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

# Isolation and Characterisation of Secondary Metabolites from Arctic, Marine Invertebrates

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# Abstract

Bioprospecting is the systematic search for and discovery of products in nature, with the purpose of developing commercial products. The marine environment displays a rich biological diversity, as well as a diversity within environmental factors. This environment has necessitated the production of potent secondary metabolites by marine organisms in their arms race against predators and pathogens, in the battle for space and to increase chances of reproduction. The resulting compounds are generally known to have unique chemical features, often unknown from terrestrial sources, as well as interesting biological activities. Due to these factors, they are believed to hold an immense potential as lead compounds in development of commercial products.

The aim of this thesis was to isolate and characterise secondary metabolites from extracts of eight Arctic, marine invertebrates. Prefractionated extracts were screened for anticancer activity, and active fractions were dereplicated to investigate if the bioactive compound(s) was novel or had been previously reported. Three compounds believed to be novel were isolated, structure elucidated and biologically characterised. A novel compound, named BI-L-665.6 in this thesis, was isolated from the organic extract of *Bryozoa indet.* In addition, Ponasterone A (Pon A) and dehydroxy-Pon A were isolated from the organic extract of *Alcyonidium gelatinosum*. Pon A was first isolated from *Podocarpus nakaii* in 1966, but this is the first time that this compound has been isolated from *A.gelatinosum*. Biological characterisation of the isolated compounds detected no anticancer or antibacterial activity at the test concentrations employed in the assays. The results from this thesis show that bioprospecting of collected marine invertebrates enables discovery of secondary metabolites with novel chemistry, as well as previously reported compounds in new species.

# Acknowledgements

# Sample names

The collected marine invertebrates were prepared into two crude extracts: an aqueous and an organic extract. Each extract was fractionated into eight fractions. The sample names in this thesis are based on an abbreviation of the species name, extract type (W: aqueous extract, L: organic extract) and flash fraction number (Table 1). Example: Flash fraction 5 of the organic extract of *A.gelatinosum* is named AG-L-05. In total, 19 fractions were included in the work conducted as part of this thesis.

Isolation of target compounds was conducted on the crude extracts named after their originating species and the extraction method. Compounds were isolated from the organic extract of *B.indet* (BI-L) and the organic extract of *A.gelatinosum* (AG-L). After isolation, the mass-to-charge ratio (*m/z*) of the isolated compounds was added to the extract name. Example: AG-L-449.4 is the name for the isolated compound with *m/z* 449.4 from the organic extract of *A.gelatinosum*.

*Table 1: **Samples were assigned a unique name containing abbreviations indicating species, extraction method and flash fraction number.***

| Organism | Extract | Fraction | Sample name |
|---|---|---|---|
| *Mycale (Mycale) lingua* | W | 5 | ML-W-05 |
| | W | 6 | ML-W-06 |
| | W | 7 | ML-W-07 |
| | L | 4 | ML-L-04 |
| | L | 5 | ML-L-05 |
| | L | 6 | ML-L-06 |
| | L | 7 | ML-L-07 |
| *Bryozoa indet* | W | 4 | BI-W-04 |
| | W | 5 | BI-W-05 |
| | W | 6 | BI-W-06 |
| *Porifera indet* | L | 6 | PI-L-06 |
| | W | 6 | PI-W-06 |
| *Alcyonidium gelatinosum* | W | 4 | AG-W-04 |
| | W | 5 | AG-W-05 |
| | L | 5 | AG-L-05 |
| *Styela rustica* | W | 5 | SR-W-05 |
| *Astarte borealis* | L | 7 | AB-L-07 |
| *Nuculana pernula* | L | 5 | NP-L-05 |
| *Strongylocentrotus droebachiensis* | L | 1 | SD-L-01 |

# Workflow

Overview of workflow and results from the individual steps conducted as part of this thesis.

| | Activity in primary anticancer screening | Activity in secondary anticancer screening | Dereplication | Isolation | HR-NMR | Structure elucidated | Bioactivity profiling |
|---|---|---|---|---|---|---|---|
| ML-W-05 | + | + | Phosphocholine | | | | |
| ML-W-06 | + | + | Phosphocholine | | | | |
| ML-W-07 | + | + | Phosphocholine | | | | |
| ML-L-04 | + | - | | | | | |
| ML-L-05 | + | + | Phosphocholine | | | | |
| ML-L-06 | + | + | Phosphocholine | | | | |
| ML-L-07 | + | + | Phosphocholine | | | | |
| BI-W-04 | + | - | | | | | |
| BI-W-05 | + | + | Suspected novel compounds | Yes: Conducted on organic extract (BI-L) | 3 isolated compounds: BI-L-665.6, BI-L-478.4, BI-L-340.3 | Novel: BI-L-665.6 | No anticancer or antibacterial activity |
| BI-W-06 | + | - | | | | | |
| PI-L-06 | + | + | Ianthelline | | | | |
| PI-W-06 | + | - | | | | | |
| AG-W-04 | + | - | | | | | |
| AG-W-05 | + | - | | | | | |
| AG-L-05 | + | + | Suspected novel compounds | Yes: Conducted on organic extract (AG-L) | 3 isolated compounds: AG-L-465.3, AG-L-449.4, AG-L-541.4 | Known: Ponasterone A, Known: Dehydroxy-Ponasterone A | No anticancer or antibacterial activity |
| SR-W-05 | + | - | | | | | |
| AB-L-07 | + | - | | | | | |
| NP-L-05 | + | - | | | | | |
| SD-L-01 | + | - | | | | | |

# Abbreviations

| | | | |
|---|---|---|---|
| 1D | One-dimensional | $IC_{50}$ | Half maximal inhibitory concentration |
| 2D | Two-dimensional | LB | Luria-Bertoni |
| ACN | Acetonitrile | LC | Liquid chromatography |
| AFM | Atomic force microscopy | m/z | Mass-to-charge |
| BPI | Base peak intensity | MALDI | Matrix-assisted laser desorption/ionization |
| Br | Bromine | MeOH | Methanol |
| cf | Confer | MHz | Megahertz |
| CFU | Colony forming unit | MIC | Minimum inhibitory concentration |
| Cl | Chlorine | Min | Minutes |
| DCM | Dichloromethane | MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| D-MEM | Dulbecco's modified media | Mw | Molecular weight |
| DMSO | Dimethyl sulfoxide | NMR | Nuclear magnetic resonance |
| EI | Electron ionization | NP | Natural product |
| E-MEM | Earle's minimal essential medium | OH | Hydroxyl |
| ESI | Electrospray ionization | Pon A | Ponasterone A |
| FA | Formic acid | Prep | Preparative |
| FBS | Fetal bovine serum | RP | Reverse phase |
| FDA | US Food and Drug Administration | RPMI | Roswell park memorial institute medium |
| HPLC | High performance liquid chromatography | UPLC | Ultra performance liquid chromatography |
| HR-MS | High resolution mass spectrometry | UV | Ultraviolet |

# Table of Contents

# 1 Introduction

## 1.1 Bioprospecting

Humans have always relied on natural resources to survive. This is true both in regard to basic needs such as food, shelter and clothes, but also in regard to medicine. The latter point can be exemplified by the traditional utilisation of terrestrial plants, either as a whole or parts of it, processed or raw, as traditional medicine against various conditions in many cultures. Natural resources have formed the basis for traditional medicine that is still being used around the world today (Newman, Cragg, & Snader, 2000). Bioprospecting is the systematic search for and discovery of natural products (NPs) with the purpose of developing commercial products (Ashforth et al., 2010; Mateo, Nader, & Tamayo, 2001). These products can fall into three categories: chemicals, genes or designs. The NPs can be utilised as agrochemicals, as lead compounds in drug development, in cosmetics, recombinant pharmaceutical proteins, enzymes and in mechanical engineering (Mateo et al., 2001).

### 1.1.1 Natural products

NPs refers to compounds produced by a living organism . Some NPs are commonly encountered in all organisms and the organism needs to be able to transform and interconvert these NPs in order to live, grow and reproduce. These NPs, called primary metabolites, are vitally important for the survival of the organism. (Dewick, 2009, p. 7-38). In contrast, there exist compounds that are distributed in a much more limited fashion in nature. These compounds are called secondary metabolites, but the term NPs is often used when referring to these compounds (and not primary metabolites). In this thesis, NPs and secondary metabolites will be used interchangeably. These compounds are not necessarily produced under all conditions since they are not necessary for the immediate survival, growth, development or reproduction of the producing organism. It is believed that the secondary metabolites affect the interaction of the organism with its surrounding environment, and that their mode of action can influence long-term survival (Agostini-Costa, Vieira, Bizzo, Silveira, & Gimenes, 2012). They may enable the organism to survive interspecies competition (Engel & Pawlik, 2000; Luter & Duckworth, 2010), they facilitate reproductive processes (coloring attractants) (Rinehart, 1992) or they can provide defensive mechanisms (toxic materials) against pathogens and predators (Cowan, 1999). Plants, bacteria, fungi and marine invertebrates are well known sources of secondary metabolites. In addition to their beneficial effect for the producing organism, the secondary metabolites have proven to be useful for a wide range of other applications. This includes applications like cosmeceuticals, insecticides, nutraceuticals and pharmaceuticals (Vaishnav &

Demain, 2010). In this thesis, the focus will be on finding secondary metabolites that have anticancer activities and potential to be developed into commercially available anticancer pharmaceuticals.

The utilisation of NPs within the field of drug discovery has been a remarkable success (Newman & Cragg, 2016). An overview of all approved drugs from 1981 to 2014 can be seen in Figure 1. The extensive data sets reviewed by Newman and Cragg highlight the key role that NPs, and structures derived from or related to NPs, have played in drug discovery in this time period. Of the drugs approved between 1981 and 2014, only 27% are synthetic drugs while 67% are derived from- or inspired by NPs. This review also highlights the important role NPs have played in anticancer drugs approved in this time period (84% of approved anticancer drugs are NP derived or inspired) (Newman & Cragg, 2016).
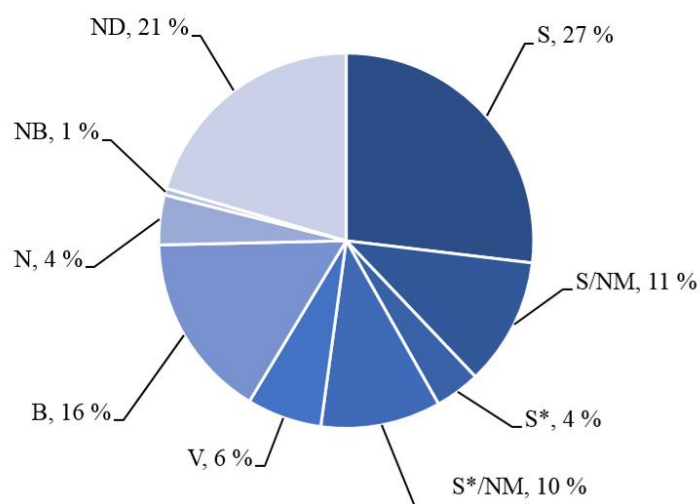


*Figure 1: **All new approved drugs from 1981 to 2014, n=1562.** B: Biological macromolecule, N: Unaltered NP, NB: Botanical drug (defined mixture), ND: NP derivative, S: synthetic drug, S*: Synthetic drug (NP pharmacophore), /NM: Mimic of NP and V: Vaccine. Figure made with inspiration from reference (Newman & Cragg, 2016).*

Despite this, many pharmaceutical companies have ceased their NPs research (David, Wolfender, & Dias, 2015; Vederas, 2009). These companies are under a lot of pressure to identify a lead compound quickly and profitably. There are certain aspects of NPs that makes this challenging, such as a slow identification process, low supply of the compound and their complex chemical structure complicating their synthesis. These companies have instead preferred screening of synthetic compounds (Vederas, 2009). However, recent advances in technology have affected this trend and now there is a re-emergence of NPs in drug discovery (Harvey, Edrada-Ebel, & Quinn, 2015).

The enormous chemical diversity present in nature suggests that NPs possess several chemical properties that make them superior as lead compound compared to synthetic compounds (Feher & Schmidt, 2003). NPs have a higher molecular weight (Mw), a higher number of ring systems, chiral centers, heavy atoms, hydrogen-bond donors and –acceptors, lower number of rotatable bonds and they are less lipophilic and more unsaturated (Clardy & Walshm, 2004; Feher & Schmidt, 2003; Muigg, Rosén, Bohlin, & Backlund, 2013). These properties make NPs more structurally diverse and more rigid than synthetic compounds (Feher & Schmidt, 2003). The NPs have evolved over time to interact with enzymes, receptors and ionic channels in plants or animals (David et al., 2015). These compounds have a greater chance of interacting with targets in the human body as well, and NPs display a much greater range of bioactivity in a larger number of targets than synthetic compounds (Battershill, Jaspars, Long, & Battershill, 2005). These characteristics make it clear that it is still important to involve NPs in drug discovery despite the time-consuming process it is to identify and develop a lead compound into a marketable drug.

### 1.1.2   The marine environment

The ocean covers 70% of earth`s surface and deep-sea environments comprise 90% of the global biosphere by volume (Snelgrove, 2016). According to Margulis and Chapman, out of the 33 known animal phyla, 32 are found in the ocean and 15 of these are exclusively marine (Margulis & Chapman, 2009). The marine environment displays a biological diversity as well as a diversity in environmental factors. These diverse conditions have affected the production of secondary metabolites, resulting in structurally novel and biologically active secondary metabolites that are unknown from terrestrial sources (de Carvalho & Fernandes, 2010). There is an abundance of bromine (Br) and chlorine (Cl) ions in seawater. This affects the secondary metabolites, and gives a higher likelihood of marine secondary metabolites being halogenated (especially brominated), a chemical feature that is uncommon in terrestrial NPs (A. Butler & Carter-franklin, 2004; Teeyapant & Proksch, 1993).

Traditionally, bioprospecting has been focused on terrestrial sources such as plants. This is mainly due to the availability of the terrestrial organisms, as well as the tradition for using them in medicine. In the 1950s, spongothymidine and spongouridine from the marine sponge *Tethya crypta* (now known as *Tectitethya crypta*) were discovered (Bergmann & Feeney, 1950, 1951). This marked the beginning of the investigation of NPs from the marine environment. In the beginning, the compounds were mainly isolated from easily accessible organisms like macroalgae. Improvements in scuba and submersible collection technologies made the physical

access to greater depths of the ocean possible. In addition, deep-water collections were made possible through dredging, trawling and remotely operated vehicles (Cragg & Newman, 2013). This resulted in an increase in the number of novel marine NPs reported every year (1340 new compounds were reported in 2015 (Blunt, Copp, Keyzers, Munro, & Prinsep, 2017)). The majority of marine NPs have been isolated from tropical and temperate waters (Leal, Madeira, Brandao, Puga, & Calado, 2012). Research now also focuses on organisms in colder climates, such as the Antarctic and Arctic. This has previously been a more unexplored habitat, but is now proving to provide valuable NPs (Blunt, Copp, Keyzers, Munro, & Prinsep, 2014).

### 1.1.3 Marine natural products

As of April 2016, there are seven FDA (U. S. Food and Drug Administration) drug approved marine compounds (Figure 2), four compounds in phase III, six compounds in phase II, two compounds in phase I/II and 12 compounds in phase I of clinical trials (Mayer, 2016). The first approved marine drug was the anticancer compound cytarabine (Cytosar-U®), isolated from the sponge *Cryptotheca crypta*, which was approved in 1969. Since then, six drugs have been FDA approved: Vidarabine (Vira-A®, no longer in use) in 1976, ziconotide (Prialt®) and omega-3-acid ethyl esters (Lovaza®) in 2004, eribulin mesylate (Halaven®) in 2010, brentuximab vedotin (Adcetris®) in 2011 and Trabectedin (Yondelis®) in 2015 (David et al., 2015; Martins, Vieira, Gaspar, & Santos, 2014; Mayer, 2016).
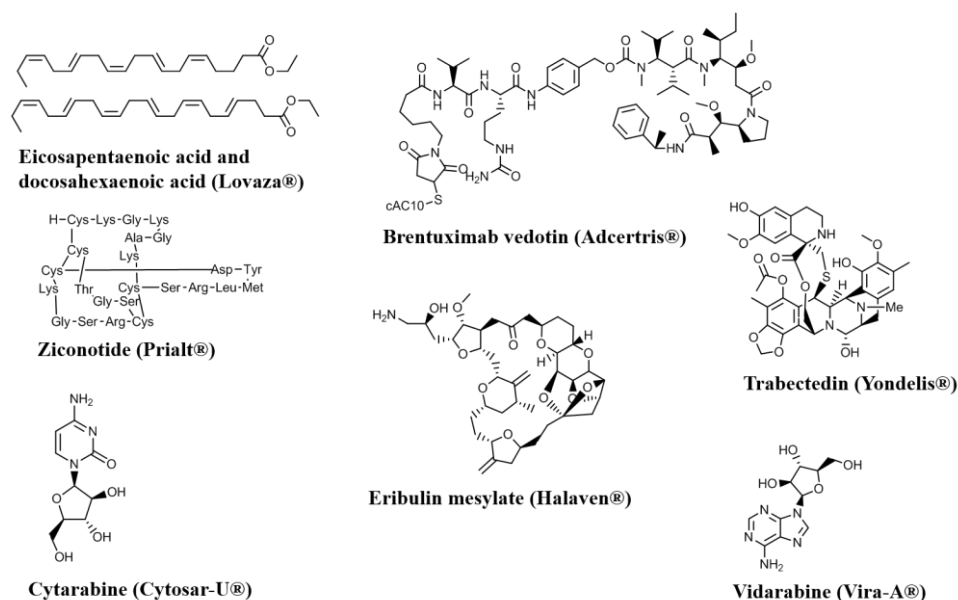


*Figure 2: **The chemical structures of the seven approved marine derived drugs as of April 2016 (Vira-A®, no longer in use).** Adcetris® is covalently attached to a monoclonal antibody (Younes, Yasothan, & Kirkpatrick, 2012). Lovaza®, Cytosar-U®, Adcertris®, Halaven® and Vira-A® have chemical structures optimised by synthesis. They are synthetic- (analogue produced by chemical synthesis) or semisynthetic (using a NP or a natural precursor as starting material) derivatives of the secondary metabolites (Gerwick & Moore, 2012). Figure made with inspiration from reference (Hanssen, 2014).*

## 1.2 Marine invertebrates

Marine invertebrates do not possess a bony or cartilaginous skeleton (Kozloff, 1990, p. 1). Many marine invertebrates are sessile and soft bodied. These organisms are unable to escape from predators and are in addition to spikes or physical structures, relying on a chemical defence (NPs) to deter predators and pathogens, keep competitors away or to paralyze prey (Leal et al., 2012). These NPs have been shown to exhibit bioactivities such as anticancer, antidiabetes and antiinflammatory (reviewed by (Senthilkumar & Kim, 2013). In this thesis, marine invertebrates from the phyla Porifera, Bryozoa, Chordata, Mollusca and Echinodermata were studied (Figure 3).
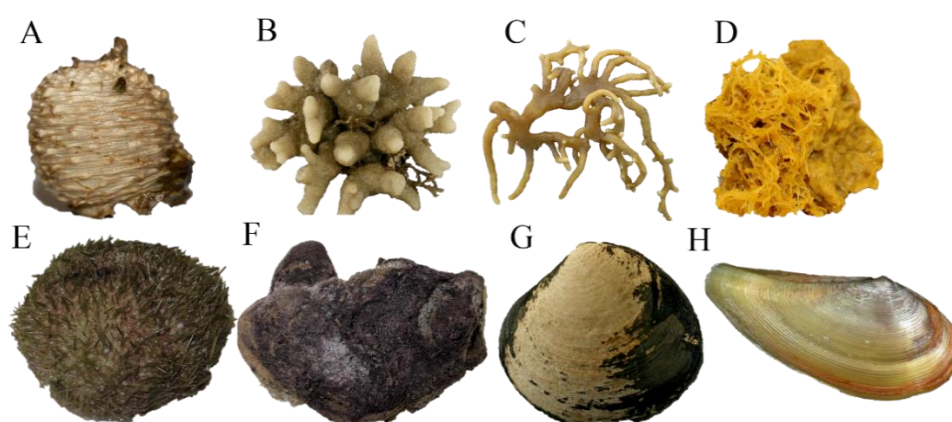


*Figure 3: **Eight marine invertebrates were studied in this thesis: S.rustica (A), B.indet (B), A.gelatinosum (C), M.lingua (D), S.droebachiensis (E), P.indet (F), A.borealis (G) and N.pernula (H).** Photo: Robert Johansen, Marbank.*

Many marine invertebrates live in symbiosis with microorganisms. These microorganisms are in many cases believed to be the true source of the bioactive secondary metabolites that previously were thought to be produced by the invertebrate (Webster & Taylor, 2012). As an example, Dolastatin 10 was first isolated from the mollusc *Dolabella auricularia* (Pettit et al., 1987), but it was later revealed that the compound was produced by a marine cyanobacterium and accumulated by *D.auricularia* through its diet (Harrigan et al., 1998). Figure 4 shows the collected source (A) and the predicted biosynthetic source (B) of marine derived or inspired drugs and clinical trial agents (Gerwick & Moore, 2012). These pie charts also illustrate the importance of marine invertebrates as the collected source for finding new chemistry with a potential for use as commercial products. Many microorganisms are host specific and they have been proven difficult to grow in culture (Hansen & Andersen, 2016; Taylor, Radax, Steger, & Wagner, 2007). The microorganisms often constitute a large part of the collected sample weight (Taylor et al., 2007) and the collected macroorganism biomass can be enough to enable

secondary metabolites isolation. Collection of marine invertebrates, as conducted for this thesis, is therefore still a feasible approach to obtain bioactive secondary metabolites, as is exemplified by the isolation of ianthelline from the Arctic, marine sponge *Stryphnus fortis* (Hanssen et al., 2014).

A

Soft corals, 5 %
Bryozoan, 5 %
Tunicates, 15 %
Mollusks, 35 %
Sponge, 20 %
Worms, 5 %
Bacteria, 5 %
Fungi, 5 %
Fish, 5 %

B

Bacteria, 60 %
Cyanobacteria, 20 %
Fungi, 5 %
Microalgae, 5 %
Macroorganisms, 10 %
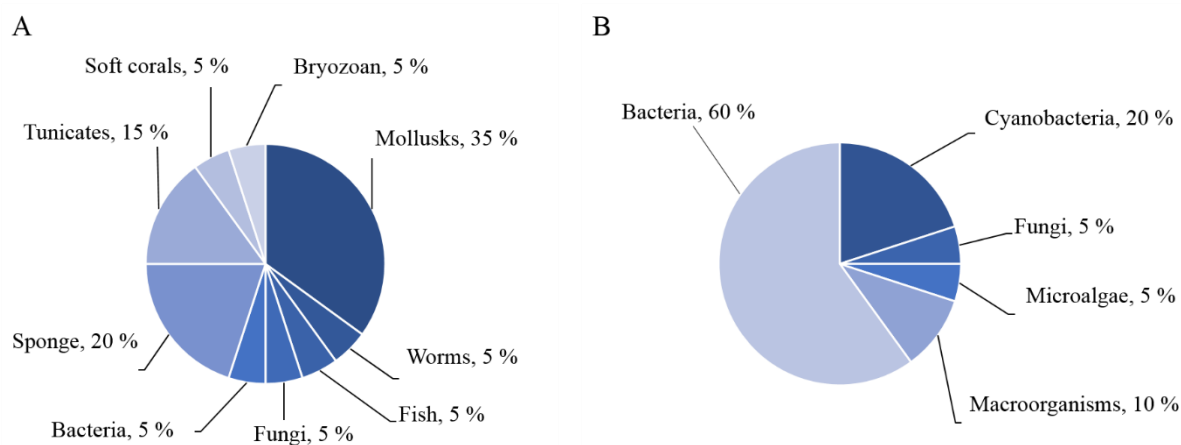
*Figure 4: **Pie charts illustrating the collected source (A) and the predicted biosynthetic source (B) of marine derived or inspired drugs and clinical trial agents.** The collected source has often been shown to or is strongly suspected of harbouring or feeding upon microorganisms that are the actual producer of the bioactive compound. Figure made with inspiration from reference (Gerwick & Moore, 2012).*

### 1.2.1 Phylum Porifera

The phylum Porifera consists of multicellular organisms more commonly known as sponges. The majority of sponges are marine, sessile organisms. Their bodies are organized around pores and chambers where water flows continually due to the beating of a flagella called choanocytes. This water current brings in oxygen and food, and takes away carbon dioxide and wastes (Kozloff, 1990, p. 73-80). Sponges have microorganisms on their body surfaces and deep inside their body. Both the sponge and the microorganisms associated with them can produce a wide variety of bioactive molecules (Webster & Taylor, 2012). Previously, the research focused on sponges from tropical and temperate waters. More recently, sponges from colder waters of the Antarctic and the Arctic have also been investigated (Abbas et al., 2011). In this thesis, *Mycale (Mycae) lingua* and *Porifera indet* (species not determined) were investigated from the phylum Porifera.

