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Isolation, structure elucidation and bioactivity profiling of lyso-ornithine lipids from the marine bacterium *Lacinutrix* sp.

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جزيل الشكر لوالدي و والدتي واختي لوقوفهم دوما بجانبي ودعمهم المستمر لي وتضحيتهم بكل الغالي والنفيس من اجل وصولي لهذه المرحله المرموقه في الدراسه. هذا وبالاضافه الي مجهوداتهم العظيمه لسنوات طويله من اجل توفير المناخ المناسب لكي اتطور واتفوق في حياتي

Tromsø, May 2021
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Abstract

Compared to the terrestrial environment, the marine environment is a field that is at large unexplored. Ever since organisms appeared in the sea, evolution has equipped the marine organisms with mechanisms to survive harsh conditions such as extreme temperatures, increased pressure, changes in salinity and low nutrition levels. These conditions have resulted in organisms developing survival mechanisms by producing secondary metabolites, to help them adapt to these conditions. This is resulting in a variety of unique potentially bioactive molecules, that for us have great pharmaceutical potential.

From previous work, it was identified two analytes of interest, produced by *Lacinutrix* sp. The two analytes are iso-branched lyso-ornithine lipids with only one CH_2 group differing between the two compounds, in the hydrocarbon chain (lysoC15:0 and C16:0). The bacterium was isolated from the marine sponge *Halicondria* sp. that was collected beside Bjørnøya, on a research cruise in 2009. The analytes were identified and nominated for isolation based on bioactivity of fractionated extract from the bacterium. Unfortunately, the isolated quantities of the two were too low to allow bioactivity profiling. In the current project, the aim was therefore to generate more of the pure compounds to further perform bioactivity profiling. This was done by performing a large-scale fermentation of the bacterium. Further, the exudates of the bacterium were extracted, fractionated, and the targeted compounds were purified then tested for bioactivity in a dose-response manner on a variety of assays, with the aim to generate bioactivity data. The isolation is therefore targeted, meaning that we already knew what compounds that are to be isolated.

The bioactivity results showed an antimicrobial activity against *S. agalactiae* and a modest antimicrobial activity against *E. faecialis* and *S. aureus*, only for compound **1.** No antimicrobial activity was displayed against the tested Gram-negative bacteria for both compounds. Cytotoxic assay was also run for the cell line A2058 (human melanoma), and activity was observed only for compound **2**. No cytotoxic activity was observed against the cell line MRC-5 (non-malignant cells). These surprising results of almost identical structures indicate that the length of the hydrocarbon chain contribute to differences in activity, as the results display a selective activity against bacterial cells for compound **1** and a selective activity against human melanoma cells for compound **2.** No cytotoxic activity was observed against the cell line MRC-5 (non-malignant cells)

Abbreviations

NP	Natural products
LS	*Lacinutrix* sp.
UV	Ultraviolet
Prep-HPLC	Preparative high-performance liquid chromatography
ESI	Electrospray ionization
MS	Mass spectrometry
HPLC	High-performance liquid chromatography
UPLC/UHPLC	Ultra-high-performance liquid chromatography
FC	Flash Chromatography
LC	Liquid chromatography
IV	Intravenous
ET	Ecteinascidin
HB	Halichondrin B
EEA	European economic area
MRSA	Methicillin resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance spectroscopy
CMC	Critical micellization concentration
OL	Ornithine lipid
LOL	Lyso-ornithine lipid

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1 Introduction

1.1 Marine biodiversity prospecting

Marine bioprospecting, or marine biodiversity prospecting involves the search, discovery and development of bioactive resources, for example natural products produced by marine micro- or macroorganisms, that has both social and economic value (1, 2). The definition is quite broad, and covers marine microorganisms such as marine bacteria, viruses, and fungi, as well as macroorganisms like sea-plants, fish and shellfish (1). Marine bioprospecting is important for many industries, not only for pharmaceutical industry, but also in engineering and construction (among others) which means that areas of application are wide (1, 2).

The Norwegian government is seeing marine bioprospecting as a focus area primarily because of the access Norway has to many different and most likely unexplored marine organisms. Norway has a long coastline and authority over large marine areas (1). There are many successful stories where we have important drugs, sourced from the marine environments that are today commercialized worldwide. The successful stories are great motivation for the search of new natural products with potential benefits. This will be addressed in the following sections.

A typical marine bioprospecting process consists of sample collection, extraction, and fractionation of the extract into several fractions. The fractions generated are further screened in both cell-based and target-based assays for e.g. cytotoxic activity, antibacterial or antidiabetic activity (3). Fractions where activity is observed are selected for dereplication in order to identify the compounds responsible for the observed activity (3). This is done by high-resolution-mass spectrometry (UHPLC-HR-MS). If it is suspected that the fraction contains a previously undiscovered compound, or a previously reported compound with novel bioactivity, isolation of the compound(s) will be performed, followed by structure elucidation by nuclear magnetic resonance spectroscopy (NMR) (3). Finally at the end, bioactivity profiling is conducted again, but this time on the pure, isolated and identified compounds (3, 4).

1.2 Natural products: primary and secondary metabolites

Natural products are described as molecules produced by a biological origin, and usually referred to as primary and secondary metabolites (5, 6). However, natural product is a wide term that also covers everything from entire organisms to pure compounds produced from organisms (6).

1.2.1 Primary metabolites

Even though living organisms have a variety of characteristics, their ability to produce, modify and degrade proteins, carbohydrates, fats and nucleotides are almost the same in all organisms (7). The process of synthetizing and modifying these compounds is called primary metabolism, and the corresponding compounds involved in the processes are called primary metabolites (7). Primary metabolism and the corresponding metabolites are crucial for the vital functions such as growth, energy storage, respiration and reproduction for all organisms (7).

1.2.2 Secondary metabolites

Secondary metabolism and the secondary metabolites are more limited to specific types or groups of organisms. The production of the secondary metabolites is a result of adapting to the surrounding environments and have been optimized chemically during the millions of years of evolution. Examples can be metabolites acting as toxins for self-defense, metabolites secreted for the purpose of attracting or repelling other different organisms or as coloring pigments to warn other organisms (3). The production of secondary metabolites is not strictly important for the organisms vital functions, but it is beneficial for the organism by increasing the fitness for survival (5, 7). Secondary metabolites are often more interesting than primary metabolites when it comes to the discovery of novel potential lead compounds suitable for further development into commercial products (7-9).

1.3 Natural products as pharmaceuticals

Many drugs that are administered today are derived from natural sources. Natural products have been used for thousands of years as treatment for many conditions and have been one of the most successful sources of potential drug leads (9). Collections of prescriptions and many pharmaceutical records have proved that natural products has been used as pharmaceuticals since 1500 B.C (9). The products in the records contain substances that are plant derived, such as licorice (*Glycyrrhiza glabra*), *Cedrus* species, *Papaver somniferum* among others (10).

One of the first pure natural products that was commercialized is **morphine** isolated from the opium poppy *Papaver somniferum, by* the German pharmacist Friedrich Sertürner around 1803-1806 (10, 11). Morphine is an analgesic that has a mechanism of action located in the nervous system. Morphine is also the origin of other semisynthetic products with a variety of potency compared to morphine, such as heroin, codeine and buprenorphine (11).

Aspirin, or acetylsalicylic acid is one of the most administered drugs in the world. It is a semisynthetic drug that was synthetized with the natural product salicin as a starting point (11). Salicin was isolated from a willow bark, *Salix alba* (11, 12). Acetylsalicylic acid is mainly administered as an antiplatelet agent, first line therapy to treat cardiovascular diseases. It has also an analgesic, anti-inflammatory and antipyretic effect in higher doses, compared to the dose for antiplatelet therapy.

Penicillin was accidentally discovered by Fleming in 1928 and is a natural product isolated from a fungus that was thought to be *Penicillium chrysogenum.* However, in 2011 Houbarken et al., discovered that the strain was *Penicillium rubens* (9, 10, 13). Fleming discovered that penicillin had an antimicrobial bacteriolytic effect on a variety of bacteria, including *Staphylococcus aureus* and *Streptococcus pyogenes* (14, 15). Later, many researchers saw the potential in microorganisms producing antimicrobial agents, which further led to the "golden era" between 1940 and 1970, where many new classes of antibiotics were discovered (16).

1.3.1 Marine natural products

It has not been long since scientists began to do research in the marine environment with the aim to find molecules with pharmaceutical value (17). The marine environment contains a wide diversity of organisms, many of them still not explored for their production of secondary metabolites. The first research and investigation that was done goes back to the early 1950, where researcher Ross Nigreli worked with a toxin extracted from sea cucumber (*Actynopyga agassizi*) (17). The toxin was later named holothurin and showed cytotoxic activity demonstrated on mice, but never reached the market as a treatment. Even though holothurin never reached the market, the research on the marine environment in general has continued and increased (17).

Fortunately, the search for bioactive molecules in the deep waters has resulted in successful stories where we have drugs sourced from marine organisms, such as Eribulin mesylate, commercialized as Halaven® (17). The original natural product that resulted in Halaven was

isolated from a Japanese sponge, called *Halichondria okadai,* in 1985 (17, 18). Halaven is administered intravenously (IV) for local or metastatic breast cancer (17). Originally, it was the polyether macrolide halichondrin B (HB) and other macrolides that were isolated from the Japanese sponge, but HB was the most potent one as it exhibited activity against cancer cells (19). Unfortunately, the availability of such HB producing sponge was low, and the yield of HB produced was also too low and impractical (20, 21). To reduce dependence on the natural source, a total synthesis of halichondrin B was proposed in 1992 by the Kishi group at Harvard University. In the same year, it was revealed that parts of the molecule were not necessary for the activity of HB under the experiments performed (3, 22). Later, a derivative of the natural molecule was developed into Eribulin, that today is used for the treatment (20) (**Figure 1-1**)

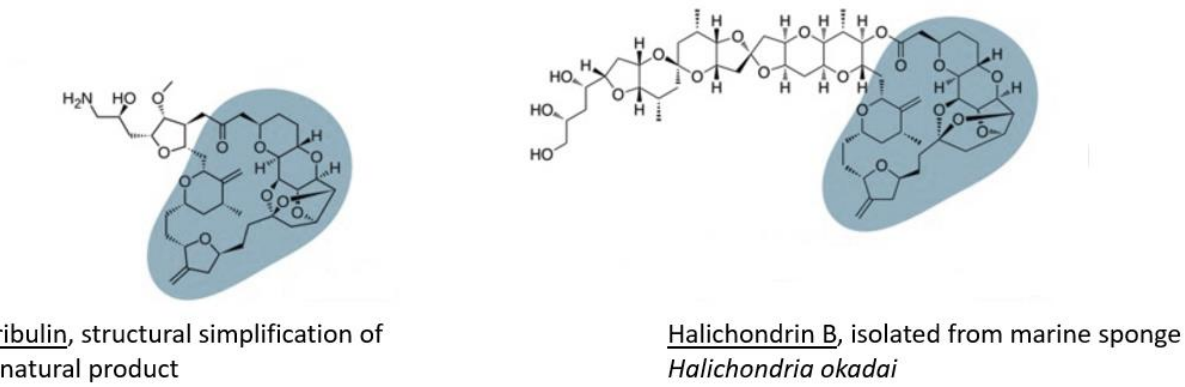


Figure 1-1 *The structures of Eribulin, the synthetic derivative, and halichondrin B, isolated from the marine sponge. The parts that are pharmacologically active are highlighted in blue. Eribulin is a derivate from Halichondrin B with a simpler structure (23).*

Trabectedin is also a natural product from the marine environment, isolated from the Caribbean tunicate called *Ecteinascidia turbinate* (24). Trabectedin is marketed as Yondelis® and is administered IV as a treatment against metastatic, advanced soft tissue sarcomas and ovarian cancer (24, 25). Trabectedin is a semisynthetic compound, and many scientists have been working with its development. Trabectedin directly extracted from the natural source is named ecteinascidins (ET). Many forms of ET's have been reported, but ET-743 was the one most prominent when it comes to the amount isolated (26). Today, trabectedin is not directly extracted from the invertebrate, since it is present in very low amounts in the natural source (26). As a consequence of this, it has been a challenge for scientists to develop a method for a total synthesis. However, in 1996, it was carried out a total synthesis of trabectedin, with the aim to circumvent the reliance on the natural source (26). This resulted in a very complex

synthesis, with many steps, that was not suitable for future development to ensure commercialization (26). Later it was proposed a semisynthetic process, where it is utilized a natural product called cyanosafrasin B, extracted from the bacterium *Pseudomonas fluorescens*, as a starting point for the synthesis of ET-743 (**Figure 1-2**) (23, 26). This semisynthetic process was successful, and the total synthesis of the complex structure that ET-743 have, is no longer executed in the labs, nor is the process dependent on the original natural source (26).

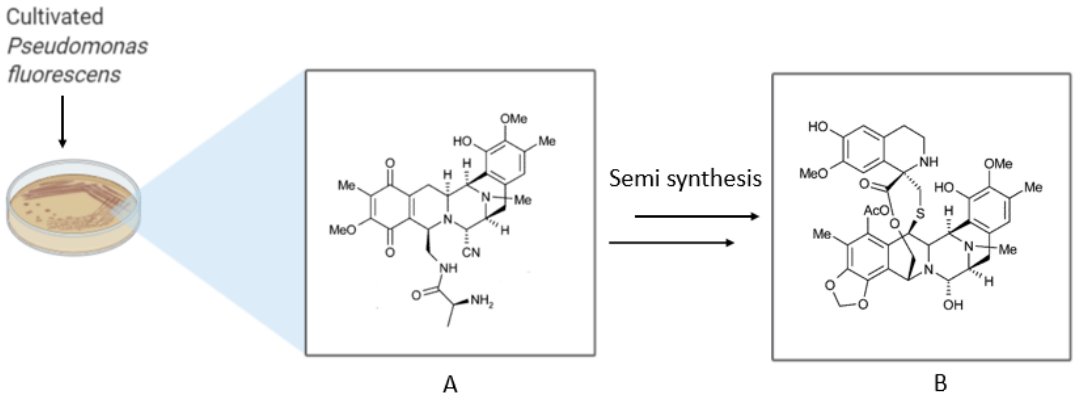


Figure 1-2 *The semisynthetic process of Yondelis. (A) = Cyanosafrasin B, and (B) = Trabectedin, Yondelis ®. The figure represents a semi synthesis where Cyanosafrasin B is employed as a starting point. (A) is extracted from the bacterium Pseudomonas fluorescens. The figure was made with inspiration from reference (23).*

1.3.2 Natural products from bacteria

We are focusing on bacteria as producer strains of secondary metabolites, because several of the e.g. antibiotics used today are produced by bacteria (27). Also, because bacteria can be grown in the laboratory (if cultivable), we can achieve unlimited amounts of biomass, circumventing the issues with supply, which has been highlighted in the examples above. Therefore, the bacteria represent an important and valuable source for discovery of new drugs. A bacterial group that have received a lot of attention over the years are actinobacteria (28). This phylum covers a group of Gram-positive bacteria, found in a variety of environments, both terrestrial and marine. The group is very large, consisting of over 30 taxonomic families of organisms (29). Many of the species from the actinomycetes class such as the *Streptomyces* sp. are beneficial sources of many kinds of pharmaceutically active ingredients (28). *Streptomyces* sp. have the ability to produce secondary metabolites that function mainly as antibiotic but also antifungal, antiviral, and anticancer agents (27). Active pharmaceutical ingredients produced by *Streptomyces* sp. that are commercialized today, are the antifungal agent nystatin, antibiotics such as erythromycin and clindamycin from the chemical class macrolides and other antibiotics

such as vancomycin, colistin, chloramphenicol, tetracyclines. The anticancer agent doxorubicin is also derived from *Streptomyces* sp. (28).

Other interesting species belonging to the actinobacteria are *Salinispora* sp. derived from the marine environment. The bacterium *Salinispora arenicola* was found to produce salinosporamide A, which is a secondary metabolite with anticancer properties. Salinosporamide A is still undergoing clinical trials under the name Marizomib, in cancer patients (28). In many cases, natural products isolated from macroorganisms are later found to actually being produced by microorganisms such as bacteria, living in a symbiotic relationship with the macroorganisms, such as an invertebrate (30). Marine sponges are among the oldest invertebrates that exist and are hosts to many secondary metabolites producing bacteria (30). The marine sponges represent an important factor for discovering bioactive natural products, either the compounds are produced by clusters of enzymes located in the sponge, or by microorganisms living in a symbiotic relationship with the sponge (31). In this thesis, the natural products that are studied originates from the marine bacteria *Lacinutrix* sp. isolated from the marine sponge *Halichondria* sp.

1.3.3 Lipids as secondary metabolites and biosurfactants

Most of the fatty acids are described as primary metabolites, but the main focus in the present project has been on the secondary metabolite lipoamino acid ornithine lipid (OL), derived from the marine bacterium *Lacinutrix* sp, only produced in a limited amount of bacterial species (7). The OLs has an amphiphilic structure, indicating that the compound might act as a biosurfactant, having surface active properties.

1.3.3.1 Biosurfactants

Surfactants are widely used compounds, not only limited to pharmaceutical application, but also in the industry, agriculture, in food and cosmetics (32, 33). The amphiphilic molecules are in lower concentrations, adsorbed in the surface of water, or between two liquids, forming a monolayer, and decrease the surface tension in water or interfacial tension between the two liquids (34). Adding surfactants in a concentration above critical micellization concentration (CMC), micelles will spontaneously form, as the surfactants start to aggregate. Incorporating insoluble compounds into micelles helps the compound to dissolve, allowing them to be absorbed within the body (35) or disrupt the cell membranes in cells, among others. The surfactants also function as e.g. pharmaceutical excipients such as detergents, wetting agents,

solubilizing agents and emulsifiers (34). Biosurfactants are compounds with hydrophobic and hydrophilic moieties produced by plants, animals and microbes (32). Introducing biosurfactants as an alternative to synthetic surfactants could be beneficial, since it is more compatible to the environment as it is more biodegradable and has lower toxicity compared to many synthetic surfactants (36). Furthermore, biosurfactants are required in smaller quantities because they display lower critical micellization concentration (CMC) compared to many synthetic surfactants (32).

Many biosurfactants are described to have various bioactivities and it has been reported that biosurfactants exhibit an additive or a synergistic effect with antibiotics, by e.g. affecting the permeability of a target cell to enhance penetration efficiency of antibiotics (33). It has also been reported that biosurfactants can disrupt biofilms (33). The role of biosurfactants in the treatment of burn wounds has also been studied previously and the results obtained showed that the biosurfactants led to wound healing and reduction of scar formation (33). The main classes of biosurfactants are glycolipids, phospholipids, polymeric biosurfactants and lipopeptides (36).

1.3.3.2 Ornithine lipids (OL)

Glycerophospholipids are membrane lipids most commonly found in the membrane of bacteria (37, 38). This class of membrane lipids consists of two fatty acids attached to the hydroxyl group of the glycerol moiety via an ester linkage. The membrane lipids also contain a phosphate group linked to the glycerol moiety, with a variable head group (37). Examples of different glycerophospholipids found in bacteria are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (37). Over the years, it has been observed that some bacterial species produce and replace already existing membrane lipids to other kinds of lipids. Under specific environmental conditions, such as limited amounts of inorganic phosphate, some bacteria are triggered to produce membrane lipids without phosphorus, such as ornithine lipids (OL) (38). However, some bacterial species produce OL regardless if the cultivation medium contains phosphate or not (even when the bacteria that produce the lipids at stress conditions are closely related to the bacteria that produce it regardless of stress conditions).The reason behind this is still not fully understood (38, 39).

OLs are found in many bacterial species but are most prominent in the outer membrane of Gram-negative bacteria (38). The structure of OL consists of a 3-hydroxy fatty acyl group

bound to the α-amino group of ornithine with an amide bond, making a lyso-ornithine lipid (LOL). Another fatty acyl group is then bound to the lipid, by an ester bond to the 3-hydroxy group of the first fatty acid (38). Two pathways for biosynthesis of OLs have been described, for some bacteria. In one pathway, the biosynthesis of OL in bacteria has two steps and are synthetized mainly by acyltransferases generated from the genes OlsA and OlsB in the bacterium (39). In step one, LOLs are formed by acyltransferase encoded by OlsB, from ornithine and 3-hydroxyacyl-fatty acids and step 2 is catalyzed by acyltransferase encoded by OlsA, forming OL, from LOL and a fatty acid (39).

The functions of amino acid containing lipids such as the OLs is still not fully understood and is being investigated, but some phenotypes have been observed. The ability of the bacteria to produce OL might be beneficial for the survival of some bacterial species in phosphate deplete habitats (39). For some other bacteria, their presence could be related to the bacteria's acid tolerance, by the formation of a positively charged "shield", that protects the cell from an acidic environment (38, 40). It is also known that these OLs can undergo hydroxylation-modifications that might also play a role in making the outer membrane of the bacteria less fluid and therefore less permeable, by increasing the extent of hydrogen-bonds between the molecules, within the bacteria (39).

The lipids studied in this project are two LOLs, which will further be abbreviated as **compound 1** for the lipid with elemental composition **$C_{20}H_{40}N_2O_4$** and **compound 2** for the one with elemental composition **$C_{21}H_{42}N_2O_4$**. Both LOLs are known to be precursors of the OLs, and are presented in **Figure 1-3**)

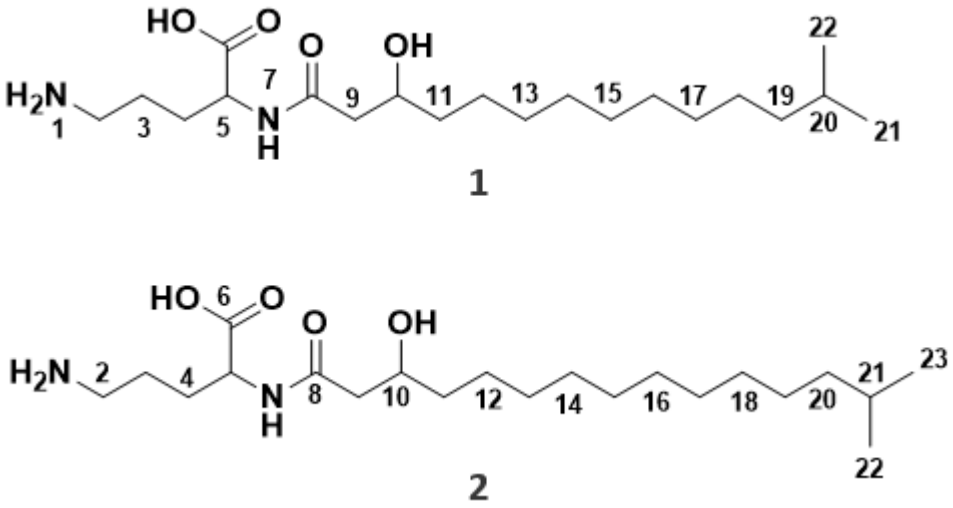


Figure 1-3 *Structure of the OLs extracted from the marine bacterium Lacinutrix sp. Figures were established in the previous project.The NMR data of the compounds are shown in Appendix B*

1.3.3.3 The genus *Lacinutrix*

The genus producing the OLs in this project is *Lacinutrix* sp. The bacterium is a Gram-negative bacterium that belongs to the family *Flavobacteriaceae* (order: Flavobacteriales, class: Flavobacteria, phylum: Bacteroidetes). The bacterial genus was first described and established in 2005, by John P Bowman and David S Nichols (41). The colonies of *Lacinutrix* sp. are often golden-yellow because of their carotenoid production, which is one of many factors that differentiate *Lacinutrix* sp. from other members of the family. The strains are also aerobic and non-motile (42, 43). Optimal growth is observed at temperatures between -2 to 25°C, in organic media that contain seawater salts (as they require Na^+ for growth) and energy sources like D-glucose or D-mannose or sucrose. No growth occurs at 30 °C or higher (42). Currently, there are ten marine Gram-negative species identified from *Lacinutrix* widely distributed in cold polar sea ice habitats, but also registered in warmer waters (42). In this thesis, the ability of *Lacinutrix* to produce secondary metabolites, will be assessed, targeting the previously mentioned OLs in particular.

