UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

Bioprospecting of Arctic marine microorganisms

Exploring microbial secondary metabolite production using the one strain-many compounds approach: isolation and characterization of secondary metabolites

Venke Kristoffersen

A dissertation for the degree of Philosophiae Doctor, October 2021

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Tromsø

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Summary

Natural products have been used by humans since ancient times as benefactors for improved health. Prior to modern medicine and chemistry, these compounds remained hidden in the plants, animals and other organisms used to heal inflammation, wounds, headache and stomachache among other conditions. Since the start of modern drug discovery with isolation of morphine in 1805, numerous natural products have been isolated from plants, animals, macroorganisms and microorganisms. Today, natural products, or their derivatives, are used as pharmaceuticals within a wide range of therapeutic areas, including cancer, pathogenic infections, inflammation and pain.

Microbial natural products have played a particularly important role in the field of antibiotics. The discovery of penicillin from the *Pencillium rubens* fungus by Alexander Fleming in 1928 marked the beginning of the "Golden Age" of antibiotics that lasted until 1962, where most antibiotic classes in clinical use today were discovered.

Several marketed drugs originate from marine microorganisms. Marine microorganisms are underexplored, thus representing a potential source for discovering novel bioactive compounds. In this project, Arctic marine microorganisms were fermented under different conditions based on the OSMAC approach and evaluated for their production of antibacterial and cytotoxic compounds.

In paper I, a *Pseudomonas* sp. bacterium was cultivated in different growth media. The fermentation extracts were fractionated and tested for bioactivity, revealing different bioactivity profiles of the fractions from the different media. Dereplication of the active fractions by UHPLC-HR-MS and molecular networking led to identification of six rhamnolipid compounds, including one novel mono-rhamnolipid. All six compounds had antimicrobial activities, while three had cytotoxic activities.

In paper II, a fractionated extract of the bacterium *Lacinutrix* sp. displayed antibacterial activity. Dereplication of the active fraction resulted in identification of two lyso-ornithine lipids, **1** and **2**. The compounds were isolated and their structures were elucidated with UHPLC-HR-MS and NMR. Bioactivity screening showed that **1** had antibacterial activity, while **2** had cytotoxic activity.

In paper III, the fungus *Digitatispora marina* was fermented under different cultivation conditions. Fermentation extracts were fractionated and bioactivity screening of the fractions revealed antibacterial and cytotoxic activities. UHPLC-HR-MS analysis of the fractions showed a compound with an isotope distribution pattern for an ion with a single chlorine atom. The compound was isolated, and structure elucidation with NMR identified it as chlovalicin B. Its bioactive properties were broadly evaluated, revealing it had weak cytotoxic activity but no antimicrobial activities.

Acknowledgement

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List of abbreviations

antiSMASH	antibiotics and secondary metabolite analysis shell
BC	before Christ
BGC	biosynthetic gene cluster
BLAST	basic local alignment search tool
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
e.g.	exempli gratia
EPA	eicosapentaenoic acid
ESI	electrospray ionization
et al.	et alia
etc.	et cetera
FDA	Food and Drug Administration
GNPS	Global Natural Product Social Molecular Networking
HPLC	high performance liquid chromatography
HR	high resolution
HTS	high throughput screening
log P	octanol-water coefficient
LPS	lipopolysaccharide
Mbp	million base pair
MMAE	monomethyl auristatin E
MMOA	molecular mechanism of action
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthase
OSMAC	one strain-many compounds
PKS	polyketide synthase
prep-HPLC	preparative high performance liquid chromatography
PRISM	prediction informatics for secondary metabolomes
Q-TOF	quadrupole-time of flight
RNA	ribonucleic acid
Ro5	Lipinski`s rule of 5
RP	reverse phase
sp.	species
spp.	species pluralis
s.s.	*sensu stricto*
UHPLC	ultra-high performance liquid chromatography
UV/Vis	ultraviolet/visible
WHO	World Health Organization

List of publications

Paper I

Venke Kristoffersen, Teppo Rämä, Johan Isaksson, Jeanette Hammer Andersen, William H. Gerwick and Espen Hansen.
Characterization of Rhamnolipids Produced by an Arctic Marine Bacterium from the *Pseudomonas fluorescence* Group
Marine Drugs **2018**, *16*(5), 163.

Paper II

Venke Kristoffersen, Marte Jenssen, Heba Raid Jawad, Johan Isaksson, Espen H. Hansen, Teppo Rämä, Kine Ø. Hansen and Jeanette Hammer Andersen.
Two Novel Lyso-Ornithine Lipids Isolated from an Arctic Marine *Lacinutrix* sp. Bacterium
Molecules **2021**, *26*(17), 5295.

Paper III

Marte Jenssen, Venke Kristoffersen, Kumar Motiram-Corral, Johan Isaksson, Teppo Rämä, Jeanette H. Andersen, Espen H. Hansen and Kine Østnes Hansen.
Chlovalicin B, a Chlorinated Sesquiterpene Isolated from the Arctic Marine Mushroom *Digitatispora marina*
In preparation

Contributions

	Paper I	**Paper II**	**Paper III**
Concept and idea	VK, TR, JHA, EH	VK, MJ, TR, EHH, JHA	MJ, VK, TR, JHA, EHH
Study design and methods	VK, TR, JHA, WHG, EH	VK, MJ, KØH, EHH, JHA	MJ, VK, TR, JHA, EHH, KØH
Data gathering and interpretation	VK, JI, EH	VK, MJ, HRJ, JI, KØH	MJ, KMC, VK, JI, EHH, KØH
Manuscript preparation	VK, TR, JI, JHA, WHG, EH	VK, MJ, HRJ, JI, TR, KØH, EHH, JHA	MJ, KMC, JHA, EHH, KØH

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1 The search for new drugs from nature

1.1 Natural products

Natural products are compounds produced by living organisms, e.g. plants, animals and microorganisms. They played an important role in ancient medicine and are also essential in today's modern medicine. Particularly plants, but also animal-derived products were used for treating diseases in the distant past [1]. Thousands of plants have been described, which have been used for millennia in Europe and Asia for treatment of cough, inflammation, wounds, headache and stomachache. Examples of ancient remedies are found in the medical work "Hippocratic Corpus" by Hippocrates from Greece, circa 460– 370 BC, who described the use of more than 300 medicinal plants, and there is a 5000-year-old description of 12 drugs developed from 250 plants from Nagpur in India. Fossils studies from Kurdistan in Iraq revealed that plants have likely been used as medicine for more than 60 000 years [2-4]. The chemicals responsible for the therapeutic effect of the medicinal plants were however not known before the development of modern chemistry in the eighteenth and nineteenth centuries. The introduction to modern drug discovery came in 1805, when morphine was isolated from the opium plant *Papaver somniferum* (Figure 1) by the German pharmacist Friedrich Wilhelm Adam Sertürner. Morphine was the first natural product used in its purified form, and it was followed by isolation of other drugs such as cocaine, codeine, digitoxin and quinine in the 1800s [4,5]. Another hallmark within drug discovery from nature was the discovery of the antibiotic penicillin in 1928 by Alexander Fleming. This marked the start of the "Golden Age" of antibiotics that followed the next decades and peaked in the 1950s. Between 1940 and 1962, more than 20 new classes of antibiotics were marketed [6].

Figure 1. *Papaver somniferum*, the plant that morphine was isolated from. Photo: copied from Alchetron [7].

Natural products are categorized into primary and secondary metabolites. Primary metabolites are necessary for the immediately survival of the organism, e.g. for growth, development and reproduction. Secondary metabolites are considered to provide the producing organism an advantage for long time survival, e.g. for defense against other organisms, competition for space and food, or as signaling molecules [8,9]. Secondary metabolites are known to display a broad range of bioactivities, including anticancer, antibiofilm, antibacterial, antifungal, antiviral and anti-inflammatory activities among others. They are structurally diverse, from the simpler structure of lovastatin to the more complex structure of palytoxin (Figure 2).

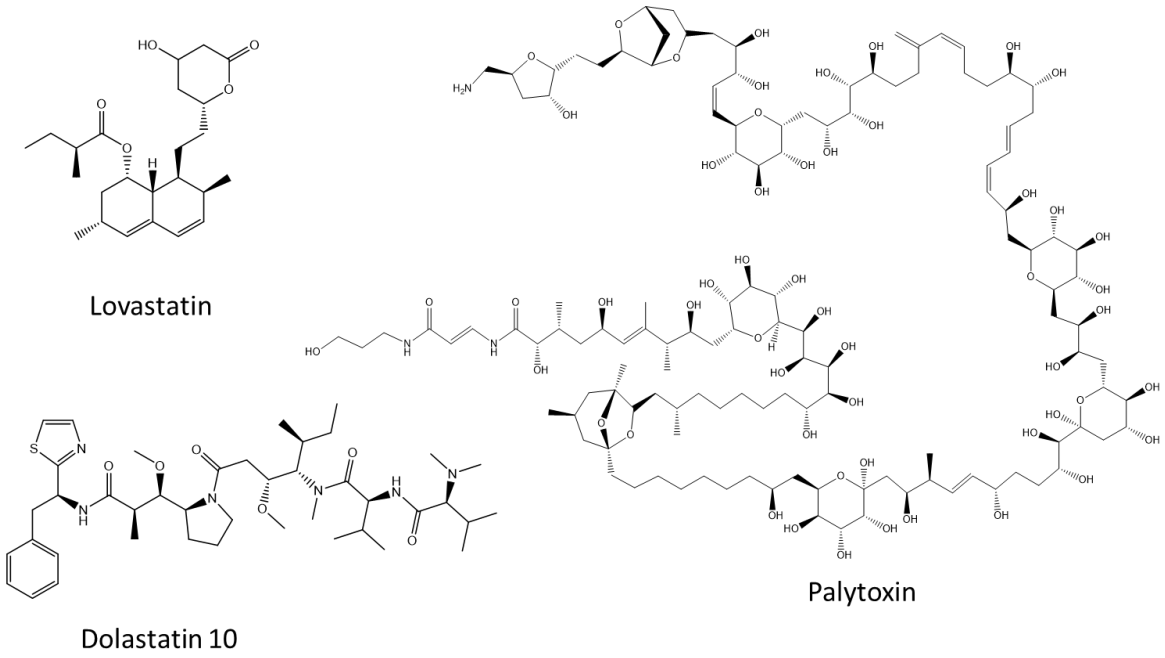


Figure 2. Structurally diverse secondary metabolites. Cholesterol-lowering lovastatin is produced by the fungus *Aspergillus terreus* [10]. Dolastatin 10 was isolated from the sea hare *Dolabella auricularia*. Synthetic analogues are used as anticancer agents [11]. Palytoxin is a highly toxic compound first isolated from marine *Palythoa tuberculosa* [12].

1.1.1 Bioprospecting

Numerous natural products have been isolated and today more than 400 000 are registered in various databases [13]. As reported by Pye et al. (2017) [14], the number of natural products isolated from microorganisms and marine derived organisms is increasing, from a few a year in the 1940s, to averagely 1600 new natural products every year for the last three decades. This is a result of bioprospecting, which can be defined as the systematic search for, and development of biologically active compounds from nature that can be commercialized [15]. Organisms commonly explored for bioactive natural products include plants, microorganisms, invertebrates, algae, fish etc. Advances in analytical technologies and development of methods within genomics, proteomics and metabolomics over the last decades have

contributed to important information regarding biosynthesis of natural products [16]. Another important development in bioprospecting was high throughput screening (HTS) that was implemented in the 1980s. Automation of screening libraries made it possible to screen a high number of samples in a short amount of time. Microtiter plates with 96 wells was the standard format in the beginning of the HTS period, but with advances in robotic systems, 384, 1536, 3456 and even 6144 well-formats are used today [17,18]. Bioprospecting has been highly successful and has resulted in a diversity of marketed drugs originating from natural products, such as anticancer drugs, antibiotics, painkillers, hypocholesterolemic agents, anti-coagulants and anti-depressant drugs among others [19].

1.1.2 Properties of natural products versus synthetic compounds

The search for, and development of drugs from nature is a time consuming and costly process. Hence, despite of many successful stories of natural products being developed into drugs, the pharmaceutical industry shifted its focus from natural product research to combinatorial chemistry in the 1980-1990s in an attempt to generate a strategy that would enable faster and cheaper development of new drugs [20]. In combinatorial chemistry, numerous chemical building blocks can be used to synthesize different compounds simultaneously, to acquire a chemical library that can contain up to millions of analogues [21]. However, the combinatorial chemistry approach did not give the wanted results and as an attempt to increase the rate of new drugs, the Lipinski`s rule of 5 (Ro5) was introduced in 1997. Lipinski et al. (1997) [22] examined the physicochemical properties of compounds that had made it from phase I to phase II in clinical trials and came up with four criteria a compound should meet to be more drug-like. According to the Ro5, a compound should have:

- mass less than 500 Daltons
- 5 or less hydrogen bond donors
- 10 or less hydrogen bond acceptors
- high lipophilicity with an octanol-water partition coefficient (log P) not greater than 5

The introduction of the Ro5 influenced the way drug research was conducted, but it is now clear that the Ro5 should be considered as guidelines and not rules. They have some limitations, and as stated by Shultz (2018) [23], the criteria would have been different if they were defined today. Schultz (2018) analyzed the FDA approved drugs from 1900-2017. He found that the mass and hydrogen bond acceptors had increased in new drugs compared to the average in 1997 when the Ro5 was introduced. A limitation with the Ro5 criteria is that they only apply to orally administered drugs, and only 50 % of FDA approved drugs are taken orally. Of these, about 20 % violate at least one of the parameters of the Ro5. The Ro5 do not apply to drugs that are substrates for transporters or administered with other routes (intravenous, intrathecal, intramuscular etc.), which natural products often are [24,25]. Ganesan (2008)

[26] analyzed 24 natural products that were developed into drugs between 1970 and 2006 according to the Ro5. He found that of the four parameters, log P seemed to be the most precise, indicating that low lipophilicity is essential for a drug. To keep a low lipophilicity with increasing molecular weight, polar functional groups are required. Nature has accomplished this, but it is not a straightforward process for synthetic compounds. Natural products can also violate the hydrogen bond donor and acceptor parameter by forming intramolecular H-bond, which is difficult to achieve through combinatorial chemistry. Feher and Schmith (2003) [27] compared combinatorial compounds with natural products and drugs. They found that combinatorial compounds on average had less chiral centers, higher molecular size and more flexibility than drugs and natural products. It is clearly not easy to compete with nature, and after two decades with combinatorial chemistry not being as successful as hoped, natural product research was brought back into focus. Between 1981-2019, of all new small-molecule drugs (excluded vaccines and large peptides (>50 residues)) around 70 % originated from natural products, including unaltered natural products, derivatives of natural products and synthetic compounds mimicking natural products, demonstrating the important contribution of natural products within the field of drug discovery [19].

1.2 The marine environment

To avoid rediscovering already known compounds, one strategy is to investigate less explored places and organisms in the search for novel compounds. As most of the search to date has been conducted in the terrestrial environment, the ocean represents a potential source for novel chemistry. More than 70 % of the earth's surface consists of ocean, with biological and chemical diversity that differs from the terrestrial environment. The ocean is less explored simply because the terrestrial environment is easier to access. In contrast to the thousands of terrestrial plants known to be used in ancient medicine, only a few algae are described [28]. It was not before the 1970s that natural product research focused on the marine environment as a result of development of diving equipment. Further development of manned submersibles in the 1980s and remotely operated underwater vehicles in the 1990s made exploration of unique places in the ocean possible [28]. The ocean contains a diversity of organisms, some that live under stressful and changing conditions, with high pressure and high salinity [29]. Adaption to these conditions has potentially led to the biosynthesis of metabolites exclusive to these environments. Since the marine environment is less explored than the terrestrial environment, the prospect is that we will see more new drugs originating from marine organisms.

1.2.1 Drugs from the marine environment

The first marketed drug originating from marine organisms was the anticancer drug cytarabine. It is a derivative of a pyrimidine nucleoside initially isolated from the Caribbean sponge *Tectitethya crypta* (Figure 3A) in 1951. It was FDA approved in 1969 and has been used to treat cancer for over 50 years

[16,30]. Other anticancer agents from the marine environment include eribulin mesylate, trabectedin and lurbinectedin. Eribulin mesylate was FDA approved in 2010 and is a derivative of halichondrin B, isolated from the marine sponge *Halichondria okadai* in 1986 [11,16]. Trabectedin was isolated from the Caribbean and Mediterranean Sea tunicate *Ecteinascidia turbinata* in the 1960s and FDA approved in 2015 [30]. Lurbinectedin is a synthetic analogue of ET-736, also isolated from *E. turbinata*, and was FDA approved in 2020 [11]. Several anticancer agents on the market are antibody conjugates, consisting of an antibody linked to monomethyl auristatin E (MMAE). MMAE is a synthetic analogue of dolastatin 10 (Figure 2), a peptide that was isolated from the sea hare *Dolabella auricularia* (Figure 3B) in 1987. These antibody conjugate drugs include brentuximab vedotin, FDA approved in 2011 [11] and polatuzumab vedotin and enfortumab vedotin, both FDA approved in 2019. Belantamab mafodotin was FDA approved in 2020 and is an antibody conjugate with an antibody linked to monomethyl auristatin F, another synthetic analogue of dolastatin 10 [11]. Drugs from marine organisms also include the anti-viral agent vidarabine and the pain killer ziconotide. Vidarabine was FDA approved in 1976 and originates from the same sponge as cytarabine. Ziconotide was isolated from the venom of the cone snail *Conus magus* (Figure 3C). It was FDA approved in 2004 and reached the market as an unaltered natural product [30].

There are also several marine drugs on the market isolated from various fish, for reducing blood triglyceride levels For example Lovaza (containing mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), Vascepa (the only omega-3-product that exclusively contains EPA) and Epanova (a mixture of carboxylic acids, mainly EPA and DHA), approved by FDA in 2004, 2012 and 2014, respectively [31].

Figure 3. Marine organisms as sources for drugs. A: The sponge *Tectitethya crypta* [32], B: Sea hare *Dolabella auricularia* [33], C: Cone snail *Conus magus* [34]. All photos copied from Alchetron.

The majority of approved drugs originating from marine organisms have been mentioned here, and they are all reported to be isolated from macroorganisms such as invertebrates and fish. However, several of these compounds are likely of microbial origin. Today it is known that trabectedin is produced by the bacterium *Candidatus Endoecteinascida frumentensis* [35], and that dolastatin 10 is produced by cyanobacteria [36,37]. Dolastatin 10 is a polyketide synthase (PKS) and nonribosomal peptide synthase

(NRPS) hybrid product. PKS and NRPS products are among the most abundant secondary metabolites isolated from fungi and bacteria [38-41]. Several of the marine compounds that have been developed into marketed drugs were isolated from sponges. Up to 40 % of the sponges' volume can consist of microorganisms, and many of the bioactive compounds from sponges are a result of symbiosis with their microorganisms, suggesting that they might be produced by symbiotic microorganisms [42,43]. This is thought to be true for the sponge-isolated halichondrin B, as the structure resembles known products from PKS [38]. The structure is shown in figure 8 in section 1.6 where the development of halichondrin B into a drug is described more in detail.

1.2.2 Drugs from microorganisms

The story of drugs from microorganisms began in 1928, when Alexander Fleming discovered penicillin. It was isolated from a terrestrial fungus for a long time believed to be *Penicillium chrysogenum* (previously known as *Penicillium notatum*) [44], but in 2011, Houbraken et al. (2011) [45] identified the strain as *Penicillium rubens*. Many important drugs used today originate from microorganisms, such as the antibiotics tetracyclines from *Streptomyce*s spp, *Dactosporangium* spp. and *Actinomadura brunnea*, the antibiotic streptomycin, produced by 1 % of soil actinomycetes, the antimigraine ergotamine from *Claviceps* spp. and the immunosuppressant cyclosporine A from *Tolypocladium* spp. [46]. Most of the microbial drugs are developed from secondary metabolites isolated from terrestrial actinomycetes and fungi. Why should we change the focus from successful terrestrial microorganisms to marine microorganisms? The antibiotic daptomycin was discovered after screening 10^7 soil actinomycetes, and to discover novel antibiotics from soil actinomycetes it is estimated that new 10^7 bacteria must be screened [47]. Hence, a good reason to focus on marine microorganisms (Figure 4) is that they are less studied. More importantly, as several of the marketed marine drugs are proved to, or believed to originate from microorganisms, it demonstrates that the marine microorganisms have already been important contributors to new drugs. Several compounds originating from marine bacteria are also in clinical trials today. The anticancer agent salinosporamide A, isolated from the marine actinomycete *Salinispora tropica,* is in phase III, and several compounds originating from marine cyanobacteria are in phase I and II in clinical trials as anticancer agents [48,49].

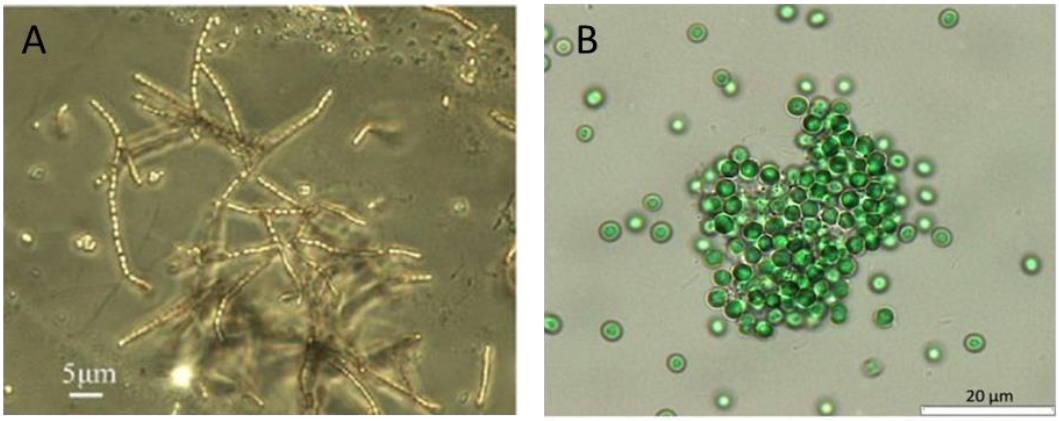


Figure 4. Marine microorganisms. A: Marine actinomycete *Streptomyces chumphonensi*. Photo: copied from Hu et al. (2019) [50]. B: Marine cyanobacteria *Synechocystis salina*. Photo: copied from Costa et al. (2014) [51].

