UiT The Arctic University of Norway

The Norwegian College of Fishery Science, UiT - The Arctic University of Norway

Secondary Metabolites from Marine Invertebrate Extracts

Discovering, isolating, and characterizing bioactive compounds using bioactivity guided isolation

Brede Bjerke Eiken

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Abstract

In drug discovery, the extensive biodiversity of natural organisms offers a rich source of bioactive compounds with significant therapeutic potential. Currently, above 1/3 of all drugs has its origin from natural sources. Bioprospecting is the process of systematically screening of organisms, such as plants, microorganisms, or invertebrates, to identify and isolate novel compounds usable for drug development and other applications. Historically, terrestrial organisms have received more thorough examination for such compounds than those in the marine environment, primarily due to accessibility. Now, with the advent of new sampling methods for marine organisms, efforts have shifted to explore the rich biodiversity below the ocean surface. Given the marine environment's comparable or arguably superior diversity, it serves as a valuable starting point for bioprospecting, leading to the emergence of marine bioprospecting. Within this work, we are utilizing the marine bioprospecting pipeline to analyse extracts of marine invertebrates, with the goal of isolating novel molecules possessing bioactivity that renders a valuable starting points for drug development.

In total, 16 extracts of marine invertebrate biomass were analysed: 12 from the Norwegian National Marine Biobank, Marbank, and four from White Point Biomarine (WPB) in the US. Each Marbank extract underwent prefractionation using flash chromatography. The resulting fractions were screened for bioactivity against a cancer cell line. Previous work, part of my bachelor's thesis, involved analysing a set of 244 extracts from WPB in similar fashion. Based on the bioactivity screening results of the Marbank and WPB extracts, 10 fractions were selected for dereplication using UHPLC-HR-MS, to identify potentially bioactive compounds. Ultimately, five compounds were isolated using prep-HPLC and submitted for nuclear magnetic resonance spectroscopy analysis to determine their structure. While partially unsuccessful, the purified compounds were re-tested for bioactivity, revealing potent cytotoxic activity in one of compounds.

In conclusion, the marine bioprospecting pipeline utilised in this study effectively prioritised samples by systematically narrowing down the sample set, enabling focused efforts towards the most promising samples during the labour-intensive and time-consuming process of marine bioprospecting. The discovery of compound MBC-414 exhibiting potent bioactivity against cancer cells (IC_{50} = 2.2 -7.1 µM) further confirms that the pipeline is a viable approach for discovering bioactive compounds from broad sample panels containing complex samples.

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Brede Bjerke Eiken

Abbreviations

ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, excretion
BHI	Brain heart infusion
BPI	Base peak intensity
C	Concentration in mol/L
D-MEM	Gibco Dulbecco's Modified Eagle Medium
ddH_2O	Double-distilled water (Milli-Q®)
DM	White Point Biomarine non-polar extract
DMSO	Dimethyl sulfoxide
e.g.	Example
ESI	Electrospray ionisation
Ext.	Extract
FA	White Point Biomarine polar extract
FBS	Fetal bovine serum
HR-MS	High-resolution mass spectrometry
HCl	Hydrogen chloride
IC_{50}	Half-maximal inhibitory concentration
Log P	Octanol-water partition coefficient
Marbank	Norway's national marine biobank
mbar	Millibar
MEM	Minimum Essentials Medium Eagle
MeOH	Methanol

MHz	Megahertz
MIC	Minimal inhibitory concentration
m/z	Mass over charge
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Mw	Molecular weight
N	Number of moles
NMR	Nuclear magnetic resonance
PAINS	Pan assay interfering compounds
PBS	Phosphate-buffer saline
Prep-HPLC	Mass-guided preparative high-performance liquid chromatography
QSAR	Quantitative structure–activity relationship
QSPkRs	Quantitative Structure-Pharmacokinetic Relationships
rpm	Rounds per minute
RP	Reversed phase
Rt	Retention time
sp.	Species
UHPLC	Ultra-high performance liquid chromatography
USAN	United States Adopted Names
V	Volume in liters
WPB	White Point Biomarine
w/v	Weight/Volume

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1 Introduction

Throughout history, humans have acquired knowledge about Nature and learned to utilize its resources. By gathering knowledge about Nature, it was possible to develop harvesting techniques and farms, which subsequently led to the formation of societies, and eventually civilizations. The research field of life science and biology has a crucial role in understanding the complexities of living organisms and the mechanisms that govern their functions. And with the integration of advanced technologies and the emerging of new knowledge, life science and biology have become an essential field in addressing both current and future challenges.

In recent decades, the fields of biology and pharmacy have seen the emergence of several new technological developments, including synthetic biology, RNA vaccines, combinatorial synthesis and the arrival of gene editing through CRISPR-Cas9. However, still to this day is biodiscovery one of the most prominent ways of discovering new pharmaceuticals. Biodiscovery has been prominent since 2600 BC in ancient Mesopotamia and remain an effective way of discovering new drugs (1).

Bioprospecting is the systematic search for new products in Nature that can be exploited for commercial purposes. It leverages knowledge of biodiversity and ecology to identify relevant organisms that can be targeted for investigation (2). The aim of bioprospecting typically involves identifying a chemical, a gene, or a biomimicry concept that can be utilized directly or as a foundation for developing products across various sectors, including pharmaceuticals, nutraceuticals, cosmetics, automotive, and renewable energy industries (3). Within this thesis, we are using the approach of bioprospecting to discover, isolate and characterize new molecules with possible novel bioactivities in extracts of marine invertebrates, with the ultimate goal of discovering compounds that can be developed into drugs.

The environment we are basing our search on is the marine, which holds vast untapped potential due to being relatively underexplored for bioactive compounds compared to the terrestrial environment. The marine environment and ocean are vital ecosystems for life to thrive on Earth, providing many of the necessary fundamental conditions for life. The ocean harbours 50-80% of all life on Earth, but only a fraction (approximately 5-10%) of its diverse life forms is believed to have been explored (4). Renowned for its rich biodiversity, the marine realm showcases a vast array of species, ecosystems, and genetic variation. This combination of high biodiversity and vast unexplored territories positions the marine environment and its lifeforms as exceptional sources of undiscovered natural products.

So, by utilizing marine natural resources in bioprospecting methodology, we get an approach with improved chances of discovering novel compounds of interest.

1.1 The Demand for Pharmaceutical Innovations

Discovering novel pharmaceutical compounds remains as pertinent today as ever. The world has just passed its biggest health crisis in modern times with the SARS-CoV-2 virus (responsible for COVID-19), which highlighted the importance of drug-discovery and -development (5). Besides the prospect of new illnesses, it exists a myriad of reasons necessitating the continuous development of new pharmaceuticals. While a wide variety of neglected diseases and other indications remains untreated, the two most prominent problems today are antibiotic drug resistance and cancer (6) (7).

The incidences of both antibiotic drug resistance and cancer have been steadily increasing. Antibiotic drug resistance is rapidly emerging and spreading, which is threatening to become a new and more fatal pandemic. This has led to abundant research to discover new antibiotics or combination therapies to weaken resistant pathogens (8). Cancer has simultaneously, with the increasingly life expectancy, become the most prevalent cause of death in Norway and many other western countries (9). It is estimated that by 2050, there will be 35 million new annual cancer cases around the world, representing a 77% increase from 2022 (10). Ongoing research continually expands our understanding of the causes, progression, and consequences of these conditions, which has facilitated the development of new and improved treatments. In the case of cancer, research has shown that marine natural products are a good source for novel anticancer drugs, which is believed to come from the production of secondary metabolites as defence to survive in competitive environments (11).

1.2 Natural Products

1.2.1 Natural Products; Primary and Secondary Metabolites

A natural product is a compound, gene or substance produced by a living organism. They are synthesized through diverse biosynthetic pathways, which gives organisms distinctive biological functions (12). The appeal of natural products is their structural ‘optimization’ by evolution, which gives them unique biological functions (13). This encompasses the regulation of endogenous defence mechanisms and competitive interactions with other organisms, which explains their heightened significance in the context of infectious diseases and cancer (14). To understand the variation of metabolites within an organism, we need to establish the difference between the two different types: primary and secondary metabolites.

Primary metabolites are natural products essential for an organism's immediate survival, including growth, development, and reproduction. They are involved in core metabolic processes and are commonly present in the cells of many or all living organisms. Examples of these are essential amino acids, nucleotides, fatty acids, carbohydrates etc (15).

Secondary metabolites on the other hand are compounds that increases the chances of long-term survival for the producing organism. Many times, they are produced by a single, or a set of closely related organisms. Like primary metabolites, secondary metabolites are enzymatically produced. The functions of secondary metabolites are diverse and includes anti-predator or -pathogen activities (16). As primary metabolites are omnipresent, they will most of the time not have the bioactivity that is needed of a drug molecule, unless it is used as a substitution like e.g. insulin. Thus, within the field of natural product drug discovery, it is these secondary metabolites that hold the greatest potential.

1.2.2 Relevance of Natural Products in Drug Discovery

The properties that make natural products interesting within the field of pharmaceuticals are their general high binding affinities for products of DNA translations (since most drug targets in the body are, e.g. receptors, ion channels and enzymes) and their abilities to do this in a selective manner (17). The reasoning behind this is that natural products have a natural affinity for amino acid-based structures, as they themselves were assembled by the enzymatic machinery of the producer. The selectivity towards the targets can be explained by comparing natural products with compounds produced through combinatorial synthesis, where natural products tend to have a higher molecular mass, more chiral centres and sp3 carbon atoms, more oxygen atoms, more H-bond acceptors and donors, higher hydrophilicity and greater molecular rigidity, all factors that are known to increase binding specificity (14) (17).

The history of natural drugs goes far back in time. Herbs and plants with therapeutic effect have been used since ancient times, and the oldest medical text are estimated to be from 2600 BC in ancient Mesopotamia with hundreds of clay tablets in cuneiform (1). In modern times, the most significant medical breakthroughs occurred with the discovery and development of antibiotics, such as penicillin (Figure 1), in the 1940s, a period when natural drugs dominated the market. These discoveries persisted into the period from the 1950s to the 1970s, which is often referred to as the golden era for the discovery of novel antibiotics (18). Approaching the 1990’s, the industry discovered new ways of developing pharmaceuticals, e.g. through combinatorial synthesis, and the area of synthetic drugs started. Since then, we have witnessed a steady decline

in natural products-based drug candidates (19). There were several reasons for this within the pharmaceutical industry: natural products often weren't compatible with traditional target-based assays popularly use by the pharma industry, running the risk of rediscovery of already known compounds, not accessing enough biological material, and the deconvolution of their molecular mechanisms of action can be time consuming (14). However, that have changed in recent years with techniques as dereplication, the resurgence of phenotype screening, the highlighted focus of natural product derivatives, the addition of high throughput screening and more (14) (19) (20) (21). Moreover, the human body is highly capable at processing natural products, both primary and secondary metabolites, as they form the basis for human life as the building blocks of our bodies, energy, vitamins, micronutrients etc. This makes secondary metabolites, unlike synthetic compounds, naturally more likely to be absorbed, distributed, and excreted in the same way as the components in our food (22).

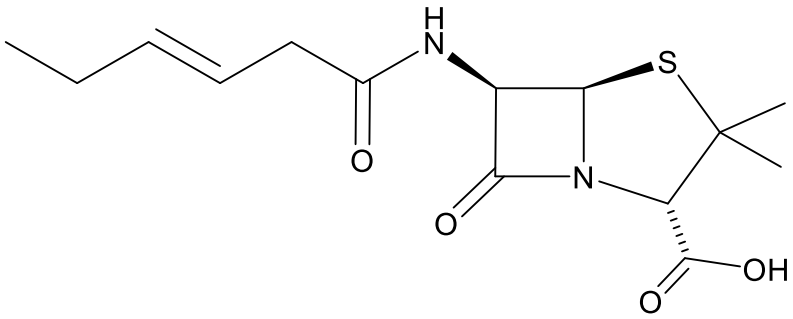


Figure 1: The molecule of Penicillin F (Created in ChemDraw).

1.2.3 The Pharmacology in Natural Products

As acknowledged, natural products have intrinsic properties making them desirable starting points for drug development. The variety of chemical classes amongst secondary metabolites are extensive, however, not all natural metabolites have the potential to become pharmaceuticals. To understand how pharmaceuticals function and how the potential of various drug candidates get recognized, it's essential to understand the two fundamental branches of pharmacology: pharmacodynamics and pharmacokinetics.

A drug candidate's pharmacodynamic properties are any effect the compound exerts on the body, which includes understanding how substances interact with their targets to elucidate the pharmaceutical's molecular, biochemical, and physiological effects (23). Within pharmacodynamics, themes such as mechanisms of action, dose-response relationships, drug

efficacy and potency, as well as therapeutic window and index, makes up a in depth understanding to how a drug works (24). In bioprospecting, the term bioactivity is used to describe a compounds pharmacodynamic effect against living matter or subcomponents of a living matter. Living matter or its subcomponents encompass a broad spectrum, including isolated drug targets (e.g. enzymes), living organisms (e.g. mice), living tissue (e.g. human cells), and more depending on the specific target being studied. In a pharmaceutical context, examples of target areas of interest include infectious diseases, cancer, the immune system, the central nervous system, metabolism, cardiovascular conditions, gastrointestinal disorders, and more (25). The bioactivity plays a crucial role in drug discovery because it indicates the potential of a compound to produce a biological effect when interacting with biological targets *in vivo* and are often the basis for further analysis (26).

However, several pharmacodynamic parameters are intricately linked to the pharmacokinetic properties of a drug molecule. Pharmacokinetic describes how medications is moving though the human body. Pharmacokinetics encompasses the assessment of a compound's absorption, distribution, metabolism, and excretion (ADME) properties. The ADME system (sometimes referred to with additional letters: LADME (Liberation-ADME) or ADMET (ADME-Toxicity)) is a well-known terminology used to describe the different aspects of the field pharmacokinetics (27). Understanding this process is the key to how the drug-candidates can be used as a pharmaceutical, and one of the key factors to a drugs pharmacokinetics is their ability to dissolve in water (hydrophilicity) or in lipids (lipophilic). Lipophilicity is dictating the ability to traverse cellular barriers, and the compound’s delivery to its cellular targets (28). Hydrophilic drug candidates come with major challenges due to their low degree of membrane penetration, but new ideas as loaded polymeric nanoparticles, has been explored to solve the problem (29). Most drugs are lipophilic, and it’s recognized as a meaningful parameter for pharmaceuticals, with numerous attributes supported by Quantitative structure–activity relationship (QSARs) and Quantitative Structure-Pharmacokinetic Relationships (QSPkRs) (30). A drug with too high lipophilicity (Log P > 5) will usually be described as a compound with rapid metabolic turnover, low solubility, and poor absorption (31).

The relationship between the pharmacodynamic-effects and the pharmacokinetic attributes is what shapes a potential drug. The pharmacodynamic effect are often straight forward, in comparison are the pharmacokinetics very complex. To help evaluating a drug-candidates pharmacokinetic-potential, are there general established criteria and attributes that can estimate a compounds drug-likeness. Drug-likeness makes for a robust tool during early stages of drug

discovery to predict if a given bioactive molecule will have desirable ADME properties (32). The most established rulesets in pharmacy are probably the *Lipinski rule of fives*, which is a set of rules to help evaluate if a compound has the potential to becoming an orally available drug (33). After establishing the pharmacodynamic effects and pharmacokinetic attributes, it becomes possible to evaluate whether a proper drug candidate has been identified.

1.2.4 Natural Products Currently Used as Pharmaceuticals

Natural products play a vital role in the pharmaceutical industry, with continuous research into new leads as well as the ongoing production of existing pharmaceuticals. Out of all the pharmaceuticals on the market is it estimated that 35 to 50% originates from nature (34). That includes direct therapeutic agents (pure drugs and phytomedicines), semi-synthetic drugs, prototypes of lead molecules, and as taxonomic leads of new drugs (35). One of the most prominent natural product pharmaceuticals in use today is morphine, which is a potent analgesic derived from *Papaver somniferum* (36). Some of the most prominent natural product drugs from the marine environment are anticancer drugs, e.g. the nucleoside cytarabine (37) and the polyketide eribulin (38).

1.3 The Marine Environment and Marine Invertebrates

1.3.1 The Potential in the Marine Environment

The ocean is estimated to contain 50-80% of all life on Earth but remains vastly unexplored by humans with less than 10% of its depths investigated. (4). The ocean covers 360 million square miles and are made up by unique ecological habitats as deep-sea hydrothermal vents, coral reefs, and polar regions. The potential of the marine environment lies in its biodiversity, which consists of vast array of organisms, including bacteria, fungi, algae, vertebrates, and invertebrates spanning across all the different marine habitats. The biodiversity reflects the myriad of adaptions and survival strategies evolved to cope with the extreme conditions. To survive, organisms must adapt to both abiotic factors, such as limited sunlight, high pressure, salinity, and UV radiation, as well as biotic factors, including predators, parasites, and competition for food (39). They have done so by producing a wide range of secondary metabolites (e.g. alkaloids, terpenoids, peptides, and polyketides), which has led the marine life to become so highly diverse with a lot of different unique adaptions and survival tactics (40). The secondary metabolites, which has been chemically optimised during millions of years of evolution, therefore holds a lot of pharmaceutical potential (41).

1.3.2 Marine Invertebrates

Marine invertebrates encompass a vast array of organisms that lack a vertebrae column, constituting a diverse and abundant group within ocean ecosystems. The lack of a vertebrae column entails that these animals do not have an adaptive immune system. Most are sessile or slow moving and they get their nutrition through filter feeding. Still, these animals thrive in their ecosystems, where the presence of pathogens and predators is constant. One widespread defence mechanism is the utilization of an arsenal of bioactive compounds to deter threats. These defence mechanisms often involve the synthesis and secretion of secondary metabolites. The chemical defences of marine invertebrates often exhibit remarkable specificity and potency, targeting specific physiological processes or biochemical pathways of predators or competitors (42). As a result, they are highly relevant targets in drug discovery.

1.3.3 Studies with Marine Invertebrates Relevant for This Study

Marbio has successfully isolated and characterized several novel compounds from marine invertebrate biomass in the past. Several of these can be published, as their commercial potential is limited, mainly due to their structures being previously published or their limited bioactivity, including ianthelline (43), the purpuroines (44) and the ponasterons (45). However, the most promising compound classes needs to be kept strictly confidential to avoid interference with later commercialization efforts. The success of these efforts has resulted in the spin-off company KinSea Lead Discovery AS, whose mission is to exploit the potential of marine bioactives for the treatment of human diseases mainly based on assets discovered and characterized at Marbio.

1.3.4 Marine Invertebrates Analysed in This Study

In this study we have two sets of marine invertebrate extracts. One set of extracts were collected and prepared by Marbank, and the other were collected and prepared by White Point Biomarine.

1.3.4.1 The Marine Invertebrates Sampled by Marbank

Marbio collaborates with Marbank (Norwegian national marine biobank, Institute of Marine Research) by receiving marine extracts from its invertebrate extract libraries. The samples received from Marbank have been collected, taxonomically classified and prepared (extracted), then provided to Marbio to be prefractionated and screened (Table 1). The marine invertebrates selected for the thesis are based on two factors: evaluations of biodiversity relevance (evaluated by Marbank), and the quantity of material obtained from the extraction.

Table 1: The marine invertebrates used in studies which were obtained by Marbank.

Marine Invertebrates from Marbank			
Sample ID:	Phylum:	Species:	Common name/ description:
M22009	Arthropoda	*Lebbeus polaris*	Polar Shrimp
M22010	Arthropoda	*Bythocaris leucopis*	Marine Shrimp
M22012	Cnidaria	*Isidella lofotensis*	Horn coral
M22013	Cnidaria	*Funiculina quadrangularis*	Tall sea pen
M22022	Echinodermata	*Ophiopleura borealis*	Brittle Star
M22024	Chordata	*Ciona intestinalis*	Vase tunicate

***Lebbeus polaris* (Polar shrimp, phylum Arthropoda)**

is a species of small shrimps and they thrive in the Eastern Pacific, Arctic, and Northeast Atlantic. It is a versatile species, being found in any depth between 0 to 900 m, but most frequently at depths between 30 to 300 m on both hard and soft bottoms. The colours consist of a pale outer layer with strong red or orange markings, which also include their legs. Its sizes vary and can reach a length up to 9 cm. These polar shrimps are well-adapted to the extreme cold and harsh conditions of their polar habitats and play an essential role in the marine ecosystem as both predators and prey. Their well-adaptions could lead to them containing unique biochemicals that may hold potential for biotechnological applications, but there is little to no research on the secondary metabolites of the species (46).

***Bythocaris leucopis* (Deepsea shrimp, phylum Arthropoda)**

is a species of deep-sea shrimps and have been found in the marine Northeast Atlantic between Norway and Greenland. It has been documented at depths as low as 2700 m below the sea. Its sizes are around 0,5 to 2 cm and it has a red outer layer. It distinguishes itself from other species of Bythocaris by three diagnostic characters, an anteriorly truncate scaphocerite, the lack of pigments on the eye, and the rostrum having the ventral margin concave. To the best of my knowledge, no research has been previously conducted to examine secondary metabolites from this species (47).

***Isidella lofotensis* (Horn koral, phylum Cnidaria)**

is a species of bamboo-coral found primarily in the cold waters of the North Atlantic Ocean, particularly around the Lofoten Islands of Norway. This species typically forms dense, bushy tufts on rocky substrates, often in intertidal or shallow subtidal zones. *I. lofotensis* is characterized by its delicate branching structure and distinctive red coloration, which can vary from pinkish red to deep crimson. As a key component of marine ecosystems, it provides habitat and shelter for various marine organisms, contributing to local biodiversity. To the best of my knowledge, no research has been previously conducted to examine secondary metabolites from this species (48).

***Funiculina quadrangularis* (Tall sea pen, phylum Cnidaria)**

is a species of colonial hydroid found in marine environments, particularly in the northeastern Atlantic Ocean and the Mediterranean Sea. It belongs to the family Sertulariidae and is characterized by its distinct branching colonies with square-shaped stems. Each stem can grow up to several centimetres in height and features alternating side branches bearing tiny polyps. *F. quadrangularis* typically inhabits rocky substrates in shallow coastal waters, where it forms dense colonies that may resemble miniature forests. As a filter feeder, it plays a role in nutrient cycling and ecosystem dynamics, contributing to the marine food web. Very limited research has been conducted to map the secondary metabolites of the species (49) (50).

***Ophiopleura borealis* (Brittle star, phylum Echinodermata)**

is a species of brittle star found in the cold waters of the North Atlantic Ocean, particularly in the Arctic and sub-Arctic regions. It belongs to the family Ophiuridae and is characterized by its five long, slender arms radiating from a central disk. This species typically inhabits soft sediments on the ocean floor, where it can bury itself partially for protection. Ophiopleura borealis is an opportunistic feeder, consuming detritus, small organic particles, and occasionally small invertebrates. It plays a role in benthic ecosystems, contributing to nutrient cycling and serving as prey for various marine organisms. There have been research on the fatty acid content of this species (51).

***Ciona intestinalis* (Vase tunicate, phylum Chordata)**

is a species of tunicates which are characterised by its soft tunic body with a pale translucent greenish/yellow colour. They are considered an invasive species and are widely distributed throughout temperate regions across the world. Its habitat is located from 0-500m depth and

usually locate areas with low exposure to water flow. It is considered a large solitary sea squirt and can grow up to 20cm tall. As a world spread filter feeder, makes it an interesting prospect for secondary metabolite characterisation, which shows in the comprehensive selection of secondary metabolites isolated from the species (52).

1.3.4.2 The Marine Invertebrates Sampled by White Point Biomarine

White Point Biomarine (WPB) provided a sample set to Marbio after the business closed due to retirement. The sample set consisted of 244 extracts, organized into pairs of two extracts per organism, making the total numbers of organisms 122. This sample set were analysed during the work with my bachelor thesis. The extracts in this set had varying levels of taxonomical assessment, ranging from full genus identification to only phylum-level classification. All the extracts were tested for anticancer and antimicrobial bioactivities. Based on this work, two of the extracts were selected for further evaluation (Table 2).

Table 2: The taxonomical information about the two marine invertebrate extracts received from Withe Point Biomarine.

Marine Invertebrates from White point Biomarine			
Sample ID:	Phylum:	Highest taxonomical evaluation:	Common name/ description
Extract-041-USA	Chordata	Aplidium sp. (Genus)	Colonial sea squirts
Extract-086-USA	Chordata	Enterogona (Order)	Tunicate

***Aplidium sp.* (Colonial sea squirts, phylum Chordata)**

is a colonial sea squirt named *Aplidium sp*. The sample was collected south of Low Island, Antarctica, and there are documented eighteen different Aplidium genuses from 239 collections in the area (based on data from the Global Biodiversity Information Facility database). Ascidians like Aplidium are known for numerous defensive strategies related to physical, nutritional, or chemical properties of the tunic (53). This has resulted in the isolation of numerous secondary metabolites from various types of Aplidium species.

