Master Thesis in Fishery Science

Seafood Science 60 credits

Bioactive molecules extracted from the marine sponge *Aplysilla sulfurea* and the marine phytoplankton *Porosira glacialis*

by Ingrid Varmedal

Department of Marine Biotechnology

Norwegian College of Fishery Science

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Sammendrag

Marin bioprospektering har blitt møtt med økende interesse både nasjonalt og internasjonal, og defineres som søken etter verdifulle kjemiske forbindelser og genetisk materiale fra det marine miljø. Målet med denne oppgaven har vært å undersøke tilstedeværelsen av bioaktive molekyler i to arktiske arter, en svamp og et fytoplankton. Det har spesielt blitt fokusert på anticancer, antibakteriell og antioksidative forbindelser.

Organiske og vandige ekstrakter av svampen *Aplysilla sulfurea* og metanolekstrakt av mikroalgen *Porosira glacialis* ble fraksjonert på preparativ HPLC til 40 fraksjoner i henhold til polariteten til komponentene i ekstraktet. Fraksjonene ble testet for antibakteriell, anticancer og antioksidativ aktivitet. Etter rekromatografi og videre undersøkelse av aktive fraksjoner ble de analysert ved hjelp av massespektrometri (LC-MS) for å forsøke å identifisere komponentene som var ansvarlige for de gitte aktivitetene. I de organiske ekstraktene fra svampen ble det påvist cytotoksiske (anticancer og antibakteriell) aktiviteter. En anticancer aktivitet ble bestemt å ha en molekylmasse på 472.9672. En slik masse tilsvarer stoffet Ianthelline, et di-bromert molekyl som tidligere er vist å ha antimikrobielle egenskaper. Det er ikke tidligere rapportert at forbindelsen er cytotoksisk ovenfor humane celler. Ut over dette ble det påvist antioksidativ aktivitet, men dette ble ikke videre undersøkt da prøvene var cytotoksiske. I de semi-vandige fraksjonene fra pellet av mikroalgen ble det påvist antioksidativ aktivitet. Det ble ikke påvist cytotoksisk aktivitet i noen andre fraksjoner i verken pellet eller supernatant fra dyrkningen av *P. glacialis*.Analyse ved hjelp av LC-MS ga ikke noe klart svar med hensyn til identifikasjon av den antioksidative forbindelsen. Data fra massespektrometrien var komplekse, og det vil kreve videre undersøkelser for å presentere oppklarende data.

Dette studiet har vist at svampen *Aplysilla sulfurea* inneholder det cytotoksiske molekylet Ianthelline, som antakeligvis har både anticancer og antibakteriell aktivitet. Dette bekrefter svamper som kanskje de mest interessante organismene i jakten på nye potensielle medikamenter. Videre undersøkelser kan føre til publikasjoner og patentering av dette molekylets egenskap som anticancerforbindelse. Videre ble det påvist vannløselige antioksidanter i ekstraktet fra *Porosira glacialis* (fytoplankton), og dette bør studeres videre.

Summary

Marine bioprospecting is a rapidly increasing field of interest worldwide, and is defined as the search for valuable chemical compounds and genetic material from marine sources. In the last decade benefits of drug development from marine resources has increased rapidly. The overall aim of this master thesis has been to investigate and study the presence of bioactive molecules in two Arctic species, a sponge and a phytoplankton. Specifically it has been focused on bioactivities with anticancer, antibacterial and antioxidant properties.

Aqueous and organic extracts of the Arctic sponge *Aplysilla sulfurea* and methanolic extracts from the Arctic phytoplankton *Porosira glacialis* were fractionated using preparative HPLC into 40 fractions according to the polarity of the components in the extract. The fractions were tested for antibacterial, anticancer and antioxidative activities. After re-chromatography, active fractions were analyzed by mass spectrometry in order to identify components responsible for the confirmed activities. In the organic extracts of the sponge, fractions containing cytotoxic activity were found. Ianthelline, a di-brominated molecule with molecular mass 472.9672 was tentatively identified as the cytotoxic compound. The antibacterial activity of this molecule has previously been published, but the cytotoxicity related to human cancer cell lines has not been reported. The antioxidative activity found in the same fractions was not further investigated due to the likely cytotoxicity of the compound. The semi-aqueous fractions from the pellet of the phytoplankton *Porosira glacialis* were found to have antioxidative activity. No cytotoxicity was found, and this made the antioxidative components interesting for further investigation by mass spectrometry in order to elucidate the structure. However, the data were complex and not clear, and further investigation will be necessary in order to come to an identification.

The present study has shown that the sponge *Aplysilla sulfurea* contains the cytotoxic molecule Ianthelline, with probably both antibacterial and anticancer activity. This confirms sponges as very interesting species when searching for possible new drug leads. The further investigation may lead to publications and patenting of the anticancer activities found in Ianthelline. Furthermore, the phytoplankton *Porosira glacialis* is possessing water-soluble antioxidants. Further investigation of the fractions of interest will be carried out.

Abbreviations

ACN	Acetonitrile
DCM	Dichloromethane
D-MEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylendiaminetetraacetate
E-MEM	Eagle's Minimal Essential Medium
ESI	Electrospray Ionization
EtOH	Ethanol
FBS	Fetal bovine serum
FRAP	Ferric reducing ability of Plasma
FWHM	Full width at half maximum
HCl	Hydrocloric acid
HPLC	High performance liquid chromatography
ISA	Iso-Sensitest-Agar
LC-MS	Liquid chromatography – mass spectrometry
MeOH	Methanol
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MTT	2H-Tetrazolium, 2-(4,5-dimetyl-2-3,5-difenyl-, bromide)
MP	Mobile phase
NaCl	Sodium chloride
NCI	National Cancer Institute
ORAC	Oxygen Radical Absorbance Capacity
OD	Optical density
PBS	Phosphate buffered saline
RPMI1640	Roswell Park Memorial Institute Medium
ROS	Reactive oxygen species
RT	Room temperature
Rpm	Rounds per minute
TOF	Time-of-flight
TPTZ	2,4,6-tripyridyl-s-triazine
TIC	Total ion chromatogram
UV	Ultraviolet

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1. Introduction

Nature has always been a resource for humans providing us among others essentials in nutrition and medicine. The interest for research based on terrestrial sources has lead to a wide range of projects concerning natural products. In 1928 Fleming's discovery of the antibiotic substance penicillin from the fungus *Penicillium notatum* revolutionized medical treatment of humans and marked the beginning of modern antibiotics. So far, research on terrestrial animals, plants, fungi and bacteria has resulted in many new drugs, and it is estimated that approximately 60% of the pharmaceutical drugs on the market today originates from these sources. However, in the past decades there has been an increasing interest towards the marine environment.

Marine bioprospecting is a rapidly increasing field of interest worldwide, and is defined as the search for valuable chemical compounds and genetic material from marine sources. Sometimes the term is used more narrowly referring only to the primary collection of biological material for subsequent use for biodiscovery, or more broadly to include a wider field of interest. Biodiscovery is the extraction and testing of molecules for biological activity, identification of compounds with promise for further development, and research on the molecular basis for the biological activity (Bailey and Dundas, 2001).

The ocean covers 70% of the surface of the earth, and is still to a great extent unexplored. Accelerated technological development in sub-sea level sampling and harvesting equipment has increased the availability to areas below sea level (Synnes, 2007). Since the 1990's an increasing interest in bioprospecting of the marine environment has resulted in the discovery of bioactive compounds from several marine species. In the last decade benefits of drug development from marine resources has increased rapidly. The interest in drugs from the marine environment resulted as early as 1951 in three reports on nucleosides from marine sponges. This lead to the development of the chemical derivatives ara-A and ara-C, nucleosides that have been in clinical use in anticancer treatment for decades (Molinski et al., 2009). Another compound in clinical use today is ziconotide (Prialt, Elan Pharmaceuticals), a peptide originally discovered in a tropical cone snail, *Conus magus*. It acts as a calcium channel blocker, inhibiting pain signals from reaching the brain, and was in 2004 the first marine-derived compound to be

approved for the treatment of chronic pain in the United States (Molinski et al., 2009). In nature the compound is used to paralyze the snail's prey or enemies.

The habitats of marine organisms incline them to make adaptive adjustments, and to develop unique systems, which make them differ from terrestrial organisms. Marine organisms living in the Arctic are exposed to extreme conditions with regard to temperatures and light. Because of this, organisms are developing unique qualities and biochemical processes in order to survive under these extreme circumstances. Such extremophile organisms are interesting in the search for unique, bioactive compounds. In addition, phytoplankton can be exposed to extreme salinity and oxygen saturation under the ice in the Arctic (McMinn et al., 2005). Different phyla have been investigated for the presence of interesting marine natural products. Figure 1 shows that the differences between the hits in each phylum are big. The most promising organisms are microorganisms, sponges and cnidarians, and microorganisms and sponges have also had a significant increase in number of discovered compounds per year from 1965 to 2007.

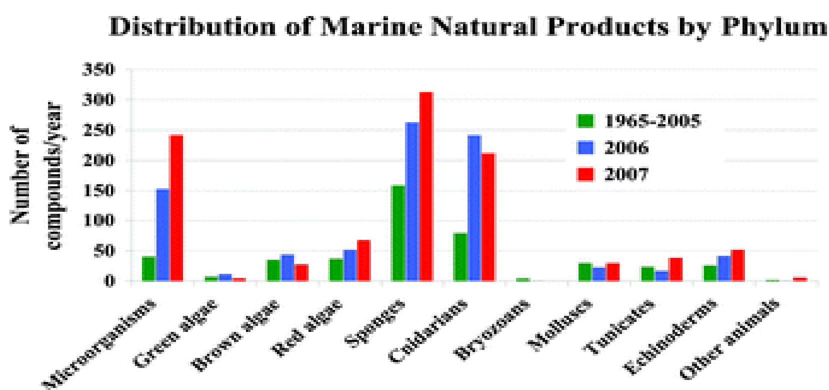

Figure 1: Distribution of marine natural products by phylum from Natural Product Reports (Blunt et al., 2006).

MabCent-SFI is situated in Tromsø, and is a Centre for Research-based Innovation (CRI) of marine bioactivities and drug discovery, established by the Research Council of Norway. MabCent-SFI is currently screening a large number of extracts from marine organisms for a diverse range of bioactivities. The centre has a focus on

automated and high-throughput screening within several areas, such as antibacterial, antitumor and anti-inflammatory activities as well as enzymes and antioxidants. MabCent-SFI is organized in close association to the Marbank and Marbio facilities. Marbank is a national marine repository, which is coordinating sampling expeditions, taxonomic determination and storage of marine organisms from Norwegian waters. After sampling, the material and extracts are available for research of biological diversity and commercial utilization. Marbio is a high-capacity screening laboratory with a focus on searching for biologically active components in marine organisms provided by Marbank.

1.1 The goal of the thesis

The overall aim of this master thesis has been to investigate and study the presence of bioactive molecules in two Arctic species, a sponge and a phytoplankton. Specifically it has been focused on:

1. Cytotoxic compounds against mammalian cancer cells (anticancer compounds) and against bacteria (antibacterial compounds)
2. Antioxidant compounds

2. Background

2.1 Marine sponges

Sponges are animals of the phylum Porifera, and are the most primitive group amongst the multicellular organisms. All sponges are sessile aquatic animals, most of which are marine and living one by one or in colonies. There is a great variety of shapes and sizes, and they occur in all oceans and have a wide distribution from tropical and temperate to Arctic regions. Commonly, all have a skeleton of spiculas and spongin, and have no digestive, circulatory, respiratory or excretory system. They have neither true tissue nor organs, but have distinct cell types such as epithelium, ciliated choanocytes (feeding cells) and a mesogloeal layer. The sponges are adapted to maintain a constant water flow through their bodies. This system enables them to obtain food and oxygen and remove waste. They feed on microorganisms like unicellular alga and bacteria and dead organic matter. Sessile organisms are lacking mobility and must defend themselves from predation or encroachment of competitors. Sponges are known to produce bioactive metabolites as part of their defensive system (Amsler et al., 2001). They occasionally develop symbiotic (mutually beneficial) relationship with both algae and microorganisms, and symbionts are to an extent the true source of secondary metabolites found in sponges (Radjasa and Sabdono, 2009). Because of their prevalence, ease of collection, and ability to biosynthesize a variety of natural product structural classes, sponges have become one of the dominant sources of biologically active marine natural products.

2.1.1 *Aplysilla sulfurea*, a marine Arctic sponge

Aplysilla sulfurea is of the phylum Porifera, in the class Demospongiae, the order Dendroceratida and the family Darwinellidae. It is found along the entire Norwegian coastline and most parts of the Atlantic Ocean, ranging from depths between the tidal zone to more than 300 meters. It is mostly found on bedrock locations with little natural light. The sponge forms three to six mm thick sheets with small, pointed “spines”. It has

one or only a few oscules (waste openings) between one and three mm in diameter. The sponge is soft and compressible.

2.2 Marine phytoplankton

Plankton are organisms in the oceans that are able to move but can not surmount the oceanic current. Phytoplankton are algae that obtain energy through photosynthesis and are living in the well-lit euphoric zone. They form the basis of most of the production of biomass and organic compounds in the oceans. Many algal metabolites have unique structures, and are among others believed to be the primary source of the secondary metabolites discovered in marine invertebrates (Radjasa and Sabdono, 2009). This connection is believed to be carried through symbiosis, association, food chain and other forms of nutrient dependency (Shimizu, 1993). The secondary metabolites in algae are generally limited to a specific species, and individual adaptations in species and organisms makes the sources almost unlimited. A number of the algae possess biological activity such as toxicity, antibacterial, antifungal, antitumor and other specific activities, and this makes algae an interesting source of useful biologically active products (Cannell, 1993).

2.2.1 *Porosira glacialis*,(Grunow 1884), a marine Arctic phytoplankton

Porosira glacialis is a diatom of the phylum Bacillariophyta in the Eukaryota empire, in the class Coscinodisocophyceae and the order Thalassiosirales. In nature it appears solitary or united in chains, and it is found in cold water in the northern and southern hemispheres.

2.3 Choice of methods

MabCent-SFI has built up a library of methods for sampling and fractionating with the purpose of ensuring the possibility of finding molecules regardless of their size or composition. The screening methods used are established at Marbio to search for a wide

array of bioactivities. These methods have been the foundation of the experiments that were carried out in this thesis.

The collected samples of the sponge *Aplysilla sulfurea* were freeze-dried and extracted in water or a mix of methanol and dichloromethane. This gives a crude separation according to the polarity of the molecules. Aqueous and organic extracts were further purified by high performance liquid chromatography (HPLC).

The samples of the microalgae *Porosira glacialis* were extracted in methanol and water (80 % methanol). The extraction was continued with adding 50% acetonitrile, to separate into an aqueous and an organic phase. The materials in both phases were separated by HPLC.

The semi-purified fractions were screened for anticancer, antibacterial and antioxidative effects. The methods used in the anticancer screening are based on the methods utilized by the National Cancer Institute (NCI, United States) in their search for anticancer drugs. In the anticancer screening the cancer cells were exposed to the semi-purified fractions and the viability was checked with cell lines from human carcinomas. A normal cell line was used as a control for toxicity. The antibacterial screening was carried out by exposing a panel of Gram-positive and Gram-negative bacteria to the semi-purified fractions, and the inhibition of bacterial growth was determined. Bacteria strains in this study were selected because they cause serious infections and frequently lead to problems for human health. The antioxidant screening was carried out by testing the ferric reducing ability of plasma in order to measure the antioxidant capacity of the extracts. Fractions that were positive in the first screening were further purified by HPLC and retested.