### 1.2.2 Phylum Bryozoa

Bryozoa is a phylum of colonial, aquatic animals. The colonies are built up by asexual reproduction, where a single individual gives rise to a new colony by budding. The colonies can form membrane-like crusts or bush-like colonies on substrates such as kelp, crustaceans, stones and rock surfaces (Moen, Svensen, Cochrane, & Pleijel, 2004, p. 393-394). Bryozoans

are attached to the substrate and the feeding in bryozoans therefore depends on ciliary activity. This ciliary activity creates a current of water that moves food particles toward the mouth. These sessile organisms are important fouling organisms on ship bottoms, floating docks and buoys (Kozloff, 1990, p. 480-482). Bryozoans have been a source for novel and/or biologically active compounds such as the bryostatins (Hornung, Pearson, Beckwith, Longo, & Hornung, 1992). In this thesis, *A.gelatinosum* and *B.indet* (species not determined) were investigated from the phylum Bryozoa.

### 1.2.3   Phylum Chordata

Chordates are bilaterally symmetrical with an internal notochord (a skeletal rod) present at some life stage. Organisms in this phylum are very adaptable and can occupy most kinds of habitats. This phylum comprises the subphylums Cephalochordata, Urochordata and Vertebrata. All chordates are deuterostomes, meaning that the anus forms before the mouth during the embryo development stage. A chordate takes in food through the mouth and has a digestive system with stomach and intestines (Hickman, 2011, p. 500-501). Bioactive secondary metabolites have been investigated in this phylum, antibacterial activity has for example been detected in a Defensin compound produced in *Branchiostoma japonicum* (Teng, Gao, & Zhang, 2012). In this thesis, *Styela rustica* was investigated from the phylum Chordata.

### 1.2.4   Phylum Mollusca

The phylum Mollusca consists of clams, snails, octopuses and their relatives. Even though the inner structure and physiology of these organisms are relatively similar, there is a great diversity when it comes to exterior body forms in this phylum. The phylum contains organisms ranging from small snails to 20 m long squids (Moen et al., 2004, p. 282). Molluscs are found in marine, freshwater and terrestrial habitats. The organisms are mostly free-living, and only occasionally parasitic. They can be burrowers, bottom feeders or pelagic, and they therefore represent a variety of lifestyles (Hickman, 2011, p. 334-336). Some secondary metabolites from molluscs have been investigated. From oysters, various bioactive peptides have been discovered with antioxidant and anticancer activities (Umayaparvathi et al., 2014). In this thesis, *Astarte borealis* and *Nuculana pernula* were investigated from the phylum Mollusca.

### 1.2.5   Phylum Echinodermata

This phylum contains sea stars, sea urchins and their relatives. All Echinoderms have a calcareous endoskeleton either as plates or as scattered tiny ossicles. Echinoderms have no freshwater or terrestrial representatives and the organisms are found at every ocean depth. Apart from a few pelagic species, almost all the organisms in this phylum are bottom dwellers

(Hickman, 2011, p. 475). The main secondary metabolites produced in this phylum are saponins (glycosides). Triterpene glycosides have been isolated from sea cucumbers. Some of these glycosides have a cytotoxic activity towards human tumour cell lines (Zou et al., 2003), viricidal activity (Maier et al., 2001) or antifungal activity (Murray, Muniaín, Seldes, & Maier, 2001). In this thesis, *Strongylocentrotus droebachiensis* was investigated from the phylum Echinodermata.

## 1.3 The Bioprospecting pipeline

An outline of the workflow in the bioprospecting pipeline conducted at Marbio, and in this thesis, is shown in Figure 5. Marbio is an analytical platform for screening, isolation and identification of bioactive NPs (Svenson, 2013). The bioprospecting pipeline at Marbio starts with bioactivity screening of prefractionated crude extracts (provided by Marbank), and active fractions from this initial screening are submitted for dereplication by high resolution-mass spectrometry (HR-MS). If the fraction contains a suspected novel compound, or a previously reported compound but with a novel bioactivity, the compound will be isolated using preparative (prep) high performance liquid chromatography (HPLC)-MS. After the isolation, the purity of the compound is examined. If the compound is pure, structure elucidation using HR-MS and nuclear magnetic resonance (NMR) spectroscopy is conducted. At the end of this pipeline, bioactivity screening is again employed to make a bioactivity profile of the isolated compound. The different steps in this isolation approach are discussed in greater detail in the subsequent paragraphs.
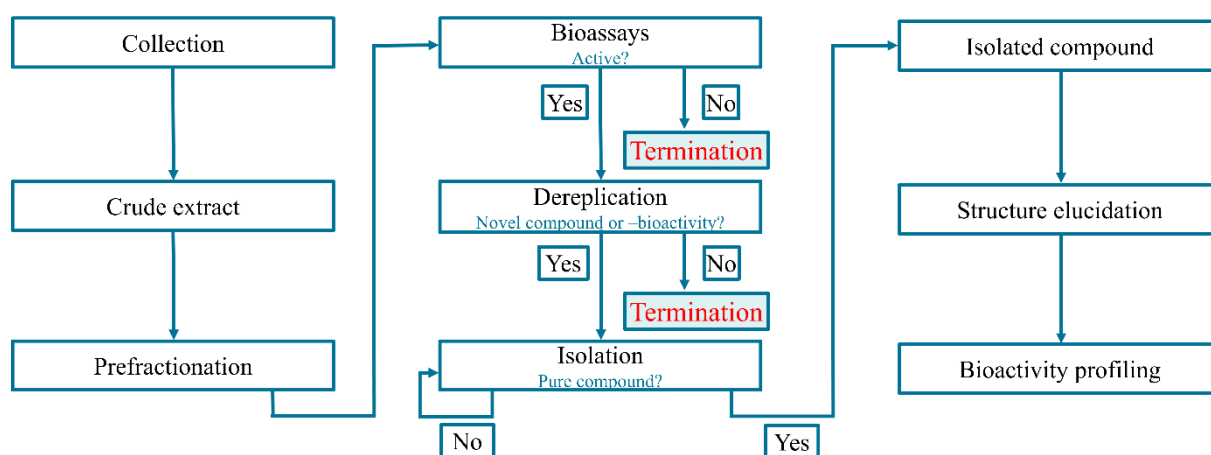


*Figure 5: **Overview of the bioprospecting pipeline employed at Marbio and in this thesis.** Figure made with inspiration from references (Hanssen, 2014; Svenson, 2013).*

### 1.3.1 Collection, extraction and prefractionation

Marbank has the responsibility for collection and preservation of marine organisms for scientific research. The material is collected during several annual research cruises in the Arctic- and sub-Arctic waters of Norway. The collected organism (invertebrate) is lyophilized and extracted to yield an aqueous and an organic (Dichloromethane (DCM): Methanol (MeOH)) extract (Svenson, 2013). These crude extracts contain a complex mixture of compounds, and prefractionation of the extracts prior to bioactivity screening is advantageous because it has been shown to increase the chance of detecting bioactive compounds in bioassays (M. S. Butler, 2004).

The prefractionation can be conducted by using an array of different techniques, but a commonly employed technique is liquid chromatography (LC), such as HPLC or flash chromatography. At Marbio, a flash chromatographic method resulting in eight fractions with known sample weight is used for prefractionation. The advantage with flash chromatography is the high loading capacity in addition to the relatively easy process of creating finished fractions that can readily be weighed. In this LC technique, the mobile phase is pumped through the stationary phase in a tightly closed glass column or in a prepacked cartridge (Bucar, Wube, & Schmid, 2013). This results in a prefractionation of the applied sample and the collected fractions are analysed using bioassays to detect the presence of bioactive compounds in the fractions.

### 1.3.2 Bioassay

A bioassay is an *in vitro* or *in vivo* system used to detect the presence of a biologically active compound in a sample (Fenner & Gerwick, 2014). Two main bioassay strategies exist: target-based screening and phenotypic screening. The target-based screening measures the compounds ability to affect a defined target. These targets can be enzymes, cellular proteins, receptors, DNA or ion channels. This type of screening does not take into consideration the compound`s ability to cross the cell membrane or the compound`s stability to cellular enzymes. Therefore, an effect in a target-based screening (*in vitro*) does not necessarily mean that the compound has an effect *in vivo* (Fenner & Gerwick, 2014).

Phenotypic screening employs whole cell, animal or organ assays. This type of screening measures the ability of a compound or a mixture of compounds to produce an effect in the cell/organism. This could for example be death of a specific cell type or inhibition of cell growth. This type of screening has been successful in discovering new therapeutics and new drug classes (Swinney & Anthony, 2011). The screening does not require any prior knowledge

about the mode of action of the target compound as it evaluates the compound`s effect on the entire system, not on a single target. It also allows the target compound to be screened against several drug targets simultaneously. The disadvantage in this type of screening is that the mode of action is not determined (Sams-Dodd, 2005).

In NP drug discovery, a combination of the two types of bioassays are often used. An example is the use of phenotypic screening in the initial stage in drug discovery, and then the use of target-based screening as follow-up screens to possibly shed light on the mode of action for the isolated compound. This enables a greater detection rate in the beginning of the bioprospecting pipeline because the extract (or prefractionated extract) is screened against several drug targets simultaneously (Swinney & Anthony, 2011). In addition, NPs can have activities with new modes of actions against unvalidated targets or targets for which no target-based assays exist, and then will not be discovered through target-based screenings. Fractions that give a positive result in the initial bioassay screening are submitted for dereplication using LC-MS.

### 1.3.3  Dereplication

Dereplication is a crucial step in NP drug discovery. This step is conducted prior to isolation and aims to identify known compounds in bioactive extracts or fractions to avoid replication of previously conducted work. When dereplication is conducted as part of the bioprospecting pipeline, the probability of rediscovery and reisolation of a previously well characterised compound is lower. Ideally, known compounds whose bioactivity have been examined previously are removed from consideration before the isolation process begins, and limited resources are therefore used more efficiently (Blunt & Munro, 2014).

A widely-used approach to dereplication is using LC-HR-MS, followed by database searches. HR-MS gives the Mw and isotopic patterns of compounds (see section 1.3.5.1 "Mass spectrometry"). The isotopic patterns and the exact mass can be used to calculate the elemental composition for compounds in the sample. Finally, the elemental composition, bioactivity profile and taxonomic information of the compound can be used to search against external or internal databases for potential matches with known compounds (Lindequist, 2016). Examples of databases that can be used for this type of search is MARINLIT, Dictionary of Natural Products, Chemspider and SciFinder. Even though dereplication lowers the probability of rediscovery and reisolation, this process is merely a calculation based on the elemental data that are available. This means that the calculated elemental composition can be incorrect and therefore, there is a chance of rediscovery and reisolation. However, the probability of this is still lower when dereplication is employed prior to isolation (Blunt & Munro, 2014).

### 1.3.4 Isolation

Compounds need to be isolated to allow for structure elucidation as well as bioactivity screening of the isolated compounds. Several chromatographic techniques are available for further fractionation and purification of NPs (Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011), and prep HPLC is commonly used for NP isolation. This technique is versatile and robust, and it provides the researcher with a high resolving power that is necessary for purifying NPs mixtures (Seger, Sturm, & Stuppner, 2013). The results from dereplication are used to decide which compound(s) to isolate. In addition, the sample is investigated for the presence of other compounds that can be included in the isolation. Even though these compounds might not have displayed bioactivity in the initial bioactivity screening, there is a chance that these compounds will display other bioactivities than what the sample was initially screened against. The researcher is in this sense an opportunist and will include compounds that are easily isolated from other impurities and seems to be present in the sample in a fairly large amount.

#### 1.3.4.1 Prep HPLC-MS

At Marbio, prep HPLC-MS is used for purification of target compounds (Figure 6). In this system, the sample is injected onto the HPLC column and compounds are separated based on their affinity for the column packing material and the mobile phase (Neue, 1997, p. 115). After separation in the column, a flow splitter splits the mobile phase to the fraction collector and the ultraviolet (UV) detector and MS detector. Only a small part of the sample (about 1%) is analysed in the UV detector and MS. The majority of the sample (about 99%) is collected in fractions. The fractions from several individual injections of the sample can be pooled and dried, and used in another round of HPLC separation, if the previous HPLC separation round was not sufficient to get a pure compound. A computer controls the entire system, and receives and processes the data coming from the HPLC and MS.
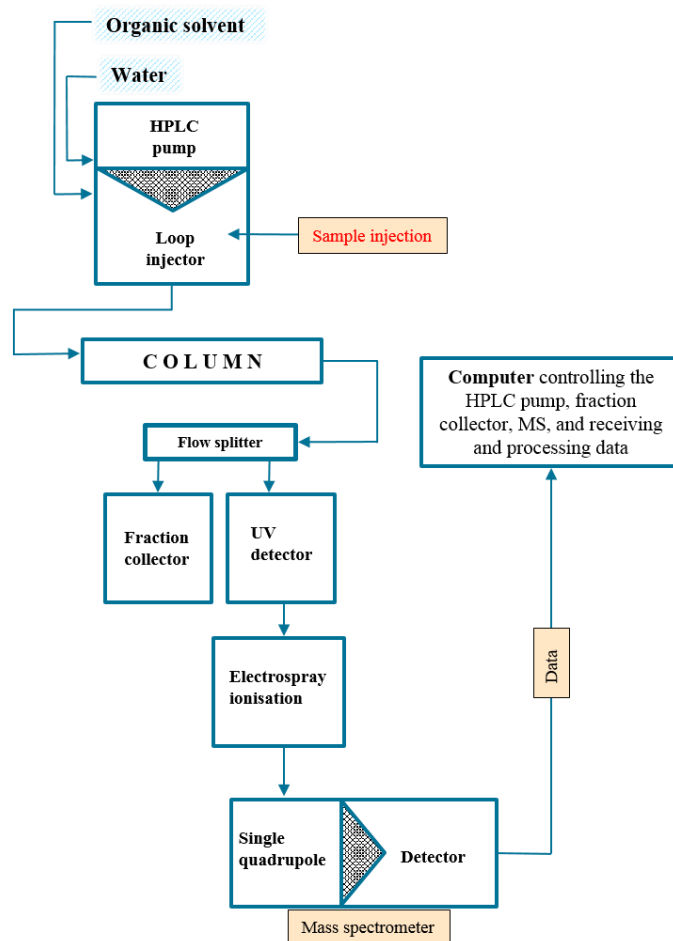
*Figure 6: **Schematic overview of the prep HPLC-MS (prep HPLC-UV-ESI-single quadrupole MS system) used for compound isolation in this thesis.** Figure made with inspiration from reference (Hanssen, 2014).*

Reverse phase (RP) prep HPLC is a popular method used in NPs isolation. In RP prep HPLC, a non-polar stationary phase and a polar mobile phase are employed to isolate NPs (Neue, 1997, p. 4). The columns are usually silica-based with additional groups coupled to the silica. It is the surface modifications of this packing material that determines the interactions that occur between the target compound(s) and the stationary phase inside the column. The solvents used to elute the compounds in RP prep HPLC is often a mixture of water and organic solvents such as acetonitrile (ACN) or MeOH. The water is used as the weak solvent and the strong organic solvent (for example ACN) is used to elute the target compound(s) from the column (Bucar et al., 2013; Latif & Sarker, 2012).

During the isolation process, several different HPLC columns and different elution gradients are generally necessary to isolate the target compound(s). One essential part of the isolation process is to establish the isolation strategy that will be used for separating the target compound(s) from the rest of the sample matrix. The strategy is often established by conducting scouting runs on a small amount of the sample with different columns to determine which

columns gives the best separation of the target compound(s) from other sample constituents. These scouting runs are the basis for the isolation strategy and determines which columns to use in the different rounds of HPLC separation in order to eliminate impurities from the target compound(s) (Latif & Sarker, 2012). The researcher can in general use a gradient of mobile phases or isocratic mobile phase conditions to isolate the target compound(s). In isocratic conditions, the solvent mixture is kept constant throughout the isolation. If the target compound(s) elutes over a broader concentration range of the mobile phase however, the isocratic conditions will not be suitable for isolation. In this case, the researcher often uses a truncated version of the initial gradient used during column investigation (scouting run). The starting conditions will be the solvent system used in the scouting run and the end conditions will be the concentration of mobile phase required to elute the last desired peak from the column. When the solvent system is decided, the injection volume can be increased until the loading and separation limits have been reached (Neue, 1997, p. 310-315).

The desired compounds will be collected in fractions. The fraction collector of the prep HPLC-MS can be programmed to collect by time or by mass triggering. Collection by mass triggering uses the MS data to trigger the collection of compounds eluting from the HPLC column. This method combines the chromatographic separation of the HPLC column with real time MS data, making it a powerful tool in NP isolation. When the system is set to collect by elution time, the same time interval is collected for all the sample injections. While collection by time triggered fractionation can be affected by drifts in the retention time between different injections, mass triggering fractionation will not be affected by this as this method is set to collect predefined masses (Latif & Sarker, 2012).

### 1.3.5 Structure elucidation

Several different techniques exist for use in structure elucidation, such as NMR, HR-MS, UV–visible spectroscopy, infrared absorption spectroscopy and atomic force microscopy (AFM) (Hanssen, Schuler, et al., 2012; Seger et al., 2013). Due to the chemical complexity of NPs, a combination of different techniques is often necessary to elucidate the structure of the isolated compound(s) (Hoffman, 2004, p. 332-394).

#### *1.3.5.1 Mass spectrometry*

MS determines the mass of a molecule and this is achieved by measuring the molecule`s *m/z* ratio. A MS consists of four components: a sample inlet, an ionization source, a mass analyser and an ion detector (Figure 7). The sample inlet introduces sample molecules to the instrument where they are converted to ions in the ionization source. Different ionization sources exist,

such as electrospray ionization (ESI), electron ionization (EI) and matrix-assisted laser desorption/ionization (MALDI). ESI is the ionization source used in the HR-MS systems found at Marbio. This ionization source creates a fine spray of highly charged droplets (dispersed into a fine spray from a metal nozzle) in the presence of an electrical field. Dry gas and heat are applied to the droplets to evaporate the solvent. ESI involves a continuous introduction of solution and it is suitable as an interface with for example HPLC. After the ions are produced, they are electrostatically pushed into the mass analyser where they are separated according to their *m/z*. Finally, the detector converts the ion energy into electrical signals that are transmitted to a computer and a mass spectrum is produced (Bouslimani, Sanchez, Garg, & Dorrestein, 2014; Siuzdak, 2003, p. 5-15). The mass spectrum is a plot of the relative abundance of the ions as a function of the *m/z* ratio. This spectrum gives information about the mass, as well as the isotopic pattern of the compound, and can be used to calculate the elemental composition of compounds (Kind & Fiehn, 2010).
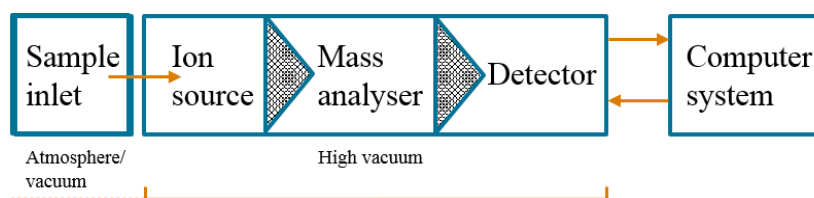


*Figure 7: **Overview of the four components of a MS system: sample inlet, ionization source, a mass analyser and an ion detector.** Figure made with inspiration from reference (Silverstein, Webster, & Kiemle, 2005, p. 419).*

### 1.3.5.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy measures the physical and chemical properties of molecules and this is achieved by exploiting the magnetic properties of certain atomic nuclei. The technique relies on NMR, a physical phenomenon where nuclei in a magnetic field absorb and re-emit electromagnetic radiation. $^1$H and $^{13}$C are the two most commonly examined nuclei. In general, the principle of NMR involves two sequential steps. First, randomly oriented nuclei are subjected to an external magnetic field which they will align either with or against (Figure 8). Alignment against the magnetic field requires the least amount of energy. Second, an electromagnetic pulse (usually radio frequency) causes the nuclei to flip, from aligning with (lower-energy spin state) to aligning against (higher-energy spin state) the magnetic field. When the radiation is switched off, the nucleus re-emits the absorbed energy and relaxes back to the lower energy state. This emitted energy signal produces a measurable signal called the resonance frequency, and the resonance frequency is affected by the molecule`s atomic properties. The resonance frequency is processed into a NMR spectrum (Mlynárik, 2016; Pauli, Jaki, & Lankin, 2005; Silverstein et al., 2005, p. 106). NMR spectrum can be either one-

dimensional (1D) or two-dimensional (2D). 1D experiments are analysis of a single nucleus, such as $^1$H NMR and $^{13}$C NMR. 2D NMR provides more information about a molecule than 1D NMR because it involves data plotted in a space defined by two frequency axes rather than just one (Silverstein et al., 2005, p. 245-251). To elucidate the structure of a complex compound, a combination of several NMR experiments, as well as other analytical data, are often required. After the structure is elucidated, the bioactivity profile of the isolated compound can be established.
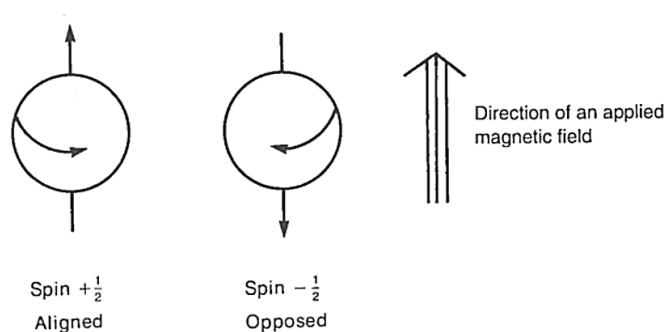


*Figure 8:* **In an applied magnetic field, nuclei are either aligned with the field or opposed to it.** *The spin state* $+\frac{1}{2}$ *is of lower energy since it is aligned with the field, while the spin state* $-\frac{1}{2}$ *is of higher energy since it is opposed to the applied field (Silverstein et al., 2005, p. 106).*

### 1.3.6 Bioactivity profiling of isolated compounds

After structure elucidation, the bioactivity profile of the isolated compound is determined. This includes confirming or disproving the initial bioactivity that was detected in the active fraction prior to isolation. In addition, the compound can be submitted to general bioactivity profiling including bioassays different from the one where the initial bioactivity was detected. It is favourable to combine the use of phenotypic screening and target-based screening in the bioactivity profiling (Swinney & Anthony, 2011). The compound can for example be screened against a wide range of targets for different disease areas or it can be screened in target-based screenings with the aim of determining the mode of action for one specific disease area.

When the bioactivity profile of the isolated compound has been investigated, the isolated compound`s efficiency is determined. This can be achieved by determining the concentration ranges for minimum inhibitory concentration (MIC) or the lowest concentration resulting in 50% inhibition ($IC_{50}$).

### 1.3.7 Commercialization of natural products

Before a NP with an elucidated structure and confirmed *in vitro* bioactivity can be made commercially available as a drug, many challenges need to be addressed. Drug development comprises all activities that are necessary for transforming a NP into a product that is approved for marketing (Rang, 2006, p. 221). A technical development of the compound is conducted as part of a lead optimisation. The safety and efficiency of the compound are investigated in pre-clinical and clinical trials, before the compound is marketed as a drug. The road from discovery of the NP to a marketable drug is a complex and time-consuming process (Rang, 2006, p. 257-269), and it was beyond the scope of this thesis.

# 2 Aim of the thesis

The overall aim of this thesis was to identify and isolate bioactive secondary metabolites from Arctic, marine invertebrates. The main target activity was anticancer, and results from a primary anticancer screening conducted at Marbio were used as a starting point for this thesis.

The key objectives of the thesis were to:

1. Confirm anticancer activity detected in an initial bioactivity screening, in a secondary anticancer screening
2. Dereplicate the bioactive fractions to identify target compounds
3. Establish an isolation strategy to enable isolation of the target compounds in sufficient amounts for further work involving structure elucidation and bioactivity profiling

# 3   Materials and methods

An overview of the various experimental steps in this thesis can be seen in the flow chart on page IV.

## 3.1   Biological material

Arctic, marine invertebrates were collected as described in Table 2, at various locations. The biomass samples were stored at -22˚C in the dark before being extracted and prefractionated as described in section 3.2.1 "Extraction" and in section 3.2.2 "Prefractionation of crude extracts (flash chromatography)".

*Table 2: **Overview of the Arctic, marine invertebrates investigated in this thesis.** The species were collected at different locations, by Marbank, as described in the table.*

| Species | Collection date | Geographic position | Location | Depth (meter) | Collection method |
|---|---|---|---|---|---|
| *Bryozoa indet* | 13.05.2014 | 79.385N, 10.0952E | Magdalenafjorden, Svalbard | 72 | Triangular scrape |
| *Styela rustica* | 15.05.2014 | 79.3428N, 10.4295E | Magdalenafjorden, Svalbard | 43 | Triangular scrape |
| *Mycale (Mycale) lingua* | 11.05.2014 | 79.3426N, 10.4451E | Magdalenafjorden, Svalbard | 72 | Triangular scrape |
| *Alcyonidium gelatinosum* | 06.05.2014 | 75.5168N, 23.9793E | Hopenbanken, Svalbard | 72 | Triangular scrape |
| *Astarte borealis* | 10.05.2014 | 79.0613N, 10.4551E | Kongsfjorden, Svalbard | 36 | Triangular scrape |
| *Nuculana pernula* | 02.10.2011 | 79.6972N, 11.12373E | Smeerenburfjorden, Svalbard | 202 | Agassiz trawl |
| *Strongylocentrotus droebachiensis* | 30.09.2011 | 78.4287N, 16.3723E | Gipshukodden, Svalbard | 57 | Agassiz trawl |
| *Porifera indet* | 03.04.2007 | 71.1498N, 18.6555E | Tromsøflaket, Troms | 190 | Beam trawl |

Each sample was assigned a unique name containing abbreviations indicating samples originating species, extraction method and flash fraction. For example, BI-W-04 is the fourth flash fraction of the water extract of *B*.indet. A complete list of sample names can be seen in Table 3. These abbreviations are used throughout the text in this thesis and can also be found on page III.

