

Antimicrobial discovery from South African marine algae

By

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**UNIVERSITY of the
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Declaration

I declare that this thesis “*Antimicrobial discovery from South African marine algae*” is a presentation of my original research work and it has not been submitted for any degree or examination in any other university. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

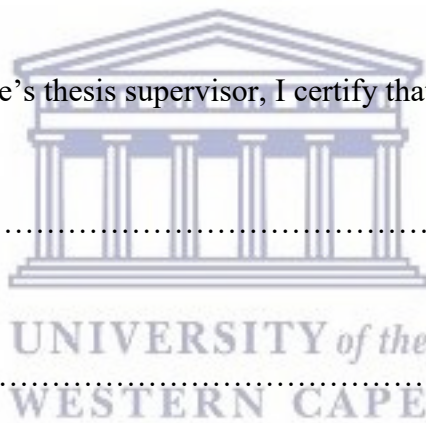
The work was done under the guidance of Professor Denzil R. Beukes, at the University of the Western Cape, South Africa.

Edmund Rufaro Mabande

In my capacity as the candidate’s thesis supervisor, I certify that the statements are true to the best of my knowledge.

Prof Denzil R Beukes

Date:.....



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Dedication

For my mother Mrs Mavis Mabande.



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List of abbreviations:

°C	Degrees Celsius
Ara-A	Vidarabine (9-β-D-arabinofuranosyladenine)
CDCl ₃	Deuterated chloroform
CH ₂ Cl ₂	Dichloromethane
COSY	¹ H- ¹ H homonuclear correlation spectroscopy
DCM	Dichloromethane
DEPT	Distortionless enhancement of polarisation transfer
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's modified eagles's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Eastern Cape
EtOAc	Ethyl acetate
FDA	Food and Drug Administration Agency
GARP	Global Antibiotic Resistance Partnership
GPP	Geranyl pyrophosphate
HEK-293	Human embryonic kidney
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMPV	Human metapneumovirus
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
HSV	Herpes simplex virus
HTS	High-throughput screening
Hz	Hertz
IC ₅₀	Half maximal inhibitory concentration
IPP	Isopentenyl pyrophosphate

IR	Infrared radiation
<i>J</i>	Spin-Spin coupling constant (Hz)
KZN	KwaZulu Natal
LD ₅₀	Lethal dose 50
MCF-7	Michigan Cancer Foundation-7
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MIC	Minimum inhibitory concentration
MVA	Mevalonic acid
MEP	2-C-methylerythritol 4-phosphate
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NADPH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear overhauser enhancement spectroscopy
OD	Optical density
PBS	Phosphate buffered saline
SCUBA	Self-contained underwater breathing apparatus
TB	Tuberculosis
TLC	Thin layer chromatography
UTI	Urinary tract infection
WST-1	Water-soluble tetrazolium salt
XDR-TB	Extensively drug-resistant tuberculosis
δ	Chemical shift (ppm)
d	Doublet
dd	Doublet of a doublet
m	Multiplet
Multi	Multiplicity
q	Quartet
s	Singlet

Abstract:

Antimicrobials are chemical compounds that destroy or inhibit the growth of microorganisms. The majority of these antimicrobials are actually natural products or natural product derived with key examples being the pioneer antibiotics penicillin and cephalosporin. Antimicrobials are an extremely important class of therapeutic agents; however, the development of drug resistance and slow pace of new antibiotic discovery is one of the major health issues facing the world today. There is therefore a crucial need to discover and develop new antibacterial agents. In this study, the potential of marine algae as a source of new antibiotics was explored.

Crude organic extracts and chromatographic fractions obtained from small-scale extraction of 17 different marine algae were used to prepare a pre-fractionated library that would be tested against several disease causing microorganisms. The activity of the pre-fractionated library and purified compounds was determined against a panel of drug resistant microorganisms namely *Acinetobacter baumannii* ATCCBAA[®]-1605[™], *Enterococcus faecalis* ATCC[®] 51299[™], *Escherichia coli* ATCC[®] 25922[™], *Staphylococcus aureus* subsp. *aureus* ATCC[®] 33591[™] and *Candida albicans* ATCC[®] 24433[™]. Finally, cytotoxicity tests of 50 selected library extracts and isolated compounds were done against two cell lines namely MCF-7 (breast cancer) and HEK-293 (kidney embryonic).

Based on their antimicrobial activity and interesting chemical profiles, the seaweeds *Plocamium* sp. and *Stypodium multipartitum* were selected for further study. Three new and unusual halogenated monoterpenes (**4.16**, **4.17** and **4.18**) were isolated from *Plocamium* sp., and an unusual meroditerpenoid (**5.8**) was isolated from *Stypodium multipartitum*. The metabolites were purified using preparative (silica gel) chromatography as well as semi-preparative normal phase HPLC. The structures of purified compounds were determined from spectroscopic data, including nuclear magnetic resonance (NMR) spectroscopy.

A small library of 153 fractions was generated from collections of South African marine algae. Pre-fractionated crude extracts showed excellent antimicrobial activity against all microbes but particularly against *Staphylococcus aureus*. The compounds were generally active against the Gram positive bacteria and the yeast. In conclusion, three antimicrobial halogenated monoterpenes and an unusual monoterpene were isolated from a *Plocamium* sp. and *Stypodium multipartitum* respectively. Antimicrobial activity of crude fractions was excellent but that of isolated compounds was not as great as anticipated.

Chapter 1

The need for new antibiotics

1.1 Background

Antibiotics are an extremely important class of therapeutic drugs. The term antibiotic generally refers to drugs that treat several different infections caused by bacteria but not viral infections such as influenza. Common diseases that are treated by antibiotics include urinary tract infections (UTIs), sexually transmitted infections (STIs), acne, otitis media, bronchitis *etc.* In South Africa antibiotics for oral use are not dispensed over-the-counter, but can only be sold or issued with the provision of a valid written or telephonic prescription (Essack *et al.*, 2011).

The discovery of antibiotics began with the serendipitous discovery of the therapeutic potential of penicillin by Sir Alexander Fleming in 1928 (Tan and Tatsumura, 2015). Large scale production of penicillin and the discovery of other antibiotics only began about a decade later, but the effect of this discovery would impact humanity forever. The antibiotic was so crucial during the Second World War, saving millions of lives and potential amputations. The drug was also a quick and effective way to treat venereal diseases such as syphilis and played a crucial role in preventing the spread of venereal disease towards the end of the war (Kardos and Demain, 2011). The discovery of penicillin changed the face of the global medical field; death threatening infections such as bacterial meningitis could now be cured (Borchardt, 2004). Today we cannot dare to imagine a world without antibiotics.

However, due to the frequent and often improper use of these clinically important drugs, the bacteria they are intended to kill develop resistance against them, a term known as antibiotic resistance. The World Health Organisation (WHO) notes antibiotic resistance as one of the greatest threats to global health today (WHO, 2017b). It is a natural phenomenon, which arises when the disease-causing microbe develops a way to protect itself against the antibiotic drug intended to kill it. A bacterial cell usually undergoes genetic mutation or sometimes exchange genes that make it resistant to the prescribed treatment from another resistant bacterium. Frequent and improper use of prescribed antibacterial agents is attributed as the main cause in antibiotic resistance (Ventola, 2015). Once bacteria develop resistance they render the antibiotic ineffective and these bacteria then multiply and make the host sick.

Antibiotic resistance is made worse by poor patient compliance to the prescribed antibiotic treatment course, which ultimately leads to sub-therapeutic doses being taken (Amabile-Cuevas, 2010). The use of counterfeit and substandard antibiotics also perpetuates the problem. Counterfeiting of drugs such as antibiotics is noted as a huge problem in South Africa (Essack *et al.*, 2011).

Antibiotic resistance can develop gradually or can be a spontaneous event. Three critical mechanisms in antimicrobial resistance are the enzymatic breakdown of the antibiotic or antimicrobial agent, changes in the antibiotic's target proteins in the bacterial cell and alterations in the bacterial cell's membrane with regards to permeability of the antibiotic. The effect of enzymatic degradation is apparent in the β -lactam antibiotics, penicillin and cephalosporins. Antibiotic resistance of these crucial antibiotics comes about as a result of the enzymatic hydrolysis by β -lactamase (Dever and Dermody, 1991).

1.2 The global challenge of antibiotic resistance

Antibiotic resistance is linked to high morbidity and mortality rates. Several people worldwide are suffering from the burden caused by drug resistant infections with over two million people infected and around 23 000 dying each year from antibiotic resistant infections in the United States alone (Centers for Disease Control and Prevention, 2016). Early in 2017, a woman from Nevada, United States died from an incurable bacterial infection. The infection did not respond to 26 different antibiotics and the Centres for Disease Control and Prevention (CDC) concluded that no drug currently available on the market could be used to treat her (McCarthy, 2017). Cases of drug-resistant gonorrhoea are found to be increasing world over, with resistance to extended spectrum antibiotics cefixime and ceftriaxone being observed in more than 50 countries. Extended spectrum cephalosporins are usually the last resort antibiotics for drug-resistant infections (WHO, 2017c). Failure in treatment of gonorrhoea can have serious implications in reproductive health and for the newborn baby. Some of the consequences have been described such as ectopic pregnancy, penile oedema, conjunctivitis, infertility and even neonatal blindness (Unemo and Shafer, 2014). Increased resistance of *Helicobacter pylori* has been recently reported in several countries around the world (Thung *et al.*, 2016). These are just few cases around the world highlighting the huge problem of antibiotic resistance.

Antibiotic resistance is also noted in several diseases e.g. cholera, pneumonia and nosocomial infections such as Klebsiella infections.

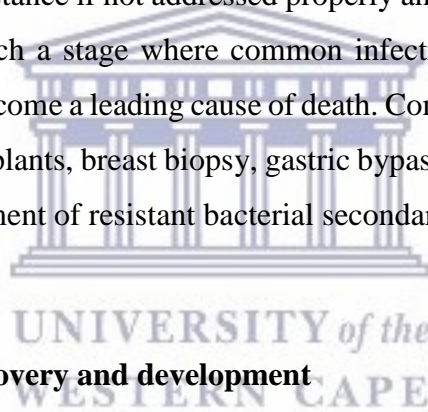
Closer to home, the problem of antibiotic resistance is noted in South Africa clearly with the tuberculosis (TB) resistance. Drug-resistant TB has been a huge problem for the country for a number of years now. The burden of drug resistant TB infections is very high in the country, with three out of every 100 TB cases being drug resistant (National Health Laboratory Services, 2017). WHO lists South Africa among the 30 high-burden-resistant TB countries. Even more worrying is the fact that the country is listed amongst the 27 high-MDR-TB-burden countries in 2015; the list also includes the populous Asian countries, China and India (WHO, 2016a). Multi drug resistant tuberculosis (MDR-TB) is a public health threat. It is defined as a TB infection that is resistant to rifampicin and isoniazid, two powerful first-line anti-TB drugs. Almost half a million people worldwide were infected with MDR-TB in the year 2015, and 9.5% of them had extensively drug-resistant tuberculosis (XDR-TB) (WHO, 2016b). XDR-TB, an even greater challenge, was found to be increasing in a study conducted in KwaZulu Natal Province, South Africa (Lim *et al.*, 2015). The problem of drug resistant TB is compounded in immunocompromised patients such as those affected by HIV or suffering from cancer. In the country, the drug resistance challenge is not only limited to TB, but also extends to several other infectious diseases. Resistance to “last resort” antibiotics such as carbapenem and colistin was noted to be increasing in South Africa, with over 2000 carbapenem-resistant infections reported between years 2000 and 2016 (Osei Sekyere, 2016).

Recently, WHO published a list of 12 bacteria that they described as a threat to human life. The organisation is appealing to pharmaceutical corporations and other relevant stakeholders to prioritise research and development for drugs effective against these bacteria. Three Gram negative bacteria namely *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* were deemed the three most critical of the 12. What is even more worrying is the fact that these bacteria are carbapenem-resistant. Carbapenems are known to be the best antibiotics against multi-drug resistant strains and are usually the last resort antibiotics against Gram negative bacteria. The list also includes *Enterococcus faecium*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter* spp., *Salmonellae*, *Neisseria gonorrhoea*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Shigella* spp., all resistant to important antibiotics (WHO, 2017a). Gram negative bacteria are especially more problematic as they are much harder to kill as compared to Gram positive bacteria. Gram negative bacteria have a thinner

peptidoglycan layer that is surrounded by many thin layers of membrane, which makes it extremely difficult for the antibiotic drug to infiltrate.

Drug resistance also has a significant negative economic impact. It is associated with longer hospital stays, increased mortality rates and higher treatment costs. Treating drug resistant infections costs the USA over \$21 billion a year (Infectious Diseases Society of America, 2015). So big is the problem of drug resistant infections that it poses an economic threat especially to third world countries. The World Bank predicts that the problem will lead to a reduction in gross domestic product (GDP) of developing countries of above 5% by the year 2050, thereby increasing poverty in these low income countries (The World Bank, 2016). Antimicrobial resistance is also noted as a threat to sustainable development goals (SDGs) with deaths from resistant infections estimated to reach 10 million a year by 2050 and economic costs expected to reach \$100 trillion (Jasovský *et al.*, 2016).

The problem of antibiotic resistance if not addressed properly and urgently, could lead us to an absolute crisis. We could reach a stage where common infections that could previously be easily treated by antibiotics become a leading cause of death. Common surgical procedures like caesarean section, organ transplants, breast biopsy, gastric bypass *etc.* could easily become life threatening upon the development of resistant bacterial secondary infections.



1.3 Decline in antibiotic discovery and development

The discovery of penicillin paved the way for a golden age of antibiotic discovery. This was the first few decades that followed the discovery and introduction of penicillin as a clinical agent. The period was characterised by the discovery of several new antibiotics, including cephalosporins, chloramphenicol, vancomycin and erythromycin. From 1980 onwards, there was a huge decline in antibiotic discovery, leaving many research and development companies with decreased returns on investments. Many companies decreased their research for new antibiotics and some have completely abandoned this research niche (Ventola, 2015). Global pharmaceutical giant, Pfizer had to close its antibiotic research unit in northeastern USA in 2011 due to financial reasons. Several other big pharmaceutical companies such as Elly Lilly have also not discovered new antibiotics for almost 30 years (Krans, 2014).

Significant progress has been noted to be mainly in structural modification of existing antibiotic analogues, rather than in the discovery of new novel compounds (Livermore *et al.*, 2011). This era of antibiotic discovery decline has been worsened by increasing antibiotic resistance. Antibiotic drugs are generally not as profitable as other drug classes such as anti-cancer and other chronic medications. Other classes of drugs are usually used for a long time, such as for the rest of a patient's life, however on the other hand, antibiotics are normally used for short course treatments which are usually a three, five or seven-day course and at times even a once off dose. This in turn means that for pharmaceutical companies the return on investments or profitability is not as huge as compared to chronic medication (Projan, 2003). Fewer new antibiotic agents are coming on to the market, due to low profitability of antibacterial agents and the ever-rising costs of developing a new drug. The process of developing a new antibiotic drug, like other drugs is very costly and takes a lot of time. The cost of developing a new prescription drug from preclinical research through clinical trials till the drug reaches the market is said to be estimated to be over US\$2.5 billion, a large increase from about \$800 million in 2003 (Avorn, 2015). The process of developing a new drug could take up to over 10 years. Yet despite these huge investments, the 'drug' being developed might not reach the market.

The antibiotic "pipeline" is literally running dry with hardly any new drugs being developed to meet the current need (Harbath and Theuretzbacher, 2015). Teixobactin has been the only new class of antibiotics that has been discovered in the last three decades. It is a new class of natural product antibiotic produced using a new technology known as the iChip. The molecule has a novel mechanism of action, killing bacteria in a different way to other antibiotics. Teixobactin inhibits peptidoglycan synthesis when it binds to Lipid II and III, which serve as crucial precursors in the bacterial cell wall synthesis. Sadly, teixobactin is not effective against the more problematic Gram negative bacteria, but is only active against Gram positive bacteria e.g. Methicillin-resistant *Staphylococcus aureus* (MRSA) and mycobacteria. What is more encouraging is that no detectable resistance to teixobactin has been found as yet. The molecule was anticipated to get to the clinical trial phase this current year, 2017 (Piddock, 2015). More research is currently underway. Its discovery certainly offered a glimmer of hope in the quest for new antibiotics.

1.4 Strategies to combat antibiotic resistance

Several measures are in place to combat the problem of drug resistance such as surveillance of antibiotic use and resistance in clinical practice at different levels, good microbial practices, and initiatives such as the Global Antibiotic Resistance Partnership (GARP) (Winters and Gelband, 2011). The GARP is an international collaboration of low to middle-income countries founded to address the problem of antibiotic resistance. It was launched in 2009 and its member countries include South Africa and India (Centre for Disease Dynamics, Economics and Policy, 2016). In South Africa there is a pharmacist-driven initiative known as the South African Antibiotic Stewardship Program. The body is comprised of human and animal healthcare experts and involves several stakeholders such as provincial health departments. It plays a leading role in the promotion and advocacy of proper use of antibiotics in both the public and private sector in South Africa in an effort to decrease antibiotic resistance. Prescribers such as clinical nursing practitioners and medical officers can play a role in decreasing the problem by prescribers' antibiotics only when necessary, and avoiding their over-usage. Vaccinations, good hygienic practices and sanitation can also play a pivotal role in the fight against antimicrobial resistance. Antibiotic use in farm animals should not be encouraged as it leads to transfer of resistance genes to the human population (Chang *et al.*, 2015). The general public need to be educated about antibiotic use and especially the dangers of drug resistance. However, all these actions and initiatives are not enough to combat the challenge.

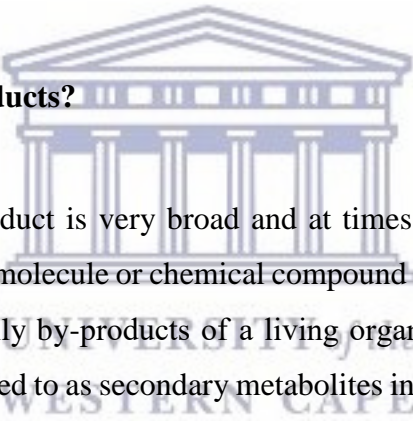
As the old adage goes, united we stand, divided we fall. Scientists, academic institutions, medical personnel, the community and research funders should join hands and work together to develop the present strategies and come up with innovative ways of tackling the problem. More regional and international collaboration is needed, and incentives to encourage pharmaceutical companies to develop new antibiotics are needed. In the year 2012, then American president Barack Obama signed into law an act promoting and incentivising antibiotic development known as the Generating Antibiotic Incentives Now (GAIN) act (Brown, 2013). The act offers additional market exclusivity making it more financially attractive for innovative pharmaceutical companies to step up and upscale their antibiotic discovery and development efforts. DRIVE-AB is a similar initiative in Europe to promote antibiotic discovery which was funded up to almost €10 million (Harbarth and Theuretzbacher, 2015). A number of related programs have been initiated in several countries in an effort to contain the problem. In 2013, the United States Biomedical Advanced Research and

Development Authority (BARDA) started funding antibiotic research by industrial companies' (Nathan and Cars, 2014). Financial investments and research incentives in the area are much needed if any headway is to be made in the battle.

Regardless of the above mentioned efforts and several other similar initiatives, there is still a critical need to discover new antibiotic drugs that could preferably have a totally new mechanism of action and are able to overcome drug resistance. It would also be fundamentally important to discover novel molecular scaffolds which could be developed into better antibacterial agents than the ones already available commercially. This research study explored the potential of natural products, specifically from marine algae as a source of new or novel antibiotic lead compounds.

1.5 Natural products as a source of new antimicrobials

1.5.1 What are natural products?



The definition of a natural product is very broad and at times confusing. In simple terms a natural product (NP) is a small molecule or chemical compound produced by a living organism (Nature, 2016). They are usually by-products of a living organism's secondary metabolism, and in some instances are referred to as secondary metabolites in the medicinal chemistry field. Secondary metabolites are compounds that are not essential for survival of the host organism, but confer an advantageous property to the organism (Demain and Fang, 2010), (Mazid, Khan and Mohammad, 2011). These small and unique molecules have great structural and chemical diversity that is not comparable to any synthetic libraries, and therefore form the richest source of drug and drug lead molecules (Demain and Fang, 2010). The term natural product is also used to refer to complex extracts, extracted from these organisms. Several studies have shown natural products to be modulators of bio-molecular function due to the structural and chemical diversity, specificity and target affinity (Hong, 2011).

Natural products have several sources; they are extracted from the cells, tissues and secretions of terrestrial and marine animals and plants, as well as from several different types of microorganisms. Generally, the natural product is extracted in a very complex mixture. The mixture should then be systematically processed using several separation techniques in a way

to isolate and purify the natural product. The amount of natural product obtained is usually very little. Sourcing drugs from natural products comes with its own challenges. Natural products are often complex molecules and usually have stability, dereplication and purification challenges *etc.* However, use of advanced and recent technologies such as whole genome sequencing and bioinformatics amongst others can aid in the discovery of new drugs from natural products (Wright, 2014).

Some natural product compounds or derivatives with good antibacterial activity have potential to be lead compounds for the discovery of novel antibiotics. Natural products have been touted as a potential source of novel antibiotics (Maloney, 2016). Several novel natural product compounds with antibacterial activity have been discovered to date. An example of such compounds is the muraymycin nucleoside peptides (Weigemann *et al.*, 2016).

1.5.2 Natural products and antibiotic discovery

Natural products have been very successful in drug discovery. The majority of antibiotics are natural products or are derived from natural products (Demain, 2009). Penicillin, the pioneer antibiotic is a classical natural product produced. The compound was produced by the fungus *Penicillium* sp. As mentioned earlier it was discovered by Sir Alexander Fleming in the beginning of the 20th century. Penicillin is used in the treatment of several bacterial infections such as gonorrhoea, bacterial endocarditis, cellulitis, meningitis *etc.* there are many antibiotics derived from natural sources approved in clinical use at present moment. A few examples are griseofulvin, gentamycin, chloramphenicol, amphotericin B, tetracyclines and cephalosporins. **Figure 1.1** shows chemical structures of the natural antibiotics. Cephalosporins were actually discovered and isolated from a marine fungus, *Cephalosporium acremonium*. Their discovery was initiated by the Italian scientist Giuseppe Brotzu, who isolated the fungus from a sewer on an island in the Mediterranean Sea and realised that it had activity against bacteria. The discovery later led to the isolation of an antibacterial compound Cephalosporin C, by another research group. Structural modifications of Cephalosporin C led to a series of several Cephalosporin antibiotics with potent activity. The discovery of natural antibiotics such as these led to the development of several synthetic and semi-synthetic antibiotics. An example is penicillin which has led to the production of semi-synthetic analogues such as the

aminopenicillins, beta-lactamase-resistant penicillins and carboxypenicillins *etc.* (Oshiro, 1999).

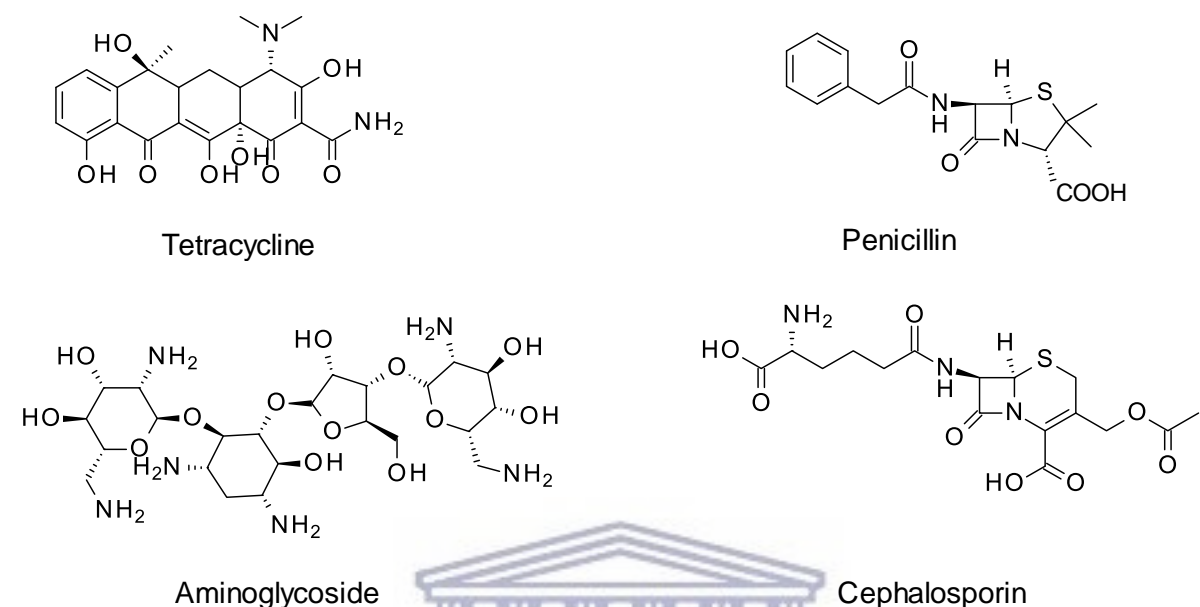


Figure 1.1 Chemical structures of selected natural product antibiotics

1.5.3 Other antimicrobials from nature

Numerous compounds with antimicrobial action have been isolated from various natural sources. It will be impractical to list each and every compound, but clearly it can be seen that nature is a prolific source of natural antimicrobials. Even natural foods such as cinnamon and honey, oil of Oregano, coconut oil, horseradish, citrus fruit and garlic contain compounds have some antibiotic properties, however little (Ibrahim, 2017). In a study, the antibacterial property of citrus fruit was found to be due to the natural product, citric acid (Tomotake *et al.*, 2006). Garlic is another food substance known to have several healthy and antimicrobial properties. The antimicrobial activity of garlic is attributed to a natural product compound called allicin (Ankri and Mirelman, 1999). Oils extracted from Basil (St Joseph’s wort), a popular culinary herb were also shown to have antimicrobial activity. They were shown to be active against both gram positive and negative bacteria and a fungus. Their antimicrobial activity highlighted their potential as preservatives in food products (Suppakul *et al.*, 2003).

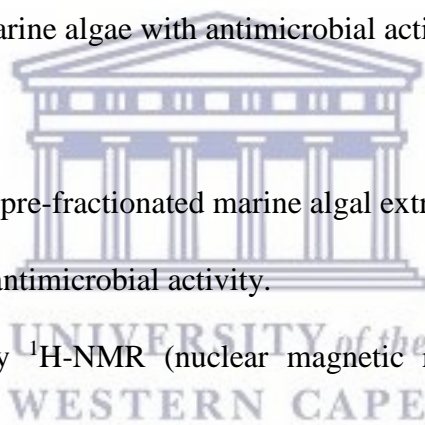
It is also important to note that several antimalarial drugs have been derived from natural sources e.g. chloroquine, quinine, mefloquine and artemisinin (Cragg and Newman, 2013). There is clearly no doubt that nature is an extremely important source of bioactive compounds with therapeutic potential. The importance of natural products, particularly from the less explored marine environment as a source of novel new antibiotics can never be overlooked. To date, seven marine derived drugs have been approved by the Food and Drug Administration agency (FDA) for clinical use (Lindequist, 2016). Perhaps, the next novel antibiotic is hidden somewhere in the earth's vast oceans, who knows? The following chapter will discuss several marine natural products isolated from different macro and microorganisms. Of particular interest are marine macro algae, from the marine environment which are identified as a less explored source of drugs with great potential.

Aim:

To isolate metabolites from marine algae with antimicrobial activity.

Objectives:

- To prepare a library of pre-fractionated marine algal extracts.
- To screen extracts for antimicrobial activity.
- To profile extracts by $^1\text{H-NMR}$ (nuclear magnetic resonance) spectroscopy and chromatography.
- To isolate active compounds from selected active extracts and determining their structures by spectroscopic methods.
- To determine the antimicrobial activity and cytotoxicity of pure compounds.



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Chapter 2

Antimicrobial Marine Natural Products: Review

2.1 Background

As seen in Chapter 1, antimicrobial resistance, particularly antibiotic resistance poses a significant and urgent health threat to the human population. Therefore, there is a need to explore several sources, particularly less explored sources like marine natural products, for new drugs to treat these infections. Drug discovery scientists are increasingly exploring the ocean for new biologically active and novel compounds that have medicinal potential. Drug discovery and development had in the past been mainly focussed on terrestrial plants, animals and minerals due to the limited accessibility of the sea, particularly the deep-sea environment. According to the United States National Oceanic and Atmospheric Administration Agency (NOAA), only about 5% of the world's ocean has been explored (NOAA, 2015). This leaves the ocean environment with an overwhelming 95% to explore. Though the exact number of marine species may never be known, it is estimated that there are over two million eukaryotic marine species (Mora, *et al.*, 2011). Clearly, the marine environment is a great reservoir of marine natural products from the several macro and microorganisms' dwelling therein.

This chapter seeks to highlight the importance or potential of marine natural products, particularly marine macro algae as a source of new or novel antimicrobial compounds. In this literature review, we discuss several compounds isolated from marine organisms that have antiviral, antibacterial, antifungal and antiparasitic activity.

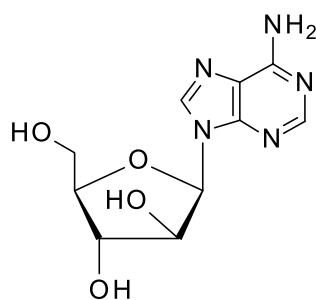
2.2 Introduction to marine natural products

2.2.1 Marine natural products

Marine natural products from several different organisms and species have been shown to contain several chemicals with biological activity. Some of these chemicals include haloforms, halogenated alkanes, hydroquinones and terpenoids. The area of marine natural product

research is increasingly gaining interest from all over the world, and several new and exciting compounds are being discovered. Marine organisms such as sponges and algae are growing in extreme conditions surrounded by several pathogenic microorganisms and small and larger predators (Speight and Henderson, 2010). The marine environment is known to have the densest populations of microbes on the entire earth with an estimated 10^{12} microbial organisms/cells per millilitre of water, with about a million of them being bacterial cells (Amaral-Zettler *et al.*, 2010). Pollution of the marine environment such as with plastic litter, have also with time made the ocean less habitable for aquatic life (Derraik, 2002). Marine organisms are also constantly exposed to extreme conditions in terms of temperature, pH, salinity *etc.* To survive these conditions would certainly not be easy for any organism. However, despite growing in these extreme conditions it is interesting to note that several marine organisms such as algae seem to thrive and are rarely attacked by these pathogenic microorganisms. How are they able to achieve such a feat? Marine organisms such as algae and sponges are known to produce biologically active secondary metabolites. Secondary metabolites are organic molecules or chemical substances that are produced by living organisms, which are not essential for the growth, development and reproduction of the organism but are known to play a vital role in the defence or survival of the host (Agostini-Costa *et al.*, 2012). In essence, secondary metabolites help a particular marine organism in carrying out a defensive duty for the host against different types of microbes such as bacteria and viruses or even insects and larger animals. Secondary metabolites are usually small molecules and may be classified either by biosynthetic origin (e.g. acetate or shikimate derived) or structural class (e.g. terpenes, flavonoids, tannins and alkaloids). Bioactive marine natural products have several different mechanisms of action some of which are yet to be known or fully studied. The biological activities include, but are not limited to cytotoxic, antibacterial, antiviral, and antifungal. Antibacterial, antiviral and antifungal activities can be collectively referred to as antimicrobial activity. Vidarabine (Ara-A, **Figure 2.1**) is one of the very few marine-derived antimicrobial drugs on the market. It is used in the treatment of herpes infections. The drug was derived from a marine natural product, the nucleoside, spongouridine (Rangel and Falkenberg, 2015). **Table 2.1** summarises several marine natural products with antimicrobial activity.

Despite their enormous pharmaceutical potential marine natural products remains a less explored source of antibiotic lead compounds.



2.1

Figure 2.1 Chemical structure of Vidarabine, a marine-derived antimicrobial drug

2.2.2 South African marine environment

Located at the tip of the African continent, South Africa has a very large coastline of approximately 2900 km (Encyclopaedia Britannica, 2017). These extensive areas are clearly a rich source of marine natural products. The coastal areas stretch from as far KwaZulu-Natal where the country borders with Mozambique all the way to the Northern Cape, where the country borders with Namibia. The unique feature of the coastal line of South Africa is that this is where the two oceans meet, the Indian Ocean with its warm waters and the South Atlantic Ocean with its cooler waters. Though yet to be fully explored, the South African marine environment could have potential pharmaceutical value as a source of bioactive metabolites. The country is also one of the world's best in terms of biodiversity with an abundance of flora and fauna both on the terrestrial landscape and more excitingly, in the coastal areas. Several marine species including sponges, algae, fish *etc.*, have been identified and some of them are endemic. South Africa is known to have over 12 000 marine species, and its coastal areas are relatively well-conserved (Griffiths *et al.*, 2010). Studies of South African marine invertebrates for their pharmaceutical potential is an area that is gaining more interest.

2.3 Why marine algae?

Marine macro-algae, also known as seaweed are plant-like organisms that are found in the marine environment and often grow attached to rocky substrates (Guiry, 2017). They are an interesting group of marine organisms and are an excellent source of novel biologically active

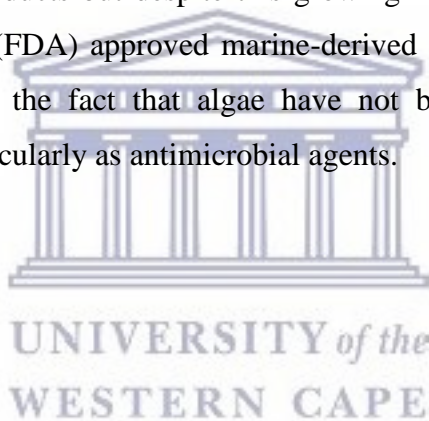
compounds. They are marine photosynthetic organisms growing in places where there is water, sunlight and nutrients, and they are responsible for about 50% of the world's photosynthetic processes. They come in different shapes and sizes and are found virtually everywhere in the earth's oceans. They are usually found in large quantities in shallow, rocky marine environments. There is about 10 000 species of marine algae world-wide, of which 6500 are red algae (Guiry, 2017). Several species exist in South Africa with an estimated number of about 800 (Bolton and Stegenga, 2002). The country is known to have one of the richer marine algae floras based on the number of species found therein, and the west coast has lower species distribution in comparison to the south or eastern coasts (Bolton and Stegenga, 2002).

Algae are responsible for approximately 9% of biologically active compounds obtained from the sea (Chauhan and Kasture, 2014). Seaweeds are important commercially as a source of food, medicines, nutraceuticals, alginates, *etc.* In Asian countries like China, Japan and Vietnam, seaweed has been a part of their diet for many years. In these countries, certain seaweeds are commonly used as crude drugs. An example is the use of the common seaweed *Sargassum* spp. to treat diseases such as goitre (Liu *et al.*, 2012). Common edible seaweed includes *Sargassum* spp., *Undaria pinnatifida*, *Mastocarpus stellatus*, *Palmaria palmate*, *Hypnea* *etc.* Red algae are known to be a rich source of vitamins and proteins, and as such are popular in Asian diets such as the Nori dish in Japan (Noda, 1993). In several countries, seaweeds are economically important and grown for exports. They form a large portion of the \$7 billion world aquaculture economy. About 2000 tonnes of *Ulva lactuca* (sea lettuce), the green seaweed is grown commercially in South Africa in a given year. Many types of seaweeds contain melatonin, the natural sleep hormone, in very large quantities which could be harvested for therapeutic use (Kandale *et al.*, 2011). Seaweeds have also been explored as a source of prebiotics for both animals and humans (O'Sullivan *et al.*, 2010). Red algae that contain carrageenan have been used for many years to treat respiratory conditions like sinus *etc.* (Kandale *et al.*, 2011). Marine algae are also known to contain a huge amount of iodine, giving them that salty taste. This enables preparations of these algae to be used in the removal of accumulation of phlegm and in iodine deficiency-goitre (Kandale *et al.*, 2011).

Marine algae are classified into three main types; red algae (Rhodophyta), green algae (Chlorophyta) and brown algae (Phaeophyta). Of the three, red algae are known as the best source of biologically active compounds (El Gamal, 2010). As mentioned earlier, these life forms are growing in a "soup of microorganisms", thus surrounded by several pathogenic microorganisms whilst producing nutritionally rich polysaccharides. Several studies have

shown that seaweeds produce biologically active secondary metabolites, which aid the seaweed in chemical defence against predation and extreme conditions. One such study was done in the USA at Georgia Institute of Technology. A research group led by Julia Kubanek isolated a unique compound called lobophorolide from the seaweed *Lobophora variegata*. The isolated compound exhibited potent antifungal activity against marine fungi. Lobophorolide is a macrolide that is known to bind with actin as part of its mechanism of action (Kubanek *et al.*, 2003). The discovery substantiates the fact that indeed seaweeds produce secondary metabolites to help fend off predators.

