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Faculty of Health Sciences, Institute of Pharmacy

Isolation and Characterization of Secondary Metabolites from Arctic Marine Hydroids

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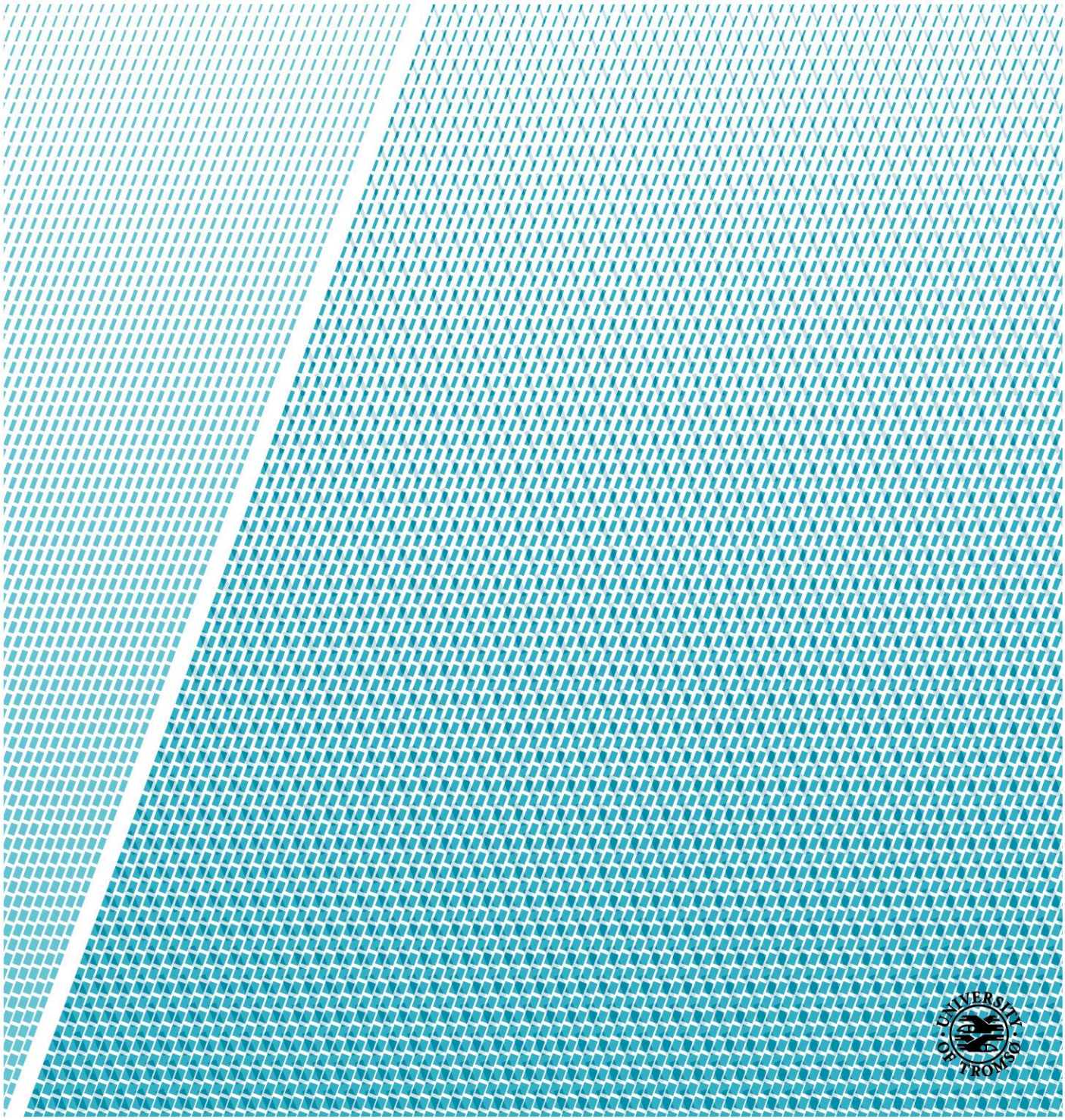

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Abstract

Marine bioprospecting is the systematic search for and discovery of products from Nature, with potential of being developed into commercially available pharmaceuticals. The ocean represents the largest habitat on Earth, and represent a great resource of organisms with unique biological and chemical diversity. The marine environment makes the living condition challenging. To survive these conditions, marine organisms produce numerous potent secondary metabolites to avoid predation and invasion by pathogenic microorganisms. Due to their natural function and chemical diversity, secondary metabolites are believed to have enormous potential as lead compounds in development of commercial products.

The aim of this thesis was to isolate and characterize halogenated secondary metabolites from organic extracts of Arctic marine Hydroids. The crude organic extracts were analyzed by using UHPLC-HR-MS, to evaluate the presence of known and already reported compounds. Based on the data from the HR-MS analysis, three brominated compounds believed to be novel were selected for isolation. Chemistry-guided isolation was conducted for the selected compounds using mass guided preparative HPLC. During the isolation process, two other compounds were selected for isolation for the reason that they appeared to be present in abundant amount and that seems easy to isolate, in addition to the preselected compounds. At the end, one compound was isolated in amounts allowing structure elucidation using NMR, and turned out to be the known compound Loliolide. Loliolide was first isolated from *Lolium perenne* in 1964, but this is the first time that this compound has been isolated from Hydroids. The compound was screened for antibacterial activity and inhibition of biofilm formation, but no bioactivity was found. The result form this thesis shows that isolation of a high enough amount of secondary metabolites for structure elucidation and bioactivity screening can be a challenge due to difficulty of collecting enough biomass. The results also shows that previously reported compounds can be discovered in new species.

List of Abbreviation

1D	One-dimensional
2D	Two-dimensional
ACN	Acetonitrile
C18	Octadecyl
COSY	Correlation spectroscopy
DMSO	Dimethyl Sulfoxide
ESI	Electrospray ionization
h	hour
HMBC	Heteronuclear multiple-bond correlation
HSQC	Heteronuclear single quantum coherence
HPLC	High performance liquid chromatography
HR-MS	High-resolution mass spectrometry
MeOH	Methanol
MS	Mass spectrometry
NMNP	New marine natural product
NMR	Nuclear magnetic resonance
NP	Natural product
Q	Quadrupole
ROESY	Rotating frame nuclear overhauser effect spectroscopy
Rf	Radio frequency
ToF	Time of Flight
UHPLC	Ultra-high performance liquid chromatography

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1 Introduction

1.1 Marine bioprospecting

Marine bioprospecting, is the systematic search for interesting and unique genes, biomolecules or designs from the marine environment. The definition is broad, and the scope of the definition is not conclusive (1). In this thesis however, the term marine bioprospecting is used to describe the search for small molecules in organic extracts of Arctic marine hydroids, with potential of being developed into commercially available pharmaceuticals. Marine bioprospecting describes the procedure of biomass collection, extraction, compound purification, bioactivity screening and structure determination of compounds from marine organisms (2). This is followed by bioactivity profiling of the compounds. The ocean represents the largest habitat on Earth, and covers more than 70 % of Earth´s surface. This represent a great resource of organisms with unique biological and chemical diversity. The Arctic marine environment makes the living condition challenging. The seasonal changes in light condition, from complete darkness during winter to 24 h sunlight during summer, and the cold waters makes the ability to adapt critical to survive. To survive these challenging condition, and to avoid predation and invasion by pathogenic microorganisms, the marine organisms produce numerous secondary metabolites (3).

Collection of marine invertebrates has until now been the most important source of new marine natural products (NMNPs). Marine invertebrates does not have an adaptive immune system, but rather a chemical defense system, and therefor produces potent natural products (NPs) to protect themselves in a hostile environment (4). These NPs have been shown to exhibit pharmacological activities such as anti-inflammatory, anti-diabetes and anti-cancer activity to mention a few (5). Increasing evidence shows that natural products (NPs) isolated from invertebrates frequently are produced by symbiotic microorganisms, such as bacteria or cyanobacteria (6). As an example, Yondelis®, an anti-cancer agent approved in 2007 in Europe for soft tissue sarcoma, was first isolated from the marine tunicate *Ecteinascidia turbinata*, which is a Caribbean Sea squirt. It was later strongly suggested that this compound was in fact produced by one of its symbionts, γ-proteobacterium *Candidatus Endoecteinascidia*

frumentensis (6, 7). Many microorganisms are host specific and they have been proven difficult to grow in culture (8, 9). Collection of marine invertebrates is therefor still important for the discovery of NMNPs in the future.

Marine organisms often produce halogenated secondary metabolites, with bromine being the most commonly incorporated halogen, due to the relative high bromine concentration in sea water. This is contrary to terrestrial organisms which often produce chlorinated compounds (10). Other halogen containing compounds are quite rare, but fluorinated an iodinated compounds are reported. From 1968 there has been discovered more than 5000 halogenated natural products and these numbers are steadily increasing. Many of the halogenated natural products discovered from marine organisms have reported a large range of biological activity, and the activity of many NP's are influenced by the presence of halogenated substituents (11, 12). The mechanisms of incorporating halogens to NP's is complex, and their biosynthesis have been studied for over 40 years. Nature has adopted a wide variety of chemistries to optimize the bioactivity of natural products by incorporation of halogens to secondary metabolites. Enzymatic halogenation through oxidative mechanisms is the most common path of producing halogenated metabolites. Halogenation via halide anion incorporation through both enzymatic and non-enzymatic pathway is also known (13, 14).

1.1.1 The marine bioprospecting pipeline

There are several ways of conducting bioprospecting. Most often, several techniques are employed in sequence. At Marbio, samples are first collected, either by bottom scraping or SCUBA diving. The aim is to collect biomass in amounts sufficient for compound isolation, structure elucidation and bioactivity characterization.

- Extraction

At Marbio all samples are by routine extracted by dicing and freeze-drying the collected biomass. The dry biomass is ground and subjected to an aqueous extraction by adding water for 24 h. The mixture is centrifuged and the supernatant and pellet are separated. The supernatant is dried and with this becomes the aqueous extract. In addition to containing the water-soluble compounds, these extracts typically contain salt, sugars and fibers. The pellet is

freeze-dried and added a 50:50 mixture of methanol (MeOH) and dichloromethane (DCM) and left for 24 h. The mixture is then filtered and the filtrate is dried under reduced pressure, resulting in the organic extract.

- Flash fractionation

The crude extracts are pre-fractionated using flash chromatography, using a column packed with Diaion HP20SS packing material. To prepare the sample, aliquots of the organic and aqueous extracts (approximately 2 g) are dissolved in hexane and mixed with 1 g column material, before the mixture is dried under reduced pressure. This allows the sample components to form strong interactions with the packing material. The mixture is applied to the top of a prepacked column and the mobile phase is flushed through the column. The mobile phase composition is changed in a step-wise manner, starting with 5 % MeOH and ending with 100 % acetone. The first fractions contain the most water-soluble substances and the later fractions contain the most lipophilic substances. All of the fractions are then dried under vacuum and redissolved in dimethyl sulfoxide and stored at -23 °C in the dark until primary activity screening is conducted.

- Primary activity screening

The finished fractions are submitted to a primary bioactivity screening. The bioassays can be divided into two groups: target-based assays and phenotypic assays. Target-based assays conducted at Marbio is kinase and anti-diabetes assays. Phenotypic assays used at Marbio are cell-based assays and include anticancer, antibacterial, antioxidant and immunostimulatory and immunosuppression assays (15). The results from this primary screening will normally be the basis for the bioactivity guided isolation routinely conducted at Marbio.

- The bioprospecting pipeline conducted as part of this thesis

As part of this thesis, chemistry-guided isolation was conducted. This isolation strategy share some common approaches as bioassay-guided isolation, but the pre-fractionation and primary activity screening steps were not used as part of this work. The crude extracts are

analyzed using Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HR-MS) to identify known an already reported compounds in the extracts (dereplication). This process reduces the risk of isolating compounds previously identified and described in the literature. By using HR-MS, the elemental composition of a compound is statistically calculated from its accurate mass and isotopic pattern. The obtained data can be used to search in online databases like MarinLit, Marine Natural Product Database and Chemspider for comparison of the data with available compound information. The isolation was initiated based on defined chemical properties of the compounds, and here a compound was determined to be interesting based on its solubility and elemental composition. Halogenated compounds are easy to identify due to their characteristic isotope pattern, and in this work brominated compounds were selected as target compounds. Novel compounds or known compounds with potential novel bioactivity were selected for isolation, structure elucidation and bioactivity screening.

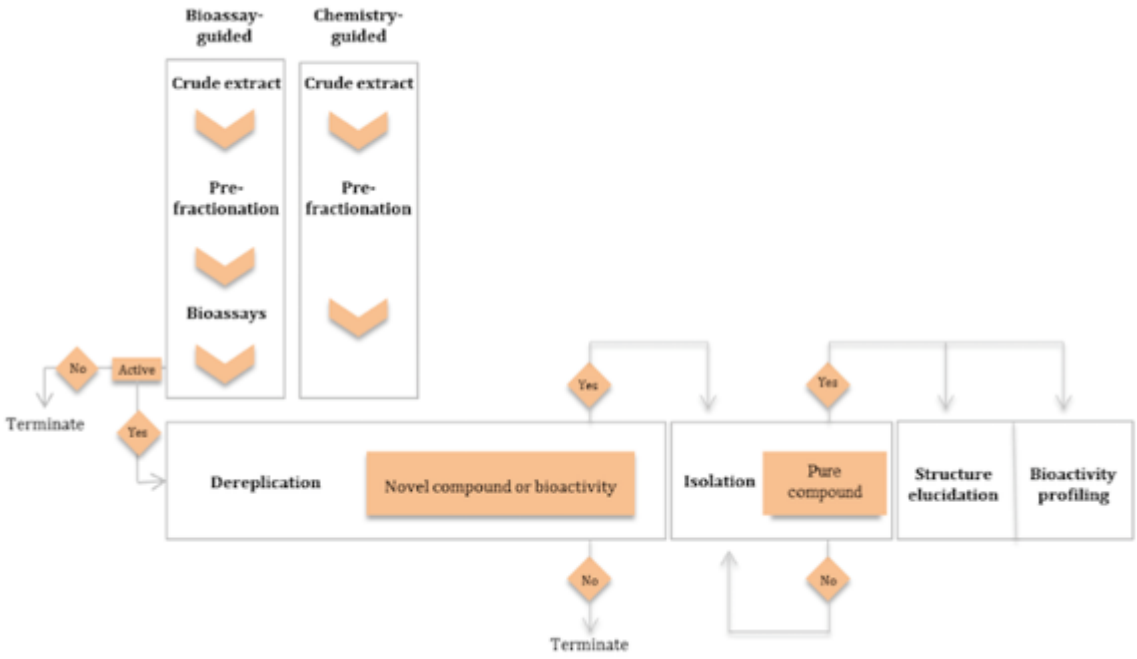

Figure 1: Illustration of bioassay- and chemistry-guided bioprospecting pipeline. Figure from (16).

1.1.2 Natural products: primary- and secondary metabolites

Natural products (NP) refer to any compounds produced by living organisms: animals, plants and microorganisms (17).

Primary metabolites

All living organisms biosynthesis organic compounds that are crucial for normal growth, development, reproduction and structure, hence the term primary metabolites. These metabolites are critical for the immediate survival of the producing organism, and include carbohydrates, proteins, fats, amino acids and nucleic acids (18).

Secondary metabolites

Other natural products are distributed much more limited in nature. These products are not needed for the immediate survival, growth and development of the organism and are therefore called secondary metabolites. The organism produce these compounds to increase their chances of long-term survival, and are often produced to affect interactions between the organism itself and the surrounding environment (19). They can act as anti-feeding agents, to repel pathogens or biofouling organisms, or to avoid overgrowth by the organisms competing for the same space (20, 21). The production of secondary metabolites is often not constant, but rather initiated as a response to an external threat (22).

1.1.3 Marine secondary metabolites

More than 20,000 compounds have been discovered from marine organisms since 1960. Weinheimer and Spragginsthe discovered prostaglandins in the Caribbean octocoral *Plexaura homomalla* at the same time as prostaglandins were discovered as an important compound in humans, this led to interest in further search for novel compounds from the sea (23).

The total number of drugs of marine origin approved by the U.S. Food and Drug Administration is seven, and 22 marine compounds are currently part of the clinical trial pipeline, as of March 2018 (24). The first FDA approved marine derived drug was the anticancer pyrimidine analogue cytarabine (Cytosar-U®) in 1969, isolated from the sponge *Cryptotheca crypta* (25). Following this, three additional marine derived anticancer agents have been approved. These are trabectedin (Yondelis ®), which is the most recently approved marine derived

pharmaceutical, approved in 2015 (26, 27), the antibody-drug conjugate brentuximab vedotin (Adcetris®) and eribulin mesylate (Halaven ®). In addition, omega-3 fatty acid ethyl esters (Lovaza ®) are approved to treat hyperglyceridemia, Ziconotide (Prialt ®) to treat severe chronic pain and vidarabine (Vidarabine ophthalmic®) to treat optical virus infections are approved (28).

1.2 Hydroids from the phylum Cnidaria

Cnidaria is a phylum that contains over 9,000 species, and most of them are found in the marine environment. Cnidarians are classified into four main classes; anthozoa, cubozoa, scyphozoa and hydrozoan. They have no organs, but they have a gastrovascular cavity which act as a mouth and anus. Most are dipoblastic phylum, which means that they are only composed of two cell layers, the outer layer is called ectoderm and the inner layer called endoderm. All the Cnidarians have explosive stinging or adhesive cells called cnidocytes (hence the name Cnidarian) in the tentacles and/or the mouth lining. A cnidocyte contains a surface cilium or modified cilium (the cnidocil), and a nematocyst that is the actual stinging element. The nematocysts contain toxins that can paralyze and kill prey and then the tentacle leads the prey to the mouth (29). Hydrozoa includes nearly 3500 species, but despite this, there are only a few of them that have yielded interesting marine natural products (30). This is probably because the secondary metabolite content of most hydrozoans has not been investigated yet.

