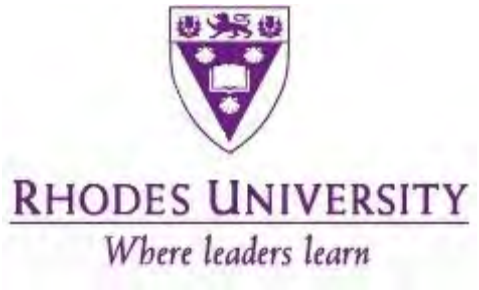


The Isolation and Characterisation of Secondary Metabolites from Selected South African Marine Red Algae (Rhodophyta)

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

By

Jameel Fakee

February 2013

Acknowledgements

There are numerous people I would like to forward heartfelt thanks to who assisted me in completing this thesis in more ways than one:

- My devoted supervisor, Dr. Denzil Beukes. A well spirited, well balanced man whose unfaltering belief in me got me this far. His incredible knowledge and ability to hold me awe inspired in this research area truly was my main source of encouragement. He is a truly respectable supervisor and a more admirable human being. Thank you!
- Professor John Bolton from the University of Cape Town for professional identification of algal samples.
- Dr. Pierre Kempgens for proficient NMR training.
- Dr. Edith Antunes for so kindly running NMR experiments for me.
- Mr and Mrs. Morley of the Pharmacy Faculty whose technical assistance over the years contributed significantly toward the completion of this thesis.
- Prudence for making the lab a much more blissful place to work.
- Dr Marietjie Stander from Stellenbosch University for assistance with mass spectrometry.
- Dr. Adrienne Edkins and her team at BioBru for conducting the cytotoxicity assays.
- Andrew Mellon foundation and Rhodes University for generous funding.
- Tich Samkange for his technical support.
- My colleagues in the Faculty of Pharmacy for their tireless support.
- My colleagues in labs G3 and G5, Mutenta, Denzel, Byron, Tatenda, Anthonia and Chiko for putting up with me.
- Special mention must go to Maynard Chiwakata. A great friend whose selfless ways have taught me not only infallible laboratory skills but also invaluable life skills. You are truly one in a million.
- To ALL my wonderful friends, in particular the M-11 crew, Mohammed Adam and Ashraf Ameen. You have all made these two years bearable and I owe the completion of this thesis to your terrific guidance, counseling and loyal friendship. Thank you!

- Hayat and Farida Ticklay. Thank you for being such wonderful mentors and outstanding human beings. Your support in the most difficult of times kept me focused and encouraged me to persevere.
- Yasmin Farrelly and family for providing constant support over the years for which I will always cherish.
- Finally to my family. My wonderful parents **Amina** and **Asgar Fakee**. Your consistent and unwavering efforts, support and sacrifices made to ensure that this world is that much easier for me to survive in undeniably goes fully noticed and well received. **I dedicate this thesis to you** and pray that I manage to do nothing less than make you proud parents of a son who loves you dearly.

  Aasif, Nasreen, Ameera, Zayd and my Cape Town family at large. Your generosity and welcoming nature always rejuvenated my soul and allowed me to get my much needed break from the labs. Ameera you always managed to put a smile on my face when I needed it the most. Thank you!

  Imraan and Kareema Fakee. Thank you for always providing a shoulder to lean on when the goings got tough.

  To ALL my loving cousins, uncles and aunties, your encouragement, support and motivation always kept my spirits high. Thank you!

Look deep into nature and you will understand everything better

Albert Einstein

Table of contents

Chapter 3

Chapter 4

Chapter 5

Appendices

List of Figures

List of Schemes

List of Tables

List of Abbreviations

ax	Axial
°C	Degrees Celsius
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
cLogP	Calculated log of the Partition Coefficient
COSY	^1H-^1H Homonuclear Correlation Spectroscopy
d	Doublet
Da	Daltons
dd	Double Doublet
DEPT	Distortionless Enhancement of Polarisation Transfer
dq	Doublet of Quartets
EtOAc	Ethyl Acetate
eq	Equatorial
gem	Geminal
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electron Spray Ionisation Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IC_{50}	Inhibitory Concentration 50%
IR	Infrared
J	Spin-Spin coupling constant (Hz)
m	Multiplet
m/z	Mass to charge ratio
MeOH	Methanol
MCF-7	Michigan Cancer Foundation - 7
MHz	Megahertz
mult	Multiplicity
M_r	Molecular Weight
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PFBHA	Pentafluoro-benzyl-hydroxylamine

q	Quartet
s	Singlet
Spp	Species
t	Triplet
TLC	Thin Layer Chromatography
UV	Ultra Violet
δ	Chemical shift (ppm)

Abstract

Secondary metabolites from natural sources are fast growing as popular drug leads. The structural novelty and favourable biological activity that these compounds display contribute to their popularity as drugs of the future. Examples of such compounds include the potent anticancer drug paclitaxel isolated from the bark of a yew tree as well as the more commonly known analgesic aspirin which stems from the bark of the willow tree. The biological activities exhibited by these secondary metabolites are vast and range from antimicrobial to anticancer activity to mention but a few. As a result, the isolation of novel compounds from natural sources is on the rise.

The South African seaboard is home to a wealth of various marine algal species which produce fascinating secondary metabolites. For example, *Portierria hornemanii* was shown to produce halomon, a halogenated monoterpene which has displayed promising cytotoxic activity. This study thus focused primarily on pursuing novel compounds from three endemic South African marine algal species which have never been analysed previously from a chemical perspective. These are *Plocamium rigidum* (Bory de Saint-Vincent), *Laurencia natalensis* (Kylin) and *Delisea flaccida* (Suhr) Papenfuss.

Four known compounds and one new halogenated monoterpene, **(2*E*,5*E*,7*Z*)-8-chloro-7-(dichloromethyl)-4-hydroxy-3-methylocta-2,5,7-trienal**, were isolated from *Plocamium rigidum*. The breast cancer (MCF-7 cell line) inhibitory activity for these compounds was assessed and it was observed that an increase in the lipophilic nature of the compounds produced more favourable IC_{50} values.

A pre-cursor to bromofucin type compounds, *cis*-laurencenyne, was isolated from *Laurencia natalensis*, as well as a new acetoxy chamigrane type compound, **4-bromo-3,10-dichloro-7-hydroxy-3,7,11,11-tetramethylspiro [6.6] undec-1-yl acetate**.

Delisea flaccida was seen to contain two known bromofuranone type compounds isolated as an isomeric mixture, **1-[(5*Z*)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl] butyl acetate** and **1-[(5*E*)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl]butyl acetate**. These compounds are famous for their ability to inhibit bacterial biofilm production and they have been isolated before from an Australian *Delisea* spp.

Chapter 1

General introduction

1.1 Natural products

A natural product or secondary metabolite is defined as any compound produced by an organism as a result of a metabolic pathway which does not directly affect growth, development or reproduction. It may however enhance the chances of survival of the organism by playing defensive or chemo attractant roles. This is in direct contrast to primary metabolites such as sugars, amino acids and lipids, which are critical for the normal functioning of the cell (Maplestone *et al*.; 1992; Dewick, 2002).

Natural products are regarded as one of the most prolific sources of prospective drug lead compounds (Mishra and Tiwari, 2011).These compounds display complex as well as novel structural attributes which are seen to be unique compared to those produced using a standard combinatorial type approach (Butler, 2004).

1.1.1 Natural products in history

There are several instances in history wherein natural product-based therapy formed the cornerstone of treatment for minor ailments and major illnesses.

Primitive records of natural product usage were found on clay tablets originating from Mesopotamia (2600 BC). These contained details of oils extracted from *Commiphora* spp. (myrrh) and *Cupressus sempervirens* (Cypress) which are still recognised today for their acclaimed effectiveness against respiratory tract infections (Cragg and Newman, 2005). The *Ebers Papyrus* (2900 BC) originating from Egypt was seen to contain over five hundred documented herbal remedies (Cragg and Newman, 2005) while the Indian traditional healing practice known as Ayurveda, which has been practiced for several hundreds of years, highlights the use of plant-based remedies amongst others for the treatment of various illnesses (Mukherjee *et al*., 2012).

The Greek philosopher and natural scientist Theophrastus (300 BC) wrote a treatise entitled *Historia Plantarium*. This collection provided physicians with details on plant taxonomy as well as plant remedies for a variety of different illnesses (Cragg and Newman, 2005).

Ibn Sina (Avicenna), a Persian physician and Islamic scholar wrote about forty books on medicine. His piece entitled *Canon Medicinae* was one of the more famous, and contained evidence of natural product usage for the cure of disease (Cragg and Newman, 2005).

1.1.2 Distinction between primary and secondary metabolism

It is important to recognise and distinguish the biosynthetic origin of these secondary metabolites. A clear definition is provided which differentiates primary metabolites within an organism from its secondary metabolites, the latter being the compounds of interest as prospective drug leads

Primary metabolites are those compounds which are produced within a living organism as direct products of biosynthetic and/or catabolic processes involving proteins, carbohydrates, nucleic acids and fats (all essential to living organisms). (Haefner, 2003).

Conversely, secondary metabolites are molecules formed *via* unique metabolic pathways which are deemed as being less essential for the survival of the organism than primary metabolites. These pathways are often seen to be unique to a particular organism or species and hence can be viewed as a form of expressing identity. The rationale for their production has come under great scrutiny over the years and the role they play in contributing toward the organism's survival must not be over looked (Maplestone *et al*., 1992).

Secondary metabolites are thought to be produced in response to environmental cues. It is hypothesised that organisms produce them for defensive purposes, as they have displayed antiherbivory, antifouling and ichthyotoxic effects amongst others (Colegate and Molyneux, 2008). The biosynthesis of these metabolites involves either the shikimate, acetate, mevalonate or methylerythritol phosphate pathway (Dewick, 2002). These pathways give rise to a chemically diverse nature of compounds.

1.1.3 Clinically significant natural products

Many well-known drugs on the market today originate from natural sources. These compounds have so far played a significant role in either the management or cure of disease. Furthermore, they have forwarded invaluable knowledge on structural originality which researchers can apply to modern day thinking in the drug discovery process (Mishra and Tiwari, 2011).

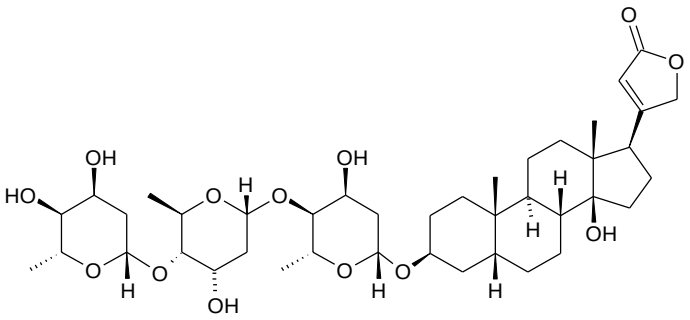
The active constituent of *Digitalis purpurea*, digitoxin (**1.1**), had been isolated in the eighteenth century. It was found to improve cardiac contractility and consequently has been used along with its analogues for the management of congestive cardiac disease (Der Marderosian and Beutler, 2002).

Perhaps one of the most renowned natural products to date is acetylsalicylic acid (**1.2**) also commonly known as aspirin. It is derived from salicin, found in the bark of a willow tree, *Salix alba*, and has made its name as an effective analgesic agent (Der Marderosian and Beutler, 2002).

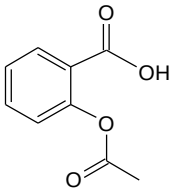
Analyses of the chemical constituents of the opium poppy, *Papaver somniferum,* displayed numerous alkaloids including morphine (**1.3**), a potent opioid analgesic whose role in pain management is well established. Other derivatives such as heroin and codeine also stem from the opium poppy (Der Marderosian and Beutler, 2002).

Quinine (**1.4**), isolated from the bark of the *Cinchona succirubra* tree, had formally been used by the British to treat malaria in the nineteenth century (Der Marderosian and Beutler, 2002).

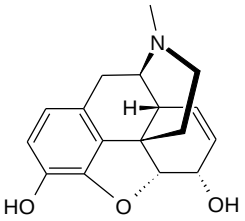
The *L*-histidine-derived alkaloid pilocarpine (**1.5**) found in *Pilocarpus jaborandi* (Rutaceae) has been used as a clinical drug for over a century in the treatment of various glaucomas. An oral preparation of pilocarpine is now available and is indicated for use in xerostomia as well as management of Sjorgen's syndrome (Der Marderosian and Beutler, 2002).



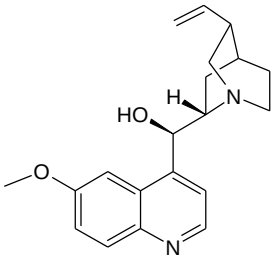
1.1
Digitoxin



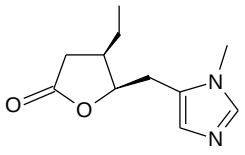
1.2
Aspirin



1.3
Morphine



1.4
Quinine



1.5
Pilocarpine

Figure 1.1: Examples of clinically significant compounds derived from natural products

1.1.4 Natural products from marine algae

Natural products are found in a variety of marine organisms such as sponges, marine algae and tunicates (Blunt *et al*., 2009).

Marine algae are classified as either microalgae or macroalgae. Over 30 000 species of macroalgae (seaweed) have been identified (Faulkner, 2002) and they are seen to play critical environmental roles such as the provision of the majority of atmospheric oxygen.

The natural product profile possessed by marine algae boasts an array of structurally diverse complex molecules. Classes of these compounds range from terpenes, linear acetogenins, peptides, indoles, cyclic polyketides and hydrocarbons to phenol type molecules (Haefner, 2003).

Unsurprisingly the majority of these secondary metabolites are halogenated, of which the most common halo-substituents are seen to be bromine and chlorine. This reflects the abundant nature of bromide and chloride ions in the ocean (Butler and Sandy, 2009).

Halogenated molecules are known to show interesting biological activity. It is for this reason that marine natural products have displayed such a vast assortment of biological activities including antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, antifouling, antifeedant, cytotoxic, ichthyotoxic, and insecticidal activity (Blunt *et al*., 2009).

1.1.5 Drug discovery and marine natural products

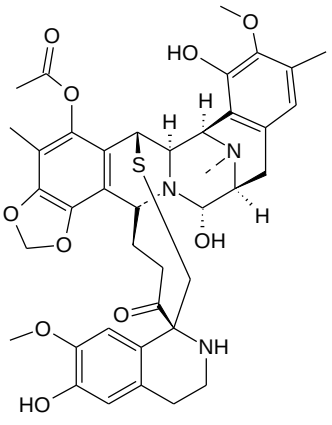
The fact that approximately 70% of the earth's surface is made up entirely of water, in addition to the serendipitous discovery of penicillin by Alexander Fleming in 1928 sparked a global pharmaceutical interest to explore natural products as potential drug leads, particularly from the marine environment. It was seen that there was a greater chance of finding exclusive biodiversity from marine sources and hence prospective drug leads (Haefner, 2003). A testament to this interest is the initiation of several natural product discovery (NPD) programs by major pharmaceutical companies in an attempt to attain lead compounds for various diseases (Ojima, 2008; Baker *et al*., 2007).

The aquatic environment became more easily accessible for investigation due to advancements in snorkelling techniques as well as the introduction of SCUBA diving in the early 1970s (Alejandro *et al*., 2010). Organisms that were scrutinised include sponges, tunicates, algae and bryozoans amongst others (Blunt *et al*., 2009).

Regrettably, the initiation of high throughput screening (HTS) encouraged the hasty demobilisation of numerous NPD programmes by pharmaceutical companies (Nussbaum *et al*., 2006). Some of the challenges faced included failure in total syntheses of the compounds due to the structural complexity of the molecules isolated. In addition, marine organisms were seen to be difficult to classify hence finding the same species and re-isolating the same compound was seen to be almost impossible (Faulkner, 1977).

Nonetheless advancements in spectroscopic techniques, amplification of instrumentation sensitivity and the advent of chemotaxonomic as well as genomic means of classifying marine organisms reinitiated the interest in natural product based drug discovery (Baker *et al*., 2007).

A prominent example of a successful compound isolated from the marine environment is Trabectedine (**1.6**) (Cuevas and Francesch, 2009). This compound was the first marine anticancer drug to be approved in the European Union (Alejandro *et al*., 2010).



1.6
Trabectedine

Figure 1.2: The anticancer drug Trabectedine

1.1.6 South African marine algae

South African seaweed flora is diverse with high levels of endemism. In excess of 12 000 marine plants and animals exist along the southern African seaboard (Branch *et al*., 2005) with more than 50% of the seaweeds found on the west coast being confined to the southern African region (Stegenga *et al*., 1997). A major contributing factor to this phenomenon is the Agulhas current (Figure 1.3). This current flows down the east coast of Africa and brings with it warm salty water from the Indian ocean to the southern African shoreline. It is regarded as one of the strongest currents in the world and its direction of flow serves to refresh the nutrients around the southern African coast. The mixing of this warm current with the colder Benguela current along the western seaboard creates a unique oceanic environment conducive for algal growth.

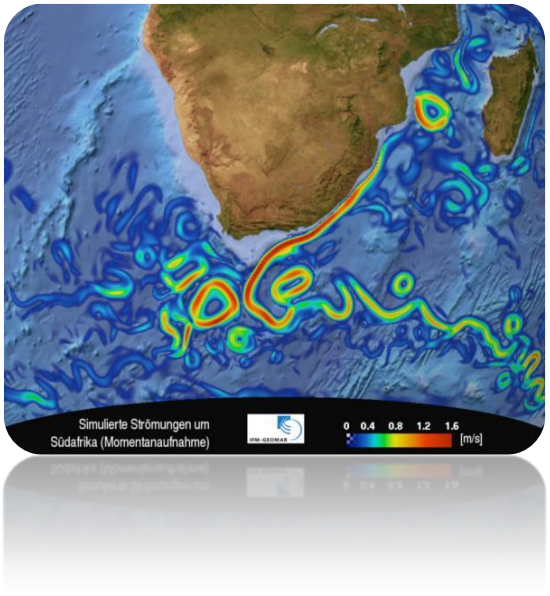


Figure 1.3: A depiction of the Agulhas current[1]

(Accessed 16/8/2012), Available at: **http://www.sciencedaily.com/releases/2008/11/081126133539.htm**

[1] Image courtesy of Leibniz Institute of Marine Sciences, Kiel, Germany.

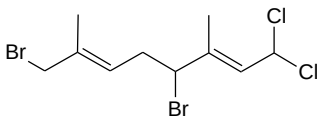
Bolton and Anderson (1997) describe South Africa to have three marine provinces along its shoreline as shown in Table 1.1.

Table 1.1: Marine provinces along the South African shoreline

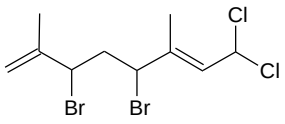
Coast	Temperature	Province
West	Cool	Benguela
South	Warm	Agulhas
East	Sub-tropical	Overlap[1]

[1] Overlap of Agulhas and Indo-West Pacific provinces

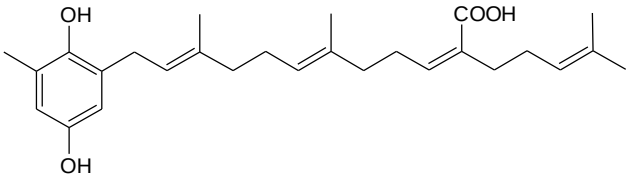
The last few years have witnessed a research boom of the natural product chemistry of South African marine algae with novel compounds such as the procoralides (**1.7** and **1.8**) (*Plocamium corallorhiza*) being isolated (Knott *et al*., 2005). Additionally, species such as *Sargassum* spp. have afforded compounds such as sargahydroquinoic acid (**1.9**) (Afolayan, 2008).



1.7



1.8



1.9
Sargahydroquinoic acid

Figure 1.4: Examples of compounds isolated from South African marine algae

1.2 Research aims and objectives

With the largely untapped marine algal biodiversity along the South African coastline available to us we have initiated a programme to systematically study the natural products produced by South African macroalgae. An important question to us is whether the high level of endemism translates into natural product structural novelty. Finally we are also interested in assessing the potential chemotaxonomic significance of natural products produced by specific species. With these questions in mind we collected three previously unstudied South African marine algae. These algal genera are known to produce diverse and biologically significant metabolites.

The specific objectives for this research were to:

- Isolate and characterise the natural products produced by three endemic South African red marine algae namely *Plocamium rigidum* (Bory de Saint-Vincent), *Laurencia natalensis* (Kylin) and *Delisea flaccida* (Suhr) Papenfuss.
- Where possible assess the biological activity of any new natural products.
- Analyse the chemotaxonomic significance of metabolites isolated.

1.3 Thesis outline

This thesis is divided into several self-contained chapters. Chapter 1 provides a general introduction while chapters 2-4 focus on the three algae studied in this project. Each chapter starts with a general introduction to the algal genus as well as metabolites isolated from that genus. New data obtained in this research is then presented. Finally, chapter 5 provides a brief conclusion and discusses the results of this study in the context of the specific study aims.

Figure 1.5: Low tide at Noordhoek collection site, Port Elizabeth, 02-08-2012

1.4 References

Afolayan, A. F. **2008**. Isolation and characterisation of anti-plasmodial metabolites from South African marine algae. M.Sc thesis. Rhodes University, Grahamstown.

Alejandro, M.; Glaser, K. B.; Cuevas, C.; Jacobs, R. S.; Kem, W.; Little, R. D.; McIntosh, J. M.; Newman, D. J.; Potts, B. C.; Shuster, D. E. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in Pharmaceutical Sciences* **2010**, *31*, 255-265.

Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V. The value of natural products to future pharmaceutical discovery. *Natural Product Reports* **2007**, *24*, 1225-1244.

Blunt, J. W.; Copp, B. R.; Hu, W. P.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. Marine natural products. *Natural Product Reports* **2009**, *26*, 170-244.

Bolton, J. J.; Anderson, R. J. Marine vegetation. In Cowling, R.M., Richardson, D.M. & Pierce, S.M. (Eds) *Vegetation* of *Southern Africa*; Cambridge, Cambridge University Press. **1997**, 348-370.

Branch, G. M.; Griffiths, C. L.; Branch, M. L.; Beckley, L. E. *Two oceans: A guide to the marine life of Southern Africa*, David Philips Publishers, South Africa, **2005**, 1-2.

Butler, M. S. The role of natural product chemistry in drug discovery. *Journal of Natural Products* **2004**, *67*, 2141–2153.

Butler, A.; Sandy, M. Mechanistic considerations of halogenating enzymes. *Nature* **2009**, *460*, 848-854.

Colegate, S. M.; Molyneux, R. J. *Bioactive Natural Products: Detection, Isolation and Structure Determination*; CRC Press: Boca Raton, FL, USA, **2008**, 421-437.

Cragg, G. M.; Newman, D. J. Biodiversity: A continuing source of novel drug leads. *Pure Applied Chemistry* **2005**, *77*, 7-24.

Cuevas, C.; Francesch, A. Development of Yondelis® (trabectedin, ET-743). A semi-synthetic process solves the supply problem. *Natural Product Reports* **2009**, *26*, 322-337.

Der Marderosian, A.; Beutler, J. A. *The Review of Natural Products*, 2nd ed.; Facts and Comparisons; Seattle, WA, USA, **2002**; 13-43.

Dewick, P. M. *Medicinal Natural Products: A Biosynthentic Approach*, 2nd ed.; John Wiley and Son: West Sussex, UK, **2002**; 520.

Faulkner, D. J. Interesting aspects of marine natural products chemistry. *Tetrahedron* **1977**, *33*, 1421-1433

Faulkner, D. J. Marine natural products. *Natural Product Reports* **2002**, *19*, 1-48.

Haefner, B. Drugs from the deep: Marine natural products as drug candidates. *Drug Discovery Today* **2003**, *8*, 536-544.

Knott, M. G.; Mkwananzi, H.; Arendse, C. E.; Hendricks, D. T.; Bolton, J. J.; Beukes, D. R. Plocoralides A-C, polyhalogenated monoterpenes from the marine alga *Plocamium corallorhiza*. *Phytochemistry* **2005,** *66*, 1108-1112.

Maplestone, R. A.; Stone, M. J.; Williams, D. H. The evolutionary role of secondary metabolites - A review. *Gene* **1992**, *115*, 151–157.

Mishra, B. B.; Tiwari, V. K. Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry* **2011**, *46*, 4769–4807.

Mukherjee, P. K.; Nema, N. K.; Venkatesh, P.; Debnath, P. K. Changing scenario for promotion and development of Ayurveda - way forward. *Journal of Ethnopharmacology* **2012,** *143*, 424-434.

Nussbaum, F. V.; Brands, M.; Hinzen, B.; Weigand, S.; Habich, D. Antibacterial natural products in medicinal chemistry - exodus or revival? *Angewandte. Chemie International Edition* **2006**, *45*, 5072-5129.

Ojima, I. Modern natural products chemistry and drug discovery. *Journal of Medicinal Chemistry* **2008**, *51*, 2587-2588.

Stegenga, H.; Bolton, J. J.; Anderson, R. J. *Seaweeds of the South African west coast*; Bolus herbarium: Cape Town, **1997**, 655.

Chapter 2

Cytotoxic, polyhalogenated monoterpenes from *Plocamium rigidum*

Abstract

The *Plocamium* genus of marine algae has been known to produce a wide array of halogenated monoterpenes. These compounds have shown fascinating antiplasmodial as well as selective cytotoxic activity. This has motivated the isolation and characterisation of related monoterpenes from *Plocamium* spp. which have not yet been chemically analysed. Additionally, several secondary metabolites have been observed to exist in more than one *Plocamium* spp. making species differentiation more challenging. This emphasises the importance of isolating metabolites unique to a particular species from a chemotaxonomic perspective.

One new halogenated monoterpene **(2*E*,5*E*,7*Z*)-8-chloro-7-(dichloromethyl)-4-hydroxy-3-methylocta-2,5,7-trienal** (**2.27**) and four known monoterpenes were isolated from the South African endemic alga, *Plocamium rigidum*. The structures of the compounds were deduced from spectroscopic data. Interestingly, compound **2.27** contained both a *gem*-dichloro moiety at C-9 and an aldehyde at C-1 which is unique to this species. Compounds bearing the *gem*-dichloro moiety exhibited moderate to good cytotoxic activity against a breast cancer cell line.

Chapter 2

Cytotoxic, polyhalogenated monoterpenes from *Plocamium rigidum*

2.1 Introduction

The *Plocamium* genus (family Plocamiaceae, order Plocamiales, phylum Rhodophyta) contains roughly forty different species which thrive in calm coastal environments (Saunders *et al*., 2005). At least six *Plocamium* species are endemic to the Southern African coastline. This includes *Plocamium robertiae* (F.Schmitz ex Mazza), *Plocamium maxillosum* (Poiret) J. V. Lamouroux, *Plocamium cornutum* (Turner) Harvey, *Plocamium corallorhiza* (Turner) J. D. Hooker & Harvey, *Plocamium rigidum* (Bory de Saint-Vincent), *Plocamium beckeri* (F.Schmitz ex Simons), *Plocamium suhrii* (Kützing) and *Plocamium glomeratum* (J.Agardh) (Lubke, 1988).

The general morphology of the *Plocamium* spp. is described by upright, oval shaped thalli that grow up to 50 cm in length. A red, distinctive tinge is common amongst the species in addition to fronds which further separate into smaller branchlets (MBARI)[1]. Since morphological characteristics of *Plocamium* species are so similar, various methods of species differentiation can be employed. This includes methods such as generating a full chemical profile of the species (chemotaxonomic approach), or analysing the genetic sequence of the algae (genomic approach), (Bino *et al*., 2004).

A classic example of this is shown by *Plocamium cartilagineum*. This constitutes the most studied *Plocamium* species and its identification has originally been done on the basis of morphological characteristics. However, recent research published by (Cremades *et al*., 2011) suggests that *P. cartilagineum* itself can be divided into a number of different species. The chemistry of these species could assist in differentiating cryptic species from one another.

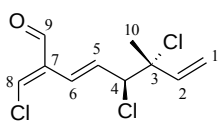
[1] **MBARI** (Monterey Bay Aquarium and Research Institute), (Accessed 07/03/2012)
Available at: http://www.mbari.org/staff/conn/botany/reds/eric/PHYS.HTM

Plocamium spp. produce an assortment of polyhalogenated monoterpenes (Mynderse and Faulkner, 1975; Crews and Kho, 1974). The first polyhalogenated monoterpenes were originally isolated from the digestive gland of a sea hare, *Aplysia californica* (Faulkner and Stallard, 1973). However, it was later discovered that the sea hare sequestered halogenated monoterpenes from *Plocamium cartilagineum* (Dixon) and stored these in its digestive glands (Mynderse and Faulkner, 1975).

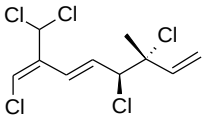
2.1.1 Monoterpenes isolated from South African *Plocamium* spp.

The chemistry of three South African *Plocamium* spp, *P. cornutum*, *P. suhrii* and *P. corallorhiza*, has previously been reported (Knott *et al*., 2005; Antunes *et al*., 2011). The major metabolites in both *P. cornutum* and *P. suhrii* contain a *gem*-dichloro moiety at C-9 (Figures 2.1 and 2.2) however the two groups of compounds may be distinguished by the terminal vinyl group in *P. cornutum* metabolites and a vinyl bromide in *P. suhrii* metabolites. A distinguishing feature of the major *P. corallorhiza* metabolites (Figure 2.3) is the *gem*-dichloro moiety at C-1. Interestingly, both *P. cornutum* and *P. corallorhiza* produce metabolites bearing aldehyde functionalities instead of dichloromethyl groups which suggests an interconversion between the aldehyde and dichloromethyl groups.

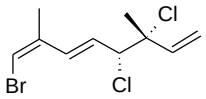
Halogenated monoterpenes from South African *Plocamium* spp have shown promising antiplasmodial (Afolayan *et al*., 2009) as well as cytotoxic (Antunes *et al.,* 2011) activity and provide further motivation for the investigation of previously unstudied *Plocamium* spp.



