht E, Palleroni NJ, Westley JW, Miller PA: **Novel polyether antibiotics X-14868A, B, C, and D produced by a *Nocardia*. Discovery, fermentation, biological as well as ionophore properties and taxonomy of the producing culture.** *J Antibiot (Tokyo)* 1983, **36:**343-350.

132. Tadashi N, Yukio T, Yuko Kea, J.P. Patent 61,260,888, 1986.

133. Sasaki T, J.P. Patent 60,130,394, 1985.

134. Hatsu M, Sasaki T, Miyadoh S, Watabe H, Takeuchi Y, Kodama Y, Orikasa Y, Kajii K, Shomura T, Yamamoto H: **SF2487, a new polyether antibiotic produced by *Actinomadura*.** *J Antibiot (Tokyo)* 1990, **43:**259-266.

135. Jones ND, Chaney MO, Chamberlin JW, Chamberlin RL, Chen S: **Structure of A204A, a new polyether antibiotic.** *J Am Chem Soc* 1973, **95:**3399-3400.

136. Dirlam JP, Cullen WP, Huang LH, Nelson TH, Oscarson JR, Presseau-Linabury L, Tynan EJ, Whipple EB: **CP-91,243 and CP-91,244, novel diglycoside polyether antibiotics related to UK-58,852 and produced by mutants of *Actinomadura roseorufa*.** *J Antibiot (Tokyo)* 1991, **44:**1262-1266.

137. Dirlam JP, Bordner J, Chang SP, Grizzuti A, Nelson TH, Tynan EJ, Whipple EB: **The isolation and structure of CP-120,509, a new polyether antibiotic related to semduramicin and produced by mutants of *Actinomadura roseorufa*.** *J Antibiot (Tokyo)* 1992, **45:**1544-1548.

138. Westley JW, Blount JF, Evans RHJ, Stempel A, Berger J: **Biosynthesis of lasalocid. II. X-ray analysis of a naturally occurring isomer of lasalocid A.** *J Antibiot (Tokyo)* 1974, **27:**597-604.

139. Nakayama H, Seto H, Otake N, Yamagishi M, Kawashima A, Mizutani T, Omura S: **Studies on the ionophorous antibiotics. XXVIII. Moyukamycin , a new glycosylated polyether antibiotic.** *J Antibiot (Tokyo)* 1985, **38:**1433-1436.

140. Liu CM, Westley J, U.S. Patent 4,137,241, 1979.

141. Cullen WP, Celmer WD, Chappel LR, Huang LH, Maeda H, Nishiyama S,

Shibakawa R, Tone J, Paul C: **CP-54, 883 a novel chlorine-containing polyether antibiotic produced by a new species of *Actinomadura*: taxonomy of the producing culture, fermentation, physico-chemical and biological properties of the antibiotic.** *J Antibiot (Tokyo)* 1987, **40:**1490-1495.

142. David L, Leal Ayala H, Tabet JC: **Abierixin , a new polyether antibiotic. Production, structural determination and biological activities.** *J Antibiot (Tokyo)* 1985, **38:**1655-1663

143. Bernan VS, Montenegro DA, Goodman JJ, Alluri MR, Carter GT, Abbanat DR, Pearce CJ, Maiese WM, Greenstein M: **Martinomycin, a new polyether antibiotic produced by *Streptomyces salvialis*. I. Taxonomy, fermentation and biological activity.** *J Antibiot (Tokyo)* 1994, **47:**1434-1441.

144. Kusakabe Y, Takahashi N, Iwagaya Y, Seino A: **Portmicin, a new antibiotic.** *J Antibiot (Tokyo)* 1987, **40:**237-238.

145. Liu C, Hermann TE, Liu M, Prosser BL, Palleroni NJ, Westley JW, Miller PA: **Novel polyether antibiotics X-14667A and X-14667B from *Streptomyces cinnamonensis* subsp. urethanofaciens. Discovery, fermentation, biological as well as ionophore properties and taxonomy of the producing culture.** *J Antibiot (Tokyo)* 1981, **34:**1241-1248.

146. Dirlam JP, Bordner J, Cullen WP, Jefferson MT, Presseau-Linabury L: **The structure of CP -96,797, a polyether antibiotic related to K-41A and produced by *Streptomyces* sp.** *J Antibiot (Tokyo)* 1992, **45:**1187-1189.

147. Ruddock J, Cornish DR, Cullen WP, Tone J, Maeda H, E.P. Patent 0169,011, 1986.

148. Delfini LF: **The first case of *Plasmodium falciparum* resistant to chloroquine treatment discovered in the Republic of Afghanistan.** *Trop Med Hyg* 1989, **83:**316.

149. **Antimalarial drug combination therapy report of a who technical consultation world health organization** [http://www.rollbackmalaria.org/cmc_upload/0/000/015/082/use_of_antimalarials2.pdf]

150. Schapira A, Beales PF, Halloran ME: **Malaria: living with drug resistance.** *Parasitol* 1993, **9:**168-174.

151. Trape JF, Pison G, Preziosi MP, Enel C, Desgrees du Lou A, Delaunay V, Samb B, Lagarde E, Molez JF, Simondon F: **Impact of chloroquine resistance on malaria mortality.** *C R Acad Sci III* 1998, **321:**689-697.

152. Kassankogno Y. et al: **Combination therapy for uncomplicated malaria in Africa?** *Liaison bulletin of the malaria programme WHO/AFRO* 2001 **4:**1-4.

153. Otoguro K, Ishiyama A, Ui H, Kobayashi M, Manabe C, Yan G, Takahashi Y, Tanaka H, Yamada H, Omura S: **In vitro and in vivo antimalarial activities of the monoglycoside polyether antibiotic, K - 41 against drug resistant strains of *Plasmodia*.** *J Antibiot (Tokyo)* 2002, **55:**832-834.

154. Fitch Coy D: **Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs.** *Life science* 2004, **74:**1957-1972.

155. Reeves DC, Liebelt DA, Lakshmanan V, Roepe PD, Fidock DA, Akabas MH: **Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms.** *Mol Biochem Parasitol* 2006, **150:**288-299.

156. Kovac L, Poliachova V, Horvath I: **Ionophores and intact cells. I Valinomycin and nigericin act preferentially on mitochondria and not on the plasma membrane of *Saccharomyces cerevisiae*.** *Biochim Biophys Acta* 1982 **721:**341-348.