Cnidarians have proven to be a fruitful source of NMNP´s, where terpenoids are most frequently reported (31). In 2012, breitfussin A and B were isolated from the Arctic hydrozoan *Thuiaria breitfussi* from Bjørnøya, these are brominated compounds, in addition, breitfussin A also contains iodine (32). In 2016, two brominated-indoles were reported from the hydroid *Abietinaria abietina*, which were found to activate NF-kB dependent transcription (33). Annulins A, B and C were isolated from the marine hydroid *Garveia annulata*, and potently inhibit indolamine 2,3-dioxygenase, which is thought to play a role in evasion of T-cell-mediated immune rejection, and Solandelactones C, D, and G isolated from the hydroid *Solanderia secunda* which inhibit farnesyl protein transferase, that could be a potential target

for novel anticancer agents (30). These are some examples of interesting NPs found in hydrozoan.

1.3 High Performance Liquid Chromatography (HPLC) and Ultra-High Performance Liquid Chromatography (UHPLC)

HPLC is a commonly used chromatographic technique to separate compounds dissolved in a liquid solvent. The LC system is assembled of a mobile phase reservoir, pump, sample injector, column and a detector. In liquid chromatography, the mobile phase is liquid, and passes through a column that is packed with a stationary sorbent, which can interact and retain analytes in the mobile phase. The analytes are injected into the flow of the mobile phase before it is forced through the separation column. The column is connected to a detector, which detects the substances eluting from the column.

In this thesis, there will be used reverse phase chromatography, where hydrophobic groups are chemically bound to silica particles inside the column. In these columns the interaction that retains the analytes is mainly based on hydrophobic interactions of Van der Waals forces. The mobile phases in reversed phase chromatography are a mixture of water and other water miscible organic solvents, where acetonitrile and methanol are most commonly used.

UHPLC is similar to HPLC, the difference is the size and shape improvements of the silica particles in the column. The diameter can be below 2 μm whereas in HPLC the particle diameter is typically 3-10 μm. The column is often shorter in UHPLC because of the smaller particles. UHPLC utilizes higher pressure (over 10,000 psi compared to about 6,000 psi for HPLC), which result in shorter analysis time, and the peak capacity and resolution are improved (34).

1.4 Mass Spectrometry

Mass spectrometry is an analytical method where the ions in gas phase are separated according to their mass-to-charge ratio (*m/z*), and detected in the detector. In this project, ESI single quadrupole mass separator preparative HPLC was used for isolation of the compounds of interest, and ESI-QTOF mass separator for analysis of compounds in the crude extracts.

1.4.1 Electrospray ionization (ESI)

ESI is an ionization technique among the group of atmospheric pressure ionization (API), and is the ionsource used in the mass spectrometers at Marbio. In this ionization technique, the mobile phase with the analytes are pressed through a charged stainless steel capillary that ionizes molecules in the mobile phase. The mobile phase is sprayed as an aerosol at atmospheric pressure and the mobile phases are rapidly evaporated while the charge is transferred to analyte molecules. ESI is a soft ionization technique (the molecule is typically not broken down into smaller fragments) that transfers ions from a solution, to gas phase at atmospheric pressure and then continue into the mass analyser under vacuum (35).

When an analyte in solution is transferred to gas phase ions, the solution undergoes four processes. (a) Electrochemical reactions (oxidation reaction when positive ion mode is used) inside the ESI capillary, which produce charge in the solution, (b) Production of charged droplets from the high-voltage capillary tip, (c) Solvent evaporation from the charged droplet (micro range size) resulting in very small charged droplets (nano range size), (d) and finally the mechanism of the formation of gas-phase ions from the very small charged droplet (36).

1.4.2 Quadrupole (Q)

The quadrupole is made up of four parallel metallic rods, that are applied a constant DC voltage to each rod pair, one pair is positive and the other is negative on opposite planes. It is also applied an alternating radio frequency (RF) oscillating voltage on each pair of rods. Typically, the DC and RF potentials are adjusted in such a way that only molecules with one *m/z* can traverse the quadrupole in stabile oscillations at a time (37).

1.4.3 Time of flight (ToF)

The ions from the ion source is introduced to a mass filter in a vacuum chamber and into the time of flight mass analyser. The mass of the compound is measured by the time it takes for the compound to reach the detector from the ion pusher. The background pressure must therefore be so small that no collision between them happens, to not interfere with the exact flight time. The ions that are emitted from the ion pusher, are accelerated so they have equal kinetic energy before entering the field free drift region (the flight tube). The kinetic energy (E) is equal to ½ mv^2, and as the equation shows, ions with smaller masses will have higher velocity, and therefor reach the detector first. Velocity (v) is equal to distance (d) divided by time (t), so by combining these two equations the mass of a compound can be calculated by measuring the flight time (37).

ToF-MS is a high-resolution mass filter, that means that ions with very similar masses can be separated, as opposed in e.g. a quadrupole which is a low-resolution mass filter (38). The instrument also have a high mass accuracy, which means that it has the ability to measure *m/z* with only a few parts per million errors of the true monoisotopic *m/z* of a compound (39). The combination of high resolution, high mass accuracy and high sensitivity in full scan mode makes HR-MS an ideal instrument to analyse complex samples such as crude extracts from marine bioprospecting (40).

1.5 Nuclear magnetic resonance spectroscopy (NMR)

This technique is mainly used to identify the carbon-hydrogen skeleton of a molecule. Some atomic nuclei behave like small magnetics by spinning around it´s own axis, called “nuclear spin”. This spin results in small magnetic fields which can be influenced by a fixed external magnetic field from a NMR instrument. In organic chemistry both proton (^{1}H) and carbone (^{13}C) have the correct spin quantum number (1/2) and can be analysed using NMR. The nuclei are randomly oriented, but will be aligned parallel when they are subjected to an external magnetic field, aligning either with (+1/2) or against (-1/2) the external field. The nuclei that are aligned against the external magnetic field are in the higher-energy spin stat. The sample is then applied energy in the radio frequency (rf) range. When a nucleus in the lower-energy spin state (aligning with external field) is subjected to a rf frequency identical to the nucleus’ Larmour frequency, the nucleus flip to the higher energy spin state, aligning against the magnetic field. When the rf is switched off, the nuclei relaxes back to the lower energy state by re-emitting the absorbed energy. The emitted energy of a particular resonance frequency produces a measurable rf signal, and is processed into a NMR spectrum (41, 42). NMR spectra can be either one-dimensional (1D) or two-dimensional (2D). 1D experiments are analyses of a single nucleus. In 2D experiments, interactions between different nuclei can be detected. The most used 2D-NMR experiments for structure elucidation is correlated spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear overhauser enhancement spectroscopy (NOESY) (43).

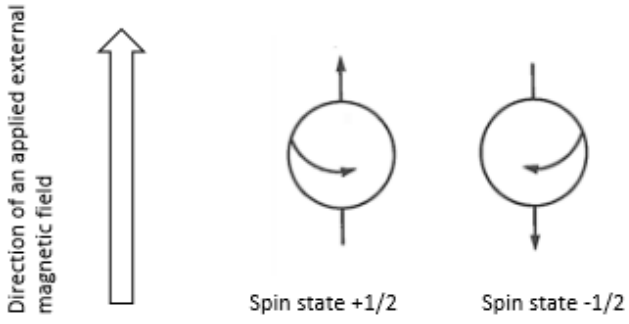

Figure 2: Illustration of nuclei aligned either with or against an applied magnetic field. The spine state +1/2 is aligned with the field and therefor is of lower energy, while the spin state -1/2 is aligned against the field and therefor is of higher energy. Figure made with inspiration from (44).

2 The aim of the thesis

The overall aim of this thesis was to identify and isolate halogenated secondary metabolites from Arctic marine hydroids, and conduct bioactivity profiling of the compounds isolated using bioassays available at Marbio.

The main objectives of the thesis were to:

- Dereplicate the crude extract in order to nominate compounds for isolation based on suspected interesting chemistry and novelty of the compound
- Isolate target compounds from organic extracts of Arctic or Sub-arctic hydroids
- Elucidate the structure of the isolated compounds
- Conduct bioactivity profiling of the isolated compounds

3 Materials and methods

As part of this thesis, sample of hydroids with low collected sample weight were used as biomass. The low collected sample weight resulted in low amount of organic extract, and therefore, the normally conducted flash fractionation and primary bioactivity screening steps were skipped. Instead, the organic extracts of the hydroids were analysed using high resolution mass spectrometry (HR-MS) to identify compounds with interesting chemistry. To conclude, the normally conducted strategy to isolate bioactive compounds (bioactivity guided isolation) was exchanged for a different approach: chemistry guided isolation. If this strategy works, this will show that samples, previously put aside to wait for the collection of additional biomass at Marbio, can be analyzed with a different approach to yield interesting compounds.

Table 1 shows the two samples used in this thesis. The M17017 sample contains one specific specie, and M17015 sample is a mixture of different species, including the same species as in M17017 sample. The M17015 sample was included in this work as it contained all the compounds isolated from the M17017 sample, and because of the higher amount of organic extract, which provide more amount of the target compounds.

Table 1: Overview over the samples of hydroids examined as part of this thesis.

Collection ID:	**Family:**	**Genus:**	**Species:**	**Quantity (WW):**	**Organic extract:**
M17015	Mix of different families			173.77 g	1.1 g
M17017	*LAFOEIDAE*	*GRAMMARIA*	*ABIETINA*	64 g	0.33 g

3.1 Extraction of hydroids

The materials and equipment used during extraction can be seen in Table 2.

Table 2: Materials and Equipment used in the extraction process.

Materials/Equipment	Supplier
MilliQ water	Millipore, Billerica, MA, USA
DCM (≥ 99.8 %)	Sigma-Aldrich, MO, USA
MeOH (≥ 99.9 %)	Sigma-Aldrich, St.Louis, MO, USA
Whatman filter paper 125 Ø (no.3)	Sigma-Aldrich, MO, USA
Centrifuge: Heraus Multifuge 3 S-R	Hanau, Germany
Freeze-drier: Heto PowerDry PL9000 and Heto PowerDry PL6000	Thermo Fisher Scientific, Waltman, MA, USA
Rotary evaporator, Heidolph Laborota	Heidolp Instruments GmbH & Co, Germany
Scale: Mettler Toledo PB3002-S Fact	Greifensee, Switzerland
A11 Basic grinder	IKA Works, Staufen, Germany

Extraction of the collected hydroid biomass

The biomass samples were stored at -22°C in the dark before being extracted. The samples were then freeze-dried for 2-3 days, and the freeze-dried samples were pulverized with a grinder and transferred to tared 1 L Duran bottles, weighed and stored in the freezer at -22°C.

Aqueous extraction

The pulverized sample was transferred to 0.6 L centrifuge bottles and added MilliQ water approximately ten times the dry weight of the sample and shaken until the suspension got a slurry consistency. The suspension was centrifuged at 4565 g, at 5 °C for 30 minutes. The supernatant was transferred to a Pyrex dish covered with aluminium foil and stored in the freezer -22°C. The pellets were re-extracted with MilliQ water, approximately half the amount used the first round, and shaken until the suspension got slurry consistency. The suspension was stored in the refrigerator at 5 °C for about 30 minutes before it was centrifuged as before. The supernatant was transferred to another Pyrex dish as before and stored in the freezer over night at -22 °C. The pellets were transferred to a Pyrex dish, covered with aluminium foil and stored in freezer over night at -22 °C. The water extracts were frozen at –80 °C for 2-3 hours before freeze-drying for 2-3 days, before grinded with mortar and pestle. The resulting powder was termed the aqueous extract, and stored in a 50 mL falcon tube in the freezer at -22 °C.

Organic extraction

The pellets were freeze dried for 2-3 days, grinded in mortar and transferred to a tared 1 L Duran bottle. The pellets were then extracted with a solvent mixture of DCM-MeOH (1:1, v/v), of approximately ten times the weight of the pellets until the suspension got a slurry consistency. The suspension was stored in the refrigerator at 5 °C to the next day, before it was vacuum filtered with Whatman filters. The material on the filter was transferred back to the bottle and the extraction was repeated one more time with approximately half of the solvent volume used in the first extraction. The suspension was shaken and stored for 30 minutes in refrigerator before it was filtered again. The filtrate was then concentrated to 10-20 mL on a rotary evaporator before it was transferred to a 50 mL Florence flask and evaporated under reducing pressure until the extract was dry, then stored at -22 °C.

3.2 Preparation of the samples for MS analysis

Aliquots of organic extract from the M17015 and M17017 samples was transferred to an Eppendorf tube and dissolved in 0.5 mL MeOH (≥ 99.9 %, Sigma-Aldrich, St.Louis, MO, USA). The dissolved sample was transferred to HPLC vials for further UHPLC-HR-MS analysis.

3.3 UHPLC-HR-MS analysis (dereplication)

The materials and equipment used in the dereplication analysis can be seen in Table 3.

Table 3: Materials and equipment used in dereplication of organic extracts employed in UHPLC-HR-MS analysis.

Materials/Equipment	Supplier
MilliQ water	Millipore, Billerica, MA, USA
MeOH LC-MS Ultra CHROMASOLV®	Thermo Fisher Scientific, MA, USA
LiChrosol® ACN (hypergrade for LC-MS)	Merck KGaA, Germany
Formic Acid ULC/MS (99%)	Biosolve B.V., Netherland
Vion® IMS qTOF	Waters, MA, USA
Acquity I-Class UPLC®	Waters, MA, USA
Acquity UPLC® BEH C18, 2.1x100 mm, 1.7µM Column	Waters, MA, USA
Acquity Sample Manager FTN	Waters, MA, USA
Acquity UPLC® I-Class Binary Solvent Manager	Waters, MA, USA
Acquity UPLC® PDA Detector	Waters, MA, USA
Acquity UPLC® Column Manager	Waters, MA, USA

All of the organic extracts of the samples M17015-M17028 (for table, see appendix A1) were analysed using UHPLC-HR-MS to look for novel compounds with interesting chemistry. The two samples that were used for further preparation and isolation, M17015 and M17017 contained interesting compounds and were two of the samples with the highest amount of extracts. The gradient and instrument parameters used in the analysis are described in table 4 and 5.

Table 4: Gradient used in UHPLC-HR-MS analysis of the organic extracts. Mobile phase A: ultra-pure water with 0.1 % formic acid (FA), and mobile phase B: ACN with 0.1 % FA.

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

Table 5: Instrument parameters used in UHPLC-HR-MS analysis.

Polarity	Positive
Low mass (*m/z*)	50
High mass (*m/z*)	2000
Source type	ESI
Source temperature (°C)	120
Desolvation temperature (°C)	450
Desolvation gas flow (L/h)	800
Capillary voltage (kV)	0.80
Cone voltage (V)	30
Cone gas flow (L/h)	50

3.4 Preparation of the organic and aqueous extract of sample M17015 and M17017

The material and equipment used during the extraction of the extract of M17015 and M17017 sample can be seen in Table 6.

Table 6: Materials and equipment used in the preparation of organic extracts for isolation.

Materials/Equipment	Supplier
MeOH (≥ 99.9 %)	Sigma-Aldrich, St.Louis, MO, USA
MilliQ water	Millipore, Billerica, MA, USA
Hexane (≥ 97.0 %)	Sigma-Aldrich, St.Louis, MO, USA
Rotary evaporator: Heidolph Laborota 4002	Nürnberg, Germany
Centrifuge: Heraeus Multifuge 3 S-R	Hanau, Germany
Scale: Mettler Toledo AB204-S Fact	Greifensee, Switzerland

Organic extract

The Florence flask containing the extract was weighed four times on an analytic scale to make the measurement as exact as possible. The Florence flasks were added 25 mL hexane, which then was transferred to a separatory funnel. The process was repeated until the Florence flask was clean. The Florence flask was then weighed again to determine the weight of the organic extract. The separatory funnel was added a volume of approximately 150 mL hexane and 100 mL 90% MeOH and shaken. When the two phases separated, the MeOH phase (the lower phase) was transferred to a new Florence flask. The shaking and transferring was repeated three times into the same Florence flask. The solvent was evaporated on a rotary evaporator until the sample was dry. The dry sample was added 4 mL MeOH and spinned on the rotary evaporator (without vacuum) to completely redissolve the dried sample, and then transferred to a glass tube. Then the Florence flask, containing residues of the prepared organic extract, was added 2 mL MeOH multiple times until all of the sample was dissolved, and transferred to the tube. The organic phase was stored in the refrigerator at 5 °C until use.

Aqueous extract

All of the dry aqueous extract was transferred to a tared falcon tube and added 20 mL 90 % MeOH, mixed and centrifuged at 4565 g, at 5 °C for 2 minutes. The supernatant was transferred to a Florence flask after centrifugation, and this process was repeated three times. The sample was then evaporated on a rotary evaporator until the sample was dry. The dry sample was added 4 mL MeOH, spun down and transferred to a glass tube, as the procedure described for the organic extract.

3.5 Scout run HPLC analysis of M17015 and M17017 extracts

Scout means searching/looking for something. In this thesis, the scout run was used to determine the optimal isolation and purification conditions. This was achived by injecting a definite volume from each samples onto different HPLC columns. Based on the results from the different columns, the best column for isolation and purification was selected. The materials and equipment used during scout run analysis can be seen in Table 7.

Table 7: Materials and equipment used in scout run analysis and isolation.

