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Isolation and characterization of bioactive compounds from the marine hydrozoans *Halecium muricatum* and *Halecium beanii*

Thesis for the degree Master of Pharmacy

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Preface

The work for this thesis was performed at MabCent- CRI, Department of Chemistry at the University of Tromsø and Norut. The work started in September 2011 and ended in May 2012. The supervisors were Terje Vasskog, Jeanette H. Andersen and Espen Hansen.

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Abstract

In the marine environment there is a great potential for discovering new commercial drugs, and marine bioprospecting has previously led to the discovery of drugs available for clinical use, like Yondelis ® and cytosine arabinoside. In this work two hydroids were tested for anticancer and antibacterial activity.

The organic extracts from the two hydroids, *Halecium muricatum* (M11046) and *Halecium beanii* (M11047), showed activity in the 1st and 2nd screening against the human melanoma cell line and *Streptococcus* group B. This resulted in dereplication and isolation of eight possible bioactive compounds. After the isolation, the two purest compounds were analyzed using TOF- MS, ^{1}H- NMR, ^{13}C- NMR, HMBC combined with HSQC NMR and MS- MS for structure elucidation. From the structure elucidation it was obvious that the two compounds were phosphocholines, however the exact structures were not confirmed. These two compounds were tested against human melanoma, normal lung fibroblasts, breast cancer and prostate cancer for confirmation of bioactivity. Compound **1** showed a weak effect against the normal lung fibroblast cell line at 100 µg/ mL. The lack of activity against the human melanoma cell line indicate that compound **1** was not responsible for bioactivity in the 1st and 2nd screening against this cell line, or that it was a synergic effect responsible for the observed bioactivity. Compound **2** showed a weak effect against human breast carcinoma and were active against normal lung fibroblasts and human melanoma at 100 µg/ mL. Activity against normal lung fibroblasts was undesirable since this cell line was used as a toxicity control. Effect against this cell line indicates no specificity against cancer cell lines versus normal cells. This work is to the best of our knowledge the first work to test the anticancer and antibacterial effects of hydroids. Another contribution of this work was the ability to detect phosphocholines present in a sample at an earlier stage. In bioprospecting the early detection of previously known compounds is essential to minimize the use of time analyzing these compounds.

Abbreviations

ACE	Angiotensin Converting Enzyme
ACN	Acetonitrile
ATL	Anti- tumor Lipids
AQOS	Aqueous One Solution
A2058	Human melanoma cell line
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
COSY	Correlation Spectroscopy
DCM	Dichloromethane
ddH$_2$O	Deionized distilled water
D- MEM	Dulbecco's Modified Media
DMSO	Dimethyl Sulfoxide
DU145	Human prostate cancer cell line
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E- MEM	Eagle Minimal Essential Media
ER	Endoplasmic Reticulum
ESI	Electrospray Ionization
EtOH	Ethanol
FA	Formic Acid
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
GC	Gas Chromatography
HCN	Hydrogen Carbon Nitrogen
HMBC	Heteronuclear Multiple- Bond Correlation
HPLC	High Performance Liquid Chromatography
HR- MS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single- Quantum Correlation
HTS	High- Throughput Screening
LB	Luria- Bertani medium
M	Molecular ion
MCF7	Human breast carcinoma cell line
MeOH	Methanol
MH	Mueller- Hinton
MIC	Minimum Inhibitory Concentration
MRC5	Normal lung fibroblasts cell line
MS	Mass Spectrometry
MS- MS	Tandem Mass Spectrometry
m/z	Mass- to- charge
NaCl	Sodium Chloride
NCI	National Cancer Institute in USA
NEAA	Non- Essential Amino Acid
NMR	Nuclear Magnetic Resonance
OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*

PBS	Dulbecco's Phosphate Buffered Saline
R & D	Research & Development
RP	Reverse Phase
RPMI- 1640	Roswell Park Memorial Institute- 1640 cell media
RPM	Rotations Per Minute
SAR	Structure Activity Relationship
S. aureus	*Staphylococcus aureus*
TOF	Time of Flight
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet light

1. Introduction

1.1 History of natural products

For many millennia nature has provided humankind with treatments for different ailments and diseases. Plants have often been the remedy for these sufferings, which is evident from the "Ebers Papyrus". This record dates back to 1500 BC, and is perhaps the best known record of ancient Egyptian medicine. The "Ebers Papyrus" documents over 700 drugs, and many of these drugs have a plant origin [1]. The Egyptians were not alone in practicing medicine in this era. There have been found records from Mesopotamia, which dates back to 2600 BC and records from China, which dates back to 1100 BC. The records from Mesopotamia documents use of about 1000 substances of plant origin [1]. What's fascinating about these records is that we still use many of these substances today thousands of years after their discovery. Even today a major part of the world's population, approximately 60%, relies on plants for medication [2]. Since plants had an indisputable role in curing various ailments, the search for new drugs started with plants, animals and microbes. The first commercial pure product was first sold in 1826 by E. Merck, and was morphine from the opium poppy *Papaver somniferum* [3]. Some of the major discoveries after morphine are the cardiac glycosides, digitoxin and digoxin, from foxglove (*Digitalis purpurea)* which are used against chronic heart failure [4]. The venom of the pit viper (*Bothrops jararaca)* led to the discovery of angiotensin converting enzyme inhibitors against various cardiovascular diseases like hypertension [4]. The ACE inhibitors are perhaps the most important class of antihypertensive drugs today. Another example of a compound found in nature with major impact on treatment of diseases is of course Alexander Fleming's discovery of penicillin from the fungus *Penicillum* [4].
The common ground for these discoveries, are that all have terrestrial origin. At this point in history, the marine environment was completely left out.

1.2 Marine bioprospecting

Bioprospecting or biodiversity prospecting is defined as the
"*process of collecting or surveying of a large set of flora (or fauna) for purpose of biological evaluation and isolation of lead compounds*" [4].

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The start of marine biodiscovery begins with Bergmanns discoveries and identification of the spongothymidine and spongouridine from the Caribbean sponge *Thetya crypta* in the early 1950s. These discoveries led to the identification of cytosine arabinoside, a compound used against leukemia [5].

How marine organisms defend themselves against predators, competition against other marine organisms and diseases were questions which arose. This led to the discovery that especially sedentary, soft- bodied marine organisms use secondary metabolites as a defensive mechanism [5]. These secondary metabolites display characteristics of effective drugs, these traits are high potency, highly targeted mechanism of action, and the ability to permeate membranes, since the metabolites need to be intracellular to be effective [5].

Other reasons pro marine bioprospecting is the high biodiversity in the marine environment and that the marine environment still is underexplored. Tropical and subtropical regions are the regions that have been explored in the largest scale, while the Arctic regions are virtually unexplored [1]. David Newman said in his lecture in Tromsø in March 2012, "the coral reef is the rainforest of the ocean". This implies the vast biodiversity in the marine environment, and in nature. Newman also said that "Mother nature doesn't make a structure unless it has a function. We just don't know what it does yet". This is obvious, since production of metabolites from an organism is energy consuming. If the organism made unnecessary molecules or metabolites, that organism would face extinction. Because of the biodiversity and the purpose behind every metabolite, the marine environment, and nature in general, is a perfect place to look for new drugs.

From statistics it is evident that a major part of new small molecule drugs come from nature, as much as approximately 50% in the years 2000 to 2010, while combinatorial chemistry has failed in the search for new drugs, or new chemical entities. Only one "de novo" new chemical entity was discovered between 1981 and 2010. However, the role of combinatorial chemistry in chemical altering of new scaffolds found in nature is undisputable, since the new chemical entities become more potent and gains greater activity [6].

Marine bioprospecting is a promising field because extracts from marine organisms are more likely to lead to anticancer drugs than extracts of terrestrial origin, and because there still probably are many undiscovered active compounds in nature [7].

1.3 Sources of marine natural products

In the early stages of marine bioprospecting before the scuba technique was available, the collection of samples were limited to intertidal areas. Some of the first creatures to be analyzed were red algae, sponges and soft corals. With the advent of the scuba technique, the diversity of creatures available for analyzing increased compared to the creatures previously available. Creatures now available for examination were marine cyanobacteria, marine fungi and marine eubacteria, which led to the realization that many compounds isolated from macroorganisms like sponges actually were secondary metabolites produced by microbes. This discovery inspired groups to cultivate marine bacteria, which gave a reliable supply of samples to analyze. However, it is estimated that today still only 1 % of bacteria from seawater have been cultured. This cultivation has obviously given results, since the majority of marine natural products which are FDA approved or in phase I- III trials originate from bacteria [8]. Sponges have for a long time been the marine invertebrate which researchers were most interested in, however lately Cnidarians have started to attract attention because of their production of venoms and toxins [7].
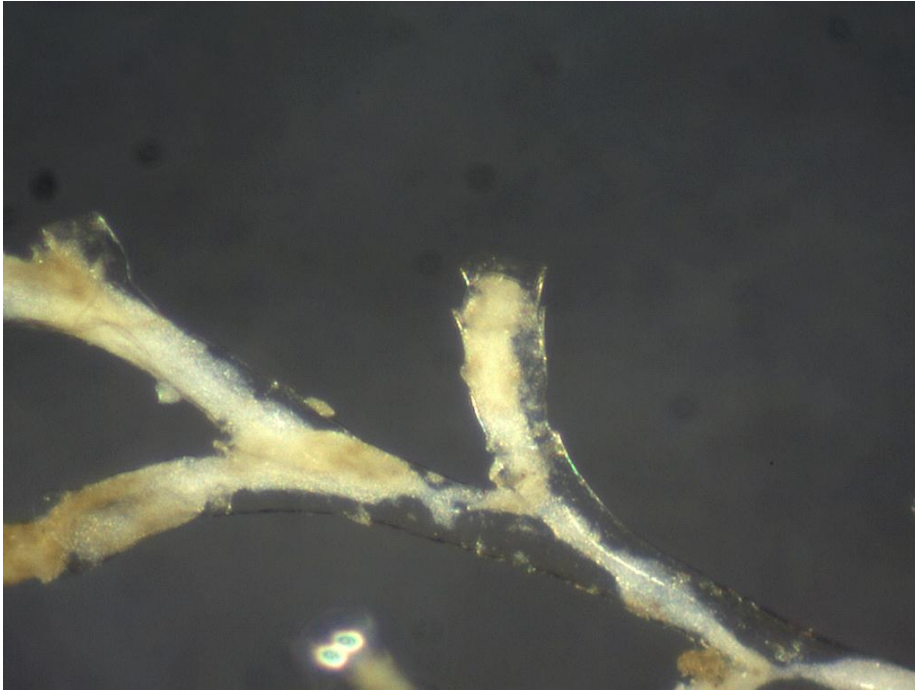
Halecium muricatum and *Halecium beanii*

The organisms analyzed in this work are *Halecium muricatum* (M11046) and *Halecium beanii* (M11047). They are closely related species, belonging to the same genus namely the class Hydrozoa (Phylum Cnidaria). Hydroids are small, abundant colonial species. In the hydroid colonies, each individual is called a polyp, which buds out from a main stem. This stalk is fastened to the substrate, often on algae, a rock or a shell. The polyps are interconnected through the stem and the whole colony shares a common gastrovascular cavity. One character trait shared by many colonial animals, including hydroids, is that the individuals exhibit morphological polymorphism. This means that the colony possesses two or more structurally and functionally different kinds of individuals. Two common kinds of individuals are the gastrozooids, which captures food and take part in digestion, and the gonozooids which plays a vital role in asexual reproduction. Medusae are budded from the gonozooids. They represent the sexually reproducing generation of the hydroid colony. Defensive individuals are common as well. The *Halecium* species are thecate hydroids, which mean they have hydrothecae. A hydrotheca looks like a goblet, which protects the individuals

in the colony. The hydrothecae in this family only envelop the lower part of the polyps, and can often take a stacked form. *Halecium* species are separated by the form of their gonothecae, which are the reproduction polyps. If these polyps are not present, the identification of species is extremely difficult [9].
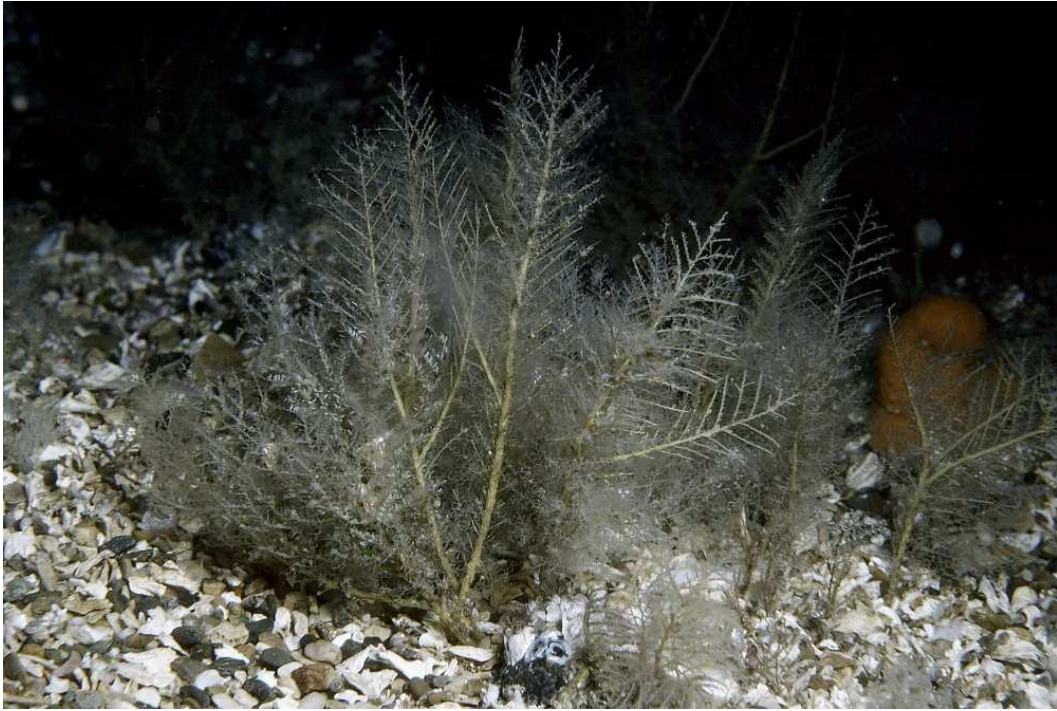
Picture 1: Picture of a colony of *Halecium muricatum* (M11046) in its natural environment. (www.habitas.org)

Picture 2: Picture of *Halecium muricatum* with a hydrothecae.

Picture 3: Picture of *Halecium muricatum* with multiple intertwined stems and the reproduction polyps (gonothecae).

1.4 Cancer

Cancer is a collective term for approximately 200 distinct types of diseases [10]. There are six hallmarks, or six alternations that lead to malignant growth. These hallmarks are self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [11]. In the year 2009, 27 520 persons got the diagnosis in Norway. Among women the most frequent type of cancer is breast cancer, and there were reported 2745 new cases in 2009 [10]. In a worldwide perspective, breast cancer is the leading cause of cancer death among women, as well as the most frequent diagnosed cancer [12]. The five year mortality is approximately 20 %, but collectively one out of three will die because of breast cancer [13]. Among males prostate cancer is most common, and in the year 2009, 4299 men got the diagnosis in Norway [10]. The mortality is approximately 3 % [14]. Malignant melanoma is in Norway the second most frequent diagnosed type of cancer in the age group 15- 54 years. In 2009 there were 1413 new cases diagnosed in Norway, and this incidence rate is one of the highest in the world [15] .The current forms of treatments for cancer are surgery, radiation and chemotherapy. Even though the incidence of breast cancer in all ages in Norway is increasing, the mortality rate for breast cancer is declining [16]. The reason for this decline in mortality may be the result of a better national screening program and more effective treatment. Yet today's treatment with cytostatics is still associated with many serious side effects. Some side- effects are constipation, diarrhea, nausea, vomiting, anemia, neutropenia and thrombocytopenia [17]. With radiation therapy one of many side- effect is second malignant neoplasms [18]. Obviously there is a need for more target specific and effective treatments. For some types of cancer the treatment is not good enough, which is the case for lung cancer. The five year survival rate after diagnosis is lower than 20 % [19].

A recently approved cancer drug in EU was Trabectidin (Yondelis ®) from the marine tunicate *Ecteinascidia turbinate*. Trabectidin function as a DNA alkylating agent, which challenge the DNA repair machinery. In EU Trabectidin's approval was as a second- line treatment for advanced soft tissue sarcoma. Paclitaxel (Taxol ®) from the bark of *Taxus brevifolia*, Pacific yew, dominates the cytotoxic marked [20] with its clinical use against ovarian cancer, breast cancer, non-small-cell lung cancer, small cell lung cancer and cancers in the head and neck [21]. However, because of resistance induced by efflux proteins and toxicities there is a need for new drugs against cancer [20].

1.5 Bacteria

A new era in the treatment of infectious diseases started with Alexander Flemings discovery of penicillin from the fungus *Penicillum notatum* in 1928 [22]. Penicillin was not utilized as an antibiotic until the 1940s, when scientists from England and USA developed the appropriate fermentation technology to yield high- purity penicillin in large enough quantities. After the discovery of the fermentation technique, and during the following decade, there was an enormous focus on antibiotic research, which led to the discovery of the better part of antibiotic classes that we currently use therapeutically [23]. In the 1960s and early 1970s, it was thought that the previous problematic infectious diseases were overcome [24]. However, the situation today is that only four new classes of antibiotics have arrived at the marked since the early 1960s [25], and the occurrence of multidrug- resistant bacteria has led to infections which have few, or none treatment options [24]. To handle the critical situation with multidrug- resistant bacteria, new classes of antibiotics needs to be developed. Only new classes of antibiotics with novel mechanisms of action can handle drug resistance [24]. In spite the fact that there is a need for new classes of antibiotics which show novel mechanisms of action, the research and development for antimicrobials are reduced or ceased by many pharmaceutical companies [26]. The reasons why pharmaceutical companies reduce or cease their R&D funding may be the fact that developing a new drug is a costly and lengthy process. The development of a new drug takes 8-15 years, and may cost 800 million USD [27]. Another reason may be that developing a new drug against an infection is less economically attractive [24]. The treatment of an infection is short term, and pharmaceutical companies focus now largely on chronic diseases prevalent among the elderly, for instance hypercholesterolemia and hypertension which is economically attractive. The new antibiotics marketed in the past years are only new generations of already existing classes of antibiotics, which are synthetic tailored and only results in marginally improved drugs [27]. Because of the lack of new antibiotics with novel mechanisms of action and the fact that infectious diseases cause 17 million deaths annually worldwide [26], the importance of research centers like MabCent is evident.

1.6 MabCent- Centre for Research based Innovation (CRI)

MabCent- CRI was established by the Research Council of Norway in March 2007, and is a centre for research- based innovation. The main objective of MabCent- CRI is to find and develop high- value bioactive products through screening of Arctic or sub- Arctic organisms for innovative and commercial use. MabCent uses High- throughput screening (HTS) in its work to detect bioactivity in different bioactivity assays. HTS is an automated process which enable the screening of large numbers of compounds for bioactivity [4]. The screening method combines chromatographic bioassay- guided fractionation with various bioactivity assays, and is essential in the discovery of new drugs or bioactive products. The organisms in focus are marine bacteria, algae and benthic invertebrates [28]. Marbank collect samples, determine the taxonomy of the samples, organize the samples and produces extracts for the high throughput screening platform Marbio. Active compounds are isolated and characterized by MabCent. Other groups at the Univeristy of Tromsø and the University Hospital in North Norway also determine mode of action of bioactive compounds. The therapeutic areas that MabCent focuses on are antibacterial activity, antitumor activity, anti- inflammatory activity, diabetes, obesity, antioxidants and immunostimulants. The obtaining of samples is done by surveys in marine waters from the coast of northern Norway to Svalbard. The methods used to obtain the samples are divers, dredging and trawling [29]. Previous discoveries made by the biotech industry in Tromsø, were not done by systematic approaches, and hopefully will the systematic high- throughput screening implemented by MabCent lead to an increase of hits and leads.

2. Aim of this study

The main goal of this work was to isolate and characterize the structure of bioactive compounds from the marine benthic organisms *Halecium muricatum* (M11046) and *Halecium beanii* (M11047). This was achieved by screening the extracts from the organisms against different cancer cell lines and bacterial strains, and to identify the structure of active compounds as well. Although the main priority was the anticancer screening, the bioactivity screening against different bacterial strains was carried out to detect if there was a general cytotoxic effect, or a specific cytotoxic effect against eukaryotic cells.

3. Theory

3.1 Extraction

The purpose of extraction was to extract as many compounds from the biomass sample as possible over in a solution. Aqueous and organic extraction was chosen to get a broad spectrum of compounds available for bioactivity testing. When the biological samples were collected in the Barents Sea by MabCent, the first part was to identify the species. The preparation of samples began with freeze drying of the biological sample, followed by pulverization of the freeze dried sample. This was done because the hydrophilic compounds can be stored in membrane pockets, be protected by lipophilic substances or be bound to membranes. Then the extraction of the pulverized and freeze- dried sample began. The solvents used in extraction were based on the characteristics of the desirable compounds we wanted to extract. For the aqueous extraction water was used to extract the hydrophilic compounds in the material. The organic compounds, or the hydrophobic compounds, were extracted using dichloromethane and methanol.

Before HPLC fractionation of the organic extract, liquid- liquid- extraction was done by using acetonitrile and hexane in a separating funnel. The acetonitrile phase is the phase where potential compounds may reside, and the hexane phase is the phase where the most lipophilic compounds reside. Drugs need to be sufficiently lipophilic to cross membranes, but should not be so lipophilic that they precipitate in the blood, which very well may be the case with compounds from the hexane phase. Drugs precipitating in the blood can have a fatal ending for the patient receiving the treatment. Therefore the highly lipophilic hexane phase was not used, while the acetonitrile phase was brought further in the process.

3.2 High Performance Liquid Chromatography and Mass Spectrometry

High Performance Liquid Chromatography (HPLC) is a chromatographic method used to separate compounds in a liquid. A HPLC setup consists typically of an injector, a column, a pump, mobile phases and a detector. The retention time is the time it takes for the compound to traverse the column, and can give an indication about the characteristics of the compound eluted. The main principle is that components in the sample are carried by the mobile phase through a column, and when the compounds have arrived at the end of the column they enter

a detector. The detector measures one or multiple characteristics of the analyte, depending on what kind of detector is used. This is converted to a signal which is displayed as a chromatogram, which shows the signal versus time. Ultra Performance Liquid Chromatography (UPLC) involves smaller particle sizes, lesser internal diameters of the column and a higher counter pressure, which results in a higher resolution of the analyses.

The column and the mobile phase used in HPLC/ UPLC separate the sample compounds. The perhaps most widely used column is the reverse phase column. The stationary phase in the reverse phase column is non- polar, compared to what historically was named normal phase chromatography where the stationary phase was polar. The column is called reverse phase since the introduction of this column reversed the elution order [30]. In the reverse phase column the hydrophobic stationary phase is bound to the silanol groups, where most often the hydrophobic group is C 18. The mechanism of retention utilized in reverse phase columns is van der Waals forces, which retard hydrophobic molecules the most, and small hydrophilic molecules the least. The small hydrophilic molecules will then elute first, which obviously gives them the shortest retention time. Hydrophobic molecules will elute last, and have the longest retention time.

Two commonly used detectors in a HPLC setup are the UV- detector and the mass spectrometry detector. In this work both detectors were used. With the mass spectrometry detector the compounds need to be ionized before they are detected. Within LC- MS electrospray ionization is a common ion source, where the compounds undergo a loss or gain of a proton, and the product is called a deprotonated or protonated molecular ion. Other possibilities are the formation of adducts, which is called a pseudo molecular ions. In this work Ultra Performance Liquid Chromatography- Mass spectrometry with electrospray ionization (ESI) in positive mode was used with a time- of- flight mass spectrometer.

3.3 Structure elucidation

Ultra violet (UV) spectroscopy

The value of utilizing UV- spectroscopy is to detect functional groups like ketones, esters and amides, as well as the presence or absence of unsaturated functional groups. The phenomenon that creates UV- peaks is the excitation of an electron from an orbital, which requires energy. This leads to an electron transition from a lower to a higher energy state [31]. UV-

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spectroscopy is based on Beer's law. Beer's law states that if monochromatic electromagnetic radiation with the intensity I_0 is directed towards a solution of an analyte, some of this radiation will be absorbed. The rest of the radiation will pass through the sample. The intensity of the radiation is measured on the back of the sample.

$A = \log (I_0/I) = a \times b \times c$,

Where A is the absorbance, a is absorbency, b is the path length and c is the concentration of the analyte [32].

UV- spectroscopy can be used for quantitative and qualitative measurements. Identification is performed by comparing the spectrum of an unknown analyte with a reference spectrum for a known compound. If the spectra are identic, it is highly probable that the analyte and the compound is the same. However multiple compounds can have nearly identical spectra, and other structure elucidation techniques are often required [32]. In this work UV spectroscopy was used as a general detector, not as a part of the structure elucidation. However as written earlier, UV spectroscopy can be used in structure elucidation.

Nuclear magnetic resonance

In the process of structure elucidation the obtaining of ^{1}H- NMR and ^{13}C- NMR is crucial. The sample in the NMR spectrometer is exposed to radiofrequency radiation in the presence of a strong external magnetic field. In ^{1}H- NMR the spectrometer measures the energy levels of the nucleus of hydrogen, which is possible because the radiation in the presence of the magnetic field can change the orientations of protons in the nucleus [33]. The NMR spectra are generated by the magnetic properties of the atomic nuclei of the analyzed elements. These magnetic properties are generated by the spinning charge of electrons [34]. This gives information about the hydrogens in the molecule. The properties of the proton of hydrogen appear also in [13]C. The ^{13}C- NMR spectroscopy is based on the presence of ^{13}C in a mixture with ^{12}C, since only ^{13}C is detected in the analysis. These spectra are often simplified by decoupling the effects of the hydrogens, and reveal the different kinds of carbon atoms in the compound [33]. Other NMR methods used in this work was HSQC (Heteronuclear Single-Quatum Correlation) and HMBC (Heteronuclear Multiple- bond Correlation). These spectra are often coupled together giving information about the correlation between two different nuclei separated by one bond (HSQC) where each unique proton coupled to a carbon gives a

peak, and correlations over longer ranges approximately 2-4 bonds (HMBC). With COSY (COrrelation SpectroscopY) one can detect which atoms are connected to each other. NMR is a non- destructive method of analysis.