Fucoidans with antiviral activity, including anti-HIV activity, are commonly isolated from brown algae. These compounds are sulphated polysaccharides of high molecular weight. Fucoidans isolated from the seaweeds, *Saccharina cichorioides* and *S. japonica* showed potent antiviral activity (Prokofjeva *et al.*, 2013). Marine algae continue to be an excellent source of biologically active natural products but despite this growing interest, none of the Food and Drug Administration agency (FDA) approved marine-derived drugs has been isolated from algae. This could be due to the fact that algae have not been fully explored for their pharmaceutical potential, particularly as antimicrobial agents.



2.4 Antimicrobial marine natural products

Table 2.1 provides a summary of some of the important marine derived antimicrobial metabolites reported in the literature.

Table 2.1: Some marine natural products with antimicrobial activity.

Compound name	Source	Antimicrobial activity	Minimum Inhibitory Concentration (MIC) ($\mu\text{g}/\text{mL}$)	Reference
Actinomycin D	<i>Streptomyces</i> sp. (marine bacteria)	Antibacterial	0.25-0.5 (<i>Mycobacterium bovis bacillus</i> Calmette-Guérin)	Chen <i>et al.</i> , 2012
Anthracimycin	<i>Streptomyces</i> sp. (marine bacteria)	Antibacterial	0.031 (<i>Bacillus anthracis</i>)	(Jang <i>et al.</i> , 2013)
Bifurcatriol	<i>Bifurcaria bifurcate</i> (marine algae)	Anti-protozoal	IC ₅₀ value 0.66 $\mu\text{g}/\text{ml}$ (<i>Plasmodium falciparum</i>)	Smyrniotopoulos <i>et al.</i> , 2017
Borrelidin	<i>Nocardiopsis</i> sp. (actinomycete)	Antibacterial	16 μM (<i>Salmonella enterica</i>)	Kim <i>et al.</i> , 2017
Chalcomycin	<i>Streptomyces</i> sp.	Antibacterial	4 (<i>Staphylococcus aureus</i>)	Jiang <i>et al.</i> , 2017
Dihydrochalcomycin	<i>Streptomyces</i> sp.	Antibacterial	32 (<i>Staphylococcus aureus</i>)	Jiang <i>et al.</i> , 2017
Dragmacidin G	<i>Spongosirites</i> (marine sponge)	Antibacterial Anti-mycobacterial	0.62 (<i>Staphylococcus aureus</i>) 0.62 (<i>Mycobacterium tuberculosis</i>)	Wright <i>et al.</i> , 2017
Hyrtinadine D	<i>Hyrtios</i> sp. (marine sponge)	Antibacterial	16 (<i>Escherichia coli</i> and <i>Bacillus subtilis</i>)	Kubota <i>et al.</i> , 2016

Essramycin	<i>Streptomyces</i> sp. (marine bacteria)	Antibacterial	1 (<i>Staphylococcus aureus</i> and <i>Bacillus subtilis</i>)	El-Gendy <i>et al.</i> , 2008
Etamycin A	<i>Streptomyces</i> sp. (marine bacteria)	Antibacterial	1-2 methicillin resistant <i>Staphylococcus aureus</i>)	(Haste <i>et al.</i> , 2010)
Gageostatins (1-3)	<i>Bacillus subtilis</i> (marine bacteria)	Antifungal	4-32 (<i>Rhizoctonia solani</i> , <i>Botrytis cinerea</i> and <i>Colletotrichum acutatum</i>)	Tareq <i>et al.</i> , 2014
Kocurin	<i>Kocuria palustris</i> (marine derived bacterium)	Antibacterial	0.25 (methicillin resistant <i>Staphylococcus aureus</i>)	Martín <i>et al.</i> , 2013
Lindgomycin	<i>Lindgomycetaceae</i> (marine fungus)	Antibacterial	IC ₅₀ Value 5.1 µM	Wu <i>et al.</i> , 2015
Lobophorolide	<i>Lobophora variegata</i> (marine alga)	Antifungal	IC ₅₀ 0.5 µg/ml <i>Candida albicans</i> (amphotericin- resistant strain)	Kubanek <i>et al.</i> , 2013
MDN-0170 (new napyradiomycin)	<i>Streptomyces</i> sp. (actinomycete)	Antibacterial	> 64 (methicillin- resistant <i>Staphylococcus aureus</i> and <i>Escherichia coli</i>)	Lacret <i>et al.</i> , 2016
Micromonohalal- mane B	<i>Micromonospora</i> sp. (marine bacteria)	Antibacterial	40 (methicillin resistant <i>Staphylococcus aureus</i>)	Zhang <i>et al.</i> , 2016
Myticalin A8	<i>Mytilus</i> spp. (marine mussels)	Antibacterial	1 (<i>Escherichia coli</i> and <i>Acinetobacter baumannii</i>)	Leoni <i>et al.</i> , 2017

Myticalin D2	<i>Mytilus</i> spp. (marine mussels)	Antibacterial	2 (<i>Bacillus subtilis</i> and <i>Acinetobacter baumannii</i>)	Leoni <i>et al.</i> , 2017
Penicibrocazine C	<i>Penicillium brocae</i> (fungus)	Antibacterial	0.25 (<i>Staphylococcus aureus</i> and <i>Micrococcus luteus</i>)	Meng <i>et al.</i> , 2015
Solwaric acid A	<i>Solwarapora</i> sp. (Actinobacteria)	Antibacterial	32 (methicillin- resistant <i>Staphylococcus aureus</i>)	Ellis <i>et al.</i> , 2014
Streptcytosine A	<i>Streptomyces</i> sp.	Anti- mycobacterial	32 (<i>Mycobacteria smegmatis</i>)	Bu <i>et al.</i> , 2014
Theonellamide G	<i>Thionella swinhoei</i> (marine sponge)	Antifungal	IC ₅₀ value of 2.0 µM (Amphotericin B- resistant <i>candida albicans</i>)	Youssef <i>et al.</i> , 2014
Trichodin B	<i>Trichoderma</i> sp.	Antibacterial	IC ₅₀ value of 4 µM (<i>Staphylococcus epidermidis</i>)	Wu <i>et al.</i> , 2014

2.5 Antimicrobial seaweed metabolites

The number of bioactive natural products being discovered from different algae continues to grow. The growth can be attributed to increasing research interest in the area and improved technologies in compound isolation and purification, and structural elucidation techniques. Metabolites isolated from macro algae have exhibited a range of biological activities ranging from antiviral, antibacterial, antitumour, antioxidant, antimalarial amongst others. The antimicrobial activity of some compounds isolated from marine algae with different degrees of potency and pharmaceutical potential will be discussed in the following section.

2.5.1 Red algae

Red algae are usually reddish in colour due to the presence of a pigment known as phycoerythrin (Van Den Hoek, Mann and Jahns, 1995). The pigments enable red algae to photosynthesise and live at greater depths than green or brown algae. Red algae are known to produce or provide the greatest variety of secondary metabolites. Several compounds isolated from red algae exhibit antimicrobial activity (**Figure 2.2**). The type and degree of biological activity differs from one seaweed to another and from one species to another, with several ecological and geographical factors coming into play.

Most biologically active natural products from red algae are usually brominated. A brominated diterpene, sphaerodactylomelol (**2.2**), isolated from the red alga, *Sphaerococcus coronopifolius*, exhibited good activity (IC₅₀ 93.3 µM) against *Staphylococcus aureus*. The compound did not show great activity against other microbes *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Rodriguez *et al.*, 2015). It is also interesting to note that an earlier study on the same alga that was collected in Morocco showed the organic extracts to have some antimicrobial and antimalarial activity. Two compounds were then isolated from the seaweed namely, bromosphaerone (**2.3**) and 12 s-hydroxybromosphaerodiol (**2.4**). Both showed good antibacterial activity against *Staphylococcus aureus*. The compounds had MIC values of 0.104 µM and 0.146 µM, respectively (Etahiri *et al.*, 2001).

In another study done in Brazil, a glycolipid, sulfoquinovosyldiacylglycerol (**2.5**) isolated from a red alga *Osmundaria obtusiloba* from south-eastern Brazilian coast showed antiviral activity against HSV-1 and HSV-2 viruses. EC₅₀ values of 42 µg/mL and 12 µg/mL were observed against HSV-1 and HSV-2 respectively. Again, previous studies by the same group had shown the crude extracts to have antiviral activity against HSV-1 (De Souza *et al.*, 2012).

A crude methanol extract of the red seaweed *Laurencia complanata* was tested for its antimicrobial activity using the agar diffusion assay. The extract exhibited potent antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Candida albicans*. Zones of inhibition were determined and found to be 19.5, 20, 20 and 11.5 mm for these microbes, respectively. The antimicrobial activity of the seaweed was thought to be due to a tetrabromoindole, which was one of the main brominated indoles isolated (Rahelivao *et al.*, 2015). Polyhalogenated indoles are also known to have antifungal activity. Halogenated indoles isolated from a red alga collected in New Zealand, *Rhodophyllis*

membranacea, 2,3,6,7-tetrabromoindole (**2.6**) and 2,6,7-tribromo-3-chloroindole (**2.7**) had activity against *Saccharomyces cerevisiae*, a fungus, with IC₅₀ values around 10 µM (Woolner *et al.*, 2016).

Laurencia species seem to be one of the best algae in producing bioactive molecules. Elatol (**2.8**) and iso-obtusol (**2.9**) are biologically active halogenated metabolites isolated from a Malaysian red seaweed *Laurencia majuscula*. Elatol showed activity against six of the eight tested pathogenic bacteria. It showed particularly good activity against *Staphylococcus epidermis*, *Klebsiela pneumonia* and *Salmonella* sp. On the other hand, iso-obtusol showed good activity against only four bacterial species, and like elatol the best activity was against *Klebsiela pneumonia* and *Salmonella* sp. (Vairappan, 2003). A sesquiterpene (**2.10**) isolated from *Laurencia tristicha* from Taiwan exhibited good antibacterial activity the compound had activity comparable to the standard antibiotic ampicillin (Chen *et al.*, 2016). Other interesting antimicrobial compounds from *Laurencia* seaweed are the laurakamins. The three compounds, laurokamin A, B (**2.11**) and C (**2.12**) were isolated from *Laurencia okamurae* in China. The compounds are known to have some antibacterial activity. Laurakamins B and C and showed activity against *E. coli*. The biological activity was not great with zones of inhibition of 6 mm inhibition diameter (Gribble, 2015). A compound known as compositacin G (**2.13**) isolated from *Laurencia composita* exhibited very good antifungal activity. The compound was isolated with ten other chamigrane halogenated sesquiterpenes, and had an MIC₈₀ value of 4 µg/mL against the fungus *Microsporium gypseum* (Yu *et al.*, 2017).

Plocamenone (**2.14**) isolated from the red alga, *Plocamium* sp. is a unique acyclic polyhalogenated monoterpene. The compound is known to be a potent mutagen (Gribble, 1996) and exhibited remarkable antimicrobial activity against *B. subtilis*, comparable to that of the antibiotic chloramphenicol (Timmers, Dias and Urban, 2012). A halogenated monoterpene (**2.15**) isolated from *Plocamium cartilagineum* showed activity against nine different oral bacteria. Greatest activity was observed against *P. gingivitis* with a zone of inhibition of 19.35 mm in comparison with a positive control of 23.32 mm (Rovirosa *et al.*, 2013).

Biologically active bromophenols have also been reportedly isolated from red algae. Five bromophenols isolated from *Rhodomela confervoides* exhibited antimicrobial activity when tested against eight strains of gram positive and gram negative bacteria. One of the bromophenols was found to have a minimum inhibitory concentration below 70 µg/mL (Xu *et al.*, 2003). 2,2',3,3'-tetrabromo-4,4',5,5'-tetrahydroxydiphenylmethane (**2.16**) another

bromophenol isolated from a red alga, *Odonthalia corymbifera* showed activity against *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum* (Oh, 2008).

In another study conducted in New Zealand, three metabolites with activity against *Mycobacterium smegmatis* were isolated from a red alga, *Osmundria colensei*. The compounds are lanosol methyl ether (**2.17**), lanosol butanone (**2.18**) and rhodomelol (**2.19**). The three compounds had the following IC₅₀ values respectively 7.8, 26.2 and 28.1 µg/mL. However, these compounds had low MIC values. Lanosol ethyl ether proved to have good bacteriostatic and fungistatic activities (Popplewell and Northcote, 2009). Related compounds; lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) (**2.20**) and 2,2',3-tribromo-3',4,4',5-tetrahydroxy-6'-methoxymethyldiphenylmethane (**2.21**), were noted as having potential for rhinovirus therapy in the future. The compounds were isolated together with five other “polybromocatechols” from the red alga, *Neorhodomela aculeata*. Lanosol was remarkably active, with an IC₅₀ value of 2.50 µg/mL against the HRV2 virus (Park *et al.*, 2012).



2.5.2 Green algae

Unlike red and brown seaweed, green algae are mostly found in freshwater sources. Most have a characteristic greenish colour due to the presence of chlorophyll A and B. However, the algae can also have different pigmentation like yellowish or brownish colours due to the presence of other pigments. Several crude extracts from green algae have been shown to possess antimicrobial activity. A number of compounds isolated from green algae have been shown to possess antimicrobial activity (**Figure 2.3**).

Caulerprenylols A (**2.22**) and B (**2.23**) isolated from a Chinese green algae *Caulerpa racemosa* were shown to exhibit good antifungal activity. These two compounds are prenylated para xylenes. Observed activity against *Candida glabrata*, *Trichophyton rubrum* and *Cryptococcus neoformans* had MIC₈₀ values of 4-64 µg/mL. (Liu *et al.*, 2013). Kahalalide F (**2.24**), isolated from the common green alga, *Bryopsis* sp. in Hawaii showed good antimicrobial activity, and were active against a number of fungi including *Aspergillus oryzae*, *Penicillium notatum* and *Candida albicans*. Observed zones of inhibition for the three fungi were 19, 26 and 16 mm respectively. Antiviral activity of kahalalide F was 0.5 µg/mL against HSV II (Hamann and Scheuer, 1993). Interestingly, kahalalide F is a potent antitumour agent that reached clinical trial stage (Rademaker-Lakhai *et al.*, 2005). This compound has a range of biological activity having been also shown to have anti-leishmania activity (Cruz, *et al.*, 2009).

Capisterones A (**2.25**) and B (**2.26**) from the green algae *Penicillus capitatus* showed fluconazole-enhancing activity against *Saccharomyces cerevisiae*. The compounds showed potential as agents in antifungal combination therapy (Li *et al.*, 2006). The compounds had been previously isolated and shown to have potent antifungal activity against *Lindra thalassiae* with LD₅₀ values of 0.03 and 0.94 µg/mL (Puglisi *et al.*, 2004). The compounds isolated from *Udotea orientalis*, a green alga, were found to have some antitrypanosome activity. The compounds are namely curcuepoxide A (**2.27**), curcuepoxide B (**2.28**) and 10α-hydroxycurcudiol (**2.29**) (Sabry, Goergen and Gerwick, 2017).

3-o-β-D Glucopyranosyl clerosterol a steroid isolated from the green alga *Ulva lactuca* exhibited antibacterial activity against *Streptococcus lactus*, *Bacillus subtilis* and *Pseudomonas putidu*. In addition, the compound also inhibited the growth of *Fusarium oxysporium* a fungi and *Saccharomyces cerevisiae*, a yeast (Awad, 2000). A novel lecithin with potent antiviral activity was isolated from the green alga, *Halimeda renscheii*. The compound was named

HRL40 and had activity against the influenza virus. The biological activity of the protein was found to be due to the excellent binding of the molecule to hemagglutinin. The molecule had an ED₅₀ of 2.45 nM (Mu *et al.*, 2017).

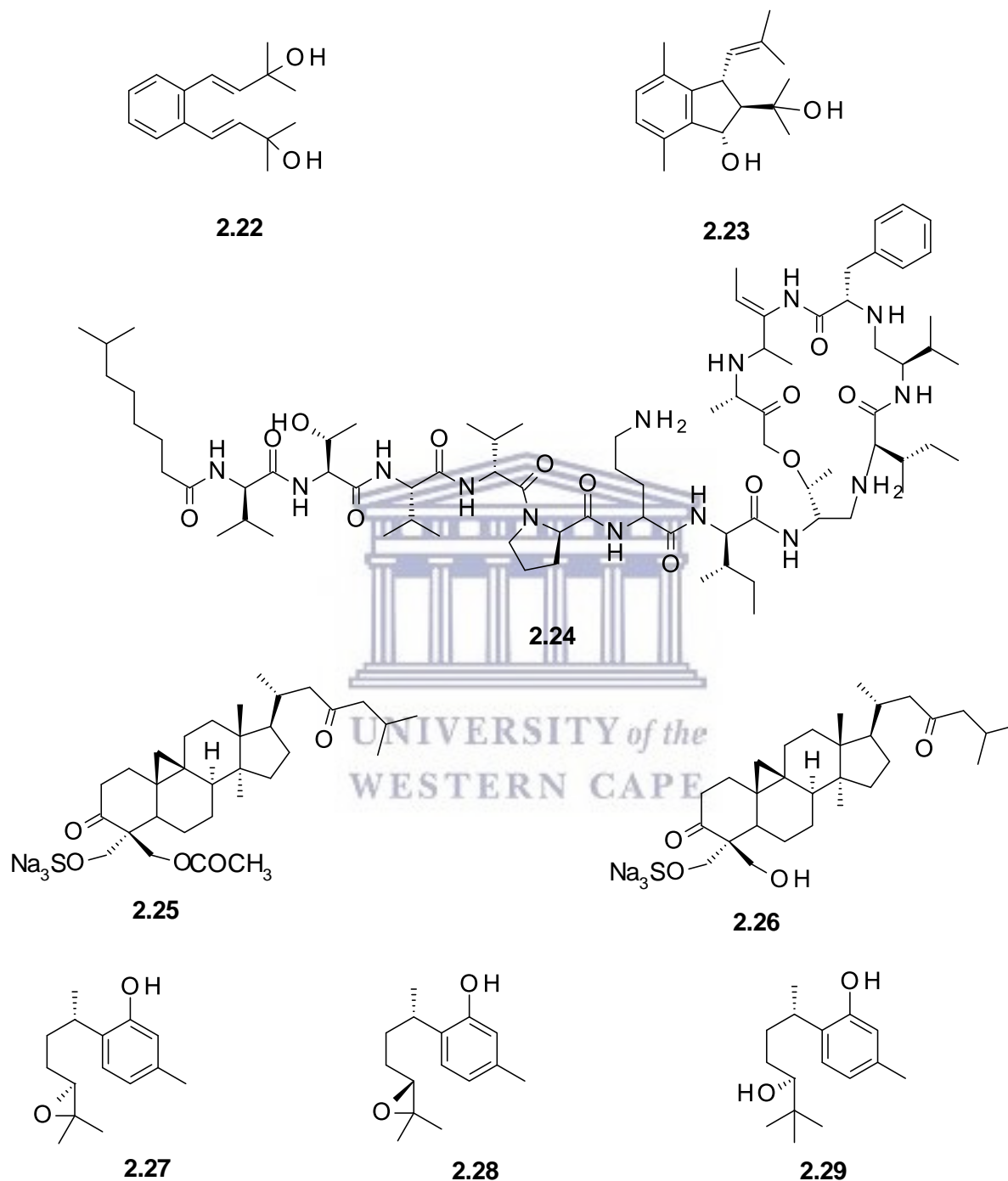


Figure 2.3. Chemical structures of some of the metabolites with antimicrobial activity from green algae

2.5.3 Brown algae

Brown algae usually have a characteristic brown colour that is due to the abundance of fucoxanthin. This compound masks the display of other pigments such as the chlorophylls (Kumar and Singh., 1979). Like red algae, several brown algae produce primary and secondary metabolites that are biologically active (**Figure 2.4**). Bifurcatriol (**2.30**), a terpene compound was recently isolated from the brown seaweed *Bifurcaria bifurcata*. The compounds were found to have some *in-vitro* antiprotozoal activity against *Plasmodium falciparum* and *Leishmania donovi*. The highest activity of the diterpene was against *P. falciparum* with an IC₅₀ value of 0.65 µg/mL. The compound was found to be slightly toxic to human cell lines (Smyrniotopoulos, 2017). Eganolone (**2.31**), another compound from *Bifurcaria bifurcata* was found to have antiplasmodial activity against *P. falciparum* with an intra-embryonic stage IC₅₀ of 2.6 µg/mL. The compound was found to be less active against *Trypanosoma brucei* (Galle *et al.*, 2013). A meroditerpenoid, atomaric acid (**2.32**) isolated from the lipophilic extract of *Styopodium zonale* was found to have anti-Leishmania activity. The compound inhibited amastigotes and had an IC₅₀ of 9.0 µg/mL. In another study atomaric acid and another meroditerpenoid, epitaondiol (**2.33**), both isolated from *Styopodium zonale* were shown to have antiviral activity against human metapneumovirus (HMPV). At least a 90% replication inhibition was observed for both compounds (Mendes *et al.*, 2011).

Dollabelladienols A (**2.34**), B (**2.35**) and C (**2.36**), three diterpenes with antiviral activity were isolated from a Brazilian brown algae *Dictyota pfaffii*. Dollabelladienol A and B had excellent anti-HIV 1 activity with IC₅₀ values of 2.9 and 4.1 µM, respectively. Interestingly, the compounds had low cytotoxic activity against human cell lines proving that they could be important lead compounds in the search of new HIV drugs (Pardo-Vargus, 2014). Dollabelladietriol (**2.37**) a related compound to the dollabelladienols, was also isolated from the alga, *Dictyota pfaffii*. The compound showed anti-HSV activity. It was observed to have an inhibitory effect on HSV lesions on mice (Garrido *et al.*, 2017). Terpenoids from brown algae seem to be very active. Several other diterpenoids were isolated from a related alga, *Dictyota plactens* from China. Five of the compounds isolated exhibited good antiviral activity with IC₅₀ ranging from 16.1 to 30.5 µM (Cheng, 2014).

An interesting group of unique compounds from brown algae are phlorotannins, which are complex polymers of the molecule phloroglucinol. The novel phlorotannin, eckol (**2.38**), was

isolated from the brown alga, *Eklonia cava*. Eckol was tested for its biological activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella* spp. The MIC values of eckol against both microbes were around 125 to 250 µg/mL. Although the antimicrobial activity was not as great, eckol when tested in combination with ampicillin was found to have greater inhibitory activity (0.31 to 0.5 µg/mL). The authors suggested that the compound could have an additive or synergist effect. It is interesting to note that the seaweed from which eckol was isolated, *Eklonia cava* also produces metabolites with other biological activities such as anticoagulant and antioxidant properties. (Choi *et al.*, 2010). Other phlorotannins isolated from brown algae have also shown antimicrobial activity against different strains such as *Bacillus subtilis* and *Escherichia coli*. Some of these phlorotannins include phloroglucinol (**2.39**), fucofuroeckol-A (**2.40**) and eckol. Surprisingly, in this research, eckol was found to be more active with an IC₅₀ value of between 32-64 µg/mL when tested against MRSA (Eom and Kim, 2013).

In another study, three meroditerpenoids isolated from a brown seaweed *Styopodium flabelliforme* were found to have some antimicrobial activity. The isolated compounds were epitandiol monoacetate (**2.41**), stypotriol triacetate (**2.42**) and stypodiol (**2.43**). Epitandiol had the best antimicrobial activity of the three. However, these compounds had MIC values that were higher than 114 µg/mL. These compounds were tested against the following microbes *Staphylococcus aureus*, *Salmonella typhimurium*, *Proteus mirabilis*, *Bacillus cereus*, *Enterococcus faecalis* and *Micrococcus luteus*. *Enterococcus faecalis* proved to be the most susceptible microorganism with an MIC of 114 µg/mL (Pereira *et al.*, 2011).

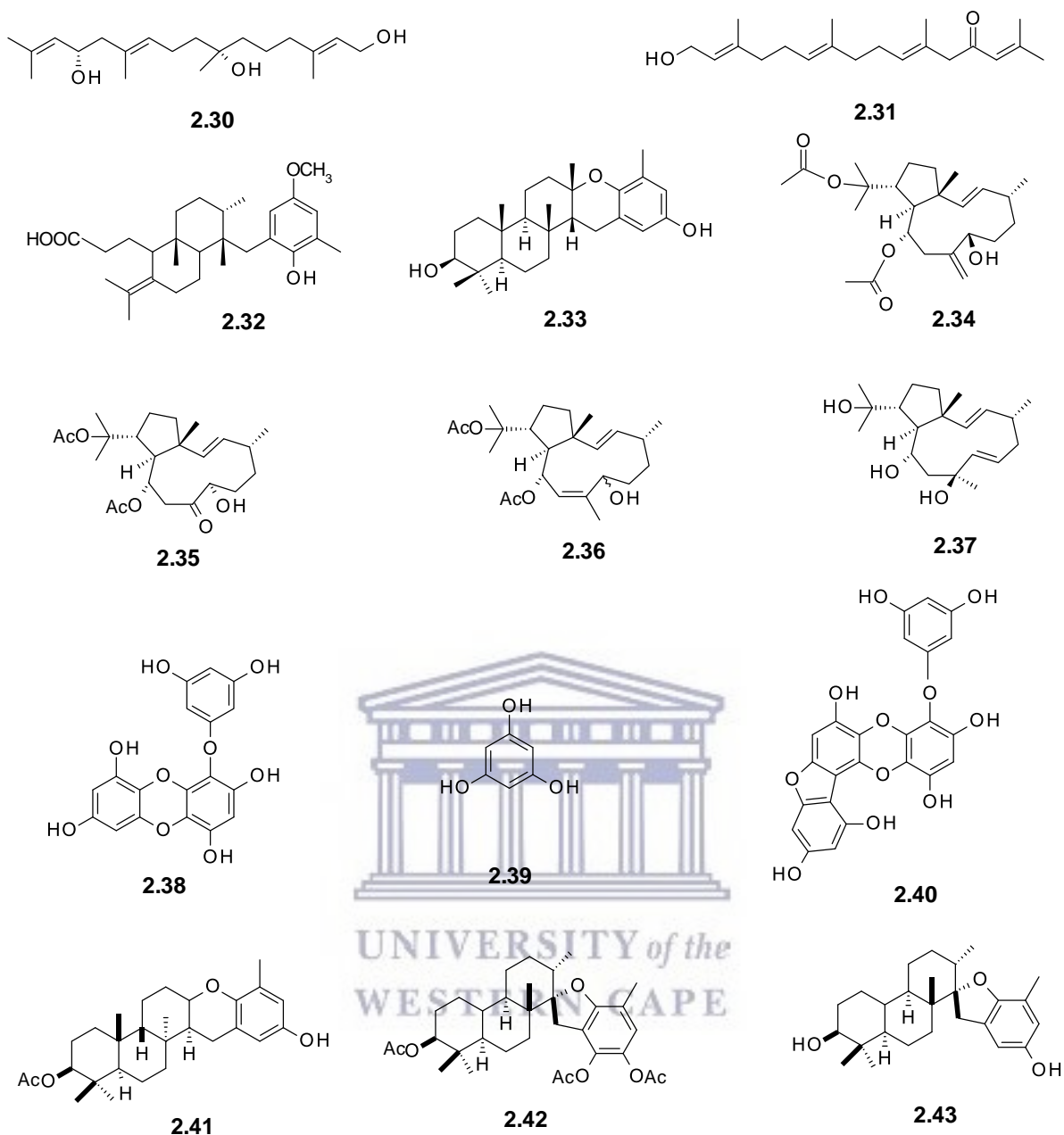


Figure 2.4. Structures of selected compounds from brown algae with antimicrobial activity

2.6 Overview of antimicrobial assays

Several methods can be employed in the *in-vitro* screening of compounds and extracts for antimicrobial activity. The choice of method depends on several factors as determined by the researcher. As the norm with other assays; reproducibility, cost and time are some of the main factors. Several factors also affect the antimicrobial susceptibility testing such as pH, moisture

and temperature. A particular microbe is considered to be susceptible to an antimicrobial agent or compound if the addition or introduction of such an agent can lead to the inhibition of the growth of the microorganism(s) in culture (Hobson and Dockrell, 2014). The greater the extent of inhibition, the greater the potency of the antimicrobial agent. Activity is usually recorded as minimum inhibitory concentration (MIC). However, activity can also be expressed in several ways such as percentage inhibition, half maximal inhibitory concentration (IC_{50}), *etc.* The three main methods of testing are; diffusion, bioautographic and microdilution methods. Diffusion assays and bioautography are qualitative techniques and thus will only give an indication of whether samples have any biological activity. These methods are more suitable for extracts. On the other hand, the micro-dilution assay is a quantitative technique and allows calculation of a sample's MIC. It is more suitable for pure compounds, but it is a method that is labour intensive.

In the disk/well diffusion method, if a microorganism is susceptible to the compound, a zone of inhibition would appear on the agar plate. The greater the zone of inhibition, the greater the potency of the test compound, and *vice versa*. The method is commonly used to assess the antimicrobial activity of marine algae extracts due to its low costs, simplicity and quickness, and the method is deemed most appropriate for fractions or samples obtained from bioassay guided fractionation (Desbois and Smith, 2015). However, this type of assay is not best for non-polar compounds (Valgas, 2007). Thin layer chromatography-bioautography is also commonly used to determine the activity of marine algal extracts. There are different types of bioautography methods including agar diffusion and thin-layer chromatography direct bioautography (TLC-DB), with the TLC-DB being the most common (Balouiri, Sadiki and Ibsouda, 2016). Direct bioautography is known as a high throughput method that enables multiple samples to be simultaneously assessed for antimicrobial activity (Choma and Jesionak, 2015). The method is known to be cheap, reproducible and simple to conduct (Valgas, 2007). In addition, it allows easy localisation of biological activity in complex matrices such as natural product extracts or fractions (Valgas, 2007). The method makes use of silica gel TLC plates, onto which samples are spotted. The mobile phase is evaporated off completely before the antimicrobial activity can be assessed. Several other methods of measuring biological activity exist such as the E-test, bioluminescence, automated antimicrobial susceptibility testing systems.

In this project, the bioautographic method was chosen for the preliminary screening of marine algal extracts, and the broth microdilution assay was used for testing isolated compounds.

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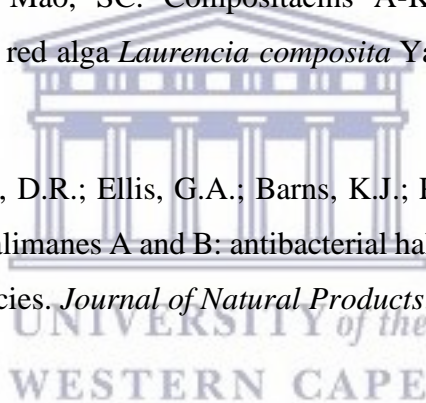
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Chapter 3

Preparation and antimicrobial screening of a marine algal extracts library

3.1 Introduction

This chapter focusses on the development of a pre-fractionated marine natural products library for medium throughput antimicrobial screening. The research looked to identify algal fractions that had the most potent antimicrobial activity and interesting chemical profiles. This library development forms part of a larger Marine Biodiscovery Research Group's (MBDR) marine natural products' library. It was anticipated that the study would yield several fractions with exciting chemical profiles and potent antimicrobial activity which could be pursued further to isolate biologically active pure compounds.



3.1.2 Pre-fractionated natural products libraries in drug discovery

Although natural products have been a very successful source of new drugs, it nevertheless suffers from significant drawbacks in a modern drug discovery context where the focus is on high throughput screening. One of the main drawbacks is that crude extracts of plants, *etc.* may contain hundreds (if not thousands) of individual compounds which makes the identification of the active component/s in an extract very challenging. In addition, the presence of ‘nuisance’ compounds that often give false positives can also frustrate the drug discovery campaign. Finally, the identification of new compounds and dereplication of known compounds are much more difficult when crude extracts are used. On the other hand, the development of pure natural product libraries, although advantageous and much more suited to high throughput screening, is very expensive and time-consuming to develop. Therefore, the development of pre-fractionated libraries, containing “crude” fractions of reduced complexity and allowing the removal of “nuisance” compounds is a useful compromise between crude extracts and pure compound libraries.

Pre-fractions are generally obtained by using different chromatographic stationary phases and eluting the crude extract with organic solvents of different polarities to obtain a set number of

fractions. It simply entails the separation of components from each other based on polarity. Several conventional and novel techniques can be applied in the fractionation process (Newman, 2017). Pre-fractionation will enable screening to be done at a higher concentration of minor components and makes the process of dereplication a lot easier (Butler, Fontaine and Cooper, 2014). Resultant fractions are known to vary greatly in terms of composition, some fractions may contain a complex mixture of several compounds and some could contain a single major compound with a purity of up to 90% or even more (Rizzo, Wakchaure and Waldmann, 2014, Quinn, 2012, Bugni *et al.*, 2008). The idea of prefractionation library development centres on the need to identify extracts or fractions with desirable characteristics before actual isolation of the pure compounds is done (Camp *et al.*, 2012). It is indeed beneficial to get an insight into the chemistry or activity of the compounds within a particular fraction or extract, before energy, time and monetary resources are channelled to isolate the compound(s). This makes pre-fractionated natural product libraries so appealing to modern day drug discovery researchers. Several companies and institutions have created natural product libraries comprising of compounds and extracts from different macro- and micro-organisms. Examples include the Natural Product Library initiative at the Scripps research institute, ChromaDex and the Greenpharma Natural Compound Library (National Center for Complimentary and Integrative Health, 2017). The development of natural product libraries in academic institutions plays a critical role in complementing similar libraries of industrial or private scientific researchers (Buss and Butler, 2013).

In this study, we aimed to develop a pre-fractionated library of marine algal extracts for antimicrobial screening. The library would contain detailed information regarding the biological activity and chemical profiles of each fraction. Active fractions with “interesting” chemistry could then be prioritised for further study.

3.2 Results and discussion

3.2.1 Preparation of a pre-fractionated library

Seventeen red and brown algae were obtained from the Marine Biodiscovery collection, University of the Western Cape. These algae were collected from sites in Kwazulu-Natal, the

Eastern Cape and the Western Cape (**Table 3.1**). Small-scale extraction and silica gel gradient fractionation was done to each of the resultant crude extracts. The fractionation protocol used had previously been developed by our research group (Afolayan *et al.*, 2008) and were designed to separate out the relatively non-polar to intermediate polarity compounds in the algal extracts. Small-scale crude extracts of the 17 different marine algae (**Table 3.1**) followed by silica gel gradient fractionation gave rise to a total of 153 fractions.

Table 3.1 Algae used in this study and their collection sites

Alga	Collection code	Alga name	Phylum	Collection site
1	NDK100328-3	<i>Sargassum incisifolium</i>	Brown	Noordhoek, EC
2	NDK130821-1	<i>Sargassum incisifolium</i>	Brown	Noordhoek, EC
3	PE07-22	Unknown	Brown	Port Edward, KZN
4	TS13022-7	<i>Oerstedtia scalaris</i>	Brown	Three Sisters, EC
5	KOS5130226	<i>Zonaria subarticulata</i>	Brown	Kenton-On-Sea, EC
6	TS130227-2	<i>Dictyoptemia macrocarpa</i>	Brown	Three Sisters, EC
7	SB20140302-3	<i>Dictyota naevosa</i>	Brown	Shaka's Beach, KZN
8	SB20141122-2	<i>Dictyopteris ligulata</i>	Brown	Shaka's Beach, KZN
9	PB20141122-1	<i>Styopodium multipartitum</i>	Brown	Palm Beach, KZN
10	PA100331-6	<i>Portieria hornemannii</i>	Red	Port Alfred, EC
11	UWC150619-1	<i>Laurencia glomerata</i>	Red	Glencairn, WC
12	GC120901-1	<i>Plocamium</i> sp. (Glencairn)	Red	Glencairn, WC
13	SB20141121-2	<i>Hypnea spicifera</i>	Red	Shaka's Beach, KZN
14	PA100331-9	Unknown	Red	Port Alfred, EC
15	PA130427-6	Unknown	Red	Port Alfred, EC
16	D124a	Unknown	Red	-
17	D1033	Unknown	Red	-

The purpose of the extract library was to enable the rapid retrieval of both the biological activity and chemical profile information of a particular fraction in order to better select promising projects. For this to happen there was need to have sufficient quantities of extract and fractions for further study. The masses of crude extracts are shown in **Table 3.2** and the percentage recovery after silica column chromatography is presented in **Figure 3.1**. The amount of extract obtained from about 5 mL of seaweed varies greatly from about 50 mg to more than 150 mg. This is to be expected since some algae are more “fleshy” and others more “wood-like”. Nevertheless, this amount of material is more than sufficient for small-scale fractionation and characterisation. After silica gel fractionation, an average percentage recovery of 81% was

obtained and more than 85% of extract was recovered for the majority of the algae while only one alga, SB20141121-2 produced recovery a percentage of below 50% (**Figure 3.1**). From this information, algae producing sufficient extract could be prioritised for further study.

