RHODES UNIVERSITY
Where leaders learn

Isolation and structure elucidation of halogenated metabolites from *Portieria hornemannii* and *Portieria tripinnata*

A Thesis Submitted in Fulfilment of the Requirements for the

Degree of

MASTER OF SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

By

Mohammed Adam

February 2015

Acknowledgements

There are numerous people I would like to thank for their immense assistance in the completion of this degree. Among the few notable ones:

- My supervisor, Prof. Denzil Beukes, a man who from day one, was always smiling, cheerful and willing to assist in any way possible. A truly great man, whose ocean of knowledge, not just in chemistry, was of great support to me. I thank you from the bottom of my heart for allowing me to be a student under your supervision.
- Professor John Bolton from the University of Cape Town for professional identification of algal samples.
- Dr. Edith Antunes for so kindly running NMR experiments for me.
- Mr. and Mrs. Morley, for always providing not only technical assistance, but also a good laugh throughout the course of this degree.
- Mr. Purdon, for driving my colleagues and I, tirelessly to and from our various conferences.
- Ma Prudence for making the lab a beautiful workplace.
- My G3 and G5 colleagues, Tafadzwa, Byron, Archibald, Emmanuel, Theoneste, and Chiko. You guys have been amazing workmates, and better friends. Thank you for always being there for me.
- My colleagues in the Faculty of Pharmacy for their tireless support.
- A special thank you to two of my closest friends, Maynard Chiwakata and Jameel Fakee. You guys have not only provided expert advice in the lab, but you have also been amazing friends and mentors. I will definitely miss the good times we've shared, from our various (and sometimes weird) conversations in the lab, to our daily lunch breaks at the Day Kaif, not to mention the fun-filled drives to Cape Town. I sincerely wish you all the best in life, and hope to one day be as good a mentor to someone, as you were to me.
- To all my amazing friends that I've made along the way, you have always been there for me through thick and thin, I thank you for all the amazing memories we've had.
- To my lovely family. My parents **Naseema** and **Abdul-Shakur Adam**, you are my strength, unwavering support and my reason for persevering through everything. Your sacrifices and efforts are what made me the man I am today, and I can only hope to one day be a son you are proud of. My brothers **Waheed** and **Suhail** and my dear

sister **Salma**, you are the shoulder I can always lean on, I will forever cherish and be grateful for your undying support. My uncle **Shiraz**, my aunt **Haseena**, my cousins **Shamaa**, **Sameer** and **Nazia**. You have always been there for me, and I cannot express how much I appreciate all that you have done for me over the years. My beautiful wife-to-be **Fathima**, thank you for always believing in me and cheering me on to the finish line, you are an amazing person, and I am looking forward to spending the rest of my life with you. **This thesis is dedicated to my family as a whole**, your support, encouragement, and guidance made this possible. THANK YOU ALL!!

Table of Contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Figures

List of Schemes

List of Tables

List of Abbreviations

°C	Degrees Celsius
$CDCl_3$	Deuterated Chloroform
DCM	Dichloromethane (CH_2Cl_2)
COSY	^1H-^1H Homonuclear Correlation Spectroscopy
d	Doublet
dd	Doublet of Doublets
ddd	Doublet of Doublet of Doublets
dt	Doublet of Triplets
EtOAc	Ethyl Acetate
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
IR	Infrared
J	Spin-Spin coupling constant (Hz)
m	Multiplet
m/z	Mass to charge ratio
MeOH	Methanol
MHz	Megahertz
mult	Multiplicity
NMR	Nuclear Magnetic Resonance
s	Singlet
spp	Species
td	Triplet of Doublets
t	Triplet
δ	Chemical shift (ppm)

Abstract

The red marine algal genus, *Portieria*, is known to produce a number of potent cytotoxic compounds with anticancer potential. The most important anticancer lead produced by this genus is the compound halomon. Unfortunately, the lack of sufficient quantities of this compound hampered its further development. Two *Portieria* species, *Portieria hornemannii* and *Portieria tripinnata*, are found along the South African coastline. Recent studies, based on DNA analysis, suggest that *Portieria hornemannii* may in fact be divided into several cryptic species. The current project is part of a larger study designed to investigate the use of secondary metabolites to identify new marine algal species. In this study ^{1}H NMR profiles of the organic extracts of selected *Portieria* spp were compared in order to identify new species. Selected compounds were then isolated and characterised as potential chemotaxonomic markers.

Four halogenated monoterpenes were isolated from *Portieria hornemannii*. Two of these were new compounds **4-(3-bromo-4-chloro-4-methylpentyl)-3-chlorofuran-2(5H)-one,** which were isomers of each other**.** The two known compounds had been previously isolated from *Portieria hornemannii* samples off the Madagascar coast. These compounds could prove to be useful as chemotaxonomic marker compounds, as they have never been isolated from any other species of marine algae.

Three known halogenated monoterpenes were isolated from *Portieria tripinnata*. These compounds had been previously isolated from different species of marine algae and therefore, could not serve as chemotaxonomic marker compounds for this species of marine alga. Further work needs to be done on *Portieria tripinnata*, with regards to its chemistry, as it is a species of marine algae that has not been previously researched.

Chapter 1

Introduction

1.1 Natural Products

A simple definition of a natural product is a small molecule that is synthesized by a biological organism. (Sheppard 2007). A more accurate definition of a natural product (secondary metabolite) is "a naturally produced substance which does not play an explicit role in the internal economy of the organism that produces it" (Mapelstone *et al*., 1992).

Secondary metabolites are only found in certain organisms or groups of organisms. The function of secondary metabolites in an organism is not always known, however, certain secondary metabolites have been found to assist the organism in adapting to its environment. Secondary metabolites could assist an organism in adapting to its environment by functioning as a toxic substance to ward of predators, as volatile attractant to attract organisms of similar or different species or as a colourant to attract or caution other organisms. Secondary metabolites provide the most pharmacologically active natural products (Dewick 2002).

Primary metabolites on the other hand, are compounds that are directly involved in the modifying and/or synthesizing of carbohydrates, fats, proteins and nucleic acids in an organism (Dewick 2002).

1.1.1 Examples of Notable Natural Products

In the past, pharmaceutical companies have made use of plant extracts as crude therapeutic formulations (Mishra *et al*., 2011). Advancement in the pharmaceutical industry in the mid-twentieth century however, has led to the use of fairly pure compounds from natural sources (Firn and Jones, 2003).

Some of the more notable natural products discovered include:

a) Salicin, which was isolated from the bark of a willow tree, *Salix alba*, which led to the synthesis of the anti-inflammatory agent aspirin (acetylsalicylic acid) (Mishra *et al*., 2011).

b) Morphine, a potent opioid analgesic, was first reported in 1803 and was isolated from the plant *Papaver somniferum* (opium poppy). In the 1870's, morphine was synthetically altered to yield both heroin and codeine. (Dias *et al*., 2012).
c) Penicillin was discovered, from the fungus *Penicillium notatum*, by Alexander Fleming in 1929 (Mann 1994).
d) Quinine, an antimalarial drug, was extracted from the bark of *Cinchona succirubra* (Der Marderosian *et al*., 2002).
e) Vancomycin, a glycopeptide antibiotic, was isolated from cultures of the bacteria *Amycolatopsis orientalis*, in 1953 by Edmund Kornfeld (Dias *et al*., 2012)
f) Erythromycin, a macrolide antibiotic, isolated from the bacteria *Saccharopolyspora erythraea* (Dias *et al*., 2012).
g) Paclitaxel (Taxol®), an anti-cancer drug used in the management of breast cancer, was isolated from the bark of *Taxus brevifolia* (Pacific Yew) (Dias *et al*., 2012).

1.1.2 Marine Natural Products

The global use of marine macro-algae is a multi-billion dollar industry which is mainly based on the farming of edible species, or the production of agar, carrageenan and alginate (Smit, 2004). Recently however, pharmaceutical companies have taken an interest in the natural products from marine organisms, including marine algae, as a potential source for new drugs. Examples of important, biologically active compounds from marine algae are:

a) Carrageenans is a family of linear sulphated polysaccharides, isolated from Rhodophyta (red marine algae). Of particular interest is λ-carrageenan isolated from *Gigartina skottsbergii*, which showed strong antiviral activity against different strains of the Herpes Simplex Virus 1 and 2 during the virus adsorption stage (Carlucci *et al*., 1997).
b) Fucoidan is another sulphated polysaccharide, which is found in many species of Phaeophyceae (brown marine algae), has been shown to possess strong anti-viral properties against the Respiratory Syncytial Virus (Malhotra *et al*., 2003), Human Immunodeficiency Virus (Sugawara *et al*., 1989) and the Herpes Simplex Virus type 1 and 2 (Feldman *et al*., 1999).
c) Kahalalide A and F were isolated form a species of *Bryopsis* which is from the Division of Chlorophyta (green algae), and showed antimicrobial activity against

Mycobacterium tuberculosis (el Sayed et al., 2000). Kahalalide F is currently in Phase II clinical trials in order to evaluate its anti-tumour activity. (Martin-Algarra *et al*., 2009)

d) Bromofuranone is a lactone from the marine red alga *Delisea pulchra*, and is a potential treatment for chronic *Pseudomonas aeruginosa* infection (Smit, 2004).

e) Halomon, a polyhalogenated monoterpene, isolated from the marine red alga *Portieiria hornemannii*, showed interesting anti-tumour activity, more specifically, halomon showed 40% of apparent cures against the highly aggressive U251 brain tumour line after being administered five times a day (50mg/kg) during an *in vivo* evaluation of the compound. (Fuller *et al*., 1994)

f) Two bromoditerpenes, bromosphaerone and 12S-hydroxybromosphaerodiol have been found to show anti-bacterial activity against *Staphylococcus aureus* at a minimum inhibitory concentration of 0.104 and 0.146 µM respectively. These compounds were isolated from the red marine algae *Sphaerococcus coronopifolus*. (Etahiri et al., 2001)

Of particular interest is the isolation of, the polyhalogenated monoterpene, halomon. Although halomon showed potent anti-tumour activity, its development has been hindered by limited amounts isolated from the alga *Portieria hornemannii*, unsuccessful re-isolation attempts, as well as an inaccessibility to a synthetic form of the compound (Fuller *et al*., 1994). The former could possibly be due to the fact that the marine alga *Portieria hornemannii*, is known to comprise several cryptic species (Payo *et al*., 2013). When two or more distinct species are classified as being one specie, the term cryptic species is used (Bickford *et al*., 2007).

1.1.3 Halogenated monoterpenes isolated from *Portieria hornemannii*

Red seaweeds (Rhizophyllidaceae) have shown to produce high amounts of secondary metabolites (Payo *et* al., 2011). The *Portieria* genus is tropical Indo-Pacific genus that falls into this family and has been shown to be an abundant source of halogenated secondary metabolites (Andrianasolo *et* al., 2006). Many halogenated monoterpenes have been isolated from *P. hornemannii.* As mentioned above, the most notable metabolite being halomon (compound **1.1**, **Table 1.1**) which showed selective anti-tumour activity (Fuller *et al*., 1994).

Halomon (compound **1.1**) and compound **1.7** (**Table 1.1**) were found to be low micromolar inhibitors of DNA methyl transferase-1 (Andrianasolo *et al.*, 2006). Compounds **1.2**, **1.3** (**Table 1.1**) and **1.16** (**Table 1.2**) show comparable panel-averaged potency against a panel of 60 human tumour cell lines (Fuller *et al*., 1994). Earlier studies carried out suggest the following Structural –Activity relationships for halogenated monoterpenes (Fuller *et al*., 1994):

1. The presence of a halogen at C-7 is not essential for anti-tumour activity
2. The absence of a halogen at C-6 resulted in weak activity, which suggests that it is required for anti-tumour activity
3. The presence of a halogen at C-2 is required for "Halomon-like" activity.

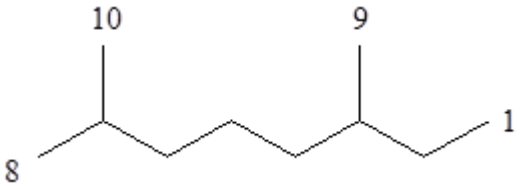


Figure 1.1: Basic structure and carbon numbering, of an acyclic monoterpene

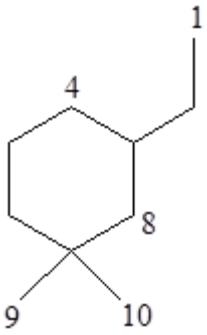


Figure 1.2: General structure of cyclic monoterpenes isolated from *Portieria hornemannii*

The *Portieria* genus is an understudied taxon and most studies carried out on this genus have been attempts to isolate pharmaceutically active compounds (Payo *et* al., 2011). Very few studies have been carried out on the *Portieria* genus in order to decipher this cryptic species in terms of inter and/or intra-specific variation of these secondary metabolites (Payo *et* al., 2011). One study tested the carbon/nutrient hypothesis, which states that the secondary metabolites produced by marine alga, are determined by the available nutrients in the

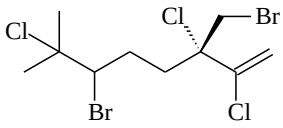
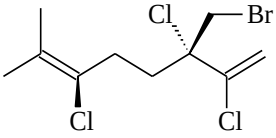
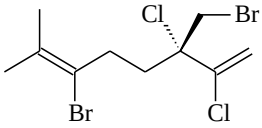
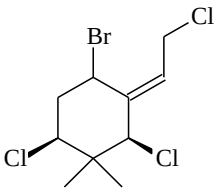
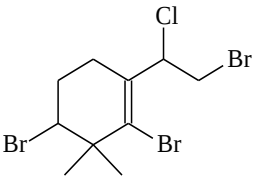
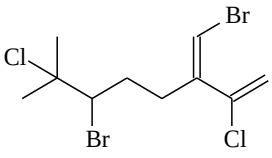
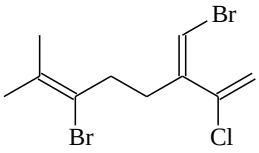
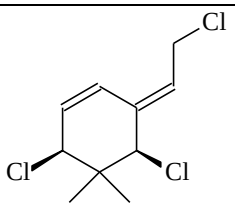
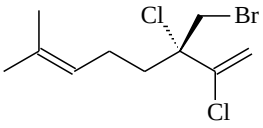
surrounding environment. Their findings showed that the differences in ochtodene concentrations found in *Portieria hornemannii* were due to differences in light availability, rather than nutrient availability (Puglisi *et al*., 1997). Some authors have suggested the evaluation the genotype of these organisms to better understand the mechanisms that regulate the production of certain types of secondary metabolites (Payo *et al*., 2011).

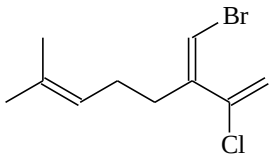
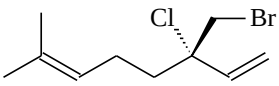
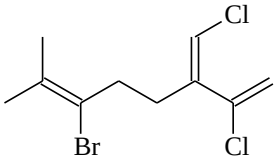
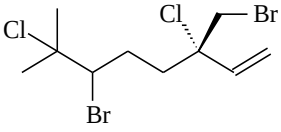
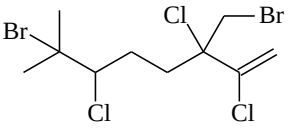
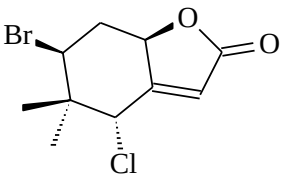
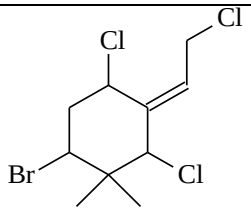
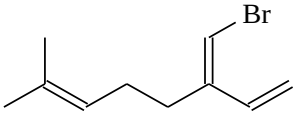
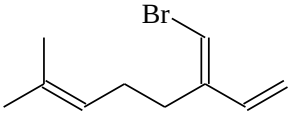
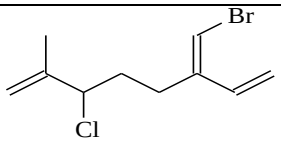
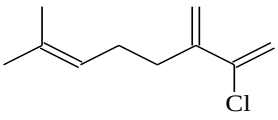
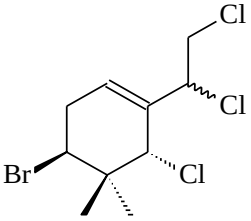
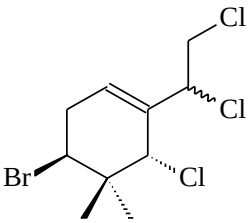
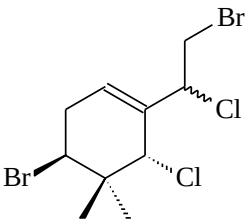
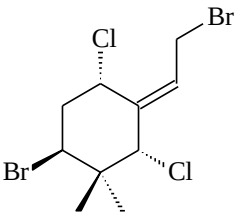
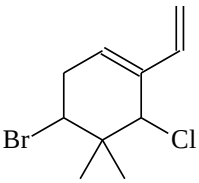
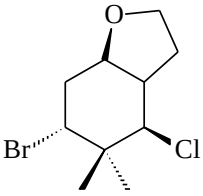
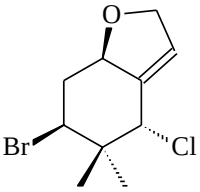
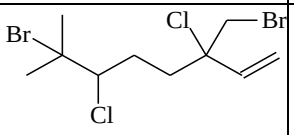
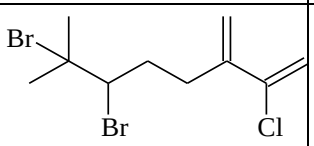
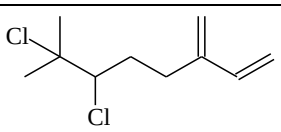
A study was done in order to determine the possible sources of variation in non-polar secondary metabolites from *Portieria hornemannii*, from two different locations in the Philippines. Different life-history stages, presence of cryptic species and/or spatiotemporal factors were investigated using Principle Component Analysis (PCA). This study resulted in the discovery of intraspecific variation in three cryptic species from six different sites, as well as the fact that life-history driven variations may influence the differences in secondary metabolite production by this species (Payo *et al*., 2011).

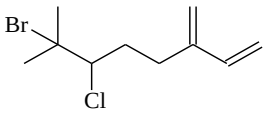
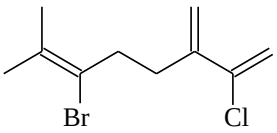
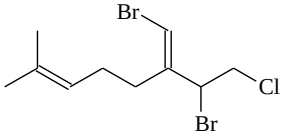
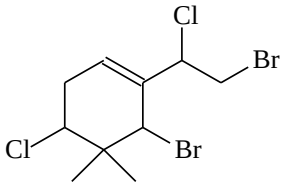
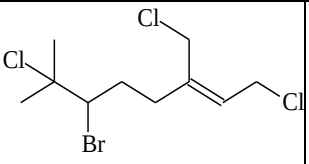
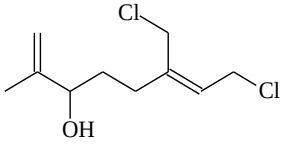
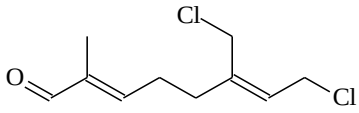
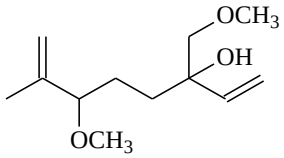
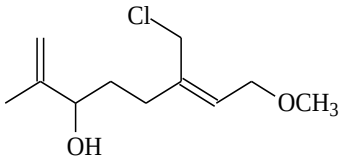
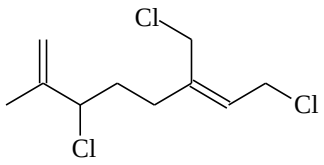
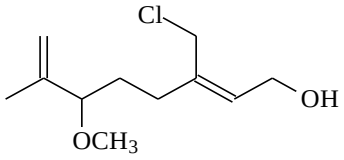
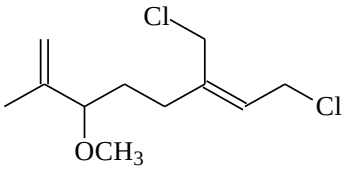
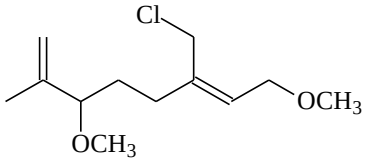
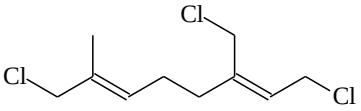
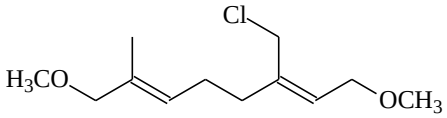
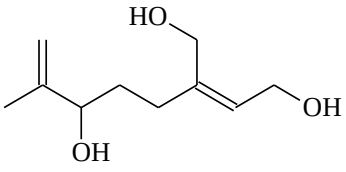
Table 1.1 shows the various secondary metabolites, in particular, halogenated monoterpenes that have been isolated from *Portieria hornemannii*, and gives us an idea of how rich this genus is, in terms of these secondary metabolites.

Table 1.1: Halogenated monoterpenes isolated from *P. hornemannii*

[a] Fuller *et al*., 1992; [b] Fuller *et al*., 1994; [c] Andrianasolo *et al*., 2006; [d] Gunatilaka *et al*., 1999; [e] Burreson *et al*., 1975; [f] Barahona *et al*., 2003; [g] Crews *et al*., 1983; [h] Woolard *et al*., 1978; [i] Wright *et al*., 1991.

No.	Structure	No.	Structure	No.	Structure
1.1[a]		**1.2**[b]		**1.3**[b]	
1.4[b]		**1.5**[b]		**1.6**[c]	
1.7[c]		**1.8**[a]		**1.9**[b]	

1.10[b]		**1.11**[b]		**1.12c**	
1.13[b]		**1.14**[b]		**1.15**[h]	
1.16[d]		**1.17**[e]		**1.18**[e]	
1.19[h]		**1.20**[f]		**1.21**[g]	
1.22[g]		**1.23**[g]		**1.24**[g]	
1.25[b]		***1.26**[e]		**1.27**[e]	
1.28[e]		**1.29**[e]		**1.30**[e]	

1.31[e]		**1.32**[e]		**1.33**[e]	
1.34[e]		**1.35**[i]		**1.36**[i]	
1.37[i]		**1.38**[i]			
1.39[i]		**1.40**[i]			
1.41[i]		**1.42**[i]			
1.43[i]		**1.44**[i]			
1.45[i]		**1.46**[i]			

*It should be noted that the compounds **1.26** and **1.27** are stereoisomers at position C6. These compounds are thought to serve as herbivore feeding deterrents based on laboratory feeding experiments (Fenical, *et al*., 1980).

1.2 Research Aims and Objectives

Apart from the apparent existence of cryptic species within *Portieria hornemannii*, we see that the taxonomy of the *Portieria* genus is extremely complex and complicated. Two *Portieria* species, *Portieria hornemannii* and *Portieria tripinnata*, are found along the South African coastline. Our interest resides primarily in the chemistry of these species, specifically the secondary metabolites that can be isolated from these species. *Portieria tripinnata* has not been chemically studied previously, and the secondary metabolites isolated from this species are of significant interest to us. The specific objectives of this study were to:

a) Compare ^{1}H NMR profiles of various *Portieria* samples collected off the South African coastline.
b) Isolate and characterise secondary metabolites from *Portieria hornemannii* and *Portieria tripinnata*.
c) Evaluate the chemotaxonomic significance of these metabolites.

1.3 References

Andrianasolo, E. H.; France, D.; Cornell-Kennon, S.; Gerwick, W. H. DNA Methyl Transferase Inhibiting Halogenated Monoterpenes From The Madagascar Red Marine Alga *Portieria hornemannii*. *Journal of Natural Products* **2006**, *69*, 576 - 579.

Barahona, L. F.; Rorrer, G. L. Isolation Of Halogenated Monoterpenes From Bioreactor-Cultured Microplantlets Of The Macrophytic Red Algae *Ochtodes secundiramea* And *Portieria hornemannii*. *Journal of Natural Products* **2003**, *66*, 743 – 751.

Bickford, D.; lohman, D. J.; Sodhi, N. S.; Ng, P. k. L.; Meier, R.; Winker, K.; Ingram, K. K.; Das, I. Cryptic Species As A Window On Diversity And Conservation. *Trends in Ecology and Evolution* **2007**, *22*, 148 - 155.

Burreson, B. J.; Woolard, F.; Moore, R. E. Chondrocole A And B, Two Halogenated Dimethylhexahydrobenzofurans From the Red Alga *Chondrococcus hornemannii* (Mertens). *Tetrahedron Letters* **1975**, *26*, 2155 – 2158.

Carlucci, M. J.; Pujol, C. A.; Ciancia, M.; Noseda, M. D.; Matulewicz, M. C.; Damonte, E. B.; Cerezo, A. S. Antiherpetic And Anticoagulant Properties Of Carrageenans From The Red Seaweed *Gigartina skottsbergii* And Their Cyclized Derivatives: Correlation Between Structure And Biological Activity. *International Journal of Biological Macromolecules* **1997**, *20*, 97 – 105.

Crews, P.; Myers, B. L.; Naylor, S.; Clason, E. L.; Jacobs, R. S.; Stall, G. B. Bio-active Monoterpenes From Red Seaweeds. *Phytochemistry* **1984**, *23*, 1449 – 1451.

Der Marderosian, A.; Beutler, J. A. The Review of Natural Products, 2nd Edition; Facts And Comparisons Publishing Group; Seattle, WA, USA, **2002**, 13–43.

Dewick, P. M. Medicinal Natural Products A Biosynthetic Approach; Primary And Secondary Metabolism; West Sussex, UK, **2002**, 7-8.

Dias, D. A.; Urban, S.; Roessner, U. A Historical Overview Of Natural Products In Drug Discovery. *Metabolites* **2012**, *2*, 303 – 336.

Etahiri, S.;, Bultel-Ponce, V.; Caux, C.; Guyot, M. New Bromoditerpenes From The Red Alga *Sphaerococcus coronopifolius*. *Journal of Natural Products* **2001**, *64*, 1024 - 1027.

Feldman, S. C.; Reynaldi, S.; Stortz, C. A.; Cerezo, A.S.; Damont, E. B. Antiviral Properties Of Fucoidan Fractions From *Leathesia difformis*. *Phytomedicine* **1999**, *6*, 335 – 340.

Firn, R. D.; Jones, C. G. Natural Products – A Simple Model To Explain Chemical Diversity. *Natural Product Reports* **2003**, *20*, 382 – 291.

Fuller, R. W.; Cardellina, 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 – 441.

Fuller, R. W.; Cardellina, J. H.; Kato, Y.; Brinen, L. S.; Clardy, J.; Snader, K. M.; Boyd, M. R. A Pentahalogenated Monoterpene From The Red Alga *Portieria hornemannii* Produces A Novel Cytotoxicity Profile Against A Diverse Panel Of Human Tumor Cell Lines. *Journal of Medicinal Chemistry* **1992**, *35*, 3007 – 3011.

Gunatilaka, A. A. L.; Paul, V. J.; Park, P. U.; Puglisi, M. P.; Gilter, A. D.; Eggleston, D. S.; Haltiwanger, R. C.; Kingston, D. G. I. Apakaochtodenes A and B: Two Tetrahalogenated Monoterpenes From The Red Marine Alga *Portieria hornemannii*. *Journal of Natural Products* **1999**, *62*, 1376 – 1378.

Malhotra, R.; Ward, M.; Bright, H.; Priest, R.; Foster, M. R.; Hurle, M.; Blair, E. Bird, M. Isolation And Characterisation Of Potential Respiratory Syncytial Virus Receptor(s) On Epithelial Cells. *Microbes and Infection* **2003**, *5*, 123 – 133.

Mann, J. *Murder, Magic, and Medicine*; Oxford University Press: New York, NY, USA, **1994**, 164–170.

Maplestone, R. A.; Stone, M. J.; Williams, D. H. The Evolutionary Role Of Secondary Metabolites - A Review. *Gene* **1992**, 115, 151–157.

Martin-Algarra, S.; Espinosa, E.; Rubio, J.; lopez, L. J. J.; Manzano, J. L.; Carrion, L. A.; Plazaola, A.; Tanovic, A.; Paz-Ares, L. Phase II Study Of Weekly Kahalalide F In Patients With Advanced Malignant Melanoma. *European Journal of Cancer* **2009**, *45*, 732 - 735.

Mishra, B. B.; Tiwari, V. K. Natural products: An Evolving Role In Future Drug Discovery. *European Journal of Medicinal Chemistry* **2011**, *46*, 4769 – 4807.

Payo, D.A.; Leliaert, F.; Verbruggen, H.; D'hondt, S.; Calumpong, H.; De Clerk, O. Extensive Cryptic Species Diversity And Fine-Scale Endemism In The Marine Red Alga Portieria In The Philippines. *Proceedings of the Royal Society B: Biological Sciences* **2013**, 280: 20122660.

Puglisi, M. P.; Paul, V. J. Intraspecific Variation In The Red Alga Portieria hornemannii: Monoterpene Concentrations Are Not Influenced By Nitrogen Or Phosphorous Enrichment. *Marine Biology* **1997**, *128*, 161 - 170.

Sayed, K. A.; Bartyzel, P.; Shen, X.; Perr, T. L.; Zjawiony, J. K.; Hamann, M. T. Marine Natural Products As Antituberculosis Agents. *Tetrahedron* **2000**, *56*, 949 – 953.

Sheppard, T. L. All Natural. *Nature Chemical Biology* **2007**, *3*, 351 - 352.

Smit, A. J. Medicinal And Pharmaceutical Uses Of Seaweed Natural Products: A Review. *Journal of Applied Phycology* **2004**, *16*, 245 – 262.

Sugawara, I.; Itoh, W.; Kimura, S.; Mori, S.; Shimada, K. Further Characterization Of Sulfated Homopolysaccharides As Anti-HIV Agents. *Experientia* **1989**, *45*, 996 – 998.

Woolard, F. X.; Moore, R. E. The Structure And Absolute Configuration Of Chondrocolactone, A Halogenated Monoterpene From The Red Alga *Chondrococcus hornemannii*, And A Revised Structure For Chondrocole A. *Tetrahedron Letters* **1978**, *27*, 2367 – 2370.

Wright, D. A.; Konig, G. M.; Sticher, O. Five New Monoterpenes From The Marine Red Alga *Portieria hornemannii*. *Tetrahedron* **1991**, *47*, 5717 – 5724.

Chapter 2

^{1}H NMR profiling of South African *Portieria* species

Abstract

The *Portieria* genus is known to include the following species; *P. dichotoma, P. harveyi, P. hornemannii, P. japonica, P. kilneri, P. spinulosa* and *P. tripinnata*. Two of these are found along the South African coastline (*Portieria hornemannii* and *P. tripinnata*). Differentiation between species is done morphologically. Recent studies, based on DNA analysis, suggests *Portieria hornemannii* may be divided into several cryptic species. A study was carried out to determine whether morphologically similar species would have the same chemical fingerprint. Of the fourteen samples analysed, seven samples had an almost identical chemical profile to each other. One sample was deemed *Portieria tripinnata*, and the six other samples had very different chemical profiles. There is a significant difference in chemical composition of samples that were all deemed *Portieria hornemannii* based on morphology. This however, could be due to seasonal, or environmental changes as samples were collected at different times and from different locations. ^{1}H NMR fingerprinting could prove to be a useful tool in the determination of species due to its simplicity and efficiency.

2.1 Introduction

Portieria hornemannii is known to comprise several cryptic species (Payo *et al*., 2011a). There is little evidence to suggest the exact number of species that fall under the genus *Portieria*. Until recently, there has been only one comprehensive study based on morphology of the genus *Portieria*, which suggested only one species exists, namely *P. hornemannii* (Payo *et al*., 2011a). However, recent research based on DNA done in the Philippine Archipelago, resulted in the discovery of 21 species that showed high levels of intra-archipelagic endemism (Payo *et al.*, 2011b). Another study was carried out to determine possible reasons for variation in non-polar secondary metabolites from *P. hornemannii*. This study established that both inter- and intra-specific variations existed. The results of this study established that variations were either life-history driven, or possibly due to micro-scale factors (Payo *et al*., 2011b).

Two species of *Portieria* are currently found along the South African coastline, *P. hornemannii* and *P. tripinnata* (De Clerck *et al.*, 2005). The use of ^{1}H NMR to determine the chemical composition of the crude organic extract of a sample of algae could prove to be useful in distinguishing between different species.

The metabolome is defined as the chemical profile or fingerprint of that organism (Aranibar *et al*., 2001). In order to identify a sample of marine algae to be species specific using its chemical composition, the complete metabolome of that sample needs to be determined. Metabolomics or metabonomics is an emerging technique in which the metabolic profiles of different organisms are determined. These techniques are then interpreted by pattern recognition chemometric statistical analysis (Weljie *et al*., 2006). The essence of this technique is in the simplifying of complex chemical information such as NMR spectra (Weljie *et al*., 2006). The use of NMR spectroscopic data has proved to be very useful due to the wealth of chemical information that it can provide, this however, also has various issues. One of the issues with NMR spectroscopic data is the case in which complex spectra show high levels of overlap in certain regions of the spectrum, this however, is commonly overcome by chemometric analysis of 1- and 2-D NMR spectra (Weljie *et al*., 2006).

A useful tool in the analysis of NMR spectral data is the use of Principal Component Analysis (PCA). PCA is a useful tool when it comes to the analysis of NMR spectra due to the fact that instead of measuring data in terms of an x-y axis, it determines the principal components of a data set and plots these data sets on a new x-y axis. These principal

components are a smaller data set which are easier to analyse and interpret, while still containing most of the information from the original data set (Dallas 2013).

2.1.1 Chapter Aims

This work forms part of a larger study that aims to discover possible cryptic species of *P. hornemannii*. Therefore, the main objective of this part of the project was to:

- Collect *Portieria* species from along South African coast
- Compare ^{1}H NMR spectra of crude extracts of selected *Portieria* species

2.2 Results and Discussion

Sixteen samples of *Portieria* were collected from various sites along the South African coastline. Voucher specimens were made for twelve of these algal samples (**Figure 2.1**). The sites at which each sample was collected can be seen in **Table 2.1**. Each sample of *Portieria* collected, was identified based on morphology.

Table 2.1: *Portieria* sample information

Sample code	**Location Collected**	**Date Collected (YY/MM/DD)**	***Name***
D1033	Mission Rocks (Northern KwaZulu-Natal)	11/09/26	*P. hornemannii*
D1052	Port Edward (KwaZulu-Natal)	11/09/28	*P. tripinnata*
D1242 A	Three Sisters (Eastern Cape)	13/02/27	Species not stated
D1242 - B	Three Sisters (Eastern Cape)	13/02/27	Species not stated
D1242 - C	Three Sisters (Eastern Cape)	13/02/27	Species not stated
D925 - A	De Hoop (Koppie Alleen – Western Cape)	11/02/17	*P. hornemannii*
D925 - B	De Hoop (Koppie Alleen – Western Cape)	11/02/17	*P. hornemannii*
KOS110914 - 3	Kenton On Sea (Eastern Cape)	11/09/14	*P. hornemannii*
NDK120802 - A	Noordhoek (Eastern Cape)	12/08/02	*P. hornemannii*
NDK120802 - B	Noordhoek (Eastern Cape)	12/08/02	*P. hornemannii*
PA100331 - 9	Port Alfred (Eastern Cape)	10/03/31	*P. hornemannii*
PA120803	Port Alfred (Eastern Cape)	12/08/03	*P. hornemannii*
PA130427 - 4A	Port Alfred (Eastern Cape)	13/04/27	*P. hornemannii*
PA130427 – 4B	Port Alfred (Eastern Cape)	13/04/27	*P. hornemannii*
PA130427 – 6 Red A	Port Alfred (Eastern Cape)	13/04/27	*P. hornemannii*
PA130427 – 6 Red B	Port Alfred (Eastern Cape)	13/04/27	*P. hornemannii*

D925 - A	D925 - B	NDK120802 -
		
NDK120802 - B	**PA130427 – 4A**	**D1242 C**
		

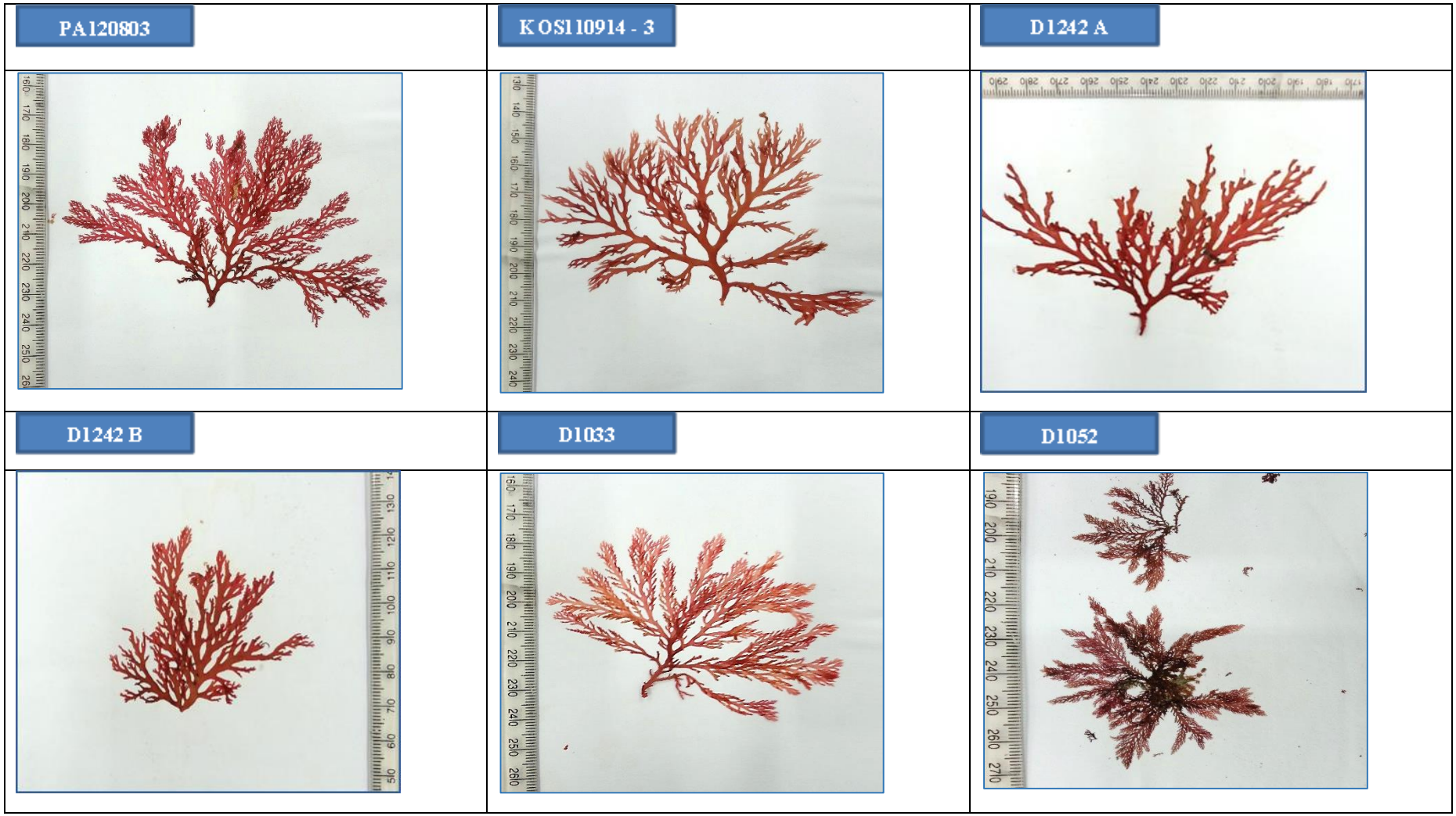


Figure 2.1: Photographs of the various *Portieria* specimens studied in this section

A small scale (~ 1g of wet alga) extraction process was used to obtain sufficient organic crude extract of each alga to be assessed using ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of the organic crude layer for each sample can be seen in **Figure 2.2**. On the analysis of the ^{1}H NMR spectra of the crude extracts (**Figure 2.2**) a clear difference in chemical constituents of the samples can be seen. Seven of the sixteen samples had similar ^{1}H NMR spectra and this can be seen in **Figure 2.3**. The remaining nine samples displayed different crude spectra. This can be seen in **Figure 2.4**.

