A study of *Plocamium corallorhiza* secondary metabolites and their Biological activity

Thesis

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Table of contents

Chapter One: General Introduction

List of Figures

List of Tables

List of Schemes

List of Abbreviations

COSY	^1H-^1H Homonuclear Correlation Spectroscopy
CC	Column chromatography
DEPT	Distortionless Enhancement of Polarisation Transfer
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EIMS	Electron Impact Mass Spectroscopy
EtOAc	Ethyl Acetate
Fctn	Fraction
GC-MS	Gas Chromatography-Mass Spectrometry
H/E	Hexane/ethyl acetate
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
IC_{50}	Inhibitory concentration 50%
IR	Infrared
LRMS	Low Resolution Mass Spectrometry
MeOH	Methanol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser enhancement spectroscopy
Prtn	Partition
SCUBA	Self Contained Underwater Breathing Apparatus
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHC01	Oesophageal cancer cells

d	doublet
dd	double doublet
eV	electron Volt
m	Multiplet
m/z	mass to charge ratio
multi	Multiplicity
q	Quartet
s	Singlet
t	Triplet

Abstract

Seaweeds of the genus *Plocamium* are known to produce a variety of halogenated monoterpenes. In addition to their ecological role as feeding deterrents, biological activities reported for these compounds include antibacterial, antialgal, antifungal and anticancer activities. An investigation of the non-polar extracts of the seaweed *Plocamium corallorhiza* resulted in the isolation of six known halogenated monoterpene compounds, 4-bromo-5-bromomethyl-1-chlorovinyl-2, 5-dichloro-methylcyclohexane (**2.68**), 1,4,8-tribromo-3,7-dichloro-3,7-dimethyl-1,5-octadiene (**2.67**), 8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (**2.66**), 4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene (**2.64**), 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (**2.65**) and 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**2.63**) as well as eight new compounds, including five halogenated monoterpene aldehydes. The new compounds were identified by 1D and 2D NMR spectroscopic techniques as: 8-Bromo-6,7-dichloro-3,7-dimethyl-octa-2,4-dienal (**2.72**), 8-Bromo-1,1,2,7-tetrachloro-3,7-dimethyl-octa-3,5-diene (**2.70**), 4,8-Dichloro-3,7-dimethyl-octa-2,4,6-trienal (**2.74**), 4-Bromo-8-chloro-3,7-dimethyl-octa-2,6-dienal (**2.76**), 8-Bromo-4-chloro-3,7-dimethyl-octa-2,4,6-trienal (**2.75**), 4-Bromo-1,3,6,7-tetrachloro-3,7-dimethyl-octa-1,4-diene (**2.71**), 8-Bromo-1,3,4,7-tetrachloro-3,7-dimethyl-octa-1,5-diene (**2.69**), 4,6-Dibromo-3,7-dimethyl-octa-2,7-dienal (**2.73**).

All compounds were screened for antimicrobial activity, brine shrimp lethality and cytotoxicity towards oesophageal cancer cells. Compound **2.68** was toxic to brine shrimp larvae at a concentration of 50 μg/mL. It also showed promising activity towards oesophageal cancer cells with an IC_{50} of 2 μg/mL.

Chapter One

General Introduction

1.1 Natural Products: What are they?

Primary metabolites (sugars, amino acids, nucleotides) are organic substances produced by all living organisms and play a central role in the metabolism and reproduction of the organism. The pathways for the metabolism of these metabolites are known as primary metabolism (Dewick, 2000). While primary metabolites are encountered in all organisms, secondary metabolites have a limited distribution. Secondary metabolites, also called natural products, are thought to be essential for the long-term survival of the organism producing them, and several hypotheses have been put forward to clarify their role (Williams *et al.*, 1989). It is because of their 'subsidiary' role in the biology of the organism that they were called secondary metabolites (Haslam, 1979; 1986). The distinction between primary and secondary metabolites is quite artificial because the two systems are intimately related, with primary metabolism producing precursors for secondary metabolism (Mann, 1987).

Secondary metabolites are generally classified by their structural characteristics and biosynthetic pathway into one of the following categories: phenolics (aromatic and polyaromatic), isoprenoids (terpenoids and steroids), polyketides or alkaloids (Haslam, 1986).

1.2 The Importance of Natural products in drug development

1.2.1 Plant Natural Products

From antiquity man has relied on nature as a source of food, clothing, shelter, means of transportation and also medicines. As early as about 2600 BC, plant derived substances, which include oils from *Cedrus* species (cedar), *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (liquorice), *Commiphora* sp. (myrrh) and *Papaver somniferum* (poppy juice) were used to treat ailments ranging from coughs and colds to parasitic infections and inflammation. Egyptian medicine dates back to 2900 BC and more than 700 drugs, mostly from plants, were recorded by the Egyptian Pharmaceutical record, "Eber Papyrus" By 1500 BC the delivery of drugs had developed to include the use of formulations such as gargles, snuffs, poultices, infusions, pills and ointments, with milk, wine, honey and beer being the common vehicles. The Chinese *Materia Medica* records about 2000 plant-derived substances used as traditional medicines since about 1100 BC (Dev, 1999). Some of these early herbal remedies are still used today.

The spectrum of conditions in which natural products are used has spread to include: anti-infectives (antibacterials, antifungals, antivirals and antimalarials), cardiovascular agents (antihypertensives and cholesterol lowering agents, Alberts *et al.*, 1980), analgesics and antineoplastics. The involvement of terrestrial natural products in the development of drugs is highlighted in brief by some examples below.

- Antiinfectives agents:

Fleming's serendipitous discovery of the antibiotic penicillin has provided the impetus to finding antiinfective agents particularly antibiotics (Mateles *et al.*, 1998). Further work and development of penicillins by modification of the parent structure lead to the synthesis of many different penicillins. However, the development of antibiotic resistance by microorganisms meant more work was needed to discover compounds, which could circumvent the enzymes (for example, penicillinases and lactamases) produced by bacteria to inactivate antibiotics. Cephalosporins, which were isolated from the actinomycete, *Cephalosporium acremonium,* were the next generation of antibiotics (Newton *et al.*, 1956) and to date up to 80% of antibiotics on the market are natural products or derivatives of natural products. The *Cinchona* alkaloid, quinine, was used as a template for the further development of antimalarial drugs of the chloroquine/mefloquine type. A more recent antimalarial agent, artemisinin, was isolated from *Artemisia annua* (wormwood) thanks to the inspection of Chinese herbal medicine data (Clark, 1996).

- Analgesics/Painkillers:

Opiates have been used for pain management as "poppy juice" (extract of *Papaver somniferum*), since about 6000 years ago, but because of their addictiveness, other sources of strong painkillers without abuse potential have been sought. Morphine was first isolated in 1806 and it is still used in management of acute and chronic pain. Attempts to synthesize compounds with the same potency as morphine but with reduced addiction potential have failed, with the exception of the semi-synthetic compound buprenorphine, which is approximately 25-50 times more potent and has a lower abuse potential. The study of the mode of action of opiates, particularly morphine, has led to the identification of opiate receptors and hence a better understanding of pain management and pharmacology (Martin, 1967; Terrenius, 1978).

- Antineoplastics:

Plants have a long history in the treatment of cancer (Hartwell, 1982), and among the best-known compounds used are the vinca alkaloids, vincristine and vinblastine, isolated from the Madagascan periwinkle, *Catharanthus roseus. C. roseus* was used by many cultures in the treatment of diabetes, and vincristine and vinblastine, together with two other active alkaloids, vinleurosine and vinrosidine, were isolated during the investigation of the plant for potential hypoglycaemic agents. Therefore, the discovery of the initial two compounds maybe indirectly attributed to the observation of an unrelated medicinal use of the plant source (Cragg *et al.*, 1994). Selective chemical modifications of these molecules has lead to two new semi-synthetic compounds, vinorelbine and vindesine, which have been approved for treatment of cancer in several European countries (Pezzuto, 1997).

Work on the roots of *Podophyllum* species, which were used by the early American and Asian cultures to treat skin cancers and warts lead to the isolation of epipodophyllotoxin, (Cragg *et al.*, 1994), which is the parent of two clinically active agents, etoposide and teniposide. One of the new anticancer drugs on the market, introduced in 1993 is paclitaxel (Taxol®), which was isolated from the bark of *Taxus brevifolis* (Cragg *et al*, 1993). There still are many natural products or their synthetic analogues at different stages of preclinical or clinical development, for example, flavopiridol, which is in phase I/II clinical trials against a broad spectrum of tumors (Christian *et al.*, 1997). Although this compound is synthetic it is based on the structure of a novel natural product, rohitukine, isolated from *Dysoxylum binectariferum* (Naik *et al.*, 1988).

1.2.2 Marine Natural Products

Terrestrial organisms have been the heart of drug development, but with the advent of SCUBA in the latter half of the twentieth century the previously unexplored marine environment has become more accessible (Munro *et al.*, 1999).

Initial investigation of the marine biota began in the 1950's with Bergmann (Bergmann and Feeney, 1950; 1951) stimulating interest through the 1960's and 1970's (Faulkner, 1979). Marine natural products chemistry developed rapidly in the late 1970's and the 1980's and has matured in the 1990's (Faulkner, 2000) yielding thousands of previously undescribed secondary metabolites, (Andersen *et al.*, 2000; Jaspars, 1999; Faulkner, 2000; 2001; 2002). These studies demonstrated the rich chemistry and bioactivity of several novel chemical classes of marine natural products (Carte, 1996).

From an early stage, chemical studies on marine organisms had been concerned with defining the ecological role of their secondary metabolites thus giving rise to the development of chemical ecology (Paul, 1992),. This collaboration of natural products chemists and biologists lead to questions such as, "why are certain marine invertebrates not prey for higher organisms?" Why do fish not prey on some algae? Why do sponges not grow over each other? The quest to answer these questions broadened the scope of marine natural products chemistry (Newman and Cragg, 2004). The first notable bioactive marine compounds, C-nucleosides, spongouridine and spongothymidine, isolated from the Carribean sponge, *Tethya crypta*, showed antiviral activity (Bergmann and Feeney, 1950; 1951). This discovery led to the change in the thinking then, that, "for a nucleoside to have biologically activity, it has to have ribose or deoxyribose as the sugar, but that the base could comprise a multiplicity of heterocycles and even carbocycles". This change in thinking precipitated in an explosion of compounds described (Suckling, 1991; Newman *et al.*, 2000). At the peak of research on marine compounds in the 1980`s, compounds where screened for cancer activity through funding made available by the National Cancer Institute (NCI). Compounds such as didemnim B and dolostatin 10 progressed to advanced clinical trials but were later withdrawn because of toxicity. Table 1.1 shows some of the many marine derived compounds with are currently in preclinical or clinical trials stage and it is probable that some will arrive on the market soon. It can be seen from the table that the spectrum of medical conditions in which these compounds are being tested is broad, but no doubt cancer is still the primary target.

Table 1.1 Status of Marine-Derived Natural Products in clinical and Pre-clinical trials (adapted from Newman and Cragg, 2004).

Name	**Source**	**Status (disease)**	**Comment**
didemnin B	*Trididemnum solidum*	Phase II (cancer)	dropped middle 90s
dolastatin 10	*Dolabella auricularia* (marine microbe derived; cyanophyte)	Phase I/II (cancer)	many derivatives made synthetically; no positive effects in Phase II trials; no further trials known
girolline	*Pseudaxinyssa cantharella*	Phase I (cancer)	discontinued due to hypertension
bengamide derivative	*Jaspis* sp.	Phase I (cancer)	licensed to Novartis, Met-AP1 inhibitor, withdrawn 2002
			cryptophycins (also arenastatin A)
	Nostoc sp. & *Dysidea Arenaria*	Phase I (cancer)	from a terrestrial cyanophyte, but also from a sponge as arenastatin A; synthetic derivative licensed to Lilly by Univ. Hawaii, but withdrawn 2002
bryostatin 1	*Bugula neritina*	Phase II (cancer)	now in combination therapy trials; licensed to GPC Biotech by Arizona State Univ.; may be produced by bacterial symbiont
TZT-1027	synthetic dolastatin	Phase II (cancer)	also known as auristatin PE and soblidotin
cematodin	synthetic derivative of dolastatin 15	Phase I/II (cancer)	some positive effects on melanoma pts in Phase II; dichotomy on fate
ILX 651, synthatodin	synthetic derivative of dolastatin 15	Phase I/II (cancer)	in Phase II for melanoma, breast, NSCLC
ecteinascidin 743	*Ecteinascidia turbinata*	Phase II/III (cancer)	in 2003licensed to Ortho Biotech (J&J); produced by partial synthesis from microbial metabolite
aplidine	*Aplidium albicans*	Phase II (cancer)	dehydrodidemnin B, made by total synthesis
E7389	*Lissodendoryx* sp	Phase I (cancer)	Eisai's synthetic halichondrin B derivative
discodermolide	*Discodermia dissolute*	Phase I (cancer)	licensed to Novartis by Harbor Branch Oceanographic Institution
kahalalide F	*Eylsia rufescens/ Bryopsis* sp.	Phase II (cancer)	licensed to PharmaMar by Univ. Hawaii; revision of structure
ES-285 (spisulosine)	*Spisula polynyma*	Phase I (cancer)	*Rho*-GTP inhibitor
HTI-286 (hemiasterlinderivative)	*Cymbastella* sp	Phase II (cancer)	synthetic derivative made by Univ. British Columbia; licensed to Wyeth
KRN-7000	*Agelas mauritianus*	Phase I (cancer)	an agelasphin derivative
Squalamine	*Squalus acanthias*	Phase II (cancer)	antiangiogenic activity as well
Æ-941 (Neovastat)	shark	Phase II/III (cancer)	defined mixture of <500 kDa from cartilage; antiangiogenic activity as well
NVP-LAQ824	Synthetic	Phase I (cancer)	derived from psammaplin, trichostatin, and trapoxin structures
Laulimalide	*Cacospongia mycofijiensis*	preclinical (cancer)	synthesized by a variety of investigators
Curacin A	*Lyngbya majuscula*	preclinical (cancer)	synthesized, more soluble combi-chem derivatives being evaluated
vitilevuamide	*Didemnum cucliferum* & *Polysyncraton lithostrotum*	preclinical (cancer)	
diazonamide	*Diazona angulata*	preclinical (cancer)	synthesized and new structure elucidated
eleutherobin	*Eleutherobia* sp.	preclinical (cancer)	synthesized and derivatives made by combi-chem; can be produced by aquaculture
sarcodictyin	*Sarcodictyon roseum*	preclinical (cancer) (derivatives)	
peloruside A	*Mycale hentscheli*	preclinical (cancer)	
salicylihalimides A	*Haliclona* sp.	preclinical (cancer)	first marine Vo-ATPase inhibitor; similar materials from microbes, synthesized
thiocoraline	*Micromonospora marina*	preclinical (cancer)	DNA polymerase R inhibitor
ascididemnin			preclinical (cancer) reductive DNA-cleaving agents
variolins	*Kirkpatrickia variolosa*	preclinical (cancer)	Cdk inhibitors

Table 1.1 cont.

Name	Source	Status (disease)	Comment
dictyodendrins	*Dictyodendrilla verongiformis*	preclinical (cancer)	telomerase inhibitors
GTS-21 (aka DMBX)		Phase I (Alzheimer's)	modification of a worm toxin; licensed to Taiho by Univ. Florida
manoalide	*Luffariaella variabilis*	Phase II (antipsoriatic)	discontinued due to formulation problems
IPL-576,092 (aka HMR-4011A)	*Petrosia contignata*	Phase II (antiasthmatic)	derivative of contignasterol;
IPL-512,602	derivative of 576092	Phase II (antiasthmatic)	with Aventis
IPL-550,260	derivative of 576092	Phase I (antiasthmatic)	with Aventis
ziconotide (aka Prialt)	*Conus magus*	Phase III (neuropathic pain)	licensed by Elan to Warner Lambert
CGX-1160	*Conus geographus*	Phase I (pain)	contulakin G
CGX-1007	*Conus geographus*	Phase I (pain & epilepsy)	conantokin G; discontinued
AMM336	*Conus catus*	preclinical (pain)	ö-conotoxin CVID
ø-conotoxin	*Conus* sp.	preclinical (pain)	conotoxin MR1A/B
CGX-1063	Thr10-contulakin G	preclinical (pain)	modified toxin
ACV1	*Conus victoriae*	preclinical (pain)	R-conotoxin Vc1.1

The other important aspect of marine natural products is that, though they may not all make it to the market they may provide important chemistry in unique "scaffolds" on which synthetic and combinatorial chemists can develop other drugs (Newman and Cragg, 2004).

1.3 South African Marine Biodiversity

The South African coastline provides a unique environment for marine organisms. This is due to the mixing of the warm Agulhas current, flowing in a south easterly direction on the Kwazulu-Natal east coast and the cold Benguela current flowing in a westerly direction on the west coast. The difference between the two currents is evident in the marine *fauna* and *flora* found on the two coasts (Branch *et al.*, 1994). Branch *et al.* (1994) also reported the presence of numerous species on the warm south east coast stretching from Port St Johns to Cape Point, and estimated that 12% of the about 10 000 species are endemic. Given this diversity, it is unfortunate that not much is reported or has been done on the natural products chemistry of most of the marine organisms found on the South African coast. Most of the studies done have focused on marine invertebrates such as sponges, soft corals and mollusks (Pika and Faulkner, 1994; Antunes *et al*, 2004; Beukes *et al.*, 1998) while very little or no work have been published on marine algae and microorganisms. A few of the publications on South African marine algae include the isolation of chamigranes from the red alga *Laurencia glomerata* (Elsworth and Thomson, 1989), and the screening of crude extracts for antimicrobial activity by Vlachos *et al.* (1997). The seaweed industry at the moment is small, recognizable activity being the harvesting of kelps, *Ecklonia maxima* and *Laminaria pallida* mainly on the west coast, for alginate. *Ecklonia* is used for the production of plant growth stimulants, and also as feed for commercially grown abalone. *Gelidium* species are harvested from intertidal shores and used in the production of agar (Lubke *et al.*, 1988).

1.4 Project Objective

In a previous investigation crude extracts of several marine algae collected along the South African coast were screened for cytotoxicity and antibacterial activity (Vlachos, 1997; Knott, 2003). Extracts of *Plocamium corallorhiza* showed moderate activity in both screens and were selected for further study. Knott (2003) isolated and characterised several new metabolites from a *Plocamium corallorhiza* collection obtained from the west coast of South Africa but reported only limited biological activity for these compounds. We have recently established a collaboration with Dr Denver Hendricks at the University of Cape Town to screen marine algal metabolites for activity against an oesophageal cancer cell line. Therefore, the main objective of this project was to study the chemistry and biological activity of secondary metabolites from *Plocamium corallorhiza* collected from the south east coast of South Africa.

References

Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V, Huff, J.; Hoffmann, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Patchett, A.; Monaghan, R.; Currie, S.; Stapley, E.; Albers-Schonberg, G.;Hensens, O.; Hirshfield, k.;Liesch, J.; Springer, J. *Proc. Natl. Acad. Sci. USA*, **1980**, 77, 3957

Andersen, R. J.; Williams, D. E., In *Issues in Environmental Science and Technology* 13; *Chemistry in the Marine Environment,* Hester, R. E.Harrison, R. M. Eds, Royal Society of Chemistry: Cambridge **2000** pp 55-59

Antunes, E. M.; Beukes, D. R.; Kelly, M.; Somaai, T.; Barrows, L. R.; Marshal, K. M.; Sincich, C.; Davies-Coleman, M. T. *J. Nat. Prod.* **2004**, 67, 1286-76

Bergmann, W. and Feeney, R. J. *J. Am. Chem. Soc.*, **1950**, 72, 2809-10

Bergmann, W. and Feeney, R. J. *J. Org. Chem.*, **1951**, 16, 981-987

Beukes, D. R.; Davies-Coleman, M. T.; Kelly-Borges, M.; Harper, M. K. and Faulkner, D. J. *J. Nat. Prod.* **1998**, 61, 699-701

Branch, G. M.; Griffiths, C. L.; Branch, M. L.; Beckley, L. E. In *Two Oceans* David Philips: Cape Town, **1994**

Carte, B. K. *Bioscience* **1996**, 271-286

Christian, M. C.; Pluda, J. M.; Ho, T. C.; Arbuck S. G.; Murgo, A. J.and Suasville, E. A. *Semin. Oncol.* **1997**, 24, 219-240

Clark, M. A. *Pharm. Res.* **1996**, 13, 1133-1144

Cragg, G. M.; Boyd, M. R.; Cardellina II, J. H.; Newman, D. J.; Snader, K. M.; McCloud, T. G. in *Ethnobotany and the Search for New Drugs*, Ciba symposium No. 185, **1994**, p. 128

Cragg, G. M.; Schepartz, S. A.; Suffness, M.; Grever, M. R. *J. Nat. Prod.* **1993**, 56, 1657-68

Dev, S. *Environ. Health Perpsect.* **1999**, *107*, 783

Dewick, P. M. In "*Medicinal Natural Products.*" Wiley; J. Bath, **2000**, 5-6, 152

Elsworth, J. F.; Thomson, R. H. *J. Nat. Prod.* **1989**, 52 , 893-5

Faulkner, D. J. *Oceanus* **1979**, 22, 44-50

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

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

Faulkner, D. J. *Nat. Prod. Rep.* **2001**, 18, 1-49

Faulkner, D. J. *Nat. Prod. Rep.* **2000**, 17, 7-55

Hartwell, J. L., Plants used against cancer, Quarterman, Lawrence, **1982**, 271-2

Haslam, E. In *Comprehensive Organic Chemistry*; Barton, D.; Ollis, W. D. Eds; Pergamon

Press; Oxford: **1979** Vol 5, part 21.1, pp 3-20

Haslam, E. *Nat. Prod. Rep.* **1986**, 3, 217-249

Jaspars, M. *Ed. Chem.* **1999**, 36, 39-41

Knott, G. M., MSc Thesis, **2003**, Rhodes University, Grahamstown, South Africa

Lubke, R. A.; Gess, F. W.; Bruton, M. N. In *The field guide for the Eastern Cape Coast,* The Grahamstown Centre of the Wildlife Society of Southern Africa: Port Elizabeth, **1988**, 35-37
Bold, H. C.; Wynne, M. J. In *Introduction to the algae,* Prentice-Hall, Inc: New Jersey, **1978**, 464-564

Mann, J. *Secondary* Metabolism (second edition); Oxford University Press: Oxford. **1987**; 374pp

Martin, W. R. *Pharmacol. Rev.* **1967**, 19, 463-521

Mateles, R. I. *Penicillin: A Paradigm for Biotechnology,* Candida Corporation, Chicago, **1998**

Munro, M. H. G.; Blunt, J. W.; Dumdei, E. J.; Dickford, S. J. H.; Lill, R. E.; Li, S.; Battershill, C. N.; Duckworth, A. R. *J. Bacteriology,* **1999**, 70, 15-25

Naik, R. G.; Kattige, S. L.; Bhat, S.V.; Alrega, B.; de Sousa, N. J. and Rupp. R. H. *Tetrahedron* **1988**, 44, 2081-2086

Newman, D. J. and Cragg, G. M. *J. Nat. Prod.* **2004**, 67, 1216-1238

Newman D. J.; Cragg, G. M.; Snader, k. M. *Nat. Prod. Rep.* **2000**, 17, 215-234

Newtown, G. G. F.; Abraham, E. P. *Biochem. J.*, **1956**, 62, 651

Paul, V. J. *Ecological Roles of Marine Natural Products;* Cornell University Press New York, **1992**, 245 pp

Pezzuto, J. M. *Biochem. Pharmacol.*, **1997**, 53, 121-133

Pika, J. and Faulkner, D. J., *Tetrahedron* **1994**, 50, 3065-3070

Suckling, C. J. *Sci. Prog.* **1991**, 75, 323-359
Terrenius, L. *Annu. Rev. Pharmacol. Toxicol.* **1978**, 18, 189-204

Vlachos, V.; Critchley, A. T.; von Holy, A. *S. Afr. J. Sci.* **1997**, 93, 328-334

Williams, D. H.; Stone, M. J.; Hauck, P. R.; Rahman, S. K. *J. Nat. Prod.* **1989**, 52, 1189-1208

Chapter Two

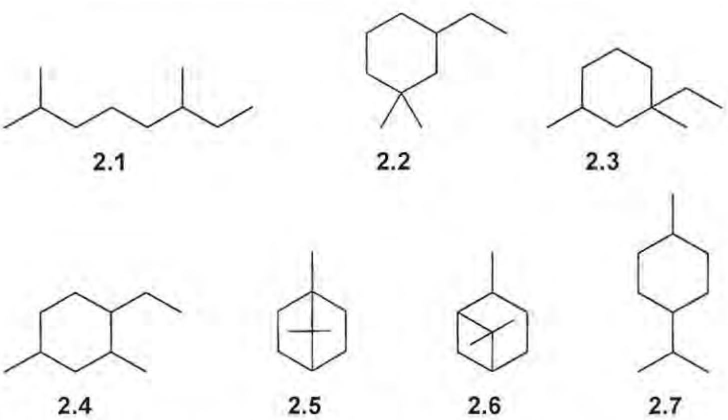
Labile halogenated monoterpenes from *Plocamium corallorhiza*

2 Introduction and literature review

Halogenated monoterpenes are unique secondary metabolites mainly produced by red algae of the Plocamiaceae and Rhizophylliadaceae families, both of the order Gigartinales (Faulkner, 1984; Naylor *et al.*, 1983). Sea hares of the genus *Aplysia,* as well as some seaweeds of the genus *Microcladia* (order Ceramiales) are exceptions from which these natural products have been isolated. It has been suggested that the latter two organisms, however, are not a direct source of halogenated monoterpenes (Naylor *et al.*, 1983). Sea hares, for example, are known to sequester toxic algal metabolites and use them to deter predators (Stallard and Faulkner, 1974; Faulkner *et al.*, 1973). Polyhalogenated monoterpenes were in fact first discovered in the digestive glands of the sea hare, *Aplysia californica* in 1973 (Faulkner and Stallard, 1973), and then later from *P. cartilagineum (*Mynderse and Faulkner, 1974; Mynderse *et al.*, 1975).

2.1 Structural features of halogenated monoterpenes.

The earliest studies on terrestrial monoterpenes date back to the 1800`s, nevertheless, this area of research remains of interest. As the simplest terpenoids, monoterpenes are formed by the dimerization of isoprene (C_5) units, and have carbon skeletons which are subdivided into regular or rearranged isoprene types (Ruzicka, 1959). In spite of their simple genesis, many different carbon frameworks have been identified. The halogenated monoterpenes (isoprene dimers) isolated from *Plocamium* species can be sub-divided into four main structural sub-classes. These consist of the head to tail acyclic skeleton (**2.1**, Figure 2.1), found in both marine and terrestrial organisms (Ruzicka, 1959), and three distinct monocyclic types, 3-ethyl-1,1-dimethyl-cyclohexane, 1-ethyl-1,3-dimethyl-cyclohexane and 1-ethyl-2,4-dimethyl-cyclohexane (**2.2** - **2.4**). The skeletons **2.3** and **2.4** are unique to marine organisms and are exclusive to *Plocamium* spp. Chemical evidence indicates that the framework **2.3** rearranges to **2.4** by ethyl migration (Naylor *et al.*, 1983). The common terrestrial monoterpenes skeletons **2.5** to **2.7** are not represented in the marine environment.



2.1 **2.2** **2.3**

2.4 **2.5** **2.6** **2.7**

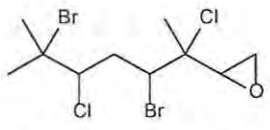
Figure 2.1 Commonly encountered skeletons of monoterpenes found in terrestrial and marine natural products

The large number of acyclic and cyclic halogenated monoterpenes from marine organisms are mainly differentiated by i) the number of double bonds in the molecule, ii) the number and type of halogens atoms (bromine or chlorine) and iii) the geometry of double bonds and configuration at chiral centres. The addition of oxygen to these structures further increases the number of halogenated monoterpenes that may be obtained. Figure 2.2 shows some of the commonly encountered halogenated monoterpenes structure-types.

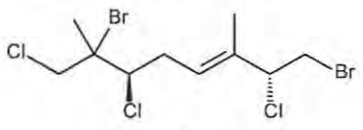
Most halogenated monoterpenes contain a labile tertiary allylic halide group which may complicate structure elucidation in that the compound may change during the isolation process. Ichikawa *et al.* (1976) found that extraction of *Chondrococcus japonicus* with acetone followed by hexane yielded compounds **2.27** and **2.28** but, when the extraction was done using methanol, only two methoxylated compounds were obtained. Furthermore, the treatment of **2.27** and **2.28** with refluxing methanol produced the same methoxylated products (Scheme 2.1).

Artefacts may also result from rearrangement of halogenated monoterpenes as illustrated by the formation of **2.34** from **2.33** at room temperature (Scheme 2.2, Stierle and Sims, 1979).