*Table 3: **Each sample was assigned an abbreviation indicating samples originating species, extraction method and flash fraction.** In total, 19 samples from eight Arctic, marine invertebrates were chosen for further analysis in this thesis.*

| Organism | Extract | Fraction | Sample name |
| --- | --- | --- | --- |
| *Bryozoa indet* | W | 4 | BI-W-04 |
| | W | 5 | BI-W-05 |
| | W | 6 | BI-W-06 |
| *Styela rustica* | W | 5 | SR-W-05 |
| *Mycale (Mycale) lingua* | L | 4 | ML-L-04 |
| | L | 5 | ML-L-05 |
| | L | 6 | ML-L-06 |
| | L | 7 | ML-L-07 |
| | W | 5 | ML-W-05 |
| | W | 6 | ML-W-06 |
| | W | 7 | ML-W-07 |
| *Alcyonidium gelatinosum* | L | 5 | AG-L-05 |
| | W | 4 | AG-W-04 |
| | W | 5 | AG-W-05 |
| *Astarte borealis* | L | 7 | AB-L-07 |
| *Nuculana pernula* | L | 5 | NP-L-05 |
| *Strongylocentrotus droebachiensis* | L | 1 | SD-L-01 |
| *Porifera indet* | L | 6 | PI-L-06 |
| | W | 6 | PI-W-06 |

## 3.2 Sample handling routinely conducted at Marbio

Marbank routinely produces crude extracts from collected organisms. These crude extracts are prefractionated as part of the routine work at Marbio. Based on results from the primary anticancer screening of such fractions, 19 fractions were chosen for further analysis in this master thesis. The procedure described in section 3.2 "Sample handling routinely conducted at Marbio" to section 3.3 "Sample selection based on primary anticancer screening" was conducted at Marbank and Marbio as part of their routine work prior to the start of this master thesis. The procedure described in section 3.4 "Bioassays" and onwards was conducted as part of this thesis.

### 3.2.1 Extraction

The material and equipment used during extraction can be seen in *Table 4*.

*Table 4: **Materials and equipment used during extraction of the marine invertebrates.***

| Materials/Equipment | Supplier |
|---|---|
| Rotary evaporator, Heidolph Laborota | Heidolph Instruments GmbH & Co, Germany |
| Whatman® qualitative filter paper, grade 3, 1003-090 | Sigma-Aldrich, MO, USA |
| Ultra-pure water | Merck KGaA, Germany |
| Dichloromethane, 34856 | Sigma-Aldrich, MO, USA |
| Methanol, 34860-M | Sigma-Aldrich, MO, USA |

The freeze-dried organisms were ground and extracted twice with ultra-pure water (24 hours and 30 minutes (min)) at 5˚C in the dark. After centrifugation (two rounds) the supernatant was removed, combined and dried. The resulting powder was termed the aqueous extract. The remaining pellet was extracted twice with a 1:1 (vol:vol) mixture of DCM and MeOH (24 hours and 30 min) at 5˚C in the dark. The mixture was vacuum-filtrated through a Whatman Ø 125 mm no. 3 filter. The resulting filtrate was reduced to a concentrated liquid under vacuum. This concentration resulted in a finished organic extract. Both the aqueous and the organic extracts were stored at -23˚C until use.

### 3.2.2 Prefractionation of crude extracts (flash chromatography)

The material and equipment used during prefractionation of crude extracts with flash chromatography can be seen in Table 5.

*Table 5: **Materials and equipment used during prefractionation of organic and aqueous extracts of marine invertebrates.***

| Materials/Equipment | Supplier |
|---|---|
| Biotage® HPFC SP4 Flash Purification System | Biotage, Sweden |
| Biotage®SNAP Cartridge KP-Sil 10 g, FSK0-1107-0010 | Biotage, Sweden |
| Universal Shaker SM 30 | Edmund Bühler GmbH, Germany |
| Rotary evaporator, Heidolph Laborota | Heidolph Instruments GmbH & Co, Germany |
| Syncore® Polyvap | Büchi, Switzerland |
| Heto PowerDry® PL9000 Freeze Dryer | Thermo Fisher Scientific, MA, USA |
| Diaion®HP-20SS, 13615-U | Sigma-Aldrich, MO, USA |
| Methanol, 34860-M | Sigma-Aldrich, MO, USA |
| Acetone, 34850 | Sigma-Aldrich, MO, USA |
| Dimethyl sulfoxide, D4540 | Sigma-Aldrich, MO, USA |
| Ultra-pure water | Merck KGaA, Germany |

Approximately 1 g of extract was transferred to a round bottom flask and dissolved in 10 mL hexane. To this mixture, 2 g Diaion® HP-20SS was added before the mixture was dried under vacuum. The dried sample was added to the top of a flash column packed with 6 g Diaion® HP-20SS equilibrated with 5:95 (vol:vol) MeOH: ultra-pure water. Fractionation was performed using Biotage HPFC SP4 flash purification system and a gradient of mobile phases (water, MeOH and acetone) (Table 6-left). The gradient was pumped with a flow of 12 mL/min, and every fraction was collected for 2 min. The fractions were combined as described in Table 6 (right), and dried under vacuum. This resulted in eight dried fractions (called flash fractions from this point on) and these flash fractions were dissolved in dimethyl sulfoxide (DMSO) in a concentration of 40 mg/mL. The fractions were stored in CRYO tubes at -23°C in the dark until further use (see section 3.3 "Sample selection based on primary anticancer screening").

*Table 6: **Left) Mobile phase gradient used during flash chromatography prefractionation. Right) After prefractionation with flash chromatography, the fractions were pooled as described in this table and dried under vacuum.** The resulting eight flash fractions were screened in the initial anticancer screening at Marbio.*

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

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

## 3.3   Sample selection based on primary anticancer screening

The samples selected to be worked with in this thesis had all shown activity against a human melanoma cancer cell line (A2058) in the ongoing primary anticancer screening (test concentration 50 µg/mL) conducted at Marbio. The results from all samples screened in this assay in the fall of 2015 were examined and all samples resulting in less than 50 % remaining cell survival were nominated for secondary anticancer screening. From these preliminary results, 19 flash fractions (from eight different Arctic, marine invertebrates) with anticancer activity were chosen for further examination in this thesis. This marks the end of the procedure conducted at Marbio prior to the start of this master thesis. The 19 fractions (Table 3) were investigated as described in section 3.4 "Bioassays" and onwards for this master thesis.

## 3.4 Bioassays

### 3.4.1 Anticancer screening

The material and equipment used in the anticancer screening are listed in Table 7.

*Table 7: **Materials and equipment used in anticancer screening.***

| Materials/Equipment | Supplier |
| --- | --- |
| DTX 880 multimode detector | Beckman Coulter, CA, USA |
| $CO_2$ Incubator, model: MCO-18AIC | Panasonic Biomedical, Japan |
| Herasafe biological safety cabinet (Class II) | Thermo Fisher Scientific, MA, USA |
| Dulbecco`s Modified Eagle Medium (D-MEM), high glucose, GlutaMAX™ Supplement, HEPES, 32430027 | Thermo Fisher Scientific, MA, USA |
| Earle`s minimal essential medium (E-MEM) with 20 mM HEPES, F4315 | Merck KGaA, Germany |
| Roswell park memorial institute medium (RPMI-1640), FG 1383 | Merck KGaA, Germany |
| Fetal Bovine Serum (FBS), S 0115 | Merck KGaA, Germany |
| Gentamycin [10 mg/mL], A2712 | Merck KGaA, Germany |
| L-Alanyl-L-glutamine [200 mM], K 0302 | Merck KGaA, Germany |
| NEA - Non essential amino acids (100x), K 0293 | Merck KGaA, Germany |
| Sodium pyruvat 100 mM, L 0473 | Merck KGaA, Germany |
| Sodium bicarbonate 7,5%, L 1713 | Merck KGaA, Germany |
| Cell Titer 96® Aqueous One Solution Reagent, G358B | Promega, WI, USA |
| DMSO, D4540 | Sigma-Aldrich, MO, USA |
| Triton™ X-100, T8787 | Sigma-Aldrich, MO, USA |
| Trypsin (1:250), 27250018 | Thermo Fisher Scientific, MA, USA |
| A2058 (ATCC® CRL-11147™) | LGC Standards, UK |
| MRC-5 (ATCC® CCL-171™) | LGC Standards, UK |
| MCF-7 (ATCC® HTB-22™) | LGC Standards, UK |
| HT-29 (ATCC® HTB-38™) | LGC Standards, UK |

Four adherent cell lines were used in the anticancer screening and these cell lines were sustained in culture (for appropriate growth medium, see Table 8). The adherent cells were split (trypsinated) twice a week. After trypsination, the cells were resuspended in appropriate growth medium. A new culture flask was prepared with fresh media, and transferring sufficient amounts of resuspended cells to reach a cell density of 70 – 80% before the next round of cell splitting. The rest of the cell suspension (not used for further growth of the cell lines) was available for use in anticancer screening (see sections 3.4.2 "Secondary anticancer screening", 3.4.3 "Tertiary anticancer screening" and 3.4.4 "Bioactivity profiling of isolated compounds"). Microtiter plates (96 wells) were prepared by seeding cells at 2000 cells/well (A2058, MCF-7 and HT-29) or 4000 cells/well (MRC-5).

*Table 8: **Cell lines and appropriate growth medium used in the anticancer screening.***

| Cell line | Cell type | Growth medium |
|---|---|---|
| A2058 | Human melanoma | DMEM with 10 % FBS, 1% L-Alanyl-L-glutamine and 0,1 % gentamycin |
| MRC-5 | Human lung fibroblast | E-MEM with 10% FBS, 0,1 % gentamycin, 1 % NEA, 1% sodium pyruvate, 1% sodium bicarbonate and 1% L-Alanyl-L-glutamine |
| MCF-7 | Human breast carcinoma | E-MEM with 10% FBS, 0,1 % gentamycin, 1 % NEA, 1% sodium pyruvate, 1% sodium bicarbonate and 1% L-Alanyl-L-glutamine |
| HT-29 | Human colon carcinoma | RPMI with 10 % FBS, 1% L-Alanyl-L-glutamine and 0,1 % gentamycin |

### 3.4.2   Secondary anticancer screening

The prefractionated samples (see section 3.2.2 "Prefractionation of crude extracts (flash chromatography)") were screened against the malignant cell line A2058. The samples were also screened against the non-malignant lung fibroblast MRC-5 to investigate the sample`s toxicity against normal human cells. After seeding of 96-well microtiter plates as described above, the plates were incubated overnight in 37˚C, 5% $CO_2$, to allow settling of the cells. The following day, the growth medium was removed from the microtiter plate wells and new growth medium with samples (preheated to 37˚C) was added to the wells. The cell lines were exposed to different concentrations of the samples: 50, 25 and 10 μg/mL. The total assay volume was 100 μL and each sample was screened in duplicates. Wells with 100 μL growth medium were used as negative control. Cells treated with 0.5% triton were used as a positive control. The cells were exposed to the samples for 72 hours (37˚C, 5% $CO_2$).

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, 2012). After 72 hours, 10 μL Aqueous One was added to each well in the microtiter plate. The plates were incubated for one hour in 37˚C, 5% $CO_2$. The cell survival was then analysed by measuring light absorbance using a DTX 880 multimode detector at 485 nm. Using a mean for the positive and negative control, the percentage of cell survival was calculated using formula 1

*Formula 1:*

$$\frac{\text{Average measurement test sample-mean positive control}}{\text{Mean negative control- mean positive control}}*100=\% \text{ Cell survival}$$

Samples, whose activity was confirmed through the secondary anticancer screening, were nominated for further investigation using ultra performance liquid chromatography (UPLC)-HR-MS analysis (see section 3.5 "Dereplication of active samples").

### 3.4.3 Tertiary anticancer screening

Collected fractions from the refractionation of AG L-05 (see section 3.6 "Refractionation of AG-L-05 with prep HPLC-MS") were analyzed in a tertiary anticancer screening. The freeze-dried fractions in deep well plates were dissolved by adding 7,5 µL DMSO to each well. The plates were incubated in room temperature with constant shaking for 30 min. After this incubation, 750 µL of E-MEM was added to each well and the plate was incubated at room temperature with shaking for 30 min. Finally, 750 µL E-MEM was added to each well and the samples were incubated at room temperature with constant shaking for 10-15 min.

The tertiary anticancer screening was performed as described in the secondary anticancer screening (section 3.4.2 "Secondary anticancer screening"), apart from the sample volume. In sample wells, 50µL E-MEM and 50 µL of the dissolved fractions were added to the cell lines.

### 3.4.4 Bioactivity profiling of isolated compounds

#### 3.4.4.1 Anticancer screening

An anticancer screening of the isolated compounds (see section 3.7 "Isolation of target compounds from extract BI-L and AG-L using prep HPLC-MS") was performed on the cell lines A2058, MRC-5, MCF-7 and HT-29. The cell lines were exposed to different concentrations of the isolated compounds (see Table 9). In addition, DMSO controls were conducted with the same percentage of DMSO as what was present in the sample wells with AG-L-465.3 and AG-L-449.4, because it exceeded the recommended DMSO concentration of 1% (Eastwood et al., 2007). The screening was conducted as described in section 3.4.2 "Secondary anticancer screening".

*Table 9: **The isolated compounds AG-L-465.3, AG-L-449.4 and BI-L-665.6 were tested in an anticancer screening.** Left) The cell lines A2058, MRC-5, MCF-7 and HT-29 were exposed to different concentrations of the samples AG-L-465.3 and AG-L-449.4. In addition, DMSO controls were screened for the percentage of DMSO that was present in the sample. Right) The cell lines A2058, MRC-5, MCF-7 and HT-29 were exposed to different concentrations of the sample BI-L-665.6.*

| Sample | Concentration (μg/mL) | DMSO (%) |
|---|---|---|
| AG-L-465.3 | 250 | 2.5 |
| | 200 | 2 |
| | 150 | 1.5 |
| | 100 | 1 |
| | 50 | 0.5 |
| | 25 | 0.25 |
| AG-L-449.4 | 250 | 2.5 |
| | 200 | 2 |
| | 150 | 1.5 |
| | 100 | 1 |
| | 50 | 0.5 |
| | 25 | 0.25 |

| Sample | Concentration (μM) |
|---|---|
| BI-L-665.6 | 10 |
| | 5 |
| | 2.5 |
| | 1.25 |
| | 0.625 |
| | 0.3125 |

### 3.4.4.2 Antibacterial screening

Materials and equipment used in the antibacterial screening can be seen in Table 10.

*Table 10: **Materials and equipment used in the antibacterial screening.***

| Material/Equipment | Supplier |
|---|---|
| Heated Incubator MIR-262 | Panasonic Healthcare, Japan |
| Incubator Unimax 1010 | Heidolph Instruments GmbH & Co, Germany |
| Victor Multilabel Counter | Perkin Elmer, MA, USA |
| Software: WorkOut 2.5 | Dazdag, UK |
| Herasafe biological safety cabinet (Class II) | Thermo Fisher Scientific, MA, USA) |
| Mueller Hinton broth (MH), 275730 | Becton Dickinson and Company, NJ, USA |
| Brain heart infusion broth (BHI), 53286 | Sigma-Aldrich, MO, USA |
| Sodium chloride, S5886 | Sigma-Aldrich, MO, USA |
| Ultra-pure water | Merck KGaA, Germany |
| Blood agar plates | University hospital of North Norway(UNN), Norway |
| Luria-Bertoni (LB) plates | University hospital of North Norway (UNN), Norway |
| Glycerol, G5516 | Sigma-Aldrich, MO, USA |
| Gentamycin (10mg/mL), A 2712 | Merck KGaA, Germany |
| *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 |

Preparation of bacterial strains

The antibacterial activity of the isolated compounds BI-L-665.6, AG-L-05-465.3 (Pon A) and AG-L-05-449.4 (dehydroxy-Pon A) (see section 3.7.2 "Isolation of target compounds from extract BI-L (prep HPLC-MS)" and section 3.7.3 "Isolation of compounds from extract AG-L (prep HPLC-MS)") were screened against five bacterial strains (Table 11). These bacterial

strains were stored in the same growth medium as they were cultivated in, with 10% glycerol at -80˚C. When in use, the bacteria were kept on blood agar plates (maximum 1 month), with re-streaks every second week for maintenance of the bacteria. When the antibacterial screening was performed, the five different bacterial strains were seeded from blood agar plates to 8 mL growth media (See Table 11 for appropriate growth medium). These bacterial suspensions were incubated over night at 37˚C.

*Table 11: **Test bacteria utilised in the antibacterial screening, as well as their appropriate growth medium and cultivation time (second day).***

| Bacteria | Growth medium | Incubation second day (hour) |
|---|---|---|
| *S. aureus* | MH broth | 2.5 |
| *E. coli* | MH broth | 1.5 |
| *E.faecalis* | BHI broth | 1.5 |
| *P.aeruginosa* | MH broth | 2.5 |
| *S. agalactiae* | BHI broth | 1.5 |

The following day, 2 mL of the overnight bacterial cultures were transferred to 25 mL fresh medium for incubation with shaking for 1.5/2 hours (see Table 11 for appropriate incubation time) until the growth reached 0.5 McFarland standard ($1.0 \times 10^8$ 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 for antibacterial screening

The compounds BI-L-665.6, AG-L-05-465.3 (Pon A) and AG-L-05-449.4 (dehydroxy-Pon A) were screened in final test concentration of 10, 5, 2.5, 1.25, 0.625 and 0.3125 µM, in duplicates, in the antibacterial assay. The compounds were dissolved in DMSO and diluted in sterile ultra-pure water to concentrations 20, 10, 5, 2.5, 1,25 and 0,625 µM. 50µL of the samples were added to 5 different microtiter plates (one for each bacterial strains) in two parallels. 50µL of the diluted bacterial suspension were added to the samples (diluting the sample 1:2, giving the previously mentioned test concentration).

For a negative control, 50 µL growth media and 50µL sterile ultra-pure water were used. For positive control, 50µL sterile ultra-pure water and 50µL of the diluted bacterial suspension were used. The microtiter plates were incubated for 22 hours at 37˚C. After this incubation, absorbance of the microtiter plates were measured at 600 nm using Victor Multilabel Counter.

Low absorbance indicated bacterial growth inhibition, and $Abs_{600}$ values were used to define the compounds as active, inactive or questionable:

$$A < 0.05$$
$$Q = 0.05\text{-}0.09$$
$$I > 0.09$$

Gentamycin control and control of colony forming unit (CFU)

A gentamycin control and control of colony forming unit (CFU) were conducted to ensure that the bacterial strains were growing properly and that the assay was working. This is conducted routinely at Marbio. The gentamycin control was conducted with final test concentrations varying from 0.01 µg/mL to 16 µg/mL, to find the MIC values for gentamycin. 50 µL of the gentamycin controls was added to 50 µL bacterial solution. The plate was incubated over-night at 37°C. If the MIC-values for gentamycin was within one titer step outside of the reference MIC-values (see Table 12), it was decided that the assay was working. The MIC-values were evaluated visually, and Table 12 shows the reference MIC-values for each bacterium.

Control of CFU was conducted by using the bacterial solutions after incubation (1.5/2.5 hour) on the second day of the antibacterial screening. This bacterial solution was diluted in 0.9% NaCl solution: First 1:100, then 1:100 again and 1:10 times two. 100 µL of the two last dilutions (1:100 000 and 1:1 000 000) were plated in two parallels on LB plates, and incubated over-night at 37°C. The next day, the number of colonies was counted and CFUs were calculated and controlled against the standard CFU ranges listed in Table 12. If the calculated CFU were within the range of the standard CFU ranges, the bacterial growth was deemed normal.

*Table 12: **Reference MIC-values and CFU ranges for the test bacteria utilised in antibacterial screening.***

| Test bacteria | Reference MIC-values for gentamycin (µg/mL) | CFU ranges |
|---|---|---|
| *S.aureus* | 0.25 | $0.5\text{-}3\text{x}10^5$ CFU/mL |
| *E.coli* | 0.5 | $0.5\text{-}3\text{x}10^5$ CFU/mL |
| *E.faecalis* | 10 | $0.5\text{-}3\text{x}10^5$ CFU/mL |
| *P.aeruginosa* | 0.5 | $3\text{-}7\text{x}10^4$ CFU/mL |
| *S. agalactiae* | 4 | $0.5\text{-}3\text{x}10^5$ CFU/mL |

## 3.5 Dereplication of active samples

The material and equipment used in UPLC-HR-MS analysis of active samples from the secondary anticancer screening are listed in Table 13.

*Table 13: **Material and equipment used in dereplication of active samples from the secondary anticancer screening employing UPLC-HR-MS analysis.***

| Material/Equipment | Supplier |
|---|---|
| Acquity UPLC ® BEH, 2.1x100 mm, 1.7 μm column | Waters, MA, USA |
| Aquity Sample Manager | Waters, MA, USA |
| Aquity Binary Solvent Manager | Waters, MA, USA |
| 2998 Photodiodide Array Detector | Waters, MA, USA |
| LCT Premier | Waters, MA, USA |
| LiChrosol® Acetonitrile (hypergrade for LC-MS), 1.00029 | Merck KGaA, Germany |
| Formic Acid ULC/MS 99%, 069141 | Biosolve B.V., Netherland |
| Methanol LC-MS Ultra CHROMASOLV®, 14262 | Thermo Fisher Scientific, MA, USA |
| Ultra-pure water | Merck KGaA, Germany |

The active samples from the secondary anticancer screening (see section 3.4.2 "Secondary anticancer screening"), as well as the fraction eluting directly before and directly after the active fraction(s), were removed from the freezer and thawed in room temperature. In a deep well plate, 5 μL of the fraction was added to 10 μL 50% ACN. This mixture was analysed using UPLC-HR-MS. A gradient of two mobile phases were used, mobile phase A: ultra-pure water with 0.1% formic acid (FA) and mobile phase B: ACN with 0.1% FA (see Table 14).

*Table 14: **Mobile phase gradient used in UPLC-HR-MS analysis of active samples from the secondary anticancer screening.** Mobile phase A: Ultra-pure water with 0.1 % FA, and mobile phase B: ACN with 0.1 % FA.*

| Time (min) | Flow (mL/min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|---|
| Initial | 0.55 | 80 | 20 |
| 3.5 | 0.55 | 0 | 100 |
| 5 | 0.55 | 0 | 100 |
| 5.1 | 0.55 | 80 | 20 |

The injection volume for each sample was 3 μL, and the run time was 6.50 min with a 0.55 mL/min flow. Instrument parameters can be found in the appendix, see A1. The mass spectrum for each target compound was used to calculate an elemental composition. This elemental composition were used as a search input in Dictionary of Natural Products to determine if the target compounds were novel or previously reported compounds.

The sample AG-L-05 was refractionated (see section 3.6 "Refractionation of AG-L-05 with prep HPLC-MS") and screened in a tertiary anticancer screening (see section 3.4.3 "Tertiary anticancer screening") to better determine the compound(s) responsible for the observed anticancer activity in the secondary anticancer screening (see section 3.4.2 "Secondary anticancer screening").

## 3.6 Refractionation of AG-L-05 with prep HPLC-MS

The material and equipment used for refractionation of sample AG-L-05 with prep HPLC-MS are listed in Table 15.

*Table 15: **Materials and equipment used for refractionation of sample AG-L-05 with prep HPLC-MS.***

| Materials/ Equipment | Supplier |
| --- | --- |
| XTerra® Prep RP18 10μm 10*300 mm coloumn | Waters, MA, USA |
| Software: MassLynx 4.1 | Waters, MA, USA |
| 600 Controller | Waters, MA, USA |
| 2996 photodiodide array detector | Waters, MA, USA |
| 3100 mass detector | Waters, MA, USA |
| 2767 sample manager | Waters, MA, USA |
| Flow splitter | Waters, MA, USA |
| Prep degasser | Waters, MA, USA |
| 515 HPLC pump | Waters, MA, USA |
| SC250 Express SpeedVac Concentrator | Thermo Fischer Scientific, MA, USA |
| Heto PowerDry® PL9000 Freeze Dryer | Thermo Fisher Scientific, MA, USA |
| Acetonitrile, 34851 | Sigma-Aldrich, MO, USA |
| Formic acid, 56302 | Sigma-Aldrich, MO, USA |
| Methanol, 34860N | Sigma-Aldrich, MO, USA |
| Ultra-pure water | Merck KGaA, Germany |

The sample AG-L-05 was prepared for refractionation with prep HPLC-MS by mixing 250 μL of sample (20 mg) with 50 μL 100% ACN. The injection volume was 280 μL (18.67 mg sample). A gradient of mobile phases, A: ultra-pure water with 0.1% FA and B: ACN with 0.1% FA, was used (Table 16). Instrument parameters can be found in the appendix, see A2.