Regarding marine fungi, the literature often states that no marketed drugs have originated from this kingdom, at least no drugs from true marine fungi. Nonetheless, in 1945, Giuseppe Brotzu discovered antibacterial compounds produced by the fungus *Cephalosporium acremonium* (now known as *Acremonium chrysogenum*), isolated from a bay by the sewer in Sardinia. This led to the isolation of cephalosporin N, P and C a few years later, marking the beginning of the development of cephalosporins, which are still used as antibiotics [52-56]. Although cephalosporin was originally isolated from a fungus isolated from the sea, *A. chrysogenum* is considered to be a marine-derived fungus and not a true marine fungus. It is therefore debatable if the cephalosporins are of marine origin or not, but they were indeed isolated from a fungus isolated from the marine environment and are therefore sometimes referred to as being the first drugs isolated from the marine environment [57].

1.2.3 Marine fungi

Many fungi found in the ocean are also found in terrestrial environments, proving they can live both in water and on land [58]. As reviewed by Pang et al. (2016) [57], various definitions have been used to classify marine fungi (Figure 5). Kohlmeyer (1974) [59] proposed a definition in the 1970s which has frequently been used. It states that obligate marine fungi are “those that grow and sporulate exclusively in a marine or estuarine habitat” and facultative marine fungi are “those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment”. (Sporulation is the production of sexual or asexual spores and is hence related to reproduction and dispersal of fungi). This implies that fungi found in both marine and non-marine environments should not be considered marine. A much broader term in fungi classification is the use of “marine-derived” fungi, which was introduced in the 1990s and has been a popular term within natural products research. This term is based on the environment the fungi are isolated from and does not reveal anything about the ecology of the fungi; if they are obligate or facultative marine, or actually terrestrial. The result of this definition is that a fungus

classified as marine-derived may not be of marine origin at all. For example, a spore from a non-marine fungus that has been blown to the sea and later been isolated from the sea can be classified as marine-derived, when it is actually a terrestrial or freshwater fungus [60,61]. In 2014, Overy et al. (2014) [60] introduced the term marine fungi *sensu stricto*, stating that marine fungi are fungi that exclusively live in marine environments, similar to the obligate marine fungi definition of Kohlmeyer (1974). In 2016, Pang et al. (2016) [57] suggested a classification in-between the strict definition of obligate (and *sensu stricto*) marine fungi, and the "marine-derived" fungi term. This classification of a marine fungus is based on the following three criteria, where a marine fungus should fall within one of them:

1. It is any fungus that is recovered repeatedly from marine habitats because it is able to grow and sporulate (on substrata) in marine environments
2. It forms symbiotic relationships with other marine organisms
3. It is shown to adapt and evolve at the genetic level or be metabolically active in marine environments

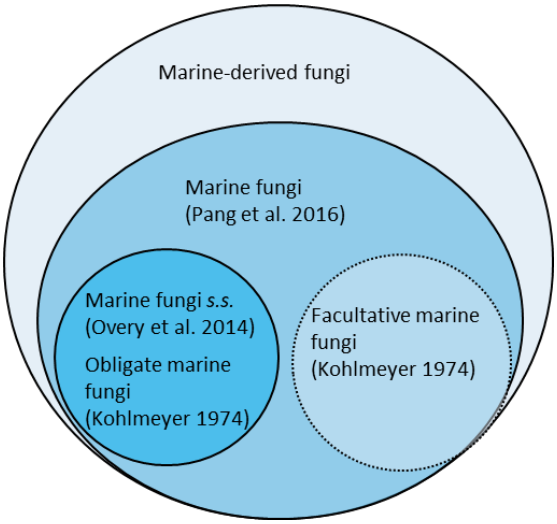


Figure 5. Classification of marine fungi.
Obligate and *sensu stricto* (*s.s.*) marine fungi: grow and sporulate only in the marine environment. Marine fungi: repeatedly isolated from the marine environment and are adapted to the marine environment. Marine-derived fungi: isolated from the marine environment, but can be non-marine fungi as the ecology might be unknown. Facultative marine fungi: non-marine fungi able to grow in the marine environment [57,59-61]. Adapted from a figure provided by Teppo Rämä.

The studies of marine fungi were limited for many years due to lack of knowledge of isolation methods and cultivation conditions, so until 2010, only 690 compounds from marine fungi were reported [62]. More focus has been put into this field the last decade, with for example 494 and 470 new secondary metabolites reported from marine fungi in 2015 and 2016, respectively [63]. In 2019, 47 % of all new reported marine natural products were from fungi, with around 700 fungal metabolites [64]. As reviewed by Overy et al. (2014) [60], of all secondary metabolites reported from marine fungi up to 2014, only a few of them were isolated from marine fungi *sensu stricto*. The relatively few secondary metabolites isolated from marine fungi, and particularly marine fungi *sensu stricto*, indicate that there is a potential

for discovering novel bioactive fungal secondary metabolites. For example is the anticancer agent plinabulin, a synthetic analogue of a halimide produced by marine and terrestrial *Aspergillus* spp., in phase III in clinical trials as anticancer agent [65].

1.2.4 Marine bacteria

Bacteria are highly abundant in the ocean, and 1 µL of seawater can contain as much as 1000 bacteria [66]. However, only a small portion of the bacteria counted with microscope can be cultivated on agar plates. This difference is known as the "great plate-count anomaly" [67]. Based on the number of known bacteria from ribosomal RNA sequences, counting of bacteria with epifluorescence microscopy, and observation on how many marine bacteria grow on standard cultivation media, it is estimated that less than 1 % of the bacteria in the ocean have been cultivated, and that many major lineages remain to be cultivated. Some bacteria may not grow in the laboratory under standard cultivation methods, as the medium can for example be toxic. In addition, bacteria are often cultivated alone in the laboratory, which can destroy the communication and interactions between other bacteria and organisms. Hence, nutrients and chemical signals that are needed for growth, which the bacteria get from interaction with other organisms in the natural environment will not be present [16,68]. The contradiction is that to cultivate the bacteria, we need to know how to cultivate them, but to obtain the knowledge on how to do that, we firstly need to cultivate them. Approaches to overcome this problem can be in situ cultivation and co-cultivation. These approaches also apply to fungi.

1.2.5 In situ cultivation

In in situ cultivation the microorganisms are taken from their natural habitat, diluted and put into diffusion chambers before they are placed back into their natural environment where nutrients and chemical signals can diffuse into the chambers [69]. The use of isolation chips has increased the microbial recovery extensively and has also been beneficial in the discovery of bioactive compounds [70,71]. The antibacterial compound teixobactin was for example isolated from the soil bacteria *Eleftheria terrae* grown in an isolation chip in 2015. Teixobactin has been active against several bacteria and has a unique mechanism of action. It is hoped that it will enter clinical trials, as it could be valuable in the fight against antibiotic resistance. Teixobactin has been widely studied since its discovery, but has not entered clinical trials yet [72,73].

1.2.6 Co-cultivation

The marine microorganisms have to compete for substrates and are exposed to chemical signals from potential competitors in nature. These interactions are thought to be the main factors for triggering the production of secondary metabolites. Two or more microorganism are cultivated together in co-culivation. The interactions between the microorganisms can mimic their natural environment and

provide substances necessary for them to grow and possibly trigger the production of bioactive secondary metabolites not observed when they are cultivated alone. Co-cultivation of marine microorganisms has been reported to enhance and induce the production of bioactive secondary metabolites [74,75].

1.3 Secondary metabolite production

In an enclosed vessel, the growth cycle of bacteria and fungi includes a lag phase, an exponential growth phase, a stationary phase and a death phase (Figure 6). In the lag phase, the microorganisms adapt to the cultivation conditions and start producing RNA, enzymes and molecules needed for cell division and growth. In the exponential growth phase, each organism cell divides to form two more cells and so on. When nutrition is depleted or waste products inhibit growth, they reach the stationary phase where there is no net decrease or increase in the number of microorganisms. After a period with decreasing nutrients and accumulation of waste products, more and more cells will die and they enter the death phase [76]. Secondary metabolite production usually happens in the late exponential growth phase and stationary phase. As a result of less available nutrients, the microorganisms switch from producing biomass to secondary metabolites [77,78].

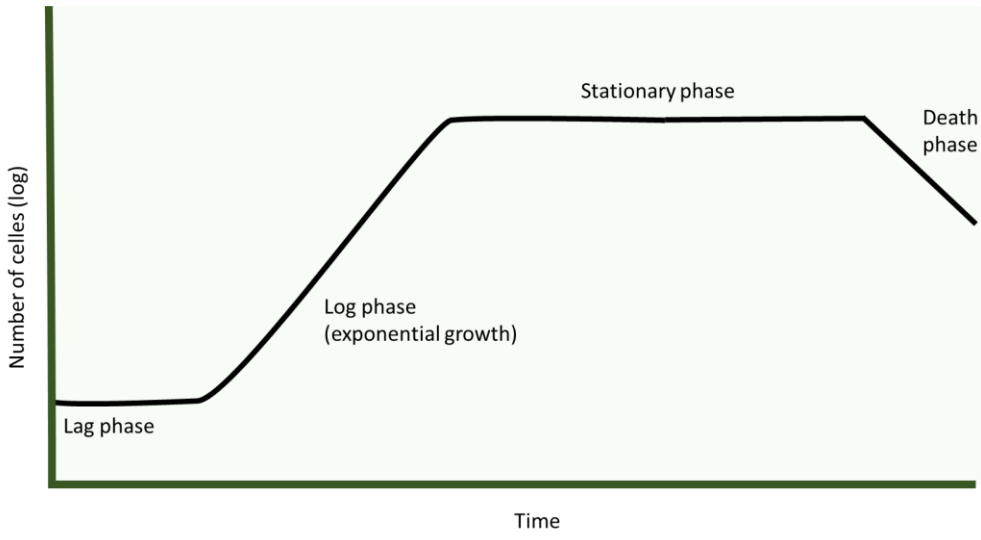


Figure 6. Growth cycle of marine microorganisms in an enclosed vessel. This includes a lag phase, an exponential growth phase, a stationary phase and a death phase. Figure adapted from Madigan and Martinko (2006) [76].

1.3.1 Biosynthetic gene cluster

Many secondary metabolites are coded by biosynthetic gene clusters (BGCs) in bacteria and fungi. A BGC is two or more genes clustered together which collectively encode a biosynthetic pathway for the production of a secondary metabolite. All the enzymes and regulatory genes necessary for the encoding of a secondary metabolite are included in the BGC. NRPS and PKS products are two major classes of secondary metabolites produced by enzymes encoded in a BGC [79,80]. They display a wide range of structural chemistry and bioactivities, and many of them have been developed into drugs. Some of the most important drugs in use are NRPS products, such as the antibiotics penicillins, cephalosporins and daptomycin, and vancomycin and cyclosporine A with immunosuppressant and anti-inflammatory activities [79]. Drugs that origin from PKS include lovastatin for lowering cholesterol, the antibiotics tetracyclines and erythromycin A, and the anticancer compound doxorubin [80]. This makes NRPS and PKS products popular targets in the search for bioactive secondary metabolites.

1.3.2 Genome mining unveils hidden potential

Genome mining has revealed that many microorganisms have the potential to produce secondary metabolites not discovered yet. Genome mining is the process of identifying conserved BGCs within the genome of a sequenced organism. It involves the identification of genes or domains that are very specific for known biosynthetic pathways, such as the major secondary metabolite classes polyketides, nonribosomal peptides, ribosomally synthesized and post translationally modified peptides, alkaloids and terpenes [81]. The genome is annotated with for example BLAST (Basic Local Alignment Search Tool) before the BGC is identified with tools such as antiSMASH (antibiotics and secondary metabolite analysis shell) and PRISM (PRediction Informatics for Secondary Metabolomes) commonly used for fungi and bacteria [82]. Conserved Domain Database can be used to predict the structures of NRPS and PKS products based on comparison of known metabolites. Since many BGCs are silenced under standard cultivation conditions, the challenge is to make the microorganisms express these genes to find the compounds corresponding to the identified BGCs. As reviewed by Rutledge and Challis (2015) [83], different strategies are used in an effort to activate these genes. This includes altering the cultivation conditions, engineering the transcription and translation machinery, manipulate transcriptional regulators, manipulate pathway-specific regulators, and the use of heterologous expression. In this project, the focus was on altering the cultivation conditions, as described in section 2.1.

1.4 The importance of new drugs

1.4.1 Antibiotic resistance

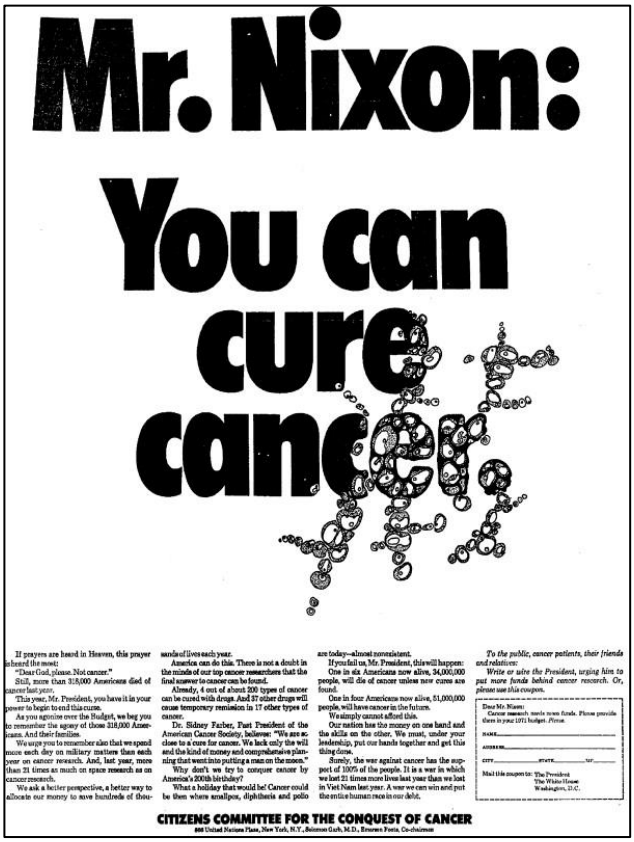
Pathogenic microorganism's resistance to antibiotics has emerged as a serious health concern. Some bacteria have resistance to most of the antibiotic classes on the market, resulting in infections that are

difficult to treat. This leads to longer hospital stays and deaths from untreatable infections. Globally, antibiotic resistant bacteria are estimated to cause 700 000 deaths every year, a number that is predicted to reach 10 million by 2050. The reasons behind this dramatic trend are many and complex, but include antibiotic drug research not being prioritized by the pharmaceutical industry, and extensive use and misuse of antibiotics within agriculture and medicine [84,85]. Between 1940 and into the 1960s, more than 20 classes of antibiotics were discovered. Major classes include e.g. sulfonamides, β-lactams, tetracyclines and cephalosporins. Over the next three decades, the new antibiotics on the market were derivatives of already known classes. The next classes on the market did not come before 2000 and 2003, with the synthetic compound oxazolidinone in 2000 (linezolid) and cyclic lipopeptide (daptomycin) in 2003 [6]. Oxazolidinones were discovered in the end of the 1970s, and linezolid was a result of a 12-year research program [86]. Daptomycin was isolated from soil actinomycetes in the 1980s. As reported by WHO (2020) [87], of 11 new approved antibiotics since 2017, only two represent a novel class (meropenem-vaborbactam and lefamulin). Meropenem is a synthetic derivative of thienamycin, isolated from *Streptomyces cattleya* in 1976 [88]. Lefamulin is a derivative of pleuromutilin, isolated from the fungus *Clitophilus scyphoides* in the 1950s [19,89]. Although considered to be new classes, the initial discovery was done long time ago, meaning no new marketed classes have been discovered recently. Alexander Fleming warned already in 1945 that the use of antibiotics would lead to resistance, and today several multidrug resistant bacteria exists [90]. Examples are the gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* [84]. In addition, groups of gram-positive bacteria: MRSA and vancomycin-resistant *enterococci* are responsible for infections that are difficult to treat. *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* species belong to a group of multidrug resistant bacteria that has been named ESKAPE pathogens. To beat infections from these bacteria, novel antibiotics are highly needed, as the new antibiotics on the market and in the pipeline do not address these pathogens [87].

1.4.2 Cancer

Cancer represents another global health problem where new drugs are needed. Cancer is an umbrella term, comprising a diverse group of complex diseases. There are many different types of cancer, and they behave different in different organs and cell types. The main development of cancer is due to oncogenes and tumor suppressor genes. Activation and inactivation of these genes by mutations can lead to uncontrolled cell growth and proliferation, resulting in cancer [91]. The “war on cancer” began the December 9th in 1969, when the Citizens’ Committee for the Conquest of Cancer published the article “Mr. Nixon: You can cure cancer” (Figure 7) in The Washington Post and The New York Times. The 23rd of December 1971, President Nixon signed the National Cancer Act. This changed the aspect of cancer research and care globally, as it led to the National Cancer Program, including research

institutes around the world [92]. Despite of great progress and development within cancer research, cancer is still a leading cause of death worldwide. Chemotherapy and radiation are great achievements within modern medicine, threating millions of cancer patients. However, with the increase in aging and population, the number of cancer deaths and new cancer cases rises. Around 19.3 million cases and 10.0 million deaths were estimated in 2020. Female breast cancer followed by lung, colorectal, prostate and stomach cancers are the main groups of cancer, responsible for 46% of new cancer cases. The main cause for death is lung cancer, followed by colorectal, liver, stomach and female breast cancers, contributing to 50.3 % of cancer deaths [93]. This shows that new cancer drugs are still highly needed. Of the cancer drugs used today, 60% originate from nature, including anticancer drugs from marine microorganisms as described in section 1.2.1 [94]. Several compounds from marine microorganisms are also in clinical trials today, showing the important contribution of drugs from marine microorganisms in the fight against cancer.


Mr. Nixon:

You can cure cancer.

If prayers are heard in Heaven, this prayer is heard the most:
"Dear God, please. Not cancer."
Still, more than 318,000 Americans died of cancer last year.
This year, Mr. President, you have it in your power to begin to end this curse.
As you agonize over the Budget, we beg you to remember the agony of those 318,000 Americans. And their families.
We urge you to remember also that we spend more each day on military matters than each year on cancer research. And, last year, more than 21 times as much on space research as on cancer research.
We ask a better perspective, a better way to allocate our money to save hundreds of thousand of lives each year.
America can do this. There is not a doubt in the minds of our top cancer researchers that the final answer to cancer can be found.
Already, 4 out of almost 200 types of cancer can be cured with drugs. And 37 other drugs will cause temporary remission in 17 other types of cancer.
Dr. Sidney Farber, Past President of the American Cancer Society, believes: "We are so close to a cure for cancer. We lack only the will and the kind of money and comprehensive planning that went into putting a man on the moon."
Why don't we try to conquer cancer by America's 200th birthday?
What a holiday that would be! Cancer could be then where smallpox, diphtheria and polio are today—almost nonexistent.
If you fail us, Mr. President, this will happen:
One in six Americans now alive, 34,000,000 people, will die of cancer unless new cures are found.
One in four Americans now alive, 51,000,000 people, will have cancer in the future.
We simply cannot afford this.
Our nation has the money on one hand and the skills on the other. We must, under your leadership, put our hands together and get this thing done.
Surely, the war against cancer has the support of 100% of the people. It is a war in which we lost 21 times more lives last year than we lost in Viet Nam last year. A war we can win and put the entire human race in our debt.
To the public, cancer patients, their friends and relatives:
Write or wire the President, urging him to put more funds behind cancer research. Or, please use this coupon.
Dear Mr. Nixon:
Cancer research needs more funds. Please provide them in your 1971 budget. *Please.*
NAME
ADDRESS
CITY STATE ZIP
Mail this coupon to: The President
The White House
Washington, D.C.
CITIZENS COMMITTEE FOR THE CONQUEST OF CANCER
200 United Nations Plaza, New York, N.Y., Solomon Gub, M.D., Eleanor Poste, Co-chairmen

Figure 7. Article published by the Citizens' Committee for the Conquest of Cancer. Published in Washington Post and The New York Times, 9th of December 1969 Photo: copied from Coleman (2013) [92].