Order Enterogona (Tunicate, phylum Chordata)

is a tunicate only named with the Order (O: Enterogona). The sample was collected outside of the King George Island at the Admiralty Bay in front of Copa Station in 1994. Tunicates are in general known for its high amounts of bioactive secondary metabolites, with more than 1200

active molecules were identified from tunicates and tunicate-associated microbial species (e.g. peptide and alkaloids) (54).

1.4 Marine Bioprospecting

Bioprospecting is the systematic search of biological resources that can be commercialized and utilized by humans. Bioprospecting uses the exploration of biodiversity to discover natural products and develops them with techniques across a multitude of different scientific disciplines. Products discovered from bioprospecting can be divided into three main categories: chemicals, genes, and biological designs. Given the potential products that can be discovered, bioprospecting is carried by numerous industries, including pharmacy, dietary supplement industry, manufactories, environmental preservation, cosmetic industry, and more (3).

Bioprospecting has mainly been focused on terrestrial organisms, mainly due to its easy accessibility. Within pharmacy, this has led to most of the natural product-based drugs have derived from terrestrial plants, fungi, or microorganisms. In later years has this been encompassed by new gathering tools, which has led to the marine environment becoming a more central source of research (48). This have led to emergences of marine bioprospecting, using highly potent biomass from the marine as the basis for the search. If bioactivity assays are used to detect bioactivity of extracts prior to compound isolation, the approach is called bioassay-guided isolation. The approach revolves around the discovery of bioactivity within extracts and continues by tracing the presence of a certain bioactivity throughout the isolation process until the molecule(s) with the desired bioactivity are isolated. Bioactivity are discovered by submitting fractionated crude extracts to bioactivity screening, which can be performed either *in vivo* or *in vitro*, were the bioactive extracts are subjected to further research continuing with dereplication and lastly compound isolation (22). This methodology led the basis for the development of the marine bioprospecting pipeline developed by Marbio.

1.4.1 The Marine Bioprospecting Pipeline

The marine bioprospecting pipeline is a strategic framework of methods designed to discover marine natural products with pharmaceutical potential. It is a top-down drug discovery approach, where they perform bioassay-guided isolation of compounds from marine organisms. The pipeline encompasses collection, extraction, fractionation, bioactivity assays, dereplication, compound isolation, structure elucidation and retesting of bioactivity (Figure 2) (55). The pipeline is designed to be able to evaluate and prioritize samples at each individual step, allowing for ruling out samples and narrowing it down to only novel natural products.

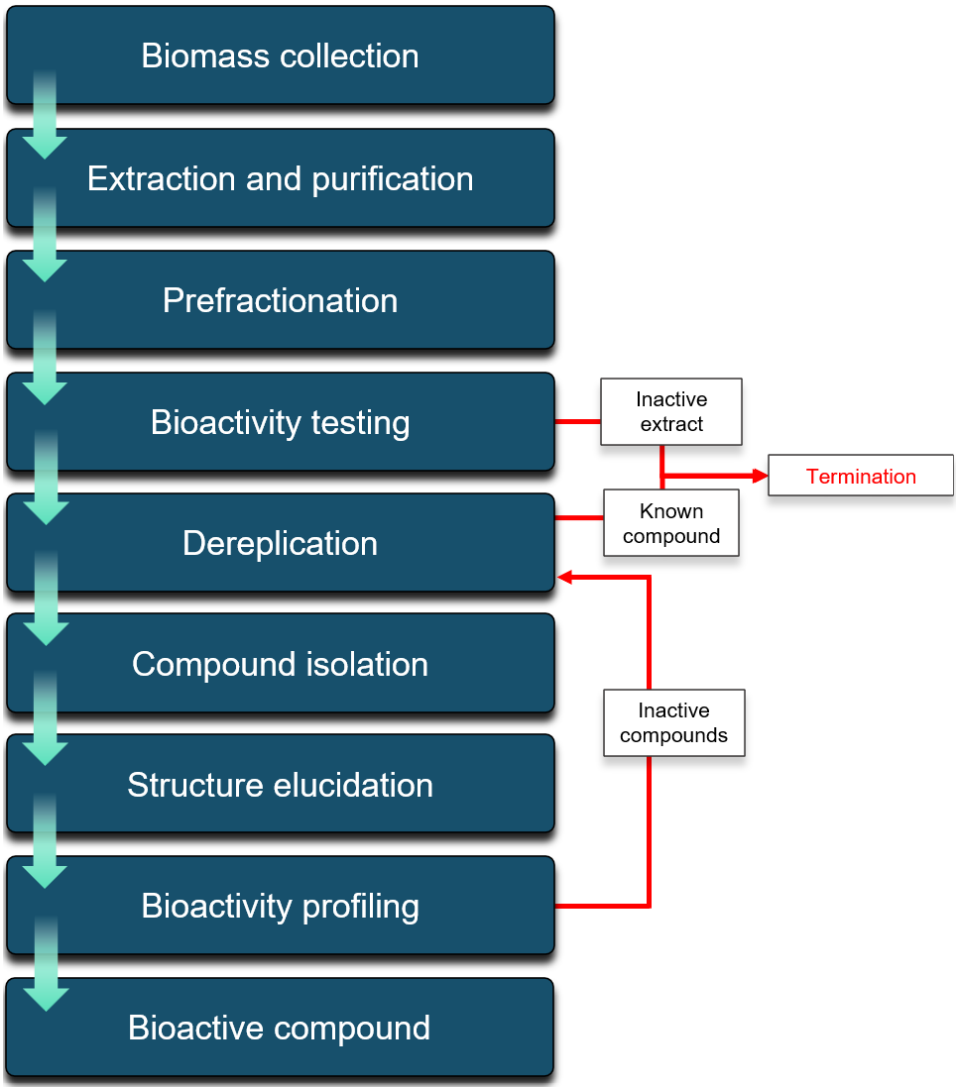


Figure 2: The workflow in this thesis is based on the marine bioprospecting pipeline, which is developed and conducted at Marbio. This marine bioprospecting pipeline is utilized or discovering novel, bioactive natural products using bioassays (55).

1.4.2 Sample Collection, Taxonomy and Extraction

Prior to the work of this thesis, the samples were collected, taxonomically evaluated, and extracted by technicians at Marbank. The other set of extracts, the WPB samples, were prepared in a similar manner by local staff. Invertebrate biomass is freeze-dried, processed by liquid-liquid partitioning to obtain both an aqueous and organic extract. These extracts are complex blends of various compounds and are initially fractionated based on polarity, with the aqueous phase containing more polar compounds and the organic phase containing more non-polar compounds (56). This general separation makes it beneficial to further fractionate them before conducting bioactivity screenings.

1.4.3 Prefractionation

In bioprospecting, fractionation of a sample prior to bioactivity testing is referred to as pre-fractionation. Studies have highlighted that such fractionation significantly enhances the probability of identifying the bioactivity of the sample, mainly due to reducing the samples complexity. The objective of the process is to eliminate components that might result in false positives and to remove substances that could mask bioactive secondary metabolites during testing, simultaneously facilitating the dereplication analysis. Sample components with low potential as drugs (e.g. highly lipophilic cell wall components and hydrophilic salts and carbohydrates) are often separated from the desired secondary metabolites, which often has a polarity that elutes them in fractions away from the e.g. lipids and salts (57).

Fractionation techniques vary, with liquid chromatography (LC) being a commonly employed method, including HPLC or flash chromatography. At Marbio, a flash chromatographic approach produces eight distinct fractions that are dried and weighed. The advantages of flash chromatography include its high loading capacity and the ease with which finished, weighable fractions can be generated. In this LC technique, a sealed column or prepacked cartridges are employed, and a pump propels the mobile phase through the stationary phase. Consequently, this segregates the sample based on its affinity for either the stationary or mobile phase (58).

After the extracts are fractionated, the fractions are standardized based on concentration and organized into systematic layouts, which are necessary for subsequent analysis. Following prefractionation, the collected fractions undergo bioassays to identify the presence of bioactive compounds. This method helps pinpoint potentially active compounds within the collected fractions.

1.4.4 Bioactivity Testing

The resulting fractions are submitted to bioactivity testing. This is done to nominate samples for further examination, where a bioactive fraction will continue through the bioprospecting pipeline. As part of this thesis, the invertebrate extracts/fractions were tested in viability assays for activity against malignant and non-malignant cell lines and against Gram-positive and Gram-negative bacteria.

To test for activity against the cell lines, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell cytotoxicity assay was used. This is a widely used colorimetric method for assessing cell viability and cytotoxicity *in vitro*. It relies on the reduction of MTS by metabolically active cells to form a coloured

formazan product, which can be quantified spectrophotometrically. This assay offers several advantages, including simplicity, speed, and compatibility with high-throughput screening. Additionally, the MTS assay is non-toxic to cells and can be used to monitor cell viability over time or following exposure to various treatments, in this case in exposure the extracts/fractions (59). Its versatility and reliability make it a valuable tool in bioprospecting aiming at detecting cytotoxic compounds in extracts/fractions.

To test for activity against bacteria, the broth microdilution antibacterial assay setup was used. This is a widely used method for determining activity of compound mixtures (like extracts and fractions) or pure compounds against bacterial pathogens. Pathogenic bacterial can be subjected to a serial dilution of pure compounds to determine their minimum inhibitory concentration (MIC) against them. The method involves diluting the extracts, fractions, or pure compounds in liquid growth medium in microtiter plates, inoculating the plates with standardized bacterial suspensions, and incubating them under controlled conditions. After incubation, bacterial growth is assessed visually and by measuring optical density, with the MIC defined as the lowest concentration of the added test agent that inhibits visible growth. This assay provides information about the potency of the tested material and their effectiveness against specific bacterial strains. Its quantitative nature and high-throughput potential make it a cornerstone in antimicrobial susceptibility testing and drug development efforts aimed at combating bacterial infections (60).

During the initial stage of bioactivity testing, all fractions undergo a single concentration screen against a sensitive cell line that is responsive to various types of bioactivities. This step aims to detect any potential bioactive compounds present. For instance, at Marbio the A2058 cancer cell line is commonly used because of its high sensitivity. If an extract or fraction demonstrates activity, it is designated as a hit, and the extract is labelled as 'bioactive'. A hit is determined based on the percentage of cell survival, were percent survival<50% is determined active and 50-60% is labelled questionable (may e.g. possibly contain an active component in low concentrations). These fractions undergo further evaluation through dose-response screening, where they are tested at lower concentrations to highlight extracts with high levels of activity. The bioactive fractions are then subjected to dereplication to conduct further analysis of their contents.

1.4.5 Dereplication

Dereplication is the process of chemically analysing bioactive fractions or extracts in an attempt to identify the individual components responsible for the observed bioactivity and nominate these for further isolation. The bioactive fraction is analysed along with the fraction eluting directly before and after. By comparing the chemical content of the active fraction with the chemical content of the neighbouring inactive fractions, compound(s) that are only found, or found in larger amounts, in the active fraction can be suspected to be the compound(s) responsible for the observed bioactivity (61). When compound(s) are identified, they are run through a database in an attempt to find more information about them. If the compounds have been previously isolated and well characterized, this will not be prioritized for further isolation. It provides as an efficient way of ruling out if a sample is containing pan-assay interference compounds (PAINS) or priorly known bioactive compounds. The point of dereplication is to contribute to more efficient use of limited resources. It makes it possible rule out and make prioritization of between samples. Phospholipids are one of the major reasons for bioactivity from invertebrates in bioassays. This compound comes from different types of cell membranes and can be highly effective in disrupting membranes of other cells (Figure 3) (62). They have the same ability as soap to dissolve the outer membrane and destroying the cell integrity. This leads them to be toxic and effective in killing cells non-specifically. As a result of this, are they recognized as molecules that we are unable to use as drugs, where specific activity rather than general toxicity is of utmost importance.

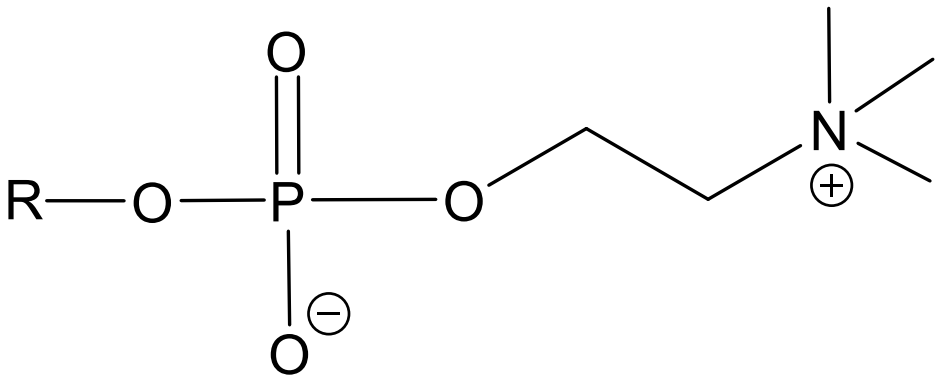


Figure 3: The chemical structure phospholipids. It's a well-known case of a pan-assay interference compounds, where this particular structure is connected to fatty acids and has a tendency to interfere with assays through processes such as oxidation, nitration, and subsequent oxidative degradation (62) (Created in ChemDraw).

Importantly, dereplication will also identify compounds expected to be novel. If these compounds turn out to be the ones responsible for the observed bioactivity, they can be protected through patenting. Patenting is an important aspect in bioprospecting, especially when the goal is to develop a new pharmaceutical product, as the development is very expensive. The clinical development is most often conducted by an established biotechnology or pharmaceutical company. To convince these companies to take on this time-consuming and expensive development, patent protection is an absolute requirement as this will enable the company to earn back the expenses when the compound enters the clinic.

Within the field of dereplication, there are there several strategies that can be applied. Dereplication techniques encompass a variety of chromatographic and spectrometric methods, but since electrospray ionization was introduced at the end of 20^{th} century, liquid chromatography in combination with HR-MS has become the tool of choice. One of the leading techniques is Ultra High-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UHPLC-HR-MS). It is a technique that combines high chromatographic performance with mass detection. In the ESI ion source, the analytes are transferred from a liquid phase to the gas phase. The ions are separated according to their mass to charge (*m/z*) ratios by the HR-MS system, and the number of the individual ions are recorded (63). The registered *m/z*-ratio from positive electrospray is usually the molecules molecular weight (Mw) plus one added proton. This is because of molecules being pronated (molecule plus H^+) during the ionization. However, there are some exceptions where compounds reacting with adducts instead (e.g. molecule plus $Na^+/K^+/NH_4^+$) (64). After the analysis the data can be viewed as a mass chromatogram where the intensity is plotted as a function of the retention time on the chromatographic system. The extracts showing promising bioactivity and containing compounds of interest are prioritized for compound isolation, while the others may be ruled out. The *m/z* values of the compound and its isotopes can be used to calculate the elemental composition and estimate fragmentation patterns.

1.4.6 Compound Isolation

Extracts and fractions of marine organisms are complex mixtures of numerous compounds. Purification entails separating the desired compound(s) from this matrix. Within this project, mass-guided preparative high-performance liquid chromatography (prep-HPLC) was employed to isolate the compounds of interest identified in the dereplication step, purifying them from undesirable impurities. This facilitated subsequent endeavours in structural determination and bioactivity assessment.

In prep-HPLC, the column is filled with a packing material (the stationary phase) which the mobile phase is pumped through. The mobile phase consists of water and an organic solvent (must be miscible with water), with additional formic acid to control the pH and provide charge of the compounds (65). When the sample, either a dissolved and cleaned extract or fraction, is injected into the system, its components are distributed between the stationary phase and the mobile phase. This distribution is based on a wide range of physicochemical factors of both the stationary and mobile phases, as well as those of the sample components. Combining these factors gives the individual components of the sample a specific retention time (Rt). The Rt is the elapsed time between the injection of a sample into the chromatographic system and the emergence of a particular compound from the column. By collecting the eluate at the time when the compound of interest elutes, the target compounds can be separated from other sample components with different Rt's (65). As the chromatographic system is attached to a mass spectrometer, the system can be programmed to trigger collection when the compound of interest elutes. Alternatively, if the Rt is known, the system can be programmed to trigger collection at a specific time frame.

1.4.6.1 Scout Run

However, before starting an isolation campaign, a scout run (or column screening) is conducted. This entails injecting the extract/fraction onto columns with different packing materials to evaluate which column gives:

- Desirable peak shape of the target compound(s): well-defined shape with a sharp leading edge and a symmetrically declining tail
- Separates the compound(s) of interest from sample impurities and, if relevant, each other to the highest degree

If the best column in the scout run fails to provide a pure sample, the results can be used to assess whether a column with a different packing material can effectively separate the compound from the co-eluting impurity in a second purification step. As part of this work, columns were evaluated against the extract to locate the compounds listed for isolation. All the columns used in this study are reversed phase (RP) columns with a C18 stationary phase (Figure 4). The columns used in this study were the Xterra, SunFire, and Atlantis C18 RP columns. The main distinction among these columns lies in their particle size: Xterra and Atlantis have a particle size of 10 μM, while SunFire Prep has a particle size of 5 μM. Xterra and SunFire columns have reduced surface silanols compared to Atlantis, making Atlantis better suited for

polar compounds. SunFire, with its smaller particle size, theoretically offers increased resolution and improved ability to separate closely eluting compounds. The column that yields the best compound separation is selected for isolating the compounds of interest.

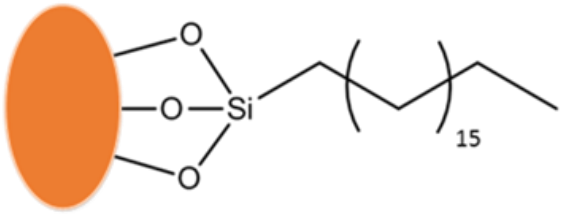


Figure 4: The C18 stationary phase packing material inside the reversed phase (RP) columns.

1.4.7 Structure Elucidation

Structure elucidation is the process of determine a compounds chemical structure, which is important for understanding properties and behaviours of compounds. Nuclear magnetic spectroscopy (NMR) exploits the physical phenomenon where magnetic nuclei in an external magnetic field absorbs and re-emit electromagnetic radiation, and is a method frequently used for conclusive structure elucidation of organic molecules(66). Nuclei exhibiting nuclear spin due to odd numbers of protons and/or neutrons, and thus have magnetic properties, can be analysed. By subjecting them to an external magnetic field, magnetic nuclei that are otherwise randomly organised, will align to the magnet, either with or against the applied magnetic field, thus generating two distinct energy states of individual nuclei. By applying a pulse of electromagnetic radiation that matches the difference between the two energy levels (usually within the radio frequency range), nuclei to absorb the electromagnetic energy and flip its spin state. When the electromagnetic pulse is discontinued, the energy absorbed by the individual nuclei is released, producing a measurable signal, called the resonance frequency. These signals, which ideally are different for all the measurable nuclei in the analysed molecule, are processed into various NMR spectra. The most studied nuclei in NMR spectroscopy are ^{1}H and ^{13}C. Logically, ^{1}H-NMR provide information regarding the protons and ^{13}C regarding the carbon atoms of the analysed molecules. 1h and 13C experiments are NMR experiments are examples of 1-dimensional (1D) NMR analysis, analysing a single nucleus, and are the most widely employed NMR analysis. Two-dimensional (2D) are analysing their relationships between the different magnetic nuclei of a molecule. A wide range of 2D experiments exists. By analysing a molecule with several of these, the data can collectively be used to determine

the structure of the analysed molecule(66). The most commonly used 2D experiments include heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY).

1.4.8 Bioactivity Profiling

After a compound have been isolated and structurally elucidated, the profiling of the compounds can start. Bioactivity profiling is the determination of the bioactivity a compound process. In the marine bioprospecting pipeline, bioactivity testing of a purified compound can be divided into two categories: retesting and characterization. Retesting involves testing the compounds in the assays that initially showed positive results during the screening of its originating fraction or extract, to confirm whether the molecule was responsible for the observed bioactivity. This step is crucial for determining whether the correct compound(s) have been isolated from the extract, or if the bioactive component(s) remain unidentified. Whether the compound is confirmed to be the bioactive component or not, the process of characterization follows up. The characterization is to study the specific effect of the compound in a broader panels of bioactivity assays. This is achieved by subjecting the compound to a wide range of bioassays encompassing various bioactivities (67). These tests help pinpoint specific effects, if the compound is cytotoxic, while also determining the efficacy of the bioactivity (e.g. through MIC or half-maximal inhibitory concentration (IC_{50})). If the compound meets the criteria for being labelled as novel, the biological and chemical mechanisms are investigated through target-based and phenotypic screening approaches.

2 Aims of the Thesis

Primary aim

The primary objective of this study was to isolate and characterize secondary metabolites from invertebrate extracts that have the potential of being developed into commercially available pharmaceuticals.

Secondary aims/interim goals

To reach the primary aim, the following secondary aims were pursued:

- Prepare a fraction screening library from invertebrate extracts
- Identify bioactive fractions of invertebrate extracts through bioactivity screening
- Identify the active components through dereplication using UHPLC-HR-MS
- Isolate compounds of interest (mass-guided preparative HPLC)
- Perform chemical characterization on compounds (NMR)
- Perform bioactivity profiling (retesting and characterization)

3 Material and Methods

The methods used in this thesis are from the marine bioprospecting pipeline established at Marbio, where the work was conducted. The biological material was supplied by Marbank and WPB. Equipment and consumables were provided by Marbio. The most basic laboratory equipment, such as glassware, Eppendorf tubes and pipette tips, will not be referred to in the equipment lists, unless a very specific variant was needed in the setup.

3.1 Sample Origin and Preparation

3.1.1 Marbank Marine Invertebrate Samples

The sample panel consists of marine invertebrate extracts supplied by Marbank (the Institute of Marine Research, Tromsø). In total, two extracts from six species (12 extracts in total) were analysed, all originated from the Artic, marine environment. They were all collected during scientific cruises with the aim of gathering marine biomaterials (Table 3). The samples were taxonomically verified, stored, and prepared (extracted) by Marbank.

Table 3: The data regarding the collection of the biomass yielding the 12 extracts from Marbank. (A: Aqueous extract, O: Organic extract)

Collection data of the marine invertebrates extracts from Marbank					
Sample ID:	Species:	Date:	Location:	Depth (m):	Extract Weight (g):
M22009	*Lebbeus polaris*	20.08.20	Bjørnøybanken	100	A: 26,1
					O: 4,5
M22010	*Bythocaris leucopis*	09.08.20	Fram Strait	2000	A: 22,1
					O: 8,0
M22012	*Isidella lofotensis*	08.05.13	Sagfjorden	368	A: 14,3
					O: 3,1
M22013	*Funiculina quadrangularis*	09.05.13	Engleøya North	422	A: 14,8
					O: 6,4
M22022	*Ophiopleura borealis*	08.08.20	Yermak Plateau	730	A: 20,4
					O: 3,3
M22024	*Ciona intestinalis*	14.08.20	Fram Strait	3000	A: 32,6
					O: 5,7

The process of preparing the samples starts onboard the reach vessel during excursions, where the biomass is sorted and frozen. The frozen material is then transported to the laboratory and stored at -28 °C in the dark until further processing. Before extraction, a biomass sample is given a sample ID in the format MYYXXX, where M informs that the sample is a marine invertebrate, YY is the year the sample was extracted (e.g 2022 would be written 22) and XXX a sequential number where the first extracted sample of a year gets the number 001. Extract production starts of by cutting the animal biomass into small pieces and freeze-drying them for 2-3 days. These are milled and stored awaiting further processing. The material (max 400g) is afterwards mix with water in a 10 mL:1 g ratio, chilled for 3 hours, and then centrifuged for 30 minutes at 4600 rpm in 5 °C. The layer of water is taken out and transferred to steel plates. The aqueous extraction is then repeated once more. After the second water fraction is transferred, the pellet is transferred to a separate steel plate, which will form the basis for the organic extract. The undried aqueous extract and the pellet are stored in a freezer overnight. The next day are the samples placed in a Heto PowerDry PL9000 freeze dryer at for 2-3 days. When the samples are completely dried, they are brushed out of the heat proof plates. The dried aqueous extract is placed in 50 ml falcon tubes, ready for further processing. The dry pellet is added 10 mL:1 g ratio of dichloromethane and methanol (MeOH, 1:1 ratio) and stored overnight. The next day the solution is filtered through a Whatman filter. The liquid from the filtering is then transferred to round bottom flasks and dried using a rotavapor. The pressure is slowly lowered from 900 mbar to 220mbar, where it is kept for 30 minutes. The vaporized product is the finished organic extract. The organic and aqueous extracts are given a sample ID in the following format: ‘Sample ID’-X-L or ‘Sample ID’X-W. Sample ID refers to the processed invertebrate biomass, X is a number to indicate if the entire biomass is processed as a whole, X = 0, or if the animal has been dissected into subsamples (e.g. a crab could be separated into shell and shell content before the extraction started, which would give X = 1 and 2 to separate the resulting extracts) and L and W labels the organic and aqueous extracts, respectively. Both the aqueous and organic extracts from all the invertebrate samples listed in Table 3 was further processed as part of this thesis. The extraction process was conducted before this project started by Marbank personnel.