157. Smith CK, Strout RG: ***Eimeria tenella*: effect of narasin, a polyether antibiotic on the ultrastructure of intracellular sporozoites.** *Exp Parasitol* 1980, **50:**426-436.

158. Flanigan TP: **Human immunodeficiency virus infection and cryptosporidiosis: protective immune responses.** *Am J Trop Med Hyg* 1994, **50:**29-35.

159. Fujikawa H, Miyakawa H, al. IKe: **Intestinal cryptosporidiosis as an initial manifestation in a previously healthy Japanese patient with AIDS.** *J Gastroenterol* 2002, **37:**840-843.

160. Lim YA, Rohela M, Sim BL, Jamaiah I, Nurbayah M: **Prevalence of cryptosporidiosis in HIV-infected patients in Kajang Hospital, Selangor. Southeast Asian.** *J Trop Med Public Health* 2005, **36:**30-33.

161. Blagburn BL, Sundermann CA, Lindsay DS, Hall JE, Tidwell RR: **Inhibition of *Cryptosporidium parvum* in neonatal Hsd:(ICR)BR Swiss mice by polyether ionophores and aromatic amidines.** *Antimicrob Agents Chemother* 1991, **35:**1520-1523.

162. Carrada-Bravo T: **Toxoplasmosis. A public health problem. Advances and perspectives.** *Bol Med Hosp Infant Mex* 1983, **40:**353-362.

163. Soete M, Fortier B, Camus D, J.F. D: **Toxoplasma gondii: kinetics of bradyzoite-tachyzoite interconversion in vitro.** *Exp Parasitol* 1993, **76:**259-264.

164. Wong SY, Remington JS: **Biology of *Toxoplasma gondii*.** *Aids* 1993, **7:**299-316.

165. Ricketts AP, Pfefferkornm ER: **Toxoplasma gondii: susceptibility and development of resistance to anticoccidial drugs in vitro.** *Antimicrob Agents Chemother* 1993, **37:**2358-2363.

166. Huskinson-Mark J, Araujo FG, Remington JS: **Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*.** *J Infect Dis* 1991, **164:**170-171.

167. Sarciron ME, Lawton P, Petavy AF, F. P: **Alterations of Toxoplasma gondii Induced by 2',3'-Dideoxyinosine In vitro.** *J Parasitol* 1998, **84:**1055-1059.

168. Otake N, Koenuma M, Kinashi H, Sato S, Saito Y: **The crystal and molecular structure of the silver salt of lysocellin, a new polyether antibiotic.** *J Chem Soc, Chem Commun* 1975**:**92-93.

169. Robin L, Dewar RL, Vasudevachari MB, Natarajan V, Salzman NP: **Biosynthesis and processing of human immunodeficiency virus type 1 envelope glycoproteins: effects of monensin on glycosylation and transport.** *J Virol* 1989, **63:**2452-2456.

170. Gyemant N, Tanaka M, Molnar P, Deli Jea: **Reversal of multidrug resistance of cancer cells in vitro: modification of drug resistance by selected carotenoids(1A):367-74.** *Anticancer Res* 2006, **26:**367-374.

171. Lage H: **ABC-transporters: implications on drug resistance from Microorganisms to human cancers.** *Int J Antimicrob Agents* 2003, **22:**188-199.

172. Simizu S, Tanabe K, Tashiro E, Takada M, Umezawa K, Imoto M: **Potentiation of paclitaxel cytotoxicity by inostamycin in human small cell lung carcinoma, Ms-1 cells.** *Jpn J Canc Res* 1998, **89:**970-976.

173. Margolis LB, Novikova IY, Rozovskaya IA, Skulachev VP: **K+/H+-antiporter nigericin arrests DNA synthesis in Ehrlich ascites carcinoma cells.** *Proc Natl Acad Sci USA* 1989 **86:**6626-6629.

174. Moins N, Gachon P, Maublant J, Duchene-Marullaz P: **Hemodynamic effects of grisorixin , a monocarboxylic ionophore.** *Arch Int Pharmaco Ther* 1982, **260:**104-114.

175. Moins N, Gachon P, Duchene-Marullaz P: **Effects of two monocarboxylic ionophores, grisorixin and alborixin, on cardiovascular function and plasma cation concentrations in the anesthetized dog.** *J Cardiovasc Pharm* 1979, **1:**659-671

176. Munir K, Muneer MA, Khan MZ: **Immunomodulatory effects of salinomycin sodium in broiler chickens.** *Pakistan Vet J* 1994, **14:**171-179.

177. Chatila T, Silverman L, Miller R, Geha R: **Mechanisms of T cell activation by the calcium ionophore ionomycin.** *J Immunol* 1989, **143:**1283-1289.

178. White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, Kokwaro G, Ouma J, Hien TT, Molyneux ME, et al: **Averting a malaria disaster.** *Lancet* 1999, **353:**1965 - 1967.

179. Dorkov P, Pantcheva IN, Sheldrick WS, Mayer-Figge H, Petrova R, Mitewa M: **Synthesis, structure and antimicrobial activity of manganese(II) and cobalt(II) complexes of the polyether ionophore antibiotic sodium monensin A.** *J Inorg Biochem* 2008, **102:**26-32.

180. Prosser BLT, Palleroni NJ: **Naturally Occurring Acid Ionophores.** In *Polyether Antibiotics. Volume* **1**. New York: Marcel Dekker; 1982: 21-41

181. Okami Y, Okazaki T, Kitahara T, Umezawa H: **Studies on marine microorganisms. V A new antibiotic, aplasmomycin, produced by a *Streptomyces* isolated from shallow sea mud.** *J Antibiot (Tokyo)* 1976, **29:**1019-1025.

182. Cheng XC, Jensen PR, Fenical W: **Arenaric acid, a new pentacyclic polyether produced by a marine bacterium (*Actinomycetales*).** *J Nat Prod* 1999, **62:**605-607.

183. Tsuji N, Nagashima K, Terui Y, Tori K: **Structure of K-41B, a new diglycoside polyether antibiotic** *J Antibiot (Tokyo)* 1979, **32:**169-172.

184. Dorman DE, Hamill RL, Occolowitz JL, Terui Y, Tori K, Tsuji N: **Structure of polyether antibiotic A28695B** *J Antibiot (Tokyo)* 1980, **33:**252-255.