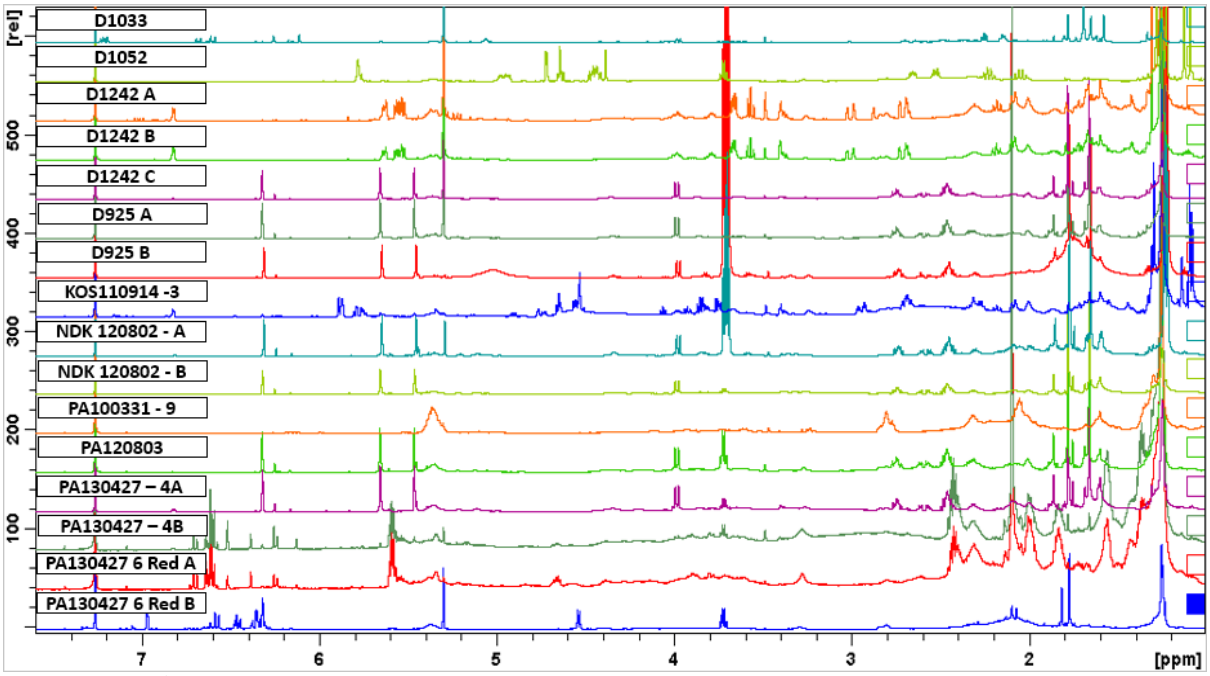


Figure 2.2: ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of the *Portieria* crude extracts

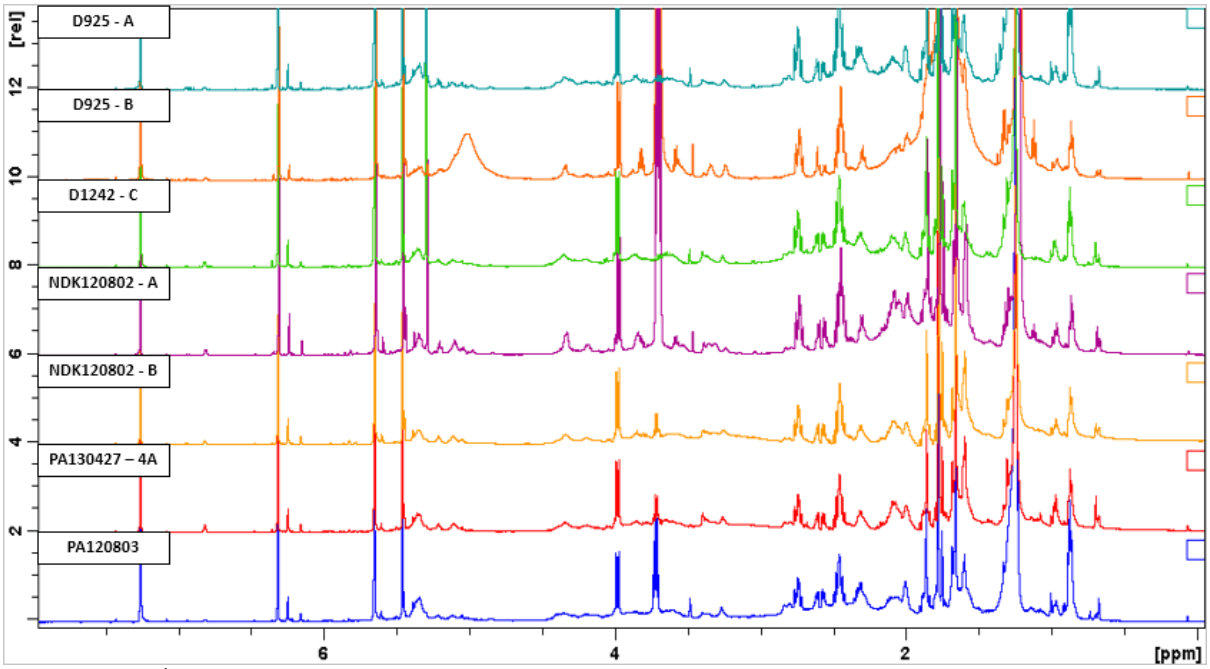


Figure 2.3: ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of crude *Portieria* extracts displaying similar spectra

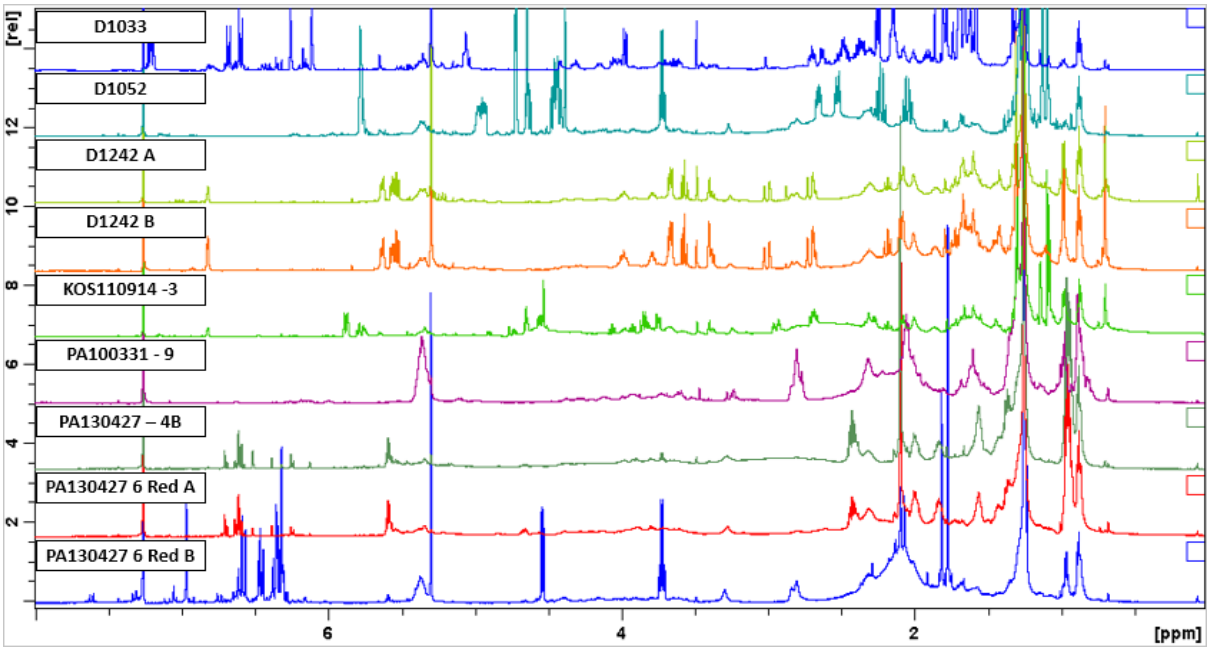


Figure 2.4 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of crude *Portieria* extracts displaying contrasting spectra

As seen from the table above, most of the samples collected were identified as *Portieria hornemannii* based on morphology. From the ^{1}H NMR profiles of the crude extracts of each sample however, we see significant differences in the chemical composition of the samples.

Figures 2.5 and **2.6** show the 1H NMR spectra of samples collected from the same location and on the same date. These show differences in the chemical profiles of their organic crude extracts. Of particular interest are samples D1242 A, D1242 B, D1242 C and samples PA130427 - 4A and PA130427 - 4B.

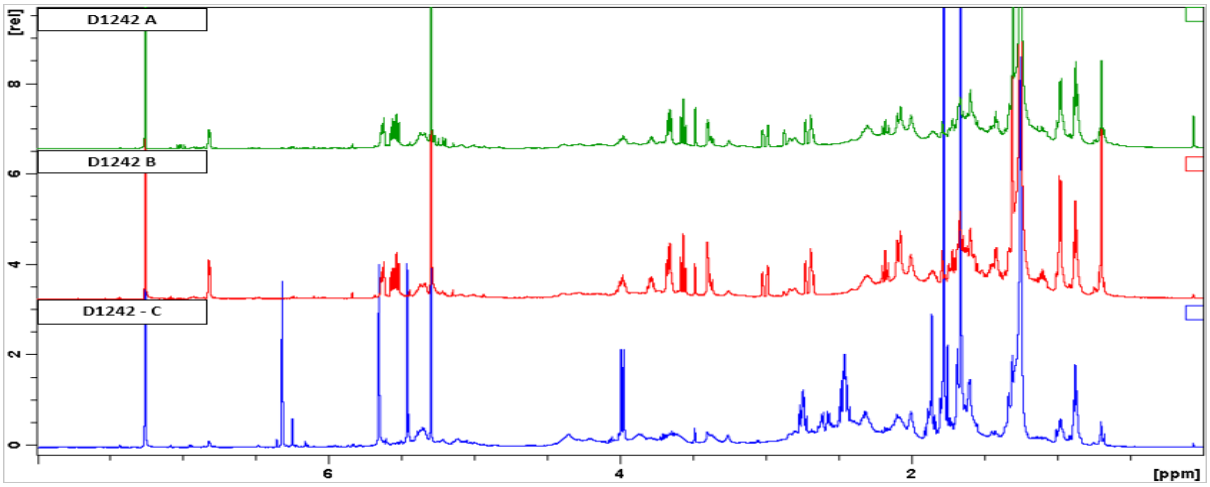


Figure 2.5: [1]H NMR profiles ($CDCl_3$, 400 MHz) of *Portieria* samples (D1242 A, D1242 B and D1242 C) collected at Three Sisters, Eastern Cape, South Africa.

From **Figure 2.5** we clearly see that the chemical composition of sample D1242 C is different to samples D1242 A and D1242 B. These differences could be due to the fact that these are entirely different species to each other since the samples were not identified as being *Portieria hornemannii* when collected. Another possible reason for the differences in chemical composition of these samples could be due to potential differences in metabolic ages of each sample. A similar situation arises in **Figure 2.6**, as can be seen with samples PA130427 – 4A and **b**.

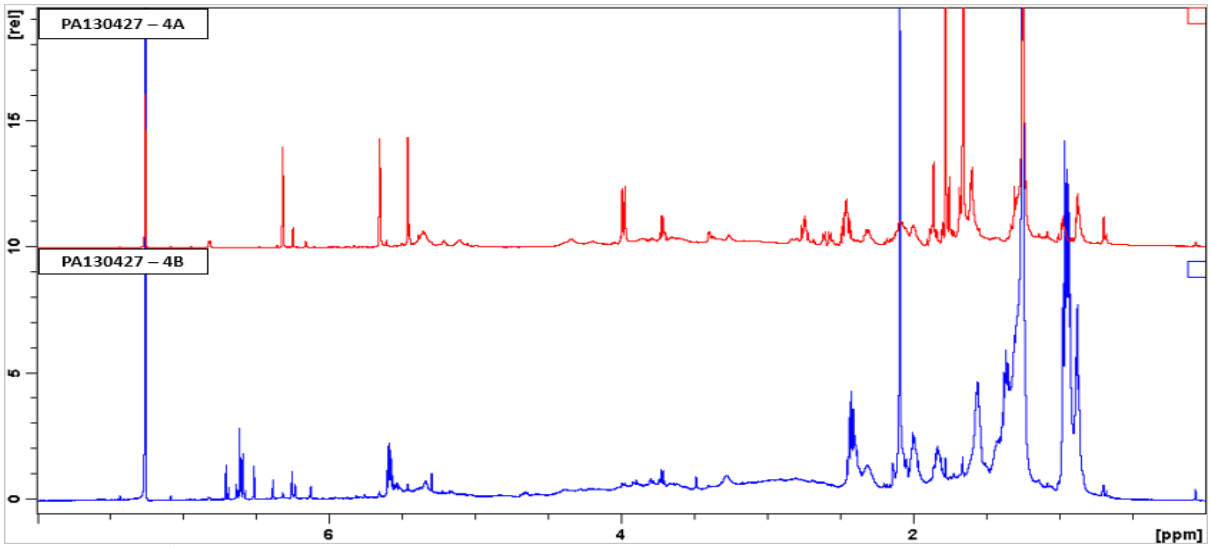


Figure 2.6: [1]H NMR profiles (400 MHz, $CDCl_3$) of *Portieria* samples (PA130427 – 4A and PA130427 – 4B) collected at Port Alfred, Eastern Cape, South Africa.

These intra-site variations in chemical composition of samples lead us to conclude that samples from Three Sisters (Eastern Cape) and Port Alfred (Eastern Cape) cannot be included in a chemotaxonomic study as these would likely result in inconsistent results. Samples D1242 C, D925 - A, D925 - B, NDK120802 – A, NDK120802 – B, PA130427 – 4A and PA120803 all showed very similar [1]H NMR spectra as seen in **Figure 2.3**. From these [1]H NMR spectra, there is a clear indication of the presence of a major metabolite in these samples. This is due to the intense signals at ~ δ 1.7, ~ δ 1.8, ~ δ 2.5, ~ δ 2.8, ~ δ 4.0, ~ δ 5.5, ~ δ 5.7 and ~ δ 6.3. These signals are seen more clearly in **Figure 2.7** which shows the [1]H NMR spectra of crude of sample D925 – A.

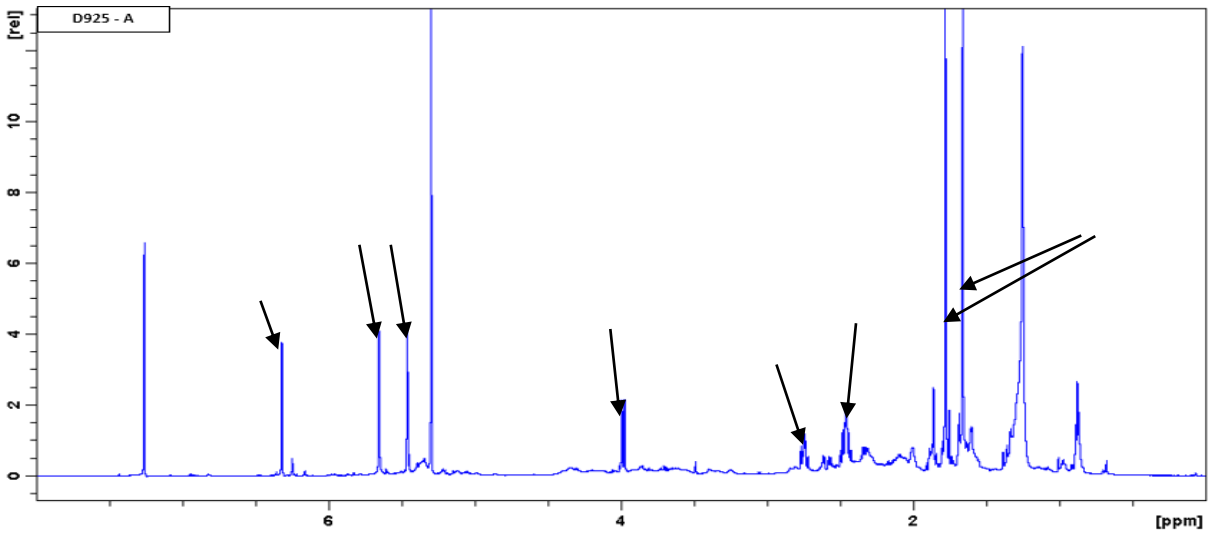


Figure 2.7: [1]H NMR profile ($CDCl_3$, 400 MHz) of sample D925 – A collected in De Hoop (Koppie Alleen – Western Cape), South Africa.

Figure 2.8 shows the ^{1}H NMR spectra of a crude extract of sample D1052 which was identified as being *Portieria tripinnata.* From this ^{1}H NMR spectrum, we note the possibility of more than one major metabolite in this algal sample. This is due to the cluster of sharp singlet peaks observed between δ 1.5 and δ 2.0, which most likely represent protons from methyl functional groups.

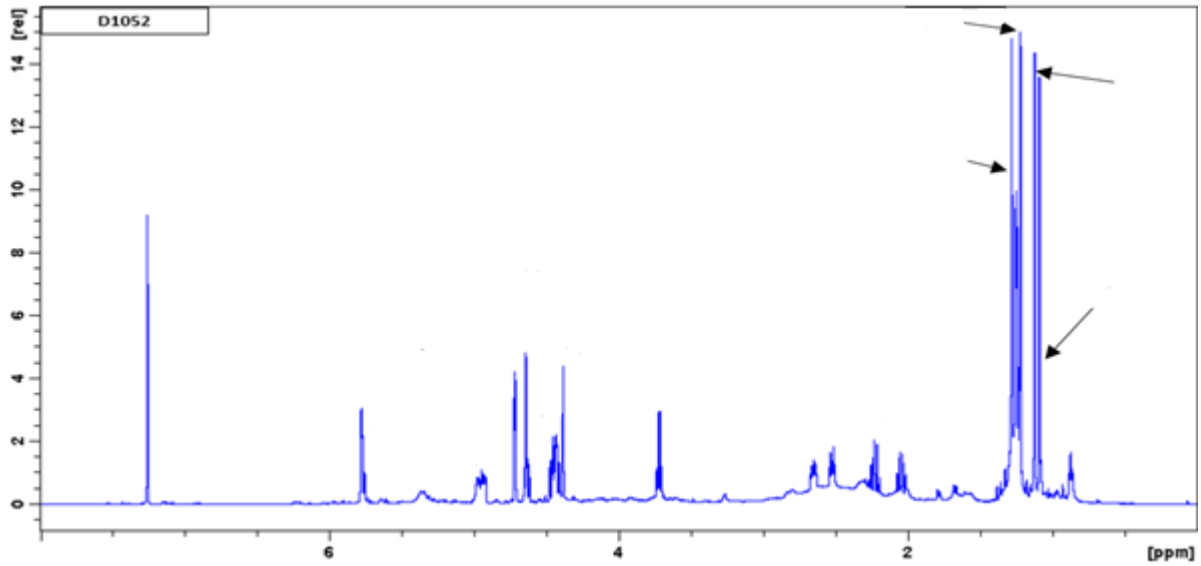


Figure 2.8: ^{1}H NMR profile ($CDCl_3$, 400 MHz) of sample D1052 collected in Port Alfred (KwaZulu-Natal), South Africa.

Stacking these two crude ^{1}H NMR spectra together, we see a significant difference in chemical composition between the *Portieria hornemannii* and *Portieria tripinnata* species (**Figure 2.9**)

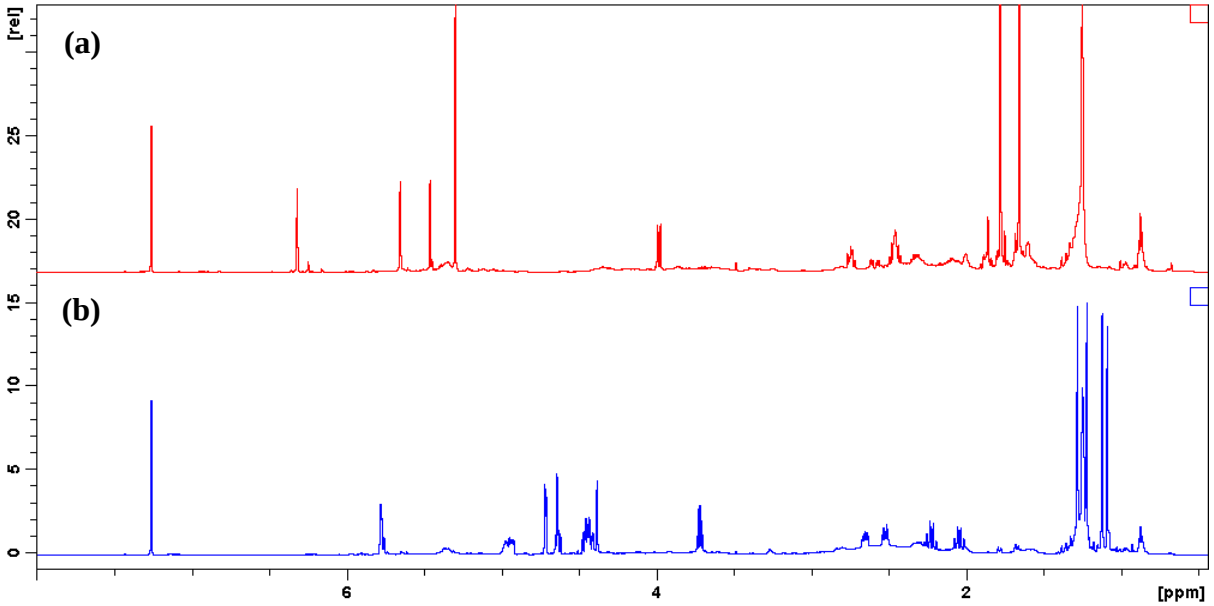


Figure 2.9: ^{1}H NMR profile ($CDCl_3$, 400 MHz) of samples D925 – A (**a**) and D1052 (**b**)

2.3 Conclusion

The algal samples collected show very different ^{1}H NMR spectra of their organic crude fractions. Due to insufficient knowledge on how to carry out a Principle Component Analysis, the results were unfortunately not analysed by this technique. This however, was not problematic as the different *Portieria* samples, could be placed into two specific groups. The first group, being the *Portieria* samples with similar crude spectra seen in **Figure 2.3**, in which the differences between these spectra are negligible and the samples could be deemed similar. The second group being the *Portieria* samples with significantly varying crude spectra, as seen in **Figure 2.4**. One of the samples placed in the latter group is sample D1052 which is *Portieria tripinnata*, exhibited a significantly different crude spectra to all other crude spectra obtained. Overall, the differences observed make the consistent isolation of chemotaxonomic marker compounds very difficult, as all the samples apart from D1052, were deemed *Portieria hornemannii* based on morphology. From these results, we can safely conclude that samples containing the major metabolite seen in **Figure 2.3** would be the preferred *Portieria hornemannii* samples to be used in this chemotaxonomic study. Samples with this compound as their major metabolite show consistent chemical compositions. Samples D925 – A and D925 – B were combined and used as the representative samples of *Portieria hornemannii* and sample D1052 was used as the representative sample of *Portieria tripinnata*, for the chemotaxonomic study of *Portieria* samples found along the South African coastline.

2.4 Experimental

2.4.1 General Experiments

Solvents

All solvents used to extract the organic crude extracts were High-Performance-Liquid-Chromatography (HPLC) grade from LiChrosolv® from Merk®, Darmstd, Germany.

Nuclear Magnetic Resonance (NMR)

All ^{1}H NMR spectra of organic crude fractions were acquired using a Bruker® Avance III™ HD 400 MHz The solvent used to obtain the spectra was deuterated chloroform ($CDCl_3$). All spectra were in reference to the $CDCl_3$ and the reference peak for this solvent was at δ 7.26 ppm.

2.4.2 Plant Material

The sixteen *Portieria* samples (D1033, D1052, D1242 A, D1242 – B, D1242 – C, D925 – A, D925 – B, KOS110914 – 3, NDK120802 – A, NDK120802 – B, PA100331 – 9, PA120803, PA130427 - 4A, PA130427 – 4B, PA130427 – 6 Red A and PA130427 – 6 Red B) were collected along the South African coast (East coast, South Eastern coast and South Western coast). Identification of samples was done based on morphological characteristics, and was carried out by Prof John J. Bolton of the department of Botany, University of Cape Town, South Africa. Samples were kept in a freezer at ~ -18^{o}C. Of the sixteen samples collected, voucher specimens were made for only twelve, this was due to some samples being damaged and thus not being suitable for use as voucher specimens. These voucher specimens are kept with the Pharmaceutical Chemistry division of Rhodes University, South Africa.

2.4.3 Small Scale Extractions

A small amount (~ 1g) of each sample was rinsed with water, after which it was allowed to air dry for ~ 5 minutes. This was then placed in a test tube. To this, 4 ml of methanol (MeOH) was added and these were sonicated for 5 minutes, thereafter, 8 ml of Dichloromethane (DCM) was added. The samples were then sonicated for a further 5 minutes. The organic layer (DCM and MeOH) was then pipetted off and placed in size six

vials. The solvent was then evaporated off using a Rotary evaporator. The crude extracts were then reconstituted in $CDCl_3$ and their ^{1}H NMR spectra were then obtained.

2.5 References

Aranibar, N.; Singh, B. K.; Stockton, G. W.; Ott, K. H. Automated Mode-Of-Action Detection By Metabolic Profiling. *Biochemical and Biophysical Research Communications* **2001**, *286*, 150 – 155.

De Clerck, O.; Tronchin, E. M.; Schils, T. Red Algae. Rhodophyceae. Guide To The Seaweeds Of KwaZulu-Natal. *Scripta Botanica Belgica* **2005**, *33*, 131 - 267.

Dallas, G. Principle Component Analysis 4 Dummies: Eigenvectors, Eigenvalues And Dimension Reduction **2013**. Accessed on: 22/09/15. Available at: https://georgemdallas.wordpress.com/2013/10/30/principle-component-analysis-4-dummies-eigenvectors-eigenvalues-and-dimension-reduction/.

Payo, D. A. Diversity Of The Marine Red Alga Portieria In The Philippines, An Integrative Approach. Ph.D. Thesis, Ghent University, Gent, Belgium, **2011a**.

Payo, D. A.; Colo, J.; Calumpong, H.; De Clerk, O. Variability Of Non-Polar Secondary Metabolites In the Red Alga *Portieria*. *Marine Drugs* **2011b**, *9*, 2438 – 2468

Payo, D.A.; Leliaert, F.; Verbruggen, H.; D'hondt, S.; Calumpong, H.; De Clerk, O. Extensive Cryptic Species Diversity And Fine-Scale Endemism In The Marine Red Alga Portieria In The Philippines. *Proceedings of the Royal Society B: Biological Sciences* **2013**, 280: 20122660.

Weljie, A. M.; Newton, J.; Mercier, P.; Carlson, E.; Slupsky, C. M. Targeted Profiling: Quantitative Analysis Of ^{1}H NMR Metabolomics Data. *Analytical Chemistry* **2006**, *78*, 4430 – 4442.

Chapter 3

Isolation of halogenated monoterpenes from *Portieria hornemannii*

Graphical Abstract

The red marine algal genus, *Portieria*, is known to produce a number of potent cytotoxic compounds with anticancer potential. The most important anticancer lead produced by this genus is the compound halomon. Unfortunately, the lack of sufficient quantities of this compound hampered its further development. The current project is part of a larger study designed to investigate the use of secondary metabolites to identify new marine algal species. Samples of *Portieria hornemannii* were collected of the South African coastline. Compounds were isolated and assessed for their potential chemotaxonomic markers. Two known halogenated monoterpenes (compound **1.6** and **1.7**) and two new halogenated monoterpenes (compound **3.1** and **3.2**) were isolated from the red marine alga *Portieria hornemannii*. The structures of the new metabolites extracted, were determined using one- and two-dimensional NMR spectroscopy and HRESIMS.

Chapter 3

Isolation of halogenated monoterpenes from *Portieria hornemannii*

3.1 Introduction

The taxonomy of the *Portieria* genus is complicated. Initially, the *Portieria* genus was referred to as *Desmia* which entailed three species namely *D. herbacea*, *D. hippurina* and *D. hornemannii*. The genus *Desmia* was then amended to only contain *D. hornemannii* and two other species namely *D. ambigua* (India) and *D. tripinnata* (South Africa). At the same time, the genus *Chondrococcus* was established which was later adopted as the new, preferred name for the genus *Desmia*. *Portieria* was the only available synonym of *Chondrococcus* and entailed one specie namely *C. coccinea*. This species was included in the synonymy of *Chondrococcus hornemannii*. *Portieria* was then established as the generic name for the genus in accordance to a change made in the ICBN in 1981. The *Portieria* genus includes the following species: *P. dichotoma*, *P. harveyi*, *P. hornemannii*, *P. japonica*, *P. kilneri*, *P. spinulosa* and *P. tripinnata*. (Catalogue of Benthic Marine Algae of the Philippines)

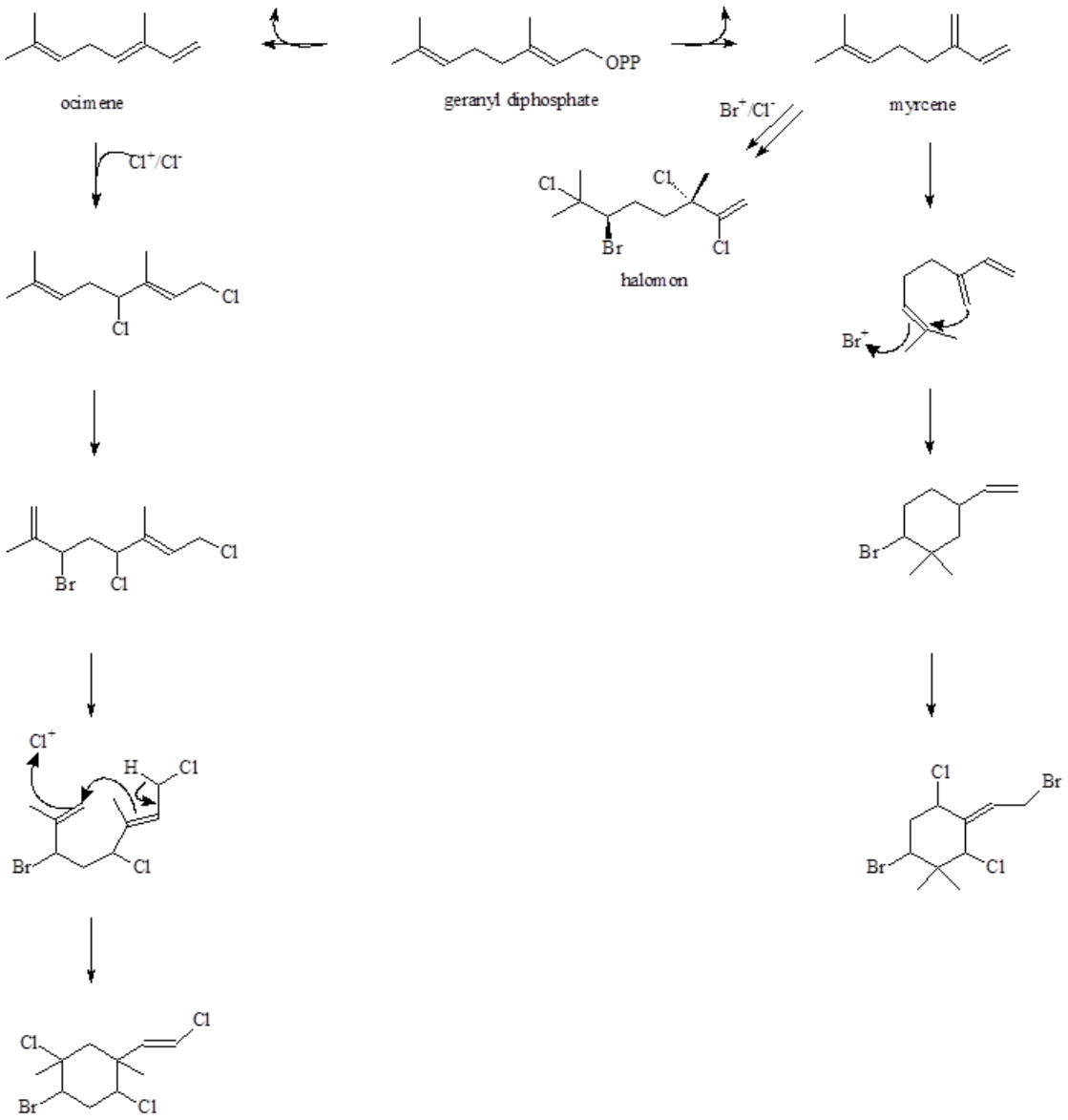
There is little evidence to suggest the exact number of species that fall under the genus *Portieria*. As mentioned in the previous chapter, a recent DNA-based study conducted in the Philippine archipelago resulted in the finding of 21 species that showed high level of intra-archipelagic endemism (Payo *et al*., 2013). This further proves *Portieria* is a complex genus of marine algae that currently has an unknown number of species.
Previously, in order to distinguish between *Portieria* species, studies were carried out based on morphology. The results of these studies are highly refuted and recently, DNA-based studies which are more reliable and the preferred method of species delimitation, have been used to distinguish between different species. (Leliaert *et al*., 2014).

The metabolome of an organism is defined as the chemical profile of the metabolites of that organism (Aranı`bar *et al*., 2001). Certain secondary metabolites that are unique to a sample of marine algae, could be used to classify that sample as being from a specific species. These metabolites are known as chemotaxonomic markers.

In a study carried out on the terrestrial plant *Ephedra*, the metabolomic profiles of different samples were assessed using ^{1}H NMR (Kim *et al*., 2005). The result of that study identified three different species, by simply assessing the ^{1}H NMR profiles of the organic crude fractions of each sample using Principle Component Analysis (PCA). ^{1}H NMR fingerprinting has the advantage of being an effective, fast and reproducible technique that requires very little sample preparation.

3.1.1 Biosynthesis of cyclic and acyclic monoterpenes

Red algae (Rhodophyta) are known to produce polyhalogenated monoterpenes (Stallard *et al*., 1974)**.** This class of compounds are however, restricted to the genera *Plocamium* and *Portieria* (Fuller *et al.,* 1992). The precursor for the formation of these monoterpenes in these red alga is myrcene or ocimene (Wise *et al.,* 2002). The marine environment is rich in chloride and bromide ions, the incorporation of these into the monoterpene structure is thought to be facilitated by the vanadium-dependent bromoperoxidase enzyme (Carter *et al.,* 2004). **Scheme 3.1** shows the proposed biosynthetic pathway of cyclic and acyclic monoterpenes proposed by (Wise *et al.,* 2002)



Scheme 3.1: Proposed biosynthetic pathway of acyclic and cyclic polyhalogenated monoterpenes (Adapted from Wise *et al.*, 2002)

3.1.2 Chapter Aims

This work forms part of a larger study that aims to identify various species that fall under the cryptic species *Portieria hornemannii*. The main objectives of this part of the project was to:

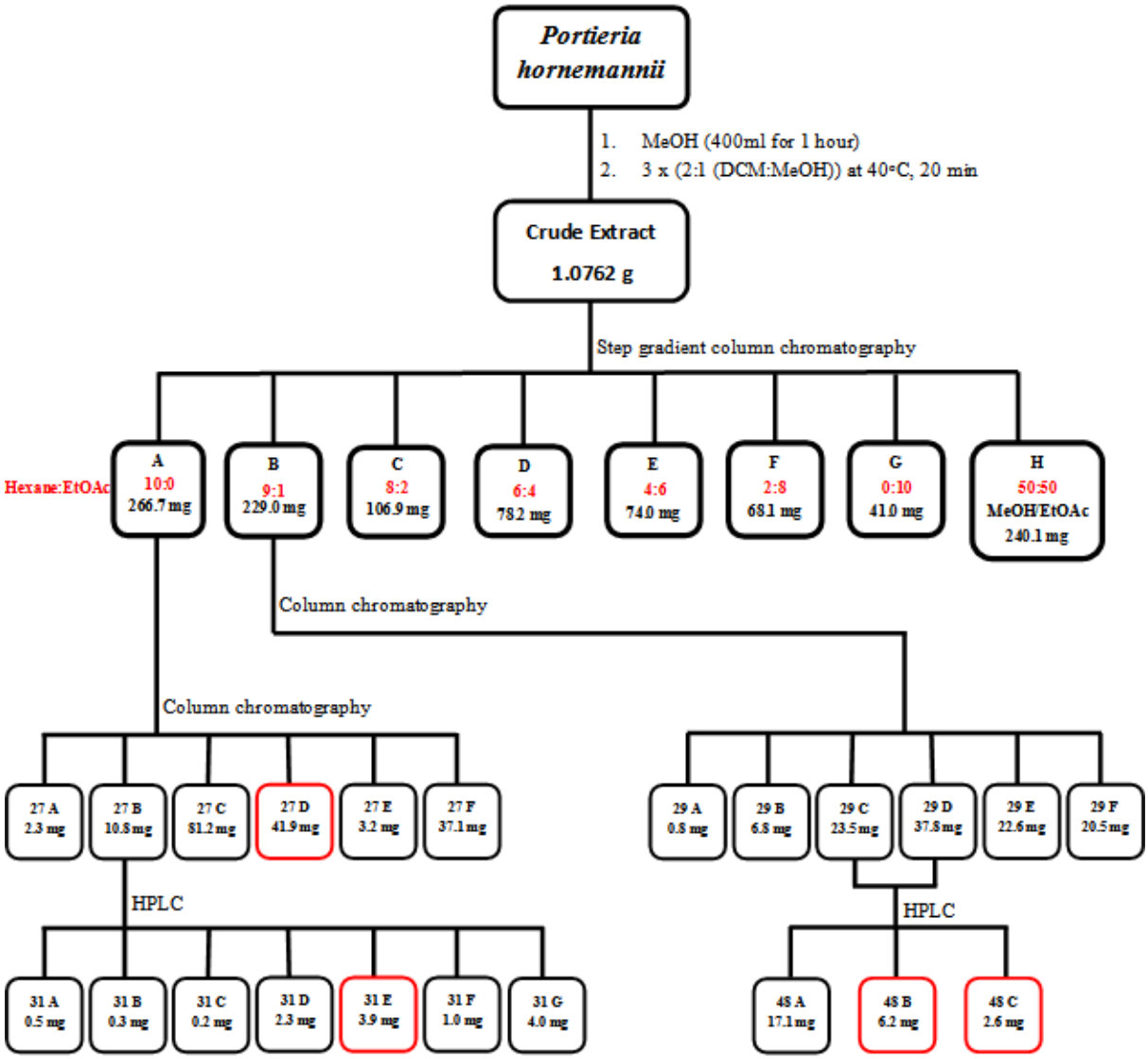
a) Isolate and characterise metabolites from *Portieria hornemannii*
b) Identify potential chemotaxonomic markers for *Portieria hornemannii*

Figure 3.1: Photograph of segment of sample D925 A (*Portieria hornemannii*)

3.2 Results and Discussion

3.2.1 Isolation and characterisation of halogenated monoterpenes from *Portieria hornemannii*

Portieria hornemannii samples D925A and D925B, were collected from the De Hoop Nature reserve (Western coast), South Africa. These two samples were combined on the basis of their similar ^{1}H NMR spectra and relabelled D925. This sample was soaked in MeOH for ~ 1 hour and then extracted three times with DCM:MeOH (2:1). The crude organic extract was separated using step-gradient column chromatography to give eight fractions of increasing polarity (**Scheme 3.2**).



Scheme 3.2: Isolation of compounds **1.6 (**27D), **1.7** (31E), **3.1** (48B) and **3.2** (48C)

Separation of the crude fraction into fractions A-H (**Scheme 3.2**) can be seen in **Figure 3.3** which shows the ^{1}H NMR spectra of these fractions.

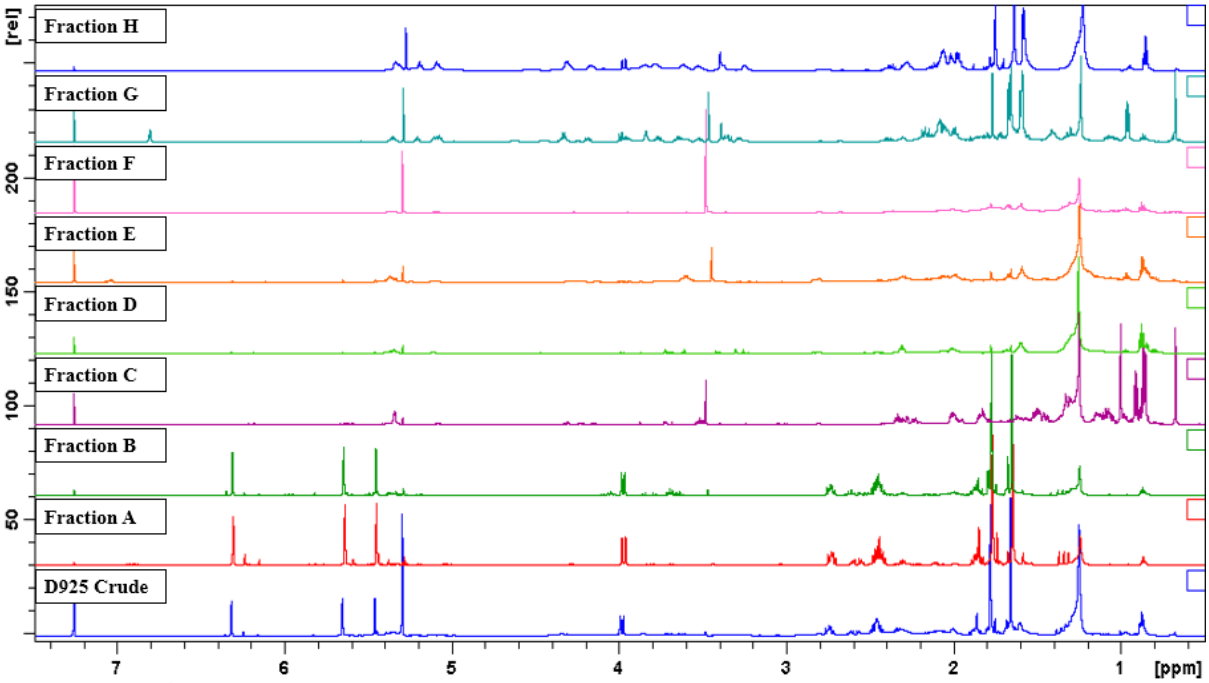


Figure 3.2: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of D925 crude and open-column silica gel chromatography fractions A-H

From **Figure 3.2** we note the presence of a major compound in the crude fraction, fraction A and fraction B. Fraction A was further separated via column chromatography and resulted in six new fractions (27A – 27 F). Fraction 27D contained pure compound **1.6** and fraction 27B was further separated using High Performance Liquid Chromatography (HPLC) to give rise to compound **1.7**. Fraction B was separated as above, via column chromatography and six new fractions were realized (29A – 29F). Fractions 29C and 29D were similar and were combined, thereafter separated and resulted in three fractions (48A – 48C). Fractions 48B and 48C contained pure compounds (**3.1** and **3.2**) respectively.

