UiT The Arctic University of Norway

Department of Pharmacy, The Faculty of Health Sciences

Isolation and characterization of secondary metabolites from Arctic, Marine Invertebrates

Amalie Rebekka Nyborg

Master thesis in Pharmacy (May 2022)

Table of Contents

Acknowledgements

This thesis was conducted at Marbio, UiT, the Arctic University of Norway from August 2021 to May 2022.

I would like to thank my great team of supervisors, Terje Vasskog, Kine Østnes Hansen and Espen Hansen for the support and feedback during my master thesis. A special thank goes to Kine for your encouragement.

I would like to thank Marte Albrigsten and Kirsti Helland for the practical help with the bioactivity screening, and Johan Isaksson for the NMR work of this thesis.

Finally, a big gratitude goes to my family, friends and my significant other, Reidar, for the support you have given me this year.

Tromsø, May 2022

Amalie Rebekka Nyborg

Abstract

The search for bioactive compounds, or genes in macro- and microorganisms from the sea, is termed marine bioprospecting. Marine bioprospecting aims to increase the utilization and value of marine biomasses and organisms with the purpose of developing commercial products. Because of the special condition of the sea, it is known that marine invertebrates have adapted chemical defense strategies to survive, such as producing secondary metabolites. Secondary metabolites are unique in diversity, functional and structural features, and it has been shown that secondary metabolites can have bioactive properties. These secondary metabolites result in the ocean offering a variety of compounds with bioactive activity that can be utilized as commercial products such as pharmaceuticals.

In this project the aim was to isolate and characterize secondary metabolites from marine invertebrates. Four flash fractions with anticancer activity against a human melanoma cancer cell line (A2058) were selected for dereplication with high-resolution mass spectrometry. Two flash fractions from *Porifera Indet* and *Lucernaria quadricornis* extracts were chosen for further isolation using preparative high performance liquid chromatography and structure elucidation using nuclear magnetic resonance spectroscopy. The purified compounds were screened for bioactivity using anticancer- and antibacterial assays. No activity was detected with the test concentration employed in the assays.

Abbreviations

A	Active
A2058	Human melanoma cancer cell line
ACN	Acetonitrile
ATCC	American type culture collection
BHI	Brain heart infusion
BPI	Base peak intensity
CFU/mL	Colony forming units/milli liter
CNS	Central nervous system
COSY	Correlated spectroscopy
dc	Constant voltage
DMEM	Dulbecco´s Modified Eagle´s Medium
DMSO	Dimethyl sulfoxide
EMA	European Medicine Agency
EI	Electron ionization
ESI	Electrospray ionization
FA	Formic acid
FDA	U.S Food and Drug Administration
FSB	Fetal Bovine serum
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High performance liquid chromatograph
HR	High resolution
HR-MS	High-resolution mass spectrometry
HTS	High throughput screening
I	Inactive
L	Organic extract
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MH	Mueller-Hinton
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
m/z	Mass to charge
NA	Not available
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement
OD	Optical density
ORF	Radio frequency oscillating voltage
PDA	Photo diode assay
PBS	Phosphate buffer saline
Q	Questionable
Rf	Radio frequency
RP	Reverse phase
Rpm	Rounds per minute
SIM	Selected ion minitoring
TIC	Total ion chromatogram
UPLC	Ultra-High-performance liquid chromatograph
W	Aqueous extract
WHO	World health organization
1D	One-dimensional
2D	Two-dimensional

1 Introduction

1.1 Natural products

Natural products are defined as a compounds that is biosynthesized by a living organism such as a plant, an animal, or a microorganism (1,2). Overall, natural products have larger structural diversity and complexity with a higher molecular mass, greater molecular rigidity, more sp^3-carbons and oxygen atoms, and a higher proportion of hydrogen-bonds acceptors and hydrogen-bond donors compared to synthetic compound libraries. Natural products have been a major source for medicinal use for a thousand years and are an excellent resource for discovering potential new drug lead compounds (3,4)

1.1.1 Primary and secondary metabolites

Natural products can be divided into primary and secondary metabolites. The processes of synthesizing and metabolizing compounds such as proteins, carbohydrates, fats, and nucleic acids are crucial for the organisms vital functions, such as growth, respiration, energy storage, and reproduction. These processes are termed primary metabolism, and the resulting products are known as primary metabolites. Variants of primary metabolites are universal to larger groups of organisms (3).

Secondary metabolites are not essential for an organisms growth, development, or reproduction. Instead, they are produced to be beneficial for long-term survival or increase the chance of reproduction. Secondary metabolites may protect against predators or pathogens or be beneficial in the competition for space and nutrition. The production of specific secondary metabolites is often triggered by environmental cues. This entails that the organism senses changes in its environment (for example the presence of pathogenic microorganisms or changing salt concentration), which initiates the production of secondary metabolites as part of adaption (3,5). The secondary metabolites are not vital for the immediate survival of the producing organism and are therefore not produced under every circumstance as primary metabolites are. Also, a specific secondary metabolite is often only produced by a single species or by a group of closely related species (3).

1.1.2 Natural products as pharmaceuticals

Many secondary metabolites or secondary metabolite derivates are used as pharmaceuticals today. The first record of natural products was dated as early as 2600 years before Christ on clay tablets in Mesopotamia, documenting the use of plant-based oil against colds, inflammation, and coughs (3). A known example of a natural product isolated from a plant is morphine isolated from *papaver somniferum*. A pharmacist named Friedrich Sertürner was the first to isolate the crystals from the opium poppy, and with this, morphine became the first natural product to be commercialized in its pure form as far back as 1803 (3,6). Even though the structure was not elucidated until 120 years later, morphine was found to be an excellent analgesic and a sedative because of its action on the central nervous system (CNS) (7). One of the most famous natural product discoveries occurred in 1929 when Alexander Fleming isolated Penicillin from the fungus *Penicillium chryogenum*. Newer studies have re-identified the strain of the fungus as *Penicillium rubens*. (3,8). Penicillin is a ß-lactam antibiotic that affects a wide range of bacteria by inhibiting the synthesis of the bacteria cell wall, resulting in an antibacterial effect (9).

1.1.3 Marine bioprospecting and marine natural products

The ocean harbors the richest biodiversity that exists on the planet. The special conditions of the sea, such as temperature fluctuations, varied pressure, salinity and lack of sunlight, have made organisms living in it develop properties such as producing secondary metabolites to survive (10-12). This results in the ocean representing a reservoir of chemical compounds/secondary metabolites that are unique in terms of diversity, functionality, and structural features. The biodiversity the ocean has to offer in addition to all the organisms producing various metabolites, making the marine environment an excellent source for discovering new drug lead compounds (11,13,14). The search for bioactive compounds, or genes in macro- and microorganisms from the sea, is termed marine bioprospecting. Marine bioprospecting aims to increase the utilization and value of marine biomasses and organisms with the purpose of developing commercial products (12,15). The first biological active marine natural product from the sea was reported late in 1950. Since then, the total number of approved drugs sourced from the marine organism has increased. In 2021 there were 13 approved drugs by the EMA (European Medicine Agency) and/or FDA (U.S Food and Drug Administration), in addition to many other compounds in the preclinical phase and phase I, II and III (16,17).

One example of a successful story of a drug with origin from marine organisms is Yondelis®. Trabectedin (Yondelis®) is originally isolated from the Caribbean tunicate, *Ecteinascidia turbinata*, and is an intravenous treatment used against metastatic, soft tissue sarcomas and ovarian cancer (18). The mechanism of action is binding to the DNA, disrupting the cell cycle and inhibiting cell proliferation (19). The medicine has shown a low toxicity profile and is now an approved treatment in over 70 countries (18). Prialt® is also a medicine based on a chemical structure from a marine organism. The active component in Prialt® is ziconotide, which is the synthetic form of ω-conotoxin MVIIA, and is the venom obtained from the marine snail *Conus magus*. The medicine is a non-opioid analgesic used to treat severe chronic pains in adults and is administrated intrathecal (20,21). It works by blocking neuronal N-type calcium channel that reduces nociceptive transmission and leads to analgesia (22).

1.2 Marine invertebrates

Marine invertebrates are defined as organisms from the sea lacking a vertebra column and are classified under different taxonomic groups such as *Porifera*, *Mollusca*, *Cnidaria*, *Arthropoda,* and *Echinodermata* (23). As they do not have a vertebra column, this entails that they lack the adaptive immune system found in, *e.g*., humans. Many are filter feeders, meaning that they are heavily exposed to the bacteria and viruses found in the ocean. In addition, many are sessile and live the majority of their life attached to one spot. To avoid pathogenic infections and pathogenic attacks and to prevent being overgrown by other species, invertebrates have developed a chemical defense strategy for protection by producing secondary metabolites. For example, these secondary metabolites can, *e.g.*, be antibacterial or toxic to predators (14,24,25). As mentioned above, it is the secondary metabolites that have been particularly successful as pharmaceuticals. Many marine natural products isolated from invertebrates have shown therapeutic effects such as antimicrobial, antihypertensive, anticancer, and anti-inflammatory, among others (25). Therefore, it is easy to understand why we use biomass from marine invertebrates as the starting point for the work conducted as part of this project.

1.2.1 Porifera

Species belong to the *Porifera* phylum are among the simplest multicellular animals that exist and are commonly known as sponges. They consist of numerous cells aggregated and organized into a recognizable organism. Their bodies consist of pores, holes, and chambers. Water is transported through these openings into something called spongocoel. In the spongocoel, food and oxygen are extracted by flagellated choanocytes, and carbon dioxide and waste exit through

a large pore called the osculum (14). Sponges also consist of microorganisms living on the surface or inside their bodies. These microbes can include bacteria, fungi, and microalgae. This symbiosis between the sponge and the microbe is advantageous for the sponges survival as it, for example, can contribute to the hosts defense by producing bioactive metabolites (26). In some cases, it has been thought that the sponges produced the isolated bioactive compound, while later, it has been confirmed that it has its origin from the bacteria living in symbiosis with it (17). In this project, a species identified as *Porifera Indet* (species is not determined) from the phylum Porifera was investigated.

1.2.2 Cnidaria

The phylum *Cnidaria* is a large taxonomic group that consists of a variety of simple but versatile marine organisms such as sea anemones, jellyfish, and corals. Their bodies are separated into an inner and an outer body with a gelatinous layer called the mesoglea. A mouth is located centrally and is surrounded by tentacles making it possible to ingest and capture food (14). Like all invertebrates, the *Cnidaria* also lack an adaptive immune system and to survive, they have provided defense mechanisms such as producing powerful venoms and toxins (27,28). In this project, the species *Lucernaria quadricornis* was investigated (Figure 1). *Lucernaria quadricornis* occurs in the shallow north Atlantic and are under the class *Stauoromedusae*, which typically are small and solitary organisms that live in shallow habitats near the shore (29).

Figure 1: *Picture of Lucernaria quadricornis. The picture is used with permission from Kåre Telnes (30).*

1.3 The bioprospecting pipeline: setup and methods

The bioprospecting pipeline applied in this thesis is based on methods used at Marbio (Figure 2). Marbio is an analytical laboratory working with identification, bioactivity screening, and purification of marine natural products. The pipeline starts with collecting biomasses on research cruises. The biomass is extracted and prefractionated before the fractions are analyzed in various inhouse bioassays. When an active fraction is detected, the fraction is subjected to chemical analysis using high-performance (HPLC) high resolution-mass spectrometry (HR-MS) to identify compounds that could potentially be responsible for the observed bioactivity. When compounds with suspected novel structures or known compounds where bioactivity has not been explored are identified, they are selected for isolation using preparative high-performance liquid chromatography (prep-HPLC). The structures of the isolated compounds are determined using nuclear magnetic resonance spectroscopy (NMR) before the compounds are retested to confirm or disprove their bioactivity (10). This project started with dereplication of fractions found to be active against the human melanoma cancer cell line A2058.

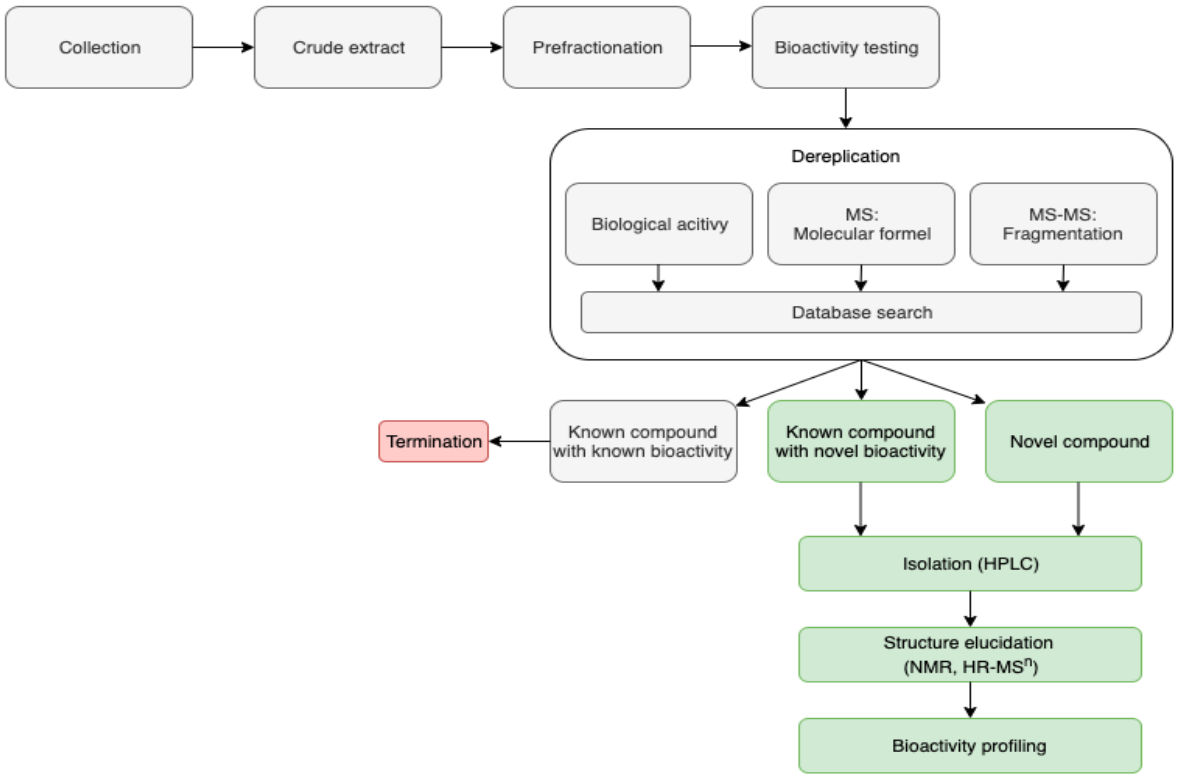

Figure 2: *An overview of the bioprospecting pipeline used at Marbio. Figure made with diagrameditor.com.*

1.3.1 Sample collection, extraction and prefractionation

Prior to the work conducted as a part of this thesis, Artic marine invertebrates were collected in the Barents Sea by UiT - The Arctic University of Tromsø research vessels. The samples were brought to the marine biobank Marbank - Institute of Marine Research Tromsø, which has national responsibility for collecting and storing marine organisms for scientific research. The crude samples were lyophilized before being extracted into organic and aqueous extracts (10). The organic and aqueous extracts were prefractionated before the bioactivity was tested on different bioassays. Prefractionation can be done in several ways, and the most used technique is liquid chromatography (LC), such as flash chromatography or high-pressure liquid chromatography (HPLC), but other techniques such as liquid-liquid partitioning can also be used. Since the crude samples are complex with many different components, it has been shown that prefractionation increases the efficiency of detecting the secondary metabolite responsible for the activity by removing components such as salt, sugar, and lipids (1,10). Prior to this project, each extract was separated into eight fractions descending in polarity from being hydrophilic to lipophilic using flash chromatography.

1.3.2 Bioassay

Bioassays can be divided into two different strategies: phenotypic screening and target-based screening. In phenotypic screening, the biological effect on living organisms or cells is used to detect an activity (31). The assays are based on knowledge of diseases and evaluate the compounds effect on the entire system instead of a single target. The approach does not require any knowledge about the compound's mode of action or the biology of the disease. This is also a disadvantage of this strategy as the mode of action is not determined. Overall, this type of screening is broadly used and is successful in discovering new drug classes and therapeutics (32,33).