Fractions that were confirmed as active after they were retested were subjected to mass spectrometry (MS) analysis. The samples were ionized by electrospray to gain mass data at high resolution in order to find accurate molecular weight and eventually elemental composition of the active compounds. This information was compared to databases to find out if it could be identified as a known structure. Molecules that were not found in the databases were subjected to further work in order to make a structure elucidation. The flow chart (figure 1a) shows the process from extraction to MS analysis.

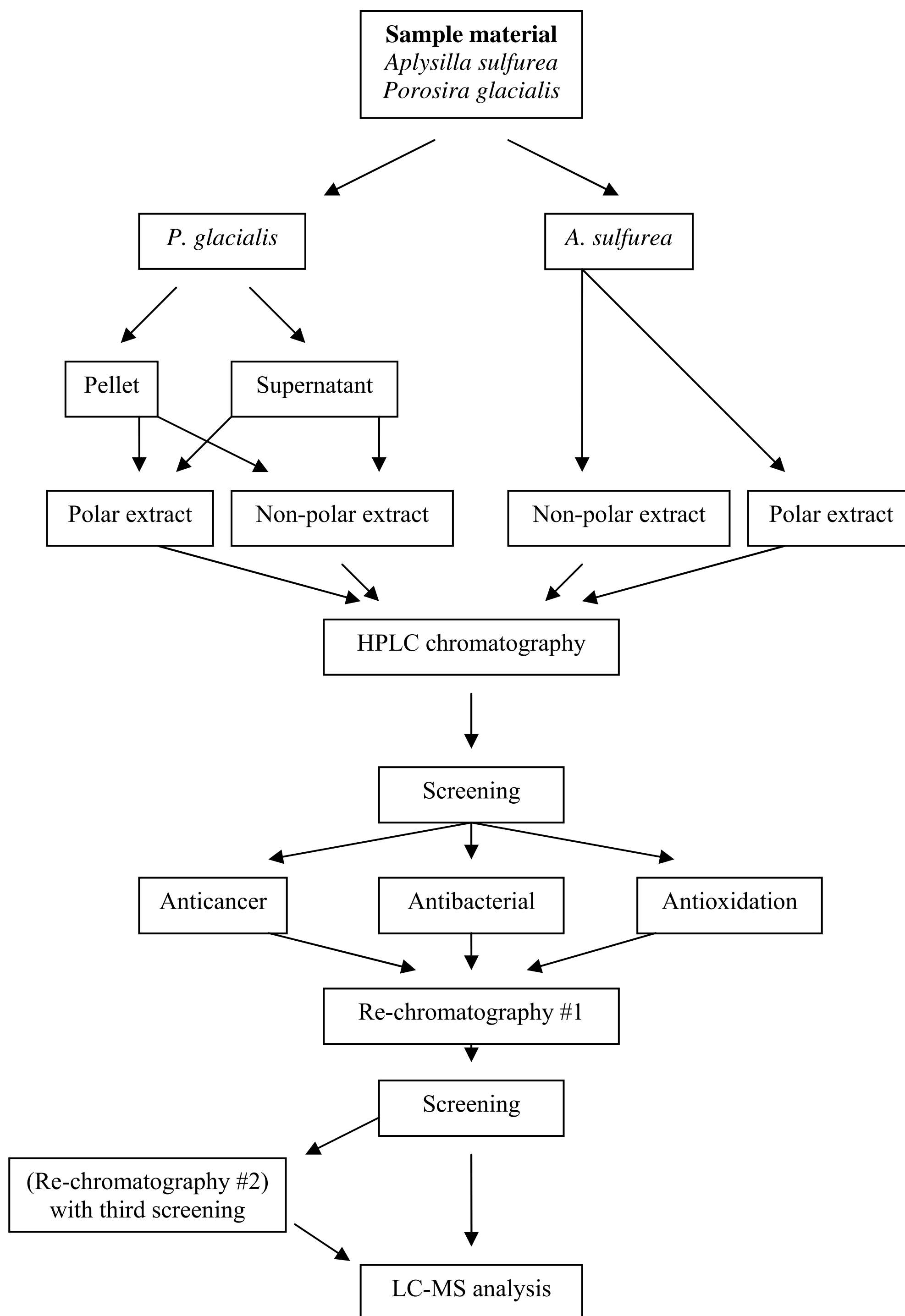

Figure 1a: Flow chart of procedures from extraction to MS analysis

2.4 Extraction and purification

The goal of the extractions was to obtain a widest possible range of compounds from the freeze-dried material. The principle behind the extraction is that molecules of the same polarity will follow solvents of the same polarity. The aqueous solution will be a solvent for water-soluble polar molecules, and dichloromethane will be a solvent for non polar fat-soluble molecules.

In the process of screening for undiscovered compounds that can lead to development of new natural products there is uncertainty concerning the size and shape of the molecules of interest. Because of this it is important to extract the widest range possible. As a result an aqueous procedure was implemented followed by an organic extraction. The extracts were complex and they may contain hundreds of different compounds.

The water-soluble components in the biological material are often unavailable because they are bound to membranes, are situated inside membranes or in membrane pockets or are protected by other lipophilic substances. By freeze-drying and pulverizing the biological material before the extraction, it is possible to destroy such membranes and pockets and make the water-soluble components available for extraction. Alternatively an organic solvent such as methanol can be added to the water in order to dissolve lipophilic pockets at the same time as the water-soluble components are extracted.

The aqueous and organic extracts prepared have a vast complexity. In order to minimize the risk of antagonistic or agonistic effects between different molecules in the extracts, the extracts are partly purified and separated by HPLC into 40 fractions prior to the screening. HPLC is a form of column chromatography used to separate, identify, and quantify compounds, and it is employed frequently in biochemistry and analytical chemistry. The column in HPLC holds chromatographic packing material (stationary phase), and the mobile phases are moved through the column by a pump. The retention time of the molecules is measured by a detector. The interactions between the stationary phase, the molecules passing trough the column, and the solvents used can cause variations in retention time. The figure below shows a schematic illustration of the HPLC unit (figure 2).

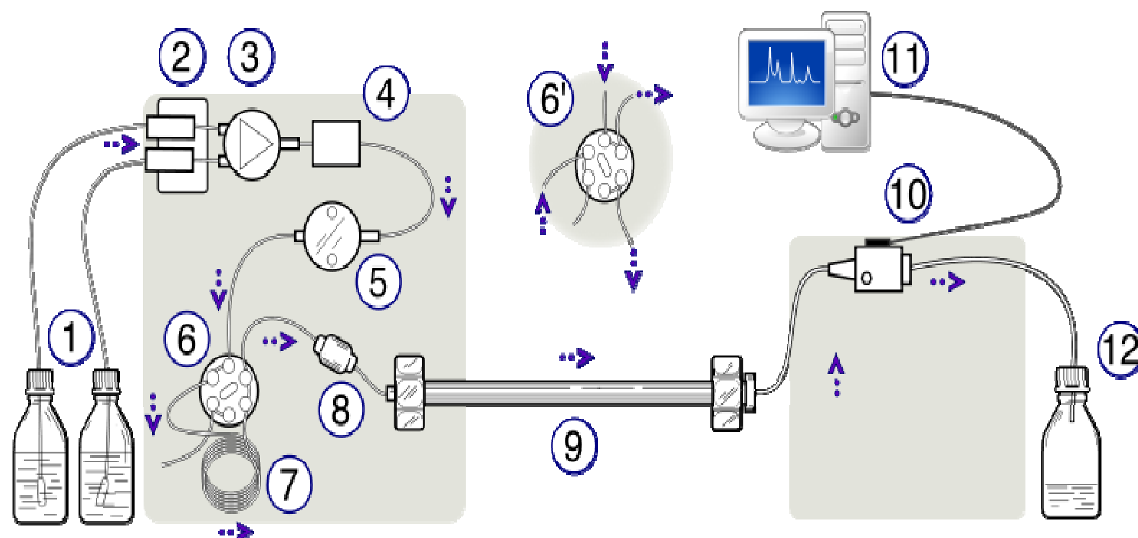

Figure 2: Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. (http://en.wikipedia.org/wiki/File:HPLC_apparatus.svg)

2.5 Cancer cell lines and screening for anticancer activities

Cancer is a disease that occurs because of uncontrolled cell division and cell survival, invasion and metastasis. The three main methods of treatment are surgery, chemotherapy and radiation therapy. The goal is to completely remove the cancer cells without damaging the healthy tissue. Almost 50% of all cancer patients today are permanently cured, but there are still needs for more effective drugs for most cancer forms. However, development of new effective drugs during the last few years has led to an increased efficiency of treatment for patients that suffer from cancer forms with traditionally few means of treatment (Vigerust, 1997).

Because there are not sufficient biological differences between normal and malignant cells it is often difficult to target the unwanted cancer cells specifically. Cancer cells divide more frequently than normal cells. Most chemotherapeutic drugs are directed against actively dividing cells, and so the malign cells will be influenced to the larger extent than normal cells. These drugs will effect normal cells in the dividing stage and therefore patients experience adverse side effects. Targeted cancer therapy uses drugs that

specifically should attack cancer cells, and hence may have fewer side effects (Health, 2009).

In this project three cancer cell lines and one normal cell line were used for the anticancer screening. One cell line from normal lung tissue was used as a control, and malign cell lines from breast, colon and melanoma cancer.

The anticancer screening was carried out using the MTT-assay. MTT (2H-Tetrazolium, 2-(4,5-dimetyl-2-3,5-difenyl-, bromide)) is a yellow tetrazolium salt. The method is based on an enzyme that can break the tetrazolium ring on the MTT. As a consequence a formazan salt with a dark blue color is formed (figure 3). This only takes place in cells that are actively respirating. Viable cells will produce a dark blue color and dead cells are indicated by a yellow color. The amount of viable cells is directly related to the formazan product that is formed and can thus be measured spectrophotometrically and calculated.

Figure 3: The reduction process from tetrazolium to formazan.

2.6 Bacteria and screening for antibacterial activities

Bacteria are a large group of unicellular microorganisms that can be divided into two main groups. The cell wall in Gram-negative cells contains relatively little peptidoglycans but have an outer membrane composed of lipopolysaccharide and other complex macromolecules. The Gram-positive bacteria have a cell wall consisting of peptidoglycans, and are lacking the outer membrane of Gram-negative cells. The structural differences can cause differences in antibiotic receptiveness. For instance, some antibiotics can kill Gram-positive bacteria but are ineffective against Gram-negative pathogens (Perry et al., 2002).

Today there is an increasing incidence concerning resistance towards antibiotics in pathogenic bacteria. Excess consumption and wrong medication using antibiotics has caused the development and dispersion of resistance. Combined with the lack of development of new antibiotics, this is causing a problem related to treatment of resistant bacteria.

The Gram-positive bacterial strains *Enterococcus faecalis*, *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) and the Gram-negative bacterial strains *Escherichia coli* and *Pseudomonas aeruginosa* were used for the antibacterial bioactivity screening. All these bacteria may cause serious infections in humans.

The Minimum Inhibitory Concentration (MIC) is a method for determination of the smallest amount of an antimicrobial agent that inhibits growth of microorganisms (Perry et al., 2002). Among others it is used in the process of monitoring resistance, comparison of new antibiotics and for screening new compounds for antimicrobial activity. A known concentration of the bacteria is inoculated with several dilutions of the test compound and tested for the ability to grow. The MIC value is indicated for the sample with the smallest concentration (mg/ml) of the antibiotic compound to prevent visible growth of bacteria. In the first screening of the HPLC fractions no dilutions are made because the concentration of antimicrobial substances in the fractions is unknown.

2.7 Antioxidants and screening for antioxidants

Substances that have the ability to restrain oxidation are called antioxidants. Proteins and enzymes represent high molecular antioxidants. Low molecular antioxidants are artificial or natural compounds that are fat-soluble (e.g. vitamin E) or water-soluble (e.g. vitamin C). Reactive oxygen species (ROS) are free radicals and non radical oxygen species constantly produced in human cells. To avoid damages from these ROS we have differentiated systems with antioxidants to minimize the problems related to this. An imbalance between the production of ROS and antioxidant defense can cause oxidative stress and lead to oxidative damage (Wolfe and Liu, 2007). Proteins, lipids and nucleic acids are prone to oxidative damage which in a worst case scenario may lead to a number of serious conditions such as cancer, cardiovascular diseases and Alzheimer’s disease

(Hermans et al., 2007). Search for natural and safe antioxidants is therefore currently a very active area of research.

The antioxidative effect of semi purified fractions was determined using the Ferric Reducing Ability of Plasma (FRAP) assay. At a low pH the Fe^{III}-TPTZ will be reduced to Fe^{II}. This causes the development of an intense blue color with an absorption maximum at 593 nm. If an antioxidant is present the conditions will promote the reduction of the compound and hence the color. The results are compared to Trolox, a water-soluble analog to vitamin E.

2.8 Mass spectrometry

Mass spectrometry (MS) is a method used for investigating the purity and structure of molecules. The technique is based on the principle that molecules with a positive or negative charge will move away from objects with the same charge. Figure 4 is an outlining of a mass spectrophotometer. The compounds are ionized by electrospray. Subsequently a separation of the ions according to the mass: charge ratio in a mass filter is performed. The number of ions and their respective mass is recorded by a detector. Several ion sources and mass filters are on the market. For this thesis a mass spectrometer with electro spray ionization (ESI) and time-of-flight (TOF) mass separator was utilized.

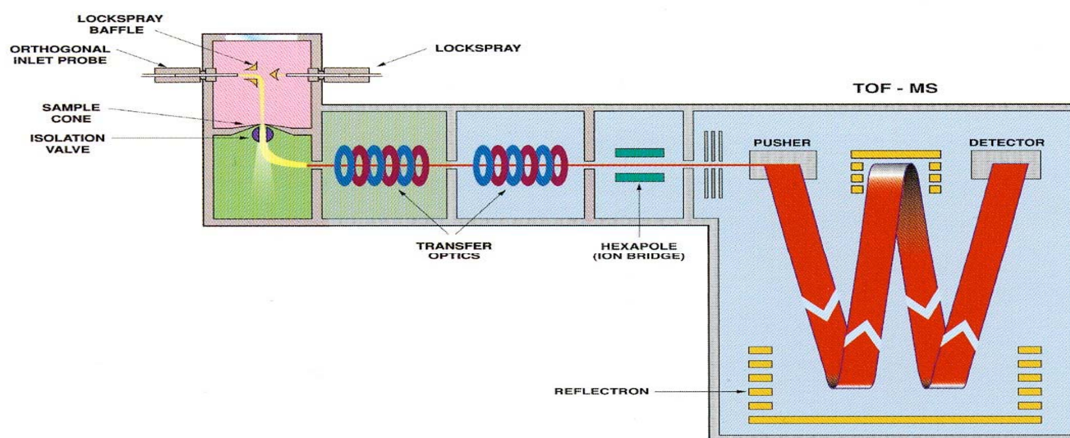

Figure 4: Outlining of an ESI-TOF mass spectrometer (www.waters.com)

3. Materials and methods

3.1 Sampling and storage

Aplysilla sulfurea was collected 15th of august 2007 in the Southwest Barents Sea at 71°35'36" N and 21°22'86" E with bottom trawl at 331 meters of depth. This sponge was attached on top of another sponge, *Geodia sp.*, and was dethatched before being stored at -20°C.