3.2.2 Structure elucidation of metabolites

3.2.2.1 Compound 1.6

Compound **1.6** displayed a relatively simple ^{1}H NMR spectrum (**Figure 3.3**) with two methyl singlets at δ 1.66 and δ 1.78, three complex multiplets at δ 2.46 (2H), 1.87 (1H) and 2.75 (1H) which are indicative of methylene protons. Two methine doublets are seen at δ 3.98 (d, *J* = 11.5 Hz) and δ 6.35 (d, *J* = 0.7 Hz). The former suggests a halomethine group, whilst the latter is suggestive of a deshielded olefinic proton. Two doublets are seen at δ 5.49 (d, *J* = 1.6 Hz) and δ 5.68 (d, *J* = 1.6 Hz) which are reminiscent of a disubstituted terminal alkene.

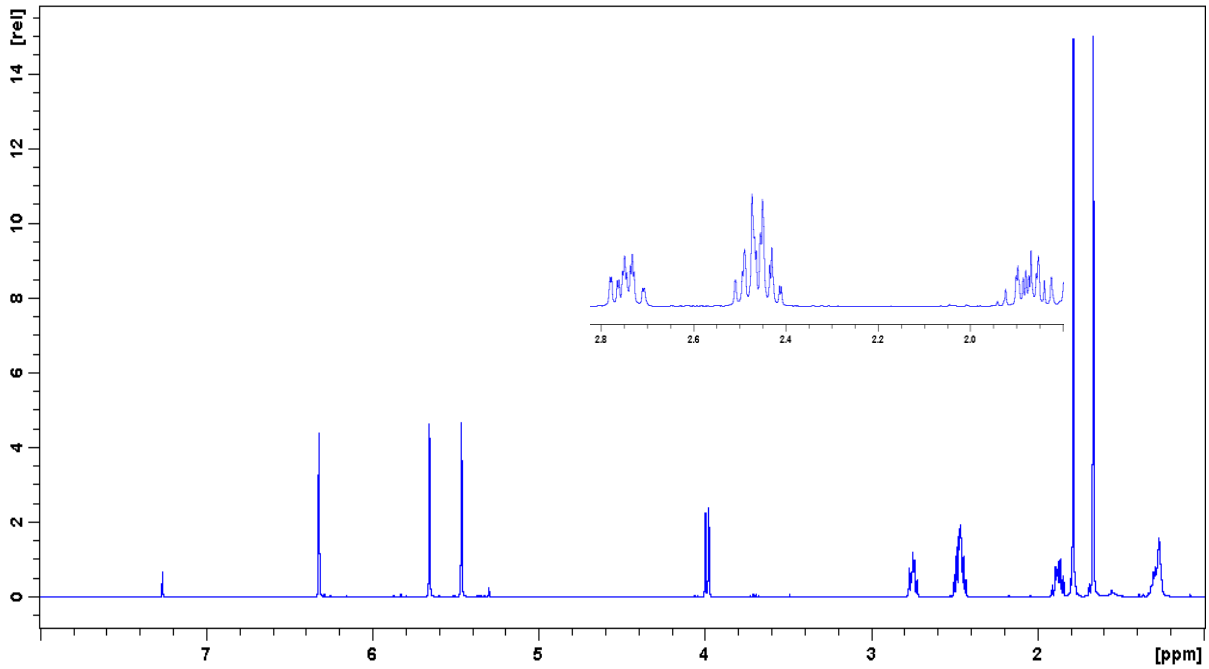


Figure 3.3: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

The ^{13}C NMR spectrum of compound **1.6** (**Figure 3.4**) showed ten carbon signals, characteristic of monoterpenes. Assessing the ^{13}C NMR spectrum along with the HSQC NMR spectrum (**Figure 3.5**) of compound **1.6**, we observe the presence of two double bonds with carbons at δ 106.2, δ 118.8, δ 135.3 and δ 141.8. Two methyl carbons are seen at δ 27.3 and δ 33.1, two methylene carbons at δ 31.8 and δ 33.9 and a methine carbon seen at δ 64.1. A quaternary carbon is also seen at δ 71.5.

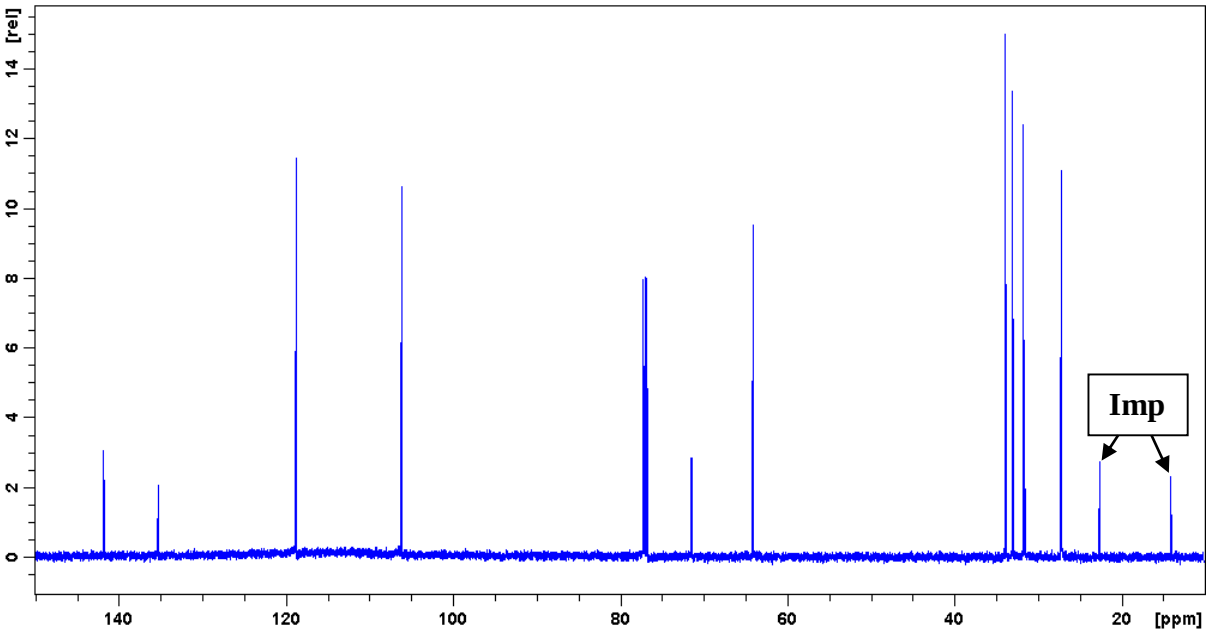


Figure 3.4: ^{13}C spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

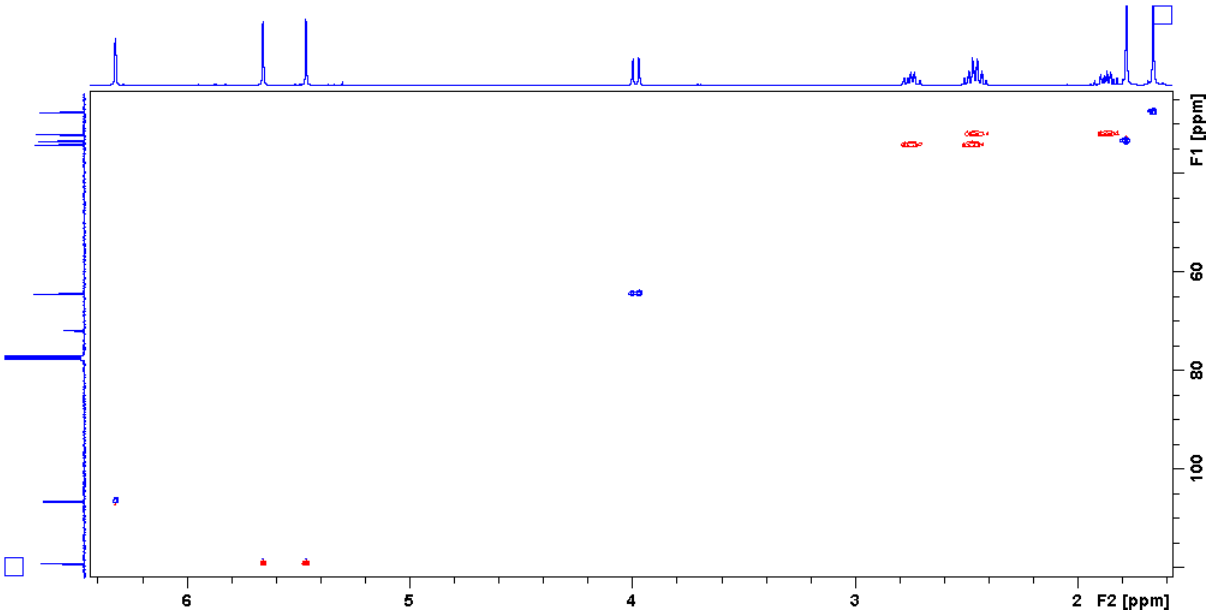


Figure 3.5: HSQC spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

From the COSY NMR spectrum (**Figure 3.6**), the methylene proton at δ 2.47 is coupled to the multiplet seen at δ 1.87 which is in turn coupled to a doublet seen at δ 3.98 (d, J = 11.5 Hz). The latter of which indicates the presence of a halomethine proton, thus giving the proposed substructure CHX-CH2-CH2 (substructure **B**, **Figure 3.7**).

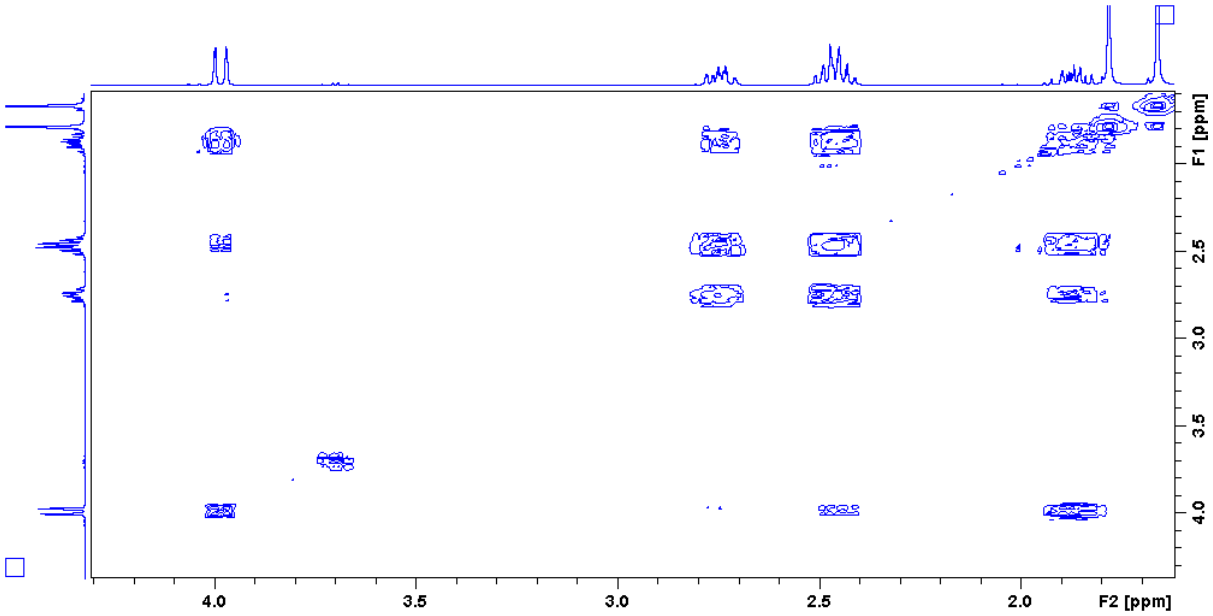


Figure 3.6: COSY NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.6**

The two methyl groups showed HMBC correlations to each other, as well as to the quaternary carbon at δ 71.5 and the halomethine carbon at δ 64.1, giving rise to substructure **A** (**Figure 3.7**). Substructure **A** was linked to substructure **B** by the HMBC correlations of the two methyl groups to the halomethine carbon described in substructure **B** above. Substructure **C** (**Figure 3.7**) was deduced from the fact that the two terminal methylene protons showed HMBC correlations to quaternary carbons at δ 135.3 and δ 141.8, as well as to the deshielded methine carbon at δ 106.2 and the methylene carbon at δ 33.9. The HMBC correlation between the terminal methylene protons to the methylene carbon at δ 33.9 connects substructure **B** and substructure **C**. The resultant planar structure obtained is presented in **Figure 3.7**.

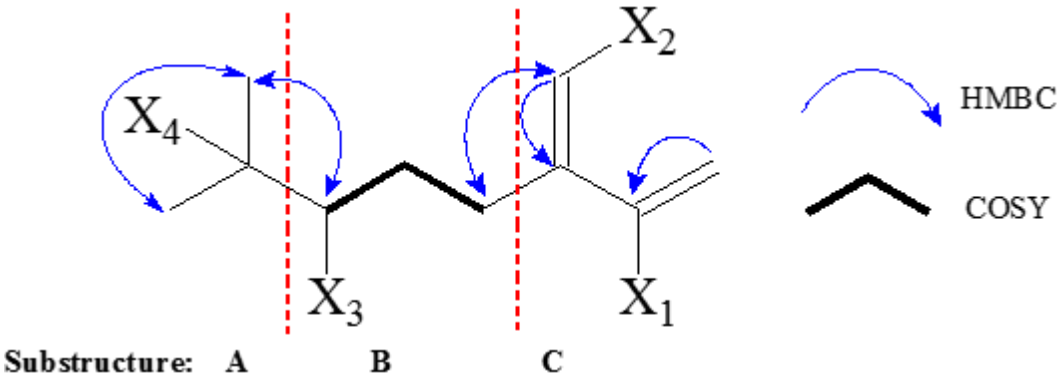


Figure 3.7: Planar structure of compound **1.6**, showing key COSY and HMBC correlations

Table 3.1: NMR spectroscopic data for compound **1.6** in $CDCl_3$

No.	**δ_C, mult**	**δ_H, mult, J (Hz)**	**COSY**	**HMBC**
1.	118.8, CH_2	5.46, (d, 1.7) 5.68, (d, 1.7)	--	C2, C3, C4, C9
2.	135.3, C	--	--	--
3.	141.8, C	--	--	--
4. a **b**	33.9, CH_2	2.47, (m) 2.75, (m)	$H5_a$, $H5_b$	C2, C3, C5, C6, C9
5.a **b**	31.8, CH_2	1.87, (m) 2.44, (m)	$H4_a$, $H4_b$, H6	C3, C4, C6, C7
6.	64.1, CH	3.98, (dd, 11.4, 1.3)	--	C7, C8, C10
7.	71.5, C	--	--	--
8.	27.3, CH_3	1.66, (s)	--	C6, C7, C10
9.	106.2, CH	6.35, (s)		C2, C3, C4
10.	33.1, CH_3	1.78, (s)	--	C6, C7, C8

The ^1H and ^{13}C NMR spectra of compound **1.6** were compared to literature values of compounds previously isolated from *Portieria hornemannii* (Adrianasolo *et al*, 2006), and were found to be consistent with the ^1H and ^{13}C NMR spectra of the known compound (**Table 3.2**). It should however, be noted that due to the absence of crystallographic data, the optical rotation of compound **1.6** could not be determined. Thus, the optical rotation of compound **1.6** could not be compared to optical rotation for the compound described in previous articles. It is therefore likely that compound 1.6 could be isomeric with the compound reported by Adrianasolo *et al* (2006).

Table 3.2: Comparison of ^{1}H and ^{13}C values of compound **1.6** to literature values (Adrianasolo *et al*, 2006)

	^{1}H NMR		**^{13}C NMR**	
Carbon no.	**Compound 1.6 δ_H (mult, J (Hz)) ($CDCl_3$)**	**Literature values δ_H (mult, J (Hz)) ($CDCl_3$)**	**Compound 1.6 δ_C ($CDCl_3$)**	**Literature values δ_C ($CDCl_3$)**
1.	5.46 (d, 1.68) 5.68 (d, 1.71)	5.49 (d, 1.7) 5.69 (d, 1.7)	118.8	118.9
2.	--	--	135.3	135.7
3.	--	--	141.8	142.3
4.	2.47 (m) 2.75 (m)	2.50 (m) 2.80 (m)	33.9	34.4
5.	1.87 (m) 2.44 (m)	1.89 (m) 2.49 (m)	31.8	32.4
6.	3.98 (dd, 11.4, 1.3)	4.03 (dd, 11.3, 1)	64.1	64.5
7.	--	--	71.5	72.4
8.	1.66	1.69	27.3	27.7
9.	6.35	6.35	106.2	106.8
10.	1.78	1.81	33.1	33.6

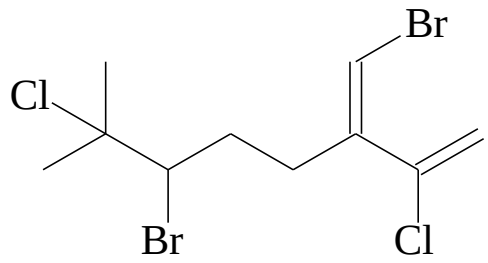


Figure 3.8: Structure of compound **1.6**

A comparison of the ^{1}H NMR spectra of the crude extract of sample D925 and compound **1.6**, it is clear that compound **1.6** was the major metabolite in this sample of *Portieria hornemannii*. Interestingly, compound **1.6** was not the major compound isolated from the Madagascar collection of *P. hornemannii* by Adrianasolo *et al*, (2006).

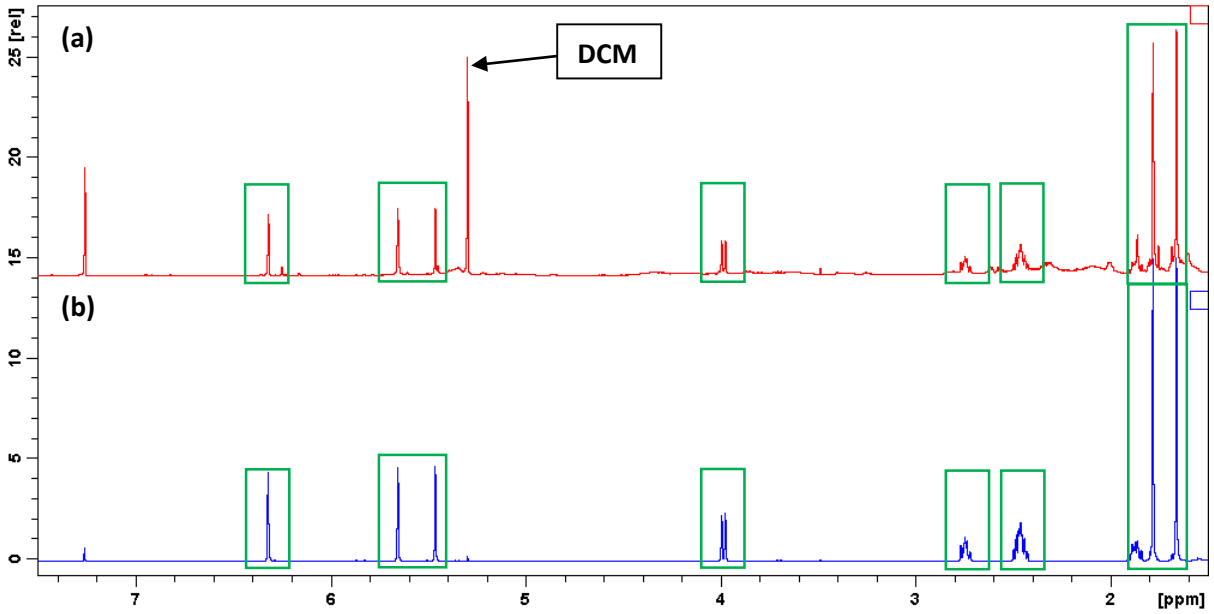


Figure 3.9: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of organic crude extract of D925 **(a)** and compound **1.6 (b)**

3.2.2.2 Compound 1.7

The main difference between the ^{1}H NMR spectra of compound **1.7** and **1.6** is the disappearance of the methine doublet at δ 4.0. The other signals include two methyl singlets at δ 1.76 and δ 1.86, one complex multiplet at δ 2.59 (4H) indicative of two methylene groups. Two doublets are observed at δ 5.45 (d, J = 1.5 Hz) and 5.65 (d, J = 1.5 Hz), that represent two terminal methylene protons are similar to the terminal methylene protons observed in compound **1.6**. A deshielded methine singlet is present at δ 6.25 which is similar to the singlet peak found in compound **1.6**, and is suggestive of a deshielded olefinic proton.

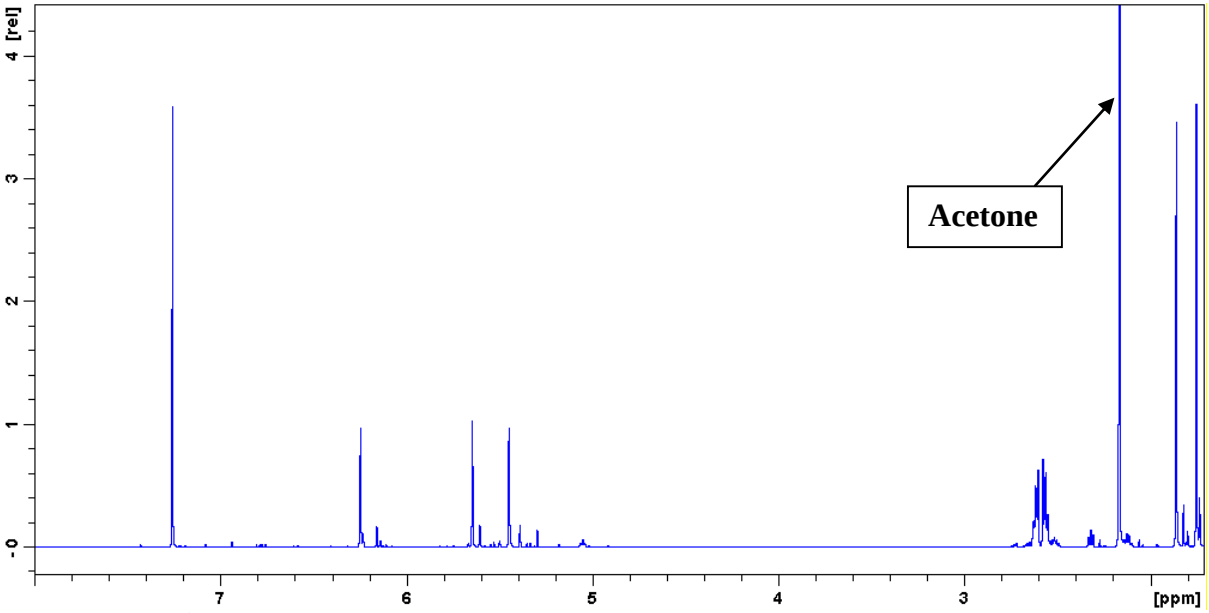


Figure 3.10: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.7**

The ^{13}C NMR spectrum of compound **1.7 (Figure 3.11)**, showed ten carbon signals including two methyl carbons at δ 20.5 and 25.4, two methylene carbons at 34.3 and 35.8 and six olefinic carbons (δ 105.7, 118.5, 120.0, 131.8, 135.7 and 141.9).

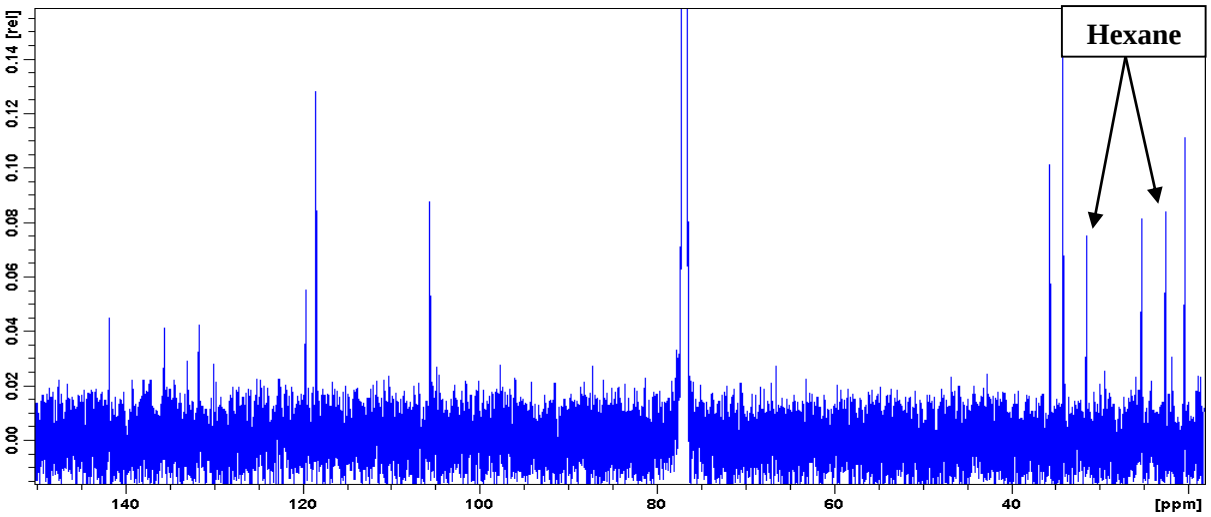


Figure 3.11: ^{13}C NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.7**

Further two dimensional NMR data could not be obtained for compound **1.7** due to its degradation. Comparison of the ^{1}H and ^{13}C NMR data of compound **1.7** with those found in literature (Adrianasolo *et al*, 2006), seen in **Table 3.3** positively identified it.

Table 3.3: Comparison of ^{1}H and ^{13}C values ($CDCl_3$) of compound **1.7** to literature values

	^{1}H NMR		**^{13}C NMR**	
No.	**Compound 1.7 δ_H (mult, J (Hz)) ($CDCl_3$)**	**Literature values δ_H (mult, J (Hz)) ($CDCl_3$)**	**Compound 1.7 δ_C ($CDCl_3$)**	**Literature values δ_C ($CDCl_3$)**
1.	5.45 (d, 1.5) 5.65 (d, 1.5)	5.48 (d, 1.6) 5.68 (d, 1.6)	118.8	118.9
2.	--	--	135.3	136.1
3.	--	--	141.8	142.3
4.	2.59 (m) 2.59 (m)	2.62 (m) 2.62 (m)	35.8	36.2
5.	2.59 (m) 2.59 (m)	2.62 (m) 2.62 (m)	34.3	34.7
6.	--	--	120.0	120.1
7.	--	--	131.8	132.2
8.	1.76 (s)	1.80 (s)	20.5	20.9
9.	6.25 (s)	6.25 (s)	106.2	106.1
10.	1.86 (s)	1.95 (s)	25.4	25.8

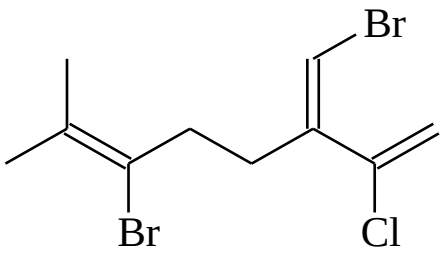


Figure 3.12: Structure of compound **1.7** as found in literature (Adrianasolo *et al*, 2006)

3.2.2.3 Compound 3.1

The ^{1}H NMR spectrum of compound **3.1** (**Figure 3.13**) lacked the olefinic methine signals observed with compounds **1.6** and **1.7**. These signals were replaced by a deshielded methylene singlet at δ 4.78 and a methine doublet of doublets at δ 4.01 (dd, *J* = 11.2, 1.6 Hz).

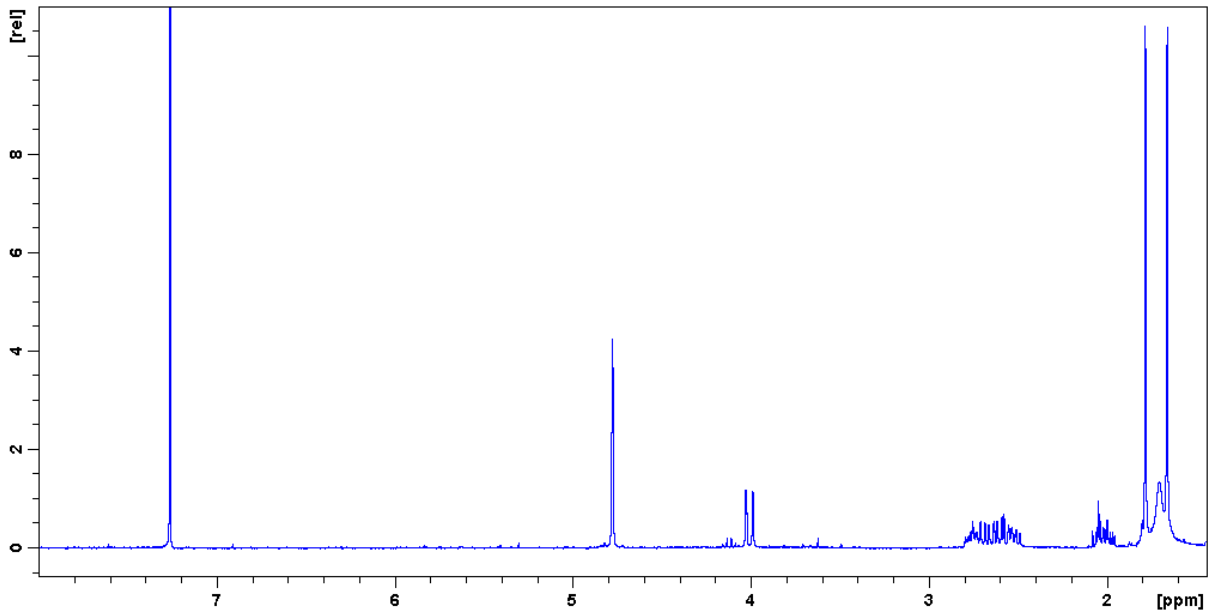


Figure 3.13: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

The ^{13}C NMR spectrum (**Figure 3.14**) of compound **3.1**, displayed ten carbon signals. Assessing the ^{13}C NMR spectrum along with the HSQC NMR spectrum (**Figure 3.15**) of compound **3.1**, indicated the presence of a tetrasubstituted double bond with carbons at δ 127.6 and δ 151.0. A carbon signal at δ 171.1 is suggestive of a carbonyl group. Two methyl carbons are seen at δ 27.2 and δ 32.9, while carbons at δ 22.6, δ 31.6 and δ 71.3 represent methylene groups. A deshielded methine carbon at δ 64.4 is suggestive of a halomethine group while the carbon signal at δ 71.5 represents a quaternary carbon bearing an electron withdrawing substituent.

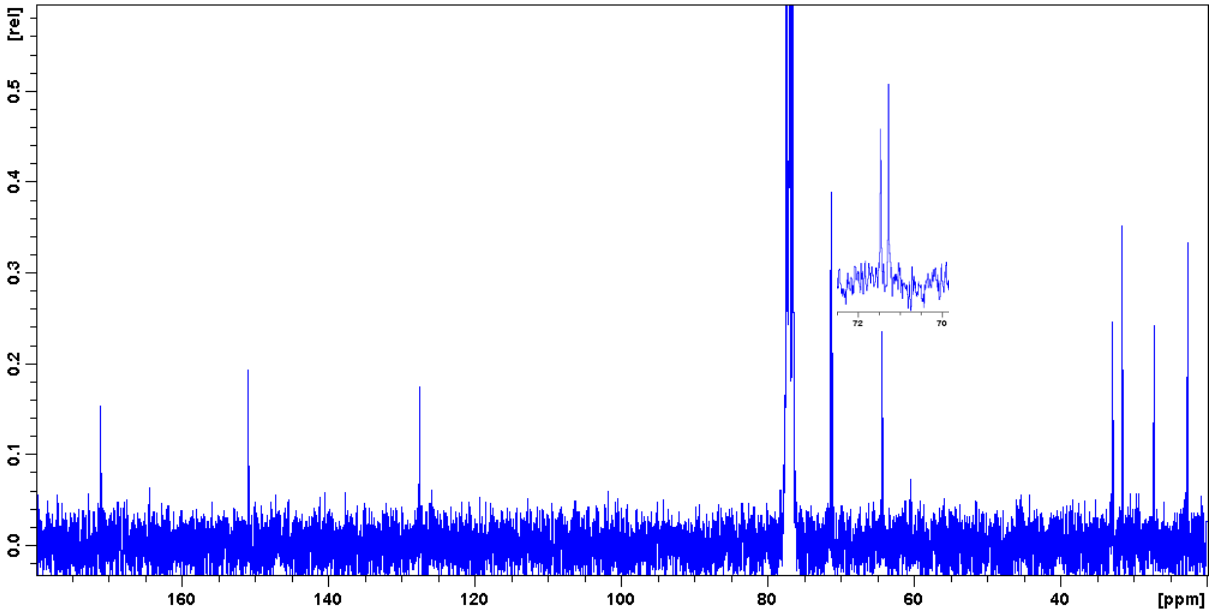


Figure 3.14: ^{13}C NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

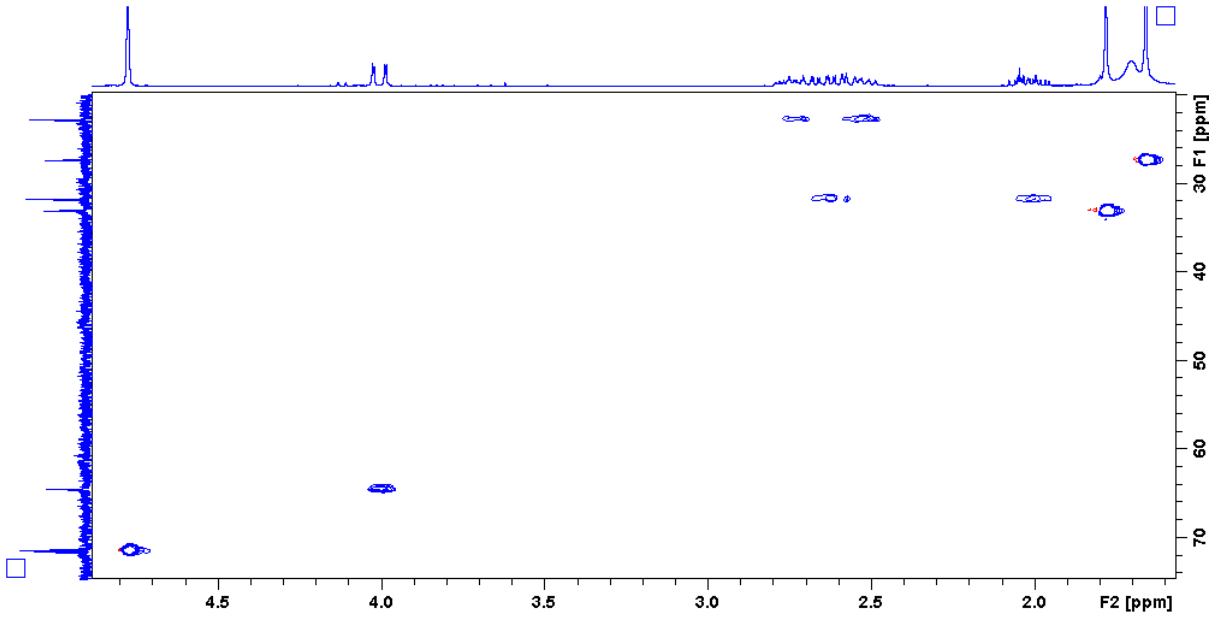


Figure 3.15: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

A CHX-CH_2-CH_2 spin-system (substructure **B**, **Figure 3.19**) was deduced from COSY correlations (**Figure 3.16**) from the methylene protons at δ 2.02 to the multiplets at δ 2.53, δ 2.63 and δ 2.75, as well as the doublet of doublets at δ 4.01

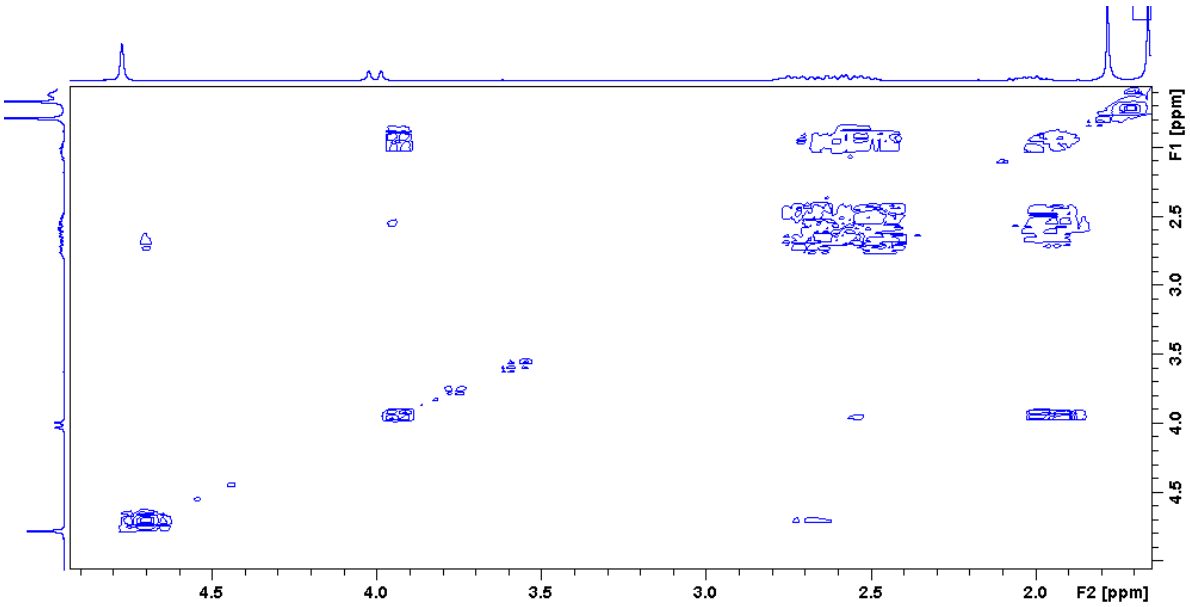


Figure 3.16: COSY NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

The two methyl groups (δ 1.66 and δ 1.78) showed HMBC correlations to each other, as well as to the quaternary carbon at 71.5 and the halomethine carbon at δ 64.4, giving rise to substructure **A** (**Figure 3.19**). Substructure **A** was linked to substructure **B** by the HMBC correlations of the two methyl groups to the halomethine carbon in substructure **B** above. The deshielded methylene group showed HMBC correlations to carbons at δ 127.6, δ 151.0 and δ 171.1. The deshielded methylene group at δ 71.3 is either attached to an oxygen or a halogen functional group. The presence of a γ-lactone was confirmed by the IR spectrum (**Figure 3.18**) of compound **3.1**. Substructure **C** (**Figure 3.19**) was thus obtained. An HMBC correlation between the methylene group (δ 2.53 and δ 2.75) and the carbon at δ 151.0, linked substructure **B** to substructure **C**.

At this point two possible structures were considered for compound **3.1** (below).

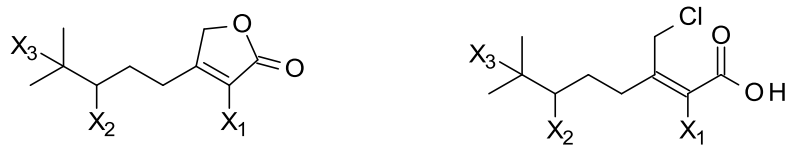


Figure 3.17: Possible planar structures of compound **3.1**

This however, was settled after observing the IR spectrum (**Figure 3.18**) of compound **3.1**, which confirmed the presence of **C-O** at 1446.58 cm^{-1} as well as **C=O** at 1750.43 cm^{-1}.