3.1.2 White Point Biomarine Marine Invertebrate Samples

The other type of extract analysed in this study were marine invertebrate samples provided from White Point Biomarine from USA. Marbio were supplied a sample-set of 244 extracts from 122 different marine invertebrates which originated from the USA and the South Pole, collected

between February 26th 1994, and March 5th 1995. The sample-set was integral to a bachelor's study previously conducted by me, where a method was developed for preparing these extracts and subsequently screening them for bioactivity against cancer cells and pathogenic bacteria. The results from two sets of the extracts caught our attention by providing promising results on the viability assays and were selected for further processing as part of this thesis.

The samples were collected during two separate cruises, both of which were sampled through a dredge box. The biomass obtained were made into two set of extracts. The public recipe made by the company in 1997 by J. P. Delvin. WPB categorized the extracts as non-polar (Shortened to DM in the sample ID) and polar (shortened to FA in the sample ID). In essence, the biomass material was extracted using 100% MeOH. When dried, the resulting pellet was added dichloromethane to extract the most lipophilic compounds, yielding the DM extract when dried. The remains of the MeOH pellet the underwent a chromatographic step to remove polysaccharides, amino acids, and inorganic salts, using HP-20 ion elution and water. The eluate was subsequently washed with acetone, yielding the polar fraction 'FA' (68). After the DM and FA fractions were dried, stored, and was sent to Marbio in 2021. An overview of the collection data from the samples used in this study is displayed in Table 4.

Table 4: The summary of the collection-data for the two extracts (two per invertebrate) from White Point Biomarine.

Collection-data of the marine invertebrates extracts from White Point Biomarine					
Sample ID:	Highest taxonomical evaluation:	Date:	Location:	Depth (m):	Extract Weight (mg):
Ext. 041	Aplidium sp. (Genus)	18.10.1994	Low Island	135-148m	DM: 82,6
					FA: 46,0
Ext. 086	Enterogona (Order)	31.10.1994	King George Island	160-200m	DM: 43,7
					FA: 26,8

3.2 Prefractionation

The samples were fractionated prior to bioactivity testing in a process termed prefractionation. As the samples weight of the WPB extracts were low (less than 100 mg for all samples), only the Marbank extract were perfectionated. The equipment used in the sample preparation, flash chromatography and sample plate preparation are listed in Table 5.

Table 5: The material used in the sample preparation, flash chromatography and plate standardization.

Equipment: Sample preparation	Supplier
Centrifuge, Heraeus® Multifuge® 3S-R	Kendro, Osterode, Germany
Rotary evaporator, Heidolph Laborota	Heidolph, Schwabach, Germany
Equipment: FLASH	**Supplier**
FLASH column, Biotage® Snap cartridge KP-Sil 10g, FSK0-1107-0010	Botage, Sweeden
FLASH purification system, Biotage® HPFC SP4	Botage, Sweeden
Vacuum manifold, VMR 57030-U	Merck KGaA, Germany
Equipment: Plate standardization	**Supplier**
Freeze dryer, FreeZone 8 Liter -50C	Labconco, MO, USA
Polyvap, Syncore®	Büchi, Switzerland
Solvents/material	**Supplier**
Aceton	Sigma-Aldrich, MO, USA
Hexane	Sigma-Aldrich, MO, USA
HP20SS resin	Sigma-Aldrich, MO, USA
Methanol (MeOH)	Sigma-Aldrich, MO, USA
Milli-Q water	Merck KGaA, Germany

3.2.1 Sample Preparation – Organic Extract

An aliquot of the organic extract (1,5 g) was transferred to an Erlenmeyer flask and dissolved in 60 ml hexane. The mixture was transferred to a separation funnel, and an extra 10 ml of hexane was added to the Erlenmeyer flask and transferred to ensure a complete transfer. When everything was transferred, 50 ml of 90% aqueous MeOH was added to the hexane solution in the separation funnel and mixed. The mixture spontaneously separated into two layers, were the bottom layer (the 90% MeOH phase) was collected. The process of adding 90% MeOH to the hexane solution was then repeated twice. The combined 90% MeOH phases were dried under reduced pressure using a rotavapor. A total of 6 organic extracts were prepared using this method.

3.2.2 Sample Preparation – Water Extract

An aliquot of the water extracts (5 g) was transferred to an Erlenmeyer flask and dissolved in 45 ml 90% aqueous MeOH. This solution rested for 4-5 hours, before it was centrifuged at 4500 rpm for 20 minutes at 18 °C. The supernatant was collected and transferred to a round bottom flask and dried under reduced pressure using a rotavapor. A total of 6 aqueous extracts were prepared using this method.

3.2.3 Drying the Samples Before Preforming Flash Chromatography

The dried samples from 3.2.1. and 3.2.2 were separately redissolved in 2 ml hexane and added 2 g of HP20SS resin. The mixtures were dried using a rotavapor. The rotavapor started at 250 mbar at 40 °C, before it gradually was lowered down to 30-40 mbar. This continued until the resin-extract samples were completely dried.

3.2.4 Preparation of Flash Columns

The preparation of the Flash columns was started by weighing 6,5 g of HP20SS resin and incubating it with 75 ml of MeOH for 20 minutes. After 20 minutes the majority of the MeOH was removed by careful decantation. An empty Flash column was then mounted on a vacuum manifold. The vacuum was set on 10-20 mHg, and Milli-Q water (˜5 mL) was added to the empty column. Then the resin was added to the water containing column and vacuumed with additional water added to preventing the column from dry out (water level kept ˜1 cm above the resin). The finished column was stored at 4 °C in the dark until used. A total of 12 columns were prepared using this method.

3.2.5 Flash Chromatography

The flash instrument was turned on and mobile phase liquid pumped through the tubing to remove air before the flash column was connected to instrument. To equilibrate the column, 80 mL of the mobile phase, holding a concentration equal to the starting condition of the mobile phase gradient (Table 6), was pumped through the column to equilibrate it. A reck of 27 numbered testing tubes are then placed in the fraction collection. The resin-extract mixture was poured onto the top of the equilibrated column from the round bottom flask. To allow complete transfer, the round bottom flask was washed with 1 ml Milli-Q water, which again was poured onto the column. The mobile phase was then started, following the composition listed in Table 6, at a flow rate of 12 ml/minute. The eluate was collected in two-minute fractions in a total of 27 fractions. A total of 12 separate flash chromatography runs were conducted using this method.

Table 6: The stepwise mobile phase composition used in the flash chromatography step.

Time (min)	Water (%)	MeOH (%)	Acetone (%)
0-6	95	5	0
6-12	75	25	0
12-18	50	50	0
18-24	25	75	0
24-36	0	100	0
36-42	0	50	50
42-54	0	0	100

3.2.6 Drying and Preparation of Sample Plate

A set of eight polyvap drying tubes were marked and weight. The contents of selected flash tubes were combined in individual polyvap tubes in three's (collected flash tube 1-3, 4-6, 7-9 and so on), apart from the last six (collected flash tube 22 – 27), which were all added to the same polyvap tube. They were then placed in a Polyvap vacuum drier, where the pressure was slowly reduced from 250 mbar down to 30 mbar at 40 °C. The samples were dried until all liquid was evaporated, which took between 1-3 days. The dried samples were weight again. By subtracting the first weighing of the polywap tubes could we find the amount of dried material. The dried material was dissolved in dimethyl sulfoxide (DSMO). The dilution ratios were based on the fraction weight as listed in Table 7.

Table 7: The dilution ratios used to dissolve the flash fractions. Amount of material refers to the weight of material after the samples were dried. (X: flash fraction weight)

Amount of material	Dilution concentration
X < 4 mg	10 mg/ml
4 mg < X < 8 mg	20 mg/ml
8 mg < X < 120 mg	40 mg/ml
120 mg < X	80 mg/ml

All the DSMO diluted samples were shaken for 30 minutes before being transferred to cryotubes, which were later stored at -28 °C in the dark. The resulting fractions were named flash fraction 1 – 8, where fraction 1 was the result of flash tube 1-3, fraction 2 of flash tube 4 – 6 and so on. The sample IDs for the flash fractions was by this expanded to e.g. M22009-0-W01-01 to indicate that this sample was flash fraction 1 of the aqueous extract of *Lebbeus polaris*. Later, an aliquot of all fractions was transferred to an individual well of three different

deep-well plates, with the volume adjusted based on the dilution rate to standardize each well to contain 250 µg of material. This amount was selected as this sample amount conveniently can be redissolved and assayed as 100 µg/mL against cancer cells as part of bioactivity screening, and later in dose-response tests if found to be active, following a standard setup at Marbio. The DMSO of the fractions were evaporated using a freeze dryer. When dry (˜4 h drying time), the plates were sealed and stored at -28 °C in the dark. The layout of the sample plates is shown in Figure 5. These plates ease future work as they allow effective conduction of the bioactivity testing.

	1	2	3	4	5	6	7	8	9	10	11	12
A	M22009 0-W03-1	M22010 0-W03-1	M22012 0-W03-1	M22013 0-W03-1	M22022 0-W03-1	M22024 0-W03-1	M22009 0-L03-1	M22010 0-L03-1	M22012 0-L03-1	M22013 0-L03-1	M22022 0-L03-1	M22024 0-L03-1
B	M22009 0-W03-2	M22010 0-W03-2	M22012 0-W03-2	M22013 0-W03-2	M22022 0-W03-2	M22024 0-W03-2	M22009 0-L03-2	M22010 0-L03-2	M22012 0-L03-2	M22013 0-L03-2	M22022 0-L03-2	M22024 0-L03-2
C	M22009 0-W03-3	M22010 0-W03-3	M22012 0-W03-3	M22013 0-W03-3	M22022 0-W03-3	M22024 0-W03-3	M22009 0-L03-3	M22010 0-L03-3	M22012 0-L03-3	M22013 0-L03-3	M22022 0-L03-3	M22024 0-L03-3
D	M22009 0-W03-4	M22010 0-W03-4	M22012 0-W03-4	M22013 0-W03-4	M22022 0-W03-4	M22024 0-W03-4	M22009 0-L03-4	M22010 0-L03-4	M22012 0-L03-4	M22013 0-L03-4	M22022 0-L03-4	M22024 0-L03-4
E	M22009 0-W03-5	M22010 0-W03-5	M22012 0-W03-5	M22013 0-W03-5	M22022 0-W03-5	M22024 0-W03-5	M22009 0-L03-5	M22010 0-L03-5	M22012 0-L03-5	M22013 0-L03-5	M22022 0-L03-5	M22024 0-L03-5
F	M22009 0-W03-6	M22010 0-W03-6	M22012 0-W03-6	M22013 0-W03-6	M22022 0-W03-6	M22024 0-W03-6	M22009 0-L03-6	M22010 0-L03-6	M22012 0-L03-6	M22013 0-L03-6	M22022 0-L03-6	M22024 0-L03-6
G	M22009 0-W03-7	M22010 0-W03-7	M22012 0-W03-7	M22013 0-W03-7	M22022 0-W03-7	M22024 0-W03-7	M22009 0-L03-7	M22010 0-L03-7	M22012 0-L03-7	M22013 0-L03-7	M22022 0-L03-7	M22024 0-L03-7
H	M22009 0-W03-8	M22010 0-W03-8	M22012 0-W03-8	M22013 0-W03-8	M22022 0-W03-8	M22024 0-W03-8	M22009 0-L03-8	M22010 0-L03-8	M22012 0-L03-8	M22013 0-L03-8	M22022 0-L03-8	M22024 0-L03-8

Figure 5: The layout of the samples in the 96-well deep well plate, which were submitted to bioactivity testing. A-H/1-12 is the layout of a standard 96-well deep well plate. The sample ID of each fraction in each well is indicated with the M22XXX-0-W/L-1-8 codes.

3.3 Bioactivity Testing

The bioactivity testing in our study was based on the survival rate of different cell types after exposure to the above-described fractions. There were two cell types used for the initial bioactivity testing, the MRC5 cells (non-malignant fibroblast cells) and the A2058 cells (malignant melanoma cells) (Table 8). The equipment used in the bioactivity assays is listed in Table 9. In addition, the WPB samples had been assayed against the same cell lines as part of the work conducted for my bachelor's thesis.

Table 8: The cell lines used as part of the bioactivity testing.

The viability assays cell lines used in bioactivity testing of extracts				
Cell-line:	Function:	Description:	Supplier:	Product number:
A2058	Test object	Melanoma/Skin cancer cells	LGC Standards	ATCC-CRL - 11147
MRC5	Cytotoxicity testing	Lung Fibroblast /Healthy human cells	LGC Standards	ATCC-CCL - 171

Table 9: All the equipment and consumables used for the cell viability assays for the bioactivity screening.

Equipment:	Supplier
Biological Safety Cabinet, Herasafe™ KS Class II, Type A2	Thermo Fisher Scientific, MA, USA
Counting chamber, Bürker	Thermo Fisher Scientific, MA, USA
Incubator, Sanyo MCO-18AIC CO2	Panasonic Biomedical, Japan
Microtiter plate, Nunc™ Microwell™ 96-well	Thermo Fisher Scientific, MA, USA
Multimode detector, DTX 880	Beckman Coulter, CA, USA
Solvents/Material	Supplier
CellTiter 96® AQueous One Solution Reagent	Promega, WI, USA
Dimethyl sulfoxide (DMSO)	VWR, PA, USA
Fetal bovine serum (FBS)	Merck KGaA, Germany
Gentamycin (10 mg/mL)	Merck KGaA, Germany
Gibco Dulbecco's Modified Eagle Medium (D-MEM)	Thermo Fisher Scientific, MA, USA
Minimum Essentials Medium Eagle (MEM)	Biochrom, Cambridge, UK
Non-essential amino acids	Biochrom, Cambridge, UK
Sodium bicarbonate (7.5%)	Biochrom, Cambridge, UK
Sodium pyruvate (100 mM)	Biochrom, Cambridge, UK
Stable Glutamine (200 mM)	Merck KGaA, Germany
Trypsin	Thermo Fisher Scientific, MA, USA
Trypan blue	Sigma-Aldrich, MO, USA

3.3.1 Single Concentration Screening

One of the standardized sample plates were used to perform the assays. Both cell lines were incubated at 37 °C with 5% CO_2. MRC5 was cultured and assayed in MEM supplemented with 10% FBS, 10 µg/mL gentamycin, 1 nM sodium pyruvate, 200 nM glutamine, 5 mL non-essential amino acids and 0,15% (w/v) sodium bicarbonate, and A2058 in D-MEM supplemented with 10% FBS, 10 µg/mL gentamycin and 200 nM glutamine stable. To make new cell cultures and to seed the cells into assay wells, the following procedure was conducted

for both cell lines: The first step of the viability assay is to check if the cells are fully grown, so they were observed and checked under a microscope. After removing the old growth medium, the cells were washed using 10 mL PBS buffer, which was removed before 3 mL trypsin was added and the cells were incubated for 2-3 minutes until the cell layer detached from the bottom of the culture flasks. While the cells were in the incubator, 14 ml fresh cell media were added to new culture flasks. The culture flask containing the cells was then taken out of the incubator and 10 ml of DMEM was added. The resulting cell suspensions were thoroughly mixed, and 1 ml transferred to the 14 ml new culture flask.

To count the cells, 100 µl of Trypan Blue was added to an Eppendorf tube. The Trypan Blue was mixed with 100 µl of the cell suspension. After mixing, 10 µl was transferred to a counting chamber and counted under a microscope. The results from the cell counting were used to calculate the volume of cell suspension needed to produce the correct cell density for the viability assay (2000 cells/100 µL for A2058 and 4000 cells/100µL for MRC5) was calculated. In total, 15 mL of cell suspension were prepared/assay plate. The finished D-MEM/MEM cell suspensions were transferred to each well of two different 96-well microtiter plates at 100 µL/well, before incubating the plates overnight.

After 24 hours the cells in the microtiter plates were observed under a microscope to ensure that the cells had adhered to the bottom. It was followed up by gently removing the media while the cells remained at the bottom of the wells. Then 50 µl of all the samples were added to three well each. For the positive control were 50 µl DMSO added, and the negative control were cell medium. The plates were then incubated at 37 ℃ for 72 hours. When the incubation was complete, 10 µl of CellTiter 96® Aqueous One Solution Reagent was added to all the wells before incubating the plate for 1 hour at 37 ℃ with 5% CO_2. The plates were then taken to the plate reader where absorbance was measured at 485 nm with a DTX 880 multimode detector. Percent survival of the cells in each well were calculated using the equation below (Equation 1). The fractions were screened in triplicates (three technical replicates).

Equation 1

$$Cell\ survival\ (\%) = \frac{(abs.treated\ well - abs.positive\ control)}{(abs.negative\ control - abs.positive\ control)} x\ 100$$

3.3.2 Dose-Response Screening

All the active samples (samples resulting in cell survival <60%) in the first run was re-run in a dose-response screening. The dose-response screening was performed using the A2058 cell

line, following the same method and data processing setup described in section 3.3.1. The selected samples were assayed at three different concentrations: 50, 25 and 10 µg/mL. This assay was conducted using three technical replicated on three different days (biological replicates).

3.4 Dereplication

The dereplication was performed using an UHPLC-qToF-MS system. The UHPLC provided chromatographic separation of sample components, which were analysed in UNIFI® Scientific Information System. The equipment used for the dereplication is listed in table 10.

Table 10: The equipment and consumables used during the dereplication.

Equipment: The UHPLC-ToF-MS	Supplier
Binary Solvent Manager, Acquity	Waters, MA, USA
PDA detector, Acquity	Waters, MA, USA
Sample Manager – FTN, Acquity	Waters, MA, USA
IMS QTOF, VION	Waters, MA, USA
Equipment: Coloumn	Supplier
Column, Acquity UPLC® C18, 2.1 x 100 mm, 1.7 µM	Waters, MA, USA
Solvents	Supplier
Acetonitrile (ACN), hypergrade for LC-MS LiChroSolv®	Merck KgaA, Germany
Formic acid, hypergrade for LC-MS LiChroSolv®	Merck KgaA, Germany
Methanol, hypergrade for LC-MS LiChroSolv®	Merck KgaA, Germany

After the viability assay results were analysed, 5 µl of the bioactive fractions and their neighbouring fractions were transferred to a MS-sample plate. Then, 95 µl 100% acetonitrile (ACN) was added. As the WPB samples only existed as extracts, the entire extract was used for dereplication without the possibility of comparing it to an inactive neighbour. The samples were transferred to the autoinjector in the UHPLC-qToF-MS. To separate the compounds in the extracts, a Waters Acquity BEH C18 column was used with a mobile phase of water and ACN (both with 0.1% formic acid). For the extracts, 5 µl was injected using a gradient that ranged from 5 to 100% ACN over 14 minutes. In the mass spectrometer (MS), positive ions were observed between 50 and 2000 Da. The ion source was heated to 350°C, and the capillary voltage was set at 800V. Nitrogen gas (600L per hour) was used to assist in evaporating the mobile phases.

3.5 Compound isolation

From the dereplication, compounds were nominated for isolated using mass-guided preparative HPLC. The extracts were prepared for the prep-HPLC in two different matters. The Marbank samples were prepared by extraction and centrifugation from the original extract, while WPB samples were prepared by completely dissolving all the remaining material in DMSO and hexane. The program used during the isolation were MassLynx® V4.2. The equipment used for the preparations and isolation is listed in Table 11.

Table 11: The equipment and consumables used during extract preparation and the compound isolation.

Equipment: Sample preparation	Supplier
Centrifuge, Heraeus® Multifuge® 3S-R	Kendro, Osterode, Germany
Rotary evaporator, Heidolph Laborota	Heidolph, Schwabach, Germany
Equipment: prep-MS	Supplier
Controller, Waters 600	Waters, MA, USA
Flow Splitter (1:100), Waters	Waters, MA, USA
HPLC Pump, Waters 515	Waters, MA, USA
Mass Detector, Waters 3100	Waters, MA, USA
Photodiode Array Detector, Waters 2996	Waters, MA, USA
Prep Degasser, Waters	Waters, MA, USA
Sample Manager, Waters 2767	Waters, MA, USA
Equipment: Coloumns	
Atlantis® Prep dC18 OBD™ Prep 100Å, 10 µM, 10 x 250 mm	Waters, MA, USA
SunFire® Prep MS C18 5 µM, 10 × 250 mm column	Waters, MA, USA
Xterra® Prep MS C18 10 µM, 10 x 250 mm column	Waters, MA, USA
Solvents	Supplier
Acetonitrile (ACN)	Sigma-Aldrich, MO, USA
Dimethyl sulfoxide (DMSO)	VWR, PA, USA
Formic acid	Sigma-Aldrich, MO, USA
Methanol (MeOH)	Sigma-Aldrich, MO, USA
Milli-Q water	Merck KgaA, Germany
Hexane	Sigma-Aldrich, MO, USA

3.5.1 Preparation of Samples for Isolation

Marbank Sample

An aliquot of the aqueous extract (2 g) was weight and transferred to an Erlenmeyer flask. 100 ml 90% aqueous MeOH was added to the flask before the mixture was lightly shaken and left to rest for 10 minutes. The mixture was equally transferred to 4 falcon tubes. The tubes were placed in a centrifuge and spun at 4000 rpm for 5 minutes. The supernatants were combined in a round bottom flask and dried using a rotavapor. The pressure was slowly reduced from 250 mbar to 30 mbar. Depending on the amount of dried material, 5-15 ml of ACN was added to re-dissolve the sample. The finished solution was spun down and transferred to an HPLC injection vial.

WPB Sample

The extract was stored in a vial dissolved in DMSO at a concentration of 100mg/ml. To ensure complete dissolution, approximately 1,5 ml of DMSO and a few drops (approx. 50 µl) of hexane were added to the sample. The dissolved sample was then transferred to an Eppendorf tube. Subsequently, 300 µl of DMSO was transferred to the original container to recover any remaining material. This solution was then transferred to the Eppendorf tube. The tube was centrifuged at 3400 rpm for 2 minutes, after which the supernatant was transferred to an HPLC injection vial.

3.5.2 Scout run – Optimization of Isolation Conditions

Marbank Sample

The samples were first injected onto 3 columns (Atlantis, Sunfire and Xterra 18) with different packing materials in a process known as a scout run. Prior to injecting the sample, the columns were equilibrated with the starting mobile phase composition. The gradient can be seen in Table 12. The results from this run set the basis for column selection for further analysis.

Table 12: Mobile phase used in the scout run as part of the scout run and first round isolation on the Marbank sample. Mobile phase A: MilliQ-H_2O with 0.1% formic acid, mobile phase B: ACN with 0.1% formic acid. Flow rate: 6 mL/minute.

Time (min)	A (%)	B (%)
Initial	90	10
15.00	0	100
20.00	0	100
20.10	90	10
22.00	90	10

WPB Sample

As the WPB sample was present at a low amount, a scout run was not prioritised from this sample as this would have required almost all if the sample to be used in this step and this would not have left enough biomaterial to be able to isolate enough sample from further work.

3.5.3 Compound Isolation

The selected column the Atlantis column for every purification step of the compounds from both the Marbank and the WPB samples.

First-Round of Isolation

The injection volume of the Marbank samples was 400 µl at the time (˜1,2 ml injected per hour) and 100 µl at the time (˜300 µl injected per hour) of the WPB sample. The mobile phase composition and gradient were the same as listed in Table 12 for the Marbank samples and Table 13 for the WPB samples. The unprotonated monoisotopic masses of the target compounds were written in the sequence to trigger collection. During each injection, the individual compounds were collected in separate glass tubes. Each of the individual compounds from the different injections were pooled in round bottom flasks, dried, and weighed. This step yielded three semi-purified compounds.

Table 13: Mobile phase used as part of the first-round isolation on the WPB sample and the purification of the isolated compounds. Mobile phase A: MilliQ-H_2O with 0.1% formic acid, mobile phase B: ACN with 0.1% formic acid. Flow rate: 6 mL/minute.