Target-based assays measure the effect a compound has on a biological target. Biological targets can be cellular proteins, enzymes, ion channels, or DNA (34). The aim is to find components that selectively affect the biological target without producing side effects (32). The interaction between the compound and the target-based assay measures the efficacy and toxicity of the bioactive compound without any knowledge of the chemical structure (33,34). Target-based screening is performed *in vitro* and does not consider the stability of cellular enzymes or the penetrance of a cell. Even though the compound has a biological effect *in vitro* does not mean the effect would be the same *in vivo* (35).

Marbio, the research group where this project was carried out, routinely performs bioactivity screening of fractions prepared from extracts of marine invertebrates. The assays used in these screening campaigns include phenotypic cytotoxicity assay using malignant and non-malignant cell lines and antibacterial assay. The setup of the screening campaign is organized to enable testing of a high number of samples in several phenotypic and biochemical assays over a relatively short period of time. This setup is termed high throughput screening (HTS) (36). HTS conducted at Marbio is designed to detect fractions with bioactivity to nominate samples for chemical investigation and simultaneously exclude furth work on biomass without bioactivity. When a sample shows biological activity, various techniques are used to identify the compound(s) and finally determine their chemical structure(s). As the last step in the bioprospecting pipeline, the bioactivity profile of the isolated compound(s) is evaluated in a selection of bioassays to confirm or disprove their biological activity

1.3.3 Dereplication

Dereplication is the analysis of a natural product, a fraction or extract for structural information and evaluating whether these compound(s) are novel, known, or known with novel bioactivity (35,37). Dereplication is often conducted using HPLC coupled to an HR-MS (*e.g.*, Time of flight). High-performance liquid chromatography high resolution-mass spectrometry (HPLC-HR-MS) is a highly accurate and sensitive method, which is an advantage as the bioactive fractions pinpointed through the initial HTS step often are complex (38). There are several ways of performing a dereplication analysis. At Marbio, a common strategy is to perform HPLC-HR-MS analysis of the bioactive fraction and two neighboring inactive fractions. This will enable to identification of compounds that are exclusively present in the bioactive fraction and thus are suspected to be the active component. The procedure is exemplified by the results of a previous dereplication round of fractions produced from the Echinodermata *Pteraster militaris* in Figure 3**.** Fraction 5 was active against human cancer cells, while fractions 6 and 4 showed no activity. Based on dereplication analysis, one compound (highlighted with arrow in Figure 3) was exclusively present in fraction 5 and was suspected of causing the observed bioactivity. Then the HPLC-HR-MS software makes it possible to calculate the elemental composition of the compound by using the isotopic pattern and the accurate mass of the target compound. Search in online databases are used to consider whether the compound is novel or not (10). Since the probability of rediscovering a known compound is relatively high, it is essential to achieve dereplication at an early stage in the bioprospecting pipeline to exclude all known

compounds and save precious time (35). Dereplication is achieved as a pointer on which compound to isolate, but it is not a guarantee that the isolated compound is responsible for the observed biological activity (39).

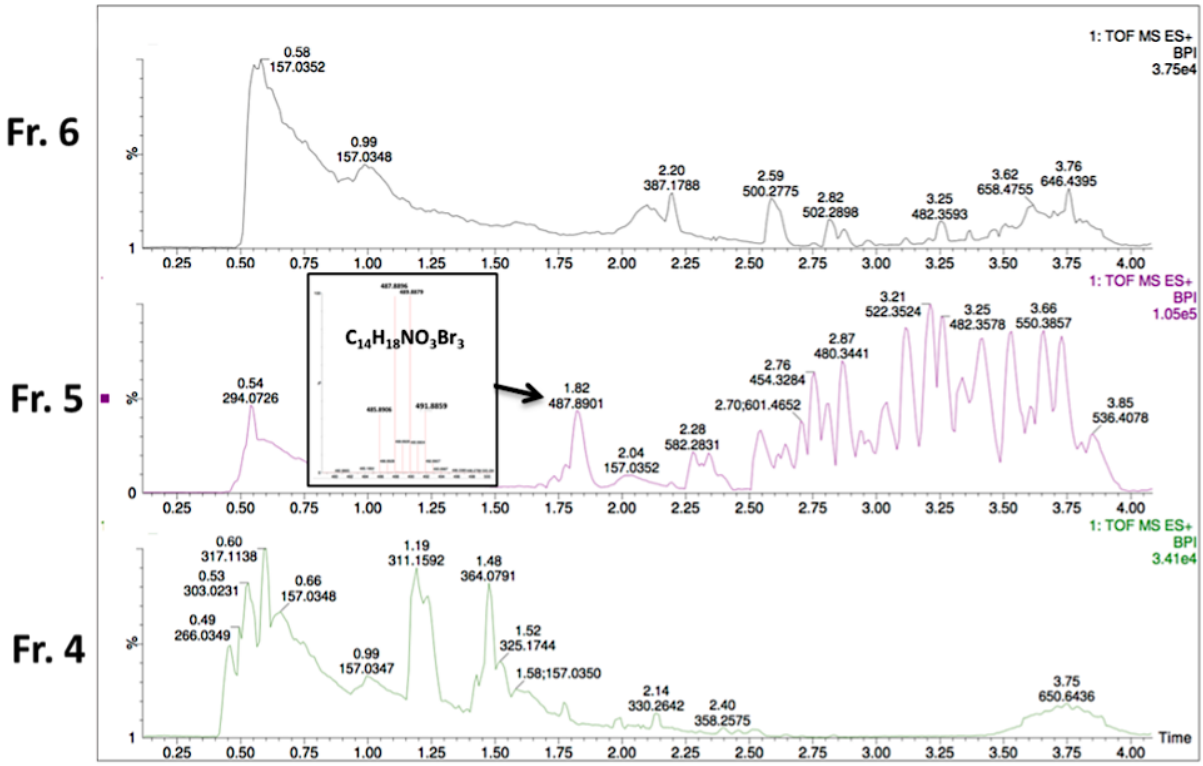

***Figure 3**: Example of a dereplication analysis previously conducted at Marbio of a bioactive fraction (Fr 5) from Pteraster militaris. Data not published.*

1.3.4 Purification of compound

Extracts of marine invertebrates are complex and consist of a matrix with many different compounds. Purification means separating the target compound(s) from the matrix by removing unwanted compounds from a sample. In this project, mass guided preparative HPLC was conducted to isolate the target compound from the unwanted impurities for structure elucidation and bioactivity profiling (40).

1.3.4.1 Preparative high performance liquid chromatography

Preparative high-performance liquid chromatography (prep-HPLC) is a chromatographic method used to separate or isolate molecules from a complex matrix (41). In prep-HPLC, the column is filled with a packing material which constitute the stationary phase. The mobile phase will flow through the column, and when the sample is injected, the analytes are distributed between the stationary phase in the column and the mobile phase. Several physicochemical

factors related to the target compound, the packing material on the stationary phase and the composition of the mobile phase results in different retention time of the compounds. Because of different strengths in interactions with the stationary phase, the compounds will move through the column with different velocities and therefore elute at different times (42,43). The aim with prep-HPLC is to isolate the target compound from the rest of the matrix, and by programming the instrument, fractions can be collected by time or by a signal such as mass (*m/z*) or UV-absorption. With "time fraction" the instrument is programmed to collect the fraction over a set time period. Collecting by mass is often referred to as "mass guided fractionation". With this method, an exact mass will trigger the system to collect the fraction (41).

The separation can occur in different chromatographic modes, such as reverse phase, normal phase, size-exclusion, and ion-pair chromatography. Reverse phase (RP) chromatography is one of the most used separation methods in LC and is usually the first technique used when purifying and analyzing unknown compounds from a complex matrix (41). Reverse phase chromatography has a hydrophobic stationary phase in the column. The mobile phase consists of water and an organic solvent miscible with water. Water is a weak mobile phase, while organic solvents such as acetonitrile and methanol are strong mobile phases. An acid or base is often added to the mobile phase to control the pH and charge of the compounds. When the sample is injected onto the column, hydrophobic interactions such as Van der Waals forces are formed between the analyte and the non-polar stationary phase. Non-polar compounds are retained stronger to the stationary phase than more polar compounds and will elute later and have a longer retention time (43).

The retention time of a reverse phase HPLC relates to the lipophilicity of a compound. The lipophilicity of a compound is important when looking for new drug lead compounds as it is desirable for a drug to have both lipophilic and hydrophilic characteristics. Compounds with a short retention time will have more hydrophilic properties and have a low permeability through biological barriers as membranes. Compounds with a long retention time will be highly lipophilic and are able to cross biological membranes but result in low bioavailability because of low solubility in the blood (44).

Before starting the isolation of the target compound with preparative HPLC, column screening is usually conducted. Columns varies in their lipophilicity which results in different affinities to the compounds. During column screening different columns are consequently tested to find the column that provides optimal separation of the target compound from the other unwanted compounds (45).

1.3.5 Mass spectrometry

A mass spectrometer (MS) is a method used for measuring the masses of molecules, atoms, or fragments of molecules (43). The molecules need to be ionized in the ion source in order to be detected by the detector. Several ion sources can be used, such as electrospray ionization (ESI), electron ionization (EI), and matrix-assisted laser desorption/ionization (MALDI), among others (46). The ionization source used in this project is ESI and will be further described below. In the mass analyzer, the ions are separated according to their *m/z*-ratio before they are detected in proportion to their abundance. The ions are converted into electrical signals transmitted to a data processing system, and a mass spectrum is generated. The mass spectrum presents the number of ions detected at each value of the *m/z*-ratio and provides information about the isotopic pattern and the mass (43).

1.3.5.1 Electrospray ionization

Electrospray ionization (ESI) is a soft ionization method that promotes molecular ions without typically producing fragments (43). In LC-MS, ions are produced by applying a strong electric field under atmospheric pressure to the mobile phase through a capillary tube. The electric potential at the capillary results in an aerosol of highly charged droplets of the mobile phase containing the analyte. The electrostatic forces in the highly charged droplets increase the surface tension in the droplets to a level that they break into smaller droplets. This process continues until the ions and mobile phase are in gas phases. A drying gas and heat help the droplets to evaporate, resulting in free ions that are drawn to the mass analyzer through a cone with an opposite charge of the ions. The charge in the capillary tube can form both positive and negative ions depending on the voltage (43,46). In positive ionization mode, the molecule is protonated and is observed as $[M+H]^+$ ions. In negative ionization mode, the molecule releases a proton and is observed as $[M-H]^-$ ions. In positive mode, adduct ions such as $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ can be observed in the spectra, while in negative mode, adduct ions such as $[M+COO]^-$ and $[M+Cl]^-$ can be observed (47,48).

1.3.5.2 Quadrupole

A quadrupole is a mass analyzer consisting of four circular rod electrodes which are placed in parallel diagonal distances from each other. The four electrodes are connected electrically with each other, where one pair is positively charged, and the other is negatively charged (49). Quadrupoles separate ions according to their *m/z*-value by applying a constant voltage (dc) and a radio frequency oscillating voltage (ORF) to the electrodes. By varying the voltage, only ions with a specific *m/z*-value are allowed to reach the detector. The rest will collide with the electrodes and not pass through the quadrupole and reach the detector. The instrument can be used in different modes, such as full scan or selected ion monitoring (SIM) (43).

1.3.5.3 Time of flight

A time-of-flight (ToF) mass spectrometer consists of an ion source, an accelerating grid, a field-free tube called flight tube and a detector (50). The ions are formed through an electric potential in the ion source. In the accelerating grid, all the ions are applied with the same kinetic energy and accelerated in pulses at the same time through the flight tube (43). Ions with a low *m/z*-value will fly with a higher velocity in the flight tube than ions with a higher *m/z*-value. The *m/z*-values will therefore be separated according to their velocities. In the ToF instrument, the *m/z*-values are calculated by the flight time of the ions, the kinetic energy, and the distance between the pusher and the detector (50,51). ToF instruments are high-resolution mass analyzers (HR-MS) and can determine the molecular weight of substances with an accuracy of up to four decimals places. This means that the instrument is extremely precise and can separate ions with similar *m/z*-value, making HR-MS valuable for researchers working with isolation of natural products and novel compounds (51).

1.3.6 Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance spectroscopy (NMR) technique records the change in energy that occurs when atomic nuclei in an external magnetic field are exposed to electromagnetic radiation. The NMR technique is most widely used to map the carbon-hydrogen framework of molecules. ^{13}C and ^{1}H are nuclei that spin around their own axis, and when they are exposed to an external magnetic field (b0), the spins of the magnetic nuclei are oriented either aligned parallel or antiparallel to the external field (52). The parallel orientation has a lower energy state which is more favored than the antiparallel orientation. Transitions between these states can be stimulated with irradiation with radio frequency (rf) waves. With irradiation the lower

energy state “spin-flips” to a higher energy state, and when the rf is switched of, the atomic nuclei in a high energy state level return to the low energy level, and the absorbed rf energy is emitted. This energy will be detected as a spectral line called resonance signal and is displayed as an NMR spectrum (52,53). Each nucleus in a molecule does not come into resonance at the same frequency of the applied field, and since the electrons in a molecule exist in different electronic environments from each other, they can shield a nearby nucleus from the applied field. Each nucleus produces a unique absorption signal called the chemical shift (δ) (51).

The NMR spectrum can be either *one*-dimensional (1D) or *two*-dimensional (2D). The 1D-NMR experiments are analyses of a single nucleus, and the spectrum has one frequency axis corresponding to the chemical shift (δ) and the other axis corresponding to the signal intensity. An example of 1D is ^{1}H and ^{13}C spectra. The 2D-NMR experiments are based on the couplings between two nuclei. The 2D-spectrum has two frequency axes, and the intensity of the signals is displayed in the third dimension (54). Examples of 2D-NMR techniques are correlated spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum correlation, (HMQC), and nuclear overhauser enhancement spectroscopy (NOESY) (1,55).

1.3.7 Bioactivity screening

1.3.7.1 Anticancer assay

In this project, it was performed an anticancer assay on a human melanoma cancer cell line (A2058). Cancer is a disease where uncontrolled proliferation of the cells in the body occurs. It is one of the leading causes of death globally, and it exists more than 200 different types of cancers where some is more lethal than others (56). Melanoma is an aggressive type of skin cancer with high metastatic potential and Norway is one of the countries in Europe with the highest incidence and mortality of this type of cancer (57). The melanoma cancer cells have also started developing resistance to cytotoxic agents and it is therefore urgent to find new compounds with anticancer activity (58).

1.3.7.2 Antibacterial growth inhibition assay

In this project, it was performed an antibacterial growth inhibition assay on five bacterial strains *E. faecalis, E. coli, P. Aeruginosa, S. agalactiae* and *S. aureus.* Bacteria is divided into Gram-positive pathogens and Gram-negative pathogens. One main difference between Gram-positive and Gram-negative pathogens, is that Gram-negative pathogens consist of an extra cell membrane that gives the bacteria protection compared to Gram positive pathogens. This makes Gram-positive pathogens generally more available to antimicrobial agents. *S. aureus, E. faecalis* and *S. agalactiae* are Gram-positive pathogens, while *E. coli* and *P. Aeruginosa* are Gram-negative pathogens (59). Antibiotic resistance is an increasing threat to humans today as some bacterial strains have evolved mechanisms to escape the effect of antibiotics. It is therefore necessary to discover new compounds against bacterial pathogens due to the antibiotic resistance. In 2017, the world health organization (WHO) presented a priority list of pathogens resistant to antibiotics ranging from priority 1 to 3. Priority 1 was listed as critical, priority 2 as high and priority 3 as medium (60).

2 Aim of the project

The aim of this project was to isolate and characterize bioactive secondary metabolites from Arctic marine invertebrates that previously had shown activity against a human melanoma cancer cell line (A2058).

The main objectives of the project were:

- Dereplication of the bioactive fractions to identify compound(s) that could potentially be responsible for the observed bioactivity.
- Isolation of the target compound(s) with preparative HPLC
- Structure elucidation using NMR
- Bioactivity screening of the isolated compound(s) in bioassays to evaluate the anticancer and antibacterial activity.

3 Materials and methods

3.1 Sample handling prior to the start of this project

The samples that were used as a starting point of the work conducted as a part of this thesis can be seen in Table 1.

