THE NATURAL PRODUCT CHEMISTRY OF SOUTH AFRICAN *PLOCAMIUM* SPECIES

THESIS

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by

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To my parents, Chris and Basia Knott

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List of Abbreviations

COSY	^{1}H-^{1}H- Homonuclear Corrlelation Spectroscopy
DEPT	Distortionless Enhancement of Polarisation Transfer
EIMS	Electron Impact Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
IR	Infrared
LRMS	Low Resolution Mass Spectrometry
NMR	Nuclear Magnetic Resonance
SCUBA	Self Contained Underwater Breathing Apparatus
TLC	Thin Layer Chromatography
UV	Ultra Violet
d	doublet
eV	electron Volt
Fn	fraction
lr	long range
m	multiplet
q	quartet
s	singlet
t	triplet

Abstract

The brine shrimp lethality assay was used as a preliminary tool to screen eighteen seaweeds collected from the South African coast. Of the seaweeds tested, the red algae *Plocamium corallorhiza* and *Hypnea rosea*, and the green alga *Halimeda* sp., showed the most potent activity. The chemical investigation of *P. corallorhiza* resulted in the isolation and structural elucidation of five previously undescribed secondary metabolites, along with three known compounds and four possible artifacts of the extraction process. Standard spectroscopic methods and comparison with known compounds were used to determine the structures of the new metabolites. The new compounds included the linear halogenated monoterpenes 4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene (**99**), 4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene (**100**), 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (**101**) and 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**102**) and the cyclic monoterpene 5-bromo-5-bromomethyl-1-chlorovinyl-2,4-dichloro-methylcyclohexane (**103**) while the known compounds were identified as 4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (**35**), 1,4,8-tribromo-3,7-dichloro-3,7-dimethyl-1,5-octadiene (**94**) and 8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (**96**). The four methoxylated compounds (**104-107**) were presumably formed *via* a standard substitution reaction between the halogenated monoterpenes **96** and **101** and MeOH, which was used as a component in the extraction solvent.

With over 100 000 natural products having been reported, it has become necessary to employ an efficient dereplication strategy to quickly identify known compounds. A simple Gas Chromatography-Mass Spectrometry (GC-MS) method for the efficient physico-chemical screening, identification and dereplication of *Plocamium* metabolites was developed. In this study the crude extracts of *P. corallorhiza*, *P. cornutum* and *P. maxillosum* were screened by GC-MS and the retention times and mass spectral fragmentation patterns of compounds **94, 96, 99 - 107** were used to quickly identify known and new compounds in the crude extracts of *P. cornutum* and *P. maxillosum*. This data indicated that compounds **99**, **100**, **103** were present in both *P. corallorhiza* and *P. cornutum*, while compound **102** was found to be present in *P. corallorhiza*, *P. cornutum* and *P. maxillosum*. These studies also indicated that ecotypes and chemotypes are not a significant feature of *P. corallorhiza* and *P. cornutum*. Different species of *Plocamium* (namely: *P. corallorhiza*, *P. cornutum*, and *P. maxillosum*) have very different chemical

profiles, and GC may therefore have appreciable taxonomic application in the identification of the different *Plocamium* spp. which are endemic to South Africa.

Chapter One

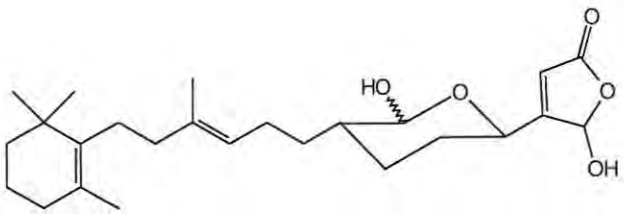
Introduction

1.1 Primary and Secondary Metabolism

All living organisms, from the smallest bacterium to the biggest mammal, make use of basic chemical reactions to transform and interconvert organic compounds in order to live, grow and reproduce. The pathways for the metabolism of carbohydrates, proteins, fats and nucleic acids are found to be generally the same in all organisms, and are collectively known as primary metabolism.[1]

While primary metabolism is encountered in all organisms, secondary metabolism is only found in specific organisms. Secondary metabolism is an expression of the individuality of the species, and in most cases the function of the compounds (secondary metabolites) produced via this pathway remains unknown. Biologically active secondary metabolites have played a significant part in the development of modern medicine.

The biodiversity of the planet is heavily weighted towards the oceans, with the majority of the phyla for the five kingdoms being aquatic. Estimates of the number of aquatic species range from 1.5 to 4.5 million, with the majority of these being undescribed.[2] With this barely explored biodiversity that is offered by the oceans, the potential for discovering new pharmaceutical compounds from the ocean is enormous. Over the past three decades natural products with potent pharmacological activities have been isolated from the marine environment. These include anti-inflammatories (e.g. manoalide, **1**),[2] antivirals (e.g. Ara-A, **2**, which was the lead compound used for the synthesis of acyclovir),[2] antibiotics (e.g. cephalosporins, **3**),[3] insecticides (e.g. nereistoxin, **4**),[3] anthelminthic agents (e.g. α-Kainic acid, **5**),[3] toxins (e.g. tetrodotoxin, **6**),[4] and anti-tumor compounds (e.g. dolastatin 10, **7**).[3]



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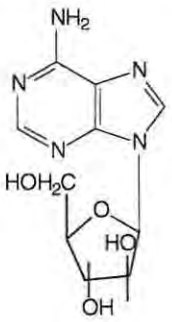

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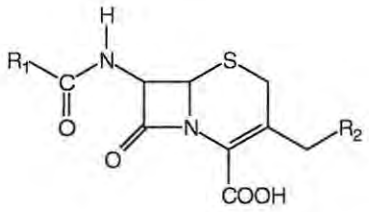

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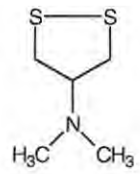

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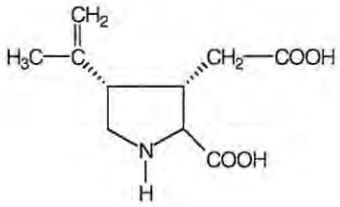

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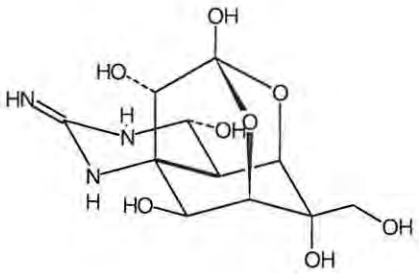

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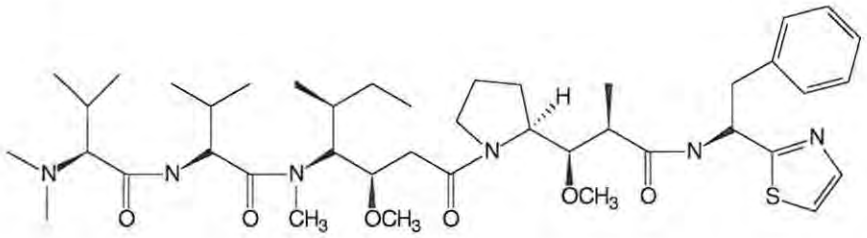

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1.2 South African Marine Algae

The south-eastern Cape has a vast diversity of endemic marine algae whose chemistry and pharmacology have never been studied. This diversity is due to unusual mixing of waters of the warm Agulhas and cold Benguela currents, creating an environment unique to this area (Figure 1.1). This feature causes the coastal area stretching from Port St. Johns to Cape Point to be characterised by a high level of endemic marine species. The coastline of southern Africa (from northern Namibia to southern Mozambique) has over 10 000 species of marine *fauna* and *flora* which amounts to approximately 15% of all the coastal marine species known worldwide. Of this amazing assemblage, 12% of these species are endemic.[5]

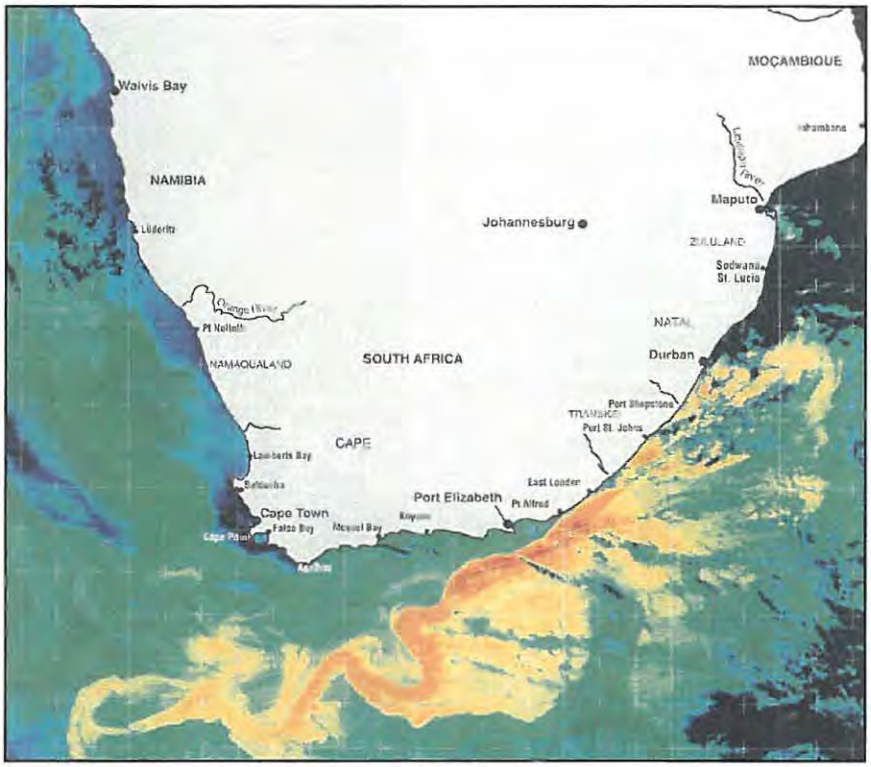


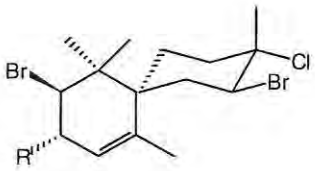
Figure 1.1 Satellite photograph of the coast of southern Africa (reproduced with permission of Prof. G. Branch) [5]

1.2.1 Commercial uses of Marine Algae

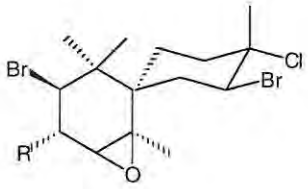
South Africa has a small seaweed industry based on the utilisation of several brown and red algae. On the west coast, the kelps *Ecklonia maxima* and *Laminaria pallida* are collected for alginate. *Ecklonia* is used for the production of plant-growth stimulant (e.g. fertilisers), and harvested as feed for commercially farmed abalone. *Gracilaria* is collected at Saldanha Bay where research into its mariculture is yielding good results while in the Eastern Cape, *Gelidium* species are harvested from intertidal shores as a good source of agar.[6]

1.2.2 Natural product chemistry of South African Marine Algae

Marine algae are classified into three classes: Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). Literature reports on the natural product chemistry of South African marine algae are very scarce. There is only one publication in the marine natural product literature describing the isolation of four halogenated chamigranes (**8 – 11**) from the South African red algae *Laurencia glomerata*.[7]



8 R = H
9 R = OH



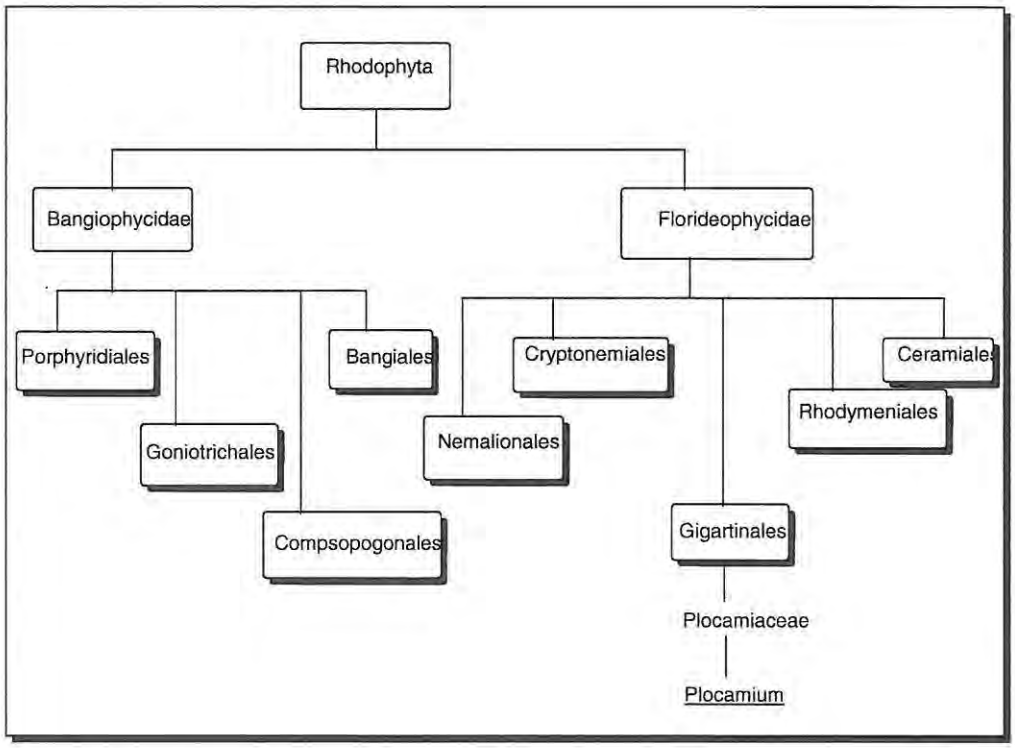
10 R = H
11 R = OH

The only other published study on South African marine algae was by Vlachos *et al.* in which 56 crude extracts were screened for antimicrobial activity.[8] This was a pharmacological study in which characterisation of the compounds responsible for the activity was not reported.

1.3 Review of *Plocamium* metabolites

1.3.1 Introduction

The classification system in use for the Rhodophyta class (red algae) is illustrated below (Scheme 1.1). Red seaweeds are the largest group of marine algae and are characterised by an outer appearance that is usually red, purple or pink. The appearance may also be pale (almost brown), green (but tinged with red), or covered with a white or pinkish calcareous crust.[6]



Scheme 1.1 Classification of red seaweed (Rhodophyta) [6]

The families Bonnemaisoniaceae (Nemalionales), Plocamiaceae (Gigartinales), Rhizophyllidaceae (Gigartinales), and Rhodomelaceae (Ceramiales) tend to be particularly rich in biologically active compounds. These compounds range in structure from simple aliphatic halo-ketones and brominated phenols to more complex monoterpenes, sesquiterpenes, and diterpenes.[9] Only the red seaweeds, of the families Plocamiaceae and Rhizophyllidaceae (Gigartinales), or sea hares of the genus *Aplysia* contain extracts rich in acyclic and cyclic monoterpenes. Yields of these compounds (mass of compound extracted per dry mass of plant) range from trace amounts to as much as 3-5% of the plant's mass.[9]

1.3.2 Pathway for the biosynthesis of monoterpenes

Monoterpenes are derived from two C_5 isoprene units joined in a head-to-tail fashion. These five-carbon molecules are either made from the condensation of three acetyl coenzyme A (CoA) units (from the glycolysis pathway) to form mevalonic acid via the mevalonic acid (MVA) pathway; or by an alternative route, whose first committed intermediate is 2-methylerythritol 4-phosphate (MEP). This is known as the methylerythritol pathway (MEP) (Figure 1.2). The C_5 isoprene unit in the form of dimethylallyl diphosphate (DMAPP) can be used to undergo alkylation reactions involving electrophilic addition, or may ionize to generate a resonance stabilized allylic carbocation (Figure 1.3). This can then react with an alkene, such as isopentenyl diphosphate (IPP) via the enzyme prenyl transferase. The resultant carbocation may then lose a proton to give the uncharged product geranyl diphosphate (GPP) (Figure 1.4).[1] Where the alkene and carbocation functions reside in the same molecule, this type of mechanism can be responsible for cyclization reactions as seen in certain *Plocamium* metabolites.

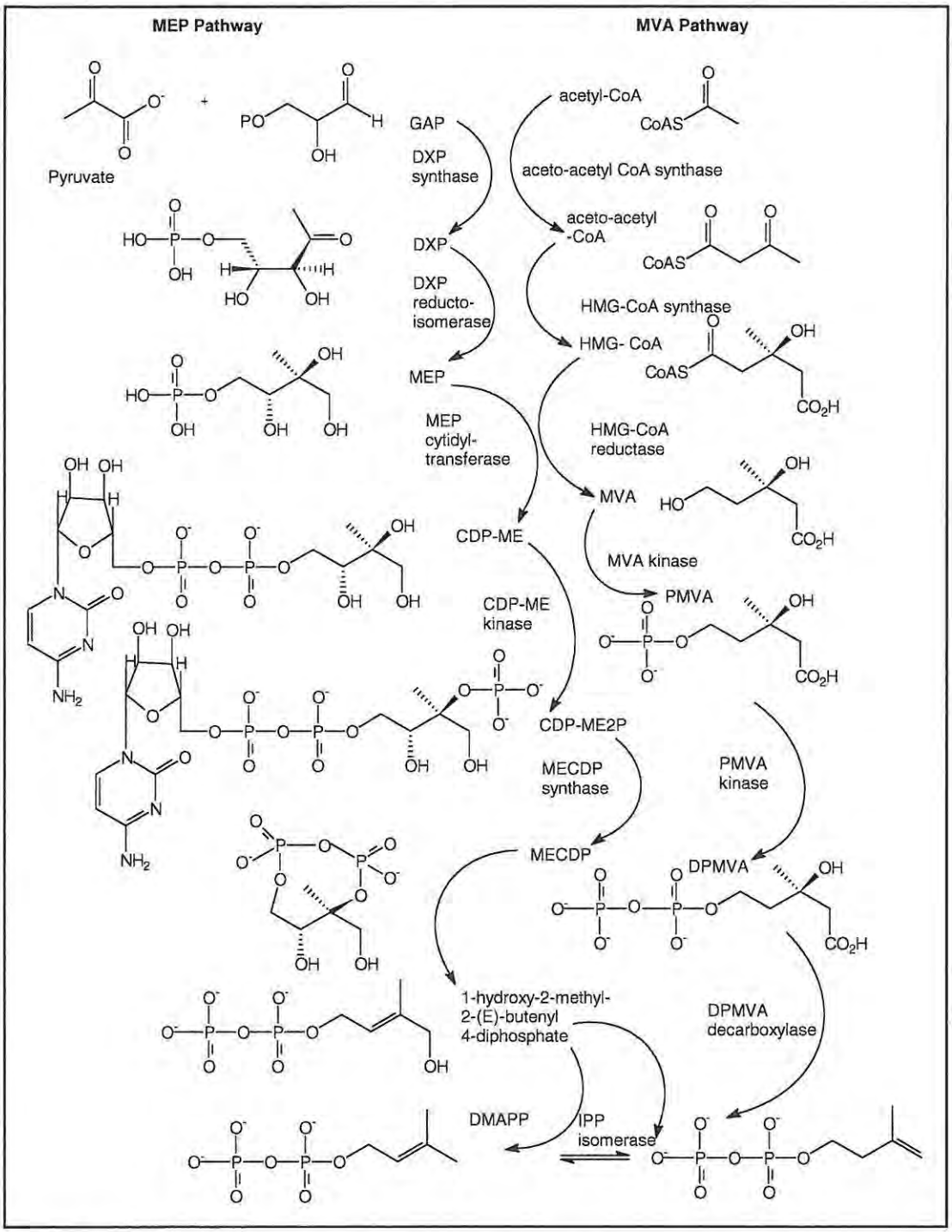


Figure 1.2 Formation of mevalonic acid (MVA) and dimethylallyl diphosphate (DMAPP) from acetyl-CoA via the MVA pathway, and the formation of DMAPP from pyruvate via the non-mevalonate or methylerythritol pathway (MEP)[1]

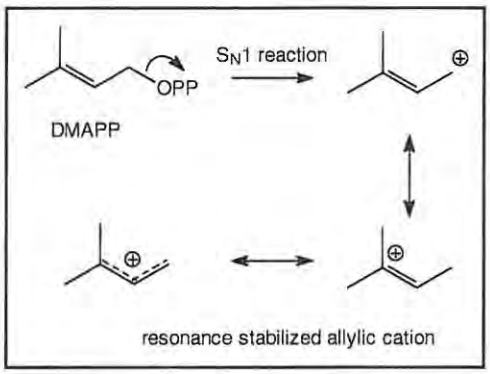


Figure 1.3 Formation of a reactive carbocation from DMAPP [1]

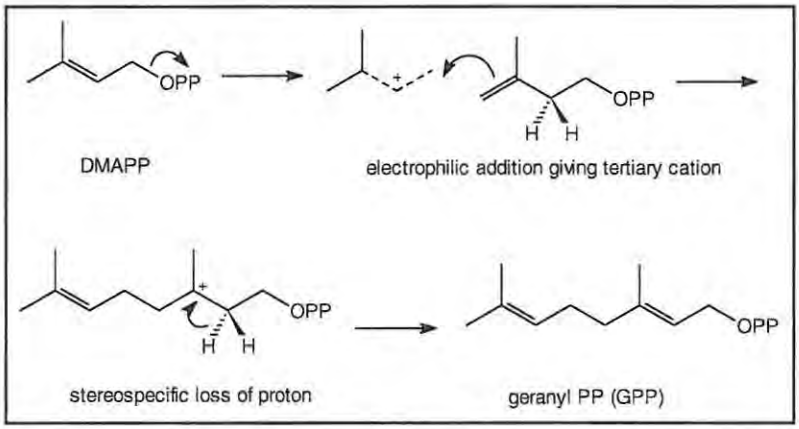


Figure 1.4 Formation of geranyl diphosphate (GPP) from DMAPP and isoprene [1]

The loss of HOPP (a diphosphate acid) from DMAPP can generate myrcene or ocimene (Figure 1.5) from which polyhalogenated monoterpenes are derived (Figure 1.6 and 1.7). It has been proposed that polyhalogenated monoterpenes are formed via an enzyme catalysed electrophilic addition reaction.[10] The Markovnikov addition of Br^+ to myrcene followed by Cl^-, leads to the formation of a dihalogenated product. An initial addition of Cl^+ to ocimene followed by H^+ loss, and the subsequent addition of Cl^+, and finally Cl^- capture leads to **(85)** or **(86)**. Halogenated myrcene derivatives are only isolated from the Rhizophyllidaceae family, while halogenated ocimene derivatives are only found in Plocamiaceae family.[10] Cyclization of linear compounds may also occur (Figure 1.8).

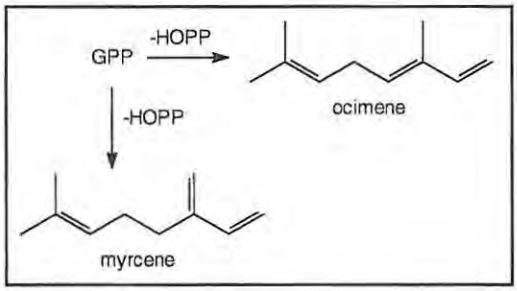


Figure 1.5 Formation of ocimene from myrcene [10]

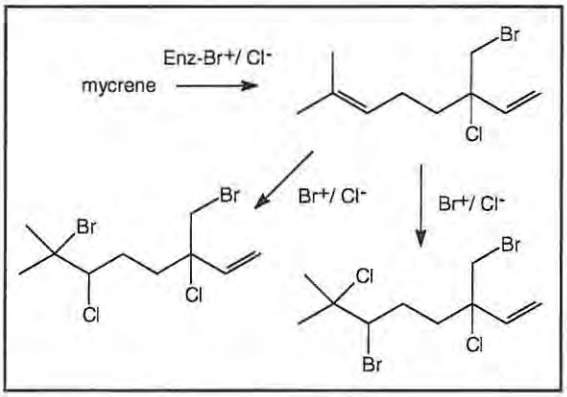


Figure 1.6 Biogenesis of halogenated myrcenes [10]

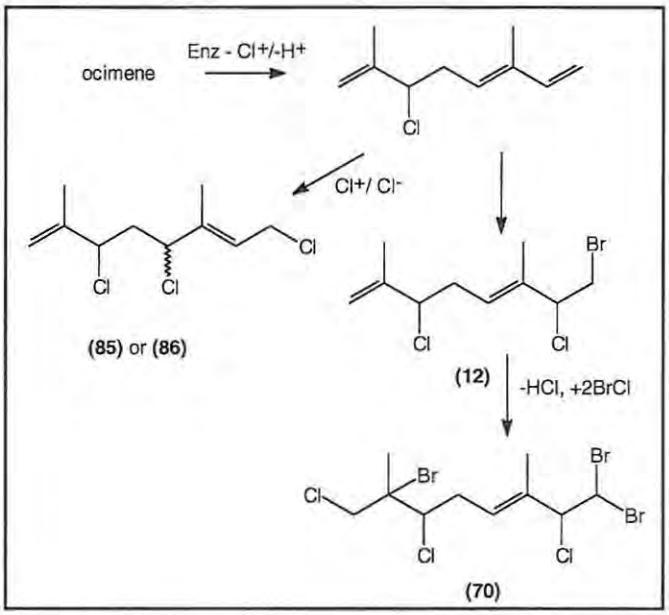


Figure 1.7 Biogenesis of halogenated ocimenes [10]

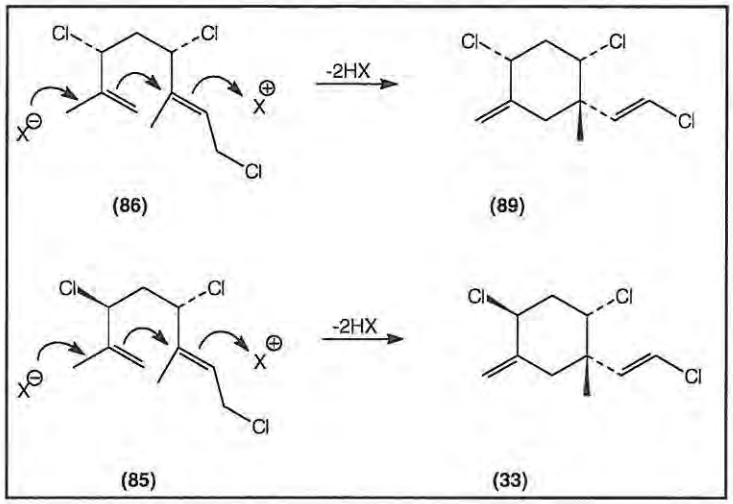


Figure 1.8 Cyclization of acyclic monoterpenes [10]

Halogenated monoterpenes are difficult to synthesize in the laboratory, due to the stereochemical requirements of the halogen substituents. Marine organisms contain haloperoxidase enzymes that can chlorinate or brominate organic compounds in the presence of chloride or bromide ions.[11] Halogens (characteristic of all *Plocamium* monoterpenes) are obtained from the ocean, which is a good source of Cl^- and Br^-. The marine environment has been estimated to contain *ca* 19 g/l of chloride and 65 mg/l of bromide.[12] Although bromine is the predominant halogen in red seaweed sesquiterpenes and diterpenes, inspection of halogenated monoterpenes shows a different picture. Halogenated monoterpenes either contain only chlorine, or a high chlorine to bromine ratio in all families, except the Rhizophyllidaceae family.[10]

1.3.3 Structural categories of monoterpenes found in red seaweeds

The halogenated monoterpenes (isoprene dimers) that predominate in the extracts of *Plocamium* spp. can be sub-classified into four main structural categories (Figure 1.9). These consist of a head to tail framework (**A**) and three monocyclic types (**B**, **C**, and **D**). Acyclic compounds are further divided into four sub-groups: monoenes, dienes, trienes, and myrcenes. Crews [10] suggested the numbering for **B**, **C**, and **D**.

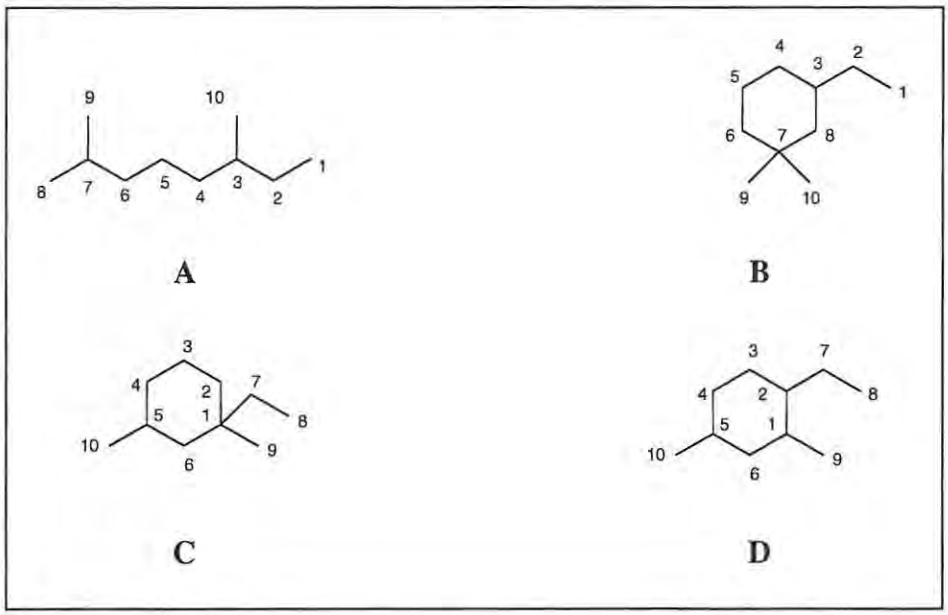


Figure 1.9 The four structural categories of monoterpenes [10]

The head to tail acyclic skeleton (**A**) is found in both marine and terrestrial organisms. Skeletons (**C**) and (**D**) are unique to marine organisms and are exclusive to *Plocamium* spp. (*Plocamium* spp. are also characterized by the presence of type (**A**) skeletons.) Chemical evidence indicates that the carbon framework of (**C**) arranges to (**D**) by ethyl migration as opposed to other alternatives such as methyl migration in a 1,5 dimethyl-1-ethylcyclohexane precursor.[10]

The Rhizophyllidaceae family produces monocyclic monoterpenes of the (**B**) type. *Microcladia* species have compounds of type (**A**), (**C**), and (**D**), although it is believed that these organisms are able to "concentrate" the halogenated metabolites produced by *Plocamium* spp.[10]

1.3.4 Metabolites isolated from various *Plocamium* spp.

Although monoterpenes have been isolated from the red seaweeds, *Chondrococcus* and *Ochtodes*, as well as certain types of sea hares; only the halogenated metabolites isolated from *Plocamium* spp. are tabulated in the following section. Structural re-assignments have substituted the original suggestions for some of the references, however outstanding re-assignments may still be present (Table 1.1-1.11).

Table 1.1 Metabolites isolated from *Plocamium angustum*

No.	Isolated Compounds	Location	Reference
12, 13	**2,6-Dimethyloctadienes**	Cape Northumberland (W. Australia)	13

Table 1.2 Metabolites isolated from *Plocamium cartilagineum*

No.	Isolated Compounds	Location	Reference
14	**2,6-Dimethyloctenes**	Kaikoura (N. Zealand)	14
15		Figueira de Foz (Portugal)	15
16	**2,6-Dimethyloctadienes**	Kaikoura (N. Zealand)	14
17	**2,6-Dimethyloctratrienes** Cartilagineal	California (USA)	16
18-28		La Jolla (USA)	17
29		L'Estartit (Spain)	18
30		Figueira da Foz (Portugal)	15
31, 32		Schouten Beach (Tasmania)	19
33, 34	**1-Ethyl-1,3-dimethyl cyclohexanes**	Antarctica	20
35-37		USA	21
38-41		Chile	22
42-44	**1-Ethyl-2,4-dimethyl cyclohexanes**	USA	21
45-50	**Hydroxy bisnor monoterpenes**	Kaikoura (N. Zealand)	14

51-53	**Polyhalodroxylated monoterpenes**	Chile	23
54-56	**Furanoid monoterpenes**	Chile	24
57,58	**Tetrahydropyran monoterpenes**	Chile	25
59-62	**Homosesquiterpenic fatty acids**	Maltese Islands, and Corsica (Mediterranean)	26
	Non-Terpenoid compounds Floridoside and Poly (β-hydroxybutyrate)	Figueira da Foz (Portugal)	27

Table 1.3 Metabolites isolated from *Plocamium coccineum*

No.	Isolated Compounds	Location	Reference
63	**1-Ethyl-1,3-dimethyl cyclohexanes**	Bastiagueiro (NW. Spain)	28
64, 65		Bastiagueiro (NW. Spain)	29

Table 1.4 Metabolites isolated from *Plocamium costatum*

No.	Isolated Compounds	Location	Reference
66	**2,6-Dimethyloctene**	Eaglehawk Neck (Tasmania)	30
67, 68	**2,6-Dimethyloctadienes** Costatol, Costatone	Port MacDononnell (S. Australia)	31
69		Robe (S. Australia)	32
	Polysaccharides	Tauranga (New Zealand)	33

Table 1.5 Metabolites isolated from *Plocamium cruciferum*

No.	Isolated Compounds	Location	Reference
70	**2,6-Dimethyloctene**	Rosy Morn (N. Zealand)	34
71	**Degraded or mixed biogenesis monoterpene**	Kaikoura (N. Zealand)	35
72		Rosy Morn (N. Zealand)	34

Table 1.6 Metabolites isolated from *Plocamium hamatum*

No.	Isolated Compounds	Location	Reference
73	**2,6-Dimethyloctene**	Palm Is. (W. Australia)	36
74	**2,6-Dimethyloctadiene**	Palm Is. (W. Australia)	36
75	**1-Ethyl-1,3-dimethyl cyclohexane**	Palm Is. (W. Australia)	36

Table 1.7 Metabolites isolated from *Plocamium mertensii*

No.	Isolated Compounds	Location	Reference
76	**1-Ethyl-1,3-dimethyl cyclohexanes** Mertensene	Australia	37
77		Carnac Is. (W. Australia)	38
78, 79	**1-Ethyl-2,4-dimethyl cyclohexanes**	Australia	37
80		Carnac Is. (W. Australia)	38

Table 1.8 Metabolites isolated from *Plocamium oregonum*

No.	Isolated Compounds	Location	Reference
81, 82	**2,6-Dimethyloctadienes** Oregonene A (**81**)	California (USA)	39

Table 1.9 Metabolites isolated from *Plocamium telfairiae*

No.	Isolated Compounds	Location	Reference
	1-Ethyl-1,3-dimethyl cyclohexane		
83	Telfairine	Fukui (Japan)	40

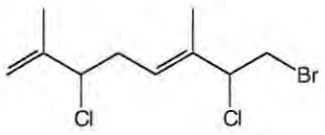
Table 1.10 Metabolites isolated from *Plocamium violaceum*

No.	Isolated Compounds	Location	Reference
	2,6-Dimethyloctadienes		
84-86	Preplocamene A, B and C	California (USA)	41
	2,6-Dimethyloctratriene		
87		California (USA)	42
	1-Ethyl-1,3-dimethyl cyclohexanes		
88	Violacene	California (USA)	43
89-91	Plocamene D, D`, and E	California (USA)	44
	1-Ethyl-2,4-dimethyl cyclohexanes		
92	Plocamene-C / Violacene-2	California (USA)	45
93	Plocamene-B	N. California(USA)	46

Table 1.11 Metabolites isolated from *Plocamium* spp.

No.	Isolated Compounds	Location	Reference
	2,6-Dimethyloctatrienes		
94-97		Antarctica	47

* Compound **94** was not isolated from a *Plocamium* spp.