Time (min)	A (%)	B (%)
Initial	90	10
11.00	24	76
11.10	0	100
13.00	0	100
13.10	90	10
15.00	90	10

Second Round of Isolation

The semi-purified compounds isolated were subjected to a second round of isolation. The purification began with a 25 µl injection to calibrate the collection, followed by 50 µl (˜150 µl injected per hour) the following injections. The mobile phase composition and gradient were the same as listed for the WPB samples in Table 13. The collection methodology was performed

the same way as in ‘First-round of isolation’ above. The samples were submitted to NMR for structure elucidation.

The setup used on the Marbank samples couldn’t be used in the second round as the impurities co-isolated with the target compounds were a result of compound overload of one of the impurities, causing it to blead out through the run. When the semi-purified sample was re-injected, the amount of the impurity was significantly reduced and thus was better retained by the column (in theory) allowing our compounds to be collected as pure samples, separated from the compound overloading the column during the first run, thus yielding a pure sample.

Third Round of Isolation

One of the compounds was subjected to a third round of isolation using the same setup as described above in ‘Second round of isolation’. When the semi-purified compound was re-injected again, the amount of the impurity was future reduced and allowing our compounds to be collected as a pure compound. The finished sample were submitted to NMR for structure elucidation.

Analysis of the Purity of the Isolated Compounds from the Marbank and WPB Samples

Normally, the purity of the isolated samples would be analysed using the UHPLC-HR-MS instrument at Mabio. This is normally an essential step, as a compound must be >90% pure prior to NMR analysis. However, due to a long-term down time of this instrument, the purity of the samples could not be analysed prior to NMR analysis.

3.6 Nuclear Magnetic Resonance

The nuclear magnetic resonance (NMR) analysis was performed by Johan Isaksson at the Intitule of Pharmacy, UiT. The resulting datasets were processed by my supervisor Kine Østnes Hansen at Marbio, using MestReNova software. The NMR experiments were acquired on a Bruker Avance III HD spectrometer operating at 599.90 MHz for protons, 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). Experiments were typically acquired using gradient selected adiabatic versions where applicable. All experiments were acquired using Top Spin 3.5 pl2 at 298 K. Total sample run time was ˜1.5 weeks.

3.7 Bioactivity Profiling

The bioactivity profiling was preformed after receiving the samples from NMR. The bioactivity profiling was conducted by testing against malignant and non-malignant cell lines and against Gram-positive and Gram-negative bacteria. In total, three cell lines (Table 14) and five bacterial strains (Table 15) were used for the bioactivity profiling. The equipment used in the bioactivity assays are listed in Table 16.

Table 14: The three cell-lines used in the bioactivity profiling of the purified compounds.

The viability assays cell lines used in bioactivity profiling of isolated compounds				
Cell-line:	Function:	Description:	Producer:	Product number:
A2058	Anti-cancer testing	Melanoma, Skin cancer cells	LGC Standards	ATCC-CRL – 11147
MCF-7	Anti-cancer testing	Breast adenocarcinoma, Breast cancer cells	LGC Standards	ATCC HTB-22
MRC5	Cytotoxic testing	Lung Fibroblast, Healthy human cells	LGC Standards	ATCC-CCL – 171

Table 15: The five bacterial strains used in the bioactivity profiling of the purified compounds.

Bacteria strains used in bioactivity profiling of isolated compounds				
Bacteria strain:	Function:	Gram type:	Producer:	Product number:
Enterococcus faecalis	Anti-bacterial testing	Gram positive	LGC Standards	ATCC 29212
Escherichia coli	Anti-bacterial testing	Gram negative	LGC Standards	ATCC 25922
Pseudomonas aeruginosa	Anti-bacterial testing	Gram positive	LGC Standards	ATCC 27853
Staphylococcus aureus	Anti-bacterial testing	Gram negative	LGC Standards	ATCC 25923
Streptococcus agalactiae	Anti-bacterial testing	Gram positive	LGC Standards	ATCC 12386

Table 16: The equipment used for the bioactivity profiling of the purified compounds.

Equipment: Cell Viability assay	Supplier
Biological Safety Cabinet, Herasafe™ KS Class II, Type A2	Thermo Fisher Scientific, MA, USA
Counting chamber, Bürker	Thermo Fisher Scientific, MA, USA
Incubator, Sanyo MCO-18AIC CO2	Panasonic Biomedical, Japan
Microtiter plate, Nunc™ Microwell™ 96-well	Thermo Fisher Scientific, MA, USA
Multimode detector, DTX 880	Beckman Coulter, CA, USA
Equipment: MIC assay	**Supplier**
Biological Safety Cabinet, Herasafe™ KS Class II, Type A2	Thermo Fisher Scientific, MA, USA
Blood agar plates	UNN, Norway
Incubator 37 °C, Panasonic Healthcare, MIR262	Panasonic Healthcare, MO, USA
Victor Multilabel Counter, PerkinElmer	Perkin Elmer, MA, USA
Solvents/Material	**Supplier**
Brain Heart Infusion broth (BHI)	Becton, Dickson and Company, NJ, USA
CellTiter 96® Aqueous One Solution Reagent	Promega, WI, USA
Dimethyl sulfoxide (DMSO)	VWR, PA, USA
Fetal bovine serum (FBS)	Merck KGaA, Germany
Gentamycin (10 mg/mL)	Merck KGaA, Germany
Gibco Dulbecco's Modified Eagle Medium (D-MEM)	Thermo Fisher Scientific, MA, USA
Mueller Hinton broth (MH)	Sigma-Aldrich, MO, USA
Minimum Essentials Medium Eagle (MEM)	Biochrom, Cambridge, UK
Non-essential amino acids	Biochrom, Cambridge, UK
Sodium bicarbonate (7.5 %)	Biochrom, Cambridge, UK
Sodium pyruvate (100 mM)	Biochrom, Cambridge, UK
Stable Glutamine (200 mM)	Merck KGaA, Germany
Trypsin	Thermo Fisher Scientific, MA, USA
Trypan blue	Sigma-Aldrich, MO, USA

3.7.1 Sample Preparation

The isolated compounds (dissolved in d_6-DMSO from the NMR experiments) were transferred to new tubes, freeze dried overnight and weight the following day. Based on the isolated compounds molecular weight and sample weight, 10 mM DMSO stock solutions were prepared for each compound. The standard equations, equation 2 (see below): number of moles of a substance (n) = mass in gram (m)/ molecular weight in g/mol (Mw) and equation 3 (see below) volume in L (V) = n/concentration in mol/L (C), were used to calculate the correct DMSO volume to add.

Example calculation for one of the compounds: Mw = 423,63 g/mol, sample weight = 1 mg:

Equation 2: $n = \frac{m}{Mw} = \frac{1\ mg}{423{,}63\ g/mol} = 0{,}00000235\ mol$

Equation 3: $V = \frac{n}{C} = \frac{0{,}00000235\ mol}{0{,}01\ mol/L} = 0{,}000236\ L = 236\ \mu L$

For this particular compound, 236 µL DMSO was added to the 1 mg of sample to produce a 10 mM (or 0,01 mol/L) stock solution.

For the initial rounds of the bioactivity profiling, the compounds were tested at concentrations of 100, 50, 25, 12,5, 6,25, 3,125 µM. The top concentration (100 µM) was prepared by adding 18 µL 10 mM stock solution to 162 µL MilliQ-water. These volumes were calculated using the 'dilution equation' as shown below in equation 4 for the calculation of the testing of all isolated compounds against two cancer cell lines. As the compound is diluted 1:10 in the assay wells, the concentration of the compound had to be 10x compared to the assay concentration. The prepared volume, 180 µL, was sufficient to produce the lower concentration point through 50:50 dilution steps while maintaining enough dilution to perform the assay (30 µL + 10 µL extra per cell line).

Equation 4: $C1 \times V1 = C2 \times C2 \rightarrow V1 = \frac{C2 \times V2}{C1} = \frac{1000\ \mu M \times 180\ \mu L}{10000\ \mu M}$ = 18 µL

C1 = the initial concentration of the solution before dilution
V1 = the initial volume of the solution before dilution
C2 = the final concentration of the solution after dilution
V2 = the final volume of the solution after dilution

It was transferred 18 µL of the 10 mM stock solution to be diluted to 180 µL to produce a 1000 µM solution. Thus, 180 µL – 18 µL = 162 µL solvent (MilliQ-water) was mixed with 18 µL stock solution. The 1000 µM concentration was the diluted 1:1 in Milliq-water (90 µL 1000 µL solution : 90 µL MilliQ-water) to produce the 500 µM solution (giving an assay concentration of 50 µM), and so on ending in final concentration step 31.25 µM (= 3,125 µM assay concentration). Similar setups were used for all calculations related to preparation of compounds for bioactivity testing.

3.7.2 Bioactivity Testing of the Purified Compounds

Cancer-Cell Viability Assay

The cytotoxicity of the isolated compounds was tested against the malignant cell lines A2058 and MCF-7 (Human adenocarcinoma cell line. Cell density 2000 cells/well, maintained and assayed in MEM Eagle) following the method described in section 3.1.1. All compounds were initially tested using a six-step two-fold dilution ranging from 100 – 3,125 µM in triplicates. One of the compounds was found to be active against A2058 and MCF-7. To get a more accurate understanding of the potency of the compound, this compound was retested against A2058 and MCF-7 using a 12-step two-fold dilution ranging from 50 µM – 40 nM. The active compound was additionally assayed against the non-malignant MRC-5 cell lines, using the assay setup described in section 3.3.1 at a two-fold dilution from 50 µM – 40 nM.

MIC Antibacterial Assay

The bacterial strains were taken out from long-time storage (stored at -80 ℃, preserved in 10% glycerol) and plated out on blood agar plates before being incubated overnight at 37 ℃. The day after were colonies of *S. aureus*, *E. coli*, and *P. aeruginosa* were added to one tube each with 8 mL of MH-broth, and colonies of *E. faecalis* and *S. agalactiae* were a were added to one tube each with 8 mL BHI-broth. The five tubes incubated overnight at 37 ℃. The following day, 50 µL of all the different concentrations of the compounds were added in duplicates to five separate stock plates. Following the compounds, each well was filled with 50 µL of bacterial suspension, with one strain per plate. The positive control comprised 50 µL of cell-medium and 50 µL of Milli-Q water, while the negative control comprised 50 µL of bacterial suspension and 50 µL of Milli-Q water. Additionally, a separate set of plates was prepared for an additional gentamicin dilution series control for all the strains. The plates were then taken to incubation for 24 hours, were the next day the absorbance was measured at 600 nm with a Victor Multilabel Counter.

4 Results and Discussion

The aim of the project was to isolate and characterize secondary metabolites from invertebrate extracts, with the intent of discovering a compound that had the potential of being developed into a pharmaceutical. In total, 16 yielded extracts of marine invertebrate biomass were analysed in the thesis, were 12 was provided by Marbank and four from WPB. Fractions of *Funiculina quadrangularis* (Marbank sample) and the extract of *Aplidium sp.* (WPB sample) showed promising activity during bioactivity testing, which led to in total five compounds being isolated. All isolated compounds were sent to NMR for structure elucidation, and later bioactivity profiled, were compound MBC-414 showed promising activity against cancer cells.

4.1 The Supply Issue

Problems surrounding low quantities of material in bioprospecting is commonly referred to as 'The supply issue'. As the bioactive compounds often are present in low concentrations, low sample quantities can be problematic as 0.5 – 1 mg is the lowest sample amount that can be used to elucidate the structure of a compound using NMR. Without a sufficient sample supply, getting enough sample to do all the desired analysis of a purified compound can be challenging. Especially if the structure of a compound cannot be elucidated, removing the option to produce the compound using chemical synthesis.

The extracts chosen from Marbank were deliberately based on not being processed in the bioprospecting pipeline at Marbio previously, that they were taxonomically classified down to the species name, and that there were enough of both aqueous and organic extract to ensure sufficient sample to conduct all the methods needed to complete this work. This latter point is an essential part of marine bioprospecting on marine invertebrates since they can't be cultivated in the same fashion as bacteria or alga. The luxury of having fresh, well characterized samples in sufficient amounts was however not the case for the WPB sample set. The WPB samples used as part of this thesis dates back to 1993-1995, making them three decades old: a very mature age for a sample that is to be used as input in a bioprospecting pipeline. Furthermore, they were present at low amounts, didn't have full the taxonomic evaluation and the production method was not conclusively described from the provider. The problem with no proper taxonomical evaluation (the evaluation could also be outdated) is that it prevents the possibility of re-collecting material in the future. This couldn't be solved by DNA isolation from the samples since they were extracted and dissolved in DMSO. This weren't made any easier by having no recipe for how the extracts were made, as well as a lack of understanding regarding

why all these extracts were organized into this particular sample set. This meant that the remaining material from the extracts was all we had to work with.

However, from the resulting bioactivity testing preformed during my Bachelor thesis showed that multiple of the WPB samples had potential to contain novel compounds, and it was a rare opportunity to analyse samples from Antarctic. Since the amount of material left was low, only the most potent samples were selected for further processing as part of this thesis. We chose to prioritize the samples were both yields of the extracts (DM and FA) had shown bioactivity. However, this could turn out that it was the wrong move, since we maybe should have based it on amount of material, level of activity and later the complexity of the sample. Either way, the samples chosen were extract 41 and 86 (out of 35 different bioactive extracts as determined through the work conducted as part of my bachelor's thesis) (Figure 6). To overcome the supply issue, we had to be as efficient with the material as possible, which led to no flash fractionation, no cytotoxicity testing on non-malignant cells and no retesting of the results from the Bachelor thesis.

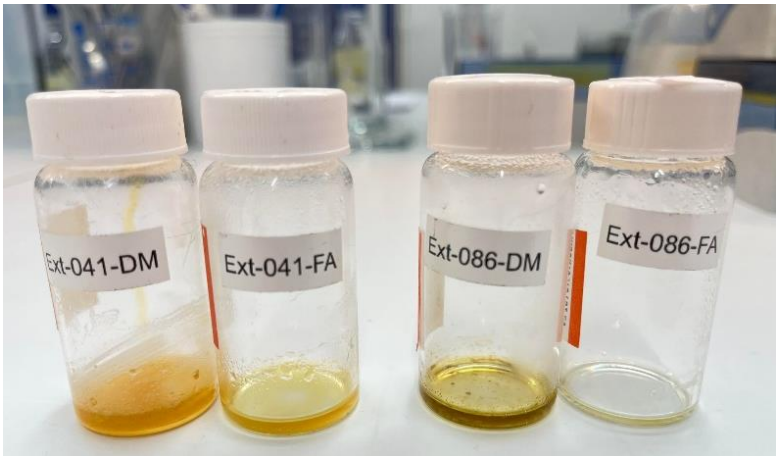


Figure 6: The starting material of Extract 41 and 86 DM/FA.

4.2 The Origin of the Bioactivity

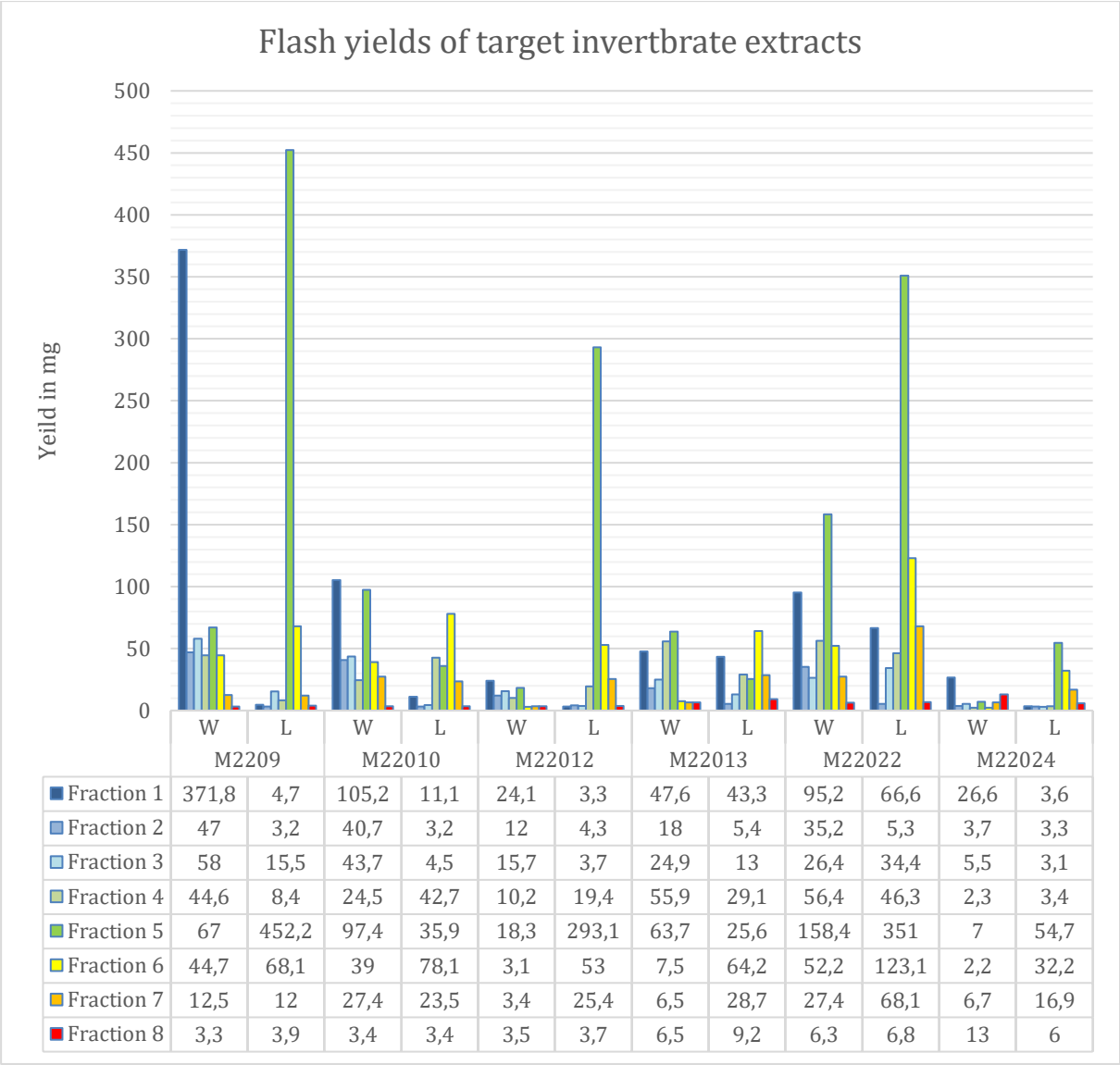
It's also important to note that even if a bioactive compound is isolated from an invertebrate extract, it doesn't necessarily mean that the compound originates from the invertebrate itself. Many microorganisms are associated with macroorganisms in these types of extracts, especially in the case of invertebrates. More often than not, these macroorganisms are closely associated with symbiotic microorganisms, which can sometimes be found in relatively large amounts (up to 50% of the collected biomass for e.g. sponges). Indeed, the bioactivity could originate from these microorganisms instead of the invertebrate itself. This also explains why so many

bioactive compounds have been isolated from invertebrates like sponges and tunicates, where the microorganisms with the bioactivity either have been pray or using the invertebrate as its host. By examining the structures of the compounds, it can become evident that many are made by enzymatic machineries not available in invertebrates. The true producers can be confirmed through DNA sequencing of the microorganisms isolated in/on invertebrate biomass (22).

4.3 Preparation of Marbank Extracts for Further Analysis

The prefractionation phase is an important step in the bioprospecting pipeline. It provides fractions, which is better fitted to detecting the bioactivity of compounds present in lower concentrations. The prefractionation also facilitates the dereplication process by enabling comparisons between active and neighbouring inactive fractions, thereby identifying compounds for isolation. In total there were 12 Marbank invertebrate extracts (one organic and aqueous extract per invertebrate). Each of these extracts were fractionated into eight fractions, making it in total 96 fractions to analyse during the bioactivity testing. The yields of the fractions varied, as shown in Figure 7**.** As established in 3.2.5, to facilitate bioactivity testing, 250 µg of each sample was transferred to a deep well plate, freeze dried, and standardized as in Fingure 5. However, due to variable yield of the fractions, spanning from 2,2 to 452,2 mg in the dilutions had to be adjusted according to Table 7.

The flash chromatography, using a reversed phase column, separate sample components based on the lipophilicity/hydrophilicity of the sample components. This will elute the hydrophilic components eluting first, followed by compounds with medium lipophilicity and last the highly lipophilic compounds. In general, do the organic extract yield most of its mass in fraction five due to the high concentration of non-polar components, the aqueous extract yields a lot in the first fraction due to high amounts of salts and highly polar or positively charged molecules (which experience no retention in HP-20 columns), and the last fraction usually contains the least amounts because of the acetone flow. However, there is no definitive answer on which fractions contain the most material. This is because the biological content of the animal extracts can vary significantly. The biological content does first get separated in the liquid-liquid partition, leading to the aqueous and organic containing vastly different compound, where the two yields again is chromatographically separated into eight fractions. For example, if an animal contains a lot of fatty acids, could we theorise that a high general outcome of yield material would be in the later fractions of the organic extract, based on the polarity of fatty acids.



	W	L	W	L	W	L	W	L	W	L	W	L
	M2209		M22010		M22012		M22013		M22022		M22024	
Fraction 1	371,8	4,7	105,2	11,1	24,1	3,3	47,6	43,3	95,2	66,6	26,6	3,6
Fraction 2	47	3,2	40,7	3,2	12	4,3	18	5,4	35,2	5,3	3,7	3,3
Fraction 3	58	15,5	43,7	4,5	15,7	3,7	24,9	13	26,4	34,4	5,5	3,1
Fraction 4	44,6	8,4	24,5	42,7	10,2	19,4	55,9	29,1	56,4	46,3	2,3	3,4
Fraction 5	67	452,2	97,4	35,9	18,3	293,1	63,7	25,6	158,4	351	7	54,7
Fraction 6	44,7	68,1	39	78,1	3,1	53	7,5	64,2	52,2	123,1	2,2	32,2
Fraction 7	12,5	12	27,4	23,5	3,4	25,4	6,5	28,7	27,4	68,1	6,7	16,9
Fraction 8	3,3	3,9	3,4	3,4	3,5	3,7	6,5	9,2	6,3	6,8	13	6

Figure 7: The yields of the flash fractions from the aqueous (W) and the organic (L) extracts. The amount of crude extract used were ˜5g of the aqueous and ˜1,5g for the organic.

On the topic of polarity, in the context of drug discovery the polarity of the compounds does play a key role in pharmacokinetics. The principles of ADME clearly highlights the preference of lipophilic drugs, but the main point is that the compound must be neither not too hydrophilic nor too lipophilic. This is due to highly lipophilic or hydrophilic compounds often will have an impaired ability to penetrate membranes, which is a necessity for perorally administered drugs. Appling this principle to the fractions, the compounds eluting in fractions 4-6 are most likely to have desirable drug-like properties.

The crude extracts of the samples contain a myriad of different components (both primary and secondary metabolites), like sugars and fatty acids. The varying concentrations of these compounds can affect the sample preparation and fractionation processes, like making the samples boil easily during the evaporation in the rotavapor and the polywap. This was the case for sample M22022-0-W, were we had a lot of difficulties during evaporation, probably because the sample contained a lot of sugars. This resulted in the sample using 3 hours (normally 20-40 minutes) in the rotavapor, and for the fraction 1 and 2 used over 3 days in the polywap, plus 1 additional day in the freeze dryer (normally 1-2 days in the polywap).

4.4 Bioactivity Discovery in Fractionated Invertebrate Extracts

The most central step of the marine bioprospecting is the discovery of bioactivity, which is done in the bioactivity screening. The bioactivity screening was performed in two rounds, first a single concentration screen of all fractions, followed up by dose-response screen of all the bioactive fractions. The cell viability assays used in the bioactivity testing are the cell-line A2058 (skin-cancer cells) and MRC5 (non-malignant fibroblasts, general cytotoxicity test).

4.4.1 Bioactive Testing of the Marbank Fractions

Primary Screening Against A2058 and MRC-5

The first bioactivity screening (also termed ‘primary screening’) was conducted by testing all the Marbio fractions (96 in total) against the malignant A2058 and the non-malignant MRC5 (toxicity testing/counter screen) cell lines using a single concentration of 100 µg/ml (Table 17). The screening revealed 10 fractions with activity against A2058 (out of which five were classified as questionable (Q)). Two of the fractions were also active against MRC-5, while the remaining 8 were exclusively A or Q against A2058. The remaining 86 fractions were inactive (I) against A2058 or MRC-5 (data not shown). This result allowed future efforts to be focused on only 10/96 initial fractions, reducing the starting sample set down by ˜90%. This clearly highlights the importance of the bioactivity screening step, as a focused investigation now can be conducted towards 10% of the initial samples rather that the sample set as a whole. The simultaneous activity against A2058 and lack of activity against MRC-5 is a positive sign, as this indicates that there might be a compound in the fraction that potentially can inhibit cancer cell survival, while having no effect on non-malignant cells. While these are very early results, this is the type of activity that is ideal for a cancer drug, as unselective cytotoxicity is what causes the devastating side effects of cytostatic agents like cytarabine and cisplatin. The classification of compounds as A, Q or I is a system developed as Marbio, and is meant to aid

in downstream processing and selection of sample to prioritise further work on. A fraction classified as A (0 -50% cell survival) or Q (51 – 60% cell survival) will always be further analysed, with the knowledge that the activity in the Q fractions is weak. If a sample is to be nominated for isolation from this sample, the apparent concentration of it needs to be on the low side.