1.4 Workflow and methodology

1.4.1 Workflow

The experiments conducted in this project are according to the methods in Marbio. In this project, a bacterium of the genus *Lacinutrix* was cultivated and the exudates were collected with Diaion HP20 resin. The analytes were then extracted with methanol before the extract was fractionated with flash chromatography. Compound **1** and **2** were subsequently isolated using reversed phase-preparative-mass guided HPLC. The isolated compounds were further subjected for bioactivity profiling. **Figure 1-4** gives an overview of the workflow conducted. The central techniques in this project are described in the next sections, such as the extraction, flash fractionation and isolation of the compounds and the bioactivity profiling. Structure elucidation by nuclear magnetic resonance was not performed as a part of this thesis.

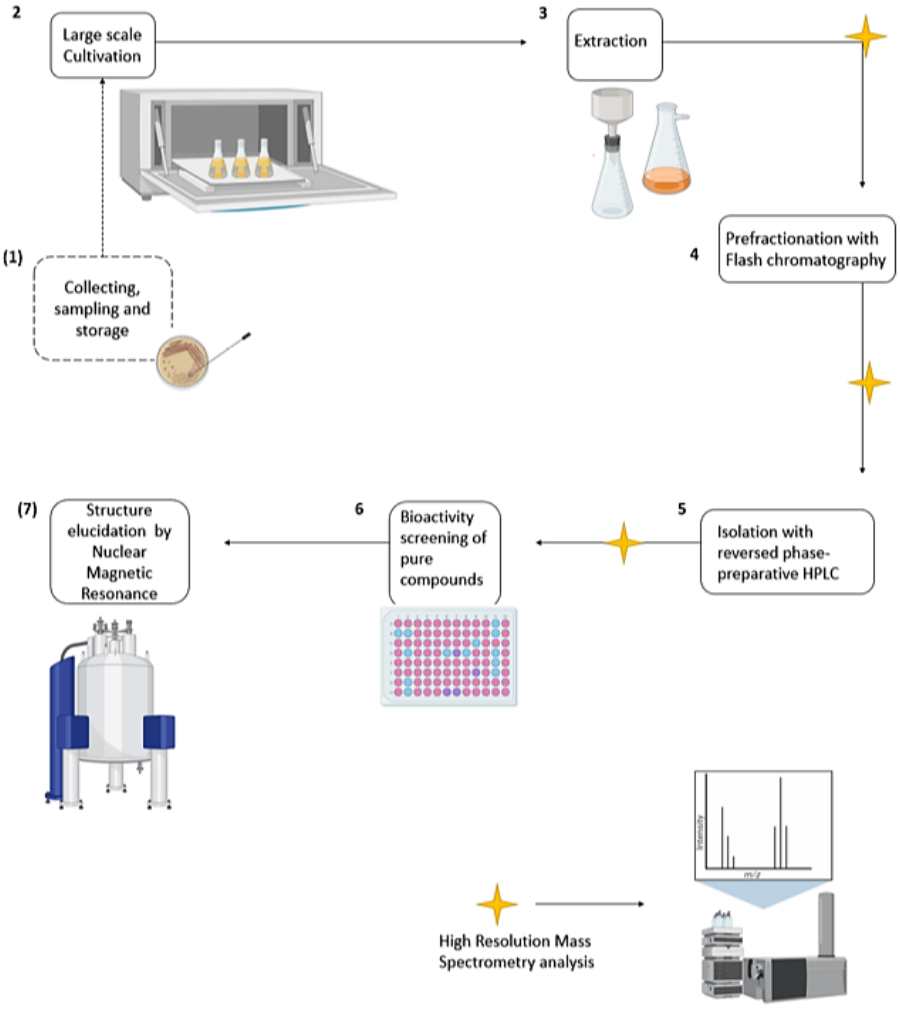


Figure 1-4 *Workflow for the master thesis. figure made with biorender.com. UHPLC-HR-MS analysis was conducted in several parts of the workflow to make sure the desired secondary metabolites were present in the extract. Collection, sampling and storage of the bacterium was performed by Marbank, the Marine biobank, Tromso.*

1.4.2 Solid phase extraction (SPE) of secreted metabolites

There are several extraction methods available for natural products. One of them is SPE, which is a very common sample preparation technique used to extract analytes from liquid cultures. SPE can be performed in several ways. It can be performed by preparing a column with the solid phase particles then apply the sample to the column, or it can be performed by adding the solid phase particles directly to the samples and do e.g. vacuum filtration. By introducing solid phase particles to the sample, the analytes (and other matrix components) are retained by different types of interactions to the solid particles (44). The analytes are afterwards extracted from the particles by utilizing a solvent that breaks the interaction between the solid phase particles and the compounds, creating an extract to be further worked with (44). When it comes to natural products, it is usual to utilize reversed phase SPE, where the solid particles are either made of modified silica-based sorbent or a polymer-based sorbent. The interactions between the solid phase and the analyte are therefore hydrophobic.

1.4.3 Separation

The separation of different analytes from matrix is referred to as *chromatography*. In analytical chemistry, the compounds of interest are usually referred to as *analytes*, and the rest of the sample, containing other unwanted compounds as *matrix*. In liquid chromatography (LC), the sample is injected and introduced to a *mobile phase* composed of one or more liquids. The mobile phase along with the sample runs through a *stationary phase* in a *column* until the eluted compounds exit the column and gets detected by a *detector* (45). In the column, the sample is distributed between a solid phase and a liquid phase. The physiochemical properties of the analyte, the stationary phase and the composition of the mobile phase determine the *retention time* of the analytes and other components (46). The stronger the analytes are retained to the stationary phase, the longer retention time (46). The graphic output from an HPLC analysis is referred to as a *chromatogram* (45). Separation can be carried out in several types of chromatographic modes, such as normal phase, reversed phase or ion exchange chromatography (43). Reversed phase chromatography is the most common separation technique in LC (44). The non-polar stationary phase is usually composed of small spherical silica particles in the size ranging from 3-10 µm making the stationary-phase tightly packed in a highly uniform and reproducible manner that leads to efficient separation. The surface of the particles is prepared with a reaction with a variety of chlorosilane reagents such as octadecyl-chain (C_{18}), octyl-chain (C_8), phenyl (C_6H_5-$(CH_2)_3$) or cyanopropyl-chain (CN-$(CH_2)_3$), where

octadecyl having the most hydrophobic character and cyanopropyl with the most hydrophilic character (44). The interaction that takes place between analyte and stationary phase is *hydrophobic interactions*. A sample containing non-polar compounds will therefore be stronger retained in the stationary phase, while the compounds of more hydrophilic character will elute faster (44). Mobile phases that are utilized in a RP-HPLC is composed of water and organic solvent(s) miscible with water, usually isopropanol, acetonitrile or methanol. It is also very common to add an acid such as formic acid, a base or a buffer to the mobile phase to control the pH and charge of the compound (44).

When isolation is carried out in complex samples, it is usually done in stepwise purification where it is utilized several chromatographic techniques with different kinds of columns. The chromatographic techniques have different capacities when it comes to the amount of sample that can be loaded in the column and purified in each run. After the extraction (e.g. SPE), a large sample quantity in the gram scale can be generated, and thus it is beneficial to initiate the isolation of the compounds with a chromatographic technique that have large capacity and columns with large particle size (3). An example of such a technique is flash chromatography (3). This will be further described in section **1.4.3.1**. The analytes of interest are further purified with a chromatographic technique using a column containing smaller particle size. The capacity of such columns is lower, but they provide higher resolution and better separation of the desired compounds (3). In this thesis, this was conducted with mass guided preparative RP-HPLC, which will be further described in section **1.4.3.2**. In other words, initially in the isolation process it is utilized techniques with columns that have large particle size to fractionate the sample into few crude fractions, but in the end, it is utilized columns with smaller particle size for more efficient resolution and separation of the desired compounds. This will purify the targeted compounds, preparing them for compound elucidation and bioactivity profiling (3).

1.4.3.1 Flash chromatography

Flash chromatography is a chromatographic technique with great capacity and with columns containing RP-particles with the size ranging between 75-150 µm. This means that large samples can be loaded (up to 2 g of sample in each run). The disadvantage with this chromatographic technique is peaks with poor chromatographic resolution and separation of compounds. However, this is not an issue as it is the first purification step, and many unwanted compounds are removed. Therefore, flash is not expected to produce the same resolution or

reproducibility as other HR-instruments, as the main goal of fractionation in this thesis is to reduce the complexity of crude extracts and is one of several purification steps in this project (3). Reducing the complexity of the extracts is important, otherwise, the bioactivity of the analytes may be masked by the interfering compounds in the matrix (46). Flash chromatography will also make the method development for the next purification step (preparative-HPLC) simpler, and also reduces the workload needed for the isolation process, saving us work hours and will cause as little abrasion as possible to the preparative-HPLC system. Additionally, flash chromatography prevents highly lipophilic compounds from entering the RP-preparative-column. This is important, since lipophilic compounds stuck in the column could be very challenging to remove (3). A maximum 2 g of the crude extract result in six fractions based on decreasing polarity (3).

1.4.3.2 High performance liquid chromatography – HPLC

To further reduce the sample complexity and to purify the compound of interest, one can change to chromatographic techniques providing better separation, e.g. preparative RP-HPLC. Compared to flash chromatography in which large sample sizes can be loaded to the column, preparative RP-HPLC has less capacity when it comes to sample load due to the small particle size of the column (approx. 3-10 µm compared to 75-150 µm in flash techniques).

However, in preparative RP-HPLC, purification is often performed on a large scale using large columns, large sample loads, and high flow rates. This allows purification of compounds from a complex matrix in high quantities (47). Further analysis (e.g. quantification, identification of elemental composition) of the isolated compounds can be achieved by using analytical HPLC-systems. Compared to preparative HPLC systems, smaller columns, with smaller capacity, having particle size down to 1.5 µm are utilized in the analytical HPLC-systems.

The RP-preparative-HPLC instrument can have an automated system to collect the analytes in tubes when they elute from the column. A small portion of the eluent (approximately 1 %) is diverted to the MS, while the rest of the eluent is directed to the collector. The analytes pass a valve that opens if the instrument is programmed to collect an analyte either by time, (e.g. after a set time period) or of a specific signal, like mass (*m/z*) and/or UV absorption. Collecting by mass is called mass guided fractionation (3). A second purification step of the eluted compounds might also be necessary. Therefore, the compounds collected in tubes in the first

run can be injected onto the same column again (or different column with other properties and different gradient) for further purification of the analytes (47).

When it comes to purifying natural products, it is common to use reverse phase columns, such as -C18 or C8-columns. (47, 48) Deciding which type of column to use can be difficult, especially when working with unknown chemical structures. However, it is useful to try different types of reverse phase columns if possible and different gradients to analyze separations achievable, to obtain the best method for isolating the analytes. this is also referred to as column screening or scout. In other words, there is a lot of trial and error (3, 47).

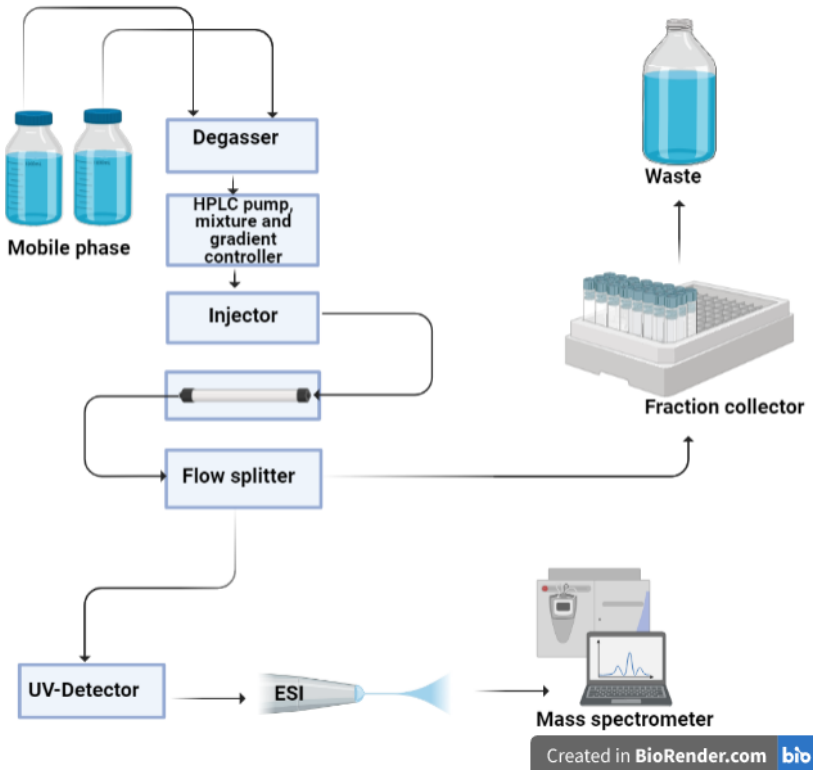


Figure 1-5 *Simple sketch of the mass guided preparative HPLC. Figure is made from inspiration from reference (49), (44) and (47)*

1.4.3.3 Ultra-high-performance LC - UHPLC

Utilization of a column with particles having a size sub-2 micron is called *Ultra high-performance liquid chromatography* (UHPLC), whereas particles with the size ranging from 3-10 µm are used in HPLC. UHPLC is a development of HPLC, and factors such as size, shape of the silica particles and operation pressure for the mobile phase are the main differences between HPLC and UHPLC. These are important factors when it comes to bandwidth as smaller and more uniform silica particles provide less bandwidth in the chromatogram. As a

consequence of utilizing smaller silica particles, higher operation pressure is used to pump the mobile phase through the column (45). The instrument is therefore equipped with pumps that have a regulation mechanism to keep a constant flow rate in the column despite of the back pressure from the small particle size of silica (44). UHPLC provides better separations and faster analysis of samples, and usually have shorter columns because of the small particle size.

1.4.4 Detection techniques

1.4.4.1 Mass spectrometry

The most powerful detector for chromatography is a mass spectrometer. Mass spectrometry have the ability to provide both qualitative and quantitative information for a given sample (44, 50). A mass spectrometer is generally composed of five parts; a *sample inlet, ion source, mass analyzer*, *detector* and a *data processing system*. (44, 51). The compounds of interest are introduced to the MS by a sample inlet. Molecules are ionized in the ion-source and separated according to their mass to charge ratio by a mass filter (e.g. Time of Flight), before they are detected by the detector where the ions are converted into electrical signals. The data processing system processes the signals from the detector and produces the output from the MS analysis, referred to as the *mass spectrum* where the number of ions detected are displayed (44). The data processing system is also utilized for controlling the instrument (51). A combination of separation and detection is used to perform isolation on the compounds, hence LC-MS.

Electrospray ionization

Before the analytes enter the MS from the HPLC, they must be ionized in order to be detected. Therefore, the first step in the MS analysis is ionization of the compounds in a sample. The sample constituents can be ionized in several ways, but in this thesis electrospray ionization (ESI) is utilized as an ionization technique. ESI is often referred to as a soft ionization method, because it does not fragment the molecular ions. In source fragmentation can occur but it is dependent on the selected voltage on the capillary during ionization (44). The ionization happens at atmospheric pressure where the mobile phase and analytes from the HPLC column passes through a thin, heated capillary tube. The capillary tube is connected to a power supply that supplies the capillary with electric charge that ionize the analytes (44, 52). At the capillary, the liquid is sprayed out and the aerosol formed contains small, charged droplets. These droplets are reduced rapidly in size because of solvent evaporation from heat of the source temperature and the desolvation gas with its temperature, until the droplets become unstable and torn apart

into even smaller droplets due to the repulsion of the sample constitutes of the same charge. This ends up with ionized analyte molecules which are then pulled to the mass analyzer through a cone, that is also connected to a power supply with the opposite charge of the analyte molecules (44). In electrospray ionization, positive or negative ions are formed depending on the applied voltage and is based on acid-base chemistry where there is proton uptake or release. In positive ionization, the analyte molecule (M) gains one proton and is observed as $[M+H]^+$ in the MS, -while negative ionization causes the analyte molecule to lose one proton and is observed as $[M-H]^-$ in the MS (44). Other ions can also be formed, such as molecular ions combined with solvent or mobile-phase components. This is termed "adducts" and forms ions such as $[M+Na]^+$ or $[M+Cl]^-$ (52) but will not be further mentioned in this thesis, since this was not observed for the compounds worked with.

Quadrupole

The quadrupole is a device made of four cylindrical rods, placed in parallel to each other, where one pair is positively charged, and the other pair is negatively charged. The rods are applied a constant voltage (dc) and an oscillating voltage radio frequency (ORF) (44, 52). The electric field generated from the dc and ORF in the rods determines which ions get stable oscillations, and thus only allow ions with a specific *m/z* to pass through the quadrupole and reach the detector, while other ions collide with one of the rods and disappear. The quadrupole can be utilized in different modes, usually in full scan, selected ion monitoring (SIM) and selected reaction monitoring (SRM) mode. The instrument can scan a wide *m/z* range for a limited time in full-scan or quickly switch between specific *m/z* values in SIM mode. This is possible because of the rapidly varying voltages that selects ions of different *m/z* values (44).

Time of flight (ToF)

In Time of flight instruments, all ions that are formed in the ion source are collected in one spot, usually referred to as the *pusher.* The ions are accelerated in pulses at the same time to an identical kinetic energy applied by the pusher (44). The ions coming from the pusher fly into a tube without any electric fields, also referred to as *the flight tube*, located between pusher and detector. Since the ions are accelerated to the same kinetic energy, they will fly at different velocities depending on their mass and charge values. Ions with a small *m/z* value will travel faster than ions with larger values and therefore will reach the detector faster (44). The *m/z* values are therefore determined based on the flight time of each ion through the field-free

region, kinetic energy and the distance from pusher to detector (44). This type of mass analyzers provides the accurate mass of the ions analyzed if the instrument is calibrated properly. The instrument also have the ability to provide high mass resolution, meaning that the instrument can separate masses that are almost identical, minimizing the possibility of overlap of the mass peaks that are close (53, 54). The instrument also provides high mass accuracy, meaning the ability of the instrument to measure an *m/z* value against the true calculated *m/z* value for the compound. This is often expressed in ppm, and for ToF instruments, the measured *m/z* will only deviate in low ppm from the true known value of the compound (55). ToF instruments also have great sensitivity in full-scan mode compared to a full scan mode in a triple quadrupole instrument. With the high resolution, high sensitivity in full scan mode and high mass accuracy, the ToF makes a great instrument for identifying the elemental composition of unknown compounds, or to confirm the elemental composition of an already known compound. This is an important part in bioprospecting, since it provides us with valuable information of active, or potentially active compounds.

Ion mobility spectroscopy (IMS)

While mass spectrometers separate the compounds based on their mass to charge ratio, in ion mobility spectrometry the compounds of interest are separated by the size, charge and three-dimensional shape. Molecules with an open structure (such as a hydrocarbon chain) will travel slower in a gas filled chamber than molecules with a more compact shape (56). The drift time can be converted to a collision cross section (CCS) value. Combining IMS with MS provides additional structural information of the compounds, e.g. isomer separation. In other words, two isomers can have the same mass, but a different CCS, because of differences in shape (56).

1.4.5 Structure elucidation by nuclear magnetic resonance – NMR

Nuclear magnetic resonance spectroscopy is a powerful tool for determining the structures of molecules. It was first developed in 1946 by two research groups at Harvard and Stanford university (57). This technique depends on energy changes that appear on molecules when they are treated with electromagnetic radiation (58). Nuclei that are important for organic structure elucidation are proton (^{1}H) and carbon (^{13}C) nucleus with nuclear spin, meaning that they behave as if they were spinning about an axis (58). The spin act like tiny magnetic fields that interacts with an external magnetic field (B_0) and thus adopt a specific orientation. Therefore, the magnetic field either aligns parallel or antiparallel to the applied external field. In the

absence of B_0, the spins are oriented randomly. The nuclei that are aligned in parallel to the external field have lower energy state, while the ones that are aligned in antiparallel to the external field have higher energy state. By introducing the nuclei with lower energy state to an electromagnetic radiation with a specific frequency, typically radio frequency (*v*) the nuclei would undergo a spin flip to a higher energy state and thus align antiparallelly to the external field (59). This phenomenon is called resonance. When the nuclei return to the lower energy state, relaxation occurs, and a specific energy is emitted. This energy will be detected and displayed as an NMR spectrum (58). When it comes to NMR, there are both *one*-dimensional (1D) and *two*-dimensional (2D) NMR, which are the most common to operate with. In the last decades we have also been introduced to *three*-dimensional (3D) NMR. This will not be further discussed in this thesis. Early after the discovery of NMR spectroscopy, measurements relied on 1D experiments. The result of 1D experiments are spectra containing one frequency axis (x-axis) that is corresponding to chemical shifts in ppm, while the second axis (y-axis) is corresponding to the intensity of the signals (57, 58). Conventionally, ^{1}H or ^{13}C NMR are referred to 1D-NMR. The development of 2D-NMR happened in the 1970s where it operates with two frequency axes, while the intensity of the signals are displayed in a third dimension (57). Examples of techniques used in 2D-NMR are correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond coherence (HMBC) (58). By performing structure elucidation, it is very common to combine several NMR techniques.

1.4.6 Bioactivity profiling

The selection and performance of bioactivity testing is a crucial part of bioprospecting. The selection of the test system should be simple, quick and relevant (60). At this stage, the testing is performed *in vitro*, on a variety of bacteria, isolated cells, and a variety of proteins such as enzymes or receptors (60). It is also possible to perform bioactivity testing *in vivo* on animals, but this is usually done in a later stage, as *in vivo* test on animals are more expensive and controversial (60). In Marbio, antibacterial-, cytotoxic-, biofilm formation inhibition-, and antidiabetic assays are performed, among others.

1.4.6.1 Antimicrobial bioactivity profiling

In 2016, the World Health Organization (WHO) was strongly requested to create a priority list of antibiotic resistant bacteria, to support the research of antimicrobial agents (61). The list is

divided into different priorities, ranging from priority 1 to 3, where there is an urgent need for new antimicrobials, in the following 1;critical, 2; high and 3; medium (62). According to WHO, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacteriaceae* bacteria such as e.g. *Klebsiella pneumonia, Eschericia coli* are all pooled in Priority 1, and are carbapenem resistant. In priority 2, *Enterococcus faecium, Staphylococcus aureus, Helicobacter pylori, Campylobacter, Salmonella* sp., and *Neisseria gonorrhoeae* are listed (62).