Table 1: *Overview of flash fractions from marine invertebrates that showed activity against a human melanoma cancer cell line (A2058). The organic extract is abbreviated L, and the water extract is abbreviated W.*

Sample name	Fractions with activity against A2058	Weight Aqueous extract (g)	Weight organic extract (g)	Family	Species
M18001	W5-6-7 L1-2-5-6	99.3	20.2	*Porifera*	*indet*
M18003	W5 L4-5-7	55.7	NA*	*Securiflustra*	*securifrons*
M18004	L4	86.2	13.6	*Lucernaria*	*quadricornis*
M18005	L7-8	58.8	4.0	*Phycodrys*	*rubens*
M18008	W5-6 L4-5	49.3	21.4	*Porifera*	*indet*
M18011	W5 L3-4-5-6	5.2	3.2	*Algae*	*mix*
M18012	L4-5-6	16.4	2.4	*Desmarestia*	*viridis*
M18014	W1-2-3-5 L2-3-4-5	12.4	13.1	*Saxifraga*	*oppositifolia*
M18015	W2-3-5 L3-5	7.4	3.0	*Viridiplantae*	*indet*
M18016	W2-3 L7	41.7	7.1	*Ascidiacea*	*indet*
M18018	W5-6-7	30.0	11.5	*Dendrodoa*	*aggregata*
M18019	W5	19.3	3.1	*Leptychaster*	*arcticus*
M18022	W5 L4	37.0	2.6	*Polysiphonia*	*stricta*
M18024	W3-6	33.6	23.7	*Fucus*	*evanescens*
M18027	W4-5-6 L4	31.8	2.9	*Porifera*	*indet*
M18029	L3-4	115.1	2.3	*Cynaea*	*capillata*
M18032	L5	9.2	1.8	*Porifera*	*indet*
M18033	L5	10.3	9.9	*Haliclona*	*sp*
M18034	W5 L5	38.3	4.9	*Porifera*	*indet*
M18035	L4	53.0	2.0	*Actinostola*	*callosa*
M18036	L7	249.4	3.0	*Gracilechinus*	*acutus*
M18040	L1-2-3	36.9	2.7	*Brada*	*inhabilis*
M18043	W5	16.4	2.8	*Tethya*	*norvegica*
M19019	W5	89.9	8.6	*Volutopsius*	*norvegicus*
M19023	W5	25.0	3.5	*Neptunea*	*despecta*

*NA=Not available

Arctic marine invertebrates are collected with different collection methods at varying locations in the Barents Sea. The crude samples are sorted and taxonomically determined by the Norwegian Biobank of Arctic Marine Organisms, Marbank. The biomass samples are given names based on their preparation year. Within each year, the individual samples are given a

production number. For instance, a sample produced in 2018 is named M18001, where 18 refers to 2018 and 001 to this sample being the one that was first extracted this year. The samples are lyophilized before being extracted into an organic and an aqueous extract and are given the same name as the biomass sample with the suffix-W (aqueous) or-L (organic). The individual fractions are named according to their originating biomass and extract names and added the suffix -1, -2 up to -8, referring to the different flash fractions. For example, flash fraction 3 of the aqueous extract produced from biomass sample M18001 would be named M18001-W-3. The bioactivity of the flash fractions was tested on a human melanoma cancer cell line (A2058).

3.2 Sample selection

In this project, four of the twenty-five samples in Table 1 were selected to proceed to dereplication. Dereplication is a technique used to identify compounds that could potentially be responsible for the observed bioactivity. The ability of the species from sample M18004 and M19019 to produce secondary metabolites has not been assessed previously, making the samples interesting. All four samples also had a high amount of extracts to work with and were therefore selected for this project. Table 2 shows the collection details of the four selected marine invertebrates.

Table 2: *An overview of the collection of the marine invertebrates selected as a part of this project. The table shows the date, location, depth, and method for collecting the different species.*

Sample name/Species	Collection date	Geographic position	Location	Depth (meter)	Collection method
M18003 *S. securifrons*	12.08.2016	79.18N 19.59E	Hinlopen, Von Otterøya	35	Triangular bottom scrape
M18004 *L. quadricornis*	05.08.2016	74.33N 22.33E	Spitsbergenbanken	105	Agassiz dredge trawl
M18008 *P. indet*	20.09.2017	80.20N 12.70E	Spitsbergen	170	Bottom trawl
M19019 *V. norvegicus*	01.10.2008	74.87N 16.93E	Northeast Barents Sea	379	Trawl

As a part of this project, ten flash fractions from the four species were selected for dereplication. A complete list with sample names used through this thesis is found in Table 3.

Table 3: *The ten flash fractions from the four different species selected for dereplication.*

Extract/Species	Extract	Fraction	Sample name
M18003 *S. securifrons*	W L L L	5 4 5 7	M18003-W-05 M18003-L-04 M18003-L-05 M18003-L-07
M18004 *L. quadricornis*	L	4	M18004-L-04
M18008 *P. indet*	W W L L	5 6 4 5	M18008-W-05 M18008-W-06 M18008-L-04 M18008-L-05
M19019 *V. norvegicus*	W	5	M19019-W-05

3.3 Dereplication

The ten active flash fractions and the non-active fractions before and after the active fractions from the four selected samples M18003, M18004, M18008 and M19019 were analyzed with HPLC-HR-MS. The equipment and solvents used for the analysis are listed in Table 4.

Table 4: *Equipment and chemicals used during dereplication analysis of flash fraction from M18003, M18004, M18008 and M19019 with HPLC-HR-MS.*

Materials/Equipment	Distributor
MilliQ-H_2O	Millipore, Billerica, MA, USA
MeOH LC-MS Ultra CHROMASOLV®	Thermo Fisher Scientific, MA, USA
LiChrosol® Acetonitrile (hypergrade for LC-MS)	Merck KGaA, Germany
Formic acid UPLC/MS (99%)	Biosolve B.V. Netherland
Vion® IMS qTOF	Waters, MA, USA
Acquity I-Class UPLC®	Waters, MA, USA
Acquity UPLC® BEH C18, 2.1x100mm, 1.7 µM Column	Waters, MA, USA
Acquity Sample Manager FTN	Waters, MA, USA
Acquity UPLC® I-Class Binary Solvent Manager	Waters, MA, USA
Acquity UPLC® PDA Detector	Waters, MA, USA
Acquity UPLC® Column Manager	Waters, MA, USA

An amount of 5 µL of the flash fractions was mixed with 100 µL methanol (MeOH) and analyzed on an Acquity I-class UPLC with a C18 column coupled to a PDA detector and a Vion IMS Q-ToF. The mobile phase gradient and instrument parameters are listed in Table 5 and Table 6.

Table 5: *Mobile phase gradient used during analysis of flash fractions from M18003, M18004, M18008 and M19019 with HPLC-HR-MS. Mobile phase A: MilliQ-H_2O with 0.1% formic acid, mobile phase B: acetonitrile with 0.1% formic acid.*

Time (min)	Flow (mL/min)	A (%)	B (%)
Initial	0.450	90	10
12.00	0.450	0	100
13.50	0.450	0	100

Table 6: *Instrument parameters for the mass spectrometer used when analyzing flash fractions from M18003, M18004, M18008 and M19019.*

Parameters	Setting
Low mass (*m/z*)	50
High mass (*m/z*)	2000
Source type	ESI+
Source temperature	120
Desolvation temperature	350
Desolvation gas flow (liters/hour)	600
Capillary voltage (kilovolt)	0.80
Cone voltage (volt)	30
Cone gas flow (liters/hour)	50

M18004-L-04 and M18008-W-05 were selected for further isolation.

M19019 showed no compounds of interest, while the compounds of interest from M18003 were already known. The two samples M19019 and M1803 were therefore terminated from the project.

3.4 Column screening

3.4.1 Sample preparation

The organic extract M18004-L-04 and the aqueous extract M18008-W-05 were prepared for column screening with preparative HPLC. The equipment and chemicals used are listed in Table 7.

Table 7: *Equipment and chemicals used to prepare the organic extract M18004-L-04 and the aqueous extract M18008-W-05 for column screening.*

Materials/Equipment	Distributor
MeOH (>99.9%)	Sigma-Aldrich, St.Louis, MO, USA
Hexane (>97.0%)	Sigma-Aldrich, St.Louis, MO, USA
Acetonitrile prepsolv ®	Merck, KGaA, Germany
Laborota 4002 – control Rotary evaporator	Heidolph Instruments GmbH & Co. KG Schwalbach, Germany
Haerus Multifuge 3-SR	Kendro Laboratory products, Odterode, Germany

<u>Aqueous extract of M18008-W-05:</u>

Around two grams of the lyophilized aqueous extract was weighed on an analytic scale and transferred to a tared Falcon tube and 30 mL 90% acetonitrile (ACN) was added. The mixture was shaken and then centrifuged at 4000 rounds per minute at 20°C for 2 minutes. The process led to a pellet and a clear liquid supernatant. The supernatant was transferred to a Florence flask with a pipette, and the process was repeated two times in an attempt to get as much as possible of the extract in the supernatant and to eliminate un-dissolvable compounds. The supernatant was removed between each centrifugation step and pooled in the same Florence flask. The sample was dried on a rotary evaporator under reduced pressure. The process was repeated under the same conditions as mentioned above, and the supernatant was transferred to the same Florence flask as in the first round. The sample was dried on a rotary evaporator until it was almost dry before being stored at 4°C in the refrigerator.

Before injecting the sample into the preparative HPLC for a column screening, the sample was dissolved in 5 mL methanol.

<u>Organic extract of M18004-L-04:</u>

Around two grams of the lyophilized organic extract was weighed on an analytic scale and transferred to an Erlenmeyer flask. The weighed extract was transferred to a 500 mL separatory funnel by dissolving the organic extract in 150 mL hexane. To get as much as possible of the

organic extract to the funnel, 20 mL hexane was added to dissolve what was left in the Erlenmeyer flask, so the total amount of hexane used was 170mL. Then 150 ml 90% ACN was added to the separatory funnel and shaken. After 10 minutes the two immiscible phases were separated, and the ACN-phase ended up at the bottom and was collected in a Florence flask. This process was repeated two times where the ACN-phase ended up at the bottom and was collected in the same Florence flask. The sample was dried on a rotary evaporator until the sample was almost dry. The process was repeated in an attempt to get enough sample and to remove un-dissolvable compounds before the sample was stored at 4°C in the refrigerator.
Before injecting the sample into the preparative HPLC for a column screening, the sample was dissolved in 3 mL methanol.

3.4.2 Column screening

Column screening was conducted to select a column with optimal separation of the compounds. The aqueous and the organic extract were dissolved in methanol and run through the preparative HPLC. The equipment and solvents used for the column screening can be seen in Table 8.

Table 8: *Equipment and chemicals used in column screening of the organic extract M18004-L-04 and the aqueous extract M18008-W-05.*

Materials/Equipment	Distributor
Xterra® Prep RP18 10 µM 10x300 mm column	Waters, MA, USA
Atlantis® Prep dC18 10 µM 10x250mm column	Waters, MA, USA
XSELECT™ CSH™ Phenyl-Hexyl Prep 5 µM 10x250 column	Waters, MA, USA
SunFire™ Prep C18 5 µM 10x250mm column	Waters, MA, USA
600 Controller	Waters, MA, USA
2996 photodiodide array detector	Waters, MA, USA
3100 mass detector	Waters, MA, USA
2767 sample manager	Waters, MA, USA
Flow splitter	Waters, MA, USA
Prep degasser	Waters, MA, USA
515 HPLC pump	Waters, MA, USA
SoftWare MassLynx 4.1	Waters, MA, USA
Acetonitrile prepsolv ® 1139758	Merck, KGaA, Germany
Formic acid	Sigma-Aldrich, MO, USA
Methanol 20864	Sigma-Aldrich, MO, USA
MilliQ-H_2O	Merck, KGaA, Germany
SC250 Express SpeedVac Concentrator	Thermo Fischer Scientific, MA, USA
Heto PowerDry® PL9000 Freeze Dryer	Thermo Fischer Scientific, MA, USA

The sample was injected into four columns: SunFire, Xterra C18, Atlantis and Phenyl-Hexyl. The gradient of the mobile phases was the same on all the tested columns and can be seen in Table 9. The instrument parameters for the preparative HPLC instrument are listed in Table 10.

Table 9: *Mobile phase gradient used during column screening of the organic extract 18004-L-04 and the aqueous extract M18008-W-05 with preparative HPLC. Mobile phase A: MilliQ-H_2O with 0.1% formic acid, mobile phase B: acetonitrile with 0.1% formic acid.*

Time (min)	Flow (mL/min)	A (%)	B (%)
Initial	6.00	90	10
15.00	6.00	0	100
20.00	6.00	0	100
20.10	6.00	90	10
21.00	6.00	90	10

Table 10*: Instrument parameters for the mass spectrometer used during column screening of the organic extract M18004-L-04 and the aqueous extract M18008-W-05.*

Parameters	Settings
Source temperature	120
Desolvation Temperature	300
Desolvation gas flow (liters/hour)	650
Low mass (*m/z*)	200
High mass (*m/z*)	2000
Source type	ESI+

3.5 Isolation

3.5.1 Sample preparation

The samples were prepared as described in section 3.4.1 “Sample preparation”. The equipment and chemicals used for the sample preparation can be seen in Table 11.

Table 11: *Equipment and chemicals used to prepare the organic extract 18004-L-04 and the aqueous extract M18008-W-05 for isolation with preparative HPLC.*

Materials/Equipment	Distributor
MeOH (>99.9%)	Sigma-Aldrich, St.Louis, MO, USA
Hexane (>97.0%)	Sigma-Aldrich, St.Louis, MO, USA
Acetonitrile prepsolv ®	Merck, KGaA, Germany
Laborota 4002 – control Rotary evaporator	Heidolph Instruments GmbH & Co. KG Schwalbach, Germany
Haerus Multifuge 3-SR	Kendro Laboratory products, Odterode, Germany

The preparation session was repeated three times for M18004-L-04 and five times for M18008-W-05 to get enough material of the extracts. Before starting the isolation of compounds from the extracts, M18008-W-05 was dissolved in 8 mL methanol and M18004-L-04 was dissolved in 5 mL methanol.

3.5.2 Isolation of target compounds from the extracts M18004-L-04 and M18008-W-05

The isolation of target compounds from the organic extract M18004-L-04 and aqueous extract M18008-W-05 were conducted with preparative HPLC. The equipment and chemicals used during isolation can be seen in Table 12.

Table 12: *Equipment and chemicals used during isolation of compounds from the organic extract M18004-L-04 and the aqueous extract M18008-W-05 with preparative HPLC.*

Materials/Equipment	Distributor
Xterra® Prep RP18 10 µM 10x300 mm column	Waters, MA, USA
Atlantis® Prep dC18 10 µM 10x250mm column	Waters, MA, USA
XSELECT™ CSH™ Phenyl-Hexyl Prep 5 µM 10x250 column	Waters, MA, USA
SoftWare MassLynx 4.1	Waters, MA, USA
600 Controller	Waters, MA, USA
2996 photodiodide array detector	Waters, MA, USA
3100 mass detector	Waters, MA, USA
2767 sample manager	Waters, MA, USA
Flow splitter	Waters, MA, USA
Prep degasser	Waters, MA, USA
515 HPLC pump	Waters, MA, USA
Acetonitrile prepsolv ® 1139758	Sigma-Aldrich, MO, USA
Formic acid	Sigma-Aldrich, MO, USA
Methanol 20864	Sigma-Aldrich, MO, USA
MilliQ-H_2O	Merck, KGaA, Germany
SC250 Express SpeedVac Concentrator	Thermo Fischer Scientific, MA, USA
Heto PowerDry® PL9000 Freeze Dryer	Thermo Fischer Scientific, MA, USA

Isolation of the target compounds from extract M18004-L-04 and M18008-W-05 was conducted on reverse phase HPLC columns. The first and the second isolation of M18004-L-04 were obtained with a Phenyl-hexyl column. The first isolation of M18008-W-05 was obtained with an Xterra column, but since the mass from dereplication could not be detected, M18008-W-05 was terminated from the project. The gradient of the mobile phase and the

instrument parameters were the same as used in the column screening (Table 9 and Table 10). The compounds were collected using "time-based fractionation" and "mass triggered fractionation". The collection method during the first isolation is listed in Table 13**.**

Table 13: *An overview of the first isolation of the target compounds from the organic extract M18004-L-04 on preparative HPLC. The table shows the protonated mass of the collected fractions and the collection method.*

Compound	Protonated mass [M+H]	Collection method
M18004-L-04	222	Mass triggered
M18004-L-04	366	Time Fraction

After the first isolation, the collected fractions were dried on a rotary evaporator and dissolved in methanol before conducting the second isolation process. Table 14 summarizes the protonated mass of the collected fractions and the collection method used in the second isolation. Each compound was given a name based on the mass that was isolated. For example, the mass 222 was isolated from extract M18004-L-04 and is named M18004-L-04-222.