1.5 Biosurfactants

Biosurfactants are compounds produced by microorganisms with surface activity due to their amphiphilic nature. Biosurfactants help to protect the microorganisms against harmful compounds and organisms, by changing the cell composition and thereby the membrane permeability [95,96]. They consist of a polar head, usually a peptide, amino acid, monosaccharide or disaccharide connected to a lipophilic tail, which can be one or more linear, branched, saturated or unsaturated fatty acid. The different building blocks lead to structural diversity and more than 2000 biosurfactants have been described [97]. Glycolipids comprising rhamnolipids and sophorolipid, lipopeptides such as surfactin and lichenysin, and fatty acids are three major classes of biosurfactants. Biosurfactants are used in the food and the cosmetic industries. In food, they are used as emulsifiers, for consistency control and stable solubilization of ingredients [98,99]. As cosmetics, they are used in anti-wrinkle creams, in deodorants,

toothpaste and nail care products due to their antimicrobial properties [100]. Biosurfactants have been studied for use in other applications, such as the pharmaceutical industry, as they are known to display antibacterial, anticancer, antifungal, antiviral and antibiofilm activities. Despite of having potential as pharmaceuticals and being extensively studied, with a few exceptions, they are not used as pharmaceuticals today. The exceptions include among a few others, the clinical used antibiotics daptomycin and polymyxin B and E [101,102]. Contrary to chemical synthetic surfactants, biosurfactants are biodegradable, less toxic and can often tolerate higher temperature and pH values [103]. Chemical surfactants are used in many industrial applications, and biosurfactants could potential be a sustainable replacement, as more than 13 million tons chemical surfactants are used yearly in the world, representing a big environmental problem [104]. Biosurfactants have been evaluated for their potential as antifouling agents and in bioremediation. Biofouling, the growth of microorganisms followed by growth of macroorganisms (algae and invertebrates) is a problem to marine and shipping industries. It can result in e.g. corrosion and degradation of the material and increase the weight and volume of the immersed structures, such as ships and oil rigs. Alemán-Vega et al. (2020) [105] showed that the biosurfactant producing *Bacillus niabensis* sp. reduced the formation of marine biofilm, and that using cell free supernatant of *B. niabensis* in antifouling paint reduced the attachment of macroorganisms with 30% on a painted frame when it was immersed in the ocean. Regarding bioremediation, it is reported that the amount of biosurfactant producing microorganisms have increased in oil contaminated waters [104]. Hence, they have the potential to be used in bioremediation to enhance oil recovery and to remove heavy metals and hydrocarbons from contaminated sites. As reviewed by Nikolova and Gutierrez (2021) [106], surfactin has been used to recover sand trapped oil, and lichenysin recovered up to 40 % of residual oil from sandstone cores compared to 10 % with chemical surfactants. Rhamnolipids have been reported to emulsify petrol and diesel. Although biosurfactants may have the potential as antifouling agents and in bioremediation, much of the research is still at laboratorial stages. To replace the chemical surfactants, the biosurfactants must perform equal or better than today's chemical surfactants, and they must be profitable. To be profitable, the yield of biosurfactants produced by the microorganism should be prominent. For example, *P. aeruginosa* has been engineered to increase its production of rhamnolipids, which are now produced in large scale for commercial use [107]. One issue with *P. aeruginosa* is that it is pathogenic. To achieve a safe production of biosurfactants, it might be possible to genetically alter the pathogenic biosurfactant producing microorganism into a non-pathogenic microorganism or use non-pathogenic hosts to express the synthesis of biosurfactants. Another option is to search for new sources for biosurfactants. Arctic marine microorganisms have been less explored for the production of biosurfactants; hence, they represent a novel source in the search for new producers of biosurfactants, both with the potential of high yield and being non-pathogenic. Another potential advantage with biosurfactants from Artic marine microorganisms is that they are

adapted to cold water, so they might have other properties than biosurfactants isolated from warmer environments. Cold adapted biosurfactants can for example be used as laundry detergents for washing clothes at low temperatures to save energy, or for bioremediation at contaminated sites in cold environments [104].

1.6 From the laboratory to a marketed drug

The road from the discovery of a bioactive metabolite to a marketed pharmaceutical is long. For the few compounds that complete this journey, development normally spans over at least 10-15 years. After isolation and structure elucidation of the bioactive secondary metabolite, there are several steps before it might become a drug. In short, this development will initially often include molecular mode of action (MMOA) determination, analogue production to improve activity and pharmacokinetic properties, and to reduce toxicity/off-targets effects and in vivo efficacy studies in e.g. mice. Some secondary metabolites reach the market as unchanged drugs, but most are synthesized and modified. Optimization can include improving absorption, distribution, metabolism, excretion and toxicity profiles. Chemical modifications can be altering the functional groups or ring systems, alter the saturation, or reduce the structure complexity of the metabolite [108]. Eribulin, a derivative of halichondrin B isolated from a marine sponge, but now known to be produced by cyanobacteria, is a good example of a modified and optimized drug (Figure 8) [109]. The active part of halichondrin B was found by testing various analogues of the molecule, which led to the synthetic eribulin with a simpler structure.

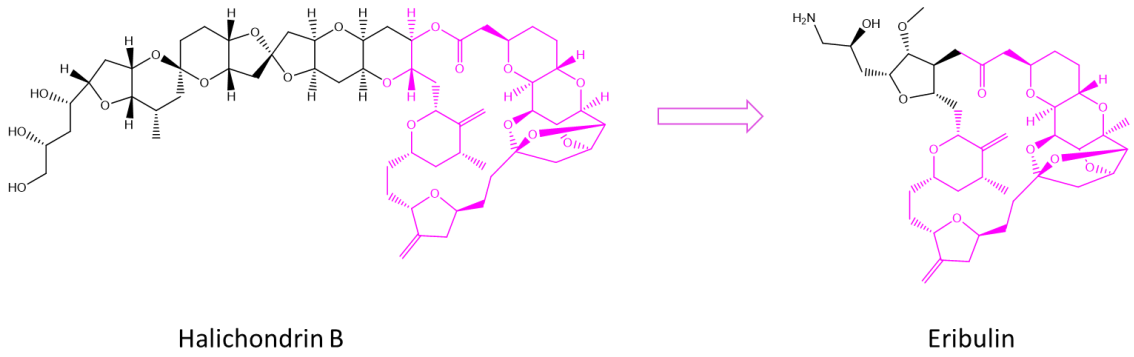


Figure 8. Structures of halichondrin B and eribulin. Halichondrin B was isolated from the marine sponge *Halichondria okadai*, but is now known to be produced by symbiotic cyanobacteria. By testing analogues, the active part of Halichondrin B was found. This led to the synthesis of eribulin, a simpler structure of halichondrin B. Eribulin is in combination with mesylate an anticancer drug. The pink structure shows which part of halichondrin B is included in the synthetic eribulin [109].

When a lead compound is selected, the next step is animal testing to make sure it is safe and truly works before potentially entering clinical trials. Clinical trials are performed on humans and involve three phases. Phase I normally consists of a small group (20-100 people) of healthy people who are given small doses of the drug to test if it is safe. In phase II, the drug is given to a bigger group of people with

the disease (100-500 people). In this phase, the goal is to find the most effective dose and the best delivery method. Most drugs that fail, fail in phase II. In phase III, the drug is usually tested on 1000-5000 people with the disease, to further evaluate the safety and benefits of the drug. If the drug passes phase III, it can be registered, approved and marketed. The drug will still be supervised while it is on the market, which is part of phase IV [110]. All the necessary steps make it difficult to get a compound from the laboratory to the pharmacy, so to develop more drugs, collaboration between academic and pharmaceutical companies is desired [111].

2 Discovery of natural products - the bioprospecting pipeline

As reviewed by Blunt et al. (2016) [112], the cold regions (Arctic and Antarctica) are little explored in the search for natural products, and only 330 of the 25 700 reported marine natural products between 1965 and 2014 were isolated from organisms originating from Arctic and Antarctica. This makes the Arctic a promising source in the search for novel bioactive compounds. In this project, marine microorganisms isolated from the Arctic ocean and the coast of the Northern Norway were cultivated under various conditions to explore their potential for production of bioactive secondary metabolites following the bioprospecting pipeline at Marbio (Figure 9). This pipeline follows a bioassay-guided isolation approach. In this approach, extracts/fractions of the fermented microorganisms are tested for bioactivities in selected bioassays and the active fractions are selected for further work. Dereplication of the active fractions/extracts is performed using ultra-high performance liquid chromatography-high resolution-mass spectrometry (UHPLC-HR-MS) and database searches to identify the compound(s) responsible for the observed activity. A compound with novel structure or novel bioactivity will be isolated with preparative-high performance liquid chromatography (prep-HPLC). The structure of the purified compound(s) is elucidated with HR-MS and nuclear magnetic resonance (NMR) spectroscopy before being retested in the bioassays to evaluate its bioactive profile.

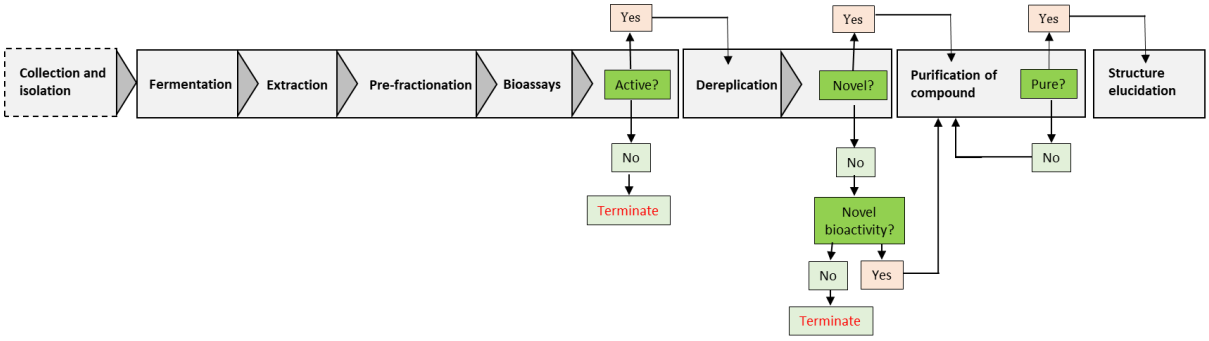


Figure 9. The bioprospecting pipeline at Marbio, which follows a bioassay-guided isolation approach. Adapted from a figure provided by Kine Ø. Hansen.

2.1 Fermentation and extraction of microorganisms

Many of the secondary metabolite encoding genes in microorganisms can be silent under standard cultivation conditions. By changing the cultivation conditions, such as altering the temperature, light, pH, medium composition, shaking, culture flasks and using co-cultivation etc., the number of secondary metabolites produced by one strain can increase. Bode et al. (2002) [113] demonstrated this strategy and named it the One Strain-Many Compounds (OSMAC) approach. The theory is that various cultivation conditions can activate different enzymes responsible for the gene expression of secondary metabolites. The genes are usually regulated at transcription level, where the DNA is transcribed into messenger ribonucleic acid (mRNA), but they can also be regulated at translation level where the mRNA is used to assemble amino acids into a protein, or at protein level where the secondary metabolites are synthesized [114]. As shown by Bode et al. (2002), the fungus *Sphaeropsidale*s sp. F-24.707, known to produce one antifungal spirobisnaphthalene, produced eight new and six known spirobisnaphthalenes when it was cultivated under different conditions. From only six different microorganisms, Bode et al. (2002) managed to isolate more than 100 compounds, demonstrating the potential of microorganisms to produce secondary metabolites and the importance of fermentation conditions.

Liquid-liquid or liquid-solid phase extraction is often used for extraction of microbial secondary metabolites from the fermentation cultures. In liquid-liquid extraction the compounds are separated based on their solubility in two solvents that are immiscible or partially miscible, most often one organic and one inorganic solvent. In liquid-solid phase extraction, the analysts are transferred to the solid phase before being eluted from the solid phase with a liquid [115]. Adsorbent resin is commonly used for recovering secondary metabolites from fermentation cultures [116]. The secondary metabolites are transferred onto the resin before being eluted with an organic solvent such as methanol. Different types of resins can be used, such as Amberlite XAD, Sepabeads SP-850 and Diaion HP-20, which are non-ionic and have affinity for hydrophobic and aromatic compounds [117].

2.2 Prefractionation

Prefractionation is a process where crude extracts are fractionated into less complex samples prior to bioactivity testing. As crude extracts often are complex mixtures of numerous compounds, this step has the following advantages:

- Lowers the chance of masking activity of secondary metabolites that most often are present in low amounts
- Lowers the chance of a sample producing an active result caused by non-specific interactions between components and the assayed subject (e.g. a cell line)

Liquid chromatography, such as FLASH chromatography and HPLC, is a common method used for prefractionation of extracts. This separates the compounds based on their polarity, so the fractions end up with compounds with similar polarity. Normally, the gradient used during prefractionation will go from being highly hydrophilic to becoming highly lipophilic (or the opposite way if a normal phase column is used), to elute all compounds from the column. This causes hydrophilic sample components like salts and carbohydrates to elute early, while lipophilic sample components, such as cell wall lipids, will elute in the later fractions. Secondary metabolites normally have a medium lipophilicity, which makes them elute in fractions collected when the mobile phase holds a medium lipophilicity. It is also important not to divide the extract into too many fractions, as the active compound can be split into too many fractions, thus having too low concentration to display activity in the following bioassays [16].

2.3 Bioactivity testing

Bioassays, an in vivo or in vitro assay setup, can be used to detect the presence of biologically active components in a sample. In the beginning of drug discovery, phenotypic bioassays were commonly used in the search for new drugs. In phenotypic screening, compounds or fractions are tested against cells, tissues or in animals to see if they have the wanted effects, without knowing their MMOA or their molecular targets. Penicillin was for example discovered because it killed bacteria, while the MMOA was determined years later [118]. With the introduction of recombinant technology and genomics, target based screening became a popular screening approach. A target can be a molecule known to be important in a disease (e.g. a kinase), a single gene or a molecular mechanism that has been identified. Compounds can be screened against these targets to see if they have an effect. Both methods have been successful in their own way, as phenotypic based screening has resulted in more first-in-class drugs, while target based screening has resulted in best-in-class drugs [119]. In this project, phenotypic screening was used. Bacterial and fungal extracts and purified compounds were tested against living bacteria, fungus and cancer cells (Figure 10) to evaluate their bioactivities.

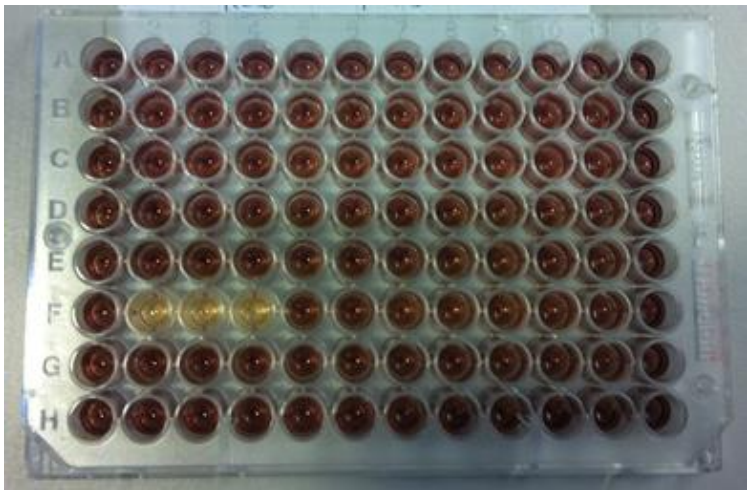


Figure 10. Anticancer assay. Metabolic active cells reduce yellow tetrazolium salt added to the wells in the microtiter plate to a formazan product that is dark blue/purple. Hence, wells with samples without cytotoxicity turn dark blue, while wells with cytotoxic microbial fractions/compounds remain yellow and represent dead cancer cells. Photo: V. Kristoffersen.

2.4 High performance liquid chromatography

HPLC/UHPLC is a valuable technique in natural products research for separating compounds in a complex mixture. The chromatographic system consists of a mobile phase and a stationary phase. The sample is dispersed into the mobile phase, which is pumped through the stationary phase. This results in the compounds in the sample being separated based on their relative affinity towards the stationary phase.

There are four primary HPLC separation methods: normal phase, reverse phase (RP), ion exchange and size exclusion. For the purpose of natural product isolation, RP-HPLC is most frequently used [120]. The most important component in the RP-HPLC system is the column packing material, which forms the base for the separation. Columns with C18 ligands attached to the matrix, commonly silica, is often used as a stationary phase for separation of natural products, where separation is mainly based on hydrophobic interactions between the compounds and the stationary phase. Other stationary phases widely used are fluorophenyl and phenyl-hexyl, which are good for separating aromatic groups due to different retention mechanisms [121]. Separation is achieved using an increasing concentration of an organic gradient, where methanol and acetonitrile are commonly used as organic solvents. The compounds in the polar mobile phase are pushed through the column and are retained by hydrophobic and Van der Waal force interactions with the C18 ligand in the column. Hydrophobic compounds such as fats and lipids are retained longer on the C18 stationary phase, while more polar compounds are eluted first [122]. To detect the compounds, ultraviolet/visible (UV/Vis) detectors or MS detectors can

be used. UV/Vis detectors measure the amount of ultraviolet or visible light that is absorbed by the compounds in the UV/Vis wavelength range (200–600 nm), while in MS the compounds are ionized and detected according to their molecular masses to charge ratio (*m/z*) [123].

For isolation of compounds, prep-HPLC is commonly used, while UHPLC is preferred for analysis of natural product samples as it separates the compounds better. The better separation is achieved as UHPLC has a higher resolution than HPLC due to smaller particles with high surface area. The small particle size in the column makes the backpressure in UHPLC is much higher than in HPLC, with 600-1400 bar versus 400-600 bar [120,124].

2.5 Mass spectrometry and dereplication

An important step when searching for novel compounds is to avoid spending time and resources on rediscovering known and redundant compounds. The process of identifying a known compound in a mixture is known as dereplication. This process normally includes UHPLC-HR-MS analysis and database searches [125,126].

HR-MS is a sensitive, fast and accurate method where a small quantity of a sample is required for good analysis. The compounds are first separated by the UHPLC before being analyzed on an MS coupled to the UHPLC. Electrospray ionization (ESI) is a widely used technique for analysis of natural products. ESI transforms the compounds eluting from the UHPLC into ions and is a soft ionization technique resulting in little fragmentation, hence the intact molecule is usually present. A sample is dissolved in a polar solvent before it is directed into the ion source in the MS as a spray via a needle. Highly charged droplets are formed by a high electrical potential applied to the needle. The droplets are then vaporized by warm nitrogen gas, leading the droplets to break down, resulting in ions that desorb into the gas phase. The ions are then sent by an electrical field to the mass analyzer, for example a Q-TOF, a system that consists of a quadrupole MS (Q) combined with a time-of- flight MS (TOF). The two mass analyzers separate the ions based on their *m/z* values. A quadrupole MS consists of four cylindrical rods parallel to each other. These are connected to radio-frequency and direct current voltages. The ion beam is sent in at one end of the rod. At one voltage setting, only one *m/z* ion will pass through the quadrupole and reach the mass detector. In a TOF MS, the *m/z* is determined by the flight time of the ions. Ions are accelerated at the start of a flight tube so that all ions have the same kinetic energy. Ions with lower *m/z* ratio travel faster than ions with higher *m/z* and will reach the detector faster [127].

The HR-MS can provide the elemental composition in addition to fragment data (when MS/MS is used), which are useful for dereplication. The elemental composition can be used to search databases such as MarinLit, SciFinder, ChemSpider and Dictionary of marine natural products. Good databases are necessary for successful dereplication. A database search based on only the elemental composition can

lead to several compounds that need to be evaluated [128,129]. With the use of MS/MS, the ion can be fragmented and give additional information about the structure. In MS/MS, two mass analyzers can be linked to a collision cell, such as Q-TOF. The ion of interest is selected from the first MS before entering the collision cell where fragmentation occurs. The fragments are then analyzed by the second MS [127].

Molecular networking is a powerful method to organize the fragment data based on fragments similarity and correlations between them, as similar compounds will have similar fragmentations. These fragment data can then be used to search MS fragment libraries like the Global Natural Products Social molecular networking (GNPS). Programs such as Cytoscape can be used to visualize the molecular network [130-132].

Molecular networking has proven to be a valuable tool for identifying various compounds from marine microorganisms. As reported by Patiño et al. (2021) [133], molecular networking was used for identification of biosurfactants. They evaluated the production of biosurfactants from five marine bacteria and identified six biosurfactants. Dereplication of the bacterial extracts was performed using GNPS libraries, where one cluster identified various compounds in the surfactin family (Figure 11).

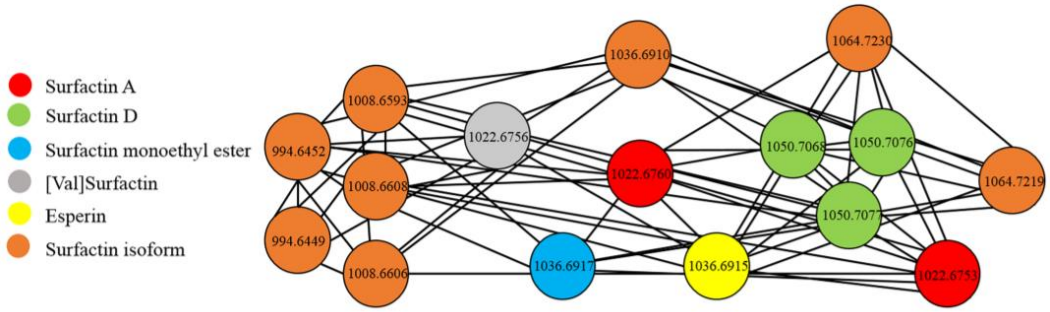


Figure 11. Molecular network of compounds from the surfactin family, copied from Patiño et al. (2021) [148].

The nodes in the network cluster are connected due to the similar fragmentation patterns of the compounds. The nodes with orange color represent surfactin isoforms not identified by search in the GNPS libraries. Compounds can only be identified if their MS/MS spectra are available in the MS/MS libraries, so if compounds are not identified, it can mean that they are novel compounds, or that they are not present in the library. Molecular networking was also used to identify the novel antibacterial amino-polyketide derivatives vitroprocines, produced by the marine bacterium *Vibrio* sp. [134], and Oppong-Danquah et al. (2018) used molecular networking to analyze the metabolite diversity between co-cultivated marine derived fungi and phytopathogens, and their respective mono-cultures. This revealed induced production of molecular network clusters in the co-cultivation cultures, including putative novel compounds [135].

2.6 Structure elucidation

Structure elucidation of natural products is an important, but not always straightforward process. As reported by Suyama et al. (2011) [136], 135 marine compounds were miss-assigned between 1981-2010, with the majority between 1996 and 2010. As described in paper I, Tedesco et al. (2016) [137] also miss-assigned a rhamnolipid structure. Structure elucidation of natural products commonly includes NMR spectroscopy combined with the structural information acquired from HR-MS analysis.

2.6.1 Nuclear magnetic resonance spectroscopy

NMR spectroscopy is a valuable technique in structure elucidation of natural products. The basis for NMR is that the nuclei of some isotopes have non-zero spins (*I*) that can be manipulated by radio-frequency pulses and detected. Regarding natural products, proton NMR and carbon NMR are the most useful NMR methods, as natural compounds contain hydrogens and carbons. The most abundant hydrogen isotope, ^{1}H (natural abundance 99.98 %) has spin number $I = ½$. The most abundant carbon isotope ^{12}C (~98.9% abundance), has zero-spin and cannot be used in NMR. Carbon isotope ^{13}C on the other hand (natural abundance 1.1 %), has spin number ½ and can be used in NMR. The number of orientations a nucleus can have in an external magnetic field is $2I + 1$. For example will ^{1}H and ^{13}C with spin number ½ have two energy levels. When the sample is placed into a strong magnetic field, and at the same time is exposed to radiofrequency radiation, the nuclei of the compound can be perturbed to create a spin coherence. Their magnetic moments will then precess at their resonance frequency and a spectrum can be acquired. Chemical shifts and spin-spin coupling give rise to signals with splitting patterns resulting from their coupling constants, and is very useful for interpreting proton NMR spectra. Another important feature in proton NMR is that integration of the signals gives the relative number of protons in each signal. In carbon spectra, proton decoupling is applied to remove the proton couplings so that ^{1}H does not split the ^{13}C signals. This, together with little spin-spin coupling due to the low ^{13}C abundances, makes the carbon NMR spectrum relatively simple, with sharp signals for each chemically nonequivalent carbon [138].