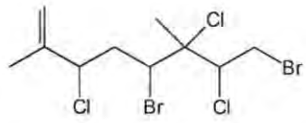
Linear monoterpenes



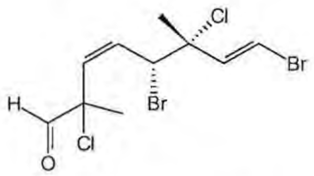
2.8 (fully saturated)



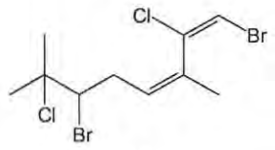
2.9 (Δ^3-ene)



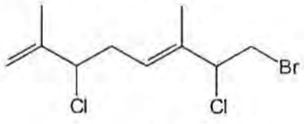
2.10 (Δ^7-ene)



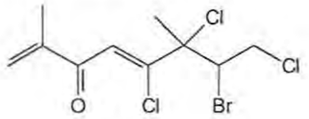
2.11 ($\Delta^{1,5}$-diene)



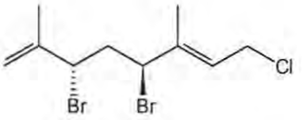
2.12 ($\Delta^{1,3}$-diene)



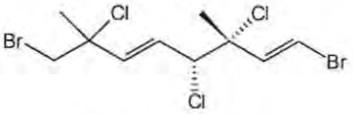
2.13 ($\Delta^{3,7}$-diene)



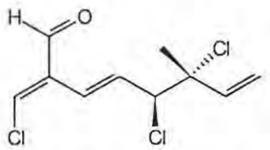
2.14 ($\Delta^{4,7}$-diene)



2.15 ($\Delta^{2,7}$-diene)

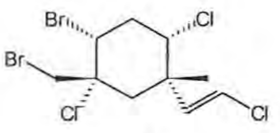


2.16 ($\Delta^{1,5}$-diene)



2.17 ($\Delta^{1,5,7}$-triene)

Cyclic monoterpenes



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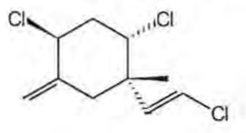

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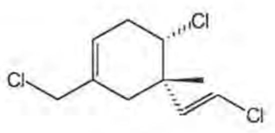

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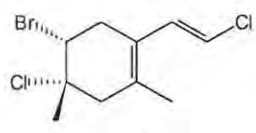

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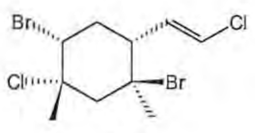

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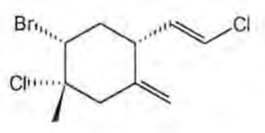

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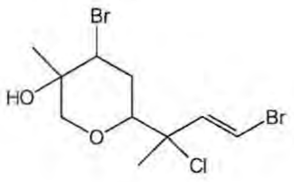

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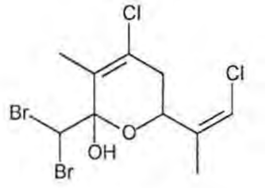

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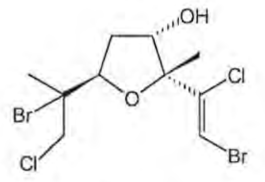
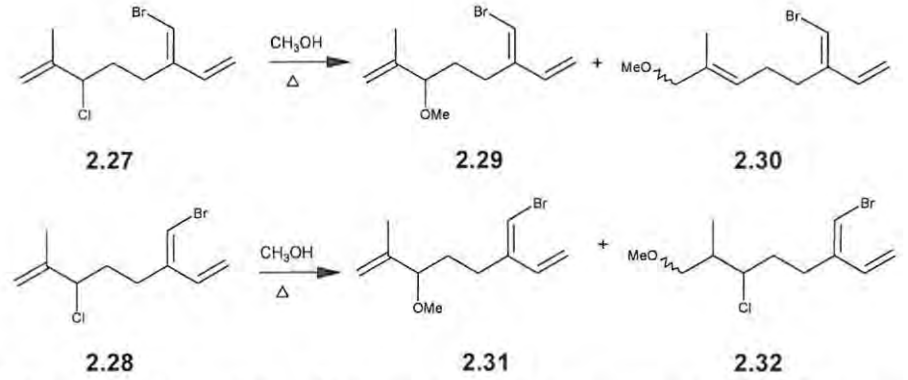


2.26

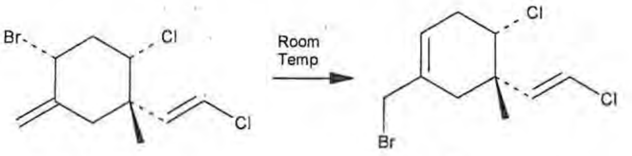
Figure 2.2 Structural variations found in halogenated monoterpenes



2.27 **2.29** **2.30**

2.28 **2.31** **2.32**

Scheme 2.1 Methoxylation of halogenated monoterpenes isolated from *C. japonicus*.



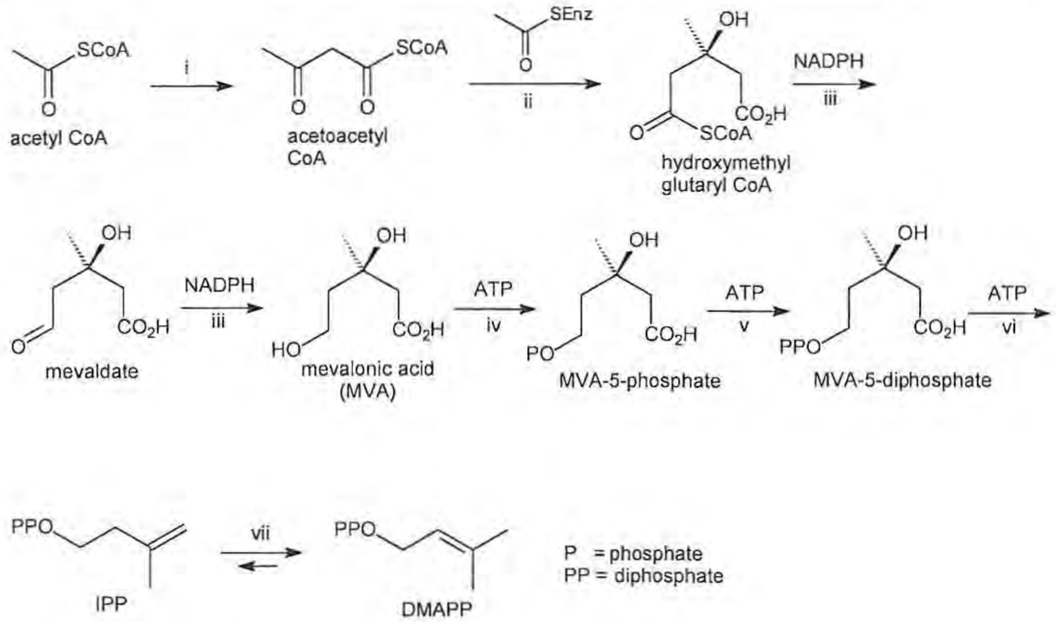
2.33 2.34

Scheme 2.2 Formation of an artefact by rearrangement in structure of a cyclic monoterpene (Stierle and Sims, 1979)

2.2 Biosynthesis of halogenated monoterpenes

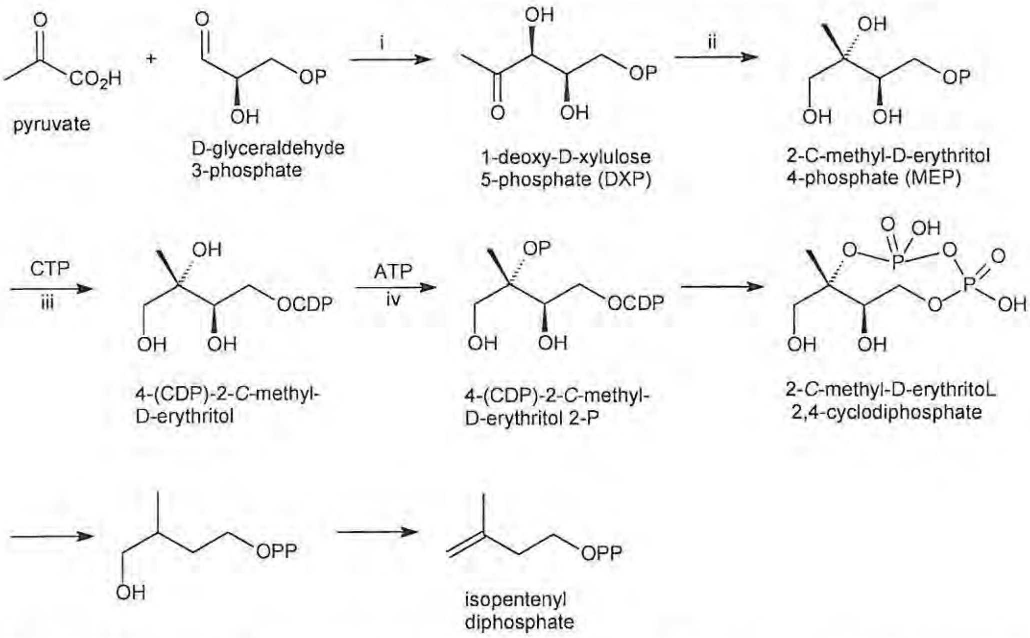
2.2.1 Formation of the terpenoid precursor isopentenyldiphosphate (IPP)

The classical mevalonate pathway (Scheme 2.3) was the first defined biosynthetic route to isopentenyldihosphate (IPP) the isoprene precursor of the monoterpenes. This pathway involves the two-step condensation of three molecules of acetyl-CoA which is catalysed by the enzymes acetyl-CoA acyltransferase and hydroxymethylglutaryl-CoA (HMG-CoA) synthase to produce HMG-CoA. This C$_6$ compound is then reduced by HMG-CoA reductase to (3*R*)-mevalonic acid, which is then transformed into IPP, in three steps, involving two sequential phosphorylation steps by mevalonate kinase and phosphomevalonate kinase and a decarboxylation step catalysed by phosphomevalonate decarboxylase. The IPP isomerase catalyses the conversion of part of the IPP in a reversible reaction to dimethylallyldiphosphate (DMAPP).



Scheme 2.3 The mevalonate pathway. *Enzymes:* i) acetoacetyl-CoA thiolase, ii) HMG-CoA synthase, iii) HMG-CoA reductase, iv) mevalonate kinase, v) phosphmevalonate kinase, vi) mevalonate 5-diphosphate decarboxylase, vii) IPP isomerase

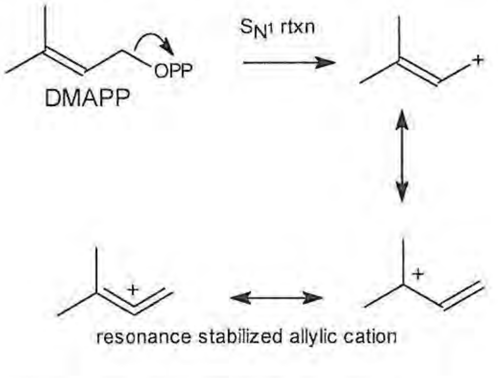
However, a number of results from incorporation studies with ^{14}C-labelled substrates and with inhibitors could not be explained by terpenoid synthesis *via* the mevalonate pathway. A new pathway leading to IPP was proposed where a C_5 precursor for IPP, 1-deoxy-D-xylulose 5-phosphate (DOXP) was formed from glyceraldehyde 3-phosphate and pyruvate (Dewick, 2002). This pathway was found to occur in eubacteria, green algae and in plastids of higher plants. The first reaction of the pathway is a condensation between pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DOXP) (Scheme 2.4). The latter is then converted by DOXP-reductase to 2*C*-methyl-D-erythritol 4-phosphate (MEP) the first committed intermediate for IPP biosynthesis. MEP is subsequently converted in several steps to the last intermediate 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate which is then converted to IPP and DMAPP (Dewick, 2002). Several names are in common use for this pathway, including mevalonate-independent pathway, nonmevalonate pathway, glyceraldehyde 3-phosphate/pyruvate pathway, deoxyxylulose phosphate (DXP or DOXP) pathway, and methylerythritol phosphate (MEP) pathway.



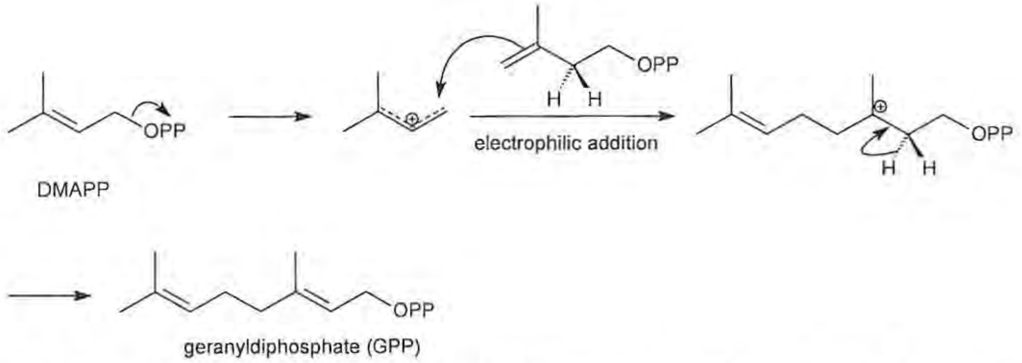
Scheme 2.4 The deoxyxylulose phosphate pathway. *Enzymes*: i) 1-deoxyxylulose 5-phosphate synthase (DXP synthase), ii) 1-deoxyxylulose 5-phosphate reductoisomerase (DXP reductoisomerase), iii) 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (CDP-ME synthase), iv) 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CDP-ME kinase).

2.2.2 Biosynthesis of ocimenes and myrcenes

All terpenoids are biosynthesized from IPP and DMAPP, originating from either of the two pathways outlined above. The head to tail condensation of one molecule of IPP with one molecule of DMAPP, catalysed by a prenyl transferase, yields the C_{10} compound geranyl diphosphate (GPP), which is the immediate precursor of the monoterpenes (Scheme 2.6) (Dewick, 2002; Rohmer, 1993). This occurs via the formation of resonance stabilized carbocation (Scheme 2.5).



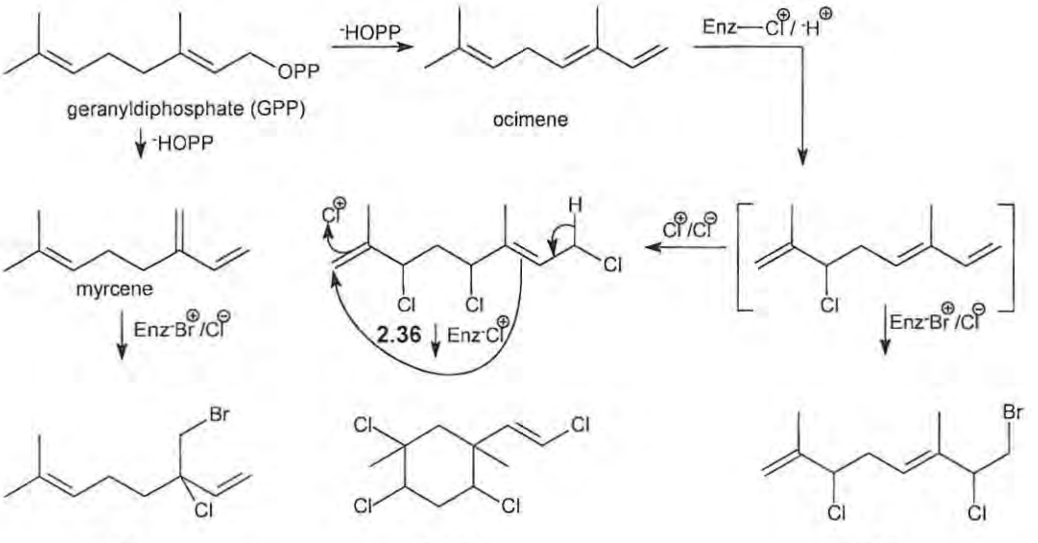
Scheme 2.5 Formation of reactive carbocation from DMAPP (Dewick, 2000; Rohmer, 1993)



Scheme 2.6 Formation of GPP from DMAPP and isoprene (Dewick, 2000; Rohmer, 1993)

The loss of HOPP (a diphosphate acid) from GPP can generate myrcene or ocimene (Scheme 2.7), from which polyhalogenated monoterpenes are derived (Naylor *et al.*, 1983).

Recently, Wise *et al.* (2002) isolated a myrcene synthase from suspension cultures of the marine red alga *Ochtodes secundiramea* and demonstrated the conversion of GPP to myrcene.



2.35 **2.37** **2.38**

Scheme 2.7 Proposed biogenesis of selected halogenated monoterpenes from myrcenes and ocimenes (adapted from Naylor *et al.*, 1983)

2.2.3 Halogenation

The halogenation process is proposed to proceed *via* enzyme-catalysed oxidation of halide by a peroxidase enzyme (chloroperoxidase, CPO; bromoperoxidase, BPO or iodoperoxidases, IPO) and hydrogen peroxide followed by subsequent attack of nucleophilic double bonds (Gribble, 1998). Butler and co-workers (Butler and Walker, 1993) showed that vanadium bromoperoxidases isolated from marine algae generated the oxidized intermediate, Br^+ which then halogenates appropriate organic substrate or oxidizes a second equivalent of hydrogen peroxide producing dioxygen.

For example, Markovnikov addition of bromine, (Br^+) to myrcene followed by chlorine (Cl^-) leads to the formation of the dihalogenated product **2.35** (Scheme 2.7). An initial addition of Cl^+ to ocimene followed H^+ loss, subsequent addition of Cl^+ and finally Cl^- capture would lead to preplocamene **2.36** (Scheme 2.7) (Naylor *et al.*, 1983). Cyclic halogenated monoterpenes such as plocamene **2.37** may be formed from **2.36** *via* initial electrophilic addition of Cl^+ to the terminal double bond followed by intramolecular nucleophilic attack on the carbocation by the second double bond and loss of H^+. The addition on the halogen is regiospecific and hence the novelty and diversity of marine natural products (Martinez et al., 2001).

2.3 Review of structure elucidation and stereochemistry determination of halogenated monoterpenes

2.3.1 Introduction

One of the main challenges faced by natural product chemists is the structural elucidation of new compounds. Natural products have provided many structurally different compounds, which have contributed to the general interest in structural analysis and development of techniques such as spectroscopy to previously un-imagined sophistication. Although halogenated monoterpenes may appear to be among the more simple compounds isolated from marine organisms, their structural elucidation has been complicated by the high degree of halogen substitution in the molecules.
The number of structural re-assignments and revisions of previously isolated compounds highlights the difficulties of structure elucidation. For example, the structure of violacene (**2.39**) was revised from **2.40** based on X-ray analysis (van Engen *et al.*, 1978; Mynderse and Faulkner, 1974) (Figure 2.3).

2.40 **2.39**

Figure 2.3 Structural revision of violacene (van Engen *et al.*, 1978)

The structures of coccinene (**2.41**), and its analogue (**2.42**) (Figure 2.4) have also been revised based on 2D NMR and NOE difference (NOED) spectroscopy to **2.43** and **2.44**, respectively (Sardina *et al.*, 1985).

2.41 X = Br **2.43** X = Br

2.42 X = Cl **2.44** X = Cl

Figure 2.4 Structural reassignment of coccinene (**2.41**) and its analogue (**2.42**)

The earlier structural assignment of **2.41** had been based on X-ray analysis (González *et al.*, 1978), but it is now known that crystals of polyhalogenated monoterpenes undergo considerable damage upon X-ray irradiation (Sardina *et al.*, 1985), and this might have been the cause of the original erroneous conclusions.

Chondrocole A (**2.45**) and B (Burreson *et al.*, 1975) also had regio- and stereochemical structural revisions at C-6 and C-8 (Figure 2.5). The initial halogen placement was based on the appearance of an intense M$^+$-Br fragment ion in the mass spectrum, which was interpreted as involving the loss of an allylic bromine atom. The structure of chondrocole A (**2.45**) was revised to **2.46** as a result of its oxidation to chondrocolactone, a known monoterpene, whose structure had been determined by X-ray analysis (Woolard *et al.*, 1978).

2.45 **2.46**

Figure 2.5 Structure revision of chondrocole A (Woolard *et al.*, 1978)

2.3.2 Structure Determination

2.3.2.1 NMR spectroscopy

The development of NMR spectroscopy, particularly 2D experiments, greatly enhanced structure elucidation and has made the process simpler and quicker, than deducing structure from degradation studies and comparison with known compounds. Advances in this field have led to solving regio- and stereochemistry problems as highlighted by numerous structural revisions. Another advantage is that the structure of a compound can be determined from only a few milligrams of sample.

^{1}H and ^{13}C NMR data have provided very important structural information for marine halogenated monoterpenes. ^{13}C NMR data has been used to determine halogen regiochemistry and substituent stereochemistry (Sims *et al.*, 1978a). Crews *et al* (Crews, 1977, Crews *et al.*, 1984) continued the work in the use of ^{13}C data to determine regio- and stereochemistry of halogenated monoterpenes, by developing models, to which comparisons of the substituent effects where made and also employed coupling constants. Substituent additivity constants were used to calculate estimated ^{13}C chemical shifts and models were developed for alicyclic as well as cyclic compounds. Stereochemistry determination using this method is based on the ^{13}C shift effects of methyl groups from α-, β- and γ-substituents. Perusal of the ^{13}C NMR data presented in Figure 2.6 clearly demonstrates the value of ^{13}C NMR in determining the placement of various halogen atoms in monoterpenes. Table 2.1 summarizes the differences in the ^{13}C chemical shifts depending on whether chlorine or bromine is primary, secondary or tertiary. From Table 2.1 it can be seen that C-

Cl and C-Br can be distinguished most reliably at a primary site in an acyclic molecule and least reliably at the tertiary site in a cyclic molecule (Naylor *et al.*, 1983). Generally primary chlorines resonate approximately 11 ppm downfield from the corresponding bromine carbon. Likewise, secondary carbons bearing chlorine resonate approximately 7 ppm downfield from the corresponding brominated carbon.

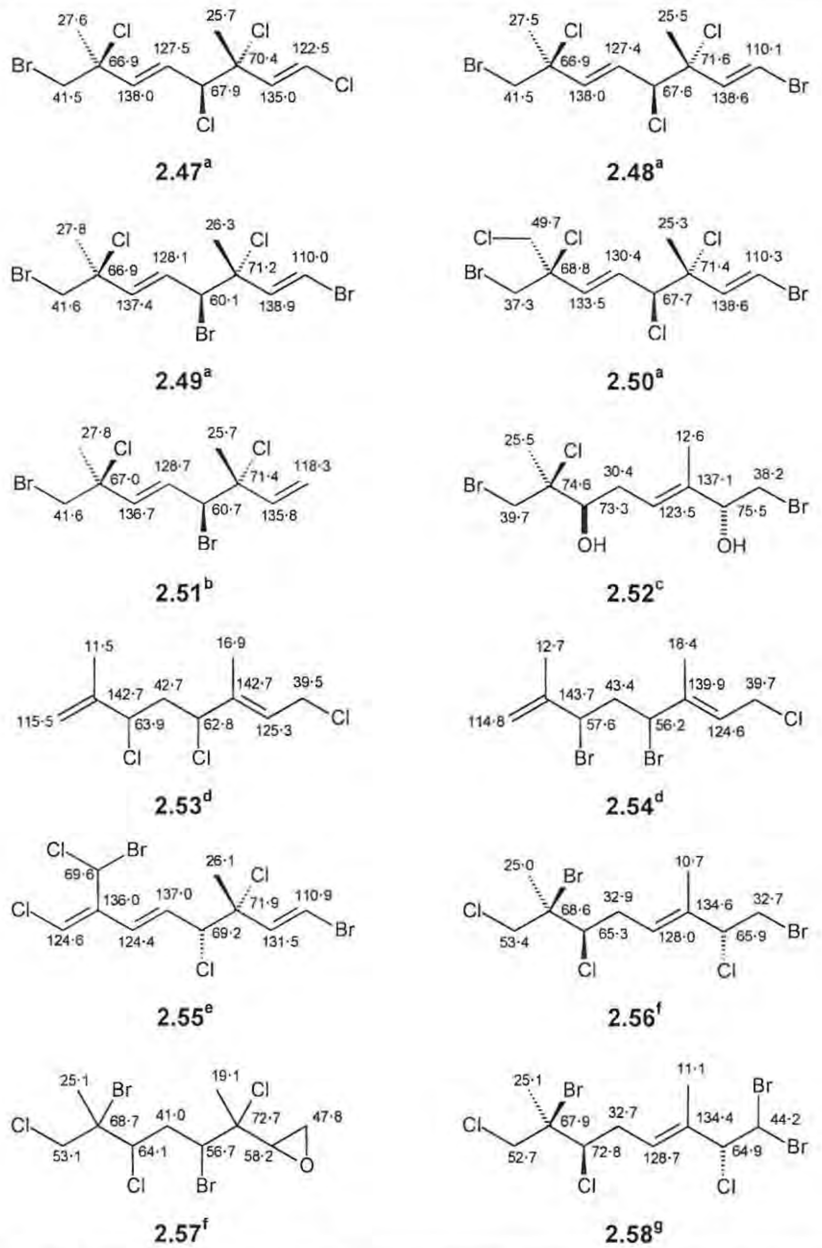


Figure 2.6 The effect of halogen substitution on ^{13}C chemical shifts in acyclic monoterpenes [a] Stierle *et al.*, 1979; [b] Coll *et al.*,1988; [d] Crews 1977; [e] Jongaramjuong and Blackman, 2000; [f] Blunt *et al.*,1985; [g] Bates *et al.*,1979

Table 2.1 ^{13}C NMR Chemical shift differences ($\Delta\delta$) for Br vs. Cl at the α-position

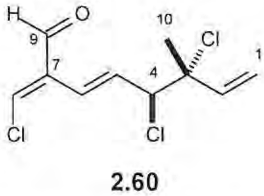
Type	Acyclic	Cyclic
RCH_2X	12	-
R_2CHX	8	4
R_3CX	3	1

One of the limitations of this method is that, standard additivity values cannot be used in ^{13}C shift analysis of molecules with vicinal polar substituents. Sims and co-workers (1978) found, however, that a good match between calculated and experimental value for methine and methylene carbon, which is attached to a polar group and vicinal to another polar group, could only be achieved after a correction factor of –6 ppm was applied. This correction factor must also be applied to calculate the shift of the carbon α to a polar group and flanked also by at least two non-polar β-substituents.

Caution has to be exercised in the use of the additive effects approach to determine stereochemistry of cyclic compounds because of geometric effects (molecular distortions) due to substituents. Thus it has been proposed that heavily substituted cyclic compounds would have chemical shifts which differ significantly from the ideal cyclohexane ring to accommodate the steric and dipolar repulsions among the substituents (Sardina *et al.*, 1986). This is important because molecular distortions are known to profoundly modify ^{13}C chemical shifts (Maciel, 1974). Another important aspect to consider is that the models do not account for important effects such as 1,3-diaxial interactions that are always present in cyclic polyhalogenated terpenes. Sardina and co-workers (1986) developed a method to deal with these problems using 2D NMR and NOED spectroscopy complemented by molecular modeling for structural analysis of cyclic monoterpenes. This method seems to account consistently for intramolecular attractions. The models developed by Crews *et al.* (1977; 1984) and comparison of structures with similar fragments from known compounds are the most commonly used method in structure determination of halogenated monoterpenes.

2.3.2.2 Stereochemistry

NMR data has given useful insight into the stereochemistry of acyclic monoterpenes (Naylor *et al.*, 1983). A rigid conjugated planar diene chromophore can be characterized by long-range ^1H NMR coupling constants of 1-2 Hz. Thus, the observation of a ^4J of 2 Hz between H-6 and H-9 in cartilagineal (**2.59**) is indicative of a conformation fixed enal chromophore (Crews and Kho, 1974).



2.60

Determination of the relative stereochemistry at chiral centers (for example C-3 and C-4 in **2.60**) represents a more important problem because chiral centers are common in these positions for linear monoterpenes. Mynderse et al. (1975) proposed proton NMR strategies to solve the relative stereochemistry at position C-3 and C-4, using the chemical shifts of the methyl groups at positions C-3 and C-7 as the main diagnostic features. The C-3 methyl shift of δ 1.79 was characteristic of 3(R*) 4(R*) whereas δ 1.73 was characteristic of 3(R*) 4(S*). Unfortunately though, this method does not work decisively for compounds whose methyl chemical shift is between 1.73 and 1.79. Crews (1977) observed a much larger ^{13}C NMR C-10 methyl shift difference. The chemical shift for C-10 in halogenated monoterpenes with a 3R,4R relative stereochemistry is 28 ppm while a chemical shift of 25 ppm is observed for monoterpenes with 3R,4S relative stereochemistry (Figure 2.7). The larger shift difference observed in ^{13}C NMR spectra makes this the method of choice when assigning relative stereochemistry.

2.62 **2.61**

Figure 2.7 The effect of C-3 stereochemistry on the C-10 ^{13}C chemical shift

2.3.2.3 X-ray crystallography

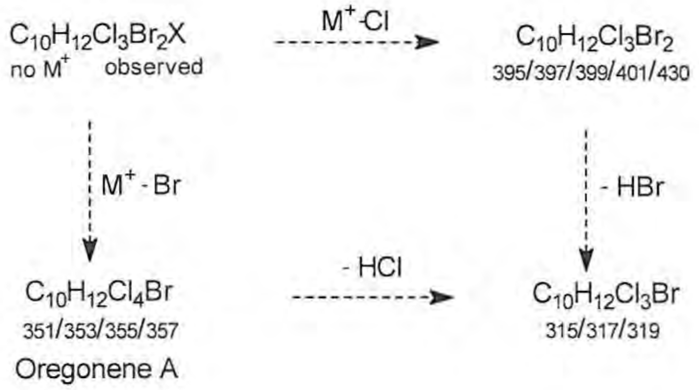
As has been mentioned previously this is the only unambiguous method of structural analysis for monoterpenes, and has been responsible for many re-assignments (Faulkner, 1984). Unfortunately, this method is limited to compounds that are in crystalline state and can be isolated in sufficient quantities e.g. violacene (**2.39**, van Engen *et al.*, 1978), costatone (**2.25**, Stierle *et al.*, 1976). Consequently, this technique finds little application with isolates that are oils.

2.3.2.4 Ultraviolet (UV) and infrared (IR) spectroscopy

These two techniques have not been widely used in the structure elucidation of halogenated monoterpenes, due to the lack of suitable chromophores. λ_{max} values of conjugated dienes and polyenes can yield insights about the chromophore present in a number of monoterpenes such as myrcene and other conjugated monoterpenes (Squillacote *et al.*, 1979). The λ_{max} values calculated from Woodward rules can give an indication of the absorbance of a compound, but substituent and solvent effects have to be accounted for (Kemp, 1975). IR spectroscopy is only of use for those compounds with C-O, C=O and O-H bonds.

2.3.2.5 Mass spectrometry

Although high-resolution mass spectrometry is extremely useful in determining molecular formulas and thus the number of halogens present in these compounds, it is often difficult to obtain a reliable molecular ion peak. However, careful evaluation of odd *m/z* fragment ion peaks generates, by construction of a fragmentation map, fairly reliable projections of unknown X (Cl vs. Br) and the non-detectable M$^+$ mass. An example is outlined in the scheme for oregonene A (**2.50**) below (Scheme 2.8).



