DISCOVERY OF ANTIBACTERIAL LEAD COMPOUNDS FROM MARINE ORGANISMS

By
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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Pharmaceutical Science in the Faculty of Natural Sciences, University of the Western Cape

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Co-supervisor: Professor Marla Trindade

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Declaration

I, Omolola Afolayan, declare that this thesis **"Discovery of antibacterial lead compounds from marine organisms"** submitted to the University of the Western Cape for the degree of Doctor of Philosophy in Pharmaceutical Chemistry in the Faculty of Natural Sciences, is my original work and it has not been submitted to any other institution for the award of an academic degree. The sources and quotes that I have used have been duly acknowledged and referenced. I am fully aware of the University of the Western Cape policy on plagiarism and I have taken every precaution to comply with the regulations of the University.

Student…….. Date…….

In our capacity as the candidate's thesis supervisors, we certify that the statements are true to the best of our knowledge.

Supervisor…… Date…….

Co-supervisor…… Date…….

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Dedication

To my Dad and Mum, Prof AJ and Mrs BT Afolayan

Table of contents

List of figures

List of tables

List of schemes

List of abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
BGCs	Biosynthetic gene clusters
BLAST	Basic local alignment search tool
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
CH_3OH	Methanol
COSY	Correlation spectroscopy
DCM	Dichloromethane
DEPT-135	Distortionless enhancement of polarisation transfer-135
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
IC_{50}	Half Maximal Inhibitory Concentration
LCMS	Liquid chromatography mass spectrometry
MeOH	Methanol
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
OD	Optical density
ORFs	Open reading frames
PCR	Polymerase chain reaction
PPM	Parts per million
rRNA	Ribosomal ribonucleic acid
TB	Tuberculosis
TLC	Thin-layer chromatography

Abstract

Marine organisms including algae and bacteria are known to produce chemically diverse secondary metabolites for survival purposes in the marine environment. Scientists have identified some of these natural products as therapeutic agents including some antibiotics. Given the increase in the resistance of pathogenic microorganisms especially methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* to commonly prescribed antibiotics, researchers have turned towards exploiting marine natural products for new antibacterial compounds. Due to the proven success of finding bioactive compounds in the marine environment this study therefore aims to discover lead compounds against MRSA and *Mycobacterium tuberculosis* from two marine sources, the marine algae and the bacteria associated with marine invertebrates referred to as bacterial isolates. The first part of this study involved a small scale library extraction of 38 different marine algae. The crude organic extracts were screened for activity against MRSA ATCC 33591 using a TLC-bioautography assay. Of these, 24 algal extracts showed inhibitory activity against MRSA. In addition, ^{1}H NMR spectroscopy of the extracts was performed. Based on antibacterial activity and interesting chemical profiles, three South African algae namely; *Dictyota naevosa*, *Laurencia pumila* and *Laurencia sodwaniensis* were prioritised for further studies. Column fractionation was carried out on the extracts for compound separation and further purification using High Performance Liquid Chromatography (HPLC) was performed. Ultimately, a known diterpene pachydictyol A and a known sesquiterpene cartilagineol was successfully isolated from *D. naevosa* and *L. sodwaniensis*, respectively. An unusual C15-acetogenin was isolated from *L. pumila*. The structure of the compounds were elucidated by analysis of the 1D and 2D NMR spectral data. Subsequently, the purified compounds were found to exhibit inhibitory activities against MRSA ATCC 33591 and *Mycobacterium aurum* A+ following a TLC-bioautography assay.

The second part of the study focussed on bacteria from the marine invertebrates. This aimed at screening some marine bacteria for natural compounds that may also act as leads for the discovery and development of antimicrobials against MRSA and *Mycobacterium tuberculosis*. For this purpose, six marine bacterial isolates were obtained from the Institute of Microbial Biotechnology and Metagenomics (IMBM), University of the Western Cape. Following a sloppy-agar overlay and TLC-bioautographic tests, three isolates coded PE05-143, PE14-104 and PE14-104 were selected for further studies as they showed inhibitory activity against MRSA ATCC 33591 and

Mycobacterium smegmatis LR222. The identities of these isolates were determined by PCR amplification and sequencing of their 16S rRNA genes prior to sequencing their genomes. The isolates, PE05-143, PE14-28 and PE14-104 showed the highest identity to *Paenibacillus glucanolyticus* NBRC 15330, *Vibrio splendidus* LGP32 and *Vibrio pomeroyi* CAIM 578 strains, respectively. The genomic sequence of the isolates were queried using antiSMASH for the identification of the biosynthetic gene clusters and potential secondary metabolites that may be responsible for the observed antimicrobial activity.

Chapter 1
General introduction

1.1. Pathogenic bacteria infection

Several human infections are caused by microscopic organisms such as bacteria. Bacteria are ubiquitous unicellular living microorganisms capable of causing fatal human illnesses. Not all bacteria are, however, pathogenic. Usually, bacterial pathogenesis begins when it gains entry into the human body either through cuts, ingestion of contaminated food or water and by breathing in the exhaled droplets when an infected person coughs or sneezes (Ribet and Cossart, 2015; Pizarro-Cerdá and Cossart, 2006). Globally, an estimated 30 million people are affected by sepsis from which 6 million of the cases reported annually are fatal (World Health Organisation, 2019). Sepsis is most commonly caused by bacteria (Martin, 2012). Examples of deadly bacterial infections include pneumonia and meningitis mainly caused by *Streptococcus pneumoniae*; and tuberculosis, an airborne bacterial infection caused by *Mycobacterium tuberculosis (*Mook-Kanamori *et al*., 2011; Smith, 2003).

1.2. Methicillin resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* sometimes referred to as the "super bug" is an example of a human pathogenic bacterium. MRSA can cause minor skin and soft tissue infections such as abscesses and impetigo to major life threatening diseases including bacteraemia and endocarditis. The bacterium is also a common cause of infections associated with implanted drains, catheters and prosthetic devices (Hassoun *et al*., 2017). Vancomycin and teicoplanin are antibiotics used to treat MRSA infections although several MRSA strains are currently considered as vancomycin intermediate-resistant *S. aureus* (Hiramatsu *et al*., 2014). This has prompted the use of ceftaroline, daptomycin and linezolid as alternative drugs for more severe infections (Geriak *et al*., 2019). Although efforts are made to provide adequate drug treatment for infected individuals, there is still a need for promising research avenues aiming to discover novel and effective compounds for the treatment of bacterial infections and associated drug resistance. Therefore, one of the aims of this research study is to discover antibacterial leads towards MRSA infections.

1.3. The menace of tuberculosis (TB)

An estimated one-third of the world population is reported to have been latently infected by *M. tuberculosis* and the approximate mortality rate from active tuberculosis is about three million annually (World Health Organisation, 2019). Hence, another aim of this study is to make some contributions towards finding some lead compounds towards the development of antibiotics against tuberculosis. It is important to note that tuberculosis infection continues to be a major cause of human mortality especially in developing countries, such as sub-Saharan Africa (Zumla *et al*., 2015). The current drug treatment is faced with several challenges including toxic side effects, high pill counts, long duration of treatment and multi-drug-resistant tuberculosis (MDR-TB) (Volmink and Garner, 2007). The available first-line treatment for tuberculosis is currently in the form of a multidrug regimen, namely, ethambutol, isoniazid, pyrazinamide and rifampicin. For example, in order to achieve high efficacy the drugs must be administered for six consecutive months (Van den Boogaard *et al.,* 2009). Furthermore, tuberculosis treatment is presently faced with challenges associated with drug resistance and the human immunodeficiency virus (HIV) epidemic (Getahun *et al*., 2010). TB drug resistance refers to the drugs to which the bacterium is not susceptible whereas multi-drug resistant TB (MDR-TB) is essentially resistance to two main drugs used for TB treatment namely isoniazid and rifampicin (Nachega and Chaisson, 2003). Factors associated with drug resistance include duration and non-adherence to prescribed treatment, side effects of anti-TB drugs and dual infection of TB and HIV. Co-infection of TB and HIV often involves toxic drug-drug interaction between the anti-TB drugs and the anti-retroviral drugs (Dworkin *et al*., 2005).

1.4. Marine environment as a main source of antibiotic leads

The ocean represents an environment rich in biodiversity covering over 70% of the earth surface. It harbours a vast array of chemically diverse natural products originating from algae and invertebrates such as corals, echinoderms, sponges and tunicates and from microorganisms including bacteria, cyanobacteria and fungi (Newman and Cragg, 2012). The natural products extracted from the marine inhabitants have produced some novel leads against diseases caused by parasites, fungi, viruses and bacteria. Although, most of the currently used natural product-derived therapeutics have been from terrestrial sources, comparative studies have shown that natural products from marine sources are chemically more diversified than terrestrial natural products (Kong *et al.,* 2010; Mondol and Shin, 2014). According to Montaser and Luesch (2011), the secondary metabolites generated as a result of ecological pressure such as pollution, salinity, nutritional scarcity and chemical defence mechanism produced by organisms that lack physical

protection play a vital role in introducing lead compounds for drug discovery for the treatment and control of diseases. A number of compounds have been identified from marine sources which have varying therapeutic functionalities. For example, salinosporamide A is sourced from a marine bacterium and is being evaluated for cancer treatment against multiple myeloma (Feling *et al*., 2003; Fenical *et al*., 2009). Vidarabine is a derivative from a sponge metabolite and used as an antiviral drug against herpes simplex (Sagar *et al*., 2010).

1.5. The focus of this project

Given the structural diversity and potent biological activity of marine natural products, this study focused on discovering antibacterial leads from marine sources. The process involved bioassay-guided fractionation of marine algae and microbial extracts whereby secondary metabolites responsible for antibacterial properties was extracted and purified. Subsequently, the structures of the bioactive compounds was elucidated by spectroscopic methods. The activity of the purified compounds was further assessed against MRSA and *Mycobacterium aurum* test strains in order to determine the potency and selectivity of such compounds hoping that this study should thus create a platform that will facilitate the development of novel antibacterial drug design and discovery.

1.6. Problem identification

The treatment of bacterial infections is complicated by the development of resistance to commonly prescribed antibiotics, particularly; infections caused by MRSA and *M. tuberculosis*. There is therefore a need for new and effective drugs for the treatment of these diseases. In addition, there are also challenges associated with natural product drug discovery from natural products. For example, there are no Food and Drug Administration (FDA) approved antimicrobial drug sources from terrestrial plants (these are mainly sourced from microbial sources), hence, the search for leads from marine organisms are warranted. Marine natural products offer unique and chemically diverse groups of compounds that may serve as potential antibacterial leads. Given that the existing MRSA and *M. tuberculosis* antibiotics are susceptible to resistance, ongoing research channelled towards finding novel and potent antibacterial compounds for their treatment is essential. Herein, the purpose of this study is to discover antibacterial leads against MRSA and *M. tuberculosis* from marine natural products. It is hoped that such leads will eventually produce new and effective drugs for these resistant pathogenic organisms.

1.7. Aims and objectives

This study was divided into two parts. Firstly, the potential of South African marine algae as a source of antimicrobial agents was explored. This was aimed at isolating natural lead compounds. Secondly, effort was made to identify natural compounds from marine bacteria that have been previously isolated from marine invertebrates and shown to exhibit activity against *M. tuberculosis* (These bacteria are referred to in this project as **bacterial isolates)**. They were coded as PE05-99, PE05-143, PE06-104, PE08-57, PE14-28 and PE14-104). The overall aim of this study was to discover new and effective antibacterial leads against MRSA and *M. tuberculosis* from marine natural products.

The specific objectives are:

- To prepare a small scale library of marine algal extracts.
- To screen the extracts for antibacterial activity against gram-positive methicillin-resistant *S. aureus* (MRSA ATCC 33591), and *Mycobacterium aurum* A+. In this study, *M. aurum* served as a substitute for *M. tuberculosis.* This was because the laboratory accessible for screening was a Biosafety Level 2 laboratory while *M. tuberculosis* must be handled in Biosafety Level 3 laboratory. *M. smegmatis* was also used as a test organism because of availability and lower pathogenicity as compared to *M. tuberculosis*. Hence, *M. smegmatis* also served as a substitute for *M. tuberculosis*.
- To isolate bioactive compounds from selected active algae extracts and to determine their structures using spectroscopic methods.
- To determine the antibacterial activity of the compounds against MRSA and *M. aurum*.
- To culture bacterial isolates and extract their metabolites by liquid-liquid extraction.
- To employ genome sequencing as a dereplication tool with respect to the secondary metabolite capacity of the isolates.

1.8. Thesis overview

Chapter one is a general introduction to bacterial infection and rationale for this study while antibacterial natural products is reviewed in chapter two. The preparation of marine algal extracts library and microbial extracts and subsequent screening of the extracts for antibacterial activity is outlined in chapter three. Bioinformatic characterisation of bacterial isolates is provided in chapter 4. Chapter five describes the isolation and structural elucidation of the purified active compounds from selected algae. Susceptibility evaluation of the active algal extracts and compounds for

antibacterial activity is assessed in chapter six. The general conclusion, limitations and recommendations is summarised in chapter seven.

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Chapter 2
Review of antimicrobial drugs from marine sources

2.1. The causes of antibiotic resistance

The first reported antibiotic, penicillin, was discovered in 1929 by Alexander Fleming (Fleming, 1929). However, it was not until 1937 that antibiotics, the sulphonamides, were first introduced for therapeutic use (Davies and Davies, 2010). This remarkable discovery of antibiotics led to the treatment of infectious diseases, such as tuberculosis and leprosy, which were previously incurable. As a result many lives have been saved. The period between 1940s and 1960s is considered the 'golden age of antibiotic discovery' whereby novel classes of antibiotics were introduced including the ones still used in the present day (Walsh and Wencewicz, 2014). Unfortunately, over the years many of these drugs have gradually become less effective due to overprescription, sub-therapeutic doses, misuse by not abiding to the recommended course of treatment and unregulated over the counter sale in some developing countries (Ventola, 2015; Michael, 2014; Viswanathan, 2014).

2.1.1. Mechanisms of resistance

The mechanisms by which bacteria acquire resistance include enzymatic inactivation of the antibiotics (e.g. inactivation of beta-lactam antibiotics by beta-lactamase), alteration of antimicrobial targets through modification of drug receptor site and changes in membrane permeability through decreased cell permeability and active efflux of antibiotics (Munita and Arias, 2016). In general, gram-negative bacteria are more resistant to antibiotics than gram-positive bacteria. Gram-negative bacteria possess an outer membrane which not only function as protein translocator and signal transductor, but also prevent the entry of drugs and antibiotics into the bacterial cell. Generally, there are four major ways by which bacteria exhibit resistance to antibiotics. These include:

Drug inactivation or modification: This happens when an antibiotic is deactivated by enzymes within the bacteria. It involves the breakdown or destruction of the beta-lactam bond in beta-lactam antibiotics, for example, penicillin by beta-lactamase enzymes in the resistant bacteria. What generally happens however is an addition of an acetyl or phosphate group to the antibiotic at a specific site. This in turn prevent the antibiotics from successfully binding and disrupting protein synthesis in the bacteria (Tooke *et al*., 2019). An illustration is shown below in Figure 2.1.

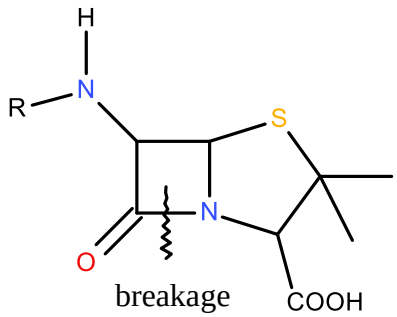


Figure 2.1: Breakdown of beta lactamase ring in penicillins.

Modification of metabolic pathway: Nucleic acids are synthesised through a pathway that uses para-amino benzoic acid (PABA) as a precursor. Sulfonamides are structurally similar to PABA and act as antibiotics by serving as a substrate for the enzyme dihydropteroate synthetase which uses PABA to manufacture nucleic/folic acid in the bacteria and therefore blocks the pathway. Sulfonamide-resistant bacteria are able to by-pass the use of PABA as a precursor and instead use pre-formed folic acid (Sköld, 2000).

Modification of binding site: This form of resistance occurs when the site to which the antibiotic would normally bind to prevent ribosomal protein synthesis has been conformationally altered. The protective protein bind to the bacteria's ribosome preventing the antibiotics from binding and thus allow the continuation of protein synthesis by the bacteria. This type of resistance is observed in MRSA (Connell, 2003).

Decreased concentration or accumulation of drugs: This occurs when the accumulation of certain antibiotics within the bacteria cell is prevented by active efflux or pumping out of the antibiotics from the cell before it is able to damage the bacteria. It can occur by decreasing the permeability of the cell membrane to the drug thereby prevents its influx into the cell. An example of this is seen with fluoroquinolone resistance (Redgrave *et al*., 2014).

Given that most antibiotics are within the category of either beta-lactam pathway or metabolic inhibitor, binding site or cell wall inhibitor, all these have rendered most of the available antibiotic useless. This therefore warrants the need for the development of novel antibiotics. Hence, this study is aimed at finding novel antibacterial compounds and agents through tapping into new diversity in endemic marine environments.

2.1.2. Antibiotic resistant Staphylococcus aureus and Mycobacterium tuberculosis

Antibiotic resistance occurs when bacteria develop mechanisms to oppose the efficacy of the drugs required for treating infections. The bacteria that withstand the treatment agents survive and continue to multiply which ultimately results in resistance, whereas, the susceptible bacteria succumb to the antibiotics and thus kill or inhibit the activity of such bacteria. Globally, an estimated 10 million human fatalities is predicted from drug resistant related infections by 2050 (O'Neill, 2014). Resistance to antibiotics can develop either by genetic mutation or by conjugation. During conjugation bacteria exchange genetic materials, including genes encoding antibiotic resistant genes (Blair *et al*., 2015). Examples of bacteria resistant to antibiotics include MRSA, MDR-TB, carbapenem-resistant *Enterobacteriaceae* (CRE) and vancomycin-resistant *Enterococcus* (VRE). This project is aimed at finding antibacterial compounds which can be used against infectious drug resistant pathogen such as MRSA and MDR-TB.

2.1.2.1. Methicillin-resistant Staphylococcus aureus (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a gram-positive bacterium that is responsible for several human infections ranging from skin, soft tissue, joint and implant-related infections to serious illnesses such as bacteraemia, endocarditis and pneumonia (Naves *et al*., 2012). The types of methicillin-resistant *Staphylococcus aureus* are healthcare or hospital acquired MRSA (HA-MRSA) and community-associated MRSA. Through evolution, MRSA has become genetically distinct from other *S. aureus* strains and has developed multi-drug resistance to a number of β-lactam antibiotics. Beta-lactam antibiotics are a broad spectrum class of antibiotic agents consisting of a β-lactam ring in the molecular structure and includes compounds such as amoxicillin, cephalosporins, methicillin, penicillin, oxacillin. MRSA produces beta-lactamase, an enzyme capable of mediating antibiotic hydrolysis, which destroys the beta-lactam ring thus causing the antibiotics to become ineffective (Peacock *et al*., 2015). Although MRSA is resistant to commonly used antibiotics such as penicillin and oxacillin, a few antibiotics are prescribed for MRSA treatment (Figure 2.2). The first-line therapy is trimethoprim-sulfamethoxazole (**2.1-2.2**). Sulfonamides are antibiotic agents that were first discovered in 1932 and registered for clinical use in 1935 (Jesman *et al*., 2011). Trimethoprim was first used for the treatment of human infections in 1962 and subsequently administered in combination with sulfonamides for clinical use in 1968 (Eliopoulos and Huovinen, 2001). Second-line therapy is clindamycin derived from lincomycins, which are lincosamide antimicrobial agents that was discovered from *Streptomyces lincolnensis* in 1962 (Mason *et al*., 1962). Subsequently, clindamycin (**2.3**) was discovered from lincomycin as a derivative in 1966 (Smieja, 1998). Third-

line therapy include tetracycline (**2.4**) or doxycycline (**2.5**). Tetracycline was discovered from *Streptomyces* and *Actinobacteria*, and was first prescribed in 1948 and doxycycline is an analogue of tetracycline that was clinically developed in 1967 (Li and Corey, 2013; Nelson and Levy, 2011). Fourth-line therapy is linezolid (**2.6**), a synthetic oxazolidinone class of antibiotics discovered in mid 1990s, and approved for commercial use in 2000 (Leach *et al*., 2011).

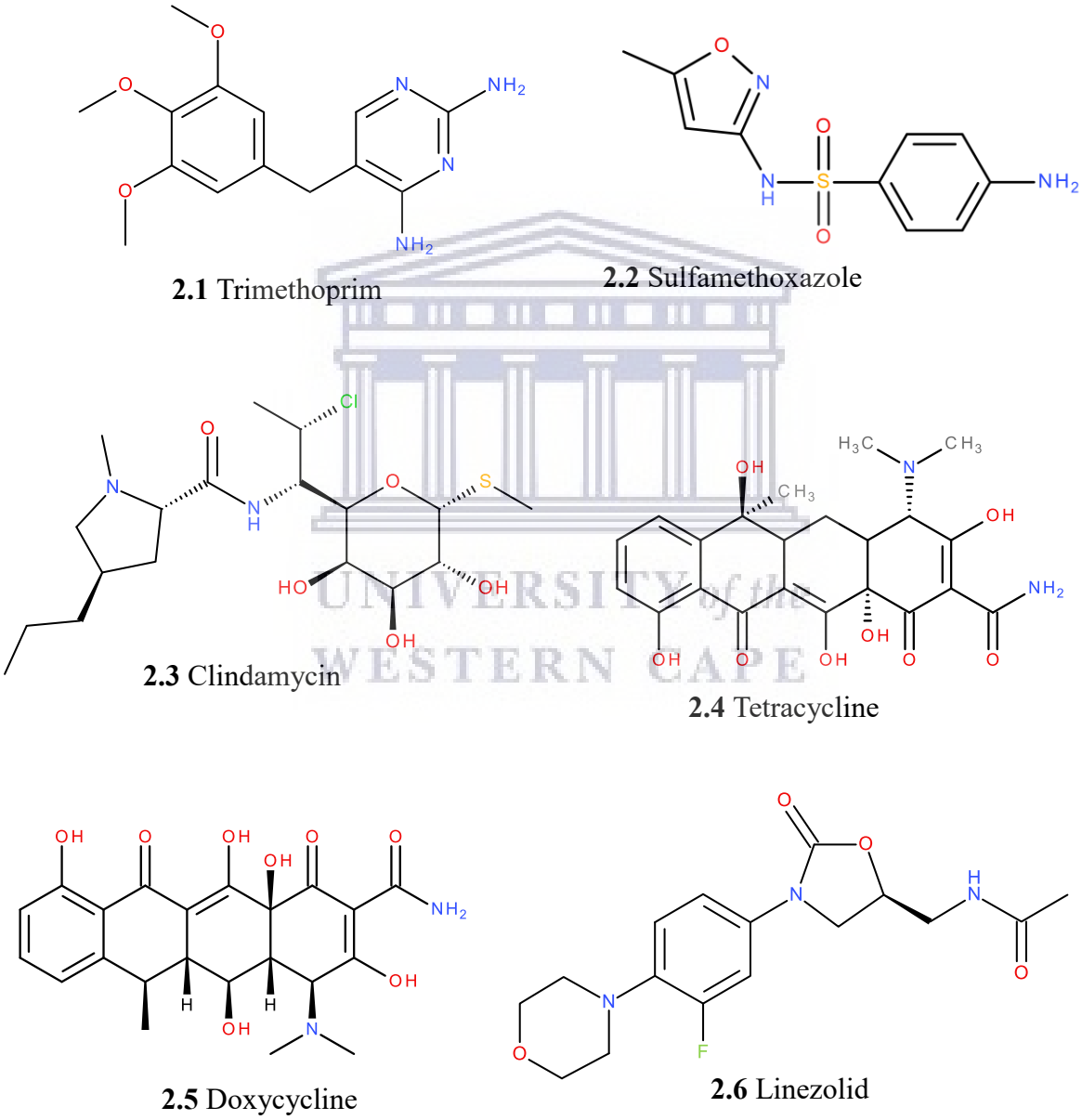
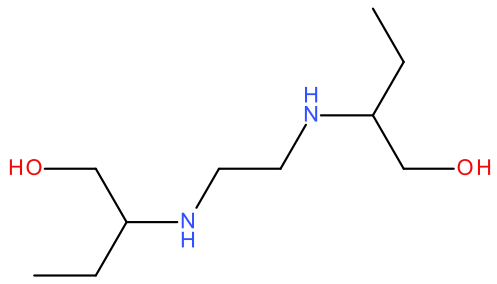


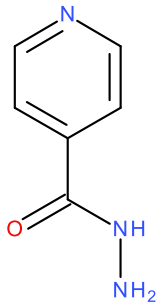
Figure 2.2: Chemical structures of antibiotic agents used for the treatment of MRSA.

2.1.2.2. Multi-drug-resistant Mycobacterium tuberculosis (MDR-TB)

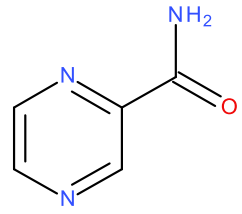
Tuberculosis, commonly referred to as TB, is an airborne bacterial infection caused by *Mycobacterium tuberculosis* which was identified by Robert Koch in 1882 (Gordon and Parish, 2018). *M. tuberculosis*, an intracellular pathogen that inhabits the host's macrophages and often remain latent after host cell invasion (Delogu *et al*., 2013). The latent TB may become active when the host has a compromised immune system such as the case of acquired immune deficiency syndrome (AIDS). Symptoms of active TB infection include chronic cough sometimes accompanied by blood stained phlegm/mucus, fever, loss of appetite, shortness of breath and chest pain. An estimated one-third of the world population is reported to have been latently infected by *M. tuberculosis* (World Health Organisation, 2019). Furthermore, the approximate mortality rate from active TB is about three million annually. The first-line treatment for TB is currently in the form of a multidrug ethambutol (**2.7**), isoniazid (**2.8**), pyrazinamide (**2.9**) and rifampicin (**2.10**) regimen (Figure 2.3). These drugs were discovered in 1961, 1952, 1952 and 1957, respectively and must be administered for six consecutive months for optimal efficacy (Van den Boogaard *et al*., 2009). Presently however, the challenges faced in TB treatment are those of drug resistance and the human immunodeficiency virus (HIV) epidemic. Co-infection of TB and HIV often involves toxic drug-drug interaction between the anti-TB drugs and the antiretroviral drugs, for example, rifampicin and the antiretroviral protease inhibitors namely nevirapine and ritonavir (Lalloo, 2009). Moreover, high pill count and risk of immune reconstitution inflammatory syndrome are also factors impacting on the problem of TB drug resistance (Narita *et al*., 1998; Volmink and Garner 2007). Multi-drug-resistant *M. tuberculosis* has led to renewed research interests in natural products with the hope of discovering new and novel anti-tubercular leads.



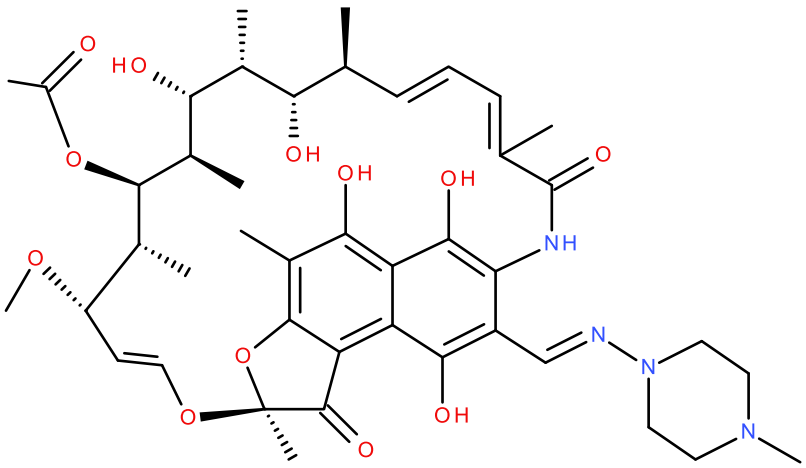
2.7 Ethambutol



2.8 Isoniazid



2.9 Pyrazinamide



2.10 Rifampicin

Figure 2.3: Chemical structures of first-line drugs used for the treatment of TB.

2.2. Bioactive natural products

Natural products are organically produced chemical compounds or substances which originate from living organisms. They continue to generate popularity as a source of templates for the development of new drug scaffolds in the treatment of human diseases. In fact, natural products account for over 60% of newly introduced drugs in the market within the past three decades (Newman and Cragg, 2013). These products may originate from individual organisms such as plants, animals and microorganisms or maybe products synthesised by biological associations between two or more living organisms (Berdy, 2005). Researchers have been investigating the associations that take place among microorganisms and their host organisms following evidence suggesting that many of the bioactive compound discoveries originally associated with host organisms are not produced by the organisms itself but are synthesised by symbiotic or associated microorganisms (Choudhary, 2017). It is becoming apparent that these associations involve the sharing of complex chemical signals through quorum sensing depending on whether they are competitive or mutually advantageous. In most cases, the signalling molecules are produced as defence mechanisms for protection against predators. These metabolites present an opportunity to discover novel therapeutic chemical scaffolds as treatment against diseases. The produced metabolites are classified as primary or secondary with the former forming the starting materials for secondary metabolites. Primary metabolites are the main products of energy metabolism, namely proteins, carbohydrates and fats. These metabolites are regarded as fundamental,

providing the basic constituents for sustaining physiological processes including growth, primary development and reproduction. Secondary metabolites, however, may not be involved in primary growth but play a role in defence mechanisms such as the development of pigments and natural antibiotics. It is believed that these secondary metabolites are generated as a result of ecological pressure such as predation, extreme pH, nutritional scarcity, space competition and chemical defence (Schmidt, 2005; Thornburg *et al*., 2010). This essentially play a role in introducing lead compounds for drug discovery.

Secondary metabolites are generally classified into five main groups namely; **terpenoids and steroids**: terpenoids are vast multicyclic structures that appears to be unrelated. They account for approximately 60% of known natural products (Firn, 2010). They are biosynthesised from isoprene units, and typically contain oxygen as a functional group. Steroids usually consist of tetracyclic carbon backbone and are biosynthesised through modification of terpenoids that are formed from the triterpene lanosterol (Davis and Croteau, 2000).

Polyketides and fatty acids: These are formed through carboxylation of acyl precusors such as acetyl-CoA, propionyl-CoA or methylmalonyl-CoA through enzymatic reactions of fatty acid synthases (Staunton and Wiessman, 2000). Fatty acids are long chains of lipid-carboxylic acids which are either straight, branched, saturated or unsaturated (Pérez *et al*., 2016). An eicosanoid biosynthesis is an example of fatty acid derived natural product.

Nonribosomal peptides: They are biosynthesised from amino acid without a direct RNA transcription. The process occurs through multifunctional enzyme complex (Süssmuth and Mainz, 2017). The antibiotic, penicillin is an example of a beta-lactam nonribosomal peptide.

Alkaloids: Alkaloids are vast and diverse class of organic compounds. Structurally, the compounds consist of basic amine functional group, and are biosynthesised from amino acid precursors (Ziegler and Facchini, 2008).

Enzyme cofactors: Are unlike the other natural product classes or categories. A cofactor is a non-protein organic molecule that is required during enzyme's activity as catalyst in a chemical reaction. An enzyme without a cofactor will remain inactive (apoenzyme) and will only be activated once it binds to a cofactor (holoenzyme). For example, pyridoxal phosphate as a coenzyme is capable of catalysing a variety of enzymatic reactions.

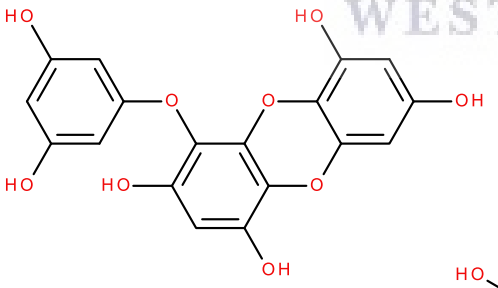
2.3. Secondary metabolites from marine algae

Algae are photosynthetic organisms mostly found in aquatic environments. These include both macro- and micro-algae. Macroalgae or seaweeds are multicellular organisms located in benthic regions as green, brown or red algae. Microalgae or microphytes are unicellular and are typically found in benthic and littoral zones in marine and freshwaters as phytoplankton (Garson, 1989). Algae generate useful natural products that play a role in several industrial applications including human foods, fuels, fertilizers, cosmetics, medicine and pharmaceuticals (Spolaore *et al*., 2006). The brown and red algae in particular are amongst the largest and most complex classes of algae from which numerous pharmacological agents have been reported. Thus, many research activities have focused on these organisms in an effort to discover more antibacterial agents.