Materials/Equipment	Supplier
ACN (≥99.8 %)	Sigma-Aldrich, St. Louis, MO, USA
Formic Acid (98.0 %)	Sigma-Aldrich, St. Louis, MO, USA
MeOH (≥ 99.9 %)	Sigma-Aldrich, St.Louis, MO, USA
MilliQ water	Millipore, Billerica, MA, USA
XSELECT™ CSH™ Prep Fluoro-Phenyl, 5 μm, 10x250 mm column	Waters, MA, USA
Atlantis® Prep C18, 10 μm, 10x250 mm column	Waters, MA, USA
XTerra® Prep C18, 10 μm, 10x300 mm column	Waters, MA, USA
XSELECT™ CSH™ Prep Phenyl-Hexyl, 5 μm, 10x250 mm column	Waters, MA, USA

Both the organic and aqueous extracts were analysed using preparative HPLC-MS with the instrument parameters and mobile phase gradient described in table 8 and 9, to look for the targeted compounds for isolation and further structure elucidation. The samples were injected on four different columns with an injection volume of 200 μL on each column, to determine which column to use for the isolation. When isolating a sample using mass guided preparative HPLC, several isolation rounds are needed to completely remove sample impurities from the compound of interest. The chromatograms from the scout runs can also be used when selecting columns for the second and third rounds of isolation. From the scout run chromatograms, the columns were evaluated after which columns separated the compounds of interest from the impurities, and also by the chromatography of the compound (peak shape, intensity etc.).

Table 8: Gradient used for the scout run analysis and isolation from the organic extracts. Mobile phase A: ultra-pure water with 0.1 % Formic acid (FA), and mobile phase B: ACN with 0.1 % FA.

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

Table 9: Overview of instrument parameters used during scout run and isolation from the organic extracts of M17015 and M17017 samples with prep-HPLC.

Source Temperature °C	120
Desolvation Temperature °C	300
Desolvation Gas Flow (L/h)	650
Polarity	ES+
Low mass (*m/z*)	200
High mass (*m/z*)	1000
Source type	ESI

3.6 Isolation of target compounds from the organic extract of M17015 and M17017 using prep HPLC-MS

The material and equipment used during isolation of the target compounds from the organic extract of M17015 and M17017 are listed under the method "3.5 scout run HPLC analysis of M17015 and M17017 extracts". The instrument parameters and mobile phase gradient used for isolation can be seen in Table 8 and 9.

The compounds isolated from the organic extract of M17017 were isolated and purified using two rounds of HPLC separation (Table 10). Compounds isolated from the organic extract of M17015 were isolated and purified using three rounds of HPLC separation (Table 11).

Table 10: All isolated compounds from the extract of M17017 according to the different columns used for the first and second isolation run. The gradient used for each isolation is illustrated in table 8.

Compound	HPLC separation round	Column
2	First	Fluoro-Phenyl
	Second	Phenyl-Hexyl
3	First	Fluoro-Phenyl
	Second	Phenyl-Hexyl
4	First	Fluoro-Phenyl
	Second	Xterra C_{18}
5	First	Fluoro-Phenyl
	Second	Xterra C_{18}

Table 11: All isolated compounds from the extract of M17015 according to the different column used for the first, second and third isolation run. The gradient used for each isolation is illustrated in table 8.

Compound	HPLC separation round	Column
1	First	Atlantis C_{18}
	Second	Xterra C_{18}
2	First	Atlantis C_{18}
	Second	Xterra C_{18}
	Third	Phenyl-Hexyl
3	First	Atlantis C_{18}
	Second	Xterra C_{18}
	Third	Phenyl-Hexyl
4	First	Atlantis C_{18}
	Second	Xterra C_{18}
	Third	Xterra C_{18}
5	First	Atlantis C_{18}
	Second	Xterra C_{18}
	Third	Xterra C_{18}

3.7 NMR analysis of isolated compounds from the organic extracts M17015 and M17017

Johan Isaksson at the Department of Chemistry at UiT The Arctic University of Norway preformed the NMR analysis, as well as the interpretation of the data.

All spectra were acquired on a Bruker Avance III HD spectrometer operating at 599.90 MHz for protons, equipped with an inverse detected cryo-probe enhanced for ^{1}H, ^{13}C and ^{2}H.
The isolated compounds (Table 17) were prepared by dissolving the sample in 500 µL dimethyl sulfoxide (DMSO). The sample was transferred into a 5mm disposable tube. Experiments were typically acquired using gradient selected adiabatic versions where applicable. All experiments were acquired using TopSpin 3.5 pl2, at 298 K.

3.8 Bioactivity testing

After isolation and NMR analysis, the one compound that was possible to structure elucidate, compound 2, was chosen for further bioactivity testing. The dried sample was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) to create a stock solution with a concentration of 3.33 µg/µL. From the stock solution it was made a series of dilutions with concentration between 1.12-40 µM, starting with 40 µM and diluted 50:50 down to 1.12 µM. The sample and dilutions were stored in the refrigerator at 4 °C until the next day.

3.8.1 Preparation of the test bacteria for both MIC and biofilm assays

The test bacteria were stored in the same growth medium that they were cultivated, with 10% glycerol at -80 °C. The solution of bacteria was transferred from cryotubes while kept on ice to an agar plate. An inoculating loop was used to transfer a small amount from the bacterial stock solution to the blood agar plates. The plates were incubated at 37 °C overnight and then stored in the fridge at 4°C for two weeks. After two weeks a colony of bacteria was transferred to a new plate and incubated overnight for maintenance, and stored at 4 °C for two weeks before they are thrown away.

3.8.2 Antibacterial screening (MIC-assay)

The materials and equipment used during antibacterial screening can be seen in Table 12.

Table 12: Materials and equipment used in the antibacterial screening (MIC) of compound 2.

Materials/Equipment	Supplier
Mueller Hinton broth (MH)	Becton, Dickinson and Company, New Jersey, USA
Brain heart infusion broth (BHI)	Sigma-Aldrich, Missouri, USA
Gentamycin (10 mg/mL) A2712	Merck KGaA, Germany
Milli-Q water	Millipore, Billerica, MA, USA
Glycerol G5516	Sigma-Aldrich, Missouri, USA
Escherichia coli ATCC® 25922	LGC Standards, UK
Streptococcus agalactiae ATCC® 12386	LGC Standards, UK
Blood agar plates	University Hospital of North Norway (UNN) Tromsø, Norway
Herasafe biological safety cabinet, Class II	Thermo Fisher Scientific, Massachusetts, USA
Incubator Unimax 1010	Heidolph Instruments GmbH & Co, Germany
Heated Incubator MIR-262	Panasonic Healthcare, Japan

Preparation of test bacteria

Compound 2, the one fully structure elucidated compound, was screened for antibacterial effect against two bacteria, *E.coli* and *S.agalacitae*. A scoop of bacteria was transferred to 8 mL growth media, and allowed to grow overnight at 37 °C in an incubator. 2 mL of the overnight cultures from the day before was transferred to 25 mL fresh cultivated medium. The bacteria culture was incubated for 1.5 h in an incubator at 37 °C to reach a turbidity of 0.5 McFarland standard (1.0 x 10^8 bacteria/mL). After incubation, the bacterial suspensions were diluted 1:1000 in fresh media before being used in the antibacterial assay.

Preparation of 96-well microtiter plates

Compound 2 was dissolved in DMSO and diluted in sterile ultra-pure water to 40, 20, 10, 5, 2.5 and 1.25 µM. 50 µL of the sample was added to two different microtiter plates (one for each bacterial strains) in three parallels. 50 µL of the diluted bacterial suspension was added to the sample (diluting the sample 1:2, giving the final test concentration half of the concentration previously mentioned). 50 µL growth media and 50 µL sterile ultra-pure water were used as negative control, while 50 µL sterile ultra-pure water and 50 µL diluted bacterial suspension was used as positive control. The microtiter plates were then incubated at 37 °C for 24 h.

The next day the plates were checked visually for growth inhibition before measuring the absorbance at 600 nm. Threshold Abs_{600} values were used to define the compound as either active, questionable or inactive.

The definition values used to define the compound:

- Active ≤ 0.05
- Questionable 0.05-0.09
- Inactive ≥ 0.09

Gentamycin control

Gentamycin controls are performed routinely as control for normal growth of the bacteria, and for the assay. The controls were performed in concentrations ranging from 16 µg/mL to 0.01 µg/mL (final test concentration), in order to determine the minimum inhibitory concentration (MIC) of gentamycin for the test bacteria. In a microtiter plate 50 µL of the gentamycin control was added to 50 µL bacterial solution, and incubated at 37 °C overnight. The MIC values were evaluated visually by looking for growth inhibition. Clear wells indicates inhibition of bacterial growth.

3.8.3 Biofilm inhibiting activity screening

The materials and equipment used during the biofilm inhibiting screening can be seen in Table 13.

Table 13: Materials and equipment used in biofilm inhibiting activity screening for compound 2.

Materials/Equipment	Supplier
Milli-Q water	Millipore, Billerica, MA, USA
Dextrose, D9434	Sigma-Aldrich, Missouri, USA
Ethanol 70%	Sigma-Aldrich, Missouri, USA
Tryptic soy broth (TSB), 105459	Merck KGaA, Germany
Crystal violet, 115940	Merck KGaA, Germany
Glucose, D9434	Sigma-Aldrich, Missouri, USA
Staphylococcus epidermidis ATCC 35984	University Hospital of North Norway (UNN) Tromsø, Norway
Staphylococcus haemolyticus	Clinical isolate 8-7A, University Hospital of North Norway (UNN) Tromsø, Norway
Blood agar plates	University hospital (UNN) Tromsø, Norway
Herasafe biological safety cabinet, Class II	Thermo Fisher Scientific, Massachusetts, USA
Incubator Unimax 1010	Heidolph Instruments GmbH & Co, Germany
Heated Incubator MIR-262	Panasonic Healthcare, Japan

One scoop of each bacteria was transferred to 5 mL TSB and incubated overnight at 37 °C. After overnight incubation, the cultures were diluted 1:100 in TSB with 1% glucose. 50 µL of dissolved and diluted compound 2, was added to the microtiter plate in three parallels with concentrations as described in "3.8.2 Antibacterial screening (MIC assay)". 50 µL of the sample was transferred to a microtiter plate and 50 µL *S.epidermidis* suspension was added to the sample wells. 50 µL *S.epidermidis* culture and 50 µL sterile ultra-pure water were used as positive control. 50 µL sterile ultra-pure water and 50 µL of the non-biofilm producing bacteria *S.haemolyticus* culture was used as negative control. A medium blank with TSB and 1% glucose was also included in the screening, with 50 µL medium and 50 µL sterile ultra-pure water. Then the microtiter plates were incubated overnight at 37 °C.

After the overnight incubation, the plates were visually examined for growth inhibition to check that antibacterial activity was not misinterpreted as biofilm formation inhibiting activity. Clear wells indicates bacterial growth inhibition instead of inhibition of biofilm formation. The bacterial suspension was poured out, and the wells were rinsed with water before incubation for 1 h at 55 °C to fixate the biofilm to the bottom of the wells. After the fixation, the biofilm was colored by adding 70 µL 0.1 % crystal violet solution to all the wells. The colored plates were incubated for 5 minutes, and then the crystal violet solution was poured off and the plates again rinsed with water. The microtiter plates were incubated again for 1 h at 55 °C for the plates to be completely dry. The dry plates were added 70 µL 70 % ethanol to all the wells, and incubated for 10 minutes at room temperature before the absorbance was measured at 600 nm. The compound was considered active if the measured Abs_{600} was below 0.25.

4 Results

4.1 UHPLC-HR-MS analysis

All of the organic extracts of the samples M17015-M17028 (for table, see appendix A1) were analysed using UHPLC-HR-MS to look for novel compounds with interesting chemistry. The two samples that were used for further preparation and isolation, M17015 and M17017 contained interesting compounds and were two of the samples with the highest amount of extracts.

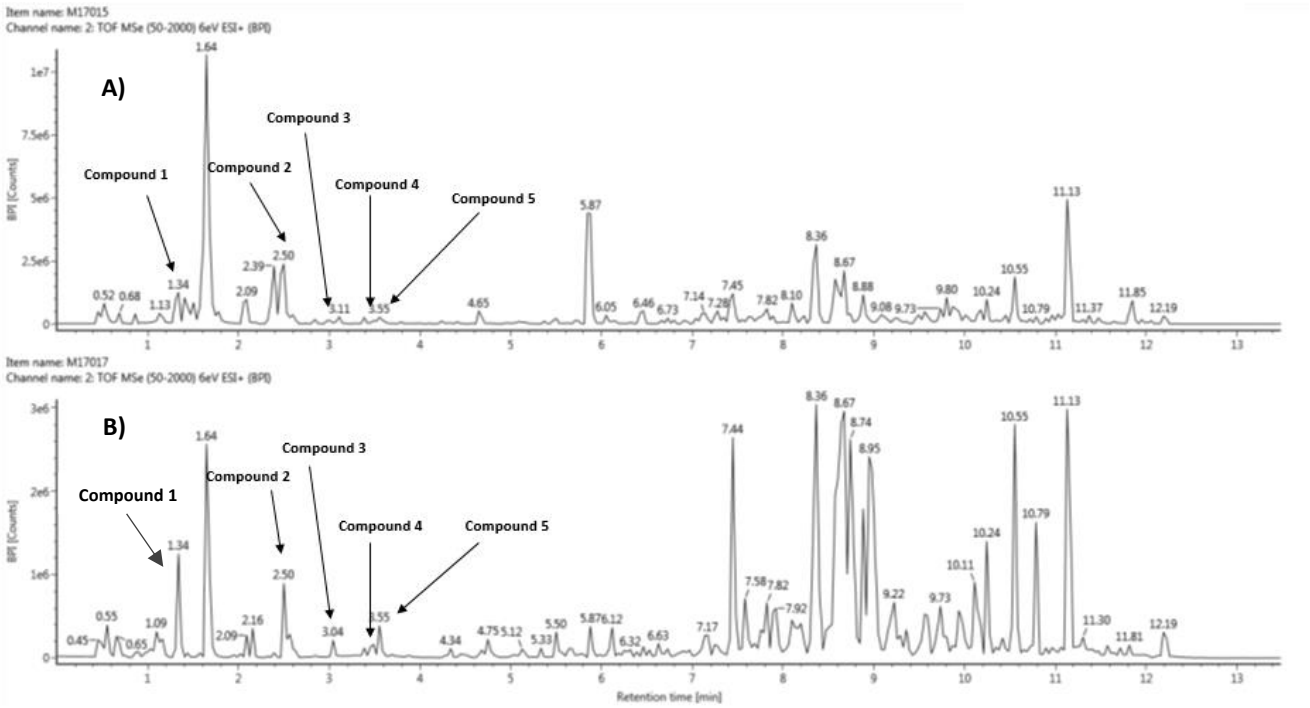

Figure 3: Base peak intensity chromatogram of the crude organic extract A) M17017 and B) M17015 as part of the dereplication run on HR-MS. Aliquots of the extract was injected onto a C_{18} column. The arrows with the compound numbers indicates each compound that was later isolated, and which peak they represent in the chromatogram.

The Figures 4-7 show the mass spectra of the five target compounds that were nominated for isolation and characterization. As can be seen in Figure 4, the mass spectrum of compound 2, from the HR-MS (ESI orbitrap MS), the most abundant ion formed was the protonated form of the compound. Other ions corresponding to different adducts of the molecule can also be observed. In addition to the protonated compound, the sodium adduct, the protonated dimer

and the sodium adduct of a dimer can be seen. This increases the probability that the most abundant signal (*m/z* 197.1169) in fact is the protonated compound.

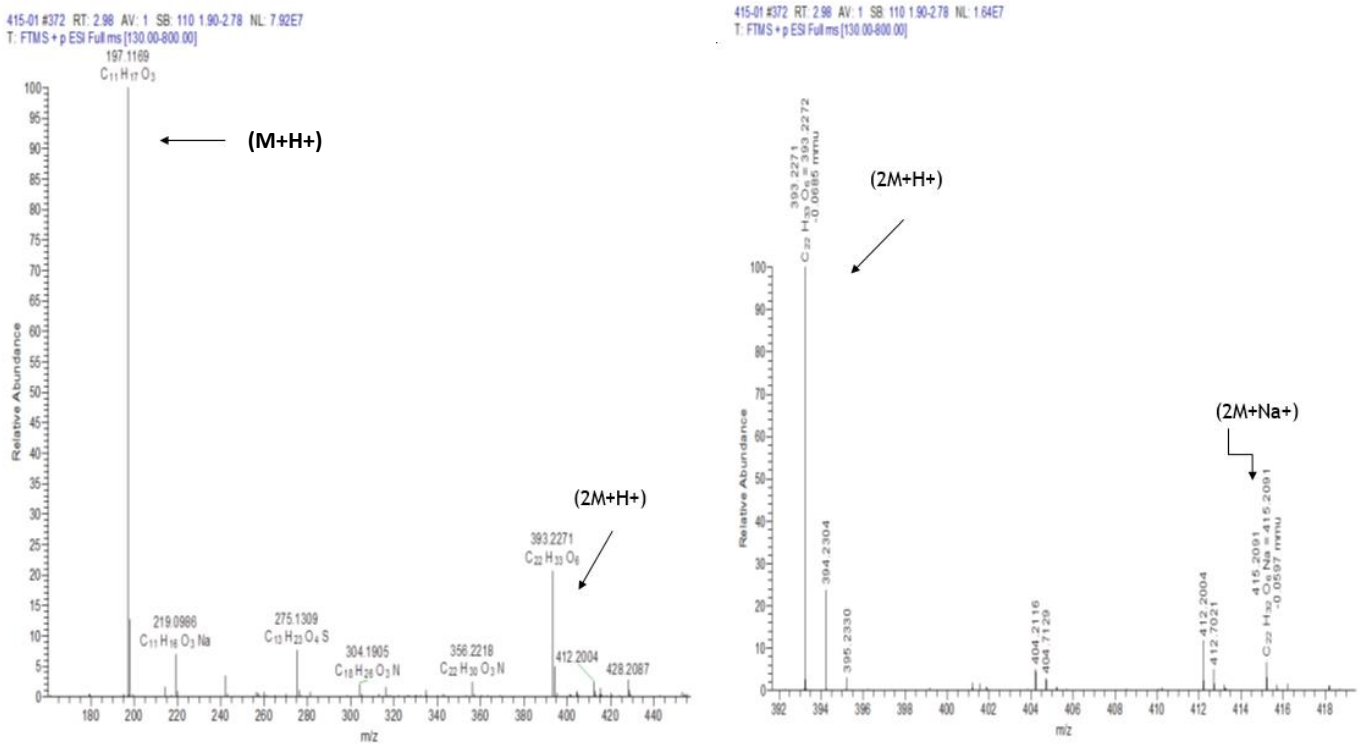

Figure 4: Mass spectrum of compound 2, discovered in the organic extracts of M17015 and M17017. The mass spectra show the protonated compound and the different adducts formed in the ion source.

