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Metabolic profiling of marine sponges as a tool to discover novel natural products

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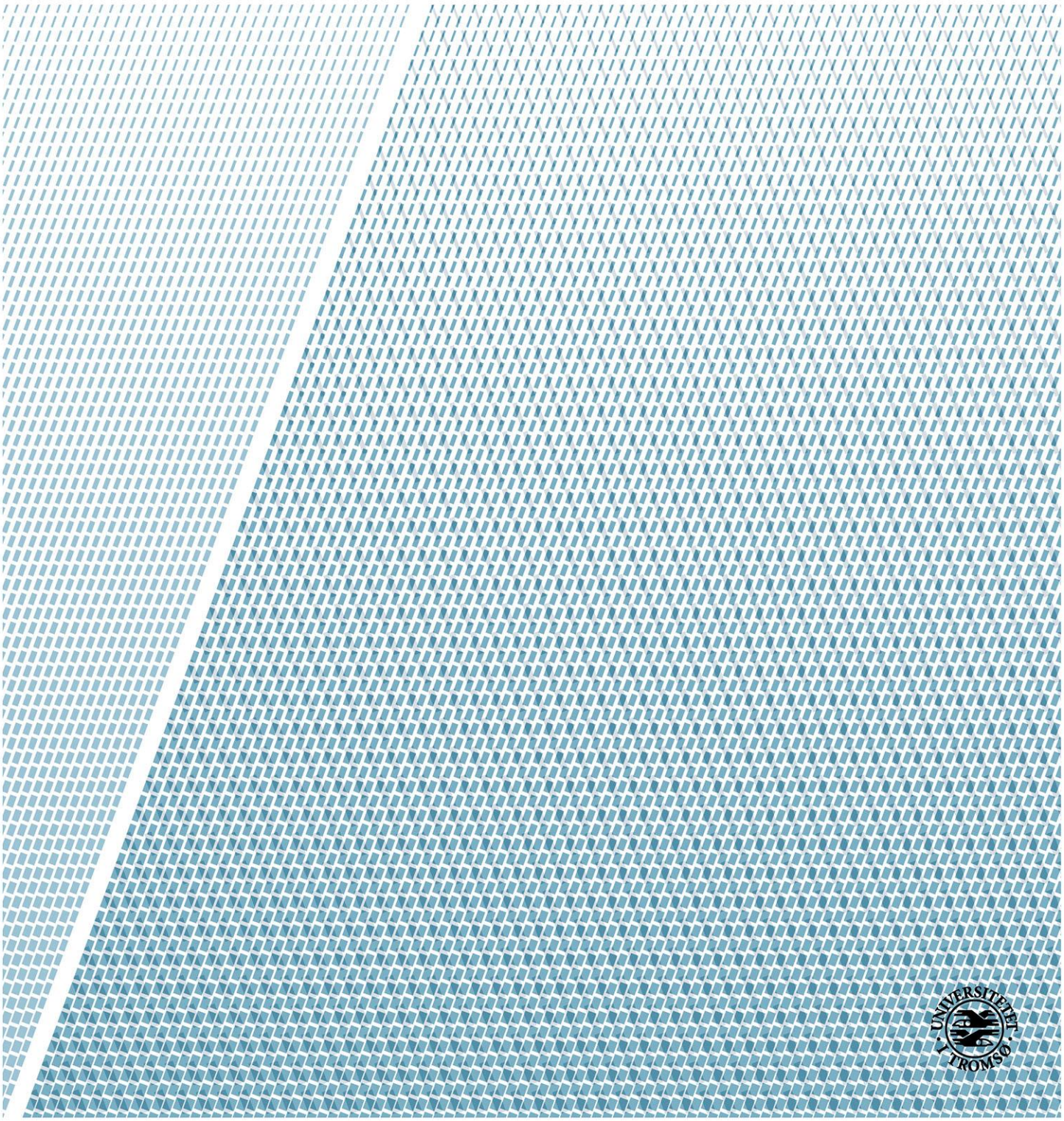

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Contents

Abstract

In this project metabolic profiling was explored as a means for finding unique and novel natural products. Natural products have had a vital role in drug discovery throughout history and in recent times, that includes the world oceans more and more. Therefore two related species of marine sponges, where the samples were collected from different locations, was chosen to test the methodology. This project was performed at MabCent, a research center which focuses on bioactive compounds from Arctic and sub-Arctic organisms. A high-resolution mass spectrometer (HR-MS) was used to provide data for the analysis and a processing software program was used for interpretation of the data. The sponge`s metabolic profiles clearly grouped according to species. A known bioactive compound, barettin, was present in all the samples although in different amounts. One of the species contained a unique compound that was not present in the samples from the other species. The compound now labeled MBC-169 had an m/z: 400 and an elemental composition of $C_{21}H_{37}NO_4S$, which was determined by the MS software. MBC-169 was isolated with the help of preparative high performance liquid chromatography (HPLC) before the structure was determined with the combination of HR-MS and nuclear magnetic resonance (NMR) data. After the structure elucidation MBC-169 was tested for antioxidant activity in two different assays as well as antibiotic activity, and for kinase inhibiting properties. In addition this thesis provides background information of challenges when natural products are used as a source for discovering novel bioactive compounds and how those challenges were overcome in this particular project.

List of abbreviation

AAPH	2,2- Azobis (2- methylpropionamidine dihydrochloride)
ACN	Acetonitrile
API	Atmospheric Pressure Ionization
CAA	Cellular Antioxidant Activity
CLPAA	Cellular Lipid Peroxidation Antioxidant Activity
COSY	Correlation Spectroscopy
DCFH-DA	2,7 dichlorofluorescin diacetat
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EMRT	Exact Mass Retention Time
EtOH	Ethanol
ESI	Electrospray Ionization
FBS	Fetal Bovine Serum
GC	Gas Chromatography
GM	Growth Medium
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Performance Liquid Chromatography
HR	High Resolution
HSQC	Heteronuclear Single-Quantum Correlation
HTS	High Throughput Screening
LC	Liquid Chromatography
MeOH	Methanol
MIC	Minimum Inhibition Concentration
MS	Mass Spectrometer
MVDA	Multivariete Data Analysis
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PBS	Phosphate Buffered Saline Solution
TM	Treatment Medium
TOF	Time of Flight
UPLC	Ultra Performance Liquid Chromatography

Introduction

Natural Products Drug Discovery

Throughout history natural products and their derivatives has undeniably been a leading source of therapeutic agents. One of the earliest documented cases, around 2600 BC in Mesopotamia, describes the use of approximately 1000 plant derived substances. An example from more recent time, is from 2007 when a complex alkaloid isolated from the colonial tunicate *Ecteinascidia turbinate*, was approved under the name Yondelis® for treatment of soft tissue sarcoma [1]. Natural products have several characteristics that make them favourable as lead structures for drug discovery, like remarkable chemical diversity and biochemical specificity [2]. These characteristics are already being utilized in approved synthetic drugs. When comparing synthetic compounds from the database WOMBAT to natural products, it is confirmed that natural products show the same bioactivity as the synthetic compound. The fact that the natural products have more complex chemical properties could prove an advantage when it comes to discovering novel leads with unique properties [3]. Their large, complex structures can provide new scaffolds with very specific target interactions. On the downside there are challenges in screening mixtures filled with several different types of these structurally complex compounds. Such challenges are considered one of the reasons for the decline in the field of natural products drug discovery. Pharmaceutical research into natural products reached its peak in western medicine in 1970-1980, since then it has slowly declined during the past 20 years. However, recent technological advances in separation and structure-determination have lowered these obstacles and combined with advances in similar fields the extraordinary chemical diversity of natural products can again offer exciting novel drug discoveries [4]. This type of discovery is also an ambitious goal with this thesis, but with an untraditional course of action.

Marine bioprospecting

Biodiversity prospecting, or -bioprospecting in short, has been defined as "The process of collecting or surveying a large set of flora (or fauna) for the purpose of the biological evaluation and isolation of lead compounds" [5]. Although the bioprospecting have by far been done mostly on land, the relatively unexplored marine environment has become more relevant in recent times [6]. Marine invertebrates have proven to be a rich source of bioactive compounds, which biotechnical potential now attracts scientific interest all over the world. The prospect of finding novel drug leads and possible profit also awakens interest from the pharmaceutical industry. For instance the anticancer agent salinosporamide A® which is a result of the cooperation of academic researchers and the pharmaceutical industry and demonstrates the oceans potential to yield novel drug leads [7]. The probability of finding previously undiscovered bioactive compounds in the world`s oceans is in its own a reason to support the field of marine bioprospecting. When in addition it has been claimed that marine organisms are more likely to yield anticancer drugs than terrestrial sources, the field is more than promising [8].

Metabolomics

Metabolomics has been defined as "the comprehensive analysis of all metabolites in a system" [9]. It is important to notify that in this particular project metabolomics is not used as previously defined. It is only the metabolites that reside in an organic extract that are subject to metabolic analysis. This is due to the extraction process and compounds it is optimised to extract. The desired compound properties will be elaborated under the extraction section.

To put metabolomics in a simpler term: it provides snapshots of the metabolites (the metabolome) present in an organism, at a given time. Several analytical methods can be put to use for this "snapshot", where the purpose is not to target a specific molecule but often to identify and quantitate multiple targets. Development in ionization and mass detection where differences in molecular weights and polarities are detected makes mass spectrometry (MS) a helpful tool in metabolomics. There are several different techniques within MS that have been found suited for metabolomics, depending on the raw material and the aim of the project. Gas chromatography (GC) coupled to MS have been used for identification and structure elucidation in several projects. Liquid chromatography (LC) has been found useful when investigating non-volatile plant metabolites. An arrangement containing a quadrupole time-of-of-flight (QTOF) - MS was developed for detection of and identification of semipolar secondary metabolites. However, there are some limitations when applying MS to metabolomics, the different sensitivity of the detection of molecules and its reproducibility due to the different detector response. Nuclear magnetic resonance (NMR) spectrometry has many advantages and fulfils the criteria for reproducibility in a metabolomics technology platform. The NMR can therefore be a useful supplement to MS in metabolomics-based projects. Another aspect of metabolomics- based work is the high dimensional data that require multivariate data analysis (MVDA). Like most of the metabolomics data, this project is also based on the classifications of samples into different groups. There are several approaches to classification, in this project principal component analysis (PCA) was used [10].

Metabolomics-based natural products drug discovery

The metabolome is in constant change due to cellular processes and environmental changes and previously the vast possibilities of bioactive compounds in these gene expression end products have not been fully understood or exploited [11]. Partially due to the traditional reductionist approach to drug development and partially caused by challenges connected to dealing with a matrix abundant with different compounds. Some examples of these challenges are the interaction between metabolites in the partially purified mixtures, or the presence of antagonisms or synergisms that might take place. Such a complex matrix requires extensive purification to isolate new compounds, and getting to the root of antagonism or synergism often poses a great intellectual challenge as well as being very time consuming [10]. Another example of the challenges when dealing with natural products is one of the efforts made to reduce the drug discovery timeline and costs, high throughput screening (HTS). HTS enables the screening of more than 100 000 compounds a day [12] but might be incompatible with natural products. The sheer amount of source material needed is often a problem in itself when it comes to natural products. The source material should ideally be found in abundance in an easily accessible area, or even better, thrive in an artificial manmade environment at a low cost. With that in mind it is not hard to appreciate that the collection of several kg of a marine sponge from 200 meters depths in the ocean is anything but ideal. Also the number of fractions needed to screen over a hundred thousand compounds will be hard for any lab to produce. However, several strategies are now being used to overcome some of the challenges dealing with natural products. Applications of new technologies, like connecting metabolomics with multivariate data analysis (MVDA) and a high quality natural products library can at least speed up limiting steps. Generating these comprehensive metabolic profiles provides a massive amount of data to process but also provides better understanding of the link between the changes in profiles and alterations in underlying biochemistry. This in turn will provide knowledge on altered physiology, disease etiology, toxic side effects and better drug targeting [11]. In this project the benefits from metabolomics is combined with bioprospecting in a new approach to search for novel drug leads, where the traditional pathway of bioassay guided fractionation has been diverted from. The main principle with bioassay guided fractionation is to split a sample into fractions and test to see which fraction contains a possible hit and then repeat that process until what is left is a small, concentrated fraction containing the hit, before structure elucidation and further testing can be done. This thesis differs from that route because metabolomics has been used to look for compounds that are unique in a sample and test that compound for bioactivity, and also to search for new and

exciting possible drug leads in a marine organism known to produce bioactive metabolites. Figure 1 illustrates the two different pathways, and the different steps for the route chosen in this project will be further elaborated in the oncoming sections.

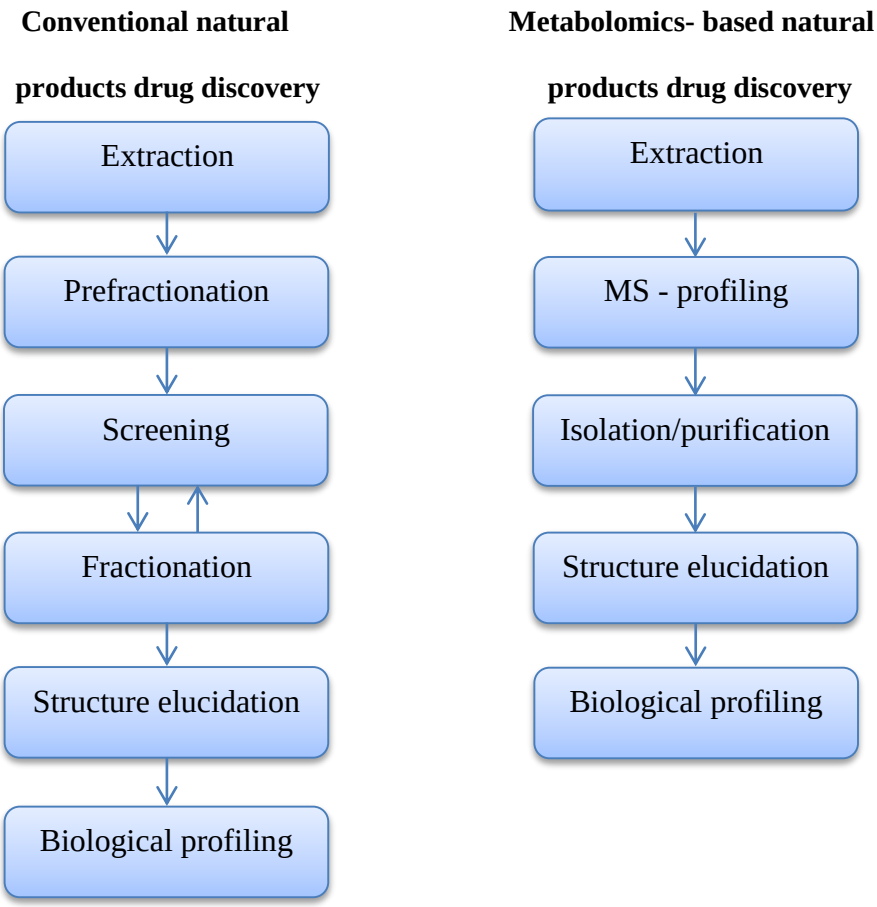

Figure 1: Comparing the conventional natural products drug discovery pathway to the metabolomics-based used in this project.

Extractions

There are several extraction methods available for marine natural products, and which method that is chosen depends on the nature of the raw material and the purpose of the extraction [13]. In general the extraction process is designed to obtain one or more compounds from a solid mass or withdrawal of a solute from a liquid to another liquid or solid mass. If the sample is solid, it is usually cut into small pieces or grounded to fine particles to facilitate solvent penetration and prevent any hydrophilic compounds from being enveloped in membrane pockets or protected by lipophilic substances. Stirring or shaking can increase the diffusion rate.

In this project we wanted to extract as many compounds with the desirable characteristics from the sample as possible. Therefore, water was first used to extract the hydrophilic compounds and thereafter a mixture of dichloromethane and methanol was used to extract the compounds with lipophilic characteristics. Another reason for separating the sample into an aqueous phase and an organic phase is the high content of salt in marine samples. The salts will reside in the aqueous phase with the rest of the hydrophilic compounds. This extraction protocol provides a broad spectrum of compounds available for bioactivity testing without the interference of hydrophilic compounds. In this case the small, lipophilic compounds are the ones of interest, since the goal is finding a novel drug lead [13]. Drugs need to be sufficiently lipophilic to cross membranes in the human body, hence the focus on the lipophilic compounds. However, if a compound is too lipophilic it will precipitate in the blood vessels[5]. This is accounted for later in the project, when preparing the extract for high performance liquid chromatography, where the extract is liquid-liquid extracted with acetonitrile and hexane in a separating funnel. The acetonitrile phase is where the compounds of interest may reside, and the hexane phase is where the most lipophilic compounds reside. Therefore the acetonitrile phase is the one used for further examination.

High-Performance Liquid Chromatography & Ultra-Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a chromatographic method used to separate compounds in a liquid. The HPLC normally consist of an injector, a column, a pump, mobile phases and a detector. It relies on the pump to pass the pressurized mobile phase containing the sample mixture through the column, which is filled with a solid adsorbent material. Different compounds in a sample will interact with the adsorbent material in the column according to their physical properties. The perhaps most commonly used column is the reverse phased column, where the stationary phase in the column is non-polar. The mechanism of retention is mainly van der Waals forces, which causes the small hydrophilic molecules to elute first. Due to the hydrophobic molecules higher affinity to the non-polar stationary phase, they will be held back. The hydrophobic molecules will therefore have the longest retention time. Retention time is the time it takes for a compound to traverse the column and enter the detector, and can give an indication on the characteristics of the compound eluted. The detector measures one or several characteristics of the compound, depending on which types of detector are being used. This is then converted to a signal and displayed as a chromatogram, showing the signal versus time of elution. Most HPLC instruments also have a column oven that allows for adjusting the temperature the separation is performed at. Ultra Performance Liquid Chromatography (UPLC) is a development of HPLC with the same principle; however it involves smaller particles, lesser internal diameters of the column and a higher counter pressure that translates into a higher resolution of the analyses. This allows for en effective separation of the many compounds in a sample in a short time, a property much needed for this project where the organic extract contains a vast number compounds. Various detectors are in common use, such as photodiode array (PDA) an UV/Vis detector, or based on mass spectrometry like the ones used for this thesis.

Ion Source & Mass Spectrometry

Mass spectrometry (MS) is an analytical method where molecules are ionized and the ions are subsequently separated according to their mass-to-charge (*m/z*) ratio and detected in a detector. There are several different ion sources and mass separators. In this project the instruments had electrospray ionization (ESI) coupled with a single quadrupole mass separator for isolation and ESI coupled with time-of-flight (TOF) mass separator for analysis. ESI is an atmospheric pressure ionization (API) technique which means that it takes place under atmospheric pressure making protonated $[M+H]^+$, or deprotonated $[M-H]^-$ molecular ions and in addition form adducts. These formations are non-covalent complexes formed between the compound of interest and any other of the compounds present in the ionized sample. Some adducts are common to find under given circumstances depending on mobile phase, which buffer are present and whether a positive or negative potential is applied. The presence of these adducts can help verify the presence of a suggested compound.

Table 1: Adducts that often occur when using ESI.

Positive ionization	**Negative ionization**
Na^+	Cl^-
K^+	CH_3COO^-
NH_4^+	
CH_3CNH^+	

<u>The Single Quadrupole</u>

The single quadrupole is made up of four parallel rods, in opposing pairs that are electrically connected. A radio frequency voltage is applied to guide the ions down the quadrupole between the rods. For a given ratio of voltage only ions with a certain *m/z* will reach the detector, other ions will have an unstable trajectory and will collide with the rods. This means the quadrupole can be used as both a specific or general detector; by varying the voltage applied it can scan for either ions with a specific *m/z* or a range of *m/z* –values. On the downside the quadrupole instruments has a lower mass unit resolution meaning the separation of ions with very similar *m/z* can be difficult. It is also a not multi-channel instrument, and therefore cannot analyze every incoming ion simultaneously.