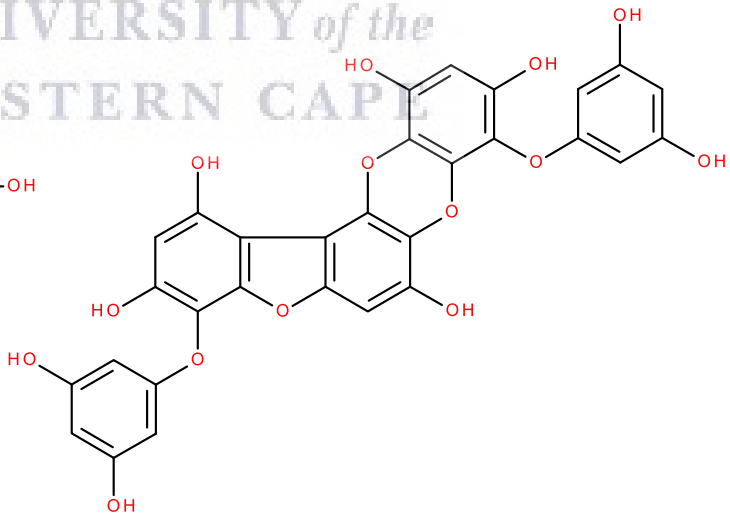
2.3.1. Bioactive compounds reported from brown algae (Phaeophyta)

Globally, there are over 1800 species of brown algae (Guiry and Guiry, 2019). The brown colour trait stems from the prevalence of xanthophyll pigments fucoxanthin which overshadows other pigments such as chlorophylls and beta-carotene (Kumar and Singh, 1979). It is estimated that at least 500 natural products have been isolated from brown algae within the past five decades (Ioannou *et al*., 2013). Among this group of algae, many genera produce diterpenes which constitutes the largest class of secondary metabolites. The members of genus *Dictyota* contribute to the many diverse secondary metabolites isolated from brown algae with over 233 diterpene compounds (Chen *et al*., 2018). Other species of brown algae that have also been reported to produce diterpenes include *Stypopodium zonale, Cystoseira myrica* and *Bifurcaria bifurcate* (Dorta *et al.,* 2002*;* Ayyad *et al*., 2003*;* Culioli *et al*., 2004). The bioactive metabolites that are produced have been the subject of extensive research. Figure 2.4 shows some of the reported bioactive metabolites. Eckol (**2.11**) is a phlorotannin that is commonly produced by the species of the brown algae of the genus *Ecklonia* such as *E. cava*. It has been reported to exhibit several biological activities including antimicrobial activity against MRSA and *Salmonella* spp at MIC values ranging from 125 to 250 μg/mL and 125 to 250 μg/mL, respectively. However, following the combination of eckol with ampicillin an improved activity was observed at MIC values of 0.31 to 0.5 μg/mL for MRSA and 0.75 to 1.0 μg/mL for *Salmonella* spp. This enhancement in activity was attributed to synergistic effect by the authors (Choi *et al*., 2010). Phlorofucofuroeckol-A (**2.12**) isolated from *E. bicyclis* was shown to exhibit antimicrobial activity against MRSA at high MIC values of 128 to 512 μg/mL (Eom *et al*., 2013). Cystodiones G and H (**2.13** and **2.14**), 11-hydroxyamentadione (**2.15**) and amentadione (**2.16)** isolated from *Cystoseira usneoides* has shown significant activity as inhibitors of the production of the proinflammatory cytokine TNF-α in LPS-

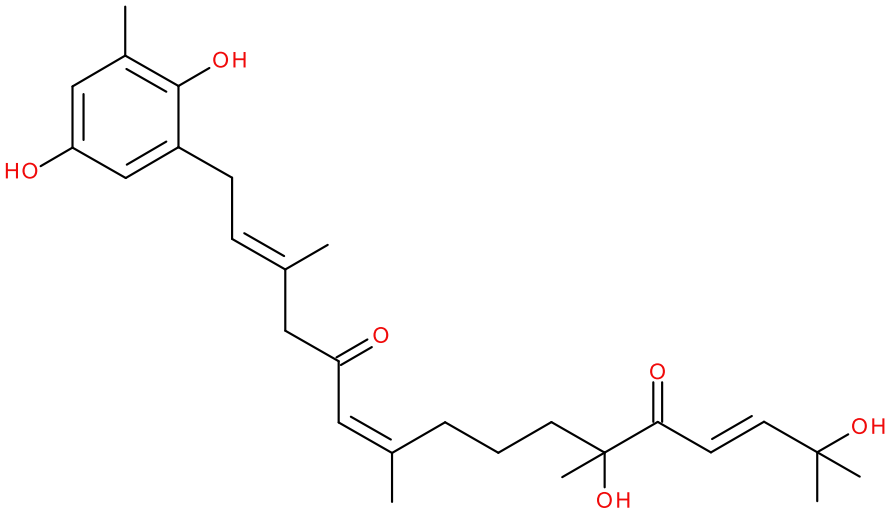
stimulated THP-1 human macrophages (De los Reyes *et al*., 2016). These metabolites were also isolated from *Dictyopteris membranacea* and were reported to display anti-inflammatory activity in LPS-elicited RAW 264.7 mouse macrophage cells (Dimou, 2016). Sargachromanol G (**2.17**), an extract of *Sargassum siliquastrum*, was shown to have anti-inflammatory effects in a murine macrophage cell line RAW 264.7 (Yoon *et al*., 2012). Trihydroxylated acyclic diterpenes isolated from *Bifurcaria bifurcata* displayed cytotoxic activity against a human lung cancer cell line NSCLC-N6 (Culioli *et al*., 2004). The ethyl acetate extract from the same algae showed trypanocidal activity against *Trypanosoma brucei rhodesiense* at IC_{50} = 0.53 µg/mL; selectivity index (SI) = 11.6 and against *Plasmodium falciparum* at IC_{50} = 7.9 µM, SI = 21.6). Eleganolone (**2.18**) was the main diterpenoid isolated from this species which was reported to have contributed to the observed activity (Gallé *et al*., 2013). Dolabelladienetriol (**2.19**) with antiviral activity was isolated from *Dictyota pfaffii*. (Soares *et al*., 2012; Cirne-Santos *et al*., 2008). The diterpenes (6R)-6-hydroxydichotoma-3,14-diene-1,17-dial (**2.20**) and (6R)-6-acetoxidichotoma-3,14-diene-1,17-dial (**2.21**) derivative from *D. menstrualis* displayed antiretroviral activity *in vitro* against human immunodeficiency virus type 1 (Pereira *et al*., 2004). Lobophorolide (**2.22**) isolated from *Lobophora variegate* showed antifungal activity against *Dendroyphiella salina*, *Lindra thalassiae* and *Candida albicans* with IC_{50} values of 0.034, 0.135 and 1.3 µg/mL, respectively (Kubanek *et al*., 2003).



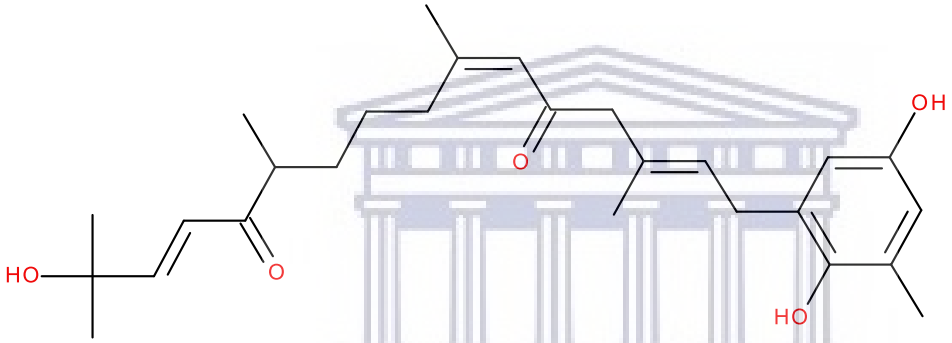
2.11 Eckol



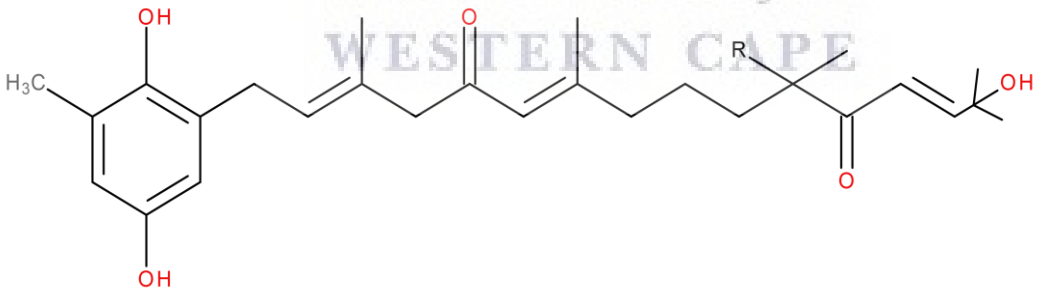
2.12 Phlorofucofuroeckol-A



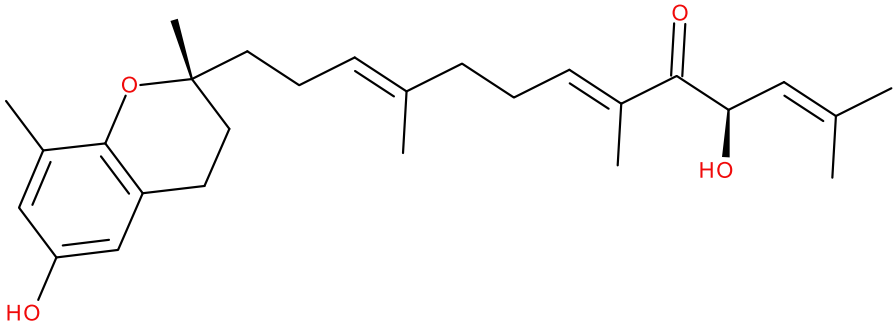
2.13 Cystodione G



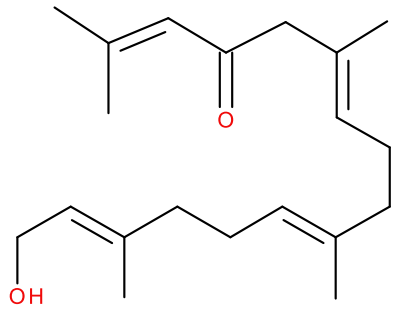
2.14 Cystodione H



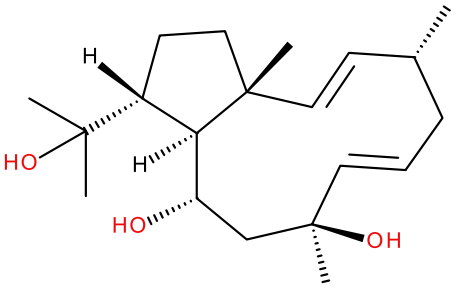
2.15 11-hydroxyamentadione; R = OH
2.16 Amentadione; R = H



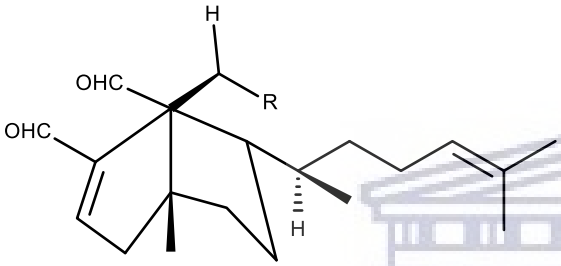
2.17 Sargachromanol G



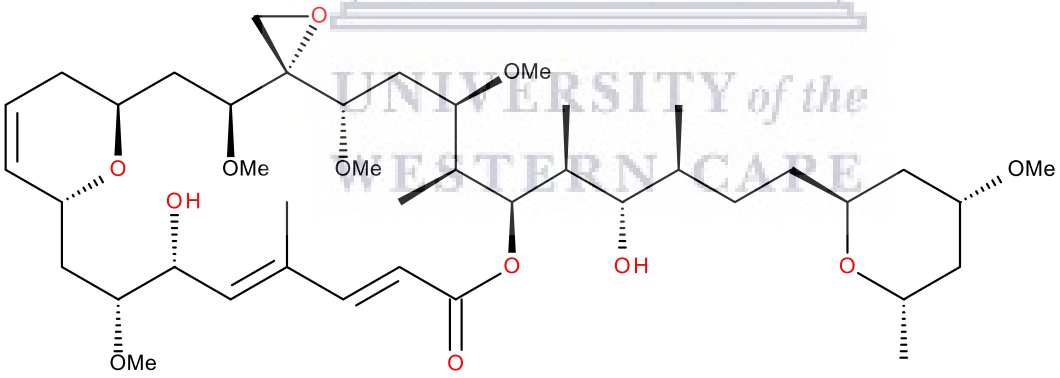
2.18 Eleganolone



2.19 Dolabelladienetriol



2.20 (6R)-6-hydroxydichotoma-3,14-diene-1,17-dial; R = OH
2.21 (6R)-6-acetoxidichotoma-3,14-diene-1,17-dial; R = OAc



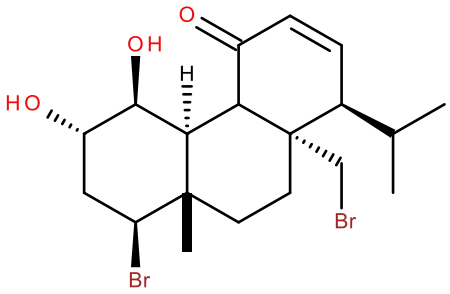
2.22 Lobophorolide

Figure 2.4: Chemical structures of some bioactive metabolites from brown algae.

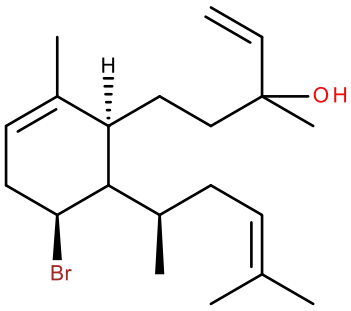
2.3.2. Bioactive compounds reported from red algae (Rhodophyta)

Red algae comprises over 8000 species (Guiry and Guiry, 2019). The red colour originates from the pigments phycoerythrin which eclipse other pigments within the algae (Bold and Wynne, 1985). Among the red algae, many genera typically produce terpenes as part of their secondary metabolite biosynthesis. The genus *Laurencia* in particular accounts for the majority of the

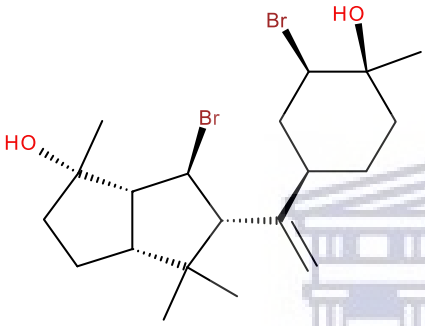
secondary metabolite produced with at least 512 sesquiterpenes and 133 diterpenes of various structures and functionalities (Harizani *et al*., 2016). Other species of red algae have also been reported to produce terpenes including *Plocamium cartilagineum*, *Plocamium cornutum* and *Sphaerococcus coronopifolius* (König *et al*., 1990; Afolayan *et al*., 2009; Rodrigues *et al*., 2015). The secondary metabolites produced by red algae have been broadly investigated for their bioactive properties some of which are included in Figure 2.5. Bromosphaerone (**2.23**) isolated from the organic extract of red alga, *Sphaerococcus coronopifolius* which showed antibacterial activity against *S. aureus* ATCC 6538 at 0.104 µM (Etahiri *et al*., 2001). Sphaerodactylomelol (**2.24**) was also identified from *S. coronopifolius* and showed antimicrobial activity against *S. aureus* at IC_{50} 96.3 µM (Rodrigues *et al*., 2015). Diterpenes neorogioldiol B (**2.25**) and prevezol B (**2.26**) were isolated from *Laurencia obtuse* and showed potent cytotoxicity against the human tumour cell lines MCF-7, PC3, HeLa, A431 and K562 (IIopoulou *et al*., 2003). (8R)-8-bromo-10-epi-β-snyderol (**2.27**) a sesquiterpene derivative isolated from the red alga *Laurencia obtusa* displayed antiparasitic activity against D6 and W2 clones of *Plasmodium falciparum* (Topcu *et al*., 2003). Moreover, the red alga *Odonthalia corymbifera* metabolite, bis (2,3-dibromo-4,5-dihydroxybenzyl) ether (**2.28**) has anti-diabetic potential (Oh *et al*., 2008). Obtusol (**2.29**) isolated from *Laurencia dendroidea* had larvicidal activity against dengue fever mosquito *Aedes aegypti* (Salvador-Neto *et al*., 2016). Similarly, omaezol (**2.30**) and hachijojimallene A (**2.31**) isolated from a *Laurencia* sp. displayed potent activities against the larvae of the barnacle *Amphibalanus amphitrite* (Oguri *et al*., 2017). Shinorine (**2.32**) and porphyra-334 (**2.33**) from *Porphyra sp*., have immunomodulatory effects (Becker *et al*., 2016). The halogenated compound, elatol (**2.34**), extracted from a number of *Laurencia* species including *Laurencia majuscula* showed antibacterial activity against disease causing human bacteria - *Staphylococcus epidermis*, *Salmonella* and *Klebsiella pneumonia* (Vairappan, 2003).



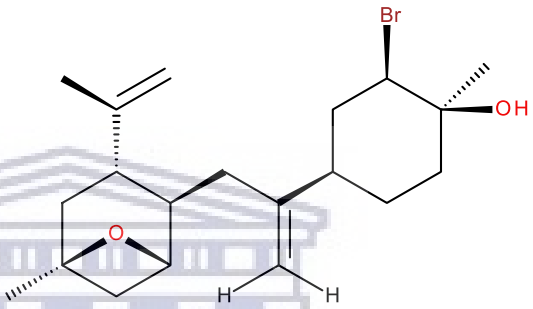
2.23 Bromosphaerone



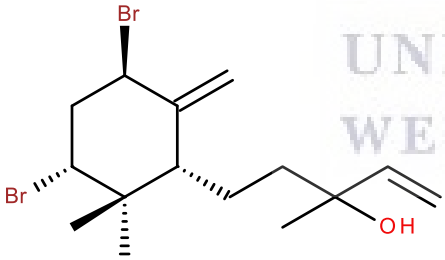
2.24 Sphaerodactylomelol



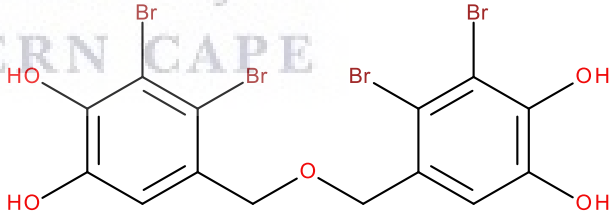
2.25 Neorogioldiol B



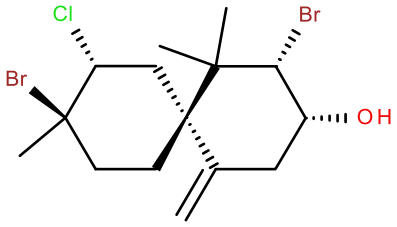
2.26 Prevezol B



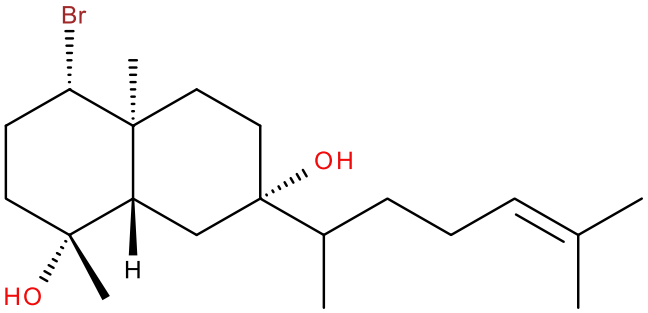
2.27 (8R)-8-bromo-10-epi-β-snyderol



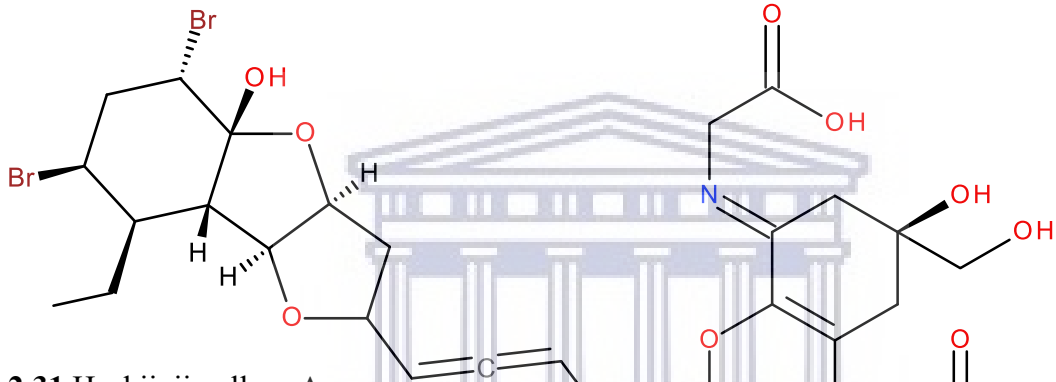
2.28 Bis (2,3-dibromo-4,5-dihydroxybenzyl) ether



2.29 Obtusol

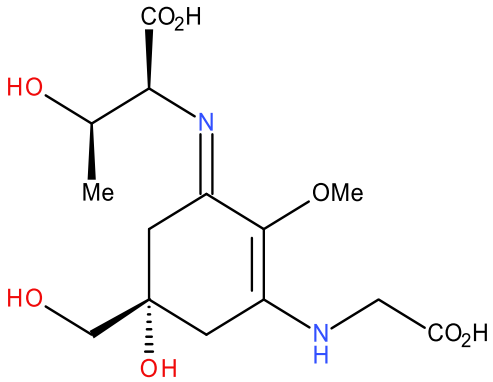


2.30 Omaezol

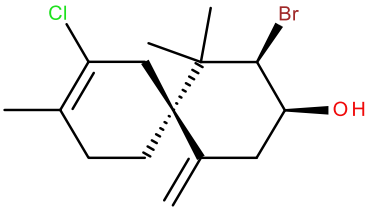


2.31 Hachijojimallene A

2.32 Shinorine



2.33 Porphyra-334



2.34 Elatol

Figure 2.5: Chemical structures of some bioactive metabolites from red algae.

2.4. Discovery of antimicrobial agents from marine bacteria

Marine bacteria are significant bioactive natural product sources and evokes much interest in drug discovery research. The bacterial phyla from which most of the metabolites are isolated include Actinomycetes, Bacteroidetes, Cyanobacteria and Firmicutes and Proteobacteria (Nikapitiya, 2012). Annual reports from 2010–2015 shows that *Actinobacteria* constitute the largest number of antimicrobials derived from marine bacteria with at least 36 reported active compounds (Schinke *et al*., 2017).

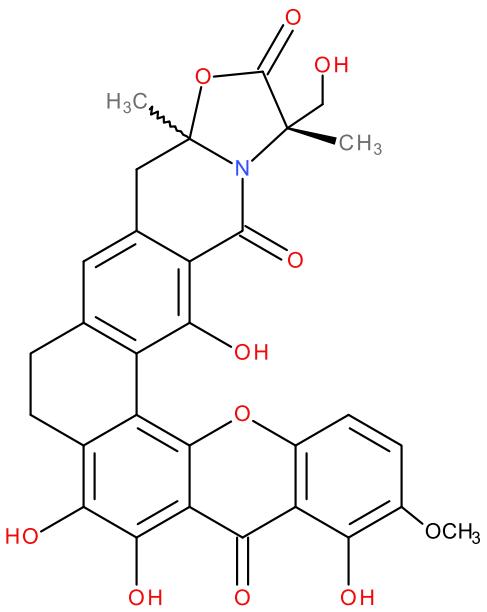
2.4.1. Examples of antibiotics extracted from marine bacteria

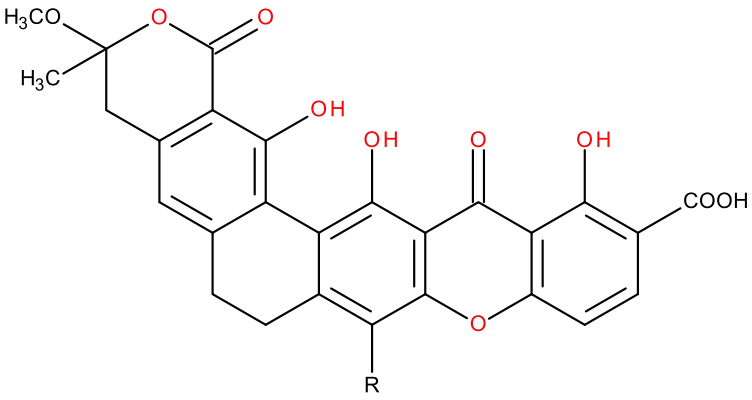
A substantial number of marine-derived antimicrobial metabolites are reported annually with varying minimum inhibitory concentration (MIC) values. In Table 2.1, a selection of these metabolites with MIC values < 10 μg/mL are listed. Their corresponding chemical structures are presented in Figure 2.6.

Table 2.1: Some of the bioactive compounds extracted from marine microorganisms

Compound name	Source	Bacterial test strains	MIC	Reference
Citreamicin θ A (**2.35**)	*S. caelestis*	*B. subtillis* 769 MRSA ATCC43300 *S. aureus* UST950701005	0.25 μg/mL 0.25 μg/mL 1 μg/mL	Liu *et al*., 2012
Citreamicin θ B (**2.36**)	*S. caelestis*	*B. subtillis* 769 MRSA ATCC43300 *S. aureus* UST950701005	0.25 μg/mL 0.25 μg/mL 1 μg/mL	Liu *et al*., 2012
Citreaglycon A (**2.37**)	*S. caelestis*	*B. subtillis* 769 MRSA ATCC43300	8 μg/mL 8 μg/mL	Liu *et al*., 2012
Dehydrocitreaglycon A (**2.38**)	*S. caelestis*	*B. subtillis* 769	8 μg/mL	Liu *et al*., 2012
Etamycin A (**2.39**)	*Streptomyces* sp	MRSA strains *S. pyogenes* *S. agalactiae*	1−2 μg/L 8 μg/L 8 μg/L	Haste *et al*., 2010
Actinomycin D (**2.40**)	*S. avermitilis*	*M. bovis* BCG *M. tuberculosis* H37Rv (ATCC27294)	0.2−0.5 μg/mL 1 - 8 μg/mL	Chen *et al*., 2012
Actinomycin X2 (**2.41**)	*S. avermitilis*	*M. bovis* BCG *M. tuberculosis* H37Rv (ATCC27294)	0.2−0.5 μg/mL 1 - 8 μg/mL	Chen *et al*., 2012
Anthracimycin (**2.42**)	*Streptomyces* sp	MRSA strains/VRSA *E. faecalis* ATCC29212 *S. pneumoniae* ATCC51916	≤ 0.25 μg/mL 0.125 μg/mL 0.0625 μg/mL	Hensler *et al*., 2014; Jang *et al*., 2013

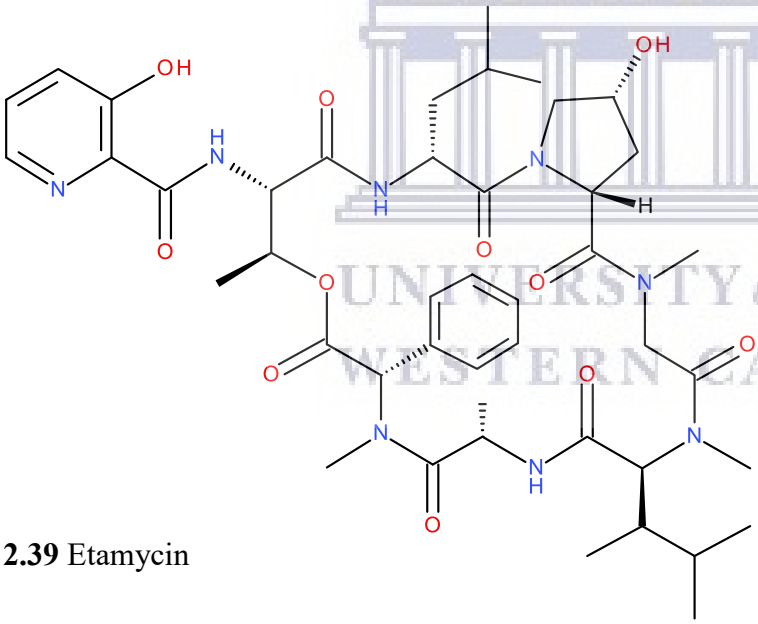
Marthiapeptide A (**2.43**)	*M. thermotolerans*	*M. luteus* *S. aureus* ATCC 29213 *B. subtilis* ATCC 6633 *B. thuringiensis*	2 µg/mL 8 µg/mL 4 µg/mL 2 µg/mL	Zhou *et al*., 2012
Caboxamycin (**2.44**)	*Streptomyces* sp	*B. subtilis*	IC_{50} = 8 µM	Hohmann *et al*., 2009
Lobophorin F (**2.45**)	*Streptomyces* sp	*S. aureus* ATCC 29213 *Enterococcus faecalis* ATCC 29212	8 µg/mL 8 µg/mL	Niu *et al*., 2011
Gageomacrolactin 1 (**2.46**)	*B. subtillis* sp	*B. subtilis* *E. coli* *P. aeruginosa* *S. aureus* *S. typhi*	0.01 µM 0.02 µM 0.01 µM 0.01 µM 0.02 µM	Tareq *et al*., 2013
Gageomacrolactin 2 (**2.47**)	*B. subtillis* sp	*B. subtilis* *E. coli* *P. aeruginosa* *S. aureus* *S. typhi*	0.06 µM 0.02 µM 0.03 µM 0.06 µM 0.02 µM	Tareq *et al*., 2013
Gageomacrolactin 3 (**2.48**)	*B. subtillis* sp	*B. subtilis* *E. coli* *P. aeruginosa* *S. aureus* *S. typhi*	0.06 µM 0.06 µM 0.03 µM 0.06 µM 0.03 µM	Tareq *et al*., 2013
Dragmacidin G (**2.49**)	*Spongosorites sp*	*S. aureus* *M. tuberculosis*	0.62 µg/mL 0.62 µg/mL	Wright *et al*., 2017
Penicibrocazine C (**2.50**)	*P. brocae*	*S. aureus* *M. luteus*	0.25 µg/mL 0.25 µg/mL	Meng *et al*., 2015



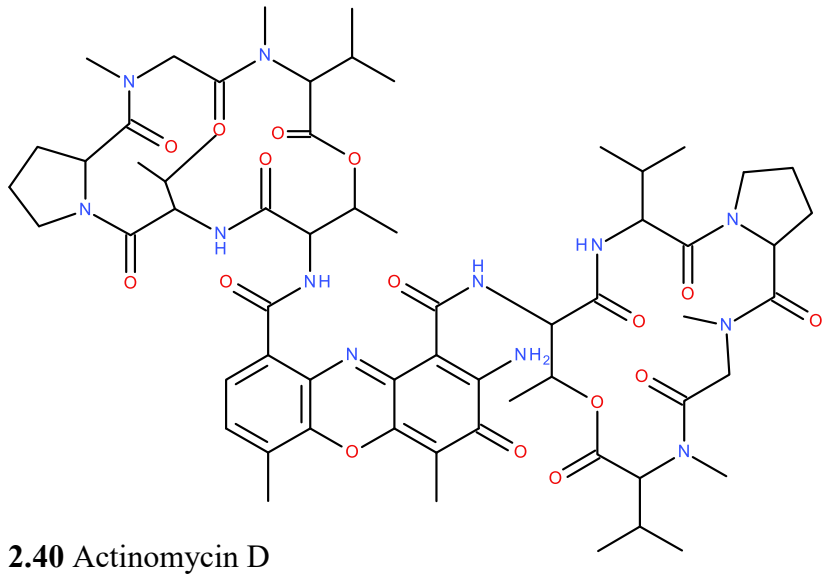


2.37 Citreaglycon A; R = OH
2.38 Dehydrocitreaglycon A; R= H

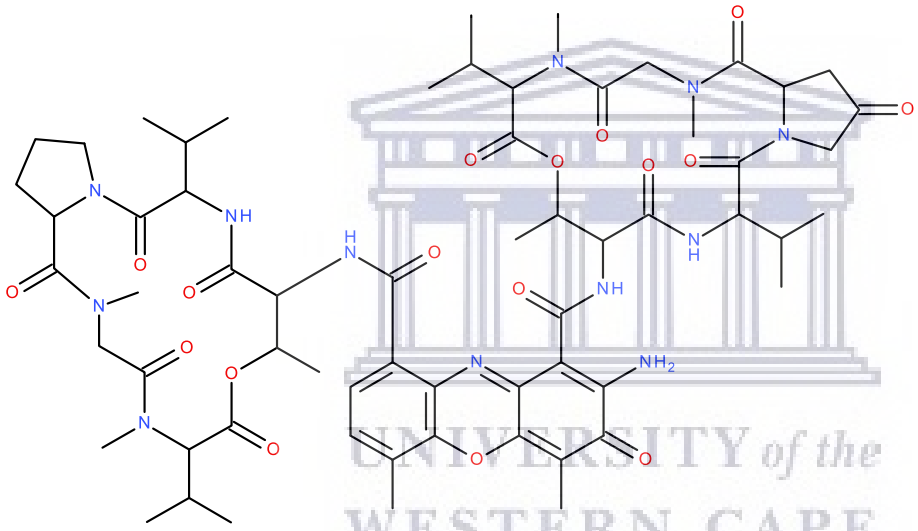
2.35 Citreamicin θ A(R)
2.36 Citreamicin θ B(S)



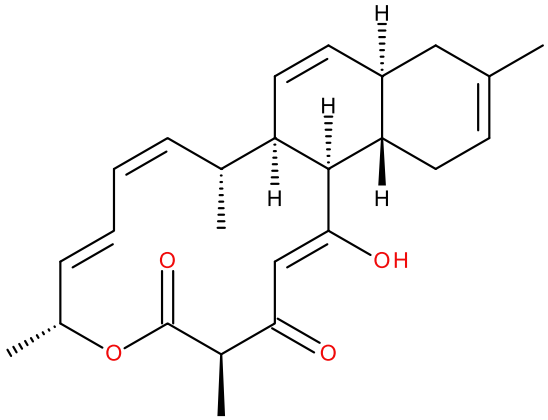
2.39 Etamycin



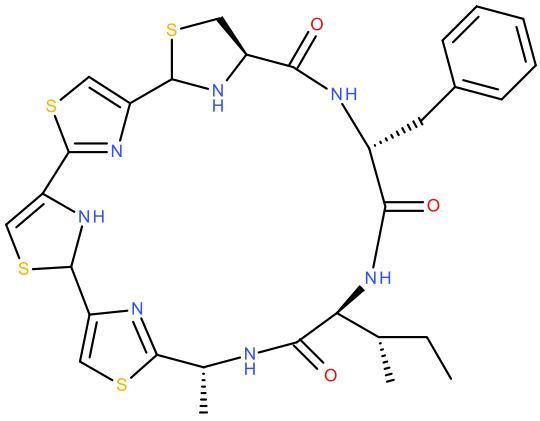
2.40 Actinomycin D



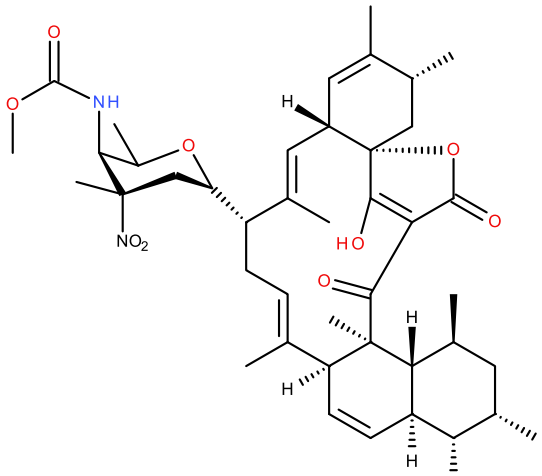
2.41 Actinomycin X2

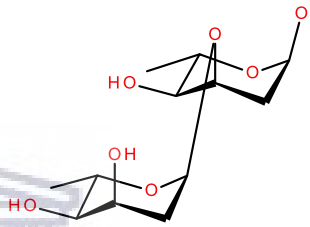


2.42 Anthracimycin

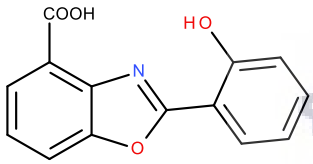


2.43 Marthiapeptide A

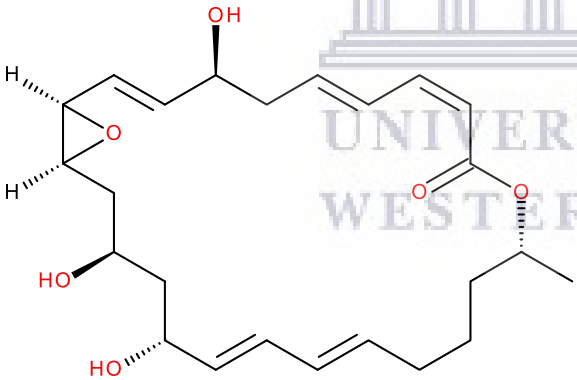




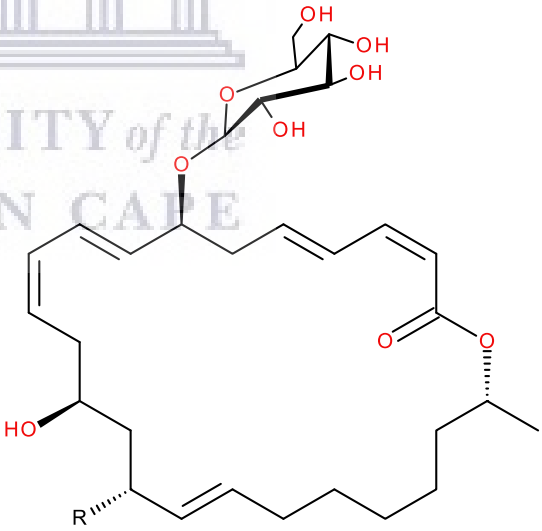
2.45 Lobophorin F



2.44 Caboxamycin

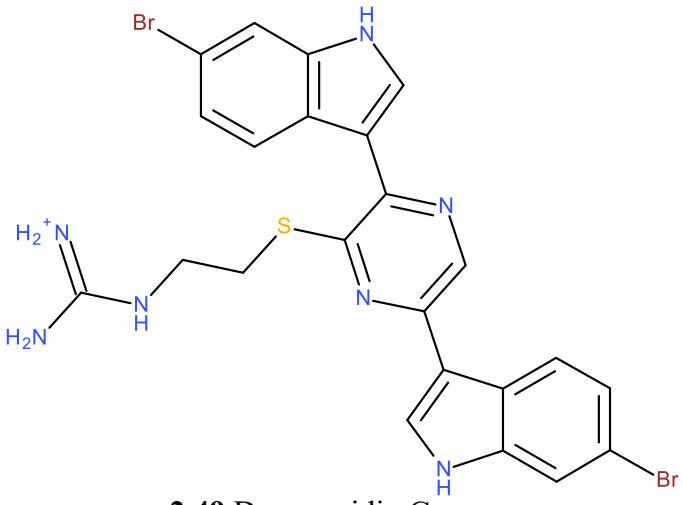


2.46 Gageomacrolactin 1

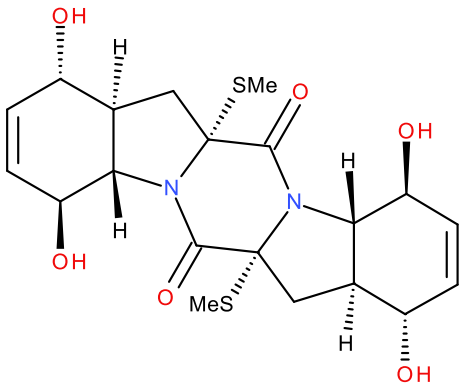


2.47 Gageomacrolactin 2; R = OCH_3

2.48 Gageomacrolactin 3; R = OH



2.49 Dragmacidin G



2.50 Penicibrocazine C

Figure 2.6. Selected examples of antibiotics from marine bacteria.