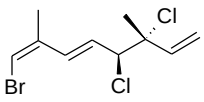
2.1



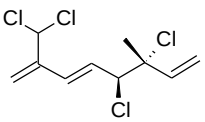
2.2



2.3

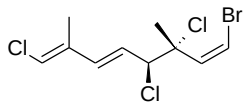


2.4

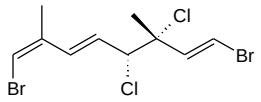


2.5

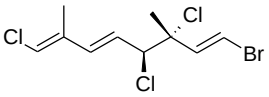
Figure 2.1: Halogenated monoterpenes previously isolated from *Plocamium cornutum* (Afolayan *et al*., 2009)



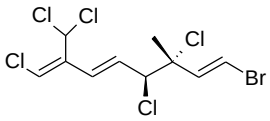
2.6



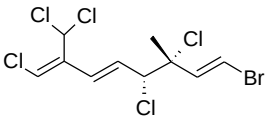
2.7



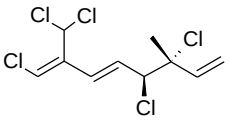
2.8



2.9

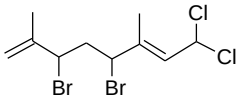


2.10

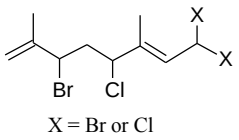


2.11

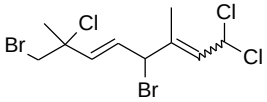
Figure 2.2: Halogenated monoterpenes previously isolated from *Plocamium suhrii* (Antunes *et al*., 2011)



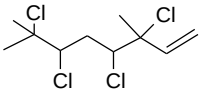
2.12



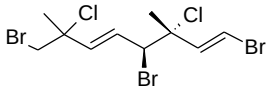
2.13



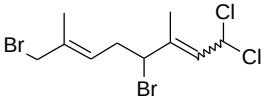
2.14



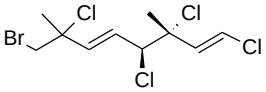
2.15



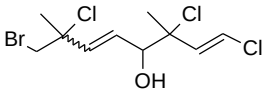
2.16



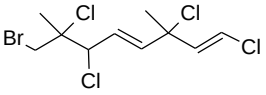
2.17



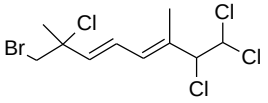
2.18



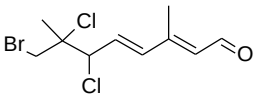
2.19



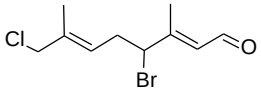
2.20



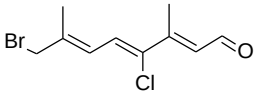
2.21



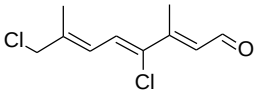
2.22



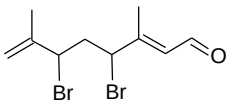
2.23



2.24



2.25



2.26

Figure 2.3: Halogenated monoterpenes previously isolated from *Plocamium corallorhiza* (Mkwananzi, 2005; Knott *et al*., 2005; Mann *et al*., 2007)

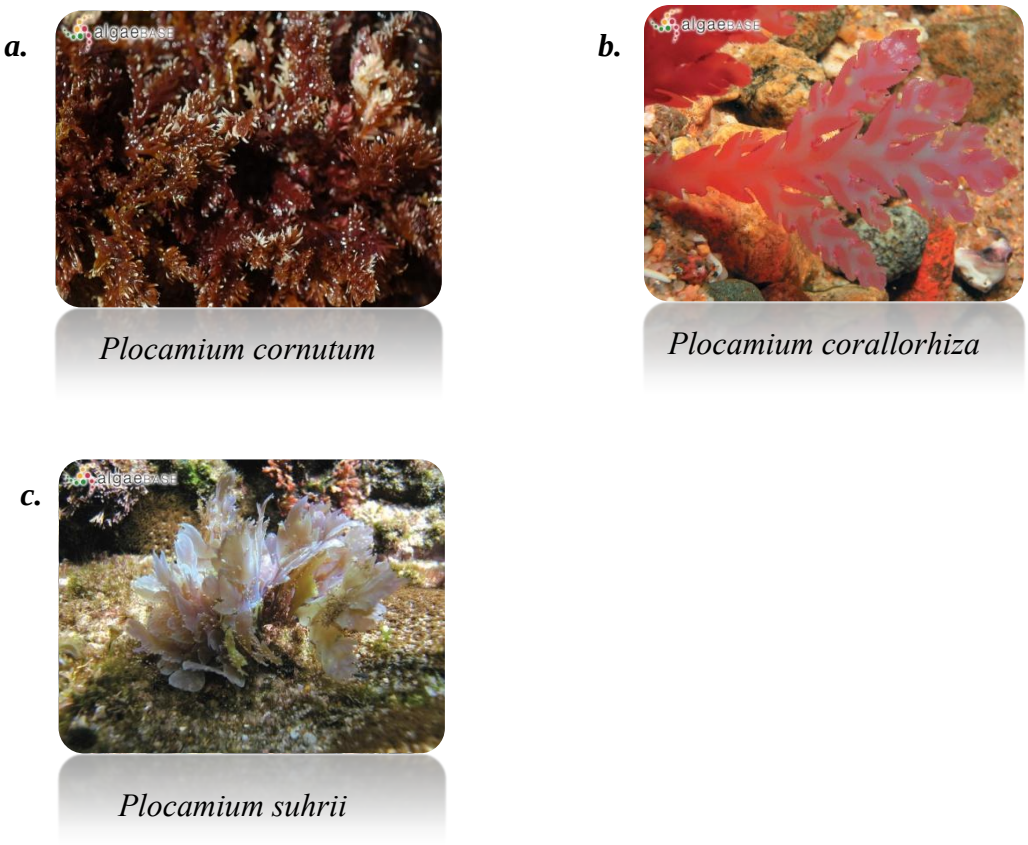


Figure 2.4: Photographs of selected South African *Plocamium* spp.[1]

a. Western Cape, South Africa.

Available at:

http://www.algaebase.org/search/species/detail/?species_id=2831&-session=abv4:92E781331db701B7E8KLx2809BB6

b. KwaZulu Natal, South Africa.

Available at:

http://www.algaebase.org/search/species/detail/?species_id=2830&session=abv4:92E781331db701B7E8KLx2809BB6

c. KwaZulu Natal, South Africa.

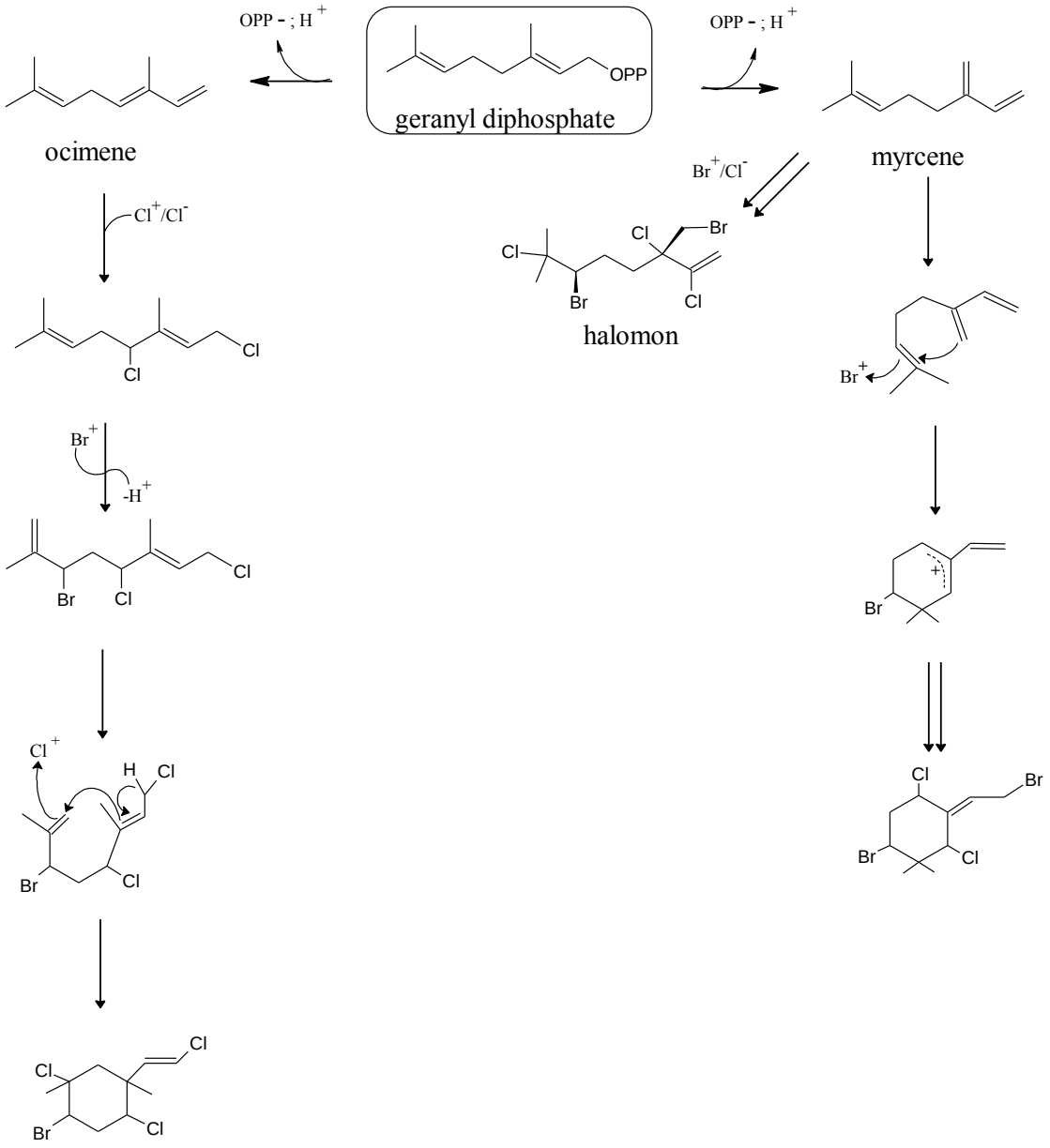
Available at:

http://www.algaebase.org/search/species/detail/?species_id=2840&-session=abv4:92E781331db701B7E8KLx2809BB6

[1] Photographs (©Robert Anderson, University of Cape Town) adapted from www.algaebase.org (Accessed 23/6/2012) with permission from Michael Guiry (Ryan Institute, Ireland).

2.1.2 Biosynthetic proposal of halogenated monoterpene formation

Most marine halogenated monoterpenes are thought to be produced by the halogenation of either ocimene or myrcene (Scheme 2.1). Consequently, myrcene and ocimene are thought to act as direct precursors to the formation of halogenated monoterpenes (Wise *et al*., 2002). The oxidising potential of haloperoxidase enzymes found in marine algae contributes to bromonium (Br^+) and chloronium (Cl^+) ion production. These ions are responsible for the addition and cyclisation reactions seen in Scheme 2.1 (Wise *et al*., 2002).



Scheme 2.1: Proposed biosynthetic mechanism of halogenated monoterpene formation involving halonium ion-induced cyclisations and addition[1]

[1] Adapted from (Wise *et al*., 2002)

2.1.3 Bioactivity of halogenated monoterpenes

Halogenated monoterpenes exhibit a range of biological activities including cytotoxic (Fuller *et al*., 1994), antiherbivory (Paul *et al*., 1980), antimicrobial (König *et al*., 1999) and insecticidal activity (San-Martin *et al*., 1991). Of interest is the impressive selective cytotoxicity that these compounds display, making them indispensable as potential anticancer leads (de Ines *et al*., 2004).

The halogen substituents present on these compounds, of which chlorinated and brominated are most common, are thought to be responsible for the bioactive properties illustrated. The shear bulkiness of the halogens allow for enhanced occupation of an active site thereby increasing binding affinity (Greenbaum *et al*., 2004; Monforte *et al*., 2009). In conjunction, halogens increase the LogP value of the compound. This amplifies lipophilicity and thus enhances membrane permeation (Gentry *et al*., 1999).

2.1.4 Chapter aims

In continuation of our studies of South African *Plocamium* spp. we have collected specimens of the endemic alga *Plocamium rigidum*. Our previous studies suggested that selected halogenated monoterpenes could be chemotaxonomically linked to individual species. In addition, these compounds have also showed potent cytotoxic activity against selected cancer cell lines (de la Mare *et al*., 2012).

The main objectives of this study were to:

1) Isolate and characterise the halogenated metabolites produced by *Plocamium rigidum*
2) Identify potential chemotaxonomic markers from this species
3) Assess the cytotoxic activity of metabolites against a breast cancer cell line (MCF-7)

Figure 2.5: Photograph of *Plocamium rigidum*[1]

Accessed (23/06/2012), Available at:
http://www.algaebase.org/search/species/detail/?species_id=2838&sk=0&from=results

[1] Photograph (©Robert Anderson, University of Cape Town) adapted from www.algaebase.org (Accessed 23/6/2012) with permission from Michael Guiry (Ryan Institute, Ireland).

2.2 Results and discussion

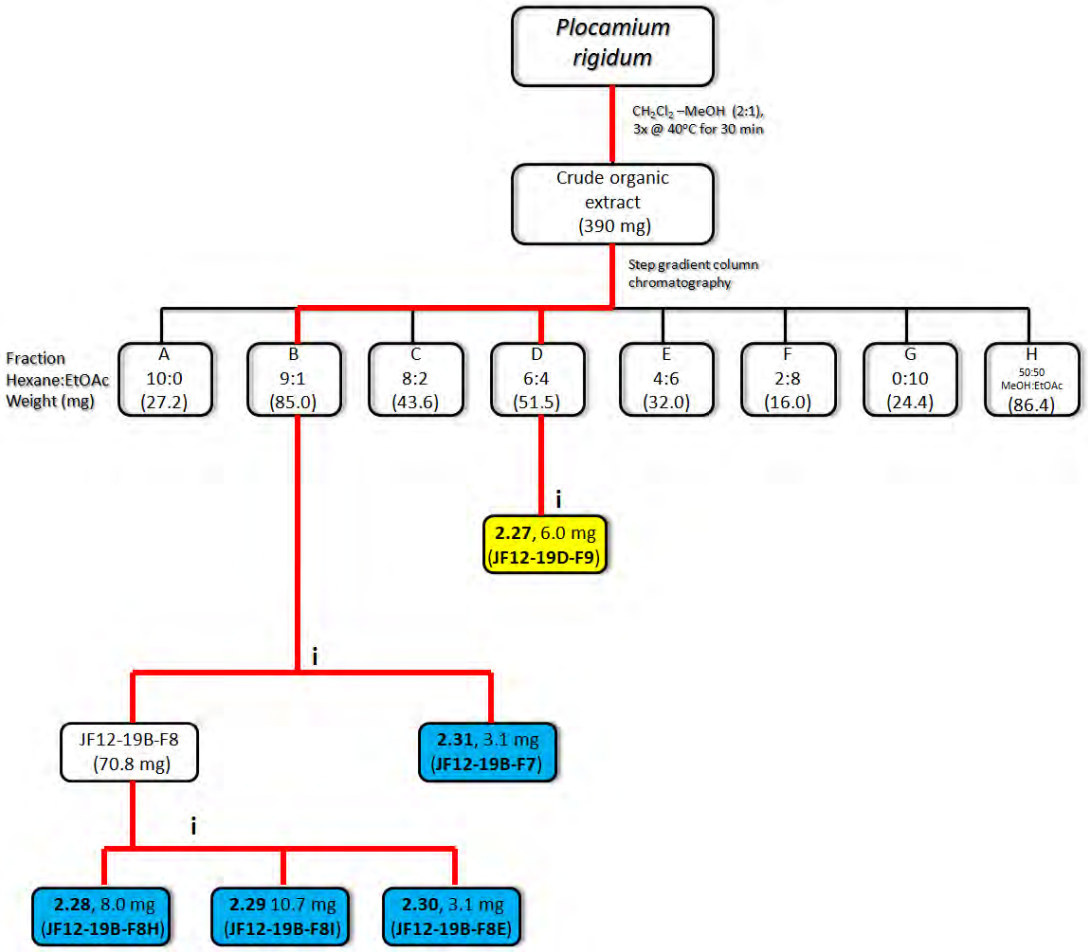
2.2.1 Extraction and isolation of metabolites

The alga was extracted initially in MeOH, after which a combination of CH_2Cl_2 and MeOH were used. These extracts were combined and the organic phase was collected and dried in vacuo to afford a crude isolate of 1.10% yield of the algal dry mass.

The crude, organic extract was fractionated into nine separate fractions on silica gel (Scheme 2.2) using a step gradient column chromatography approach.

The ^{1}H NMR spectra (Figure 2.6) shows the crude and column fractions A-H. The first point of recognition is how step gradient column chromatography separates out the crude fraction into distinct sub-fractions, some of which are undetectable in the crude ^{1}H NMR spectrum alone before purification. It was clear that typical halogenated monoterpene peaks were present in fractions B (non polar) and D (intermediate polarity) (Figures 2.7 and 2.8). When compared to the crude ^{1}H NMR spectrum, fractions B and D seemed to possess major metabolites. Fraction C was dark green in colour and contained characteristic chlorophyll peaks. The remaining fractions contained no interesting peaks and hence were not pursued.

Both fractions B and D were further purified via normal phase semi-preparative HPLC (100% hexane). This ultimately afforded pure compounds **2.27** -**2.31**.



Scheme 2.2: Isolation of compounds **2.27-2.31** from *Plocamium rigidum*

Conditions: i) Normal phase HPLC (100% hexane)

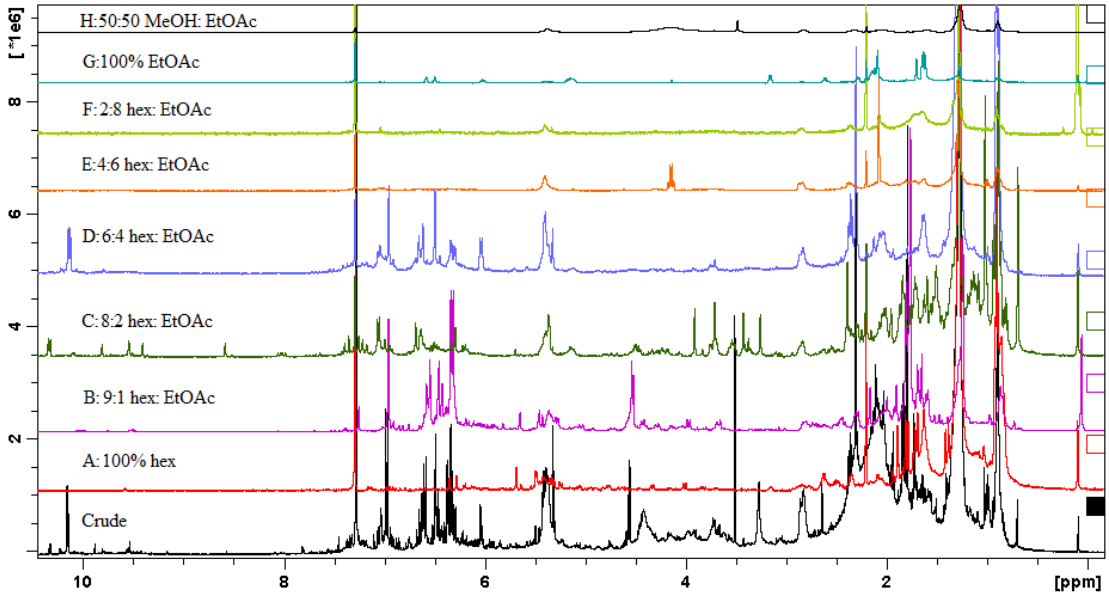


Figure 2.6: ^{1}H NMR ($CDCl_3$, 600 MHz) spectra of the crude and fractions A-H

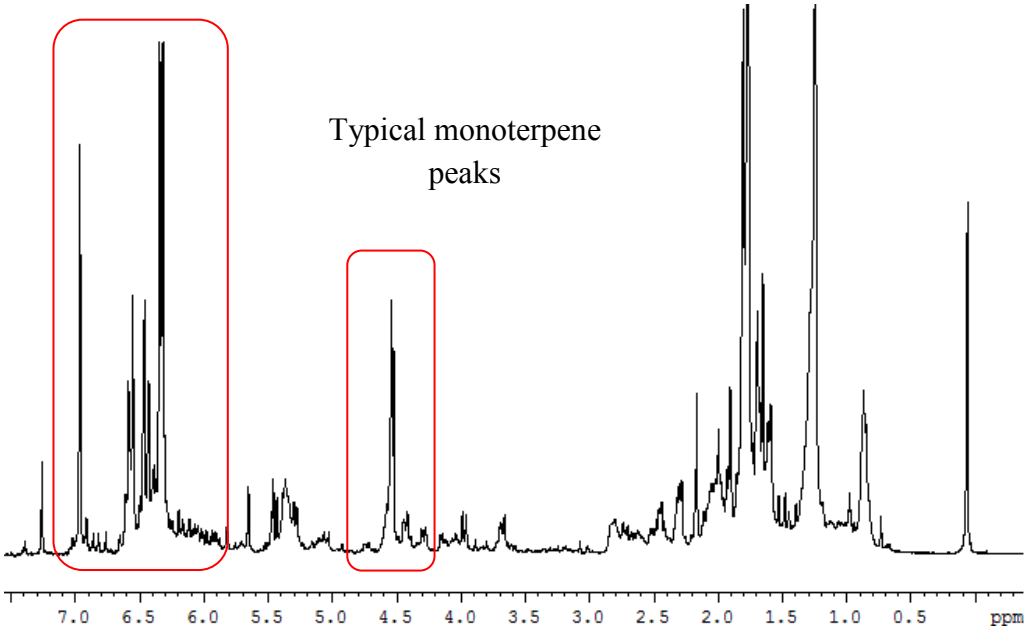


Figure 2.7: ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of fraction B

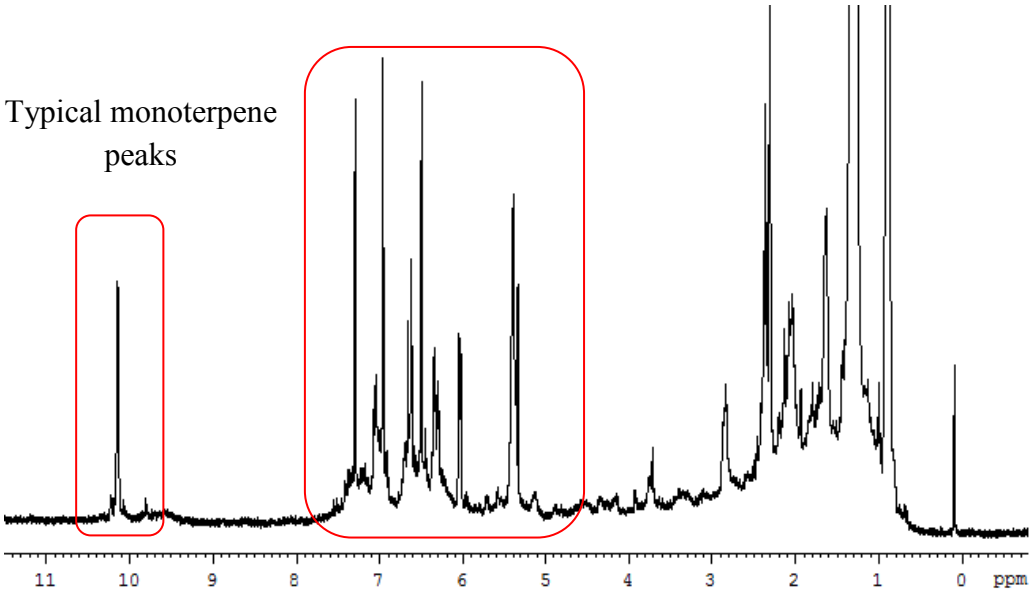


Figure 2.8: ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of fraction D

2.2.2 Structure elucidation of metabolites

2.2.2.1 Compound 2.27

Compound **2.27** was isolated as a brown oil. Its ^{1}H NMR spectrum (Figure 2.9) displayed a methyl singlet at δ 2.29 as well as a deshielded singlet at δ 6.47. A deshielded methine signal was also observed at δ 6.29 (dd, *J* = 15.6, 5.6 Hz,) which was coupled to a doublet at δ 6.62 (d, *J* = 15.6 Hz,) as well as a broad deshielded methine proton at δ 5.40, producing a -CH=CH-C(X) - structural motif. Moreover, a deshielded carbonyl signal was observed at δ 10.14 (d, *J* = 7.9 Hz) coupled to a methine proton at δ 6.02 (d, *J* = 7.8 Hz), characteristic of an α, β-unsaturated aldehyde. The ^{13}C NMR spectrum (Figure 2.10) together with the DEPT-135 spectrum of compound **2.27** showed seven –CH groups (δ 191.4, 136.0, 134.2, 130.6, 122.5, 69.3, and 66.5), two quaternary carbons (δ 152.9, 140.6) and one methyl carbon (δ 13.2).

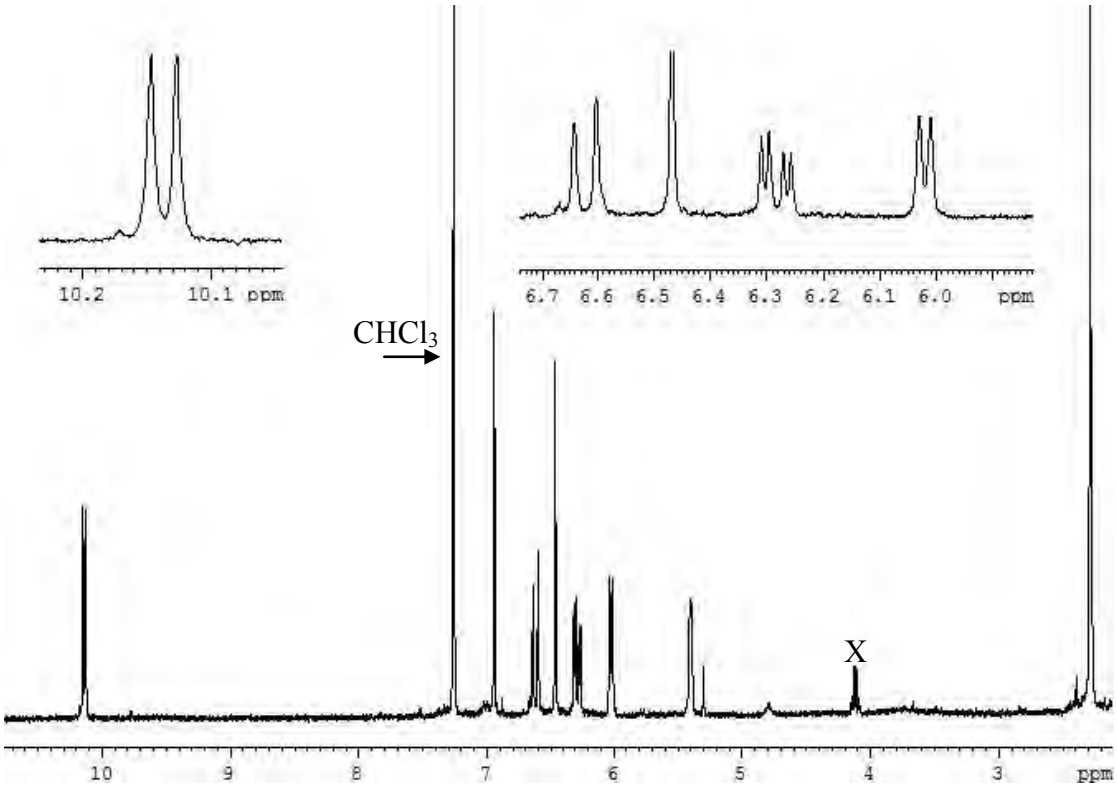


Figure 2.9: ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **2.27** [1]

[1] X denotes peaks not due to the proposed compound

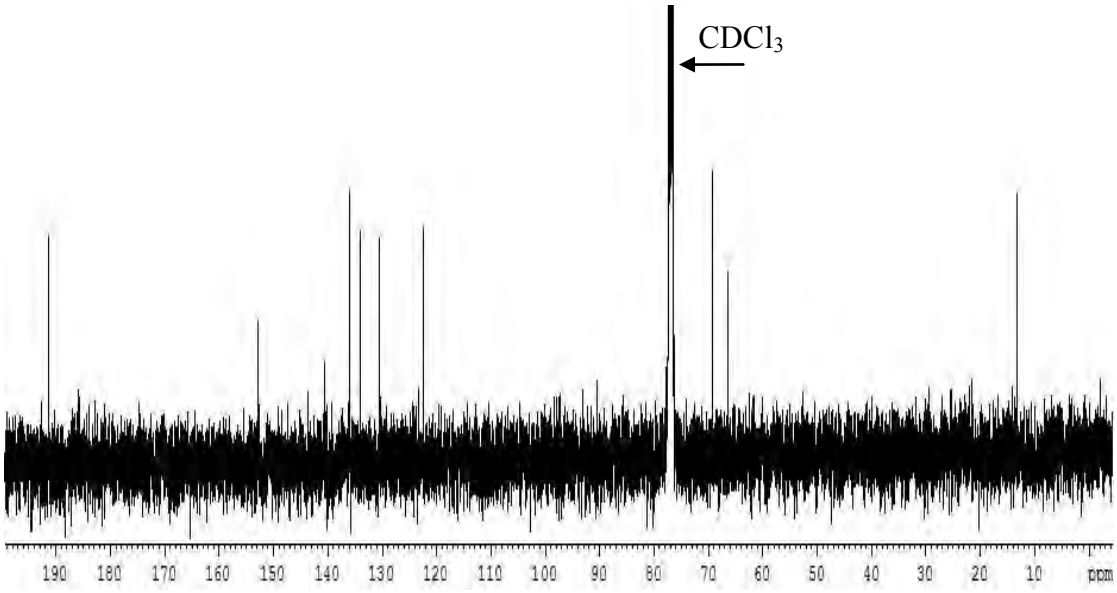


Figure 2.10: ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **2.27**

The deshielded methine at δ 6.94 showed HMBC correlations to carbon signals at δ 122.5 and δ 140.6 to give substructure **A** (Figure 2.11).

Substructure **B** (Figure 2.11), comprising of a -CH=CH-CHX- spin system, was easily deduced from ^1H NMR and COSY correlations from δ 6.29 to the olefinic methine at δ 6.62 and the deshielded methine at δ 5.40.

Substructure **C** (Figure 2.11) was deduced from correlations between the methyl signal at δ 2.29 to the quaternary carbon at δ 152.9, the methine carbon at δ 130.6 as well as the aldehyde carbon at δ 191.4.

Partial structure **A** did not show any HMBC correlations to the other partial structures however the most sensible assumption is that it is attached to partial structure **B** as is most often the case in this series of compounds**.** This is further substantiated as NOE correlations existed between H-9 and H-6 as well as H-8 and H-6. The resultant planar structure was obtained (Figure 2.11).

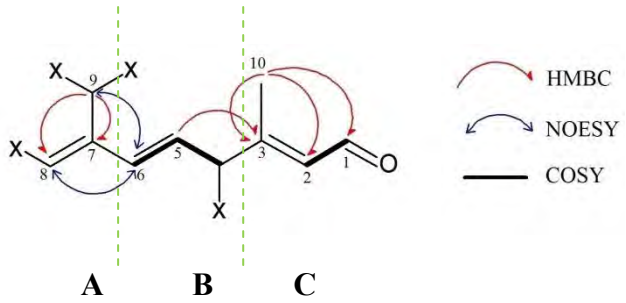
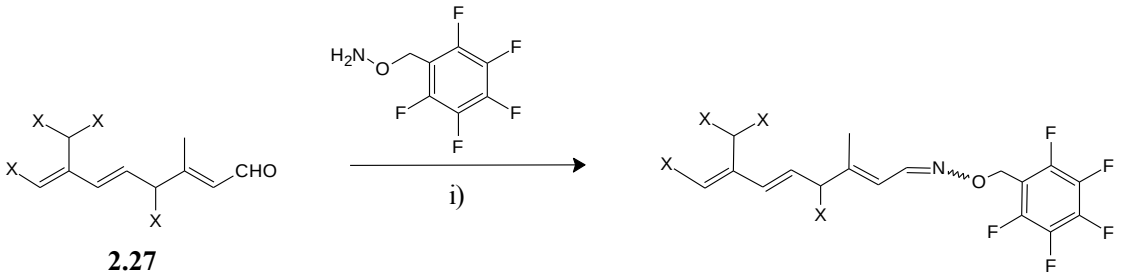


Figure 2.11: Key HMBC and COSY correlations of compound **2.27**

Compound **2.27** is related to the plocoraldehydes previously isolated from *P. corallorhiza* (Mann *et al*., 2007). Difficulties in obtaining molecular ion peaks for the plocoraldehydes necessitated the synthesis of pentafluorobenzylhydroxylamine (PFBHA) derivatives which gave stable molecular ions under ESIMS conditions (Mann *et al*., 2007). Thus compound **2.27** was treated with PFBHA (Scheme 2.3), of which the product was isolated and analysed by HRESIMS to give a molecular ion of *m/z* 464.0018 corresponding to a molecular formula of $C_{17}H_{13}NO_2Cl_3F_5$. The HRESIMS (Figure 2.12) of the PFBHA derivative of compound **2.27** showed, as the base peak (**A**), the molecular ion (M+1) of 464.0018 Da. This complemented the initial isotopic prediction of three ^{35}Cl atoms (Table 2.1). Isotopic peaks were also seen at 465.9992 Da (**B**) and 467.9965 Da (**C**) analogous to predictions 2 and 3 from Table 2.1. The isotopic peak cluster **A**, **B** and **C** followed a typical tri-chloro isotopic abundance pattern. The predicted molecular formula of the derivative of compound **2.27**, $C_{17}H_{13}NO_2Cl_3F_5$, was consequently justified. This implied a molecular formula of $C_{10}H_{11}Cl_3O_2$ for compound **2.27**.