185. Seto H, Mizoue K, Otake N: **Studies on the ionophorous antibiotics. XXVI The assignements of the ^{13}C-NMR spectra of lonomycin A and mutalomycin.** *J Antibiot (Tokyo)*1980, **33:**979-988.

186. Westley JW: ***Polyether antibiotics: naturally occurring acid ionophores.*** New York: Marcel Dekker Inc.; 1982.

187. Loo I, Huijsdens X, Tiemersma E, Neeling A, Sande-Bruinsma N, Beaujean D, Voss A, Kluytmans J: **Emergence of Methicillin-Resistant *Staphylococcus aureus* of Animal Origin in Humans.** *Emerg Infect Dis* 2007 **13:**1834-1839.

188. Kevin II DA, Meujo DAF, Hamann MT: **Polyether ionophores: broad-spectrum and promising biologically active molecules for the control of drug-resistant bacteria and parasites.** *Expert Opin Drug Discov* 2009, **4:**109-146

189. Tsuji N, Nagashima K, J.P. 57,014,597, 1982.

190. Collins L, Franzblau SG: **Microplate alamar blue assay versus BACTEC 460 system for high- throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*.** *Antimicrob Agents Chemother* 1997, **41:**1004-1009.

191. Faulkner D: **Marine natural products.** *J Nat Prod Rep* 1998, **15:**113-158.

192. Gochfeld DJ, Hamann MT: **Isolation and biological evaluation of filiformin, plakortide F, and plakortone G from the Caribbean sponge *Plakortis* sp.** *J Nat Prod* 2001, **64:** 1477-1479.

193. Chen Y, Killday KB, McCarthy PJ, Schimoler R, Chilson K, Selitrennikoff C, Pomponi SA, Wright AE: **Three new peroxides from the sponge *Plakinastrella* species.** *J Nat Prod* 2001, **64:**262-264.

194. Berrue F, Thomas OP, Le Bon CF, Reyes F, Amade P: **New bioactive cyclic peroxides from the Caribbean marine sponge *Plakortis zyggompha*.** *Tetrahedron* 2005, **61:**11843-11849.

195. Harrison B, Crews P: **Cyclic polyketide peroxides and acyclic diol analogues from the sponge *Plakortis lita*.** *J Nat Prod* 1998, **61:**1033-1037.

196. Rudi A, Afanii R, Gravalos LG, Aknin M, Gaydou E, Vacelet J, Kashman Y: **Three new cyclic peroxides from the marine sponge *plakortis* aff simplex.** *J Nat Prod* 2003, **66:**682-685.

197. Sperry S, Valeriote FA, Corbett TH, Crews P: **Isolation and cytotoxic evaluation of marine sponge-derived norterpene peroxides.** *J Nat Prod* 1998, **61:**241-247.

198. Chen Y, McCarthy PJ, Harmody DK, Schimoler-O'Rourke R, Chilson K, Selitrennikoff C, Pomponi SA, Wright AE: **New bioactive peroxides from marine sponges of the family *Plakiniidae*.** *J Nat Prod* 2002, **65:**1509-1512.

199. Qureshi A, Salva J, Harper MK, J. FD: **New cyclic peroxides from the Philippine sponge *Plakinastrella* sp.** *J Nat Prod* 1998, **61:**1539-1542.

200. El Sayed KA, Hamann MT, Hashish NE, Shier WT, Kelly M, A. KA: **Antimalarial, antiviral, and antitoxoplasmosis norsesterterpene peroxide acids from the red sea sponge *Diacarnus erythraeanus*.** *J Nat Prod* 2001, **64:**522-524.

201. Davies-Coleman MT, Cantrell CL, Gustafson KR, Beutler JA, Pannell LK, Boyd MR: **Stolonic acids A and B, new cytotoxic cyclic peroxides from an Indian Ocean *Ascidian stolonica* species.** *J Nat Prod* 2000, **63:**1411-1413.

202. Holzwarth M, Trendel JM, Albrecht P, Maier A, Michaelis W: **Cyclic peroxides derived from the marine sponge *plakortis simplex*.** *J Nat Prod* 2005, **68:**759-761.

203. Perry TL, Dickerson A, Khan AA, Kondru RK, Beratan DN, Wipf P, Kelly M, Hamann MT: **New peroxylactones from the Jamaican sponge *Plakinastrella onkodes*, with inhibitory activity against the AIDS opportunistic parasitic infection *Toxoplasma gondii*.** *Tetrahedron* 2001, **57:**1483-1487.

204. Donald B. Stierle DJF: **Metabolites of three marine sponges of the genus *Plakortis*.** *J Org Chem* 1980, **45:**3396-3401.

205. Samoylenko V, Ashfaq MK, Jacob MR, Tekwani BL, Khan SI, Manly SP, Joshi VC, Walker LA, Muhammad I: **Indolizidine, Antiinfective and Antiparasitic Compounds from *Prosopis glandulosa* var. *glandulosa*** *J Nat Prod* 2009, ***72*:**92-98.

206. Reed LJ, Muench H: **A simple method of estimating fifty per cent endpoints** *Am J Hyg* 1938 **27:**493-497.

207. Stuyver L, Whitaker T, McBrayer T, Hernandez-Santiago B, Lostia S, Tharnish P, Ramesh M, Chu C, Jordan R, Shi J, et al: **Ribonucleoside analogue that blocks replication of bovine viral diarrhea and hepatitis C viruses in culture.** *Antimicrob Agents Chemother* 2003 **47:**244-254.

208. Makler MT, Hinrichs DJ: **Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitaemia.** *Am J Trop Med Hyg* 1993, **48 :**205-210.

209. Macedo MLR, Garcia VA, Freire MGM, Richardson M: **Characterization of a Kunitz trypsin inhibitor with a single disulfide bridge from seeds of *Inga laurina* (SW.) Willd.** *Phytochemistry* 2007, **68:**1104-1111.

210. ***Inga laurina*. In International Legume Database & Information Service** [http://www.ildis.org/LegumeWeb?version~10.01&LegumeWeb&tno~15672&genus~Inga&species~fagifolia#1]

211. Lokvam JC, T.P.; Grapov, D.; Coley, P.D.; Kursar, T.A.: **Galloyl depsides of tyrosine from young leaves of *Inga laurina*.** *J Nat Prod* 2007, **70:**134-136.