2.5. Limitations in the culturing of marine microorganisms

Although marine microorganisms are rich sources for biodiscovery, there are limitations associated with acquisition of chemical leads from these organisms. A major constraint is cultivation of some of the microorganisms. These limitations include;

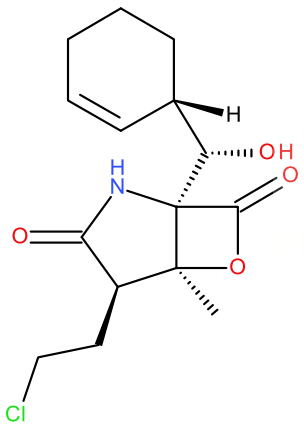
Quorum sensing: This involves cell-to-cell communication through production of signalling molecules that diffuse amongst cells. Diffusion mechanism of signalling molecules determines a bacterium's ability to use quorum sensing (QS) constitutively, enabled by three inherent characteristics such as secreting a signalling molecule, detecting the change in its concentration and regulating gene transcription. It should be noted that molecular diffusion is a function of cell density. In the marine environment, the most common QS class of compound produced by *Proteobacteria* is acylated homoserine lactones (AHLs) ((Lazdunski *et al*., 2004; Eberhard *et al*., 1981).

Metabolic consortia: Another difficulty may be attributed to metabolic consortia. A microbial consortium involves production of an intermediate through metabolism of one microbial species which in turn can be further metabolised by another microbial species. This phenomenon was demonstrated by distributing metabolic pathways among *Escherichia coli* and *Saccharomyces cerevisiae* and through co-culture of the engineered organisms to produce taxanes, sesquiterpenes and tanshinone precursors (Zhou *et al*., 2015). Problems associated with laboratory culturing of abundant bacteria includes disruption of the natural interactions that takes place between the microbes. For instance, the fastest growing species may overpower the slower

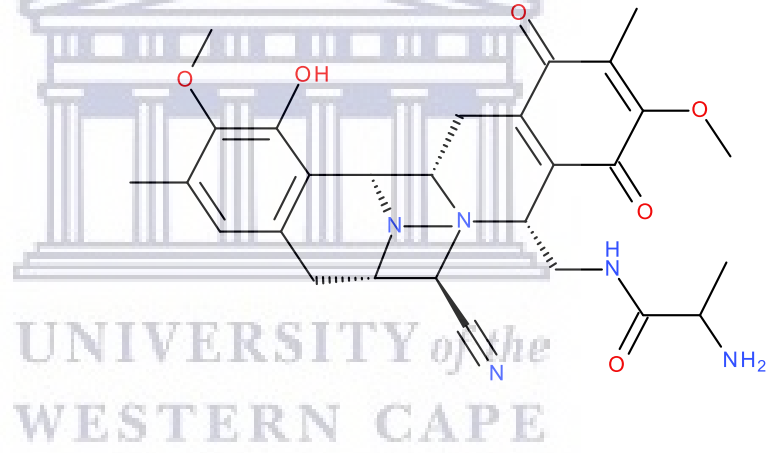
cultured dividing species due to favourable growth conditions. In other instances, the cells may become inactive due to inhibitory compound production by other microbes.

Another common limitation is that marine microbes may be incapable of growing in the available nutrient substrate due to lack of knowledge of the appropriate growth temperature, nutrient concentrations and substrate combinations. These factors may hinder successful laboratory culturing of marine microbes.

Despite the challenges, a number of culturable strains with bioactive compounds have been reported. For example, as shown in Figure 2.7 salinosporamide A (**2.41**) was isolated from a marine sediment-derived actinomycete culture, *Salinispora tropica* (Fenical *et al*., 2009). The marine metabolite anti-cancer drug, trabectedin's supply is dependent on initial fermentation of the starting material cyanosafracin B (**2.42**) from *Pseudomonas fluorescens*, and subsequent semi-synthetic steps to the final products (Cuevas and Francesch, 2009).



2.51 Salinosporamide A



2.52 Cyanosafracin B

Figure 2.7: Chemical structures of salinosporamide A **2.51** and cyanosafracin B **2.52**.

2.6. Genome- guided drug discovery

The advancement in analytical tools coupled with biosynthetic genetic engineering have made it possible to identify potential bioactive secondary metabolite producers. These tools for example, antibiotics & Secondary Metabolite Analysis Shell (antiSMASH), NaPDoS and ClusterFinder have enabled *in silico* characterisation of putative secondary metabolite biosynthetic gene clusters (BGCs) and pathways (Blin *et al*., 2013; Medema *et al*., 20110; Cimermancic *et al*., 2014; Ziemert *et al*., 2012). This bioinformatics aided approach facilitates the genome mining process through which an unknown natural product biosynthetic gene cluster in an organism is identified and characterised by genome sequencing analysis (Corre and Challis, 2009). This can inform on

possible detection and isolation strategies of the natural product encoded by the BGCs that may be responsible for bioactivity (Ziemert *et al*., 2016). This technique has been used in the discovery of several bioactive metabolites including antibiotics such as paenimacrolidin and elgicins (Wu *et al*., 2011; Teng *et al*., 2012).

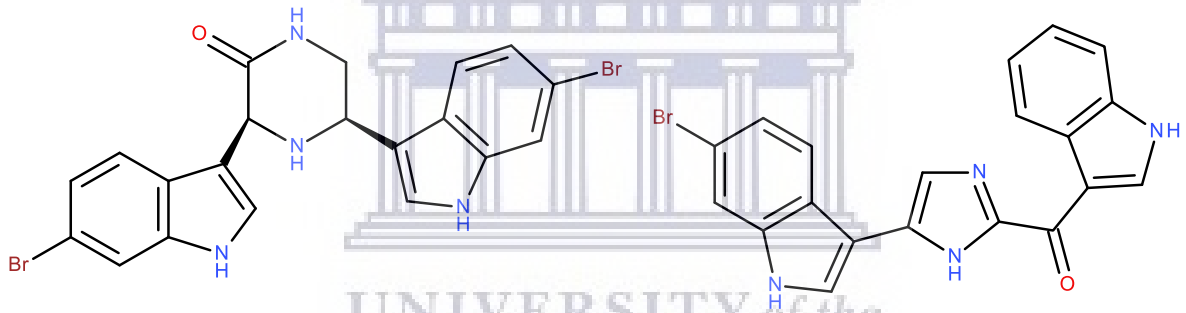
2.7. Techniques used in the bioactivity assessment of lead compounds for drug discovery

An example of a commonly used assay technique for bioactivity assessment of lead compounds for drug discovery is bioautography. There are three types of bioautographic techniques namely; direct, contact and agar overlay or immersion. However, the most commonly used is the direct bioautography method (Choma and Grzelak, 2011). It involves the application of a bacterial suspension on a developed thin-layer chromatography plate, subsequent incubation of the plate in humid and conducive temperature, then, the plate is sprayed with dehydrogenase reagent such as tetrazolium salt. Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes reduces the tetrazolium salt to formazan facilitated by dehydrogenase of living microorganism for the visualisation of the inhibition zone. The result is a clear spot which appears against the purple background of the TLC plate indicative of bioactivity. This method is advantageous because of its simplicity, reproducibility, rapidity and high sensitivity. Contact bioautography requires the transfer of bioactive compounds from a TLC plate onto an inoculated agar surface to enable diffusion. Following diffusion, the agar layer is incubated which results in the presence of inhibition zones in areas where the active compounds were in direct contact with the agar layer. Agar overlay or immersion bioautography involves immersion of the TLC plate in agar medium. Thereafter, the seeded agar medium together with the test microorganisms are incubated for diffusion purposes and subsequent detection of bioactive compounds. The latter method is a fusion of contact and direct bioautography (Islam *et al*., 2003). Bioautography is an effective assay for rapid detection of biological active substances in complex mixtures as it enables direct localisation of the active compounds. The zones of activity are then targeted for the isolation of the active constituents. The assay is useful in avoiding isolation of inactive compounds which is time consuming and tedious (Suleimana *et al*., 2009).

2.8. South African coastline

South Africa is located on the southern tip of the African continent and presides over a vast coastline of approximately 3650 km (Lombard, 2004). The coastline is particularly unique because it is surrounded by two oceans stretching from the warm Indian Ocean in the east to the cooler Atlantic Ocean in the west (Branch *et al*., 2017). The merging of the two currents has consequently

bring about marine diversity in the region. The South African coastline has been dubbed marine biodiversity 'hotspot' with a plethora of endemic flora and fauna in the marine and terrestrial coastline environment (Le Roux, 2002; Davis-Coleman and Beukes, 2004). At least 12914 marine species have been documented (Griffiths *et al*., 2010). There are several documented marine species some of which include algae, sponges and tunicates that are endemic to South Africa. This has made the coastline the ideal niche for natural product discovery. Examples of reported bioactive compounds shown in Figure 2.8 include cis-3,4-dihydrohamacanthin B (**2.53**) and bromodeoxytopsentin (**2.54**) that were identified from the South African marine sponge *Topsentia pachastrelloides* and displayed antibacterial activity against MRSA 252 at MIC values of 12.5 and 6.25 µg/mL, respectively (Zoraghi *et al*., 2011). South African marine has been established as a rich source of natural products based on the endemicity and diversity of the organisms. This study examined bacterial isolates from sponges and tunicates as well as algae that were collected off the Algoa and KwaZulu Natal coast of South Africa, respectively.



2.53 cis-3,4-Dihydrohamacanthin B **2.54** Bromodeoxytopsentin

Figure 2.8: Chemical structures of cis-3,4-Dihydrohamacanthin B **2.53** and Bromodeoxytopsentin **2.54**.

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Chapter 3

Screening of extracts from marine algae and bacterial isolates for antibacterial activities: A contribution to the development of a natural products library

3.1. Introduction

A natural product library is a repository containing series of stored chemically diverse compounds used in high-throughput screening. Based on the challenges associated with lead compound discovery such as limited availability of resources, expenses and requiring significant amount of time, a chemical library is generated to expedite and improve the process (Kingston, 2010). In addition, library establishment of natural compounds assists in filtering compounds with complex physicochemical properties and problematic functionalities including issues pertaining to solubility in carrier and assay solvents at applicable concentrations. Usually, the library compounds are tested for bioactive 'hits' against multiple disease models or drug targets. Once a 'hit' has been identified based on activity and reproducibility, an alternative means of producing the desired compound by synthetic means may be considered. Such libraries may consist of purified natural products, extracts, fractions and synthetic or semisynthetic analogues (Dandapani *et al*., 2012).

This chapter describes the development of a small scale natural products library of 38 marine algal extracts from the Marine BioDiscovery (MBD) collection at the University of the Western Cape (representing the total number of algae available at the beginning of this study). The extracts from the algae were screened for activity against gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591. Several algae have been reported to produce bioactive antimicrobial compounds and are sources of novel chemical and pharmacological agents (El Gamal, 2010). This provided the rationale for the selection of algae for this study. In addition to the search for antibacterial leads from the algae, six selected marine bacteria extracts were also screened. The bacteria were previously isolated from marine invertebrates and have been documented in the database at the Institute for Microbial Biotechnology and Metagenomics (IMBM) at the University of the Western Cape as active against *M. tuberculosis* H37Ra. The bacterial isolates that were used in this study were supplied by IMBM.

3.2. Experimental

3.2.1. General experimental procedures

All solvents that were used for extraction were redistilled before use. Deuterated chloroform, dichloromethane, ethyl acetate, hexane and methanol and silica gel 60 were purchased from Merck (Darmstadt, Germany). Silica gel 60 (0.040-0.063mm) was obtained from Merck KGaA (Germany). Diaion® HP20SS was purchased from Supelco (USA). NMR data were recorded on a Bruker Avance spectrometer at 400 MHz in deuterated chloroform. Chemical shifts were measured in parts per million (ppm) and referenced to undeuterated solvent signals at δ_H 7.26. Other reagents including bacteriological agar, D-glucose, calcium carbonate, calcium chloride, magnesium chloride, malt extract, peptone, sodium chloride and yeast extract were purchased from Merck (Darmstadt, Germany). Middlebrook 7H9 and potassium chloride were obtained from Sigma-Aldrich (USA).

3.2.2. Collection of marine algae

The algae samples (Table 3.1), collected over a period of five years from the South African coastline, are part of the Marine Biodiscovery (MBD) collection hosted at the School of Pharmacy, University of the Western Cape. This collection includes five red (*Portieria*, *Laurencia*, *Plocamium*, *Hypnea*, *Heringia*), four brown (*Sargassum*, *Dictyota*, *Stypopodium*, *Zonaria*) and two green (*Bryopsis*, *Codium*) algae genera. The algae were stored at -20 °C following collection. Algae identification was done by Professor John J. Bolton at the University of Cape Town, South Africa. Taxonomic reference samples of algae were deposited at the Bolus Herbarium and the MBD collection (See Table 3.1).

3.2.3. Extraction of the algae

A total of 38 frozen algae stored at -20 °C were thawed, and approximately 2 g of each alga was macerated in 9 mL methanol (MeOH) overnight at 4 °C in a 15 mL centrifuge tube. Following overnight extraction, the extracts were sonicated for approximately 10 minutes. The MeOH extracted algae were filtered using cotton wool and decanted through a funnel into pre-weighed 30 mL polytop glass vials. The extracts were dried using a R-210 rotary evaporator (BUCHI, Switzerland) and subsequently placed in a desiccator for complete dryness. Thereafter, the algae were soaked in 9 mL dichloromethane-methanol (2:1) in 15 mL vials overnight at 4 °C for further extraction after which the above step was repeated. The algae were again extracted twice with DCM-MeOH (2:1), combined with the previous MeOH extract, dried and weighed. The masses

were reported in mg/g dry weight. The dried extracts were reconstituted in 0.5 mL chloroform-D1 ($CDCl_3$) and ^1H NMR spectra obtained (Bruker, Germany). ^1H NMR spectroscopy analysis was performed using Bruker TopSpin 3.5 pl7 software. The analysed extracts were decanted back into the vials, dried and stored at -20 °C for future use.

3.2.4. Thin-layer bioautography of crude algae extracts

Crude algal extracts were diluted from a concentration of 10 mg/mL to a concentration of 1 mg/mL in DCM. Thereafter, 20 µg of each extract was spotted on a 1x1 cm square-partitioned blocks on 20x10 cm TLC silica gel plate in duplicate. Four antibiotic standards (10 µL), ampicillin, chloramphenicol, cycloheximide and vancomycin with concentrations of 10 mg/mL were spotted on each silica TLC gel plates as positive controls and 20 µg of DCM was used as a negative control. Sterile cotton wool swab was used to apply MRSA 33591 onto the silica gel plates. The plates were incubated at 37 °C in an enclosed plastic container containing moist paper towel for 24 h. Thereafter, the plates were treated with MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolium dye) and re-incubated for 3 h, then evaluated for the appearance of purple spots (indicative of organism growth) or white spots (indicative of organism growth inhibition).

3.2.5. Marine bacteria used in this study

Six marine bacteria code-named PE05-99, PE05-143, PE06-104, PE08-57, PE14-28 and PE14-104 were obtained from the IMBM library. These bacteria have previously been shown to inhibit *M. tuberculosis* H37Ra established within the PharmaSea consortium of which IMBM was a partner. The bacterial isolates PE05-143, PE14-28 and PE14-104 were selected for further study based on their antimycobacterial activity. The isolates PE05-99, PE06-104 and PE08-57 were excluded from the study due to lack of bioactivity.

3.2.6. Culturing of bacteria

3.2.6.1. Preparation of Middlebrook 7H9 broth and agar

Middlebrook 7H9 broth was prepared by suspending 2.35 g of Middlebrook 7H9 broth base in 450 mL of distilled water in an autoclavable container while the agar medium was Middlebrook 7H9 broth supplemented with 10 g bacteriological agar. Thereafter, 1 mL of glycerol was added to the broth and agar media. In a separate container, 10 g of D-glucose was dissolved in 100 mL of distilled water. Sterilisation of the broth, agar and D-glucose was then carried out by autoclaving

at 121 °C for 15 min. The media were left to cool at 55 °C before aseptically adding 50 mL of D-glucose into Middlebrook 7H9 broth and agar. The agar medium was poured into Petri dishes and stored together with the broth at 4 °C until needed.

3.2.6.2. Preparation of GYM streptomyces broth and agar

GYM *streptomyces* broth was prepared with 0.4% D-glucose, 0.2% calcium chloride, 0.53% magnesium chloride, 1% malt extract, 0.07% potassium chloride, 2,4% sodium chloride, 0.4% yeast extract, pH 7.2 while GYM *streptomyces* agar was GYM *streptomyces* medium supplemented with 1.5% bacteriological agar and 0.2% calcium carbonate, pH 7.2. The broth and agar were autoclaved at 121 °C for 30 min at 15 psi of pressure. Thereafter, the broth was placed on the workbench and left to cool at room temperature for further use. The agar was cooled at 55 °C, then poured into Petri dishes and stored at 4 °C for future use.

3.2.6.3. Preparation of Zobell marine broth and agar

Zobell marine broth was prepared with 0.0075% calcium chloride, 0.2% magnesium chloride, 0.375% peptone, 0.0525% potassium chloride, 1.8% sodium chloride, 0.125% yeast extract, pH 7.5 while Zobell agar was Zobell medium supplemented with 1.5% bacteriological agar, pH 7.5. The broth and agar were autoclaved at 121 °C for 30 min at 15 psi of pressure. The agar was subsequently left to cool at 55 °C, then decanted into Petri dishes and stored at 4 °C until required.

3.2.6.4. Culturing of MRSA 33591 test strain

Luria-Bertani (LB) broth consisting of 0.5% tryptone, 0.5% sodium chloride, 0.25% yeast extract in 500 mL distilled water at pH 7.0 was prepared and autoclaved at 121 °C for 30 min. MRSA ATCC 33591 was inoculated into 5 mL LB broth and incubated at 37 °C for 24 h, shaking at 160 rpm. The 5 mL cell culture was diluted into 250 mL LB broth and incubated as per the afore-mentioned growth parameters. The optical density (OD) of the culture for use in TLC-bioautography was measured at 600 nm and the culture OD was adjusted to 0.5 for TLC-bioautography.

3.2.6.5. Culturing of M. aurum A+ test strain

The *M. aurum* A+ was inoculated into 5 mL of Middlebrook 7H9 broth and incubated at 37 °C for 72 hours, shaking at 160 rpm. The 5 mL cell culture was diluted into 250 mL Middlebrook 7H9

and grown at the same growth parameters. The optical density of the culture was measured at 600 nm and the culture OD was adjusted to 0.5 for TLC-bioautography.

3.2.7. Fermentation for the extraction of the bacterial isolates

The isolates PE05-143, PE14-28 and PE14-104 were streaked on separate Zobell agar plates and incubated for two weeks at 15 °C. A single colony of each isolate was inoculated into two separate 5 mL GYM broths and grown shaking at room temperature overnight. The one overnight 5 mL cell culture was diluted into 250 mL GYM broth and grown at room temperature for 14 days shaking at 150 rpm. The remaining 5 mL cell culture was stored at -80 °C in aliquots containing 15% glycerol for long-term strain archival. Furthermore, and in order to achieve higher biomass yields, the isolates were grown at 15 °C in a litre GYM broth using a bioreactor at 400 rpm for 7 days.

3.2.8. Extraction of metabolites from the bacterial cell culture

The cell culture of isolates PE05-143, PE14-28 and PE14-104 were decanted into a sterile flask. Diaion HP20SS (1 g) was added to the cell culture, placed on a shaker at 150 rpm for 24 h. Thereafter, the cell culture was harvested by centrifugation at 6000x*g* for 30 minutes at 15 °C. The supernatant and pellet with the adsorbed Diaion HP20SS were separated for subsequent extraction. The extraction of metabolites from bacterial fermentations was carried out as described by Ong *et al*., (2018) with slight modifications. Briefly, the pellet with the adsorbed Diaion HP20SS was extracted with 100 mL methanol, and the supernatant with 100 mL ethyl acetate using separating funnels. The extracts were collected in separate round bottom flasks and dried using a rotary evaporator (Buchi, Switzerland). The dried extracts were reconstituted in deuterated chloroform and analysed by ^{1}H NMR spectroscopy (Bruker, Germany). Ultimately, the bacterial isolates supernatant extracted with ethyl acetate were further investigated for bioactivity following a well diffusion activity screening of the isolates cell culture. The bacterial isolates PE05-143, PE14-28 and PE14-104, from the ethyl acetate extracts yielded 29.3 mg, 211.9 mg, and 23.8 mg, respectively.

3.2.9. Sloppy-agar overlay

A colony of *M. smegmatis* LR222 was used to inoculate 5 mL of Middlebrook 7H9 broth. The cell culture was grown for 72 h in a shaking 37 °C incubator. Following incubation, the optical density was measured at 600 nm. The cell culture was pipetted into 6 mL sloppy-agar tubes and gently

vortexed. Finally, sloppy-agar containing the cell culture was distributed evenly over the entire surface of GYM-growing bacterial isolates agar plate. Thereafter, a clear zone surrounding the bacterial isolates which represented the zone of inhibition was observed. *M. smegmatis* was initially used as a test organism for sloppy-agar overlay assays because of availability. However, following a change in laboratory *M. aurum* was used as a substitute for MIC determination for TLC-bioautography assays.

3.2.10. Thin-layer bioautography of the bacterial isolates

Following the extraction of the metabolites from the bacterial cell culture as described in section 3.2.8, the extracts were prepared to a concentration of 10 mg/mL in DCM. The TLC-bioautography assay of the extracts were then carried out using MRSA 33591 and *M. aurum* A+ test strains as described in section 3.2.4.

3.3. Results and discussion

3.3.1. Extraction of algae

The frozen algae were allowed to thaw at room temperature, washed with fresh water and extracted with MeOH and CH_2Cl_2-MeOH (2:1) at 4 °C. The extracts were dried under reduced pressure using a rotary evaporator. The yield of extract from the algae, milligram per gram varied considerably ranging from 5.2 mg/g (dry weight) in D1242C to 441.4 mg/g in SB20140302-3 (See Table 3.1). According to Zhang *et al*. (2018), yields of extracts depends on a number of factors namely, the solvent of extraction, the polarity of the combination of the extractants, the method of extraction and the temperature. In this work, combinations of methanol and dichloromethane were used. The experimental set up has extracted more from SB20140302-3 (*Dictyota naevosa*) than in any other alga. Table 3.1 shows a list of 38 algae that were extracted and the yield of extract per alga. The small scale library of the marine algae extracts that were generated is shown in Figure 3.1.

Table 3.1: Yield of crude extract per alga

Collection code	Yield extract (mg)	Yield per g of alga (mg/g)	MBD code	Species
D1242C	10.4	5.2	D1242C	*Portieria hornemannii*
DJ20140301-1	35.1	17.5	DJ140301-1	*Portieria hornemannii*
DJ20140301-2	12.5	6.2	DJ140301-2	*Bryopsis* sp

DJ20140301-3	23.4	11.7	DJ140301-3	*Laurencia* sp
DJ20140301-4	44.7	22.3	DJ140301-4	*Sargassum* sp
GC180104-1	94.7	48.8	GC180104-1	*Plocamium* sp
GC180104-2	38.7	19.3	GC180104-2	Unidentified
GC180104-3	93.6	46.8	GC180104-3	*Dictyota* sp
GC180104-4	72.4	36.2	GC180104-4	*Plocamium* sp
GC180104-5	101.2	50.6	GC180104-5	Unidentified
GC180104-7	70.8	35.4	GC180104-7	*Plocamium* sp
KZN13104-1	11.5	5.5	KZN131004-1 (*Laurencia* sp)	*Laurencia* sp
KZN13107-1 (*L. pumila*)	24.2	12.1	KZN131007-1 (*L. pumila*)	*Laurencia pumila*
KZN1311-1 (*L. sodwaniensis*)	32	16	KZN1311-1 (*L. sodwaniensis*)	*Laurencia sodwaniensis*
SB20130303-1	47.9	23.9	PB141122-1	*Stypopodium mulitpartitum*
SB20140302-1	180.5	90.2	SB140302-1	*Laurencia flexuosa*
SB20140302-3 (*D. naevosa*)	882.9	441.4	SB140302-2 SB140302-3	*Hypnea spicifera* *Dictyota naevosa*
SB20140302-4	60.3	30.1	SB140302-4	*Zonaria subarticulata*
SB20140302-5	59.1	29.5	SB140302-5	*Heringia mirabilis*
SB20140302-6	11.3	5.6	SB140302-6	*Callythamnon stiposum*
SB20141121-2	40.9	20.4	SB140303-1	Unidentified
SB20141122-1	561.2	280.6	SB141122-1	Unidentified
TS090311-1	45.5	22.7	TS090311-1	*Laurencia elata*
UWC20180112-1	18.5	9.2	UWC180112-1	*Portieria hornemannii*
UWC20180112-10	22.1	11	UWC180112-10	*Codium* sp
UWC20180112-11	14.4	7.2	UWC180112-11	*Sargassum incisifolium*
UWC20180112-12	52.2	26.1	UWC180112-12	Unidentified
UWC20180112-13	40	20	UWC180112-13	*Laurencia* sp
UWC20180112-14	79.6	39.8	UWC180112-14	*Laurencia* sp
UWC20180112-15	26.2	13.1	UWC180112-15	*Laurencia* sp
UWC20180112-2	33.6	16.8	UWC180112-2	*Laurencia* sp
UWC20180112-3	65.2	32.6	UWC180112-3	*Plocamium corallorhiza*
UWC20180112-4	130.2	65.1	UWC180112-4	Unidentified
UWC20180112-5	39.2	19.6	UWC180112-5	*Sargassum incisifolium*
UWC20180112-6	63.2	31.6	UWC180112-6	*Sargassum incisifolium*
UWC20180112-7	90.5	45.2	UWC180112-7	*Sargassum* sp

UWC20180112-8	51.4	25.7	UWC180112-8	*Sargassum* sp
UWC20180112-9	20	10	UWC180112-9	Unidentified

Figure 3.1: A library of dried 38 marine algae extracts.

3.3.2. Antibacterial activity of the algae extracts

From the 38 algae screened, 24 crude extracts showed inhibitory activities against MRSA. The active extracts were represented by the presence of clear spots on the TLC plates (Figure 3.2). This suggests that the active extracts contain bioactive compounds that effectively inhibited the growth of MRSA. The distinct zone of inhibition of the active extracts are represented by the well-defined clearer zone of inhibition and relatively bigger size of the zones as compared to the positive controls (chloramphenicol and vancomycin). These active extracts appear to be effectual in killing MRSA than the positive controls as the size and intensity of the controls were less apparent though effective. Similar observations were made by Jassbi *et al*. (2013) who used TLC bioautography to determine the antibacterial activity of extracts of red algae, *Hypnea flagelliformis*, and two brown algae, *Cystoseira myrica* and *Sargassum boveanum* on *S. aureus*. The 14 algal extracts that did not exhibit activity stained purple. The lack of bioactivity in these extracts does not preclude that they may be active in synergistic or in additive interactions. Similar observations were made by Suleimana *et al*. (2010) who used TLC bioautographic procedure to screen hexane, acetone, dichloromethane and methanol extracts of seven South African plants. Also, in this study chloramphenicol and vancomycin antibiotics impeded MRSA growth. According to Wang *et al*., (2018) chloramphenicol is known to inhibit bacterial protein synthesis by disrupting peptide bond formation by binding to A2451 and A2452 in the 23S rRNA of the 50S ribosomal subunit whereas

vancomycin inhibits bacterial cell wall synthesis by binding to the D-Ala-D-Ala of polymers of N-acetylmuramic acid and N-acetylgucosamine which are the cell wall formation core strands.

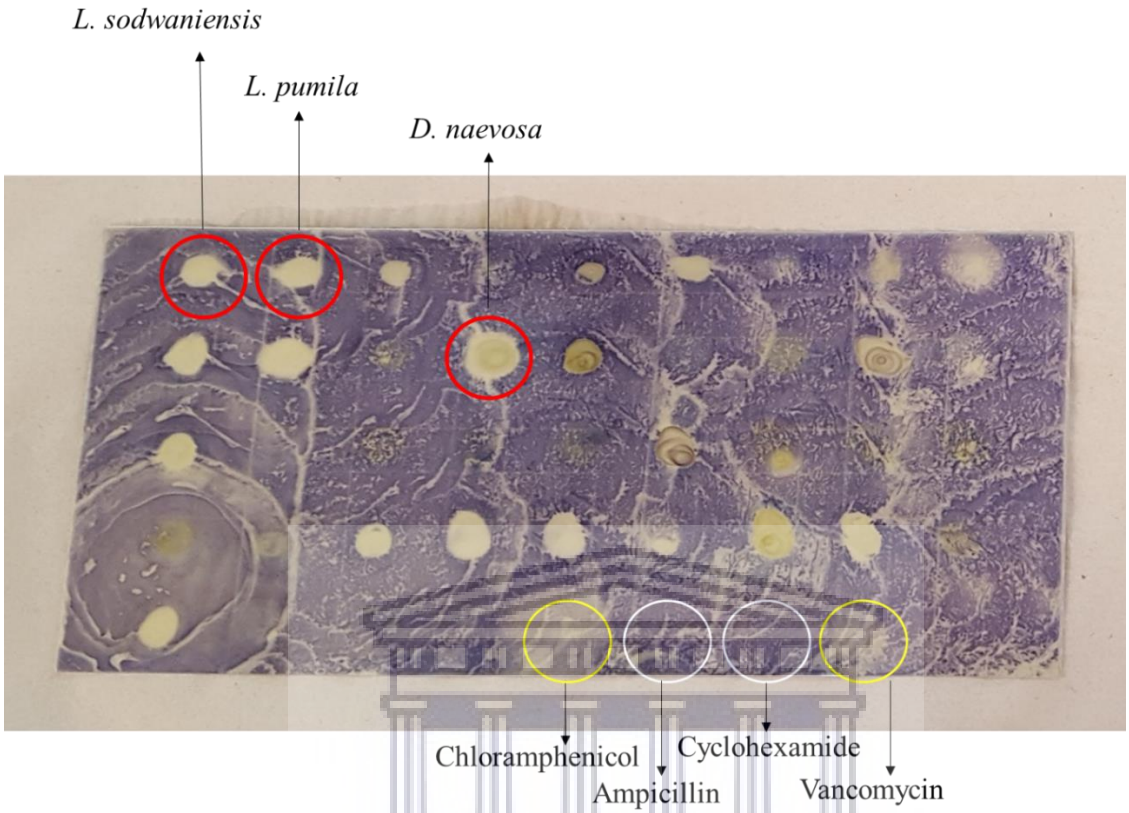

Figure 3.2: The thin-layer bioautography plate of the tested crude algae extracts showing the zones of inhibition from the 24 active species. The zones for *L. sodwaniensis, L. pumila* and *D. naevosa* are highlighted in red circles. The zones of inhibition from the two controls that showed activity against MRSA namely; chloramphenicol and vancomycin are highlighted in yellow circles. The negative controls, that is, ampillicin and cyclohexamide are highlighted in white circles.

A representative of the beta lactam penicillin group, ampicillin, was also used along with the antifungal cyclohexamide as negative controls. Ampicillin showed no activity against MRSA (Figure 3.2). This was probably because MRSA secretes beta-lactamases in the cell wall that cleave the beta-lactam ring of ampicillin and through acquisition of penicillin-binding protein 2A (PBP2A) capable of cell wall reformation with low affinity for beta-lactams (Guignard *et al*., 2005). According to Lim and Strynadka (2002), this property of some bacteria has been the basis for their current resistance to some drugs. Ampicillin was unable to inhibit MRSA growth which would mean that beta lactams in general would not be active against MRSA thus emphasising the issue of resistance of infectious bacterial commonly prescribed antibiotics. Since cyclohexamide is an antifungal it was not expected to have an activity against MRSA (Yin *et al*., 2018).

3.3.3. ^{1}H NMR profiling of the metabolites in the extracts

Chemical profiling by ^{1}H NMR spectroscopy was performed on the 38 algae extracts to give an indication of the chemical components or the types of metabolites present within the crude algae extracts. Figure 3.3 represents the selected ^{1}H NMR spectra of the crude algae extracts that was analysed using TopSpin 3.5. The array of peaks between 0 – 2 ppm is generally indicative of the presence of fatty acids and sterols which are often more prominent than other metabolites within the extracts (Knothe, 2003). Moreover, the spectra in particular *L. sodwaniensis* showed interesting mix of multiplets between 2 and 5 ppm which is indicative of a potential electronegative groups in the structure of metabolites present.

In literature, the genera *Dictyota* and *Laurencia* have been reported to possess natural products which are responsible for diverse bioactivities (Ioannou *et al*., 2013; Da-Silva-Machado *et al*., 2014). On this basis, *D. naevosa*, *L. pumila* and *L. sodwaniensis* were selected for further chemical and structural studies on the basis of their effective antimicrobial activities and interesting ^{1}H NMR spectrum. Next is the isolation and detailed analyses of 2D NMR spectroscopy data which will enable the identification of the active compounds present in the crude extracts.

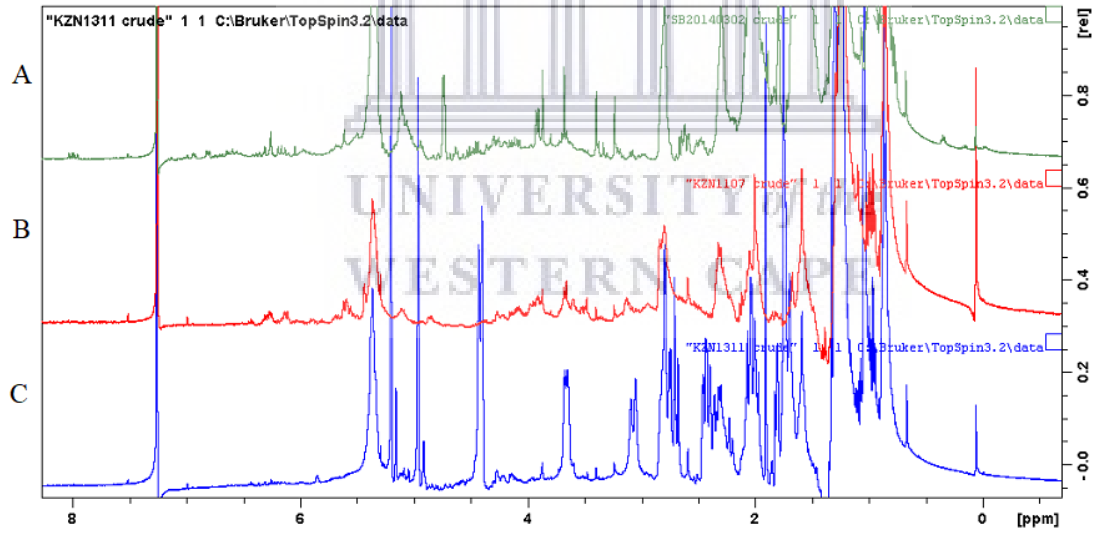

Figure 3.3: ^{1}H NMR spectroscopy of crude algae extracts. **(A)** *D. naevosa* **(B)** *L. pumila* **(C)** *L. sodwaniensis.*

3.3.4. Testing for bioactivity of the extracts from the bacterial isolates

The results from the screening of the ethyl acetate extract of the six bacterial isolates has revealed that the secondary metabolites only three of the isolates extracts inhibited the growth of *M. smegmatis*. This is represented by the clear inhibition zones around the inoculated isolates (Figure

3.4). The inhibitory activity is likely linked to a defect in the biosynthesis of the cell wall barrier of *M. smegmatis*. According to Lui and Nakaido *et al.* (1999), it was established that due to defective mutants in the mycolic acid biosynthesis, an increased uptake and sensitivity to antibiotics was observed, causing the antibiotics to permeate the cell wall barrier. This is interesting because, it has validated the initial aim of the study, which was to investigate possible antibacterial compounds from these marine bacteria. In addition, *M. aurum* A+ was also used as a test strain and as a substitute for *M. tuberculosis* because it has mycolic acids in the cell wall which are similar to those of *M. tuberculosis* (Belisle *et al*., 1997). *M. aurum* is also a fast-growing microbe with low pathogenicity (Phelan *et al*., 2015). However, the extracts from the marine bacterial isolates did not show a clear inhibition of *M. aurum* growth (Figure 3.5) suggesting that the mycolic acid in the *M. aurum* cell barrier makes the organism resistant to the effectiveness of the antibiotic (Gebhardt *et al*., 2007). While PE05-143 showed slight activity against *M. aurum* A+, PE14-28 and PE14-104 were not active against the test strain even at high concentration of 10 mg/mL. Vancomycin which served as a positive control showed clear inhibition. Distilled water was used as a negative control thus showed no activity.