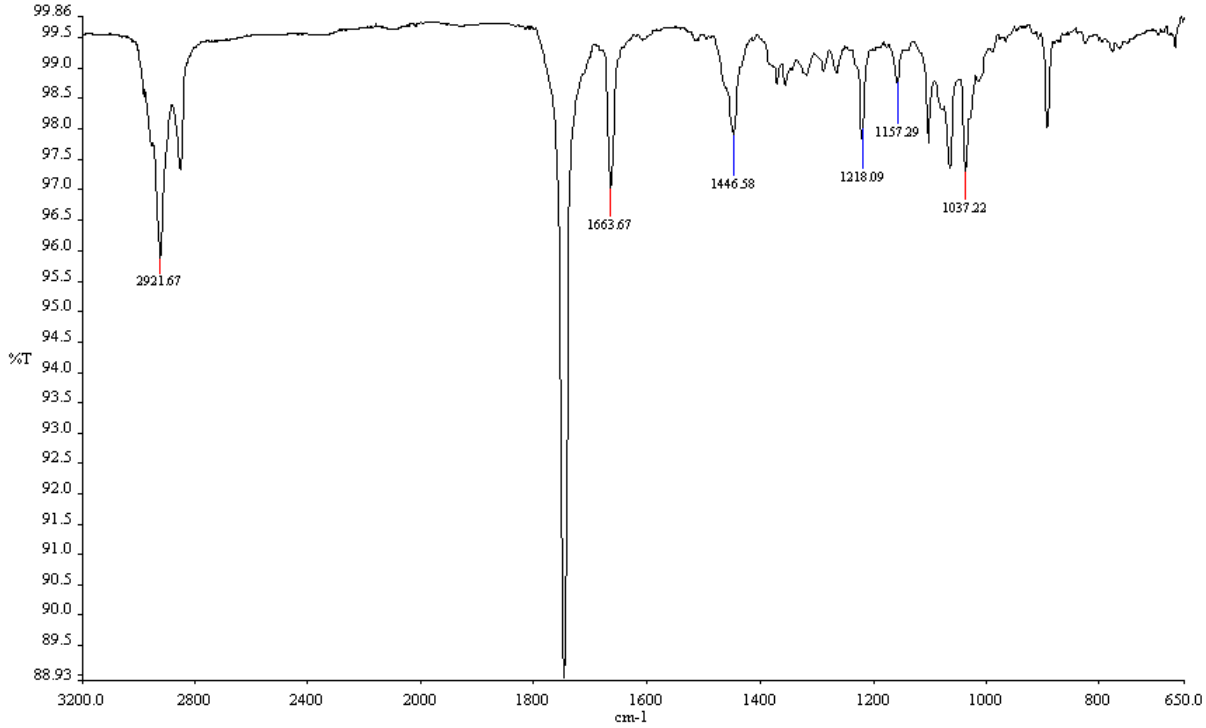


Figure 3.18: IR spectrum of compound **3.1**

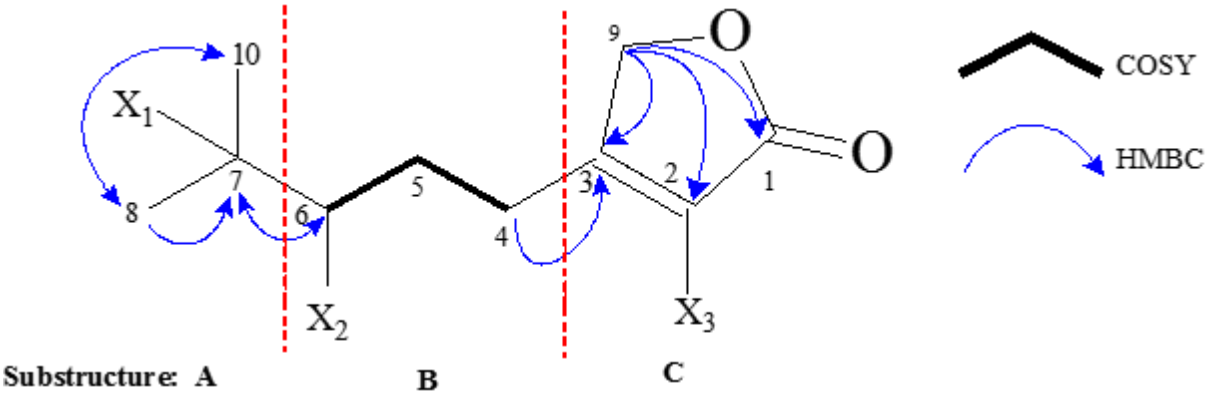


Figure 3.19: Planar structure of compound **3.1**, showing key COSY and HMBC correlations

Table 3.4: NMR spectral data for compound **3.1** in $CDCl_3$

No.	**δ_C, mult**	**δ_H, mult, J (Hz)**	**COSY**	**HMBC**
1.	171.1, C	--	--	--
2.	127.6, C	--	--	--
3.	151.0, C	--	--	--
4.	22.6, CH_2	2.53, m 2.75, m	H5	C1, C2, C3, C5, C6
5.	31.6, CH_2	2.02, m 2.63, m	H4	C4, C6, C8
6.	64.4, CH	4.01, dd, 11.5, 1.6	H5	C4, C5, C7, C10
7.	71.5, C	--	--	--
8.	27.2, CH_3	1.66, s	--	C6, C7, C10
9.	71.3, CH_2	4.77, s		C1, C2, C3
10.	32.9, CH_3	1.78, s	--	C6, C8

The HRESIMS spectrum of compound **3.1** gave a pseudomolecular ion peak of *m/z* 338. 9339 corresponding to a molecular formula of $C_{10}H_{13}Cl_2BrO_2Na$ which is consistent with a butenolide structure for compound **3.1**. This resulted in the structure of compound **3.1** seen in **Figure 3.20**

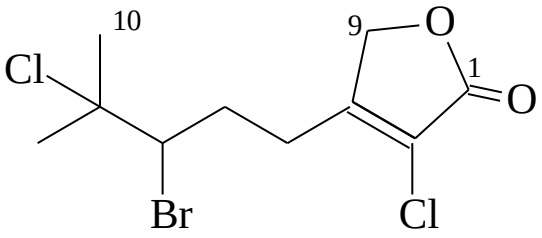


Figure 3.20: Structure of compound **3.1**

3.2.2.4 Compound 3.2

The ^{1}H NMR spectrum of compound **3.2** is very similar to that of compound **3.1** varying by a very slight difference in chemical shifts, as can be seen in **Figure 3.21**.

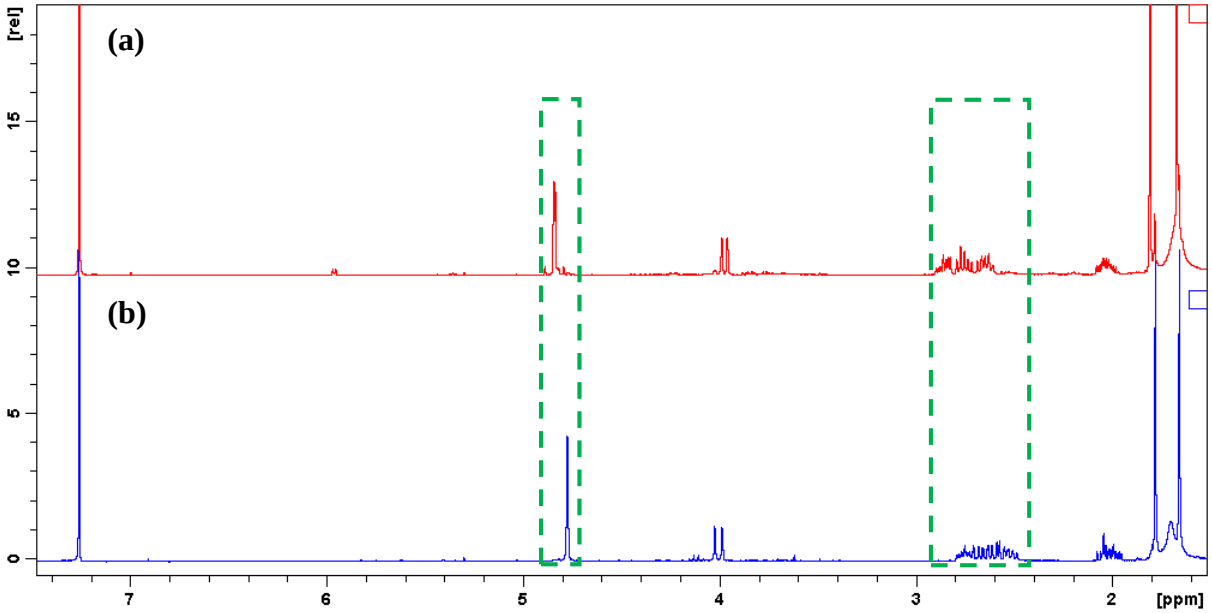


Figure 3.21: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2** (a) and ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1** (b)

The ^{1}H NMR spectrum of compound **3.2** displays two singlet peaks at δ 1.67 and δ 1.81 which represent protons from two methyl groups. Four complex multiplets are seen at δ 2.03 (1H), δ 2.65 (1H), δ 2.75 (1H) and δ 2.86 (1H) which are indicative of protons belonging to methylene groups. A doublet of doublets at δ 3.97 (dd, J = 11.2, 1.4 Hz,) suggestive of a halomethine group. A doublet is seen at δ 4.84 (d, J = 3.1), which is similar to the proton signal seen in the ^{1}H NMR spectrum of compound **3.1** at δ 4.77, indicating a deshielded methylene proton.

Looking at the carbon spectrum of compound **3.2** in **Figure 3.22**, we observe the presence of nine carbon signals. This is unusual as monoterpenes are ten carbon compounds. Along with the Edited HSQC NMR spectrum (**Figure 3.23**), the ^{13}C NMR spectrum of compound **3.2** revealed the presence of one double bond with carbons at δ 120.7 and δ 157.8. Two methyl carbons are seen at δ 26.6 and δ 33.3, while carbons at δ 25.9, δ 31.6 and δ 71.1 represent methylene groups. A deshielded methine carbon is seen at δ 63.3 suggesting a halomethine group. The carbon signal at δ 71.5 represents a quaternary carbon.

Since the ^{1}H NMR spectrum of compound **3.2** is similar to that of compound **3.1**, the ^{13}C spectra of these two compounds were compared, as seen in **Table 3.5**.

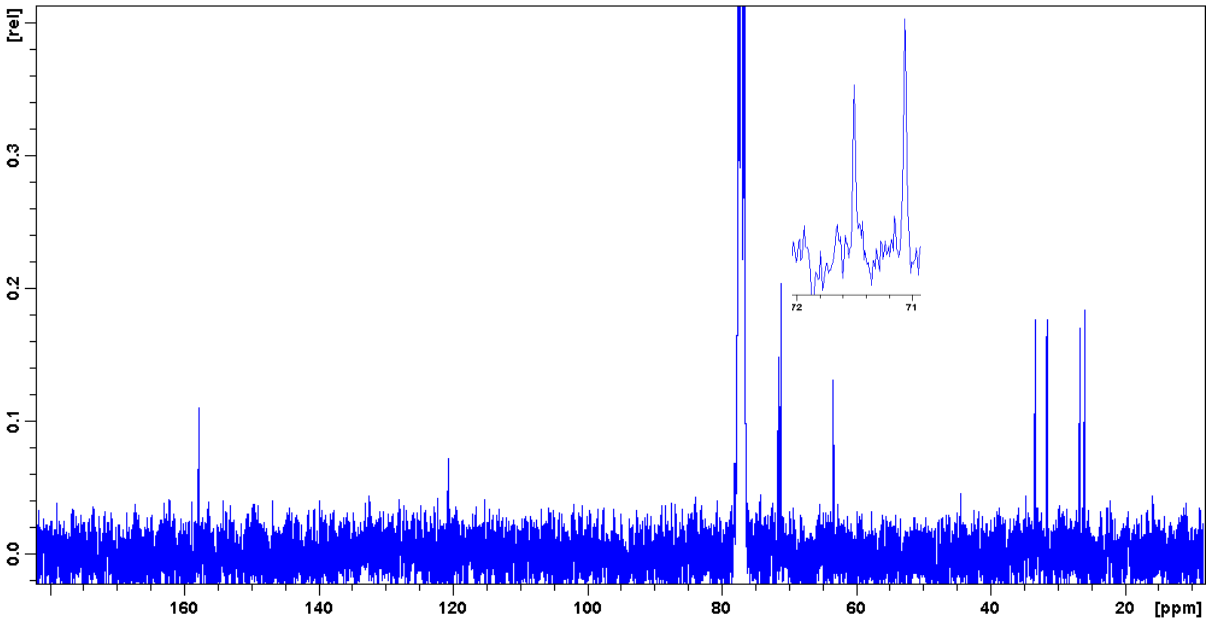


Figure 3.22: ^{13}C NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

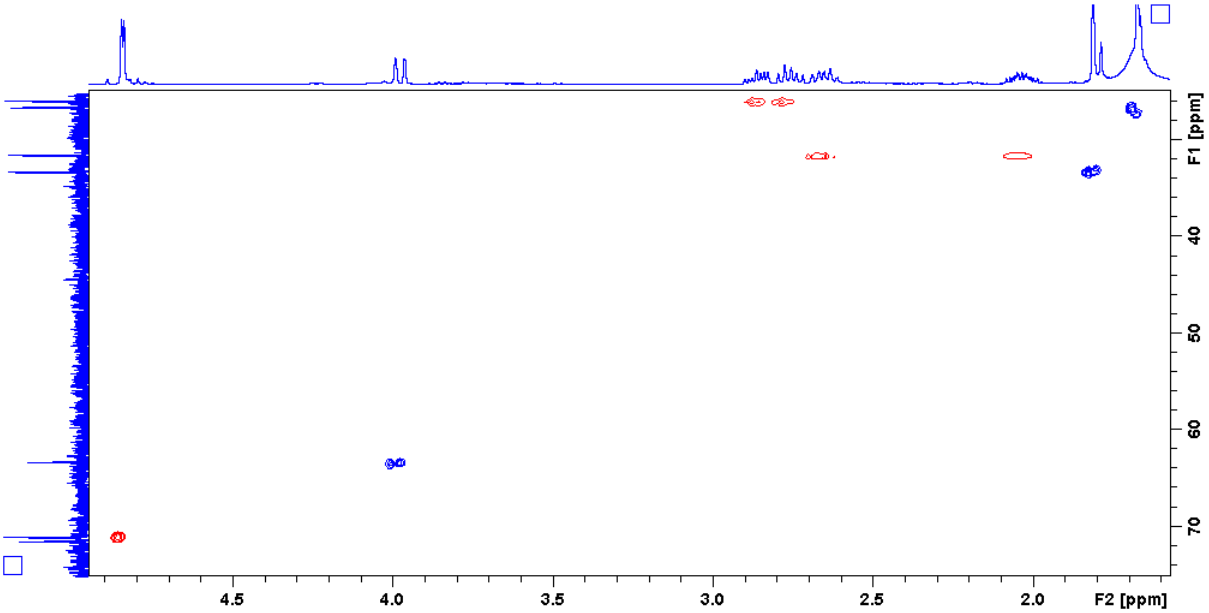


Figure 3.23: Edited HSQC NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

Table 3.5: ^{13}C spectral data of compound **3.1** and compound **3.2** in $CDCl_3$

	Compound 3.1	**Compound 3.2**
No.	**δ ^{13}C**	**δ ^{13}C**
1.	171.1	168.4
2.	127.6	120.7
3.	151.0	157.8
4.	22.6	25.9
5.	31.6	31.5
6.	64.4	63.3
7.	71.5	71.5
8.	27.2	26.6
9.	71.3	71.1
10.	32.9	33.3

From **Table 3.7** we see that the ^{13}C values of compound **3.2** are very similar to those of compound **3.1**. A carbon signal similar to the carbon signal representing the carbonyl group seen in compound **3.1** (δ 171.1) is not seen in the ^{13}C NMR spectrum of compound **3.2**. From the HMBC NMR spectrum (**Figure 3.25**) of compound **3.2**, however, we do note the presence of a correlation (circled in red) of the proton at δ 4.84 to a carbon at δ 168.4. This suggests compound **3.2** is a possible isomer of compound **3.1**.

Examining the COSY NMR spectrum (**Figure 3.24**) of compound **3.2**, we observe that the methylene proton at δ 2.03 is coupled to the multiplets seen at δ 2.65, δ 2.75, and δ 2.86, as well as to the doublet of doublets seen at δ 3.97 (dd, J = 11.2, 1.4 Hz,). This gives the proposed substructure **B** seen in **Figure 3.26**.

The two methyl groups (δ 1.67 and δ 1.81) showed HMBC correlations to each other, as well as to the quaternary carbon at δ 71.5 and the halomethine carbon at δ 64.4, giving rise to substructure **A** (**Figure 3.26**). Substructure **A** was linked to substructure **B** by the HMBC correlations of the two methyl groups to the halomethine carbon described in substructure **B** above. The deshielded methylene group showed HMBC correlations to carbons at δ 120.7, δ 157.8 and δ 168.4. The deshielded methylene group at δ 71.3 suggested the presence of either an ester, or a halomethylene functional group. The presence of an ester group was confirmed by the IR spectrum (**Figure 3.27**) of compound **3.1**. Substructure **C** (**Figure 3.26**) was thus

obtained. An HMBC correlation between the methylene group (δ 2.75 and δ 2.86) and the carbon at δ 157.8, linked substructure **B** to substructure **C**.

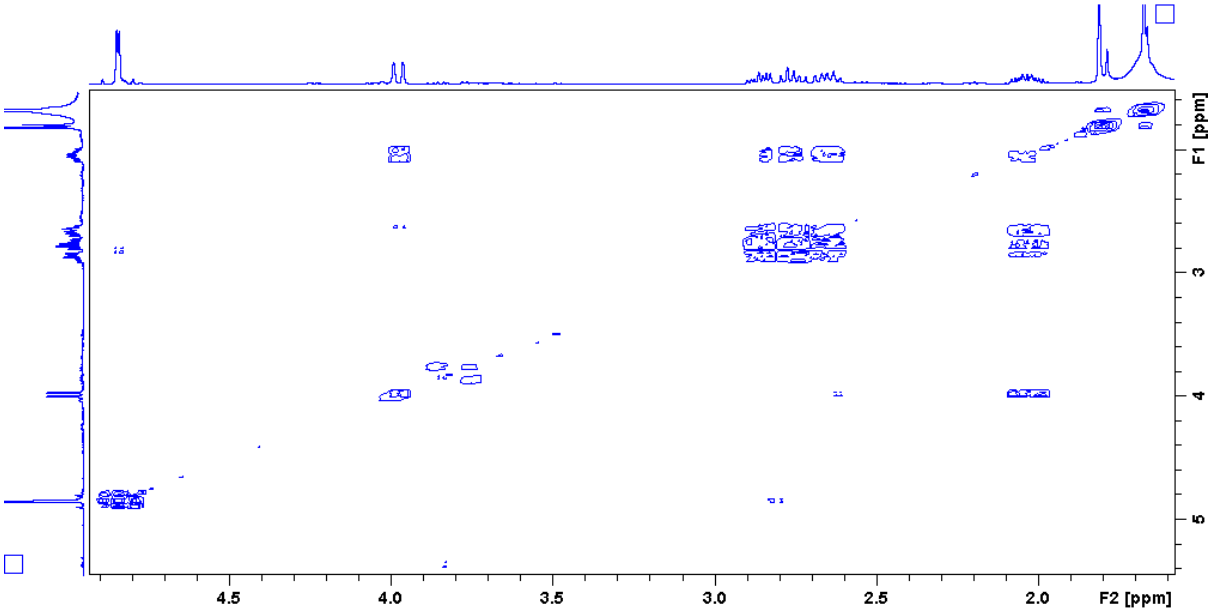


Figure 3.24: COSY NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

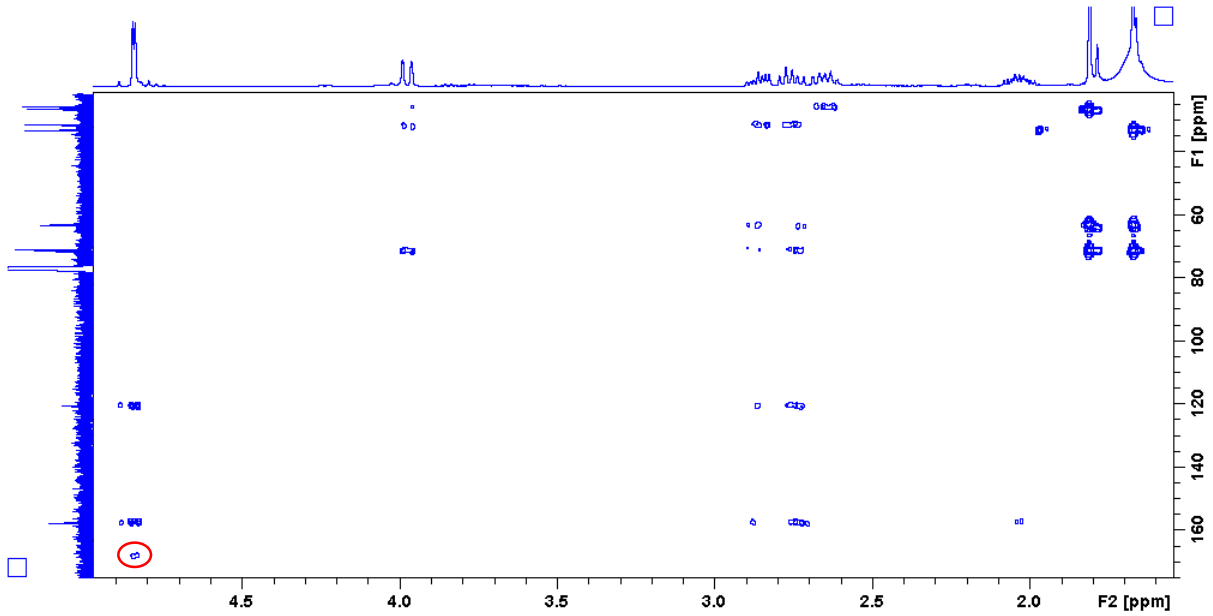


Figure 3.25: HMBC NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

As with compound **3.1**, the IR spectrum of compound **3.2** (**Figure 3.27**) indicated the presence of a γ-lactone due to signals found at 1443.00cm^{-1} and 1753.34cm^{-1} as seen with compound **3.1**, and thus, the resulting proposed planar structure is observed:

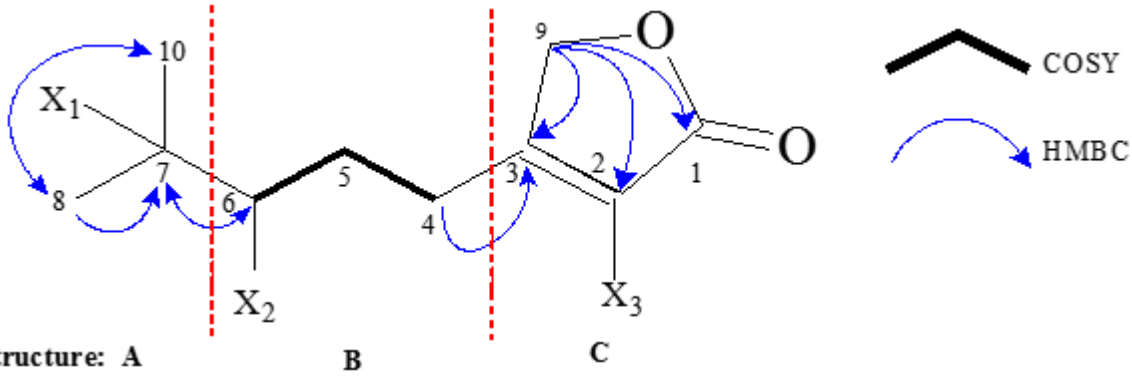


Figure 3.26: Planar structure of compound **3.2**, showing key COSY and HMBC correlations

Table 3.6: NMR spectral data for compound **3.2** in $CDCl_3$

No.	**δ ^{13}C, mult**	**δ ^{1}H, (mult, J (Hz))**	**COSY**	**HMBC**
1.	168.4, C	--	--	--
2.	120.7, C	--	--	--
3.	157.8, C	--	--	--
4.	25.9, CH_2	2.75,(m) 2.86, (m)	H5a, H5b	C2, C3, C5, C6, C9
5.	31.5, CH_2	2.03, (m) 2.64, (m)	H4a, H4b, H6	C3, C4, C6,
6.	63.3, CH	3.97, (dd, 11.2, 1.4)	H5a, H5b	C4, C5, C7
7.	71.5, C	--	--	--
8.	26.6, CH_3	1.67, (s)	--	C6, C7, C10
9.	71.1, CH_2	4.84, (s)		C1, C2, C3
10.	33.3, CH_3	1.81, (s)	--	C6, C7, C8

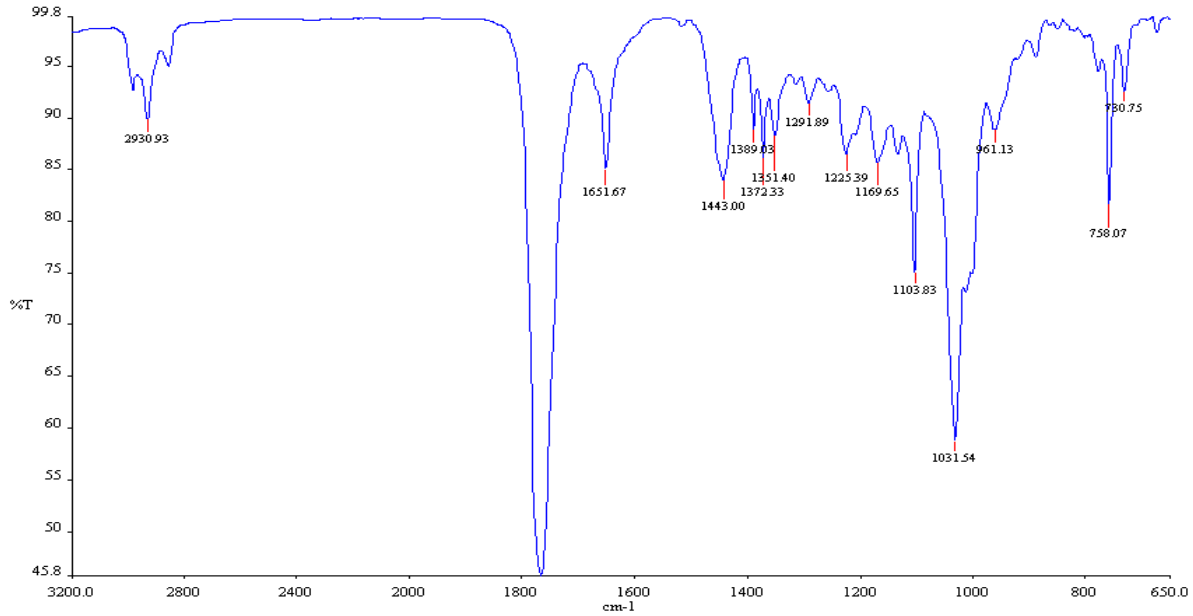


Figure 3.27: IR Spectrum of compound **3.2**

The HRESIMS spectrum of compound **3.2** gave a pseudomolecular ion peak of *m/z* 316.9511 corresponding to a molecular formula of $C_{10}H_{13}Cl_2BrO_2H$ which is consistent with a butenolide structure as with compound **3.1**. From this, we confirm that compound **3.1** and compound **3.2** are isomeric. However, to confirm what type of isomerism these compounds display, we would require optical rotation data for both of the compounds. This was unfortunately not obtained due to very minute quantities isolated of each compound, which was further decreased after carrying out the various NMR spectroscopic experiments. Compounds **3.1** and **3.2** both did not present in the form of crystals, and thus crystallographic data could not be obtained, which would assist in the determination of the type of isomerism displayed. Upon observing the ^{13}C NMR spectra of the two compounds however, we note the only significant differences being between the carbons that form the ring, with the rest of the molecule being almost identical.

Without any additional information on the compound, we thus propose the structure of compound **3.2** seen in **Figure 3.28**.

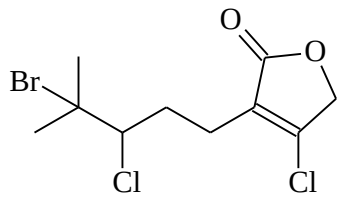


Figure 3.28: Structure of compound **3.2**

3.3 Experimental

3.3.1 General Experiments

Solvents

All solvents used to extract and separate the organic crude extract of sample D925 were High-Performance-Liquid-Chromatography (HPLC) grade from LiChrosolv® from Merk®, Darmstd, Germany.

Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR spectrum of the organic crude extract of sample D925, as well all NMR spectra of compound **3.2** were acquired using a Bruker® Avance III™ HD 400 MHz spectrometer. All NMR spectra of compounds **1.7** and **1.6** were acquired on a Bruker® 600 MHz Avance spectrometer. All NMR spectra of compound **3.1** were acquired using a Bruker® UltraShield™ 300 MHz spectrometer. The solvent used to obtain the spectra was deuterated chloroform ($CDCl_3$). All ^{1}H and ^{13}C spectra were in reference to the $CDCl_3$ and the reference peaks for this solvent were at δ_H 7.26 ppm and δ_C 77.00 ppm respectively.

3.3.2 Plant Material

Samples were collected on 11/02/17 from De Hoop (Koppie Alleen), Western Cape, South Africa. Identification of samples was done by Prof John J. Bolton of the department of Botany, University of Cape Town, South Africa. Samples were kept in a freezer at ~ -18^{o}C. Voucher specimens were made for the samples collected and are kept with the Pharmaceutical Chemistry division of Rhodes University, South Africa.

3.3.3 Extraction and Isolation of metabolites

Sample D925A and D925B were combined (based on results discussed in Chapter 2) to give total wet mass of 115.7 g (after extraction, dry mass 27.8 g).

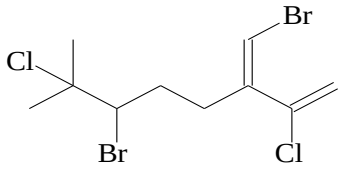
The algal sample was rinsed with water and then left to air dry for ~ 10 minutes and thereafter, it was placed in a 1 litre beaker, to this 400 ml MeOH was added and this was allowed to stand for one hour. The MeOH was then decanted into a 2 litre conical flask. 400 ml 2:1 (DCM/MeOH) was added to the sample and heated to 37 ^{o}C for 20 min, this was then decanted into the conical flask containing the MeOH. This last step was repeated three times after which the solution was placed in a separating funnel, distilled water was added to this in sufficient quantity to cause phase separation. The aqueous layer was discarded and the organic layer was collected and dried off under reduced pressure using a Rotavap®. 1.0762 g of crude was extracted (3.7 % yield).

The crude extract was separated via open-column silica gel chromatography, using Hex/EtOAc as solvents, to give rise to eight fractions of increasing polarity. Fraction A (266.7 mg) was further fractionated via column chromatography, using Hexane as the solvent, and resulted in six fractions, of which the fourth fraction contained pure compound **1.6** (41.9 mg). The second of these fractions (10.8 mg) was purified via HPLC, using Hexane as the solvent, to result in compound **1.7** (3.9 mg). Fraction B (229.0 mg) was fractionated, using a 9:1 Hexane/EtOAc solvent system, via column chromatography to achieve six fractions. Two of these fractions were combined and purified via HPLC, using a 9:1 Hexane/EtOAc solvent system, resulting in three fractions. The second and third of these fractions contained compounds **3.1** (6.2 mg) and **3.2** (2.6 mg) respectively.

3.3.4 Compounds Isolated

*Naming of compounds was done with ACD/ChemSketch® (2012), therefore, the ^{13}C numbering does not follow the numbering from this chapter.

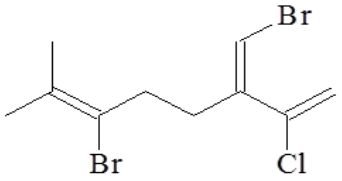
Compound 1.6



1.6

*(3Z)-6-bromo-3-(bromomethylidene)-2,7-dichloro-7-methyloct-1-ene (**1.6**): colourless oil; NMR data available in **Table 3.1**. (As previously reported by Adrianasolo *et al*., 2006)

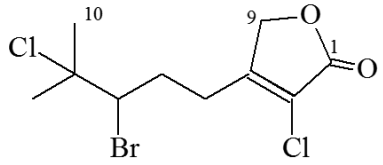
Compound 1.7



1.7

*(3Z)-6-bromo-3-(bromomethylidene)-2-chloro-7-methylocta-1,6-diene (**1.7**): colourless oil; NMR data available in **Table 3.3**. (As previously reported by Adrianasolo *et al*., 2006)

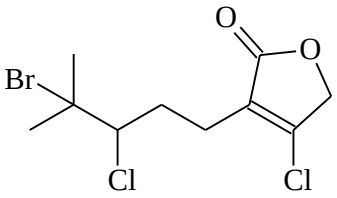
Compound 3.1



3.1

*4-(3-bromo-4-chloro-4-methylpentyl)-3-chlorofuran-2(5H)-one: Off-white solid; NMR data available in **Table 3.4**. HRESIMS compound **3.1**, m/z 338.9339 **[M+Na]**$^+$ (calculated 338.9344) $C_{10}H_{13}{}^{35}Cl^{37}Cl^{79}BrO_2Na$/ 336.9373/ 342.9294/ 340.9315.

Compound 3.2



3.2

*4-(3-bromo-4-chloro-4-methylpentyl)-3-chlorofuran-2(5H)-one: Colourless oil; NMR data available in **Table 3.6**. HRESIMS compound **3.2**, m/z 316.9511 **[M+H]**$^+$ (calculated 316.9524) $C_{10}H_{14}{}^{35}Cl^{37}Cl^{79}BrO_2$/ 314.9554/ 320.9474/ 318.9495.

3.4 References

Andrianasolo, E. H.; France, D.; Cornell-Kennon, S.; Gerwick, W. H. DNA Methyl Transferase Inhibiting Halogenated Monoterpenes From The Madagascar Red Marine Alga *Portieria hornemannii*. *Journal of Natural Products* **2006**, *69*, 576 - 579.

Aranibar, N.; Singh, B. K.; Stockton, G. W.; Ott, K. Automated Mode-Of-Action Detection By Metabolic Profiling. *Biochemical and Biophysical Research Communications* **2001**, *286*, 150 – 155.

Carter-Franklin, J. N.; Butler, A. Vanadium Bromoperoxidase-Catalyzed Biosynthesis Of Halogenated Marine Natural Products. *Journal of the American Chemical Society* **2004**, *126*, 15060 – 15066.

Fuller, R. W.; Cardellina, J. H.; Kato, Y.; Brinen, L. S.; Clardy, J.; Snader, K. M.; Boyd, M. R. A Pentahalogenated Monoterpene From The Red Alga *Portieria hornemannii* Produces A Novel Cytotoxicity Profile Against A Diverse Panel Of Human Tumor Cell Lines. *Journal of Medicinal Chemistry* **1992**, *35*, 3007 – 3011.

Kim, H. K.; Choi, Y. H.; Erkelens, C.; Lefeber, A. W. M.; Verpoorte, R. Metabolic Fingerprinting Of *Ephedra* Species Using 1H-NMR Spectroscopy And Principal Component Analysis. *Chemical and Pharmaceutical Bulletin* **2005**, *53*, 105 – 109.

Leliaert, F.; Verbruggen, H.; Vanormelingen, P.; Steen, F.; Lopez-Bautista, J. M.; Zuccarello, G. C.; De Clerk, O. DNA-Based Species Delimitation In Algae. *European Journal of Phycology* **2014**, *49*, 179 – 196.

Payo, D. A.; Leliaert, F.; Verbruggen, H.; D'hondt, S.; Calumpong, H. P.; De Clerk, O. Extensive Cryptic Species Diversity And Fine-Scale Endemism In The Marine Red Alga Portieria In The Philippines. *Proceedings of the Royal Society B: Biological Sciences* **2013**, 280: 20122660, 1 – 2.

Silva, P. C.; Menez, E. G.; Moe, R. L. *Catalog Of Benthic Marine Algae Of The Philippines*; Smithsonian Institution Press: Washington, D.C, USA, 1987, Smithsonian Contributions to the Marine Sciences Number 27, 128 – 129 (Accessed from the Internet at : http://www.seaweedafrica.org/pdf/562E29B402eeb341B1okN1F55350/6675.pdf)

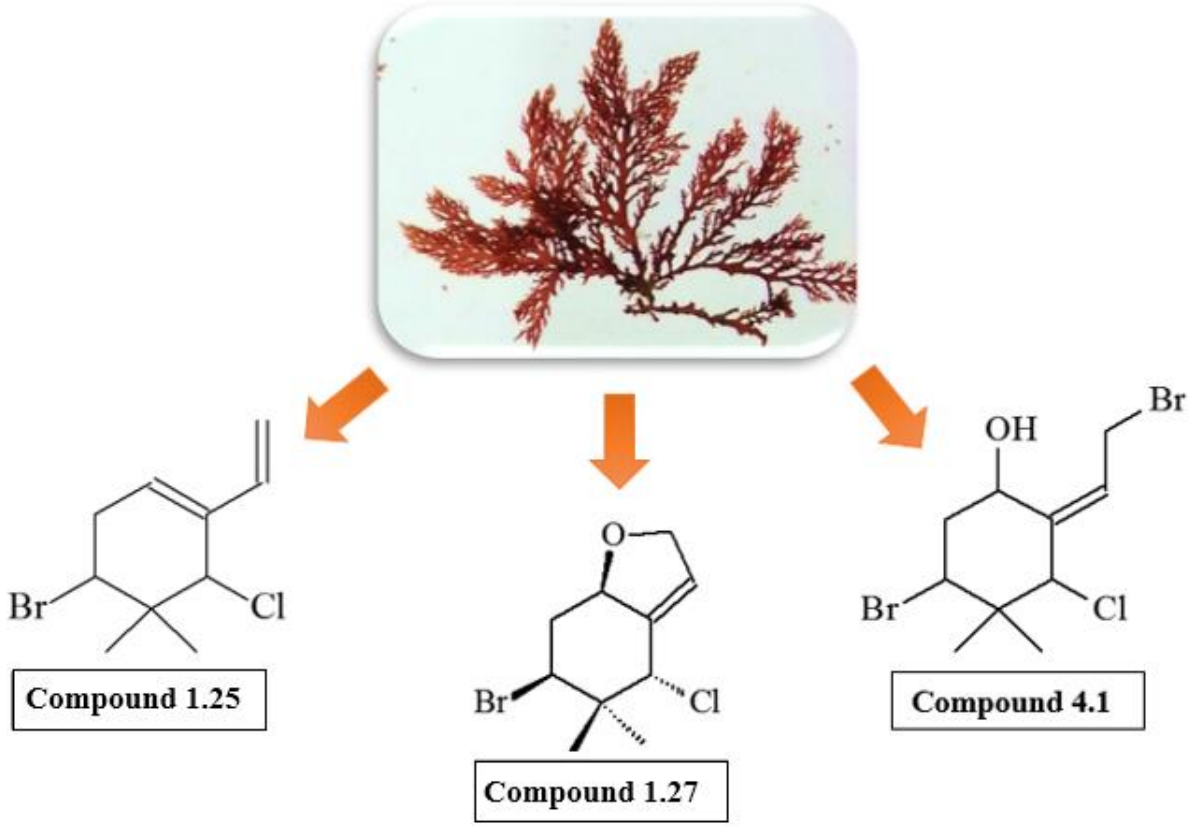
Stallard, M. O.; Faulkner, D. J. Chemical Constituents of the Digestive Gland Of The Sea Hare *Aplysia californica-I.* Importance Of Diet. *Comparative Biochemistry and Physiology Part - B* **1974**, *49*, 25-35.

Wise, M. L.; Rorrer, G. L.; Polzin, J. J.; Croteau, R. Biosynthesis Of Marine Natural Products: Isolation And Characterization Of A Myrcene Synthase From Cultured Tissues Of The Marine Red Alga *Ochtodes secundiramea*. *Archives of Biochemistry and Biophysics* **2002**, *400*, 125 – 132.

Chapter 4

Isolation of halogenated monoterpenes from *Portieria tripinnata*

Graphical Abstract



A study of *Portieria tripinnata* yielded three known halogenated monoterpenes. The structures of these metabolites were determined using standard spectroscopic techniques.

Chapter 4

Isolation of halogenated monoterpenes from *Portieria tripinnata*

4.1 Introduction

As previously discussed in Chapter 3, the taxonomy of the genus *Portieria* and its species, is complex. *Portieria tripinnata* is synonymous with *Rhodymenia tripinnata* (Hering, 1846), *Sphaerococcus tripinnatus* (Hering) (Kutzing 1849), *Desmia tripinnata* (Hering) (Agardh 1852), *Chondrococcus hornemannii* var. *tripinnatus* (Hering) (Tyson, 1910) and *Chondrococcus tripinnatus* (Hering) (Delf and Michell, 1921) (Guiry *et al*, 2014).

Portieria tripinnata is described as being "erect, up to 5cm high, forming dense little tufts, composed of complanate fronds". Its habitat being described as "epilithic, rarely epiphytic, predominantly in high and mid intertidal regions, becoming exposed at low tide or growing in very shallow rock pools and gulleys." (De Clerk *et al*., 2005).

Seeing as the *Portieria* genus is not very well understood, it is possible that *Portieria tripinnata* has previously been studied as a different species of *Portieria*. In this chapter, we look at the isolation of secondary metabolites and identifying possible chemotaxonomic marker compounds. Being novel research, the results found in this section could prove to be very useful in assisting with the differentiation of *Portieria tripinnata* from the cryptic species of *Portieria hornemannii*.