Table 14: *An overview of the second purification of target compounds from the organic extract M18004-L-04. The table show the protonated mass of the collected fractions and the collection method.*

Compound	Protonated mass [M+H]	Collection method
M18004-L-04-222	222	Mass triggered
M18004-L-04-255-1	255	Time fraction
M18004-L-04-366	366	Time fraction
M18004-L-04-255-2	255	Mass triggered
M18004-L-04-243	243	Mass triggered

The collected fractions after the second isolation were dried on a rotary evaporator. Target compounds from extract M18004-L-04 were isolated in two rounds with the same column as listed in Table 15.

Table 15: *An overview of the columns used in the first and second isolation of target compounds from extract M18004-L-04 with preparative HPLC.*

Compound	HPLC separation round	Column packing material
M18004-L-04-222	First	Phenyl-hexyl
	Second	Phenyl-hexyl
M18004-L-04-243	First	Phenyl-hexyl
	Second	Phenyl-hexyl
M18004-L-04-255-1	First	Phenyl-hexyl
	Second	Phenyl-hexyl
M18004-L-04-255-2	First	Phenyl-hexyl
	Second	Phenyl-hexyl
M18004-L-04-366	First	Phenyl-hexyl
	Second	Phenyl-hexyl

3.6 Nuclear magnetic resonance spectroscopy

The compounds M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-366 were isolated in a sufficient quantity and were sent for structure elucidation with NMR. M18004-L-04-222 and M18004-L-04-243 were not isolated in a sufficient amount to obtain NMR data and were terminated from the project. This part of the project and the interpretation of the data was performed by Dr. Johan Isaksson at the Department of Chemistry at UiT – the Arctic University of Norway.

3.7 Bioactivity screening

A stock solution of M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-366 was made by dissolving the samples in (DMSO) to a concentration of 10 mM. The stock solution was further diluted with Milli-Q-H_20 to 20, 100 and 200 μM for the antibacterial growth inhibition assay. For the anticancer assay, the stock was diluted in Dulbecco´s Modified Eagle´s Medium (DMED) to 20, 100 and 200 μM.

3.7.1 Anticancer assay

In the anticancer screening, the samples were tested on a human melanoma cancer cell-line A2058. The assay was performed in a class II safety cabinet to provide aseptic conditions. The materials and equipment used to perform the anti-cancer assay are listed in Table 16.

Table 16: *An overview of the materials and equipment used when performing anti-cancer assay.*

Materials/Equipment	Distributor
DMEM (Dulbecco´s Modified Eagle´s Medium), D6171-500ML	Merck Life science AS (sigma)
Gentamycin, A2712	VWR (Biochrom)
Glutamine stable 200 mM, X0551-100	VWR (Biowest)
FBS (Fetal Bovine Serum), S1810-500	VWR (Biowest)
Trypsin, X0930	VWR (Biowest)
Trypan blue, T8154	Merck Life Science AS
PBS (phosphate buffer saline)	-
DMSO (Dimethyl sulfoxide), D4540	Merck Life Science AS
CellTiter 96® Aqueous One Solution reagent, G3581	Nerliens (Promega)

3.7.1.1 Preparation of cell culture

The human melanoma cancer cells (A2058) were kept in an appropriate cell culture medium called Dulbecco´s Modified Eagle´s Medium (DMEM) as seen in Table 17.

Table 17*: An overview of cell type, cell line and ATCC number with appropriate cell culture media used when performing anti-cancer assay.*

Cell type (ATCC number)	Cell line	Cell culture media
A2058 (ATCC CRL-114)	Human melanoma, malignant cell	Dulbecco´s Modified Eagle´s - 10% Fetal Bovine serum (FBS) - 1% L-Alanyl-L-glutamine - 0.1% gentamycin

When the cells had a density of 70-80%, the cell-line was split in ratio 1:10-1:20 to preserve the culture. Since the cells were attached to the bottom of the flask, the cell culture was removed from the flask, and phosphate buffer saline (PBS) was added to remove the excess of the cell culture. The next step was adding trypsin to help the cells detach from the bottom of the flask. To ensure each well in the microtiter plate had an amount of 2000 cells, 100 µL trypan blue and

100 µL cell suspension were measured with a Bürker chamber. The trypan blue penetrates the cell membrane of cells with damaged cell membrane or dead cells, binds to the DNA and colors the cell blue. This makes it possible to only count the healthy cells. The cell suspension was diluted with a cell culture medium to make sure the 96-well microtiter plates had $2x10^4$ cells/mL in each well. 100 µL of the diluted cell suspension was transferred to each well, and the plate was incubated at 37°C with 5% CO_2 over the night to allow the settling of cells to the well.

3.7.1.2 Sample preparation for anticancer screening

The target compounds M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-366 were as mentioned diluted in DMEM to a concentration of 20, 100 and 200 µM. When transferring the samples to the cell lines in the 96-well microtiter plates, they were diluted in the ratio 1:2, resulting in a final test concentration of the compounds of 10, 50 and 100 µM. First, the cell culture was removed from the wells, and new fresh cell culture was added in an amount of 50 µL. An amount of 50 µL of the compounds was added in triplicate to the wells. The microtiter plate also had a negative and positive control. The negative control consisted of 100 µL fresh cell culture, which attended to give the cell 100% cell survival. The positive control contained 10% DMSO and 90% fresh cell culture, attending to give the cell 0% cell survival. The plates were incubated at 37°C with 5% CO_2 for 72 hours.

3.7.1.3 Reading the results

After 72 hours of incubation, 10 µL of CellTiter 96® Aqueous One Solution reagent (AQOS) was transferred to each well in the microtiter plate. The plate was incubated at 37°C with 5% CO_2 for 1 hour. The optical density (OD) was measured with DTX 880 Multimode Detector at 490 nm. The equation for calculating the percentage of cell survival is presented below (Equation 1). The cytotoxic activity was scored to the cell survival and was divided into three groups as active (A), inactive (I) or questionable (Q).

Equation 1: *Cell survival is calculated using the given equation. Fractions represent the average OD-value measured in the wells with fractions. Positive control represents the average OD-value measured in the wells with positive controls, while negative controls represent the average OD-value measured in the wells with negative controls.*

$$\text{Cell survival (\%)} = \frac{(\text{Fraction} - \text{Positive control}) \cdot 100\%}{(\text{Negative control} - \text{Positive control})}$$

3.7.2 Antibacterial growth inhibition assay

In the antibacterial growth inhibition assay, the samples were tested on five different pathogens *E. faecalis, E. coli, P. Aeruginosa, S. agalactiae* and *S. aureus.* The materials and equipment used to perform the assay is listed in Table 18.

Table 18: *An overview of the materials and equipment used when performing antibacterial growth inhibition assay.*

Materials/Equipment	Distributor
Enterococcus faecalis, ATCC 29212	LGC Standards
Escherichia coli, ATCC 25922	LGC Standards
Pseudomonas aeruginosa, ATCC 27853	LGC Standards
Staphylococcus aureus, ATCC 25923	LGC Standards
Streptococcus agalactiae, ATCC 12386	LGC Standards
MilliQ-H_2O	Merck Millipore
Mueller-Hinton (MH) broth, 53286	Sigma Aldrich
Brain Heart Infusion (BHI), 275730	Difco
Blood Agar plate	Media Kitchen UNN
Gentamicin, A2712	VWR
Victor Multilabel Counter	PerkinElmer
Shaking incubator 37°C	Heidolph Instrument GmbH
Incubator 37°C, MIR-262	Panasonic healthcare
Microtiter plates, 734-2073	VWR International

3.7.2.1 Preparation of bacterial strains

The five bacterial strains *E. faecalis, E. coli, P. Aeruginosa, S. agalactiae* and *S. aureus* were kept on blood agar plates and were stored in the refrigerator at 4°C. When performing antibacterial growth inhibition assays, a scoop of the five bacterial strains from the five different blood agar plates was transferred to 8 mL growth medium, as shown in Table 19. The bacterial cultures were incubated at 37°C for approximately 20 hours.

Table 19: *An overview of the growth medium and American type culture collection (ATCC) number of the five bacterial strains.*

Bacterium	Growth media
Enterococcus faecalis (ATCC 29212)	Brain heart infusion (BHI)
Escherichia coli (ATCC 25922)	Mueller-Hinton (MH)
Pseudomonas aeruginosa (ATCC 27853)	Mueller-Hinton (MH)
Staphylococcus aureus (ATCC 25923)	Mueller-Hinton (MH)
Streptococcus agalactiae (ATCC 12386)	Brain heart infusion (BHI)

An amount of 2 mL of the bacterial cultures of each pathogen was transferred to an Erlenmeyer flask with 25 mL growth medium and was incubated at 37°C at 100 rounds per minute. After incubation for 1.5 hours for *E. faecalis, E. coli* and *Streptococcus agalactiae* and 2.5 hours for *E. coli* and *P. Aeruginosa* (Table 20), each strain was diluted in the ratio 1:1000 in growth medium so the cultures reached a McFarland standard at 0.5.

Table 20: *An overview of the incubation time and colony forming units/mL (CFU/mL) of the five bacterial strains*

Bacterium	Incubation time (hours)	CFU/mL
Enterococcus faecalis	1.5	$0.5\text{-}3x10^5$
Escherichia coli	1.5	$0.5\text{-}3x10^5$
Pseudomonas aeruginosa	2.5	$3\text{-}7x10^4$
Staphylococcus aureus	2.5	$0.5\text{-}3x10^5$
Streptococcus agalactiae	1.5	$0.5\text{-}3x10^5$

3.7.2.2 Sample preparation for antibacterial growth inhibition assay

The compounds M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-366 were as mentioned diluted with MilliQ-H_2O to a concentration of 20, 100 and 200 µM. When transferring 50 µL the samples to the 96-well microtiter plates (one for each bacterial strain), they were diluted in the ratio 1:2, and the final test concentration of the compounds was 10, 50 and 100 µM. Bacterial suspension (50 µL) was added in duplicates to five different microtiter plates. Each microtiter plate had a negative control and a growth control. The negative control consisted of 50 µL growth media and 50 µL Milli-Q-H_2O and was applied to ensure that the growth medium or Milli-Q-H_2O were not contaminated. The growth control (positive)

consisted of 50 µL Milli-Q-H_2O and 50 µL bacterial suspension and was applied to ensure the bacteria grew normally.

A gentamycin control was prepared in a separate 96-well microtiter plate at a concentration of 0.02-16 µg/ml. 50 µL of gentamycin and 50 µL of the bacterial suspension were added to each well. The gentamycin control was conducted to make sure the bacterial strains had normal reaction to antimicrobial compounds. This was done by finding the lowest concentration of gentamycin that inhibits the growth of the five pathogens. Each bacteria strain has its own minimal inhibitory concentration (MIC) value, and the measured MIC-value for gentamycin for each bacterium was compared to the reference listed in Table 21.

Table 21: *Reference minimal inhibitory concentration (MIC)-value for gentamycin of the five bacterial strains.*

Bacterium	Reference MIC-value for gentamycin (µg/mL)
Enterococcus faecalis	8
Escherichia coli	0.13
Pseudomonas aeruginosa	0.25
Staphylococcus aureus	0.06
Streptococcus agalactiae	4

All the six 96-well microtiter plates with the five different bacteria and the gentamycin control were incubated overnight at 37°C. After 19-20 hours, the optical density (OD) was measured at 600nm in the wells using the Victor plate reader. The antibacterial activity was scored to the measured absorbance and was divided into three groups as active (A), inactive (I), or questionable (Q).

4 Results and discussion

4.1 Dereplication

Flash fractions from the four samples M18003, M18004, M18008 and M19019 (Table 1) showed bioactivity against the human melanoma cancer cell line (A2058) and were chosen for dereplication. The fractions were analyzed with HPLC-HR-MS to identify the target compound(s) that potentially were responsible for the observed bioactivity in the anticancer assay and evaluate whether these compound(s) were novel, known, or known with novel bioactivity (47). The chromatograms of the inactive fractions that eluted before and after the active fractions were compared to the chromatogram of the active fraction to investigate whether there were peaks only present in the active fractions. When a unique peak was observed, the MS software was used to calculate a possible elemental composition by using the isotopic pattern and the accurate mass for the target compound. The elemental composition was used to search in databases to consider if the compound was novel or not. Most compounds that are dereplicated are usually already known, so when a compound with suspected novel structure or known compounds where the bioactivity has not been explored is identified, they are selected for further investigations (10).

M18003 showed a series of peaks with higher abundances in the active fraction compared to the inactive fraction. Searches in databases were found to match compounds previously isolated from *S. securifrons*. This included securidine A and securamine C and E (structures in Figure 4). As the identified securamine family of compounds are known to be potent cytotoxic compounds, as well as several members of the securidine family of compounds have been isolated and their bioactivities have been thoroughly investigated, M18003 was not selected for further examination.

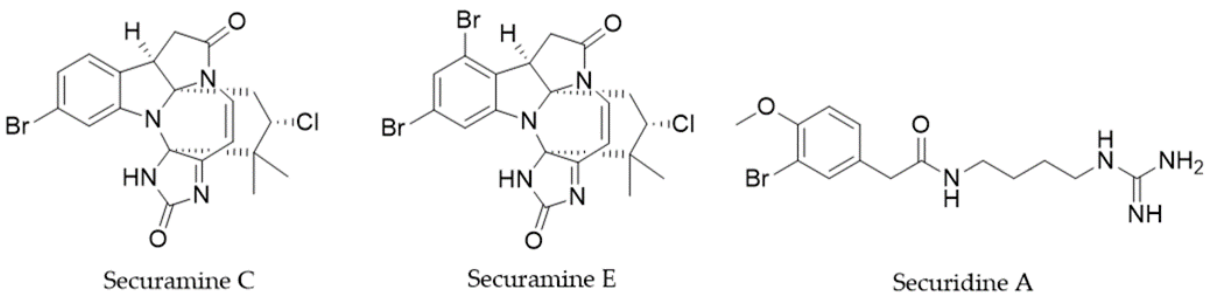

Figure 4: *Structures of securamine C, securamine E and securidine A, which were all present in the active fractions of M18003 (61).*

M18019 showed no obvious compounds of interest in the active fraction and this sample was therefore terminated from the project. Since some molecules only ionize in negative mode (ESI^-) and some only in positive mode (ESI^+), the analysis should be run with both modes to capture as much information about the components as possible. In this project, the fractions from the four samples (Table 3) were only tested in positive mode, and molecules that only ionize in negative mode will not be identified. Due to the time limitations of a master project, it was not prioritized to reanalyze the M18019 using negative mode ionization. M18004 and M18008 were selected for further isolation. The explanation is described in the sections below.

4.1.1 Dereplication of flash fractions from M18004-L

Figure 5 shows the base peak intensity (BPI) chromatogram of the active fraction M18004-L-04 (middle panel) and the inactive fractions M18004-L-03 (top panel) and M18004-L-05 (bottom panel). The peak that eluted with a retention time of 3.08 (Figure 5**)**, and mass of *m/z* 366 (Figure 7) was present at a higher relative abundance in the active fraction than in the inactive fractions as can be seen in the ion chromatogram (Figure 6). The peaks eluting to the right in the chromatogram are most likely lipids, fatty acids, or other compounds not of interest because of lipophilicity. It is desirable for an active substance in a drug to have lipophilic and hydrophilic properties. Thus, is the compound eluting in the middle of the gradient with a retention time of 3.08 and an *m/z* 366 selected for further isolation (44). The elemental composition was determined to be $C_{15}H_{20}BrN_5O$. Searches in relevant databases did not result in any hits that matched this compound.