Table 17: The active and questionable hits from the initial round of bioactivity testing. The table shows the cell survival (%) for A2058 and MRC-5 cell-lines for all the fractions that were classified as either active (A) or questionable Q).

The results from the single concentration bioactivity testing				
Sample ID:	A2058 Survival Rate %:	A2058 Results:	MRC5 Survival Rate %:	MRC5 Results:
M22009-0-W03-07	56	Q	91	I
M22012-0-W03-05	58	Q	92	I
M22013-0-W03-05	6	A	46	A
M22013-0-W03-07	27	A	61	I
M22009-0-L03-04	49	A	121	I
M22010-0-L03-03	56	Q	116	I
M22010-0-L03-04	50	Q	113	I
M22012-0-L03-03	52	Q	125	I
M22024-0-L03-04	4	A	62	I
M22024-0-L03-05	49	A	28	A

Secondary Screening Against A2058

To further evaluate this activity of the fractions listed in Table 17, all were re-tested at four different concentrations against A2058 using in three biological replicates (three technical replicates each time). The results from this dose-response screening are listed in Table 18. As the fractions are included in this assay setup based on activity from the primary screening, this step is referred to as secondary screening.

Table 18: The secondary (dose-response) screening result for all the fractions with bioactivity. The table shows the survival rates (%) of A2058 from the individual plates (plate 1, 2 and 3) and the average across the three plates at all exposure concentrations. The results are classified as active (A), questionable (Q) and inactive (I).

The results from the dose-response screening of the bioactive fractions							
Sample ID:	Conc. (µg/ml)	A2058 Survival Rate %			Std.dev.	Average survival (%)	Result:
		Plate 1	Plate 2	Plate 3			
M22009-0-W03-07	100	67	74	68	4,2	70	I
	50	99	94	112	9,0	101	I
	25	110	104	109	3,3	108	I
	10	100	111	109	5,8	107	I
M22012-0-W03-05	100	20	18	21	1,5	20	A
	50	97	97	92	2,5	95	I
	25	99	103	103	2,2	102	I
	10	115	118	140	14,0	124	I
M22013-0-W03-05	100	5	8	6	1,6	6	A
	50	26	28	29	1,6	28	A
	25	54	48	46	3,8	49	A
	10	93	102	102	5,0	99	I
M22013-0-W03-07	100	15	17	16	1,0	16	A
	50	63	63	53	5,8	60	Q
	25	96	92	100	3,7	96	I
	10	102	92	104	6,6	100	I
M22009-0-L03-04	100	27	39	49	11,0	38	A
	50	51	65	61	7,2	59	Q
	25	75	92	88	9,1	85	I
	10	84	101	106	11,7	97	I
M22010-0-L03-03	100	81	98	92	8,5	90	I
	50	83	110	108	15,0	100	I
	25	90	113	114	13,7	105	I
	10	94	120	114	13,5	109	I
M22010-0-L03-04	100	85	96	92	6,0	91	I
	50	88	103	100	7,9	97	I
	25	89	94	94	3,0	93	I
	10	84	88	95	5,5	89	I
M22012-0-L03-03	100	8	10	9	0,9	9	A
	50	89	105	98	8,3	97	I
	25	99	114	108	7,8	107	I
	10	105	112	111	3,6	109	I
M22024-0-L03-04	100	2	5	6	2,0	4	A
	50	59	56	64	4,1	60	Q
	25	96	97	93	2,4	95	I
	10	94	107	102	6,9	101	I
M22024-0-L03-05	100	78	61	72	8,8	70	I
	50	100	112	103	5,9	105	I
	25	91	102	88	7,3	93	I
	10	100	113	112	7,2	108	I

The results from the dose-response screening had some variations compared with the single concentration screening. First of all, the fractions from M22009-0-W03-07, M22010-0-L03-03, M22010-0-L03-04 and M22024-0-L03-05 did not show any activity, which they originally did in the primary screening, which eliminates them from further analysis. The reason why samples lose activity from one screening round to the next are unclear. However, several reasons can be suspected. The secondary screening is conducted on the same samples as the primary screening (the same fraction plate), the samples were stored in a freezer diluted in water for 3 weeks between the assay runs. It is possible that the active component(s) were unstable in waters and were broken down when entering the secondary screening. Another possibility is that the active components in the primary screening were of lipid nature. When stored in water, these lipids may aggregate, and thus not be evenly distributed throughout the sample volume. Even if the sample apparently is screened at the same concentration in both rounds (100 µg/mL), the actual concentration in assay plate can be different. Both because the lipid particles might not be transferred from the fraction plate to the assay plate, but also because they may not interfere with their cellular targets in the assay well when they are chemically bound to each other. Both these alternatives might seem like a disadvantage to the bioprospecting pipeline, as bioactive components are lost along the way. However, as the overall goal of the work conducted at Marbio is to discover compounds that can be developed into drugs, highly unstable or lipophilic compounds are not the most desired candidates.

The rest showed varying amount of activity, were none of the extracts showed activity below 50 µg/ml, except fraction M22013-0-W03-05. This highlights M22013-0-W01 as a particularly interesting sample going into the dereplication step. From the secondary screening of the Marbank extract sample set, six fractions were selected for dereplication:

- M22009-0-L03-04
- M22013-0-W03-05
- M22013-0-W03-07
- M22012-0-W03-05
- M22012-0-L03-03
- M22024-0-L03-04

Compared to the starting point of 96 sample, this is a manageable amount to dereplicate and further analyse as part of this work.

4.4.2 Bioactivity Screening of the White Point Biomarine Extracts

The results regarding the WPB samples are taken from my Bachelor thesis. As a short context: 244 extracts were received from WPB. These were pairs of extracts (DM and FA) from 122 marine invertebrates. All were screened for activity against A2058, out of which 35 extracts showed activity. Out of these, four were selected for further analysis. The reasoning for these samples being elected as valuable enough to be analysed further (out of all the 35 extract that showed activity) are the activity shown in Table 19 and that both extracts from one samples (DM and FA) showed activity. The extract showing activity in the primary screening against A2058, were nominated to the secondary (dose-response) screening. These assays were performed in the spring of 2022. The samples were only tested once per concentration (in two technical replicates) and the general cytotoxicity of the samples against MRC-5 were not tested.

Table 19: The summery of the results of extract 41 and 86 from WPB. The first run was a single concentration assay, and later were tested in a dose-response assay. The table shows the four different concentrations the samples were tested on, the survival rate and the result.

The bioactivity shown from chosen White point Biomarine samples			
Samples	Concentration (µg/mL)	A2058 Survival Rate %	Results:
Ext. 041-DM	100	55	Q
	75	51	Q
	50	49	A
	25	65	I
Ext. 041-FA	100	59	Q
	75	1	A
	50	4	A
	25	90	I
Ext. 086-DM	100	5	A
	75	6	A
	50	7	A
	25	16	A
Ext. 086-FA	100	16	A
	75	12	A
	50	21	A
	25	60	I

The following samples were nominated:

- Ext. 41-DM
- Ext. 41-FA
- Ext. 86-DM
- Ext. 86-FA

4.5 Dereplication

These samples selected in paragraphs 4.4.1 and 4.4.2 were analysed using the UHPLC-HR-MS system. The output of this analysis was one chromatogram per injected sample. Furthermore, useful information, like elemental composition and fragmentation patterns, for the individual compounds can be extracted. The analytical and bioactivity data can be used as input in database searches in an attempt to identify the sample components. The active Marbank fractions were analysed together with their neighbouring inactive fractions and the active WPB extracts were analysed without the possibility of comparing active and inactive fractions.

4.5.1 Selecting Compounds

An important part of the marine bioprospecting pipeline is to prioritize samples for further work and focus on the samples with the most potential. The dereplication phase is both a super useful tool which could help both rule out samples and make educated guesses on bioactive compounds. It is also a very complex analysation tool. A set of criteria was therefore defined, to help efficiently select extracts/fractions to work further with. While it is always helpful to conduct work efficiently, this is especially important during a project with a defined end point, like a master thesis. Therefore, when searching through the different samples where these the main evaluation criteria:

- Does the bioactive sample contain PAINS or known compounds with known bioactivity? If yes: sample is most likely discontinued.
- Does the sample contain any obvious compounds of interest? If yes: Work with this sample continues.
- Is the sample too complex to make successful compound isolation likely? If yes: The sample is most likely discontinued.

These rules are not absolute. E.g. a sample might contain compounds that are known to produce unspecific cytotoxicity against cells, but also compounds assumed to be novel. In this case, the complexity and number of more promising compounds would determine if this sample would be a candidate for isolation or not.

The process of identifying bioactive compounds involves pinpointing the most probable retention time window where the compound is expected to elute. The content of the active Marbank fractions were compared to the content of the neighbouring inactive fractions. Since the fractions are chromatographically separated, the bioactive compound would most likely

elute between the main group of peaks amidst its neighbouring fractions. If a compound in this time window is only present in the active fraction, or present in higher amounts than in the inactive fractions, this compound would be considered a candidate for isolation.

Another point to consider is that we mainly focused on molecules eluting before Rt = ˜8 min. Compounds eluting after Rt = ˜8 min are often too lipophilic to be viable as drugs. Based on experience from Marbio, molecules after this retention time have mainly been lipids. This is again a trade-off to decomplex the dereplication step, as there indeed might be lipophilic compounds with intriguing properties suitable for e.g. topical formulations, but this is not the main focus for Marbio. There are also examples of compounds highly lipophilic cyclic peptides (e.g., cyclosporine used as a highly efficient immunosuppressive drug (69)), but for this analysis have the focus area been on compounds below Rt = ˜8 min. Again, this is not an absolute rule. If a novel compound elutes after 8 min, it might be included in the isolation if e.g. the complexity of the sample is low.

4.5.2 Sample Ext. 86-DM/FA

Analysis of these samples resulted in a few very weak signals, most likely because of too little material injected into the UHPLC (chromatogram of 86-FA in Figure 8). In addition, these extracts were provided at a low amount (86-DM: 43,7 mg and 86-FA: 26,8 mg), making it unlikely that a compound could be isolated from these samples in amount sufficient for NMR analysis. No compound(s) of interest was nominated in these extracts, and the extracts were thus not prioritized for further work.

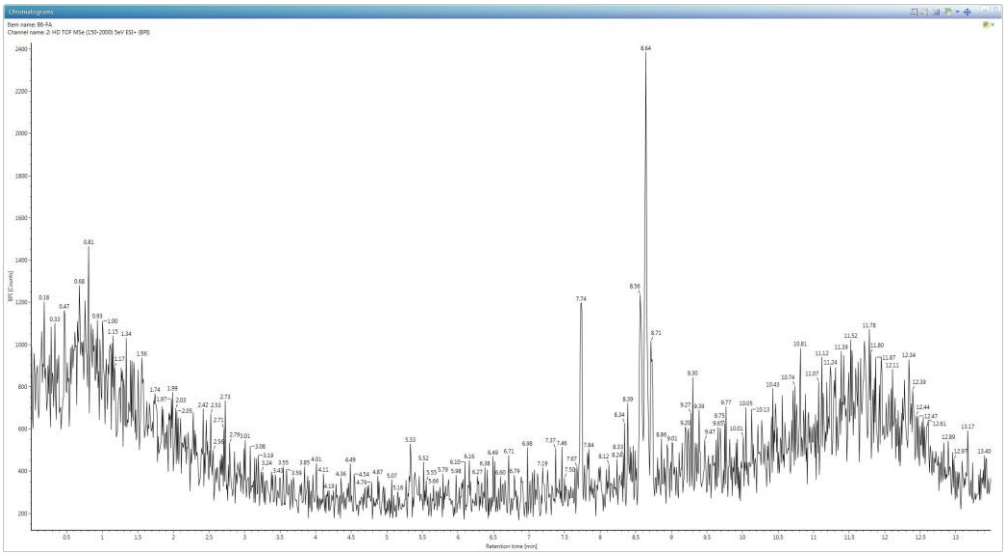


Figure 8: The mass chromatogram of 86-FA. The sample added to MS were of a too low concentration to provide a proper chromatography. The total amount left of the sample did not support a second round of MS. The sample were then dismissed. The chromatogram of 86-DM looked similar.

4.5.3 Marbank Fraction M22012-0-W03-05

Sample M22012-0-W03-05 had activity in the dose-response assays. When comparing the chromatogram of the active fraction to its inactive neighbours, a lot of overlapping (the same compound found in two or all three fractions) was observed. One compound did however stand out in the active fraction (Rt = 8,75 min in Figure 9, left). This peak had a *m/z* of 466,38 and a calculated elemental composition of $C_{24}H_{52}NO_5P$ (Figure 9, right). When this compound was searched for in the ChemSpider database, this compound was identified as a phospholipid. As mentioned earlier, phospholipids are known to have unselective toxicity against cancer cells, which means the fraction was not prioritised for further work.

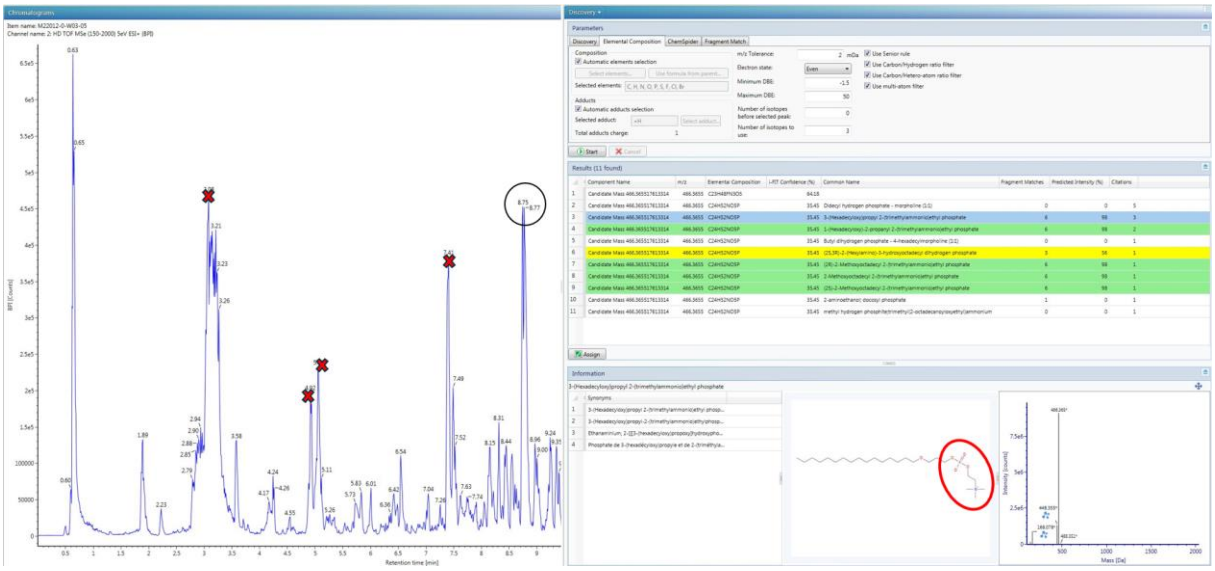


Figure 9: A segment of the UHPLC chromatogram of fraction M22012-0-W03-05 (left) and the searches in the ChemSpider databases (right). On the left have crosses been used to illustrate the overlapping peaks, while the most dominant peak that's not overlapping is marked by the circle, located at Rt = 8,75 min. The compound responsible for this peak was used as input for a database search. The result from this search (right) in the ChemSpider database, indicated that the compound was a phospholipid.

4.5.4 Marbank Fraction M22013-0-W03-05

Sample M22013-0-W03-05 were one of the more active samples with a low survival rate at low concentrations in Table 18, but it was also one of the samples that showed activity against the non-malignant MRC5 cell line. When comparing the active fraction to the neighbouring fraction, two peak clusters caught our attention (Figure 10).

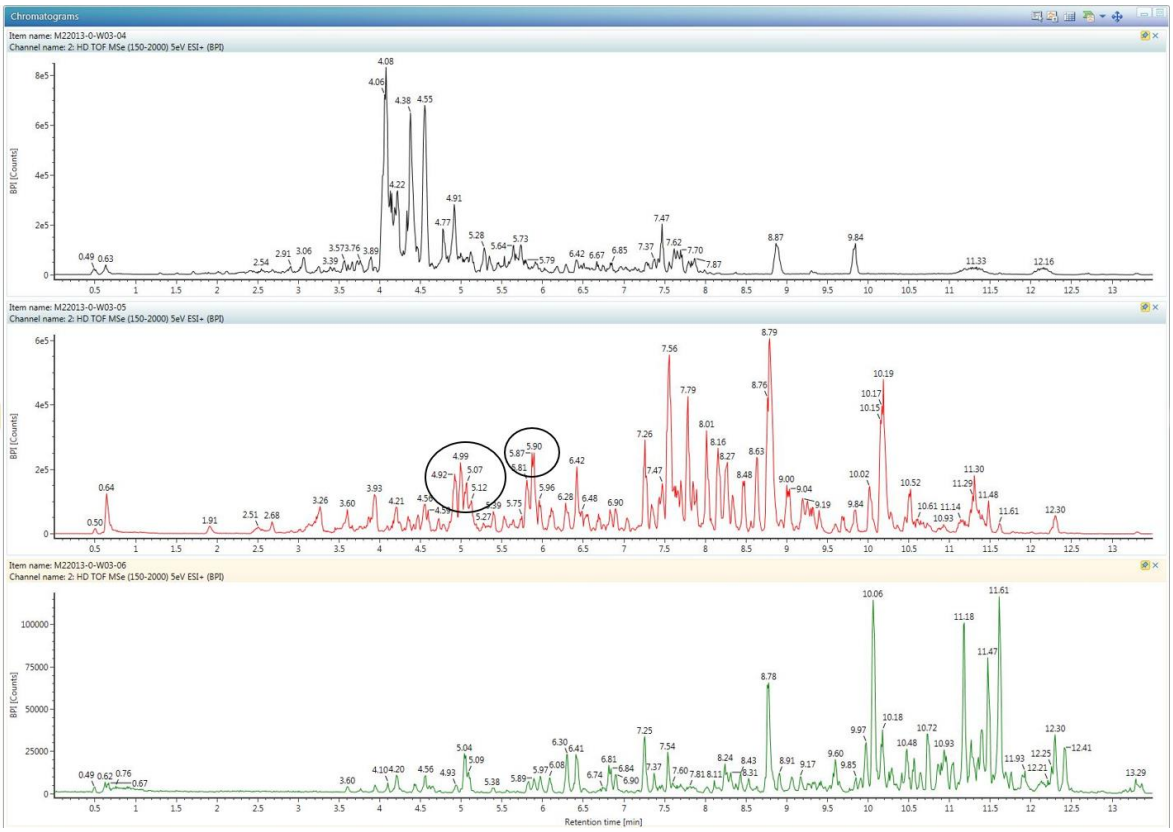


Figure 10: MS chromatography of fraction M22013-0-W03-05, with neighbour fractions above and below. The two circles illustrate the most promising peak clusters, which eluted at Rt = 4,92-5,06 and 5,81-5,90 min, respectively.

The first peak cluster appeared at Rt = 4,92-5,07 and the second at Rt = 5,81-5,90 min. The first peak cluster consisted of three major peaks eluting at Rt = 4,92, 4,99 and 5,06 min (Figure 11). Two of the peaks, Rt = 4.92 and 5.07 min, consisted of what appeared to be isomers of the same compound, both with an *m/z* value of 424.34. This *m/z* is also evident in smaller neighbouring peaks. The other peak in the cluster, appearing at Rt = 4,99 min, had a *m/z* of 374,26. Neither compound matched any known compounds in the ChemSpider database, and based on both compounds' potential novel chemistry, both were marked as compounds of interest.

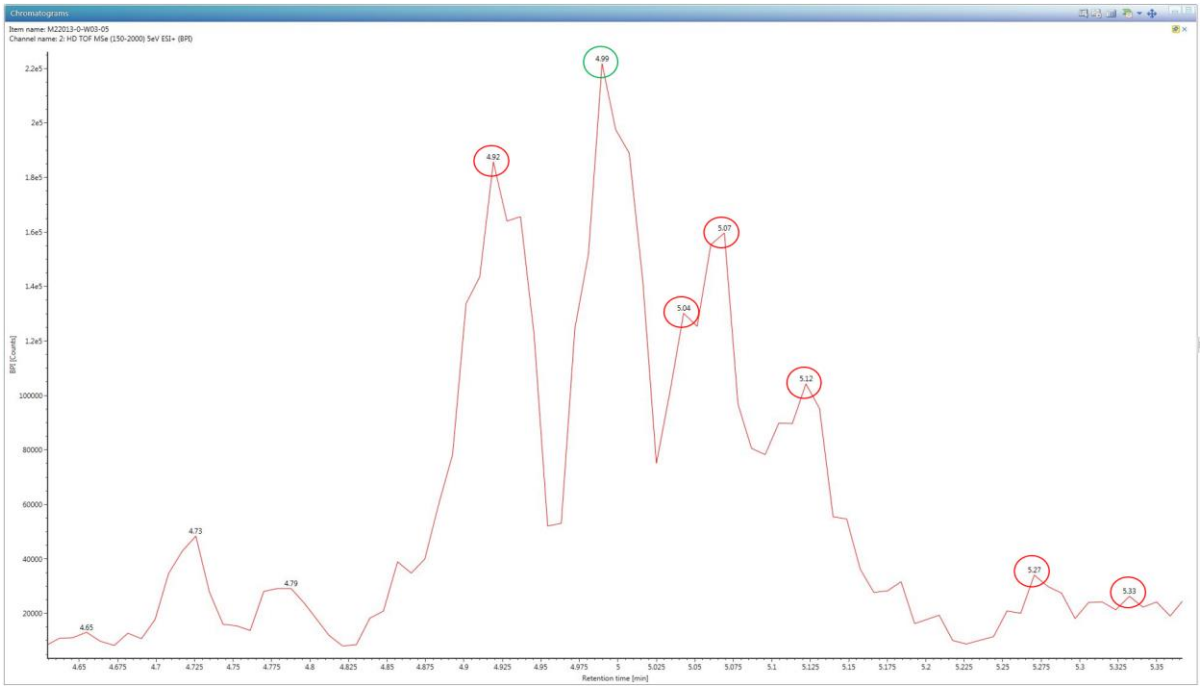


Figure 11: The peak structure of interest between Rt = 4,92-5,07 min in M22013-0-W03-05. The structure consists of three major peaks located at Rt = 4,92, 4,99 and 5,06 min. The red markers show the peaks where the molecule with 424.34 *m/z* is the most predominant, and the green marker shows where the molecule with 374.26 *m/z* is the most predominant.

The second peak cluster consisted of three major peaks eluting at Rt = 5,81, 5,87 and 5,90 min. The three peaks are all closely connected, highlighting a possible connection. Upon further inspection does the peaks appear to consist of the same compound with *m/z* at 452,37 (Figure 12). Again, showing the possibility for one compound being dispersed during the separation process in the column, making one dominant peak appear as many smaller once. Upon further analysis, no molecule fully matches the compound in the ChemSpider database. Based on the potential peak size, no match in the database, appearing in the desired retention time frame (below Rt = ˜8 min), the molecule marked as compounds of interest.