3 Aim of the project

The aim of this project was to use the OSMAC approach to explore Arctic marine microorganisms for their production of bioactive secondary metabolites and characterize isolated metabolites.

The objectives were

- Ferment Arctic marine microorganisms under different cultivation conditions
- Screene fractionated fermentation extracts for antibacterial and cytotoxic activities
- Dereplicate active fractions to identify bioactive compound(s)
- Isolate the active compound(s)
- Structure elucidate the isolated compound(s)
- Screen purified compound(s) for antibacterial and cytotoxic activities

4 Summary of papers

4.1 Paper I

Characterization of Rhamnolipids Produced by an Arctic Marine Bacterium from the *Pseudomonas fluorescence* Group

Venke Kristoffersen, Teppo Rämä, Johan Isaksson, Jeanette Hammer Andersen, William H. Gerwick and Espen Hansen

Marine Drugs **2018**, *16*(5), 163

An Arctic marine bacterium from the *Pseudomonas fluorescence* group, strain M10B774, isolated from an Atlantic halibut in the Norwegian Sea, was fermented in four different media, M19, VR_1, VR_2 and SGC, using the OSMAC approach in an attempt to induce the production of antibacterial and cytotoxic compounds. Fermentation extracts were fractionated and tested for antibacterial and cytotoxic activities, revealing different bioactivity profiles of the fractions from the four media. One M19 fraction was active against the three tested gram-positive bacteria and had also cytotoxic activities against cancer cells. One VR_2 fraction was active against two gram-positive bacteria, while one VR_1 fraction was active against one gram-positive bacterium. The fractions from the SGC medium did not have any bioactivities, and none of the tested fractions had antibacterial activities against gram-negative bacteria.

MS/MS network analysis of the active VR_2 fraction resulted in a molecular network with one cluster exclusive to the active fraction where one node matched a rhamnolipid standard present in the GNPS library. Clustering of the compounds indicated that they were likely related rhamnolipids. UHPLC-HR-MS analysis revealed that the suspected related rhamnolipids were present both in active M19 and VR_1 fractions, but not in the inactive SGC fractions, and that highest amount of rhamnolipids was found in the M19 fraction. Hence, this medium was selected for upscaling and isolation. Six related compounds were isolated. The structures were elucidated with NMR and were confirmed to be one rhamnolipid lipid moiety and five mono-rhamnolipids, including one mono-rhamnolipid with novel structure (Figure 12).

The isolated compounds were tested for antibacterial, antibiofilm and cytotoxic activities. All six compounds had antibacterial and antibiofilm activities, while **2**, **4** and **6** also had cytotoxic activities.

Using the OSMAC approach on *Lacinutrix* sp. resulted in the production of different amounts of rhamnolipids in the four different media, and subsequently different bioactivity profiles, demonstrating the effect of altering cultivation conditions. Molecular networking proved to be a good approach for

identifying related compounds, and it was the first time rhamnolipids from a *Pseudomonas fluorescence* group bacterium were described.

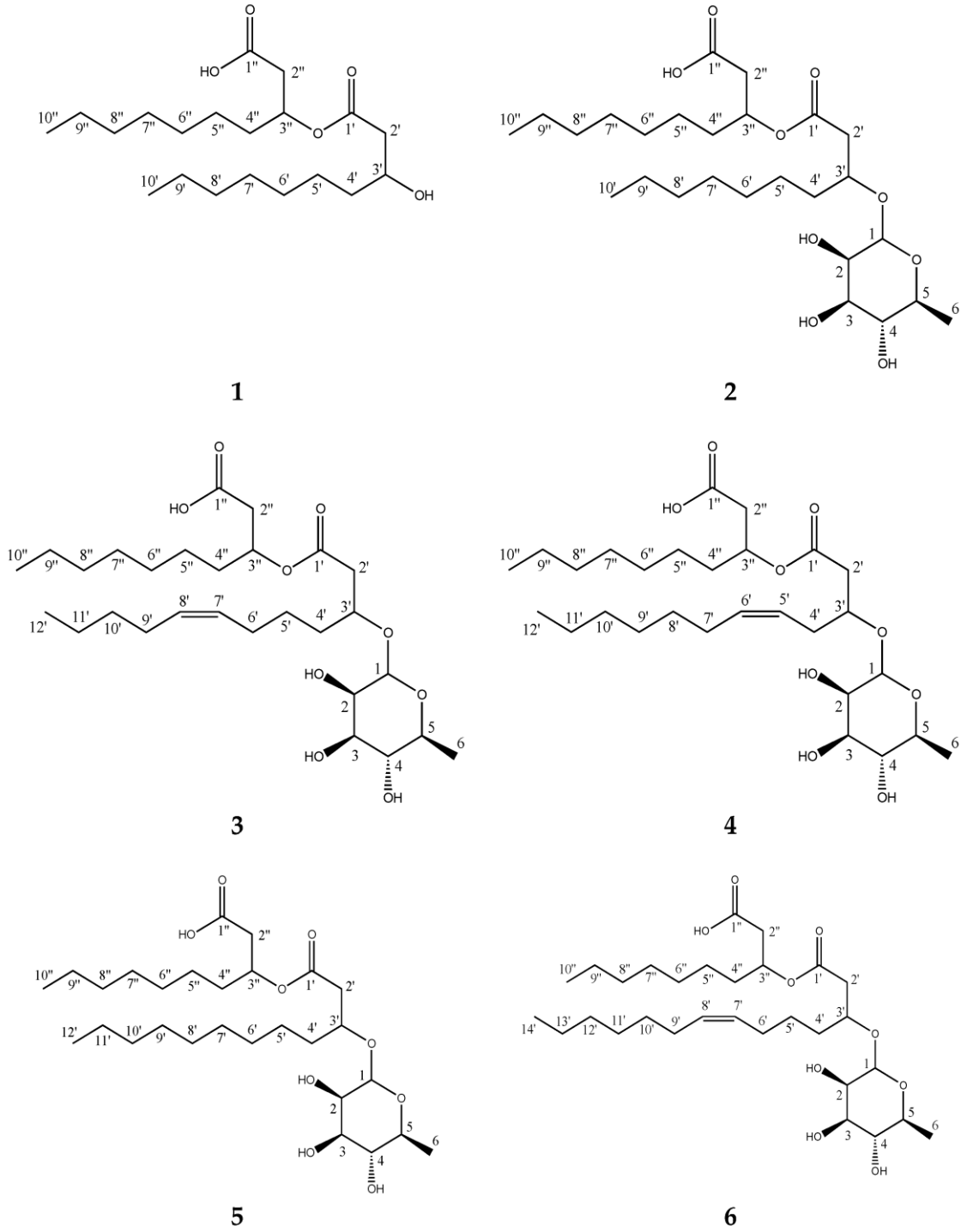


Figure 12. Structures of compounds **1**–**6** isolated from *Pseudomonas* sp. Compound **3** was described for the first time.

4.2 Paper II

Two Novel Lyso-Ornithine Lipids Isolated from an Arctic Marine *Lacinutrix* sp. Bacterium

Venke Kristoffersen, Marte Jenssen, Heba Raid Jawad, Johan Isaksson, Espen H. Hansen, Teppo Rämä, Kine Ø. Hansen and Jeanette Hammer Andersen.

Molecules **2021**, *26*(17), 5295.

The Arctic marine bacterium *Lacinutrix* sp. strain M09403 was isolated from a *Halichondria* sp. sponge collected in the Barents Sea. The bacterium was cultivated in M19 medium before fractionated fermentation extract was screened for antibacterial activities, revealing one fraction with activities against gram-positive bacteria. UHPLC-HR-MS analysis of the active fraction and the inactive fractions 4 and 6 revealed two compounds, **1**, with elemental composition $C_{20}H_{40}N_2O_4$ and **2**, with elemental composition $C_{21}H_{42}N_2O_4$ exclusively present in the active fraction. UHPLC-HR-MS analysis showed that **1** eluted as three peaks and **2** as two peaks. NMR analysis and fragmentation pattern from the UHPLC-HR-MS suggested that they were stereoisomers. It was not possible to separate the individual isomers on the prep-HPLC due to low chromatographic resolution. The 2-dimentional structures of the isolated compounds, two lyso-ornithine-lipids, are shown in Figure 13. The difference between the structures is one CH2 group more in the lipid chain of **2**. The purified compounds were tested for antibacterial and cytotoxic activities. Compound **1** had antibacterial activities against gram-positive bacteria, in particular against *Streptococcus agalactiae*, while **2** had cytotoxic activity against A2058 human melanoma cells. The different activities between the two compounds show the effect of different lipid chain length. As the lyso-ornithine lipids are amphiphilic, their activities are suspected to be due to interactions with the membranes of the target cells. This was the first bioactive molecules reported from *Lacinutrix* sp., showing lyso-ornithine lipids with cytotoxic activity and with antibacterial activity against gram-positive bacteria.

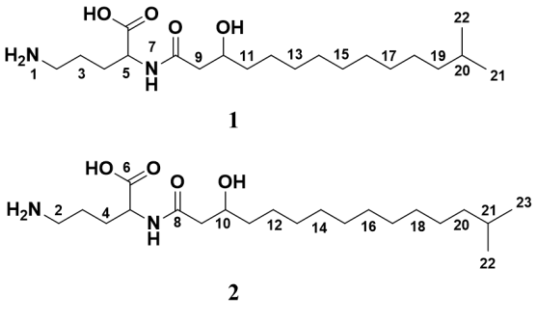


Figure 13. Structures of lyso-ornithine lipids **1** and **2**.

4.3 Paper III

Chlovalicin B, a Chlorinated Sesquiterpene isolated from the Arctic marine mushroom *Digitatispora marina*

Marte Jenssen , Venke Kristoffersen, Kumar Motiram-Corral , Johan Isaksson , Teppo Rämä , Jeanette H. Andersen , Espen H. Hansen and Kine Østnes Hansen.
In preparation

Digitatispora marina, a marine basidiomycete fungus was isolated from *Betula* sp. driftwood collected at Vannøya, Norway. The fungus was cultivated under different conditions as part of a screening program at Marbio. Fractionated extracts of the fungus had antibacterial and cytotoxic activities. UHPLC-HR-MS analysis of the fractions showed a compound with the characteristic chlorine isotopic pattern in a 3:1 ratio with elemental composition $C_{15}H_{23}O_5Cl$. It was isolated with prep-HPLC before structure elucidated with NMR. The compound was determined to be chlovalicin B (Figure 14), a novel variant of the previously isolated compound chlovalicin. Chlovalicin, differing from chlovalicin B with a methoxy group in the C3 position where chlovalicin B has a hydroxyl group had previously been isolated from the fungus *Sporothrix* sp. Chlovalicin B was screened for antibacterial activity in a growth inhibition assay against five pathogenic bacterial strains, the gram-positive *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae* and the gram-negative *Pseudomonas aeruginosa* and *Escherichia coli*. It was tested for inhibition of biofilm formation of *Staphylococcus epidermidis* and for cytotoxicity against the malignant A2058 human melanoma cell line and the non-malignant lung fibroblast MRC-5 cell line in an anticancer assay. It was also tested for antifungal activity against *Candida albicans*. Chlovalicin B had no antimicrobial activities at concentrations up to 100 µM and weak activity against the A2058 cells, with ~50% cell survival at 50 µM, the highest test concentration. It had no activity against the MRC-5 cell line at 50 µM. Chlovalicin had previously been reported to display activity against the mouse melanoma cell line B16, with IC_{50} = 37 µM, which can indicate that the chlovalicins affect a common cellular target in melanoma cell lines.

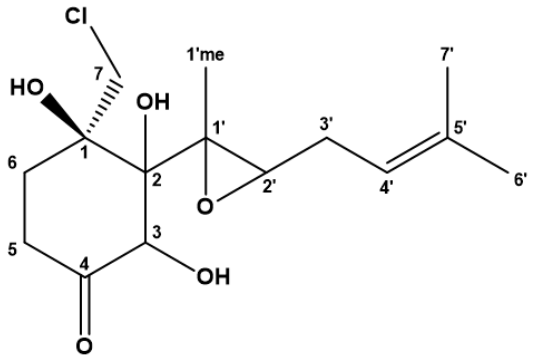


Figure 14. Structure of chlovalicin B.

5 Discussion

As described throughout the introduction, miccrooganisms have been essential sources for antibiotics and anticancer drugs for many years. Most of the drugs originate from terrestrial species, but marine microorganisms have also contributed to vital drugs used today. Arctic marine microorganisms were grown under different cultivation conditions in this project. This resulted in the isolation of rhamnolipids in paper I, one with novel structure, two novel lyso-ornitine lipids in paper II, and the novel compound chlovalicin B in paper III, isolated from the bacteria *Pseudomonas* sp. and *Lacinutrix* sp., and the fungus *Digitatispora marina*, respectively. The rhamnolipids and lyso-ornithine lipids had moderately antibacterial and cytotoxic activities, while chlovalicin B had some cytotoxic activity.

5.1 Selection of microorganisms

The microorganisms used in this project were sampled from the Arctic. The bacteria were collected from the Arctic waters by Marbank, the national marine biobank of Norway, while the fungus was collected as a project to explore the diversity of fungi growing on wooden logs along the coast of Northern Norway [139]. The foundation for selecting marine microorganisms in the search for novel antibacterial and cytotoxic compounds was that microorganisms have been the source for most of the antibiotics used today, in addition to several anticancer agents as mentioned in section 1.2. As the marine environment is less explored than the terrestrial environment, marine microorganisms represent an underexplored source, which can increase the chances of discovering novel bioactive compounds from these microorganisms.

Diverse Arctic marine microorganisms were screened for antibacterial and cytotoxic activities as part of a screening program at Marbio. The three microorganisms used in this project displayed antibacterial and/or cytotoxic activities in this initial screening and were thus selected for further studies.

Pseudomonas spp. belonging to the *Gammaproteobacteria* class have been isolated from both marine and terrestrial environments and are known to produce various bioactive compounds [140-143]. The two other microorganisms, *Lacinutrix* sp. from the *Flavobacteriia* class and *Digitatispora marina* from the *Basidiomycota* division have exclusively been isolated from the marine environment and had as reported in paper II and III, respectively, not previously been assessed for their production of secondary metabolites. The examination of the three microorganisms resulted in the isolation of several bioactive novel compounds. This demonstrates that exploring Artic marine microorganisms is a valid strategy for discovering compounds with novel chemistry.

One objective of this project was to isolate compounds with antibacterial and cytotoxic activities. It was not stated in the aim of the project, but the overall goal was as might be expected to discover compounds

that could potentially become anticancer agents or drugs. However, the activities of the isolated compounds were not sufficient to be considered as potential drug leads compared to marketed antibiotics and cancer drugs [144,145]. The microorganisms were selected for further work based on observed bioactivities of fractionated extracts. Other criteria could have been considered, for example the genome size and the presence of BGCs. Although no BGCs were described and no NRPS and PKS products were isolated in this project, they have been the source for many drugs and are central in the search for new drugs as mentioned in section 1.3.1, and will thus be included in this discussion.

As reported by Donadio et al. (2007) [41], the genome size is important for the presence of BGCs. Donadio et al. (2007) analyzed 223 bacterial strains and found that bacteria with genome size less than three Mbp seem to not display the genes for PKS and NRPS. Considering the genome size of the bacteria used in this project, the assembled genome of *Lacinutrix* sp was 3.6 Mbp, while *Pseudomonas* sp. was not fully genome sequenced. Cho et al. (2015) [146] analyzed 11 different strains within the *Pseudomonas fluorescence* group where the genomes ranged from 5.9-7.1 Mbp. Assuming the genome of *Pseudomonas* sp. is within this range, the genome sizes of the two bacteria indicate that they should have the potential to produce a diversity of secondary metabolites. *Lacinutrix* sp. was subjected to genome mining and was analyzed with antiSMASH and PRISM for the presence of PKS and NRPS gene clusters, but no such structures were predicted (data was not included in paper II). This shows that even if the genome size is within the range of containing the genes for NRPS and PKS, they may not be part of the genome.

Considering the genome size and presence of BGCs of bacteria known to have produced numerous bioactive secondary metabolites, actinomycetes from the *Actinobacteria* class have large genomes, ranging from five to nine Mbp [147]. Around 40-50 % of all bioactive compounds discovered today are produced by actinomycetes, mainly *Streptomyces*, and 75% of marketed antibiotics are derived from *Streptomyces* [147-149]. The genus *Streptomyces* has genome size around 8-9 Mbp and is known to have at least 20-30 BGCs [150,151]. Low et al. (2018) reported a *Streptomyces* sp. with 52 BGCs [152], hence the genetic diversity within one species can differ substantially. *Cyanobacteria* have also been the source for numerous secondary metabolites, as more than 2010 secondary metabolites, including 450 from marine species have been characterized [153,154]. Leao et al. (2017) [155] analyzed four strains from the genus *Moorea*, which has produced more than 40% of all natural products reported from cyanobacteria. These four strains had the genome size ranging from 8.37-9.71 Mbp, with 33 to 44 BGCs per genome. Dolastatin 10, which is a component in several anticancer drugs as described in section 1.2.1 is for example produced by cyanobacteria, and several compounds from cyanobacteria are in clinical trials as anticancer drugs [49]. Myxobacteria have also been a rich source of secondary metabolites, mainly PKS and NRPS products, with various bioactivities which includes antibacterial,

anticancer, antifungal and antioxidant activities. Myxobacteria have some of bacteria's biggest genomes, the *Sorangium* strain So ce56 has for example a 13Mbp genome with 17 BGCs [156,157].

The genome of *Digitatispora marina* was sequenced and the assembled genome was 36.89 Mbp. It was not analyzed for the presence of BGCs, but comparing the genome size with fungi known to be prolific producers of bioactive secondary metabolites, such as *Penicillium, Aspergillus* and *Fusarium*, there is no reason it should not have BGCs and the potential to produce various secondary metabolites. *Penicillium, Aspergillus* and *Fusarium* are well studied filamentous fungi from the *Ascomycota* division [158] with varying genome sizes and number of BGCs. Yang et al. (2016) [159] analyzed 13 *Penicillium* spp. and found that their genome size ranged from 24.2 to 39.5 Mbp. The genome size of *Aspergillus* ranges from 28 to 40 Mbp [160] and the *Fusarium* genome ranges from 36.4 Mbp to 50.4 Mbp [161]. Vesth et al (2018), [162] analyzed 37 *Aspergillus* and *Penicillium* genomes and identified 2717 BGCs, which can explain the remarkable production and diversity of secondary metabolites from these fungi. *Penicillium* has been the source for various drugs, including the antibiotics penicillins, the antifungal griseofulvin and mevastatin for lowering cholesterol [163]. Numerous compounds with various bioactivities have been isolated from *Aspergillus*, including the cholesterol lowering drug lovastatin [164]. Li et al. (2020) [161] reviewed the secondary metabolite production from *Fusarium* and found that 678 secondary metabolites had been isolated as of December 2019, with 272 exclusively to *Fusarium*. These metabolites include NRPS and PKS products [165].

Digitatispora marina belongs to the *Basidiomycota* division which is less studied than *Ascomycota*. Basidiomycetes are known to produce compounds belonging to the major secondary metabolite classes. Many compounds are produced by terrestrial fungi from this division, including pleuromutilin, which is the source for lefamulin, a new class of antibiotics that was FDA approved in 2019 [166]. There are also promising anticancer drugs originating from *Basidiomycota*, such as irofulven, a semisynthetic compound initially isolated from a terrestrial *Omphalotus illudens*. It did not pass the clinical trials, but it shows the potential of basidiomycetes as producers of bioactive metabolites [167,168].

5.2 One strain-Many compounds

As biosynthetic gene clusters can be silent under standard cultivation conditions, the OSMAC approach was used in this project as an attempt to induce or/and enhance the expression of these genes, and thereby the production of their corresponding secondary metabolites. As reported in paper I, *Pseudomonas* sp. was fermented in four different media M19, VR_1, VR_2 and SGC. The components of the four media are depicted in table 4 in paper I. The three first media are rich nutrient media where the main differences are the carbon source and nitrogen source which are known to affect the production of secondary metabolites [148,169]. In the VR_2 medium, iron and bromine were added. Iron contributes to regulate

the genes responsible for secondary metabolite synthesis, which affects the production of secondary metabolites [170,171]. Brominated compounds from the marine environment are known to display various biological activities such as anticancer [172,173], antibacterial [174,175] and anti-inflammatory [176] activities. Hence, the theory was that adding bromine and iron to the VR_2 medium would increase the production of brominated and bioactive secondary metabolites. The SGC medium was a low nutrient medium. The metabolite production occurs in the end of the growth phase and in the stationary phase when nutrition is depleted [77,78], hence, the concept was that a medium with little nutrients would stress the bacteria into producing secondary metabolites.

The use of the OSMAC approach on the *Pseudomonas* sp. resulted in fractionated fermentation extracts with various bioactivities, where a fraction from cultivation in the M19 medium had most activities. *Lacinutrix* sp. in paper II was fermented in the same media as *Pseudomonas* sp., excluding the SGC medium. The fraction from fermentation in the M19 medium had strongest antibacterial activities and was selected for further work. Based on the observed bioactivities from the flash fractions of *Pseudomonas* sp. and *Lacinutrix* sp., adding bromine and iron to the VR_2 medium did not increase the production of more antibacterial and cytotoxic compounds.

Many bacteria were fermented in the SGC medium as part of the screening project of Artic marine microorganisms at Marbio. The biomass yield was in general low, and the fractions showed little bioactivities (data not shown). It is possible that the medium had too little nutrition and that the bacteria never entered a proper exponential growth phase. However, low nutrition media could have the potential for stressing the bacteria into producing secondary metabolites, if a suitable low nutrient medium is used.