Time of flight (TOF) mass spectrometry

The TOF- MS is a high- resolution (HR) mass filter, meaning that ions with very similar masses can be separated. The principle behind the TOF is that a population of ions is accelerated with the same kinetic energy; the ions with the lower mass will have greater velocity when travelling and therefore reach the detector faster than the ions with a greater mass. Each ion with a particular *m/z* ratio will then have a unique time of flight. Even though the exact same energy is applied to the ions, minor differences in initial kinetic energy will occur due to position of the ions etc. That is why a reflector, called the reflectron, is used to compensate for these differences [14]. The reflectron will focus the beam as it redirects the ions towards the detector. When the ions strike the detector, their mass-dependent flight time will be recorded and the instrument can calculate the mass. This technique can be more accurate in terms of separation than the quadrupole because it will provide a higher resolution. It also means that the instrument can detect all ions that are being produced by an ionization event, due to the separation by time-of-flight, and therefore has a so-called multi-channel advantage. For this project separating compounds with similar mass is crucial. The aim is to detect potential unknown natural compounds in a complex mixture of other similar compounds. To achieve this one is depended on an instrument with high resolution to separate these compounds. By using the TOF-MS it is possible to separate the compounds and determine their exact mass, and in turn calculate the elemental composition.

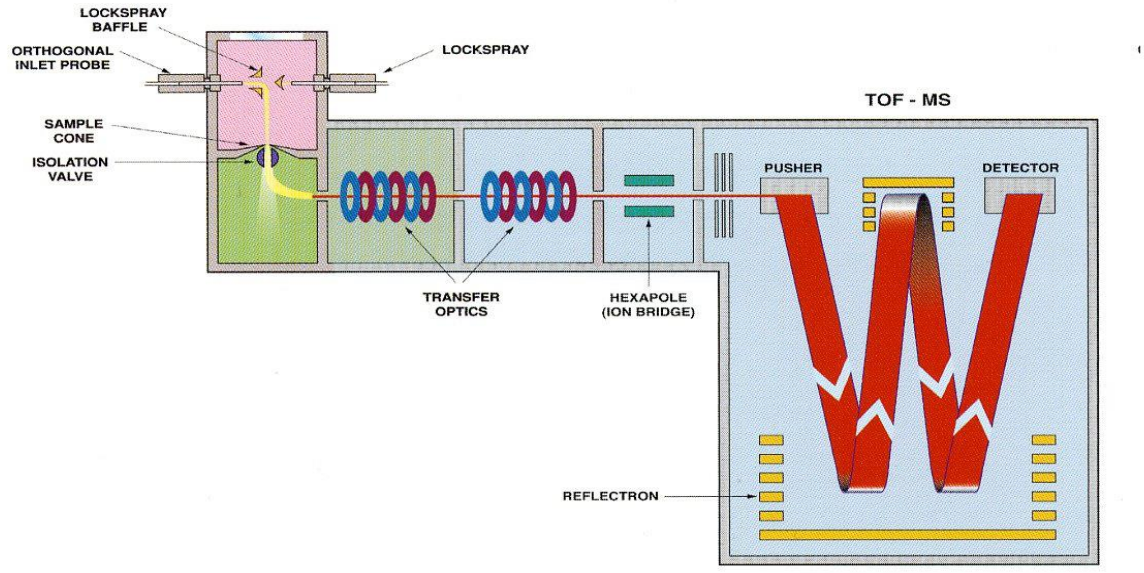

Figure 2: ESI-TOF MS with double reflectors, illustration provided by Waters Corporation.

Data processing (MarkerLynx)

The UPLC-HR-MS analysis supplies a lot of information and it generates a vast amount of data to process, therefore an efficient processing tool is needed. MarkerLynx is a post-acquisition processing package that can be applied to MS and LC/MS data files. Its operation can be divided into three steps where the first is detecting chromatographic peaks. The second step is organizing the results in a table, and the last step is conducting principal components analysis (PCA). This is a multivariate statistical approach that facilitates the identification of differences or similarities between groups. The MarkerLynx identifies peaks and builds a results matrix of exact mass, retention time (EMRT) and intensity pairs. The PCA allows us to identify the EMRTs that are responsible for dissimilarities in the samples.

Nuclear magnetic resonance spectroscopy (NMR)

When determining the molecular structure of a novel compound the use of NMR is crucial. It is a non-destructive analytical method that is based on the magnetic spin in the nucleus of magnetically active elements and NMR provides information about the molecular bond network and about conformation and configuration that cannot be acquired with other methods for structure elucidation. In a NMR spectrometer the sample is placed in a static magnetic field making all the active nuclei in the sample spin with a frequency from 60 to 1000 MHz, depending on the strength of the magnetic field (in this project the instrument used operated at 599.934 MHz for ^{1}H). The sample is then irradiated by radiofrequency pulses. This causes the orientation (the spin) of the protons in the nucleus to change, and thus change their energy levels, which in turn is measured by the ^{1}H-NMR [15]. The spectrum that the NMR provides is absorption of energy as a function of frequency, often referred to as resonance frequency. The radiofrequency pulses perturb the spins of all nuclei and rotate them out of plane. When the pulses are turned off, the spins slowly return to equilibrium by a rotating motion that is recorded by a detector in the instrument and converted to a readable spectrum. For the spectrum to have more than one single peak for all the protons a compound, it is necessary to be able to tell them apart from another. This is possible because every proton in a molecule is shielded by its electron cloud, which density will differ depending on the chemical surroundings. Meaning the degree of shielding of a proton will depend on the inductive effect of other groups attached to the carbon atom. The NMR instrument will exploit this effect and measure the difference in the resonance frequency of a particular proton from the resonance frequency of a reference proton. These measurements are called the chemical shift of the particular nuclei, labeled as δ or with the unit ppm and are independent

of the spectrometers operated frequency [16]. This explains how the NMR can give information about the number of H in a compound and by using the chemical shift values it can give indirect information about other possible elements the carbon is attached to. Therefore it can indicate element composition, especially when the molecular weight has been established in prior experiments. However, to determine the molecular structure it is also necessary to know how the "building blocks" are put together. Different radiofrequency pulsing schemes will produce different kinds of NMR experiments in one or more dimensions. HSQC (Heteronuclear Single-Quantum Correlation) and HMBC (Heteronuclear Multiple-Bond Correlation) are 2D Heteronuclear experiments that are often acquired together to provide information. HSQC demonstrates correlation between two different nuclei separated by one bond, for example, where each unique proton coupled to a carbon has a peak. The HMBC will show correlations between protons and carbons over a longer range approximately 2-4 bonds. Another technique often used is COSY (Correlation Spectroscopy). This is a 2D homonuclear experiment that uses 2-3 bonds ^{1}H-^{1}H correlations ("neighbor-hydrogen") to reveal which protons are located on neighboring carbons in the molecule.

Marine Sponges

In this project, marine sponges were chosen to test if it was possible to use metabolomics to search for unique and novel compounds from the sponges extract. Marine sponges are animals of the phylum Porifera (meaning "pore bearers") and are sessile metazoans (multicellular immobile animals).The sponges comes in many different shapes and sizes, the smallest is only 2 millimeters high, while others can become more than 2 meters in height/diameter. They are the most primitive of the multicellular organisms, with no actual tissue or organs and they lack nervous, digestive and circulatory systems. Instead, the marine sponges rely on water flowing through their pores to obtain oxygen, nutrients and to dispose of waste. With their complex cell types and their symbiosis with a great number of bacteria, they have developed a very efficient defense system against harmful bacteria, fungi and virus [17]. Research show that several bioactive compounds previously thought to be of animal origin may be produced by their symbiotic microorganism instead [5]. It is this complex defense system that has awakened scientific interest and it represents a great possibility for new drug leads [17]. It is also the reason why, in addition to the fact that sponges are the foremost providers of marine bioactive compounds, two related species of the organism has been selected for this project [8].

Geodia barretti

Geodia barretti is a large, globular sponge in the class Demospongiae. Their normal size is 30 cm in diameter, but they can grow up to 50 cm in diameter and weigh up to 24 kg. They are light brown or grey in color and the smooth surface is only broken by a few large pores (osculum). The sponge is found in the north-east Atlantic Ocean, on the north side of Spitsbergen, by the coast of Greenland and on the south coast of northern Spain [17].
As previously mentioned marine sponges have an efficient chemical defense system, which is sorely needed for the immobile organisms to keep their surface free from fouling organisms. One of *G. barretti*`s defenses is the compound barettin, which has previously been proven to have anti-fouling properties [18]. This compound has also showed antioxidant activity as well as anti-inflammatory activity in biochemical assays and cell assays, and the combination of these two properties are of interest as drug leads. For instance as treatment of cardiovascular diseases such atherosclerosis, for which there are no direct targeting treatment in western medicine today (with the exception of cholesterol lowering drugs) [19].

Figure 3: The picture shows the marine sponge *Geodia barretti.* Photo: © Sten-Richard Birkely, Marbank/IMR

Geodia macandrewii

Geodia macandrewii is another marine sponge in the class of Demospongiae that resembles *G. barretti*. These two sponges are equal in size, but *G. macandrewii* has got a more symmetrical shape and the osculus are usually located on a flattened top of the sponge. It thrives in deeper waters (below 150 meter) but can occasionally be found in the shallow waters (depth 20 meter) in the fjords [17]. The close relations to *G. barretti* makes the sponge interesting since its "cousin" has known bioactive metabolites, it could mean *G. macandrewii* also contains or produce metabolites with similar properties.

Figure 4: Marine sponge, *Geodia macandrewii*. Photo: © Erling Svensen

The aim of the thesis

The aim of this project was to use metabolomics to investigate organic extracts from two marine sponges, one with known bioactive metabolites and one closely related to the first one for potential novel and unique compounds. If possible the molecular structure of novel compounds would be determined and tested for antioxidant and antibacterial activity, as well as kinase inhibiting properties.

MabCent-SFI

The project was carried out at MabCent-SFI, which is a centre for research-based innovation on marine bioactives and drug discovery. It was established by the Research Council of Norway in March 2007 and is hosted by the University of Tromsø. Here scientists within MabCent have focused their efforts on the study of marine organisms inhabiting the Arctic waters with the long term goal of novel drug discovery and development. In the Mabcent-SFI program, Marbank collects and stores marine organisms for further taxonomy studies and extract production. Purification, screening and identification of bioactive compounds are performed by the analytical platform Marbio. Here they screen for novel bioactivities against bacteria, viruses and tumours as well as antioxidant, anti-diabetics, immunostimulants and anti-inflammatory activities. Over 260 000 screenings have been performed so far and in cooperation with four commercial partners, Mabcent-SFI has identified and characterized the structure of more than 40 bioactive molecules, out of which 2 have been patented [20].

Materials and methods

Sampling and storage

Table 2: Date, depth and location of the sampling of *G. Barretti* and *G. Macandrewii*

Species	Sampling date	Location	Depth (meter)	Labelling
G. barretti	11.05.13	Saltfjorden, Nordland	127	1
G. barretti	11.05.13	Bliksvær, Nordland	125	2
G. barretti	13.05.13	Lekshammeren, Trondheimsfjorden	193	3
G. barretti	14.05.13	Borgenfjorden, Trondheimsfjorden	246	4
G. macandrewii	11.05.13	Bliksvær, Nordland	127	5
G. macandrewii	09.05.13	Saltfjorden, Nordland	129	6

All the biomass samples were stored at -22ºC in the dark.

Extractions of *G. baretti* and *G. macandrewii*

Materials used

DCM (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)

MeOH (≥99.9 %, Sigma-Aldrich, St. Louis, MO, USA)

MilliQ water (Millipore, Billerica, MA, USA)

Equipment used

A11 Basic grinder (IKA Works, Staufen, Germany)

Centrifuge: Heraus Multifuge 3 S-R (Hanau, Germany)

Freeze-drier: Heto PowerDry PL9000 and Heto PowerDry PL6000 (Thermo Fisher Scientific, Waltman, MA, USA)

Mortar and pestle: MTC Haldenwanger (W.Haldenwanger Technische Keramik, Berlin, Germany)

Rotary evaporator: Heidolph Laborata 4002 (Nürnberg, Germany)

Scale: Mettler Toledo PB3002-S Fact (Greifensee, Switzerland)

Whatman filter paper 125 Ø (no.3) (Springfield Mill, England)

Sample preparation

The sponges were cut into approximately 1 cm^3 pieces while still frozen before transferring the material to Pyrex dishes. The dishes were covered with perforated aluminium foil and freeze-dried for 2 days.

Aqueous extraction

The freeze-dried sponges were pulverized and transferred to tared 1 L Duran bottles and weighed. The material was then transferred to 0.6 L centrifuge bottles and MilliQ water was added, approximately ten times the weight of the dry weight of the material, and shaken until the suspension got a slurry consistency. The extraction was performed at 5ºC to minimize the risk of bacterial decomposition of the compounds and to maintain sample stability. The suspension was centrifuged at 3400 g, at 5ºC for 30 minutes. The water supernatant was transferred to Pyrex dishes and the pellet re-extracted with MilliQ water, approximately half of the amount used in the first round but enough to give the suspension the slurry consistency. The suspension was shaken and put in the refrigerator for a minimum of approximately 40 minutes before it was centrifuged again at 3400 g, at 5ºC for 30 minutes. After the centrifugation the supernatant was first transferred to Pyrex dishes, and then the pellet was transferred to separate Pyrex dishes. All the dishes were covered with perforated aluminium foil and stored at -22ºC until freeze-drying. The water extracts were frozen at -80ºC for 30-45 minutes before freeze-drying for 3 days. The material was grinded with mortar and pestle, transferred to 50 ml centrifuge tubes and stored at -22ºC.

Organic extract

The freeze-dried pellet left over from the aqueous extraction was grinded and transferred to tara 1 L Duran bottles before extraction with the solvent mixture DCM-MeOH (1:1, v/v), approximately ten times the weight of the material, until the suspension got a slurry consistency. The suspension was stored in the refrigerator until the next day when it was vacuum filtrated with Whatman filters. Afterwards the solid material was transferred back into the Duran bottle and re-extracted with the solvent mixture DCM/MeOH (1:1, v/v), approximately half of the amount used in the first round but enough to give the suspension the slurry consistency. The suspension was shaken and stored in the refrigerator for approximately 40 minutes before repeating the filtration process. The filtrate was then concentrated to 10-20 mL using a rotavapor before transferred to a 50 ml round flask and evaporated under reduced pressure in the rotavapor until dry and stored at -22ºC.

Preparation of the samples for MS analysis

Materials used

MeOH (≥99.9 %, Sigma-Aldrich, St. Louis, MO, USA)

Approximately 150 mg of the organic extract from each of the samples was dissolved in sufficient amount of MeOH to achieve 100 mg/ml of sample.

Table 3: Amount of organic extract dissolved in amount of MeOH and the concentration of the solution.

Organic extract (mg)	MeOH (mL)	Sample concentration (mg/mL)
150,1	1,501	100
150,2	1,502	100
150,6	1,506	100
151,2	1,512	100
150,3	1,503	100
151,2	1,512	100

UPLC-HR-MS analysis

Materials used

ACN (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)

Formic Acid (98.0%, Sigma-Aldrich, St. Louis, MO, USA)

MeOH (≥99.9 %, Sigma-Aldrich, St. Louis, MO, USA)

MilliQ water (Millipore, Billerica, MA, USA)

Equipment used

Acquity UPLC ® BEH, 2.1×100 mm, 1.7 µm column (Waters, Milford, MA, USA)

Waters Acquity UPLC

Waters LCT Premier detector

Mobile phases:

A: 0.1 % formic acid in Milli-Q water

B: 0.1 % formic acid in acetonitrile

Table 4: Gradient used for UPLC-HR-MS analysis

Time	**Flow (mL/min)**	**% A**	**% B**
Initial	0.6	90	10
10	0.6	0	100
12	0.6	0	100
13	0.6	90	10

Injection volume: 100 µL

Each sample was injected 6 times.

Instrument parameters

Polarity: ES+

Analyser: W Mode

Capillary (V): 2600.0

Sample Cone (V): 50.0

Desolvation Temp (C): 300.0

Source Temp (C): 120.0

Cone Gas Flow: 5.0

Desolvation Gas Flow: 500.0

MarkerLynx processing parameters

Mass: 150-1500 Da

Marker treshold: 500 counts

Retention time window: 0.2 minutes

Mass window: 0.1 Da

The program was set to de-isotope, meaning it would remove signals from common isotopes such as ^{13}C.

The MarkerLynx analysis detected a potential novel compound that was labelled MBC-169 in sample 5 and 6. Therefore, MBC-169 had to be isolated and sample 5 was the chosen sample.

Preparation of sample 5 for HPLC isolation of MBC-169

Materials used

ACN (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)

Formic Acid (98.0%, Sigma-Aldrich, St. Louis, MO, USA)

Hexane (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)

MilliQ water (Millipore, Billerica, MA, USA)

Rotary evaporator: Heidolph Laborata 4002 (Nürnberg, Germany)

4.0031 g of the organic extract was dissolved in 200 ml hexane, and subsequently liquid-liquid extracted twice with 100 ml 90% ACN. Under the first liquid-liquid extraction there was a slight problem separating the two phases when the ACN phase also showed a cluster of particles due to saturation. This was removed and added 50 ml ACN and shaken until the particles was dissolved and the ACN phases was then combined before the second liquid-liquid extraction. Both phases were collected and concentrated using a rotavapor, the hexane phase until dryness and the ACN phase to approximately 10 ml. The hexane phase was then stored in the freezer and the ACN phase was used in the continued process. 1 ml of sample from the ACN phase was then added 2 ml MeOH and used in HPLC isolation.

1st HPLC isolation of MBC-169

Materials used

ACN (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)

Formic Acid (98.0%, Sigma-Aldrich, St. Louis, MO, USA)

MeOH (≥99.9 %, Sigma-Aldrich, St. Louis, MO, USA)

MilliQ water (Millipore, Billerica, MA, USA)

Equipment used

Atlantis ® Prep C18, 10 µm, 10×250 mm column

Freeze-drier: Heto PowerDry PL9000 (Thermo Fisher Scientific, Waltman, MA, USA)

Rotary evaporator: Heidolph Laborata 4002 (Nürnberg, Germany)

Software: MassLynx 4.1

Waters 600 Controller (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

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

The make-up pump maintains the flow from the splitter necessary to provide a stable signal.

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

Wash solution 1: Acetonitrile

Injection volume: 250 µL

Conditions:
Polarity: ES+
Source Temp (C): 120
Desolvation Temp (C): 300
Desolvation Gas Flow (L/hr): 650

After 7 injections attempting to adjust a linear gradient which could isolate the compound in best way possible without succeeding, an isocratic gradient was chosen consisting of 65 % mobile phase B and 35 % mobile phase A to the continued isolation.
All the collected fractions were transferred to a round-bottomed flask and concentrated in the rotary evaporator before being transferred to a glass tube and freeze-dried for 48 hours.

2nd HPLC isolation of MBC-169

Materials used
ACN (≥99.8 %, Sigma-Aldrich, St. Louis, MO, USA)
Ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA)
Formic Acid (98.0 %, Sigma-Aldrich, St. Louis, MO, USA)
MilliQ water (Millipore, Billerica, MA, USA)

The same set-up from the first isolation was used for the second isolation but with a different method where the mobile phase and the gradient were changed.

Mobile phases:
A: Aqueous phase with buffer
B: 0.1 % formic acid in acetonitrile
Mobil phase A: 1.58 g of ammonium bicarbonate was dissolved in 2 L Milli-Q water and the pH adjusted with formic acid to 7.8.

Make-up pump solution: 80 % aqueous MeOH w/ 0.2 % formic acid
Wash solution 1: 5 % MeOH in Milli-Q water
Wash solution 1: Acetonitrile

Table 5: Gradient used for isolation of MBC-169

Time	Flow (mL/min)	% A	% B
Initial	6	55	45
10	6	45	55
11	6	0	100
15	6	0	100
16	6	55	45

Injection volume: 100 µL

Afterwards all of the tubes containing the isolated compound were transferred to a round-bottomed flask and concentrated in the rotary evaporator before being transferred to a glass tube and freeze-dried for 24 hours.

H^{1}-NMR-analysis

Johan Isaksson at Smallstruct, the Department of Chemistry at the University of Tromsø performed this procedure and interpretation of data.

The NMR data was acquired on a Varian (now Agilent) Inova spectrometer operating at 599.934 MHz for ^{1}H, equipped with a cryogenically enhanced inverse triple resonance probe (HCN).