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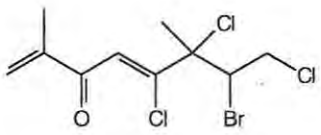

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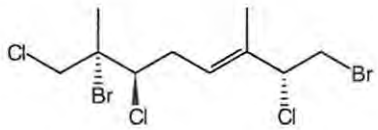

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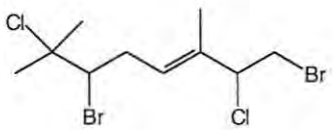

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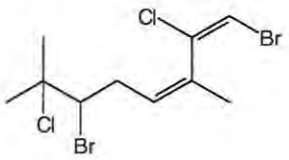

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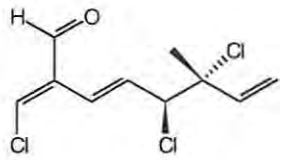

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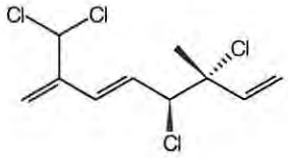

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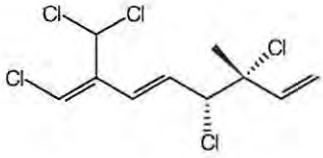

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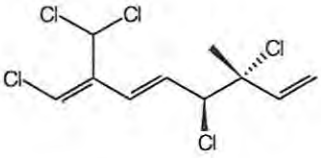

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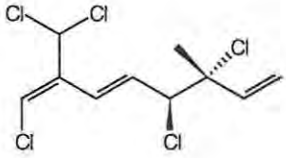

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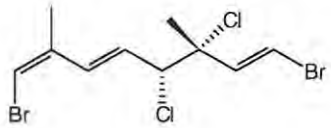

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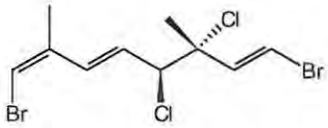

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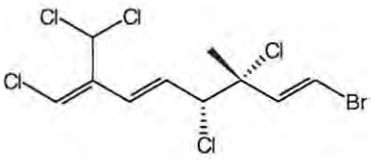

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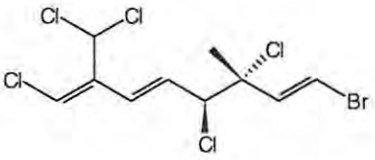

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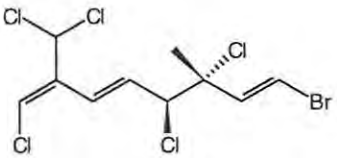

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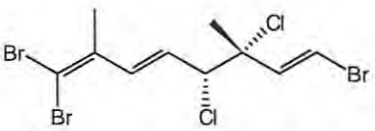

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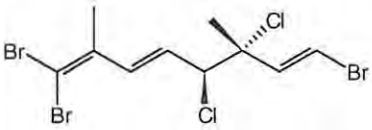

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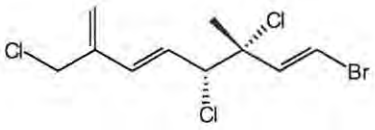

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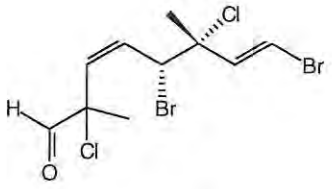

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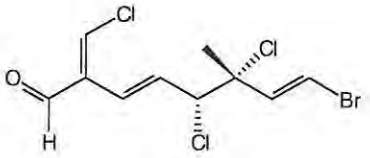

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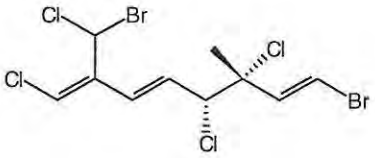

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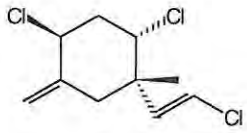

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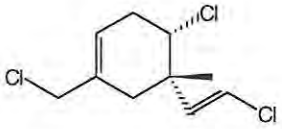

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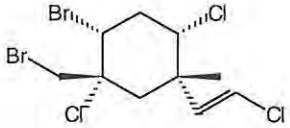

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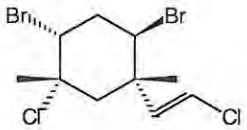

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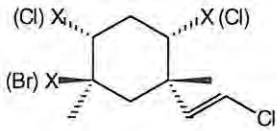

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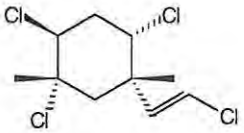

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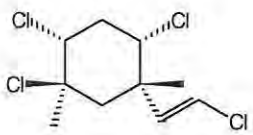

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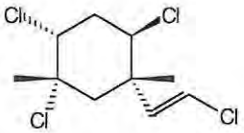

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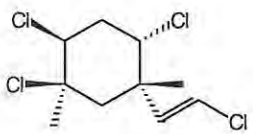

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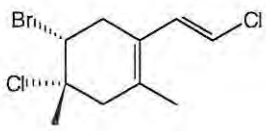

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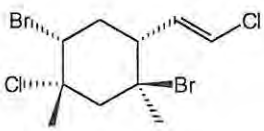

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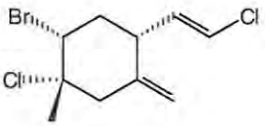

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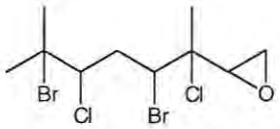

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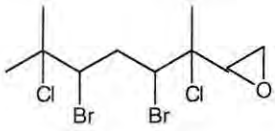

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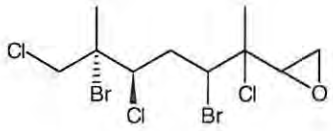

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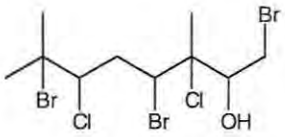

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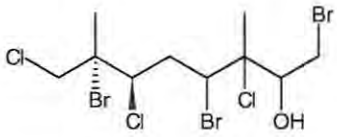

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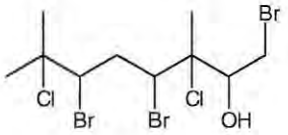

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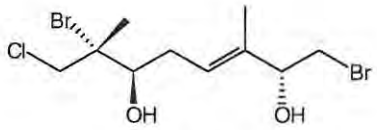

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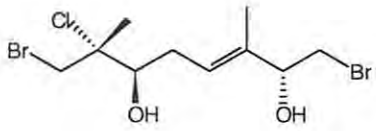

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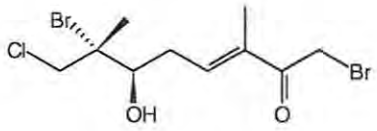

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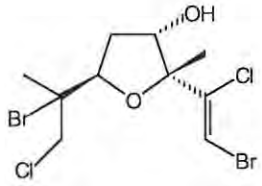

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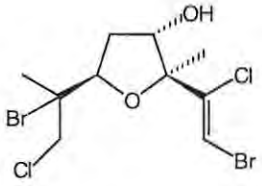

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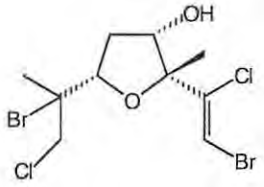

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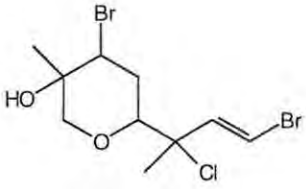

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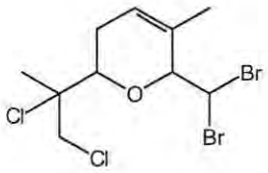

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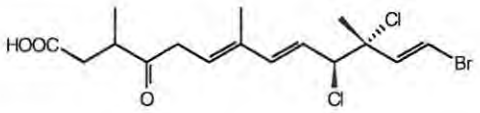

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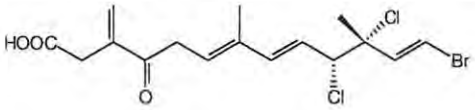

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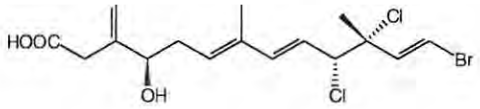

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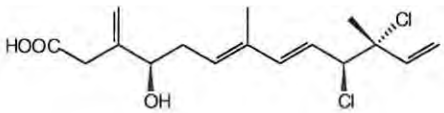

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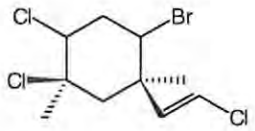

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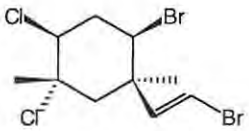

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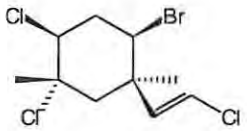

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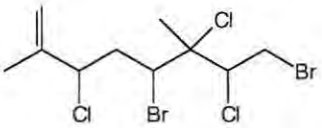

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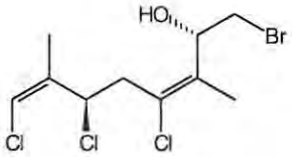

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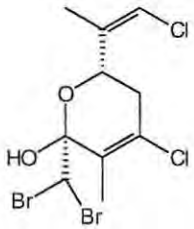

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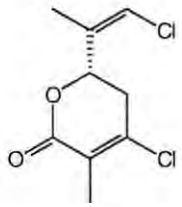

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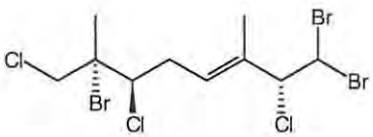

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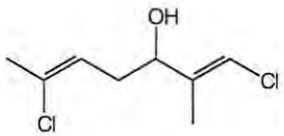

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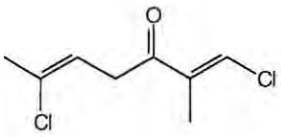

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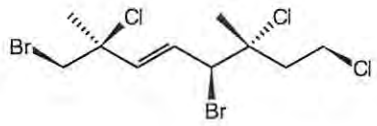

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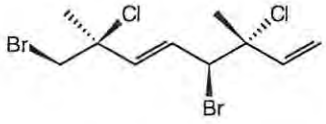

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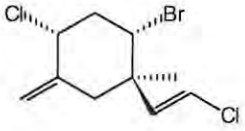

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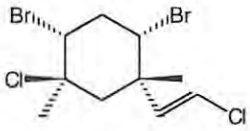

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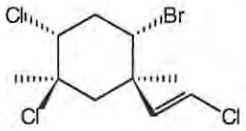

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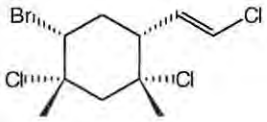

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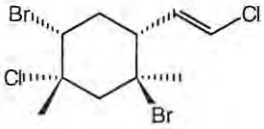

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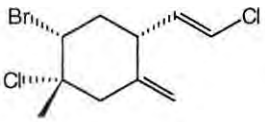

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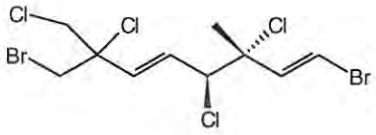

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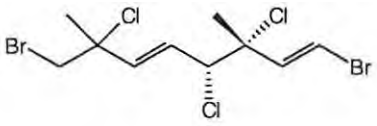

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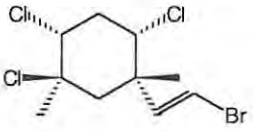

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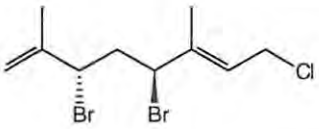

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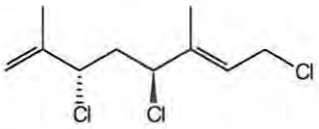

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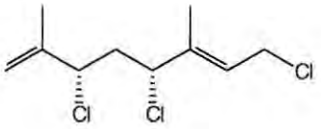

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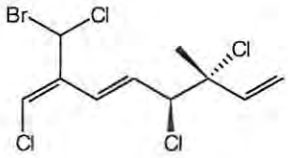

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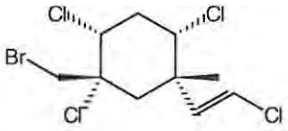

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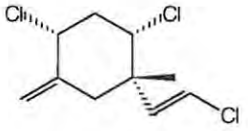

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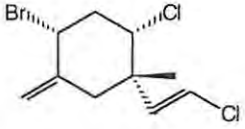

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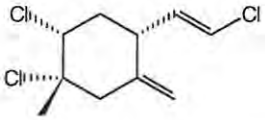

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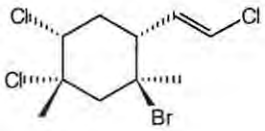

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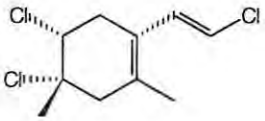

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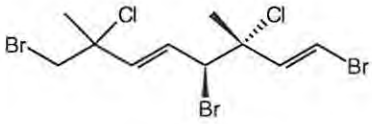

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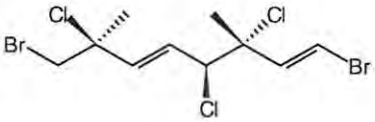

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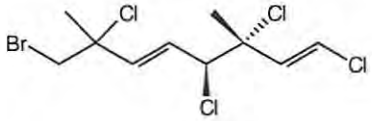

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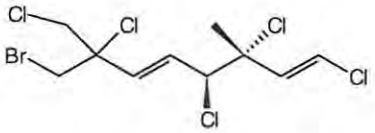

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Some polyhalogenated monoterpenes are common to more than one *Plocamium* spp (Table 1.12).[10]

Table 1.12 Monoterpenes common to more than one *Plocamium* spp. [10]

No.	Algae	Location
81	*Plocamium* spp.	Antarctica
	P. oregonum	Whidby Island, Washington
19, 20	*P. cartilagineum*	San Diego, California
	P. sandvicense	Maui, Hawaii
22, 23	*P. cartilagineum*	San Diego, California; South Wales, UK
	P. oregonum	Whidby Island, Washington

* This table was complied in 1983 and may contain some omissions

1.4 Thesis Objective

With the high degree of endemic marine algae found along the South African coast, and the relatively little work done that has been done on investigating the chemical and pharmacological properties of seaweeds in South Africa, the potential for finding novel pharmaceutical compounds from the ocean is fairly high.

The main objectives of this study were:

(a) To screen South African marine algae for biological activity.

(b) To extract, purify and characterise natural products responsible for biological activity.

(c) The development of a suitable method for the rapid screening and dereplication of marine algal metabolites.

Chapter Two

Halogenated monoterpenes from the endemic South African red algae, *Plocamium corallorhiza*

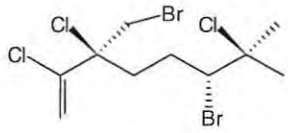
2.1 Introduction

Monoterpenes are unique secondary metabolites that are produced by red algae of the Plocamiaceae and Rhizophyllidaceae families, both of the order Gigartinales.[48] Sea hares of the genus *Aplysia*, as well as some seaweeds of the genus *Microcladia* (order Ceramiales) are exceptions. However, these organisms are not a direct source for the production of halogenated monoterpenes.[10] A number of cyclic (cyclohexane derivatives) and acyclic monoterpenes, as well as tetrahydropyran and tetrahydrofuran derivatives have been reported from the genus *Plocamium*.[48]

Polyhalogenated monoterpenes were first discovered in the digestive glands of the sea hare (*Aplysia californica*) in 1973,[49] and then later from *P. cartilagineum*.[43] The search for novel halogenated monoterpenes was very competitive until about 1985, then interest started to decline. Reasons for this may be the increased frequency at which known metabolites were being isolated from marine algae, and the routine use of SCUBA to collect some of the less accessible marine invertebrates such as soft corals and sponges.

With the discovery of halomon (**98**), a polyhalogenated monoterpene which demonstrated a selective and potent cytotoxicity profile against a panel of cancer cell lines,[50] there has been a renewed interest into the chemistry and pharmacology of polyhalogenated monoterpenes.

Collections for the chemical investigation of the *Plocamium* genus have taken place on every continent, including Africa. However, the chemical and pharmacological characteristics of *Plocamium* spp. from southern Africa have never been investigated or published. With the exception of various *Plocamium* spp. from Australia,[13, 31, 36, 38] most collections have focussed primarily on the secondary metabolites from *P. cartilagineum*.[14-27] This seaweed [51] has demonstrated the greatest variety of halogenated monoterpenes, which are known to vary between individual species and location.[52] This should be no surprise since *P. cartilagineum* is abundant, and has displayed at least three distinct morphological forms.[52]



98

At least six endemic *Plocamium* spp. (Figure 2.1) [53] occur along the South African coast.[6] These include *P. beckeri*, *P. corallorhiza*, *P. cornutum*, *P. glomeratum*, *P. rigidum*, and *P. suhri*. Members of the Plocamiaceae family have a characteristic thallus which is broadly flattened, as well as claw-like leaflets with teeth on the margins. The two most common species of *Plocamium* in South Africa are *P. corallorhiza* and *P. cornutum*.

P. corallorhiza (or coral *Plocamium*) illustrated below (Figure 2.2), is described as having, a broad thallus, flattened fronds with claw-like leaflets (10 mm wide) and serrated margins. *P. corallorhiza* has a deep-pink colour, which becomes an iridescent blue-purple underwater. Leaflets are arranged in alternating pairs, while the reproductive bodies form rosettes of tiny leaflets on the thallus. *P. cornutum* (or horny *Plocamium*) has a course and untidy thallus with crowded and cylindrical branchlets. The branchlets are arranged in series of not more than two at a time. *P. cornutum* is brown, up to 14 cm tall, and has claws of about 1 mm wide. Both *P. corallorhiza* and *P. cornutum* grow very well in areas exposed to waves or heavy surf. They are consequently located in the cochlear zone, which is the zone between low water neap and low water spring tide.[6]

Plocamium corallorhiza

Plocamium cornutum

Plocamium suhrii *Plocamium rigidum*

Figure 2.1 Four endemic species belonging to the Plocamiaceae family (with permission from Prof G. Branch) [53]

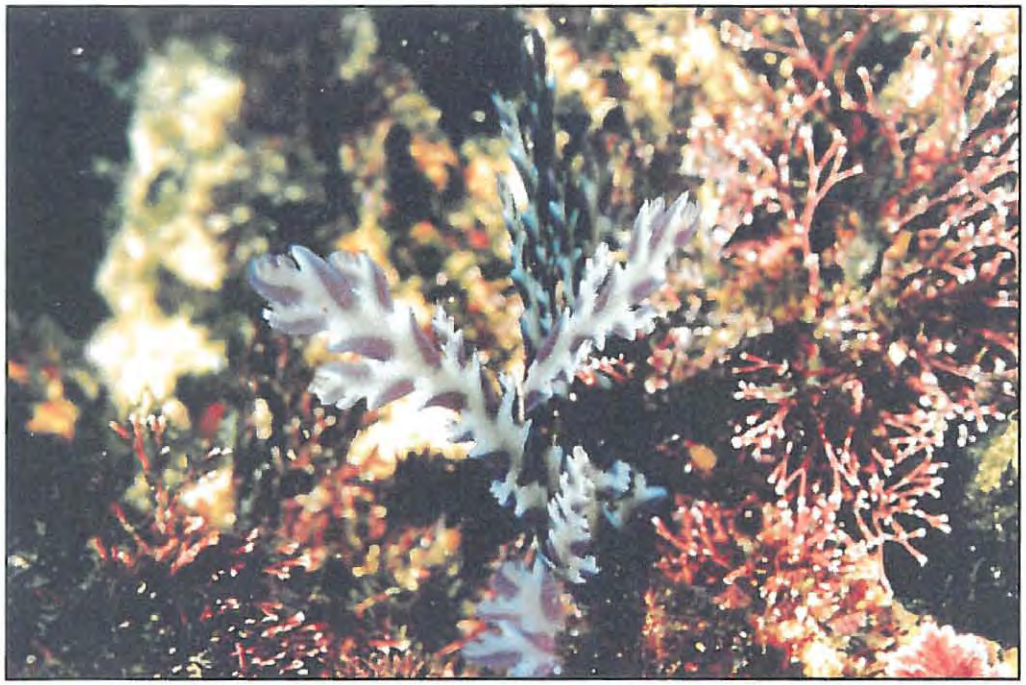


Figure 2.2 *Plocamium corallorhiza* (with permission from Prof M.T. Davies-Coleman)

Structural elucidation of halogenated monoterpenes

Structure determination of polyhalogenated monoterpenes is not trivial due to the large number of halogen atoms (up to six halogens) that are incorporated into a ten-carbon chain or a six membered ring. This has led to a large number of structural reassignments. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are routinely used to characterise marine monoterpenes. Ambiguities from NMR and MS data are remedied by X-ray analysis or by chemical inter-conversion and degradation pathways.

i) *NMR spectroscopy*: ^{1}H and ^{13}C NMR data have provided very important structural information for all marine monoterpenes. However, ^{1}H NMR chemical shift data should be interpreted with caution, owing to the deshielding and through space effects from neighbouring halogen atoms. Although the ^{13}C NMR substituent effects of halogens are much larger than the corresponding ^{1}H NMR substituent effects, assignment of the ^{13}C NMR spectrum can be ambiguous unless ^{13}C and ^{1}H NMR chemical shifts are correlated. ^{13}C NMR data has also been used (with much success) in addressing the regiochemical and stereochemical problems associated with marine monoterpenes.

Determination of the total number of halogens and their location or regiochemistry, remains one of the most difficult problems associated with the structural determination of polyhalogenated monoterpenes. Comparison of known (provided that the structure was correctly assigned) and unknown compounds using ^{13}C NMR spectroscopy data is far superior to ^{1}H NMR when determining halogen regiochemistry, and is of more value in acyclic polyhalogenated monoterpenes.

Although ^{1}H chemical shifts have not been useful in differentiating between CH-Br and CH-Cl substituents, the ^{1}H coupling constants have proved to be extremely useful in deducing the stereochemistry around a double bond. For example, proton and carbon NMR strategies have been used to solve the C_5 – C_6 stereochemistry of acyclic compounds.

ii) *Mass spectrometry*: Although high resolution mass spectrometry is extremely useful in determining the molecular formula and thus the number of halogens present in these monoterpenes, it is often difficult to obtain a reliable molecular ion peak. Intense ion peak clusters from mass spectral fragmentation patterns, have been of value for assignment of Br and Cl in type (**A**) compounds. However, fragmentation patterns obtained from mass

spectrometry must be used with great care, since halogen atoms on the vicinal carbon atoms can rearrange on heating.[48]

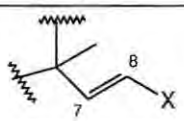
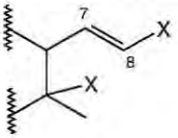
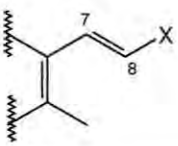
iii) *Ultraviolet and infrared spectroscopy*: Due to a lack of suitable chromophores, both ultraviolet (UV) and infrared (IR) spectra have not been of great assistance with regards to the structural elucidation of marine monoterpenes.

iv) *X-ray crystallography*: X-Ray crystallography is the only unambiguous method of structural analysis for halogenated monoterpenes, and this method has been responsible for many structural reassignments.[48] Unfortunately, the use of X-Ray crystallography is limited to compounds that are in a crystalline state and can be isolated in sufficient quantities. Consequently, this technique finds little application with isolates that are oils.

v) *Physical characteristics*: Both optical rotation and melting points have shown to vary significantly for the same compound isolated from different laboratories. This may be due to the presence of impurities in the samples.

Naylor *et al.*[10] have developed some guidelines for the structural elucidation of halogenated monoterpenes. These are summarised in Table 2.1 and provide simple parameters for rapidly determining whether a newly isolated monoterpene falls into an existing structural class, or represents a new discovery.

Table 2.1 Correlation of structure types (**A**), (**B**), (**C**), and (**D**) with diagnostic features [10]

Structure Type	Key Sub-Units	Important Spectroscopic Properties
(**A**)		Unsaturation number equal to the number of C = C (^{13}C NMR)
(**B**)		^{13}C (CH_3's δ 20 – 30) ^{1}H (CH_3's δ 0.8 – 1.4)
(**C**)		^{13}C (CH_3's δ 26 – 30; C_7 δ 134 – 140) ^{1}H (CH_3's δ 1.2 – 1.3; H_7 δ 5.9 – 6.6)
(**D**)	 	^{13}C (CH_3's δ 28 – 32; C_7 δ 130 – 133) ^{1}H (CH_3's δ 1.9; H_7 δ 5.9 – 6.1, dd) m/z = 131 (base peak) ^{13}C (CH_3's δ 18 – 19; C_7 δ 6.7 – 6.8) ^{1}H (CH_3's δ 1.7; H_7 δ 5.7 – 5.9, d) m/z = 131 (base peak)

2.2 Results and Discussion

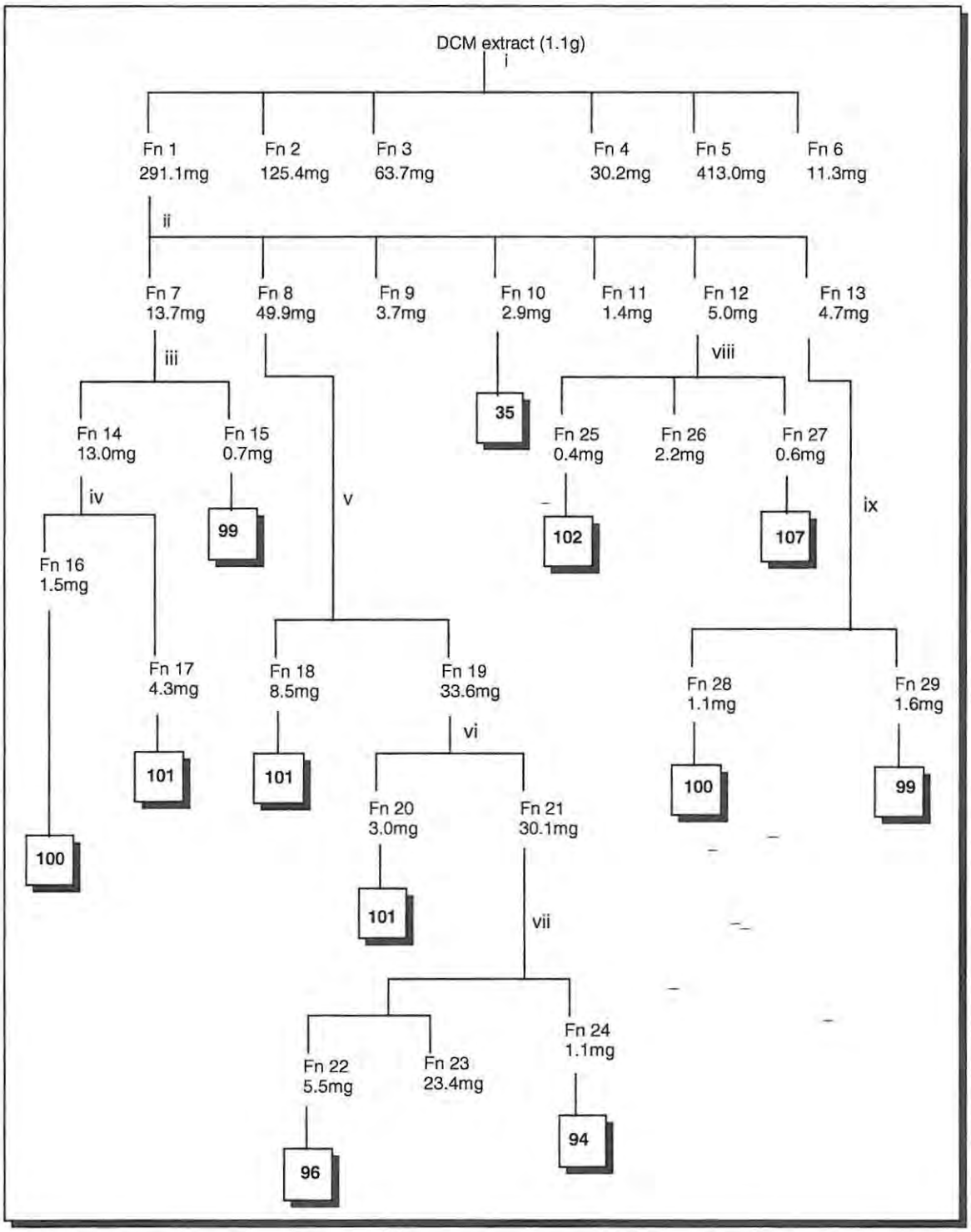
2.2.1 Collection, extraction and isolation

P. corallorhiza (Kalk Bay)

During March of 2002 specimens of *P. corallorhiza* were collected from Kalk Bay, near Cape Town and stored frozen. The frozen sample (14.3 g dry weight) was left in cold methanol (650 ml) for 6 hours. The methanolic extract was drained off, and the seaweed was extracted three times with dichloromethane (DCM) (500 ml) for 24 hours per extraction, to give 1.1 g of crude DCM extract. The ^{1}H NMR spectra of the crude methanolic and DCM extracts were found to be very similar and these extracts were thus combined.

Column chromatography of the crude DCM extract employing a hexane-EtOAc step-gradient (hexane to ethyl acetate) gave six fractions, which were grouped on the basis of TLC and ^{1}H NMR data. (Scheme 2.1). Fraction one consisted of compounds obtained from a solvent gradient varying between hexane (100%) to EtOAc-hexane (3:7). Fraction two resulted from the use of 70-50% hexane, fraction three had a hexane concentration of between 50-30% while fraction four contained hexane from 30-0%. Fractions five and six were obtained using DCM and MeOH respectively.

Fraction 1 (291.1 mg) was further fractionated by repeated normal phase HPLC (EtOAc-hexane, 5:95) to give pure compounds **35** (2.9 mg, 0.020% dry wt), **99** (2.3 mg, 0.016% dry wt), **100** (2.6 mg, 0.018% dry wt), **101** (15.8 mg, 0.111% dry wt), **96** (5.5 mg, 0.039% dry wt), **94** (1.1 mg, 0.007% dry wt), **102** (0.4 mg, 0.003% dry wt), and **107** (0.6 mg, 0.004% dry wt) (Scheme 2.1).



Scheme 2.1 Extraction and isolation scheme for fraction one of the DCM extract of *P. corallorhiza* (Kalk Bay) i) Column chromatography, solvent gradient, EtOAc-hexane; ii) Normal phase HPLC, EtOAc-hexane (5:95) iii) Normal phase HPLC, EtOAc-hexane (3:97); iv) Normal phase HPLC, hexane (100%); v) Normal phase HPLC, EtOAc-hexane (8:92); vi) Normal phase HPLC, EtOAc-hexane (3:97); vii) Normal phase HPLC, hexane (100%); viii) Normal phase HPLC, hexane (100%); ix) Normal phase HPLC, EtOAc-hexane (3:97)

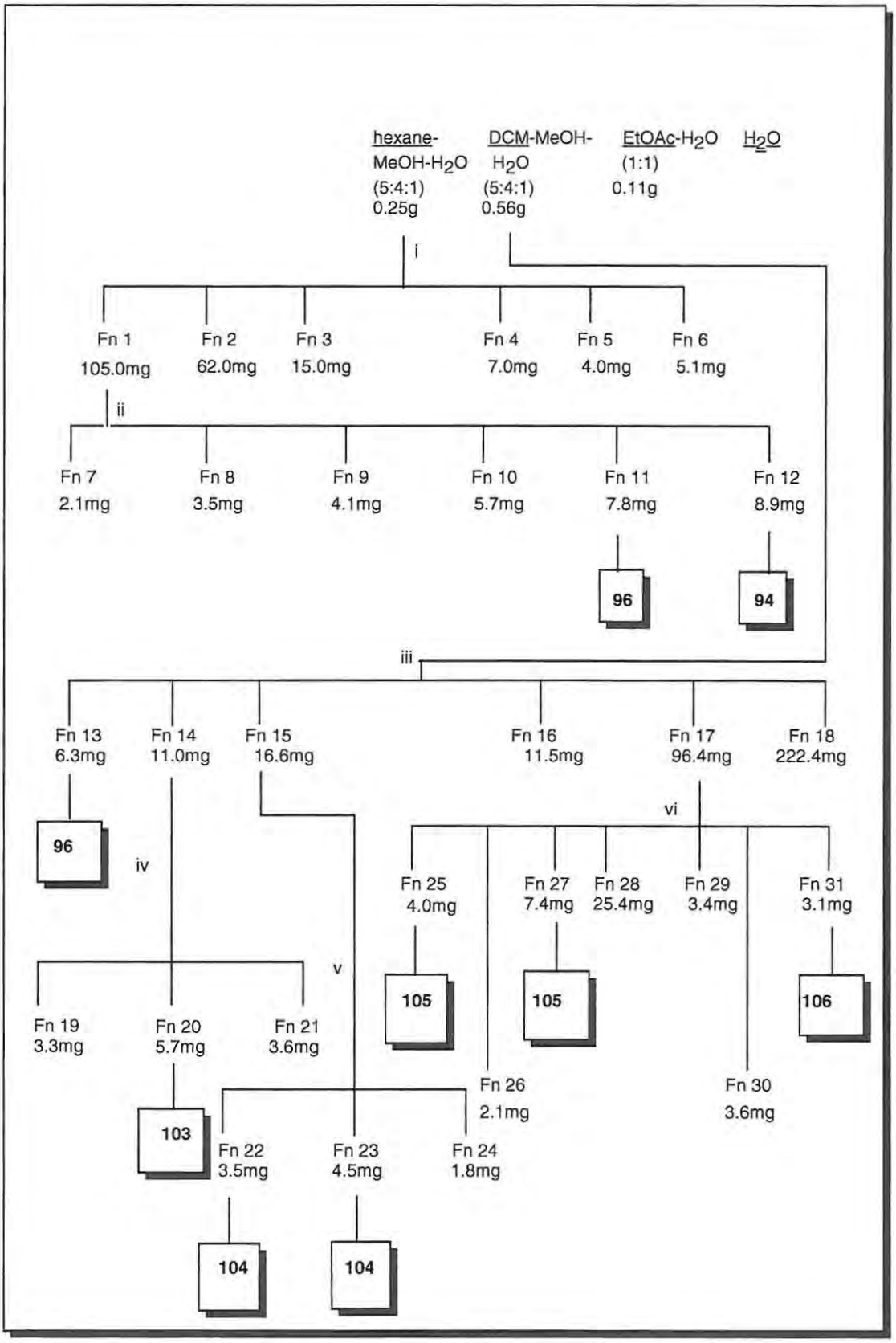
P. corallorhiza (Riet River)

P. corallorhiza was also obtained from Three Sisters (Riet River) near Port Alfred in April of 2001. The frozen sample (15.6 g dry weight) was extracted with three different solvent systems: MeOH (1 x 900 ml), DCM-MeOH (2:1 x 900 ml), and H_2O-MeOH (1:3 x 900 ml) for three days each. These three extracts were combined, and dried under reduced pressure to produce a dark, viscous oil. The oil was partitioned between hexane (50 ml) and MeOH (40 ml) containing 10 ml water. TLC and ^1H NMR analysis demonstrated that the hexane partition contained compounds of greater quantity and variation than that of the MeOH partition.

Combined hexane extracts were dried over anhydrous Na_2SO_4, filtered and concentrated to give 0.25 g of crude extract. The aqueous methanol partition was dried under vacuum and partitioned further between DCM and 10% aqueous MeOH. Combined DCM extracts were dried over Na_2SO_4, filtered and concentrated to give 0.56 g of crude extract. The MeOH was removed under reduced pressure, and the crude extract was partitioned with EtOAc-H_2O (1:1 x 3) to give 0.11 g of EtOAc extract.

The crude hexane extract was chromatographed (over silica gel) to give six fractions that were analysed by TLC and ^1H NMR spectroscopy (Scheme 2.2). Selected fraction one was purified by normal phase HPLC to give **96** (7.8 mg, 0.050% dry wt), and **94** (8.9 mg, 0.057% dry wt). ^1H NMR data of fractions two to six indicated that they contained the same compounds as in fraction one, but in much smaller quantities. These fractions were not investigated.

Column chromatography of the crude DCM extract (0.56 g) over silica gel, using a solvent gradient of EtOAc-hexane, gave six fractions that were analysed by TLC and ^1H NMR spectroscopy (Scheme 2.2). Selected fractions were purified by normal phase HPLC to give **96** (6.3 mg, 0.040 % dry wt), **103** (5.7 mg, 0.036% dry wt), **104** (8.0 mg, 0.051% dry wt), **105** (11.4 mg, 0.073% dry wt), and **106** (3.1 mg, 0.020% dry wt) respectively (Scheme 2.2).

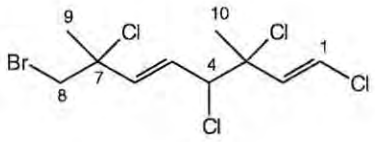


Scheme 2.2 Extraction and isolation scheme for *P. corallorhiza* (Riet River) i) Column chromatography, EtOAc-hexane (1:3); ii) Normal phase HPLC, EtOAc-hexane (3:17); iii) Column chromatography, solvent gradient, EtOAc-hexane; iv) Normal phase HPLC, EtOAc-hexane (2:98); v) Normal phase HPLC, EtOAc-hexane (1:9); vi) Normal phase HPLC, EtOAc-hexane (43:57)

2.2.2 Structure determination of isolated compounds

2.2.2.1 Structural elucidation of linear halogenated monoterpenes

*8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (**96**):*



96

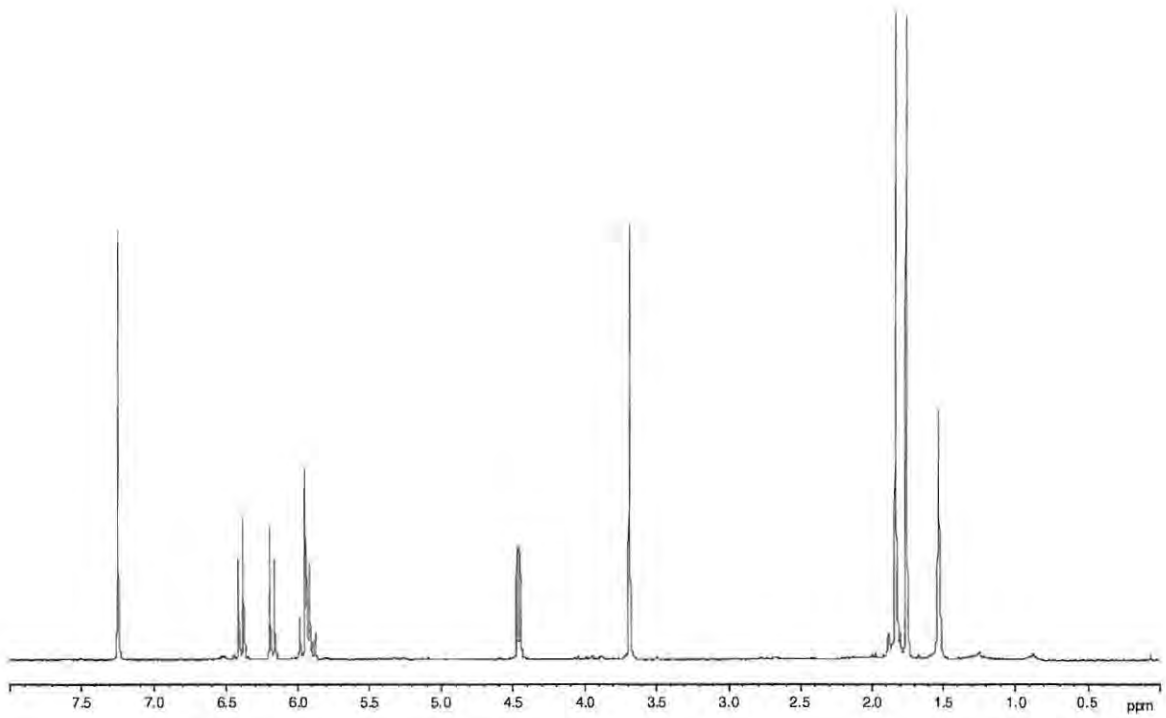


Figure 2.3 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (**96**)

The ^{1}H NMR spectrum of compound **96** (Figure 2.3) showed two coupled, deshielded methines at δ 6.40 (J = 13.2 Hz, H-1) and 6.18 (J = 13.2 Hz, H-2), a two proton multiplet at δ 5.93 (H-5, H-6) and a one proton doublet at δ 4.56 (J = 7.6 Hz, H-4). The two proton singlet at δ 3.69 (H-8) indicated a terminal bromide, while the signals at δ 1.83 (H-9) and 1.76 (H-10) were consistent with methyl groups. The ^{13}C spectrum indicated the presence of ten carbons; four were sp^2 hybridized (δ 137.9, 134.9, 127.4 and 122.4), while the other six included one methine (δ 67.9), one methylene (δ 41.5), two methyls (δ 27.3 and 25.6),

and two quaternary carbons (δ 70.4 and 66.9) (Table 2.2). The low resolution mass spectrum (LRMS) did not reveal a molecular ion, but showed a base peak ion cluster at *m/z* 123, 125, 127 characteristic of the following fragment (Figure 2.4):

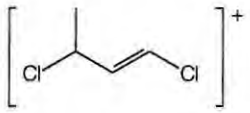


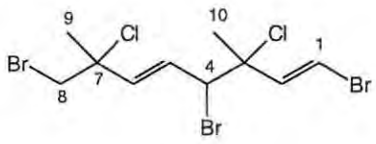
Figure 2.4 Mass spectral fragment of compound **96**

All spectroscopic data were consistent with the structure of 8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene which had been previously reported.[47]

Table 2.2 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC correlations for compound **96** in $CDCl_3$ (Literature data are included for comparison.)