2.27

Scheme 2.3: Derivitisation of compound **2.27** using PFBHA

Conditions: i) Acetonitrile, anhydrous $MgSO_4$, stir for 5 h at room temp.

Table 2.1: Predicted molecular masses (Da) for various isotopic combinations of Cl in the PFBHA derivative of compound **2.27**

	Isotopic pattern	Predicted Mr (M+1)
1	**^{35}Cl ^{35}Cl ^{35}Cl**	**464.001028**
2	**^{35}Cl ^{35}Cl ^{37}Cl**	**465.998078**
3	**^{35}Cl ^{37}Cl ^{37}Cl**	**467.995128**
4	^{37}Cl ^{37}Cl ^{37}Cl	469.992178

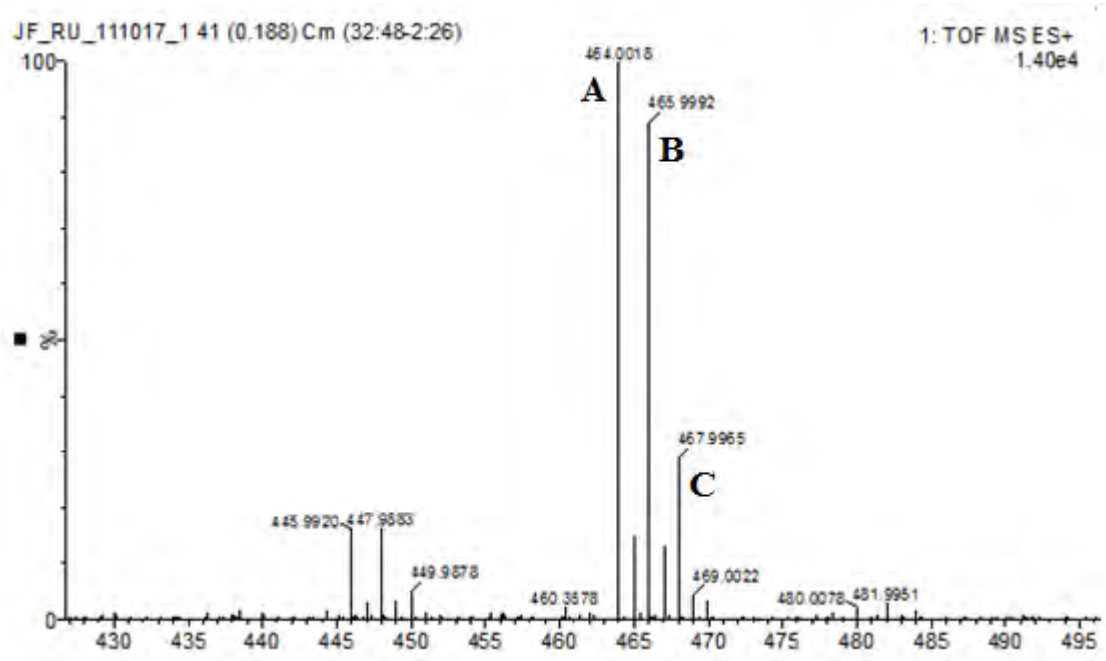


Figure 2.12: HRESMS of PFBAH derivative of compound **2.27**

The mass spectrometry information together with the carbon chemical shifts of carbons C-8 and C-9 suggested the presence of a vinyl chloride and *gem*-dichloro substituents at these carbons respectively. Finally, the substituent at C-4 must therefore be an -OH as required by the molecular formula. This was also confirmed by the presence of a strong O-H stretching vibration in the infrared spectrum of compound **2.27** at 3510 cm^{-1} (Figure 2.13).

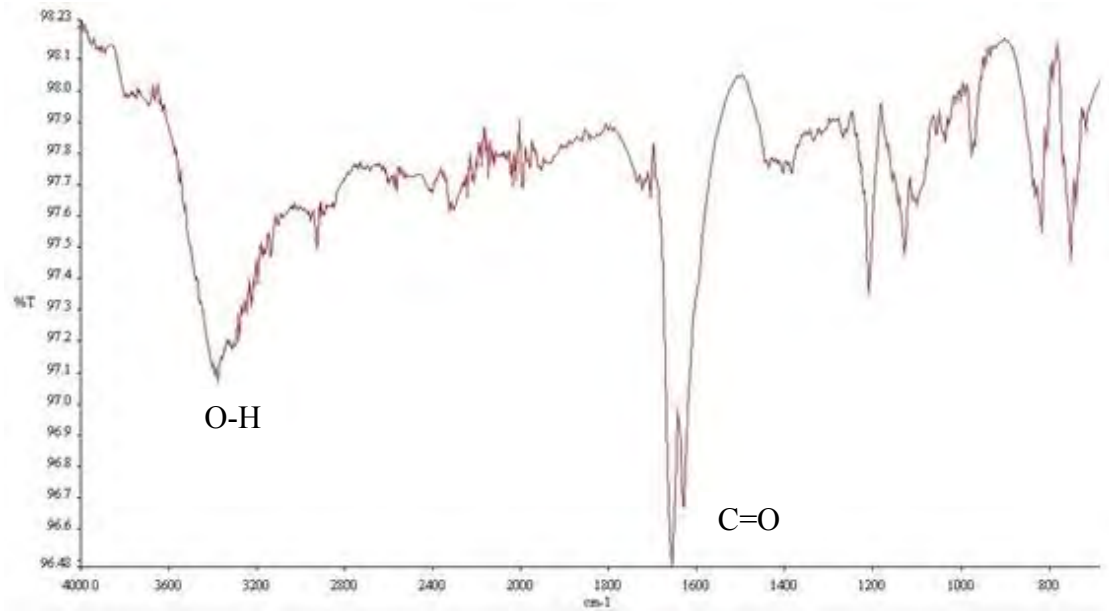
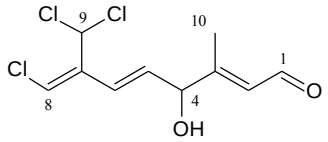


Figure 2.13: IR spectrum of compound **2.27**

The geometry of the $\Delta^{1,2}$, $\Delta^{5,6}$ and $\Delta^{7,8}$ double bonds were assigned by the presence of NOESY cross peaks between H-1 and H-10, H-5 and H-10 and between H-6 and H-8 The vicinal coupling constant ($^3J_{5,6}$ = 15.6 Hz) was also considered.

The presence of both an aldehyde as well as a *gem*-dichloro moiety within the same molecule has not been seen before within this compound class.



2.27

Table 2.2: NMR spectroscopic data of compound **2.27** in $CDCl_3$

Carbon No	δ_C	δ_C **mult**	δ_H**, mult, J (Hz)**	**COSY**	**HMBC**	**NOESY**
1	191.4	CH	10.14, d, 7.9	H-2	C-2	H-10
2	130.6	CH	6.02, d, 7.8	H-1, H-10	C-10	-
3	152.9	C	-	-	-	-
4	69.3	CH	5.40, br s	H-5, H-10	-	H-10
5	136.0	CH	6.29, dd, 15.6, 5.6	H-4, H-6	C-3, C-4	H-10
6	134.2	CH	6.62, d, 15.6	H-4, H-5	C-4	H-8
7	140.6	C	-	-	-	-
8	122.5	CH	6.47, s	-	C-9	H-5
9	66.5	CH	6.94, s	-	C-7, C-8	H-6
10	13.2	CH_3	2.29, s	-	C-1, C-2, C-3	-

2.2.2.2 Compounds 2.28 and 2.29

The complex cluster of peaks between δ 6.3 and 6.4 in the 400 MHz ^{1}H NMR spectra for **2.28** and **2.29** were initially somewhat confusing. Fortunately, the ^{1}H NMR spectra at 600 MHz clarified the situation by resolving the signals due to three separate protons (δ 6.31, dd, J = 15.6, 5.7 Hz; δ 6.32, d, J = 15.6 Hz; δ 6.32, s) as in compound **2.28** (Figure 2.15 and Table 2.3). It was therefore clear that **2.28** and **2.29** contained the same -CH=CH-CHX- moiety as in compound **2.27**. However, signals for the conjugated aldehyde have been replaced by a disubstituted double bond at δ 6.59 (d, J = 13.5 Hz) and 6.46 (d, J = 13.5 Hz) as in compound **2.28**. In addition, signals corresponding to the *gem*-dichloro group (δ 6.96, s) and the vinyl chloride (δ 6.32, s) were also present. The most notable difference between the ^{1}H NMR spectra (Figure 2.14) of **2.28** and **2.29** was the upfield shift of the methyl signal from δ 1.82 in compound **2.28** to δ 1.77 in compound **2.29** suggestive of diastereomers (Mynderse and Faulkner, 1975).

The replacement of the conjugated aldehyde by a disubstituted double bond was confirmed by the loss of the aldehyde signal and the appearance of an additional olefinic carbon signal in the ^{13}C NMR spectrum at δ 110.7 as in compound **2.28**. The differences in the planar structures of **2.27** and **2.28/2.29** can thus be narrowed down to carbons C-1 to C-3.

A comparison of the spectroscopic data of compound **2.28** and **2.29** to literature values confirmed it to be *(1E,3R*,4**R***,5E,7E)-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene* and *(1E,3R*,4**S***,5E,7E)-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene*, respectively. These two compounds have previously been isolated from *Plocamium cartilagineum* (Mynderse and Faulkner, 1975) and *Plocamium suhrii* (Antunes *et al*., 2011).

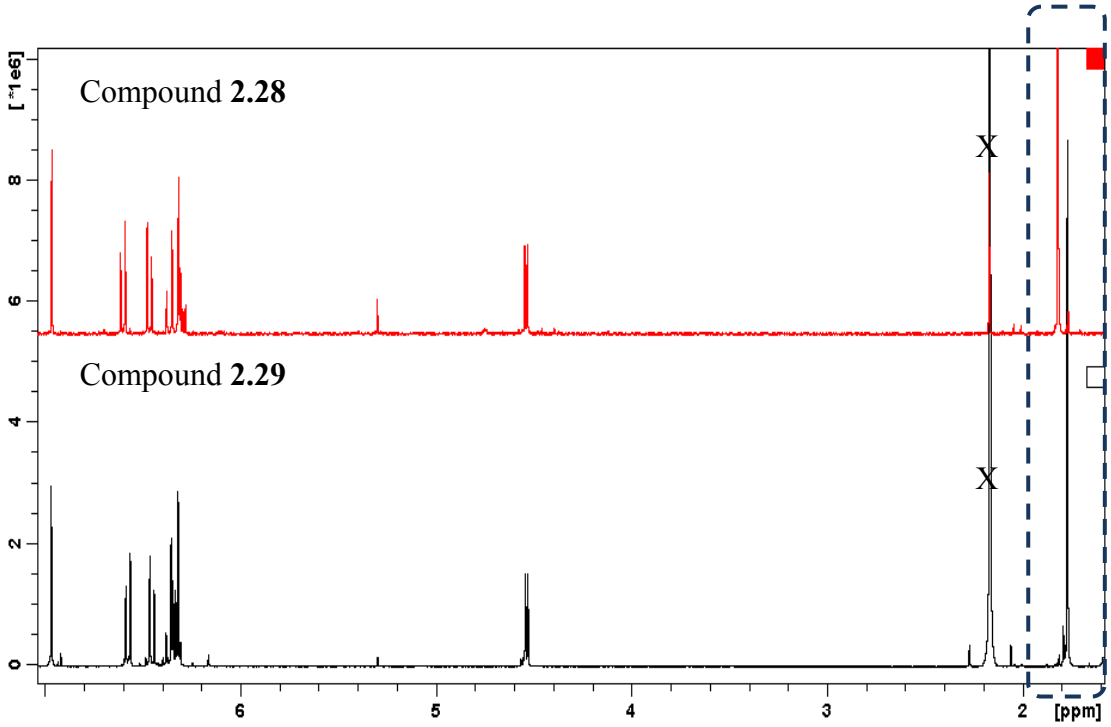


Figure 2.14: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of compound **2.28** and compound **2.29**

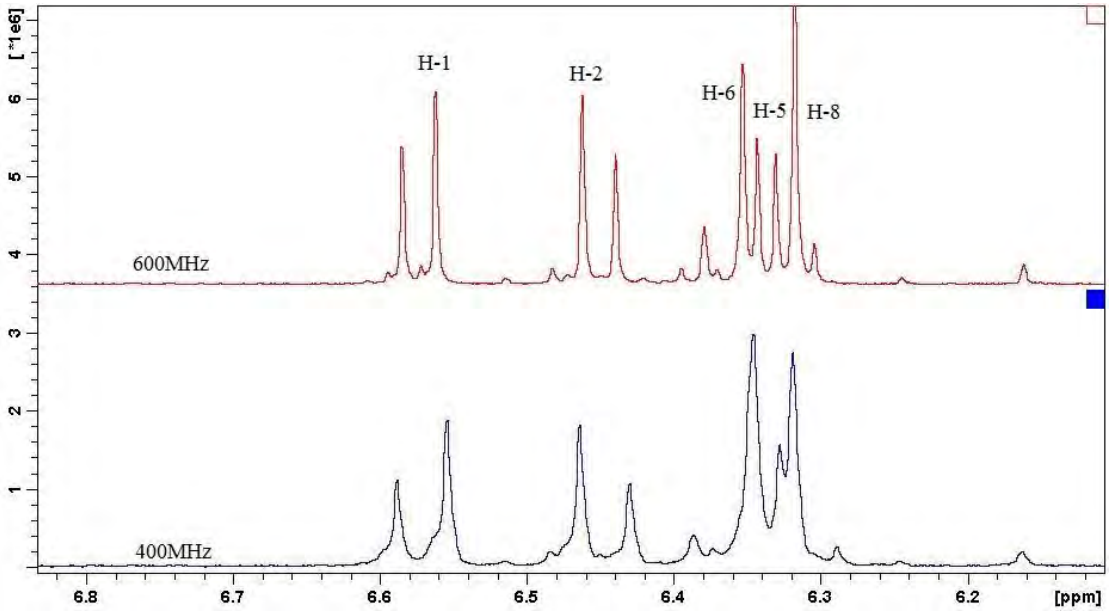


Figure 2.15: ^{1}H NMR expansion of compound **2.29** showing comparisons of peak splitting using different NMR field strengths

Compounds **2.29** and **2.27** are the major metabolites produced by *Plocamium rigidum*. Figure 2.16 clearly shows the prominent signals of the two compounds in the crude ^{1}H NMR spectrum.

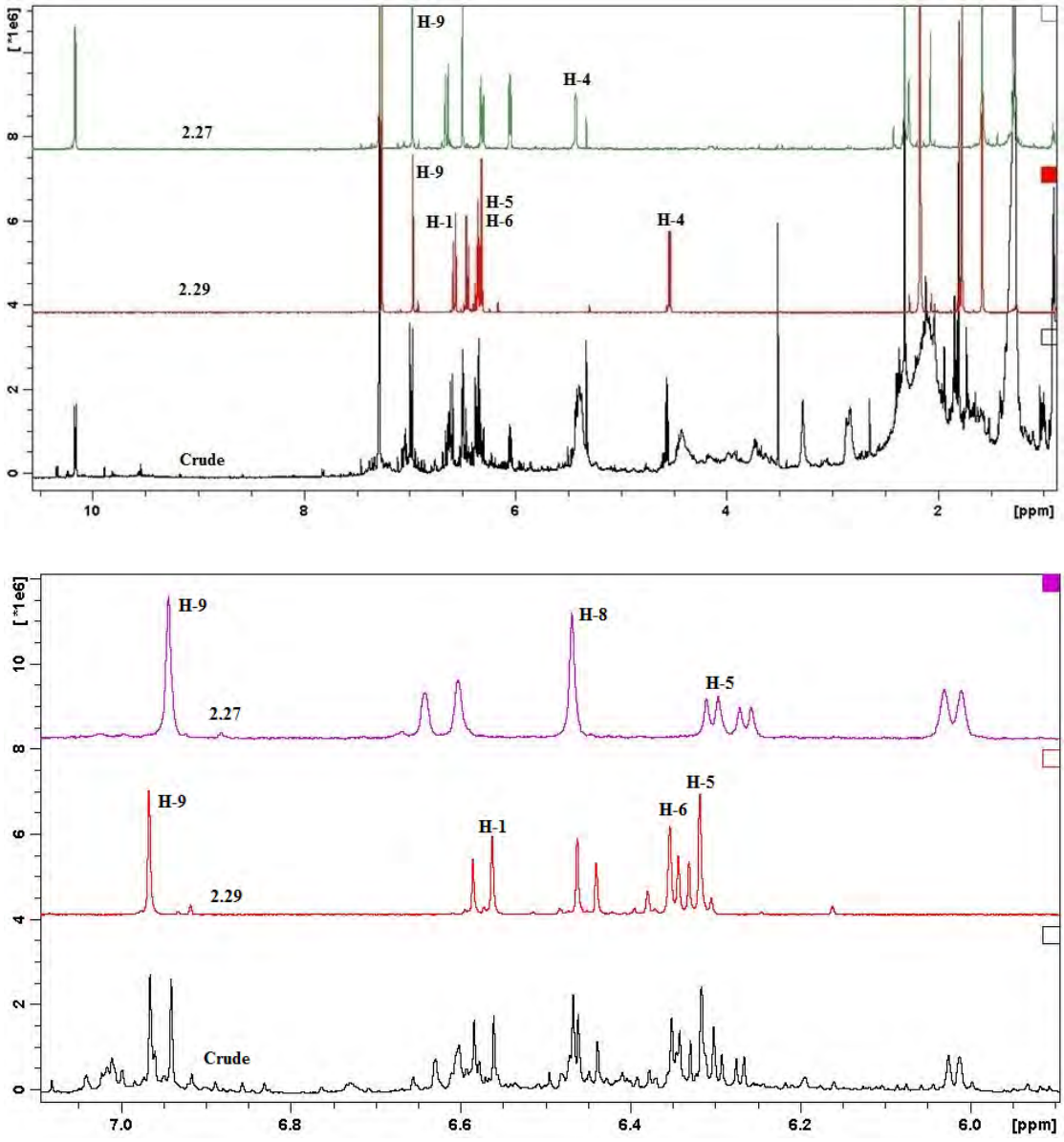
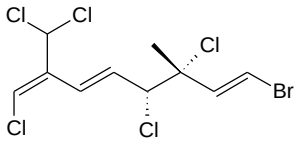
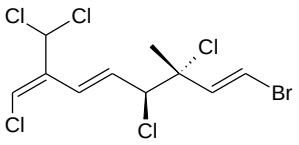


Figure 2.16: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of the crude extract *vs.* the proposed major metabolite **2.29** *vs.* compound **2.27**, with expansion (5.9 ppm -7.2 ppm) shown in the bottom spectrum



2.28



2.29

2.2.2.3 Compound 2.30

The ^{1}H NMR spectrum of **2.30** (Figure 2.17) was similar to that of compound **2.29** except for replacement of the signals due to the vinyl bromide with those of a terminal alkene (δ 5.42, d, *J* = 17.4 Hz; 5.30 d, *J* = 10.6 Hz and 6.09, dd, *J* = 17.1, 10.6 Hz). Interestingly, and unlike in **2.28** and **2.29**, the overlapping ^{1}H NMR signals at δ 6.3 could not be resolved even at 600 MHz. The ^{13}C NMR spectrum of **2.30** showed resonances due to ten carbons which included one methyl (δ 25.0), one methylene (δ 116.5) corresponding to the terminal alkene, six methine (δ 65.5, 68.8, 119.3, 126.8, 130.4 and 139.5) and two quaternary carbons (δ 71.9 and 137.7) (Table 2.4).

The above spectroscopic data is consistent with that of (*3R*,4S*,5E,7Z)-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene*, a penta-chloro monoterpene previously isolated from *Plocamium cartilagineum* (Mynderse and Faulkner 1975) and *Plocamium suhrii* (Antunes *et al*., 2011).

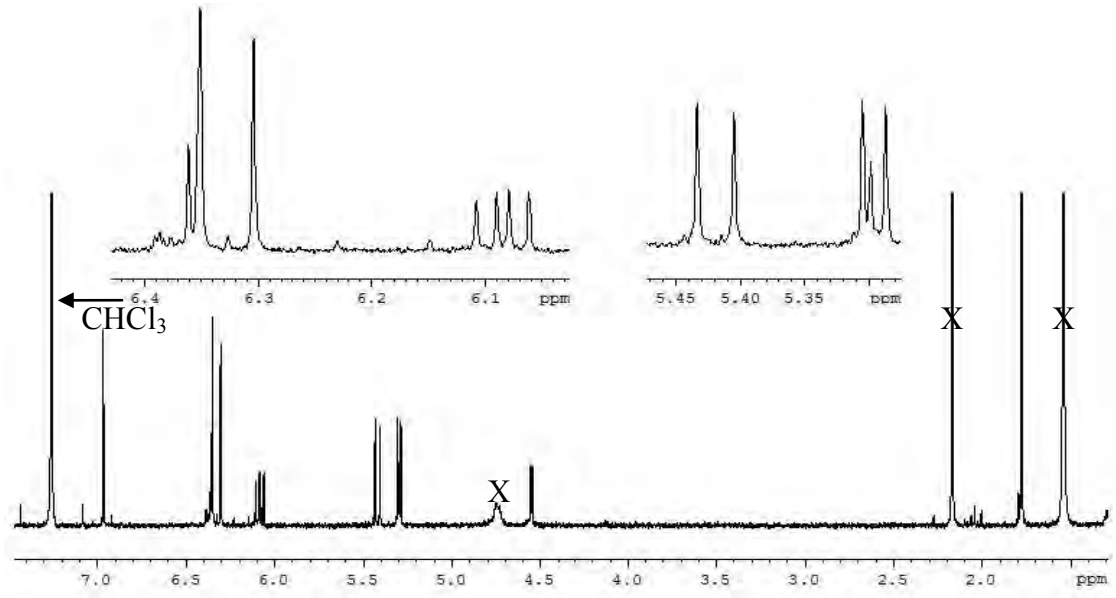
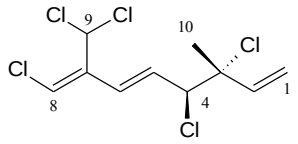


Figure 2.17: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **2.30**



2.30

2.2.2.4 Compound 2.31

Compound **2.31**, isolated as a colourless oil, showed in its ^{1}H NMR spectrum (Figure 2. 18 and Table 2.3) a clear olefinic doublet at δ 6.85 (d, *J* = 15.8 Hz,), a double doublet at 5.92 (dd, *J* = 15.8, 9.2 Hz), and a doublet at 4.57 (d, *J* = 9.2 Hz). This data is consistent with a -CH=CH-CHX- moiety as seen in compounds **2.27**-**2.30**. Methine doublets at δ 6.59 (d, *J* = 13.5 Hz) and 6.49 (d, *J* = 13.5 Hz) suggest a *trans*-disubstituted alkene in **2.31**. Prominent differences in the ^{1}H NMR spectrum of **2.31** when compared to those of compounds **2.28-3.30** include a more shielded olefinic methine singlet at δ 6.20 (*cf* δ 6.3 in compounds **2.28-2.30**), and the absence of the *gem*-dichloro signal (normally at δ 7.0). The latter was replaced by a methyl singlet at δ 1.91.

The ^{13}C NMR spectrum of **2.31** showed resonances due to ten carbons which included two methyl (δ 19.5 and 28.5), six methine (δ 69.2, 108, 110.4, 128.1, 132.9 and 135.5) and two quaternary (δ 71.9 and 137.3) carbon atoms(Table 2.4).

The geometry about the $\Delta^{7,8}$ double bond was confirmed by NOE correlations between H-8 and H-9. The relative configuration of C-4 was assigned based on the δ_C of C-10 as governed by empirical evidence established by Crews, (1977). The above spectroscopic information is consistent with the structure of *(1E,3R*,4R*,5E,7Z)-1,8-dibromo-3,4-dichloro-3,7-dimethylocta-1,5,7-triene* previously reported by (Mynderse and Faulkner, 1975) from *Plocamium cartilagineum* and from *Plocamium suhrii* (Antunes *et al*., 2011).

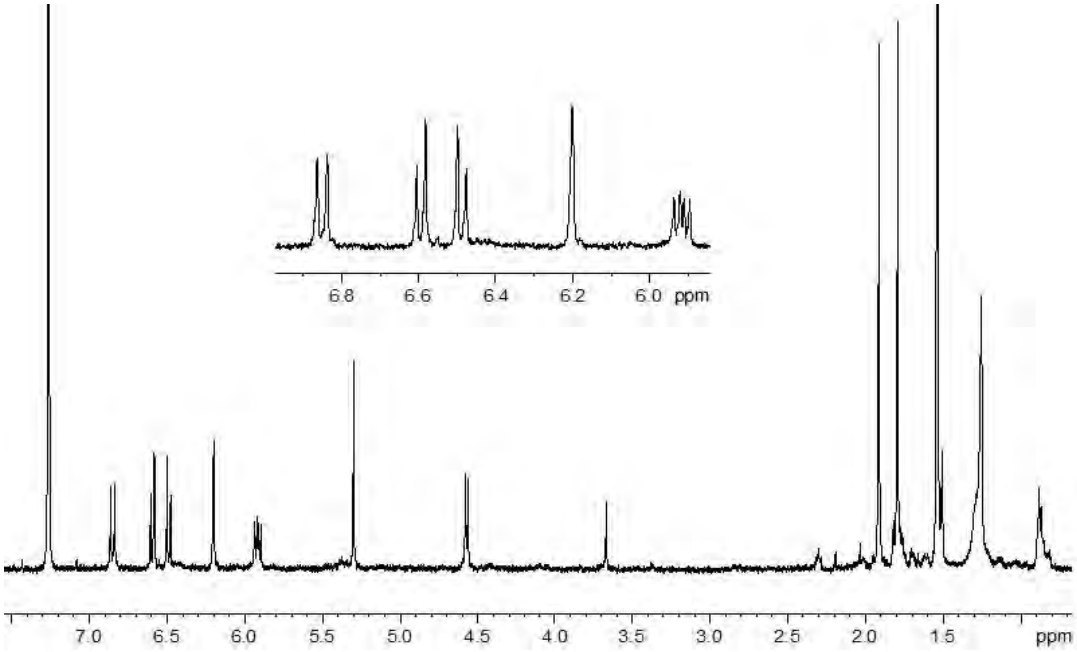
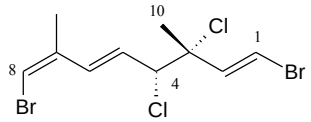
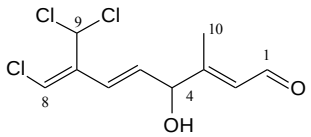


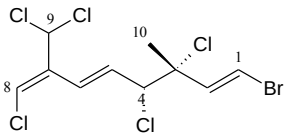
Figure 2.18: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **2.31**



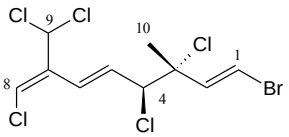
2.31



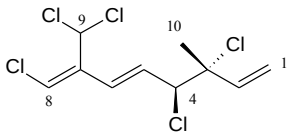
2.27



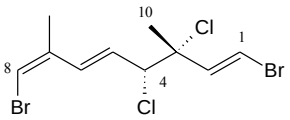
2.28



2.29



2.30



2.31

Table 2.3: ^{1}H NMR data ($CDCl_3$, 600 MHz) for compounds **2.27-2.31**

Compound	H-1	H-2	H-4	H-5	H-6	H-8	H-9	H-10
2.27	10.14, d, 7.9	6.02, d, 7.8	5.40, s	6.29, dd, 15.6, 5.6	6.62, d, 15.6	6.47, s	6.94, s	2.29, s
2.28	6.59, d, 13.5	6.46, d, 13.6	4.54, d, 8.4[1]	6.31, dd, 15.6, 5.7	6.32, d, 15.6	6.32, s	6.96, s	1.82, s
2.29	6.57, d, 13.6	6.45, d, 13.6	4.54, d, 6.8[1]	6.33, dd, 15.5, 5.3	6.34, d, 15.6	6.32, s	6.96, s	1.77, s
2.30	5.42, d, 17.4	6.09, dd, 17.4, 10.6	4.55, d, 6.6[1]	6.35, dd, 15.5, 5.6	6.36, d, 15.5	6.30, s	6.97,s	1.78, s
	5.30, d, 10.6	-	-	-	-	-	-	-
2.31	6.59, d, 13.5	6.49, d, 13.5	4.57, d, 9.2	5.92, dd, 15.8, 9.2	6.85, d, 15.8	6.20, s	1.91, s	1.79, s

[1] Previously reported by (Antunes *et al*., 2010) as multiplets however the multiplicity was ascertained in this study. Compounds **2.29-2.31** show virtual coupling due to the small Δδ for H-5 and H-6. This makes determination of coupling constants for H-4, H-5 and H-6 unattainable and these coupling patterns are thus reported as multiplets.

Table 2.4: ^{13}C NMR data ($CDCl_3$, 150 MHz) for compounds **2.27-2.31**

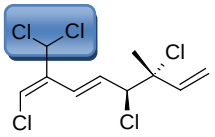
Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
2.27	191.4	130.6	152.9	69.3	136.0	134.1	140.6	122.5	66.5	13.2
2.28	110.7	137.0	71.8	68.5	127.3	129.8	137.6	119.5	65.5	28.1
2.29	110.2	137.5	71.5	68.2	127.4	129.6	138.5	119.7	65.5	25.3
2.30	116.5	139.5	71.8	68.8	126.8	130.4	137.7	119.3	65.5	25.0
2.31	110.4	135.5	71.9	69.2	128.1	132.9	137.3	108.0	19.5	28.5

2.2.3 Cytotoxicity of compounds 2.27 – 2.31

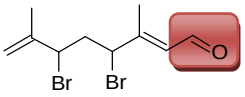
Halogenated monoterpenes containing *gem*-dichloro or aldehyde moieties at either C-1 or C-9 have been shown to produce favourable IC_{50} values in cytotoxic assays (Table 2.5) (de la Mare *et al*., 2012). It was therefore of interest to assess the cytotoxicity of compound **2.27** which contained both functionalities. Ideally, it would have been useful to include the *P. corallorhiza* metabolites in this study. An attempt was made to re-isolate these metabolites however, the collected specimens of *P. corallorhiza* showed the presence of different metabolites in both intra- and inter-site samples. This data is summarised in Appendix 6.2.

Of particular interest in this series of compounds was the importance of the aldehyde and dichloromethyl groups. These groups are likely to exert their cytotoxic effect through non-specific interactions with biological macromolecules. For this reason, citral was also tested in the same cytotoxicity assay.

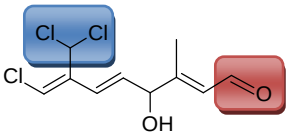
The cytotoxicity assay was carried out against the MCF-7 breast cancer cell line. Surprisingly, compound **2.27**, which contains both a dichloromethyl and an aldehyde group, showed lower cytotoxic activity (IC_{50} value of 116.3 μM, Table 2.6) relative to other reference compounds within this series (Table 2.5). Citral, bearing the same aldehyde moiety as seen in **2.26** and **2.27** proved to be non-toxic suggesting that the aldehyde moiety alone is not a sufficient requirement for cytotoxic effects (de la Mare *et al*., 2012). It therefore seems that the cytotoxicity of halogenated monoterpenes cannot simply be explained on the basis of isolated functional groups. This idea is reinforced when considering that other dichloromethyl containing halogenated monoterpenes such as **2.28** – **2.30** (tested in the same assay) showed good activity.