Table 3.2 Masses of extracts and fractions obtained for each alga

Alga	Collection code	Crude extract mass	Fractions								
			A mg	B mg	C mg	D mg	E mg	F mg	G mg	H mg	I mg
1	NDK100328-3	64.9 mg	1.8	2.0	9.6	5.8	7.7	10.8	4.3	3.0	11.7
2	NDK130821-1	82.0 mg	0.8	2.5	9.0	17.9	13.5	7.6	3.0	1.8	17.0
3	PE07-22	79.6 mg	3.5	4.9	7.4	9.3	3.1	2.7	2.1	3.0	17.9
4	TS13022-7	67.3 mg	3.9	8.1	9.9	9.2	2.1	1.8	1.4	3.2	19.0
5	KOS5130226	106.1 mg	4.2	5.2	7.2	17.7	18.7	14.4	8.8	9.0	29.5
6	TS130227-2	76.9 mg	2.4	6.5	6.3	4.6	3.7	1.8	1.5	5.3	20.9
7	SB20140302-3	114.3 mg	5.5	18.9	11.9	14.0	12.2	13.2	6.4	3.9	24.0
8	SB20141122-2	116.8 mg	2.8	6.6	6.5	8.4	5.6	3.1	3.3	3.8	27.2
9	PB20141122-1	143.4 mg	1.4	3.7	7.0	18.7	29.3	24.9	13.7	10.1	24.4
10	PA100331-6	103.6 mg	7.5	20.3	15.0	5.4	4.8	3.5	3.0	6.0	25.8
11	UWC150619-1	102.0 mg	0.7	6.2	14.7	19.0	9.7	6.6	2.7	1.1	26.8
12	GC120901-1	60.4 mg	2.4	4.0	5.9	5.2	4.6	2.5	1.5	3.0	6.7
13	SB20141121-2	100.0 mg	2.1	3.0	3.1	3.4	3.5	2.2	2.0	1.6	14.1
14	PA100331-9	65.0 mg	2.7	3.3	7.2	11.0	11.2	7.7	4.5	3.7	11.3
15	PA130427-6	99.1 mg	3.0	4.7	15.4	13.3	8.1	6.6	3.7	3.3	28.9
16	D124a	158.1 mg	2.4	2.9	5.7	8.4	9.6	8.4	13.7	21.4	52.4
17	D1033	150.9 mg	2.4	20.2	33.4	25.1	9.1	6.3	11.5	10.1	32.5

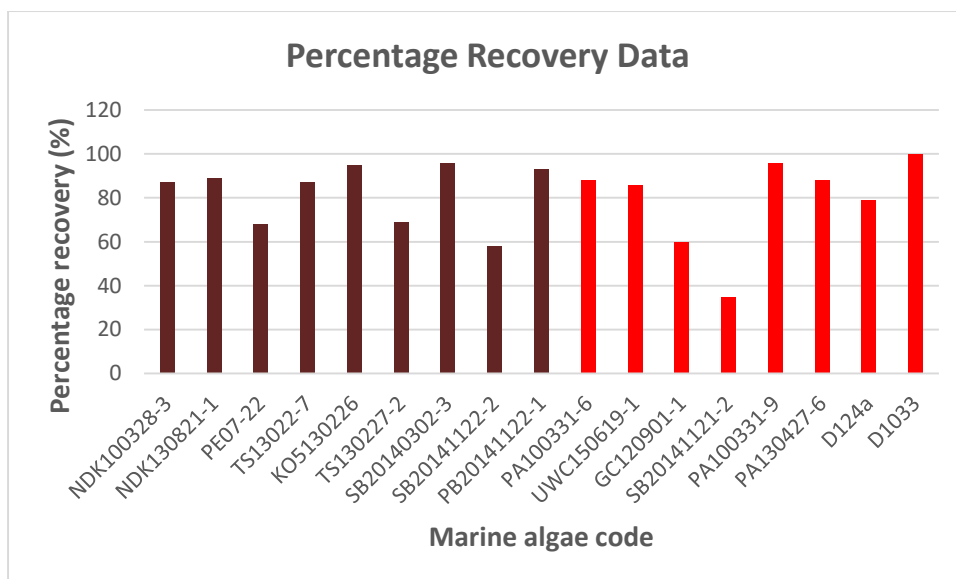


Figure 3.1 Percentage recovery after silica gel fractionation of the 17 marine algae

All fractions generated were dissolved in DMSO (dimethyl sulfoxide) and stored as stock solutions of 10 mg/mL. DMSO is known as a solvent of choice when it comes to short and long term storage of organic compounds or extracts. It is also advantageous as it dissolves both polar and non-polar compounds. Samples were stored in solution in tightly sealed and properly labelled screw-cap vials. The vials were neatly arranged in vial boxes that were appropriately labelled and sealed with additional layers of plastic foil to exclude any moisture. Samples were then stored frozen at -20°C. **Figure 3.2** shows some of the pre-fractionated samples of the library in their vials in sealed vial boxes.



Figure 3.2. A photograph of marine algal fractions prepared and stored as part of the MBDR natural products library ready for high throughput screening

3.2.2 Chemical profiling of marine algal library by $^1\text{H-NMR}$ spectroscopy

Several techniques may be used to provide insight into the chemical components of crude or fractionated extracts. Two of the most popular are gas chromatography-mass spectrometry and high performance liquid chromatography-mass spectrometry. Alternatively, $^1\text{H-NMR}$ (proton nuclear magnetic resonance) spectroscopy may also be used. Over the past few decades, NMR has become the leading method in determining the structure of organic compounds. NMR has also become a vital tool in identifying plant metabolites, both *in-vivo* and in extracts, and is very important in metabolite fingerprinting (Krishnan, Kruger and Radcliffe, 2005).

In this study, we used $^1\text{H-NMR}$ spectroscopy because of availability and cost. All 17 crude extracts and their resultant fractions were analysed using $^1\text{H-NMR}$ spectroscopy. **Figure 3.3** shows an example of the complexity of the $^1\text{H-NMR}$ spectrum of the crude extract of the seaweed *Laurencia glomerata* (UWC150619-1). The $^1\text{H-NMR}$ spectra of the first three chromatographic fractions are shown in **Figure 3.3**. Although there is some overlap between components in the fractions it also shows significant differences. Depending on availability and biological activity, this is a seaweed that would be prioritised for further study.

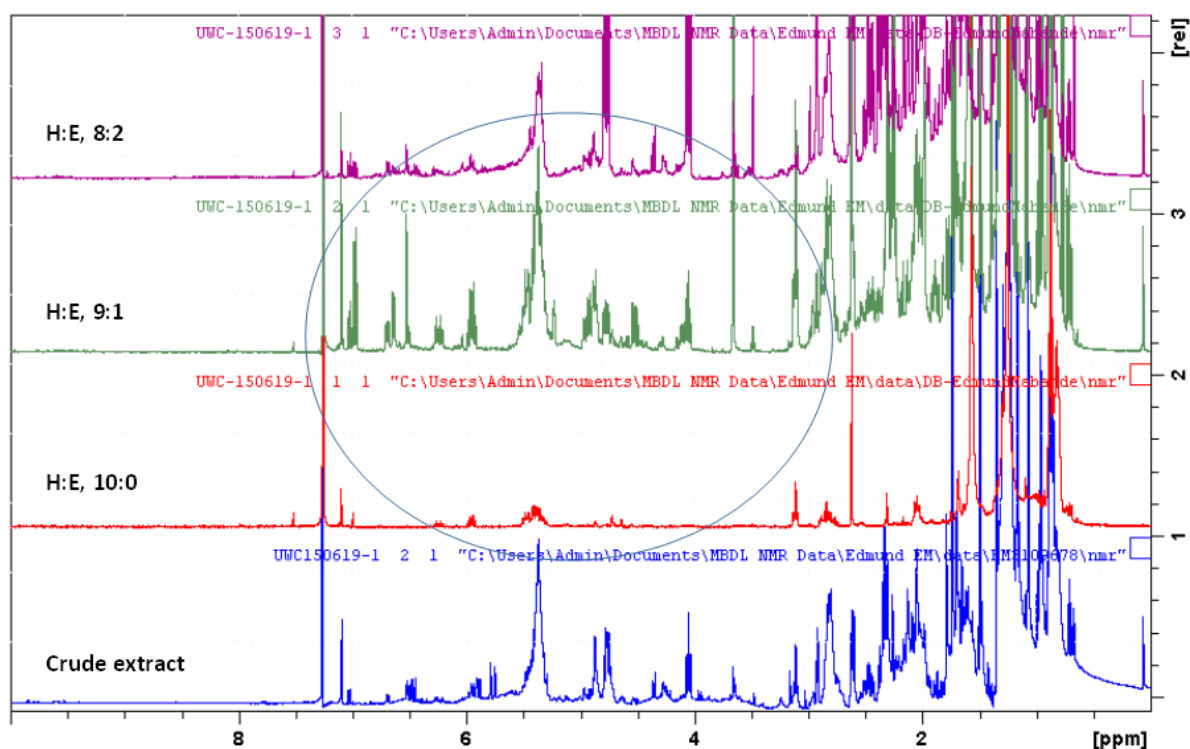
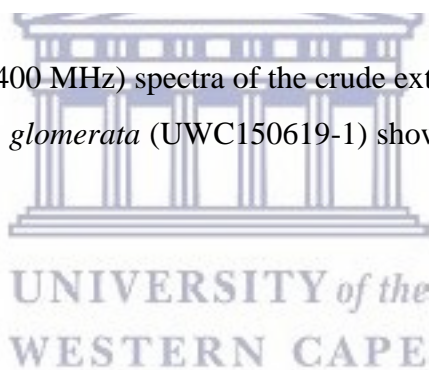


Figure 3.3 $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) spectra of the crude extract and fractions A (10:0), B (9:1) and C (8:2) of *Laurencia glomerata* (UWC150619-1) showing the effect of eluting with different solvent combinations



3.2.3 Biological studies

3.2.3.1 Antimicrobial activity of algal extracts

From a natural products based drug discovery perspective, the biological activity is the most important factor in deciding which extracts to prioritise. In this study, all fractions were evaluated for activity against five clinically relevant microbes, namely *Acinetobacter baumannii* ATCC BAA-1605, *Enterococcus faecalis* ATCC 51299, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* subsp. *aureus* ATCC 33591 *Staphylococcus aureus* ATCC, and the fungus *Candida albicans* ATCC 24433 using a modification of the popular thin layer chromatography bioautography method. This assay provides a rapid assessment of the antimicrobial activity of the extracts and fractions. A particular advantage of this assay is that compounds need not be soluble in aqueous-based solvents which is a disadvantage of the agar

well-diffusion method. This method can also be readily adapted for the screening of larger numbers of samples.

Samples (20 µg) and standard antibiotics were applied to squares (1 cm x 1 cm) on a silica gel (20 cm x 10 cm) thin layer chromatography plate. The plate was covered with the microorganism of interest and incubated overnight. Live cells were visualised by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). A clear zone indicates absence of microbial growth and provides an indication of antimicrobial activity (**Figure 3.4**). Although this assay only provides qualitative information we nevertheless tried to provide an indication of the activity by looking at the zone of inhibition: (red), complete clearing of 1 cm x 1 cm square suggests excellent activity; (orange), good activity, almost complete clear zone and (yellow), moderate activity, partial clear square, blank indicates no activity (**Figure 3.5**).

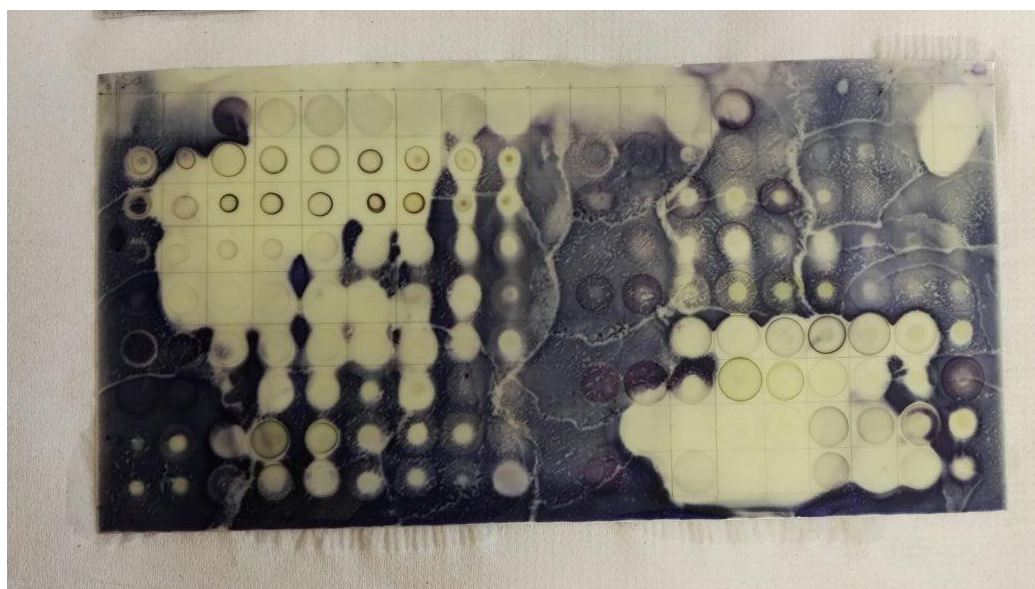


Figure 3.4: Activity of fractions against methicillin-resistant *Staphylococcus aureus* showing the zones of clearing against the purple background

The test organisms included two Gram positive and two Gram negative bacteria to evaluate broad spectrum activity of extracts. A fungus, *Candida albicans* was also chosen to evaluate the effect of the compounds on a eukaryote and to also observe the spectrum of activity. The bacteria selected and used, are listed by the World Health Organisation as leading resistant pathogens (WHO, 2017). *Acinetobacter baumannii* is a problematic opportunistic microbe that leads to serious nosocomial infections, e.g. meningitis and hospital-acquired pneumonia and novel treatment for it is badly needed (Howard *et al*, 2012). *Enterococcus faecalis* is a multi-drug resistant Gram positive bacterium. It also causes several nosocomial infections especially

in the urinary tract system (Kau *et al.*, 2005). *Escherchia coli* is usually not harmful, but some strains are very pathogenic and lead to food borne disease or poisoning. MRSA is a *S. aureus* strain that is resistant to the common antibiotic methicillin. The microbe is not known to cause infection on a healthy skin surface, but once it enters the host's bloodstream or internal areas, infection can result (Taylor and Unakal, 2017). *Staphylococcus aureus* therefore leads to serious bacterial infections such as abscesses (Kobayashi, Malachowa and DeLeo, 2015). *Candida albicans* is also evidenced to becoming resistant to choice antifungal drugs such as the azole antifungals (Whaley *et al.*, 2016). The pathogen is especially problematic in immunocompromised patients and is implicated in nosocomial infections as well.

The majority of the samples were active against the Gram positive bacteria (*S. aureus* and *E. faecalis*) while fewer samples were active against the Gram negative bacteria (*E. coli* and *A. baumannii*) and the yeast (*C. albicans*). Highest activity was noted against *S. aureus*, with 78% of the fractions being active, 46% of them potently. Activity of fractions against Gram negative bacteria was very poor, with 79% of the fractions not active at all against *E. coli* as well as the fungus, *C. albicans*. About 76% of the fractions were not active against *A. baumannii*. Standard antibiotics were used as controls in the experiment. Some of the activity observed was better than that of standard antibiotics used as controls: vancomycin or ampicillin, cyclohexamide, penicillin and chloramphenicol. Our results are in accordance with those of Val *et al.* (2001) and Vlachos *et al.* (1997) in that most fractions were active against Gram positive bacteria.

Certain seaweeds were more active than others and the spectrum of activity was variable. Algae that exhibited high activity were NDK100328-3 (*Sargassum incisifolium*), NDK130821-1 (*Sargassum incisifolium*), PA100331-6 (*Portieria hornemannii*), UWC150619-1 (*Laurencia glomerata*) GC120901-1 (*Plocamium* sp.), PB20141122-1 (*Styopodium multipartitum*), PA130427-6, D124a and D1033. Most of the activity as stated earlier was against *S. aureus*. Red algae were found to be generally more active in comparison to brown algae. This came as no surprise as red algae are known to be the best in producing biologically active compounds (El Gamal, 2010). The findings also seem to tally with another study by Kaaria *et al* (2015) that discovered that red algae are associated with the most biological activity. Epiphytes and endophytes isolated from red algae had greater antimicrobial activity than those from green or brown algae, and the high activity could be attributed to the high production of halogenated compounds by red algae (Kaaria *et al.*, 2015). Certain seaweeds such as *Portieria hornemannii* were expected to have good activity as a previous study on its

extracts had shown growth inhibition over 75% against *S. aureus* (Muregesan *et al.*, 2017). *Sargassum* spp. had also previously been shown to exhibit antimicrobial activity and its activity did not come as a surprise (Moorthi and Puthamohan 2014; Tajbakhsh *et al.*, 2015; Chiao-Wei, Siew-Ling and Ching-Lee, 2011). The two *Sargassum* species and *Styopodium multipartitum* were the most active of the brown algae particularly against *S. aureus*. Seaweeds that showed activity against both gram positive and gram negative bacteria were not many. PA100331-6, PA130427-6 and D124a were some of the few seaweeds that had broad spectrum activity, killing both Gram positives, both Gram negatives and the *Candida albicans*. From the results presented here, it is clear that the marine algae under study are potentially useful sources of antimicrobial substances.



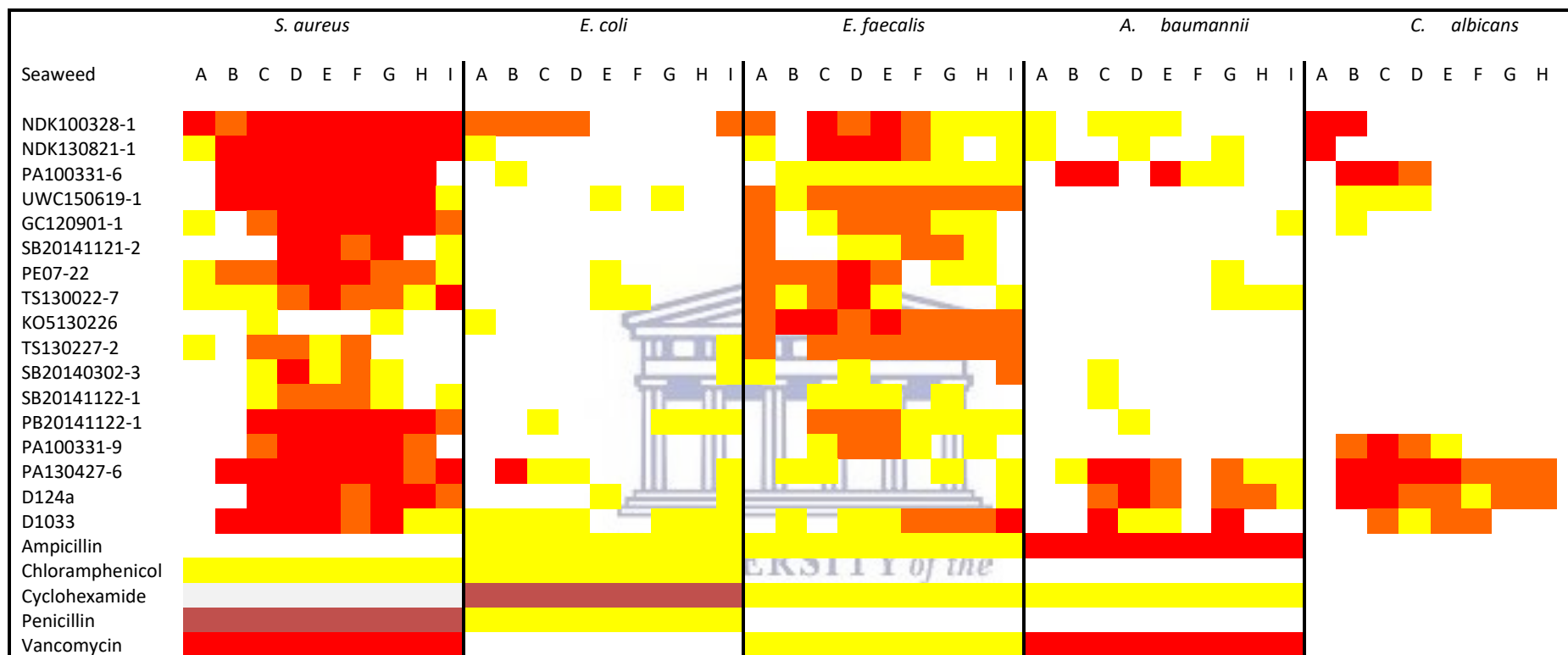


Figure 3.5. Antimicrobial activity (represented as a heat map) of the marine algal fractionated library against five human pathogens

Red = excellent activity, orange = good activity, yellow = moderate activity and white = no activity.

3.2.3.2 Cytotoxicity activity of selected fractions

As part of a new antibiotic discovery programme it is critically important that the antimicrobial lead extracts pursued show selective antimicrobial activity and are not generally cytotoxic. A desirable antimicrobial compound would be one with good antimicrobial activity whilst not being cytotoxic. The 50 most active antimicrobial fractions were evaluated for their antiproliferative effects against two human cell lines, the breast cancer cell line (MCF-7) and human embryonic kidney cells (HEK-293).

For this study, the WST-1 (water-soluble tetrazolium salt) assay. WST-1 cell proliferation reagent provides an easy and accurate way to measure cell viability, cytotoxicity and cell proliferation. This method is not radioactive, is extremely fast, much more sensitive in comparison with other assays. Other key advantages of the reagent are that it is safe and ready to use. WST-1 is a tetrazolium salt which is converted to formazan by enzymes that belongs to the respiratory chain of the mitochondria (Riss *et al.*, 2016). This is only active in live or metabolically active cells. The absorbance directly correlates to the number of live cells. The formazan dye produced by live cells can be quantitatively measured by measuring the absorbance at 440 nm.

Most fractions (66%) only showed moderate effects (greater than 50% viability at a concentration of 50 µg/mL) against the MCF-7 cell line (**Figure 3.6**). The HEK-293 cell line was slightly more sensitive to the algal fractions with 40% showing greater than 50% cell viability (**Figure 3.7**).

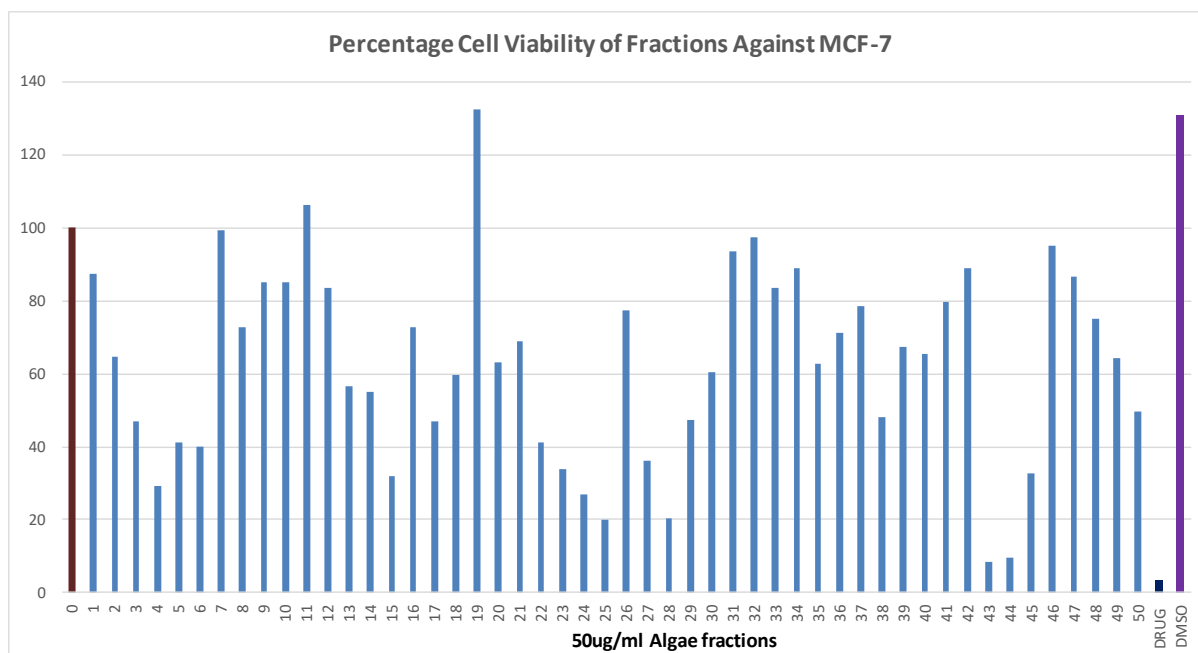


Figure 3.6 Percentage cell viability of algal fractions against MCF-7 cell line. Fraction 1-9 are GC120901-1 (A-I), Fr 10-18 are PB20141122-1 (A-I), Fr 19-23 are NDK130821-1 (B-F), Fr 24-28 are PA100331-6 (B-F), Fr 29-31 are PE07-22 (D-F), Fr 32-36 are KOS130226 (A-E), Fr 37-41 are PA100331-9 (B-F), Fr 42-46 are PA130427-6 (B-F) and Fr 47-50 are D124a (B-E)

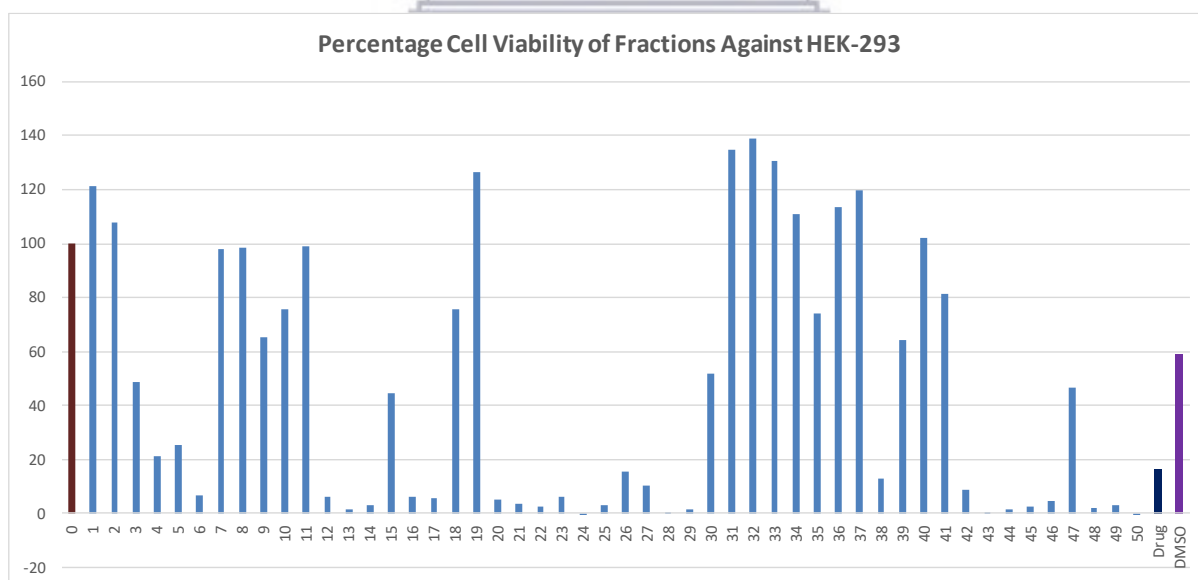
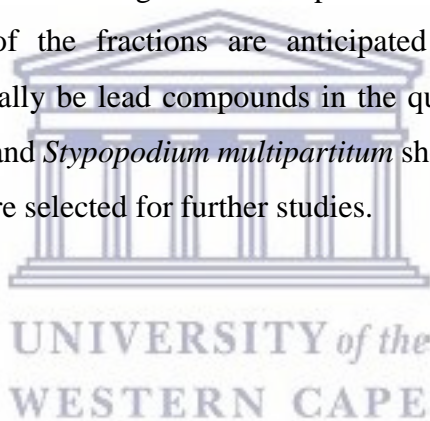


Figure 3.7 Percentage cell viability of algal fractions against HEK-293 cell line. Percentage cell viability of algal fractions against MCF-7 cell line. Fraction 1-9 are GC120901-1 (A-I), Fr 10-18 are PB20141122-1 (A-I), Fr 19-23 are NDK130821-1 (B-F), Fr 24-28 are PA100331-6

(B-F), Fr 29-31 are PE07-22 (D-F), Fr 32-36 are KOS130226 (A-E), Fr 37-41 are PA100331-9 (B-F), Fr 42-46 are PA130427-6 (B-F) and Fr 47-50 are D124a (B-E)

3.2.4 Conclusion

A pre-fractionated library of 153 pre-fractionated extracts from 17 different marine algae was successfully obtained and profiled using $^1\text{H-NMR}$ spectroscopy. Percentage recovery during silica gel fractionation was generally very high. All fractions were evaluated for antimicrobial activity and most were active particularly against *S. aureus*. Fractions were barely active against gram negative bacteria and fungus. A total of 50 selected fractions was evaluated for cytotoxic activity, and found to be generally non-toxic. Fractions of selected seaweeds had very good antimicrobial activity and exciting $^1\text{H-NMR}$ spectra. Based on their chemical and biological profiles, several of the fractions are anticipated to contain novel chemical compounds that could potentially be lead compounds in the quest for new antibiotics. Two algae namely, *Plocamium* sp. and *Styopodium multipartitum* showed very good antimicrobial activity and chemistry and were selected for further studies.

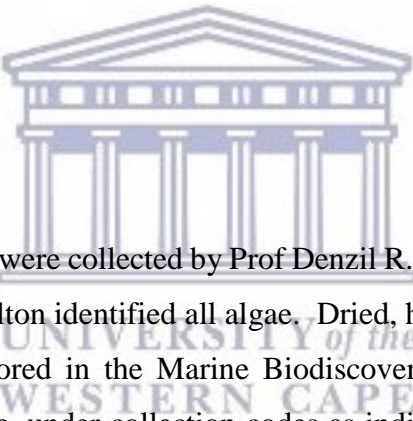


3.3 Experimental

3.3.1 General experimental

All solvents used for extraction and chromatography was either redistilled or of HPLC grade (Sigma Aldrich). Spectroscopic data of crude extracts and fractions was recorded on a Bruker Avance 400 MHz spectrometer with CDCl_3 as solvent. Chemical shifts were measured in part per million (ppm) and referenced to undeuterated solvent residues (δ_{H} 7.26 and δ_{C} 77.0). Silica gel chromatography was done using Merck Kieselgel 60 (0.040-0.063 mm). Absorbance reading for cytotoxicity studies was measured using a POLAR star Omega plate reader, sourced from BMG Labtech. The DMSO and N-acetyl-D-sphingosine (ceramide) used in the experiment was sourced from Sigma Aldrich.

3.3.2 Biological material



Marine algae used in this study were collected by Prof Denzil R. Beukes (UWC) and Prof John J. Bolton (UCT). Professor Bolton identified all algae. Dried, herbarium specimens (voucher specimens) of all algae are stored in the Marine Biodiscovery Research group collection, University of the Western Cape, under collection codes as indicated in **Table 3.1**. The algae were collected at various sites along the South African coast, from KwaZulu Natal to the Western Cape shoreline. Upon collection, the algae were immediately frozen and transported under appropriate conditions to the laboratory. Each alga was given a unique collection code which would serve as its identity and they were all labelled and photographed *ex situ*.

3.3.3 Extraction and fractionation

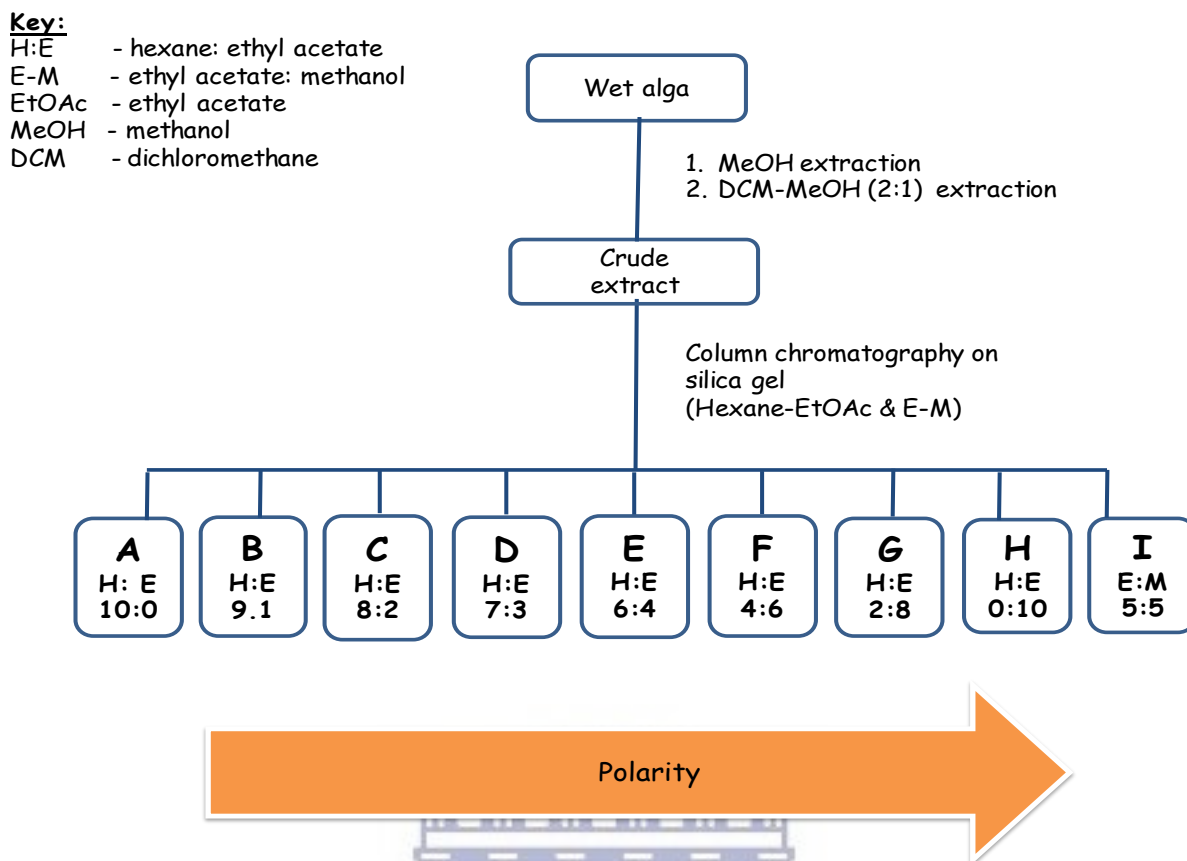
The frozen algae were allowed to thaw at room temperature and roughly chopped (**Figure 3.8**). Approximately 3 mL of chopped algae were then placed in a 50 mL centrifuge tube and submerged in MeOH (~ 3 mL). This was placed on a sonicator for about 10 minutes and then left in a dark closet for about two hours. The extract was transferred to a pre-weighed 50 mL

round bottom flask and dried *in vacuo*. The same alga was then re-extracted in CH₂Cl₂-MeOH (2:1) overnight. This re-extraction process was repeated twice. The MeOH and DCM-MeOH extracts were combined and dried *in vacuo* and its weight recorded. Each extract obtained was chemically profiled using ¹H-NMR spectroscopy and stored at -20°C before silica gel fractionation.