4.1.2 Chapter Aims

The main objectives of this part of the project was to:

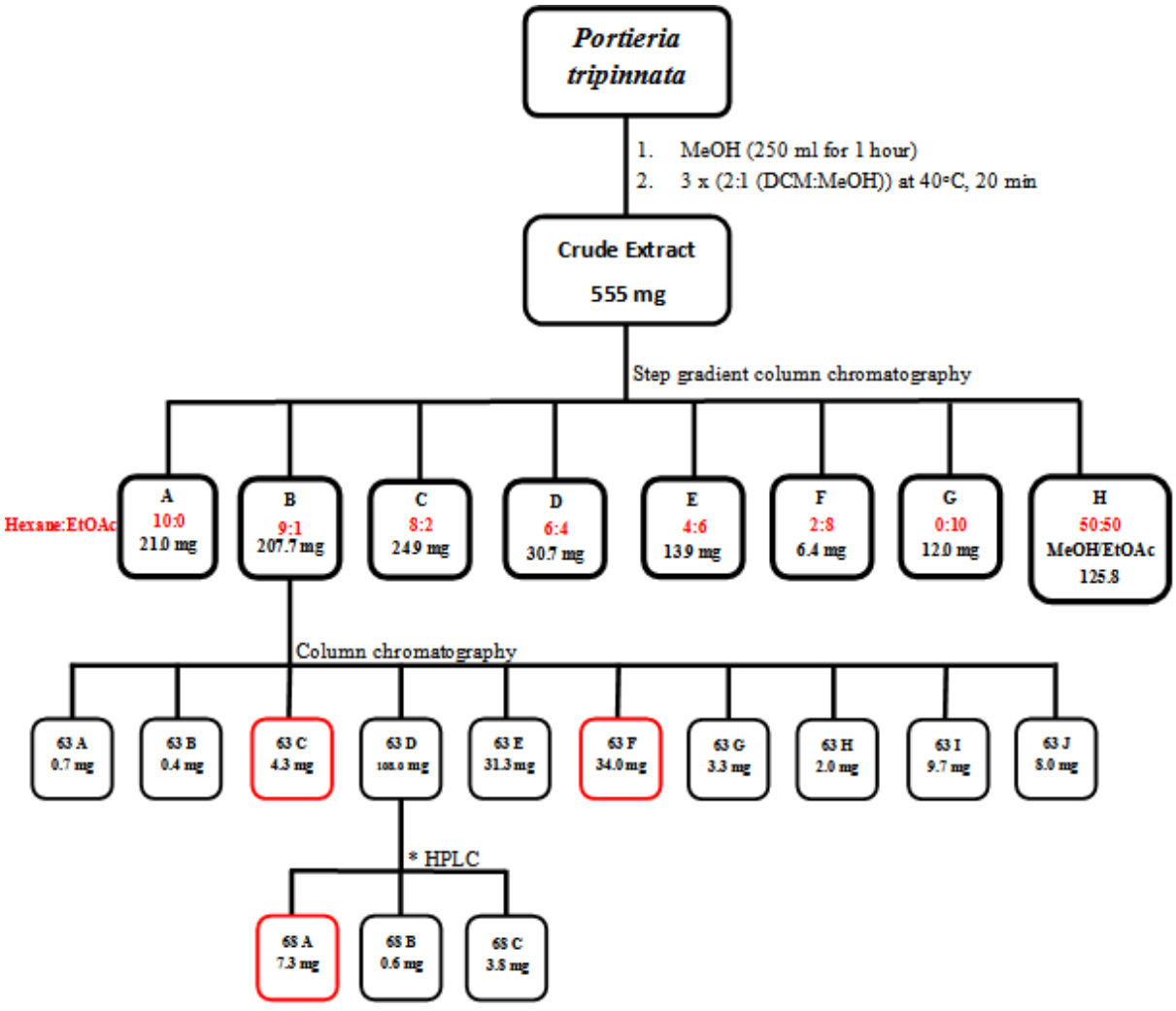
a) Isolate and characterise metabolites from *Portieria tripinnata*
b) Identify potential chemotaxonomic markers for *Portieria tripinnata*

Figure 4.1: Photograph of *Portieria tripinnata* (KwaZulu-Natal, South Africa)

4.2 Results and Discussion

4.2.1 Isolation and characterisation of halogenated monoterpenes from *Portieria tripinnata*

Portieria tripinnata (D1052) was collected from Port Edward (KwaZulu-Natal), South Africa. The algal sample was soaked in MeOH for one hour and then extracted with DCM:MeOH (2:1). The crude extract was chromatographed *via* step-gradient column chromatography and resulted in eight fractions of increasing polarity (**Scheme 4.1**). The ^{1}H NMR spectra of the resulting fractions is seen in **Figure 4.2**.



Scheme 4.1: Isolation scheme of compounds **1.25** (63 C), **4.1** (63 F) and **1.27** (68 A)

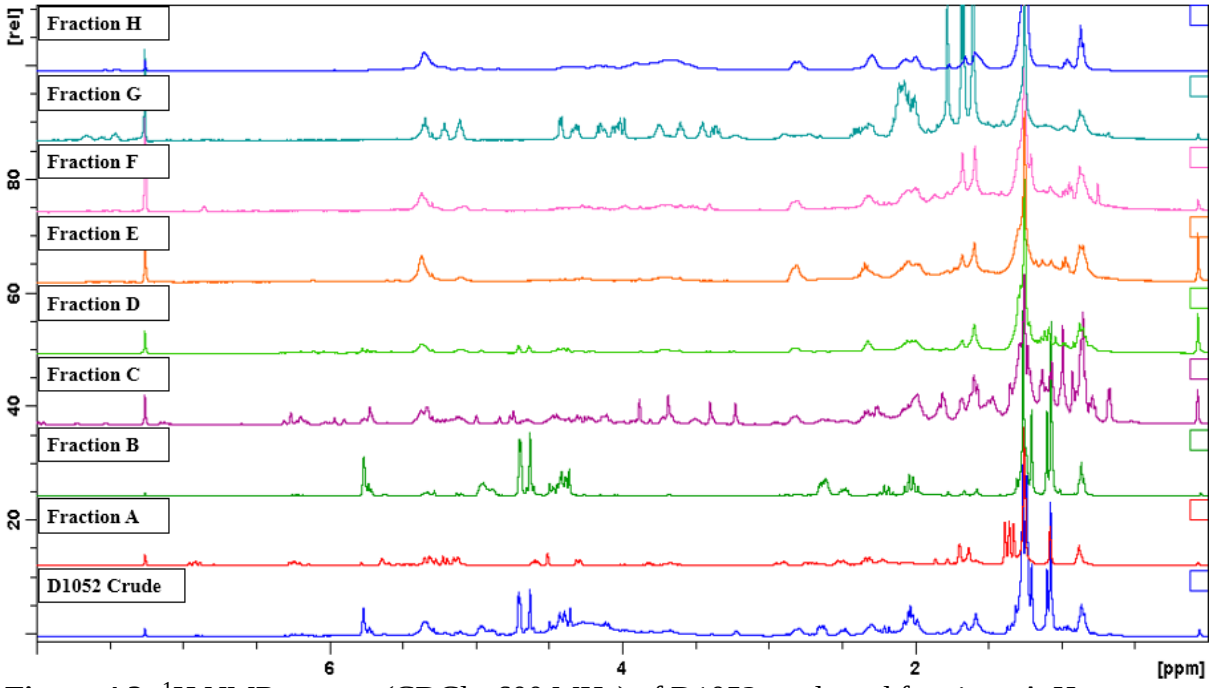


Figure 4.2: ^{1}H NMR spectra ($CDCl_3$, 600 MHz) of D1052 crude and fractions A-H

Compound **1.27** was the major component in fraction B (9:1, Hexane/EtOAc). Fraction B was further separated *via* column chromatography, using Hexane-EtOAc (9:1) resulting in ten new fractions (63 A -63 J). Fractions 63 C and 63 F contained pure compounds **1.25** and **4.1** respectively. Fraction 63 D was separated using HPLC and resulted in three new fractions (68 A – 68 C) of which fraction 68 A contained pure compound **1.27**.

4.2.2 Structure elucidation of metabolites

4.2.2.1 Compound 1.25

Compound **1.25** revealed a relatively complex ^{1}H NMR spectrum (**Figure 4.3**) in which two sharp singlet peaks were observed at δ 1.08 and 1.33, representing methyl groups. Two multiplets are seen at δ 2.71 (1H, m) and 2.93 (1H, dt, *J* = 19.4, 5.75 Hz), representing two diastereotopic protons from a methylene group. Two halomethine signals are seen at δ 4.51 and 4.60 (dd, *J* = 6.27, 11.04 Hz,) as well as two doublets at δ 5.14 (d, *J* = 11.1) and at 5.33 (d, *J* = 17.5 Hz), reminiscent of a monosubstituted alkene. Two olefinic protons are observed at δ 5.64 (t, *J* = 4.03 Hz,) and at 6.24 (dd, *J* =11.1, 17.6 Hz,).

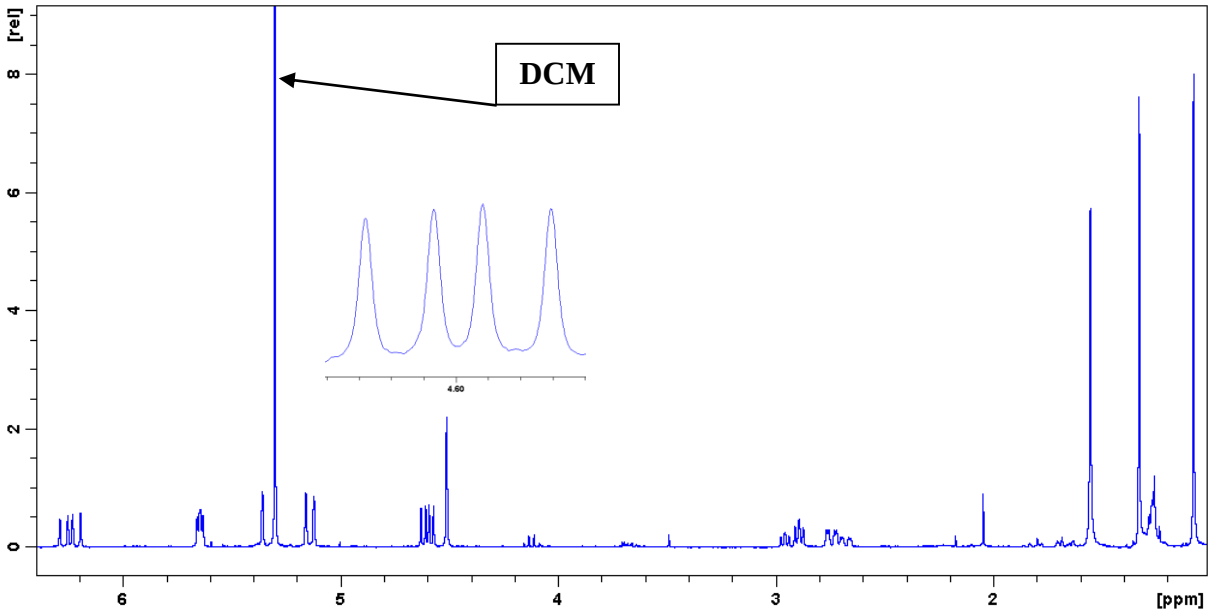


Figure 4.3: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

The ^{13}C NMR spectrum of compound **1.25** (**Figure 4.4**) showed ten carbon signals. Along with the HSQC NMR spectrum (**Figure 4.5**) two double bonds were noted with carbon resonances at δ 113.3, 129.3, 135.6 and 136.3. A further two methyl carbons were observed at δ 19.7 and 28.2 in addition to one methylene carbon (δ 36.0), two halomethine carbons (δ 55.1 and 63.0) and a quaternary carbon (δ 39.5).

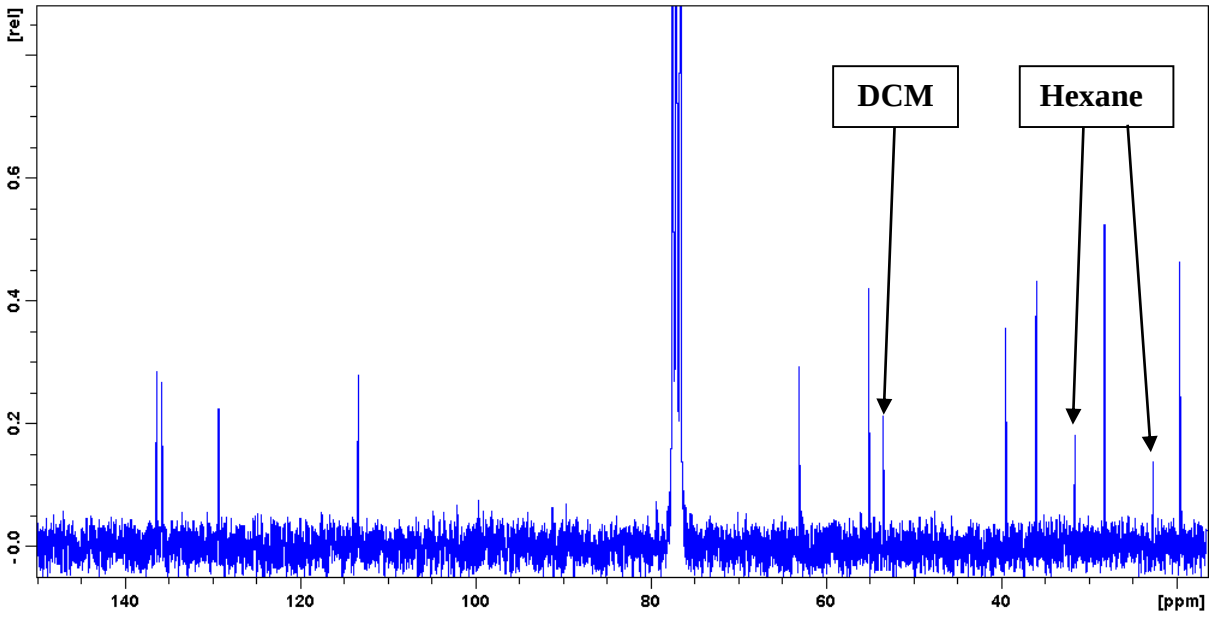


Figure 4.4: ^{13}C NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

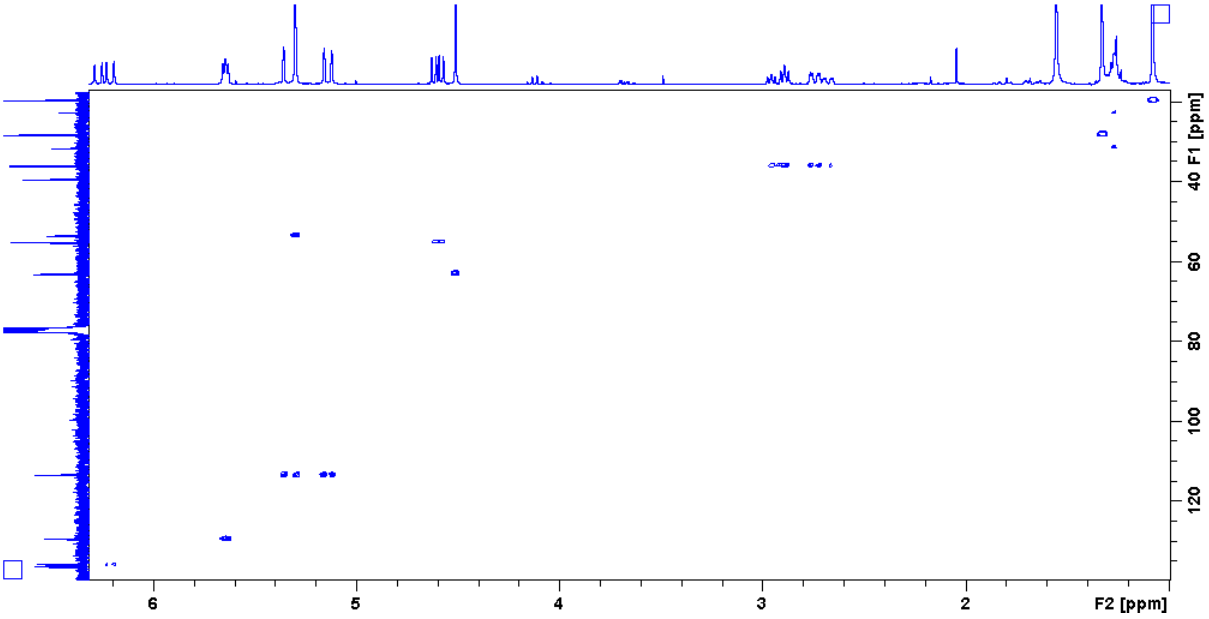


Figure 4.5: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

Substructure **A** (**Figure 4.7**) was constructed from the COSY NMR correlations from the two methylene protons at δ 2.71 and 2.93 to both the halomethine proton at 4.60 (dd, J = 6.27, 11.04 Hz) and the olefinic proton at 5.64 (t, J = 4.03 Hz,).

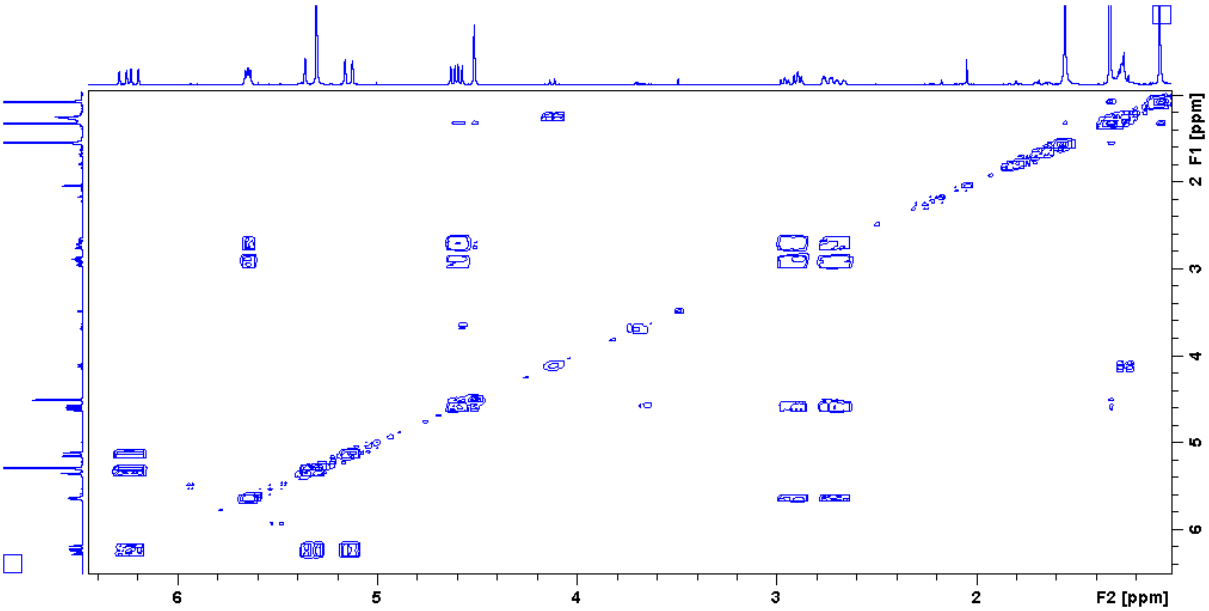


Figure 4.6: COSY NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

The two methyl groups showed HMBC correlations to each other as well as to the quaternary carbon seen at δ 39.5, and the two halomethine groups at δ 55.1 and δ 63.0. This gives rise to possible substructure **B** (**Figure 4.7**).

The two terminal alkene protons showed HMBC correlations to the carbon at δ 135.6 which in turn showed HMBC correlations to the carbon at δ 136.3 as well as the halomethine carbon at δ 63.0 and the olefinic carbon at δ 129.3. This results in the possible substructure **C** (**Figure 4.7**).

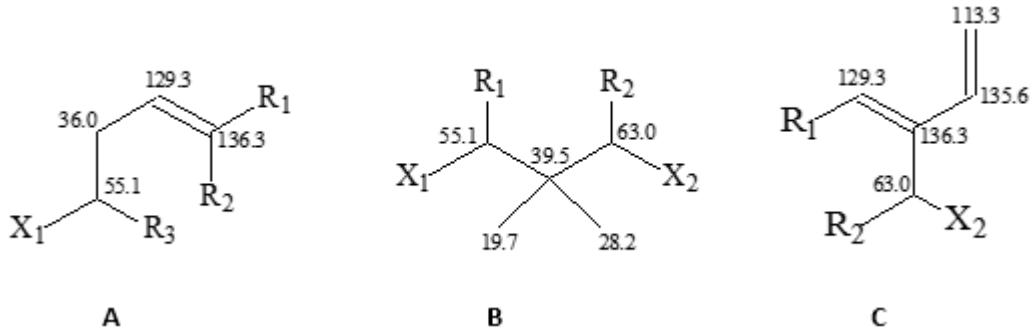


Figure 4.7: Substructure **A-C** of compound **1.25** constructed from COSY and HMBC data

Combining substructures **A** – **C** results in the planar structure seen in **Figure 4.8**.

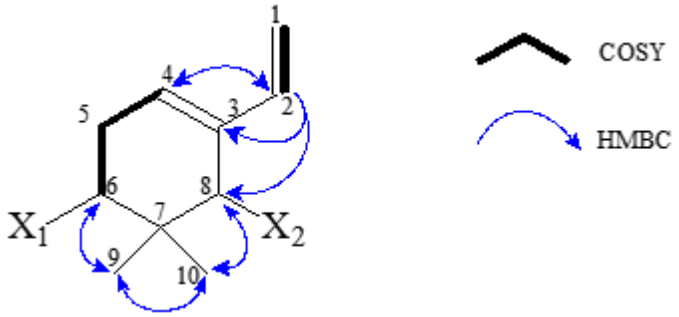


Figure 4.8: Planar structure of compound **1.25**, showing key COSY and HMBC NMR correlations

The ^{1}H NMR spectroscopic data of compound **1.25** were compared to those of a cyclic halogenated monoterpene previously isolated from *Ochtodes secundiramea* (Fenical *et* al., 1978) (**Table 4.1**).

Table 4.1: NMR spectroscopic data for compound **1.25** in $CDCl_3$ and as found in literature (Fenical *et* al., 1978)

No.	**δ_C, mult**	**δ_H, mult, J (Hz)**	**Literature values δ_H (mult, J (Hz))**	**COSY**	**HMBC**
1.	113.3, CH_2	5.14, (d, 11.1) 5.33, (d, 17.5)	5.14 (d, 11.2) 5.33 (d, 17.5)	H2	C2
2.	135.6, CH	6.24, (dd, 11.1 17.6)	6.24 (dd, 11.2, 17.5)	$H1_a$; $H1_b$	C3, C4, C8
3.	136.3, C	--		--	--
4.	129.3, CH	5.64, (t, 4.0)	5.65	$H5_a$; $H5_b$	C2, C6, C8
5.	36.0, CH_2	2.71, (m) 2.93, (dt, 19.4, 5.8)	2.73 (m) 2.93 (m)	H4; H6 H4; H6	C2, C3, C4, C6, C7
6.	55.1, CH	4.60, (dd, 6.3, 11.0)	4.59 (dd, 6.1, 12.0)	$H5_a$; $H5_b$	C5, C7, C10
7.	39.5, C	--	--	--	--
8.	63.0, CH	4.51, (s)	4.51 (s)	--	C3, C4, C6, C7, C9, C10
9.	28.2, CH_3	1.33, (s)	1.33 (s)		C6, C7, C8, C10
10.	19.7, CH_3	1.08, (s)	1.08 (s)		C6, C7, C8, C9

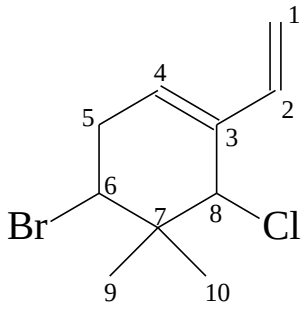


Figure 4.9: Structure of compound **1.25**

4.2.2.2 Compound 1.27

Upon assessing the ^{1}H NMR spectrum (**Figure 4.10**) of compound **1.27** two methyl singlet peaks were observed at δ 1.09 and 1.29 as well as two multiplets at δ 2.05 (1H) and δ 2.65 (1H) representing two protons from a methylene group. Two sets of halomethine protons were observed at δ 4.42 (dd, *J* = 13.06, 4.01) and at δ 4.64 together with a deshielded methylene signals at δ 4.72 (2H) (dd, *J* = 5.01, 1.43). Finally, a complex multiplet at δ 4.98 (1H), and a methine at δ 5.78 (1H) completed the spectrum.

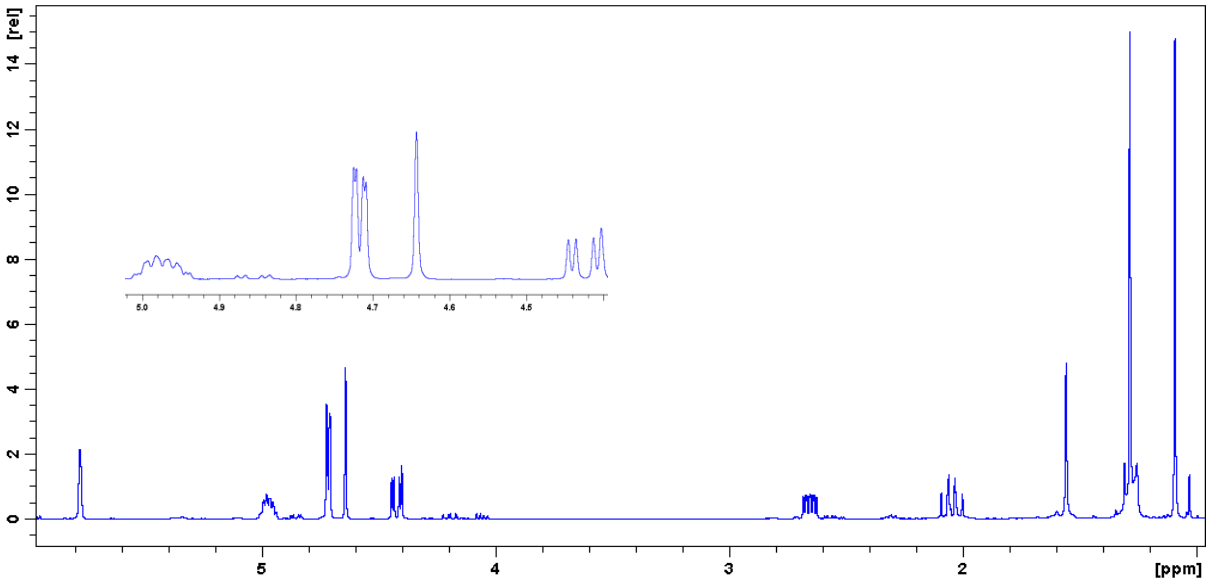


Figure 4.10: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

The ^{13}C NMR spectrum (**Figure 4.11**) of compound **1.27** showed ten carbon signals including one double bond (δ 122.5 and 137.8), two methyl carbons (δ 21.0 and 27.6), two methylene carbons (δ 41.7 and 75.5), three methine carbons (δ 54.6, 63.8 and 80.8) and a quaternary carbon at δ 41.8. The Edited HSQC NMR spectrum of compound **1.27** is seen in **Figure 4.12**.

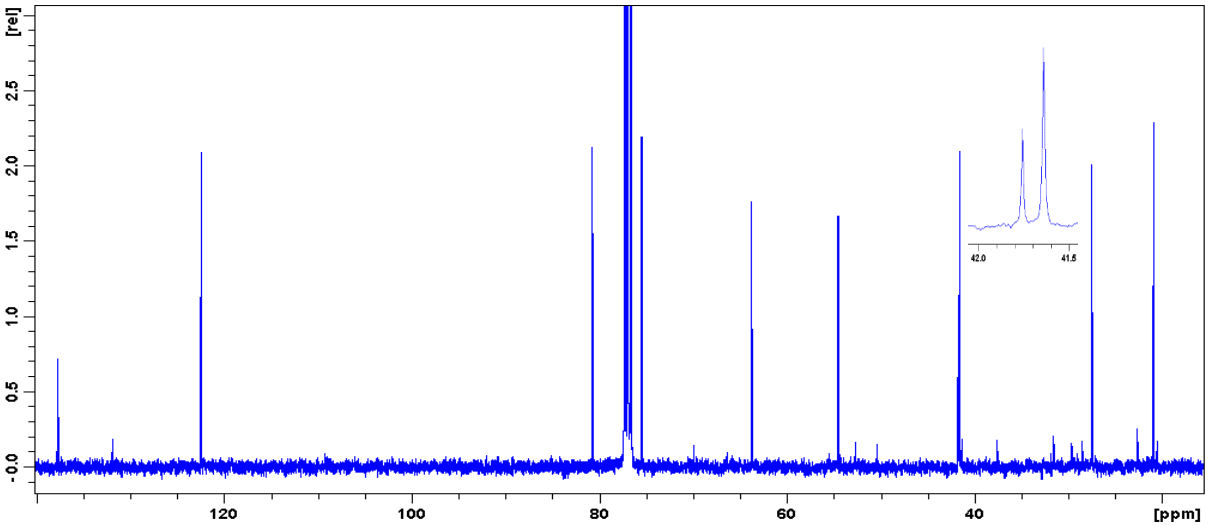


Figure 4.11: ^{13}C NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

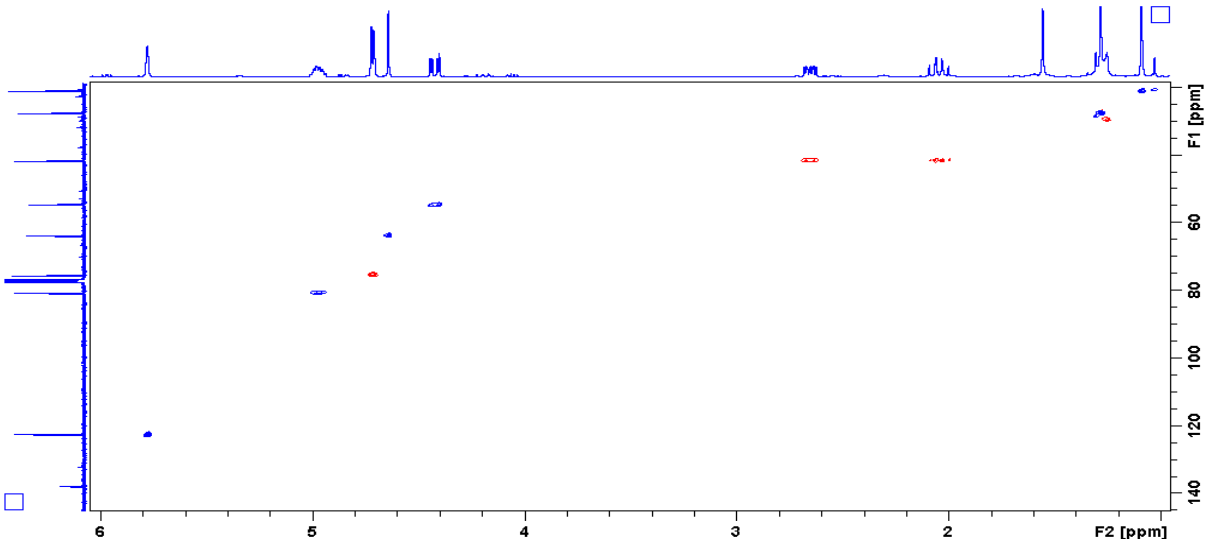


Figure 4.12: Edited HSQC NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

The COSY NMR spectrum (**Figure 4.13**) of compound **1.27**, displayed correlations between the two methylene protons at δ 2.05 and δ 2.65 , to the halomethine signal at δ 4.42 and the deshielded methine multiplet at δ 4.98 to give the substructure **A** (**Figure 4.14**). The deshielded methylene protons at δ 4.72 are also seen to show COSY correlations to the olefinic proton at δ 5.78.

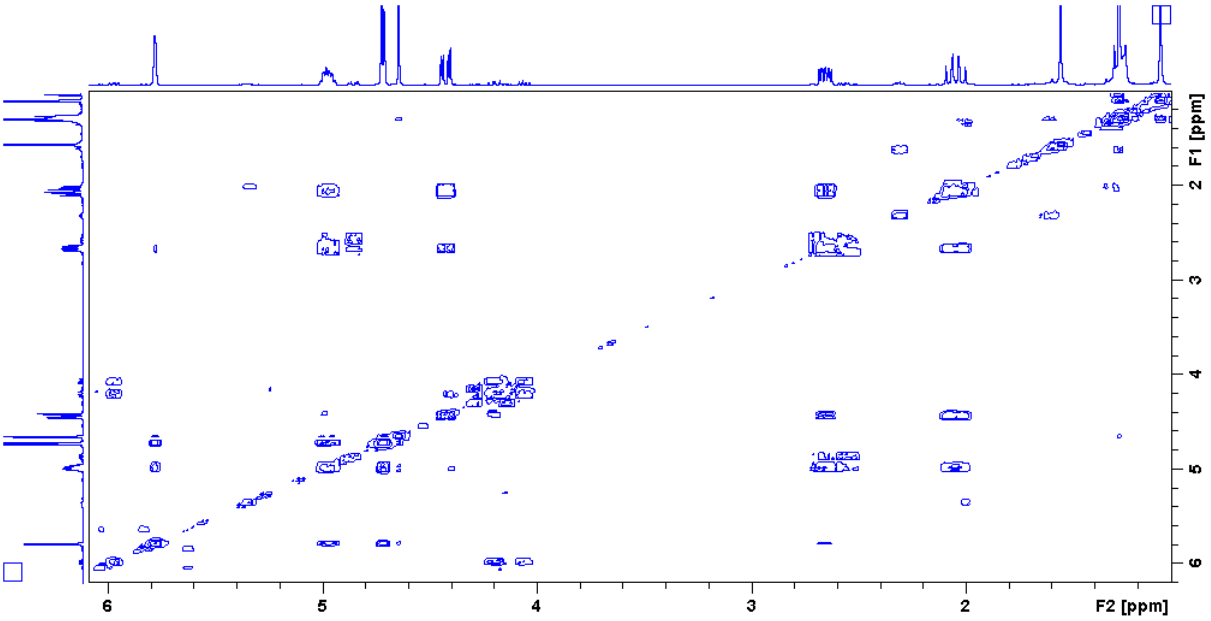


Figure 4.13: COSY spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

The two methyl groups showed HMBC correlations to each other, as well as to the two halomethine groups at δ 54.6 and 63.8 and to the quaternary carbon at δ 41.8 to give substructure **B** (**Figure 4.14**).

Substructure **C** (**Figure 4.14)** was constructed from HMBC correlations from the olefinic proton at δ 5.78 to the deshielded methylene group at δ 75.5, the quaternary carbon at δ 137.8, and the halomethine carbon at δ 63.8.

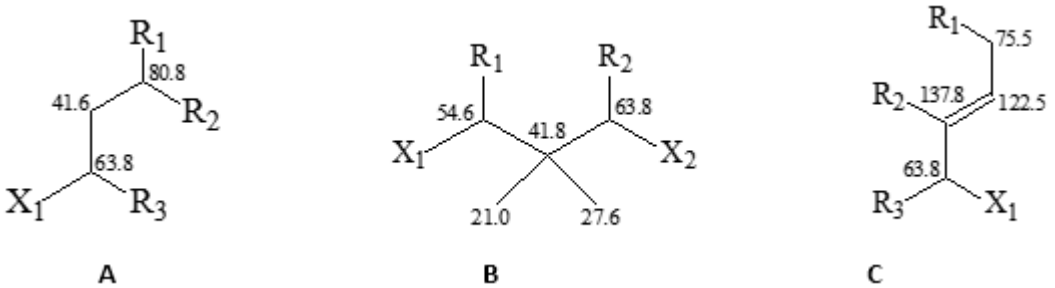


Figure 4.14: Substructures **A-C** of compound **1.27**

The deshielded methylene group (δ 4.72) and the deshielded methine group (δ 4.98) suggested the presence of a highly electronegative group. Combining the substructures **A** – **C** gave the planar structure for compound **1.27** (**Figure 4.15**).

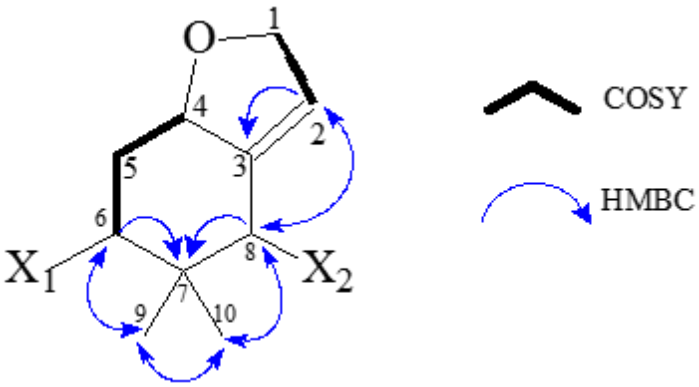


Figure 4.15: Planar structure of compound **1.27** showing key COSY and HMBC correlations

Table 4.2: NMR spectroscopic data for compound **1.27** in $CDCl_3$ and as found in literature (Burreson, *et al*., 1975)

No.	**δ_C, mult**	**Literature values δ_C**	**δ_H, mult, J (Hz)**	**Literature values δ_H, (mult)**	**COSY**	**HMBC**
1.	75.5, CH_2	75.4	4.72, (dd, 5.0, 1.4)	4.72, (m)	H2	C2, C3, C4, C5, C8
2.	122.5, CH	122.3	5.78, (m)	5.78 (m)	--	C1, C3, C4, C8
3.	137.8, C	137.6	--	--	--	--
4.	80.8, CH	80.7	4.98, (m)	5.0 (m)	H1	C2, C3
5.	41.6, CH_2	41.7	2.05, (m) 2.65, (m)	2.05 (m) 2.65 (m)	H4, H5 H4, H5	C3, C4, C6, C7
6.	54.6, CH	54.4	4.42, (dd, 13.1, 4.0)	4.45 (m)	--	C3, C4, C7, C8, C9, C10
7.	41.8, C	41.7	--	--	--	--
8.	63.8, CH	63.8	4.64, (s)	4.64 (s)	--	C2, C3, C4, C6, C7, C9, C10
9.	27.6, CH_3	27.6	1.29, s	1.33 (s)	--	C6, C7, C8, C10
10.	21.0, CH_3	21.0	1.09, s	1.15 (s)	--	C6, C7, C8, C9

The ^{1}H and ^{13}C NMR spectroscopic data of compound **1.27** were identical to a halogenated dimethylhexahydrobenzofuran previously reported by Burreson *et al*. (1975).

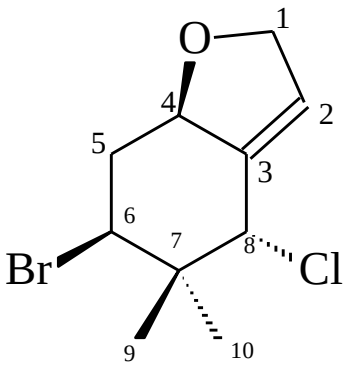


Figure 4.16: Structure of compound **1.27** (Chondrocole A)

4.2.2.3 Compound 4.1

The ^{1}H NMR spectrum (**Figure 4.17**) of compound **4.1** is relatively complex displaying two methyl singlets (δ 1.12 and δ 1.22), two methylene multiplets (δ 2.22 and δ 2.53) and a sharp singlet at δ 4.38 reminiscent of a halomethine proton. It also displays a doublet of doublets at δ 4.44 (dd, *J* = 12.3, 5.1) which possibly represents a second halomethine group. Two doublet of doublet of doublets are seen at δ 4.46 (ddd, *J* = 11.9, 7.7, 1.2) and δ 4.64 (ddd, *J* = 12.3, 7.9, 1.0) which represent protons from a deshielded methylene group. A multiplet is seen at δ 4.94 (1H) representing a deshielded methine group. Finally, a triplet of doublets is seen at δ 5.77 (td, *J* = 15.6, 1.7)

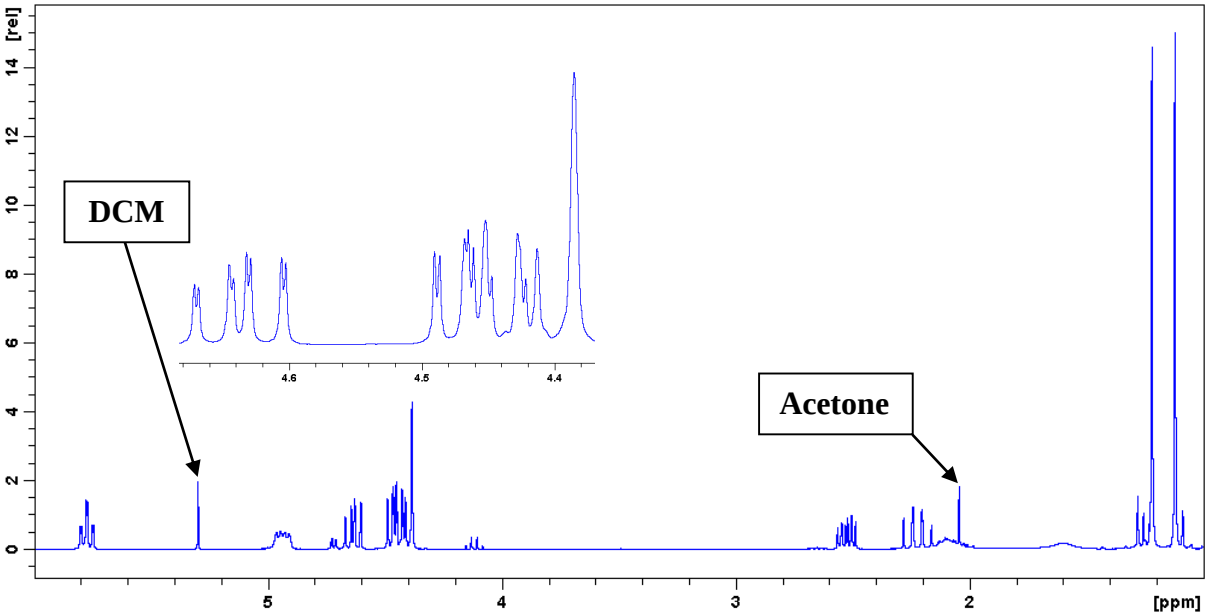


Figure 4.17: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

The ^{13}C NMR spectrum (**Figure 4.18**) of compound **4.1** displayed ten carbon signals which in combination with the HSQC NMR data (**Figure 4.19**) of compound **4.1**, revealed two methyl carbons seen at δ 21.9 and 26.6, two methylene carbons at δ 39.6 and 42.8, a quaternary carbon at δ 42.2, and three deshielded methine carbons at δ 55.0, δ 69.4 and δ 73.6. The presence of one double bond was noted with carbons at δ 126.1 and δ 139.7.