Scheme 2.8 Determination of halogen content in oregonene A (**2.50**) when no M$^+$ is observed (Naylor *et al.*, 1983)

Intense ion peak clusters from the mass spectral fragmentation patterns have been of value for assignment of Br and Cl in compounds with framework (**2.1**), (Figure 2.1). However, fragmentation patterns obtained from mass spectrometry must be interpreted with caution, since halogen atoms on the vicinal carbon atoms can rearrange on heating (Faulkner, 1984). The success in the use of mass spectrometry in the study of halogenated monoterpenes results from the fact that Cl and Br have two naturally abundant isotopes. Comparing observed multiple clusters for both M$^+$ and fragment ion clusters with those expected for different halide atom combinations have been standard and extremely useful practice (Figure 2.8).

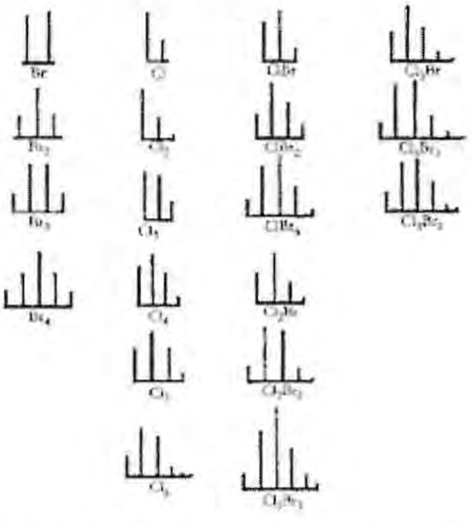


Figure 2.8 Mass spectral isotope cluster patterns observed for halogenated compounds (Shrader, 1971)

2.4 Total synthesis of halogenated monoterpenes

Some attempts have been made to synthesize halogenated monoterpenes, but the enzymatic regio- and stereoselectivity of halogenation has proved a great challenge for synthetic chemists. In an attempt to synthesise violacene (**2.39**), Williard and co-workers (1985) employed a β-halomethacrolein as a dienophile in their Diels-Alder approach for the synthesis of the halogenated ring. However, since cycloaddition with monosubstituted dienes such as isoprene gave inseparable mixtures of regioisomers, a straightforward construction of the terpenoid skeleton was not feasible. Other attempts to synthesise monoterpenes include synthesis of costanolide (Williard *et al.*, 1983) and costatone (**2.25**) (Williard and Grab, 1984)

The following section describes the isolation and structure elucidation of several new and known halogenated monoterpenes from the marine alga *Plocamium corallorhiza*.

2.5 Results and discussion

In continuation of our investigation of the chemistry of South African marine algae and microorganisms we have screened several algal extracts for cytotoxicity and antimicrobial activity. Extracts obtained from several collections of *P. corallorhiza* have shown moderate activity in these screens and were chosen for further study.

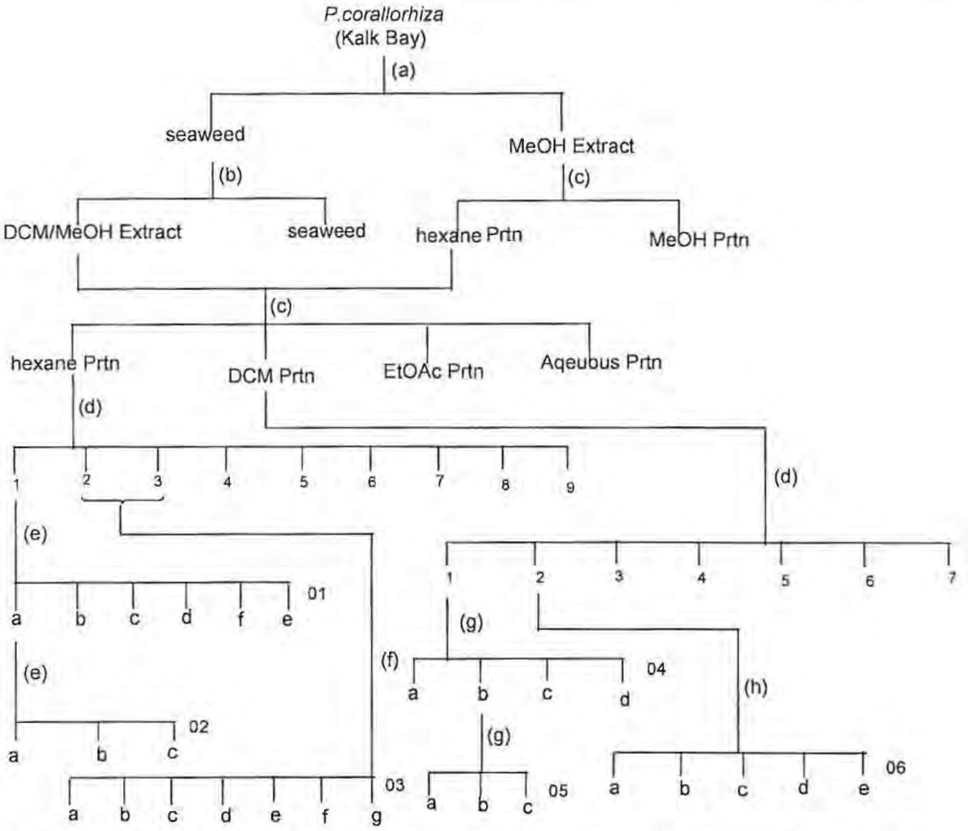
2.5.1 Collection, extraction and isolation

***P. corallorhiza* (Kalk Bay)**

The extraction and isolation protocol used to isolate the halogenated monoterpenes in this study was developed in order to reduce the formation of artifacts from the substitution of halogens by nucleophilic solvents (e.g. MeOH) and loss of volatile metabolites. Therefore, the frozen seaweed is either steeped in MeOH or isopropanol for three hours followed by extraction with a MeOH-DCM (1:1) mixture. Solvents are removed on a rotavap at a temperature of about 30°C.

Extraction of *P. corallorhiza* collected from Kalk Bay (Figure 2.9), near Cape Town, resulted in the isolation and purification of six known compounds, (**2.63** - **2.68**), and two new compounds, (**2.70**) and (**2.71**) (Scheme 2.10). Although **2.70** was isolated from this collection the structure of this compound was determined from subsequent isolations.

Figure 2.9 Author collecting *P. corallorhiza* (March 2004).



Scheme 2.10 Extraction and isolation of metabolites from *P. corallorhiza* (Kalk Bay)

Conditions: a) Methanol extraction (3 hours), b) 50% DCM/MeOH extraction, c) solvent partitioning, d) silica column chromatography, e) HPLC 100% hexane, f) HPLC 85% hexane –EtOAc, g) HPLC 80% hexane-EtOAc.

P. corallorhiza (Woody Cape)

During October of 2003 *Plocamium corallorhiza* was collected from Woody Cape, near Kenton-On-Sea, on the southeast coast of South Africa in order to obtain more material for our biological studies. The seaweed was sequentially extracted with isopropanol and DCM-MeOH (1:1), the extracts concentrated *in vacuo* and the resulting aqueous fractions partitioned with DCM. The ^{1}H NMR spectra of the DCM extracts indicated, for the first time, the presence of compounds containing aldehyde functionalities. Column chromatography of this extract employing a hexane-EtOAc (H/E) step-gradient gave 9 fractions. The ^{1}H NMR spectrum of fraction 1, (90% H/E) showed easily distinguishable halogenated monoterpene signals and was further purified by repeated normal phase HPLC (100% hexane, Figure 2.10) to give six pure compounds **2.63 - 2.68**. The fraction obtained from the ethyl acetate wash showed aldehyde signals[1] in the ^{1}H NMR spectrum and so it was re-injected using 10% ethyl acetate as mobile phase. Four pure halogenated monoterpene aldehydes,[2] **2.69 - 2.75** were isolated but they degraded before any further spectral data could be obtained.

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[1] These signals were also observed in the ^{1}H NMR spectrum of the crude DCM partition.

[2] As determined from their ^{1}H NMR spectra.

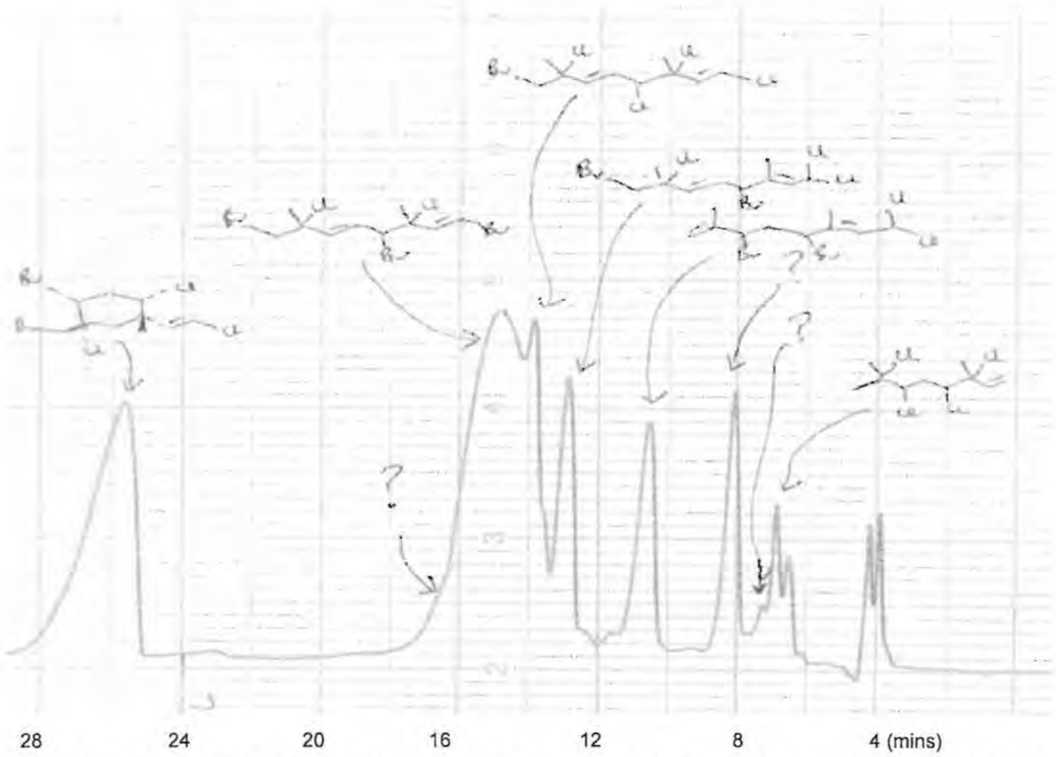


Figure 2.10 HPLC chromatogram (90% hexane) of the ethyl acetate partition of the DCM extract. *Conditions*: Whatman Partisil 10 silica column (1 x 50 cm), RI detection.

The ^1H NMR spectrum of the second fraction obtained from the silica column (80% H/E) also showed the presence of halogenated monoterpenes including aldehyde compounds. Normal phase HPLC of this fraction gave three halogenated monoterpene aldehydes **2.72**, **2.74** and **2.73** and three additional compounds **2.70**, **2.69** and **2.77** which did not contain aldehydes. Again, the aldehydes degraded before any further information could be obtained.

Table 2.2 Masses and the percentage yield relative to the wet weight of the seaweed for the first extraction.

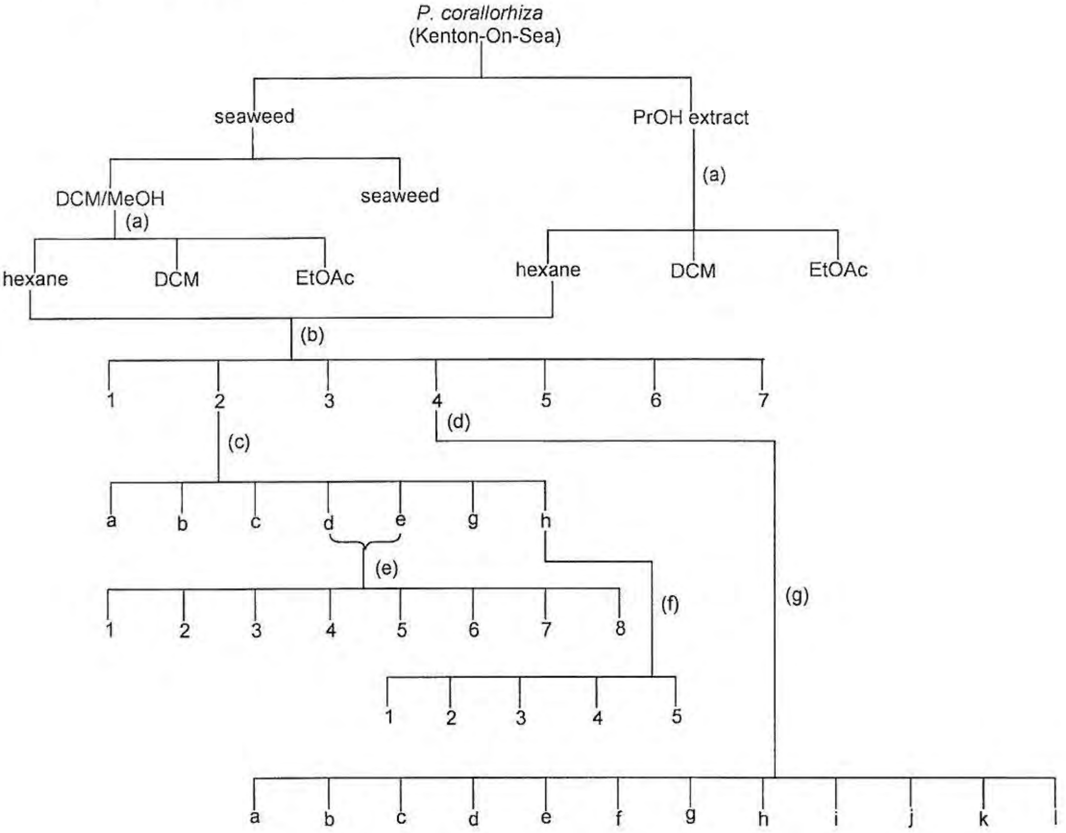
Isolation code	*Compound #*	*Mass (mg)*	*% Yield*
01-d	**2.63**	4.5	0.0015
01-f	**2.64**	4.4	0.0015
01-g	**2.65**	2.3	0.0008
01-i	**2.66**	3.9	0.0013
01-j	**2.67**	4.7	0.0016
01-k	**2.68**	2.7	0.0009
02-d	**2.76**	10.7	0.0036
02-f	**2.75**	5.5	0.0018
02-g	**2.74**	6.9	0.0023
02-i	**2.73**	2.7	0.0009
03-c	**2.75**	4.8	0.0016
03-d	**2.74**	4.6	0.0015
03-g	**2.73**	4.1	0.0014
03-j	**2.69**	4.2	0.0014

Silica column chromatography of the crude hexane partition of DCM/MeOH employing a hexane-EtOAc step-gradient (hexane to ethyl acetate) gave 5 fractions, which were grouped on the basis of TLC and ^{1}H NMR data. Fraction 1 consisted of compounds obtained from 90-100% hexane, fraction 2 compounds resulting from 70-80% hexane, fraction 3 compounds obtained from hexane concentration between 30 and 50 %. The last two fractions consisted of compounds from 100% ethyl acetate and methanol respective. Normal phase HPLC with 100% hexane eluent led to the isolation of 4 compounds **2.63**, **2.66**, **2.67** and **2.68**. Repeated normal phase chromatography of fraction 2 led to purification of four aldehydes, but because of the small quantities isolated the structures was could not be determined. No further analysis was done on the rest of the fractions because either, their ^{1}H NMR spectrum did not show of the signals of interest or there was too little material.

P. corallorhiza (Kenton on Sea)

The small quantities and decomposition of aldehydes isolated necessitated further extraction to get sufficient material for structure determination and bioassays. A further collection of *P. corallorhiza* was therefore made at a more accessible site near Kenton-On-Sea (see Figure 2.11. The extraction and isolation protocol used was slightly modified in order to prevent degradation of aldehydes. The seaweed was extracted with isopropanol and DCM/MeOH as before except that the extraction was done at 4°C (Scheme 2.12). The extracts were then concentrated at 30 °C *in vacuo* and the aqueous mixture partitioned with hexane, DCM and ethyl acetate. The corresponding partitions from the isopropanol extract and DCM/MeOH extracts were combined based on the similarity of their ^{1}H NMR spectra. The hexane extract was a dark green oil and showed the signals typical of aldehydes in its ^{1}H NMR spectrum. Since the pale brown oil obtained from the DCM partition only contained minute quantities of the elusive aldehydes we focused our attention on the hexane partition. Silica column chromatography followed by HPLC gave compounds **2.64**, **2.69**, **2.70** **2.72**, **2.73**, **2.74**, **2.76**, **2.78**, **2.79** and **2.80**. Unfortunately compounds **2.79** and **2.80** degraded before their structures could be determined (Scheme 2.11 and Table 2.3).

Figure 2.11 Collection site at Kenton-On-Sea.



Scheme 2.11 Extraction and Isolation scheme of *P. corallorhiza* collected from Kenton-On Sea. *Conditions:* a) solvent partitioning, b) silica column chromatography, c), e) and f) HPLC 90% hexane-EtOAc, d) HPLC 70% hexane-EtOAc.

Table 2.3 The masses and percentage yield (calculated relative to dry mass) of compounds isolated from *P. corallorhiza*.

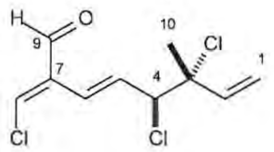
Isolation code[a]	*Compound #*	*Mass (mg)*	*% Yield*
2a	**2.75**	4.7	0.0045
2c	**2.70**	4.5	0.0043
2g	**2.78**	0.9	0.0009
2ed3	**2.74**	9.8	0.0094
2ed5	**2.79**	6.3	0.0061
2ed8	**2.76**	1.1	0.0010
3c	**2.76**	3.7	0.0036
4a	**2.64**	2.7	0.0026
4f	**2.73**	4.5	0.0043
4h	**2.74**	7.3	0.0070
4l	**2.69**	6.0	0.0058

[a] isolation code: silica frn, hplc fn, hplc fraction e.g 2a1 silica column fn 2 hplc fn 1 followed by another hplc fn 1

2.5.2 Structure determination of the new compounds

The instability of these compounds frustrated our efforts to obtain good quality high resolution mass data (HRMS). All new compounds were submitted for high resolution mass measurement on three different occasions but degraded before good spectra could be obtained. Our structure elucidation of the new metabolites therefore depends entirely on NMR and low resolution mass data. Once compounds had been purified samples were made up in $CDCl_3$ and these solutions were kept at 4 °C. Further manipulation of samples was kept to a minimum since evaporation of solvent and dry storage seemed to increase the rate of degradation of these compounds.

The compound numbering used for all acyclic compounds is based on the following system:



2.5.2.1 Structure elucidation of 2.69

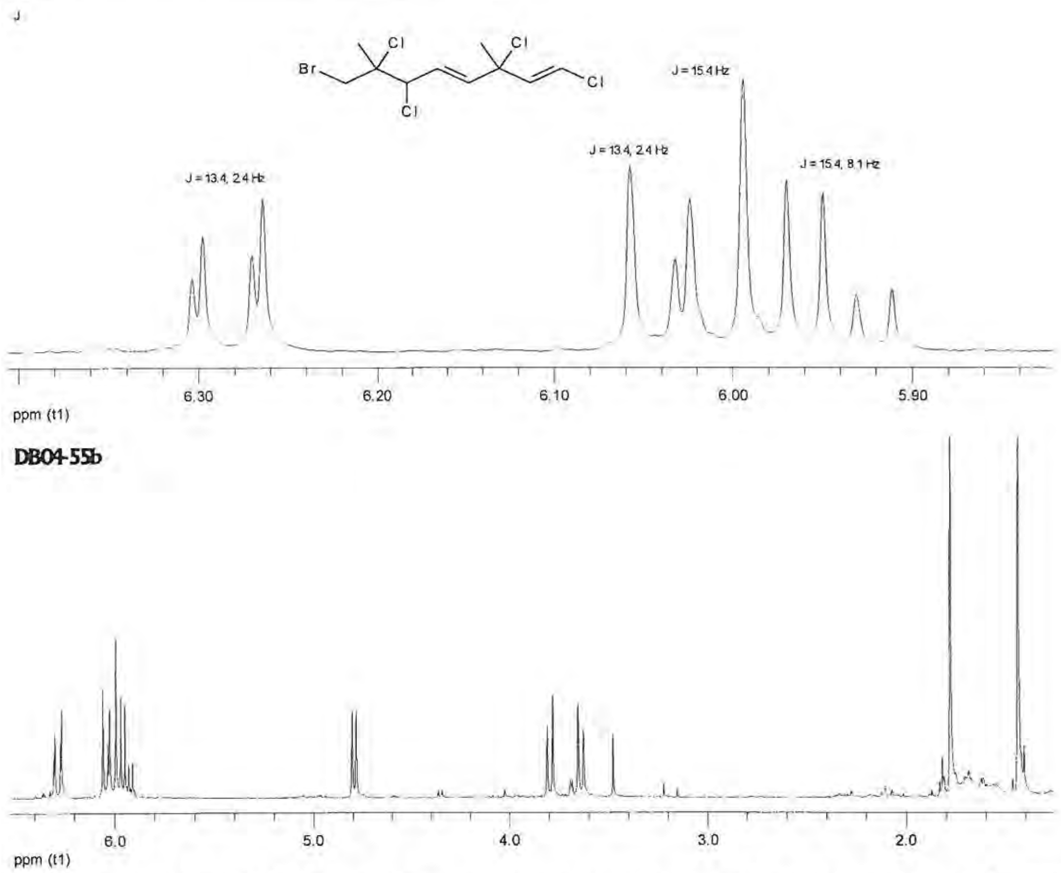


Figure 2.12 (a) ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **2.69** (b) expanded ^{1}H NMR spectrum showing overlapping signals

Compound **2.69** was isolated as an optically active, colorless oil. The ^{1}H NMR spectrum of this compound (Figure 2.12a) was showed three partially overlapped olefinic signals between δ 5.9 and 6.1. The expansion of this portion of the ^{1}H NMR spectrum (Figure 2.12b) clearly showed two mutually coupled olefinic methine signals at δ 6.28 (d, J = 13.4, 2.4[3] Hz) overlapping doublets, and 6.05 (d, J = 13.4 Hz). A second spin system was deduced from the observation of coupling between olefinic methine signals at δ 6.01 (d, J = 15.4 Hz) and 5.94 (dd, J = 13.4, 8.1 Hz). The latter proton also showed coupling to a methine at δ 4.8 ppm. A further two mutually coupled proton signals were observed at δ 3.8 (d, J = 11 Hz) and 3.65 (d, J = 11 Hz) in addition to two methyl singlets at δ 1.78 and 1.43.

At first glance the ^{13}C (Figure 2.13) and DEPT-135 spectra showed ten well-resolved signals, which indicated the presence of two double bonds, three sp^3 hybridized carbons bearing halogens, a methylene and two methyl signals. However, closer inspection of the ^{13}C (Figure 2.13b) and

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[3] The coupling constant of 2.4 Hz may be due to either long range coupling to CH$_3$-10 methyl or the presence of an isomer.

DEPT-135 spectra indicated that almost all of the carbons signals were duplicated[4], indicating the presence of an isomeric mixture. This isomeric mixture could not be separated by HPLC and showed a single peak when analyzed by gas chromatography (Figure 2.14).

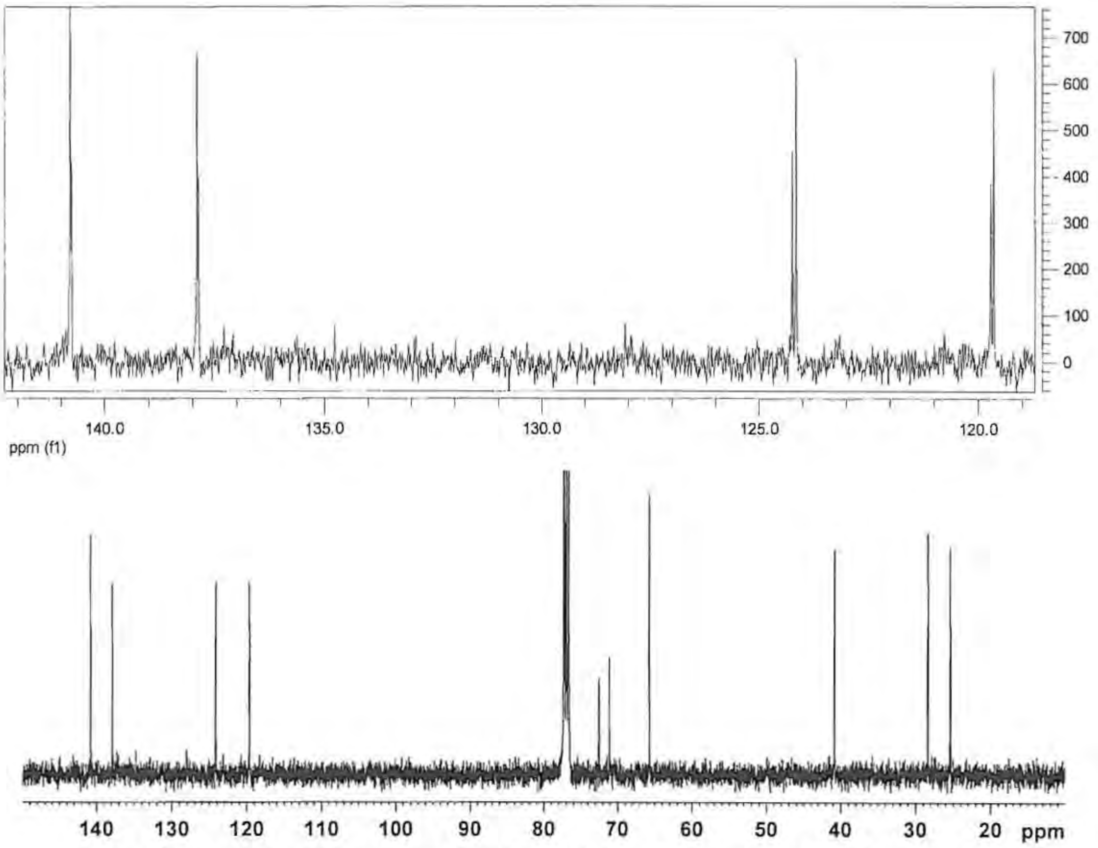

Figure 2.13 (a) Full ^{13}C NMR ($CDCl_3$ 100 MHz) spectrum of **2.69** and (b) Expanded ^{13}C NMR spectrum showing duplication of signals.

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[4] The duplication of signals may be due to the presence of a mixture of isomers which are epimeric at C-3.

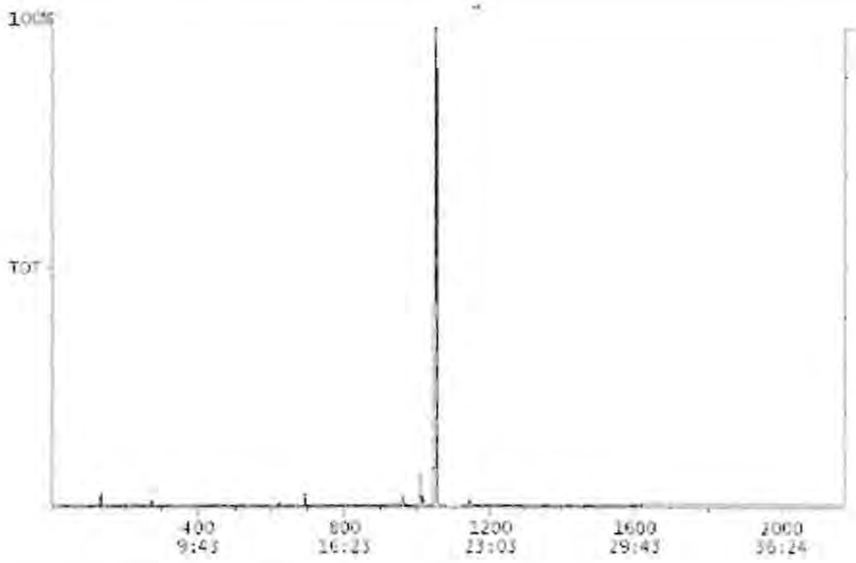

Figure 2.14 GC trace of isomeric mixture of compound **2.69**

Nevertheless, the duplication of ^{13}C NMR signals did not complicate the structure elucidation of **2.69**. The planar structure was elaborated mainly by interpretation of ^{1}H-^{1}H COSY and HMBC data and the identity and positions of the halogen atoms by comparison of ^{1}H and ^{13}C data with that of known compounds. The methyl protons at δ 1.43 (CH$_3$-10) showed clear HMBC correlations (Figure 2.15, Table 2.4) to carbon signals at δ 119.6 (C-1), 137.9 (C-2), 72.5 (C-3), 124.2 (C-5) and 140.8 (C-4). The latter carbon signal was correlated to the olefinic methine signal at δ 6.02 (1H, d, J = 15.4 Hz, H-4), which in turn, showed vicinal coupling to the methine signal at δ 5.97 (1H, dd, J = 15.4 Hz and 8.1, Hz, H-5). The latter methine also showed vinylic coupling to a halomethine proton at δ 4.80 (1H, d, J =8.1 Hz, H-6). Finally, strong and unambiguous HMBC correlations from the CH$_3$-9 methyl (δ 1.78) to carbons at δ 65.7 (C-6), 71.1 (C-7) and 40.8 (C-8) completed the planar structure of **2.69** (Figure 2.15).

