UiT The Arctic University of Norway

Department of Pharmacy, The Faculty of Health Sciences

Isolation and Biological Evaluation of Phomenins from the Marine Fungus *Parafenestella* sp.

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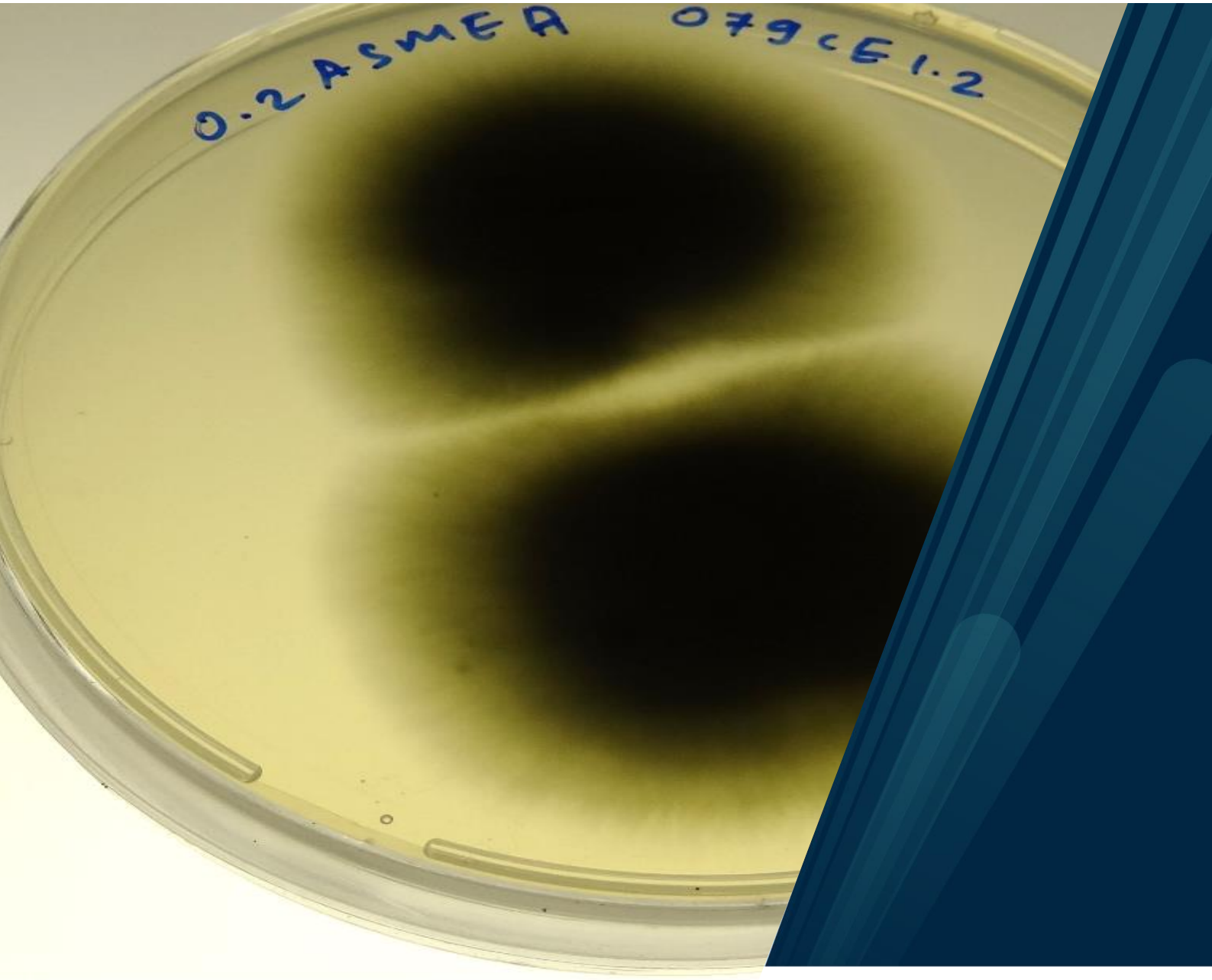
Master's thesis in pharmacy (FAR-3911) May 2024



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Tromsø, May 2024

Julie Marie Lesjø

Abstract

Background: Marine fungi are prolific producers of secondary metabolites. Phomenin A (**2**) and B (**3**) are secondary metabolites produced by *Parafenestella* sp. Previous studies have described them as bioactive, but due to the limited availability of pure compounds, their bioactivity has yet to be extensively characterised. These compounds were identified and isolated at Marbio, but the quantities obtained were insufficient for proper bioactivity profiling. Therefore, the aim of this thesis was to produce larger amounts of the compounds and isolate them to perform proper bioactivity profiling. Additionally, 22 marine fungi extracts were screened for bioactivity to identify new bioactive compounds.

Method: *Parafenestella* sp. and a selection of other marine fungi were inoculated and cultivated for one month in liquid media. The resulting biomass was filtered and extracted using liquid-liquid extraction. The 22 marine fungi extracts were screened for cytotoxic, antibacterial, and antidiabetic properties. The *Parafenestella* sp. extract was fractionated using flash chromatography and then analysed using high-resolution mass spectrometry (HR-MS) to verify the presence of the target compounds. The phomenins were subsequently isolated using preparative HPLC in the fractions from *Parafenestella* sp. Pure isolated compounds were tested for cytotoxic, antidiabetic and antibacterial growth inhibition properties.

Results: Phomenin A (**2**) and B (**3**) were successfully isolated and characterised. The isolation yielded 0.6 mg of phomenin A (**2**) and 0.4 mg of phomenin B (**3**). Phomenin A (**2**) was considered antibacterial at 200 µM when tested against *S. agalactiae.* A cytotoxic assay was also run using the cell lines MCF7 (malignant cells) and MRC-5 (non-malignant cells), but no activity was observed. In addition, the isolated compounds were tested for antidiabetic activity in DPP-IV and PTP1B assay but were not categorised as active. A selection of marine fungi from the 22 isolates showed cytotoxic and antibacterial activity.

Conclusion: The present study isolated and characterized phomenin A (**2**) and B (**3**) from *Parafenestella* sp., demonstrating their bioactivity against bacterial strains. The study found them, though, to be too unstable and not potent enough for commercial drug development. Of the 22 marine fungi, four showed promising cytotoxic and antibacterial properties. Further research is needed to explore and identify the therapeutic potential of the secondary metabolites in these fungal extracts.

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Abbreviations

BPI	Base peak intensity
CID	Collision-induced dissociation
DCM	Dichloromethane
EA	Ethyl acetate
EI	Electron ionization
EMF	Erlenmeyer flask
ESI	Electrospray ionization
FA	Formic acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HR	High resolution
IMS-Q-ToF	Ion Mobility Spectrometry Quadrupole Time-of-Flight
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoproteins
LLE	Liquid-liquid extraction
m/z	Mass to charge ratio
Marbank	The Norwegian national marine biobank
MCF7	Michigan Cancer Foundation-7 cell line
MeCN	Acetonitrile
MeOH	Methanol
MRC-5	Medical Research Council cell strain 5
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NMR	Nuclear magnetic resonance
NP	Natural product
OD	Optical density
Prep-HPLC	Preparative high-performance chromatography
PTP1b	Protein tyrosine phosphatase 1B
Rs	Resolution
R_t	Retention time
SNR	Signal-to-noise ratio
SPE	Solid phase extraction
TIC	Total ion chromatogram
ToF	Time of Flight
UHPLC	Ultra-high-performance liquid chromatography
UiT	The Arctic University of Norway

1. Introduction

1.1 Natural Products: Primary and Secondary Metabolites

A natural product (NP) is a substance produced by living organisms, such as plants, animals, and microorganisms (1). They are categorized into primary and secondary metabolites (2). NPs exhibit high structural diversity and unique pharmacological or biological activity due to the influence of natural selection and evolutionary processes that have occurred for millions of years (3). Since ancient times, humans have used NPs, primarily plants and plant extracts, - to treat various diseases and illnesses (2). A long history of using cinchona bark for fever in Peru can be traced back to the 1600s. Since then, cinchona bark has been used in its crude state until the early 1800s, when chemists became convinced that the curative power was due to its active compounds. By 1820, two French chemists had isolated quinine alkaloid from the bark of a cinchona tree and recognized it as the main treatment for malaria. Despite the effectiveness of this medicinal natural product, it has been replaced by another potent anti-malaria drug because of its side effects and narrow therapeutic index. It is no longer the first-line treatment (4). The cinchona tree is presented in **Figure 1**, with the complex chemical structure of quinine.

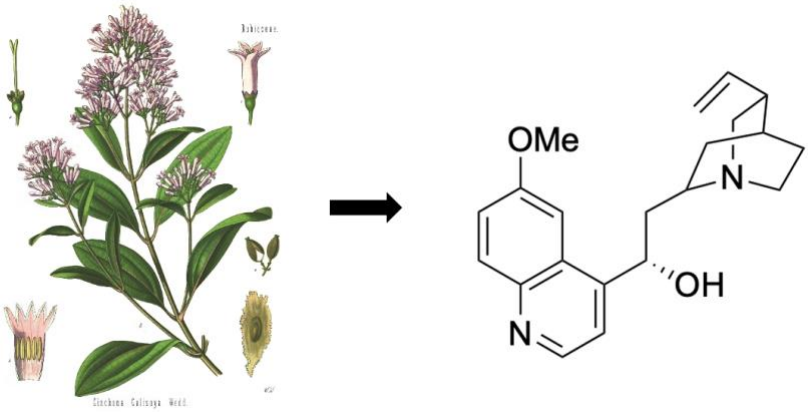


Figure 1: *The cinchona tree (flower) (5) and the chemical structure of quinine (created in ChemDraw).*

Despite having access to modern technology that can predict favourable structures for drug target interactions and synthetic approaches to produce them, NPs are often used as lead compounds for drug discovery (6). For instance, docking software can screen and predict the binding affinity of compounds to specific drug targets (7). The advantage of using NP as a lead compound comes with a variety of chemical structures and is structurally "optimized" by evolution to serve biological functions (6, 8).

1.1.1 Primary Metabolites

Primary metabolites are essential organic compounds, comprising the foundation for the basic life processes of a living organism. The primary metabolism includes biosynthesis and the breakdown of the four fundamental components of life: amino acid, nucleic acids, lipids, and carbohydrates. Most primary metabolites are common to all, or many, organisms and play an essential role in maintaining growth, development, and all-around functionality (2).

1.1.2 Secondary Metabolites

Secondary metabolites, on the other hand, are organic compounds that are not necessary for the organism's immediate survival. A smaller taxonomic group of organisms produces them and has, therefore, a narrow species distribution (9). These compounds confer competitive advantages, e.g., toxic accents against predators and colouring agents to attract a mate or warn other species off. They are produced when the organism adapts to a hostile environment or competes for resources. Such challenges can vary based on environmental factors, i.e., threats from pathogens or predators or stress from fluctuating salinity, pH or temperatures (2). Most marine animals (invertebrates) and microorganisms do not have an adaptive immune system, relying on innate immunity or a chemical defence to avoid predation, pathogenic attacks, and overgrowth (10). The components of the chemical defence strategy are of particular interest within the field of marine bioprospecting. In practice, the term "natural product" usually refers to secondary metabolites, and these terms are often used synonymously (2, 9).

1.1.3 The Challenge of Natural Products Structures

Organic chemistry, which dates to the early 19th century, has its roots in the exploration of natural products and their structural diversity and complexity. Researchers were intrigued by the interesting compounds and studied them to understand their chemical composition and properties. However, the molecular structure remained a challenge. Therefore, isolating and elucidating their molecular structures was a crucial task. Friedrich Wöhler and Justus von Liebig independently studied silver salts of identical elemental composition but observed distinct properties. This difference was eventually resolved through Berzelius's theory of isomers. Isomerism highlighted that properties and chemical reactivity are not solely determined by the number and type of elements but also by the arrangement of atoms within a molecule (11).

This final alternation of the chemical structure contributes to the high degree of chemical diversity observed among the secondary metabolites. Primary metabolic pathways synthesize small single products; for example, the tryptophan pathway produces only tryptophan. Secondary metabolic pathways often produce several, varying from one or two to more than 100 products. One example is the gibberellin biosynthetic pathway, which leads to the production of 136 closely related products (12). This abundance of end products may contribute to the presence of isomers among NPs. Their specific chemical structures determine their pharmacological properties, hence the importance of understanding their structural diversity in drug discovery and development (13).

1.2 Where To Find Natural Products

The ocean is a vast and diverse environment covering more than 70% of the Earth´s surface (14). However, most oceans have not yet been explored by humans, leaving large areas unexplored and many species unknown (15). The ocean is home to several unique marine organisms, such as fungi, bacteria, and sponges. Some microorganisms and invertebrates live in extreme and often challenging conditions, with low nutrient levels, high pressure, and low temperatures (14, 16). Exploring the biodiversity of the oceans could be the solution to global challenges and human health issues. For example, many marine organisms produce compounds with potent antimicrobial properties as part of their chemical strategies. By identifying these compounds, we can develop new treatments for various infections and bacteria resistance (17).

1.2.1 Marine Bioprospecting

Bioprospecting is the discovery, processing/refinement, and commercialisation of products from nature, including genes, enzymes, and NPs (18). However, it is not limited to biological materials only, as it also includes biomimetics that explores natural designs or principles found in the environment that can be applied to various techniques (19). For instance, it can be used to optimize the aerodynamic properties of cars or planes. When organisms in the marine environment are studied, the process is called marine bioprospecting (20, 21). Marine bioprospecting aims to discover novel NPs that offer promising starting points for developing commercially available drugs.

These isolated bioactive compounds offer a promising resource for developing commercial therapeutic agents and play an essential role in drug discovery (6). A study from 2020, has provided a comprehensive analysis of the role of natural products as a source of new drugs. NP derived from biological sources such as plants, marine organisms, and microorganisms have emerged as valuable reservoirs of bioactive compounds with therapeutic potential. **Figure 2** is a pie chart of all newly approved drugs from 1981 to 2019, showing that synthesized drugs still make up a significant portion of newly approved medicines. However, NP structures still play a crucial role as being the source of inspiration for many synthesized drugs, and their structure often serves as a starting point for drug development (22).

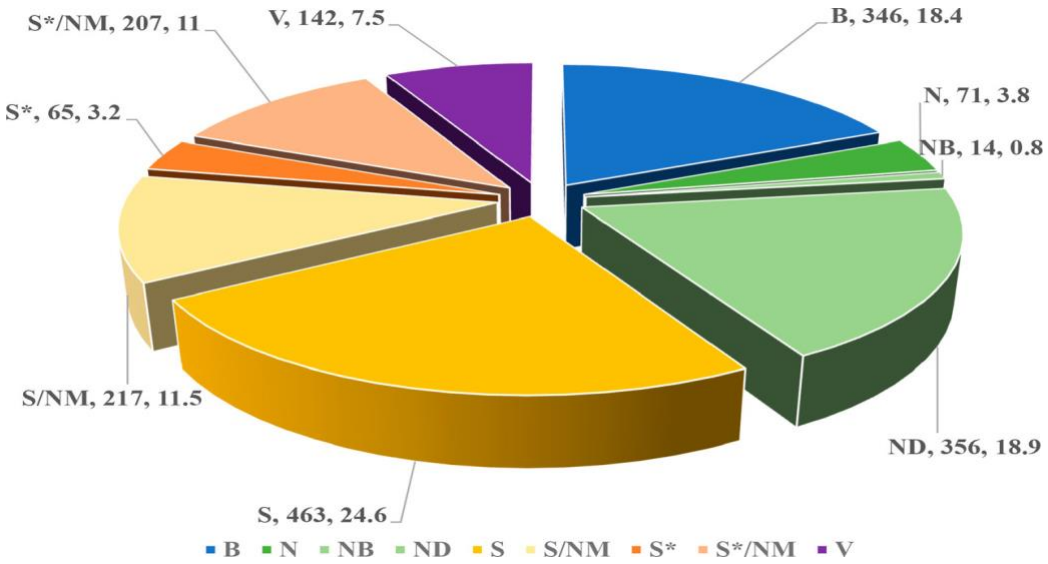


Figure 2: *A pie chart of all new approved drugs from 1981 to 2019, n=1881 (22). "B" = biological (usually large peptide or protein), "N" = NP, "NB" = NP "Botanical", "ND" = derived from NP and usually semisynthetic modification, "S" = synthetic, "S*" = synthetic but pharmacophore was from a NP, "NM" = NP mimic, "V" = vaccine.*

1.2.2 Marine Bioprospecting at Marbio

The marine bioprospecting pipeline at Marbio starts with collecting and preparing marine biomass, a task conducted by the Norwegian National Marine Biobank (Marbank) (20). Further processing is done by Marbio, where the collected biomass is extracted and fractionated. The fractions are tested for bioactivity in cell- and target-based assays, i.e., for cytotoxic or antibacterial activity and activity against kinases. Bioactive fractions then undergo chromatographic and spectrometric analyses using UHPLC- high-resolution mass spectrometry (HR-MS) to identify and recognise previously isolated substances. The compound(s) of interest are then isolated using the preparative HPLC and analysed using HR-MS and nuclear magnetic resonance spectroscopy (NMR) for molecular structural elucidation and identification. Then, the pure compound(s) are finally submitted to another round of bioactivity profiling (23), potentially for target deconvolution (mode-of-action) and the evaluation of their pharmacokinetic properties.

Marine organisms can produce bioactive compounds as a valuable resource for drug discovery due to their unique and diverse structures. However, these compounds often require refinement to enhance their specific properties necessary for therapeutic use. Medicinal chemistry plays an essential role in refining these bioactive compounds to enhance their efficacy, safety, and pharmacokinetic properties. Furthermore, medicinal chemistry can optimize molecular structure to improve its potency, selectivity, and metabolic stability in drug applications (24).

1.2.3 Marine Bioprospecting in Norway

Norway´s long coastline and extensive marine territories offer vast opportunities for accessing marine resources and biodiversity. There is limited knowledge of marine organisms' molecular and genetic traits, particularly those inhabiting cold waters. The Norwegian government presented the first national strategy for marine bioprospecting in 2009, aiming to increase the exploitation of Norwegian marine resources (20). One specific project that aims to collect and register marine fungi is the Norwegian Marine Fungi (NMF) project, which is done in collaboration with Marbank as part of Havforskningsinstituttet (HI). They have collected marine fungi from Svalbard, Jan Mayen, and the Norwegian coast and focused on collecting in counties with few to no registered marine fungi. The project will describe known and unknown marine fungal species in various marine substrates, from the tidal zones to seabed habitats (25).

1.3 Marine Microorganisms

Microorganisms, also known as microbes, are microscopic living organisms that can only be observed under a microscope (26). The size ranges depending on which microbes it is, but most of them are around 1 µm. Living organisms are divided into two main groups: prokaryotes and eukaryotes. Prokaryotes are single-celled organisms lacking internal membrane-bound organelles and a nucleus. This group include bacteria and archaea. In contrast, eukaryotic cells can be much larger and contain membrane-bound organelles and a nucleus. They can be single-celled, like protists, or multicellular, like fungi, plants, and animals (27).

Microbes play essential roles in sustaining life on our planet. For instance, they contribute to the oxygen, nitrogen, carbon, and sulphur cycles and are responsible for nearly half of all photosynthesis. Animals and plants have a close relationship with microorganisms, hosting about ten microbes for every human cell. These microbes perform vital functions, such as helping digestion and supporting the development of the immune system (28).

Microorganisms are also found in the ocean and are responsible for more than 98% of ocean biomass (29). These marine organisms can exist in practically any environment and gather energy from various sources, from solar radiation to chemosynthesis. In addition, they have many different roles, such as being the base of the food chain and contributing to the ecosystem. Another important task they have is symbiotic relationships with marine invertebrates, providing nutrients, waste recycling and protection. Marine invertebrates give back a habitat that supports the growth of the microorganisms (30). Without microbes, the balance of natural processes would be thrown off, causing problems for living organisms and ecosystems. Earth would face significant challenges without these tiny but crucial organisms (31).

1.3.1 Kingdom Fungi

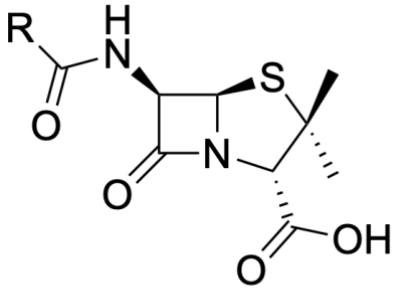
Fungi are heterotrophic, eukaryotic organisms. Heterotrophs are organisms that cannot produce their nourishment. Instead, fungi acquire their food by absorbing dissolved organic matter (i.eg. cellulose) from their surroundings, making them essential decomposers in the ecosystem. The kingdom contains a wide range of organisms, including single-celled organisms (like mould and yeast) and multicellular organisms with fruiting bodies (like mushrooms). The cell wall of fungi consists primarily of a structural polysaccharide called chitin. Chitin is a distinguishing feature of fungi and provides rigidity and support (32).

Despite their often-overlooked presence, fungi have profound impacts on ecosystems, human health, and industry. The decomposer process is essential for nutrient cycling and soil formation. Fungi also have symbiotic relationships with plants called mycorrhizae, where they exchange nutrients for carbohydrates. This enhances the plant roots' ability to absorb water and nutrients from the soil. The microbe is important for humans, generating the food we eat and drink and providing medicine derived from secondary metabolites (32). Some are very toxic, and others have found great utility in medicine.

1.3.2 Pharmaceuticals Derived from Fungi

Fungi are a rich source of secondary metabolites and are a great foundation for potential drug leads. Throughout history, they have made significant contributions to pharmacotherapy and provide unique structural diversity compared to synthetic compounds (2). Their various biological activities, including protection and toxicity, make them potential candidates for drug discovery research (33).

One of the most famous natural product discoveries derived from fungi is the antibiotic penicillin (**Figure 3**), isolated from the fungi *Penicillium notatum* by Fleming in 1929. Penicillin works by interfering with the bacterial cell wall, weakening it and causing the cell wall to collapse (34). The breakthrough won the 1945 Nobel Prize in Physiology and Medicine, and the interest in discovering new antibiotics from microorganisms and bioactive natural products increased worldwide (2).



Penicillin

Figure 3: *The core chemical structure of the antibiotic penicillin (created in ChemDraw).*

Another class of pharmaceuticals that originate from fungi is the statins. Statins are a class of hypolipidemic drugs that lower cholesterol by inhibiting a rate-limiting enzyme of the mevalonate pathway of cholesterol biosynthesis. The drug also upregulates low-density lipoproteins (LDL) receptors, increasing the clearance of LDL from the bloodstream. The first commercial statin, lovastatin (**Figure 4**), was derived from *Monascus rubra* and *Aspergillus terrus* and was approved in 1987 by the US Food and Drug Administration (FDA) (35).

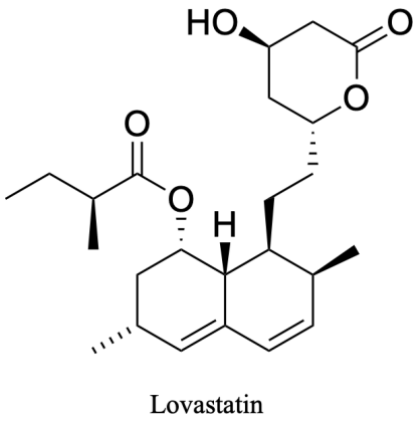


Figure 4: *Chemical structure of lovastatin (made in ChemDraw).*

Marine fungi are likewise genetically diverse as their terrestrial counterparts but much less studied. Thus, the widespread belief is that by looking for bioactive NPs in marine fungi, novel lead compounds from pharmaceutical development can be discovered (36).