In this project, five bacteria were used for the assessment of the bioactivity. *Staphylococcus aureus, Enterococcus faecalis* and *Streptococcus agalactiae,* all Gram-positive bacteria, and the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. The antimicrobial effect of desired compounds is tested *in vitro* on living bacterial cells, by measuring how well they kill or inhibit the growth of the living organism (60). Prior to the profiling, dilution series of the compounds are prepared. Then, a known amount of the bacteria is inoculated with the solutions of the compound of varying concentrations, on a 96-well microtiter plate. After 24 hours, the plates are checked visually for growth, then the absorbance is measured, of each well (described in more detail in section **3.9.1.2**) A cloudy liquid medium indicates that there is no antimicrobial effect, while clear transparent medium indicates that there is an antimicrobial effect. To obtain more detailed results from the antimicrobial assay, the optical density at 600 nm is measured in each well in the 96-well microtiter plates. Here, the amount of light that passes through a well is measured. The more bacteria present in a solution/higher bacterial density, the more light gets scattered, and the less light pass through the well. This means that the optical density value is increased if there are more bacteria in the solution.

1.4.6.2 Cytotoxic activity profiling

Cancer is a general term of various types of diseases with irregular cell growth, and can affect any part of the body (63). Mutations occurring in the DNA of normal cells could potentially turn cells to cancerous ones. Those mutations are either inherited or acquired (64). Throughout the years, cancer have been studied a lot, and it is found four characteristics of cancerous cells that are absent in normal cells. These include uncontrolled proliferation, de-differentiation and loss of function, invasiveness, and metastasis (64). The uncontrolled proliferation is not strictly related to the rate of proliferation (as some cancerous cell proliferate slower than normal cells), but rather that the cells do not have the mechanisms regulating cell division and tissue growth the normal way (64). Dedifferentiation and loss of function is a process where the cells return

to an earlier state, become less specialized and lose their function (64). Invasiveness of the cells is defined as the cancerous cell's capacity of invading surrounding tissues by secreting enzymes that breaks down the extracellular matrix (64). Metastasis is defined as secondary tumors where parts of a primary tumor located at a specific place in the body, travels to another part of the body by e.g. blood vessels or lymphatics. This is also considered the major problem for cancer therapy and is the main cause of mortality (64). In 2020, ten million cancer related deaths have been reported, mainly to breast, lung, colon & rectum, skin, and stomach cancer, and it is an increasing problem worldwide (63).

In this thesis, the cytotoxic activity of the isolated compounds was assessed with Aqueous One Solution Cell Proliferation assay, which is a colorimetric method to determine the amount of viable cells in a 96-well microtiter plate (65). The reagent of Aqueous one solution is a tetrazolium salt compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt), which is also called MTS. The assay is performed by adding a small volume of MTS in each well, then incubating for 1-4 hours, before the absorbance/OD is measured in a plate reader (65). Metabolically active cells (living cells) will reduce MTS (yellow colored) to a formazan, coloring the liquid in the well to a dark purple color. Metabolically non-active (dead cells) in the wells will not be able to reduce the MTS compound, and will be observed as a yellow colored solution in the well (65) (**Figure 1-6).** The formazan products generated, is directly proportional to the number of cells alive in the cell culture, and is therefore recorded spectrophotometrically, as the formazan compound product absorbs radiation at 490 nm (65).

The cell lines that are to be tested on this project are A2058 (human melanoma cancer) and MRC-5 (lung-fibroblasts, non-cancerous cells). A2058 is utilized to investigate the compounds effect against cancerous cells, while MRC-5 was utilized to investigate the compounds toxicity against normal cells.

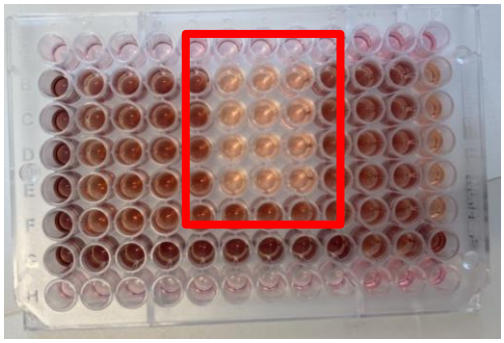


Figure 1-6 *A2058 cell lines incubated for three days, then an amount of 10 µL of Aqueous One solution was added, and incubated for 1 hour. The highlighted area corresponds to non-metabolic active, dead human melanoma cells, as these cells do not reduce MTS to formazan.*

1.5 Background of the project

The ability of bacteria of the genus *Lacinutrix* to produce secondary metabolites has not been published previously, making the bacterium an interesting project. At Marbio, an isolate of *Lacinutrix* sp. has been studied, and two compounds were nominated for isolation (**Figure 1-3**). The compounds were successfully isolated with preparative-HPLC, and their structures elucidated by NMR (data in appendix) and HR-MS. There were not sufficient amounts isolated of the compounds to do thorough bioactivity profiling and obtain bioactivity data. Due to the insufficient amount of compounds, new rounds of cultivation and re-isolation had to be performed, in order to generate more of the pure compounds **1** and **2**, to further perform bioactivity. This was the basis for the current master project. As a consequence of this, the current project is a targeted isolation, meaning that we already knew the structures of the compounds we are interested in isolating.

2 Aim of this project

The aim of this thesis was to perform a large scale fermentation of the bacterium *Lacinutrix* sp. in order to isolate two targeted natural products, lyso-ornithine lipids. This was done to generate sufficient amounts of pure compounds to further perform bioactivity profiling, using bioactivity assays available at Marbio.

The main objectives of the project are:

- ➔ Large scale fermentation of the bacterium, extraction of metabolites and fractionation of the extract generated into less complex samples of descending polarity.
- ➔ Run the samples on UHPLC-HR-MS to confirm the presence of the analytes.
- ➔ Isolate the two target compounds from the fractions.
- ➔ Perform bioactivity profiling of the isolated compounds.

3 Materials and methods:

3.1 Sampling locations, sampling, and storage

In this project, a marine bacterial strain of the genus *Lacinutrix* (M09B143) was used. The bacterium was provided by the marine biobank, Marbank, Institute of Marine Research, Tromsø. This bacterium was isolated from a sponge of the *Halichondria* sp. in the Barents Sea, on a research cruise in 2009 (Longitude 19.19 East, latitude 74.37 North). The bacterium was classified as part of the genus *Lacinutrix* by Marbank, based on sequencing of the 16S rRNA gene. Species is not determined.

For long-term storage, the liquid FMAP medium with 30% glycerol (v/v, sigma Aldrich St. Louis, MO, USA) was inoculated with the *Lacinutrix* sp, then stored in -80ºC in a cryo-tube. Cryo-tubes with bacterium were provided by Marbank for the purpose of this project, see appendix for glycerol stock preparation.

3.2 Preparation of cultivation medium

Materials:

Table 1 *Equipment and chemicals used when preparing medium*

Equipment and chemicals	Equipment and chemical specifications	Distributor
D-Mannitol	63560	Sigma Aldrich (Merck, Darmstadt, Germany)
Peptone from casein, enzymatic digested	82303	Sigma Aldrich (Merck, Darmstadt, Germany)
Milli-Q gradient A10 ultrapure water		Merck KGaA (Germany)
Filtered sea water (FSW)		Norwegian College of Fishery Science, University of Tromso – the Arctic University of Norway
Panasonic Labo Autoclave	MLS-3781L	Sanyo Techno Solutions (Tottori, Japan)
Stuart heat-stir SB162		Stuart equipment (United Kingdom)

Method:

For the large-scale cultivation of *Lacinutrix* sp, M19 medium was prepared. An amount of 20 g/L D-Mannitol and 20g/L peptone from casein was prepared in Milli-Q water and filtered sea water in the ratio 1:1. The medium was then dissolved and autoclaved for 25 minutes, at 121 °C. The medium was stored in a cool, dark place, until further use.

3.3 Large-scale cultivation of *Lacinutrix* sp. for extraction

Materials:

Table 2 *Equipment and chemicals used for the cultivation of Lacinutrix*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Lacinutrix sp.	M09B143	Marbank, Institute of Marine research (Norway)
HERA safe KS15 safety cabinet	Class II	Kendro (Germany)
Infors HT Multitron pro incubation shaker	S-000121591	Infors HT (Switzerland)

Method:

The M19 medium described previously (section **3.2**) was used for cultivation of *Lacinutrix* sp. Inoculation was performed in a class II safety cabinet. The bacterium was provided as glycerol stock, and from the glycerol stock of *Lacinutrix* sp. 200 µL was added to each flask. *Lacinutrix* sp. was cultivated in 1 L Erlenmeyer flasks with 400 mL medium. The flasks were then sealed with aluminum foil (flasks and foil sterilized) and incubated at 130 rpm shaking, at 13 °C for 14 days before resin was added. There were also prepared media controls in parallel to the bacterial cultures to check for possible contamination of the medium. The extraction was performed if there were no visible signs of contamination in the media controls. The media control was also prepared to compare the medium to the bacterial extract when performing HR-MS, to confirm that the compounds were produced by the bacterium. The bacterium was cultivated in several rounds, under the same conditions. The total volume of bacterial culture was 22.4 L.

To check for contamination, the glycerol stock was plated on FMAP agar using an inoculation loop, then stored and checked for growth after a few days of incubation at 10ºC. The plates were visually checked for contamination (colonies with different pigmentation than yellow and/or different morphology). The cultures were extracted if there was no obvious contamination on the plates.

3.4 Extraction of bacterial cultures

Materials:

Table 3 *Equipment and chemicals used in the extraction*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Methanol	34860	Sigma Aldrich (Merck, Darmstadt, Germany)
Milli-Q gradient A10 ultrapure water		Merck KGaA (Germany)
Diaion®HP-20	13607	SUPELCO, Sigma Aldrich (Merck, Darmstadt, Germany)
Cheesecloth filter, fine mesh	1057	Dansk Hjemmeproduktion (Denmark)
Whatman® qualitative filter paper, grade 3	1003-090	GE Healthcare Life sciences (Buckinghamshire, UK)
Heraeus Biofuge pico	75003235	Kendro Laboratory products (Osterode, Germany)
Laborota 4002 – control Rotary evaporator		Heidolph Instruments GmbH & Co. KG (Schwalbach, Germany)

Method:

For this extraction, a reversed phase resin with affinity for non-polar compounds was used, called Diaion HP-20 (copolymer styrene-divinylbenzene). It was added to the cultures in the amount of 40 g/L. Resin-beads were first activated by adding 100% methanol and leaving the resins swelling in the methanol for 30 minutes. The methanol was then carefully removed and replaced with Milli-Q water and left for 15 minutes. The activated resin was added to the cultures. All cultures were incubated with resin for 4 days before the extraction, which means that the total incubation time for the cultures was 18 days.

Before the extraction, subsamples from the bacterial cultures were taken for DNA analysis, to store in case there was a suspicion of contamination in the cultures. The DNA analysis makes it possible to do an identity check for the culture. A sample of 400 µL bacterial culture was transferred to Eppendorf-tubes and centrifuged at 13000 rpm for 5 minutes. The supernatant was decanted, and the pellet was further washed with autoclaved Milli-Q water. The centrifugation step was repeated under the same conditions as previously. The clear liquid was decanted, and the pellet left in the Eppendorf tube was stored at -20ºC. No DNA analysis was performed as a part of this project.

The bacterial culture with resin beads was filtered under vacuum using a cheesecloth filter. The resin beads in the cheesecloth filter were then washed with 200 mL Milli-Q to remove the remaining cultivation medium. The metabolites were extracted from the resin beads twice. The first extraction was carried out by adding 150 mL methanol, leaving it for minimum one hour, shaking at 100 rpm. The extract was thereafter vacuum filtered through Whatman filter no. 3. The second extraction was done with adding 150 mL methanol, leaving it for 15 minutes, shaking at 100 rpm. Then the extract was filtered into the same flasks as the first extraction. The methanolic extract was dried under reduced pressure at 40ºC using a rotavapor. The dried extract was stored at -20ºC until used. The extracts were named X0078K for the first extraction round, X0078L for the second extraction round and X0078M for the third extraction round. Before the extracts were completely dried, 200 µL sample was taken for a UHPLC-HR-MS analysis to check that the compounds were present in the extracts. Further description on the UHPLC-HR-MS is presented in chapter **3.8.**

3.5 Fractionation of extract with flash-chromatography

Materials:

Table 4 *Equipment and chemicals used in the preparation of the columns and the fractionation*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Diaion® HP-20SS	13615-U	Supelco, Sigma Aldrich (Merck, Germany)
Biotage® SNAP Ultra (10 g)	FSUL-0442-0010	Biotage (Sweden)
Methanol	34860	Sigma Aldrich (Merck, Germany)
Visi prep® SPE manifold		VWR International (Radnor, Pennsylvania, USA)
Laborota 4002 – control Rotary evaporator		Heidolph Instruments GmbH & Co. KG (Schwalbach, Germany)
Biotage HPFC SP4 Flash Purification System		Biotage (Sweden)

3.5.1 Preparation of the columns

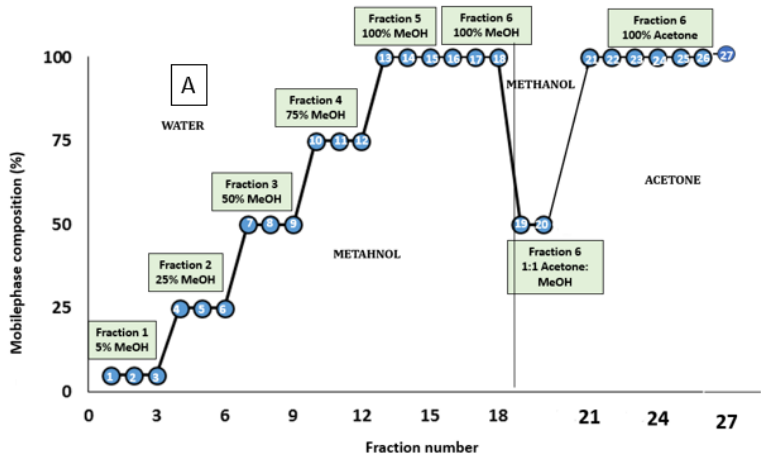
The extracts were fractionated by flash-chromatography. First, SNAP ultra-flash columns were prepared, by packing the column with Diaion®HP-20SS resin. This packing material acts like a reverse phase stationary phase. An amount of 6.5 g Diaion® HP-20SS resin was transferred to an Erlenmeyer flask, and activated in 75 mL methanol for approximately 20 minutes. The methanol was subsequently removed and replaced with water, using a vacuum manifold. The resin was then poured into the column. Water was added to the column to make sure that resins were continuously covered in liquid. The columns were stored at 4ºC until used.

3.5.2 Fractionation of the extracts

Fractionation was performed using a Biotage SP4 Flash system. The maximum amount of extract per flash run was 2 g. For preparation of the extracts, the extract was first dispersed in 90% methanol. For each run, 2 g of Diaion® HP-20SS was added to each sample and dried with rotavapor at 40ºC. Adding resin to the samples before loading it to the columns is important for a well performed flash chromatography, since it forms interactions between sample components and the stationary phase. The Diaion HP-20SS with the dried extract was then loaded on top of the packing material of the previously prepared column (see chapter **3.5.1).** Before loading the sample extract, the column was equilibrated with 5% methanol.

The mobile phases were composed of water, methanol and acetone, and the flow rate was 12mL/min. Each tube collected 24 mL of eluent. The solvents were added in a stepwise elution, as shown in **Figure 3-1**. The first mobile phase step was composed of 5% methanol and was held for 6 minutes. Afterwards, the stepwise gradient increased to 25% methanol and held for 6 minutes each time, up to a final step consisting of 100% methanol, held for 12 minutes. Then a step consisting of methanol:acetone in a ratio 1:1 was added and held for 4 minutes, before the last step was added consisting of 100% acetone, and run for 14 minutes. A total of 27 tubes were produced per run, and these were pooled into six fractions of decreasing polarity, like shown in **Figure 3-1**B.

As shown in **Figure 3-1**B, tubes 1-3 were pooled to one fraction (fraction 1) while tube 4 to 6 were pooled to another separate fraction (fraction 2), and so on. All tubes from 16 to 27 were merged into one fraction (fraction 6). The first fractions (1 to 3) contained mostly polar compounds. Fractions 4, 5 and 6 were collected individually as displayed in table **Figure 3-1**B and were dried under reduced pressure at 40ºC. They were also analyzed on the analytical UHPLC-HR-MS to determine which fractions to use for further purification of the compounds of interest.



Tube number	Fraction
1,2,3	1
4,5,6	2
7,8,9	3
10,11,12	4
13,14,15	5
16,17,18,19,20, 21,22,23,24,25, 26,27	6

B

Figure 3-1 *Overview of gradients used (A) and their fractions of reducing polarity from 1-6 (B)*

3.6 Purification of the LOLs with preparative system 1

Materials:

Table 5 *Equipment and chemicals utilized when mass guided RP-Preparative HPLC was performed, in preparative system 1.*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Ultra-High-Performance Centrifuge Tubes 50 mL with flat cap		VWR International (Radnor, Pennsylvania, USA)
Methanol	34860	Sigma Aldrich (Merck, Germany)
Heraeus Multifuge 3-SR	75004371	Kendro Laboratory products (Osterode, Germany)
Acetonitrile Prepsolv ®	1.13358.2500	Merck KGaA (Germany)
Milli-Q gradient A10 ultrapure water		Merck KGaA (Germany)
Formic acid ≥98%	33015-2.5L-M	Sigma Aldrich (Merck, Germany)
Methanol HiPerSolv Chromanorm	20864.320	VWR International (Radnor, Pennsylvania, USA)
Sample Manager	2767	Waters (USA)
HPLC Makeup Pump	515	Waters (USA)
Controller	600	Waters (USA)
Flow Splitter		Waters (USA)
Prep Degasser		Waters (USA)
Photodiode Array Detector	2996	Waters (USA)
Mass detector 3100	3100	Waters (USA)
Masslynx (software) V4.1		

Method for preparing the samples:

Fraction five with the total weight of 1.0212g from extract X0078K, X0078L and X0078M was resuspended in methanol, then transferred to a centrifuge tube and centrifuged at 4000 rpm for 2 minutes at 20ºC. This process resulted in a clear liquid and a pellet. The liquid was transferred to a new centrifuge tube, while methanol was added to the previously formed pellet and mixed. Following this, the methanol:pellet suspension was centrifuged three times under the same conditions (as mentioned above) in an attempt to dissolve as much as possible from the fraction five, while also removing un-dissolvable components from the sample before injecting it into the preparative HPLC column. The supernatant formed was removed between each centrifugation and collected. To purify the analytes from the matrix of the fraction, preparative

HPLC-MS was used. The preparative HPLC-MS system used for the first purification of the compounds will be called “preparative system 1” throughout the thesis.

First purification step on preparative system 1 of the target compounds and development of the method for the optimal separation conditions

Purification of the two compounds was conducted on a reversed phase preparative HPLC column. Instrument specifications are given in **Table 5**. The parameters and optimized gradient are given in **Table 6** and **Table 7**. The protonated molecular ions $[M+H]^+$ 373.4 and 387.5 was used to trigger the mass collection of compound **1** and **2**, respectively. As mentioned previously, a suitable column and an optimal gradient must be selected specifically for the analytes before purification is carried out. After some trial and error (further described in the result and discussion section), a preparative SunFire C18 OBD column (5µm, 10mmx250mm, Waters) was chosen. Separation was achieved by using a gradient starting with 50% of mobile phase B (consisting of 0.1 % formic acid in acetonitrile) and 50% of mobile phase A (consisting of 0.1% formic acid in Milli-Q), increasing linearly to 85% of mobile phase B run over 10 minutes. The flow rate was set to 6 mL/min and the injection volume was set to 150 µL for each run and with the optimized gradient. After the first purification, the collected fractions were dried under reduced pressure and weighed. Before the samples were completely dried, an amount of 10 µL was taken for UHPLC-HR-MS analysis. Compound **1** did not require a second purification; however, a second purification was required for compound 2, which will be addressed in section **3.7.**

Table 6 GRADIENT 1: *Mobile phase gradients used for the preparative system1 consisting of A, Milli-Q and 0.1% formic acid and B, consisting of acetonitrile and 0.1% formic acid.*

Time (minutes)	Flow (mL/min)	A%	B%
Initial	6	50	50
10	6	15	85
10.10	6	0	100
13	6	0	100

Table 7 *The parameters used when running the RP-Preparative-HPLC for the purification of the analytes from the complex matrix, in preparative system 1*

Mass range	250-500 *m/z*
Capillary voltage (kV)	3
Cone voltage (V)	30
Source type/polarity	ESI+
Source temperature (ºC)	120
Desolvation temperature (ºC)	300
Cone gas flow (L/h)	5
Desolvation gas flow (L/h)	650
Scan time (s)	0.5

3.7 Purification of the target compound 2 with preparative system 2

Materials:

Table 8 *Equipment and chemicals utilized when RP-Preparative HPLC was performed in preparative system 2*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Methanol	34860	Sigma Aldrich (Merck, Germany)
Acetonitrile Prepsolv ®	1.13358.2500	Merck KGaA (Germany)
Milli-Q gradient A10 ultrapure water		Merck KGaA (Germany)
Formic acid ≥98%	33015-2.5L-M	Sigma Aldrich (Merck, Germany)
Acquity Arc Sample Manager FTN-R		Waters (USA)
Acquity Arc Quaternary Solvent Manager-R		Waters (USA)
Acquity Arc Column manager		Waters (USA)
Acquity QDa Detector		Waters (USA)
Photodiode Array Detector	2998	Waters
Isocratic solvent manager		Waters (USA)
Fraction manager		Waters (USA)
Masslynx (software) V4.2		

Method for preparing the samples:

Since compound **2** required a second purification step, dry sample from the first purification of the compound (23.2 mg) was resuspended in a suitable amount of methanol and transferred to a test tube. This process was repeated twice. The supernatant was transferred to an MS vial prior to the second purification. As mentioned above, compound **1** did not require a second purification. The preparative HPLC-MS system used for the second purification of compound **2** will be called "preparative system 2" throughout the thesis.

The second purification on preparative system 1 of target compounds and development of the method for the optimal purification conditions

The second purification of compound **2** was performed on a reversed phase preparative HPLC, Waters instrument. Parameters and optimized gradient are given in table **Table 9** and **Table 10**. Since some of compound **1** co-eluted with compound **2**, it was decided to also isolate a fraction

of compound **1** in the second purification. For the second purification, a preparative Atlantis T3, C18 column (3µm, 3mmx150mm, Waters) was utilized. After some trial and error, separation was achieved by using a gradient starting with 35% of mobile phase B (0.1% formic acid in acetonitrile), and 65% A (0.1% formic acid in Milli-Q) increasing linearly to 55% of mobile phase B over 12.5 minutes. The flow rate was set to 1.5 mL/min. The injection volume was set to 25-30 µL for each run. With the optimized gradient, there were in total 133 runs. After the second purification step, the samples were dried under reduced pressure, and weighed. The amount of compound **1** that was collected from the second purification was pooled with the rest of compound **1** that did not undergo a second purification step. The samples were then completely dried and weighed. Before the samples were completely dried, an amount of 10 µL was taken for HR-MS analysis. An overview of the isolation is presented in table **Table 11**.