The co-cultivation approach was used on *Digitatispora marina* in paper III. The fungus was cultivated both as mono-cultures and co-cultures with an Arctic marine bacterium. HR-MS analysis revealed that *D. marina* did not show a clear response to the co-cultivation, hence, the co-cultivating data was not included in paper III. Our experience was that it takes time and effort to find the correct ratio of the fungus and the bacterium for how to induce the production of secondary metabolites. The results show that the co-cultivation procedure used in this project could be optimized and confirms that co-cultivation does not necessarily affect the production of secondary metabolites. Other co-cultivation conditions could have resulted in the production of more secondary metabolites, as co-cultivation of marine microorganisms has been reported to enhance and induce the production of anticancer, antibacterial and antifungal compounds among others [74,177-179]. Options could be to add the co-cultivating microorganism at different ratios or at different times, co-cultivate several microorganisms together, use different growth media etc. Another factor to consider, particularly for the slow-growing *D. marina*, is

that slow growth increases the chances of bacteria outgrowing the fungi. One way to overcome this issue is adding bacterial lipopolysaccharide (LPS) instead of living bacteria cells. LPS is the major component present in the outer membrane of gram-negative bacteria and are known to affect the production of secondary metabolites in some fungi [180,181]. By using LPS instead of living bacteria, the bacteria will not overtake the cultures. In addition, by using LPS, you will know which organism is the true producer of observed secondary metabolites. It is also possible to co-cultivate the microorganisms without physical contact between them, for example by growing them in a vessel where they can be separated with a filter that allows for exchange of chemical signals and metabolites between the different microorganisms [182].

In this project, liquid cultures were used in the OSMAC approach, followed by extraction. This is a common method for upscaling and harvesting microbial secondary metabolites [183-190]. VanderMolen et al. (2013) evaluated terrestrial fungi grown on solid media and in liquid cultures. They found that the biomasses of fungi grown on solid medium were larger compared to the biomasses obtained from fermentation in liquid medium. The production of two marker compounds were also lower in the liquid cultures [191]. Shomura et al. (1979) [192] reported that some soil actinomycetes only produced antibiotics during solid medium fermentation, and not in liquid cultures. These studies involved terrestrial species, but the results might also apply to marine microorganisms. Hence, including solid fermentation in the OSMAC approach could potentially lead to the discovery of more bioactive secondary metabolites.

5.3 Bioassay-guided isolation

5.3.1 Identification of compounds

The bioassay-guided isolation approach was used in this project, and identification of the isolated compounds was performed by UHPLC-HR-MS analysis of bioactive fractions. The compounds isolated in the three papers were identified based on prominent peaks in the HR-MS chromatograms, molecular networking and isotopic pattern. Rhamnolipid **2** in paper I was a prominent peak exclusive in the HR-MS chromatogram of the active fraction from the M19 medium, shown in Figure 15.

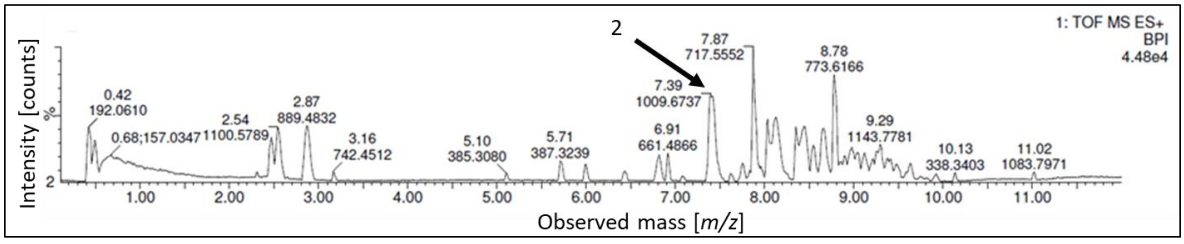


Figure 15. Base peak intensity HR-MS chromatogram of fraction 5 from M19 medium. Rhamnolipid **2** was one of the major peaks in the active fraction, shown as the dimer [2M + H], m/z 1009.6737.

The active VR_2 fraction which also contained **2** was subjected to molecular networking for further analysis and dereplication (Figure 16). The nodes in the molecular network were connected due to similar fragmentation patterns of the compounds. Molecular networking proved to be an efficient approach for identifying similar compounds based on MS/MS fragmentation pattern, as several rhamnolipids were identified in the fraction.

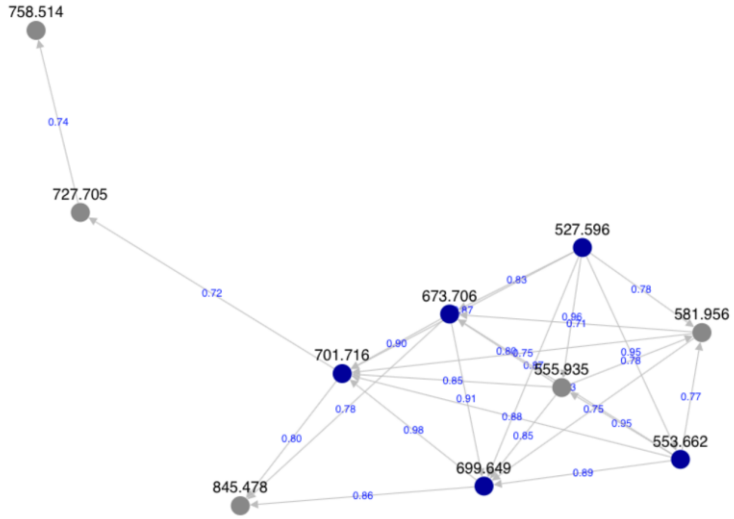


Figure 16. Molecular network cluster of rhamnolipids from *Pseudomonas* sp. The five blue nodes and 555.935 and 581.956 represent rhamnolipids.

The lyso-ornithine lipids isolated in paper II were among the most prominent peaks in the HR-MS chromatograms of the active fraction as displayed in Figure S3 in paper II. On the UHPLC-HR-MS it was possible to detect **1** as three isomers and **2** as two isomers. However, the isomers could not be separated from each other on the prep-HPLC due to lower chromatographic resolution. It is possible that HPLC column with a chiral stationary phase could have separated them, but this was not tested [193].

It was decided to isolate chlovalicin B described in paper III as the mass specter displayed the chlorine isotopic 3:1 ratio pattern (Figure 17). Many marine secondary metabolites contain halogens, mainly bromine and chlorine, due to the relatively high concentrations of these halogens in the sea, compared to the soil [194,195]. The presence of chlorine(s) is known to affect the bioactivity of some compounds. For example two chlorines are needed in the antibiotic vancomycin for its bioactivity, and salinosporamide A in phase III clinical trial as anticancer agent has one chlorine substituent that is required for its activity [196].

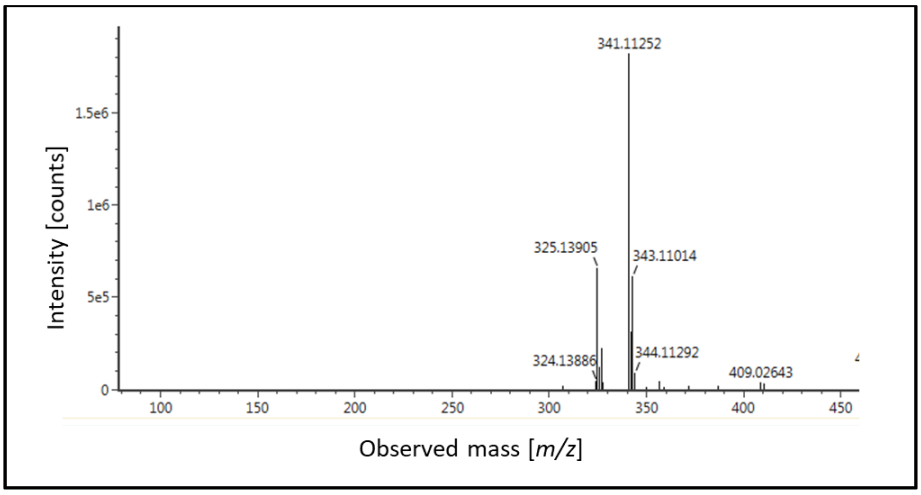


Figure 17. Chlorine isotopic pattern of chlovalicin B: $[M+Na]^+$, m/z 341.1132 and 343.1103.

The formation of non-covalent dimers and proton and sodium adducts are often observed in electrospray ionization and can complicate the assignment of the molecular mass in the MS-spectra [197]. The formation of adducts depends on the concentration and on the energies in the ion source [128]. The microorganisms were fermented with sea salt and/or seawater containing sodium, possibly increasing the concentration of sodium in the samples Sodium from glassware were samples are prepared and stored can also be transferred to the samples. The base peak of chlovalicin B was the sodium adduct, while the proton adducts were the base peaks for the lyso-ornithine lipids. Different adduct pattern due to different ion sources was observed, e.g. the protonated dimer of rhamnolipid **2** was the base peak in the Q-TOF, while the sodium adduct was the base peak on the prep-HPLC-MS.

Additional, as described in paper I, some of the nodes in the rhamnolipid molecular network represented di-rhamnolipid not present in the sample. Their appearance were likely due to dimerization in the HR-MS, as the rhamnose moiety can detach from the lipid moiety in the ion source and react with a mono-rhamnolipid to form a di-rhamnolipid.

Another factor complicating the identification of the active compound is that the size of the peaks in the chromatograms depends on the ionization efficiency of the compounds. With the use of ESI, the compounds´ efficiency to ionize in a solvent can give results that differ by several orders of magnitude from the same concentration of different compounds, so the highest peak is not necessary the most abundant compound in a sample [198]. Hence, compounds that might be bioactive can have low ionization efficiency, which can lead to no signal on the HR-MS, or they can have so low signal that they are considered too minor to be essential for the bioactivity and consequently not selected for isolation.

The compounds isolated in this project were isolated based on their MS signals. Some compounds do not ionize and cannot be detected on the MS. To overcome this problem, UV/Vis can sometimes be used. To be detected by the UV/Vis detector, the compounds must have a chromophore so that they can absorb light in the UV/Vis region (wavelength 200-600 nm) [123].

5.3.2 Bioactivity of isolated compounds

The bioassays used in this project were phenotypic, as the screening included gram-positive and gram-negative bacteria, fungus and human cells, where the MMOA of the bioactive compounds were unknown. This is an advantage as compounds with various MMOA can be identified.

The rhamnolipids and lyso-ornithine lipids described in paper I and II, respectively, had various antimicrobial activities against gram-positive bacteria and cytotoxic activities against cancer cells. No activities were seen against the gram-negative bacteria for any of the tested compounds, which is likely due to the outer LPS layer of gram-negative bacteria, making it more difficult for the bioactive compounds to access the target`s membrane [180].

The rhamnolipids and lyso-ornithine lipids are biosurfactants and were isolated without knowing their MMOA. However, biosurfactants are known to affect the membrane of target cells due to their amphiphilic structure, by binding to the membrane of the targets and disrupt it [199]. Biosurfactants have been studied for use in the pharmaceutical industry, but only a few are used as drugs, such as the antibiotics daptomycin and polymyxin B and E. These antibiotics are cyclic lipopeptides and structurally very different from the biosurfactants discovered in this project, with a much bigger head group and consequently a higher molecular weight. Daptomycin consists of 13 amino acids, 10 that form a circle, linked to a fatty acid [200], while polymyxin B and E consist of 10 amino acids where seven form a circle, linked to a fatty acid [201]. Although biosurfactants are not widely used as pharmaceuticals today, they might have potential in the future as the research continues.

Chlovalicin B described in paper III had weak cytotoxic activity against cancer cells and no antimicrobial activities. Chlovalicin (with a methoxy group in the C-3 position of the cyclohexane ring versus a hydroxyl group for chlovalicin B) has showed cytotoxic activity against a different melanoma cell line used in this project, in addition to osteoclastogenesis inhibition activity [202,203]. The different substituents of the cyclohexane ring can potentially affect the bioactivities, but even though chlovalicin B was not very active in the tested bioassays, it is possible that it has other bioactivities.

The bioassay-guided isolation approach used in this project did not lead to the isolation of compounds with potential to become antibiotic or anticancer drug leads, but this approach has previously been successful in the discovery of potential drug leads. Fractionated extract of the cnidarian *Thuiaria*

breitfussi showed cytotoxic activity in the anticancer bioassay, leading to the identification and isolation of breitfussins in 2012 (personal communication with Kine Ø. Hansen). The breitfussins are now being developed and investigated as kinase inhibitors [204].

Bioassay-guided isolation is preferred when you are looking for a compound with specific bioactivity. However, it means that compounds with interesting chemistry that are not active in the selected bioassays are not detected. As chlovalicin B only had very weak cytotoxic activity, it was not responsible for the observed activity of the *D. marina* fraction in the initial screening and would not have been discovered if other compounds in the fraction had not been active. Another approach frequently used for isolation of compounds is chemistry-guided isolation. In this approach, UHPLC-HR-MS can be used to examine the fraction or extract for compounds with interesting chemistry, such as halogenated compounds, or if you are looking for specific functional groups. The isolation of the compounds is then motivated by the chemistry of sample components, versus bioassay-guided isolation where isolation is performed based on observed activity of a fraction or extract.

In the fraction where chlovalicin B was isolated from, two other compounds were also suspected to be responsible for the activities in the initial screening (data not shown). However, isolation of these two compounds proved to be challenging as they eluted very close to each other. Due to the slow growing *D. marina*, low yield of biomass and metabolites, time limitations and the fact that we isolated chlovalicin B that was suspected to be a bioactive component, this project was put on hold.

5.4 Sample supply and yield

In natural products research, one issue is the sample supply. Regarding plants and invertebrates that are the origin of many drugs, large amounts of the organisms must sometimes be sampled to obtain enough material for research. One example is bryostatin 1, isolated from the bryozoan *Bugula neritina* collected in waters off the California coast. To obtain 18 g of bryostatin 1, 13 000 kg of the bryozoan was collected over several years [205]. Collecting organisms is expensive, and the conditions in the sea varies. Different environmental conditions affect both the production of secondary metabolites and the presence of living organisms, so the macroorganism itself may not be harvested in the next sampling, and if it is harvested, the wanted compound may not be produced [206]. When working with microorganisms, it is in theory possible to overcome the supply issue by growing as much of the microorganisms in the laboratory as needed to achieve enough of the wanted compound. However, some microorganisms cannot be grown, or are difficult to grow in the laboratory. Some microorganisms grow slowly and produce small amounts of the secondary metabolite, making large-scale production laborious.

For example, basidiomycete cultures are known to grow slowly and to have low yields [207]. This was also the experience with *Digitatispora marina* in paper III. The fungus was cultivated for more than four

months, and from a total of 30 L culture, 0.6 mg of chlovalicin B was isolated. Comparing with the bacteria, *Pseudomonas* sp. in paper I and *Lacinutrix* sp. in paper II were cultivated for 2-3 weeks before extraction. Rhamnolipid **2** produced by *Pseudomonas* sp. was the compound with highest yield/ L culture of all the isolated compounds, with around 300 mg isolated from around 17 L culture. This demonstrates that the yield of secondary metabolites produced by the microorganisms can differ largely.

One solution for slow growing microorganisms and/or with low yield can be genetic modification. Bailey et al. (2016) [208] increased the yield of pleuromutilin after reconstruction of the biosynthetic pathway into the heterologous host *Aspergillus oryzae*, a fungus that also grows faster than the original pleuromutilin producing basidiomycete.

Cultivation and isolation challenges are reasons why marine myxobacteria have not been explored much. They grow slowly and are thus outgrown by other organisms during the isolation process, and they cannot be cultivated in rich media as this results in poor cell density [157]. Hence, promising microorganisms may not be investigated for the production of secondary metabolites, as it demands a lot of work and resources.

6 Conclusion

The use of the OSMAC approach showed that different growth media affected the production of bioactive compounds. This approach led to the isolation of compounds with novel chemistry. The isolated compounds may not seem too interesting since they did not display sufficient antibacterial or cytotoxic activities to have the potential to become drug leads. However, it is important to publish these results and include the compounds in the databases. This will help to make the dereplication process faster for all natural products researchers. In addition, studying them, so-called basic research, to get a better understanding of their function and properties, may result in the compounds being useful in the pharmaceutical industry or in other applications one day. In addition, we acquire more knowledge about what compounds the various microorganisms are capable of producing.

Molecular networking proved to be a valuable tool for dereplication, as related compounds clustered together based on their similar MS/MS fragmentation patterns. This was visualized as connected nodes in a molecular network, which resulted in rapid identification of several rhamnolipids.

Working with *Digitatispora marina* revealed some challenges in the search for novel secondary metabolites. The fungus grew slowly, had low yield, and promising compounds were challenging to isolate. In addition, co-cultivation with a bacterium did not result in the detection of more secondary metabolites. However, *D. marina* was only cultivated in one medium and in co-cultivation with one

bacterium, which led to the isolation of the novel compound chlovalicin B, indicating it is a promising source for novel secondary metabolites.

The relatively small genome size of *Lacinutrix* sp. and the genome mining not identifying any BGCs for NRPK and PKS may not make it the best choice in the search for secondary metabolites. *Pseudomonas* sp. and *Digitatispora marina* on the other hand are still considered to have the potential to produce even more interesting chemistry than was discovered in this project, if other cultivation conditions are used. There is no reason to believe that the chemical diversity of marine organisms is not equally rich to that of terrestrial organisms, so the likelihood of discovering novel secondary metabolites may not be limited by the capacity of the microorganisms to produce them, but by the research approach.

7 Further work and personal considerations

Rhamnolipids are widely studied, so no more effort will be put into this project. *Pseudomonas* sp. produced relatively large amounts of rhamnolipid **2**, so it could be possible to replace it with the pathogenic *P. aeruginosa* for industrial production of rhamnolipids. This is out of the scope of our research group, but it is worth to have in mind.

The lyso-ornithine lipids were isolated as isomers as it was not possible to separate them on our prep-HPLC system. It would be possible to try to separate them with HPLC using a chiral stationary phase [193]. However, as the compounds did not have strong antibacterial or cytotoxic activities, the project has at the moment ended.

Chlovalicin B will be tested in other bioassays. As two other interesting compounds produced by *Digitatispora marina* have not been isolated yet, more effort will be put into isolating them and more of chlovalicin B. If any of the compounds turn out to be bioactive, MMOA studies will be performed. *D. marina* was only grown in one medium and in co-cultivation with one bacterium, so other media and co-cultivation conditions will be tried, including solid medium. This might induce and/or enhance the production of secondary metabolites, increase the yield or make the fungus grow faster.

After working with microorganisms, gaining more experience and knowledge, I think an important object in the search for microbial secondary metabolites is the selection of microorganisms. Going through the collection of microbial strains at Marbank, it contains few strains of the mentioned microorganisms known to be prolific producers of bioactive secondary metabolites. This shows that if the aim is to explore specific microorganisms, it is important to start the selection already in the sampling and isolation process, by using isolation methods specific for the wanted microorganisms [209-211].

Another important approach in my opinion is to sequence the microbial genomes and analyze them for BGCs. At least if you work with microorganisms were the genome size and presence of BGCs are not known. As long as the microorganisms display BGCs, they will be interesting to explore for the production of secondary metabolites. For example teixobactin isolated from *Eleftheria terrae* is synthesized by two NRPSs [212]. This potential antibiotic would not have been discovered if the strategy was to focus on species known to be prolific producers of secondary metabolites. Genome mining of *Penicillium*, *Aspergillus* and *Streptomyces* have revealed that they all have BGCs, including NRPS, PKS and hybrid PKS-NRPS, where the corresponding products are not known [213-215]. In a study of Grijseels et al. (2017) [163], genome mining identified 10 *Penicillum* spp. with a high diversity of BGCs. Analysis of secondary metabolite production revealed that the production varied when the *Penicillium* spp. were cultivated in two different media. Hence, I believe the combination of genome mining and OSMAC represents a promising approach to investigate both well studied and less studied microorganisms for the production of novel bioactive compounds. With the evolving crisis of antibiotic resistant microbes and the increase in cancer deaths, new drugs are in high demand and all potential sources should be explored.

"*Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less*." –Marie Curie

8 Works cited

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Paper 1

marine drugs

MDPI

Article

Characterization of Rhamnolipids Produced by an Arctic Marine Bacterium from the *Pseudomonas fluorescence* Group

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Abstract: The marine environment is a rich source of biodiversity, including microorganisms that have proven to be prolific producers of bioactive secondary metabolites. Arctic seas are less explored than warmer, more accessible areas, providing a promising starting point to search for novel bioactive compounds. In the present work, an Arctic marine *Pseudomonas* sp. belonging to the *Pseudomonas (P.) fluorescence* group was cultivated in four different media in an attempt to activate biosynthetic pathways leading to the production of antibacterial and anticancer compounds. Culture extracts were pre-fractionated and screened for antibacterial and anticancer activities. One fraction from three of the four growth conditions showed inhibitory activity towards bacteria and cancer cells. The active fractions were dereplicated using molecular networking based on MS/MS fragmentation data, indicating the presence of a cluster of related rhamnolipids. Six compounds were isolated using HPLC and mass-guided fractionation, and by interpreting data from NMR and high-resolution MS/MS analysis; the structures of the compounds were determined to be five mono-rhamnolipids and the lipid moiety of one of the rhamnolipids. Molecular networking proved to be a valuable tool for dereplication of these related compounds, and for the first time, five mono-rhamnolipids from a bacterium within the *P. fluorescence* group were characterized, including one new mono-rhamnolipid.

Keywords: arctic bacteria; bioactive; OSMAC (one strain, many compounds); molecular networking; rhamnolipids

1. Introduction

It is estimated that only a small percentage of the existing marine bacterial diversity has been cultivated to date. As a result, there is a strong likelihood to isolate previously uncultured bacterial strains and some of these will produce new secondary metabolites (SMs) [1]. It is also likely to find novel SMs from already cultivated bacteria by applying the OSMAC (one strain many compounds) approach [2]. The concept behind this approach is that some metabolic pathways remain silent during standard cultivation conditions and the corresponding SMs are not synthesized. Introducing small changes into the cultivation conditions can activate different metabolic pathways which may lead to the production of numerous SMs from a single strain [2].