Microalgae were collected in the surroundings of Tromsø using a plankton net or niskin bottles. These cultures were stored with light and temperature simulating *in situ* conditions. From this, *Porosira glacialis* was selected and cultivated further as a monoculture. Natural seawater was used as medium after being filtered and enriched with 0,25 ml/l Substral, 12.3 µmol/l $Si(OH)_4$, and 10 µL/ litre $^{-1}$ soil extract (for trace metals). The seawater was filtered through three filters, first of 0,5µm, secondly an UV filter and finally a 0,22 µm Millipore Cartridge 0.22µm filter (Guillard and Ryther, 1962, Ryther and Guillard, 1962b, Ryther and Guillard, 1962a). Monocultures were maintained and kept on test tubes at relatively low temperature and low light to keep the growth rate at a minimum. The monocultures were cultivated in a 600 liter column under artificial conditions, in order to expose the cultures to the desired CO_2, O_2 and light conditions. The culture in this project was cultivated at low temperature (3°C) and high light (160 µmol Quanta m-2 s-1) in seawater medium. The algae were harvested by filtering the water from the dense algae culture by slowly tapping the culture from the 600 litre mass-culture columns into 20µm plankton nets. The filtrate was centrifuged at 2500 rpm for 20 minutes, and pellet and supernatant were transferred into 50ml Falcon polystyrene tubes (BD Biosciences, USA), frozen in liquid N_2 and stored at -80°C.

The cultivation of *P. glacialis* was performed by Professor H. C. Eilertsen and his technicians at Institute of Aquatic Biology at the University of Tromsø.

3.2 Freeze-drying

The frozen biomass samples of *A. sulfurea* were chopped into pieces of approximately 1 cm^3 on a wooden board and placed in glass containers. The samples were weighed, and immediately stored at -20°C. The samples were then freeze-dried using a Heto PowerDry (Thermo; Waltham, USA) until dryness. The dried samples were pulverized with an IKA A11 Basic (IKA Works; Staufen, Germany) mill, and transferred to Duran bottles and weighed.

The frozen biomass samples of *P. glacialis* were separated into a pellet and a supernatant by centrifugation as described in chapter 3.1. The samples were freeze-dried and stored at -20°C.

3.3 Extraction

Organic and aqueous extracts were made from the freeze-dried samples of *Aplysilla sulfurea*. The organic extraction was performed with dichloromethane: methanol (1:1), and the aqueous extraction with Milli-Q water (Millipore; MA, USA). From the *Porosira glacialis* an extraction with 4:1 methanol (MeOH) and water was performed.

Dichloromethane (p.a.), ethanol (p.a.), hexane (HPLC), acetonitrile (HPLC) and MeOH (p.a.) were supplied by Merck (Darmstadt, Germany).

3.3.1 Aqueous extracts of *Aplysilla sulfurea*

Milli-Q water (900 ml) was added to the 362 g freeze-dried material. The samples were vigorously stirred three times during six hours and left overnight. The next day they were transferred to 0.7 liter centrifugation bottles and centrifuged for 30 minutes at 4000 rpm (5°C) with a Multifuge 3SR (Kendro; Osterode, Germany). The supernatant was decanted and stored, and the extraction was repeated with an extraction time of 30 minutes. The two supernatants were pooled and freeze-dried.

3.3.1.1 Preparation for HPLC

The freeze-dried extracts were pulverized and stored in centrifugation tubes at -20 °C. Approximately 200 mg of the freeze-dried supernatant was transferred to a 13 ml polystyrene centrifugation tube, and 2 ml Milli-Q water was added before mixing on a Universal SM 30 (Edmund Bühler GmBH; Hechingen, Germany) shaker for 90 minutes at 100 rpm. The aqueous phase (2 ml) was transferred to a new centrifugation tube, added 8 ml 96 % ethanol (EtOH) and kept overnight in a freezer (-20°C). The samples were taken out of the freezer and kept at room temperature for 5-10 minutes before centrifuging using a Multifuge 3SR at 4000 rpm (5°C) for 30 minutes. The supernatant was transferred to a rotary evaporation bulb and was vacuum evaporated at 40°C using a Laborota 4002 (Heidolph;Nürnberg, Germany) evaporator to approximately 2 ml at 70 mbar pressure. This volume was then divided equally between 2 centrifugation tubes and adjusted to 1 ml with Milli-Q water. The tubes were centrifuged at 13000 rpm for 30 minutes with a Multifuge 3SR. The supernatant was filtered trough a 0.22 μm Millex GS (Millipore) filter into an HPLC tube, mixed well and adjusted to 1 ml with Milli-Q water. The product of this procedure was water soluble components ready for separation by HPLC and testing of bioactivities.

3.3.2 Organic extracts of *Aplysilla sulfurea*

The pellet from the final aqueous extraction was freeze-dried and subsequently pulverized before being transferred to a Duran bottle. Dichloromethane (DCM) and MeOH (1:1) was added to the sample in a 10:1 ratio. The samples were stirred vigorously three times in a period of six hours and left overnight at 5°C. The next day the samples were vacuum filtered with a Whatman no 3 (Whatman; Madistone, England) filter. The extraction was repeated, now with a 30 minute extraction time. The two filtrates were pooled, and a Laborota 4002 rotary evaporator was used to vaporize the solvents. The evaporation was carried out at 40°C and atmospheric pressure (~1000 mbar) and gradually reducing the pressure to 200 mbar. The pressure was further reduced till the solvents were sufficiently vaporized from the organic extracts with 10 to 20 ml remaining, and they were stored at -20°C.

3.3.2.1 Preparation for HPLC

Organic freeze-dried extracts (395.3 mg) were transferred to test tubes of glass and mixed with 3 ml hexane. Then 3 ml 90% acetonitrile (ACN) was added, mixed and the test tube was centrifuged at 300 rpm for 3 minutes using a Multifuge 3SR centrifuge. The hexane phase was transferred to a new test tube and once again mixed with 3 ml 90% ACN. The centrifugation was repeated, and the combined ACN phases were vacuum evaporated using a Laborota 4002 by gradually reducing the pressure to 150 mbar at 40°C until approximately 1.5 ml remained. This was equally divided between two 1 ml centrifugation tubes and the volume was adjusted to 1 ml with 90% ACN, and the tubes centrifuged at 13000 rpm for 30 minutes. The supernatant was transferred to HPLC tubes and adjusted to 1 ml with 90% ACN.

3.3.3 Extracts of *Porosira glacialis*

The freeze-dried material was pulverized and transferred to Duran bottles and extracted with approximately 50 ml 80% MeOH per gram of dry weight for 18 hours at 4°C. The samples were centrifuged at 4000 rpm using a Multifuge 3SR. The supernatant was stored at -20°C. The pellet was extracted a second time with approximately 20 ml 80% MeOH per gram dry weight. The extract was vacuum filtered with a Whatman no.2 filter, and the filtrate was mixed with the supernatant from the first extraction. The extracts were vaporized using a Laborota 4002 rotary evaporator at approximately 40 mbar until all MeOH was removed. The samples were freeze-dried, pulverized and transferred to test tubes and stored at -20°C.

From the freeze-dried extracts 200 mg were transferred to test tubes and 2 ml of 50% ACN was added. Two phases were formed and transferred to two Eppendorf tubes and centrifuged for 5 minutes at 10 000 rpm using a Multifuge 3SR. The supernatant was transferred to HPLC tubes.

3.4 Purification by HPLC

3.4.1 Purification

The extracts were fractionated using a Waters 600E Multisolvent Delivery HPLC with a Prep Degasser and Waters 2767 Sample Manager (Waters; MA, USA). The HPLC was fitted with an XTerra® Prep RP18, 10µm (10x300mm, 10x10mm guard column) column (Waters). The mobile phase gradients were composed of acidic water (MP A: 0.1% FA) and ACN (MP B). The separations were performed with individual gradients for the aqueous, organic and *P. glacialis* extractis by using a gradient from 5 to 100 % MP B for 40 minutes (Table 1, 2 and 3). The flow rate was 6 ml/min and the injection volume 900µl for the organic and aqueous extracts, and volumes according to table 4 were injected from extracts of *P. glacialis*.

Table 1: Mobil phase gradient for the organic extracts of *A. sulfurea*

ml/min	Time(min)	MP A	MP B
6	initial	80	20
	2	80	20
	30	0	100
	40	0	100

Table 2: Mobile phase gradient for aqueous extracts of *A. sulfurea*

ml/min	Time(min)	MP A	MP B
6	initial	95	5
	3	95	5
	30	50	50
	35	5	95
	40	5	95

Table 3: Mobile phase gradient for extracts of *P. glacialis*

ml/min	Time(min)	MP A	MP B
6	initial	95	5
	2	95	5
	30	0	100
	40	0	100

Table 4: Injection volume for extracts of *P. glacialis*

Sample	Injection volume
Pellet, ACN fase	350 µl
Pellet, aqueous fase	550 µl
Supernatant, ACN fase	250 µl
Supernatant, aqueous fase	400 µl

3.5 Anticancer screening

3.5.1 Anticancer screening

The anticancer activities were tested using a melanoma cell line (MCF7), a colon carcinoma cell line (HT29) and a breast adenocarcinoma cell line (A2058). Human fibroblasts (MRC-5) were used as a control of cytotoxicity. The cell lines were cultivated in their respective media (Table 5) with 10 % Fetal Bovine Serum (Biochrom) and 10 µg/ml gentamicin (Aventis Pharma; Lysaker, Norway). When the cell confluency was 70 – 80 % the cultures were trypsinated with a solution of trypsin/ EDTA in PBS (Invitrogen; CA, USA), and 100 µl was seeded out in 96 well Microtiter (Nunc; Roskilde, Denmark) plates in a concentration of approximately 2 x 10^4 cells/ml. The cells were incubated for 24 hours at 37ºC, 5% CO_2 before addition of HPLC fractions. The addition of HPLC fractions and cells to the assay plates was performed by automatic liquid handler (Biomex Minicore System, Beckman Coulter).

Table 5: Cell lines and their respective media

Cell lines	Origin of cell	Growth media
A2058 (Promochem AB; Borås, Sweden)	Melanoma	D-MEM (Biochrom; Cambridge GB)
HT29 (ECACC; Wiltshire, GB)	Colon carcinoma	RPMI1640 (Biochrom)
MCF7 (ECACC)	Breast carcinoma	E-MEM(Biochrom)
MRC-5 (Promochem AB)	Normal fibroblasts	E-MEM

The organic HPLC fractions were dissolved in 7.5 µl Dimethyl sulfoxide (DMSO, Sigma Aldrich; München, Germany) and 750 µl RPMI-1640. The aqueous fractions were dissolved in 750 µl RPMI-1640.

After the incubation time of 24 hours the cells in the Microtiter plates were inspected with a microscope. The growth media was removed, and 50 µl RPMI-1640 with 10 % Fetal Bovine Serum (FBS) was added to all wells before 50 µl of the HPLC fractions was added in triplicates (figure 5). Triton-X100 (Sigma Aldrich) 1% (50 µl) was added as a positive control and RPMI-1640 (50 µl) as a negative control. The plates were incubated for 72 hours at 37ºC, 5% CO_2. Fractions were defined as active when cell survival was less than 50 % compared to untreated cells.

	Fraction 1-7 (21-27)			Fraction 8-14 (28-34)			Fraction 15-20 (35-40)			Control	
	1	1	1	8	8	8	15	15	15	N	N
	2	2	2	9	9	9	16	16	16	N	N
	3	3	3	10	10	10	17	17	17	N	N
	4	4	4	11	11	11	18	18	18	N	N
	5	5	5	12	12	12	19	19	19		
	6	6	6	13	13	13	20	20	20		
	7	7	7	14	14	14	C	C	C		

Figure 5: HPLC fractions in anticancer screening in assay plate. The shaded areas are not in use.

Aqueous One Solution (10 µl) from Sigma Aldrich was added to each well by liquid handler, and the plates were incubated for 1 hour at 37ºC, 5% CO_2. The absorbance was measured at 590 nm in DTM 880 Multimode Detector (Beckman Coulter).

Active fractions from the first screening were retested to find dose-response relationship in 1:1, 1:2 and 1:4 dilutions. Active fractions that were confirmed in 1:1 and 1:2 dilutions were pooled and further purified before being screened for anticancer activity according to the procedure from the first screening. Active fractions from the further purified material were subjected to structure elucidation by LC-MS.

3.6 Antibacterial screening

3.6.1 Dissolving of HPLC fractions

Organic fractions were dissolved in 7.5µl DMSO before shaking at 200 rpm for 2 hours at room temperature (RT) using a Titramax 1000 (Heidolph). Sterile Milli-Q water (750 µl) was added and the fractions were shaken for 30 minutes at RT. Another 750 µl of sterile Milli-Q water was added and shaken for 30 minutes.

Aqueous fractions were dissolved in 750 µl sterile Milli-Q water and shaken for 30 minutes at RT. Another 750 µl sterile Milli-Q water was added and the fractions were shaken for 2 hours under similar conditions.

3.6.2 Experimental procedures

The bacterial strains to be tested were inoculated in 8 ml cultivation media (table 6), and incubated over night at 37ºC. After the incubation 2 ml was transferred to 25 ml fresh media for enrichment to achieve exponential growth. The suspension was incubated at 37 ºC and shaken at 400 rpm for 1 to 3 hours depending on bacterial strain on a Titramax 1000. Some strains needed to be incubated for a longer time before they enter the log-face (table 6). The bacteria were cultivated until they entered log-face at a turbidity of 0.5 McFarland standard ($1.0x10^8$ bacteria/ml). The cultivation media were from Oxoid LTD (Cambridge, England).

Table 6: Strains, incubation time and cultivation media

Bacteria strains	**Cultivation medium**	**Inc. period**	**Density of bacteria**
S.aureus ATCC 25923	Mueller-Hinton (MH)	2,5 h	$0,5\text{-}3x10^5$ CFU/ml
E.coli ATCC 25922	MH	1,5 h	$0,5\text{-}3x10^5$ CFU/ml
E.faecalis ATCC 29212	Brain Heart Infusion (BHI)	1,5 h	$0,5\text{-}3x10^5$ CFU/ml
P.aeruginosa ATCC 27852	MH	2,5 h	$3\text{-}7x10^4$ CFU/ml
MRSA* ATCC 33591	MH	2,5 h	$0,5\text{-}3x10^5$ CFU/ml

* Methicillin resistant *S. aureus*

The HPLC fractions (50 µl) were added to 96 well Microtiter plates in two parallels (Figure 6). The bacterial suspension (50 µl) was added to the fractions after being diluted 1:100 and finally 1:10 in cultivation media. The bacterial suspension must be added to the fractions within 30 minutes to maintain a viable cell density. The plates were incubated for 19-20 hours (37 ºC) depending on bacterial strain.

N	1	1	9	9	17	17	25	25	33	33	**P**
N	2	2	10	10	18	18	26	26	34	34	**P**
N	3	3	11	11	19	19	27	27	35	35	**P**
N	4	4	12	12	20	20	28	28	36	36	**P**
N	5	5	13	13	21	21	29	29	37	37	**P**
N	6	6	14	14	22	22	30	30	38	38	**P**
N	7	7	15	15	23	23	31	31	39	39	**P**
N	8	8	16	16	24	24	32	32	40	40	**P**

Figure 6: HPLC fractions in antibacterial screening in assay plate. N= negative control, P= positive control, 1-40= the different HPLC fractions added to the wells in parallels.

The negative control in column 1 consisted of cultivation medium and sterile Milli-Q water 1:1, and the positive control consisted of sterile Milli-Q water and bacteria suspension 1:1 in column 12. Gentamicin was used as control for the setup and precision between the tests. To estimate the number of bacteria, the suspension must be diluted to approximately $1x10^2$-$1x10^3$ bacteria/ml. The suspensions were plated out on ISA (Iso-Sensitest-Agar) plates and incubated at 37 ºC for 19-20 hours depending on bacterial strain. The assay plates were subject to a photometric reading at OD 600nm using a Victor 3 TM 1420 Multilabel Counter (Perkin Elmer Instruments; Shelton, USA) spectrophotometer. Visible clouding implicates bacterial growth and inactive (I) fractions. Wells without visible clouding implicates exclusion of bacteria and active (A) fractions. The photometric values for active fractions were defined as A<0.05, and values between 0.05 and 0.09 are considered borderline cases. For inactive fractions I>0.09.