2.2



2.26



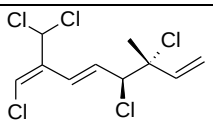
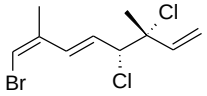
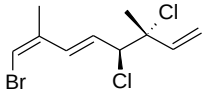
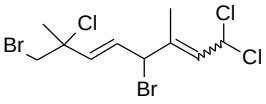
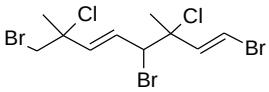
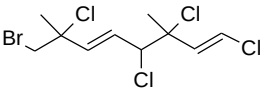
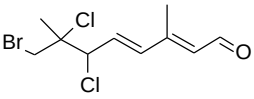
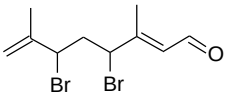
2.27

Figure 2.19: Compound **2.27** possesses both a *gem*-dichloro as well as an aldehyde functionality that have been seen to exist separately in other isolated halogenated monoterpenes i.e. Compounds **2.2** and **2.26**

Not surprisingly, compound **2.31** was found to be non-toxic. This followed the same trend as previously reported (compounds **2.3** and **2.4**, Table 2.5) where a methyl substituent at C-9 is thought to be the reason for reduced activity.

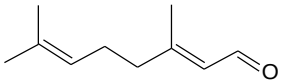
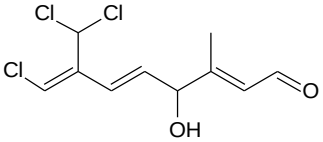
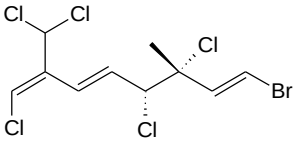
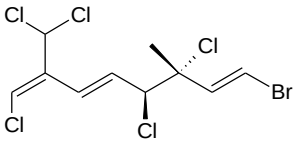
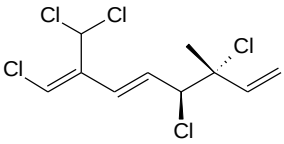
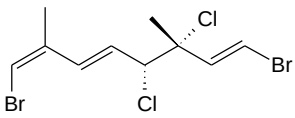
The *gem*-dichloro containing compound, **2.30**, showed an IC_{50} value of 54.9 µM. The relative cytotoxicity values of the diastereomers **2.28** and **2.29** were 16.2 and 13.7 µM respectively indicating that the configuration at C-4 bears little to no influence on cytotoxicity.

Table 2.5: Previously reported IC_{50} values (μM) of halogenated monoterpenes isolated from *P. cornutum, P. suhrii* and *P. corallorhiza* on the MCF-7 breast cancer cell line (Lawson, 2009; de la Mare *et al*., 2012)

Compound	Structure	IC_{50} (μM)
2.2		3.6
2.3		Non-toxic [1]
2.4		Non-toxic
2.14		65.1
2.16		69.7
2.18		90.0
2.22		28.9
2.26		33.0

[1] IC_{50} values greater than 200 μM were considered non-toxic

Table 2.6: IC_{50} values of compounds **2.27-2.31** against the MCF-7 breast cancer cell line

Compound	Structure	IC_{50} (µM)
Citral		Non-toxic [1]
2.27		116.3
2.28		16.2
2.29		13.7
2.30		54.9
2.31		Non-toxic

[1] IC_{50} values greater than 200 µM were considered non-toxic

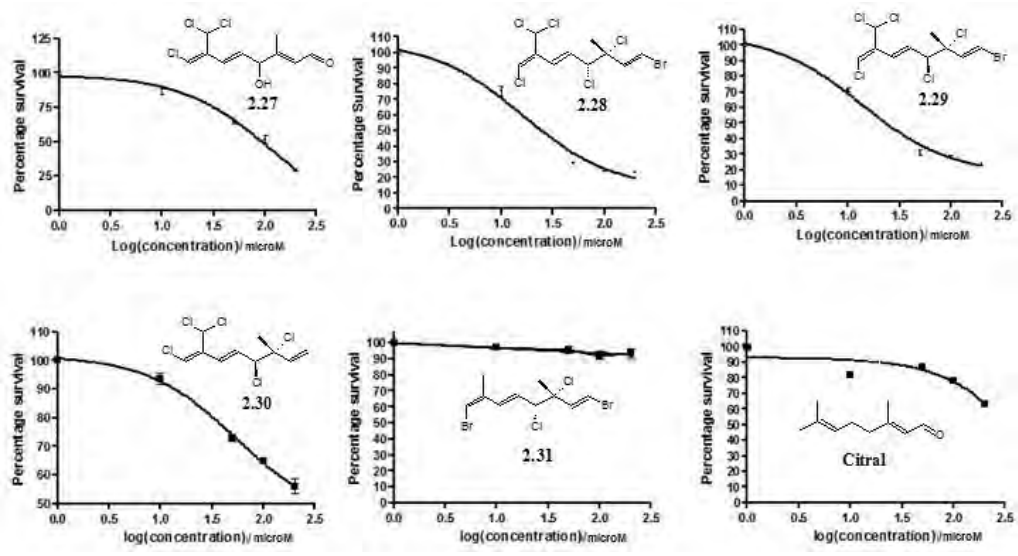


Figure 2.20: Graphs of % survival of MCF-7 cells *vs.* conc. for compounds **2.27-2.31**

A graph of cLogP *vs.* IC_{50} (Figure 2.21) was plotted to explore the possible relationship between partition coefficient and cytotoxicity of compounds **2.27** -**2.30**. Unsurprisingly the plot suggests that an increase in the lipophilicity, which enhances membrane permeation of the molecule, increases the cytotoxic potential. This could further explain why compound **2.27** displayed less favourable results than the other compounds.

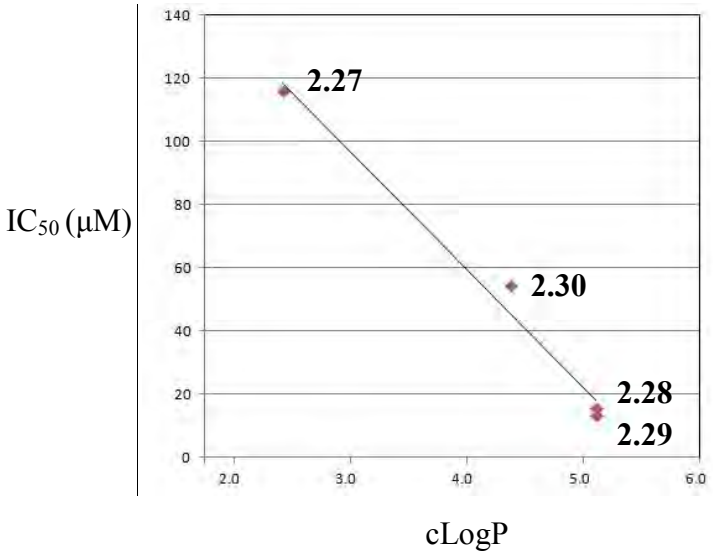
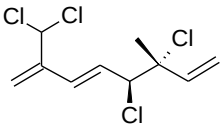


Figure 2.21: Plot of cLogP *vs.* IC_{50} value of compounds **2.27-2.30**

2.2.4 Chemotaxonomic significance of metabolites from *Plocamium rigidum*

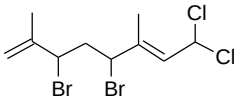
Before making analyses using a chemotaxonomic approach it should be mentioned that at times even homologous characters cannot be used as signs of phylogenetic associations. This is because metabolic convergence of different species is a very common feature (Hegnauer, 1986). Moreover, many secondary metabolites perform diverse ecological roles. The less secondary metabolites deviate from essential metabolites, and the more important secondary metabolites are from an ecological aspect, the greater the chance is of their occurrence in unrelated taxa.

That being said, from a chemotaxonomic perspective, it is interesting to observe that South African *Plocamium* spp can be classified on the basis of their major metabolites. Figure 2.22 highlights the major metabolites produced by selected South African *Plocamium* spp. The significance of the same metabolite occurring in more than one species, for example compounds **2.28** and **2.29** found in both *P. suhrii* and *P. rigidum*, is unclear. Table 2.7 summarises halogenated monoterpenes isolated from selected *Plocamium* spp and highlights their occurrence in different species. It would be interesting to explore the genetic relationship between these algae.



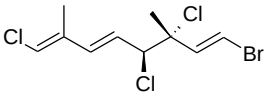
2.5

Plocamium cornutum



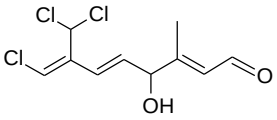
2.12

Plocamium corallorhiza



2.8

Plocamium suhrii



2.27

Plocamium rigidum

Figure 2.22: Major distinguishing metabolites present in *Plocamium* spp.

Table 2.7: Chemotaxonomic overview of halogenated monoterpenes[1]

Compound	Alga(e)	Reference
2.1	*P. cornutum*	(Afolayan *et al*., 2009)
	P. cartilagineum	(Crews and Kho, 1974)
2.2	*P. cornutum*	(Afolayan *et al*., 2009)
	P. cartilagineum	(Faulkner and Mynderse, 1975)
2.3	*P. cornutum*	(Afolayan *et al*., 2009)
2.4	*P. cornutum*	(Afolayan *et al*., 2009)
2.5	*P. cartilagineum*	(Faulkner and Mynderse, 1975)
2.6	*P. suhrii*	(Antunes *et al*., 2011)
2.7	*P. suhrii*	(Antunes *et al*., 2011)
	P. rigidum	**(This study)**
2.8	*P. suhrii*	(Antunes *et al*., 2011)
2.9	*P. suhrii*	(Antunes *et al*., 2011)
	P. cartilagineum	(Faulkner and Mynderse, 1975)
	P. rigidum	**(This study)**
2.10	*P. suhrii*	(Antunes *et al*., 2011)
	P. cartilagineum	(Faulkner and Mynderse, 1975)
	P. rigidum	**(This study)**
2.11	*P. suhrii*	(Antunes *et al*., 2011)
	P. cartilagineum	(Faulkner and Mynderse, 1975)
	P. rigidum	**(This study)**
2.12	*P. corallorhiza*	(Knott *et al*., 2005)
2.13	*P. corallorhiza*	(Mann *et al*., 2007)
2.14	*P. corallorhiza*	(Mkwananzi, 2005)
2.15	*P. corallorhiza*	(Mkwananzi, 2005)
2.16	*P. corallorhiza*	(Mann *et al*., 2007)
2.17	*P. corallorhiza*	(Knott *et al*., 2005)
2.18	*P. corallorhiza*	(Knott *et al*., 2005)
2.19	*P. corallorhiza*	(Mkwananzi, 2005)
2.20	*P. corallorhiza*	(Mkwananzi, 2005)
2.21	*P. corallorhiza*	(Mkwananzi, 2005)
2.22	*P. corallorhiza*	(Mann *et al*., 2007)
2.23	*P. corallorhiza*	(Mann *et al*., 2007)
2.24	*P. corallorhiza*	(Mkwananzi, 2005)
2.25	*P. corallorhiza*	(Mann *et al*., 2007)
2.26	*P. corallorhiza*	(Mann *et al*., 2007)
2.27	***P. rigidum***	**(This study)**

[1] Refer to Appendix 6.1 for structures of compounds **2.1**-**2.27**

2.3 Experimental

2.3.1 General Experimental

All NMR experiments were performed using a Bruker® Avance 600 MHz spectrometer using standard pulse sequences. ^{1}H and ^{13}C experiments were run at 600 MHz and 150 MHz respectively using $CDCl_3$ as the NMR solvent. Column chromatography was performed using Merck® Silica gel 60 (0.040-0.063 mm), Germany. All solvents used were chromatography grade (LiChrosolv®), obtained from Merck®, Darmstadt, Germany.

HPLC was performed using a semi-preparative normal phase Whatman Partisil® 10 M9/50 (9.5mm x 500mm) column and a Rheodyne® containing Waters® HPLC pump with a Spectra physics® RI detector attached to a Ridenki® chart recorder.

High resolution mass spectra were obtained at the University of Stellenbosch using a Waters® Synapt G2 spectrometer. The sample was introduced via direct injection (1 μL into a stream of 80% acetonitrile and 0.1% formic acid using a Waters UPLC at flow rate of 0.2 mL/min. The ionisation source was ES+ with a capillary voltage of 3 kV and a cone voltage of 15 V. The lock mass was set with Leucine enkephalin.

2.3.2 Plant material

(DH110219-3)

Plocamium rigidum was collected by hand at de Hoop beach in the southern coastal region of South Africa in February 2011. A voucher specimen has been stowed away with the Division of Pharmaceutical Chemistry, Rhodes University. Identification of the algae was done by Professor John Bolton with the Department of Botany, University of Cape Town, South Africa.

2.3.3 Extraction and isolation

The algal material (wet mass 125.6 g; dry mass 35.0 g) was initially steeped in MeOH at room temperature for 1 hour, after which it was extracted in triplicate with CH_2Cl_2-MeOH (2:1,770 mL) at a constant temperature of 40 ^{o}C. The combined organic phases were collected (after addition of an adequate amount of water for phase separation) and concentrated under reduced pressure to produce a crude extract of 0.390 g which

corresponds to a 1.10% yield [Weight of crude/(Weight of dry mass + Weight of Crude)]. Fractionation was further carried out via hexane-EtOAc step gradient column chromatography to yield eight fractions. Fraction B and D were further purified via normal phase HPLC using 100% hexane to yield **2.27** (6.0 mg, 0.015%), **2.28** (8.0 mg, 0.02%), **2.29** (10.7 mg, 0.0271%), **2.30** (3.1 mg, 0.0078%) and **2.31** (3.1 mg, 0.0078%).

2.3.4 MCF-7 assay details

The effect of the compounds on MCF-7 cells (obtained from the University of Cape Town, originally supplied by ATCC, cat no HTB-22) was determined using the chromogenic compound MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Roche®).

The assay is based on the cleavage of the yellow MTT salt to form purple crystals in metabolically active cells, which are then solubilised and the concentration determined by optical density at 550 nm.

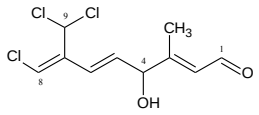
Plates are seeded with 6000 cells/well in 50 µL and incubated overnight at 37 ºC and 9% CO_2. Thereafter the cells are dosed with a range of concentrations of the test compound (dissolved in DMSO) in 50 µL and incubated for 96 hours as before, after which 10 µL of 5 mg/mL MTT reagent is added before incubating for a further 4 hours.

Finally, 100 µL of Solubilisation Solution (10% SDS in 0.01 M HCl) is added and the cells are incubated overnight before reading their absorbances at 550 nm. The chemotherapeutic drug paclitaxel (Sigma®) is used as a control cytotoxic compound at 100 nM.

All treatments are carried out in triplicate. The absorbance *vs.* concentration curve is used to determine the percentage survival at a particular concentration, from which the IC_{50} value may be calculated relative to the DMSO vehicle-treated negative control.

2.3.5 Compounds isolated[1]

Compound 2.27 (isolation code JF12-19D-F9)



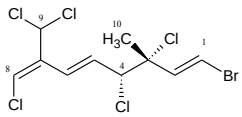
2.27

(2E,5E,7Z)-8-chloro-7-(dichloromethyl)-4-hydroxy-3-methylocta-2,5,7-trienal (**2.27**): brown oil; $[\alpha]_D$+158; IR V_{max} 1650, 3510 cm^{-1}; NMR data available in Table 2.2; HRESIMS of PFBHA derivative, *m/z* 464.0018 [M+1] (calculated 464.0010) $C_{17}H_{13}NO_2{}^{35}Cl_3F_5$ / 465.9992 / 467.9965; 445.9920 [M+1-H_2O].

*Derivitisation of compound **2.27** for mass spectrometry*

Compound **2.27** (3 mg) was weighed out and dissolved in acetonitrile (1.5 mL). This was then added to PFBHA.HCl (3 mg) (PFBHA – O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride, ≥98%, Sigma Aldrich®, Switzerland), previously dissolved in acetonitrile (1.5 mL). Anhydrous magnesium sulphate (1.5 mg) was also added. The reaction mixture was stirred for 5 hours at room temperature. The reaction mixture was concentrated in vacuo and silica gel column chromatography was then used to purify the resultant aldehyde derivative using (9:1 hexane: EtOAc).

Compound 2.28 (isolation code JF12-19B-F8H)

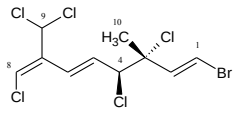


2.28

(1E,3R,4R*,5E,7E)-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene* (**2.28**): colourless oil; NMR data available in Tables 2.3 and 2.4. As previously reported by (Mynderse and Faulkner, 1975).

[1] The systematic name for compounds **2.27**-**2.31** was based on the 3-methyl-1,5,7-octatriene skeleton which is most commonly used in literature

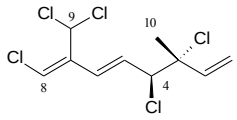
Compound 2.29 (isolation code JF12-19B-F8I)



2.29

(1E,3R,4S,5E,7E)-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene (**2.29**): colourless oil; NMR data available in Tables 2.3 and 2.4. As previously reported by (Mynderse and Faulkner, 1975).

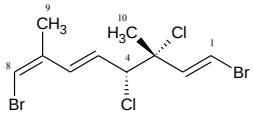
Compound 2.30 (isolation code JF12-19B-F8E)



2.30

(*3R*,4S*,5E,7Z)-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene* (**2.30**): colourless oil; NMR data available in Tables 2.3 and 2.4. As previously reported by (Mynderse and Faulkner, 1975).

Compound 2.31 (isolation code JF12-19B-F7)



2.31

(1E,3R,4R*,5E,7Z)-1,8-dibromo-3,4-dichloro-3,7-dimethylocta-1,5,7-triene* (**2.31**): colourless oil; NMR data available in Tables 2.3 and 2.4. As previously reported by (Mynderse and Faulkner, 1975).

2.4 References

Afolayan, A. F. **2008**. Isolation and characterisation of anti-plasmodial metabolites from South African marine algae. M.Sc thesis. Rhodes University, Grahamstown.

Afolayan, A. F.; Mann, M. G. A.; Lategan, C. A.; Smith, P. J.; Bolton, J. J.; Beukes, D. R. Antiplasmodial halogenated monoterpenes from the marine red alga *Plocamium cornutum*. *Phytochemistry* **2009**, *70*, 597-600.

Antunes, E. M.; Afolayan, A. F.; Chiwakata, M. T.; Fakee, J.; Knott, M. G.; Whibley, C. E.; Hendricks, D. T.; Bolton, J. J.; Beukes, D. R. Identification and *in vitro* anti-esophageal cancer activity of a series of halogenated monoterpenes isolated from the South African seaweeds *Plocamium suhrii* and *Plocamium cornutum*. *Phytochemistry* **2011**, *72*, 769-772.

Bino, R. J.; Hall, R. D.; Fiehn, O.; Kopka, J.; Saito, K.; Draper, J.; Nikolau, B. J.; Mendes, P.; Roessner-Tunali, U.; Beale, M. H.; Trethewey, R. N.; Lange, B. M.; Wurtele, E. S.; Sumner, L. W. Potential of metabolomics as a functional genomics tool. *Trends in Plant Science* **2004**, *9* , 418-425.

Cremades, J.; Barrero, R.; Maneiro, I.; Saunders, G. A New Taxonomic Interpretation of the Type of *Plocamium cartilagineum* (Plocamiales, Florideophyceae) and its Consequences. *European Journal of Phycology* **2011**, *46*, 125–142

Crews, P.; Kho, E. Cartilagineal. An unusual monoterpene aldehyde from marine alga. *Journal of Organic Chemistry* **1974**, *39*, 3303.

Crews, P.; Kho, E. Plocamene B. New cyclic monoterpene skeleton from a red marine alga. *Journal of Organic Chemistry* **1975**, *40* , 2568-2570.

Crews, P. Monoterpene halogenation by the red alga *Plocamium oregonum*. *Journal of Organic Chemistry* **1977**, *42*, 2634-2636.

Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R. Halogen regiochemistry and substituent stereochemistry determination in marine monoterpenes by carbon-13 NMR. *Journal of Organic Chemistry* **1984**, *49*, 1371-1377.

Davies Coleman, M. T.; Beukes, D. R. Ten years of marine natural products research at Rhodes University. *South African Journal of Science* **2004**, *100,* 539-544.

de Ines, C.; Argandoña, V. H.; Rovirosa, J.; San-Martin, A.; Diaz-Marrero, A. R.; Cueto, M.; Gonzalez-Coloma, A. Cytotoxic activity of halogenated monoterpenes from *Plocamium cartilagineum*. *Journal of Biological Sciences* **2004**, *59*, 339-344.

de la Mare, J. A.; Lawson, J.; Chiwakata, M.; Beukes, D.; Edkins, A.; Blatch, G. Quinones and halogenated monoterpenes of algal origin show anti-proliferative effects against breast cancer cells in vitro. *Investigational New Drugs* **2012**, *30* , 2187-2200.

Faulkner, D. J.; Stallard, M. O. 7-chloro-3,7-dimethyl-1,4,6-tribromo-1-octen-3-ol, a novel monoterpene alcohol from *Aplysia californica*. *Tetrahedron Letters* **1973**, *14*, 1171-1174.

Fuller, R. W.; Cardellina II, J. H.; Jurek, J.; Scheuer, P. J.; Alvarado-Lindner, B.; McGuire, M.; Gray, G. N.; Steiner, J. R.; Clardy, J.; Menez, E.; Shoemaker, R. H.; Newman, D. J.; Snader, K. M.; Boyd, M. R. Isolation and structure/activity features of halomon related antitumor monoterpenes from the red alga *Portieria hornemannii*. *Journal of Medicinal Chemistry* **1994**, *37*, 4407–4411.

Gentry, C. L.; Egleton, R. D.; Gillespie, T.; Abbruscato, T. J.; Bechowski, H. B.; Hruby, V. J.; Davis, T. P. The effect of halogenation on blood brain barrier permeability of a novel peptide drug. *Peptides* **1999**, *20* , 1229-1238.

Greenbaum, D. C.; Mackey, Z.; Hansell, E.; Doyle, P.; Gut, J.; Caffrey, C. R.; Lehrman, J.; Rosenthal, P. J.; McKerrow, J. H.; Chibale, K. Synthesis and Structure Activity Relationships of Parasiticidal Thiosemicarbazone Cysteine Protease Inhibitors against *Plasmodium falciparum*, *Trypanosoma brucei*, and *Trypanosoma cruzi*. *Journal of Medicinal Chemistry* **2004**, *47* , 3212-3219.

Hegnauer, R. Phytochemistry and plant taxonomy-an essay on the chemotaxonomy of higher plants. *Phytochemistry* **1986**, *25*, 1519-1535.

Knott, M. G. **2003**. The natural product chemistry of South African *Plocamium* species, M.Sc thesis. Rhodes University, Grahamstown.

Knott, M. G.; Mkwananzi, H.; Arendse, C. E.; Hendricks, D. T.; Bolton, J. J.; Beukes, D. R. Plocoralides A-C, polyhalogenated monoterpenes from the marine alga *Plocamium corallorhiza*. *Phytochemistry* **2005**, *66* , 1108-1112.

König, G. M.; Wright, A. D.; Linden, A. D. *Plocamium hamatum* and its monoterpenes: chemical and biological investigations of the tropical marine red alga. *Phytochemistry* **1999**, *52*, 1047–1053.

Lawson, J.; M.Sc thesis; Analysis of the anti-cancer activity of novel indigenous algal compounds in breast cancer: towards the development of a model for screening anti-cancer stem cell activity, **2009**, Rhodes University, Grahamstown.

Lubke, R. A.; Gess, F. W.; Bruton, M. N. *A field guide to the Eastern Cape coast*; The Grahamstown Centre of the Wildlife Society of Southern Africa: Grahamstown, **1988**, 56-57.

Mann, M. G. A.; Mkwananzi, H.; Antunes, E. M.; Whibley, C. E.; Hendricks, D. T.; Bolton, J. J.; Beukes, D. R. Halogenated monoterpene aldehydes from the South African marine alga *Plocamium corallorhiza*. *Journal of Natural Products* **2007**, *70*, 596–599.

Mann, M. G. A. **2008**. An investigation of the antimicrobial and antifouling properties of marine algal metabolites. M.Sc thesis. Rhodes University, Grahamstown.

MBARI (Monterey Bay Aquarium and Research Institute), Phycological Society of America; "*Plocamium*: the delicate beauty" Accessed (07/03/2012). Available at http://www.mbari.org/staff/conn/botany/reds/eric/PHYS.HTM

Mkwananzi, H. B. **2005**. The study of *Plocamium corallorhiza* metabolites and their biological activity. M.Sc thesis. Rhodes University, Grahamstown.

Monforte, A. M.; Logoteta, P.; Ferro, S.; Luca, L. D.; Iraci, N.; Maga, G.; Clercq, E. D.; Pannecouque, C.; Chimirri, A. Design, synthesis, and structure activity relationships of 1,3-dihydrobenzimidazol-2-one analogues as anti-HIV agents. *Bioorganic & Medicinal Chemistry* **2009**, *17* , 5962-5967.

Mynderse, J. S.; Faulkner, D. J. Polyhalogenated monoterpenes from the red alga *Plocamium cartilagineum*. *Tetrahedron* **1975**, *31* , 1963-1967.

Paul, V. J.; McConnell, O. J.; Fenical, W. Cyclic monoterpenoid feeding deterrents from the red marine alga *Ochtodes crockeri*. *Journal of Organic Chemistry* **1980**, *45*, 3401–3407.

San-Martin, A.; Negrete, R.; Rovirosa, J. Insecticide and acaricide activities of polyhalogenated monoterpenes from Chilean *Plocamium cartilagineum*. *Phytochemistry* **1991**, *30*, 2165–2169.

Saunders, G. W.; Lehmkuhl, K. V. Molecular divergence and morphological diversity among four cryptic species of *Plocamium* (Plocamiales, Florideophyceae) in northern Europe. *European Journal of Phycology* **2005***, 40*, 293–312.

Wise, M. L.; Rorrer, G. L.; Polzin, J. J.; Croteau, R. Biosynthesis of Marine Natural Products: Isolation and Characterisation of a Myrcene Synthase from Cultured Tissues of the Marine Red Alga *Ochtodes secundiramea*. *Archives of Biochemistry and Biophysics* **2002**, *400* , 125-132.

Chapter 3

Isolation of secondary metabolites from *Laurencia natalensis*

Abstract

The *Laurencia* genus of marine algae produces an assortment of structurally unique halogenated and non-halogenated secondary metabolites. These vary considerably and range from C_{15} acetogenins to terpene type compounds.

This chapter discusses the isolation and characterisation of two metabolites from the the organic extract of the South African endemic marine alga *Laurencia natalensis* namely a C_{15} acetogenin, laurencenyne, as well as a new chamigrane sesquiterpene. The structures of the compounds were determined by spectroscopic methods.

Chapter 3

Isolation of secondary metabolites from *Laurencia natalensis*

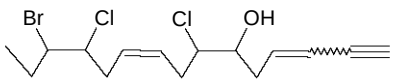
3.1 Introduction

Laurencia, a genus of red marine algae of the Rhodomelaceae family, is one of the most widely studied macroalgae in the aquatic environment. This is due to the vast range of structurally unique halogenated metabolites produced by this genus (Faulkner, 1977; Blunt *et al*., 2009). These include C_{15} acetogenins, sesquiterpenes, diterpenes and triterpenes (Suzuki and Vairappan, 2005). The C_{15} acetogenins give rise to a diverse population of cyclic ethers with ring sizes ranging from five to nine atoms. Side chains possessing terminal enyne or allene functionalities are also common (Erickson, 1979). Metabolites isolated from *Laurencia* spp. show various halogenation patterns; however brominated molecules are most common (Suzuki and Vairappan, 2005). Figure 3.1 demonstrates the diverse nature of some of the metabolites produced by *Laurencia* spp.

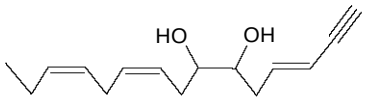
Laurencia spp. can be characterised by a particular set of compounds they produce (Suzuki and Vairappan, 2005). This is owing to the fact that these molecules are unique to each species and are not seen as being widespread among other members within the genus (Fenical, 1976).

Studies have revealed that a single *Laurencia* species, although morphologically identical, can produce different secondary metabolites. *Laurencia nipponica* (Yamada) has shown such a trait. This has led to the concept of further sub-dividing species into chemical races (Masuda *et al*., 1997a, Harborne and Turner, 1984). It would be interesting to further study the phenomenon of chemical races in *Laurencia* spp. and its potential as a chemotaxonomic tool.

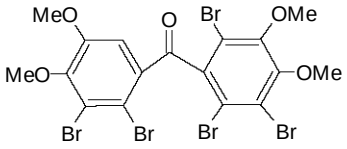
Given the structural diversity and complexity of the metabolites found in *Laurencia* spp. it comes as no surprise that diverse biological activities have been reported. These include antibacterial (Vairappan *et al*., 2001), antiviral (Sakemi *et al*., 1986), cytotoxic (Kladi *et al*., 2009), antiasthmatic (Jung *et al*., 2009) and analgesic (Chatter *et al*., 2009) activities.



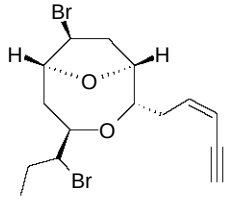
3.1



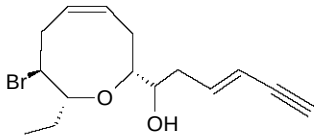
3.2



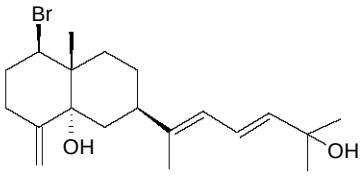
3.3



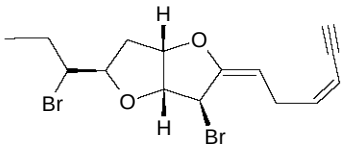
3.4



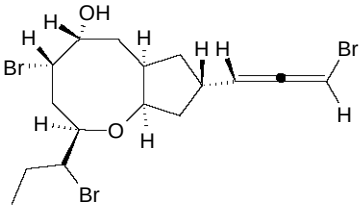
3.5



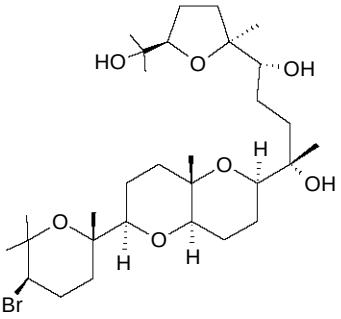
3.6



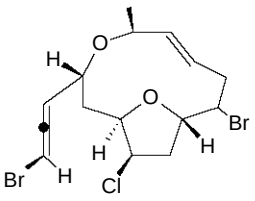
3.7



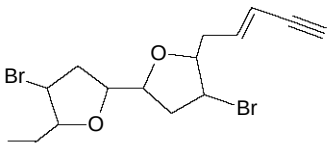
3.8



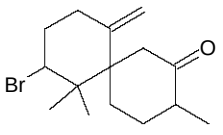
3.9



3.10



3.11



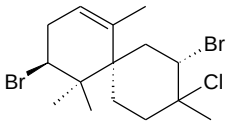
3.12

Figure 3.1: Selected compounds isolated from various *Laurencia* spp.[1]

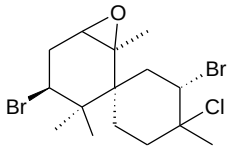
[1] **3.1, 3.10** (Gutierrez-Cepeda *et al*., 2011); **3.2, 3.4, 3.5** (Suzuki *et al*., 2009); **3.3** (Qin *et al*., 2010); **3.8** (Suzuki *et al*., 2005); **3.9** (Sakemi *et* al., 1986); **3.11** (Dias and Urban, 2011); **3.12** (De Nys *et al*., 1993)

3.1.1 South African *Laurencia* spp.

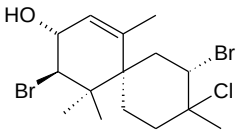
Laurencia spp. found on the South African coastline include *Laurencia natalensis* (Kylin), *Laurencia glomerata* (Kützing) *Laurencia flexuosa* (Kützing) and *Laurencia obtusa* (Hudson) J.V. Lamouroux (Stegenga *et al*., 2007). Of these, only *L. glomerata* and *L. flexuosa* had been studied previously. *L. glomerata* produces chamigrenes and chamigranes **3.13** – **3.16** (Elsworth and Thomson, 1989), while the sesquiterpene **3.17** and (*Z*) and (*E*)-bromofucin (**3.18** and **3.19**) have been isolated from *L. flexuosa* (Mann, 2008).