Carbon no.	δ_C	δ_C [47]	δ_H, multi, J (Hz)	δ_H [47]	^{1}H-^{1}H COSY	HMBC
1	122.4,d	122.5,d	6.40,d, 13.2	6.42,d	H-2	C-3, C-2
2	134.9,d	135.0,d	6.18,d, 13.2	6.16,d	H-1	C-10, C-4, C-3, C-1
3	70.4,s	70.4,s	-	-	-	-
4	67.9,d	67.9,d	4.56,d, 7.6	4.47,dd	H-5	C-10, C-3, C-5, C-6
5	127.4,d	127.5,d	5.95,m	5.99	H-4	C-7, C-4
6	137.9,d	138.0,d	5.92,m	5.93	H-4	C-7
7	66.9,s	66.9,s	-	-	-	-
8	41.5,t	41.5,t	3.69,s	3.70,s	H-9	C-9, C-7, C-6
9	27.3,q	27.6,q	1.83,s	1.84,s	-	C-10, C-8, C-7, C-6
10	25.6,q	25.6,q	1.75,s	1.76,s	-	C-9, C-4, C-3, C-1, C-2

*1,4,8-tribromo-3,7-dichloro-3,7-dimethyl-1,5-octadiene (**94**):*



94

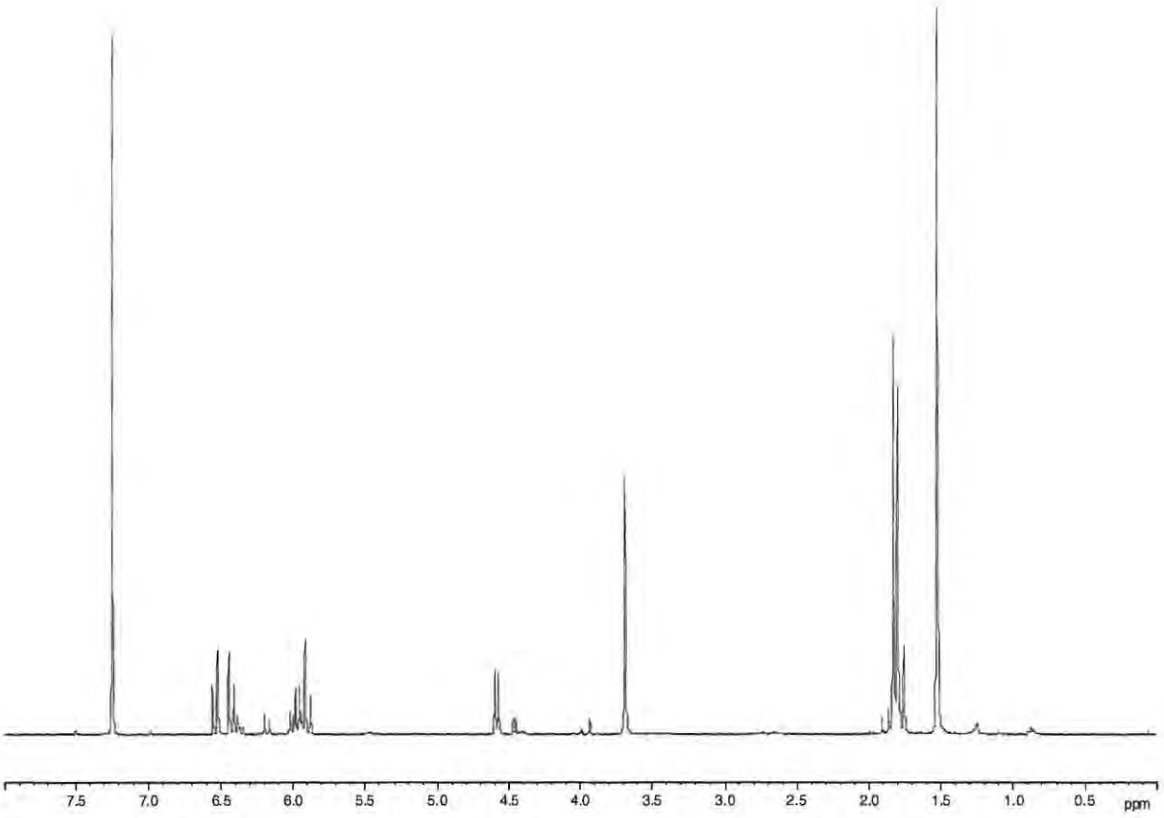


Figure 2.5 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 1,4,8-tribromo-3,7-dichloro-3,7-dimethyl-1,5-octadiene (**94**)

The ^{1}H NMR spectrum of compound **94** (Figure 2.5) showed some similarities to that of **96**. Two coupled, deshielded methines at δ 6.54 (J = 13.6 Hz, H-1) and 6.43 (J = 13.6 Hz, H-2) were representative of a vinylic bromide, while the one proton multiplet at δ 5.97 (H-5), as well as 5.92 (H-6), and the doublet at δ 4.57 (J = 9.4 Hz, H-4) indicated the presence of an allylic bromide. The two proton singlet at δ 3.69 (H-8) was consistent with a terminal bromide moiety (as seen previously), while proton peaks at δ 1.83 (H-9) and 1.80 (H-10) indicated the presence of two methyl groups. The ^{13}C spectrum revealed the presence of ten carbons of which four were sp^2 hybridized (δ 138.9, 137.3, 128.1, and

110.0); one methine (δ 60.0), one methylene (δ 41.5), two methyls (δ 27.2 and 26.4), and two quaternary carbons (δ 71.1 and 66.9) were also observed (Table 2.3). The low resolution mass spectrum of **94** indicated the presence of a large halogen cluster at *m/z* 167, 169, 171 (Figure 2.6) which supported the proposal of Br at C-1.

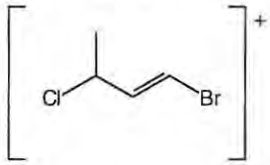


Figure 2.6 Mass spectral fragment of compound **94**

After association of all protons with their directly bonded carbon atoms by means of a ^{1}H-^{13}C one bond shift-correlated 2D NMR (HMQC) experiment, it was possible to use a ^{1}H-^{1}H COSY (Table 2.3) spectrum to discern the fragments of three possible spin systems: CH_3CX-CH=CHX at δ 1.80, 6.43, 6.54 (Figure 2.7a); CX-CH= at δ 4.57 and 5.97, and CH_2X-$C(CH_3)X$ at δ 3.69 and 1.83 (Figure 2.7b). The methine proton at δ 6.43 (H-2) was directly coupled to the methine proton at δ 6.54 (H-1) and the methyl protons at 1.80 (H-10). The proton at δ 5.97 (H-5) showed coupling to the methine proton at δ 4.57 (H-4), while the methyl protons at δ 1.83 (H-9) correlated with the methylene protons at δ 3.69 (H-8).

(a) (b)

Figure 2.7 Selected COSY-90 correlations for compound **94**

Correlations observed in the ^{1}H-^{13}C multiple-bond shift–correlated 2D NMR (HMBC) spectrum (Table 2.3) confirmed the above assignments and allowed these fragments to be linked to generate structure **94** as CH_2X-$C(CH_3)X$-CH=CH-CHX-$C(CH_3)X$-CH=CHX (Figure 2.8).

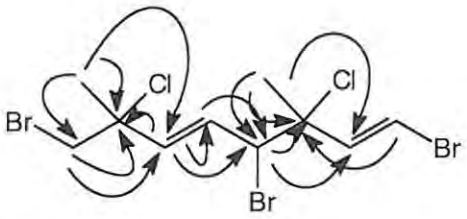


Figure 2.8 HMBC correlations for compound **94**

In a comparison of the structure of **94** to that of **96** it was noted that the resonance of the terminal olefinic carbon at C-1, and the resonance at C-4 both experienced an upfield shift from δ 122.4 to 110.0, and from δ 67.9 to 60.1 respectively. These spectral differences are consistent with the proposal of a Br at both C-1 and C-4. Halogens at C-3, C-7, and C-8, for compound **94**, displayed similar carbon shifts to that of compound **96**. This implied that compound **94** has the same halogen distribution as compound **96** for these three positions.

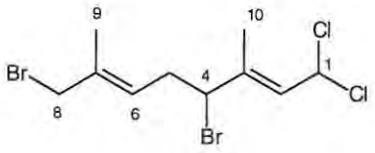
Compound **94** has not been previously reported from a *Plocamium* spp., but has been isolated from the sea hare *Aplysia californica*.[54] This compound was later shown, by means of X-ray diffraction and spectral differences,[47] to have a Br at C-4, and not a Cl as previously suggested.

In this work, it was shown that compound **94** is an actual metabolite of the *Plocamium* genus. Naylor *et al.*[10] have argued in favour of certain biosynthetic transformations that the sea hare may perform to produce **94** *in vivo*. This was based on labelling studies that have shown that *A. californica* can carry out *in vivo* synthetic modifications on halogenated sesquiterpenes.[55] Further support for this, "sea hare biosynthetic theory," was based on the isolation of various polyhalogenated monoterpenes that contained acetate groups (which at the time, were exclusive to sea hares). In over two hundred samples of *Plocamium* collected in the vicinity of the sea hare, compound **94** has never been isolated.[10] However, Faulkner[54] pointed out that the algal source of the metabolites had probably not yet been discovered, our results indicate that it is just as plausible that *A. californica* could obtain **94** from its diet.

Table 2.3 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **94** in $CDCl_3$ (Literature data are included for comparison.)

Carbon no.	δ_C	δ_C [47]	δ_H, multi, J (Hz)	δ_H [47]	^{1}H-^{1}H COSY	HMBC
1	110.0,d	110.0,d	6.54,d, 13.6	6.58,d	-	C-3
2	138.9,d	138.9,d	6.43,d, 13.6	6.40,d	H-1, H-10	C-10, C-4, C-3, C-1
3	71.1,s	71.2,s	-	-	-	-
4	60.0,d	60.1,d	4.57,d, 9.4	4.54,dd	-	C-10, C-3, C-5, C-6
5	128.1,d	128.1,d	5.97,m	6.02	H-4	C-7, C-6
6	137.3,d	137.4,d	5.92,m	6.89	-	C-8, C-4, C-7, C-5
7	66.9,s	66.9,s	-	-	-	-
8	41.5,t	41.6,t	3.69,s	3.70,s	H-9	C-9, C-7, C-6
9	27.2,q	27.8,q	1.83,s	1.82,s	-	C-8, C-7, C-6
10	26.4,q	26.3,q	1.80,s	1.79,s	-	C-4, C-3, C-2

*4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene (**99**):*



99

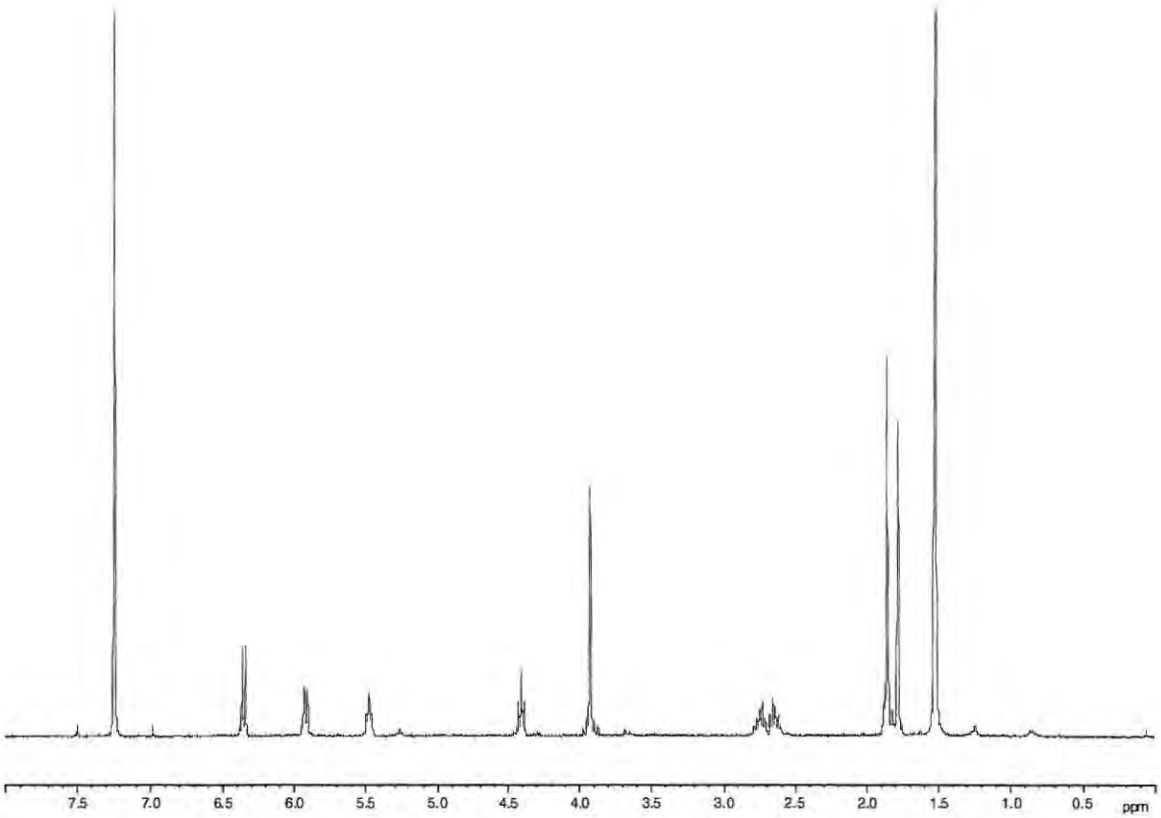


Figure 2.9 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene (**99**)

The HREIMS mass spectrum of **99** provided a molecular formula of $C_{10}H_{14}Br_2Cl_2$. The ^{1}H NMR spectrum of compound **99** (Figure 2.9) was significantly different to those of compounds **94** and **96** and showed three, deshielded methines at δ 6.35 (J = 9.4 Hz, H-1), 5.93 (J = 9.4 Hz, H-2) and δ 5.48 (J = 6.8, 6.6 Hz, H-6). Further signals included a two proton singlet at δ 3.93 (H-8) indicating a terminal bromide, a methine proton at δ 4.41 (J = 7.6 Hz, H-4), a two proton multiplet at δ 2.67 (H-5) and two methyl groups at δ 1.78 and 1.88 respectively. The ^{13}C spectrum indicated the presence of only eight of the expected ten carbons, of which four were methine carbons (δ 128.3, 125.8, 66.6 and 56.8), two

methylene carbons (δ 40.2 and 35.6), and two vinylic methyls (δ 15.2 and 12.9) [56] (Table 2.4). A further two carbon signals at δ 138.3 and 135.7 were deduced from the HMBC spectrum (Figure 2.12).

By means of HMQC and ^{1}H-^{1}H COSY measurements, it was possible to discern the following two discrete spin systems $CH_3C{=}CH{-}CHX_2$ at δ 1.88, 5.93, 6.35 (Figure 2.10a); and $CH_2X{-}C(CH_3){=}CH{-}CH_2{-}CHX$ at δ 3.93, 1.78, 5.48, 2.67, and 4.41 (Figure 2.10b). The vinylic methine proton at δ 5.93 (H-2) showed correlations to methyl protons at δ 1.88 (H-10) as well as a methine proton at δ 6.35 (H-1). The proton at δ 5.48 (H-6) showed direct proton-proton coupling with two methylene groups at δ 3.93 (H-8) and 2.67 (H-5), as well as a methyl group at δ 1.78 (H-9). These two fragments (Figure 2.10a, Figure 2.10b) were then linked together by the COSY correlation between δ 4.41 (H-4) and 2.67 (H-5).

(a) (b)

Figure 2.10 Selected COSY-90 correlations for compound **99**

The HMBC data (Figures 2.11 and 2.12, Table 2.4) confirmed the above structural assignment.

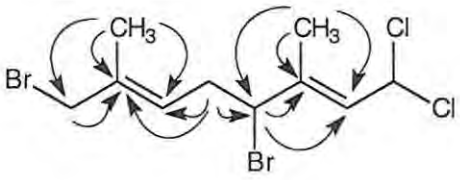


Figure 2.11 HMBC correlations for compound **99**

The four halogen atoms were positioned by ^{13}C NMR chemical shift comparisons made between compound **99**, and data from compounds **20**, [17] **29**, [18] and **94**. [47] A dichloride substituent attached to a double bond has a typical ^{13}C chemical shift of δ 65.5 [17] which is consistent with the observed chemical shift of C-1 (δ 66.6) in **99**. Similarly, the chemical

differed significantly from compound **29** [18] which contained a similar terminal fragment. Compound **29** has a chloride moiety at C-8 (δ 43.9), which led to the proposal of a bromine substituent at C-8 (δ 40.2).

Since a Z-configuration requires an olefinic methyl chemical shift of δ 20.9,[57] the E-configuration for the double bonds at C-2 and C-6 were proposed from the ^{13}C shifts of the olefinic methyls of δ 15.2 (C-9) and 12.9 (C-10).

* The stereochemistry of new compounds was not assigned.

All spectroscopic data of **99** were consistent with the proposed structure of (2E, 7E) 4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene.

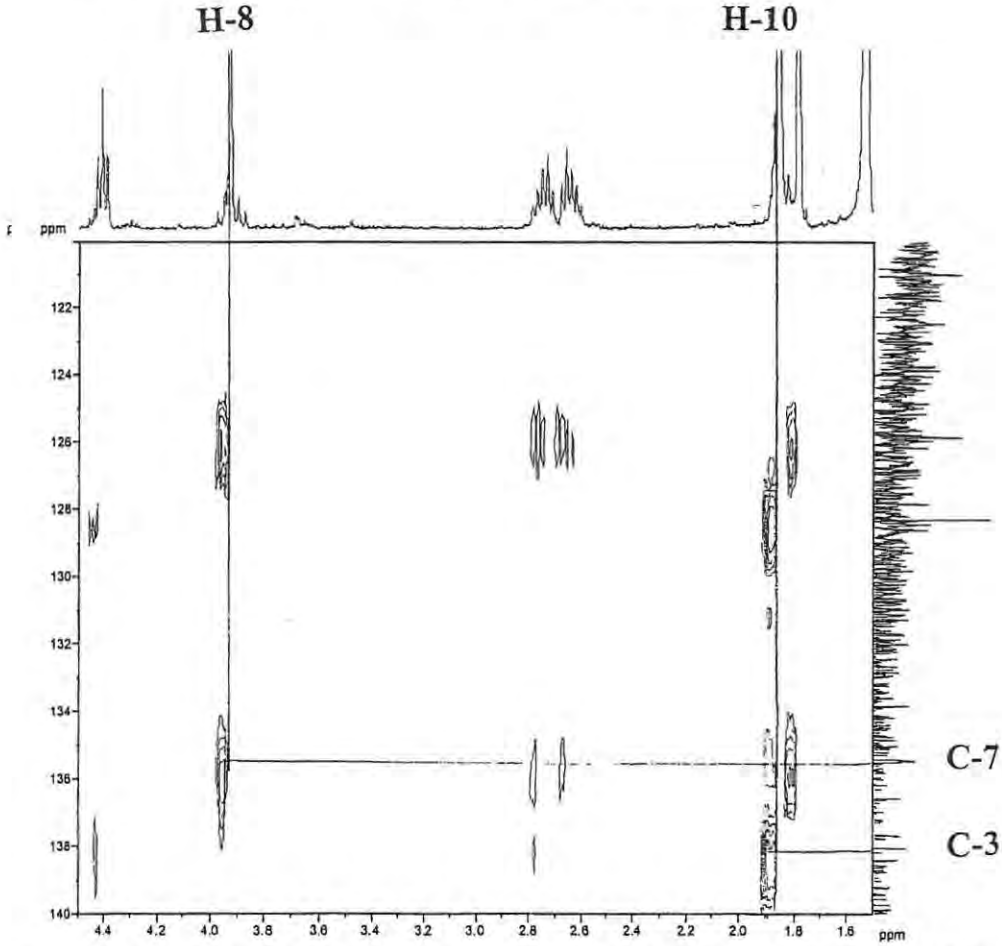
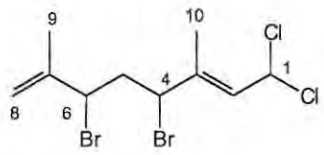


Figure 2.12 HMBC spectrum of 4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene ($CDCl_3$, 400 MHz) indicating two quaternary carbons at δ 138.3 and 135.7

Table 2.4 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **99** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	66.6, d	6.35, d, 9.4	H-2	-
2	128.3, d	5.93, d, 9.4	H-10	-
3	138.3, s	-	-	-
4	56.8, d	4.41, t, 7.6	H-5	C-10, C-2, C-3
5	35.6, t	2.67, m	H-4, H-6, H-9	C-4, C-6, C-7
6	125.8, d	5.48, t, 6.8, 6.6	H-5, H-9 (w), H-8	-
7	135.7, s	-	-	-
8	40.2, t	3.93, s	H-6 (w)	C-9, C-6, C-7
9	15.2, q	1.78, s	H-6, H-5	C-8, C-6, C-7
10	12.9, q	1.88, s	H-2	C-4, C-2, C-3

4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene ***(100)****:*



100

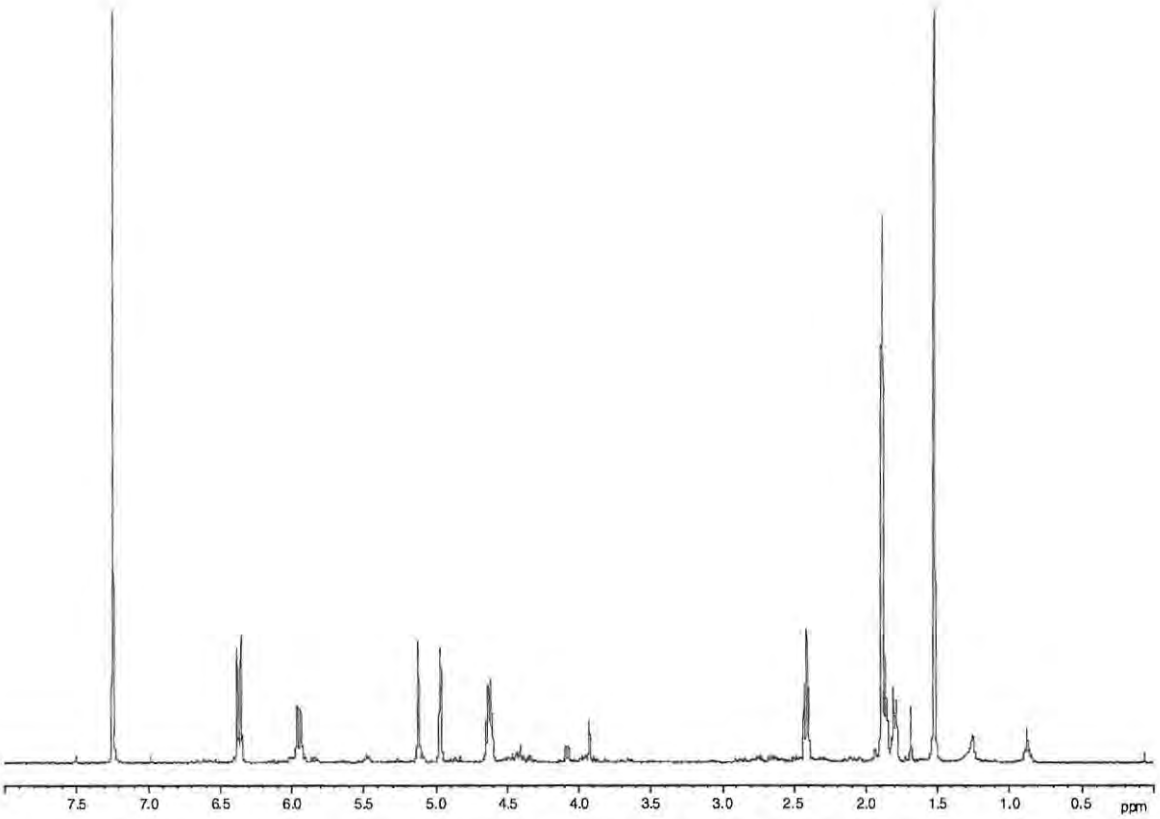


Figure 2.13 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene (**100**)

A molecular formula of $C_{10}H_{14}Br_2Cl_2$ was deduced from HREIMS data. The ^{1}H NMR spectrum of compound **100** (Figure 2.13) showed the same two mutually coupled doublets at δ 6.36 (J = 9.4 Hz, H-1) and 5.95 (J = 9.4 Hz, H-2) as seen in **99** thus suggesting a similar dichloro substituent attached to a double bond. Other signals in the ^{1}H NMR spectrum included two singlets at δ 5.12 (H-8a) and 4.97 (H-8b), a two proton multiplet at δ 4.62 (H-4, H-6), a methylene at δ 2.41 (t) (J = 7.1 Hz, H-5) and a six proton singlet at δ 1.89 (H-9, H-10). At first glance the ^{13}C spectrum indicated the presence of only nine carbons of which two quaternary carbons (δ 143.5 and 137.4), three methine groups (δ

128.6, 66.5, 56.0), two methylene groups (δ 115.2, 43.1) and two methyls (δ 18.3 and 13.2) were present (Table 2.5). However, on closer inspection it was observed that the signal at δ 56.0 was split into two (Figure 2.16). This was confirmed by DEPT-135 data and an HMQC correlation of this signal (δ 56.0) to a two proton multiplet at δ 4.62.

The number of overlapping signals in the ^{1}H and ^{13}C spectra of **100** complicated its structural elucidation, but a comparison of the ^{1}H and ^{13}C chemical shift data of **99** and **100** immediately suggested that these two compounds maybe structurally related. A combination of COSY and HMBC data confirmed that **100** contained that same vinylic dichloride moiety, and allylic bromide substituent as in **99**. Thus, the methine proton at δ 5.95 (H-2) showed coupling to both the methyl protons at δ 1.89 (H-10), and the methine proton at δ 6.36 (H-1). Methylene protons at δ 2.41 (H-5) demonstrated coupling to protons at δ 4.62 (H-4, H-6); while the methyl protons at δ 1.89 (H-9) correlated to the terminal double bond proton at δ 5.12 (Figure 2.14, Table 2.5). The remaining structural characteristics of **100** were identified as follows.

^{13}C, DEPT-135 and HMQC NMR data indicated that the signal at δ 115.2 (C-8) was a terminal methylene carbon. This terminal methylene (C-8) showed COSY and HMBC correlations to the methyl group at δ 1.89 / 18.3 (C-9). Further HMBC correlations from C-8a (δ 5.12) to carbon signals at δ 143.5 (C-7) and 56.0 (C-6), and from the latter to a methylene signal at δ 56.0 (C-4) completed the gross structural assignment of **100** (Figure 2.15, Table 2.5). The symmetrical fragment from C-3 to C-7 accounted for the overlapping signals.

(a) (b)

Figure 2.14 Selected COSY-90 correlations for compound **100**

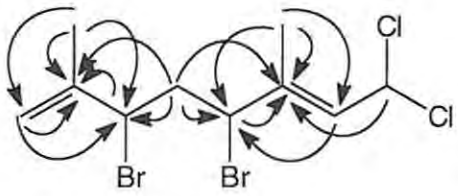


Figure 2.15 HMBC correlations for compound **100**

With the exception of the dichloro group at C-1, which has only previously been reported in octatriene skeletons, compound **100** has the same structure to that of Preplocamene A (**84**).[41] The halogen substituents at C-4 and C-6 are therefore consistent with bromines at both positions.[10]

Naylor *et. al*[10] suggested that for compounds of this structural type (**84**), C-4 (δ 56.2) has an upfield shift over C-6 (δ 57.6). The same rationale was applied when determining the C-4 and C-6 carbon shifts of compound **100**. The E-configuration of the double bond between C-2 and C-3, followed from the ^{13}C chemical shift of the olefinic methyl at δ 13.2 (C-10).[57]

Compound **100** is another new compound, and is thus assigned as 4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene.

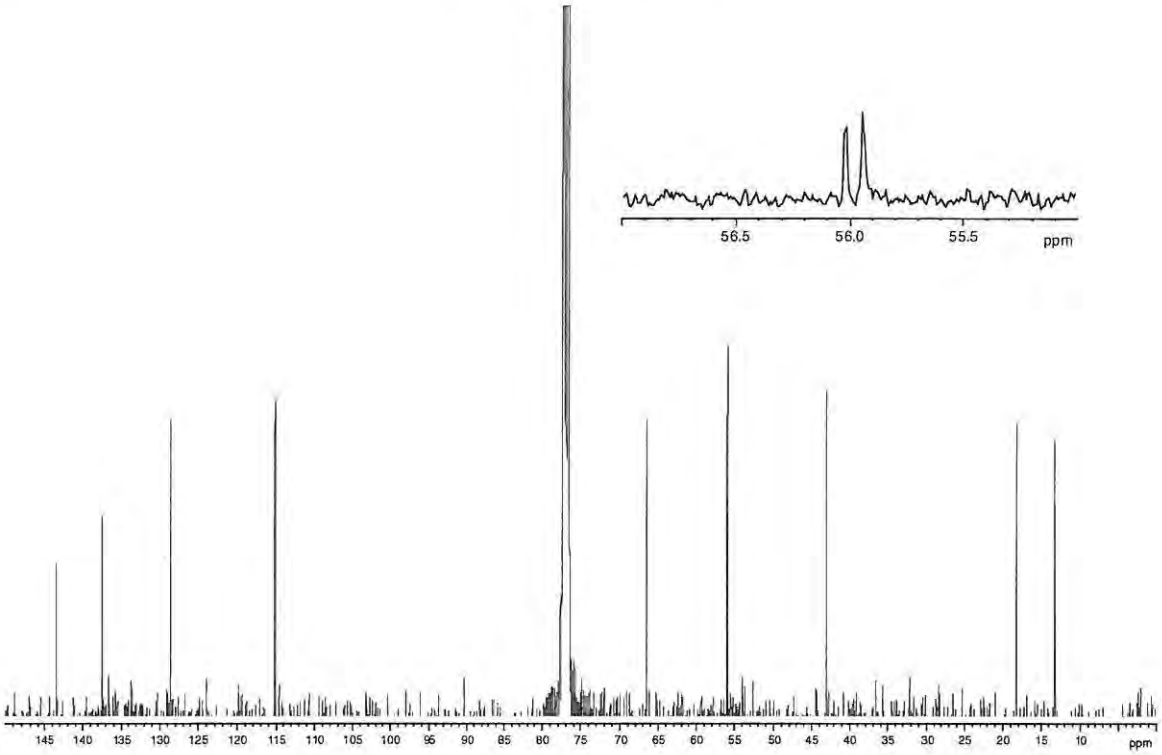


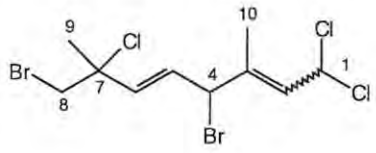
Figure 2.16 ^{13}C ($CDCl_3$, 100 MHz) NMR spectra of compound **100**

Table 2.5 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **100** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	66.5, d	6.36, d, 9.4	H-2	C-3
2	128.6, d	5.95, d, 9.4	H-1, H-10 (w)	C-10, C-4
3	137.4, s	-	-	-
4	55.95*, d	4.62, m	H-5	C-10, C-5, C-6, C-3
5	43.1, t	2.41, t, 7.1	H-4, H-6	C-4, C-6, C-3, C-7
6	56.03*, d	4.62, m	H-5	C-9, C-5, C-8, C-7
7	143.5, s	-	-	-
8a 8b	115.2, t	5.12, s 4.97, s	H-9	C-9, C-6, C-7 C-9
9	18.3, q	1.89, s*	H-8	C-6, C-8, C-7
10	13.2, q	1.89, s*	H-2	C-4, C-2, C-3

*The carbon shifts at δ 56.03 and 55.95, and the proton shifts at δ 1.89 and 1.89 maybe interchangeable

*4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (**101**):*



101

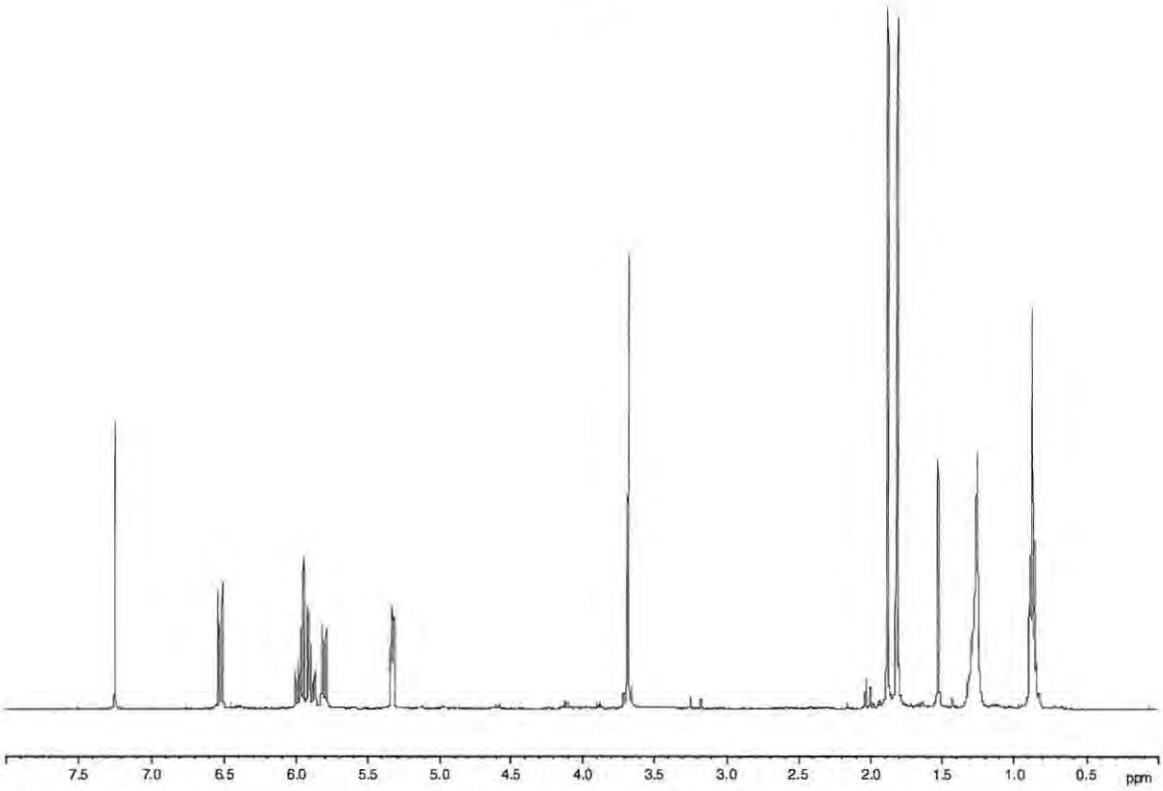


Figure 2.17 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (**101**)

Compound **101** did not give a molecular ion peak in the HREIMS spectrum; however, a molecular formula of $C_{10}H_{13}Br_2Cl_3$ was deduced from HREIMS, ^{13}C and DEPT data. The ^{1}H NMR spectrum of compound **101** (Figure 2.17) showed the same two coupled, deshielded methines at δ 6.52 (J = 9.7, 1.3 Hz, H-1) and 5.80 (J = 9.7, 1.3 Hz, H-2) which were encountered in compounds **100** and **99**, and are consistent with a dichloro substituent attached to a double bond. A two proton multiplet at δ 5.97 (H-5) and 5.91 (H-6), together with a multiplet at δ 5.33 (H-4) were also observed, while a two proton singlet at δ 3.68 (H-8) indicated a terminal bromide. Two methyl groups were indicated by signals at δ 1.88 (H-10) and 1.81 (H-9). Ten carbons were observed in the ^{13}C spectrum, of which four were sp^2 hybridized (δ 136.7, 135.8, 129.1, and 128.6), this suggested the presence of two

double bonds. Additional signals in the ^{13}C spectrum included two methine groups (δ 65.3 and 56.8), one methylene (δ 41.7), two methyls (δ 27.7 and 18.6) and a quaternary carbon (δ 67.0) (Table 2.6). A combination of HMQC, COSY and HMBC data were used to determine the overall structure of compound **101**. All protons were assigned to their associated carbons by means of an HMQC experiment. This was especially important in the highly congested region around δ 5.97 where a number of signals were overlapped. An expansion of this area (Figure 2.19) clearly showed coupling of the proton multiplet at δ 5.91 (H-6) to the carbon at δ 135.8 (C-6), the proton multiplet at δ 5.97 (H-5) to the carbon at δ 128.6 (C-5), and the double doublet at δ 5.80 (H-2) to the carbon at δ 129.1 (C-2).
The methyl singlet at δ 1.81 (H-9) showed HMBC correlations to carbons at δ 41.7 (C-8), 67.0 (C-7) and 135.8 (C-6). The latter signal showed vicinal coupling, in the COSY spectrum, to the signal at δ 5.97 (H-5), as well as HMBC correlations to signals at δ 67.0 (C-7) and 56.8 (C-4). The vinylic methyl a δ 1.88 (H-10) showed HMBC correlations to signals at δ 56.8 (C-4), 136.7 (C-3), 129.1 (C-2) and 65.3 (C-1). An expansion of the HMBC spectrum is shown in Figure 2.20. The above data allowed the following fragment to be constructed (Figure 2.18):

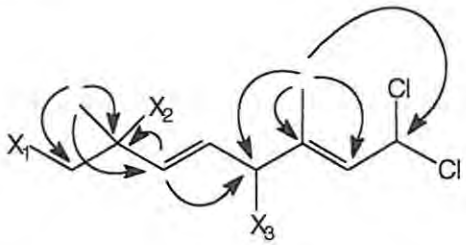


Figure 2.18 Selected HMBC correlations for compound **101**

The nature of the halogen substituents was determined by an analysis of the ^{13}C chemical shifts and comparison to those of known compounds. A chemical shift of δ 65.3 (C-1) suggested a dichloro substituent as seen before. Compound **101** shows an identical fragment from C-5 to C-9 as compound **94,** this information, in combination with a high coupling constant of 15.5 Hz for C-6 and 15.3 Hz for C-5 was used to determine the geometry of the C5-C6 double bond. Very similar chemical shifts in this region also suggested a similar halogenation pattern. This therefore implied the presence of a bromine substituent at δ 41.7 (C-8), and a chlorine substituent at δ 67.0 (C-7). The ^{13}C chemical shift of C-4 (δ 56.8) corresponds to a Br substituent at this position

(a chemical shift of ~ δ 63.0 suggests a Cl moiety).[41] Compared to compounds **99** and **100**, the carbon shift at C-10 has a relatively downfield value for a methyl group coupled to a quaternary carbon. This may suggest an altered stereochemistry around the C-2 double bond. Compound **101** is a third new compound and is known as 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene.