*Table 16: **Mobile phase gradient used during refractionation of sample AG-L-05 with prep HPLC-MS.** Mobile phase A: Ultra-pure water with 0.1% FA, mobile phase B: ACN with 0.1% FA. Flow was 5 mL/min.*

| Time (min) | Flow (mL/min) | Mobile phase A (%) | Mobile phase B (%) |
| --- | --- | --- | --- |
| initial | 6 | 70 | 30 |
| 30 | 6 | 30 | 70 |
| 40 | 6 | 0 | 100 |

Total run time was 56 min and the eluting mobile phase was collected in 1 min fractions (collection time 40 min). This resulted in 40 collected fractions that were divided between three deep well plates, 2 mL per plate. The fractions were dried under vacuum and subsequently freeze-dried. The dried fractions were redissolved and analysed in a tertiary anticancer screening (see section 3.4.3 "Tertiary anticancer screening").

## 3.7 Isolation of target compounds from extract BI-L and AG-L using prep HPLC-MS

The material and equipment used during isolation of target compounds from extract AG-L and BI-L are listed in Table 17. The instrument parameters can be seen in the appendix, A2. Isolation of target compounds was conducted on the crude extracts named after their originating species and the extraction method. Compounds were isolated from the organic extract of *B.indet* (BI-L) and the organic extract of *A.gelatinosum* (AG-L).

*Table 17:* ***Materials and equipment used during isolation of target compounds using prep HPLC-MS.***

| Materials/ Equipment | Supplier |
|---|---|
| XTerra® Prep RP18 10μm 10*300 mm column | Waters, MA, USA |
| Atlantis® Prep dC18 10μm 10x250mm column | Waters, MA, USA |
| XSELECT™ CSH™ Phenyl-Hexyl Prep 5μm 10x250 column | Waters, MA, USA |
| XSELECT CSH™ Prep Fluoro-Phenyl 5μm 10x250 mm column | Waters, MA, USA |
| SunFire™ Prep C18 5μM 10x250mm column | Waters, MA, USA |
| Software: MassLynx 4.1 | Waters, MA, USA |
| 600 Controller | Waters, MA, USA |
| 2996 photodiodide array detector | Waters, MA, USA |
| 3100 mass detector | Waters, MA, USA |
| 2767 sample manager | Waters, MA, USA |
| Flow splitter | Waters, MA, USA |
| Prep degasser | Waters, MA, USA |
| 515 HPLC pump | Waters, MA, USA |
| SC250 Express SpeedVac Concentrator | Thermo Fischer Scientific, MA, USA |
| Heto PowerDry® PL9000 Freeze Dryer | Thermo Fisher Scientific, MA, USA |
| Acetonitrile, 34851 | Sigma-Aldrich, MO, USA |
| Formic acid, 56302 | Sigma-Aldrich, MO, USA |
| Methanol, 34860N | Sigma-Aldrich, MO, USA |
| Ultra-pure water | Merck KGaA, Germany |

### 3.7.1 Pre-treatment of extract BI-L and AG-L (liquid-liquid partitioning)

A liquid-liquid partitioning was performed on the organic extract of *A.gelatinosum* (AG-L) and *B.indet* (BI-L) (see section 3.2.1 "Extraction")*.* Approximately 1.5-2 g (1985.6 mg of AG-L and 1601.6 mg of BI-L) of organic extract was transferred to an Erlenmeyer flask and 50 mL

hexane was added. This mixture was poured into a separatory funnel, and the Erlenmeyer flask was washed twice with 50 mL hexane (added to the separatory funnel after each washing).

To the mixture in the funnel, 150 mL 90% MeOH was added, and the mixture was allowed to separate into two distinct phases. Following collection of the MeOH phase, fresh 150 mL 90% MeOH was added to the separatory funnel. This was repeated twice. The MeOH phase was dried under vacuum. The resulting sample was kept at -23˚C until it was submitted to isolation using prep HPLC-MS (see Section 3.7.2 "Isolation of target compounds from extract BI-L (prep HPLC-MS)" and section 3.7.3 "Isolation of compounds from extract AG-L (prep HPLC-MS)").

### 3.7.2 Isolation of target compounds from extract BI-L (prep HPLC-MS)

The observed bioactivity in the *B.indet* was detected in the aqueous extract BI-W, and dereplication pointed to compound with *m/z* 478.4 as a possible responsible compound for this bioactivity. The organic extract was investigated for the presence of this target compound, and because of the amount present of the compound with *m/z* 478.4 in this extract, it was decided to isolate compound *m/z* 478.4 from the organic extract BI-L instead of the aqueous extract BI-W.

The dried sample (1080mg) from liquid-liquid partitioning (see section 3.7.1 "Pre-treatment of extract BI-L and AG-L (liquid-liquid partitioning)") was dissolved in 22 mL 100% MeOH (concentration 49.1 mg/mL). To determine the optimal purification conditions, 300 µL (14.70 mg sample) of the dissolved sample was injected onto five different RP prep HPLC columns. A gradient of mobile phases was used (Table 18), named elution gradient 1. Mobile phase A: ultra-pure water with 0.1 % FA, and mobile phase B: ACN with 0.1% FA. Total run time was 27 min.

*Table 18: **A gradient of mobile phase A: ultra-pure water with 0.1 % FA, and mobile phase B: ACN with 0.1% FA was used (named elution gradient 1) during isolation of compounds utilising prep HPLC-MS.** The flow was 6 mL/min and total run time was 27 min.*

| Time (min) | Flow (mL/min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|---|
| Initial | 6 | 90 | 10 |
| 20.00 | 6 | 30 | 70 |
| 20.10 | 6 | 0 | 100 |
| 25.00 | 6 | 0 | 100 |
| 25.10 | 6 | 90 | 10 |

*3.7.2.1 First and second round of separation using prep HPLC-MS (BI-L)*

Compounds BI-L-340.3, BI-L-665.6 and BI-L-478.4 were isolated using one or two rounds of HPLC separation (Table 19). The utilised elution gradients were versions of the elution gradient 1 in Table 18. The column and gradient were changed for each round of HPLC separation. The injection volume was 500µL for the first round of HPLC separation and 100µL for the second round of HPLC separation. The compounds were collected in fractions using either mass triggered or time triggered fractionation. The resulting fractions were pooled and dried under vacuum, before being used in the next HPLC separation round, or finally before HR-MS and NMR analysis (see section 3.8 "HR-MS analysis of isolated compounds from extract BI-L and AG-L" and section 3.9 "NMR analysis of isolated compounds from extract BI-L and AG-L").

*Table 19: **The compounds BI-L-340.3, BI-L-665.6 and BI-L-478.4 were isolated under conditions described in the table.** In gradient, B= ACN with 0.1% FA.*

| Compound | HPLC separation round | Column | Gradient | Sample (mg) |
|---|---|---|---|---|
| BI-L-340.3 | First | Phenyl-hexyl | 10-67 % B over 19 min, then 100% B for 2 min | 3.35 |
| BI-L-665.6 | First | Phenyl-hexyl | 10-67 % B over 19 min, then 100% B for 2 min | 2.25 |
| | Second | Fluoro-phenyl | 10-58 % B over 16 min, then 100% B for 2min | 0.60 |
| BI-L-478.4 | First | Phenyl-hexyl | 10-67 % B over 19 min, then 100% B for 2 min | 5.30 |
| | Second | Fluoro-phenyl | 10-64 % B over 18 min, then 100% B for 2min | 0.90 |

### 3.7.3 Isolation of compounds from extract AG-L (prep HPLC-MS)

The dried AG-L sample (1400 mg) from liquid-liquid partitioning (see section 3.7.1 "Pre-treatment of extract BI-L and AG-L (liquid-liquid partitioning)") was dissolved in 31 mL 100% MeOH. To determine the optimal purification conditions, 300 µL (13.55 mg sample) of the dissolved sample was injected onto five different RP prep HPLC columns. The utilised mobile phase gradient can be seen in Table 18 (elution gradient 1). Mobile phase A: ultra-pure water with 0.1 % FA, and mobile phase B: ACN with 0.1% FA. Total run time was 27 min.

*3.7.3.1 First, second and third round of separation using prep HPLC-MS (AG-L)*

Compounds AG-L-465.3, AG-L-449.4 and AG-L-541.4 were isolated using three rounds of HPLC separations (Table 20). The gradients used was a version of elution gradient 1 in Table 18. The column and elution gradients were changed for each round of HPLC separation. The

injection volume in the first round of HPLC separation was 700 µL, 70-150 µL (100µL for AG-L-05-465.3, 70µL for AG-L-05-449.4 and 150 µL for AG-L-05-541.4) for the second round and 100 µL for the third round of HPLC separation. Compounds were collected in fractions using either mass triggered or time triggered fractionation. The resulting fractions were pooled and dried under vacuum, before being used in the next HPLC separation round or finally before HR-MS and NMR analysis (see section 3.8 "HR-MS analysis of isolated compounds from extract BI-L and AG-L" and section 3.9 "NMR analysis of isolated compounds from extract BI-L and AG-L").

*Table 20: **The compounds AG-L-05-465.3, AG-L-05-449.4 and AG-L-05-541.4 were isolated under conditions described in this table.** In gradient, B= ACN with 0.1% FA. The flow was 6 mL/min.*

| Compound | HPLC separation round | Column | Gradient | Sample (mg) |
|---|---|---|---|---|
| AG-L-05-465.3 | First | Phenyl-hexyl | 10-70% B over 20 min, then 100% B for 1 min | 12.50 |
| | Second | Fluoro-phenyl | 10-46% B over 12 min, then 100% B for 1 min | 4.20 |
| | Third | Atlantis® | 10-52% B over 14 min, then 100% B for 1 min | 1.25 |
| AG-L-05-449.4 | First | Phenyl-hexyl | 10-70% B over 20 min, then 100 % B for 1 min | 33.60 |
| | Second | Fluoro-phenyl | 10-52% B over 14 min, then 100% B for 1 min | 14.20 |
| | Third | Xterra® | 10-61% B over 17 min, then 100% B for 1 min | 5.47 |
| AG-L-05-541.4 | First | Phenyl-hexyl | 10-70% B over 20 min, then 100 % B for 1 min | 21.75 |
| | Second | SunFire™ | 10-76% B over 22 min, then 100% B for 1 min | 6.10 |
| | Third | Atlantis® | 10-76% B over 22 min, then 100% B for 1 min | 3.03 |

## 3.8 HR-MS analysis of isolated compounds from extract BI-L and AG-L

The material and equipment used in HR-MS analysis for isolated compounds from the extract BI-L (section 3.7.2 "Isolation of target compounds from extract BI-L (prep HPLC-MS)") and AG-L (section 3.9 "NMR analysis of isolated compounds from extract BI-L and AG-L") are shown in Table 21.

*Table 21:* ***The material and equipment used in UPLC-QToF-MS analysis of the isolated compounds from extract AG-L and BI-L.***

| Materials/Equipment | Supplier |
|---|---|
| ACQUITY UPLC® PDA Detector | Waters, Milford, MA, USA |
| ACQUITY UPLC® Column Manager | Waters, Milford, MA, USA |
| ACQUITY UPLC® I-Class Sample Manager FTN | Waters, Milford, MA, USA |
| ACQUITY UPLC® I-Class Binary Solvent Manager | Waters, Milford, MA, USA |
| Vion IMS QToF | Waters, Milford, MA, USA |
| ACQUITY UPLC® BEH C18 1.7µM 2.1*100m column | Waters, Milford, MA, USA |
| Software: UNIFI | Waters, Milford, MA, USA |
| Methanol LC-MS Ultra CHROMASOLV®, 14262 | Thermo Fisher Scientific, MA, USA |
| LiChrosol® Acetonitrile (hypergrade for LC-MS), 1.00029 | Merck KGaA, Germany |
| Formic Acid ULC/MS 99%, 069141 | Biosolve B.V., Netherland |
| Ultra-pure water | Merck KGaA, Germany |

The samples were dissolved and diluted in 80% MeOH, and injected onto the UPLC column (injection volume 1 µL). A gradient of mobile phases, A: ultra-pure water with 0.1% FA, and B: ACN with 0.1% FA, was used (see Table 22). For instrument parameters, see appendix A3.

*Table 22:* ***Mobile phase gradient used in HR-MS analysis of isolated compounds from extract AG-L and BI-L.*** *Mobile phase A: ultra-pure water with 0.1% FA, mobile phase B: ACN with 0.1% FA.*

| Time (min) | Flow (mL/min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|---|
| 0.00 | 0.50 | 90.0 | 10.0 |
| 7.00 | 0.50 | 0.0 | 100.0 |
| 8.00 | 0.50 | 0.0 | 100.0 |

## 3.9 NMR analysis of isolated compounds from extract BI-L and AG-L

Johan Isaksson at the Department of Chemistry at UiT The Arctic University of Norway performed this procedure, 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 $^1H$, $^{13}C$ and $^2H$.

The NMR samples (see section 3.8 "HR-MS analysis of isolated compounds from extract BI-L and AG-L" and section 3.9 "NMR analysis of isolated compounds from extract BI-L and AG-L") were prepared by dissolving the sample in 500 µL DMSO (as described in Table 23). 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.

*Table 23: **Isolated compounds were analysed in DMSO as described in this table.***

| Isolated compound | Weight (mg) | DMSO (µL) |
|---|---|---|
| AG-L-05-465.3 | 1.25 | 500 |
| AG-L-05-449.4 | 5.47 | 500 |
| AG-L-05-541.4 | 3.03 | 500 |
| BI-L-05-340.3 | 3.48 | 500 |
| BI-L-05-665.59 | 0.6 | 500 |
| BI-L-05-478.4 | 0.9 | 500 |

# 4 Results

## 4.1 Primary anticancer screening

Flash fractions analysed in the fall of 2015 causing remaining cell survival of the malignant cell line A2058 to drop below 50% in the primary anticancer screening, were chosen for further examination in this thesis. Table 24 shows the result from the primary anticancer screening for the 19 fractions (eight different invertebrates) that were chosen for further examination in this thesis. An overview of sample names can be seen on page III.

*Table 24: **The results of the 19 flash fractions screened in the fall of 2015 that were found to inhibit cell survival of A2058 in an ongoing anticancer screening campaign at Marbio.** Cell survival (%) was determined by the MTS assay after 72 hours of exposure to the samples.*

| Sample | Cell survival (%) | Sample | Cell survival (%) |
|--------|-------------------|--------|-------------------|
| ML-W-05 | 0 | PI-L-06 | 10 |
| ML-W-06 | 0 | PI-W-06 | 49 |
| ML-W-07 | 3 | AG-W-04 | 11 |
| ML-L-04 | 44 | AG-W-05 | 1 |
| ML-L-05 | 0 | AG-L-05 | 17 |
| ML-L-06 | 6 | SR-W-05 | 2 |
| ML-L-07 | 12 | AB-L-07 | 34 |
| BI-W-04 | 50 | NP-L-05 | 0 |
| BI-W-05 | 0 | SD-L-01 | 12 |
| BI-W-06 | 21 | | |

## 4.2 Secondary anticancer screening

To confirm the results obtained in the primary anticancer screening, and to avoid further work with false positive samples, a secondary anticancer screening (A2058) was conducted. The fractions were also screened against non-malignant lung fibroblasts (MRC-5) to investigate the flash fraction`s toxicity against normal human cells.

### 4.2.1 *Mycale (Mycale) lingua (ML)*

Seven fractions from *M.lingua* were screened in the secondary anticancer screening and the results can be seen in Figure 9. All seven fractions showed anticancer activity in the secondary screening. The fraction ML-L-04 showed less anticancer activity towards A2058 than the other fractions from the organic extract of *M.lingua*. Furthermore, many of the fractions also showed a higher activity against A2058 compared to MRC-5. A dose-response effect could be observed for some of the fractions (exemplified by fraction ML-W-05 in Figure 9). As the activity of the ML-L-04 was weaker compared to its adjacent fractions and the fractions of the water extract,

this fraction was not submitted for dereplication. All the other fractions were dereplicated in an attempt to identify the compounds causing the observed bioactivity.
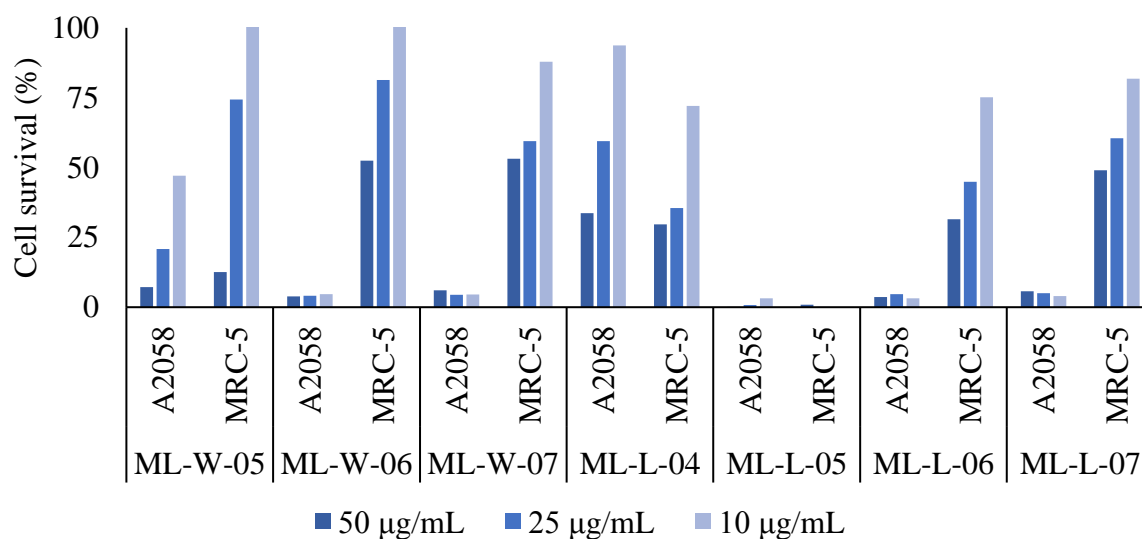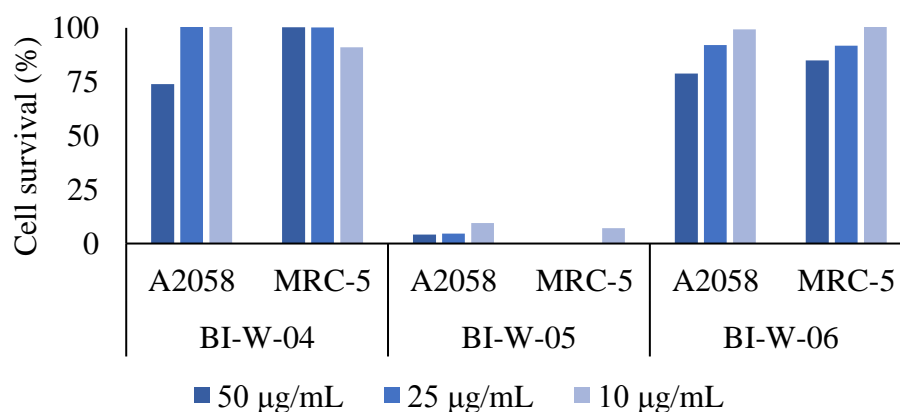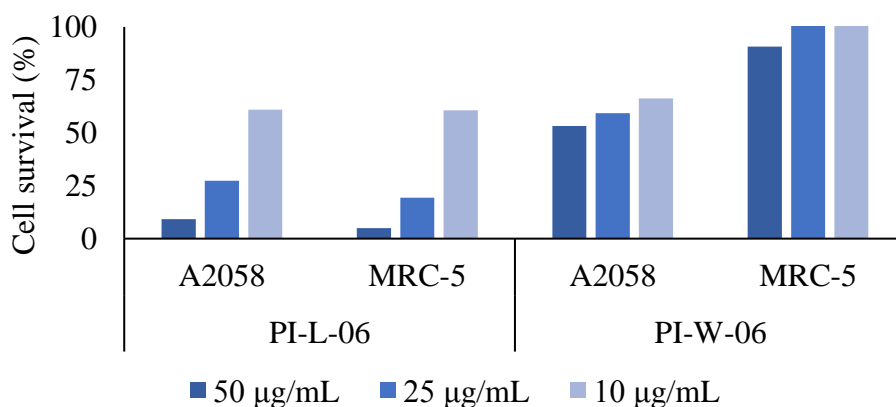


*Figure 9: **Results from the secondary anticancer screening of fractions from M.lingua.** The fractions were screened against the A2058 and MRC-5 cell line. The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the flash fractions and the values are means of two parallel fractions. Concentration points with no apparent bar had 0% cell survival.*

### 4.2.2    Bryozoa indet (BI)

Results from the secondary anticancer screening of fractions from *B.indet* can be seen in Figure 10. Fraction BI-W-05 showed activity against the A2058 and MRC-5 cell line. The activity of this fraction was confirmed, and the fraction was therefore submitted for dereplication using UPLC-HR-MS. Fractions BI-W-04 and BI-W-06 were not deemed active against the A2058 and MRC-5 cell line. Therefore, no further work was done with these fractions.
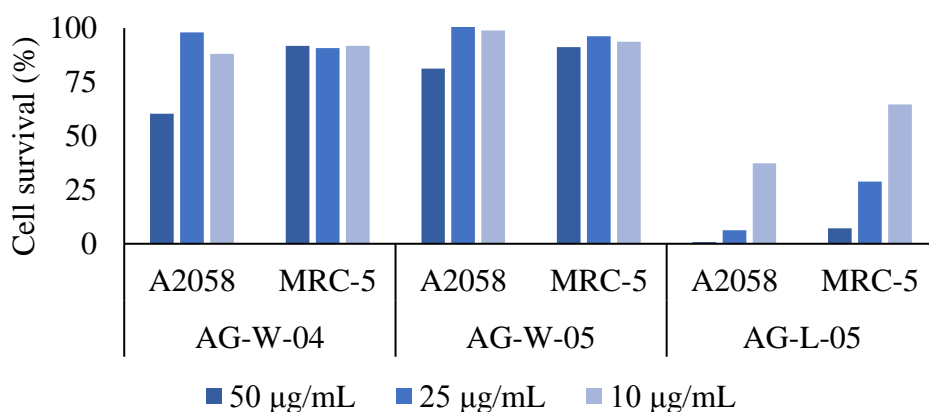


*Figure 10: **Results from the secondary anticancer screening of fractions from B.indet.** The fractions were screened against the A2058 and MRC-5 cell line. The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the fractions, and the values are means of parallel fractions. Concentration points with no apparent bar had 0% cell survival.*

### 4.2.3  Porifera indet (PI)

Results from the secondary anticancer screening of fractions from *P.indet* can be seen in Figure 11. The fraction PI-L-06 showed activity against the A2058 and the MRC-5 cell line. Furthermore, a dose-response effect could also be observed for this fraction. The activity of this fraction was confirmed, and the fraction was submitted for dereplication using UPLC-HR-MS. The fraction PI-W-06 showed a weak activity towards A2058, but was deemed not active in the secondary anticancer screening. No further work was done with this fraction.



*Figure 11:* **Results from the secondary anticancer screening of fractions from P. indet.** *The fractions were screened against the A2058 and MRC-5 cell line. The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the fractions, and the values are means of parallel fractions.*

### 4.2.4  Alcyonidium gelatinosum (AG)

In Figure 12, results from the secondary anticancer screening of fractions from *A.gelatinosum* are shown. The fraction AG-L-05 showed activity against the A2058 and MRC-5 cell line. Furthermore, a dose-response effect could be observed for both of the exposed cell lines. The fraction was submitted for dereplication using UPLC-HR-MS. The fractions AG-W-04 and AG-W-05 were deemed not active and no further work was done with these fractions.