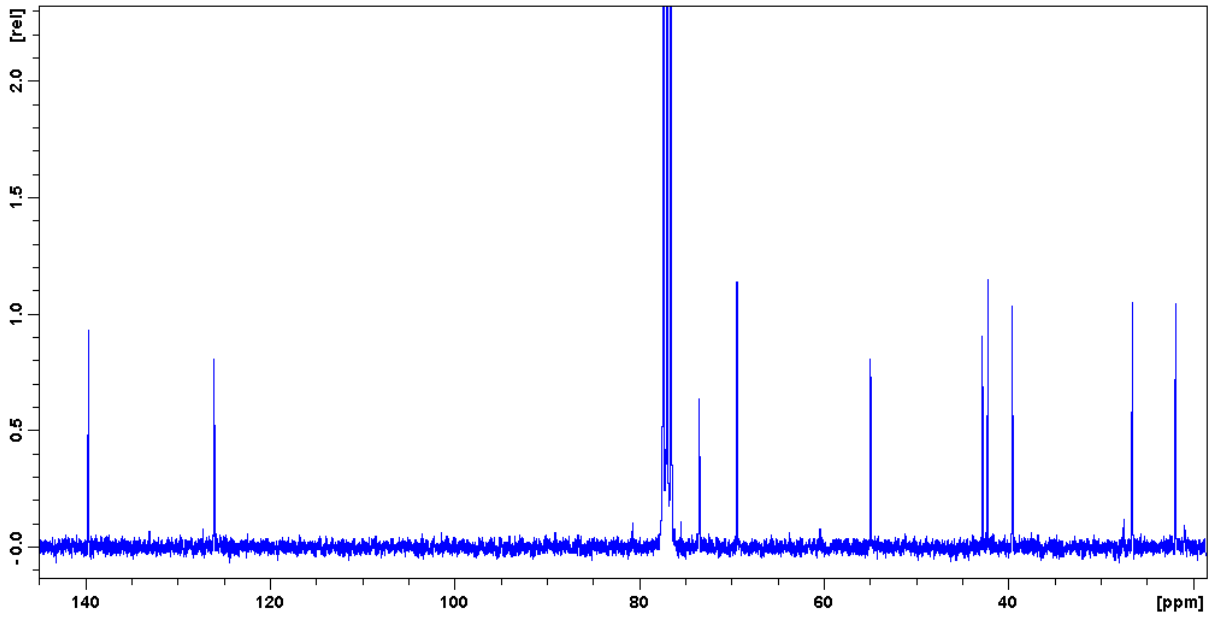


Figure 4.18: ^{13}C NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

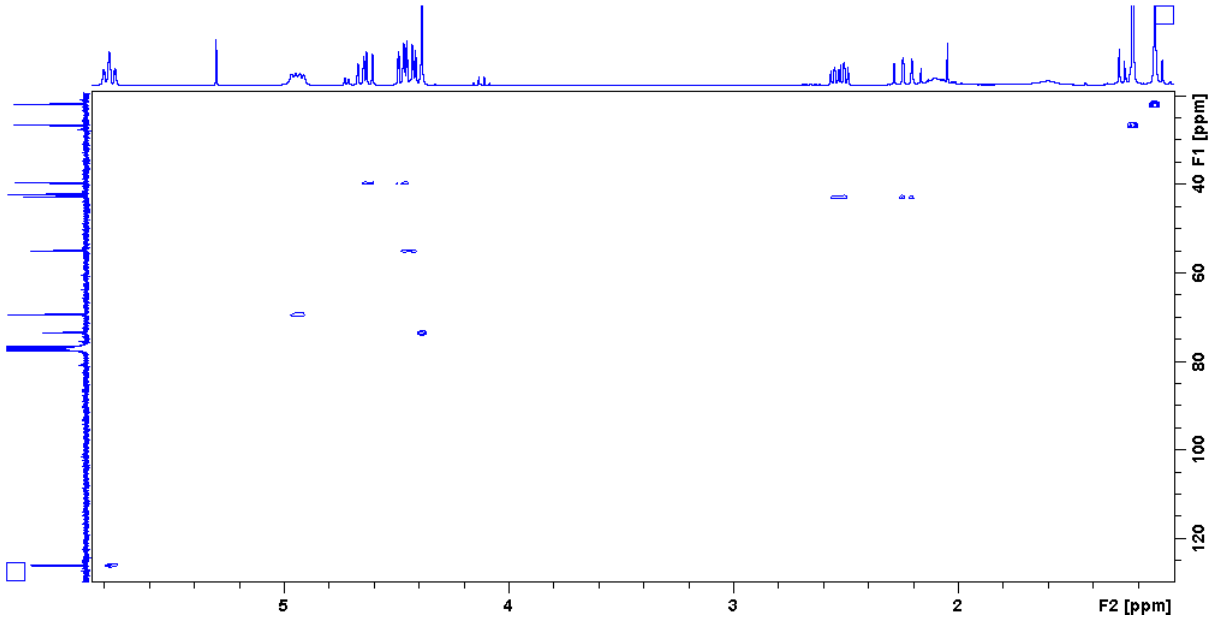


Figure 4.19: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

The COSY NMR spectrum (**Figure 4.20**) of compound **4.1**, showed correlations between the two methylene protons (δ 2.22 and 2.53) and the two halomethines at δ 4.44 and 4.94 to give substructure **A** (**Figure 4.21**).

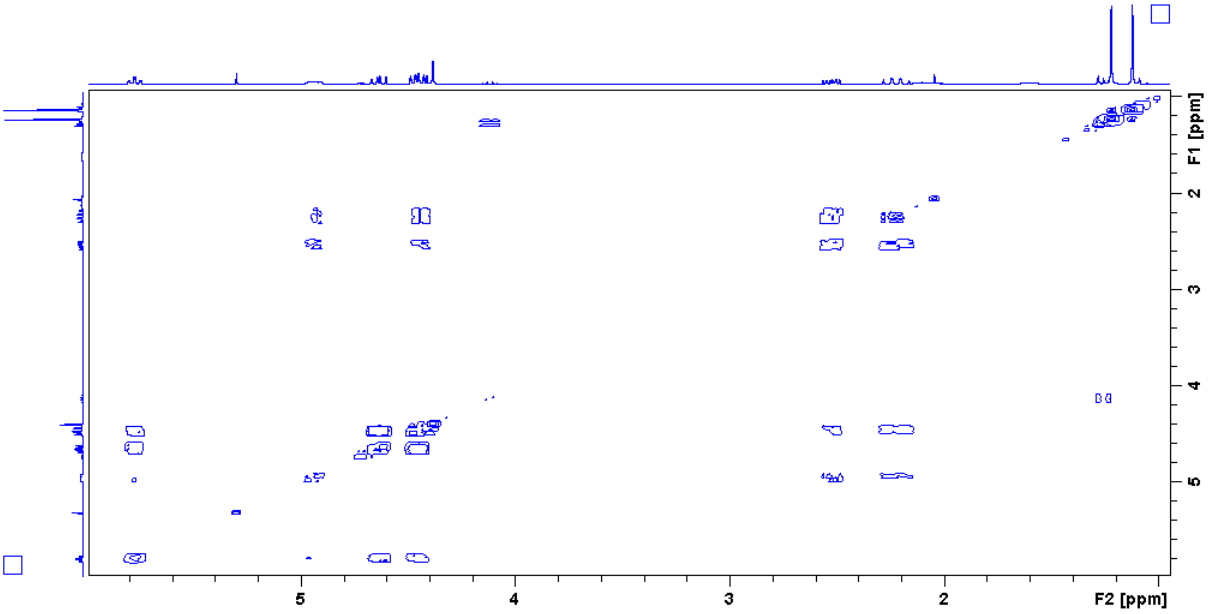
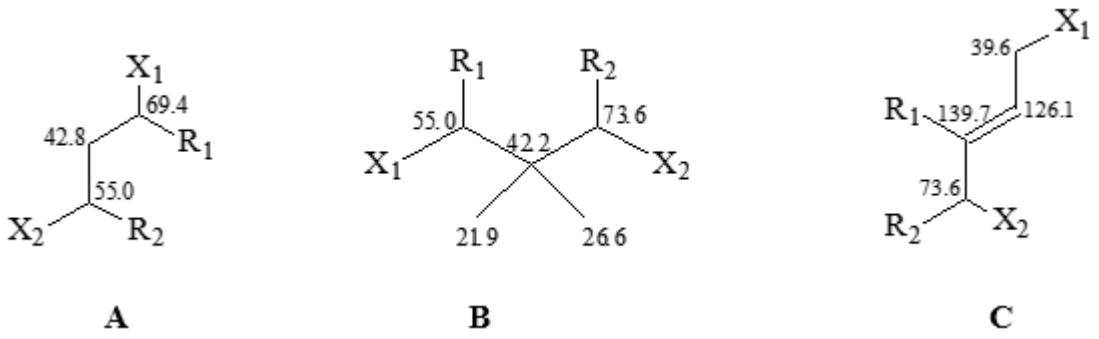


Figure 4.20: COSY NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

The two methyl groups showed HMBC correlations to each other, as well as to the quaternary carbon at δ 42.2, and the two halomethine groups at δ 55.0 and δ 73.6 to give substructure **B** (**Figure 4.21**).

A COSY correlation between the methylene protons (δ 4.46 and δ 4.64) and olefinic proton at δ 5.77 together with HMBC correlations from the former to carbons at δ 126.1, 139.7 and 73.6 gave substructure **C** (**Figure 4.21**).



A **B** **C**

Figure 4.21: Substructures **A-C** of compound **4.1**

Combining all three substructures of compound **4.1** resulted in the cyclic planar structure (**Figure 4.22**).

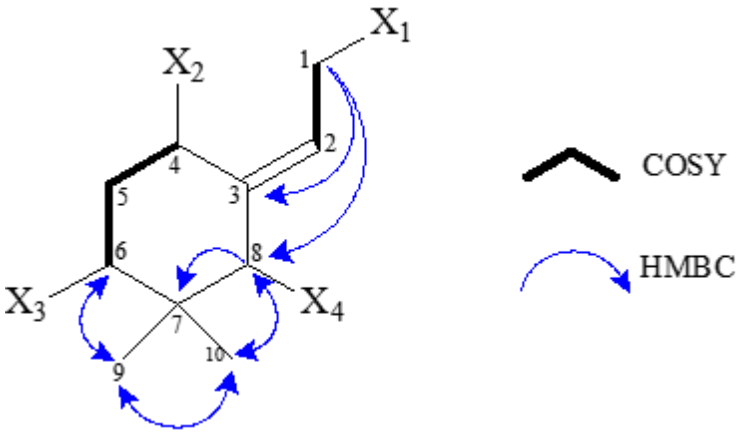


Figure 4.22: Planar structure of compound **4.1** showing key COSY and HMBC NMR correlations

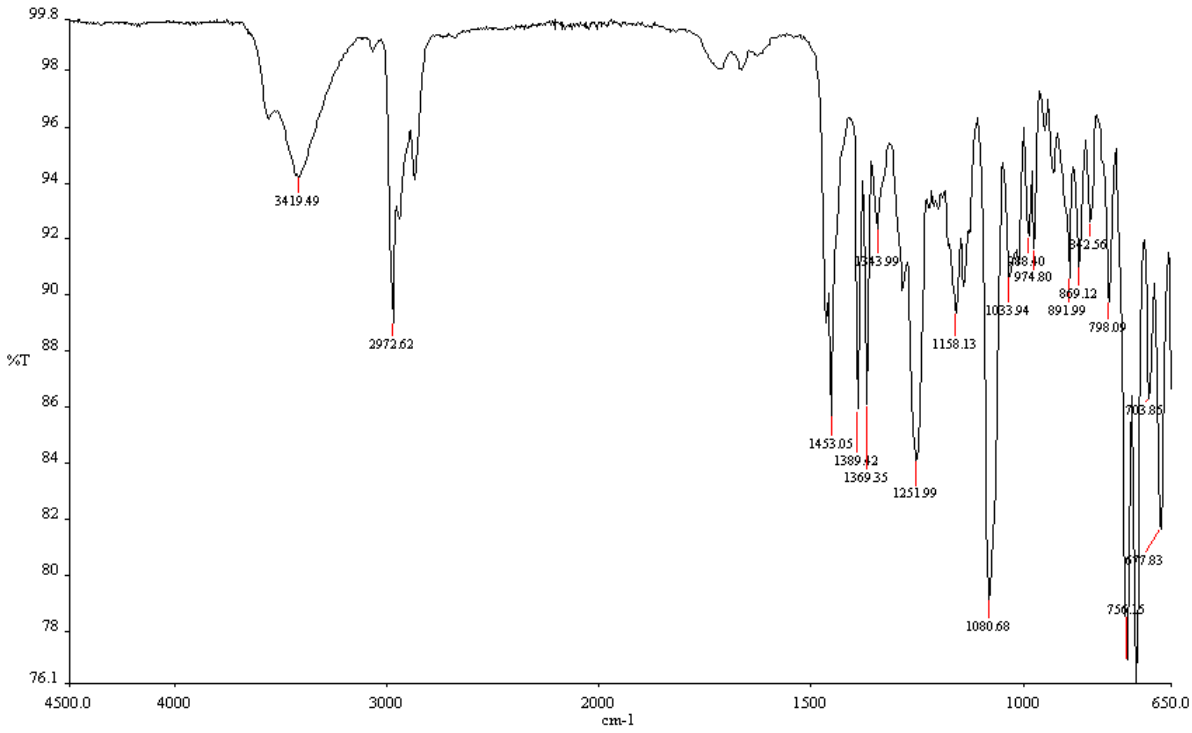


Figure 4.23: IR spectrum of compound **4.1**

With the planar structure of **4.1** in hand we next turned our attention to assigning substituents X_1, to X_4. This is not a trivial exercise and researchers generally rely on chemical shift arguments to assignment halogenated substituents in these systems. From **Figure 4.23**, a broad peak is noted at 3419.49 cm^{-1} indicating the presence of a hydroxyl group. **Table 4.4** shows a comparison of ^{13}C NMR assignment for a series of related systems. Clearly, compound **4.1** is most closely related to monoterpene **1.51**. The key differences lie in the chemical shifts of C4 and C8. These discrepancies can most likely be explained by stereochemical differences between the two compounds.

The structure of compound **4.1** can only be truly resolved with the use of optical rotation and HRESMS, which unfortunately have not been determined. Based on our current evidence we suggest compound **4.1** has the structure as shown in **Figure 4.24** and is most probably a diastereoisomer of monoterpene **1.51**. With the optical rotation and HRESMS, we would be able to determine whether our conclusion is true or not.

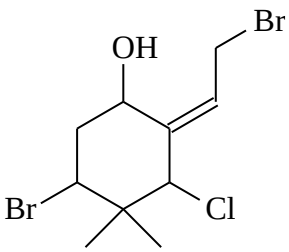


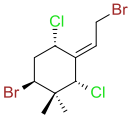
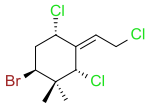
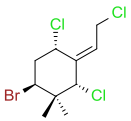
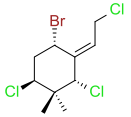
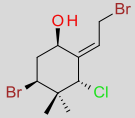
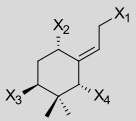
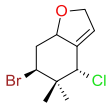
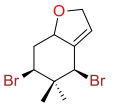
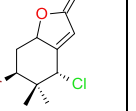
Figure 4.24: Proposed structure of compound **4.1**

Table 4.3: NMR spectral data for compound **4.1** in $CDCl_3$

No.	**δ ^{13}C, mult**	**δ ^{1}H, mult, J (Hz)**	**COSY**	**HMBC**
1.	39.6, CH_2	4.46, (ddd, *J* = 11.9, 7.7, 1.2) 4.64, (ddd, *J* = 12.3, 7.9, 1.0)	H2	C8, C2, C3, (C6)
2.	126.1, CH	5.77, (td, *J* = 15.6, 1.7)	$H1_a$; $H1_b$	C8, C4
3.	139.7, C	--	--	--
4.	69.4, CH	4.94, (m)	--	C5, C2, C3
5.	42.8, CH_2	2.22, (m) 2.53, (m)	H4; H6 H4; H6	C6, C4, C7, C3
6.	55.0, CH	4.44, (dd, *J* = 12.3, 5.1)	--	C9, C10, C7, C4, C2, C3
7.	42.2, C	--	--	--
8.	73.6, CH	4.4, (d, *J* = 8.2)	--	C9, C10, C7, C6, C4, C2, C3
9.	26.6, CH_3	1.2, (s)	--	C7, C10, C6, C8
10.	21.9, CH_3	1.1, (s)	--	C7, C9, C6, C8

Table 4.4. Comparison of ^{13}C NMR chemical shifts for cyclic halogenated monoterpenes of the ochtodane class

[a] Maliakal *et al*., 2001; [b] Gunatilaka *et al*., 1999; [c] Fuller *et al*., 1994; [d] Crews *et al*., 1984; [e] Burreson *et al*., 1975; [f] Woolard et al., 1978

									
	1.47[a]	**1.48[b]**	**1.49[b]**	**1.50[c]**	**1.51[d]**	**4.1**	**1.52[e]**	**1.53[a]**	**1.54[f]**
1	37.6	37.4	37.6	37.6	39.6	39.6	75.3	75.3	171.0
2	131.9	131.7	131.9	131.8	125.0	126.1	124.8	124.8	115.4
3	137.7	138.2	137.8	137.9	-	139.7	137.6	138.3	164.4
4	50.4	59.6	50.4	50.4	73.6	69.4	80.7	82.6	76.9
5	41.3	41.8	41.3	41.3	42.6	42.8	41.7	41.4	40.0
6	52.7	52.7	52.7	52.7	54.9	55.0	54.4	55.7	51.0
7	41.3	41.0	41.4	41.4	-	42.2	41.7	43.6	42.3
8	70.0	60.7	70.0	70.0	68.6	73.6	63.8	54.8	60.8
9	20.4	20.6	20.5	20.5	21.5	21.9	21.0	16.0	20.5
10	28.5	28.6	28.5	28.5	26.6	26.6	27.6	29.1	26.9

4.3 Experimental

4.3.1 General Experiments

Solvents

All solvents used to extract and separate the organic crude extract of sample D1052 were High-Performance-Liquid-Chromatography (HPLC) grade from LiChrosolv® from Merk®, Darmstd, Germany.

Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR spectrum of the organic crude extract of sample D1052 was acquired on a Bruker® 600 MHz Avance spectrometer. All NMR spectra of compound **1.25** and compound **4.1**, were acquired using a Bruker® UltraShield™ 300 MHz spectrometer. All NMR spectra of compound **1.27** were acquired on a Bruker® Avance™ III HD 400 MHz Avance spectrometer. The solvent used to obtain the spectra was deuterated chloroform ($CDCl_3$). All ^{1}H and ^{13}C spectra were in reference to the $CDCl_3$ and the reference peaks for this solvent were at δ_H 7.26 ppm and δ_C 77.00 ppm respectively.

4.3.2 Plant Material

Sample **D1052** was collected on 11/09/28 from Port Edward, KwaZulu-Natal, South Africa. Identification of samples was done based on morphological traits, and was carried out by Prof John J. Bolton of the department of Botany, University of Cape Town, South Africa. Samples were kept in a freezer at ~ -18ºC. A voucher specimen was made for the collected sample and is kept with the Pharmaceutical Chemistry division of Rhodes University, South Africa.

4.3.3 Extraction and Isolation of metabolites

Sample **D1052** had a total wet mass of 52.3 g (after extraction, dry mass 9.8 g).

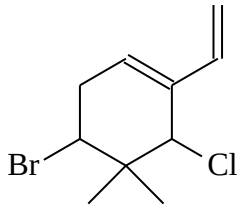
The algal sample was initially rinsed under tap water and thereafter, air-dried for ~ 10 minutes and placed in a 1 litre beaker, to this 400 ml MeOH was added and this was allowed to stand for one hour. The MeOH was then decanted into a 2 litre conical flask. 400 ml 2:1 (DCM/MeOH) was added to the sample and heated to 37 ^{o}C for 20 min, this was then decanted into the conical flask containing the MeOH. This last step was repeated three times after which the solution was placed in a separating funnel, distilled water was added to this in sufficient quantity to cause phase separation. The aqueous layer was discarded and the organic layer was collected and dried off under reduced pressure using a Rotavap®. 0.5552 g of crude was extracted (5.4 % yield).

The crude extract was separated via open-column silica gel chromatography, using Hex/EtOAc as solvents, to give rise to eight fractions of increasing polarity. Fraction B (207.7 mg) was further fractionated via column chromatography, using 9:1 (Hexane/EtOAc) as the solvent system, and resulted in ten fractions, of which the third and sixth fractions contained pure compounds **1.25** (4.3 mg) and **4.1** (34.0 mg). The fourth of these fractions (108.0 mg) was purified via HPLC, using a 9:1 (Hexane/EtOAc) solvent system, to result in compound **1.27** (7.3 mg).

4.3.4 Compounds Isolated

*Naming of compounds was done with ACD/ChemSketch® (2012), therefore, the 13C numbering does not follow the numbering from this chapter.

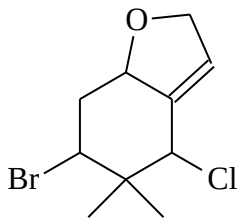
Compound 1.25



1.25

* 4-bromo-6-chloro-1-ethenyl-5,5-dimethylcyclohexene (**1.25**): colourless oil; NMR data available in **Table 4.1**. (As previously reported by Fenical *et* al., 1978)

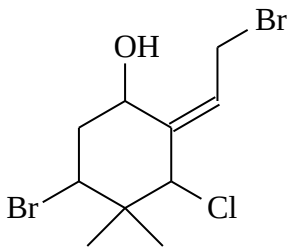
Compound 1.27



1.27

* 6-bromo-4-chloro-5,5-dimethyl-2,4,5,6,7,7a-hexahydro-1-benzofuran (**1.27**): colourless oil; NMR data available in **Table 4.2**. (As previously reported by Burreson, et al., 1975)

Compound 4.1



(2E)-5-bromo-2-(2-bromoethylidene)-3-chloro-4,4-dimethylcyclohexanol (**4.1**): colourless oil; IR (neat) 3419.49, 2972.62 cm^{-1}; NMR data available in **Table 4.3**.

4.4 References

Burreson, B. J.; Woolard, F.; Moore, R. E. Chondrocole A and B, Two Halogenated Dimethylhexahydrobenzofurans from the Red Alga *Chondrococcus hornemannii* (Mertens). *Tetrahedron Letters* **1975**, *26*, 2155 – 2158.

Crews, P.; Naylor, S.; Hanke, F. J.; Hogue, E. R.; Kho, E.; Braslau, R.J. Halogen Regiochemistry and Substituent Stereochemistry Determination in Marine Monoterpenes by 13C NMR. *Organic Chemistry* **1984**, *49*, 1371-1377.

De Clerk, O; Bolton, J. J.; Anderson, R. J.; Copperjans, E. *Guide to the seaweeds of KwaZulu-Natal*, *Scripta Botanica Belgica 33*. National Botanic Garden of Belgium, VLIZ: Flanders Marine Institute and Flemish Community**2005**; pp. 1 – 294.I

Fenical, W.; McConnell, O. J. Ochtodene and Ochtodiol: Novel Polyhalogenated Cyclic Monoterpenes from the Red Seaweed *Ochtodes secundiramea*. *Journal of Organic Chemistry* **1978**, *43*, 4238 – 4241.

Fuller, R. W.; Cardellina, 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 – 441.

Gunatilaka, A. A. L.; Paul, V. J.; Park, P. U.; Puglisi, M. P.; Gilter, A. D.; Eggleston, D. S.; Haltiwanger, R. C.; Kingston, D. G. I. Apakaochtodenes A and B: Two Tetrahalogenated Monoterpenes from the Red Marine Alga *Portieria hornemannii*. *Journal of Natural Products* **1999**, *62*, 1376 – 1378.

Guiry, M. D. in Guiry, M. D. & Guiry, G.M. 2014. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; Accessed on 29 December 2014.

Maliakal, S.; Cheney, D. P.; Rorrer, G. L. Halogenated Monoterpene Production in Regenerated Plantlet Culture of *Ochtodes secundiramea* (Rhodophyta, Cryptonemiales). *Journal of Phycology* **2001**, *37*, 1010–1019.

Woolard, F. X.; Moore, R. E. The Structure and Absolute Configuration of Chondrocolactone, A Halogenated Monoterpene from the Red Alga *Chondrococcus hornemannii*, and a Revised Structure for Chondrocole A. *Tetrahedron Letters* **1978**, *27*, 2367 – 2370.

Chapter 5

Conclusion

The main objectives of this project, were to assess the chemical profiles of different samples of *Portieria* collected along the South African coastline; to isolate and characterise compounds from both *Portieria hornemannii* and *Portieria tripinnata*, as well as to assess the chemotaxonomic relevance of these compounds. These objectives were successfully achieved.

The *Portieria* samples collected revealed significantly different ^{1}H NMR spectra of their organic crude fractions. These differences make the consistent isolation of chemotaxonomic marker compounds very difficult. Some of the algal samples that displayed similar ^{1}H NMR profiles were collected at different times during the year, and therefore, seasonal variations do not appear to affect the chemical profiles of *Portieria*. Certain algal samples were collected at the same location and on the same day, however, displayed contrasting profiles. Based on the findings in this part of the project, the differences in chemical composition of samples could possibly be due to the following reasons:

a) Samples collected are not *Portieria hornemannii*. This is because as mentioned, *Portieria hornemannii* has been found to be comprised of several cryptic species.

b) Samples collected are of different metabolic ages, and therefore, different metabolites are present.

Four halogenated monoterpenes were isolated from *Portieria hornemannii*. Compounds **1.6** and **1.7** had been previously isolated from *Portieria hornemannii*. From previous research, compound **1.7** was found to be a low micromolar inhibitor of DNA methyl transferase-1. Compounds **3.1** and **3.2** showed very interesting structures. These halogenated monoterpenes possessed γ-lactone groups, and this is a phenomenon that has not been observed previously. These compounds could prove to be useful as chemotaxonomic marker compounds, as they have never been isolated from any other species of marine algae. However, before these compounds could be classified as being chemotaxonomic marker compounds, further tests such as the optical rotation of the compounds needs to be determined in order to distinguish the two molecules as being isomers of each other.

The *Portieria tripinnata* species has not been assessed previously and therefore, the type of compounds isolated from this species were of great interest to us. Three halogenated monoterpenes were isolated from this specie of *Portieria*, compounds **1.25**, 1**.27** and **4.1**. Compound **1.25** had previously been isolated from *Portieria hornemannii*, and therefore, could not serve as a chemotaxonomic marker for this species. Compound **1.27** is thought to serve as an herbivore feeding deterrent. **Compound 1.27** had been previously isolated from *Portieria hornemannii*, *Ochtodes crockery*, as well as *Ochtodes secundiramea*. Compound **1.27** thus, confirms the link between the two genera, which have the same order (Gigartinales) as well as family (Rhizophyllidaceae). A stereoisomer of Compound **4.1** had previously been isolated from *Ochtodes secundiramea*. All three compounds can therefore, not be used as chemotaxonomic marker compounds. It should be noted however, that a future study should be conducted on *Portieria tripinnata* as these three compounds, are unlikely to be the only metabolites from this species.

Future studies are necessary on this genus. Some suggestions to be considered are as follows:

a) To include the use of PCA, to get a more statistically accurate result of any differences found when assessing the chemical profiling of *Portieria*.
b) The use of larger amounts of marine alga when assessing the full metabolome of this genus. This would result in the isolation of larger quantities of secondary metabolites and thus, facilitate the identification or structure elucidation of these metabolites.
c) Look into purifying all fractions obtained after the initial crude separation as there is a possibility of some secondary metabolites being present in these fractions.
d) Due to the high complexity with regards to the cryptic species of *Portieria hornemannii*, one should include the use of genomic studies together with chemical analysis to obtain a better understanding of the species.

RHODES UNIVERSITY
Where leaders learn

Isolation and structure elucidation of halogenated metabolites from *Portieria hornemannii* and *Portieria tripinnata*

A Thesis Submitted in Fulfilment of the Requirements for the

Degree of

MASTER OF SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

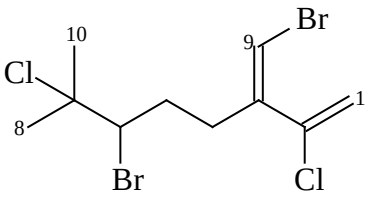
By

Mohammed Adam

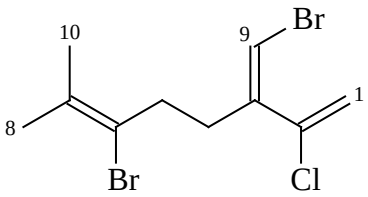
February 2015

Supplementary data

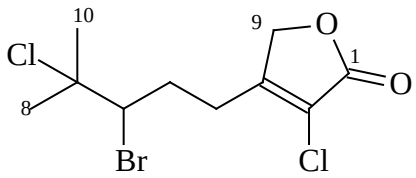
Chapter 3



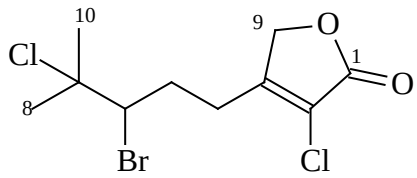
1.6



1.7



3.1



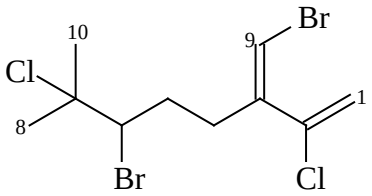
3.2

Table S3.1: ^{1}H NMR data ($CDCl_3$) for compounds 1.6-3.2

Compound	H1	H4	H5	H6	H8	H9	H10
1.6	5.46 (d, 1.68) 5.68 (d, 1.71)	2.47 (m) 2.75 (m)	1.87 (m) 2.44 (m)	3.98, (dd, 11.4, 1.3)	1.66 (s)	6.35 (s)	1.78 (s)
1.7	5.45 (d, 1.51) 5.65 (d, 1.51)	2.59 (m) 2.59 (m)	2.59 (m) 2.59 (m)	--	1.76 (s)	6.25 (s)	1.86 (s)
3.1	--	2.53, m 2.75, m	2.02, m 2.63, m	4.01, (dd, 11.5, 1.6)	1.66, s	4.77, s	1.78, s
3.2	--	2.75,(m) 2.86, (m)	2.03, (m) 2.64, (m)	3.97, (dd, 11.2, 1.4)	1.67, (s)	4.84, (s)	1.81, (s)

Table S3.2: ^{13}C NMR data ($CDCl_3$) for compounds 1.6-3.2

Compound	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
1.6	118.8	135.3	141.8	33.9	31.8	64.1	71.5	27.3	106.2	33.1
1.7	118.8	135.3	141.8	35.8	34.3	120.0	131.8	20.5	106.2	25.4
3.1	135.3	127.6	151.0	22.6	31.6	64.4	71.5	27.2	71.3	32.9
3.2	141.8	168.4	157.8	25.9	31.5	63.3	71.5	26.6	71.1	33.3



1.6

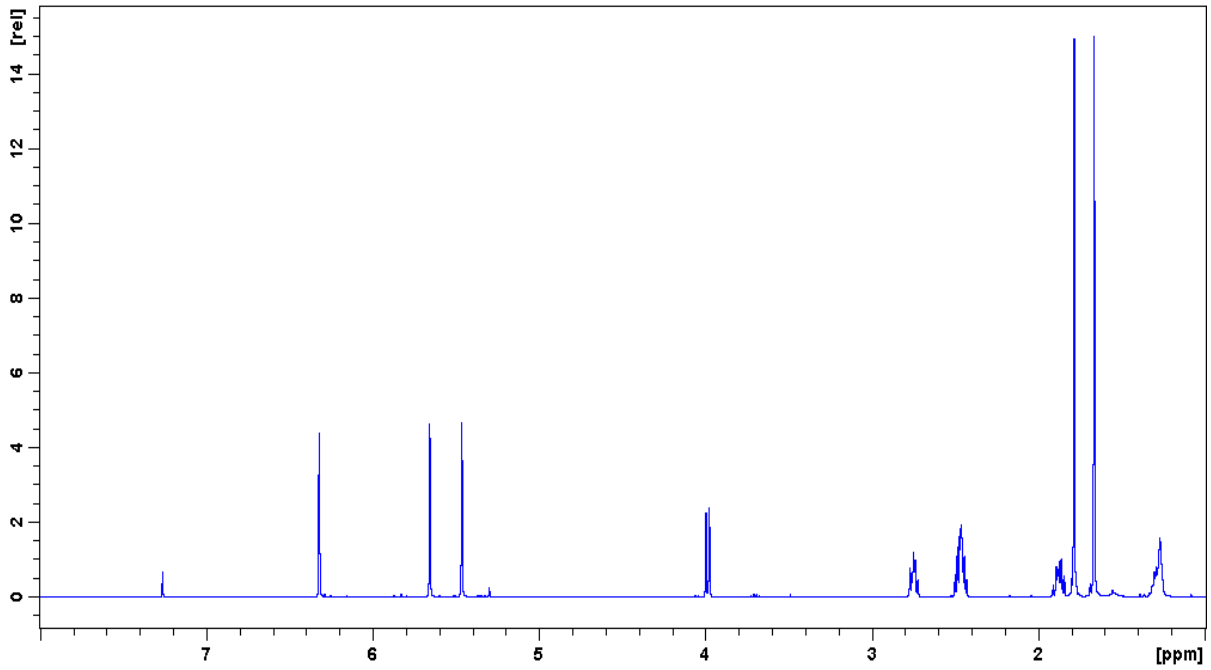


Figure S3.1: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

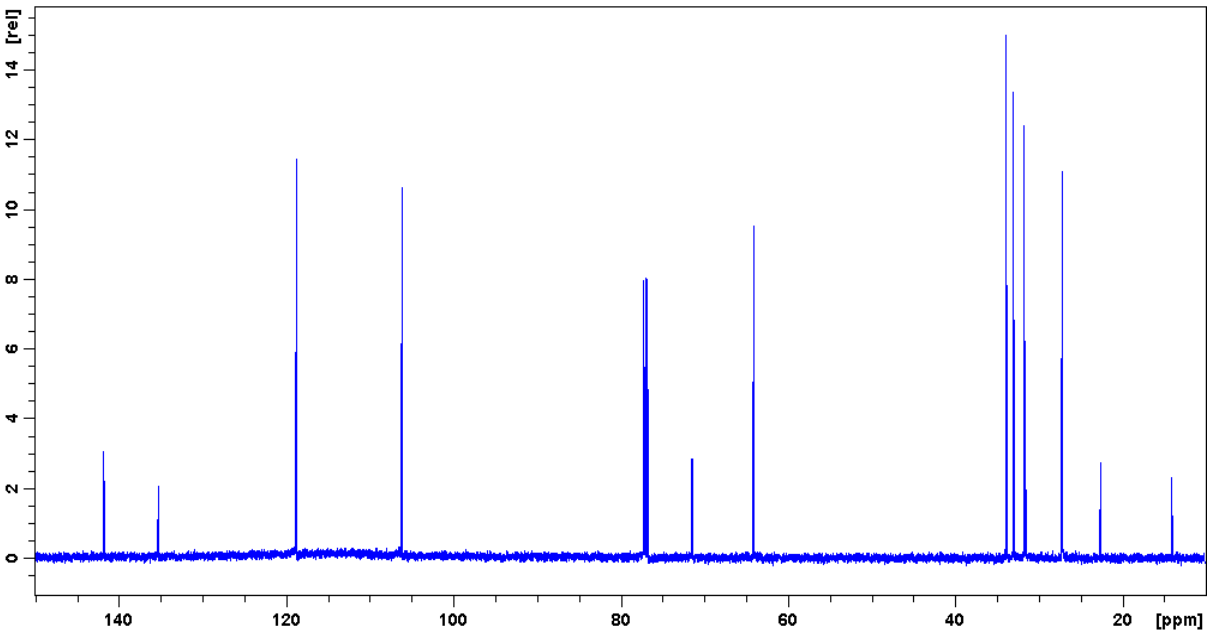


Figure S3.2: ^{13}C spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

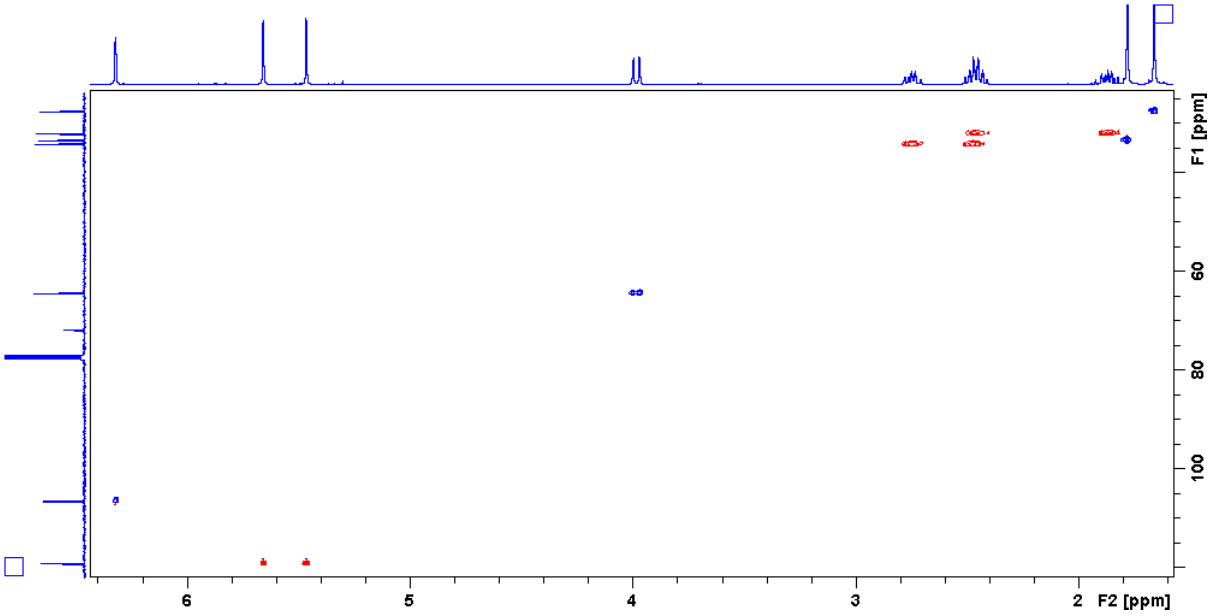


Figure S3.3: HSQC spectrum ($CDCl_3$, 600 MHz) of compound **1.6**

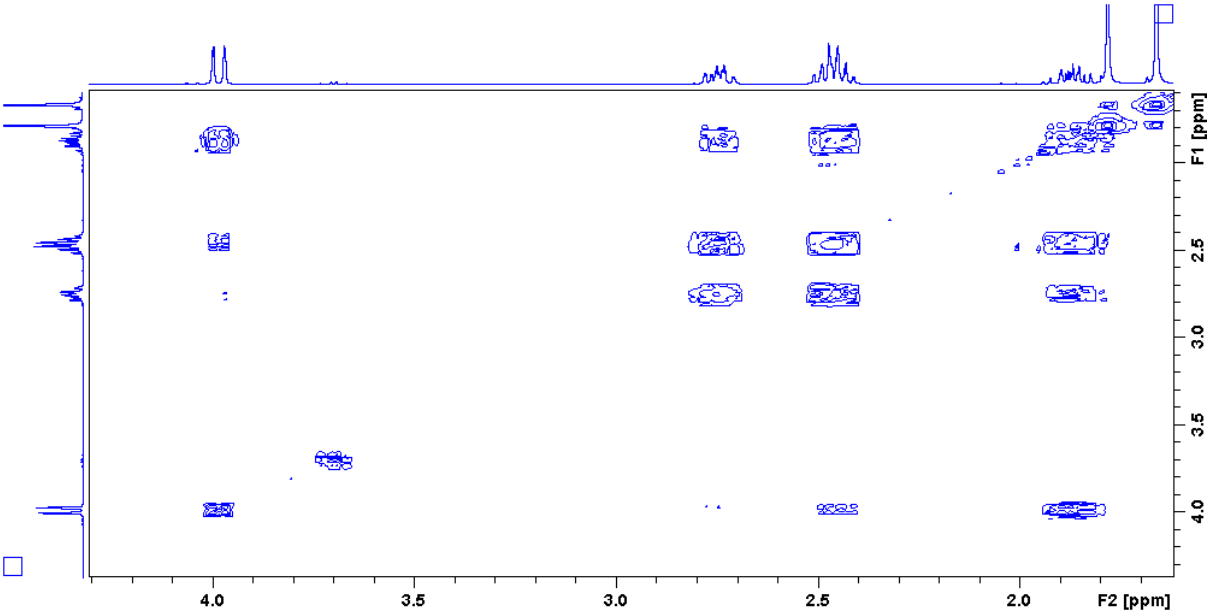


Figure S3.4: COSY NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.6**

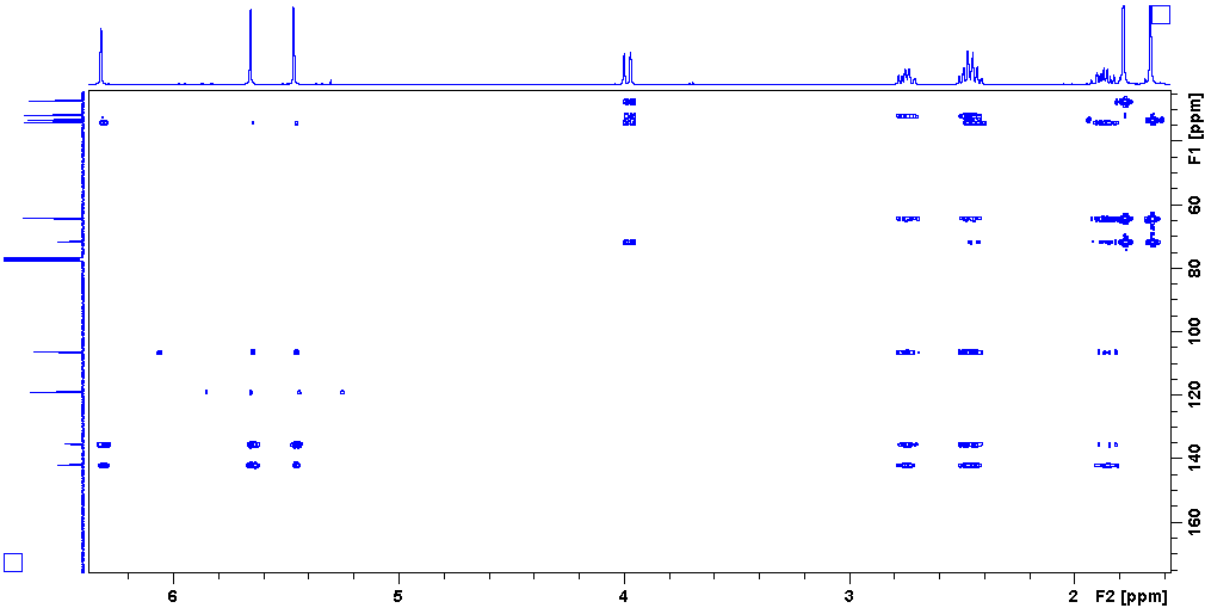
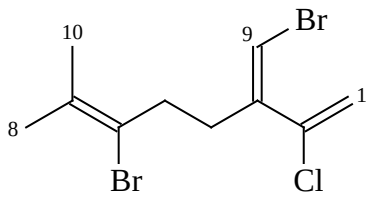