The mass spectra for the four other target compounds, named in this thesis as compound 1, 3, 4 and 5, are shown in Figure 5-7. All the target compounds had isotopic patterns indicative of bromine, and this was the reason why they were selected for isolation.

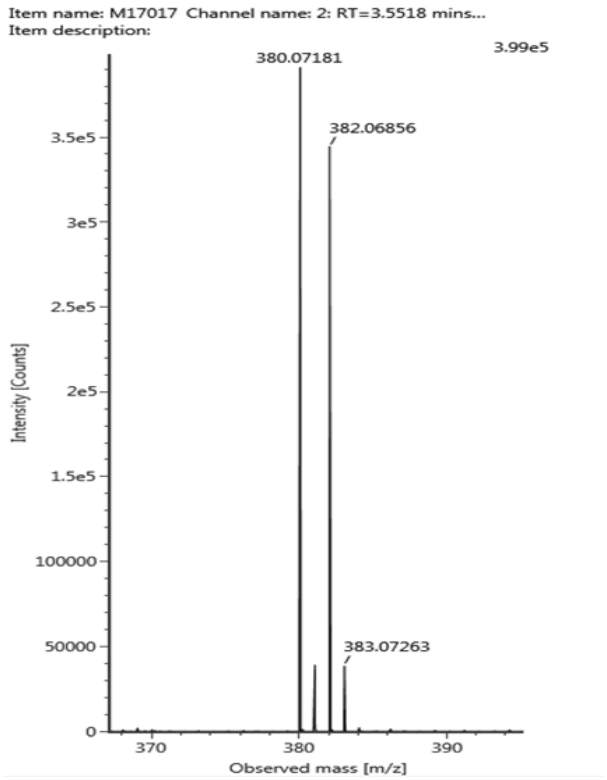

Figure 5: Mass spectrum of compound 5, showing the isotopic pattern of the mono-brominated compound found in the organic extracts of M17017 and M17015.

The isotope pattern of the spectrum in Figure 5 shows two signals with similar intensity that are two mass units apart (1:1 ratio), m/z 380 and 382. There are also two signals with considerably lower intensity two mass units apart, m/z 381 and 383. This comes from the occurrence of carbon isotopes and bromine isotopes that provide an isotope pattern that is easily recognised when analysed by mass spectrometry. The natural abundances of carbon isotopes are ^{12}C: 98.93 % and ^{13}C: 1.07 %, Bromine isotopes are ^{79}Br: 50.69% and ^{81}Br: 49.31% (45). This indicates that this compound contain one Br in the structure.

Table 14: Isotope patterns in relation to the different signals in the spectrum illustrated in Figure 5.

Signal (*m/z*)	C/Br isotope
380	$^{12}C/^{79}Br$
381	$^{13}C/^{79}Br$
382	$^{12}C/^{81}Br$
383	$^{13}C/^{81}Br$

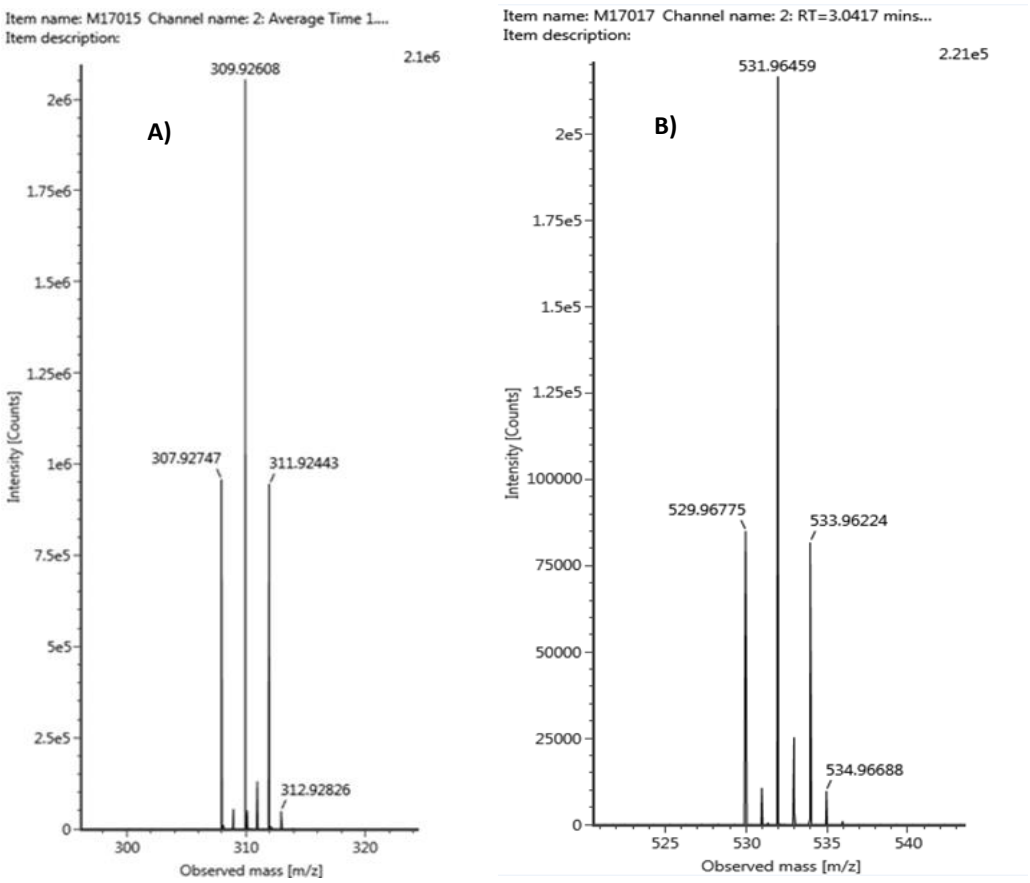

Figure 6: Mass spectrum of the compounds 1 and 3, showing the isotopic pattern of the di-brominated compounds found in the organic extracts of M17017 and M17015. A) Compound 1, and B) compound 3.

The MS spectrum in Figure 6 shows a pattern with three signals with a 1:2:1 ratio separated by two mass units, *m/z* 529, 531 and 533 for compound 3, and *m/z* 307, 309 and 311 for compound 1. There are also three signals with considerably lower intensity two mass units apart, *m/z* 530, 532 and 534 for compound 3, and *m/z* 308, 310 and 312 for compound 1, with the same ratio as described above. This pattern indicates that this compound contain two Br in the structure. The isotope pattern-signals relation for the compound in Figure 6B, is shown in Table 15, the same applies for the compound in Figure 6A.

Table 15: Isotope patterns in relation to the different signals in the spectrum illustrated in Figure 6B.

Signal (*m/z*)	C/Br isotope
529	$^{12}C/^{79}Br+^{79}Br$
530	$^{13}C/^{79}Br+^{79}Br$
531	$^{12}C/^{79}Br+^{81}Br$
532	$^{13}C/^{79}Br+^{81}Br$
533	$^{12}C/^{81}Br+^{81}Br$
534	$^{13}C/^{81}Br+^{81}Br$

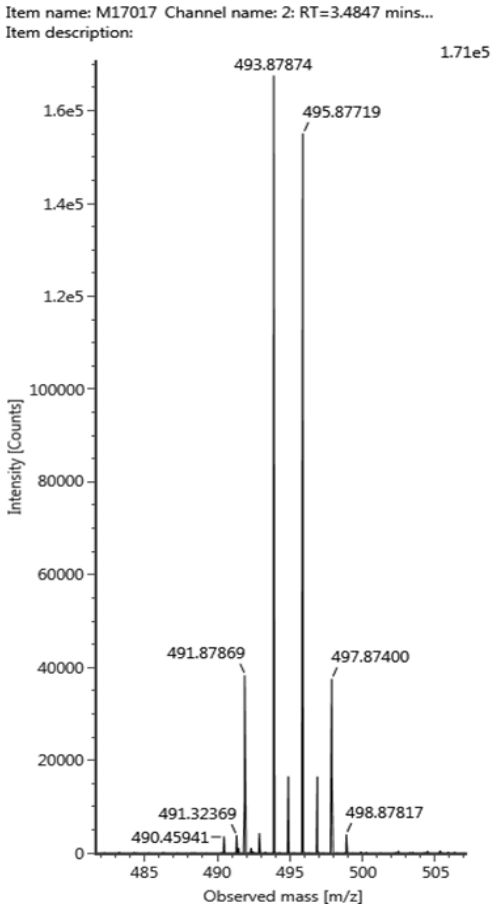

Figure 7: Mass spectrum of compound 4, showing the isotopic pattern of the Tri-brominated compound found in the organic extracts of M17017 and M17015.

The MS spectrum in Figure 7 shows a pattern with four signals with a 1:3:3:1 ratio separated by two mass units, m/z 491, 493, 495 and 497 with high intensity signals. There are also four signals with considerably lower intensity two mass units apart, m/z 492, 494, 496 and 498 with the same ratio as described above. This pattern indicates that this compound contain three Br in the structure.

Table 16: Isotope patterns in relation to the different signals in the spectrum illustrated in Figure 7.

Signal (*m/z*)	C/Br isotope
491	$^{12}C/^{79}Br+^{79}Br+^{79}Br$
492	$^{13}C/^{79}Br+^{79}Br+^{79}Br$
493	$^{12}C/^{79}Br+^{79}Br+^{81}Br$
494	$^{13}C/^{79}Br+^{79}Br+^{81}Br$
495	$^{12}C/^{79}Br+^{81}Br+^{81}Br$
496	$^{13}C/^{79}Br+^{81}Br+^{81}Br$
497	$^{12}C/^{81}Br+^{81}Br+^{81}Br$
498	$^{13}C/^{81}Br+^{81}Br+^{81}Br$

Table 17: Overview of the compounds, with the exact m/z, elemental composition and retention time from HR-MS analysis. Basis of selection describes when the compounds were selected as target compounds for isolation. Compound 1-2 was selected after column selection for the first isolation round and compound 3-5 was selected in the HR-MS analysis.

Compound	*m/z*	Elemental composition	Retention time (min)	Basis of selection
1	307.928	$C_9H_{11}Br_2NO$	1.34	Scout run
2	197.116	$C_{11}H_{16}O_3$	2.50	Scout run
3	529.968	$C_{17}H_{17}Br_2N_5O_5$	3.04	HR-MS
4	491.879	$C_{16}H_{16}Br_3NO_2$	3.48	HR-MS
5	380.072	$C_{15}H_{18}BrN_5O_2$	3.55	HR-MS

4.2 Compound isolation

Compounds 3-5 from the organic extracts of M17015 and M17017 samples were selected from the dereplication analysis, for isolation and purification. The M17015 sample was included to increase the amount of target compounds isolated from the M17017 sample.

4.2.1 Preparation of the organic extract for isolation

After the crude extracts of M17015 and M17017 were partitioned between hexane and MeOH, the resulting dried samples had reduced quantity. This is a result of removal of lipophilic compounds from the crude extract.

4.2.2 Column selection: Scout run

Following dereplication, three compounds were selected for isolation, compound 3-5 shown in Table 17. In order to select the best column for compound isolation from the organic extract, and aid further isolation of the selected compounds, a series of scout runs were conducted. This entails the injection of a set volume (400 µL) onto columns with different packing materials. The chromatography of the compound of interest, as well as co-elution of impurities were evaluated for each column. For the M17017 extract, the Fluoro-phenyl column was found to be the column that provided the best separation of the compounds of interest from the impurities and from each other, and that retained all of the compounds. For the M17015 sample, Atlantis C_{18} column was found to be the best column that provided the best separation of the compounds of interest from the impurities and from each other, and that retained all of the compounds.

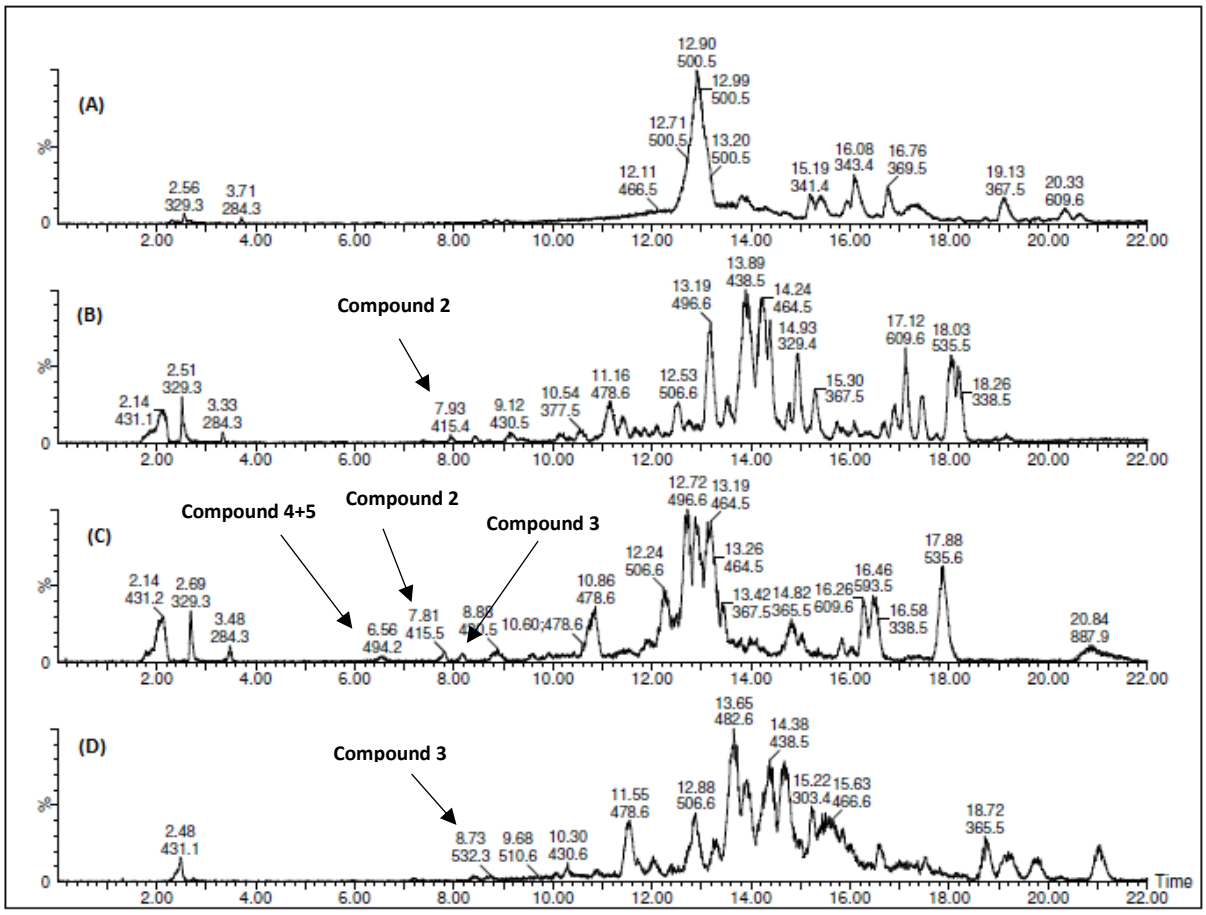

Figure 8: Base peak intensity chromatograms of the organic extract M17017, as part of a scout run. Aliquots of the extract were injected onto four different columns: (A) C_{18} Atlantis, (B) Phenyl-Hexyl, (C) Fluoro-Phenyl, (D) C_{18} Xterra. The masses of interest are the compounds called 1-5 as shown in Table 17.