Table 9 GRADIENT 2*: Mobile phase gradients used for preparative system 2 with A, consisting of Milli-Q and 0.1% formic acid and B, consisting of acetonitrile and 0.1% formic acid.*

Time (minutes)	Flow (mL/min)	A%	B%
Initial	1.5	65	35
12.50	1.5	45	55
12.60	1.5	0	100
16.00	1.5	0	100

Table 10 *The parameters used when running the RP-Preparative-HPLC in preparative system 2 for the purification of the analytes from the complex matrix*

Mass range	250-500 *m/z*
Capillary voltage (kV)	1.5
Cone voltage (V)	20
Source type/polarity	ESI +
Source temperature (ºC)	120
Desolvation temperature (ºC)	350
Cone gas flow (L/h)	Fixed values
Desolvation gas flow (L/h)	Fixed values
Scan time (s)	0.5

Table 11 *An overview of the number of purification rounds, columns and gradients used for the purification process performed for both compounds. Gradient 1 and 2 are presented in Table 6 and Table 9, respectively.*

Purification-round	Compounds		Gradient used
	Compound 1	Compound 2	
1st	Sunfire*	Sunfire*	1
2nd	-	Atlantis**	2

** SunFire C18 OBD Preparative column (5µm, 10mmx250mm), **: Atlantis T3, C18 (3µm, 3mmx150mm)*

3.8 Analysis with UHPLC-QToF-MS

Materials:

Table 12 *Equipment and chemicals used when running the UHPLC-QToF-MS samples*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Waters LC-MS certified vials		Waters (USA)
Acquity UPLC Class-I Binary solvent manager		Waters (USA)
Acquity UPLC Class-I Sample manager FTN		Waters (USA)
Acquity UPLC Column manager		Waters (USA)
Acquity UPLC® Bridged Etylen hybrid (BEH) C18 1.7 µm, 2.1x100 mm column		Waters (USA)
Acquity UPLC PDA Detector		Waters (USA)
VION ® Ion Mobility Spectroscopy (IMS) QToF		Waters (USA
Methanol LiChrosolv ®	67-561	Merck KGaA (Germany)
Formic Acid 99% ULC/MS	33015-2.5L-M	Merck KGaA (Germany)
LiChrisolv ® Acetonitrile Hypergrade for LC-MS	75-05-8	Merck KgaA (Germany)

Method:

Before the samples were completely dried, an amount varying from 10-200 µL was taken for a MS analysis from the crude extracts, from fraction 4, 5 and 6, and from the final isolated compounds **1** and **2**. If necessary, the samples were transferred to an Eppendorf tube, and centrifuged at 13000 rpm for 5 minutes prior to injection. The clear liquid was transferred to a LC-MS vial and analyzed on the UHPLC-HR-MS (ToF). The chromatographic separation was conducted on an ACQUITY UPLC BEH C18 column (1.7µm, 2.1mmx100mm) with the gradient 10-100% acetonitrile containing 0.1% formic acid (A) and Milli-Q water containing

0.1% formic acid (B). Instrument parameters and gradient used when running the samples are given in **Table 13** and **Table 14**.

Table 13 *Instrument parameters used when running the UHPLC-QToF-MS samples*

Mass range	250-2000 *m/z*
Capillary voltage (kV)	0.8
Cone voltage (V)	30
Source type/polarity	ESI (+ and -)
Source temperature (ºC)	120
Desolvation temperature (ºC)	350
Cone gas flow (L/h)	50
Desolvation gas flow (L/h)	600
Scan time (s)	0.2
Flow (mL/min)	0.450

Table 14 *Mobile phase gradient of (A) consisting of Milli-Q + 0.1% FA and (B) consisting of Acetonitrile + 0.1% FA*

Time (minutes)	Flow (mL/min)	A%	B%
Initial	0.450	90	10
12	0.450	0	100
13.50	0.450	0	100

3.9 Sample preparation for bioactivity profiling

Since the transference of the samples to the NMR was delayed, the sample was divided into two sample parts, where the first part was stored for future NMR experiments, and the second part was prepared for bioactivity profiling.

3.9.1 Bioactivity profiling of compound 1 and 2

Compound **1** and **2** were tested for antimicrobial and cytotoxic assays, which will be further described in the next sections. Two adherent cell lines were utilized in the cytotoxic assay. The cell line A2058 (human melanoma) and the cell line MRC-5 (human lung fibroblast) were used to investigate the compounds toxicity against cancer cells and non-malignant human cells. A range of compound concentrations were used to investigate the potential dose-response nature of the detected activity. The bioactivity profiling was performed in a 96-well microtiter plates, and the results were evaluated by using optical density measurements. Prior to the bioactivity profiling, samples of both isolated compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 20 mM. Then a stock solution was prepared by diluting the samples into a concentration of 1 mM in water. The stock solution was further utilized to make the dilution series of 10, 50, 100 and 150 µM for bioactivity profiling.

3.9.1.1 Cytotoxic activity profiling

Materials:

Table 15 *Equipment and chemicals used in the cytotoxic activity profiling*

Equipment and chemicals	Equipment and chemical specifications	Distributor
A2058	ATCC CRL-11147	LGC Standards (UK)
MRC-5	ATCC CCL-171	LGC Standards (UK)
Dulbecco's Modified Eagle's medium	D6171	Merck KGaA (Germany)
Minimum Essential Medium Eagle	M7278	Merck KgaA (Germany)
CellTiter 96® Aqueous One Solution Reagent	G3581	Promega (Wisconsin, USA)
Dimethyl sulfoxide (DMSO)	D4540	Merck (KgaA (Germany)
Trypan Blue solution	T8154	Merck KgaA (Germany)
TRYPSIN-EDTA 10X Phosphate buffered saline (PBS)	X0930-100	Biowest, the serum specialist (Nuaillé – France)
Glutamine stable 100X, 200mM	X0551-100	Biowest, the serum specialist (Nuaillé – France)
Non essential amino acids	K0293	Merck KgaA (Germany
Sodium pyruvate solution	L0473	Merck KgaA (Germany)
Sodium bicarbonate solution	L1713	Merck KgaA (Germany)
Fetal Bovine Serum (FBS)	S1810-500	Biowest, the serum specialist (Nuaillé – France).
Gentamycin	G1272	Merck KgaA (Germany)
HERA cell vios 160i		Thermo Scientific
DTX 880 Multimode Detector		Beckman Coulter (California, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
Nuclon Delta Surface 96-well	734-2073	Thermo scientific
Nunc™ EasYFlask™ Cell Culture Flasks	734-2066	Thermo scientific
Bürker chamber		

Maintenance of the cell cultures/splitting of the cells

All work was performed in aseptic conditions, with a class II safety cabinet. The cell-line A2058 was cultivated in D-MEM, and MRC-5 was cultivated in MEM eagle, both with additions in the medium, as displayed in **Table 16**. Both growth medium, PBS and the trypsin were first heated to 37ºC before use. Prior to use, the cells were stored in the specific growth medium with 10% DMSO, in liquid nitrogen at -196 ºC. The cells were first thawed and then cultured in their suitable medium, until they reach a cell-density of approximately 70-80%. Then, cell-culture splitting was performed. Before splitting, the cells are arranged in a monolayer attached at the bottom of the flask. When splitting was performed, the growth medium was carefully removed from the cell-culture flask. The cell-layer was then washed with phosphate buffered saline (PBS) to remove the remaining growth medium. The PBS was subsequently removed before an amount of trypsin (approximately 3 mL) was added to the cells and further incubated at 37ºC. This step was performed to help the cells detach from the bottom of the flask, making the splitting of the cell culture possible. After the incubation, the cells were examined under the microscope to determine whether or not they have detached from the flask. The detached cells were further suspended in approximately 10 mL of growth medium. A sufficient amount of cell culture was transferred to a new culture flask with fresh medium to continue growing until the next round of splitting. The cell-line A2058 was split approximately 1:10-1:20 and MRC5 is split approximately 1:3-1:4. The cell-cultures were further incubated at 37ºC with 5% CO_2. The rest of the cell suspension was transferred to a 50 mL centrifugation tube, for further cytotoxic activity profiling.

Table 16 *Cultivation medium for the cell line A2058 and MRC-5.*

Cell line and type	Growth medium
A2058 (Human melanoma, malignant cells)	• Dulbecco's Modified Eagle's medium • Fetal Bovine Serum (FBS) • Gentamycin • Glutamine stable 100X, 200mM
MRC-5 (Human lung fibroblast, normal cells)	• Earle's Minimal Essential Medium • Fetal Bovine Serum (FBS) • Gentamycin • Glutamine stable 100X, 200mM • Non essential amino acids • Sodium pyruvate solution • Sodium bicarbonate solution

Seeding of cells in the 96-well plates

To make sure each well in the microtiter plate had the sufficient number of cells for each cell-line, a Bürker chamber was used to determine the amount of cells per mL in the cell suspension. An amount of 100µL Trypan Blue solution and cell suspension were transferred to an Eppendorf tube and mixed. Here, the trypan blue was added to label the dead cells, to easily distinguish between the cells that are alive, since the trypan blue penetrates the membrane of cells with a damaged cell membrane and binds to their DNA. Therefore, the trypan blue solution dye the dead cells/the cells that are about to die in the color blue, so the cells were not taken into account in further calculations. An amount of 10 µL of the trypan blue solution and cell suspension mix were transferred to the Bürker chamber, and the cells that were not blue were counted. The cell suspension was further diluted and adjusted to the correct number of cells per mL, corresponding to an amount of $4x10^4$ cells/mL in fresh growth medium for the cell line MRC-5 and $2x10^4$ cells/mL in fresh growth medium for the cell line A2058. An amount of 100 µL of the diluted cell-suspension for A2058 and MRC5 was transferred to each well in a 96-well microtiter plates, corresponding to 4000 cells each well for MRC5 and 2000 cells each well for A0258, one plate for each cell line. The plates were further incubated at 37ºC with 5% CO_2 for 24 hours, to make sure that the adherent cells attach to the bottom of the wells.

Adding compound 1 and 2 to the cell lines in the 96-well plates

The next day, the cells were ready for the addition of compound **1** and **2** in the 96-well microtiter plates. Prior to the adding, dilution series were made of the stock solution with the growth medium of each cell line (see chapter **3.9.1** for the preparation of stock solution).

First, the growth medium in the 96-well microtiter plates was removed, before the test-compounds were added to the wells with cells. This way, the cells were arranged in a monolayer in the bottom of the wells. The compounds were tested for activity in the final concentrations of 10µM, 50µM, 100µM and 150µM in quadruplicates, for both compounds. Negative and positive controls were also included with the cells. The negative control was composed of fresh growth medium, which will give approximately 100% cell survival, whereas the positive control contained 10% DMSO in fresh growth medium, which will give approximately 0% cell survival. The controls were used to calculate the cell survival of each concentrations. The plates were further incubated for approximately 72 hours at 37ºC and 5% CO_2.

Reading of results

After approximately 72 hours, an amount of 10 µL CellTiter 96® Aqueous One Solution Reagent was added to each well, and the plates were incubated for approximately 1 hour, at 37ºC and 5% CO_2. The plates were then put in a DTX 880 Multimode Detector, and the optical density (OD) was measured at 485 nm in each well to investigate the fraction of reduced formazan. The OD was presented in cell survival (%). The equation for the calculation of cell survival is presented below:

$$Survival\ (\%) = \frac{OD_{C1\ or\ C2} - OD_{PC}}{OD_{NC} - OD_{PC}} \cdot 100\%\ (E.1)$$

Equation 1*: Calculation of cell survival (%) in the wells where compound 1 and 2 were added to the cells. Optical density measurements from these wells are used (ODC1 or C2), together with positive and negative control. Optical density of the positive control (OD_{PC}) corresponds to the average of the optical densities of the wells that were treated with 10% DMSO corresponding to 0% cell survival. Optical density of the negative control (OD_{NC}) corresponds to the average of the optical densities in the wells that had neither sample compounds nor DMSO corresponding to 100% cell survival.*

In total, the cytotoxic activity profiling was conducted in three independent biological replicates, each containing four technical replicates (n=12) for each cell line.

3.9.1.2 Antibacterial activity profiling

Materials

Table 17 *Equipment and chemicals used when performing the antibacterial activity profiling*

Equipment and chemicals	Equipment and chemical specifications	Distributor
Staphylococcus aureus	ATCC 25923	LGC Standards (UK)
Escherichia coli	ATCC 25922	LGC Standards (UK)
Enterococcus faecalis	ATCC 29212	LGC Standards (UK)
Pseudomonas aeruginosa	ATCC 27853	LGC Standards (UK)
Streptococcus agalactiae	ATCC 12386	LGC Standards (UK)
Milli-Q gradient A10 ultrapure water		Merck KGaA (Germany)
Milli-Q ultrapure water with 0.9% NaCl		
Mueller Hinton broth (MH)	275730	Becton, Dickinson and company (New Jerzey, USA)
Brain Heart Infusion broth (BHI)	53286	Sigma Aldrich (Merck, Darmstadt, Germany)
Blood agar plates	SUMP Media kitchen	University hospital of North Norway, UNN (Norway)
Gentamicin	A2712	Merck KGaA (Germany)
Heated incubator MIR-262		Panasonic Healthcare (Japan)
Incubator Unimax 1010		Heidolph Instruments GmbH, Co (Germany)
Victor multilabel Counter		Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)
96-well Micro titer plates	734-2073	VWR VWR International (Radnor, Pennsylvania, USA)

Preparation of the test bacteria on agar plates and maintenance

The five bacterial strains were stored in a 10% glycerol stock preparation at -80ºC prior to use. The strains were streaked on a blood agar plate from the glycerol stock with an inoculation loop, and subsequently incubated at 37°C over night. Then the plates were stored at 4ºC for two

weeks, before a new bacterial colony were transferred to a new plate and incubated as mentioned above. After a total of 4 weeks of storage, the blood agar plates were thrown away, and strains from the glycerol stock preparations were transferred and streaked in a new plate. This was done routinely, to make sure the bacteria are maintained in the same environment. Over time, water can condense and settle over the agar and the bacterium. The cells and agar can also dry out over time if they are kept in the same agar plate for a longer period. In addition, this was done routinely to reduce the risk of using bacterial strains in the plate that might be contaminated by other bacteria. All these factors mentioned above could contribute to abnormal or non-optimal growth conditions to the bacteria. This could have an impact in the results when the assays are run.

Profiling

When antibacterial profiling was conducted, one bacterial colony was picked from the blood agar plate and transferred to 8 mL of growth medium suitable for the bacterium that was to be tested. The bacterial cultures were further incubated at 37ºC over night. Then an amount of 2 mL of the bacterial cultures of each strain were transferred to 25 mL of fresh cultivation medium. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were incubated for 2.5 hours with shaking, while *Escherichia coli*, *Enterococcus faecalis* and *Streptococcus agalactiae* were incubated for 1.5 hours with shaking until the bacterial cultures reaches a turbidity of a 0.5 McFarland standard (**Table 18**). Afterwards, the bacterial suspensions were further diluted 1:1000 in the suitable growth medium, before they were transferred to the 96-well microtiter plate.

Table 18 *Overview of incubation time required for each bacterium strain to reach a turbidity of 0.5 McFarland*

Bacterium	Cultivation medium recommended	Incubation time (hours)	CFU/mL
Staphylococcus aureus	Mueller Hilton broth	2.5	$0.5\text{-}3\times10^5$
Pseudomonas aeruginosa	Mueller Hilton Broth	2.5	$3\text{-}7\times10^4$
Enterococcus faecalis	Brain Heart Infusion broth	1.5	$0.5\text{-}3\times10^5$
Escherichia coli	Mueller Hilton broth	1.5	$0.5\text{-}3\times10^5$
Streptococcus agalactiae	Brain Heart Infusion broth	1.5	$0.5\text{-}3\times10^5$

Dilution series with the concentrations 150 µM, 100 µM, 50 µM and 10 µM for both compound **1** and **2** were prepared with Milli-Q water, before each biological replicate. Since the compounds were going to be diluted 1:2 in the 96-well microtiter plates, concentrations of 300 µM, 200 µM, 100 µM and 20 µM were prepared in test deep well plates. An amount of 50 µL of the prepared dilution series were transferred from the deep-well plates into the 96-well microtiter plate in triplicates. From the prepared bacterial solutions, an amount of 50 µL was added to each well, meaning that the bacterial solutions were diluted in total 1:2000 with the compounds. When antimicrobial profiling was performed, one 96-well microtiter plate was used for each bacterial strain tested. Each plate was incubated with a growth control (G) and a negative control (blank) (N). The negative control (N) was utilized to make sure there were no contaminants in the growth medium nor the water. The control consisted therefore of sterile broth and Milli-Q water in the ratio 1:1, applied in the first column on the plate. The positive control (G) was utilized to make sure that the bacteria grew normally, and consisted of Milli-Q water and bacterial broth in the ratio 1:1. If there was visible growth in the negative control, and/or if there was no growth in the growth control, the testing had to be run again.

The gentamycin controls

The gentamycin control was prepared in a separate 96-well plate, where each bacterial strain was cultivated with concentrations of gentamicin diluted in autoclaved Milli-Q water ranging from 0.02 µg/mL to 16 µg/mL. This is done routinely, to make sure that the strains that are tested have normal growth. The MIC of gentamicin was determined visually and then compared to the normal MIC values of gentamicin, displayed in **Table 19**. If the growth of the bacteria differed more than one titer step from the expected value, the results from the assay would have to be discarded. The 96-well microtiter plates of the compounds to be tested and the gentamicin control were all incubated at 37ºC for 24 hours.

Table 19 *Normal MIC values of gentamicin against the tested bacteria.*

Bacterial strain	**MIC value (µg/mL)**
Staphylococcus aureus	0.06
Pseudomonas aeruginosa	0.25
Escherichia coli	0.13
Enterococcus faecalis	8
Streptococcus agalactiae	4

After approximately 19-20 hours, the optical density at 600 nm for each well in the 96-well microtiter plates were measured in a Victor plate reader and the antibacterial activity of compound **1** and **2** was evaluated. In total, the antibacterial activity profiling was conducted in three independent biological replicates, each containing three technical replicates (n=9)

4 Results and discussion

In a previous study at Marbio, two compounds were isolated from a bacterium and their structures were elucidated using HR-MS, 1D and 2D NMR. However, there were not enough isolated quantities available of the two isolated compounds for a thorough bioactivity profiling. In the current project, the aim was therefore to isolate more of the two compounds in order to test them for bioactivity against bacteria and human cells. The isolation was therefore targeted, meaning that we already knew what compounds we wanted to isolate.

4.1 Extraction

4.1.1 Weight of the crude extracts

A bacterium of the genus *Lacinutrix* was fermented in a large-scale cultivation and an overview of the weight of the crude extracts for *Lacinutrix* are presented in **Table 20**, resulting in a total of 28.02 grams from 22.4 L of bacterial culture, when all the three extracts (X0078K-M) were pooled together. A medium control was also extracted in order to compare with the bacterial culture using UHPLC-HR-MS (qToF).

Table 20 *Overview of the weight of the dry extracts X0078K, X0078L, X0078M and the volume used for each cultivation. Each bacterial culture flask contained 400 mL of the prepared liquid medium M19.*

Extract name	Weight (g)	Culture volume
X0078K	9.7614	8 L
X0078L	8.2176	7.2 L
X0078M	10.0364	7.2 L
SUM	**28.0154**	**22.4 L**

4.1.2 Crude extracts and UHPLC-HR-MS analysis

All the crude extracts were analyzed with UHPLC-HR-MS to make sure that the targeted compounds were present in the extracts. In this section, the UHPLC-HR-MS results will be demonstrated with extract X0078K. In **Figure 4-1**B, the two compounds that were isolated and purified are highlighted. **Figure 4-1**A shows the chromatogram of the medium control and **Figure 4-1**B shows the chromatogram of X0078K (crude bacterial extract). Both compounds were found in all three bacterial extracts X0078K, X0078L and X0078M (data for the two latter not shown but were comparable to X0078K) and not in the media control, which means that their presence is a result of the compounds being produced by the bacterium. The chromatograms of the crude extract after the cultivation process looked complex compared to the medium control.

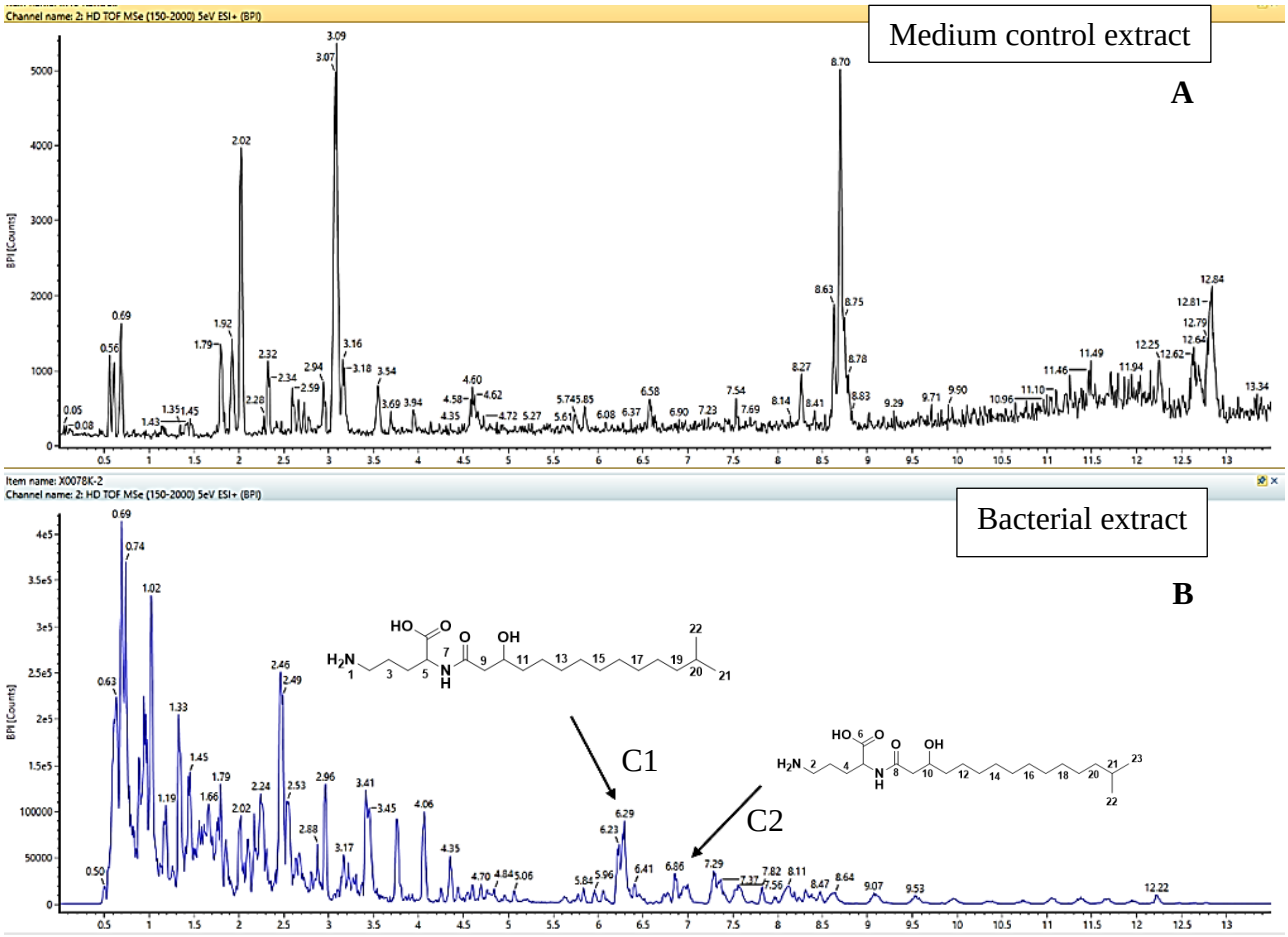


Figure 4-1 *UHPLC-HR-MS chromatogram of medium control extract (A) and bacterial extract X0078K (B). Compounds **1** and **2** are highlighted in B with arrows indicating their elution peaks. The compounds were not found in the medium control extract.*

The elemental composition of compound **1** was calculated to be $C_{20}H_{40}N_2O_4$ with *m/z* value observed to be 373.3062 $[M+H]^+$ (calculated *m/z* 373.3066) and $C_{21}H_{42}N_2O_4$ with *m/z* value observed to be 387.3218 $[M+H]^+$ for compound **2** (calculated *m/z* 387.3223). **Figure 4-2** and **Figure 4-3** display the extracted mass chromatogram (EIC) of compound **1** and **2**, with their mass spectra. The calculated elemental compositions, the exact masses of the compounds, the observed retention times and the collision cross section (CCS) values observed in the HR-MS of both compounds **1** and **2** matched the results from the previous isolated compounds, clearly indicating that the same compounds from the previous project had been produced by the bacterium. The elemental compositions and the structures from the NMR data indicate that there is only one CH_2 group differing between the two compounds. Extracted mass chromatograms showed that compound **1** eluted in three peaks, while compound **2** eluted in two peaks.