1.3.3 *Parafenestella* sp.

The marine fungus is classified under the *Cucurbitariaceae* family in the order *Pleosporales.* The species are mainly fungicolous (associated with perithecial ascomycetes), saprobic or necrotrophic on woody plants (37). The family typically produce fruiting bodies called ascomata, which contain sac-like structures called asci that hold the ascospores. Many species within the family are saprophytic (absorbing organic matter). However, some species are also known to be plant pathogens and cause diseases, including cucurbits, which is where the family name originates from. Like many other fungi, the taxonomy of the family is still being researched (38).

1.4 Workflow and Methods

The pipeline in this project is based on methods used at Marbio and shown in **Figure 5**. The marine fungus *Parafenestella* sp. and other selected marine fungi isolates were cultivated, and the fungal biomass was extracted using liquid-liquid extraction (LLE) with ethyl acetate. The *Parafenestella* sp. extract was fractionated using flash chromatography based on the compounds polarity. Target compounds were purified and isolated with preparative high-performance liquid chromatography coupled with mass spectrometry (prep-HPLC-MS). The isolated compounds were structure elucidated with nuclei magnetic resonance (NMR) and tested for bioactivity in different bioassays. The selected marine fungi extracts were screened for bioactivity to identify potential novel or known secondary metabolites. The following section provides a more detailed description of the methods used in this master thesis.

1.4.1 Graphical overview of the workflow used in this master project

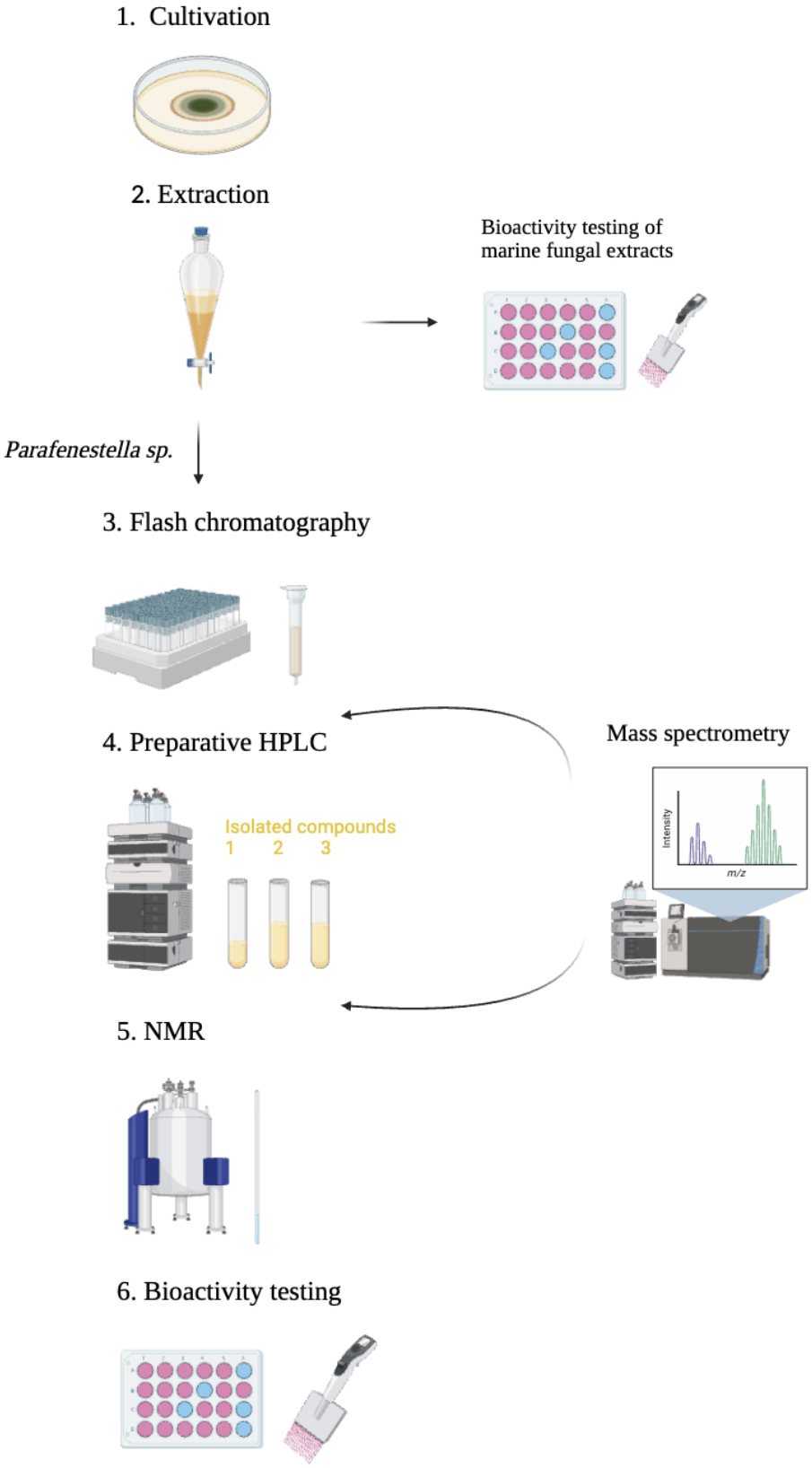


Figure 5*: A broad overview of the methodological pipeline used in this master project (created in Biorender.com)*

1.5 Inoculation and Cultivation

Inoculation is vital in microbiology and biotechnology as the starting point for cultivating microorganisms in various environments. It involves introducing microorganisms to a culture media or a host organism to initiate growth or infection (39). Culture media is designed for rapid microbial growth and contains essential nutrients like vitamins, water, carbon, and nitrogen. The microorganisms introduced to the fermentation medium are called inoculum. The inoculum can be any part of the microorganism that causes infection. Specifically, for fungi, the inoculum could be small pieces of mycelia or spores (40). It is performed in an aseptic condition to prevent or minimise the risks of contamination, which can kill or out-compete the microorganisms (39).

1.6 Sample Preparation and Extraction

Biological sources are the primary type of samples used as a starting point for marine bioprospecting. These are usually a complex mixture of many substances, where only one or a few (at best, for many extracts, none) hold the potential to be developed into a commercially available product. In a complex mixture, primary metabolites can often interfere with the detection of the secondary metabolites. For example, the analyte might be present in such low concentration that it can't be detected, or there could be harmful substances to the chosen mass spectrometer. These issues need to be taken into consideration during sample preparation. Sample preparation involves all the necessary steps required before the sample can undergo analysis on an instrument. Sample preparation depends on the sample, but centrifuging blood sample is one example. The step can address these challenges by serving two purposes: isolating the analyte from the matrix and concentrating low-concentrated analytes. The step can be time-consuming and prone to errors, but it is crucial to obtain accurate analytical results (41, 42).

Liquid-liquid extraction (LLE) is a sample preparation technique (**Figure 6**), which is a process of separating a substance from a mixture. These substances are dissolved in one solvent and extracted into another solvent. Two solvents used in LLE should be immiscible. Water and an organic solvent such as dichloromethane (DCM) or ethyl acetate (EA) are usually used as two immiscible phases. The immiscible phases must be shaken well to achieve good contact between solvent and extractant, allowing the dissolved substances to distribute between the two phases (42).

The distribution of the substances depends on their partition coefficient (*P*). It is defined as the ratio of the compound concentration in two specific phases. An example is the octanol/water partition (*logP*). Octanol is somewhat like biota lipids, where the log*P* value can provide the distribution of organic compounds between environmental compartments. In general, a log*P* < 0 means high hydrophilic, and log*P* > 10 means high lipophilic and a greater affinity for lipid environments (43, 44).

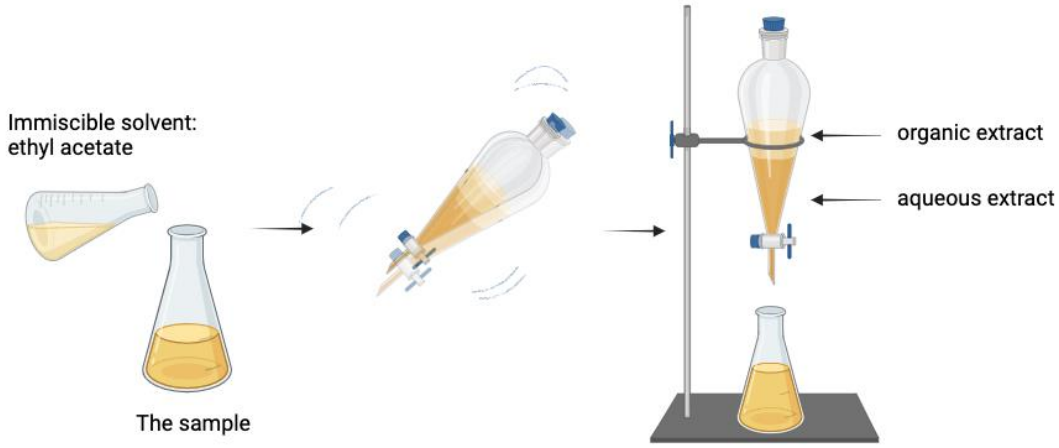


Figure 6: *A graphic overview of liquid-liquid extraction (LLE) (created in Biorender.com).*

1.7 Chromatography and Mass Spectrometry

1.7.1 Liquid Chromatography

Chromatography is an analytical technique to separate compounds from a mixture. The method relies on the principle that different substances in a mixture move at distinct speeds as they travel through a medium. A chromatographic system consists of a stationary phase and a mobile phase. The separation of compounds is achieved based on differences in their interaction between two phases, which depends on the chemical and physical properties of the analyte. These variances among the molecules will cause some analytes to move slowly due to their extended retention caused by their strong interaction with the stationary phase. In contrast, others quickly move into the mobile phase, interacting less with the stationary phase, leading them to exit the system earlier (45).

In liquid chromatography (LC), the mobile phase consists of a solvent or a mixture of solvents, and the stationary phase is a packed column of solid particles, usually silica-based particles. The analytes are injected in the mobile phase and will travel through the column. Analytes interacting more with the solid phase will stay longer in the column than those interacting less. The time the analytes travel through is termed retention time (Rt) (45). The elution conditions are divided into gradient elution and isocratic elution. An isocratic eluent means that the mobile phase mixture is consistent throughout the analysis. In contrast, a gradient elution implies that the composition of the mobile phase changes during the chromatographic run (46).

1.7.1.1 High – and Ultra – High – Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation method to separate substances using high pressure to push the mobile phase through a chromatographic column. This column is packed with solid particles that are small and uniform, with a particle size often around 3-5 micrometres. Ultra-high-performance Liquid Chromatography (UHPLC) operates with a higher pressure, shorter column, and smaller particle size (< 2 um), which allows even higher efficiency and faster separation (47, p. 187-188, 48).

1.7.1.2 Reversed-Phase Chromatography

Reversed-phase (RP) chromatography is the most used technique in liquid chromatography. In RP chromatography, the stationary phase is hydrophobic and contains modified silica particles with an attached octadecylsilane (C_{18}) ligand (**Figure 7**). The C_{18} column is one example, and other columns have different substances attached to the alkyl chain. The mobile phase often consists of polar solvents, such as a mixture of water and organic solvents soluble in water, like methanol or acetonitrile. The weak mobile phase is unable to break the primary (lipophilic) interactions between the stationary phase and the analytes, while the strong mobile phase can break these interactions. The higher the proportion of organic solvent in the mobile phase, the stronger it becomes in RP chromatography. Since the stationary phase is hydrophobic, the non-polar analytes will interact more with the surface of the stationary phase, leading to a longer retention time (47, p. 160-168). There are hydrophobic interactions (van der Waals) between the column and the analytes.

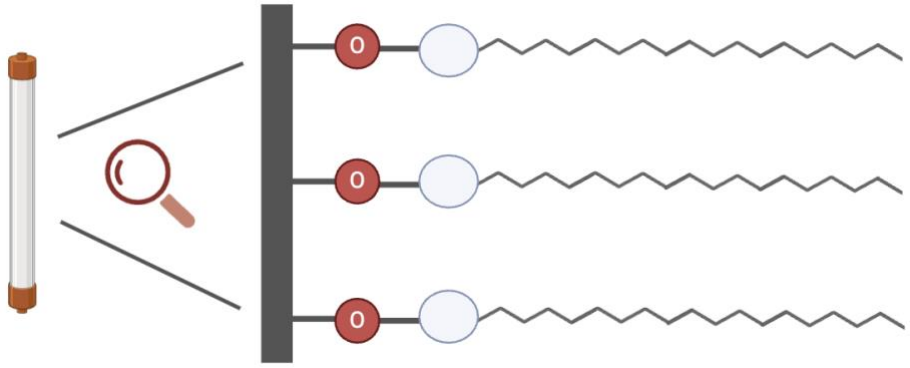


Figure 7: *A C_{18} column. The thick line represents the "silica backbone" (created in Biorender.com).*

Different RP chromatographic columns typically separate specific compounds due to their varied interactions with different stationary phases. The phenyl-hexyl (PH) column is one example, with a phenyl attached to the carbon chain (**Figure 8**). The aromatic is associated better with aromatic compounds, interacting through π-π interactions. The column also has hydrophobic interactions, but compared to the C_{18} column, it is weaker (49).

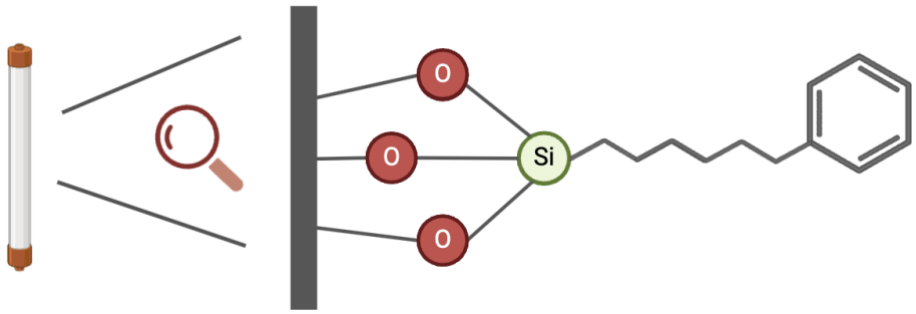


Figure 8: *The stationary phase of the PH column. The thick line represents the "silica backbone" (created in Biorender.com).*

1.7.2 Flash Chromatography

Flash chromatography (**Figure 9)** is another chromatographic technique that separates and purifies compounds from a mixture. The method includes a stationary phase, often a prepacked column with a solid absorbent material (e.g., silica), organic solvent, or a mix of organic solvents (e.g., methanol and acetonitrile) as a mobile phase and our sample. Compared to other chromatographic methods like HPLC, this method operates on a larger scale. The columns have larger diameters with a larger particle size (40-62 µm). Because of the greater scale, the column operates at a higher flow rate, allowing it to load the column with more sample material. The downside of this method is that it has less resolution. After the separation, the compounds in the mixture are sorted into fractions based on their polarity. In RP chromatography, most polar compounds go quickly through the column, while more non-polar compounds are more bound to the stationary phase (50).

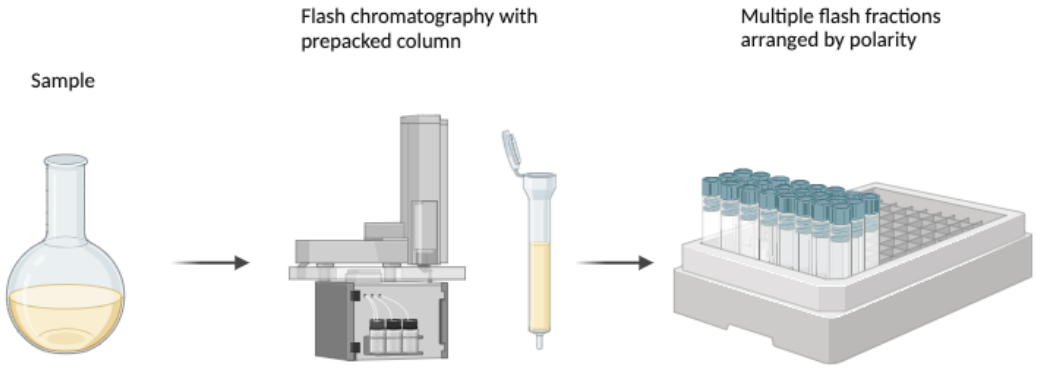


Figure 9: *The flash chromatography workflow (created in Biorender.com).*

1.7.3 Preparative High-performance Liquid Chromatography

Preparative HPLC (prep-HPLC) is based on the same fundamental principle as HPLC, yet it serves a different purpose. Prep-HPLC is used to isolate compounds from a complex mixture (41). The system usually has larger columns (particle size 10-20 µm), higher sample loading, and a high flow rate (5-100 mL/min). The prep-HPLC is a robust, versatile, and usually rapid technique where compounds can be purified from complex mixtures (51). The prep-HPLC can collect separated compounds by setting a retention time window or pairing it with a mass spectrometer, where the compound gets separated when a specific *m/z* value is detected.

1.7.4 Mass Spectrometry

Mass spectrometry (MS) is an analytical tool to measure the mass of atoms, molecules, and fragments of molecules. The instrument used for these measurements is a mass spectrometer. Since it uses electric and magnetic fields to accelerate the analytes into the instrument, only charged particles can be analysed. The MS measures the mass-to-charge ratio of the molecule (*m/z*) and is used to identify and quantify chemical compounds by helping study the structure and elemental composition of the molecules. The mass spectrometer itself consists of an ion source, a mass analyzer, and a detector. The instrument is typically combined with an inlet, as shown in **Figure 10.** The two most common types of inlets are gas chromatography (GC) and liquid chromatography (LC). The inlet separates compounds in complex samples before introducing them to the mass spectrometer, simplifying the interpretation of the results (52).

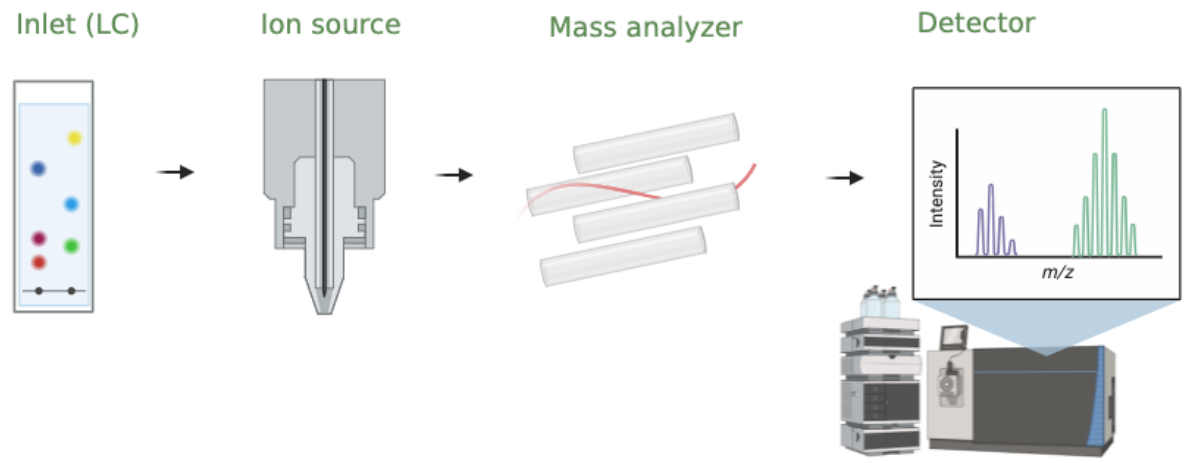


Figure 10: *An overview of MS composition with an ion source, mass analyzer and detector, combined with an inlet (created in Biorender.com).*

1.7.4.1 Ion Source

The analytes are ionized in the ion source so they can accelerate into the instrument before the mass spectrometer analysis. There is a variety of ion sources. Some ionization techniques are highly energetic, leading to extensive fragmentation (e.g., electron ionization), while others are softer, producing molecular ions (e.g., electrospray ionization). In gas chromatography, the most common technique is electron ionization (EI), where molecules from the sample are bombarded with high-energy electrons, removing an electron from the analytes.

The most common ionisation technique for LC is electrospray ionisation (ESI). As illustrated in **Figure 11**, to produce ions using an electrospray, a high voltage is applied to a liquid to generate an aerosol (52, p. 15-16). In the ion source, a stainless-steel capillary can carry either a positive or negative charge, ionising the analytes either positively or negatively. This ionisation process occurs at atmospheric pressure. A high voltage is applied to the capillary outlet where the samples are introduced, nebulising the particles into tiny, charged droplets. With help from heat and nitrogen gas, the solvent evaporates, and the droplets decrease in size. When the charge within the droplets comes too close, they repel each other. The energy generated from this eventually becomes greater than the surface tension, resulting in a coulombic explosion. As a result, we are left with analytes carrying a charge in the gas phase that is guided into the mass spectrometer by an electric field (52, p. 43-46, 53).

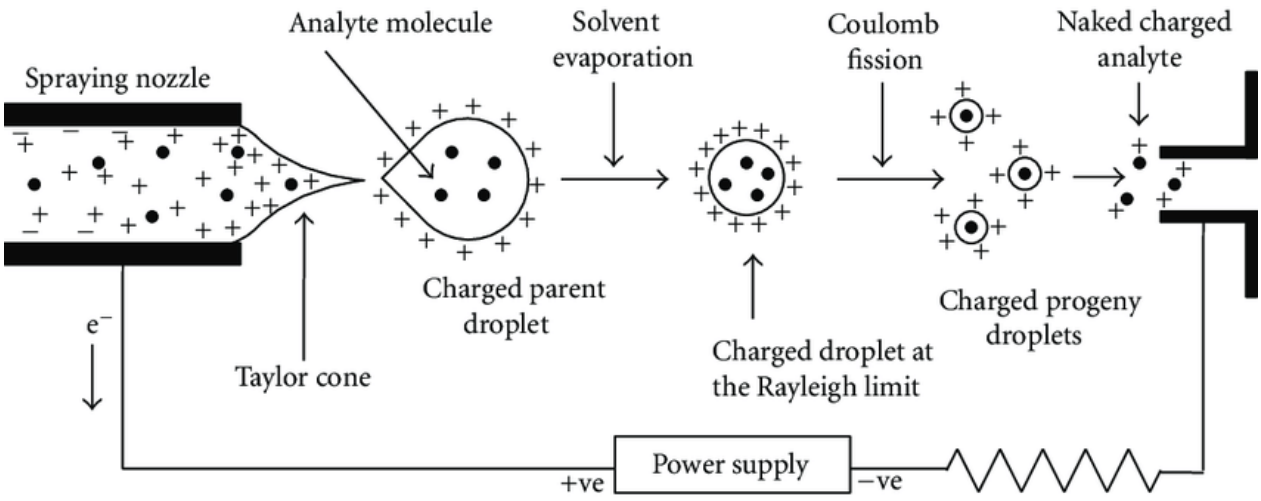


Figure 11: *Illustrating the mechanism of ESI (54).*

When energy from ionisation is added to these analytes, the molecule can break into smaller particles called fragments, and this is referred to as in-source fragmentation. In ESI, the degree of in-source fragmentation is low. To generate fragments in ESI, the ionised molecules are fragmented in a collision cell in a process called collision-induced dissociation (CID). CID transfers energy by collisions with the collision gas molecules, typically argon. In this case, not all bonds in a molecule will break, resulting in moderate fragmentation. Usually, the bond between a carbon and heteroatoms (such as C-O, C-N) and bonds between carbon-carbon in alpha-position to a function group break.