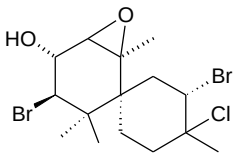
3.13



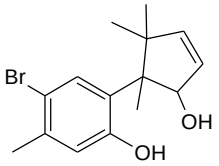
3.14



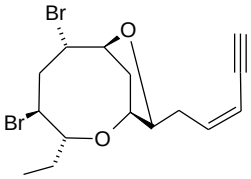
3.15



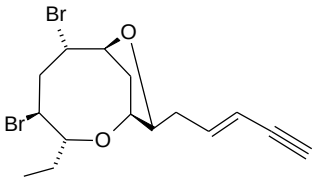
3.16



3.17



3.18



3.19

Figure 3.2: Selected compounds isolated from South African *Laurencia* spp.

3.1.2 Chapter aims

As part of a larger study to assess the chemotaxonomic significance of South African *Laurencia* metabolites we collected the endemic South African marine alga, *Laurencia natalensis*. The main objective of this study was to isolate and characterise the metabolites produced by this alga as a prelude to their assessment as chemotaxonomic markers.

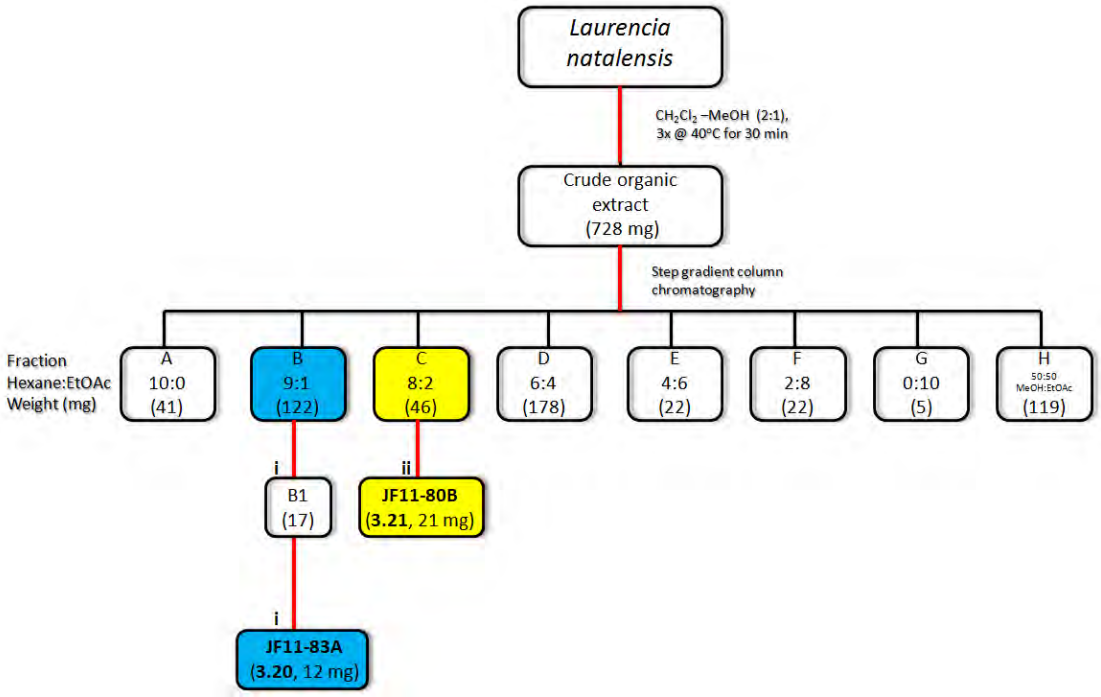
Figure 3.3: Photograph of *Laurencia natalensis*[1]

[1] Photograph (© Robert Anderson, University of Cape Town) adapted from www.algaebase.org (Accessed 23/6/2012) with permission from Michael Guiry (Ryan Institute, Ireland).

3.2 Results and discussion

3.2.1 Extraction and isolation of metabolites

The extraction and fractionation procedures performed on *L. natalensis* (KOS110914-1), collected from Kenton-on-Sea on the eastern coast of South Africa, are summarised in Scheme 3.1. The alga was initially steeped in MeOH and then further extracted with a combination of CH_2Cl_2 and MeOH. The crude organic extract of yield 0.82% was obtained and fractionated into nine separate fractions on silica gel using a step gradient solvent system (hexane-EtOAc) of increasing polarity. Upon analysis of the ^{1}H NMR spectra of these fractions peaks of interest where observed in fractions B and C which were then further purified using silica gel column chromatography to yield compounds **3.20** and **3.21**.



Scheme 3.1: Isolation of compounds **3.20** and **3.21** from *Laurencia natalensis*

Conditions: i) Silica gel column chromatography (9:1 hex: EtOAc)
ii) Silica gel column chromatography (8:2 hex: EtOAc)

3.2.2 Structure elucidation of metabolites

3.2.2.1 Compound 3.20

Compound **3.20**, was isolated as a colourless oil. The ^{1}H NMR spectrum (Figure 3.4) of compound **3.20** showed a complex cluster of olefinic methine signals between δ 5.0 and δ 6.0 indicating the presence of several alkene functionalities. An ethyl moiety (–CH_2–CH_3) was deduced from a triplet signal at δ 0.97 (t, J = 7.6 Hz) which was coupled to a methylene signal at δ 2.08 (dq, J = 7.4, 7.4 Hz). A broad singlet was also observed at δ 3.12.

The ^{13}C NMR spectrum of compound **3.20** (Figure 3.5), in combination with the DEPT-135 spectrum, showed fifteen signals which included resonances characteristic of an enyne system (δ 143.6, 108.3, 81.8 and 80.2) in addition to signals due to a further three double bonds (δ 132.1, 129.6, 128.7, 127.7, 127.0, 126.0).

Compound **3.20** was identified as laurencenyne by comparison of spectroscopic data with literature values. This linear C_{15} acetogenin with a terminal alkyne moiety was previously isolated by Kigoshi *et al*. (1981) and successfully synthesised by Flock and Skattebol (2000).

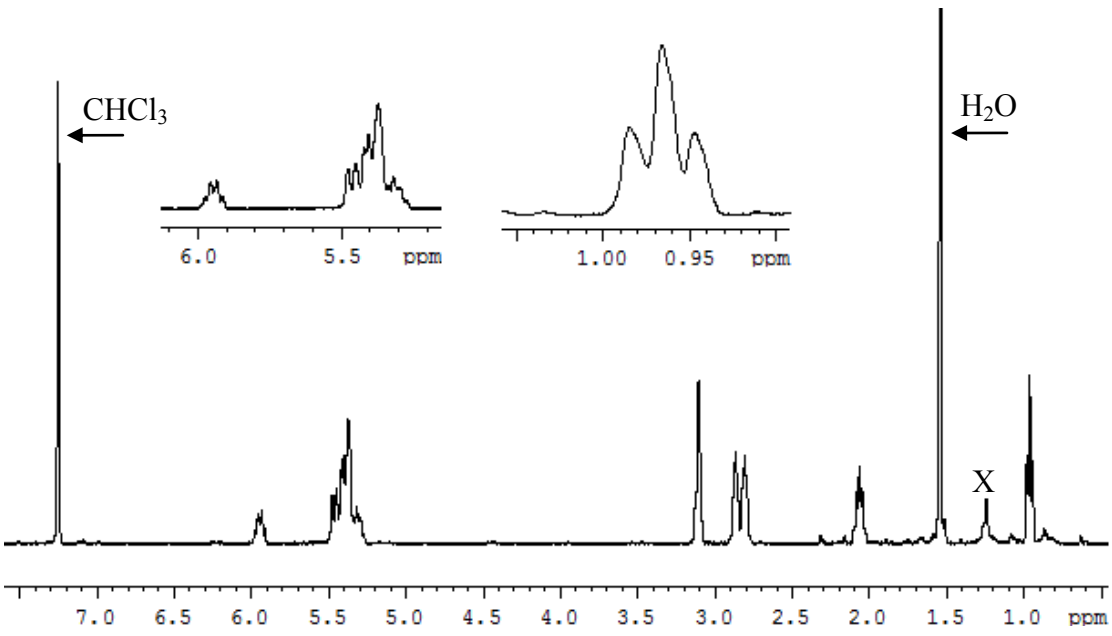


Figure 3.4: ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of compound **3.20**

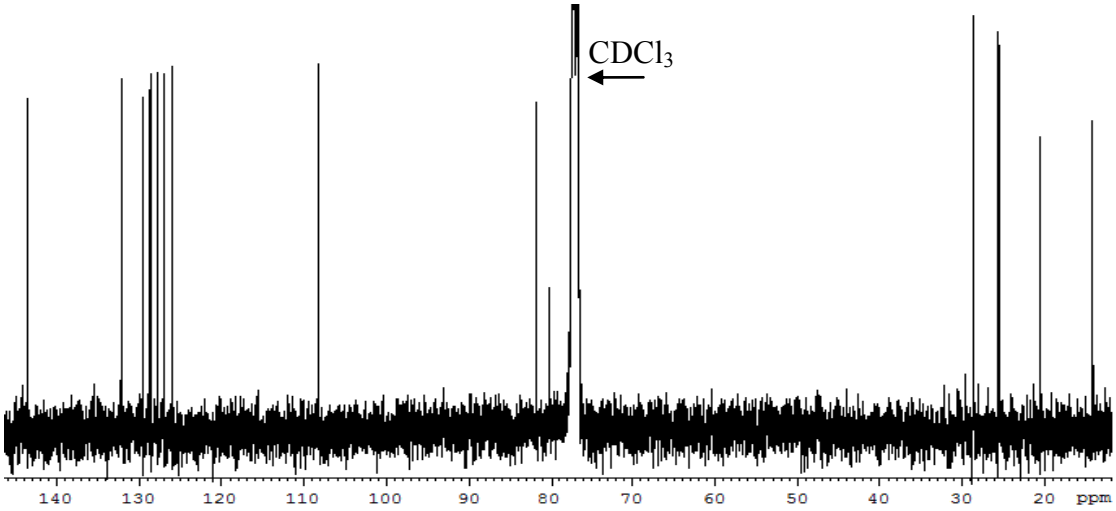
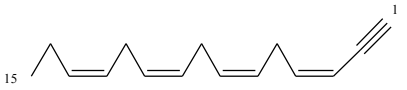


Figure 3.5: ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of compound **3.20**



3.20

Table 3.1: NMR spectroscopic data of compound **3.20** ($CDCl_3$)[1]

Carbon No	δ_C	δ_C mult	δ_H, mult, J (Hz)
1	81.8	CH	3.12, s
2	80.2	C	-
3	108.3	CH	5.48, m
4	143.6	CH	5.96, m
5	28.7	CH_2	3.12, m
6	129.6	CH	5.43, m
7	128.7	CH	5.38, m
8	25.7	CH_2	2.88, m
9	127.7	CH	5.38, m
10	127.0	CH	5.32, m
11	25.5	CH_2	2.83, m
12	126.0	CH	5.43, m
13	132.1	CH	5.39, m
14	20.5	CH_2	2.08, dq, 7.4, 7.4
15	14.3	CH_3	0.97, t, 7.6

[1] The majority of signal multiplicity in the ^1H NMR spectrum of compound **3.20** could not be established due to the abundant overlapping of peaks. Such is a common phenomenon associated with hydrocarbon chains attributable to the presence of several magnetically equivalent spin-systems. The resultant signals were thus reported as multiplets.

3.2.2.2 Compound 3.21

Compound **3.21**, the major component of *L. natalensis*, was isolated as a white crystalline solid. The HRESIMS spectrum of compound **3.21** provided a molecular ion cluster at *m/z* 428.0521, 430.049 and 432.0474 (Figure 3.6) which implied a molecular formula of $C_{17}H_{27}BrCl_2O_3$ and required three degrees of unsaturation. This information, together with the presence of five methyl singlet resonances (δ 0.92, 1.11, 1.23, 1.76 and 2.08) in its ^{1}H NMR spectrum (Figure 3.7) immediately suggested a chamigrane type structure for compound **3.21**.

The ^{1}H NMR spectrum (Figure 3.7) also showed a broad methine singlet at δ 5.02 and a methine double doublet at δ 4.83 (dd, *J* = 12.8, 4.43 Hz). Two sets of diastereotopic methylene resonances were observed at δ 2.52 (t, *J* = 13.1 Hz) and 2.28 (d, *J* = 13.1 Hz) and at δ 2.77 (dd, *J* = 14.9, 3.0 Hz) and 2.62 (dd, *J* = 14.9, 2.9 Hz).

The ^{13}C NMR spectrum (Figure 3.8) of **3.21** showed only one unsaturated carbon signal at δ 169 which was distinctive of an ester moiety. A further five methyls (δ 21.3, 21.5, 22.5, 22.5 and 27.4), four methylenes (δ 30.6, 32.4, 40.2 and 42.0), three methines (δ 59.6, 63.3 and 74.3) and four quaternary carbons (δ 47.7, 50.7, 69.6 and 78.2) were deduced from ^{13}C NMR, DEPT-135 and HSQC data. The HSQC spectrum in particular, was useful in identifying the overlapping carbon signals at δ 22.5 (Figure 3.9). The above information together with the three degrees of unsaturation implied by the molecular formula confirmed the presence of a chamigrane type skeleton.

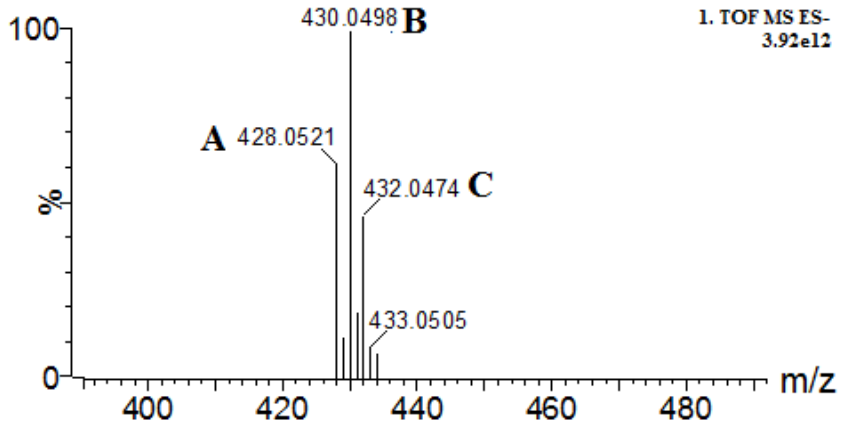


Figure 3.6: HRESMS of compound **3.21**

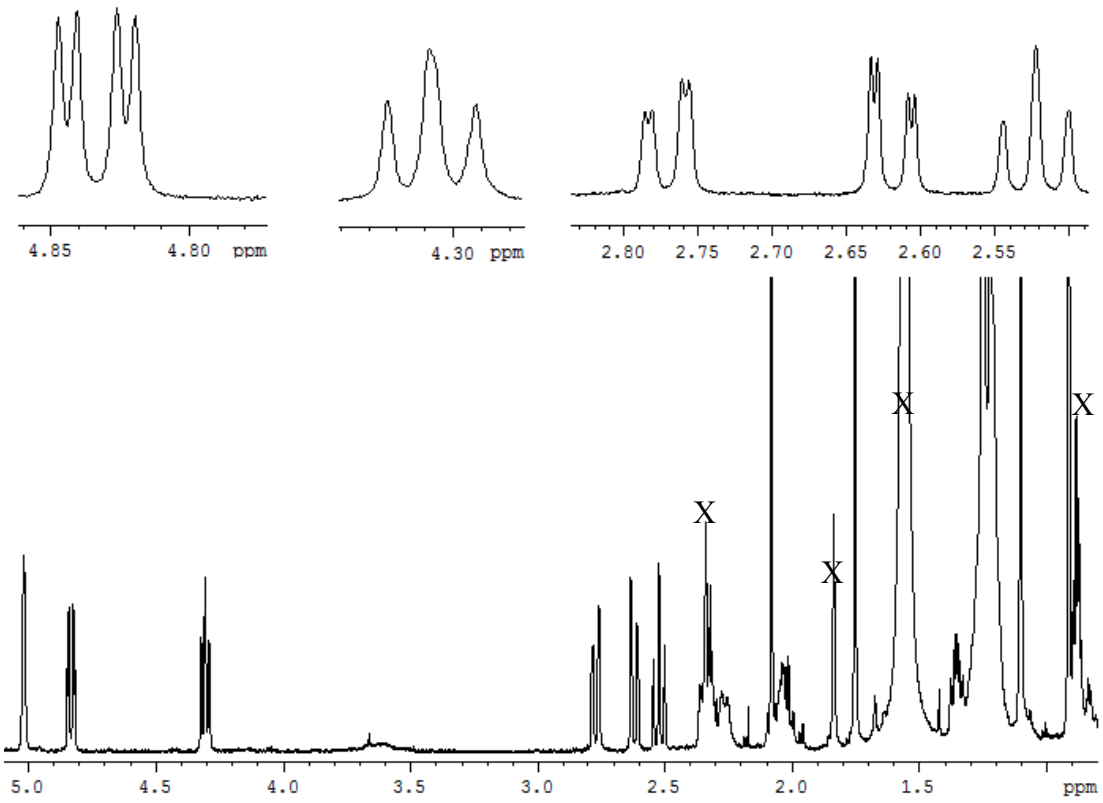


Figure 3.7: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **3.21**

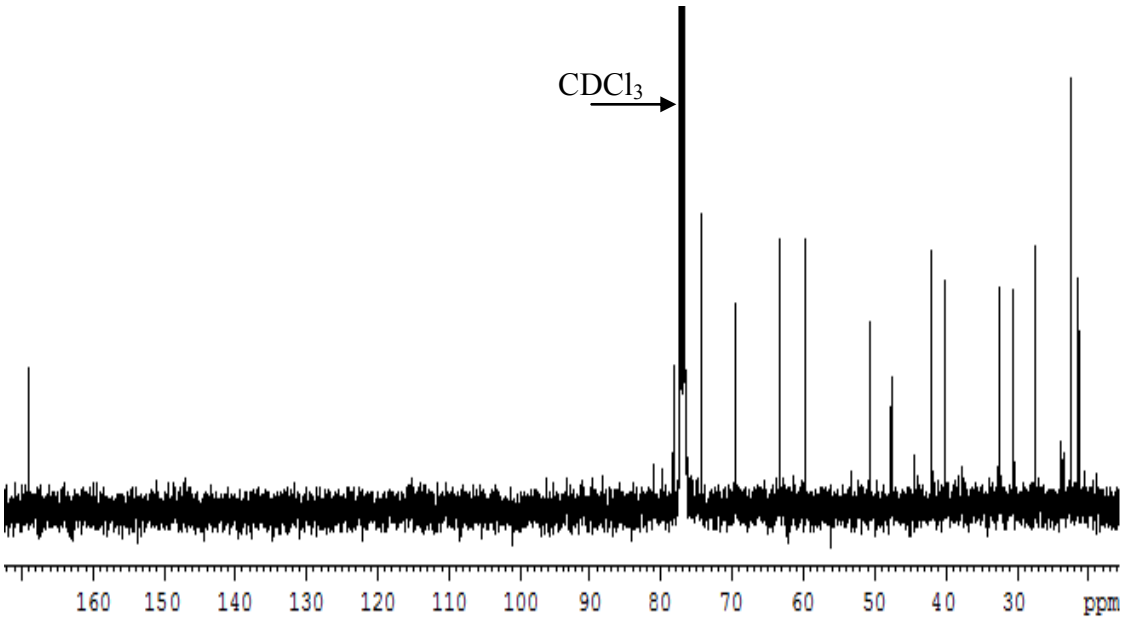


Figure 3.8: ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of compound **3.21**

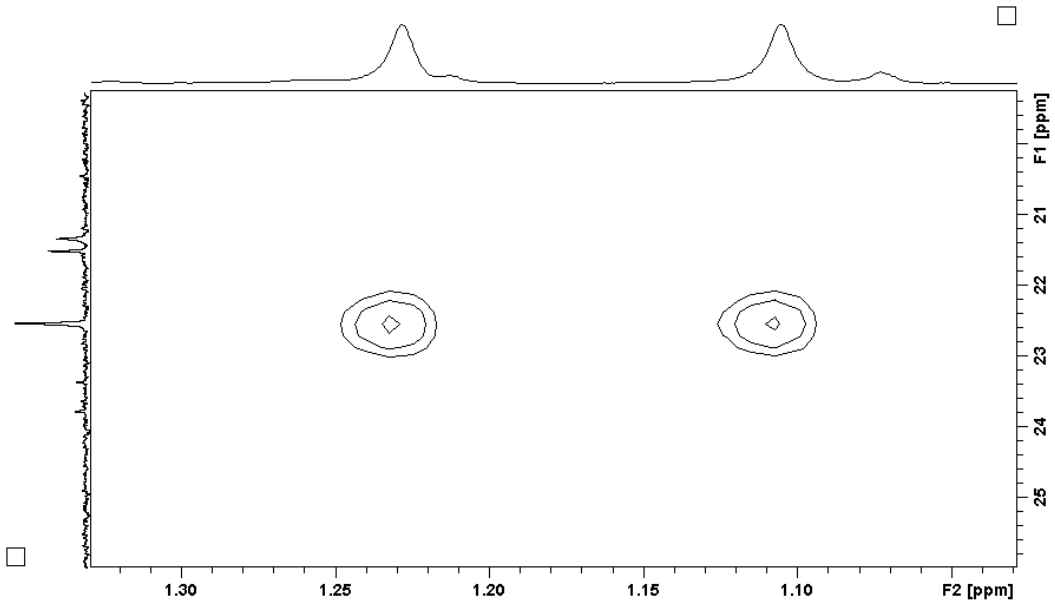


Figure 3.9: HSQC expansion of compound **3.21** showing two signals a δ_C 22.5

Since a chamigrane type system was assumed, two cyclohexane substructures were generated and 2D NMR correlations (COSY, HMBC) were assigned as shown below (Figures 3.10 and 3.11).

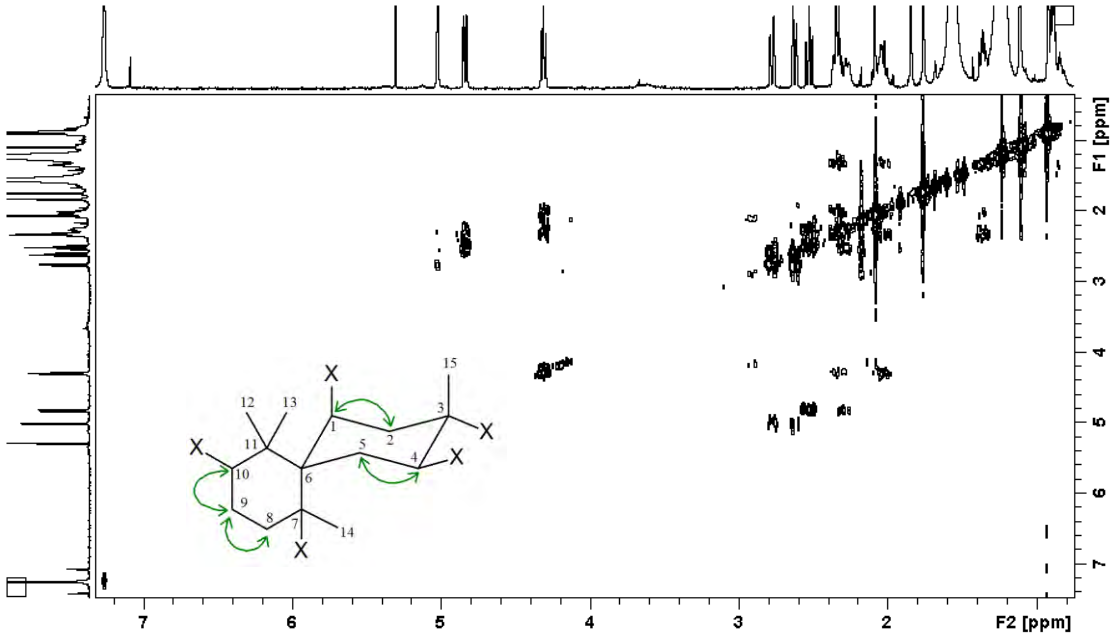


Figure 3.10: COSY NMR spectrum of compound **3.21**

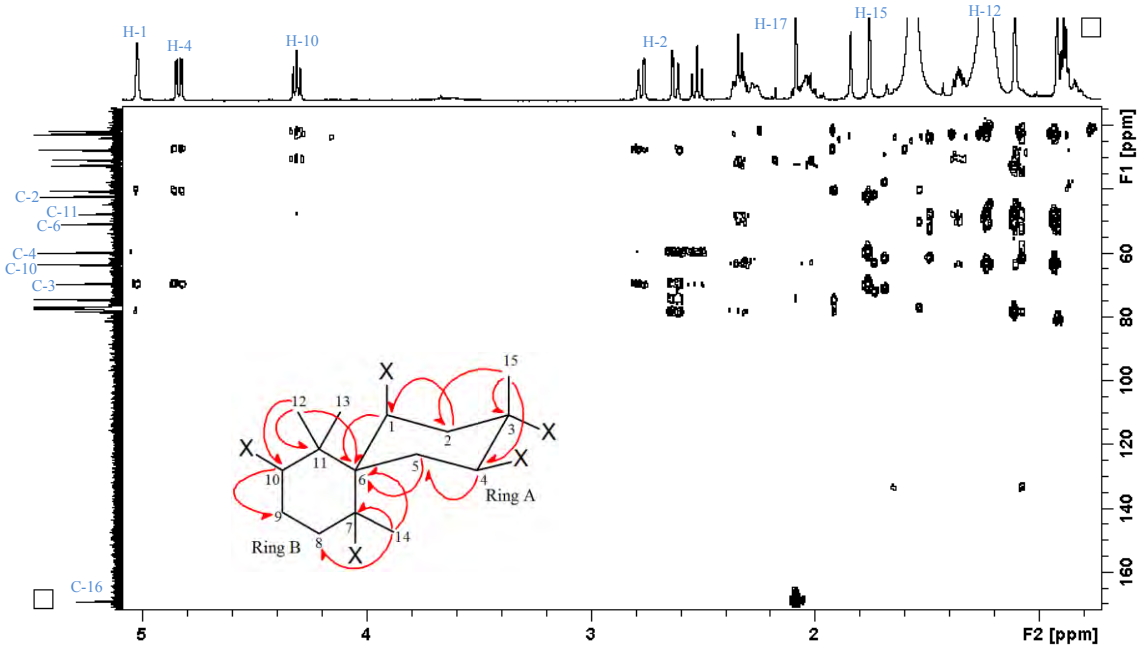


Figure 3.11: HMBC NMR spectrum of compound **3.21**

Ring A was constructed by observation of key HMBC correlations from methyl protons at δ 1.75 (C-15) to carbon atoms with chemical shifts of δ 42.0 (C-2), 59.6 (C-4) and 69.6 (C-3).

Ring B was generated based on key HMBC correlations between methyl protons at δ 1.23 (C-12) and carbon atoms δ 47.7 (C-11), 50.7 (C-6) and 63.3 (C-10). Methyl protons at δ 1.11 (C-14) also showed HMBC correlations to carbon atoms at δ 32.4 (C-8), 50.7 (C-6) and 78.2 (C-7). The carbon at δ 50.7 (C-6) was assigned as the ring junction due to the converging HMBC correlations as well as by comparison of the chemical shift of C-6 in related systems.

Unusual HMBC correlations were also seen to exist between positions H-14 to C-11 and H-15 to C-1 (Figure 3.12). This can be explained by a phenomenon known as W coupling or $^{4\text{-}5}J$ coupling. Such coupling is commonly seen in alicyclic systems wherein atomic bond rotation is hindered. Figure 3.13 illustrates an example of such wherein coupling only occurs between proton H_a and H_c due to a "W" like arrangement ($^4J_{ac}$ = 7 Hz). Neither H_a and H_d nor H_b and H_d show any signs of coupling ($^4J_{ad}$ = 0 Hz; $^4J_{bd}$=7 Hz) (Pretsch *et al.*, 2000).

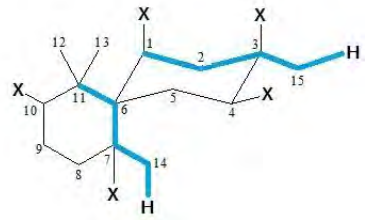


Figure 3.12: Compound **3.21** showing (^4J) coupling between (H-14 and C-6; H-15 and C-11)

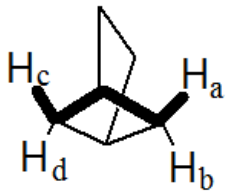


Figure 3.13: Illustration of W coupling in an alicyclic system

Considering the planar structure and the HRESIMS spectrum a -$BrCl_2$ halogenation pattern was assumed. This then corresponds to a molecular formula of $C_{17}H_{27}BrCl_2O_3$ Gonzalez *et al*. (1979) show how various halogens, as well as the relative configuration of these substituents, affect carbon chemical shifts in chamigrane type systems (Figure 3.14).

Since the halogenation pattern was already ascertained to be –$BrCl_2$ from mass spectroscopy data, determining the halogens present at the deshielded carbons C-3 and C-4 would consequently establish the halogen present at C-10.

From Table 3.2 it is seen that the ^{13}C NMR chemical shifts of compound **3.21** for C-3, C-4 and C-10 are δ 69.6, δ 59.6, and δ 63.3 respectively. From Figure 3.14 at position C-4, a bromo-substituent is seen to produce a chemical shift range of δ 62.3-63.6 ppm and a chloro-substituent δ 65.2-68.0 ppm. Since the δ_C at C-4 (59.6) for compound **3.21** was closest to the 62.3-66.6 range, it was assumed that a bromo-substituent existed at C-4. This then leaves two unassigned chlorine atoms hence C-3 and C-10 were assigned as possessing chloro substituents. Furthermore Kennedy *et al*. (1988) isolated a 3-chloro, 4-bromo system with δ_C 70.20 and δ_C 59.79 respectively complementing the NMR spectral data of compound **3.21**. Elsworth and Thomson (1989) also report a similar halogenation pattern showing δ_C 70.7 and δ_C 61.8 at positions C-3 and C-4 respectively (Figure 3.2, compound **3.16**).