*Figure 12: **Results from the secondary anticancer screening of fractions from A.gelatinosum.** The fractions were screened against the A2058 and MRC-5 cell line. The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the fractions, and the values are means of parallel fractions. Concentration points with no apparent bar had 0% cell survival.*

### 4.2.5 *Styela rustica* (SR)*, Astarte borealis* (AB)*, Nuculana pernula* (NP) and *Strongylocentrotus droebachiensis* (SD)

Results from the secondary anticancer screening of fractions from *S.rustica, A.borealis, N.pernula* and *S.droebachiensis* are shown in Figure 13. Fractions SR-W-05, AB-L-07, NP-L-05 and SD-L-01 were deemed not active against A2058. Consequently, no further work was done with these fractions.
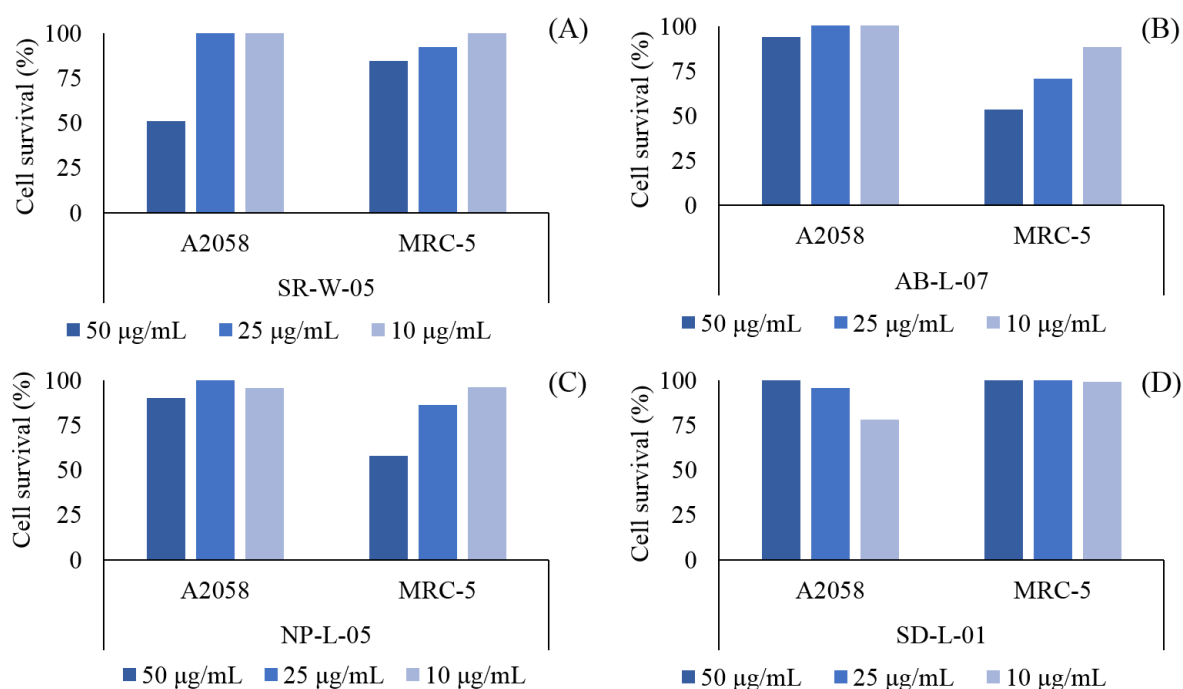


*Figure 13: **Results from the secondary anticancer screening of fractions from S.rustica (A), A.borealis (B), N.pernula (C) and S.droebachiensis (D).** The fractions were screened against the A2058 and MRC-5 cell line. Cell survival (%) was determined by the MTS assay after 72 hours of exposure to the fractions, and the values are means of parallel fractions.*

### 4.2.6 Summary anticancer screening

Based on the results from the secondary anticancer screening, the fractions that were chosen for dereplication with UPLC-HR-MS were

- ML-W-05, ML-W-06 and ML-W-07
- ML-L-05, ML-L-06 and ML-L-07
- BI-W-05
- AG-L-05
- PI-L-06

## 4.3 Dereplication

Fractions with a positive result in the secondary anticancer screening, as well as the fraction eluting directly before and directly after the active fraction(s), were analysed with UPLC-HR-MS. The results from this analysis were processed in an attempt to determine possible active compound(s). This was done by trying to identify compound(s) found exclusively, or in a significantly higher concentration, in the active fraction. An elemental composition was calculated for the target compounds, and database searches were performed to assess if the compound was novel or previously reported.

### 4.3.1 *Mycale (Mycale) lingua (ML)*

In the active fractions, ML-W-05, ML-W-06 and ML-W-07, a peak with *m/z* 482.35 were detected (marked with a black arrow for ML-W-05 in Figure 14). The elemental composition was calculated, $C_{23}H_{48}NO_7P$, and this gave a hit with a phosphocholine when searching in the Dictionary of Natural Products. Phosphocholines are compounds that are known to interact with the cell membrane and are not considered interesting for drug development (Hansen & Andersen, 2016). As a consequence of this, it was decided to terminate further work with these fractions.
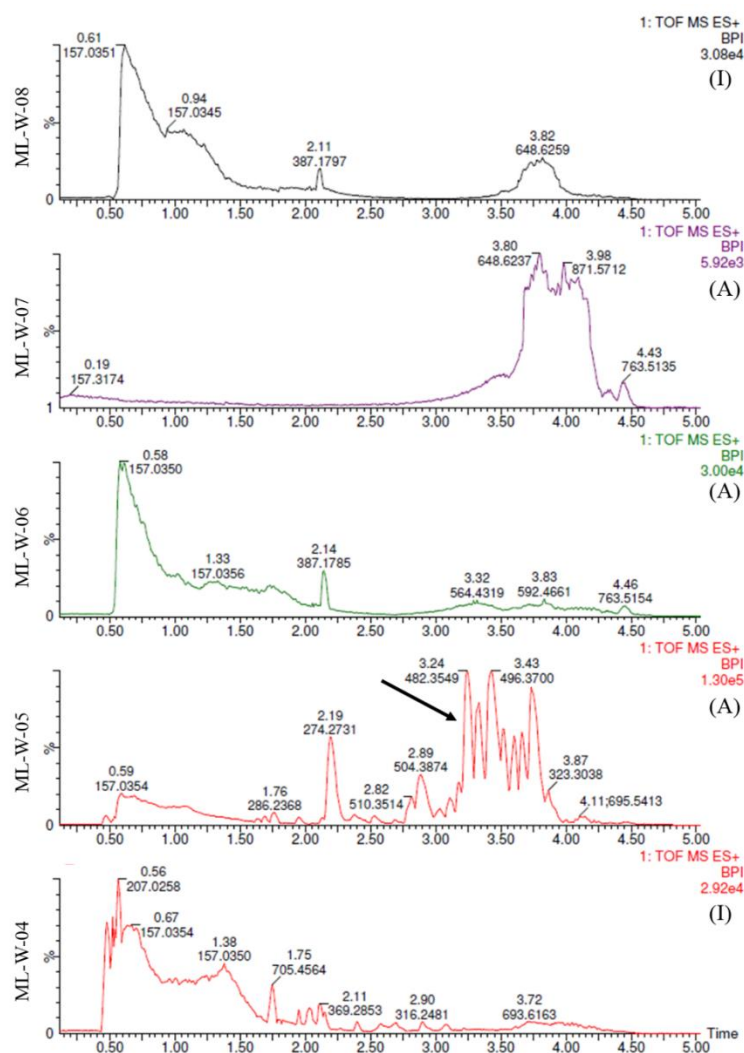
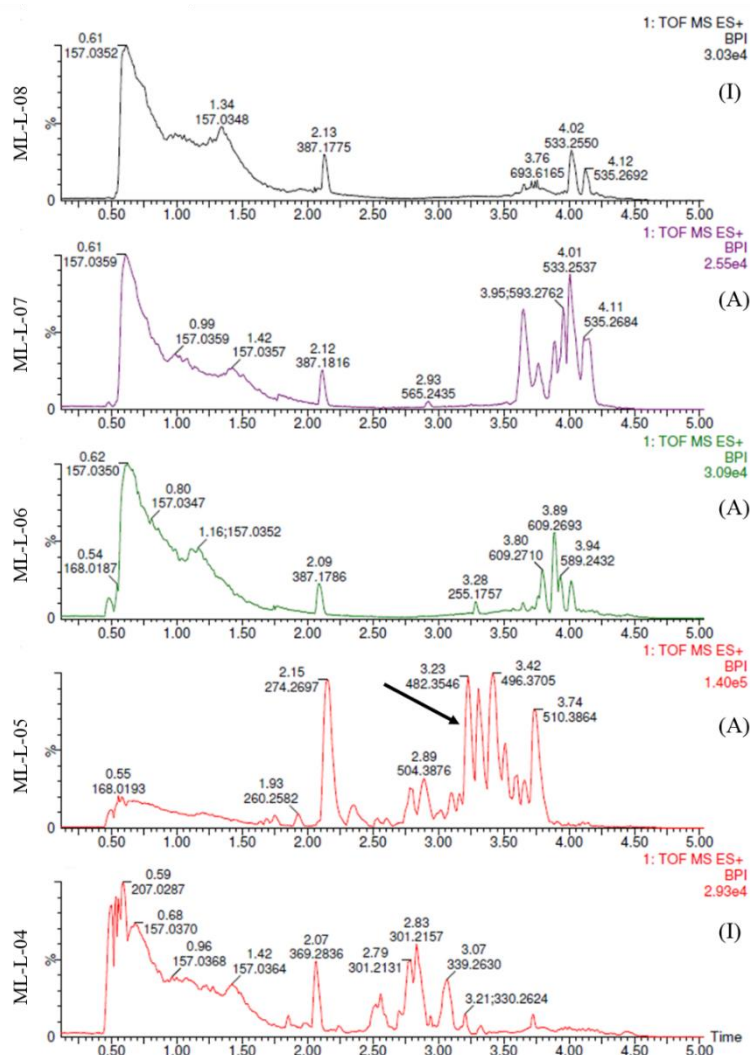*Figure 14: **UPLC-HR-MS Base peak intensity (BPI) chromatograms of fractions ML-W-04, W-05, W-06, W-07 and W-08.** The fractions were injected onto a Waters Aquity UPLC ® (2.1x100 mm, 1.7 μm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 0.5 mL/min (20-100% ACN with 0.1% FA over 3.5 min, then 100 % ACN over 1.5 min). Fractions marked "I" were inactive and "A" were active in the secondary anticancer screening. A phosphocholine was detected in the three active fractions (marked with a black arrow in fraction ML-W-05).*

Similar to ML-W-05, ML-W-06 and ML-W-07, a peak with *m/z* 482.35 was detected in the active fractions ML-L-05, ML-L-06 and ML-L 07 (Figure 15, marked with a black arrow in fraction ML-L-05). The elemental composition was calculated to be the same as the peak with *m/z* 482.35 in the active fractions of the aqueous extract of this invertebrate. Due to the presence of a phosphocholine, no further work was conducted with these fractions.

41

*Figure 15: **UPLC-HR-MS BPI chromatograms of fractions ML-L-04, -L-05, -L-06, -L-07 and -L-08.** The fractions were injected onto a Waters Aquity UPLC ® (2.1x100 mm, 1.7 μm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 0.5 mL/min (20-100% ACN with 0.1% FA over 3.5 min, then 100 % ACN over 1.5 min). Fractions marked "I" were inactive and "A" were active in the secondary anticancer screening. A phosphocholine was detected in the active fractions (marked with a black arrow in fraction ML-L-05).*

### 4.3.2   Bryozoa indet (BI)

In the active fraction BI-W-05, one compound in particular (labelled BI-L-478.4) stood out in the resulting chromatogram and was only observed in the active fraction (Figure 16-left). As a result, this compound was believed to be responsible for the observed bioactivity in the secondary anticancer screening. The mass spectrum for this compound can be seen in Figure 16 (right). The calculated elemental composition for BI-L-478.4 was $C_{25}H_{51}NO_7$. This formula gave no hits in Dictionary of Natural Products. The compound was submitted for isolation using prep HPLC-MS and was named BI-L-478.4. This name was used because the compound was

42

isolated from the organic extract of *B.indet*, where it was present in a larger amount than in the aqueous extract.
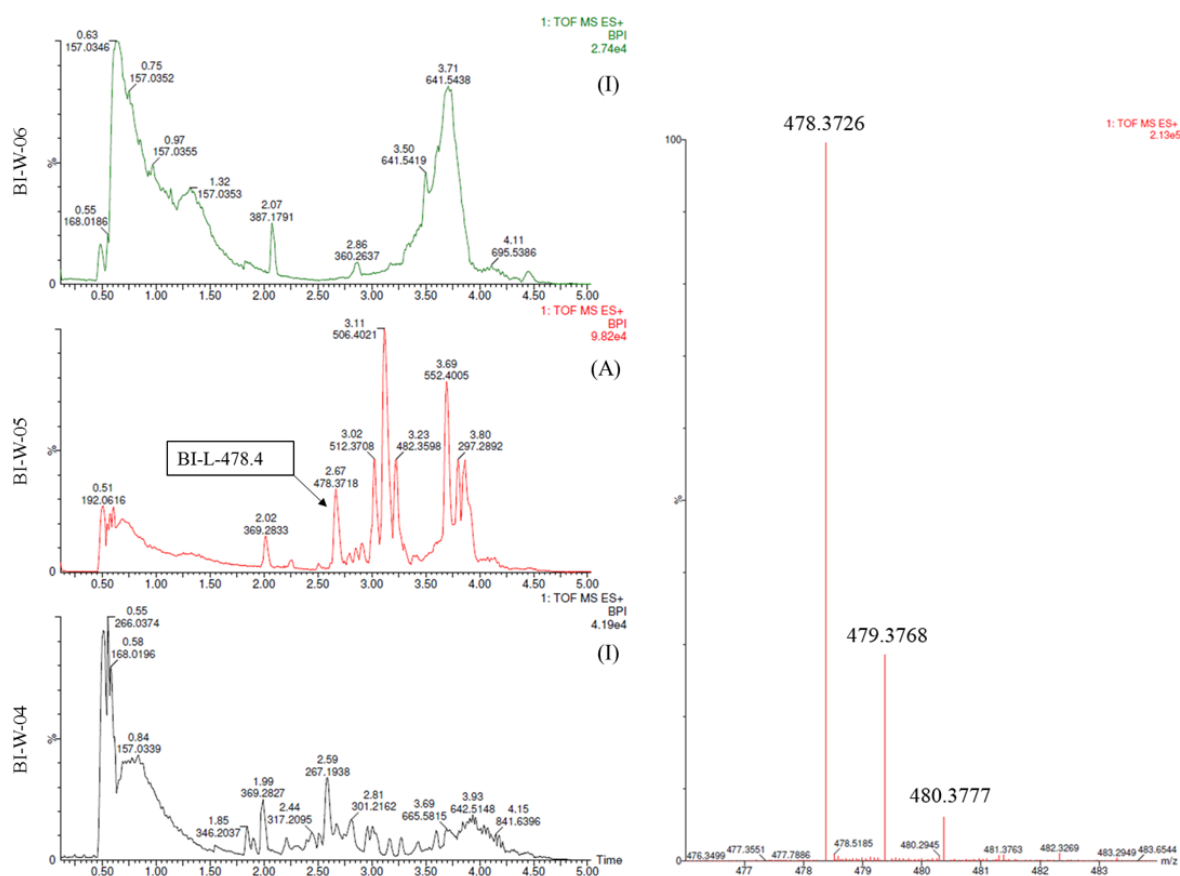


*Figure 16: **Left) UPLC-HR-MS BPI chromatograms of active fraction BI-W-05, and inactive fractions BI-W-04 and BI-W-06.** The fraction was injected onto a Waters Aquity UPLC ® (2.1x100 mm, 1.7 μm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 0.5 mL/min (20-100% ACN with 0.1% FA over 3.5 min, then 100 % ACN over 1.5 min). Compound BI-L-478.4 was exclusively found in the active fraction. Right) Mass spectrum for BI-L-478.4. The calculated elemental composition for BI-L-478.4 was $C_{25}H_{51}NO_7$. This formula gave no hits in Dictionary of Natural Products.*

### 4.3.3  Porifera indet (PI)

The active fraction PI-L-06 was analysed without comparison with the inactive fractions (PI-L-05 and PI-L-07) because there was no more sample available of these fractions. One peak stood out in the resulting chromatogram (Figure 17-left), highlighted as ianthelline, and was believed to be responsible for the observed bioactivity. Its elemental composition was calculated to be $C_{15}H_{17}Br_2N_5O_3$. This elemental composition gave a hit with iantheline when searching in Dictionary of Natural Products. Ianthelline has previously been isolated from the sponge *Stryphnus fortis* and its structure is published (Shearman, Myers, Beale, Brenton, & Ley, 2010). In addition, its bioactivity has been thoroughly investigated at Marbio (Hanssen,

43

Andersen, et al., 2012; Hanssen et al., 2014). As a consequence of this extensive previous work, no further work was conducted with this fraction.
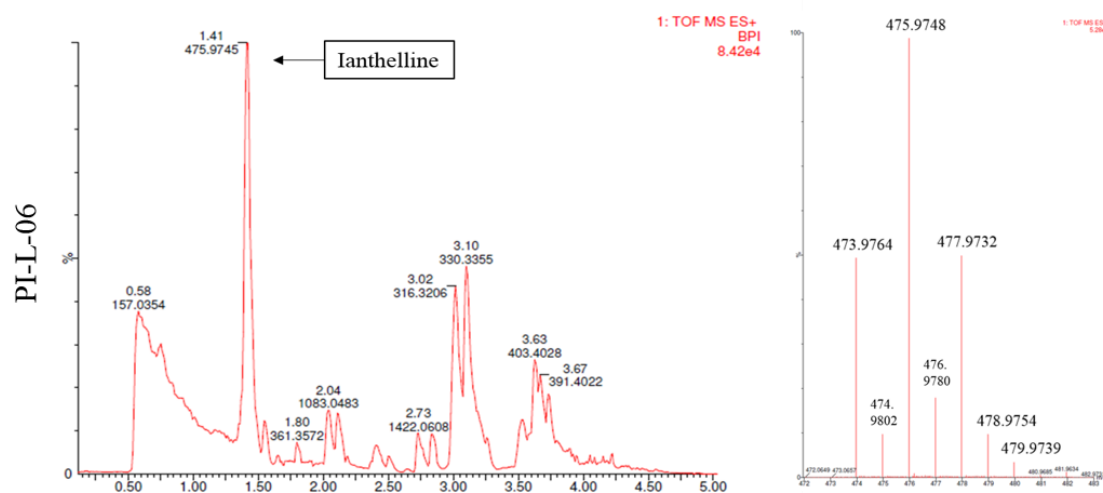


*Figure 17: **Left) UPLC-HR-MS BPI chromatograms of fraction PI-L-06**. The fraction was injected onto a Waters Aquity UPLC ® (2.1x100 mm, 1.7 μm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 0.5 mL/min (20-100% ACN with 0.1% FA over 3.5 min, then 100 % ACN over 1.5 min). Ianthelline was believed to be responsible for the observed bioactivity. Right) Mass spectrum for the compound marked ianthelline in fraction PI-L-06. The calculated elemental composition for this compound was $C_{15}H_{17}Br_2N_5O_3$. This elemental composition gave a hit with ianthelline in Database of Natural Products. Consequently, no further work was done with this fraction.*

### 4.3.4 Alcyonidium gelatinosum (AG)

In the active fraction AG-L-05, it was difficult to determine compound(s) responsible for the observed bioactivity (Figure 18). As a consequence of this, this fraction was further fractionated using prep HPLC-MS in an attempt to identify bioactive compound(s) in this fraction.
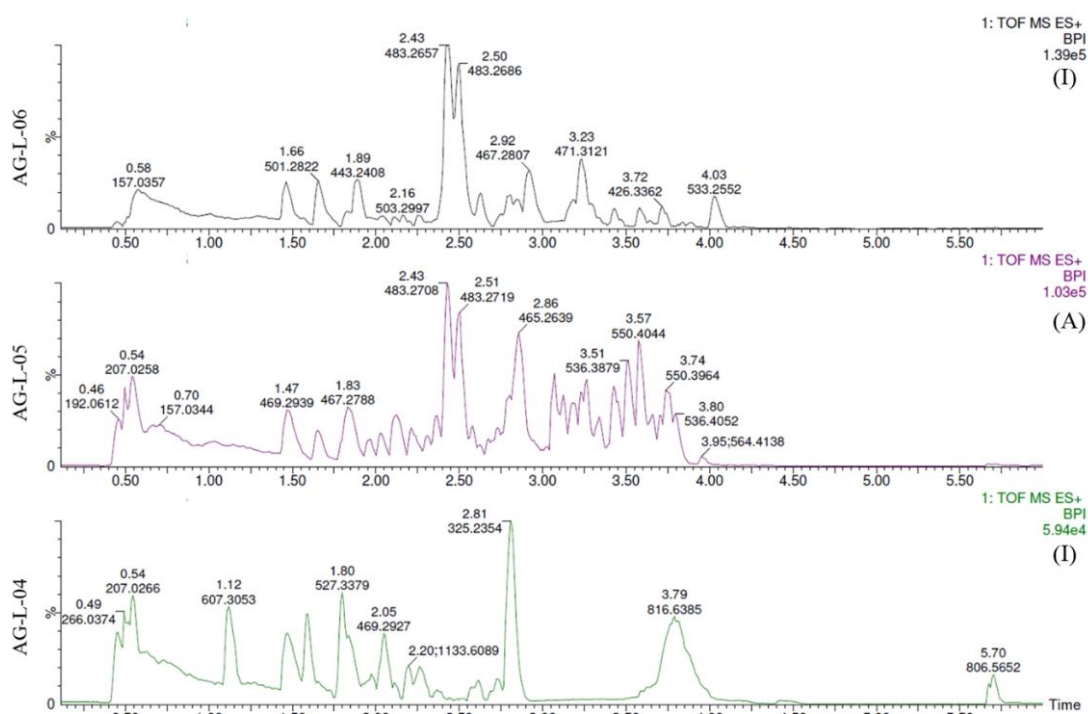
*Figure 18: **UPLC-HR-MS BPI chromatograms of fraction AG-L-05**. The fraction was injected onto a Waters Aquity UPLC ® (2.1x100 mm, 1.7 μm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 0.5 mL/min (20-100% ACN with 0.1% FA over 3.5 min, then 100 % ACN over 1.5 min). In this fraction, compound(s) responsible for the observed bioactivity could not be determined solely based on these results.*

### 4.3.4.1 Refractionation of AG-L-05 using prep HPLC-MS

Flash fraction 5 of the organic extract of *A.gelatinosum* was injected onto a RP C18 column and the eluting mobile phase was collected in 1 min fractions. This resulted in 40 collected fractions that were dried, re-dissolved and screened for activity against A2058. The resulting chromatogram can be seen in Figure 19.
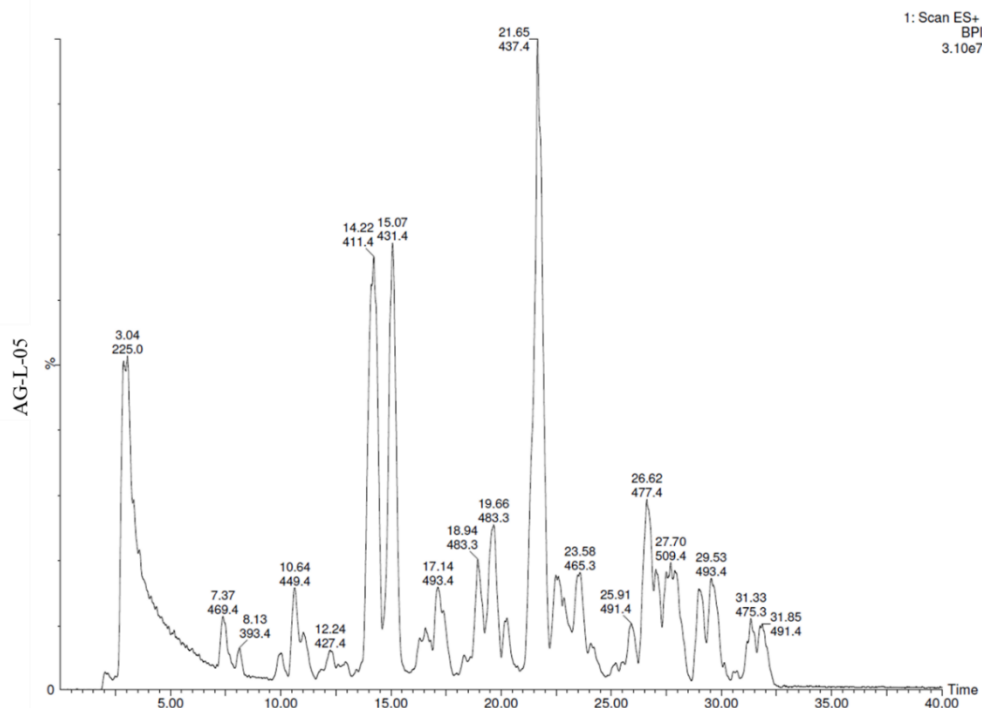
*Figure 19: **BPI chromatogram from the refractionation of fraction AG-L-05 with prep HPLC-MS.** The fraction was injected onto a Waters XTerra® Prep RP18 (10µm 10x300 mm) column. A gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 6 mL/min (30-70% ACN with 0.1% FA over 30 min, then 70-100% ACN with 0.1% FA over 10 min). During this refractionation, 40 1 min fractions were collected for further analysis in a tertiary anticancer screening.*

Anticancer activity in the refractionated fraction AG-L-05

The results from the tertiary anticancer screening against A2058 can be seen in Figure 20. Fractions 15, 17-22, 24, 27, 28, 33, 34, 36 and 37 were found to result in cell survival lower than 50%, and these fractions were therefore deemed active.
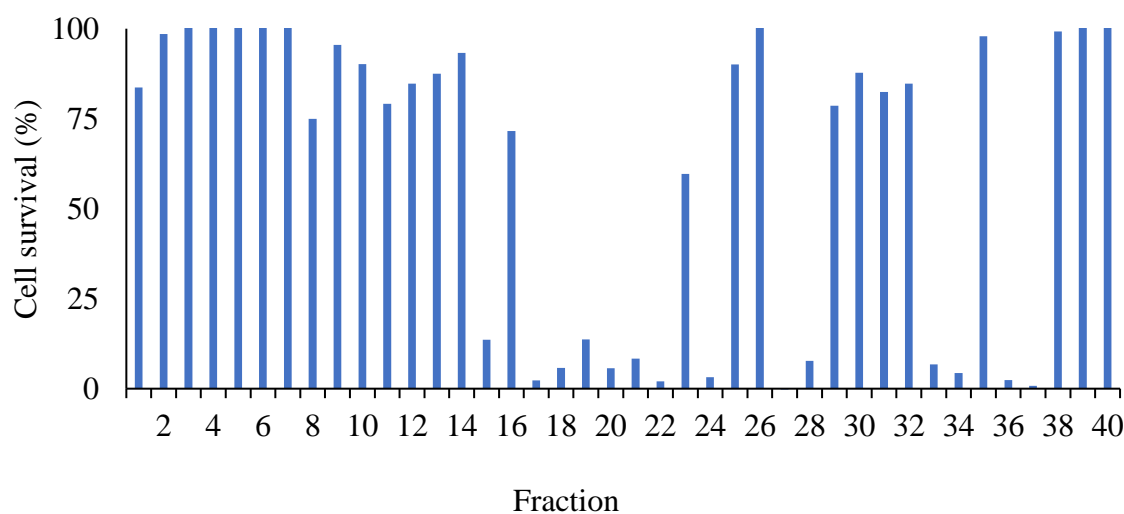