High resolution Mass spectrometry

High resolution mass spectrometry is used to accurately determine the mass of the molecular ion in structure elucidation to identify or confirm the molecular formula for a compound. The spectrometers have evolved over time to overcome limitations of this technique like peak broadening and interfering ions. This evolution led to the more recent techniques like electrospray ionization (ESI), matrix- assisted laser desorption/ ionization (MALDI) and time- of- flight (TOF). In this work ESI and TOF was used [35].

MS- MS

In MS- MS, or tandem mass spectrometry, two mass analyzers are coupled in series. The first MS detects the mass of a selected ion (for example *m/z* 482) out of a mixture of ions created in the ion source of the first MS. Collision activated decomposition (CAD) occurs in an intermediate region, which leads to fragmentation of the selected ion. These fragments created by CAD are detected by the second MS. These fragment ions from the parent ions gives information about substructures and functional groups [36]. MS- MS instruments are often Quadrupole – Time of Flight instruments (Q- ToF) or Tandem Quadrupole instruments (TQ).

4. Chemicals and equipment

4.1 Chemicals

Table 1: List of chemicals used in this work.

Chemical	Manufacturer/ producer/Firm
Acetonitrile (HPLC grade)	Merck, Darmstadt, Germany
Acetonitrile (UPLC grade)	Merck
Aqueous one cell solution	Promega, USA
Brain heart infusion	Oxoid, Cambridge, England
Dichloromethane	Merck
DMSO	Sigma- Aldrich, St. Louis, MO, USA
Dulbecco's Phosphate Buffered Saline	Biochrom, Berlin, Germany
D- MEM	Invitrogen, California, USA
E- MEM	Biochrom
Ethanol 96 %	Sigma- Aldrich
Fetal bovine serum	Biochrom
Formic acid	Merck
Gentamicin	Biochrom
Hexane	Sigma- Aldrich
Methanol	Sigma- Aldrich
MilliQ Water	Millipore, Billerica, MA, USA
Müeller Hinton bullion	Merck
NEAA	Biochrom
RPMI- 1640	Biochrom
Trypsin	Gibco, NY, USA

4.2 Equipment

<u>Preparative HPLC- MS</u>

Waters 600 E (Milford, MA, USA)

Waters 2996 photodiode array detector

Waters 3100 mass detector

Waters 2767 Sample manager

Waters flow splitter

Waters prep degasser

Waters 515 HPLC pump

Software: MassLynx V4.1 SCN714

Column: Aqueous extracts: Atlantis ® Prep dC18 10µm 10 x 250 mm

                 Organic extracts: Xterra ® Prep RP18 10 µm 10 x 300 mm

                 Purification: XSelect CSH Prep Fluoro- Phenyl 5µm 10x 250 mm

Pre- column: XTerra ® Prep RP 18 10 µm 10 x 10 mm

<u>High Resolution- MS</u>
Waters Acquity UPLC

Detector: Waters LCT Premier

Software: MassLynx V4.1 SCN639

Column: Acquity UPLC ® BEH C18 1.7 µm 2.1 * 50 mm

<u>NMR:</u>

Varian Spectrometer 600MHz (Varian, Palo Alto, USA)

Probe: inverse detection HCN cryoprobe

<u>MS-MS</u>
Waters Xevo TQ MS

Programvare: Masslynx 4.1

Collision gas: Argon 4.0 (Aga, Oslo, Norway)

Desolvation gas: Nitrogen from generator (Peak, Glasgow, UK)

<u>Miscellaneous:</u>

Minishaker (VWR International, Radnor, Pennsylvania, USA)

Rotary evaporator Heidolph Laborata 4002 (VWR International)

Edmund Bühler GmBH 5m- 30 control shaker (Hechingen, Germany)

Centrifuges: Heraeus Multifuge 3 S-R (Thermo Fisher Scientific, Waltham, MA, USA)

Heraeus Biofuge Pico (Thermo Fisher Scientific)

Freeze- dryer: Heto Power Dry PL9000 (Thermo Fisher Scientific)

IKA ® A11 basic grinder (IKA works, Staufen, Germany)

SC250 Express SpeedVac Concentrator (Thermo Fisher Scientific)

RTV 4104 Refrigerated Trap (Thermo Fisher Scientific)

Victor 3 Multilabel Counter (PerkinElmer, Waltham, MA, USA)

- Software: WorkOut 2.5 (dazdaq, Brighton, England)

Heidolph Incubator 1000 (VWR International)

DTX multimode detector (Beckman Coulter, USA)

5. Method

5.1 Retrieving the specimens, extraction and fractionation

5.1.1 Retrieving the specimens

The specimens of *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) were collected with an Agassiz dredge trawl at 30 m depth northwest of Spitsbergen (74º19'N,19º8'E) in May 2009. Identification was performed by Robert Andre Johansen, Marbank and confirmed by Ninel Panteleeva, Institute for Marine biology in Murmansk. The biomass sample was stored in the dark at -22 °C until further used and a reference specimen was deposited as a sub-sample in the Norwegian National Marine Biobank (Marbank), University of Tromsø, Norway.

5.1.2 Extraction of *Halecium muricatum and Halecium beanii*

Pre- treatment of samples before extraction

The purpose of the pre- treatment of samples was to prepare the sample for extraction. This method is appropriate for samples in the weight range of 100g – 500 grams. The frozen organism was stored in the freezer, and thawed in room temperature or in the refrigerator. The samples were dissected, transferred to glass flasks on an ice bath and frozen overnight at – 20 °C. The next day the samples were cut into 1 cm dices, weighed and frozen. Afterwards the samples were freeze- dried for 2-3 days, and grinded. The grinded samples were weighed and transferred to a flask.

Extraction

The purpose of the extraction was to produce aqueous and organic extracts. The aqueous extract was prepared first.

Aqueous extract:

First ultra-pure water (Milli-Q water) was added to the samples. The amount added was approximately ten times of the dry weight of the pre- treated samples. Then the flasks were shaken by hand, and stored in the refrigerator at 5 °C. At the end of the day the samples were

shaken again, and stored overnight in the refrigerator. The next day the samples were distributed to two 0.7 L centrifuge flasks, and centrifuged for 30 minutes at 3400 g at 5 °C. The supernatant was removed and transferred to a flask. Then there was a second round of extraction, where 750 mL of Milli- Q water was added to the centrifuge flasks for *Halecium muricatum,* and 1200 mL for *Halecium beanii*. The samples were mixed with a spoon, and were stored for 30 minutes in the refrigerator at 4 °C. After the storage, the samples were centrifuged by the same conditions as the first extraction. After the second round of extraction each sample had two supernatants. The two supernatants were pooled, frozen at – 20 °C and freeze dried overnight. The two pellets from the two rounds of extraction were transferred to two Pyrex dishes, one for each sample, frozen, freeze dried and weighed.

Organic extract:

The pellet from the aqueous extraction was used to produce the organic extract. Extraction agent was added slowly, until the ratio extraction agent: sample (dry weight) was 10:1. For *Halecium muricatum* 800 mL of extraction agent was used, and for *Halecium* beanii 1400 mL was used. The extraction agent used was DCM and methanol, mixed to a 1:1 ratio (volume-volume). After the extraction agent was added, the sample was shaken and stored in the refrigerator at 5 °C. At the end of the day, the sample was shaken, and stored overnight in the refrigerator. The next day the sample was vacuum filtrated (Whatmann no. 3 filter). The extraction and vacuum filtration of the pellets was repeated, only with half of the amount of extraction agent that was used in the first round. Before the last vacuum filtration, the sample was stored in the refrigerator for 30 minutes with the extraction agent. The two filtrates were transferred to a flask (without the screw cap, because of the vapor pressure). Then the organic extract was concentrated by vacuum evaporation in the rotavapor at 40 °C in a water bath, until the sample was nearly dry. The organic extract was then transferred to a 13 mm test tube, and stored at 4 °C.

5.1.3 HPLC fractionation of *Halecium muricatum* and *Halecium beanii*

Preparation of the extracts from *Halecium muricatum* and *Halecium beanii* before HPCL fractionation

Aqueous extract:

The samples were weighed in polystyrene centrifuge tubes, approximately 200 mg from *Halecium muricatum* and *Halecium beanii*, and 2000 µL Milli-Q water was added to each tube. Afterwards the samples were shaken for 90 minutes at 100- 160 RPM. Then the samples were transferred to a new centrifuge tube, and frozen overnight. The next day the samples were thawed for 5- 10 minutes, mixed well and centrifuged at 3400 g for 30 minutes at 5°C. Afterwards the supernatants were transferred to 25 mL round flasks, and evaporated in the rotavapor to less than 1 mL at approximately 70 mbar pressure in a 40 °C water bath. Then the supernatants were filtrated through a 0.22 µm Millex GS filter in HPLC tubes. Afterwards the samples were mixed well with a pipette and the volumes were adjusted to 1000 µL with Milli- Q water (if the volume was below 1000 µL).

Organic extract:

The samples were weighed in 13 mm glass test tubes, approximately 200 mg from *Halecium muricatum* and *Halecium beanii*, and dissolved in 3 mL hexane. Afterwards the samples were shaken with the mini shaker, 3 mL acetonitrile was added, and the samples were shaken in the mini shaker again. Then the samples were centrifuged at 20 g for 3 minutes. The hexane-phase (on top) was transferred to another test tube, and 3 mL acetonitrile was added. Afterwards the samples were shaken on the mini shaker, and centrifuged again. The two acetonitrile phases were transferred to 25 mL round flasks, and evaporated under low pressure with a gradual pressure reduction down to 150- 180 mbar. The evaporation was stopped when there was approximately 1 mL left in the flask. The volume of the samples was adjusted to 1000 µL with 90 % acetonitrile and centrifuged at 16200 g for 30 minutes. Afterwards the supernatants were then transferred to HPLC vials and adjusted to 1000 µL with 90 % acetonitrile.

HPLC fractionation of *Halecium muricatum* and *Halecium beanii*

Mobile phases:

A: 0.1 % formic acid in Milli- Q water

B: 0.1 % formic acid in acetonitrile

Make-up pump solution: 80 % aqueous MeOH w/ 0.2 % formic acid

Wash solution 1: 5 % MeOH in Milli-Q water

Wash solution 2: Acetonitrile (HPLC grade)

Injection volume: 900 μL

Injector: 1000 μL

Column:

Aqueous extract: Atlantis ® Prep T3 OBD 10μm 19x250 mm.

Organic extract: XTerra® Prep MS C18 OBD 10 μm 19x250 mm.

Conditions:

Source temperature: 120 °C

Desolvation temperature: 300 °C

Desolvation gas flow: 500 L/ hour

During the HPLC fractionation 99 % of the mobile phase flow goes to the collection of fractions, and 1 % goes to the UV- and MS- detectors. The flow directed to the detectors is very low compared to the flow to the fractionation, and would lead to a time lag between the detector signal and fractionation if left unattended. A make- up pump increases the flow to the detectors and eliminates this problem. The wash solutions wash the injector, and the first wash solution (weak wash) washes out water soluble compounds. The second wash solution (strong wash) washes out lipophilic compounds.

Table 2: Gradient used for the fractionation of the aqueous extract:

Time (min)	Flow (mL/ min)	% A	% B
Initial	6	95	5
3	6	95	5
30	6	50	50
35	6	5	95
40	6	5	95

Table 3: Gradient used for the fractionation of the organic extract:

Time (min)	Flow (mL/ min)	% A	% B
Initial	6	80	20
2	6	80	20
30	6	0	100
40	6	0	100

Each extract was fractionated into 40 1 minute fractions, each fraction was 6 mL. The fractions were transferred to four identical deep well plates, with 1.33 mL from each fraction in each deep well plate. The deep well plates were dried in a SpeedVac to evaporate the acetonitrile and formic acid, and then freeze- dried to remove the water.

Since the aqueous and organic extracts were not previously fractionated or purified a pre-column was used.

Dissolution of HPLC fractions for bioactivity screening

Growth inhibition and antitumor assay:

There were two samples, M11046 and M11047, and each sample had an aqueous and organic extract. In total eight deep well plates were used in the growth inhibition and antitumor assay. First 7.5 µL of DMSO were added only to the deep well plates with organic fractions, followed by shaking of the samples for 2.5 hours. Then 750 µL of autoclaved Milli- Q water

was added to the plates used in the growth inhibition assay. The same amount of RPMI- 1640 was added to the plates in the antitumor assay, and the deep well plates were shaken for 30 minutes. Afterwards 750 µL of autoclaved Milli- Q water was added again (RPMI- 1640 for antitumor assay), and the deep well plates were shaken for 3 minutes. DMSO was only added to the deep well plates with organic fractions to dissolve the fractions, since the organic extracts do not dissolve in pure water or RPMI- 1640.

5.2 Bioactivity testing

5.2.1 Growth inhibition assay

Growth inhibition assay – 1st screening

Table 4: Bacterial strains used in the growth inhibition assay.

Bacterial strain
Staphylococcus aureus ATCC 25923
Escherichia coli ATCC 25922
Enterococcus faecalis ATCC 29212
Pseudomonas aeruginosa ATCC 27853
Streptococcus gr. b ATCC 12386

Day 1:

The strains of bacteria (see table 4) were plated from freeze stock (- 80 °C) to blood agar plates overnight. Then the bacterial strains were sown out from blood agar plates to 8 mL growth medium (see table 5) and incubated overnight, 18-20 hours, at 37 °C and 5 % CO_2.

Table 5: The appropriate growth medium, incubation time and bacterial density of the different strains of bacteria.

Bacterial strain	Growth medium	Incubation time (h)	Bacterial density
S. aureus	MH- broth	2,5	$0,5 – 3 \times 10^5$ CFU/ml
E. coli	MH- broth	1,5	$0,5 – 3 \times 10^5$ CFU/ml
E. faecalis	BHI – broth	1,5	$0,5 – 3 \times 10^5$ CFU/ml
P. aeruginosa	MH – broth	2,5	$3 – 7 \times 10^4$ CFU/ ml
Streptococcus gr. b	BHI – broth	1,5	$0,5 – 3 \times 10^5$ CFU/ml

Day 2:

For exponential growth, 2 mL from the bacterial suspension from day one were transferred to 25 mL fresh growth medium. Then the bacterial suspensions were incubated (see table 5 for appropriate incubation times). After the incubation, 100 µL of this bacterial "stock" suspension was added to a glass tube containing 9.9 mL of growth medium. The glass tube was shaken with the mini shaker, and 2 mL was transferred to a falcon tube containing 18 mL of growth medium. In total, the suspension of bacteria was diluted 1000 times. This dilution was done for each strain of bacteria. Then 50 µL of the HPLC fractions and 50 µL of the bacterial suspension were added to each well in a micro titer plate. Each bacterial strain had one micro titer plate, and each HPLC fraction was added in two parallels. The micro titer plates were incubated overnight at 37 °C and 5 % CO_2.

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 1: HPLC fraction setup in micro titer plates for growth inhibition assay. N = negative control, P = positive control, 1- 40 was the different HPLC fractions.

Controls:
- Negative control: 50 µL growth medium and 50 µL sterile distilled water.
- Positive control: 50 µL sterile distilled water and 50 µL bacterial suspension.

Day 2 – bacterial control counting:

For control counting, 100 µL of the bacterial "stock" suspension was diluted 1 000 000 times in autoclaved 0.9 % NaCl Milli- Q water. Then 100 µL from the diluted suspension was seeded on two LB- plates for control counting the next day.

Day 2 – MIC determination of gentamicin

To control the validity of the assay, it is essential to determine the MIC- values of gentamicin against the bacterial strains used in the assay. Diluted gentamicin, 50 µL, was added in the following concentrations; 0.01 µg/mL, 0.03 µg/mL, 0.06 µg/mL, 0.12 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL. The micro titer plate was incubated at 37 °C and 5 % CO_2 overnight, and visually inspected the next day.

Day 3: Reading of results

The plates containing the HPLC fractions were visually inspected for inhibition of growth, and fractions with growth inhibition were noted. The plates were also inspected

photometrically by the Multilabel Counter VICTOR^3$_{TM}$. The Multilabel Counter measures light absorbance at 600 nm, and the absorbance for the HPLC fractions were compared with the positive and negative controls. The fractions are defined active, questionable and inactive with the following absorbance:

- o Active < 0,05
- o Questionable: 0,05- 0,09
- o Inactive: > 0,09

The term questionable activity is just a cutoff value between the terms active and inactive. Fractions that show activity in this term show weak activity.

The gentamicin control plate was visually inspected, and MIC values were noted and compared to the expected MIC values for gentamicin against these strains of bacteria. The bacterial colonies on the LB- plates were counted, to check the concentration of bacteria.

Growth inhibition assay – 2nd screening

The purpose of the 2nd screening was to confirm antibacterial effect found in the 1st screening by testing the active fractions from the 1st screening. The active fractions were only tested against bacterial strains they showed growth inhibition against in the 1st screening. The setup of this method was the same as the 1st screening. The only difference was that the active fractions were tested undiluted, diluted 1:2 and diluted 1:4 in two parallels (see figure 2 below).

N	A(U)	A(1:2)	A(1:4)	E(U)	E(1:2)	E(1:4)	I (U)	I(1:2)	I(1:4)		P
N	A(U)	A(1:2)	A(1:4)	E(U)	E(1:2)	E(1:4)	I (U)	I(1:2)	I(1:4)		P
N	B(U)	B(1:2)	B(1:4)	F(U)	F(1:2)	F(1:4)	J(U)	J(1:2)	J(1:4)		P
N	B(U)	B(1:2)	B(1:4)	F(U)	F(1:2)	F(1:4)	J(U)	J(1:2)	J(1:4)		P
N	C(U)	C(1:2)	C(1:4)	G(U)	G(1:2)	G(1:4)	K(U)	K(1:2)	K(1:4)		P
N	C(U)	C(1:2)	C(1:4)	G(U)	G(1:2)	G(1:4)	K(U)	K(1:2)	K(1:4)		P
N	D(U)	D(1:2)	D(1:4)	H(U)	H(1:2)	H(1:4)	L(U)	L(1:2)	L(1:4)		P
N	D(U)	D(1:2)	D(1:4)	H(U)	H(1:2)	H(1:4)	L(U)	L(1:2)	L(1:4)		P

Figure 2: Setup for active HPLC fractions in the micro titer plate. A-L are active fractions and the dilution is in the parenthesis. N and P are positive and negative controls.

5.2.2 Anticancer assay

Anticancer assay 1st Screening:

Thawing of cells from nitrogen container

The cells were stored in a liquid nitrogen container, and when needed for assays the cell lines were thawed and maintained at 37 °C in 5 % CO_2. The cryo tube containing the cell line was directly transferred to preheated sterile Milli- Q water, for quick thawing. The tube containing the cells was sterilized with 70 % ethanol, and the cells were resuspended in the ampoule with a pipette. The cell suspension was transferred to a 50 mL centrifuge tube, and 10- 20 mL growth medium (see table 6) was added. The tube was centrifuged for 7 minutes at 200 g. After the centrifugation the medium was removed and replaced with new medium, and mixed well to dissolve the cell pellet. The cell suspension was transferred to a culture flask and incubated at 37 °C and 5 % CO_2. After 1-2 days the medium was changed to fresh preheated medium. When the cell culture had established a cell monolayer they were split into a new culture flask as described below.

Table 6: Cell line, origin and appropriate medium.

Cell line	ATCC ® number	Origin	Medium
A2058	CRL- 11147™	Human melanoma	D- MEM + 10 % FBS + 10 µg/mL gentamicin
MRC5	CCL- 171™	Normal lung fibroblasts	E-MEM + 10 % FBS + 1 % NEAA
MCF7	HTB- 22™	Human breast carcinoma	E- MEM + 10 % FBS + 1 % NEAA
DU145	Unknown	Prostate cancer	RPMI- 1640 + 10 % FBS

Splitting and seeding of cell lines on plates

The purpose of cell splitting was to maintain cell lines in culture, and the purpose of seeding cells into micro titer plates was for bioactivity assays. Splitting of cells was done approximately twice per week.

Splitting:

The medium and chemicals were first preheated to 37 °C in an incubator. Then the cell culture flask was inspected in the microscope to determine the amount that needed to be transferred to the new cell culture flask, since the amount that was transferred depended on the cell density. Afterwards the growth medium was removed from the old cell culture flask, and 10 mL PBS was added to wash the cell monolayer. The cells were washed with PBS to remove serum that inactivates trypsin, and PBS was removed after approximately 1 minute. Trypsin 0.25 %, 5 mL, was added. It was important to tilt the flask so that the whole cell layer was covered. Trypsin was removed after approximately 15 seconds, and afterwards the flask was incubated for approximately 5 minutes at 37 °C, and the monolayer was inspected to see if the cells had loosened. The cells were resuspended in 10 mL growth medium. Then 15 mL growth medium was added to the new cell culture flask, and 0.5- 1 mL of the resuspended cell culture was added to the new cell culture flask. The amount of resuspended cell culture that needed to be added to the new cell culture flask depended on the cell density of the old cell culture. The new cell culture flask was incubated at 37 °C and 5 % CO_2.

Seeding:

To ensure an appropriate cell concentration in the wells of the microtiter plate, which was 2x 10^4 cells/ mL, Burkes' counting chamber was used to count and calculate the appropriate cell concentration for further use. Then 100 µL of the new cell suspension with the appropriate cell concentration was added to each well in the microtiter plate, and incubated overnight at 37 °C and 5 % CO_2. The next day the medium in the microtiter plate wells was removed and 50 µL fresh RPMI- 1640 medium was added. Afterwards 50 µL of the HPLC fractions was added to the microtiter plates (see figure 3) in three parallels. The microtiter plates were incubated at 37 °C and 5 % CO_2 for 72 hours.

	1	1	1	8	8	8	15	15	15		
	2	2	2	9	9	9	16	16	16	N	
	3	3	3	10	10	10	17	17	17	N	
	4	4	4	11	11	11	18	18	18	N	
	5	5	5	12	12	12	19	19	19	N	
	6	6	6	13	13	13	20	20	20	N	
	7	7	7	14	14	14					

Figure 3: Setup of HPCL fractions in anticancer assay. N = negative control, which is only medium. 1- 20 = fraction 1-20 in three parallels. The grey areas indicate wells only containing medium.

The positive control is an average value calculated from separate setups with Triton X- 100. The value of the positive control is 0.181446. This positive control was used to calculate the cell survival of each fraction, using the equation below.

$$\frac{X_1 - \bar{X}_{\sim p}}{\bar{X}_N - \bar{X}_{\sim p}} \times 100\ \% = cell\ survival\ (\%)$$

Where X_1 was the optical density of sample well
$\bar{X}_{\sim p}$ is the positive control
$\bar{X}_N$ is the mean OD values of the negative control

Aqueous One Solution cell proliferation assay of *Halecium muricatum* and *Halecium beanii*

Aqueous one solution (AQOS) was preheated to 37 °C in an incubator, then 10 μL AQOS was added to each well. The plates were incubated for one hour at 37 °C and 5 % CO_2. Absorbance was measured at 485 nm by the DTX 880 Multimode Detector. Metabolic active cells reduced the tetrazolium salt from AQOS (yellow color) to a formazan product (dark blue). Number of viable, or surviving, cells was directly proportional to the amount of formazan product formed. The color concentration was read by a spectrophotometer. The fractions were defined as active, questionable or inactive with the following percentage of cell survival:

- Active: < 50 %
- Questionable: 50- 60 %
- Inactive: > 60 %

Anticancer assay – 2nd Screening of *Halecium muricatum* and *Halecium beanii*

Active fractions from the 1st screening were tested in the 2nd screening. The 2nd screening was carried out the same way as the 1st screening, except that the active fractions were tested against the A2058 cell line undiluted, diluted 1:2 and diluted 1:5 in three parallels (see figure 4 below)

	A(U)	A(U)	A(U)	A(1:2)	A(1:2)	A(1:2)	A(1:5)	A(1:5)	A(1:5)		
	B(U)	B(U)	B(U)	B(1:2)	B(1:2)	B(1:2)	B(1:5)	B(1:5)	B(1:5)	N	
	C(U)	C(U)	C(U)	C(1:2)	C(1:2)	C(1:2)	C(1:5)	C(1:5)	C(1:5)	N	
	D(U)	D(U)	D(U)	D(1:2)	D(1:2)	D(1:2)	D(1:5)	D(1:5)	D(1:5)	N	
	E(U)	E(U)	E(U)	E(1:2)	E(1:2)	E(1:2)	E(1:5)	E(1:5)	E(1:5)	N	
	F(U)	F(U)	F(U)	F(1:2)	F(1:2)	F(1:2)	F(1:5)	F(1:5)	F(1:5)	N	
	G(U)	G(U)	G(U)	G(1:2)	G(1:2)	G(1:2)	G(1:5)	G(1:5)	G(1:5)	N	

Figure 4: Display of HPCL fractions in the 2nd anticancer screening. A-G = different fractions, U = undiluted, 1:2 = diluted 1:2, 1:5 = diluted 1:5. The grey areas indicate wells only containing medium.

5.3 Dereplication

Mobile phases:

A: H_2O + 0.1 % FA

B: ACN + 0.1 % FA

Wash solutions:

Weak wash solution: H_2O + 10 % ACN.

Strong wash solution: 50 % ACN + 50 % H_2O.

Table 7: Gradient used in the dereplication of *Halecium beanii*.

Time (min)	Flow (mL/ min)	% A	% B
Initial	0.350	80	20
3.5	0.350	0	100

Standard conditions for TOF- MS with ESI+:

Capillary: 2.6 kV

Cone 35 V

Source temperature: 120 °C

Desolvation temperature: 300 °C

Cone gas flow: 5L/ hour

Desolvation gas flow: 550 L/ hour

Dereplication is the identification and subsequent removal of known compounds from the biodiscovery pipeline. Because of the bioactivities from the 1st and 2nd anticancer assays, compounds from *Halecium beanii* (M11047) were chosen for dereplication. Inactive and active fractions were analyzed using UPLC- TOF- MS with ESI in positive mode in order to find compounds that could be correlated to the observed bioactivity. The bioactive fractions should contain other compounds than inactive HPCL fractions. Alternatively the bioactive fractions should contain more of the bioactive compound, or compounds, than the fractions

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that did not show bioactivity. The elemental composition was calculated by Masslynx from the accurate mass and isotope distribution of the selected compounds. This elemental composition was used to search the Dictionary of Marine Natural Products for known natural products. If it was an unknown compound, or a known compound which had not previously shown bioactivity, then that was an interesting discovery. If the compound was known, and it had activity in the assays performed, then the discovery was unimportant, and this step prevents further waste of time.