212. Blake SF: **Review of the genus *Diplostephium*.** In *Am J Bot. Volume* 15; 1928: 43-64

213. ***Diplostephium rhododendroides* Hieron** [http://data.gbif.org/species/15127100/]

214. Bout D, Aucouturier J, Ganne V, Hoebeke J, W.O. Patent 9,815,288 A1, 1998.

215. Ganne VA, J W.O. Patent 96/32964, 1996.

216. Trouve G, Dupuis L, W.O. Patent 2,002,080,840, 2002.

217. **Appendix 5 - FDA & EPA safety levels in regulations and guidance fish and fisheries products hazards and controls guidance**

218. **Outbreak alert 2008** [http://www.cspinet.org/new/pdf/outbreak_alert_2008_report_final.pdf]

219. **Outbreak alert 2009** [http://cspinet.org/new/pdf/outbreakalertreport09.pdf]

220. **Fish and seafood utilization** [http://www.fao.org/fishery/topic/424/en]

221. **What you need to know about mercury in fish and shellfish, March 2004, EPA-823-R-04-005** [http://www.fda.gov/food/resourcesforyou/consumers/ucm110591.htm]

222. Naliwaiko K, Araújo RL, da Fonseca RV, Castilho JC, Andreatini R, Bellissimo MI, Oliveira BH, Martins EF, Curi R, Fernandes LC, Ferraz AC: **Effects of fish oil on the central nervous system: a new potential antidepressant.** *Nutr Neurosci* 2004, **7:**91-99.

223. Morris MC, Evans DA, Bienias JL, Tangney CC, Bennett DA, Wilson RS, Aggarwal N, Schneider J: **Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease.** *Arch Neurol* 2003, **60:**940-946.

224. Barberger-Gateau P, Letenneur L, Deschamps V, Pérès K, Dartigues JF, Renaud S: **Fish, meat, and risk of dementia: cohort study.** *BMJ* 2002, **325:**932-933.

225. **Food is getting healthier and better, thanks to EU research** [http://europa.eu/rapid/pressReleasesAction.do?reference=IP/06/1759&format=HTML&aged=0&language=EN&guiLanguage=en]

226. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al: **Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomized open-label, blinded endpoint analysis.** *Lancet* 2007, **369:**1090-1098.

227. Cohen JT, Bellinger DC, Connor WE, Kris-Etherton PM, Lawrence RS: **A quantitative risk-benefit analysis of changes in population fish consumption.** *Am J Prev Med* 2005, **29:**325-334.

228. Mozaffarian D, Rimm EB: **Fish intake, contaminants, and human health: evaluating the risks and the benefits.** *JAMA* 2006, **296:**1885-1899.

229. **Fish & Fish Products Market Report 2008** [http://www.researchandmarkets.com/reportinfo.asp?cat_id=0&report_id=597257&q=seafood market&p=1]

230. **Food outlook - global market analysis - Global Information and early warning system on food and agriculture** [http://www.fao.org/docrep/012/ak341e/ak341e00.htm]

231. Delgado CL, Wada N, Rosegrant MW, Meijer S, Ahmed M: **Outlook for Fish to 2020: Meeting Global Demand. Washington DC: International Food Policy**

Research Institute & The WorldFish Center. 2003.

232. **Review of the state of world marine fishery resources Food and Agriculture Organization of the United Nations, FAO fisheries technical paper 457** [http://www.fao.org/docrep/009/y5852e/Y5852E00.htm#TOC]

233. **Food-related illness and death in the United States** [http://www.cdc.gov/ncidod/EID/vol5no5/mead.htm]

234. **Report to congress food and drug administration amendments Act of 2007 Public Law 110-85 Section 1006 - Enhanced aquaculture and seafood inspection. enhanced aquaculture and seafood inspection - Report to congress** [http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/SeafoodRegulatoryProgram/ucm150954.htm]

235. Campas M, Beatriz PS, Jean-Louis M: **Biosensors to detect marine toxins : Assessing seafood safety.** *Talanta* 2007, **72:**884-895.

236. Fleming LE, Broad K, Clement A, Dewailly E, Elmir S, Knap A, Pomponi SA, Smith S, Gabriele HS, Walsh P: **Oceans and human health: Emerging public health risks in the marine environment.** *Mar Pollut Bull* 2006, **53:**545-560.

237. **Marine Toxins** http://www.cdc.gov/ncidod/dbmd/diseaseinfo/marinetoxins_g.htm]

238. **Marine Biotoxins** [http://www.fao.org/docrep/007/y5486e/y5486e00.HTM]

239. **BBB - Various shellfish-associated toxins -Bad Bug Book: Foodborne pathogenic microorganisms and natural toxins handbook various shellfish-associated toxins** [http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodborne PathogensNaturalToxins/BadBugBook/ucm070008.htm .]

240. **Assurance of seafood quality** [http://www.fao.org/docrep/003/t1768e/T1768E00.htm#TOC]

241. Clark RF, Williams SR, Nordt SP, Manoguerra AS: **A review of selected seafood poisonings.** *Undersea Hyperb Med* 1999, **26:**175-184.

242. **Assessment and Management of Seafood Safety and Quality** [http://www.fao.org/docrep/006/y4743e/y4743e00.htm#Contents]

243. Patockaa J, Stredab L: **Brief review of natural nonprotein neurotoxins.** In *The ASA Newsletter*, vol. 88: Applied science and analysis Inc.; 2002.

244. García C, Pereira P, Valle L, Lagos N: **Quantitation of diarrhetic shellfish poisoning toxins in Chilean Mussel using pyrenyldiazomethane as fluorescent labeling reagent.** *Biol Res* 2003, **36:**171-183.

245. Fernández-Ortega JF, Morales-de los Santos JM, Herrera-Gutiérrez ME, Fernández-Sánchez V, Loureo PR, Rancaño AA, Téllez-Andrade A: **Seafood intoxication by tetrodotoxin: first case in Europe.** *J Emerg Medicine* 2009, **39:**612-617

246. Hwang DF, Noguchi T: **Tetrodotoxin poisoning.** *Adv Food Nutr Res* 2007, **52 :**141-236

247. Bachvaroff TR, Adolf JE, Squier AH, Harvey HR, Place AR: **Characterization and quantification of karlotoxins by liquid chromatography-mass spectrometry.** *Harm Algae* 2008, **7:**473-484.

248. Van Wagoner RM, Deeds JR, Satake M, Ribeiro AA, Place AR, Wright JLC: **Isolation and characterization of karlotoxin 1, a new amphipathic toxin from *Karlodinium veneficum*.** *Tetrahedron Lett* 2008, **49:** 6457-6461.