Data & software: All data was acquired using VnmrJ 3.2A and chempack 6, using standard pulse sequences, where applicable, gradient- and adiabatic pulse- versions with homospoils between scans were utilized. For heteronuclear experiments, BIP versions with gradient selection and sensitivity enhancement were used. All spectra processing and spectra simulations were made in Mnova 8.1.2.

Cellular Antioxidant Activity Assays

Materials and equipment used

2, 7 dichlorofluorescin diacetat (DCFH-DA, Sigma-Aldrich, St.Louis, MO, USA)

AAPH (Cayman)

Bodipy ® 581/591 C11 (Lipid peroxidation Sensor, Invitrogen)

Bürkes Countingchamber

CumOOH (Sigma-Aldrich, St.Louis, MO, USA)

DMSO (99.5 %, Sigma-Aldrich, St.Louis, MO, USA)

Ethanol (EtOH, 96 % Sigma-Aldrich, St.Louis, MO, USA)

Fetal Bovine Serum (Biochrom)

Growth medium (GM): E-mem (Biochrom) with 10 % FBS, 500 mL:

- Gentamycin 500 µL
- NEA 5 mL
- Sodium Pyruvate 5 mL
- L-Alanyl-glutamin 5 mL
- FBS 50 ml

Hank`s saline solution (Biochrom)

Heidolph Incubator 1000 (VWR International)

Luteolin (Cayman)

Milli-Q water (Millipore, Billerica, MA, USA)

Microscope

Microtiter plates; black 96-well plates with optical bottom (Costar, Cornings)

Multichannel pipette

Phosphate buffered saline solution (PBS)

Shaking apparatus: Edmund Bühler GmbH SM 30

Treatment medium without FBS

- Same as growth medium, only without FBS

Trypan blue (VWR International)

Trypsin (Gibco, NY, USA)

Victor 3 Multilabel Counter (Perkin Elmer, Waltham MA, USA)

Cell splitting and seeding

HepG2- cells are humane hepatocellular cells from a 15 year old boys liver tissue. The cells are epithelial, chromosome no.55.

Phosphate buffered saline solution (PBS) and treatment medium (TM) were preheated to 37ºC in the incubator, and trypsin to room temperature. The cells were inspected in a microscope to ensure a healthy cell population before the cell medium was decanted and 10 mL PBS was added to the cell layer. The container with cells and PBS was gently tilted from side to side to make sure all the cells were washed before the PBS was pipetted out. 4 mL trypsin was added, the container tilted from side to side to cover the cells with trypsin and then the surplus of trypsin was poured out carefully to avoid loss of cells. The container was incubated at 37ºC for 5 minutes and inspected in the microscope to ensure that the cells had unlatched from the surface of the container. Then 20 mL growth medium (GM) were added and the suspension was resuspended several times to avoid the cells to clump together. In order for the cells to be counted 200 µL of the cell suspension was added to 200 µL of Trypan blue and mixed together. 10 µL was put in a Bürkes counting chamber and counted under the microscope. From the grid, the cell numbers in 5 squares were counted and the average from those 5 was used to calculate cells/mL:

$$\textbf{2 (adjusting for trypan blues contribution) x no. cells counted x } 10^4$$

The formula used to calculate cell concentration:

$$\mathbf{C_1 \times V_1 = C_2 \times V_2}$$

C_1 = concentration of cells in the suspension till this point
V_1 = the unknown volume
C_2 = desired concentration of cells (CAA = 80 000 cell/well, CLPAA = 90 000 cell/well)
V_2 = desired volume of cell suspension

The suspension was then again suspended several times before transferred to the plates, 100 µL to each well. The plates were then incubated over 24 hours in 37ºC and 5 % CO_2.

Cellular Antioxidant Activity (CAA) Assay

1st screening:

PBS and TM were heated up (10 mL for each plate). The cells were inspected in a microscope. A healthy cell population will form peninsula shapes and cover most of the bottom of the wells with little space between each cell. If the cells are circular and far apart, the cells are damaged and cannot be used in this assay. Cell medium from the day before was removed from the cells using a vacuum pipette. The pipetting was done carefully to avoid damage or loss of cells. TM with DCFH-DA was made immediately prior to use. 15.6 µL of DCFH-DA was added to 10 mL TM (for each plate). The wells were then washed with 100 µL PBS. 20 µL of the compound being tested were added to four wells, 80 µL TM was added to the same wells and to the control wells. The rest of the wells not containing the compound being tested for this thesis are being used to test other compounds by Mabcent with slightly different concentrations (5 µL of a given test compound and 95 µL TM). 20 µL of the known antioxidant Luteolin (250 µL/mL) were added to four wells, also 20 µL MQ-H_2O to the (+) and (-) controls. The plates were then incubated for 1 hour at 37ºC and 5 % CO_2. See table # for a schematic set up.

Reading:

The instrument was set to 37ºC. A 600 µM AAPH in Hank`s solution was prepared (30 µL AAPH was added to 10 mL Hank`s for each plate). The cell medium added an hour earlier was then removed with a vacuum pipette and the plate washed with 100 µL PBS. 100 µL of Hank`s was added the negative control wells (-). Then 100 µL of the prepared Hank`s with AAPH was added to all the remaining wells. Fluorescence was measured at 485/520 nm in Victor plate reader with 12 readings every 240th second. The plates were then incubated for 1 hour at 37ºC and 5 % CO_2 and the absorbance was measured again.

Cellular Lipid Peroxidation Antioxidant Activity (CLPAA) Assay

C11-BODIPY were dissolved in DMSO to 6.25 mM and stored in -80ºC prior to the experiment. Immediately prior to use 15.9 µL BODIPY (6.25 Mm) was added 11 mL TM, which resulted in 10 µM concentration on the plate. The CumOOH was also prepared immediately prior to use as following; 10 µL 6M CumOOH was added to 590 µL 70% EtOH. Then diluted to 50 µM by adding 5 µL 0.1 M CumOOH to 9995 µL Hanks.

1st screening:

PBS and TM were heated up (11 mL for each plate). The cells were inspected in a microscope. (A healthy cell population will form peninsula shapes and cover most of the bottom of the wells with little space between each cell. If the cells are circular and far apart, the cells are damaged and cannot be used in this assay). Cell medium from the day before was removed from the cells using a vacuum pipette. The pipetting was done carefully to avoid damage or loss of cells. Then the wells were washed with 100 µL PBS. TM with 10 µM C_{11}-Bodipy was made immediately prior to use. 100 µL TM with Bodipy was added to the control wells and then the plates were incubated 37ºC and 5 % CO_2 for 30 minutes to enable the cells to absorb the fatty compound Bodipy. Afterwards the TM was removed from the wells with a manual pipette. 80 µL of TM without Bodipy was added to all the sample wells, and 80 µL of TM without Bodipy was added to the control wells. 20 µL of the compound being tested were added to four wells. (The rest of the wells not containing the compound being tested for this thesis are being used to test other compounds by Mabcent). 20 µL Luteolin (250 µL/mL) were added to four wells, also 20 µL MQ-H_2O to the (+) and (-) controls. The plates were then incubated for 1 hour at 37ºC and 5 % CO_2. See table # for a schematic set up.

<u>Reading:</u>

The instrument was set to 37ºC. A 50 µM CumOOH in Hank`s solution was prepared.
The cellmedium added earlier was removed with a pipette and the plate was washed with 100 µL PBS. 100 µL of the prepared Hank`s with CumOOH was added to all the wells except the four control wells, where 100 µL of Hank`s without CumOOH was added. Fluorescence was measured at 590/632 nm in a Victor plate reader (CW-lamp at 10 000) for red fluorescence with a reading every 3rd minute for an hour, 21 readings total. Followed by a new measuring at 485/520 nm (CW-lamp at 20 000) for green fluorescence also every 3rd minute for an hour.

Table 6: Assay set up for CAA and CLPAA. The outer wells were not used for the samples due to edge effect. Negative controls are marked with (-), positive controls with (+). The known antioxidant Luteolin were marked (L). Compounds tested for this thesis: (C) and the rest marked (X) were compounds tested by MabCent.

	C	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**-**	**-**	
	C	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**-**	**-**	
	C	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**+**	**+**	
	C	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**+**	**+**	
	X	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**L**	**L**	
	X	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**L**	**L**	

Kinase RR-analysis: PKA and ABL inhibition testing

Materials and equipment used

ABL (ProQinase, Boston, MA, USA)

Centrifuge: Heraeus multifuge 1 S

Dithriotreitol (DTT, Sigma-Aldrich, St.Louis, MO, USA)

DMSO (99.5 %, Sigma-Aldrich, St.Louis, MO, USA)

Glasscontainer

Kinase RR-kit (Biothema)

Milli-Q water (Millipore, Billerica, MA, USA)

Peptidsubstrate (KEMP-TID, ABL-TID)

Pipettes (10 µL, 100 µL, 1000 µL)

PKA (ProQinase, Boston, MA, USA)

Plates: 384- well plates with flat, white bottoms (Perkin Elmer Optiplate -384)

Shaking apparatus: Edmund Bühler GmbH SM 30

Staurosporin (10 µM, Sigma-Aldrich, St.Louis, MO, USA)

EnVision (Perkin Elmer, Waltham MA, USA)

Controls:

- Blank: No peptidsubstrate (KEMP-TID or ABL-TID) = Inactive mix + Milli-Q water
- Control: Active mix + Milli-Q water
- Positive control: Active mix + 1 µM staurosporin

MBC-169 was already dissolved in 100% DMSO to 10mg/mL. It was diluted to 500 µg/mL, 250 µg/mL and 5 µg/mL, making the concentration in the wells 100 µg/mL, 50 µg/mL and 1 µg/mL. 5 µL of each concentration was added to individual wells on the plates (one plate for PKA and one plate for ABL). The master mix was prepared according to the Kinase RR- kit. The content is presented in table 7.

Table 7: PKA- and ABL- master mix

PKA- master mix	**ABL-master mix**
7500 µL Kinase RR-buffer	7500 µL Kinase RR-buffer
1200 µL ATP-reagent-SL	1200 µL ATP-reagent-SL
37.5 µL 0.4 M DTT	37.5 µL 0.4 M DTT
74161 µL Millie-Q water	161 µL Milli-Q water
7.8 µL PKA-enzyme (dilute to 1:10 from stock) →Take out 117.5 µL for blank control Ad 2.5 µL Milli-Q water for this	19 µL ABL-enzyme →Take out 117.5 µL for blank control Ad 2.5 µL Milli-Q water for this
184 µL KEMP-TID (KEMP2)-substrate (2 mM)	92 µL ABL-TID-substrate (4 mM)

15 µL of the master mix was pipetted to each well except for the blank controls. The controls were added as following:

Blank (b): 5 µL Milli-Q water + 15 µL inactive mix were added to well B7, B8 and B9.

Control (c): 5 µL Milli-Q water + 15 µL active mix were added to well D7, D8 and D9.

Positive control (pc): 5 µL staurosporin + 15 µL active mix were added to well F7, F8 and F9.

Table 8: Set-up for PKA and ABL inhibition testing, where (x) is MBC-169 and (b), (c), (pc) are the controls. The shaded wells are used by MabCent to test other compounds.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
A																							
B							**b**	**b**	**b**														
C																							
D							**c**	**c**	**c**														
E																							
F							**pc**	**pc**	**pc**														
G																							
H							**x**	**x**	**x**														
I																							
J							**x**	**x**	**x**														
K																							
L							**x**	**x**	**x**														
M																							
N																							
O																							
P																							

5 µL of ATP was added to each well before the plates were centrifuged for 1 minute at 190 G, then shaken for 2 minutes in the minishaker. The plates were read in the platereader.

Minimum Inhibiting Concentration (MIC) Assay

Materials and equipment used

Bacteria (ATCC):

- *Enterococcus coli*
- *Enterococcus faecalis*
- *Pseudomonas aeruginosa*
- *Streptococcus aureus*
- *Streptococcus agalactiae*

Bloodplate

Brain Heart Infusion (BHI broth, Merck)

Erlenmeyerflask (sterile)

Falcontubes

LB-plates

Microtiterplates

Milli-Q water (sterile, Millipore, Billerica, MA, USA)

Mueller-Hinton (MH broth, Oxoid Ltd)

Multichannelpipette

NaCl, 0.9% (sterile, Sigma-Aldrich, St.Louis, MO, USA)

Loops

Shaking incubator: Heidolph Unimax 1010, Heidolph Inkubator 1000)

Victor 3 Multilabel Counter (Perkin Elmer, Waltham MA, USA)

The sample was diluted until the final concentration on the plate was 50µg/mL.
On the first day bacterial strains were sown from the blood plates to 8 mL growth medium and incubated in suitable atmosphere overnight. The growth medium was chosen by two criteria. The first is that it had to be a good growth environment for the bacteria. The second is that it could not have any influence on the sample being tested.

On the second day 2 mL of the bacterial suspension from day one were transferred to 25 mL fresh medium for exponential growth. The bacterial suspension was incubated in the shaking incubator according to table 9. The differences in incubation time in the table are due to differences in the bacterial strains; some take longer time to go into log-phase and the incubation time was adapted accordingly. Bacterial growth was sufficient when the turbidity reached 0.5 McFarland standards (1.0×10^8 bacteria/ mL).

Table 9: Demonstrating incubation time and growth medium for each of the different bacterial strains and the appropriate bacterial density.

Bacterial strain	Growth medium	Incubation time	Bacterial density
S.aureus	MH- broth	2.5 hours	0.5-3×10^5 CFU/mL (2500-15000 CFU/ well)
E.coli	MH- broth	1.5 hours	0.5-3×10^5 CFU/mL (2500-15000 CFU/ well)
E.faecalis	BHI- broth	1.5 hours	0.5-3×10^5 CFU/mL (2500-15000 CFU/ well)
P.aeruginosa	MH- broth	2.5 hours	3-7×10^4 CFU/mL (2500-15000 CFU/ well)
S.agalactiae	BHI- broth	1.5 hours	0.5-3×10^5 CFU/mL (2500-15000 CFU/ well)

50 µL of sample were added in 4 parallels on the microtiterplates for each strain (the rest of the places were used for testing of other compounds by MabCent). The different bacterial strains were placed on different mikrotiterplates, so that each plate only tested one bacterial strain. The bacterial suspension was diluted, first 1:100 and then 1:10 in the growth medium before adding 50 µL of the diluted suspension to the plates. To uphold viable cell density, the bacterial suspension was added within 30 minutes after the standardization. The plates were incubated for approximately 20 hours at 37ºC.

Table 10: Assay set up for MIC. Positive controls are marked with (P), negative controls with (N). The compound tested in this thesis is marked (C), and the rest are fractions tested by Mabcent marked (X).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**N**	X	X	X	X	X	X	X	X	X	X	**P**
B	**N**	X	X	X	C	X	X	X	X	X	X	**P**
C	**N**	X	X	X	C	X	X	X	X	X	X	**P**
D	**N**	X	X	X	C	X	X	X	X	X	X	**P**
E	**N**	X	X	X	X	X	X	X	X	X	X	**P**
F	**N**	X	X	X	X	X	X	X	X	X	X	**P**
G	**N**	X	X	X	X	X	X	X	X	X	X	**P**
H	**N**	X	X	X	X	X	X	X	X	X	X	**P**

Controls:

Negative controls: 50 µL growth medium + 50 µL sterile Milli-Q water.

Positive controls: 50 µL sterile Milli-Q water + 50 µL bacterial suspension.

Gentamycin was used as control for the set up and for precision between each experiment. To achieve measurable values of the bacteria the solution was diluted to approximately 1×10^2- 1×10^3 bacteria/mL. The dilution was done in 0.9% NaCl as following: 1) 1:100, 2) 1:100, 3) 1:10, 4) 1:10. Then 100 µL were plated out from the final dilution in two parallels on LB-plates and incubated overnight

The plates were read visually, where visual turbidity indicates inactive compound (I). Wells without visual turbidity indicates inhibition of bacteria and active compound (A). When in doubt, the compound is set as questionable (Q). A photometric reading using the Victor platereader was performed, where absorbance units (AU) was used to classify the compound. AU: < 0.05 = A, AU: 0.05-0.09 = Q, AU: > 0.09 = I.

Results

Extractions

The biomass of each sample was weighed before and after freeze-drying and afterwards all the samples show a far lower dry weight. The dry biomass sample was used for the aqueous extraction which yielded from 65% to 80% in the samples. The pellet from the aqueous extraction was freeze dried and used in the organic extraction which yielded from 1.4% to 2.5% in the samples. The exact weight from each step of the extraction for each species is described in table 11.

Table 11: Sample no. for this project, species, sample location, sample weight, dry weight, yield from aqueous and organic extraction.

Sample no.	Species	Location	Sample weight (g)	Sample dry weight (g)	Aqueous extract (g)	Organic extract (g)
1	*G. barretti*	Saltfjorden, Nordland	2935.70	602.94	405.65	10.1078
2	*G. barretti*	Bliksvær, Nordland	2081.46	382.29	251.63	6.6433
3	*G. barretti*	Lekshammeren, Trondheimsfjorden	748.73	164.19	123.36	4.0741
4	*G. barretti*	Borgenfjorden, Trondheimsfjorden	3381.71	599.46	425.55	13.7616
5	*G. macandrewii*	Bliksvær, Nordland	2230.52	370.14	239.15	8.6572
6	*G. macandrewii*	Saltfjorden, Nordland	3490.29	954.52	766.02	14.0362

UPLC-HR-MS analysis of sponge extracts

The six samples were analyzed using UPLC- HR- MS to look for novel compounds for potential drug discovery and the data from this analysis are the base for the metabolic profile that are presented in this thesis. A chromatogram from each sample is included in the appendix, see figures 28-33. In all the chromatograms the cluster of peaks eluted after approximately eight minutes are mostly residue from the plastic containers previously used. The signals before the eight-minute mark are components of interest and the biomarker software MarkerLynx was used to analyze the data, so the manual inspection of the data was kept to a minimum prior to the analysis.

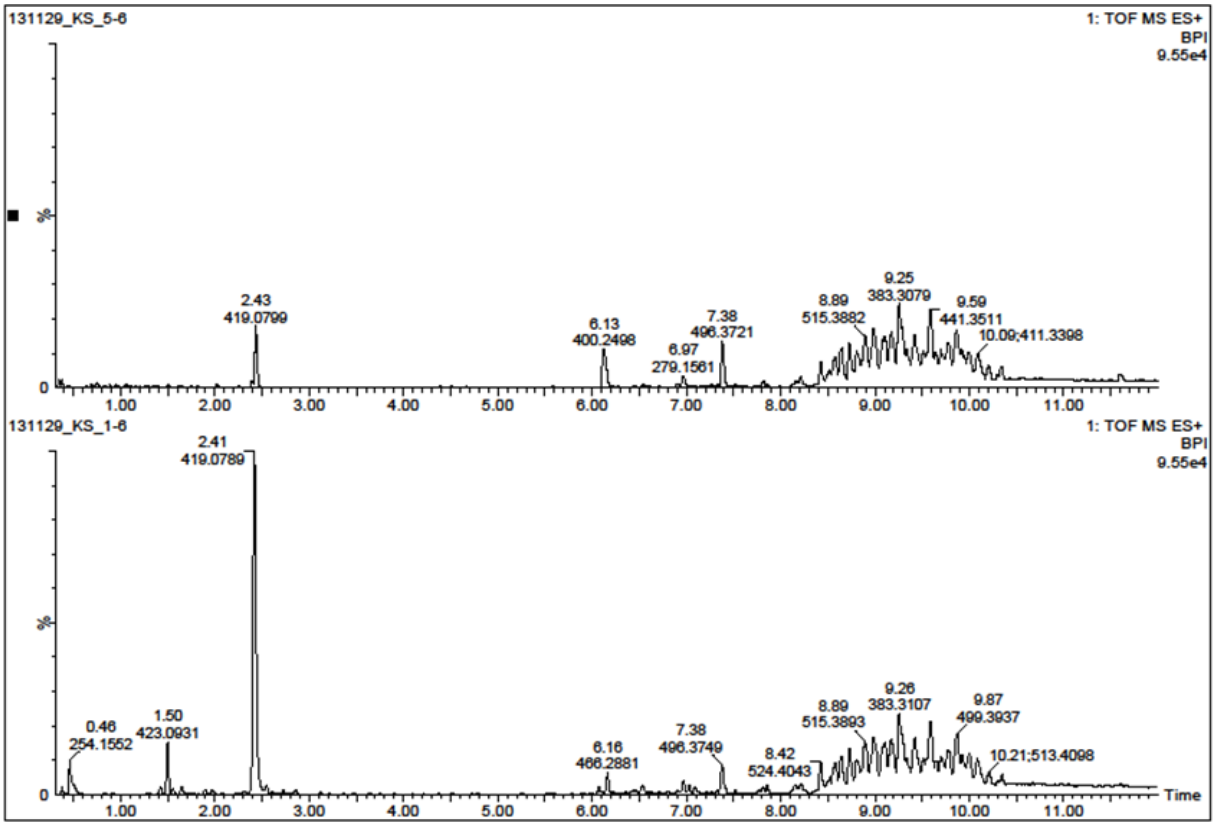

Figure 5: Top chromatogram: *G. macandrewii* collected in Bliksvær, Nordland 11.05.13.
Bottom chromatogram : *G. barretti* collected in Saltfjorden, Nordland 11.05.13. ESI in positive mode.