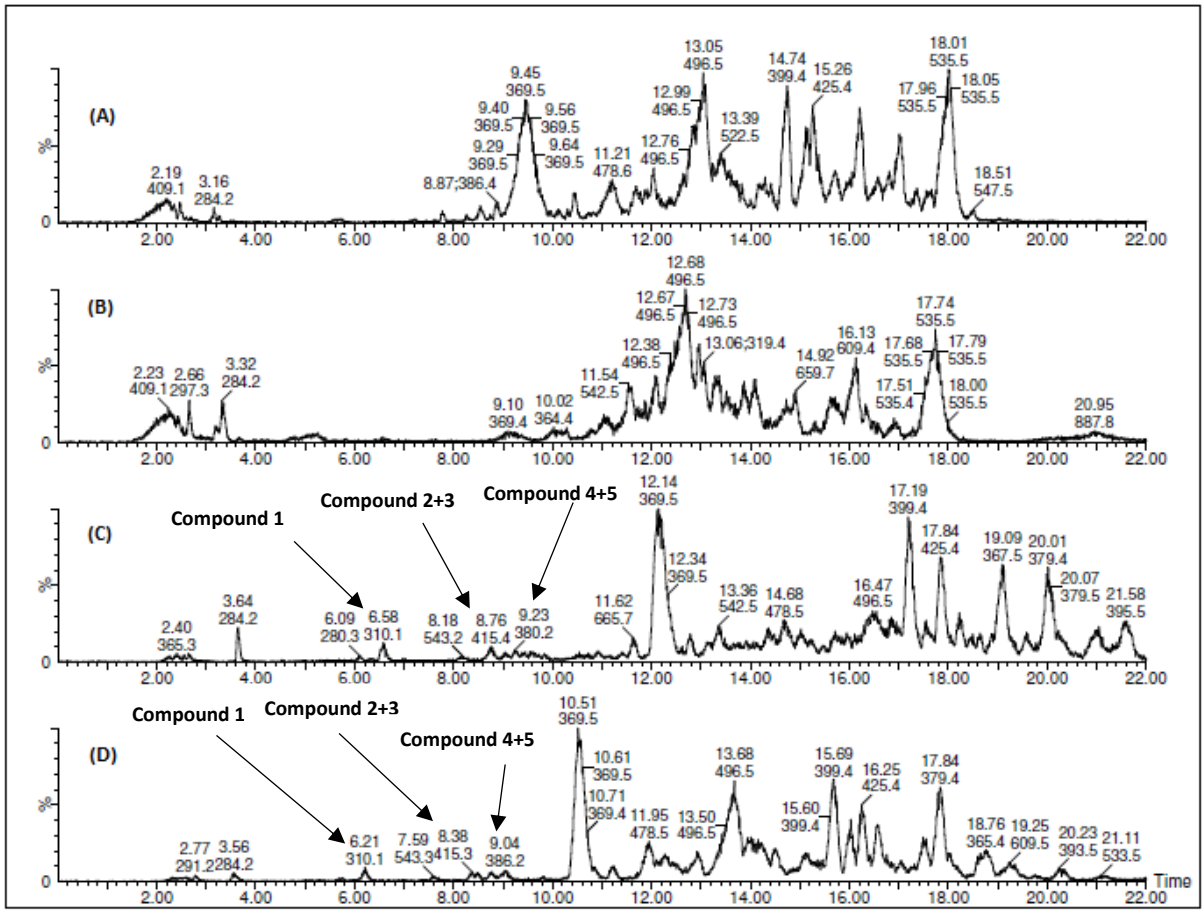

Figure 9: Base peak intensity chromatograms of the organic extract M17015, as part of a scout run. Aliquots of the extract were injected onto four different columns: (A) Phenyl-Hexyl, (B) Fluoro-Phenyl, (C) C_{18} Atlantis, (D) C_{18} Xterra. The masses of interest are the compounds called 1-5 as shown in Table 17.

The results from the scout runs aided in column selection for the first round of crude compound isolation. In addition, the results were used to select the optimal column for further purification of the crudely isolated compounds from the first isolation round. After column selection for first round isolation of the target compounds, two other compounds (compound 1 and 2 shown in Table 17) was also included in the study as they could easily be isolated.

4.2.3 Isolation of compound 1

Compound 1 was isolated from the organic extract of M17015. The compound eluted after 6.82 minutes on the Atlantis C_{18} column (Figure 10A). In total 34 injections (400 µL per injection) were performed to inject all of the extract. The collected fractions from the first isolation were pooled, dried, redissolved in MeOH and injected on the C_{18} Xterra column to remove impurities co-eluting on the Atlantis C_{18} column (Figure 10B). In total, 0.2 mg of compound 1 was isolated.

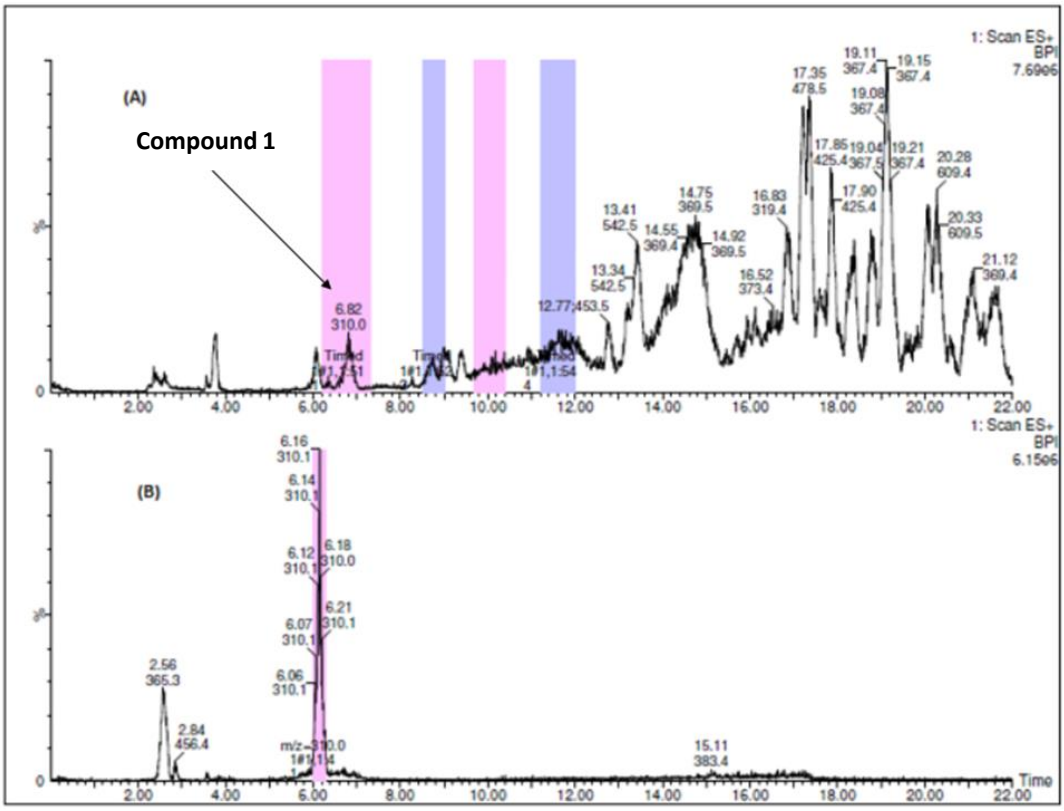

Figure 10: Isolation of compound 1 from the M17015 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Atlantis C_{18} column. (B) BPI chromatogram from the second isolation on the Xterra C_{18} column, conducted to remove impurities from the crudely isolated sample. The colored areas indicate the collected fractions.

4.2.4 Isolation of compound 2

Compound 2 was isolated from both the organic extract of M17015 and M17017. From the M17015 extract the compound eluted after 8.67 minutes on the Atlantis C_{18} column (Figure 11A). In total 34 injections (400 µL per injection) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co-eluting in the Atlantis C_{18} column (Figure 11B). The collected fractions from the second isolation were then again pooled, dried, redissolved in MeOH and injected onto the Phenyl-Hexyl column to remove more impurities (Figure 11C). From the M17017 extract, the compound eluted after 7.74 minutes on the Fluoro-Phenyl column (Figure 12A). In total 46 injections (300 µL) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Phenyl-Hexyl column to remove impurities co-eluting in the Fluroro-Phenyl column (Figure 12B). In total, 0.2 mg of compound 2 was isolated.

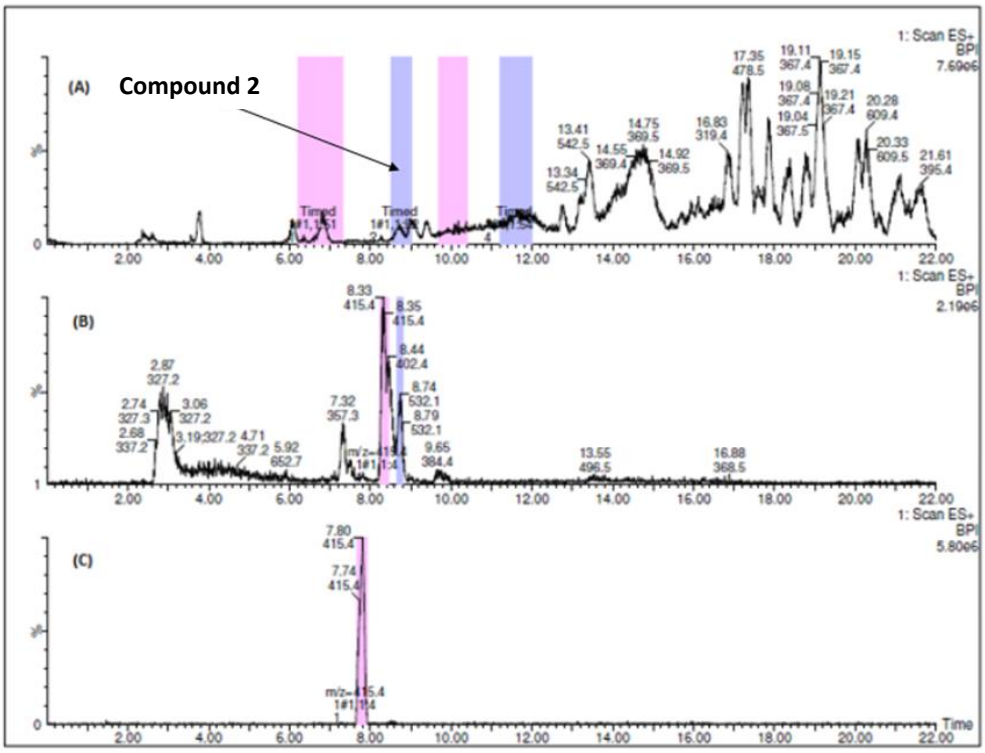

Figure 11: Isolation of compound 2 from the M17015 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Atlantis C18 column. (B) BPI chromatogram from the second isolation on the Xterra C18 column. (C) BPI chromatogram from the third isolation run on Phenyl-Hexyl column conducted to remove impurities from the first and second isolation of the sample. The colored areas indicate the collected fractions.

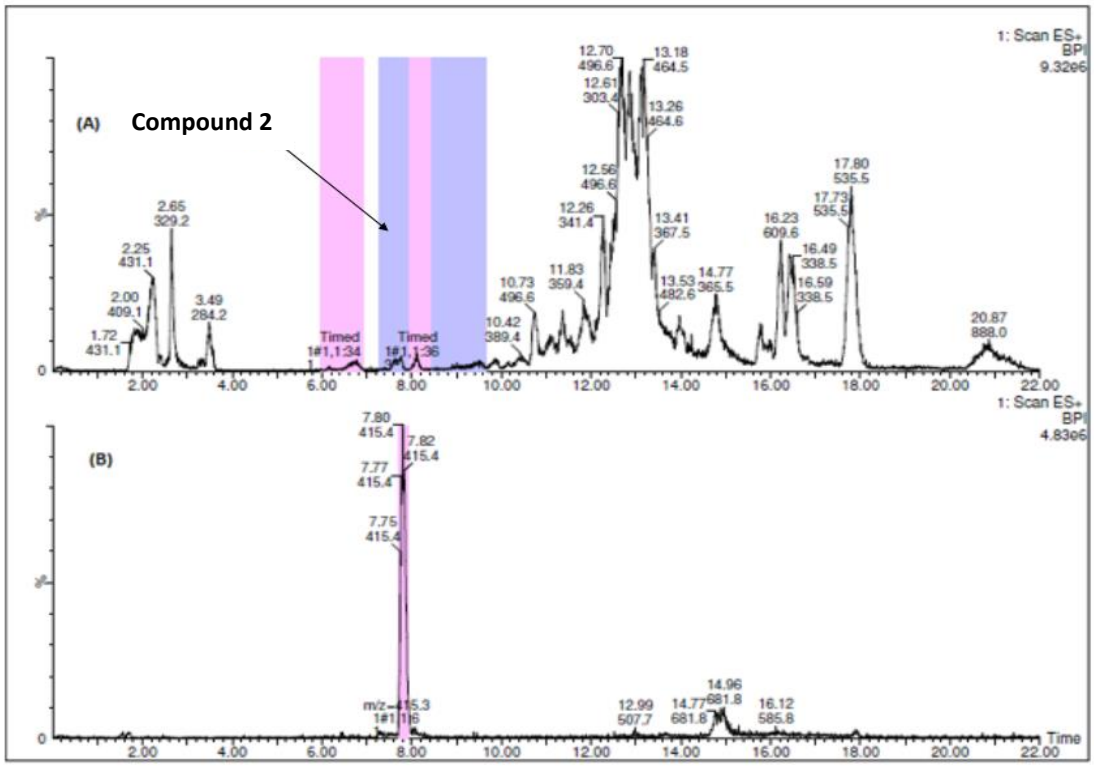

Figure 12: Isolation of the compound 2 from M17017 extract. (A) Base peak intensity (BPI) chromatogram from the first crude isolation from the organic extract using Fluoro-Phenyl column. (B) BPI chromatogram from the second isolation on the Phenyl-Hexyl column, conducted to remove impurities from the crudely isolated sample. The colored areas indicated the collected fractions.

4.2.5 Isolation of compound 3

Compound 3 was isolated from both the organic extract of M17015 and M17017. From the M17015 extract the compound eluted after 8.42 minutes on the Atlantis C_{18} column (Figure 13A). In total 34 injections (400 µL per injection) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co-eluting in the Fluroro-Phenyl column (Figure 13B). The collected fractions from the second isolation were then again pooled, dried, redissolved in MeOH and injected on the Phenyl-Hexyl column to remove more impurities (Figure 13C). From the M17017 extract the compound eluted after 8.08 minutes on the Fluoro-Phenyl column (Figure 14A). In total 46 injections (300 µL) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Phenyl-Hexyl column to remove impurities co-eluting in the Fluroro-Phenyl column (Figure 14B). In total, 0.2 mg of compound 3 was isolated.

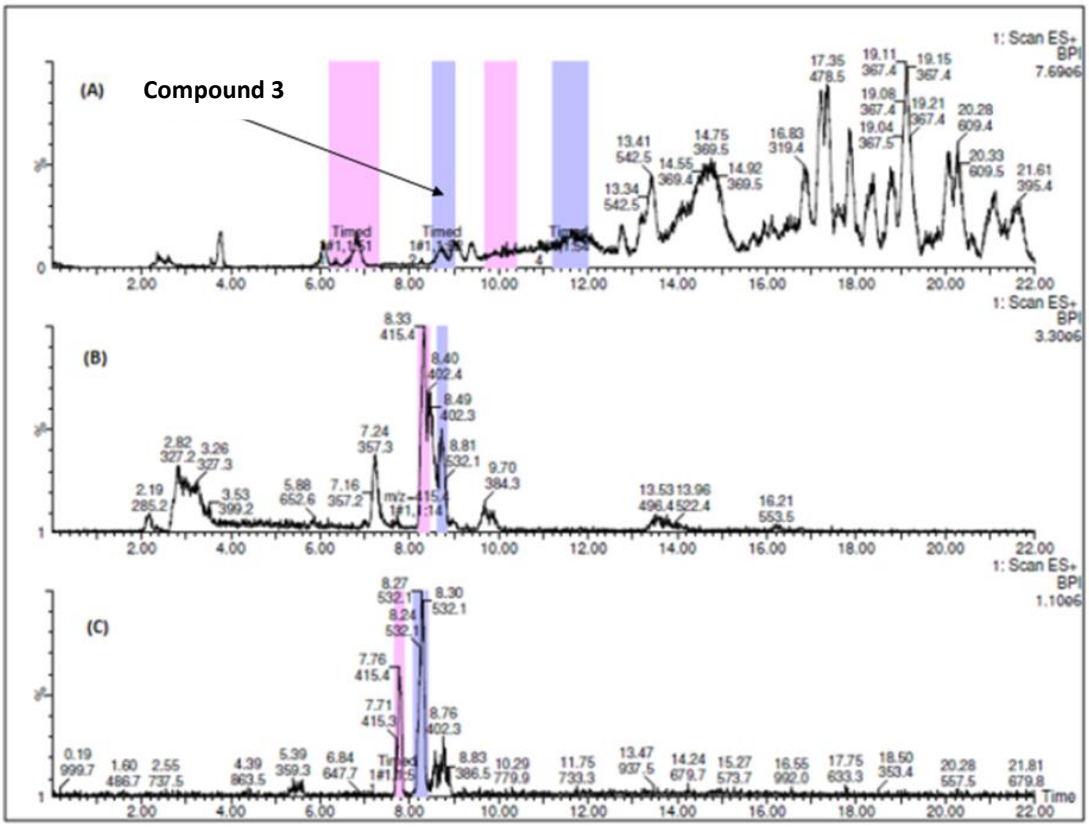

Figure 13: Isolation of compound 3 from the M17015 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Atlantis C_{18} column. (B) BPI chromatogram from the second isolation on the Xterra C_{18} column. (C) BPI chromatogram from the third isolation on the Phenyl-Hexyl column conducted to remove impurities from the first and second isolation of the sample. The colored areas indicate the collected fractions.

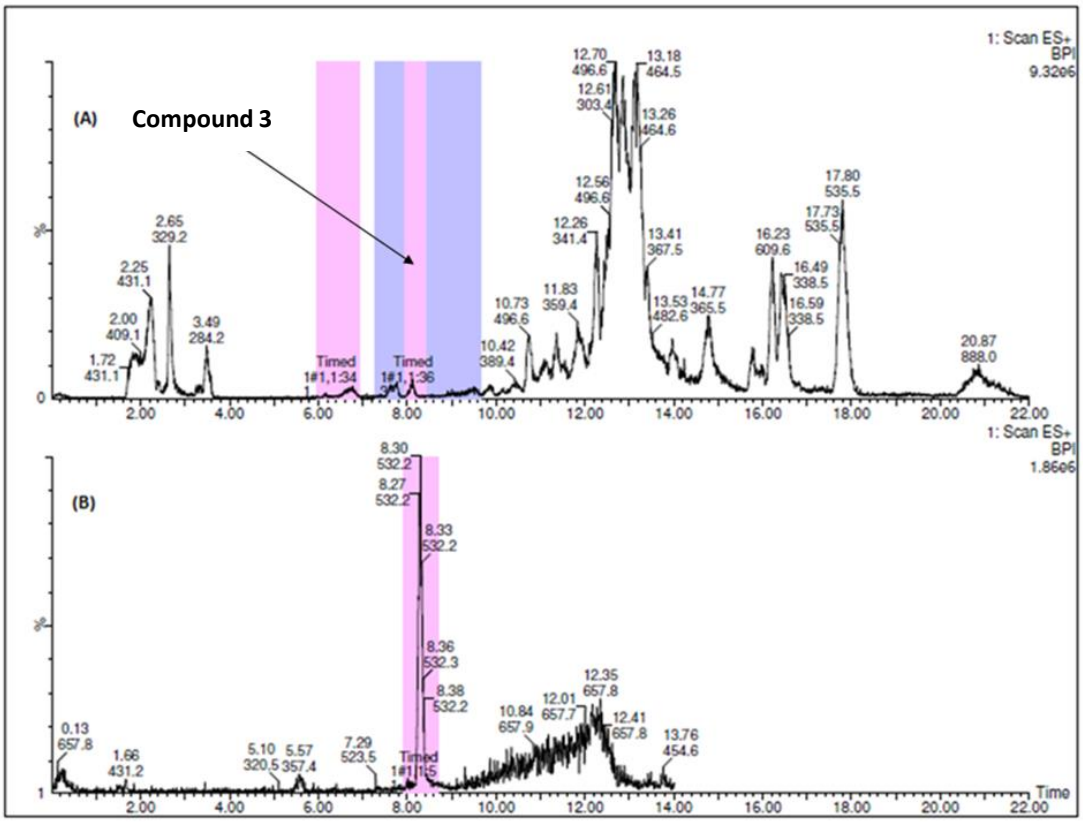

Figure 14: Isolation of compound 3 from the M17017 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Fluoro-Phenyl column. (B) BPI chromatogram from the second isolation on the Phenyl-Hexyl column, conducted to remove impurities from the crudely isolated sample. The colored areas indicate the collected fractions.