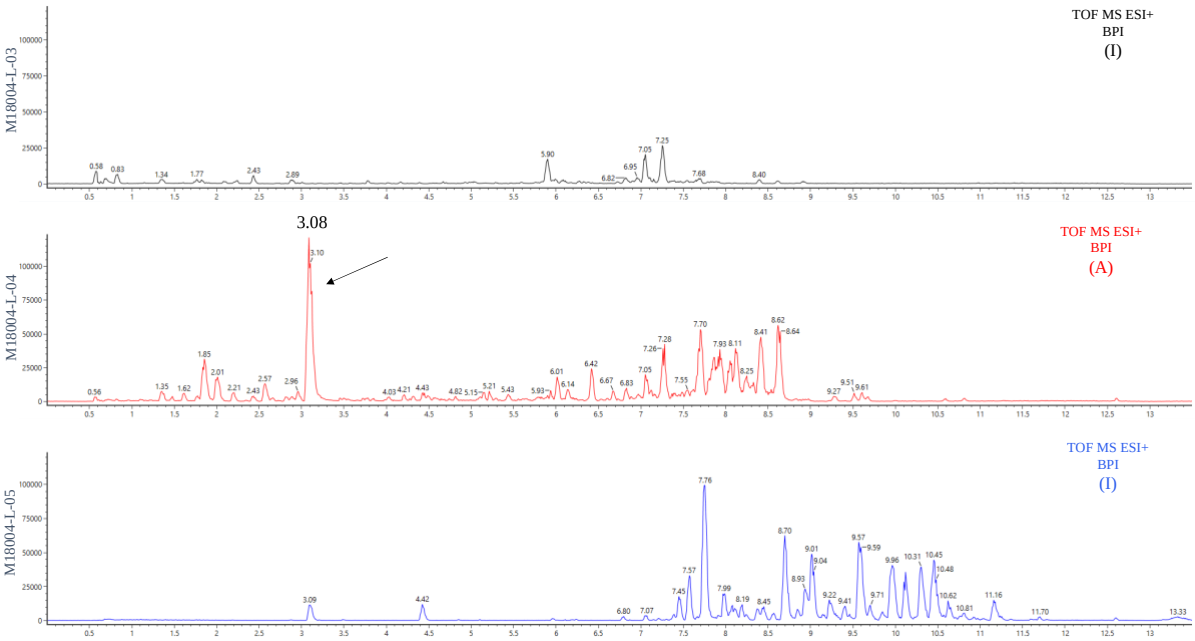

Figure 5: *HPLC-HR-MS BPI chromatogram of fraction M18004-L-03, M18004-L-04 and M18004-L-05. The chromatogram of the active fraction is marked (A), while the chromatogram of the inactive fraction is marked (I). The x-axis shows retention time (min) and the y-axis the signal intensity.*

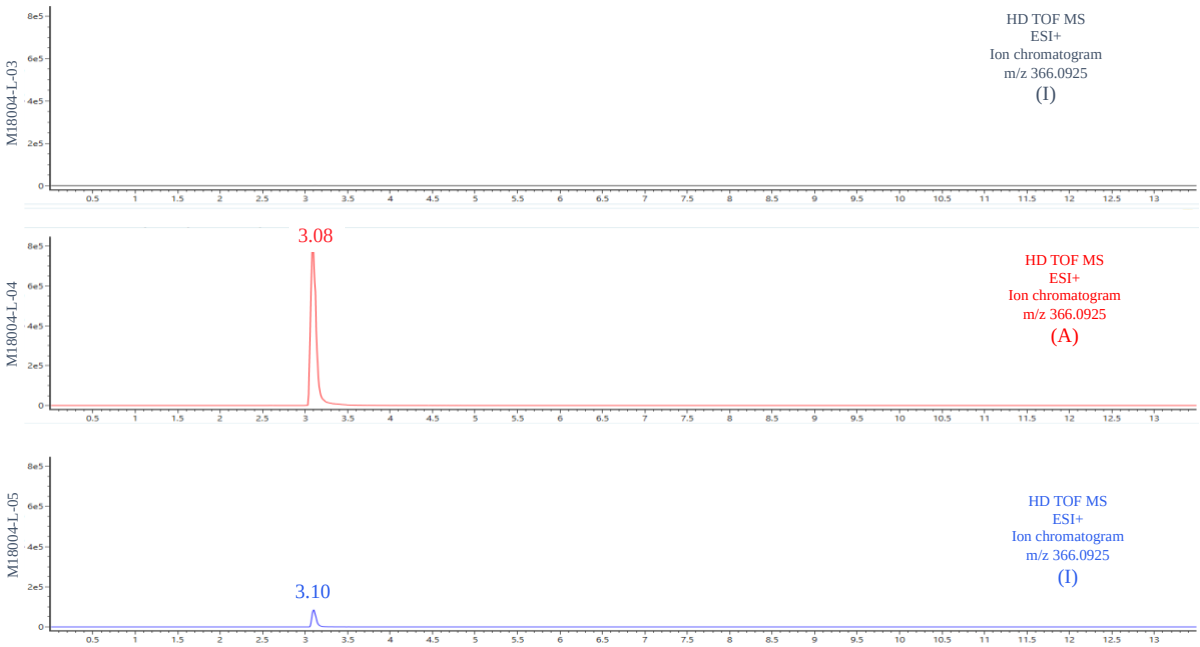

Figure 6: *HPLC-HR-MS ion chromatogram of m/z 366 in fraction M18004-L-03, M18004-L-04 and M18004-L-05. The chromatogram of the active fraction is marked (A), while the chromatogram of the inactive fraction is marked (I). The x-axis shows retention time(min) and the y-axis the signal intensity.*

The mass spectrum of M18004-L-04-366 can be seen in Figure 7, showing an isotopic pattern with two similar peaks only two mass units apart. The natural abundance of bromine isotopes in nature is in the ratio of 51:49, where 50.69% are ^{79}Br and 49.31% are ^{81}Br. The isotopic pattern indicated that the eluted compound was mono-brominated, where the peak with *m/z* 366.09260 is ^{79}Br and the peak with *m/z* 368.09128 is ^{81}Br. The two other peaks in the spectrum at *m/z* 367.09492 and *m/z* 369.09389 are most likely carbon isotopes. The natural abundance of carbon is in the ratio of 99:1, where 98.89% is ^{12}C and 1.11% is ^{13}C (62). The elemental composition was calculated to be $C_{15}H_{20}BrN_5O$ by the software, which underlines that bromine is included in the structure. It is relatively common for marine natural products to biosynthesize products that contain halogens such as bromine (63). Halogen atoms in chemical structures can affect the biological activity and generate an antagonistic or agonistic effect with conformational changes in the active site on the target molecule. They can also inhibit cellular processes associated with cancer, such as stimulating apoptosis (64). Since the extract M18004-L-04 had shown biological activity against a cancer cell line, it was not unlikely that this could be the compound responsible for the activity. The combination of the chemical structure with halogen and that there are, to my knowledge, no compounds reported isolated from the species *L. quadricornis* previously, made this compound interesting.

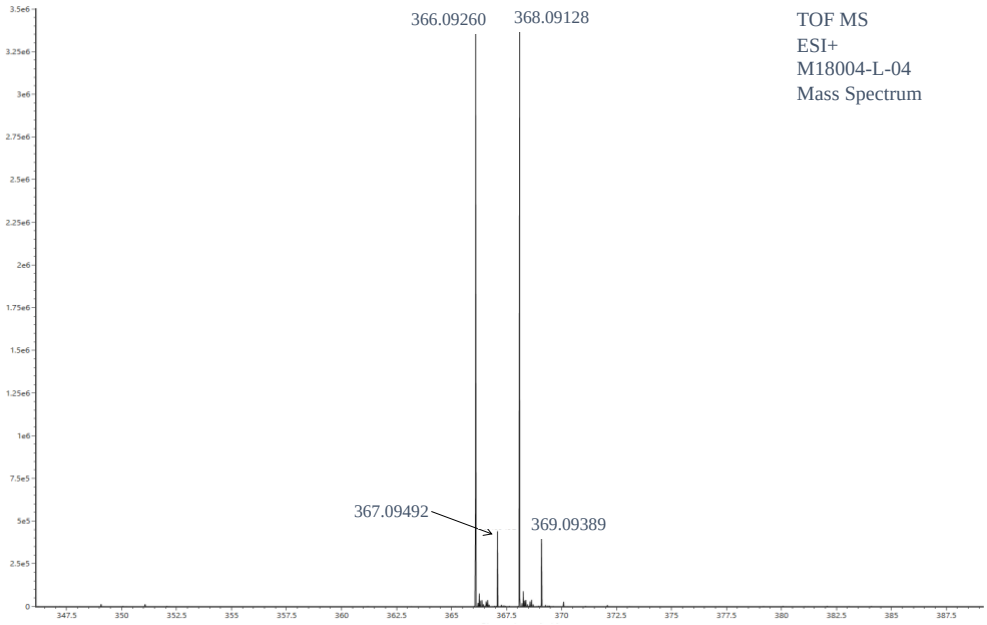

Figure 7: *HPLC-HR-MS mass spectrum of compound m/z 366 in the active fraction M18004-L-04. The x-axis shows the mass (m/z), and y-axis shows the signal intensity.*

4.1.2 Dereplication of flash fraction from M18008-W

Figure 8 shows the base peak intensity (BPI) chromatogram of the active fraction M18008-W-05 (bottom panel) and the inactive fraction M18008-W-04 (top panel). In the active fraction, most peaks eluted late in the chromatogram, but one peak stood out and was separated from the other peaks. This peak eluted with a retention time of 3.46 (Figure 8) and *m/z* 556 (Figure 10) and was present at a higher relative abundance in the active fraction than in the inactive fraction as seen in the ion chromatogram **(**Figure 9**)**. The charge state of this peak was 5 and the neutral mass was determined to be 2777.5 Da. The elemental composition was calculated to be $C_{119}H_{204}N_{36}O_{40}$, but the statistics of this calculation was not very good. Nevertheless, the compound was a candidate for bioactivity testing and was chosen for isolation.

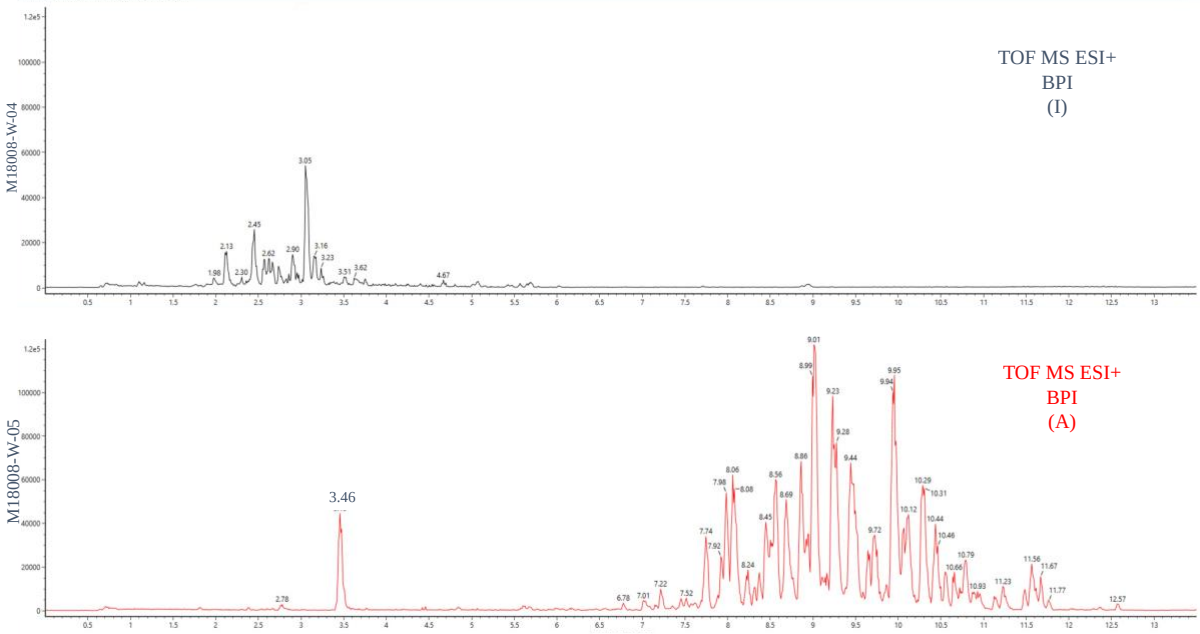

Figure 8: *HPLC-HR-MS BPI chromatogram of fraction M18008-W-04 and M18008-W-05. The chromatogram of the active fraction is marked (A), while the chromatogram of the inactive fraction is marked (I). The x-axis shows retention time(min) and the y-axis the signal intensity.*

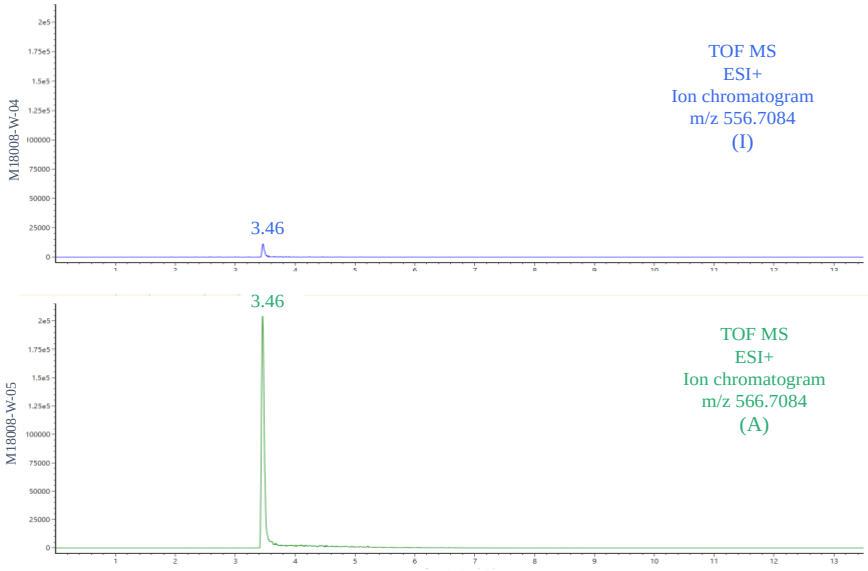

Figure 9: *HPLC-HR-MS ion chromatogram of m/z 566 in fraction 18008-W-04 and M18008-W-05. The chromatogram of the active fraction is marked (A), while the chromatogram of the inactive fraction is marked (I). The x-axis shows retention time(min) and the y-axis the signal intensity.*

Figure 10 shows the mass spectrum of the peak eluting at retention time 3.46 in the active fraction M18008-W-05. The three major peaks in the mass spectrum represent the different charge states of the compound, as indicated by the number above their *m/z* values. By multiplying the *m/z* values with their charge states and subtracting the respective numbers of protons (assuming that the compound is protonated), the neutral mass of the compound is obtained to be 2777 Da. Because of the high weight of the molecular ion and multiple charges, it may indicate that this molecule is a peptide. Peptides have shown to be capable of having cytotoxic activity against cancer cells without affecting healthy normal cells. The mechanism behind the selectively anticancer effect is not fully known. One suggestion is that cancer cells are negatively charged as they contain anionic molecules on their membranes. Since peptides are cationic and healthy normal cells are zwitterionic, the electrostatic interactions between the peptide and the cancer cell will lead to target selectivity and cell death by membrane disruption. Peptides have also expressed an antibacterial effect against both Gram-negative and Gram-positive pathogens. Bacteria also have a negatively charged membrane that works as a target for the peptide leading to membrane disruption and cell death (65). The properties of peptides to potentially have activity against cancer cells and bacteria, made this compound interesting and it was therefore selected for isolation.

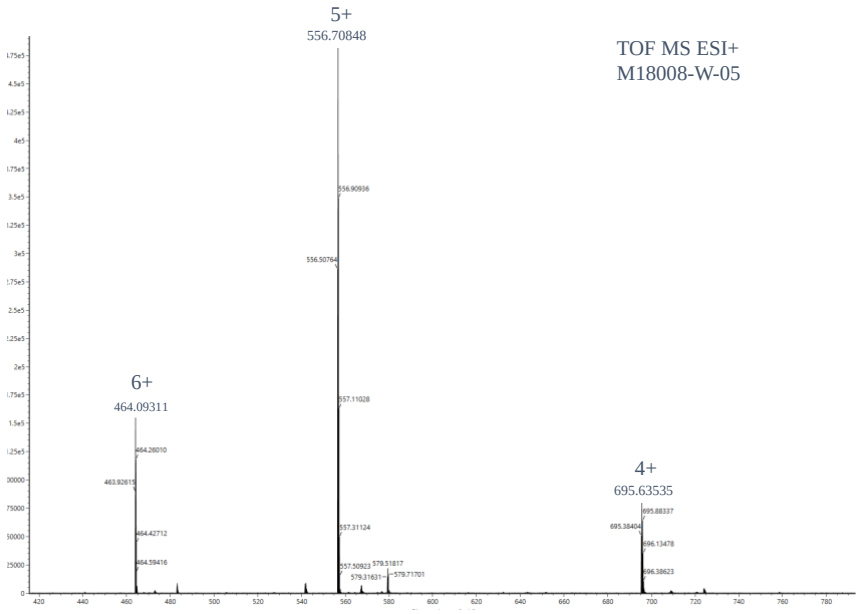

Figure 10: *HPLC-HR-MS mass spectrum at retention time(min) 3.46 of the active fraction M18008-W-05. The x-axis shows the mass (m/z), and y-axis shows the signal intensity.*

Figure 11 is a section of the mass spectrum of the peak eluting at 3.46 in the active fraction M18008-W-05. The fraction has 4 charges and 0.25 mass units between each peak. The same pattern would be seen in the mass spectrum with 5 charges and 0.2 mass units between each peak, and in a spectrum with 6 charges it would have been 0.16 mass units between each peak.