When searching for novel SMs from natural sources, it is important to reduce the time and resources spent on rediscovering known compounds. The process of identifying known compounds is known as "dereplication". The most common method of dereplication in natural product (NP) drug discovery employs mass spectrometry (MS) in combination with liquid chromatography, as this combination is both sensitive and well suited for analyzing complex mixtures. Further, when using high-resolution MS, the accurate mass of the compound can be used to calculate the elemental composition which can then be used to search databases such as MarinLit, Dictionary of Natural Products, and SciFinder to identify known molecules. However, this approach will only recognize compounds that are identical to those in the databases, and any that are similar but non-identical to existing compounds will not be identified [3–5]. A strategy to overcome this limitation is to include information on MS/MS fragmentation in the dereplication process, as fragments will be characteristic for common structural features in a molecular class. These fragment data can be used to search MS fragment libraries such as Global Natural Products Social molecular networking (GNPS) [6]. As the number of NPs included in these fragmentation libraries is rapidly increasing, this method is becoming very useful for dereplication as well as compound class identification. The MS fragmentation data from compounds in a given sample can also be organized into molecular networks, a feature which also displays the mass differences between compounds in a network cluster. Therefore, compounds with similar structures will give similar fragmentation patterns and group together [7,8].

In the current study, we cultivated in four different media a newly isolated Arctic marine *Pseudomonas* sp. strain M10B774 that is affiliated with the *P. fluorescence* group. Fractions of the culture extracts were screened for antibacterial activity against the pathogenic bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae*, *Escherichia coli* and *Pseudomonas aeruginosa* in a growth inhibition assay. Cytotoxic activity of the fractions was also evaluated against three cancer cell lines, human melanoma (A2058), human breast carcinoma (MCF7) and human colon carcinoma (HT29), as well as the non-malignant normal lung fibroblast cell line (MRC5). Further, the project demonstrated the use of MS/MS-based molecular networking as a dereplication strategy to identify known compounds, their analogs and related compounds. The use of this strategy led to the isolation of one new and four known mono-rhamnolipids as well as the lipid moiety from one of the rhamnolipids.

2. Results

2.1. Identification of the Isolate M10B774

The bacterium isolate M10B774 was isolated from an Atlantic halibut in the Norwegian Sea on a medium containing Difco Marine Broth 15 g/L, peptone 5 g/L, 300 mL filtered seawater and 700 mL Milli-Q water (FMAP). To identify the bacterium, 16S rRNA sequencing and Basic Local Alignment Search Tool (BLAST) searches against reference sequences in GenBank were performed [9]. Based on these results, a set of related sequences were selected and a phylogenetic tree created (Figure S1). This phylogenetic analysis showed that the isolate is closely related to *P. gessardii* and belongs to the *P. fluorescence* group. The identity was not fully resolved, but it is suggested that the isolate is a new species or perhaps conspecific with *P. gessardii*.

2.2. Bioactive Extracts

The *Pseudomonas* sp. isolate was evaluated for its potential to produce antibacterial and cytotoxic compounds. It was cultivated in four different growth media: M19, VR_1, VR_2 and SGC (media compositions are listed in Section 4.2), in volumes of 2 × 200 mL. Compounds excreted into the medium were collected by adding Diaion® HP20ss resin beads to the cultures. The resin was collected and extracted with methanol (MeOH). Dried extracts were fractionated with flash chromatography into six fractions and screened for antibacterial activity in a growth inhibition assay against *E. coli*, *S. aureus*, *P. aeruginosa*, *E. faecalis* and *S. agalactiae.* The fractions were screened for cytotoxic activity

against three cancer cell lines, A2058, HT29 and MCF7, as well as the non-malignant MRC5 cell line, using a viability assay.

With six fractions obtained from each of the four extracts, 24 fractions were screened for bioactivity. Fraction 5, eluting in 100% methanol from the M19, VR_1 and VR_2 growth media showed activity in both the antibiotic and cytotoxicity assays. The screening results showed that cultivating this *Pseudomonas* sp. in the four different growth media led to different bioactivity profiles (Table 1). Fraction 5 from the VR_1, VR_2 and M19 media showed activity in the antibacterial assay ($OD_{600\,nm} < 0.05$), whereas no activity was observed in the SGC fractions. The M19 Fraction 5 was active against all three of the Gram-positive bacteria, whereas the VR_2 Fraction 5 showed activity against just two of them, *S. agalactiae* and *E. faecalis*. Further, Fraction 5 from the VR_1 culture was active against only one bacterium, *S. agalactiae*. None of the tested fractions had any effect on the assayed Gram-negative bacteria (*E. coli* and *P. aeruginosa*).

In the cytotoxicity assay, only the M19 Fraction 5 was active against all the four tested cell lines (Table 1). Based on these bioactivity results, active Fraction 5 from the VR_2 media was analyzed using LC-MS/MS to generate molecular networks for the compounds present in this fraction.

Table 1. The antibacterial activities of chromatography Fraction 5 (eluting with 100% MeOH) from the extracts of *Pseudomonas* sp. grown in four different media were tested in a growth inhibition assay. Cytotoxic activities of the fractions were evaluated with a cell viability assay. Test concentration for both assays was 50 µg/mL.

	Growth Inhibition Assay					**Viability Assay**			
Media	*E. coli* ^{N}	*S.aur* ^{P}	*P.aer* ^{N}	*E.F* ^{P}	*S.aga* ^{P}	A2058	MCF7	HT29	MRC5 *
VR_1	−	−	−	−	+	−	−	−	−
VR_2	−	−	−	+	+	−	−	−	−
M19	−	+	−	+	+	+	+	+	+
SGC	−	−	−	−	−	−	−	−	−

Antibacterial assay: +, $OD_{600\,nm} < 0.05$ and active; −, $OD_{600\,nm} > 0.05$ and inactive. ^{P} Gram-positive; ^{N} Gram-negative. *S.aur*, *S. aureus*; *P.aer*, *P. aeruginosa*; *E.F*, *E. faecalis*; *S.aga*, *S. agalactiae*. Viability assay: +, >50% cell death; −, <50% cell death. * Non-malignant cell line.

2.3. Identification of Bioactive Compounds

A molecular network-based approach using MS/MS data from active Fraction 5 (sample VR_2) as well as the inactive Fractions 4 and 6 utilized the GNPS platform in an attempt to identify the compounds responsible for the observed antibacterial activity. The molecular networking gave rise to 183 clusters. One of the clusters was especially promising because the nodes (compounds) were exclusively present in active Fraction 5. Moreover, one of the nodes matched with that of a rhamnolipid standard that was present in the GNPS library.

Rhamnolipids are secondary metabolites that consist of one or two rhamnose moieties linked to one or two saturated or unsaturated fatty acids [10,11], and are known to have potent surfactant properties [11]. The clustering of the compounds indicated that they were likely related rhamnolipids. UHPLC-HR-ESI-MS analysis of the fraction suggested that the compounds were present as Na^{+} adducts. UHPLC-HR-ESI-MS of active Fraction 5 from the M19 and VR_1 samples revealed that the same compounds were present (i.e., identical retention times, accurate mass and collisional cross sections), whereas the inactive Fraction 5 from the SGC sample did not contain detectable amounts of any of these compounds. Based on the chromatographic and culture condition distribution of these rhamnolipids, it was suspected that they were responsible for the observed activity; resultingly, they were selected for isolation and structure elucidation.

2.4. Characterization of the Isolated Compounds

Compounds **1–6** were isolated as viscous liquids. Their molecular formulae were calculated using accurate mass and isotope distribution from HR-ESI-MS. The structures shown in Figure 1 were determined using 1D and 2D NMR as well as and MS/MS fragmentation. Compound **1** was found to be the lipid moiety of compound **2**, and compounds **2–6** were found to be mono-rhamnolipids with different fatty acids. Proton scalar coupling constants, as well as chemical shifts, were in close agreement with the previously reported relative configuration of the rhamnose moiety; ^{3}J(1,2 1.7 Hz), ^{3}J(2,3 3.3 Hz), ^{3}J(3,4 9.5 Hz), and ^{3}J(4,5 9.5 Hz). The observed NOESY/ROESY patterns with two overlapping anti-phase zero quantum coherence artifacts for H3–H4 and H4–H5 suggests that extra care should be taken when interpreting these results. This conclusion is consistent with a rhamnose sugar since the zero quantum coherences (ZQCs) suggest that H4, H5 and H6 are sequentially anti and axial to each other, thus giving rise to strong scalar couplings with very little ROE contribution, but with significant ZQC due to their similar chemical shifts. Together with chemical shifts and coupling constants, all sugar moieties in **2–6** are fully consistent with rhamnose in α position. HSQC, HMBC, H2BC and HSQC-TOCSY were successfully employed to fully assign the resonances of the lipid chains and the positions of unsaturation.

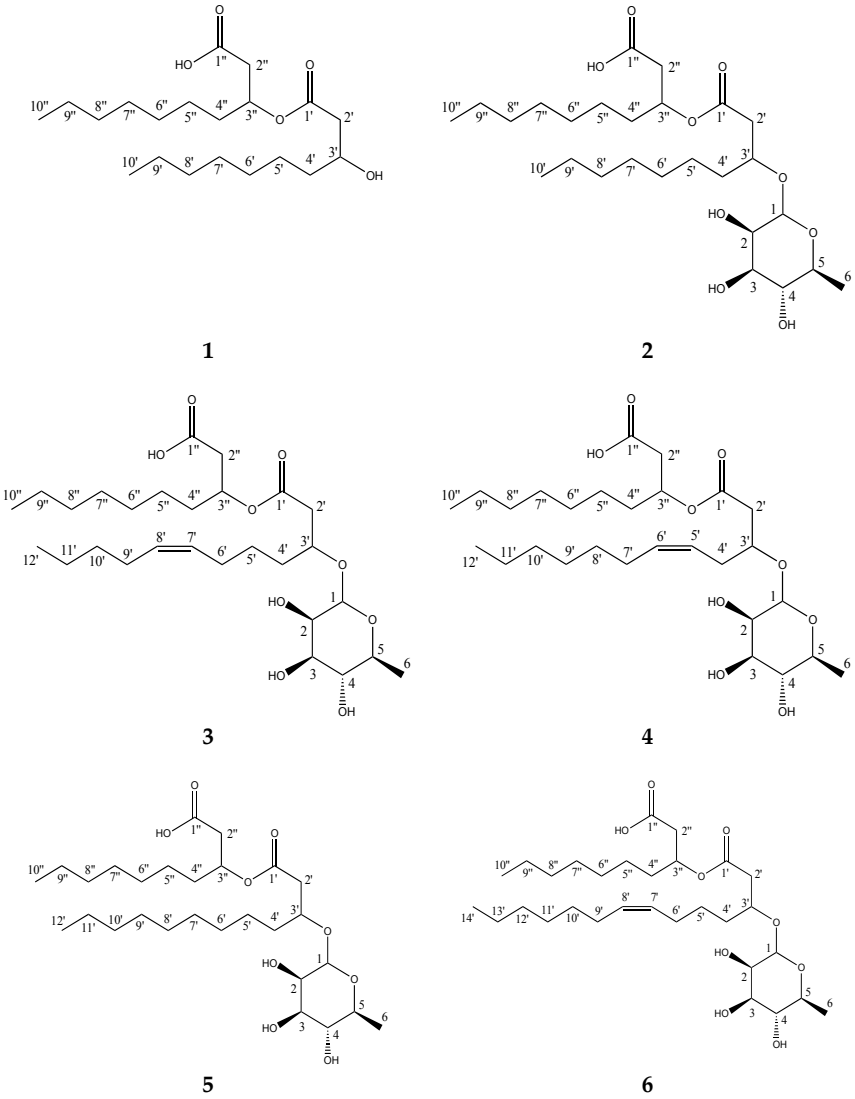

Figure 1. Structures of compounds **1–6** isolated from *Pseudomonas* sp.

The molecular formula of **1** was calculated to be $C_{20}H_{38}O_5$ (*m*/*z* 381.2609, $[M + Na]^+$, calcd 381.2611), suggesting two degrees of unsaturation. 1D and 2D NMR spectra (Figures S4–S8) showed that the compound was a di-lipid comprised of two-saturated 3-hydroxydecanoic acids that were linked through an ester bond. MS/MS fragmentation confirmed that each fatty acid consisted of 10 carbon atoms (Figure S46).

The molecular formula of **2**, $C_{26}H_{48}O_9$ (*m*/*z* 527.3192, $[M + Na]^+$, calcd 527.3191), indicated three degrees of unsaturation. 1D and 2D NMR data (Figures S9–S13) revealed that it was the known rhamnolipid Rha-C_{10}-C_{10} [11], consisting of one rhamnose moiety with the same fully saturated C_{10}-C_{10} di-lipid moiety as in **1**. The size and saturation of the lipid chains were confirmed with MS/MS fragmentation data (Figure S47).

Compound **3** had the same molecular formula as that of compound **4**, $C_{28}H_{50}O_9$ (*m*/*z* 553.3344, $[M + Na]^+$, calcd 553.3347), but a different retention time, suggesting that it had a different unsaturation pattern. 2D NMR data (HSQC + HMBC) indicated that **3** was indeed a rhamnolipid very similar to **4**, but with the double bond at position 7′–8′ instead of 5′–6′ (Figure 1). The structures of the lipid chains were assembled by HMBC and H2BC correlation data as a result of the central placement of the double bond which induced good spectral dispersion throughout the 12-carbon chain and allowed for the unambiguous identification of all carbon resonances. MS/MS fragmentation data confirmed the length of the lipid chains to be C_{10} and C_{12}, with an unsaturation on the C_{12} chain (Figure S48). The assignments are summarized in Table 2 and Figure 2. The configuration of the olefinic protons of compound **3** could not be directly assessed because of spectral overlaps in both the proton and the carbon dimensions for 7′/8′ as well as 6′/9′. It is reported here in a *cis* configuration by analogy to the other rhamnolipids isolated in this work. See below for the determination of the configuration of compounds **4** and **6**.

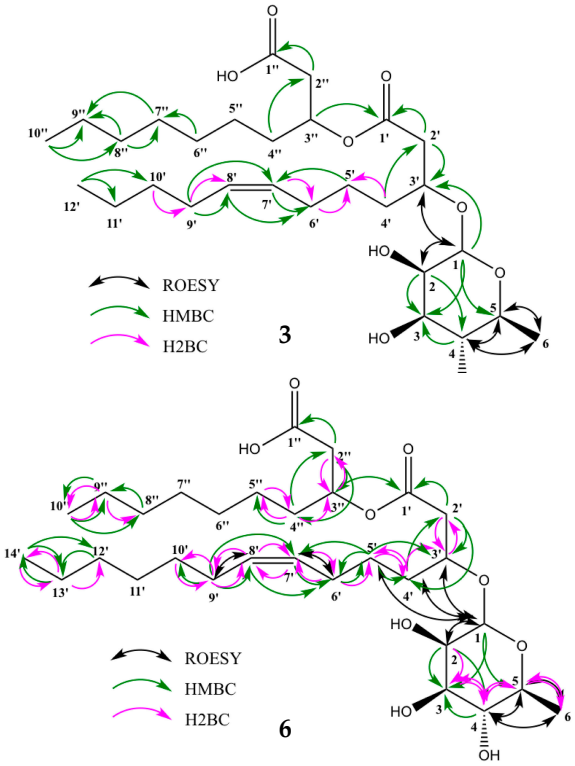

Figure 2. Selected 2D-NMR correlations for compound **3** and **6**. HMBC and H2BC revealed the position of unsaturation in the lipid chain, and the full lipid spin systems were identified in HSQC-TOCSY. HMBC and ROESY correlations confirmed the rhamnose moiety structure, while ROESY as well as homo- and heteronuclear coupling constants determined the olefinic protons to be in *cis* configuration.

Table 2. ^{1}H and ^{13}C NMR assignments for compound **3** and the observed HMBC and H2BC correlations (^{1}H→^{13}C).

Position	δ_C, Type	δ_H (*J* in Hz)	COSY	HMBC	H2BC	ROESY
1	98.7, CH	4.62, s		3′,3,5		3′,2,4′
2	70.3, CH	3.52 [o]		3,4		
3	69.3, CH	3.41 [o]	4		4	
4	71.9, CH	3.12, t (9.1)	3,5	10,5	5	5
5	69.0, CH	3.43 [o]	4,6	4	6	3′,4,6
6	17.8, CH_3	1.08, d (6.1)	5	4,5	5	4,5
1′	170.3, C					
2′	40.1, CH_2		3′	1′,3′	3′	3′
3′	72.9, CH	3.91, d (5.6)	2′,4′	1′	2′	2′,1,4′
4′	32.1, CH_2	1.45, dt (10.0, 6.4)	5′,3′	2′,3′	5′	o
5′	24.3, CH_2	1.30 [o]	6′,4′	7′		o
6′	26.3, CH_2	1.98 [o]	7′,5′	8′,7′	7′,5′	o
7′	129.9, CH	5.33 [o]	6′	9′,6′	8′,6′	o
8′	129.3, CH	5.32 [o]	9′	9′,6′	9′,7′	o
9′	26.6, CH_2	2.00 [o]	10′,8′	11′,10′,8′,7′	10′,8′	o
10′	31.3, CH_2	1.27 [o]	9′		9′	o
11′	21.7, CH_2	1.27 [o]	12	12′,10′	12′	o
12′	13.8, CH_3	0.86, t (6.9)	11′	11′,10′	11′	o
1″	170.6 *, C					
2″	40.4, CH_2	2.38 [o]		1″		
3″	71.0, CH	5.11, s [b]	2″,4″	1′		
4″	33.7, CH_2	1.52, s [b]	3″	2″		o
5″	24.7, CH_2	1.20 [o]				o
6″	28.6 **, CH_2	1.23 [o]		7″		o
7″	28.8 **, CH_2	1.23 [o]		9″		o
8″	31.2, CH_2	1.22 [o]		9″,7″		o
9″	22.1, CH_2	1.25 [o]	10″	10″,8″	10″	o
10″	14.0, CH_3	0.85, t (7.0)	9″	9″,8″	9″	o

* Not detectable in 1D, extracted from 2D HMBC ** Assignments could not be unambiguously distinguished [b] Broad peak [o] Overlapping peak in ^{1}H.

Compound **4**, which was recently isolated and identified from *Pseudomonas* sp. [12], was assigned the molecular formula $C_{28}H_{50}O_9$ (*m*/*z* 553.3348 [M + Na]$^+$, calcd 553.3347). 1D and 2D NMR (Figures S19–S26), together with MS/MS fragmentation (Figure S49), confirmed the lipid chains to be 10 and 12 carbon atoms long, with the unsaturation present in the C_{12} chain at position 5′–6′ (Figure 1). Upon closer examination, it was found that the configuration of the olefinic protons was in a *cis* configuration, which is in disagreement to what has been previously reported [12]. The vicinal $^{3}J_{HH}$ coupling constant between the two vinyl protons was determined to be roughly 10.9 Hz from deconvolution and simulation of the 1D proton multiplets (dtt, *J* = 10.9, 7.3, 1.5 Hz) (Figure S27). The ROE between the two protons has a dominant antiphase character (Zero Quantum artifact) and is close to the diagonal, making it inconclusive as it could be present in both configurations. However, a ROE/NOE connectivity can be traced from 4′→5′→6′→7′ as well as a direct 4′→7′ consistent with *cis* (Figure S26). Furthermore, there are no direct ROE/NOE from 4′→6′ or 7′→5′, which would have been expected in a *trans* configuration. The $^{3}J_{CH}$ couplings involving the olefinic protons were estimated to be between 9–10 Hz which also favors a *cis* configuration over *trans* (Figures S28 and S29).

The molecular formula of compound **5**, $C_{28}H_{52}O_9$ (*m*/*z* 555.3503, [M + Na]$^+$, calcd 555.3504), indicated structural similarity to **3** and **4**, but without the unsaturation on one of the lipid chains, as it had one less degree of unsaturation. 1D and 2D NMR (Figures S30–S34) as well as MS/MS fragmentation (Figure S50) confirmed it was a Rha-C_{10}-C_{12}, hence, the same lipid chain lengths as **3** and **4**, but fully saturated. A database search revealed that it was a known compound, previously identified from *Pseudomonas aeruginosa* [13].

The molecular formula of **6** was determined to be $C_{30}H_{54}O_9$ (*m*/*z* 581.3660, $[M + Na]^+$, calcd 581.3660), indicating four degrees of unsaturation and one lipid chain two carbons longer than compounds **3**, **4** and **5**. The four degrees of unsaturation indicated that one lipid chain possessed a double bond. 1D and 2D NMR data (Figures S35–S43) established **6** to be the mono-rhamnolipid Rha-$C_{14:1}$-C_{10}, with the unsaturation at position 7′–8′. From MS/MS fragmentation (Figure S51), the lipid chain lengths were confirmed to be 10 and 14 carbons long, with the unsaturation being present in the longer chain. A database search showed that rhamnolipids with the composition Rha-$C_{14:1}$-C_{10} are indeed known, but neither the position of unsaturation nor the order of the lipid chains were assigned in the previous studies [11]. However, comparing the NMR and MS/MS fragmentation data with the data from Tedesco et al. [12], it seems probable that their compound 3 has the same structure as our compound **6**. Our 1D and 2D NMR data were nearly identical to that reported, with the mean error of carbon chemical shifts = 0.69 ppm. Furthermore, the MS fragmentation data showed an identical pattern. However, they interpreted their data differently and described a different structure (Rha-$C_{12:1}$-C_{12}). We believe that the key fragment at *m*/*z* 265.18 represents the sodium adduct of the first fatty acid (i.e., 1′–14′) which indicates that the two lipid chains are C_{10} and C_{14} instead of both being C_{12}. This is in agreement with the fragmentation mechanism of compounds **3**, **4**, **5** and **6**. The difference in mass of the fragments between **6** and **3**, **4**, and **5** correspond to C_2H_4, suggesting that the additional C_2H_4 is added to the unsaturated chain instead of the saturated chain as Tedesco et al. reported for their compound 3. Simulations in Mass Frontier 7.0 were not conclusive as both tentative structures of **6** could form fragments of the correct mass within a reasonable number of steps. Careful examination of the HSQC-TOCSY data for **6** allowed us to unambiguously identify all 14 carbons in the spin system of the suggested unsaturated lipid chain (Figures S41 and S42), thus conclusively establishing the identity of the rhamnolipid with two chains of 10 and 14 carbons, respectively, and where the longer chain possesses a double bond at position 7′–8′. The assignments are summarized in Table 3 and Figure 2. Analogous to compound **3**, the configuration of the olefinic protons was found to be in a *cis* configuration. The vicinal $^3J_{HH}$ coupling constant between the two nearly overlapping olefinic protons was determined to be roughly 10.9 Hz from deconvolution and simulation of the 1D proton multiplets (dtt, *J* = 10.9, 6.6, 0.6 Hz) (Figure S15). The ROE/NOE pattern is less dispersed because of the greater distance to the branching point, but careful inspection allowed us to identify that all observable correlations did indeed follow the same pattern as in compound **4** (Figure S44). Most importantly there are no direct ROE/NOE from 4′→6′ or 7′→5′, which would have been expected in a *trans* configuration.