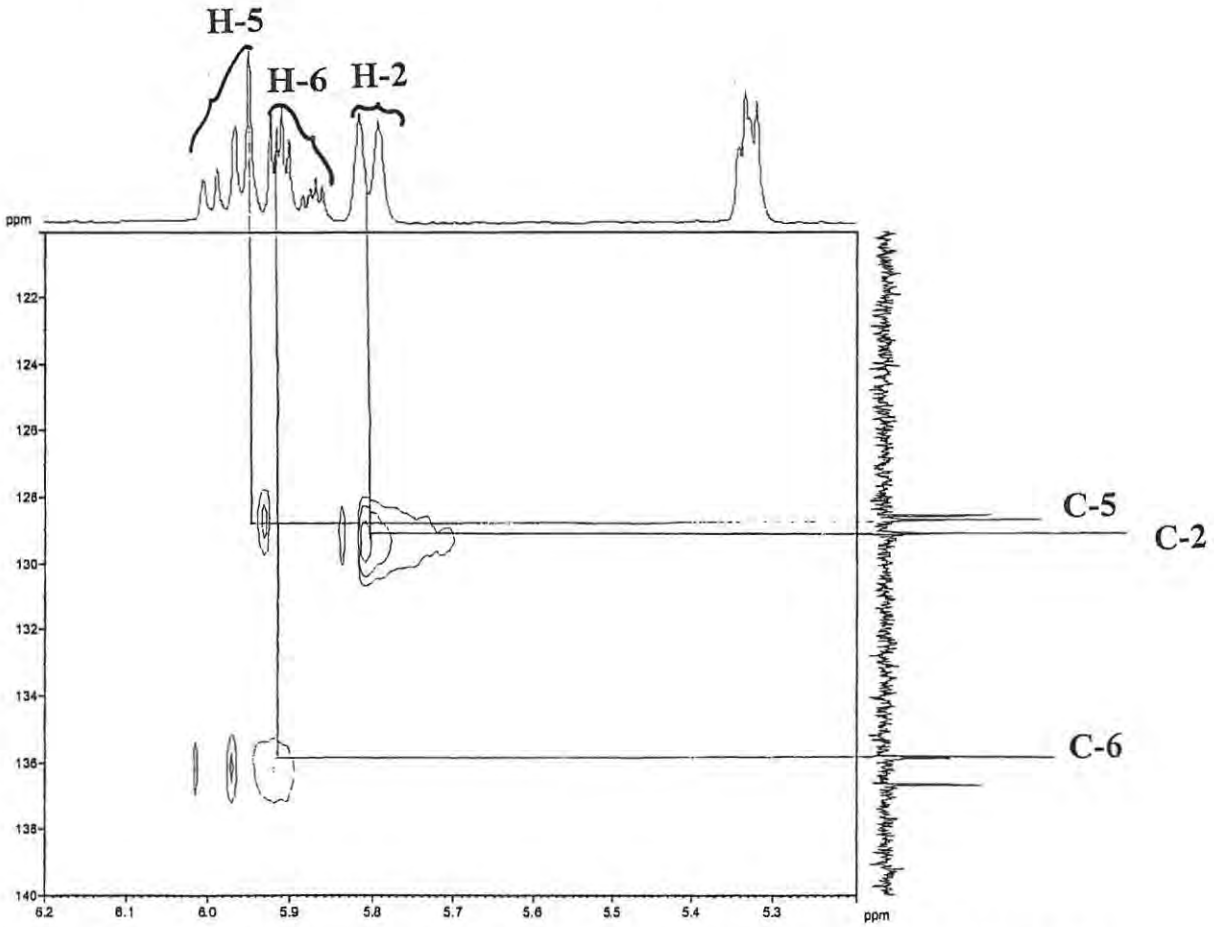


Figure 2.19 HMQC spectrum ($CDCl_3$, 400 MHz) of 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene **(101)**

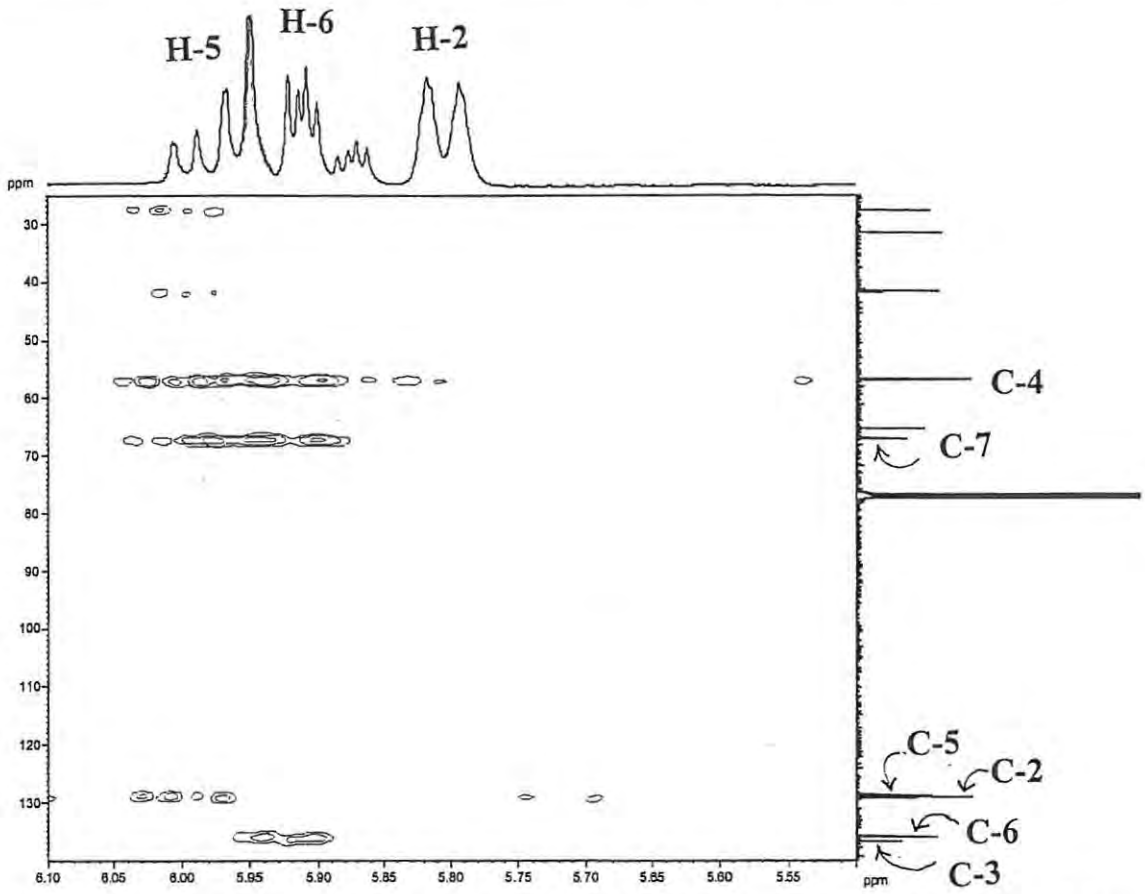
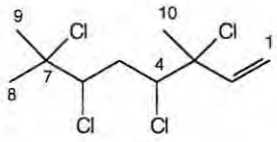


Figure 2.20 HMBC spectrum ($CDCl_3$, 400 MHz) of 4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (**101**)

Table 2.6 ^1H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **101** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^1H-^1H COSY	HMBC
1	65.3, d	6.52, dd, 9.7, 1.3	H-2, H-10 (w)	C-3
2	129.1, d	5.80, dd, 9.7, 1.3	H-1, H-10 (w)	C-10
3	136.7, s	-	-	-
4	56.8, d	5.33, m	H-5	C-10, C-5, C-3
5	128.6, d	5.97, m	H-4, H-6	C-4, C-7, C-6
6	135.8, d	5.91, m	H-5	C-4, C-7
7	67.0, s	-	-	-
8	41.7, t	3.68, s	H-9	C-9, C-7, C-6
9	27.7, q	1.81, s	H-8	C-8, C-7, C-6
10	18.6, q	1.88, s	H-2, H-1 (w)	C-4, C-2, C-3

*3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**102**):*



102

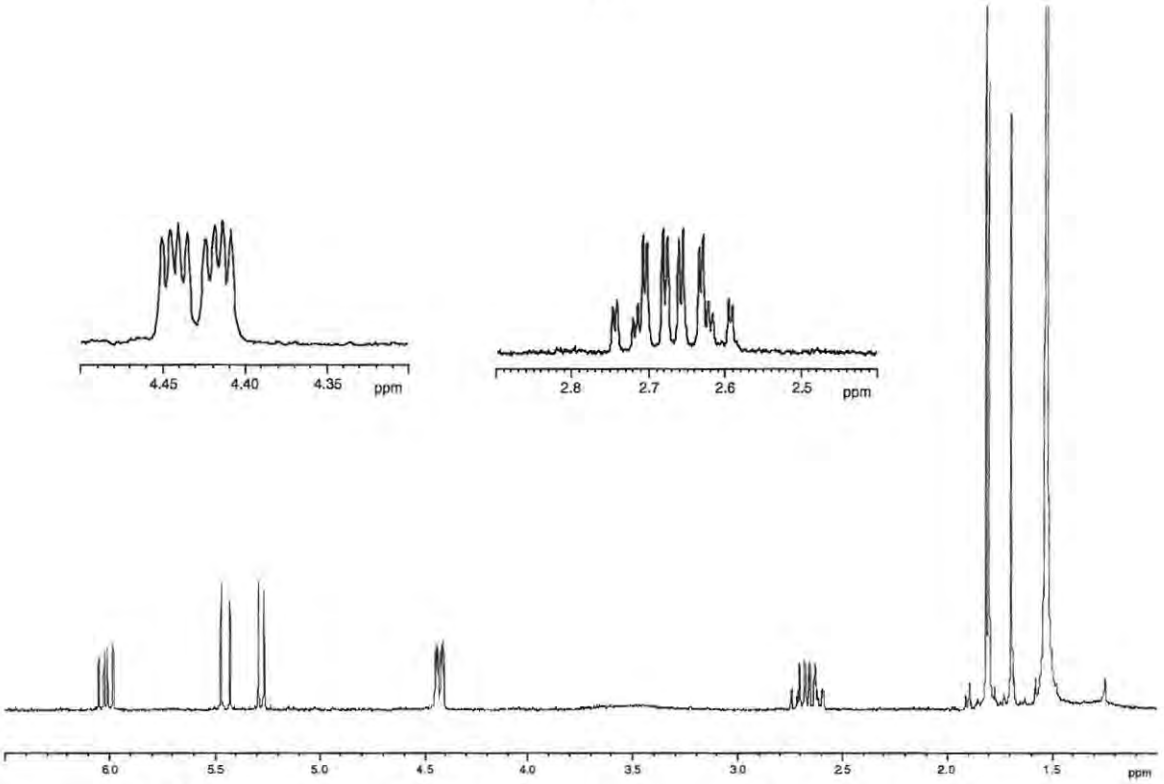


Figure 2.21 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**102**)

Compound **102** was a minor metabolite in the crude extract (~0.5 mg), which made its structure elucidation quite challenging. In addition, the HREIMS spectrum for this compound did not show a molecular ion peak (M^+) or (M^+-X). These data are crucial to the unambiguous assignment of the structure of **102**. Nevertheless, in this section the structure elucidation of **102** based on the NMR data is reported. The ^{1}H NMR spectrum of compound **102** (Figure 2.21) showed deshielded resonances at δ 6.02 (J = 16.8, 10.4 Hz, H-2), 5.45 (J = 16.8 Hz, H-1a) and 5.28 (J =10.4 Hz, H-1b) (indicative of a terminal double bond) as well as two proton signals at δ 4.43 (J = 4.0, 3.8 Hz, H-4, H-6) and δ 2.67 (J = 2.8, 2.0 Hz, H-5) and three methyl groups are represented by shifts at δ 1.82 (H-10), 1.81 (H-8) and 1.70 (H-9).

Although the ^{13}C NMR and DEPT-135 spectra were very weak, due to the limited sample available, a combination of HMBC and HMQC data, allowed the identification of ten carbons. These included two sp^2 hybridized carbons (δ 140.7 and 116.2), two methine groups (δ 63.4 and 61.8), one methylene group (δ 39.2), three methyl groups (δ 32.8, 28.3 and 26.4) and two quaternary carbons (δ 71.9 and 68.9) (Table 2.7). The LRMS data did not reveal a molecular ion but showed a prominent ion cluster at *m/z* 89, 91 (Figure 2.22).[16]

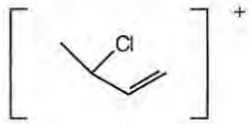


Figure 2.22 Mass spectral fragment of compound **102**

Analysis of COSY and HMBC data (Figures 2.23, 2.24, and 2.25, Table 2.7) were used to determine the structure of **102**. Key HMBC correlations from the methyl group at δ 1.70 (H-9) to a methyl at δ 32.8 (C-8), a quaternary carbon at δ 68.9 (C-7) and a deshielded methine at δ 63.4 (C-6), and from the latter to a methylene at δ 39.2 (C-5) allowed the assignment of the fragment from C-5 to C-9. A second fragment followed from HMBC correlations from the methyl signal at δ 26.4 (C-10) to a deshielded methine at δ 61.8 (C-4), a quaternary carbon at δ 71.9 (C-3) and the vinyl carbon at δ 140.7 (C-2). The structural assignment was confirmed by COSY correlations (Figure 2.23, Table 2.7) observed for compound **102**.

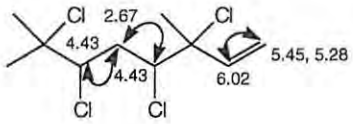


Figure 2.23 Selected COSY-90 correlations for compound **102**

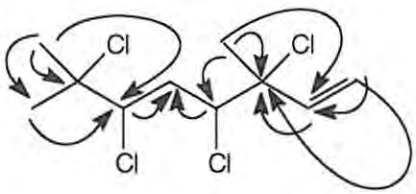


Figure 2.24 HMBC correlations for compound **102**

The chemical shifts of C-3 (δ 71.9) and C-4 (δ 61.8) together with the observation of a mass fragment at *m/z* 89 / 91 are consistent with chlorine atoms at these positions.[16] Similarly, the ^{13}C chemical shifts of C-6 and C-7 are in agreement with published values for chlorine atoms at these positions.[14, 15]

The unusual 8,9,10-trimethyl substitution pattern in **102** is also found in compounds **16**, **45**, **46**, **48** and **50** while the monosubstituted alkene has also been reported in compounds **18-21**, **74** and **87**.

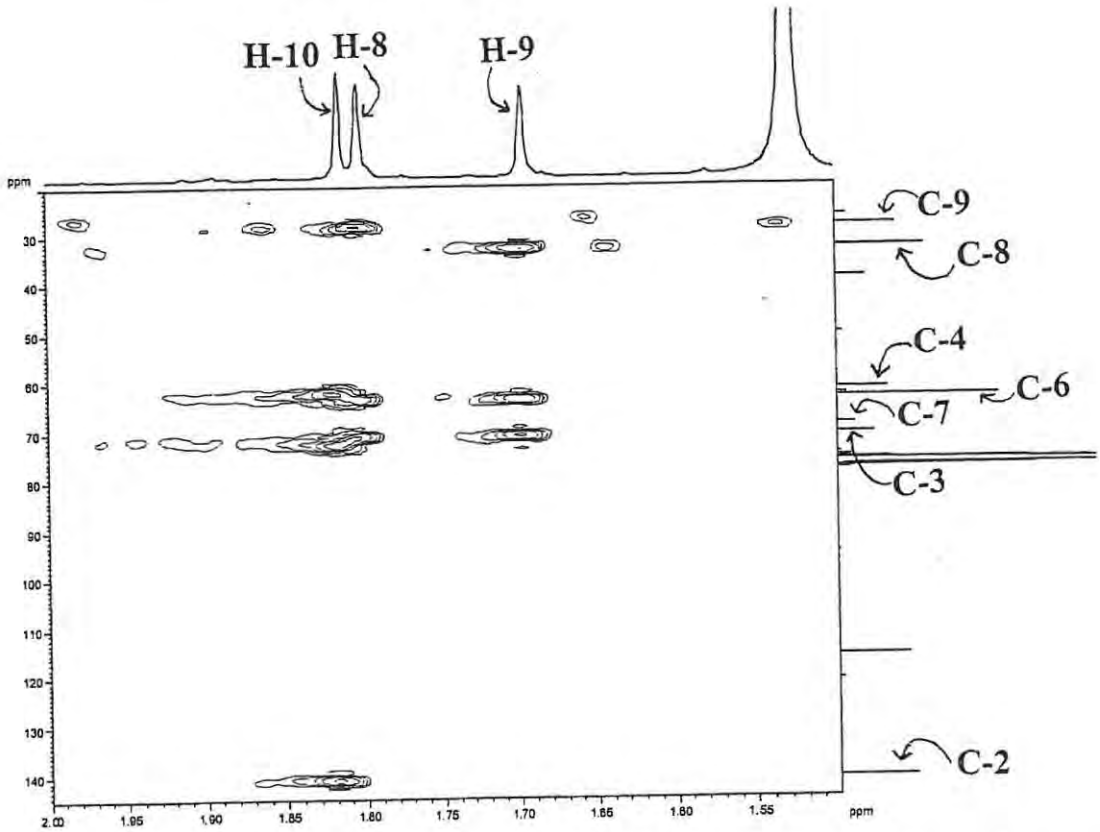


Figure 2.25 Expanded HMBC spectrum of 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene ($CDCl_3$, 400 MHz)

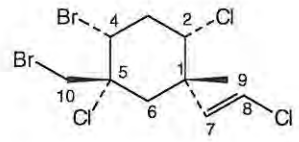
Table 2.7 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **102** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1a	116.2, t	5.45, d 16.8 (trans)	H-2	C-3, C-2
1b		5.28, d 10.4 (cis)	H-2	
2	140.7, d	6.02, dd, 16.8, 10.4	H-1a, H-1b	C-3
3	71.9, s	-	-	-
4	61.8, d	4.43, dd, 4.0, 3.8*	H-5	C-5
5	39.2, t	2.67, ddd, 2.3, 2.0	-	-
6	63.4, d	4.43, dd, 4.0, 3.8*	H-5	C-5
7	68.9, s	-	-	-
8	32.8, q	1.81, s	-	C-9, C-6, C-7
9	28.3, q	1.70, s	-	C-8, C-6, C-7
10	26.4, q	1.82, s	-	C-4, C-3, C-2

* Carbon shift assignments maybe interchangeable

2.2.2.2 Structural elucidation of cyclic halogenated monoterpenes

4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (35):



35

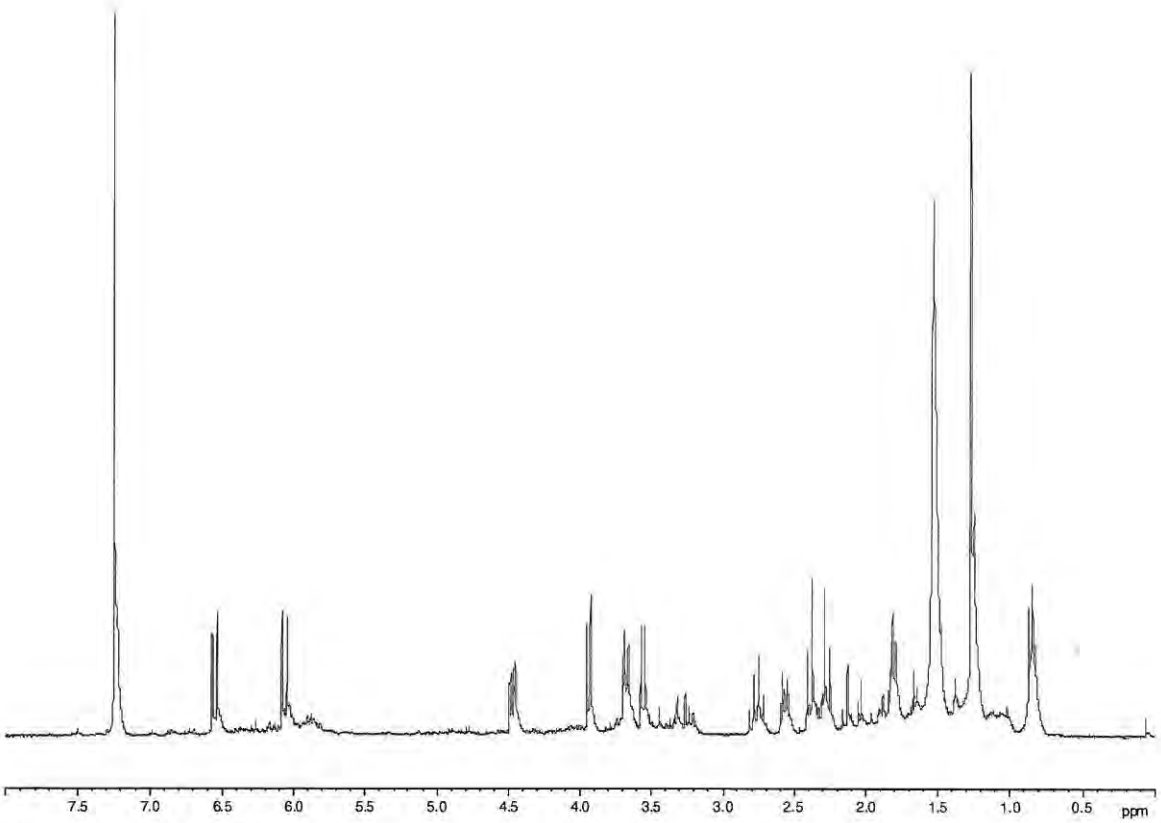


Figure 2.26 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (**35**)

The ^{1}H NMR spectrum of **35** (Figure 2.26, Table 2.8) exhibited four pairs of clearly distinguishable coupled methines at δ 6.55 (J = 13.6 Hz, H-7) and 6.06 (J = 13.6 Hz, H-8), δ 4.45 (J = 12.4, 4 Hz, H-4) and 3.70 (J = 12.4, 4 Hz, H-2), δ 3.94 (J = 10.8 Hz, H-10b) and 3.57 (J = 10.8 Hz, H-10a), and δ 2.39 (J = 15.2 Hz, H-6b) and 2.27 (J = 15.2 Hz, H-6a); two additional methines at δ 2.75 (H-3a) and 2.57 (H-3b), and a single methyl resonance at δ 1.26 (H-9) were also noted.

The ^{13}C spectrum (Table 2.8) indicated ten carbons of which two were sp^2 hybridized (δ 135.4 and 119.2), while the other shifts included two deshielded methine groups (δ 64.6 and 51.2), three methylene functions (δ 48.6, 40.4 and 39.2), one methyl (δ 27.5), and two quaternary carbons (δ 70.1 and 42.1) (Table 2.8). The LRMS data showed a molecular ion cluster at 404/402/400/398/396, which suggested a molecular formula of $C_{10}H_{13}Br_2Cl_3$.

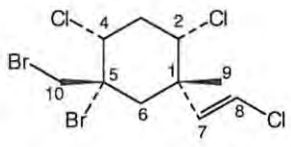
All the above spectroscopic data were consistent with 4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (**35**) which had previously been isolated from *P. cartilagineum*.[21]

Table 2.8 ^{1}H (400MHz, $CDCl_3$) and ^{13}C (100MHz, $CDCl_3$) NMR data of compound **35**, as well as expected literature values

Carbon No.	35		35 [21]	
	δ_H	δ_C	δ_H	δ_C
1	-	42.1, s	-	42.0
2	3.70, dd,, 12.4, 4	64.6, d	3.70, dd	64.6
3a 3b	H-3ax 2.75, m H-3eq 2.57, m	39.2, t*	H-3ax 2.78, q H-3eq 2.57, dt	39.1*
4	4.45, dd, 12.4, 4	51.2, d	4.49, dd	51.1
5	-	70.1, s	-	70.8
6a 6b	H-6ax 2.27,d, 15.2 H-6eq 2.39,d, 15.2	48.6, t	H-6ax 2.27, d H-6eq 2.40, d	48.5
7	6.55, d, 13.6	135.4, d	6.53, d	135.3
8	6.06, d, 13.6	119.2, d	6.08, d	119.1
9	1.26, s	27.5, q	1.26, s	27.4
10a 10b	3.57, d, 10.8 3.94, d, 10.8	40.4, q*	3.57, d, 3.95, d	40.3*

* Carbon shift assignments maybe interchangeable

*5-bromo-5-bromomethyl-1-chlorovinyl-2,4-dichloro-methylcyclohexane (**103**):*



103

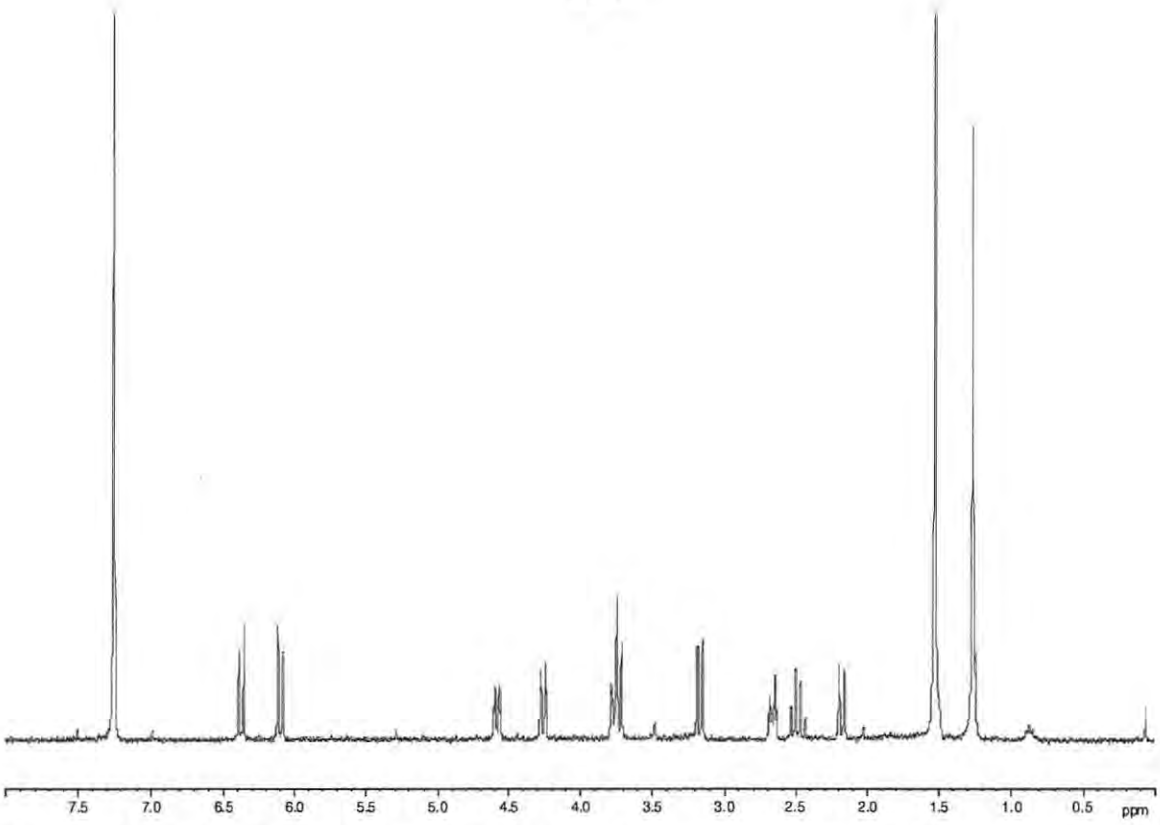


Figure 2.27 ^{1}H NMR spectrum ($CDCl_3$) of 5-bromo-5-bromomethyl-1-chlorovinyl-2,4-dichloro-methylcyclohexane (**103**)

HREIMS data gave a molecular ion peak at *m/z* 395.844046 corresponding to a molecular formula of $C_{10}H_{13}Br_2Cl_3$ and two degrees of unsaturation. Similarities between the ^{1}H NMR spectrum of **103** (Figure 2.27) and that of **35** were immediately apparent and suggested a similar cyclic monoterpenes structure. Several well-dispersed resonances were observed in the ^{1}H NMR spectrum at δ 6.37 (1H, d, *J* = 13.6 Hz, H-8), 6.10 (1H, d, *J* = 13.6 Hz, H-7), 4.57 (1H, dd, *J* = 13.0, 4.4 Hz, H-4), 3.78 (1H, dd, *J* = 14.0, 4.0 Hz, H-2), 4.25 (1H, d, *J* = 13.2 Hz, H-10a), 3.73 (1H, d, *J* = 12.8 Hz, H-10b), 2.66 (1H, dt, *J* = 14.0, 4.0 Hz, H-3a), 2.48 (1H, m, H-3b), 3.17 (1H, d, *J* = 14.8 Hz, H-6a), 2.17 (1H, d, *J* = 14.8 Hz, H-6b) and 1.26 (3H, s, H-9).

^{13}C and DEPT analyses showed the expected ten carbon resonances of which two were sp^2 hybridized methines (δ 131.5 and 122.2) thus indicating the presence of a single double bond. Compound **103** therefore must by cyclic. Further carbon resonances include two sp^3 methines (δ 64.7 and 57.8), three methylene functions (δ 51.2, 49.8, and 41.2), one methyl (δ 29.4), and two quaternary carbons (δ 67.2 and 43.9) (Table 2.9).

Detailed analysis of COSY (Figure 2.28, Table 2.9) and HMBC (Figure 2.29, Table 2.9) correlation data allowed the assignment of the structure of **103** as follows. The presence of the cyclohexane ring structure was confirmed by significant HMBC correlations from the methylene protons at δ 3.17 and 2.17 (H-6) to carbons resonances at δ 43.9 (C-1), 64.7 (C-2), 57.8 (C-4) and 67.2 (C-5), and COSY correlations from δ 2.66 and 2.48 (H-3) to methine signals at δ 3.78 (H-2) and 4.57 (H-4). Further HMBC correlations from the methyl signal at δ 1.26 (H-9) to carbons at δ 43.9 (C-1), 64.7 (C-2), 131.5 (C-7) and 49.8 (C-6) placed the methyl signal at C-1; while a strong HMBC correlation from the methylene at H-10 to the signal at δ 67.2 (C-5) placed it at position 5. This data confirmed that **103** has the same 1,5-dimethyl-1-ethylcyclohexane structure as **35**. The differences in chemical shifts between **103** and **35** could therefore only be accounted for by a different halogenation arrangement and possibly stereochemistry.

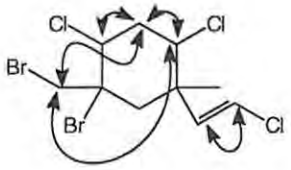


Figure 2.28 Selected COSY-90 correlations for compound **103**

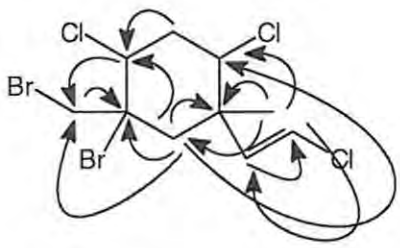
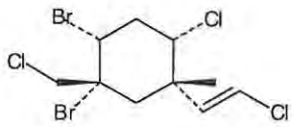


Figure 2.29 HMBC correlations for compound **103**

The analysis of ^{1}H and ^{13}C chemical shift data to deduce regio- and relative stereochemistry, in acyclic monoterpenes, is a commonly employed strategy. Accordingly, the ^{13}C chemical shifts of C-8 (δ 122.2) and C-2 (δ 64.7) are consistent with chorine substituents at these positions.[21] The downfield shift of the C-4 resonance in **103** (δ 57.8) when compared to that of **35** (δ 51.1) (Table 2.10) is suggestive of another chlorine substituent at this position. This proposal was also supported by the halogen substituent at C-9 of compound **81**.[39] Therefore, it appears, that the differences in spectral characteristics between **103** and **35** are a consequence of a structural change at C-5. Simple arithmetic thus suggests that the two bromine atoms required by the molecular formula must therefore be accommodated at C-5 and C-10. However, the chemical shift of the methylene at δ 51.2 (C-10) is inconsistent with a bromine substituent at this position. An alternative explanation, to account for the spectral characteristics of **103** could involve different relative stereochemistry. An examination of the vicinal coupling constants of the halomethines at δ 3.78 (1H, dd, J = 14.0, 4.0, H-2) and δ 4.57 (1H, dd, J = 13.0, 4.4, H-4), together with the chemical shift of the C-9 methyl indicates that both hydrogens must be axial and could therefore not explain the spectral differences. Similarly, the expected *trans* stereochemistry around the C-7 and C-8 double bond was deduced from the vicinal coupling constant (J = 13.6 Hz). An alternative structure **(103b)** with bromine atoms at C-4 and C-5, is also possible. It is apparent that only X-ray analysis would satisfactorily resolve the structure for **103**.



103b

Table 2.9 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **103** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	43.9, s	-	-	-
2	64.7, d	3.78, dd, 14.0, 4.0	H-3a, H-3b	-
3a 3b	41.2, t	2.66, dt, 14.0,4.0 2.48, m	-	C-4
4	57.8, d	4.57, dd, 4.4, 13.0	H-3a, H-3b	C-10
5	67.2, s	-	-	-
6a 6b	49.8, t	3.17, d, 14.8 2.17, d, 14.8	H-6b	C-1, C-4, C-2, C-5 C-1, C-10, C-5
7	131.5, d	6.10, d, 13.6	-	C-8
8	122.2, d	6.37, d, 13.6	H-7	C-7
9	29.4, q	1.26, s	-	C-1, C-6, C-2, C-5, C-7
10a 10b	51.2, t	4.25, d, 13.2 3.73, d, 12.8	H-2 H-3a, H-3b	C-5 C-5

Table 2.10 ^{13}C (100MHz, $CDCl_3$) NMR data of compound **103**, as well as the literature values of compound **88** and **35**

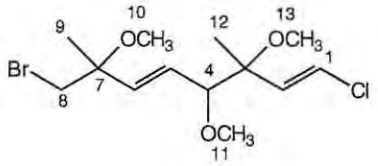
Carbon No.	**108**	**88** [43]	**35** [21]
	δ_C	δ_C	δ_C
1	43.9, s	42.0	42.0
2	64.7, d	64.1	64.6
3	41.2, t	38.3	39.1*
4	57.8, d	59.0	51.1
5	67.2, s	71.3	70.8
6	49.8, t	48.8	48.5
7	131.5, d	135.4	135.3
8	122.2, d	119.5	119.1
9	29.4, q	27.4	27.4
10	51.2, t	38.8	40.3*

* Carbon shift assignments maybe interchangeable

2.2.2.3 Structural elucidation of artifacts

Several methoxylated compounds were also isolated from the *P. corallorhiza* extract and were presumably formed during the extraction process in which methanol was used. No attempt was made to obtain elemental analyses for the compounds and there structures were determined from NMR data and comparison to compounds previously isolated.

*8-bromo-1-chloro-3,4,7-trimethoxy-3,7-dimethyl-1,5-octadiene (**104**):*



104

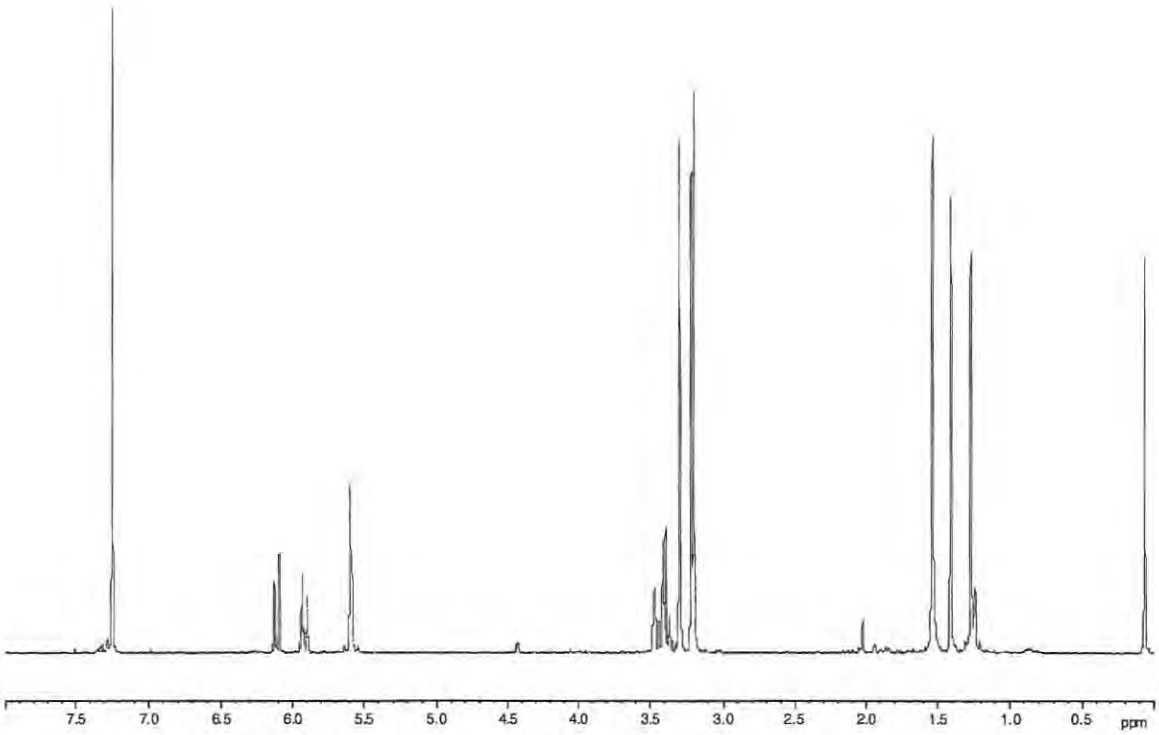


Figure 2.30 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1-chloro-3,4,7-trimethoxy-3,7-dimethyl-1,5-octadiene (**104**):

Both carbon and proton NMR spectra for compound **104** (Figure 2.30) indicated that it had the same carbon skeleton, and terminal halogen distribution as that of compound **96**. However at positions C-3, C-4, and C-7 the Cl halogens had been substituted for methoxy groups. Methoxy groups were easily identified by distinct ^{13}C NMR shifts at δ 50.6 (C-

13), 57.3 (C-11), and 50.9 (C-10), and by distinct proton shifts between δ 3.2-3.3 (Table 2.11). The presence of methoxy groups at C-13 and C-10 also resulted in a downfield carbon shift of between δ 7-8 for methyl groups situated at C-9 and C-12 (when compound **104** was compared to compound **96**).