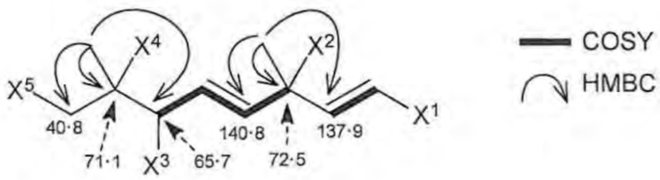

Figure 2.15 Key HMBC correlations for **2.69**

The halogen substitution pattern was assigned by comparing the ^{13}C chemical shifts of **2.69** with those of known compounds. The chemical shifts of C-1 and C-8 are consistent with a vinyl chloride[5] and methylene bromide functionalities,[6] respectively, while the chemical shifts of C-3, C-

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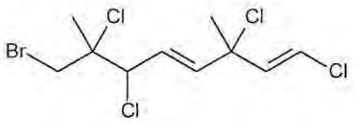
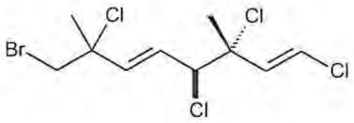
[5] The chemical shift of a vinyl bromide is *ca.* 110 ppm (Stierle *et al.*, 1979)

[6] The chemical shift of a methylene chloride is *ca.* 50 ppm (Naylor *et al*, 1983)

6[7] and C-7 are indicative of chlorine substituents at these positions (Stierle *et al.*, 1979, Naylor *et al*, 1983; Diaz-Marrero *et al*, 2002; Crews *et al*, 1984).[8] The *E*-geometry of the Δ^1 and Δ^4 double bonds were deduced from the $J_{1,2}$ (14 Hz) and $J_{4,5}$ (15.4 Hz) coupling constants and confirmed by the observation of NOESY correlations between H-5 and H-10, and H-2 and H-4 (Table 2.4). The configurations of the chiral centers at C-3, C-6 and C-7 were not assigned. Compound **2.69** is isomeric with 8-bromo-1,3,6,7-tetrachloro-3,7-dimethyl-octa-1,4-diene (**2.66**) previously reported from a *Plocamium* sp. Collected from Antarctica (Stierle *et al.*, 1979).



2.69



2.66

Table 2.4 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations of **2.69** in $CDCl_3$

Carbon No:	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
1	119.6	6.28, d, 13.4	H-1	C-2, C-3	H-4, H-10
2	137.9	6.05, d, 13.4	H-1	C-1, C-3, C-4, C-10	H-10
3	72.5				
4	140.8	6.02, d, 15.4	H-5	C-3, C-5, C-6	H-2, H-6
5	124.2	5.94, dd, 8.1, 15.4	H-4, H-6	C-2, C-3, C-4, C-6	H-8
6	65.7	4.80, d, 8.1	H-5	C-4, C-5, C-7, C-8, C-9	H-5, H-8, H-9
7	71.1				
8	40.8	3.65, d, 11.0 3.80, d, 11.0	Hb-8 Ha-8, H-9	C-6, C-7, C-9	H-9
9	25.3	1.78, s	Hb-8	C-6, C-7, C-8	H-8, H-6, H-5
10	28.3	1.43, s		C-1, C-2, C-3, C-4, C-5	H-1, H-5

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[7] The chemical shift of a bromomethine is ca. 60 ppm (Stierle *et al*, 1979; Naylor *et al*. 1983)

[8] The assignment at C-6 was based on comparison with compounds containing the -$CHClCCH_3Cl$- fragment e.g. oregonene A (Crews, 1977; Stierle, 1975) and cartilagineal (Crews, 1974).

2.5.2.2 Structure elucidation of 2.70

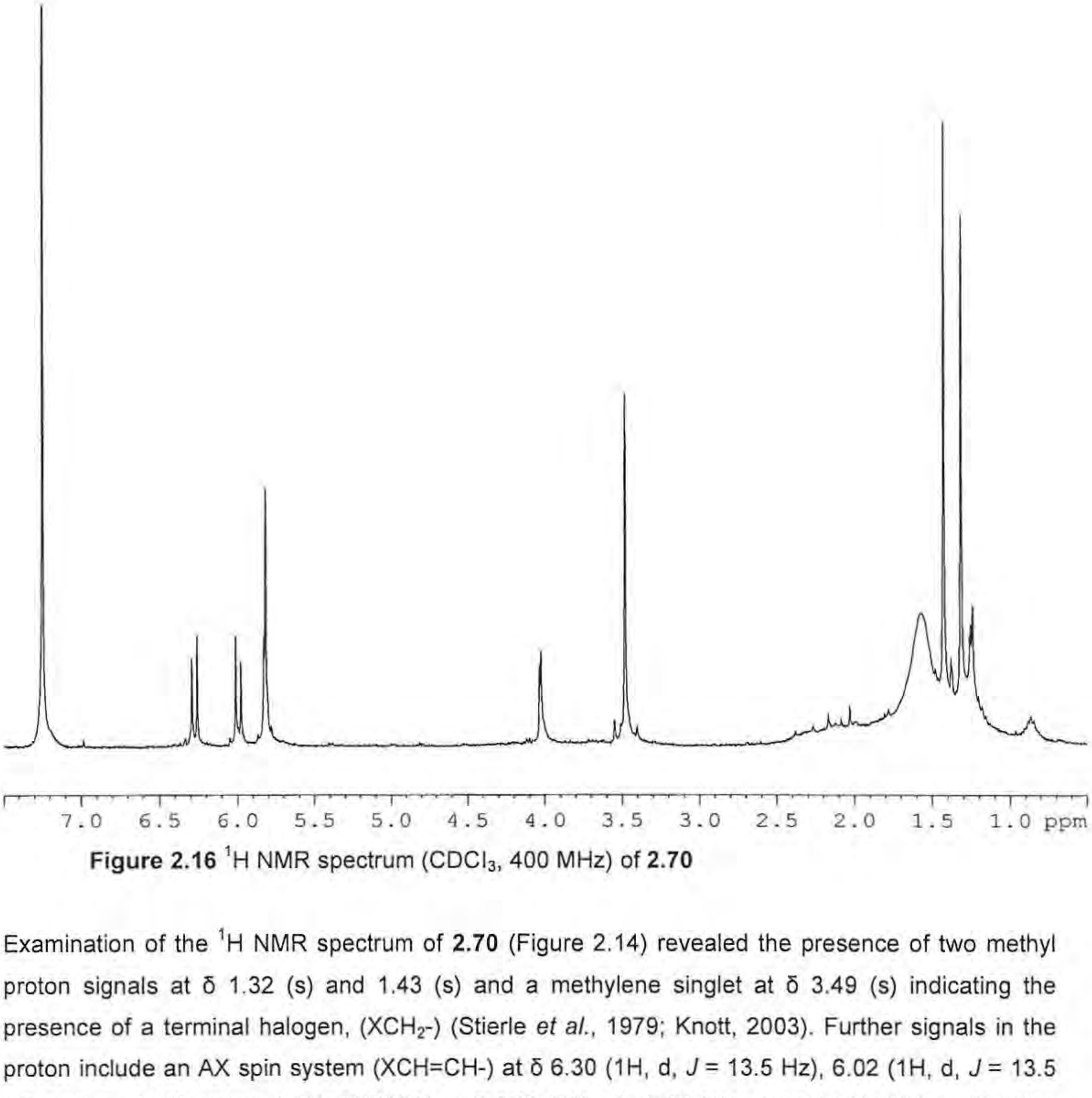


Figure 2.16 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **2.70**

Examination of the ^{1}H NMR spectrum of **2.70** (Figure 2.14) revealed the presence of two methyl proton signals at δ 1.32 (s) and 1.43 (s) and a methylene singlet at δ 3.49 (s) indicating the presence of a terminal halogen, (XCH_2-) (Stierle *et al.*, 1979; Knott, 2003). Further signals in the proton include an AX spin system (XCH=CH-) at δ 6.30 (1H, d, J = 13.5 Hz), 6.02 (1H, d, J = 13.5 Hz) and an ABX system (-CH=CHCHX-) at δ 5.82 (1H, m), 5.83 (1H, s) and 4.03 (1H, m, X of the ABX system). The A and B protons in this system are close together and at first glance the signal at δ 5.82 appears as if it is a doublet. On close analysis, and with the aid of HMQC data, it was clear that this seeming doublet was in fact due to two proton signals close to each other. The first order AMX system is the more common spin system for three coupled protons for halogenated monoterpenes (Abreu *et al.*, 1996). However, as the difference between the chemical shifts of protons A and M (Δδ) becomes smaller (# J_{AB}), a non-first order spectrum results. Compounds with the non-first order ABX system have also been reported (Crews, 1975; Kazlauskas *et al.*, 1976; Crews, 1977). The chemical shift positions and coupling constants are not easy to extract from ABX systems (Kemp, 1991). However, it is theoretically possible to change the non-first order spectrum to a first order spectrum by either increasing the field strength of the spectrometer used, or changing the NMR solvent. Fortunately, the complex ^{1}H NMR spectrum did not significantly complicate the interpretation of the 2D NMR data.

The ^{13}C spectrum of **2.70** showed ten signals, which indicated the presence of two double bonds (δ 120.0, 135.6, 127.9, and 137.2), three sp^3 hybridised carbons at δ 75.2, 71.5, and 78.8, a bromomethylene carbon at δ 44.9 and two methyl carbon singlets δ 26.2 and 24.5. The single bond C-H connectivities were established by 2D HMQC experiment.

The COSY data confirmed the presence of fragments XCH=CH- and -CH=CHCHX- (Table 2.5). Again we found the HMBC data particularly useful in elucidating the structure of **2.70**. Key HMBC correlations from the methyl resonating at δ 1.43 (CH$_3$-9) to the methylene carbon (δ 44.9, C-8), the quaternary carbon (δ 71.5, C-7) and the vinylic carbon (137.2, C-6) and from the methyl at δ 1.32 (CH$_3$-10) to the vinylic carbon C-2 (δ 135.6) and quaternary carbons C-3 (δ 75.2) and C-4 (78.1) provided the planar structure are shown below (Figure 2.17)

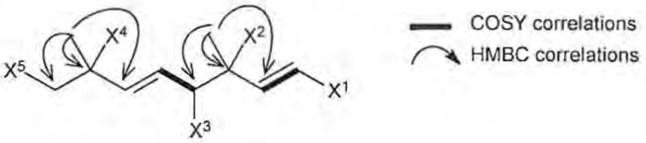
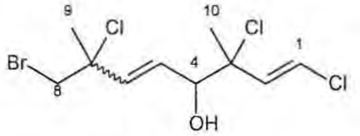


Figure 2.17 Key HMBC correlations for **2.70**

As before, the halogens were placed by comparing the ^{13}C chemical shifts with those of known compounds. The chemical shifts of C-1 and C-7 are consistent with chlorine substituents at these positions while a bromine atom was assigned to C-8. A chemical shift of 78.1 ppm at C-4 is consistent with a hydroxyl group at this position (Diaz-Marrero *et al.*, 2002b). The presence of the hydroxyl group was confirmed by IR data that displayed a broad O-H stretch (3437cm^{-1}) and a sharp C-O stretch (1213cm^{-1}). The geometry of the Δ^1 double bond was assigned as *trans* on the basis of the coupling constants, J = 13.4 Hz. The assignment at Δ^5 could not be made on this basis because of the distorted coupling due to the transition from an AMX to an ABX system. NOESY data (Table 2.5) was however used to determine assign the *trans* configuration and also confirm the assignment at $\Delta^{1,2}$. All the spectral data are consistent with the structure of **2.70**. The relative configuration at C-3, C-4 and C-7 was not determined.



2.70

Table 2.5 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) COSY and HMBC correlations of **2.70** in $CDCl_3$

Carbon No:	δ_C	δ_H, multi, J(Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
1	120.0	6.30, d, 13.4	H-2	C-2, C-3	H-10
2	135.6	6.02, d, 13.4	H-1	C-3, C-1	H-4
3	75.2				
4	78.1	4.03,m	H-5[9]	C-5, C-6	H-2, H-6, H-10
5	127.9	5.83,m	H-6	C-1, C-3, C-10	H-9
6	137.2	5.82,m	H-4, H-8, H-10	C-4, C-7	H-4, H-8, H-9
7	71.5				
8	44.9	3.49, s	H-9	C-6, C-7, C-9	H-6, H-9
9	26.2	1.43, s	H-8, H-6	C-4, C-7, C-8	H-5, H-6, H-8
10	24.5	1.32, s		C-3, C-2	H-1, H-4

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[9] A definite COSY correlation between H-4 and H-5 is observed. However, due to non-first order spectrum the coupling constant cannot be determined. The non-first order spectra also affects the chemical shift of X (H-4) as shown by simulation of the spectra which also it's confirms non-first order nature.

2.5.2.3 Structure elucidation of 2.71

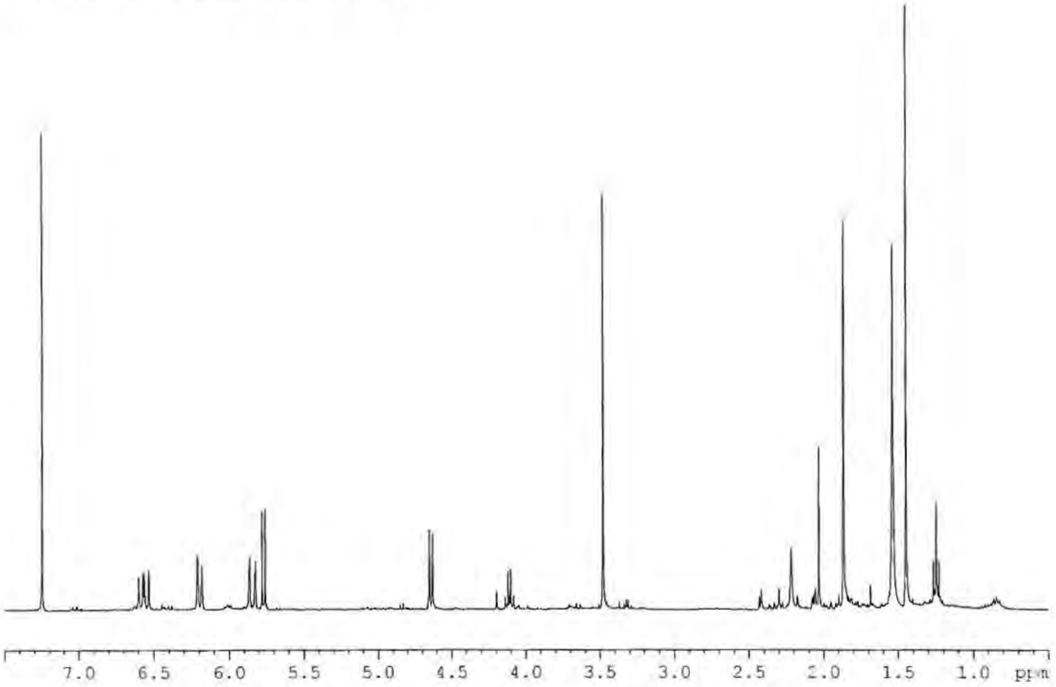


Figure 2.18 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **2.71**

The ^{1}H NMR spectrum of **2.71** (Figure 2.18) showed vinylic methine resonances at δ 5.86 (d, J = 15 Hz), 6.56 (dd, J = 15, 11 Hz) and 6.20 (d, J = 11 Hz) with a coupling pattern suggestive of an AMX system. A further two mutually coupled methine resonances were observed at δ 5.78 (d, J = 9 Hz) and 4.63 (d, J = 9 Hz) as well as a deshielded methylene singlet at δ 3.48 (s) and two methyl singlets at δ 1.45 (s) and 1.86 (s). Ten signals were discernable from the ^{13}C NMR and DEPT-135 spectra, consistent with a monoterpene, and comprised of four olefinic carbons, three halocarbons and two methyl carbons. All C-H single bond connections were determined from HSQC data (Figure 2.19, Table 2.6).

^{1}H-^{1}H COSY and HMBC data were used to delineate the planar structure of **2.71**. HMBC correlations from the methyl protons to the neighboring carbons were particularly helpful. Thus, the methyl signal at δ 1.45 (H_3-9) showed HMBC correlations to carbons resonating at δ 139.6 (C-6), 71.8 (C-7), 44.7 (C-8) and δ 124.6 (5) while the methyl protons at δ 1.86 (H_3-10) showed correlations to carbons at δ 132.6 (C-3), 72.7 (C-2) and 131.4 (C-4) (Figure 2.31, Table 2.20).

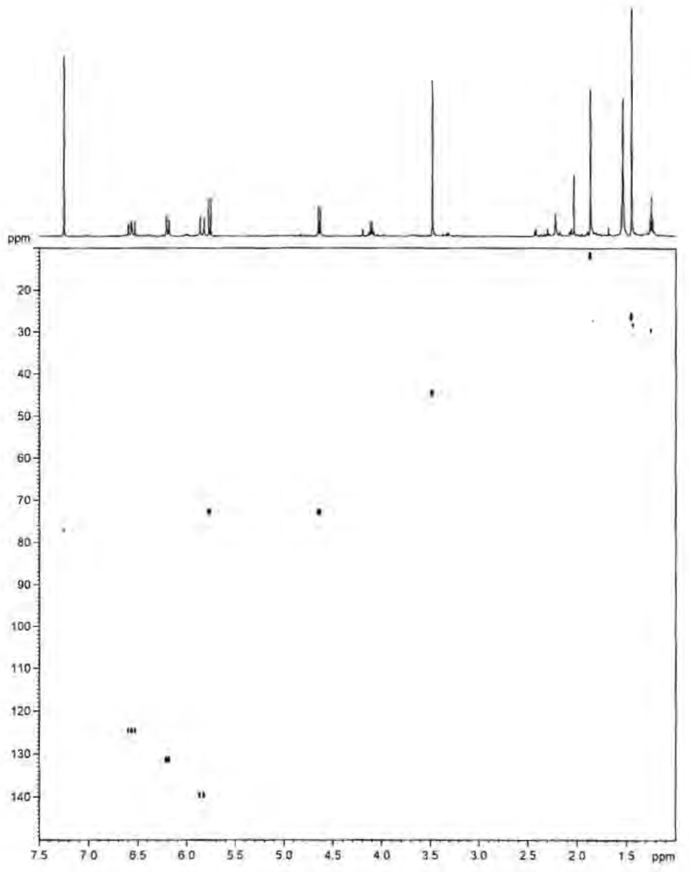


Figure 2.19 HSQC spectrum of **2.71**

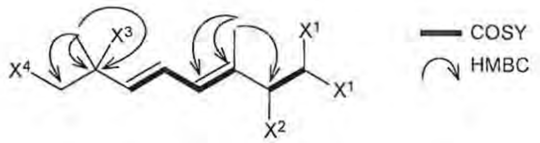
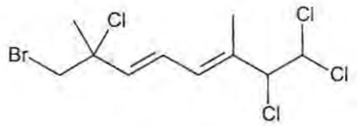


Figure 2.20 Key COSY and HMBC correlations for **2.71**

Having established the skeleton for **2.71** we now turned our attention to determining the identity and positions of the halogen atoms. This was again done by comparing the ^{13}C chemical shifts of **2.71** to those of known compounds.

The halogen at C-7 and C-8 were assigned to be Cl and Br respectively (Stierle *et al.*, 1979; Knott, 2003) and while the chemical shift of δ 72.9 at C-1 is consistent with two chlorine atoms at this position (Imperato *et al.*, 1977; Mynderse *et al.*, 1975). Similarly, the C-2 chemical shift (δ 72.7) is suggestive of a chlorine atom at this position (Bates *et al.* 1979). The geometry at $\Delta^{5,6}$ was assigned *trans* based on the observed coupling constant of 15 Hz between the protons and NOESY data (Figure 2.19 and Table 2.6). The stereochemistry at C-7 was not assigned. All available spectroscopic data are consistent with the proposed structure for **2.71**.



2.71

Table 2.6 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations of **2.71** in $CDCl_3$

Carbon No:	**δ_C**	**δ_H, multi, *J* (Hz)**	**^{1}H-^{1}H COSY**	**^{1}H -^{13}C HMBC**	**^{1}H -^{1}H NOESY**
1	72.9*	5.78, d, 9	H-2	C-2, C-3	H-2, H-10
2	72.7*	4.63, d, 9	H-1, H-10	C-1, C-3, C-10	H-4, H-10
3	132.6				
4	131.4	6.20, d, 11	H-2, H-5, H-10	C-2, C-3, C-5, C-6, C-10	H-2, H-6
5	124.6	6.56, dd, 11 and 15	H-4, H-6	C-3, C-4, C-6, C-7, C-9	H-4, H-8, H-10
6	139.5	5.86, d, 15	H-5, H-9	C-4, C-5, C-7, C-8, C-9	H-4, H-8, H-9
7	71.8				
8	44.7	3.48, s	H-6, H-9	C-5, C-6, C-7,C-9	H-5, H-6, H-9
9	26.4	1.45, s	H-8	C-5, C-6, C-7, C-8	H-6, H-8
10	11.6	1.86, s	H-2, H-4	C-2, C-3, C-4	H-1, H-5

* carbon chemical shifts may be interchangeable.

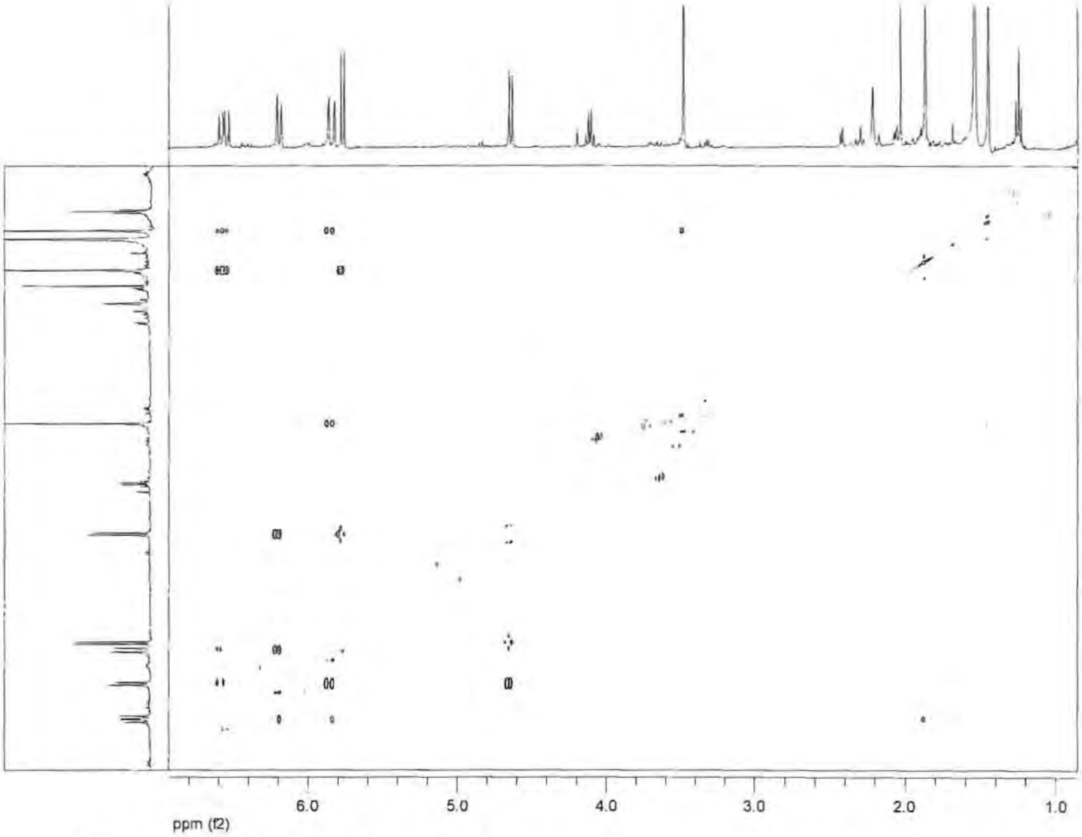


Figure 2.21 NOESY spectrum of **2.71**

2.5.2.4 Structure elucidation of 2.72

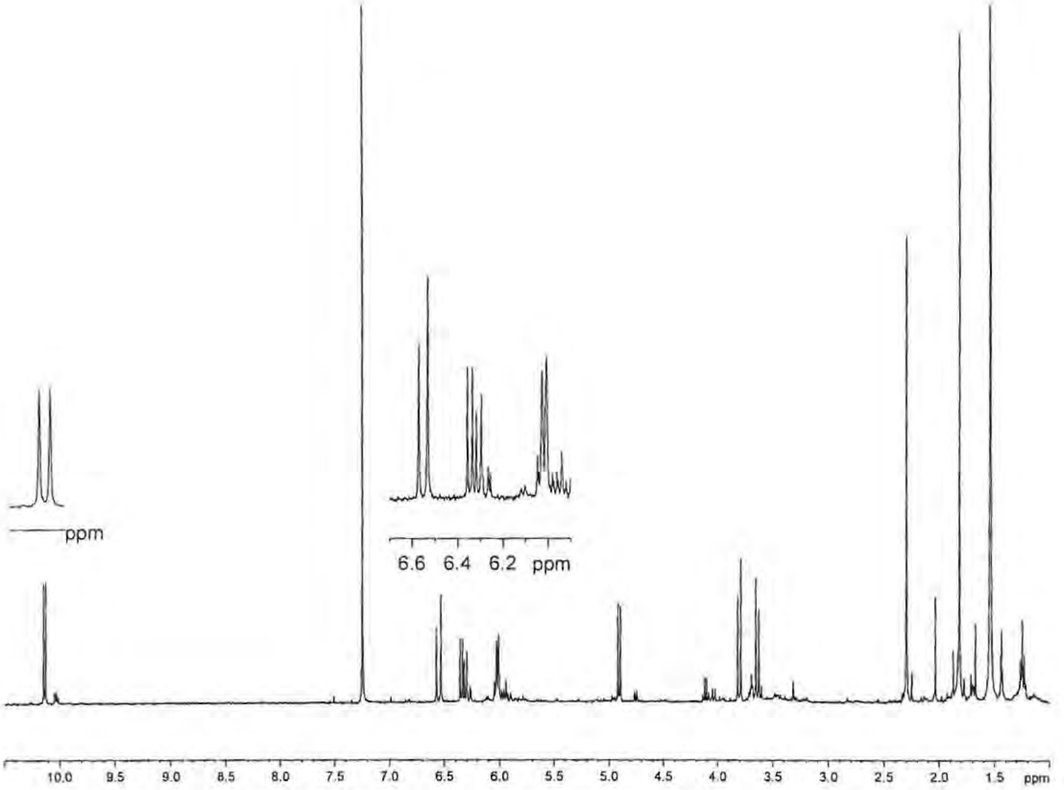


Figure 2.22 ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **2.72**

Compound **2.72** was isolated as a pale green oil. This colour suggested the presence of a conjugated chromophore, which was confirmed by a λ_{max} of 266 nm (hexane) in the UV spectrum. Low resolution mass spectroscopy showed the presence of cluster at m/z 263/265/267 (8:10:2) corresponding to the M^+-Cl ion. The most apparent difference between the ^{1}H NMR spectrum of **2.72** (Figure 2.22) and that of **2.69** was the fact that the vinyl chloride had been replaced by a deshielded proton signal at δ 10.15 (d, J = 8.0 Hz) which immediately suggested the presence of an aldehyde. This was confirmed by IR data, which showed a peak at 1664 cm^{-1} typical of the aldehyde carbonyl. Vinyl methine signals at δ 6.53 (d, J = 16.0 Hz) and 6.33 (dd, J = 16.0 and 9.0 Hz) together with the halomethine at δ 4.91 (d, J = 9.0) were indicative of a similar –CH=CH-CHX-moiety as seen in **2.69**. Interestingly, as was the case for **2.69**, the signal due to the bromomethylene appeared as a doublet of doublets [δ 3.82 (d, J = 11.0) and δ 3.65 (d, J = 11.0 Hz)]. It is likely that the presence of the heavy halogen atoms may force the molecule to adopt a more rigid conformation which results in the two methylene protons being in difference electronic environments[10].

The ^{13}C NMR data revealed the presence of two double bonds (δ 131.4, 150.0, 138.1, and 131.0), three halogen bearing carbons (δ 65.8, 71.1 and 40.6), an aldehyde carbon (δ 191.1) and two

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[10] The presence of the two chiral centres together with the stereoelectronic effects of the halogens cause the two methylene protons to be in different environments.

methyl groups (δ 25.6, and 13.2). ^{1}H-^{1}H COSY, HSQC and HMBC data allowed the structure of **2.72** to be determined. The methyl proton at δ 2.30 (CH$_3$-10) showed HMBC correlations (Figure 2.23 and Table 2.7) to carbons at δ 131.0, 150.0, 138.1 (C-2, C-3 C-4, respectively) while the proton at C-2 (δ 6.00 (d, *J* = 8.0 Hz) showed a COSY correlation to the aldehyde proton at δ 10.15 (H-1, d, *J* = 8.0 Hz) and a weak correlation to the methyl protons at δ 2.30 (s, C-10). The protons of the methyl group at δ 1.83 (s, CH$_3$-9) showed HMBC correlations to carbons at δ 65.8, 71.1 and 40.6 (C-6, C-7 and C-8). The methylene protons at C-8 (δ 3.83 and 3.65) appeared as two doublets geminally coupled (*J* = 11 Hz) indicating that they exist in chemically different environments evidence of which is shown by the fact that only H$_b$ shows a COSY correlation to methyl proton at δ 1.83 (C-9).

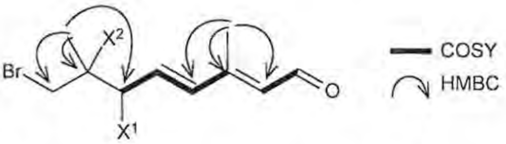
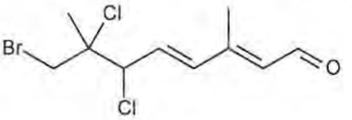


Figure 2.23 Key COSY and HMBC correlations for **2.72**

As before, the identity of the halogen atoms at position C-7, C-8 and C-9 were assigned by comparison of ^{13}C chemical shifts with those of known compounds. The chemical shifts of C-6 and C-7 (δ 65.8 and 71.1, respectively) are consistent with chlorine atoms at these positions while a bromine atom must be accommodated at C-8 to account for the chemical shift of δ 40.6. The *E*-geometry of the Δ^4 double bond, was deduced from the $J_{4,5}$ (16 Hz) coupling constant and confirmed by NOESY data, with H-4 showing correlation to H-2 and H-6, thus indicating they are on the same side of the double bond. An NOE between H-1 and H-10 indicated *E*-geometry for the Δ^2 double bond. The stereochemistry of the chiral centres at C-6 and C-7 was not determined. All spectroscopic data are consistent with the proposed structure for **2.72**.