Active fractions from the first screening were retested to find dose-response relationship in 1:1, 1:2 and 1:4 dilutions. Fractions that were confirmed active in 1:1 and 1:2 dilutions were pooled and further purified before being screened in two parallels

according to the procedure from the first screening. Active fractions were subjected to structure elucidation by LC-MS.

All bacterial strains in the screening are within risk group 2, and can be handled under normal conditions. To avoid the risk of contamination, the work was carried out in a laminar flow hood, and aseptic techniques were employed to avoid contamination of the bacteria suspensions and samples.

3.7 Antioxidant screening

3.7.1 Dissolving of HPLC fractions

Organic fractions were dissolved in 7.5µl DMSO before shaking at 200 rpm for 2 hours at room RT using a Titramax 1000 (Heidolph). Milli-Q water (292.5 µl) was added and the fractions were shaken for 30 minutes at RT.

Aqueous fractions were dissolved in 300 µl Milli-Q water and shaken for 2.5 hours at RT.

3.7.2 Antioxidant screening

Total antioxidant activity was measured by the ferric reducing ability of plasma (FRAP) assay after Benzie and Strain (Benzie and Strain, 1996). Fe-solution was made of 51.4 mg $FeCl_3$ * $6H_2O$ in 10 ml Milli-Q water and was mixed with TPTZ-solution made of 31.2 mg 2,4,6-tripyridyl-s-triazine (TPTZ) in 10 ml 40 mM HCl, 5 ml of each mixed with 50 ml acetate buffer pH 3.6 forming the FRAP reagent.

HPLC fractions (5 µl), Milli-Q water (15 µl) and FRAP reagent (150 µl) was added (Figure 7). A stock solution of Trolox (F. Hoffmann–La Roche, Ltd.; Basel, Switzerland) giving 1000 µM was made of 31.25 mg Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in 1.25 ml MeOH and diluted with water. For the standard curve this was diluted further to 250, 125, 62.5, 31.25 and 15.625 µM. Water was used as blank. The plates were read colorimetrically at 595 nm and 37 ºC using a

DTM 880 Multimode Detector. In the first screening fractions were defined as active when the Trolox equivalent was above 50 µM.

Fraction 1-8		Fraction 9-16		Fraction 17-24		Fraction 25-32		Fraction 33-40		Trolox (uM)	
1	1	9	9	17	17	25	25	33	33	0	0
2	2	10	10	18	18	26	26	34	34	15,625	15,625
3	3	11	11	19	19	27	27	35	35	31,25	31,25
4	4	12	12	20	20	28	28	36	36	62,5	62,5
5	5	13	13	21	21	29	29	37	37	125	125
6	6	14	14	22	22	30	30	38	38	250	250
7	7	15	15	23	23	31	31	39	39		
8	8	16	16	24	24	32	32	40	40	Blank	Blank

Figure 7: HPLC fractions on assay plate in antioxidative screening

Active fractions from the first screening were retested to find dose-response relationship in 1:1, 1:2 and 1:4 dilutions. Fractions that were confirmed active in 1:1 and 1:2 dilutions were pooled and further purified before being screened in two parallels according to the procedure from the first screening. Active fractions were subjected to structure elucidation by LC-MS.

3.8 Pooling and second purification of active fractions

The active fractions from the bioactivity screening were indicators for the area of interest for further analysis. The fractions were dissolved in 100 to 200 µl 90 % ACN and the volume was adjusted to 1 ml with ACN. From this volume 900 µl was separated using a Symmetry® Prep C18, 7µm (7,8x300mm) column with a flow rate of 4 ml/min and individually adjusted mobile phase (MP) gradients according to the retention time of the pooled fractions. The MP gradients were adjusted to achieve the best separation possible for the components in each fraction. The 40 fractions were collected using a fraction collector. Fractions were transferred to deep well plates in an equal manner and these were freeze dried as described in chapter 3.2.

Table 7: Mobile phase gradients for second and third purification of active HPLC fractions from the first and second screening of antibacterial and anticancer activity

ml/min	Time (min)	MP A	MP B
4	initial	70	30
	30	35	65
	31	0	100
	40	0	100

Table 8: Mobile phase gradients for re-chromatography of active HPLC fractions from the first screening of antioxidants

ml/min	Time (min)	MP A	MP B
4	initial	95	5
	10	95	5
	30	70	30
	35	5	95
	40	5	95

3.9 Structure elucidation by LC-MS

3.9.1 ESI-TOF-MS

The active purified fractions were analyzed by a Waters Micromass LCT Premier time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source. The samples were introduced to the mass spectrometer using a Waters 2795 analytical HPLC with an XTerra® MS C_{18} 3.5µm (2.1x150mm) column (Waters). The mobile phase consisted of Milli-Q water (MP A) and acetonitrile (MP B), both containing 0.1 % (vol:vol) formic acid (puriss, Sigma Aldrich), and a gradient running for 15 min was used to elute the compounds (table 9). Leucine-enkephalin (Sigma Aldrich) was infused trough the reference probe and used as lock mass for internal calibrations throughout the data acquisitions. Every morning when the analysis took place, the instrument was tuned to a resolution of at least 10 000 FWHM and calibrated using sodium formate. Data were acquired in the positive ion (ES+) mode, and the mass range was set to 100-2000 *m/z*. The mass spectrometer was operated in the W-mode (both reflectrons active), and at

capillary and cone voltages of 2600 and 70 V, respectively. The desolvation chamber was kept at 250°C and the ion source at 150°C, while the desolvation gas flow rate was 700 Lh^{-1} and the nebulizer gas flow rate was 5 Lh^{-1}.

Table 9: HPLC conditions for MS.

ml/min	Time (min)	MP A	MPB
0,2	Initial	95	5
	10	30	70
	11	5	95
	15	5	95

4. Results

4.1 Aplysilla sulfurea

4.1.1 Samples, extracts and fractions

The entire sponge (1227.5 g) was freeze-dried to a dry weight of 362 grams g giving the sponge a dry weight content of 29.5%. The dry biomass underwent an aqueous extraction followed by an organic extraction. This resulted in a dry weight yield after freeze-drying of 14.5 g and 29.5 g respectively.

Freeze-dried aqueous extract (200mg) and freeze-dried organic extract (396.3mg) were the basis for the HPLC separations. The fractions (40) from each HPLC run were tested for bioactivities as described in the Materials and Methods section. Fractions with bioactivities were pooled and freeze-dried before undergoing re-chromatography by HPLC and screening for bioactivities. Some samples were pooled, re-chromatographed and screened a third time.

4.1.2 Antibacterial screening

Five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus*) were used in the antibacterial screening. The fractions were considered active at a photometric measurement of less than 0.05 OD600nm (MIC value).

4.1.2.1 Organic HPLC fractions

Fractions 8 to 15 from *A. sulfurea* displayed antibacterial activity as demonstrated in figure 8. The effect of the fractions against the five bacterial strains differed slightly. *S. aureus* and MR *S. aureus* was inhibited by all the fractions from 8 to 15, while *P. aeruginosa* was only inhibited by fraction 10 and 11. *E. faecalis* and *E. coli* were inhibited by fractions 9 to 12.

The active fractions were retested in dilutions of 1:1, 1:2 and 1:4 in order to confirm the activity. The fractions that showed activity in 1:1 and 1:2 dilutions (fractions

8 to 15) were pooled and re-chromatographed into 40 fractions in order to obtain a better separation of the components in the active fractions.

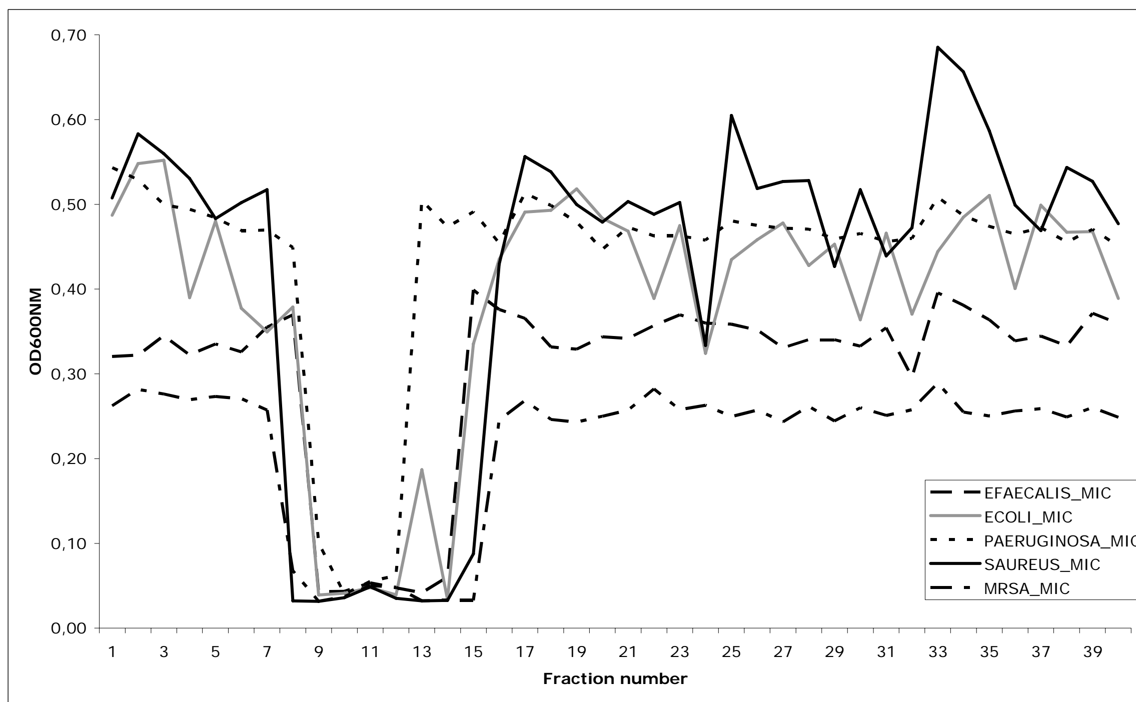

Figure 8: Antibacterial activity of the 40 organic fractions from HPLC of extracts from *A. sulfurea* against five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus)*. Values below 0.05 OD600nm indicate active fractions.

The second screening of the re-chromatographed fractions displayed antibacterial activity in fewer fractions (figure 9). MRSA was inhibited by fraction 3 and 5 to 11. *S. aureus* was inhibited by fraction 5 to 9, *P. aeruginosa* by fractions 5 to 7, *E. coli* by fractions 5 to 8 and *E. faecalis* by fraction 5 to 9. However, a better separation of the active fractions was not achieved due to an overloading of the column, and fractions 8 to 15 were re-chromatographed again to achieve a better separation. The third screening for antibacterial activity revealed no activity distinctively differing from the activity of the inactive fractions, and all fractions were defined as inactive (figure 10).

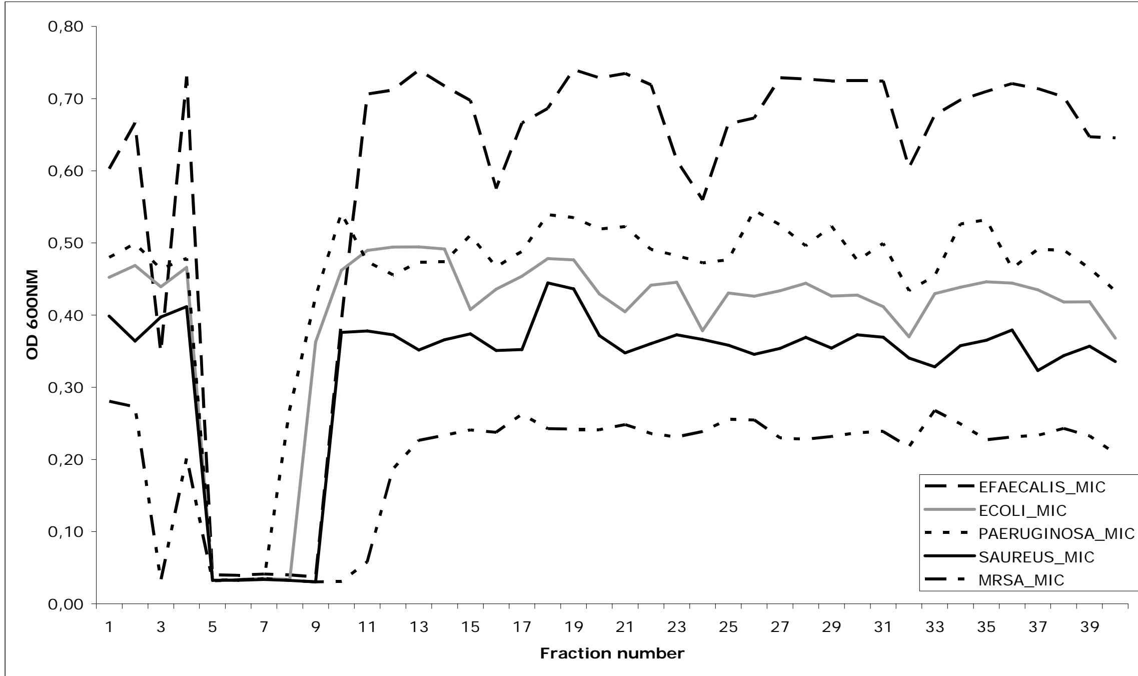

Figure 9: Antibacterial activity of the 40 organic fractions from re-chromatographed fractions (8-15 figure 8) from *A. sulfurea,* against five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus)*. Values below 0.05 OD600nm indicate active fractions (second screening).

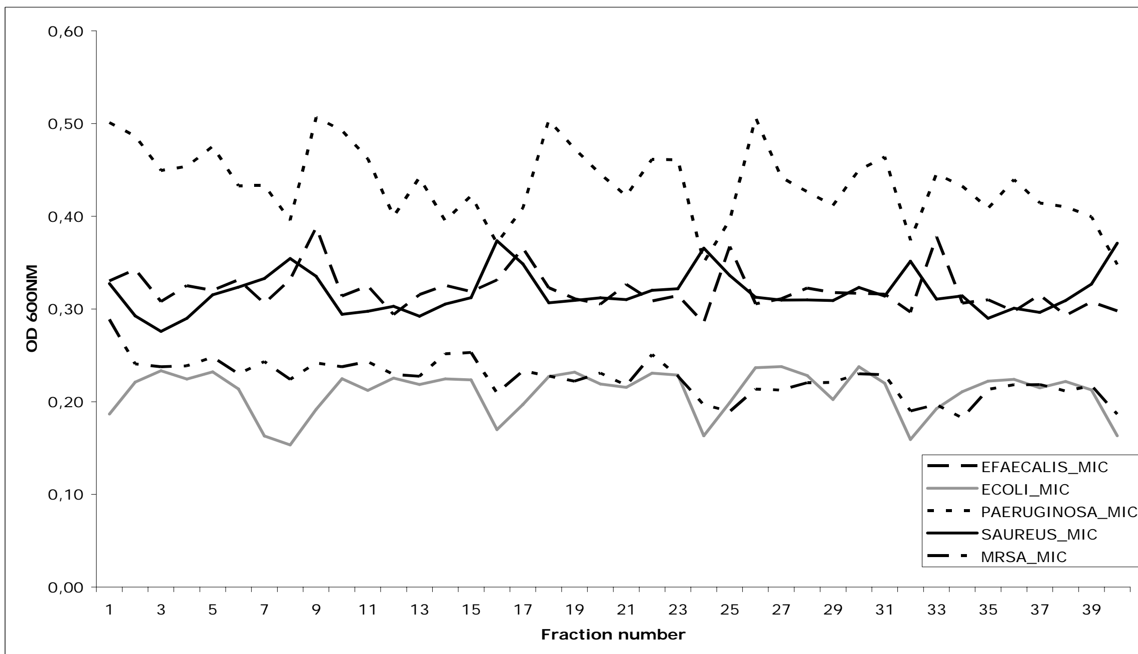

Figure 10: Antibacterial activity of the 40 organic fractions from re-chromatographed fractions (3-11 figure 9) from *A. sulfurea,* against five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus)*. Values below 0.05 OD600nm indicate active fractions (third screening).