The ^{1}H shift at δ 3.40 (H-8) was illustrated by a double doublet, and not as a singlet (as is mostly characteristic of the linear dienes of non-methoxy compounds). The cause of this large splitting could be due to the methoxy group at C-10. HMQC, COSY and HMBC (Figure 2.31) correlations also supported the proposed structure (Table 2.11).

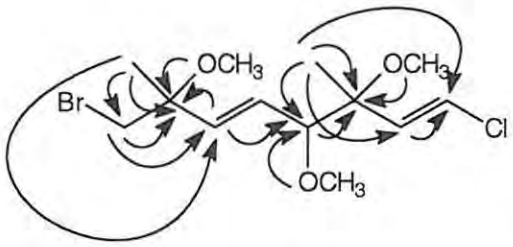
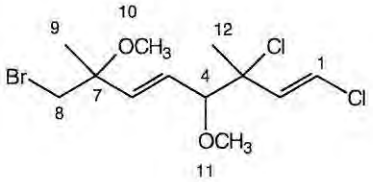


Figure 2.31 HMBC correlations for compound **104**

Table 2.11 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **104** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	120.6, d	6.11, d, 13.6	-	C-3, C-2
2	135.0, d	5.92, d, 13.6	-	C-12, C-1
3	79.1, s	-	-	-
4	87.4, t	3.47, d, 5.8	-	C-11, C-3, C-5, C-6
5	129.0, d	5.61, m	H-4	-
6	136.6, d	5.59, m		C-7, C-4
7	76.0, s	-	-	-
8	40.4, t	3.40, dd, 10.5, 18.1	-	C-9, C-7, C-6
9	21.2, q	1.41, s	-	C-8, C-7, C-6
10	50.9, q	3.22, s	-	C-7
11	57.3, q	3.30, s	-	C-4
12	18.1, q	1.27, s	-	C-3, C-4, C-1, C-2
13	50.6, q	3.20, s	-	C-3

*8-bromo-1,3-dichloro- 4,7-dimethoxy-3,7-dimethyl–1,5-octadiene (**105**):*



105

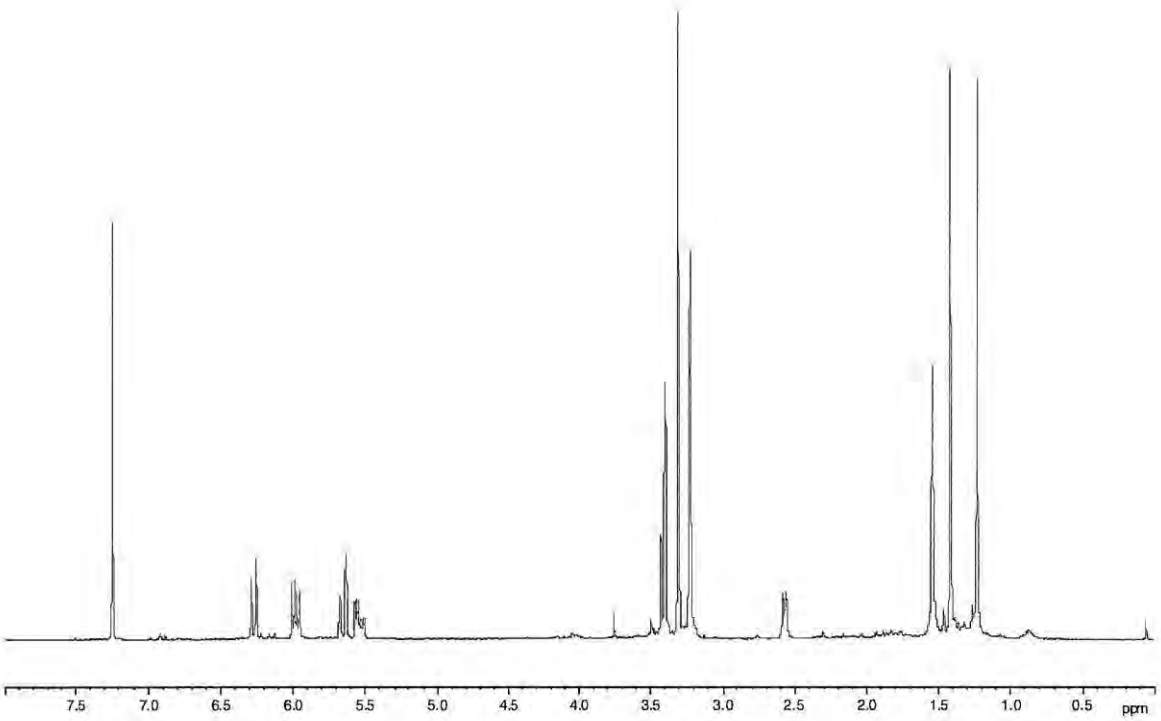


Figure 2.32 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1,3-dichloro- 4,7-dimethoxy-3,7-dimethyl–1,5-octadiene (**105**)

Compound **105** (Figure 2.32) demonstrated similar carbon and proton NMR characteristics to that of compound **104**. However it lacked a methoxy substituent at C-3. Only two methoxy peaks at δ 50.8 (C-10) and δ 56.9 (C-11) were observed in the ^{13}C spectrum (Table 2.12). HMQC, ^{1}H-^{1}H COSY and HMBC (Figure 2.33) measurements supported the structural assignment (Table 2.12).

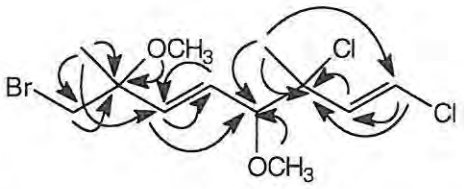
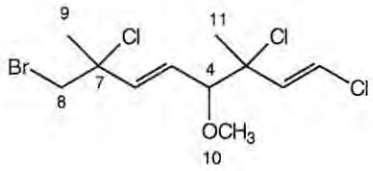


Figure 2.33 HMBC correlations for compound **105**

Table 2.12 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **105** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	119.2, d	6.27, d, 13.6	-	C-3, C-2
2	137.1, d	5.98, dd, 6.8, 6.8	-	C-12, C-3, C-1
3	74.4, s	-	-	-
4	87.8, d	3.45, d, 8.0	-	C-12, C-11, C-3, C-6
5	128.4, d	5.54, m	-	C-7, C-4, C-6
6	138.1, d	5.67, d, 16.0		C-9, C-8, C-7, C-4, C-5
7	76.0, s	-	-	-
8	40.1, t	3.40, d, 7.2		C-9, C-7
9	20.8, q	1.41, s	-	C-8, C-7, C-6
10	50.8, q	3.23, s	H-8	C-8, C-7
11	56.9, q	3.31, s	-	C-4
12	23.2, q	1.26, s	-	C-3, C-4, C-1

*8-bromo-1,3,7-trichloro-4-methoxy-3,7-dimethyl–1,5-octadiene (**106**):*



106

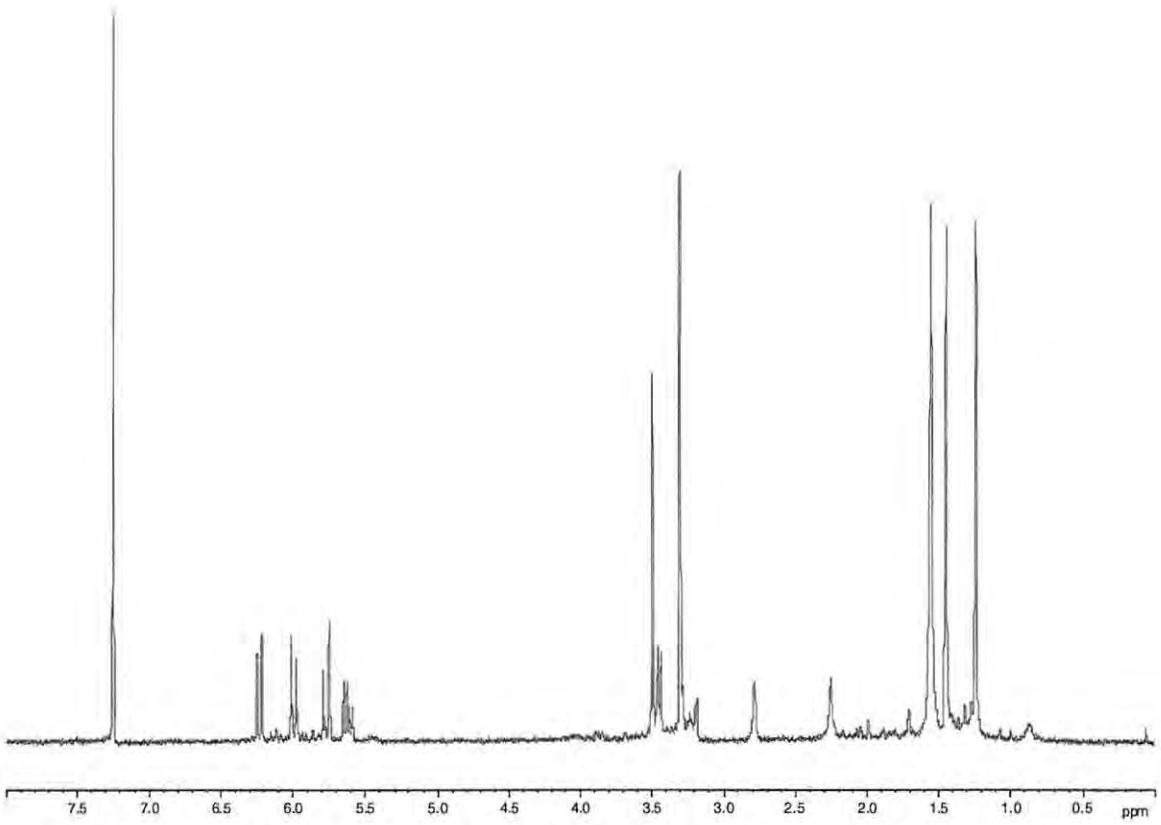


Figure 2.34 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1,3,7-trichloro-4-methoxy-3,7-dimethyl–1,5-octadiene (**106**)

Compound **106** indicated the presence of a single methoxy group situated at δ 56.9 (C-10) (Table 2.13). This compound displayed similar NMR spectra (Figure 2.34) to that of compound **96** and **105**. Although H-8 was correctly expressed as a singlet at δ 3.50, concern was expressed over the corresponding carbon shift at δ 44.9 (C-8), which is high when compared to known values. 2D NMR (Figure 2.35) data confirmed the structure of compound **106** (Table 2.13).

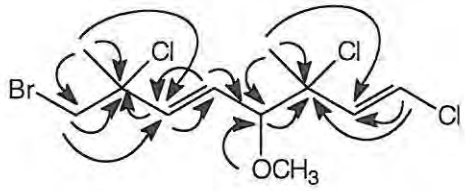
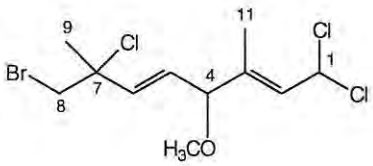


Figure 2.35 HMBC correlations for compound **106**

Table 2.13 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **106** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	119.2, d	6.23, d, 13.2	-	C-3, C-2
2	137.1, d	5.99, d, 13.2	-	C-11, C-3, C-1
3	74.5, s	-	-	-
4	87.5, d	3.45, d, 8.0	-	C-11, C-10, C-3, C-6
5	125.9, d	5.62, dd, 8.0, 16.0	-	C-7, C-4, C-6
6	140.4, d	5.76, d, 16.0	-	C-8, C-7, C-4, C-5
7	71.6, s	-	-	-
8	44.9, t	3.50, s	H-10, H-11	C-9, C-7, C-6
9	26.3, q	1.45, s	H-10, H-11	C-8, C-7, C-6
10	56.9, q	3.30, s	-	C-4
11	23.4, q	1.24, s	-	C-3, C-4, C-2

*8-bromo-1,1,7-trichloro-4-methoxy-3,7-dimethyl-2,5-octadiene (**107**):*



107

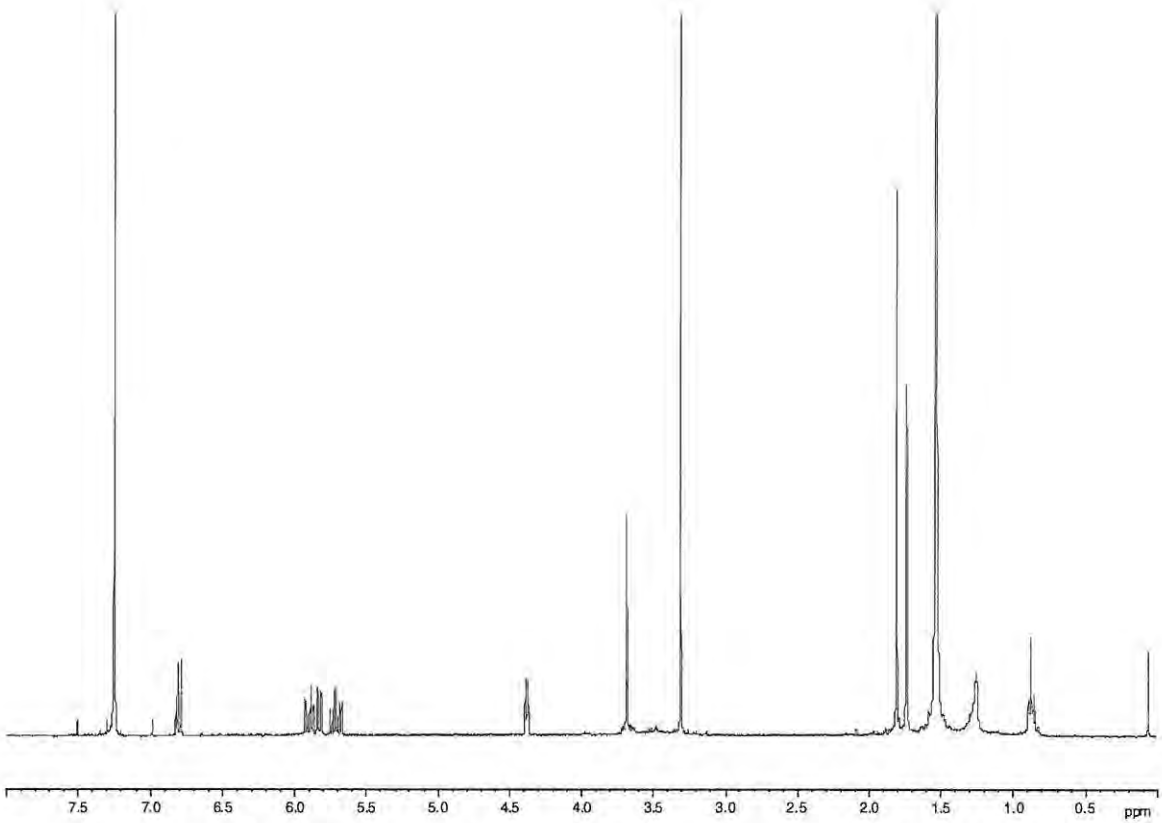


Figure 2.36 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of 8-bromo-1,1,7-trichloro-4-methoxy-3,7-dimethyl-2,5-octadiene (**107**)

Compound **107** (Figure 2.36) displayed a similar structure to that of compound **101**. The only significant difference was the substitution of a Br group at C-4 with a methoxy moiety. This substitution was identified by a characteristic carbon shift of δ 56.7 (C-10) and a proton shift of δ 3.31 (H-10), which was coupled to the methine group at δ 80.1 (C-4). 2D NMR (Figure 2.37) experiments agreed with the nature of the proposed structure (Table 2.14).

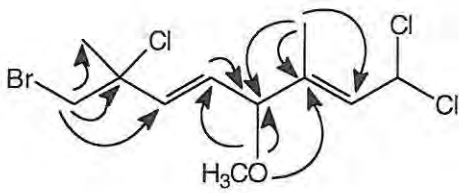


Figure 2.37 HMBC correlations for compound **107**

Table 2.14 ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY and HMBC data for compound **107** in $CDCl_3$

Carbon no.	δ_C	δ_H, multi, J (Hz)	^{1}H-^{1}H COSY	HMBC
1	66.3, d	6.80, dd, 10.0, 4.0	H-2	-
2	129.2*, d	5.83, dd, 11.2, 1.6	H-11, H-4 (w)	C-4
3	137.4, s	-	-	-
4	80.1, d	4.38, d, 5.6	-	C-11, C-10, C-5, C-2, C-6, C-3
5	129.0*, d	5.71, m	H-4	C-4
6	134.6, d	5.90, dd, 6.4,1.2	H-5, H-4 (w)	-
7	67.6, s	-	-	-
8	42.0, t	3.68, d, 3.6	H-9	C-9, C-7, C-6
9	27.4, q	1.81, s	-	-
10	56.7, q	3.31, s	-	C-4, C-5, C-3
11	19.5, q	1.74, s	-	C-4, C-2, C-3

* The carbon shifts of 129.0 and 129.2 maybe interchangeable

Ichikawa [58] found that extraction of *Chondrococcus japonicus* with acetone followed by hexane, yielded monoterpenes without methoxy groups. But when methanol was used methoxy groups appeared. When reacting the acetone extracts (by refluxing with methanol) methoxy products were once again formed. The following reaction scheme has been suggested (Figure 2.38):

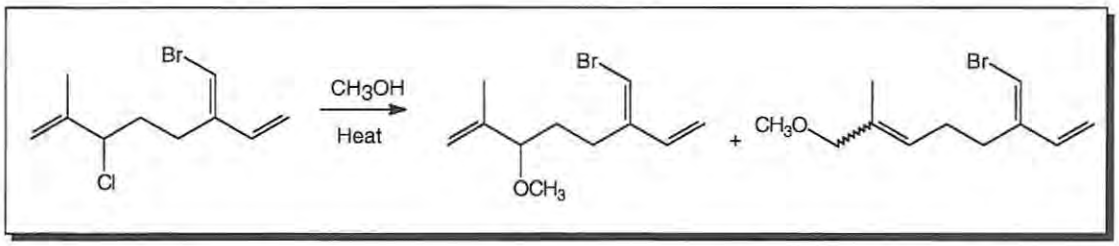


Figure 2.38 Proposed reaction scheme for the production of methoxy groups [10]

Although, MeOH and MeOH / DCM are commonly used as extraction solvents for red algae, it is interesting that not more artefacts had been reported in the literature. The allylic halogen atoms could be replaced by a good nucleophile, such as MeOH via an S_N1 reaction.

By comparing the two different extraction methods employed in the isolation of compounds from *P. corallorhiza* (collected at Kalk Bay and Port Alfred) the following was noted; the crude extract from Port Alfred, which was left in MeOH at room temperature for 3 days, contained significantly more methoxy artifacts than the crude extract from Kalk Bay, which was only left in cold MeOH for 6 hours. This could indicate that MeOH was responsible for the production of methoxy artifacts.

To avoid methoxy groups from being formed as artifacts of the extraction method, it is best to reduce the conditions that lead to S_N1 reactions. This can be done by reducing the amount of water, salt and MeOH which is used for the extraction procedure. For example, freeze-drying would be a good option for reducing the amount of water present, and if MeOH is used, it should be used cold and for a short period of time.

Chapter Three

Development of a method for the rapid chemical screening of *Plocamium* spp. using Gas Chromatography-Mass Spectrometry (GC-MS)

3.1 Introduction

Conventional methods of chromatography using solvent partitioning, column chromatography (CC), and high performance liquid chromatography (HPLC), are time consuming and expensive. Scientists searching for novel compounds often waste much of their time extracting and purifying known compounds, for example Stierle *et al.* [49] reported two compounds that were also isolated from *P. corallorhiza* (See Chapter 2). In the search for new compounds, it is important to know that the particular species under investigation does not contain secondary metabolites that have already been discovered. Furthermore, with the large number of metabolites that have been isolated from seaweeds, an effective dereplication strategy is essential. Being able to provide this information, both rapidly and accurately, is extremely valuable to the natural product chemist.

Polyhalogenated monoterpenes are relatively volatile compounds, which makes them well suited to investigation by gas chromatography (GC). By comparing the retention time and characteristic mass spectra (MS) of known compounds, with those obtained from the crude extract of different *Plocamium* spp., rapid chemical screening can be accomplished. For such a system to work, the following criteria must be met: crude extracts must be obtained in standard manner and, once an optimised GC method has been established, the same method must be used throughout the screening procedure.

Several investigators have used GC and GC-MS during routine natural product investigations of seaweed extracts,[39, 41, 52] however, none of these researchers have used these techniques in a systematic manner to screen for new compounds or identify known compounds. The advantages of using GC-MS are summarised below:

- Decreased impact on ecology
- GC-MS has an increased sensitivity over NMR
- Decreased cost in exploring natural product chemistry
- Increased scientific output by rapidly identifying known compounds and prioritizing extracts containing new compounds

The aim of this study was thus to develop a method that could be routinely used to screen small samples of seaweed extracts, and identify known and possibly new compounds present in these extracts.

3.2 Results and Discussion

In this study two separate instruments were used. A Hewlett Packard 6890 Gas Chromatograph equipped with a HP-5 Phenyl Methyl Siloxane column was used to develop standard methods before samples were run on a Finnigan GCQ mass spectrometer. GC and MS parameters can be found in Tables 3.1- 3.3.

Table 3.1 GC parameters (HP 6890)

Oven Initial temp: 75 °C; Final temp: 250 °C; Ramp: 5 °C/minute; Post temp: 250 °C; Run time: 35.00 min
Front inlet Mode: Split; Initial temp: 270 °C; Pressure: 73 kPa; Split ratio: 75:1; Split flow: 150.7 ml/min; Total flow: 155.3 ml/min; Gas saver: On; Saver flow: 20.0 ml/min; Saver time: 2.00 min; Gas type: Helium
Column 1 Capillary Column; Model Number: HP 19091J-413; HP-5 5% Phenyl Methyl Siloxane; Max temperature: 325°C; Nominal length: 30.0m; Nominal diameter: 320.00µm; Nominal film thickness: 0.25µm; Mode: constant flow; Initial flow: 2.0ml/min; Nominal initial pressure: 73kPa; Average velocity: 35 cm/sec; Inlet: Front Detector Outlet: Front Detector; Outlet pressure: ambient
Front Detector (FID) Temperature: 270 °C; Hydrogen flow: 40.0 ml/min; Air flow: 450.0 ml/min; Mode: Constant column makeup flow; Combined flow: 45.0 ml/min; Makeup flow: On; Gas type: Nitrogen; Flame: On; Electrometer: On; Lit offset: 0.0
Signal 1 Data rate: 50 Hz; Type: front detector; Save Data: On; Attenuation: 0
Signal 2 Data rate: 20 Hz; Type: front detector; Save Data: On; Attenuation: 0
Post Run Post Time: 2.00 min; Oven Temperature: 250 °C; Column 1 Flow: 2.0 ml/min
Solvent injected Hexane (HPLC grade) (1µl injection)

Table 3.2 GC parameters using the Finnigan (GCQ) method

Oven Initial temp: 75 °C; Final temp: 220 °C; Ramp: 5 °C/min; Post temp: 250 °C; Run time: 39.00 min
Front inlet Mode: Splitless; Initial temp: 200 °C; Split close time: -0.20 min; Split open time: 1.00 min
Column 1 Capillary Column; Model Number: DB-1; Max temperature: 320 °C; Mode: constant flow; Constant velocity: 40 cm/sec; Surge pressure off
Post Run Post Time: 10.00 min; Oven Temperature: 220 °C
Solvent injected Hexane (HPLC grade) (1µl injection)

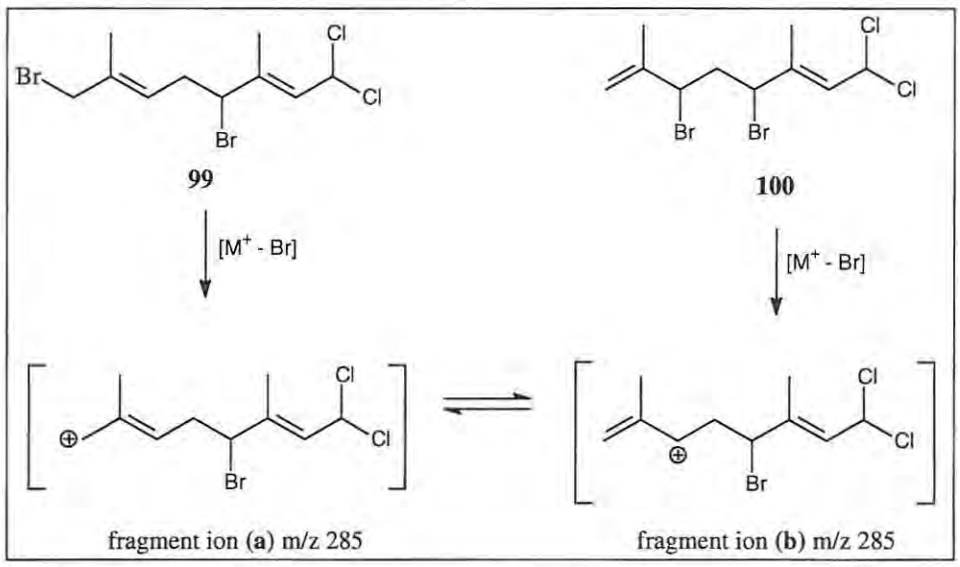
Table 3.3 GCQ MS method

Acquisition Time: GC Run Time
Seconds Per Scan: 1.00 sec
Mass Defect: 0.0 mmu/amu
Acquire Cal Gas: No
Source Temp: 200 °C
Transfer Line: 275 °C
Segment: 1 **Start Time:** 3.00 min
Polarity: POS
AcqThresh: 0 **Mult Offset:** +0 **uScans:** 7
Scan Mode: Full Scan 50-440

3.2.1 GC-MS analysis of a *P. corallorhiza* extract

A single, fresh leaf (340 mg) of *P. corallorhiza* (collected from Riet River, near Port Alfred) was placed in MeOH (200 ml) for 4 hours. The MeOH was evaporated, and the extract was dissolved in 1 ml of hexane, and then passed through a cotton wool plug. One microliter (1 μl) of the crude solution was then analysed by GC-MS. Figure 3.1 shows the expanded GC chromatogram for the crude *P. corallorhiza* extract. The retention times and mass spectra of the major and minor peaks were recorded (Table 3.4). Purified compounds (described in Chapter 2) were also analysed and their retention times, and fragmentation patterns recorded (Table 3.5). These fragmentation patterns were then compared to the fragmentation patterns from the crude extract of *P. corallorhiza*. Thus, the presence of the pure compounds could be confirmed in the crude extract from their characteristic fragmentation patterns. This is illustrated using two examples (Figures 3.2-3.3).

It was noted that the GC retention times for purified compounds were slightly different when injected separately. This is to be expected, as several factors (for example, concentration, variations in flow rate and interaction with other compounds in the crude extract) may affect the retention time of a substance. This did not present a problem since the retention times were only used as a guide while identification relied on mass spectral fragmentation patterns. In general, each compound should give a characteristic mass spectral fragmentation pattern. However, this does not take into account several complicating factors, such as the presence of enantiomers, which would give identical mass fragmentation patterns. In addition, knowledge of the fragmentation mechanism could be useful in rationalizing the presence of various fragment ions. For example, the presence of fragment ions due to $[M^+ - X]$ is common in halogenated monoterpenes. Therefore, if hypothetical compounds A and B only differ in the presence of a single halogen at a specific position then $[M^+ - X]$ will give identical mass spectra for these compounds. Compounds **99** and **100** present another interesting case. Both **99** and **100** gave essentially identical mass spectra even though they had been assigned different structures based on NMR data. Both compounds would tend to undergo very similar fragmentations which would include loss of X or HX and allylic cleavage. Loss of Br at C-8 in **99** and at C-6 in **100** would give the same resonance stabilised allylic carbocation which could then fragment further thus explaining the similar mass spectra (Scheme 3.1).



Scheme 3.1 Proposed mass spectral fragmentation scheme for compounds **99** and **100**

Using GC-MS we were able to rapidly identify known compounds from the crude *P. corallorhiza* extract. The MS fragmentation data and retention times of the known compounds were used to build up a database, which could be used for the rapid chemical screening of various *Plocamium* spp. This information was used to determine which compounds still needed to be isolated.

Caution should be exercised when using GC-MS, as not all the peaks present are indicative of monoterpenes. Some peaks are residual amounts of solvent, or "other" artifacts of the extraction procedure, however polyhalogenated monoterpenes are easily identified by the presence of isotopic halogen clusters. HPLC grade solvents, which were used in the extraction processes were found to be "clean," and only contributed in a small or insignificant manner to the overall chromatographic trace. Impurities (Table 3.6) may also contribute to artificial results on the MS chromatogram.

Interestingly, only one methoxylated compound (**107**) was found in the crude trace of *P. corallorhiza*. This argues in favour of the methoxy groups being by-products of the extraction process. Perhaps different extraction procedures could be explored to minimise the presence of any possible contaminants. Further chromatography (on *P. corallorhiza*) still needs to be done to determine the identity of the other peaks present in the crude trace.

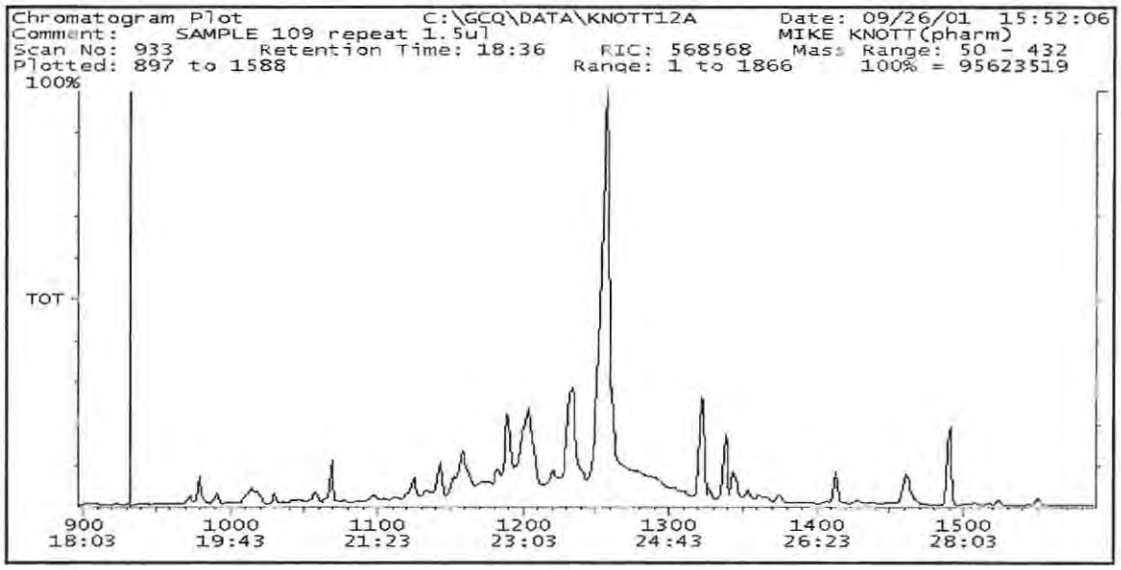


Figure 3.1 Expanded GC chromatogram of *P. corallorhiza* collected from Riet River (near Port Alfred) (Retention time from 18.03 to 30.03 minutes)

Table 3.4 GC retention times and mass spectral data of different peaks from the crude extract of *P. corallorhiza*

Rt. Time (minutes)	**Range/Intensity 100%=95623519**	**m/z (absorbance %)**
19.16	971 – 975 (188708)	249/247 (3, 2); 213/211 (2, 2); 205/203 (7, 9); 169/167 (17, 49); 141/139 (14, 15); 131 (100); 117/115 (11, 12); 105 (34); 91 (44); 77 (21); 65 (8); 51 (7)
19.22	977 – 981 (204897)	269/267/265/263 (4, 29, 100, 76); 229/227 (29, 31); 185/183 (18, 48); 179 (22); 148 (84); 129 (45); 119 (91); 105 (87); 91 (58); 79/77 (87, 69); 65 (47); 51 (30)
19.34	989 – 993 (67657)	267/265/263 (27, 100, 75); 229/227 (31, 30); 186/184/182 (19, 47, 35); 165 (15); 149(69); 148/146 (81, 60); 129 (53); 119(88); 105(89); 91 (59); 79/77 (73, 59); 65 (45); 51 (28)
19.57	1012 – 1016 (161777)	261/263 (6, 5); 229/227 (41, 40); 205/203 (9, 12); 179 (33); 169/167 (32, 65); 148/146 (88, 54); 133/131 (47, 65); 119/117/115 (47, 28, 59); 105 (98); 91 (55); 79/77 (100, 51); 65 (19); 53 (19)
20.13 Compound **102**	1028 – 1032 (284773)	297/295/293 (2, 4, 2); 253/251/249 (5, 20, 15); 215/213 (13, 13); 197/195 (8, 7); 171/169 (13, 37); 134 (22); 133 (100); 115 (7); 107/105 (7, 8); 93/91 (10, 13); 79/77 (29, 14); 53 (7)
20.41	1056 – 1060 (69924)	403/401 (3, 4); 357/355 (4, 6); 267/265/263 (22, 84, 63); 229/227 (22, 17); 184 (45); 183 (47); 167/165 (12, 22); 149 (48); 148 (66); 145 (41); 129 (44); 119 (79); 110 (85); 95 (41); 85 (72); 71 (84); 57/55 (100, 33)

20.52	1067 – 1071 (648814)	242 (15); 213 (25); 199 (100); 185 (53); 171 (34); 157 (56); 143 (100); 129 (32); 115 (13); 101 (54); 87 (19); 73 (14); 55 (44) *suspected contaminant
21.20	1095 – 1099 (134438)	261/259 (6, 6); 229/227 (43, 43); 179 (38); 161 (13); 148/146 (100, 56); 137 (19); 119 (54); 105 (78); 95 (27); 79/77 (30, 32); 67/65 (12, 15); 53 (15)
21.48	1123 – 1127 (455841)	297/295/293 (2, 4, 3); 253/251/249 (5, 23, 17); 227/225 (11, 9); 215/213 (12, 12); 197/195 (4, 9); 171/169 (8, 25); 145 (8); 133 (100); 111 (28); 91 (27); 81 (16); 79/77 (33, 22); 67 (9); 53 (4)
21.57	1132 – 1136 (198137)	279/277 (3, 3); 225 (37); 253/251 (5, 4); 229/227/225 (12, 38, 27); 197/195/193/191/189 (12, 32, 22, 12, 14); 165 (8); 145 (26); 113/111/109 (31, 100, 48); 91 (12); 81 (51); 79/77 (77, 46); 67 (15)
22.06 Compound **99** or **100**	1141 – 1145 (593456)	287/285/283/281 (10, 19, 13, 4); 251/249/247 (9, 32, 25); 213 (17); 211 (17); 205/203 (25, 34); 182/180 (9, 7); 169 (40); 168 (23); 167 (100); 141/139 (10, 14); 133 (24); 132 (17); 131 (42); 127 (15); 115 (13); 105 (33); 91 (42); 79/77 (17, 17); 67/65 (36, 21); 51 (8)
22.21	1156 – 1160 (590004)	317/315/313 (1, 2, 2); 279/277 (4, 4); 229/227/225 (27, 100, 76); 197/195/193/191/189 (24, 78, 61, 18, 24); 167/165 (12, 8); 147/145 (26, 66); 131 (11); 115/113/111/109 (32, 45, 59, 57); 91 (17); 81 (31) 79/77 (62, 43); 67 (18); 53 (16)
22.45	1180 – 1184 (288540)	315/313/311 (3, 2, 2); 257/255 (5, 5); 229/227/225 (27, 100, 72); 197/195/193/191/189 (19, 78, 58, 17, 13); 159/157 (6, 7); 147/145 (18, 63); 125/123 (13, 9); 115/113 (33, 42); 110 (39); 109 (34); 91 (10); 79/77 (24, 22); 67 (11); 51 (7)
22.52	1187 – 1191 (933616)	333/331/329 (2, 5, 3); 313/311/309 (14, 65, 48); 299/297/295/293 (2, 2, 5, 3); 281/279/277/275/273 (6, 15, 11, 5, 5); 247/245/243/241 (4, 4, 12, 8); 233/231/229/227 (3, 10, 10, 7); 203/201/199/197 (8, 28, 25, 6); 154 (52); 123 (25); 109 (20); 91 (22); 89 (100); 79/77/75 (21, 14, 48); 59 (12)
23.07 Compound **107**	1202 – 1206 (520966)	317/315/313 (12, 24, 13); 285/283/281/279/277 (7, 16, 17, 20, 16); 267/265 (13, 10); 247/245/243/241/239/237/235/233/231/229/227/225 (18, 12, 8, 9, 11, 18, 26, 30, 9, 11, 15, 15); 219/217 (15, 16); 203/201/199/197/195/193 (31, 47, 30, 52, 41, 51); 186 (21); 169 (18); 167/165/163/161/159/158/157/155/153/151/150/149 (18, 53, 68, 33, 40, 40, 25, 100, 27, 32, 23, 16, 21); 131/129/127 (44, 43, 20); 119 (76); 105 (34); 91 (53); 79 (62); 65 (20); 51 (20)
23.24	1219 – 1223 (177781)	317/315 (2, 3); 285/283/281 (2, 5, 2); 253/251/249/247/245 (4, 9, 7, 7, 7); 202/201 (8, 13); 183 (7); 169/167/165/163 (40, 47, 19, 9); 149 (10); 137/135/133/131 (37, 54, 32, 22); 127/125/123 (26, 100, 90); 105 (15); 103/101/99 (15, 23, 37); 91 (35); 79/77 (62, 34); 65 (21);