2.72

Table 2.7 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations of **2.72** in $CDCl_3$

Carbon No:	**δ_C**	**δ_H, multi, *J* (Hz)**	**^{1}H-^{1}H COSY**	**^{1}H-^{13}C HMBC**	**^{1}H-^{1}H NOESY**
1	191.1	10.15, d, 8	H-2	C-2	H-10
2	131.0	6.00, d, 8	H-1, H-10	C-10, C-4	
3	150.				
4	138.1	6.53, d, 16	H-5	C-10, C-6, C-5, C-3	H-2, H-6
5	131.0	6.33, dd, 9, 16	H-4, H-6	C-4, C-3	H-6
6	65.8	4.91, d, 9	H-5	C-4, C-5, C-8, C-9	H-5, H-8
7	71.1				
8a	40.6	3.82, d, 11	H_b-8, H-9	C-9, C-6, C-7	H-6
8b		3.65, d, 11	H_a-8		
9	25.6	1.83, s	H_A-8	C-8, C-6, C-7	H-8
10	13.2	2.30, s	H-2	C-2, C-3, C-4	H-1, H-5

2.5.2.5 Structure elucidation of 2.73

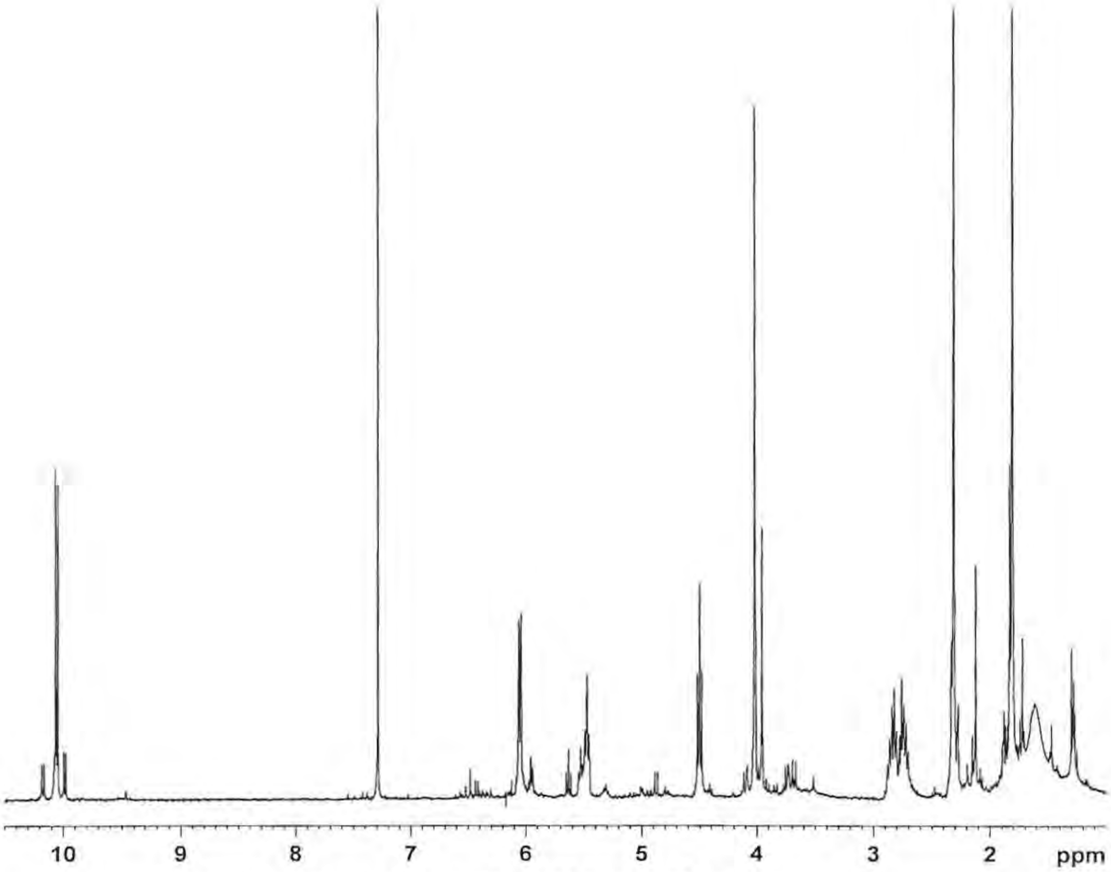


Figure 2.24 ^1H NMR spectrum ($CDCl_3$, 400 MHz) of **2.73**

This compound was isolated as a colourless oil. The ^1H NMR spectrum of compound **2.73** (Figure 2.35) showed an aldehyde proton signal at δ 10.3 (d, *J* =7.3 Hz) coupled to a methine proton at δ 6.02 (d, *J* = 7.3 Hz) which immediately suggested the presence of the same α,β-unsaturated aldehyde moiety as seen in **2.72**. Further signals included a vinylic methine at δ 5.43 (t, *J* = 7.6 Hz) coupled to a methylene multiplet centred at δ 2.78 (m, *J* = 7.6 Hz), a methylene singlet at δ 3.98 (s) indicating a terminal halides, while signals at δ 2.25 (s) and δ 1.81 (s) were consistent with methyl groups. The ^{13}C spectrum showed ten distinct peaks suggesting a monoterpene with two double bonds (δ 157.9, 135.6, 127.5, and 124.9) The other six signals included an aldehyde carbon resonating at δ 191.3, a halomethine (δ 56.5), two methylenes (δ 51.2 and 34.8) and two methyls (δ 14.7 and 13.8).

Use of COSY and HMBC (Figure 2.25 and Table 2.8) data was crucial in determining the structure of **2.73**. COSY correlations were observed between the aldehyde proton δ 10.1 (H-1) and the olefinic methine at δ 6.02 (H-2) while the methylene protons at δ 2.78 (H-5) showed correlations to signals at δ 4.4 (H-4) and δ 5.43 (H-6). HMBC correlations from methyl protons at δ 2.25 (CH_3-10) to carbon signals at δ 56.5 (C-4), 157.6 (C-3) and 127.5 (C-2) was consistent with the fragment –CHX-C(CH_3)=CH-CHO. A further fragment, X-CH_2-C(CH_3)=CH-] was constructed from the observation of HMBC correlations from the methyl signal at δ 1.81 (CH_3-9) to carbons at δ 51.2 (C-8), 135.6 (C-7) and 124.9 (C-6). The above observations are consistent with the planar structure of **2.73** (Figure 2.25).

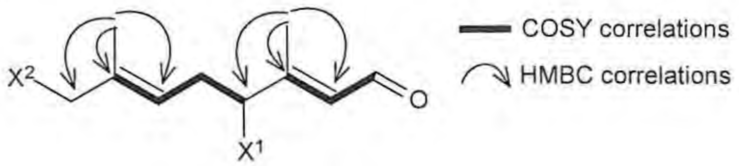
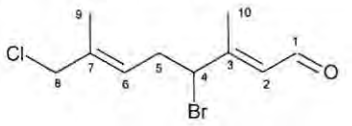


Figure 2.25 Important correlations for **2.73**

The halogen substituents at C-4 (δ 56.5) and C-8 (δ 51.2) were established as bromine[11] and chlorine, respectively, by comparison of proton and carbon NMR data of known compounds (Crews, 1977; Crews *et al.*, 1984). The geometry of the Δ^2 and Δ^6 double bonds are proposed as *trans*[12] due to the shielding effect of the γ-substituent on these double bonds (Crews, 1977). This assignment was confirmed by NOE correlations between H-1 and H-10 and between H-6 and H-8. All available spectroscopic data are consistent with the proposed structure for **2.73**.



2.73

Table 2.8 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations for **2.73** in $CDCl_3$

Carbon No:	δ_C	δ_H, multi *J* (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H HMBC	^{1}H-^{1}H NOESY
1	191.2	10.03, d, 7.33	H-2	C-2	H-2, H-10
2	127.5	6.02,d, 7.33	H-1, H-10	C-2, C-3, C-5, C-10,	H-4, H-5
3	157.9				
4	56.5	4.47, t, 7.58	H-5	C-10, C-5	H-2, H-5, H-6, H-10
5	34.8	2.78, m,	H-4, H-6, H-9	C-3, C-4, C-6, C-7	H-4, H-9, H-10
6	124.9	5.43, t, 6.57	H-5, H-8, H-9	C-8, C-9	H-4, H-5, H-8
7	135.6				
8	51.2	3.98, s	H-5, H-6	C-6, C-7, C-9	H-6, H-9
9	14.7	1.81, s	H-6, H-5	C-6, C-7, C-8	H-8
10	13.8	2.25, s	H-2	C-2, C-3, C-4	H-1, H-4

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[11] A chlorine substutent at this position will give a chemical shift of ca. 62 ppm (Crews *et al*, 1977)

[12] The chemical shift for a methyl group trans to the γ-alkyl substituent is ca. 22-24 (Crews *et al*, 1977)

2.5.2.6 Structure elucidation of 2.74

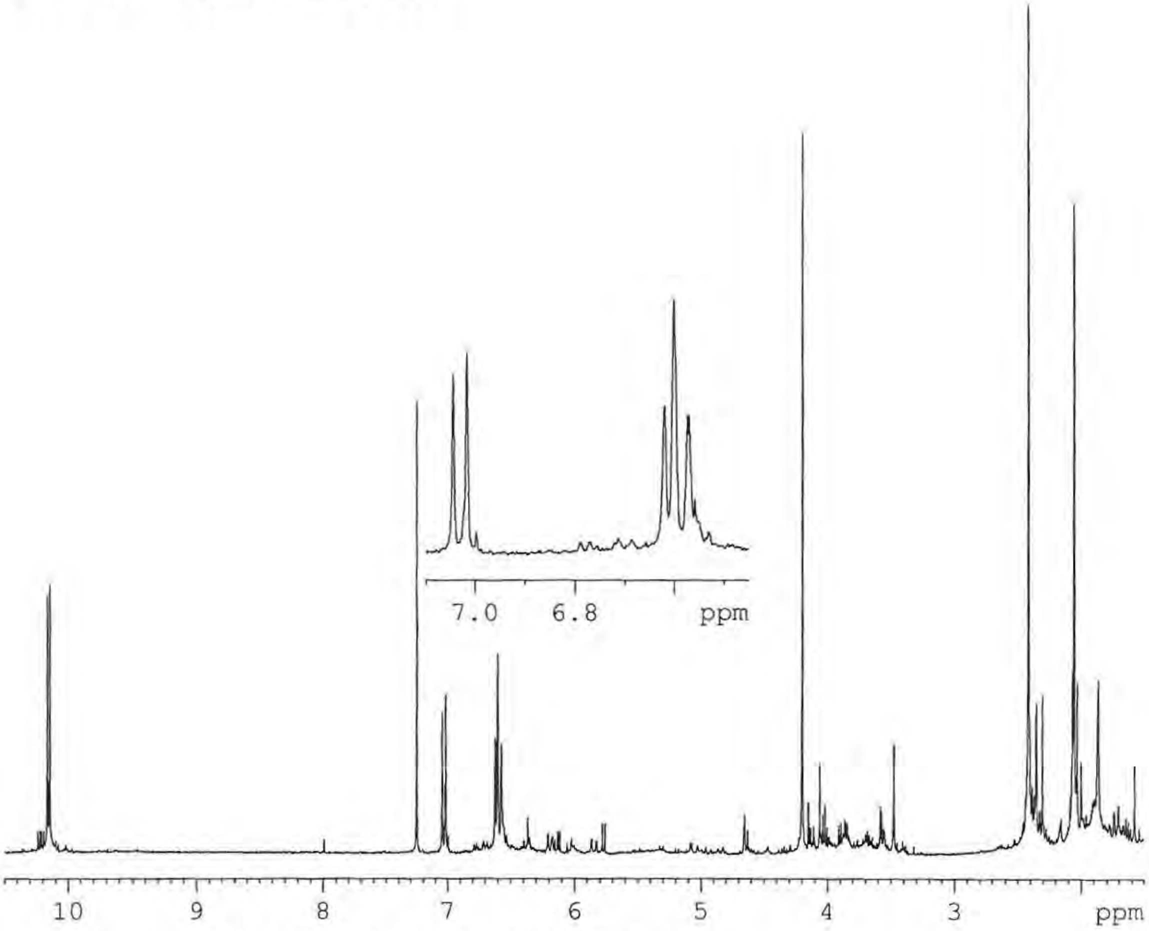

Figure 2.26 ^{1}H NMR (CDCl$_3$, 400 MHz) spectrum of **2.74**

Compound **2.74** was extracted as a yellowish oil. The ^{1}H NMR and ^{1}H-^{1}H COSY data (Figure 2.26 and Table 2.9) indicated the presence a methine proton resonating at δ 7.04 (d, J = 10.9 Hz) which was coupled to another methine proton at δ 6.58 (d, J = 10.9 Hz). The HSQC spectrum (Figure 2.27) clearly shows that the cluster at δ 6.6 is due to two protons attach to carbon atoms close to each other. The other olefinic proton resonating at δ 6.61 (d, J = 7.4 Hz) in the cluster is coupled to an aldehyde proton at δ 10.16 (d, J = 7.4 Hz). The presence of the aldehyde was further confirmed by IR data with a carbonyl stretch at 1668 cm^{-1}. The other signals in the ^{1}H NMR spectrum included a methylene proton at δ 4.19 (s) and two methyl singlets at δ 2.05 and 2.41. The ^{13}C NMR data revealed the presence of three double bonds (δ 128.6, 134.4, 150.7, 125.1, 125.8 and 141.9), a bromomethylene carbon at δ 42.3 (Stierle *et al.*, 1979) and, two methyl carbons (δ 22.9 and 14.7). Information deduced from the ^{1}H and ^{13}C NMR data envisaged the presence of the following fragments in the molecule: -CH$_2$Br-, -CH=CH-C-, C=CH-CHO, -C(CH$_3$)=C-. 2D NMR data enabled the planar structure of this compound to be determined. The fragments constituting the structure of the compound were discerned from the COSY and HMBC correlations from the methyl protons (Table 2.9 and Figure 2.28).

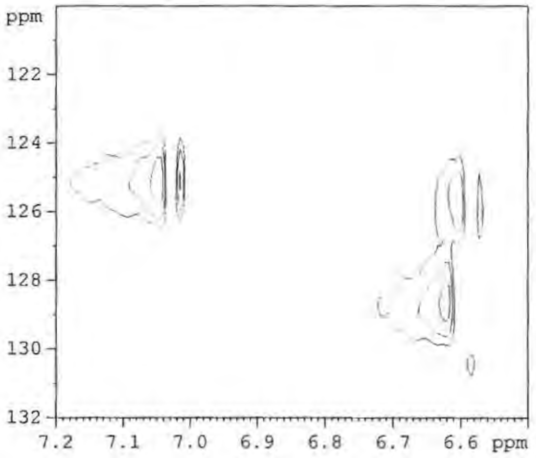

Figure 2.27 Expanded HSQC spectrum of **2.74**.

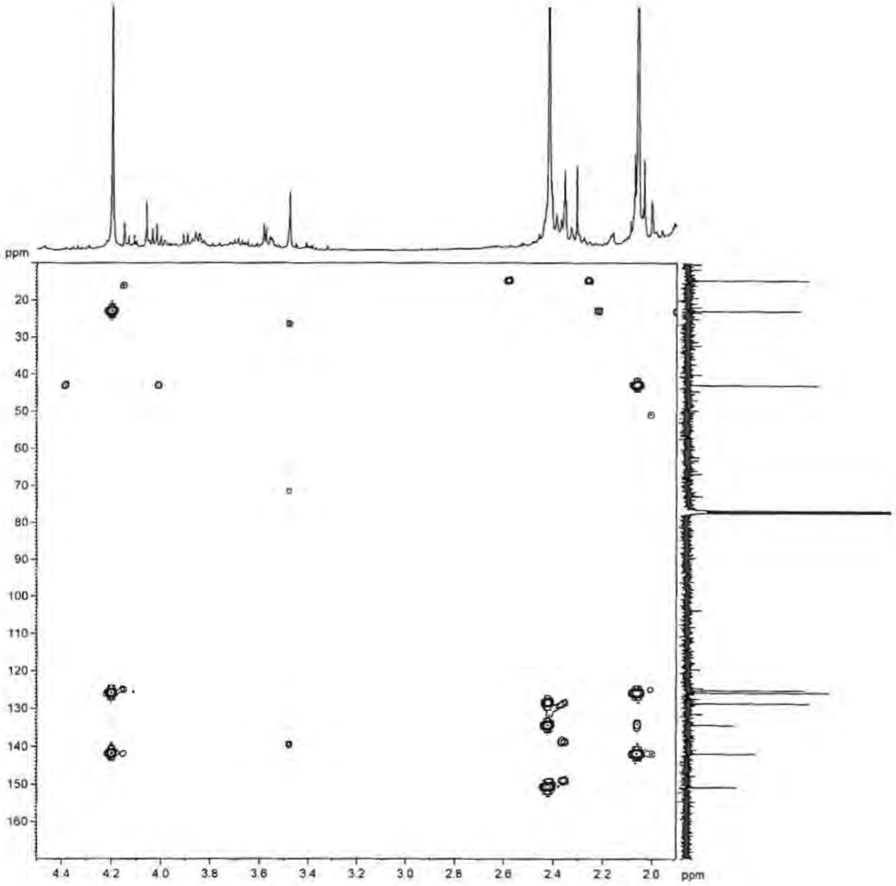

Figure 2.28 Expanded HMBC showing important correlations used in elaborating the fragments of **2.74**.

The C-10 methyl protons (δ 2.41) showed clear HMBC correlations to carbons at δ 128.6 (C-2)[13], 134.4 (C-3) and 150.7 (C-4). The C-9 methyl protons (δ 2.05) showed HMBC to carbons at δ 141.9 (C-7), 125.8 (C-6) and 42.9 (C-8). Long range COSY correlations were observed with protons linked to the latter two carbons, H-6 and H-8 as well as H-5. The two fragments derived from the methyl groups were joined by COSY and HMBC correlations of δ 7.04 (H-5). COSY correlations to protons resonating at δ 6.58 (H-6) and 2.05 (CH_3-9) and HMBC correlations with carbons at δ 134.4 (C-3), 150.7 (C-4) and 141.9 (C-7) were also observed. Important correlations in the configuration and linkage of fragments are shown in Figure 2.29 below, double ended and single ended arrows representing COSY and HMBC respectively.

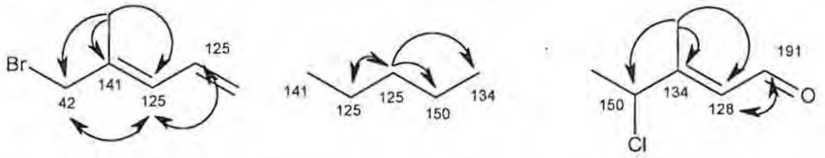

Figure 2.29 Selected COSY and HMBC correlations important in elaborating the fragments of the structure (**2.74**) and linking together.

The chemical shift at C-8 (δ 42.9) indicated a bromomethylene carbon (Stierle *et al.*, 1979) whereas the chemical shift at C-4 indicated the presence of a chlorine (Dunlop *et al*, 1979; Sims *et al*, 1978). The double geometry for Δ^2 and Δ^6 were assigned as *trans* based on NOESY data (Figure 2.30).

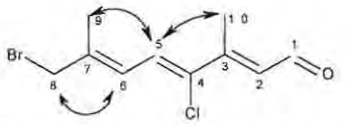

Figure 2.30 Structure of **2.74** showing important NOESY correlations

Table 2.9 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY, HMBC and NOESY data for **2.74**

Carbon No:	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
1	191.2	10.16, d, 7.4	H-2	C-2	H-10
2	128.6	6.61, d, 7.4	H-1, H-10	C-3, C-10	
3	150.7				
4	134.4				
5	125.1	7.04, d, 10.9	H-6, H-9	C-3, C-4, C-7	H-9, H-10
6	125.8	6.58, d, 10.9	H-5, H-8, H-9	C-8, C-9	H-8
7	141.9				
8	42.9	4.19, s	H-6, H-9	C-6, C-7, C-9	H-6
9	22.9	2.05, s	H-5, H-6, H-8	C-6, C-7, C-8	H-5
10	14.7	2.41, s	H-2	C-2, C-3, C-4	H-1, H-5

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[13] , a long range COSY correlation was also observed with the proton linked to this carbon.

2.5.2.7 Structure elucidation of 2.75

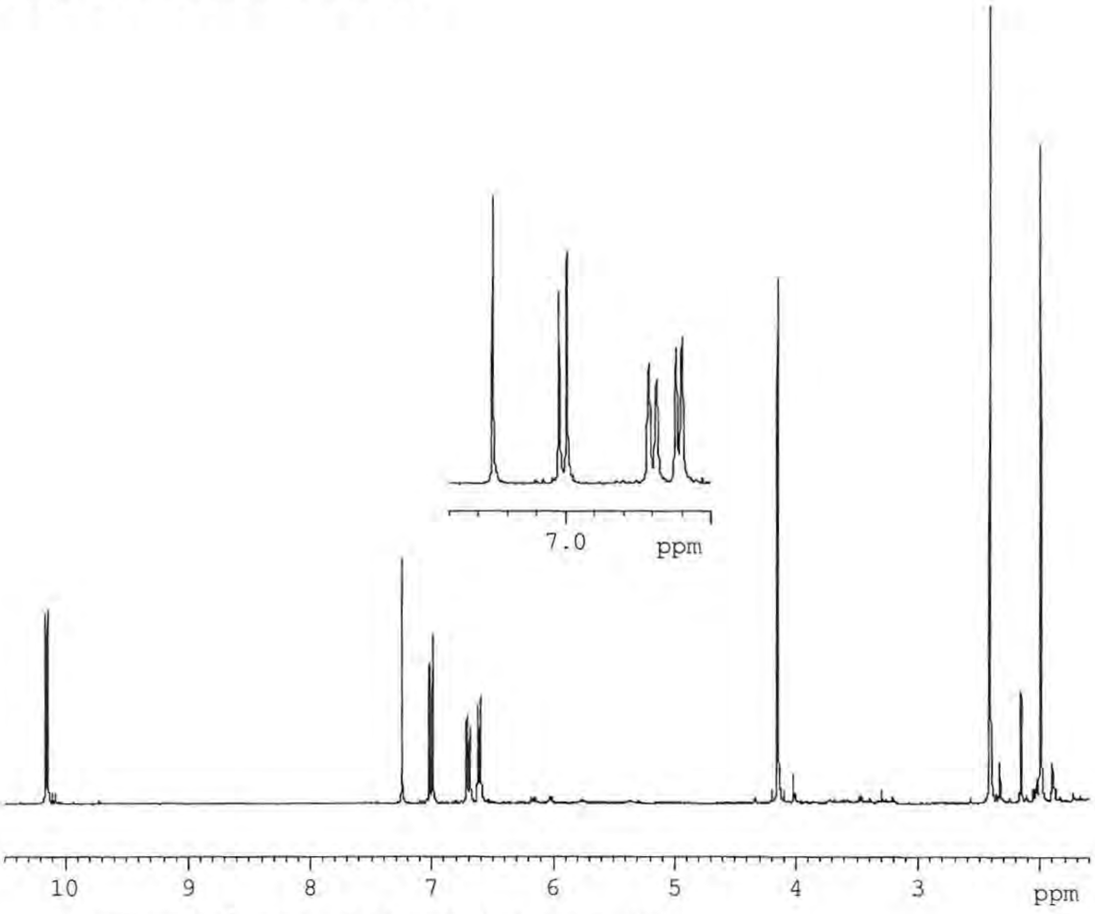


Figure 2.31 ^{1}H NMR ($CDCl_3$, 400 MHz) spectra of **2.75**

Compound **2.75** was isolated from the organic extract as a yellowish green oil with chromophores absorbing at λ_{max} of 330.6, 316.4 and 231.6 nm.

The ^{1}H NMR spectrum of **2.75** (Figure 2.31) displayed two vinylic methyl signals at δ 2.00 (s) and 2.41 (s), a singlet due to methylene protons at δ 4.15 (s), an aldehyde proton doublet at 10.15 (d, J = 7.7 Hz), coupled to a methine proton at δ 6.62 (d, J = 7.7 Hz) by COSY data (Table 2.10) .The remaining signals included two coupled vinylic protons at δ 7.02 (d, J = 10.6 Hz) and δ 6.71 (d, J = 10.6 Hz). Ten signals were seen in the ^{13}C carbon spectrum (Figure 2.32), with six assigned as sp^2 olefinic carbons resonating at δ 125.1, 126.2, 128.5, 135.0, 142.0 and 150.6, two methyl carbons δ 15.9 and 14.9 and a halomethylene carbon at 51.2. The presence of four double bonded carbons meant the structure could only be acyclic.

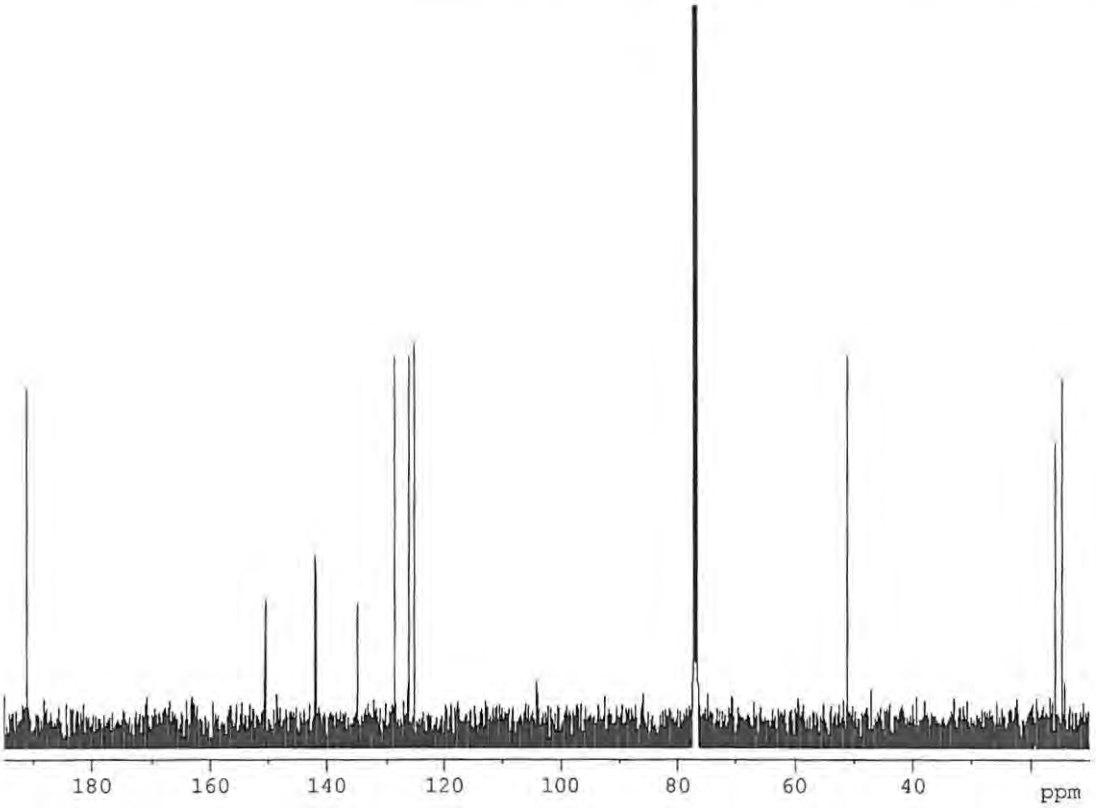


Figure 2.32 ^{13}C NMR ($CDCl_3$, 100 MHz) spectra of **2.75**

Interpretation of HSQC, HMBC, and ^1H-^1H COSY data (Table 2.10) led to the discernment of important fragments of the molecule and ultimately the elucidation of the planar structure. The HMBC correlations of the methyl group protons again defined the fragments. Clear HMBC correlations (Figure 2.33 and 2.34) from the methyl protons at δ 2.00 (CH_3-9) to carbon atoms at 125.1 (C-6), 142.0 (C-7) and 51.2 (C-8) were seen. The proton coupled to the carbon at δ 125.1 (H-6) displayed long range COSY correlations to protons at δ 4.15 (CH_2-8) and 2.00 (CH_3-9), respectively. The methyl protons at δ 2.41 (CH_3-10) displayed clear correlations to carbons at 191.2 (C-1), 128.5 (C-2), 135.0 (C-3) and 150.6 (C-4) as well as a weak COSY correlation to the proton at δ 6.62 (H-2), which in turn showed a strong COSY correlations to a signal at δ 10.15 (H-1). HMBC correlations from the methine at δ 7.02 (H-5) to carbons at δ 135.0 (C-3), 150.6 (C-4) and 142.0 (C-7) as well as COSY correlations to the proton at δ 6.71 joined the fragments. Selected important correlations are shown (Figure 2.33) below.

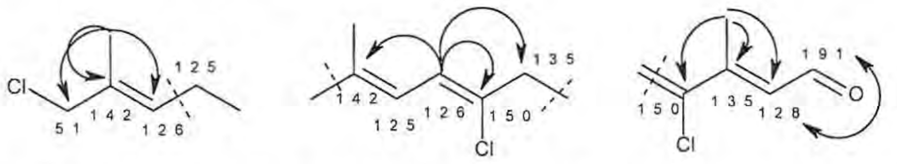
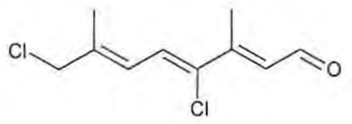


Figure 2.33 Selected COSY and HMBC correlations used in determination of, and, linking of fragments in **2.75**.

Table 2.10 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations of **2.75** in $CDCl_3$

Carbon No:	δ_C	δ_H, multi, *J* (Hz).	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
1	191.2	10.15, d, 7.66 Hz	H-2	C-2	H-2, H-10
2	128.5	6.62, d, 7.66 Hz	H-1, H-10	C-3, C-4, C-10	H-1
3	150.6				
4	135.0				
5	126.2	7.02, d, 10.61 Hz	H-6, H-8, H-9	C-3, C-4, C-7	H-9, H-10
6	125.1	6.71, d, 10.61 Hz	H-9, H-8, H-5	C-9, C-8, C-3	H-8
7	142.0				
8	51.2	4.15, s	H-6, H-9	C-6, C-7, C-9	H-6
9	15.9	2.00, s	H-6, H-8	C-6, C-7, C-8	H-5
10	14.7	2.41, s	H-2	C-2, C-3, C-4	H-1, H-5

The chemical shifts of C-4 (135.0 ppm) and C-8 (51.2 ppm) are consistent wit chlorine atoms at these positions (Bates *et al.*, 1979; Crews *et al.*, 1984). The double bonds were assigned *trans* from chemical shift values of the methyl groups and confirmed by NOEs between H-5 and H-9 and H-10 and between H-1 and H-9.