In figure 5 two HR-MS chromatograms from each species are displayed for comparison. There is a signal with *m/z*: 419 in both chromatograms, however *G. macandrewii* with a less intense signal than *G. barretti*. The signal *m/z*: 400 are only present in the *G. macandrewii* chromatograms. The other signals in these chromatograms, such as *m/z*: 496, 466 and 423, have been analyzed by MarkerLynx and are present in all the samples, although in varied amounts.

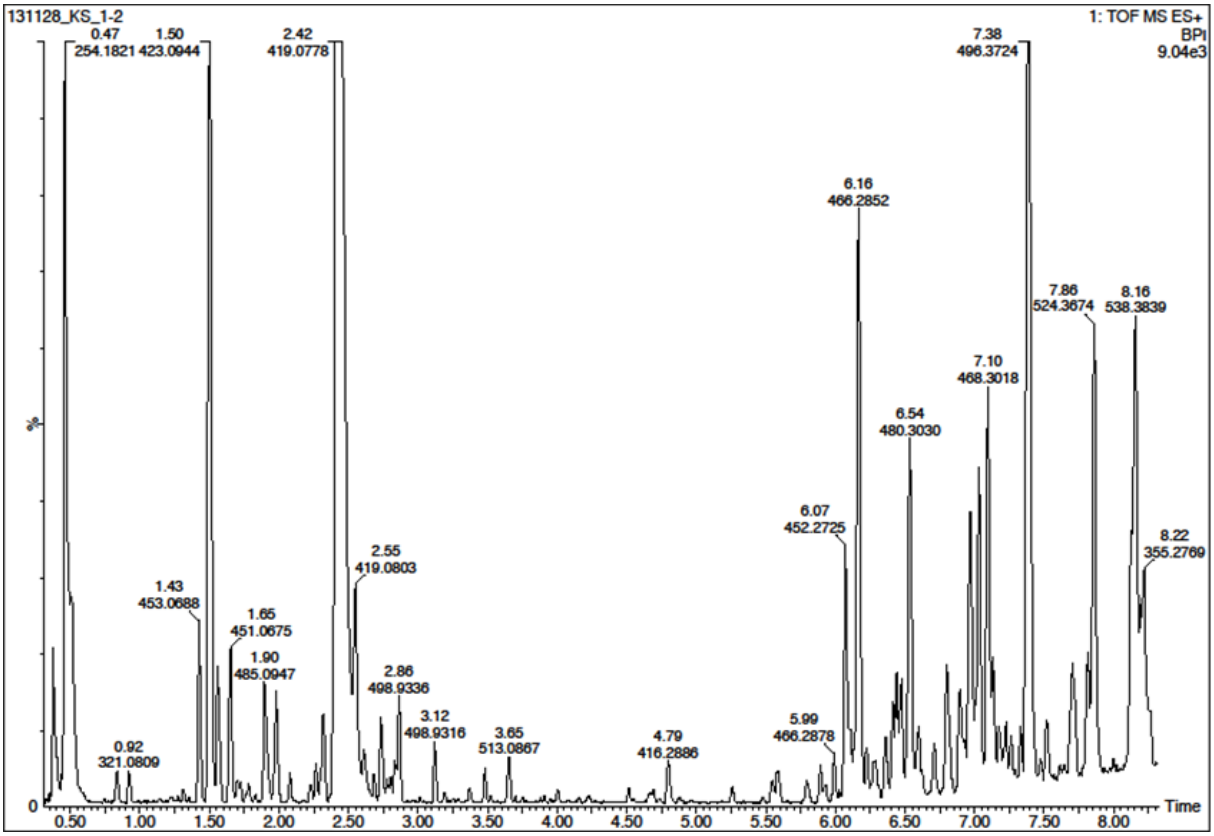

Figure 6: Chromatogram of one of the *G. baretti* samples demonstrating the number of compounds found in an organic extract from a marine sponge. ESI in positive mode.

In figure 6 a chromatogram from a selected sample is shown to illustrate the amount of different signals detected from the organic extract of a marine sponge. The rest of the chromatograms have been normalized to the highest peak, hence the signals that have very low intensity will not be visible. The potentially unique signals will still be detected and analyzed by the processing program MarkerLynx.

Identification of biomarkers using MarkerLynx

The MarkerLynx with its principal components analysis separates the samples into groups based on the similarities in their metabolic profiles, which provides an opportunity to pin point exactly which metabolites that extinguish one sample from another. Three injections of each sample have been done to compensate for instrument variation, where a data point represents each injection. The MarkerLynx have been set to de-isotope and common isotopes such ^{13}C isotopes will not be taken into account during the analysis, however the software is not sophisticated enough to distinguish all isotopes, such as the ones that occur with brominated molecules like barettin.

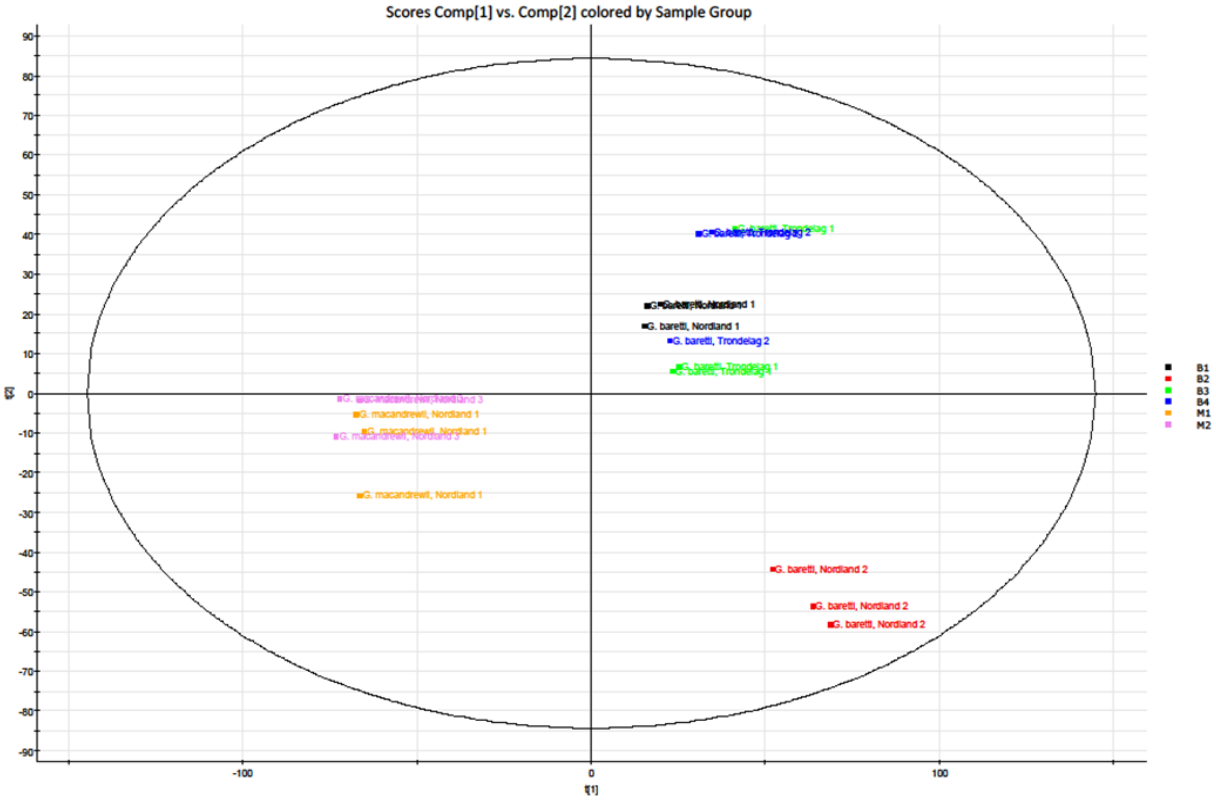

Figure 7: Scores plot of all the samples where; *G. barretti* collected in Saltfjorden, Nordland = *G.baretti*, Nordland 1, *G. barretti* collected in Bliksvær, Nordland = *G.baretti*, Nordland 2, *G. barretti* collected in Lekshammeren, Trondheimsfjorden = *G.baretti*, Trøndelag 1, *G. barretti* collected in Borgenfjorden, Trondheimsfjorden = *G.baretti*, Trøndelag 2, *G. macandrewii* collected in Bliksvær, Nordland = *G.macandrewii*, Nordland 1, *G. macandrewii* collected in Saltfjorden, Nordland = *G.macandrewii*, Nordland 3

In figure 7 the scores plot describes the samples relationships, comparing similarities and dissimilarities in the metabolites from the samples organic extract. It is apparent that both the

G.macandrewii samples, represented with six data points, are similar to each other in metabolic profile but differs from the metabolic profile of the *G.baretti* samples. The projection and the direction of the *G.macandrewii* data points from the variable origin indicate dissimilarities from the *G.baretti* samples and the two *G.macandrewii* samples are therefore placed in one group as shown in figure 7. The *G.baretti* samples also demonstrate a similarity in metabolomic composition and are placed in their own group. There is one *G.baretti* sample (*G.baretti*, Nordland 2), represented by three data points to the right in negative part in figure 7, that differs from the other *G.baretti* samples, meaning there is something in that sample that distinguishes it from the others.

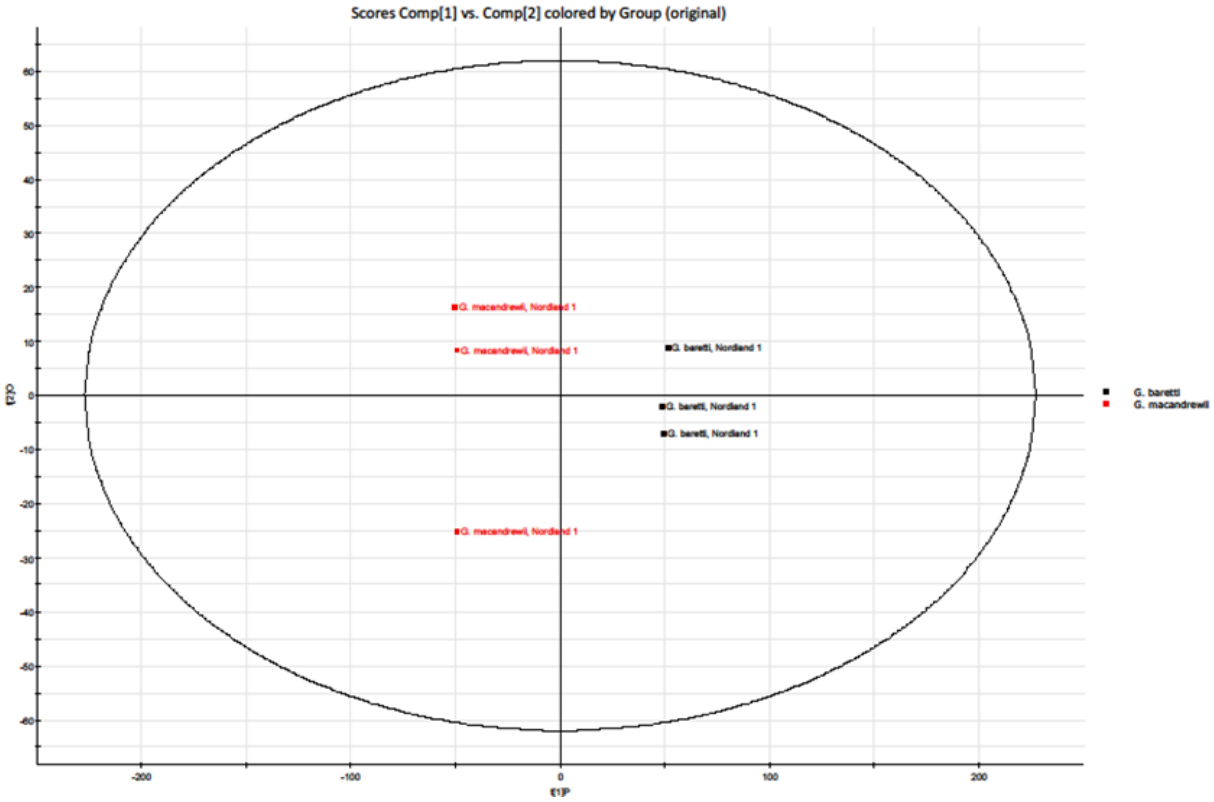

Figure 8: Scores plot of *Geodia macandrewii* collected in Bliksvær, Nordland 11.05.13 and *Geodia barretti* collected in Saltfjorden, Nordland 11.05.13.

In order to find the biomarkers responsible for the dissimilarities between the species, two and two samples have to be compared. A sample of each species collected from the same place at the same time was chosen for this comparison. In figure 8, the two samples are represented with three data points each and again the two species are clearly separated from each other. The variation in the data points from the same species is caused by instrument variation.

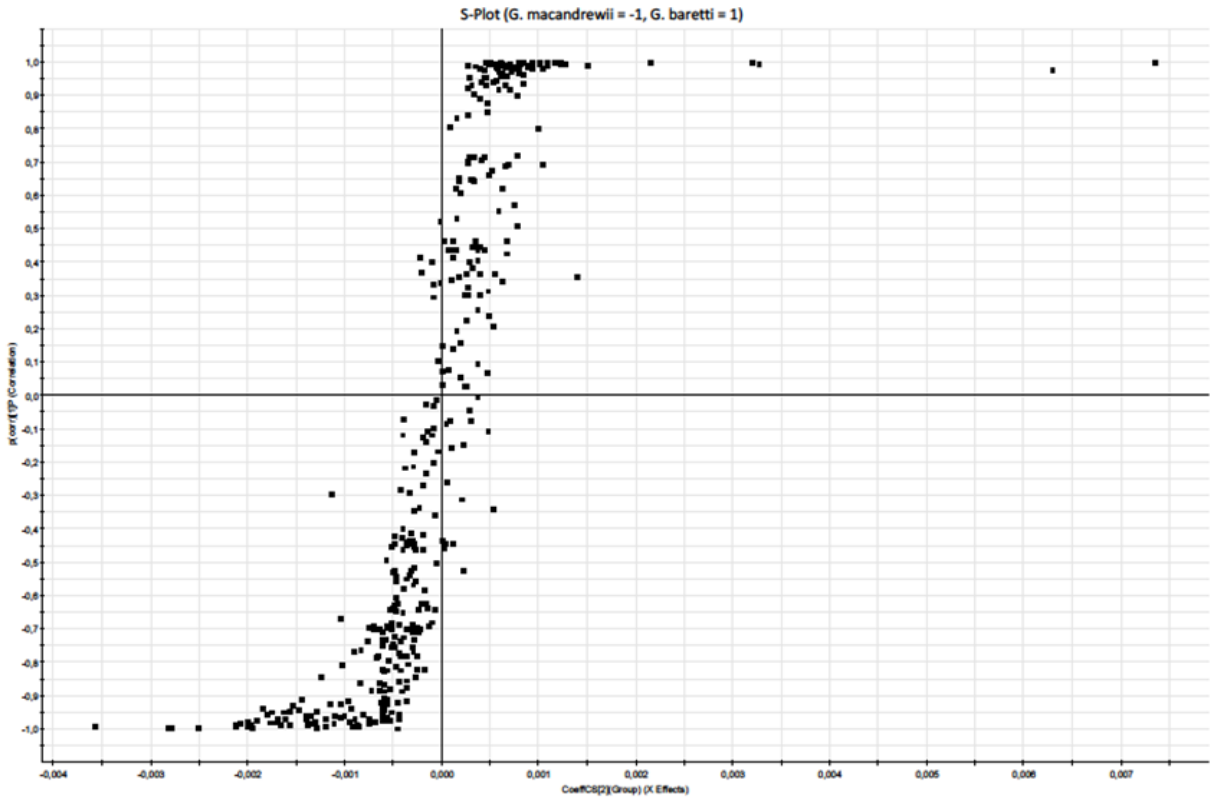

Figure 9: S-plot of *Geodia barretti* collected in Saltfjorden, Nordland 11.05.13 in the positive range and *Geodia macandrewii* collected in Bliksvær, Nordland 11.05.13 in the negative range.

The S-plot describes the variables relationship but it is also a means for further interpretation by demonstrating which of the variables are causing similarities and dissimilarities. In the plot in figure 9 each variable is representing a metabolite from the chosen sample`s organic extract and the plot describes similarities and dissimilarities in their metabolic profile. The further away from the variable origin and the cluster of marks the more significant the variable is for dissimilarity. In this case the *G.baretti* sample is in the positive range and the variables marked on the far right is *m/z*: 419 and m/z: 421. The other two variables on the right, clustered closely together, are *m/z*: 421 and *m/z*: 423. In the negative range is the *G.macandrewii* sample and the variable marked to the far left is due to the compound with *m/z*: 400. The S-plot confirms that this biomarker makes a considerable contribution to the differences in metabolic profiles previously demonstrated in the scores plot (figures 7 and 8) and the compound is subject to bioactivity testing, structure elucidation, and therefore needs to be isolated.

Identification:

The marine sponge *G.baretti* is known to produce the bioactive compound barettin with following elemental composition $C_{17}H_{19}BrN_6O_2$. To help verify the *m/z*: 419 as the protonated molecule barettin, $[M+H]^+$, an isotope spectrum in figure 10 has been used to demonstrate the different isotopes of the monobrominated compound.

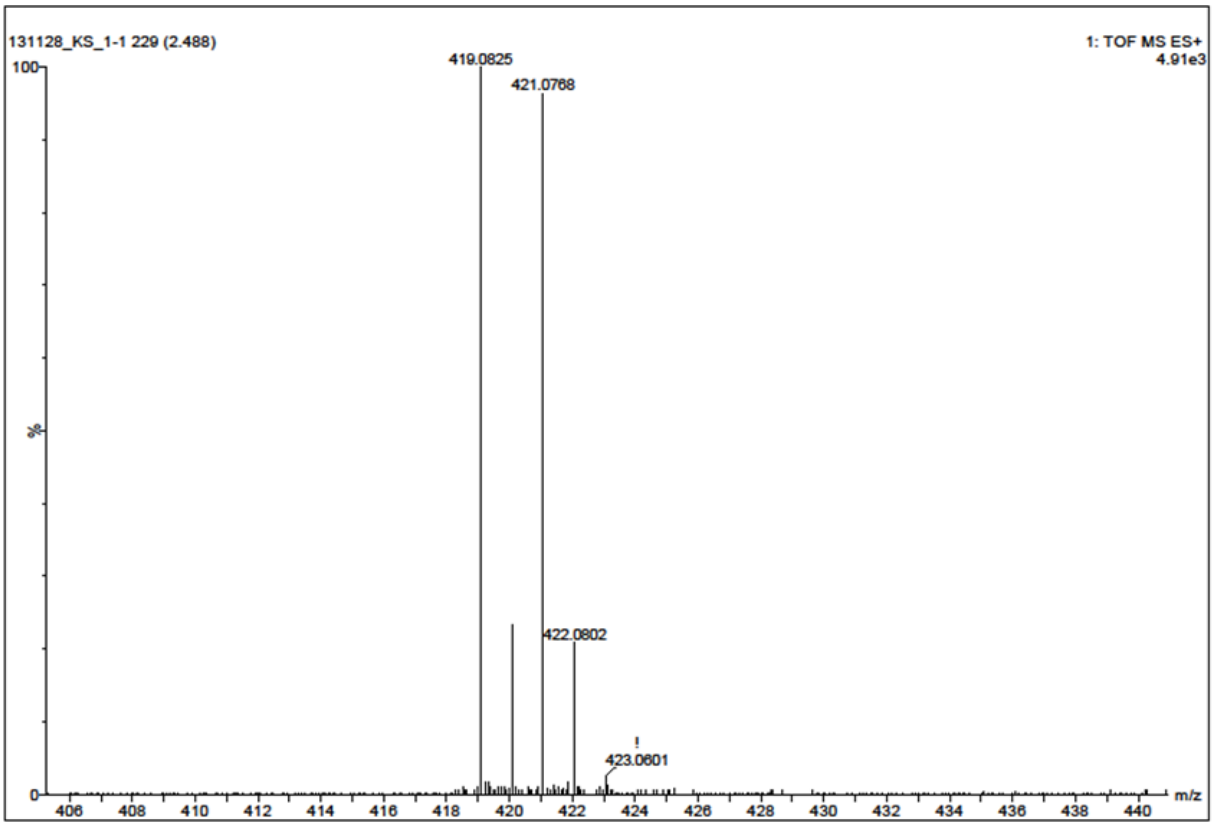

Figure 10: HR-MS isotope spectrum of barettin from the *G. barretti* sample collected in Saltfjorden, Nordland 11.05.13. ESI in positive mode.