Table 3. ^1H and ^{13}C NMR assignments for compound **6** and the observed HMBC and H2BC correlations ($^1H \rightarrow ^{13}C$).

Position	δ_C. type	δ_H (*J* in Hz)	COSY	HMBC	H2BC	R/NOESY
1	99.8, CH	4.78, d (1.5)	2	3′		2,3′,4′,5′
2	72.8, CH	3.74, dd (3.3, 1.7)	1,3			1, **
3	72.0, CH	3.67 º	2,4	2,4	2,4	**
4	74.2, CH	3.31 º	3,5	5,6	3,5	**
5	70.1, CH	3.67 º	4,6	6	4,6	**
6	17.9, CH_3	1.25, d (6.1)	5	4	5	4,5
1′	172.8, C			2′		
2′	41.2, CH_2	2.56, dd (15.1, 7.6) 2.47 º	3′	4′ w	3′	3′,4′
3′	74.7, CH	4.11, dq (7.5, 5.6)	2′,4′	2′,5′	2′,4′	1,2′,4′,5′
4′	33.5, CH_2	1.56 º	3′,5′	2′,5′	5′	1,2′,3′,5′,6′
5′	25.9, CH_2	1.30, qd (7.4, 1.5)	4′,6′	6′	4′,6′	1,3′,4′,6′
6′	28.1, CH_2	2.05 º	5′,7′	5′,(7′,8′)	7′	4′,5′,7′w,7′
7′	130.3, CH	5.34 º	6′	5′,(6′,9′)	6′,8′	6′

Table 3. *Cont.*

Position	δ_C. type	δ_H (*J* in Hz)	COSY	HMBC	H2BC	R/NOESY
8′	131.5, CH	5.37 ^{o}	9′	(6′,9′),10′ ^{w}	7′,9′	9′
9′	28.2, CH_2	2.07 ^{o}	8′,10′	(7′,8′)	8′,10′	8′,10′,11′
10′	30.8, CH_2	1.31 ^{o}	9′	9′	9′	9′
11′	30.1, CH_2	1.32 ^{o}				9′
12′	32.99, CH_2 *	1.30 ^{o}		14′	13′	
13′	23.7, CH_2 *	1.31 ^{o}	14′	12′,14′	14′	14′
14′	14.45, CH_3 *	0.91, t (7.0) *	13′	13′	13′	13′
1″	177.1, C			2″		
2″	42.3, CH_2	2.47 ^{o}	3″	4″ ^{w}	3″	3″,4″
3″	73.6, CH	5.31, m	2″,4″	2″	2″,4″	2″
4″	35.4, CH_2	1.61, q (6.6)	3″,5″	2″	5″	2″,5″
5″	26.3, CH_2	1.33 ^{o}	4″	4″	4″	4″
6″	30.6, CH_2	1.31 ^{o}				
7″	30.4, CH_2	1.31 ^{o}				
8″	32.95, CH_2 *	1.28 ^{o}		10″	9″	
9″	23.7, CH_2 *	1.31 ^{o}	10″	8″,10″	10″	10″
10″	14.46, CH_3 *	0.90, t (7.0) *	9″	9″	9″	9″

* Assignments could not be chain-specifically distinguished; ^{o} overlapping peak in ^{1}H; shift extracted from 2D HMBC; ^{w} weak.

*2.5. Bioactivity of Compounds **1**–**6***

2.5.1. Antibacterial Activity

The six isolated compounds were tested for antibacterial activity in a growth inhibition assay and in a biofilm formation inhibition assay. Test concentrations in both bioassays were 50, 100 and 150 μM. In the growth inhibition assay, the compounds were tested against five pathogenic bacteria. All of the compounds were active against the three Gram-positive bacteria (Figure 3); however, none showed activity against the two Gram-negative bacteria *E. coli* and *P. aeruginosa* (Figure S2). Compounds **1**–**5** also showed a dose dependent activity against *E. faecalis*. Compared to the control, **1** had some effect at all three test concentrations, but it was less active than the other five compounds. Compound **2** was highly active ($OD_{600\,nm} \leq 0.05$) against *E. faecalis* at the two highest concentrations, while compound **3** showed high activity against *E. faecalis* only at the highest concentration of 150 μM. Compounds **4** and **5** were highly active at the two highest concentrations, while **6** had high activity at all three concentrations.

Against *S. aureus,* all compounds displayed a dose dependent activity. Compounds **1**, **3** and **6** had some effect at all concentrations compared to the control, but they did not show a high level of activity even at 150 μM. Compounds **2** and **5** were highly active at 150 μM, and **4** was active at the two highest concentrations. All compounds were highly active against *S. agalactiae* from 50 μM.

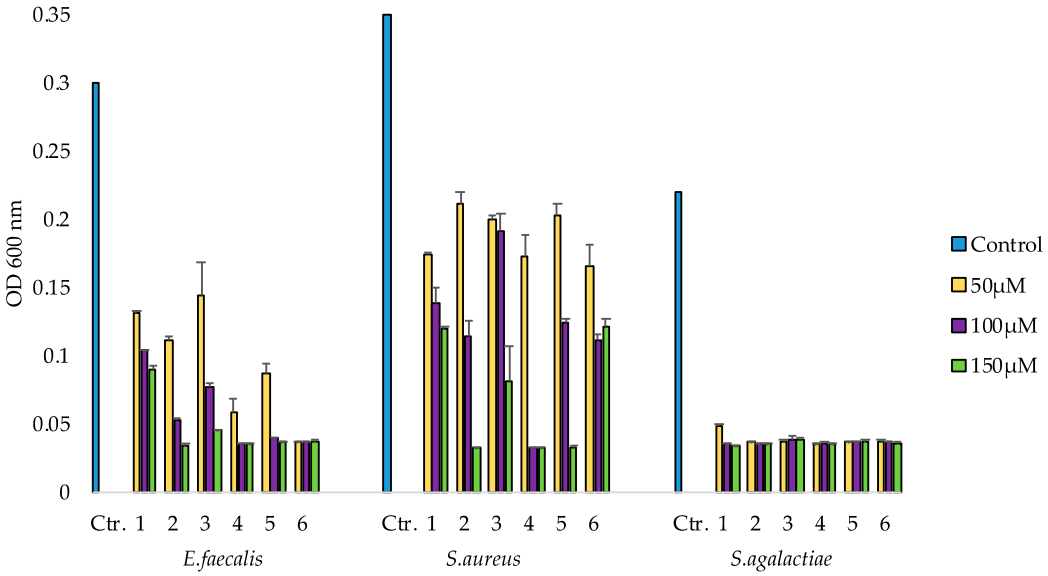

Figure 3. Growth inhibition assay of **1–6** tested at three concentrations against the Gram-positive bacteria *E. faecalis*, *S. aureus* and *S. agalactiae*. Bacteria and medium (50:50) were used as negative growth controls. Values are means of two replicates, error bars indicate standard deviation.

2.5.2. Inhibition of Biofilm Formation

The ability of the six compounds to inhibit biofilm formation was tested using the Gram-positive bacterium *Staphylococcus epidermidis*. All compounds displayed a dose dependent activity (Figure 4). Compounds **1** and **2** displayed high activity with $OD_{600\,nm}$ values below 0.2 (controls had OD ~1.0) at 50 μM, whereas the other compounds had high activity at 100 μM and above (Figure 4). Compound **3** seemed to have higher effect at 100 μM compared to 150 μM, but that is likely due to variations in the assay.

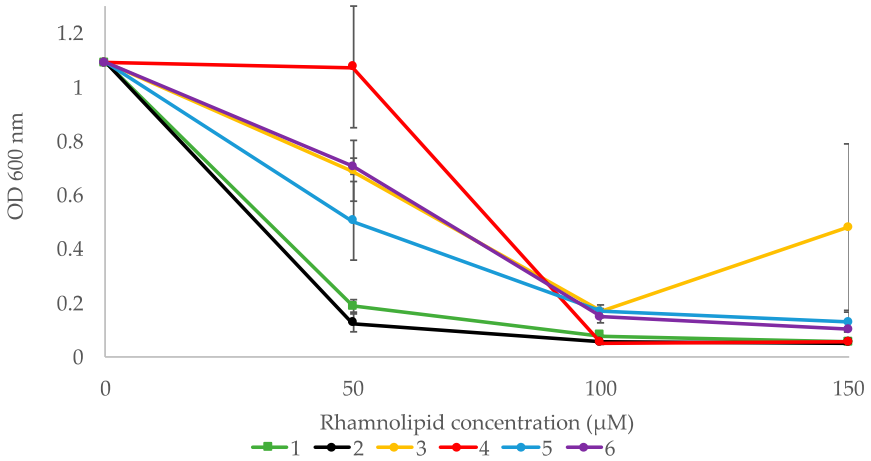

Figure 4. Biofilm formation inhibition assay performed on *S. epidermidis*. Values are mean of three replicates, ± standard error.

2.5.3. Cytotoxic Activity

The human melanoma cancer cell line A2058 and the non-malignant MRC5 cell line were used to test compounds **1–6** for activity in an MTS cell viability assay (Figure 5). Compounds **2**, **4** and **6** showed a dose-dependent activity against A2058 cells. They had no effect at the lowest concentration,

but compound **6** had some activity at 100 µM, with around 40% cell survival. At 150 µM, compounds **2**, **4** and **6** showed high activity with 0% cell survival. Compounds **1**, **3** and **5** did not display any activity against the A2058 cells. While compounds **2**, **4** and **6** also displayed activity against MRC5 cells at 150 µM, with 0% cell survival, compounds **1**, **3** and **5** showed no effect against this cell line at the tested concentrations.

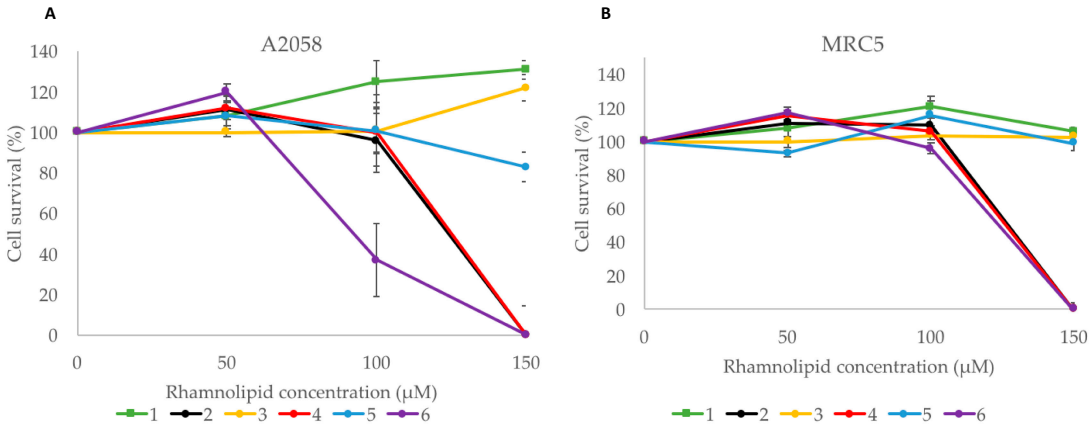

Figure 5. A cell viability MTS assay was used to evaluate the cytotoxicity of compounds **1**–**6**: (**A**) Human melanoma cells (A2058); (**B**) non-malignant cells (MRC5). Values are mean of three replicates, ± standard error.

3. Discussion

This newly isolated *Pseudomonas* sp. strain was cultured in four different media, and the culture extracts were subsequently fractionated into six fractions each prior to bioactivity screening. SMs are often produced in small quantities, and other compounds, such as media components and primary metabolites, can mask their activities. This risk is mitigated when the extracts are pre-fractionated, which generally increases the hit rate in bioassays [11]. The bioactivity screening of the fractions from the four media revealed that the selected media influenced the production of bioactive compounds. Activity was observed in Fraction 5 from the M19, VR_1 and VR_2 media. These are all nutrient rich media wherein the main difference is the energy source, a feature which is known to affect the production of secondary metabolites [11,14]. The M19 medium has D-mannitol as the energy source, and Fraction 5 from this medium showed activity against all three of the tested Gram-positive bacterial strains. In addition, it was the only fraction that showed activity in the cancer cell viability assay, where it was active against all three of the cancer cell lines as well as the non-malignant cell line. The fractions deriving from the extracts formed from growth in the VR_1 and VR_2 media were similar; both media contain yeast and malt extracts as energy source. The difference between them is that the VR_2 medium contains iron sulfate and potassium bromide, which are components of seawater [15]. Adding trace elements to a growth medium is known to effect the production of secondary metabolites [16], and this modification seemed to have some effect in our study, as the VR_2 Fraction 5 was active against both *E. faecalis* and *S. agalactiae*, whereas the VR_1 Fraction 5 was active only against *S. agalactiae* in the antibacterial assay. No samples from the SGC medium had any activity in the bioactivity screening. This was the only low nutrient medium used; we had hypothesized that stressing the *Pseudomonas* sp. might induce the production of new secondary metabolites. As the samples from this growth medium did not have any activity, it may be that the nutrient level was too low to allow the production of energetically costly antibacterial and anticancer compounds. These results demonstrate that a diverse selection of growth media is important when searching for bioactive compounds from cultured microorganisms.

HR-ESI-MS analyses of the fractions showed that the isolated rhamnolipids were present in the samples from the M19, VR_1 and VR_2 media, but not in the inactive SGC sample. Rhamnolipids

are known to have antibacterial and cytotoxic activities, so these compounds were suggested and later confirmed to be responsible for the observed bioactivity [17]. Yield, diversity and ratios of rhamnolipids depend on cultivation conditions [18–20], so differences in the rhamnolipid content and composition due to different media composition can explain why the three samples were active in the different bioassays. However, it is also possible that non-identified compounds were responsible for some of the observed bioactivity. The effect of the media composition was clearly observed for compound **1**, as it was among the most abundant peaks in the HR-ESI-MS of the M19 extract, while it was found only in minute amounts in the VR_1 and VR_2 extracts.

MS/MS fragmentation followed by molecular networking proved to be an effective way to dereplicate these related rhamnolipids. Using HR-ESI-MS for dereplication of bioactive compounds is a powerful tool, as the elemental composition can be used to search databases of known compounds. However, subtle changes in the chemical structure of a known compound can be difficult to recognize, such as position of unsaturation and relative carbon chain length of fatty acid chains. Using MS/MS fragmentation patterns to establish relationships between molecules within a sample as well as between unknown compounds and library references can facilitate the dereplication process.

The molecular network cluster also suggested that the VR_2 Fraction 5 contained di-rhamnolipids. From HR-ESI-MS analysis, it appeared that the di-rhamnolipids had the same retention time as the mono-rhamnolipids with the same lipid chains, the only difference being an extra rhamnose moiety in the di-rhamnolipidc (e.g., Rha-Rha-C_{10}-C_{10}, and Rha-C_{10}-C_{10}). The same feature was observed in the prep-HPLC-MS data obtained during isolation of the mono-rhamnolipids from the M19 extracts; it appeared that the mono-rhamnolipids and traces of the corresponding di-rhamnolipids had the same retention times. However, when analyzing the purified compounds by NMR, di-rhamnolipids were not detected. This suggests that the di-rhamnolipids were likely generated in the ion source of the MS. Rhamnose moieties are easily removed from the lipid moiety in the ion source, resulting in free rhamnose moieties which can react with a mono-rhamnolipid, forming a di-rhamnolipid species. Indeed, considering the structural differences of mono- and di-rhamnolipids, they are not expected to have the same retention times. Déziel et al. [21] and Behrens et al. [22] showed that mono-rhamnolipids and the corresponding di-rhamnolipids had different retention times on reversed-phase HPLC columns, supporting the idea that the proposed di-rhamnolipids were generated in the ion source.

Rhamnolipids were first discovered in 1946 by Bergstrøm et al. [23] as a product of *P. aeruginosa*. Subsequently, other *Pseudomonas* sp. and bacteria from the genus *Burkholderia* have been discovered to produce rhamnolipids, but the known producers are still limited to only a few species [11,24,25]. Rhamnolipids have been widely studied, and today more than 60 congeners and isomers have been identified and characterized, as reviewed by Abdel-Mawgoud et al. in 2010 [11]. In addition to having antibacterial and cytotoxic activity, rhamnolipids have also shown antiviral, antifungal and anti-biofilm activities. Most studies have focused on *P. aeruginosa*, which is currently used for the industrial production of rhamnolipids. However, one issue arising from use of this bacterium for commercial production is its human pathogenicity [26–28]. Bacteria from the *P. fluorescence* group are not known to be human pathogens, so the *Pseudomonas* sp. strain used in this study could be a candidate to replace *P. aeruginosa* for industrial production of rhamnolipids. Hence, it is important to gain insight into which rhamnolipids this M10B744 strain produces.

The *Pseudomonas* sp. strain M10B744 was partly identified by phylogenetic analysis of the 16S rRNA gene, and is either a *P. gessardii*, or a new species closely related to *P. gessardii. P. gessardii* is not well studied, but *P. fluorescence* and *P. synxantha*, belonging to the *P. fluorescence* group, are reported to produce rhamnolipids [29–33]. However, the only rhamnolipid structurally characterized from this group is the di-rhamnolipid Rha-Rha-C_{10}-C_{10} isolated from a *P. fluorescence* strain [34]. Thus, the five mono-rhamnolipids we isolated in the current study are the first mono-rhamnolipids structurally characterized from the *P. fluorescence* group.

In this study, we were able to describe the fatty acids and their order for all the isolated rhamnolipids, including the position and stereochemistry of the double bonds. However, the absolute

stereochemistry of C-3′ and C-3″ remains unresolved. The structure of compound **3** is described for the first time in this study. Searches in databases indicate that it is a new compound. Rhamnolipids with the same elemental composition and lipid chain lengths have been reported in several studies [21,22,35], but without the position of unsaturation or order of lipid chains identified. The previously reported structures are not necessarily identical to **3**, as it contains an unsaturation that in principal can be present in different positions. This is illustrated for compound **4** which had the same elemental composition and lipid chain lengths as **3**, C_{10}–$C_{12:1}$, but with the unsaturation at a different position (Figure 2). Compound **4** was recently described by Tedesco et al. as an isolate from an Antarctic *P. aeruginosa* [12].

We identified compound **6** as a mono-rhamnolipid with lipid chains C_{10} and $C_{14:1}$. Rhamnolipids with these chains have previously been reported, but the position of the unsaturation and order of chain lengths have not been previously assigned [36]. However, comparison of our NMR and MS/MS fragmentation data with data from the study by Tedesco et al. revealed that the data were identical, and that compound **6** is the same rhamnolipid as their compound 3, which they described as a novel rhamnolipid with C_{12} and $C_{12:1}$ lipid chains. MS/MS analysis of **6** gave a key fragment at m/z 411.24, and this mass corresponds the loss of a C_{10} lipid chain (Figure S51). Although this fragment was also present in the data of Tedesco et al., it was not assigned to any specific loss. In conclusion, both the NMR data (Figures S35–S45) and the MS/MS data (Figure S51) strongly indicated that the lipid chains are C_{10} and $C_{14:1}$, and not C_{12} and $C_{12:1}$ as reported by Tedesco et al. [12].

Much of the previous bioactivity screening of rhamnolipids has been performed on mixtures or on non-characterized rhamnolipids [37–42]. In the current study, we assessed the bioactivity of these natural products individually, and tested the isolated compounds in their pure form. In the antibacterial assay, all compounds showed some effect against the three Gram-positive bacteria strains. However, no activity was observed against the two Gram-negative bacteria strains, which usually are less sensitive to antimicrobial agents due to their outer cell wall that contains lipopolysaccharides acting as an extra barrier [43]. All isolated compounds were active in the biofilm formation inhibition assay against Gram-positive *S. epidermidis*.

A number of antimicrobial agents are amphiphilic compounds, such as daptomycin [44] and brilacidin [45], that function by binding to membranes as detergents, leading to membrane lysis. Rhamnolipids are amphiphilic due to their lipophilic lipid chain and hydrophilic rhamnose moiety, and are reported to act by affecting the membrane of target cells [46,47]. Sotirova et al. [48] found that rhamnolipids are inserted into the phospholipid membrane of cells, thus affecting their structure and function, which can lead to cell death. Al-Tahhan et al. [49] reported that rhamnolipids lead to the loss of lipopolysaccharides (LPS) and subsequent alteration of the outer membrane in the Gram-negative bacterium *P. aeruginosa*. Jiang et al. [50] reported that rhamnolipids can also induce cytotoxicity by reducing the surface tension of the culture medium, and this is also an effect of their amphiphilic nature [51,52].

As the rhamnose moiety is the same for all five of the mono-rhamnolipids studied herein, the variations in bioactivity between these compounds must be a result of differences in the lipid chains. The difference in activity in the cytotoxicity assay between **3** and **5** (not active) and **4** (highly active with 0% cell survival for both A2058 and MRC5) is somewhat surprising. Compounds **3**, **4** and **5** are structurally very similar to one another, as they have the same lipid chain lengths, C_{10}-C_{12}, but **3** and **4** have an unsaturation at different positions in chain B, and **5** is fully saturated. On the other hand, it is possible that there are some inaccuracies in the test concentrations, a matter that should be considered when working with small amounts of isolated natural products.