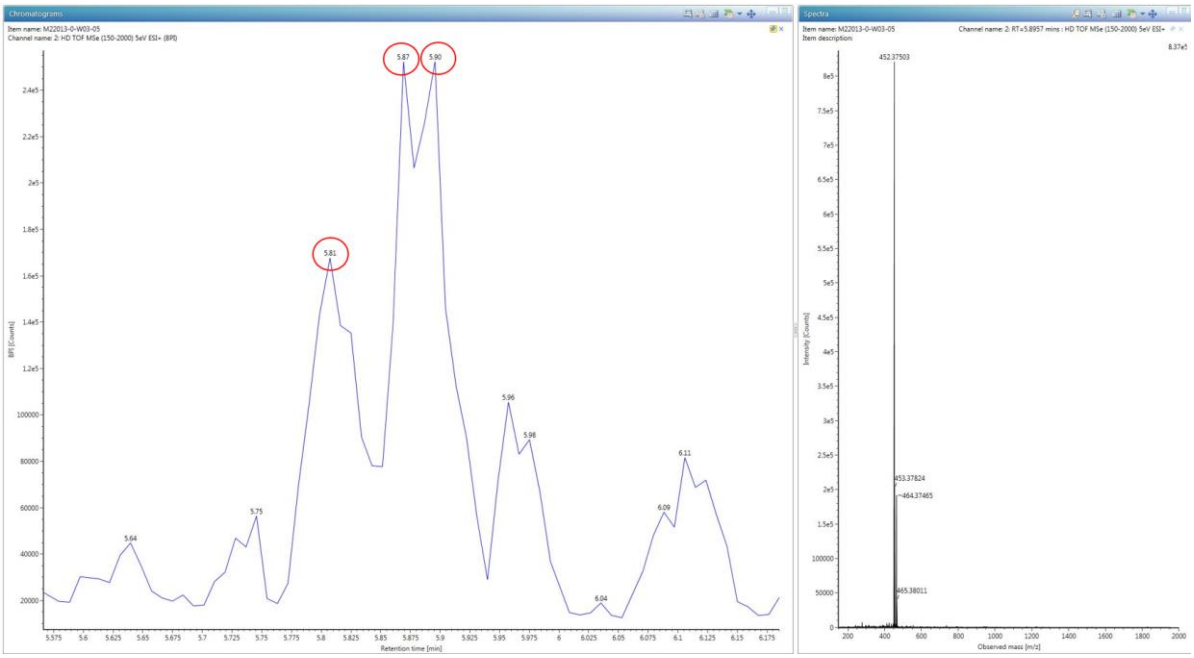


Figure 12: The peak structure of interest between 5,81-5,90 Rt in M22013-0-W03-05. The chromatogram shows the peaks on interest with the following spectra of peak 5,90. The red rings marks the peaks of interest with the m/z 452,37.

Thus, in total where tree compounds of interest were listed for isolation from fractions M22013-0-W03-05.

4.5.5 Sample Ext. 41-DM/FA

The WPB samples Ext. 41 DM/FA were analysed in a different way compared with the Marbank samples since we didn't have any neighbour fractions to compare them with. The only comparison they had were the two fractions (DM/FA). That meant that there was no narrowing down the number of compounds through elimination of overlapping peaks, making every peak relevant. No compound of interest was nominated from Ext. 41-FA due to the complexity of the sample. The DM fraction on the other hand were less complex and head some clear candidates. Going through the sample was it apparent that one peak and one peak cluster of were dominant in the sample (RT = 2,17 and 1,83 min in Figure 13, respectively).

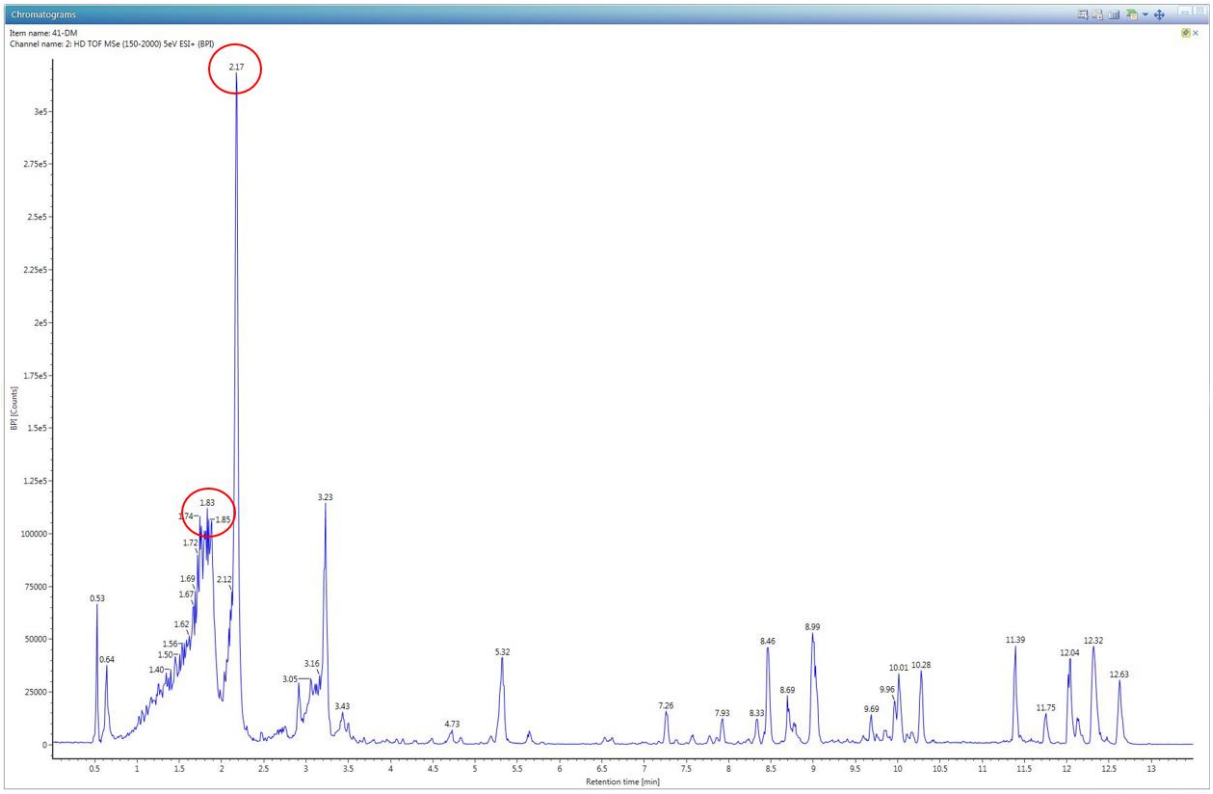


Figure 13: UHPLC-MS chromatography of sample Ext. 41-DM. The sample had a one major peak eluting at Rt = 2,17 min and a cluster of multiple peaks around Rt = 1,83 min. Both the peak and the cluster had a predominant molecule with an *m/z* value of 395,18. The two red circles illustrate the peaks of interest.

Both of the peaks contained the same molecule which had *m/z* = 395,18. Based on the area-under-curve for the of the cluster and the high, sharp peak combined compared to the remaining peaks, it appeared that *m/z* = 395,18 made up the majority of the Ext. 41-DM extract. Searches in the ChemSpider database rewarded no hits for this compound, making this a suspected novel compound. The compound was not discovered in Ext. 41-FA either. This made it a compound of interest and was selected for isolation.

4.5.6 The Remaining Marbank Fractions

No compounds were nominated from the remaining four extracts: M22009-0-L03-04, M22012-0-L03-03/4 and M22013-0-W03-07. The reasoning behind this were the had no clear compounds of interest, due to either complexity and the inability to nominate one or a few compounds to isolate from these samples. Although, fraction M22013-0-W03-07 had no clear candidates, since the fraction is a part of the same extract as one of the fractions M22013-0-W03-05, is there still a chance to discover the compound during the scout run before the compound isolation.

4.5.7 Compounds of Interest from the Dereplication

Four compounds from the dereplication were selected for isolation, coming from two samples: the Marbio fraction M22013-0-W03-05 and the WPB sample Ext. 41-DM. The compounds chosen are educated guesses of the which molecules could be responsible the bioactivity discovered during the bioactivity testing (Table 20).

Table 20: The overview of all the compounds targeted for isolation. The table contain the relevant samples, the peaks retention time (Rt), the compounds *m/z* values and calculated elemental composition.

Compounds of interest listed form the dereplication			
Sample ID:	Peak (Rt), min	Mass over charge (*m/z*) value	Calculated elemental Composition
M22013-0-W03-05	4,92 and 5,07	424.34	$C_{25}H_{45}NO_4$
M22013-0-W03-05	4,99	374,26	$C_{23}H_{35}NO_3$
M22013-0-W03-05	5,87 and 5,90	452,37	$C_{27}H_{49}NO_4$
Ext. 041-DM	1,85 and 2,17	395,18	$C_{19}H_{26}N_2O_7$

4.6 Compound Isolation

To isolate the different compounds of interest the original, non-fractionated extract M22013-0-W01 and all the remaining material of Ext. 41-DM were used.

4.6.1 Scout Run and Locating Target Compounds

To isolate the compounds of interest, a suitable preparative HPLC column needs to be selected. The selection process is conducted by performing a series of scout runs. The scout runs entails injecting the extract on columns with different packing materials to examine which column provides the most favourable chromatography for the isolation of the compounds selected in the dereplicate step. As the Ext. 41-DM was available at a low amount, scout runs were not prioritised for this sample. After completing scout runs for the M22013-0-W01 extract on the Atlantis, Sunfire and Xterra 18 columns, the column of Atlantis was selected (Figure 14). As the compound of interest in the Ext. 41-DM sample eluted early in the UHPLC gradient, it is indicative of it being a more hydrophilic compound. In this case, it was hypostasized that the column that in theory is best suited for separating hydrophilic compounds (the Atlantis column) would be the best of the three. This column was therefore also selected for the isolation of the compound from Ext. 41-DM.

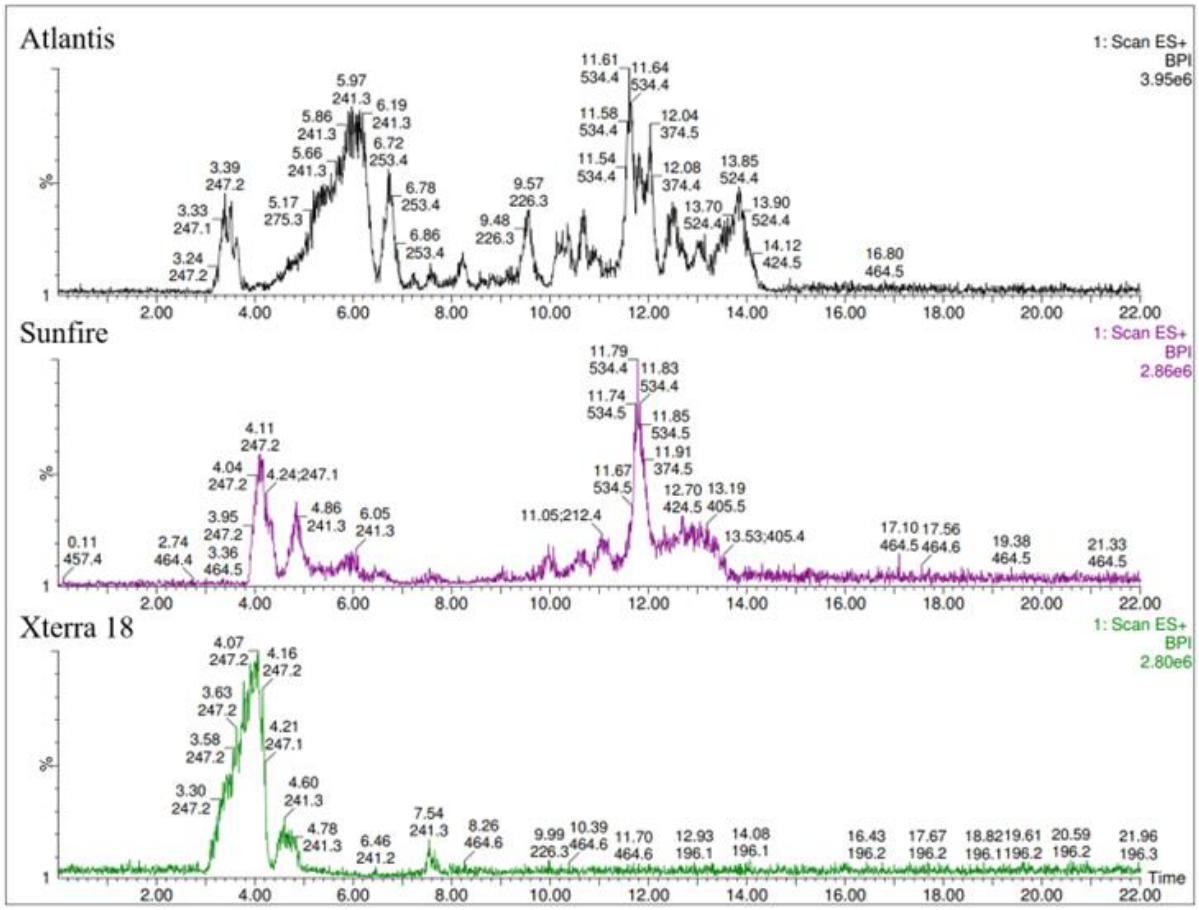


Figure 14: Base peak intensity (BPI) chromatogram of the scout runs for the M22013-0-W01 extracts. The sample was injected onto three columns (Atlantis, Sunfire and Xterra 18)

In the scout run of M22013-0-W01, compound 424 *m/z* and 374 *m/z* from the dereplication were rediscovered using all columns, but not 452 *m/z*, which therefore could not be isolated. While 'losing' compounds from the UHPLC to the HPLC analysis is uncommon, it has happened before and might be cause by several factors, including being present at too low concentrations to be detected by the less sensitive MS used as part of the mass-guided prep-HPLC runs. Compound 424 *m/z* was visible as two peaks, which likely means the compound was present as two isomers. In addition to the two compounds of interest, a separate compound with a *m/z* of 241 was isolated due to its evident presence. Adding compounds that easily can be isolated during an isolation run is common practice, as isolating additional compounds requires little extra efforts, but might provide high rewards if it turns out to be active in its pure form in later bioactivity tests. The compound selected were noted down and given names listed in Table 21.

Table 21: The overview of the compounds isolated from M22013-0-W01.

Compounds isolated from M22013-0-W01			
Compounds isolated (*m/z*):	Elemental composition	New compound name:	Reasoning for isolation:
424,4 (v.1)	$C_{25}H_{45}NO_4$	MBC-410	Compound of interest
424,5 (v.2)	$C_{25}H_{45}NO_4$	MBC-410 (v.2)	Possible isomer of compound of interest
374,4	$C_{23}H_{35}NO_4$	MBC-411	Compound of interest
241,2	To be determined	MBC-412	Easy accessed compound

During the first round of isolating compounds from Ext. 41-DM, were compound 395 was the main compound of interest, an additional compound was clearly visible, with an *m/z* value of 431.2. As this stood out as an easy target for isolation, the compound was added to the list of compounds that were to be isolated from Ext. 41-DM (Figure 15 and Table 22).

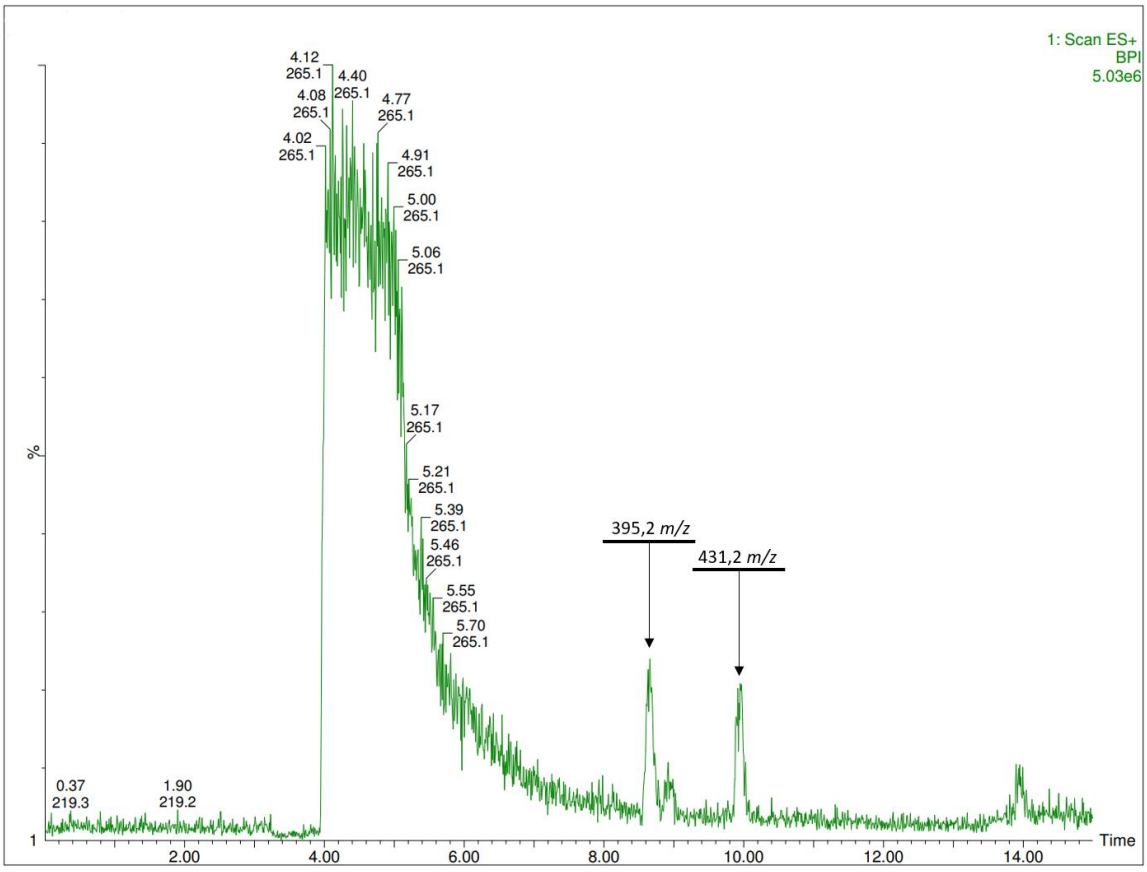


Figure 15: BPI chromatogram of the first-round isolation of the compounds of interest from Ext. 41-DM. *m/z* = 395,2 was the original target compound, appearing after ca. 8,5 min. Based on its clear visibility and seemingly unproblematic isolation, *m/z* = 431.2 appearing after ca. 10 min was also isolated.

Table 22: The overview of the compounds isolated from Ext. 41-DM

Compounds isolated from Ext. 41-DM			
Compounds isolated (*m/z*):	Elemental composition	New compound name:	Reasoning for isolation:
395,2	$C_{19}H_{26}N_2O_7$	MBC-413	Compound of interest
431,2	$C_{18}H_{25}ClN_6O_3$	MBC-414	Easy accessed compound

While both compounds elute after Rt = ˜8 min, the high activity, low sample complexity, their apparent novelty and the intriguing background of the WPB samples, nominated these samples for isolation. From this point onward, all nominated compounds will be referred to by their new compound names (MBC-XXX, shown in Table 21 and Table 22).

4.6.2 Isolation of Compounds From Extracts

Marbank Sample: M22013-0-W01

For the M22013-0-W01 extract 10g of dried material was extracted using 20ml ACN. The supernatant from this extraction step was dried, redissolved, and used in the isolation step. In each injection round, between 150-400 µL was injected. The injection volume was increased when it was confirmed that the isolation protocol was shown to run smoothly. The injector of the prep system was able to inject 1000 µL/run. However, the pressure sensor on the prep-system was not working. This means that if a column got clogged due to too high amount sample injected, the pressure could increase to a level where the HPLC pump could get damaged, as it would not shut down when the upper pressure limit of the system (6000 psi) was reached. The isolation was thus proceeded with caution. The base peak intensity (BPI) chromatogram from the first isolation round of compounds from M22013-0-W01 can be seen in Figure 16. As is apparent, this was a complex sample, with multiple compounds, many of which does not have base line separation from neighbouring peaks. What was not equally apparent in the BPI chromatogram, was that all compounds that are eluting next to the compounds of interest were only partially overlapping with the compounds of interest. In theory, these compounds would thus be separated from the target compounds if the collected samples were re-injected onto the same column using the same setting, as the impurities would be present in lower amounts in the semi-purified sample. The results from this isolation run were four semi-purified samples of our target compounds.

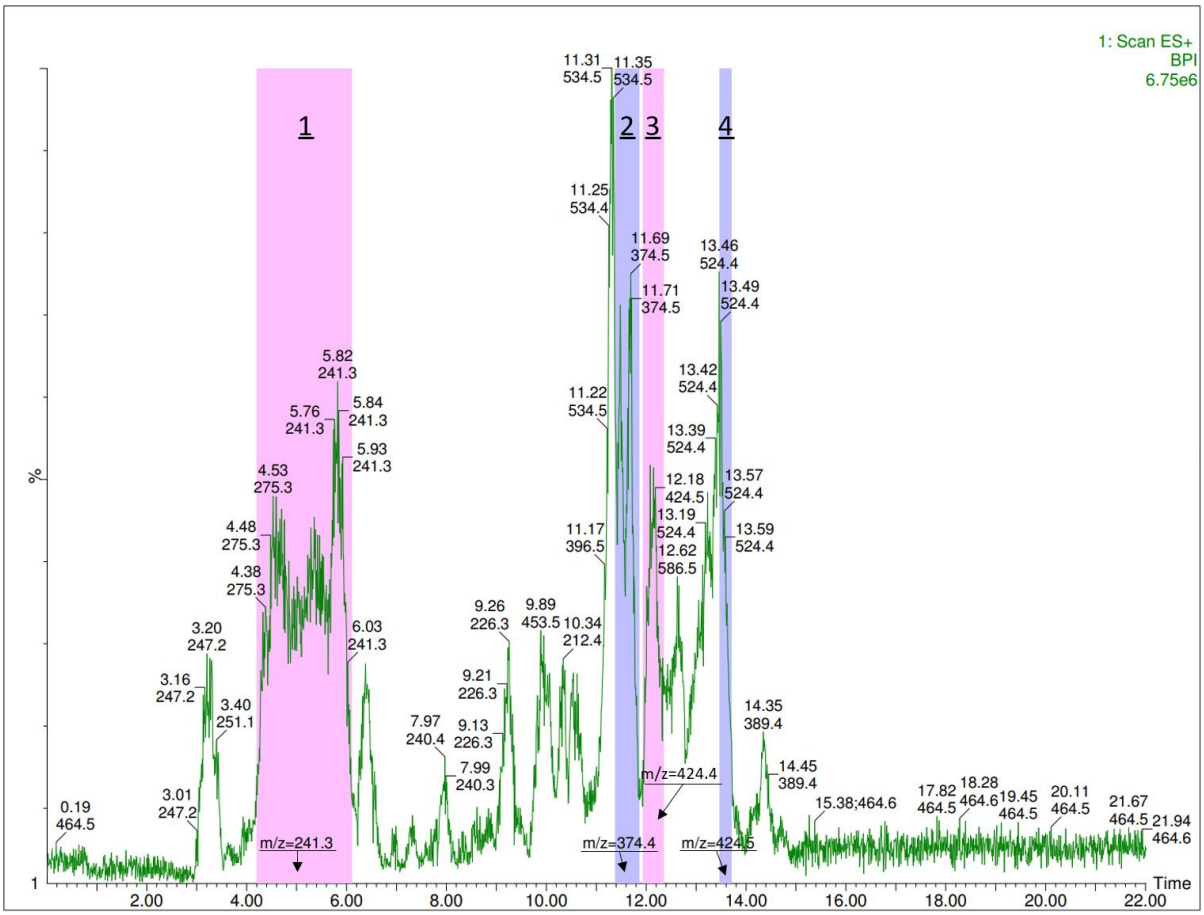


Figure 16: BPI chromatogram of the first-round isolation of compounds from Marbank extract M22013-0-W01. The coloured areas represent the individual collected areas. Compounds: 1. MBC-412 2. MBC-411 3. MBC-410 4. MBC-410 (v.2).

WPB Sample: Ext. 41-DM

For Ext. 41-DM around 700 µL of the extract (concentration 100mg/ml in DMSO) was dissolved in 2 ml ACN and 50 µL hexane. The hexane was needed to dissolve the most lipophilic sample constituents. The sample was transferred to HPLC injection vials and placed in the prep-HPLC injector. In each run, 25 – 75 µL was injected (volume as increased for some of the later samples when it was observed that the isolation was proceeding without any complications). The runtime for each injection was 15 minutes. The collection was triggered using timed events due to the inability of the instrument to trigger based on mass. Looking back on the process of prioritising a WPB sample for further work, the selection should have been based not only on bioactivity but also the amount of material left. Having more material at hand would have made this process easier and left a higher chance of isolation enough of the target compound(s) to run record proper NMR data. Sample Ext. 41-DM were on the border on what

were possible to isolate from. According to Marbio colleagues, it is not normal for an extract to mainly consist of one compound, which appeared to be the case based in the dereplication analysis. During the isolation step, it became apparent the sample contained a significant amount of an impurity that was not visible using UHPLC-HR-MS. While Ext. 41-DM would still have been chosen, including another WPB sample with higher available amounts would increase the chances of isolating compound in higher amounts (Figure 17).