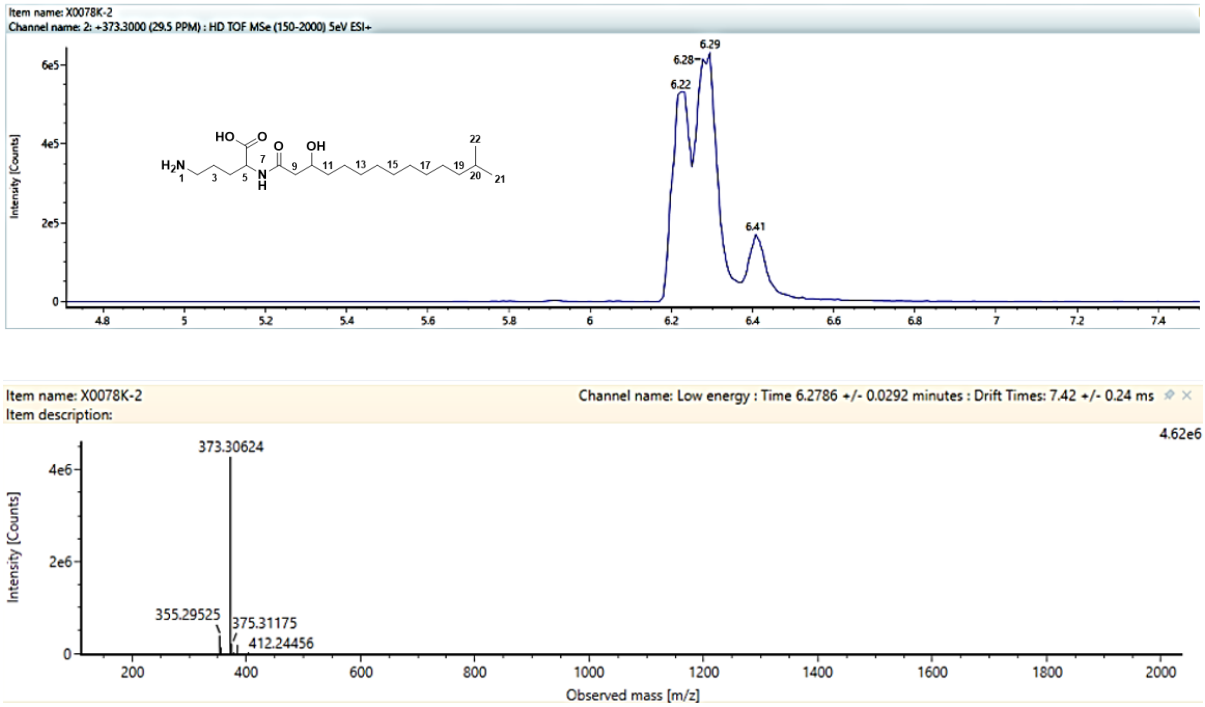


Figure 4-2 *Extracted mass chromatogram and mass spectra for compound **1** from the crude extract X0078K*

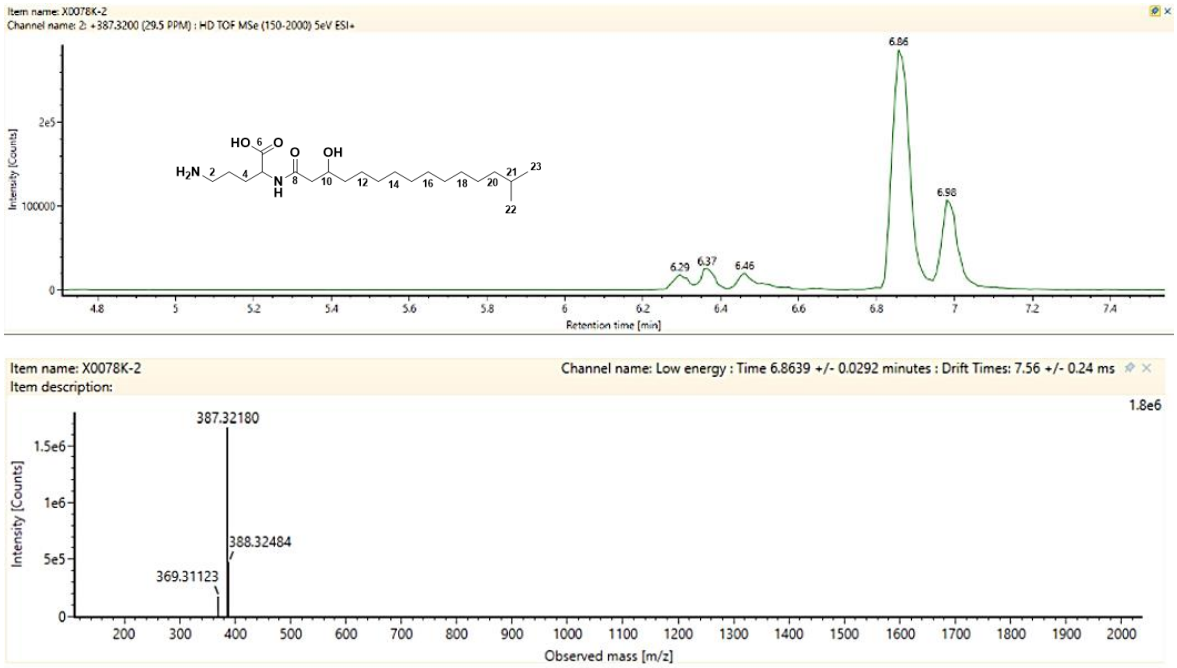


Figure 4-3 *Extracted mass chromatogram and mass spectra for compound **2** from the crude extract X0078K*

Since compound **1** eluted in three peaks, and compound **2** eluted in two peaks (as observed in the UHPLC-HR-MS **Figure 4-2** and **Figure 4-3**), there was a suspicion that there might be different kinds of structural isomers of compound **1** and **2**, differing with the branching on the hydrocarbon chains. An attempt to separate the different variants from each other were made during the isolation procedure, by altering the mobile phase composition and slope of the gradient (data not shown). Unfortunately, it was not possible to separate the isomers with the preparative HPLC as it has lower chromatographic resolution compared to the HR-MS. In other words, in the preparative HPLC, all the isomers eluted in the same peak. Additionally, it was not possible to tell whether the peaks were isomers or not based on the CCS values of the compounds from the ion mobility spectroscopy separation (data not shown), as all the values were similar for all the peaks within compound **1** and **2**. The samples were also analyzed on an Orbitrap ID-X Tribid mass spectrometer by Terje Vasskog at the Department of Pharmacy at UiT the Arctic University of Norway to investigate whether the several peaks observed in the chromatograms in **Figure 4-2** and **Figure 4-3** corresponded to different isomers of compound **1** and **2**. It was not possible to assess whether the observed peaks for the same monoisotopic mass were isomers from the fullscan, MS^2 and MS^3 data obtained from the Orbitrap, as shown with compound **2**, displayed in **Figure 4-4**, **Figure 4-5** and **Figure 4-6**. The results of the analysis of compound **1** (not shown) on the orbitrap was similar as shown for compound **2**.

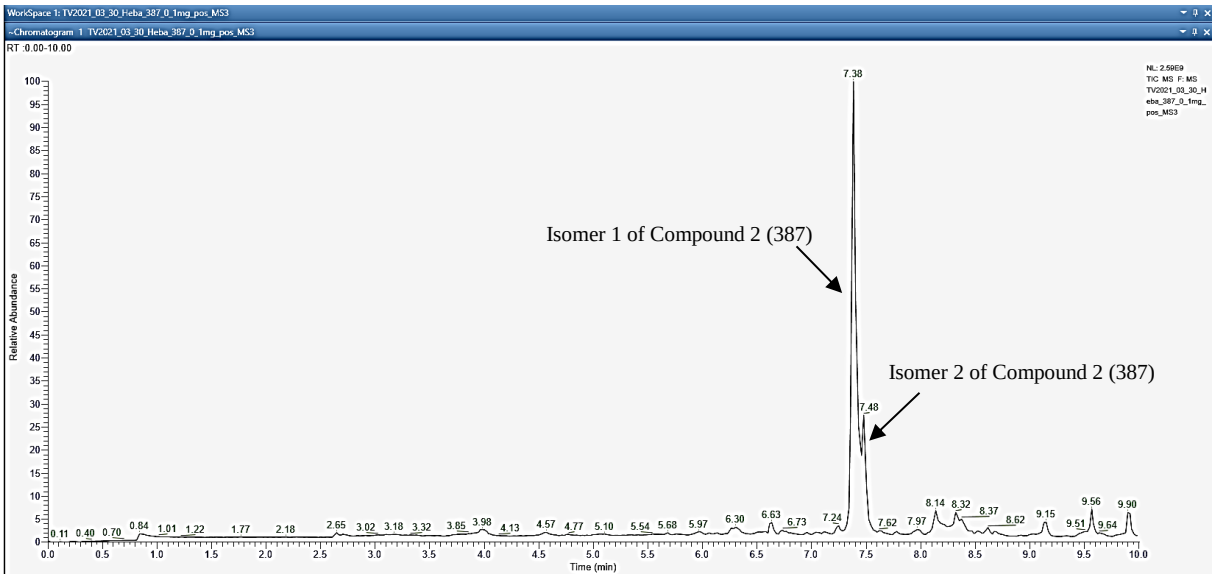


Figure 4-4 *Total ion chromatogram of the isolated compound **2** as a part of the isomer assessment in the orbitrap. The two suspected isomers are highlighted with the arrows and have the retention time 7.38 for isomer 1 and 7.48 for isomer 2. Samples were run in positive mode, ESI+.*

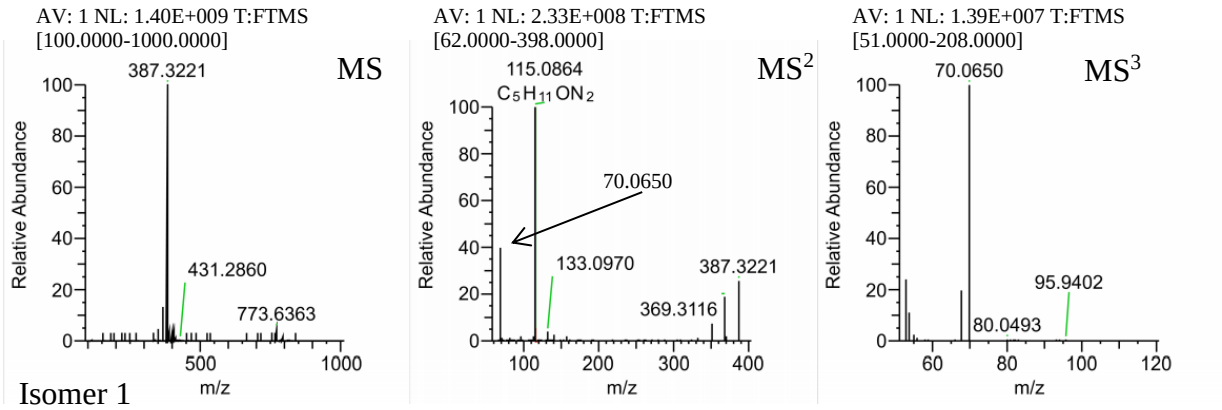


Figure 4-5 *Mass spectra of compound **2** in MS/fullscan, MS^2 and MS^3 for **isomer 1** with the retention time of 7.38 min**,** run with ESI in positive mode**.** The mass spectra (MS) display the intact protonated molecule of compound **2**, while the MS^2 data display the fragmentation of the compound. The MS^3 data display the compounds that are obtained when the fragment 115.0864 is fragmented again.*

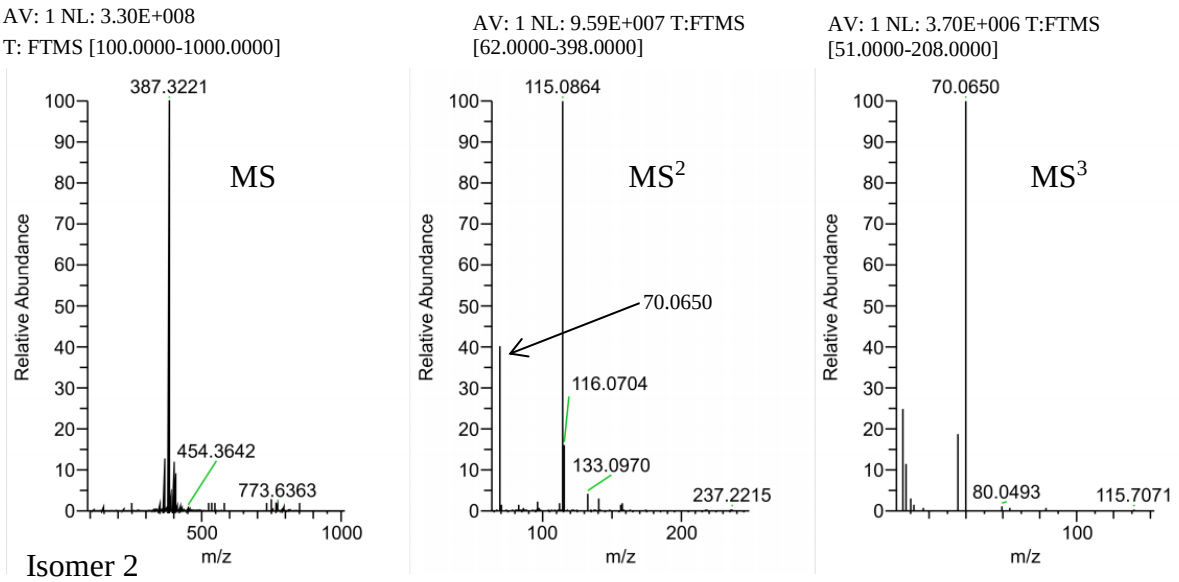


Figure 4-6 *Mass spectra of compound **2** in MS/fullscan, MS^2 and MS^3 for **isomer 2,** with the retention time of 7.48, run with ESI in positive mode. The mass spectra (MS) display the intact protonated molecule of compound **2**, while the MS^2 data display the fragmentation of the same compound. The MS^3 data display the compounds that are obtained when the fragment 115.0864 is fragmented again. The same results were obtained from compound **1**, as in compound **2**.*

The fragmentation did not appear on the branching of the hydrocarbon chain of the molecules, where the differences between the possible isomers were suspected. The fragments of *m/z* 70 and 115 were observed in the MS 2 and 3 data for both possible isomers 1 and 2 for compound **2** (**Figure 4-5** and **Figure 4-6**), which means that fragmentation happened most likely in the ornithine moiety of the compounds. The same fragments (*m/z* = 70 and 115) for the three isomers of compound **1** were also observed (data not shown). This indicates that the fragmentation of compound **1** also appeared in the ornithine moiety. The fragmentation patterns obtained by running MS2 is not surprising, as it is normally difficult to break carbon-carbon bonds with this fragmentation technique (higher energy collisional dissociation, HCD). To obtain structural information of the isomers for both compounds, it could be an option to perform mass spectrometry by using a hard ionization method, such as electron ionization in GC-MS systems, since branching on the chain changes the ratio of height between the individual fragments. The analytes will be bombarded with electrons, that will break the carbon-carbon bonds in the hydrocarbon chain, making us able to differentiate between the possible isomers and providing us with additional structural information of the possible isomers. However, the samples were not run in a GC-MS with electron ionization, because such instruments were not available in the laboratories of Marbio nor Department of Pharmacy, and also because of the limits of time and prioritization of tasks performed as a part of this thesis. Although the chromatographic data display that the mass of compound **1** elutes in three peaks,

indicating that there could be three isomers of the compound, and that the mass of compound **2** elutes in two peaks, indicating that there could be two isomers of the compound, they will be referred to as compound **1** and **2** throughout this thesis, as it was not possible to separate the isomers from each other nor identify what the structural differences within the potential isomers are.

4.2 Fractionation with flash chromatography

4.2.1 Weight of the fractions

Since the compounds were present in the extracts, the three extracts X0078K-X0078M were fractionated with flash chromatography, before further purification. The extracts were fractionated into six fractions of descending polarity. Through HR-MS analysis, both compound **1** and **2** were found in fraction 4, 5 and 6. The weight of these fractions is displayed in **Table 21**. This is a large reduction in biomass weight (from 28.02 g), which means that the complexity of the sample was already reduced a lot by performing this step. Isolation is a time-consuming process, mostly due to the restricted amounts of sample that can be injected in each sample run. After flash fractionation, only approximately 6.5% was found to contain the detectable amounts of compounds **1** and **2** of the total biomass produced. This reduction in sample amount substantially reduces the workload needed to isolate the compounds, saving both work hours and expenses for consumables and will cause the as little abrasion as possible to the isolation system.

Table 21 *Weight of fractions 4,5 and 6 after flash chromatography of the bacterial culture extracts.*

Fraction number	Weight of dried fractions (mg)
4	618.6
5	1021.2
6	182.6

4.2.2 Fractions and UHPLC-HR-MS analysis

Since the compounds appeared to be present in larger amounts in fraction five (which was also the fraction with the most biomass weight out of the three fractions) this fraction was prioritized for isolation. The UHPLC-HR-MS chromatograms for the three fractions are shown in **Figure 4-7**. Fractions 4 and 6 were stored in -20ºC, until further use in case more sample was needed for compound isolation.

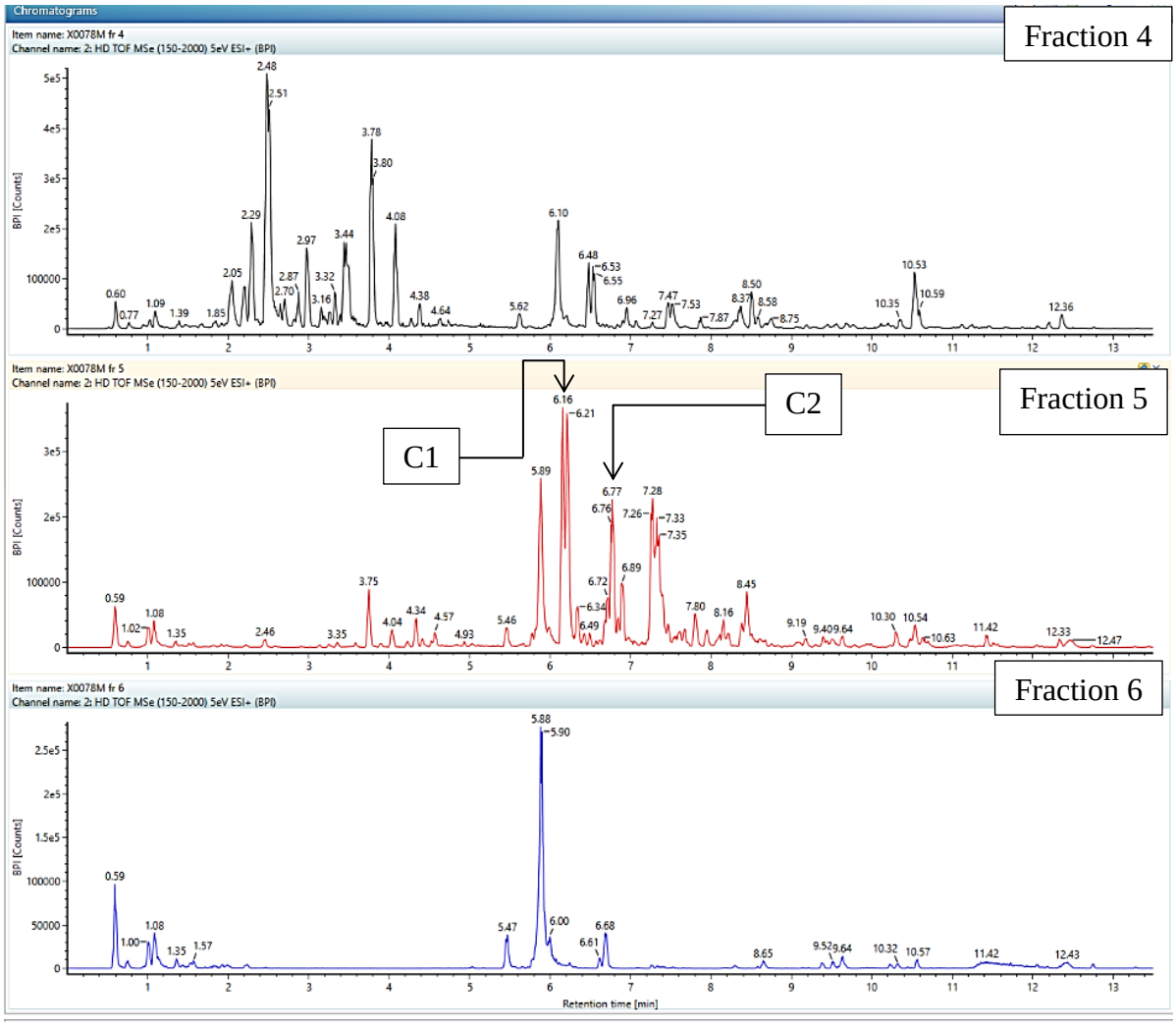


Figure 4-7 *UHPLC-HR-MS chromatograms of flash fractions 4, 5 and 6. All fractions contained compound* ***1*** *and* ***2,*** *but flash fraction 5 was prioritized for further use for the purification of compound* ***1*** *(C1) and compound* ***2*** *(C2). The analytes are presented in larger amounts in this fraction, compared to fraction 4 and 6.*

4.3 Purification of the LOLs with preparative system 1

The total amount of 1.0212 g of flash fraction 5 was used to isolate compound **1** and **2** by mass guided preparative HPLC. Different HPLC columns and gradients were tested, to evaluate the optimal chromatographic conditions in each column. This was done by running fraction five with different reversed phase columns with different modifications on the silica particles. This was done to assess which of the columns that provided best separation of the two compounds, and also to get a better understanding of the sample and the separations achievable (47). This process is also referred to as column screening or scout. In the first column screening, compound **1** and **2** eluted simultaneously when using the Atlantis column (data not shown). This can be explained by similar structures of the analytes, out of the NMR data obtained from the previous project. The only difference between compound **1** and **2**, is that the latter compound has one extra carbon in the carbon chain, making it difficult to separate these compounds. Another column was therefore tested, XSelect CSH preparative Fluoro-Phenyl column (5µm, 10mmx250 mm) with the gradient 10-100% acetonitrile and 0.1% formic acid run over 15 minutes. Compound **2** was not well enough separated from compound **1**, as displayed with overlapping peaks in **Figure 4-8**. This column was not chosen for isolation. This is an example of the results that are obtained when performing column screening.