4.1.2.2 Aqueous HPLC fractions

The aqueous fractions from extracts of *A. sulfurea* revealed no active fractions in the antibacterial screening.

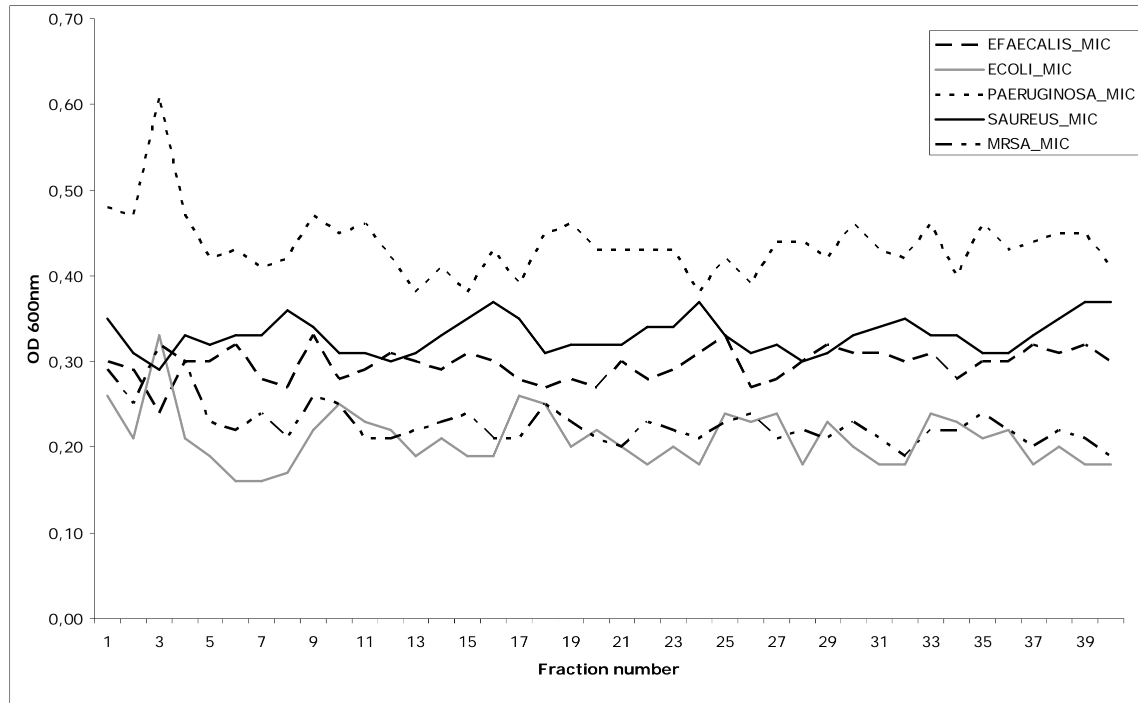

Figure 11: Antibacterial activity of the 40 aqueous fractions from HPLC of freeze-dried extracts from *A. sulfurea* determined by testing the effect on five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus)*. Values below 0.05 OD600nm indicate active fractions.

4.1.3 Anticancer screening

Three cancer cell lines (A2058, MCF7 and HT29) were used in the anticancer screening. One normal cell line from human lung tissue (MRC-5) was used as a toxicity control. The fractions were considered active when the cell survival was less than 50 %.

4.1.3.1 Organic HPLC fractions

The HPLC fractions tested for antibacterial activities (figure 8) were also tested for anticancer activities (figure 12). Two of the 40 organic fractions from *A. sulfurea* showed cytotoxic activity in the first screening. All cell lines including the normal fibroblast cell line had less than 5 % survival when exposed to fractions 9 and 10. Some fractions (21 –

27) appeared to specifically stimulate growth of the normal cell line. However, this was not further investigated. Fraction 9 and 10 from the first HPLC chromatography were retested in dilutions of 1:1, 1:2 and 1:4 to confirm the effect.

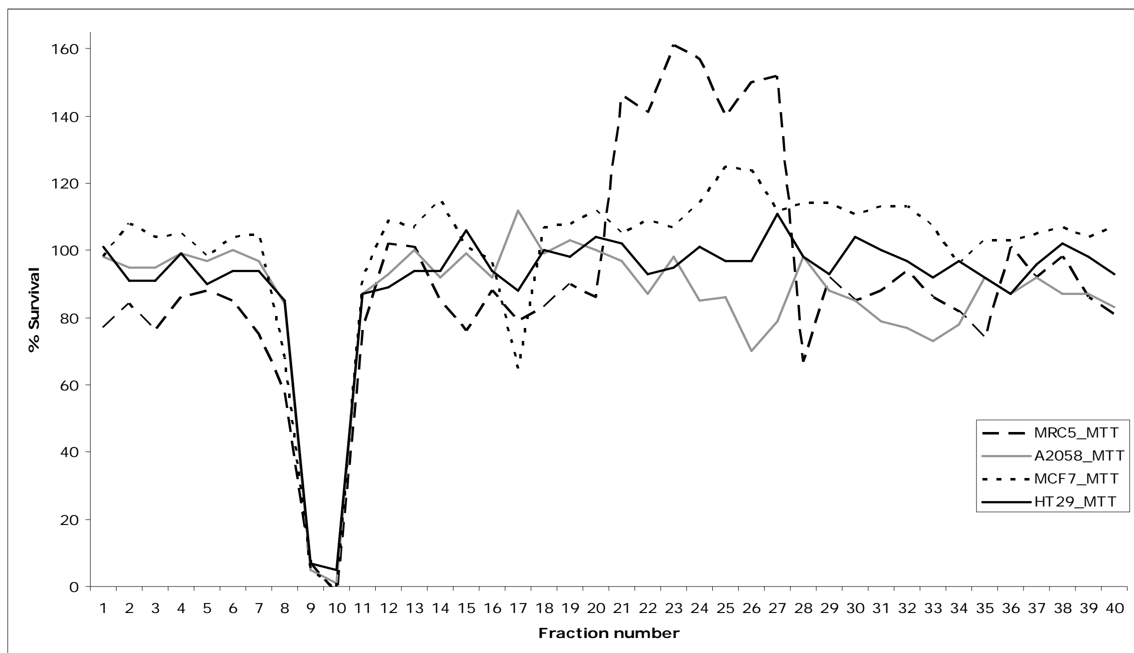

Figure 12: Anticancer activities of the 40 organic fractions from *Aplysilla sulfurea* was determined by testing the effect on three cancer cell lines (A2058, MCF7 and HT29) and control cell line (MRC-5). Cell survival below 50 % is defined as active fractions.

The pooled and re-chromatographed fractions tested for antibacterial activities (figure 9) were also tested for anticancer activities (figure 13). All cell lines including the normal cell line had less than 10 % survival after exposure to fraction 5 and 6.

Fractions 3 to 11 from the second screening of antibacterial activities (figure 9) were selected and pooled for a third chromatography by HPLC and separated into 40 fractions. In addition to antibacterial activities, the fractions were also screened for anticancer activities (figure 14). Fraction 6 had activity against the breast carcinoma cell line. No activity against the other three cell lines including the normal fibroblast cell line was detected. The bioactivity data from the anticancer, antioxidant and antibacterial screening were compared and were used in the structure elucidation by LC-MS.

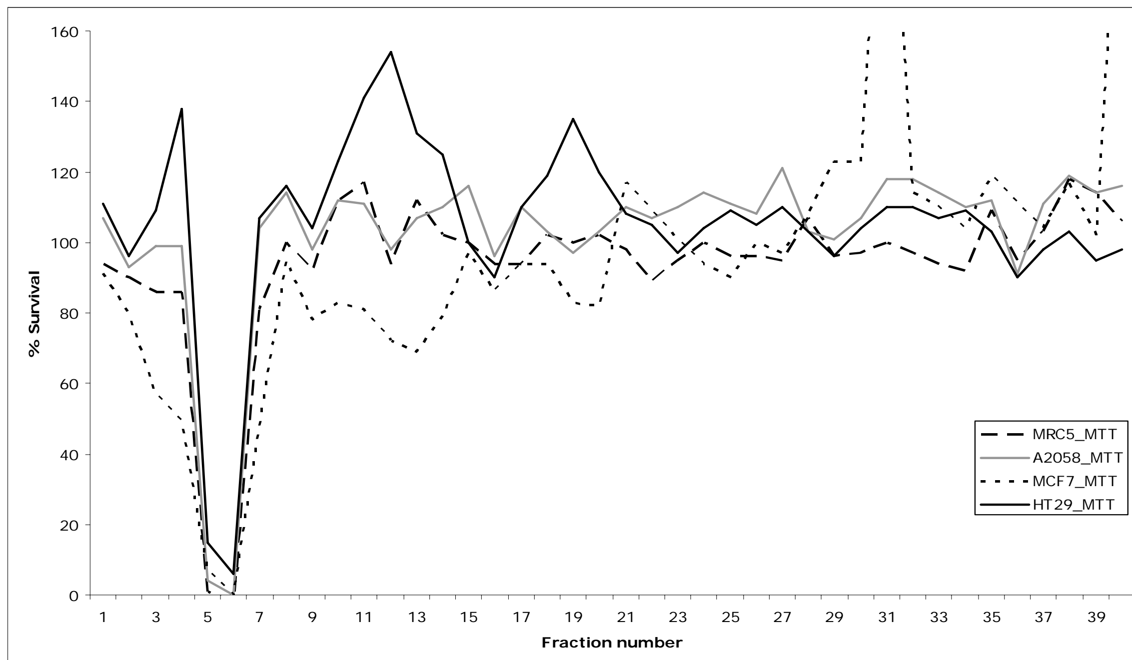

Figure 13: Anticancer activities of the 40 re-chromatographed organic fractions from *Aplysilla sulfurea* (fractions 8-15 figure 12) was determined by testing the effect on three cancer cell lines (A2058, MCF7 and HT29) and control cell line (MRC-5). Activities below 50 % indicate active fractions (second screening).

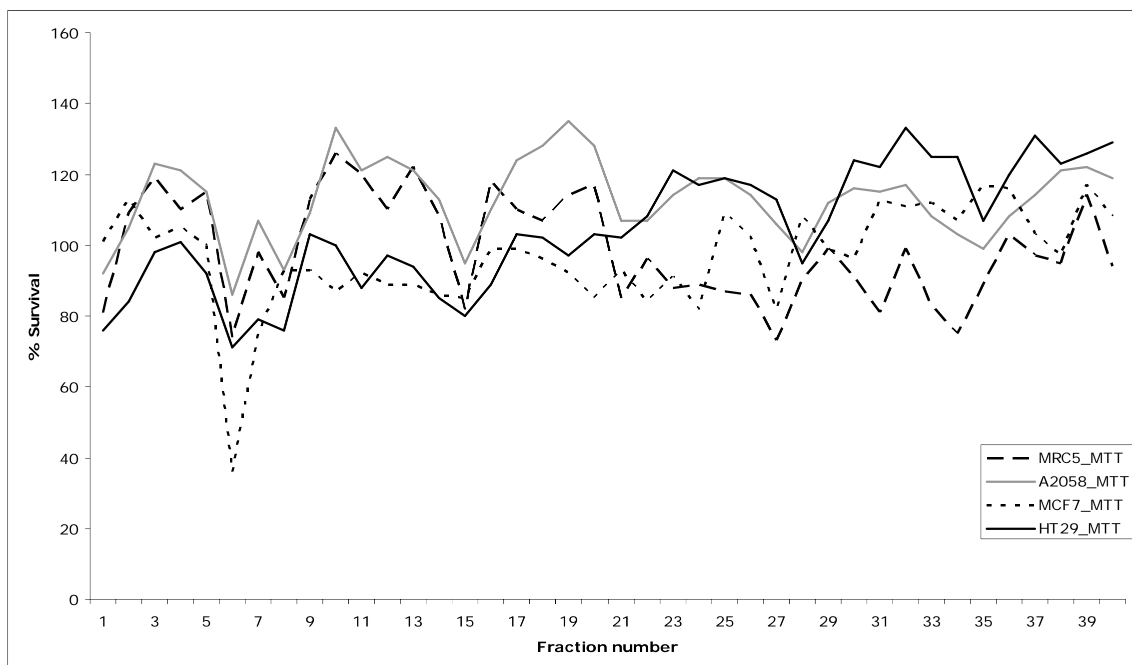

Figure 14: Anticancer activities of the 40 re-chromatographed organic fractions from *Aplysilla sulfurea* (fractions 3-11 figure 13) was determined by testing the effect on three cancer cell lines (A2058, MCF7 and HT29) and control cell line (MRC-5). Activities below 50 % indicate active fractions (third screening).

4.1.3.2 Aqueous HPLC fractions

None of the fractions obtained from the HPLC chromatography of the aqueous extract of *A. sulfurea* were active against the cancer cell lines (Fig. 15). Fraction 3 appeared to have some activity against MRC5, A2058 and HT29 reducing survival to 55 to 65 %. The activity was however above the defined cut-off value for active fractions and did not lead to a further purification or retesting.

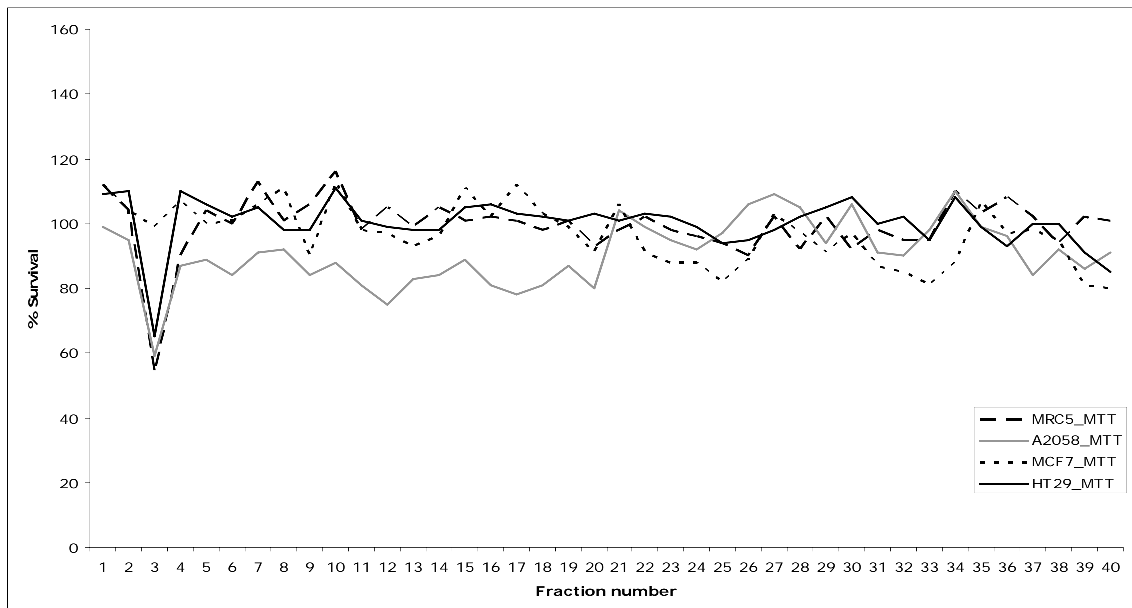

Figure 15: Anticancer activities of the 40 aqueous fractions from *Aplysilla sulfurea* was determined by testing the effect on three cancer cell lines (A2058, MCF7 and HT29) and control cell line (MRC-5). Activities below 50 % indicate active fractions.