Figure 3.8: Photograph of five different algae just before the process of small-scale extraction

Approximately 100 mg of crude extract was loaded onto a 1 g silica gel column and eluted with solvents of increasing polarity (**Scheme 3.1, Figure 3.9**). A total of nine fractions were obtained for each alga; Fraction A - 100% hexane, Fraction B – 90% hexane: 10% ethyl acetate, Fraction C – 80% hexane: 20% ethyl acetate, Fraction D – 70% hexane: 30% ethyl acetate, Fraction E – 60% hexane: 40% ethyl acetate, Fraction F – 40% hexane: 60% ethyl acetate, Fraction G – 20% hexane: 80% ethyl acetate, Fraction H – 100% ethyl acetate and finally the most polar Fraction I – 50% ethyl acetate: 50% methanol. The fractions were dried *in vacuo*, weighed and all chemically profiled using ¹H-NMR spectroscopy.



Scheme 3.1. General pre-fractionation scheme used in the library development

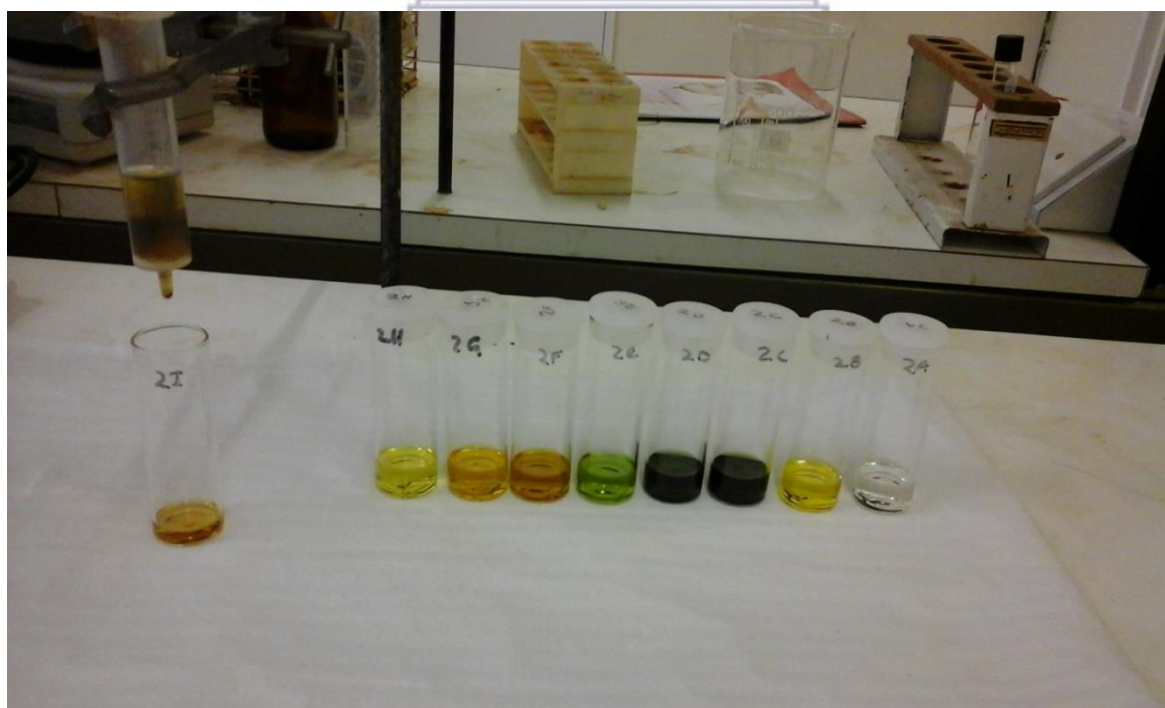


Figure 3.9: Silica gel fractionation of an alga

3.3.4 ¹H-NMR Spectroscopy

All crude extracts and their resultant fractions were profiled using ¹H-NMR spectroscopy. Samples to be tested were transferred dissolved in minimum amount of CDCl₃ into 5 mm NMR tubes. The tube(s) was tightly sealed with a polyethylene cap, appropriately labelled, and then inserted into the spectrometer. Samples were run at 32 scans per minute. Tetramethylsilane (TMS) was used as an external reference at 0 parts per million. The CDCl₃ was used an internal reference at a chemical shift of 7.2600 ppm, and all samples were ran at constant magnetic field strengths. The spectra generated were processed and analysed using Topspin® software. After NMR spectroscopy, all samples were transferred back to their original containers, evaporated of the CDCl₃, repackaged and stored in the freezer.

3.3.5 Antimicrobial assay

3.3.5.1 Preparation of samples for antimicrobial assays

From the 10 mg/mL stock solutions in the natural products library, samples of each fraction were transferred for the assays. A 20 cm x 10 cm thin layer chromatography (TLC) silica gel plate was divided into several 1 cm x 1 cm square grids (**Figure 3.10, Table 3.3**) into which the different samples were spotted before the test. Each of the 153 fractions was diluted in ethyl acetate to make a final concentration of 1 mg/mL. 20 µL of extract was spotted on each TLC plate square, thus a total of 20 µg of extract was spotted on each square. This was done twice for each microorganism to have a duplicate template. The TLC plates were air dried to remove organic solvent.

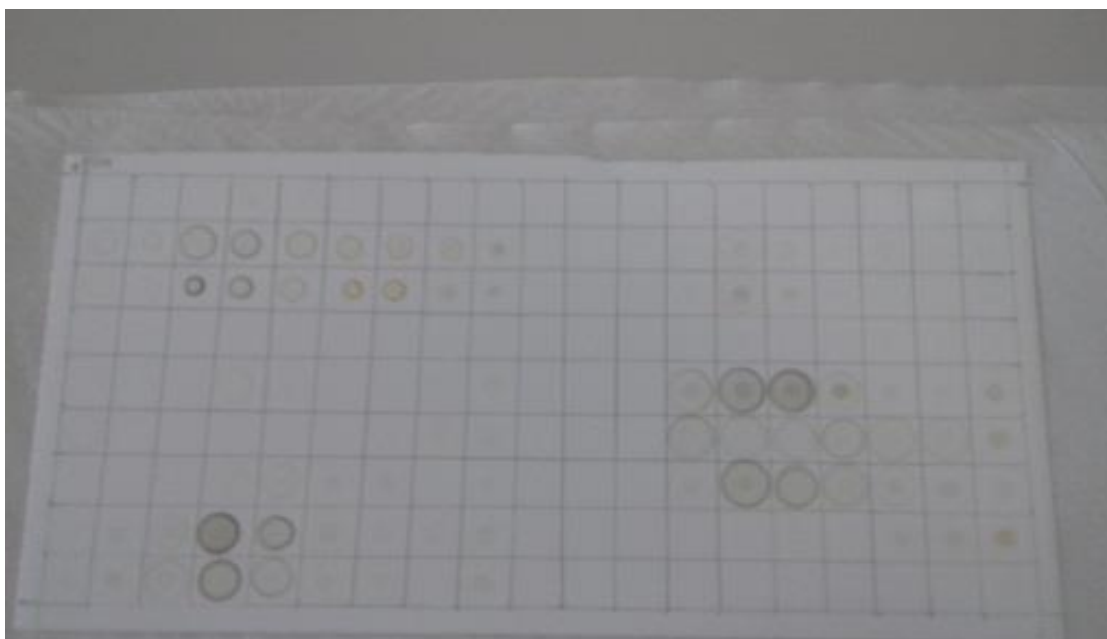


Figure 3.10: A photograph of a TLC plate just before inoculation

Table 3.3 A general layout of where each sample and the test antibiotic agent were placed in the TLC for the bioautographic method

Code																			Code		
D1033	Amp	Chl	A	B	C	D	E	F	G	H	I							Cyc	Pen	Van	
NDK...3	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	KOS...6		
NDK...1	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	TS...2		
PA...6	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	SB...3		
UWC...1	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	SB...2		
GC...1	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	PB...1		
SB...1-2	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	PA...9		
PE...22	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	PA...6a		
TS...7	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	D124a		
	Fractions									Fractions											

After incubation of the five test microorganisms overnight in culture media of choice (**Table 3.4**), they were transferred from the nutrient agar to the TLC plates with the aid of sterile cotton wool. The wool was gently dipped into the nutrient broth and softly dabbed onto the TLC plate. The TLC plates were carefully placed into big plastic boxes. A moist paper towel was added to each of the boxes used and the boxes were closed. This was done to mimic the conditions where the bacteria would thrive. This was then incubated for 24 hours at the microorganisms' optimal conditions. After 24 hours, the TLC plates were then treated with tetrazolium salt (MTT) in phosphate buffer solution. After this, they were re-incubated again in the microorganisms' optimal conditions for 3 hours. After this time period elapsed each of the TLC plates was checked for appearance of clearing zones against a background purple colour. The clear spots observed in some areas showed areas where the extracts had antimicrobial activity and the square that remained purple or dark showed areas where the microorganism persisted or were not killed. Activity was graded based on the extent of the colour change.

Table 3.4 Microorganisms used in the antimicrobial assays

Microorganism	ATCC Number	Growth media
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>	ATCC® 33591™	Nutrient broth (37 °C)
<i>Enterococcus faecalis</i>	ATCC® 51299™	Brain heart infusion broth (37 °C)
<i>Escherichia coli</i>	ATCC® 25922™	Tryptic soy broth (37 °C)
<i>Acinetobacter baumannii</i>	ATCC® BAA-1605™	Tryptic soy broth (37 °C)
<i>Candida albicans</i>	ATCC® 24433™	Nutrient broth (30 °C)

3.3.6 Cytotoxicity assays

3.3.6.1 The WST-1 assay

There are several ways to measure the cytotoxicity or cell viability of test samples. These methods include MTT, XTT, MTS and the WST-1 assay among others. WST-1 cell proliferation reagent provides an easy and accurate way to measure cell viability, cytotoxicity and cell proliferation. The method is non-radioactive and allows “spectrophotometric quantification” of cell viability or growth (Schantz and Woei, 2010). The WST-1 reagent is known to be more stable and is associated with more rapid colour changes in comparison with other salts e.g. XTT and MTS (Rampersad, 2012). The reagent is relatively safe and ready to use. This WST-1 assay is much more sensitive in comparison with other assays. One of its biggest advantages is that it is very quick, easy to use and convenient, and method can be performed in the same plates that the cells are grown in (Nissen, Pauli and Vollenbroich, 1997). WST-1 is a tetrazolium salt. Tetrazolium salts are cleaved to water-soluble formazan by certain enzymes in the mitochondria. Another alternative salt, MTT forms a formazan salt that is not water soluble, which makes it less desirable for use in assays as compared to WST-1 (Tominaga *et al.*, 1999). This is only active in live or metabolically active cells. The absorbance directly correlates to the number of cells WST-1 facilitates the reduction of the tetrazolium into the colored formazan product (Riss *et al.*, 2016). The formazan dye produced by live cells can be quantitatively measured by measuring the absorbance at 440 nm. The WST-1 reagent has a starting colour of pink. A pink colour would indicate presence of living cells and on the other hand a colour change from pink to a yellowish/orange colour would signify the presence of dead cells (**Figure 3.7**).



Figure 3.11. Photography of a 96-well plate showing the activity of some the algal fractions against human cells.

3.3.6.2 Preparation of samples for cytotoxic assay

The 50 marine algal extracts to be evaluated for antiproliferative activity were drawn out from the 10 mg/mL DMSO stock solutions. The frozen samples were left to thaw before they were diluted to afford to a standard concentration. Crude extract fractions were added to the media and cancer cells already in the well plate to afford a concentration of 50 µg/mL in the media. To do this 1.75 µL of an algal fraction was added to 348.25 µL of media to give a total of 350 µL. Human breast cancer MCF-7 and kidney HEK-293 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) in 96-well plates. The media contains 4.5 g/L glucose with L-glutamine and was stored at 2-8°C before the experiment was conducted. The experiment was done in triplicate per each alga fraction with 100 µL added into each well per fractions. Therefore, each fraction needed a total of 300 µL, leaving an excess of 50 µL. Dimethyl sulfoxide (DMSO) was diluted from a concentration of 100% to 50 µM and used as the negative control. N-acetyl-D-sphingosine (ceramide) was also diluted from a concentration of 14.6 mM to a concentration of 50 µM and used as the positive control. The ceramide and DMSO were also added to their designated wells in the 96-well plates. This was also done in triplicate per each control. The 96-well plates were then placed in an incubator at 37°C, 5% CO₂ and a humid environment for 24 hours.

After 24 hours of incubation, the cells lines removed from the incubator. WST-1 reagent was added to the cells in the 96-well plates. The plates were then placed back into the incubator under the same conditions for a further three hours. The experiment was performed in a fume cupboard and WST-1 as a light-sensitive reagent and was always protected by aluminum foil during the experiment.

After the three hours had elapsed the 96-well plates were taken from the incubator and into a plate reader to be analysed for absorbance. The absorbance for each algae fraction was recorded and compared to the untreated cells and controls to give a cell viability value. Untreated cells, thus live cells had 100% cell viability and dead cells would give less viability. A large cell viability value would indicate actually indicate an increase in the cell growth.



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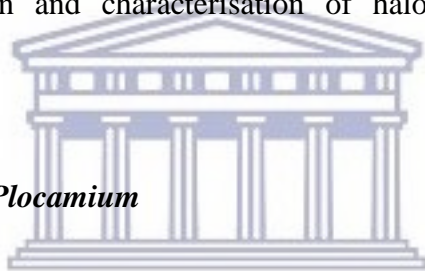
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Chapter 4

Halogenated tetrahydrofuran monoterpenes from a South African *Plocamium* sp.

4.1 Introduction

Following the screening of the fractionated library for antimicrobial activity (Chapter 3), we selected the seaweed GC120901-1, for further study on the basis of its biological activity and interesting $^1\text{H-NMR}$ profile. Most of its fractions showed good antimicrobial activity against the test microbes. This seaweed was identified as a new South African *Plocamium* sp. In this chapter we will briefly review the chemistry of seaweeds of the genus *Plocamium* followed by a discussion of the isolation and characterisation of halogenated monoterpenes from GC120901-1.



4.1.1 Seaweeds of the genus *Plocamium*

Plocamium is a genus of red algae that belongs to the family Plocamiaceae, order Plocamiales. Approximately 47 known *Plocamium* species are widely distributed throughout the world and is reportedly found in both cold and temperate seas (Francis, 2009; Nilauro, 1999). *Plocamium* species have been found in oceans in both the southern and northern hemispheres in countries such as the U.S.A, Canada, Iceland, Chile, South Africa, Mozambique, New Zealand, Australia and the Pacific islands (Guiry, 1998). The seaweed has erect fronds with a bright red or scarlet colour. The fronds are branched in either a regular or an irregular pattern with each branch having several tiny branchlets on them (Fish and Fish, 2011). The branching is very delicate giving a beautiful colourful appearance (**Figure 4.1**). The seaweeds are known to be found from the intertidal zone to a depth of over 35 m (Crews, 1977). *Plocamium* species found along the South African coast include *P. glomeratum*, *P. membranaceum*, *P. beckeri*, *P. rigidum*, *P. cornutum*, *P. suhrii*, *P. corallorhiza* and *P. telfairiae* (Simon, 1964). Species of the genus are known to be more diverse in the Southern hemisphere (Cremades *et al.*, 2011) and are known to be a rich source of halogenated terpenoids (Abreu and Galindro, 1996).



Figure 4.1: Photograph of *Plocamium cartilagineum* showing the bright red delicately branched fronds (Fernandez, 2011)

4.1.2 Natural products produced by *Plocamium* spp.

Plocamium species produce an array of halogenated monoterpenes (Rovirosa *et al.*, 2013). Monoterpenes are made of two isoprene (five carbon) units and are a class of chemically interesting compounds. Studies on monoterpenes begun as far back as the 19th century and has continued leading to the discovery of a large number of new monoterpenes (Naylor *et al.*, 1983). Monoterpenes isolated from *Plocamium* spp. usually contains a number of chlorine and bromine atoms (Gunatilaka *et al.*, 1999) and can be acyclic, cyclic and tetrahydropyran derivatives (Jongaramruong and Blackman, 1999). **Figure 4.2** shows the most common carbon frameworks of natural products isolated from *Plocamium* spp.

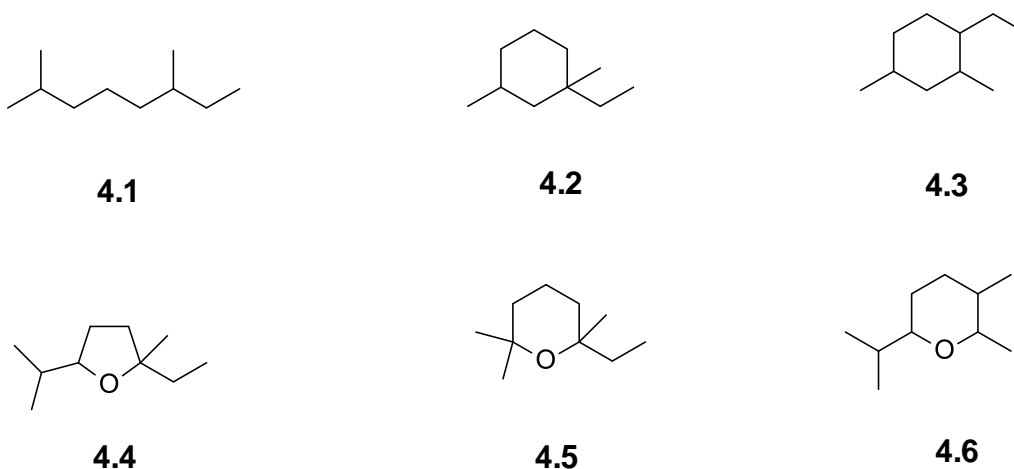


Figure 4.2 The basic carbon skeletons identified in *Plocamium* halogenated monoterpenes

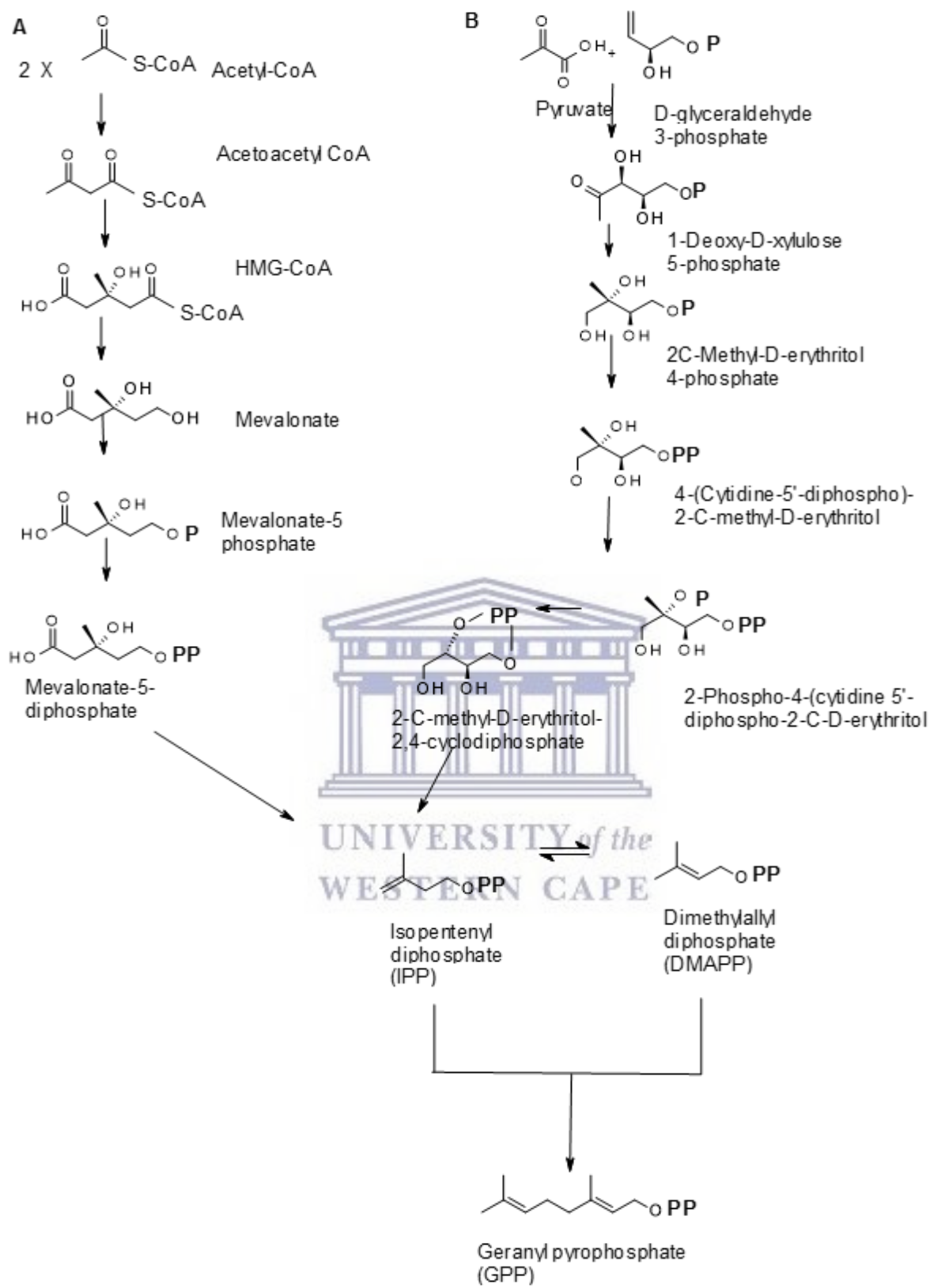
There are two main biosynthetic pathways responsible for the synthesis of terpenoids: the mevalonate pathway and the non-mevalonate pathway (**Scheme 4.1**).

4.1.2.1 The mevalonate pathway

The production of mevalonic acid (MVA) begins with the enzymatically catalysed condensation reaction of acetyl-CoA and acetoacetyl-CoA to form an intermediate compound known as 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then sequentially reduced to mevalonic acid by NADPH in the presence of HMG-CoA reductase until MVA is produced. MVA is then phosphorylated by ATP in the presence of mevalonate kinase to give MVA 5-phosphate, which also undergoes another phosphorylation reaction with ATP and phosphomevalonate kinase to give MVA 5-diphosphate. MVA 5-diphosphate is then enzymatically converted to the two foundational isoprene units; isopentyl diphosphate (IPP) and dimethyl-allyl diphosphate (DMAPP) (Dewick, 2002). These two intermediate compounds have a five carbon skeleton. They are joined together in a head-to-tail fashion to form geranyl pyrophosphate (GPP), which is known as the universal precursor of all monoterpenes (**Scheme 4.2**) (Zebec *et al.*, 2016; Demissie *et al.*, 2013).

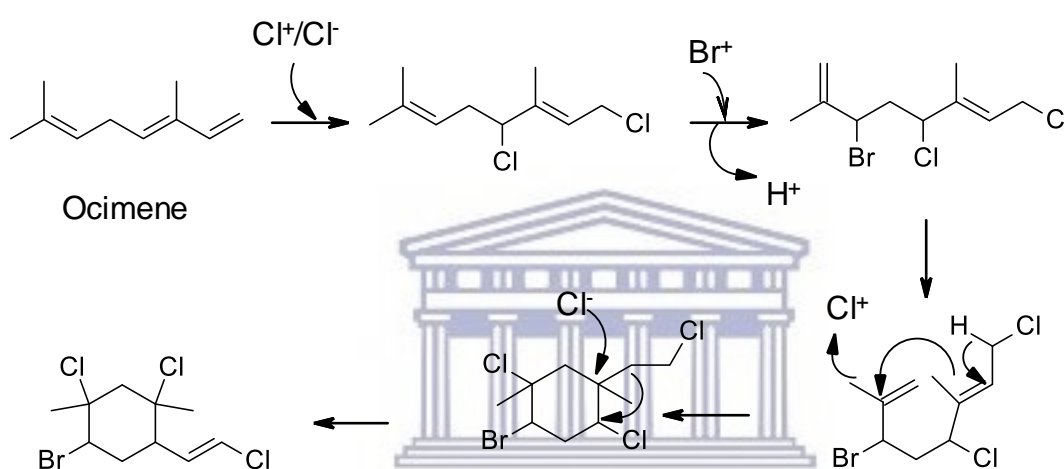
4.1.2.2 The non-mevalonate or methylerythritol phosphate (MEP) pathway

The non-mevalonate pathway is known to begin with the condensation of pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate which is then converted to 2C-methyl-D-erythritol 4-phosphate (MEP) in an isomerisation reaction (Lange *et al.*, 1999). MEP is then converted to the precursor molecule dimethyl diphosphate (IPP) via a series of enzymatically catalysed reactions (Dubey, Bhalla and Luthra, 2003). As with the mevalonate pathway IPP can isomerise to DMAPP and the two molecules can similarly condense together to form geranyl pyrophosphate (GPP) (**Scheme 4.1**).



Scheme 4.1 Initial steps in the biosynthesis of monoterpenes showing the production of the precursor isoprene unit dimethyl diphosphate (A) via the mevalonate pathway and (B) via the non-mevalonate pathway. Note: (letter P) in bold represents a phosphate group. (Scheme adapted from: Lange et al., 2000)

The process of halogenation in these compounds is known to start with the oxidation of halides e.g. chloride or bromide by haloperoxidases, which are enzymes which have been found in all classes of seaweed (Butler and Walker, 1993). Electrophilic attack on one of the double bonds in myrcene or ocimene by the enzymatically generated electrophilic Br^+ or Cl^+ produces an intermediate halonium ion which is subsequently attacked by either chloride (Cl^-), bromide (Br^-) or an oxygen nucleophile (e.g. H_2O). Further electrophilic addition of halogens and or halogen induced electrophilic cyclisation give rise to the more than 100 halogenated monoterpenes reported from *Plocamium* spp. (Scheme 4.2, Wise *et al.*, 2002). Figure 4.3 shows a few selected halogenated monoterpenes isolated from different *Plocamium* species.



Scheme 4.2: Steps showing the halogenation of monoterpenes from marine algae. (Wise *et al.*, 2002)

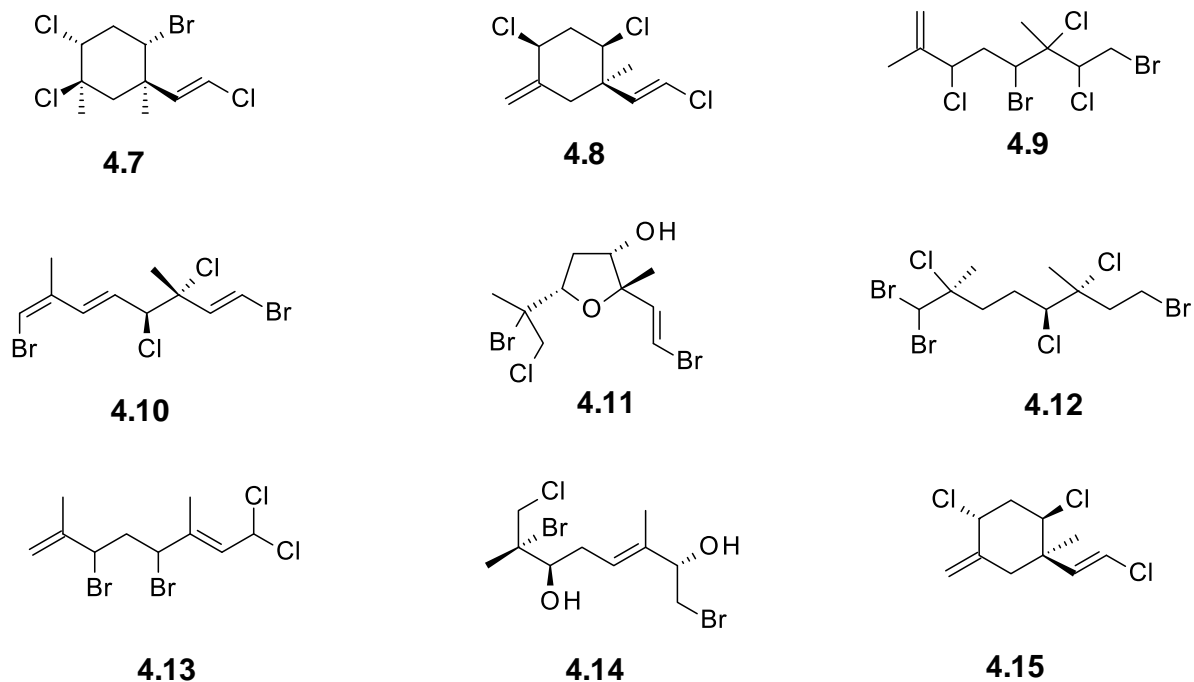


Figure 4.3 Chemical structures of selected halogenated monoterpenes isolated from different *Plocamium* species

4.2 Results and discussion

In this research study on South Africa marine algae, several pre-fractionated extracts from 17 different marine algae showed excellent antibacterial activity against pathogens including methicillin-resistant *staphylococcus aureus*. We decided to pursue the alga *Plocamium* sp. for further studies as it showed exciting chemistry, good biological activity, and that it had not been previously studied. This led to the isolation and identification of three new halogenated furanoid monoterpenes.

4.2.1 Extraction and isolation of compounds from *Plocamium* sp.

A small sample of *Plocamium* sp. (collection code: GC120901-1), **Figure 4.4** was collected from Glencairn, near Cape Town on 1 September 2012 and the sample stored frozen until workup.



Figure 4.4: Photograph of *Plocamium* sp. used in this study

The $^1\text{H-NMR}$ spectra of the MeOH and MeOH- CH_2Cl_2 (1:2) extracts (**Figure 4.5**) of the alga showed one major compound and was combined before further fractionation. Fractionation of the organic extract by silica gel column chromatography yielded nine fractions (A-I) of which the $^1\text{H-NMR}$ spectra of fractions A and B exhibited typical signals of halogenated monoterpenes (**Figure 4.6**). Further fractionation of these fractions yielded three compounds (**4.16**, **4.17** and **4.18**) (**Scheme 4.3**).

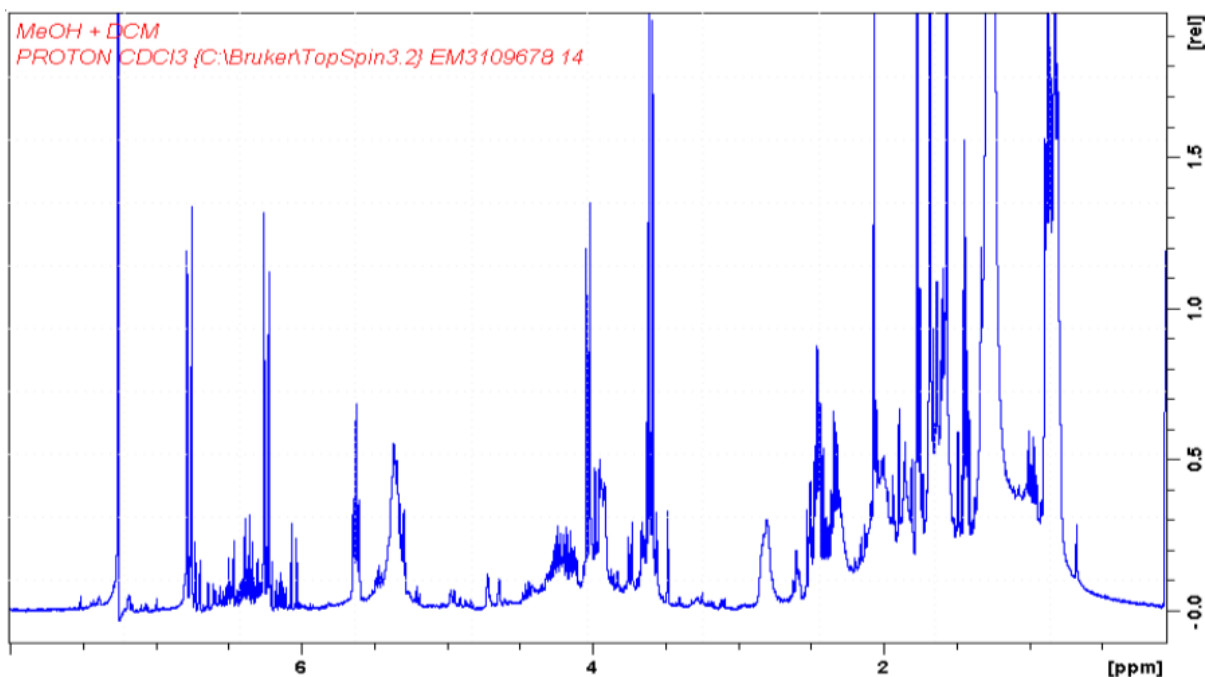
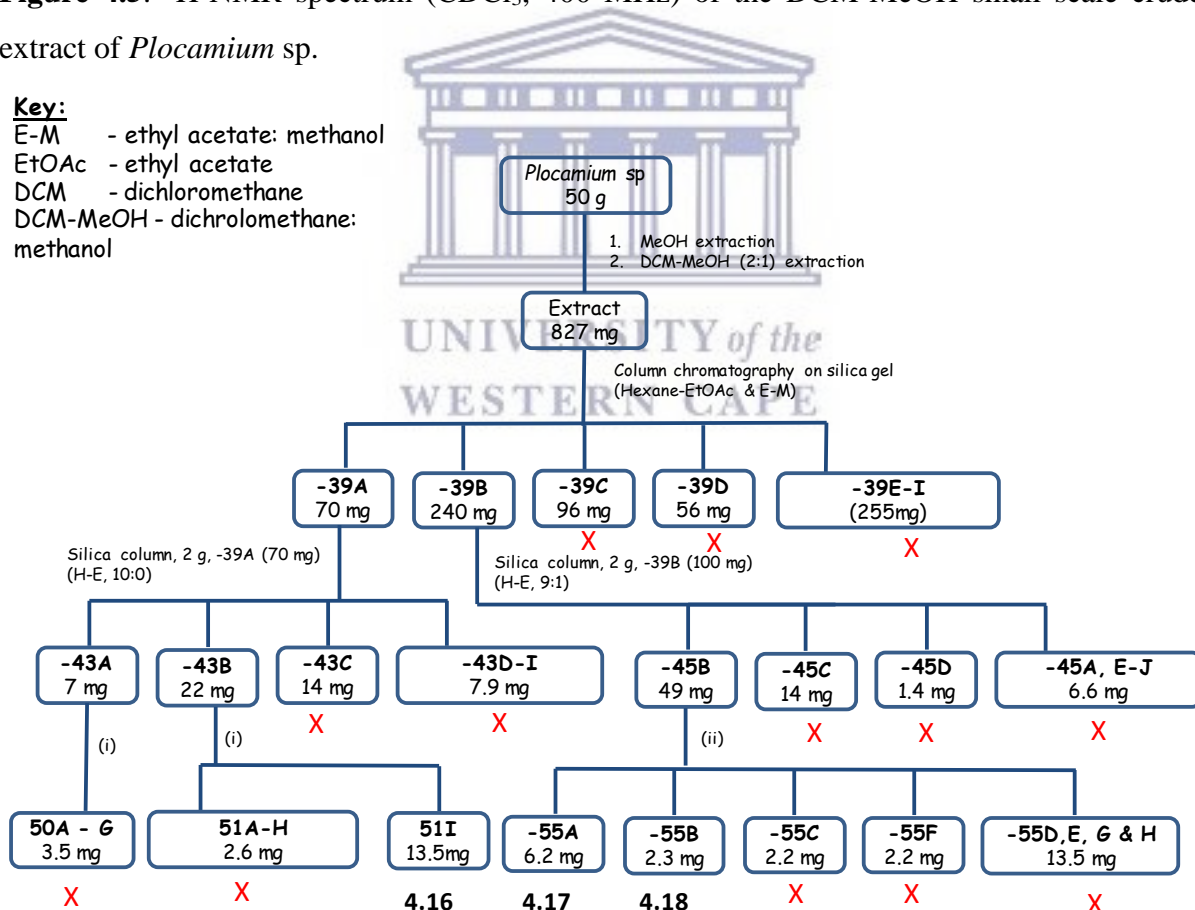


Figure 4.5: $^1\text{H-NMR}$ spectrum (CDCl_3 , 400 MHz) of the DCM-MeOH small scale crude extract of *Plocamium* sp.