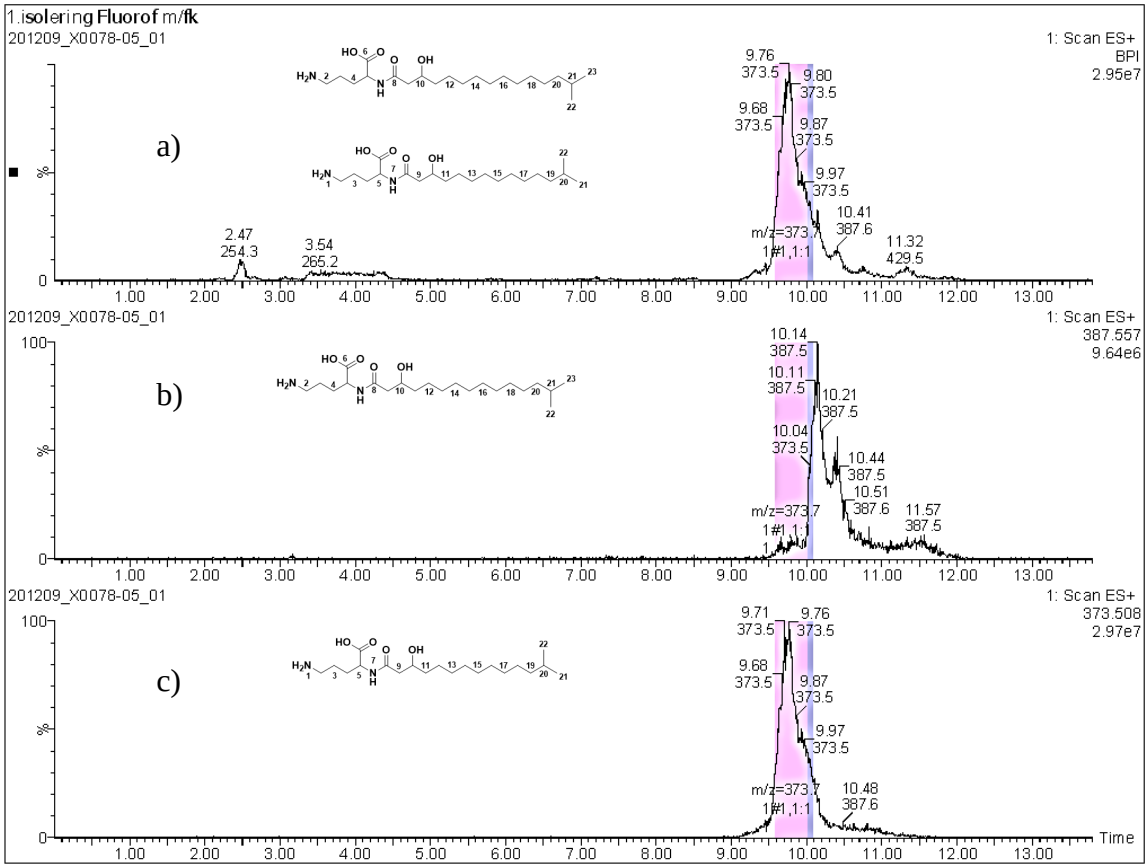


Figure 4-8 *Chromatogram from the mass guided preparative HPLC of fraction five applied to the Fluoro-Phenyl based column, which did not give sufficient separation of compound **1** and **2** under the tested settings. Chromatogram a) displays the base peak intensity chromatogram of both compounds, b) displays the extracted ion chromatogram of compound **2** while c) displays the extracted ion chromatogram of compound **1**.*

After several columns were tested in the column screening, SunFire C18 column was chosen for the initial purification. The column with its 5 µm particle size and the gradient mentioned in section 3.6 displayed both desired analytes and provided adequate separation between them. After some trial and error with the method optimalisation, the threshold, gradient and the mass range was altered to provide the best separation achievable for these compounds, as can be seen in **Figure 4-9**.

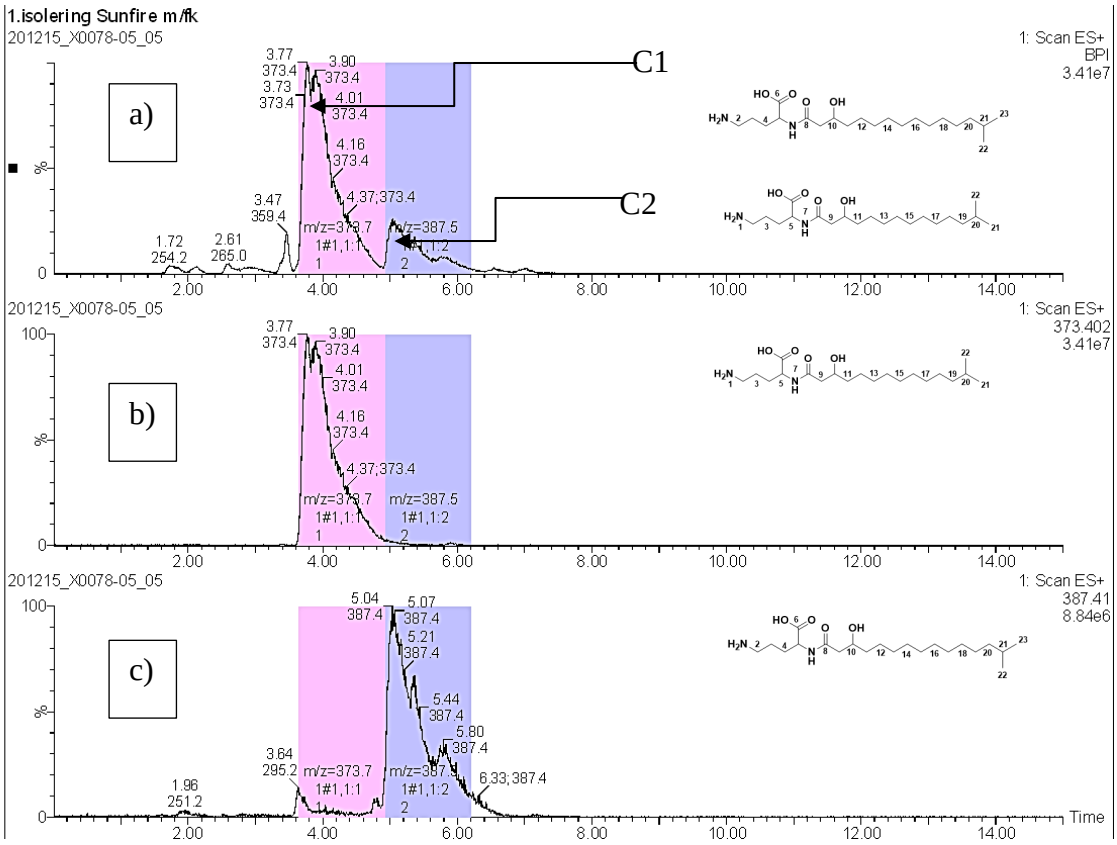


Figure 4-9 *First purification of compound **1** and **2** in the preparative HPLC using SunFire C18 OBD Preparative column (5µm, 10mmx250mm). a): BPI chromatogram of fraction five, b): Extracted ion chromatogram of compound **1,** c) extracted ion chromatogram of compound **2**. Each color corresponds to collected compounds.*

Compound **1** eluted between 3.8-4.8 minutes corresponding to an acetonitrile concentration of approximately 25%, while compound **2** eluted between 4.9-6.2 minutes, corresponding to an acetonitrile concentration of approximately 30%. In total, there were 70 injections, with 150 µL per injection. After the purification, the samples were dried and weighed. This resulted in 26.8 mg of compound **1** and 23.2 mg of compound **2**, as presented in **Table 22**.

Table 22 *Elution time and amount of compounds purified from the first round of purification*

Compound 1		**Compound 2**	
Elution time (min)	**Amount of compound after first purification (mg)**	**Elution time (min)**	**Amount of compound after first purification (mg)**
3.8-4.8	26.8	4.9-6.2	23.2

4.4 Purification of the target compound 2 with preparative system 2

According to the data obtained from preparative-HPLC and UHPLC-HR-MS (data not shown), compound **2** required additional purification to remove remaining impurities and also to collect a fraction of compound **1** that co-eluted with compound **2** in the first round of purification. Hence, the amount of 23.2 mg compound **2** was subjected to a second purification process by mass triggered preparative HPLC. For this step, another preparative HPLC instrument from Waters was used. Column screening was not possible in the preparative system 2, as only the column Atlantis C18 (3µm, 3mmx150mm) was available in the laboratory. The gradient from the previous preparative system was used as a starting point, but the gradient and the threshold for collecting was altered to fit our sample and to provide the optimal separation between the compounds. Compound **1** eluted between 4.1-5 minutes, while compound **2** eluted between 5.2-7 minutes (**Table 23**). In total there were 133 injections, with 25-30 µL per injection. After the second purification, the samples were dried under reduced pressure and weighed. The rest of compound **1** was merged with the samples that did not undergo a second purification step, before they were dried (**Figure 4-10**). In total, 4.9 mg of compound **2** was isolated. Thus, after the second purification round, 21.1% of the original sample from the first purification round of compound **2** was recovered. This clearly shows that compound **2** was not sufficiently pure following the first purification step.

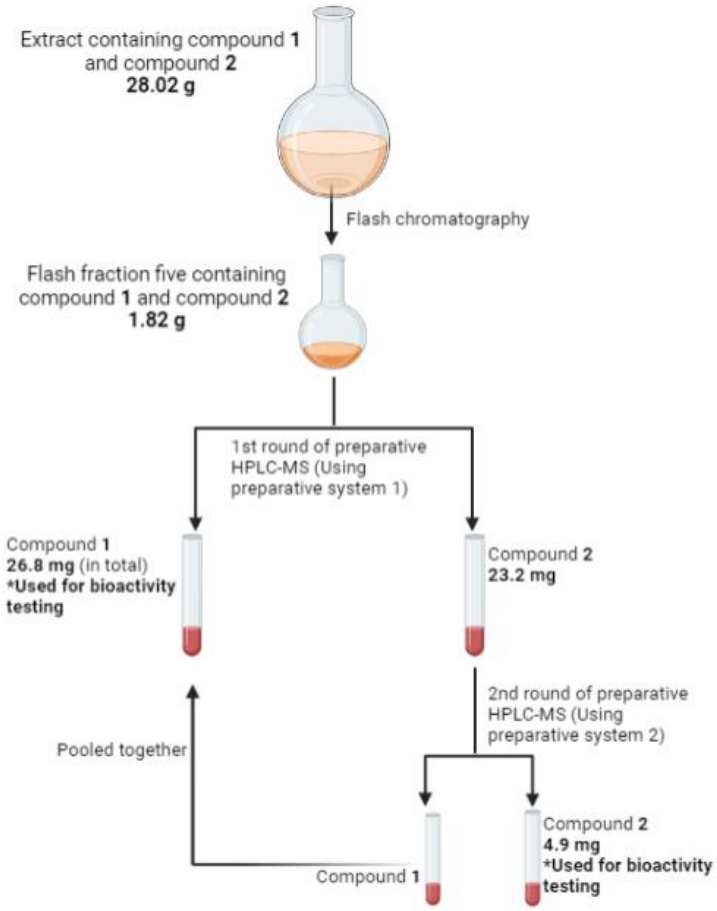


Figure 4-10 *The isolation process of compound* ***1*** *and* ***2. Figure made with biorender.com***

The differences between the two preparative HPLC-systems is that the columns for this instrument have smaller particle size, and smaller dimensions. The preparative column in the second run provided therefore higher resolution and more efficient purification of both compounds **1** and **2**. However, the system that was used in the second purification step had a lower capacity for the amount of sample that can be loaded at each run, meaning a smaller injection volume had to be injected at each run.

Table 23 *Elution time and total amount of compounds isolated from second HPLC round of purification*

Compound 1		**Compound 2**	
Elution time (min)	**Amount of compound after second purification (mg)**	**Elution time (min)**	**Amount of compound after second purification (mg)**
4.1-5	26.8	5.2-7	4.9

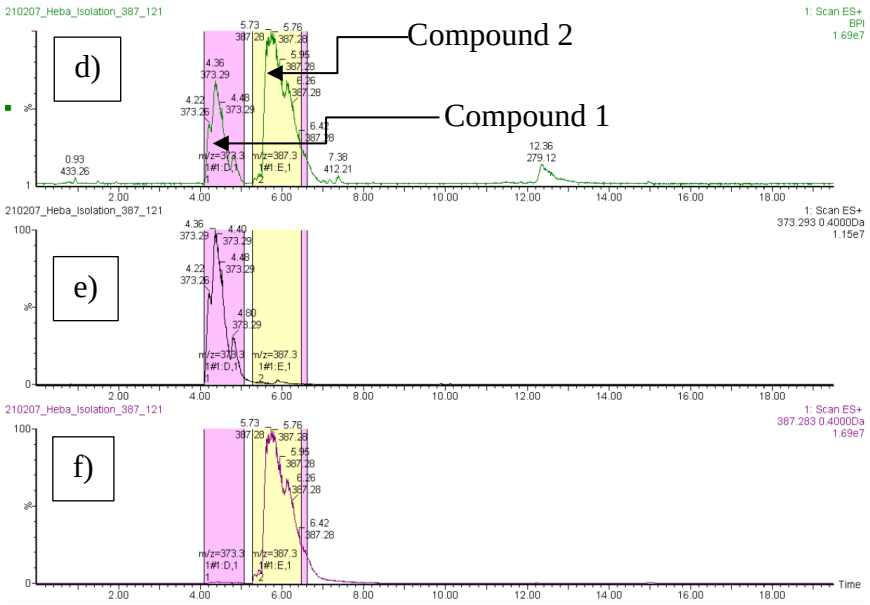


Figure 4-11 *second purification of compound **2** in the preparative HPLC using Atlantis T3, C18 (3µm, 3mmx150mm). d): BPI chromatogram of both compound **1** and **2** of the sample that did undergo the first purification step, e): Extracted ion chromatogram of compound **1**, f) extracted ion chromatogram of compound **2**. Each color corresponds to collected compounds.*

Figure 4-12 and **Figure 4-13** below represent chromatograms of the purification process that was required to purify compound **1** and **2**. Purity analysis with UPLC-UV was attempted, but the compounds did not absorb UV-radiation. As a consequence of this, purity assessment by UPLC-UV was not possible to perform. However, after the final UHPLC-HR-MS analysis, the compounds were visually deemed ready for bioactivity profiling. As can be seen in the chromatograms (**Figure 4-12** and **Figure 4-13**) the peaks of the compounds from the HR-MS chromatograms are of higher relative abundance. The compounds appear to be pure enough for further profiling.

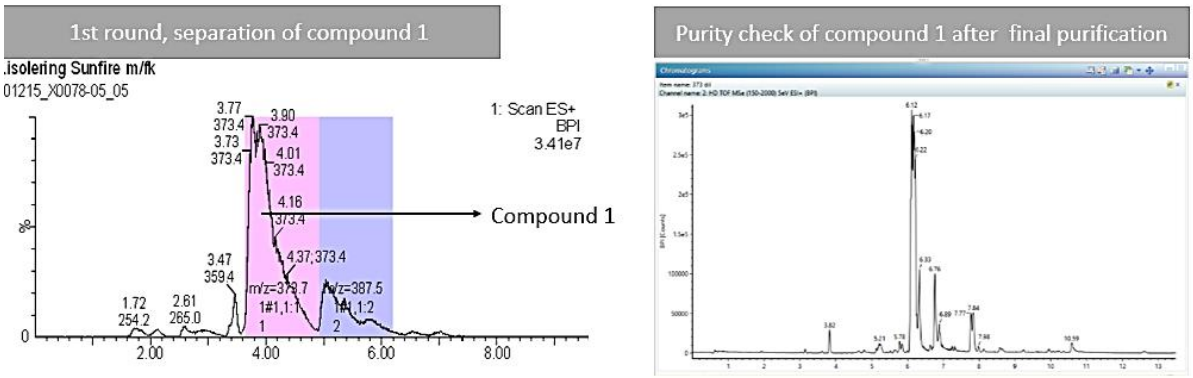


Figure 4-12 *Purity check of compound **1**. Left: 1st round of purification where compound **1** is separated from compound **2** and other impurities in fraction five. Right: Purity check of the isolated compound assessed in using HR-MS*

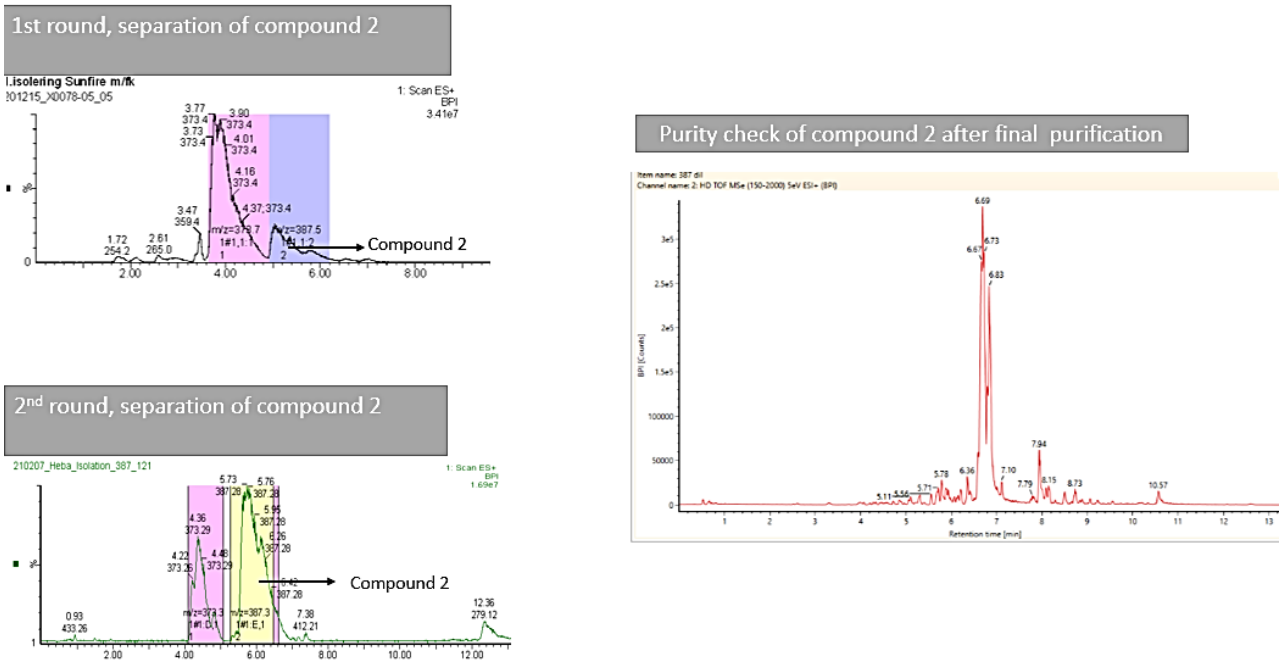


Figure 4-13 *Purity check of compound **2**. Top left: 1st round of purification where compound **2** was separated from compound **1** and other impurities in fraction five. Bottom left: 2nd round of purification where the remaining impurities from compound **1** was removed and other impurities left over from the first round of purification. Right: Purity check of isolated compound was assessed with HR-MS*

4.5 Bioactivity profiling of the isolated compounds

4.5.1 Antimicrobial activity

The compounds were tested for activity against five bacterial strains, *E. faecalis, E. coli, P. aeruginosa, S. agalactiae and S. aureus.* The compunds were tested in consentrations of 10, 50, 100 and 150 µM, corresponding to 3.725, 18.627, 37.254 and 55.881 µg/mL for **compound 1** and 3.87, 19.33, 38.6567 and 57.99 µg/mL for **compound 2,** respectively. The optical density was measured at 600 nm in each well to assess whether or not the bacterial growth had been inhibited after overnight incubation with the compounds. The assay was performed using three technical replicates in three independent experiments (biological replicates) for each bacterial strain (n=9). As shown in **Figure 4-14**, compound **1** displayed activity against the Gram positive bacteria *S. agalactiae* at the highest concentrations tested. A modest activity at the highest concentration was also seen against *E. faecialis* and *S. aureus*. No activity was observed for neither compound **1** nor compound **2** against the Gram-negative bacteria *P. aeruginosa* and *E.coli.* Since compound **1** displayed no cytotoxic activity, it is indicating that there might be selective activity against bacterial cells for this compound.