In figure 10, the isotope spectrum shows two signals with similar intensity that are two mass units apart, *m/z*: 419 and 421. There are two other signals with lower intensity, *m/z*: 420 and 422, which are also two mass units apart. The natural occurrence of carbon isotopes (^{12}C: 98.93% and ^{13}C: 1.07%) and bromine isotopes (^{79}Br: 50.69% and ^{81}Br: 49.31%) provides a very characteristic isotope pattern when analyzed. The same pattern as displayed in figure 10 and the connection between isotopes and signals is illustrated in table 12.

Table 12: Connecting the different signals from the spectrum illustrated in figure 10 to different isotopes of barettin.

Signal (m/z)	419	420	421	422
C/Br isotope	$^{12}C/^{79}Br$	$^{13}C/^{79}Br$	$^{12}C/^{81}Br$	$^{13}C/^{81}Br$

Since barettin is an already known compound, a search in the database was done to compare retention time. Previously performed experiments at MabCent demonstrated the same retention time for barettin as the retention time for *m/z*: 419 in this project. Therefore, it can be concluded that *m/z*: 419 is barettin. The structure and exact mass of barettin are illustrated in figure 11.

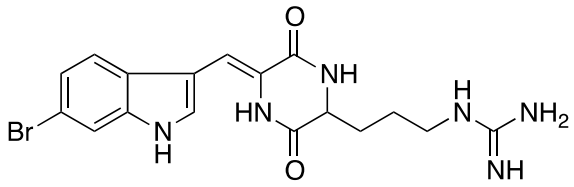

Figure 11: Structure of barettin where the exact mass is 418.0753 Da and chemical formula: $C_{17}H_{19}BrN_6O_2$.

The *G. macandrewii* samples had a prominent signal at *m/z*: 400, a signal for a novel compound that was not present in any of the four *G. barretti* samples and therefore a target for further examination. To help verify the *m/z*: 400 as a protonated molecule (and not an adduct of another molecule) the spectrum from the UPLC-HR-MS analysis in figure 12 is used to demonstrate the presence of common adducts often found when using ESI in positive mode. The *m/z*: 400 is the protonated molecule $[M+H]^+$, meaning the actual molecule has a mass of 399 Da. The less intense signal at *m/z*: 401 suggest the same molecule`s ^{13}C isotope. The signal at *m/z*: 422 is likely the common adduct $[M+Na]^+$ and the *m/z*: 441 indicates the acetonitrile adduct $[M+CH_3CNH]^+$. Another signal indicating that the *m/z*: 400 represents an actual molecule is *m/z*: 799, which shows a protonated dimer of the molecule, $[2M+H]^+$.

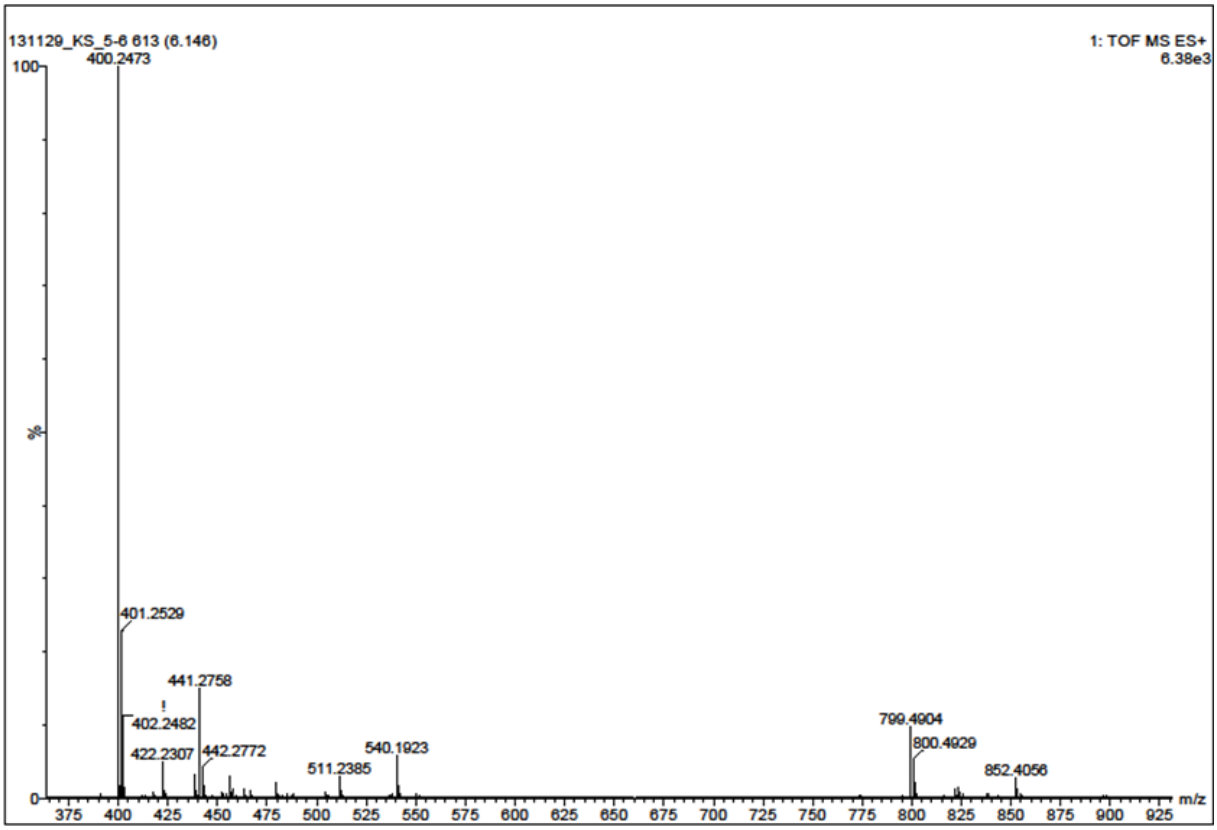

Figure 12: HR-MS spectrum of the compound m/z: 400 from the *G. macandrewii* sample collected in Bliksvær, Nordland 11.05.13. ESI in positive mode.

The spectrum in figure 13 from the UPLC-HR-MS analysis with ESI in negative mode are also presented to help verify the *m/z*: 400 as a protonated molecule of an actual molecule with 399 Da. In negative mode the intense signal *m/z*: 398 are the deprotonated molecule, and the *m/z*: 399 and *m/z*: 400 represents the ^{13}C isotopes of the same molecule. The deprotonated dimer of the 399 Da compound with *m/z*: 797 is also found in the spectrum in figure 13.

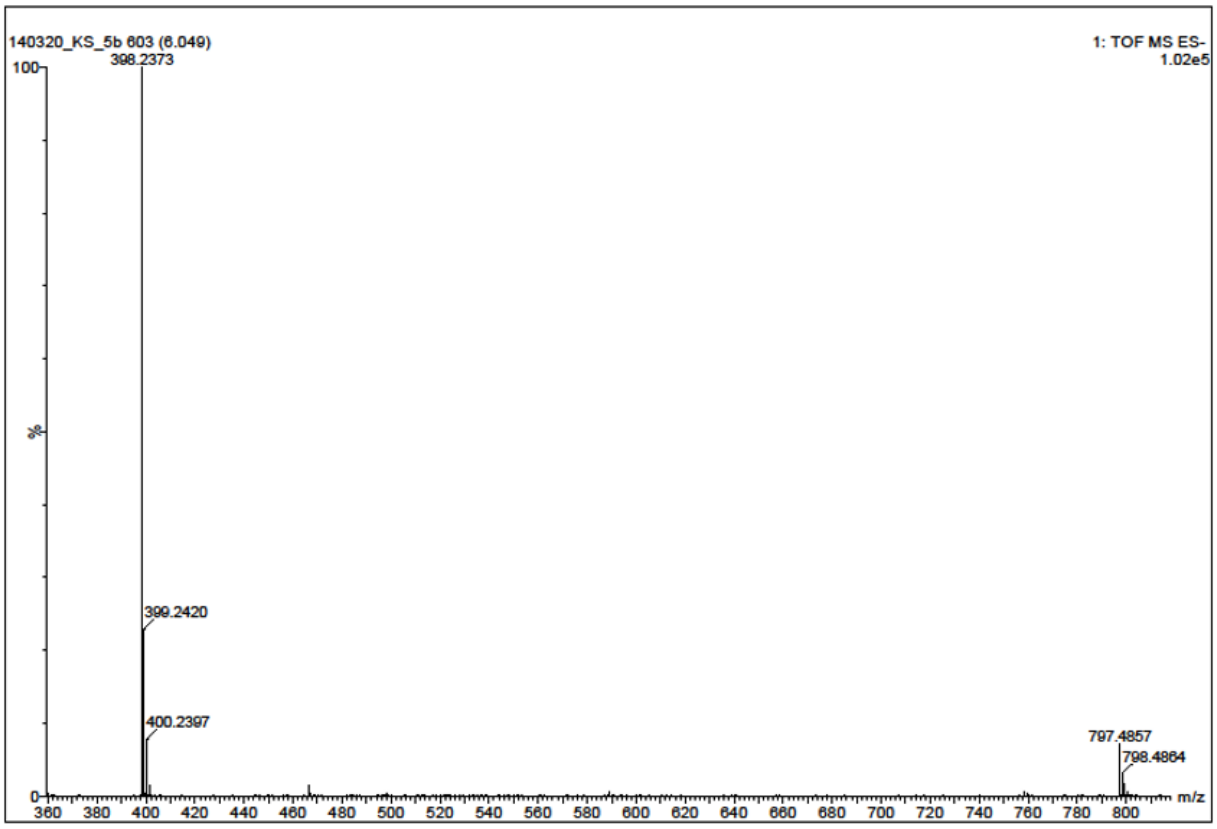

Figure 13: HR-MS spectrum of the compound m/z: 400 from the *G. macandrewii* sample collected in Bliksvær, Nordland 11.05.13. ESI in negative mode.

All of these signals help confirm the *m/z:* 400 signal as a protonated molecule, the *m/z*: 398 as the deprotonated molecule and that these signals represent an actual compound with 399 Da. The software was used to process the signals from the UPLC-HR-MS analysis with ESI in negative mode to suggest the elemental composition. Figure 14 displays the results from the software with suggestions to elemental composition, where $C_{21}H_{36}NO_4S$ are ranged as the most likely elemental composition. This means the actual elemental composition when the compound is not deprotonated would be $C_{21}H_{37}NO_4S$.

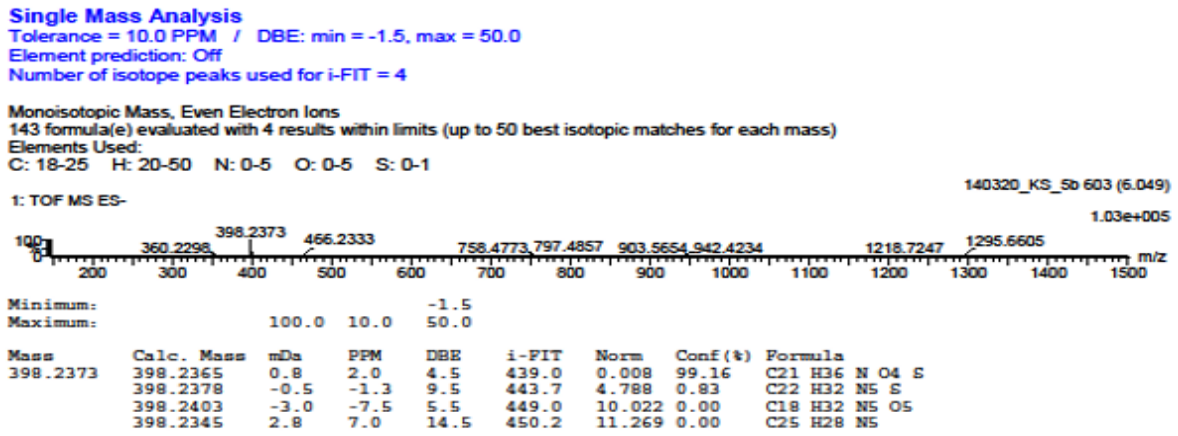

Figure 14: Elemental composition suggestions based on the data from the analysis performed with the UPLC-HR-MS with ESI in negative mode. The elemental compositions are ranged after percentage of likelihood.

With a probable elemental composition in place, structure elucidation are the next natural step before bioactivity testing of the compound. In order to determine the structure the compound needs to be isolated.

HPLC Isolation of MBC-169

For the bioactivity testing and structure elucidation the compound had to be isolated. The first round of purification was not sufficient; it was difficult to get the compound to elute as a symmetrical peak and instead the peak was broad and asymmetrical. Therefore a second round of purification was needed in order to obtain a pure compound. Achieving a satisfying chromatogram with clearly separated signals for MBC-169 was challenging at first and use of different columns were attempted: XTerra ® Prep RP18, 10 µm, 10×250 mm, XTerra ® Prep C8, 10µm, 10×250 mm and X-Select CSH ® Prep, Fluoro-phenyl, 5 µm, 10×250 mm, Atlantis ® Prep C18, 10 µm, 10×250 mm column without a satisfying result. However, when using the same column as used in the first round of isolation (Atlantis ® Prep C18, 10 µm, 10×250 mm) and changing to a basic mobile phase and thereby changing (pH = 8), the chromatography improved significantly. The second round of purification therefore had a better result as the compound eluted in a narrow peak and the signal *m/z*: 400 were dominating the fraction collected by the instrument. The HPLC isolation of MBC-169 yielded 14.0 mg after the first round and 0.7 mg after the second round.

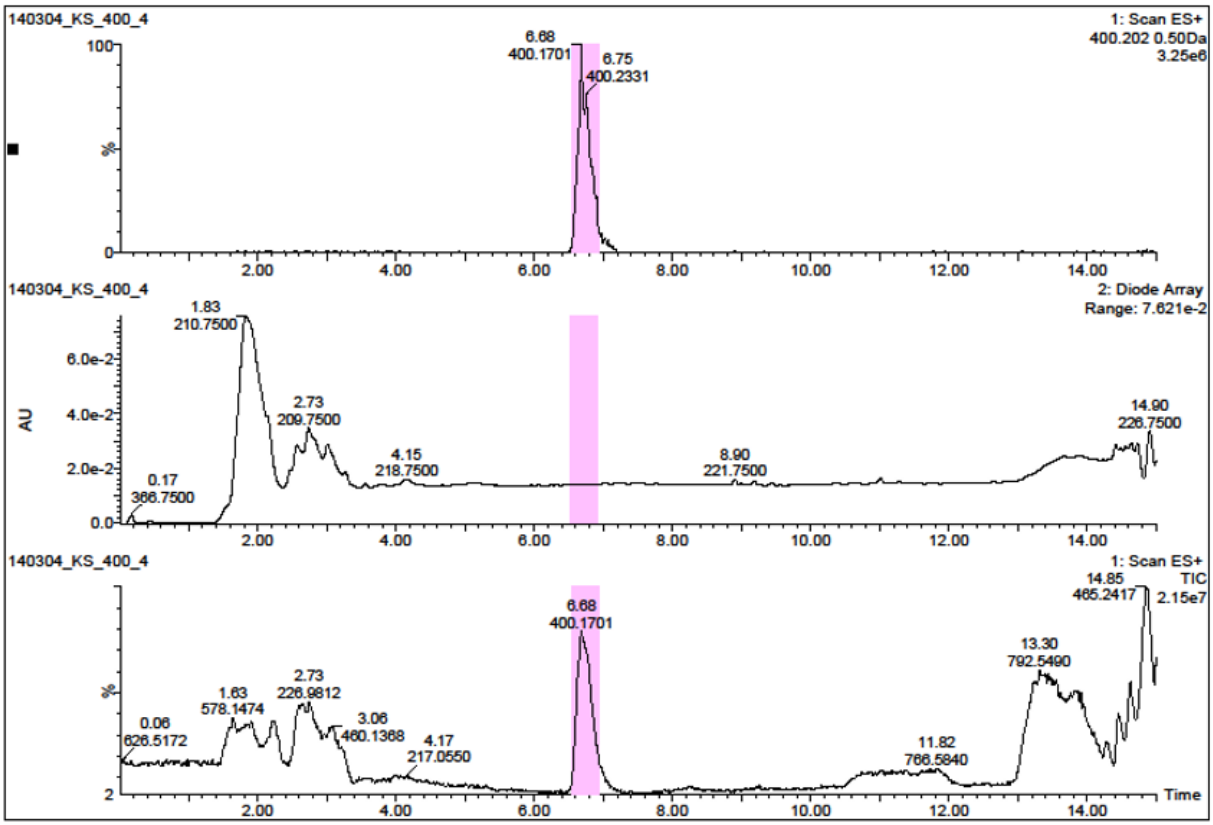

Figure 15: Chromatograms from the second round of isolation of the compound. Top chromatogram with ESI in positive mode and set to show compounds with 400 m/z. The middle chromatogram UV 250-500 nm and the bottom chromatogram with ESI in positive mode and set to show total ion count (TIC).

Structure Elucidation

Figure 16 of the ^{1}H-NMR reveals the number of hydrogen (H) in the compound with the intensity of the peaks. The chemical shift value ppm indicates the chemical surroundings of an H, meaning the H with the same ppm have the same chemical surroundings.