5.4 Isolation of possible bioactive compounds from *Halecium beanii*

After the dereplication, eight possible bioactive compounds were found which needed to be isolated. Before isolation on HPLC, more of the organic extract was needed. Preparation of samples for HPLC was done according to section 5.1.3 under organic extract. This was done twice to assure that enough of the sample was available to complete further bioactivity screening on the isolated compounds. The isolation of the eight different compounds was done on the preparative HPLC, with an optimized gradient.

Mobile phases:
A: 0.1 % formic acid in Milli- Q water
B: 0.1 % formic acid in acetonitrile

Make-up pump solution: 80 % MeOH w/ 0.2 % formic acid
Wash solution 1: 5 % MeOH in Milli-Q water
Wash solution 2: Acetonitrile (HPLC grade)

Injection volume: 100- 410 µL
Injector: 1000 µL

Table 8: Optimized gradient used for isolation of dereplicated compounds.

Time (min)	Flow (mL/ min)	% A	% B
Initial	6	70	30
2	6	70	30
2.10	6	60	40
20	6	50	50

The MS- detector was programmed to trigger fraction collection by the protonated molecular weights $[M+H]^+$ of the eight different compounds. After repeated HPLC injections the fractions were transferred to one flask for each compound. The compounds were then concentrated by evaporation under low pressure with a gradual pressure reduction.

After the isolation the compounds were analyzed using UPLC- TOF- MS, with ESI in positive mode, to determine the purest compounds for further bioactivity testing. The mobile phases and wash solutions were the same as in section 5.3.
Afterwards the samples were transferred to a pre-weighed glass tube and were vacuum evaporated. This was performed to find the amount of each compound available for further bioactivity testing.

Table 9: Gradient used for determining the purest of the compounds using UPLC- TOF- MS.

Time (min)	Flow (mL/min)	% A	% B
Initial	0.35	90	10
10	0.35	0	100

5.5 Identification of possible bioactive compounds from *Halecium beanii*

NMR

The compounds with protonated masses of 454.3 and 482.3 were dissolved in 500 μL deuterated MeOH. The NMR spectra were provided on a Varian Inova spectrometer operated at 599.934 MHz for ^{1}H- NMR, and for ^{13}C- NMR it was operating at 150.863 MHz. The probe that was used was a cryogenically cooled inverse detection HCN probe with an enhanced proton channel (2nd generation). All spectra, WET1D (^{1}H- NMR), ^{13}C- NMR, HSQC (Heteronuclear Single- Quatum Correlation), HMBC (Heteronuclear Multiple- bond Correlation), COSY (COrrelation SpectroscopY), were acquired with a resolution of 1440 x 200 complex data points. All spectra were acquired at 298 K.

Wet1D: 256 scans, sweepwidth: 16 ppm, complex datapoints: 16k
Carbon: 20000 scans, sweepwidth: 240ppm, proton decoupling and noe enhancement
gCOSY: 16 scans @ 200x1439 complex points
gHSQCAD: 64 scans @ 200x1024 complex points
gHMBCAD: 64 scans @ 256x1024 complex points

MS- MS

The two compounds with protonated masses of 454.3 and 482.3 were analyzed by MS- MS for further structure elucidation, and were directly infused to the mass spectrometer by a built-in syringe. Cone voltage was optimized to get the highest possible signal for the protonated molecular ion, and the collision energy was optimized during the infusion to get the best possible daughter specter. The cone and desolvation gas used was nitrogen, and the collision gas used was argon.

5.6 Confirmation of bioactivity

The protonated mass of the compounds chosen for further bioactivity testing were 454.3 and 482.3. After the dereplication and isolation of the two potential active compounds, the bioactivity needed to be confirmed. This was done due to the possibility that the compounds chosen were not the active compounds that showed initial activity. Since the main focus in

this work was bioactivity against cancer cells, only the anticancer assay was performed. The setup for this test was done as described in section 5.2.2. The only difference was the concentration of the isolated compounds used and the number of cell lines, to examine the possibility that the isolated compounds show cell line specificity. The cell lines used in this assay were A2058, MRC5, MCF7 and DU145. For the concentration gradient setup for this assay see figure 5 below.

A (2,5 µg/mL)	A (2,5 µg/mL)	A (2,5 µg/mL)	B (2,5 µg/mL)	B (2,5 µg/mL)	B (2,5 µg/mL)				N	N
A (5 µg/ mL)	A (5 µg/ mL)	A (5 µg/ mL)	B (5 µg/ mL)	B (5 µg/ mL)	B (5 µg/ mL)				N	N
A (10 µg/ mL)	A (10 µg/ mL)	A (10 µg/ mL)	B (10 µg/ mL)	B (10 µg/ mL)	B (10 µg/ mL)				N	N
A (25 µg/mL)	A (25 µg/mL)	A (25 µg/mL)	B (25 µg/mL)	B (25 µg/mL)	B (25 µg/mL)				N	N
A (50 µg/mL)	A (50 µg/mL)	A (50 µg/mL)	B (50 µg/mL)	B (50 µg/mL)	B (50 µg/mL)				N	N
A (75µg/ mL)	A (75µg/ mL)	A (75µg/ mL)	B (75µg/ mL)	B (75µg/ mL)	B (75µg/ mL)				N	N
A (100 µg/ mL)	A (100 µg/ mL)	A (100 µg/ mL)	B (100 µg/ mL)	B (100 µg/ mL)	B (100 µg/ mL)				N	N

Figure 5: Setup for confirmation of bioactivity. A = 454,3, B = 482,3. Compound concentration in brackets. The grey area indicates wells only containing medium.

6. Results

6.1 Extraction and fractionation

6.1.1   Extraction

Halecium muricatum **(M11046)**

For *Halecium muricatum* the weight of the biomass sample collected was 927 grams. After the freeze- drying and grinding, the dry weight of the biomass sample was 194 grams. This dry biomass sample was used first in the extraction of the aqueous extract which yielded 34.5 grams and gave a percentage yield of 17.8 %.
The biomass sample used in the aqueous extraction was then dried before the organic extraction. The dry weight of the biomass sample used for organic extraction was 148.0 g, and the extraction yielded 4.5 grams, which gave a percentage yield of 3.0 %.

Halecium beanii **(M11047)**

For *Halecium beanii* the weight of the biomass collected was 1293 grams. After freeze-drying and grinding the dry weight was 258.2 grams. This dry biomass sample was used in the aqueous extraction which yielded 49.5 grams, with the percentage yield of 19.2 %. The biomass sample used in the aqueous extraction was dried, before used in the organic extraction. The dry weight of the biomass sample used for organic extraction was 193.9 g, and this extraction yielded 9.0 grams, which gave a percentage yield of 4.6 %.

6.1.2   Fractionation

Before fractionation, preparation of the extracts from *Halecium muricatum* and *Halecium beanii* was performed (see section 5.1.3). For the preparation of the aqueous extract 200.7 mg from *H. muricatum* and 201.1 mg from *H. beanii* was used. For the organic extract 218.1 mg from *H. muricatum* and 209.5 mg from *H. beanii* was used.

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In the fractionation of the aqueous exctracts the injection peaks eluted at 3.35 minutes for *Halecium muricatum* (see figure 6, section A) and at 3.68 minutes for *Halecium beanii* (see figure 7, section A) in the UV chromatograms. The injection peak often consists of different salts with nearly no retention time. In the UV- chromatogram of *H. muricatum* many compounds were detected during the first 11 minutes. For *Halecium beanii* nearly all compounds were detected during the first 15 minutes (see figure 7, section A). In the ESI chromatograms for *Halecium muricatum* (M11046) (see figure 6, B and C) many compounds were detected during the first 10 minutes, but compounds were detected until 35 minutes. For *Halecium beanii* (M11047) the ESI chromatograms (figure 7, section B and C) showed that the eluted compounds were detected throughout the whole gradient.
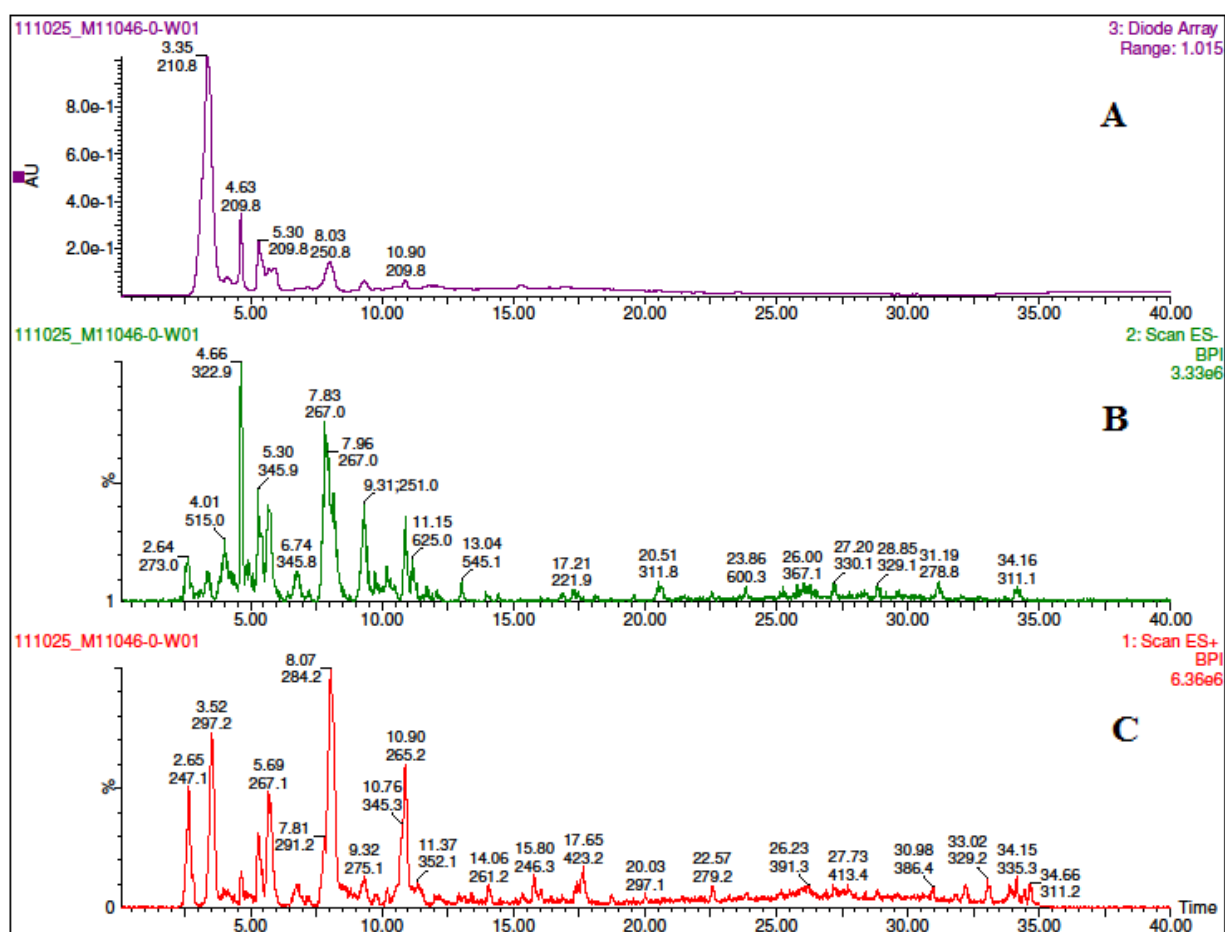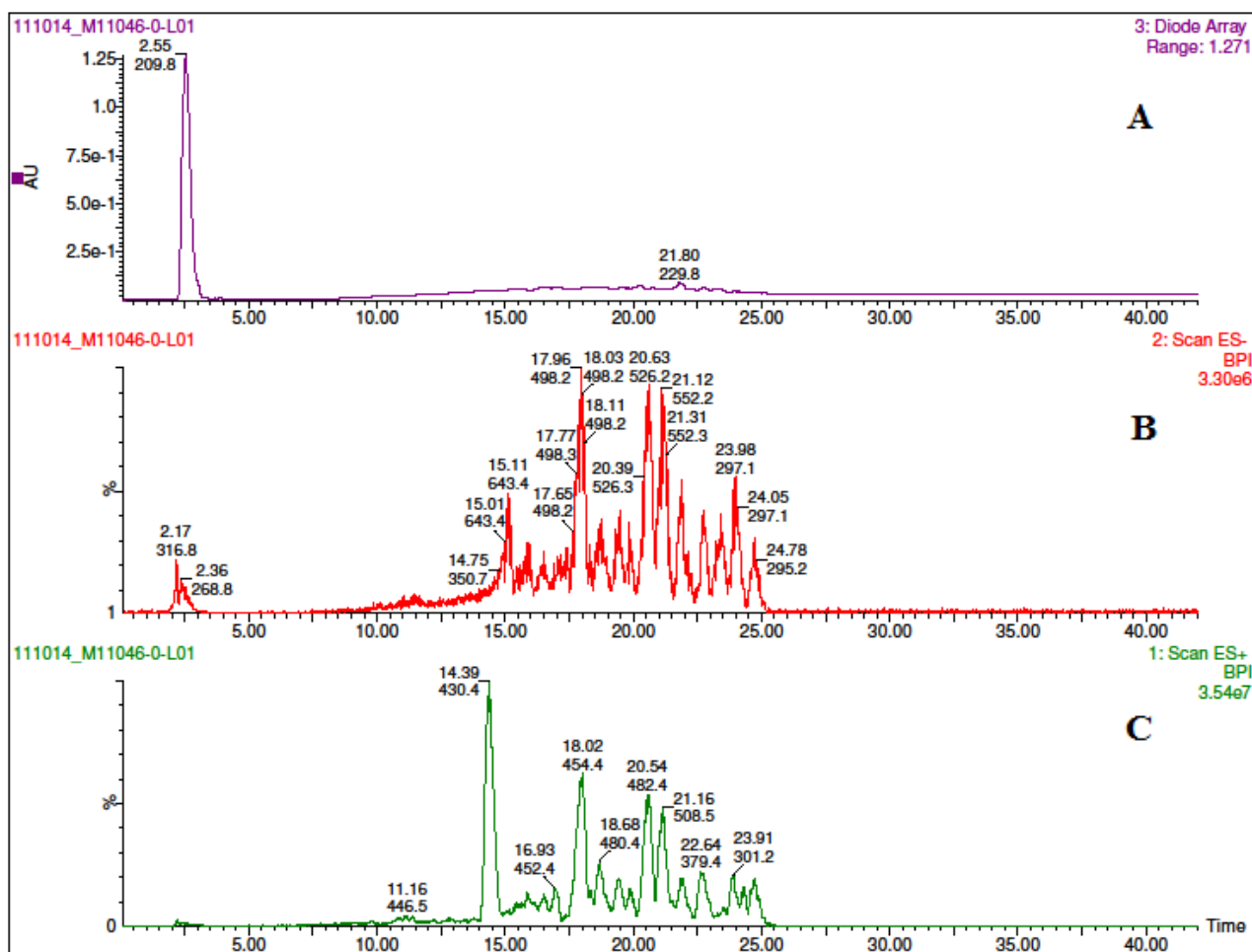

Figure 6: Chromatograms of preparative HPLC of the aqueous extract of *Halecium muricatum* (M11046). A: UV- data measured at 200- 600 nm. B and C: ESI in negative and ESI in positive mode measured at *m/z* 100 – 1500. The peaks are annotated with retention time (top) and UV absorbance (bottom) in the UV chromatogram, and retention time (top) and *m/z* (bottom) in the ESI chromatograms.
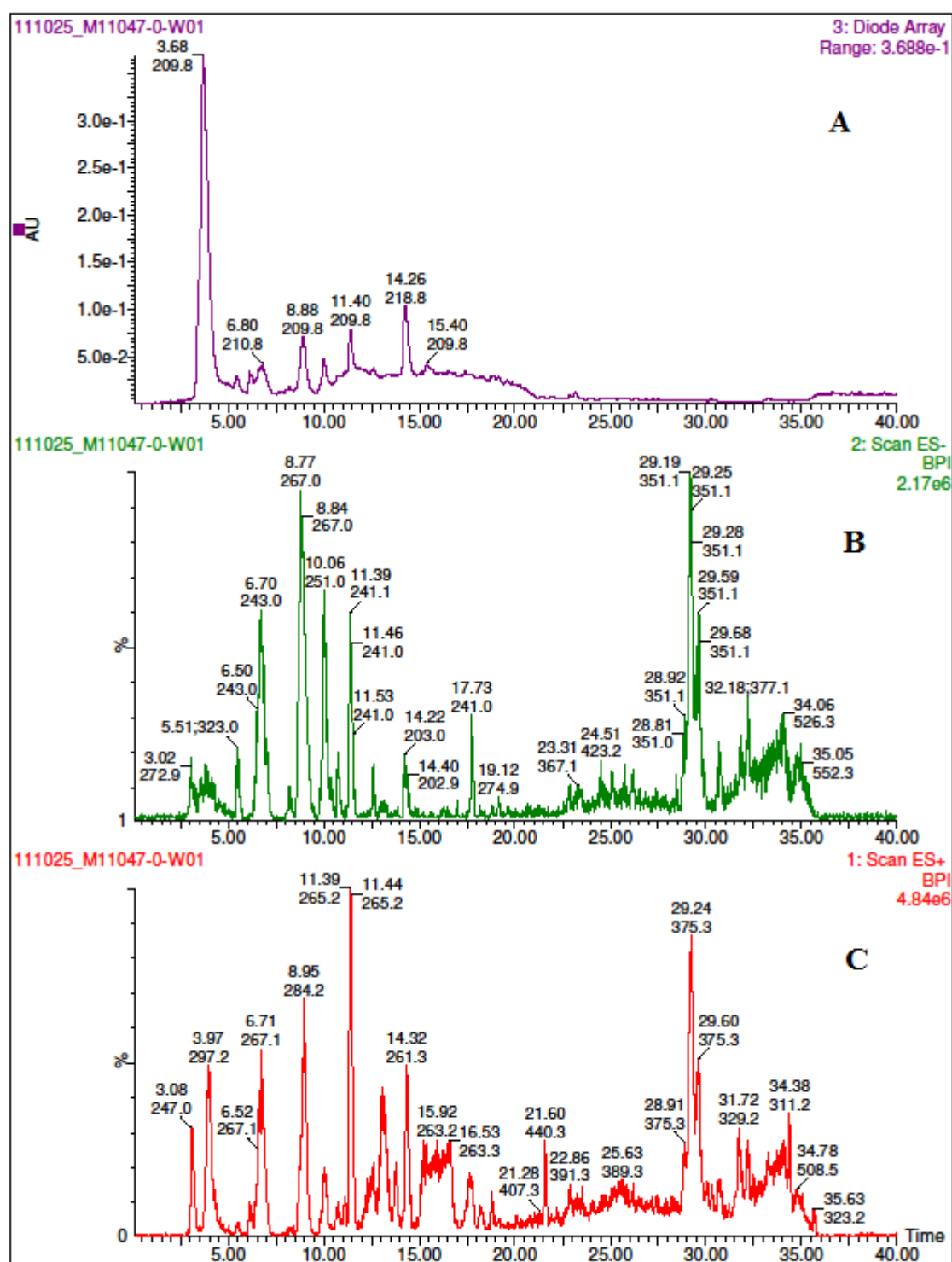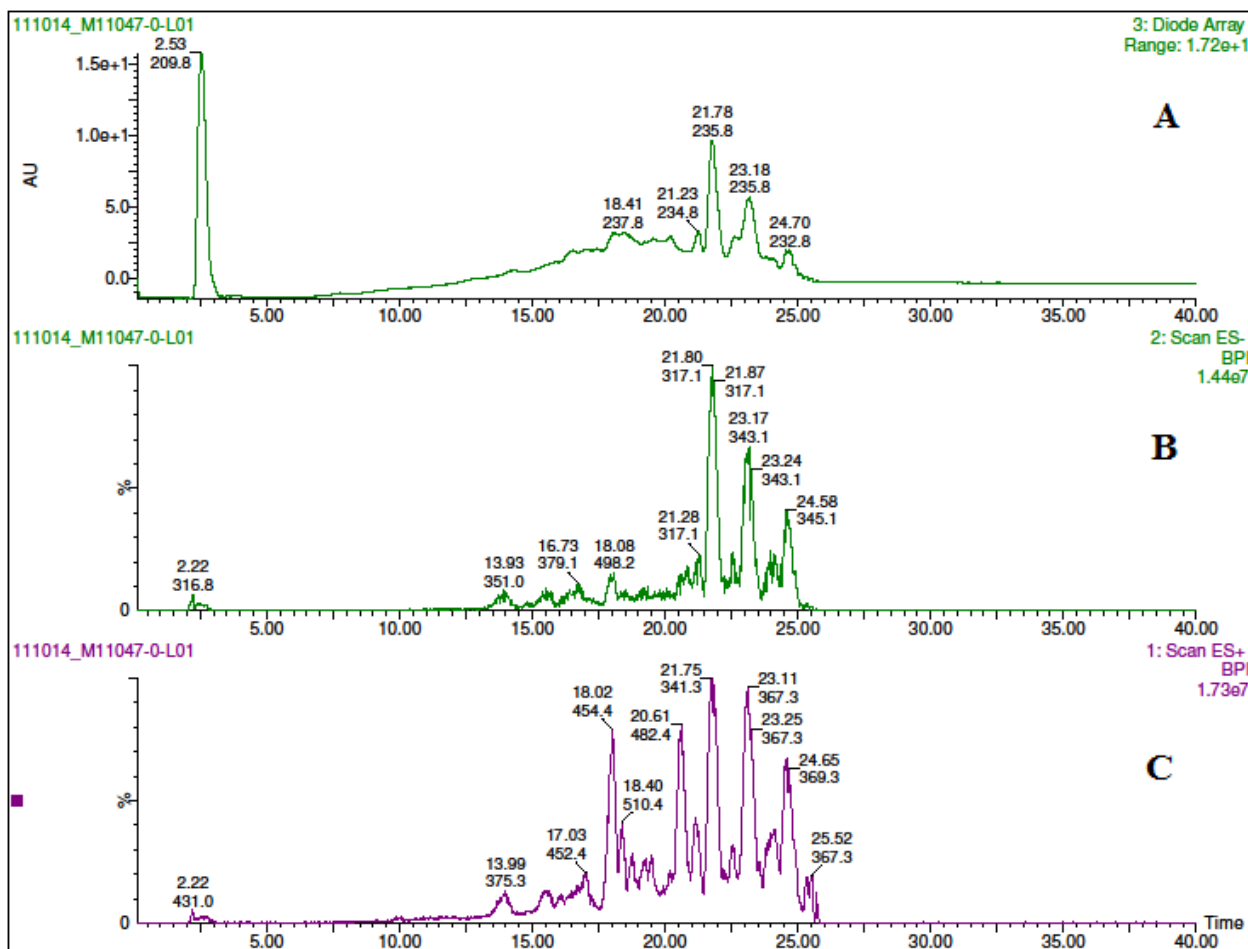
Figure 7: Chromatograms of preparative HPLC of the aqueous extract of *Halecium beanii* (M11047). A: UV-data measured at 200- 600 nm. B and C: ESI in negative and ESI in positive mode measured at *m/z* 100 – 1500. The peaks are annotated with retention time (top) and UV absorbance (bottom) in the UV chromatogram, and retention time (top) and *m/z* (bottom) in the ESI chromatograms.

In the UV chromatogram of the organic extract of *Halecium muricatum* (M11046) the injection peak had the retention time of 2.55 minutes and was dominating (figure 8, section A). For *Halecium beanii* (M11047) the UV chromatogram showed a dominating injection peak at 2.53 minutes, and the majority of compounds were detected between 15 and 25 minutes. In the ESI chromatograms most compounds were detected between 15 and 25 minutes for both *H. muricatum* and *H. beanii* (figure 8 and 9, section B and C).

Figure 8: Chromatograms of preparative HPLC of the organic extract of *Halecium muricatum* (M11046). A: UV- data measured at 200- 600 nm. B and C: ESI in negative and ESI in positive mode measured at *m/z* 100 – 1500. The peaks are annotated with retention time (top) and UV absorbance (bottom) in the UV chromatogram, and retention time (top) and *m/z* (bottom) in the ESI chromatograms.

Figure 9: Chromatograms of preparative HPLC of the organic extract of *Halecium beanii* (M11047). A: UV-data measured at 200- 600 nm. B and C: ESI in negative and ESI in positive mode measured at *m/z* 100 – 1500. The peaks are annotated with retention time (top) and UV absorbance (bottom) in the UV chromatogram, and retention time (top) and *m/z* (bottom) in the ESI chromatograms.