The three bacterial isolates prevented MRSA growth as shown in Figure 3.6. The potency of the extracts is represented by the clear white spots on the TLC plates. Vancomycin served as a positive control hence MRSA growth was inhibited. Distilled water was used as a negative control thus there was no activity as MRSA growth is stained purple.

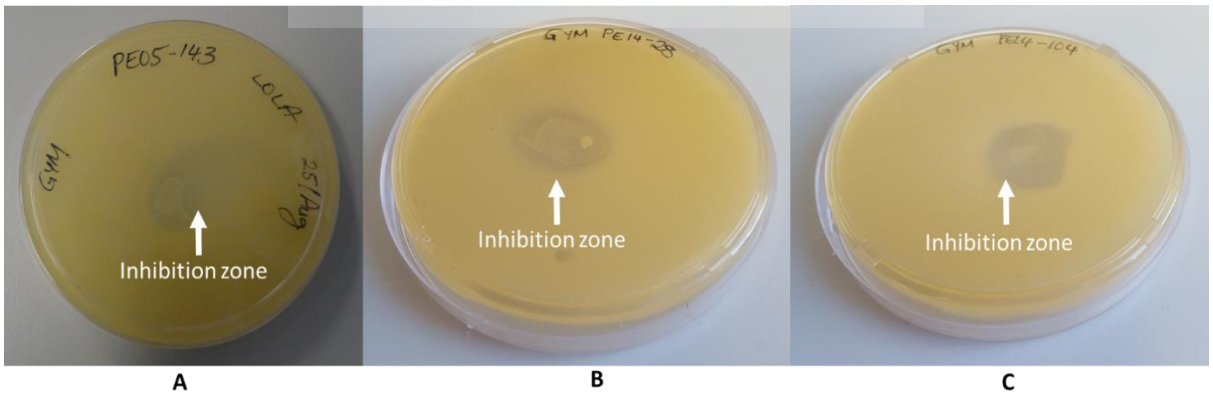

Figure 3.4: Sloppy-agar overlay of isolates with *M. smegmatis*. **(A)** PE05-143 **(B)** PE14-28 **(C)** PE14-104. The white arrows represent the inhibition zones produced by metabolites from the isolates.

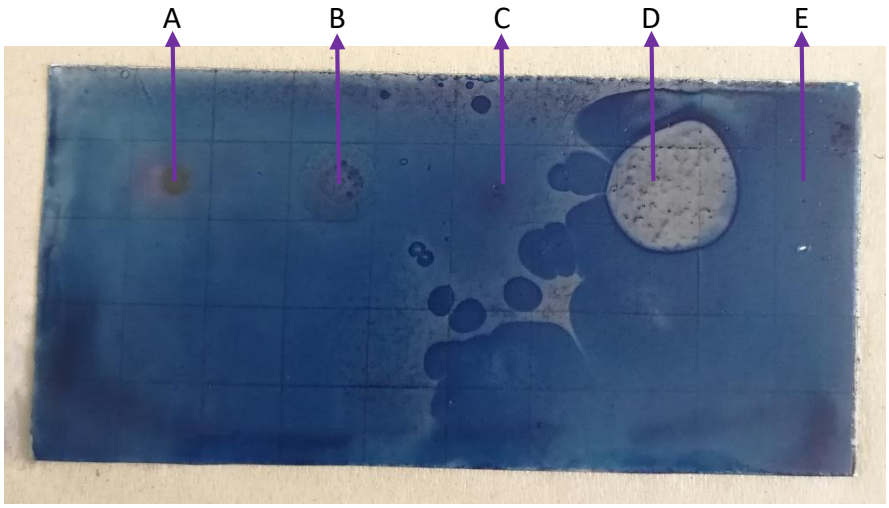

Figure 3.5: Thin-layer bioautography assay of marine microorganisms extract against *M. aurum*. **(A)** PE05-143 **(B)** PE14-28 **(C)** PE14-104 **(D)** Vancomycin **(E)** H_2O. All assays were in duplicates; clear zone-inhibition of *M. aurum* by extracts.

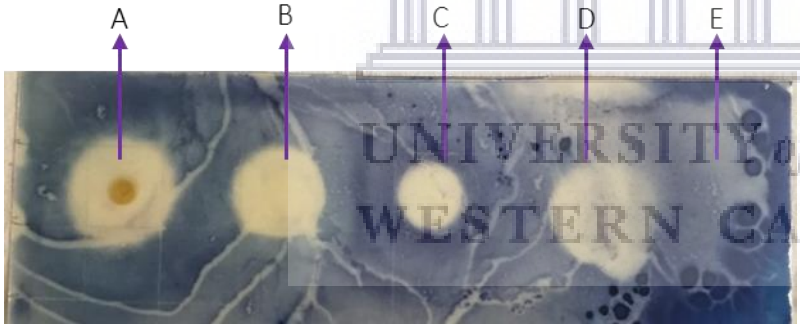

Figure 3.6: Thin-layer bioautography assay of marine microorganisms extract against MRSA **(A)** PE05-143 **(B)** PE14-28 **(C)** PE14-104 **(D)** Vancomycin **(E)** Distilled water. All assays were in duplicates; clear zone-inhibition of MRSA by extracts.

The ^{1}H NMR spectra of PE05-143, PE14-28 and PE14-104 (Figure 3.7) did not give an indication of any significant peak other than possible fatty acids in the region between 0 - 2 ppm. Although under the initial growth conditions for PE05-143, there appears to be interesting peaks between 6.5 - 7 ppm but these peaks were not replicated following subsequent fermentations. PE14-28 and PE14-104 peaks were corresponding mostly to the GYM medium control. Although these extracts showed some activities (See Figure 3.4 and 3.6), this was only exhibited at very high concentrations which reduces their viability. Furthermore, it is highly likely that the active metabolites are produced in minute concentrations and undetected by ^{1}H NMR spectroscopy as these metabolites may have been over-shadowed by prevalent compounds. Several attempts were made to scale up the bacterial isolates culture which resulted in the production of inactive metabolites.

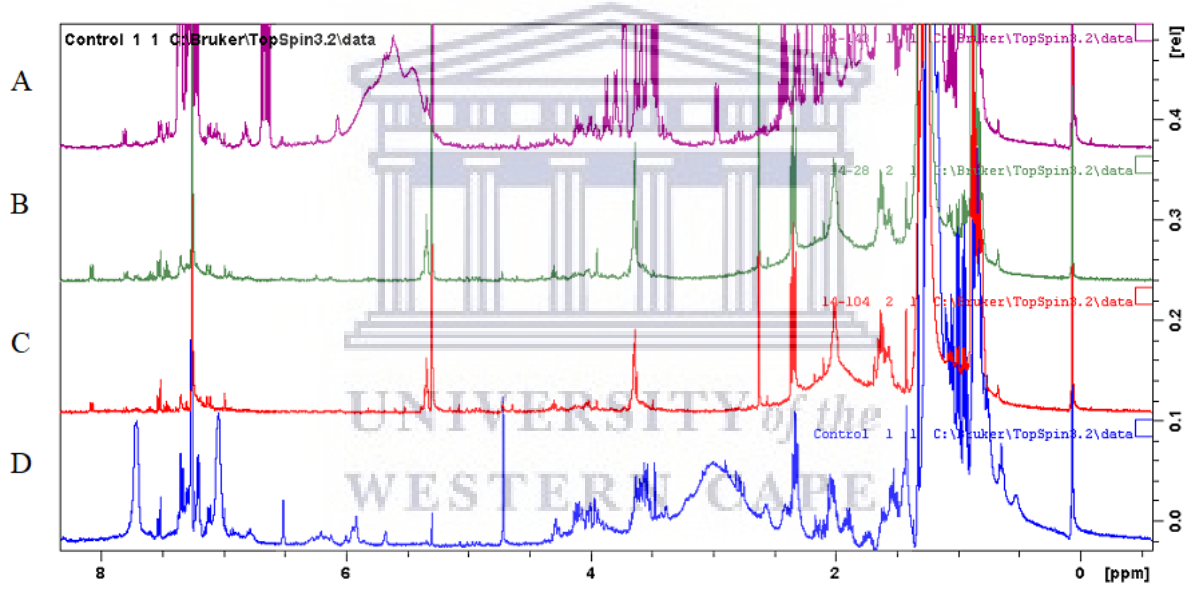

Figure 3.7: ^{1}H NMR spectroscopy of marine bacterial isolates. **(A)** PE05-143 **(B)** PE14-28 **(C)** PE14-104 **(D)** GYM medium.

3.4. Conclusion

This chapter established the marine algae library of 38 species. The secondary metabolites of the algal species were successfully extracted. Twenty-four out of 38 crude extracts showed observable antibacterial activity against MRSA. The ^{1}H NMR spectra of the extracts revealed "interesting features". Three of the algae were selected for further study on the basis of their activity against MRSA, interesting chemical profile and lack of previous studies. In addition, the metabolites from the marine bacterial isolates also showed inhibitory activity against *M. smegmatis* and MRSA

using sloppy-agar overlay and TLC-bioautography techniques. However, there was no activity observed when tested against *M. aurum*.

3.5. References

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Chapter 4
Bioinformatic aided characterisation of bioactive metabolites produced by the marine bacterial isolates

4.1. Introduction

The development of genomic sequencing technology has facilitated the mining of marine bacterial secondary metabolite biosynthetic pathways for the analysis and potential characterisation of the metabolite that is produced (Challis, 2008). There are various tools available in order to implement this bioinformatics research. These include antibiotics & Secondary Metabolite Analysis Shell (antiSMASH), ClusterFinder, CLUSEAN and ClustScan for the analysis of secondary metabolite biosynthetic gene clusters (BGCs) (Blin *et al*., 2013; Medema *et al*., 2011; Cimermancic *et al*., 2014; Starcevic *et al*., 2008; Weber *et al*., 2009). These tools particularly antiSMASH predicts the existence of the secondary metabolite biosynthetic pathways in the genomes by aligning the identified regions at the gene cluster level to their nearest relatives from a database containing all other known gene clusters, and integrates or cross-links all previously available secondary metabolite gene analysis methods in one interactive view (Blin *et al*., 2017). antiSMASH is capable of identifying biosynthetic loci covering a whole range of known secondary metabolite compound classes such as polyketides, non-ribosomal peptides, terpenes and alkaloids (Medema *et al*., 2011). Apart from core biosynthetic enzyme encoding genes, secondary metabolite BGCs generally also harbour genes encoding enzymes to synthesise specialised monomers, transporters, and regulatory elements that are involved in gene expression (Walsh and Fischbach, 2010). This technique has been used in various research as was the case with Teng *et al*. (2012) in the discovery of lantibiotics, elgicins AI, AII, B and C.

This chapter therefore aims to identify the secondary metabolic biosynthetic gene clusters found in the sequenced genomes of the marine bacterial isolates PE05-143, PE14-28 and PE14-104. Owing to the fact that the extracts from the isolates showed antimicrobial activity against *M. smegmatis* LR222 and MRSA ATCC 33591 test strains (Chapter 3, section 3.3.4), a bioinformatic aided characterisation was carried out to identify potential metabolites responsible for the activity. The identities of the three isolates were determined through amplification and sequencing of their 16S rRNA genes prior to sequencing their genomes. The sequenced and the *de novo* assembled draft genomes of the isolates were then queried in antiSMASH 4.2.1 for the identification of the gene clusters and potential secondary metabolites.

4.2. Experimental

4.2.1. Polymerase chain reaction

The PCR reaction was set up in 50 µL volumes containing 100 ng template DNA of isolates, 1 x NEB OneTaq buffer, 1.25 units of NEB OneTaq DNA polymerase , 200 µM dNTPs, 0.2 µM each of the universal primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') (Farrelly *et al*., 1995) and U1501R (5'-GGTTACCTTGTTACGACTT-3') (Reysenbach and Pace, 1995). The reaction volume was adjusted using nuclease free water. The amplification was performed in a Bio-Rad PCR T100 Thermal Cycler (Singapore) according to the following cycling parameters: initial denaturation at 95 °C for 4 min (1 cycle); 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 90 sec and final extension at 72 °C for 7 min.

4.2.2. Agarose gel electrophoresis

The amplified 16S rRNA PCR products were analysed using 1% (w/v) agarose gel electrophoresis in Tris-acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) containing 0.5 µg/mL ethidium bromide. A DNA ladder of λ DNA digested with PstI was used as a molecular marker. The amplified PCR products were run at 100 V for approximately 1 hour, and the DNA bands visualised using a MultiImage™ Light Cabinet (Alpha Innotech, USA). The amplified PCR products were sequenced at the Central Analytical Facility in Stellenbosch, South Africa.

4.2.3. Sequence analysis

The PCR amplified 16S rRNA sequences of the three isolates were queried against the 16S ribosomal RNA sequences (Bacteria and Archaea) database through the National Center for Biotechnology Information website (NCBI; http://www.ncbi.nlm.nih.gov) using the BLAST tool (Altschul *et al*., 1990) to identify the closest related species of the isolates.

4.2.4. Extraction of DNA from isolates

DNA was extracted from the isolates using the method described by Wang *et al*. (1996). Briefly, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to an aqueous solution of bacterial isolate cells (1 mL:1 mL), vortexed and separated by centrifugation (Eppendorf 5810R) at 10 000x*g* for 5 minutes at room temperature. The upper aqueous layer was carefully pipetted and transferred into a sterile 2 mL Eppendorf tube, then 1 mL of chloroform was added to the aqueous solution, vortexed and centrifuged at 10 000x*g* for 5 minutes at room temperature. The upper aqueous layer was pipetted and transferred into a sterile 2 mL Eppendorf

tube. Thereafter, 2 volumes of absolute isopropanol, and 1/10 volume of 3 M NaOAc (pH 5.2) were added for DNA precipitation overnight at 4 °C , centrifuged at 10 000x*g* for 10 minutes, and the pellet washed twice with 70% ice cold ethanol. The pelleted DNA was resuspended in 50 µL of TE buffer and cleansed with the Qiagen Gel Extraction Kit (Qiaex II, cat. no 20021).

4.2.5. Illumina sequencing and de novo assembly

The extracted genomic DNA was sequenced at the sequencing facility, Institute of Microbial Biotechnology and Metagenomics, University of the Western Cape, South Africa. Sequencing libraries were prepared with the Illumina Nextera XT library preparation kit. Libraries were individually indexed, pooled in equimolar concentrations and sequenced with a MiSeq reagent V3 kit (2×300 bp) (Illumina). The fastq files generated were analysed using CLC Genomics version 6.5 (CLC, Denmark). The reads were *de novo* assembled using the default CLC parameters (contig length-200, mismatch cost-2, insertion cost-3, deletions cost-3, length fraction-0.5 and similarity fraction-0.8). Thereafter, 71, 60 and 43 contigs were generated for PE05-143, PE14-28, and PE14-104, respectively. The contigs were uploaded into antiSMASH 4.2.1 to determine possible secondary metabolite biosynthetic pathways.

4.2.6. High resolution liquid chromatography mass spectrometry

The HR-LCMS data for the crude extracts prepared from the isolates were generated at the Central Analytical Facility at Stellenbosch University, South Africa. The extracts and blank media control were prepared to a concentration of 1 mg/mL in methanol solution before injecting 10 µL aliquots into the Waters Synapt G2 mass spectrometer system attached to an Acquity binary solvent manager to obtain the mass spectrometry data. The chromatographic analysis was done with Waters BEH (C18, 2.1 x100 mm) column with a 0.400 mL/min flow and a gradient elution mobile phase of acetonitrile and water with 0.1% formic acid. The program was set to 100% water for the first 5 min, 100% acetonitrile over 7:30 min, and lastly 100% water for 2:30 min before the column was equilibrated back to the starting conditions. The mass spectrometry was carried out in positive ionisation mode with a spray voltage of 15 V. The data files generated from the HR-LCMS were analysed using Masslynx 4.2 software.

4.3. Results and discussion

Query of the 16S rRNA sequences amplified from the three isolates in the curated NCBI 16S rRNA BLASTn database (Altschul *et al*., 1997) returned the top blast hits shown in Table 4.1. The isolates PE05-143, PE14-28, and PE14-104 showed highest identity to *Paenibacillus*

glucanolyticus NBRC 15330, *Vibrio splendidus* LGP32, and *Vibrio pomeroyi* CAIM 578 strains with percentage similarities of 97.84%, 97.87%, and 95.82%, respectively. Bacterial strains are considered as novel if they share less than 98.65% 16S rRNA sequence similarity to any known species (Kim *et al*., 2014). The percentage similarities of the closest relatives for the strains were all below 98.65% suggesting that these isolates were potentially novel species.

Table 4.1: Identified marine bacterial isolates

Marine isolate code	16S rRNA amplicon size bp	% Query Cover	Closest related bacterial species	% similarity
PE05-143	1181	97%	*Paenibacillus glucanolyticus* NBRC 15330	97.84%
PE14-28	1020	96%	*Vibrio splendidus* LGP32	97.87%
PE14-104	936	98%	*Vibrio pomeroyi* CAIM 578	95.82%

Paenibacillus is a genus of facultative anaerobic bacteria of the order of Bacillales in the Paenibacillaceae family (Grady *et al*., 2016). *Paenibacillus* was originally a member of the *Bacillus* genus, but was reclassified as its own genus in 1993, and subsequently modified in 1997 (Ash *et al*., 1993; Shida *et al*., 1997). The species of this genus can be characterised as either Gram-positive, negative or variable. The prominent ones are the Gram-positives, in attribute to their *Bacillus* heritage (Roux *et al*., 2008; Horn *et al*., 2005; Djordjevic *et al*., 2000). At least 56 species of *Paenibacillus* have been documented according to the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov). Among the *Paenibacillus*, some species have been reported to produce bioactive secondary metabolites including antimicrobial agents. Examples of some of the antibiotics identified include polymyxin A to E, gavaserin, saltavalin and fusaricidin A to D, all of which are produced by *Paenibacillus polymyxa* (Benedict and Langlykke, 1947; Stansly *et al*., 1947; Koyama, 1950; Pichard *et al*., 1995; Kajimura and Kaneda, 1997). Paenibacillin and paenimacrolidin antibiotics identified from *P. polymyxa* OSY-DF, and *Paenibacillus* sp. F6-B70, respectively, have been shown to exhibit antimicrobial activity against MRSA (Wu *et al*., 2011; He *et al*., 2007).

Vibrio is a genus of facultative anaerobic bacteria of the order Vibrionales in the Vibrionaceae family (Thompson *et al*., 2005). The species of this genus are Gram-negative, rod-shaped bacteria (Thompson and Swings, 2004). *Vibrio*s are motile microorganisms, and are widespread within marine environment (Thompson and Swings, 2004). There are approximately 128 species of

Vibrios that have been identified (http://www.ncbi.nlm.nih.gov; Mansson *et al*., 2011). There are some *Vibrio* species that have been reported to produce bioactive compounds of chemically varied structures. (Kobayashi *et al*., 1994). These include antimicrobials such as trisindoline from *Vibrio* sp, andrimid, 2,2-Di-(3-indolyl)-3-indolone and magnesidin A from *V. coralliilyticus* (strains S2052 and S4053), *V. parahaemolyticus* Bio249 and *V. gazogenes* ATCC29988, respectively (Wietz *et al*., 2010; Veluri *et al*., 2003; Kobayashi *et al*., 1994).

In silico characterisation of the potential bioactive metabolites

On the account of the isolates being potentially novel species, there is prospect that a new biosynthetic pathway might be present which may facilitate novel secondary metabolite production. Given the number of cryptic, and novel biosynthetic pathways that have been discovered with the aid of genome sequencing and analysis such as those reported from *Paenibacillus* and *Vibrio* genera for elgicins, paenimacrolidin holomycin and andrimid antibacterial production (Teng *et al*., 2012; Wu *et al*., 2011; Mansson *et al*., 2011, Wietz *et al*., 2010), the genomes of the three isolates were sequenced, and their *de novo* assembled draft genome sequences queried in antiSMASH for the identification and characterisation of BGCs.

The output of the isolates' BGCs is summarised in Table 4.2. The result for PE05-143 revealed gene similarities to pathways within 4 types of clusters. This included the nonribosomal peptide synthase (NRPS), ectoine, transatpks and NRPs-Transatpks. Except for the NRPs, the type of clusters exhibited in PE14-28 were different to the clusters observed in PE105-143. PE14-28 showed gene similarity to pathways in the arylpolene, siderophore and pufa, hgIE-KS gene clusters. This difference between the two isolates was not unexpected as the two belong to different genera. Isolate PE14-104 belonging to the same genus as PE14-28 shared pathways in the cluster type arylpolyene, NrPs, and pufa-hgIE-KS.

Table 4.2: Summary of antiSMASH 4.2.1 output from the genome sequence analysis for PE0143, PE14-28 and PE14-104 with 12, seven and six different secondary metabolite gene clusters identified, respectively.

PE05-143					
		Pathway size			
Cluster	Type	From	To	Most similar known cluster	Gene similarity (%)‡
1	Lassopeptide	53752	76240	-	
2	Bacteriocin	13521	24429	-	
3	Terpene	60737	82662	-	
4	Other	73959	117675	-	
5	Terpene	23730	45586	-	
6	Nrps-Transatpks	23466	91544	Chejuenolide A/ Chejuenolide B; polyketide	7
7	Nrps	145644	195542	Bacillibactin; Nrp	53
8	Transatpks	190803	273111	Difficidin; Polyketide + Nrp	20
9	Terpene	43274	64107	-	
10	T3pks	1*	22256	-	
11	Bacteriocin	382153	392392	-	
12	Ectoine	22590	33000	Ectoine; other	50

PE14-28					
		Pathway size			
Cluster	Type	From	To	Most similar known cluster	Gene similarity (%)
1	Arylpolyene	1*	42011	APE Vf; other	85
2	Bacteriocin	90510	101553	-	
3	Siderophore	116972	131844	Vibrioferrin; other	54
4	Nrps	1*	54341	Crochelin A; Nrp + polyketide	11

5	Betalactone	182740	215231	-	
6	Pufa, hgIE-KS	70514	126258	Eicosapentaenoic acid; other	26
7	Bacteriocin	16941	27801	-	

PE14-104

		Pathway size			Gene similarity
Cluster	Type	From	To	Most similar known cluster	(%)
1	Betalactone	69741	102118		
2	Bacteriocin	20077	30937	-	
3	Pufa, hgIE-KS	51719	107559	Eicosapentaenoic acid; other	26
4	Bacteriocin	75387	86400	-	
5	Nrps	185575	242154	Crochelin A; Nrp + polyketide	11
6	Arylpolyene	13854	57452	APE Vf; other	85

* refers to pathways that starts at position 1 of a contig may potentially be incomplete or truncated.

‡ refers to the % number of genes within the isolates with shared similarities to known clusters

The gene clusters predicted by antiSMASH analysis of the isolates' genome with similar known gene clusters are presented in Figure 4.1. A1 shows the gene sequence of cluster 6 from PE05-143. From the open reading frames (ORFs) present on the cluster, only 2 of the 26 genes showed similarities to chejuenolide A and B resulting in 7% (2/26 =7%, Table 4.2). Likewise, gene similarities was observed for bacillibactin, difficidin and ectoine at 53%, 20% and 50%, respectively (Table 4.2; Figure 4.1). None of the genes represented the core biosynthetic genes for the pathways and therefore unlikely to result in the biosynthesis of the active compound. PE14-28 and PE14-104 also mostly showed low gene similarity except for arylpolyene at 85% in each isolate. However, upon further analysis of the arylpolyene gene cluster for PE14-28 (B) and PE14-104 (C), none of the genes represented the core biosynthetic gene. This indicate that the arylpolyene pathway with it high gene similarity could not be responsible for producing the compound to which activity was observed. This further suggests that a novel biosynthetic pathway may exist in these isolates.

Both PE14-28 and PE14-104 encode similar pathways, and based on the isolates' identification results, they are different species and yet harbour the same pathway with the exception of a siderophore that is present in PE14-28 but absent in PE14-104. This further highlights the importance of a full genome sequence when evaluating strains for activity and provides justification for strain choice and for eliminating strains that produce known compounds (dereplication). Moreover, both strains were active against MRSA ATCC 33591 which suggests that the siderophore pathway (cluster 3 in PE14-28) could not have been responsible for the observed activity.

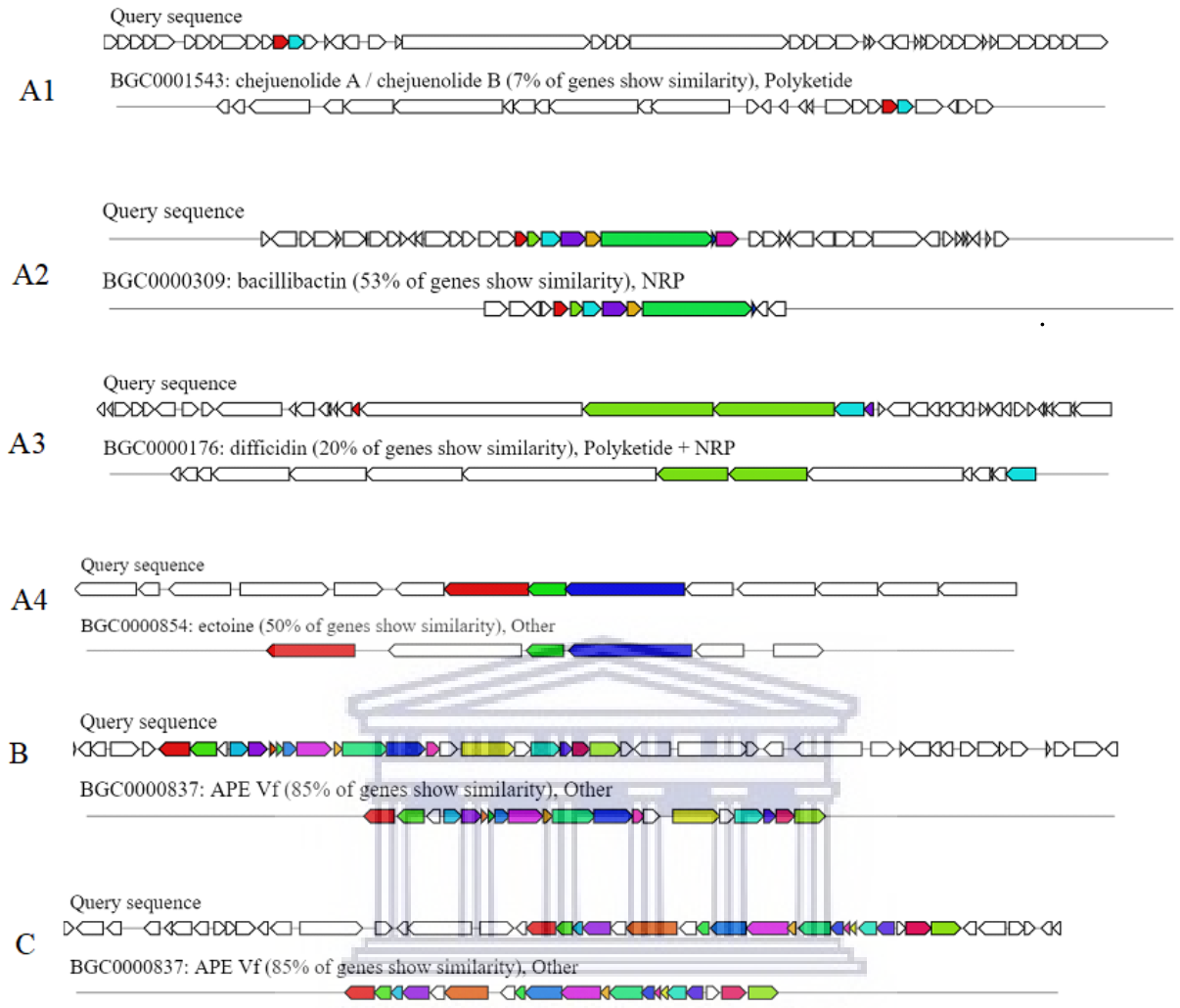
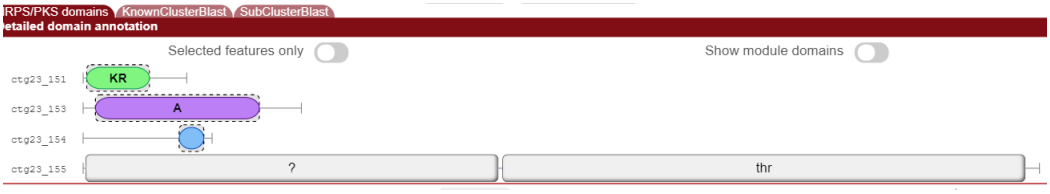


Figure 4.1: Comparison of the isolates' genomic cluster with similar known gene cluster. PE05-143: (**A1-A4**), **A1**: Cluster 6; **A2**: Cluster 7; **A3**: Cluster 8; **A4**: Ectoine; **B**: Cluster 1 from PE14-28; **C**: Cluster 6 from PE14-104 (See Table 4.2).

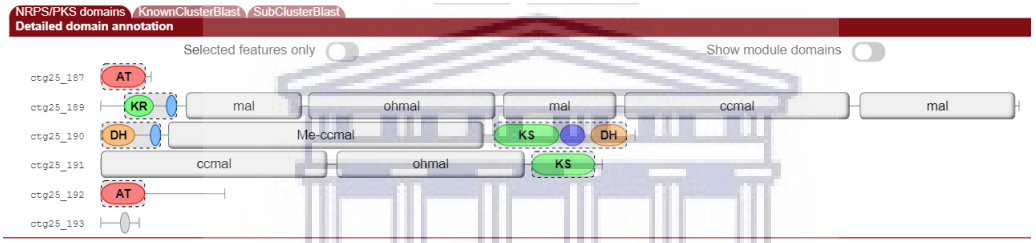
The output from antiSMASH for the three isolates, had "KnownClusterBlast" for all clusters showing gene similarity. Conversely, although all the clusters also had a "SubClusterBlast" tab, none showed any gene sequence in this regards. The NRP cluster however is the only cluster type which has a third tab, "NRPS/PKS domains" (Figure 4.2). This details the amino acid used by the synthases in the pathway to build the peptide compound. The amino acid details of the NRP clusters in all three isolates however were incomplete, that is, some of the amino acids in the cluster were unknown. This further implies that the biosynthetic pathways in the isolates are novel.

A1

A2



A3



B

C

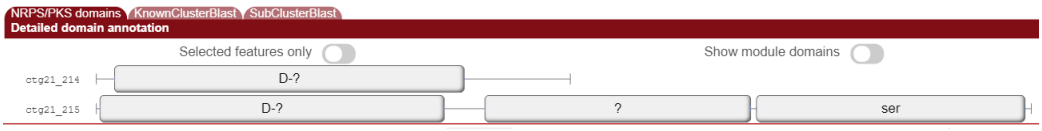


Figure 4.2: NRP amino acid domain of isolates. PE05-143: (**A1-A3**) **A1**: Cluster 6; **A2**: Cluster 7; **A3**: Cluster 8; **B**: Cluster 4 from PE14-28; **C**: Cluster 5 from PE14-104.

Figure 4.3 shows that the pathway of cluster 4 from PE14-28 is widely spread among many different *Vibrio* species including *V. lentus*, *V. tasmaniensis*, *V. splendidus*, *V. cyclitrophicus*, and PE14-104 with a high % number of genes shared. This pathway has not yet been

characterised based on the genome sequence analysis report. Once the compound from this pathway is described from 1 strain, it will likely be highly similar in all these other species.

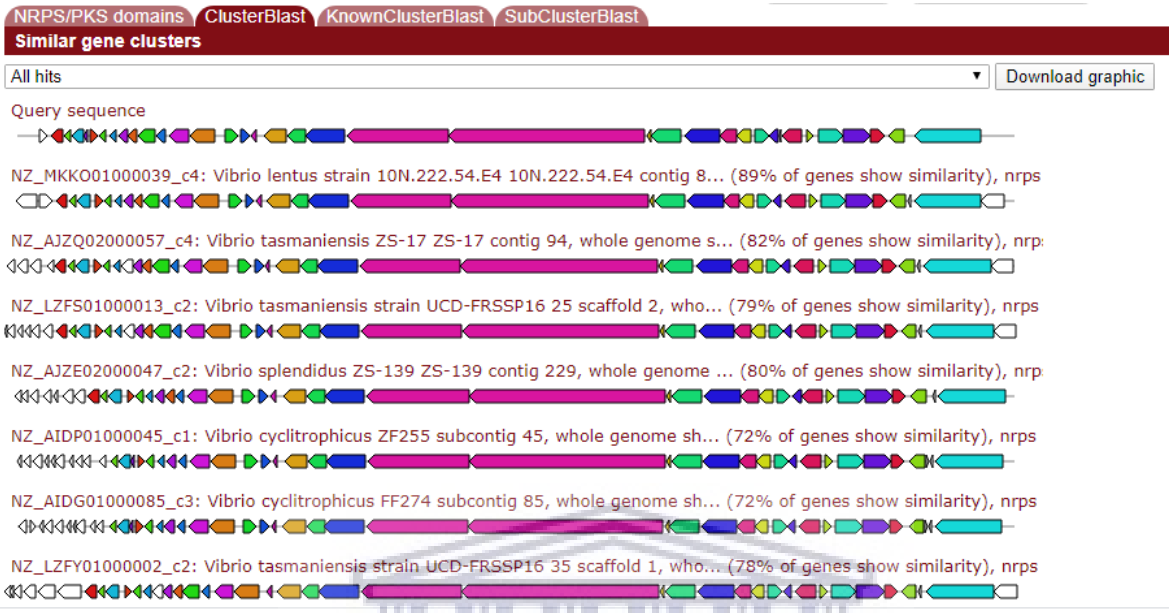


Figure 4.3: Different *Vibrio* species with similar gene pathway.

High resolution liquid chromatography mass spectrometry (HR-LCMS) analysis was carried out on the isolates extracts to give an indication of the presence of the classes of the compounds. The data was compared to the bio-informatics prediction of the biosynthetic pathways to confirm that the classes of compounds are in fact in the extracts. This was supposed to assist to provide more definite indication of the active ingredients in the extract responsible for biological activity. However, the isolates mostly showed similarities to the medium in which they were prepared. This may suggest that the potentially active compounds were not produced by the isolates prior to extraction.