The fragment pattern can be used to confirm the chemical structure of analytes and provide information about their functional groups and connectivity (53). During ionisation, it is also possible that other ions than $[M+H]^+$ and $[M+H]^-$ are formed. The presence of other ions depends on the mobile phase composition and what is present in the sample. Na^+, K^+ and $NH4^+$ are the most common adducts for positive ionisation. Cl^- and CH_3COO^- are the most usual for negative ionisation. If there is a Na^+ adduct, the MS will measure $[M+Na^+]$, with a +23 *m/z* difference. This information can be used to calculate the molecule's weight or figure out if the signal is a fragment or an adduct (53).

1.7.4.2 Mass Analyzers

In a MS, the mass analyzer separates ions with different *m/z* ratios. The quadrupole, an orbitrap or a Time-of-Flight are common types of mass analyzers. The quadrupole consists of four parallel metal rods with an electric field applied to them, which can be adjusted based on the voltage. Ions with a specific *m/z* ratio that aligns with the quadrupole´s settings will successfully travel through this field and proceed to the detector. Ions with different *m/z* values fail to follow a stable path within the quadrupole and will eject, not reaching the detector (**Figure 12**) (52, 55).

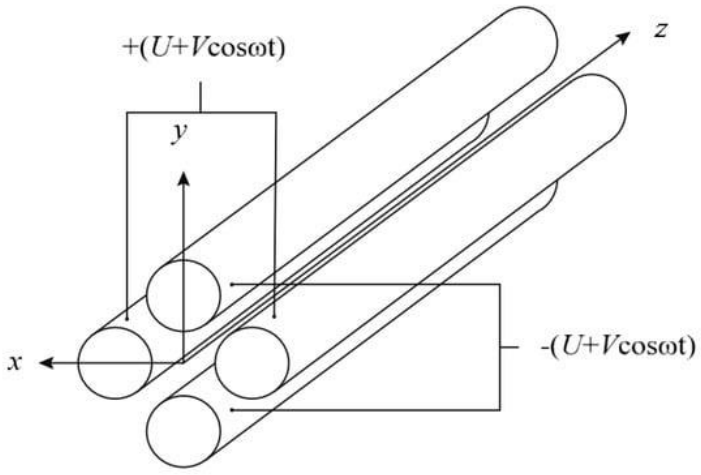


Figure 12: *The quadrupole mass analyzer showing cylindrical rod assembly. Ions with specific m/z values will follow the z-axis to reach the detector (56).*

The performance of a mass analyzer varies in its resolution, mass accuracy, and sensitivity. Resolution (Rs) is defined by how well the instrument separates ions with slight differences in the *m/z* ratio. A higher resolution in an MS allows for more narrow peaks and better separation between two ions with similar *m/z* values. Mass accuracy is how well the MS measures the correct *m/z* value of an ion. Continuous calibration is often used to achieve high mass accuracy, which includes a compound with a known mass, such as the peptide leucine enkephalin (Waters). The difference between the known mass and the measured mass of the compound is used to correct the mass measurements of the analytes in a sample (57). Finally, sensitivity refers to the instrument's detection limit. High sensitivity means how well the MS can accurately detect low concentrations of analytes and distinguish them from the background noise. The reliability of an observation can measured with a signal-to-noise ratio (SNR). The observation can be trusted if the SNR > 3 (54). All three are important to determine the element composition of an analyte.

1.7.4.3 Time of Flight

Time of Flight (ToF) is a mass analyzer that measures the time it takes for ions to travel a known distance. Their initial acceleration by an electric field allows the time-of-flight instrument to separate the analytes. The accelerated ions are transported into a flight tube that is a vacuum chamber. Ions are accelerated with the same kinetic energy, resulting in the ToF separating depending on their mass-to-charge ratio (*m/z*). Lighter ions travel faster compared to heavier ions that travel more slowly. This mass analyzer is known for its high mass accuracy and resolution, which can help find the exact element composition and separate two very close *m/z* (52, p. 126, 58).

A Vion IMS-Q-ToF (**Figure 13**) was used to analyse the samples. The instrument has an ion mobility spectrometry (IMS) filter, a gas-phase separation technique. The technique separates based on the time an ion crosses a drift tube filled with an inlet gas, usually nitrogen gas (59). The drift time (DT) depends on the ions´ size, shape, and charge. This technique adds an extra level of separation and can help distinguish ions with similar *m/z* values (60).

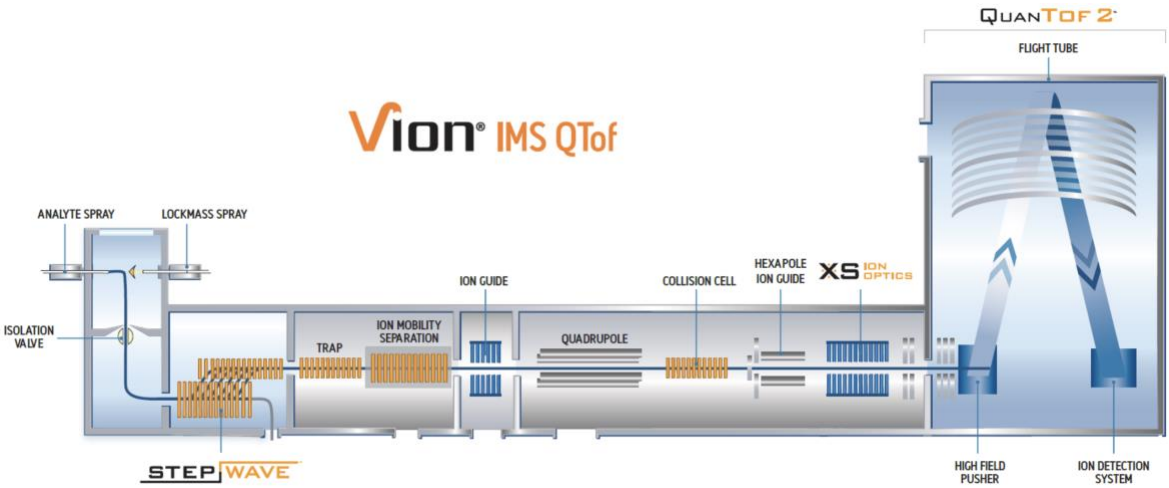


Figure 13: *Schematic figure of the Vion IMS-Q-ToF instrument.*

1.8 Nuclear Magnetic Resonance Spectroscopy

Spectroscopy involves examining the absorption and emission of electromagnetic radiation by substances. Nuclear magnetic resonance (NMR) spectroscopy is a widely used technique for precisely determining the structures of organic compounds. This method capitalizes on the behaviour of nuclear spins within magnetic fields, exploiting the phenomenon where atomic nuclei absorb and subsequently emit electromagnetic radiation when placed in an external magnetic field. Specific atomic nuclei possess nuclear spin owing to their odd numbers of nucleons (protons and neutrons). When exposed to an external magnetic field, these nuclei align either parallel or antiparallel to it, resulting in two distinct energy states and corresponding spin states. During NMR spectroscopy, a tailored electromagnetic pulse, typically in the radio frequency range, is administered, prompting the nuclei to absorb the energy and alter their spin states. Following this pulse, the nuclei emit the absorbed energy, returning to their original states. This emitted energy generates a measurable signal known as resonance frequency, which furnishes valuable insights into the physical, chemical, and structural attributes of the molecule under investigation (61).

NMR spectroscopy includes various techniques that provide insight into molecular structure and dynamics. *One*-dimensional (1D) NMR spectroscopy is an analysis of a single nucleus, where one axis corresponds to the chemical shift (δ) and the other corresponds to the signal intensity. This includes proton (^{1}H) and carbon (^{13}C) spectra, that can describe characteristic signals corresponding to different types of hydrogen or carbon atoms. *Two*-dimensional (2D) NMR spectroscopy techniques provide additional information, with correlating interaction between pairs of nuclei. 2D NMR has two frequency axes, and the signal intensity is the third axis. This includes correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY or ROESY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC). The techniques provide different information. COSY describes the connectivity between coupled nuclei, while ROSEY tells us if two protons are close to each other in space within the molecule. HSQC correlates proton and carbon nuclei that are one bond apart, telling us which proton is attached to a specific carbon. HMBC correlates carbon that is separated by multiple bonds (62). Therefore, NMR is a powerful tool for structure elucidation of small molecules.

1.9 Bioactivity Profiling

Bioassay is used to detect biological activity for a constituent in a sample or a pure compound. This process is vital for discovering bioactive compounds as lead structures for future drugs and medicine (63). Samples can undergo screening through either phenotypic or target-based assays. Phenotypic screening is a method where the interesting compound's activity is tested on living cells or organisms. These screenings evaluate how the compound affects the observable characteristics or phenotype of cells or organisms, such as cell growth. An example of this is the cell viability assay. Target-based screening, on the other hand, involves testing compounds against specific targets known to be involved in a specific disease (64). An example of a target-based assay is an enzyme activity assay, such as a PTP1B bioassay.

Five different bioassays at Marbio were used to screen the compounds' activity. These assays encompass a wide range of experimental techniques designed to assess the effects of compounds. One common bioassay involves testing compounds' impact on cell function and behaviour to see if the compounds have cytotoxic properties against immortalized cells and cancer cells. Additionally, bioassays can also focus on the activity of pathogens, for example, testing for antibacterial properties. Furthermore, biochemical assays provide insight into how the compounds interact with specific biological molecules or enzymes.

1.10 Background of the Project

The primary objective of this master project was to focus on bioactive secondary metabolites produced by the marine fungus *Parafenestella* sp. In a previous screening campaign at Marbio, the compounds phomenin A (**2**) and B (**3**) were identified and isolated from a liquid culture of *Parafenestella* sp. However, the compounds were unstable, and they were not isolated in sufficient amounts to do a proper bioactivity profiling of the pure compounds. In this thesis, the aim was, therefore, to re-cultivate the marine fungus and to isolate the two compounds in sufficient amounts to confirm their structures and to characterize their bioactivity using specific bioassays. The secondary objective was to cultivate marine fungi and investigate the bioactivity of the extracts.

2. Aim of the Thesis

The primary objective was to isolate and evaluate the bioactivity of phomenin A (**2**) and phomenin B (**3**) from the marine fungi *Parafenestella* sp. This objective was accomplished by achieving several specific secondary objectives as mentioned below:

→ Extract secondary metabolites from marine fungus *Parafenestella* sp. using LLE

→ Fractionate the extract by using flash chromatography

→ Identify the flash fractions consisting of targeted compounds utilising UPLC-MS

→ Purify and isolate target compounds using preparative HPLC

→ Evaluate the bioactivity of the isolated compounds

Secondary objective

A side project was initiated to gain broader experience in marine bioprospecting and the pipeline. This project entailed culturing, extracting, and screening a panel of 22 marine fungal isolates from the NMF project. The specific objectives of this project were as below:

→ Inoculate and culture 22 fungi in liquid media for one month

→ Extract the fungal biomass using LLE

→ Screen the fungal extracts for cytotoxic, antidiabetic and antibacterial activity

3. Materials and Methods

3.1 Biological Material

The materials used in this project were the marine fungus *Parafenestella* sp. and fungi from the NMF project. **Table 1** lists all the fungi. The fungi were collected and identified by Teppo Rämä as a part of the NMF project, which was funded by the Norwegian Biodiversity Information Centre.

The *Parafenestella* sp. was discovered in Ytre Syltevika, Norway, in September 2010. It was collected from a conifer log in the breaker zone on a rocky shore and measured 5.1 cm in length. The NMF samples were collected from Jan Mayen in August 2022. Most of these fungi were collected from macroalgae and driftwood (65).

For long-term storage, pieces of each fungal culture on a solid medium were preserved in cryotubes with 30% sterile filtered glycerol solution and stored in the freezer at -80 ºC.

Table 1: *Sampling information of biological material.*

Name	Collection ID	Taxonomy	Date	Location
079cE1.2	SU079	*Parafenestella* sp.	08.9.2010	Ytre Syltevika, Finnmark, Norway
X7212	TRä2022-44	*Cladosporium* sp.	13.8.2022	Båtsvika, Jan Mayen
X7213	TRä2022-17a	*Cladosporium* sp.	12.8.2022	Sjuhollendarbukta, Jan Mayen
X7214	TRä2022-17a	*Cladosporium* sp.	12.8.2022	Sjuhollendarbukta, Jan Mayen
X7215	TRä2022-85a	*Didymella finnmarkica*	16.8.2022	Kvallrosbukta, Jan Mayen
X7216	TRä2022-85a	*Alternaria* sp.	16.8.2022	Kvallrosbukta, Jan Mayen
X7217	TRä2022-17b	*Sprodiobolus* sp.	12.8.2022	Sjuhollendarbukta, Jan Mayen
X7218	TRä2022-17b	*Leucosporidium*	12.8.2022	Sjuhollendarbukta, Jan Mayen
X7219	TRä2022-44	*Cladosporium* sp.	13.8.2022	Båtvika, Jan Mayen
X7220	TRä2022-85b	*Cladosporium* sp	16.8.2022	Kvallrosbukta, Jan Mayen
X7221	TRä2022-51a	*Cladosporium* sp.	14.8.2022	Rekvedbukta, Jan Mayen
X7222	TRä2022-51a	*Cadophora luteo-olivaceum*	14.8.2022	Rekvedbukta, Jan Mayen
X7223	TRä2022-51a	*Cadophora luteo-olivaceum*	14.8.2022	Rekvedbukta, Jan Mayen
X7224	TRä2022-84a	*Cladosporium* sp	16.8.2022	Kvallrosbukta, Jan Mayen
X7225	TRä2022-84b	*Holtermanniella festucosa*	16.8.2022	Kvallrosbukta, Jan Mayen
X7226	TRä2022-58	*Nereiospora cristata*	15.8.2022	Ullringlaguna, Jan Mayen
X7227	TRä2022-50a	*Dactylospora* sp	13.8.2022	Båtvika, Jan Mayen
X7228	TRä2022-10b	*Paradendryphiella salina*	10.8.2022	Båtvika, Jan Mayen
X7229	M22MAR0001D2.2	*Fairmania singularis*	29.7.2022	Rakkfjorden, Jan Mayen
X7230	TRä2022-10b	*Paradendryphiella salina*	10.8.2022	Båtvika, Jan Mayen
X7231	TRä2022-80	*Cladophialophora* sp.	16.8.2022	Kvallrosbukta, Jan Mayen
X7232	M22MAR0001D2.3	*Pseudogymnoascus pannorum*	29.7.2022	Rakkfjorden, Jan Mayen

3.2 Inoculation and Cultivation of Fungi

The material used to cultivate fungi are summarized in **Table 2**. A total of 6 L of medium was prepared with a concentration of 4 g/L malt extract and 40 g/L NaCl. The mixture was poured into 24x 250 mL Erlenmeyer flasks (EMF), including a medium control, and autoclaved for 2 hours. 1-2 small pieces of the agar plug with the mycelia of the fungus from the agar plate were scraped out using an inoculation loop and placed in each liquid media. The cultures were then incubated at 16 ºC in static condition for approximately one month.

Table 2: *Chemicals and equipment used in the inoculation method.*

Chemicals and equipment	Specifications	Distributor
Malt extract		Merck KGaA, Sigma-Aldrich (Darmstadt, Germany)
Agar		Merck KGaA, Sigma-Aldrich
NaCl	Instant ocean	Aquarium systems
Milli-Q® Ultrapure water (ddH_2O)		Merck Millipore (Burlington, MA, USA)
Glycerol	20%	VWR International (Radnor, PA, USA)
Autoclave	MLS-3781L	Panasonic (Osaka, Japan)
NMF isolates	Norwegian Marine Fungi	Marbank (Tromsø, Norway)
Inoculation loop		Copan Group (Brescia, Italy)

3.2.1 Large-scale Cultivation of *Parafenestella* sp.

Parafenestella sp. was nominated to be the focus of this project based on literature and previous bioactivity testing at Marbio. Therefore, the fungi were cultivated on a larger scale. The method is the same as described in **Chapter 3.2**, but the total culture volume was 11 L.

3.3 Extraction Method

Fungal cultures were extracted using ethyl acetate by the liquid-liquid partitioning method. First, the fungal biomass was filtered, and then broth was extracted using ethyl acetate. The ethyl acetate extract was dried using a rotary evaporator at 40 ºC. The equipment and chemicals used in the extraction method and flash chromatography are given in **Table 3.**

Table 3: *Materials used in the extraction method and flash chromatography.*

Chemicals and equipment	Specifications	Distributor
Acetone		VWR International
Acetonitrile (MeCN)		VWR International
Diaion® HP-20SS		Supelco® Analytical Products, Sigma-Aldrich (Darmstadt, Germany)
Ethyl acetate		Sigma-Aldrich
Flash chromatographic instrument	Biotage SP4	Biotage (Uppsala, Sweden)
Methanol (MeOH)		VWR International
Milli-Q Ultrapure water (ddH_2O)		Merck Millipore
Rotavapor	Laborota 4002 control	Heidolph Instruments HmbH & Co. KG (Schawabach, Germany)
Separatory funnel	500 mL	
Filter papers (d 90 mm)	Lot.No. 17039487	GE Healthcare Life Science, Whatman
PolyVap Tubes		Biotage

3.4 Fractionation of Extract

3.4.1 Column and Extract Preparation

The reversed-phase column used in this method was prepared with a resin called Diaion® HP20-SS. The resin (6,5 g) was weighed and incubated with 70 mL methanol for approximately 20 minutes. After the incubation, the majority of MeOH was removed by decantation. The rest of the MeOH had to be removed and replaced with MQ-water by a vacuum manifold connected to a tap water hose, where the water pump would decrease the pressure, allowing rapid flowthrough. The empty column was mounted on top of the hose with a small amount of MQ-water before they were packed with column material (activated resin). The column material was washed at least two rounds and kept wet with at least 1 cm MQ-water level above the resin. The prepared columns were stored at 4ºC for later use. The fungal extract (0.51 g) was resuspended in 10 mL 90% MeOH. When the solution achieved dissolution, 2 g of Diaion® HP20-SS was added to the extraction. The extract-resin mixture was dried under reduced pressure using a rotavapor at 40 ºC.

3.4.2 Flash Chromatography

The system was first primed to remove air bubbles and then equilibrated with the starting conditions of the flash gradient. After equilibration, the dried extract-resin sample was loaded onto the pre-packed column and kept wet while the analyses ran. The flow rate was set at 12 ml/min. The flash instrument used was a Biotage SP4 system, and the aim was to separate

aqueous and organic compounds. A total of 27 glass tubes were filled after the fractionation. To collect fractions with similar properties and characteristics, the content of three and three glass tubes were pooled into eight PolyVap tubes, except one that was a collection of six fractions (the final six glass tubes).

The overview of glass tubes, resulting flash fraction and the composition of the mobile phase when the content of the glass tube is eluted is presented in **Table 4**. For the flash chromatography, the mobile phase composition was changed in a stepwise manner from 5–100% methanol for 18 glass tubes (yielding the finished fractions 1-6) to a combination of 50% methanol and 50% acetone for fraction 7 and 100% acetone for faction 8. The filled PolyVap tubes were stored at -20 ºC until later use.

Table 4*: Overview of the composition of fractions and mobile phase for the flash fraction.*

Glass tubes	**Fractions**	**Methanol (%)**	**Water (%)**	**Acetone (%)**
1-3	1	5	95	-
4-6	2	25	75	-
7-9	3	50	50	-
10-12	4	75	25	-
13-15	5	100	-	-
16-18	6	100	-	-
19-21	7	50	-	50
22-27	8	-	-	100

3.4.3 Drying the Flash Fractions

Eight tared PolyVap tubes were filled with fractions from the flash chromatography based on where the glass tubes belonged. A PolyVapor vacuum concentrator operated at 40 ºC and 60 rpm with reduced vacuum pressure dried the collected fractions. After drying, the tubes were weighed, and the yield of the individual fractions was calculated. The calculated volumes of DMSO were pipetted onto the PolyVap tubes and put on a shaker until complete dissolution. They were dissolved in DMSO to a known concentration, typically 20, 40 or 80 mg/mL, depending on the sample quantity. The aliquot volume of flash fractions was diluted in 90% methanol for chemical analysis, and the remaining flash fractions in cryotubes were stored at -20 ºC for later use.

3.5 Chemical Analysis

3.5.1 Sample Preparation of Flash Fractions

A total of eight dried sample fractions were weighed and dissolved in 90% MeOH. The concentration was 80 mg/ml. Each sample was pipetted from PolyVap tubes to Eppendorf tubes and centrifuged at 2000 rpm for 2 minutes. 100 µl of each flash fraction was pipetted into an HPLC vial and stored at -20 ºC for later use. It was also prepared a blank containing 400 µl 90% MeOH.

3.5.2 Dereplication of Flash Fractions

For the chromatography step, a Waters Acquity First Class UHPLC was used. The column used was an Acquity UPLC-BEH C_{18} with a 2.1 mm x 100 mm diameter and a particle size of 1.7 µm. The mobile phases contained A and B, where A was MQ-water and 0.1% formic acid (FA), while B consisted of acetonitrile and 0.1% formic acid (FA). FA helped increase the ionization of basic compounds by increasing the donation of protons (H^+) that can attach to an analyte to ionize them. The mobile phase was an isocratic eluent from 10% to 100% B for 13.50 minutes. Then, the column was equilibrated with starting conditions for 1 minute to be ready for the next analysis (see **Table 5**). The flow rate was 0.450 mL/min, the injection volume was 8 µl, and the temperature was 40 ºC.

The MS was conducted using a Waters Vion IMS-Q-ToF set on a positive ESI mode with a capillary voltage of 0.80 kV. For the MS^2 method, both low (5 eV) and high collision (20-60 eV) energy were set. However, since the structures of the compounds were known, only the low collision energy spectrum was used to confirm the presence of the interesting compounds. The column was washed with 100% B and equilibrated with starting conditions. Leucine-enkephalin with a 100 µg/µl concentration was used as a "lock mass". In **Table 6,** other key parameters are presented.

Table 5: *Mobile phase settings for the MS method.*

Time (minutes)	Composition A (%)	Composition B (%)
0.00	90	10
12.00	0	100
13.50	0	100
14.00	90	10
15.00	90	10

Table 6: *Parameters set in IMS-Q-ToF method.*

IMS-q-tof MS parameters	Value
Collision gas	Nitrogen
Cone voltage	30 V
Ionization mode	Positive (+)
Scan range	150 – 2000 *m/z*
Source temperature	120 ºC
Desolvation temperature	350 ºC

3.6 Isolation Method

The isolation of the target compounds was done by using a Waters Preparative HPLC system, consisting of a Make-up Pump, 600 Controller, 2996 Photodiode Array Detector, 3100 Mass Detector, Flow Splitter, Prep Degasser, and a 2767 Sample Manager. The software used was MassLynx 4.1. The UHPLC-MS identified the interesting compounds in flash fractions 5,6 and 7. Given these compounds' known chemical structure and mass, the prep-HPLC was set to trigger fraction collection when the mass *m/z* 253.3 was detected. Due to uncertain concentration levels, the detection threshold ranged from 500.000 to 800.000, and each injection was adjusted depending on the isolation. After isolation, glass tubes with samples were pipetted into round flasks and the sample volume was reduced using a rotavapor at 30 ºC. The volume was reduced to dryness using a Freeze Dryer, and the flasks were stored at -20 ºC until further use.