*Figure 20: **Results from tertiary anticancer screening of the 40 collected fractions from the refractionation of fraction AG-L-05.** The fractions were screened against A2058. Cell survival was determined by the MTS assay after 72 hours of exposure to the fractions and the values are means of triplicates.*

Dereplication of the active fractions from the refractionated AG-L-05

The results from the tertiary anticancer screening were compared to the chromatogram from the refractionation of the fraction AG-L-05 (Figure 21) in an attempt to identify bioactive compounds. Based on the results in Figure 21, compounds were selected as possible candidates for isolation. Extracted ion chromatograms (from Figure 19) for the target compounds were generated and mass spectra were analysed to calculate the elemental composition of the target compounds. The green bars in Figure 21 highlight the fractions that contained the compounds nominated for isolation.
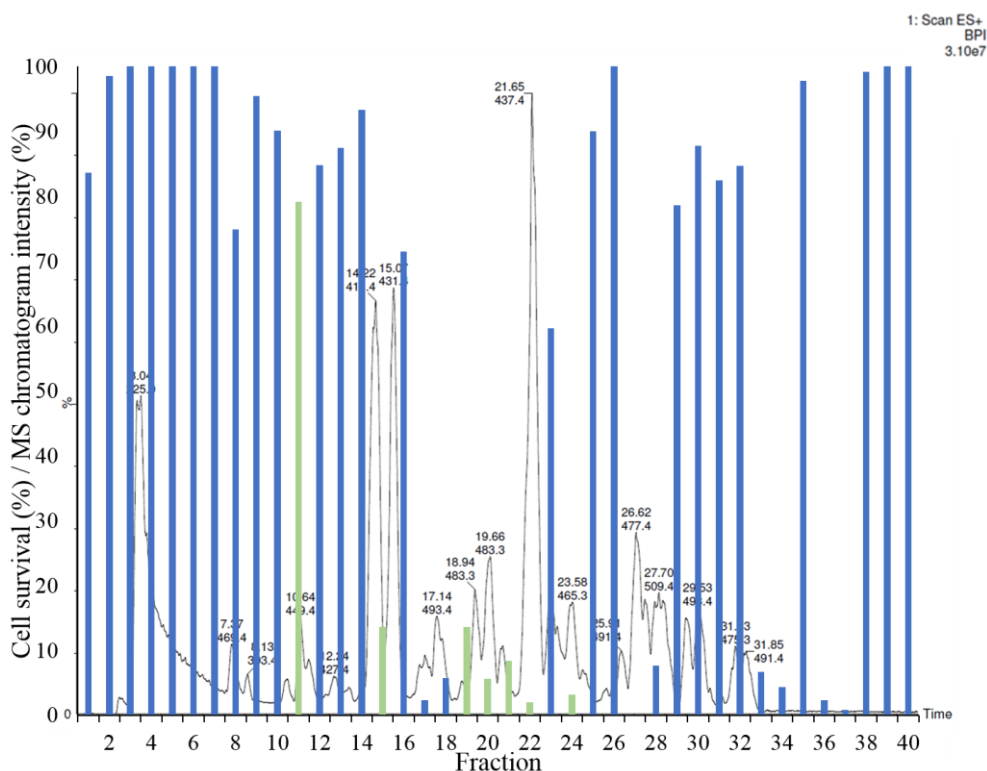


*Figure 21: **The results from the tertiary anticancer screening of the 40 fractions from refractionation of fraction AG-L-05 overlaid the prep HPLC-MS chromatogram from when the fractions were produced.** The green bars highlights the fractions that contained the compounds nominated for isolation. Compounds from fraction 11, 15, 19-22 and 24 selected for isolation.*

Fraction 11

A compound found in fraction 11 was submitted for isolation even though it did not result in significant inhibition of cell survival. This was done because this target compound (*m/z* 449.4) was believed to be structurally related to target compound *m/z* 465.3 (see Fraction 24). These two compounds differed with 16 mass units, indicating that *m/z* 449.4 (no elemental composition calculated) was a dehydroxylated version of *m/z* 465.3. The compound was named AG-L-449.4.

Fraction 15

Fraction AG-L-05-15 gave 14% cell survival of A2058. In this fraction (time 14-15 min in the chromatogram) two clear peaks were visible. The *m/z* of these peaks were 411.4 and 431.4 (Figure 22-left). Only one of these compounds, *m/z* 431,4, could however be found in the chromatogram from the UPLC-HR-MS analysis of AG-L-05. The mass spectrum for *m/z* 431.4 can be seen in Figure 22-right. The elemental composition of this compound was calculated to be $C_{28}H_{39}N_4$. A search in Dictionary of Natural Products gave no hits with this elemental composition. This compound was selected for isolation. In addition, the compound with *m/z* 411.4 was also selected for isolation. The compounds were named AG-L-431.4 and AG-L-411.1.
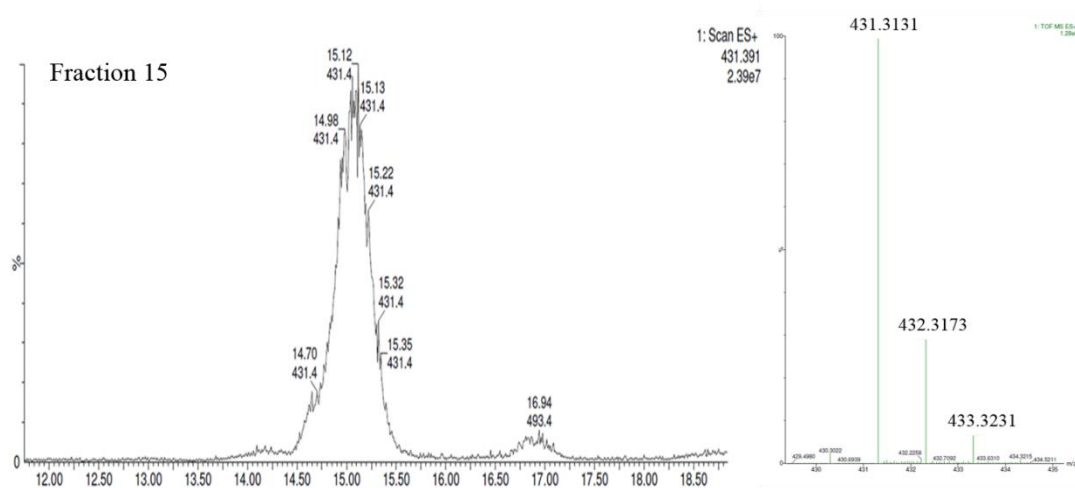


*Figure 22: **Left) Ion chromatogram for the compound from fraction 15 that was believed to be responsible for the observed bioactivity in the tertiary anticancer screening.** Right) Mass spectrum for this compound, the calculated elemental composition was $C_{28}H_{38}N_4$. A search in Dictionary of Natural Products gave no hits with this elemental composition.*

Fractions 19-21

Fractions 19, 20 and 21 gave 14%, 6% and 8% remaining cell survival, respectively, of A2058. Two clear peaks were visible in these fractions (time 19 to 20.5 min in the chromatogram), and these compounds had identical masses, *m/z* 483.3 (Figure 23-left). These compounds were found in the resulting chromatogram from the UPLC-HR-MS analysis of AG-L-05 and the mass spectrum for *m/z* 483.3 can be seen in Figure 23-right. Both compounds were calculated to have the same elemental composition: $C_{30}H_{34}N_4O_2$. A search in Dictionary of Natural Products gave no hits with this elemental composition. This compound, named AG-L-483.3 was submitted for isolation.
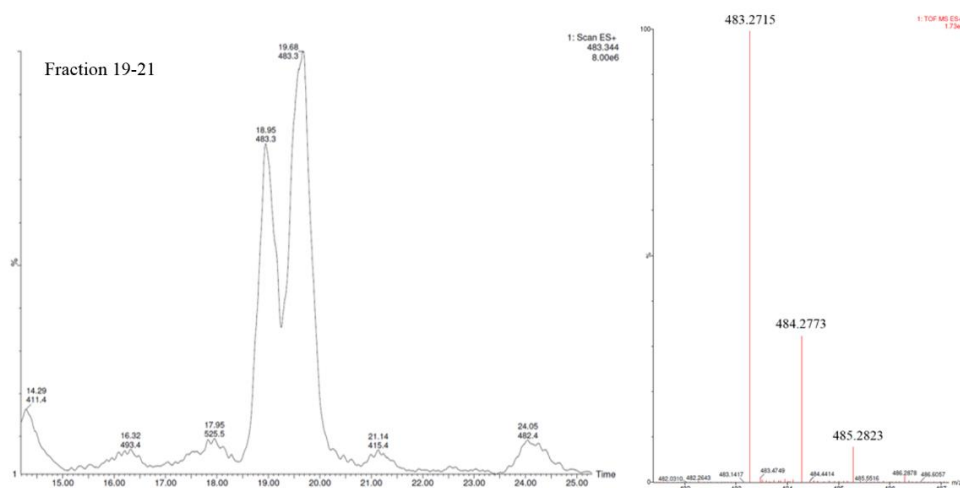
*Figure 23: **Left) Ion chromatogram for the compounds from fraction 19-21 that were believed to be responsible for the observed bioactivity in the tertiary anticancer screening.** Right) Mass spectrum for this compound, the calculated elemental composition was $C_{30}H_{34}N_4O_2$. A search in Dictionary of Natural Products gave no hits with this elemental composition.*

Fraction 22

Fraction 22 gave 2% remaining cell survival in the tertiary anticancer screening. In this fraction (time 21-22 min in the chromatogram) a big peak was visible (Figure 24-left). The *m/z* for this compound was 437.4. This compound was found in the resulting chromatogram from the UPLC-HR-MS analysis of AG-L-05 and the mass spectrum for *m/z* 437.4 can be seen in Figure 24-right. The elemental composition of this compound was calculated to be $C_{27}H_{27}N_4F$. A search in Dictionary of Natural Products gave no hits with this elemental composition. The compound, named AG-L-437.4 was submitted for isolation.
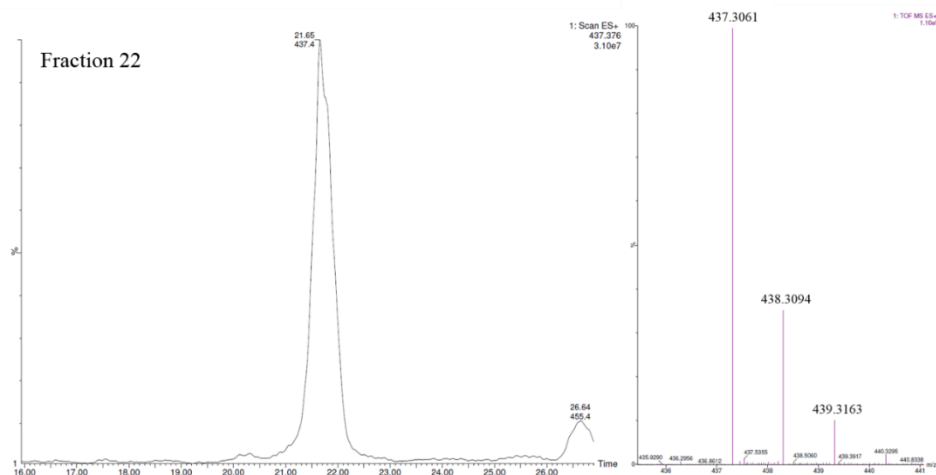


*Figure 24: **Left) Ion chromatogram for the compound from fraction 22 that was believed to be responsible for the observed bioactivity.** Right) Mass spectrum for this compound, the calculated elemental composition was $C_{27}H_{27}N_4F$. A search in Dictionary of Natural Products gave no hits with this elemental composition.*

49

Fraction 24

Fraction AG-L-05-24 gave 3% remaining cell survival in the tertiary anticancer screening. In this fraction (time 23-24 min in the chromatogram), two clear peaks were visible (Figure 25-left). These peaks had identical masses, *m/z* 465.3. These compounds were found in the resulting chromatogram from the UPLC-HR-MS analysis of AG-L-05. The mass spectrum for *m/z* 465.3 can be seen in Figure 25-right. Using Mass Lynx software, the elemental compositions for these compounds were calculated. Both compounds were calculated to have the same elemental composition: $C_{29}H_{36}O_5$. A search in Dictionary of Natural Products gave hits with four different compounds with the same empirical formula. Despite this, it was decided to isolate the compound, named AG-L-465.3, using prep HPLC-MS.
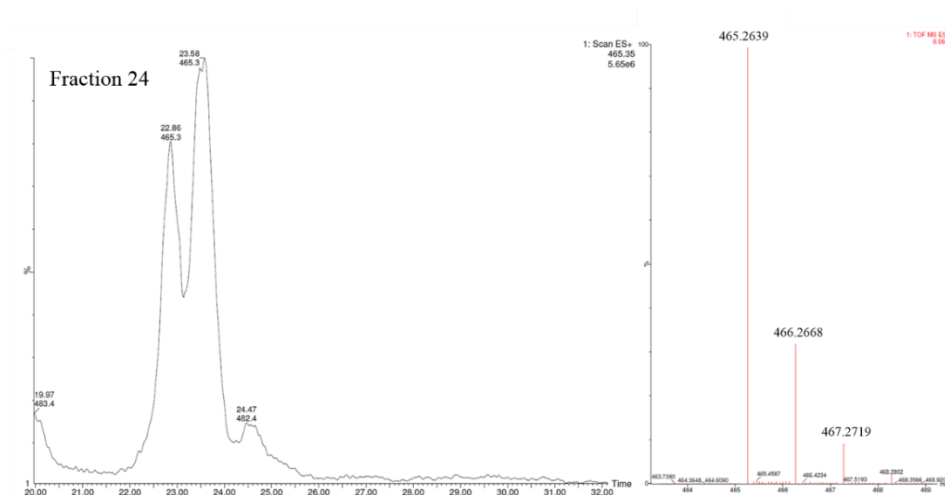


*Figure 25: **Left) Ion chromatogram for the compound from fraction 24 that was believed to be responsible for the observed bioactivity.** Right) Mass spectrum for this compound, the calculated elemental composition was $C_{29}H_{36}O_5$. A search in Dictionary of Natural Products gave hits with four different compounds with the same empirical formula.*

### 4.3.5   Summary dereplication

In total, seven compounds were nominated for isolation using prep HPLC-MS: A compound with *m/z* 478.4 in sample BI-W-05 and six compounds from sample AG-L-05. The target compound in BI-W-05 was isolated from the organic extract of *B.indet* and was therefore named BI-L-478.4. An overview of the target compounds and target compound names can be seen in Table 25.

*Table 25: **Compounds nominated for isolation from the fractions BI-W-05 and AG-L-05.** In total, seven compounds were nominated for isolation using prep HPLC-MS.*

| Fraction name | Target compound (*m/z*) | Name |
|---|---|---|
| BI-W-05 | 478.4 | BI-L-478.4 |
| | 449.4 | AG-L-449.4 |
| | 411.4 | AG-L-411.4 |
| | 431.4 | AG-L-431.4 |
| AG-L-05 | 483.3 (1) | AG-L-483.4 (1) |
| | 483.3 (2) | AG-L-483.4 (2) |
| | 437.4 | AG-L-437.4 |
| | 465.3 | AG-L-465.3 |

## 4.4 Prep HPLC-MS separation of target compounds from the organic extract BI-L

To isolate compounds from fractions that showed anticancer activity in the secondary anticancer screening, prep HPLC-MS purification was conducted on the organic extract BI-L.

### 4.4.1 Crude separation of the target compounds from BI-L

Five columns with different surface chemistry were evaluated for use in the first round of HPLC separation (for chromatograms see appendix, A4). Based on these results, the phenyl-hexyl column was chosen for the first round of HPLC separation (Figure 26) and the fractions were collected by time triggered fractionation. The compound in fraction 4 was selected for isolation based on the results from the secondary anticancer screening (Figure 10) and dereplication (Figure 16). The other remaining compounds were included in the isolation due to the amount present in the fraction and the possibility of achieving a pure compound within the time frame of this thesis. The target compounds, and the weight of the resulting dried fractions, were as followed:

Fraction 1: BI-L-340.3        (3.35 mg)

Fraction 2: BI-L-369.3        (0.85 mg)

Fraction 3: BI-L-665.6        (2.25 mg)

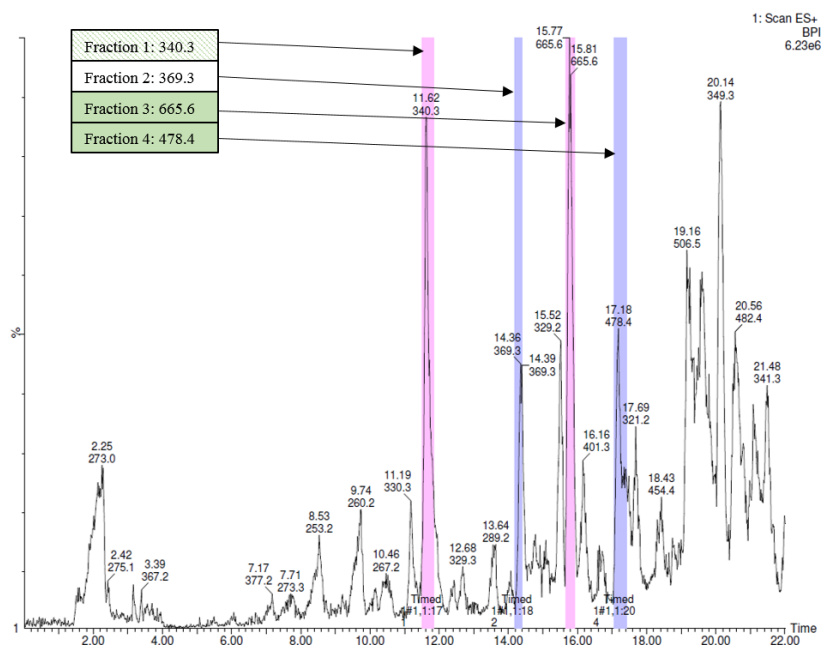Fraction 4: BI-L-478.4        (5.3 mg)

*Figure 26: **BPI chromatogram for the first round of HPLC separation of target compounds from the organic extract BI-L.** The compounds were separated using a phenyl-hexyl column. An elution gradient of ultra-pure water with 0.1 % FA and ACN with 0.1 % FA was used with a flow rate of 6 mL/min (10-67 % ACN with 0.1% FA over 19 min, then 100% ACN with 0.1% FA for 2min). Fractions 1-4 were collected by time triggered fractionation. The green boxes show the fractions that were selected for further purification (fraction 3 and 4). Fraction 1 was submitted for NMR analysis after this crude separation.*

It was decided to further purify BI-L-665.6 from fraction 3 and BI-L-478.4 from fraction 4. In addition, compound BI-L-340.3 from fraction 1 was submitted for NMR analysis after this crude separation because of the limited amount isolated of this compound.

## 4.4.2   Purification of BI-L-665.6 from fraction 3

To remove impurities from compound BI-L-665.6, a version of elution gradient 1and the fluoro-phenyl column were used. Fraction collection was triggered by retention time and the BPI chromatogram from this isolation can be seen in Figure 27. This isolation resulted in 0.6 mg of compound and it was therefore decided to not purify the compound further.
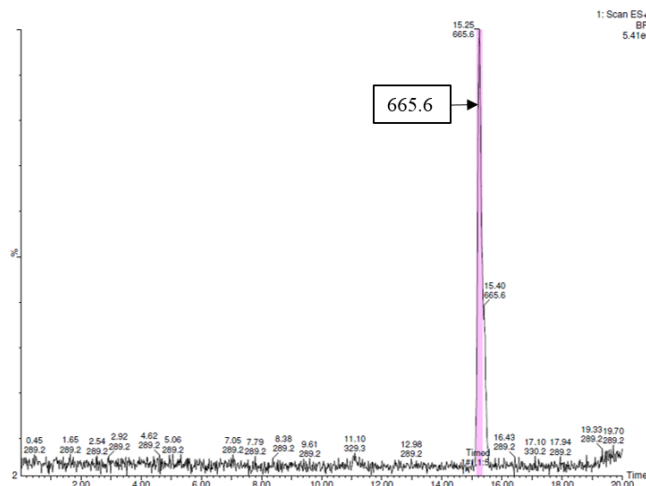
*Figure 27: **BPI chromatogram from the second round of HPLC separation of BI-L-665.6 from the organic extract BI-L.** The collected fraction from the first round of HPLC separation was further purified using a fluoro-phenyl column and a shortened version of elution gradient 1 (10-58 % ACN with 0.1 % FA over 16 min, then 100% ACN with 0.1% FA for 2min). This isolation resulted in 0.6 mg of compound.*

### 4.4.3 Purification of BI-L-478.4 from fraction 4

To remove impurities from compound BI-L-478.4, a version of elution gradient 1 and a fluoro-phenyl column were used. Fraction collection was triggered retention time and the BPI chromatogram from this isolation can be seen in Figure 28. This isolation resulted in 0.9 mg of compound and it was therefore decided to not purify the compound further.
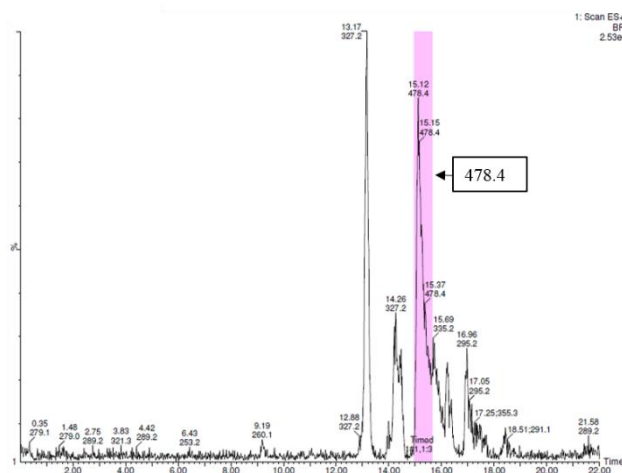


*Figure 28: **BPI chromatogram of the second round of HPLC separation of BI-L-478.4 from the organic extract BI-L.** The collected fraction from the first round of HPLC separation was further purified by using a fluoro-phenyl column and a shortened version of elution gradient 1 (10-64 % ACN with 0.1 % FA over 18 min, then 100% ACN with 0.1% FA for 2min). The isolation resulted in 0.9 mg of compound.*

### 4.5 Prep HPLC-MS separation of target compounds from the organic extract AG-L

To isolate compounds from fractions that showed anticancer activity in the tertiary anticancer screening, purification utilising prep HPLC-MS was conducted on the organic extract AG-L.

### 4.5.1 Crude separation of the target compounds

Five columns with different surface chemistry were evaluated for use in the first round of HPLC separation (for chromatograms, see appendix A5). Based on these results the phenyl-hexyl column was chosen for the first round of HPLC separation (Figure 29) and the compounds were collected by time triggered fractionation. The target compound in each fraction was nominated for isolation based on the results from the bioactivity screening and dereplication, as previously explained (Figure 21). The target compounds and the weight of the resulting dried fractions were as followed:

Fraction 1: AG-L-465.3          (12.15 mg)

Fraction 2: AG-L-449.4          (33.6 mg)

Fraction 3: AG-L-411.4          (56.7 mg)

Fraction 4: AG-L-431.4          (33.9 mg)

Fraction 5: AG-L-483.4 (1)      (21.75 mg)

Fraction 6: AG-L-483.4 (2)      (32.8 mg)
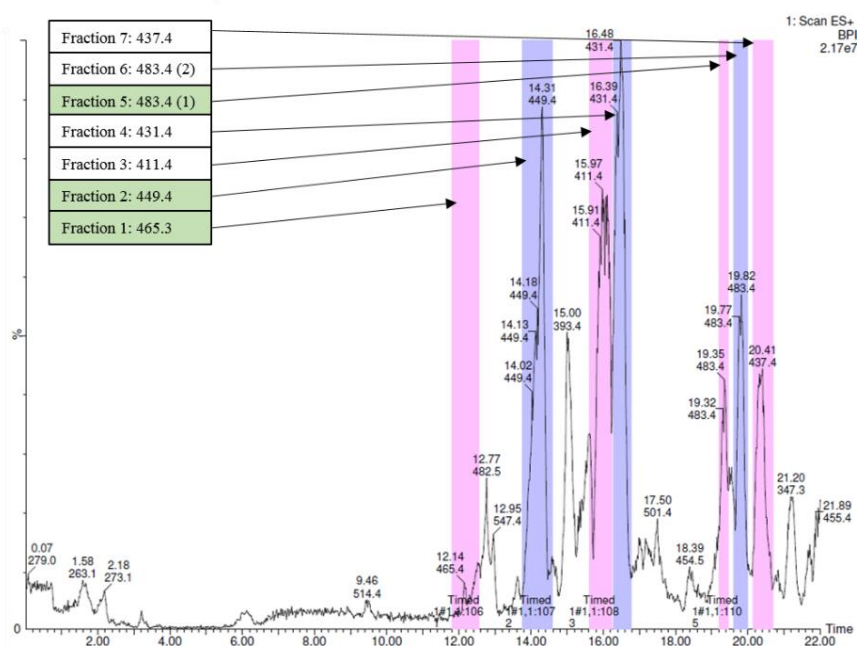
Fraction 7: AG-L-437.4          (38.8 mg)



*Figure 29: **BPI chromatogram for the first round of HPLC separation of target compounds from the organic extract AG-L.** The compounds were separated using a phenyl-hexyl column. An elution gradient of ultra-pure water with 0.1% FA and ACN with 0.1% FA was used with a flow rate of 6 mL/min (10-70% ACN with 0.1 % FA over 20 min, then 100 % ACN with 0.1% FA for 1 min). Fractions 1-7 were collected by time triggered fractionation. The green boxes show the fractions that were selected for further purification.*

It was decided to further purify AG-L-465.3 from fraction 1, AG-L-449.4 from fraction 2 and AG-L-541.4 from fraction 5 (AG-L-541.4 was present in fraction 5 from the first HPLC separation round).