4.4. Conclusion

The three isolates used in this study were identified as potentially novel based on their 16S rRNA percentage sequence similarity to closely related strains in the NCBI 16S rRNA database. AntiSMASH was used to predict the biosynthetic pathways of the isolates. Although the exact biosynthetic pathways of the potentially active metabolite were not established, the isolates may have possible novel pathways. Prediction of the biosynthetic pathways to confirm the classes of compounds present in the isolates extract was not possible because they mainly showed similarities to the control medium.

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Chapter 5
Isolation and structural characterisation of antibacterial compounds from *Dictyota naevosa*, *Laurencia pumila* and *Laurencia sodwaniensis*

5.1. Introduction

Following the preparation of the algal extracts library, bioactivity screening and ^{1}H NMR profiling, three South African marine algae, namely, *Dictyota naevosa, Laurencia pumila* and *Laurencia sodwaniensis* which showed good antibacterial activity and interesting chemical trait were selected. In this chapter, a brief review of the chemistry of natural products reported from these genera will be highlighted followed by the isolation and structural characterisation of metabolites from the selected algae.

5.1.1. Algae species of the genus Dictyota

Dictyota is a genus of brown algae of the order Dictyotales in the Dictyotaceae family. The species of this genus are located in warm tropical and subtropical waters (Tronholm, 2012). They are found in most regions of the world in countries such as Australia, Bermuda, France, Jamaica, Mozambique, Philippines, Hawaii (USA) and South Africa (De Clerck *et al*., 2006). Presently, 91 species of *Dictyota* are recognised worldwide of which four including *D. naevosa*, *D. dichotoma* and *D. liturata* (Figure 5.1) can be found in South Africa although one has not been described (Guiry and Guiry, 2019; Anderson *et al*., 2016). They are represented by a flattened thallus consisting of a layer of large medullary and smaller cortical cells. The blades are dichotomously or irregularly branched arising from a rhizoidal holdfast, and growth is by means of division of a single apical cell (Guiry and Guiry, 2019).

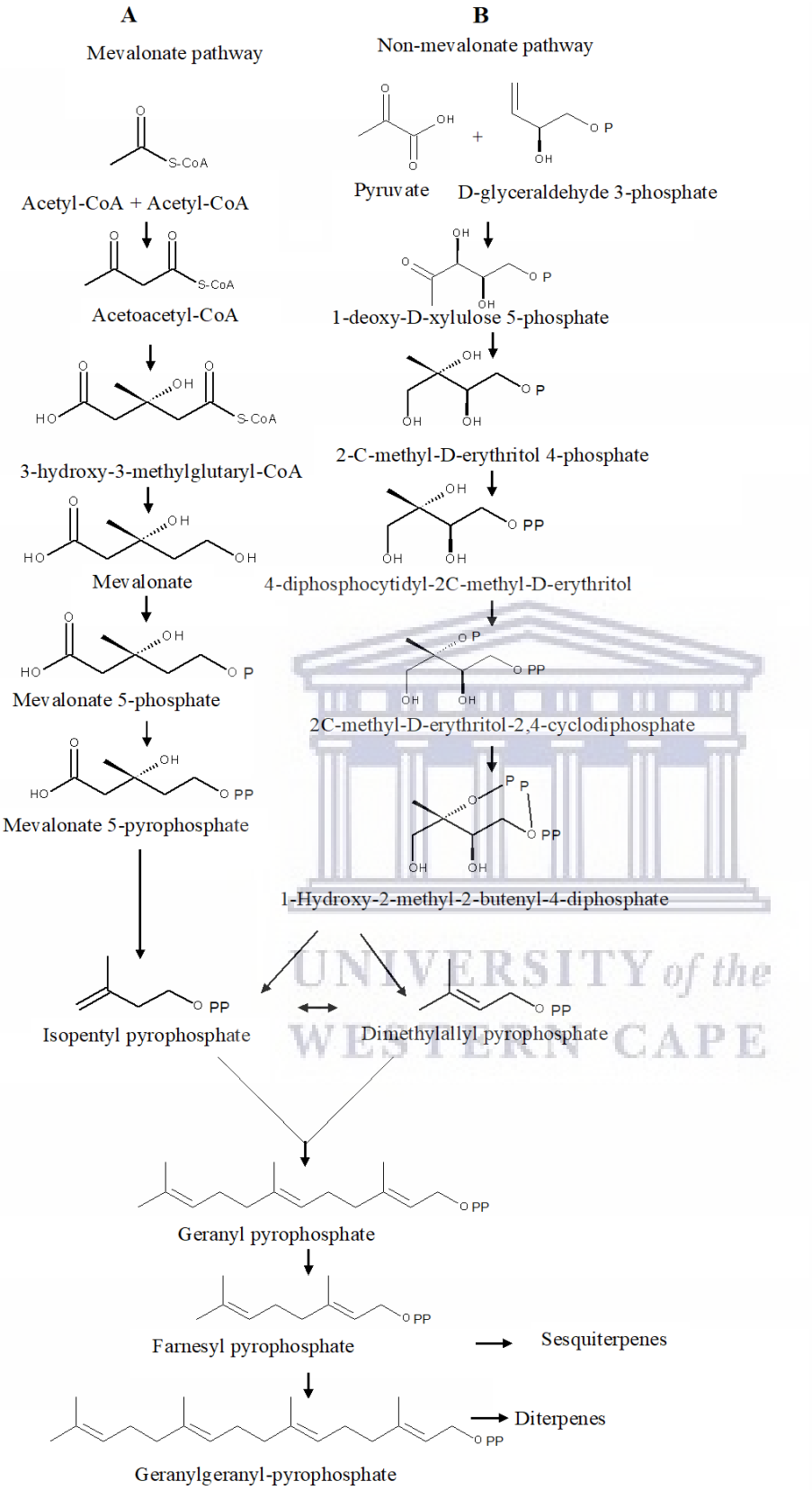
In this study, the South African brown alga *Dictyota naevosa* was examined. The alga has been recorded in Algoa Bay, False Bay and Mission Rocks in KwaZulu Natal (Anderson *et al*., 2016; Silva *et al*., 1996). Global distribution include Canary Islands, Japan Senegal and South and Western Australia (Guiry and Guiry, 2019). *D. naevosa* has been described by Tronchin and De Clerck (2005) and Stegenga *et al*. (1997) to possess either scattered or grouped tetrasporangiate sori of about 1.5 mm. It has elongated-ovoid oogonial sori of approximately 400x300 µm, and oogonia clavate which is around 90x40 µm. It also has antheridial sori surrounded by paraphyses distributed evenly over blade surface.

Dictyota naevosa *Dictyota dichotoma* *Dictyota liturata*

Figure 5.1: Selected *Dictyota* species from South Africa; photographs accessed from http://southafrseaweeds.uct.ac.za/descriptions/brown/dictyota_genus.php

5.1.2. Natural products produced by Dictyota species

Among the *Dictyota*, many species are known to produce diterpene secondary metabolites. Over 233 diterpene natural products have been reported most of which have been isolated from *D. dichotoma* (Chen *et al*., 2018). This class of natural product has since generated much interest in drug discovery research because of the diverse structural features and biological activities of their metabolites. Diterpenes have four isoprene ($C_{20}H_{32}$) units and are biosynthesised either through the mevalonate or non-mevalonate pathways (Scheme 5.1).



Scheme 5.1: Mevalonate and non-mevalonate biosynthetic pathways. (**A**) Mevalonate (**B**) Non-mevalonate. (*Scheme adapted from Lange et al., 2000*).

5.1.2.1. The mevalonate pathway

This pathway is also known as the isoprenoid pathway or the HMG-CoA pathway. The initial step in mevalonate pathway involves enzyme catalysed condensation reaction of acetyl-CoA and acetoacetyl-CoA to yield an intermediate 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Thereafter, HMG-CoA is reduced by NADPH to form mevalonate (MVA). Subsequently, mevalonate undergoes phosphorylation by ATP mediated by mevalonate kinase to yield mevalonate 5-phosphate. Hereafter, mevalonate 5-phosphate is phosphorylated by phosphomevalonate kinase to produce mevalonate 5-pyrophosphate which is then decarboxylated to yield precursor molecule isopentyl pyrophosphate (IPP). An IPP isomerase then produces dimethylallyl prophosphate (DMAPP) form the IPP. These two intermediates, IPP and DMAPP condenses to form geranyl pyrophosphate (GPP). Finally, enzymatic incorporation of one IPP unit to farnesyl pyrophosphate (FPP) to form geranylgeranyl-pyrophosphate (GGPP) by diterpene synthase results in the production of diterpenes (Abdallah and Quax, 2017; Rohmer, 1999).

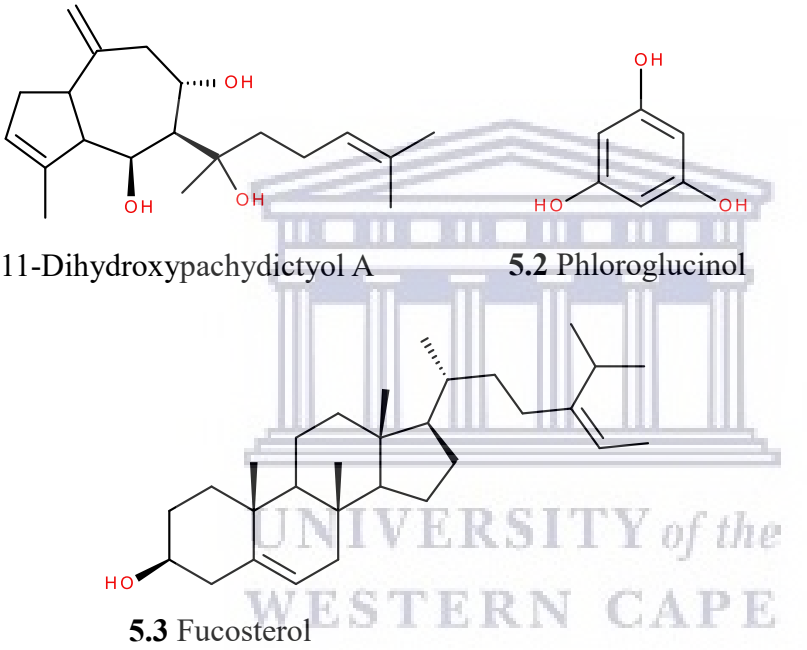
5.1.2.2. The non-mevalonate pathway

The non-mevalonate pathway is initiated with the condensation reaction of pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DOXP) which is then converted to 2C-methyl-D-erythritol 4-phosphate (MEP). Subsequently, in a series of enzyme catalysed reactions MEP is converted to precursor molecules isopentyl pyrophosphate (IPP) and dimethylallyl prophosphate (DMAPP). Similarly with mevalonate pathway, these two intermediates merge to form geranyl pyrophosphate (GPP). Lastly, a single IPP unit is added to farnesyl pyrophosphate (FPP) to form geranylgeranyl-pyrophosphate (GGPP) (Dubey *et al*., 2003; Rohmer *et al*., 1996).

5.1.3. Natural products from brown algae

Hundreds of bioactive natural products such as terpenes, phenols, sterols have been isolated from brown algae. Many of the metabolites produced have been reported to exhibit biological activities including antimicrobial, cytotoxicity, antiplasmodial, antiviral and anti-inflammatory (Ioannou *et al*., 2013). Some of the chemical compounds with diverse bioactive functionalities are represented in Figure 5.2. Diterpene, 8α,11-Dihydroxypachydictyol A (**5.1**) isolated from *Dictyota* sp., Bangsaen Beach (Thailand) and from *D. plectens* in the South China Sea showed cytotoxicity against the Vero monkey kidney cell line (ATCC CCL-81) and antiplasmodial

against *Plasmodium falciparum* (K1, multi drug resistant strain) and anti-HIV-1 activity (Cheng *et al*., 2014; Jongaramruong and Kongkam, 2007). Phloroglucinol (**5.2**), a phenolic compound isolated from brown algal *Ecklonia cava* plays a role as an antioxidant on oxidative stress markers in human HepG2 cell line (Quéguineur *et al*., 2012), and has also been reported as an antimicrobial agent against MRSA (Mittal *et al*., 2019; Yoon *et al*., 2017). Fucosterol (**5.3**) isolated from *Eisenia bicyclis* and *Ecklonia stolonifera* is a very common sterol in brown algae which plays various bioactive roles including anti-inflammatory in LPS-stimulated RAW264.7 macrophages, anti-acetylcholinesterase, and anti-adipogenic in 3T3-L1 adipocytes (Jung *et al*., 2014; Jung *et al*., 2013; Yoon *et al*., 2008).



5.1 8α,11-Dihydroxypachydictyol A **5.2** Phloroglucinol

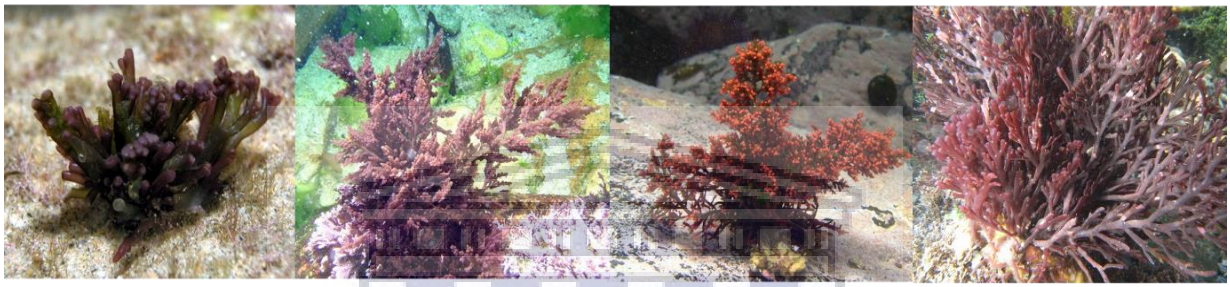
5.3 Fucosterol

Figure 5.2: Chemical structures of bioactive compounds from brown algae.

5.1.4. The genus Laurencia

Laurencia is a genus of red algae of the order Ceramiales in the Rhodomelaceae family. Members of the *Laurencia* species grow in tropical and in mild temperature regions by the shoreline. Globally, species of *Laurencia* have a wide geographical distribution and are located in countries such as Australia, Brazil, China, Japan, Philippines, (Hawaii and California) USA and South Africa (Al-Massarani, 2014; Francis, 2017). Currently, approximately 430 species have been identified, however, only 134 are taxonomically recognised (Guiry and Guiry, 2019). From the *Laurencia* complex, nineteen species have been

documented in South Africa (Francis, 2014). These include *L. pumila*, *L. glomerata*, *L. natalensis* and *L. complanata* (Figure 5.3). They are represented by erect or decumbent thallus with thickened medullary cell walls outer cortical cells has one or several intracellular colourless inclusions (*corps en cerise*) which depending on the number per cell is used to characterize each species. The blades are distichous or whorled arising from a discoid, rhizoidal or encrusting holdfast. Reproductive structures, tetrasporangia, is typically on terminal branchlet and sometimes depending on the species is on main axes (Guiry and Guiry, 2019).



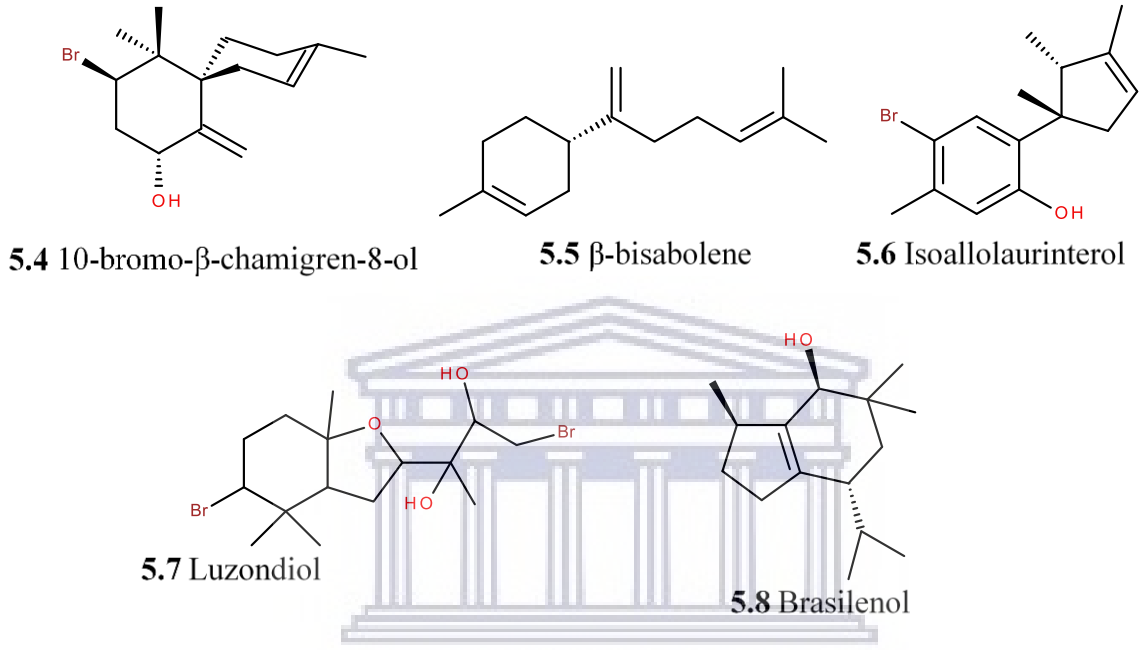
Laurencia pumila *Laurencia glomerata* *Laurencia natalensis* *Laurencia alfredensis*

Figure 5.3: Selected *Laurencia* species from South Africa; photographs accessed from http://southafrseaweeds.uct.ac.za/descriptions/red/laurencia_genus.php

5.1.5. Natural products produced by Laurencia

The *Laurencia* genus has been widely studied with over 700 isolated natural products (Wang *et al*., 2013). The major metabolites produced comprises of halogenated, typically bromine and some chlorine atoms chamigrane-type sesquiterpenes (Suzuki *et al*., 2009). However, there are few reports of diterpenes, triterpenes, steroids, alkaloids and C_{15}-acetogenins. Sesquiterpenes are made up of three isoprene ($C_{15}H_{24}$) units which are either in linear, cyclic, bicyclic or tricyclic derivatives or in form of a lactone ring (Başer and Demirci, 2007). This class of natural products serves as promising drug candidates in lead discovery because they are structurally unique and diverse, and exhibit significant pharmacological activities. This structural diversity also consists of bisabolane, laurane, snyderane and brasilan sesquiterpenes with varied backbone (Al-Massarani, 2014). Examples of some chemical structures from *Laurencia* with distinct backbone includes 10-bromo-β-chamigren-8-ol (**5.4**), β-bisabolene (**5.5**), isoallolaurinterol (**5.6**), luzondiol (**5.7**) and brasilenol (**5.8**) shown in Figure 5.4 (Iliopoulou *et al*., 2002; Kazlauskas *et al*., 1976; Kladi *et al*., 2007; Li *et al*., 2012; Makhanu

et al., 2006). Sesquiterpenes are biosynthesised via the mevalonate pathway as described in section 5.1.2.1 whereby isopentenylpyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) precursors condense to form geranyl pyrophosphate (GPP) and addition of FPP by FPPS results in sesquiterpene formation.



5.4 10-bromo-β-chamigren-8-ol **5.5** β-bisabolene **5.6** Isoallolaurinterol

5.7 Luzondiol **5.8** Brasilenol

Figure 5.4: Chemical structures of some sesquiterpene compounds from *Laurencia*.

The two *Laurencia* species of interest for this study are the Southern Africa algae, *Laurencia pumila* and *Laurencia sodwaniensis*. Geographical distribution of *L. pumila* include Tsitsikamma, northern KwaZulu Natal and southern Mozambique (Tronchin and De Clerck, 2005; Silva *et al*., 1996). *L. sodwaniensis* locality has been recorded in KwaZulu-Natal, Jesser Point and Sodwana Bay (Francis *et al*., 2017). At a microscopic level, *L. pumila* cells are circular with a linear decrease in the diameter from the innermost cortical cells to the outer cells. The outer cortical cells appears smaller in size than the innermost and medullary cells which appears to be twice the size of the outermost cells (Francis, 2014).

Given that the species of the afore-mentioned genera are known to produce compounds with a range of biological activities including antibacterial, and preliminary screening of the *D. naevosa*, *L. pumila* and *L. sodwaniensis* extracts have exhibit this functionality (Chapter 3), the chemical constituents of these algae extracts were therefore examined as potential antibacterial lead compounds.

5.2. Experimental

5.2.1. General procedures

Column chromatography was carried out using silica gel 60 (0.040-0.063 mm) purchased from Merck (Darmstadt, Germany). HPLC separations were performed using Agilent technologies equipped with a UV 100 detector at 250 nm and a Whatman 10 µm semi-preparative column (50 cm). All NMR spectra were measured with Avance Bruker spectrometer operating at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C NMR in $CDCl_3$. Chemical shifts were referenced as described in Chapter 3, section 3.2.1. The HR-LCMS instrumentation was the same as described in Chapter 4, section 4.2.6.

5.2.2. Algal material

A sample of *Dictyota naevosa* (collection code: SB20140302-3), *Laurencia pumila* (collection code: KZN13107-1) and *Laurencia sodwanienisis* (collection code: KZN1311-1) were collected by hand at the coast of KwaZulu-Natal in March 2014, October 2013 and November 2013, respectively. Botanical identification was done by Professor John J. Bolton at the University of Cape Town, South Africa. Voucher specimens were deposited at the MBD collection, University of the Western Cape. Samples were stored at -20 °C until further use. *Dictyota naevosa* is a brown alga with flat, broad lamina and dichotomy axes (Anderson, *et al*., 2016). *Laurencia pumila* is green with several branches emerging from a rhizoidal holdfast, sparsely branched thallus and club-shaped apices (Francis, 2014). *Laurencia sodwaniensis* is yellowish-brown alga connected by discoid holdfast with sparsely branched fronds (Francis, 2014; Tronchin and De Clerck, 2005).

5.2.3. Extraction and isolation

Frozen specimens of *Dictyota naevosa* (3.3 g), *Laurencia pumila* (2.3 g) and *Laurencia sodwaniensis* (23.2 g) were submerged in CH_3OH (75 mL) at 4 °C overnight. The solvents were decanted and the algae re-extracted three times with CH_3OH-CH_2Cl_2 (1:2, 75 mL) at 36 °C. The two organic fractions were combined and the solvents were dried under reduced pressure to yield 529.6 mg, 105.7 mg and 180.7 mg of organic extracts, respectively.

Dictyota naevosa crude extract (388.7 mg) was separated by silica gel column chromatography and eluted in *n*-hexane (100 %), *n*-hexane–EtOAc (90:10; 80:20; 60:40; 40:60; 20:80) EtOAc (100 %), EtOAc–MeOH (50:50) and MeOH (100 %) to obtain 9 fractions (Fr1–Fr9). Fraction

2 (40.5 mg) eluted with *n*-hexane–EtOAc (90:10) was further purified with Agilent normal phase HPLC which consist of a UV100 detector at 250 nm and Whatman 10 µm semi-preparative column (50 mm), and eluted with *n*-hexane–EtOAc (90:10) at a rate of 3 mL/min resulting in the isolation of compound **5.9** (4.90 mg).

Laurencia pumila crude extract (105.7 mg) was chromatographed by silica gel column and eluted in *n*-hexane (100 %), *n*-hexane–EtOAc (90:10; 80:20; 60:40; 40:60; 20:80) EtOAc (100 %), EtOAc–MeOH (50:50) and MeOH (100 %) to obtain 9 fractions (Fr1–Fr9). Fraction 3 (7.0 mg) eluted with *n*-hexane–EtOAc (80:20) was further purified with Agilent normal phase HPLC which consist of a UV100 detector at 250 nm and Whatman 10 µm semi-preparative column (50 mm), and eluted with *n*-hexane–EtOAc (80:20) at a rate of 3 mL/min resulting in the isolation of compound **5.10** (1.10 mg).

Laurencia sodwaniensis crude extract (180.7 mg) was purified by silica gel column chromatography with *n*-hexane (100 %), a mixture of *n*-hexane–EtOAc (90:10; 80:20; 60:40; 40:60; 20:80), EtOAc (100 %), EtOAc–MeOH (50:50) and MeOH (100 %). As a result, 9 fractions were obtained. Compound **5.11** (86.9 mg) was identified in fraction 3 [(*n*-hexane–EtOAc (80:20)].

5.2.4. Isolated compounds

Pachydictyol A **(5.9)**: colourless oil, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$ 100 MHz) see Table 5.1.

C_{15}-acetogenin (**5.10**): colorless oil, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$ 100 MHz) see Table 5.2.

Cartilagineol (**5.11**): white solid, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$ 100 MHz) see Table 5.3.

5.2.5. TLC-bioautography assay

The TLC-bioautography tests of algae crude extracts and algae column fractions were carried out against MRSA ATCC 33591 at a concentration of 20 µg/mL. The assay was performed as described in Chapter 3, section 3.2.4.

5.3. Results and discussion

Figure 5.5 shows thin-layer bioautography result of algae column fractions. Vancomycin serves as a positive control with a clear well-defined and larger inhibition zone against MRSA as compared to the algae fractions. Dichloromethane serves a negative control showing no effect on MRSA as the TLC plate retained the purple stain. *L. sodwaniensis*: Numbers 3 - 6 shows distinct inhibitory activity against MRSA by the presence of clear white spots at the zones of inhibition as compared to vancomycin; Numbers 1, 2, 7 - 9 shows minimal visible activity against MRSA. *L. pumila*: Numbers 3 - 7 shows defined inhibition against MRSA by the presence of visible white spots; Numbers 8 and 9 shows minimal visible activity; Numbers 1 and 2 does not show activity against MRSA; *D. naevosa*: Numbers 1, 3 - 8 shows distinct inhibitory activity against MRSA; Number 9 shows minimal visible inhibitory activity against MRSA; Number 2 does not show activity against MRSA. The crude algae extracts shows inhibitory activity against MRSA by the presence of well-defined clear white spots at the zones of inhibition. The majority of the algae fractions were potent in killing MRSA growth. This suggests that the compounds present in the fractions were capable of carrying out this inhibitory activity and may not require the synergistic effect that is brought about by the complex crude extract mixture. However, the few ineffective fractions may simply not possess the compound needed to impede bacterial growth or necessitate the presence of other molecules in the mixture for efficacy.

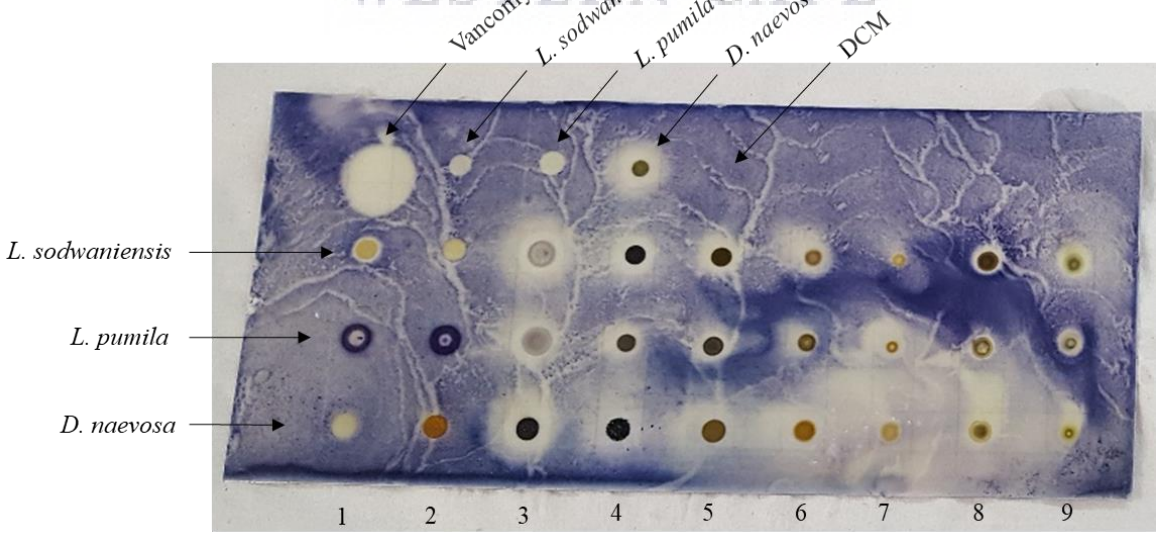


Figure 5.5: Thin-layer bioautography of algae column fractions against MRSA.

5.3.1. Extraction and isolation of compound **5.9** from *D. naevosa*

Figure 5.6: A photograph of the *D. naevosa* specimen used in this study.

The extract was chromatographed by silica gel column chromatography using variable mixtures of hexane, EtOAc and MeOH to obtain 9 fractions (Frs1-9). Each of the fractions were analysed with ^{1}H NMR as shown in Figure 5.7. Fractions 2, 4, and 5 displayed proton signals at δ 4.5 – 5.5 ppm with Fr 2 showing an additional interesting signal at 4.0 – 4.5 ppm. The remaining fractions mainly contains what appears to be fatty acids. Fraction 3 was at least 40 mg but the most interesting peaks in this fraction mainly indicated the presence of chlorophyll between 3 – 4 ppm and 6 – 6.5 ppm. Attempt was made to further purify fractions 2, 4 and 5 using a semi-preparative normal phase HPLC. All fractions obtained from further purifying Fr 4 and 5 revealed what appears to be impurities in the fractions. Purification of Fraction 2, eluted with hexane–EtOAc (9:1) through semi-preparative HPLC however afforded compound **5.9.** The extraction and isolation of compound **5.9** is summarised below in Scheme 5.2. In addition, following detailed analysis of 1D and 2D NMR spectroscopy and comparison with literature data, the structure of compound **5.9** was elucidated.

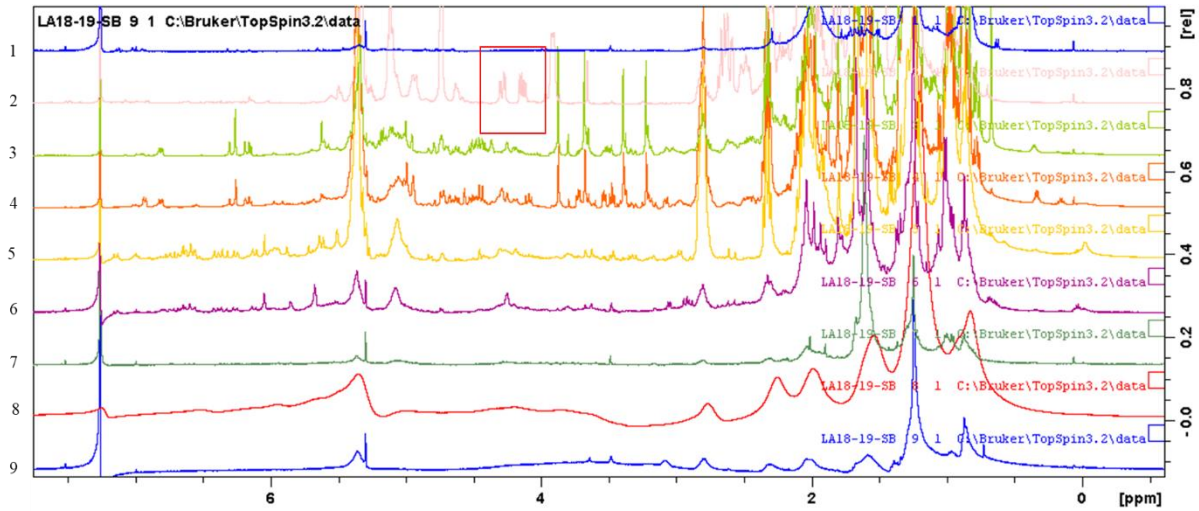
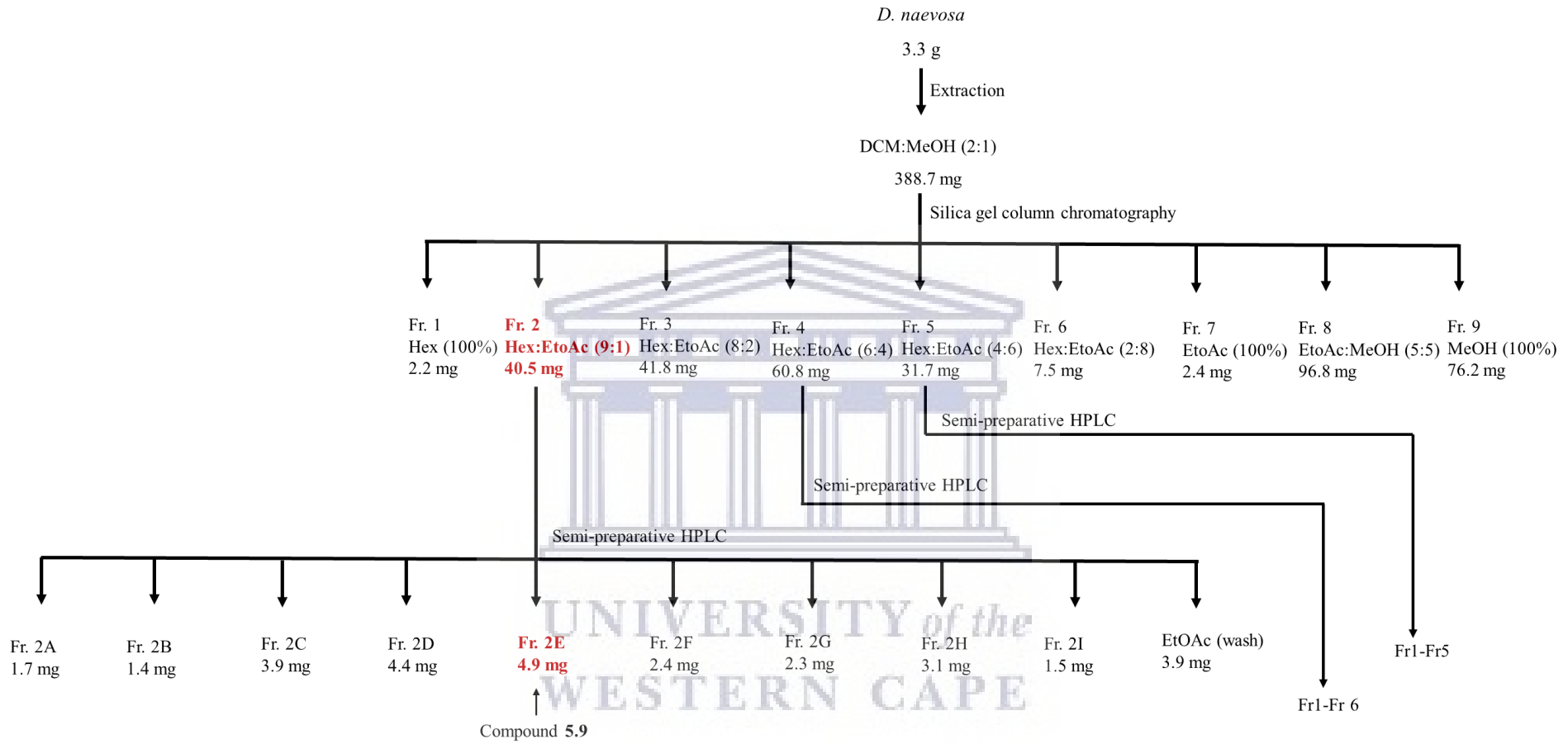


Figure 5.7: ^{1}H NMR of *D. naevosa* fractions 1-9.



Scheme 5.2: The outline of the isolation of compound **5.9** from *D. naevosa*.