4.1.4 Antioxidant screening

A chemical FRAP assay was used in the antioxidant screening, and read-out of the assay was measured colorimetrically. The fractions in the first screening were considered active when they showed an activity compared to a Trolox equivalent of more than 100 μM. Further purified fractions were assessed individually.

4.1.4.1 Organic HPLC fractions

The fractions obtained from the HPLC chromatography and the two re-chromatography separations (figure 8, 9 and 10) were also tested for antioxidant activities. The antioxidant screening of the 40 organic fractions showed that fraction 9 and 10 were active with a

Trolox equivalent above 100 μM (figure 16). The fractions were retested in 1:1, 1:2 and 1:4 dilutions and the activities were confirmed in fractions 9 and 10. The fractions from the first re-chromatography (figure 9) were also tested for antioxidative activities and fractions 5 and 7 were defined as active (figure 17). The bioactivity data from the anticancer, antioxidant and antibacterial screenings was compared and used in the structure elucidation by LC-MS.

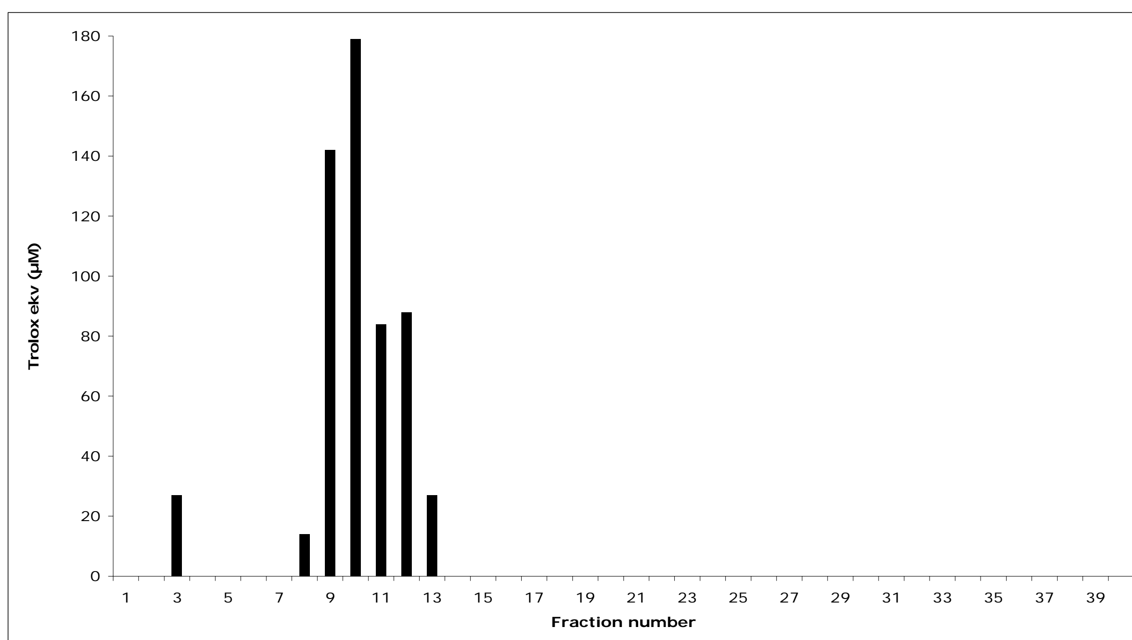

Figure 16: Antioxidative activity of chromatographed organic samples from *A. sulfurea*. Each fraction was analyzed by testing the effect in the FRAP assay. Measurements of activity above Trolox equivalents 100 μM were defined as active (first screening).

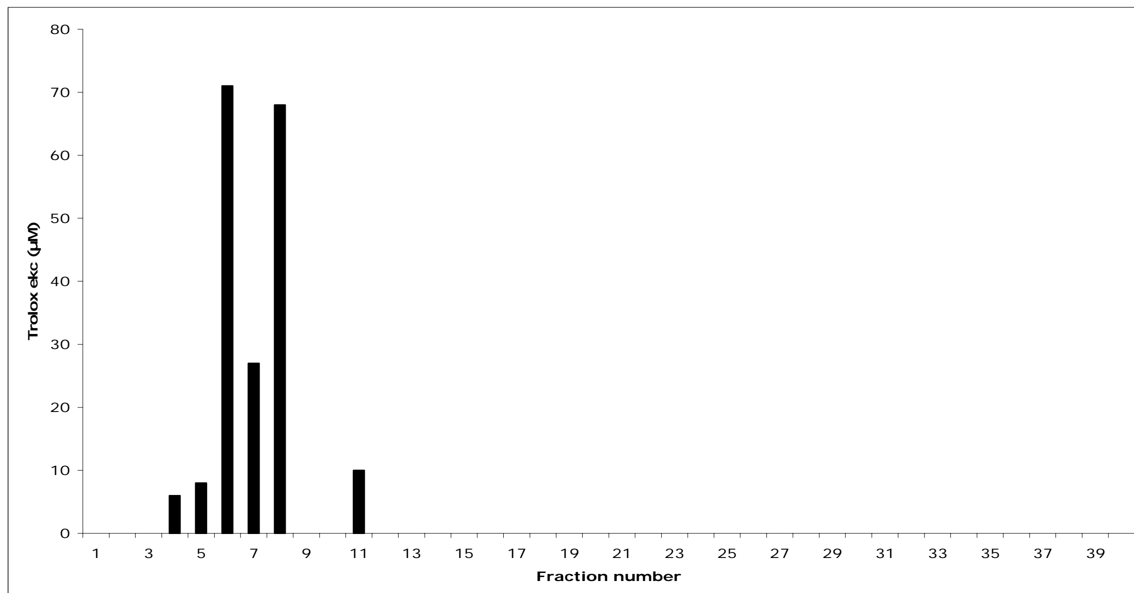

Figure 17: Antioxidative activity of re-chromatographed organic samples from *A. sulfurea* (fractions 8-15, figure 16). Each fraction was analyzed by testing the effect in the FRAP assay (second screening).

4.1.4.2 Aqueous HPLC fractions

The antioxidant screening of the aqueous fractions from *A. sulfurea* showed that some activity was found in fraction 3. However the Trolox equivalent was below the defined cut-off value for active fractions and no fractions were further investigated.

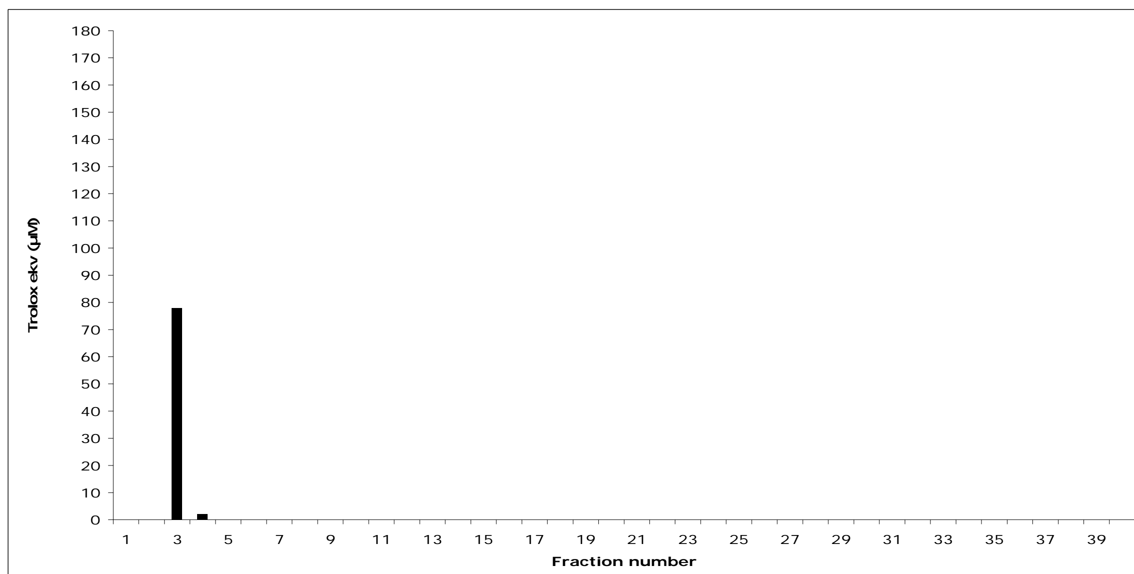

Figure 18: Antioxidative activity of chromatographed aqueous samples from *A. sulfurea.* Each fraction was analyzed by testing the effect in the FRAP assay. Measurements of activity above Trolox eqivalents 100 µM were defined as active (first screening).

4.1.5 Structure elucidation by LC-MS

The bioactivity data from the screening of anticancer and antibacterial activity was the background for evaluating fractions containing interesting activities for structure elucidation. Fraction 6 from the third anticancer screening of organic fractions from *A. sulfurea* displayed inhibiting activity against one cancer cell line (figure 14). In addition fractions in the same area gave an indication of antibacterial activity (figure 8 and 9), although the activity was not displayed in the third antibacterial screening of organic fractions from *A. sulfurea*.

The organic fractions of interest from *A. sulfurea* were investigated by mass spectrometry in order to find the composition and structure of the components. The samples were ionized by electrospray, and accurate mass data at high resolution were acquired. The data were used to calculate the elemental composition of the active compounds.

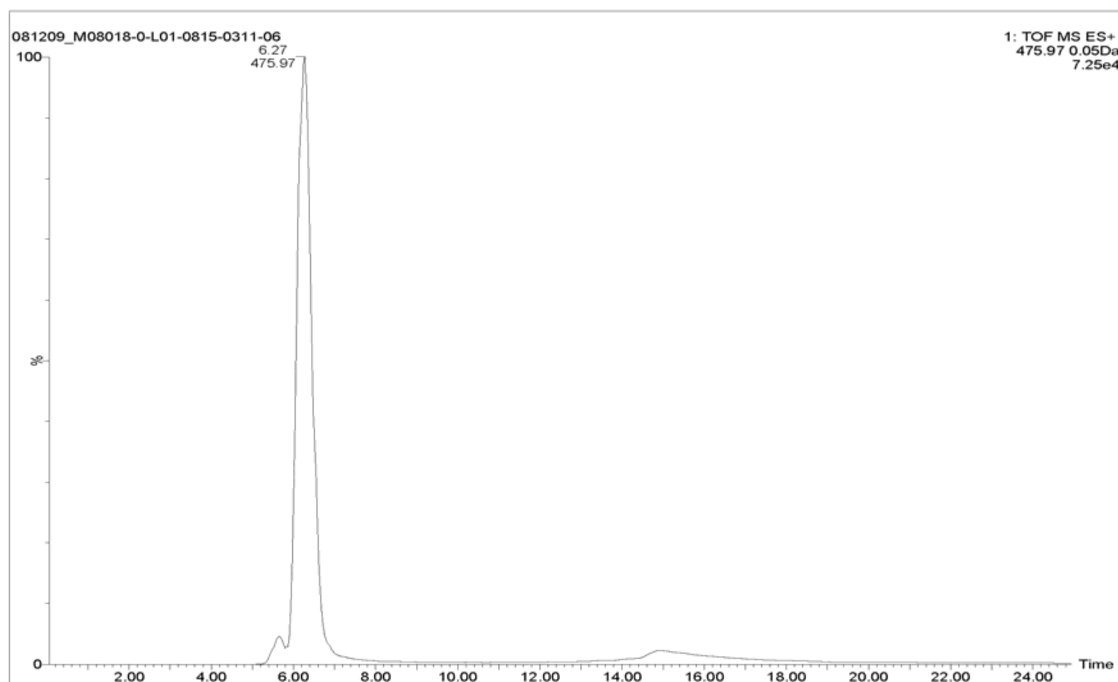

Figure 19: Fraction 6 in total ion chromatogram from the third bioactivity screening (fractions 3 to 11).

The total ion chromatogram (TIC) of fraction 6 revealed one dominating peak after 6 minutes with an m/z value of 475.97 (figure 19). It is highly likely that the compound can be linked to the observed bioactivity.

The ESI MS spectra (figure 21) of the peak at 6.27 min revealed one major peak of m/z 475.9727 and two smaller peaks at m/z 473.9745 and 477.9707. The peak with m/z of 473.9745 originates from the protonated molecule, and the peaks to the right are isotopes of the same molecule. The isotopic pattern (1:2:1 two mass units apart) is typical for compounds containing two bromine atoms, and the elemental composition analysis suggested that the molecular formula was $C_{15}H_{17}Br_2N_5O_3$. Searches in CHEMnetBASE revealed that a compound called Ianthelline, with antibacterial properties and identical molecular formula, had been identified in a related Arctic sponge (Litaudon and Guyot, 1986). Hence, Ianthelline (figure 20) seems to be responsible for the anticancer and antioxidative activities in the organic extracts from *A. sulfurea*.

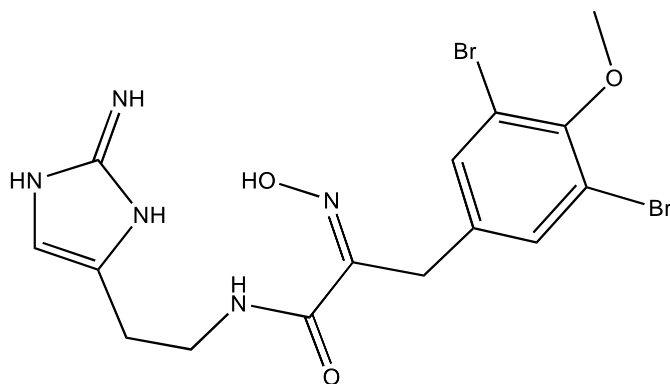

Figure 20: Ianthelline (CHEMnetBASE.com, 2009). The oxygen molecule on the di-brominated ring is bound to a methyl group (methoxygroup).

The peak with m/z of 473.9745 is protonated Ianthelline, and the peaks to the right are the isotopes of Ianthelline. The molecular mass of Ianthelline was calculated to 472.9672 (theoretical value 472.9698), the m/z for the molecule from the spectra minus the weight of one proton (1.0073 Da).

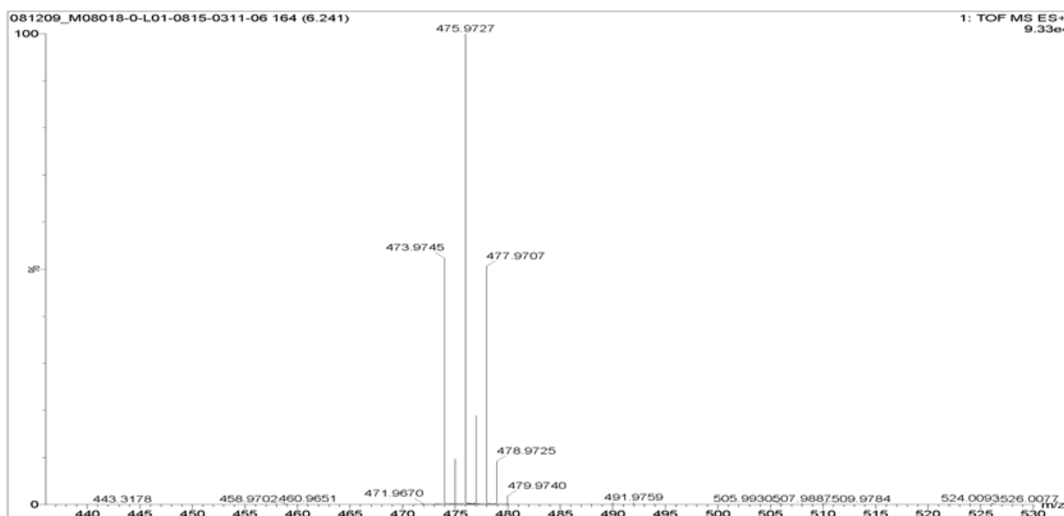

Figure 21: Positive mass spectrum of fraction 6 and the compound eluting after 6.27 min (fractions 3 to 11 figure 9).