## 4.5.2 Purification of AG-L-465.3 from fraction 1

To remove impurities from compound AG-L-465.3 in fraction 1, a version of elution gradient 1, and the fluoro-phenyl and Atlantis® column were used. Fraction collection was triggered by mass and the BPI chromatograms from these isolations can be seen in Figure 30. This isolation resulted in 1.25 mg of isolated compound after the third round of HPLC separation.
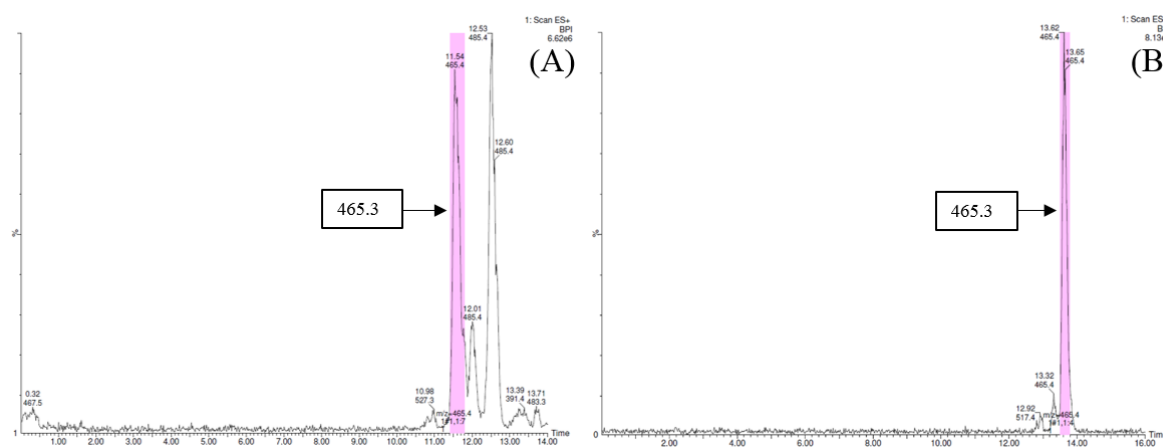


*Figure 30:* **BPI chromatogram of the second and third round of HPLC separation of AG-L-465.3 from the organic extract AG-L.** *A) The collected fraction from the first round of HPLC separation was further purified by using a fluoro-phenyl column and a shortened version of elution gradient 1 (10-46% ACN with 0.1% FA over 12 min, then 100% ACN with 0.1% FA for 1 min). B) In the third round of HPLC separation, the collected fraction from the second round of HPLC separation was further purified by using an Atlantis® column and a shortened version of elution gradient 1 (10-52% ACN with 0.1% FA over 14 min, then 100% ACN with 0.1% FA for 1 min). This isolation resulted in 1.25 mg of isolated compound after three rounds of HPLC separation.*

## 4.5.3 Purification of AG-L-449.4 from fraction 2

To remove impurities from compound AG-L-449.4 in fraction 2, a version of elution gradient 1, and the fluoro-phenyl and XTerra® column were used. Fraction collection was triggered by time (second round) or mass (third round), and the BPI chromatograms from these isolations can be seen in Figure 31. This isolation resulted in 5.47 mg of isolated compound after the third round of HPLC separation.
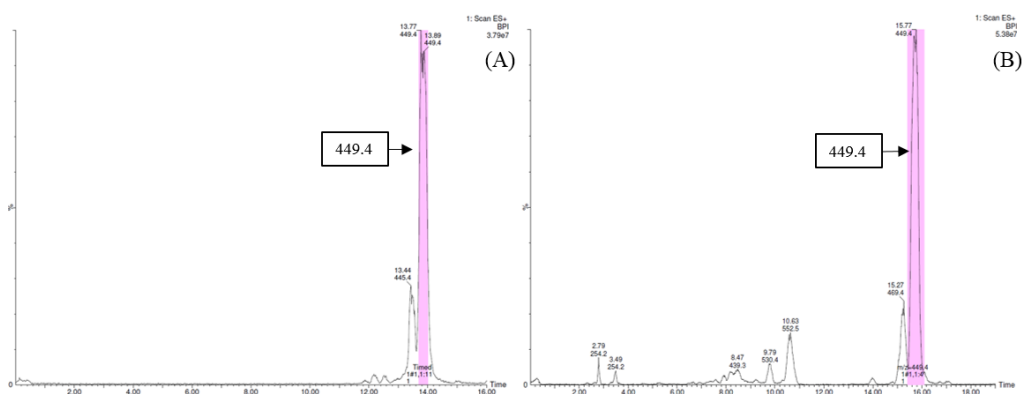
*Figure 31: **BPI chromatograms of the second and third round of HPLC separation of AG-L-449.4 from the organic extract AG-L.** A) The collected fraction from the first round of HPLC separation was further purified by using a fluoro-phenyl column and a shortened version of elution gradient 1 (10-52% ACN with 0.1% FA over 14 min, then 100% ACN with 0.1% FA for 1 min). B) The collected fraction from the second round of HPLC separation was further purified by using XTerra® column and a shortened version of elution gradient 1 (10-61% ACN with 0.1% FA over 17 min, then 100% ACN with 0.1% FA for 1 min). This isolation resulted in 5.47 mg of isolated compound.*

### 4.5.4   Purification of AG-L-541.4 from fraction 5

Fraction 5 from the first round of HPLC separation was collected in an attempt to start isolation of compound AG-L-483.4 (1). In addition to this compound, fraction 5 contained a compound with *m/z* 541.4 (named AG-L-541.4). It was decided to further purify AG-L-541.4 from this first round of HPLC separation. To remove impurities from AG-L-541.4, a version of elution gradient 1, and the Sunfire™ and Atlantis® column were used. Fraction collection was triggered by time and the BPI chromatograms from these HPLC separations can be seen in Figure 32. This isolation resulted in 3.03 mg of isolated compound after the third round of HPLC separation.
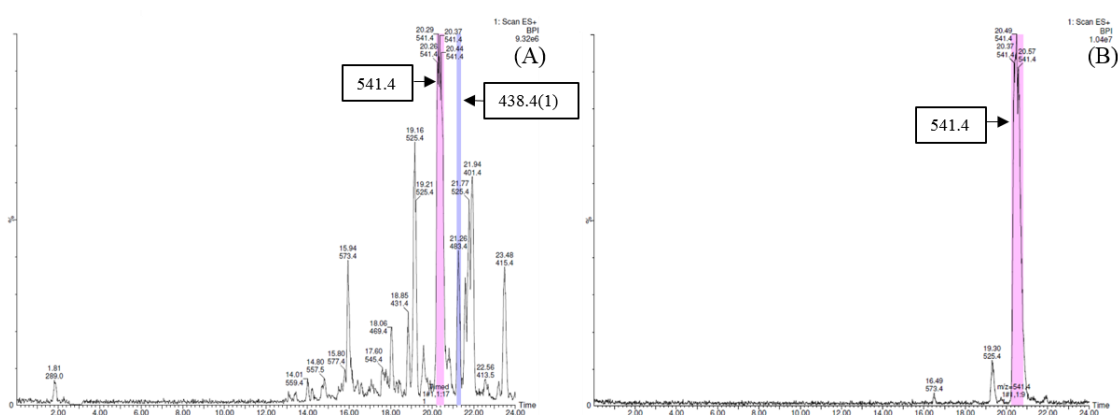


*Figure 32: **BPI chromatograms of the second and third round of HPLC separation of AG-L-541.4 from the organic extract AG-L.** A) The collected fraction from the first round of HPLC separation was further purified by using a SunFire™ column and a shortened version of elution gradient 1 (10-76% ACN with 0.1% FA over 22 min, then 100% ACN with 0.1% FA for 1 min). B) The collected fraction from the second round of HPLC separation was further purified by using a Atlantis® column and a shortened version of elution gradient 1 (10-76% ACN with 0.1% FA over 22 min, then 100% ACN with 0.1% FA for 1 min). This isolation resulted in 3.03 mg of isolated compound.*

### 4.5.5 Summary isolation

Three isolated compounds from BI-L and three isolated compounds from AG-L were submitted for structure elucidation employing NMR and HR-MS analyses. Table 26 shows the isolated compounds, as well as the calculated elemental composition and criteria for why the compounds were chosen for isolation. Some of these compounds were included in the isolation due to the amount of compound present (an elemental composition was not calculated for these compounds).

*Table 26: **Overview of isolated compounds from the extracts BI-L and AG-L.** In total, six compounds were isolated and submitted for structure elucidation employing NMR and HR-MS, A calculated elemental composition can be seen for AG-L-465.3 and BI-L-478.4.*

| Extract | Isolated compound ($m/z$) | Calculated elemental composition | Amount isolated (mg) | Criteria for isolation |
|---|---|---|---|---|
| BI-L | 340.3 | - | 3.35 | Amount of compound |
| | 478.4 | $C_{25}H_{51}NO_7$ | 0.9 | Bioactivity |
| | 665.6 | - | 0.6 | Amount of compound |
| AG-L | 465.3 | $C_{29}H_{36}O_5$ | 1.25 | Bioactivity |
| | 449.4 | Assumed to be $C_{29}H_{36}O_4$ | 5.47 | Believed to be structurally related to AG-L-465.4 |
| | 541.4 | - | 3.03 | Amount of compound |

## 4.6 NMR analysis of isolated compounds from BI-L and AG-L

The isolated compounds BI-L-340.3 (one round of HPLC separation), BI-L-478.4 (two rounds of HPLC separation) and BI-L-665.6 (two rounds of HPLC separation) from the extract BI-L, as well as AG-L-465.3, AG-L-449.4 and AG-L-541.4 (three rounds of HPLC separation for each compound) from the extract AG-L, were analysed using HR-MS and NMR. The structure was elucidated for BI-L-665.6, AG-L-465.3 and AG-L-449.4, while the other compounds in Table 26 were not purified enough to enable elucidation of the structure.

### 4.6.1 BI-L-665.6

The structure of compound BI-L-665.6 can be seen in Figure 33. The purity of the isolated product was 70-85%. The structure seen in Figure 33 was the most probable structure based on data simulations, but it was not possible to rule out other possible structures. The hydroxyl (OH)-group at carbon 19 could also be placed on carbon 20. The OH-group placed at carbon 28 could be placed anywhere from carbon 24 to carbon 31. The molecular formula of the compound was $C_{37}H_{69}O_8$. No hits in Dictionary of Natural Products or in SciFinder were detected, when searching with the formula $C_{37}H_{69}O_8$ or with the structure (allowing for structure similarity).
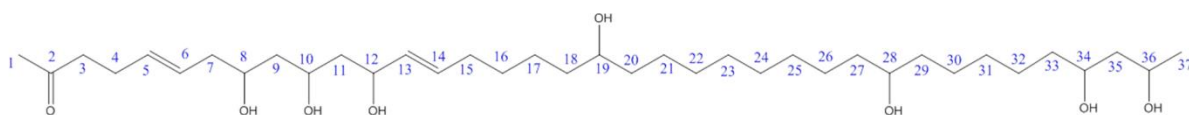
*Figure 33: **Molecular structure of BI-L-665.6 and atomic numbering of the molecule.** The molecular formula was $C_{37}H_{69}O_8$.*

### 4.6.2 AG-L-465.3 (Pon A)

The structure for compound AG-L-465.3 can be seen in Figure 34. The purity of the isolated product was ~90 % and the chemical formula was $C_{27}H_{44}O_6$. The structure elucidation shows that the isolated product was Pon A. The calculated elemental composition from dereplication of this compound did not match the molecular formula for Pon A.
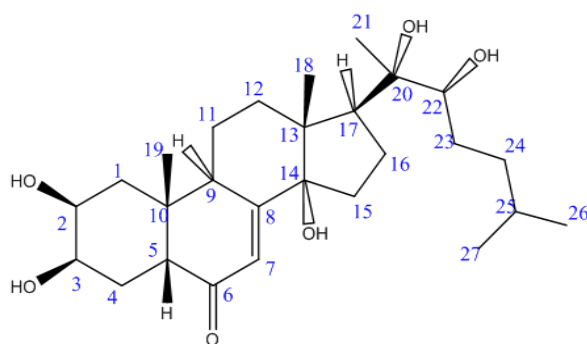


*Figure 34: **Molecular structure of compound AG-L-465.3 and atomic numbering of the molecule.** The structure elucidation shows that the isolated product is a steroid called Pon A.*

### 4.6.3 AG-L-449.4 (Dehydroxy-Pon A)

The structure of compound AG-L-449.4 can be seen in Figure 35 and the purity of the isolated compound was ~85 %. The molecular formula was $C_{27}H_{44}O_5$ and the isolated compound was a 14-deoxy version of Pon A.
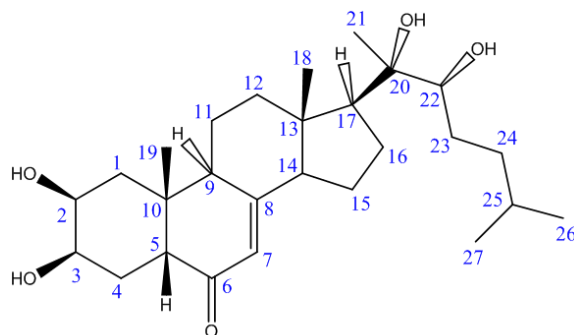


*Figure 35: **Molecular structure of compound AG-L-449.4 and atomic numbering of the molecule.** This molecule is a 14-deoxy version of Pon A (14-OH -> 14-H).*

## 4.7 Bioactivity profile of BI-L-665.6

The antibacterial activity of BI-L-665.6 was screened against the bacterial strains *S.aureus*, *E.coli*, *E.faecalis*, *P.aeruginosa* and *S.agalactiae*. No antibacterial activity was detected at the test concentrations. The compound`s anticancer activity was screened against three different cancer cell lines (A2058, HT-29 and MCF-7) and no anticancer activity was detected against these cell lines at the test concentrations. In addition, the compound`s toxicity towards normal human cells (MRC-5) was screened at the same concentrations, and the compound was not toxic against the cell line at the test concentrations.

## 4.8 Bioactivity profile of Pon A (AG-L-465.3) and dehydroxy-Pon A (AG-L-449.4)

The antibacterial activity of Pon A was screened against the bacterial strains *S.aureus*, *E.coli*, *E.faecalis*, *P.aeruginosa* and *S.agalactiae*. No antibacterial activity was detected at the test concentrations. The anticancer activity of Pon A and dehydroxy-Pon A was screened against A2058 (with DMSO controls). The results can be seen in Figure 36. Both Pon A and dehydroxy-Pon A had an effect on cell survival in some of the higher test concentrations. However, the DMSO controls show that the cells were affected by the amount of DMSO present in some of the test concentrations. Dehydroxy-Pon A had a slightly larger effect on the cell survival of A2058 than Pon-A in this screening, even at test concentration 150 µg/mL where the DMSO did not affect the cell survival.
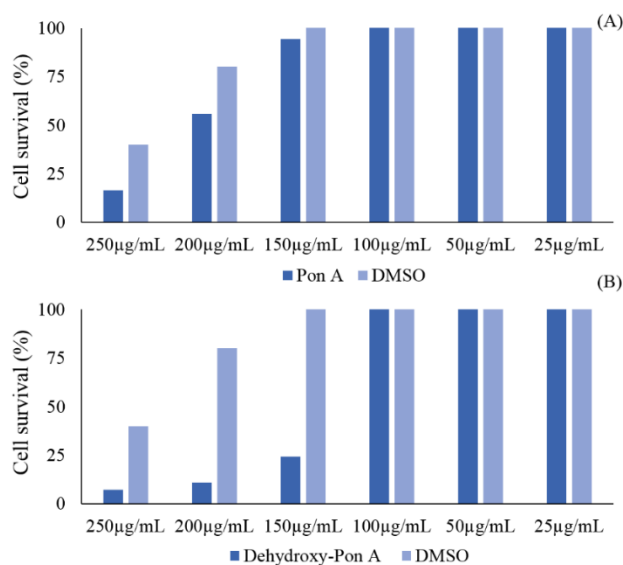


*Figure 36: **The effect of Pon A and dehydroxy-Pon A on the cell line A2058 were analysed in test concentrations ranging from 0.25 µg/mL to 250 µg/mL, with DMSO controls for each test concentration.** The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the compounds, and the values are means of parallel samples.*

The toxicity of Pon A and dehydroxy-Pon A towards MRC-5 was analysed (with DMSO controls). The results can be seen in Figure 37. Pon A and dehydroxy-Pon A an effect on cell survival of the normal human cells at some of the higher test concentrations. However, the DMSO controls showed that the cells were also affected by the amount of DMSO present in the test concentrations. Dehydroxy-Pon A had a slightly larger effect on the cell survival of MRC-5 than Pon-A in this screening.
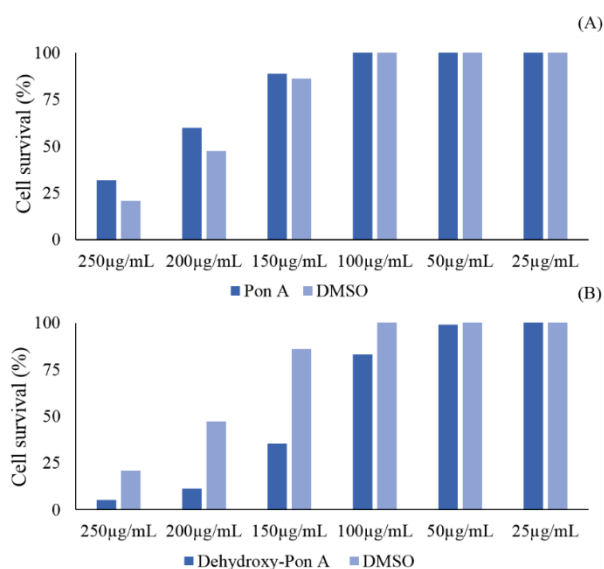


Figure 37: **The toxicity of Pon A and dehydroxy-Pon A towards MRC-5 were analysed in test concentrations ranging from 0.25 μg/mL to 250 μg/mL, with DMSO controls for each test concentration.** The cell survival (%) was determined by the MTS assay after 72 hours of exposure to the compounds, and the values are means of parallel fractions.

# 5 Discussion

Nature is a source of bioactive compounds with great chemical diversity, and the utilisation of NPs within the field of drug development has been a remarkable success. Historically, bioprospecting efforts have been focused on terrestrial species, while organisms living in the marine habitat largely were left unexplored. As the marine environment has been made more easily accessible (by improvements in scuba and submersible collection technologies), researchers have been able to focus on bioprospecting of marine species, including organisms harvested from the previously rather unexplored Arctic and Antarctic areas.

The overall aim of this thesis was to identify and isolate bioactive secondary metabolites from Arctic, marine invertebrates. The invertebrates were collected and extracted by Marbank, and prefractionated and screened in a primary anticancer assay as part of the ongoing workflow at Marbio, before work with this thesis started. Through secondary anticancer screening, dereplication, isolation and NMR analysis, three compounds were isolated and their structures were elucidated. Two previously reported compounds, Pon A and dehydroxy-Pon A, were isolated from *A.gelatinosum* for the first time. One novel compound, named BI-L-665.6 in this thesis, was isolated from *B.indet* (species not determined, *Celleporina surcularis* cf). The bioactivities of the isolated compounds were explored. The strong anticancer effects observed in their originating fractions could not be explained by the purified compounds.

## 5.1 Selection criteria for determining target compounds for isolation

The target compound needs to be isolated in amounts that are sufficient for structure elucidation, bioactivity confirmation and preferably also for bioactivity profiling. In order to achieve this in the short time period available for this thesis, several criteria were used in order to select compounds that were possible to isolate in adequate amounts. The bioactivity focus in this thesis was anticancer and activity in a secondary anticancer screening was one of the criteria used for determining target compounds for isolation. In the initial anticancer screening, 19 fractions were deemed active. While the primary anticancer screening tests the fractions at 50 µg/mL, the secondary anticancer screening tests the fractions at 50, 25 and 10 µg/mL in order to eliminate false positives or to reveal if the activity is lost at lower concentrations. By conducting a secondary anticancer screening on the fractions included in this thesis, 10 out of 19 fractions were eliminated. The reason behind the observed loss of activity is largely unknown, but might be due to sample content degradation, precipitation or other formation/decay of sample component aggregates (e.g. micelles) during storage (Di & Kerns,

2006; Eastwood et al., 2007). The results from the secondary anticancer screening allowed a focus on fractions with higher probability of containing compounds with activity against cancer cells and compounds that were stable enough to endure the treatment they would experience throughout the isolation process.

The nine active fractions (from four different invertebrates) from the secondary anticancer screening were dereplicated using UPLC-HR-MS. An elemental composition was calculated for relevant compounds found in the fractions and database searches were conducted. In the dereplication results obtained in this thesis, three of the most common outcomes of dereplication analysis are nicely illustrated. The fractions ML-L-05, -L-06, L-07, W-05, W-06 and W-07 contained phosphocholins (Figure 14 and Figure 15) and further work with these fractions was terminated. The fraction BI-W-05 contained a target compound that appeared to be novel and it was selected for isolation. The fraction PI-W-06 contained ianthelline that has reported anticancer activity (Hanssen, Andersen, et al., 2012; Hanssen et al., 2014), and work with this fraction was terminated. For fraction AG-L-05 it was difficult to determine the responsible bioactive compound(s) solely based on the results from UPLC-HR-MS analysis. A refractionation and tertiary anticancer screening was necessary to determine that AG-L-05 contained several compounds that were believed to be novel, and these compounds were selected for isolation. As mentioned in the introduction, dereplication is conducted to lower the probability of isolation of already reported compounds (Blunt & Munro, 2014). By employing dereplication before isolation in this thesis, it was possible to eliminate further work with the fractions containing already reported bioactive compounds (phosphocholine and ianthelline).

The elemental composition calculated in dereplication is a statistical calculation based on the mass spectrum of the target compounds. The researcher can influence this calculation based on the elements (and amount of the different elements present) that are included in the element list for calculation. E.g., including halogens if these are present in the mass spectrum or removing halogens if they are not present will narrow the number of possible candidates and make it more plausible that the calculation matches the actual elemental composition. There exist several challenges with using UPLC-HR-MS for dereplication, such as the difficulty in calculating an unambiguous elemental composition for compounds that only contain carbon, hydrogen, nitrogen and oxygen. This was experienced in this thesis with Pon A (AG-L-465.3) and the resulting database searches did not match with the already reported Pon A because the calculated elemental composition did not match with Pon A. The database searches gave four hits when the calculated elemental composition was used as search input. Based on the fact that

the calculated elemental composition only contained carbon, hydrogen and oxygen it was hypothesized that it would be difficult to calculate an unambiguous elemental composition. The compound was therefore nominated for isolation despite four hits in Dictionary of Marine Natural Products. Another challenge with using UPLC-HR-MS for dereplication is that the formation of adducts can affect the calculation of elemental composition (Kind 2010), and database searches. In this thesis, the formation of a sodium adduct complicated the calculation of the elemental composition for BI-L-665.6. This was not detected until after the compound was isolated. During dereplication, the three mentioned compounds were believed to be novel and were selected for isolation based on the criterias already mentioned.

Two other inclusion criteria affected the evaluation of dereplication results. First, a compound was included because it was believed to be structurally similar to target compounds. The metabolic pathways producing secondary metabolites often result in the production of several similar secondary metabolites (Fischbach & Clardy, 2007). Isolation of closely related compounds is highly valuable because it can be used to make an assumption regarding the pharmacophore of bioactive compounds (structure-activity relationship analysis) (Guha, 2013; McKinney, Richard, Waller, Newman, & Gerberick, 2000). In the extract AG-L, a compound (named AG-L-449.4) was present and was believed to be a structural variant of the target compound AG-L-465.3 (see Figure 19 and Figure 21). Second, some compounds were included in the isolation due to their abundant presence in the crude extracts, e.g. AG-L-541.4 (see Figure 32). This is normally not a major contributing factor in the process of selecting compounds for isolation at Marbio, but the limited time frame of this thesis necessitated strict inclusion criteria to ensure that compounds would be isolated in sufficient amounts for NMR analysis and bioactivity profiling. By considering factors like bioactivity, novel chemistry, possibility for structural variants and amount present in the extract, 11 compounds were isolated in the first crude separation of extracts BI-L and AG-L (Figure 26 and Figure 29).