5.3.2. Structure elucidation of compound **5.9**.

High resolution liquid chromatography mass spectrometry (HR-LCMS) of **5.9** showed a protonated molecular ion peak at m/z 289.2535 corresponding to a molecular formula of $C_{20}H_{32}O$ (calcd for 289.2533) . Following evaluation of the 1D and 2D NMR spectral data for compound **5.9** in $CDCl_3$ and comparison with literature data, the spectroscopic values for all the signals were assigned. The ^{1}H NMR spectrum including the dublet at 3.9 ppm of **5.9** showed the presence of two methine signals at δ 5.33 (H-3) and 5.01 (H-14), four methyl signals at δ 1.76 (H-17), 1.60 (H-16), 1.55 (H-20), 0.94 (H-19) and six methylene signals at δ 4.70 (H-18), 2.23 (H-9), 2.20/2.48 (H-2), 1.60/1.80 (H-13), 1.80 (H-8) and 1.19/1.52 (H-12) (Figure 5.8). The ^{13}C NMR spectrum revealed 20 carbon signals consistent with a diterpene skeleton (Figure 5.9).

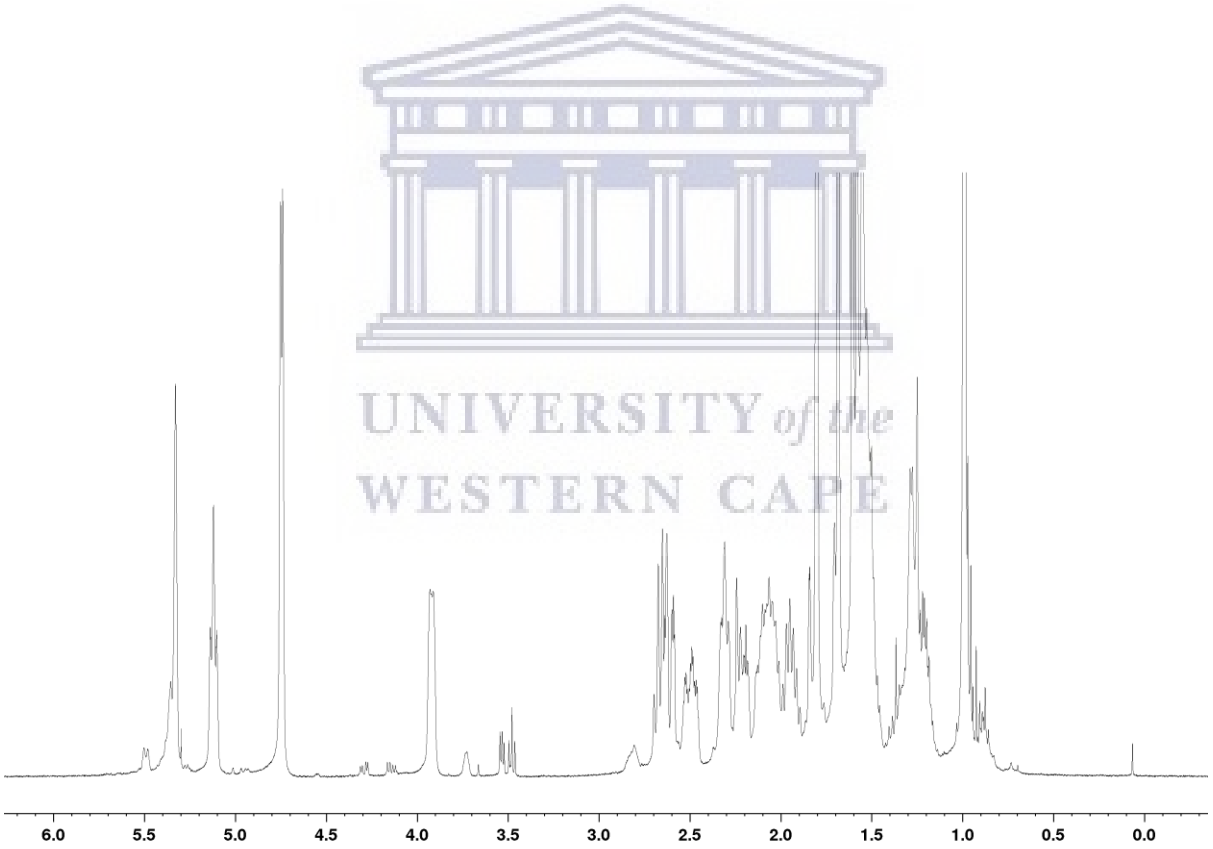


Figure 5.8: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **5.9**.

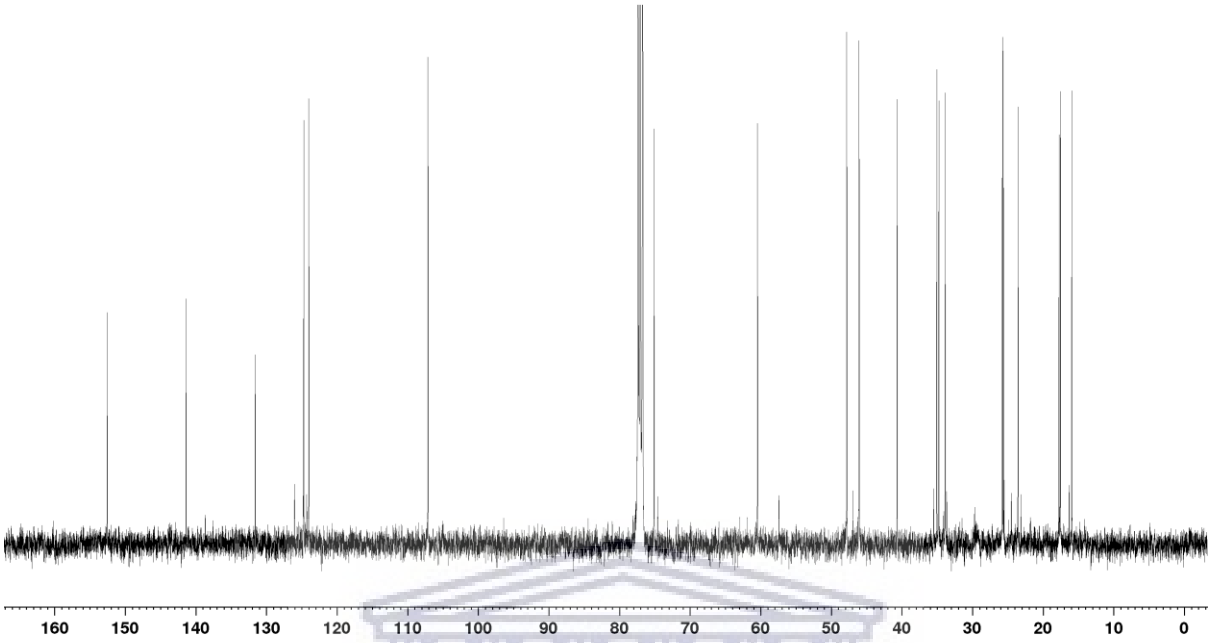


Figure 5.9: ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **5.9**.

The HSQC spectrum showed olefinic protons at δ 5.3 (H-3) and 5.01 (H-14) were attached to carbons at δ 124.0 (C-3) and 124.7 (C-14), respectively (Figure 5.10 and Figure S5.4). The COSY spectrum showed correlations of 2.62 (H-1) to 2.20/2.48 (H-2) and 2.20 (H-5), 2.20/2.48 (H-2) to 5.33 (H-3), and 1.55 (H-7) to 3.91 (H-6) and 1.52 (H-11). The pentene ring is established based on a COSY correlation (See Table 5.1 and Figure S5.6) between 2.20 (H-5) and 3.91 (H-6). The isoprenyl is then linked to the ring through a COSY bond between 1.19/1.52 (H-12) and 1.60/1/80 (H-13). The methylene proton at 4.70 (H-18) showed HMBC correlation (See Table 5.1 and Figure S5.5) to 40.6 (C-9), and 2.23 (H-9) showed a correlation to 47.7 (C-7). The HMBC correlations of the methyl proton at 1.76 (H-17) to 124.0 (C-3) and 60.4 (C-5) completed the methylated pentene ring of the diterpene (Figure 5.11). All spectroscopic data for compound **5.9** (Figure 5.12) are in agreement with literature data reported for pachydictyol A (Gedara *et al*., 2003; Ayyad *et al*., 2011). Compound **5.9** was first isolated from brown alga *Pachydictyon coriaceum* by Hirschfeld *et al*. (1973). Its occurrence in other algae species have been reported from *Dictyota flabellate* (De Andrade Moura *et al*., 2014), *Cystoseira myrica* (Ayyad *et al*., 2003) and *Pachydictyon coriaceum* (Robertson and Fenical, 1977).

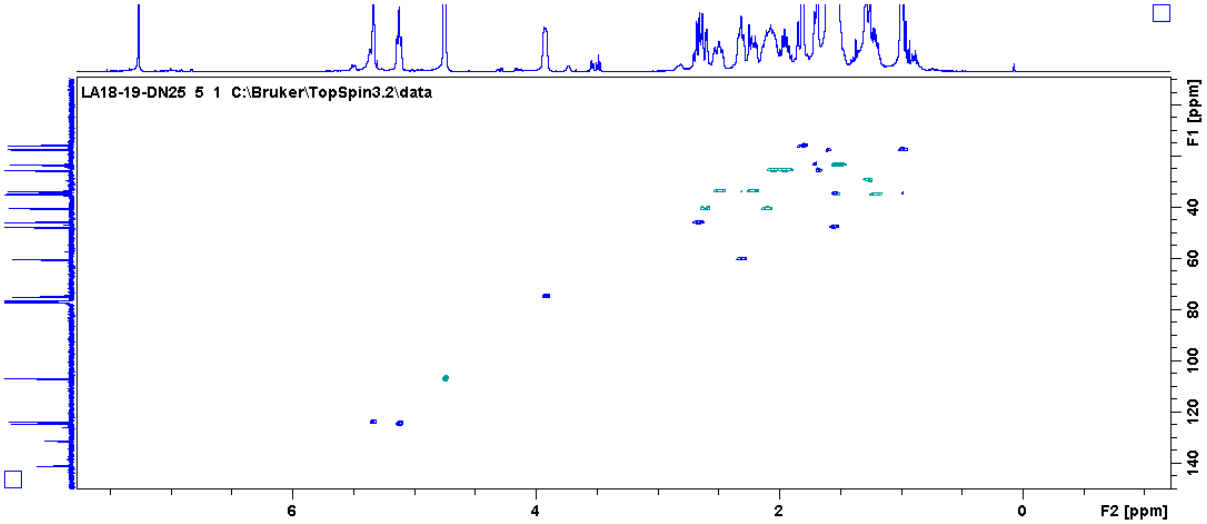


Figure 5.10: A segment of the HSQC spectrum for compound **5.9**.

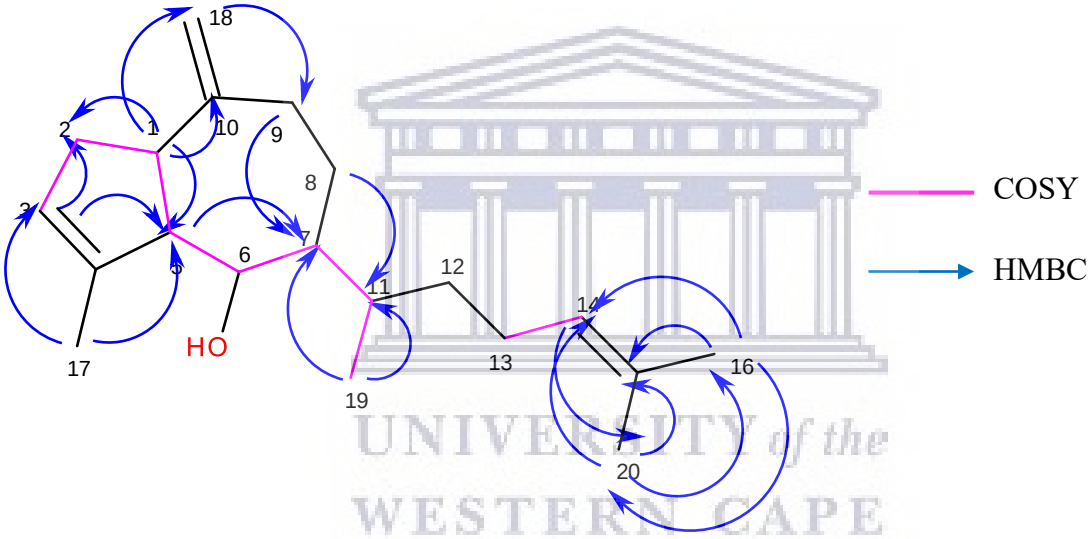


Figure 5.11: COSY and HMBC correlations of compound **5.9**; (See Figures S5.5 and S5.6 in supplementary material)

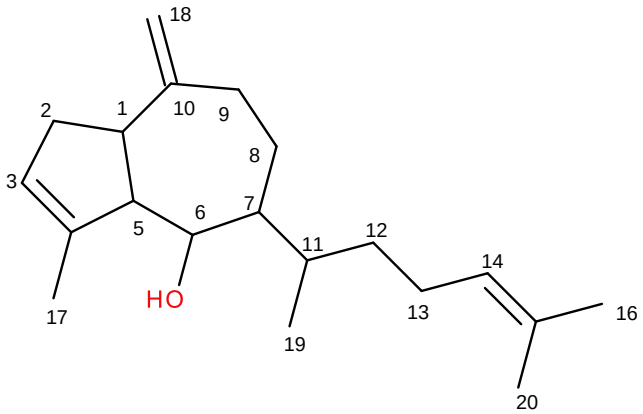
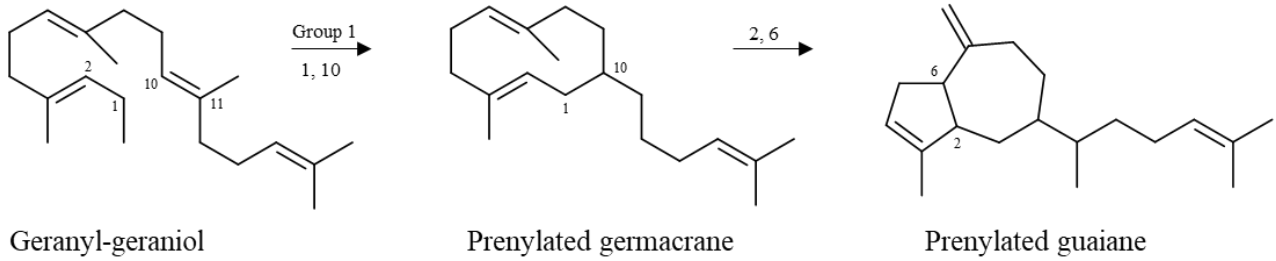


Figure 5.12: Chemical structure of compound **5.9**.

The biosynthesis of pachydictyol A in the *Dictyota* genus is thought to occur via one of the three diterpene biosynthetic pathways. Pachydictyol A is proposed to be synthesised through cyclisation of geranyl-geraniol between positions 1 and 10, and a second cyclisation between positons 2 and 6 (Scheme 5.3).



Scheme 5.3: The proposed biosynthesis of pachydictyol A (*Scheme adapted from De-Paula et al., 2012*).

Table 5.1: ^{1}H and ^{13}C NMR data in $CDCl_3$ (400 and100 MHz, respectively) for compound **5.9**

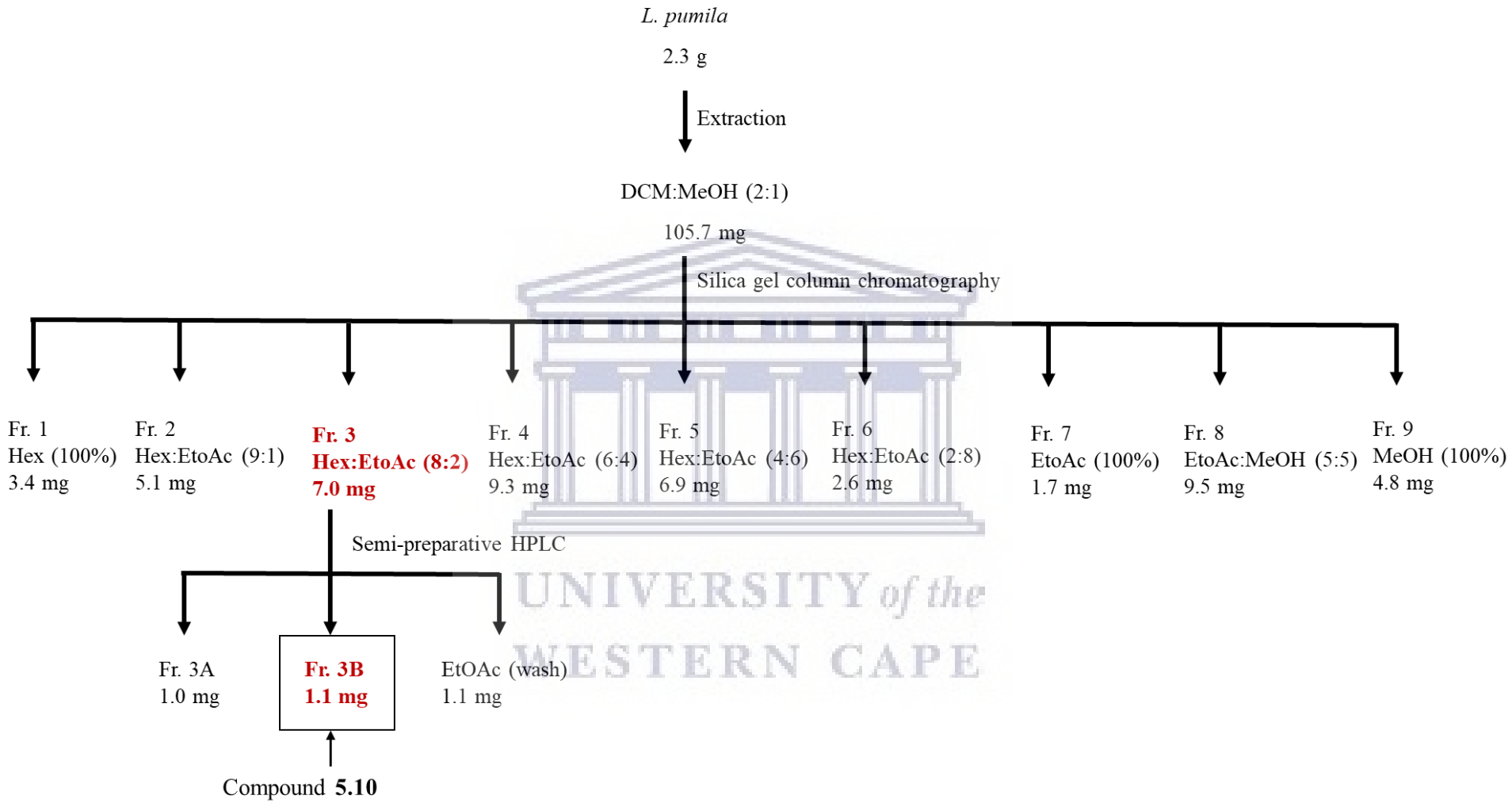
Position	δ_C	δ_C (literature)*[1]	δ_H (multiplicity, J, Hz)	δ_H (multiplicity, J, Hz) literature*[2]	COSY	HMBC
1	46.8	46.9	2.62 (m)	2.6 (1H, m)	H-2	C-2, C-5, C-10, C-18
2a b	34.7	34.7	2.20 (2H, m) 2.48 (2H, m)	2.2 (2H, m) 2.4 (2H, m)	H-1, H-3	
3	124.0	124.0	5.33 (brs)	5.2 (1H, brs)	H-2	C-2, C-5
4	141.4	141.4	---	---		
5	60.4	60.4	2.20 (m)	2.2 (1H, m)	H-1, H-6	C-7
6	75.0	75.0	3.91 (1H, d, *J* = 6.9)	3.8 (1H, d, *J* = 7)	H-5, H-7	
7	47.7	47.8	1.55 (m)	1.5 (1H, m)	H-6	
8	23.5	23.5	1.80 (m)	1.8 (2H, m)		C-11
9	40.6	40.7	2.23 (m)	2.25 (2H, m)		C-7
10	152.5	152.6	---	---		
11	35.0	35.0	1.52 (m)	1.4 (1H, m)	H-7, H-19	
12a b	35.4	35.4	1.19 (m) 1.52 (m)	1.1 (2H, m) 1.4 (2H, m)		
13a b	25.6	25.8	1.60 (m) 1.80 (m)	1.6 (2H, m) 1.8 (2H, m)	H-14	
14	124.7	124.6	5.10 (1H, brt, *J* = 7.1)	5.02 (1H, br. t, *J* = 7.5)	H-13	C-16, C-20
15	131.5	131.6	---	---		
16	25.6	25.6	1.60 (3H, s)	1.61 (3H, s)		C-14, C-15, C-20
17	15.9	15.9	1.76 (brs)	1.75 (3H, brs)		C-3, C-4, C-5
18	107.1	107.1	4.74 (br)	4.6 (2H, br)		C-9
19	17.5	17.5	0.99 (d, *J* = 5.8)	0.94 (3H, d, *J* = 6.0)	H-11	C-7, C-11
20	17.7	17.7	1.60 (3H, s)	1.54 (3H, s)		C-14, C-15, C-16

* [1]Ayyad *et al*., 2011 (^{13}C measured in $CDCl_3$ at 150 MHz); [2]Gedara *et al*., 2003 (^{1}H NMR measured in $CDCl_3$ at 400 or 500 MHz)

5.3.3. Extraction and isolation of compound **5.10** from *L. pumila*

Figure 5.13: A photograph of *L. pumila* specimen used in this study.

The organic extracts were combined and dried under reduced pressure to obtain 105.75 mg crude extract. The crude extract was chromatographed to obtain 9 fractions (Fr1-Fr9) at varying solvent mixture of hexane, EtOAc and MeOH. Fraction 3 was particularly interesting because based on the ^{1}H NMR spectrum it contained one main compound and it was also active. Fraction 3 eluted with Hex:EtOAc (8:2) was injected into a semi-preparative normal phase HPLC for further separation and purification to afford compound **5.10**. The extraction and isolation of compound **5.10** is shown below in Scheme 5.4. The 2D NMR spectroscopic results for structural elucidation of compound **5.10** are discussed and compared to literature data.



Scheme 5.4: The outline of the isolation of compound **5.10** from *L. pumila*.

5.3.4. Structure elucidation of compound **5.10**.

As previously mentioned, the DCM-MeOH of the crude extract was purified by column chromatography. This yielded a cleaner fraction which was further purified by HPLC with solvent ratio of Hexane:EtOAc (8:2) to obtain compound **5.10**. The total mass obtained for compound **5.10** was 1.1 mg which could not be further purified as the quantity was too small. Compound **5.10** was submitted for HR-LCMS analysis but this compound is known to have difficulty in ionising hence reproducible result was not obtained. In addition, the facilities to optimise the ionisation of **5.10** in order to improve on the result was not available. The ^{1}H and ^{13}C NMR spectra of fraction 3B (Fr. 3B) shows a possibility of two compounds with similar structure. This would explain why separation had not occurred with the HPLC. The fraction however showed dominant signals which was attributed to compound **5.10**. The complete structure elucidation of **5.10** could not be done due to insufficient availability of material. However, the layout/skeletal backbone of the structure could be determined from the NMR data obtained. The ^{1}H NMR spectrum (Figure 5.14) indicated the presence of a terminal methyl signal at δ 0.89 (H-15) and four methylene signals at δ 2.83 (H-5), 2.48 (H-8), 2.58 (H-11) and 1.88 (H-14). An acetylenic signal at δ 3.16 (H-1), and further upfield, four olefinic proton signals were exhibited at δ 5.61 (H-10), 5.62 (H-9), 5.56 (H-3) and 6.33 (H-4). The ^{13}C NMR spectrum displayed fifteen carbon signals (Figure 5.15). This included four methylene signals at δ 34.3 (C-5), 33.3 (C-8), 29.8 (C-11) and 29.1 (C-14), an alkyne carbon signal δ 81.6 (C-2) and nine methine carbon signals at δ 82.3 (C-1), 111.9 (C-3), 141.6 (C-4), 81.6 (C-6), 63.2 (C-7), 129.3 (C-9), 130.0 (C-10), 63.5 (C-12) and 63.2 (C-13).

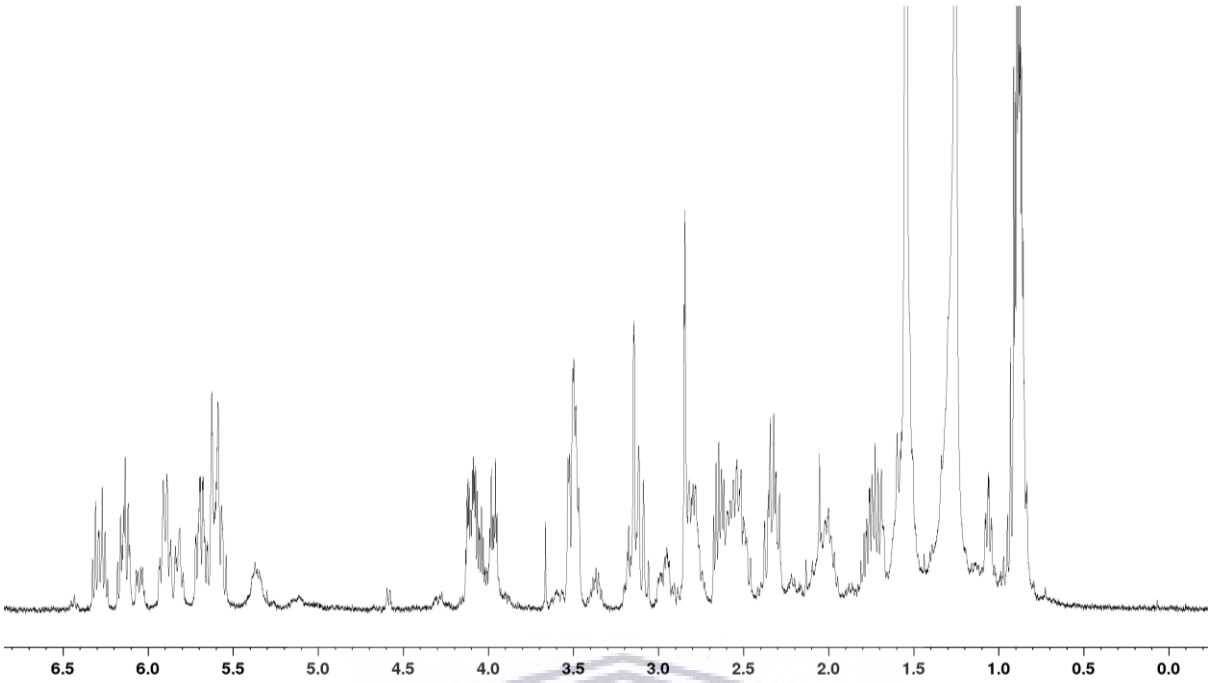


Figure 5.14: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **5.10**.

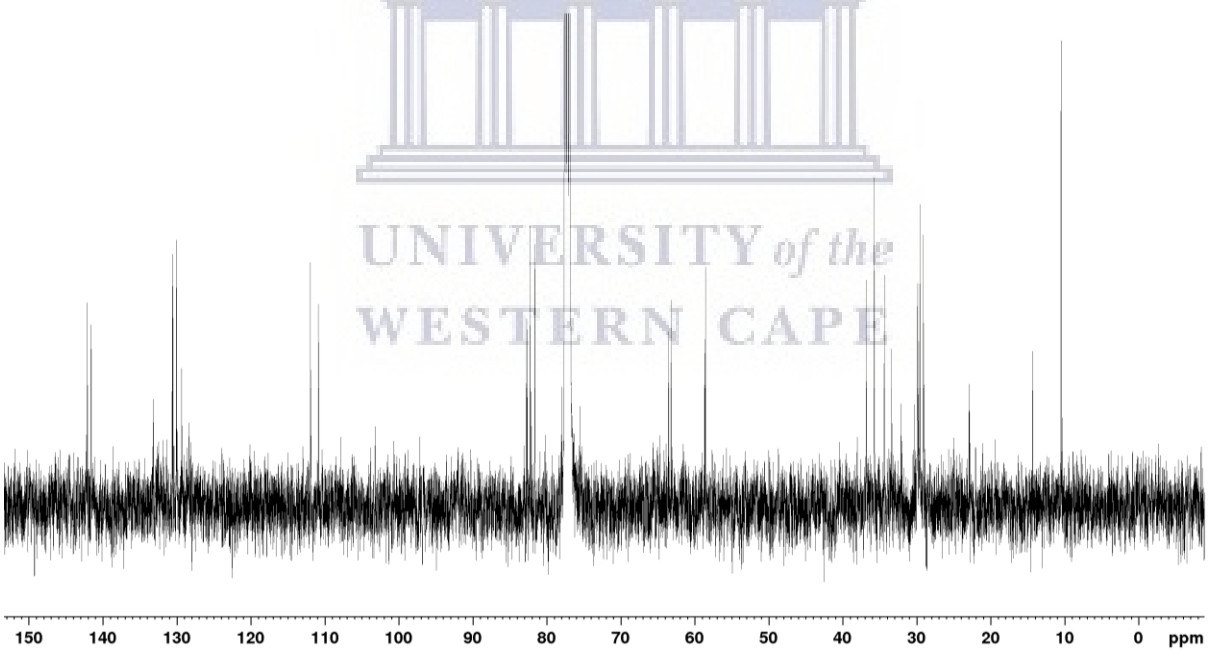


Figure 5.15: ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **5.10**.

The presence of four methine carbons signals δ 111.9 (C-3), 141.6 (C-4), 129.3 (C-9) and 130.0 (C-10) is indicative of two double bond for which 111.9 (C-3) and 141.6 (C-4) forms part of a linear chain while 129.3 (C-9) and 130.0 (C-10) is indicative of a double bond in a ring. The methine carbons 81.6 and 63.5 confirms an ether linkage. A nine membered ring was established based on the HMBC (Figure 5.16 and Figure S5.8) correlation of δ 2.68 (H-13) to the carbon δ 81.6 (C-6). The enyne side chain of the ring was established by long range correlation of δ 6.33 (H-4) to δ 81.6 (C-2) and a COSY correlation of δ 6.02 (H-4) and δ 5.56 (H-3). The skeleton is indicative of a C_{15}-acetogenin which is not uncommon in the *Laurencia* species. Literature search of the spectral data revealed a close relationship to 3Z, 12R, 13R-obtusenyne as reported by Awakura *et al*. (1999) and Norte *et al*. (1991). The major difference observed between **5.10** (Figure 5.17) and 3Z, 12R, 13R-obtusenyne were the ^{13}C chemical shift at δ C-11, C-12 and C-13 (Table 5.2). This suggest the presence of a different electronegative compound or halogen at position 12 other than bromine. This suggest the presence of a different electronegative compound or halogen at position 12 other than bromine. It has been reported that the structure of halogenated metabolites may be difficult to assign correctly due to the quaternary carbons bearing halogens atoms. A number of compounds have had their structures reviewed following detailed synthesis experiments including report by Kutateladze and Reddy, 2017. ^{13}C NMR spectrum prediction of compound **5.10** on ChemDraw 18.2, that is, substituting a bromine at position 12 for chlorine gave a very similar a spectrum to the obtained compound **5.10**. Literature search for compound **5.10**, a dichloronated version of 3Z, 12R, 13R-obtusenyne did not yield any result. ^{13}C NMR spectrum prediction of compound **5.10** on ChemDraw 18.2, that is, substituting a bromine at position 12 for chlorine gave a very similar a spectrum to the obtained compound **5.10**. Literature search for compound **5.10**, a dichloronated version of 3Z, 12R, 13R-obtusenyne did not yield any result. Further experiment to confirm the structure could not be carried out due to the small quantity of material available and the fact it requires further purification. This could in fact generate a novel halogenated C_{15}-acetogenin compound from *Laurencia pumila*.

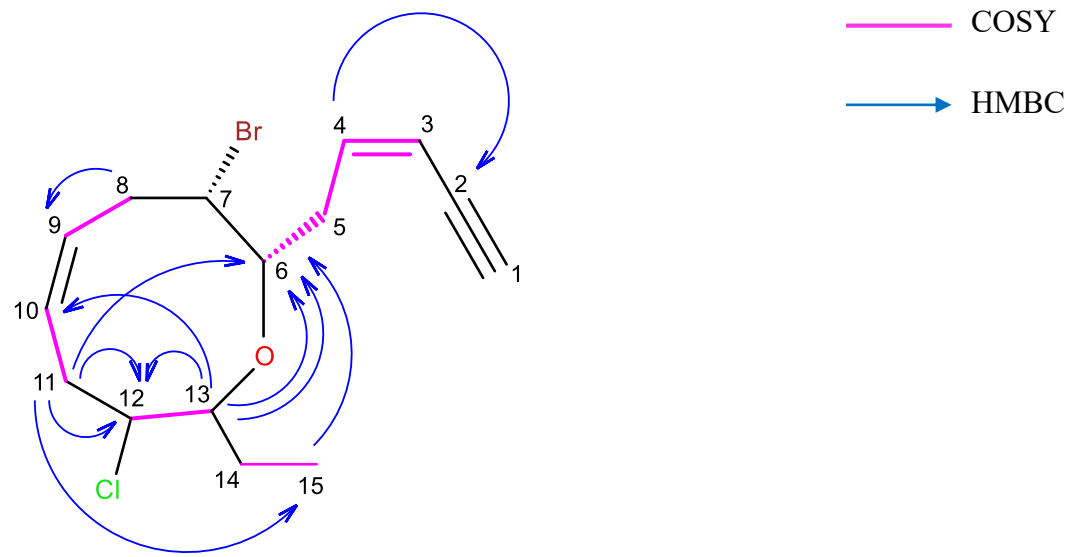


Figure 5.16: COSY and HMBC correlations of compound **5.10** (See Figures S5.8 and S5.9 in supplementary material).

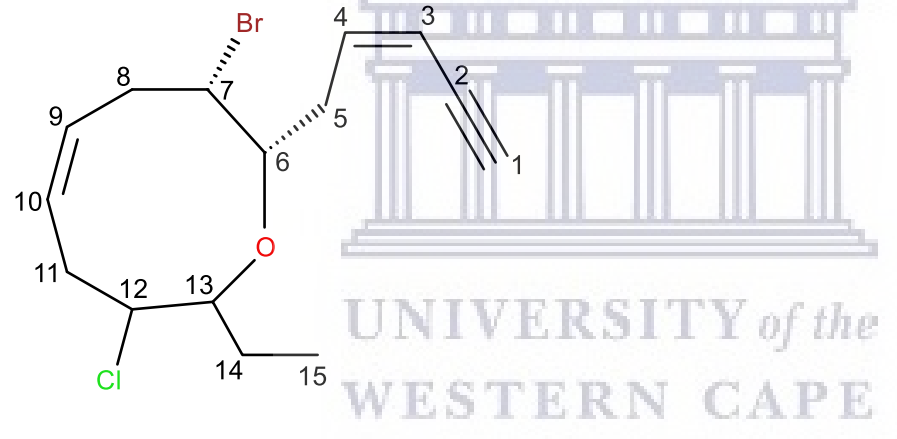


Figure 5.17: Chemical structure of compound **5.10**.