4.2 *Porosira glacialis*

4.2.1 Samples, extracts and fractions

Pure culture of *P. glacialis* had been grown in tanks under controlled conditions at the Norwegian College of Fishery Science. Both supernatant (growth medium) and pelleted cells were obtained. Complete extractions were performed on both samples as described in the Materials and Methods section.

The total weight of the supernatant before freeze-drying was 932.2 grams, but was not registered for the pellet. The pellet had a dry weight of 37.4 grams and the supernatant 34.1 grams after the first freeze-drying.

Extraction was performed on both the supernatant and the pellet from the phytoplankton samples. The yield from the extraction was 18.9 grams for the pellet and 20.9 grams for the supernatant. The extracts were separated into an aqueous and an organic phase before HPLC. From the supernatant extract 203 mg was the basis for the

HPLC separation. This resulted in 40 organic and 40 aqueous HPLC fractions. From the pellet extract 205.3 mg was the basis for the HPLC separation. This resulted in 40 organic and 40 aqueous HPLC fractions. Fractions with bioactivities were pooled and freeze-dried before undergoing re-chromatography by HPLC and screening for bioactivities.

4.2.2 Antioxidant screening

A chemical FRAP assay was used in the antioxidant screening, and the assay was measured colorimetrically. The fractions in the first screening were considered active when they showed an activity compared to a Trolox equivalent of more than 100 µM. The re-chromatographed fractions were evaluated individually without using strict cut-off values for determining activity.

4.2.2.1 Aqueous HPLC fractions from pellet

Fractions 3 and 4 had an activity above a Trolox equivalent of 100 µM, and were defined as active (figure 22). The fractions were retested in 1:1, 1:2 and 1:4 dilutions, and fractions 3 and 4 were confirmed as active.

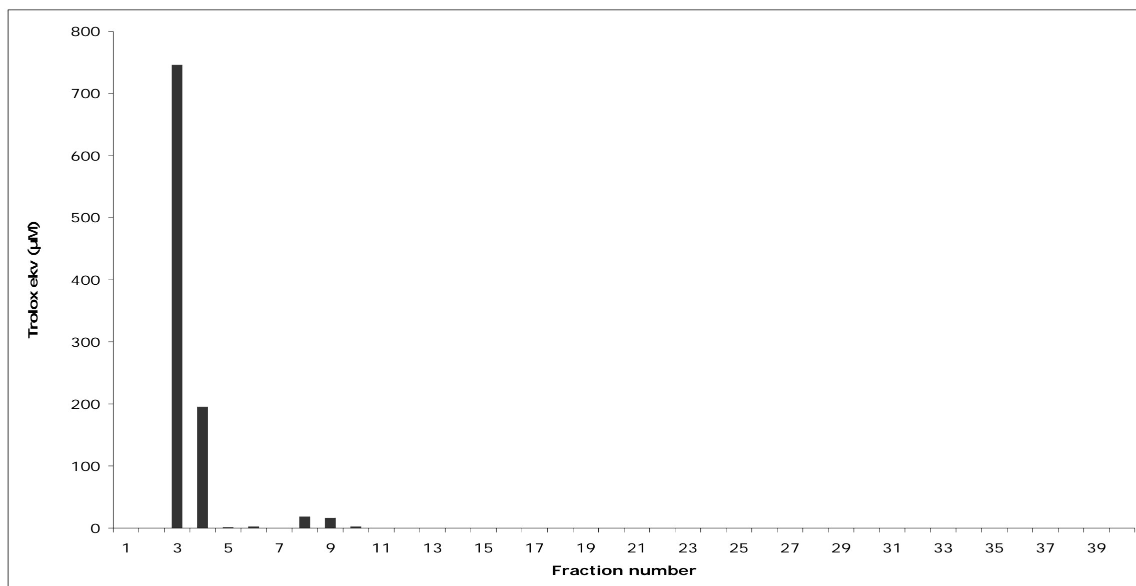

Figure 22: Antioxidative activities of chromatographed aqueous fractions from the pellet of *P. glacialis.* Each fraction was determined by testing the effect in the FRAP assay. Measurements of activity above Trolox eqivalents of 100 µM were defined as active (first screening).

Fractions 3 and 4 from the first chromatography (figure 22) were pooled and re-chromatographed. The fractions were subjected to a second screening. In the interpretation of the results from the second screening fractions 2 and 3 were defined as active. Active fractions were subjected to structure elucidation by LC-MS.

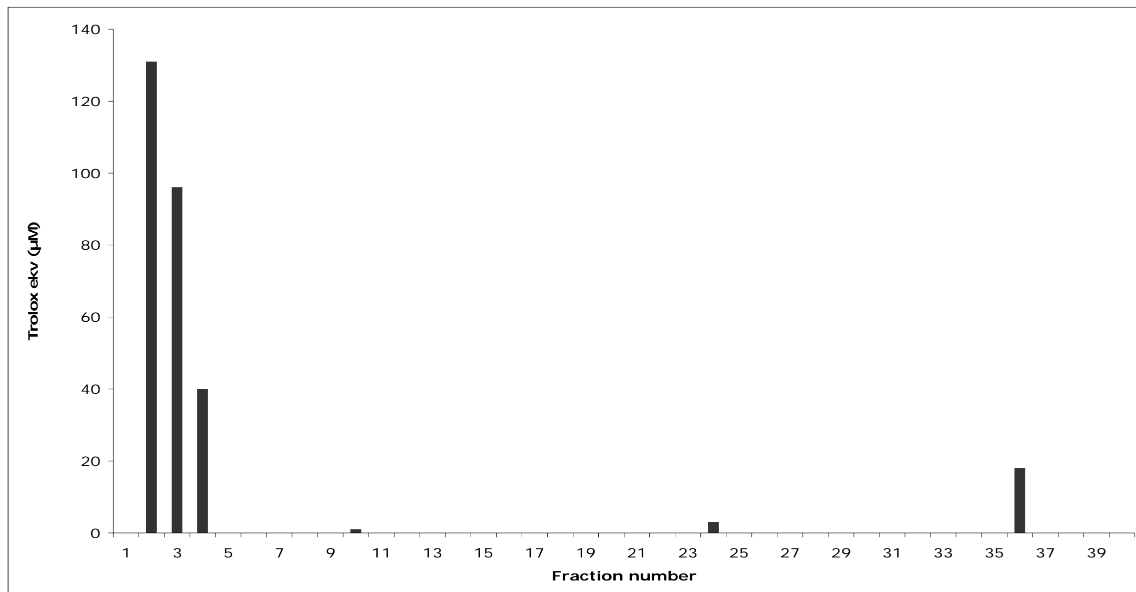

Figure 23: Antioxidative activities after re-chromatography of fractions 3 and 4 (figure 22) from *P. glacialis.* Activity in each fraction was determined by testing the effect in the FRAP assay (second screening).

4.2.2.2 Organic HPLC fractions from pellet and supernatant and aqueous HPLC fractions from supernatant

No active fractions were found in the antioxidant screening of HPLC fractions from organic pellet and supernatant or in the aqueous supernatant from *P. glacialis*. These fractions were not further investigated.

4.2.3 Antibacterial screening

Five bacterial strains (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MR *S. aureus*) were used in the antibacterial screening. The fractions were considered active at a photometric measurement of less than 0.05 OD600nm (MIC value).

4.2.3.1 Organic and aqueous HPLC fractions from pellet and supernatant

No active fractions were defined in the antibacterial screening of HPLC fractions from pellet and supernatant from *P. glacialis*.

4.2.4 Anticancer screening

Three cancer cell lines (A2058, MCF7 and HT29) were used in the anticancer screening. One normal cell line from human lung tissue (MRC-5) was used as a toxicity control. The fractions were considered active with less than 50 % survival.

4.2.4.1 Organic and aqueous HPLC fractions from pellet and supernatant

No active fractions were identified in the anticancer screening of HPLC fractions of pellet and supernatant from *P. glacialis*.

4.2.5 Structure elucidation by LC-MS

The bioactivity data from the antioxidant screening was the background for evaluating fractions containing interesting activities for structure elucidation. Fractions 2 and 3 from the second screening of aqueous fractions from P. glacialis displayed antioxidative activity (figure 23).

The aqueous fractions of interest from *P. glacialis* were investigated by mass spectrometry in order to find the composition and structure of the components. The samples were ionized by electrospray, and accurate mass data at high resolution were acquired (figure 24). The data were used to calculate the elemental composition of the active compounds.

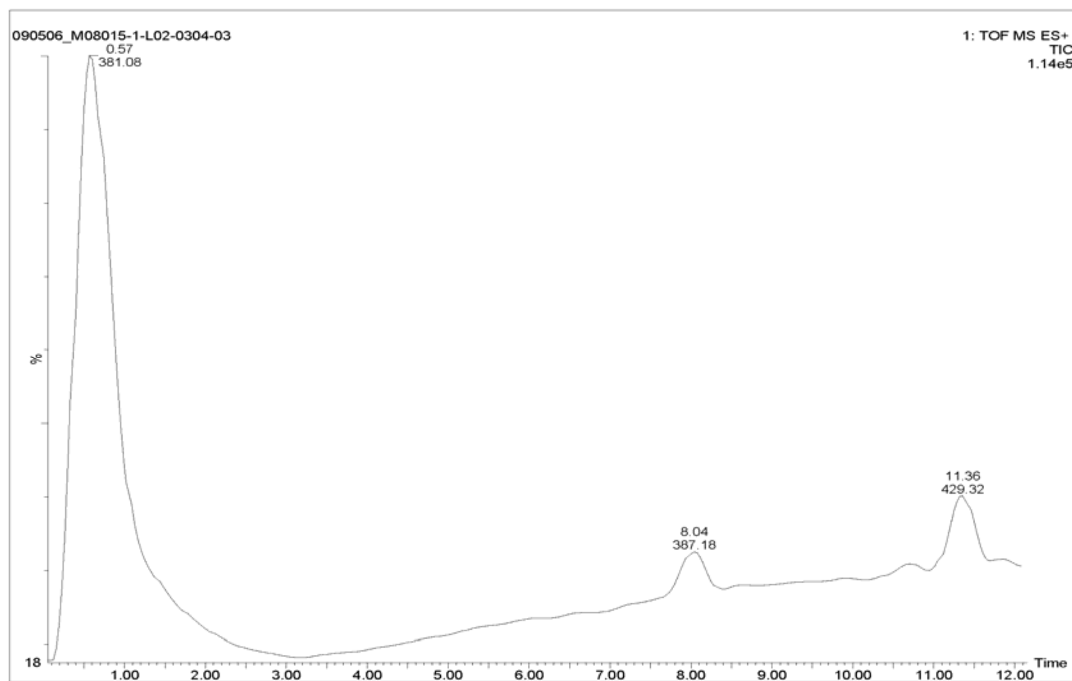

Figure 24: Total Ion Chromatogram for aqueous HPLC fraction 2 and 3 from pellet from the second antioxidative screening.

The total ion chromatogram (TIC) of fraction 3 and 4 revealed one dominating peak at 57 seconds with an m/z value of 381.08. It is highly likely that the compound can be linked to the observed bioactivity in the antioxidative screening. The pattern of the TIC indicated that several components had eluated at an early stage of the MS. Further investigation revealed two dominating components (figure 25) with an m/z of 365.11 and 381.08.

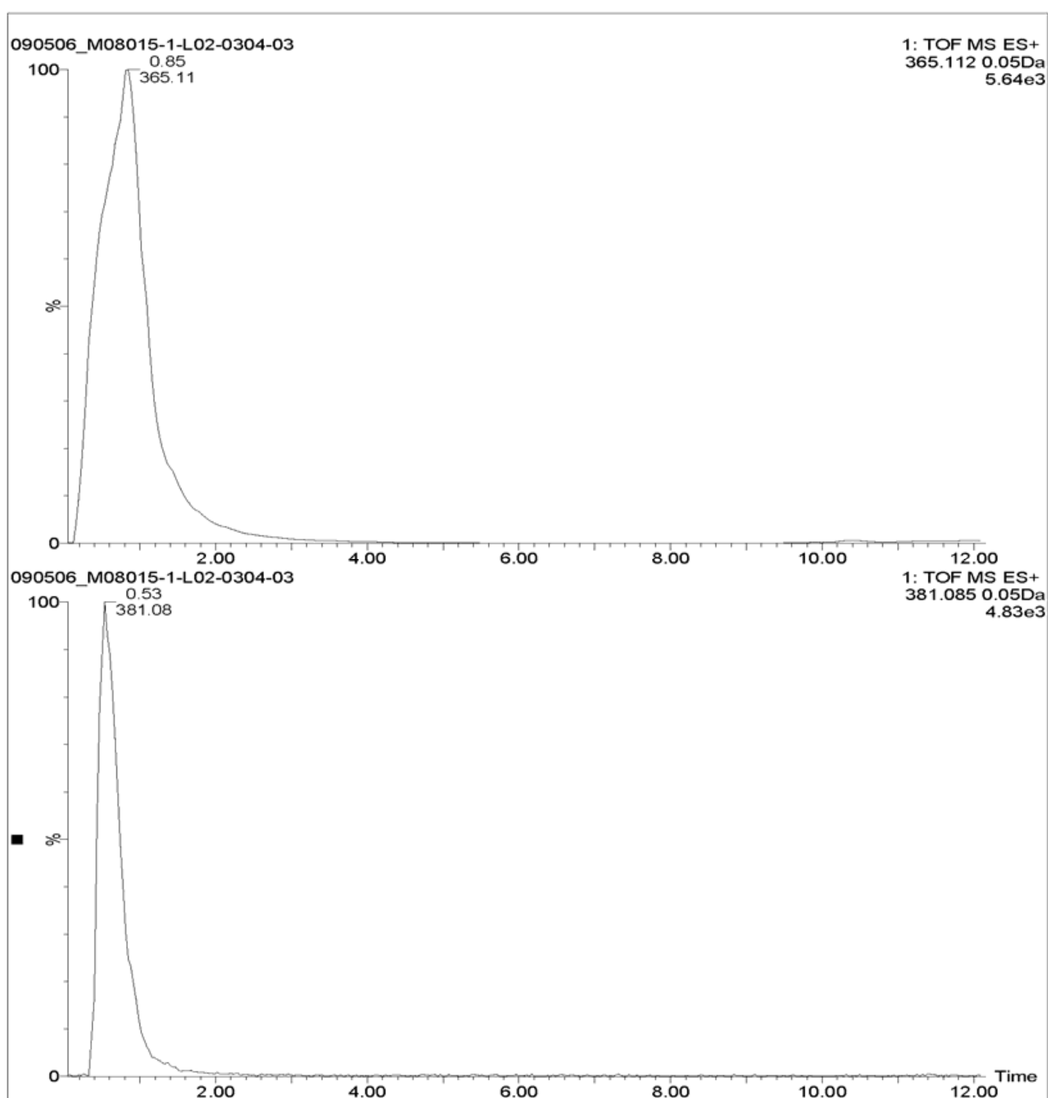

Figure 25: TIC for aqueous HPLC fraction 2 and 3 from pellet from the second antioxidative screening showing two dominant components.

The ESI MS spectra (figure 26 and 27) of the peaks revealed one major peak in each spectra. This gave a possibility of several components being responsible for the antioxidant effect. Searches in CHEMnetBASE did not give an unambiguous candidate for either one. Hence, identification could not be made at this stage, and a continued work in order to identify the active components is required.