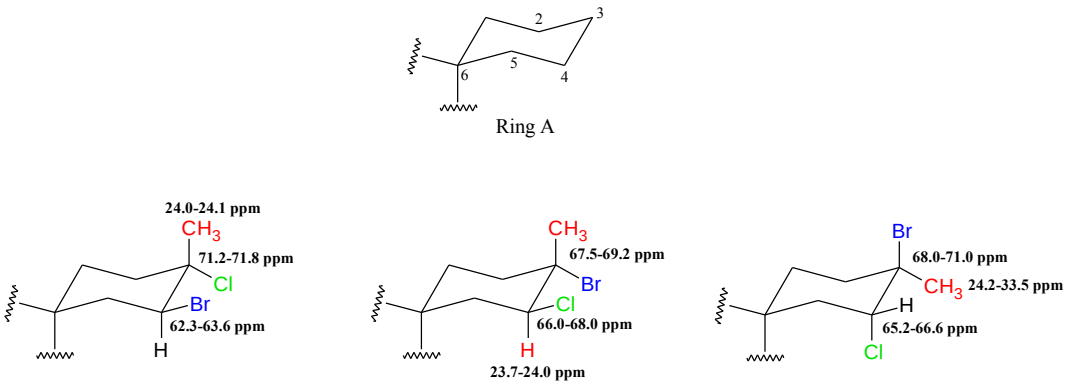


Figure 3.14: The effect of stereochemistry and halogenation pattern on ^{13}C NMR chemical shifts at C-3 and C-4 (Gonzalez *et al*., 1979).

Having assigned the halogens in compound **3.21** we now turned our attention to identifying the X substituents at carbons C-1 and C-7. The IR spectrum (Figure 3.15) showed the presence of a hydroxyl group (3513 cm^{-1}) and a carbonyl (1723 cm^{-1}) which together with carbon NMR chemical shifts at C-1 and C-7 identified the presence of a hydroxyl group and an acetoxy group in compound **3.21**. HMBC correlations could not be used to unambiguously assign the positions of the acetoxy and hydroxyl groups, however a consideration of the chemical shifts and their comparison to those of compound **3.22** also known as algoane (McPhail *et al*., 1999) suggested an acetoxy group at C-1 and a hydroxyl group at C-7 (Figure 3.16). Compound **3.21** is therefore a chlorinated analogue of acetoxyintricatol (compound **3.23**) previously isolated from *Laurencia intricata* from the Atlantic ocean (McMillan and Paul, 1974). The acetoxy group must be in the axial position based on the chemical shift and coupling constant of H-1 and confirmed by NOE correlations to both H-2a and H-2b.

The bromo substituent at C-4 was assigned as being equatorial after considering the vicinal coupling constants of H-4 (dd, J = 12.8, 4.4 Hz) and H-5 (t, J = 13.1 Hz). Furthermore H-4, which is axial, also showed NOE correlations to the axial proton at H-2b (δ 2.77) (Figure 3.17).

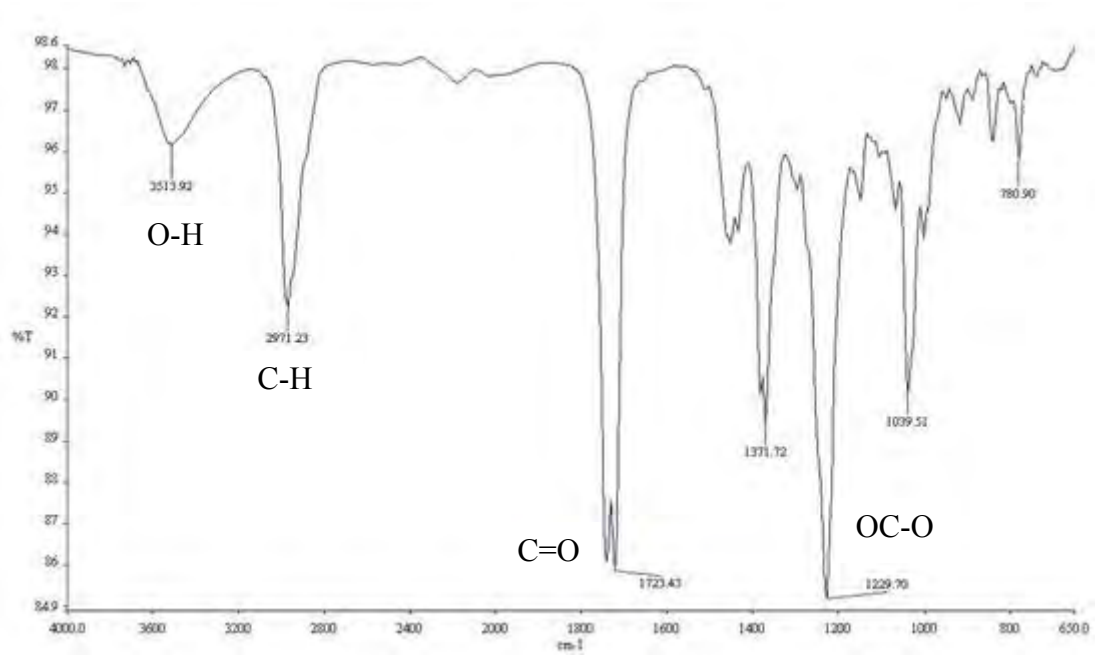
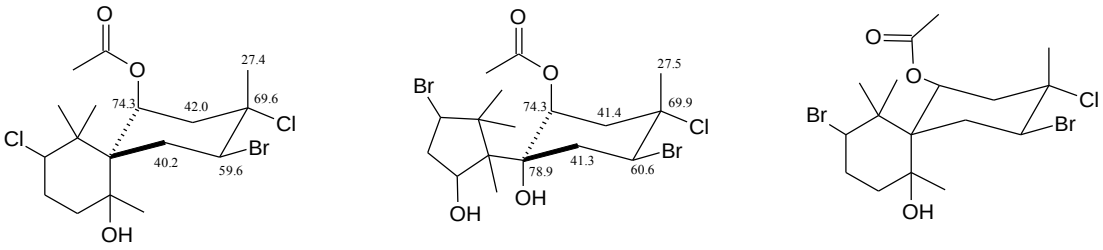


Figure 3.15: IR spectrum of compound **3.21**



3.21 **3.22** **3.23**

Figure 3.16: A comparison of related chamigrane systems to compound **3.21**

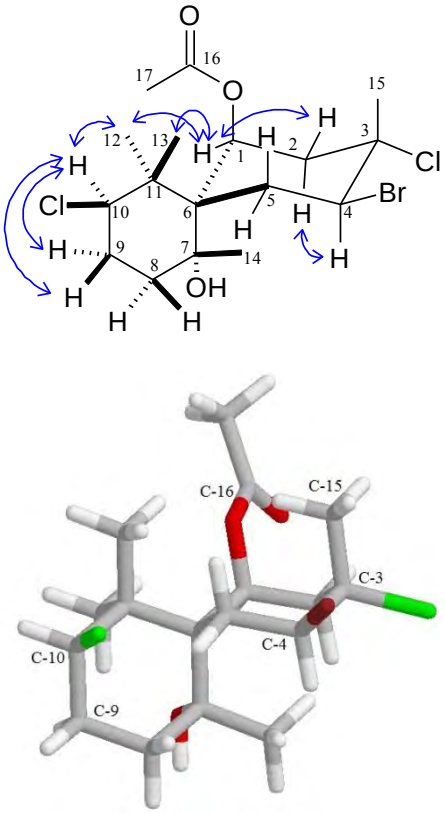
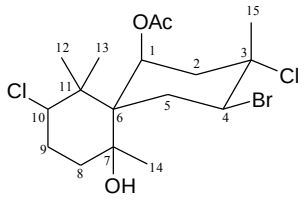


Figure 3.17: Key NOE correlations of compound **3.21**



3.21

Table 3.2: NMR spectroscopic data of compound **3.21** ($CDCl_3$)

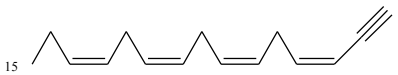
Carbon No	δ_C	δ_C mult	δ_H, mult, J (Hz)	COSY	HMBC	NOESY
1	74.3	CH	5.02, br s	H-2b	C-3, C-5, C-6	H-2a, H-2b, H-12, H-13
2a	42.0	CH_2	2.62, dd, 14.9, 2.9	-	C-1, C-3, C-4, C-15	-
2b			2.77, dd, 14.9, 3.0	H-1	C-3, C-15	H-14
3	69.6	C	-	-	-	-
4	59.6	CH	4.83, dd, 12.8, 4.4	H-5a	C-3, C-5, C-15	H-2b
5a	40.2	CH_2	2.52, t, 13.1	H-4	C-3, C-4	-
5b			2.27, d, 13.1	-	-	-
6	50.7	C	-	-	-	-
7	78.2	C	-	-	-	-
8a	32.4	CH_2	2.34, m	-	-	H-9
8b			1.35, m	H-9a	C-6, C-9, C-10, C-14	-
9a	30.6	CH_2	2.34, m	H-8b	C-8, C-10	-
9b			2.02, m	H-10	-	-
10	63.3	CH	4.31, t, 9.5	H-9b	C-8, C-9, C-13	H-15
11	47.7	C	-	-	-	-
12	22.5	CH_3	1.23, s	-	C-6, C-10, C-11	-
13	21.3	CH_3	0.92, s	-	C-6, C-10, C-12, C-14	H-5, H-10
14	22.5	CH_3	1.11, s	-	C-6, C7, C-8	-
15	27.4	CH_3	1.75,s	-	C-2, C-3, C-4, C-11	H-3
16	169	O**C**OCH_3	-	-	-	-
17	21.5	OCO**C**H_3	2.08, s	-	C-16	-

3.2.3 Significance of compound 3.20

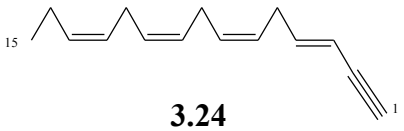
Kigoshi *et al*. (1986) have reported the isolation of both *cis* and *trans* isomers of laurencenyne (Compounds **3.20** and **3.24**, Figure 3.18) as well as compounds **3.25-3.27** from *Laurencia okamurai* (Yamada). The isomers were distinguished by the ^{1}H NMR chemical shift of H-1 (*cis*-isomer δ 3.12, *trans*-isomer δ 2.81). This allowed for the deduction of *cis*-geometry about the $\Delta^{3,4}$ double bond in compound **3.20**.

Laurencenyne is thought to be a precursor to more complex bromo-ethers as shown in Scheme 3.2. Compounds **3.20** and **3.24-3.27** were isolated alongside cyclic bromo-ethers in *L. okamurai* however, no linear C_{15} acetogenins were isolated together with bromofucin in *Laurencia flexuosa* (Mann, 2008).

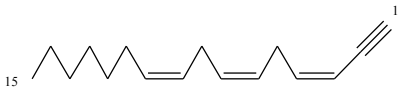
Furthermore, compound **3.20** was isolated as the major metabolite in *L. natalensis* and no bromo-ether type compounds were isolated from the alga. This could suggest that the alga lacks the enzyme systems required for cyclisation of the linear acetogenins to cyclic ether compounds. More in-depth analyses of crude extracts of *L. natalensis* are needed to confirm the presence or absence of cyclic ether compounds.



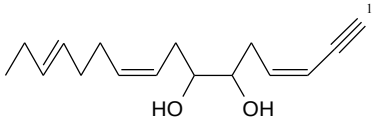
3.20



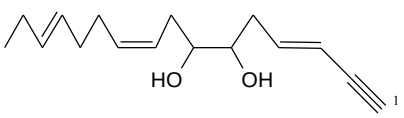
3.24



3.25

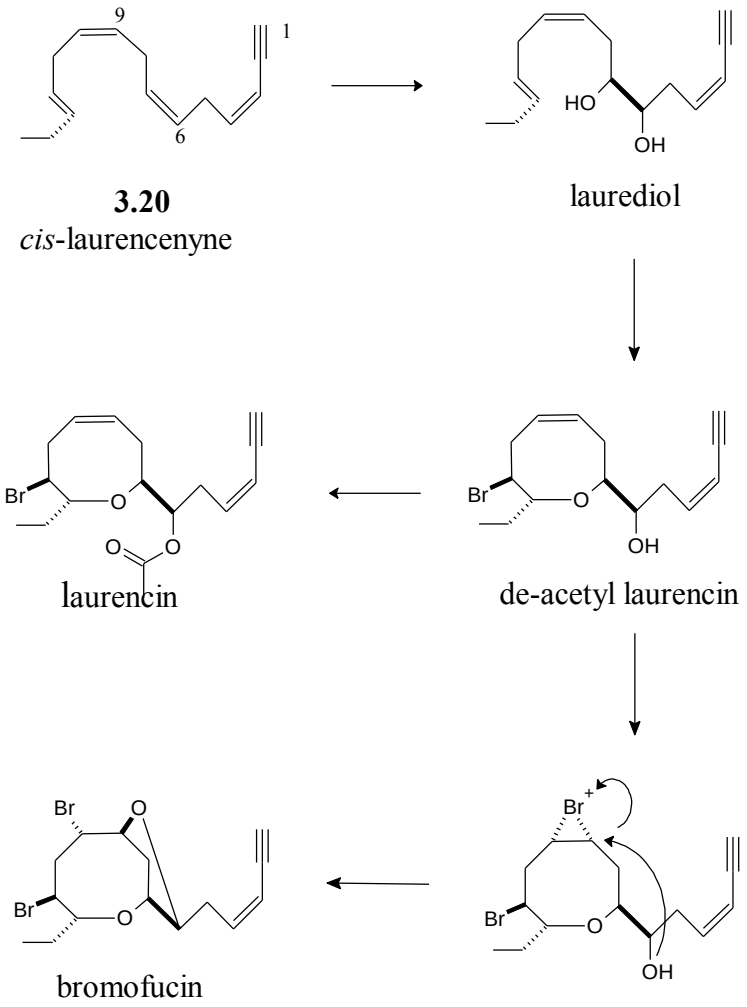


3.26



3.27

Figure 3.18: Linear C_{15} acetogenins isolated from *Laurencia* spp.



3.20
cis-laurencenyne

laurediol

laurencin

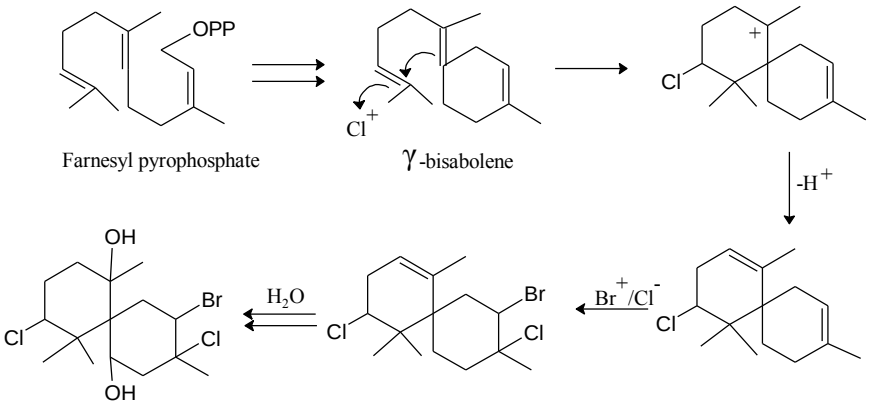
de-acetyl laurencin

bromofucin

Scheme 3.2: Proposed biosynthetic pathway of bromofucin type compounds from compound **3.20** (*cis*-laurencenyne)

3.2.4 Significance of compound 3.21

Compound **3.21** resembles a sesquiterpene chamigrane type compound which appears to be the most generalised metabolite produced by *Laurencia* spp. Over one hundred of these compounds have been isolated from different species of *Laurencia.* (Dorta *et al*., 2004). The proposed biosynthetic pathway of this class of compounds involving Farnesyl pyrophosphate as the initial precursor is as shown below (Scheme 3.3).



Scheme 3.3: Proposed biosynthetic pathway of a chamigrane type compound from Farnesyl pyrophosphate (Suzuki *et al*., 2001)

Compound **3.21** has never been seen before within the halogenated-chamigrane series and this bears great significance when analysing *Laurencia natalensis* from a chemotaxonomic perspective.

Compounds which are unique to a species or race ease the identification process which may be difficult at times due to striking morphological similarities. The presence of an acetyl moiety in compound **3.21** provides further information on the genetic material contained in *L. natalensis*. More specifically, *L. natalensis* is now suggested to possess genome which caters for the manufacture of acetylating enzymes.

3.3 Experimental

3.3.1 General Experimental

NMR experiments were performed using a Bruker® Avance 400 MHz and a Bruker® Avance 600 MHz spectrometer using standard pulse sequences. The NMR solvent used was $CDCl_3$. Column chromatography was performed using Merck® Silica gel 60 (0.040-0.063 mm), Germany. All solvents used were chromatography grade (LiChrosolv®), obtained from Merck®, Darmstadt, Germany

High resolution mass spectra were obtained from the University of Stellenbosch using a Waters® Synapt G2 spectrometer. The ionization source was ES- with a capillary voltage of 3 kV and a cone voltage of 20 V. The lock mass was set with Leucine enkephalin.

Sample introduction: Waters® UPLC in 1% formic acid, to 100% acetonitrile gradient over a 1m peak tubing at a flow rate of 0.2 mL/min.

3.3.2 Plant material

(KOS110914-1)

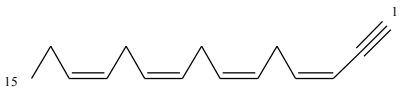
Laurencia natalensis was collected by hand at Kenton-on-Sea in the Eastern Cape, South Africa in September 2011. A voucher specimen (KOS110914-1) is being kept at the Division of Pharmaceutical Chemistry, Rhodes University. Identification of the algae was done by Professor John Bolton with the Department of Botany, University of Cape Town, South Africa.

3.3.3 Extraction and isolation

The algal material (wet mass 215 g; dry mass 88.1 g) was initially steeped in MeOH at room temperature for 1 hour, after which it was extracted in triplicate with CH_2Cl_2-MeOH (2:1,770 mL) at a constant temperature of 40 ^oC. The combined organic phases were collected (after addition of an adequate amount of water for phase separation) and concentrated under reduced pressure to produce a crude extract of 0.728 g which corresponds to a 0.82% yield [Weight of crude/(Weight of dry mass + Weight of Crude)]. Fractionation was further carried out via hexane-EtOAc step gradient column chromatography to yield eight fractions. Fractions B and C showed spectra of interest and were further purified via silica gel column chromatography to yield **3.15** (12 mg, 0.014%) and **3.16** (21 mg, 0.024%).

3.3.4 Compounds isolated

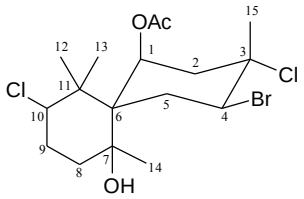
Compound 3.20 (isolation code JF11-83A)



3.20

(3Z,6Z,9Z,12Z)-pentadeca-3,6,9,12-tetraen-1-yne (**3.20**): Colourless oil; ^{1}H and ^{13}C NMR data available in Table 3.1. As previously reported by (Kigoshi *et al.,* 1981).

Compound 3.21 (isolation code JF11-80B)



3.21

4-bromo-3,10-dichloro-7-hydroxy-3,7,11,11-tetramethylspiro [6.6] undec-1-yl acetate (**3.21**): White crystals; $[\alpha]_D$ +97.6 ; IR V_{max} 3513 cm^{-1}; 2971 cm^{-1}; 1723 cm^{-1}; 1371 cm^{-1}; 1229 cm^{-1}; ^{1}H and ^{13}C NMR data available in Table 3.2; HRESIMS, *m*/*z* 428.0521 (calculated 428.0521) $C_{17}H_{27}{}^{79}Br^{35}Cl_2O_3$ / 430.0498 / 432.0474.

3.4 References

Bernart, M.; Gerwick, W. H. Isolation of 12-(S)-HEPE from the red marine alga *Murrayella periclados* and revision of the structure an acyclic eicosanoid from *Laurencia hybrida*. Implications to the biosynthesis of the marine prostanid hybridalactone. *Tetrahedron* **1988**, *29*, 2015-2018.

Bittner, M. L.; Silva, M.; Paul, V. J.; Fenical, W. A rearranged chamigrene derivative and its potential biogenetic precursor from a new species of the marine red algal genus *Laurencia* (rhodomelaceae). *Phytochemistry* **1985**, *24,* 987-909.

Blunt, J. W.; Copp, B. R.; Hu, W. P.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. Marine natural products. *Natural Product Reports* **2009**, *26*, 170–244.

Butler, A.; Sandy, M. Mechanistic considerations of halogenating enzymes. *Nature* **2009**, *460*, 848-854.

Jung, W. K.; Choi, I.; Oh, S.; Park, S. G.; Seo, S. K.; Lee, S. W.; Lee, D. S.; Heo, S. J.; Jeon, Y. J.; Je, J. Y.; Ahn, C. B.; Kim, J. S.; Oh, K. S.; Kim, Y. M.; Moon, C.; Choi, I. W. Anti-asthmatic effect of marine red alga (*Laurencia undulata*) polyphenolic extracts in a murine model of asthma. *Food and Chemical Toxicology* **2009**, *47*, 293-297.

Chatter, R.; Kladi, M.; Tarhouni, S.; Maatoug, R.; Kharrat, R.; Vagias, C.; Roussis, V. Neorogioltriol: A brominated diterpene with analgesic activity from *Laurencia glandulifera*. *Phytochemistry* **2009**, *2*, 25-28.

De Nys, R.; König, G. M.; Wright, A. D.; Sticher, O. Two metabolites from the red alga *Laurencia flexilis*. *Phytochemistry* **1993**, *34*, 725-728.

Dias, D. A.; Urban, S. Phytochemical studies of the southern Australian marine alga, *Laurencia elata*. *Phytochemistry* **2011**, *72*, 2081-2089.

Dorta, E.; Diaz-Marrero, A. R.; Cueto, M.; D'Croz, L.; Mate, J. L.; Darias, J. Chamigrenelactone, a polyoxygenated sesquiterpene with a novel structural type and devoid of halogen from *Laurencia obtuse*. *Phytochemistry* **2004**, *45*, 7065–7068.

Elsworth, J. F.; Thomson, R. H. A new chamigrane from *Laurencia glomerata*. *Journal of Natural Products* **1989**, *52*, 893-895.

Erickson, K. L. *Marine Natural Products: Chemical and Biological Perspectives*; Academic Press: New York, **1979**, *5*, 131-257.

Faulkner, D. J. Interesting aspects of marine natural products chemistry. *Tetrahedron* **1977**, *33*, 1421-1433.

Fenical, W. Chemical variation in a new bromochamigrene derivative from the red seaweed *Laurencia pacifica*. *Phytochemistry* **1976**, *15*, 511-512.

Fenical, W.; Norris, J. N. Chemotaxonomy in marine algae: chemical separation of some *Laurencia* species (Rhodophyta) from the Gulf of California. *Journal of Phycology* **1975**, *11*, 104-108.

Flock, S.; Skattebol, L. Syntheses of three metabolites of icosapentaenoic and docosahexaenoic acids. *Journal of the Chemical Society* **2000**, 3071-3076.

Fukuzawa, A.; Aye, Mye, Takasugi, Y.; Nakamura, M.; Tamura, M.; Murai, A. Enzymatic bromo-ether cyclisation of laurediols with bromoperoxidase. *Chemistry Letters* **1994,** 2307–2310.

Gonzalez, A. G.; Martin J. D.; Martin, V. S.; Norte, M. Carbon 13 NMR applications to *Laurencia* polyhalogenated sesquiterpenes. *Tetrahedron,* **1979,** *23*, 2713 – 2722.

Gutierrez-Cepeda, A.; Fernandez, J. J.; Gil, L.V.; Lopez-Rodriguez, M.; Norte, M.; Souto, M. L. Nonterpenoid C_{15} Acetogenins from *Laurencia marilzae*. *Journal of Natural Products* **2011**, *74*, 441-448.

Harborne, J. B.; Turner, B. L. *Plant Chemosystematics*; Academic Press: London, **1984**, 562.

Ji, N. Y.; Li, X. M.; Li, k.; Gloer, J. B.; Wang, B. G. Halogenated sesquiterpenes and non-halogenated linear C_{15}-acetogenins from the marine red alga *Laurencia composita* and their chemotaxonomic significance. *Biochemical Systematics and Ecology* **2009**, *36*, 938-941.

Juagdan, E. G.; Kaldindi, R.; Scheuer, P. Two new chamigranes from an Hawaiian red alga, *Laurencia cartilaginea. Tetrahedron* **1997**, 53*,* 521-528.

Kennedy, D. J.; Selby I. A.; Thomson R. H. *Chamigrane metabolites from Laurencia obtusa* and *L. scoparia .Phytochemistry* **1988**, *27*, 1761.

Kigoshi, H.; Shizuri, Y.; Niwa, H.; Yamada, K.. Laurencenyne, a plausible precursor of various nonterpenoid C_{15}-compounds, and neolaurencenyne from the red alga *Laurencia okamurai*. *Tetrahedron Letters* **1981**, *2,* 4729.

Kigoshi, H.; Shizuri, Y.; Niwa, H.; Yamada, K. Four new C_{15} acetylenic polyenes of biogenetic significance from the red alga *Laurencia okamurai*: structures and synthesis. *Tetrahedron* **1986**, 42, 3781.

Kladi, M.; Vagias, C.; Furnari, G.; Moreau, D.; Roussakis, C.; Roussis, V. Cytotoxic cuparene sesquiterpenes from *Laurencia microcladia*. *Tetrahedron Letters* **2005**, *46*, 5723-5726.

Kolb, H. C.; Sharpless, K. B. The growing impact of click chemistry on drug discovery. *Drug Discovery Today* **2003**, *8*, 1128-1137.

Mann, M. G. A. **2008**. An investigation of the antimicrobial and antifouling properties of marine algal metabolites. M.Sc thesis. Rhodes University, Grahamstown.

Masuda, M.; Takahashi, Y.; Okamoto, K.; Matsuo, Y.; Suzuki, M. Morphology and halogenated secondary metabolites of *Laurencia snackeyi* (Weber–van Bosse) stat. nov. (Ceramiales, Rhodophyta). *European Journal of Phycology* **1997a**, *32*, 293.

McMillan, J.; Paul, I. C.; White, R. H.; Hager, L. P. Molecular structure of acetoxyintricatol: a new bromo compound from *Laurencia intricate*. *Tetrahedron* **1974**, *23*, 2039-2042.

McPhail K. L.; Davies-Coleman M. T.; Copley R. C. B.; Eggleston D. S. New halogenated sesquiterpenes from South African specimens of the circumtropical sea hare *Aplysia dactylomela. Journal of Natural Products* **1999**, *62*, 1618–1623.

Pretsch, E.; Buhlmann, P.; Affolter, C. *Structure determination of organic compounds*; Springer, **2000**, 178.

Qin, J.; Su, H.; Zhang, Y.; Gao, J.; Zhu, L.; Wu, X.; Pan, H.; Li, X. Highly brominated metabolites from marine red alga *Laurencia similis* inhibit protein tyrosine phosphatase 1B. *Bioorganic & Medicinal Chemistry Letters* **2010**, *20*, 7152–7154.

Sakemi, S.; Higa, T.; Jefford, C. W.; Bernardinelli, G. Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta*. *Tetrahedron Letters* **1986**, *27*, 4287-4290.

Solveig, F.; Skattebol, L. Syntheses of three metabolites of icosapentaenoic and docosahexaenoic acids. *Journal of the Chemical Society* **2000,** *1*, 3071-3076.

Stegenga, H.; Bolton, J. J.; Anderson, R. J. *Seaweeds of the South African west coast*; Bolus herbarium: Cape Town, **1997**, 530.

Suzuki, M.; Daitoh, M.; Vairappan, C.; Abe, T.; Masuda, M. Novel Halogenated Metabolites from the Malaysian *Laurencia pannosa*. *Journal of Natural Products* **2001**, *64*, 597-602.

Suzuki, M.; Vairappan, C. S. Halogenated secondary metabolites from Japanese species of the red algal genus *Laurencia* (Rhodomelaceae, Ceramiales). *Current Topics in Phytochemistry* **2005**, *7*, 1–34.

Suzuki, M.; Takahashi, Y.; Nakano, S.; Abe, T.; Masuda, M.; Ohnishi, T.; Noya, Y.; Seki, K. An experimental approach to study the biosynthesis of brominated metabolites by the red algal genus *Laurencia*. *Phytochemistry* **2009,** *70,* 1410-1415.

Vairappan, C. S.; Suzuki, M.; Abe, T.; Masuda, M. Halogenated metabolites with antibacterial activity from the Okinawan *Laurencia* species. *Phytochemistry* **2001**, *58* , 517-523.

Chapter 4

Isolation of halogenated furanones from *Delisea flaccida*

Abstract

Delisea spp. have shown fascinating compounds based on the 4-halo-3-butyl-5 halo-methylene-2(5H)-furanone skeleton. These compounds have shown interesting antifouling activity and are popular templates for antimicrobial drug design.

The South African coast possesses an endemic alga, *Delisea flaccida* (Suhr) Papenfuss, which is thought to be a descendent of *Delisea* spp. found in Australian waters.

This chapter discusses the isolation and characterisation of two isomeric bromo-furanones from *Delisea flaccida* which have been previously isolated from other *Delisea* spp. The *cis*-isomer **1-[(5*Z*)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl]butyl acetate** and the *trans*-isomer **1-[(5*E*)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl]butyl acetate**.

Chapter 4

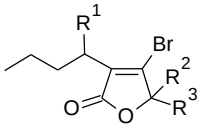
Isolation of halogenated furanones from *Delisea flaccida*

4.1 Introduction

Marine red algae of the genus *Delisea* (family Bonnemaisoniaceae) is renowned for producing polyhalogenated secondary metabolites of which 2(5H)-furanones (Figure 4.1) are the most common (McCombs *et al*., 1988). A unique characteristic associated with *Delisea* spp. is the algae's ability to prevent bacterial as well as epiphyte colonisation (Manefield *et al*., 1999). As a result, *Delisea* spp. have been observed to be a food source in an environment where algae are generally not preferred by obligate herbivores (De Nys *et al*., 1995).

It is understood that fimbrolide type compounds such as compounds **4.1-4.7** (Figure 4.1) produced by *Delisea* spp. give rise to these unique traits, and studies do infact reveal the undisputed antifouling properties shown by these metabolites (Manefield *et al*., 1999).

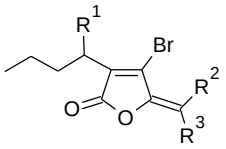
These fimbrolides are effective in reducing bacterial phenotype expression while having negligible effect on bacterial growth or protein synthesis (Ren *et al*., 2002). Moreover, the onset of resistance in bacteria to fimbrolides has yet to be observed. This undoubtedly makes these *Delisea* furanones potential drug leads for quorum sensing mediated bacterial infections. It comes as no surprise to see numerous attempts to develop antimicrobial agents based on the *Delisea* furanone motif (Goh *et al*., 2007).