		51 (26)
23.37 Compound **96**	1232 – 1236 (1726502)	321/319 (1, 1); 285/283/281 (1, 2, 1); 249/247/245 (1, 2, 2); 231/229 (4, 3); 203/201 (5, 6); 196/195/194 (10, 4, 8); 169/168/167/165 (4, 7, 10, 8); 155/153/151/149 (7, 8, 8, 11); 139 (5); 136 (6); 133/131/129/127/125/123 (10, 12, 7, 16, 70, 100); 117/116/115/113 (26, 12, 66, 11); 105 (11); 91 (20); 89/87 (19, 50); 79/77 (66, 28); 65 (11); 51 (21)
24.01 Compound **101**	1254 – 1258 (2693135)	323/321/319/317 (6, 23, 38, 23); 285/283/281 (17, 35, 20); 273/271/269 (6, 13, 7); 249/247/245/243/241/239/237/235/233 (9, 28, 25, 18, 20, 26, 30, 11, 9); 223/221 (9, 7); 209 (14); 207 (14); 205 (22); 204/203/202/201/199/197/195/193/191/189/187 (14, 14, 22, 22, 71, 33, 100, 11, 17, 13, 11, 10, 16, 8); 183/181 (18, 12); 173 (11); 170 (13); 169/168/167/166/165/164/163/162/161/ 157/155/154/153/151/150/149 (11, 13, 23, 44, 51, 37, 165, 11, 34, 19, 44, 10, 30, 26, 55, 27, 13, 37); 141/139/138/136 (18, 29, 14, 18); 133/132/131/130/129/128/127 (22, 17, 36, 27, 40, 20, 36); 123 (20); 115 (41); 105/103/101 (23, 16, 13); 91 (42); 77 (24); 65 (27); 51 (16)
25.05	1320 – 1324 (1125441)	272/270 (11, 45); 241 (44); 227 (97); 213 (57); 199 (89); 185 (87); 171 (100); 157 (68); 143 (98); 129 (45); 115 (29); 101 (62); 83 (28); 69 (17); 55 (55) * Suspected contaminant
25.10	1325 – 1329 (619579)	361/359/357 (2, 3, 1); 223 (9); 205 (9); 167 (5); 149 (100); 121 (4); 65 (4) * Suspected contaminant
25.21	1336 – 1340 (604220)	345/343/341 (4, 10, 7); 323/321/319 (2, 3, 2); 303/301 (7, 18); 299 (11); 285/283/281 (22, 45, 27); 267/265/263 (25, 100, 80); 247 (12); 229 (26); 227 (28); 201 (29); 185/183 (14, 43); 165 (25); 147 (58); 129 (27); 119 (48); 95 (42); 77 (17); 51 (7)
25.26	1341 – 1345 (340826)	363/361/359/357 (1, 3, 3, 2); 316/314/312 (4, 8, 5); 281/279/277 (7, 21, 18); 263/261 (7, 12); 253/251 (20, 28); 235/233 (11, 17); 221/219 (33, 47); 197 (25); 187/185/183 (29, 35, 100); 165 (26); 151 (39); 129 (23); 115 (34); 91 (28); 89 (26); 75 (25); 59 (9)
25.36	1351 – 1355 (74414)	365/363 (3, 7); 359/357 (27, 11); 345 (3); 321 (9); 319/317 (14, 7); 285/283/281 (12, 29, 18); 249/247/245/243 (17, 36, 28, 17); 221 (8); 203/201 (43, 68); 167/165 (77, 68); 149 (49); 133/131 (82, 78); 115 (94); 91 (100); 79/77 (62, 56); 65 (30); 51 (34)
25.58	1373 – 1377 (93283)	413/411/409/407 (2, 3, 3, 2); 377/375/373 (2, 4, 2); 331 (14); 329/327 (19, 8); 312/310/308 (11, 67, 70); 295 (7); 287/285/283 (5, 11, 4); 251/249/247 (11, 24, 17); 239/237 (75, 71); 225/223 (100, 93); 213/211 (16, 19); 199 (17); 169/167 (29, 49); 159 (53); 145 (18); 129 (14); 115 (28); 91 (13); 79 (18); 65 (5)

26.36	1411 – 1415 (494845)	337/335/333 (3, 4, 2); 279 (2); 271/269 (8, 8); 259/257/255 (32, 98, 100); 201 (7); 189/187/185 (12, 17, 12); 165 (8); 153/151/149 (18, 16, 18); 137 (19); 115 (19); 105 (8); 91 (17); 79 (6); 59 (4)
26.51	1426 – 1430 (22352)	401 (2); 358 (3); 345/343/341 (4, 7, 3); 329/327 (5, 8); 310/308 (20, 23); 282/280 (19, 15); 267/265/263 (23, 90, 57); 247 (18); 239/237 (27, 28); 201 (22); 183 (29); 167 (22); 149/147 (35, 35); 129 (28); 119 (38); 105 (55); 85 (70); 71 (90); 57 (100)
27.24 Compound **94**	1459 – 1463 (258848)	367/365/363/361 (3, 7, 8, 3); 331/329/327/325 (2, 5, 6, 3); 295/293/291/289/287/285/283/281 (2, 4, 3, 2, 3, 6, 11, 9); 249/247/245 (8, 20, 13); 215/214/213/212/211 (7, 26, 18, 22, 15); 197/195/193 (3, 10, 6); 171/169/167/165 (10, 31, 35, 13); 133/131 (100, 33); 117/115 (43, 41); 105 (76); 91 (62); 79/77 (23, 18); 65 (12); 51 (10)
27.54 Compound **35** or **103**	1489 – 1493 (541719)	402/400/398/396 (14, 26, 24, 8); 367/365/363/361 (6, 20, 19, 6); 329/327/325/324/322/319/317/315/313 (16, 21, 10, 12, 13, 17, 62, 80, 35); 287/285/283/281/279 (6, 26, 55, 33, 7); 269/267/265 (8, 8, 5); 249/247/245/243/241/239/237/235/233 (25, 100, 82, 13, 15, 11, 25, 58, 46); 205 (18); 203/201/199/197 (18, 48, 68, 15); 187/185 (12, 15); 168 (12); 167/166/165 (39, 35, 83); 161 (13); 155/154/153 (19, 14, 54); 139 (16); 131/130/129/128/127/126/125 (19, 29, 48, 13, 25, 19, 43); 117/115 (12, 13); 105 (12); 91 (31); 77 (13); 65 (10); 51 (9)
28.26	1521 – 1525 (26084)	401 (3); 355 (4); 327 (4); 296 (17); 264 (62); 246 (17); 235 (26); 221 (31); 207 (19); 193 (15); 180 (17); 166 (34); 148 (46); 134 (38); 109 (46); 95 (95); 81 (100); 67 (97); 55 (64) * Suspected contaminant
28.53	1548 – 1552 (78888)	299 (12); 298 (63); 269 (17); 255 (67); 241 (38); 227 (27); 213 (49); 199 (100); 185 (54); 171 (32); 157 (48); 143 (78); 129 (25); 115 (13); 101 (43); 83 (16); 67 (14); 55 (34) * Suspected contaminant

Table 3.5 Retention time and corresponding mass spectral data for the purified compounds of *P. corallorhiza*

No.	GC (HP-5) Time (min.)	GC-MS (DB-1) Time (min.)	m/z (absorbance %)
102	19.22	19.38	297/295/293 (2, 4, 2); 253/251/249 (5, 20, 15); 215/213 (13, 13); 197/195 (8, 7); 171/169 (13, 37); 134 (22); 133 (100); 115 (7); 107/105 (7, 8); 93/91 (10, 13); 79/77 (29, 14); 53 (7)
106	21.04	21.33	209 (34); 207 (37); 177 (53); 175 (50); 149 (6); 147 (5); 127 (53); 111 (50); 95 (100); 79 (28); 67 (39); 55 (9)
105	21.36	22.37	223/221 (20, 19); 191/189 (11, 13); 143/141 (8, 79); 127 (3); 111 (29); 109 (100); 95 (7); 79 (15)
107	22.19	22.53	317/315/313 (12, 24, 13); 285/283/281/279/277 (7, 16, 17, 20, 16); 267/265 (13, 10); 247/245/243/241/239/237/235/233/231/229/227/ 225 (18, 12, 8, 9, 11, 18, 26, 30, 9, 11, 15, 15); 219/217 (15, 16); 203/201/199/197/195/193 (31, 47, 30, 52, 41, 51); 186 (21); 169 (18); 167/165/163/161/159/158/157/155/153/151/150/149 (18, 53, 68, 33, 40, 40, 25, 100, 27, 32, 23, 16, 21); 131/129/127 (44, 43, 20); 119 (76); 105 (34); 91 (53); 79 (62); 65 (20); 51 (20)
101	23.06	23.29	323/321/319/317 (6, 23, 38, 19); 285/283/281 (17, 35, 20); 273/271/269 (6, 13, 7); 249/247/245/243/241/239/237/235/233 (9, 28, 25, 18, 20, 26, 30, 11, 9); 223/221 (9, 7); 209 (14); 207 (14); 205 (22); 204/203/202/201/199/197/195/193/191/189/187 (14, 14, 22, 22, 71, 33, 100, 11, 17, 13, 11, 10, 16, 8); 183/181 (18, 12); 173 (11); 170 (13); 169/168/167/166/165/164/163/162/161/ 157/155/154/153/151/150/149 (11, 13, 23, 44, 51, 37, 165, 11, 34, 19, 44, 10, 30, 26, 55, 27, 13, 37); 141/139/138/136 (18, 29, 14, 18); 133/132/131/130/129/128/127 (22, 17, 36, 27, 40, 20, 36); 123 (20); 115 (41); 105/103/101 (23, 16, 13); 91 (42); 77 (24); 65 (27); 51 (16)
96	23.13	23.31	321/319 (1, 1); 285/283/281 (1, 2, 1); 249/247/245 (1, 2, 2); 231/229 (4, 3); 203/201 (5, 6); 196/195/194 (10, 4, 8); 169/168/167/165 (4, 7, 10, 8); 155/153/151/149 (7, 8, 8, 11); 139 (5); 136 (6); 133/131/129/127/125/123 (10, 12, 7, 16, 70, 100); 117/116/115/113 (26, 12, 66, 11); 105 (11); 91 (20); 89/87 (19, 50); 79/77 (66, 28); 65 (11); 51 (21)
100	24.06	24.15	287/285/283/281 (10, 19, 13, 4); 251/249/247 (9, 32, 25); 213 (17); 211 (17);
99	24.13	24.27	205/203 (25, 34); 182/180 (9, 7); 169 (40); 168 (23); 167 (100); 141/139 (10, 14); 133 (24); 132 (17); 131 (42); 127 (15); 115 (13); 105 (33); 91 (42); 79/77 (17, 17); 67/65 (36, 21); 51 (8)
35	27.13	27.24	402/400/398/396 (14, 26, 24, 8); 367/365/363/361 (6, 20, 19, 6); 329/327/325/324/322/319/317/315/313 (16, 21, 10, 12, 13, 17, 62, 80, 35); 287/285/283/281/279 (6, 26, 55, 33, 7); 269/267/265 (8, 8, 5);

			249/247/245/243/241/239/237/235/233 (25, 100, 82, 13, 15, 11, 25, 58, 46); 205 (18); 203/201/199/197 (18, 48, 68, 15); 187/185 (12, 15); 168 (12); 167/166/165 (39, 35, 83); 161 (13); 155/154/153 (19, 14, 54); 139 (16); 131/130/129/128/127/126/125 (19, 29, 48, 13, 25, 19, 43); 117/115 (12, 13); 105 (12); 91 (31); 77 (13); 65 (10); 51 (9)
94	26.36	27.37	367/365/363/361 (3, 7, 8, 3); 331/329/327/325 (2, 5, 6, 3); 295/293/291/289/287/285/283/281 (2, 4, 3, 2, 3, 6, 11, 9); 249/247/245 (8, 20, 13); 215/214/213/212/211 (7, 26, 18, 22, 15); 197/195/193 (3, 10, 6); 171/169/167/165 (10, 31, 35, 13); 133/131 (100, 33); 117/115 (43, 41); 105 (76); 91 (62); 79/77 (23, 18); 65 (12); 51 (10)
103	27.11	28.09	404/402/400/398/396 (2, 6, 8, 8, 4) (Same MS as compound **35** above)
104	-	29.31	388/386 (28, 100); 372/370/368 (38, 19, 55); 353 (44); 326 (9); 301 (73); 275/273 (22, 24); 255 (30); 246 (10); 231 (34); 213 (37); 199 (18); 185 (16); 161 (21); 159 (21); 145 (25); 131 (15); 119 (12); 105 (17); 95 (16); 67 (7)

* Compounds **99** and **100** had the same MS fragmentation pattern, but slightly different retention times

** Compounds **35** and **103** had the same MS fragmentation pattern, however differed slightly at the highest halogen cluster from 404-396

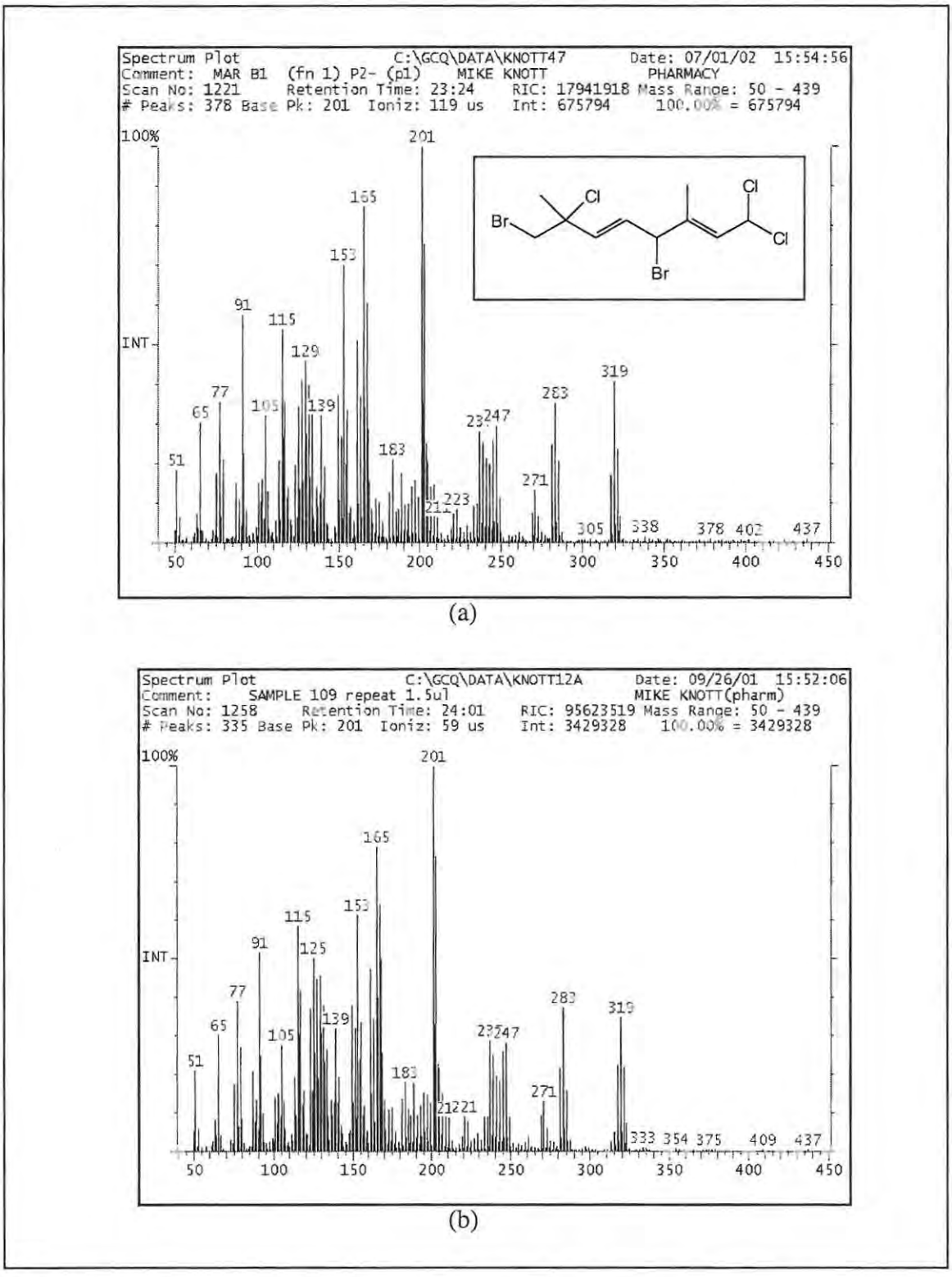
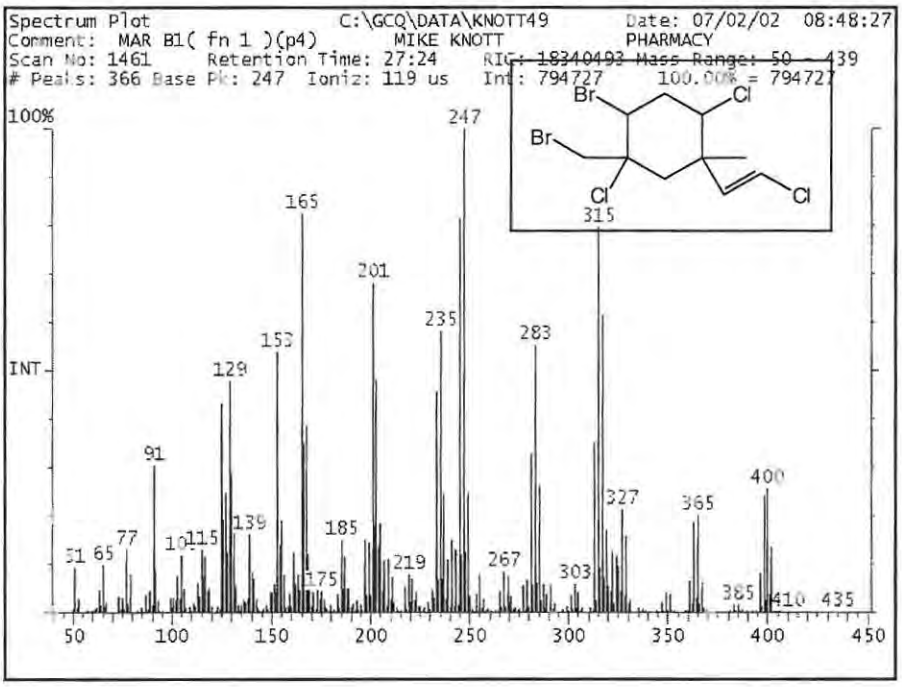
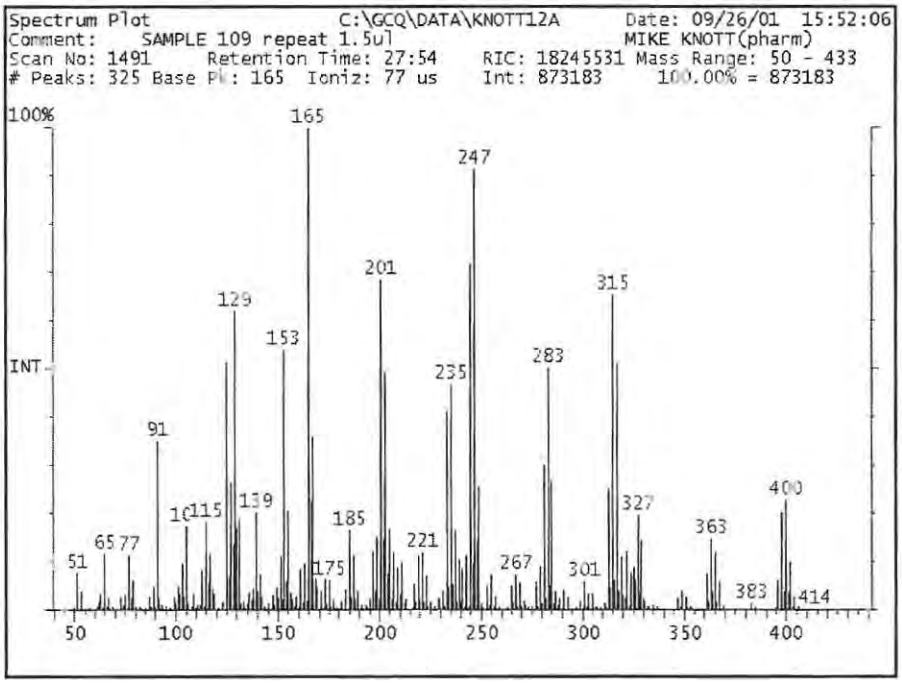


Figure 3.2 Mass spectrum of pure compound **101** (a) was the same as the MS from the major peak at 24.01 minutes (b) seen in the crude trace of *P. corallorhiza*



(a)



(b)

Figure 3.3 Mass spectrum of pure compound **35** (a) was the same as the MS from the major peak at 27.54 minutes (b) seen in the crude trace of *P. corallorhiza*

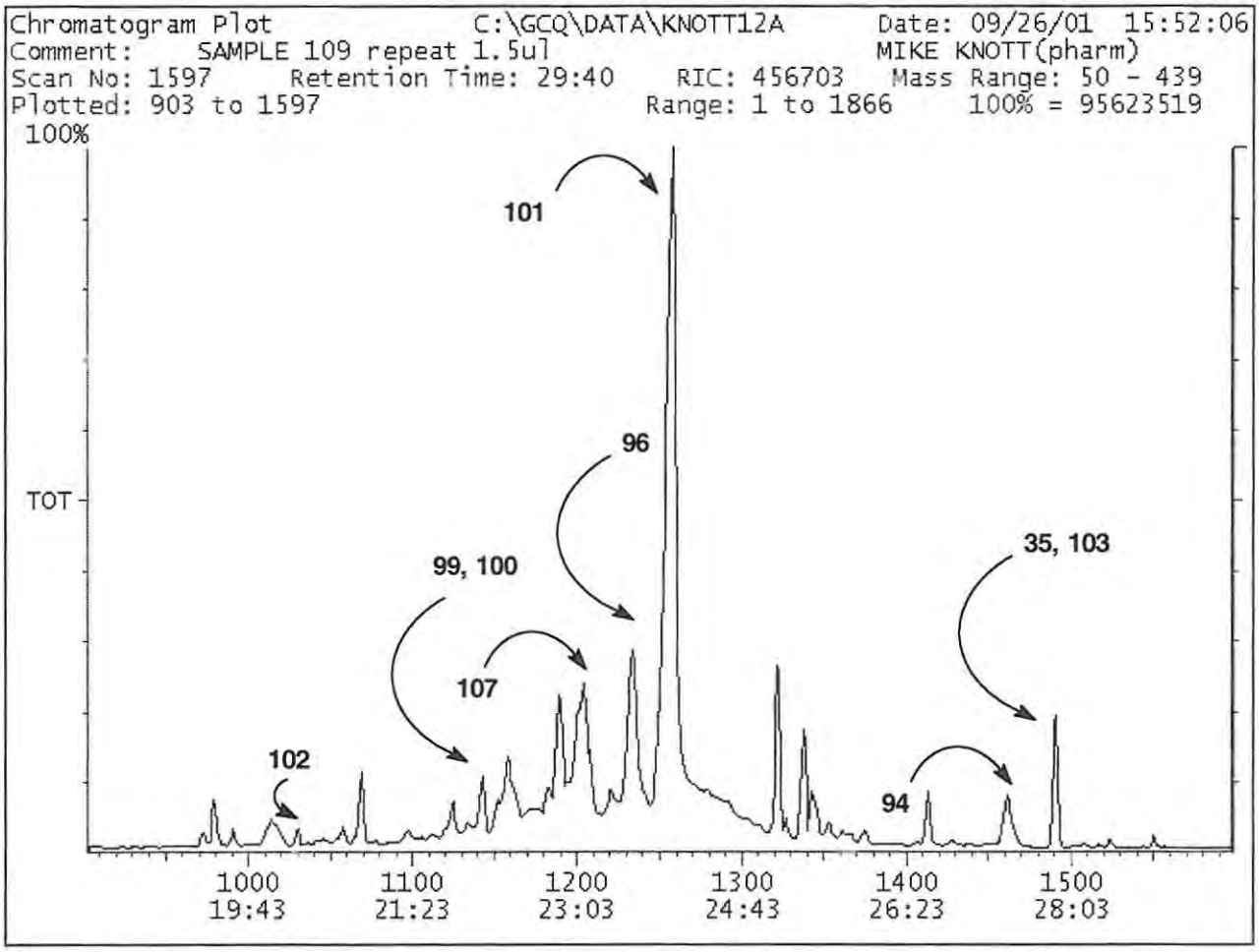


Figure 3.4 Purified compounds were identified on the crude trace by exact MS fragmentation patterns and similar retention times (Retention time from 18.03 to 29.43 minutes)

Table 3.6 Common impurities characteristic of some MS fragmentation patterns include the following:[56]

m/z Values	Cause
149, 167, 279	Plasticizers (phthalic acid derivatives)
129, 185, 259, 329	Plasticizer (tri-n-butyl acetyl citrate)
133, 207, 281, 355, 429	Silicone grease
99, 155, 211	Plasticizer (tributyl phosphate)

3.2.2 A comparison of the metabolite profiles of *Plocamium* spp.

3.2.2.1 Chemo-taxonomy of the *Plocamium* spp.

South Africa has a large population of endemic marine algae that present a formidable challenge to marine taxonomists. Polyhalogenated monoterpenes have been isolated from several species of red algae, including: *Plocamium*, *Desmia*, *Microcladia* and *Ochtodes*. No simple correlation exists between the patterns of occurrence of such compounds and their taxonomy, especially in the Plocamiaceae family. Taxonomists specialising in the identification of marine algae often struggle to differentiate between certain members of the Plocamiaceae family. In an attempt to address these taxonomic complications, we have investigated the possibility of using halogenated monoterpenes as chemotaxonomic markers for three *Plocamium* spp. (obtained from southern Africa).

P. cartilagineum demonstrates chemical variation amongst individuals[52] and thus illustrates a complete lack of any chemotaxonomic relationship; while *P. violaceum* was found to be chemically homogenous amongst individuals.[59] Although the chemical concentration of *P. violaceum* was constant with location, and did not exhibit seasonal variation, it did display three distinct chemical forms. Studies on *P. cartilagineum* from the Chilean coast have revealed little individual variation (for a particular location), however like *P. violaceum*, the Chilean *P. cartilagineum* displays ecotypes.[60] *P. hamatum* was obtained from two different localities in Australia, and both these samples contained different chemical constituents.[36]

P. coccineum was collected at various times of the year (either intertidally or by dredging) from different locations off the coast of Spain.[28] In contrast to other studies (mentioned above), the composition of *P. coccineum* remained unchanged.

3.2.2.2 A comparison of the metabolite profiles of *P. corallorhiza* collected from three different locations along the South African coast (Riet River, Kalk Bay and Holbaai)

Three different collections were made to obtain *P. corallorhiza*. Differences in geographical location, and seasonal variance were essential to the study. Collection sites included: Riet River (Sept. 2001) (Figure 3.5), Kalk Bay (Mar. 2002) (Figure 3.6) and Holbaai (Nov. 2001) (Figure 3.7). The samples were analysed in duplicate and were extracted according to a standard procedure. The chromatographic trace of each geographically different sample was expanded, and overlaid (Figure 3.8). Table 3.7 indicates the dry and wet weight of the samples that were used.

GC traces obtained from crude methanolic extracts of *P. corallorhiza* (from the same geographical location) contained the same compounds with similar concentration (based on retention time, and peak height). This illustrates a lack of individual variation. Once it was established that little variation between the same plant, or plants, of the same location was evident (in terms of compound type and concentration), the next step was to compare the same species from different locations by overlaying the data. Although a slight difference in concentration existed between *P. corallorhiza* from different locations, each specimen still contained the same major secondary metabolites. This was an unusual find, since chemical variation between individual species of *P. cartilagineum* is well documented. These results are also in contrast to those of *P. violaceum*, which demonstrate a significant difference in the monoterpene concentration (from the same species) collected from different locations.

Although the monoterpene concentration of *P. corallorhiza* varies slightly between different locations, it can be suggested that this has little do to with seasonal or geographical variation. These preliminary results also indicate that *P. corallorhiza* from different parts of the south coast of South Africa all contain the same major metabolites.

Table 3.7 Mass of *P. corallorhiza* used for extraction

Location and date of collection	**Wet weight of frozen material**	**Dry weight of hexane fraction**	**Comment/ appearance**
Riet River (Sept.2001)	9.88g	14.5mg	Dark purple
Riet River (Sept.2001)	10.37g	16.9mg	Dark purple
Kalk Bay (Mar. 2002)	10.34g	32.8mg	Fresh sample
Kalk Bay (Mar. 2002)	9.6g	32.3mg	Fresh sample
Holbaai (Nov. 2001)	10.32g	12.1mg	Dull/pinkish colour
Holbaai (Nov. 2001)	10.62g	15.6mg	Dull/pinkish colour

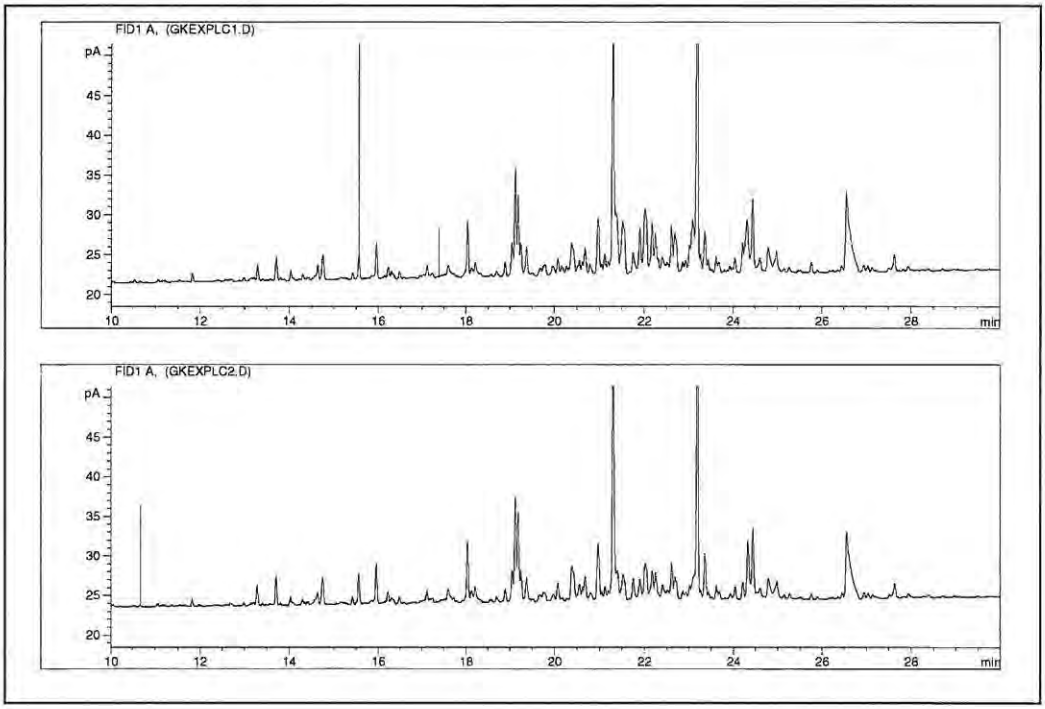


Figure 3.5 GC chromatogram of *P. corallorhiza* (Sept. 2001) Riet River (Retention time from 10 to 30 minutes)

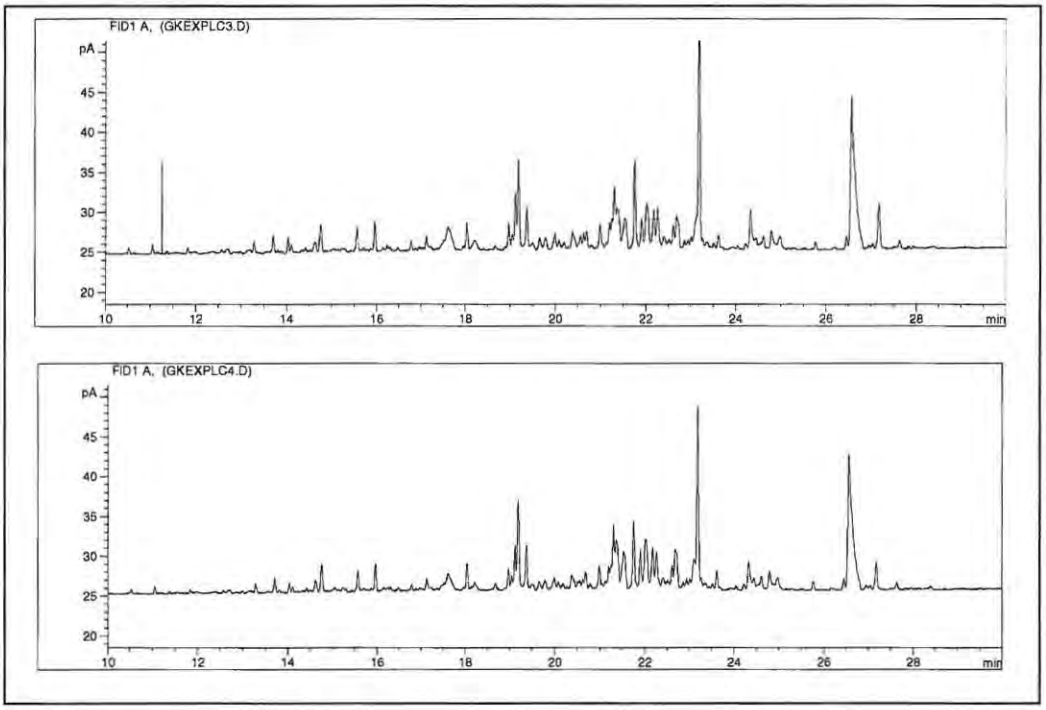


Figure 3.6 GC chromatogram for *P. corallorhiza* (Mar. 2002) Kalk Bay (Retention time from 10 to 30 minutes)

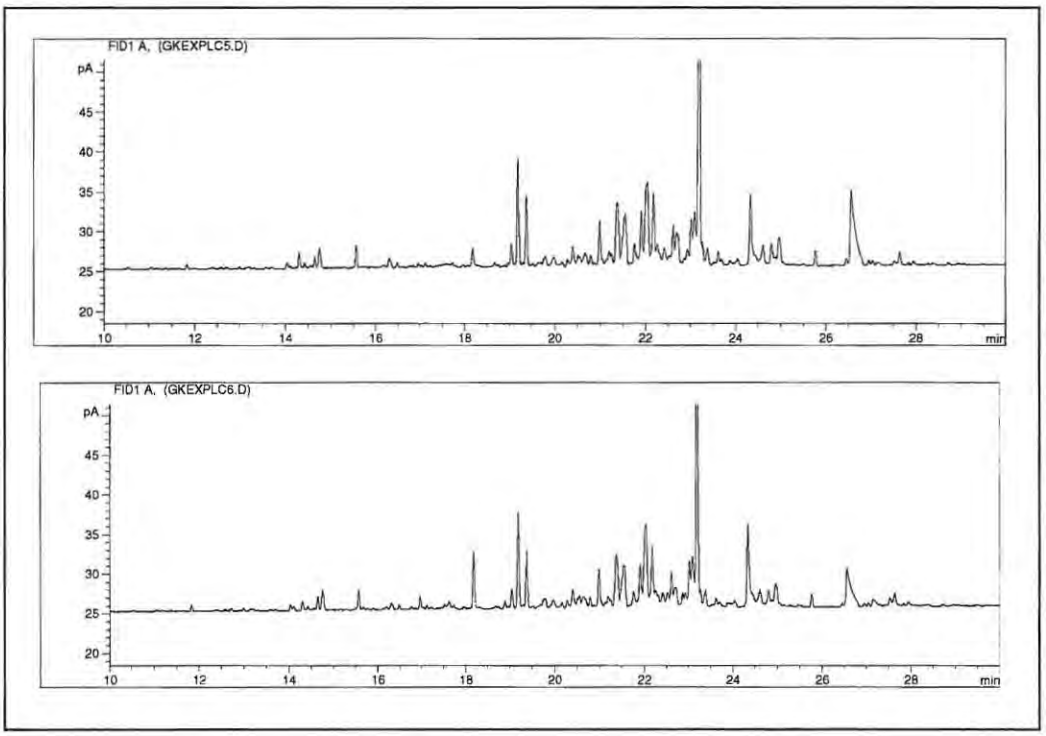


Figure 3.7 GC chromatograms for *P. corallorhiza* (Nov. 2001) Holbaai (Retention time from 10 to 30 minutes)

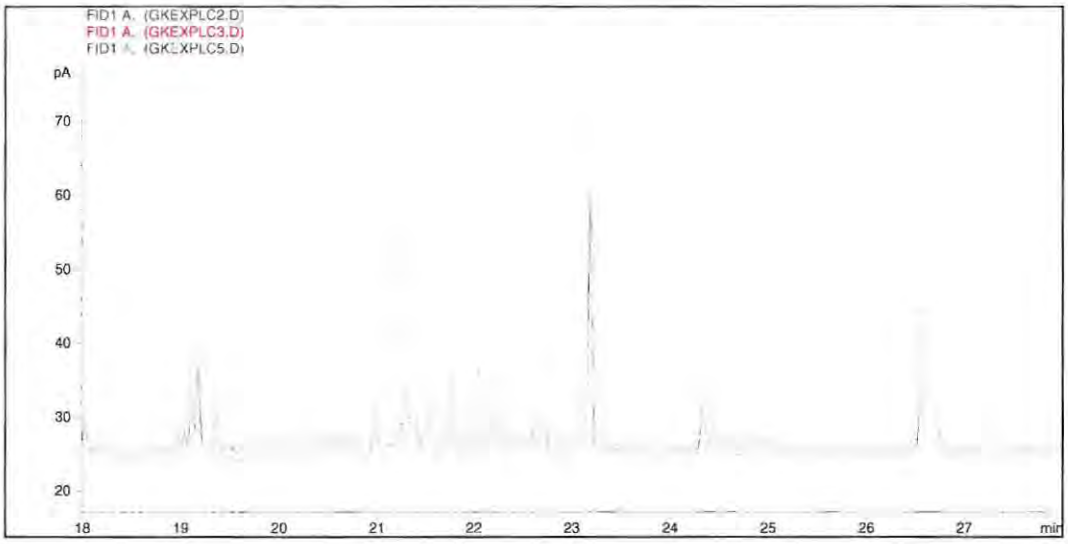


Figure 3.8 Three GC chromatograms of *P. corallorhiza* collected from different locations at different times of the year. (Retention time from 18 to 28 minutes) [*P. corallorhiza* (Sept. 2001) Riet River (blue), *P. corallorhiza* (Mar. 2002) Kalk Bay (red), *P. corallorhiza* (Nov. 2001) Holbaai (green)]

3.2.2.3 A comparison of the metabolite profiles of *P. cornutum* from Kalk Bay and Holbaai

Two different collections for *P. cornutum* were undertaken. These collections took place at Holbaai (Nov. 2001) (Figure 3.9) and Kalk Bay (Mar. 2002) (Figure 3.10) respectively. The chromatographic trace of each geographically different sample was expanded, and overlaid (Figure 3.11).