4.2.6 Isolation of compound 4

Compound 4 was isolated from the organic extract of M17015 and M17017. From the M17015 extract, the compound eluted after 10.3 minutes on the Atlantis C_{18} column (Figure 15A). In total 34 injections (400 µL per injection) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co-eluting on the Atlantis C_{18} column (Figure 15B). The fraction from the second isolation was then again pooled, dried redissolved in MeOH and re-injected on the Xterra C_{18} column to remove more impurities (Figure 15C). From the M17017 extract, the compound eluted after 6.78 minutes on the Fluoro-Phenyl (Figure 16A). In total 46 injections (300 µL) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co-eluting in the Fluroro-Phenyl column (Figure 16B). In total, 0.2 mg of compound 4 was isolated.

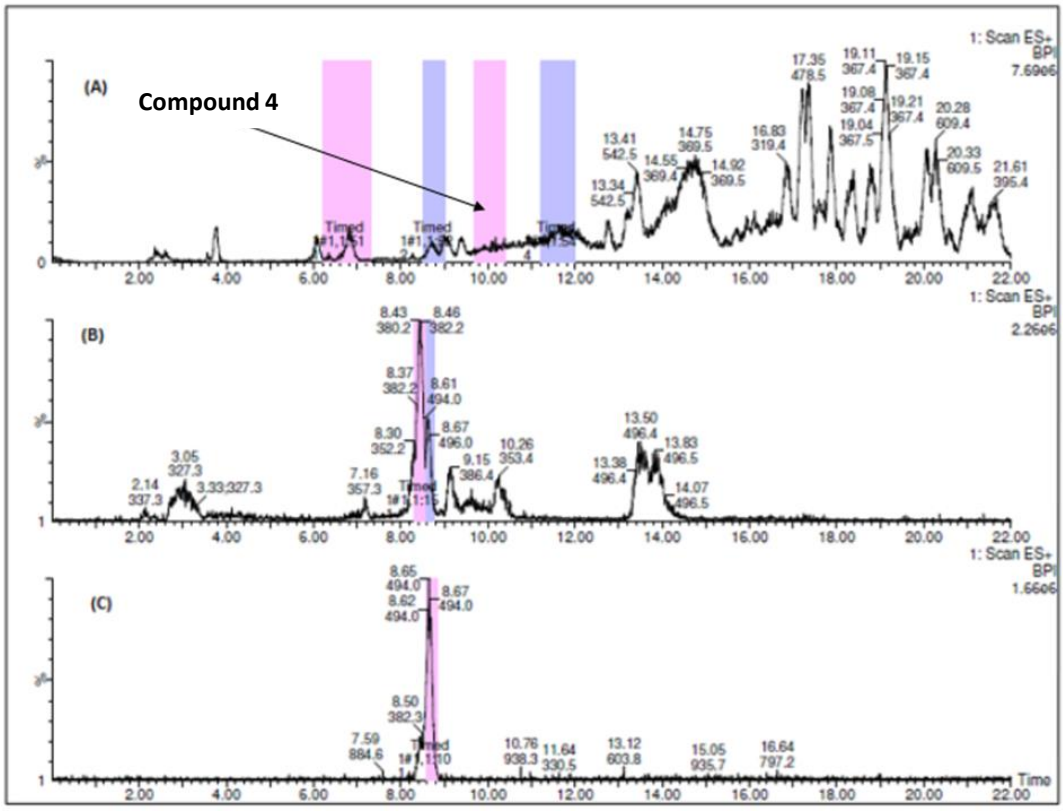

Figure 15: Isolation of compound 4 from the M17015 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Atlantis C_{18} column. (B) BPI chromatogram from the second isolation on the Xterra C_{18} column. (C) BPI chromatogram from the third isolation run a second time on the Xterra C_{18} column conducted to remove impurities from the first and second isolation of the sample. The colored areas indicate the collected fractions.

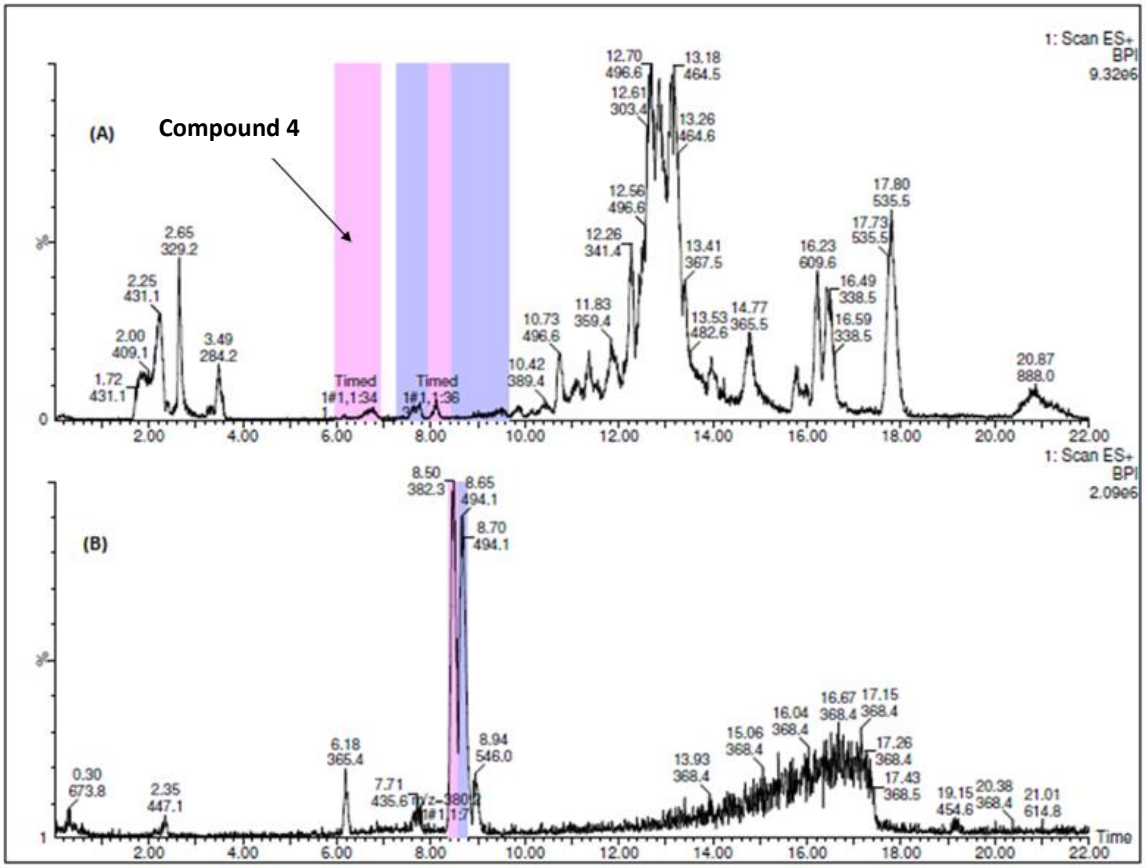

Figure 16: Isolation of the compound 4 from the M17017 extract. (A) Base peak intensity (BPI) chromatogram from the first crude isolation from the organic extract using Fluoro-Phenyl column. (B) BPI chromatogram from the second isolation on the Xterra C_{18} column, conducted to remove impurities, and to isolate compound 4 and 5 from each other from the crudely isolated sample. The colored areas indicate the collected fractions.

4.2.7 Isolation of compound 5

Compound 5 was isolated from the organic extract of M17015 and M17017. From the M17015 extract, the compound eluted after 9.91 minutes on the Atlantis C_{18} column (Figure 17A). In total 34 injections (400 µL per injection) were performed to inject all of the extract. The fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co eluting on the Atlantis C_{18} column (Figure 17B). The fraction from the second isolation was then again pooled, dried redissolved in MeOH and re-injected on the Xterra C_{18} column to remove more impurities (Figure 17C). From the M17017 extract, the compound eluted after 6.94 minutes on the Fluoro-Phenyl column (Figure 18A). In total 46 injections (300 µL) were performed to inject all of the extract. The collected fractions from the first round of isolation were pooled, dried, redissolved in MeOH and injected on the Xterra C_{18} column to remove impurities co-eluting in the Fluoro-Phenyl column (Figure 18B). In total, 0.2 mg of compound 5 was isolated.

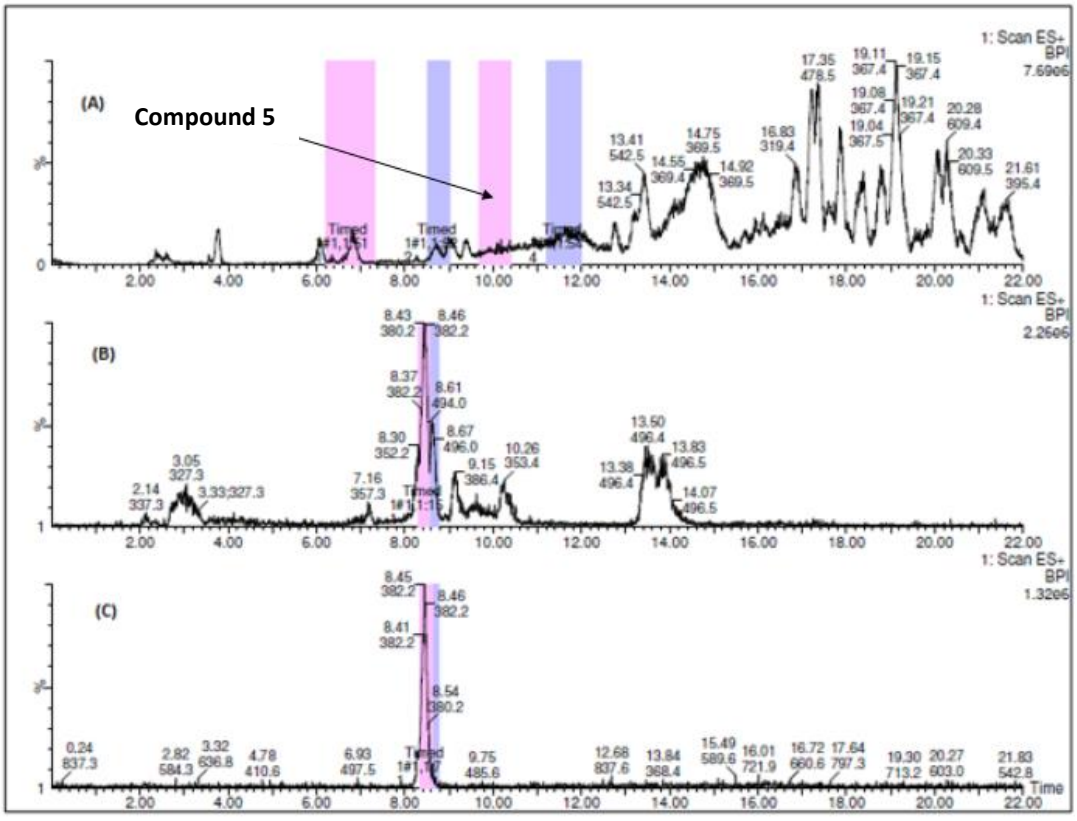

Figure 17: Isolation of compound 5 from the M17015 extract. (A) BPI chromatogram from the first crude isolation from the organic extract using Atlantis C_{18} column. (B) BPI chromatogram from the second isolation on the Xterra C_{18} column. (C) BPI chromatogram from the third isolation run a second time on the Xterra C_{18} column conducted to remove impurities from the first and second isolation of the sample. The colored areas indicate the collected fractions.

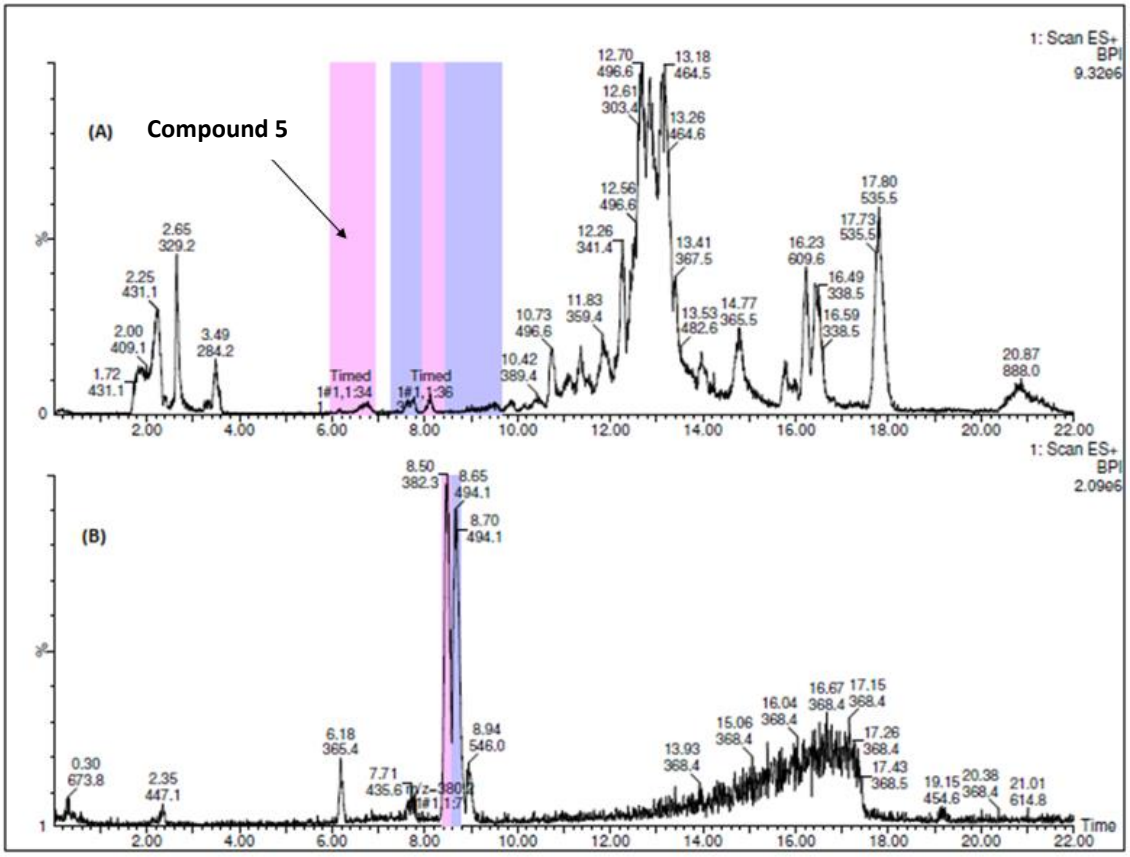

Figure 18: Isolation of compound 5 from the M17017 extract. (A) Base peak intensity (BPI) chromatogram from the first crude isolation from the organic extract using Fluoro-Phenyl column. (B) BPI chromatogram from the second isolation on Xterra C18 column, conducted to remove impurities, and to isolate the compound 4 and compound 5 from each other from the crudely isolated sample. The colored areas indicate the collected fractions.

Table 18: Overview of the compound isolated from the organic extra and the total isolated amount of each compound.

Compound	Amount isolated (mg)
1	0.2
2	0.2
3	0.2
4	0.2
5	0.2

4.3 Structure elucidation

All of the isolated compounds (Table 17) were analysed on NMR for structure elucidation. In the end only one compound was isolated in sufficient amount and purity for structure determination using NMR. This was compound 2 from the prep-HPLC isolation.

4.3.1 NMR analysis

Various 1D (Figures 19 and 20) and 2D (Figures 21-23) NMR experiments were used to determine the structure of compound 2, as shown in Figure 19-23. Compound 2 turned out to be the previously known compound loliolide.