4.1 $R^1 = OH, R^2 = OCH_3, R^3 = CH_2I$

4.2 $R^1 = OAc, R^2 = CH_3, R^3 = OCH_3$

4.3 $R^1 = OAc, R^2 = OCH_3, R^3 = CHBr_2$

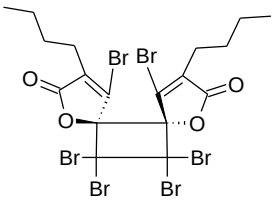


4.4 $R^1 = OH, R^2 = OCH_3, R^3 = CH_2I$

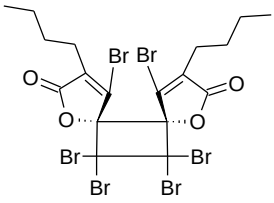
4.5 $R^1 = OAc, R^2 = H, R^3 = Br$

4.6 $R^1 = OAc, R^2 = Br, R^3 = H$

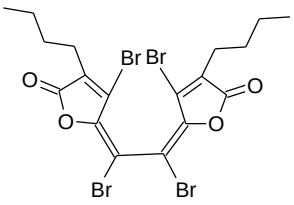
4.7 $R^1 = OH, R^2 = H, R^3 = Br$



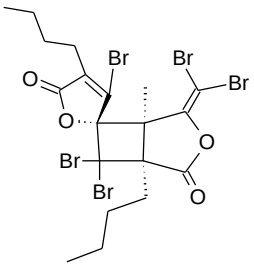
4.8



4.9



4.10



4.11

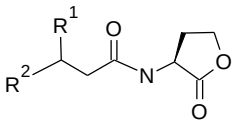
Figure 4.1: Compounds isolated from *Delisea* spp.[1]

[1] **4.1-4.7** (De Nys *et al*., 1993); **4.8-4.11 (**McCombs *et al*., 1988)

4.1.1 Biofilm inhibitory activity of furanones from *Delisea* spp.

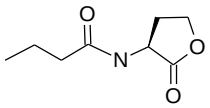
4.1.1.1 Bacterial biofilms

Bacteria have been seen to cluster when invading an organism so as to increase the chance of survival. Minimal virulence can occur if they exist discretely (Chen *et al*., 2002). They produce a protective polysaccharide based extracellular matrix known as a biofilm. This allows for increased adhesion on surfaces and enhances the ability of signalling molecules to diffuse between bacterial cells (Harshey and Matsuyama, 1994). A greater efficiency in signalling aptitude allows for a more co-ordinated biofilm formation as well as augmented swarming potential. It has been determined that N-acylated homoserine lactone (AHL) mediated systems (Figure 4.2) are key in regulating colony populations (Kjlleberg and Steinberg, 2001). AHL derivatives have been seen to exist in over fifty bacterial species, examples of which are shown below (Figure 4.2).

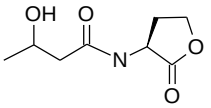


R^1 = alkyl
R^2 = O, OH, H

Acyl-homoserinelactone (AHL)



Butanoyl HSL (*P. aeruginosa*)



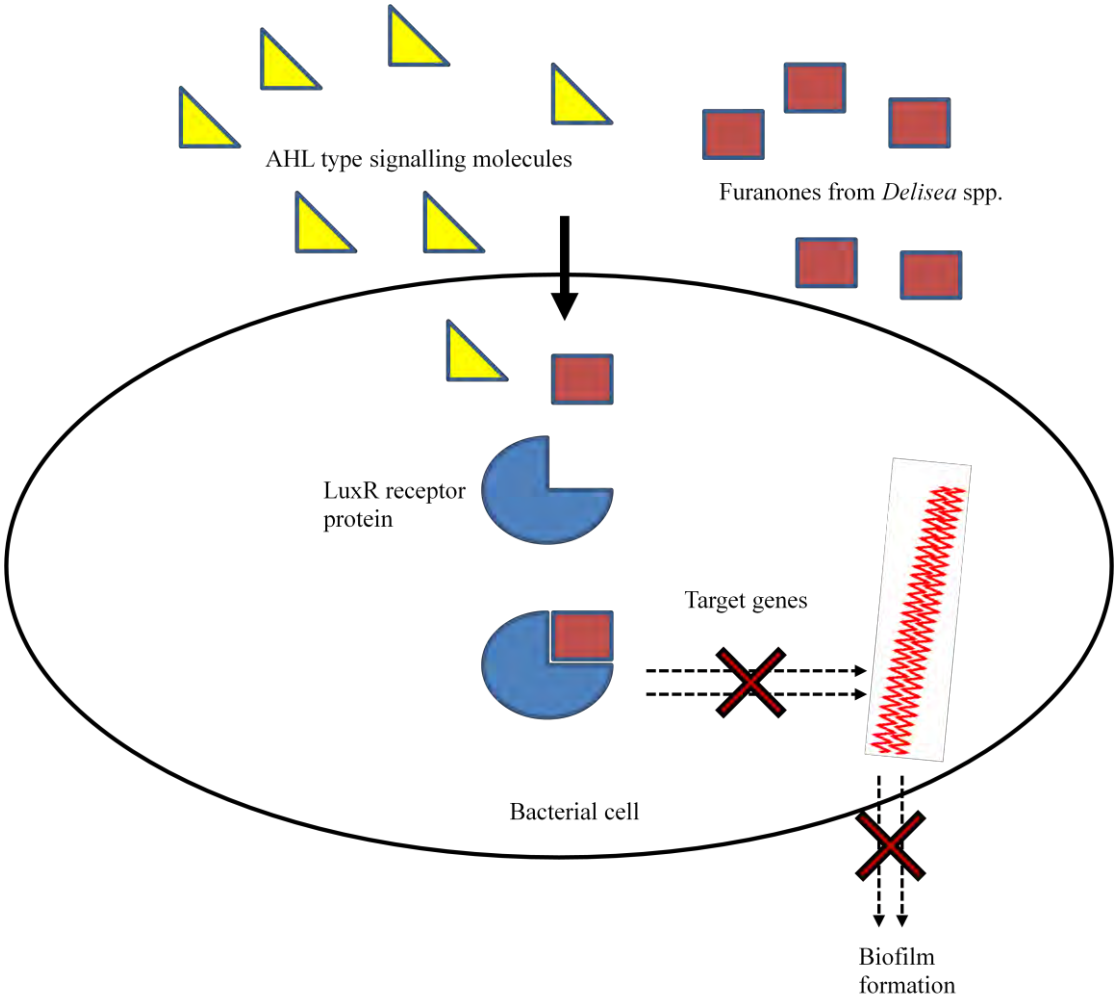
3-Hydroxybutanoyl HSL (*V. harveyi*)

Figure 4.2: AHL and AHL derivatives. The furanone ring is seen to remain an integral structural trait with variations only occurring on the alkyl side chain (Ohno, 2004)

4.1.1.2 Anti-fouling mechanism of *Delisea* spp. furanones

The AHL signalling molecule is agonistic at a particular receptor protein (LuxR) which then initiates a cascade of events ultimately leading to colonisation and hence fouling (Scheme 4.1) adapted from Whitehead *et al*., 2001. The striking structural similarity of furanones isolated from *Delisea* spp. to AHL type signalling molecules provides the rationale behind the antifouling activity that these marine organisms display.

The furanones act as direct competitive antagonists on receptors to which AHL molecules typically bind. AHL mediated gene expression is consequently inhibited leading to a reduction in phenotypes associated with colonisation (Goh *et al*., 2005; Schaefer *et al*., 1996).



Scheme 4.1: Mechanism of furanone anti-fouling activity

The *Delisea* furanones have been observed to be more potent antifouling agents with acetate (-OAc) or hydroxy (-OH) substituents on the alkyl side chain. The rigid nature of the furan ring within these compounds serves as an integral structural feature for antifouling properties. Furthermore, the vinyl bromide substituent on the furanone ring does not appear to be critical for antifouling activity (Han, 2008).

4.1.2 Chapter aims

In continuing our studies on South African endemic Rhodophyta, the chemical profile of *Delisea flaccida*, which was anonymous until now, was assessed. This alga is known for producing furanone type compounds and hence these in particular were targeted, as these compounds have displayed promising antifouling activity.

Figure 4.3: Photograph of *Delisea flaccida*[1]

[1] Obtained from Dr. D. R. Beukes, Department of Pharmaceutical Chemistry, Rhodes University, Grahamstown

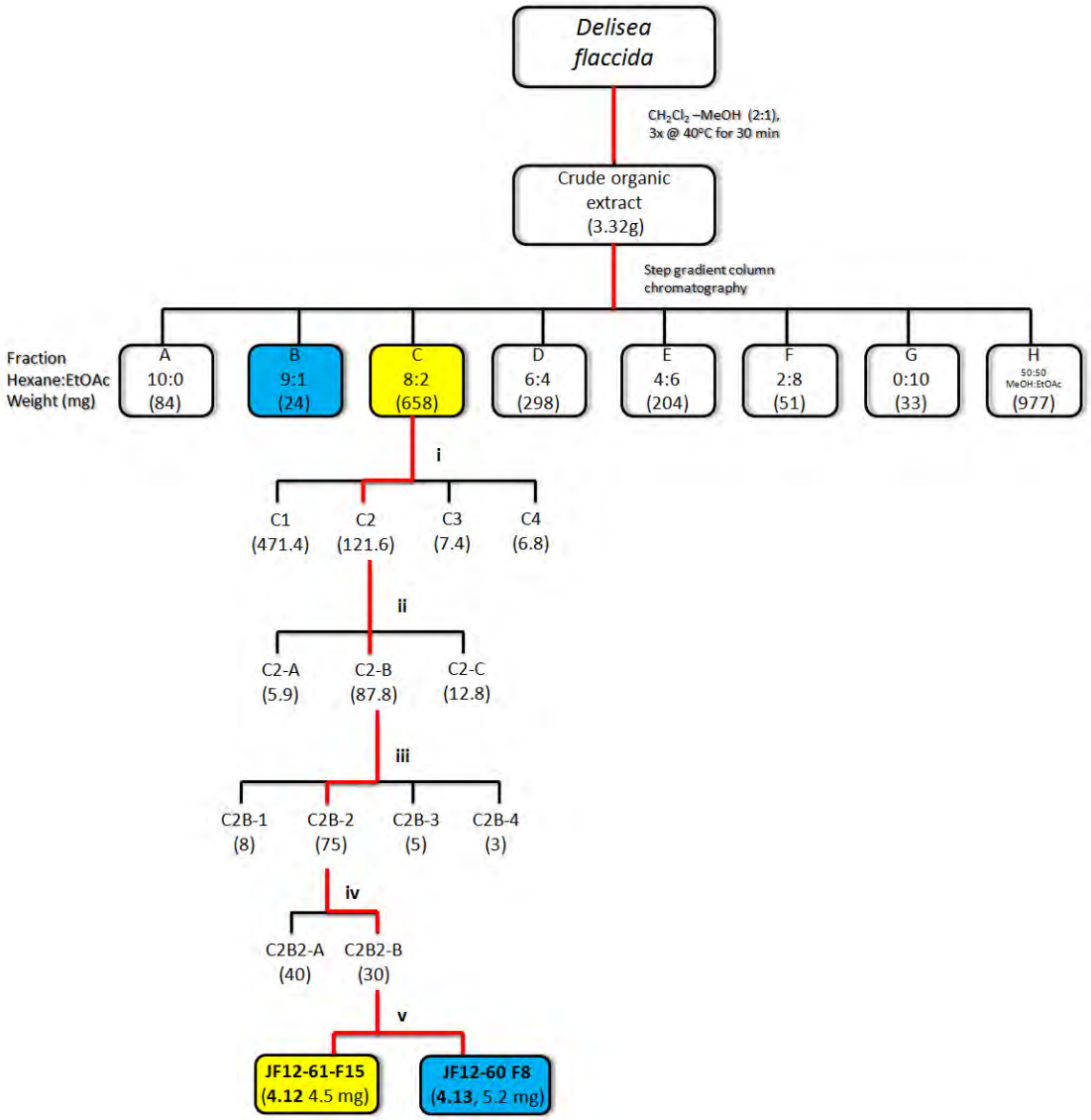
4.2 Results and discussion

4.2.1 Isolation and structure elucidation of metabolites

The extraction and fractionation procedures performed on *Delisea flaccida*, collected from Port Alfred on the eastern coast of South Africa, are summarised in Scheme 4.2. The crude extract was fractionated on silica gel using a step gradient (hexane-EtOAc) of increasing polarity into nine separate fractions.

Upon analysis of the ^{1}H NMR spectra of these fractions, peaks of interest were observed in fraction C. Fraction D showed characteristic chlorophyll signals and the remainder of the fractions did not display any appealing peaks.

As a result, Fraction C alone was further purified. This was done using silica gel column chromatography with differing solvent systems (see section 4.4.3), until finally normal phase HPLC afforded compounds **4.12** and **4.13**.



Scheme 4.2: Isolation of compounds **4.12** And **4.13** from *Delisea flaccida*

Conditions: i-iv) Repeated silica gel column chromatography (see section 4.3.3)

v) Normal phase HPLC (hexane-EtOAc, 4:1)

4.2.1.1 Compound 4.12

Compound **4.12** was isolated as a yellow oil, the ^{1}H NMR spectrum of which showed multiplets at δ 1.83 and δ 5.55 as well as a methyl triplet at δ 0.96 (t, J = 7.4 Hz) which was assumed to be neighbouring a methylene group due its multiplicity i.e. triplet. A substituted methine singlet was also observed at δ 6.38.

The ^{13}C NMR spectrum (Figure 4.5) showed an ester signal at δ 170.1 as well as a methyl signal δ 20.6 complementing an acetate functionality. Signals were also observed at δ 130.6, 131.4, 149.8 and 163.6.

The DEPT-135 spectrum of compound **4.12** showed two –CH_3 groups, two –CH_2 groups, two –CH groups and five quaternary carbons.

Compound **4.12** was identified as a previously isolated acetoxyfimbrolide (Pettus *et al*., 1977) which has been shown to inhibit AHL mediated gene expression in *E. coli* (Manefield *et al*., 1999).

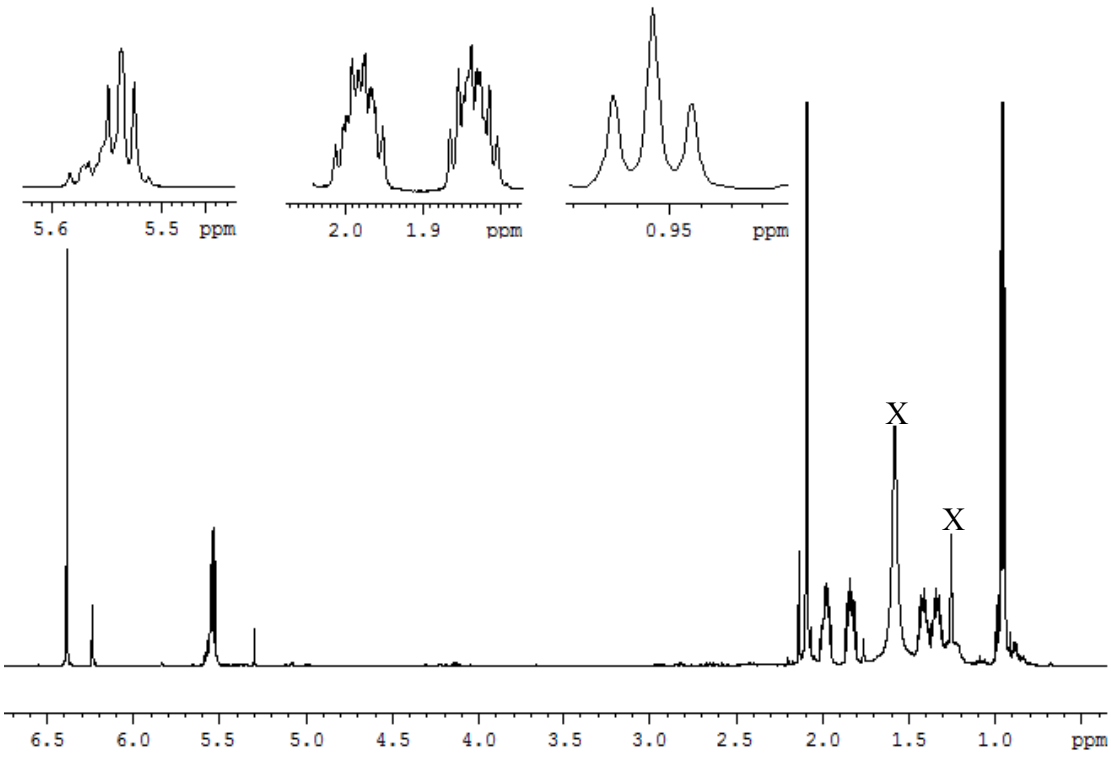


Figure 4.4: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **4.12**

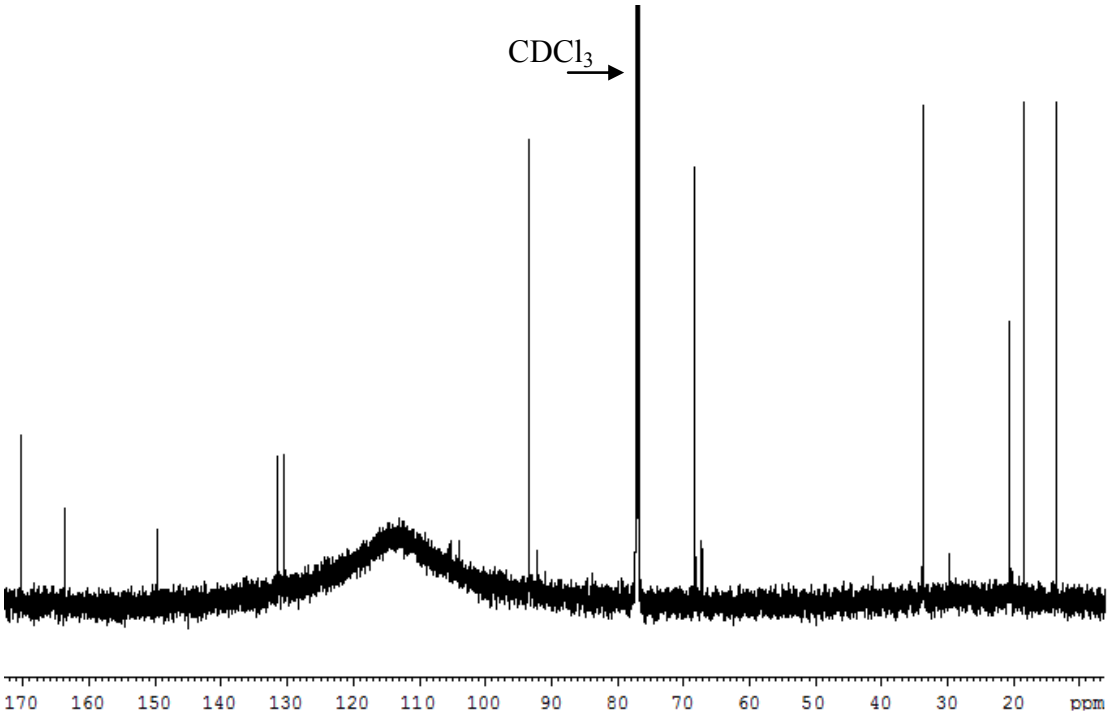


Figure 4.5: ^{13}C NMR spectrum ($CDCl_3$, 150 MHz) of compound **4.12**

The infrared spectrum (Figure 4.6) of compound **4.12** showed characteristic absorption bands for both C=O and C-O stretches. These bands were observed at 1785 cm^{-1} and 1228 cm^{-1} respectively.

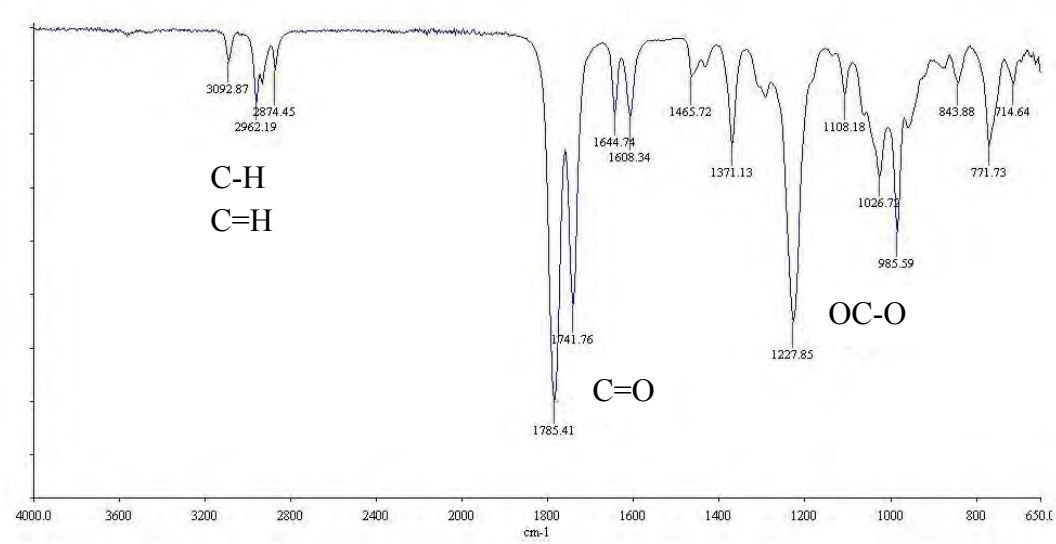
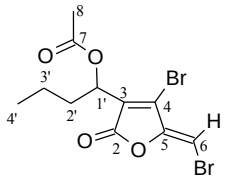


Figure 4.6: IR spectrum of compound **4.12**



4.12

Table 4.1: NMR spectroscopic data ($CDCl_3$) of compound **4.12**

Carbon No	δ_C	δ_C **mult**	δ_H **, mult, J (Hz)**
2	163.7	C	-
3	131.3	C	-
4	149.6	C	-
5	130.4	C	-
6	93.3	CH	6.38, s
7	170.1	C	-
8	20.5	CH_3	2.09, s
1’	68.1	CH	5.55, q,
2a’	33.6	CH_2	1.83, m
2b’			1.98, m
3a’	18.4	CH_2	1.36, m
3b’			1.41, m
4’	13.4	CH_3	0.95, t

4.2.1.2 Compound 4.13

Compound **4.13** was isolated as a mixture with compound **4.12**. The ^{1}H NMR spectrum of compound **4.13** (Figure 4.7) was identical to compound **4.12** apart from a halo-methine singlet being observed at δ 6.24 instead of δ 6.38. In addition, the carbon NMR spectrum displayed a halo-methine signal at δ 105.4 instead of δ 93.3 as seen in compound **4.12**.

This is suggestive of the presence of geometric isomers due to a difference in geometry about the exocyclic double bond $\Delta^{5,6}$. The bromo-methine proton of the (*Z*)-acetoxyfimbrolide isomer has been reported to be δ 6.38 while the (*E*)-isomer showed a proton chemical shift of δ 6.24 (Silverstein *et al*., 1991; De Nys et al., 1993).

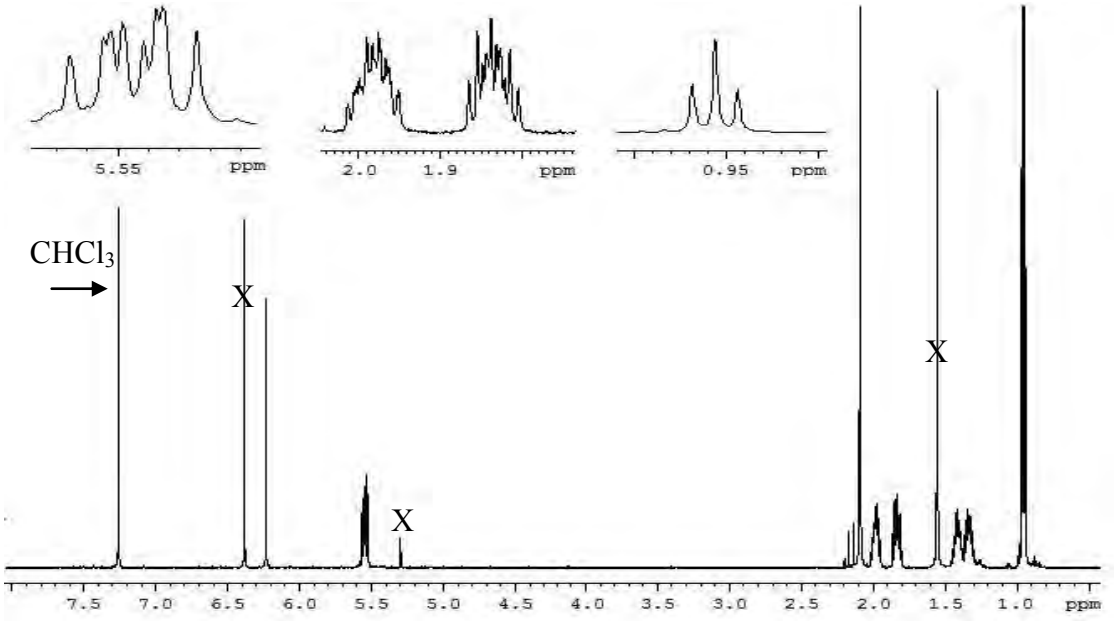


Figure 4.7: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **4.13**

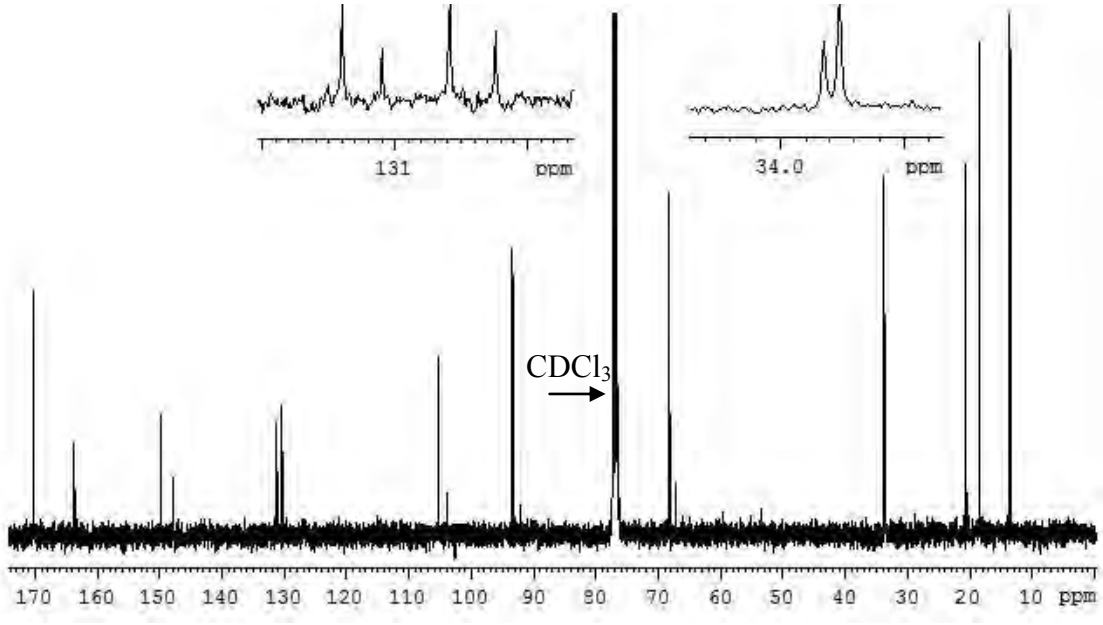


Figure 4.8: ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of compound **4.13**

Figure 4.9 below shows an expansion of the HSQC spectrum of compound **4.13**. The correlations clearly show how the carbon chemical shift at position C-6 is influenced by the geometry about the $\Delta^{5,6}$ double bond. The ^{13}C NMR shift for the (*Z*)-acetoxyfimbrolide isomer (compound **4.12**) is δ 93.4 while the (*E*)-isomer (compound **4.13**) is δ 105.2.

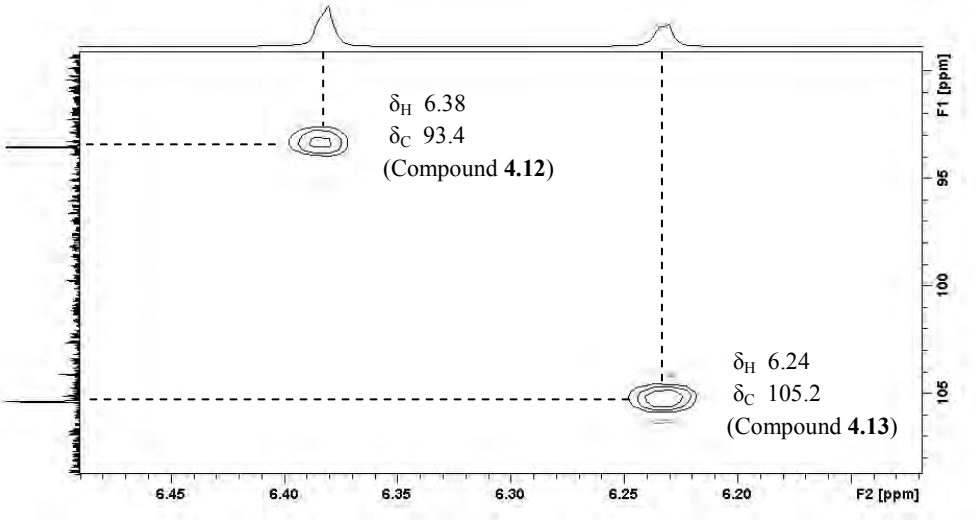


Figure 4.9: HSQC NMR spectrum of compound **4.13**

Further evidence of the existence of an isomeric mixture is shown the proton spectrum below (Figure 4.10). When integrated, the two bromo-methine singlets at δ 6.24 and δ 6.38 show integrals of ~1 corresponding to 1 proton, while the $–CH_3$ triplet at δ 0.96 has an integral of 6 showing that the triplet is due the $–CH_3$ group on each isomer.

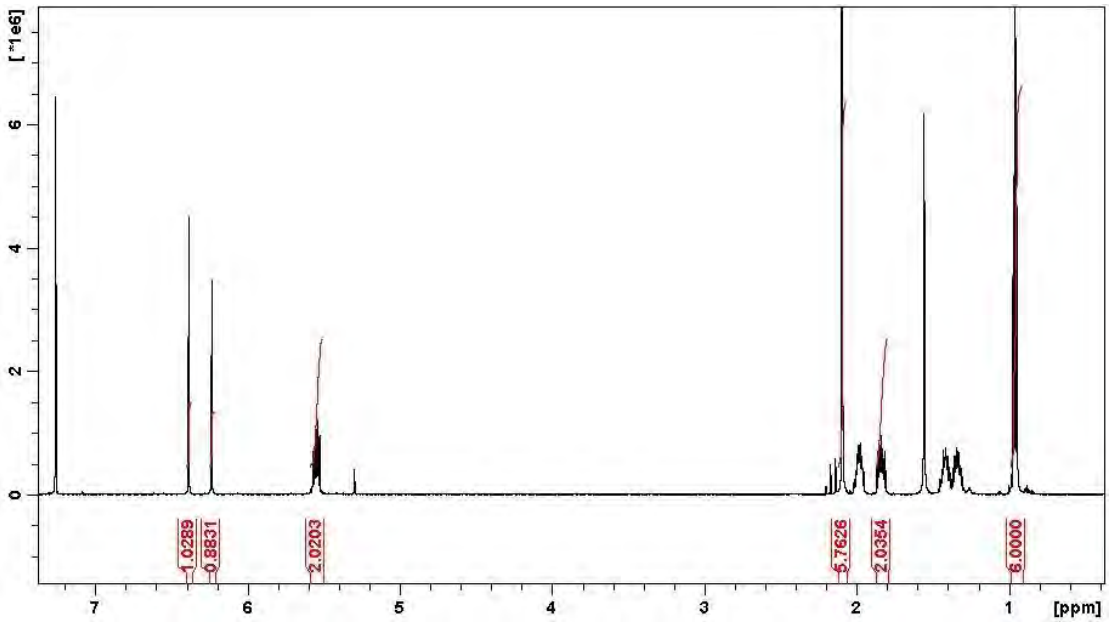
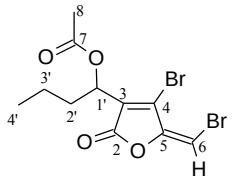


Figure 4.10: ^1H NMR spectrum ($CDCl_3$, 600 MHz) of compound **4.13** showing integrals



4.13

Table 4.2: NMR spectroscopic data ($CDCl_3$) of compound **4.13**

Carbon No	δ_C	δ_C **mult**	δ_H **, mult, J (Hz)**
2	163.6	C	-
3	131.1	C	-
4	149.8	C	-
5	130.3	C	-
6	105.4	CH	6.24, s
7	170.1	C	-
8	20.6	CH_3	2.09, s
1’	68.3	CH	5.55, q,
2a’	33.8	CH_2	1.83, m
2b’			1.99, m
3a’	18.5	CH_2	1.36, m
3b’			1.42, m
4’	13.6	CH_3	0.96, t

4.3 Significance of metabolites isolated from *Delisea flaccida*

It has been assumed by Hommersand (1986) that the majority of red algal taxa found along the west coast of South Africa originate from species found on Australian shorelines. These species are thought to have been carried away by means of a seaway across West Antarctica during an era when glaciers were not so common, i.e. Oligocene and Miocene periods. They were then distributed to South Africa *via* oceanic currents.