Based on retention time and peak height, each GC trace (for *P. cornutum*) from the same location contained similar concentrations of the same compound. Little difference in the composition of metabolites was noted from different parts of the same marine algae, or marine algae from the same location. This phenomenon was also observed in *P. corallorhiza*.

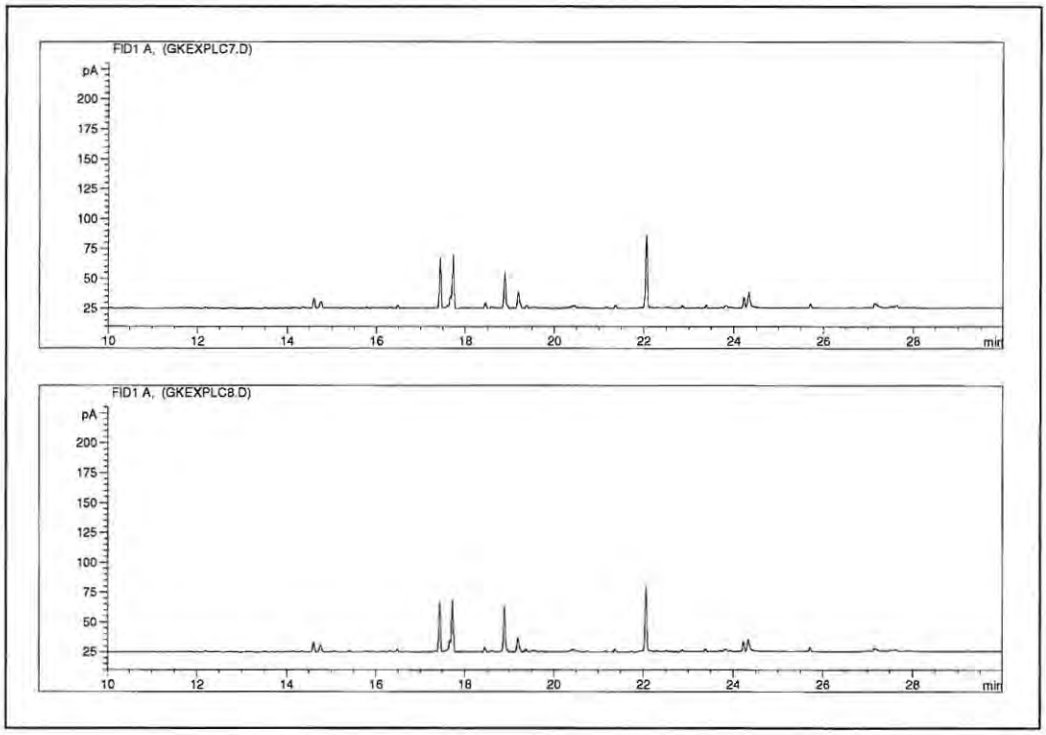


Figure 3.9 GC chromatogram for *P. cornutum* (Nov. 2001) Holbaai (Retention time from 10 to 30 minutes)

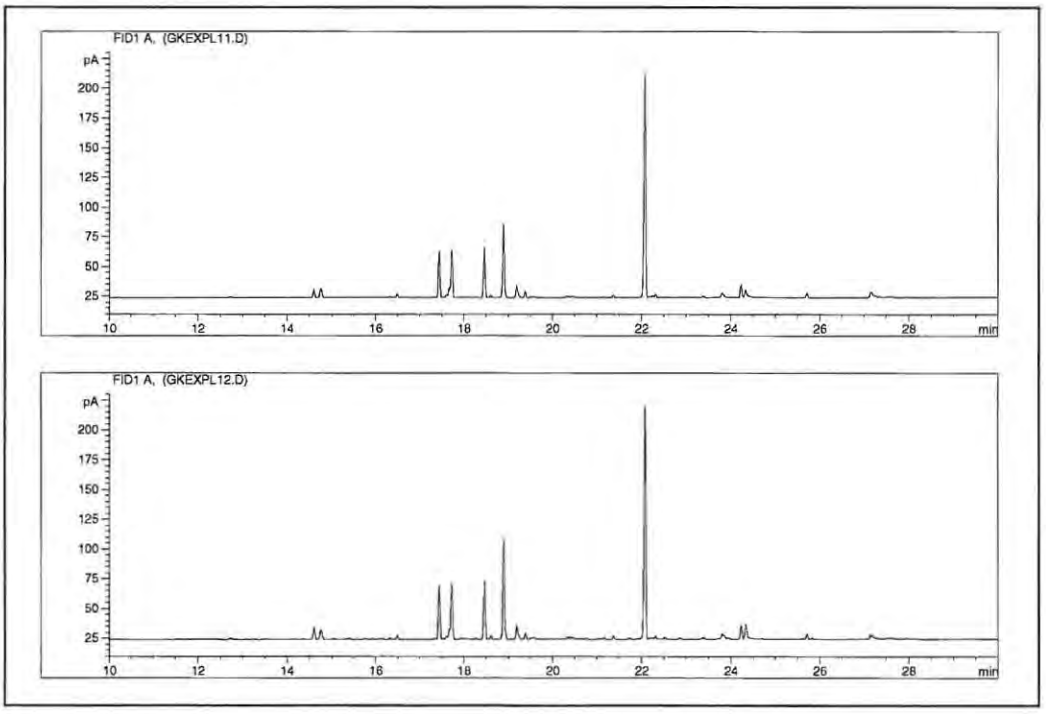


Figure 3.10 GC chromatogram for *P. cornutum* (March 2002) Kalk Bay (Retention time from 10 to 30 minutes)

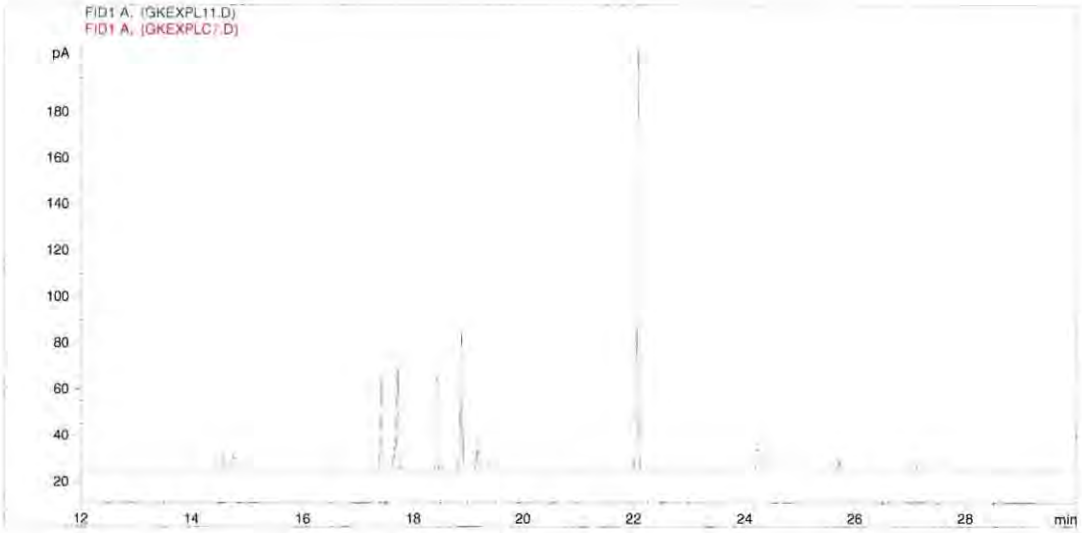


Figure 3.11 GC chromatogram for *P. cornutum* collected from two separate locations (Retention time from 12 to 30 minutes) [*P. cornutum* (Mar. 2002) Kalk Bay (blue), *P. cornutum* (Nov. 2001) Holbaai (red)]

3.2.2.4 A comparison of the metabolite profiles of different *Plocamium* spp.

A collection of different *Plocamium* species was made at Kalk Bay (Cape Town) during March 2002, *P. corallorhiza* (Figure 3.12), *P. cornutum* (Figure 3.13) and *P. maxillosum* (Figure 3.14) were collected, and identified by Prof. J. Bolton. Table 3.8 indicates both the dry, and wet weight for each of the samples used.

Duplicate results from the same *Plocamium* spp. from Kalk Bay contained the same compounds with approximately the same concentration. Of the three different species of *Plocamium* that were tested, each species was found to have its own characteristic GC trace. GC could therefore be used as a quick and reliable method to determine the characteristic monoterpene patterns exhibited by these three *Plocamium* spp. These characteristic patterns could therefore be used as a method for the chemotaxonomic identification of different *Plocamium* species.

These studies indicate that ecotypes and chemotypes are not a significant feature of *P. corallorhiza* and *P. cornutum*. A comparison between *P. cornutum* from Kalk Bay (Mar. 2002) and Holbaai (Nov. 2001) showed that slight chemical variations do occur. Seasonal or geographical aspects may affect the concentration of these secondary metabolites.

Differences in monoterpene concentration were more significant for *P. cornutum* than *P. corallorhiza*.

Different species of *Plocamium* (namely: *P. corallorhiza*, *P. cornutum*, and *P. maxillosum*) were found to have very different chemical profiles. GC may therefore have appreciable taxonomic application in the identification of different *Plocamium* spp. which are endemic to South Africa.

Table 3.8 Mass of *Plocamium* spp. used for extraction

Name of Plocamium species tested	Wet weight of frozen material	Dry weight of hexane fraction	Comment/ availability
P. corallorhiza	10.34g	32.8mg	Abundant
P. corallorhiza	9.6g	32.3mg	Abundant
P. cornutum	10.07g	36.9mg	Present
P. cornutum	10.68	42.2mg	Present
P. maxillosum	2.10g	9.9mg	Scarce
P. maxillosum	2.20g	9.9mg	Scarce

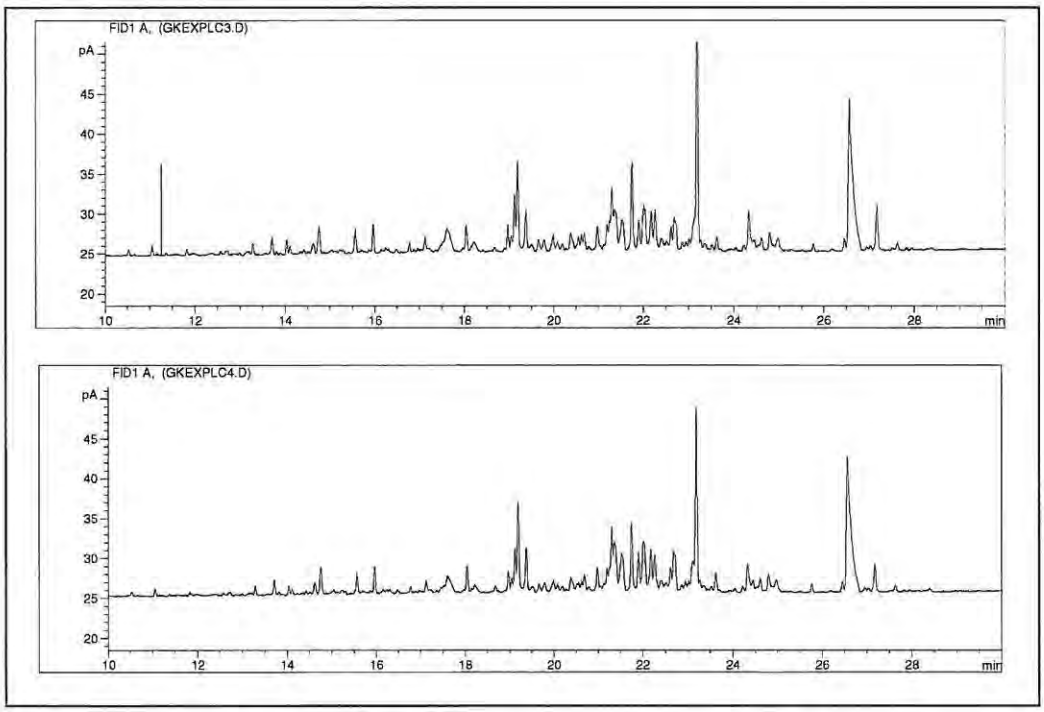


Figure 3.12 GC chromatogram for *P. corallorhiza* (Mar. 2002) Kalk Bay (Retention time from 10 to 30 minutes)

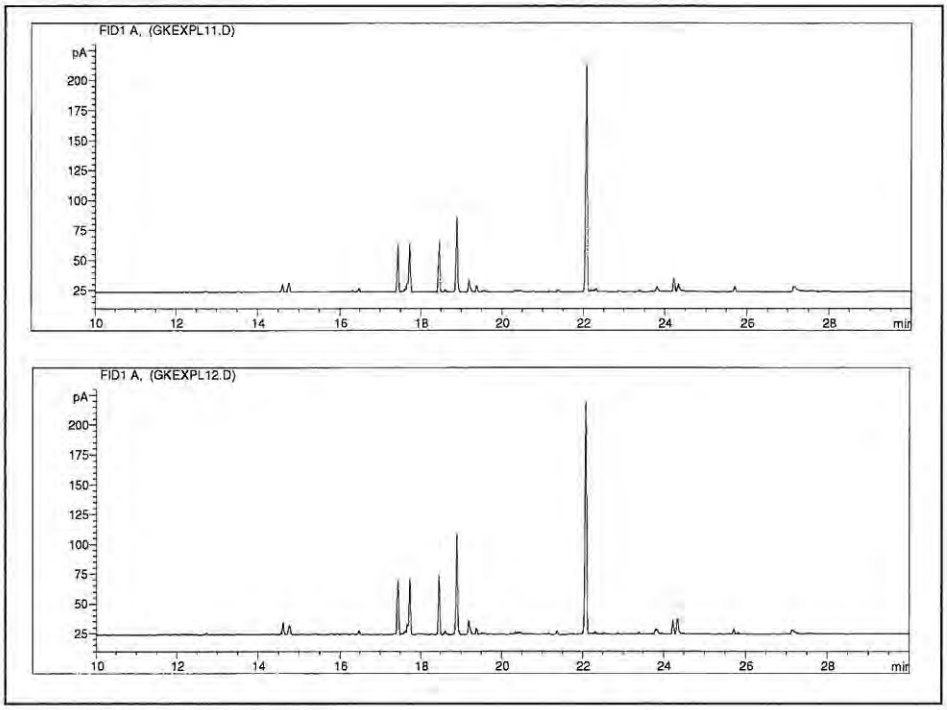


Figure 3.13 GC chromatogram for *P. cornutum* (Mar. 2002) Kalk Bay (Retention time from 10 to 30 minutes)

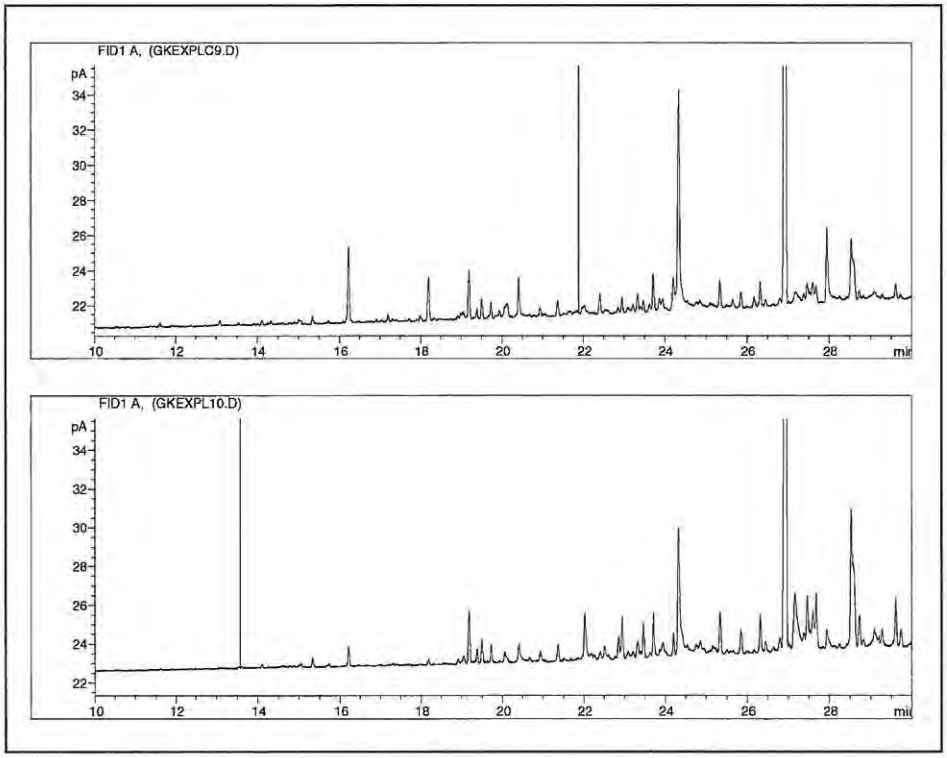


Figure 3.14 GC chromatogram for *P. maxillosum* (Mar. 2002) Kalk Bay (Retention time from 10 to 30 minutes)

3.2.2.5 A comparison of the metabolite profiles from different *Plocamium* spp. using Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS was used to explore the possibility of using monoterpene content to show phyletic differences within the Plocamiaceae family. The following criteria need to be fulfilled before this could be accomplished: [10]

i) There should be no ecotypes or chemotypes respectively

ii) A number of monoterpenes must exist, and the structure of these metabolites must be known

Comparing variation in chemical content and concentration between plants (of *Plocamium*) collected from the same location by overlaying GC traces is reasonable, since compounds with the same retention time should indicate the same compound.

GC can be used as a valuable technique when comparing the metabolite profile of the same species, as well as the metabolite profiles of different species (as seen in 3.2.2.4).

Although interspecies metabolite variation between three different *Plocamium* spp. was noted, further investigation was required to determine the chemical characteristics of the minor metabolites for each *Plocamium* spp. By comparing the fragmentation patterns of known secondary metabolites to the fragmentation patterns obtained from a crude trace, in conjunction with relative retention times for each metabolite; the identity of a known compound in a crude trace becomes possible.

The GC-MS metabolite profiles of three different species of *Plocamium* spp. were compared by using the same samples that were used in Sections 3.2.2.2, 3.2.2.3 and 3.2.2.4. The crude extract of each *Plocamium* spp. was made up to exactly the same concentration. The samples that were tested include: *P. corallorhiza* (Sept. 2001, Riet River) (Figure 3.15), *P. cornutum* (Nov. 2001, Holbaai) (Figure 3.16), and *P. maxillosum* (Mar. 2002, Kalk Bay) (Figure 3.17). These samples where specifically selected because of their complete lack of geographical and seasonal similarity. The mass spectrum of each peak in the crude extract was then compared to the mass spectrum of the known metabolites to determine any similarities between different species from the same genus (Table 3.9, Figures 3.18-3.24).

Possible differences in the chromatogram of *P. corallorhiza* illustrated in section 3.2.1 and that of section 3.2.2.5 are attributed (amongst other differences in the extraction procedure) to the duration of the solvent extraction procedure (which differs by more than 10 hours) (See Experimental).

Although the major metabolites of *P. corallorhiza*, *P. cornutum* and *P. maxillosum* differ significantly, these results indicate that minor metabolites may be common to different *Plocamium* spp. (for example, compound **102**). Each compound has its own unique characteristic fragmentation pattern, however, different compounds may sometimes have the same mass spectra. This unusual characteristic was *only* seen with compounds **99** and **100** (Table 3.9). Methoxy compounds (**104-107**) were not observed to be present in any of the crude extracts. This could therefore indicate that these compounds are artifacts of the extraction procedure.

GC-MS therefore proves to be an effective tool in rapidly determining the chemical content of various *Plocamium* spp., as long as the MS of known compounds are available and that each compound has its own unique MS.

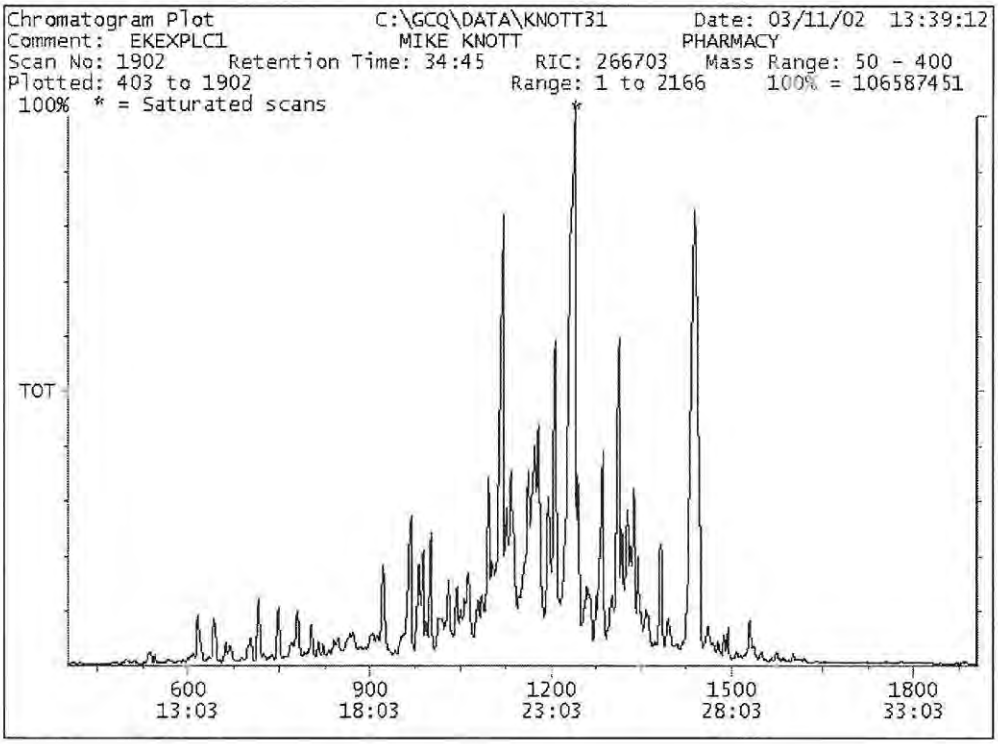


Figure 3.15 GC-MS trace for *P. corallorhiza* (Sept. 2001, Riet River) (Retention time from 11.48 to 34.18 minutes)

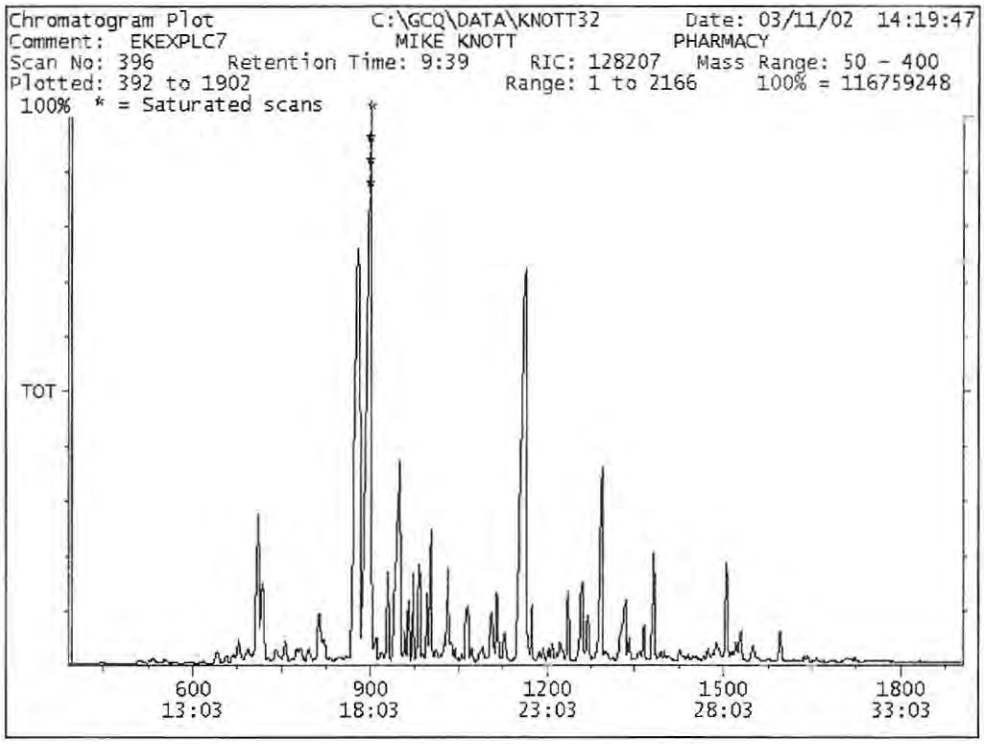


Figure 3.16 GC-MS trace for *P. cornutum* (Nov. 2001, Holbaai) (Retention time from 11.48 to 34.18 minutes)

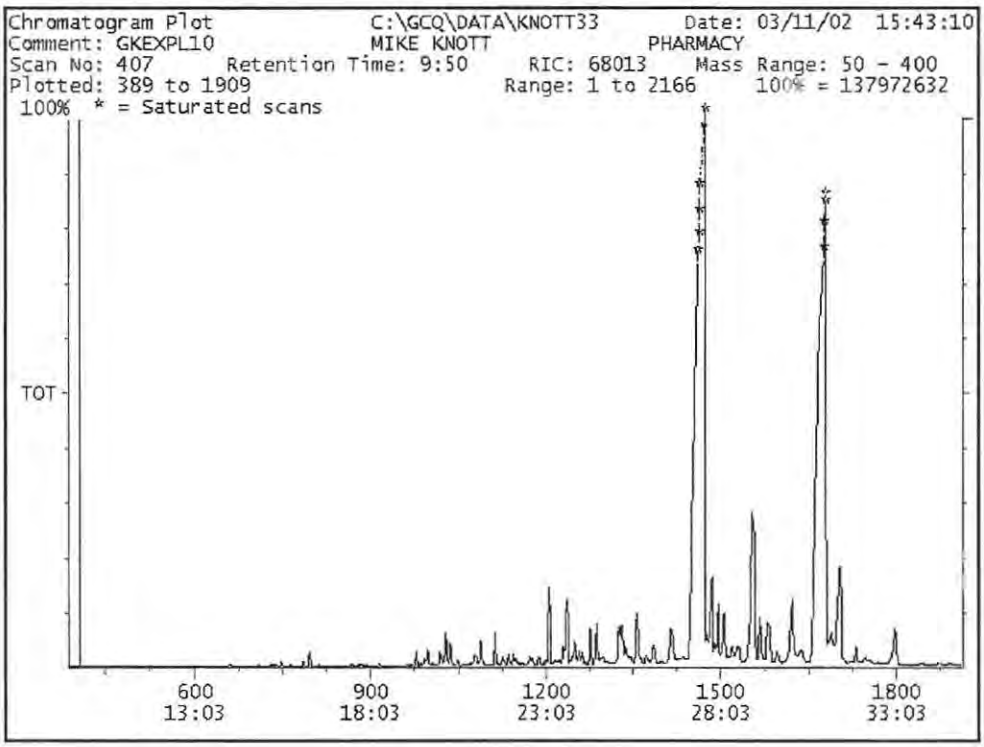


Figure 3.17 GC-MS trace for *P. maxillosum* (Mar. 2002, Kalk Bay) (Retention time from 11.48 to 34.18 minutes)

Table 3.9 Purified compounds were identified from the crude trace for each *Plocamium* spp. by means of exact MS fragmentation patterns and similar retention times

Pure Compound No./ retention time (min.)	***P. corallorhiza*/ retention time (min.)**	***P. cornutum*/ retention time (min.)**	***P. maxillosum*/ retention time (min.)**
102 (19.38)	19.45	19.46	19.41
101 (23.29)	23.44		
96 (23.31)	22.58		
100 (24.15)*	24.29*	24.12*	
99 (24.27)*	24.29*	24.12*	
35 (27.24)	27.24		
94 (27.37)	27.02		
103 (28.09)	27.41	25.08	

* Compound **99** and **100** have the same MS fragmentation patterns

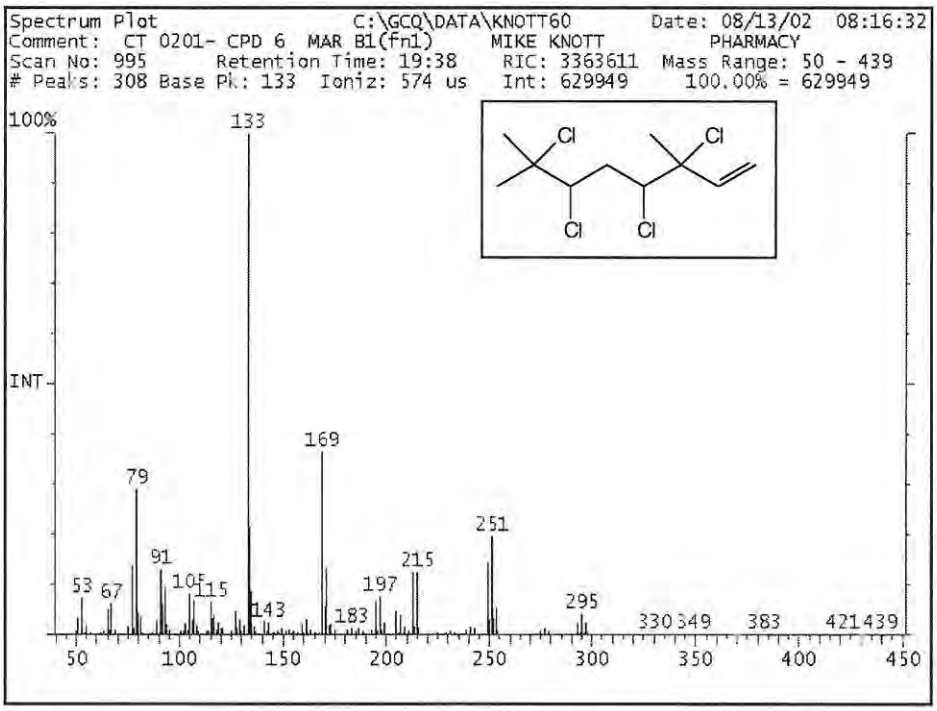


Figure 3.18 Mass spectrum of 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**102**)

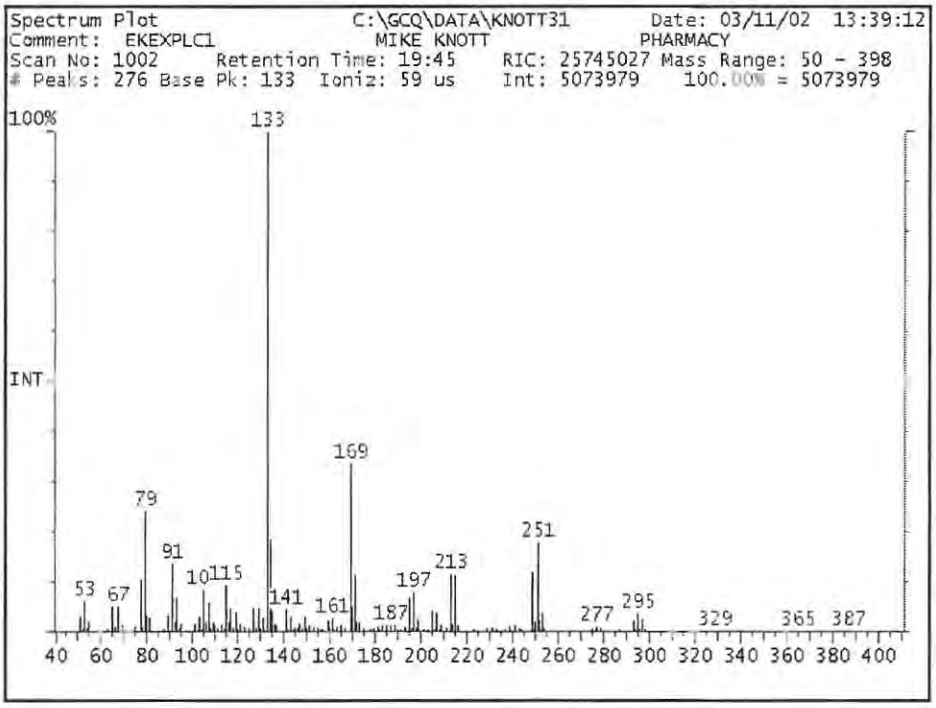


Figure 3.19 Mass spectrum of a peak at 19.45 minutes from a crude extract of *P. corallorhiza*

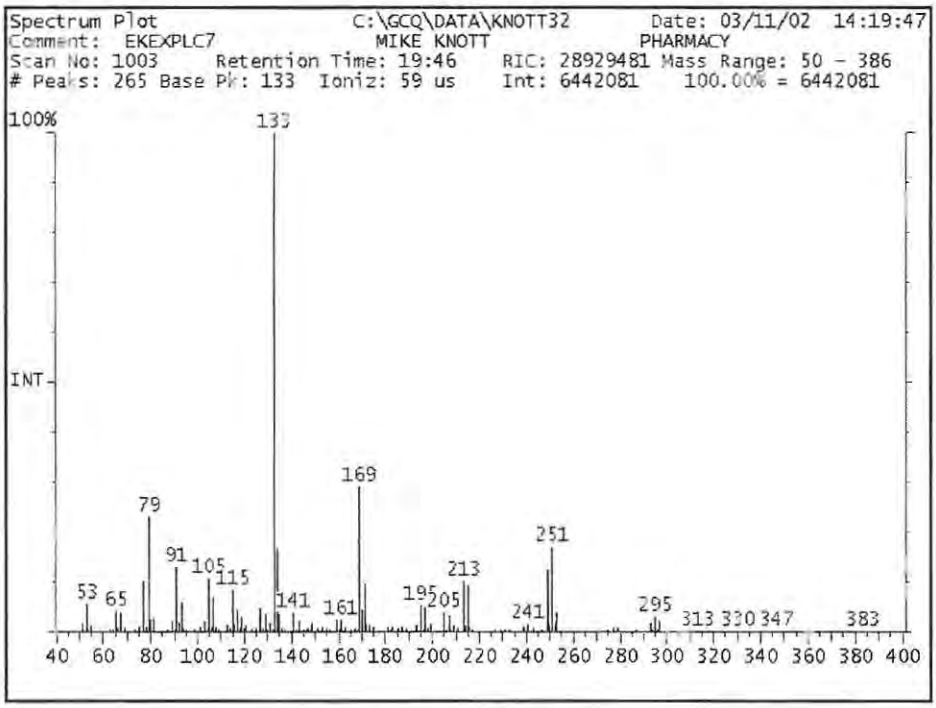


Figure 3.20 Mass spectrum of a peak at 19.46 minutes from a crude extract of *P. cornutum*

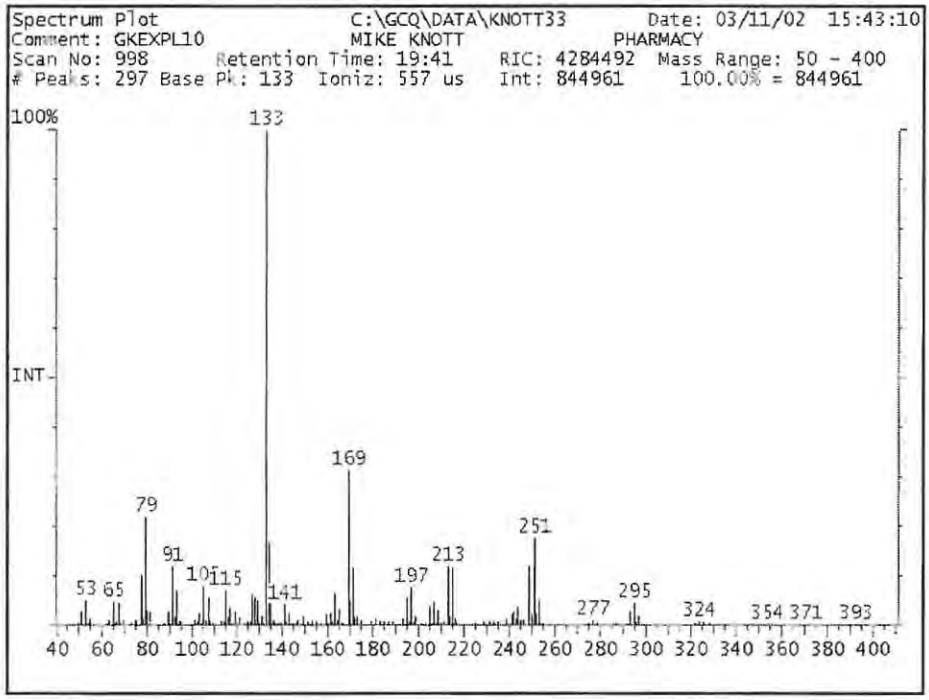


Figure 3.21 Mass spectrum of a peak at 19.41 minutes from a crude extract of *P. maxillosum*