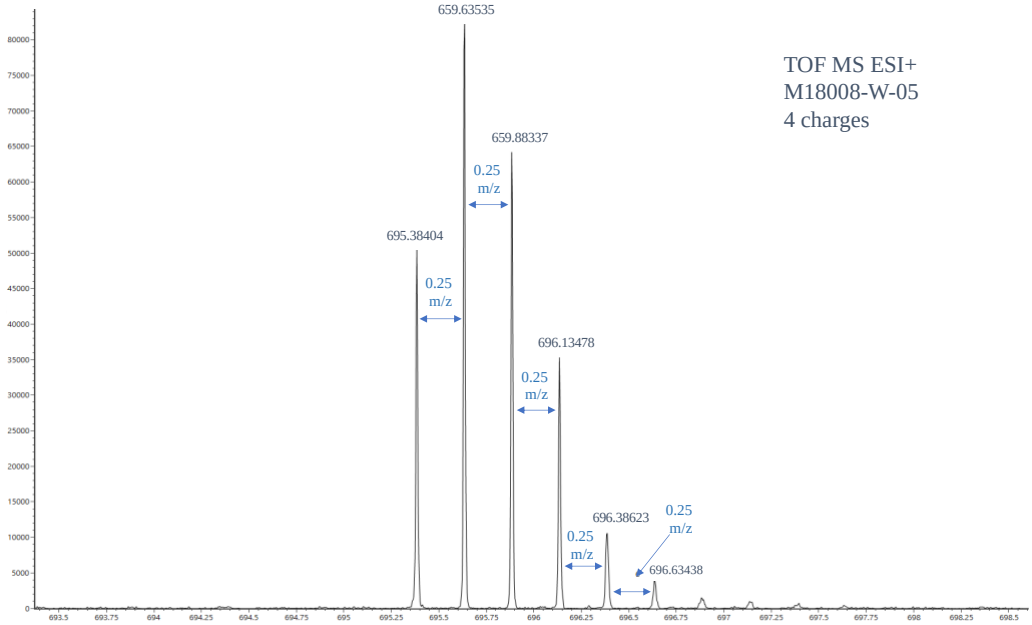

Figure 11: *HPLC-HR-MS mass spectrum at retention time(min) 3.46 of the active fraction M18008-W-05. The x-axis shows the mass (m/z), and y-axis shows the signal intensity.*

4.2 Column screening

Before starting the isolation of the target compound with preparative HPLC, a column screening was conducted to optimize the conditions for the isolation process. Four different columns were used: SunFire, Xterra C18, Atlantis and Phenyl-Hexyl (Figure 12).

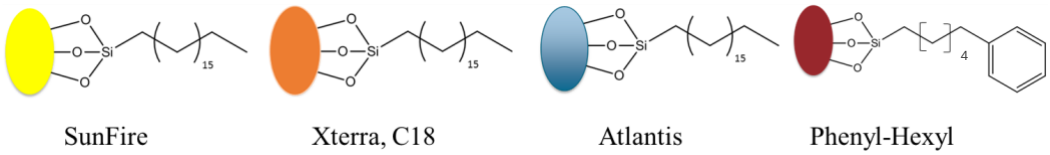

Figure 12: *Overview of the four different reverse-phase columns used during column screening. Figure made by Kine Østnes Hansen.*

The difference in the columns can be utilized when isolating the target compound using preparative HPLC. The stationary phase in the columns is different and varies in its lipophilicity. Xterra, Sunfire and Atlantis have a silica backbone with a C18 hydrocarbon attached. However, they are not entirely similar because of small modifications on the silica-particles, as well as different sizes and shapes of the particles. Phenyl-hexyl also has a silica backbone, but has an aromatic ring attached to the hydrocarbon chain. This makes it possible to have pi-pi interactions with the analyte that will affect the strength of the interaction. The column dimension, such as the length and diameter will also affect the separation (66). Since M18004-L-04 and M18008-W-05 are extracts from crude samples from the sea, they can consist of hundreds of components. Therefore, it is essential to select the column that gives optimal separation of the compounds. If the target compounds need to be purified in several steps, it is common to utilize the difference in the columns and change it between each isolation. Evaluating the chromatograms obtained in the column screening will then help evaluate which column to select.

Figure 13 shows the total ion chromatogram (TIC) of the column screening of M18004-L-04 (left) and M18008-W-05(right) on the four columns. For the M18004-L-04 extract, the Phenyl-hexyl (A) column was chosen for isolation as the peak was narrow and was the column that gave the best separation between the target compounds and the impurities. For the M18008-W-05 extract, the Xterra C18 (D) column was chosen for isolation as the peak of the target compound was narrow and did not overlap with the other compounds.

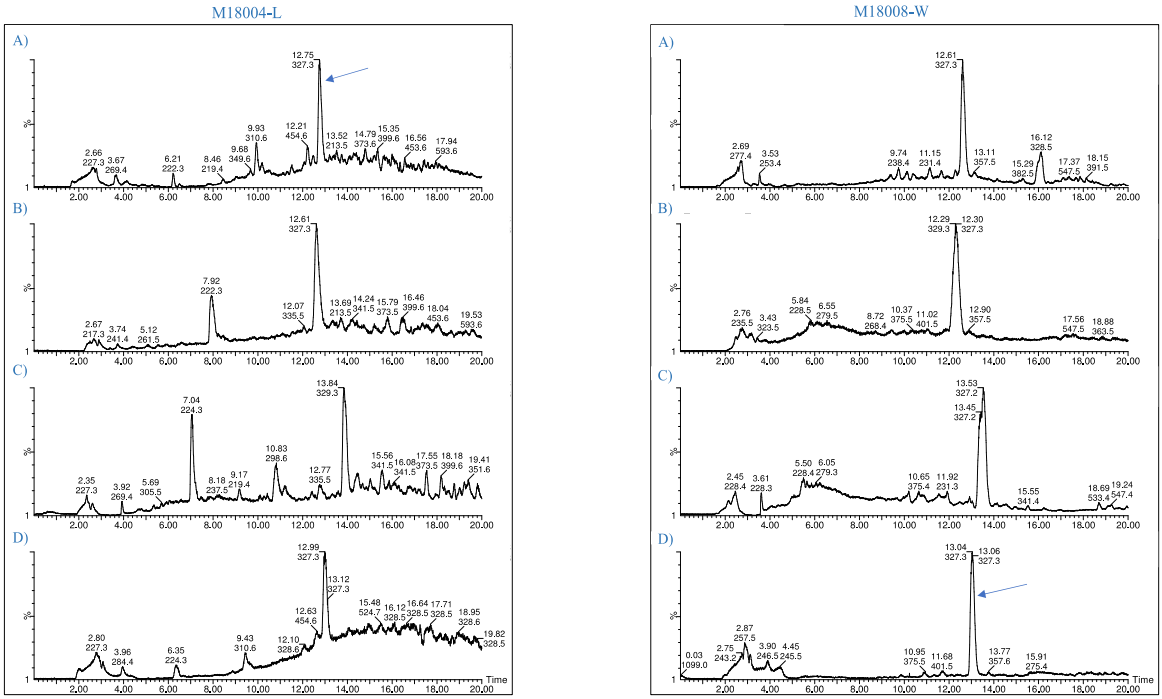

***Figure 13**: Total ion chromatogram (TIC) of the aqueous extract of M18004-L-04 and organic extract M18008-W-05 on four different columns: A) Phenyl-hexyl, B) Atlantis, C) Sunfire, D) Xterra C18. The x-axis shows the retention time (min) and the y-axis the signal intensity.*

4.3 Isolation

The isolation process on the preparative HPLC is conducted to get purified material for structure elucidation and bioactivity profiling. From dereplication with HR-MS the *m/z* 366 from M18004-L-04 and *m/z* 566 from M18008-W-05 were selected for isolation. During the isolation process of M18004-L-04 other masses of interest were selected to be isolated, while *m/z* 566 from M18008-W-05 could not be detected and was terminated from the project. An overview of the isolated compounds is illustrated in Figure 14.

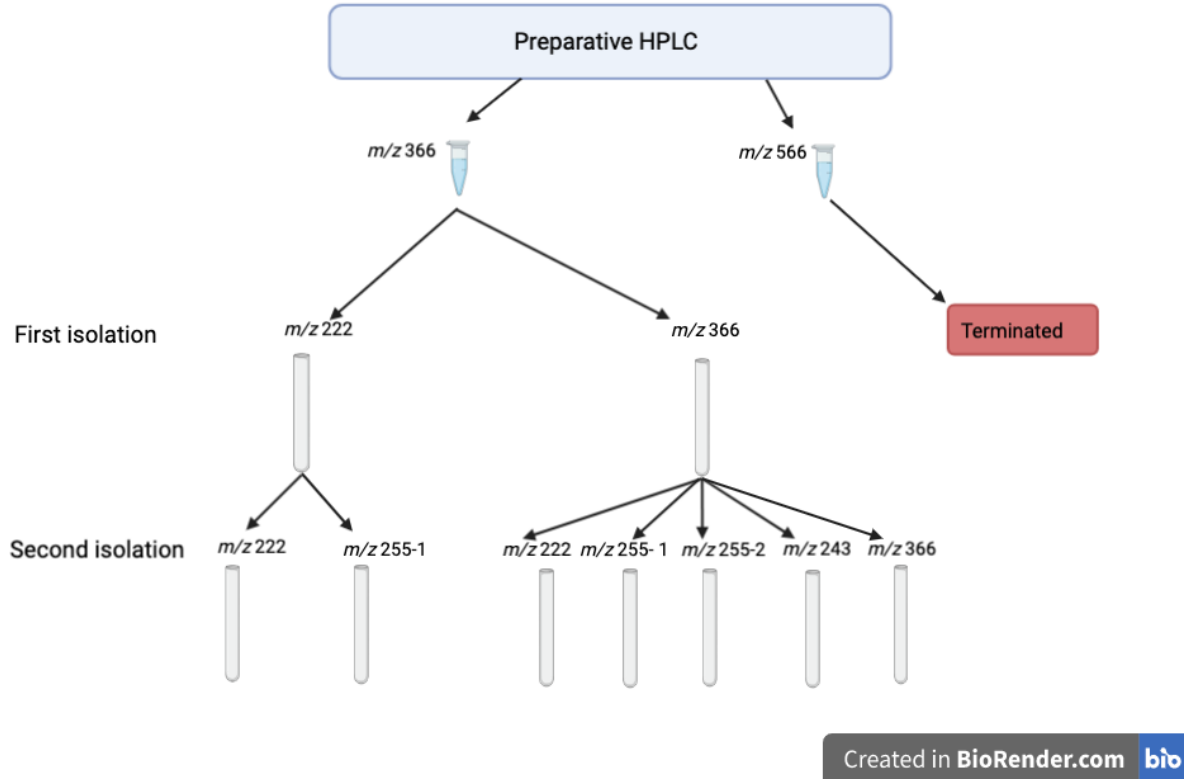

Figure 14: *An illustration of the isolated compounds during the first and second isolation with preparative HPLC.*

4.3.1 Isolation of the target compounds from the organic extract M18004-L-04

M18004-L-04

The first round of compound isolation from the M18004-L-04 extract was performed using preparative HPLC equipped with a phenyl-hexyl column where the aim was to isolate the compound found with HR-MS analysis with a *m/z* 366 (M18004-L-04-366). The compound with *m/z* 222 (M18004-L-04-222) was also collected since the mass spectrum of this compound from the preparative HPLC indicated that it contained a similar mono brominated isotopic pattern. Mass triggered fractionation was used to collect M18004-L-04-222 and time

fractionation to collect M18004-L-04-366. Since the mass of the target compound from dereplication is known, it is natural to use mass triggered fractionation when isolating the target compounds. No compound was collected when performing isolation with mass triggered fractionation on M18004-L-04-366. Therefore, time fractionation was used to isolate this compound. In retrospect, it is conceivable that the threshold for mass collection maybe was too high and it should have been tried adjusted, especially since the peak of M18004-L-04-366 showed a low signal intensity. The base peak intensity chromatogram of this isolation is seen in Figure 15.

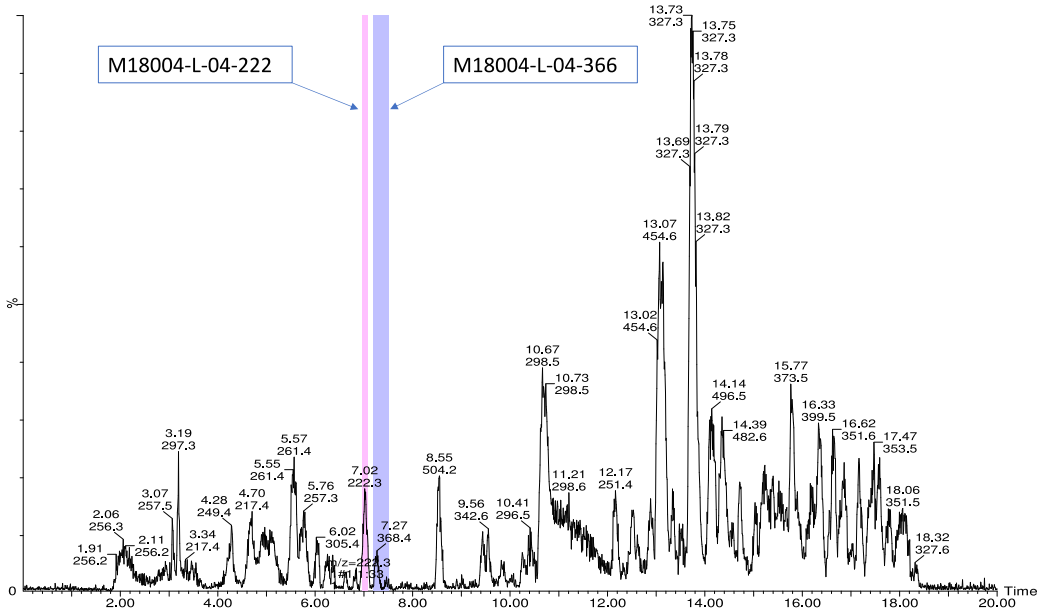

Figure 15: *Base peak intensity chromatogram of the first isolation of compounds from M18004-L-04 using a phenyl-hexyl column. The colored areas mark individually collected fractions. The x-axis shows the retention time (min) and the y-axis the signal intensity.*

M18004-L-04-366

M18004-L-04-366 was isolated in a second round to remove impurities that followed from the first isolation. In the isolation of M18004-L-04-366, a Phenyl-hexyl column was used. The signal intensity of the peak of M18004-L-04-366 was still poor and other peaks with higher signal intensity appeared. The mass spectrum from the preparative HPLC displayed that these compounds contained the same isotopic pattern. Since these compounds also had a mono brominated isotopic pattern, they were suspected of having an association with M18004-L-04-366. Therefore, *m/z* 222 (M18004-L-04-222), *m/z* 243 (M18004-L-04-243) and *m/z* 255 (M18004-L-04-255-1 and M18004-L-04-255-2) were also selected to be isolated. M18004-L-04-255-1 and M18004-L-04-255-2 have the same *m/z*-value but different retention times and

are therefore suspected to be isomers. Isomers are when two compounds have the same elemental composition with some structural differences, such as different positions of functional groups. Isomers are common in natural products and are important to be aware of as minor differences in the structure can give different physiological activities (67). M18004-L-04-366 was collected using time triggered fractionation, while mass triggered fractionation was used to collect M18004-L-04-222, M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-243. The base peak intensity chromatogram of this isolation is presented in Figure 16.