Key:

- E-M - ethyl acetate: methanol
- EtOAc - ethyl acetate
- DCM - dichloromethane
- DCM-MeOH - dichloromethane: methanol



Scheme 4.3: Isolation scheme of *Plocamium* sp. that led to the isolation of compounds **4.16**, **4.17** and **4.18**. Conditions: (i) HPLC hexane-ethyl acetate (10:0). (ii) HPLC hexane-ethyl acetate (9:1). Note: X denotes fractions that were not pursued further

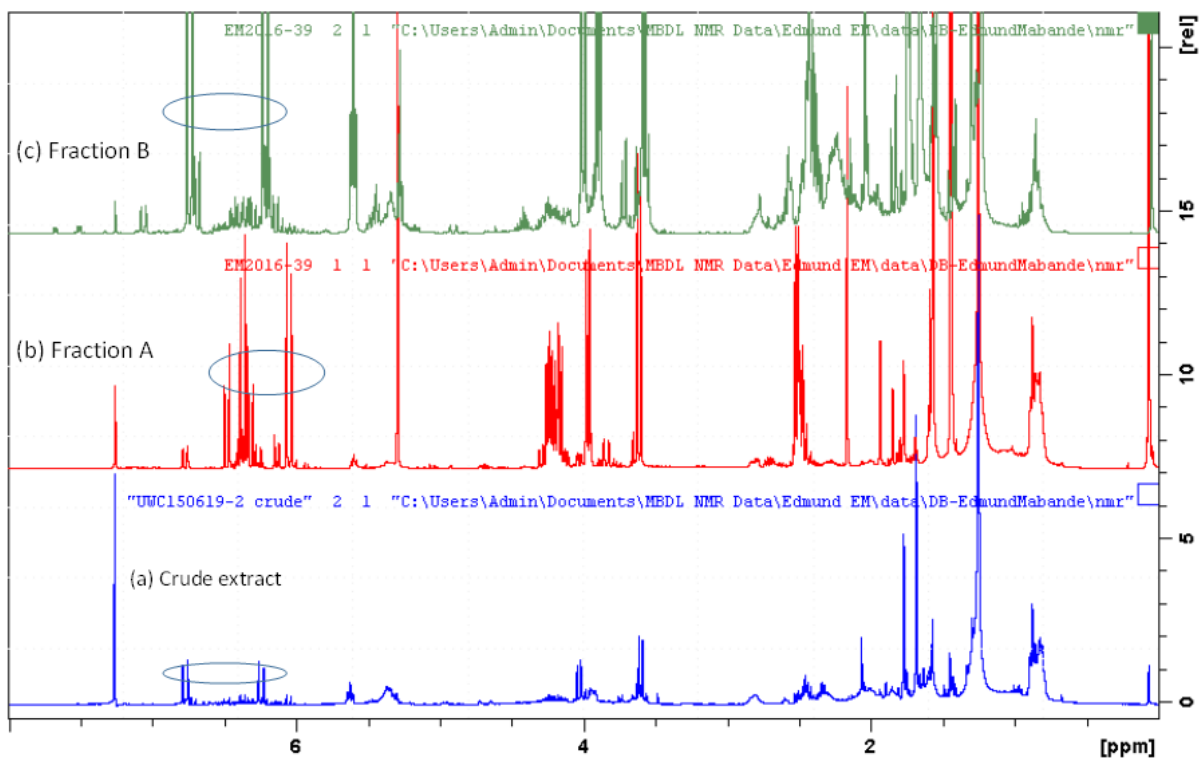
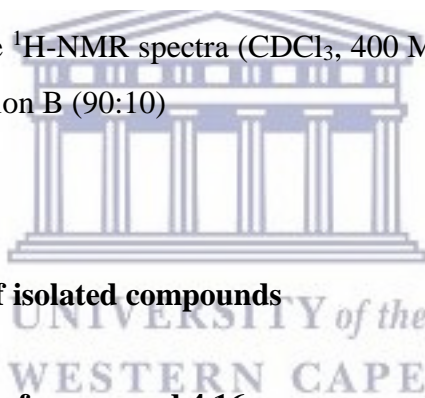


Figure 4.6: Comparison of the ¹H-NMR spectra (CDCl₃, 400 MHz) of the a) crude extract b) fraction A (100:0) and c) fraction B (90:10)



4.2.2 Structure elucidation of isolated compounds

4.2.2.1 Structure elucidation of compound 4.16

Compound **4.16** contained another minor compound in minute quantity, which could not be removed even after several rounds of normal phase HPLC. This impurity did not adversely affect the structure elucidation of compound **4.16**. The ¹H-NMR spectrum of compound **4.16** (**Figure 4.7**) in combination with its edited-HSQC spectrum, indicated the presence of a trans double bond (δ_{H} 6.38, 1H, d, $J = 13.2$ Hz and 6.09, 1H, d, $J = 13.2$ Hz) two deshielded methine signals (δ_{H} 4.17 and 4.21), two diastereotopic methylene protons (δ_{H} 4.00 and 3.65) and two methyl groups (δ_{H} 1.44 and 1.57). The ¹³C-NMR spectrum (**Figure 4.8**), together with the edited-HSQC spectrum showed ten carbons and confirmed the presence of a single, double bond (δ_{C} 119.2 and 136.0), two deshielded methine carbons (δ_{C} 51.9, 71.4), a methylene (δ_{C} 37.5) and two methyl signals (δ_{C} 25.6 and 25.8).

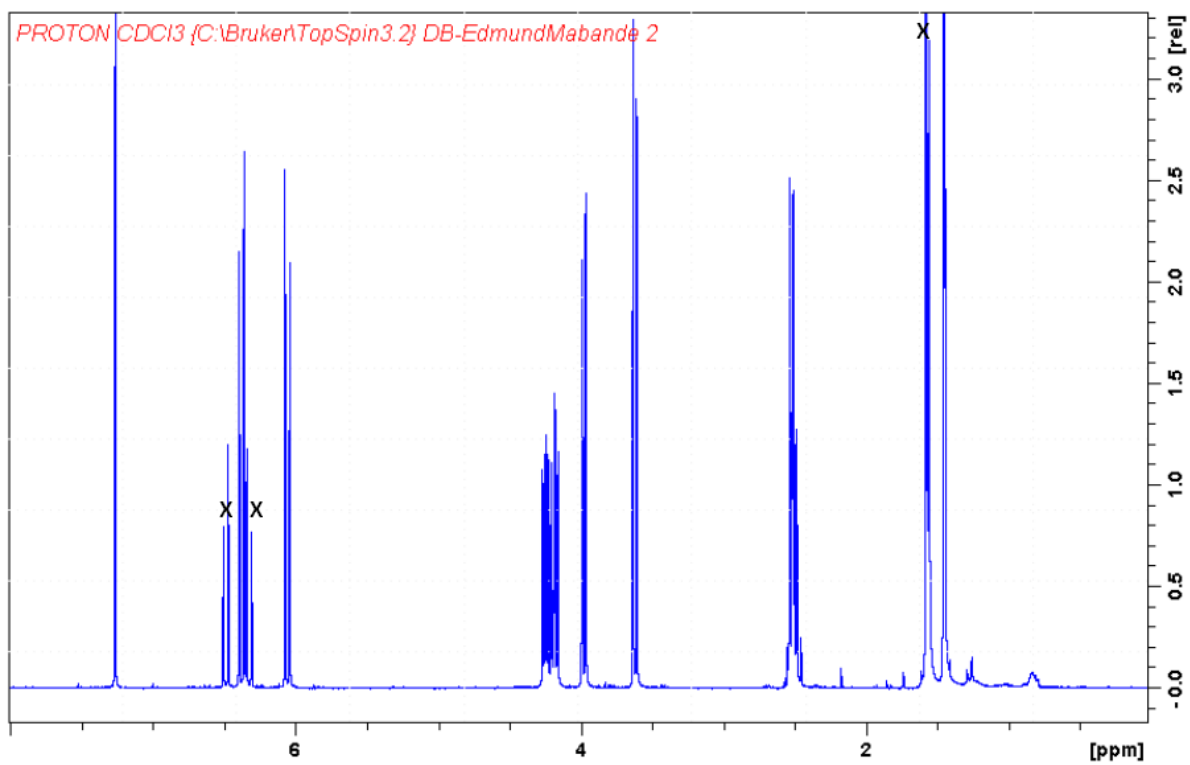


Figure 4.7: ¹H-NMR spectrum of compound **4.16** (CDCl₃, 400 MHz)

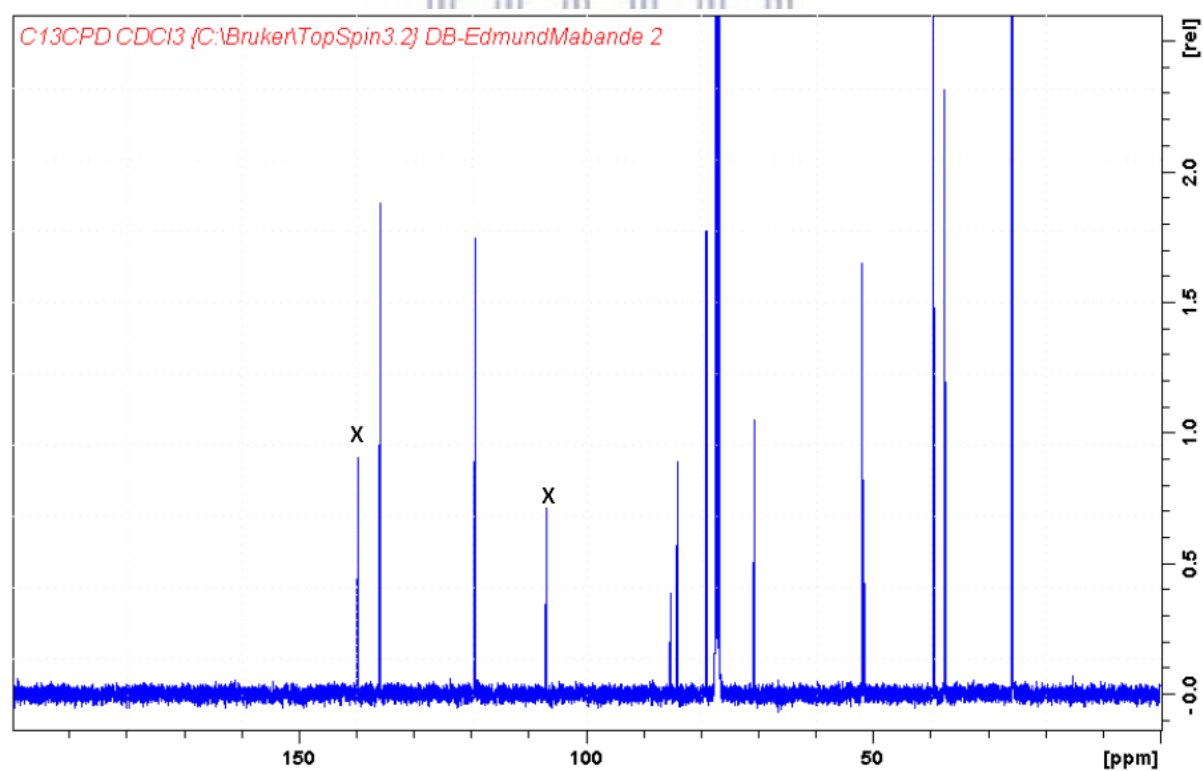


Figure 4.8: ¹³C-NMR spectrum of compound **4.16** (CDCl₃, 100 MHz)

The HMBC spectrum was used to outline the planar structure of compound **4.16** as follows: HMBC correlations from the methyl signal at δ_{H} 1.44 (Me-9) to the olefinic carbon signal at δ_{C} 136.0 (C-2), the deshielded quaternary carbon signal at δ_{C} 84.0 (C-3) and the deshielded methine carbon at δ_{C} 51.9 (C-4) indicated the presence of a $-\text{CH}(\text{X})-\text{CX}(\text{CH}_3)-\text{CH}=\text{C}$ moiety. Further HMBC correlations of the second methyl signal δ_{H} 25.6 (Me-10) to the methylene carbon at δ_{C} 39.5 (C-8) and the methine carbon at δ_{C} 78.9 (C-6) as well as the quaternary carbon at δ_{C} 71.4 (C-7) provided the second part structure, $-\text{CH}_2-\text{CX}(\text{CH}_3)-\text{CH}(\text{X})-$ and therefore, suggested a regular monoterpene skeleton for compound **4.16** (**Figure 4.9**).

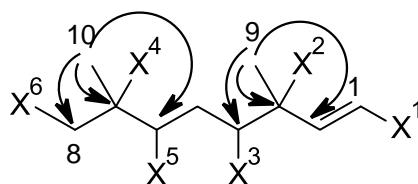


Figure 4.9. Key HMBC correlations and planar structure of compound **4.16**

With the planar structure of compound **4.16** in hand we next turned our attention to the assignment of the heteroatoms X^1-X^6 . This is typically done by a consideration of the ^{13}C NMR chemical shifts of the carbon atoms directly attached. To facilitate this process, we compiled a table of ^{13}C NMR chemical shifts of related natural products (**Table 4.1**). The electron withdrawing substituents at C-1 (δ_{C} 119.2), C-4 (δ_{C} 51.9), C-7 (δ_{C} 71.4) and C-8 (δ_{C} 39.4) can be assigned unambiguously as Cl, Br, Cl and Br, respectively, on the basis of their ^{13}C NMR chemical shifts. The ^{13}C NMR chemical shifts of C-3 (δ_{C} 84.0) and C-6 (δ_{C} 78.9) are suggestive of oxygen atoms attached to these positions. Finally, a bromine substituent was assigned at C-7 based on its chemical shift (**Table 4.1**) (Naylor 1983, Diaz-Marrero 2003). The relative configuration of the chiral centres in the compounds was probed by a NOESY NMR experiment (**Table 4.1**). A NOE cross-peak between Me-9 and H-6 suggests that these protons are on the same side of the ring. Similarly, the lack of NOE between Me-9 and H-4 indicates that these protons are on opposite sides of the ring. All of the above information can be accommodated by the proposed structure for **4.16** (**Figure 4.10**). Unfortunately, several attempts at obtaining high resolution mass spectrometric data for compound **4.16** was unsuccessful. We are currently trying to recollect the seaweed for further studies.

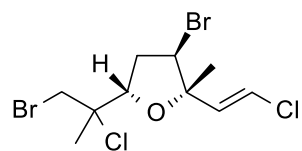
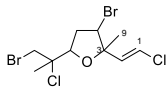
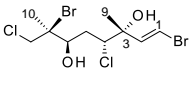
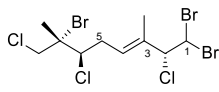
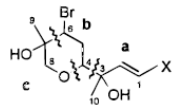
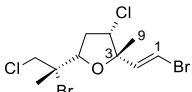
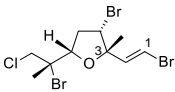
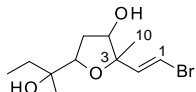
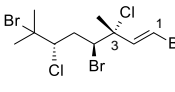


Figure 4.10: Proposed structure of compound **4.16**



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Table 4.1. Comparison of ^{13}C -NMR chemical shifts of selected halogenated monoterpenes

C #								
	(4.16)							anverene (8)
1	119.2	108.0	44.2	120.8	108.2	106.7	105.9	109.7
2	136.0	139.9	64.9	136.2	138.4	139.7	141.3	139.9
3	84.0	76.5	134.4	75.9	85.3	90.0	88.3	71.9
4	51.9	67.1	128.7	64.2	62.9	51.5	75.4	59.8
5	37.5	36.5	32.7	37.4	38.4	38.9	34.7	39.2
6	78.9	70.9	72.8	57.5	77.7	78.5	83.7	69.2
7	71.4	73.8	67.9	75.9	67.8	68.0	71.7	66.3
8	39.4	51.0	52.7	70.5	50.9	50.9	26.4	28.8
9	25.8	25.7	11.1	21.8	26.1	25.7	27.6	33.4
10	25.6	25.3	25.1	26.0	25.1	25.9	21.0	25.5
		(Diaz-Marrero <i>et al.</i> , 2002)	(Naylor <i>et al.</i> , 1983)	(Cueto <i>et al.</i> , 1998)	(Diaz-Marrero <i>et al.</i> , 2002)	(Diaz-Marrero <i>et al.</i> , 2002)	(Cueto <i>et al.</i> , 1998)	(Pasaribu <i>et al.</i> , 2006)

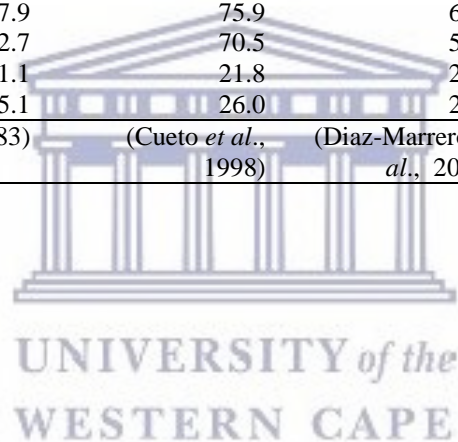


Table 4.2: NMR spectroscopic data for compound **4.16**

Carbon atom	δ_C	Type	δ_H , mult. J (Hz)	COSY	HMBC	NOESY
1	119.2	CH	6.38, d, 13.2	H2	C3	
2	136.0	CH	6.09, d, 13.2	H1	C1, C3, C9	
3	84.0	C	-			
4	51.9	CH	4.18, m			
5	37.5	CH ₂	2.50, m	H6	C3, C4, C6, C7	H6
6	78.9	CH	4.24, t, 6.7	H5		
7	71.4	C				
8	39.4	CH ₂	4.00, d, 9.9 3.65, d, 9.9			
9	25.8	CH ₃	1.44, s		C1, C2, C3	H6, H10
10	25.6	CH ₃	1.57, s	H7	C6, C7, C8	H6

4.2.2.2 Structure elucidation of compound 4.17

Compound **4.17** proved to be the minor component that was present in compound **4.16**. The ¹H-NMR and HSQC spectra of compound **4.17** showed the presence of a trans double bond, (δ_H 6.31, 1H, d, J = 13.3 Hz and δ_H 6.20, d, J = 13.3 Hz), one methine signal at δ_H 4.24, two diastereotopic methylene protons observed at δ_H 4.00 and 3.60 and two methyl groups (δ_H 1.36 and 1.58) (Table 4.2, Figure 4.11). Two mutually coupled methylene signals which are shielded are seen resonating at δ_H 1.89 and 2.01. Unlike compound **4.16**, in this compound, a total of three methylene groups instead of two can be seen from the HSQC spectrum. The main difference between compound **4.16** and **4.17** is the different halogen substituents on the double bond, with a chlorine in compound **4.16** and a bromine in compound **4.17**, and also the disappearance of the one methine signal in compound **4.16**, which is replaced by a methylene group in compound **4.17**. The ¹³C-NMR and edited-HSQC spectra showed the presence of ten peaks indicating the presence of a monoterpene and also the presence of a single double bond (δ_C 104.8 and 142.4), a deshielded methine carbon (δ_C 72.6), methylene carbons (δ_C 27.1, 37.4 and 39.4) and two methyl signals (δ_C 25.6 and 26.7) (Figure 4.12).

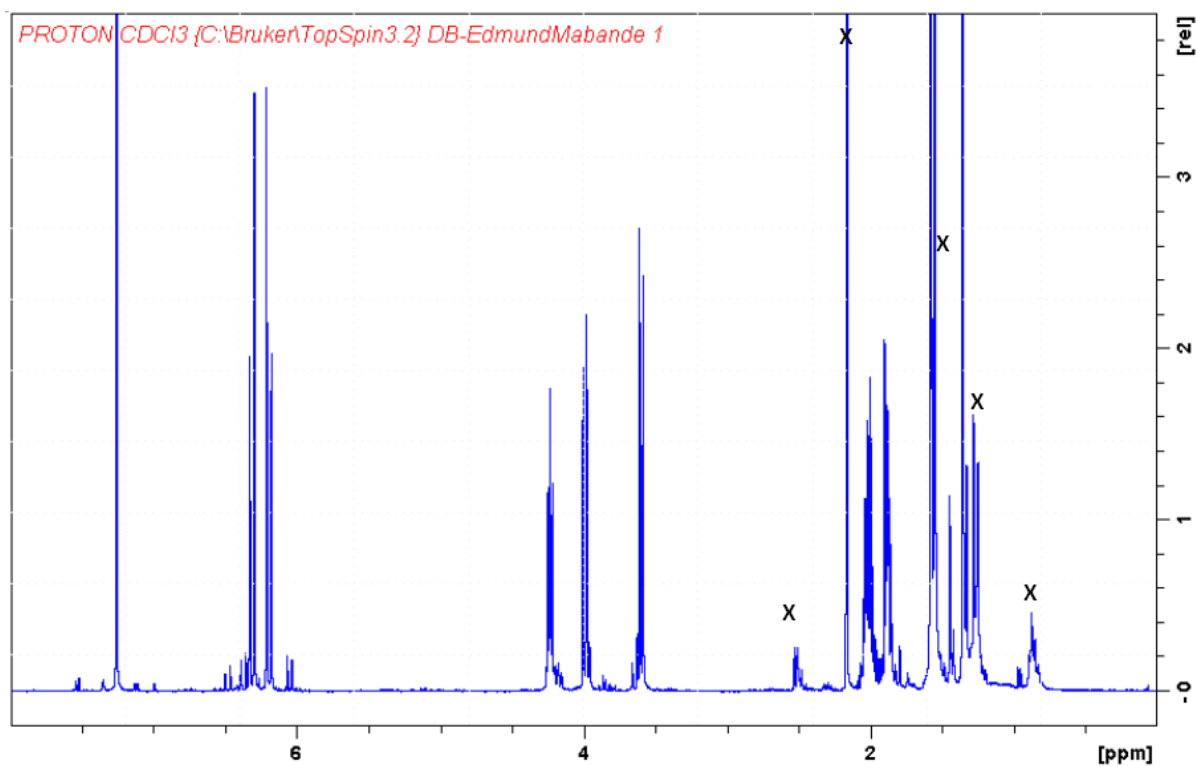


Figure 4.11: ^1H -NMR spectrum of compound **4.17** (CDCl_3 , 400 MHz).

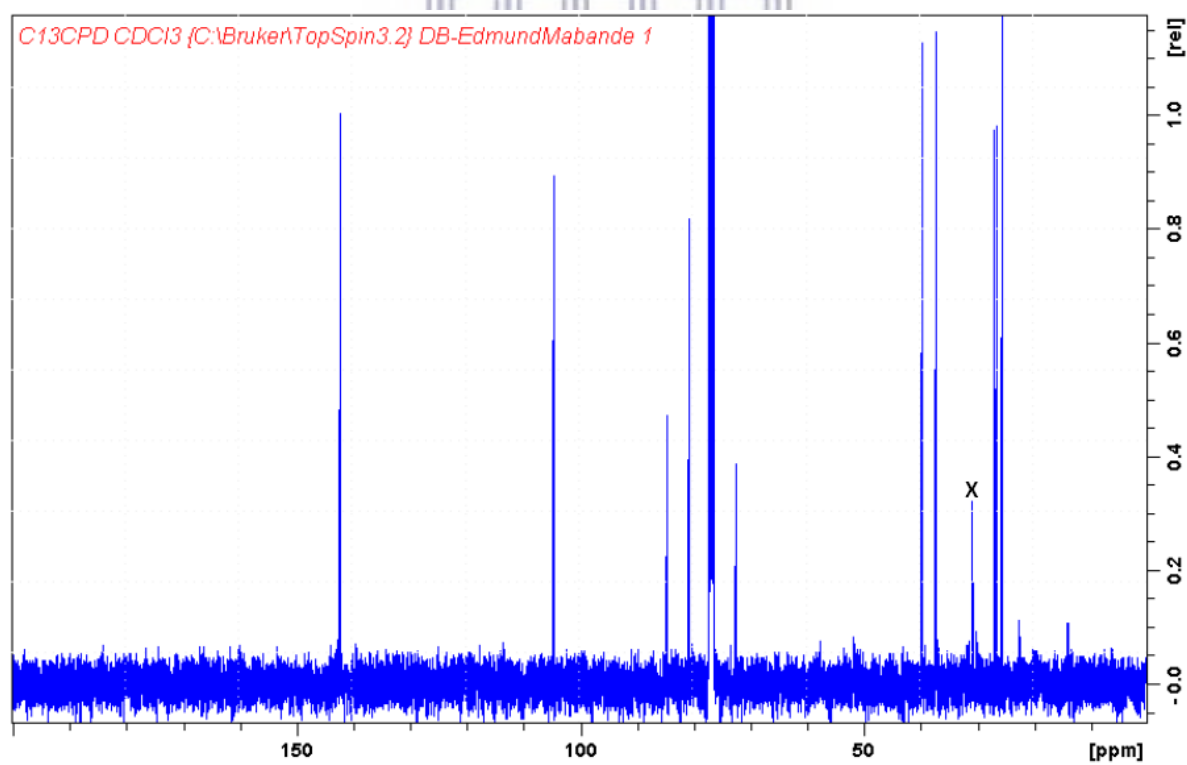


Figure 4.12: ^{13}C -NMR data of compound **4.17** (CDCl_3 , 100 MHz)

HMBC correlations observed in this compound (**Table 4.13**) are similar to those observed in compound **4.16**. HMBC correlations of δ_{H} (Me-9) to the carbon at δ_{C} 142.4 (C2), δ_{C} 84.8 (C3) and δ_{C} 37.4 (C4) are observed giving rise to the $-\text{CH}(\text{X})-\text{CX}(\text{CH}_3)-\text{CH}=\text{C}-$ moiety. HMBC correlations are also observed on the other methyl signal δ_{H} 1.58 (Me-10) and δ_{C} 80.9 (C6), δ_{C} 72.9 (C7) and δ_{C} 39.8 (C8), similarly giving rise to the $-\text{CH}_2-\text{C}(\text{X})-\text{CH}_3-\text{CH}(\text{X})-$ moiety as the other part of the molecule.

Assignment of halogen atoms on compound **4.17** was done by comparing ^{13}C chemical shift values of carbon atoms directly attached to the halogen. Substituents at C-1 ($\delta_{\text{C}} = 104.8$) and C-8 (δ_{C} 39.8) were assigned as bromines and the substituent at C-7 (δ_{C} 72.6) was assigned as chlorine, similarly to compound **4.16**. Similarly, the ^{13}C chemical shifts C-3 (δ_{C} 84.8) and C-6 (δ_{C} 80.9) are indicative of an oxygen atom directly attached to these carbon atoms. There is no halogen substituent at C-4 (δ_{C} 37.4) in compound **4.17**. The NOESY data for compound **4.17** was weak and inconclusive.

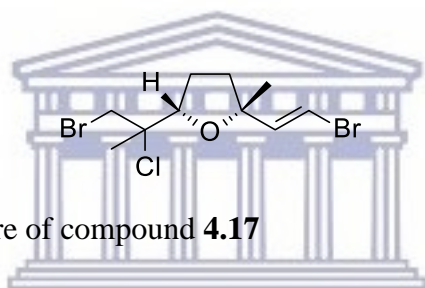


Figure 4.13: Proposed structure of compound **4.17**

Table 4.3: NMR spectroscopic data for compound **4.17**

Carbon atom	δ_{C}	Type	δ_{H} , mult.	J (Hz)	COSY	HMBC
1	104.8	CH	6.31, d,	13.3		C3
2	142.4	CH	6.20, d,	13.3		C1, C3
3	84.8	C	-			
4	37.4	CH ₂	1.89, m		H5	
5	27.1	CH ₂	2.04, m		H4, H6	C3, C4, C6, C7, C8
6	80.9	C	4.24, t,	7.0	H5	C7, C8
7	72.6	C	-			
8	39.8	CH ₂	4.00, d,	9.7		C6, C7, C10
			3.60, d,	9.9		
9	26.7	CH ₃	1.36, s		H10	C1, C2, C3
10	25.6	CH ₃	1.58, s		H9	C6, C7, C8

4.2.2.3 Structure elucidation of compound 4.18

Compound **4.18** appeared to be an isomer of compound **4.17**. $^1\text{H-NMR}$ and HSQC spectra of compound **4.17** showed the presence of a trans double bond, (δ_{H} 6.31, ^1H , d, $J=13.6$ Hz and δ_{H} 6.20, d, $J = 13.6$ Hz), a deshielded methine at δ_{H} 4.25 and methylene protons at δ_{H} 1.28 and 2.02 and diastereotopic methylene protons at δ_{H} 3.96 (10.0 Hz) and 3.64 (10.0 Hz) and two methyl groups at δ_{H} 1.31 and 1.59 (**Figure 4.14**). $^{13}\text{C-NMR}$ and edited HSQC spectra again showed presence of 10 carbon atoms, with a single double bond (δ_{C} 105.5 and 142.4), a deshielded methine carbon (δ_{C} 72.3), three methylene carbons (δ_{C} 37.8, 40.2 and 27.3) and two methyl carbons (δ_{C} 26.1 and 25.9).

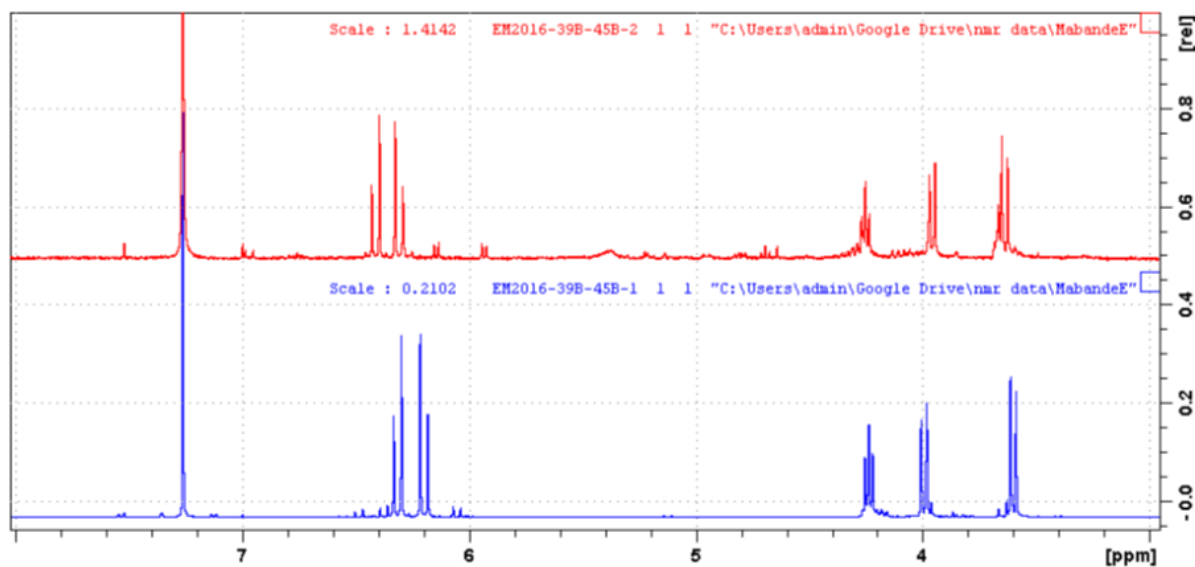


Figure 4.14: Comparison of $^1\text{H-NMR}$ spectra of compounds **4.17** (bottom) and **4.18** (top) (CDCl_3 , 400 MHz) showing the downfield shift of the olefinic proton signals between δ_{H} 6 and 6.6

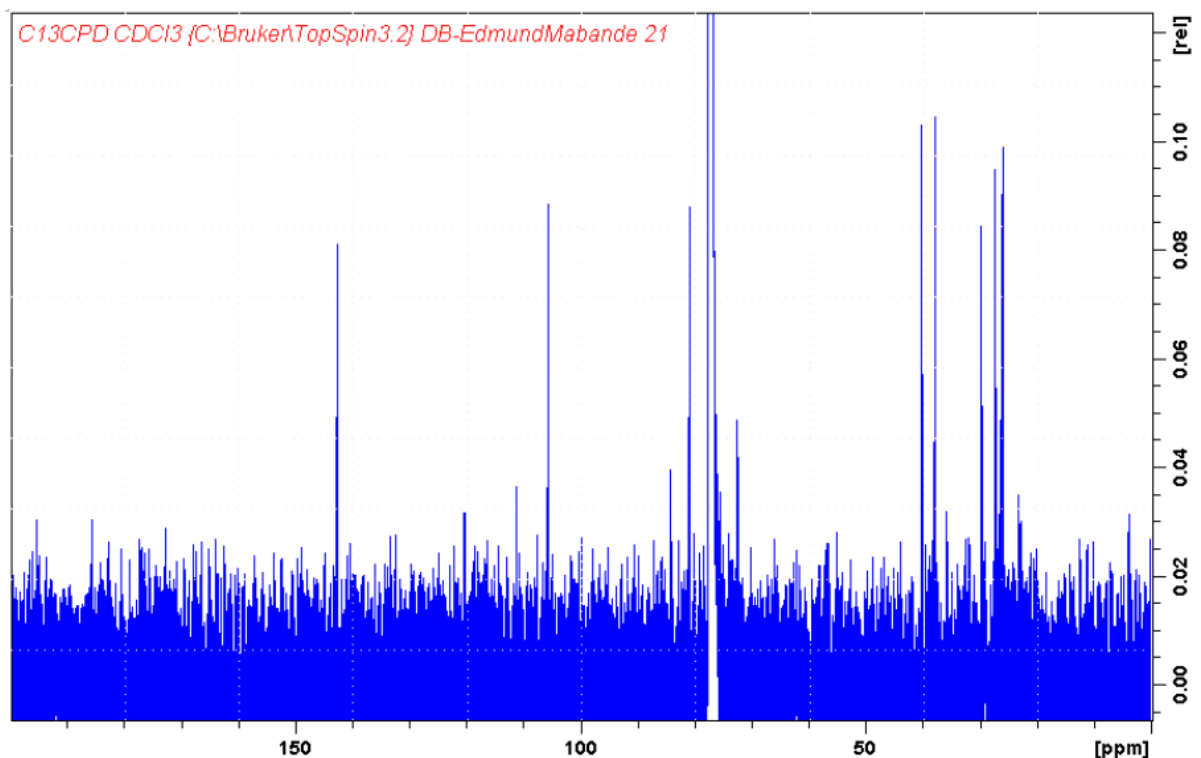


Figure 4.15: ^{13}C -NMR of compound **4.18** (CDCl_3 , 100 MHz)

Key HMBC correlations were also found to be similar to compound **4.16** and **4.17** and gave a regular monoterpene structure (**Figure 4.16**). The methyl signals at δ_{H} 1.39 (Me-9) is correlated to the olefinic carbons at δ_{C} 142.4 (C2) and to the carbons at δ_{C} 84.1 (C3) and 37.8 (C4). This again gives rise to the $-\text{CH}(\text{X})-\text{CX}(\text{CH}_3)-\text{CH}=\text{C}-$ moiety. HMBC correlations observed between the second methyl group δ_{H} 1.59 and carbons at δ_{C} 80.8 (C6), 72.3 (C7) and 40.2 (C8) gives the other part of the molecule the $-\text{CH}_2-\text{C}(\text{X})\text{CH}_3-\text{CH}(\text{X})-$ (**Table 4.4**). Halogen atoms of the structure were also assigned due to the ^{13}C chemical shift at the carbon atom directly attached to the halogen. The substituents at C1 (δ_{C} 105.5) and C8 (δ_{C} 40.2) were assigned as bromines and the substituents at C7 (δ_{C} 72.3) as chlorine. Due to similarities in the monoterpene skeleton and almost identical chemical shift values between corresponding protons and carbons of compound **4.17** and compound **4.18** it is proposed that these compounds are isomers at C-3.

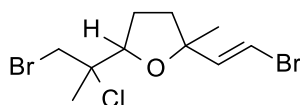


Figure 4.16: Proposed structure of compound **4.18**

Table 4.4: NMR spectroscopic data for compound **4.18**

Carbon atom	δ_C	Type	δ_H , mult. J (Hz)	COSY	HMBC
1	105.5	CH	6.41, d, 13.6	H2	
2	142.4	CH	6.31, d, 13.6	H1	C1
3	84.1	C	-		
4	37.8	CH ₂	1.86, m	H5	
5	27.3	CH ₂	2.05, m	H4, H6	C2, C3, C6, C7
6	80.8	CH	4.25, t, 6.9	H5	
7	72.3	C	-		
8	40.2	CH ₂	3.96, d, 10.0 3.64, d, 10.0		C6, C7, C10
9	26.1	CH ₃	1.31, s		C2, C3, C4
10	25.9	CH ₃	1.59, s		C6, C7, C8

4.2.3 Biological activities of the isolated monoterpenes

The isolated compounds **4.16**, **4.17** and **4.18**, were generally active at a concentration of 50 $\mu\text{g/mL}$ against Gram positive bacteria (*E. faecalis* and methicillin resistant *S. aureus*) and the fungus *C. albicans*. Antimicrobial activity was recorded as percentage inhibitions relative to the negative control. DMSO was opted for as a positive control, as these resistant bacteria respond poorly to standard antibiotics controls. DMSO at a concentration of approximately 15% has been shown to have potent antimicrobial activity, and thus a good positive control for antimicrobial assays (Ansel, Norred and Roth, 1969). Methicillin-resistant *Staphylococcus aureus* was the most susceptible microbes, with compound **4.16** having a percentage inhibition of 99% at a concentration of 50 $\mu\text{g/mL}$. Compounds **4.17** and **4.18**, were similarly very active with percentage inhibitions of greater than 100% at 50 $\mu\text{g/mL}$. Against *E. faecalis*, the compounds had percentage inhibitions of 40, 33 and 35% respectively at a concentration of 50 $\mu\text{g/mL}$ respectively for the aforementioned microbes (**Figure 4.17**). No activity or inhibition was observed at all for any of the compounds against Gram negative bacteria (*A. baumannii* and *E. coli*) (**Figure 4.18**, **Figure 4.19**). Of the susceptible microbes, *Candida albicans* was the least susceptible with percentage inhibitions of 23, 7 and 37% respectively at 50 $\mu\text{g/mL}$.