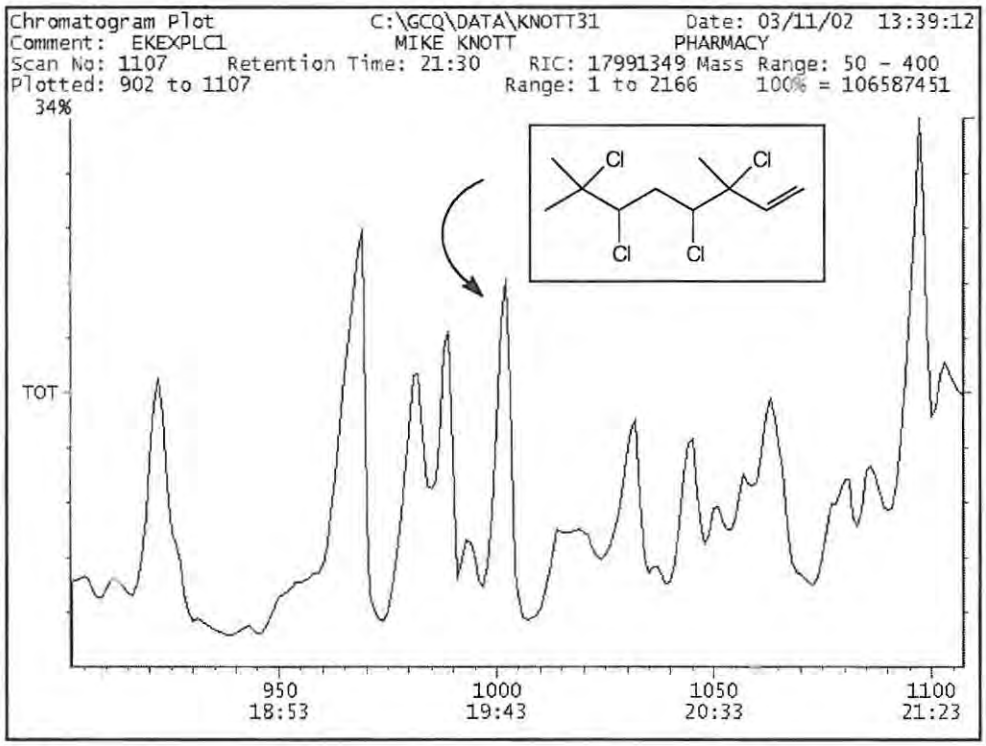


Figure 3.22 GC expansion of the crude extract from *P. corallorhiza* (from Figure 3.15)

(Retention time from 18.03 to 21.30 minutes)

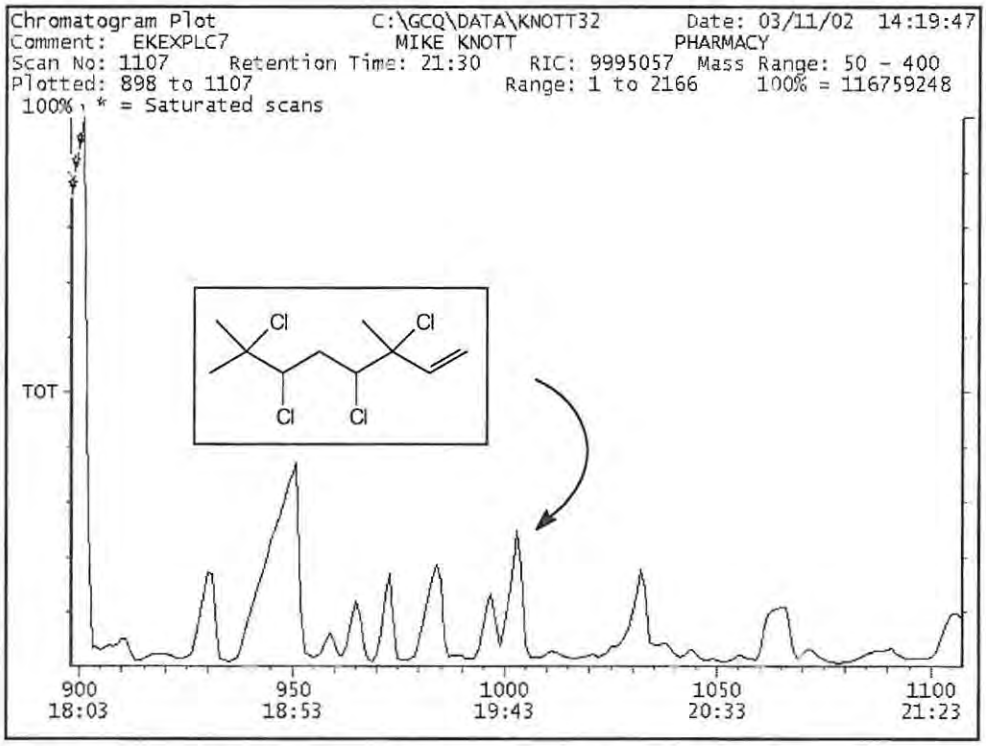


Figure 3.23 GC expansion of the crude extract from *P. cornutum* (from Figure 3.16)

(Retention time from 18.00 to 21.30 minutes)

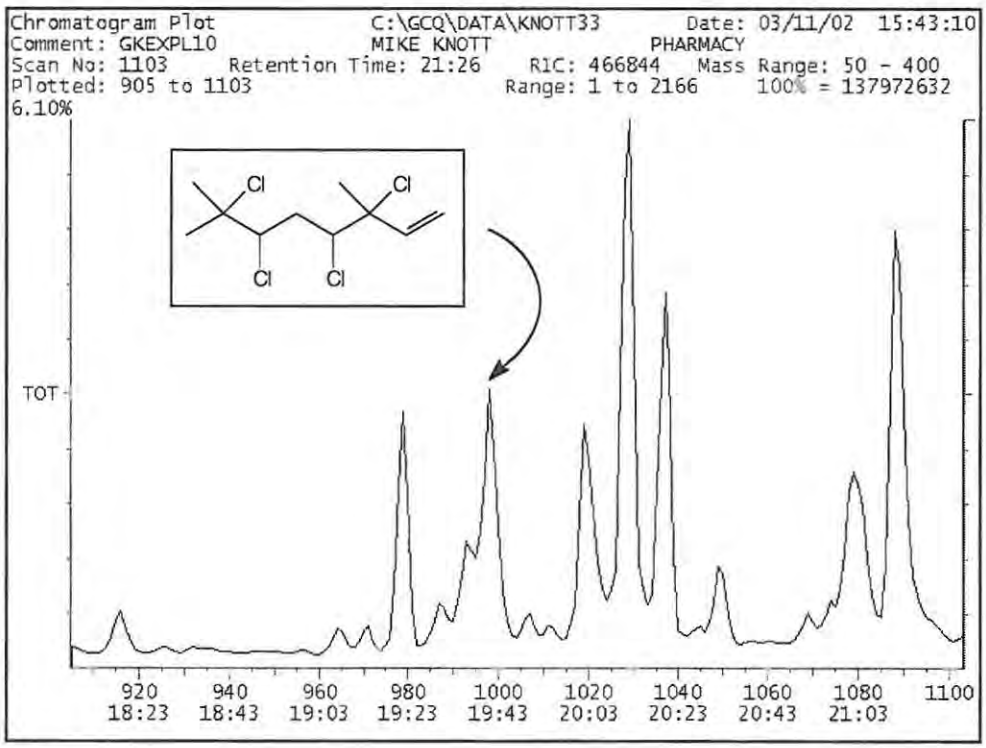


Figure 3.24 GC expansion of the crude extract from *P. maxillosum* (from Figure 3.17)

(Retention time from 18.09 to 21.25 minutes)

Chapter Four

Bioactivity of South African Marine Algae

4.1 Introduction

The relative bioactivity of crude extracts obtained from known or unknown marine algae can be extremely useful as a preliminary guide in the search for pharmacologically active marine natural products. The brine shrimp (*Artemia salina*) assay, [61] as well as various other antimicrobial tests, can be useful tools for indicating the presence of potentially cytotoxic or antimicrobial compounds which are present in the crude extract. Both these assays are routinely used as cheap, simple, and "relatively" reliable methods for screening. By means of bioassay-guided fractionation, these techniques also find application in the isolation of active compounds that are present in crude extracts. Organohalogen compounds (typically found in marine organisms) are thought to act as feeding deterrents, irritants, or poisons.[11] A direct correlation has been observed between the presence of organohalogen compounds and antimicrobial activity. [11] In addition to the widespread antibiotic activity of organohalogen compounds, other biological activity such as anti-tumor, antifungal, insecticidal, and herbicidal activity have also been reported.

The discovery of halomon (**98**) sparked off a renewed interest into polyhalogenated monoterpenes. This acyclic monoterpene exhibited a novel mode of action in the primary screen used by the National Cancer Institute (NCI). It was also shown to be one of the most extreme examples of differential cytotoxicity yet observed for that particular primary screen.[50]

Initial pharmacological studies on polyhalogenated monoterpenes (**13, 95, 96, 97**) warned of the potent mutagenicity or carcinogenicity of natural products from marine algae.[62] Investigations during the early 1980's indicated that polyhalogenated monoterpenes (**42, 43, 44, 64**) had slight cytotoxic and antimicrobial activity.[63] This cytotoxic activity was attributed to the high Br content of polyhalogenated monoterpenes. Insecticidal activity was indicated after Plocamene B (**85**) was shown to be three times more effective than the commercial pesticide lindane against mosquito larvae,[64] which implies that Plocamene B has a potency which is equal to that of dichloro-diphenyl-trichloroethane (DDT).

Telfairiae (**83**) was later shown to be 100% lethal to mosquito larvae at 10 ppm.[40] This activity is extraordinary, and a renewed interest into the chemical characteristics of the Plocamiaceae family occurred. Violaceum (**88**) was later shown to demonstrate potent insecticidal activity, however only mild antifungal activity was present.[22] König *et al.* have dominated the pharmacological investigations of various *Plocamium* spp. during the 1990's.[30, 65-67] These studies have shown that acyclic (**29, 81**) and cyclic monoterpenes (**43, 65**) from *P. cartilagineum* showed little antibacterial activity. However, these experiments showed that these compounds exhibited antifungal properties against *Penicilium. oxalicum* at low levels.[65] Cyclic monoterpenes (**43, 65**) exhibited potent molluscicidal and brine shrimp toxicity against *Biomphalaria glabrata* and *Artemia salina* respectively, while acyclic compounds (**29, 81**) at the same concentration where non-toxic.[65] Acyclic compounds (**12, 14, 66**) from *P. costatum*, have shown little antimicrobial or antialgal inhibitory activity. However, compound (**14**) did demonstrate weak effects toward *Artemia salina*.[30] A cyclic monoterpene (**35**) isolated from *P. hamatum* showed strong antialgal activity, as well as moderate antitubercular and cytotoxic activity. It was suggested that its mode of action was structure specific.[66] In the search for new antimycobacterial leads, a series of marine natural products were tested. One of the compounds that showed activity towards *Mycobacterium tuberculosis* and *M. avium*, was a cyclic monoterpene (**35**) from *P. cartilagineum*. However, the same activity was not present in any of the linear monoterpenes.[67]

Antimicrobial compounds range from acrylic acid, and diterpenoids in green seaweed, halogenated terpenoids in red seaweeds, and metabolites of mixed terpenoid-aromatic origin in brown seaweeds.[8] In a study done by Vlachos *et al* [8], the *Plocamium* genus was represented by *P. corallorhiza* (collected from Port Elizabeth and Isipingo) and *P. rigidum* (collected from Swakopmund). Only the inhibitory activity of the ten most active seaweeds from each of the three divisions: Phaeophyta, Rhodophyta and Chlorophyta were discussed. The Rhodophyta extracts inhibited the majority of Gram-positive bacteria. Little activity was noted against the yeasts, moulds and Gram-negative bacteria, with the exception of *Acinetobacter lwoffi* by the rhodophyte extracts. The *Carradoriella virgata* extract displayed the largest inhibition zones in this division (against the micro-organisms tested in this study). *Portiera hormemannii* and *Plocamium rigidum* showed the broadest spectrum of antimicrobial activity by inhibiting all the Gram-positive bacteria tested. *Laurencia complanata* produced the largest zones of inhibition, but inhibition was evident

against only five of the six Gram-positive bacteria. This indicated that the *Laurencia complanata* extract had a high degree of antibacterial activity, but a narrower spectrum of activity than, for example, *Portiera hormemannii* and *Plocamium rigidum*.

A study done at the University of the Western Cape,[68] tested the antimicrobial activity of South African red algal extracts. Seventeen red algal species were screened for antimicrobial activity against; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, and *Candida albicans*, using agar overlay bioautography. It was found that extracts of *P. corallorhiza* and *Polysiphonia virgata* produced the greatest inhibition zones. In addition to this, *P. corallorhiza* was the only alga that inhibited the growth of *C. albicans*, and proved to be a very broad-spectrum antimicrobial agent.[68]

Both South African studies conducted by Vlachos[8] *et al.* and Cameron[68] (in contrast to König *et al.*[30, 65]), indicate that the genus Plocamiaceae has antimicrobial activity, but these studies failed to identify the compounds responsible for this activity.

4.2 An investigation into the bioactivity of various marine algae using brine shrimp

The cytotoxic activity of a broad range of compounds is manifested as being toxic to brine shrimp. This simple bioassay is used to screen the crude extract for substances that are toxic to biological systems. Once the purified compounds responsible for such activity have been isolated, a battery of more sophisticated and specific bioassays are then employed. This is because the brine shrimp bioassay is not specific for antitumor, or indeed, any particular physiological action.

4.2.1 Results and Discussion

Frozen samples were placed into MeOH (300 ml) for 24 hours, after which they were drained, and then left in MeOH:DCM (1:1) (300 ml) for a further 24 hours. Solvent extracts were pooled, and each sample was dried under reduced pressure. Stock solutions of 10 mg/ml (ethanol) were prepared for each extract of crude seaweed. An ultrasonic bath was used to aid dissolving. An aliquot (100 µl) of the ethanol solution was then removed and added to 650 µl of filtered seawater to give a bioassay stock-solution of 1.0 mg test fraction per 750 µl. Filtered seawater was used in a number of serial dilutions, and 100 µl of filtered seawater (containing 10-20 *Artemia* nauplii) was added to each well. The final

volume of each well was 250 µl, with test concentrations ranging from 400 to 50 µg/ml per sample. Each micro-well plate was covered, and left in the dark at room temperature.[61] The addition of ethanol to the stock solution often caused the solution to become cloudy, while the suspension particles in the wells were occasionally seen to be "eaten" by the brine shrimp. The following samples were tested for cytotoxic activity (Table 4.1).

Table 4.1 Samples screened using the brine shrimp lethality assay

Sample #	**Full/Partial* identification**
TS0105	*P. corallorhiza*
TS0105	*P. corallorhiza*
TS0105	*P. corallorhiza*
TS0105	*P. corallorhiza*
TS0102	Unknown (red algae)
TS0103	*Gracilaria*
TS0104	*Galaxaura*
TS0106	*Gigartina*
TS0107	Unknown (red algae)
TS0108	*Carpophyllum*
TS0109	*Sargassum*
TS0110	*Epymenia*
TS0111	*Caulerpa*
TS0112	*Dictyopteris*
TS0113	*Codium*
TS0114	*Hypnea rosea*
TS0115	*Hypme*
TS0116	Unknown
TS0118	*Ecklonia*
TS0120	*Dictyota*
TS0124	*Halimeda*
Control	-

After 15 hours, only *Hypnea rosea* displayed cytotoxic activity (Table 4.2). It was also noted that brine shrimp that contained a solution of *Halimeda* extract, were very sluggish, but still alive. Table 4.3 lists the percentage mortality for all the samples that were collected.

Table 4.2 Brine shrimp percentage mortality (%) after 15 hours (for *Hypnea rosea*)

Sample #		**Concentration**			
	methanolic extract	**400µg/ml**	**200µg/ml**	**100µg/ml**	**50µg/ml**
TS0114	P_1	90%	100%	82%	0%
TS0114	P_2	100%	92%	67%	0%

* Each extract was tested in duplicate

Table 4.3 Brine shrimp percentage mortality after 24 hours (for the algal extracts of 18 samples)

Sample #		**Concentration**			
	methanolic extract	**400µg/ml**	**200µg/ml**	**100µg/ml**	**50µg/ml**
TS0105	A_1	50%	22%	33%	13%
TS0105	A_2	50%	25%	20%	0%
TS0105	B_1**	77%	20%	30%	50%
TS0105	B_2**	66%	28%	36%	66%
TS0105	C_1**	55%	25%	25%	20%
TS0105	C_2**	66%	50%	0%	25%
TS0105	D_1**	23%	13%	0%	0%
TS0105	D_2**	20%	0%	0%	0%
TS0102	E_1	0%	0%	11%	0%
TS0102	E_2	0%	33%	0%	0%
TS0103	F_1	50%	13%	0%	0%
TS0103	F_2	66%	14%	38%	13%
TS0104	G_1	50%	17%	0%	0%
TS0104	G_2	50%	20%	0%	0%
TS0106	H_1	50%	0%	14%	13%
TS0106	H_2	44%	0%	0%	0%
TS0107	I_1	33%	10%	0%	0%
TS0107	I_2	45%	0%	13%	14%
TS0108	J_1	44%	0%	0%	0%
TS0108	J_2	63%	8%	0%	14%
TS0109	K_1	33%	0%	0%	0%
TS0109	K_2	50%	0%	0%	0%

TS0110	L_1	0%	0%	10%	0%
TS0110	L_2	0%	0%	0%	0%
TS0111	M_1	0%	0%	0%	0%
TS0111	M_2	0%	0%	0%	0%
TS0112	N_1	0%	0%	0%	0%
TS0112	N_2	10%	0%	0%	0%
TS0113	O_1	0%	10%	0%	0%
TS0113	O_2	0%	0%	10%	0%
TS0114	P_1	100%	100%	100%	0% active
TS0114	P_2	100%	100%	100%	0% active
TS0115	Q_1	0%	0%	0%	0%
TS0115	Q_2	0%	0%	10%	0%
TS0116	R_1	0%	0%	0%	0%
TS0116	R_2	10%	0%	0%	0%
TS0118	S_1	0%	0%	0%	0%
TS0118	S_2	0%	0%	0%	0%
TS0120	T_1	0%	0%	0%	0%
TS0120	T_2	0%	0%	0%	0%
TS0124	U_1	67%	53%	0%	0%
TS0124	U_2	85%	62%	0%	0%
Control	V_1	0%	0%	0%	0%
Control	V_2	0%	0%	0%	0%

* Each extract was tested in duplicate (ie A_1-A_2)

** These are not methanolic extracts; B (Hexane partition of MeOH extraction); C (MeOH partition from B above), D (Crude EtOAc extract)

Hypnea rosea, *P. corallorhiza* and *Halimeda* indicated cytotoxic activity. These three marine algae require further investigation to determine the active constituents responsible for their potentially cytotoxic action. Results obtained from $A_{1\text{-}2}$, $B_{1\text{-}2}$, and $C_{1\text{-}2}$ respectively, indicate that slightly more of the potentially active metabolites were extracted in the MeOH extract of the hexane / MeOH solvent partition. $D_{1\text{-}2}$ demonstrated that EtOAc is a very poor solvent for the extraction of active metabolites from *P. corallorhiza*.

Conclusion

It is anticipated that this study has identified some of the compounds responsible for the pharmacological activity displayed by *P. corallorhiza*, and that this work will enable the chemical screening of other *Plocamium* spp. to take place at a quicker pace.

Chapter Five

Experimental Section

5.1 General Experimental

All solvents were distilled before use; HPLC (HiPerSolv ™) grade solvents from BDH Laboratory Supplies were used for all GC experiments. Normal phase TLC was performed on DC-Alufolien Kieselgel $60F_{254}$, and viewed under UV light (254 nm). Column chromatography was performed using Kieselgel 60 (230-400 mesh) silica.

Compounds where purified using a Spectra-Physics IsoChrom LC HPLC system, which was equipped with a rheodyne injector, a Waters R401 differential refractometer, and a Rikadenki chart recorder. In all cases normal phase chromatography was performed using a Whatman Magnum 10-Partisil 9 column. Gas Chromatography was performed using a Hewlett Packard 6890-Series.

The ^{1}H (400 MHz), ^{13}C (100 MHz), DEPT-135, HMQC, HMBC, COSY-90 NMR spectra where all recorded on a Bruker Advance 400 NMR spectrometer. Chemical shifts are reported in δ units (ppm) and referenced to residual undeuterated solvent resonances ($CHCl_3$ δ_H 7.25, δ_C 77.0). All coupling constants are reported in Hz.

Optical rotation was measured on a Perkin-Elmer 141 polarimeter. Ultra violet measurements were obtained from a GBC UV/ VIS 916 spectrometer, while infrared spectra were obtained from a Perkin-Elmer Spectrum 2000 FT-IR spectrometer (films) on KBr discs (applied with $CHCl_3$).

Low resolution mass spectra (EIMS) were recorded on Finnigan MAT GCQ system at 70eV. High resolution (HREIMS) spectra were obtained from Dr. Louis Fourie at the Mass Spectrometry Unit at Potchefstroom University.

5.2 Chapter two: Experimental

Collection, extraction and isolation

P. corallorhiza (Kalk Bay)

P. corallorhiza (identified by Prof. J. Bolton of the Botany Dept., University of Cape Town) was collected during March of 2002 from Kalk Bay (near Cape Town), and stored frozen. The frozen sample (14.3 g dry weight) was left in cold MeOH (650 ml) for 6 hours. The MeOH was drained and *P. corallorhiza* was extracted three times with DCM (500 ml) for 24 hours per extraction. Oil from the DCM extract weighted 1.1 g. The ^{1}H NMR spectra of the crude methanolic and DCM extracts were found to be identical, except for a large water peak that was present in the methanolic extract. Partial separation (via column chromatography) employing a hexane-EtOAc step-gradient (hexane to ethyl acetate) of the crude DCM extract (1.1 g) gave six fractions. Fraction one consisted of compounds obtained from a solvent gradient varying between hexane (100%) to EtOAc-hexane (3:7). Fraction two resulted from the use of 70-50% hexane, fraction three had a hexane concentration of between 50-30%, while fraction four contained hexane from 30-0%. Fractions five and six were the remains of the MeOH and DCM wash. Fractions were grouped on the basis of TLC and ^{1}H NMR data.

Fraction 1 (291.1 mg) was further purified using normal phase HPLC and a solvent system of EtOAc-hexane (5:95), to give compound **35** (2.9 mg, 0.020% dry wt). Fraction 3 (63.7 mg) was made up with hexane injected into the GC-MS. Five major peaks or metabolites were present. The fragmentation patterns of each of these five compounds were not new. Compounds isolated in fraction 1 demonstrated exactly the same fragmentation patterns for each of the five major peaks present in fraction 3. Fractions 7, 8, 12 and 13 underwent further chromatography using HPLC, because they demonstrated different and interesting ^{1}H NMR spectra (Scheme 2.1). HPLC of fraction 7 yielded three compounds namely: **99** (0.7 mg, 0.005% dry wt), **100** (1.5 mg, 0.010% dry wt) and **101** (4.3 mg, 0.030% dry wt). Three different compounds were obtained after HPLC of faction 8; these were compound **101** (15.8 mg) (combined weight 0.111% dry wt), **96** (5.5 mg, 0.039% dry wt) and **94** (1.1 mg, 0.008% dry wt). Fraction 12 contained compound **102** (0.4 mg, 0.003% dry wt) and **107** (0.6 mg, 0.004% dry wt), while fraction 13 contained compound **99** and **100**, bringing the total mass of these two metabolites to 2.3 mg (0.016% dry wt), and 2.6 mg (0.018% dry wt) respectively (Scheme 2.1).

P. corallorhiza (Riet River)

P. corallorhiza (identified by Prof. R. Lubke of the Botany Dept., Rhodes University) was collected during April of 2001 from Three Sisters (Riet River, near Port Alfred), and stored frozen. The frozen sample (15.6 g dry weight) was extracted with MeOH (1 x 900 ml), DCM-MeOH (2:1 x 900 ml), and H_2O-MeOH (1:3 x 900 ml) for three days per extraction. These three extracts were combined, and dried under reduced pressure to produce a dark, viscous oil. The oil was partitioned between hexane (50 ml) and MeOH (40 ml) containing 10 ml water. TLC analysis demonstrated that the hexane partition contained compounds of greater quantity and variation than that of the MeOH partition.

Combined hexane extracts were dried over anhydrous Na_2SO_4, filtered and concentrated to give 0.25 g of crude extract. The H_2O-MeOH partition layer was dried under vacuum, and partitioned between DCM (50 ml) and MeOH (40 ml) containing 10 ml water. Combined DCM extracts were dried over Na_2SO_4, filtered and concentrated to give 0.56 g of crude extract. The aqueous MeOH partition was dried under vacuum, and partitioned with EtOAc (50 ml) and water (50 ml). The EtOAc extract weighted 0.11 g. The crude hexane extract (0.25 g) was purified (over silica gel) to give six fractions (Scheme 2.2). Fraction one was purified further by normal phase HPLC to give **96** (7.8 mg, 0.050% dry wt), and **94** (8.9 mg, 0.057% dry wt).

^{1}H NMR data of fraction two indicated that it contained the same compounds as in fraction one. Fractions three, four, five and six contained very small quantities and were not investigated.

Column chromatography of the crude DCM extract (0.56 g) over silica gel, using a solvent gradient system of EtOAc-hexane, gave six fractions that were analysed by TLC and ^{1}H NMR spectroscopy (Scheme 2.2). Fraction thirteen was obtained from a pure hexane wash, fraction fourteen contained hexane (97.5%), fraction fifteen contained hexane (95%), fraction sixteen contained hexane (90%), fraction seventeen contained hexane (50%), and fraction eighteen was a MeOH wash. (EtOAc was used in combination with hexane in all the fractions obtained). Fractions 13, 14, 15, and 17 were purified by normal phase HPLC to give **96** (14.1 mg, 0.090 % dry wt), **103** (5.7 mg, 0.036% dry wt), **104** (8.0 mg, 0.051% dry wt), **105** (11.4 mg, 0.073% dry wt), and **106** (3.1 mg, 0.020% dry wt) (Scheme 2.2).

4-bromo-5-bromomethyl-1-chlorovinyl-2,5-dichloro-methylcyclohexane (35):

Colourless oil; $[\alpha]^{28}_{D}$ -27° (c 0.03, $CHCl_3$); UV (Hexane) λ_{max} 206 (ε 5132); IR (dry film) ν_{max} cm^{-1}(KBr): 3449, 2928, 2855, 1714, 1614, 1453, 1426, 1381, 1329, 1247; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.8); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.8); GC-MS (70 eV) (See Table 3.5)

1,4,8-tribromo-3,7-dichloro-3,7-dimethyl-1,5-octadiene (94):

Colourless oil; $[\alpha]^{28}_{D}$ -59° (c 0.02, $CHCl_3$); UV (Hexane) λ_{max} 208 (ε 8167); IR (dry film) ν_{max} cm^{-1}(KBr): 3086, 2930, 2361, 1617, 1448, 1380, 1229, 1046, 968, 941; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.3); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.3); GC-MS (70eV) (See Table 3.5)

8-bromo-1,3,4,7-tetrachloro-3,7-dimethyl-1,5-octadiene (96):

Colourless oil; $[\alpha]^{28}_{D}$ -62° (c 0.03, $CHCl_3$); UV (Hexane) λ_{max} 216 (ε 5501); IR (dry film) ν_{max} cm^{-1}(KBr): 3088, 2986, 2934, 2362, 2346, 1616, 1448, 1380, 1230, 969; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.2); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.2); GC-MS (70eV) (See Table 3.5)

4,8-dibromo-1,1-dichloro-3,7-dimethyl-2,6-octadiene (99):

Colourless oil; UV (Hexane) λ_{max} 216 (ε 3205); IR (dry film) ν_{max} cm^{-1}(KBr): 1380, 1207, 707; ^1H NMR ($CDCl_3$, 400 MHz) (Table 2.4); ^{13}C NMR ($CDCl_3$, 100 MHz) (Table 2.4); GC-MS (70eV) (See Table 3.5); HREIMS *m/z* 361.883985 (calcd for $C_{10}H_{14}Br^{79}Cl_3{}^{35}$, 361.883928)

4,6-dibromo-1,1-dichloro-3,7-dimethyl-2,7-octadiene (100):

Colourless oil; $[\alpha]^{28}_{D}$ -15° (c 0.02, $CHCl_3$); UV (Hexane) λ_{max} 219 (ε 7664); IR (dry film) ν_{max} cm^{-1}(KBr): 2919, 2343, 2361, 1654, 1437, 1389, 1206, 1107, 865, 707; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.5); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.5); GC-MS (70eV) (See Table 3.5); HREIMS *m/z* 361.884122 (calcd for $C_{10}H_{14}Br_2{}^{79}Cl_2{}^{35}$, 361.883928)

4,8-dibromo-1,1,7-trichloro-3,7-dimethyl-2,5-octadiene (101):

Colourless oil; $[\alpha]^{28}_{D}$ -43° (c 0.03, $CHCl_3$); UV (Hexane) λ_{max} 226 (ε 6590); IR (dry film) ν_{max} cm^{-1}(KBr): 2984, 2925, 2361, 2342, 1653, 1447, 1435, 1380, 966, 866; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.6); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.6); GC-

MS (70eV) (See Table 3.5); HREIMS *m/z* 316.926153 [M^+-Br] (calcd for $C_{10}H_{13}Br^{79}Cl_3^{35}$, 316.926620)

3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (102):

Colourless oil; UV (Hexane) λ_{max} 202 (ε 4171); IR (dry film) ν_{max} cm^{-1}(KBr): 1373, 1105, 931; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.7); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.7); GC-MS (70eV) (See Table 3.5); HREIMS *m/z* (calcd for $C_{10}H_{16}Cl_4^{35}$ *not observed*)

5-bromo-5-bromomethyl-1-chlorovinyl-2,4-dichloro-methylcyclohexane (103):

Colourless oil; UV (Hexane) λ_{max} 204 (ε 4393); IR (dry film) ν_{max} cm^{-1}(KBr): 3445, 2927, 2856, 1723, 1615, 1456, 1382, 1291, 1125, 1074; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.10); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.10); GC-MS (70eV) (See Table 3.5); HREIMS *m/z* 395.844046 (calcd for$C_{10}H_{13}Br_2^{79}Cl_3^{35}$, 395.844956)

8-bromo-1-chloro-3,4,7-trimethoxy-3,7-dimethyl-1,5-octadiene (104):

Colourless oil; UV (Hexane) λ_{max} 208 (ε 3758); IR (dry film) ν_{max} cm^{-1}(KBr): 1052; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.11); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.11); GC-MS (70eV) (See Table 3.5)

8-bromo-1,3-dichloro- 4,7-dimethoxy-1,5-3,7-dimethyl –octadiene (105):

Colourless oil; UV (Hexane) λ_{max} 206 (ε 2538); IR (dry film) ν_{max} cm^{-1}(KBr): 3461, 2983, 2935, 2828, 1720, 1623, 1455, 1375, 1085, 983; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.12); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.12); GC-MS (70eV) (See Table 3.5)

8-bromo-1,3,7-trichloro-4-methoxy-3,7-dimethyl–1,5-octadiene (106):

Colourless oil; UV (Hexane) λ_{max} 202 (ε 3856); IR (dry film) ν_{max} cm^{-1}(KBr): 3437, 2981, 2934, 1748, 1624, 1452, 1374, 1234, 1095, 979; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.13); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.13); GC-MS (70eV) (See Table 3.5)

8-bromo-1,1,7-trichloro-4-methoxy-3,7-dimethyl-2,5-octadiene (107):

Colourless oil; UV (Hexane) λ_{max} 213 (ε 5666); IR (dry film) ν_{max} cm^{-1}(KBr): 2931, 1656, 1448, 1379, 1096, 971, 857, 704, 615; ^1H NMR ($CDCl_3$, 400 MHz) (See Table 2.14); ^{13}C NMR ($CDCl_3$, 100 MHz) (See Table 2.14); GC-MS (70eV) (See Table 3.5)

5.3 Chapter three: Experimental

Results and Discussion

GC parameters: GC parameters (HP 6890) (See Table 3.1)
GC parameters using the Finnigan (GCQ) method (See Table 3.2)
GCQ MS method (See Table 3.3)

GC-MS analysis of a *P. corallorhiza* extract

A single, fresh leaf (340 mg) of *P. corallorhiza* (collected from Port Alfred) was placed in 200 ml of MeOH for 4 hours. The methanolic extract was evaporated under reduced pressure, and then made up to 1 ml with HPLC grade hexane. 1 ml of the crude solution was passed through a cotton plug, and 1 µl of this was injected onto the GC-MS. (A separate solution of HPLC grade hexane, and HPLC grade MeOH was injected into the GC-MS, these experiments proved that the peaks from the crude extract were not due to the solvent systems being used.) To characterise the unique retention time, and fragmentation patterns of the purified compounds that were isolated, hexane solutions (<10 mg/ml) were run on both the GC (HP-5 column) and GC-MS (DB-1 column) (Table 3.5).

A comparison of the metabolite profiles of *P. corallorhiza* collected from three different locations along the South African coast (Riet River, Kalk Bay and Holbaai)

Three different collections were made for *P. corallorhiza*. These included collections at Riet River (Sept. 2001), Holbaai (Nov. 2001) and Kalk Bay (Mar. 2002) respectively. Marine algae were placed in a plastic "zip lock" bag for a maximum of 3 hours before being frozen. (Identification of the marine algae was done by Prof. J. Bolton or Prof. R. Lubke.) A random portion of frozen *P. corallorhiza* was broken off from the frozen bulk, and briefly rinsed in distilled water to remove any particulate matter. The leaves were dried lightly on filter paper before being weighted. The wet algae (~10 g) was then added to a mixture of 100 ml HPLC grade methanol (with 10% distilled water) and left to stand for 15 hours. (A duplicate set of experiments was done for each sample.) Methanolic extracts were partitioned twice with 50 ml of HPLC grade hexane. Hexane extracts were dried under a vacuum and later under nitrogen gas. The dried extract was made up with hexane (1 ml) and filtered through a cotton wool plug. Extracts were dried again over nitrogen gas and accurately weighted (see Table 3.7). Each of the dried extracts was made

up to exactly the same concentration of 20 mg/ml using HPLC grade hexane. 1 µl of each crude sample was injected into the GC (HP 6890). The integration events (Table 5.1), and calibration table (Table 5.2) that were used for the HP 6890 are listed below:

Table 5.1 Integration Events

Default Integration Event Table (Event)		
Event	**Value**	**Time**
Initial Area Reject	0.000	Initial
Initial Threshold	-2.000	Initial
Initial Peak Width	0.040	Initial
Initial Shoulders	OFF	Initial
Signal Specific Integration Event Table (Event FID1B)		
Event	**Value**	**Time**
Initial Area Reject	4.209	Initial
Initial Threshold	4.475	Initial
Initial Peak Width	0.011	Initial
Initial Shoulders	OFF	Initial
Signal Specific Integration Event Table (Event FID1A)		
Event	**Value**	**Time**
Initial Area Reject	2.000	Initial
Initial Threshold	0.001	Initial
Initial Peak Width	0.001	Initial
Initial Shoulders	OFF	Initial
Integrator	OFF	0.000
Integrator	ON	2.000
Threshold	0.100	2.000

Table 5.2 Calibration Table

Calculate: Area Percent
Rel. Reference Window: 5.000%
Abs. Reference Window: 0.000min
Rel. Non-ref. Window: 5.000%
Abs. Non-ref. Window: 0.000min
Uncalibrated Peaks: not reported
Partial Calibration: Yes, identified peaks are recalibrated
Correct All Ret. Times: No, only for identified peaks
Curve Type: Linear
Origin: Included

A comparison of the metabolite profiles of *P. cornutum* from Kalk Bay and Holbaai

P. cornutum was identified by Prof. J. Bolton. The extraction procedure, and GC parameters remained unchanged from 3.2.2.2 (above).

A comparison of the metabolite profiles of different *Plocamium* spp.

A collection for different *Plocamium* species was made in Mar. 2002 at Kalk Bay (Cape Town). *P. corallorhiza*, *P. cornutum* and *P. maxillosum* was collected and identified by Prof. J. Bolton. Exactly the same extraction procedure was followed in 3.2.2.2. However, because the sample of *P. maxillosum* was so small only 20 ml of MeOH was used with 10% distilled water in the extraction procedure, and only 10 ml x 2 of HPLC grade hexane was used during the solvent partition.

A comparison of the metabolite profiles from different *Plocamium* spp. using Gas Chromatography Mass Spectrometry (GC-MS)

Exactly the same extraction procedure was followed in 3.2.2.2 (same samples), while the GC-MS method remained unaltered from that of section 3.2.

5.4 Chapter four: Experimental

Nineteen samples of marine algae were frozen for 6 months. Each sample (~20 g wet weight) was placed into MeOH (300 ml) for 24 hours, drained, and then left in MeOH:DCM (1:1) (300 ml) for a further 24 hours. This process was repeated twice.
All solvent extracts were pooled, and each sample was dried under vacuum and nitrogen gas. Samples were stored in vials at 4°C.

Culture of *Artemia salina*

Artemia salina cysts were obtained from PRO 100, Ocean Star, International Inc., Snowville, Utah, 84336, USA; and were stored under dark conditions in a refrigerator. The cysts were cultured for approximately 24 hours in normal seawater (NSW), which had been filtered. Cysts were separated from the egg cases by phototaxis, which was implemented using a desk lamp. Newly born brine shrimp were used immediately.

Preparation

Stock solutions of 10 mg/ml (ethanol) were prepared for each crude seaweed extract. An ultrasonic bath was used to aid in the dissolving of any solid material. 100 µl of this solution was then removed and added to 650 µl of filtered seawater to give a bioassay stock-solution of 1.0 mg test fraction per 750 µl. In 96 micro-well plates, 75 µl of filtered seawater was placed into wells B-D, and in as many columns as there are fractions to be tested. 150 µl was pipetted from the stock solution into well A. Serial dilutions of the test fractions were made by removing 75 µl of solution in well A, and adding it to well B, followed by adding 75 µl from B to C, and C to D. The 75 µl from well D was discarded, so that each well contained 75 µl of solution. Following this, 75 µl of NSW was added to all wells.

Adding *Artemia salina*

100 µl of filtered seawater (containing 10-20 *Artemia* nauplii) was added to each well. The final volume of each well was 250 µl, with test solutions being 400, 200, 100 and 50 µg/ml per sample. Each micro-well plate was covered, and left in the dark at room temperature. The plates were examined after 15 and 24 hours. Each plate was examined under a binocular dissecting microscope (using 10 x magnification).

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