Figure S3.5: HMBC spectrum ($CDCl_3$, 600 MHz) of compound **1.6**



1.7

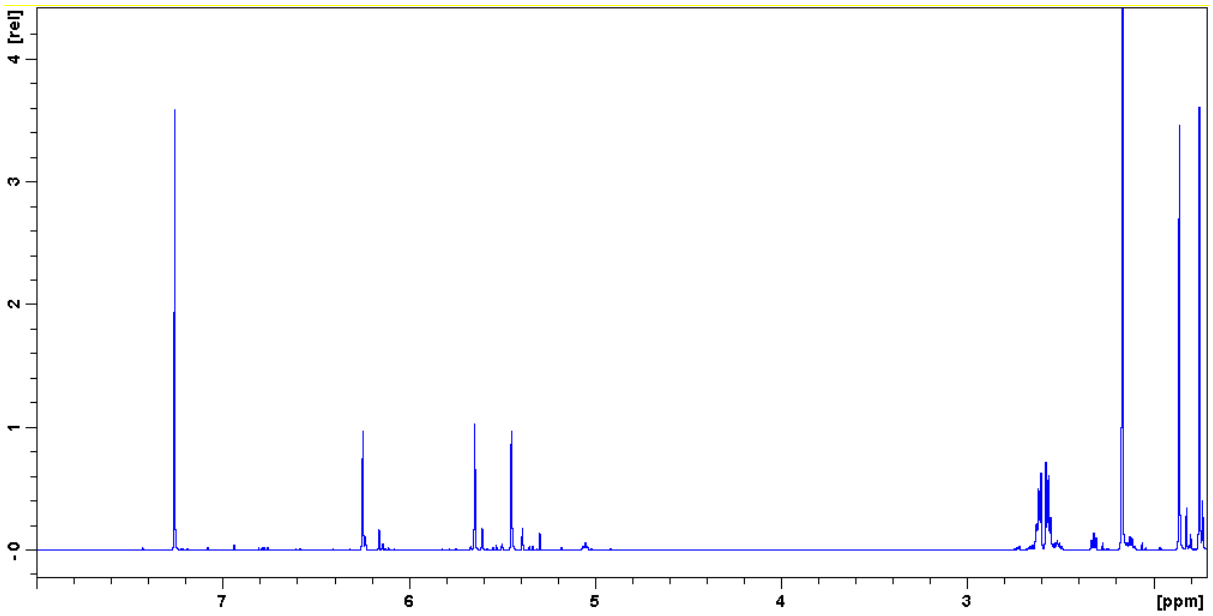


Figure S3.6: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.7**

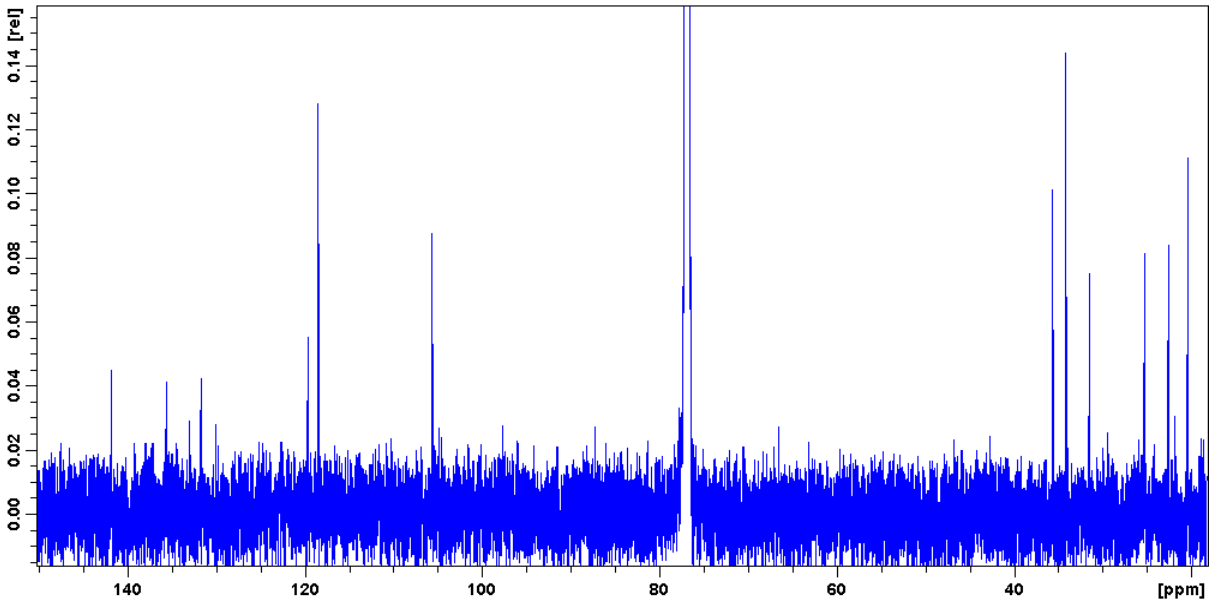
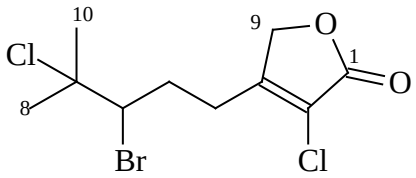


Figure S3.7: ^{13}C NMR spectrum ($CDCl_3$, 600 MHz) of compound **1.7**



3.1

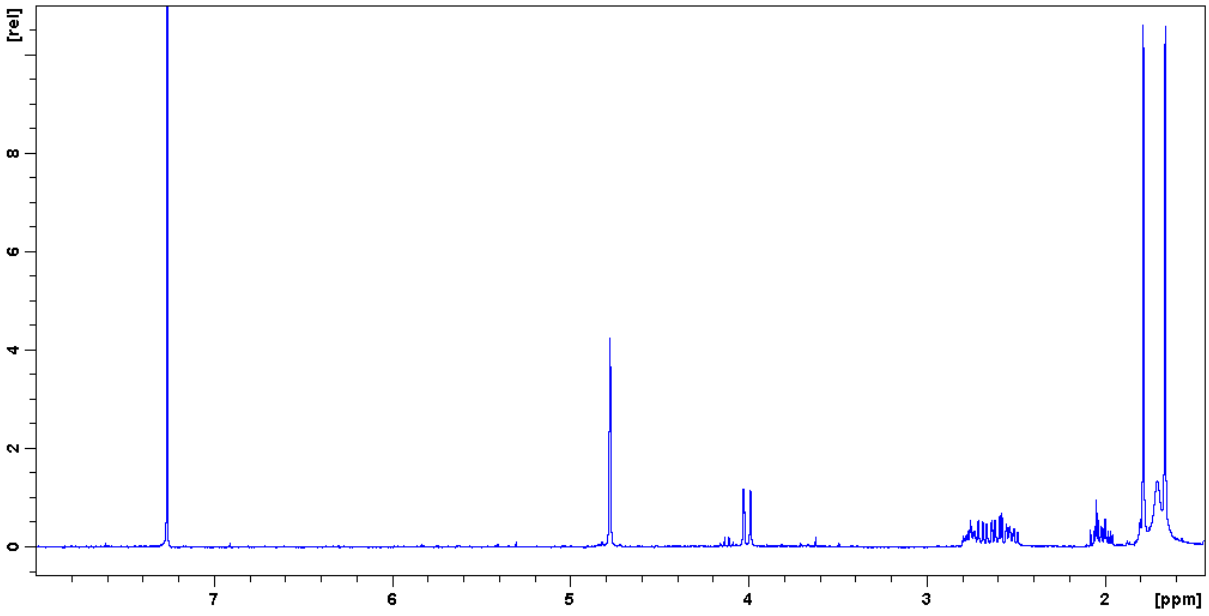


Figure S3.8: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

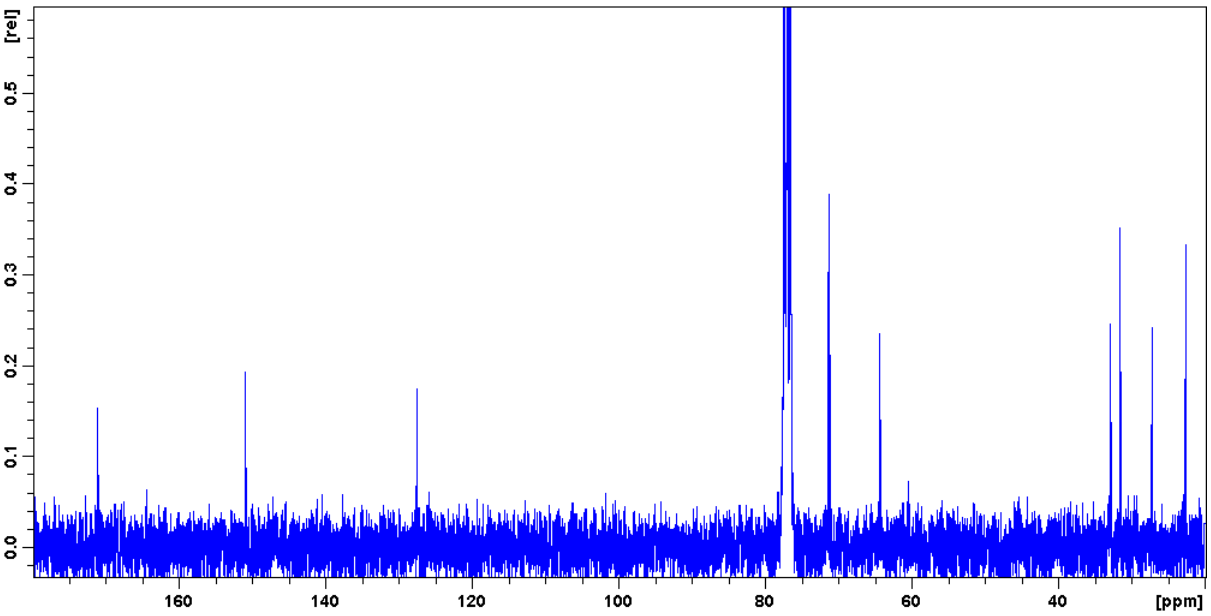


Figure S3.9: ^{13}C NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

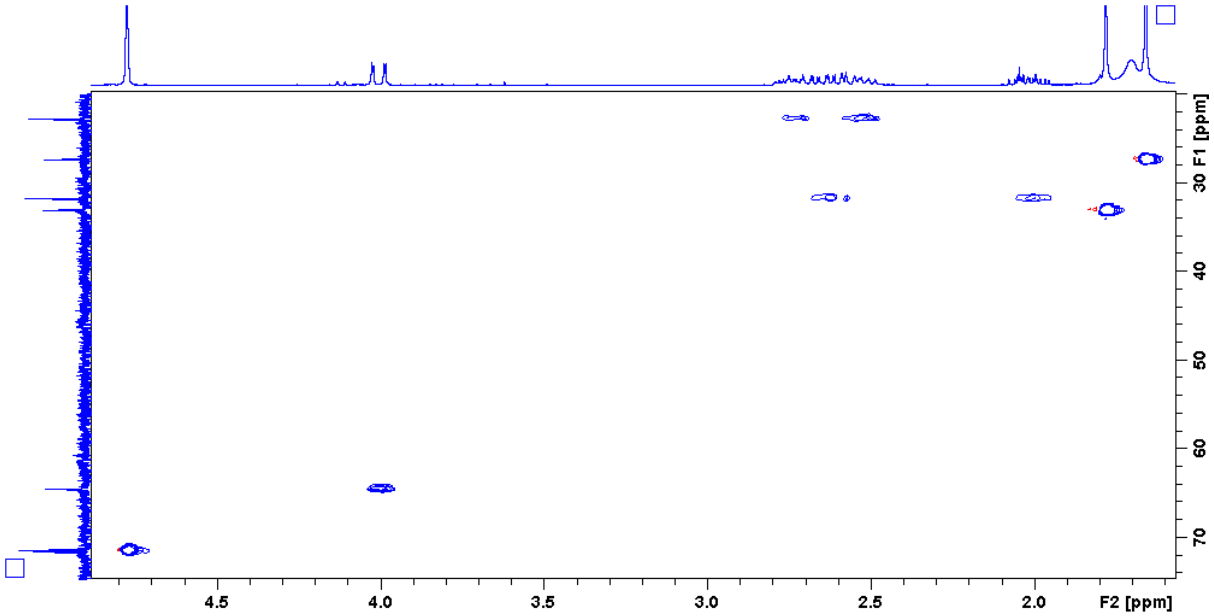


Figure S3.10: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

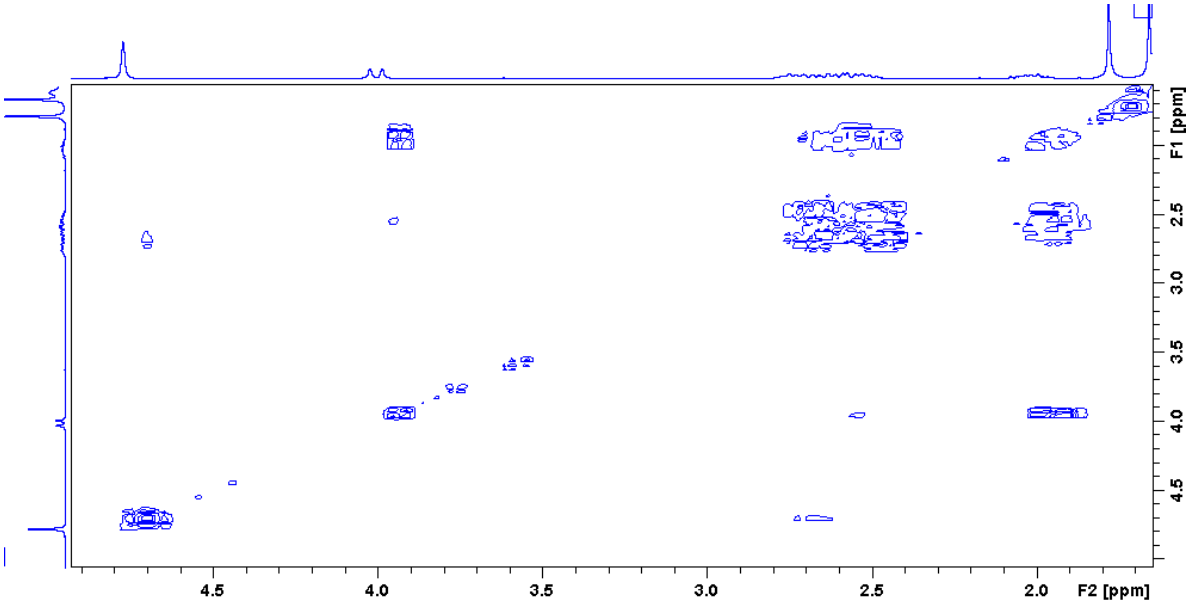


Figure S3.11: COSY NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

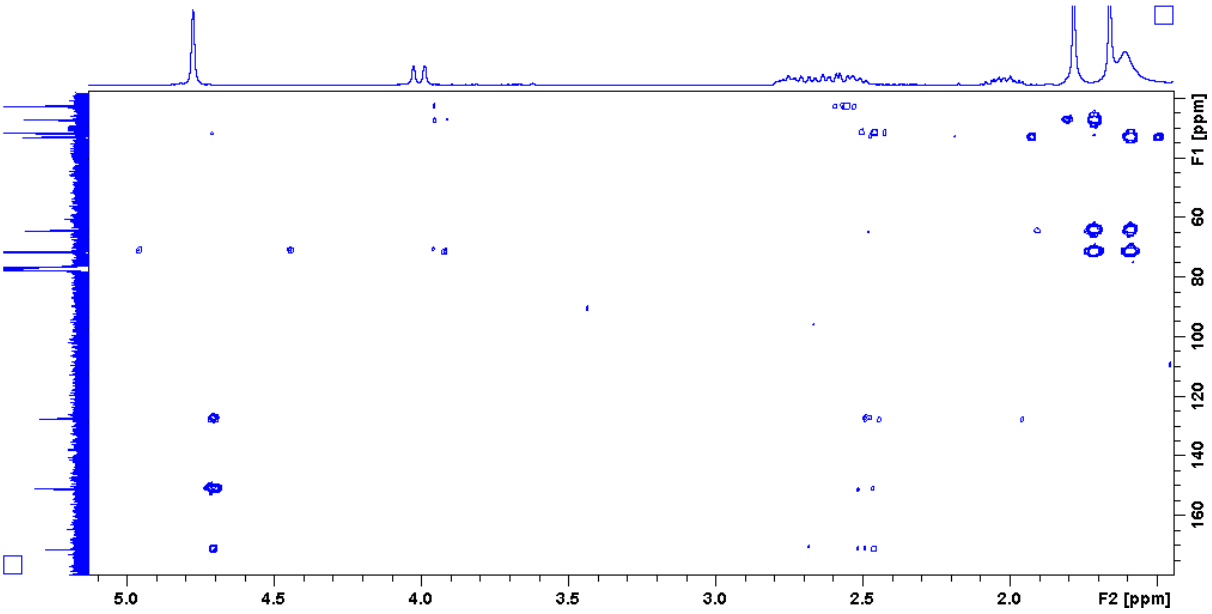


Figure S3.12: HMBC NMR spectrum ($CDCl_3$, 300 MHz) of compound **3.1**

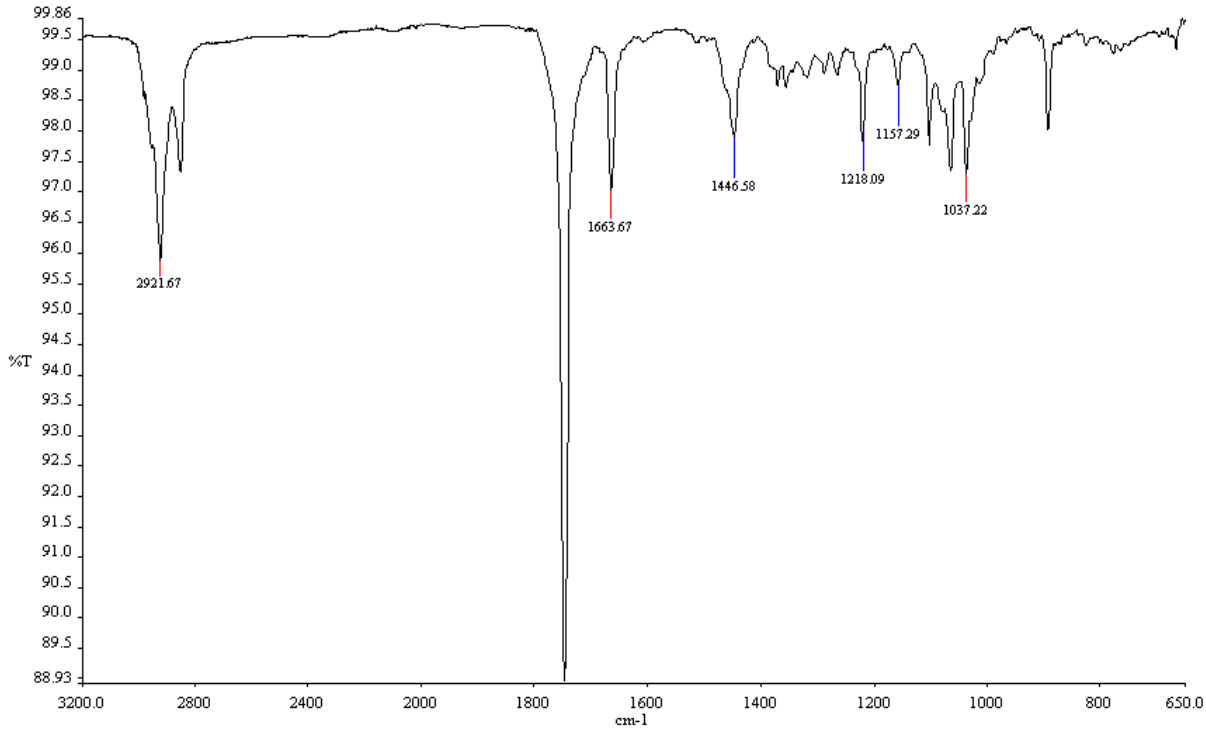


Figure S3.13: IR spectrum of compound **3.1**

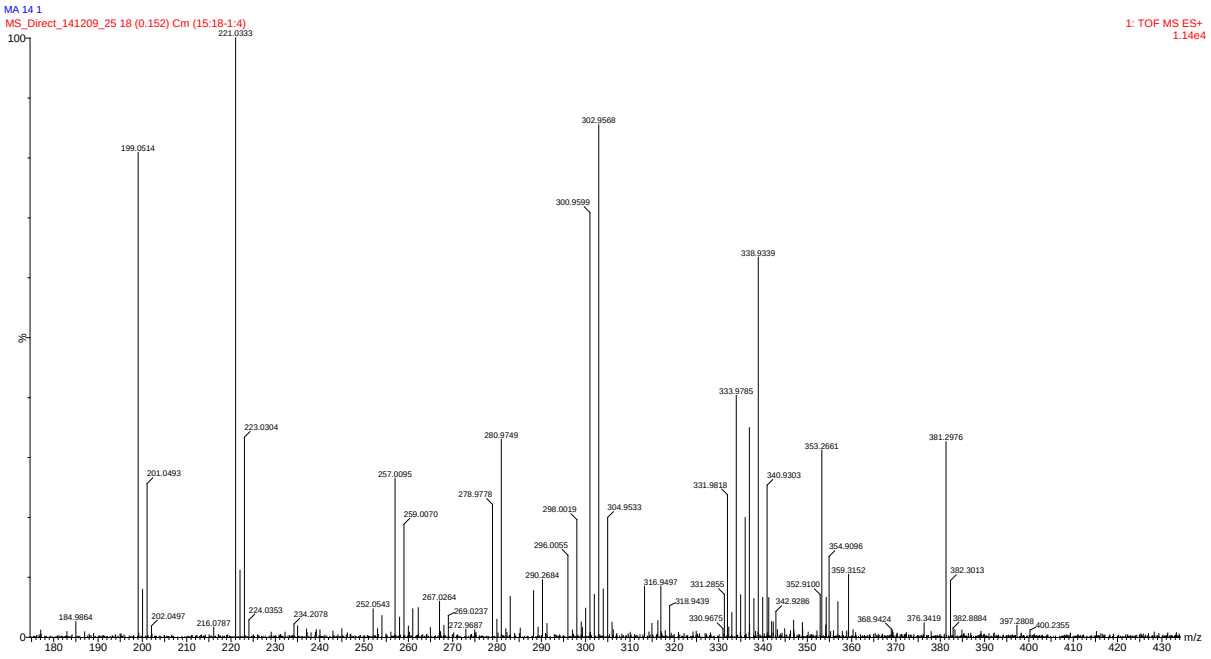
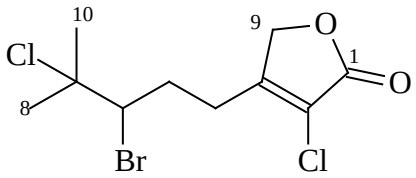


Figure S3.14: HRESIMS spectrum of compound **3.1**



3.2

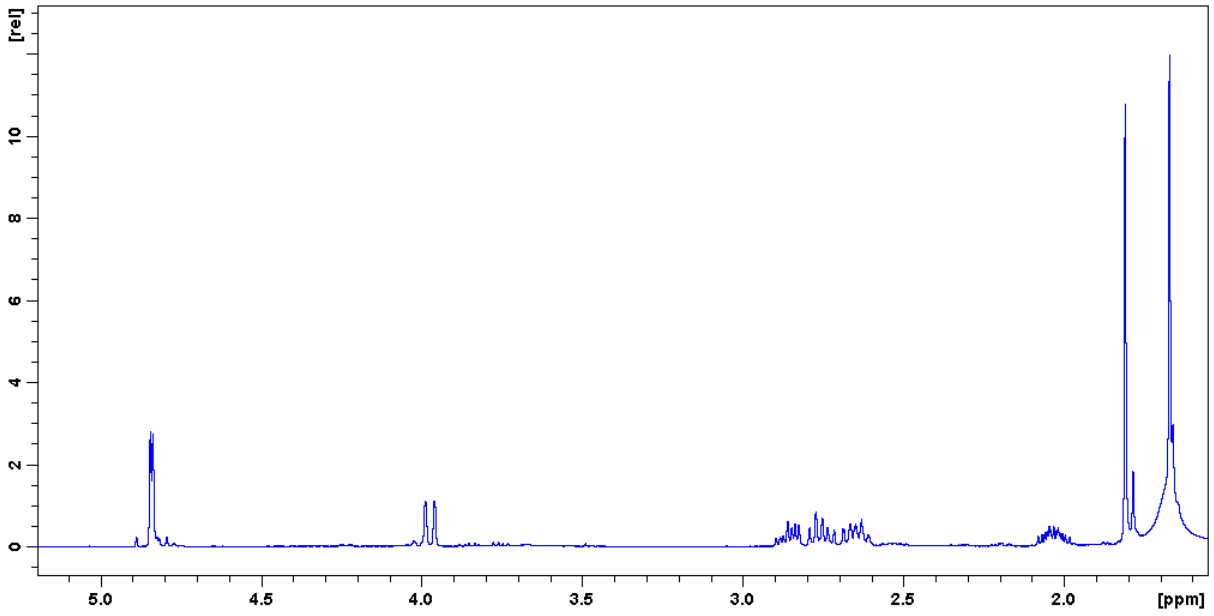


Figure S3.15: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

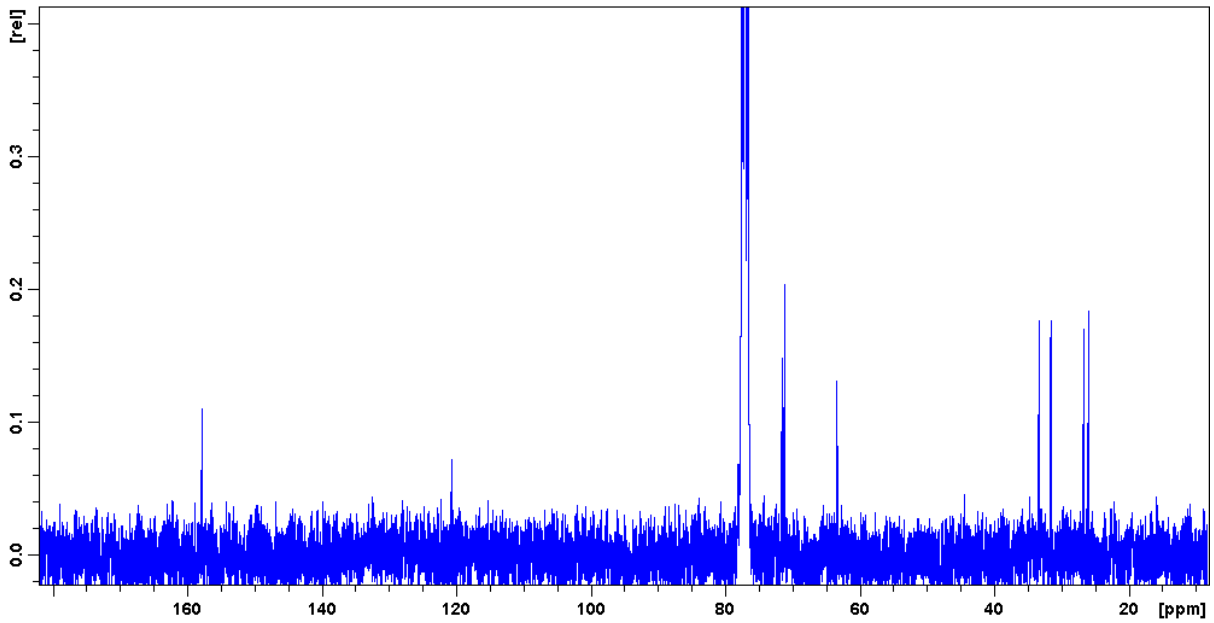


Figure S3.16: ^{13}C NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

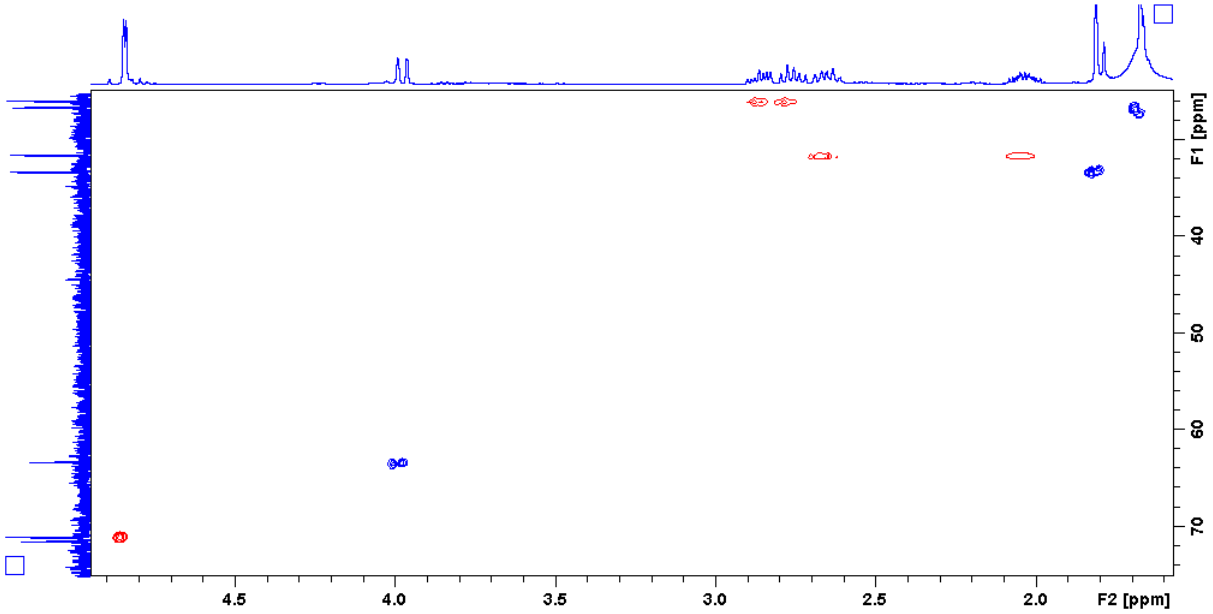


Figure S3.17: HSQC NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

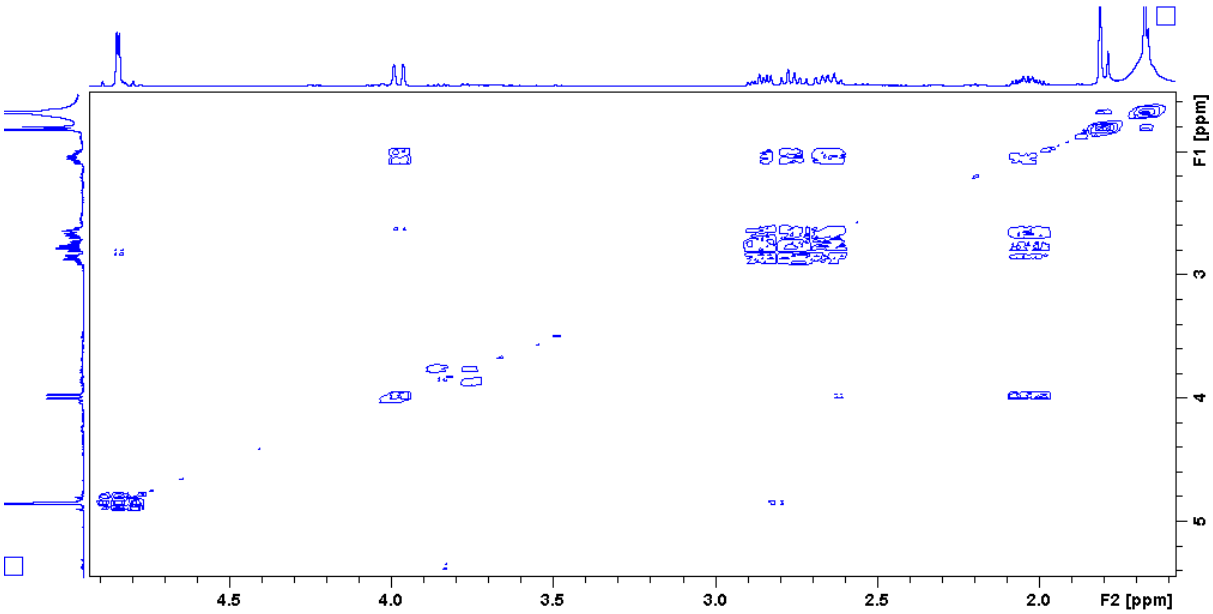


Figure S3.18: COSY NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

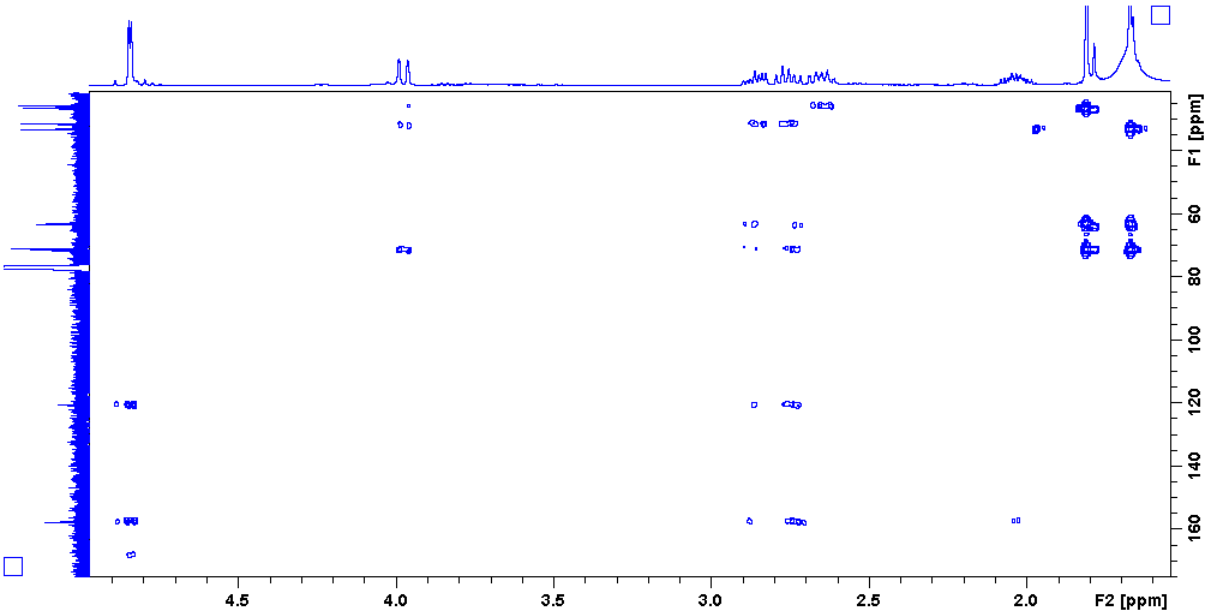


Figure S3.19: HMBC NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.2**

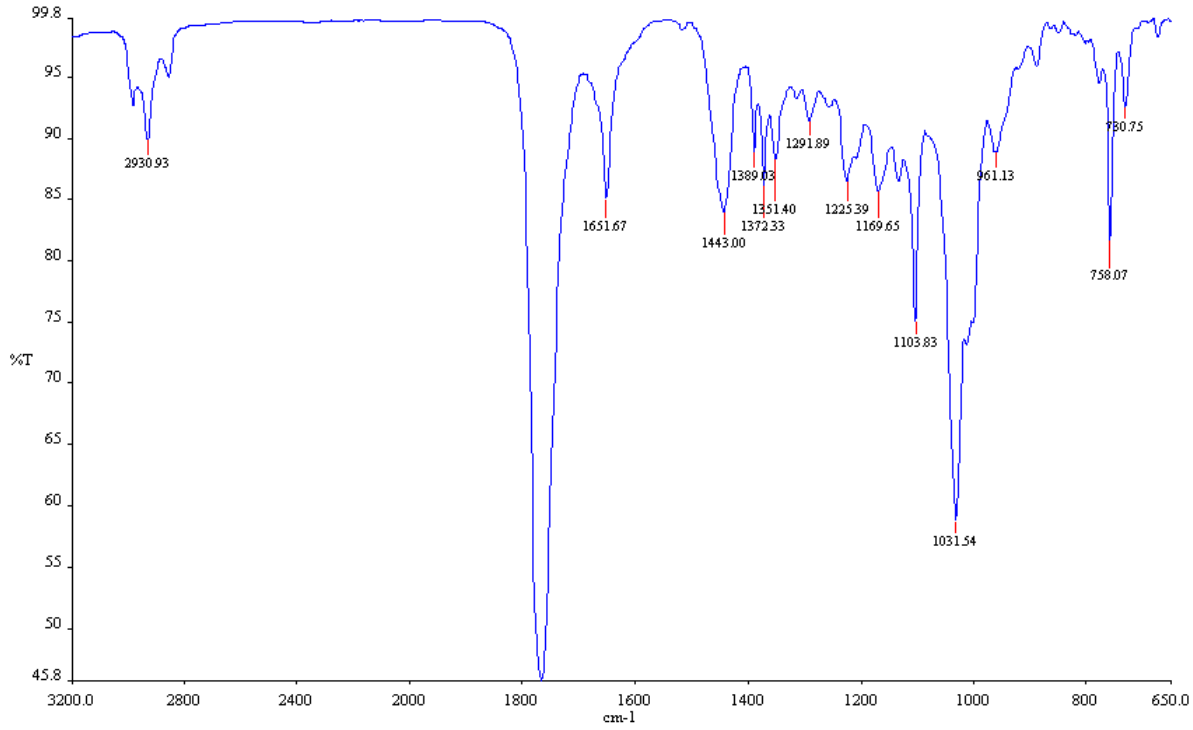


Figure S3.20: IR spectrum of compound **3.2**

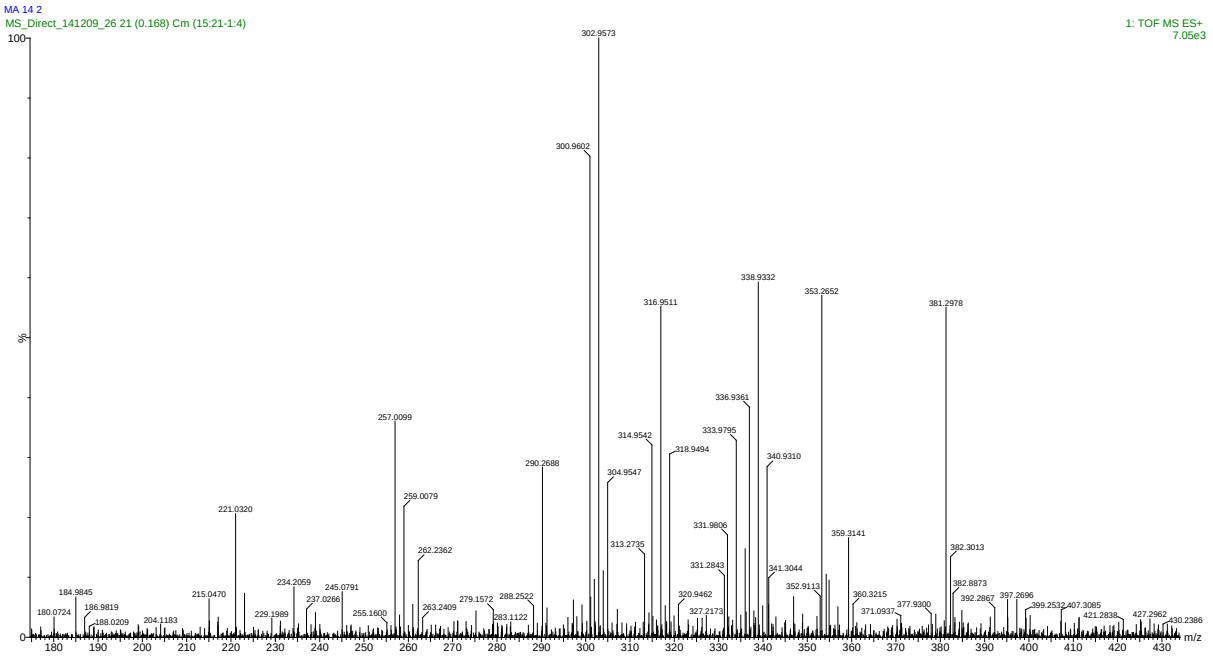


Figure S3.21: HRESIMS spectrum of compound **3.2**

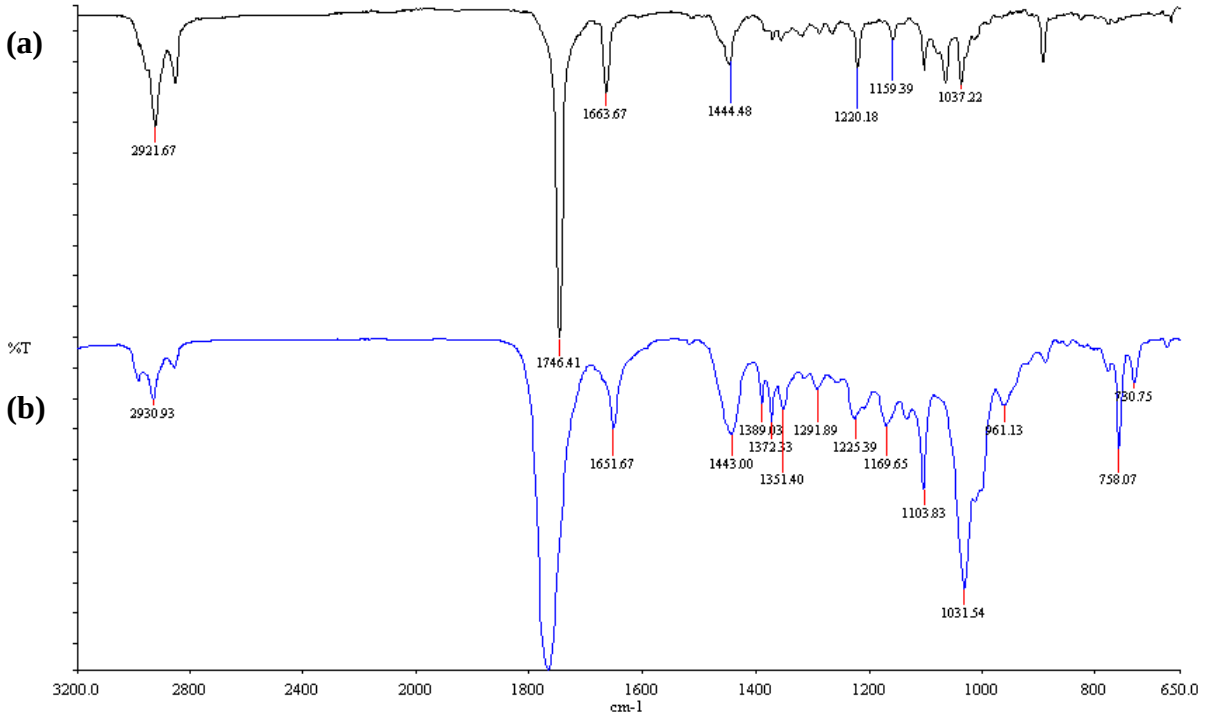
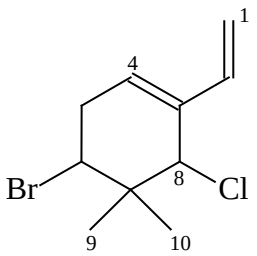
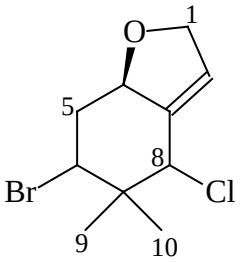


Figure S3.22: Comparison of IR spectrum of compounds **3.1 (a)** and **3.2 (b)**

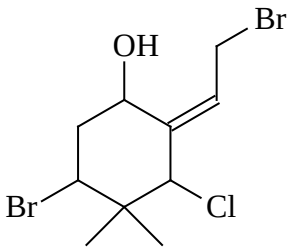
Chapter 4



1.25



1.27



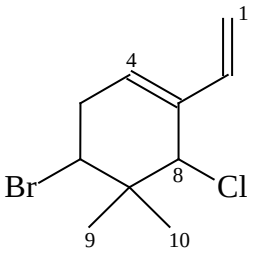
4.1

Table S3.3: ^{1}H NMR data ($CDCl_3$) for compounds 1.25, 1.27 and 4.1.