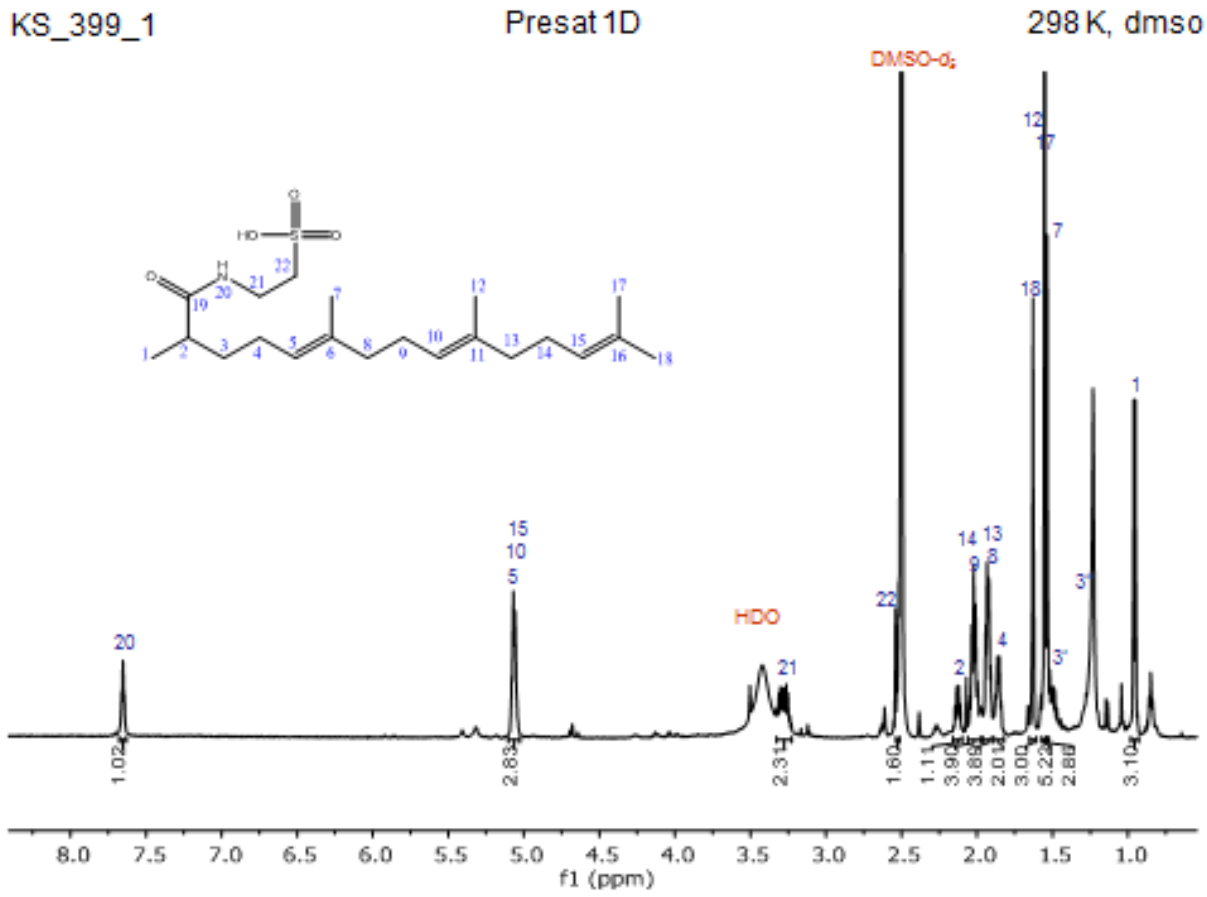

Figure 16: One dimensional ^{1}H-NMR specter of the purified compound. The peaks are numbered according to the structure of MBC 169 and the solvents are indicated. Chemical shift (ppm) is on the x-axis and the peak intensity is on the y-axis.

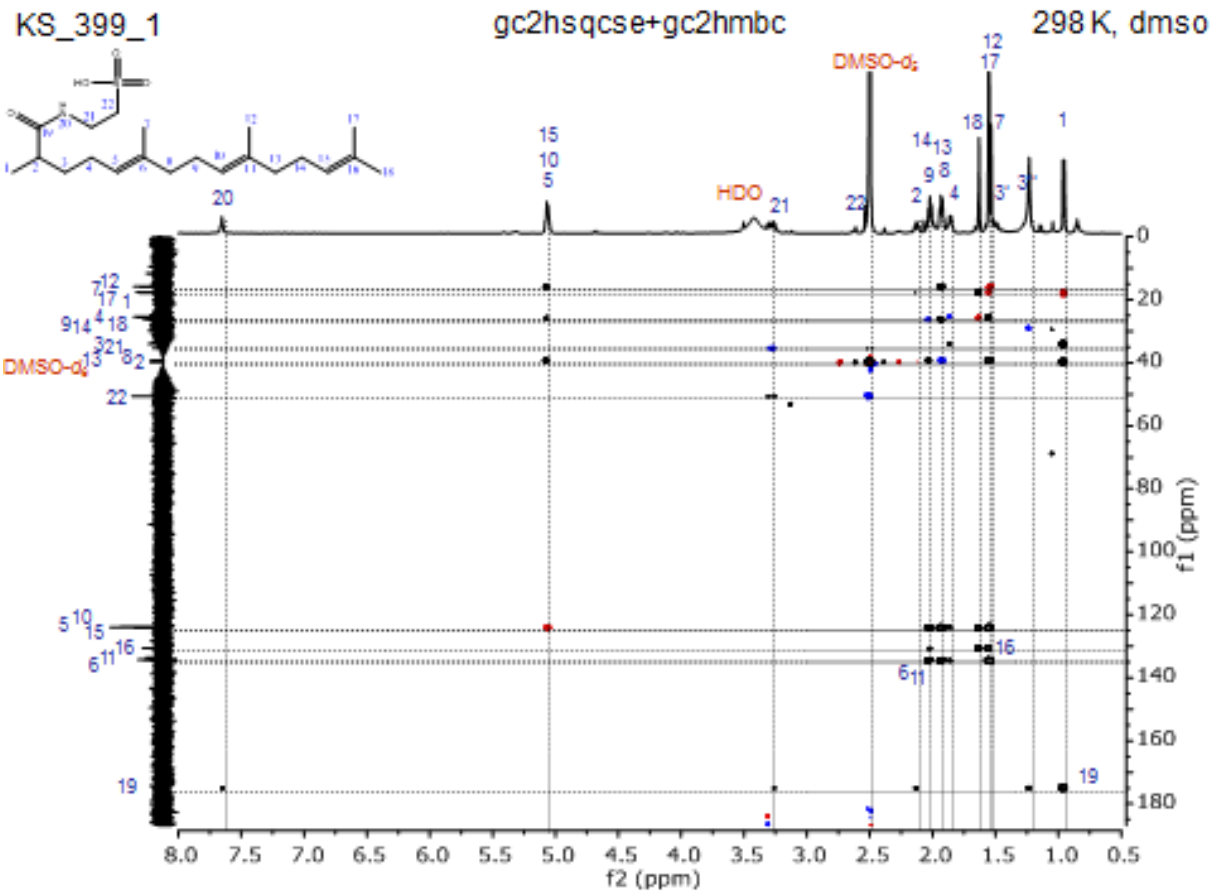

Figure 17: HSQC superimposed on HMBC. The peaks are numbered according to the structure of MBC 169 and the solvents are indicated. The HSQC experiment is displayed in red and blue markings, where red = CH and CH^3 groups, and blue= CH^2 groups. The HMBC experiment is displayed as black markings. ^1H chemical shift (ppm) is on the x-axis and the ^{13}C chemical shift (ppm) is on the y-axis. HSQC peaks indicate HC pairs coupled to each other through 1 bond and HMBC peaks indicate HC pairs coupled to each other through 2-4 bonds.

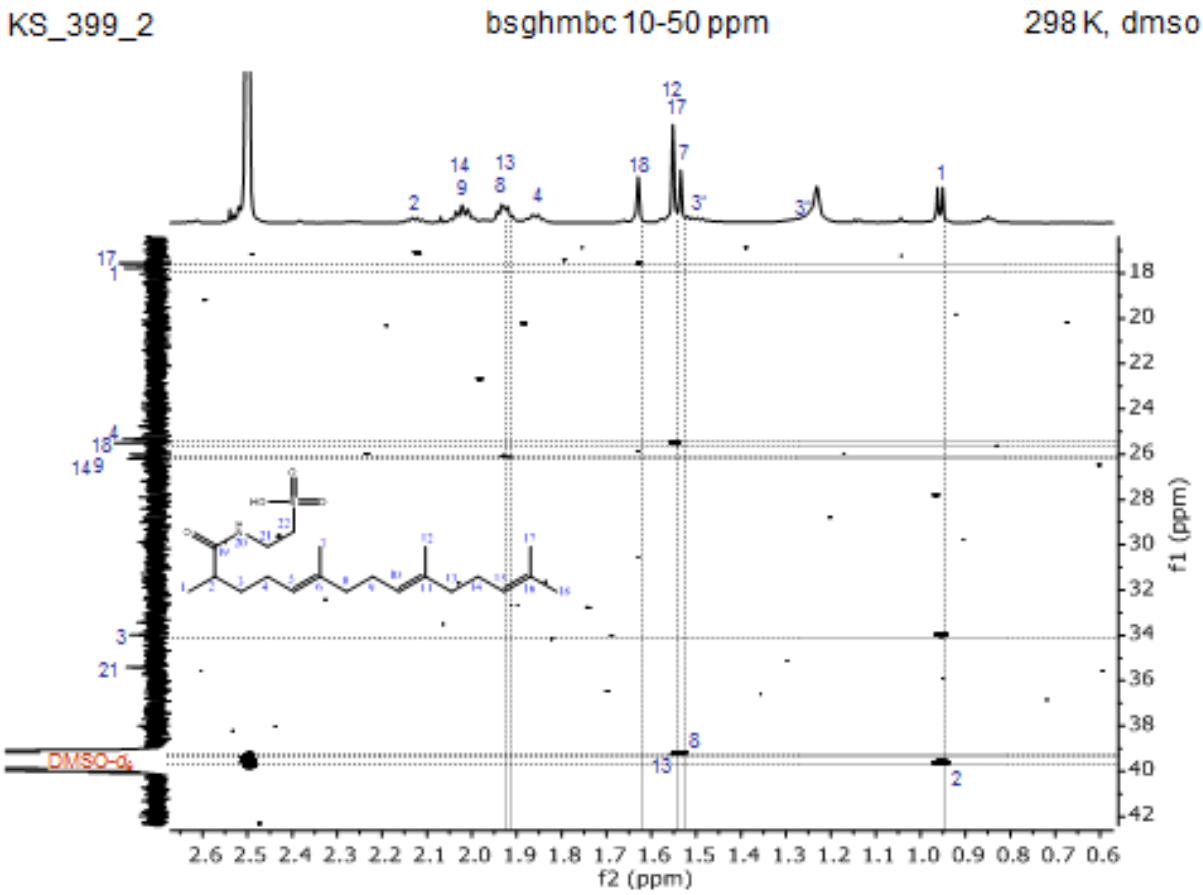

Figure 18: Band selective (15-45) ppm) HMBC specter to assign nearly overlapped carbon resonances. The peaks are numbered according to the structure of MBC 169 and the solvents are indicated. The chemical shift (ppm) for proton is on the x-axis and the chemical shift (ppm) for carbon is on the y-axis.

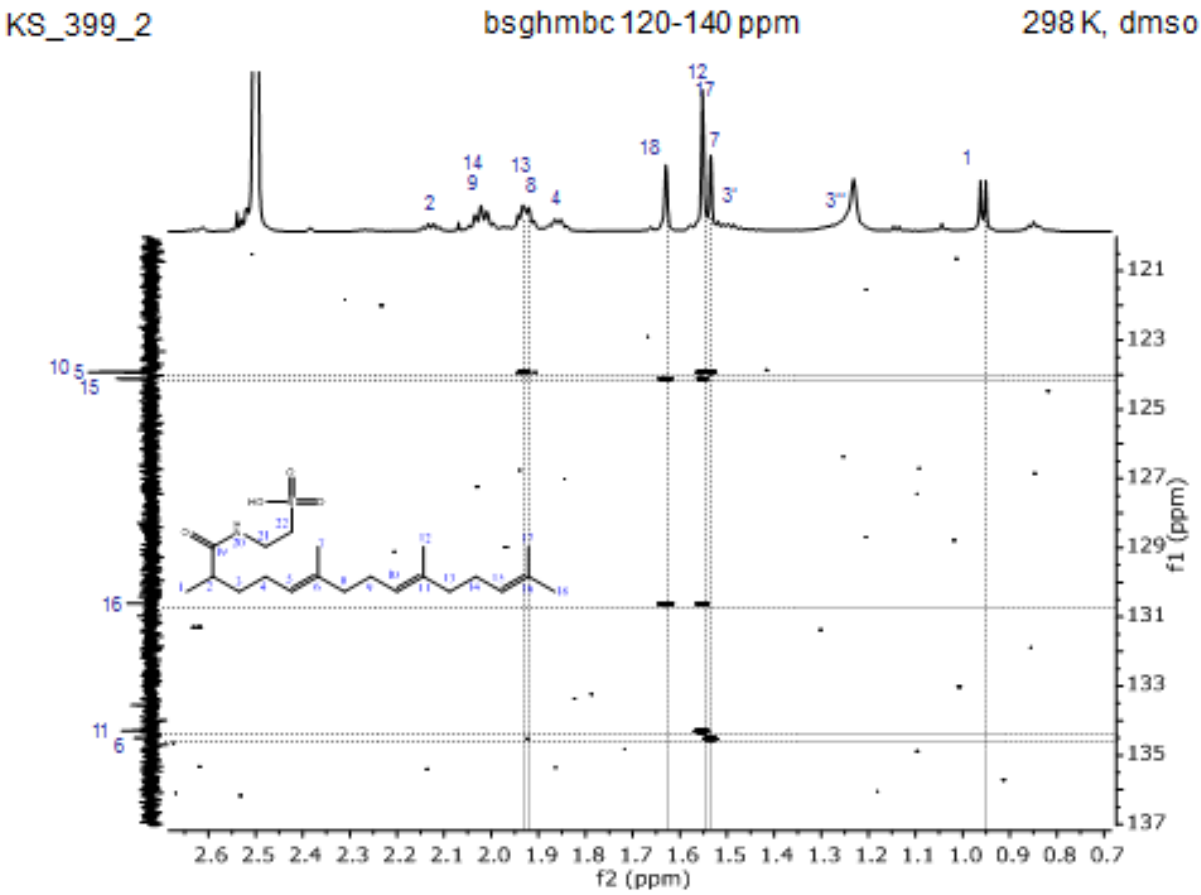

Figure 19: Band selective (120-140) ppm) HMBC specter to assign nearly overlapped carbon resonances. The peaks are numbered according to the structure of MBC 169 and the solvents are indicated. The chemical shift (ppm) for proton are on the x-axis and the chemical shift (ppm) for carbon are on the y-axis.

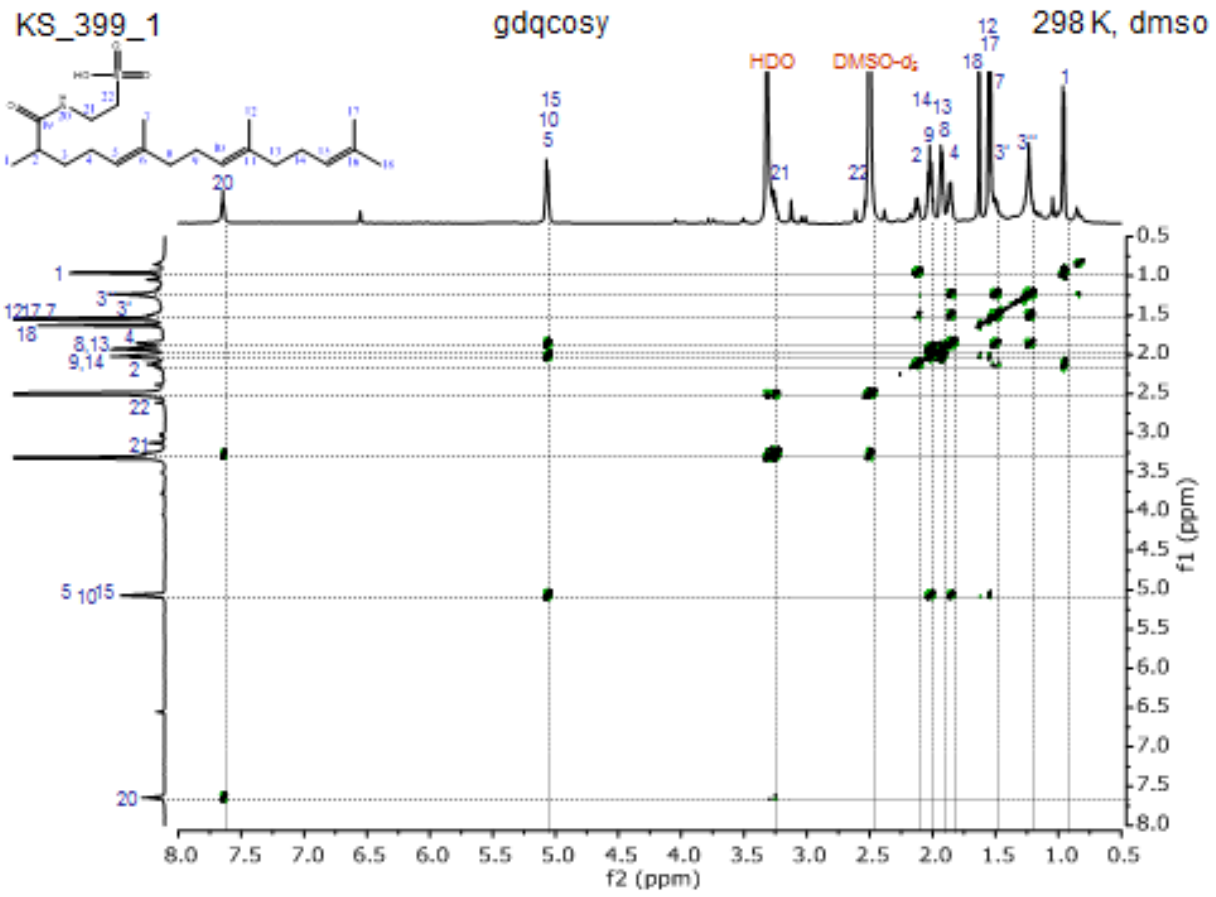

Figure 20: Two-dimensional COSY spectrum where the peaks are numbered according to the structure of MBC 169 and the solvents are indicated. Cross peaks indicate neighboring protons coupled through 2-3 bonds.

To further verify the structure of MBC-169 as illustrated in figure 22, a structure with selected COSY and HMBC correlations have been made. The ^{1}H- NMR and ^{13}C-NMR assignments of MBC-169 with HMBC-and COSY correlations are also presented in table 13.

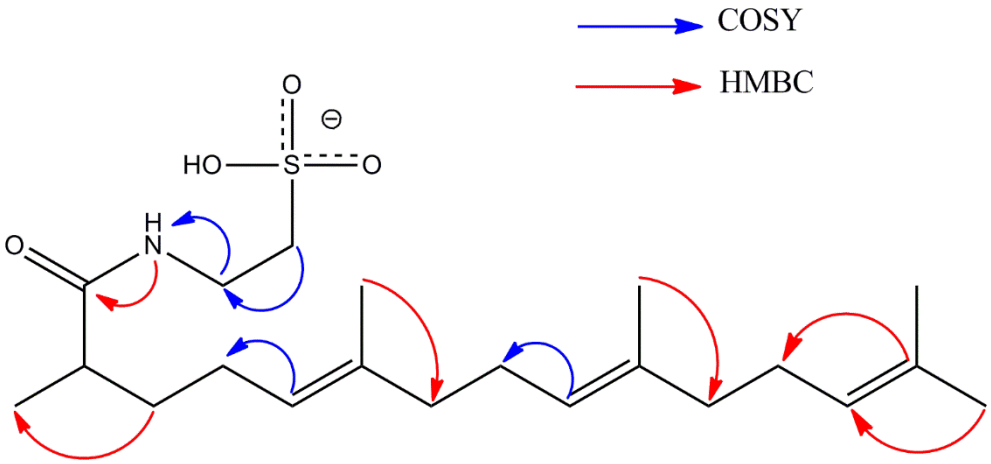

Figure 21: Structure of MBC-169 with selected COSY -and HMBC- NMR correlation indicated. The blue arrows indicates COSY correlation and the red arrows indicates HMBC correlations.

Table 13: ^{1}H- NMR and ^{13}C-NMR assignments of MBC-169 with HMBC- and COSY correlations. The position of the atoms numbered in the table can be found in figure 22.

Atom no.	δ^{13}C (ppm)	δ^{1}H (ppm)	COSY ^{1}H↔^{1}H	HMBC ^{13}C↔^{1}H
1	17.77	0.96	2	
2	39.61	2.13	1, 3	1
3	34.00	1.51/ 1.24	2, 4	1, 4
4	25.36	1.86	5	5
5	123.92	5.07	4	4, 7
6	134.52			4, 7, 8, 9
7	15.74	1.54		5, 8
8	39.16	1.93	9	7, 9, 10
9	26.04	2.02	8, 10	8, 10
10	123.92	5.07	9	8, 9, 12
11	134.30			12, 13, 14
12	17.58	1.55		10, 13
13	39.19	1.93	14	12, 14, 15
14	26.19	2.02	13, 15	13, 15
15	124.12	5.07	14	13, 14, 17, 18
16	130.63			14, 17, 18
17	15.84	1.54		18
18	25.52	1.63		15, 17
19	174.77			1, 2, 3, 20, 21
20		7.65	21	
21	35.43	3.31/ 3.26	20, 22	22
22	50.65	2.51	21	21

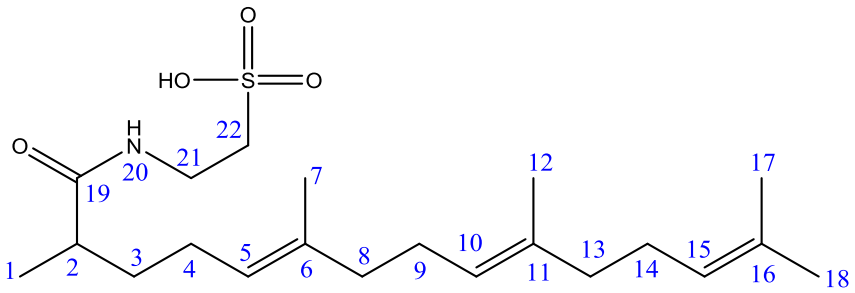

Figure 22: Molecular structure of MBC 169. The atoms numbered correlates to the atoms numbered in table 13.