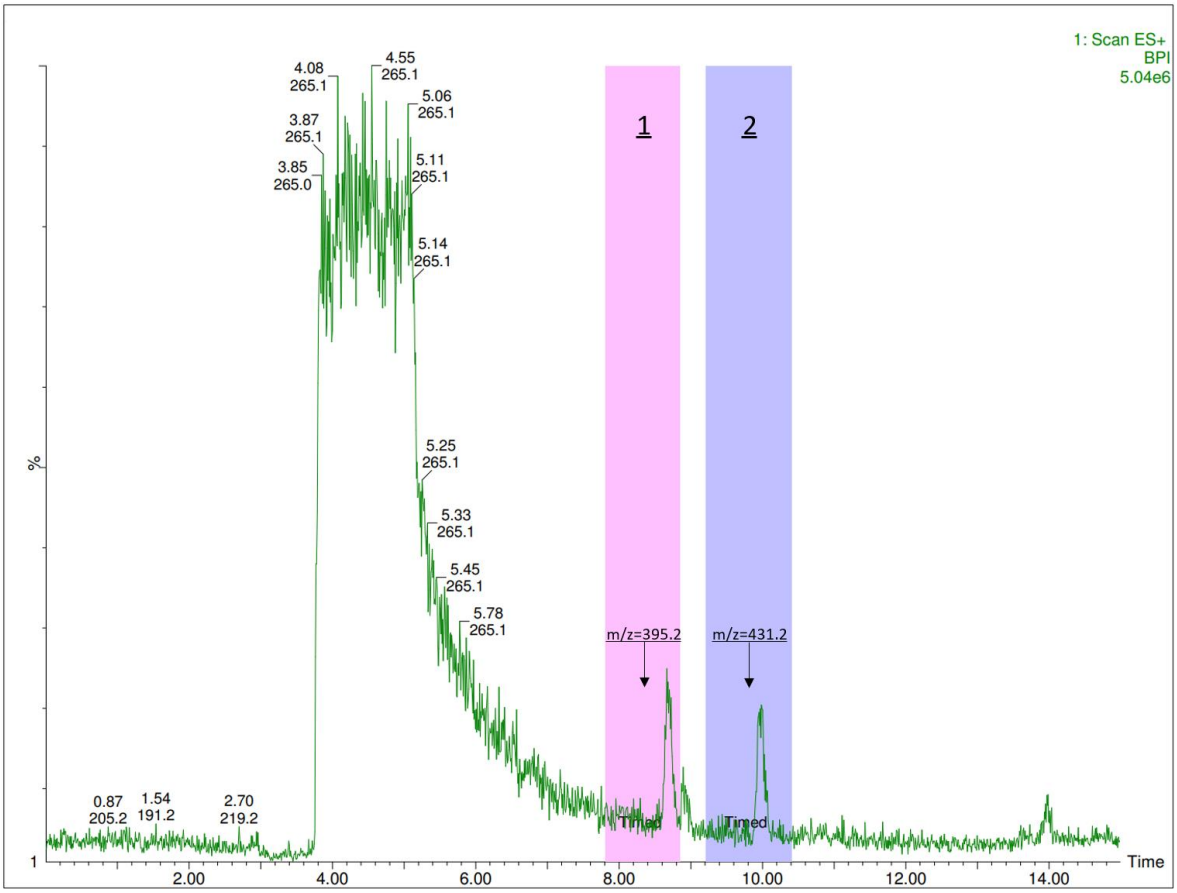


Figure 17: BPI chromatogram of the first-round isolation of compounds from Ext. 41-DM. The coloured areas show the collected timestamps. Compounds: 1. MBC-413 2. MBC-414.

4.6.3 Second (and Third) Isolation Rounds

Based on results from the first round of isolation, the same collum (Atlantis) was used for second and third round of isolation of all compounds described below. Due to time restraints weren't isomer of MBC-410 (the v.2) purified through second-round isolation and were stored in case MBC-410 showed activity in the bioactivity profiling.

Compound MBC-410

After drying, MBC-410 was dissolved in 600 µL ACN before it was transferred to prep-HPLC injection vials and placed in the prep-HPLC injector. The injection run time was set to 15 minutes, injecting 50-75 µL in each round. The base peak intensity (BPI) chromatogram in Figure 18 gives an image of the semi-purified compound following the first isolation round. As is apparent, the sample was not purified during the first isolation step. There was one challenging compound, which eluted ˜8 minutes before the target compound, but was present in high amount. The impurity peak had significant tailing, which made it coelute with our compound of interest. However, the amount of the impurity was significantly reduced by conducting another round of isolation. In addition, minor components that eluted closer to the compound if interest was also successfully removed. After the second round of isolation, 1 mg of the compound was isolated. The sample was dried using a freeze dryer, before it was submitted to NMR for structure elucidation.

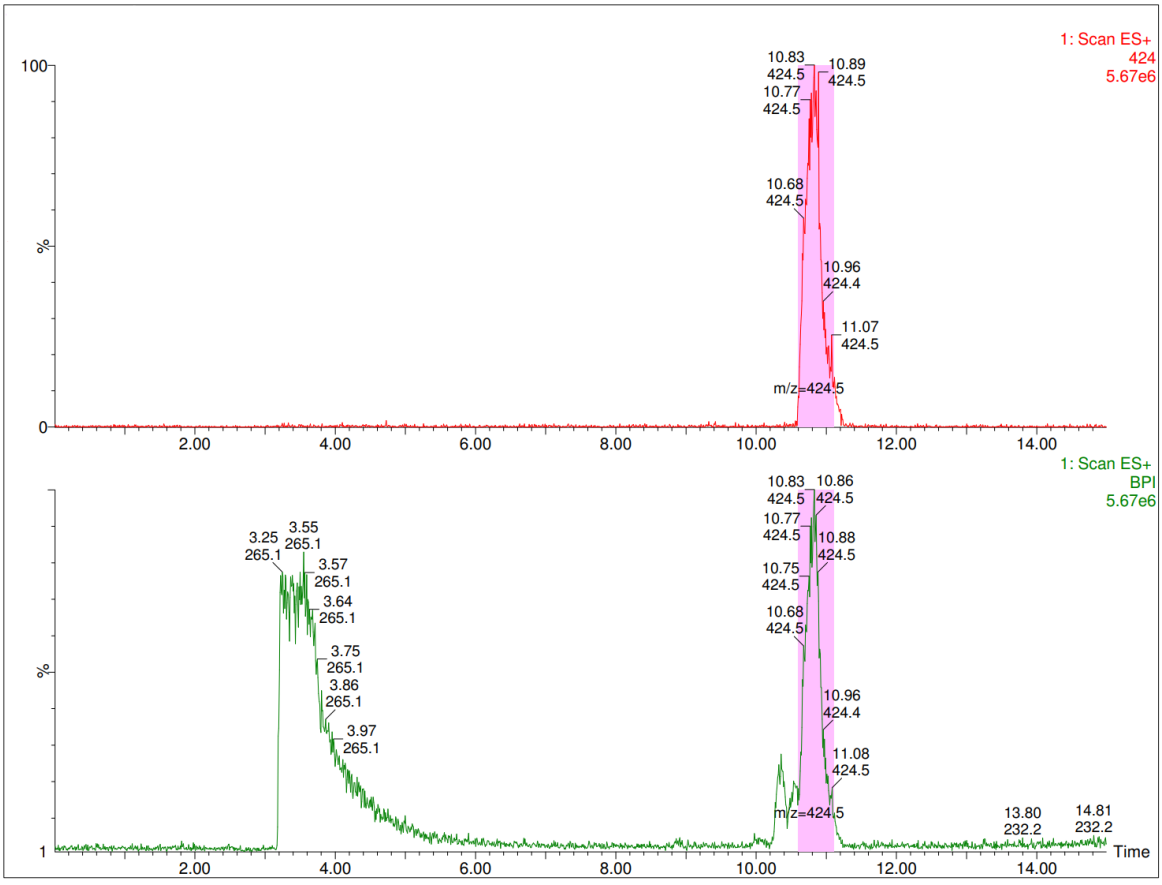


Figure 18: BPI and ion chromatograms from the second-round isolation of MBC-410. The coloured areas show the collected timestamp.

Compound MBC-411

MBC-411 was dissolved in 650 µL ACN before placed in the prep-MS. The injection run time were set to 15 minutes using 50-75 µL in each injection. The BPI chromatogram in Figure 19 gives an image of the semi-purified compound following the first isolation round. As seen in the chromatogram, the sample still contained impurities from the first isolation step. One major component did elute close to the compound if interest, but based on the chromatogram showed that the component was successfully removed. After the second round of isolation, 1,4 mg of the compound was isolated. The sample was dried using a freeze dryer, before it was submitted to NMR for structure elucidation.

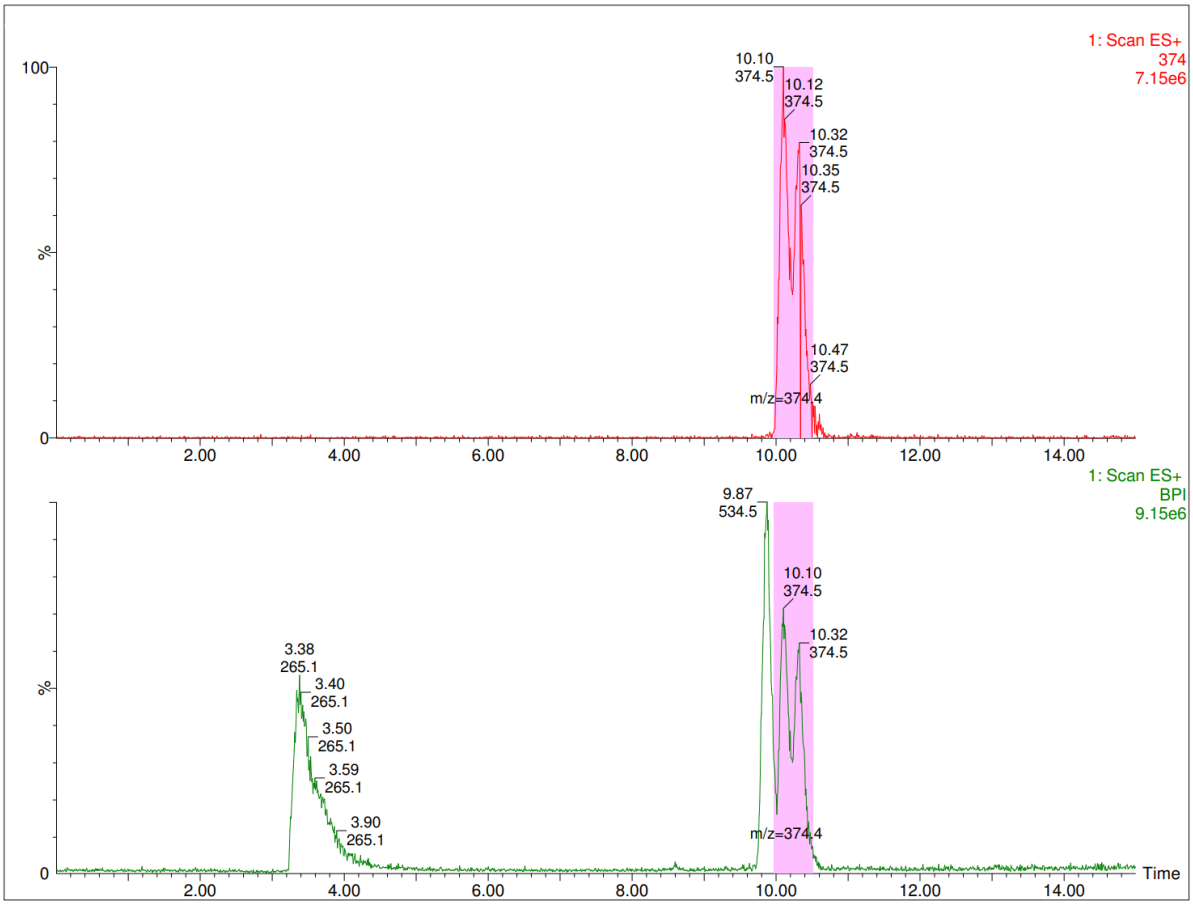


Figure 19: BPI and ion chromatograms from the second-round isolation of compound MBC-411. The coloured areas show the collected timestamp.

Compound MBC-412

From the first round of isolation were 195,4 mg of the purified compound isolated. Purely based on weight, was it highly unlikely that the compound could be pure. After the drying of the isolated product did the sample have thick oily consistency. This led to approximately 10 mg of the semi-purified mixture being used for the second-round isolation. The isolated mixture was also really hydrophilic, not dissolving in normal isolation solvents (e. g. ACN, DMSO, MeOH, Hexane), only dissolving in water. So, for the compound were ca.10 mg dissolved in 1000 µL MilliQ water before placed in the prep-MS. The injection run time were set to 15 minutes using 50-150 µL in each injection (Figure 20). After the second round of isolation, 2,1 mg of the MBC-412 was isolated. Due to down time of the UHPLC were no elemental composition of the compound calculated. The sample was dried using a freeze dryer, before it was submitted to NMR for structure elucidation.

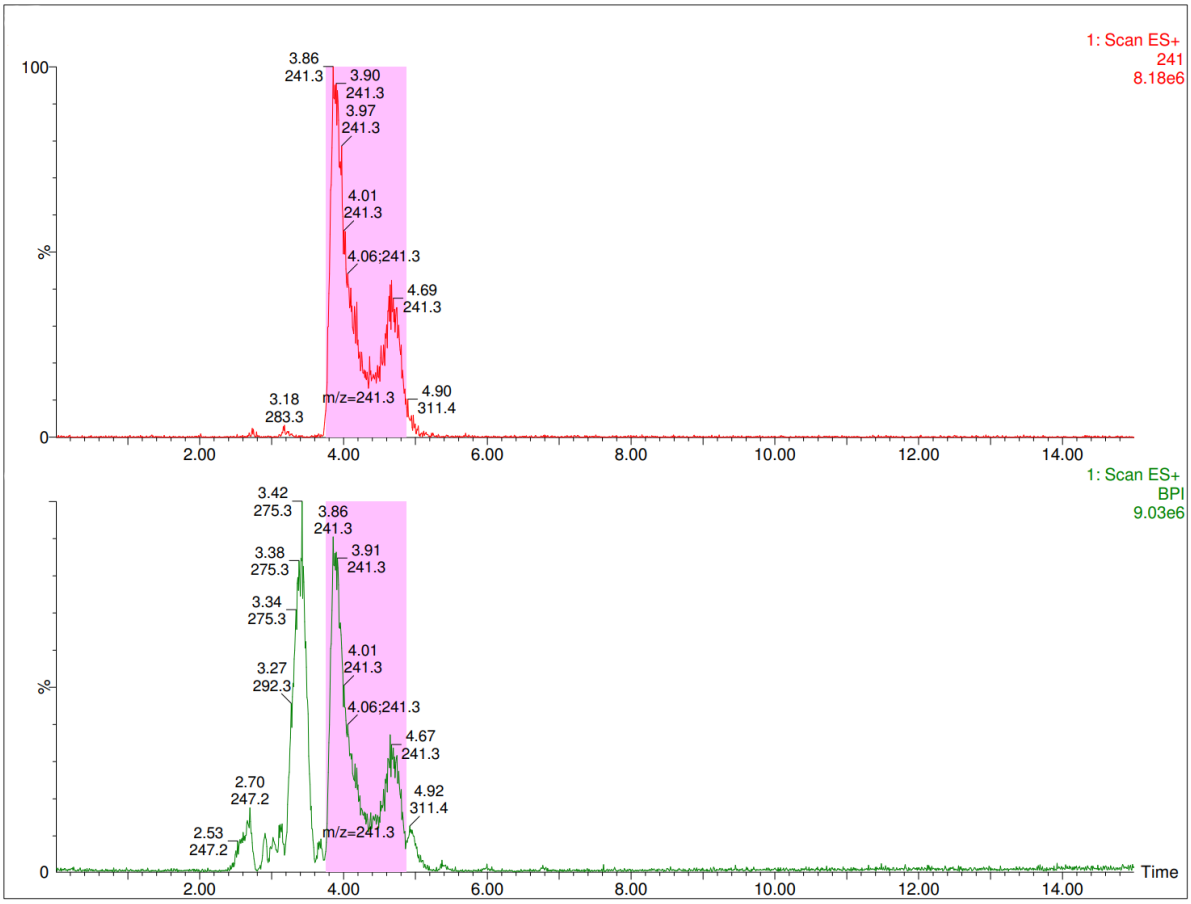


Figure 20: BPI and ion chromatograms from the second-round isolation of compound MBC-412. The coloured areas show the collected timestamp.

Compound MBC-413

Compound MBC-413 was dissolved in 550 µL ACN before placed in the prep-MS. The injection run time were set to 15 minutes using 25-50 µL in each injection (Figure 21). After the second round of isolation, 0,5 mg of the compound MBC-413 was isolated. The sample was dried using a freeze dryer, before it was submitted to NMR for structure elucidation.

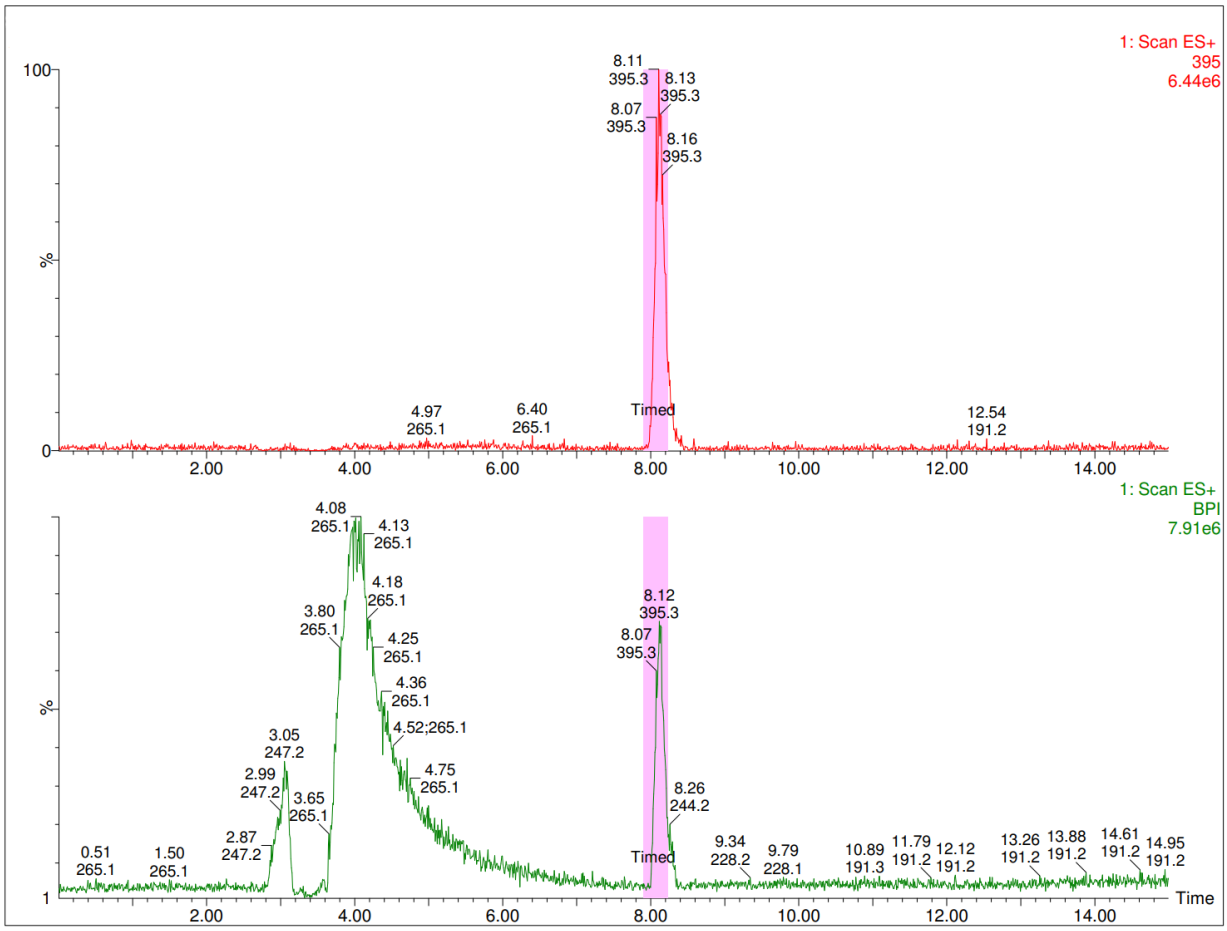


Figure 21: BPI and ion chromatograms from the second-round isolation of compound MBC-413. The coloured areas show the collected timestamp.

Compound MBC-414

Compound MBC-414 was initially not analysed in the UHPLC chromatogram during dereplication. However, following its selection, the compound was thoroughly examined in the UHPLC chromatogram of Ext. 41-DM, to determine its elemental composition and assess its previous identification status. The analysis revealed that the compound had been ionized by a

Na^+ adduct instead of H^+, revelling that the molecular weight is 408,88 g/mol and an elemental composition of $C_{18}H_{25}ClN_6O_3$.

For the purification of MBC-414, both a second and a third-round isolation were performed. MBC-414 was the first compound submitted for the second-round isolation, leading us to believe that the significant impurity was inherent to the semi-purified sample. This prompted us to perform a third-round isolation, where it became apparent that the compound was a 'bleed-through' compound, likely coming from the column. In both rounds were the compound dissolved in 700 µL ACN before placed in the prep-HPLC. The injection run time was set to 15 minutes using 25-50 µL in each injection (Figure 22). After the third round of isolation, 0,7 mg of the compound MBC-414 was isolated. The sample was dried using a freeze dryer, before it was submitted to NMR for structure elucidation.

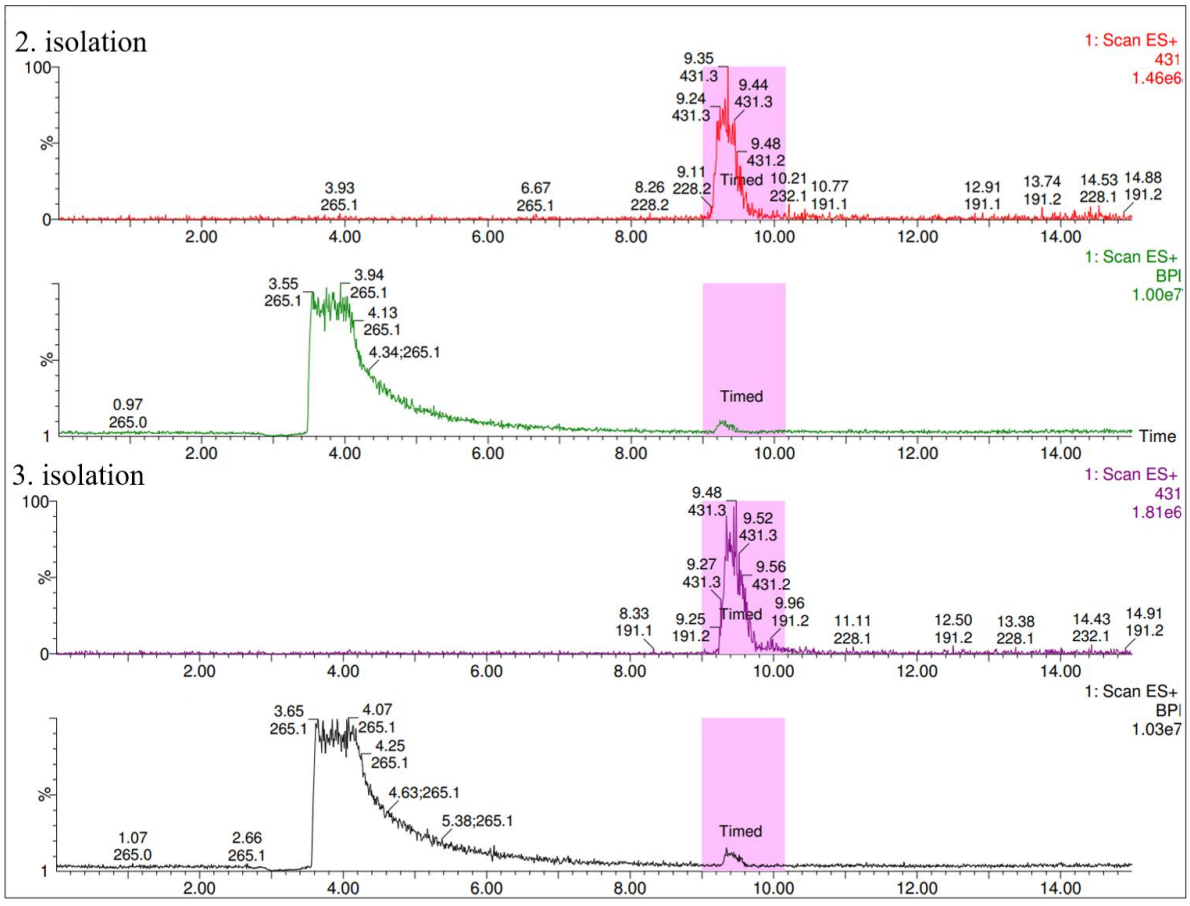


Figure 22: BPI and ion chromatograms from the second and third round of isolation of compound MBC-414. The coloured areas show the collected timestamps.