Table 5.2: ^{1}H and ^{13}C NMR data in $CDCl_3$ (400 and100 MHz, respectively) for compound **5.10**

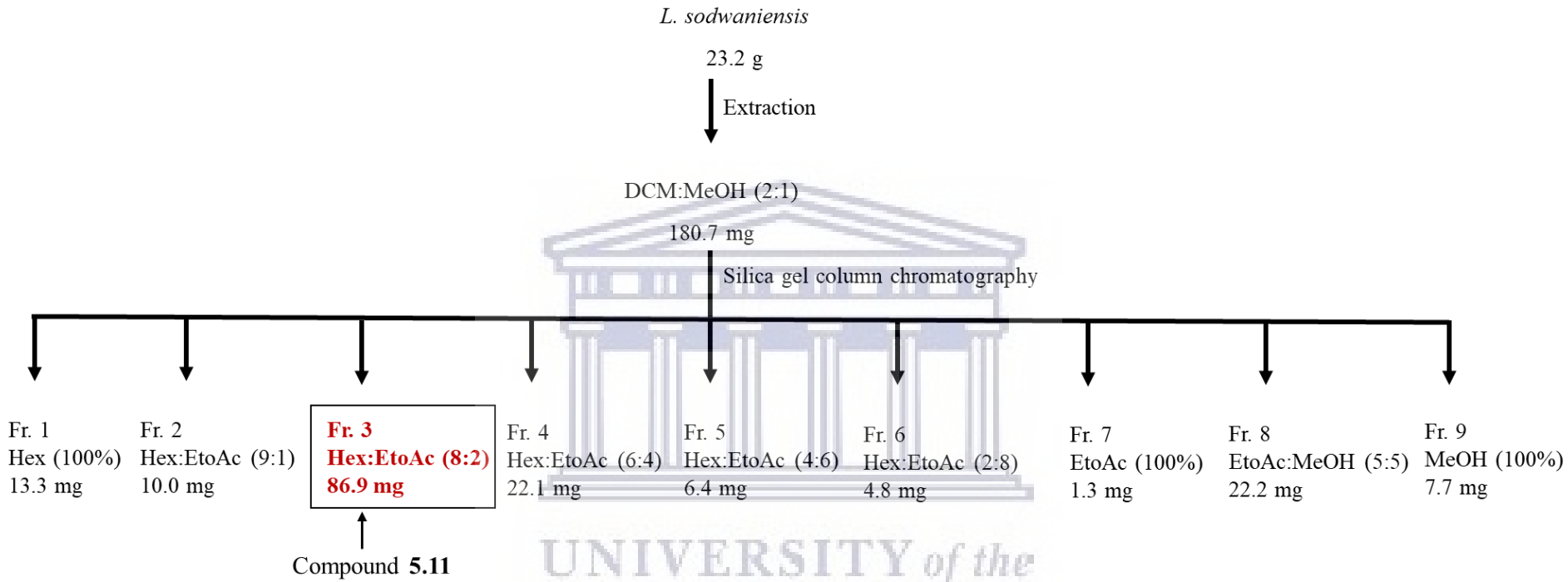
Position	**δ_C**	**δ_C (literature)***	**δ_H (multiplicity, J, Hz)**	**δ_H (multiplicity, J, Hz) literature***	**COSY**	**HMBC**
1	82.3	82.5	3.16 (m)	3.16 (brd, 1.5)		
2	81.6	---	---	---		
3	111.9	111.3	5.56 (m)	5.55 (brd, 9.5)	H-4	
4	141.6	140.3	6.33 (dt, *J* = 6.8, 15.6)	6.03 (dtd, 10.8, 7.6, 0.9)	H-3, H-5	C-2
5	34.3	34.6	2.83 (m) 2.87 (m)	2.77 (m) 2.95 (m)	H-4, H-6	
6	81.6	81.4	3.59 (m)	3.59 (brddd, 8.8, 5.6, 3.3)	H-5, H-7	
7	63.2	62.2	4.06 (m)	4.07 (ddd, 10.6, 5.4, 3.3)	H6	
8	33.3	33.5	2.48 (m) 3.22 (m)	2.46 (brddd, 13.0, 5.4, 5.4) 3.18-3.49 (m)	H-9	C-9
9	129.3	128.5	5.62 (m)	5.52-5.69 (m)	H-8	
10	130.0	130.0	5.61 (m)	5.52-5.69 (m)	H-11	
11	29.8	34.3	1.75 (m) 1.53 (m)	2.58 (brddd, 13.0, 5.4, 5.4) 3.18-3.49 (m)	H-10	C-12, C-15
12	63.5	54.7	4.25 (m)	4.27 (ddd, 10.5, 5.4, 3.1)	H-13	
13	63.2	84.0	2.68 (m)	3.23 (ddd, 8.4, 5.9, 3.1)	H-12	C-6, C-10, C-12
14	29.1	27.9	1.88 (m)	1.82-1.98 (m)	H-15	C-6
15	10.4	9.7	0.86 (m)	0.86 (t, 7.5)	H-15	C-5, C-6, C-14

* Awakura *et al*., 1999

5.3.5. Extraction and isolation of compound **5.11** from *L. sodwaniensis*

Figure 5.18: A photograph of *L. sodwaniensis* specimen used in this study.

A sample of thawed *L. sodwaniensis* (23.22 g) was extracted with CH_3OH (75 mL) at 4 °C overnight. The alga was further extracted thrice with CH_3OH-CH_2Cl_2 (1:2, 75 mL) at 36 °C. The extracts were combined and dried *in vacuo* to yield 180.70 mg crude residue. The extract was applied to a silica gel column and successively eluted with variable mixtures of hexane, EtOAc and MeOH ratios (as shown in Scheme 5.5) to obtain 9 fractions, Frs1-9. Fraction 3 (hexane–EtOAc (8:2) from which compound **5.11** was isolated was further analysed by 2D NMR spectroscopy.



Scheme 5.5: The outline of the isolation of compound **5.11** from *L. sodwaniensis*.

5.3.6. Structure elucidation of compound **5.11**.

Compound **5.11** was submitted for HR-LCMS analysis but ionisation was also a challenge, and as a result reproducible data was not obtained as the facilities to optimise ionisation of **5.11** in order to acquire better result was not available. The ^{1}H NMR spectrum (Figure 5.19) of compound **5.11** showed three distinct methyl signals at δ 1.03 (H-13), 1.32 (H-12) and 1.74 (H-15). Five overlapping methylene proton signals were observed at δ 1.74/2.06 (H-1), 1.82/2.39 (H-2), 2.78/3.08 (H-5), 2.45/2.70 (H-8), 4.95/5.19 (H-14) as well as three methine signals at δ 4.42 (H-10), 3.67 (H-9) and 4.42 (H-4). The integration of signals above 4.5 suggests the presence of impurity signals due to the smaller peaks close to 5.0 ppm in Figure 5.19. The signals at around 1.3 ppm on the same spectral data are absent in the spectra in supplementary material reported by Da-Silva-Machado *et al*., 2011. The ^{13}C NMR spectrum showed 15 carbon signals consistent with sesquiterpenes (Figure 5.20). Five of these carbon signals were confirmed as methylene carbons with a ^{13}C DEPT-135 NMR spectrum showing signals at δ 24.3 (C-1), 32.4 (C-2), 33.8 (C-5), 39.3 (C-8), and 114.7 (C-14) (Figure 5.21).

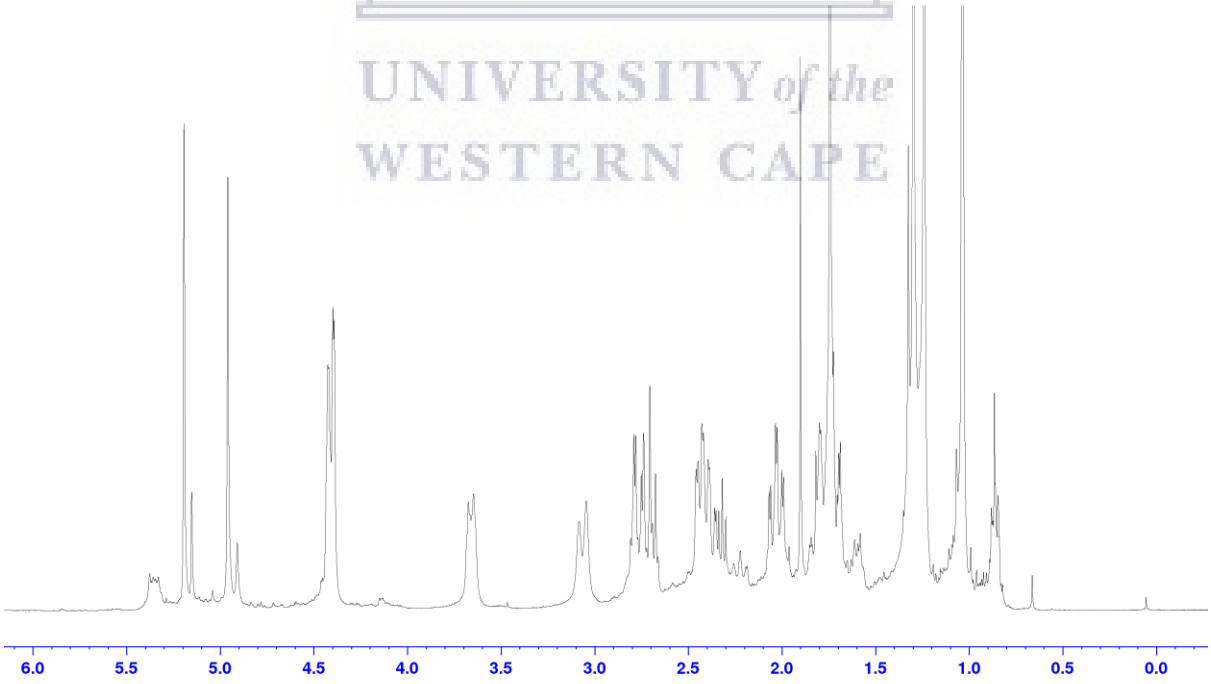


Figure 5.19: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **5.11**.

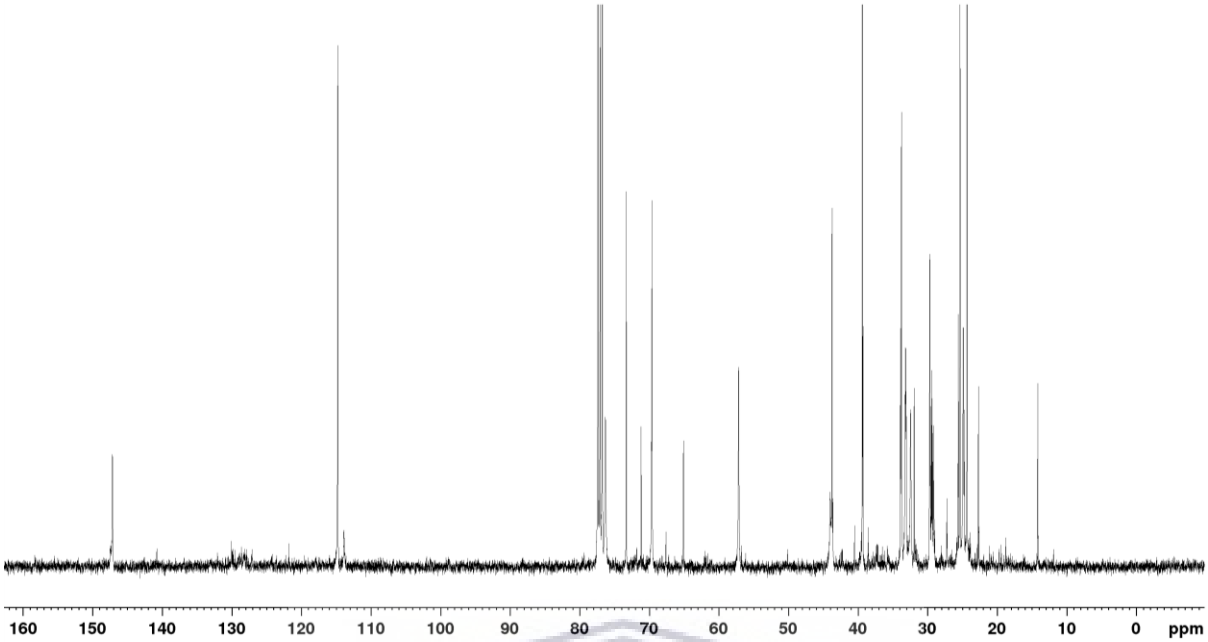


Figure 5.20: ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **5.11**.

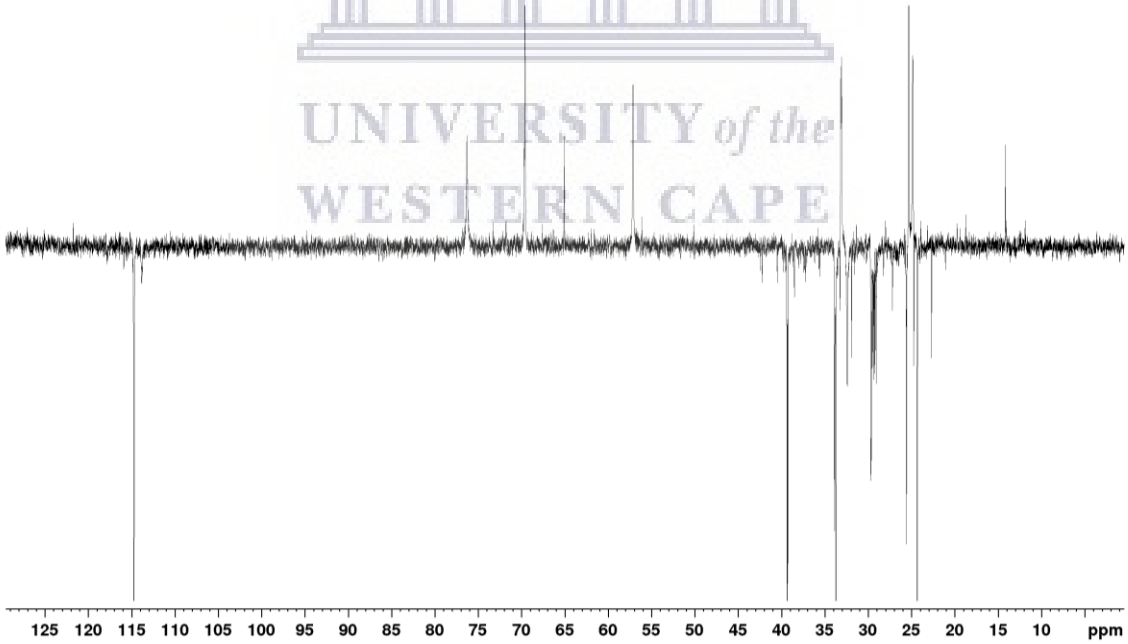


Figure 5.21: ^{13}C DEPT-135 of compound **5.11**.

Three methyl signals were observed at δ 24.8 (C-12), 25.3 (C-13) and 33.1 (C-15). The presence of halogenated carbons was established by the three carbon signals observed at 57.1 (C-4), 73.3 (C-3) and 76.3 (C-10). The deshielding of the methyl group at δ 33.1 (C-15) is indicative of the close proximity of a halogen to the methyl group. The HMBC spectrum showed correlation of 4.42 (H-4) to 33.1 (C-15) (Figure 5.22). Since a brominated methine appears generally upfield from its chlorinated analog (Crews *et al*., 1984), the ^{13}C signal at 57.1 (C-4) is indicative of a bromomethine at 57.1 (C-4) and a chlorination at 73.3 (C-3). All spectroscopic data for compound **5.11** (Figure 5.23) were in agreement with literature data reported for cartilagineol by Francisco *et al*. (1998) through establishing the correct structure by X-ray crystallography and 2D NMR experiments. It is through this findings that Da-Silva-Machado et *al*. (2011) and Kutateladze et *al*. (2017) validated the identification of the structure of cartilagineol. The HR-LCMS of fraction 3 from which **5.11** was derived showed a base peak at m/z 305.1527 which is not consistent with any of the fragments expected from the ionisation of **5.11** as reported in literature. The molecular ion of **5.11** was also expected at 414.6 g/mol, however, it is not present in the HR-LCMS.

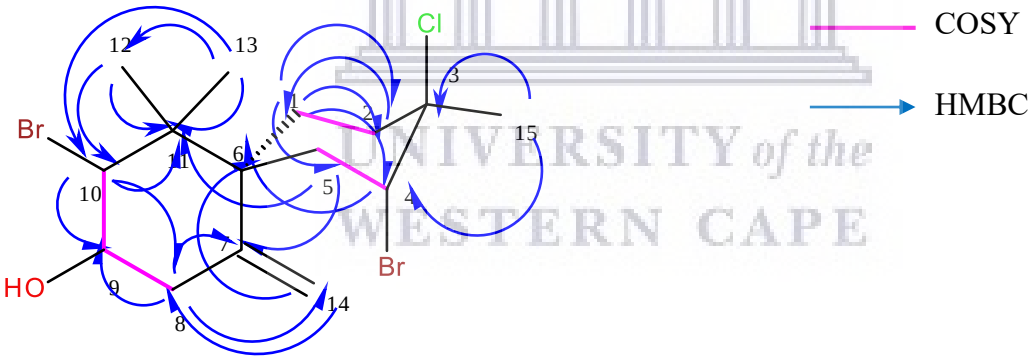


Figure 5.22: COSY and HMBC correlations of compound **5.11** (See Figures S5.11 and S5.12 in supplementary material).

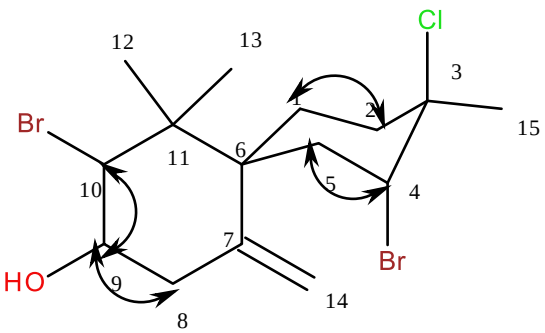


Figure 5.23: Chemical structure of compound **5.11**.

Table 5.3: ^{1}H and ^{13}C NMR data in $CDCl_3$ (400 and100 MHz, respectively) for compound **5.11**.

Position	**δ_C**	**δ_C (literature)***	**δ_H (multiplicity, J, Hz)**	**δ_H (multiplicity, J, Hz) literature***	**COSY**	**HMBC**
1	24.3	24.3	2.06 (td, 13.6, 3.8) 1.74 (m)	2.05 (dt, 13.6, 4.2) 1.70 (dd, 6.0, 3.2)	H-2	C-2, C-3, C-4, C-5
2	32.4	32.5	2.39 (m) 1.82 (m)	2.38 (dd, 13.6, 2.8) 1.82 (dd, 6.0, 3.2)	 H-1	C-1
3	73.3	73.3	---	---		
4	57.1	57.2	4.42 (m)	4.45 (m)	H-5	C-6
5	33.8	33.8	3.08 (d, 16.4) 2.78 (m)	3.08 (d, 16.0) 2.78 (dd, 16.0, 4.0)	H-4	C-7, C-11
6	43.9	44.0	---	---		
7	147.1	147.2	---	---		
8	39.3	39.4	2.70 (t, 11.9) 2.45 (m)	2.70 (t, 11.9) 2.45 (m)		C-7, C-9, C-14
9	69.6	69.6	3.67 (dt, 11.9, 4.1)	3.68 (dt, 11.9, 4)	H-8, H-10	
10	76.3	76.4	4.42 (m)	4.41 (q, 3.7, 1.6)	H-9	C-8, C-9, C-11
11	43.7	43.7	---	---		
12	24.8	24.8	1.32 (s)	1.31 (s)		C-10, C-11
13	25.3	25.3	1.06 (s)	1.04 (s)		C-10, C-11, C-12
14	114.7	114.7	5.19 (sl) 4.95 (sl)	5.20 (sl) 4.97 (sl)		C-6, C-8
15	33.1	33.1	1.74 (sl)	1.75 (sl)		C-2, C-3, C-4
OH	---	---	2.25 (m)	2.27 (d, 11.3)		

* Da-Silva-Machado *et al*., 2011

5.4. Conclusion

This chapter describes the isolation of a diterpene pachydictyol A (**5.9)** from *D. naevosa*, a C_{15}-acetogenin (**5.10**) from *L. pumila* and a chamigrane sesquiterpene cartilagineol (**5.11**) from *L. sodwaniensis* for the first time in these species. Structural elucidation of the compounds were based on detailed analysis of the spectroscopic data generated with reference to literature data.

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Chapter 6

The susceptibility evaluation of methicillin resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium aurum* to metabolites from *Dictyota naevosa*, *Laurencia pumila* and *Laurencia sodwaniensis*

6.1. Introduction

One of the earliest and commonly used antimicrobial susceptibility test methods is broth microdilution. The advantages of this technique include reproducibility, multiple testing of antibiotics at once, generation of quantitative result such as the MIC (lowest concentration of antibiotics that can inhibit bacterial growth) and low cost of test materials such as the microtiter plate (Jorgensen and Ferraro, 2009). This technique however can be tedious and labour intensive. Other methods including disk or agar diffusion and TLC-bioautography may also be used for susceptibility test. Cytofluorometric and bioluminescent methods are alternatives but are not widely used because they require specified equipment and further evaluation for reproducibility and standardisation despite being effective means of determining impact of the antimicrobial agent on the test strain microorganism (Balouiri *et al*., 2016).

This chapter describes the susceptibility assessment of extracts and isolated compounds from *D. naevosa*, *L. pumila* and *L. sodwaniensis* against MRSA and *M. aurum* by TLC-bioautographic and microdilution methods. The tests were carried out to determine the efficacy of the metabolites on the bacteria grown on a solid and in a nutrient broth media. The susceptibility activity evaluation on these platforms provides possible applications of the identified compounds as antibacterial agents.

6.2. Experimental

6.2.1. Preparation of MRSA ATCC 33591 and M. aurum A+ test strains

The algae extracts and compounds were tested for bioactivity using MRSA ATCC 33591 and *M. aurum* A+. The MRSA ATCC 33591 and *M. aurum* A+ test strains were prepared as described in Chapter 3, section 3.2.6.4 and 3.2.6.5, respectively. The optical density of the cultures were

measured at OD_{600} and the cultures OD were adjusted to 0.8 prior to use in microdiultion assay and 0.5 for TLC-bioautography assay.

6.2.2. Microdilution assay

A 500 µg/mL stock solution of each algae extract and compound were prepared by dissolving in DMSO. Serial dilution to concentrations of 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 µg/mL was established for each sample. A 20 µL of the serial dilution were added to 96-well plate containing 180 µL of bacterial inoculum per well for a final volume of 200 µL per well, with each one tested in triplicates. Positive control included 180 µL of culture plus 20 µL of vancomycin (10 mg/mL). Negative control was 180 µL of culture and 20 µL of DMSO, and the sterile control was made up of 180 µL of broth and 20 µL of DMSO. Following dispensing of samples and controls into the plate as per setup, the plate was covered with a breathe-easy sealing membrane and was incubated for 24 h at 37 °C. Following incubation, 20 µL of 0.25% (w/v) MTT was added to each well and further incubated at 37 °C for 3 h. The plate was placed in a microplate reader and the optical density was read at OD_{570}. The readings were used to calculate the percentage growth of each sample using the following formulae outlined:

$$\% \text{ growth} = \text{Final value } \frac{(\text{mean of triplicate dilution concentration})}{\text{MRSA (mean of triplicate negative control)}} \times 100$$

$$\% \text{ growth positive control} = \frac{(\text{mean of triplicate vancomycin dilution concentration})}{\text{MRSA (mean of triplicate negative control)}} \times 100$$

$$\% \text{ growth negative control} = \frac{(\text{mean of triplicate DMSO dilution concentration})}{\text{MRSA (mean of triplicate negative control)}} \times 100$$

6.2.3. TLC-bioautography assay

The TLC-bioautography tests of the algae crude extracts was carried out against *M. aurum* A+ at a concentration of 10 mg/mL. The purified compounds were tested against MRSA ATCC 33591 at a concentration of 20 µg. The assay was carried out as described in Chapter 3, section 3.2.4.

6.3. Results and discussion

The susceptibility of MRSA to *D. naevosa, L. pumila* and *L. sodwaniensis* extracts and isolated compounds is shown Figure 6.1. Neither the extracts nor the compounds showed striking activity against MRSA when tested by the microdilution method. This is in direct contrast to the results obtained from the TLC-bioautography assay. The result shows that the dilution concentration at which *D. naevosa* extract appear to show some activity against MRSA growth was at 1.56 µg/mL and with the least percentage growth of 93.74 %. MRSA growth was only slightly suppressed at 3.12 µg/mL and 0.78 µg/mL. Ideally, it is expected that the higher concentration would show increased MRSA inhibition, however, these concentrations appear to enhance the growth of the organism. Similar observation was reported by Luziatelli *et al*. (2019) who observed that vegetal derived bioactive compounds and extracts from a tropical lettuce stimulated the growth of epiphytic bacteria with biological control activity against pathogens. In addition, the extract may not be fully solubilised in liquid medium (Delgado *et al*., 2005). The result also showed that MRSA growth was not susceptible to *L. pumila* and *L. sodwaniensis* extracts as the percentage growth were above 100% when tested within the set concentration range evaluated. Similar to *D. naevosa*, the highest concentrations of *L. pumila* and *L. sodwaniensis* extracts resulted in the highest MRSA percentage growth. Based on these observations, it appears the susceptibility of MRSA to algal extracts is not dose-responsive. Perhaps higher concentrations could have been tested but it is likely that the poor solubility of the compounds is the reason for the poor activity. Vancomycin has been reported as effective antimycobacterium, therefore, it was used as the standard (Rens *et al*., 2014). Figure 6.1 also shows the susceptibility of MRSA to compounds **5.9**, **5.10** and **5.11**. The result shows that MRSA growth was not susceptible to **5.9** as the percentage growth was above a 100% across the dilution concentration ranges. The *D. naevosa* extract from which **5.10** was isolated shows inhibitory activity against MRSA growth at 3.12 µg/mL, 0.78 µg/mL and 0.39 µg/mL whereas an increase in growth was observed at these concentrations with the purified compound. This suggests that **5.9** may be insoluble, or that the dilution concentration at which **5.10** suppresses MRSA growth is at 12.5 µg/mL. The growth of MRSA is also susceptible to **5.11** at 3.12 µg/mL, 1.56 µg/mL, 0.78 µg/mL and 0.39 µg/mL. It is also quite possible that the antibacterial activity is dependent on the synergistic action of other bioactive components present in the extracts (Pérez *et al*., 2016). It has been well documented that taking the whole plant or extracts with no isolation of components, as practiced in traditional medicine, produces a better

therapeutic effect than individual compounds. According to Thomford *et al*. (2018), this is important as most of the plant metabolites likely work in a synergistic fashion or concurrently to give the plant extract its therapeutic effect.

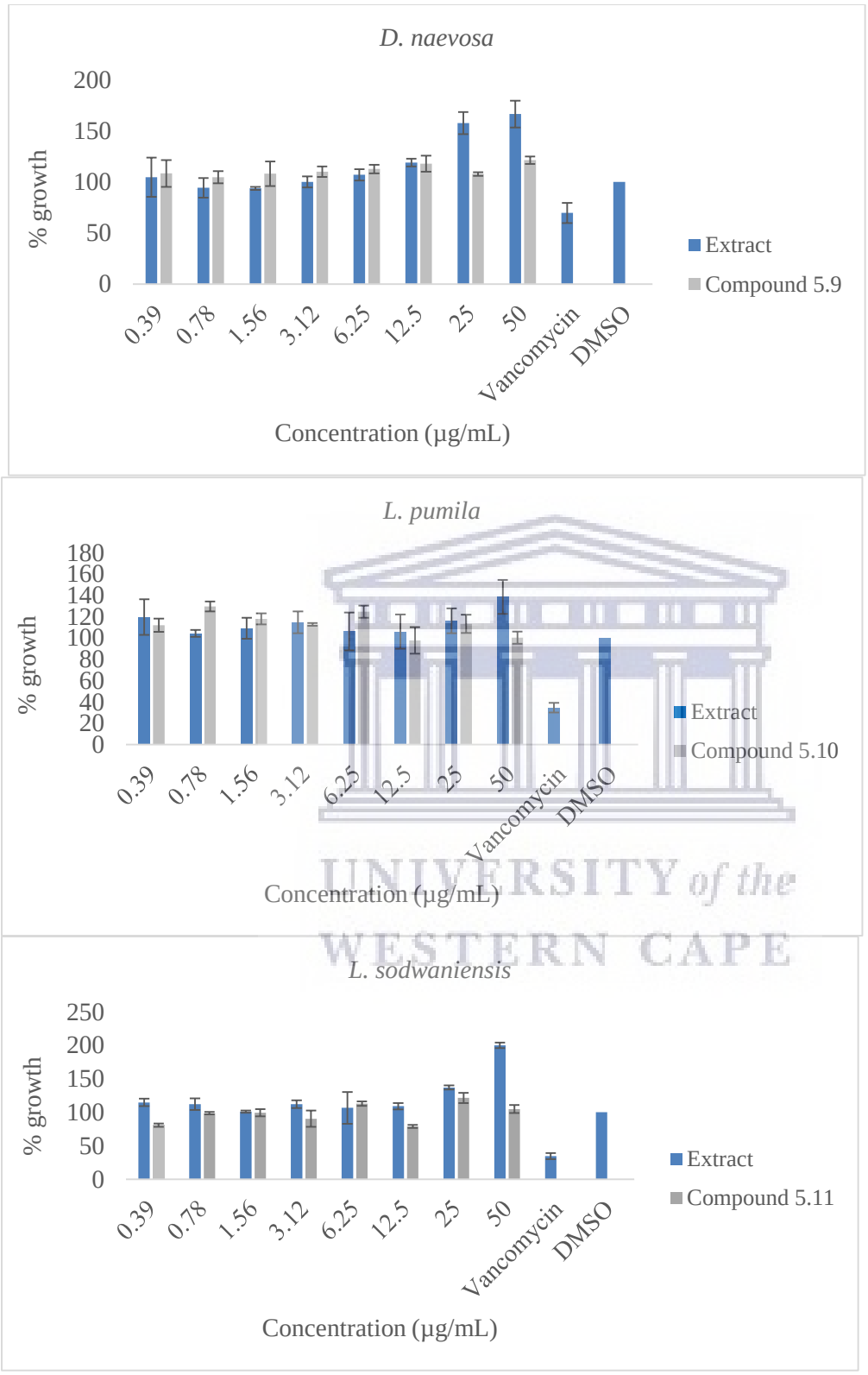

Figure 6.1: Evaluation of susceptibility of MRSA to extracts and compounds of *D. naevosa*, *L. pumila* and *L. sodwaniensis* by microdilution method.

A contrast in the activity of these metabolites in nutrient broth and TLC silica plate is observed. On the TLC silica plates the extracts showed inhibition of MRSA growth whereas an increase in growth rate was detected in the liquid medium. Factors which can influence growth include the medium in which the metabolites were applied whether solid or liquid and also the nutrients present in the liquid medium (Wiegand *et al*., 2008). The application of MRSA on a solid surface such as the silica gel plate permits for direct contact with the metabolites whereas the liquid medium with nutrients may have caused MRSA to thrive.

Figure 6.2 shows the microdilution of *M. aurum* to extracts of *D. naevosa*, *L. pumila* and *L. sodwaniensis*. The extracts of *D. naevosa* did not show inhibition of *M. aurum* at any of the tested concentrations instead an increase in growth was observed. Although the observed activity was poor, *L. pumila* extracts did show slight inhibition of *M. aurum* with the most activity observed at 88.21% at 3.12 µg/mL. An increase in growth was exhibited at 25 µg/mL. *M. aurum* was susceptible to the extracts of *L. sodwaniensis* across all the tested concentration with the highest inhibitory activity observed at 88.98% at 0.78 µg/mL.

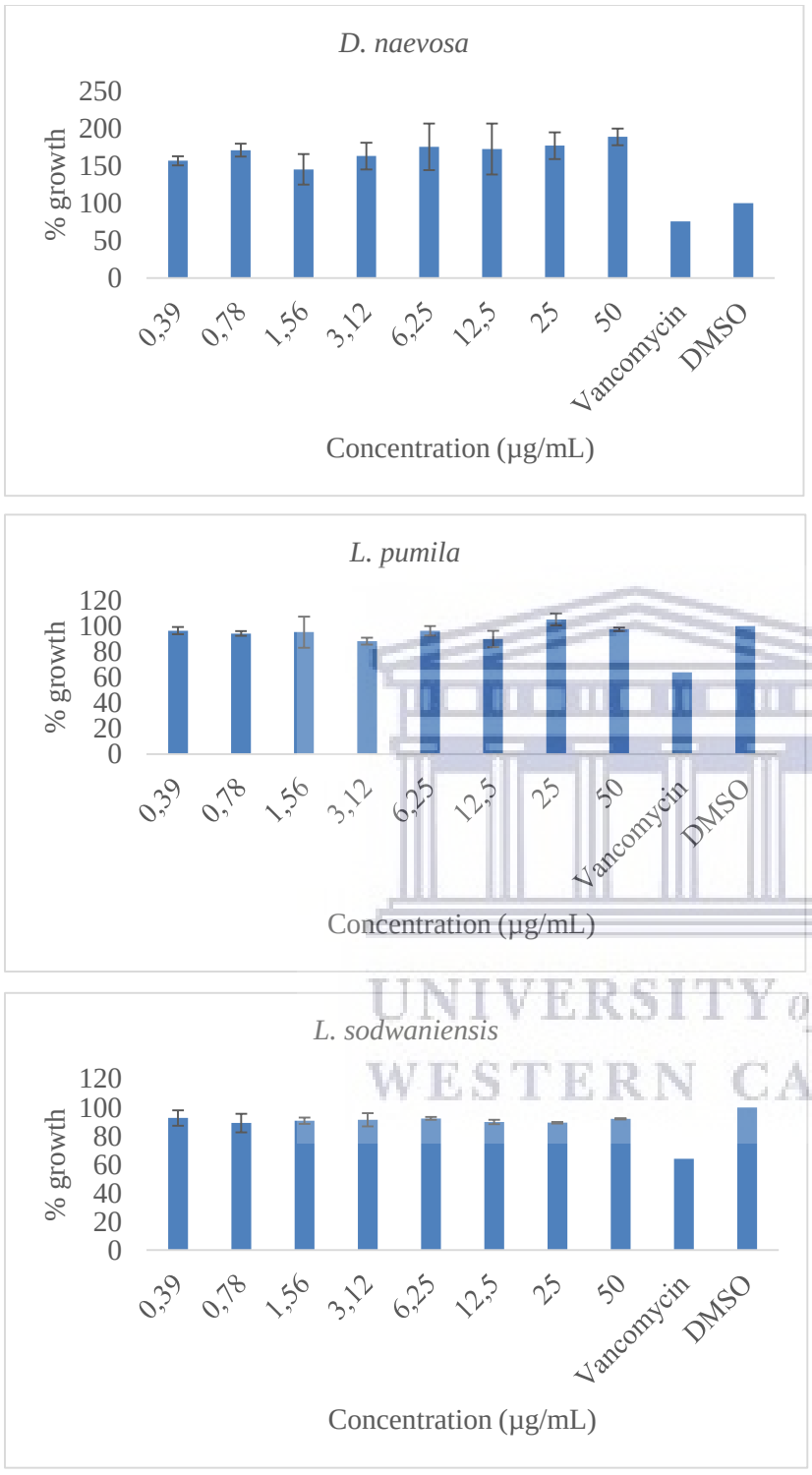

Figure 6.2: Evaluation of susceptibility of *M. aurum* to extracts of *D. naevosa*, *L. pumila* and *L. sodwaniensis* by microdilution method.

Figure 6.3 shows TLC-bioautographic result of the extracts of *D. naevosa* (**A**), *L. pumila* (**B**) and *L. sodwaniensis* (**C**) against *M. aurum*. Distinct antibacterial activity was observed represented by the clear white spot at the zone of inhibition. Rifampicin (**D**) was used as a positive control against *M. aurum* because it is a known antimycobacterial agent (Koch *et al*., 2014). Dichloromethane (E) served as a negative control.

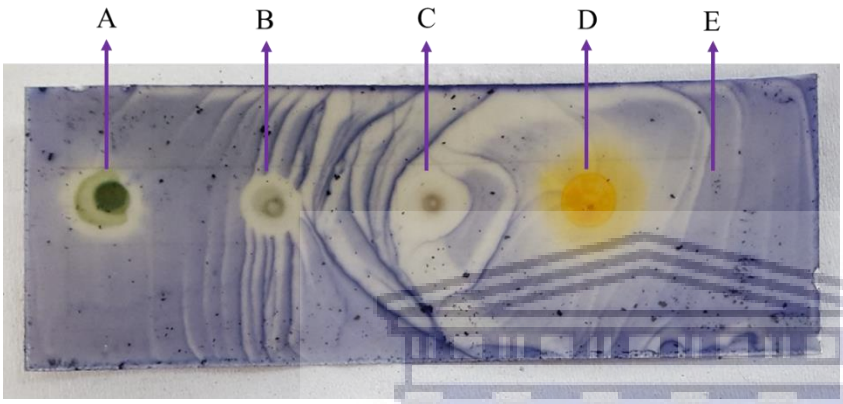

Figure 6.3: Thin-layer bioautography of algae extracts against *M. aurum*. (**A)** *D. naevosa*. (**B**) *L. pumila* (**C**) and *L. sodwaniensis* **(D)** Rifampicin **(E)** Dichloromethane. All assays were in duplicates; clear zone-inhibition of *M. aurum* by compounds.