Bioactivity

MBC-169 was tested for bioactivity in four different assays, two assays tested for antioxidant activity, one for kinase inhibiting properties and one for antibacterial activity.

Antioxidant Activity Assays

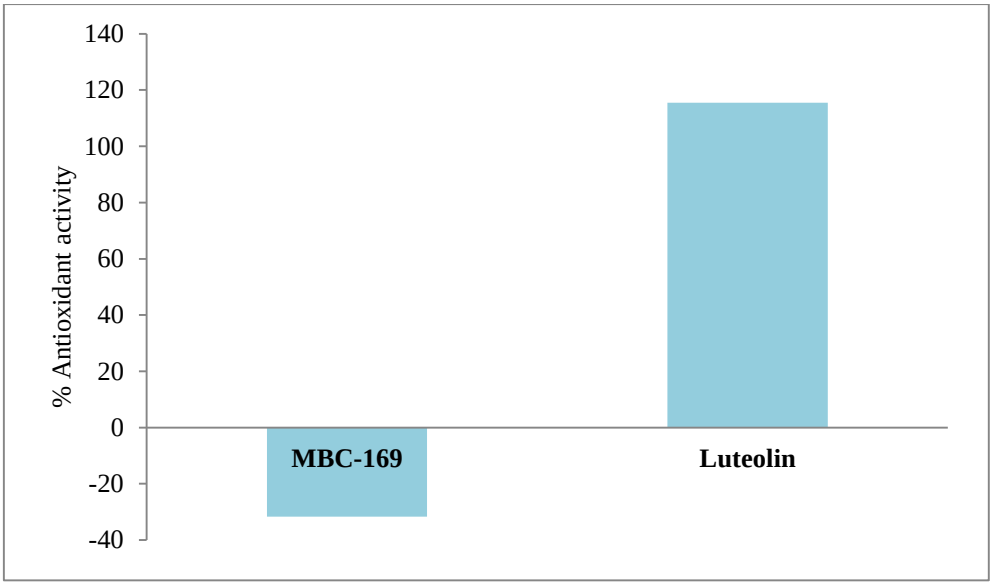

Figure 23: Column chart of the CLPAA assay illustrating antioxidant activity with 50 µg/mL MBC-169 and the control 50 µg/mL Luteolin.

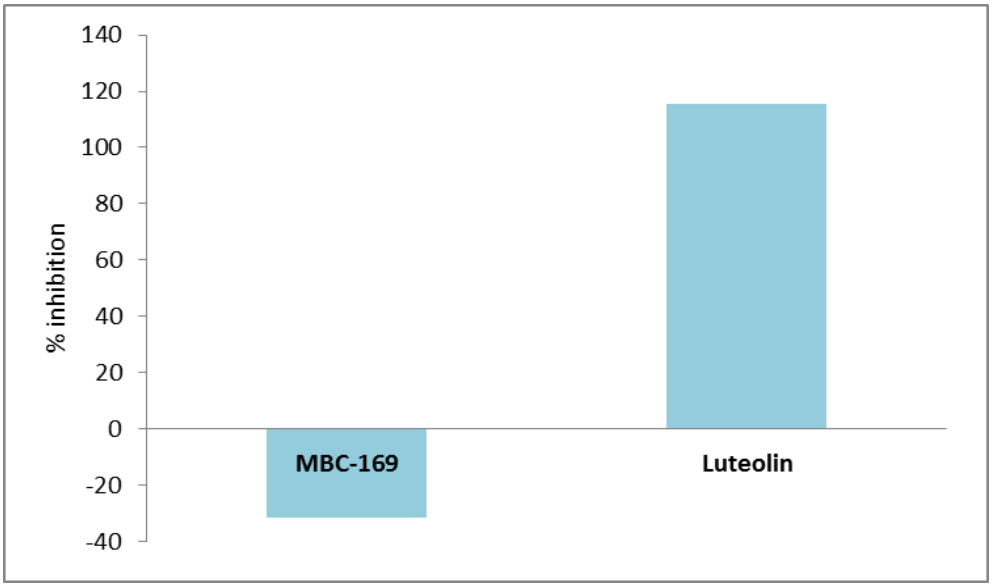

Figure 24: Column chart of the CAA assay illustrating antioxidant activity with 50 µg/mL MBC-169 and the control 50 µg/mL Luteolin.

In the antioxidant assays the control used was 50 µg/mL Luteolin. As demonstrated in figure 23 and 24 Luteolin show strong antioxidant activity. The negative value for MBC-169 was

caused by oxidative activity due to the lack of antioxidant effect. This means that both the CLPAA- and the CAA assay show no significant antioxidant activity for the MBC-169

Kinase RR-analysis: Inhibition of PKA and ABL

The inhibition was calculated as relative percentage of kinase activity of PKA/ABL compared to the amount kinase activity in the control.

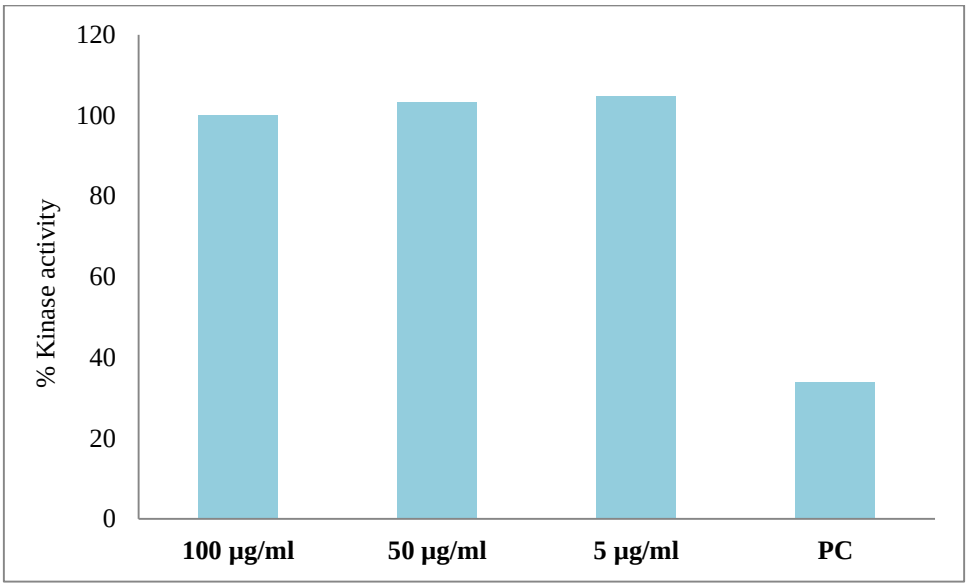

Figure 25: Column chart illustrating relative percentage kinase activity of PKA with different concentration of MBC-169. PC is the positive control where the known inhibitor staurosporin is added.

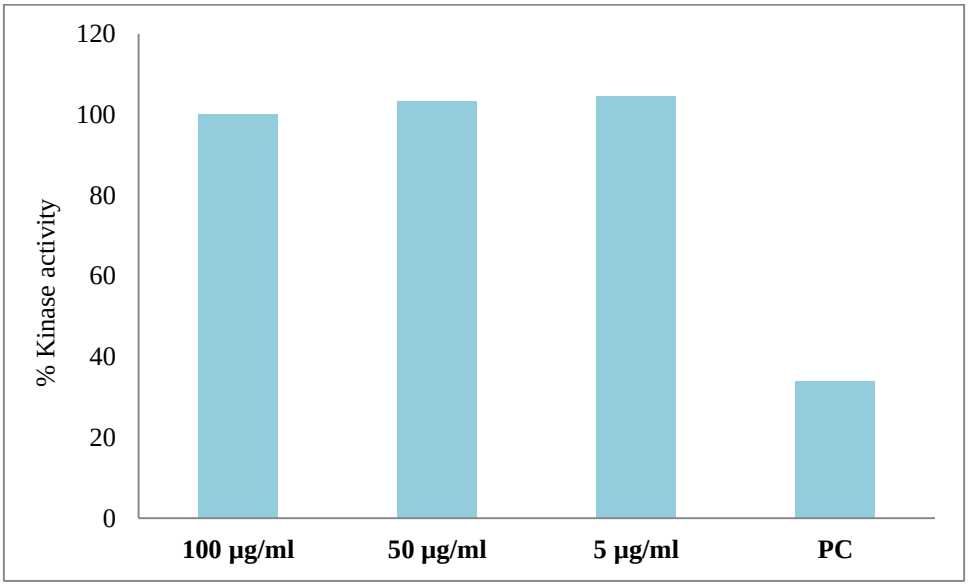

Figure 26: Column chart illustrating relative percentage kinase activity of ABL with different concentration of MBC-169. PC is the positive control where the known inhibitor staurosporin was added.

The positive control containing staurosporin show 34 % kinase activity in both column charts in figure 25 and 26. The MBC-169 demonstrates approximately 100 % kinase activity in all three concentrations, in other words MBC-169 does not show any kinase inhibition.

MIC

The minimum inhibition concentration assay tests a compounds ability to inhibit growth in bacterial strains. At MabCent the known antibiotic compound gentamycin is used as a negative control and for precision between testing. The assay is visually inspected and absorbance that reflects bacterial density is measured. Since the degree of inhibition will vary from bacterial strain to bacterial strain the negative control does not have a specific cut-off value. Instead a cut-off absorbance value that can be applied to all the bacterial strains used by MabCent have been set. The cut-off absorbance value for being classified as an active compound is <0.05 absorbance units (AU).

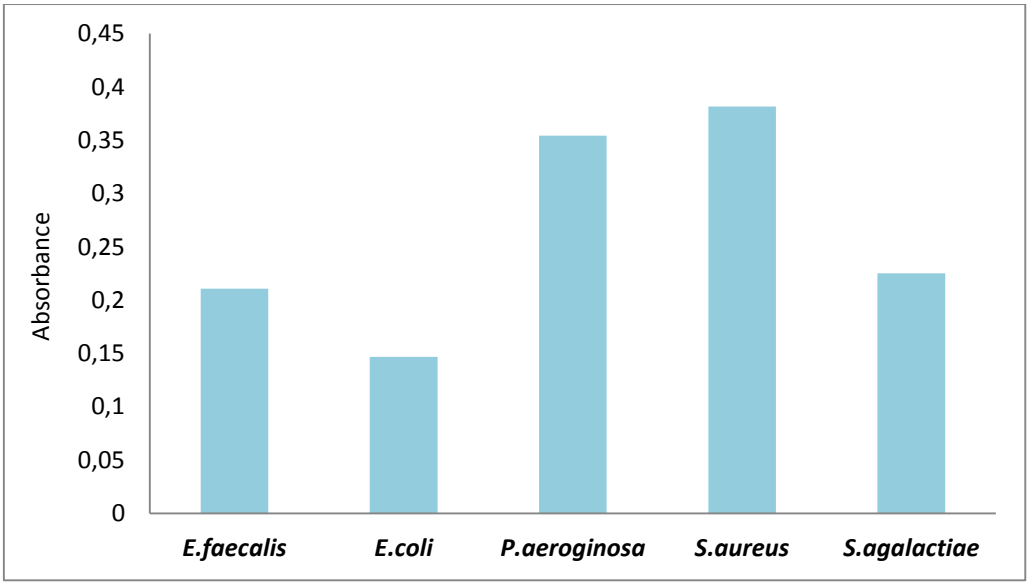

Figure 27: Column chart of the MIC assay illustrating absorbance after overnight incubation of 50 µg/mL MBC-169 with the following bacterial strains: *S.aureus*, *E.coli*, *E.faecalis*, *P.aeruginosa*, *S.agalactiae*.

The column chart in figure 27 illustrates that none of the bacterial strains shows efficient enough inhibition for the MBC-169 to be classified as an active compound. The *E.faecalis* shows 0.21 AU, the *E.coli*: 0.15 AU, the *P.aeruginosa*: 0.35 AU, the *S.aureus*: 0.38 AU and the *S.agalactiae*: 0.23 AU. Absorbance at 0.05-0.09 AU is classified as questionable, and absorbance >0.09 AU is classified as inactive. Since all of the strains show absorbance over 0.09, the MBC-169 (50 µg/mL) is classified as an inactive compound.

Discussion

This thesis focuses on the use of metabolic profiling in the investigation of a marine organism for novel compounds. There are many ways one can approach the search for novel natural products however none would be without its challenges. The previously described bioassay guided fractionation, based on finding bioactivity in a sample as a first step and as a second step finding the specific compound that are responsible for the bioactivity, is the conventional approach. When this approach is used on complex natural products an apparent loss of activity during the fractionation can often be observed. As a result the isolated compounds from the bioassay guided fractionation bioactivity would appear weak. Natural products are often extracts from a complex matrix where the chemical diverse compounds that are found to be bioactive, are also often found to be secondary metabolites rather than primary. The matrix contains a wide variety of compounds, the sheer amount and the difference in concentration means that some compounds can mask the activity of a bioactive compound. This also means that the bioactivity can come from not only one compound but several compounds in the natural products matrix. However when combining metabolomics to bioassay guided fractionation, or setting the course using metabolomics as a starting point, one can capture the chemical complexity of the metabolites [21]. Another advantage with the use of metabolomics with its heightened sensitivity is the ability to focus on the secondary metabolites, which often are responsible for the bioactivity, instead of certain strain or species. When two strains that appear different by morphology can produce the same secondary metabolites and two strains that appear identical can produce different secondary metabolites it makes sense to focus on the root of the bioactivity [22]. Although metabolic profiling offers better understanding of these metabolites it also generates a large amount of data where one is dependent on a good processing tool. Also a limited amount of samples can be compared by the processing tool; the statistics would be too complicated if too many samples were compared. Therefore it would take several analyses and time to process the data from a large set of samples.

To achieve proper data another key factor is that the MS have to be able to detect as many compounds as possible. It is therefore important that the compounds are ionized during the experiment, and that the necessary precautions have been taken. The MS instrument have several possibilities when it comes to ionization and earlier studies show that comparing data from ESI in positive mode with ESI in negative mode can reveal large amounts of ions that are uniquely detected in negative mode [23]. In this project the use of both positive and negative mode, helps to verify the novel compound as an actual molecule.

A different issue when using metabolomics to look for novel, bioactive compounds is that the compounds bioactivity is tested as a "final" step. Therefore the potentially time consuming task of purification and structure elucidation may result in a novel but inactive compound.

When it came to isolating, or rather purifying, MBC-169 it was not done without difficulty. The purification was done in two steps. In the first step finding the proper gradient of mobile phases was an issue, after trying out several different gradients an isocratic one was chosen. The chromatography was not ideal, with a broad peak for m/z: 400 (MBC-169) and clearly not completely separated from other compounds in the mixture, still it was better than other gradients. The column used was a C18 column and the MBC-169 had a long retention time. To improve the chromatography the use of several different columns with higher polarity were attempted but none provided a better result. In order to obtain a pure compound another step of purification was done with a basic mobile phase. The second step was far more successful, the chromatography showed that the compound MBC-169 eluted in a narrow peak. One theory could be that this is caused by the MBC-169s chemical properties and more specifically its change in affinity to the lipophilic material in the column. As illustrated in figure 22 the compound is made up by a rather large non-polar part and a polar part with two potentially "chargeable" groups. The non-polar part seemed to have a high affinity to the column first of all because the compound took a long time to elute. Second, it would also explain, to some extent why it would not elute in a narrow peak under the first purification and why the other mobile phase gradients resulted in an even wider peak and a poor chromatography. As mentioned the polar part consists of two polar groups where one is a sulfonic acid group (pKa: 0-1 and thereby a relatively strong weak acid [5]) which is likely to be charged in both steps of the purification. A theory as to why the purification was problematic is that the other chargeable group, the amide- group (NH) was charged by the mobile phase in some molecules and an equilibrium between molecules with charged and uncharged amid-groups occurred. This would result in a wide peak in the chromatogram. Another, more plausible theory is that the amide-group was not charged in the first step of the purification. However, when the mobile phase was changed and the pH increased in the second step, the amide-group was charged and therefore resulted in better chromatography. Even though a common reason is often different types of interactions between the column material and a molecule, it is a close to impossible to pinpoint exactly which interaction caused the improved chromatography in the second step of the purification.

The structure of MBC-169 that might be causing these effects on the chromatography have got a positive aspect when taking into account that the non-polar part might be able cross

membranes in the human body and the polar part might interact with different targets. Meaning the compound has got druglike qualities. Although, having a druglike structure it does not necessarily mean the compound is bioactive. The MBC-169 was only bioactivity tested with a selected handful of assays, where none of them showed bioactivity at an interesting level. The MIC assay was only tested with MBC-169 50 µg/mL, due to the small amount left of the MBC-169. However, it could have effect at a higher concentration. Therefore effect as an antibacterial agent cannot be completely ruled out. Other fields such as enzymes, immunomodulation, type- II diabetes and anti-inflammation are also being tested at the moment at MabCent and the MBC-169 could be bioactive within these fields [20].

The dissimilarity in one of the *G.baretti* samples was caused by different levels of barettin. The sample that stood out from the rest had a higher level of the barettin compound. Barettin has previously shown strong antioxidant activity in biochemical assays and in a lipid peroxidation cell assay [19]. It seems the higher level of barettin in the *G. barretti* collected in Lekshammeren, Trondheimsfjorden in the 13th of May in 2013 compared to the other *G. barretti* samples is connected to their respective geographic location. Lots of bioactive compounds are a result of the organism's natural habitat, secondary metabolites initiated as defense mediators due to hostile environment [24]. This production might be lowered or completely stopped when grown in monoculture without competing species or predators providing a selection pressure. If barettin is to be utilized as a drug in the future obtaining information about the natural production of barettin could be important. In general it could prove valuable to know more about which type of environment causes a marine organism to produce chemical defenses, especially when these defenses also have bioactive properties and are potential drug leads. With this information one possibility is to cultivate the sponge that produces barettin instead of collecting it from a remote location. Experiences with cultivation of an organism in order to obtain its secondary metabolites have shown that recreating the natural habitat with all of the particular organisms competing species and predators can be a challenge. Therefore synthesizing barettin seems a far more cost friendly and timesaving option. Several drugs on the marked today are after all just simple synthetic modifications or copies of natural product derived secondary metabolites [25].

Conclusion and future perspective

The aim of this project was to use metabolomics to investigate organic extracts from two species of the marine sponge *Geodia* for unique and potential novel compounds. If possible the molecular structure of any active compounds would be determined and tested for antioxidant and antibacterial activity, as well as kinase inhibiting properties. In the extracts from *G. macandrewii* a novel compound was discovered. The structure of the compound was determined and its bioactivity was tested. A handful of assays were selected; cellular antioxidant activity (CAA), cellular lipid peroxidation antioxidant activity (CLPAA), minimum inhibiting concentration (MIC) and kinase RR-analysis: PKA and ABL inhibition testing, but sadly none of the assays displayed the desired bioactivity.

The project has produced a novel natural product and proven that it is not only possible but also useful to use metabolomics in the search for novel compounds and also to identify which compounds are responsible for dissimilarities between samples. With the high sensitivity of metabolomics, one is more likely to avoid missing out on novel bioactive compounds that resides in a complex matrix. Although the bioactivity testing with MBC-169 did not have positive results, further testing can be executed. Testing with a higher concentration than 50 µg/mL in the MIC assay and testing for bioactivity in other assays available will probably be performed in the future. Another finding in this project was the elevated level of the known bioactive compound barettin in the *G. barretti* collected in Lekshammeren, Trondheimsfjorden compared to the other samples, indicating geographical differences of barettin production. Further investigation of the reasons behind this high level of barettin can be useful not only to understanding the compound barettin, but also to provide a better understanding of the environment that causes marine sponges to produce bioactive secondary metabolites.