## 5.2 Establishment of isolation strategies to enable isolation of target compounds

To establish an isolation strategy for the selected compounds, and to enable easy selection of columns for isolation steps after the first HPLC separation round, an initial scouting run was conducted. Here, the crude extract (pre-treated with a liquid-liquid partitioning step) was injected onto all five available prep HPLC columns. Implementation of a scouting run offers three main advantages: 1) The researcher is able to pick the best column for the first HPLC separation round. 2) If the compound is not pure enough after the first round, the scouting run chromatograms can be used to evaluate which columns offer the best separation of the desired

compound, and the compounds that co-eluted with it in the first round. 3) A semi-purified sample will mainly contain your valuable target compound. Using the crude extract for the scouting runs instead of this sample avoids wasting valuable sample on HPLC separation optimisation.

Different columns can be utilised to remove impurities and separate target compounds in several rounds of separation using prep HPLC-MS (Figure 38 and Figure 39). The columns have different packing material, but they may also differ in particle size and column dimensions, resulting in different separations of the sample components. In this thesis, five RP columns were used for HPLC separation (Figure 38). XTerra®, SunFire™ and Atlantis® have a $C_{18}$ hydrocarbon attached to their silica backbone, but they give rise to different separations because of differences in particle size and in silica-modifications. Fluoro-phenyl and Phenyl-hexyl share the same silica-backbone but differ in the groups coupled to this backbone, and therefore give rise to different separations (Waters, 2016). Scouting runs to determine which column gives the optimal separation of sample components should be conducted for each sample (Latif & Sarker, 2012).
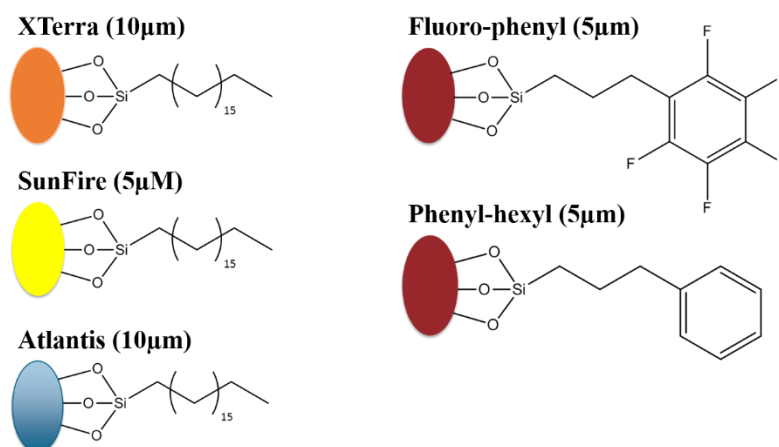


*Figure 38: **Overview of the column material of the five different RP columns used in this thesis.** The different coloured circles are representing the different silica backbone of the columns. Made with inspiration from reference (Waters, 2016).*

By combining several columns in the separation using prep HPLC-MS, we were able to purify three compounds in amounts that enabled elucidation of the structure: Pon A (AG-L-465.4), dehydroxy-Pon A (AG-L-449.4) and BI-L-665.6 (novel). Pon A and dehydroxy-Pon A were isolated using three rounds of separation with prep HPLC-MS, while BI-L-665.6 was isolated using two rounds of HPLC separation. Figure 39 exemplifies the effect of utilising different columns in several rounds of HPLC separation.
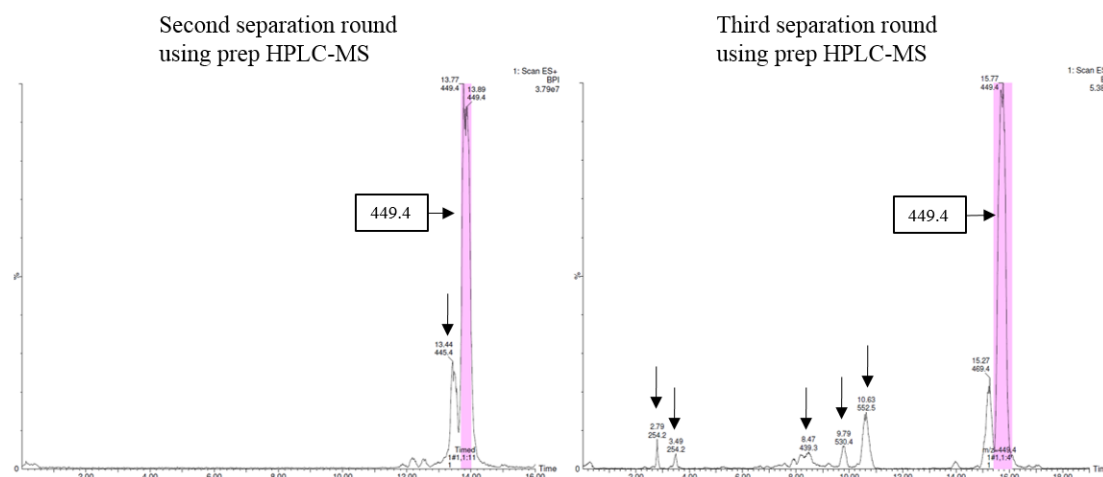
*Figure 39: **Chromatograms of the second and third separation round of AG-L-449.4, using prep HPLC-MS.** Left) Chromatogram showing the second HPLC separation round of dehydroxy-Pon A (AG-L-449.4). In this round, the highlighted impurity (black arrow) was separated from the target compound dehydroxy-Pon A. Right) Chromatogram showing the third HPLC separation round of dehydroxy-Pon A. The semi-purified sample from the second separation, collected as seen in the chromatogram to the left, was injected onto a fluoro-phenyl column. The highlighted impurities (black arrows) all co-eluted with dehydroxy-Pon A in the second HPLC separation round, but were separated by employing a column with a different stationary phase in the third HPLC separation round. This shows the strength of employing columns with different stationary phases as part of the isolation.*

## 5.3    Characterisation of isolated compounds

One of the major hurdles in marine NP drug discovery is the uncertainties surrounding supply of sufficient sample material and the difficulties regarding re-supply if more sample is needed on a later stage in the development process (David et al., 2015). Due to the low amount present of BI-L-665.6 in the sample after two HPLC separation rounds (Table 27), the compound was submitted for structure elucidation without any further purification. Elucidating the structure of BI-L-665.6 was complicated as the sample was not fully purified, and an unambiguous structure could not be decided. By using data simulations, a most probable structure was determined and this structure appears to be novel (even when allowing for structural dissimilarity in database searches). Due to the limited amounts available of BI-L-665.6, bioactivity profiling of this compound had to be done at low concentrations, and 10 µM was chosen as the highest assay concentration for this compound. The compound was not active at these concentrations, but it is still possible that it could be active at higher concentrations or in other bioassays than bioassays conducted in this thesis. Future work with this compound would involve isolating more of the compound (and purifying it further) from the aqueous extract of *B.indet*, a more conclusive elucidation of the structure and more biological characterisation of this novel compound.

*Table 27: **Collected wet weigh of the organism, weight of the isolated compounds and isolation yield of the isolated compounds BI-L-665.6, Pon A and dehydroxy-Pon A, isolated from B.indet and A.gelatinosum.***

| Organism | Wet weight | Weight isolated compound | Isolation yield |
|---|---|---|---|
| *Bryozoa indet* | 1.87 kg | BI-L-665.6: 0.6mg | 0.04 % |
| *Alcyonidium gelatinosum* | 5.7 kg | Pon A: 1.25mg | 0.06 % |
| | | Dehydroxy-Pon A: 5.47mg | 0.28 % |

Pon A and dehydroxy-Pon A were isolated in an amount that enabled a proper elucidation of the structure (Table 27). As mentioned, the calculated elemental composition for Pon A was not the same as the actual composition and the compound was falsely believed to be novel under dereplication. This compound has previously been isolated from the terrestrial plant *P.nakaii* in 1966 (Nakanishi, Koreeda, Sasaki, Chang, & Hsu, 1966). Pon A is an insect hormone, involved in regulating metamorphosis, and it shows activity in moulting assay (moulting is the manner in which an animal routinely shed parts of its body). Variants of this compound (differing only in the position of one OH group, 14-OH or 9-OH) have also been reported (Nakanishi, 1992). Pon A is used as an inducer for gene-switch systems (suitable as an inducer of ecdysone-inducible mammalian expression systems) and can be purchased from Sigma-Aldrich (Ponasterone A, P3490). To my knowledge, no other bioactivities have been reported for this steroid. In addition, Pon-A does not appear to have been isolated from *A.gelatinosum* previously. It could be discussed if the reason behind production of Pon A, with an effect against moulting, in this species could be predation pressure experienced in the marine environment.

The antibacterial and anticancer activity of Pon A and dehydroxy-Pon A were analysed. The compounds displayed no antibacterial activity at the test concentrations employed (0.3125 µM to 10µM). Sample availability of Pon A and dehydroxy-Pon A was not a limiting factor in the biological characterisation conducted for this thesis, and test concentrations up to 250 µg/mL were used in the anticancer screening. Anticancer activity was detected for Pon A and dehydroxy-Pon A at some of the higher test concentrations (Figure 36). Generally, the DMSO controls also showed an effect on cell survival at these concentrations and it can be discussed if the anticancer activity was a result of the compounds, or the DMSO concentration. In this anticancer screening, it appears to be a result of both the compound`s anticancer activity and the DMSO present in the test sample. Pon A was detected in a fraction with anticancer activity in the secondary anticancer screening, but this initial activity was only confirmed at very high test concentrations in the bioactivity profiling of the compound. It is therefore likely that a

sample component other than Pon A, or in synergy with Pon A, was responsible for the anticancer activity in the secondary screening (Hay et al., 1998). It would be possible to test the anticancer activity in the fractions after each HPLC separation round (bioassay-guided fractionation) to counteract the possibility of isolating compounds that are not biological active when isolated (Guo, Wang, Zhu, & Xu, 2016). This is a time consuming process, sample is consumed during the bioactivity screening after each separation, and it was not prioritised for the work conducted in this thesis.

In addition to information about the anticancer activity of the compounds, the results from the bioactivity profiling show that Pon A and dehydroxy-Pon A were toxic against normal human cells (see Figure 37). This is valuable information if these compounds show bioactivities in "non-cancer related" bioassays in the future, and therefore become drug candidates for other areas than cancer. New ways of delivering drugs (drug-antibody conjugates) may enable these compounds to be used as drugs despite this toxic effect against normal human cells (Ducry & Stump, 2010).

The results from this thesis indicated that dehydroxy-Pon A had a greater activity towards the cancer cell line,as well as the normal human cells, compared to Pon A. It is possible that the OH group at carbon 14 affects the activity displayed by these compounds. Earlier studies have showed that removal of the 14-OH increased the binding affinity to receptors five- to eightfold (Cherbas, Trainor, Stonard, & Nakanishi, 1982). Investigating the structure-activity relationship for these compounds would be a possible next step for characterising these compounds further. In addition, the compounds could be screened in other bioassays to fully characterise their biological activity.

# 6  Conclusions

By following strict criteria to initiate isolation of compounds in amounts that enabled structure elucidation and biological characterisation, three compounds were isolated and characterised in this thesis. One novel compound (BI-L-665.6) was isolated from *B.indet*, and a previously reported compound (Pon A), as well as a structural variant of this compound (dehydroxy-Pon A), were isolated from *A.gelatinosum*. BI-L-665.6 did not display antibacterial or anticancer activity at the concentrations screened in the bioassays. Pon A and dehydroxy-Pon A did not display antibacterial activity, but the compounds displayed minor anticancer activity at the test concentrations employed in the bioassay.

The results in this thesis demonstrate:

- The importance of dereplication to eliminate samples which should not be prioritised in a bioprospecting pipeline

- The importance of establishing isolation strategies to enable isolation of target compounds in amounts sufficient for structure elucidation and biological characterisation

- That structural variants of target compounds can be isolated together with the target compound, if a thorough dereplication is conducted prior to isolation

- That bioprospecting of collected marine invertebrates enables discovery of secondary metabolites with novel chemistry, as well as previously reported compounds in new species

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# 8  Appendix

A1: UPLC-HR-MS

Instrument parameters used for UHPLC-HR-MS analysis of active samples from the secondary anticancer screening can be seen in Table 28.

*Table 28:* **Instrument parameters for UHPLC-HR-MS.**

| Polarity | Positive |
|---|---|
| Low mass | 150 $m/z$ |
| High mass | 1500 $m/z$ |
| Scan time | 0.2 s |
| Source type | ESI |
| Source temperature | 120 ˚C |
| Desolvation temperature | 300 ˚C |
| Desolvation gas flow | 600 L/hour |
| Cone gas flow | 5 L/hour |
| Cone voltage | 110 V |
| Capillary voltage | 2.6 kV |

A2: Prep-HPLC-MS

Instrument parameters used for prep HPLC-MS refractionation of sample AG-L-05 and isolation of sample AG-L and BI-L can be seen in Table 29.

*Table 29:* **Instrument parameters for prep HPLC-MS.**

| Polarity | Positive |
|---|---|
| Low mass | 250 $m/z$ |
| High mass | 1100 $m/z$ |
| Scan time | 1 s |
| Source type | ESI |
| Source temperature | 120 ˚C |
| Desolvation temperature | 300 ˚C |
| Desolvation gas flow | 650 L/hour |
| Cone gas flow | 5 L/hour |
| Cone voltage | 42 V |
| Capillary voltage | 3 kV |

A3: UPLC-QToF-MS

Instrument parameters used in UPLC-QToF-MS analysis of isolated compounds can be seen in Table 30.

*Table 30: **Instrument parameters for UPLC-QToF-MS.***

| Polarity | Positive |
|---|---|
| Low mass | 50 *m/z* |
| High mass | 1500 *m/z* |
| Scan time | 0.20 s |
| Source type | ESI |
| Source temperature | 120˚C |
| Desolvation temperature | 500˚C |
| Desolvation gas flow | 800 L/h |
| Cone gas flow | 50 L/h |
| Cone voltage | 30 V |
| Capillary voltage | 0.8 kV |

A4: Isolation using prep-HPLC-Ms, sample BI-L

Five different columns were evaluated for their ability to separate the compound of interest from each other as well as from impurities in extract BI-L (Figure 40).
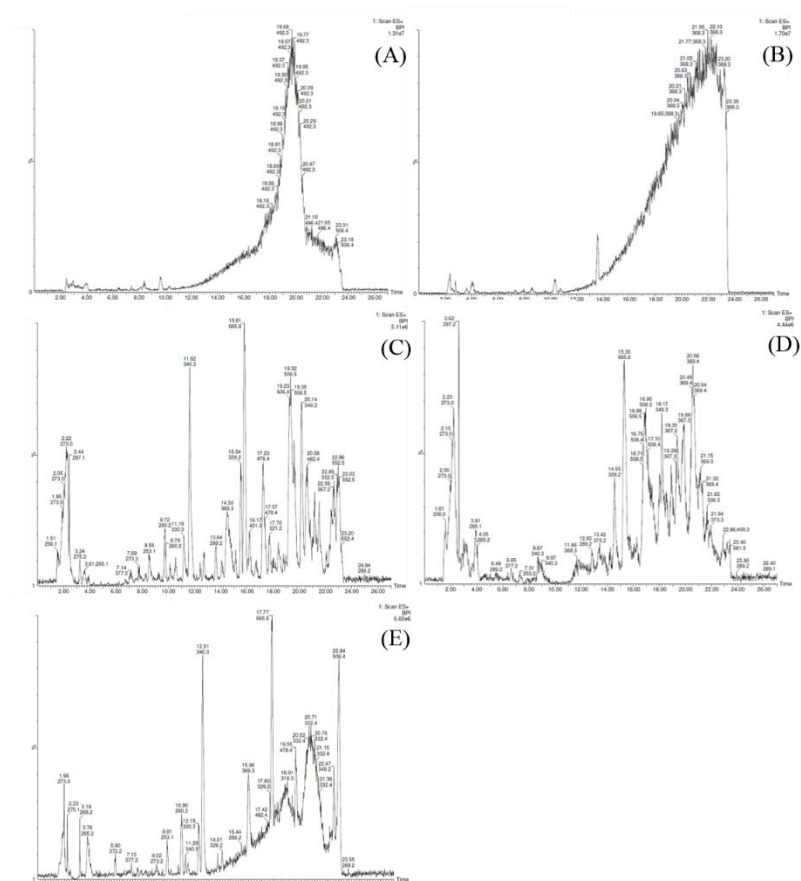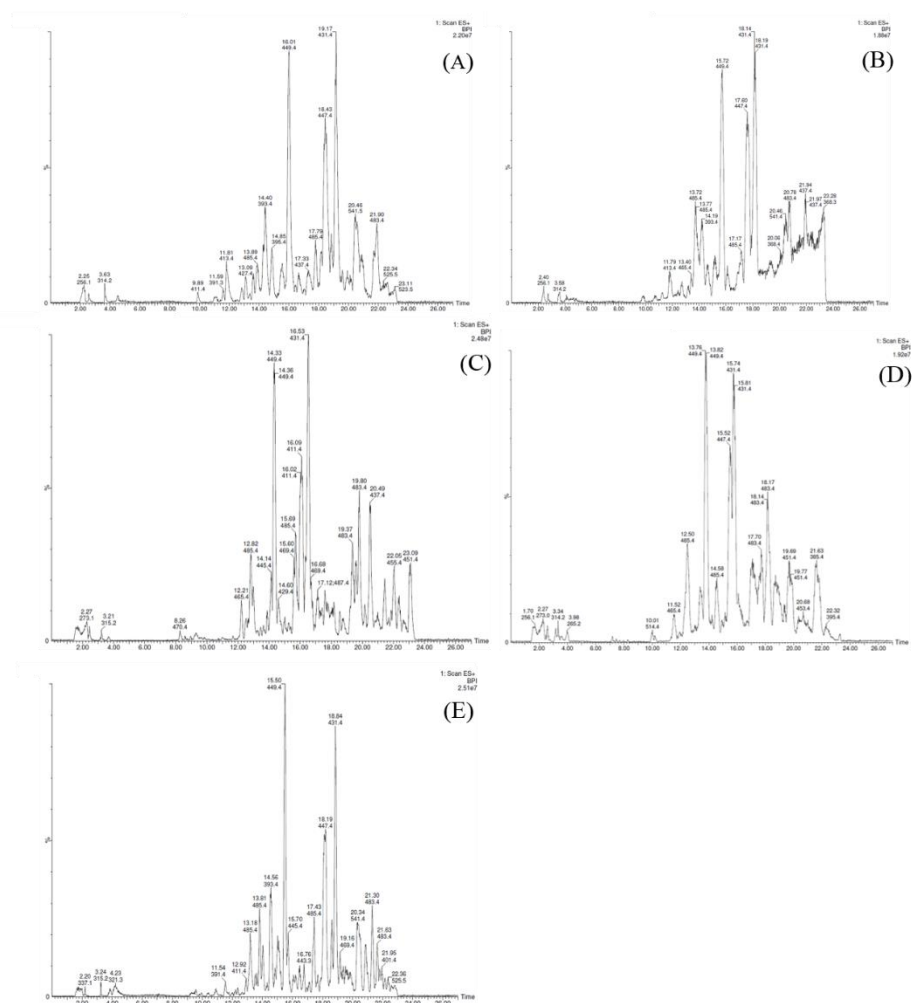


*Figure 40: **BPI chromatogram of isolation of the organic extract BI-L on five different columns with elution gradient 1**. A: Atlantis column, B: XTerra column, C: Phenyl-hexyl column, D: Fluoro-phenyl, E: SunFire. Phenyl-hexyl was used for the first round of HPLC separation.*

## A5: Isolation using prep-HPLC-MS, sample AG-L

Five different columns were evaluated for their ability to separate the compound of interest from each other as well as from impurities in extract AG-L (Figure 41).



*Figure 41: **BPI chromatograms of isolation of the organic extract AG-L on five different columns with elution gradient 1.** A: Atlantis column, B: XTerra column, C: Phenyl-hexyl column, D: Fluoro-phenyl, E: SunFire. Pheny-hexyl was used for the first round of HPLC separation.*

Isolation and Characterization of Secondary Metabolites from Extracts of Arctic Marine Invertebrates

Steroids isolated from the bryozoa *Alcyonidium gelatinosum*

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## 1 AIM OF STUDY

The aim of this study was to isolate and characterize secondary metabolites from extracts of eight Arctic marine invertebrates by using bioassay-guided isolation. The work is exemplified with the results from the organic extract of *Alcyonidium gelatinosum*.

## 2 INTRODUCTION

Marine invertebrates are known to produce a wide variety of secondary metabolites, many of which have interesting bioactivities. Anticancer screening was conducted on fractions of the organic extract of *A.gelatinosum*. In an attempt to identify the compound(s) responsible for the observed activity, the active fraction was dereplicated using UHPLC-HR-MS. In this poster, the isolation and structure elucidation of two compounds from *A.gelatinosum* are presented.
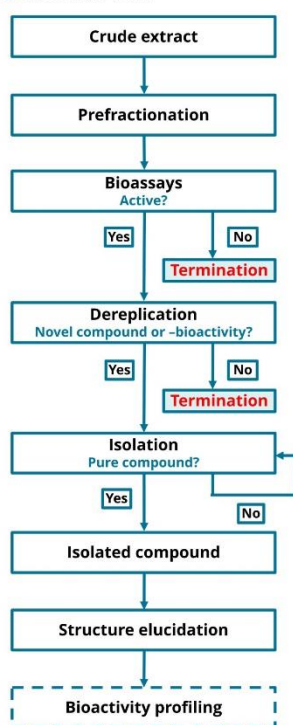
## 3 WORKFLOW



Figure 1: Overview of the workflow in this study. Further work will involve bioactivity profiling of the pure compounds.

- Fraction 5 of the organic extract of *A.gelatinosum* was found to be cytotoxic against the human melanoma cell line A2058.

- To determine compound(s) responsible for this observed bioactivity, this fraction was refractionated and fractions (40 in total) were again tested against A2058 (fig. 2).

## 4 DEREPLICATION

The anticancer screening results of the 40 fractions were compared to the chromatogram from the refractionation (Fig 2):

- **Target compound 1**: Steroid structure, gave no likely hits in database searches. Chosen for isolation.
- **Target compound 2**: Believed to be structurally related to target compound 1. Chosen for isolation.
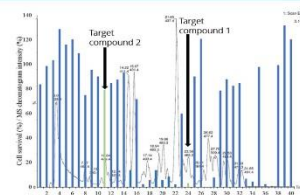


Figure 2: Overlay of anticancer screening results with the refractionation chromatogram. Compounds chosen for isolation are marked with a black arrow.

## 5 ISOLATION - PREP-HPLC-MS

Mass guided preparative HPLC was used in order to isolate target compound 1 and 2. The two compounds were isolated using three rounds of isolation, changing the column and elution gradient in each isolation round (exemplified with isolation rounds for target compound 1 in figure 3). The target compounds were isolated in sufficient amounts for NMR analysis.
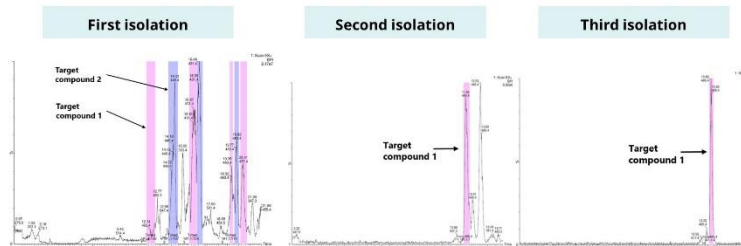


First isolation    Second isolation    Third isolation

Figure 3: Base Peak Intensity (BPI) chromatograms of the three isolation rounds of target compound 1.

## 6 STRUCTURE ELUCIDATION - NMR

The structure of target compound 1 and 2 was determined by analysing various 1D and 2D-NMR experiments, including HMBC, COSY and ROESY:
- **Target compound 1**: Ponasterone A (Fig. 4 and 5)
- **Target compound 2**: Dehydroxy-Ponasterone A

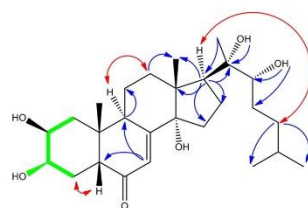Target compound 1: R=OH
Target compound 2: R=H



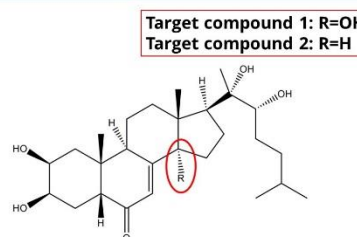Figure 4: Key HMBC, COSY and ROESY correlations for target compound 1 (Ponasterone A).

Figure 5: The structure of the isolated compounds with a red ring marking the structural difference between them. Target compound 1 (Ponasterone A): R=OH. Target compound 2 (dehydroxy-Ponasterone A): R=H

## 7 SUMMARY AND FURTHER WORK

- Cytotoxic activity towards the human melanoma cell line A2058 was detected in the organic extract of *A.gelatinosum* (fraction 5).
- The dereplication showed that a steroid, that gave no likely hits in databases, could be responsible for this observed bioactivity.
- This compound, as well as a suspected structurally related version of this compound, was isolated.

- The structures of these compounds were elucidated using HR-MS and NMR. The isolated compounds were Ponasterone A and dehydroxy-Ponasterone A.
- Further work will involve bioactivity profiling of these two compounds.

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