Compound	H1	H2	H4	H5	H6	H8	H9	H10
1.25	5.14, (d, 11.1) 5.33, (d, 17.5)	6.24, (dd, 11.1 17.6)	5.64, (tr, 4.03)	2.71, (m) 2.93, (d of tr, 19.4, 5.75)	4.60, (dd, 6.27, 11.04)	4.51, (s)	1.33, (s)	1.08, (s)
1.27	4.72, (dd, 5.01, 1.43)	5.78 (m)	4.98 (m)	2.05 (m) 2.65 (m)	4.64 (s)	4.42 (dd, 13.06, 4.01)	1.29, s	1.09, s
4.1	4.46, (ddd, *J* = 11.85, 7.70, 1.20) 4.64, (ddd, *J* = 12.25, 7.91, 1.04)	5.77, (td, *J* = 15.55, 1.72)	4.94, (m)	2.22, (m) 2.53, (m)	4.44, (dd, J = 12.25, 5.10)	4.38, (s)	1.22, (s)	1.12, (s)

Table S3.4: ^{13}C NMR data ($CDCl_3$) for compounds 1.25, 1.27 and 4.1

Compound	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
1.25	113.3	135.6	136.3	129.3	36.0	55.1	39.5	63.0	28.2	19.7
1.27	75.5	122.5	137.8	80.8	41.6	54.6	41.8	63.8	27.6	21.0
4.1	39.6	126.1	139.7	69.4	42.8	55.0	42.2	73.6	26.6	21.9



1.25

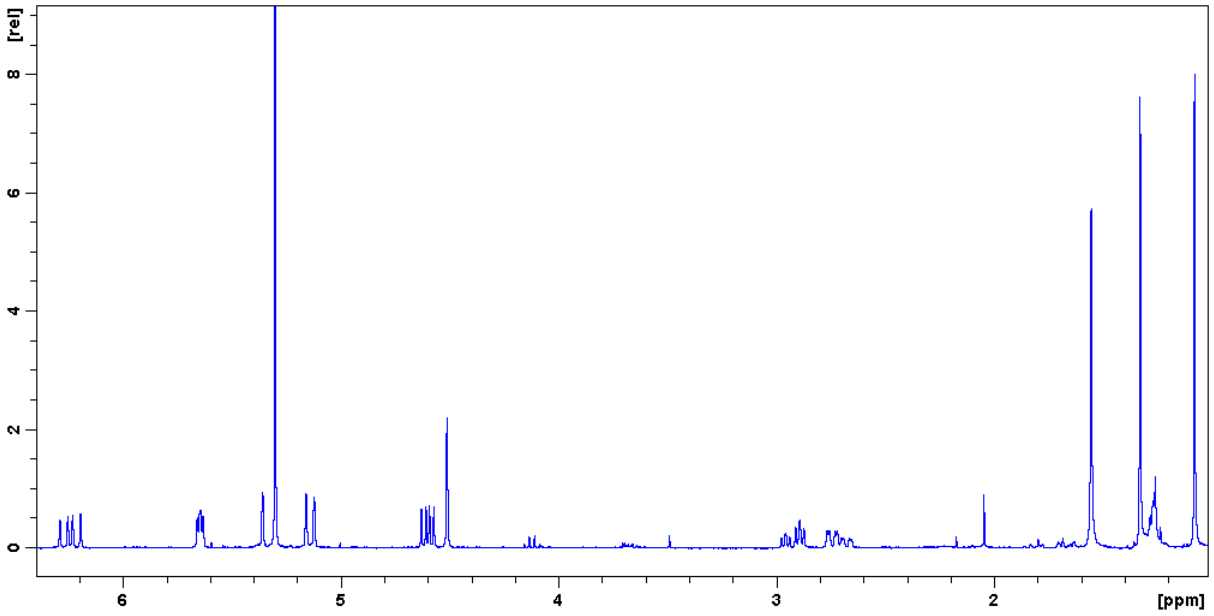


Figure S3.23: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**.

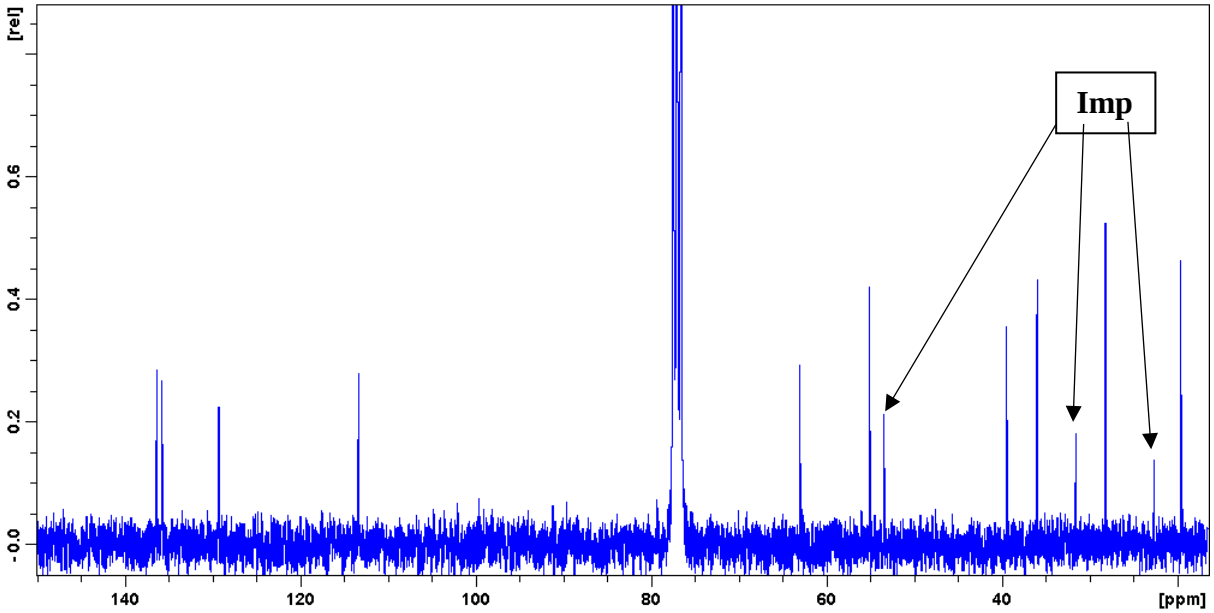


Figure S3.24: ^{13}C spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

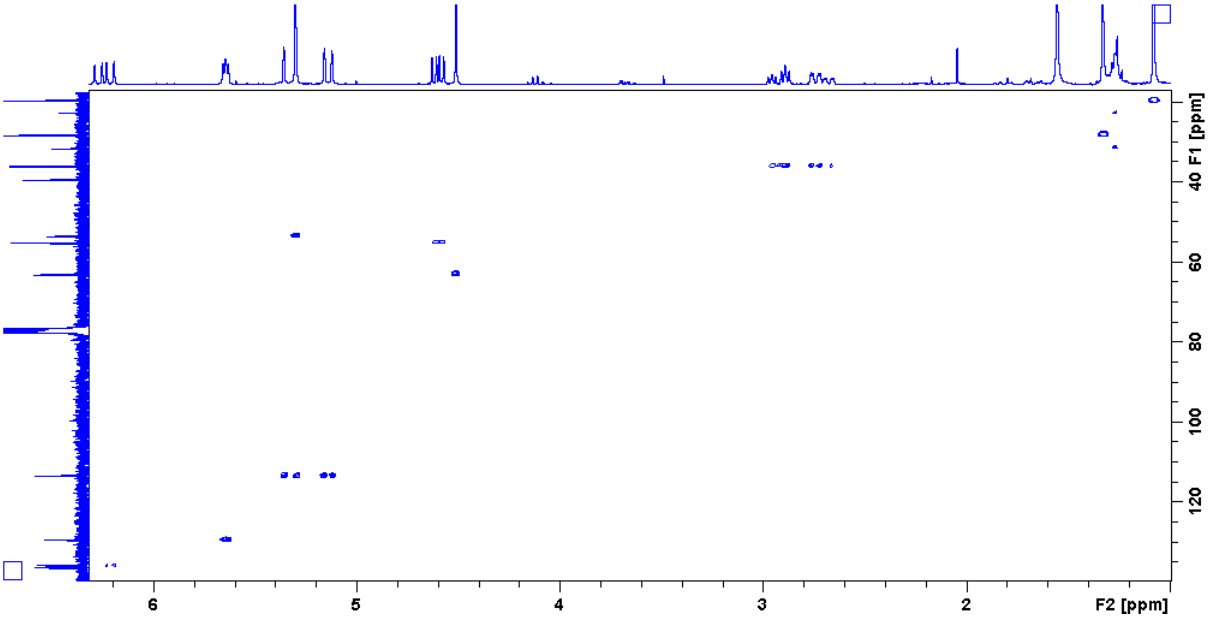


Figure S3.25: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

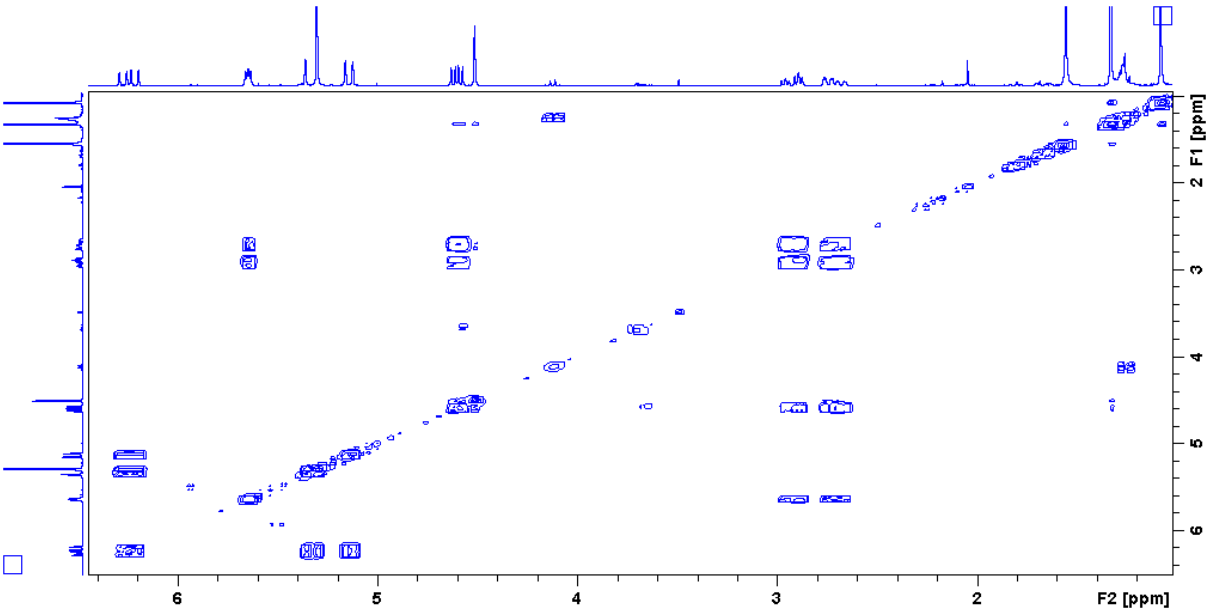


Figure S3.26: COSY spectrum ($CDCl_3$, 300 MHz) of compound **1.25**

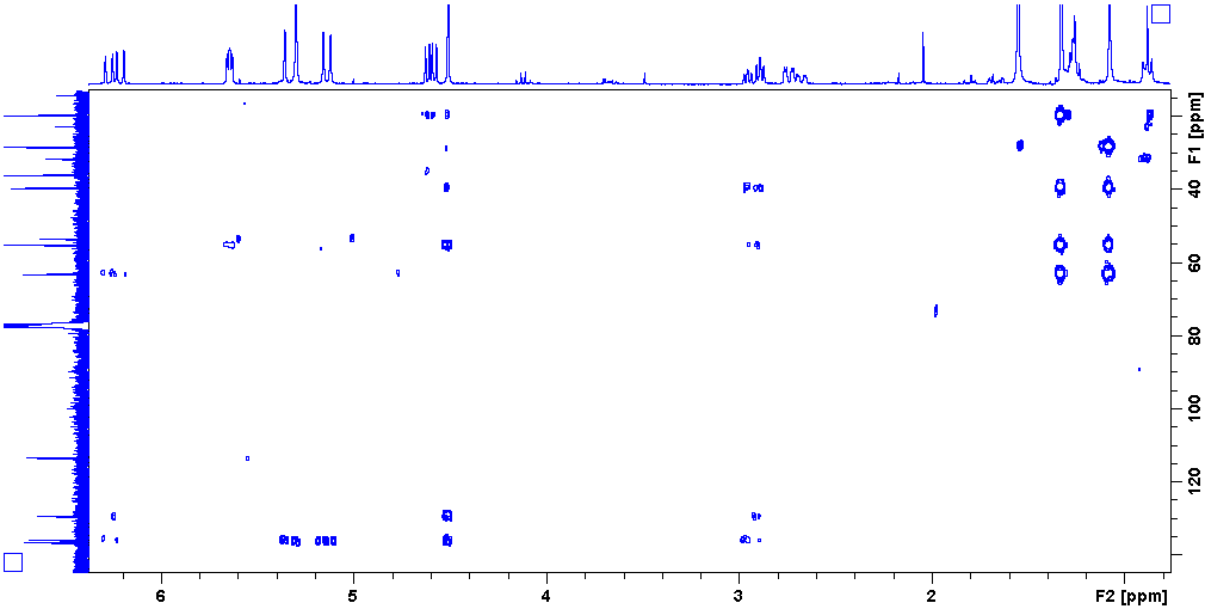
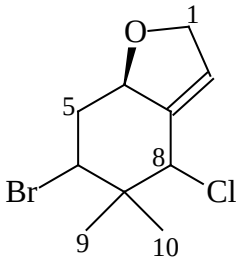


Figure S3.27: HMBC NMR spectrum ($CDCl_3$, 300 MHz) of compound **1.25**



1.27

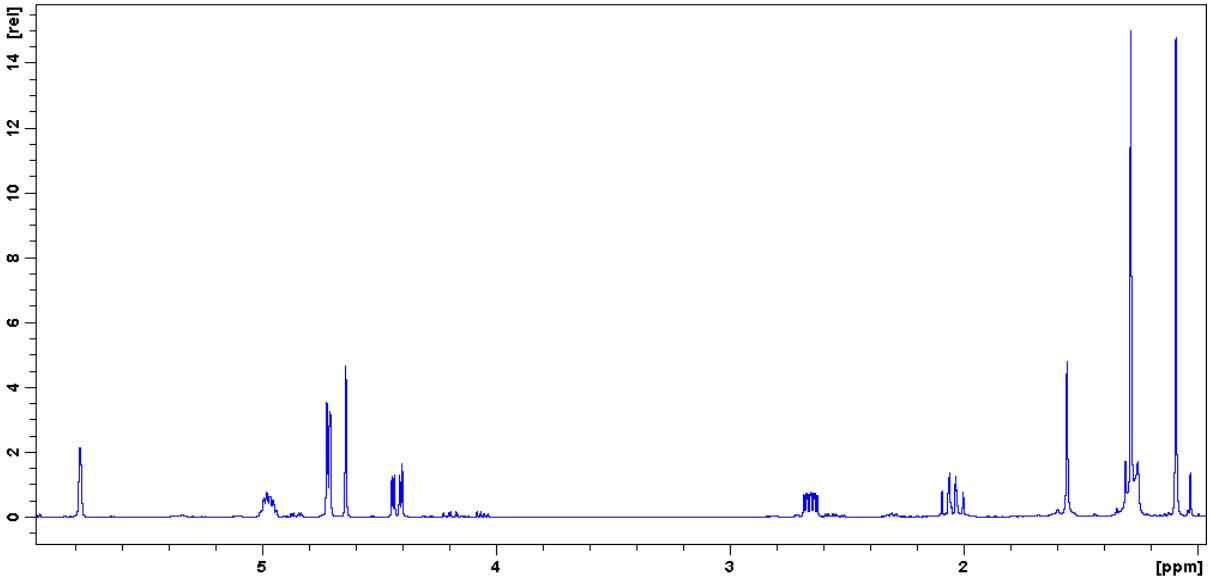


Figure S3.28: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

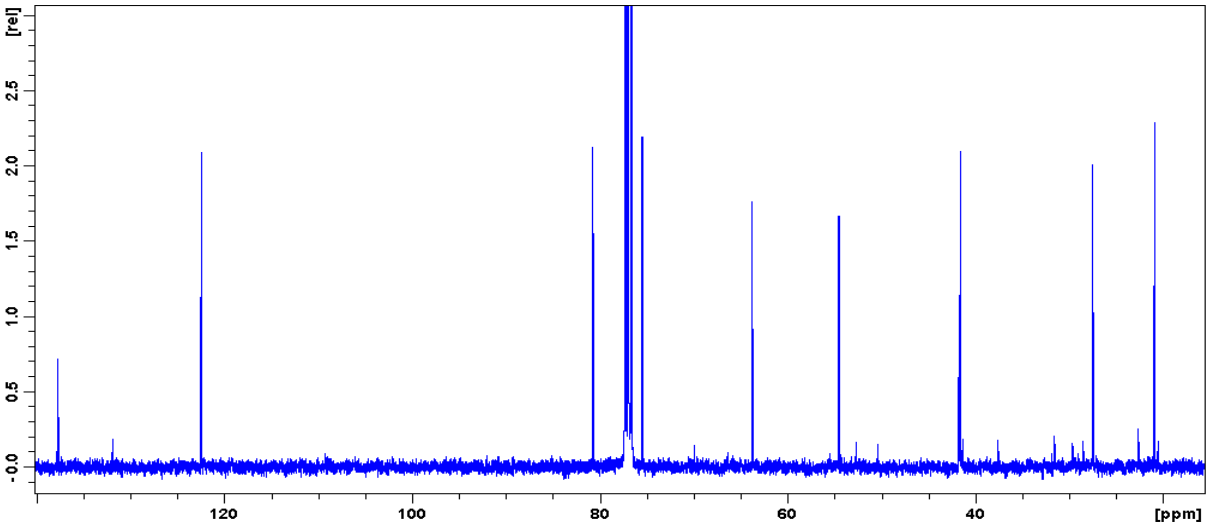


Figure S3.29: ^{13}C spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

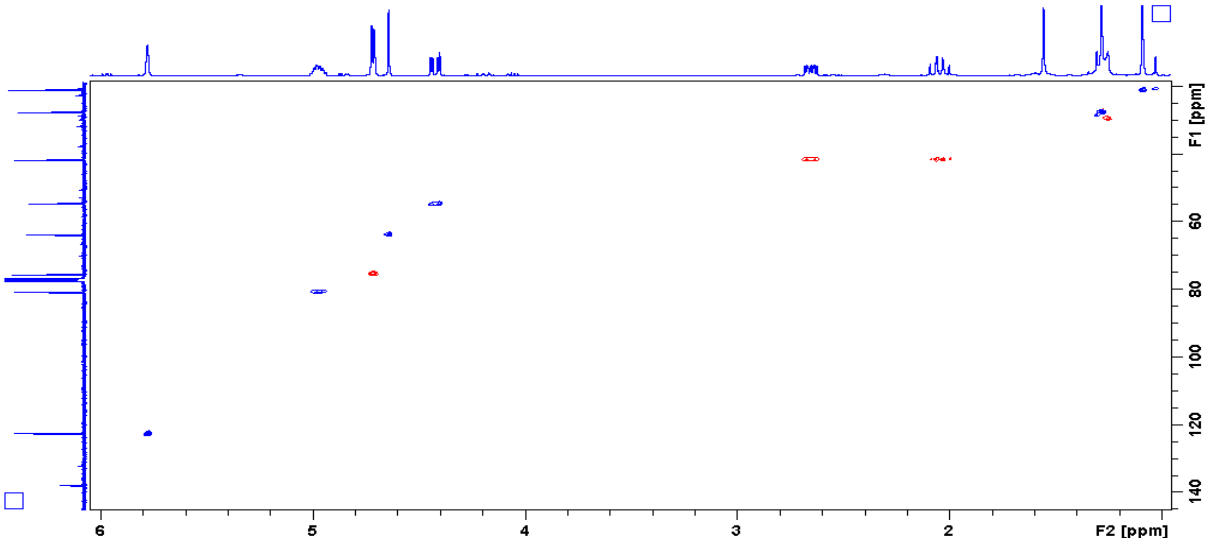


Figure S3.30: Edited HSQC NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

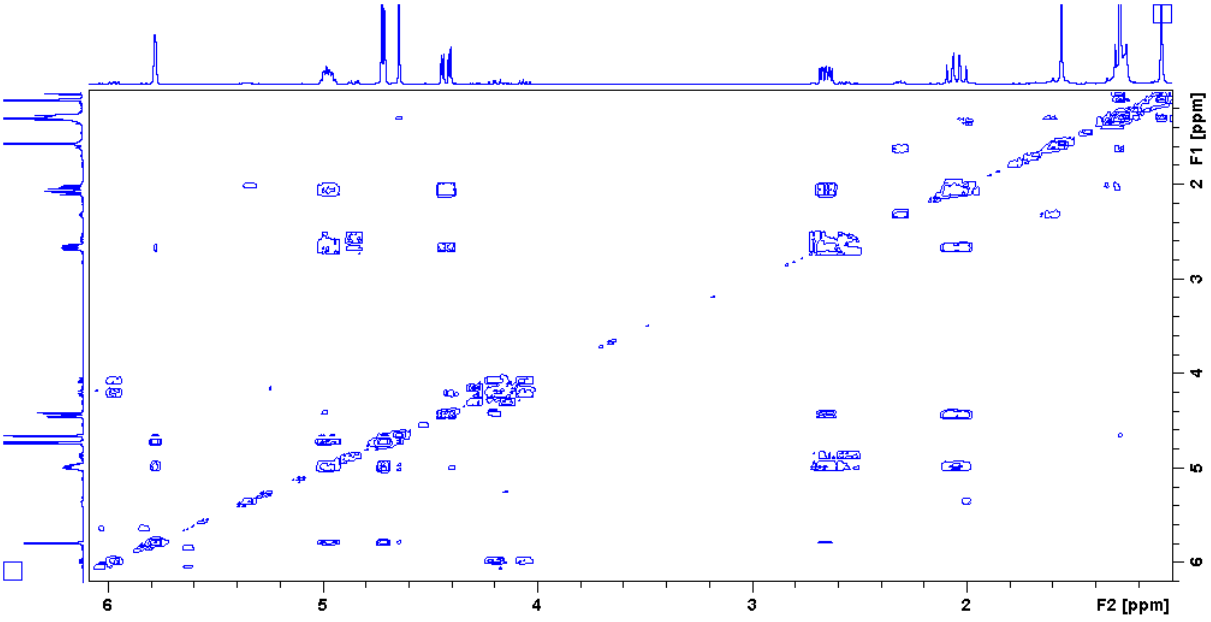


Figure S3.31: COSY spectrum ($CDCl_3$, 400 MHz) of compound **1.27**

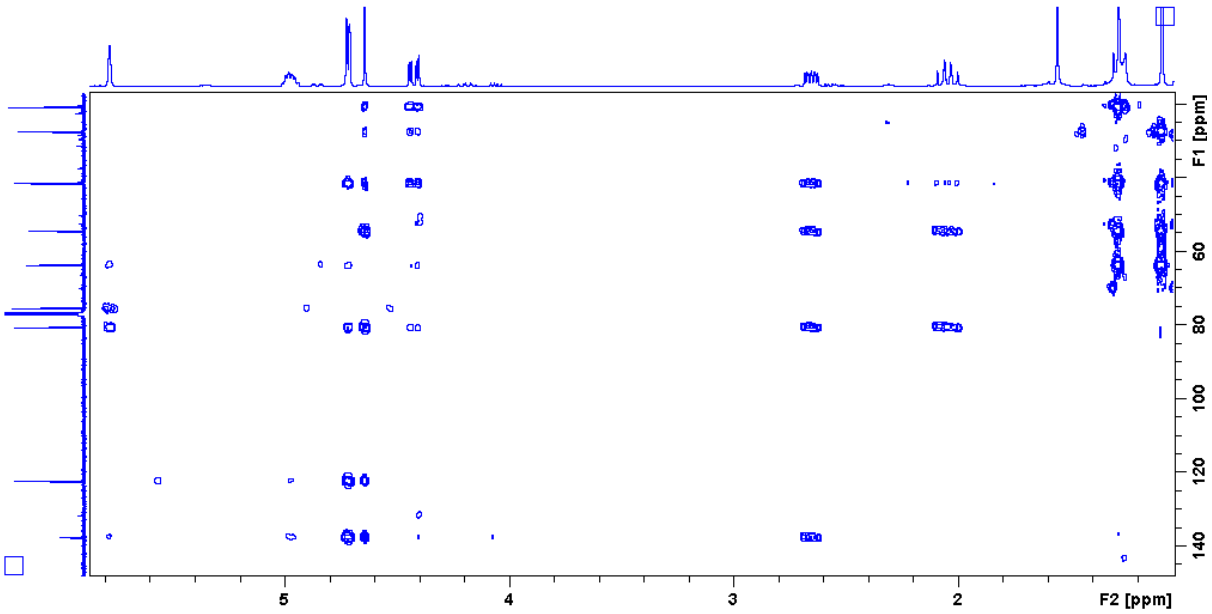
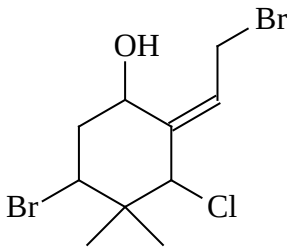


Figure S3.32: HMBC NMR spectrum ($CDCl_3$, 400 MHz) of compound **1.27**



4.1

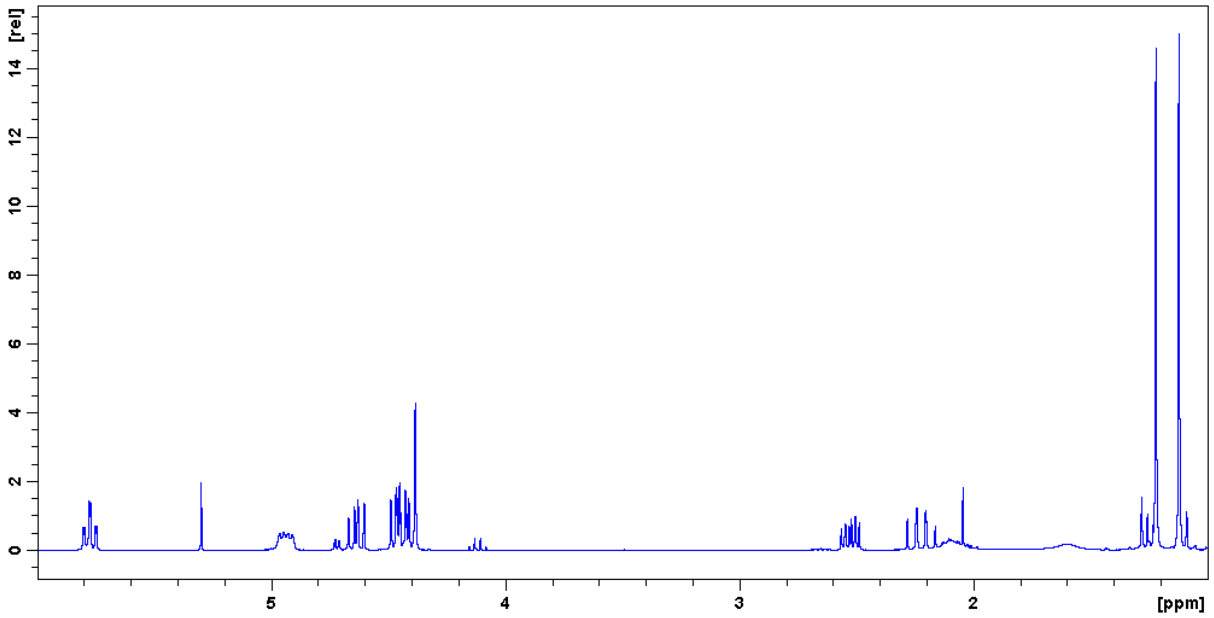


Figure S3.33: ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

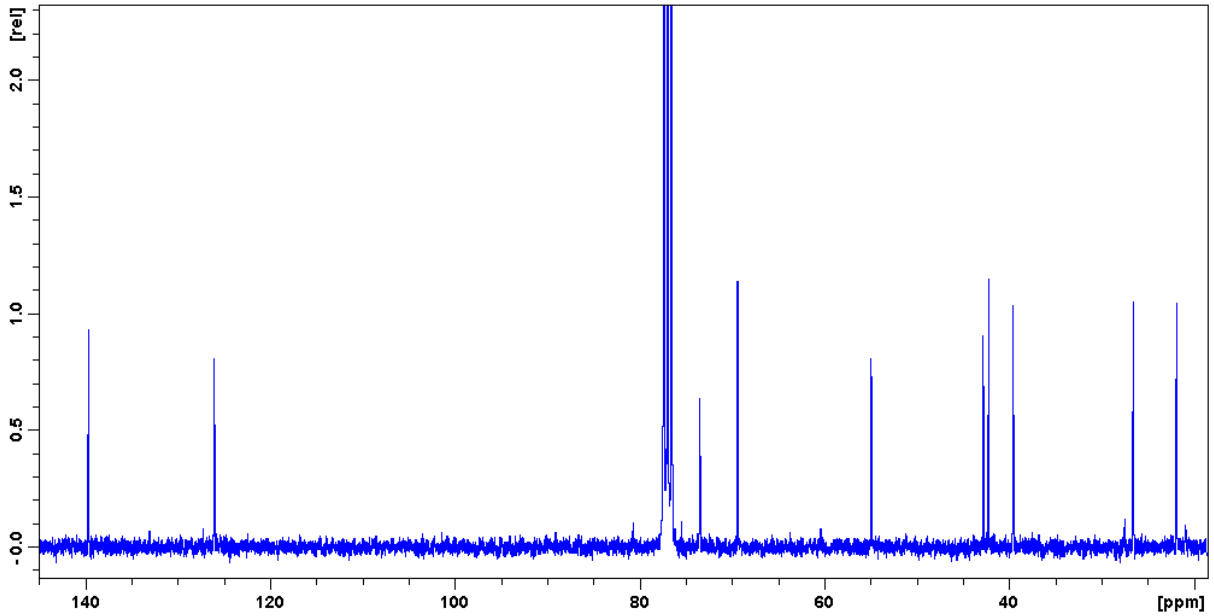


Figure S3.34: ^{13}C NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

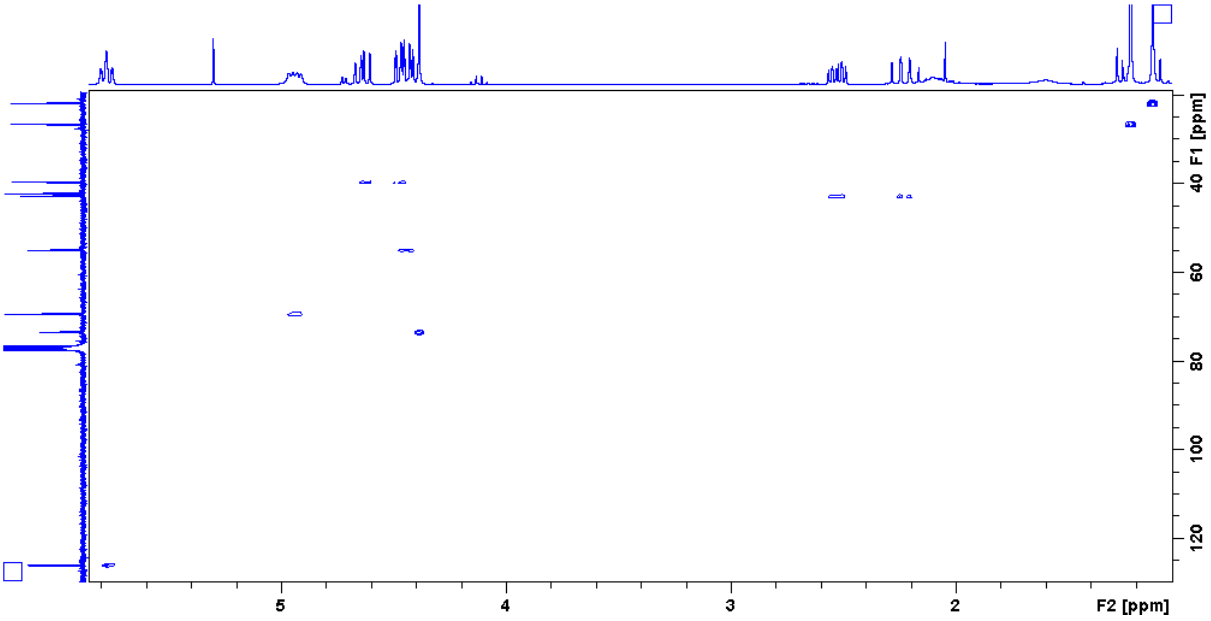


Figure S3.35: HSQC NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

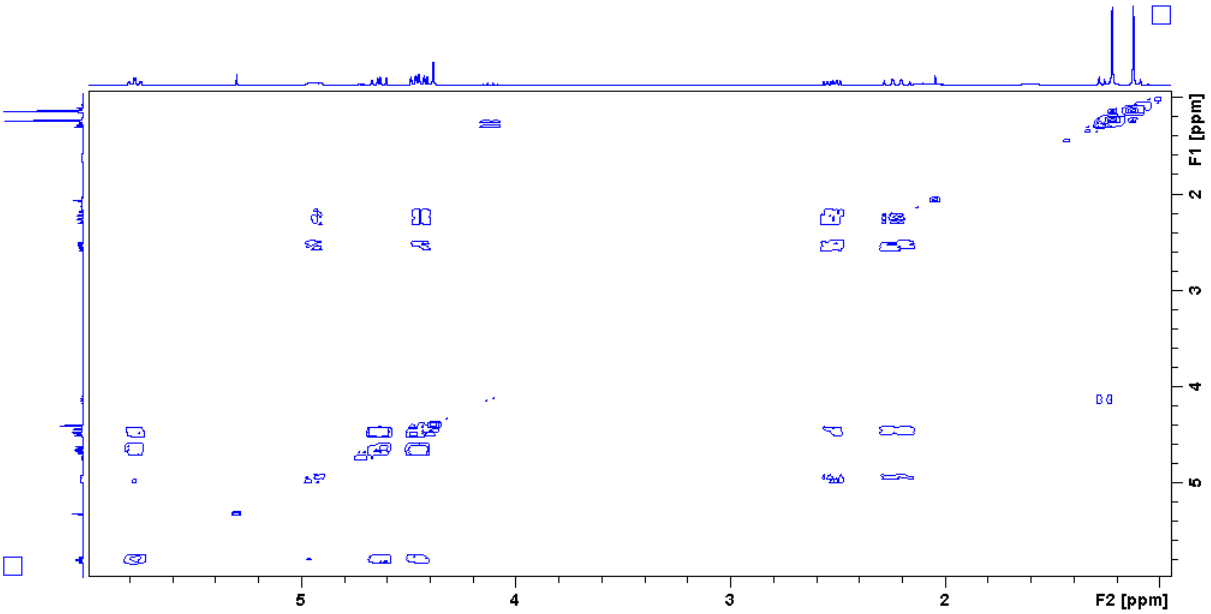


Figure S3.36: COSY NMR spectrum ($CDCl_3$, 300 MHz) of compound **4.1**

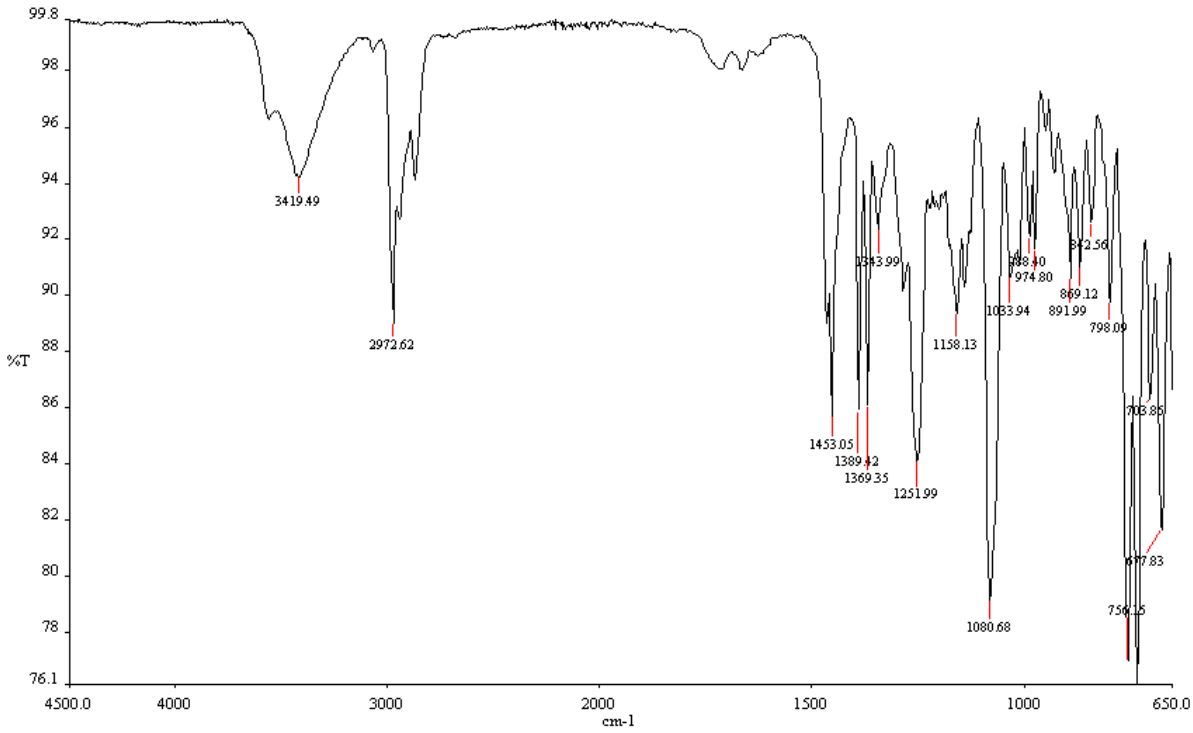


Figure S3.37: IR spectrum of compound **4.1**