The isolated compounds were tested for their cytotoxicity activity against two different cell lines namely, MCF-7, a breast cancer line and HEK-293, a kidney cell line. The WST-1 assay was carried out. Activity was measured as percentage cell viability. Compounds showed moderate toxicity to the human cell lines. Estimated IC₅₀ values of compounds **4.16**, **4.17** and **4.18** were 22, 18, and greater than 100 µg/mL, respectively for the HEK-293 cell lines (**Figure 4.20**, **Figure 4.21**). Estimated IC₅₀ values for MCF-7 were 75, 30, and above 100 µg/mL respectively for the three compounds.

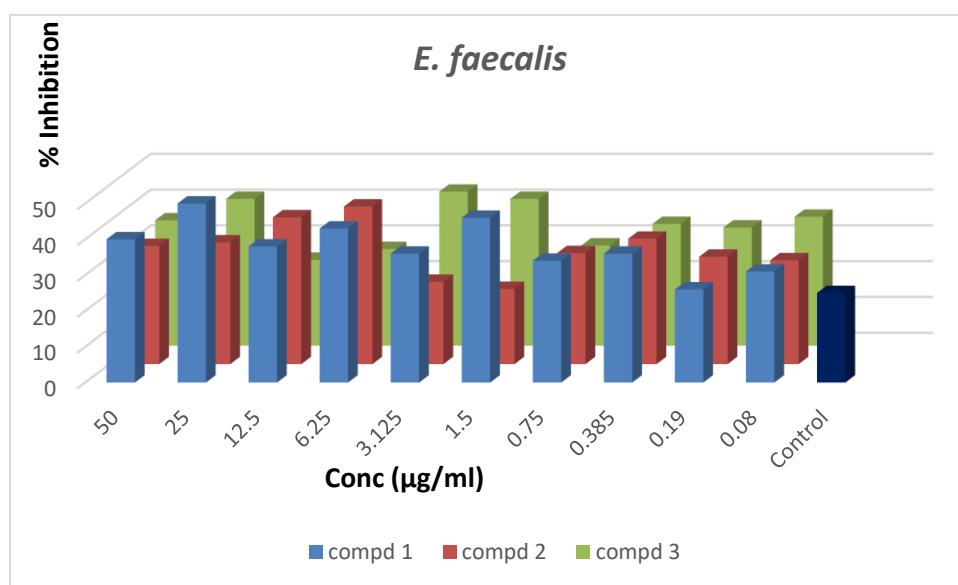


Figure 4.17. Percentage inhibition of compounds **4.16** (1), **4.17** (2) and **4.18** (3) against *Enterococcus faecalis*. (Note: DMSO was opted for as the control)

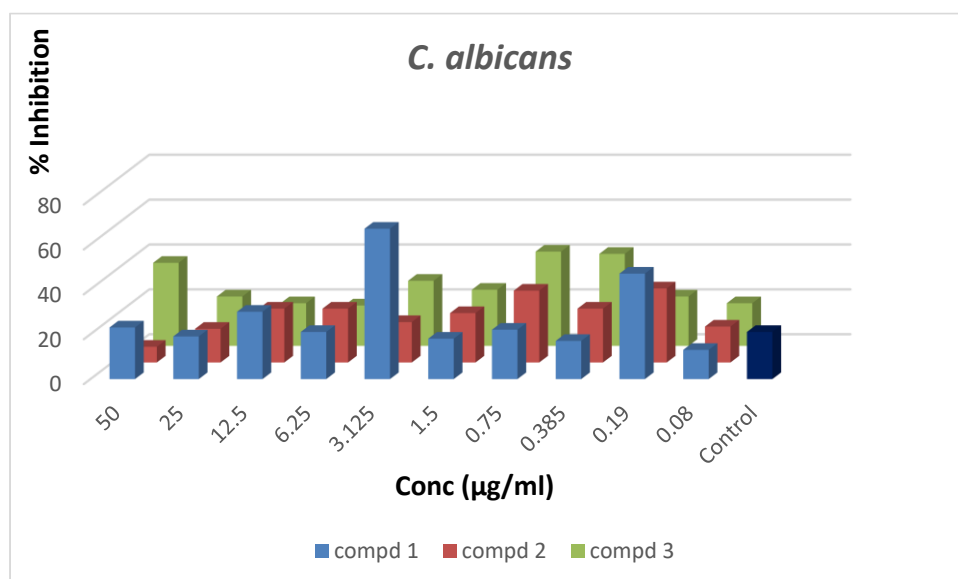


Figure 4.18. Percentage inhibition of compound **4.16** (1), **4.17** (2) and **4.18** (3) against *Candida albicans*

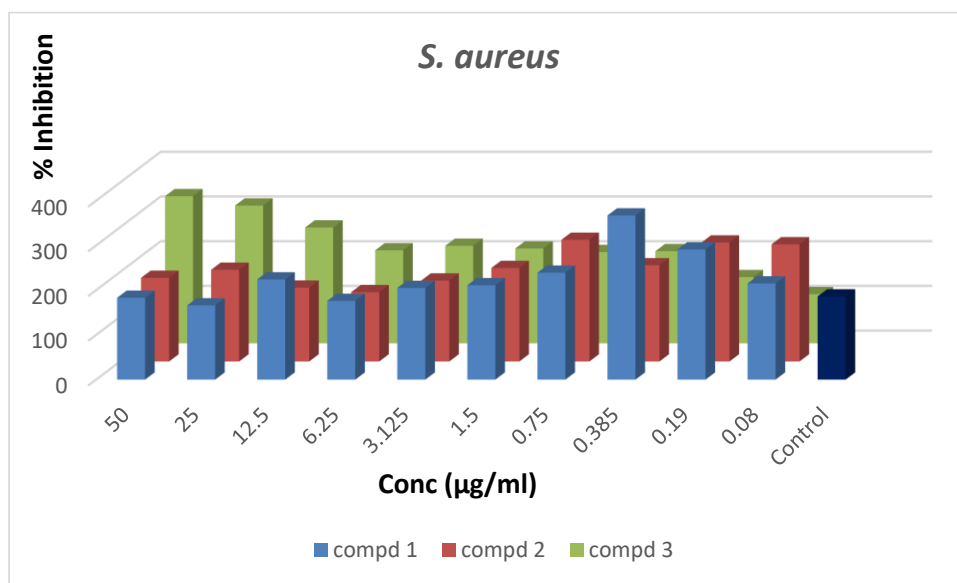


Figure 4.19. Percentage inhibition of compound **4.16** (1), **4.17** (2) and **4.18** (3) against *Staphylococcus aureus*

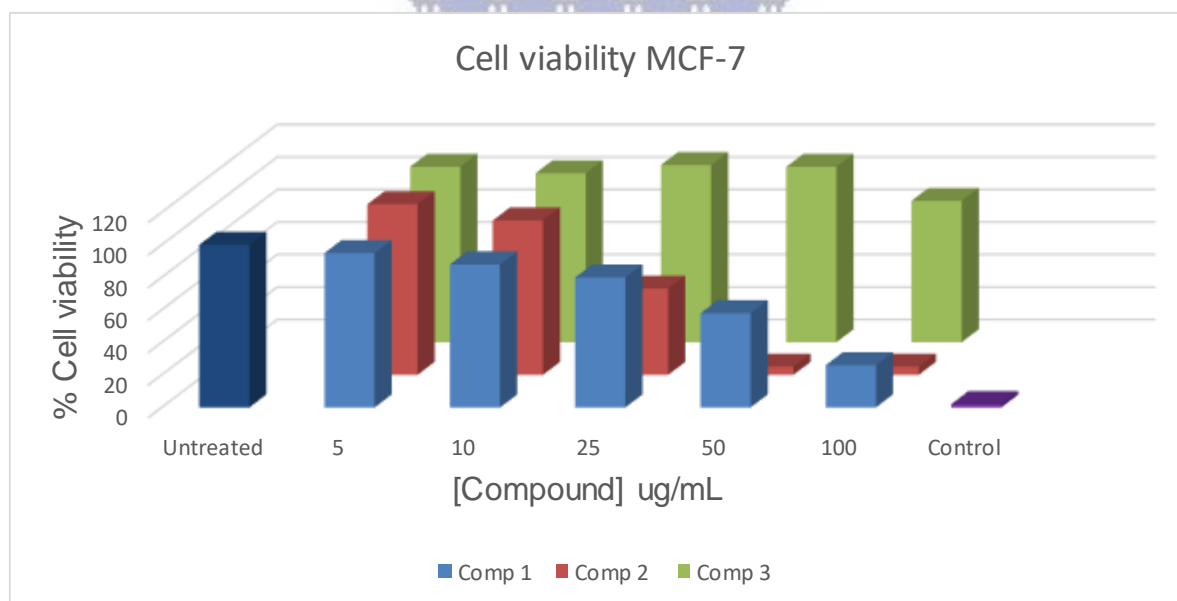


Figure 4.20. Cytotoxic activity of compound **4.16** (1), **4.17** (2) and **4.18** (3) against MCF-7 cell line. (Positive control = ceramide)

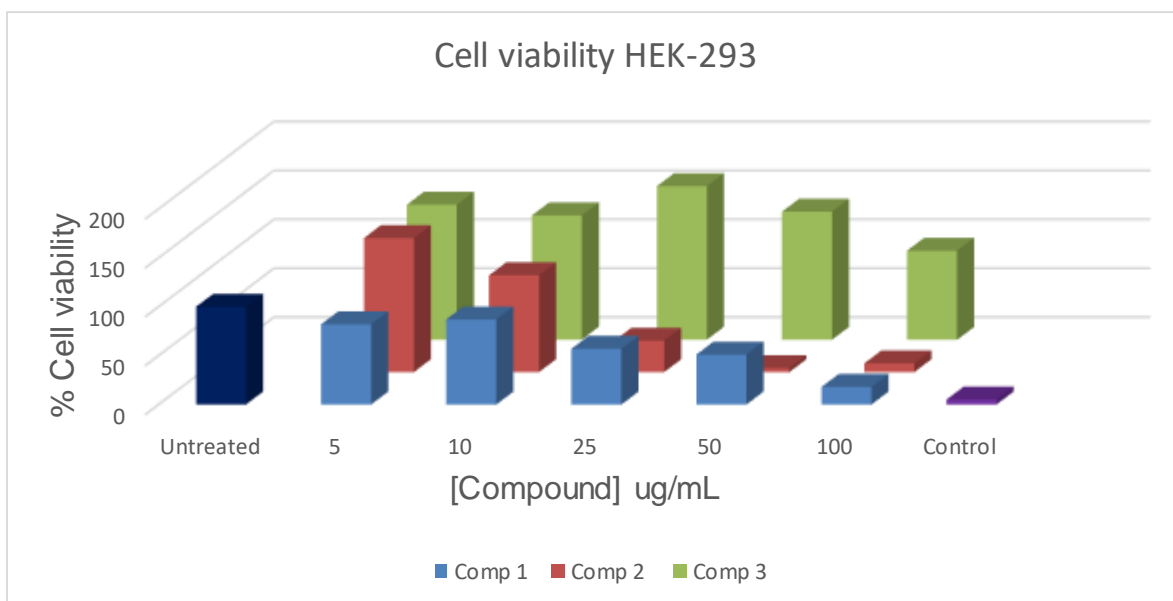


Figure 4.21. Cytotoxic activity of compound **4.16** (1), **4.17** (2) and **4.18** (3) against HEK-293 cell line. (Positive control = ceramide)

4.2.4 Conclusion

Three monoterpenes were isolated from the red alga, *Plocamium* sp. The structures of the compounds have been proposed as new halogenated tetrahydrofuran monoterpenes. The compounds all exhibited moderate antimicrobial activity against gram positive bacteria and a fungus. However, the antimicrobial activity was not as great as anticipated possibly due to the relatively low aqueous solubility of the compounds. The compounds had low cytotoxicity, and could be explored further for their therapeutic potential or their application in other areas e.g. preservatives.

4.3 Experimental

4.3.1 General experimental

All solvents used were either redistilled or HPLC grade (Sigma Aldrich). Optical rotations were measured on a Perkin Elmer Polarimeter (Precisely) at 589 nm and 20 °C. All NMR spectra was recorded on a Bruker Avance Spectrometer 400 and all spectra referenced to residual solvent signals (δ_{H} of 7.2600 ppm and δ_{C} of 77.00 ppm) with deuterated chloroform (CDCl_3) as a reference. Chemical shifts were measured in parts per million (ppm). The IR spectrum was

recorded on a Perkin Elmer Spectrum 400 spectrometer fitted with a diamond attenuated total reflectance attachment. Compounds were purified using Agilent HPLC normal phase which comprised of a UV100 detector at 250 nm and Whatman 10 μm semi-preparative column (50 cm). Merck Kieselgel 60 (0.040-0.063) was used for silica gel chromatography. Absorbance readings for cytotoxicity studies were measured on a POLAR star Omega plate reader (BMG Labtech). Absorbance readings for antimicrobial assays were measured using the Spectra Max M2 plate reader (Molecular Devices).

4.3.2 Plant material

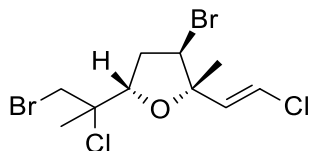
Plocamium sp. (GC120901-1) was collected from the rocky shore at Glencairn, near Cape Town on 1 September 2012 and stored frozen at $-20\text{ }^{\circ}\text{C}$ until extracted. The alga was identified by Prof J.J Bolton and a voucher specimen is stored at the School of Pharmacy, University of Western Cape. The wet seaweed was red-brown in colour. It had cartilaginous fronds of about 5 cm in length. Each frond led to several tiny branches. It had the appearance of a pink or reddish small bushy plant (**Figure 4.4**). The water thawed from the plastic package containing the seaweed had a deep red colour, signifying that the alga had an even brighter reddish colour.

4.3.3 Extraction and isolation

The frozen alga of approximately 50 g was extracted with MeOH (400 mL) overnight, and then extracted twice with MeOH- CH_2Cl_2 (1:2, 400 mL) overnight. The two extracts were combined and dried *in vacuo* to give 827 mg of organic extract. 100 mg of the organic extract was fractionated by silica gel column chromatography using a step gradient of increasing polarity as previously reported Fractions A and B, eluting with 100% hexane and hexane-EtOAc (9:1), respectively, showed typical $^1\text{H-NMR}$ signals of halogenated monoterpenes. Fraction A (70.8 mg) consisted of one main compound, and fraction B (240.7 mg) had at least two compounds. Other fractions did not seem to show any presence of monoterpenes. After normal phase HPLC (95:5, H:E) to fraction A and B, compounds **4.16** (13.5mg), **4.17** (6.2mg) and **4.18** (2.3mg) were isolated.

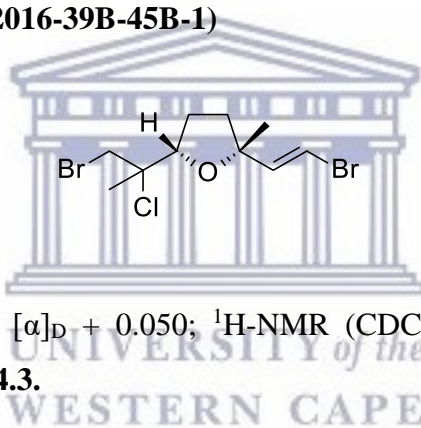
4.3.4 Isolated compounds

4.3.4.1 Compound 4.16 (EM2016-39A-B9)



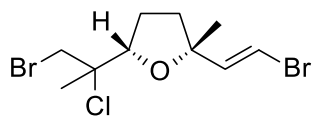
$C_9H_{12}Br_3ClO$: Colourless oil, $[\alpha]_D +0.118$; IR (ν_{max}): 1380, 1447, 2986; 1H -NMR ($CDCl_3$, 400 MHz) and ^{13}C -NMR ($CDCl_3$, 100 MHz) see **Table 4.2**.

4.3.4.2 Compound 4.17 (EM2016-39B-45B-1)



$C_9H_{13}Br_2ClO$: Colourless oil, $[\alpha]_D + 0.050$; 1H -NMR ($CDCl_3$, 400 MHz) and ^{13}C -NMR ($CDCl_3$, 100 MHz) see **Table 4.3**.

4.3.4.3 Compound 4.18 (EM2016-39B-45B-2)



$C_9H_{13}Br_2ClO$: Colourless oil, $[\alpha]_D - 0.006$; 1H -NMR ($CDCl_3$, 400MHz) and ^{13}C -NMR ($CDCl_3$, 100 MHz) see **Table 4.4**.

4.4. Biological activity studies

4.4.1 Preparation of samples for antimicrobial assays

Antimicrobial tests of isolated compounds were conducted, by the author, at Cape Peninsula University of Technology (CPUT) using the broth microdilution assay. Test strains were prepared ahead of the experiment. The following microbes and their optimal growth conditions were cultured and used in the antimicrobial assay:

Acinetobacter baumannii ATCCBAA-1605 (tryptic soy broth, 37°C);

Enterococcus faecalis ATCC 51299 (brain heart infusion broth, 37°C);

Escherichia coli ATCC 25922 (tryptic soy broth, 37°C);

Staphylococcus aureus subs. *aureus* ATCC 33591 (nutrient broth, 37°C);

Candida albicans ATCC 24433 (nutrient broth, 30°C)

A 500 µg/mL stock solution was made for each compound by dissolving the compound in DMSO. Serial dilutions were done to obtain 10 different concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5, 0.75, 0.385, 0.19 and 0.08 µg/mL for each compound. These were then dispensed to the 96-well plates in triplicate. Each well had a total of 180 µL of sample. Optical density was measured at OD₅₇₀ and were all adjusted by dilution of the cultures to give an OD₆₀₀ of about 0.5. Positive control was made up of 80 µL of culture plus 100 µL of DMSO. Negative control was comprised of 160 µL of culture and 20 µL of DMSO, and the sterile control was made up of 160 µL of broth and 20 µL of broth. All controls were tested in duplicate. When all samples and controls had been dispensed to the 96-well plates, breathe-ease membranes were used to cover each plate. Incubation was then done overnight at 37 °C. After the 24 hours had elapsed, 20 µL of 0.25% MTT was added to each plate, and the plates were then incubated again for three hours at 37 °C. After that 100 µL of DMSO was added to each plate and incubation done again for four hours. Finally, optical density of the plates was measured at OD₅₆₀. The readings were then used to calculate the percentage inhibition of each sample using the following formula.

$$\% \text{ Inhibition} = \frac{[1 - (\text{Absorbance of sample} - \text{blank})]}{[\text{Absorbance of negative Control} - \text{blank}]} \times 100\%$$

4.4.2 Preparation of samples for cytotoxicity assays

The cytotoxicity experiment was conducted at the Tissue Culturing lab at the Biotechnology department, University of the Western Cape. N-acetyl-D-sphingosine (ceramide) used as a control in the assay.

WST-1 cell proliferation reagent provides an easy and accurate way to measure cell viability, cytotoxicity and cell proliferation. This method is not radioactive, is extremely fast, much more sensitive in comparison with other assays. Other key advantages of the reagent are that it is safe and ready to use. WST-1 is a tetrazolium salt. Tetrazolium salts are cleaved to formazan by a certain enzyme system that belongs to the respiratory chain of the mitochondria. This is only active in live or metabolically active cells. The absorbance directly correlates to the number of cells. WST-1 facilitates the reduction of the tetrazolium into the colored formazan product (Riss *et al.*, 2016). The formazan dye produced by live cells can be quantitatively measured by measuring the absorbance at 440 nm. The WST-1 reagent has a starting colour of pink. A pink colour would indicate presence of living cells and on the other hand, a colour change from pink to a yellowish/orange colour would signify the presence of dead cells.

Human breast cancer MCF-7 and kidney cancer HEK-293 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) in 96-well plates. The media contains 4.5 g/L glucose with L-glutamine, and was stored at 2-8 °C before the experiment was conducted. Crude extract fractions were added to the media and cancer cells already in the well plate at a concentration of 50 µg/mL in the media. To do this 1.75 µL of an algae fraction was added to 348.25 µL of media to give a total of 350 µL. The experiment was done in triplicate per each pure compound. Dimethyl sulfoxide (DMSO) was diluted from a concentration of 100% to 50 µM and used as the negative control. N-acetyl-D-sphingosine (ceramide) was also diluted from a concentration of 14.6 mM to a concentration of 50 µM and used as the positive control. The ceramide and DMSO were also added to their designated wells in the 96-well plates. This was also done in triplicate per each control. The 96-well plates were then placed in an incubator at 37 °C, 5% CO₂ and a humid environment for 24 hours. This was done to mimic conditions of the body and thus provide optimal conditions for cells growth.

After 24 hours in an incubator, after 24 hours the cells lines removed from the incubator. WST-1 reagent was added to the cells in the 96-well plates. The plates were then taken back into the incubator under the same conditions for a further three hours. The experiment was performed in a fume cupboard and WST-1 as a light-sensitive reagent and was always protected by aluminum foil during the experiment.

After the three hours had elapsed the 96-well plates were taken from the incubator and into a POLAR star Omega plate reader to be analyzed for absorbance. The absorbance for each compound was recorded and compared to the untreated cells and controls to give a cell viability value. Untreated cells, thus live cells had 100% cell viability and dead cells would give less viability. A large cell viability value would actually indicate an increase in the cell growth.



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Chapter 5

An antimicrobial meroditerpene from *Styopodium multipartitum*

5.1 Introduction

Styopodium multipartitum (PB20141122-1) (**Figure 5.1**) was another alga that demonstrated promising activity against methicillin resistant *Staphylococcus aureus*. This alga is a southern African endemic alga and has been recorded from Arniston to northern Kwazulu-Natal (Anderson, Stegenga and Bolton., 2016). It has not been previously studied for its phytochemistry.

5.1.1 Seaweeds of the genus *Styopodium*

Styopodium is a genus of brown alga belonging to the family Dictyotaceae, the order Dictyotales and class Phaeophyceae (Smithsonian Tropical Research Institute, 2017). The seaweed is found in shallow seas in tropical and sub-tropical areas. The countries where it can be found include Pakistan, India, Australia and South Africa. About 19 species are known including *S. australasicum*, *S. flabelliforme*, *S. schimperi* and *S. multipartitum* (Guiry and Guiry, 2017). *S. multipartitum*, the species explored in this study, was previously known (incorrectly) as *S. zonale* and has reportedly been found on the shores of the South African and Mozambican coasts (Anderson, Stegenga and Bolton, 2016).

Styopodium multipartitum has a yellow-brown colour, and a bluish appearance can be seen in younger seaweeds particularly under the water. The thallus is erect and can grow up to 30 cm in length at times are stretched outwards, facing downwards. The seaweed has fan-like blades that are attached to a rhizoidal holdfast (Anderson, Stegenga and Bolton, 2016). Reproductive organs of the seaweed are located on the underside of the thallus (Alia and Mustafa, 2014). The seaweed is found in large quantities in shallow waters often attached to rocky substrates. *Styopodium* species from South Africa have not been well studied.



Figure 5.1 Photograph of *Stypopodium multipartitum* used in this study

5.1.2 *Styopodium* metabolites

Styopodium algae are known to produce a wide range of organic compounds e.g. phlorotannins. The algae are also known to be an excellent source of meroterpenoids (e.g. **5.1** – **5.7**) (**Figure 5.2**). Meroditerpenoids are natural products that are derived via mixed biogenesis, typically, the mevalonic acid and the shikimate pathways. Some of the meroterpenoids isolated from *Styopodium* species have been described as unique or unusual (Areche *et al.*, 2009; Areche *et al.*, 2015 and Valls *et al.*, 1993).

Metabolites from *Styopodium* are also associated with a range of biological activities including, antiproliferative activity (Pereira *et al.*, 2011), anti-leishmanias activity (Soares *et al.*, 2016) and also antimicrobial activity (Nagle and Wedge, 2002). *Styopodium* algae are also known to produce compounds that are toxic to fish (Searles and Schneider, 1986). The biological activities of *Styopodium* sp. metabolites seem to highlight the ecological ability of this seaweed in chemical defence.

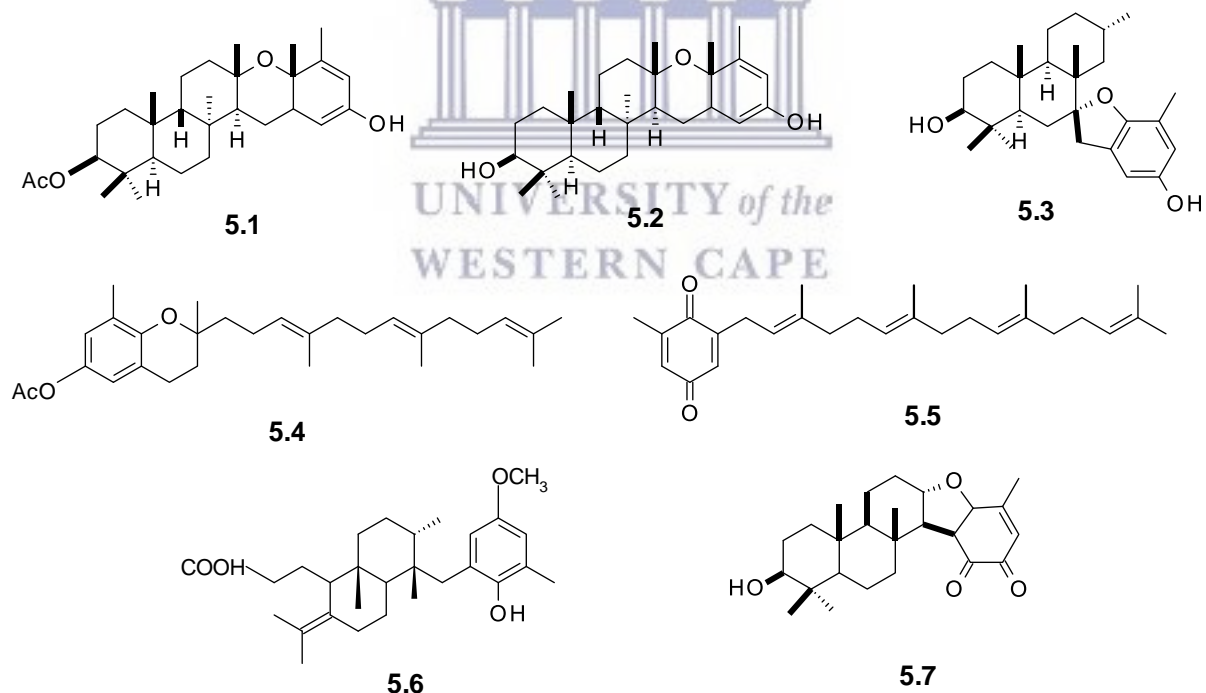


Figure 5.2. Some chemical structures of compounds isolated from *Styopodium* algae.

5.2 Results and discussion

Styopodium multipartitum (PB20141122-1) crude extracts exhibited very good antimicrobial activity and also had $^1\text{H-NMR}$ spectra which showed interesting chemistry. We decided to select this alga as well for further studies. Large scale extraction, and subsequent silica gel column chromatography led to the isolation of a new and unusual meroditerpenoid (compound **5.8**) with moderate antimicrobial activity.

5.2.1 Extraction and isolation of compound 5.8 from *Styopodium multipartitum*

A sample of *S. multipartitum* was collected at Palm Beach along the KwaZulu Natal coast in November 2014 and frozen at -20°C before extraction. The seaweed was given the collection code PB20141122-1. The $^1\text{H-NMR}$ spectrum of the MeOH and MeOH- CH_2Cl_2 (1:2) extracts (**Figure 5.3**) of the alga showed the presence of meroditerpenoids. Further silica gradient silica gel chromatography yielded fractions A-I (**Scheme 5.1**).

Fraction E showed the presence of a meroditerpenoid compound of about 80% purity and initial NMR data were obtained for this partially pure compound. The $^1\text{H-NMR}$ spectrum of compound **5.8** resembled that of sargahydroquinic acid, a compound previously isolated by our group. However, closer scrutiny of the 2D NMR data suggested that the compound was not sargahydroquinic acid and was therefore further purified by preparative TLC.

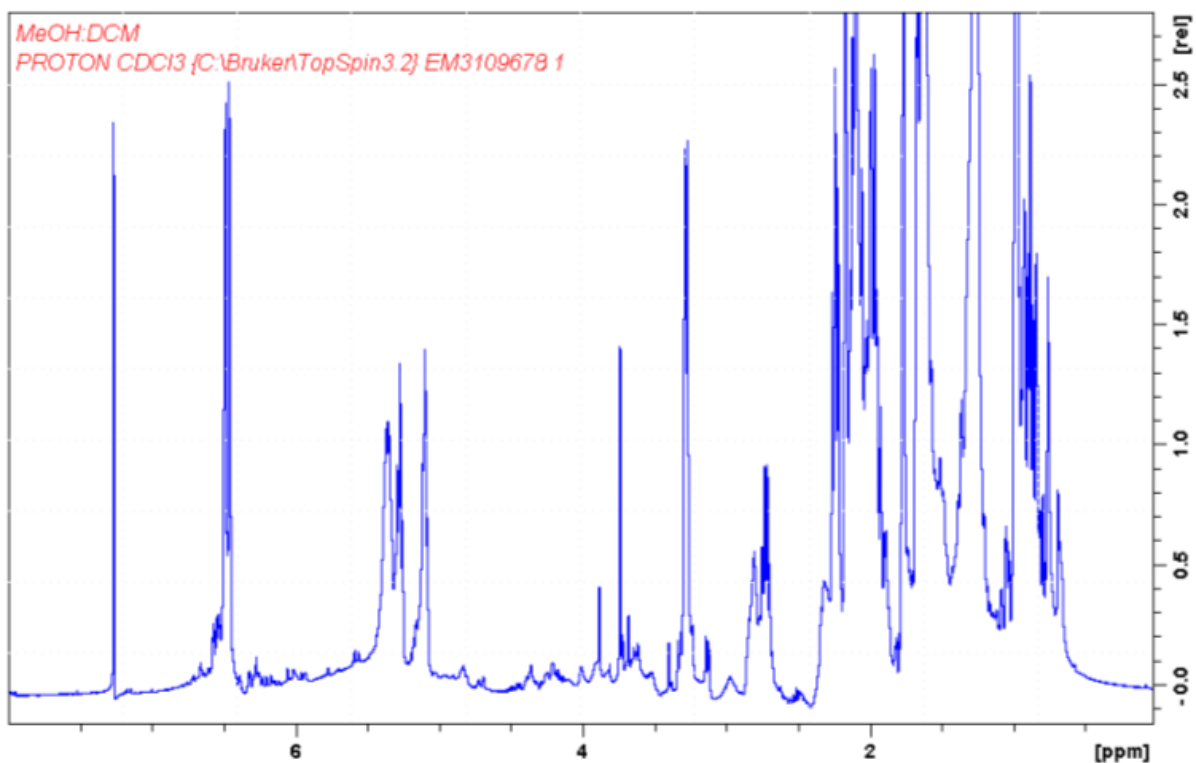
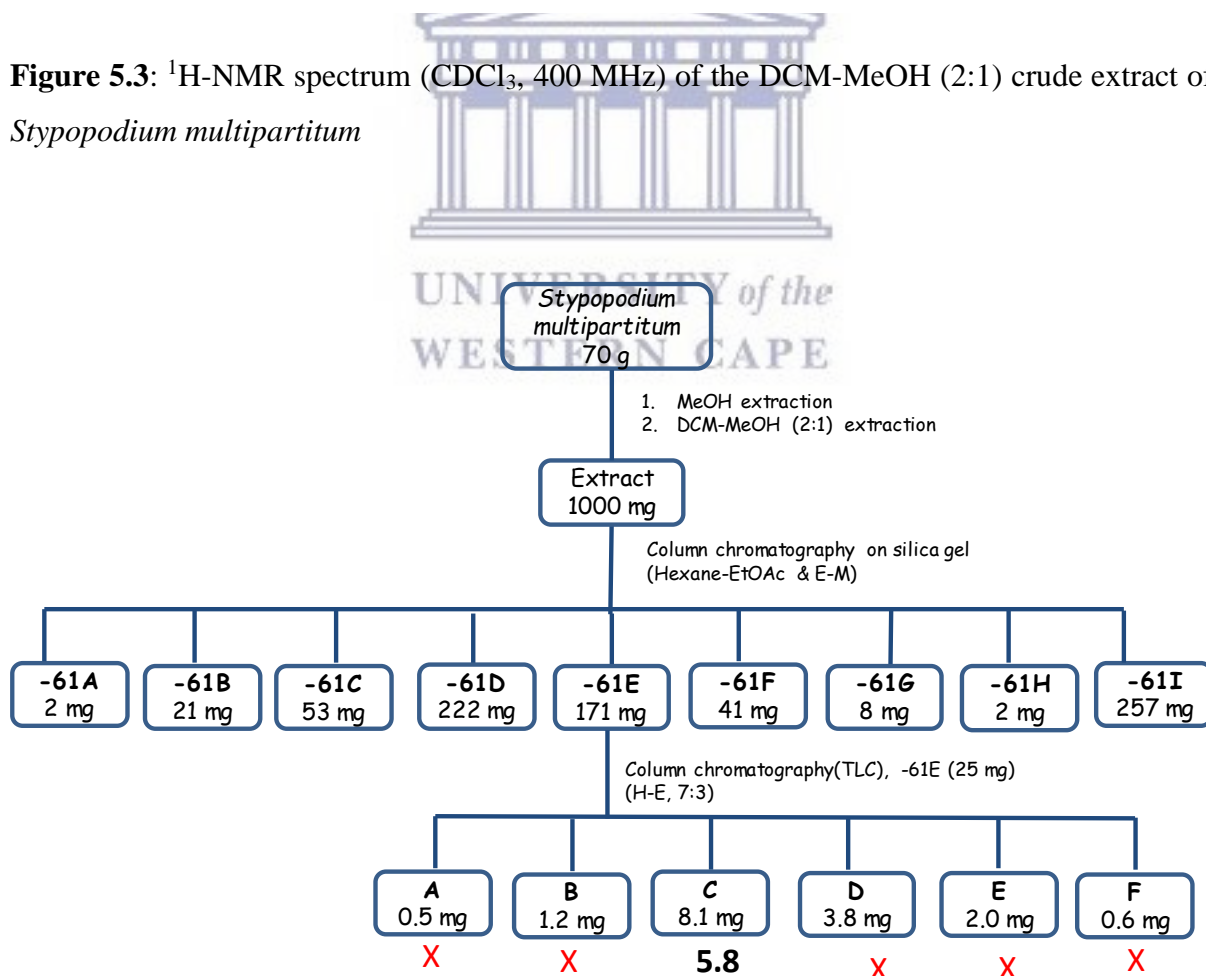


Figure 5.3: $^1\text{H-NMR}$ spectrum (CDCl_3 , 400 MHz) of the DCM-MeOH (2:1) crude extract of *Stypopodium multipartitum*



Scheme 5.1 Isolation scheme of *Stypopodium multipartitum* to yield compound **5.8**

5.2.2 Structural elucidation of compound 5.8

The ^1H NMR spectrum of **5.8** showed the presence of two aromatic singlets at δ_{H} 6.55 ppm and 6.49 ppm (**Figure 5.5**). The ^{13}C NMR spectrum exhibited typical signals indicative of the presence of a carboxylic acid group (δ_{C} 179.4) (**Figure 5.6**) and a total of 27 carbon resonances, which gave an indication that the compound is of a similar series of sargaquinoic acid (**5.10**) and its derivatives, or at least somewhat related. The chemical shifts at δ_{C} 149.1 ppm and δ_{C} 146.3 ppm showed the presence of two hydroxyl groups on the aromatic ring. A total of six methyl groups could also be seen from the spectra. The position of the carboxylic acid was determined by HMBC correlations as follows. The correlations between Me-15' (δ_{H} 0.98, d, 1H, $J = 6.9$) to δ_{C} 35.3 (C-11'), δ_{C} 30.4 (C-12'), δ_{C} 32.4 (C-13'), δ_{C} 134.5 (C-10') and δ_{C} 179.4 (C-14') were particularly important. Further HMBC correlations were observed between Me-16' (δ_{H} 1.63, s), and δ_{C} 134.5 (C-10') and δ_{C} 126.4 (C-17'). Similarly, Me-18' (δ_{H} 1.66, s) was correlated to δ_{C} 134.5 (C-10') and δ_{C} 126.4 (C-17'). On the hydroquinone moiety, Me-7 (δ_{H} 2.15, s) had HMBC correlations with δ_{C} 146.3 (C-1), δ_{C} 115.6 (C-5) and to δ_{C} 125.6 (C-6) (**Figure 5.4**). Another methyl group, Me-20' (δ_{H} 1.74, s) was correlated to δ_{C} 121.8 (C-2') and δ_{C} 138.2 (C-3'). The other critical HMBC correlation was noted between H-1' (δ_{H} 3.27, 1H, d, $J = 7.1$) to δ_{C} 30.1 (C-1'), δ_{C} 127.9 (C-2) and δ_{C} 114.1 (C-3). Compound **5.8** is thus a new meroditerpene (**Figure 5.7**).