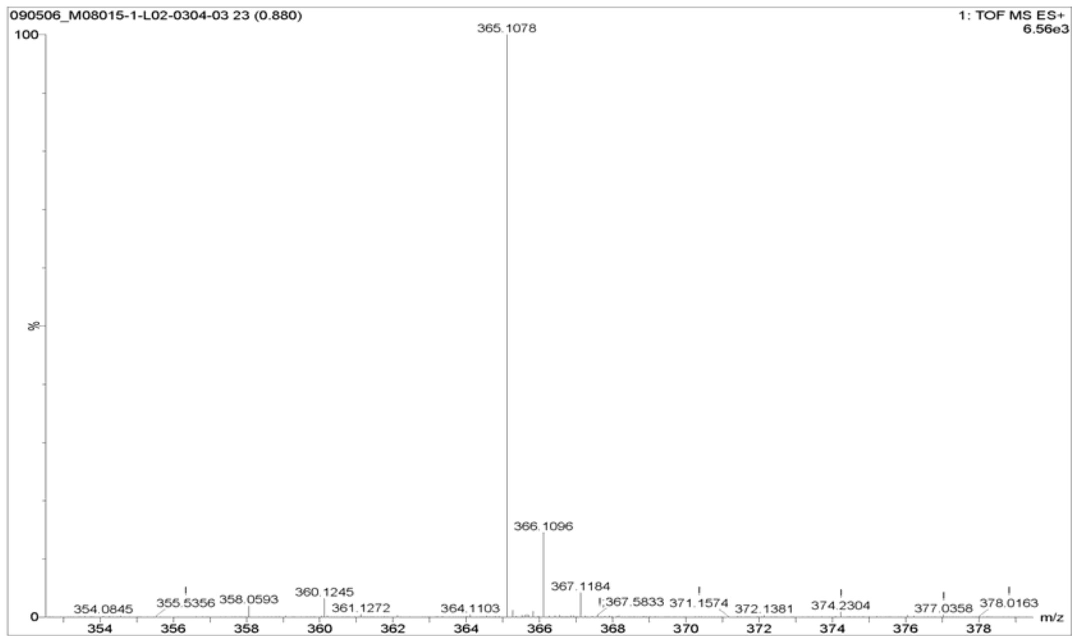

Figure 26: Positive mass spectrum of the compound eluating after 0.88 min.

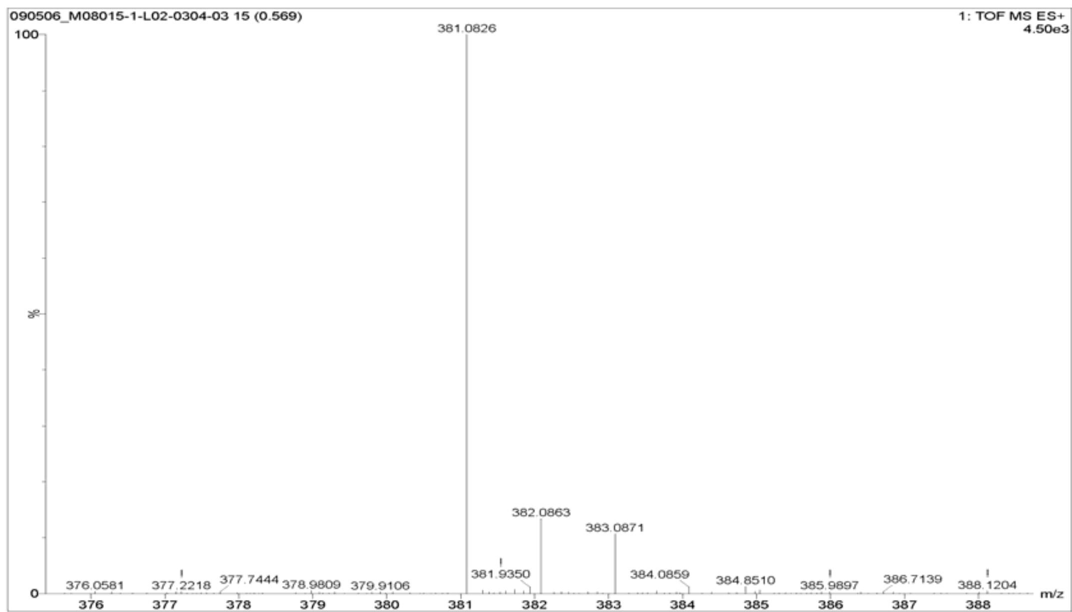

Figure 27: Positive mass spectrum of the compound eluating after 0.56 min.

5. Discussion

Marine bioprospecting has in the last decades lead to the development of several drugs based on natural products. The need for novel drugs is increasing due to issues concerning microbial resistance and curing of diseases. The nature has been a source of natural products almost as long as man has walked the earth, and now the attention has turned towards the Arctic and the marine environment in the search for new products in unexplored areas.

The overall aim of this master thesis has been to search for the presence of bioactive molecules in two Arctic marine species, a sponge and a phytoplankton. Previously it has been shown that marine sponges and microorganisms contain interesting compounds. In this study the main focus was on anticancer, antioxidant and antibacterial bioactivities in these two organisms.

All bioactivities were tested on the same fractions, and this made a comparison of the various bioactivities possible. The results showed that the organic extract from *A. sulfurea* contained compounds with cytotoxic properties towards both mammalian and prokaryotic (bacterial) cells. In addition, compounds with antioxidative properties were also found.

All the observed bioactivities in organic extracts of the sponge were found in almost the same fractions. This indicates that the same compounds may have a number of properties. It is well known that phenolic compounds like flavenoids have antioxidative properties and also may exhibit various physiological activities including anti-inflammatory, anticarcinogenic, antiallergic, antihypertensive, antiarthritic and antimicrobial activities (Puupponen-Pimia et al., 2001, Vaquero et al., 2007). The cytotoxicity towards mammalian cell lines is also cytotoxic for prokaryote cells, causing the antibacterial effect. It also seems that the prokaryote bacterial strains are more sensitive to the active components from the extracts of the sponge, because a wider range of fractions are inhibiting growth in the antibacterial screening. In the third screening of the re-chromatographed HPLC fractions the breast carcinoma cell line was inhibited by fraction 6. These results indicate that some cell types, in this case the breast carcinoma, is more sensitive to Ianthelline, compared to the other cell lines. Through the different chromatography steps the compounds in the extract can be diluted or lost, and the effect

seen in the organic re-chromatographed fractions of *A. sulfurea* in the third screening is a result of this. The re-chromatography of the active fractions also resulted in an earlier elution of the active compounds in the second purification step. The reason for this was the choice of a different mobile phase gradient in the re-chromatography. According to the fraction position of the bioactivities detected and hence the fractions chosen for re-chromatography, the bioassay guided separation was performed by choosing mobile phases according to this. This resulted in a shorter retention time, and as a result of change in mobile phase composition and retention time the activity was detected in different (earlier) fractions. In the third screening of anticancer activity in the organic extracts of *A. sulfurea* the activity was much reduced due to lower yield, but the activity could still be found in one of the fractions. The same gradient was used in both re-chromatography runs.

Using the FRAP assay two antioxidative positive fractions with the same retention times as the cytotoxic positive fractions, were found. This is a chemical assay, and a further investigation of the activity using another chemical assay, such as the Oxygen Radical Absorbance Capacity (ORAC) assay (Davalos et al., 2004), or a cellular assay such as the Cellular Antioxidant Activity (CAA) assay (Wolfe and Liu, 2007) would be necessary in order to confirm the activity and investigate the cellular applicability. However, these antioxidative fractions also contained cytotoxic compounds, and no further investigation with regard to the antioxidative activity was pursued.

Bioactivity data was used in order to guide the identification of the active compound for structure elucidation by MS. The activity from the third anticancer screening of the organic fractions of *A. sulfurea* was subjected to structure elucidation by MS in order to try to determine the active component. The Total Ione Chromatogram of fraction 6 came out with clear signals for the molecular mass of one molecule and a search pointed to Ianthelline, a brominated compound, as the active molecule in this fraction. Ianthelline has previously been described in a related sponge with regard to antibacterial and antifungal properties (Litaudon and Guyot, 1986). This is consistent with our data from the antibacterial screening. Marine sponges are rich in brominated compounds and molecules similar to Ianthelline have recently been described in sponges

from tropical waters (Nunez et al., 2008). It is well known from medical history and literature that halogenated small molecules may have antibacterial actions e.g. chloraphenicol. Specifically a recent study on brominated antimicrobials can be cited (Raimondi et al., 2006). Ianthelline has a phenolic part (methoxy-substituted dibromo-tyrosine) which might account for antioxidant property found.

The anticancer activity has not previously been reported for the molecule Ianthelline. This will need confirmation of the results and further investigation with purified Ianthelline in known concentrations.

Previously, not many studies on bioactivities in microalgae such as *Porosira glacialis* have been carried out, due to the difficulties concerning collection of pure samples. With the cultivation of pure cultures in tanks, new opportunities for studying these promising organisms and their defense mechanisms to the exposure of e.g. UV-radiation, is possible. However, it has been reported antimicrobial activity in extracts from microalgae (Engel et al., 2006), and microalgae are interesting with regard to medicinal and commercial development (Apt and Behrens, 1999). In this project there was access to both supernatant and pellet from the cultivation of *P. glacialis*. The supernatant was investigated in order to search for compounds secreted by the phytoplancton as part of their metabolism or defensive mechanisms. However, no positive results were obtained from the investigation of the supernatant by the selected bioactivity screenings.

Two fractions from the HPLC purification of the aqueous extracts from *P. glacialis* (pellet) had antioxidant properties. After re-chromatography, the activity was reduced, due to the loss of bioactive components through this step. No cytotoxic activity was found. Purification and mass spectrometry structure elucidation did not reveal any clear candidate molecules. Further work is needed in order to identify the active components. No other activity was discovered in extracts from supernatant or in the organic phase of the pellet.

The present study has shown that the sponge *Aplysilla sulfurea* contains the cytotoxic molecule Ianthelline with probably both antibacterial and anticancer activity. This confirms sponges as very interesting species when searching for possible new drug leads.

The further investigation may lead to publications and patenting of the anticancer activities found in Ianthelline. Furthermore, the phytoplankton *Porosira glacialis* is possessing water-soluble antioxidants that do not appear to be cytotoxic. The data contained in the mass spectrometry is complex, and further investigation of the fractions of interest will be carried out.

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BIOACTIVE METABOLITES IN MARINE SPONGES

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Background

Marine sponges, of the phylum **Porifera,** are believed to be the most primitive of the large animal phyla, and are sessile marine or freshwater animals with no organs, head, mouth or gut cavity. They have proven to be a rich source of bioactive metabolites.

Photo: Bernard Picton

The goal of this project was to search for and identify bioactive components from an Arctic sponge

Extraction/purification

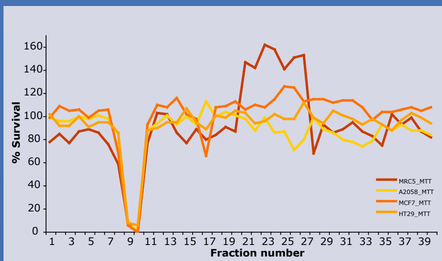
Aqueous and organic (dichloromethane: methanol) extracts were prepared from freeze dried samples of Aplysilla sp. The extracts were purified on a preparative HPLC (High Pressure Liquid Chromatography) system and separated into 40 different fractions based on the polarity of the molecules. The process was performed using a C18-column with a gradient of acidic water and acetonitrile.

Anticancer screening

Screening for bioactivity in marine extracts for the ability to kill or inhibit the growth of cancer cells was performed using the CellTiter 96® AQueous One Solution Cell Proliferation kit. The method determines the number of viable cells.

Cell lines:
A2058 cells (melanoma)
HT29 cells (colorectal adenocarcinoma)
MCF7 cells (breast adenocarcinoma)
MRC-5 cells (normal fibroblast)

Results



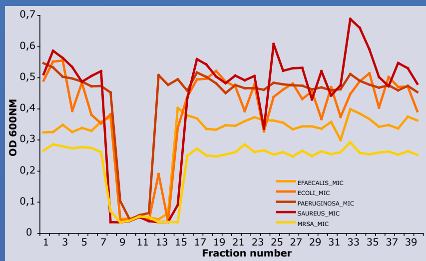
Fractions are defined as active when cell survival is less than 50 % compared to untreated cells. The figure shows the activity of 40 different fractions. The active fractions were pooled and further purified and retested for biological activity.

Antibacterial screening

Antibacterial screening was done by assaying inhibition of growth of Gram-positive and negative reference strains.

Bacterial strains:
E.coli
S.aureus
methicillin resistant S.aureus (MRSA)
P.aeruginosa
E. faecalis

Results



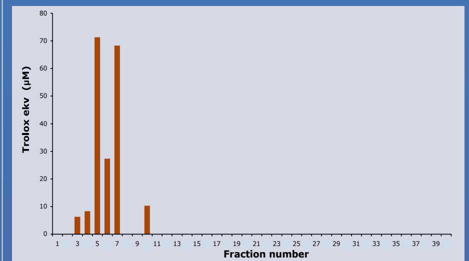
Fractions are defined as active when the optical density value is below 0,05 OD600nm. The active fractions were pooled and further purified and retested for biological activity.

Antioxidant screening

Screening for antioxidative activity was performed using the FRAP (Ferric Reducing Ability of Plasma) method.

The FRAP assay is a measurement of the power of antioxidants in the extracts. The reaction measures antioxidant reduction of ferric 2,4,6 – tripyridyl-s-triazine (TPTZ) to a colored product.

Results



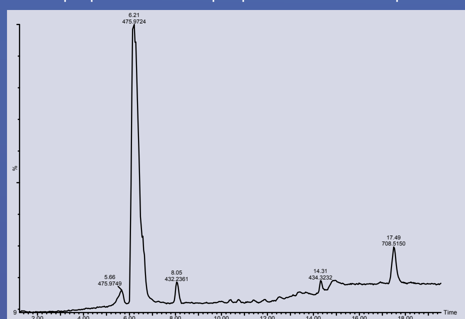
The results are presented as Trolox (vit. E analog) eqivalents. Fractions 5 and 7 are considered as active. The active fractions were pooled and further purified and retested for biological activity.

Structure elucidation by LC-MS

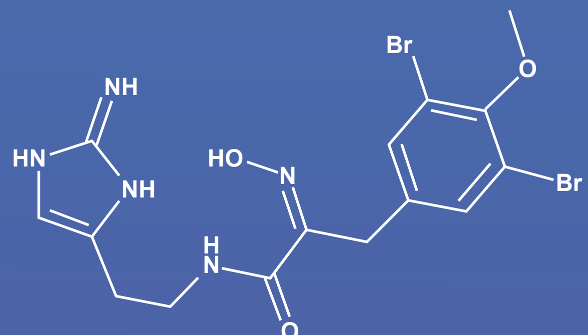
The purified active fractions from the bioactivity screening were investigated with LC-MS. The samples were ionized by electrospray, and accurate mass data at high resolution were acquired for both negative and positive ions. The data were used to calculate the elemental composition of the active compound(s).

Results

The total ion current chromatogram of the purified fraction active against bacteria revealed one major peak. The isotopic pattern for this compound was typical for compounds containing two bromine atoms, and the elemental composition analysis suggested that the molecular formula was $C_{15}H_{17}Br_2N_5O_3$.



Ion chromatogram from the LC-MS analysis of the purified bioactive fraction of the extract from *Aplysilla sp.*



Searches in databases revealed that a component with antibacterial properties and identical molecular formula had been identified in a related arctic sponge. Thus, we conclude that we have found the same compound, ianthelline, in the extracts from *Aplysilla sp.*

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