This study reveals two metabolites, Compounds **4.12** and **4.13**, isolated from a South African endemic *Delisea* spp. which have been isolated previously in other *Delisea* spp. namely *Delisea pulchra* (De Nys *et al*., 1993) and *Delisea elegans* (McCombs *et al*., 1988), both collected from the Australian coast.

This could bear great phylogenetic significance as both South African and Australian coastlines show *Delisea* spp. which produce similar metabolites. A broad inference can be made using the aforementioned data in that South African *Delisea* spp. could possess evolutionary ancestors seen along the Australian shorelines.

Furthermore, Compounds **4.12** and **4.13** belong to the bromofuranone chemical class. These compounds are promising anti-bacterial leads and have shown the following biological activity:

1. Inhibition of swarming motility of Proteus mirabilis (Gram *et al*., 1996)
2. Inhibition of AHL mediated gene expression in *E. coli* (Manefield *et al*., 1999)
3. Inhibition of carbapenem synthesis and exoenzyme virulence factor production in *Erwinia carotovora* (Manefield *et al*., 2001)

4.4 Experimental

4.4.1 General Experimental

All NMR experiments were performed using a Bruker® Avance 600 MHz spectrometer using standard pulse sequences. ^{1}H and ^{13}C experiments were run at 600 MHz and 100 MHz respectively using $CDCl_3$ as the NMR solvent. Column chromatography was performed using Merck® Silica gel 60 (0.040-0.063 mm), Germany. All solvents used were chromatography grade (LiChrosolv®), obtained from Merck®, Darmstadt, Germany.

HPLC was performed using a semi-preparative normal phase Whatman Partisil® 10 M9/50 (9.5mm x 500mm) column and a Rheodyne® containing Waters® HPLC pump with a Spectra physics® RI detector attached to a Ridenki® chart recorder.

4.4.2 Plant material

(PA110406)

Delisea flaccida was collected by hand at Port Alfred in the Eastern Cape, South Africa in April 2011. A voucher specimen (PA110406) is being kept at the Division of Pharmaceutical Chemistry, Rhodes University. Identification of the algae was done by Professor John Bolton with the Department of Botany, University of Cape Town, South Africa.

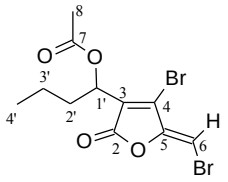
4.4.3 Extraction and isolation

The algal material (wet mass 185.6 g; dry mass 50.1 g) was initially steeped in MeOH at room temperature for 1 hour, after which it was extracted in triplicate with CH_2Cl_2-MeOH (2:1,770 mL) at a constant temperature of 40 ^{o}C. The combined organic phases were collected (after addition of an adequate amount of water for phase separation) and concentrated under reduced pressure to produce a crude extract of 3.32 g which corresponds to a 6.21% yield [Weight of crude/(Weight of dry mass + Weight of Crude)]. Fractionation was further carried out *via* hexane-EtOAc step gradient column chromatography to yield eight fractions.

Fraction C showed a spectrum of interest and was further purified *via* silica gel column chromatography using various solvent systems (**i**: 9:1 hex: EtOAc; **ii**: 19:1 hex: EtOAc, **iii**: 3:1 hex: CH_2Cl_2, **iv**: 100% CH_2Cl_2) and finally by normal phase HPLC (4:1 hex: EtOAc) to yield **4.12** (4.5 mg, 0.0084%) and **4.13** (5.2 mg, 0.0097%).

4.4.4 Compounds isolated

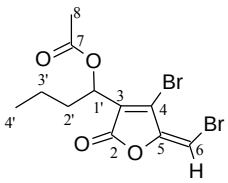
Compound 4.12 (isolation code JF12-61 F15)



4.12

1-[(5Z)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl]butyl acetate (**4.12**) :Yellow oil; NMR data available in Table 4.1. As previously reported by (Pettus *et al*., 1977)

Compound 4.13 (isolation codeJF12-60 F8)



4.13

1-[(5E)-4-bromo-5-(bromomethylidene)-2-oxo-2,5-dihydrofuran-3-yl]butyl acetate (**4.13**) :Yellow oil; NMR data available in Table 4.2. As previously reported by (Pettus *et al*., 1977)

4.5 References

Chen, X.; Schauder, S.; Potier, N.; Dorsselear, A. V.; Pelczer, I.; Bassler, B. L.; Hughson, F. M. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **2002**, *415*, 545-549.

De Nys, R.; Steinberg, P. D.; Willemsen, P.; Dworjnyn, S. A.; Gabelish, C. L.; King, R. J. Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. *Biofouling* **1995**, *8*, 259-271.

De Nys, R.; Wright, A. D.; Konig, G. M.; Sticher, O. New halogenated furanones from the marine alga *Delisea pulchra* (cf. *fimbriata*). *Tetrahedron* **1993**, *49*, 1213-1220.

Goh W. K.; Black, D. StC.; Kumar, N. Synthesis of novel 7-substituted 5, 6-dihydroindol-2-ones via Suzuki-Miyaura cross-coupling strategy. *Tetrahedron Letters* **2007**, *48*, 9008-9011.

Goh W. K.; Rice, S. A.; Kumar, N. Theoretical Study of Molecular Determinants Involved in Signal Binding to the TraR Protein of *Agrobacterium tumefaciens Molecules* **2005**, *10*, 1263-1271.

Gram, L.; De Nys, R.; Maximilien, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming motility of *Proteus mirabilis*. *Applied and Environmental Microbiology* **1996**, *62*, 4284-4287.

Han, Y.; Hou, S.; Simon, A. K.; Ren, D.; Luk, Y. Y. Identifying the important structural elements of brominated furanones for inhibiting biofilm formation by *Escherichia coli*. *Bioorganic and Medicinal Chemistry Letters* **2008**, *18*, 1006-1010.

Harshey, R. M.; Matsuyama, T. *Proceedings of the National Academy of Sciences of the United States of America* **1994**, *91*, 8631-8635.

Hommersand, M. H. The biogeography of the South African marine red algae: a model, *Botanica Marina* **1986**, *29*, 257-270.

Kjlleberg, S.; Steinberg, P. Surface warfare in the sea. *Microbiology Today* **2001**, 28, 134-135.

Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; P. Steinberg, P.; Kjelleberg, S. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* **1999**, *145*, 283-291.

Manefield, M.; Welch, M.; Givskov, M.; Salmond, G.; Kjelleberg, S. Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. *Microbiology Letters* **2001**, *205*, 131-138.

McCombs, J. D.; Blunt, J. W.; Chambers, M. V.; Munro, M. H. G.; Robinson, W. T. Novel 2(5H)-furanones from the red marine alga *Delisea elegans*. *Tetrahedron* **1988**, *44*, 1489-1502.

Pettus, J. A.; Wing, R. M.; Sims, J. J. Marine Natural Products XII Isolation of a Family of Multihalogenated Gamma-Methylene Lactones from the Red Seaweed *Delisea fimbriata. Tetrahedron Letters* **1977**, *1*, 41-44.

Ren, D.; Sims J. J.; Wood T. K. Inhibition of biofilm formation and swarming of *Bacilus subtilis* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. *Letters in Applied Microbiology* **2002**, *34*, 293-299.

Silverstein, R. M.; Bassler, G. C.; Morrill, C. T. Proton magnetic resonance spectrometry, *Spectrometric Identification of Organic Compounds*; John Wiley & Sons: New York **1991**.

Schaefer, A. L.; Hanzelka B. L.; Eberhard, A.; Greenberg, E. P. Quorum sensing in *Vibrio fischeri*: probing auto inducer-LuxR interactions with auto inducer analogues. *Journal of Bacteriology* **1996**, *178*, 2897-2901.

Whitehead, N. A.; Barnard, A. M.; Slater, H.; Simpson, N. J.; Salmond, G. P. Quorom sensing in Gram negative bacteria. *FEMS Microbiology Reviews* **2001**, *25*, 365-404.

Chapter 5

Conclusion

The need for new drug leads from marine sources cannot be over-emphasised. The Southern African coastline boasts a wide variety of marine algal genera creating a structurally diverse collection of compounds. Understanding these species as well as the compounds they produce can assist in targeting those which show fascinating chemistry making them prospective sources for drug leads. This study has exposed the plush variety of secondary metabolites available in selected marine macroalgae along the South African coastline.

The main research aim was to isolate and characterise compounds from the three South African endemic algae *Plocamium rigidum, Laurencia natalensis* and *Delisea flaccida*, and this was successfully achieved.

Five halogenated monoterpenes were isolated were isolated from *Plocamium rigidum* of which compound **2.27** is the first of its kind within this series. Compounds **2.28-2.31** have previously been isolated from other species of *Plocamium* suggesting that the species possess a common evolutionary ancestor. Compound **2.27** will allow for easier chemo-identification of *Plocamium rigidum* as its isolation has not been reported in any other *Plocamium* spp.

The cytotoxicity of compounds **2.27-2.31** was assessed against MCF-7 breast cancer cells and the results showed more potent IC_{50} values obtained with increasing compound lipophilicity. The dichloro moiety at C-9 once again proved to be essential for biological activity in the halogenated monoterpene series. An investigation into the effects of an aldehyde functionality concluded that the moiety alone does not exert cytotoxic effects.

A three site geographical variation study done on *Plocamium corallorhiza* showed no significant difference in metabolite production. The major metabolite, compound **2.33**, was different to that usually isolated in this species.

Seasonal variation studies of *Plocamium corallorhiza* should be done to assess whether or not different metabolites are produced at different times of the year.

Compound **3.20**, a C_{15} acetogenin, was isolated from *Laurencia natalensis*. This compound is thought to be a direct precursor to the production of bromofucin type compounds and compound **3.20** has been isolated before in other *Laurencia* spp.

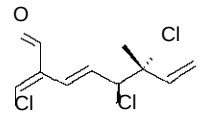
Cyclic bromo-ethers however were not isolated from the *L.natalensis* in this study suggesting a probable lack of critical enzymes required for such transformation or merely no ecological rationale to do so. Conversely, taking into account the small amounts of algal material that were used it is possible that these compounds could have gone unnoticed due to limitations in instrument sensitivity. Future studies could be done on possible halonium-ion induced cyclisation reactions of compound **3.20**.

Compound **3.21**, a new halogenated acetoxy chamigrane was also isolated and seen to be the first of its kind within the chamigrane class. A similar molecular skeleton to compound **3.21** has been isolated before from *Laurencia intricata* however acetylation as well as halogenation patterns differ. This bears great chemotaxonomic implication as it is unique to this species, allowing for easier chemo-identification.

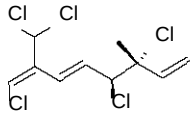
The chemical profile of *Delisea flaccida* has never been explored before until now. It has been demonstrated that this alga produces the well known acetoxyfimbrolides which have been isolated in other *Delisea* species such as the Australian *Delisea pulchra*. These compounds are well known for their antifouling ability and are popular templates for new synthetic antibacterial derivatives. In addition, an evolutionary link could be assumed to exist amongst these species on the basis that similar metabolites are being formed. From a chemotaxonomic perspective, seeing that *Delisea* spp. produce similar metabolites could bear classification challenges. It should be mentioned however that a re-isolation of metabolites from *Delisea flaccida* would be useful as compounds **4.12** and **4.13** may not be the only metabolites the alga produces.

This study has also proved that a chemically diverse range of secondary metabolites are obtained when assessing a biologically diverse selection of marine algae. This consolidates the raison d'être for pursuit of novel lead compounds from marine natural products.

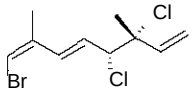
Appendix 6.1 – Metabolites from *Plocamium* spp.



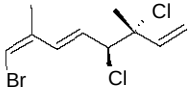
2.1



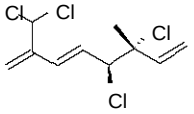
2.2



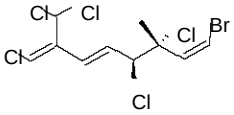
2.3



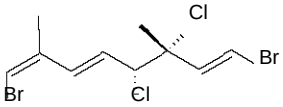
2.4



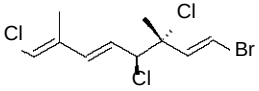
2.5



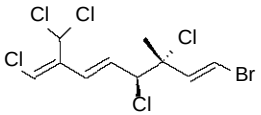
2.6



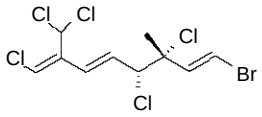
2.7



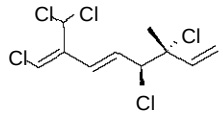
2.8



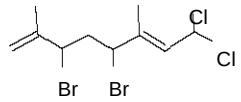
2.9



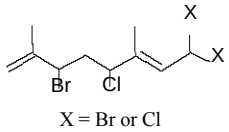
2.10



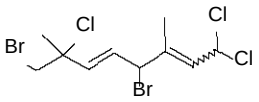
2.11



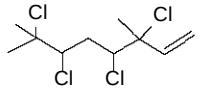
2.12



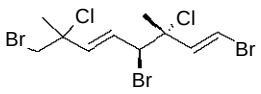
2.13



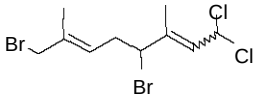
2.14



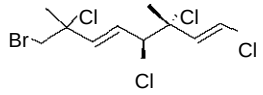
2.15



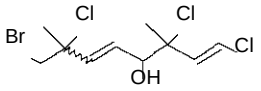
2.16



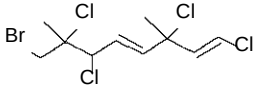
2.17



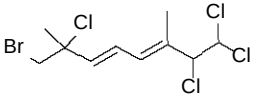
2.18



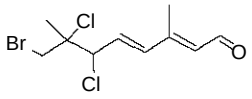
2.19



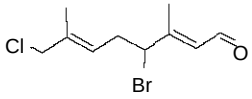
2.20



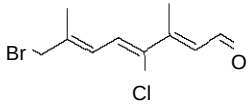
2.21



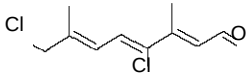
2.22



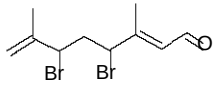
2.23



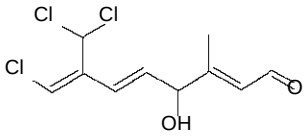
2.24



2.25



2.26

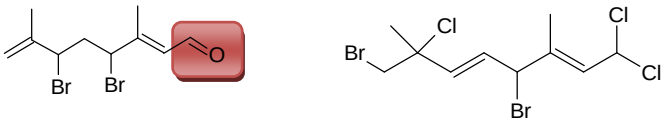


2.27

Appendix 6.2

6.2.1 Geographical variation study on *Plocamium corallorhiza*

After the isolation of compound **2.27**, a detailed cytotoxicity assay was required wherein comparisons could be made with other compounds containing isolated functional groups similar to those present in **2.27** e.g. an aldehyde moiety. The major metabolite **2.26** previously seen in *Plocamium corallorhiza* was one of interest as it contained a terminal aldehyde functionality similar to that in **2.27**. For this reason it was decided that a collection be made in order to isolate compound **2.26** and include it in the cytotoxicity assay. On closer inspection however, it was noticed that compound **2.26** was no longer being produced as the major metabolite by *Plocamium corallorhiza*. This *Plocamium* species has previously shown significant geographical variation in metabolite profile (Davies-Coleman and Beukes, 2004). An intra-site as well inter-site variation study was consequently conducted. *Plocamium corallorhiza* samples were collected from Noordhoek, Port Alfred and Kenton-on-Sea beaches and the crude organic extracts were examined by a series of standardised ^{1}H NMR spectroscopy experiments. It was observed that a previously isolated compound, compound **2.33** (Mkwananzi, 2005), was now being produced as the major metabolite.



2.26 **2.33**

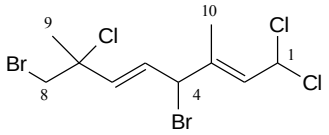
Figure 6.2.1: Major metabolites isolated in *Plocamium corallorhiza*

A ^{1}H NMR based investigation into the intra-site variation of metabolite production as shown in Figures 6.2.3, 6.2.4 and 6.2.5 display little to no variance, with compound **2.33** being the major metabolite present within the samples.

An inter-site variation ^{1}H NMR analysis (Figure 6.2.6) also reflects no significant differences in major metabolite production, with compound **2.33** seen as the major metabolite in all the extracts. Characteristic proton signals (of lower intensity as compared to those due to **2.33**) at ~ δ_H 6.4 were also seen reflecting the presence of the well known procoralides (Knott *et al*., 2005).

One key factor which may have influenced production of **2.33** was the observation of fertile like bodies known as cystocarps on the algal fronds at the time of collection. A seasonal variation study is thus recommended in order to assess the influence of time of the year on metabolite production.

In the ^{1}H NMR spectrum of **2.33** (Figure 6.2.2), a unique double doublet was seen at δ 3.69 (dd, J = 15.1, 10.5 Hz). Two singlets were observed at δ 1.83 and 1.89.The ^{13}C NMR spectrum of **2.33** showed ten distinct signals with four de-shielded resonances at δ 136.9, 136.0, 129.3 and 128.7. The DEPT-135 spectrum of **2.33** indicated the presence of five –CH, one $–CH_2$, two $–CH_3$ and two quaternary carbon groups



2.33

Table 6.2.1: NMR spectroscopic data ($CDCl_3$) of compound **2.33**

Carbon No	δ_C	δ_H, mult, J (Hz)	COSY	NOESY
1	65.3	6.53, d, 9.6	H-2	H-4
2	129.1	5.82, d, 9.9	H-10	H-10
3	136.7	-	-	-
4	56.8	5.35, d, 5.9	-	-
5	128.5	5.90, dd, 15.4, 6.3	H-4	H-9, H-4
6	135.9	5.99, d, 15.4	H-4	H-9, H-8, H-4
7	67.0	-	-	-
8	41.6	3.69, dd, 15.1, 10.5	H-9	H-9
9	27.6	1.83, s	-	-
10	18.5	1.89, s	-	-

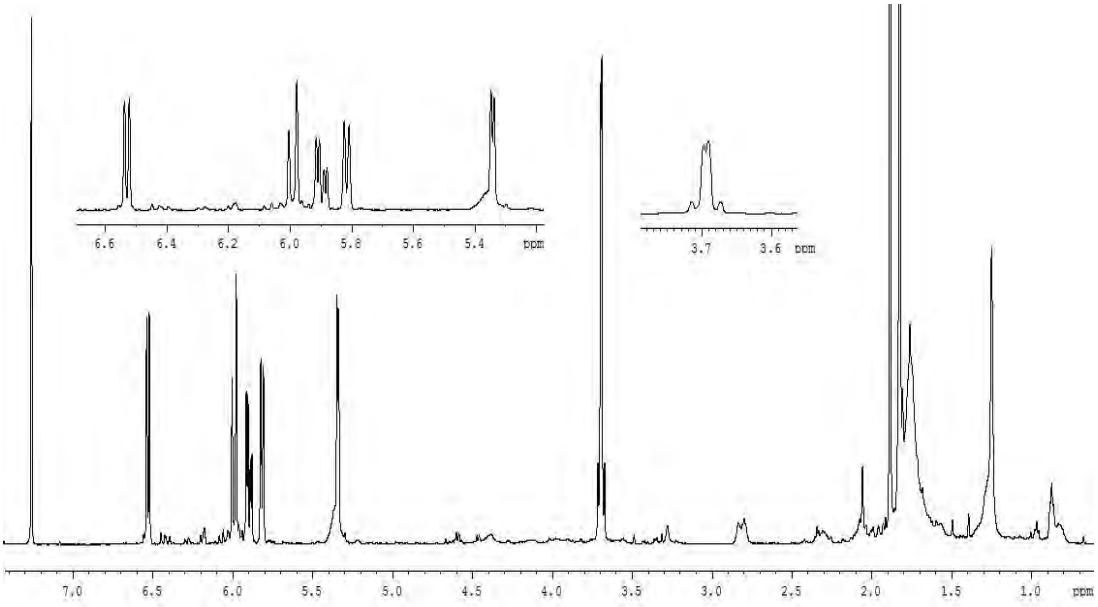


Figure 6.2.2: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **2.33**

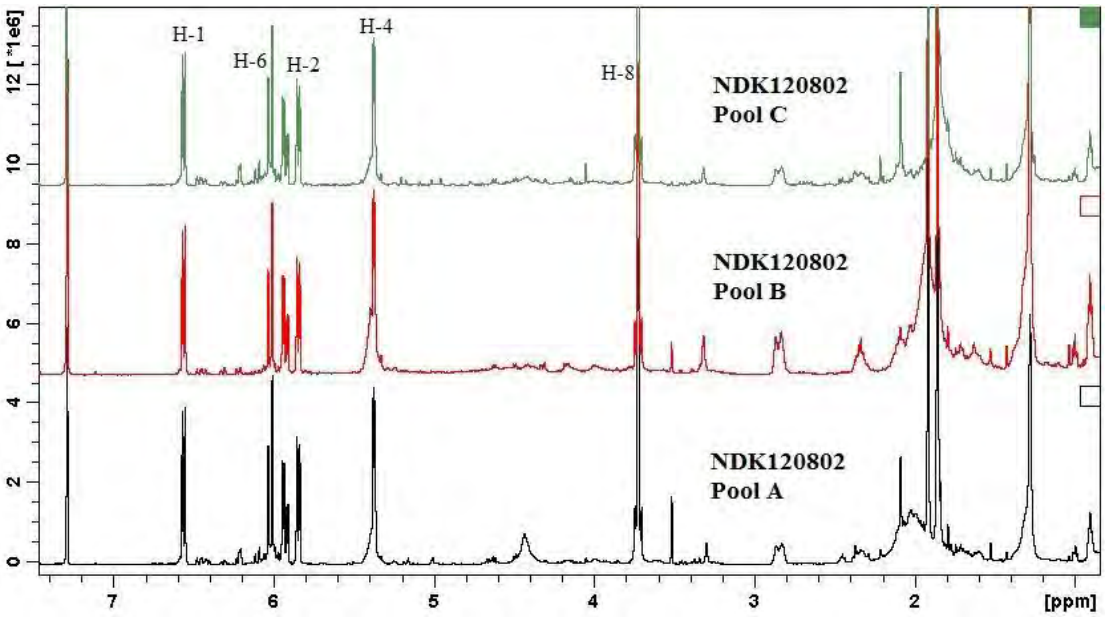


Figure 6.2.3: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of three intra-site extracts of *Plocamium corallorhiza* from Noordhoek

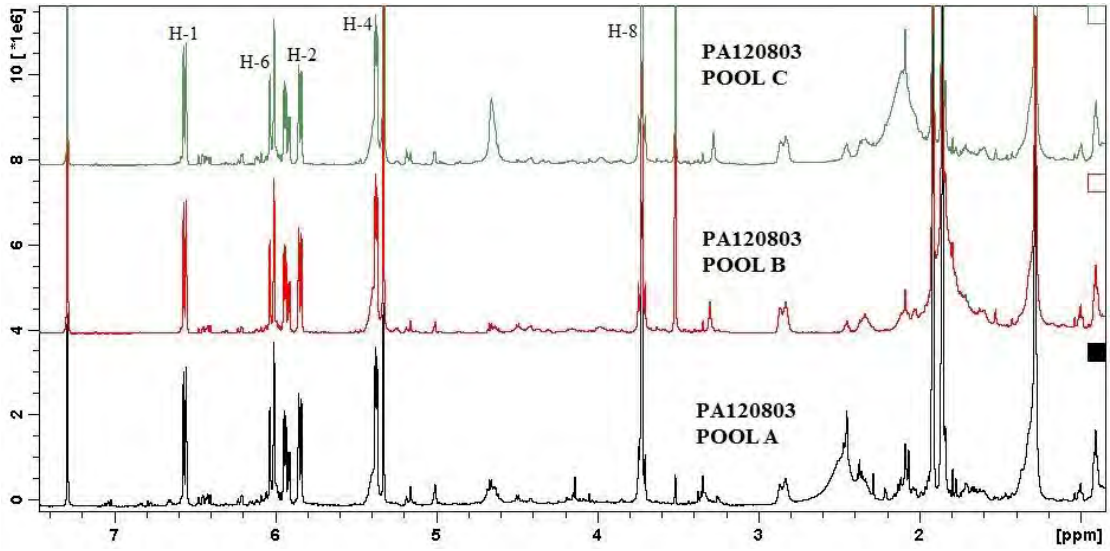


Figure 6.2.4: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of three intra-site extracts of *Plocamium corallorhiza* from Port Alfred

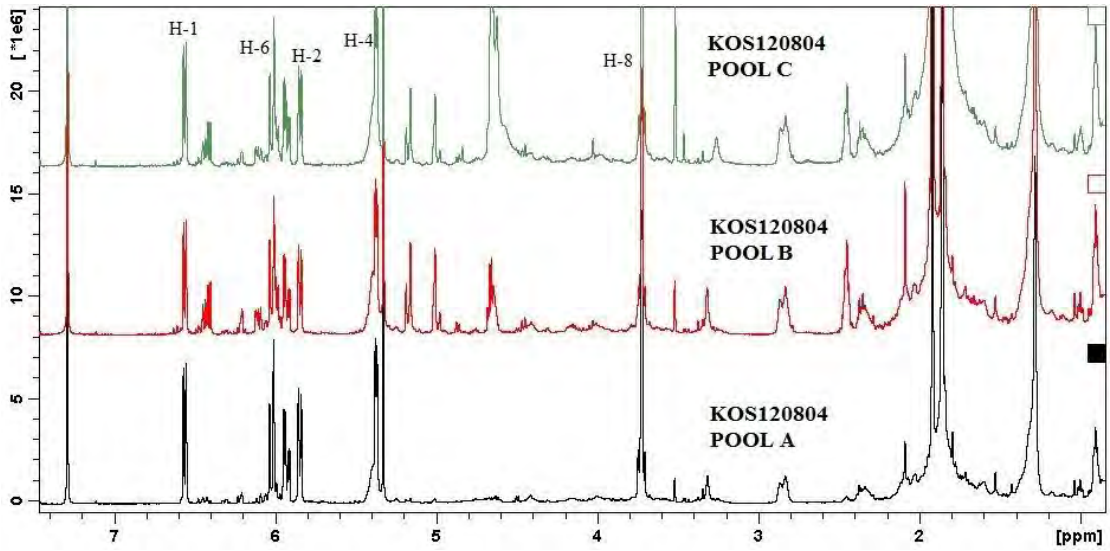


Figure 6.2.5: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of three intra-site extracts of *Plocamium corallorhiza* from Kenton-on-Sea

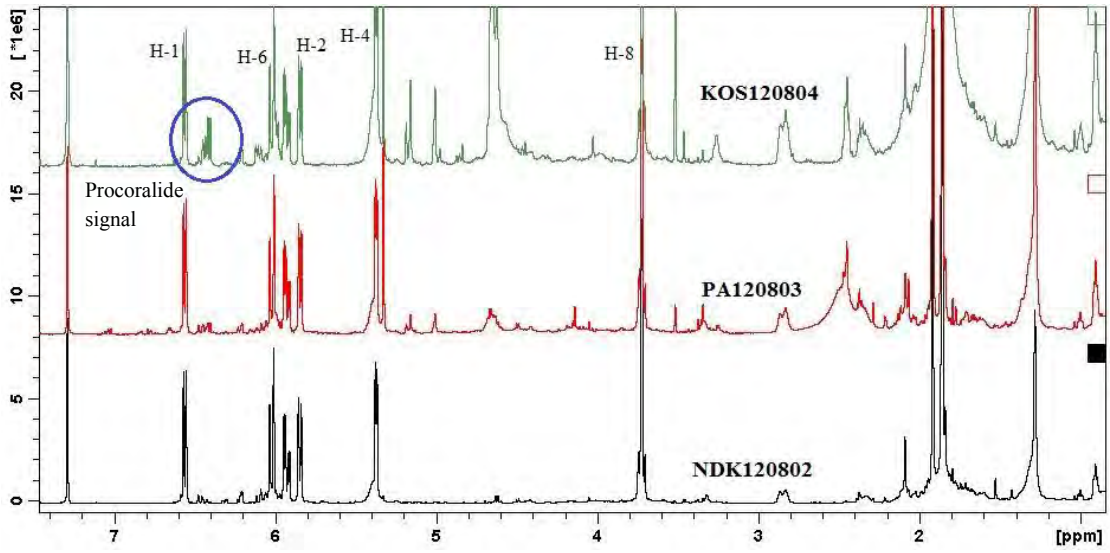


Figure 6.2.6: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of three inter-site extracts of *Plocamium corallorhiza* from Noordhoek, Port Alfred and Kenton-on-Sea

6.2.2 Geographical variation study experimental

Five algal samples were collected from three different pools within the intertidal zone at each site. The algal samples were full plants and contained fertile bodies on the fronds. The samples were flash frozen on site with liquid nitrogen and crushed to a relatively fine powder. The powdered sample (10 mL) was placed in Corning® centrifuge tubes (50 mL). To this MeOH (10 mL) and CH_2Cl_2 (20 mL) were added as the extraction solvents. The samples were then submerged in ice and transported back to the lab.

The organic layer (10 mL) in each centrifuge tube was collected and dried in vacuo. The ^{1}H NMR spectra were then obtained for these extracts using $CDCl_3$ (0.5 mL) for each analysis (See supplementary data for NMR parameters).

6.2.2.1 Plant material

(NDK120802)

Plocamium corallorhiza was collected by hand at Noordhoek beach in the Eastern Cape, South Africa in August 2012. A voucher specimen has been stowed away with the Division of Pharmaceutical Chemistry, Rhodes University

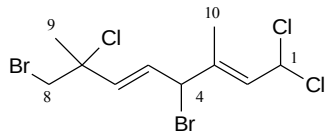
(KOS120804)

Plocamium corallorhiza was handpicked at Kenton-on-Sea beach in the Eastern Cape, South Africa in August 2012. A voucher specimen has been stowed away with the Division of Pharmaceutical Chemistry, Rhodes University

(PA120803)

Plocamium corallorhiza was handpicked at Port Alfred beach in the Eastern Cape, South Africa in August 2012. A voucher specimen has been stowed away with the Division of Pharmaceutical Chemistry, Rhodes University

6.2.2.2 Compound isolated



2.33

(2E,5E)-4,8-dibromo-1,1,7-trichloro-3,7-dimethylocta-2,5-diene (**2.33**): colourless oil; NMR data available in Table 6.2.1. As previously reported by (Mkwananzi, 2005)

6.2.3 References

Davies Coleman, M. T.; Beukes, D. R. Ten years of marine natural products research at Rhodes University. *South African Journal of Science* **2004,** *100,* 539-544.

Knott, M. G.; Mkwananzi, H.; Arendse, C. E.; Hendricks, D. T.; Bolton, J. J.; Beukes, D. R. Plocoralides A-C, polyhalogenated monoterpenes from the marine alga *Plocamium corallorhiza*. *Phytochemistry* **2005,** *66,* 1108-1112.

Mkwananzi, H. B. **2005**. The study of *Plocamium corallorhiza* metabolites and their biological activity. M.Sc thesis. Rhodes University, Grahamstown.