3.6.1 The First Round of Isolation

For the first round of isolation, compounds **1**, **2** and **3** were isolated. In total, 44 injections were done with an injection volume varying from 50 – 300 µl. The column used was a Waters Sunfire reversed phase C_{18} with a dimension of 10 mm x 250 mm and a particle size of 5 µm. The mobile phase was an isocratic eluent. Mobile phase A was MQ-water with 0.1% FA, and B consisted of acetonitrile with 0.1% FA. The composition of the isocratic eluent varied between injections, but it was set to around 56% B depending on how far or close the peaks eluted.

3.6.2 Identification of the Isolated Compounds

The isolated compounds were analysed using the IMS-Q-ToF to check their purity. MeOH was used as a solvent to dissolve the compounds before injecting. The same parameters were used as described in **Chapter 3.5.2**. The injection volume was 4 µl for compound **2** and 5 µl for compound **3**.

3.6.3 The Second Round of Isolation

The NMR analysis of compound **3** showed some impurities, resulting in another round of isolation with the preparative HPLC. This round, another column was used, a Waters XSELECT CSH phenyl-hexyl with a 10 mm x 250 mm diameter and a particle size of 5 µm. Before injecting, the sample was diluted with 3:1 with 90% MeOH. The same mobile phase compositions from **Chapter 3.6.1** were used. The mobile phase was an isocratic eluent consisting of approximately 38% MeOH, with an injection volume varied from 50 – 150 µl depending on the chromatography. The second round isolated both compounds **2** and **3**.

3.7 Nuclear Magnetic Resonance

NMR was used to confirm the structure of both compounds. Johan Isaksson, who was at the Department of Pharmacy at UiT—The Arctic University of Norway, performed the NMR. PhD fellow Sailesh Maharjan, at Marbio, interpreted the NMR data. The NMR experiments were performed using the MestReNova software on a Bruker Avance III HD spectrometer operating at 599.90 MHz for protons. The spectrometer was equipped with an inverse-detected cryo-probe enhanced for ^{1}H, ^{13}C, and ^{2}H. The NMR samples were prepared in *d*$_6$-DMSO or deuterated water. Experiments were typically acquired using gradient-selected adiabatic versions where applicable. All experiments were acquired using Top Spin 3.5 pl2 at 298 K.

3.8 Bioactivity Testing

Phomenin A (**2**) and B (**3**) were tested in various bioassays to evaluate their bioactivity. Since the yield of phomenin B (**3**) was lower, it was only tested in a few bioassays. **Table 7** shows the test concentrations of both compounds in each bioassay.

Table 7: *An overview of phomenin A (**2**) and B (**3**) test concentration for each bioassay."x" signifies that the compound was tested in the specified assay at det designated concentrations.*

Bioassays	Test concentrations (µM)	Phomenin A testing	Phomenin B testing
Cell proliferation	100, 50, 25, 12.5	x	x
PTP1B Inhibition	100, 50, 25, 12.5	x	x
DPP-IV Inhibition	100, 50, 25, 12.5	x	
Antibiofilm formation	200, 100, 50	x	
Antibacterial	200, 125, 100	x	x

3.8.1 Cell Proliferation Assay

The pure compounds were tested on two different human cell lines: normal lung fibroblast (MRC-5) and human breast adenocarcinoma (MCF7). The samples were tested using The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. This colourimetric assay was used to measure mitochondrial activity in cells by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (yellow) to formazan product (violet). The formazan product is proportional to the number of active metabolically active cells. By measuring the absorbance of the formazan product, the cell viability can be calculated by comparing this to the same value measured in negative (100% survival) and positive (0% survival) cell wells (66). **Table 8** presents the materials used in the cell proliferation assay.

Table 8: *Materials for cell proliferation assay.*

Reagents and equipment	Specifications	Distributor
CellTiter 96® Aqueous one solution reagent	G3581	Nerliens (Promega)
Dimethyl sulfoxide (DMSO)	D4540	Merck Life Science AS
FBS (Fetal Bovine Serum)	S1810-500	VWR (Biowest)
Gentamycin	A2712	VWR (Biochrom)
Glutamine stable 200 mM	X0551-100	VWR (Biowest)
Incubator	37 ºC, 5% CO_2	
MEM (Minimum essential Medium) Eagle	M7278-500ML	Merck Life science AS
Microtiterplate 96-well (Nunlon delta surfac)	734-2073	VWR International
Non-essential amino acids	K0293	VWR (Biochrom)
Nunc easy flask 75 cm^2	734-2066	VWR International
Phosphate Buffered saline (PBS)		Produced in-house
Tecan Spark platereader	Absorbance 490 nm	Tecan (Männedorf, Switzerland)
Trypsin	X0930	VWR (Biowest)
MCF7	Human breast adenocarcinoma	ATCC HTB-22
MRC-5	Lung fibroblast, normal	ATCC CCL-171

Cell Culture Media Preparation

To prepare the cell culture media, MEM Eagle was preheated to 37 ºC and supplemented with 50 mL FBS, 500 µL gentamycin, 5 mL of glutamine stable, non-essential amino acids, and sodium pyruvate. The media was then transferred to a Nunc Easy Flask, where the cells were allowed to grow. All steps were conducted in an aseptic environment throughout the cell-seeding process. The cell seeding process began with preheating cell culture media, trypsin, and PBS to 37 ºC. The cells were examined in a microscope to ensure their health before continuing. The cell culture media was removed, and the cell layer was washed with PBS. Trypsin was pipetted over the cell layer and incubated at 37 ºC for 2-5 minutes until the cells detached. Upon detaching, the cell was resuspended in 10 mL culture media. A mixture of 10 mL trypan blue and 100 µL suspension was prepared, and 10 mL was transferred to Burker counting camber for cell counting under a microscope.

Samples Preparation

The cell suspension was diluted based on cell density (2000 cells/wells for MCF7 and 4000 cells/well for MRC-5) for a total of 15 mL. 100 µL cell suspension was pipetted to a 96-well microtiter plate. The growth media was removed from all wells and replaced with 90 µL pre-warmed (37 ºC) culture media. Next, 10 µL of the samples were added in triplicate. Two rows contained 100 µL negative control (cell culture media), and one row was a positive control (10% DMSO).

Reading the Results

The plate was incubated at 37 ºC and 5% CO_2 for 72 hours. 10 µL CellTiter 96® AQ$_{uesous}$ One Solution Reagent was added to each well, and the plates were incubated for 1 hour at 37 ºC. The optical density (OD) at 490 nm was measured using a Tecan Spark plate reader. To evaluate the bioactivity of the compounds at Marbio, the OD is used to calculate health cell survival (%). The equation for calculation is presented below:

$$Cell\ survival\ (\%) = \frac{OD_{sample} - OD_{pos}}{OD_{neg} - OD_{pos}} * 100$$

Equation 1: *Calculation of cell survival (%) in viability assay. OD_{pos} = 10% DMSO, OD_{neg} = cell culture media, OD_{sample} = target compounds.*

The results at Marbio are scored as Active (A), Questionable (Q), or Inactive (I) according to the cell survival (%) of the wells shown in **Table 9**.

Table 9: *Cut-off values for the cell proliferation assay.*

Category	Cell survival (%)
Active (A)	< 50
Questionable (Q)	50 – 60
Inactive (I)	> 60

3.8.2 PTP1B Inhibition Assay

The pure compounds were tested for antidiabetic effects by inhibiting the enzyme protein tyrosine phosphatase 1B (PTP1B), which is associated with developing type 2 diabetes. **Table 10** presents the reagents and equipment used in the PTP1B enzyme assay.

Table 10: *Materials for PTP1B assay.*

Equipment and reagents	Specifications	Distributor
Bovine serum albumin (BSA)	A2153	Merck Life Science AS
Dithiothreitol (DTT)	D5545	Merck Life Science AS
DMSO		Merck Life Science AS
Ethylendiamin tetraacetic acid (EDTA)	E1644	Merck Life Science AS
Hepes	H3375	Merck Life Science AS
Milli-Q® Ultrapure water (ddH_2O)		Merck Millipore
Sodium Chloride (NaCl)	S9625	Merck Life Science AS
NaOH-solution - for adjustment of pH		
Protein tyrosin phosphatase 1B (PTP1B)	539735	Calbiochem/Merck Life Science AS (Burlington, Massachusetts, USA)
Protein tyrosin phosphatase inhibitor IV	540211	Calbiochem/Merck Life Science AS
6, 8-difluoro-4- metylumbelliferylfosfat (DiFMUP)	D6567	Thermo Fisher Scientific (Waltham, Masssachusetts, USA)
Nunc™ black polystyrene 96-well MicroWell™ plate	734-2073	VWR International
Tecan plate reader		Tecan

The assay buffer consisted of 50 mM NaCl, 25 mM Hepes, 2.5 mM EDTA, 2 mM DTT and 0.01 mg/mL BSA. The chemicals were dissolved in 500 mL and pH was adjusted to pH 7.2. A stock solution of PTP1B (15 ng/ml), PTP inhibitor IV (0.41 µg/mL), and DiFMUP (10 µM) was diluted using assay buffer to achieve the final assay concentration. 25 µL sample, inhibitor, or negative control was pipetted to a black polystyrene microtiter plate to assigned wells**.** 50 µL PTP1B was added to all wells. The plate was incubated at 37 ºC for 30 minutes. Then 25 µL DiFMUP was added to all wells and incubated at 37 ºC for 10 minutes. Fluorescence measurements were conducted using a Tecan plate reader with excitation at 360 nm and emission at 465 nm. The results at Marbio are scored as Active (A), Questionable (Q), or Inactive (I) according to the remaining enzyme activity of the wells, shown in **Table 11**.

Table 11*: Cut-off values for PTP1B assay.*

Category	Remaining enzyme activity (%)
Active (A)	< 30
Questionable (Q)	30 – 40
Inactive (I)	> 40

3.8.3 DPP-IV Inhibition Assay

Phomenin A (**2**) was tested for DPP-IV inhibitory activity, which is linked to developing type 2 diabetes. **Table 12** presents the reagents and instruments used in the DPP-IV inhibitor assay.

Table 12: *Material for DPP-IV inhibition assay.*

Equipment and reagents	Specifications	Distributor
AMC (7-amino-4-methyl-coumarin)	4001606-0001	Bachem AG (Bubendorf, Switzerland)
Black 96-well Immuno Plates Universal Binding	9502867	Thermo Fisher Scientific
Diprotin A (Ile-Pro-Ile)	4009723.0050	Bachem AG
DPP-IV	SLCG0713	Sigma
Gly-Pro-AMC HBr (7-amino-4-methyl-coumarin hydro bromide)	4002520.0050	Bachem AG
Victor® Multilabel Plate Reader (360 nm (ex), 460 nm (em))		PerkinElmer (Waltham, Massachusetts, USA
EDTA (Ethylene Diamine-Tetra-acetic Acid)	Assay buffer	
NaCl	Assay buffer	
Tris-HCl	Assay buffer	

Samples were prepared in triplicates, diluted in assay buffer, and shaken for 30 minutes. The test concentrations were 50, 100 and 200 µM. 30 µL assay buffer and 50 µL 100 uM Gly-Pro-AMC were loaded for 5 minutes at 37 ºC. A standard curve of AMC was made. The fluorescence was measured by a Victor microplate reader at excitation 360 nm and emission 460 nm as T_0. 10 µL of DPP-IV was pipetted, and the plate was incubated for 30 minutes at 37 ºC, and the fluorescence was measured again at the same wavelength as T_{30}.

3.8.4 Antibiofilm Formation Assay

The assay aimed to determine if the test compound (Phomenin A (**2**)) can inhibit the bacteria biofilm created by *Staphylococcus epidermis*. A bacterial biofilm increases the resistance to antibiotics and other antimicrobial treatments, which is why new treatment options are needed to inhibit its formation (67). *Staphylococcus haemolyticus*, a non-biofilm-forming bacteria, was used as a control. The reagents and equipment used in this assay are presented in **Table 13.**

Table 13: *Materials for antibiofilm formation assay.*

Equipment and reagents	Specifications	Distributor
Milli-Q® Ultrapure water (ddH_2O)		Merck Millipore
96% ethanol		UiT (Tromsø, Norway)
Blood agar plates	Media kithcen	SUMP (UNN, Tromsø, Norway)
Crystal violet solution 1%	V5265	Merck Life Science
Glucose	D9434	Sigma Aldrich
Nunc™ Microwell™ 96-well microtiter plate	734-2073	Thermo Fisher Scientific
Tryptic soy broth (TBS)	105459	Merck KGaA
Victor Multilabel Counter		PerkinElmer
Staphylococcus epidermidis	ATCC 35984	LGC Standards (UK, Teddington)
Staphylococcus haemolyticus	A gift	Host-microbe interaction research group UiT, Tromsø, Norway

Bacterial Strains Preparation

The pathogens from the biofreezer stock were streaked on blood agar plates and incubated overnight at 37 ºC. This assay runs over three days. A scoop of pathogens from the blood agar plates was introduced to 5 mL growth media and incubated at 37 ºC, 100 rpm for approximately 20 hours. Both bacteria strains were diluted 1:100 in the overnight cultures, which consisted of 1 g glucose and 100 mL growth media.

Samples Preparation

The samples (50 µL) were pipetted in triplicates on a microtiter plate with 50 µL diluted bacterial suspension of *S. epidermis*. One column contained 50 µL bacterial suspension of *S. epidermis* and 50 µL ddH_2O (growth control), and another was the same but with S. *haemolyticus* (non-biofilm-forming control). One column was a medium blank containing 50 µL growth medium + 50 µL ddH_2O. The microtiter plate was incubated at 37 ºC overnight.

Reading the Results

The microtiter was tapped dry against cell paper and rinsed with tap water repeatedly, then fixed at 65 ºC for 1 hour. After that, all wells were stained with a 0.1% crystal violet solution for 5 minutes before being rinsed again. 70 µL 70% ethanol was added to all wells, incubated on a shaker for 5 minutes, and the OD was measured using a Victor plate reader. The OD is used to calculate the biofilm formation (%), presented below (**Equation 2**):

$$Biofilm\ formation\ (\%) = \frac{OD_{testwells} - OD_{blankwells}}{OD_{growthcontrol} - OD_{blankwells}} * 100$$

Equation 2: *Calculation of biofilm formation (%) in the wells. Optical density (OD) measurements from the test well with samples, blank well, and growth control are used.*

The results are scored at Marbio as Active (A), Questionable (Q), or Inactive (I) according to the percentage of biofilm in the wells compared to the control wells (**Table 14**).

Table 14: *Cut-off values for the biofilm formation inhibition assasy.*

Category	Biofilm formation (%)
Active (A)	< 30
Questionable (Q)	30 – 40
Inactive (I)	> 40

3.8.5 Antibacterial Assay

Phomenin A (**2**) and B (**3**) were tested for antibacterial activity. **Table 15** shows the reagent, equipment, and the five pathogens used in the assay.

Table 15: *Material for antibacterial assay.*

Equipment and reagents	Specifications	Distributor
Blood agar plates		Sump UNN (Tromsø, Norway)
Brain Heart Infusion (BHI)	53286	Sigma Aldrich
Gentamicin	A2712	VWR International
Microtiter plates	734-2073	VWR
Milli-Q® Ultrapure water (ddH_2O)		Merck Millipore
Victor multilabel counter		PerkinElmer
Enterococcus faecalis	ATCC 29212	LGC Standards
Escherichia coli	ATCC 25922	LGC Standards
Pseudomonas aeruginosa	ATCC 27853	LGC Standards
Staphylococcus aureus	ATCC 25923	LGC Standards
Streptococcus agalactiae	ATCC 12386	LGC Standards

Pathogens Preparation

Five bacteria stains were used as pathogens. The pathogens were streaked on separate blood agar plates and incubated at 37 ºC overnight. The assay took place over three days. On the first day, a small scoop of pathogens was transferred from blood agar plates to 8 mL of growth media and incubated at 37 ºC for approximately 20 hours.

Performing the Assay

On day two, 50 µL of the samples in duplicate was pipetted to a microtiter plate. 50 µL ddH_2O was added to all columns, while 50 µL medium blank (growth medium) was pipetted to a column. A dilution series of gentamycin was also prepared on a microtiter plate as a control.

On the same day, the dilution of overnight cultures was done. 2 mL of overnight culture was transferred to a separate EMF containing 25 mL growth media and incubated for 2.5 hours (100 rpm). Then, 100 µL of the bacterial suspension was transferred to a 9.9 mL growth medium. Finally, 4 mL of this mixture was pipetted to a 36 mL growth medium. At last, 50 µL was pipetted to columns accordingly (2-11 as well as 1 or 2 rows in the gentamycin plate).

Reading the Results

On the final day, the OD in the wells was measured using a Victor plate reader. The results at Marbio are scored as Active (A), Questionable (Q), or Inactive (I) according to the measured OD presented in **Table 16**.

Table 16: *Cut-offs values for the antibacterial growth inhibition assay*

Category	Absorbance
Active (A)	< 0.05
Questionable (Q)	0.05 – 0.09
Inactive (I)	> 0.09

4. Result and Discussion

Previously at Marbio, both compounds (phomenin A (**2**) and B (**3**)) have been identified and isolated from *Parafenestella* sp., and the structures were elucidated using NMR. However, the compounds were unstable, resulting in insufficient amounts to do proper bioactivity profiling and confirming the structures. Therefore, the compounds were nominated to be re-cultivated, isolated, and screened for bioactivity. Since the compounds had already been isolated, the chemical structure was known, making this a target analysis. The secondary objective focused on the cultivation and extraction of selected marine fungal isolates to get acquainted with the techniques. As it is not the primary objective, the data are included in **Appendix 5.3**.

4.1 Extraction Yield

Parafenestella sp. was extracted using liquid-liquid extraction with ethyl acetate. The larger-scale cultivation of the fungus method yielded 0.51 g of fungal extract.

4.2 Fractions Yields

After drying the flash fractions, the yields were calculated by weighing tared empty glass tubes and glass tubes containing the sample. For analysis on UHPLC-MS, 90% MeOH was added to achieve a final concentration of approximately 40 mg/mL. Since the yields of flash fractions 6 and 8 were relatively lower than the others, the concentration was set at 20 mg/ml. While flash fractions are typically diluted in DMSO that has good solubility, it is not recommended for samples intended for UHPLC-MS analysis due to the potential damage DMSO can cause to the column. This is the reason why methanol was used instead. The results are given in **Table 17**.

Table 17: *Yields and concentration for each flash fraction from flash chromatography.*

Flash fraction	Yield (mg)	Final concentration (mg/mL)	Volume 90% MeOH (µL)
1	59.6	45.9	1300
2	42.6	38.7	1100
3	43.6	39.6	1100
4	29.8	33.1	900
5	78.2	52.1	1500
6	13.1	16.4	800
7	36.8	36.8	1000
8	13	18.6	700

4.2.1 Chemical Analysis of Flash Fractions

The flash fractions 1- 8 were analysed on the IMS-Q-ToF. Fraction 1 is the most polar, while fraction 8 is the most non-polar. The target compounds (MW 234.3) were found with the highest intensity in fractions 5-7. Secondary metabolites are usually moderately non-polar, suggesting they are likely found within fractions 4-7. **Figure 14** shows the base peak intensity (BPI) chromatogram from the UHPLC-HR-MS for flash fractions 2 (red) and 5 (green). Noticeably, the intensities of the targeted compounds were significantly higher in fraction 5 than in fraction 2. The protonated mass of targeted compounds (*m/z* [M+H] 235.13) was well detected with positive ionization mode. The compounds have an RT = 6,55-6,64 minutes. As the phomenins had already been identified at Marbio, the mass was known, which made it easier to find the compound in the chromatograms. Two closely separated peaks on the fraction 5 chromatogram indicated that the compound was an isomer. This was further investigated using prep-HPLC.

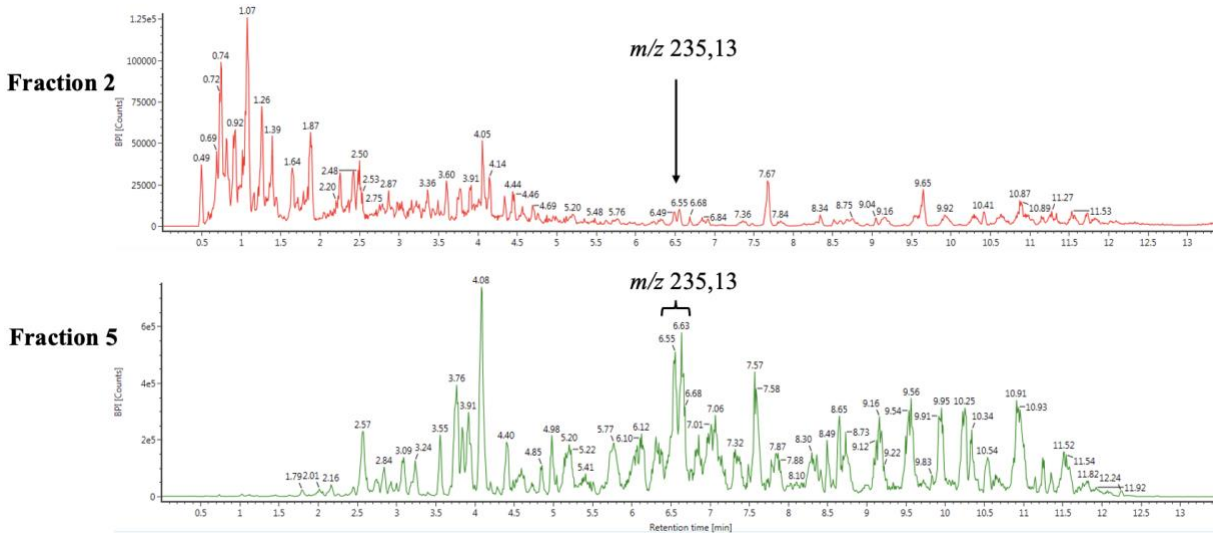


Figure 14: *UHPLC-HR-MS BPI chromatograms of fractions 2 (red) and 5 (green). The analytes are represented in larger amounts in fraction 5 than in fraction 2. ESI$^+$ mode.*

4.3 Isolated Compounds

The target compounds from fractions 5 – 7 were isolated with prep-HPLC. The concentration was unknown since the samples were diluted based on the intensity in the chromatogram. The prep-HPLC was set to find *m/z* 235.2. Numerous factors can influence a chromatogram. If the pressure is too high (above 3000 psi) due to a poorly washed column, it can cause a shift in the chromatogram and a longer retention time. The threshold was set between 500 000 – 800 000. If the sample was well diluted, the threshold was lower to ensure the prep-HPLC isolated the interested compounds. The injection also varied, depending on how far apart the compounds eluted. If they were nicely separated, the injection volume was increased.