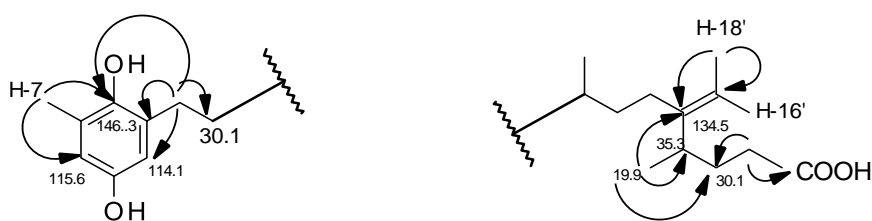


Figure 5.4 Key HMBC correlations for compound **5.8**

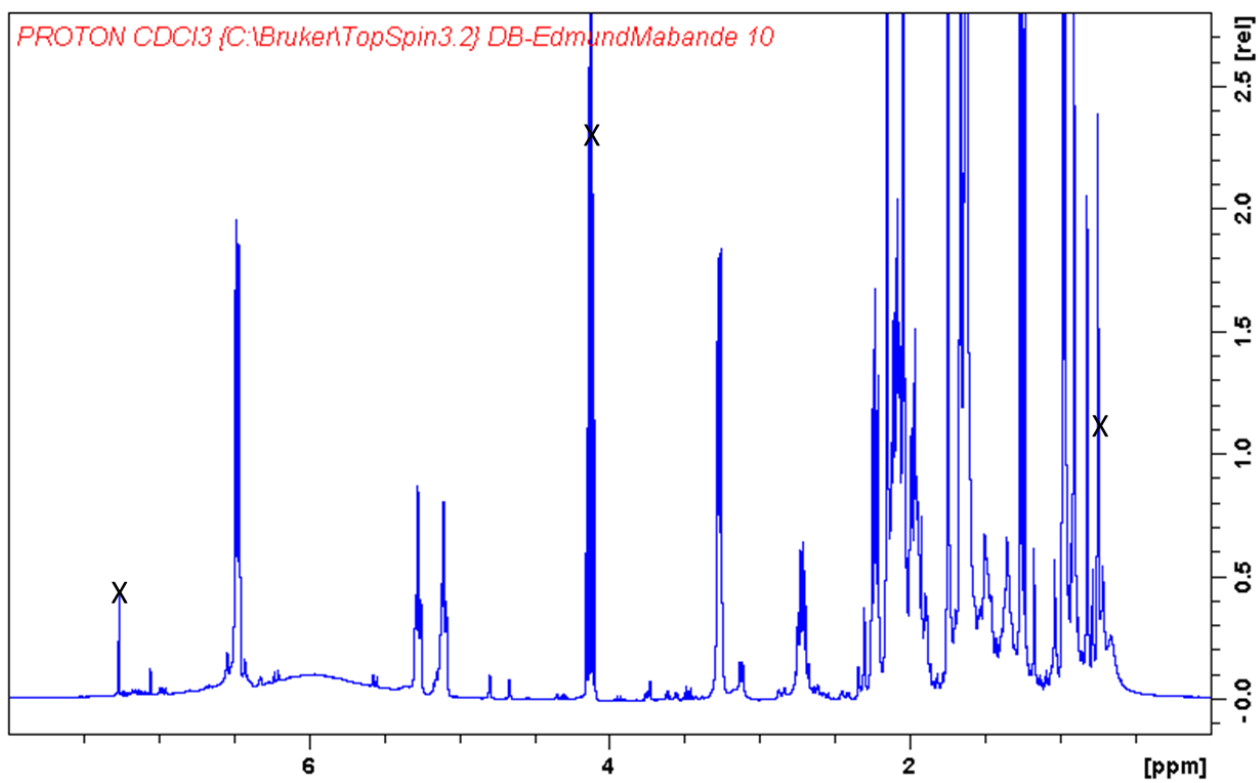


Figure 5.5 ^1H -NMR spectra of compound **5.8** (CDCl_3 , 400 MHz)

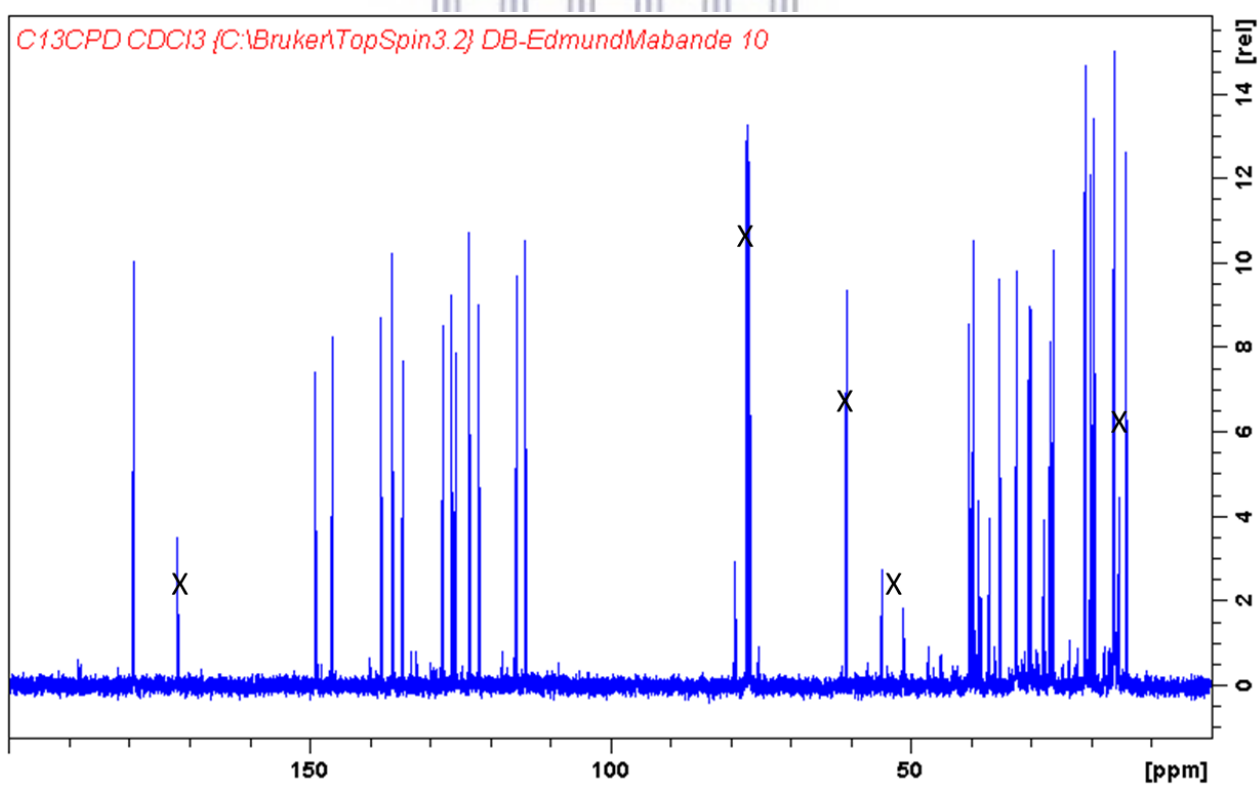


Figure 5.6 ^{13}C -NMR spectrum of compound **5.8** (CDCl_3 , 100 MHz)

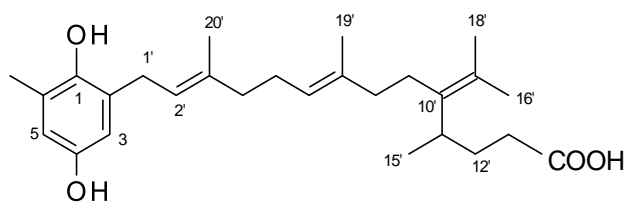


Figure 5.7 Proposed structure of compound **5.8**

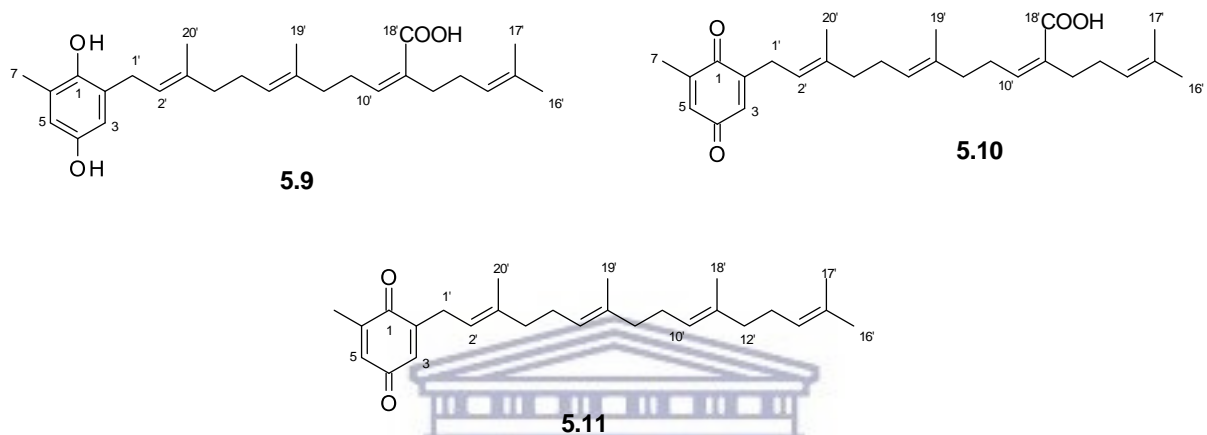


Figure 5.8 Chemical structures of related compounds

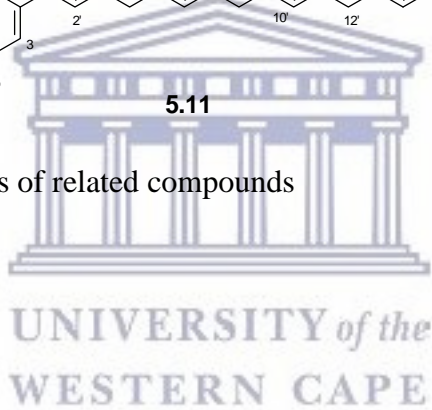


Table 5.1 NMR spectroscopic data for compound **5.8**

Carbon no.	C	C mult [']	H mult, <i>J</i> (Hz)	COSY	HMBC
1	146.3	C			
2	127.9	C			
3	114.1	CH	6.46, s		C4, C5, C1'
4	149.1	C			
5	115.6	CH	6.49, s		C3, C7
6	125.6	CH			
7	16.2	CH ₃	2.15, s		C1, C5, C6
1'	30.1	CH ₂	3.27, d, 7.1	H2'	C1, C2, C2', C3, C3'
2'	121.8	CH	5.30, t, 6.9	H1'	C1', C4', C7, C2, C20'
3'	138.2	C			
4'	39.6	CH ₂	2.10, m	H5'	
5'	26.8	CH ₂	1.97, m	H4'	
6'	123.4	CH	5.13, t, 6.9	H5'	C9', C8'
7'	136.2	C			
8'	40.3	CH ₂	1.97, m		
9'	26.2	CH ₂	2.10, m		C6'
10'	134.5	C			
11'	35.3	CH	2.72, m	H12', H15'	C10', C12', C13', C15', C17'
12'	30.4	CH ₂	1.64, m	H11'	
13'	32.4	CH ₂	2.23, t, 7.7		C11', 12', C14'
14'	179.4	COOH			
15'	19.5	CH ₃	0.98, d, 6.9	H11'	C10', C11', C13', c14'
16'	20.1	CH ₃	1.63, s		C10', C17'
17'	126.4	C			
18'	21.1	CH ₃	1.66, s		C10', C17'
19'	16.0	CH ₃	2.05, s		
20'	16.2	CH ₃	1.74, s		C2', C3'

Table 5.2. Comparison of ^{13}C -NMR data of isolated compound with that of sargahydroquinone acid, sargaquinone acid and sargaquinone

Carbon #	Compound 5.8	Sargahydroquinone acid (5.9) (<i>Brkljača and Urban, 2015</i>)	Sargaquinone acid (5.10) (<i>Ham et al., 2010</i>)	Sargaquinone (5.11) (<i>Amico et al., 1985</i>)
1	146.3	146.0	188.3	187.5
2	127.9	130.4	148.7	148.5
3	114.1	114.4	132.4	133.2
4	149.1	150.6	188.2	187.5
5	115.6	115.6	133.4	132.3
6	125.6	126.9	146.1	145.8
7	16.2	(not observed)	16.1	16.1
1'	30.1	29.3	27.6	27.6
2'	121.8	123.4	118.2	118.1
3'	138.2	137.1	140.0	134.9
4'	39.6	40.2	39.7	39.7
5'	26.8	(not observed)	26.4	26.5
6'	123.4	125.6	124.7	123.8
7'	136.2	135.4	135.0	139.8
8'	40.3	39.6	39.2	39.7
9'	26.2	28.4	28.4	26.6
10'	134.5	142.6	145.3	124.2
11'	35.3	132.5	130.7	134.4
12'	30.4	35.3	34.8	39.7
13'	32.4	28.3	28.1	26.8
14'	179.4	124.3	123.6	124.4
15'	19.5	133.1	132.4	131.1
16'	20.1	17.7	25.7	25.6
17'	126.4	25.7	17.9	17.6
18'	21.1	170.9	172.1	16.0
19'	16.0	16.2	16.1	16.0
20'	16.2	15.9	16.3	16.1

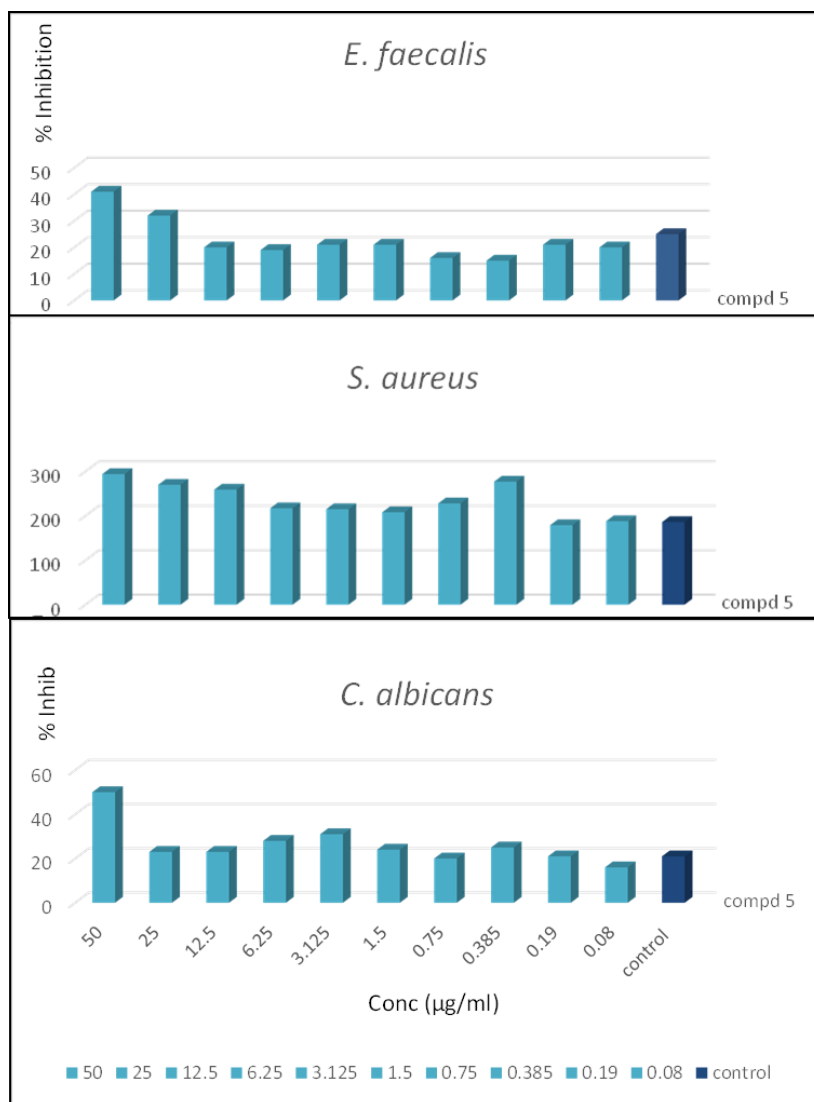
5.2.3 Biological activity of compound 5.8

The compound was assessed for its antimicrobial activity as well as its cytotoxicity. The broth microdilution assay was employed for the antimicrobial assay (as described before), and activity was recorded as percentage inhibition (**Figure 5.9**). It was found to be moderately active against *S. aureus*, *E. faecalis* and *C. albicans*. Once again, *S. aureus* was found to be the most susceptible microbe, with a percentage inhibition of greater than 100% noted at a concentration of 50 µg/mL. Percentage inhibitions of 41% and 50% were observed at 50 µg/mL for *E. faecalis* and *C. albicans* respectively (**Figure 5.9**). The degree of variability was not as pronounced as in the monoterpenes (Chapter 4). This could be due to the better solubility of the compound giving antimicrobial inhibition data which was somewhat more consistent. No inhibitory activity was observed at all for this compound against both Gram negative bacteria, *E. coli* and *A. baumannii*.

The WST-1 assay was employed for the cytotoxicity studies. The compound was quite toxic against both cell lines with estimated IC₅₀ of about 5 µg/mL for HEK-293 and about 17 µg/mL for MCF-7 (**Figure 5.10**).

The antimicrobial activity of the compound was a good discovery as *Styopodium* species are not particularly known for producing antimicrobial metabolites. Very few antimicrobial metabolites have been reported from *Styopodium* seaweed in literature. Epitandiol monoacetate, stypotriol acetate and stypodiol are a few of the compounds isolated from *Styopodium* sp. that have shown antimicrobial activity. However, these three compounds were weakly active, all having MICs greater than 100 µg/mL against different bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Proteus mirabilis* (Pereira *et al.*, 2011).

On the other hand, the high cytotoxicity of the compound was a bit of a drawback. However, it was not a surprise as the algae is known to produce some cytotoxic compounds. *Styopodium* algae has been shown to produce meroterpenoids with antiproliferative activity to several human cancer cell lines (Pereira *et al.*, 2011).



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Figure 5.9. Antimicrobial activity of compound **5.8** against *E. faecalis*, *S. aureus* and *C. albicans*

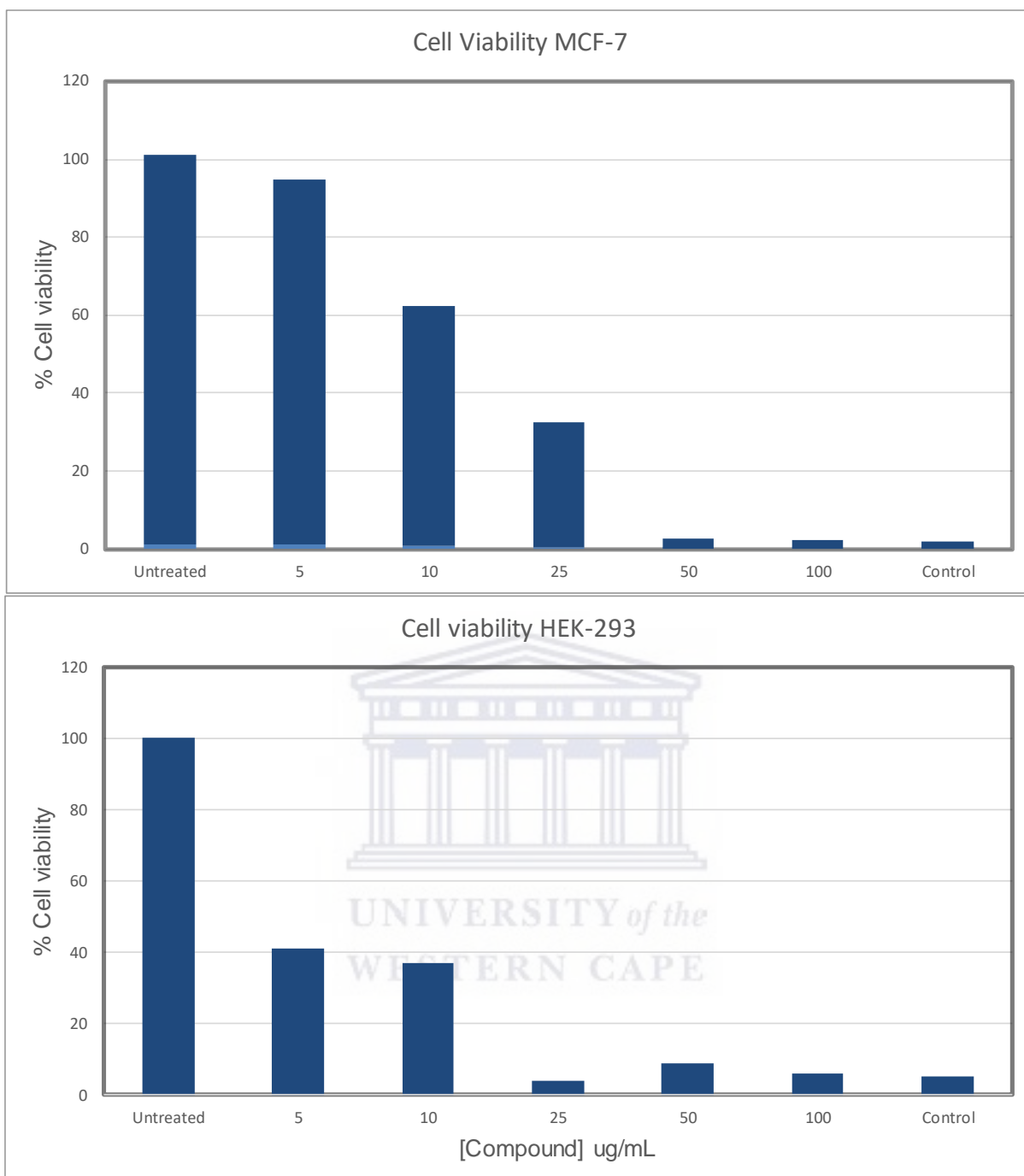


Figure 5.10. Cytotoxic activity of compound **5.8** against MCF-7 cell line (top), and HEK-293 (bottom)

5.2.4 Conclusion

A new and unusual meroditerpenoid was isolated from *Styopodium multipartitum*. The compound exhibited moderate antimicrobial activity, but was also toxic to human cell lines.

Because of this, it does not show much promise as a lead compound in our quest for new antimicrobial drugs or lead compounds. However, its cytotoxic activity should be explored as an antitumour lead compound.

5.3 Experimental

5.3.1 General experimental

All solvents and equipment used is as described in Chapter 4. High resolution mass spectroscopy (HR-ESI-MS) was performed at the CAF analytical lab at Stellenbosch University on a Waters Synapt G2 spectrometer and the following chromatographic conditions Column: Waters BEH C18, 2.1x100 mm, 1.7 μm , Solvent A1: 0.1% formic acid, solvent B1: 0.1% formic acid in acetonitrile.



5.3.2 Plant material

Styopodium multipartitum (PB20141122-1) was collected from Palm Beach, KwaZulu Natal in South Africa, and kept frozen at -20°C . The alga was identified by Prof John J Bolton and a voucher specimen is stored at the School of Pharmacy, University of the Western Cape. The alga had a dark brown colour (**Figure 5.1**). It had the appearance of a fleshy and leafy, dark brown-coloured small plant. The blades were relatively large, mostly measuring above 15 cm. When viewed against the light, the blades had a beautiful deep golden-brown colour. The surface of the blades looked bumpy or roughly, but the feel of the blades was velvety or smooth. The edges of the blades were somewhat irregular. Some the large blades divided into smaller blades or segments. The blades were attached to a small, similarly-coloured rhizoidal holdfast. Some dark brown to black-coloured sporangia were seen scattered over the blades.

5.3.3 Extraction and isolation

A frozen alga of about 70 g was extracted initially by 400 ml of MeOH overnight. This was followed by extraction by 400 ml of CH₂Cl₂:MeOH, 2:1 twice (**Scheme 5.1**). This extract was dried *in vacuo* to afford a combined total of 4.5 g of crude extract. 1000 mg of this extract was fractionated by silica gel chromatography of increasing polarity as previously reported starting from the most non-polar 100% hexane. A total of 9 fractions A-I were obtained and chemically profiles using ¹H-NMR. Further silica gel chromatography was performed on fraction E using solvents; hexane: ethyl acetate (7:3), leading to the isolated compound **5.8**.

4.3.4 Isolated compound

Compound **5.8** (EM2016-61E-93C)



C₂₇H₄₀O₄: Yellow oil, [α]_D 0; ¹H-NMR (CDCl₃, 400 MHz) and ¹³C-NMR (CDCl₃, 100 MHz) see **Table 5.1.**; HRESIMS: Calcd for C₂₇H₃₇O₄ [M-H]⁻: 425.2692, found 425.2677 (C₂₇H₃₇O₄).

5.4 Biological activity studies

5.4.1 Preparations of samples for antimicrobial and cytotoxicity assays

Antimicrobial and cytotoxicity tests were performed for Compound **5.8**. Preparation of samples, test microorganisms, assays, and conditions were exactly as described in Chapter 4 (4.4.1 and 4.4.2).

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Chapter 6

Conclusion

6.1 General summary

Interest in marine natural products is increasing and could be very valuable in the search for new antimicrobial agents. South Africa, being blessed with an abundance of marine biodiversity harbours a rich diversity of marine algae, sponges, invertebrates *etc.* that can be harnessed by drug discovery scientists in a more intensive way to discover metabolites with biological activity. The current research study showed that South African marine algae could be a valuable source of new antibiotic lead compounds.

Overall, the study was a success as compounds with some antimicrobial activity were isolated from marine algae. All set objectives were met. A library of pre-fractionated marine algal extracts was created and evaluated for antimicrobial activity against different pathogens, and selected algal extracts were evaluated for cytotoxicity. Several marine algal extracts exhibited antimicrobial activity against Gram positive bacteria, particularly methicillin-resistant *S. aureus*. It was exciting to note that some extracts showed activity greater than that of standard antibiotics. Three new halogenated monoterpenes and a new and unusual meroditerpene were isolated from a *Plocamium* sp. and *Styopodium multipartitum*, respectively. Isolated compounds showed moderate antimicrobial activity. The monoterpenes from *Plocamium* showed moderate cytotoxicity, but the meroterpenoid was more cytotoxic. The low antimicrobial activity and inconsistent values of *Plocamium* compounds was possibly due to the highly non-polar nature of the compounds. There is no doubt that continued exploration of marine resources like algae could lead to compounds with therapeutic potential. These compounds could also be used as scaffolds for the design of new antimicrobial compounds. In addition, the development of novel drug delivery technologies such as solid lipid nanoparticles could improve on the pharmaceutical potential of these compounds.

All extracts in the library were successfully chemically profiled using ¹H-NMR. Algal fractions could be clearly differentiated based on the uniqueness of their spectra. The spectra also allowed us to get a glimpse into what class of compounds were contained in a certain alga or fraction. Spectral data was stored in a readily retrievable format, should there be any extract(s) of interest. The method used for fractionation proved to be excellent as the recovery yielded was

generally very high, and chemical profiles of resultant fractions reasonably distinct. Creation of the marine natural products library will simplify the process of screening algae against new microbes. Samples were put in a ready to screen format and relevant chemical profiles and known biological activity data readily available. The library could go on to be tested for other biological activity such as antiviral, anti-mycobacterial, anti-cancer and so forth depending on assay availability and the need.

6.2 Limitations of study

The study had some limitations which were encountered. The solubility of isolated compounds and extracts was one of the the main issues of concern. Most of the extracts and the isolated compounds were highly lipophilic in nature. The solvent used for storage and bioassays was DMSO, which is a polar and aprotic solvent. Therefore the solubility of these non-polar molecules in this solvent would not be guaranteed to be 100%. Efforts were done to try to increase the solubility such as using a vortex-mixer or sonication before bioassays. DMSO was the only appropriate solvent available for carrying out the bioassays and for the short to long term storage of the compounds. High resolution mass spectroscopy (HRMS) of the *Plocamium* compounds was unfortunately not obtained in time. This would have confirmed the proposed structures of the unusual monoterpenes isolated from *Plocamium* sp. The amount of sample of pure compound isolated was very little which leads to problems with respect to structural characterisation and further biological assessment. This became a challenge as several tests had to be done resulting in low recovery of sample. As a result the amount of sample used in polarimetry tests was very little to draw a definitive conclusion about the molecules' optical rotations. However, the data obtained helped shed some light on the molecules.

6.3 Recommendations

In addition to acting as molecular scaffolds for the design of new antimicrobial drugs, the isolated compounds could also be explored for their potential in other applications such food preservatives *etc.* Extracts from other algae other than *Plocamium* sp and *Styopodium multipartitum* that exhibited good antimicrobial activity should be pursued further. Greater

priority should be placed on those extracts that had potent activity against Gram negative bacteria *A. baumannii* and *E. coli*, which are the bacteria of most concern according to the World Health Organisation. Some of the 153 fractions showed activity against *E.coli* and *A. baumannii*. Re-isolation of compounds from *Plocamium* species should be done at a larger scale. This will enable compounds to be yielded in greater quantities to allow all possible structural elucidation tests and HRMS to be carried out. We also anticipate that the process will lead to more unusual monoterpenes that could have exciting biological activity. We also recommend that the extracts and the purified compounds be tested for their anti-cancer properties, as most of the extracts had some cytotoxic activity against the HEK-293 cell line. Cytotoxicity profiles of only 50 selected extracts were obtained as resources and time were limited. It would be ideal to have each and every extract's cytotoxicity profile in our MBDR marine natural products library.