6.2 Bioactivity testing

6.2.1 Growth inhibition assay

Growth inhibition assay 1st screening of the aqueous extracts

The first screening of the aqueous extracts of both *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) showed no activity against the bacteria in the assay (see figure 10 and 11).
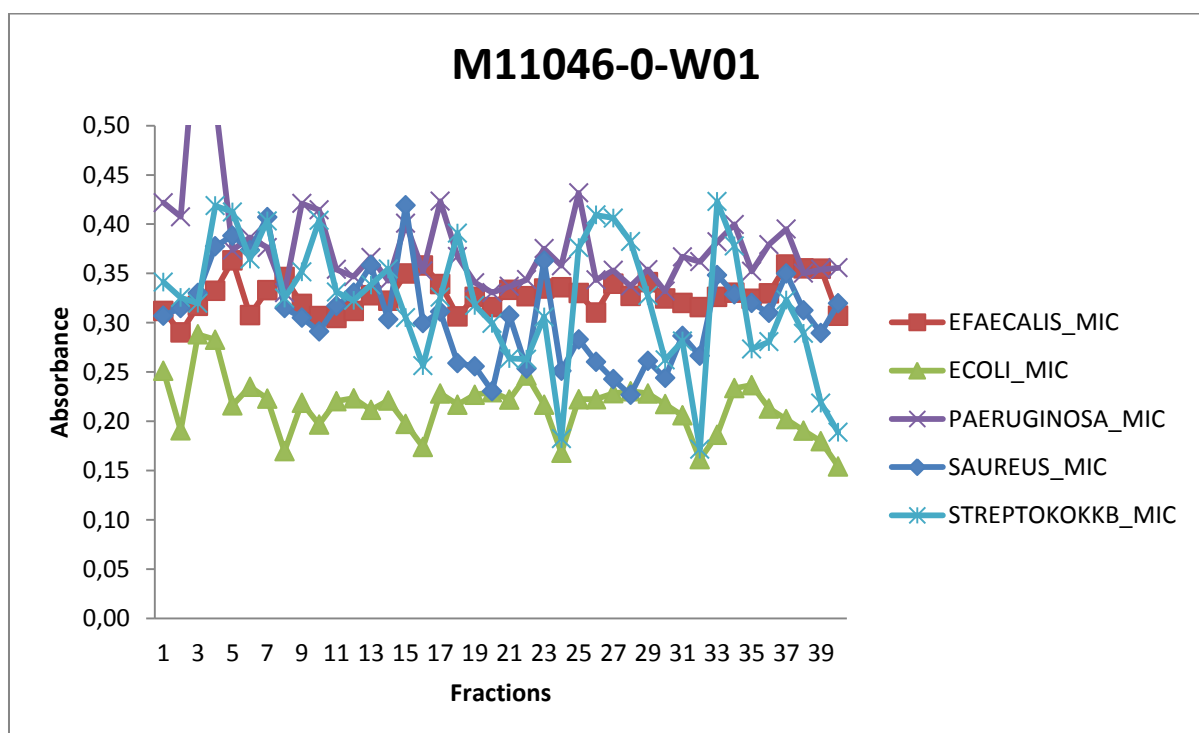
Figure 10: First growth inhibition screening of the aqueous extract of *Halecium muricatum* (M11046) against *E. faecalis*, *E. coli*, *P. aeruginosa, S. aureus* and *Streptococcus* gr. b.
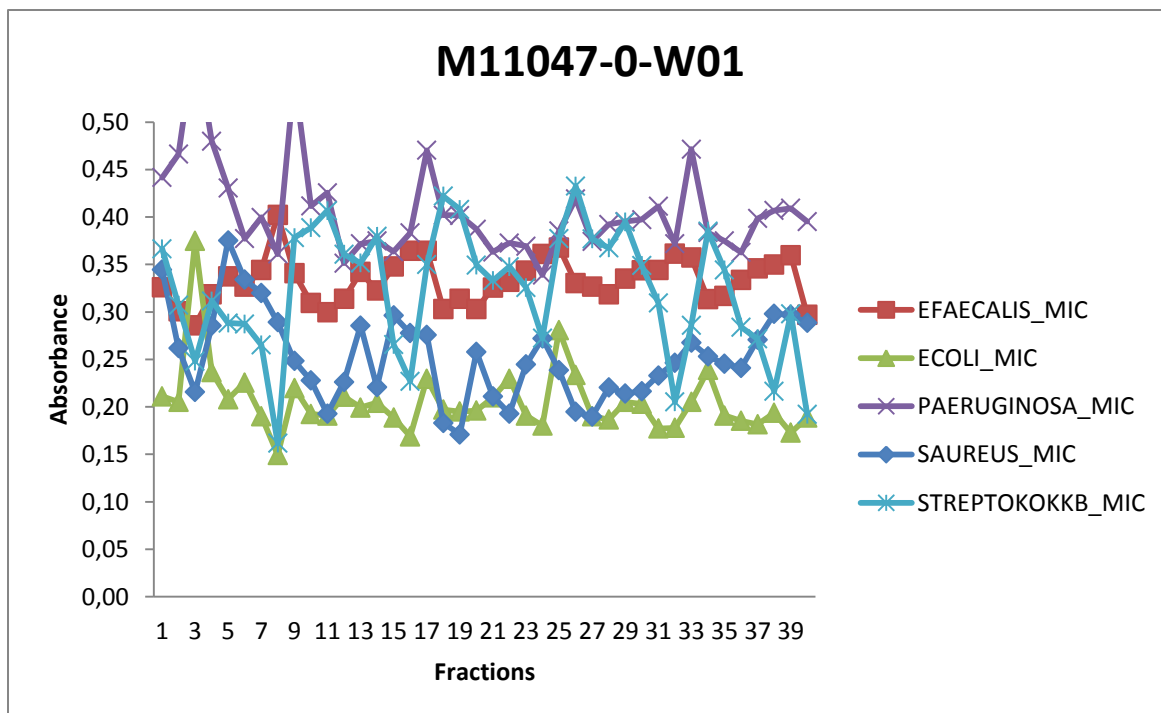
Figure 11: First growth inhibition screening of the aqueous extract of *Halecium beanii* (M11047) against *E. faecalis*, *E. coli*, *P. aeruginosa, S. aureus* and *Streptococcus* gr. b.

Growth inhibition assay 1st screening of the organic extracts:

The 1st screening of the organic extracts of *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) showed many fractions with activity against *Streptococcus* gr. b (see figure 12 and 13 below).

The results from *Halecium muricatum* (M11046) showed that many fractions only were active against one of the five different strains of bacteria, respectively *Streptococcus* gr. b (see figure 12). The following fractions showed activity against this bacteria; 20-22 and 26-30.

The results for *Halecium beanii* (M11047) showed that fewer fractions had antibacterial activity compared to the organic extract of *Halecium muricatum* (see figure 13). Fraction 27, 28, 29 and 30 showed activity against *Streptococcus* gr. b, and fraction 27 also showed activity against *E. faecalis* and *S. aureus.* These three bacteria are Gram positive bacteria.
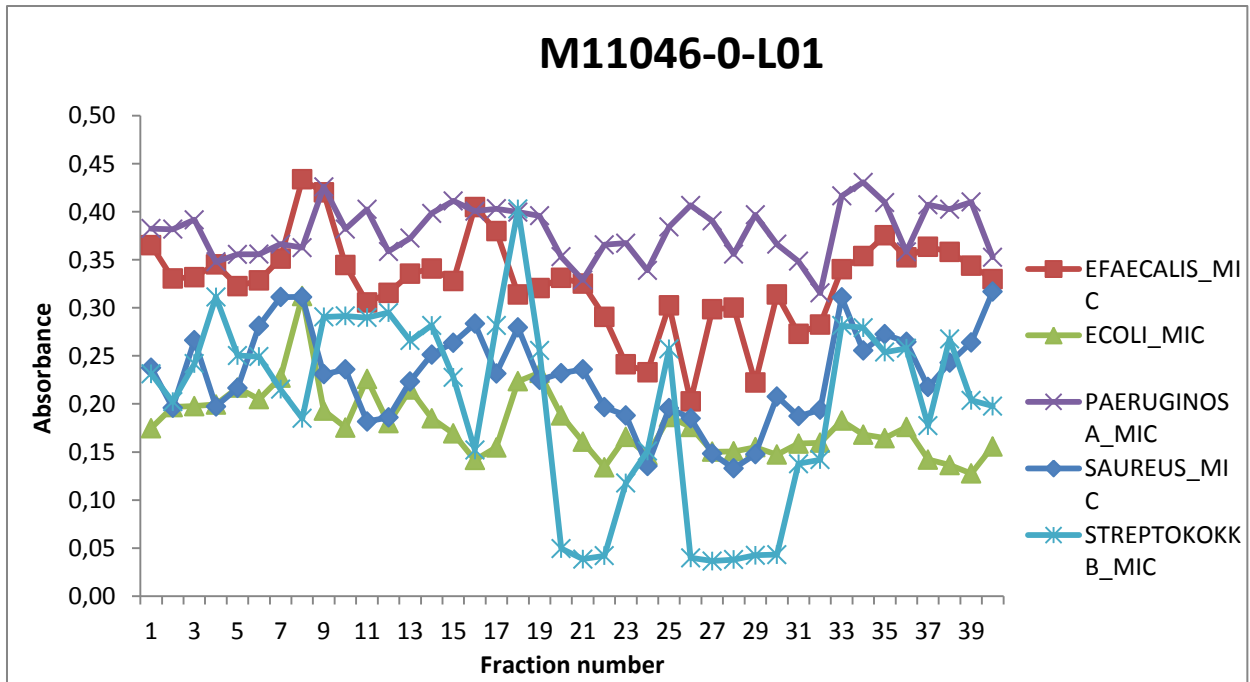
Figure 12: 1st growth inhibition screening of the organic extract from *Halecium muricatum* (M11046) against *E. faecalis*, *E. coli*, *P. aeruginosa, S. aureus* and *Streptococcus* gr. b.
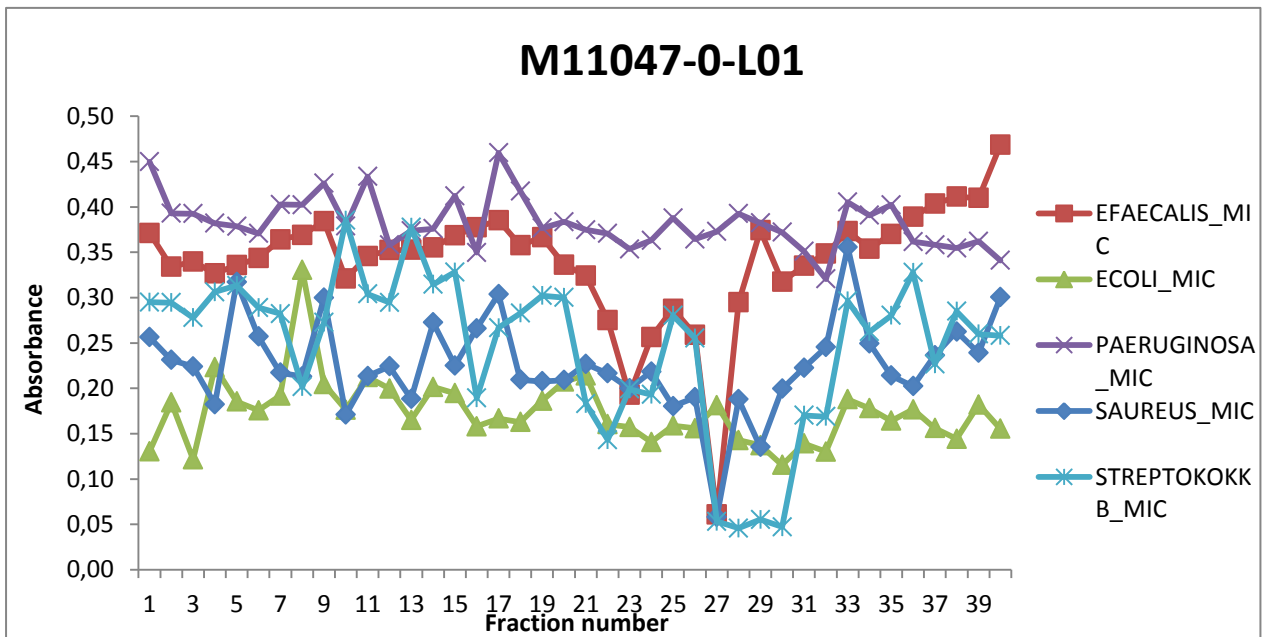

Figure 13: 1st growth inhibition screening of the organic extract from *Halecium beanii* (M11047) against *E. faecalis*, *E. coli*, *P. aeruginosa, S. aureus* and *Streptococcus* gr. b.

Growth inhibition assay 2nd screening of active fractions from the organic extracts

The second screening of the active fractions from the organic extracts of *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) showed that some of the fractions that showed activity initially, was not active in the 2nd screening (see figure 14 and 15 below). The purpose of the 2nd screening was to confirm the activity from the 1st screening and check for a dose-response relationship between the three different dilutions of the fractions. There was no clear dose- response relationship established. However, some of the fractions that showed activity in the first screening still showed activity undiluted, and fraction 29 from *Halecium beanii* showed activity undiluted and diluted 1:2 (see figure 15).
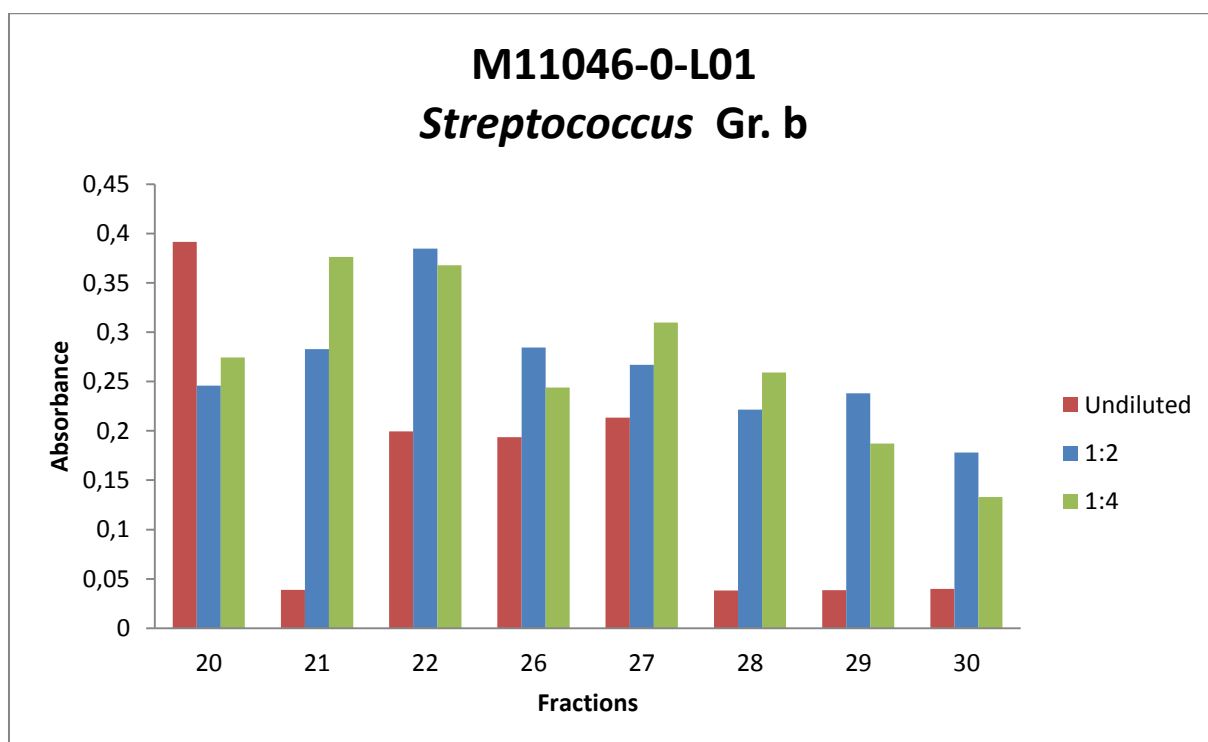
Figure 14: 2nd growth inhibition screening of the organic extract from *Halecium muricatum* (M11046) against *Streptococcus* Gr. b.

In the 2nd screening of *Halecium muricatum* (M11046) four of the initially eight active organic fractions showed activity against *Streptococcus* gr. b in the undiluted form. These fractions were fraction 21, 28, 29 and 30 (see figure 14).
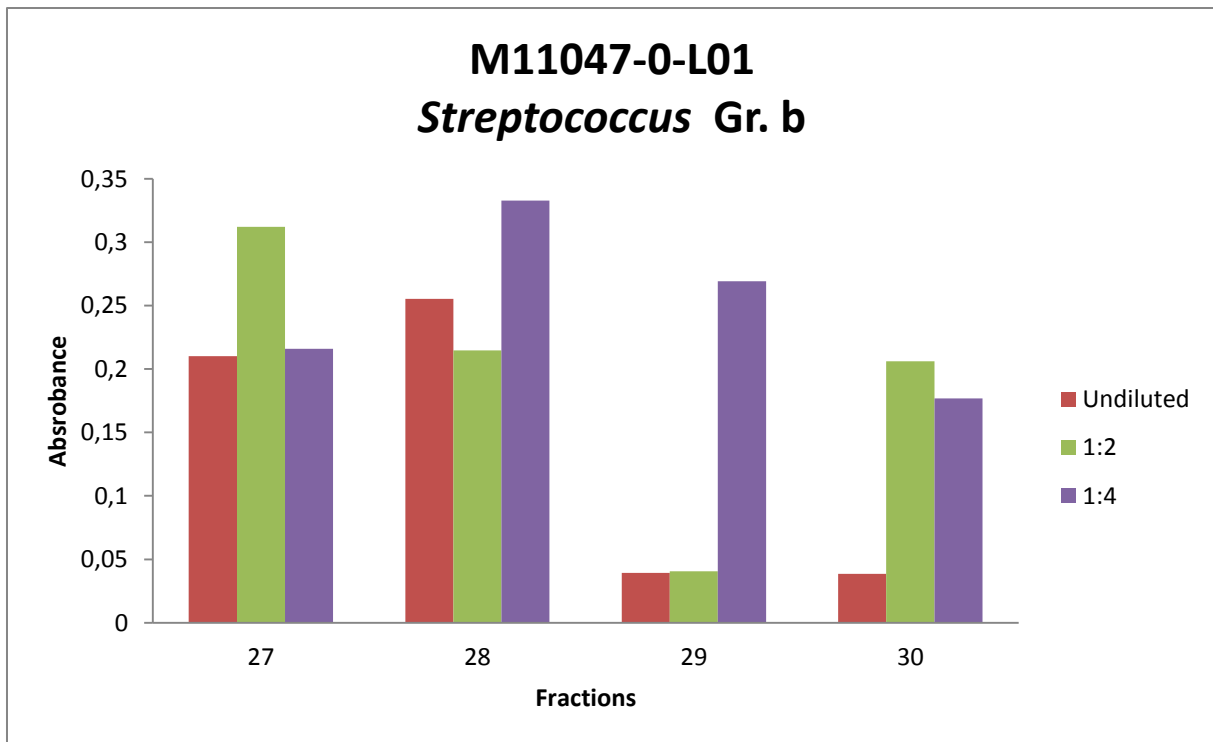
Figure 15: 2nd growth inhibition screening of the organic extract from *Halecium beanii* (M11047) against *Streptococcus* gr. b.

The 2nd screening of *Halecium beanii* (M11047) against *Streptococcus* gr. B resulted in two active fractions, fractions 29 and 30. Fraction 29 showed activity in the undiluted form, and diluted 1:2, whereas fraction 30 only showed activity in the undiluted form.
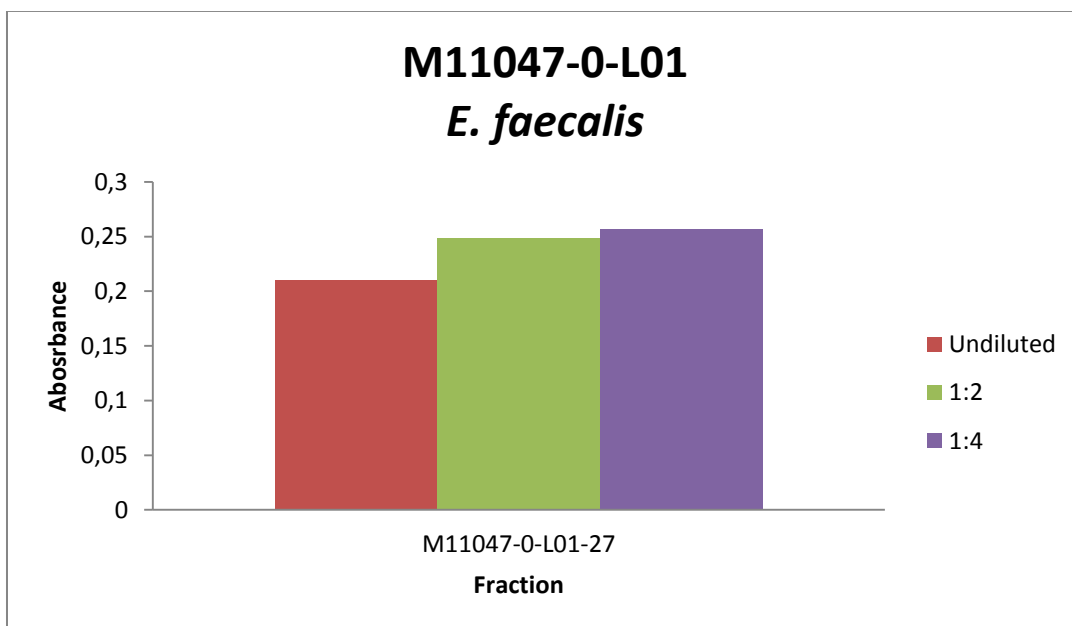

Figure 16: 2nd growth inhibition screening of the organic extract from *Halecium beanii* (M11047) against *Enterococcus faecalis*.

Figure 15: 2nd growth inhibition screening of the organic extract from *Halecium beanii* (M11047) against *Streptococcus* gr. b.

The 2nd screening of *Halecium beanii* (M11047) against *Streptococcus* gr. B resulted in two active fractions, fractions 29 and 30. Fraction 29 showed activity in the undiluted form, and diluted 1:2, whereas fraction 30 only showed activity in the undiluted form.

Figure 16: 2nd growth inhibition screening of the organic extract from *Halecium beanii* (M11047) against *Enterococcus faecalis*.

Initially fraction 27 from *Halecium beanii* (M11047) showed weak activity against *Enterococcus faecalis*, and this was retested. The results from the 2nd screening showed that this fraction was not active against this bacterium (see figure 16).

6.2.2 Anticancer assay

Anticancer assay of the aqueous fractions – 1st screening

In the 1st screening of the aqueous extract of *Halecium muricatum* (M11046) against the human melanoma cell line, A2058, six fractions showed activity. These fractions were fraction 3, 4, 5, 6, 7 and 13. However fraction 7 and 13 showed weak activity (see figure 17).
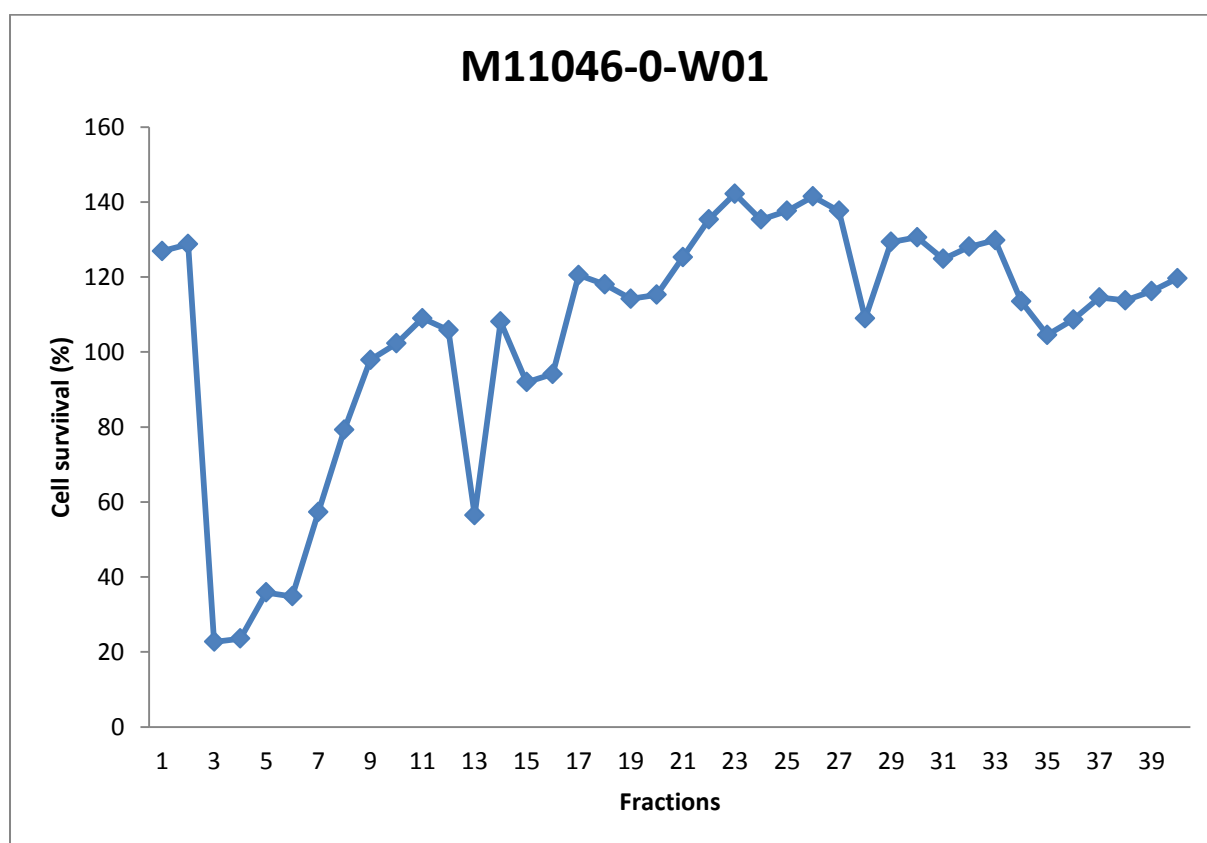
Figure 17: 1st anticancer assay of the aqueous extract of *Halecium muricatum* (M11046) against the A2058 cell line.

In the 1st screening of the aqueous fractions of *Halecium beanii* (M11047) there was only one active fraction, namely fraction 3 (see figure 18).
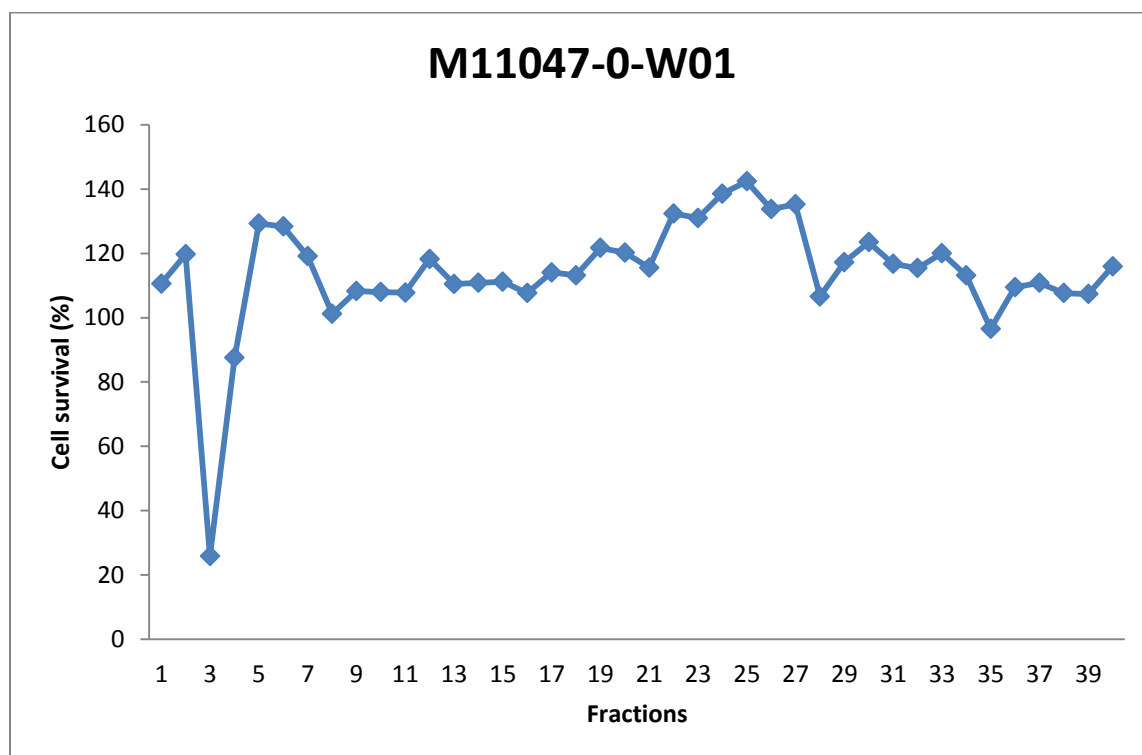
Figure 18: 1st anticancer assay of the aqueous extract of *Halecium beanii* (M11047) against the A2058 cell line.