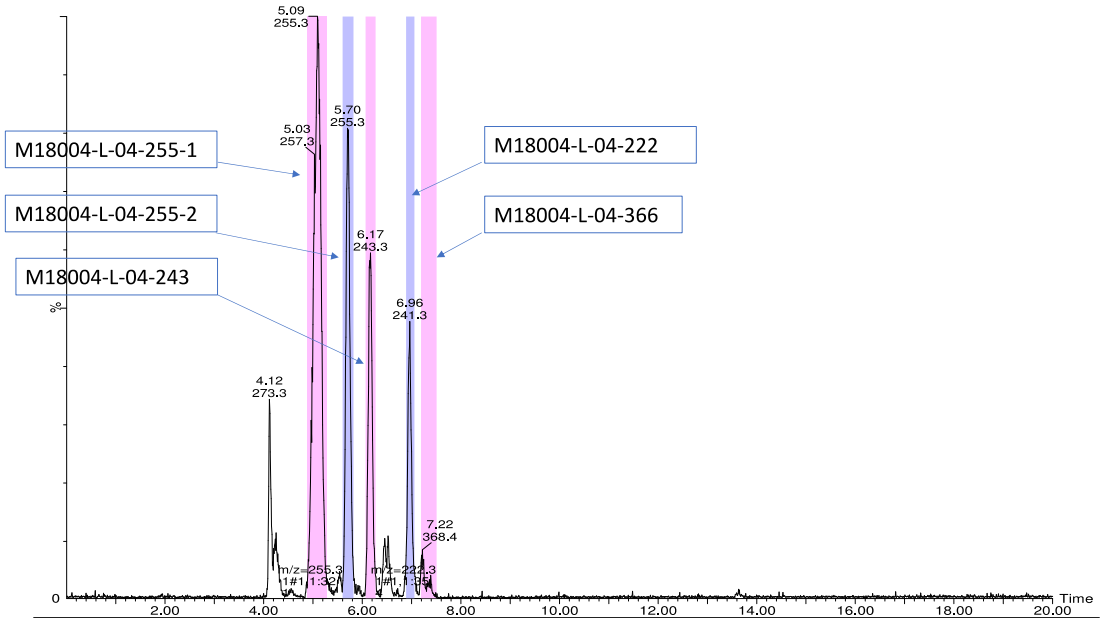

Figure 16: *Base peak intensity chromatogram of the second isolation of M18004-L-04-366 using a phenyl-hexyl column. The colored areas mark individually collected fractions. The x-axis shows the retention time (min) and the y-axis the signal intensity.*

M18004-L-04-222

M18004-L-04-222 was isolated in a second round to remove impurities that followed from the first isolation. In the isolation of M18004-L-04-222 a Phenyl-hexyl column was used. The mass spectrum from the preparative HPLC displayed another compound with *m/z* 255 (M18004-L-04-255-1) containing the same isotopic pattern. Since the compound also had a mono brominated isotopic pattern, it was suspected of having an association with M18004-L-04-222 and was therefore selected to be isolated. M18004-L-04-255-1 was also found in the second isolation of M18004-L-04-366, and the possibility that they are related makes it extra interesting. M18004-L-04-222 and M18004-L-04-255-1 were collected using time fractionation. The base peak intensity chromatogram of this isolation is presented in Figure 17.

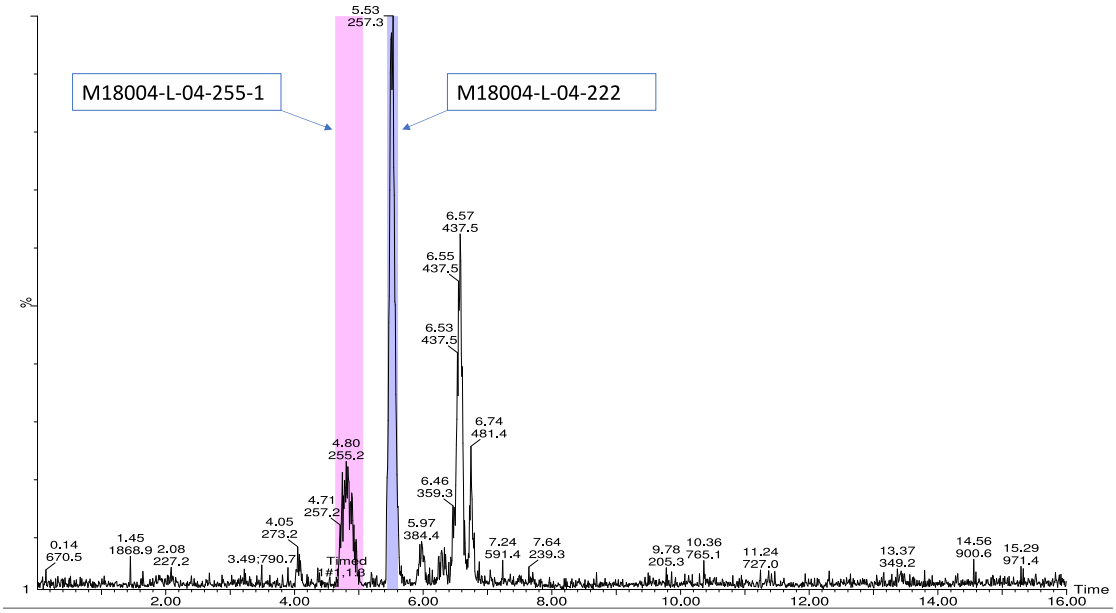

Figure 17: *Base peak intensity chromatogram of the second isolation M18004-L-04-222 using a phenyl-hexyl column. The colored areas mark individually collected fractions. The x-axis shows the retention time (min) and the y-axis the signal intensity.*

The isolation resulted in 0.76mg of M18004-L-04-255-1, 2.33 mg of M18004-L-04-255-2 and 2.69 mg of M18004-L-04-366 that were analyzed on NMR. It was not enough isolated product of M18004-L-04-222 and M18004-L-04-243 to obtain useful NMR data and they were therefore terminated from the project.

4.3.2 Isolation of the target compounds from aqueous extract M18008-W-05

M18008-W-05

The first round of compound isolation from the M18008-W-05 extract was performed using preparative HPLC equipped with an Xterra C18 column. The aim was to isolate the compound selected from the dereplication (*m/z* 566). This compound could not be found during the isolation process, and therefore was M18008-W-05 terminated form the project.

4.4 NMR

NMR is a non-destructive method which is preferable as molecules are kept intact and can be further used for bioactivity screening after analysis. Recording and interpretation of NMR data was performed by Dr. Johan Isaksson, at the Department of Chemistry at UiT – the Arctic University of Norway. The following compounds were submitted to structure elucidation using NMR: M18004-L-04-255-1, M18004-L-04-255-2, M18004-L-04-366.

4.4.1 NMR analysis of M18004-L-04-366

The compound of interest from dereplication was calculated to have the elemental composition $C_{15}H_{20}BrN_5O$ with a protonated mass of 366 Da. The result from NMR showed that the isolated structure M18004-L-04-366 had an elemental composition $C_{10}H_{12}BrN_2$ with a molecular mass of 240 Da. From the isolation with preparative HPLC to structure elucidation with NMR, the sample had a structural loss of $C_5H_6N_3O$. Further in this thesis, this sample would be named M18004-L-04-240. The structure of M18004-L-04-240 can be seen in Figure 18**.** The structure of the compound was used as input in the ChemSpider and PubChem databases. This showed that the structure was the previously known compound named 6-bromotryptamine. 6-bromotryptamine is a known natural product isolated from the marine organisms *Lissoclinum* and *Didemnum candidum* (68). From isolation with preparative HPLC to structure elucidation with NMR, the sample had a structural loss, the mechanism behind this change in elemental composition is unknown. It is however a well-known fact that several compounds are liable to degradation in the presence of acid. During the preparative HPLC isolation step, 0.1% FA is used in the mobile phase. The boiling point of FA is 100.8°C and entails that FA will be concentrated during the first parts of the rotavapor drying step as the organic solvent is evaporated before both FA and water. This might cause an acid-catalyzed hydrolysis of the compound. An article has been published about compounds from nature that degraded in line with the concentration of FA in the mobile phase due to acid catalyzed reactions (69).

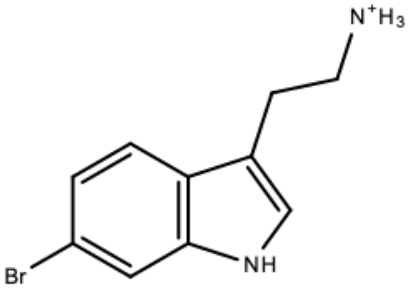

Figure 18: *Chemical structure of the isolated product M18004-L-04-240.The molecular formula was* $C_{10}H_{12}BrN_2$.

4.4.2 NMR analysis of M18004-L-04-255-1 and M18004-L-04-255-2

The results from NMR of M18004-L-04-255-1 and M18004-L-04-255-2 showed a structure with the elemental composition $C_{10}H_{11}BrN_2O$ and a mass of 254 Da and can be seen in Figure 19**.** No hits were detected in searches with structure in databases such as ChemSpider and PubChem. The compounds were suspected as isomers during the isolation process as they contained the same mass but eluted at different retention times. The result from NMR showed that M18004-L-04-255-1 and M18004-L-04-255-2 have the same chemical structure and mass, but there was no information about the stereochemistry. If the compounds are isomers of each other is therefore still unfortunately unknown.

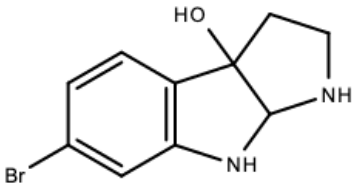

Figure 19: *Structure of the isolated products M18004-L-04-255-1 and M18004-L-04-255-2. The molecular formula was* $C_{10}H_{11}BrN_2O$.

4.5 Bioactivity screening

4.5.1 Anticancer assay

The isolated compounds M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-240 were tested against A2058 (human melanoma cancer cell line) for cytotoxic activity. The samples were tested using an Aqueous One Solution Cell Proliferation Assay. This is a colorimetric method used to determine cell survival in the wells. The yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt), also called MTS is added to each well as a reagent. MTS reduces to a purple formazan product by the mitochondria of living cells (Figure 20).

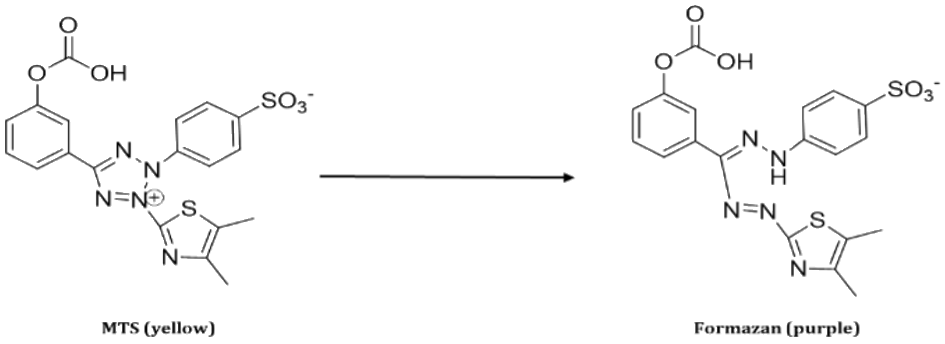

Figure 20: *Reduction of MTS to formazan by the mitochondria of metabolically active (living) cells (70).*

Metabolically non active cells will not be able to reduce MTS to formazan and will be observed as a yellow liquid. A high degree of transformation of MTS molecules into the formazan variant indicated that cells are not affected as seen in the 96-well-microtiter plate from this project in Figure 21. If a low percentage of the MTS molecules are reduced (the wells remain yellow), this indicates that a percentage of the cells has not survived exposure to the test compound. Formazan absorbs light at 490 nm which makes it possible to determine the cell viability since the quantity of formazan that is produced is directly proportional to the level of viable cells in the well (70).

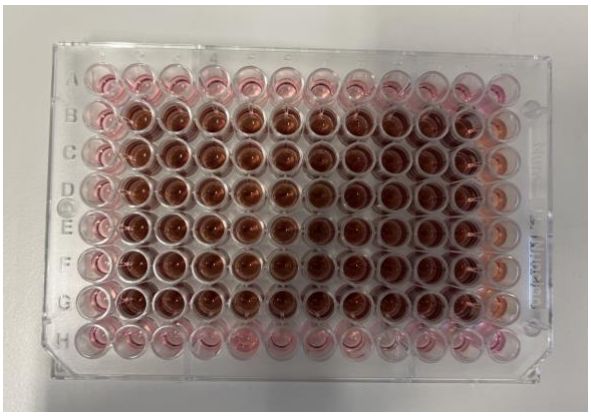

Figure 21: *The wells in the middle of the 96-well-microtiter plate is colored dark purple. This indicates that the metabolically active cells have reduced MTS into formazan, and the cells are not affected by M18004-L-04-255-1, M18004-L-04-255-2 or M18004-L-04-240.*

As seen in Figure 22, the cell survival of the tested isolated compounds on A2058 was approximately 100% and there was no cytotoxic activity detected. Since the graph of the three different concentrations is almost flat, no dose-response is identified.

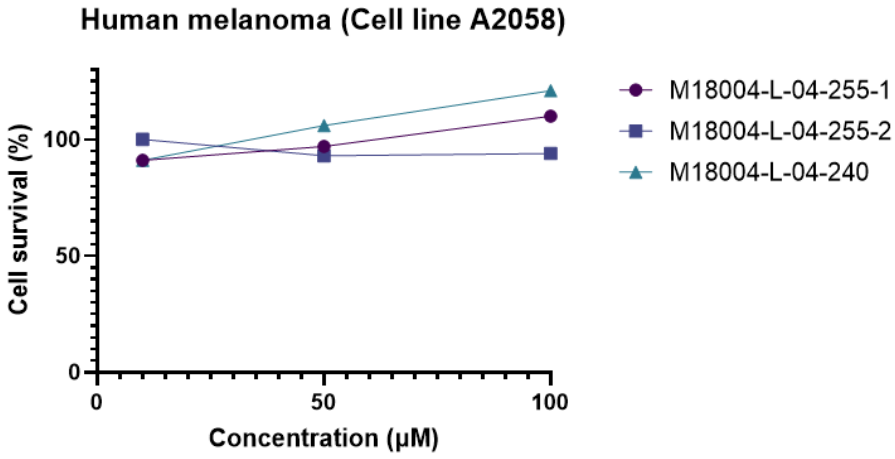

Figure 22: *Results from the anti-cancer assay of M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-240 against human melanoma cell line A2089. The isolated compounds were tested in concentration 10, 50 and 100 µM.*

4.5.2 Antibacterial growth inhibition assay

The isolated compounds M18004-L-04-255-1, M18004-L-04-255-2 and M18004-L-04-240 were tested against the bacterial strains *E. faecalis*, *E. coli*, *P. Aeruginosa*, *S. agalactiae* and *S. aureus*. *E. coli* and *P. Aeruginosa* are two Gram-positive pathogens listed as priority 1 by WHO. *S. aureus* is a Gram-negative pathogen and is listed in priority 2 by WHO. As mentioned, the priority 1 is listed as critical and priority 2 is listed as high. This specifies that new antibacterial agents against these pathogens are urgent to overcome the resistance and is one of the reasons

why this assay were selected in this project. The optical density (OD) was measured with an absorbance at 600 nm. It is desirable with a low OD-value since a clear liquid where the light passes through indicates few bacteria in the well. The OD-value will increase the more bacteria there are in the well. As seen in Figure 23, the OD-value of the tested isolated compounds on the five different pathogens was over 0.05 and no antibacterial activity was detected. Since the graph of the three different concentrations is almost flat, no dose-response is identified.