2.75

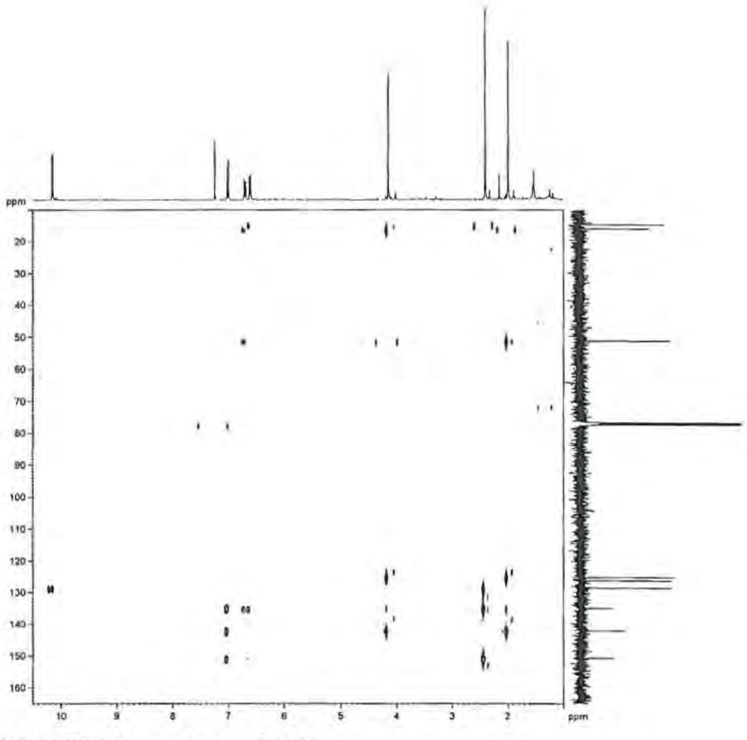


Figure 2.34 HMBC spectrum of **2.75**

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2.5.2.8 Structure elucidation of 2.76

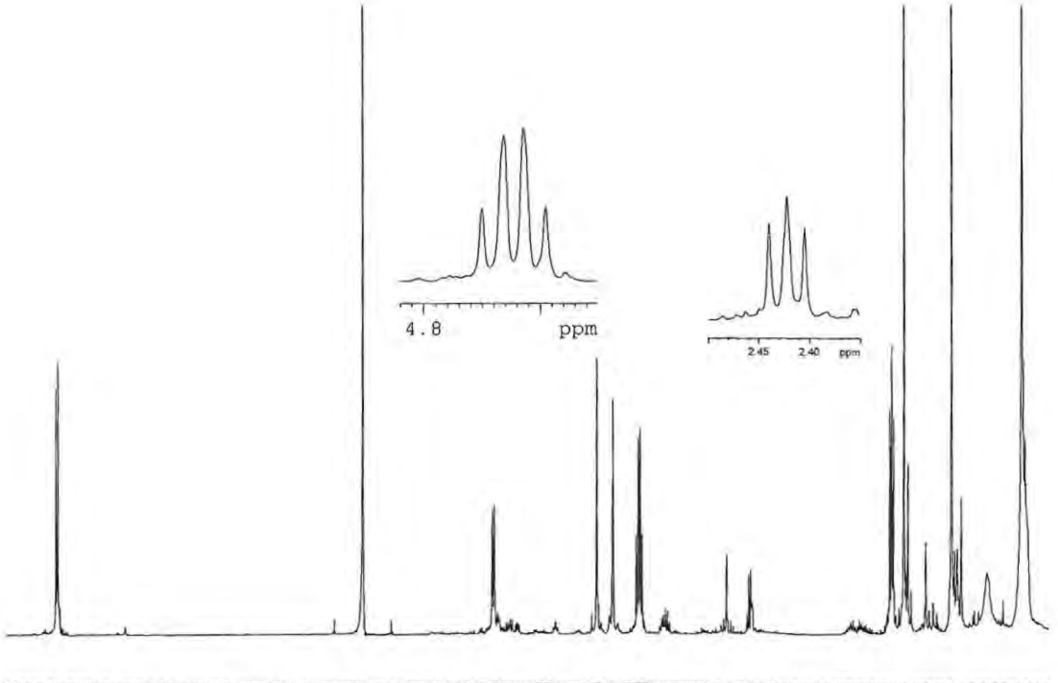


Figure: 2.35 ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **2.76**

Compound **2.76** was isolated as a clear oil from the DCM/MeOH fraction. The ^{1}H NMR spectrum (Figure 2.35) of this compound showed a quartet and triplet, which initially suggested the presence of ethyl acetate, used as one of the solvents in HPLC. Upon closer inspection this was discounted on the basis of observed chemical shifts. The quartet consisted of two protons δ 4.72 (1H, t, *J* = 7.6 Hz) and δ 4.72 (1H, t, *J* = 7.6 Hz), as shown by the integration, and is in fact due to two overlapping methine triplets coupled to a methylene at δ 2.42 (1H, t, *J* = 7.6 Hz). An aldehydic proton signal seen at δ 10.01 (1H, d, *J* = 7.33 Hz) was coupled to a vinylic proton at δ 6.05 (1H, d, *J* = 7.3 Hz). The other signals in the proton spectrum included two methyl proton singlets at δ 1.88 (1H, s) and 2.31 (1H, s), and two vinylic protons singlets at δ 5.11 (1H, s) and δ 4.96 (1H, s), which were attached to the same carbon atom, 115.0 (C-8). The ^{13}C NMR spectrum (Figure: 2.36) showed ten distinct signals at a glance, but after close analysis eleven signals where seen because they were two signals, which were almost superimposed resonating at 56 (C-4) and 56.1 (C-6) ppm respectively. Data from 2D NMR experiments however showed the signals at δ 32.0 to be from an impurity.

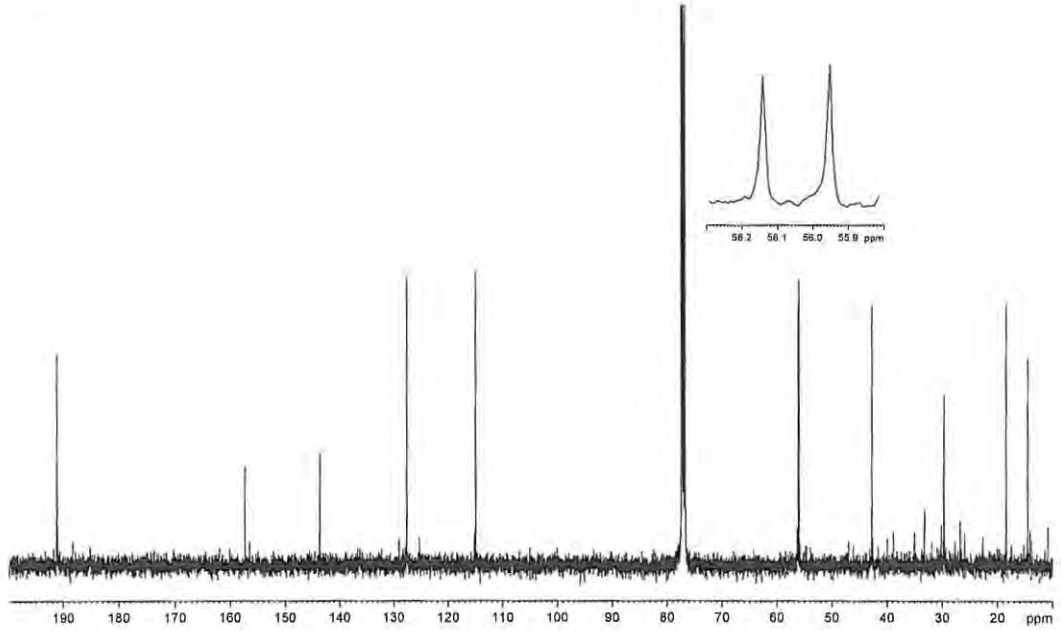


Figure: 2.36 ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **2.76**

From the ^{13}C NMR and DEPT-135 data, four vinylic carbons (two double bonds) were depicted at δ 115.0 (C-8, CH_2), 127.8 (C-2, CH), 143.7 (C-7, C) and 157.3 (C-3, C), with the latter two being quaternary carbons, four methine carbons at δ 56.1 (C-4), 56.0 (C-6), 42.7 (C-5) and the aldehyde proton at 191.2 ppm (C-1). The methyl carbons resonated at 14.5 and 18.5 ppm.

As with the previous structure elucidations in this section the planar structure of this compound was also elaborated mainly by interpretation of COSY and HMBC data, (Figure 2.37 and Table 2.11) with the correlations of the methyl groups playing a significant role in describing fragments (Figure 2.38). The methyl protons at δ 1.88 (CH_3-9) showed HMBC correlations to carbons at δ 56.0 (C-6), 143.7 (C-7) and 115.0 (C-8), while COSY correlations were seen from CH_3-9 to δ 5.11 (H-8a) and 4.96 (H-8b). The methyl protons at δ 2.31 (CH_3-10) showed HMBC correlations to carbons at δ 157.3 (C-3), 56.1 (C-4) and 127.8 (C-2). A long range COSY correlation was observed from CH_3-10 to the proton at δ 6.05 (H-2), which in turn showed a COSY correlation to the aldehyde proton at δ 10.01 (H-1). The two fragments were coupled by HMBC correlations observed from the protons at δ 2.42 (H_2-5) to 157.3 (C-3), 56.1 (C-4), 56.0 (C-6) and 143.7 (C-7) as shown in Figure: 2.39.

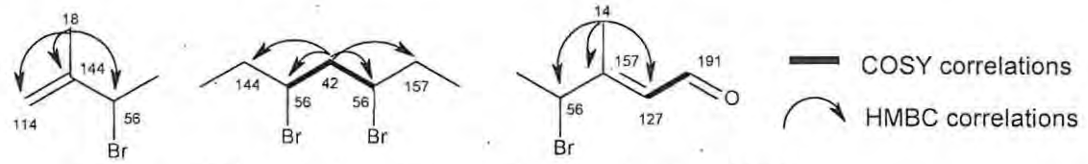
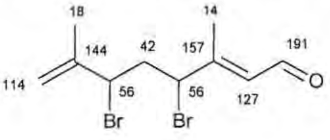
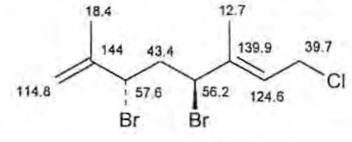


Figure: 2.37 Important correlations elaborating structure of **2.76**

The halogens at C-4 and C-6 were assigned as bromine by comparison with preplocamene A (Crews and Kho-Wiseman, 1977), whose structure is similar to this but with a terminal chlorine instead of an aldehyde. The presence of the aldehyde group was confirmed by IR data with a signal at 1678 cm^{-1}. The stereochemistry at C-4 and C-6 was not assigned. The geometry of the Δ^2 was assigned as *trans* on the basis of the chemical shift of the methyl group (Crews and Kho-Wiseman, 1977) and confirmed by the observation of an NOE between H-1 and CH$_3$-10.



2.76



2.36

Table: 2.11 ^{1}H NMR (400 MHz), ^{13}C (100 MHz), COSY, HMBC correlation of **2.76** in CDCl$_3$)

Carbon No:	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
1	191.2	10.01, d, 7.33	H-2	C-2	H-2, H-10
2	127.8	6.05, d, 7.33	H-1, H-10	C-4, C-10	H-1
3	157.3				
4	56.1*	4.72, t, 7.58	H-10	C-2, C-3, C-5	H-5, H-10
5	42.7	2.42, t	H-4, H-6	C-3, C-6, C-7	H-4, H-6
6	56.0*	4.72, t, 7.58	H-5	C-5, C-7, C-10	H-5, H-9
7	143.7				
8	115.0	5.11, s	H-9	C-6, C-7, C-9	H-9
		4.96, s	H-9	C-6, C-9	
9	18.5	1.88, s	H-8	C-6, C-7, C-8	H-8, H-6
10	14.5	2.31, s	H-2	C-2, C-3, C-4	H-1, H-4

* carbon chemical shifts interchangeable

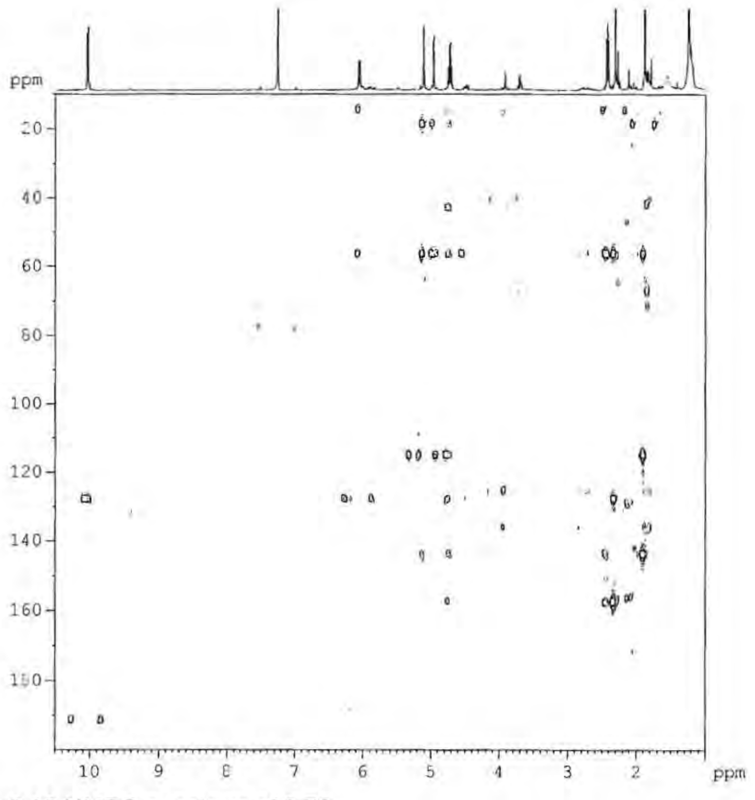
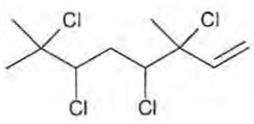


Figure: 2.38 HMBC spectrum of **2.76**

2.5.3 Known compounds

2.5.3.1 3,4,6,7-tetrachoro-3, 7-dimethyl-1-octene (2.63)



2.63

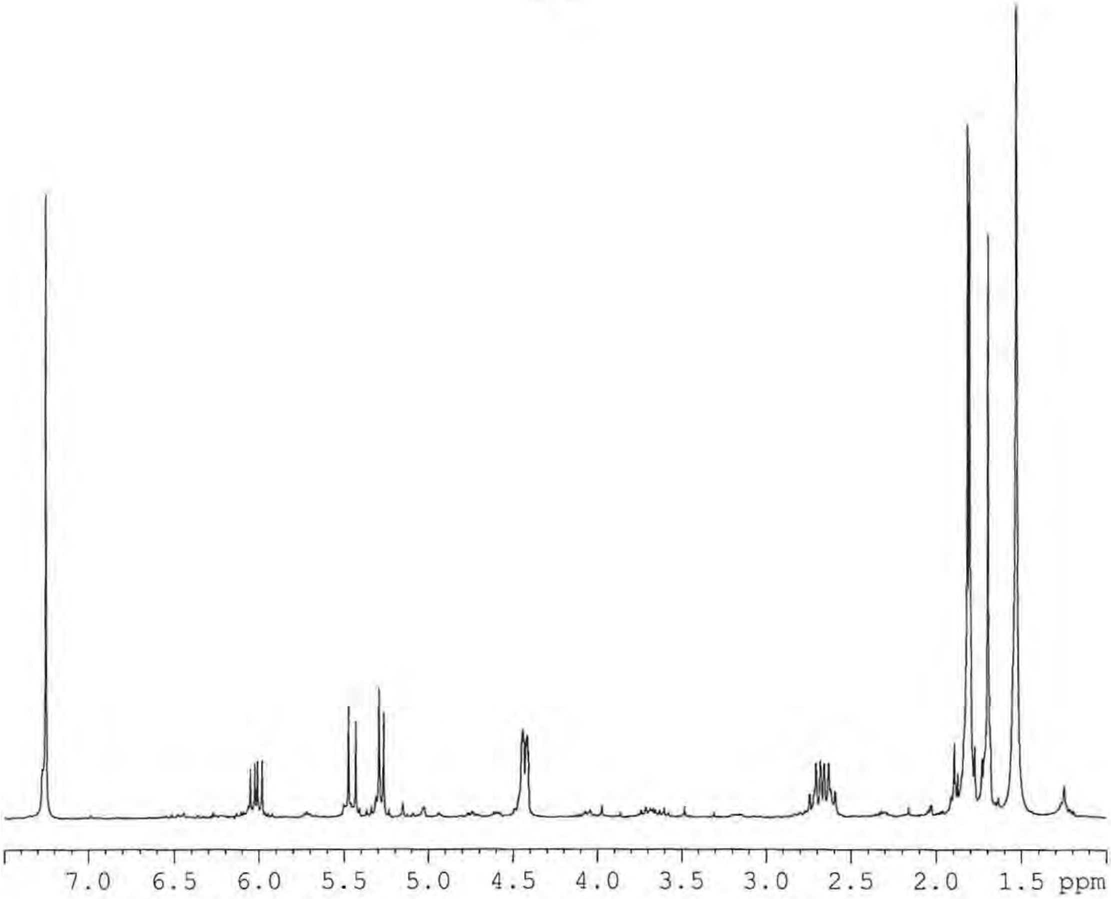
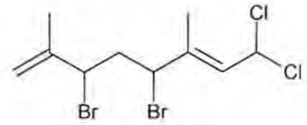


Figure 2.39 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 3, 4, 6, 7-tetrachoro-3, 7-dimethyl-1-octene (**2.63**)

The ^{1}H NMR spectrum of **2.63** showed deshielded resonances at δ 6.02 (dd, *J* = 16.8 and 10.4 Hz), δ 5.45 d, *J* = 16.8 Hz) and at δ 5.28 (d, *J* = 10.4 Hz) (Figure 2.39), which were indicative of a terminal double bond. In addition, a methine at δ 4.43 (ddd, *J* = 3.8 and 4.0 Hz) as well as a multiplet at 2.67 and three methyl proton signals (δ 1.70, 1.81 and 1.82) were also observed. These data are consistent with those of **2.63** previously isolated by Knott (2003) from the same species.

2.6.3.2 4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene (2.64)



2.64

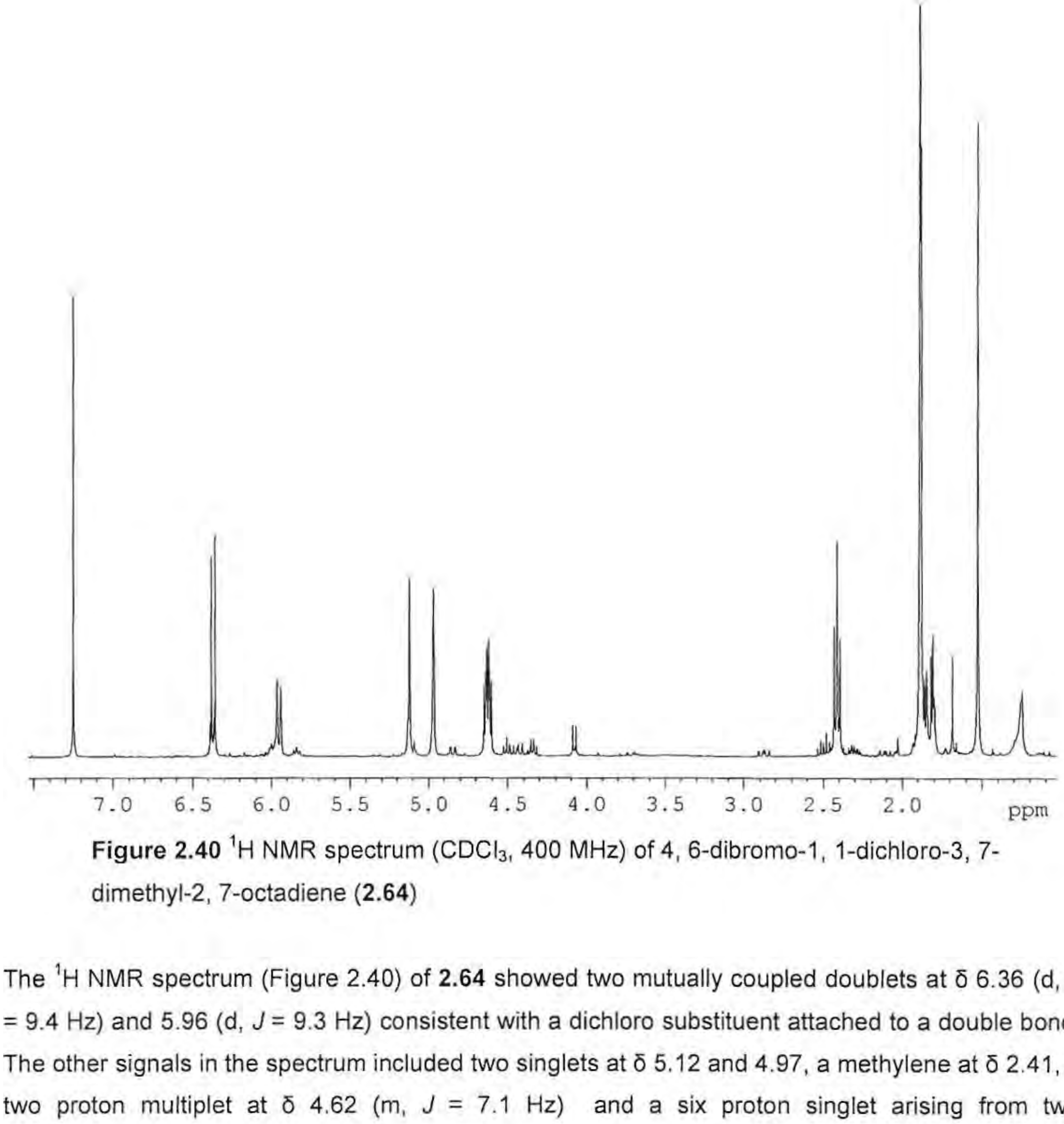
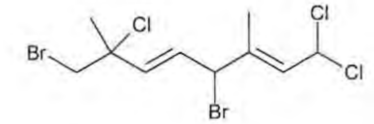


Figure 2.40 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4, 6-dibromo-1, 1-dichloro-3, 7-dimethyl-2, 7-octadiene (**2.64**)

The ^{1}H NMR spectrum (Figure 2.40) of **2.64** showed two mutually coupled doublets at δ 6.36 (d, *J* = 9.4 Hz) and 5.96 (d, *J* = 9.3 Hz) consistent with a dichloro substituent attached to a double bond. The other signals in the spectrum included two singlets at δ 5.12 and 4.97, a methylene at δ 2.41, a two proton multiplet at δ 4.62 (m, *J* = 7.1 Hz) and a six proton singlet arising from two superimposed methyl groups at δ 1.89. This data is consistent with **2.64** previously isolated by Knott (2003).

2.5.3.3 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (2.65)



2.65

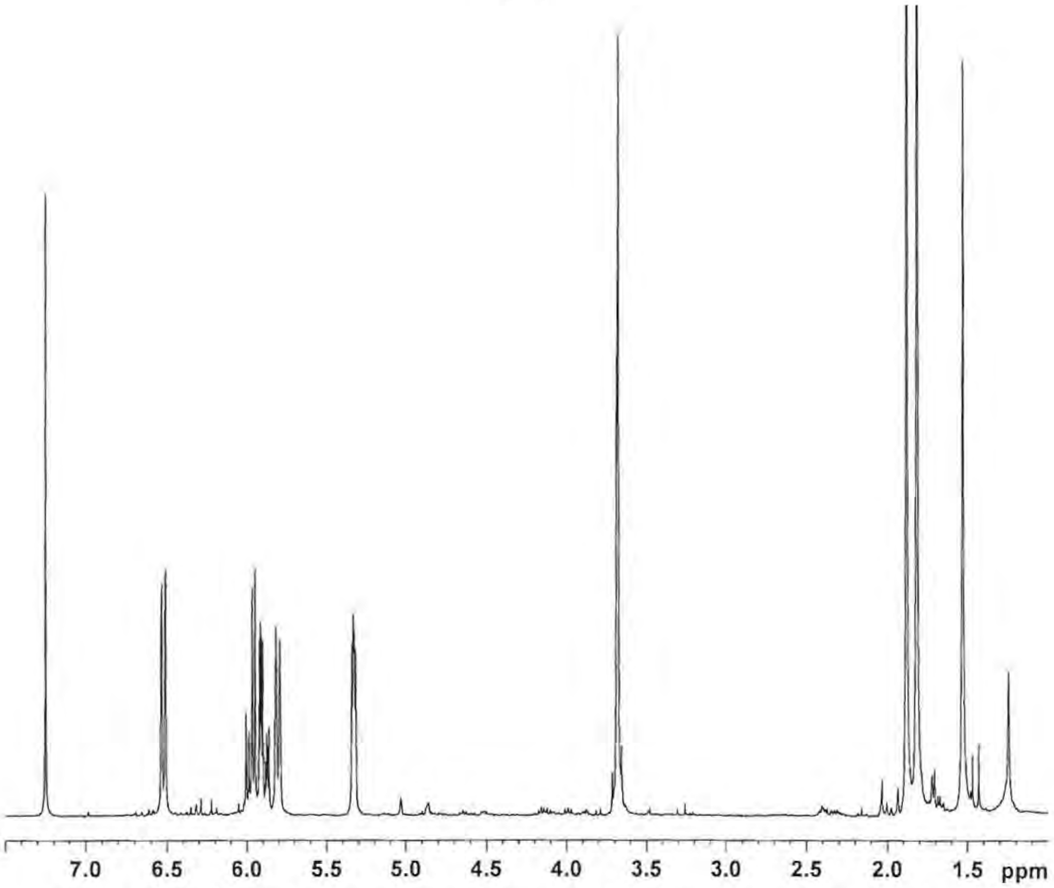
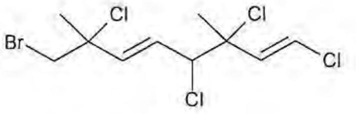


Figure 2.41 ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of 4, 8-dibromo-1, 1, 7-trichloro-3, 7-dimethyl-2, 5-octadiene (**2.65**)

The ^{1}H NMR spectrum (Figure 2.41) of **2.65** showed coupled deshielded methines signals at δ 6.53 (dd, J = 9.6 and 1.8 Hz) and 5.81 (dd, J = 9.6 and 1.3 Hz), indicating a terminal dichloro substituent attached to vinylic group. A terminal bromine singlet was observed at δ 3.68, with other signals including three single proton multiplets at δ 5.98, 5.91 and 5.34. This data is consistent with **2.65** previously isolated (Knott, 2003).

2.5.3.4 8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (2.66)



2.66

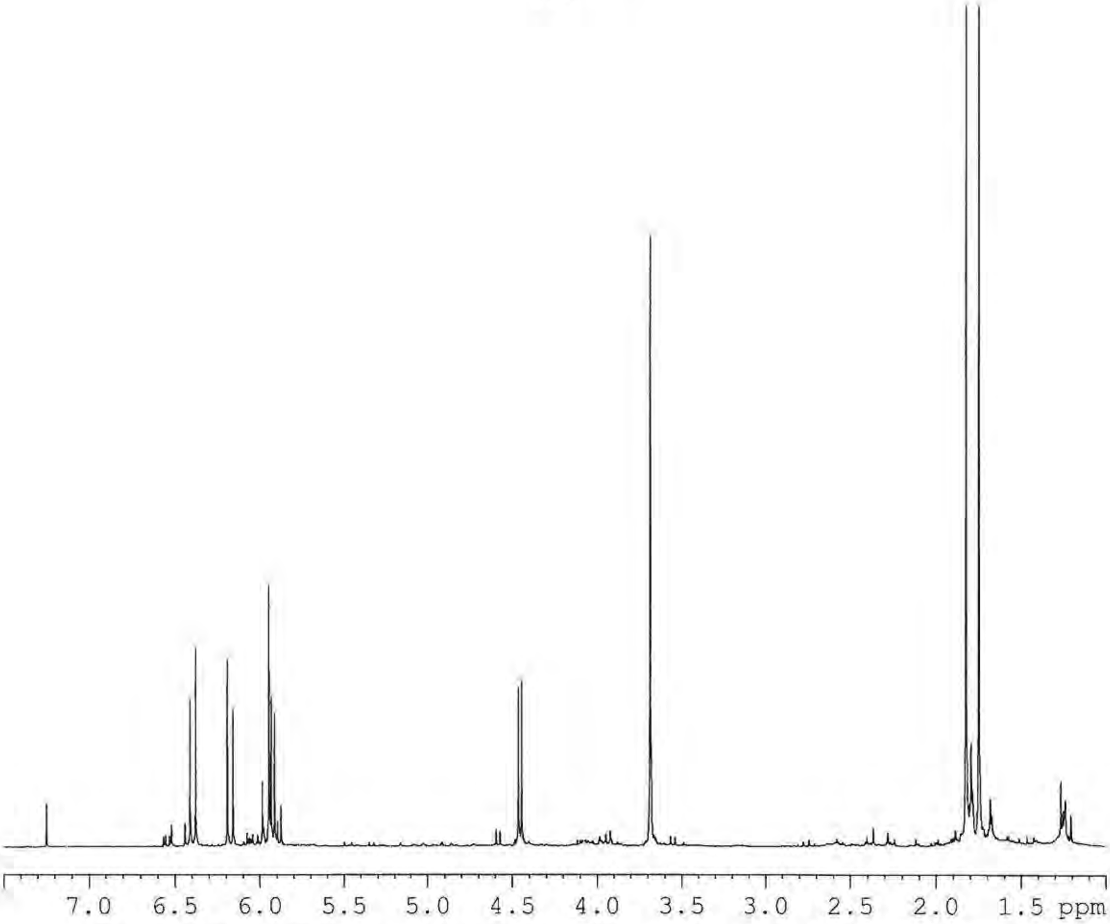
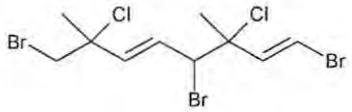


Figure 2.42 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1, 3,4,7-tetrachloro-3, 7-dimethyl-1, 5-octadiene (**2.66)**

The ^{1}H NMR spectrum of **2.66** (Figure 2.42) showed two coupled, deshielded methines at δ 6.40 (d, J = 13.2 Hz) and 6.18 (d, J = 13.2 Hz), a two proton multiplet at δ 5.92 and a one proton doublet at δ 4.47 (d, J = 7.6 Hz). The singlet at δ 3.69 indicates the presence of a terminal bromine while the signals at δ 1.83 and 1.75 are consistent with methyl groups. This data was consistent with that of 8-bromo-1, 3,4,7-tetrachloro-3, 7-dimethyl-1, 5-octadiene (Knott, 2003; Stierle *et al.*, 1979).