4.3.1 Isolation from the First Round

Figure 15 shows the BPI chromatogram for a single injection on the prep-HPLC. This led to separating and isolating four compounds with different retention times. The isolation of four compounds with the same molecule weight implies the existence of isomers. The first and second isolated compounds were combined into compound **1** and then compounds **2** and **3**. For this round, a Waters Sunfire reversed phase C_{18} (5 µM, 250x10 mm) column was used.

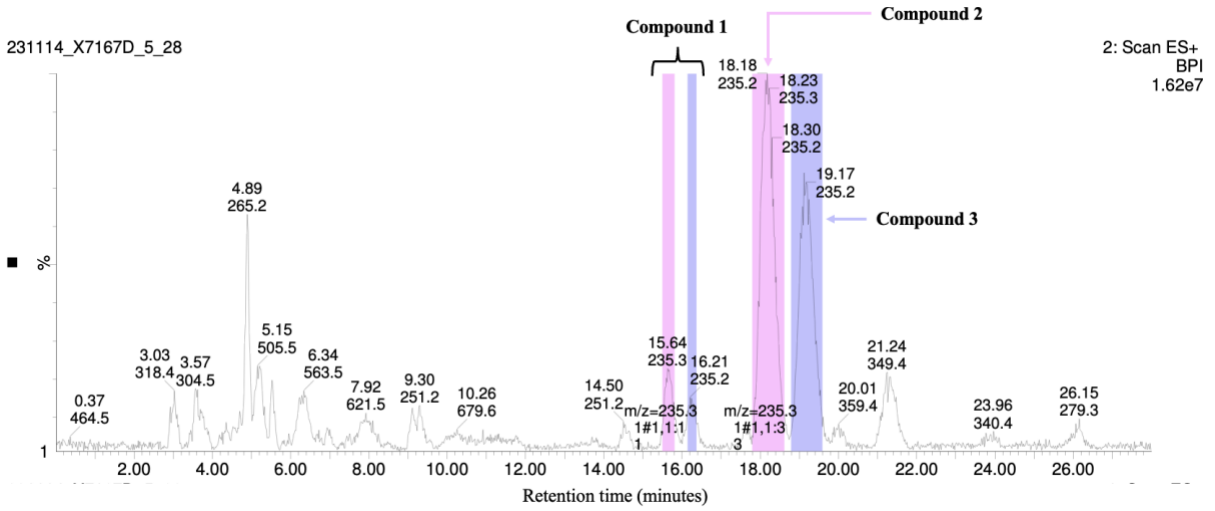


Figure 15: *Prep-HPLC BPI chromatogram from the initial isolation of the target compounds. The pink and purple lines indicate the collected area. In the first round of isolation, the prep-HPLC isolated four different compounds. The first two isolated compounds were pooled into compound **1**.*

In the first round of isolation, the purity of the compounds was evaluated using IMS-Q-ToF. Compound **1** was analysed on IMS-Q-ToF, as seen in **Figure 16**. The compound had a significantly low yield, and several impurities were detected. Additionally, the isomer of the compound has converted to compounds **2** and **3**, likely due to external factors like temperature. Specifically, the compound was dried using a SpeedVac at 30 ºC, which may have contributed to the conversion. Since the compound was unstable and had a low yield, it was not further studied.

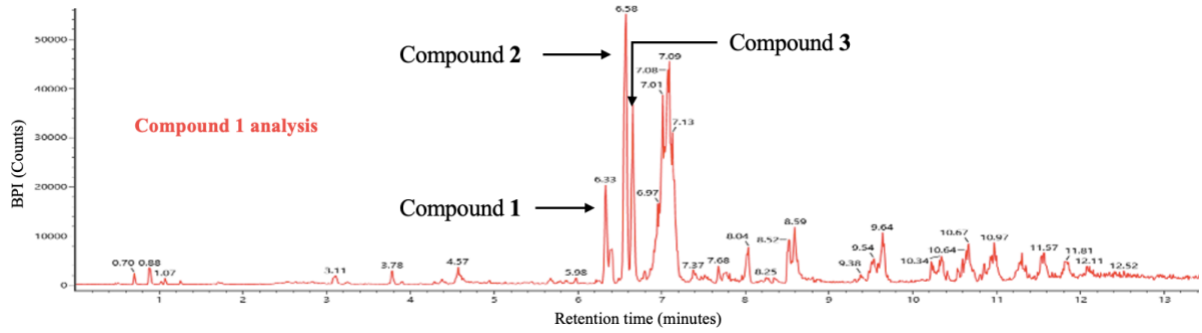


Figure 16: *UHPLC-HR-MS BPI chromatogram of compound **1** in ESI⁺ mode. Compounds **2** and **3** are more abundant than **1**.*

Figure 17 shows the UHPLC-HRMS BPI chromatogram of compounds **2** (blue) and **3** (red) in ESI⁺ mode. Compound **2** (blue) was pure enough to continue the bioactivity profiling. The BPI chromatogram of compound **3** (red) showed that the sample contained more of **2** and other impurities, which resulted in another round of isolation. This may be because compound **2** is more stable and occurs more frequently in nature, while **3** is more unstable and converts to **2**.

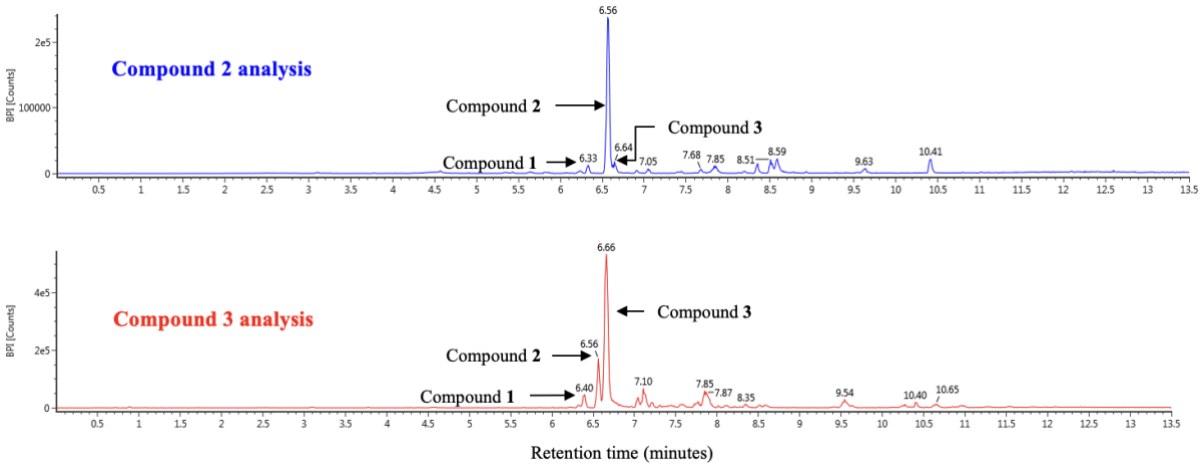


Figure 17: *UHPLC-HR-MS BPI chromatograms of target compounds **2** (blue) and **3** (red). Compound **2** is pure, while the chromatogram of **3** shows more impurities. It was obtained in ESI⁺ mode.*

4.3.2 Purification from the Second Round

Based on the IMS-Q-ToF analysis of compound **3**, it was not pure enough to continue with bioactivity testing. For a second step of isolation, a phenyl hexyl column was used. The column has an aromatic ring attached to the hydrocarbon chain, increasing the affinity for aromatic compounds, and resulting in a better separation of the target compounds. The prep-HPLC isolated both compounds **2** and **3**, shown in **Figure 18**.

Compounds **2** and **3** had already been separated and isolated from the first round of isolation. Given this, the intensity for compound **3** should be significantly higher than for compound **2** in the second step of isolation of compound **3**. As shown in **Figure 18**, the intensity of compound **2** is relatively high. This again can confirm that compound **3**, which is more unstable, has converted to compound **2**. External factors, such as heat from the drying process, may caused this. After the isolation of compounds **2** and **3**, each yield was 0.6 mg and 0.4 mg, respectively.

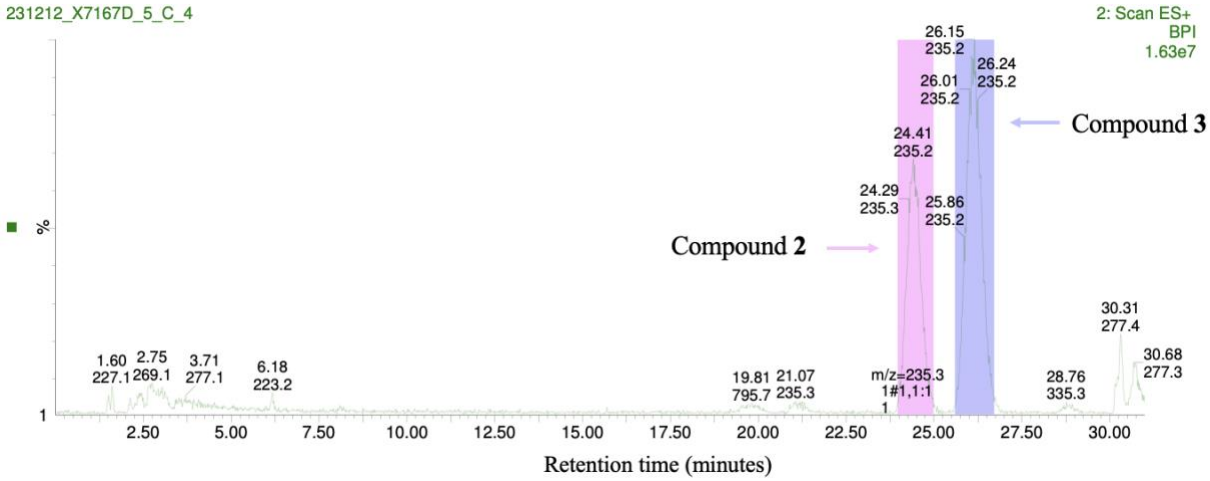


Figure 18: *BPI chromatogram from prep-HPLC for one injection. The pink and purple lines indicate the collected area at R_t = 24.41 and 26.24, respectively. Compounds **2** and **3** were isolated with m/z of 235.2.*

4.4 Structure Elucidation of the Isolated Compounds

Compound **2** was obtained as a light reddish-brown solid. The molecular formula of **2** was calculated to be $C_{14}H_{18}O_3$, based on HRESIMS *m/z* 235.1335 $[M+H]^+$ (calcd. for 235.1334), corresponding to six degrees of unsaturation that suggested a presence of a polyene or aromatic structure. The complete structure of **2** was elucidated by the interpretation of 1D (^1H and ^{13}C) and 2D (HSQC, HMBC, COSY, and ROESY) NMR spectra. The ^1H NMR spectrum (Appendix A**5.1 NMR Spectra)** of **2** showed the presence of four methyls (δ_H, 1.74, 1.80, 1.84, and 2.05), one methoxy (δ_H 3.95), three methine protons (one vinylic (δ_H 5.68 (q), J = 7.0 Hz) and two aromatic protons (δ_H 6.54, and 6.81)). The ^{13}C NMR spectrum (**5.1 NMR Spectra**) showed 14 carbon signals, attributable to four methyl carbons (δ_C, 8.67, 13.8, 14.0, 16.4), one methoxy (δ_C 56.8), nine sp^2 hybridized carbons (two tetrasubstituted olefinic (δ_C 93.6, 100.0), four olefinic (δ_C 124.6, 129.4, 132.6, 135.2), and three carbonyl-like (δ_C 165.6, 166.2, 159.7)). The HSQC spectrum supported the interpretation of all these caron signals. The HMBC correlation signals observed are displayed in (**Figure 19**). The correlations observed from methyl protons H_3-14 (δ_H 6.50) to C-3 (δ_C 100.0), C-2 (δ_C 165.6), and C-4 (δ_C 166.2); from methoxy proton H_3-4 (δ_H 3.95) to C-4; and from H-5 (δ_H 6.54) to C-2, C-4, C-6 (δ_C 159.7) allowed assignment of a α-pyrone moiety. This confirmed the attachment of methyl (H_3-14) and methoxy groups to C-3 and C-4, respectively. Moreover, HMBC correlations from H-15 to C-7 (δ_C 124.6) and from Methyl proton (CH_3-13, δ_H 13.8) to C-6 and C-7 established attachment of diene substituent at C-6. The key HMBC correlations shown in **Figure 19** indicated the partial structure of the diene substituent. Further, this partial structure of the diene substituent and configurations of the double bond at C-7 and C-9 were deduced by the ROESY experiment. The ROE correlations between H-5/H_3-13, H-8/H-10, H_3-12/H_3-16 and the absence of other correlations H_3-13/H-8 and H_3-12/H-10 provided evidence for *E*-configuration at Δ^7 and Δ^9-double bonds. Hence, the structure of **2** was assigned to Phomenin A. The ^1H and ^{13}C NMR data of Phomenin A summarized in **Table 20** was in good agreement with the previously reported data. It has been isolated from other fungi such as *Phoma tracheiphila* (68), *Leptosphaeria maculans* (69, 70), and *Alternaria infectoria* (71). However, it has not been previously isolated from *Paranestella* sp.

Compound **3** was isolated as a light reddish-brown solid. It is an isomer of **2** as indicated by the same protonated mass (*m/z* 235.1335 $[M+H]^+$) and the molecular formula ($C_{14}H_{18}O_3$) determined by HRESIMS analysis. ^1H and ^{13}C NMR spectroscopic data of **2** were similar to **3**

except for slightly deshielded methyl carbon (CH_3-12, δ_C 23.2) in the ^{13}C spectrum of **3** as shown in **Table 20**. ROESY experiment provided evidence that they differ only in the stereochemistry of the C-9 double bond. The configuration of Δ^9-double bonds was assigned as 9*Z* based on ROE correlations between H_3-13/H_3-12, H_3-12/H-10, H-8/H-11, and the absence of the H-8/H-10 correlation. Consequently, the structure of **3** was assigned to Phomenin B (68).

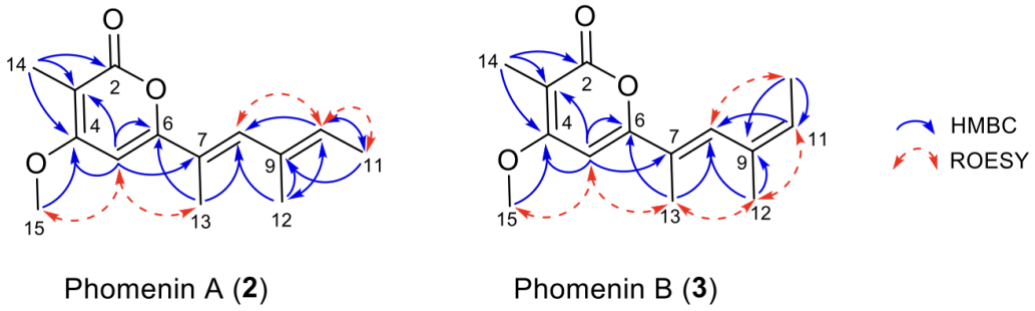


Figure 19: *Key HMBC and ROESY correlations of compounds **2** and **3**.*

4.4.1 Purified Compounds (Phomenins)

Using NMR analysis of the purified confirmed their identities as phomenin A (**2**) and B (**3**). The molecule structure is shown in **Figure 20**. They belong to the polypropionate α-pyrones class found in many biologically active metabolites (68). These compounds are stereoisomers; phomenin A (**2**) is a *9*E isomer, while phomenin B (**3**) is a *9*Z isomer. In general, the E isomer tends to be more stable and lower in energy compared to Z isomers (72). The total energy of the phomenins has not been calculated. Therefore, there is an uncertainty about which of the isomers has the lower energy. Since phomenin A (**2**) is a *9*E isomer, it could be the reason why it occurs more frequently in nature and wherefore phomenin B (**3**) converts to this isomer.

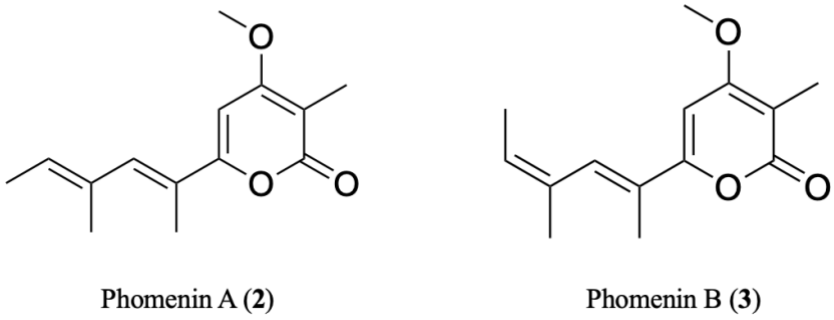


Figure 20: *The chemical structure of phomenin A (**2**) and phomenin B (**3**) (created in ChemDraw).*

4.5 Bioassays Results

The pure compounds were tested in five different bioassays to evaluate their bioactivity. **Table 7** provides an overview of the test concentrations and bioassays in which the compounds were tested. They were tested for cytotoxic, antibacterial and antidiabetic activity. The results from the testing at Marbio are categorised as Active (A), Questionable (Q) or Inactive (I). The data was visualised using GraphPad Prism Version 10.2.3.

Data on the phomenins bioactivity are discrepant. One study from 1993 detailed that the phomenins had been toxic to *Artemia salina* in a brine shrimp bioassay, which is known to be related to cytotoxic properties. Only phomenin A (**2**) was tested in a tomato-cutting assay for phytotoxicity with a minimum active concentration between 100 – 200 µg ml^{-1} (68). One year later, in 1994, another study found three interesting metabolites producing bright yellow pigments. In this study, the phytotoxicity of phomapyrones A, B, and C was not determined because of the small amounts; however, the crude extracts they were isolated from had a phytotoxic effect on *Brassica species* (73). Another study used a mixture of phomenin A (**2**) and B (**3**) (10:1) that displayed low toxicity in MRC-5 cells at a concentration of 0.8 uM. A higher concentration could not be tested because of solubility problems. The neutral red uptake decreased by 55% compared to the solvent control, but the study confirmed that the compounds were not cytotoxic based on Alamar Blue and LDH leakage assays (74).

4.5.1 Cell Proliferation Assay

The cytotoxic effect of phomenin A (**2**) and B (**3**) was assessed against normal lung fibroblasts (MRC-5) and human breast adenocarcinoma (MCF7). The test concentration was 25, 50, 100 and 100 µM. Phomenin A (**2**) and B (**3**) were considered not cytotoxic. The cell survival (%) values for both MRC-5 and MCF7 cells with both compounds were generally above 100%, indicating a potential increase in cell proliferation rather than cytotoxicity (**Figure 21**). While none of the compounds was categorised active, phomenin B (**3**) at 100 µM had the lowest % cell survival in each cell line. No dose response was identified.

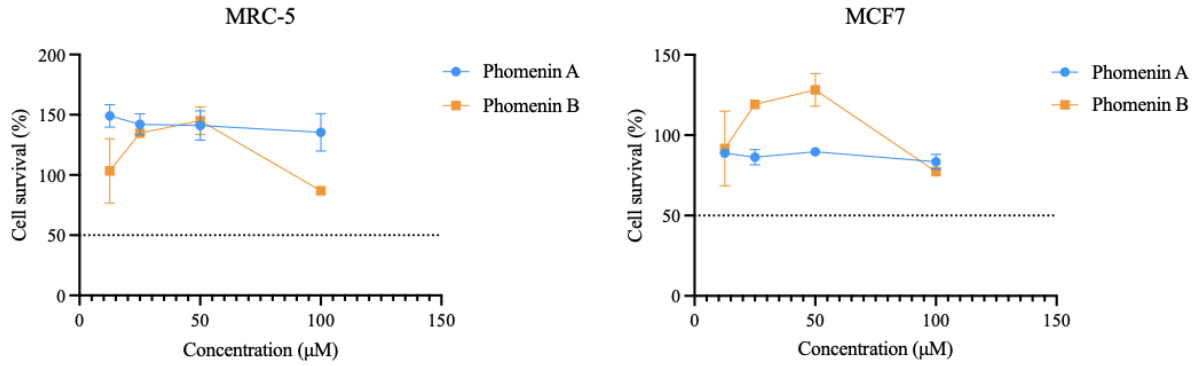


Figure 21: *Results from the viability assay. The compounds were inactive (I). The black line represents the cut-off value at 50% cell survival.*

After comparing this study with previous literature, specific differences were observed. While earlier literature reports variable cytotoxicity of the phomenins, this study did not observe cytotoxic effects at the tested concentrations. It was suggested that the low cytotoxic activity observed may have been influenced by the mixture of phomenin A and B (10:1), a factor not present in this study due to the separation of the phomenins. Furthermore, the bioactivity was tested using a different MRC-5 assay in the previous study, which has different readouts.

4.5.2 PTP1B Inhibition Assay

The PTP1B enzyme is associated with the development of type 2 diabetes. PTP1b enzyme catalyses the dephosphorylation of phosphotyrosine residues on insulin proteins, inhibiting the effect of insulin (75). In this biochemical assay, PTP1B reduces DiFMUP to its fluorescent form DiFMU. The enzyme activity is proportional to the fluorescent activity (76). None of the compounds showed an antidiabetic effect in the assay. The results showed no significant difference between phomenin A (**2**) and B (**3**). The results are represented in **Figure 22**.

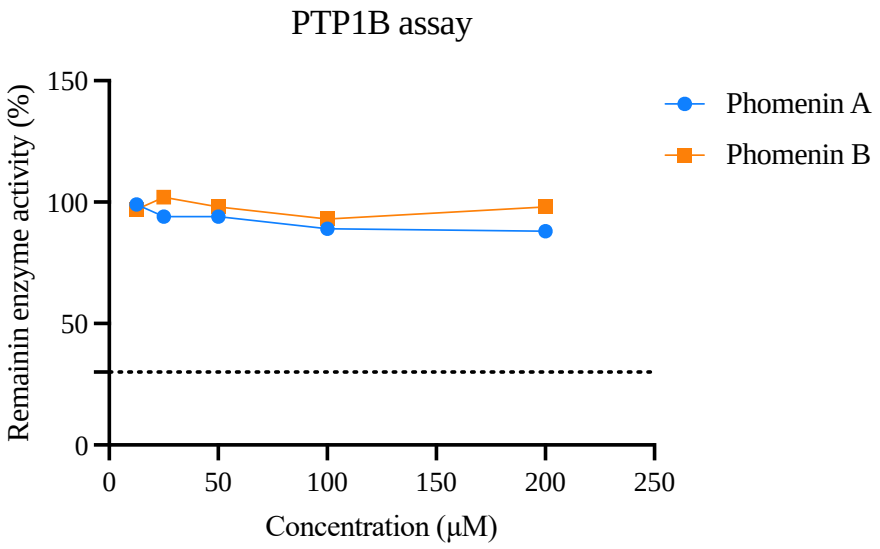


Figure 22: *Results from the PTP1B assay. None of the compounds were active (I). The black line represents the cut-off value at 40% remaining enzyme activity.*

4.5.3 DPP-IV Inhibition Assay

The DPP-IV inhibitor assay is also linked to type 2 diabetes. The DPP-IV enzyme is a competitive inhibitor of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). These hormones help stimulate the release of insulin. By hindering the inhibitor, the level of incretin hormones will increase (77). Only phomenin A (**2**) was tested in this assay at 100, 50, 25, and 12,5 µM and showed no antidiabetic activity. **Figure 23** shows that there is no significant difference between the concentrations.