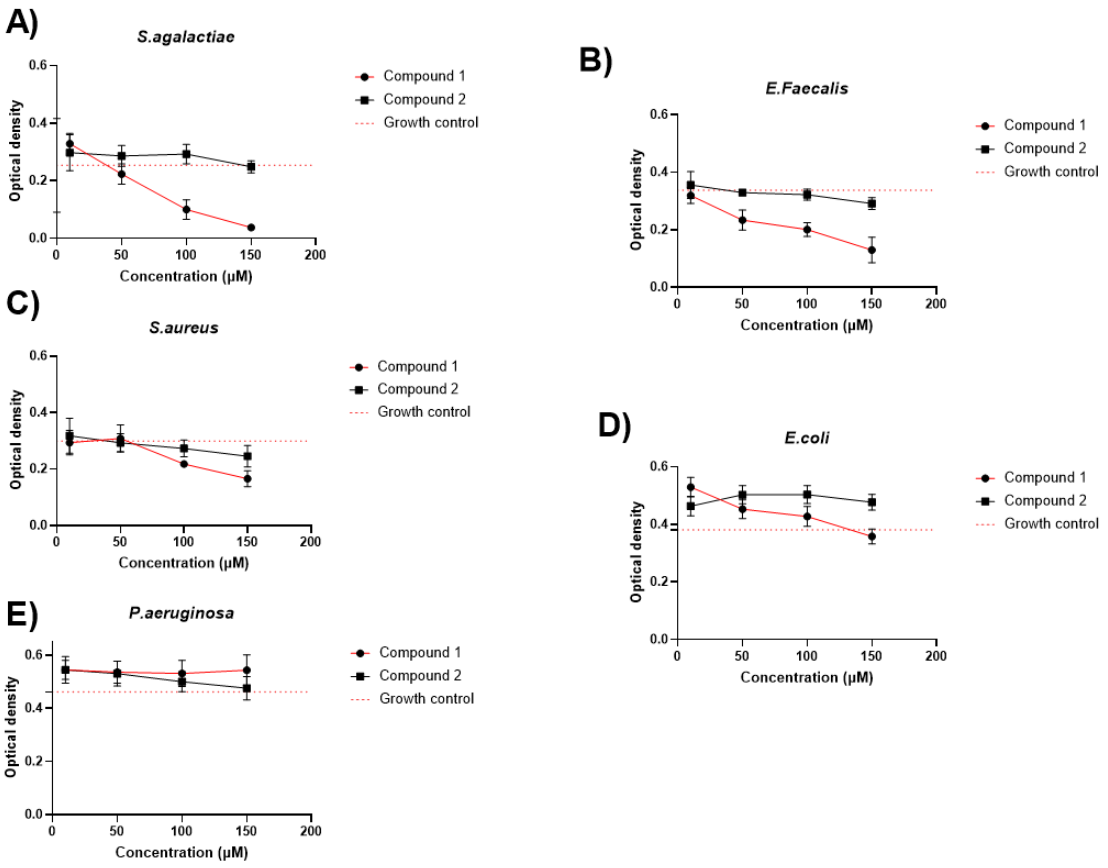


Figure 4-14 *The results obtained from the antimicrobial assay of both isolated compounds. All bacteria presented are tested at concentrations of 10, 50, 100, and 150µM. For each concentration, the optical density is presented as an average of all the technical replicates (n=9), with the standard deviations shown in the dot lines. The growth control for all corresponds to the mean value of the controls for the three individual experiments.*

If a descending trend in the growth of the bacteria is obtained in the dilution series, the compound will be considered to have activity against the strain, a so called dose-response activity. For *S. agalactiae* there is a clear activity of compound **1** at 150 µM and a dose-response can be seen in **Figure 4-14A**. *E. faecalis* and *S. aureus* exposed to the highest concentrations of compound **1** showed decreased growth (lower OD) compared to the growth control (**Figure 4-14B and C**). However, the activity did not appear to be very potent since growth was observed in the wells (higher OD) and the wells also appeared to be opaque. The slight activity observed (trend in dose-response) against *S. agalactiae* and *E. faecialis* for compound **1** may imply that a stronger antibacterial activity could have been displayed at even higher concentrations. Tahara et al. reported some interesting results regarding the antimicrobial effect of OLs and LOLs in 1977 (66). In this study, the activity of N-3-OH-palmitoyl ornithine was assesed against several Gram negative and Gram positive bacteria. N-3-OH-palmitoyl ornithine has the exact same elemental composition as compound **2** in this project, but in contrast to the

compounds in this project which are both isobranced, N-3-OH-palmitoyl ornithine has an unbranched fatty acid. In the study by Tahara et al., it was observed that the Gram negative bacteria *E. coli* and *P. aeruginosa* were completeley inhibited at concentrations of 360 and 480 µg/mL respectively, (66) which is a quite high concentration compared to this project, where the highest concentration tested for both compounds was in the range of approximately 50-60 µg/mL. The antimicrobial agents on the market today also display much lower MIC values compared to the OLs, ranging from e.g 0.004-8 µg/mL for *E. coli* (67). The concentrations of compounds **1** and **2** needed to produce antibacterial activity is therefore not considered suitable for a compound that are to be used in a clinical setting. This implicates that the antimicrobial effect of compound **1** was weak for all the strains.

Gram-positive bacteria are in general more susceptible to antimicrobial agents compared to Gram negative bacteria. This is usually due to the constituents of the cell membranes of the bacterial groups (68). The Gram-negative bacteria consist of two cell membranes, one inner membrane, a thin peptidoglycan layer and one additional outer membrane consisting of lipo-poly saccharides (LPS), phospholipids and protein structures. In order for a drug to reach the cytoplasmic membrane or the interior of the cell and hence exert its effect, it must pass through the additional outer cell membrane with LPS (68).When it comes to some of the Gram positive bacteria, the peptidoglycan layer is linked together in a specific way, it more accessible to compounds, helping them cross the layer to reach the inner membrane (68). In addition, Gram-positive bacteria have almost no periplasm, making it easier for compounds with a positive charge to accumulate to the negatively charged surface of the bacterial cell membrane (68). This could explain why activity was only observed for the Gram-positive bacteria in this project. This phenomenon of activity against the Gram positives and not the Gram negatives is also something that is often observed in the screening process of compound mixtures and pure compounds at Marbio.

The difference in the activity between compound **1** and **2** was surprising since the compounds were similar, with compound **2** only having an extra CH_2 group in the hydrocarbon moiety. The ornithine moiety for both compounds is the same, meaning that the differences in the bioactivity most likely was a result of the length of the hydrocarbon chains of the compounds. It is been known for a long time that small alterations in a molecule can result in major differences in bioactivity. However, performing cell-based assay will not provide any further information about the mode of action of the compounds nor their target.

To investigate the effect of alterations in the hydrocarbon chain, it could be necessary to do structure activity relationship (SAR) studies to investigate if further elongation, shortening or other adjustments such as one or more unsaturation at different positions of the hydrocarbon chain could affect the antimicrobial activity and the selectivity of the compound. A study by Touré et al., described the SAR investigation on various lipoamino acid analogues of phenylalanine. Here, the hydrocarbon chain length of the analogues and the number of double bonds were altered, among others, and the compounds with a saturated C14 or C12 fatty acid chain exhibited the strongest antimicrobial activity (69). However, in a review by Desbois and Smith (2010) it was highlighted that unsaturated free fatty acids often displayed greater potency than their saturated counterparts, and that antibacterial efficacy often increases with the number of double bonds in the chain. However, literature on SAR of free fatty acids does not always follow this trend. Investigations on the SAR of different LOLs would further broaden our knowledge of these compounds antimicrobial potential (70).

4.5.2 Cytotoxic activity profiling of different concentrations

The cytotoxic effect of the compounds was assessed against two cell lines, A2058 which is a human melanoma cell line and the human, non-malignant lung fibroblast MRC-5 cell line. The compounds were tested in the same concentrations as in the antibacterial assay (10, 50, 100 and 150 µM) (**Figure 4-15**). The optical density was measured at 485 nm in each well to investigate the fraction of reduced formazan. Since the quantity of formazan product is directly proportional to the amount of living cells, it was investigated whether or not the cells had survived the incubation with the compounds. The assay was performed using four technical replicates in three independent experiments (biological replicates) for each cell-line (n=12). Cytotoxic activity against the A2058 cell-line was observed for compound **2** at all tested concentrations above 50 µM, but no cytotoxic activity was observed for compound **1** (**Figure 4-15**). No cytotoxic activity was observed against the MRC-5 cells for both compounds (data not shown), implying that the compounds were non-toxic against normal cells at the given concentrations.

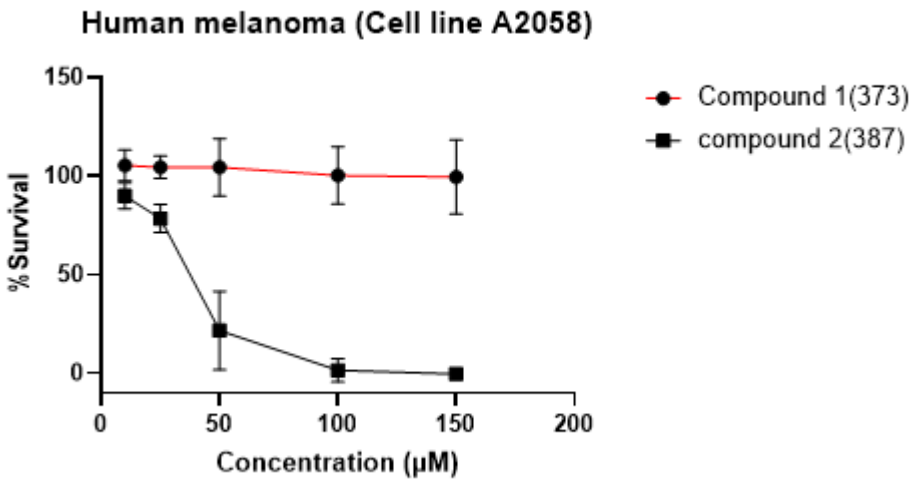


Figure 4-15 *The results obtained from the cytotoxic assay of both isolated compounds. For each concentration, the optical density is presented as an average of four technical replicates, in three experiments conducted, with the standard deviations presented in interval for each dot (n=12 for each concentration). Standard deviations are shown in the dot lines*

Since compound **1** and compound **2** have very similar structures, similar activity of these two compounds against the tested human melanoma cell line was also expected. This was not the case, as compound **1** displayed no activity while compound **2** did in the cytotoxic assay. In a recent study by Kristoffersen et al., on various rhamno lipids, cytotoxic activity was determined for three different compounds with similar structures (abbreviated as compound 3 to 5) (**Figure 4-16**). The results from the study showed a variation in cytotoxic activity of the tested compounds, just like the results obtained in this current master project. Compound **4** from the study by Kristoffersen et al., was highly active with 0% cell survival in the A2058, while compound **3** and **5** were not active (71). The study from Kristoffersen et al., showed that compound **3** and **4** have the same structures and elemental compositions, only differing in the position of the unsaturation, whereas compound **5** have saturation in these positions (**Figure 4-16**). Introduction of a double bond at a specific position resulted in other words differences in the activity. It could be necessary to perform structure activity relationship studies to investigate how alterations in the lipid chains could affect the cytotoxic activity of both compound **1** and **2**. Since compound **2** from this project displayed no antimicrobial activity, there might be selective activity against melanoma cells for this compound.

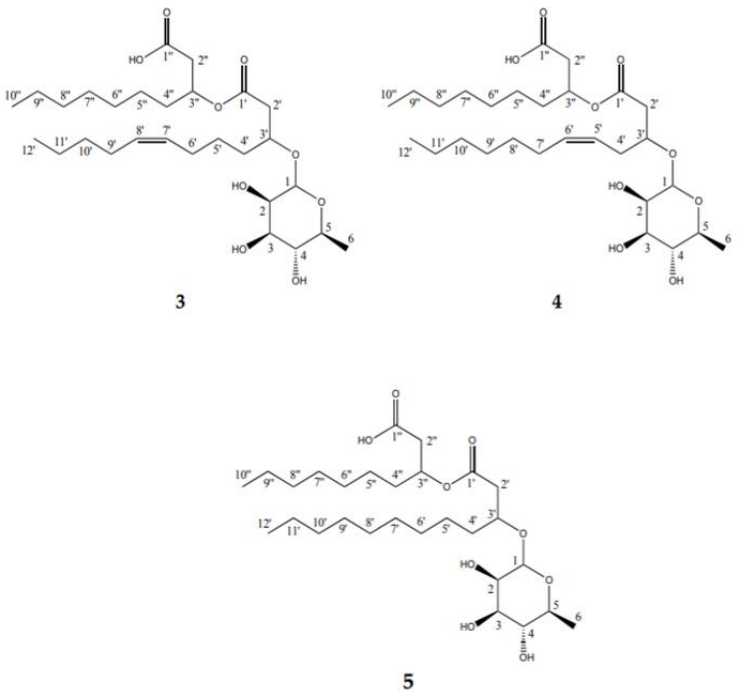


Figure 4-16 *Three of the rhamnolipids from the recent study by Kristoffersen et al. (72)*

A large standard deviation was observed for compound **2** at 50 µM (**Figure 4-15** and **Figure 4-17**). Since these compounds are lipids, their solubility in water is somewhat limited, which could be a factor contributing to the variations within the experiments. When preparing the stock solutions, the compounds were dissolved in water. The compounds were incubated with the cells for approximately 72 hours, in contrast to the bacteria, where they were incubated only overnight. This means that the compounds have stayed longer in an aqueous solution, which may have affected their stability, and thus the percentage of cell survival. To improve the experiment, the compounds could have been dissolved in the specific growth medium before serial dilution, instead of water. It also seemed like this concentration was borderline for the activity, meaning that a small variation in the biological system could result in a major difference in calculated cell survival. The cancer-cell line is a complex system which is more sensitive to alterations in the surrounding environment and has strict requirements for nutrition and CO_2 during the cultivation.

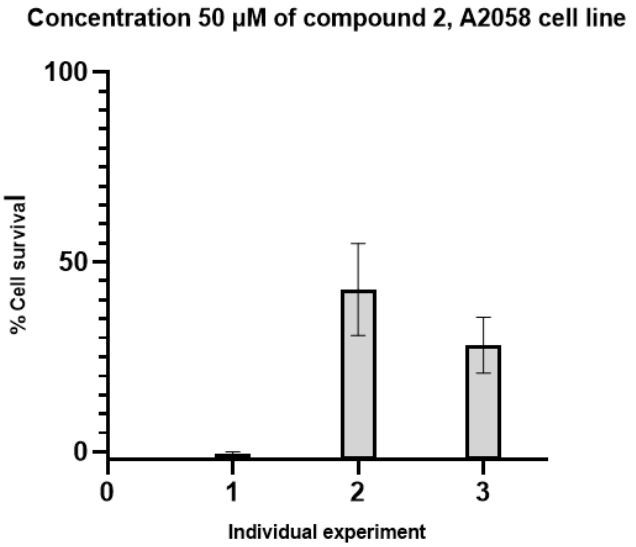


Figure 4-17 *An overview of the percentage in the cell survival for each individual experiment (1st , 2nd and 3rd) in the cytotoxic assay of compound* ***2*** *against the malignant cell line A2058. In the first independent experiment, compound* ***2*** *had activity at 50 µM as there was almost no survival of the malignant cells. In the second and third independent experiments however, cell survival was approximately over 30%. This contributed to the broad standard deviation seen for these replicates when all the data from the three independent experiments were pooled, in* ***Figure 4-15****.*

5 Conclusion and future perspectives

In this project, two compounds were isolated from the marine bacterium *Lacinutrix* sp. by extraction, followed by utilizing several purification steps in flash chromatography and preparative HPLC. UHPLC-MS provided valuable information of the compounds such as the exact masses, followed by the elemental composition calculation, retention time and CCS values, helping us confirm that the right compounds were isolated and tested for activity (as the observed results in this project matched the results from the previously isolated compounds). After the isolation, the compounds were tested for antimicrobial and cytotoxic activity. The bioactivity profiling showed antimicrobial activity against the Gram positive bacteria *S. agalactiae* at the highest concentrations (100 and 150 µM) which resulted in approximately no bacterial growth only for compound **1** and a modest activity was observed against *E. faecalis* and *S. aureus*, also only for compound **1**. No antimicrobial activity was displayed against the tested Gram-negative bacteria for both compounds. Cytotoxic assay was also run for the cell line A2058 (human melanoma) and activity was observed only for compound **2.** The highest concentrations (100 and 150 µM) resulted in 0% cell survival. The compounds did not display activity against the normal cell line (MRC-5). The results indicate that the difference in the carbon chain of the two compounds does have an effect on the bioactivity of the two compounds

against bacteria and human cells, as the results display a selective activity against bacterial cells for compound **1** and a selective activity against human melanoma cells for compound **2.**

For future perspectives, since it was not possible to assess the reason why compound **1** and **2** eluted in several peaks, it could be an option to run these compounds in an electron ionization (e.g. GC-MS), which is a harder ionization technique. This could provide valuable information about the possibility of different isomers in the sample, as discussed previously. As can be currently interpreted by the modest activity and potency of the compounds against the bacteria and the human cells, this project ends at Marbio. The results are however important and will be published, as it provides novel information about the compounds as well as their activity. This allows others to continue working with the compounds by testing them in other assays or by synthetizing analogues of the compounds. The compounds may display stronger activity in other assays that are not included in this study. The isolated compounds do not have complex structures, and it is believed that the compounds can be synthetized. Since the observed activity against human cells and bacterial cells appeared to be selective for the highly similar compounds **1** and **2**, it could be an option to further perform SAR studies, where different analogues can be synthetized, for further investigation of how alterations in the hydrocarbon chain could affect the specific activity of the compounds. This could also provide information about how the activity against the bacterial and human cells can be improved.

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Appendix A

Glycerol stock preparation

FMAP is an abbreviation for "diluted Marine Agar-peptone", the FMAP medium was composed of 15 g Difco marine broth (Becton, Dickinson and company, NJ USA) 5 g peptone (sigma Aldrich, St. Louis, MO, USA), 300 mL filtrated seawater (Norwegian College of Fishery Science, The Arctic University of Tromso) and 700 mL in house Milli-Q water system (Merck KGaA, Germany). The FMAP agar was made of the same components as FMAP medium, with additional of 15 g/L agar (Sigma Aldrich, St. Louis, MO, USA). For glycerol stock preparation, the bacterium was streaked on a FMAP agar, and incubated at 4-12 °C for 3-7 days or to visible satisfactory growth. One bacterial colony was picked from the FMAP agar and transferred to FMAP medium. The bacterial cultures were then further incubated at 4-12°C with 300 rpm shaking for 3 days, or until visible satisfactory growth in the falcon-tubes. From the bacterial culture, 500 µL was transferred to a cryo-tube, containing FMAP medium and 30% of glycerol. The falcon-tubes were then vortexed and stored at -80 °C until further use.

Appendix B

5.1 Structure elucidation

5.1.1 NMR method

The NMR experiments were performed by Johan Isaksson at the Department of Chemistry, UiT, and the structures were elucidated by Kine Ø. Hansen (The Norwegian College of Fishery Science, UiT) and Johan Isaksson. The structures of **1** and **2** were established by 1D and 2D NMR experiments. NMR spectra were acquired in CD_3OD on a Bruker Avance III HD spectrometer operating at 600 MHz for protons, equipped with an inverse TCI cryo-probe enhanced for ^{1}H, ^{13}C, and ^{2}H.

5.1.2 Results

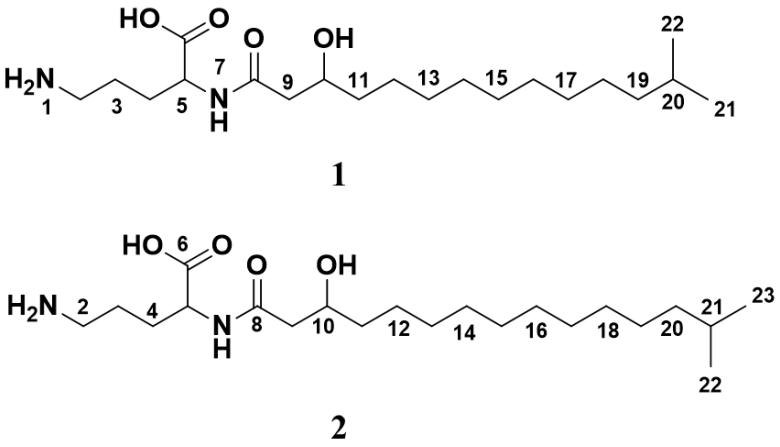

Figure 0-1 *The structures of* ***1*** *and* ***2***

Compounds **1** and **2** were isolated as light brown waxes. The structures of **1** and **2** (**Figure 0-1**) were elucidated using 1D (^{1}H and ^{13}C, table Z) and 2D (HSQC, HMBC, HSQC-TOCSY) NMR experiments and HR-MS analysis. The compounds were determined to consist of a polar ornithine head group linked to a mono-hydroxylated 15:0 (**1**)/16:0 (**2**) iso-fatty acid through an amid bond. The molecular formula of **1** was calculated to be $C_{20}H_{40}N_2O_4$ by HRESIMS, suggesting a presence of 2 degrees of unsaturation. The ornithine substructure (atoms 1 to 7) of **1** was assembled through correlations found in the HMBC spectrum (**Figure 0-2** and **Figure 0-5**) Deschielding of carbon atom CH_2-2 (δ_C 40.2) places the NH_2 group at the delta carbon of the amino acid. The carbonyl group was determined to be located at carbon atom C-6 (δ_C 178.0), due to its characteristic deshielded shift value. The fatty acid chain was found to be linked to the polar head group through an amid bond between NH-7 (δ_H 7.63) and CO-8 (δ_C 173.3) based on a HMBC correlation between the two. Furthermore, carbon atoms CO-8 to CH_2-12 (δ_C 26.6) were linked through HMBC and HSQC-TOCSY experiments (**Figure 0-2** and **Figure 0**-6). A hydroxy group was placed at carbon atom CH-10 (δ_C 69.9) based on HSQC data (**Figure 0-5**) and the deshielded shift value of the carbon atom. In agreement with previously reported data for similar compounds (30, 73) the central methines (CH_2-13 to CH_2-17) could not be unambiguously assigned due to significant signal overlap (**Figure 0-3** and **Figure 0-4**). The two equivalent CH_3 groups (CH_3-21 and CH_3-22) of the iso-terminal of the fatty acid were assigned based on ^{1}H and HMBC spectrum analysis, and were furthermore linked to a -CH-CH_2-CH_2- fragment (CH-20 (δ_C 29.0), CH_2-19 (δ_C 40.1) and CH_2-18 (δ_C 28.4) through HMBC

correlations. Consequently, the structure of **1** was assigned as 5-amino-2-(3-hydroxy-13-methyltetradecanamido)pentanoic acid.

Through HRESIMS analysis, **2** was determined to have an elemental composition of $C_{21}H_{42}N_2O_4$. The structure of **2** (**Figure 0-1**) was assigned based analysis of data from on ^{1}H, ^{13}C, HSQC, HMBC, HSQCTOCSY and COSY NMR experiments (Figure S5 – S9). The structure of **2** was unambiguously assigned in a similar manner as described above for compound **1** and was found have an elongation of the fatty acid chain by a CH_2-group compared to **1** and was consequently assigned as 5-amino-2-(3-hydroxy-14-methylpentadecanamido)pentanoic acid.