Anticancer assay of the active aqueous fractions – 2nd screening

The active fractions from the 1st anticancer screening were retested undiluted, diluted 1:2 and 1:5, to investigate if the results were reproducible and to check for a dose- response relationship. All of the initially active fractions of *Halecium muricatum* (M11046) showed activity in the undiluted form in the 2nd screening (see figure 19). When diluted 1:2 fraction 3, 4, 5 and 6 showed activity, whereas fraction 7 and 13 showed weak activity. From figure 19 it was obvious that fraction 3 and 4 were the fractions that showed the strongest activity, and they showed activity when diluted 1:5 as well.
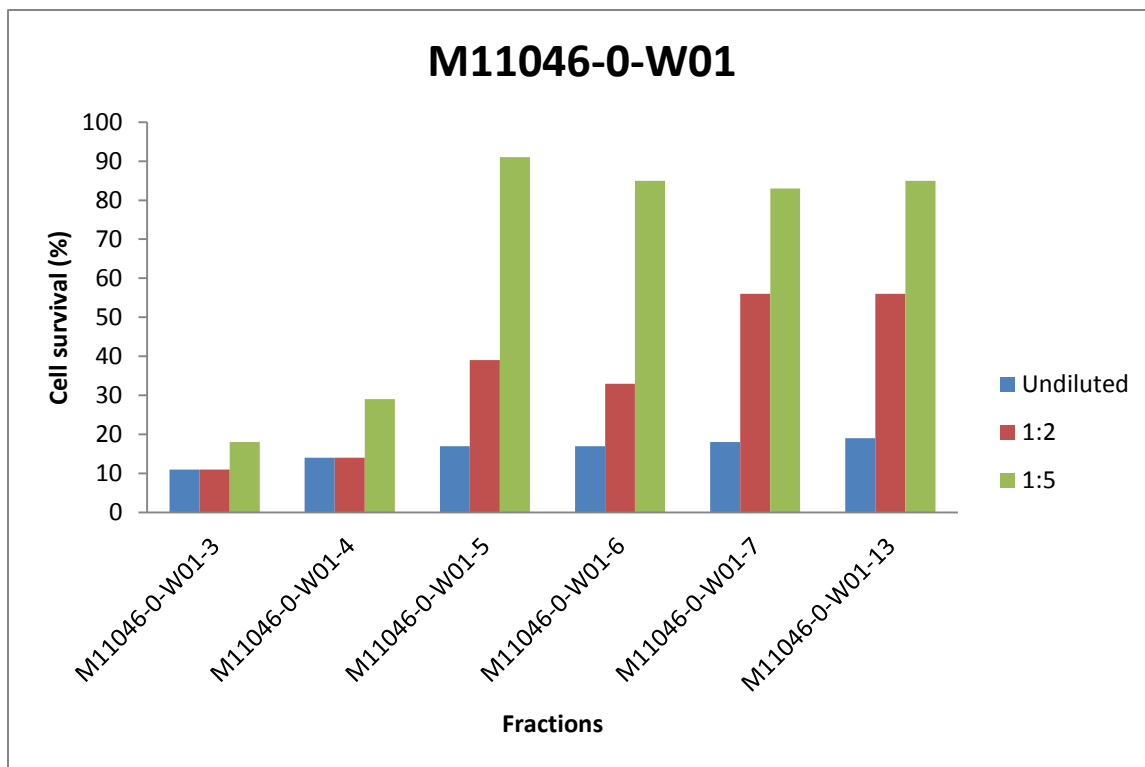
Figure 19: 2nd anticancer assay of the initially active fractions from the aqueous extract of *Halecium muricatum* (M11046) against the A2058 cell line.

Fraction 3 from the aqueous extract of *Halecium beanii* (M11047) showed a high activity in all degrees of dilution in the 2nd screening against the A2058 cell line (see figure 20).
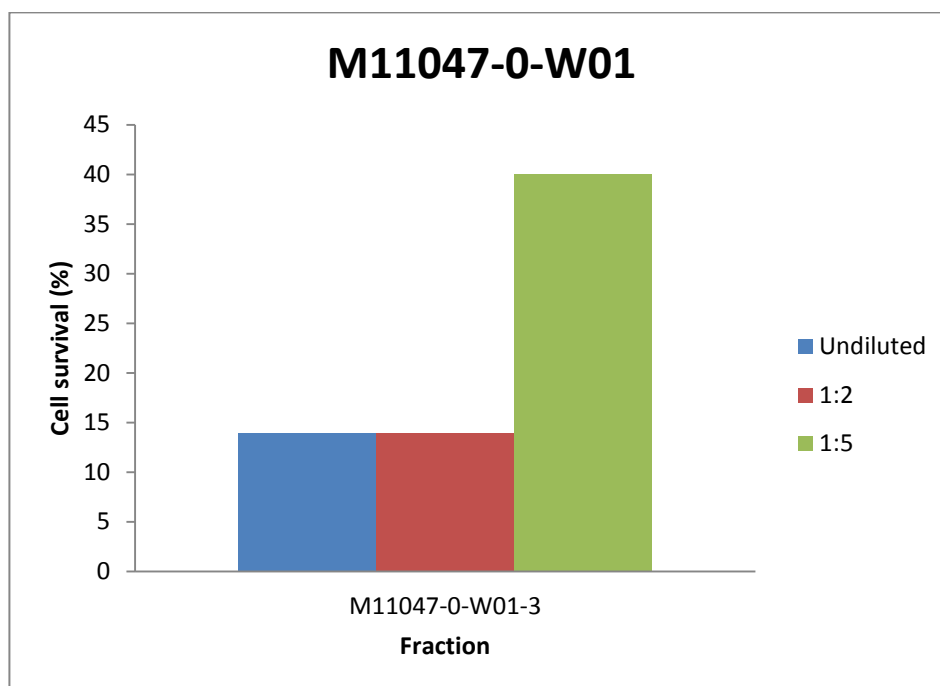

Figure 20: 2nd anticancer assay of the initially active fraction 3 from the aqueous extract of *Halecium beanii* (M11047) against the A2058 cell line.

Anticancer assay of the organic fractions - 1st screening

The 1st screening of the organic fractions showed numerous fractions with activity against the human melanoma, A2058, cell line. The two figures below (21 and 22) show that almost the same fractions of *Halecium muricatum* and *Halecium beanii* were effective against A2058. The main difference was that *Halecium muricatum* had a couple more active fractions.
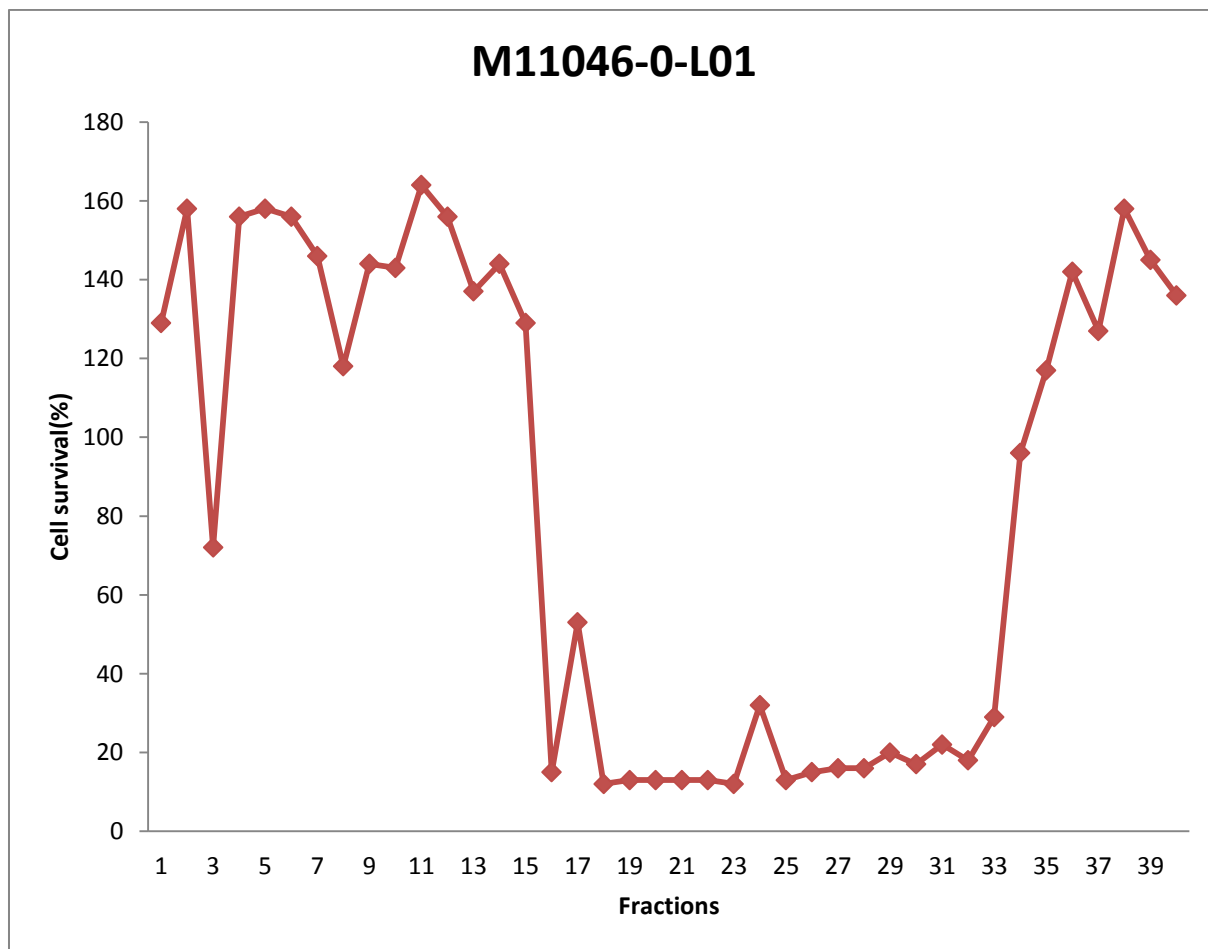

Figure 21: 1st anticancer assay of the organic extract of *Halecium muricatum* (M11046) against the A2058 cell line.

For *Halecium muriatum* (M11046) fractions 16-33 were active, with the exception of fraction 17 which showed weak activity (see figure 21).
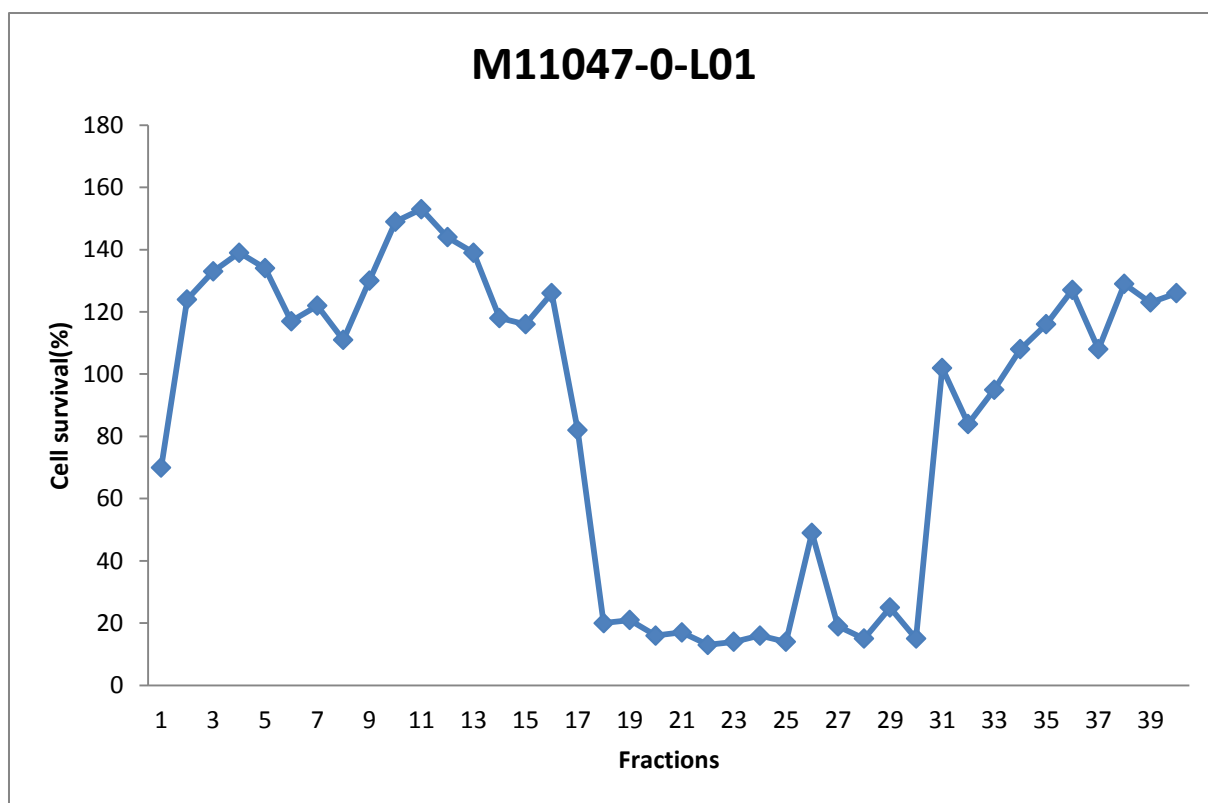
Figure 22: 1st anticancer assay of the organic extract of *Halecium beanii* (M11047) against the A2058 cell line.

For *Halecium beanii* (M11047) fractions 18-30 from the organic extract of *Halecium beanii* (M11047) showed activity (see figure 22).

Anticancer assay of the active organic fractions – 2nd screening

The fractions from the 1st screening that showed activity was retested in the 2nd screening undiluted, diluted 1:2 and diluted 1:5. As we can see from figure 23 and 24, the majority of the initially active fractions of *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) were active in the 2nd screening in the undiluted form.
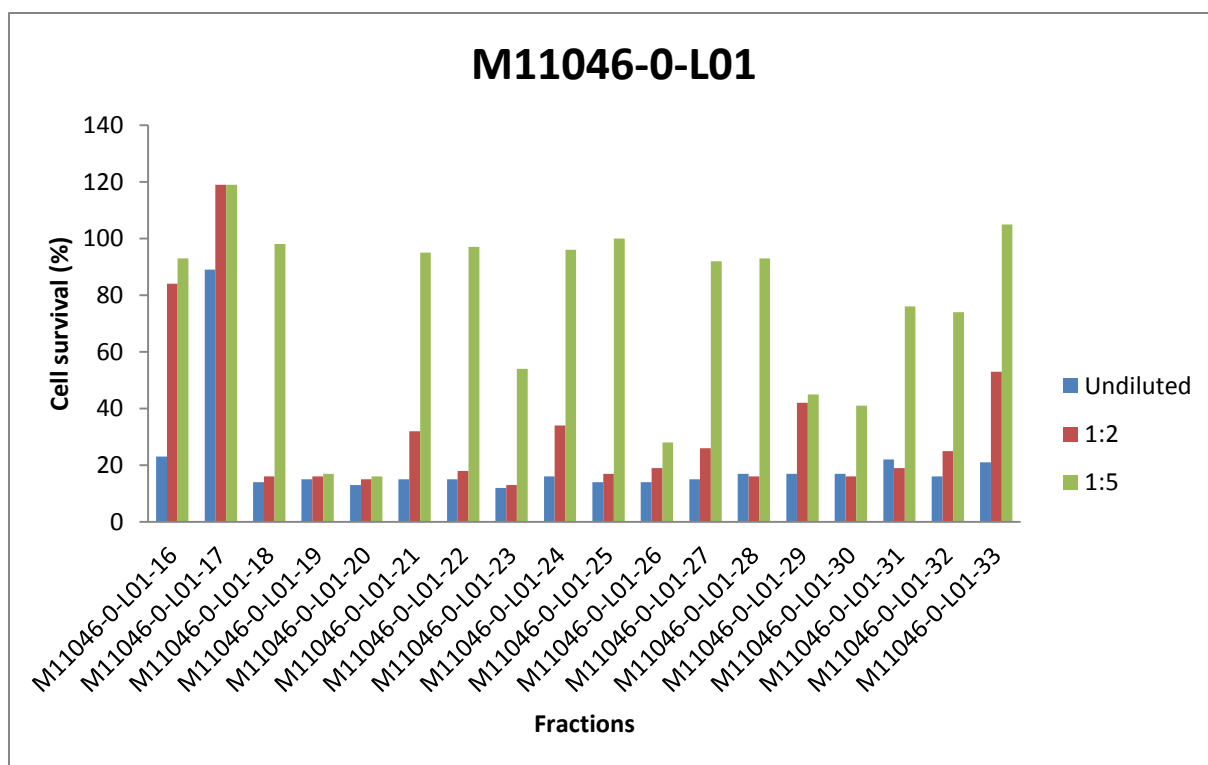
Figure 23: 2nd screening of the organic extract from *Halecium muricatum* (M11046) against the human melanoma cell line A2085.

The results from the 1st screening of *Halecium muricatum* (M11046) were highly reproducible (see figure 23), since all initially active organic fractions, except fraction 17, showed activity against A2058 in the 2nd screening in the undiluted form. All fractions except fraction 16 and 17 showed activity when diluted 1:2. When diluted 1:5, fraction 19, 20, 26, 29 and 30 showed strong activity, whereas fraction 23 showed weak activity (see figure 23).
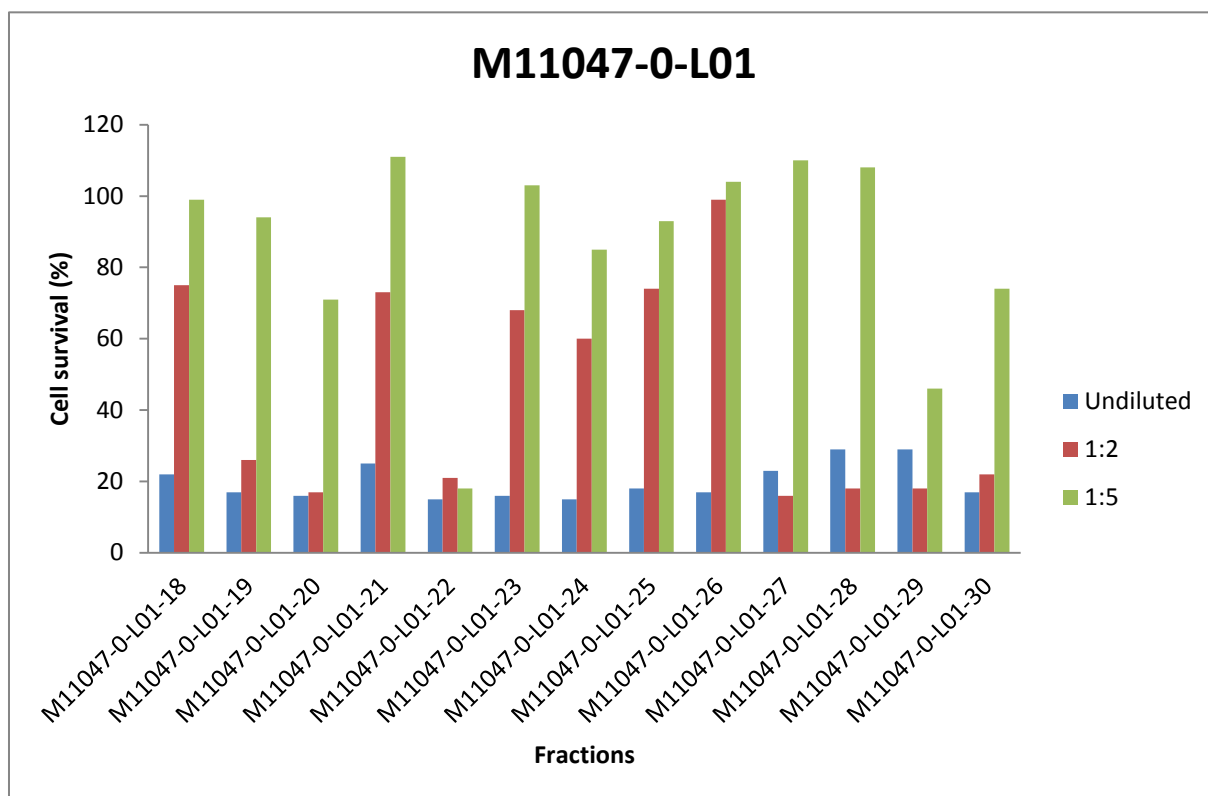
Figure 24: 2nd screening of the organic extract from *Halecium beanii* (M11047) against the human melanoma cell line A2085.

In the 2nd screening of *Halecium beanii* (M11047) no clear dose- response relationship was established (see figure 24). All fractions showed activity in the undiluted form. Fractions 19, 20, 22, 27, 28, 29 and 30 showed activity when diluted 1:2. Two fractions showed activity against cell line A2058 over the grades of dilution, fraction 22 and 29 respectively.

6.3 Dereplication

The analysis of the organic HPLC fractions from *Halecium beanii* (M11047) by HR- MS showed the presence of many compounds. Eight compounds were present in relatively large amounts in the active fractions, compared to the inactive fractions. These compounds had the *m/z* values 452.3, 454.3, 480.3, 482.3, 508.3, 508.3, 510.3 and 512.3 as the protonated molecular ions. One example is shown where the active fraction 22 from *Halecium beanii* (M11047) was compared to the inactive fraction 15 from the same organism (see figure 25). Compounds with protonated masses 482.3, and two probable isomers of 508.3 were present in

the active fraction and not in the inactive fraction. It was therefore likely that one or more of these three compounds contributed to the bioactivity observed in fraction 22.

From the accurate protonated mass of compound **1** (454.3273, see figure 29), the elemental composition was calculated to be $C_{22}H_{49}NO_6P$, and from the accurate protonated mass of compound **2** (482.3582, see figure 29) the elemental composition was calculated to be $C_{24}H_{53}NO_6P$. These protonated masses and the masses to the six other compounds correlated with molecular masses of different phosphocholines from searches in the Dictionary of Marine Natural Products. Compound **1** correlate with 1- Tetradecylglycero-3-phosphocholine, and compound **2** correlate with 1- Hexadecylglycero-3-phosphocholine.
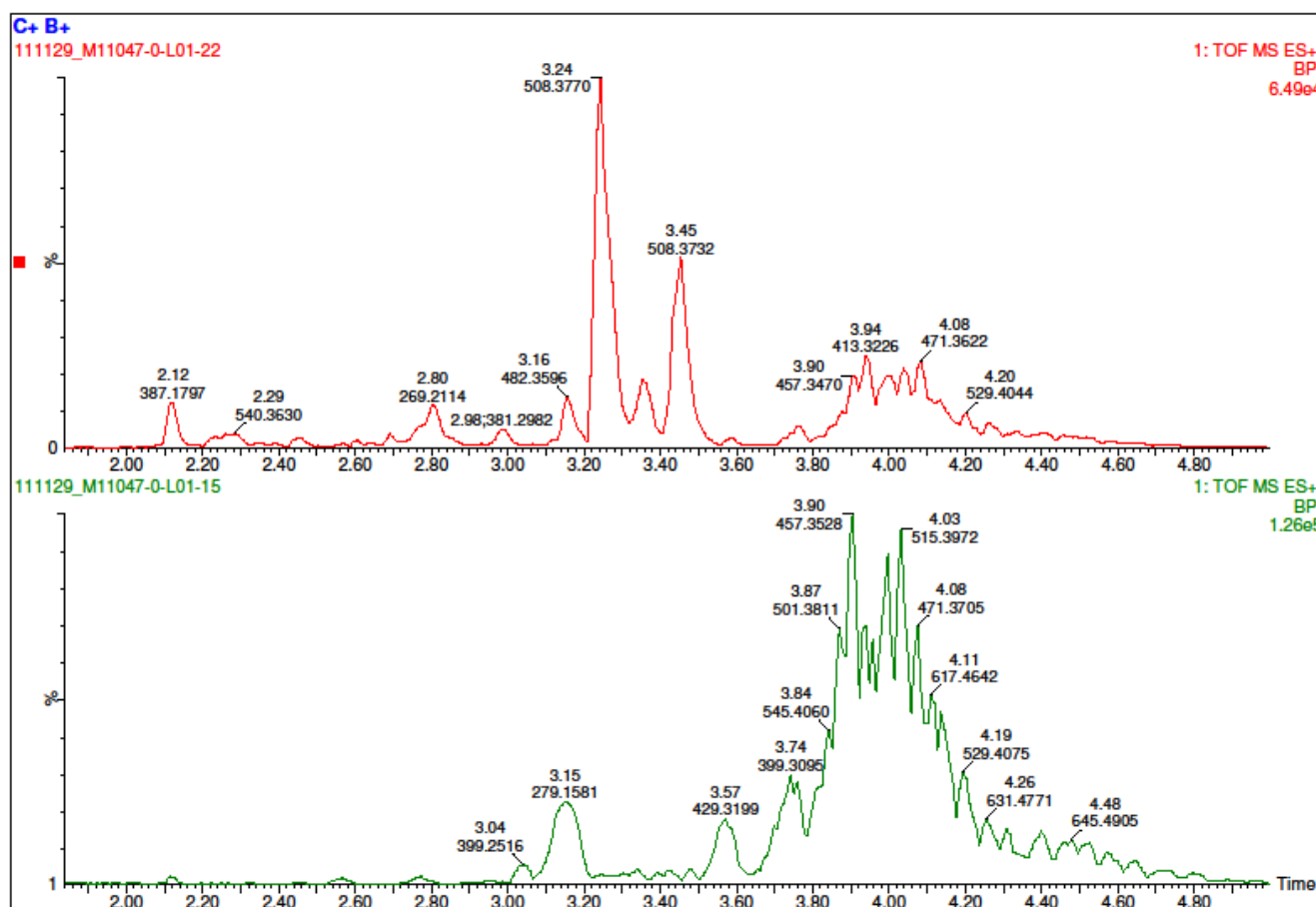

Figure 25: Chromatogram ESI+ (*m/z* 150-1500) comparing active fraction 22 (M11047) with the inactive fraction 15 from *Halecium beanii* in the dereplication process.
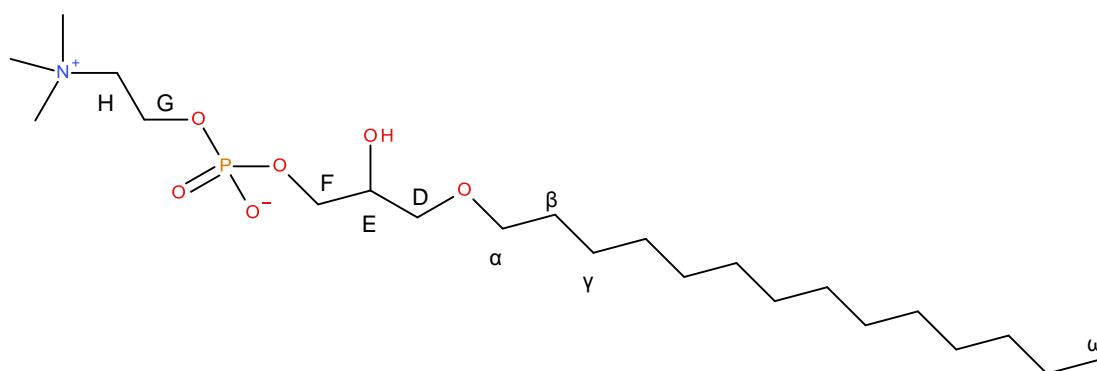
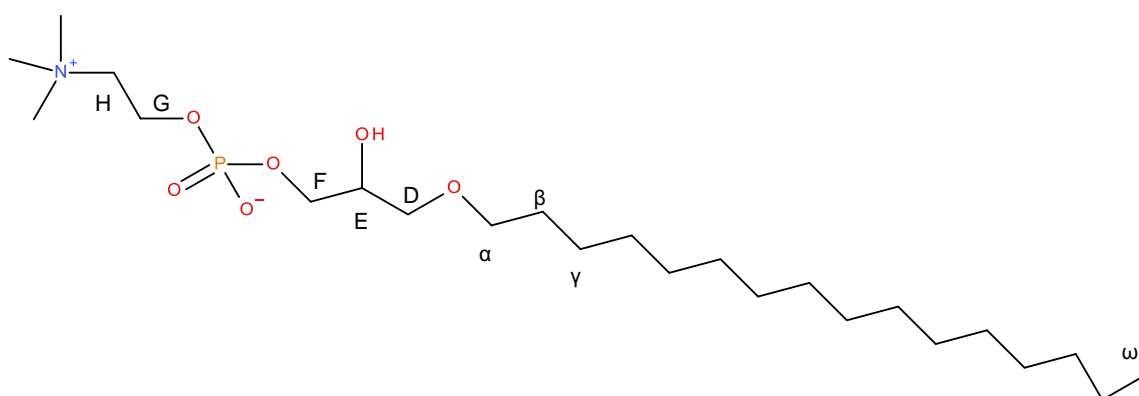Figure 26: Compound **1**, 1- Tetradecylglycero-3-phosphocholine

Figure 27: Compound **2**, 1- Hexadecylglycero-3-phosphocholine.