249. Van Wagoner RM DJ, Tatters AO. Place AR, Tomas CR, Wright JLC: **Structure and relative potency of several karlotoxins from *Karlodinium* v*eneficum*.** *J Nat Prod* 2010, **73:**1360-1365.

250. Peng J, Place AR, Yoshida W, Anklin C, Hamann MT: **Structure and absolute configuration of karlotoxin-2, an ichthyotoxin from the marine dinoflagellate *Karlodinium veneficum*.** *J Am Chem Soc* 2010, **132:** 3277-3279.

251. Abbott BC, Ballantine D: **The toxin from *Gymnodinium veneficum* Ballantine.** *J Mar Biol Assoc UK* 1957, **36:** 169-189.

252. Adolf JE BT, Krupatkina DN, Nonogaki H, Brown PJP, Lewitus AJ, Harvey HR, Place AR: **Species specificity and potential roles of *Karlodinium micrum* toxin** *Afr J Mar Sci* 2006, **28:** 415 - 419.

253. Sheng J ME, Katz J, Adolf JE, Place AR: **A dinoflagellate exploits toxins to immobilize prey prior to ingestion.** *Proc Natl Acad Sci USA* 2010, **107:** 2082-2087.

254. Su YC, Liu C: ***Vibrio parahaemolyticus*: A concern of seafood safety.** *Food Microbiol* 2007, **24:**549-558.

255. Feldhusen F: **The role of seafood in bacterial foodborne diseases.** *Microb Infect* 2000, **2:**1651-1660.

256. Shirai H, Ito H, Hirayama T, Nakamoto Y, Nakabayashi N, Kumagai K, Takeda Y, Nishibuchi M: **Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis.** *Infect Immun* 1990, **58:**3568-3573.

257. Andrews LS, DeBlanc S, Veal CD, Park DL: **Response of *Vibrio parahaemolyticus* O3: K6 to a hot water/cold shock pasteurization process**

Food Add Contam 2003, **20:**331-334.

258. **Interstate Shellfish Sanitation Conference *Vibrio vulnificus* fact sheet-Health care providers** [http://www.issc.org/client_resources/Education/VvFactSheet.pdf]

259. Samir M, Haq BS, Hari HD: **Chronic liver disease and consumption of raw oysters: A potentially lethal combination — A review of *Vibrio vulnificus* septicemia.** *Am J Gastroenterol* 2005, **100:**1195- 1199

260. Norhana MNW, Poole SE, Deeth HC, Dykes GA: **Prevalence, persistence and control of *Salmonella* and *Listeria* in shrimp and shrimp products: A review.** *Food Control* 2010, **21:**343-361.

261. FDA: **CPG Sec. 555.300 foods, except dairy products - Adulteration with *Salmonella*.** In *Sec 555300 Compliance Policy Guide* (FDA ed., vol. Sec 555.300 Compliance Policy Guide; 1980.

262. FDA: **CPG Sec. 540.600 Fish, shellfish, crustaceans and other aquatic animals - Fresh, frozen or processed - Methyl mercury.** (FDA ed., vol. Sec 540.600 Compliance Policy Guide; 2005.

263. FDA: **Domestic Fish and Fishery Products Inspection Program.** In *Compliance Program 7303842 or Sec 540250* (FDA ed., vol. Compliance Program 7303.842 or Sec 540.250 Compliance Policy Guide; 2007.

264. **Committee on evaluation of the safety of fishery products. Seafood safety (1991) institute of medicine (iom). Food and nutrition board institute of medicine** [http://www.nap.edu/openbook.php?record_id=1612&page=30]

265. Potasman I, Paz A, Odeh M: **Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective.** *Clin Infect Dis* 2002, **35:**921-928.

266. Halliday ML, Kang LY, Zhou TK, Hu MD, Pan QC, Fu TY, Huang YS, Hu SLI: **An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China.** *J Infect Dis* 1991, **164:**852-859.

267. **Norovirus: Technical Fact Sheet** [http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm]

268. Kar D, Sur P, Mandal SK, Saha T, Kole RK: **Assessment of heavy metal pollution in surface water.** *Int J Environ Sci Tech* 2008, **5** 119-124.

269. **Heavy metal pollution - heavy metal pollution is more common than you think** [http://www.fairfaxcounty.gov/nvswcd/newsletter/heavymetal.htm]

270. **Scientific opinion on arsenic in food question number: EFSA-Q 2008-425** [

http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902959840.htm]

271. Daniels JL, Longnecker MP, Rowland AS, Golding J: **Fish intake during pregnancy and early cognitive development of offspring.** *Epidemiology* 2004, **15:**394-402.

272. Oken E, Wright RO, Kleinman KP, Bellinger D, Amarasiriwardena CJ, Hu H, Rich-Edwards JW, Gillman MW: **Maternal fish consumption, hair mercury, and infant cognition in a U.S. Cohort.** *Environ Health Perspect* 2005, **113:**1376-1380.

273. Myers GJ, Davidson PW, Cox C, Shamlaye CF, Palumbo D, Cernichiari E, Sloane-Reeves JW, G.E., Kost J, Huang LS, Clarkson TW: **Prenatal methylmercury exposure from ocean fish consumption in the Seychelles child development study.** *Lancet* 2003, **361:**1686-1692.

274. Smith AG, Gangolli SD: **Organochlorine chemicals in seafood: occurrence and health concerns.** *Food Chem Tox* 2002, **40:** 767-779.

275. Hites RA, Foran JA, Carpenter DO, Hamilton MC, Knuth BA, Schwager SJ: **Global assessment of organic contaminants in farmed salmon** *Science* 2004, **303:**226-229.

276. **How FDA regulates seafood: FDA detains imports of farm-raised Chinese seafood** [http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm094558.htm]

277. FDA: **CPG Sec. 615.200 Proper Drug Use and Residue Avoidance by Non-Veterinarians.** In *Sec 615200 Compliance Policy Guide* (FDA ed., vol. Sec 615.200 Compliance Policy Guide; 1993.

278. FDA: **Chorionic gonadotropin for injection; chorionic gonadotropin suspension.** In *21 CFR 5221081* (FDA ed., vol. 21 CFR 522.1081; 2005.

279. FDA: **Formalin solution.** In *21 CFR 5291030* (FDA ed., vol. 21 CFR 529.1030. Washington, DC.: U.S. Government Printing Office; 1999.