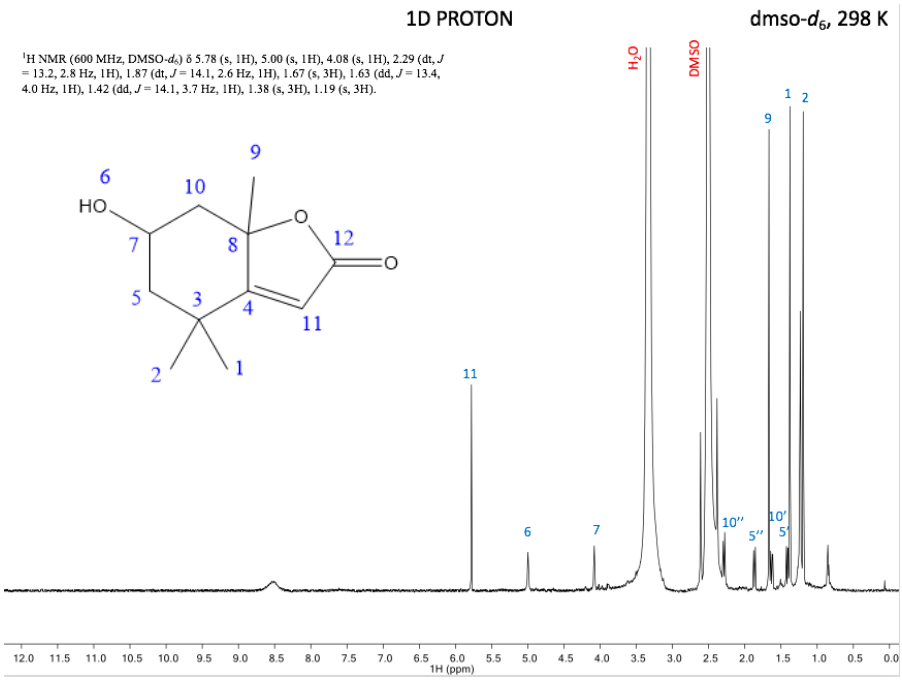

Figure 19: One dimensional ^{1}H-NMR spectrum of the purified compound. The peaks are numbered according to the structure of compound 2 and the solvents are indicated. Chemical shift (ppm) is on the x-axis, and peak intensity is on the y-axis.

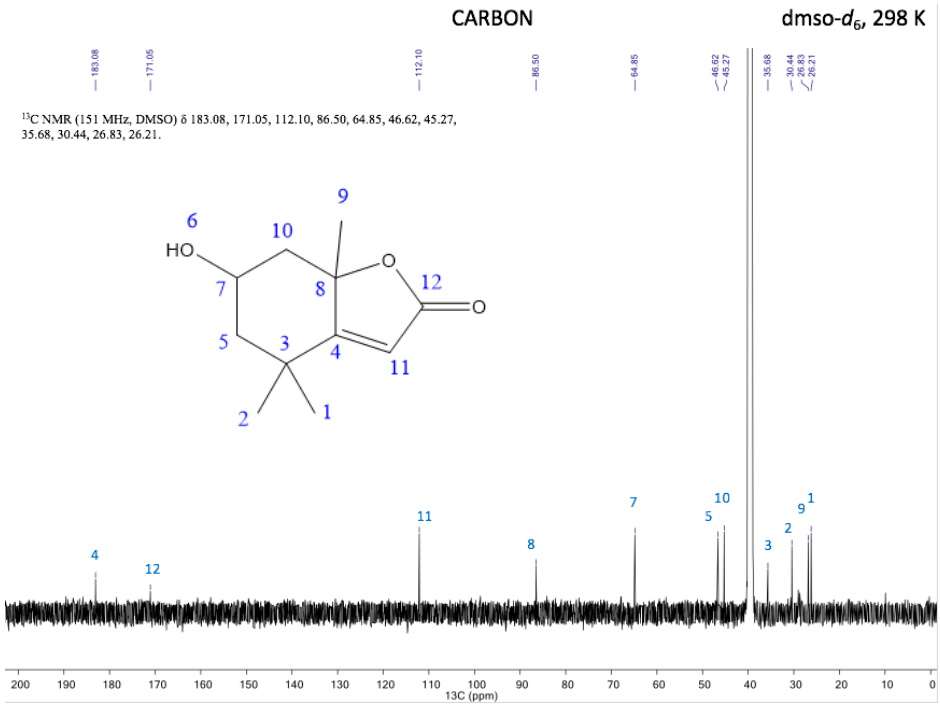

Figure 20: One dimensional ^{13}C-NMR spectrum of the purified compound. The peaks are numbered according to the structure of compound 2. Chemical shift (ppm) is on the x-axis and peak intensity on the y-axis.

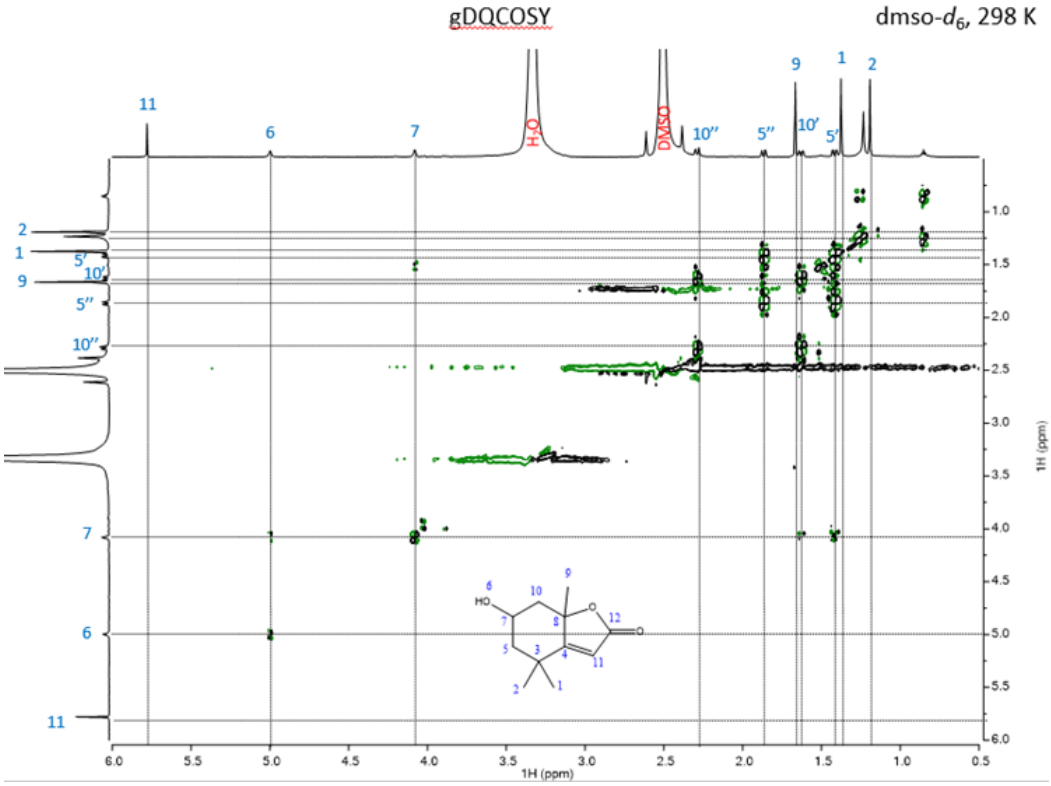

Figure 21: Two-dimensional COSY spectrum of 1H, where the peaks are numbered according to the structure of compound 2 and the solvents are indicated. Cross peaks indicate neighbouring protons coupled through 2-3 bonds.

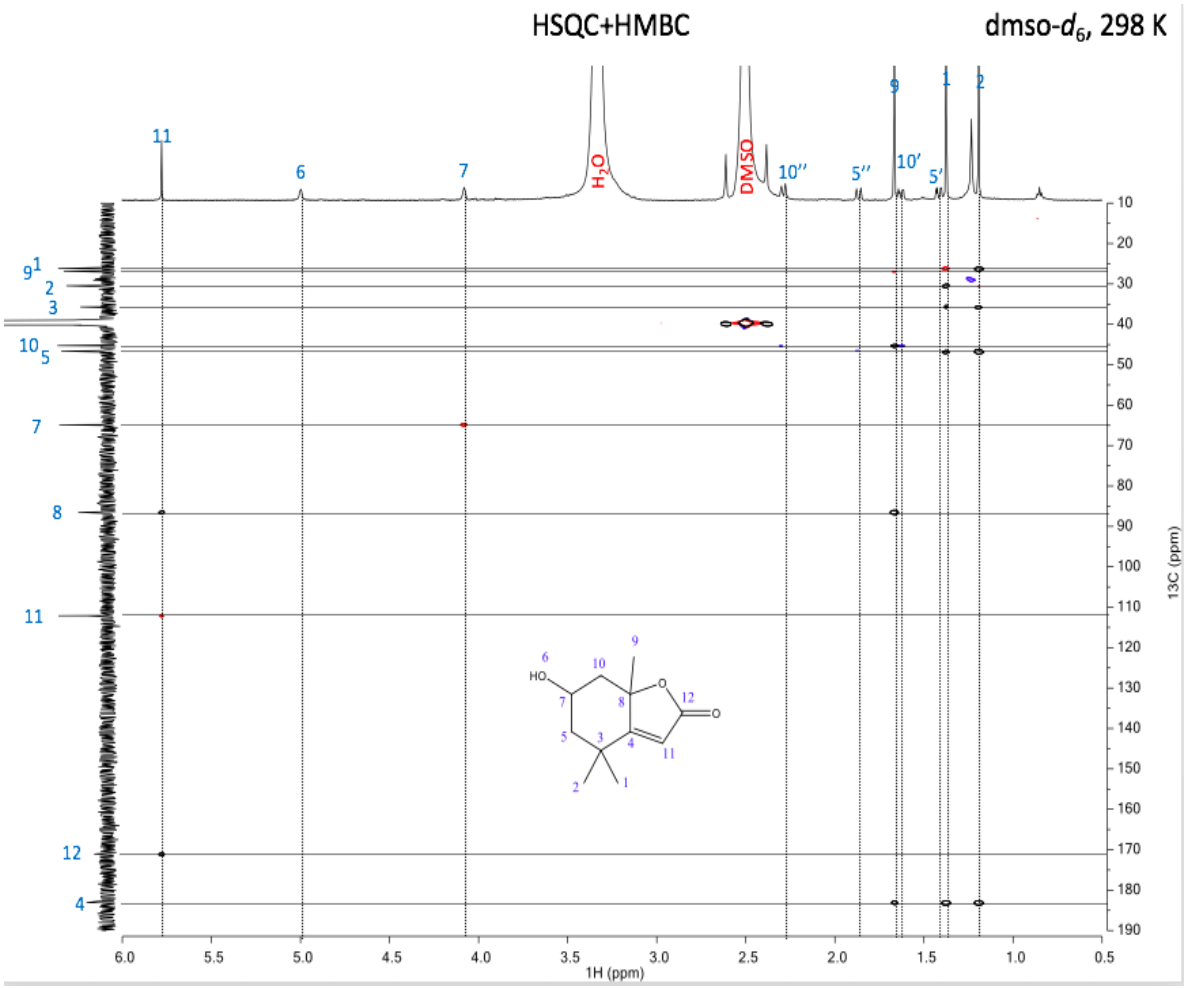

Figure 22: HSQC and HMBC 2D-spectrum. The peaks are numbered according to the structure of compound 2 and the solvents are indicated. The HSQC is displayed in red and blue markings, where CH and CH_3 groups are red and CH_2 is blue. HMBC is displayed as black markings. ^{1}H chemical shift (ppm) is on the x-axis and ^{13}C chemical shift (ppm) is on the y-axis**.**

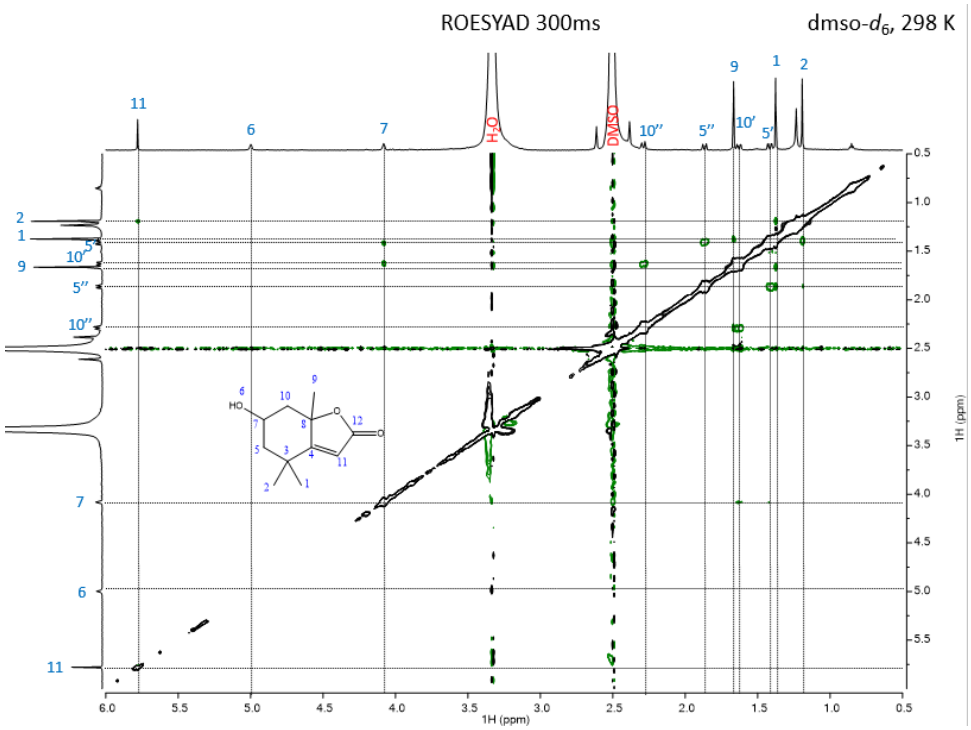

Figure 23: ROESY 2D-spectrum. The peaks are numbered according to the structure of compound 2 and the solvents are indicated. ^{1}H-^{1}H correlation mediated by dipolar coupling. Determining correlation between protons that are close to each other in space.

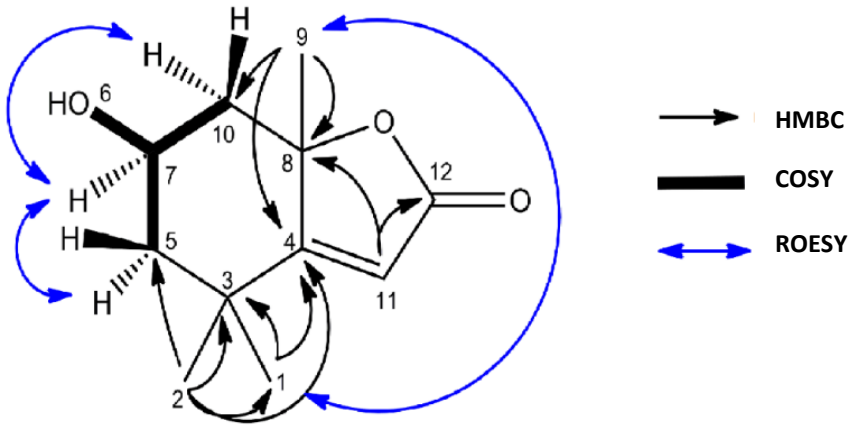

Figure 24: Key HMBC, COSY and ROESY correlations used to determine the structure of compound 2. After NMR analysis, the compound was found to be the known compound loliolide.

4.4 Bioactivity screening

It was only compound 2 that was selected for bioactivity screening. This compound was the only one that was isolated in sufficient amount and purity to determine the structure using NMR. The other compounds were not pure enough to determine the structure, and a very low amount were obtained. These compounds were therefor not selected for bioactivity screening.

4.4.1 MIC assay

The minimum inhibition concentration (MIC) assay is used to screen for the ability of a compound to inhibit growth of bacterial strains. The assay is visually inspected for growth, and the absorbance is measured. The absorbance is measured at 600 nm and the value reflects the bacterial density in each well. The known antibiotic compound gentamycin is used as a negative control (for normal growth of bacteria) and precision between each testing. The cut-off absorbance value for classifying active compounds is <0.05.

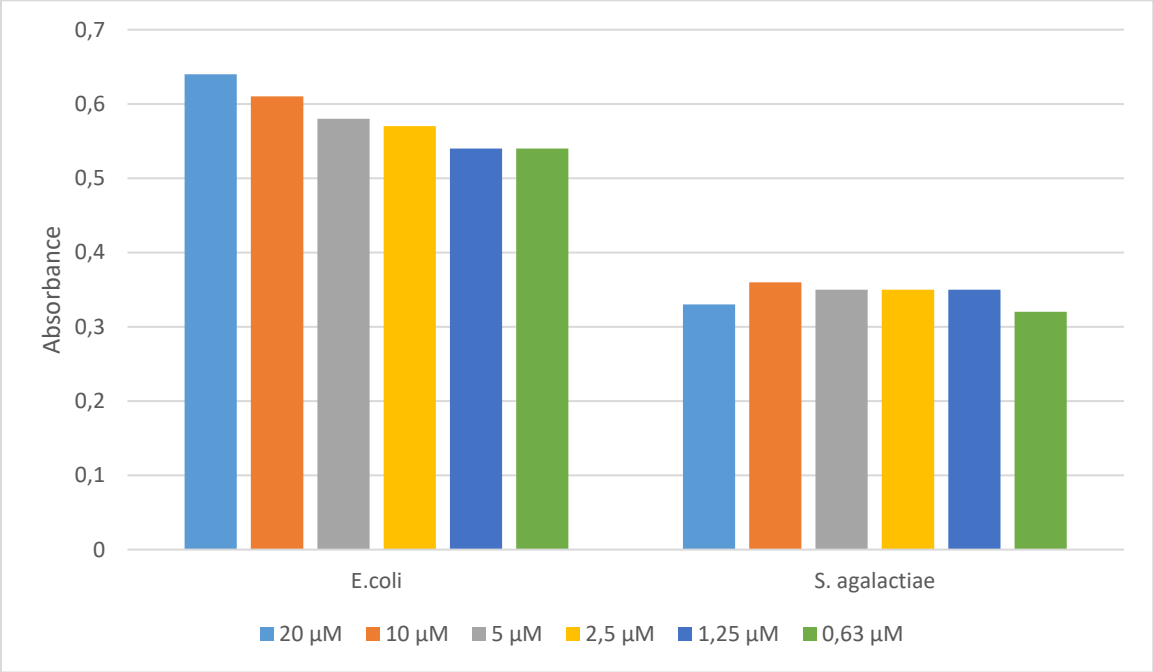

Figure 25: Column chart of the MIC assay run of loliolide with different concentrations. The figure illustrate the absorbance value measured at each concentration of loliolide with the bacteria *E.coli* and *S.agalactiae*.