General Remarks

Following all rounds of isolation, the normal procedure would be to analyse the resulting sample using UHPLC-HR-MS to assess the purity of the isolated compounds. With this knowledge at hand, the results from the scout runs can be revisited to identify a column that would separate the impurities that co-eluted with the compound of interest using the Atlantis column. In addition, a sample would not be sent to NMR analysis before the purity was confirmed. Unfortunately, the UHPLC-HR-MS system at Marbio was unavailable for a long period of time while this work was ongoing, making this analysis of impurities unavailable. Thus, the sample were isolated to they appeared pure based on the data from the prep-system.

4.7 Structure Elucidation and Bioactivity Profiling of the Isolated Compounds

In bioassay-guided isolation from crude extracts, it is established that out of five million primary screenings of compounds, 1,000 hits will be yielded. This translates to a hit rate of 0.02%, or 1 in 5,000 (70). This shows the unlikeliness of actually finding a bioactive compound. The five compounds isolated were first analysed in NMR and then submitted to a retest in the initial bioactivity assay were the original fraction showed activity and the MIC antibacterial assay. Bioactive compounds were further subjected to bioactivity characterization.

4.7.1 Structure Elucidation

The NMR analysis preformed to uncover the chemical structures of the compounds were to a degree determined to be unsuccessful, analysis listed in appendix 7.1. MBC-411, MBC-412, MBC-413 were not possible to discover anything due to either to low amounts of the compounds or the samples were not pure enough. MBC- 410 and 414 were both more promising, but due to the time restraints were these not finished analysed for the deadline of this thesis. Regardless of the structure elucidation results were the compound submitted to bioactivity retesting. If some of the Marbank compounds turned out to be active, there are still more extract available to try isolate more in the future.

4.7.2 Bioactivity Retesting

To confirm that the bioactive compounds were isolated from the extracts, the isolated compounds were submitted to the bioassay were the bioactive fraction initially showed activity. The compounds were diluted to six concentrations, from 100 to 3,125 µM. They were all both submitted to the initial assay A2058 (Table 23), but also to the relevant MIC antibacterial assay (Appendix 7.2).

Table 23: The results from the bioactivity retesting for all the compounds analysed by NMR. The table shows the survival rates (%) of A2058 and MCF-7 on average from a triplicate across six different concentrations. The results are classified as active (A), questionable (Q) and inactive (I).

The results from the bioactivity retesting of the isolated compound							
Sample	Conc. (µM)	A2058			MCF-7		
		Avg. Surv %	Std.dev	Result:	Avg. Surv %	Std.dev	Result:
MBC-410	100	105	7,9	I	64	2,6	I
	50	136	13,1	I	77	12,1	I
	25	131	19,7	I	115	7,4	I
	12,5	118	8,3	I	129	2,1	I
	6,25	131	2,3	I	118	1,2	I
	3,125	142	4,4	I	112	1,9	I
MBC-411	100	116	2,7	I	77	7,5	I
	50	117	7,6	I	87	10,8	I
	25	104	4,4	I	116	7,2	I
	12,5	102	3,5	I	137	7,6	I
	6,25	102	2,1	I	128	2,2	I
	3,125	115	5,5	I	122	4,3	I
MBC-412	100	111	11,9	I	71	7,7	I
	50	95	4,2	I	90	6,4	I
	25	94	4,0	I	85	3,6	I
	12,5	96	1,0	I	89	4,5	I
	6,25	108	11,9	I	87	8,6	I
	3,125	114	9,9	I	98	2,5	I
MBC-413	100	129	3,1	I	79	2,1	I
	50	126	6,7	I	85	6,0	I
	25	114	11,7	I	88	1,2	I
	12,5	119	9,2	I	86	2,5	I
	6,25	111	7,1	I	89	6,2	I
	3,125	117	8,8	I	90	5,6	I
MBC-414	100	1	1,5	A	7	0,1	A
	50	-1	0,2	A	13	2,0	A
	25	-1	2,0	A	7	0,7	A
	12,5	4	2,5	A	10	0,5	A
	6,25	8	0,8	A	15	0,8	A
	3,125	9	0,9	A	21	0,9	A

The results from the A2058 screening of the compounds showed that compound MBC-414 had high levels of activity against the cells, even surpassing the positive control (DMSO) at concentrations as low as 25 µM (Figure 23). MBC-414 did also show some degree of activity against bacteria in the MIC assay, by having an active fraction against *Streptococcus agalactiae* at 100 µM. Since this compound was bioactive it was submitted for further bioactivity characterization.

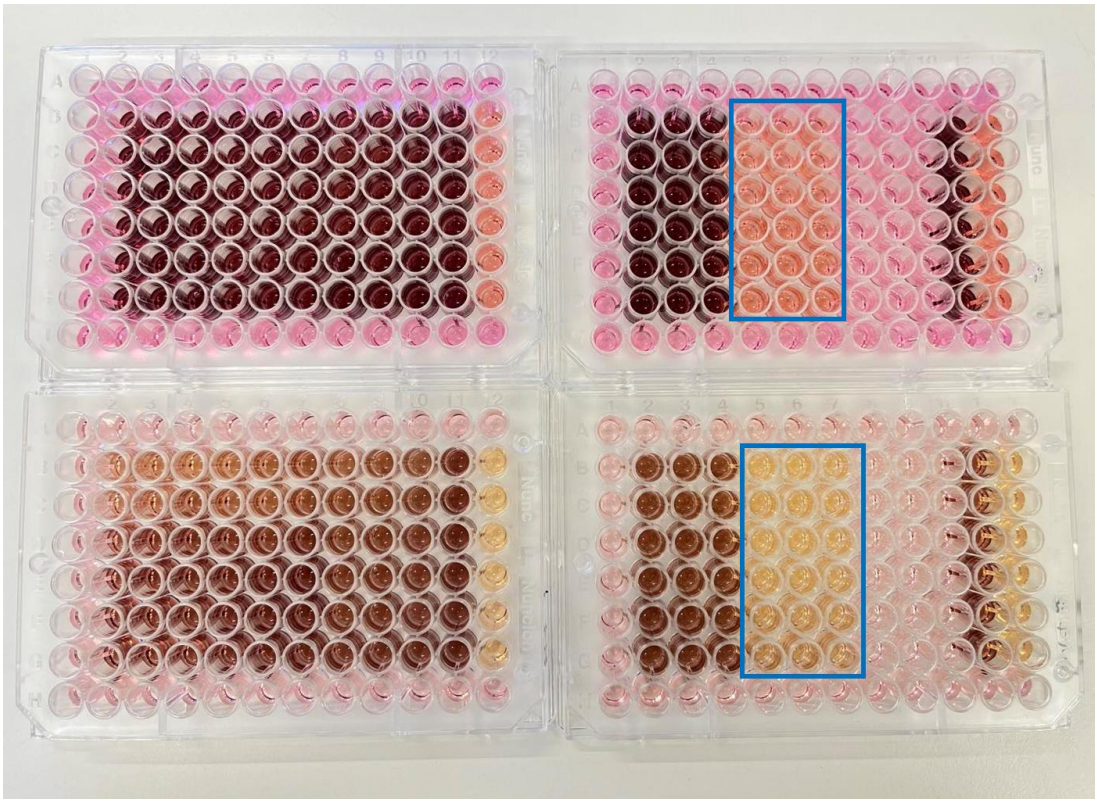


Figure 23: The A2058 (two plates above) and MCF-7 (two plates below) viability testing plates of the five isolated compounds. The blue marker showes the active wells were compound MBC-414 is tested.

On the other hand, compound MBC 410, 411, 412 and 413 didn't show any kind of bioactivity. In biodiscovery and bioprospecting, there is the mindset that even though a compound is not active in the assay it is tested in, does it not mean that the compound is not bioactive. In theory, compounds could still possess some kind of bioactivity that we haven't discovered yet, but based on the current analysis, are they are ruled out. An important mention is that M22013-0-W01 still have undefined bioactive compounds. By going back to the dereplication process to locate new compounds of interest could potentially lead to the discovery of the bioactive compound. Including that the compound with an *m/z* of 452 weren't discovered during the isolation, and which still could be the compound we are looking for.

4.7.3 Bioactivity Characterisation of MBC-414

For the characterisation of MBC-414 three sets of assays was preformed, the two extra runs of A2058, two extra rounds of MCF-7, and two additional runs of MCR-5 cytotoxicity assay (Figure 24). The concentration was lowered in these assays due to the high amount of activity shown in the retesting. The concentrations tested were 50 µM down to 0,024 µM.

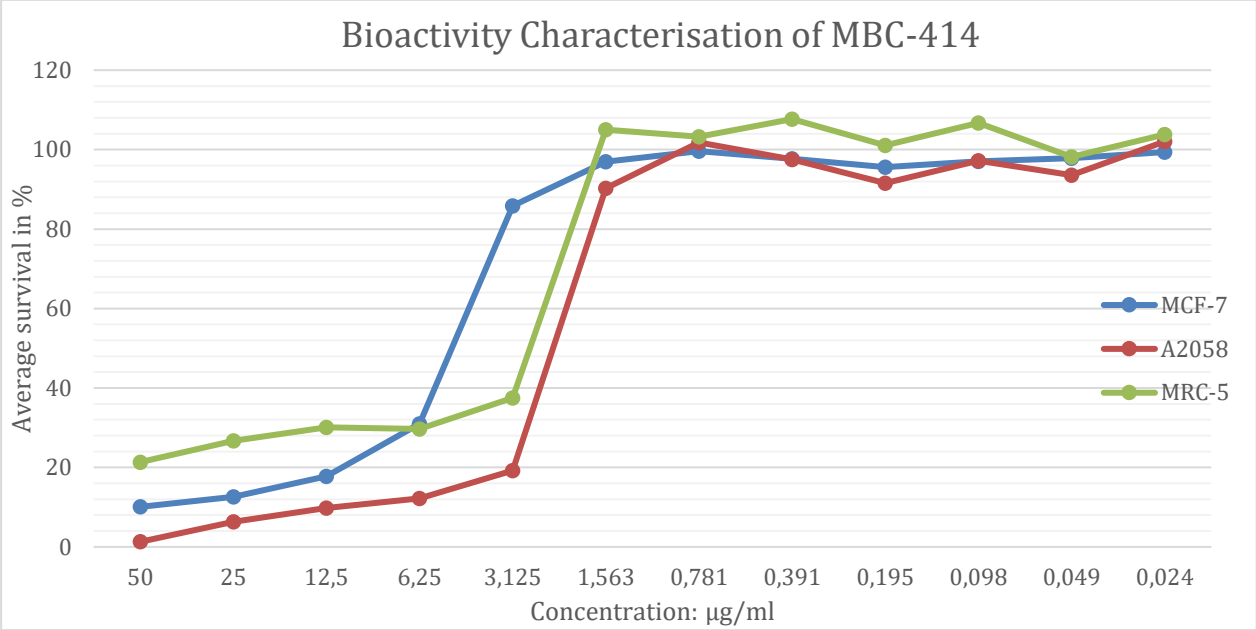


Figure 24: Diagram of the average survival rate of the different cell-lines at different concentrations of MBC-414.

From the characterisation rounds we see that the compound had about the same levels of activity as in the bioactivity retesting (4.7.2). MBC-414 had IC_{50} values of 2,2 (± 0,2) µM against A2058, 6,8 (±1,0) µM against MCF-7 and 7,1 (±0,3) µM against MRC-5. Based on the characterisation work performed on MBC-414 can we confirm that it possesses a cytotoxic bioactivity. The measured IC_{50} values places MBC-414 in a potency range which makes it interesting as a starting point for further development. However, while the cancer cell line A2058 was slightly more affected compared to the cancer cell line MCF-7 and the non-cancer cell line MRC-5, this activity does not significantly discriminate between the different cell lines. This indicates that the compound affects a cellular process common to all living cells, rather than a process that is a specific driver of malignancy in a specific cancer type. This is generally not what is required in an age where cancer therapy is moving from a general cytotoxicity approach to targeted therapies.

4.8 Future work

Using the marine bioprospecting pipeline has resulted in isolation of the bioactive compound MBC-414 from Ext.41-DM. Even with a relatively low number of extracts and a restricted time frame, was an isolated toxic compound discovered, showing the efficiently of the pipeline. However, compound MBC-414 turned out to be cytotoxic, which makes it very unlikely to be used as any kind of pharmaceutical. On the other hand, extract M22013-0-W01 was confirmed to contain at least one bioactive component during the bioactivity testing, but none of the compounds isolated from the extracts showed any bioactivity. This means that the extract still contains undiscovered compounds with bioactivity, which calls for new rounds of inspections of the UHPLC chromatogram, followed up by new rounds of compound isolation and bioactivity profiling. The prospect of the compound with 452 *m/z* is something to follow up. But this also depends on external factors like the amount of extract left, because a lot of the original yield were used during the isolation, which lowers the chance of isolating a new compound and sustain enough material to preform NMR. This could also be a great opportunity to investigate fraction M22009-0-L03-04 and M22012-0-L03-03/4, which still have undiscovered bioactive molecules in them.

When a compound with bioactive properties is discovered, there are still a lot of work required before the compound can be labelled as a drug candidate. First, further bioactivity profiling is required to map the specificity of the bioactivity and the mode of action of the compound. There are two types of approaches you could develop a drug from, a target-based approach or a phenotypic screening approach (20). A target-based approach focuses on directly identifying a solution for a specific medical condition and is the main approach in the pharmacy industry (71). A phenotypic approach focuses on the identification of molecules exhibiting promising bioactivities and explore their potential therapeutic effects (72). Bioprospecting is heavily based on this strategy, by discovering bioactive natural products and study its mode of action to find good therapeutic matches.

Regardless of the approach, the next essential step is ADME testing, to analyse if and how your compound behaves in the body. There are central guidelines like the *Lipinski's rules of five*, which can help determine the likelihood of success for a compound on beforehand. There are also other predicting tools like molecular docking to simulate how your compounds interact with the target of the illness. Combing these strategies with different *in vitro* and *in vivo* analysis's gives a clear indication if a compound could become possible pharmaceutical. Natural products are also often combined with synthetic optimalisation, either as inspiration as

a completely new synthetic compound, as natural product derivatives, or as a method for producing the purified natural compound (73).

4.9 Realistic Expectations of Developing a Pharmaceutical

A discussed earlier, compounds isolated from crude extracts yield only a 0,02% discovering bioactive compounds. Additionally, out of ten thousand compounds with activity is it estimated that only one compound ends up as a finalised and commercially available drug (70). The fraction of potential pharmaceuticals that ultimately reach the market is very low. In the world of finance, are pharmaceutical and biotechnology companies often looked upon as a gamble, where the companies often either fail or makes a great profit. Out of all potential drugs during phase 1 of clinical trials does up to 90% fail (74). If you also take into a count the competition within pharmacy industry, good, cheap, and effective pharmaceuticals are hard to come by.

This underscores the necessity for effective pipelines to 'mine' large quantities of bioactive compounds, with the marine bioprospecting pipeline serving as a prime example.

5 Conclusion

In total, 16 yielded extracts from marine invertebrates were analysed in the marine bioprospecting pipeline. After fractionation and bioactivity testing were six fractions deemed bioactive without any PAINS or known bioactivity with justifiable amounts of material left. Two extracts were prioritized for containing the most promising compounds, the aqueous extract of *Funiculina quadrangularis* and the organic (DM) extract of *Aplidium sp.* This led to five compounds isolated from the extract of *Funiculina quadrangularis* and *Aplidium sp.* The compounds MBC- 413 and 414 were isolated from *Aplidium sp.*, were MBC-414 was identified as the bioactive compound being cytotoxic with an IC_{50} in the 2.2 – 7.1 µM range against three cell lines. Together with the challenges of the compound being cytotoxic, there were nothing left of the original extracts. This makes the compound highly unlikely to become any kind of drug candidate. The compounds MBC-410, MBC-411 and MBC-412, isolated from *F. quadrangularis*, were all deemed non-active. This leaves the bioactive compound yet to be discovered in this extract. This makes room for new rounds of dereplication and compound isolation, or the restarting the investigation of the other bioactive fractions, M22009-0-L03-04 and M22012-0-L03-03/4.

The result in this thesis shows that the marine bioprospecting pipeline is a great tool to discover bioactive compounds. It also highlights:

- The importance of having sufficient amounts of material before analysing samples.
- The need for fractionation and standardisation of crude extracts, as they facilitate the discovery of bioactive compounds during bioactivity testing and dereplication.
- The strength of chromatographical analysis, through separation of samples with flash, the discovery of compounds during dereplication using UHPLC-HR-MS, and isolation of compounds using prep-HPLC

Despite the robustness of the pipeline, its application reveals the ongoing challenge of isolating and characterizing secondary metabolites with pharmaceutical potential. This difficulty persists due to the chemical and biological criteria that a compound must meet to become a viable drug candidate.

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7 Appendix

7.1 NMR Results

MBC-410 and MBC-414

The finalised analysis for these compounds weren't ready before the due date of this thesis. The data collected from the samples gives us an early indication that it should be possible to tell something about structures of the compounds. If the complete structure is discovered upon completing my thesis work, potential publication of the compounds may ensue.

MBC-413

This sample was isolated with a yield of 0.5 mg, which falls within the lower range of compound quantity typically required for NMR detection. NMR experiments were thus not expected to provide a strong dataset. This was confirmed when the data was analysed, as exemplified with the ^{1}H, ^{13}C and HSQC data shown in figure 25. The calculated elemental composition for the compound was $C_{19}H_{26}N_2O_7$.Out of this, only 11 hydrogen atoms (Figure 25A) and 7 carbon atoms (Figure 25B) were visible. 2D-NMR data did not provide any help in solving the structure as only a few correlations were detected in the HMBC, HSQC, COSY, H2BC and ROESY data, as exemplified by the HMBC data in Figure 25C. The structure of sample MBC-413 could thus not be elucidated.

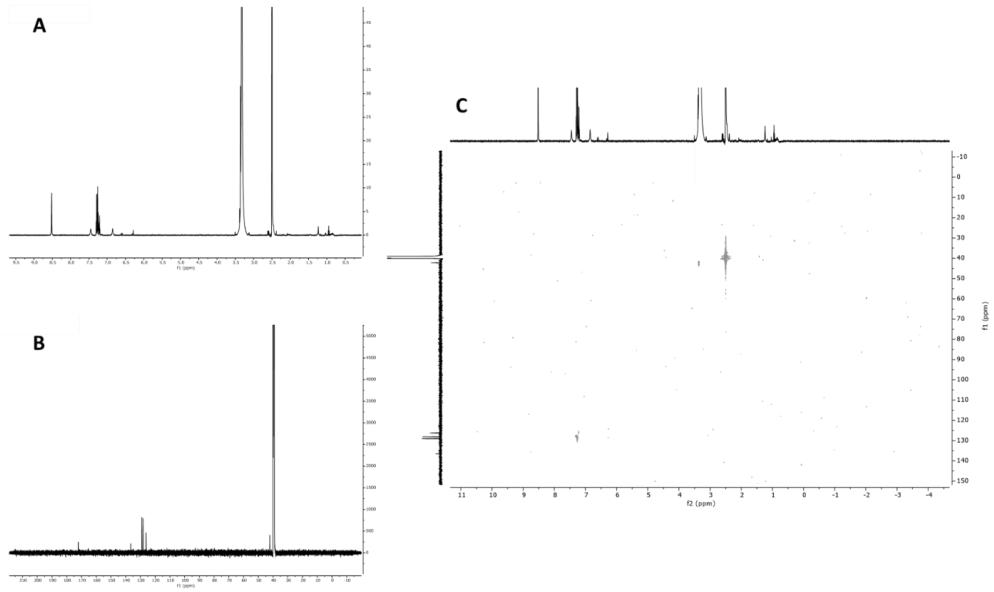

Figure 25: Selected NMR data for compound MBC-413. A) ^{1}H-NMR data gave seven clear signals, totalling 11 hydrogen atoms. B) The ^{13}C-NMR data gave clear signals for 7 carbon atoms. C) All 2D experiments provided few or no signals, as exemplified with the HMBC data. The clearly visible peak is the solvent peak.

MBC-412

The sample was isolated with a yield of 2.1 mg, which would be sufficient to obtain good quality NMR data if a sample is pure. The NMR data of MBC-412 revealed that this was not the case for this compound. The ^{1}H and ^{13}C spectra showed the presence of 50+ signals in both datasets. The elemental composition of MBC-412 could not be calculated using HR-MS analysis, however with a monoisotopic mass of ˜240 Da, this number of detected atoms is conclusive of an impure sample as is illustrated using the HMBC data in Figure 26.

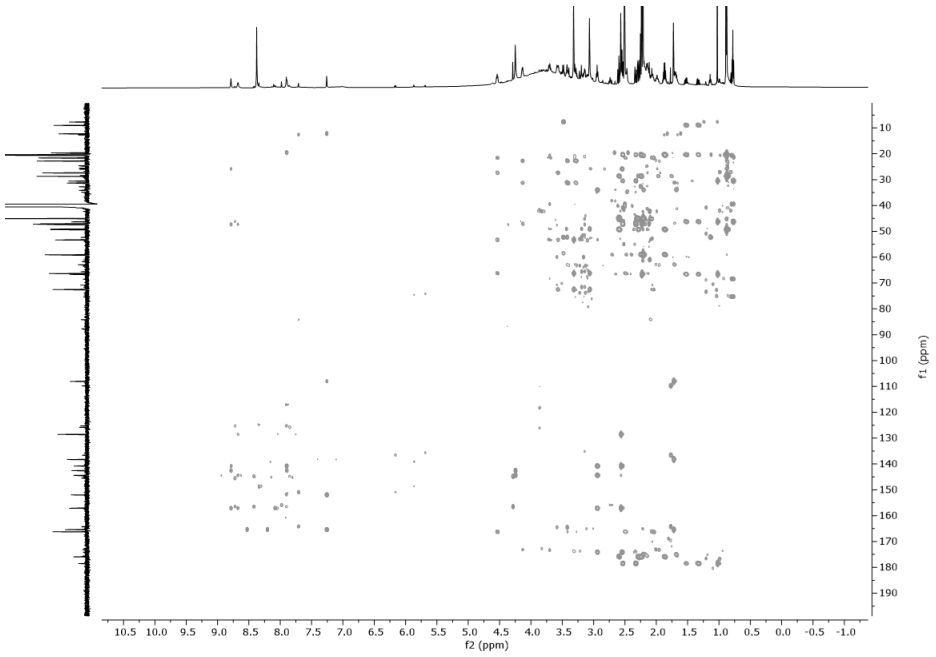

Figure 26: HMBC data of compound MBC-412. The X-axis represents the proton signals, and the Y-axis represents the carbon signals. As more the 50 signals were detected for both atom types, and 300+ correlations were visible in this experiment, it was impossible to elucidate the structure of the target compound in the mixture.

MBC-411

This compound was isolated with a yield of 1.4 mg and had a calculated elemental composition of $C_{23}H_{35}NO_4$ based on HR-MS analysis. The NMR data of this compound provided a similar result as MBC-412, and the structure of the compound could not be elucidated.

7.2 MIC Assay Results

The MIC assay results analysed during the bioactivity profiling is listed in Table 24.

Table 24: The results of the MIC assay on the five isolated compounds,

The results from the MIC testing of the isolated compound						
Sample	Conc. (µM)	Activity Results (A/Q/I)				
		E. faecalis	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. agalactiae*
MBC-410	100	I	I	I	I	I
	50	I	I	I	I	I
	25	I	I	I	I	I
	12,5	I	I	I	I	I
	6,25	I	I	I	I	I
	3,125	I	I	I	I	I
MBC-411	100	I	I	I	I	I
	50	I	I	I	I	I
	25	I	I	I	I	I
	12,5	I	I	I	I	I
	6,25	I	I	I	I	I
	3,125	I	I	I	I	I
MBC-412	100	I	I	I	I	I
	50	I	I	I	I	I
	25	I	I	I	I	I
	12,5	I	I	I	I	I
	6,25	I	I	I	I	I
	3,125	I	I	I	I	I
MBC-413	100	I	I	I	I	I
	50	I	I	I	I	I
	25	I	I	I	I	I
	12,5	I	I	I	I	I
	6,25	I	I	I	I	I
	3,125	I	I	I	I	I
MBC-414	100	I	I	I	I	A
	50	I	I	I	I	I
	25	I	I	I	I	I
	12,5	I	I	I	I	I
	6,25	I	I	I	I	I
	3,125	I	I	I	I	I