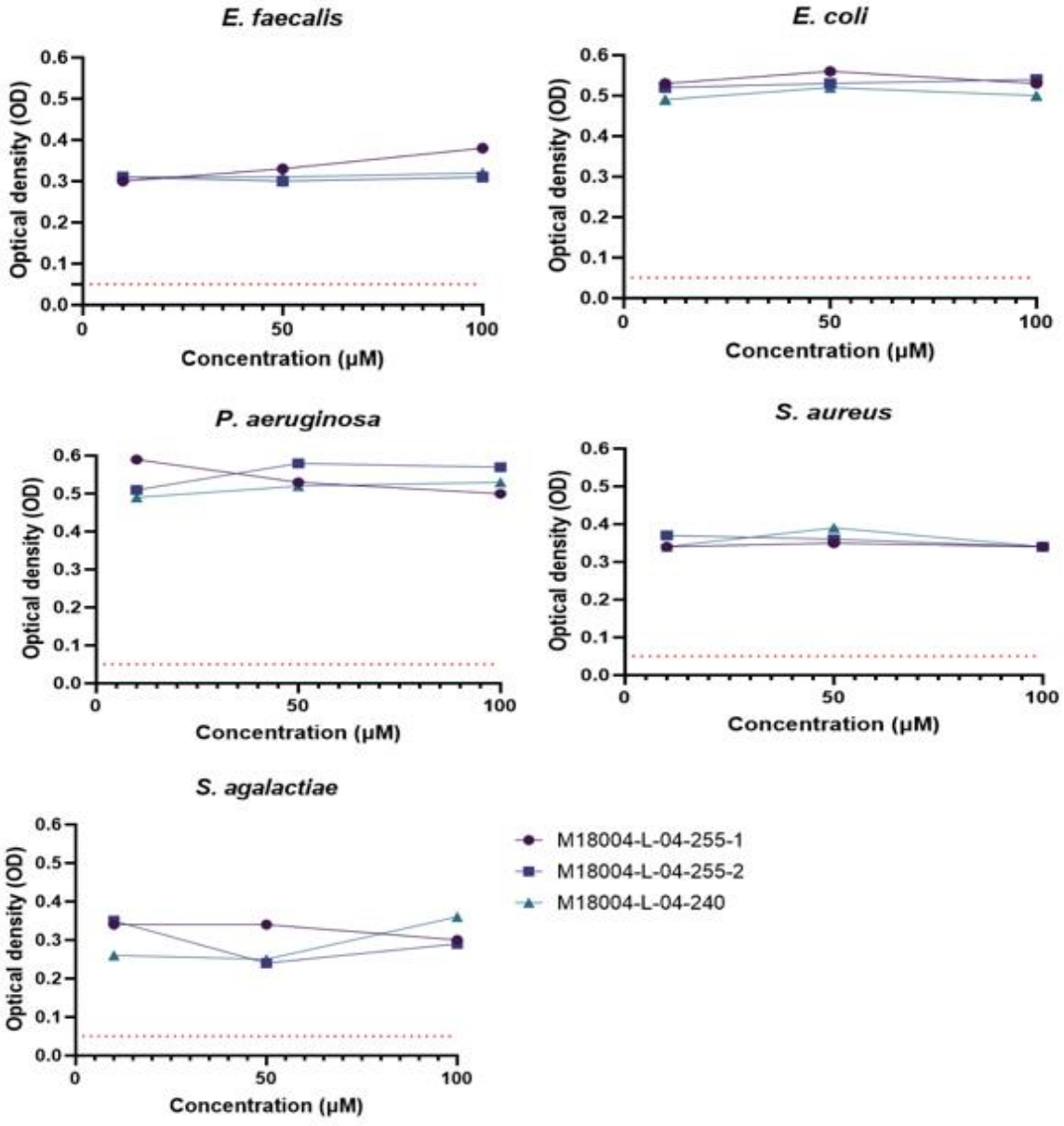

Figure 23: *Results from the antibacterial growth inhibition assay of M18004-L-04-255-1 and M18004-L-04-255-2, M18004-L-04-240 against E. faecalis, E. coli, P. Aeruginosa, S. agalactiae and S. aureus. The isolated compounds were tested in concentrations 10, 50 and 100 µM, and the optical density was measured at 600nm. The red dot line illustrates the cut off-value, where the absorbance <0.05 indicates activity against the bacteria.*

5 Conclusions

The result from this study shows that new compounds can be isolated from marine invertebrates. It furthermore highlights some of the challenges associated with this process, such as challenges related to isolation of the active component of an extract/fraction and sample degradation during sample handling. For fractions from *S. securifrons* the securamines were observed as some of the main components in the active fractions during dereplication. The securamine are known to be potent cytotoxic compounds. For the remaining fractions from *L. quadricornis*, *P. indet* and *V. norvegicus*, the bioactive component was not identified and thus remains to be determined. None of the isolated compounds were found to be bioactive against human melanoma cancer cells (A2058) or against the bacterial strains *E. faecalis, E. coli, P. Aeruginosa, S. agalactiae* and *S. aureus.*

6 References

1. Hanssen KØ. Isolation and Characterisation of Bioactive Secondary Metabolites from Arctic, Marine Organisms: UiT The Arctic University of Norway; 2014.
2. Kerr RG. Natural Products: Their Chemistry and Biological Significance. Economic Botany 1995;49(3):308-.
3. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. Metabolites 2012;2(2):303-36.
4. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov 2021;20(3):200-16.
5. Dewick PM. Medicinal Natural Products: A Biosynthetic Approach. 3. Aufl. utg. New York: New York: Wiley; 2009.
6. Krishnamurti C, Rao SSC. The isolation of morphine by Serturner. Indian J Anaesth 2016;60(11):861-2.
7. Gach K, Wyrębska A, Fichna J, Janecka A. The role of morphine in regulation of cancer cell growth. Naunyn Schmiedebergs Arch Pharmacol 2011;384(3):221-30.
8. Houbraken J, Frisvad JC, Samson RA. Fleming's penicillin producing strain is not Penicillium chrysogenum but P. rubens. IMA Fungus 2011;2(1):87-92.
9. Silva Soares GM, Figueiredo LC, Faveri M, Cortelli SC, Duarte PM, Feres M. Mechanisms of action of systemic antibiotics used in periodontal treatment and mechanisms of bacterial resistance to these drugs. J Appl Oral Sci 2012;20(3):295-309.
10. Svenson J. MabCent: Arctic marine bioprospecting in Norway. Phytochem Rev 2013;12(3):567-78.
11. Romano G, Costantini M, Sansone C, Lauritano C, Ruocco N, Ianora A. Marine microorganisms as a promising and sustainable source of bioactive molecules. Mar Environ Res 2017;128:58-69.
12. Bhatia P, Chugh A. Role of marine bioprospecting contracts in developing access and benefit sharing mechanism for marine traditional knowledge holders in the pharmaceutical industry. Global ecology and conservation 2015;3(C):176-87.
13. Zamani NP, Rahman L, Rosada RL, Tirtama W. Overview of bioactivity studies on marine natural products. IOP Conf Ser: Earth Environ Sci 2021;944(1):12029.
14. Sumich JL, Collard S. An Introduction to the Biology of Marine Life: WCB/McGraw-Hill; 1999. Available from: https://books.google.no/books?id=B2VfQgAACAAJ
15. Capon Robert J. Marine Bioprospecting − Trawling for Treasure and Pleasure. Eur J Org Chem 2001;2001(4):633-45.
16. Ahmed I, Asgher M, Sher F, Hussain SM, Nazish N, Joshi N, et al. Exploring Marine as a Rich Source of Bioactive Peptides: Challenges and Opportunities from Marine Pharmacology. Mar Drugs 2022;20(3):208.
17. Cappello E, Nieri P. From Life in the Sea to the Clinic: The Marine Drugs Approved and under Clinical Trial. Life (Basel) 2021;11(12):1390.
18. Petek BJ, Loggers ET, Pollack SM, Jones RL. Trabectedin in Soft Tissue Sarcomas. Mar Drugs 2015;13(2):974-83.
19. Carter NJ, Keam SJ. Trabectedin: A Review of its Use in the Management of Soft Tissue Sarcoma and Ovarian Cancer. Drugs 2007;67(15):2257-76.
20. Lyseng-Williamson KA, Perry C. Ziconotide. CNS Drugs 2006;20(4):331-8.
21. Miljanich GP. Ziconotide: Neuronal Calcium Channel Blocker for Treating Severe Chronic Pain. CMC 2004;11(23):3029-40.
22. McDowell GC, Saulino MF, Wallace M, Grigsby EJ, Rauck RL, Kim P, et al. Effectiveness and Safety of Intrathecal Ziconotide: Final Results of the Patient

Registry of Intrathecal Ziconotide Management (PRIZM). Pain Med 2020;21(11):2925-38.
23. Kim S-K. Marine medicinal foods : implications and applications, animals and microbes. 1st ed. utg. Waltham, Mass.: Academic Press; [lest]. Advances in food and nutrition research.
24. Leal MC, Madeira C, Brandao CA, Puga J, Calado R. Bioprospecting of Marine Invertebrates for New Natural Products - A Chemical and Zoogeographical Perspective. Molecules 2012;17(8):9842-54.
25. Lewbart GA. Invertebrate Medicine. Newark: Newark: John Wiley & Sons, Incorporated; 2022.
26. Webster NS, Taylor MW. Marine sponges and their microbial symbionts: love and other relationships: Marine sponge microbiology. Environmental microbiology 2012;14(2):335-46.
27. Rocha J, Peixe L, Gomes NCM, Calado R. Cnidarians as a Source of New Marine Bioactive Compounds-An Overview of the Last Decade and Future Steps for Bioprospecting. Mar Drugs 2011;9(10):1860-86.
28. Stabili L, Rizzo L, Caprioli R, Leone A, Piraino S. Jellyfish Bioprospecting in the Mediterranean Sea: Antioxidant and Lysozyme-Like Activities from Aurelia coerulea (Cnidaria, Scyphozoa) Extracts. Mar Drugs 2021;19(11):619.
29. Collins AG, Daly M. A New Deepwater Species of Stauromedusae, Lucernaria janetae (Cnidaria, Staurozoa, Lucernariidae), and a Preliminary Investigation of Stauromedusan Phylogeny Based on Nuclear and Mitochondrial rDNA Data. Biol Bull 2005;208(3):221-30.
30. Telnes K. Stalked Jellyfish - Lucernaria quadricornishttp://www.seawater.no/fauna/cnidaria/quadricornis.html[lest].
31. Moffat JG, Rudolph J, Bailey D. Phenotypic screening in cancer drug discovery - past, present and future. Nat Rev Drug Discov 2014;13(8):588-602.
32. Samsdodd F. Target-based drug discovery: is something wrong? Drug Discov Today 2005;10(2):139-47.
33. Swinney DC. Phenotypic vs. Target-Based Drug Discovery for First-in-Class Medicines. Clinical Pharmacology & Therapeutics 2013;93(4):299-301.
34. Santillo MF. Trends using biological target-based assays for drug detection in complex sample matrices. Anal Bioanal Chem 2020;412(17):3975-82.
35. Osbourn A, Goss RJ, Carter GT. Marine Bioprospecting. Ames, Iowa :2014.
36. Claeson P, Bohlin L. Some aspects of bioassay methods in natural-product research aimed at drug lead discovery. Trends Biotechnol 1997;15(7):245-8.
37. Sarker SD, Nahar L. Natural Products Isolation. 3rd ed. 2012. utg. Totowa, NJ: Humana Press : Imprint: Humana; [lest]. Methods in Molecular Biology.
38. Gaudêncio SP, Pereira F. Dereplication: racing to speed up the natural products discovery process. Nat Prod Rep 2015;32(6):779-81.
39. Wagenaar MM. Pre-fractionated microbial samples - The second generation natural products library at Wyeth. Molecules 2008;13(6):1406-26.
40. Bucar F, Wube A, Schmid M. Natural product isolation – how to get from biological material to pure compounds. Natural Product Reports 2013;30(4):525-45.
41. Latif Z, Sarker SD. Isolation of Natural Products by Preparative High Performance Liquid Chromatography (Prep-HPLC). I: Sarker SD, Nahar L, red. Natural Products Isolation. Totowa, NJ: Humana Press; 2012. s. 255-74. Available from: https://doi.org/10.1007/978-1-61779-624-1_10
42. Reid RG, Sarker SD. Isolation of Natural Products by Low-Pressure Column Chromatography. Methods Mol Biol 2012;864:155-87.

43. Pedersen-Bjergaard S, Gammelgaard B, Halvorsen TG. Introduction to Pharmaceutical Analytical Chemistry. Newark, UNITED KINGDOM: John Wiley & Sons, Incorporated; 2019. Available: http://ebookcentral.proquest.com/lib/tromsoub-ebooks/detail.action?docID=5683582
44. Giaginis C, Tsopelas F, Tsantili-Kakoulidou A. The Impact of Lipophilicity in Drug Discovery: Rapid Measurements by Means of Reversed-Phase HPLC. Methods Mol Biol 2018;1824:217-28.
45. Kazakevich YV, LoBrutto R. HPLC for Pharmaceutical Scientists. Hoboken: Hoboken: John Wiley & Sons, Incorporated; 2007.
46. Hoffmann Ed, Stroobant V. Mass spectrometry : principles and applications. 3rd ed. / Edmond de Hoffmann, Vincent Stroobant. utg. Chichester, West Sussex, England ;,Hoboken, N.J.: J. Wiley; [lest].
47. Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO. Dereplication of Microbial Natural Products by LC-DAD-TOFMS. J Nat Prod 2011;74(11):2338-48.
48. Demarque DP, Crotti AEM, Vessecchi R, Lopes JLC, Lopes NP. Fragmentation reactions using electrospray ionization mass spectrometry: an important tool for the structural elucidation and characterization of synthetic and natural products. Nat Prod Rep 2016;33(3):432-55.
49. Wanner KT, Höfner G. Mass spectrometry in medicinal chemistry. Weinheim :,Chichester: Wiley-VCH ; John Wiley distributor; [lest]. Methods and principles in medicinal chemistry.
50. Kool J, Niessen WMA. Introduction to Mass Spectrometry, a Tutorial. Weinheim, Germany :2015.
51. Pavia DL. Introduction to spectroscopy. 4th ed. utg. Belmont, Calif: Brooks/Cole; 2009.
52. McMurry J. Organic chemistry : with biological applications. 3rd ed. utg. Stamford, Conn: Cengage Learning; 2015.
53. Günther H. NMR spectroscopy : basic principles, concepts and applications in chemistry. Third completely revised and updated edition. utg. Weinheim an der Bergstrasse, Germany: Wiley-VCH; [lest].
54. Friebolin H. Basic one- and two-dimensional NMR spectroscopy. 4th completely rev. and updated ed. utg. Weinheim: Wiley-VCH; 2005.
55. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. Nat Rev Drug Discov 2005;4(3):206-20.
56. Martínez Andrade KA, Lauritano C, Romano G, Ianora A. Marine Microalgae with Anti-Cancer Properties. Mar Drugs 2018;16(5):165.
57. Robsahm TE, Helsing P, Nilssen Y, Vos L, Rizvi SMH, Akslen LA, et al. High mortality due to cutaneous melanoma in Norway: a study of prognostic factors in a nationwide cancer registry. Clin Epidemiol 2018;10:537-48.
58. Kim AD, Lee Y, Kang S-H, Kim GY, Kim HS, Hyun JW. Cytotoxic Effect of Clerosterol Isolated from Codium fragile on A2058 Human Melanoma Cells. Mar Drugs 2013;11(2):418-30.
59. Breijyeh Z, Jubeh B, Karaman R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules 2020;25(6):1340.
60. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18(3):318-27.
61. Hansen KØ, Hansen IKØ, Richard CS, Jenssen M, Andersen JH, Hansen EH. Antimicrobial Activity of Securamines From the Bryozoan Securiflustra securifrons. Natural product communications 2021;16(2):1934578.

62. Smith RM. Understanding mass spectra : a basic approach. 2nd ed. utg. Hoboken, N.J.: Wiley Interscience; [lest].
63. Bidleman TF, Andersson A, Jantunen LM, Kucklick JR, Kylin H, Letcher RJ, et al. A review of halogenated natural products in Arctic, Subarctic and Nordic ecosystems. Emerging Contaminants 2019;5:89-115.
64. Matulja D, Wittine K, Malatesti N, Laclef S, Turks M, Markovic MK, et al. Marine Natural Products with High Anticancer Activities. CMC 2020;27(8):1243-307.
65. Wang G. Antimicrobial peptides: discovery, design and novel therapeutic strategies. 2 utg. Wallingford, UK: Wallingford, UK: CABI; 2017.
66. Chitta KR, Van Meter DS, Stalcup AM. Separation of peptides by HPLC using a surface-confined ionic liquid stationary phase. Anal Bioanal Chem 2009;396(2):775-81.
67. Tong C, Xu J, Fu Q, Long R, Peng S, Shi S. Rapid extraction, discrimination and quantification of thermally unstable isomeric acteoside and isoacteoside in natural products by online extraction-quadrupole time-of-flight tandem mass spectrometry. Analytical methods 2019;11(16):2148-54.
68. Scott Wiens P, Johnson JL, Gribble GW. Concerning the preparation of 6-bromotryptamine. Tetrahedron 2021;85:132055.
69. Schneider Y, Jenssen M, Isaksson J, Hansen KØ, Andersen JH, Hansen EH. Bioactivity of Serratiochelin A, a Siderophore Isolated from a Co-Culture of Serratia sp. and Shewanella sp. Microorganisms (Basel) 2020;8(7):1042.
70. Protocol AG. CellTiter 96® AQueous One Solution Cell Proliferation Assay. Promega, USA 2001.

7 Appendix

3.6 Nuclear magnetic resonance spectroscopy

In brief, 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- dimethyl sulfoxide (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.