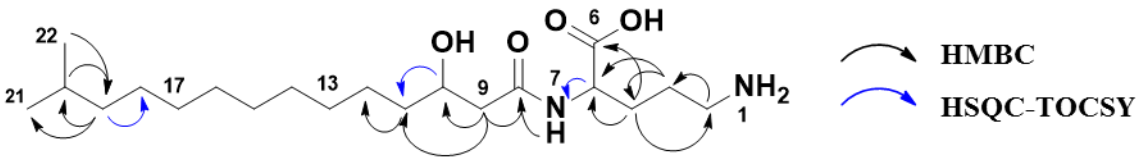

Figure 0-2 *Selected 2D NMR correlations obtained for 1.*

Table 24 *^{1}H and ^{13}C assignments for **1** and **2***

	Compound **1 (373)**		Compound **2** (387)	
position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2	40.2, CH_2	2.95, t (7.3)	40.2, CH_2	2.95, t (7.3)
3	24.6, CH_2	1.71, dtd (17.1, 9.5, 8.5, 4.2)	24.6, CH_2	1.77 – 1.64, m[e]
4	30.9, CH_2	1.91, ddd (10.0, 8.4, 4.8)	30.9, CH_2	1.90, m
5	54.8, C	4.28, dq (9.9, 3.9, 2.6)	54.8, CH	4.28, d (5.4)
6	178.0, C	-	178.0, C	-
7	-	7.63, d (8.0)	-	7.62, d (8.0)
8	173.7, C	-	173,7, C	-
9a	45.0, CH_2	2.39, dd (14.3, 3.9)	45.0, CH_2	2.39, dd (14.4, 4.0)
9b		2.30, dd (14.4, 9.2)		2.30, dd (14.4, 9.2)
10	69.9, CH	3.95, ddt (8.9, 5.8, 3.1)	69.9, CH	3.95, m
11	38.4, CH_2	1.49, m[b]	38.4, CH_2	1.52, m
12	26.6, CH_2	1.35, m[c]	26.6, CH_2	1.48, dq (7.1, 4.4, 3.9)
13	30.7 - 30.6, CH_2[a]	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
14	30.7 - 30.6, CH_2[a]	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
15	30.7 - 30.6, CH_2[a]	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
16	30.7 - 30.6, CH_2[a]	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
17	30.7 - 30.6, CH_2[a]	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
18	28.4, CH_2	1.40 – 1.22, m[c]	30.7 - 30.6, CH_2[d]	1.40 – 1.22, m[f]
19	40.1, CH_2	1.16, qd (7.5, 4.2)	28.4, CH_2	1.40 – 1.22, m[f]
20	29.0, CH	1.52, m[b]	40.1, CH_2	1.17, q (7.1)
21	22.9, CH_3	0.86, dd (10.9, 6.7)	29.0, CH	1.77 – 1.64, m[e]
22			23.6, CH_3	0.87, d (6.8)
23	-	-		

[a-f]Signals are overlapping

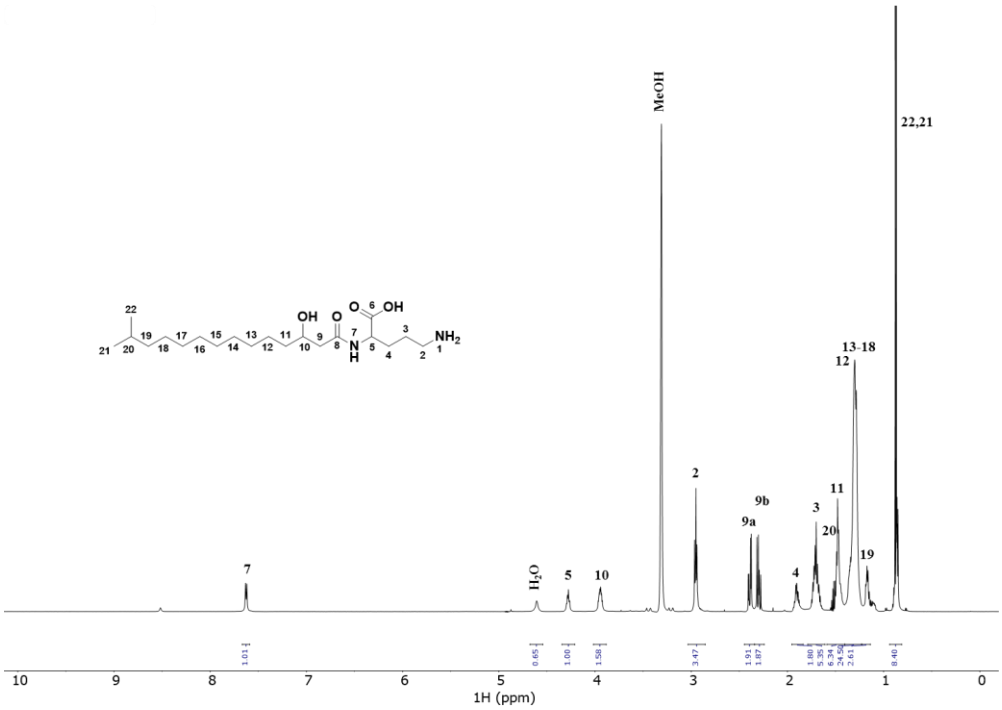

Figure 0-3 *1H NMR (600 MHz, CD3OD) spectrum of compound 1*

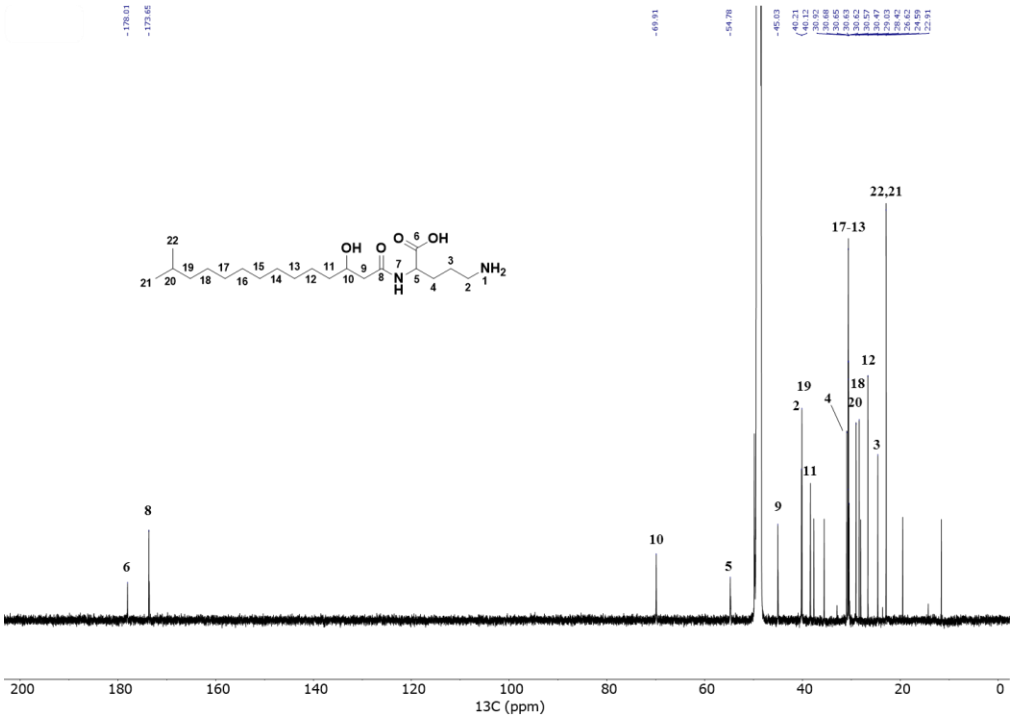

Figure 0-4 *13C (151 MHz, CD3OD) spectrum of compound 1*

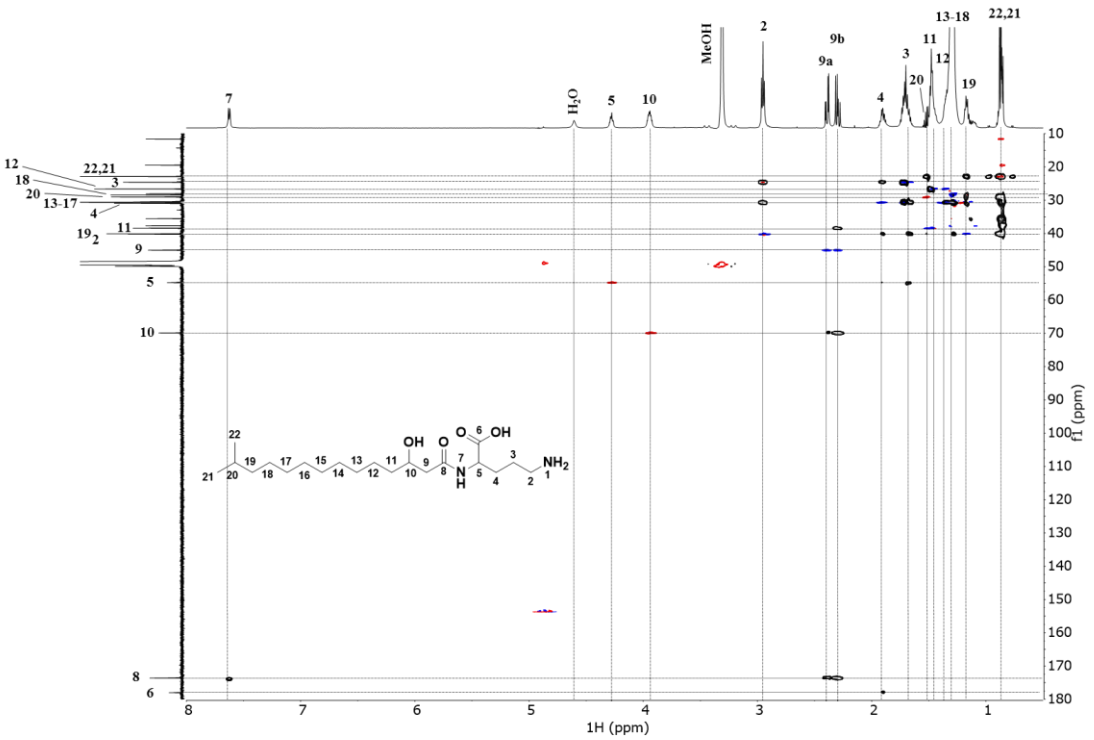

Figure 0-5 *HSQC + HMBC (600 MHz, CD3OD) spectrum of compound 1*

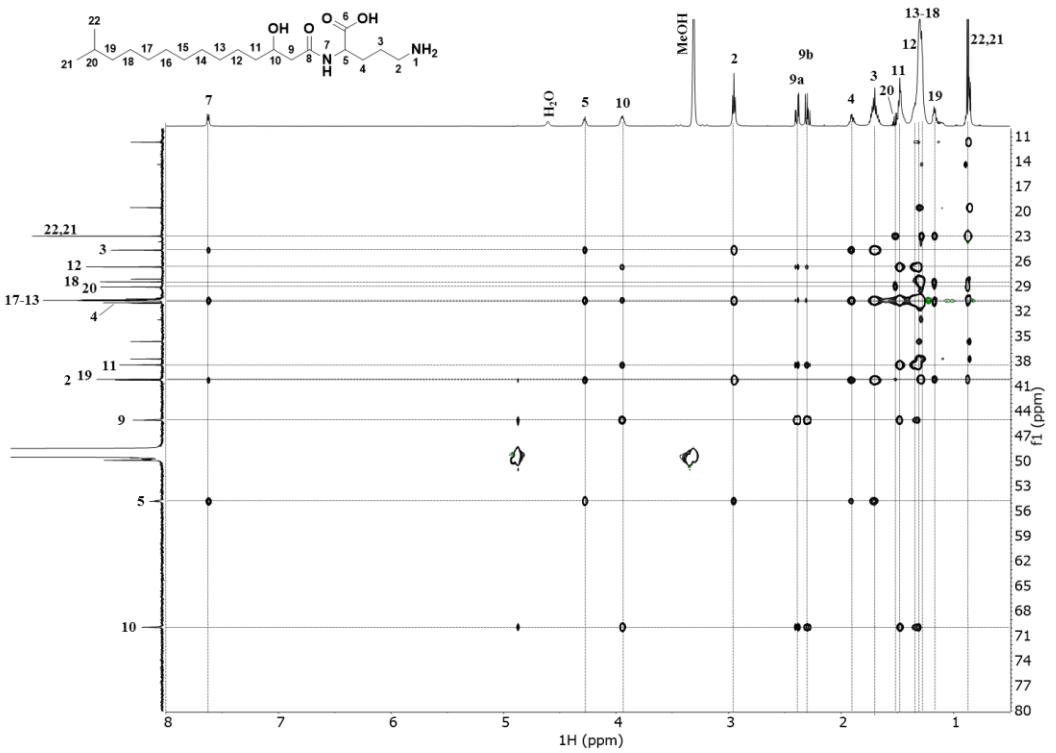

Figure 0-*6 HSQCTOCSY (600 MHz, CD3OD) spectrum of compound 1*

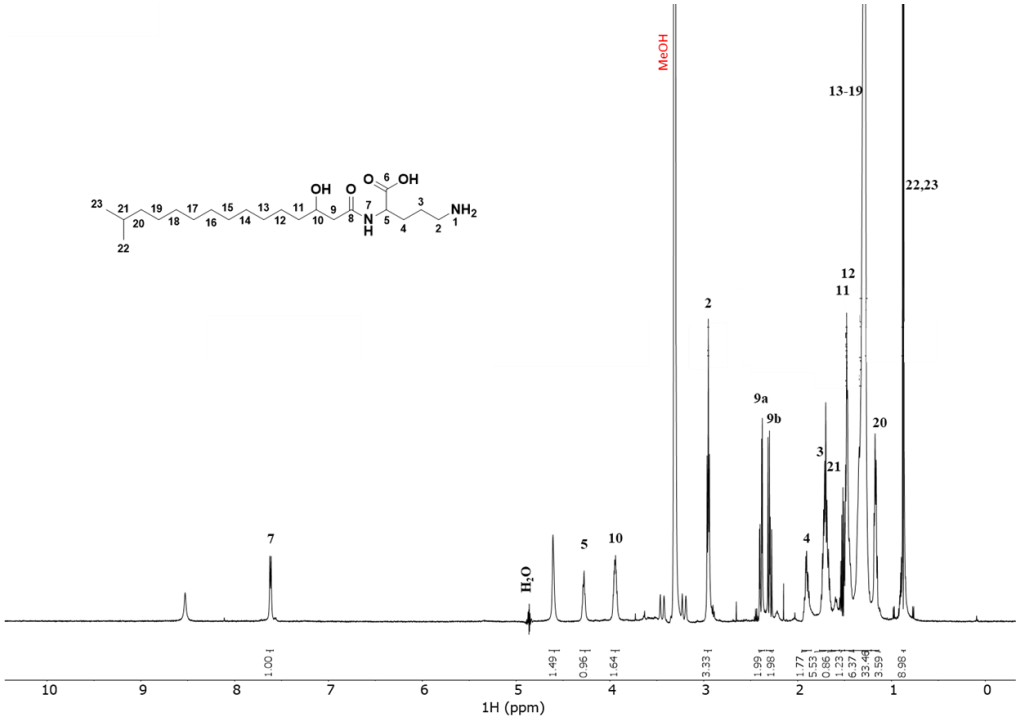

Figure 0-7 *S5. 1H NMR (600 MHz, CD3OD) spectrum of 387*

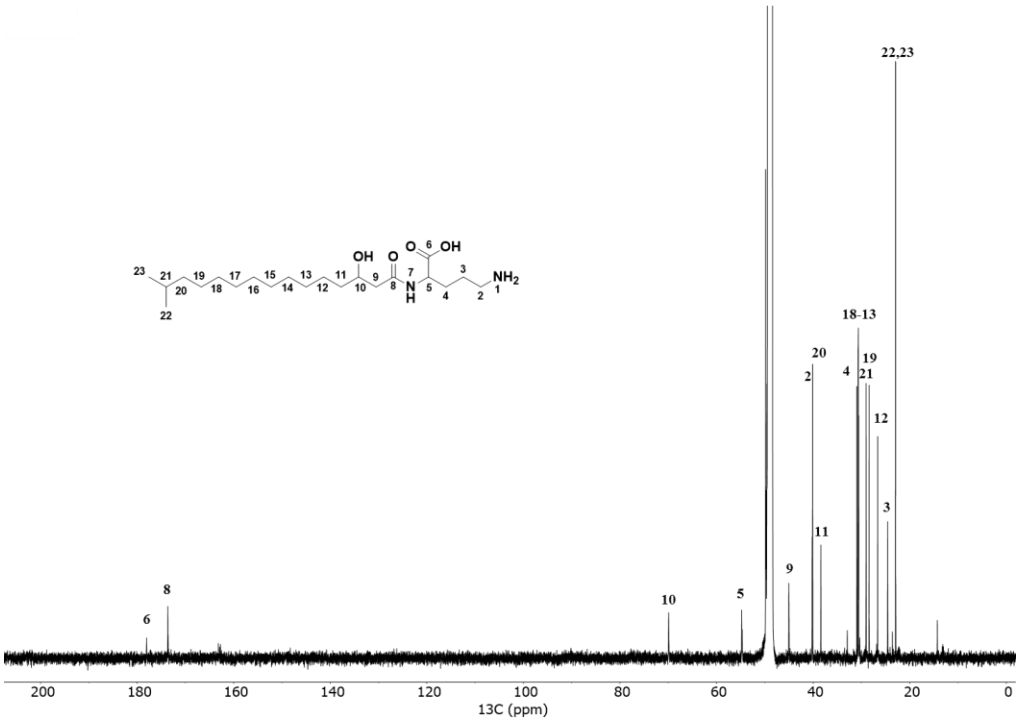

Figure 0-8 *13C (151 MHz, CD3OD) spectrum of compound 2*

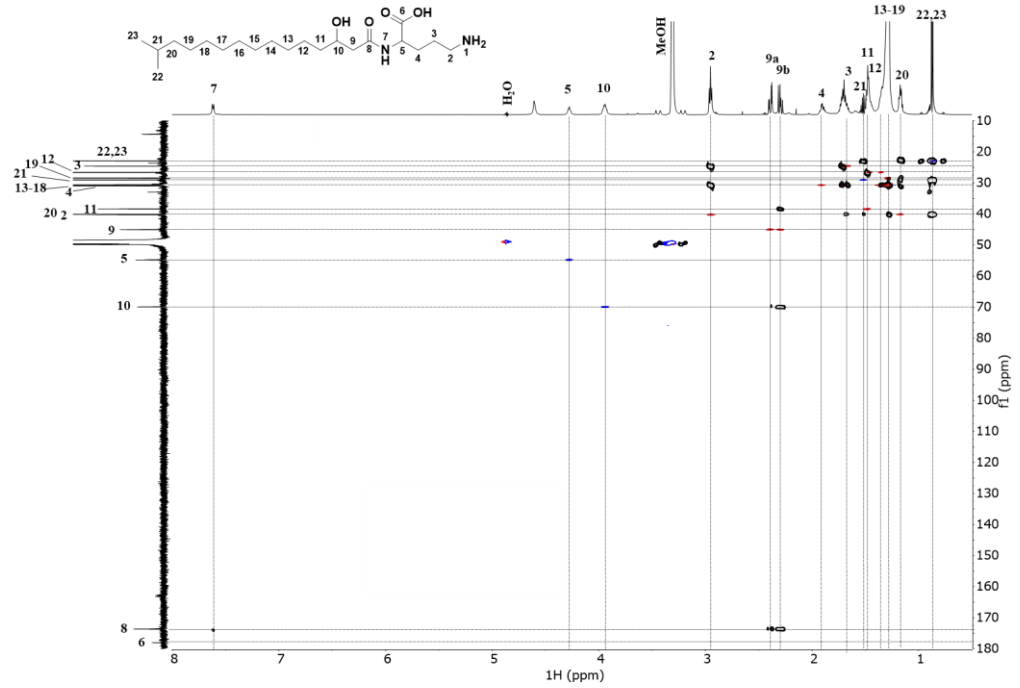

Figure 0-9 *HSQC + HMBC (600 MHz, CD3OD) spectrum of compound 2*

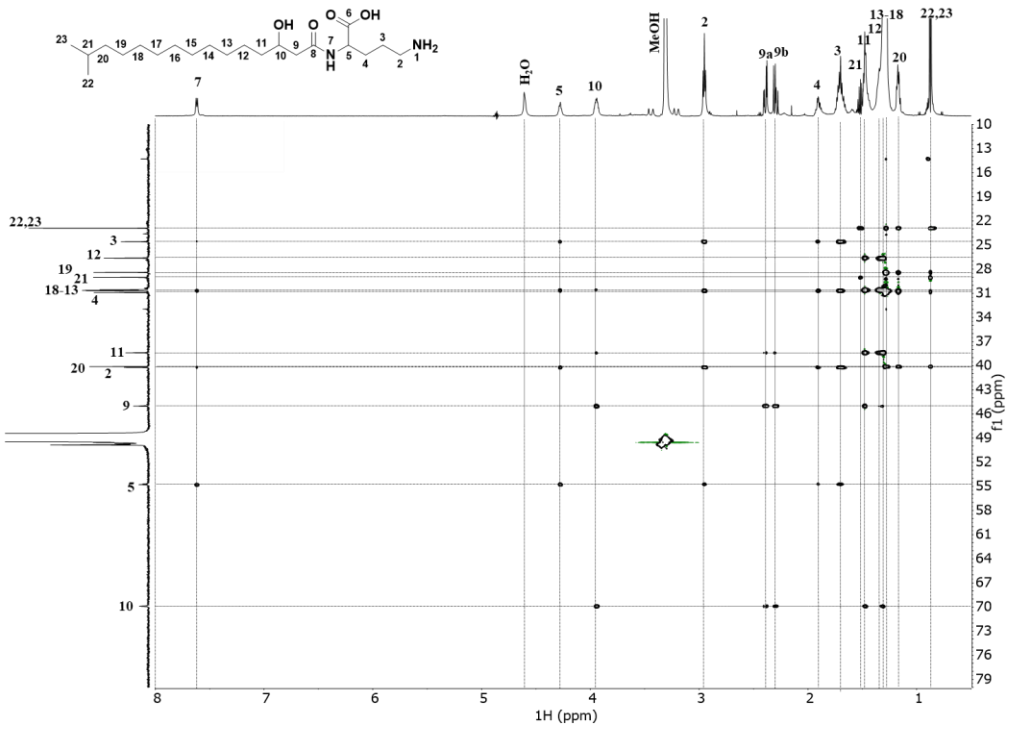

Figure 0-10 *HSQCTOCSY (600 MHz, CD3OD) spectrum of compound 2*

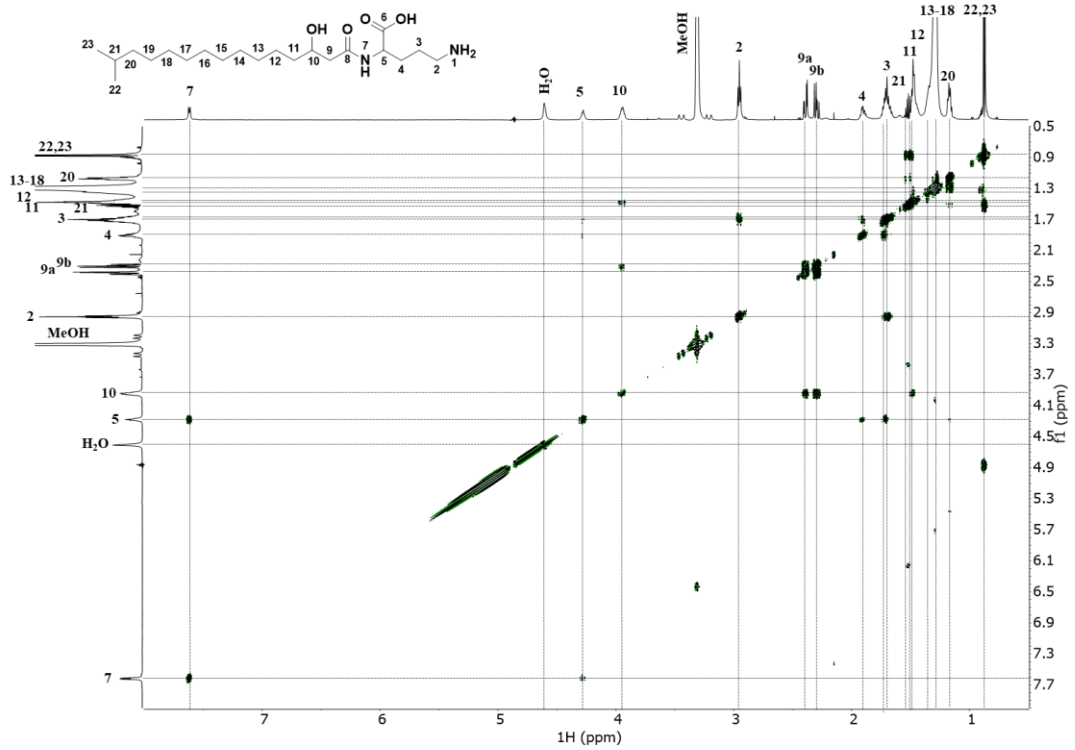

Figure 0-11 *COSY (600 MHz, CD3OD) spectrum of compound 2*