6.4 Isolation of possible bioactive compounds from *Halecium beanii*

After the dereplication of the eight different compounds, the compounds needed to be isolated to confirm their structure and bioactivity. In total 412.9 mg of organic extract from *Halecium beanii* (M11047) was used in the isolation process. The gradient used on the HPLC in the isolation process was optimized. The starting point for this gradient was from the gradient used during fractionation, and was further optimized resulting in chromatograms where the different compounds were separated satisfactory, and the fraction collection did not overlap (see figure 28). The molecular weights of the eight compounds triggered fraction collection.
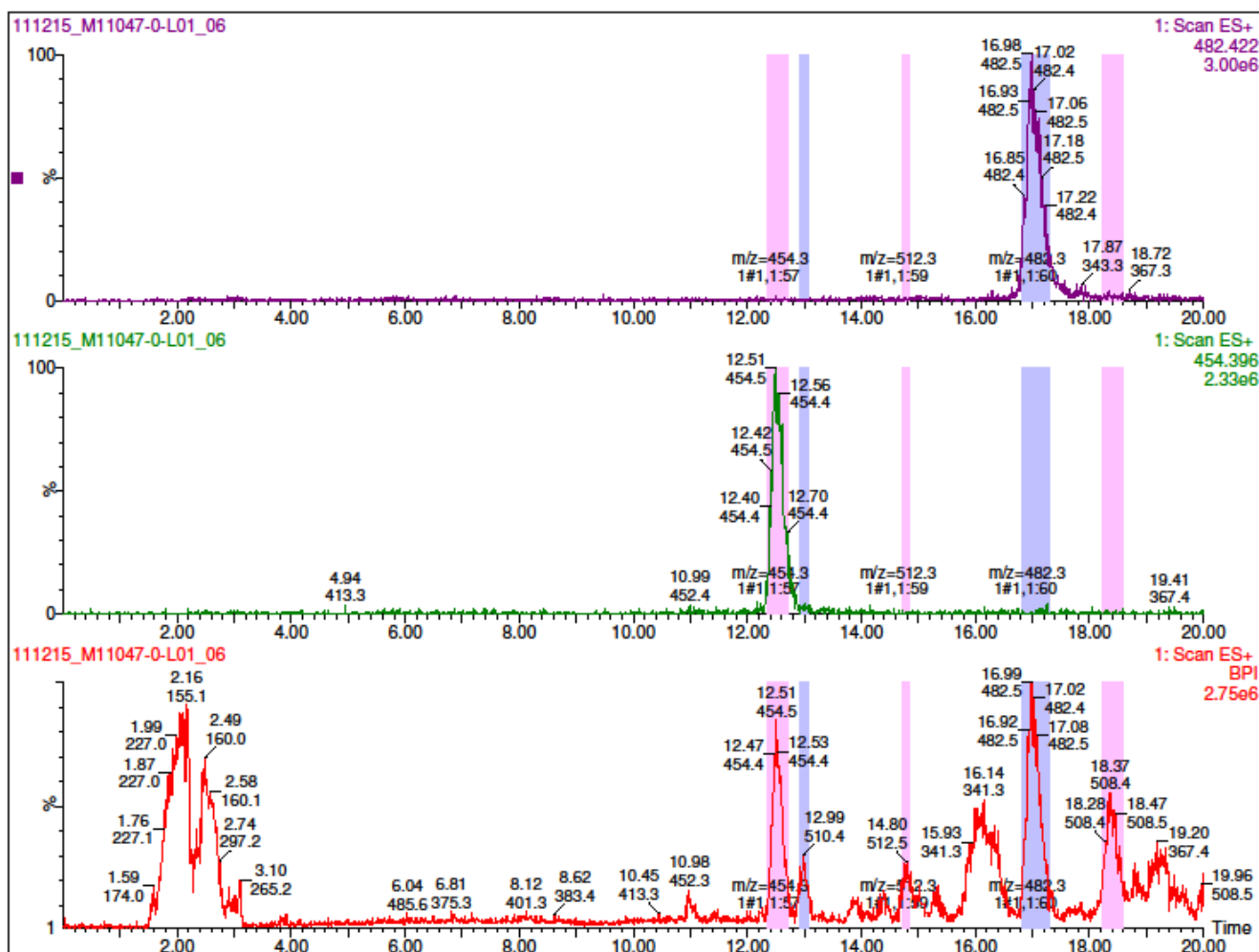
Figure 28: Example from the isolation of compounds from the organic extract from *Halecium beanii* (M11047).

After isolation, the compounds were analyzed with TOF- MS to determine the purity of the compounds. Due to time constrains, we could not investigate all eight compounds, and therefore we chose to proceed with the two purest compounds. Compound **1** and compound **2** were purest (see figure 29), and were chosen for further bioactivity testing. Figure 29 indicates that compound **1** and **2** were relatively pure. After the isolation and drying process the amount available of the two compounds was 0.7 mg for compound **1** and 1.1 mg for compound **2**.
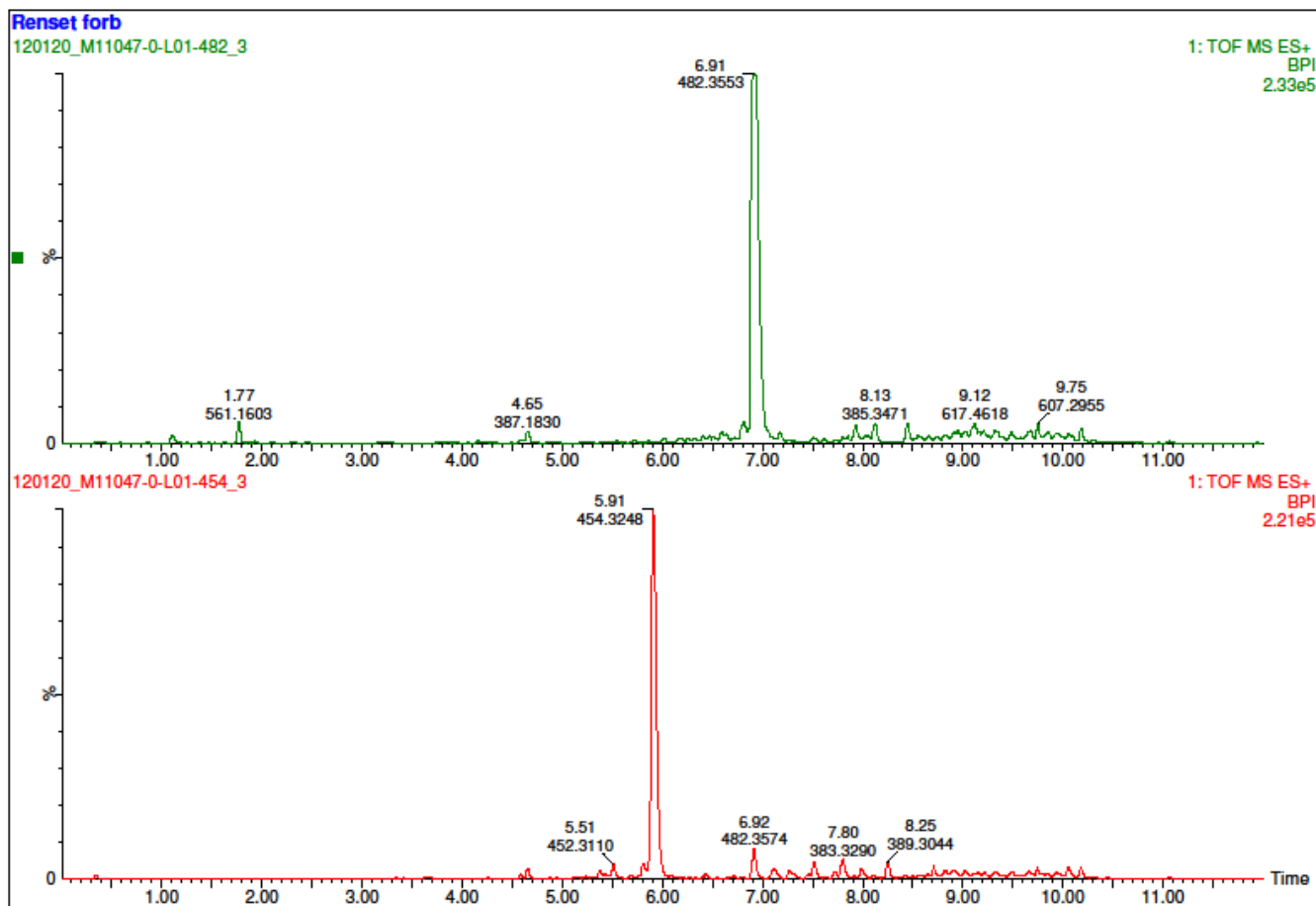
Figure 29: Compound **1** (bottom) and compound **2** (top) analyzed with MS- TOF in the isolation process.

Figure 30 displays the MS spectra for compound **1** and **2**. For compound **1** the peak with the protonated mass 454.3 was the molecular ion with an additional hydrogen ([M+H]$^+$), the protonated mass 455.3 was the isotope of the molecular ion containing one ^{13}C, and the peak with the protonated mass 456.3 was the isotope of the molecular ion containing two ^{13}C. The peak with the protonated mass 907.6 was [2M+H]$^+$, and the protonated masses 908.6 and 909.6 were the isotopes of [2M+H]$^+$ containing one and two ^{13}C. The mass 1360.7 was [3M+H]$^+$. The same pattern goes for compound **2**, where the protonated mass 482.3 was the molecular ion and a proton ([M+H]$^+$), mass 483.3 and 484.3 were the protonated molecular ion containing one and two ^{13}C. The mass 963.7 was [2M+H]$^+$, and the masses 964.7 and 965.7 were [2M+H]$^+$ containing one and two ^{13}C. The mass 1445.07 was [3M+H]$^+$.
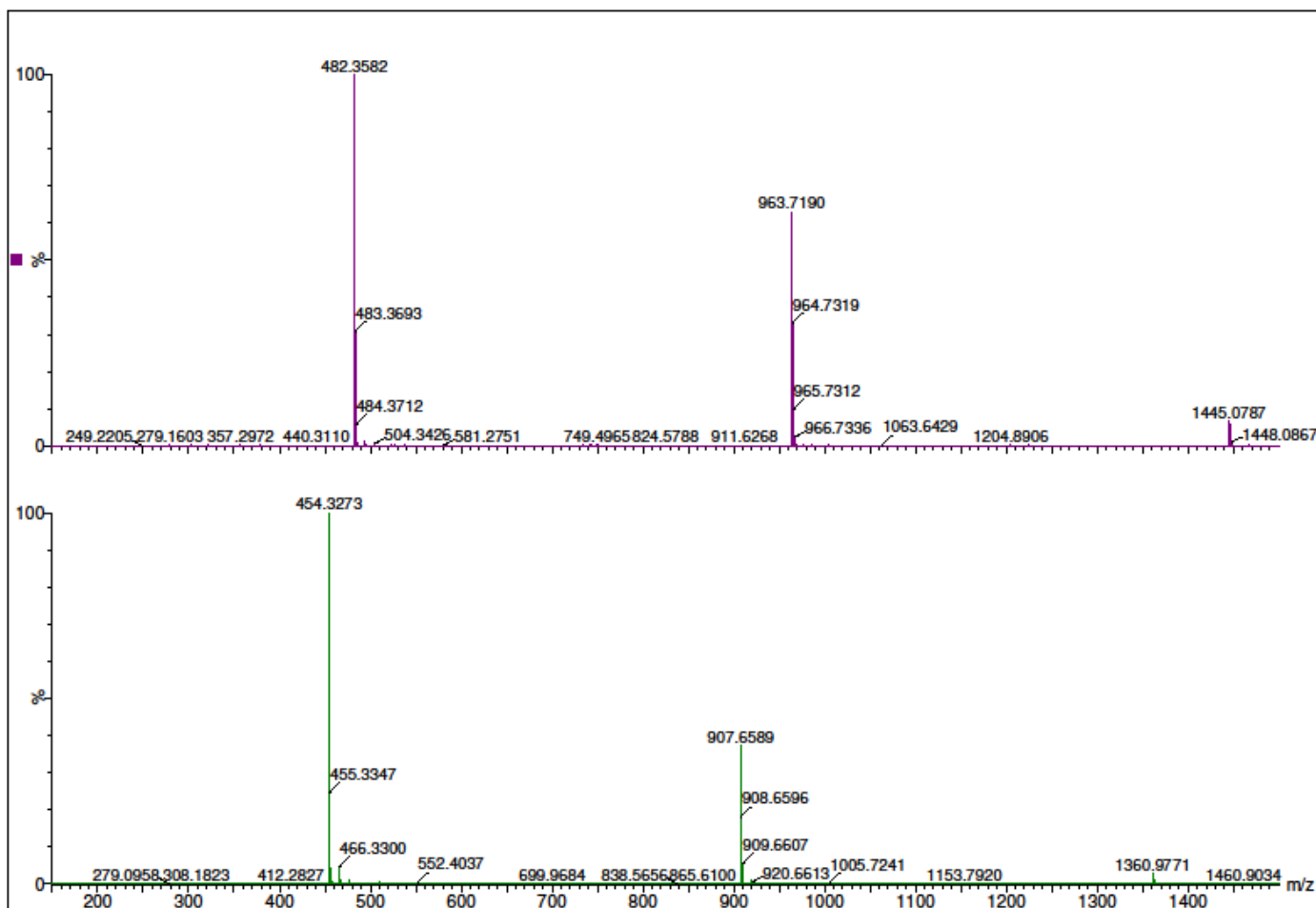
Figure 30: MS specter for compound **1** (bottom) and compound **2** (top).

6.5 Identification of possible bioactive compounds from *Halecium beanii*

HR- MS

HR- MS was used to confirm the accurate mass for compound **1** and **2** after the dereplication.

NMR

Compound **1**:

The spectrum that gave most information was the Wet1D (^{1}H- NMR) spectrum (see figure 31). 1D 1H- NMR is quantitative, which means that the area under each peak is proportional to the number of protons giving rise to the resonance. The peaks in this spectrum were integrated, by setting one peak as a reference peak. The integrals were normalized on the peak

of the identical hydrogen atoms adjacent to the nitrogen atom peak, corresponding to nine protons. They have the intensity of nine hydrogen atoms. When compared with the hydrogen atoms on the methyl- tail to the ω- carbon the ratio should be 9:3, if this sample was pure. However, the ratio was 6:3. This implies that there was a mixture of different phosphocholines in the sample. A ratio of 6:3 could mean two things. One, that the sample contained a mixture of different phosphocholines that had one and two tails. Two, that the sample contained a mixture of different phosphocholine- like compounds without the NC_3- group. Both of these possibilities would shift the ratio from 9:3 to 6:3. The gHSQCAD, gHMBCAD and gCOSY spectra did not give any additional information, other than the head group was consistent with a choline, but the resonances of the tail(s) were severely crowded preventing explicit assignment. At this point, there was no evidence which states that the compound in question was not 1- Tetradecylglycero-3-phosphocholine. It was certain however that the sample contains a mixture of different phosphocholines. To further identify the phosphocholines present, and confirm or disprove that this mixture contains 1- Tetradecylglycero-3-phosphocholine, further structure elucidation was needed.
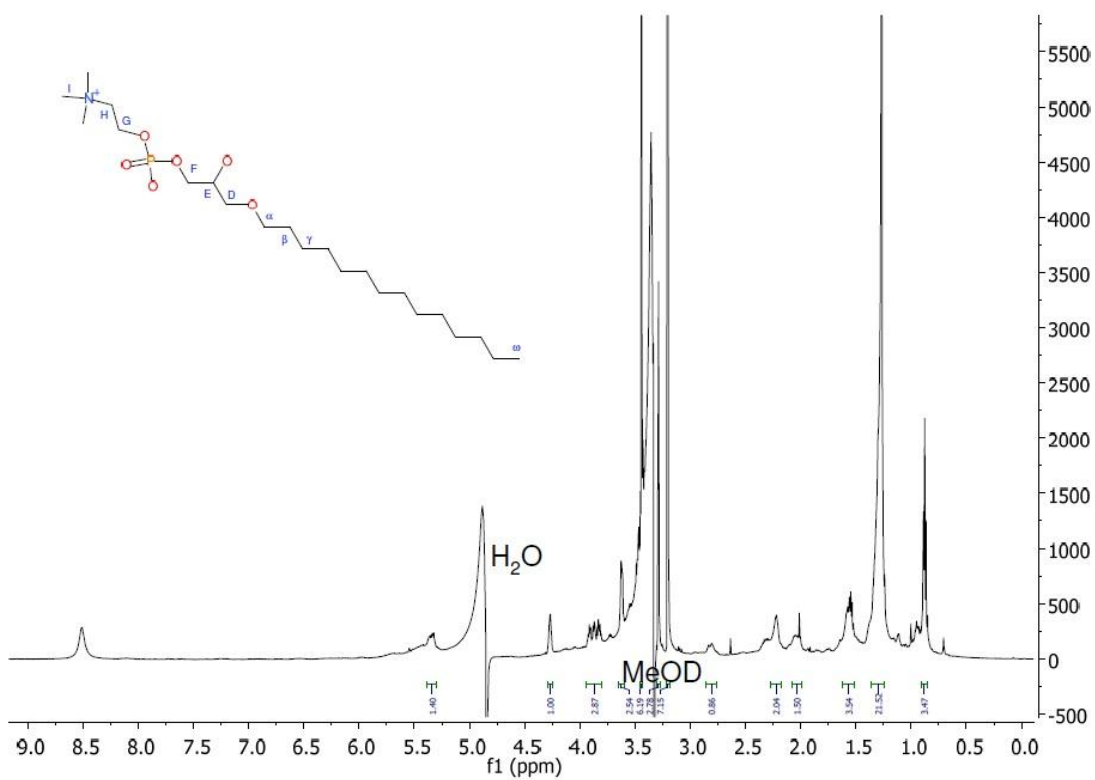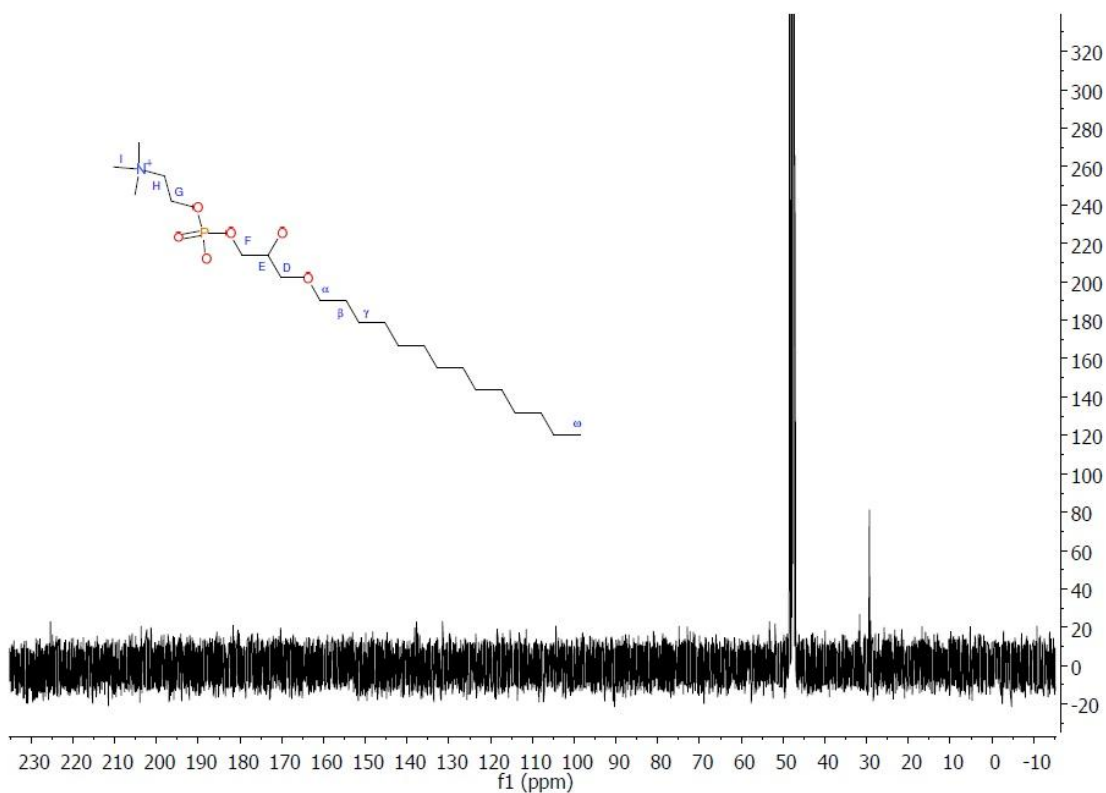
Figure 31: Wet1D specter for compound **1**.

Figure 32: ^{13}C – NMR specter for compound **1**.

Compound **2**:

Again the spectrum that gave most information was the Wet1D (^{1}H- NMR) spectrum (see figure 33). The peaks in this spectrum were integrated, by setting one peak as a reference peak. In this case the reference peak was the hydrogen atom on the E carbon. If this sample was pure, the ratio between the hydrogen atom on the E- carbon and the hydrogen atoms on the ω- carbon would be 1:3. Instead the ratio was 1: 7.5. This again implies that there was a mixture of phosphocholines in the sample. A source of error occurs when a peak consisting of one hydrogen atom is used as the reference peak, so the ratio of 1:7.5 really might be 1:6 (see section 7 for further discussion about sources of error). That implies that in this sample, there were phosphocholines with two tails. Another possibility was one tail, but with a different head group or some other difference near E, and this could also shift the ratio between E and the tail. Another example that supports this was the intensity of the integrated peak of the hydrogen atoms on the γ- carbons. If this sample only contained 1- Hexadecylglycero-3- phosphocholine the ratio would be 1:24. Instead the ratio is 1:51. This supports the theory that there was a mixture of different phosphocholines, with different numbers of tails. When the ratio between the hydrogen atom on the E- carbon and the hydrogen atoms on the NC_3- group were compared the ratio was 1:6.5. As with the other sample, this could imply that in this mixture, there was some phosphocholine- like compounds which lack the NC_3- group.