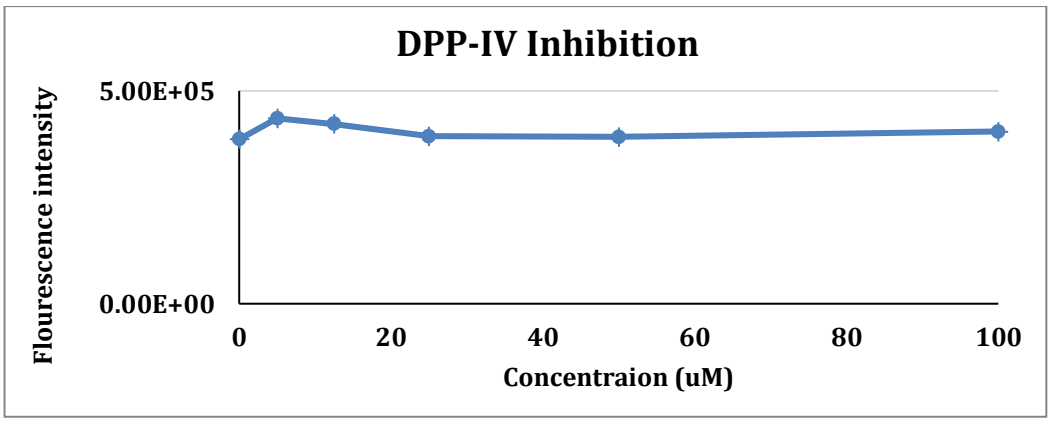


Figure 23: *Results from the DPP-IV inhibition assay when Phomenin A (2) was tested. There is no significant difference between the concentrations.*

4.5.4 Antibiofilm Formation Assay

This assay aims to see if the compound can inhibit biofilm created by *Staphylococcus epidermidis*. Crystal violet solution is added to wells to stain the biofilm. *Staphylococcus haemolyticus* does not generate a biofilm and is used as a control. Only phomenin A (**2**) was tested for antibiofilm at formation activity at 200, 100 and 50 µM and was considered inactive (I) (**Table 18**).

Table 18: *Results from the antibiofilm formation assay showing no activity (I).*

Antibiofilm formation	**Phomenin A**		
Test concentration (µm)	50	100	200
Biofilm formation (%)	106	93	75
Category	I	I	I

For this assay, biofilm formation can be seen visually. If biofilm is present, the well will be dark blue with stains. The clear wells indicate no biofilm formation. **Figure 24** shows the assay layout, where the isolated compound is pipetted in the red square. Row B2-4 is 200 µM might have some activity, but it is above the Marbio cut-off value.

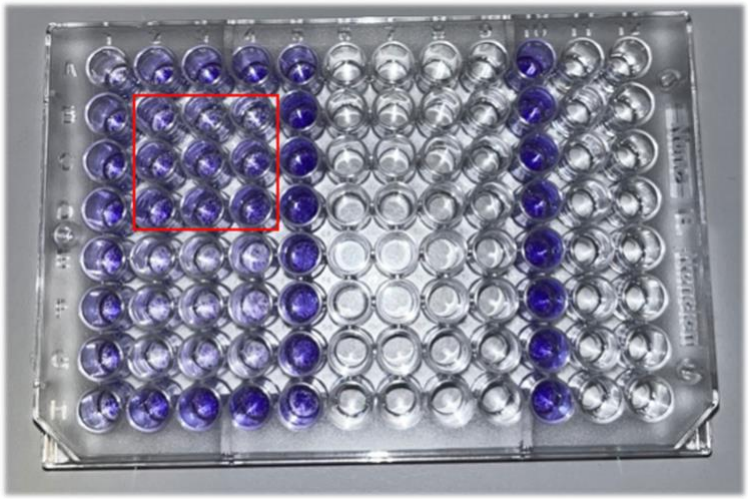


Figure 24: *Visual results from the antibiofilm formation assay. Columns B02-04, C02-04, and D02-04 are test columns. Column 10 is a positive control, column 11 is a negative control, and column 12 is a media control. The dark blue indicates biofilm formation.*

4.5.5 Antibacterial Assay

Phomenin A (**2**) was tested against five bacterial strains: *Streptococcus agalactiae, Enterococcus faecalis, Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli.* Phomenin B (**3**) was tested against one bacterial strain, *S. aureus. S. agalactiae, E. faecalis, and S. aureus* are Gram-positive bacteria, while *P. aeruginosa and E.coli* are Gram-negative bacteria. The main difference between these is the composition of the cell wall. Gram-positive bacteria generally have a single outer membrane surrounded by a thick peptidoglycan layer. In contrast, Gram-negative bacteria typically have a thin layer of peptidoglycan but are located between two outer membranes (78). Many of these bacteria strains are included in the WHO´s list of bacteria for which new antibiotics are urgently needed. *P. aeruginosa*, *E. faecium,* and *S. aureus,* are high priority on that list (79).

The optical density (OD) was measured with an absorbance of 600 nm. The suspension will appear clear if the compound(s) is effective. This gives a lower OD-value where the light passes through, indicating fewer bacteria in the well. If the compound does not have an inhibitory effect, the bacterial suspension in the well will appear opaque, resulting in a higher OD-value. Based on the cut-off values, Phomenin A (**2**) was active at a 200 μM concentration and questionable at 150 μM when tested on *S. agalactiae.* Phomenin B (**3**) was inactive (I). The results are presented in **Figure 25**. The concentration at 200 μM is relatively high, and the concentration should be lower to make it interesting for further analysis. The difference between the activity for phomenin A (**2**) and B (**3**) can be because the variance in chemical structures is making phomenin A (**2**) more specific. Earlier studies did not investigate antibacterial properties. However, this thesis suggests that phomenin A (**2**) exhibits an antibacterial effect, although at a high concentration.

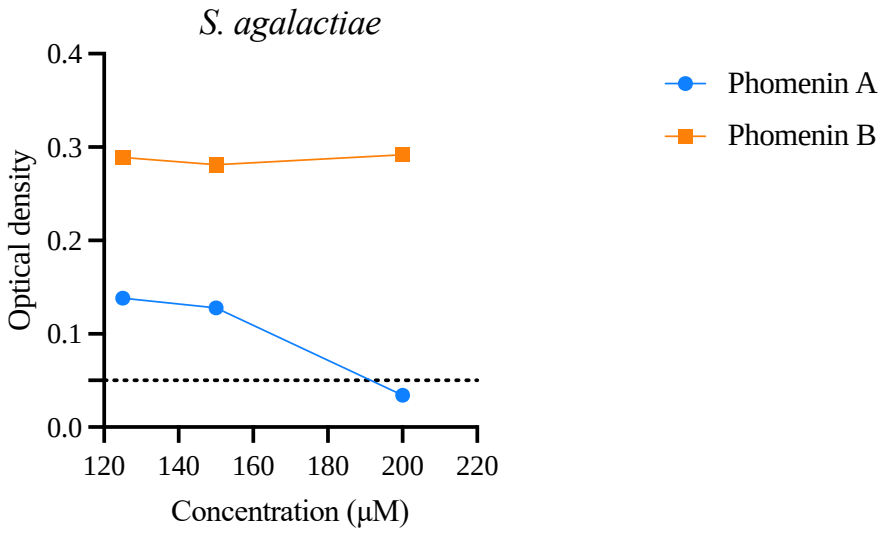


Figure 25: *Results from the antibacterial assay tested on the S. agalactiae strain. Phomenin A (**2**) was activity (A) at 200 µM and questionable (Q) at 150 µM. The black line represents the cut-off value at 0.05 OD.*

Figure 26 presents the results from the four other bacterial strains. While categorised as inactive (I), phomenin A (**2**) has the most activity against *S. aureus* at 125 µM. Based on the results, the compound is more active against Gram-positive bacteria. This can likely stem from their shared characteristics, including similarities in cell wall composition. Phomenin A (**2**) may target components of the peptidoglycan layer.

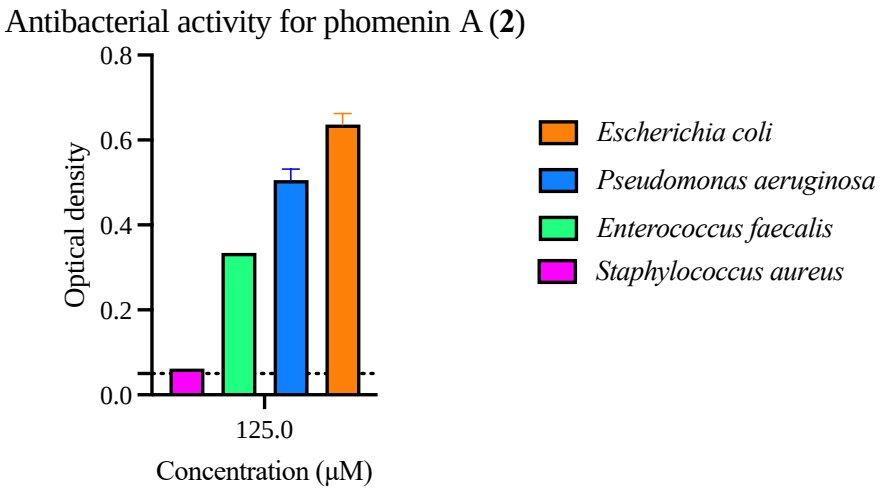


Figure 26: *Results from the antibacterial assay when Phomenin A (**2**) was tested on E. coli, P. aeruginosa, E. faecalis, and S. aureus. The black line represents the cut-off value at 0.05 OD. The compound was categorised as inactive (I).*

4.5.6 Marine Fungal Extract Summary

Table 19 provides an overview showing the bioactivity of each marine fungi tested in viability, antibacterial, and antidiabetic assays. The purpose of the primary screening is to identify potential bioactive extracts that exhibit interesting properties. Many extracts are screened, and a cut-off value is set to select which extract to prioritise for further analysis. Some marine fungi produce bioactive secondary metabolites even if they do not meet the cut-off value. The bioactivity data is presented in Appendix B (**NMF Extracts Results).**

There were two fungal extracts that showed great potential and are worth exploring. These extracts, documented as X7217 and X7218, were effective against the Gram-positive bacteria *S. agalactiae.* They may contain new bioactive secondary metabolites and may also exhibit a higher potency against other bacterial strains. The bioactive extract can be dereplicated using HPLC-HR-MS to see if the compound(s) are novel, known, or known with novel activity. Once the bioactive compound(s) have been identified, they can be isolated and their structure elucidation or characterised. Finally, the bioactivity of the target compound(s) can be characterised.

Table 19*: An overview of the bioactivity of the marine fungi extracts. Green indicates activity (A), blue indicates inactivity (I), and orange indicates activity that does not meet the cut-off value.*

NMF isolates	Cytotoxic activity		Antibacterial activity	Antidiabetic activity
	MCF7	MRC-5		
X7212	–	–	–	–
X7213	–	–	–	–
X7214	–	–	–	–
X7215	–	–	–	–
X7216	+	+	–	–
X7217	–	–	+	–
X7218	–	–	+	–
X7219	–	–	+	–
X7220	–	–	–	–
X7221	+	+	+	–
X7222	–	–	–	–
X7223	–	–	–	–
X7224	–	–	–	–
X7225	–	–	–	–
X7226	–	–	–	–
X7227	–	–	–	–
X7228	+	+	–	–
X7229	–	–	–	–
X7230	+	+	–	–
X7231	–	–	–	–
X7232	–	–	–	–

4.6 Limitations

While this study has provided valuable insights into the biological activities of phomenins, several limitations to the compounds and the experimental methods have impacted the interpretation of results in this thesis. First, the low yield of the compounds presents a significant challenge. The low yield hindered the ability to replicate experiments, such as bioassays, potentially affecting the reliability of the results. The low yield also affects the precision of concentration measurements and assay accuracy. Even minor variations in technical procedures can significantly impact the results at low concentrations.

During the thesis, it was observed that the phomenins had poor solubility, which made them challenging to work with. Since there were a lot of dissolving and drying steps during the work, it could mean losing compounds when transferring, which this study could not afford. A good solubility is usually desirable in a drug candidate for several reasons, such as bioavailability and stability. A drug should be able to dissolve in the body before it can be absorbed into the bloodstream and distributed to its target site. A poorly soluble compound may precipitate out of its solution, which can cause formulation issues.

Phomenins are unstable molecules that are more sensitive to external factors like temperatures, oxygen, and light. Previous studies at Marbio have noticed degradation of the phomenins at relatively high temperatures. Therefore, when drying the samples, the temperature was set at 30 ºC instead of the usual 40 ºC. The freeze dryer was sometimes used instead of heat to minimize the exposure to temperature, despite the risk of losing compounds.

5. Conclusion and Future Perspectives

In conclusion, our study successfully isolated and characterised phomenin A (**2**) and B (**3**), secondary metabolites derived from *Parafenestella* sp. We demonstrated that these compounds possess bioactivity, with phomenin A (**2**) exhibiting weak antibacterial activity (200 μM). The measured activities against the bacterial strains in this thesis are not potent enough to justify further development of these compounds towards becoming commercially available drugs. In addition, the phomenins were identified as unstable isomers, which is not ideal for a drug candidate. A total of 22 marine fungal extracts were cultivated for one month, extracted with LLE, and screened for bioactivity. Most of the fungi were collected from microalgae and driftwood. Four fungi were considered cytotoxic and antibacterial, making them interesting for further analysis.

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Appendix A

5.1 NMR Spectra

Table 20: *^{1}H and ^{13}C NMR of compounds **2** and **3**.*

	Compound 2		Compound 3	
Position	**δ_C, type**	**δ_H, mult. (*J* in Hz)**	**δ_C, type**	**δ_H, mult. (*J* in Hz)**
1				
2	165.6, C		163.4, C	
3	100.0, C		100.5, C	
4	166.2, C		166.02, C	
5	93.6, CH	6.54, s	93.97, CH	6.58, s
6	159.7, C		158.7, C	
7	124.6, C		127.2, C	
8	135.2, CH	6.81, s	130.7, CH	6.78, s
9	132.7, C		132.3, C	
10	129.4, CH	5.68, q (*J* = 7.0 Hz)	124.4, CH	5.51, q (*J* = 7.0 Hz)
11	14.0, CH_3	1.74, d (*J* = 7.0 Hz)	14.98, CH_3	1.52, d (*J* = 6.9 Hz)
12	16.4, CH_3	1.84, s	23.2, CH_3	1.82, s
13	13.8, CH_3	2.05, s	13.9, CH_3	1.88, s
14	8.6, CH_3	1.80, s	8.7, CH_3	1.81, s
15	56.7, CH_3	3.95, s	56.8,CH_3	3.96, s