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Appendix

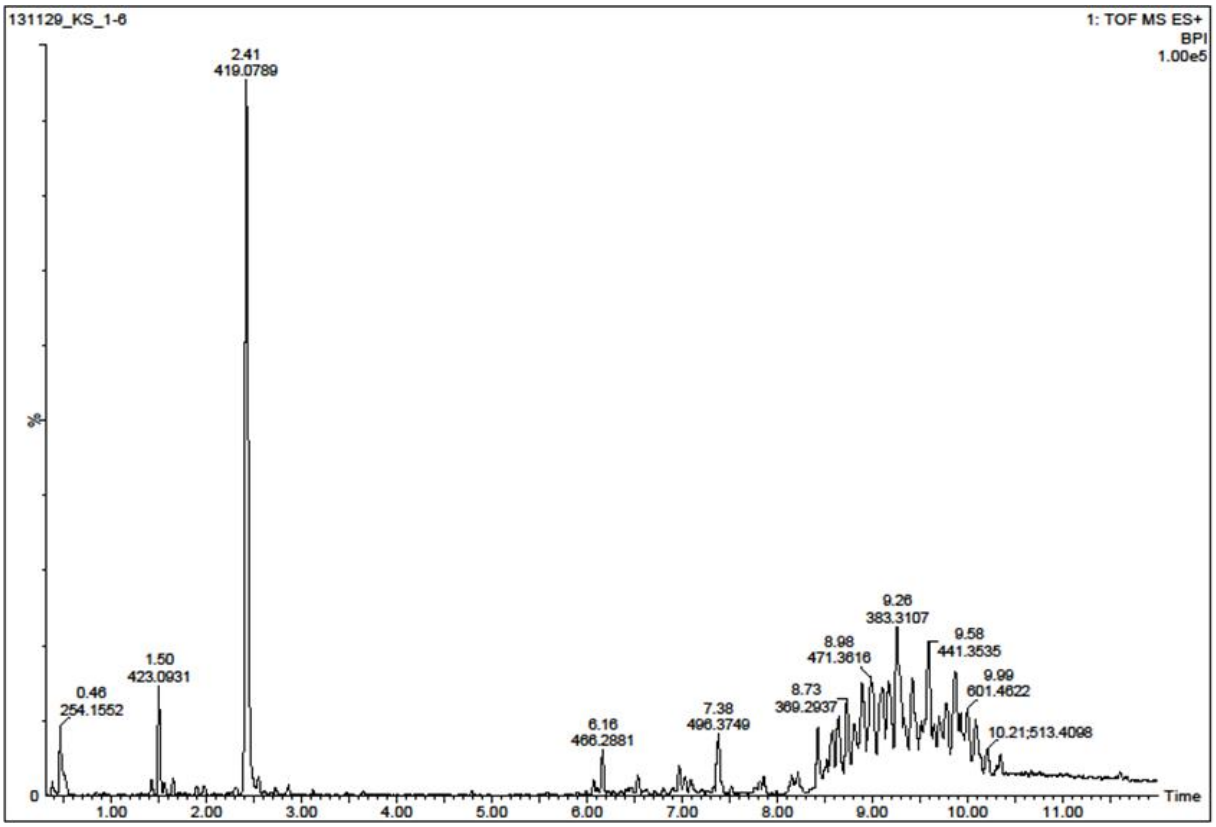

Figure 28: HR-MS chromatogram of *G. barretti* collected in Saltfjorden, Nordland 11.05.13. ESI in positive mode.

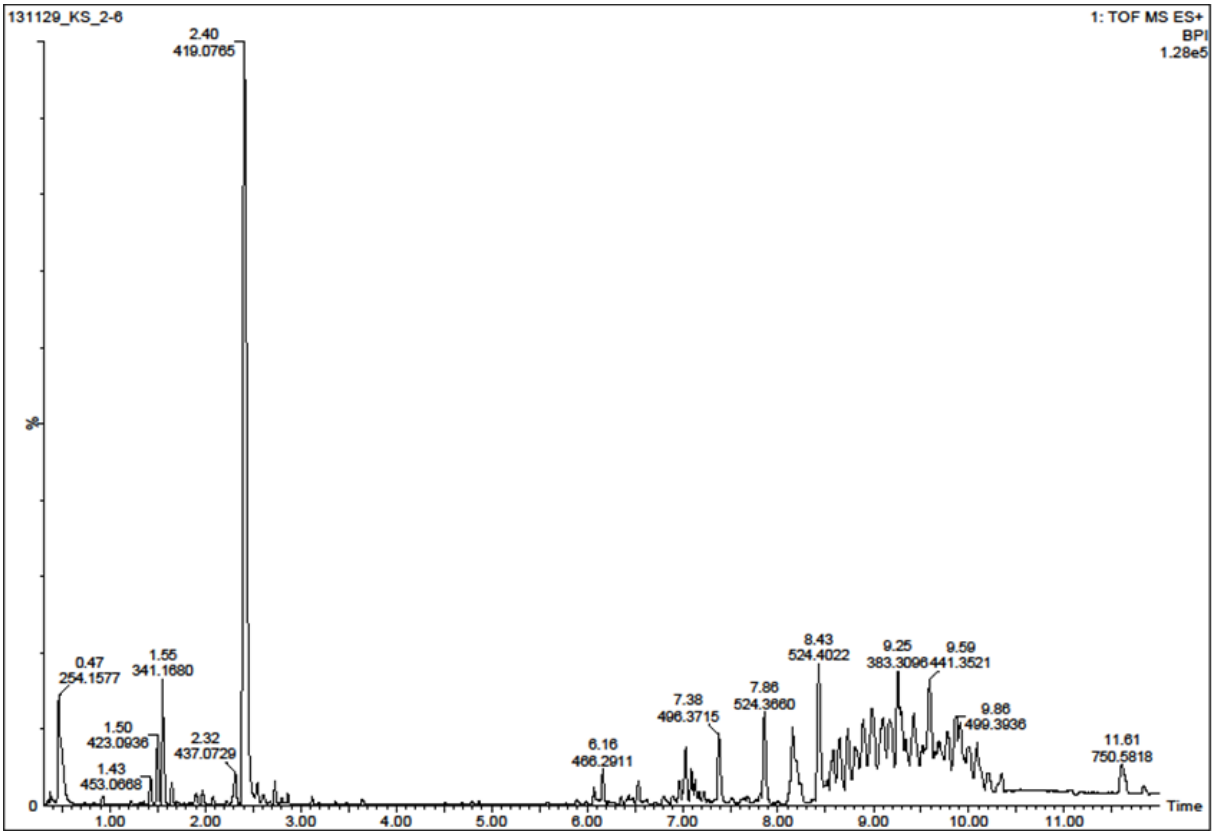

Figure 29: HR-MS chromatogram of *Geodia barretti* collected in Bliksvær, Nordland 11.05.13. ESI in positive mode.

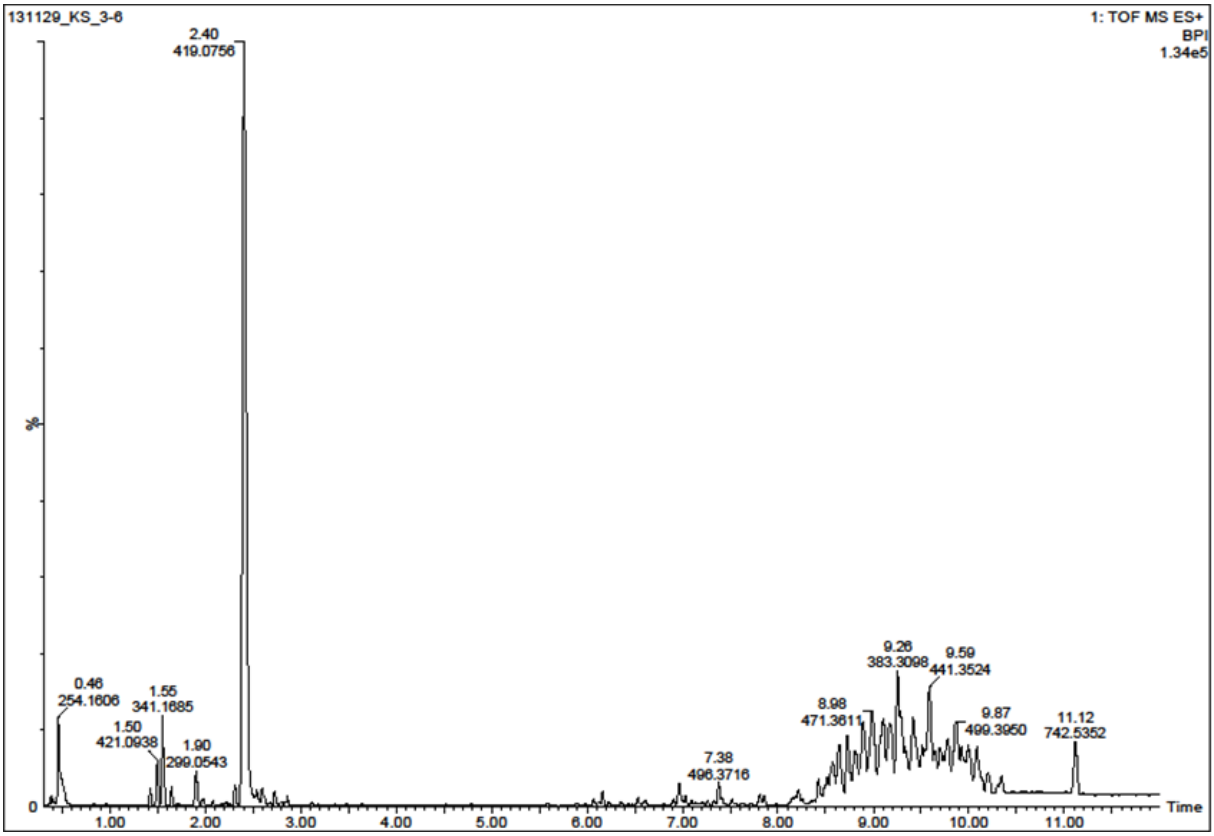

Figure 30: HR-MS chromatogram of *G. barretti* collected in Lekshammeren, Trondheimsfjorden 13.05.13. ESI in positive mode.

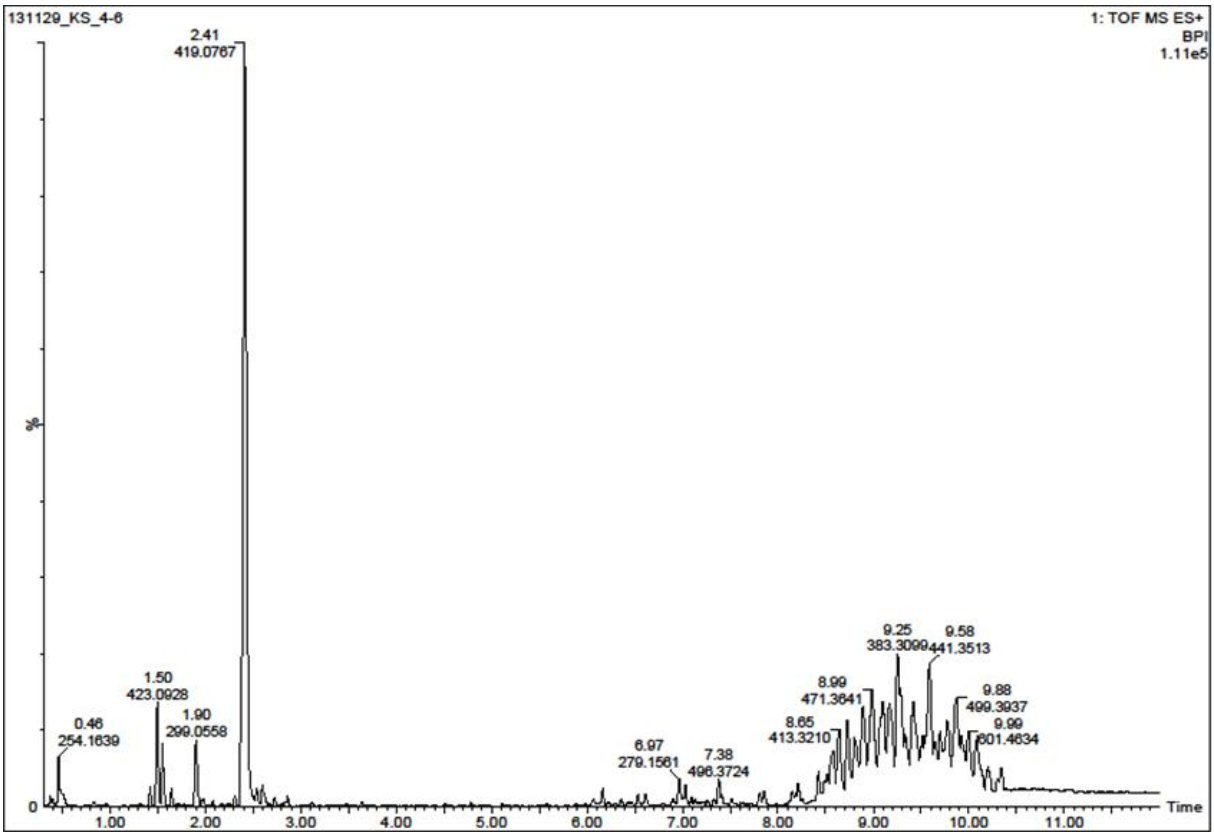

Figure 31: HR-MS chromatogram of *G. barretti* collected in Borgenfjorden, Trondheimsfjorden 14.05.13. ESI in positive mode.

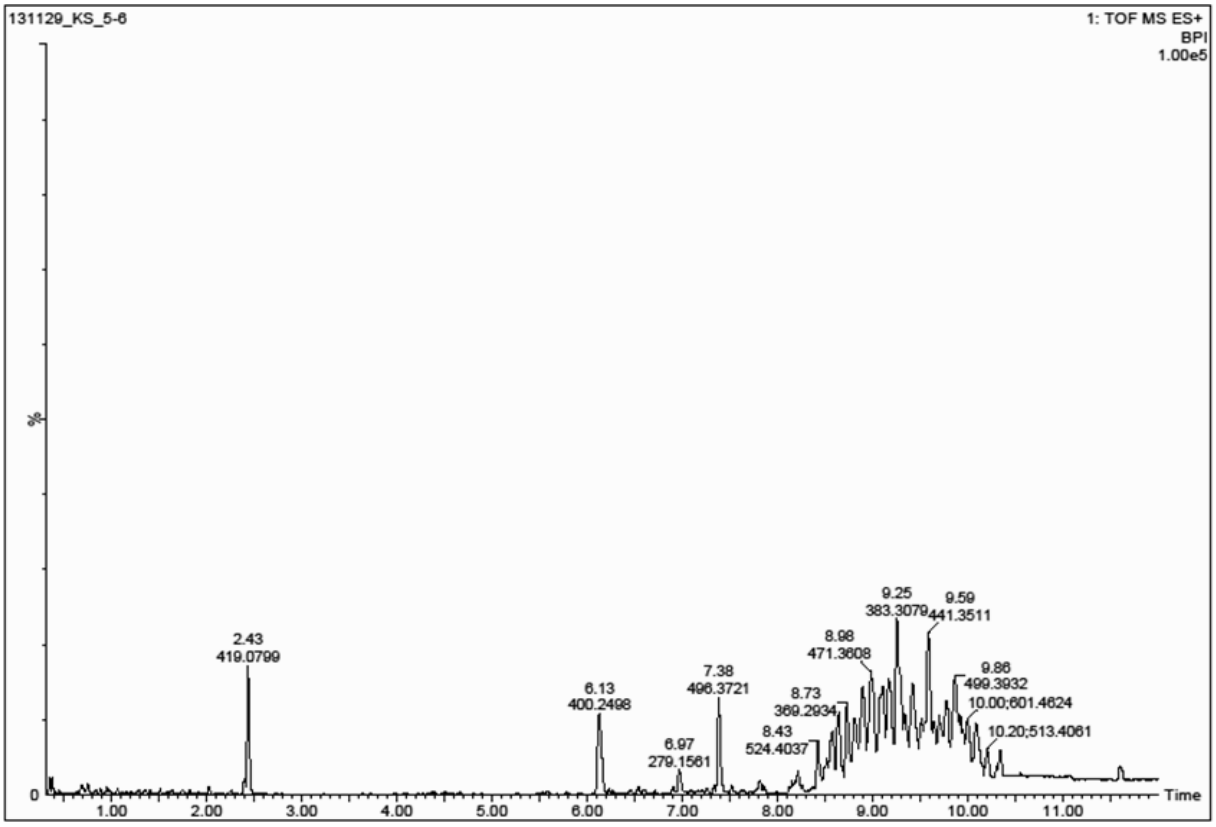

Figure 32: HR-MS chromatogram of *G. macandrewii* collected in Bliksvær, Nordland 11.05.13. ESI in positive mode.

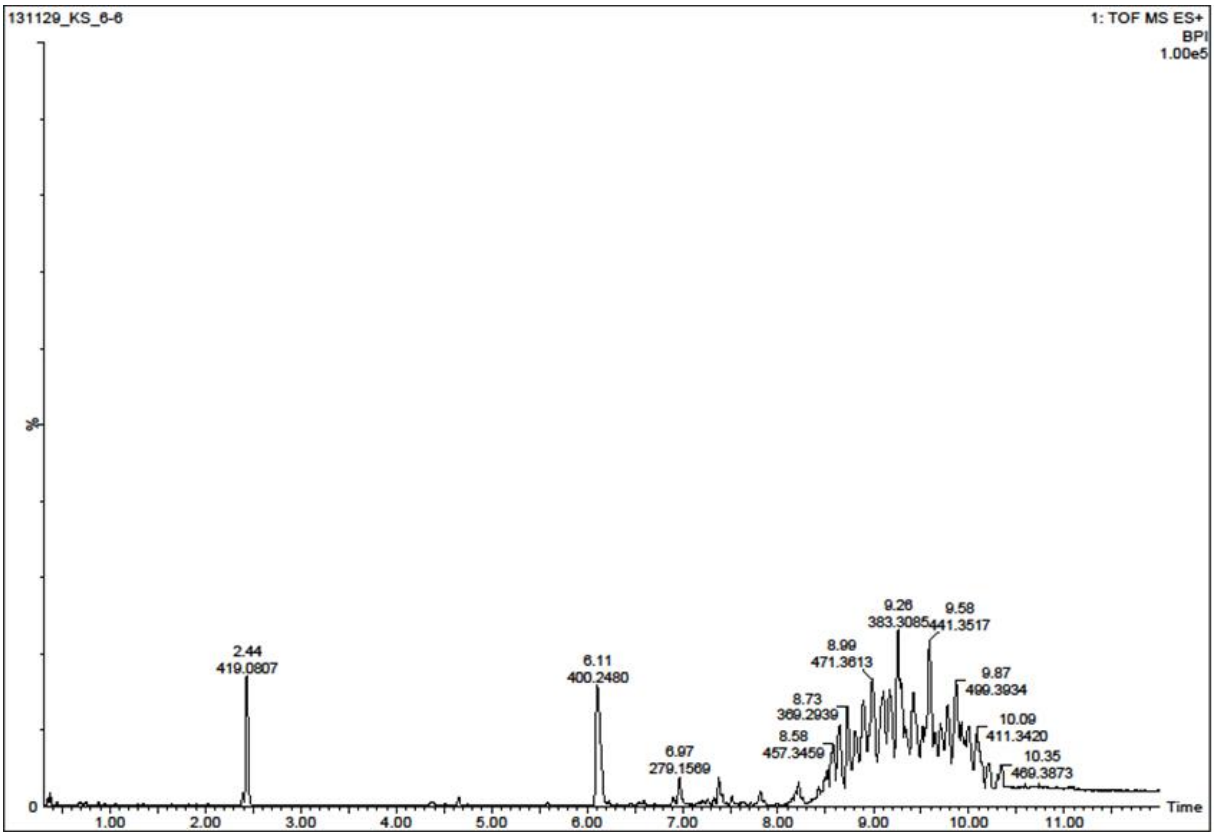

Figure 33: HR-MS chromatogram of *G. macandrewii* collected in Saltfjorden, Nordland 09.05.13. ESI in positive mode.