Figure 6.4 shows TLC-bioautographic result of isolated compound **5.9**, **5.10** and **5.11** (A, B and C) against MRSA tested at a concentration of 20 µg, respectively. The antibacterial activity of the compounds is represented by the distinct well-defined clear spots present at the zones of inhibition. The efficacy of the compounds are also shown by the bigger size of the inhibitory zones as compared with a positive control, Vancomycin (D), with a clear but smaller size of inhibitory zones. Dichloromethane (E) serves a negative control showing no effect, hence the TLC plate retained the purple colour which signifies MRSA growth.

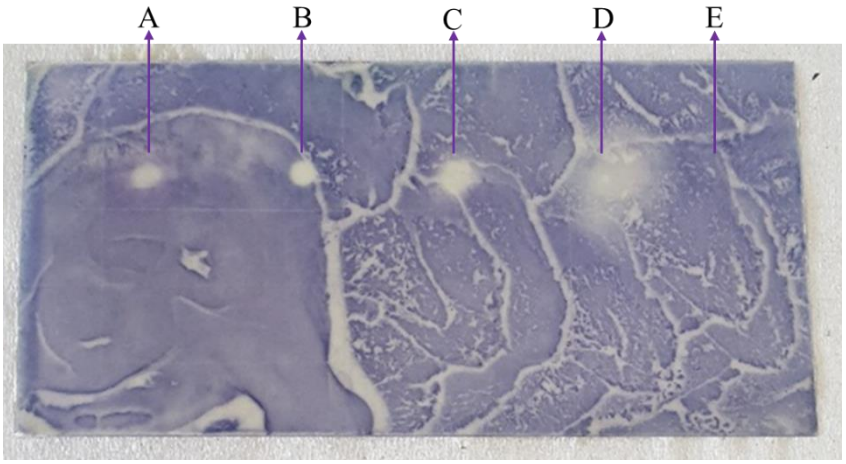

Figure 6.4: Thin-layer bioautographic assay of compounds **5.9**, **5.10** and **5.11** against MRSA. **(A)** 5.9 **(B)** 5.10 **(C)** 5.11 **(D)** Vancomycin **(E)** Dichloromethane. All assays were in duplicates; clear zone-inhibition of MRSA by compounds.

6.4. Conclusion

The extracts and compounds displayed good antibacterial and antimycobacterial activities against MRSA and *M. aurum*, respectively from the TLC-bioautographic assay. However, antibacterial activity was not exhibited in microdilution assay which is likely due to insolubility of the metabolites in a liquid medium. The metabolites also displayed poor antimycobacterium activity against *M. aurum* in an aqueous medium. It is probably more likely that the low tested concentration is responsible for the poor activity. These compounds and it is typical that algae compounds are lipophilic hence the poor MIC data obtained is more likely due to the poor water solubility of the compounds. Perhaps an alternative approach would be to incorporate the metabolites into a delivery systems such as solid lipid nanoparticle which may potentially aid in diffusion of the metabolites in broth medium thereby improving solubility.

6.5. References

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Chapter 7
General conclusion and recommendations

7.1. Summary

The emergence of antibiotic resistance of pathogenic microorganisms continues to be a major health challenge (World Health Organisation, 2020). The current available antimicrobial drugs are becoming less effective. In particular, MRSA and TB are becoming less susceptible to current antibiotic treatment. Therefore, continued research studies towards finding new antibacterial leads is essential. Marine organisms have been documented in literature as producers of secondary metabolites with a broad spectrum of pharmacological acivities including antimicrobial (Schinke *et al*., 2017). Therefore, this research study sought to explore marine organisms for antibacterial lead compounds.

In this study, 38 marine algae that were available in the MBD collection were extracted to establish a library of algal extracts. The extracts were screened for antimicrobial activity against MRSA. Of these, 24 showed good activity. Based on their antimicrobial activity and interesting chemical profiles, three South African algae species namely *Dictyota naevosa*, *Laurencia pumila* and *Laurencia sodwaniensis* were selected for chemical and structural characterisation. A known diterpene (pachydictyol A) and a known sesquiterpene (cartilagineol) were isolated from *D. naevosa* and *L. sodwaniensis*, respectively. A small quantity of an unusual C_{15}-acetogenin was also isolated from *L. pumila*. Broth dilution assay of the algae extracts and compounds against MRSA was carried out and poor antimicrobial activity was observed. Due to insufficient material availblity, additional tests could not be carried out.

In addition, six unidentified marine bacterial isolates were obtained from the IMBM collection. DNA sequencing was carried out to determine their identity. Three of the isolates (PE05-143, PE14-28 and PE14-104) showed highest identity to *Paenibacillus glucanolyticus* NBRC 15330, *Vibrio splendidus* LGP32 and *Vibrio pomeroyi* CAIM 578 strains, respectively. Sloppy-agar overlay test and TLC-bioautography assay of the isolates using MRSA ATCC 33591 and *Mycobacterium smegmatis* LR222 were carried out and showed antimicrobial activity. Genome sequencing of the isolates was also carried out to facilitate mining of the secondary metabolite biosynthetic pathways for analysis, and to characterise the predicted compounds that may have been responsible for the observed antimicrobial activity. Although no final compound was

predicted, the analysis suggested novel biosynthetic pathways in the isolates with these being similar for PE14-28 and PE14-104. The novel pathway could also result in novel bioactive compounds.

7.2. Limitations of study

The main factors which caused hindrance to this study were attributed to the small amount of metabolites isolated from the algae. Due to insufficient availability of algae start material, large scale extraction could not be carried out to further isolate more compounds. Another issue encountered was lack of activity of the extracts and isolated compounds in a liquid medium as it pertains to broth dilution assays. It could be that both the extracts and compounds were insoluble in the DMSO solvent used. However, DMSO is a polar aprotic solvent capable of dissolving polar and non-polar compounds and is miscible with water.

7.3. Recommendations

The extraction and re-isolation of the compounds on a larger scale from the algae species is important. Greater quantity of extracts will enable further separation and purification of the compounds by chromatographic means to better characterise the compounds structurally. In addition, the compounds can then be incorporated into solid lipid nanoparticles to improve solubility. Following which further biological and cytotoxicity assays can take place to gain clarity on the mode of action of isolated compounds.

In vivo characterisation of the bacterial isolates could be carried out to culture and extract potential active metabolites. Identification of the possible active metabolites can be determined using HR-LCMS and NMR spectroscopy.

7.4. References

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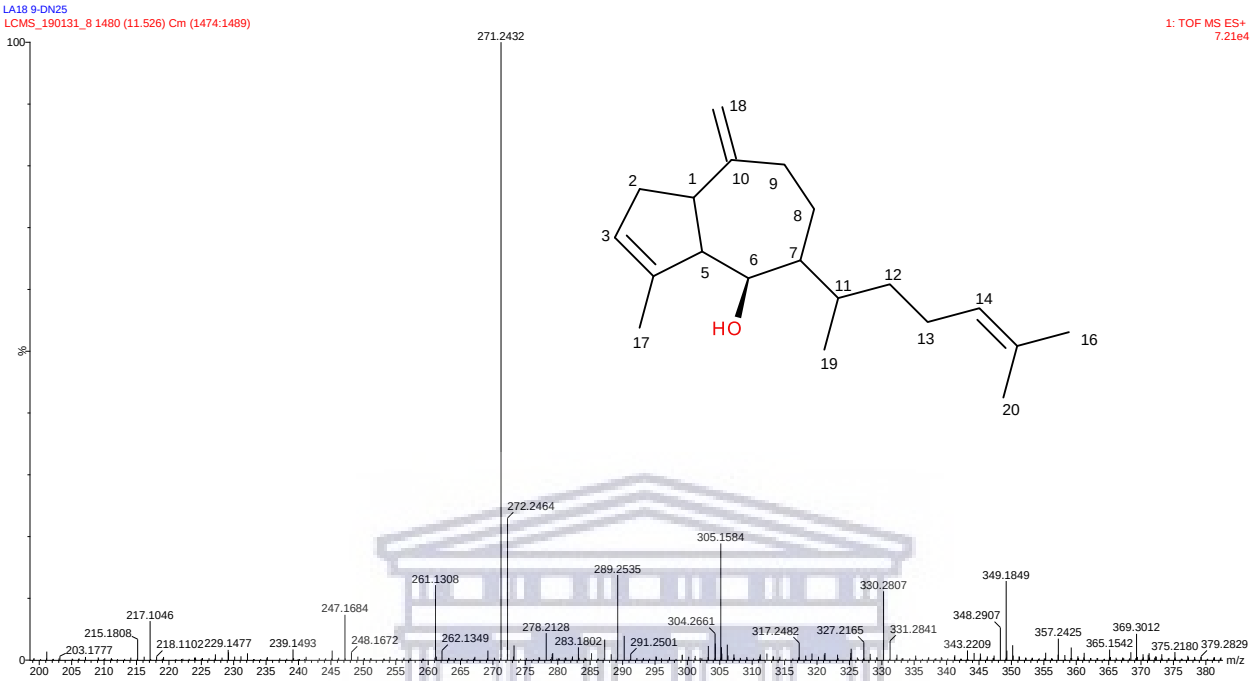

Figure S5.1: Liquid chromatography mass spectrometry of compound **5.9**.

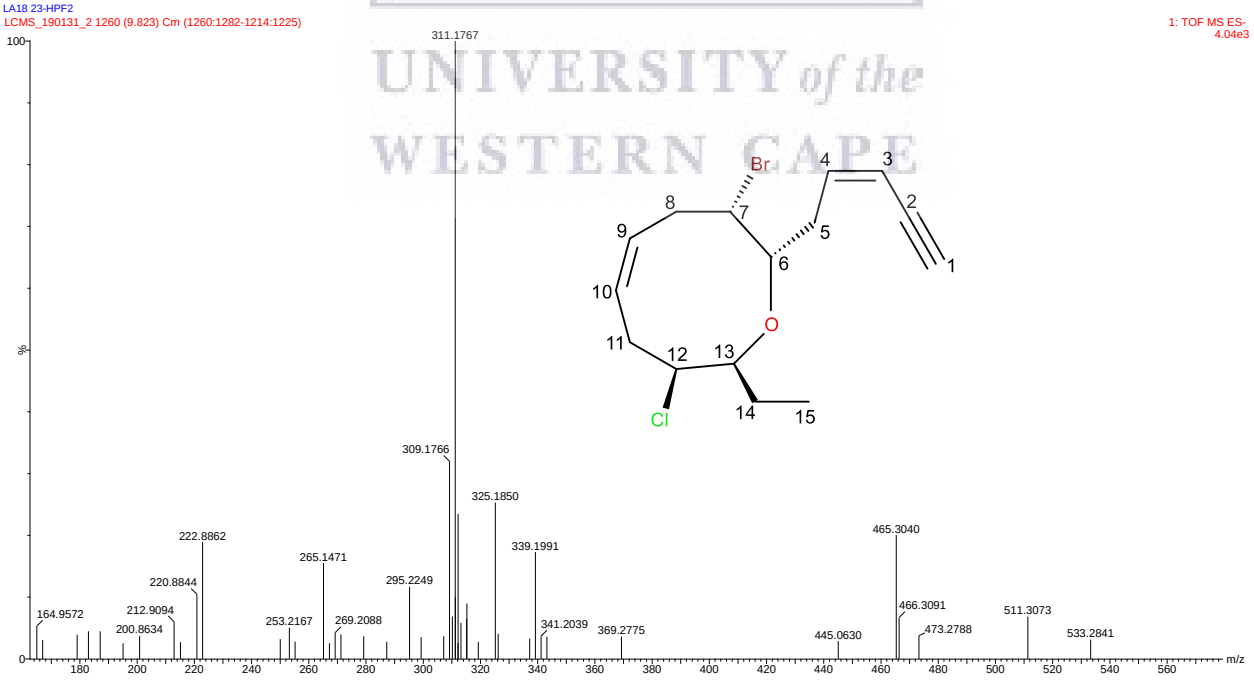

Figure S5.2: Liquid chromatography mass spectrometry of compound **5.10**.

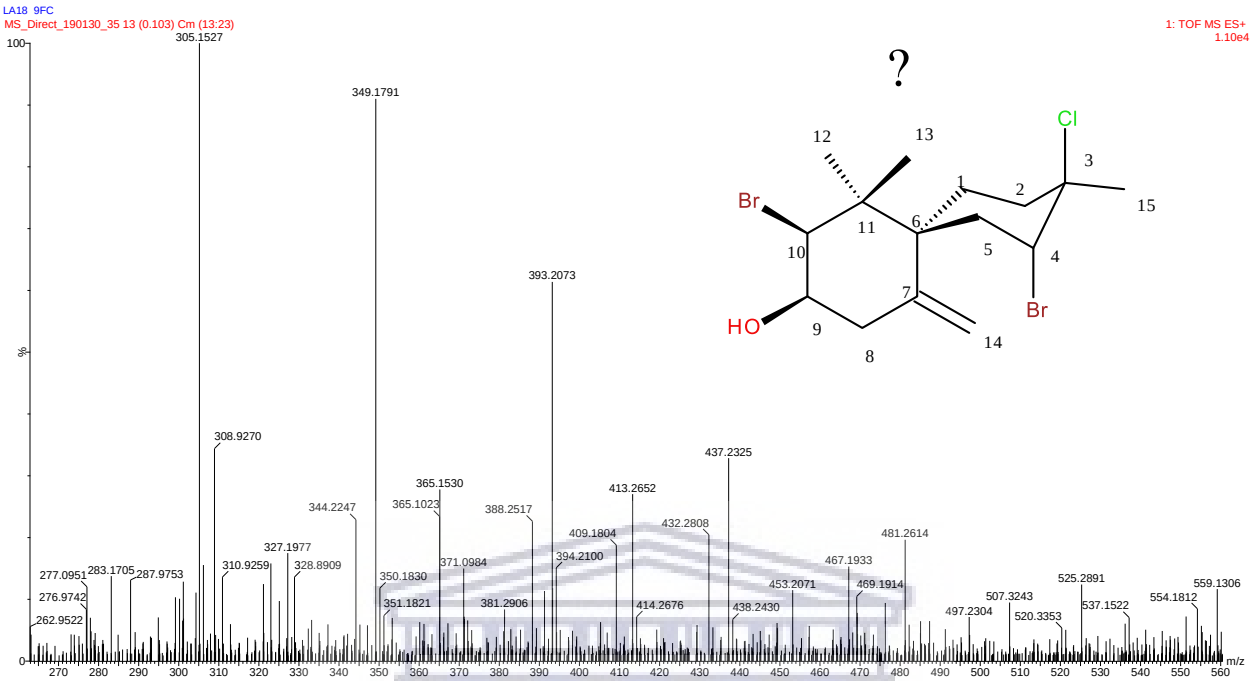

Figure S5.3: Liquid chromatography mass spectrometry of *L. sodwaniensis* fraction **3**.

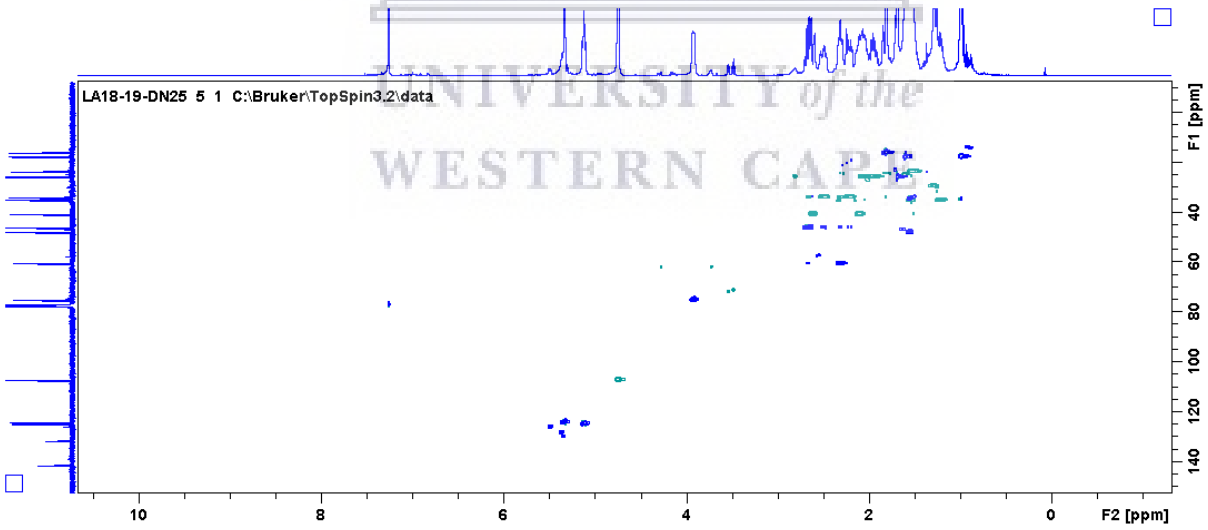

Figure S5.4: HSQC spectrum of compound **5.9**.

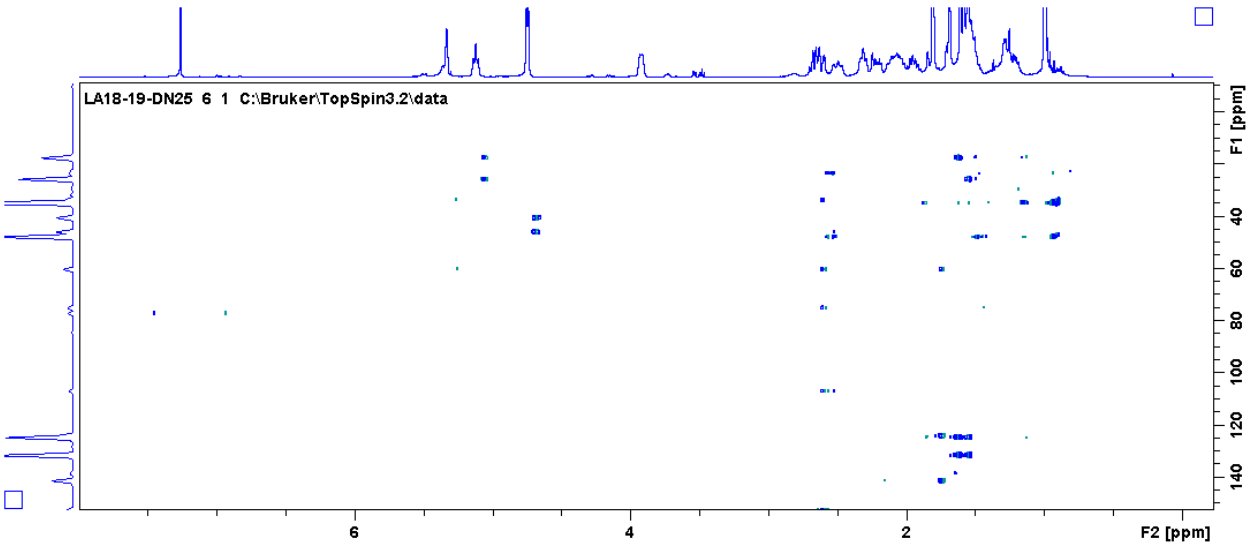

Figure S5.5: HMBC spectrum of compound **5.9**.

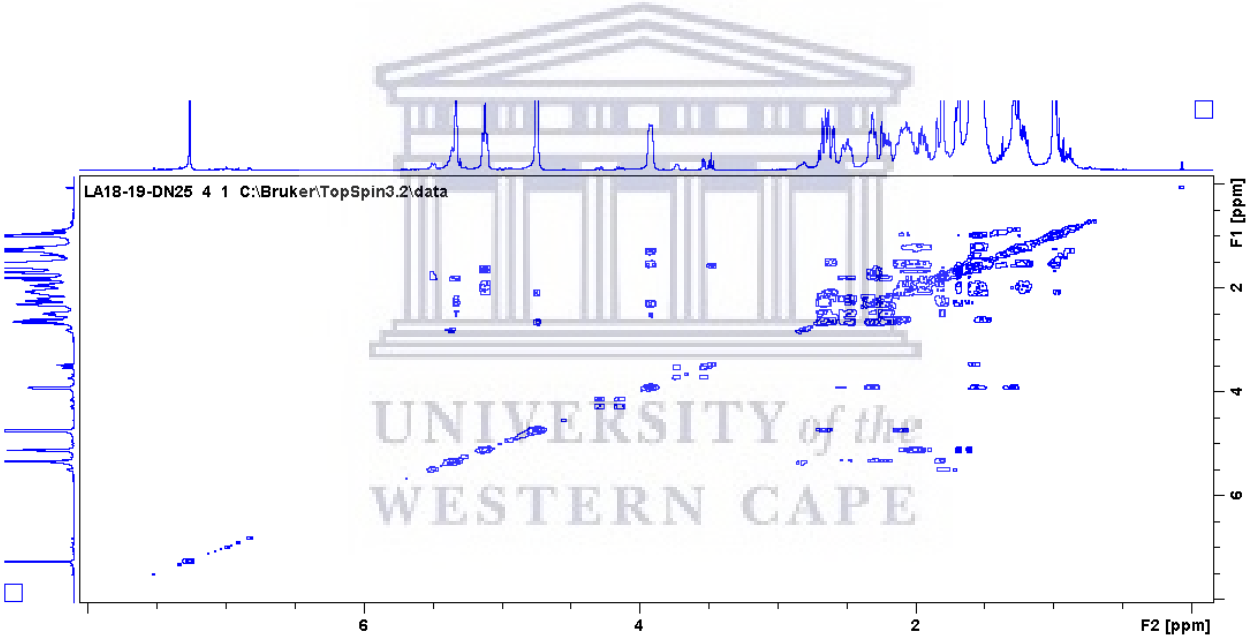

Figure S5.6: COSY spectrum of compound **5.9**.

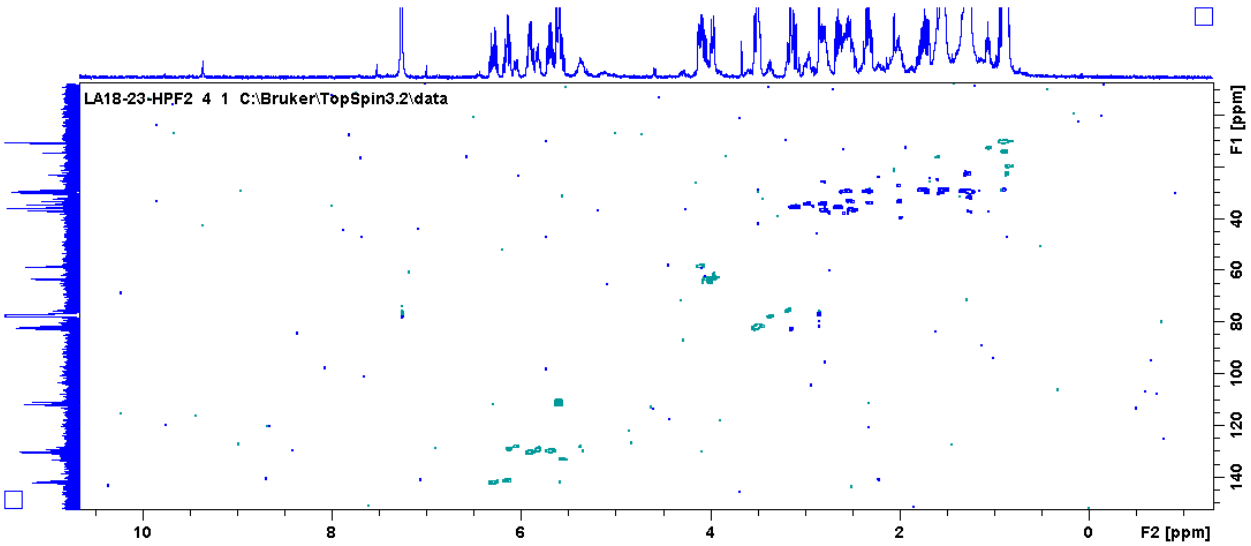

Figure S5.7: HSQC spectrum of compound **5.10**.

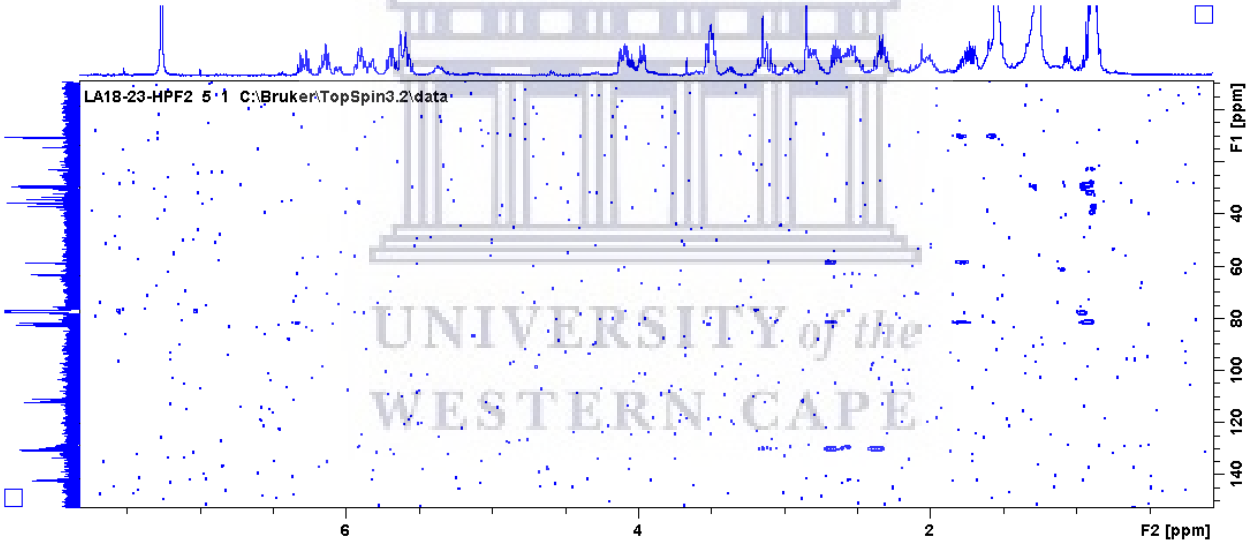

Figure S5.8: HMBC spectrum of compound **5.10**.

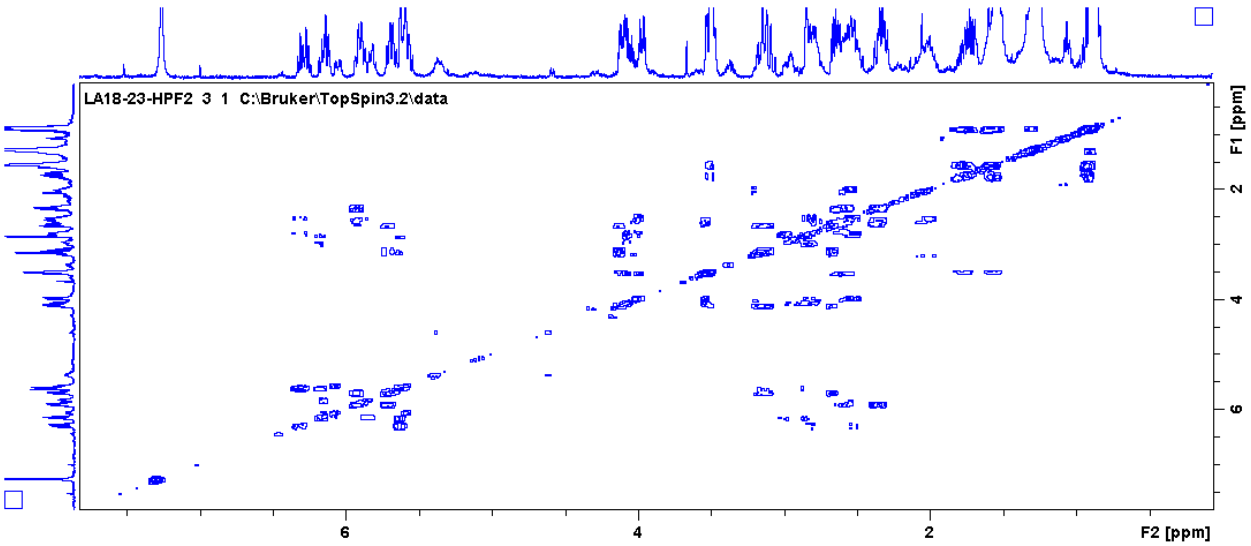

Figure S5.9: COSY spectrum of compound **5.10**.

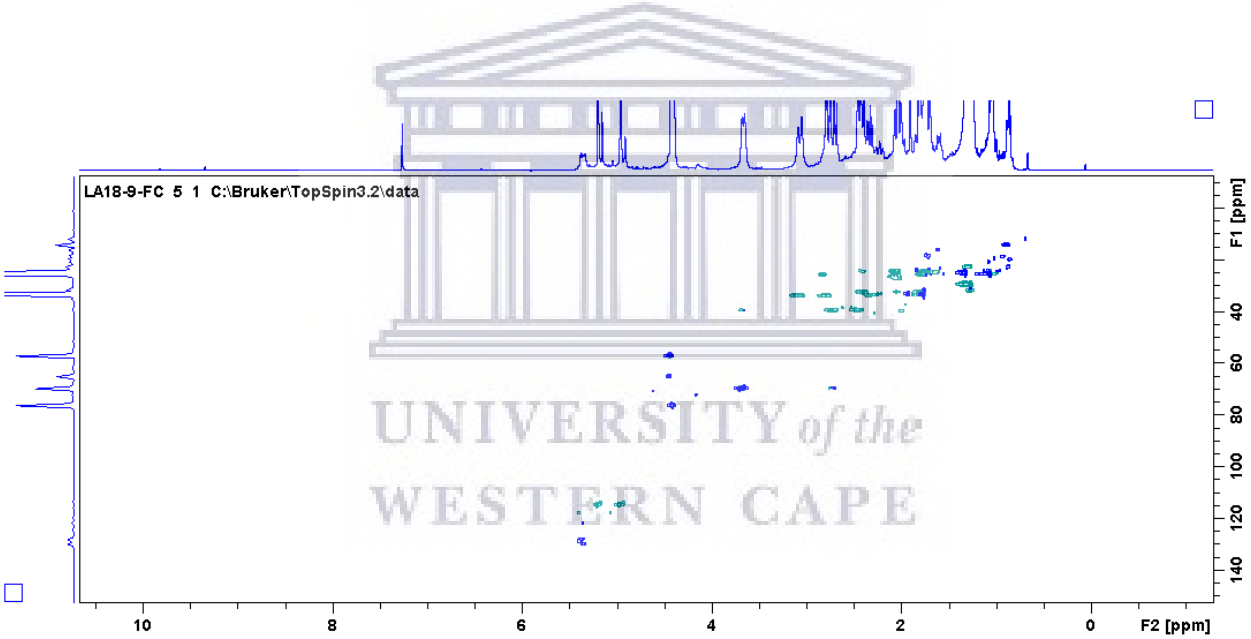

Figure S5.10: HSQC spectrum of compound **5.11**.

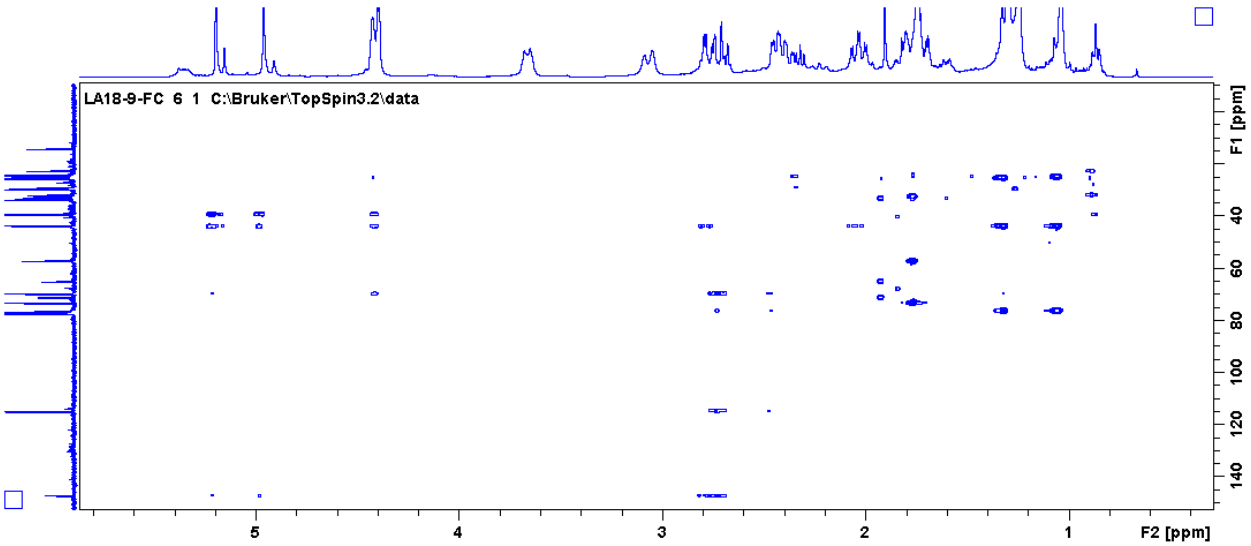

Figure S5.11: HMBC spectrum of compound **5.11**.

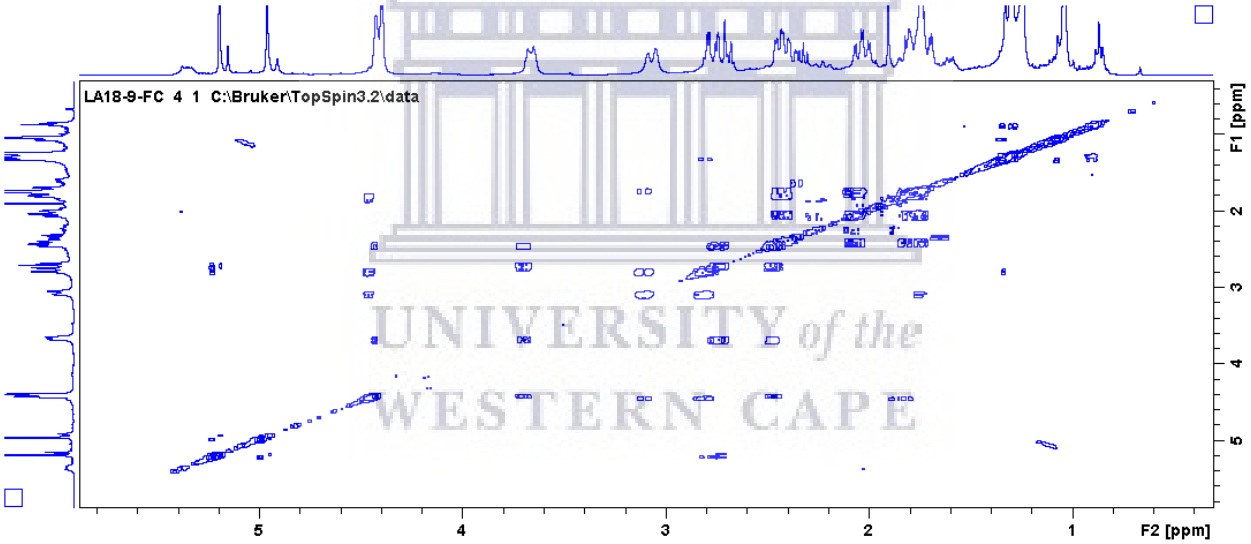

Figure S5.12: COSY spectrum of compound **5.11**.