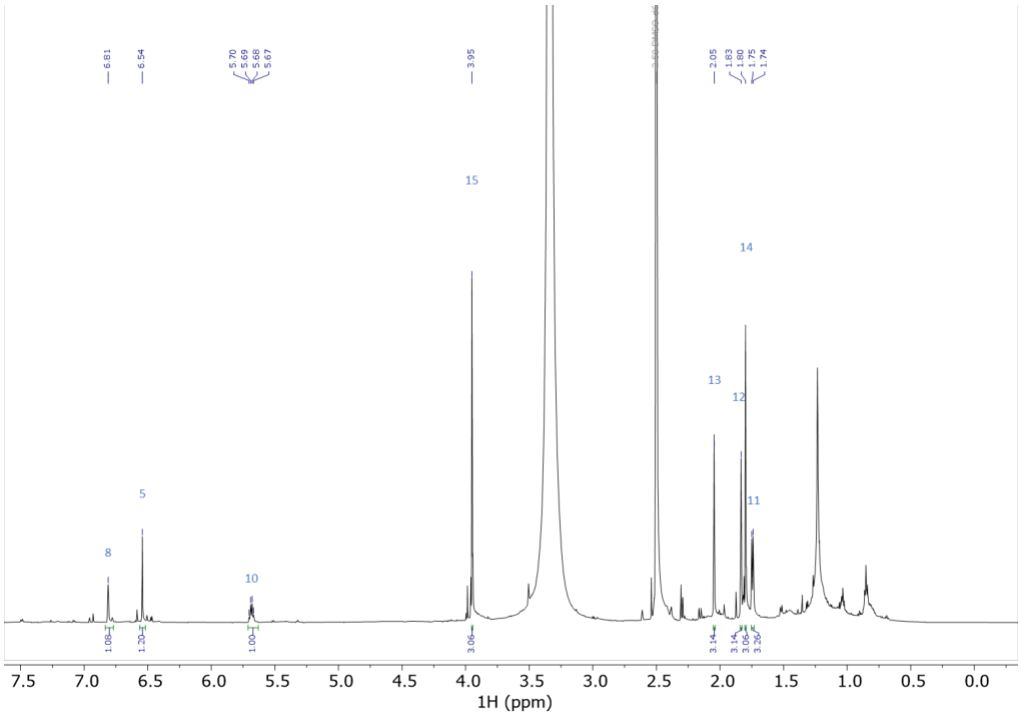


Figure 27: *^{1}H NMR of compound **2** (Batch 1).*

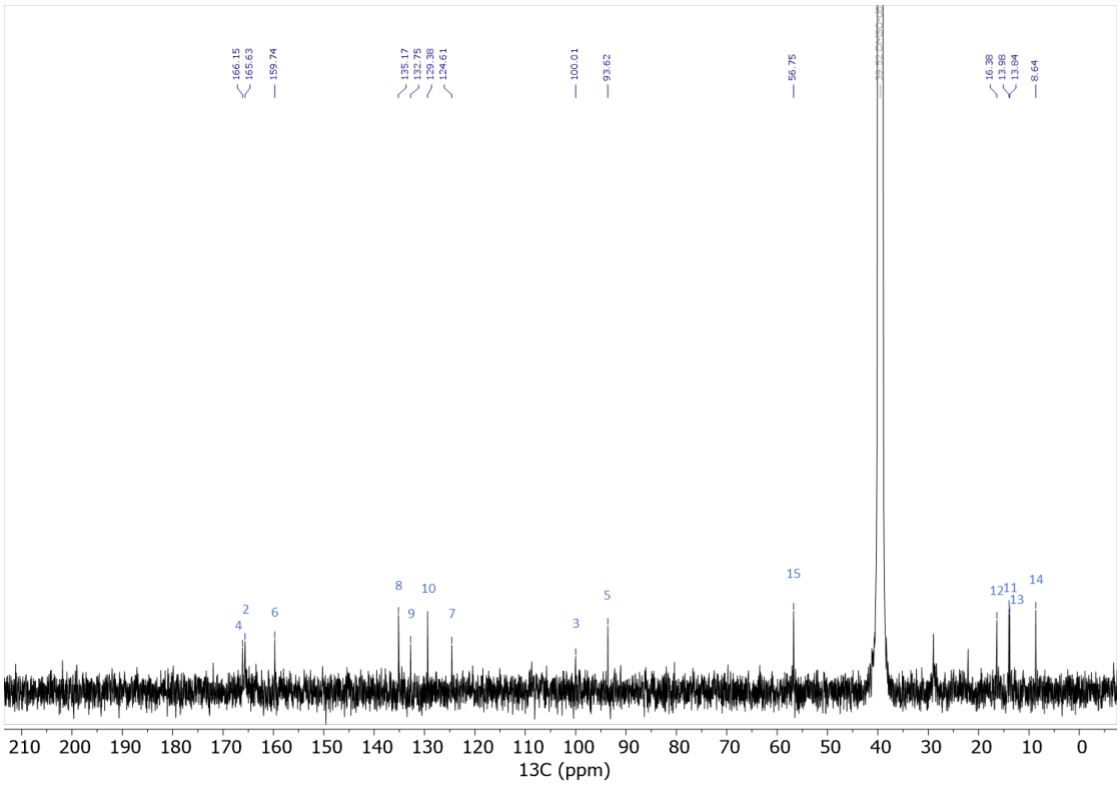


Figure 28: *^{13}C NMR of compound **2** (Batch 1).*

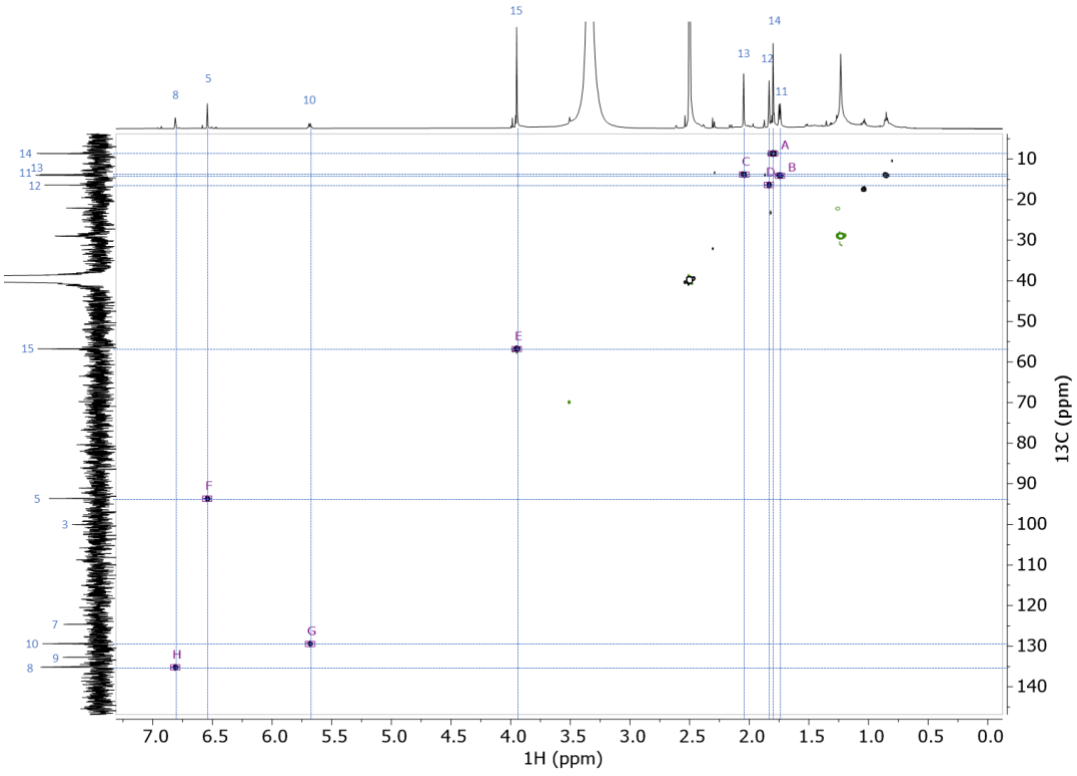


Figure 29: *HSQC of compound **2** (Batch 1).*

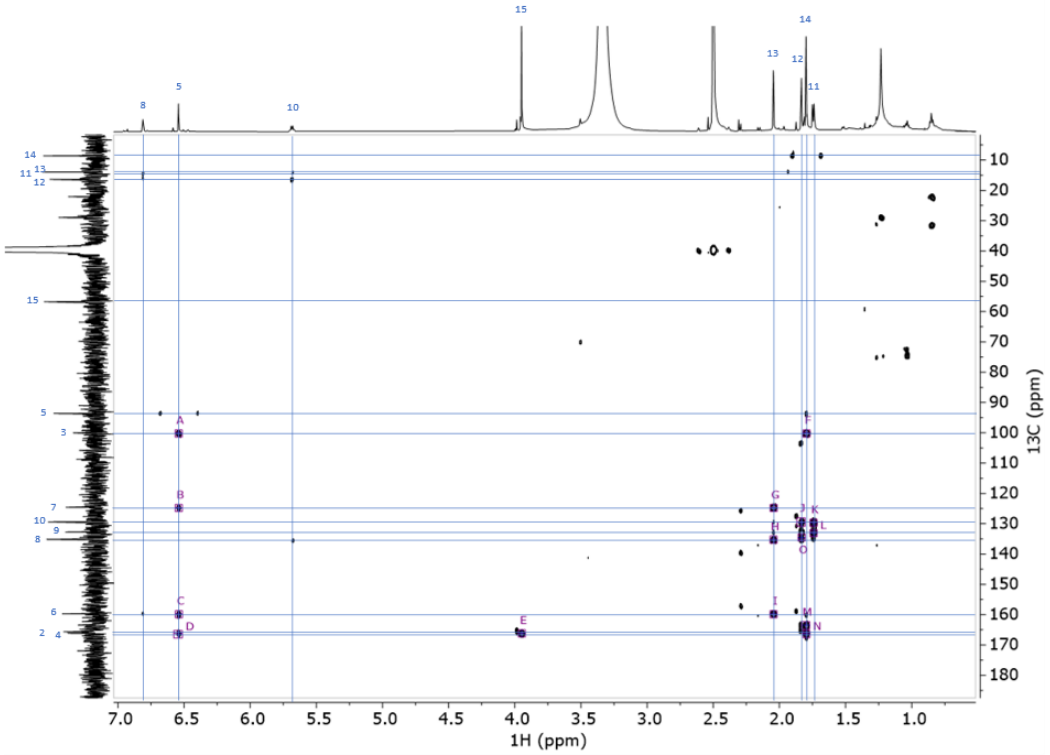


Figure 30: *HMBC of compound **2** (Batch 1).*

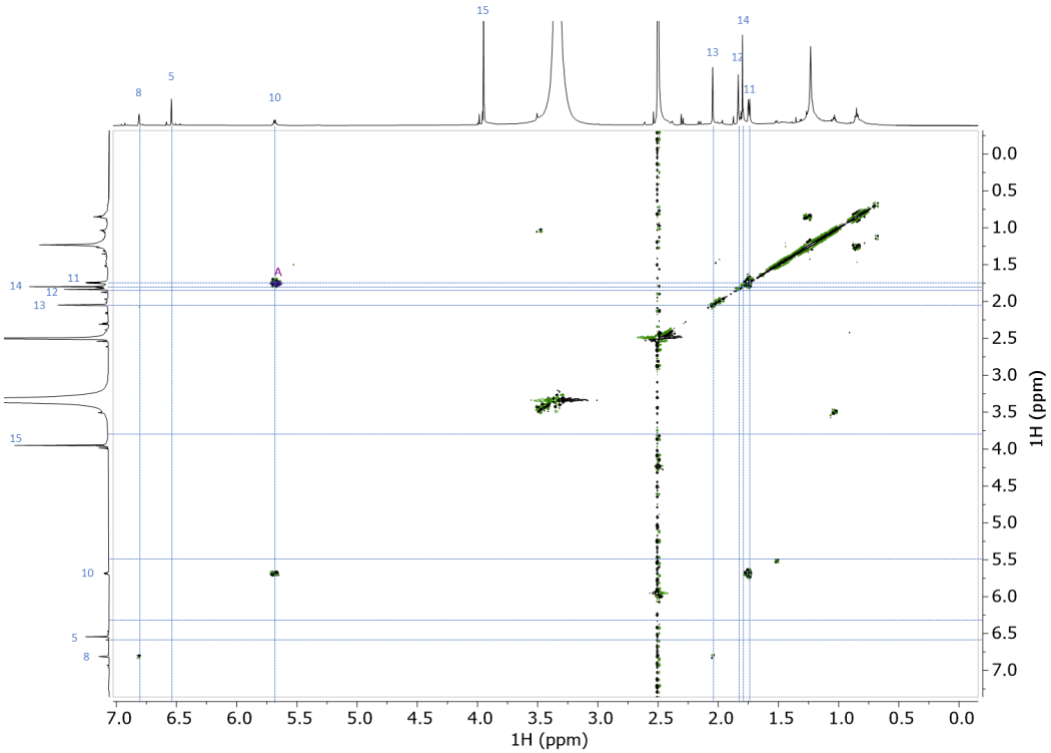


Figure 31: *COSY of compound **2** (Batch 1).*

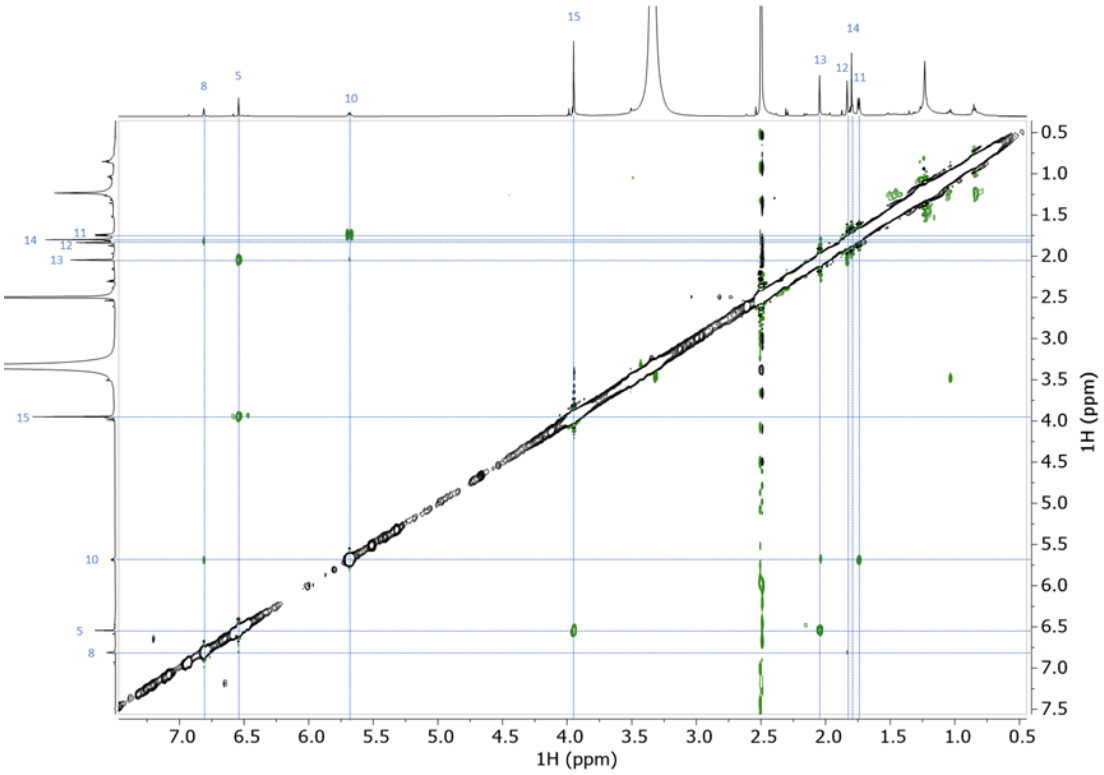


Figure 32: *ROESY of compound **2** (Batch 1).*

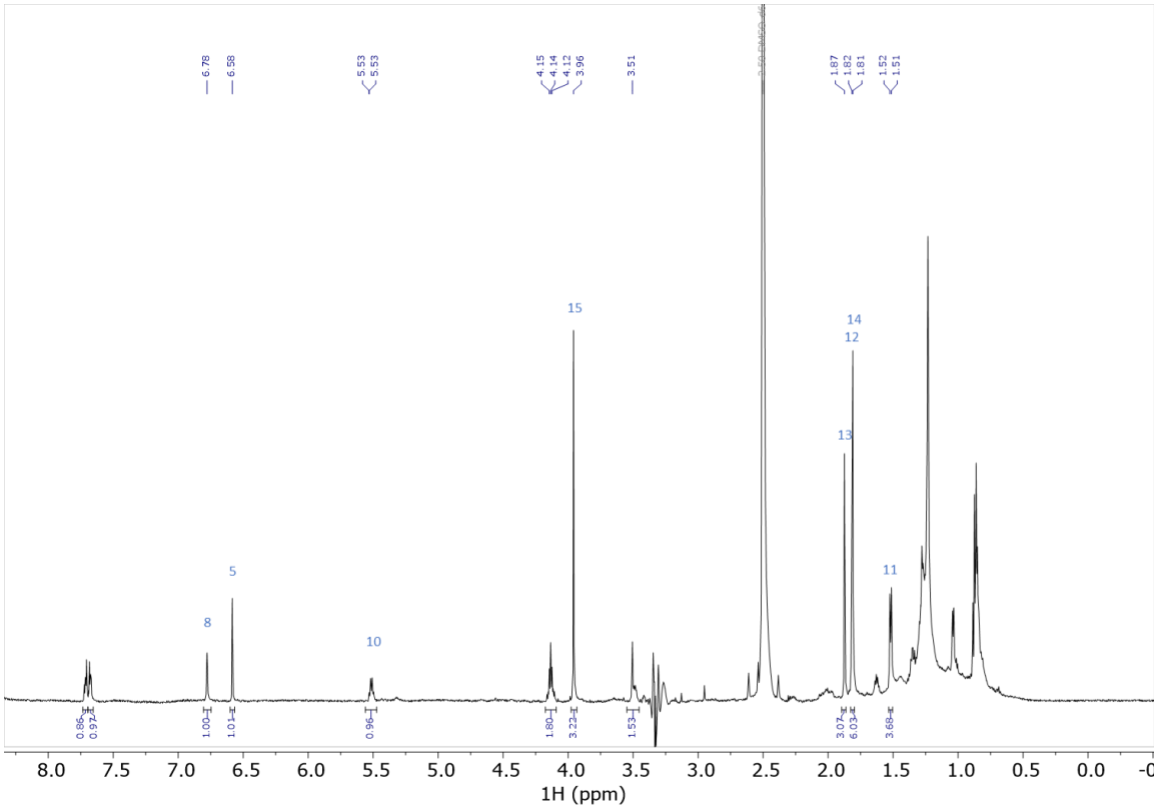


Figure 33: *^{1}H NMR of compound **3** (Batch 1).*

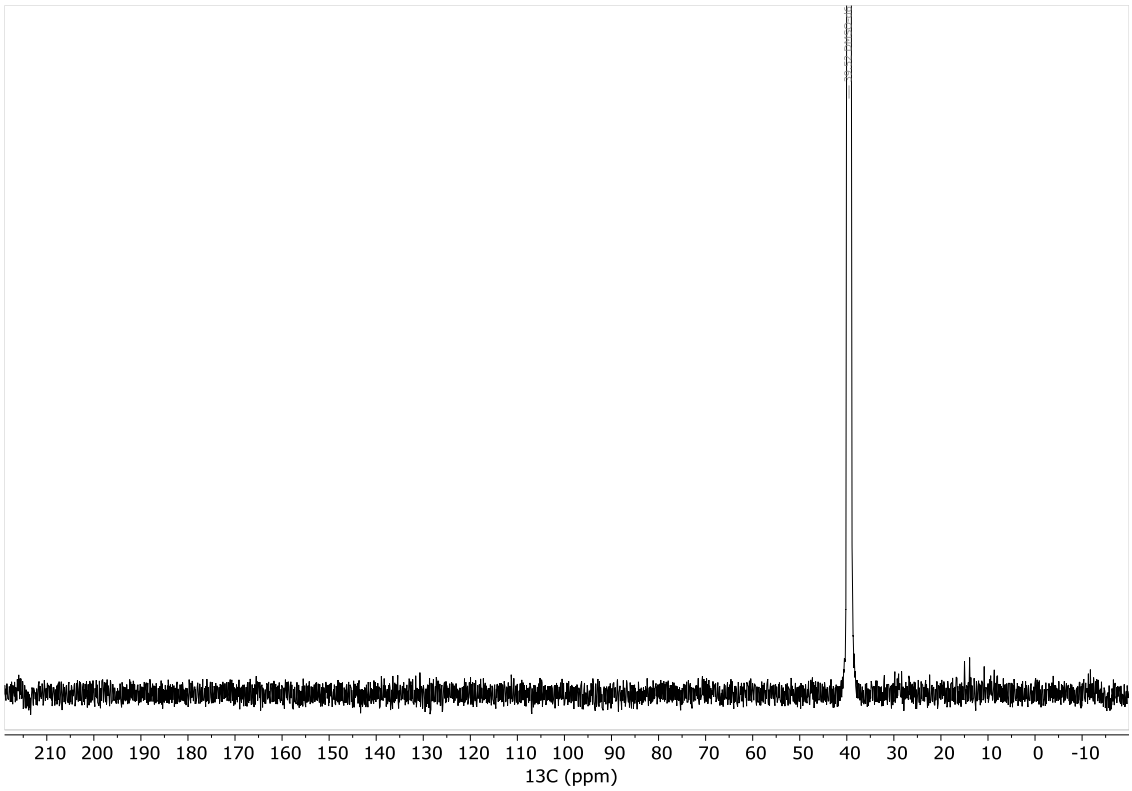


Figure 34: *^{13}C NMR of compound **3** (Batch 1).*

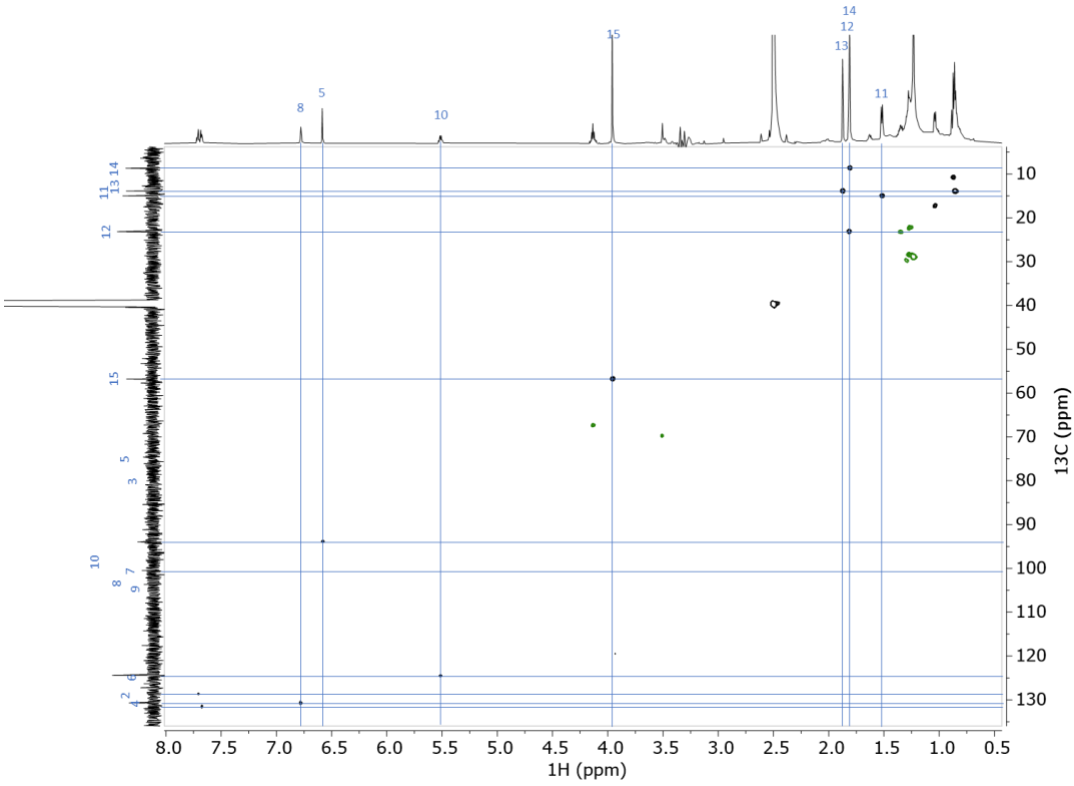


Figure 35: *HSQC of compound **3** (Batch 1).*

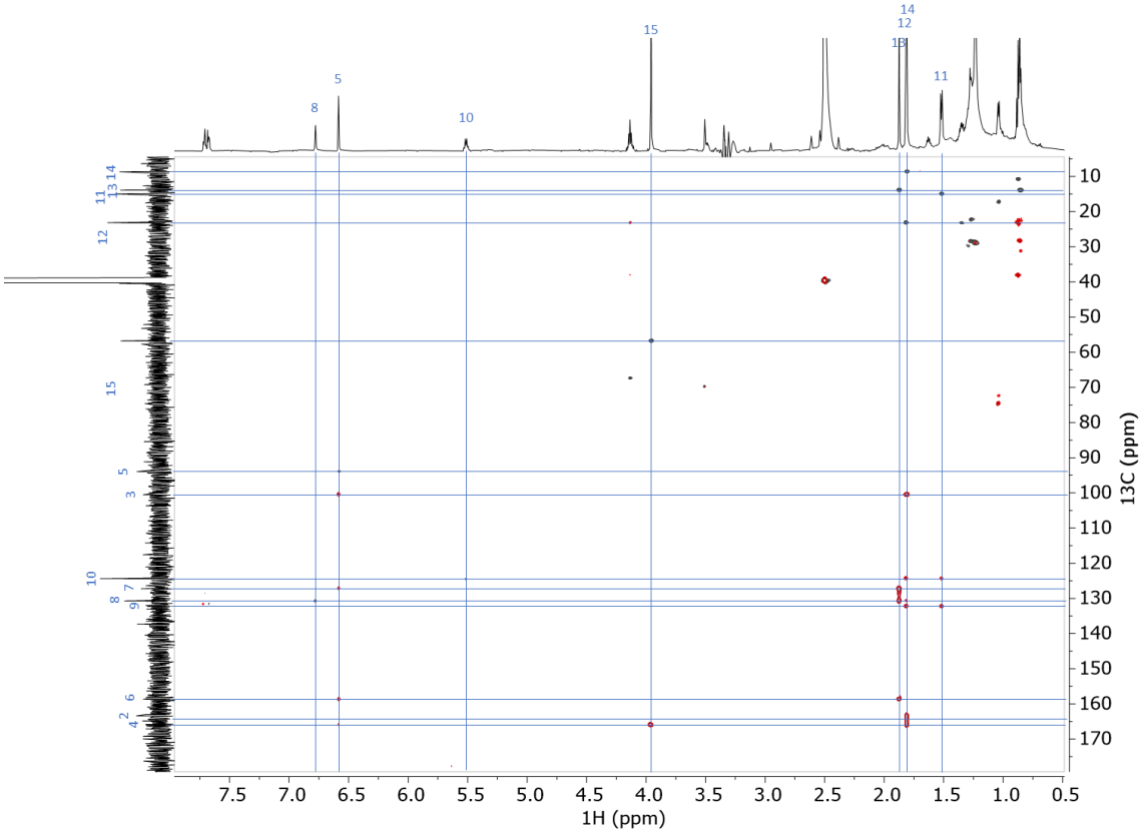


Figure 36: *HSQC + HMBC of compound **3** (Batch 1).*

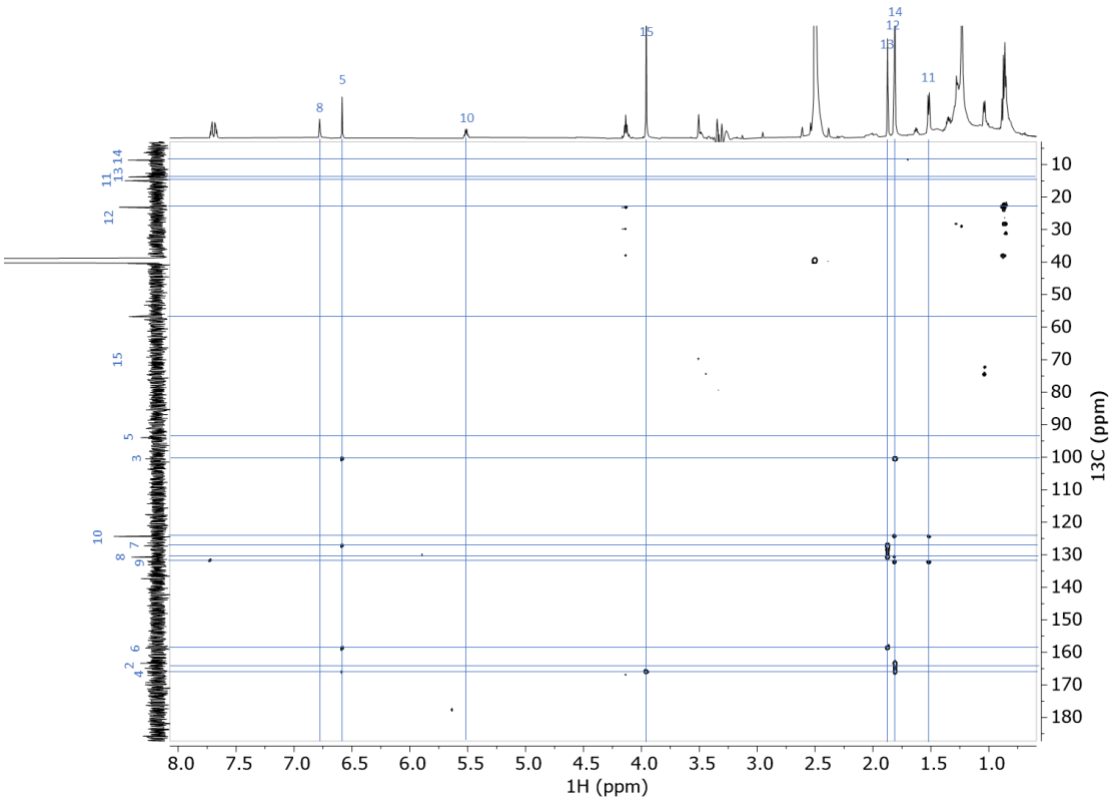


Figure 37: *HMBC of compound **3** (Batch 1).*

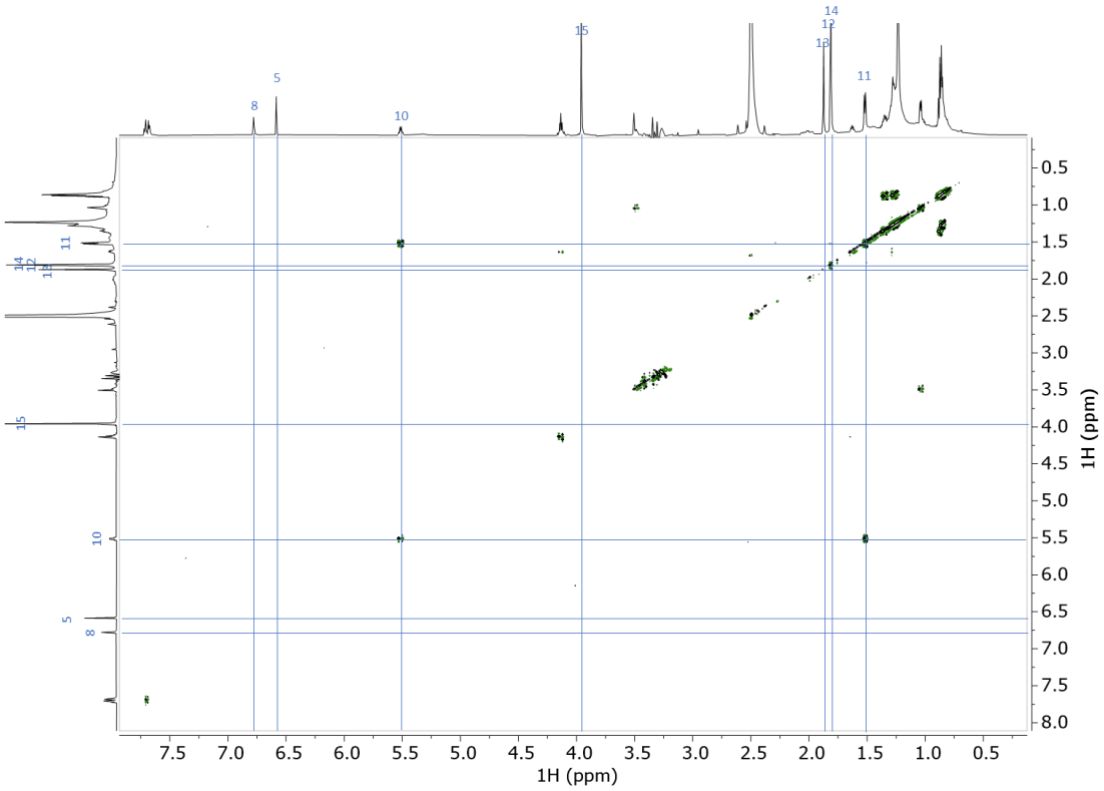


Figure 38: *COSY of compound **3** (Batch 1).*

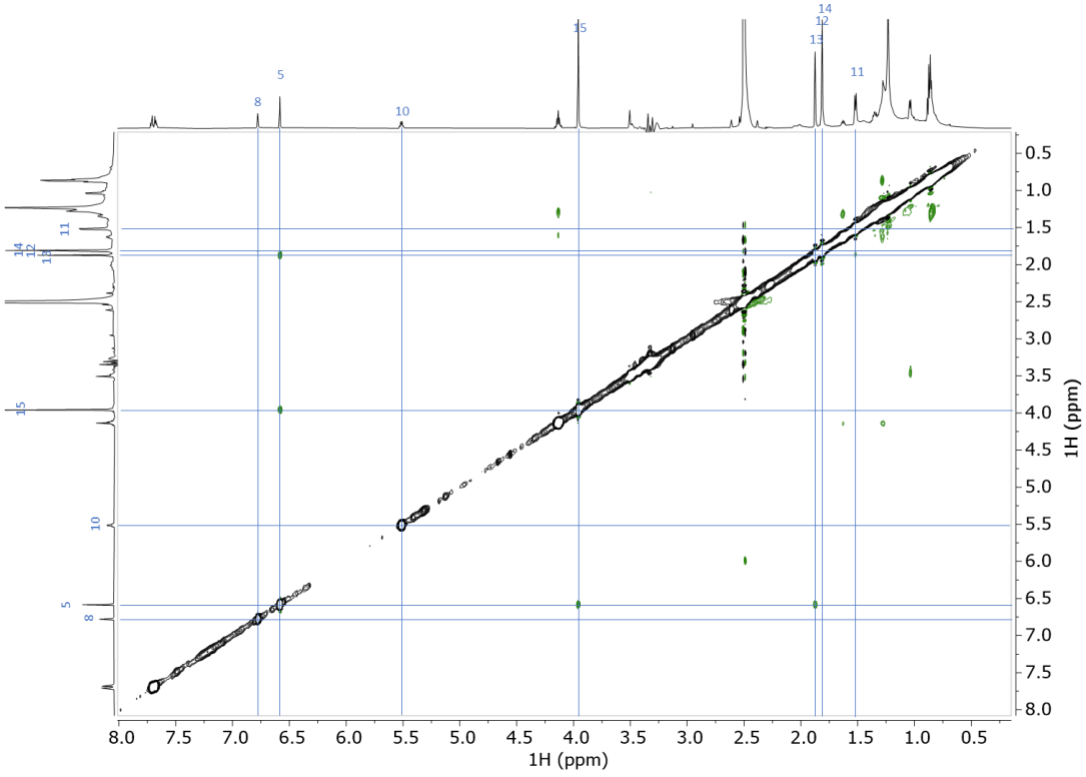


Figure 39: *ROESY of compound **3** (Batch 1).*

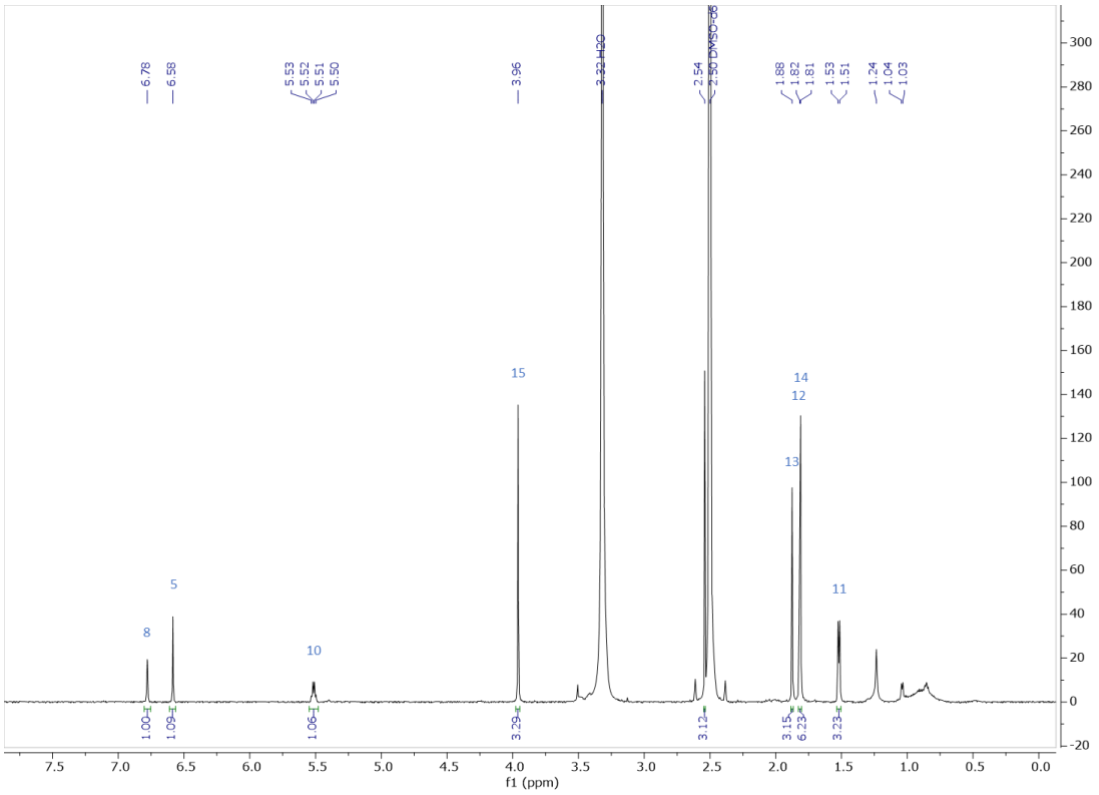


Figure 40: *[1]H NMR of compound **3** (Batch 2).*

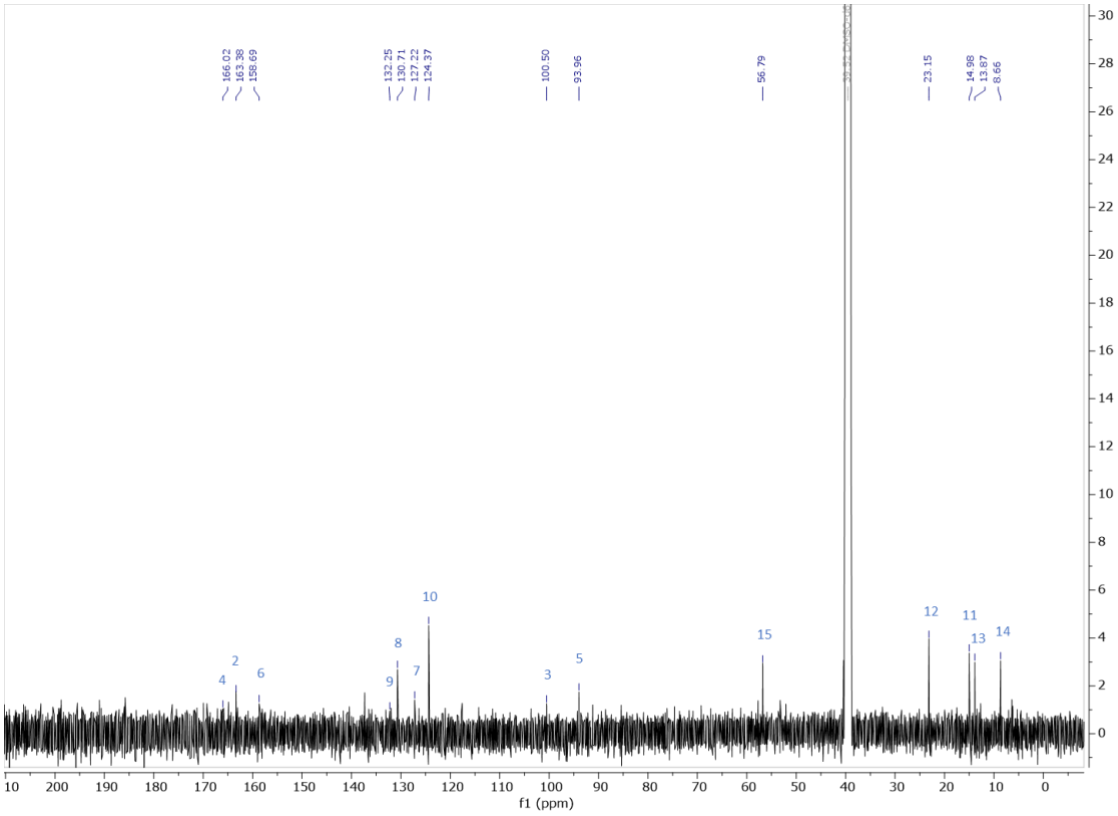


Figure 41: [13]C NMR of compound **3** (Batch 2).

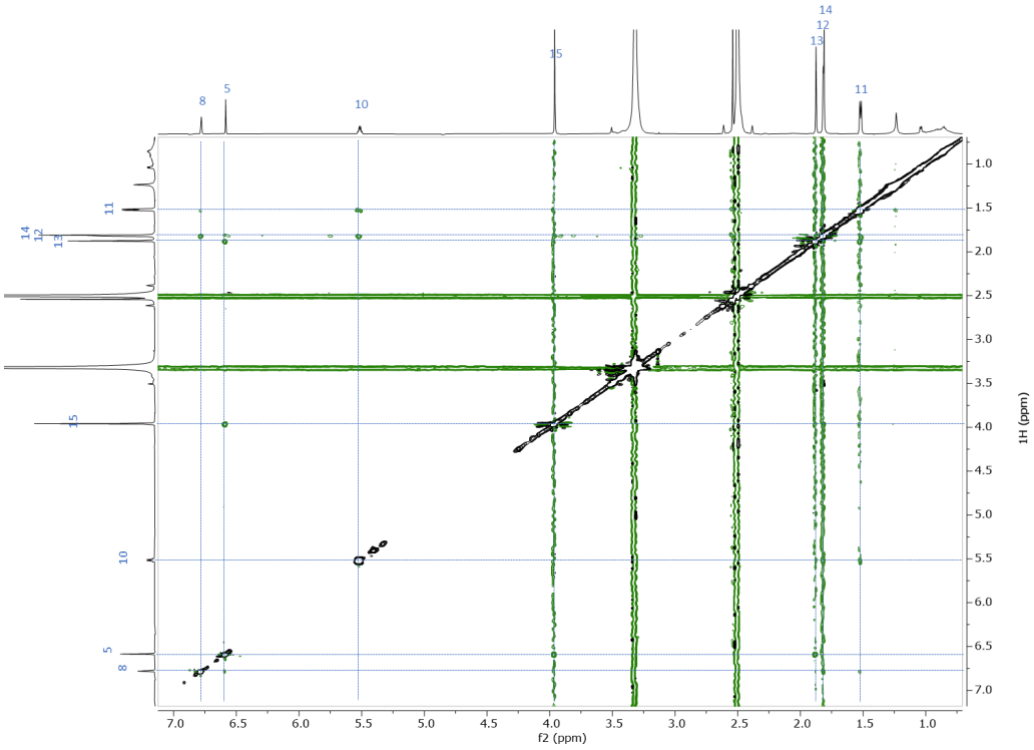


Figure 42: *ROESY of compound **3** (Batch 2).*

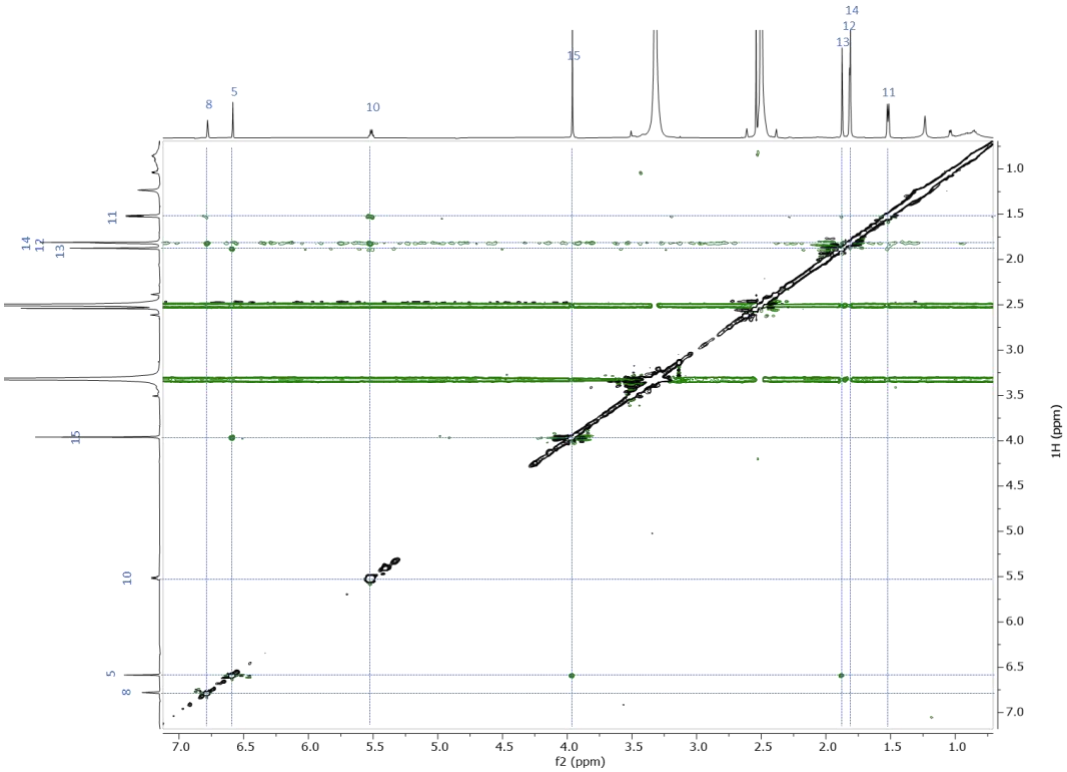
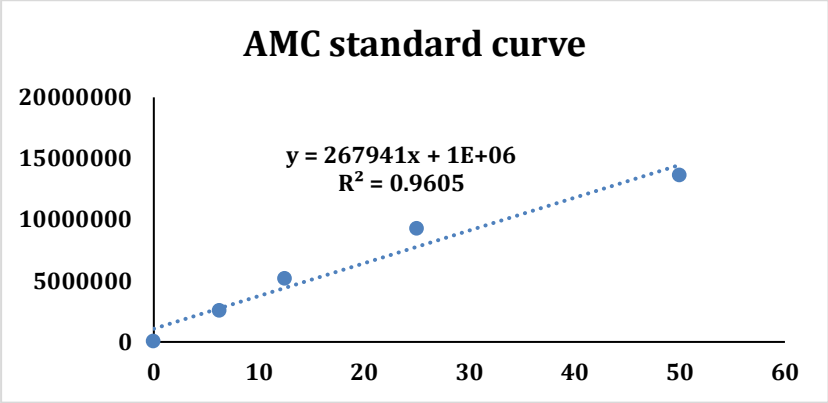


Figure 43: *ROESY (Reduced t1 noise) of compound **3** (Batch 2).*

Appendix B

5.2 Standard curve of AMC in DPP-IV Inhibition Assay

According to the analysis of T0 and T30 (**Figure 44**), the max reaction (GP-AMC with DPP-IV minus GP-AMC) is 3,93E+05. The IC50 of Diprotin A (positive control) is about 50uM (5µM final concentration), which agrees with the literature. The half readout of the max reaction is 1,96E+05.



uM	Samples
0	3,86E+05