Supplementary data

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Chapter 3: Supplementary Data



Figure S3.1: Antimicrobial activity of fractions against *Acinetobacter baumannii*

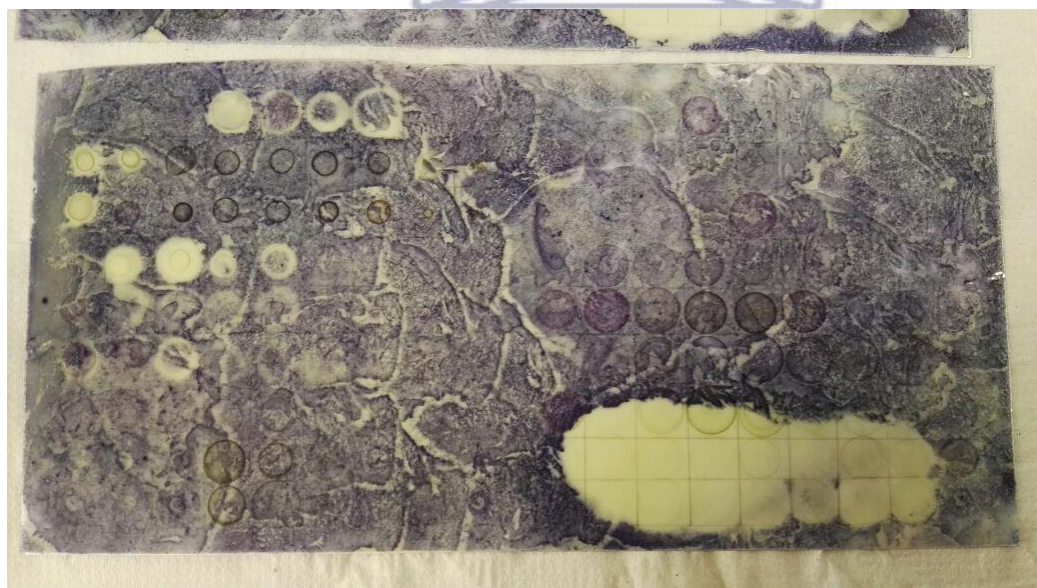


Figure S3.2: Antimicrobial activity of fractions against *Candida albicans*

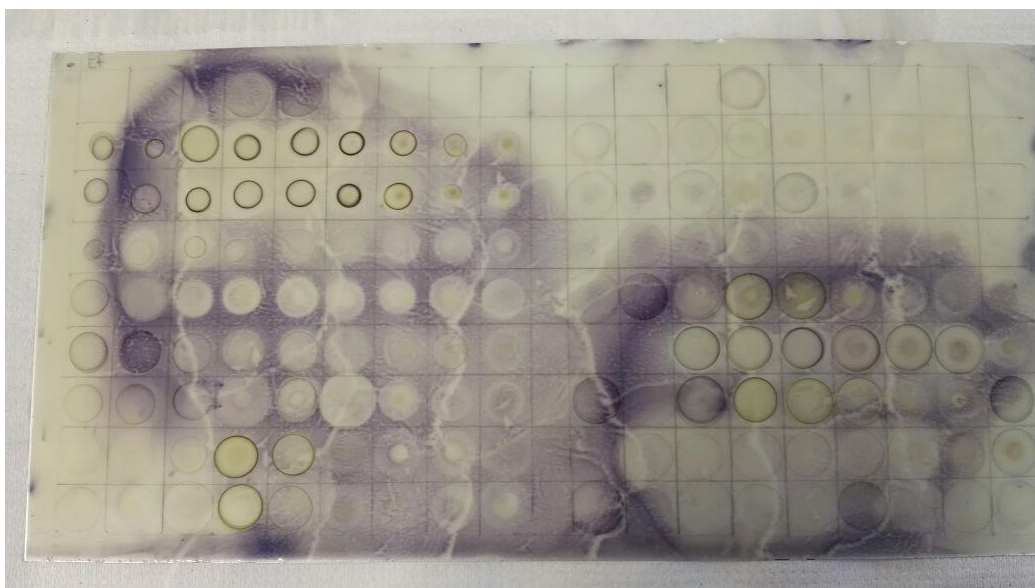


Figure S3.3: Antimicrobial activity of fractions against *Enterococcus faecalis*

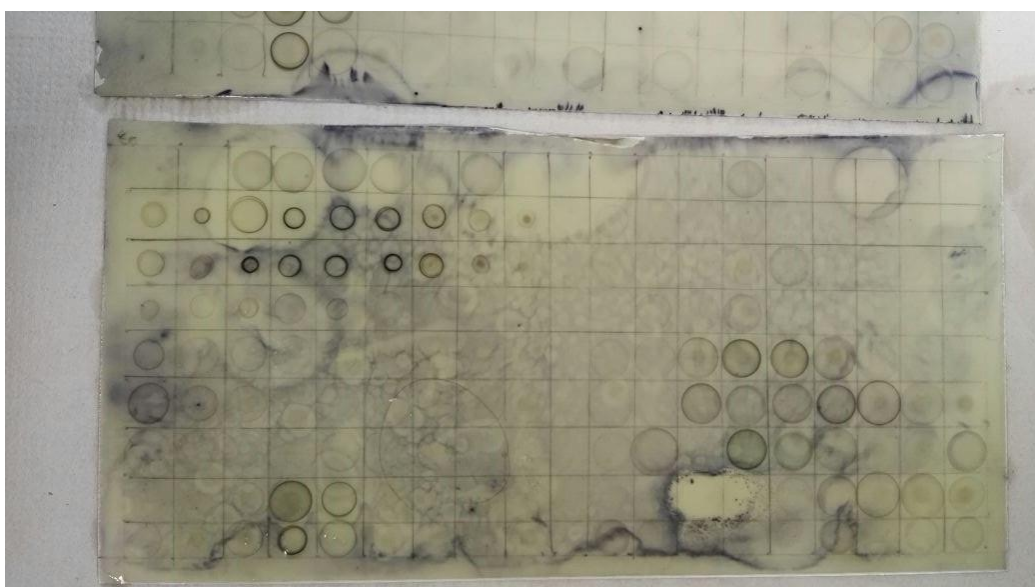


Figure S3.4: Antimicrobial activity of fractions against *Escherichia coli*

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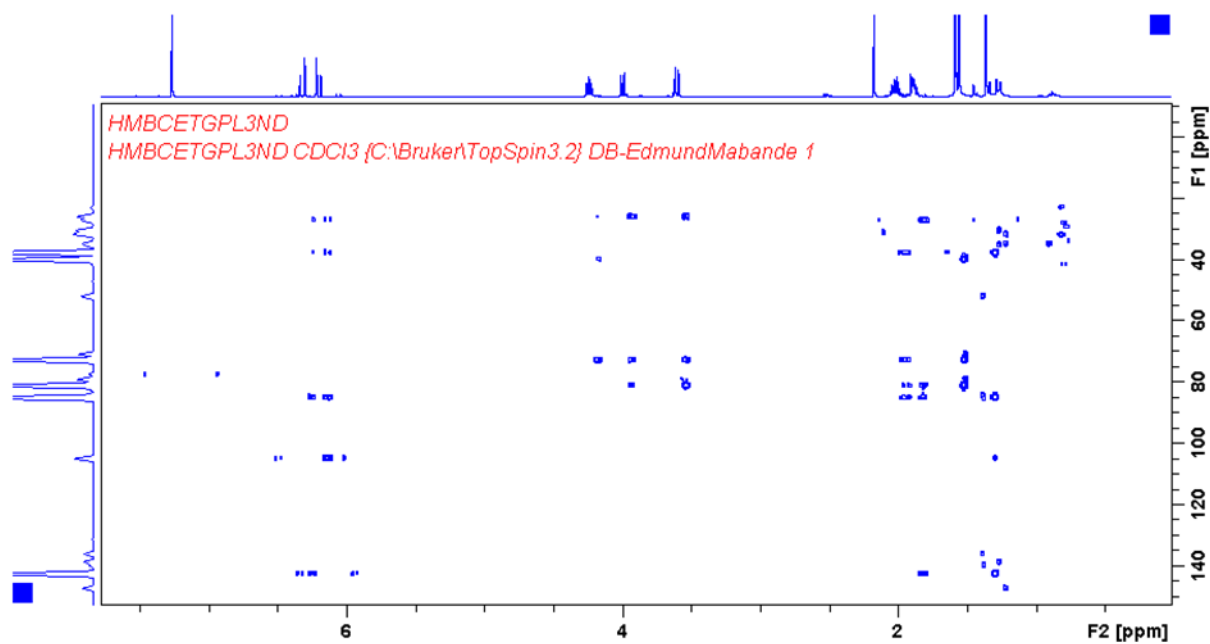


Figure S4.1: HMBC spectra of compound **4.16** (CDCl₃, 400MHz)

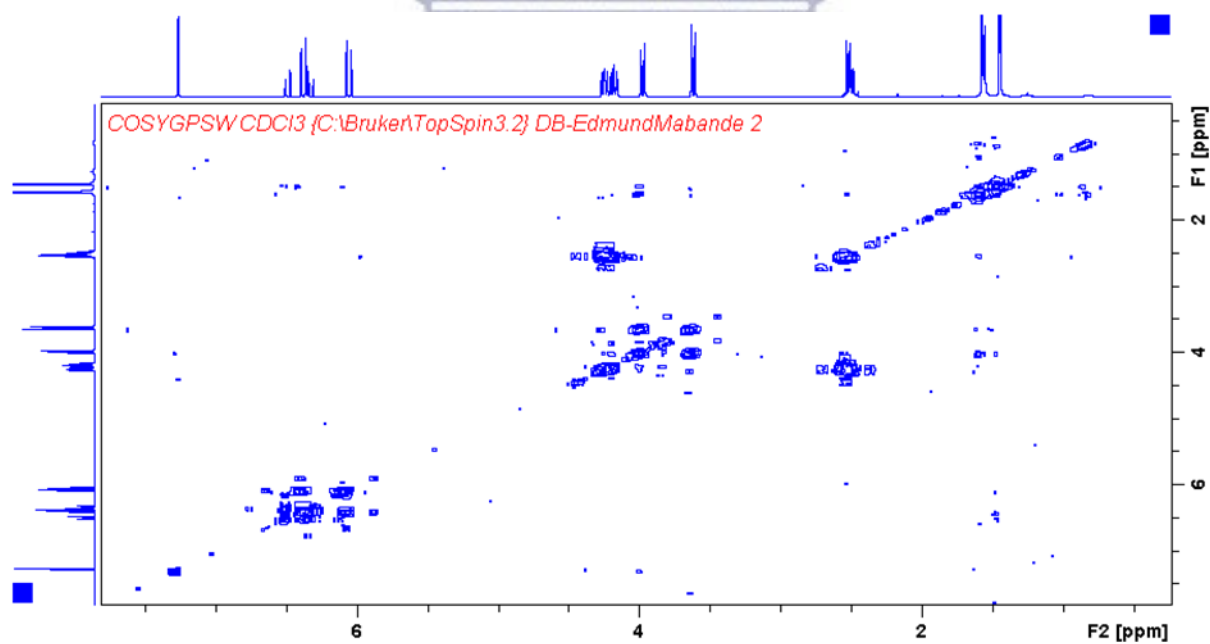


Figure S4.2: COSY spectra of compound **4.16** (CDCl₃, 400MHz)

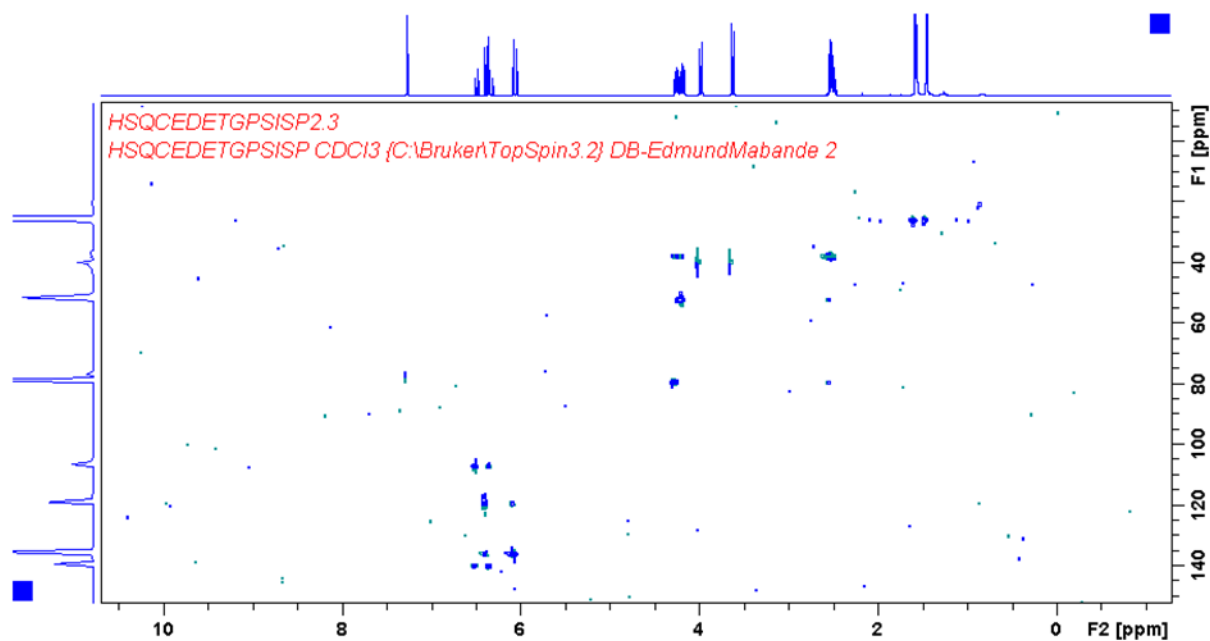


Figure S4.3: HSQC spectra of compound **4.16** (CDCl_3 , 400MHz)

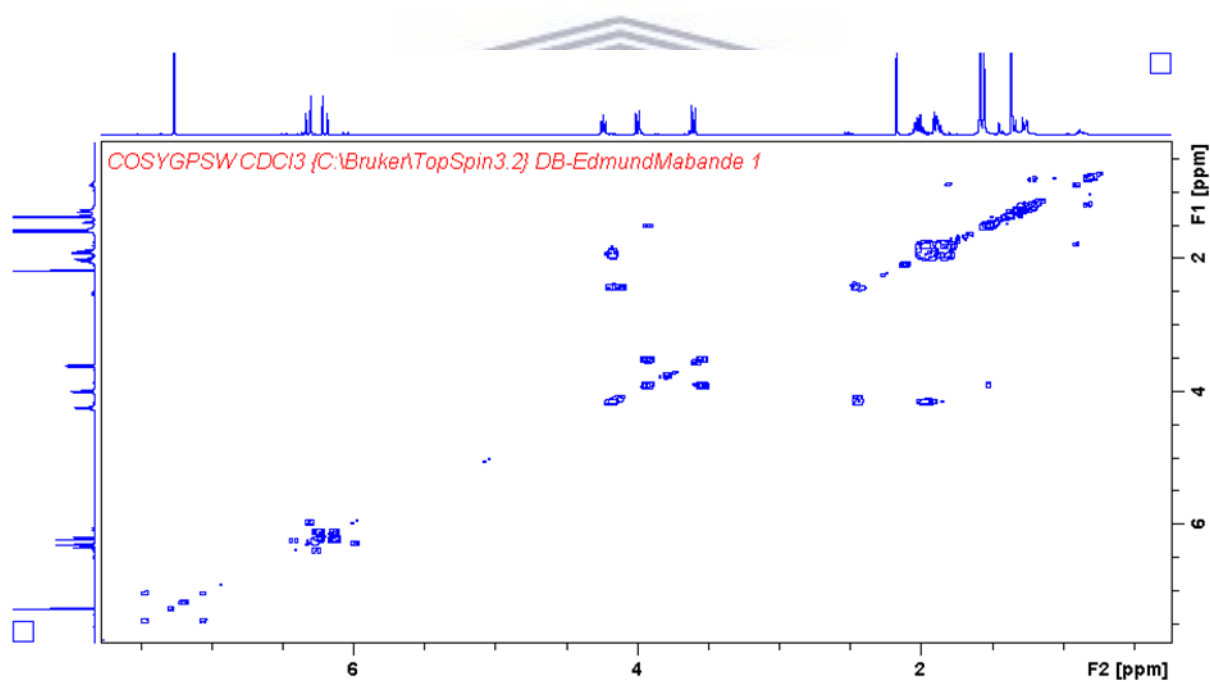


Figure S4.4: COSY spectra of compound **4.17** (CDCl_3 , 400MHz)

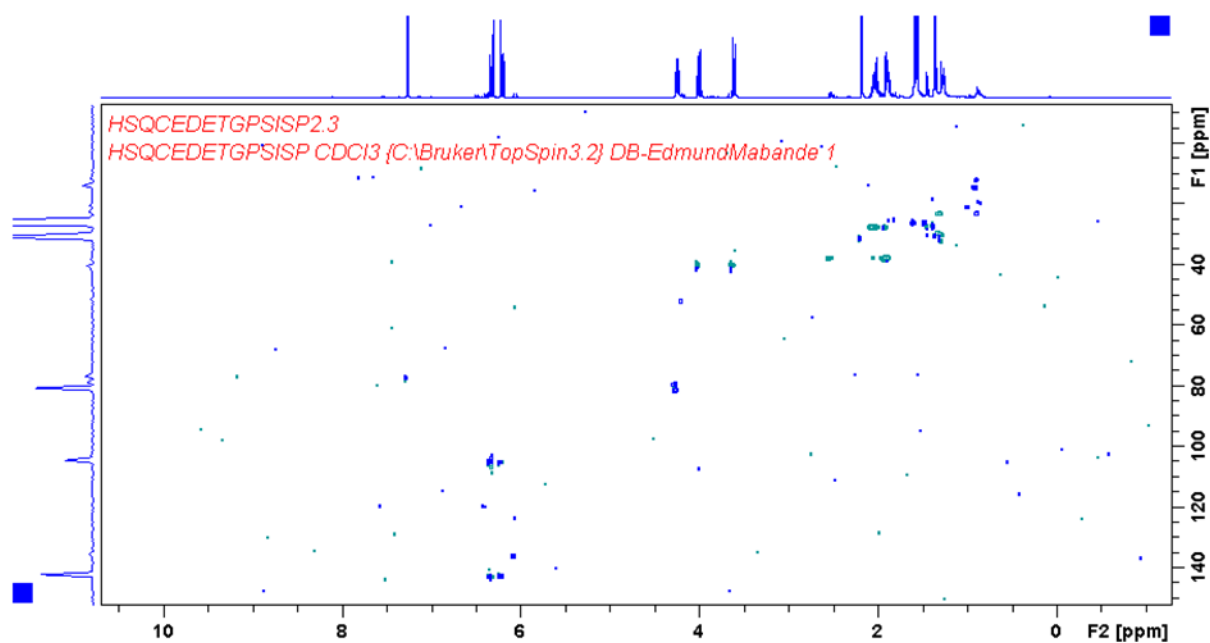


Figure S4.5: HSQC spectra of compound **4.17** (CDCl₃, 400MHz)

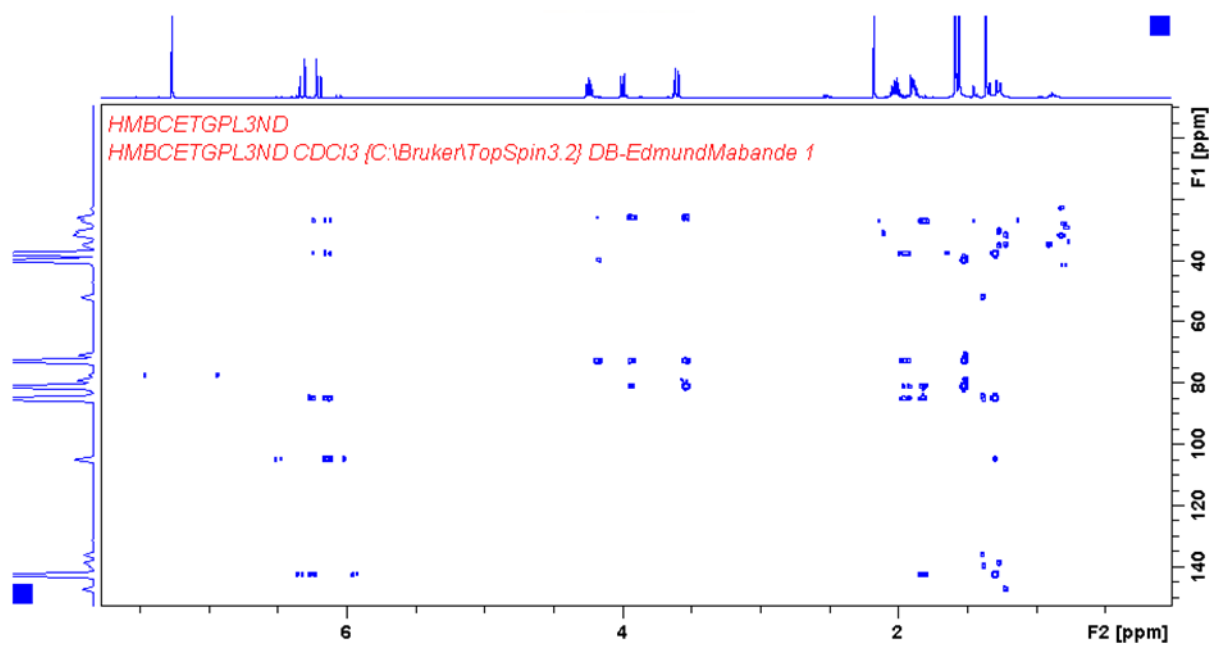


Figure S4.6: HMBC spectra of compound **4.17** (CDCl₃, 400MHz)

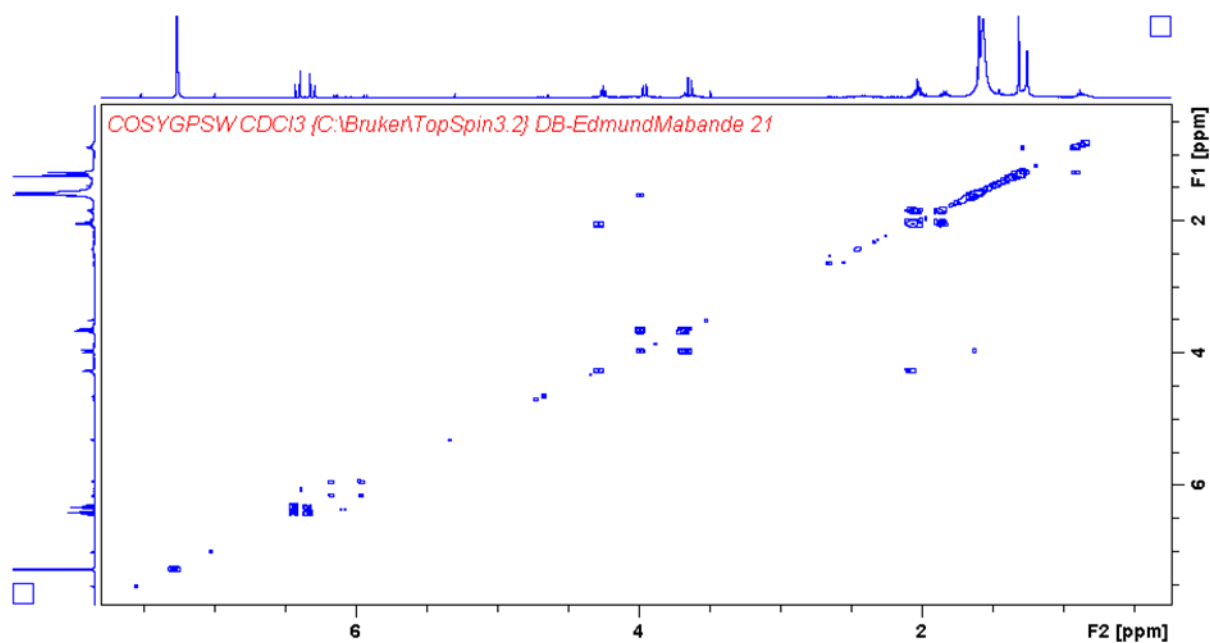


Figure S4.7: COSY spectra of compound **4.18** (CDCl_3 , 400MHz)

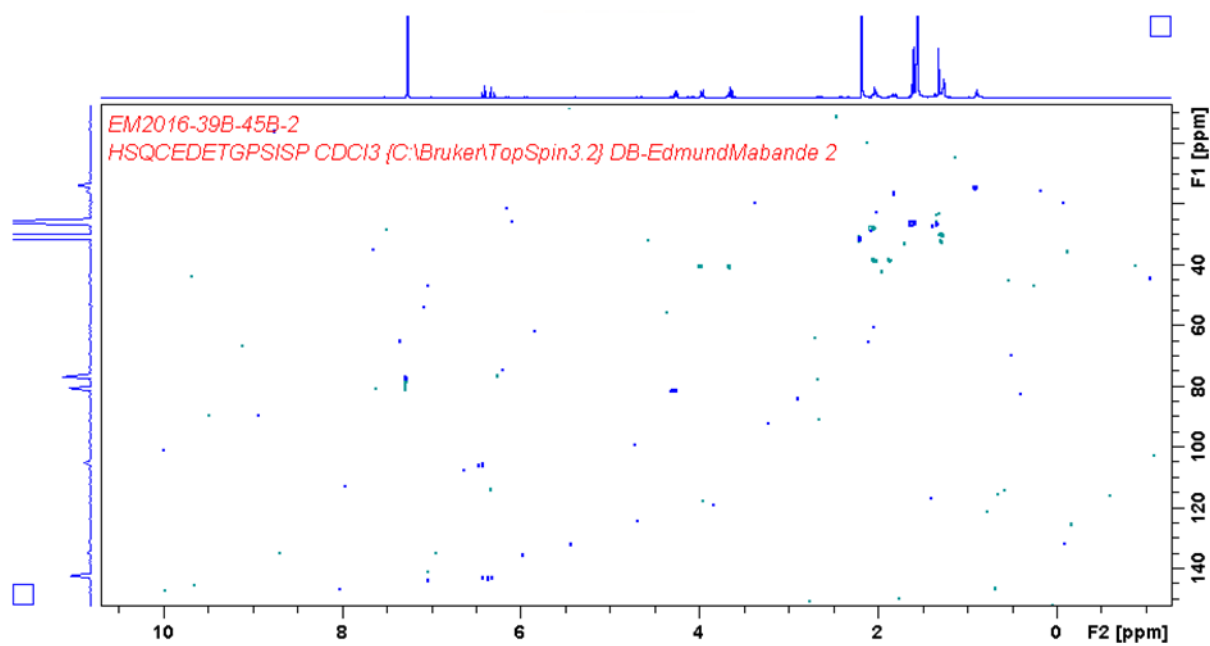


Figure S4.8: HSQC spectra of compound **4.18** (CDCl_3 , 400MHz)

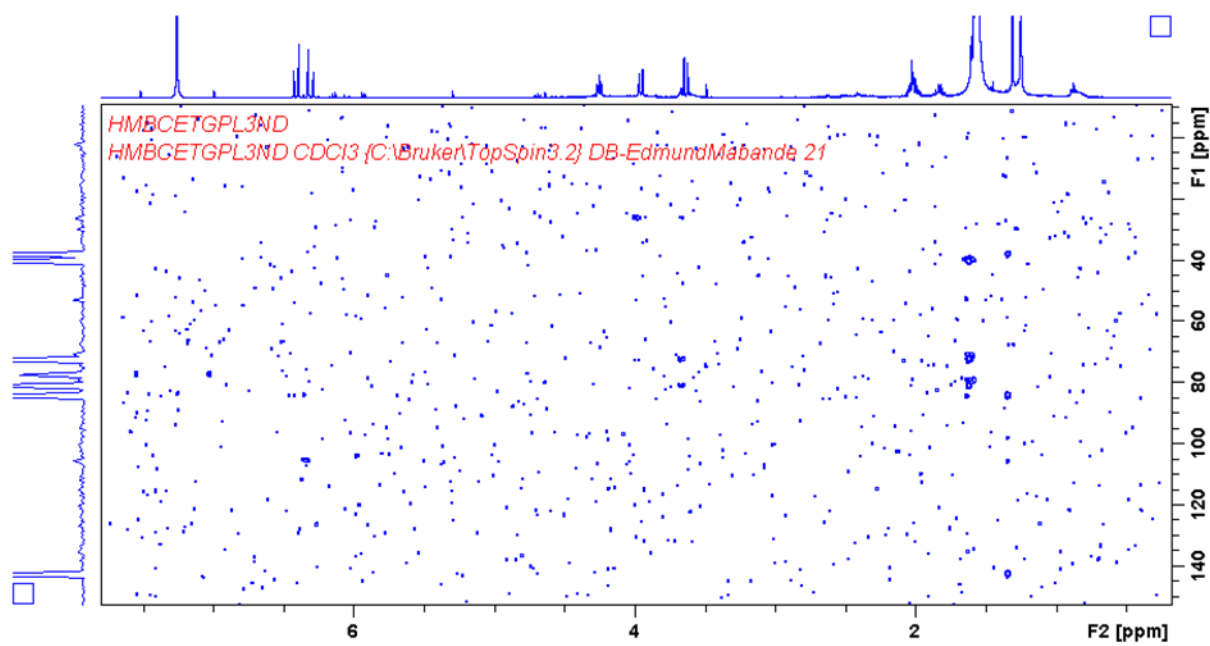


Figure S4.9: HMBC spectra of compound **4.18** (CDCl_3 , 400MHz)



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Chapter 5: Supplementary data:

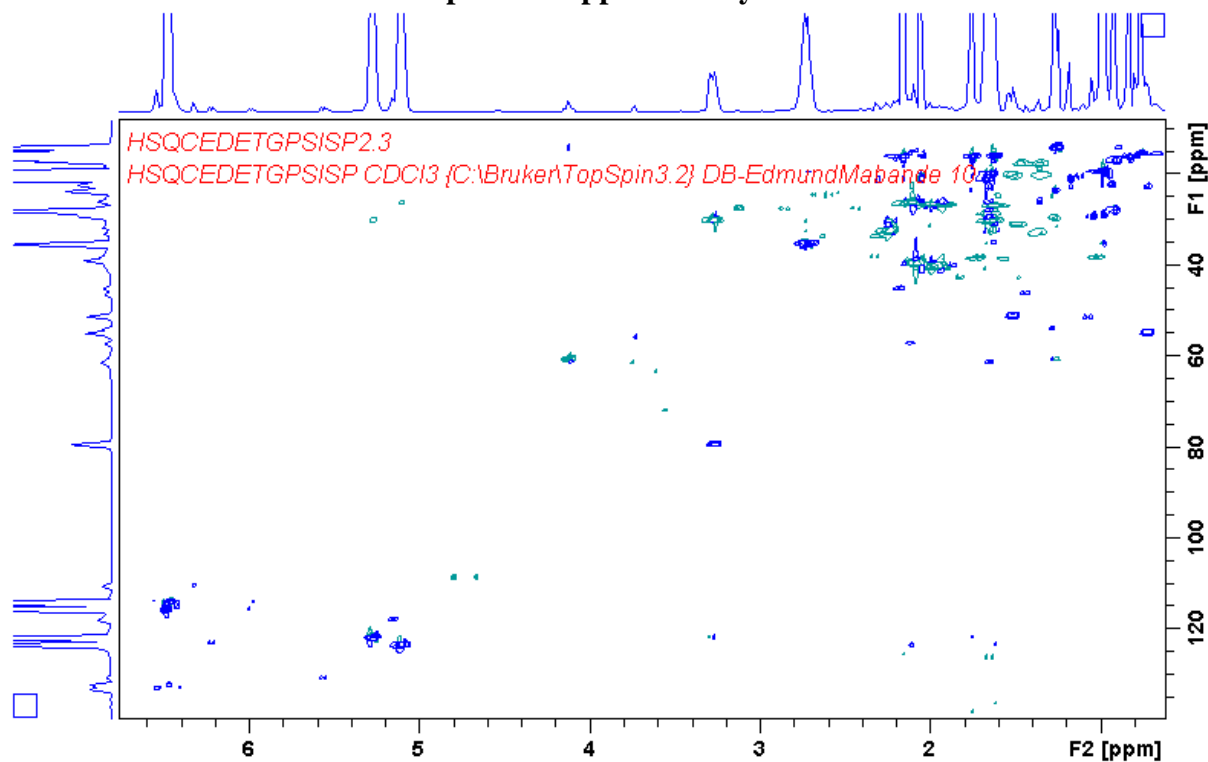


Figure S5.1 HSQC spectra of compound 5.8 (CDCl₃, 400 MHz)

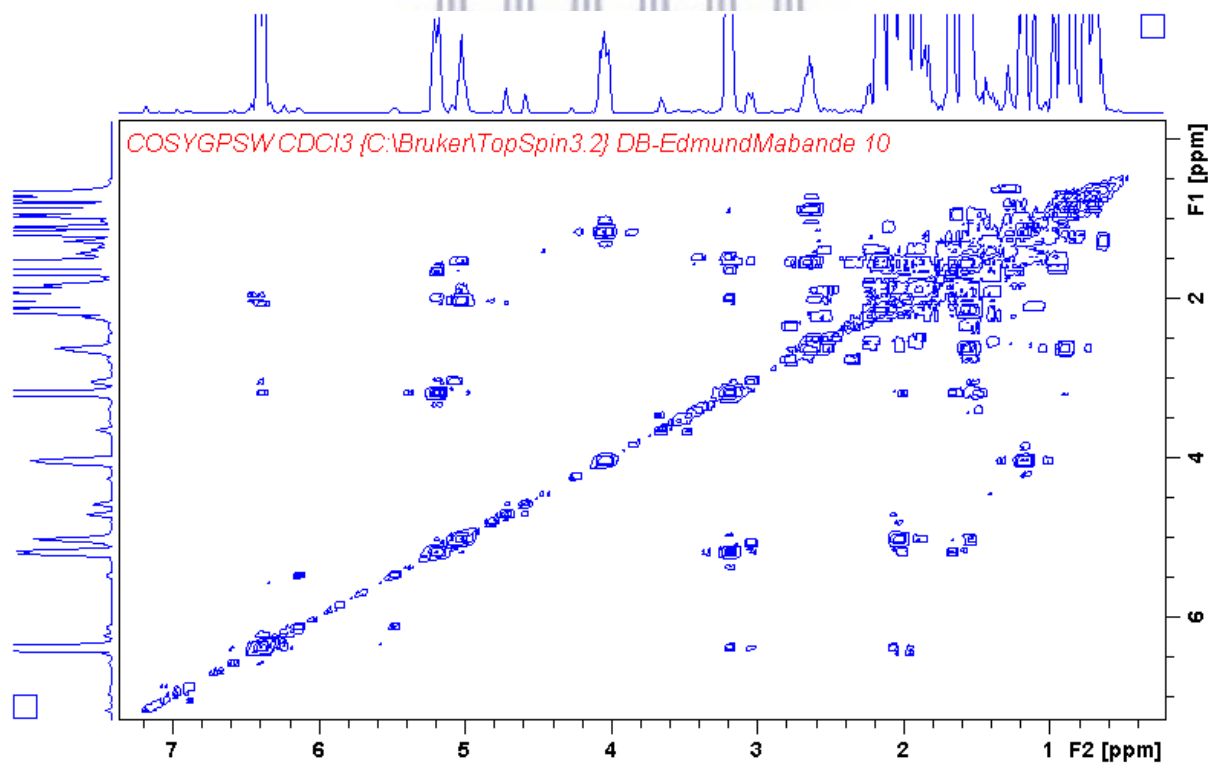


Figure S5.2 COSY spectra of compound 5.8 (CDCl₃, 400 MHz)

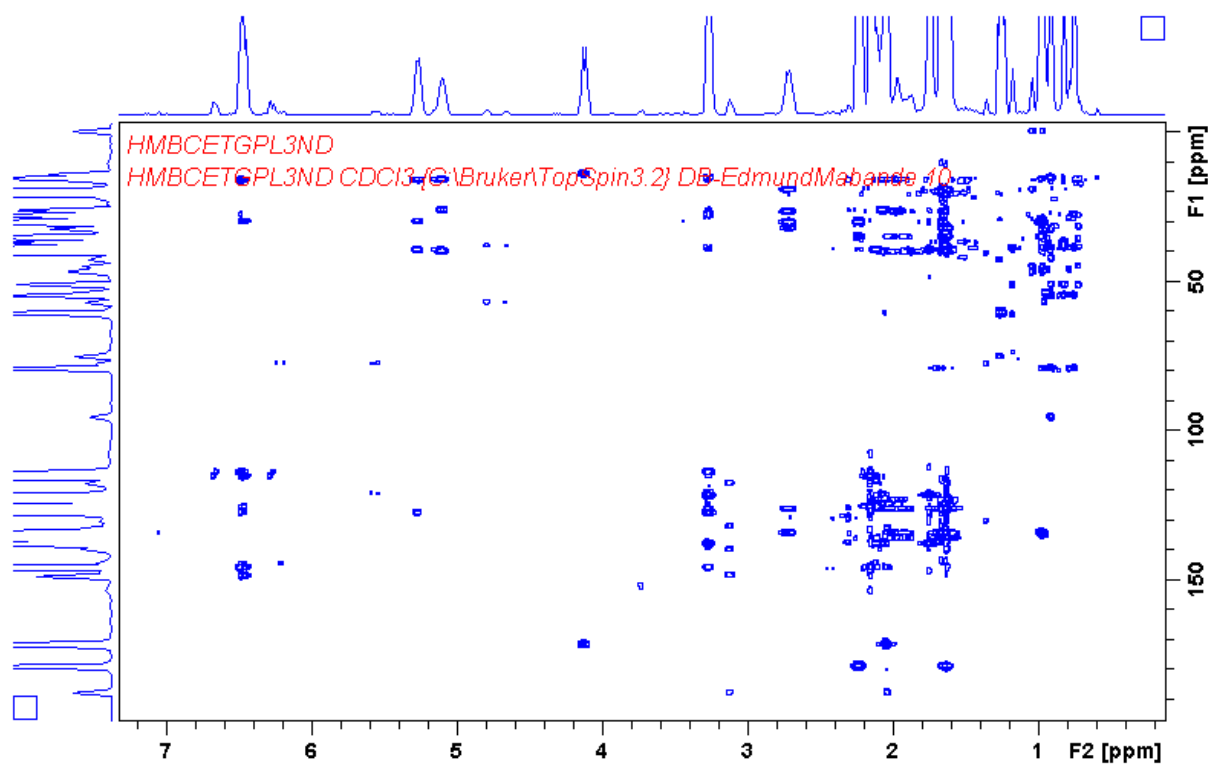


Figure S5.3 HMBC spectra of compound 5.8 (CDCl₃, 400 MHz)

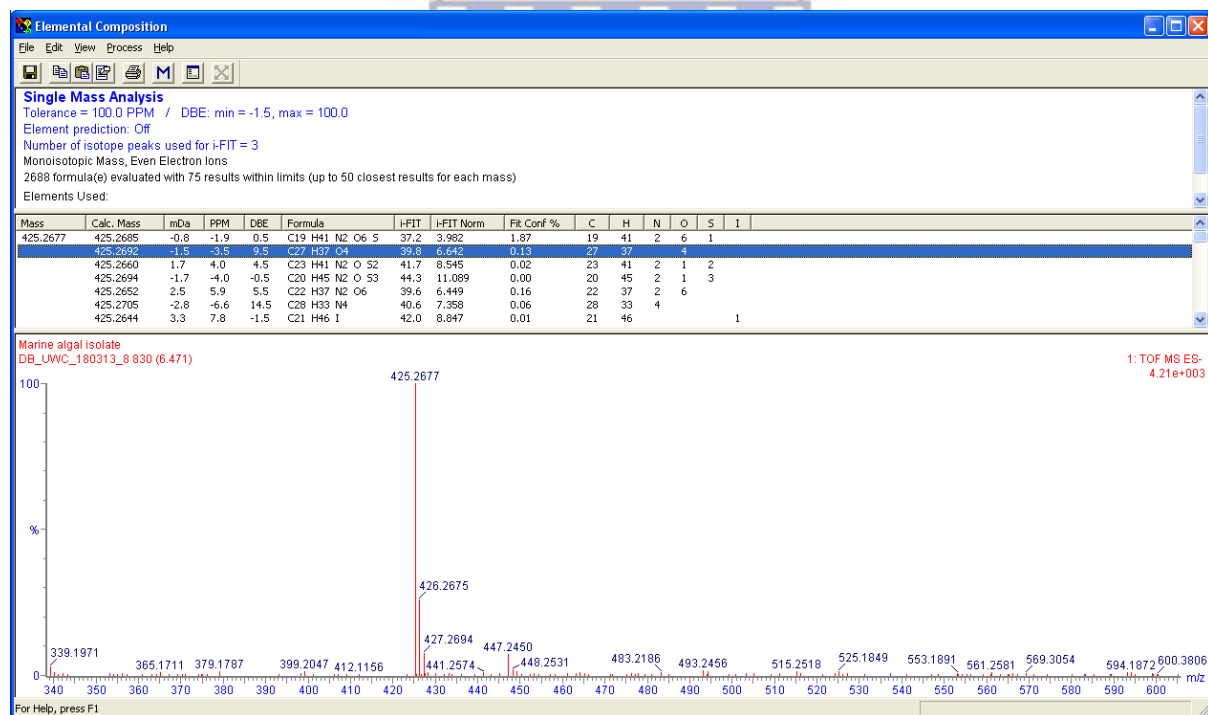


Figure S5.4 HRMS of compound 5.8