Information from the gHSQCAD+ gHMBCAD spectra implies the presence of a carbonyl- group on the α- carbon. However, it was difficult to say for sure if it was on the same molecule, an overlapping impurity or something in the sample mixture. It may not be the main component. When these results are added up, the conclusion was that there was a mixture of phosphocholines in the sample. To confirm or disprove the presence of 1- Hexadecylglycero-3-phosphocholine, further structure elucidation was needed.
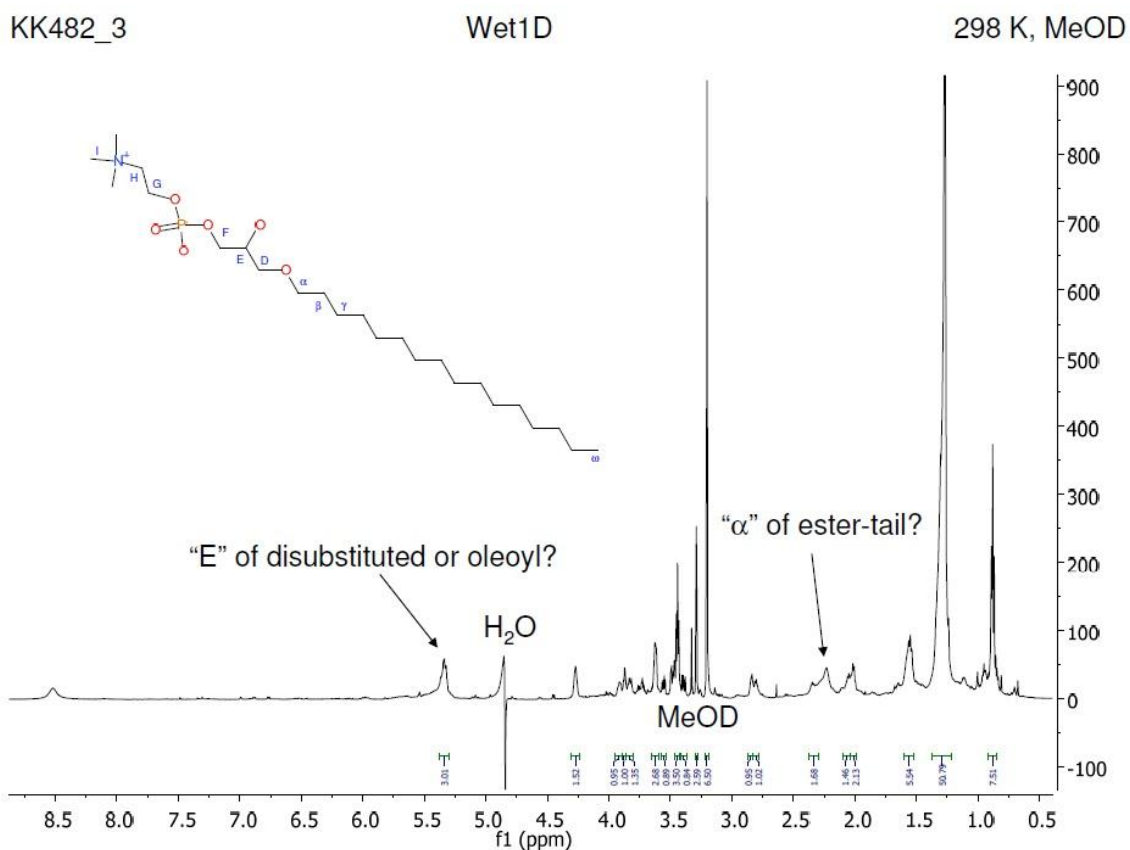
Figure 33: Wet1D specter for compound **2**.
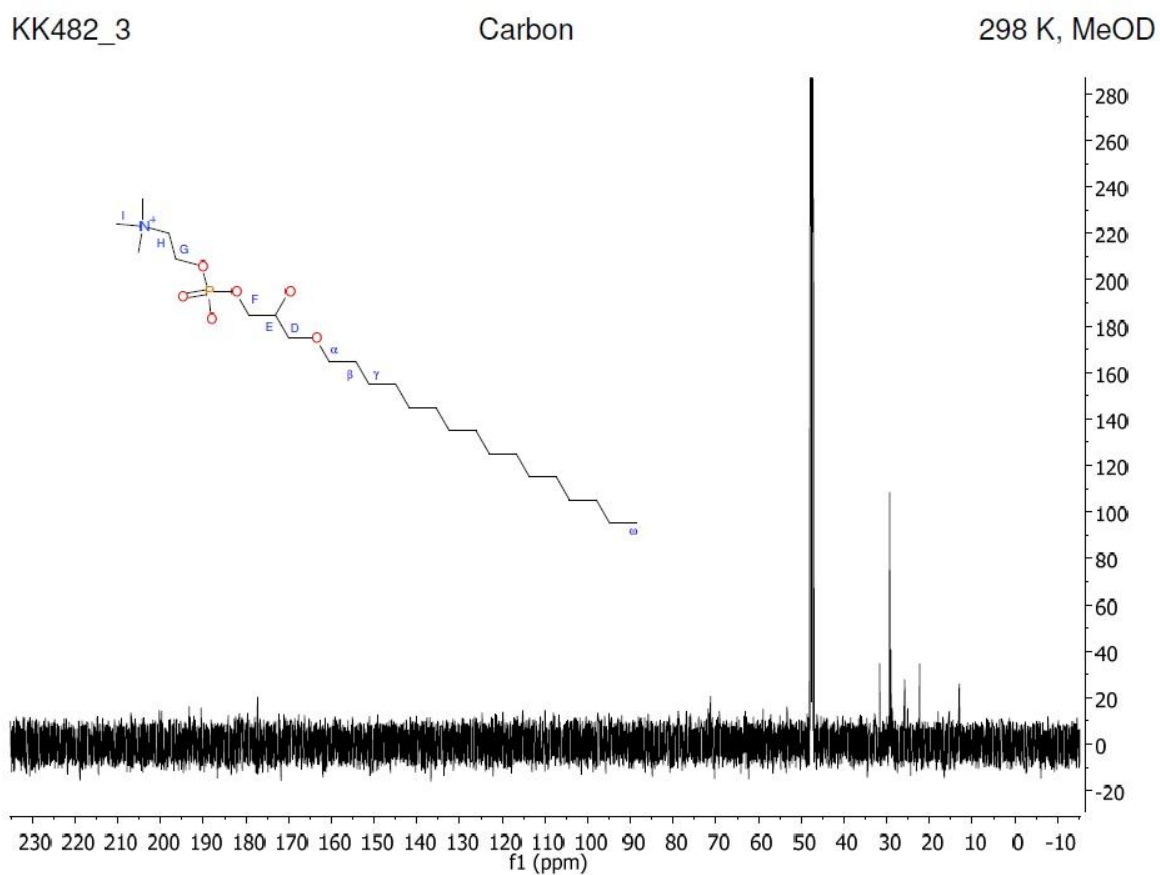
Figure 34: ^{13}C- NMR specter for compound **2**.

60
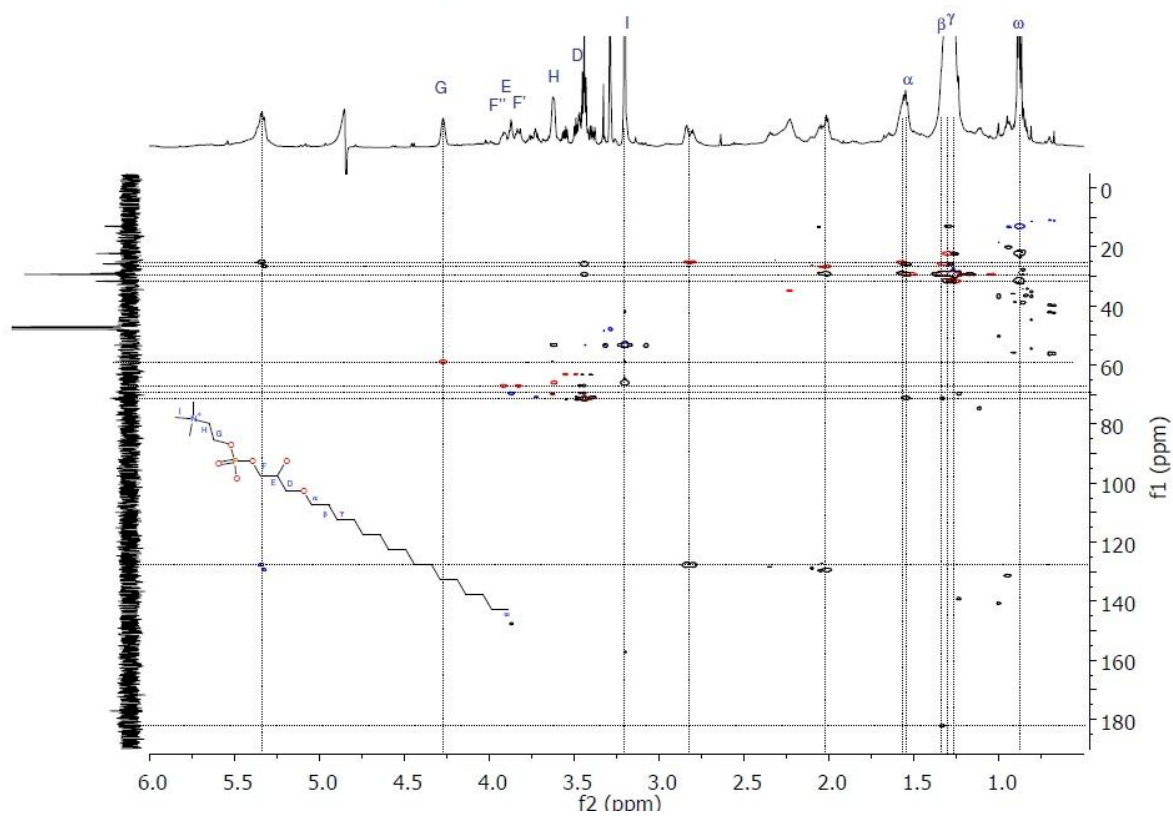
Figure 35: 2D specter (HSQC and HMBC) for compound **2**.

MS- MS:

Compound **1**:

From the MS-MS spectrum below of compound **1** (see figure 36), three relatively large peaks in addition to the protonated molecular ion 454 ([M+H]$^+$) were observed. The three fragments were 86, 104 and 184. There was a small peak at *m/z* 437 indicating a loss of a hydroxyl group, since the *m/z* difference between 454 and 437 was 17.
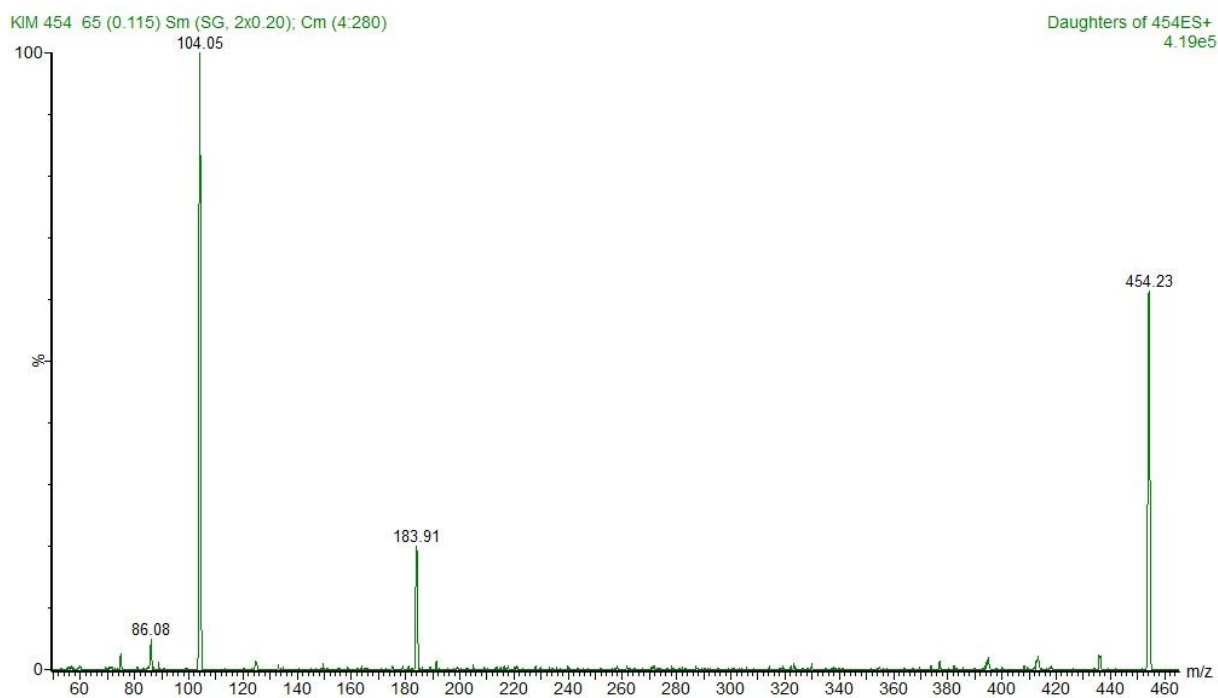
Figure 36: MS-MS spectrum of compound **1**.

Compound **2**:

For compound **2** the three first fragments in the spectrum were the same fragments found in the spectrum for compound **1**. In addition to two other fragments, and the protonated molecular ion 482 ([M+H]$^+$) (see figure 37).
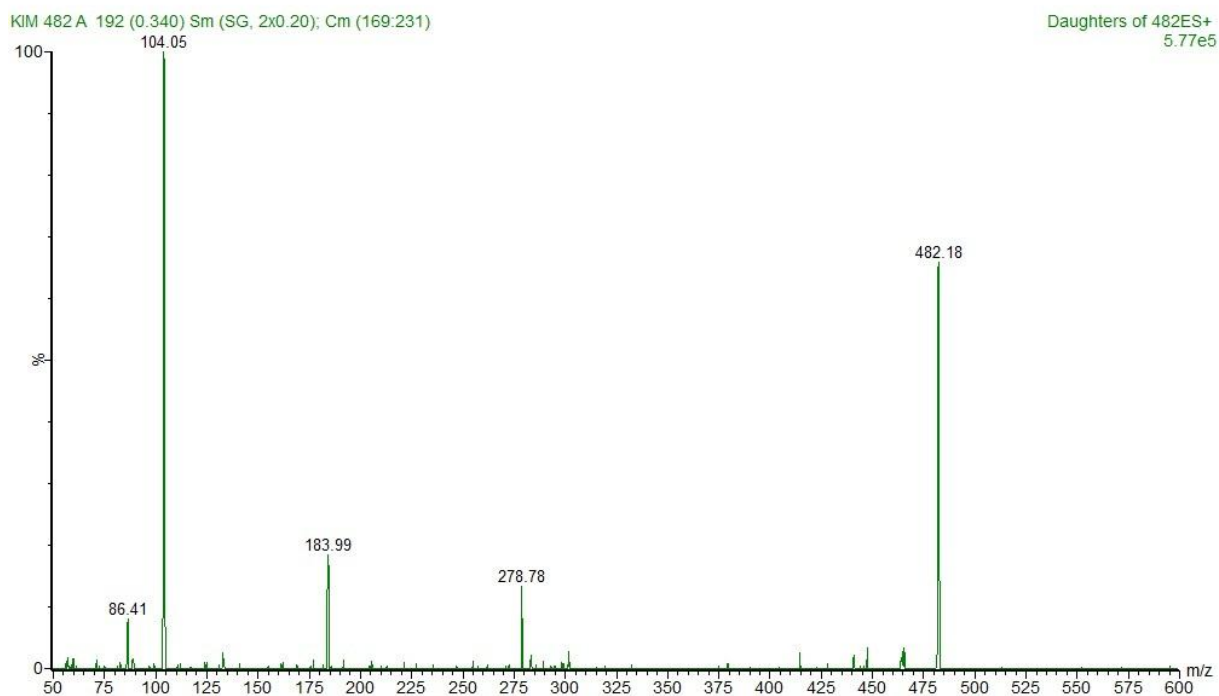
Figure 37: MS- MS spectrum of compound **2**.

The fragments with protonated masses 86, 104 and 184 are often seen when analyzing phosphocholines [37].
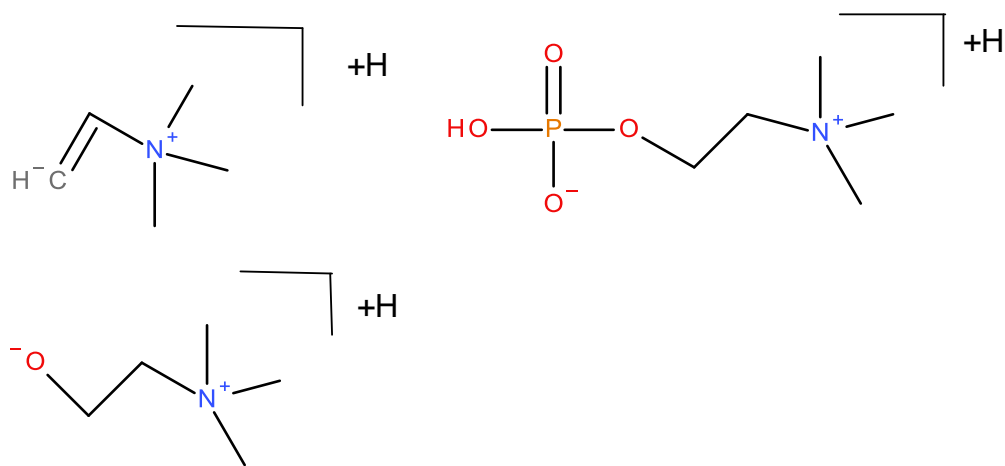
Figure 38: Proposed mass fragmentation from phosphocholines. Top left: *m/z* 86, top right: *m/z* 184, and on the bottom: *m/z* 104.

These fragments were fragments from the phosphocholine head group. The fragment with *m/z* 278 was difficult to interpret. However this fragment only appeared in one scan, and was therefore most likely an artifact, not a fragment from compound **2** (see figure 39).
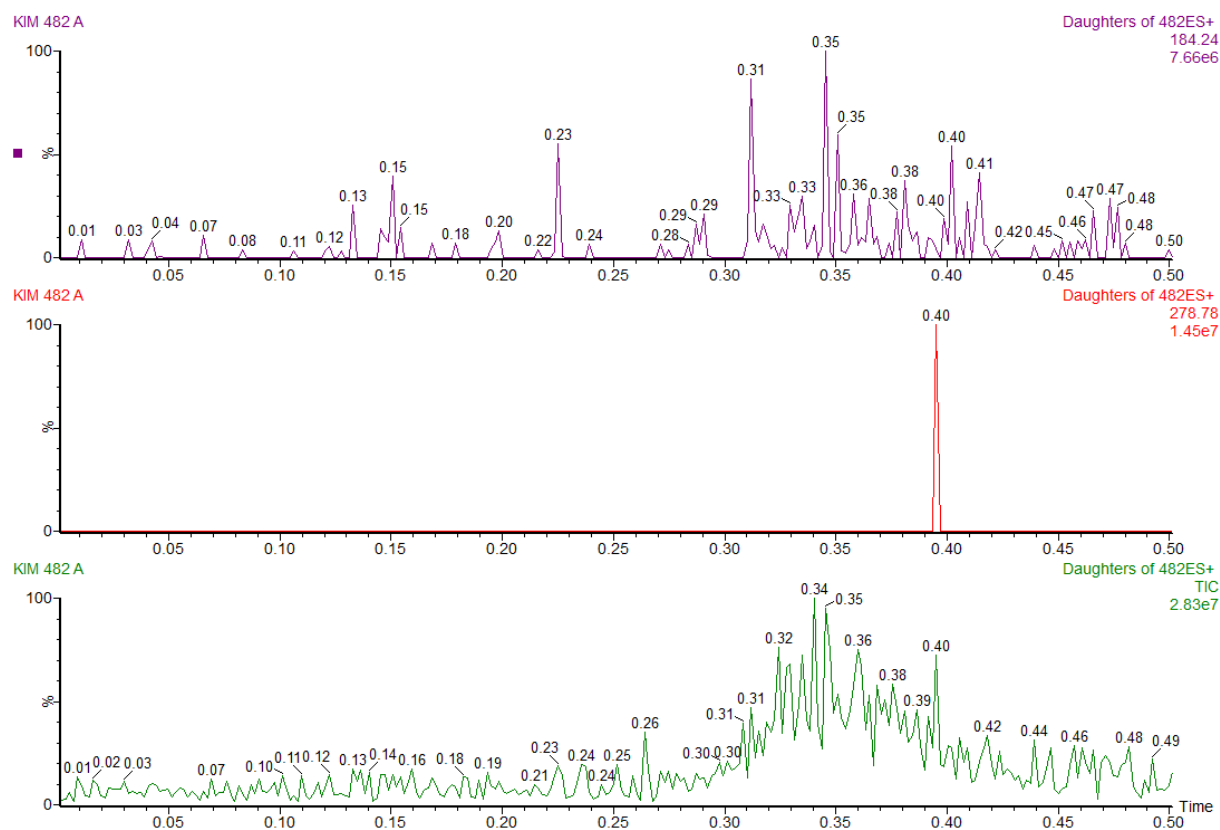
Figure 39: Data from the MS-MS scan, showing the real fragment with *m/z* 184 (top), one scan detecting the artifact with *m/z* 278.78 (middle) and TIC (bottom).

Figure 39 also displays that the fragment with *m/z* 184 occur throughout the whole analysis and with higher intensity when the TIC intensity was increased.

The peak with *m/z* 465 was compound **2** after the loss of a hydroxyl group, since the mass difference between compound **2** and *m/z* 465 was 17 (see figure 37).

6.6 Confirmation of bioactivity

As stated earlier the confirmation of bioactivity was necessary, since the compounds isolated might not be the compounds responsible for bioactivity or the bioactivity can be the result of a synergic effect from multiple compounds. In the case of compound **1**, there were no concentrations that were defined as active, according to the preset values, against either cell line (see figure 40 below). There was not activity against the A2058 (human melanoma) cell line at the concentrations tested (2.5 – 100 µg/ mL), which the active fractions showed bioactivity against in the 1st and 2nd screening. It is therefore highly likely that the compound was not the compound responsible for bioactivity, or was part of a synergic effect. Against the cell line MRC5 compound **1** showed a weak effect at 100 µg/ mL (74 % cell survival).
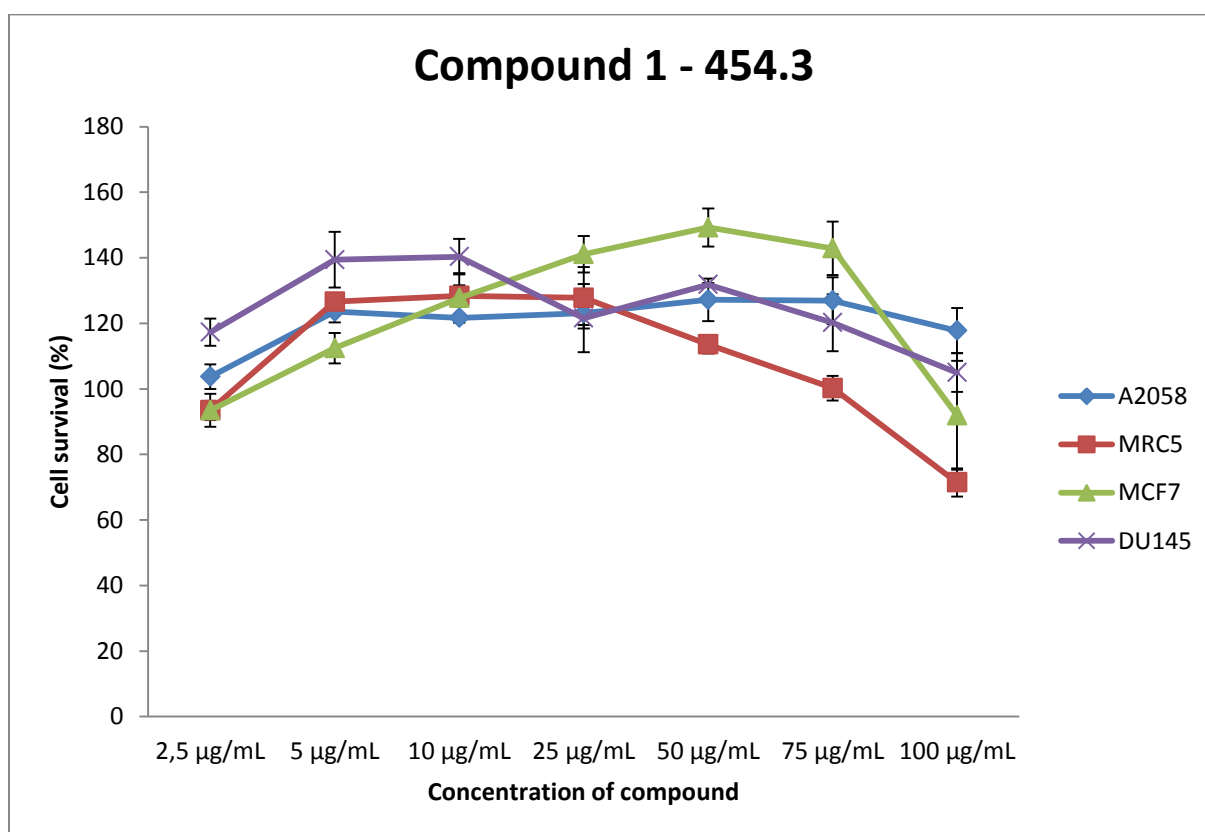
Figure 40: Result from bioactivity testing of compound **1** against four cell lines with standard deviations from three parallels.
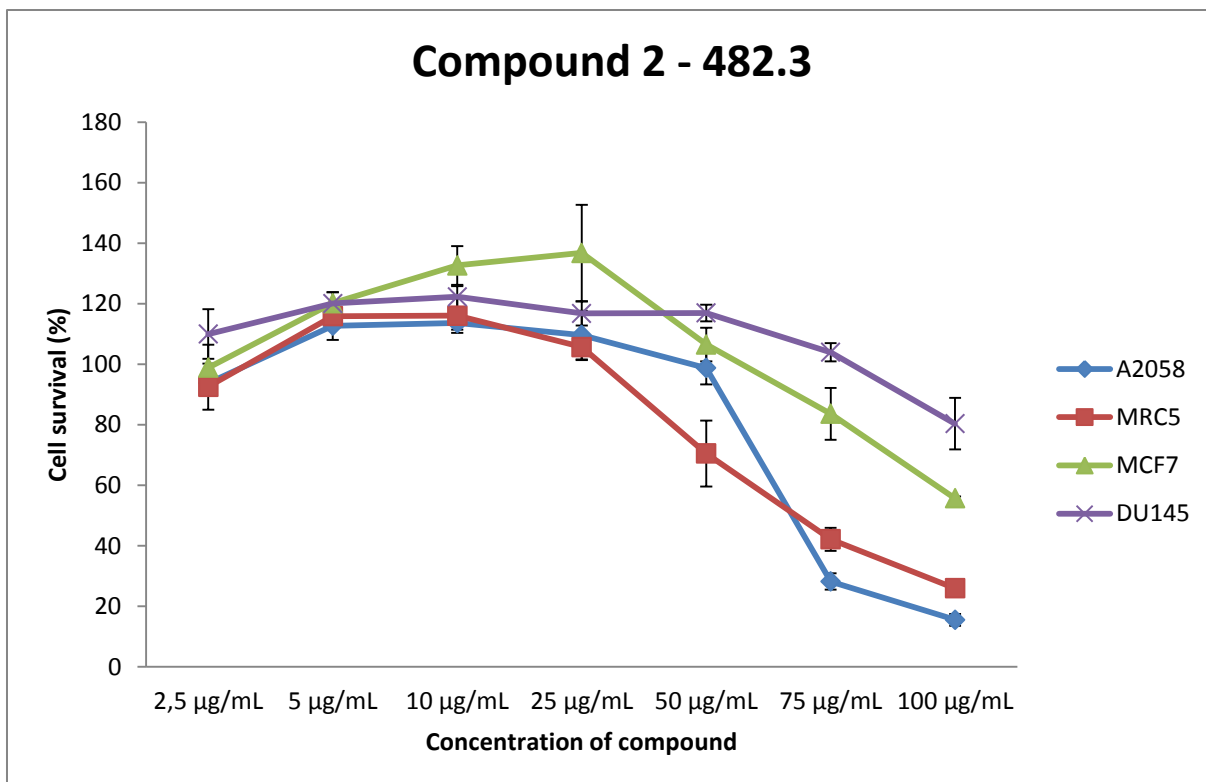
Figure 41: Result from bioactivity testing of compound **2** against four cell lines with standard deviations from three parallels.