The column chart in figure 23 illustrate that none of the concentrations of loliolide show any inhibition in the growth of the two tested bacteria. Absorbance at 0.05-0.09 is classified as questionable, and absorbance above 0.09 is classified as inactive. Since all the absorbance

values are over 0.09 for both bacterial strains at all concentrations, loliolide is classified as an inactive compound for inhibition of bacterial growth in these two bacteria.

4.4.2 Biofilm inhibition assay

The biofilm production inhibition assay screens a compounds ability to inhibit biofilm formation of *S.epidermidis*. The microtiter plate is visually inspected for inhibition of biofilm, and absorbance in measured. The absorbance is measured at 600 nm and the value reflects the density in each well. The cut-off absorbance value for classifying active compounds is <0.25.

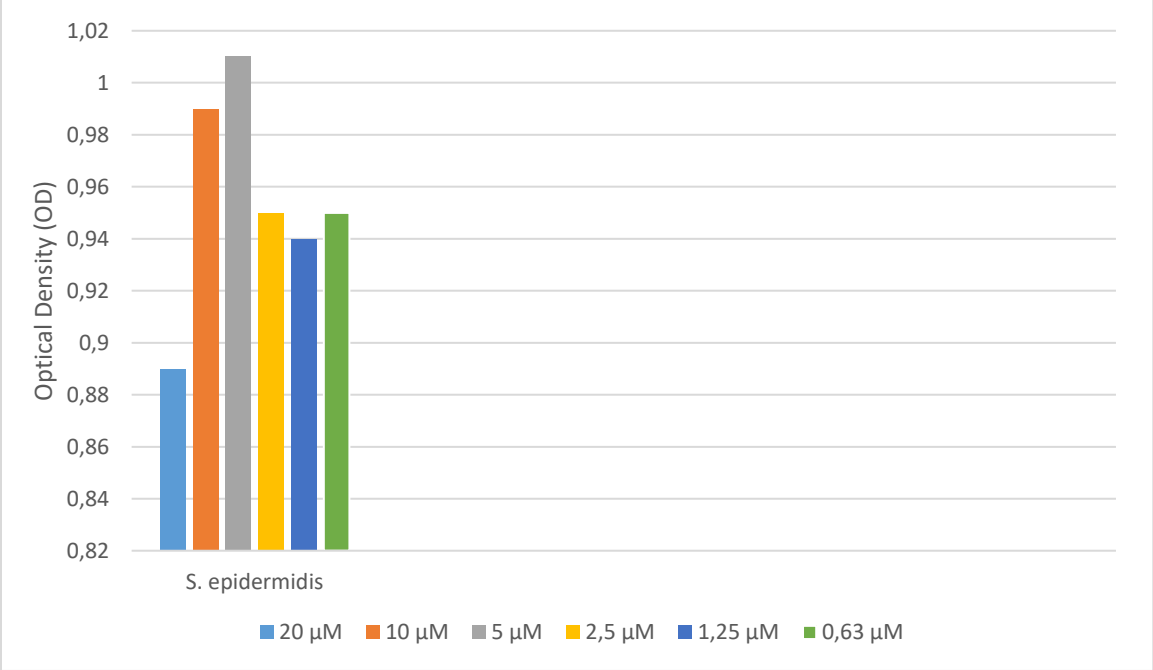

Figure 26: Column chart of the biofilm production inhibition assay for loliolide at different concentrations. The figure illustrate the OD value measured at each concentration of loliolide with the biofilm production bacteria *S.epidermidis*.

The column chart in figure 24 illustrate that none of the concentrations of loliolde show any inhibition of biofilm formation of the test bacteria *S.epidermidis*. Absorbance at 0.25-0.30 is classified as questionable, and absorbance above 0.30 is classified as inactive. Since all the absorbance values are over 0.30, loliolide is classified as an inactive compound for inhibition of biofilm formation.

5 Discussion

5.1 The content of secondary metabolites in the examined hydroids

As a result of the dereplication process, interesting compounds were found in all the different samples. A compound was determined to be interesting based on its lipophilisity, elemental composition and whether it is believed to be a novel compound. The HPLC chromatograms of all the samples, generally looked quite similar, with an intense injection peak in the beginning, followed by an area of less intense peaks, and several intense peaks towards the end of the gradient. It is the compounds eluting in the middle of the chromatogram that is interesting, as the compounds in the beginning is highly water soluble like sugars with low molecular weight, and at the end generally highly lipophilic compounds like phospholipids. Secondary metabolites with commercial potential to become a drug, usually have medium lipophilicity, and are therefore eluted in the middle of the gradient in a reversed phase HPLC system. Secondary metabolites may be present in abundant amount in an extract, this is however most often not the case. As mention earlier, the secondary metabolites are produced by the organism in small amounts because it is often highly potent and potentially toxic for the organism itself in large amounts. In addition, they are not continuously produced, and the secondary metabolite content will vary based on the ever-changing environment of the producer (46, 47). These terms may be possible reasons for the less intense signal peaks in the analysis. The results from the dereplication process therefor confirmed the assumption made when the hydroids were selected to be analyzed, that they are important source of novel secondary metabolites, as part of this thesis. In addition to the compounds nominated for isolation, it was identified several minor sample components that would be interesting to isolate if more sample was available.

5.2 Compound isolation

Dereplication

The crude extract was first analyzed using UHPLC-HR-MS to search for novel compounds for isolation, structure elucidation and screening for bioactivity. The chromatogram and mass spectra were used to nominate compounds for chemistry-guided isolation. All of the

nominated compounds were halogenated. Primary metabolites are generally not brominated, and-, when brominated compounds are found in the extracts, the chances of it being a secondary metabolite is high. The elemental composition calculated during dereplication of a compound is statistically calculated based on the mass spectrum (the accurate mass and isotopic pattern of the compound), and is used as the first step off structure elucidation (48). In figures 5-7 the mass spectra are shown for all of the isolated compounds that were brominated. Halogenated compounds (especially bromine and chloride), have very characteristic isotopic patterns in the mass spectrum and are easily discovered. This isotopic pattern is due to their relative atomic mass and isotopic composition (or natural abundance of stable isotopes).

Scout run

Before isolation, scout run analysis of the extracts were performed by injecting the extract onto four different columns to see how the chromatography of the target compounds were affected by the different column chemistries. The same gradient was used for all the columns. The scout run worked out well, as it was possible to use the results from this analysis when conducting the first, second and third round of isolation. By conducting the scout runs, one could see which column that gave narrow peaks and separation of the target compounds, in addition to impurities that eluted simultaneously. This helped decide which column was best to separate two compounds that eluted simultaneously and to remove impurities, to achieve a pure compound for further structure elucidation using NMR. In order to get a compound pure enough for NMR analysis, several rounds of mass guided preparative HPLC isolation, using columns with different packing material for each run, is most often necessary. Once a compound is isolated from the extract during the first round of isolation, it is concentrated and thus highly valuable. The scout run results enables the selecting of columns for the second and third isolation rounds, without having to inject the sample onto several just to see how the compound elute compared to sample impurities. The amount of target compounds in the extracts is already low, and injection of the extract as part of the scout run analysis most often causes significantly lower sample loss- compared to injection of a semi-pure compound in an attempt to identify a column and a gradient optimal to separate it from impurities co-eluting with the compound in the previous isolation round.

Isolation

From the scout runs, the selection of column for the first round of purifications was based on the chromatography of the target compounds. In addition to the compounds selected for isolation based on the HR-MS analysis, two other compounds selected for isolation from the chosen column. Compounds that elute in the middle of the chromatogram, and appears to be present in abundant amount (high peak intensity) and that seems easy to isolate (the compound elutes in narrow peak and does not co-elute with too many impurities), will always be collected. This opportunistic isolation strategy has previously led to the isolation of several novel products at Marbio, including securidine A (49). Isolating an additional compound is not labor intensive, and it is better to isolate an additional compound that turns out to be uninteresting compared to losing a compound that could be important and have potential for further drug development. When doing chemistry-guided isolation, compounds with potential of being developed into commercially available pharmaceutical may be left out, because the bioactivity screening is not conducted and there is no information of potential bioactive compounds in the extract.

5.3 Sample amount

The organic extracts of M17015 and M17017 were available in low amounts, 1.10 g and 0.33 g, respectively. This is considered to be a low sample weight, as the ideal is to have at least 6 – 10 g of organic extract to be able to conduct bioactivity guided isolation, and around 4 - 8 g to conduct chemistry guided isolation. Chemistry guided isolation requires less sample because the need for 2 g of extract normally used for flash fractionation and bioactivity screening in the Marbio bioactivity screening pipeline is eliminated. As mentioned in the introduction, the supply of sufficient amounts of biomass is a constant problem when working with isolation of compounds from marine invertebrates. Marbio is co-located with Marbank, the Norwegian national biobank. Marbank collect samples and prepares them into organic extracts that can be processed further by the staff at Marbio. Over the years, several samples have been left unprocessed due to low weight of the collected biomass, which would result in low weight of their corresponding organic extracts. This would furthermore entail difficulties with regards to the quantities of purified compounds that can be isolated from the extracts. Marbio has not previously examined the lower limits of biomass required to obtain sufficient

amounts of purified compounds. Because of this, many potential “high value” organisms (like hydroids) have been left unexamined for their content of bioactive secondary metabolites.

The amount of the isolated compounds were very low from these extracts, and the weights of each compound were too low to be measured accurately on an analytical scale, and they were all measured to be approximately 0.2 mg. The amount was too low to get good signals on the NMR, except from compound 2, which turned out to be the known compound loliolide. However, the chemistry guided isolation approach used in this work has given Marbio valuable information for future evaluation of how to handle samples of low mass from the biobank.

The wet weight of the collected samples M17015 and M17017 used for isolation in this thesis were 173.77 g and 63.00 g, respectively, and the total amount of each isolated compound was 0.2 mg. To get sufficient amount of compound to determine the structure on NMR and for bioactivity screening, the ideally amount of pure compound would be minimum 2 mg. To estimate how much wet weight that should be collected to reach the minimum amount of the target compounds, the biomass needed is 10 times higher than already collected. Assuming that the amount of the target compounds was isolated from the M17015 sample only, 1.7 kg biomass must be collected to be able to isolate the target compound in amounts allowing its structure to be elucidated using NMR.

The weight of loliolide accounted for 0.0001 % of the sample wet weight, which is a very low amount. Trabectedin (marketed as Yondelis ®) initially isolated from the Caribbean tunicate *Ecteinascida turbinate*, had a yield of 0.0001 % (50), and Ianthelline isolated from the sponge Stryhnus fortis yielded 0.00453 % (51). The first one represent a worst-case scenario, and the latter one a best-case scenario regarding compound yield from an extract. Trabectedin is currently marketed as antitumor chemotherapy drug for advanced soft-tissue carcinoma. The yield of Loliolide and the other target compounds isolated in this thesis, was approximately the same as Trabectedin. This proves that it is important to also focus on the compounds present in small amounts, even though their isolation and structure elucidation process might not be straight forward. The results from this thesis highlight the importance of sufficient sample collection. As recollection is difficult, these results might motivate heavy sampling in one area if hydrozoans are found, in order to maximize the collected weight of the animal.

Even though it is challenging, it is possible to work further with such compounds.

5.4 Compound 2: loliolide

Compound 2 was the only compound isolated during this project in amounts enabling structure determination by various 1D and 2D-NMR experiments. From the HR-MS analysis shown in figure 4, the most intense ion formed was the protonated ion of compound 2, but other adducts were also visible, like the sodium adduct of the dimer of the compound ($2M+Na^+$) which has *m/z* 415. During prep-HPLC-MS isolation, compound 2 gave a signal at *m/z* 415, this means that the ion formed in prep-HPLC-MS was the sodium adduct of the dimer. The mass range in the prep-HPLC-MS isolation was set to start at *m/z* 200 and end at *m/z* 1000, so the mass of the protonated compound was too low to be detected, and one was fooled into thinking the compound had a higher mass than it actually had. Compound 2 was not selected during dereplication, and the isolated ion with *m/z* 415 was not found in the previously run HR-MS analysis (dereplication). The purified isolated compound was run on another HR-MS instrument to find the elemental composition to help determining the structure by NMR analysis. If the purified compound had been dereplicated at an earlier stage, one might have found that the compound was the previously known loliolide from the databases used for searching after known compounds.

The mass of compound 2 is 196.116, which is significantly lower compared to the other compounds isolated as part of this thesis. This means that the numbers of moles in the sample was higher than for the other compounds that were isolated, even though the weight was approximately the same. The higher number of moles of compound 2 relative to the other isolated compounds and higher purity can be reasons why compound 2 was the only one that was possible to structure elucidate by NMR.

5.5 Microorganisms associated with the collected macroorganisms

Most of the compounds that are isolated from collected samples of marine invertebrates currently on the marked as a drug or in different phases of clinical trials, are produced or predicted to be produced by microorganisms (especially bacteria) living in symbiosis with the invertebrates (52). In many cases, the bacteria are often specific one, or a selected few host, and are dependent of the host to survive. It therefor makes sense that the bacteria are producing secondary metabolites that insures the survival of its invertebrate host. To access these compounds, it is often needed to collect the invertebrate hosts. Most bacteria cannot be cultured in the lab, and recreating the environment in which the bacteria produces the desired secondary metabolites is also challenging (8, 9). Loliolide has been isolated from several sources, both terrestrial and marine (53). These varying sources can indicate that microorganisms produce the compound. It can be that same microorganism lives in symbiosis with both terrestrial and marine organisms. However, it may be more likely that the producer are different bacteria containing the same genetic material enabling them to produce the same compound.

5.6 Bioactivity screening

The Loliolide was screened for bioactivity in two different bioassays, one antibacterial screening (MIC assay) testing on two different bacteria, one gram positive (*S.agalactiae*) and one gram negative (*E.coli*). The other was an anti-biofilm inhibiting activity screening using the biofilm producing bacteria *S.epidermidis*. No activity was found in any of the screenings.
Loliolide was for the first time isolated from the English Ryegrass *Lolium perenne* in 1964, the structure was elucidated and named after the English Rye grass (54). After this discovery, loliolide has been identified in queens of the red ant *Solenopsis invictra* in 1983 and in the brown algae *Sargassum crassifolium* in 1985 (53).

6 Conclusion

Five compounds were isolated from the organic extracts of hydroids using the chemistry-guided isolation approach. After isolation, one compound was pure enough and isolated in sufficient amount to allow structure elucidation using NMR analysis. This compound turned out to be the previously reported compound Loliolide. Loliolide was screened for antibacterial activity and anti-biofilm formation activity, but did not display any bioactivity at the concentration screened in the bioassays.

The results in this thesis demonstrate:

- The potential of hydroids as basis for secondary metabolite isolation and the challenges regarding the supply of sufficient hydroid biomass to allow isolation of the compounds in amounts sufficient for structure elucidation and bioactivity evaluation
- The importance of using dereplication to nominate compounds for isolation from complex samples such as crude extracts, based on compound novelty
- How amount of collected biomass is crucial for the success of chemistry-guided isolation, but also that chemistry-guided isolation requires less biomass compared to bioactivity-guided isolation and thus is a valid approach when isolating compounds from samples where large amount of biomass is difficult to collect, as is the case when collecting hydroids
- That bioprospecting of collected marine hydroids enables discovery of novel halogenated secondary metabolites, as well as previously reported compounds from other species
- Further work: aim to collect the minimum amounts of biomass suggested by the result of this project (1.7 kg) of herein analysed hydroids to enable isolation of target compounds in sufficient amounts for structure elucidation and bioactivity screening

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8 Appendix

A1: Overview of all the samples of the different family, genus and species of Hydroids collected and stored at Marbio. All samples were analyzed using UHPLC-HR-MS to search for novel compounds, but due to the weight of the extracts only the M17015 and M17017 samples were used as part of this project.

Table 19: Overview over the samples of the different family of hydroids examined as part of this thesis.

Collection ID:	**Family:**	**Genus:**	**Species:**	**Quantity (WW):**	**Organic extract:**
M17015	Mix of different families			173.77 g	1.1 g
M17016	*SERTULARIIDAE*	*ABIETINARIA*	*ABIETINA*	142 g	1.0 g
M17017	*LAFOEIDAE*	*GRAMMARIA*	*ABIETINA*	64 g	0.33 g
M17018	*SERTULARIIDAE*	*ABIETINARIA*	*ABIETINA*	55 g	0.28 g
M17019	*SERTULARIIDAE*	*SYMPLECTOSCYPHUS*	*TRICUSPIDATUS*	40 g	0.13 g
M17020	*HALECIIDAE*	*HALECIUM*	*MURICATUM*	55 g	0.29 g
M17021	*SERTULARIIDAE*	*SERTULARIA*	*MIRABILIS*	15.32 g	0.05 g
M17022	*LAFOEIDAE*	*GRAMMARIA*	*ABIETINA*	18.64 g	0.04 g
M17023	*SERTULARIIDAE*	*ABIETINARIA*	*ABIETINA*	21 g	0.09 g
M17024	*SERTULARIIDAE*	*THUIARIA*	*SP I*	18.07 g	0.11 g
M17025	*HALECIIDAE*	*HALECIUM*	*LABROSUM*	21.82 g	0.13 g
M17026	*LAFOEIDAE*	*LAFOEA*	*DUMOSA*	9 g	0.16 g
M17027	*CAMPANULARIIDAE*	*RHIZOCAULUS*	*VERTICILLATUS*	13 g	0.14 g
M17028	*SERTULARIIDAE*	*ABIETINARIA*	*FILICULA*	4 g	0.2×10^{-6} g