280. FDA: **Tricaine methanesulfonate.** In *21 CFR 5292503* (FDA ed., vol. 21 CFR 529.2503. Washington, DC: U.S. Government Printing Office; 1999.

281. FDA: **Oxytetracycline.** In *21 CFR 556500* (FDA ed., vol. 21 CFR 556.500. Washington, DC: U.S. Government Printing Office; 1999.

282. FDA: **Sulfamerazine.** In *21 CFR 558582* (FDA ed., vol. 21 CFR 558.582. Washington, DC: U.S. Government Printing Office; 1999.

283. FDA: **Sulfamerazine.** In *21 CFR 556660* (FDA ed., vol. 21 CFR 556.660. Washington, DC: U.S. Government Printing Office; 2005.

284. FDA: **Sulfadimethoxine, ormetoprim.** In *21 CFR 558575* (FDA ed., vol. 21 CFR 558.575. Washington, DC: U.S. Government Printing Office; 1999.

285. FDA: **Sulfadimethoxine.** In *21 CFR 556640* (FDA ed., vol. 21 CFR 556.640. Washington, DC: U.S. Government Printing Office; 1999.

286. Council Regulation E: **Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.** In *No 2377/90* ((EEC) CR ed., vol. No 2377/90 1990.

287. The European Parliament and the Council of the European Union: **Corrigendum to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.** In *Regulation (EC) No 853/2004 (European standards)*; 2004.

288. The Council of the European communities: **Council directives 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve molluscs.** In *EU Directive 91/492/EEC*; 1991.

289. The Council of the European communities: **Health conditions for the production and the placing on the market of fishery products.** In *Council directives 91/493/EEC* (EEC ed.; 1991.

290. FDA: **Tolerances for polychlorinated biphenyls (PCB's).** In *21 CFR 10930* (FDA ed., vol. 21 CFR 109.30 Washington, DC.: U.S. Government Printing Office; 1998.

291. FDA: **PG Sec. 575.100 pesticide residues in food and feed - Enforcement criteria.** In *Sec 575100 Compliance Policy Guide* (FDA ed., vol. Sec 575.100 Compliance Policy Guide; 1986.

292. Agency UEP: **Diquat; tolerances for residues.** (Agency UEP ed., vol. 40 CFR 180.226: U.S. Government Printing Office; 2005.

293. FDA: **Glyphosate; tolerances for residues.** In *40 CFR 180364* (FDA ed., vol. 40 CFR 180.364 Washington, DC.: U.S. Government Printing Office; 1997.

294. FDA: **Fluridone; tolerances for residues.** In *40 CFR 180420* (FDA ed., vol. 40 CFR 180.420 Washington, D.C.: U.S. Government Printing Office; 1997.

295. FDA: **Simazine; tolerances for residues.** In *40CFR180213a* (FDA ed., vol. 40CFR180.213a. Washington, DC.: U.S. Government Printing Office; 1997.

296. Quilliam MA: **The role of chromatography in the hunt for red tide toxins.** *J Chromat A* 2003, **1000:** 527-548.

297. Gerssen A, Mulder PPJ, McElhinney MA, de Boer J: **Liquid chromatography-**

tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A* 2009, **1216:** 1421-1430.

298. Syaifudin ARM, Jayasundera KP, Mukhopadhyay SC: **A low cost novel sensing system for detection of dangerous marine biotoxins in seafood.** *Sens Actuators, B* 2009, **137:**67-75.

299. Zhou Y, Li YS, Pan FG, Zhang YY, Lu SY, Ren HL, Li ZH, Liu ZS, Zhang JH, : **Development of a new monoclonal antibody based direct competitive enzyme-linked immunosorbent assay for detection of brevetoxins in food samples.** *Food Chem* 2010, **118:** 467-471.

300. Önal A: **Analytical, nutritional and clinical methods a review: current analytical methods for the determination of biogenic amines in foods.** *Food Chemist* 2007, **103:**1475-1486.

301. Peters RJB, Bolck YJC, Rutgers P, Stolker AAM, Nielen MWF: **Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry.** *J Chromat A* 2009, **1216:** 8206-8216.

302. Smith S, Gieseker C, Reimschuessel R, Decker CS, Carson MC: **Simultaneous screening and confirmation of multiple classes of drug residues in fish by liquid chromatography-ion trap mass spectrometry.** *J Chromat A* 2009, **1216:** 8224-8232.

303. **Bacteriological Analytical Manual (BAM) - Revision A. Last Updated: 05/14/2009**
[http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm]

304. Espiñeira M, Atanassova M, Vieites JM, Santaclara FJ: **Validation of a method for the detection of five species, serogroups, biotypes and virulence factors of *Vibrio* by multiplex PCR in fish and seafood.** *Food Microbiol* 2010, **27:**122-131.

305. Kural AG, Shearer AE, Kingsley DH, Chen H: **Conditions for high pressure inactivation of Vibrio parahaemolyticus in oysters.** *Int J Food Microbiol* 2008, **127:**1-5.

306. Calci KR, Meade GK, Tezloff RC, Kingsley DH: **High-pressure inactivation of hepatitis A virus within oysters.** *Appl Environ Microbiol* 2005, **71:** 339-343.

307. Kingsley DH, Chen H: **Influence of pH, salt, and temperature on pressure inactivation of hepatitis A virus.** *Int J Food Microbiol* 2009, **130:**61-64.

308. Li D, Tang Q, Wang J, Wang Y, Zhao Q, Xue C: **Effects of high-pressure processing on murine norovirus-1 in oysters (Crassostrea gigas) in situ.**

Food Control 2009, **20:**992-996.

309. Murchie LW, Kelly AL, Wiley M, Adair BM, Patterson M: **Inactivation of a calicivirus and enterovirus in shellfish by high pressure.** *IFSET* 2007, **8:**213-217.

310. Meujo DAF, Kevin D, Peng J, Bowling JJ, Liu J, Hamann MT: **Reducing oyster-associated bacteria levels using supercritical fluid CO_2 as an agent of warm pasteurization.** *Int J Food Microbiol* 2010 **138:**63-70.

311. Quan Y, Choi KD, Chung D, Shin IS: **Evaluation of bactericidal activity of weakly acidic electrolyzed water (WAEW) against *Vibrio vulnificus* and *Vibrio parahaemolyticus*.** *Int J Food Microbiol* 2010, **136:**255-260

312. Wang D, Zhang D, Chen W, Yu S, Shi X: **Retention of *Vibrio parahaemolyticus* in oyster tissues after chlorine dioxide treatment.** *Int J Food Microbiol* 2010, **137:**76-80.