Compound **2** showed activity against all the cell lines, except the DU145 cell line at 100 µg/ mL (see figure 41). Against the cell lines A2058 and MRC5 the compound showed good activity at the two highest concentrations, 75 µg/ mL and 100 µg/ mL respectively. Against MCF7 (human breast carcinoma) the highest concentration of compound **2** showed weak activity (57 % cell survival). Compound **2** showed a dose- response relationship against the cell line MRC5.

7. Discussion

New anticancer agents are needed for many reasons. Today the treatment is accompanied by many side effects, and one wish to increase the rate of survival. At the same time the population of the western world gets older and the cancer incidence is increasing. There is also a need for new antibiotics with novel mechanisms of action because of the development of bacterial resistance. To battle this development of resistance the funding of R & D needs to be increased, yet the pharmaceutical companies decrease their funding. The marine environment is underexplored and has an unparalleled biodiversity, which is one reason for establishing new centers in the field of marine bioprospecting. Academics are establishing new centers, but the pharmaceutical industry is still on the sideline, despite the previous discoveries and the potential in marine bioprospecting.

The aim of this work was to isolate and characterize the structure of bioactive compounds from *Halecium muricatum* and *Halecium beanii*. After the different bioactivity assays in the 1st and 2nd screening against human cancer cell lines and different bacterial strains, both organisms had many bioactive fractions, especially fractions from the organic extract. To our best knowledge there is no work about *Halecium* muricatum and *Halecium beanii* and their bioactivities, only literature about the identification of hydroids.

Extraction was obviously an important step to produce extracts that can be used for bioactivity assays. The method used for extraction was a specific protocol utilized by the National Cancer Institute (NCI) to preserve the biologically active molecules the biomass sample contain. The aqueous extraction was carried out first since this extraction was quicker than the organic extraction, which minimizes the time the sample was not frozen. This timeframe was very important, since marine samples deteriorate rapidly when not frozen. The maximum time the sample can be out of the freezer is two hours [38]. The weight of the aqueous extract typically exceeds by far the weight of the organic extract. The reasons for this high yield for the aqueous extract is the presence of high molecular weight compounds like polysulfated polysaccharides, peptides, proteins and the sea salt from the sea water. The extraction yield for the organic extract is typically 3-5 % after using DCM and MeOH, thus the yield from *Halecium muricatum* (M11046) and *Halecium beanii* was normal. However, the yield would be higher if the organic extraction was carried out first, but this increase in yield would be associated with the increased amount of sea salts and other substances in the organic extract, since they are partly soluble in MeOH. Therefore when doing the aqueous

extraction before the organic extraction, the constituents in the organic extract are dominated by smaller, moderately polar and non- polar compounds, which are the compounds most likely to exhibit drug- like properties. Performing the aqueous extraction before the organic extraction minimize the amount of salts present in the organic extract, which leads to a higher concentration of compounds with possible drug- like properties in the micro titer plate when conducting bioactivity assays. This because the amount of salts would comprise a large part of the approximately 200 mg of organic extract used in the bioactivity assays. A higher concentration makes it more likely to detect the bioactivity in the organic extract [38].

Before the bioactivity assays, the organic and aqueous extracts from the two organisms needed to be fractionated. The fractionation was satisfactory, based on the fact that it was possible to distinguish between compounds present in inactive and active fractions (see figure 25). This also indicated that the gradient developed for use in the fractionation was working as intended. The UV chromatograms, perhaps with the exception of the chromatogram of the organic extract of *Halecium beanii* (M11047), did not give much information, because of the few signals detected. The compounds that often show bioactivity are eluted in the middle of the gradient used in the fractionation of organic extracts. The reason for this is that these compounds are not too polar or not to lipophilic. If the compound is too hydrophilic or hydrophobic it would not be able to cross membranes, and if it is too lipophilic it would precipitate in the blood. Therefore a trait of an effective drug is a balanced lipophilic/ hydrophilic character.

In this work the aqueous and organic extracts go through bioactivity assays before isolation, instead of isolation of the compounds from the extracts without the support of bioactivity assays. The disadvantage for the latter course of action is the fact that isolation is a time consuming process, and when the results from these bioactivity assays are available there is a possibility that the compounds tested show no sign of bioactivity. With the course of action in this work, results from bioactivity assays are available at an early stage, which support the use of resources for further isolation and structure elucidation. However there are disadvantages to go through with bioactivity assays before isolation as well. The one minute fractions that undergo bioactivity assays contain many compounds, and the possibility of a synergic effect is present, where multiple compounds working together are the reason for the observed bioactivity.

The bioactivity assays done in this work were against cancer and bacteria, with anticancer assays as the main focus. After the 1st screening it was obvious that the number of active organic fractions exceeded by far the number of active aqueous fractions. Few fractions from the aqueous extracts showed bioactivity against the cell line A2058 in the 1st screening, and no fractions showed bioactivity against the different strains of bacteria. Many organic fractions showed bioactivity in the 1st screening against the A2058 cell line, and a couple of fractions showed good activity against *Streptococcus* group B in the growth inhibition assay. Fraction 27 from the organic extract of *Halecium beanii* (M11047) also showed weak activity against *E. faecalis* and *S. aureus,* but in the 2nd screening the result for *E. faecalis* was not reproducible. Because of the limited timeframe and since cancer was the main focus, the 2nd screening of *S. aureus* was not performed. The reason for the 2nd screening, after the 1st screening, was to confirm the bioactivity found in the 1st screening and check for a dose-response relationship. In the 2nd screening of the aqueous extracts against cell line A2058, fraction 3 and 4 from *Halecium muricatum* (M11046) and fraction 3 from *Halecium* beanii (M11047) showed activity. This was expected, since fraction 3 from the aqueous extracts often shows activity. The reason for this may be that sea salt, polysulfated polysaccharides, peptides and proteins elute in high concentrations in this fraction, leading to an extremely unsuitable environment for cells to survive. In the 2nd screening of the organic extracts many fractions showed activity, even in the most diluted form. All fractions from *Halecium muricatum* (M11046) except fraction 17 showed activity in the undiluted form, indicating that the results were reproducible. Fractions 19, 20 and 26 showed good activity in the undiluted and diluted forms. All fractions from *Halecium beanii* (M11047) in the 2nd screening showed activity in the undiluted form, seven fractions showed activity when diluted 1:2. Fraction 22 and 29 showed activity over the grades of dilution. *Halecium muricatum* (M11046) had a larger number of active fractions compared with *Halecium beanii* (M11047), but nearly the same fractions showed activity in the anticancer assay and in the growth inhibition assay. This indicated that it might be the same compounds responsible for the observed bioactivity, and the results from the fractionation support this because many of the isolated compounds from *Halecium beanii* (M11047) were found in *Halecium muricatum* (M11046) as well. *Halecium muricatum* and *Halecium beanii* are closely related species so this was not surprising.

The organic extract from *Halecium beanii* (M11047) was chosen for dereplication because of the amount of sample available for further testing and the observed bioactivity. The amount of sample available was the double of that available for *Halecium muricatum* (M11046) (see

section 6.1.1). Both organic extracts from the two species showed many fractions with strong bioactivity in the anticancer screening. Thus, according to a bioactivity point of view, both *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) were very similar. However, the number of active fractions after the 2nd screening obviously was in favor of *Halecium muricatum* (M11046), but that does not make up for the amount of sample available, which clearly was in favor of *Halecium beanii* (M11047).

In bioprospecting the amount of sample available is a common problem, and is the reason for cultivating marine bacteria which gives a reliable resupply of samples. It is more difficult to cultivate marine invertebrates, and are therefore dependent on that an adequate amount of samples are collected to perform all tests. However, it was likely that the same compounds are responsible for the bioactivity observed, so the obvious choice was to choose *Halecium beanii* (M11047) for dereplication.

After the isolation of the eight compounds, compound **1** and **2** were purest. However, more of compound **1** and **2** were required for confirmation of bioactivity and structure elucidation. An optimized isolation gradient was used to isolate the two compounds from the organic extract from *Halecium beanii* (M11047). This optimized gradient was found by trial and error, and ensured a satisfactory separation of the eight compounds.

The accurate masses of compound **1** and **2** were used with the isotope patterns to calculate molecular formulas for these compounds. These molecular formulas were used to search the Dictionary of Marine Natural Products for known compounds. At this point it was believed that the two compounds were two different phosphocholines, but further structure elucidation was needed to confirm this. NMR was utilized first to check the relationship between the hydrogen and carbon atoms, but from the NMR data it was evident that the samples were less pure than expected. From the different integrals from ^{1}H- NMR and ^{13}C- NMR it was obvious that in these samples there was a mixture of other compounds, perhaps different phosphocholines, where some had two tails and others one tail. This made it impossible to rely on the NMR data alone. The optimized gradient from the isolation process could have been further improved, and the result would have been more pure compounds. However, optimizing a gradient is time consuming, and the extended manipulation of samples could give a lower yield.

There were several potential sources of error using a single proton as a reference peak. If a single- proton used as a reference measured a three proton resonance, it gives three times the

error on the 3- peak, and six times the error on the 6- peak. Another source of error is overlapping peaks. If there is something hidden under the peak that will add up with the peak of interest, the peak of interest will look bigger than it actually is. The third source of error is the different relaxation properties of different protons which can affect their signal as well. This did not concern ^{1}H- NMR in the same degree as ^{13}C-NMR, which was not quantitative because of the way it was acquired.

From the MS- MS spectra of compound **1** and **2** it was obvious from the fragments from the phosphocholine head- group with *m/z* 86, 104 and 184 that these two compounds were phosphocholines. These fragments are typical fragments when analyzing MS- MS data from phosphocholines. However, the aliphatic part of the molecules was impossible to determine from the MS-MS spectra. It was fairly certain that compound **1** and **2** were present in the two samples, but it could be a mixture of different phosphocholines in the samples as well. From these results it could have been advantageous to have isolated the two compounds further and worked more with the isolation gradient. However, further isolation could have led to a lower yield and a shortage of sample for further bioactivity assays and analysis. If the structure elucidation was to be taken further GC- MS with electron ionization would be the next step. With GC- MS with electron ionization the bonds between the carbons in the aliphatic part of the molecule would break, and the fragments would appear in the spectrum. Possible side chains in the aliphatic moiety would be detected, and the molecule could be identified.

When the isolated compounds were tested against the different cell lines, compound **1** showed weak activity against the MRC5 cell line, and no activity against the DU145, MCF7 and A2058 cell lines. The organic fractions from *Halecium beanii* (M11047) showed activity against the A2058 cell line in the 1st and 2nd screening, so the explanation of the results may be that compound **1** was not responsible of the observed bioactivity or synergy between multiple compounds in the organic extract.

Compound **2** showed weak activity against the MCF7 cell line and good activity against the cell lines MRC5 and A2058 in higher concentrations. However, it was not desirable that compounds show activity against the MRC5 cell line, since this cell line consisted of normal lung fibroblasts, and was a toxicity control. The observed strong activity against this cell line was an indication of general cytotoxicity which obviously was undesirable, since this effect would lead to serious side- effects if used clinically.

Literature about phosphocholines, especially alkylphosphocholines and their synthetic analogs are previously published. In these works a wide spectrum of activity has been connected with these compounds and especially hexadecylphoshocholine, namely antineoplastic, antibacterial, antimycotic, antiprotozoal and antiviral activities [39]. There is also a registered drug, miltefosine (see figure 42), a synthetic analog of hexadecylphosphocholine, which is used clinically against visceral and cutaneous leishmaniasis and in clinical studies miltefosine showed promising results against cutaneous metastases of mammary carcinomas when administered topically [40]. One work proposed that alkylphosphocholines' and other synthetic anti- tumor lipids (ATLs) main site of action is at the plasma membrane and interferes with cellular phospholipid metabolism. It was also proposed that ATLs alter the fluidity of cell membranes and lead to membrane pore formation which permeabilised cells [41]. Newer work indicates that miltefosine lead to an increased cholesterol uptake, synthesis and accumulation of cholesterol inside the cell. This is achieved by impairing the transport of cholesterol into the endoplasmic reticulum (ER), but not the transport of cholesterol from the ER. It is also indicated that phosphatidylcholine and sphingomyelin synthesis was reduced by miltefosine, and these effects together affected the membrane stability and function, which can inhibit tumor cell growth, and can induce apoptosis [40]. However the main mechanism of action remains unclear, but it is a possibility that there are multiple events acting in concert to inhibit tumor cell growth. It was also proposed that the different effects are dose-dependent [41]. The work that indicated that alkylphosphocholines have their main site of action at the plasma membrane fits with the observed activity of compound **2**. Compound **2** showed no cancer cell specificity, which indicated a general cytotoxicity, probably by affecting the cell membrane leading to cell death. The observation that some fractions from the organic extract of *Halecium beanii* (M11047) also exerted antibacterial activity indicated that there was a general cytotoxic effect which did not differentiate between eukaryotic and prokaryotic cells.
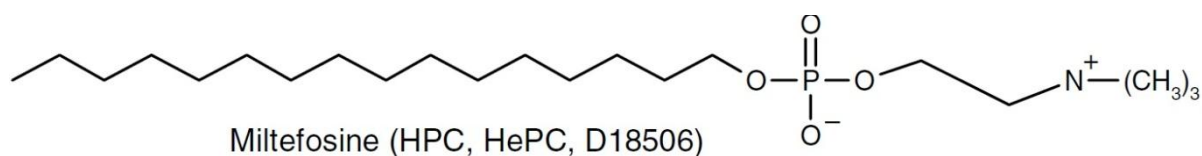

Figure 42: Structure of Miltefosine [42],

The accurate masses of the eight compounds found after the dereplication fits for different phosphocholines, but without NMR and MS- MS data their exact structures were uncertain. These compounds could have been tested for bioactivity, to see if they had a more specific activity than compounds **1** and **2**. Before this bioactivity testing, they could have been analyzed by MS- MS to see if they had the recognizable fragments with *m/z* 86, 104 and 184 which indicates phosphocholines. If they had these fragments, further bioactivity testing would give additional information about possible selectivity differences. Synthetic phosphocholines like the alkyl phosphocholines show a broad spectrum of bioactivity and the phosphocholine head group (main group) could be used as a scaffold for combinatorial chemistry. This could make their effect more selective, and structure activity relationships (SAR) could be used as well to optimize the selectivity and bioactivity against different bioactivity assays. However there is one drawback. One alkyl phosphocholine is a registered drug, miltefosine as mentioned above. The pharmaceutical company who own the rights have most assuredly taken out patents for a vast array of similar compounds, which limits the probability of making new discoveries. Before a potential synthesis of a synthetic phosphocholine, information about which compounds that is protected by patents are necessary, to limit wasting of resources.

The probability of finding previously known compounds are increasing since bioprospecting has been done for quite some time. Therefore new methods for detecting known compounds earlier in the bioprospecting process are advantageous to minimize the use of resources.

8. Conclusion and further perspectives

The organic extracts from *Halecium muricatum* (M11046) and *Halecium beanii* (M11047) showed activity in the 1st and 2nd screening against the A2058 cell line and *Streptococcus* group B. Several compounds were isolated from the active fractions, and based on purity two compounds (compound **1** and **2** from *Halecium beanii*) were selected for further activity testing and structure elucidation. Compound **1** showed weak activity against MRC5 at the highest concentration, whereas compound 2 showed a weak effect against MCF7 and good effect against MRC5 and A2058 at 100 µg/ mL. Activity against MRC5 which was normal lung fibroblasts was undesirable since this cell line was used as a toxicity control. The aqueous extracts did not show any activity against the different strains of bacteria, but some fractions showed good activity against the A2058 cell line. The structure elucidation led to the confirmation that compound **1** and **2** were phosphocholines, however the exact structures were not confirmed.

Phosphocholines are interesting compounds responsible for a wide range of bioactivities, for example versus bacteria, cancer and viruses. It is thought that phosphocholines exert their activity on the cell membrane, or that cell death is a result of apoptosis. In future studies the other six of the eight compounds from the dereplication could be tested for their bioactivity against cancer to check for specificity against cancer cell lines. Further structure elucidation utilizing GC- MS with electron ionization for determination of possible side chains in compound **2** could be done as well, followed by synthesis of synthetic phosphocholines. Different side chains and length of the side chains of the synthetic phosphocholines could be tested for their bioactivity, to detect a potential selectivity for cancer cell lines. However patented phosphocholines needs to be checked, to know which compounds are patented. Optimization of side chains may lead to selectivity against cancer cell lines and higher potency, while the toxicity is minimized.

The results of this work can change the methods used by MabCent for bioactivity testing. MS-MS data of the organic extract would give the previous mentioned recognizable fragments from phosphocholines at an earlier stage, before bioactivity assays. If there is a patent on a phosphocholine against the target used in the assay, there is no point in doing the assay, because a potential hit would not lead to a new discovery. This work has therefore contributed to shorten the time before detecting the presence of phosphocholines in the sample. To the best of our knowledge, there is no work about the bioactivity of hydroids published. Therefore

this work may be the first to describe the bioactivity of the hydroids *Halecium muricatum* and *Halecium beanii* against tumor cells and different bacterial strains.

9. References

1.   Cragg, G.M., P.G. Grothaus, and D.J. Newman, *Impact of natural products on developing new anti-cancer agents.* Chem Rev, 2009. **109**(7): p. 3012-43.
2.   Harvey, A., *Strategies for discovering drugs from previously unexplored natural products.* Drug Discov Today, 2000. **5**(7): p. 294-300.
3.   Newman, D.J., G.M. Cragg, and K.M. Snader, *The influence of natural products upon drug discovery.* Nat Prod Rep, 2000. **17**(3): p. 215-34.
4.   Clark, A., *Natural Products*, in *Foye's Principles of Medicinal Chemistry*, T.L.L. David A.Williams, Editor 2002, Lippincott Williams & Wilkins. p. 24-36.
5.   David J. Newman, G.M.C., Christopher N. Battershill, *Therapeutic agents from the sea: biodiversity, chemo- evolutionary insight and advances to the end of Darwin's 200th year.* Diving and Hyperbaric Medicine, 2009. **39**(4): p. 216-225.
6.   Newman, D.J. and G.M. Cragg, *Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010.* Journal of Natural Products, 2011. **75**: p. 311-335.
7.   Rocha, J., et al., *Cnidarians as a Source of New Marine Bioactive Compounds—An Overview of the Last Decade and Future Steps for Bioprospecting.* Marine Drugs, 2011. **9**: p. 1860-1886.
8.   Gerwick, W.H. and B.S. Moore, *Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology.* Chemistry & Biology, 2012. **19**: p. 85-98.
9.   Robert L. Dorit, W.F.W.J., Robert D. Barnes, *Cnidarians*, in *Zoology*, J.L. Alexander, Editor 1991, Saunders College Publishing. p. 595-619.
10.   NHI. 2011 [cited 2012 08.03]; Available from: http://www.kreftforeningen.no/om_kreft/hva_er_kreft.
11.   Weinberg, D.H.a.R.A., *The Hallmarks of Cancer.* Cell, 2000. **100**: p. 57-70.
12.   Ahmedin Jemal, F.B., Melissa M. Center, Jacques Ferlay, et al, *Global Cancer Statistics.* CA: A Cancer Journal for Clinicians, 2011. **61**(2): p. 69-89.
13.   NHI. 2010 [cited 2012 08.03]; Available from: http://nhi.no/sykdommer/kreft/brystkreft/brystkreft-noen-fakta-16139.html.
14.   NHI. 2012 [cited 2012 08.03]; Available from: http://nhi.no/sykdommer/kreft/mannlige-kjonnsorganer-kreft/prostatakreft-2363.html?page=2
15.   Dolven-Jacobsen, K. *Føflekkreft*. 2012 [cited 2012 04.05]; Available from: http://oncolex.no/en/Foflekk.aspx.
16.   C. Héry, J.F., M. Boniol and P. Autier, *Quantification of changes in breast cancer incidence and mortality since 1990 in 35 countries with Caucasian-majority populations.* Annals of Oncology, 2008. **19**(6): p. 1187-1193.
17.   Whitlock, K.B., *Supportive Care Therapies for Patients with Cancer*, in *Textbook of Therapeutics, Drug and Disease Management*, D.j.Q. R.A. Helms, E.T. Herfindal, D.R. Gourley, Editor 2006, Lippincott Williams & Wilkins: USA. p. 2267-2296.
18.   Phillip J Taddei, R.M.H., Sunil Krishnan, Sarah B Scarboro, Dragan Mirkovic and Wayne D Newhauser, *Risk of second malignant neoplasm following proton versus intensity-modulated photon radiotherapies for hepatocellular carcinoma.* Physics in Medicine and Biology, 2010. **55**: p. 7055-7065.
19.   K.M. Augustin, J.S.G., *Lung Cancer*, in *Textbook of Therapeutics, Drug and Disease Management*, D.j.Q. R.A. Helms, E.T. Herfindal, D.R. Gourley, Editor 2006, Lippincott Williams & Wilkins: USA. p. 2439-2462.

20.   Bailly, C., *Ready for a comeback of natural products in oncology.* Biochemical Pharmacology, 2009. **77**: p. 1447-1457.

21.   Dewick, P.M., *The Mevalonate and Deoxyxylulose Phosphate Pathways: Terpenoids and Steroids*, in *Medicinal Natural Products*2001, John Wiley & Sons: England. p. 167-289.

22.   Nelson Kardos, A.L.D., *Penicillin: the medicine with the greatest impact on therapeutic outcomes.* Appl Microbiol Biotechnol, 2011. **92**: p. 677-687.

23.   Bush, K., *The coming of age of antibiotics: discovery and therapeutic value.* Annals of the New York Academy of Sciences, 2010: p. 1-4.

24.   Brad Spellberg, J.H.P., Eric P. Brass, Loren G. Miller, John E. Edwards Jr, *Trends in Antimicrobial Drug Development: Implications for the Future.* Clinical Infectious Disease, 2004. **38**(9): p. 1279-1286.

25.   Michael A. Fischbach, C.T.W., *Antibiotics for Emerging Pathogens.* Science, 2009. **325**: p. 1089-1093.

26.   Mark S. Butler, A.D.B., *Natural products — The future scaffolds for novel antibiotics?* Biochemical Pharmacology, 2006. **71**(7): p. 919-929.

27.   Michael N. Gwynn, A.P., Stephen F. Rittenhouse, David J. Payne, *Challenges of antibacterial discovery revisited.* Annals of the New York Academy of Sciences, 2010: p. 5-19.

28.   SFI, M., *A Centre for Research-based Innovation (CRI) on marine bioactives and drug discovery*, 2007, MabCent: Tromsø. p. 16.

29.   MabCent. 2011 [cited 2011 14.12]; Available from: www.mabcent.no.

30.   Imre Molnár, C.H., *Reverse-Phase Chromatography of Polar Biological Substances: Separation of Catechol Compounds by High-Performance Liquid Chromatography.* Clinical Chemistry, 1976. **22**(9): p. 1497-1502.

31.   P. Crews, J.R., M. Jaspars, *Optical and Chiroptical Techniques: Ultraviolet Spectroscopy*, in *Organic Structure Analysis*1998, Oxford University Press: USA. p. 349-374.

32.   S. Pedersen-Bjergaard, K.E.R., *UV- spektroskopi*, in *Legemiddelanalyse*2004, Fagbokforlaget: Norway. p. 93-108.

33.   Johnson, A.W., *Determination of Structure: Spectroscopy*, in *Invitation to Organic Chemistry*, A.W. Johnson, Editor 1999, Jones and Bartlett Publishers: USA. p. 351-400.

34.   P. Crews, J.R., M. Jaspars, *Introduction to Nuclear Magnetic Resonance*, in *Organic Structure Analysis*1998, Oxford University Press: USA. p. 23-52.

35.   D. H. Russel, R.D.E., *High-resolution Mass Spectrometry and Accurate Mass Measurements with Emphasis on the Characterization of Peptides and Proteins by Matrix-assisted Laser Desorption/Ionization Time-of-Ñight Mass Spectrometry.* Journal of Mass Spectrometry, 1997. **32**: p. 263-276.

36.   P. Crews, J.R., M. Jaspars, *Additional Techniques of Mass Spectrometry*, in *Organic Structure Analysis*1998, Oxford University Press: USA. p. 281-315.

37.   Fengguo Xu, L.Z., Quingsong Lin and Choon Nam Ong, *Use of Liquid Chromatography/ tandem mass spectrometry and online databases for identification of phosphocholines and lysophosphatidylcholines in human red blood cells.* Rapid Communications In Mass Spectrometry, 2009. **23**: p. 3243-3254.

38.   McCloud, T.G., *High Throughput Extraction of Plant, Marine and Fungal Specimens for Preservation of Biologically Active Molecules.* Molecules, 2010. **15**: p. 4526-4563.

39.   Milos Lukac, M.G., Martin Mrva, Marian Bukovsky, Frantisek Ondriska,Eszter Mariassy, Ferdinand Devinsky, Ivan Lacko, *Relationship between aggregation*

properties and antimicrobial activities of alkylphosphocholines with branched alkyl chains. International Journal of Pharmaceutics, 2011. **423**: p. 247-256.

40. J.M. Jiménez-López, P.R.-M., C. Marco, J.L. Segovia, M.P. Carrasco, *Alterations in the homeostasis of phospholipids and cholesterol by antitumor alkylphospholipids.* Lipids in Health and Disease, 2010. **9**(33): p. 10.

41. Lohmeyer, M. and P. Workman, *Growth arrest vs direct cytotoxicity and the importance of molecular structure for the in vitro anti-tumor activity of ether lipids.* British Journal of Cancer, 1995. **72**: p. 277-286.

42. Mollinedo, F., *Antitumour ether lipids: proapoptotic agents with multiple therapeutic indications.* Expert Opinion Therapeutic Patents, 2007. **17**(4): p. 385-405.