2.5.3.5 1,4, 8-tribromo- 3,7-dichloro-3,7-dimethyl-1,5-octadiene (2.67)



2.67

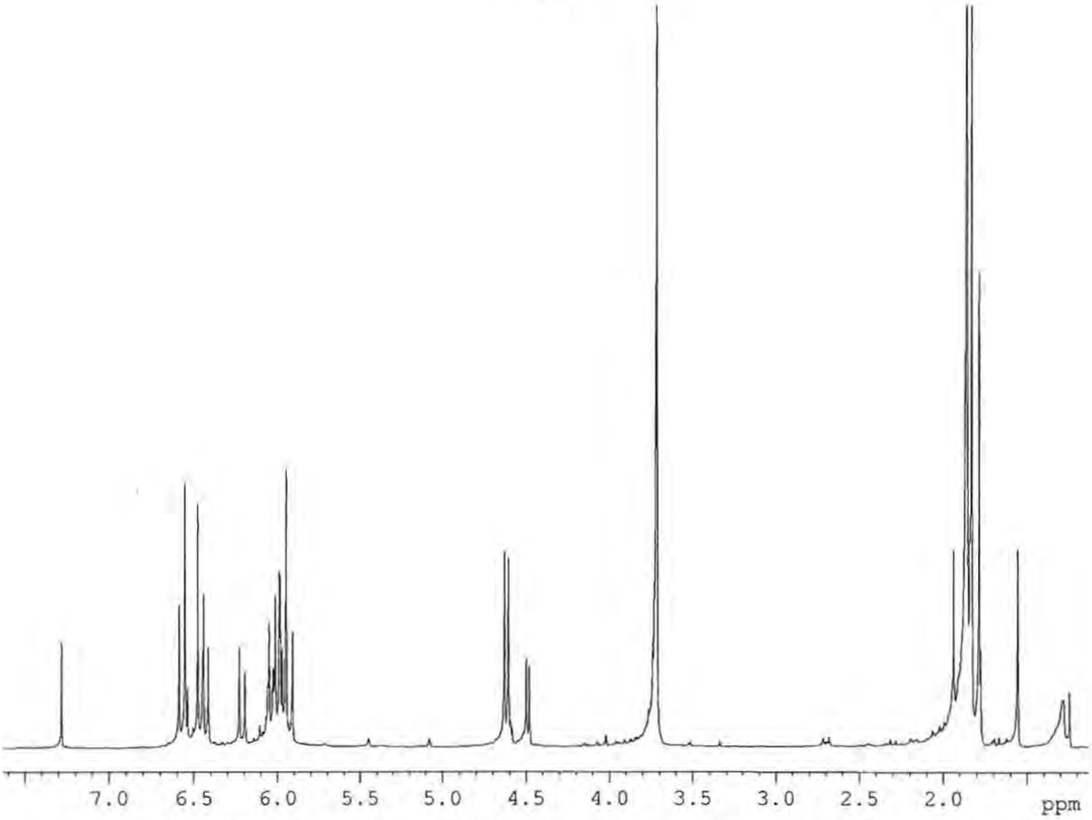
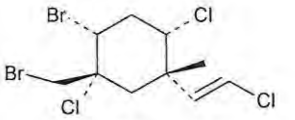


Figure 2.43 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 1, 4, 8- tribromo- 3, 7- dichloro-3, 7-dimethyl-1, 5-octadiene (**2.67**)

This spectrum of **2.67** (Figure 2.43) showed similarities to that of **2.66**, i.e. two coupled, deshielded methine protons at δ 6.53 (d, J = 13.6 Hz) and 6.42 (d, J = 13.6 Hz). These signals occur further upfield compared to those seen in **2.66**, and are typical vinylic bromide signals. The methine multiplet at δ 5.97 and the doublet at 4.57 (J = 9.6 Hz) indicated the presence of an allylic bromide, and the singlet at δ 3.67 indicated a terminal bromide (as seen previously) as well as the methyl signals at δ 1.83 and 1.79. This data was consistent with **2.67** previously reported (Knott, 2003 and Stierle *et al.*, 1979)

2.5.3.6 4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloromethylcyclohexane (2.68)



2.68

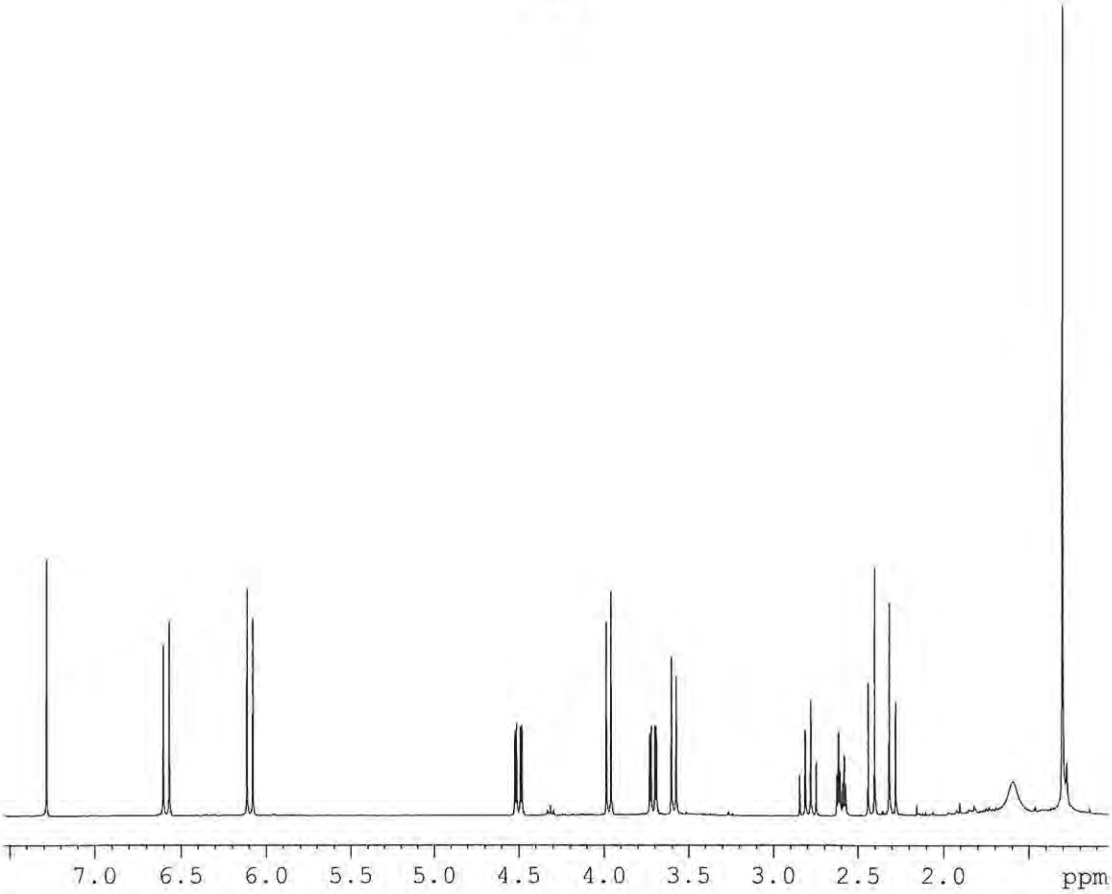


Figure 2.44 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4-bromo-5-bromomethyl-1-chlorovinyl-2, 5-dichloro-methylcyclohexane (**2.68**)

The ^{1}H NMR spectrum (Figure 2.44) of **2.68** exhibited four pairs of clearly distinguishable coupled methines at δ 6.55 (d, J = 13.6 Hz) and 6.02 (d, J = 13.6 Hz), δ 4.46 (dd, J = 12.4 and 9 Hz) and 3.67 (dd, J = 12.4 and 9 Hz), δ 3.93 (d, J = 10.8 Hz) and 3.57 (d, J = 10.8 Hz) and 2.38 (d, J = 15.2 Hz) and 2.27 (d, J = 15.2 Hz) while two additional methines at δ 2.76 and 2.57 and a single methyl resonance at δ 1.27 were also noted. These data corresponds to that of the previously isolated compound 4-bromo-5-bromomethyl-1-chlorovinyl-2, 5-dichloro-methylcyclohexane (**2.68**).

2.6 Experimental

2.6.1 General

NMR spectra were recorded on a Bruker Avance 400 spectrometer using standard pulse sequences. Chemical shifts are reported in ppm (parts per million) and referenced to residual undeuterated solvent resonances ($CHCl_3$ δ_H 7.25, δ_C 77.0). Coupling constants are reported in Hz and have been recorded directly from the spectra.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter. UV measurements were obtained on a GBC UV/ VIS 916 spectrometer, while infrared spectra were obtained on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer as films on KBr discs (applied with chloroform). Low-resolution mass spectra (EIMS) were recorded on Finnigan MAT GCQ system at 70eV.

HPLC separation was accomplished using a Spectra-Physic IsoChrom LC HPLC system, equipped with a Rheodyne injector, a Waters R401 differential refractometer, and a Rikadenki recorder. A Whatman Magnum 10-Partisil 9 column (1 x 50cm) was used for all normal phase chromatography. Gas chromatography was performed using a Hewlett Packard 6890-Series gas chromatograph (HP-5 column and GC-MS used DB-1 column) . Normal phase TLC was performed on DC-Alufolien Keiselgel $60F_{254}$, and viewed under UV light (254 nm) and Keiselgel 60 (0.040-0.063 mm) was used for column chromatography.

All solvents were either distilled before use or were of HPLC grade (HiPerSolv ^{TM}).

2.6.2 Biological material

Collection, extraction and isolation

***P. corallorhiza* (Kalk Bay)**

P. corallorhiza (identified by Prof. J. Bolton, Botany Dept., University of Cape Town) was collected in March 2002 from Kalk Bay (near Cape Town), and stored frozen. 204.68 g of the seaweed was steeped in 600 ml methanol and left in a freezer for 3 hours, after which the methanol was drained and partitioned with hexane, giving a hexane and an aqueous methanol extract. The seaweed was then extracted with dichloromethane/methanol (1:1, 3x), the extracts where concentrated *in vacuo* at 37°, and pooled to give 935 mg of a green DCM/MeOH extract. The hexane partition layer of the methanol extract was combined with the DCM/MeOH extract and further partitioned between aqueous methanol and hexane, dichloromethane, ethyl acetate and aqueous partitions. The organic partitions where dried over anhydrous sodium sulphate prior to evaporation to dryness.

The hexane partition was passed through a silica column (2.5x10 cm) producing nine fractions (hexane-EtOAc step-gradient) of which fractions 2 and 3 were combined based on similar ^1H NMR spectra. Normal phase HPLC (mobile phase 100% hexane) of fraction 1 produced three compounds, two known compounds, **2.63** and **2.64** and one new compound **2.71**. Three compounds **2.65**, **2.66**, and **2.68** were identified after normal phase HPLC of the mixture of fraction 2 and 3, using 90 % hexane-EtOAc mobile phase, as well as one new compound **2.71**.

Silica column chromatography of the DCM partition employing a hexane-EtOAc step-gradient (between 100-40% hexane) produced 9 fractions. Purification of fraction 1 (100% hexane) and fraction 2 (90% hexane-EtOAc), by normal phase HPLC using the mobile phase at which the fractions eluted in column chromatography, led to the isolation five known compounds, **2.64**, **2.65**, **2.66**, **2.67** and **2.70** (Scheme 2.10).

P. corallorhiza (Woody Cape)

P. corallorhiza was collected in October 2003 from Woody Cape near Kenton on Sea, along the southeast coast of South Africa and stored frozen. The extraction protocol used here was different from that used to extract the *Plocamium* collected from Kalk Bay. The change in the protocol was to reduce the probability of forming methoxylated artefacts. 90g (wet mass) of *Plocamium* was sonicated in isopropanol for 20 minutes and the left overnight. After two isopropanol extractions the seaweed was extracted with 1:1 DCM-MeOH (x3). The extractions were done at 4°C (in a fridge). The isopropanol extract was concentrated to an aqueous fraction which was then partitioned with DCM and the DCM partition combined with the DCM-MeOH extract. This mixture was partitioned giving hexane, DCM, EtOAc and aqueous fractions.

All organic partitions were dried with anhydrous sodium sulphate prior to concentration *in vacuo* at 37°C. The ^{1}H NMR spectra of the hexane and DCM partitions showed presence of aldehydes. Silica column chromatography of the hexane partition yielded 9 fractions which were pooled on the basis of TLC and ^{1}H NMR spectra. Normal phase HPLC of fraction 1 (100% hexane) resulted in the isolation of six known compounds, **2.63**, **2.64**, **2.65**, **2.66**, **2.67** and **2.68**. The ^{1}H NMR spectra of the EtOAc wash of this fraction showed aldehyde signals and was re-chromatographed employing a 90% hexane-EtOAc mobile phase leading to the isolation of three new aldehyde halogenated monoterpenes, **2.73**, **2.74** and **2.75**. HPLC of fraction 2 (80% hexane-EtOAc eluent) led to the isolation of five new compounds, consisting of two non-aldehyde compounds, **2.69** and **2.70**, and three halogenated monoterpene aldehydes, **2.73**, **2.74** and **2.75**.

P. corallorhiza (Kenton on Sea)

This collection was done because the new compounds isolated from the Woody Cape collection decomposed, and thus this was an attempt to re-isolate, elucidate the structures and carry out biological tests on these compounds. *P. corallorhiza* was collected from Kenton on Sea. The extraction protocol was similar to that employed for the Woody Cape collection.

HPLC (90% hexane-EtOAC) of fraction 2 from silica column chromatography led to the purification of three new compounds, **2.71, 2.75** and **2.78**. Re-injection of fraction 4 and 5 at a flow rate of 2 mL/min resulted in the isolation of another new compound **2.74**. HPLC of fraction 3 (80% hexane-EtOAc) from the silica column using the same eluent led to the isolation of one new aldehyde compound, **2.76** with four new compounds being isolated from fraction 4, **2.64**, **2.73**, **2.74** and **2.69** (Scheme 2.11).

3,4,6,7-tetrachloro-3,7-dimethyl-1-octane (2.63)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.02, dd, 16.9, 10.6; 5.45, d, 16.9; 5.28, d, 10.6; 4.43, ddd, 3.8, 4.3; 2.67, ddd, 2.0, 2.3; 1.82, s; 1.80, s; 1.70, s

4, 6-dibromo-1, 1-dichloro-3, 7-dimethyl-2, 7-octadiene (2.64)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.34, d, 9.4 Hz; 5.95, d, 9.4 Hz; 5.12, s; 4.97, s; 4.62, m; 2.41, t, 7.1; 1.89, s; 1.89, s

4, 8-dibromo-1, 1, 7-trichloro- 3, 7-dimethyl-2, 5-octadiene (2.65)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.53, dd, 9.6, 1.8; 5.98, m; 5.91, m; 5.96, m; 5.81, dd, 9.6, 1.3; 3.68, s; 1.88, s; 1.81, s

8-bromo-1, 3, 4, 7-tetrachloro-3, 7-dimethyl-1, 5 -octadiene (2.66)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.40, d, 13.1; 6.18, d, 13.1; 5.98, m; 5.92, m; 4.47, d, 7.6; 3.69, s; 1.83, s; 1.75, s

1, 4, 8-tribromo-3, 7-dichloro-3, 7-dimethyl-1, 5-octadiene (2.67)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.54, d, 13.6; 6.43, d, 13.6; 5.97, m; 5.92, m; 4.57, d, 9.4; 3.69, s; 1.83, s; 1.80, s

4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (2.68)

^{1}H NMR ($CDCl_3$, 400 MHz) 6.55, d, 13.4 Hz, 6.02, d, 13.4 Hz, 4.46, dd, 3.4, and 12.4 Hz, 3.93, d, 10.6 Hz, 3.67, dd, 3.8 and 12.3 Hz, 3.54, d, 10.6 Hz, 2.76, m, 2.56, m, 2.38, d, 15.4 Hz, 2.26, d, 15.4 Hz, 1.27, s

4-Bromo-1,3,6,7-tetrachloro-3,7-dimethyl-octa-1,4-diene (2.69)

Colourless oil; $[\alpha]^{28}_D$ - 70° (c 0.0027, $CHCl_3$) UV (Hexane) λ_{max} 203 (ε 6413); IR (dry film, KBr) ν_{max} 3429, 3019, 1215, 755, 670 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.4**) 87/89/91/93/95 (11, 13, 30, 6, 23); 103/105/107 (25, 100, 13); 113/115/117/119 (8, 25, 10, 27); 125/127/129/131/133 (14, 11, 28, 11); 141/143 (22, 12); 147/149/151/153 (17, 20, 10, 8); 184/186 (19, 7); 227/229 (13, 14); 263/265/267 (49, 63, 16); 299/301/303 (24, 37, 17)

8-Bromo-1,3,4,7-tetrachloro-3,7-dimethyl-octa-1,5-diene (2.70)

Colourless oil; $[\alpha]^{28}_D$ - 7° (c 0.0004, $CHCl_3$) UV (Hexane) $\lambda_{max} < 200$ (ε 1096); IR (dry film, KBr) ν_{max} 3428, 3019, 1215, 755, 669 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.5**) EIMS (70 eV) *m/z* (rel. int) 89/91/93/95/97/99 (70, 18, 16, 43, 100, 6); 103/105/107/109

(5, 68, 25, 6); 119/121/123 (9, 15, 7); 135/137/139 (8, 9, 5); 147/149/151 (10, 7, 5); 163/165/167 (8, 10, 2); 235/237/239 (6, 10, 4)

8-Bromo-1,1,2,7-tetrachloro-3,7-dimethyl-octa-3,5-diene (2.71)

Yellowish oil; $[\alpha]^{28}_{D}$ - 16° (c 0.0036, $CHCl_3$) UV (Hexane) λ_{max} 246 (ε 6732); IR (dry film, KBr) ν_{max} 3436, 3018, 1216, 756, 699 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.6**) EIMS (70 eV) *m/z* (rel. int) 91/93/95 (21, 10, 7); 103/105/107/109 (6, 34, 12, 6); 141/143 (24, 14); 169/171 (20, 9), 205/207/209 (38, 28, 8); 241/243/245/247 (100, 92, 31, 8); 299/301/303 (10, 16, 7)

8-Bromo-6,7-dichloro-3,7-dimethyl-octa-2,4-dienal (2.72)

Greenish oil; $[\alpha]^{28}_{D}$ - 68° (c 0.0006, $CHCl_3$), UV (Hexane) λ_{max} 266 (ε 4371); IR (dry film, KBr) ν_{max} 2251, 1667, 1378, 908, 733, 650 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.7**) EIMS (70 eV) (rel. Int) 93/95 (4, 100); 129131/133 (12, 5, 4); 263/265/267 (8, 10, 2)

4-Bromo-8-chloro-3,7-dimethyl-octa-2,6-dienal (2.73)

Yellow oil; $[\alpha]^{28}_{D}$ - 24° (c 0.001, $CHCl_3$) UV (Hexane) λ_{max} 235 (ε 4321); IR (dry film, KBr) ν_{max} 2254, 1674, 960, 870 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.8**) EIMS (70 eV) *m/z* (rel. int) 91/93/95 (71, 99, 28); 103/105/107 (12, 38, 29); 115/117/119/121 (15, 13, 40, 100); 129/131/133 (7, 30, 4); 147/149/151 (1, 18, 1); 155/157/159/161 (11, 68, 24, 2)

8-Bromo-4-chloro-3,7-dimethyl-octa-2,4,6-trienal (2.74)

Yellowish oil; $[\alpha]^{28}_{D}$ - 2° (c 0.010, $CHCl_3$) UV (Hexane) λ_{max} 330, 315, 279 (ε 9325); IR (dry film, KBr) ν_{max} 2254, 1668, 906, 732, 650 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.9**) EIMS (70 eV) *m/z* (rel. int) 89/91 (5, 100); 103/105/107 (23, 28, 3); 115/117/119 (25, 22, 48); 127/129/131/133 (5, 19, 4, 7); 145/147/149 (2, 23, 1); 165/167 (11, 3); 183/185 (78, 28);

4,8-Dichloro-3,7-dimethyl-octa-2,4,6-trienal (2.75)

Green oil; $[\alpha]^{28}_{D}$ + 2° (c 0.001, $CHCl_3$) UV (Hexane) λ_{max} 330, 316, 231 (ε 8730); IR (dry film, KBr) ν_{max} 2254, 1662, 1168, 980, 939, 907, 852, 792, 736, 655 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.10**) EIMS (70 eV) *m/z* (rel. int) 91/93 (31, 4); 103/105/107 (8, 15, 2); 117/119/121 (9, 44, 2); 127/129/131/133 (4, 17, 4, 6); 145/147/149 (1, 26, 1); 165/167 (11, 5); 183/185 (100, 34)

4,6-Dibromo-3,7-dimethyl-octa-2,7-dienal (2.76)

Green oil; $[\alpha]^{28}_{D}$ - 27° (c 0.0028, $CHCl_3$) UV (Hexane) λ_{max} 231 (ε 6597); IR (dry film, KBr) ν_{max} 2923, 2851, 2253, 1676, 1378, 1112, 907, 731, 650 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400MHz), ^{13}C NMR ($CDCl_3$, 100MHz) (see **Table 2.11**)

References

Abreu, P. M.; Galindro, J. M. *J. Nat. Prod.* **1996**, 59, 1159-62

Anderson, J. E.; Goetz, C. M.; McLaughlin, J. L. *Phytochem. Anal.* **1991**, 2, 107-111

Bates, P.; Blunt, J. W.; Hartshorn, M. P.; Jones, A.; Munro, M. H. G.; Robinson, W. T.; Yorke, S. C. *Aust. J. Chem.* **1979**, 32, 2545-54

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

Burreson, B. J.; Woolard, F. X. and Moore, R. E. *Tetrahedron Lett.* **1975**, 16, 2155-8

Butler, A.; Walker, J. V. *Chem. Rev.* **1993**, 93, 1937-44

Coll, J. C.; Skeleton, B. W.; White, A. H.; Wright, A. D. *Aust. J. Chem.* **1988**, 41, 1743-53

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

Crews, P.; Kho, E. *J. Org. Chem.* **1974**, 3303-4

Crews, P.; Kho-Wiseman, E.; Montana, P. *J. Org. Chem.* **1978**, 43, 116-20.

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

Dewick, P. M. *Nat. Prod. Rep.* **2002**, 19, 181-222

Diaz-Marrero, A. R.; Rovirosa, J.; Darias, J.; San-Martin, A.; Ceuto, M. *J. Nat. Prod.* **2002**, 65, 585-8

Dunlop, R. W.; Murphy, P. T.; Wells. R. J. *Aust. J. Chem.* **1979**, 32, 2735-9

Faukner, D. J.; Stallard, M. O. *Tetrahedron Lett.* **1973**, 14, 1171-4

Faulkner D. J. *Nat. Prod. Rep.* **1984,** 251-280

Faulkner D. J.; Stallard, M. O.; Fayos, J.; Clardy, J. *J. Am. Chem. Soc.* **1973**, 95, 3413-20

Faulkner, D. J. *Tetrahedron* **1977**, 33, 1421-43

González, A. G. Artega, J. M.; Martin, J. D.; Rodriguez, M. L.; Fayos, J.;Martinez-Ripolls, M. *Phytochemistry* **1978**, 17, 5, 947-48

Gribble, G. W. *Acc. Chem. Res.* **1998**, 31, 141-152

Higgs, M. D.; Vanderah, D. J.; Faulkner, D. J. *Tetrahedron* **1977**, 33, 21, 2775-80

Ichikawa, N.; Naya, Y.; Hirose, Y. *Chem. Lett.* (Japan) **1976**, 839-46

Imperato, F.; Minale, L.; Riccio, R. *Experientia* **1977**, 33, 1273-4

Jongaramruong, J.; Blackman, A. J. *J. Nat. Prod.* **2000**, 63, 272-5

Kazlauskas, R.; Murphy, P. T.; Quinn, R. J.; Well, R. J. *Tetrahedron Lett.* **1976**, 17, 4451-4

Kemp, W. Organic Spectroscopy. **1975** Macmillan Education Ltd, London

Knott, M. K. MSc Thesis. **2003**, Rhodes University, Grahamstown, South Africa

Martinez, J. S.; Carroll, G. L.; Tschirret-Guth, G.; Altehoff, G.; Daniel Little, R.; Butler, A. *J. Am. Chem. Soc.* **2001**, 123, 3289-94

Mynderse, J. S.; Faulkner, D. J. *J. Am. Chem. Soc.* **1974**, 96, 6771-2

Mynderse, J. S.; Faulkner, D. J.; Finer, J.; Clardy, J. *Tetrahedron Lett.* **1975**, 16, 2175-8

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

Naylor, S.; Hanke, F. J.; Manes, L. V.; Crews, P. *Prog. Chem. Nat. Prod.* **1983**, 44, 197-241

Ruzicka, L. *Proc. Chem. Soc.* **1959**, 341-9

Sardina, F. J.; Quinoa, E.; Castedo, L.; Riguera, R. *Chem Lett,* **1985**, 697-700.

Sardina, J. S.; Quinoa, E.; Castedo, L.; Riguera, R.; Mosquera, R. A.; Vazquez, S. *J. Org. Chem,* **1986**, 51, 4970-3

Shrader, S. R., Introductory Mass Spectroscopy, Boston: Allyn and Bacon **1977**.

Sims, J. J.; Rose, A. F.; Izac, R. R. In "*Marine Natural Products. Chemical and Biological Perspectives*", Scheuer, P. J.; Ed.; Academic Press: New York, Vol. II, pp 297-378, **1978**.