313. Phuvasate S, Su YC: **Effects of electrolyzed oxidizing water and ice treatments on reducing histamine-producing bacteria on fish skin and food contact surface.** *Food Control* 2010, **21:**286-291.

314. Huang YR, Hung YC, Hsu SY, Huang YW, Hwang DF: **Application of electrolyzed water in the food industry.** *Food Control* 2008, **19:**329-345.

315. Tahiri I, Desbiens M, Kheadr E, Lacroix C, Fliss I: **Comparison of different application strategies of divergicin M35 for inactivation of *Listeria monocytogenes* in cold-smoked wild salmon.** *Food Microbiol* 2009, **26:** 783-793.

316. Matamoros S, Pilet MF, Gigout F, Prévost H, Leroi F: **Selection and evaluation of seafoodborne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria.** *Food Microbiol* 2009, **26:** 638-644.

317. Pinto AL, Fernandes M, Pinto C, Albano H, Castilho F, Teixeira P, Gibbs PA: **Characterization of anti-*Listeria* bacteriocins isolated from shellfish: Potential antimicrobials to control non-fermented seafood.** *Int J Food Microbiol* 2009, **129:**50-58.

318. Song HP, Kim B, Jung S, Choe JH, Yun H, Kim YJ, Jo C: **Effect of gamma and electron beam irradiation on the survival of pathogens inoculated into salted, seasoned, and fermented oyster.** *LWT - Food Sci Technol* 2009, **42:** 1320-1324.

319. Kim B, Song HP, Choe JH, Jung S, Jang A, Kim YJ, Jo C: **Application of electron-beam irradiation on the production of salted and seasoned short-necked clam, Tapes Pilippinarum, for safe distribution.** *Rad Phys Chem* 2009, **78:** 585-587.

320. Medina M, Cabeza MC, Bravo D, Cambero I, Montiel R, OrdóñezNuñez JAM, Hoz LA: **Comparison between E-beam irradiation and high pressure treatment for cold-smoked salmon sanitation: Microbiological aspects.** *Food Microbiol* 2009, **26:**224-227.

321. Mahmoud BSM: **Effect of X-ray treatments on inoculated *Escherichia coli* O157: H7, *Salmonella enterica*, *Shigella flexneri* and *Vibrio parahaemolyticus* in ready-to-eat shrimp.** *Food Microbiol* 2009a, **26:**860-864.

322. Mahmoud BSM: **Reduction of *Vibrio vulnificus* in pure culture, half shell and whole shell oysters (*Crassostrea virginica*) by X-ray.** *Int J Food Microbiol* 2009b, **130:**135-139.

323. Gudbjornsdottir B, Jonsson A, Hafsteinsson H, Heinz V: **Effect of high-pressure processing on *Listeria* spp. and on the textural and microstructural properties of cold smoked salmon.** *LWT - Food Sci Technol* 2010, **43:** 366-374.

324. Brutti A, Rovere P, Cavallero S, D'Amelio S, Danesi P, Arcangeli G: **Inactivation of Anisakis simplex larvae in raw fish using high hydrostatic pressure treatments.** *Food Control* 2010, **21:**331-333.

325. Schirmer BC, Heiberg R, Eie T, Møretrø T, Maugesten T, Carlehøg M, Langsrud S: **A novel packaging method with a dissolving CO_2 headspace combined with organic acids prolongs the shelf life of fresh salmon.** *Int J Food Microbiol* 2009, **133:** 154-160.

326. Norhana MNW, Azman AMN, Poole SE, Deeth HC, Dykes GA: **Effects of bilimbi (*Averrhoa bilimbi* L.) and tamarind (*Tamarindus indica* L.) juice on *Listeria monocytogenes* Scott A and *Salmonella typhimurium* ATCC 14028 and the and the sensory properties of raw shrimps.** *Int J Food Microbiol* 2009, **136:** 88-94.

327. Chaiyakosa S, Charernjiratragul W, Umsakul K, Vuddhakul V: **Comparing the efficiency of chitosan with chlorine for reducing *Vibrio parahaemolyticus* in shrimp.** *Food Control* 2007, **18:**1031-1035.

328. Ye M, Neetoo H, Chen H: **Effectiveness of chitosan-coated plastic films incorporating antimicrobials in inhibition of *Listeria monocytogenes* on cold-smoked salmon.** *Int J Food Microbiol* 2008, **127:** 235-240

329. Terio V, Martella V, Moschidou P, Di Pinto P, Tantillo G, Buonavoglia C: **Food Norovirus in retail shellfish Microbiology.** *Food Microbiol* 2010, **27:** 29-32.

330. Bakar J, Yassoralipour A, Bakar FA, Rahman RA: **Biogenic amine changes in barramundi (*Lates calcarifer*) slices stored at 0 °C and 4 °C.** *Food Chem* 2010, **119:**467-470

331. **Post-harvest oyster processing technologies - fact sheet for seafood dealers and processors** [http://www.dmr.state.ms.us/Fisheries/Seafood-Technology/pdfs/fact-sheet-postharvest-oyster-processing.pdf]

332. Prapaiwong N, Wallace RK, Arias CR: **Bacterial loads and microbial composition in high pressure treated oysters during storage.** *Int J Food Microbiol* 2009, **131:** 145-150.

333. Mahmoud BS, Burrage DD: **Inactivation of *Vibrio parahaemolyticus* in pure culture, whole live and half shell oysters (*Crassostrea virginica*) by X-ray** *Lett Appl Microbiol* 2009c, **48:**572-578.

334. De Roda Husman AM, Lodder-Verschoor F, van den Berg HH, Le Guyader FS, van Pelt H, van der Poel WH, Rutjes SA: **Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks.** *J Food Prot* 2007, **70:**967-974.

335. Le Guyader FS, Parnaudeau S, Schaeffer J, Bosch A, Loisy F, Pommepuy M, Atmar RL: **Detection and Quantification of Noroviruses in Shellfish** *Appl Environ Microbiol* 2009, **75:**618-624.

336. Gentry J, Vinjé J, Lipp EK: **A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex environmental samples.** *J Virol Methods* 2009, **156:**59-65.