5	4,36E+05
12,5	4,22E+05
25	3,93E+05
50	3,92E+05
100	4,04E+05

Figure 44: *Standard curve of AMC used in DPP-IV inhibition assay. R^2 = 0.9605.*

5.3 NMF Extracts Results

A total of 22 NMF isolates were cultivated for one month and extracted using LLE, the same method used for *Parafenestella* sp. The screening program included testing for their cytotoxic, antibacterial, and antidiabetic properties. The samples were tested at a concentration of 100 µg/mL. When screening the fungal extracts for bioactivity, a cut-off value is set to determine which extract to further analyse. Note that an extract may be active even if it does not meet the cut-off value.

The fungi's cytotoxic effect was assessed against normal lung fibroblasts (MRC-5) and human breast adenocarcinoma (MCF7). X7216, X7228 and X7230 were categorised as active (A) in both cell lines, while X7221 showed weaker cytotoxic activity and did not meet the cut-off value (**Figure 45**). Extracts X7228 and X7230 are highly cytotoxic, almost killing all living cells, while X71221 are more specific towards MCF7 than MRC-5.

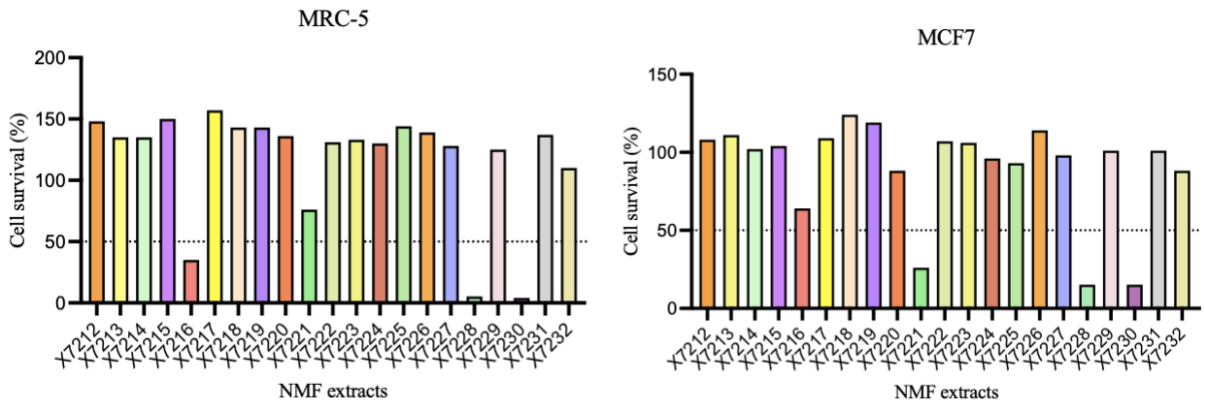


Figure 45: *Viability result for fungi extracts. The black line represents the cut-off value at 50% cell survival.*

The fungi extracts were also tested for antibacterial growth inhibition activity against the *S. agalactiae* bacteria (**Figure 46**). Two fungi extracts, X7217 and X718, were categorised as active (A). X7219 and X7221 had low antibacterial activity but were categorised as inactive (I).

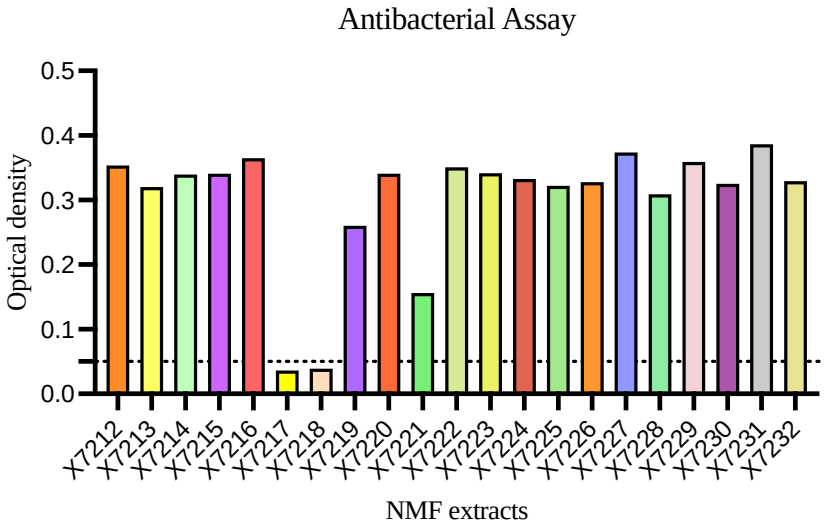


Figure 46: *Antibacterial result for fungi extracts. The black line represents the cut-off value at 0.05 OD.*

The antidiabetic assay focused on PTP1B inhibition. All fungal extracts were categorised as inactive (I) (**Figure 47**).

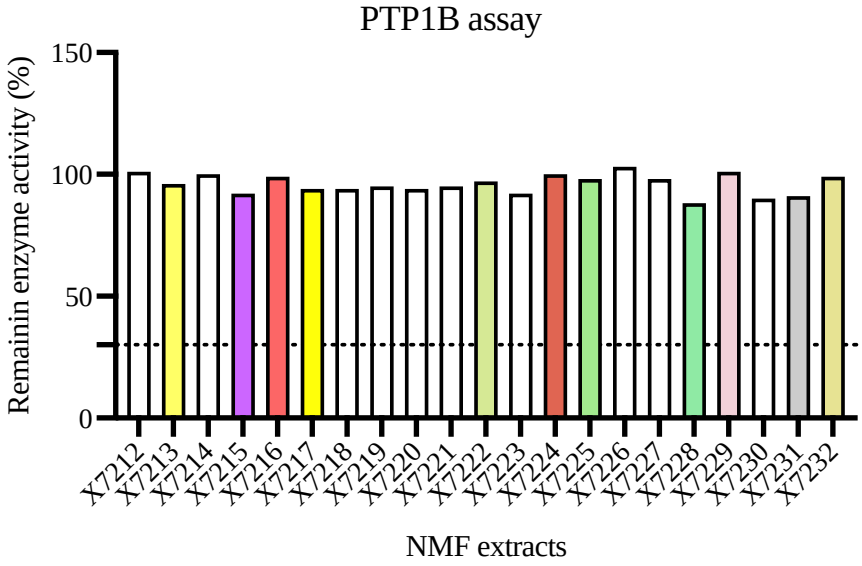


Figure 47: *PTP1B results for fungi extracts. All extracts were categorised inactive (I). The black line represents the cut-off value at 30 % remaining enzyme activity.*