Sims, J. J.; Rose, A. F.; Izac, R. R. In "*Marine Natural Products Chemistry*", P. J.; Ed.; Academic Press: New York, Vol 1, Chapter 5, **1978**

Siquiera, M. J.; Bomm, D. M.; Pereira, N. F. G.; Garcez, W. S.; Boaventura, M. A. D. *Quim. Nova.* **1998**, 21, 557-9

Squillacote, M. E.; Sheridan, R. S.; Chapman, O. L.; Anet, F. A. L. *J. Am. Chem. Soc.* **1979**, 101, 3657-59

Stierle, D. B. and Sims, J. J. *Tetrahedron* **1979**, 35, 1261-5

Stierle, D. B.; Wing, R. M. and Sims, J. J. *Tetrahedron Lett.* **1976**, 47, 4455-8

Stierle, D. B.; Wing, R. M.; Sims, J. J. *Tetrahedron* **1979**, 35, 2855-9

Stothers, J. B. *Carbon-13 NMR Spectroscopy.* Academic Press. New York (**1972**)

Van Engen, D; Clardy, J.; Kho-Wiseman, E.; Crews, P.; Higgs, M. D.; Faulkner, D. J. *Tetrahedron Lett.* **1978**, 19, 29-32,

Williard, P. G.; de Laszlo, S. E. *J. Org. Chem.* **1985**, 50, 3738-49

Williard, P. G.; Grab, L. A. *Tetrahedron Lett.* **1984**, 24, Vol 44, 5009-12

Williard, P. G.; Grab, L. A.; de Laszlo, S. E. *J. Org. Chem.* **1983**, 48, 1123-5

Wise, M. L.; Urbansky, M.; Helms, G. L.; Coates, R. M.; Croteau, R. *J. Am. Chem. Soc.* **2002**, 124, 8546-7

Woolard, F. X.; Moore, R. E.; van Engen, D. and Clardy, J. *Tetrahedron Lett.* **1978**, 19, 2367-70

Chapter Three

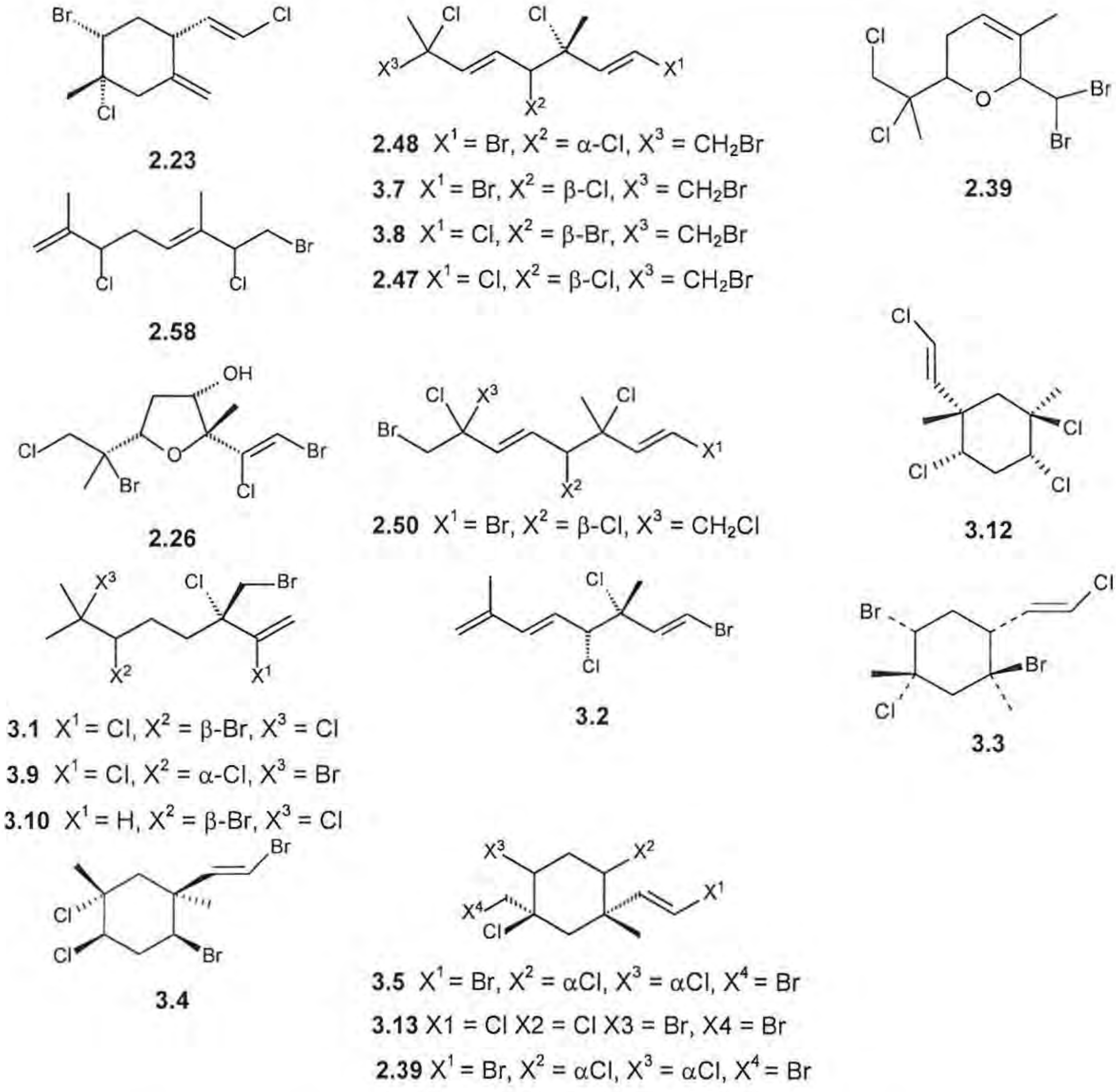
Biological activity of *P. corallorhiza* metabolites

3 Introduction

The ecological function of many of seaweed metabolites is yet unknown or unclear, although some of these compounds appear to have considerable activity in the natural environment and have been proven to be active pharmacologically and agro-chemically (Gonzalez *et al.*, 1982: Crews *et al.*, 1984a). It has been suggested that seaweed metabolites form part of a chemical defense strategy against predators and parasites (Fenical, 1975; Scheuer, 1990). The biological activities of most of these compounds appear to be related to the presence of halogens with activity increasing with an increase in the number of halogens atoms. For example, an increase in the number of bromine atoms is thought to increase the antibacterial and cytostatic activity of polyhalogenated monoterpenes (Gonzalez *et al.*, 1982). The halogens are suspected to be involved in enzymatic halogenation reactions producing active moieties, which kill bacteria, fungi and even tumour cells (Klebanoff, 1968; Weiss, 1986). Interest in the chemistry and pharmacology of *Plocamium* species has been revived with the discovery of halomon (**3.1**), a polyhalogenated monoterpene that showed a selective and potent cytotoxicity profile against a panel of cancer cell lines (Fuller *et al.*, 1992). The biological activities and structures of some halogenated monoterpenes are shown in Table 3.1 and Figure 3.1.

Table 3.1 Summary of some of the biological activities of halogenated monoterpenes isolated from *Plocamium* species.

Compound No	Biological activity	Reference:
2.48, 3.2, 3.3, 3.4	Antifungal against *P. oxacilium* Cytotoxic towards *Biomphalaria glabrata* and *Artemia salina*	Konig *et al.*, 1990
2.23, 3.3, 3.5	Cytostatic in *in vitro* on HeLa 299 cells with ID$_{50}$ ranging between 1-10 µg/ml	Gonzalez *et al.*, 1982
2.48	Slightly cytotoxic Lu1 (IC50 12.9 µg/ml), ZR-75-1 (7.8 µg/ml) Antialgal against *Chlorella fusca*	Konig *et al.*, 1999
2.58	Weak effect in brine shrimp assay at concentration of 500 µg/mL	König *et al.*, 1999
2.39	Insecticidal against *Macrosteles facifrons* Antifungal	Aurelio *et al.*, 1991
3.5, 3.6	Insecticidal against mosqioto larvae (*Culex pipiens pallens*)	Watanabe *et al.*,1989
3.7, 3.8	Mutagenic towards *S. typhimurium* strain TA100	Leary *et al*, 1979
3.1 (halomon)	Cytotoxic in NCI *in vitro* primary antitumour screen with average LC$_{50}$ = 11.5 µM	Fuller *et al.*, 1994
3.9	Cytotoxic in NCI *in vitro* primary antitumour screen with average LC$_{50}$ = 16.2 µM	Fuller *et al.*, 1994
3.10	Cytotoxic in NCI *in vitro* primary antitumour screen with average LC$_{50}$ > 100 µM	Fuller *et al.*, 1994
2.47, 2.48, 2.50, 3.11	Moderately antifungal against *Cladosporium cucumerimum*	Stierle et al., 1979
2.23, 2.26, 3.12	Anti-feedants mainly against *L. decemlineata* EC50 9-40.6 µg/cm^2 Insecticidal/cytotoxic and insect-derived SF9 cell cells (ED$_{50}$ 0.023-0.03 µg/ml)	Argandona *et al.*, 2002
3.13	Moderate cytotoxicity towards KB cells (IC$_{50}$ 12-14 µg/ml)	Konig *et al.*, 2000



2.23

2.58

2.26

3.1 X^1 = Cl, X^2 = β-Br, X^3 = Cl

3.9 X^1 = Cl, X^2 = α-Cl, X^3 = Br

3.10 X^1 = H, X^2 = β-Br, X^3 = Cl

3.4

2.48 X^1 = Br, X^2 = α-Cl, X^3 = CH_2Br

3.7 X^1 = Br, X^2 = β-Cl, X^3 = CH_2Br

3.8 X^1 = Cl, X^2 = β-Br, X^3 = CH_2Br

2.47 X^1 = Cl, X^2 = β-Cl, X^3 = CH_2Br

2.50 X^1 = Br, X^2 = β-Cl, X^3 = CH_2Cl

3.2

3.5 X^1 = Br, X^2 = αCl, X^3 = αCl, X^4 = Br

3.13 X1 = Cl X2 = Cl X3 = Br, X4 = Br

2.39 X^1 = Br, X^2 = αCl, X^3 = αCl, X^4 = Br

2.39

3.12

3.3

Figure 3.1 Structures of selected biologically active halogenated monoterpenes

3.1.1 Biological assays

Selected crude fractions and pure compounds isolated in this study were screened for brine shrimp lethality, antibacterial activity and activity against oesophageal cancer cells.

3.1.1.1 Brine shrimp (*Artemia salina*) lethality assay

The brine shrimp (*Artemia salina*) assay is a rapid and inexpensive test, which has been used for screening of biological and cytotoxic activities (De Rosa *et al.*, 1994). The high sensitivity of the brine shrimp larvae to a variety of chemicals makes this preliminary screening assay very useful. This test was developed by Michael *et al.* (1956), and adapted by Meyers *et al.* (1982) and Solis *et al.* (1993) and shows a good correlation with activities of some cancer cell lines (Anderson *et al*, 1991). Active extracts, fractions or compounds from this assay can further be tested in cultured tumour cells, antimicrobial or antiparasitic assays with good correlation (Sahpaz *et al.*, 1994; Colman-Saizarbitoria *et al.*, 1995; Siquiera *et al.*, 1998). This is important because the brine shrimp assay is not specific for antitumor, or indeed, any particular pharmacological action.

3.1.1.2 Oesophageal cancer screening

The oesophageal cancer assay was chosen for two reasons: 1) availability of the assay, through our collaborators and 2) because of the high prevalence of the condition in the Eastern Cape, particularly the Transkei area, of South Africa, where the incidence rate for men is among the highest in the world (Somdyala *et al.*, 2003). It is thought that this is because of the presence of the fungal metabolite, fumonisin, which has been linked with the aetiology of the disease (Rheeder *et al.*, 1992). *Fusarium verticillioides* (the fungus that produces fumonisin) contamination of maize grown for home consumption is the main route of transmission of the mycotoxin, (Marasas *et al.*, 1988). The risk of consumption of fumonisins and hence the high risk potential of developing oesophageal cancer in the rural communities is as such because maize is part of the staple food.

3.1.1.3 Antibiotic activity

The search for newer antimicrobial agents continues because of the continuous development of resistance to the antimicrobial agents in clinical use currently. Vlachos *et al.* (1997) investigated the antimicrobial activity of marine macroalgal extracts from Southern Africa. Their results indicated that red seaweed extracts inhibited the majority of Gram-positive bacteria used in the study and exhibited little to no activity against yeasts, moulds and Gram-negative bacteria, with the exception of *Acinetobacter lwoffii*. *Plocamium rigidium* showed the broadest spectrum of activity to the Gram-positive bacteria tested. In another study at the University of the Western Cape (Cameron, 2002), seventeen red algal species were screened for antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeroginosa, Mycobacterium smegnatis, and Candida albicans* using agar overlay bioautography. It was found that the extracts of *Polysiphonia virgata* and *Plocamium corallorhiza* produced the greatest zones of inhibition. In addition to this, *P.corallorhiza* was found to be the only alga that inhibited the growth of *Candida albicans* and proved to have a very broad-spectrum of antimicrobial action.

3.2 Results and Discussion

3.2.1 Brine shrimp assay

The samples tested in this assay include crude extracts, silica column chromatography fractions and pure compounds. All samples were tested in duplicate at concentrations of 400, 200, 100 and 50 µg/mL. Activity was measured as the percentage of dead relative to live larvae in each well. The percentage mortality observed after 24 hours was noted for the test metabolites and is shown in Tables 3.2-3.4. The nauplii that were lethargic were not counted as dead.

The general trend observed was that at high concentration most of the samples exhibited 100% lethality/mortality, and as the concentrations decreased so did the mortality; this was expected on the premise that toxicology is pharmacology at high doses/concentrations (Anderson *et al.*, 1991).
Further inspection of the brine shrimp activity results immediately drew attention to the fact that the fractions from the silica column and at least one of the pure compounds had significantly higher activity compared to the crude extracts. This is not surprising since the active compounds will be more concentrated in the crude silica column fractions. These results indicate that it may be worthwhile to assay not only crude extracts but also chromatographic fractions of crude extracts when searching for biologically active compounds from natural sources.
Samples HM09, HM15, HM16 and HM23 displayed average percentage lethality greater that 70% at a concentration of 50 µg/mL thereby indicating cytotoxic activity. The crude samples HM15 and HM16 (90 and 80%H/E fractions respectively, of DCM/MeOH extract of *P.corallorhiza*) which showed cytotoxic activity, were good candidates for bioassay-guided fractionation. These were further fractionated by repeated normal phase HPLC to isolate pure compounds, **2.71**, **2.69**, and **2.72**, which were also tested for oesophageal cancer activity.
The crude extracts of *P. corallorhiza* collected from Kenton-On-Sea (HM04 and HM05) and Port Alfred (HM06 and HM07) did not show much activity though it was noted that the isopropanol extracts potentially contained more active metabolites as they were relatively more active than the DCM/MeOH extracts. The Port Alfred extracts were relatively more active than Kenton extracts.
On comparing cytotoxic activity of different *Plocamium* species the order of cytotoxicity was as follows: *P. rigidium* > *P.corallorhiza* > *P. cornutum*. *Chondsia capensis* (not a *Plocamium* spp) showed activity greater than *P. corallorhiza*. Unfortunately, the paucity of material and precluded the further investigation of *P. rigidium* and *C. capensis*.

Table 3.2 Brine shrimp activity of crude extracts of different *Plocamium* species and *C. capensis*

Fn #	Description	400[a]	200[a]	100[a]	50[a]
HM01	DCM/MeOH extract of *P. corallorhiza*	75%	57%	13%	0%
HM02	DCM partition of Isopropanol extract of *P. corallorhiza*	100%	100%	72%	23%
HM03	**EtOAc partition of *P. corallorhiza***	**100%**	**95%**	**56%**	**44%**
HM04	Isopropanol extract of *P. corallorhiza* from Kenton.	86%	12%	12%	0%
HM05	Aqueous Partition of *P. corallorhiza* from Kenton	60%	43%	0%	4%
HM06	Isopropanol extract of *P. corallorhiza* from Port Alfred	100%	17%	5%	0%
HM07	DCM/MeOH extract of from *P. corallorhiza* Port Alfred	90%	60%	0%	0%
HM08	Isopropanol extract of *P. rigidium*	100%	100%	44%	10%
HM09	**DCM/MeOH extract of *P. rigidium***	**100%**	**100%**	**100%**	**70%**
HM10	Isopropanol extract of *C. capensis*	100%	85%	9%	0%
HM11	DCM/MeOH extract of C. *capensis*	100%	80%	3%	0%
HM12	Isopropanol extract of *P. cornutum*	100%	82%	29%	0%
HM13	DCM/MeOH extract of *P. cornutum*	88%	44%	27%	5%

[a] sample concentrations in μg/mL

Table 3.3 Brine shrimp activity of fractions from silica column chromatography

Fn #	Description	400[a]	200[a]	100[a]	50[a]
HM14	100% hexane fraction of DCM/MeOH extract	100%	75%	11%	0%
HM15	**90% H/E fraction of DCM/MeOH extract**	**100%**	**100%**	**100%**	**88%**
HM16	**80% H/E fraction of DCM/MeOH extract**	**100%**	**100%**	**100%**	**75%**
HM17	70% H/E fraction of DCM/MeOH extract	100%	100%	17%	38%
HM18	50% H/E fraction of DCM/MeOH extract	100%	93%	24%	40%
HM19	30% H/E fraction of DCM/MeOH extract	100%	100%	42%	11%
HM20	100% EtOAC fraction of DCM/MeOH extract	91%	100%	13%	19%

Table 3.4 Brine shrimp activity of selected pure compounds

Fn #	Description	400[a]	200[a]	100[a]	50[a]
HM21	**2.66**	86%	69%	38%	0
HM22	**2.67**	84%	84%	77%	9%
HM23	**2.68**	**100%**	**100%**	**100%**	**100%**
HM24*		80%	56%	9%	0%

* HM23 with stock solution prepared in DMSO.

3.2.2 Oesophageal cancer screening

Having observed brine shrimp activity for some extracts and fractions, we then further screened pure metabolites isolated from these fractions for activity against oesophageal cancer cells.
The results are shown in Table 3.5. These compounds showed some activity against oesophageal cell lines (WHC01) particularly, compounds and **2.68**, which had IC_{50}, values less than 3.5 μg/mL. Samples **2.69** - **2.76** did not have their IC_{50} values determined, but compound , **2.69**, **2.73**and **2.76** showed greater toxicity.

Table 3.5 Oesophageal cancer activity results.

compound #	50[a]	1[a]	IC_{50}[a]
2.63	0	0.04	12.0
2.64	0	0.05	3.4
2.65	0	0.075	13.9
2.66	0	0.05	5.5
2.67	0	0.02	6.6
2.68	0	0.04	2.0
2.69	0	0.07	-
2.71	0.075	0.08	-
2.73	0	0.11	-
2.74	0.02	0.125	-
2.76	0	0.175	-
2.69	0	0.12	-

[a] concentration in μg/mL

3.2.3 Antibacterial assay

The antimicrobial test was done on extracts and some pure compounds isolated from *P. corallorhiza* collected from the east coast of South Africa near Kenton on Sea.
The blank controls did not show any activity, i.e. no zones of inhibition were observed with any of the test bacteria. The standard antibiotic however, produced zones of inhibition with all the test organisms depicting their sensitivity to it. After 16 hours zones of inhibition or lack thereof, were evident and their diameter measured by a ruler, these observations did not change much after 24 hours. Ten compounds were tested in this assay and the results are shown in Table 3.6.

Table 3.6 The zones of inhibition observed after 16 hours

	Test micro-organism			
Sample[a]	*S. aureus*	*B. subtilis*	*Serratia* sp.	*E. coli*
2.63	6.5[c]	10	6.5	6
2.64	6.5	8.5	5.5	6.5
2.65	6	8	6.5	6
2.66	6	10.5	6	6.5
2.69	6.5	10	16.5	5.5
2.72	10	11.5	7	6
2.73	10.5	12	8	6
2.74	11.5	13	8	6.5
2.76	7.5	13	6	6.5
2.69	7.5	12	6	6
Standard[b]	30	30	16	16

[a] test concentration of samples 200 µg, [b] diameter of inhibition zone of standard antibiotic (ampicillin), mm [c] diameter of zone of inhibition is average of duplicates

All standard antibiotics exhibited zones of inhibition greater than 15mm, (15-30mm), indicating susceptibility of the microbes (Figure 3.2). It was interesting to note that the halogenated monoterpenes are relatively more active against Gram-positive organisms (*S. aureus* and *B. subtilis*) than the Gram-negative organisms (*Serratia marcescens*. and *E. coli*), with the exception of **2.69**, which showed activity against *S. marcescens* and no activity against *E. coli. B. subtilis* was more susceptible than *S. aureus*. The aldehyde halogenated monoterpenes showed relatively greater activity to *S. aureus* than the non-aldehyde compounds. These results concur with previous reports (Vlachos *et al.*, 1997) that Rhodophyta extracts are more active against Gram positive than Gram-negative bacteria.

Figure 3.2 Petri dishes showing (a), *Serratia* spp on nutrient agar and (b), zones of inhibition of test compounds and the standard on *B. subtilis* lawn after 16hours **2.69** and **2.72**

3.3 Experimental

3.3.1 Antibacterial assay

Liquid culture of test bacteria

5 mL of nutrient agar broth was inoculated with a loopful of test bacteria from nutrient agar plate. The broth was the incubated at 28°C overnight, after which the broth appeared cloudy indicating growth of the bacteria. The test microorganisms employed in the test were: Gram positive, (*S. aureus* and *B. subtilis)* and Gram negative (*Serratia marcescens* and *E. coli*) supplied by the Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa.

Preparation of test samples

The samples were made up to a concentration of 10 mg/mL (stock solution) in chloroform. 20 µL (approx 200 µg) of this solution was then applied to a disc of sterile filter paper (Whatman®, diameter 6mm) and allowed to dry in a laminar flow hood. Chloroform was used because the compounds dissolve completely in it and it quickly evaporates off. The control discs were prepared by loading 20 µL chloroform on sterile disc.

Standards

Erythromycin and ampicillin discs (15 and 25 µg/disc, Davis diagnostics, Randburg, 2125, Johannesburg) were used as standards.

Inoculating agar plates

120 µL of the liquid culture of test bacteria was spread over nutrient agar plate, using a glass rod. The plates were left to stand for about 15 minutes in a flow hood to dry.

Applying discs on the agar plates

The discs were applied onto "dry" inoculated agar plates using a pair of sterilised forceps, and gentle tapped to ensure good adherence to the agar and enhance diffusion. A control and standard disc was applied onto all the agar plates. The closed plates were incubated at 27°C, and zones of inhibition noted after 16 and 24 hours. The assay was performed in duplicate for each sample.
The presence of an inhibitory zone (mm) indicated activity of test sample.

3.3.2 Cytotoxicity Assay (Brine shrimp)

The procedure employed was a modified version of the brine shrimp toxicity assay developed by Meyers *et al.* (1982)

Hatching of *Artemia salina* cysts

Brine shrimp cysts (PRO 100, Ocean Star, International Inc., Snowville, Utah, 84336, USA) were hatched by incubation in filtered seawater at room temperature (approx 25°C) under constant aeration in light for 24 hours. Hatched were separated from unhatched cysts and cyst shells by phototaxis, using a desk lamp, and used immediately.

Preparation of test samples

Stock solutions of 10 mg/mL in ethanol were prepared for each sample to be tested. An ultrasonic bath was used to aid the dissolution process. 100 µL (1 mg) of this solution was removed and added to 650 µL of filtered seawater to provide a bioassay stock solution of 1 mg per 750 mL. In 96 micro-well plates, 75 µL of filtered seawater was pipetted into wells B-D and 150 µL (200 µg) of stock solution into well A. 75 µL was removed from well A, added to well B and mixed well. 75 µL was transferred from well B to C, and from C to D and the final 75 µL from wells D was discarded. Following the dilutions, 75 µL of filtered seawater was added to all the wells to a final volume of 150 µL per well.

Incorporation of *Artemia salina*

100 µL of sea water containing 10-20 *Artemia* nauplii was added to each well, giving a final volume of 250 µL per well with test solutions at concentrations 400, 200, 100 and 50 µg/mL in wells A to D respectively. The micro well plates were cover and left in a dark cupboard for observation after 12 and 24 hours. Each plate was examined under a hand lense (magnification x10) and the number of dead larvae in each well recorded. 100 µL of MeOH was added to kill live larvae and the total number of larvae per well counted. Results are recorded as a percentage of larvae killed by extract per well.

The results were obtained by recording the number of dead nauplii after 24 hours, then adding 50 µL of methanol to each well, killing the remaining nauplii, and getting a ratio of the nauplii killed by the test compound.

3.3.3 Oesophageal cancer screening

The WHC01 (oesophageal) cells lines were prepared by Catherine Arendse (Department of Medical Biochemistry, University of Cape Town, South Africa). IC_{50} were carried out using MTT kit from Roche (cat # 1465007), according to the manufacturers instructions. Briefly, 1500 cells were seeded per well in 90 µL in Cellstar 96-well plates. Cells were incubated for 24 hours then the test compounds (halogenated monoterpenes) plated at various concentrations in 10 µL (constant final concentration of DMSO = 0.1%). After 48 hours incubation, observations were made, and 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazilium bromide (MTT) solution, added to each well. After further 4 hours incubation, 100 µL of solubilisation solution was added to each of the wells and the plates incubated again overnight. The following morning, plates were read at 595nm on an Anthos micro plate reader 2001.

3.3.4 Extraction of other *Plocamium* spp

Frozen *Plocamium rigidium* (12g) and *Plocamium cornutum* (13g) collected from Clovelly and Dalebrook, False Bay, respectively, were extracted with isopropanol and 1:1 dichloromethane/methanol respectively. The crude extracts were then tested for cytotoxicity by the brine shrimp lethality assay.

Chondsia. capensis collected from Clovelly was also extracted in the same way and tested for cytotoxicity using the brine shrimp assay.

REFERENCES

Anderson, J. E.; Goetz, C. M.; McLaughlin, J. L. *Phytochem Anal.* **1991**, 2, 107-11

Argandona, V. H.; Rovirosa, J.; San-Martin, A.; Riquelme, A.; Daiz-Marrero, A. R.;Cueto, M.; Darias, J.; Santana, O.; Gaudano, A.; Gonzalez-Coloma, A. *J. Agric. Food. Chem.* **2002**, 50, 7029-33

Cameron, D. MSc *Thesis*, **2002** University of the Western Cape, Cape Town, South Africa

Capon, R. J.; Engelhardt, E.; Ghisalbrti, E. L.; Jeffries, P. R.; Patrick, V. A.; White, A. H. *Aust. J. Chem.* **1984**, 37, 537-42

Colman-Saizarbitoria, T.; Gu, Z. M.; Zhao, Z. X.; Zeng, L.; Kozlowski, J. F.; McLaughlin, J. L. *J. Nat. Prod.* **1995**, 58, 532-9

Crews, P.; Myers, B. I.; Naylor, S.; Clason, E.L.; Jacons, R. S.; Staal, G. B. *Phytochemistry* **1984**, 23, 1449-51

De Rosa, S.; De Giulio, A.; Iodice, C. *J. Nat. Prod.* **1994**, 57, 1711-6

Fenical, W. *J. Phycol,* **1975**, 31, 245-51

Fuller, R. W.; Cardellina II, J, H.; Jurek, J.; Scheuer, P. J.; Alvarado-Lindner, B.; McGuire, M.; Gray, G. N.; Steiner, G. J.; Clardy, J.; Menez, E.; Shoemaker, R. H.; Newman,D. J.; Snader, K. M.;Boyd, M. R. *J. Med. Chem.* **1994**, 37, 4407-11

Fuller, R. W.; Cardellina II, M. O.;Kato, Y.; Brinen, L. S.; Clardy, J.; Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1992**, 35, 3007-11

González, A. G. Artega, J. M.; Martin, J. D.; Rodriguez, M. L.; Fayos, J. and Martinez-Ripolls, M. *Phytochemistry* **1978**, 17, 947-8

Gonzalez, G.; Daria, V.; Estevez, E. *Planta Med.* **1982**, 44-6

Klebanoff, S. J. *J. Bacteriol.* **1968**, 95, 2131-8

Konig, G. M.; Wright, A. D.; de Nys, R. *J. Nat. Prod.* **1999**, 62, 383-5

Konig, G. M.; Wright, A. D.; de Nys, R.; Sticher, O. *Planta Med.* **1990**, 56, 559-60

Konig, G. M.; Wright, A. D.; Franzblau, S. G. *Planta Med.* **2000**, 66, 337-42

Leary, J. V.; Kfir, R.; Sims, J. J.; Fulbright, D. W. *Mutation Research* **1979**, 68, 301-5

Marasas, W. F. O.; Jaskiewicz, K.; Venter, F. S.; van Schalkwyk, D.J. *S. Afr. Med. J.* **1988**, 74:110–4

Meyers, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; Mclaughlin. J. L. *Planta Med.* **1982**, 45, 31-4

Michael, A. S.; Thompson C. G.; Abramovitz, M. *Science.* **1956**, 123, 464-7

Rheeder, J. P.; Marasas, W. F. O.; Thiel, P. G.; Sydenham, E. W.; Shephard, G. S.; van Schalkwyk, D. J. *Phytopathology,* **1992**, 82, 353–7

Sahpaz, S.; Bories, C.; Loiseal, P. M.; Cortes, D. *Planta Med.* **1994**, 60, 471-3:

San-Martin, A.; Negrete, R.; Rovirosa, J. *Phytochemistry* **1991**, 30, 2165-9

Scheuer, P. J. *Science* **1990**, 248, 173-8

Somdyala, N. I.; Marasas, W. F.; Venter, F. S.; Vismer, H. F.; Gelderblom, W. C.; Swanevelder, S. A. *S. Afr. Med.J.* **2003**, 93 (2) 144-8

Squillacote, M. E.; Sheridan, R. S.; Chapman, O. L.; Anet, F. A. L. *J. Am. Chem. Soc.* **1979**, 101, 3657-

Stallard, M. O.; Faulkner, D. J. *Biochem. Physiol.* **1974**, 49, 25-9

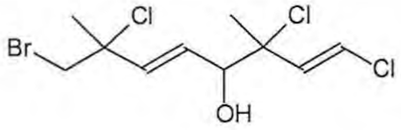
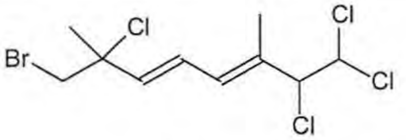
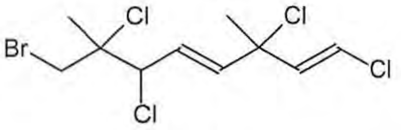
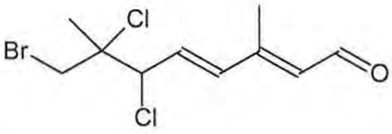
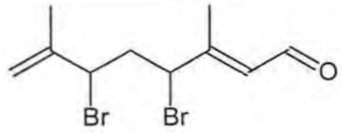
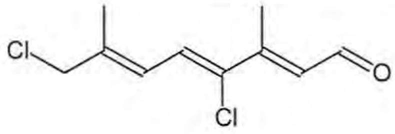
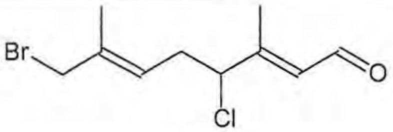
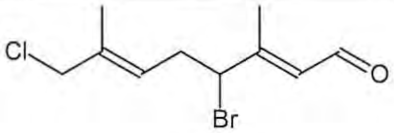
Vlachos, V.; Critchley, A. T.; von Holy, A. *S. Afr. J. Sci.* **1997**, 93, 328-32

Wanatabe, K.; Miyakado, M.; Ohno, N.; Okada, A.; Yanagi, K.; Moriguchi, K. *Phytochemistry* **1989**, 28, 77-8

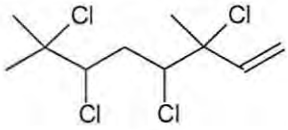
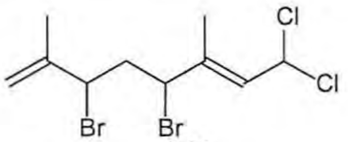
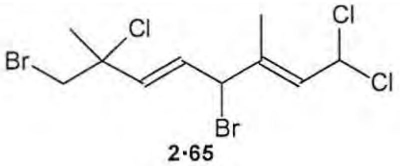
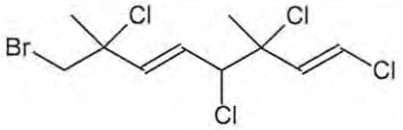
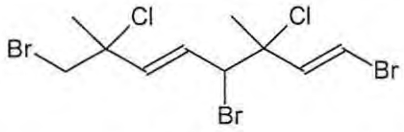
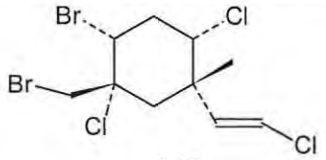
Weiss, S. J.; Test, S. T.; Eckmann, C. M.; Roos, D.; Regiani, S. *Science* **1986**, 234, 200-7

APPENDIX 1: SUMMARY OF STRUCTURES OF ISOLATED COMPOUNDS.

NEW COMPOUNDS

 **2.69**	 **2·70**
 **2.71**	 **2·72**
 **2·73**	 **2·74**
 **2·75**	 **2·76**

KNOWN COMPOUNDS

 **2·63**	 **2·64**
 **2·65**	 **2·66**
 **2·67**	 **2·68**

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