337. Xiaoxia K, Qingping W, Dapeng W, Jumei Z: **Simultaneous detection of norovirus and rotavirus in oysters by multiplex RT-PCR** *Food Control* 2008, **19:**722-726.

338. Kamihira M, Taniguchi M, Kobayashi T: **Sterilization of microorganisms with supercritical carbon dioxide.** *Agr Biol Chem* 1987 **51:** 407-412.

339. Vugia D, Hadler J: **Preliminary foodnet data on the incidence of foodborne illnesses** pp. 325-329: MMWR; 2002:325-329.

340. Hu X, Mallikarjunan P, Koo J, Andrews LS, Jahncke ML: **Comparison of kinetic models to describe high pressure and gamma irradiation used to inactivate *Vibrio vulnificus* and *Vibrio parahaemolyticus* prepared in buffer solution and in whole oysters.** *J Food Prot* 2005, **68:** 292-295.

341. Linton M, Mc Clements JMJ, Patterson MF: **Changes in the microbiological quality of shellfish, brought about by treatment with high hydrostatic pressure.** *Int J Food Sci Tech* 2003, **38:** 713-727.

342. Fraser D: **Bursting bacteria by release of gas pressure** *Nature* 1951, **167:**33-34.

343. Fages J, Poirier B, Barbier Y, Frayssinet P, Joffret ML, Majewski W, Bonel G,

Larzul D: **Viral inactivation of human bone tissue using supercritical fluid extraction.** *Am Soc Artif Intern Organs J* 1998, **44:** 289-293.

344. Hemmer JD, Drews MJ, LaBerge M, Matthews MA: **Sterilization of bacterial spores by using supercritical carbon dioxide and hydrogen peroxide.** *J Biomed Mater Res Part B* 2006, **80B:**511-518.

345. Damar S, Balaban MO: **Review of dense phase CO_2 technology: microbial and enzyme inactivation, and effects on food quality.** *J Food Sci* 2006, **71:** R1-11.

346. Garcia-Gonzalez L, Geeraerd AH, Spilimbergo S, Elst K, Van Ginneken L, Debevere J, Van Impe JF, Devlieghere F: **High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future.** *Int J Food Microbiol* 2007, **117:** 1-28.

347. Spilimbergo S, Bertucco A: **Non-thermal bacteria inactivation with dense CO_2.** *BiotechBioeng* 2003, **84:** 627-638.

348. Dunlap PV: **Regulation of luminescence by cyclic AMP in cya-like and crp-like mutants of *Vibrio fischeri*.** *J Bacteriol* 1989, **171:** 1199-1202.

349. Klein G, Mijewshi M, Krezewska J, Czeczatka M, Lipinska B: **Cloning and characterization of the dnaK heat shock operon of the marine bacterium *Vibrio harveyi*.** *Mol Genet Genomics* 1998, **259:** 179-189.

350. Lee DJ, Xu X, Lane RM, P. Z: **Shape Analysis for an Automatic Oyster Grading System".** *Proceedings of SPIE* 2004, **5606-05**.

351. Collins CH, Lyne PM, Grange JM: *Microbiological methods.* Oxford, UK: Butterworth Heinemann; 1995.

352. Damar S, Balaban MO: **Review of dense phase CO_2 technology: microbial and enzyme inactivation, and effects on food quality.** *J Food Sci* 2006, **71:**R1-R11.

353. Dillow AK, Dehghani F, Hrkach JS, N.R. F, Langer R: **Bacterial inactivation by using near- and supercritical carbon dioxide.** *Proc Natl Acad Sci USA* 1999, **96:**10344-10348.

354. Smelt JPPM, Rijke GGF: **High pressure treatment as a tool for pasteurization of foods.** *High Pressure and Biotechnology* 1992, **224:**361-364.

355. Lin H-M, Cao NJ, Chen L-F: **Antimicrobial effect of pressurized carbon dioxide on *Listeria monocytogenes*.** *J Food Sci* 1994, **59:** 657-659.

356. **Cold Storage Chart. National Food Safety Month. USDA food Safety and Inspection Service/FDA Center for Food Safety and Applied Nutrition**

[http://www.foodsafety.gov/~fsg/f01chart.html]

357. Jakabi M, Gelli DS, Torre JC, Rodas MA, Franco BD, Destro MT, Landgrafi M: **Inactivation by ionizing radiation of *Salmonella enteritidis*, *Salmonella infantis*, and *Vibrio parahaemolyticus* in oysters (*Crassostrea brasiliana*).** *J Food Prot* 2003, **66:**1025-1029.

358. Quan C, Li S, Tian S, Xu H, Lin A, Gu L: **Supercritical fluid extraction and clean-up of organochlorine pesticides in ginseng.** *J Supercrit Fluids* 2004, **31:**149-157.

359. Brooks MW, Uden PC: **The determination of abamectin from soil and animal tissue by supercritical fluid extraction and fluorescence detection.** *Pestic Sci* 1995, **43:** 141-146.

VITA

I was born in the small Cameroon town named Nkongsamba, the second child of five siblings. As a result of a medical malpractice, I became physically disabled (5-10%) at the age of two, an event which changed the course of my life forever. Far from holding me back, my disability had a positive impact on my life. As the public health of countries around the globe worsen, the need for well-trained professionals in the field of drug discovery is urgent. I chose this field several years ago because of a deep desire to make a significant contribution towards the improvement of our degrading health situation. This desire has turned into a passion that has driven me to excel in this field for several years now. So far, I have earned several outstanding awards.

I have been fortunate to have a very broad/multidisciplinary education. Topics covered as part of my training include biochemistry, microbiology, cell molecular biology, genetics and pharmaceutical sciences (Pharmacognosy). I received my first university degree, a BS in Biochemistry, from The University of Yaoundé-Cameroon in 1998. Next, I received a Masters degree (2000) and then a DEA (2001), both in Biochemistry, from the same university. In April 2003, I was awarded a fully-funded two years Fulbright scholarship, an opportunity to pursue my education in the USA. I joined the microbiology program of The University of Hawaii at Manoa during the fall of 2003 and graduated with a second Masters degree in December 2005. Finally, on January 1, 2006, I joined the pharmaceutical sciences (pharmacognosy) PhD program of the School of Pharmacy - University of Mississippi.

In addition to my extensive biomedical education, I have an outstanding bench

experience in the field of pharmaceutical sciences. In fact, I possess over 10 years of research experience (drug discovery).