The effect of the rhamnose moiety was seen when comparing the activity of **1** and **2**, as they had the same lipid moiety but **2** also contained a rhamnose moiety. Fatty acids are known to have surfactant activity and to exhibit antibacterial activity by affecting the membrane of cells [53,54]. This was verified in the antibacterial assays, as **1** was active in both the growth inhibition and anti-biofilm assays, similar to the rhamnolipids, indicating that the presence of a rhamnose moiety in compound **2** did not

substantially enhance the antibacterial activity. However, in the viability assays, compound **1** did not show any activity, whereas **2** was active against both cell lines; thus, it is clear that including a rhamnose moiety had an effect on the activity against the human A2058 and MRC5 cells.

In conclusion, using different cultivation media for the *Pseudomonas* sp. strain M10B744 gave extracts with different bioactivity profiles, appearently due to changes in the production of rhamnolipids. The rhamnolipids were initially identified by the use of MS/MS fragmentation data and molecular networking, demonstrating the utility of this approach for dereplication. Five mono-rhamnolipids were characterized for the first time from a bacterium within the *P. fluorescence* group. One of the rhamnolipids was a new molecule, demonstrating that Arctic marine bacteria can be a valuable resource for new bioactive molecules.

4. Materials and Methods

4.1. Microorganism

Isolation: *Pseudomonas* sp. strain M10B774, was isolated from an Atlantic halibut (*Hippoglossus hippoglossus*) in the Norwegian Sea, dd° N 77,46707333 and dd° E 10,609719 in January 2010. It was streaked onto FMAP agar consisting of: 15 g Difco marine broth (279110, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 15 g agar (A1296, Sigma-Aldrich, St. Louis, MO, USA), 700 mL Milli-Q water (Merck Millipore, Darmstadt, Germany), 300 mL filtrated seawater (FSW, 5 µm pore size, ceramic membrane filter 0.2 µm, UV filter) and 5 g peptone from caseine (82303, Sigma-Aldrich). After isolation the strain was stored in FMAP broth (without agar) and 30% glycerol (G5516, Sigma-Aldrich) at −80 °C.

Identification: The isolate was stored at −80 °C, plated on FMAP agar plate and grown at 10 °C for 7 days before a single colony was inoculated into an Eppendorf tube with 100 µL of Milli-Q and boiled for 5 min. PCR was performed on a thermal cycler (Mastercycler epgradient S, Eppendorf, Hamburg, Germany) using 1 µL of the bacterial lysate as template, 1 µM of forward primer (27F, AGAGTTTGATCMTGGCTCAG), 1 µM of reverse primer (1492R, CGGTTACCTTGTTACGACTT) and 12.5 µL of ThermoPrimeTM 2× ReddyMix PCR master mix (ThermoFisher Scientific, Waltham, MA, USA) in a total volume of 25 µL. PCR was carried out using the following program: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gel and documented with Bioimaging system, Syngene. The PCR product of 16S rRNA gene was purified with QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The primers 27F or 1492R were employed to sequence the purified PCR product. Sequence data were collected by the sequencing lab at University Hospital of North Norway (Tromsø, Norway). Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) provided by the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and the strain was identified using phylogenetic interference. See detailed description of the identification process in Supplementary Information Figure S1.

4.2. Fermentation and Extraction of Secondary Metabolites

Pseudomonas sp. was grown in 2 × 1 L Erlenmeyer flasks at 10 °C at 140 rpm in 200 mL M19, VR_1, VR2 and SGC medium (Table 4). All medium components were from Sigma-Aldrich, except Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and potassium bromide (KBr) from Merck. SGC medium were suspended in 100% FSW, whereas the three other media were in 50:50 FSW and Milli-Q.

Table 4. Components of growth media used for fermentation of *Pseudomonas* sp. with product numbers. The amounts of medium ingredients are given in g/L.

Medium	D-Mannitol (63560)	Peptone (82303)	D-Glucose (D9434)	Casein Hydrolase (22090)	Malt Extract (70167)	Yeast Extract (Y1625)	$FeSO_4 \cdot 7H_2O$ (1.03965)	KBr (22186)
M19	20	20	-	-	-	-	-	-
VR_1	-	11.11	-	-	6.67	6.67	-	-
VR_2	-	11.11	-	-	6.67	6.67	0.044	0.044
SGC	-	-	4	3	-	-	-	-

The bacterium was cultivated in the four different media until growth was visible (1–2 weeks). To collect secondary metabolites excreted into the medium, Diaion®HP-20 resin beads (13607, Supelco Analytica, Bellefonte, PA, USA), 40 g/L, which were soaked in MeOH (34860, Sigma-Aldrich) for 20 min and washed extensively in Milli-Q water, were added to the cultures 3–4 days before extraction. Extraction was performed by filtrating the cultures under vacuum, using a fine mesh cheesecloth (1057, Dansk Hjemmeproduktion, Ejstrupholm Danmark)). Resin beads captured on the cheesecloth were washed with 100 mL Milli-Q and extracted twice with 150 mL MeOH before vacuum filtered through Whatman Ø 90 mm No. 3 filter (Whatman plc, Buckinghamshire, UK). The extracts were dried under pressure and stored at −20 °C.

4.3. Fractionation

Extracts of *Pseudomonas* sp. cultivated in the four media were dissolved in 8 mL 90% MeOH. Then, 2 g Diaion® HP-20ss resin beads were added before the mixture was dried under pressure. Resin (6.5 g) was soaked in MeOH for 20 min before being exchanged with Milli-Q water and packed in a flash cartridge (Biotage® SNAP Ultra, Biotage, Uppsala, Sweden). The cartridge was equilibrated in 5% MeOH before the extract/resin mixture was loaded on top. Fractionation was performed using a Biotage SP4™ system with flow rate 12 mL/min and gradient 5–100% MeOH over 32 min, and MeOH:acetone (34850, Sigma-Aldrich) to 100% acetone over 18 min. This resulted in six fractions that were dried under pressure at 40 °C.

4.4. Bioactivity

4.4.1. Growth Inhibition Assay

Media used in the growth inhibition assay include Muller Hinton broth (MH, 275730, Becton, Dickinson and Company) and Brain Heart Infusion broth (BHI, 53286, Sigma-Aldrich). Bacteria strains that were cultured in MH medium included *S. aureus* (ATCC 25923), *E. coli* (ATCC 259233) and *P. aeruginosa* (ATCC 27853), and in BHI medium included *E. faecalis* (ATCC 29122) and *S. agalactiae* (ATCC 12386). Fresh bacteria colonies were inoculated in respective growth medium and incubated overnight at 37 °C. The number of cells was adjusted in fresh medium to reach the log phase, and added to a 96-well microtiter plate (734-2097, Nunclon™, Thermo Scientific) with 1500–15,000 CFU/well, total volume 100 µL/well. Flash fractions in the primary screening were dissolved in Milli-Q water with 1% dimethyl sulfoxide (DMSO, D4540, Sigma-Aldrich) to 1 mg/mL and tested in duplicates at concentrations 50 µg/mL. The isolated compounds **1**–**6** were dissolved in Milli-Q water with 1% DMSO and added to the wells in duplicates, at the final concentrations 50 µM, 100 µM and 150 µM. The plate was incubated overnight at 37 °C before the growth was measured my assessing the absorbance for at 600 nm with 1420 Multilabel Counter VICTOR^3$_{TM}$ (Perkin Elmer, Waltham, MA, USA). Bacterium suspension diluted with water (1:1) was used as growth control. A dilution series of gentamycin from 32 to 0.01 µg/mL were used as positive assay controls; the growth medium was used as a negative growth control.

4.4.2. Biofilm Inhibition Assay

Staphylococcus epidermidis (ATCC 35984) grown in Tryptic Soy Broth (TSB, 105459, Merck, Kenilworth, NJ, USA) overnight at 37 °C was diluted in fresh medium with 1% glucose (D9434, Sigma-Aldrich) before being transferred to a 96-well microtiter plate; 50 µL/well were incubated overnight with 50 µL of compound **1**–**6** dissolved in Milli-Q water added in duplicates. The bacteria were then removed from the plate and the plate washed with tap water. The biofilm was fixed at 65 °C for 1 h before 70 µL 0.1% crystal violet (115940, Merck Millipore) was added to the wells for 10 min of incubation. Excess crystal violet solution was then removed and the plate dried for 1 h at 65 °C. Seventy microliters of 70% EtOH were then added to each well and the plate incubated on a shaker for 5–10 min. Biofilm formation inhibition were assessed by the presence of violet color and was measured at 600 nm absorbance using a 1420 Multilabel Counter VICTOR$^3{}_{TM}$. Fifty microliters of a non-biofilm forming *Staphylococcus haemolyticus* (clinical isolate 8-7A, University hospital, UNN, Tromsø, Norway) mixed in 50 µL autoclaved Milli-Q water was used as a control; 50 µL *S. epidermidis* mixed in 50 µL autoclaved Milli-Q water was used as the control for biofilm formation; and 50 µL TSB with 50 µL autoclaved Milli-Q water was used as a medium blank control.

4.4.3. Cytotoxicity Assay

Cell viability of fractions and pure compounds was tested in an MTS in vitro cell proliferation assay against three cancer cell lines; human melanoma A2058 (ATCC, CRL-1147^{TM}), human breast carcinoma MCF7 (ATCC HTB-22^{TM}) and human colon carcinoma HT29 (ATCC HTB-22^{TM}) and one non-malignant cell line, normal lung fibroblasts MRC5 (ATCC CCL-171^{TM}). The cells were seeded in a 96-well microtiter plate in Roswell Park Memorial Institute (RPMI-1640 medium, FG1383, Merck) with 10% Fetal Bovine serum (FBS, S0115, Biochrom, Cambridge, UK) at a concentration of 2000 cells/well for the three cancer cell lines and 4000 cells/well for MRC5. After incubation for 24 h at 37 °C and 5% CO_2, the medium was replaced with fresh RPMI-1640 medium which included 10% FBS and gentamycin (10 µg/mL. A2712, Merck). The samples were added in triplicate, fractions at a concentration of 50 µg/mL, and isolated compounds **1**–**6** at concentrations of 50, 100 and 150 µM, to form a total volume of 100 µL/well. After an additional 72 h incubation at 37 °C and 5% CO_2, 10 µL CellTiter 96$^{®}$AQ$_{ueous}$ One Solution Reagent (G3581, Promega) with tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and phenazine ethosulfate were added to each well before incubation for 1 additional hour. The absorbance was measured at 485 nm with a DTX 880, and cell viability calculated. RPMI-1640 with 10% FBS and 0.5% Triton^{TM} X-100 (Sigma-Aldrich) were used negative controls.

4.5. Dereplication, Isolation and Structure Elucidation

4.5.1. LC-MS/MS and Molecular Networking

LC-MS/MS data for molecular networking were obtained with a system consisting of a Thermo Finnigan Surveyor Autosampler Plus, LC-Pump-Plus and PDA Plus coupled a Thermo Finnigan LCQ Advantage Max mass spectrometer. The flash chromatography fractions were dissolved in MeOH to a concentration of 1 mg/mL, and 20 µL of each fraction was injected onto a Kinetex C18 column (5 µm, 4.6 mm × 100 mm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile (ACN) and H_2O (both containing 0.1% formic acid) with a flow of 0.7 mL/min, and the components were eluted with the following gradient: 30% ACN for 5 min, increase to 99% ACN over 17 min, hold at 99% ACN for 4 min. The MS was run in positive electrospray, and data from m/z 190 to 2000 was recorded with automated full dependent MS/MS scan enabled. The chromatograms were converted to .mzxml files using msConvert (www.proteowizard.sourceforge.net), and the chromatograms were submitted to GNPS for analysis (www.gnps.ucsd.edu). Cytoscape 3.6.0 (www.cytoscape.org) was used to visualize the molecular networks. A cosine value of 0.7 was used to generate the molecular network.

4.5.2. HR-MS/MS

High-resolution mass spectrometry was run with ESI+ ionization using UPLC-QToF-MS. It was performed on an Acquity UPLC I-class and a Vion IMS QToF with an Acquity UPLC C18 column (1.7 µm, 2.1 mm × 100 mm) (all from Waters). The samples were run with a 12 min gradient increasing from 10% to 90% acetonitrile (ACN, 75-05-08, Merck) with 1% formic acid (FA, 069141, Biosolve, Dieuze, France) in ultra pure water (7732, Merck) and a flow rate 0.45 mL/min. Waters UNIFI 1.8.2 Scientific Information System software was used to process the data.

4.5.3. Isolation of Compounds **1**–**6**

Purification of the rhamnolipids was performed using a prep-HPLC system (Waters) consisting of a 600 HPLC pump, a 3100 mass spectrometer, a 2996 photo diode array detector and a 2767 sample manager. The system was controlled with MassLynx version 4.1. Various columns were used (all from Waters): X-Terra RP-18 Prep Column (10 µM, 10 mm × 300 mm), Atlantis Prep dC18 Column (10 µM, 10 mm × 250 mm), XSelect CSH Prep Fluoro-Phenyl (5 µM, 10 mm × 250 mm). Gradients were optimized using Milli-Q water with 0.1% FA (33015, Sigma-Aldrich) and acetonitrile (34851, Sigma-Aldrich) with 0.1% FA as mobile phase. Flow rate was constant at 6 mL/min. Flash Fraction 5 was resuspended in 100% MeOH, and the initial separation of the rhamnolipids was done on the Atlantis dC18 column using a gradient from 50% to 100% ACN over 15 min. The combinations of gradients and columns used for the final isolation of each compound are listed in Table 5.

Table 5. Column, gradient and run-time used for isolation of compound **1**–**6**.

Compound	Column	Gradient (%) ACN	Time (min)
1	XSelect	55–57	7.00
2	Atlantis	70–78	10.00
3	Atlantis	68–72	10.00
4	Atlantis	70–80	12.30
5	X-Terra	70–78	10.00
6	Atlantis	80–96	12.00

4.5.4. NMR

All NMR spectra were acquired on a Bruker Avance III HD spectrometer equipped with an inverse detected TCI probe with cryogenic enhancement on ^{1}H, ^{2}H and ^{13}C, operating at 599.90 MHz and 150.86 MHz for ^{1}H and ^{13}C, respectively. Samples were prepared in DMSO-d_6 and methanol-d_4, and recorded at 298 K.

All experiments were acquired using standard pulse sequences for Proton, Presat, Carbon, DQFCOSY, ECOSY, HSQC (bip), HMBC (bip), H2BC (bip), HSQCTOCSY (mlev), TOCSY (clean mlev), NOESY and ROESY (adiabatic) in Topspin 3.5pl7, using gradient selection where applicable, and processed in Mnova 12.0.0. Spectra were referenced on the residual solvent peak of methanol-d_4 (δ_H = 3.31 and δ_C = 49.00) or DMSO-*d6* (δ_H = 2.50 and δ_C = 39.52).

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/16/5/163/s1. Figures S1–S45, including a phylogenetic three of *Pseudomonas* sp., strain M10B774, bioactivity data as well as 1D and 2D NMR data, Figures S46–S51, MS/MS data of compounds **1**–**6**, and HR-ESI-MS spectra of compounds **1**–**6** can be found online.

Author Contributions: V.K. fermented the bacterium and prepared extracts and Flash fractions, tested for bioactivity and isolated the compounds; E.H. dereplicated the compounds; J.I., V.K. and E.H. elucidated the structures; T.R. J.H.A., W.H.G. and E.H. designed the study; V.K., T.R., J.I., J.H.A., W.H.G. and E.H. prepared the manuscript.

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Supplementary Materials

Characterization of rhamnolipids produced by an Arctic marine bacterium from the *Pseudomonas fluorescence* group

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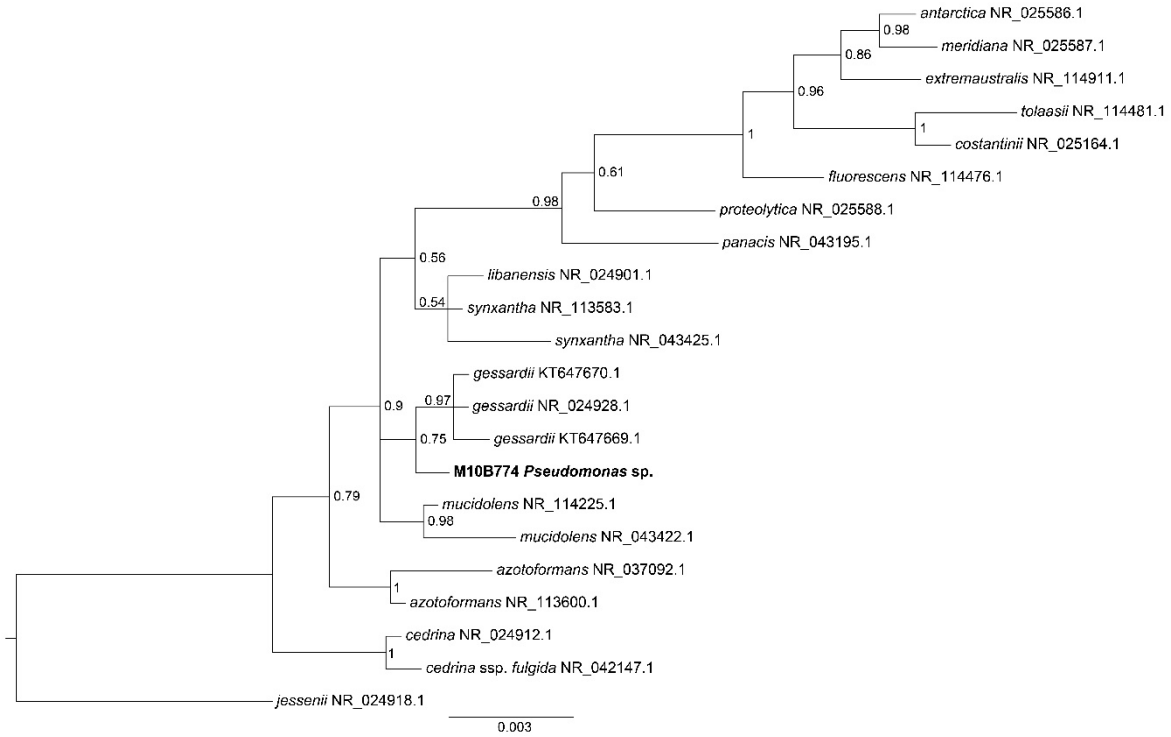


Figure S1. Phylogenetic tree of *Pseudomonas* species in the *P. fluorescence* group based on 16S rRNA gene sequences. The studied isolate M10B774 is shown in bold. Branch labels represent identification and accession codes of the Pseudomonas sequences in GenBank, node support is given as posterior probabilities (PP). *P. jessenii* was used as an outgroup for the other taxa. The accurate identity and placement of M10B774 remains unresolved, because of the adjacent polytomy and lack of node support at nearby nodes of the tree. However, the study isolate seems to be most closely related, if not conspesific, with *P. gessardii.*

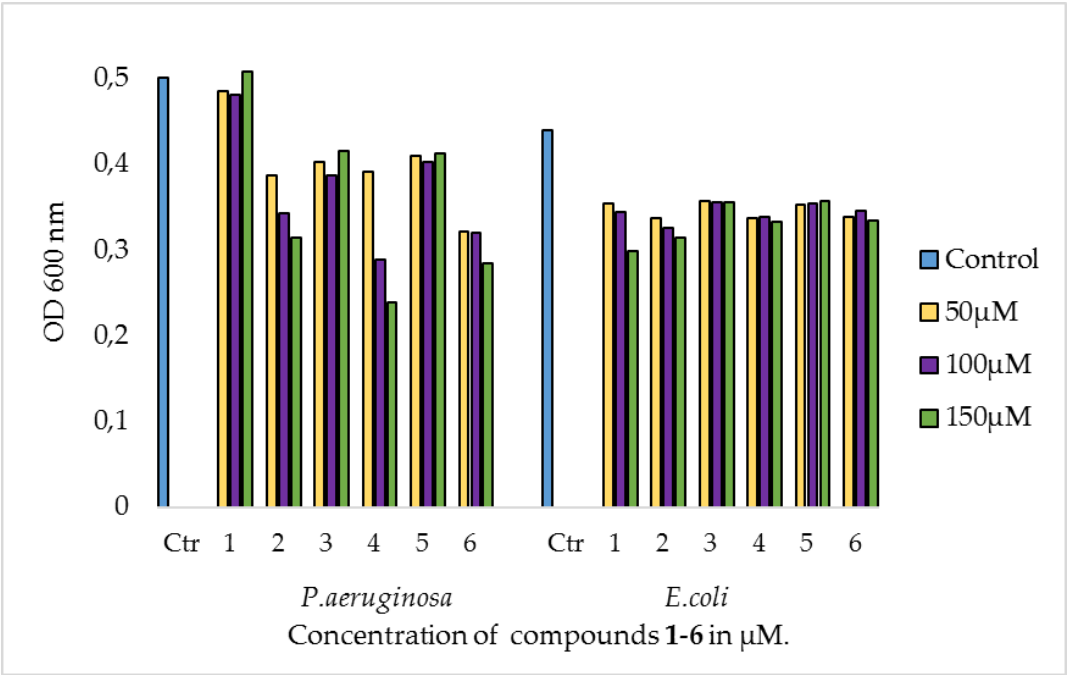


Figure S2. Antibacterial growth inhibition assay of compounds **1-6** tested at three concentrations against Gram-negative *P. aeruginosa* and *E.coli*. Bacteria cells and medium (50:50) was used as negative control (0µM). Values are mean of two parallels.

Table S3. ^{13}C NMR assignments for compounds **1-6**.

	1 (dmso-d_6)	**2** (methanol-d_3)	**3** (dmso-d_6)	**4** (dmso-d_6)	**5** (dmso-d_6)	**6** (methanol-d_3)
1		100.62	98.7	98.75	98.69	99.82
2		72.29	70.3	70.54	70.97	72.79
3		72.19	69.3	70.47	70.31	72.03
4		74.04	71.9	71.85	71.87	74.24
5		70.00	69.0	68.76	68.93	70.14
6		17.79	17.8	17.77	17.83	17.92
1′	170.59	172.31	170.3	170.03	170.32	172.77
2′	42.76	41.18	40.1	39.54	40.06	41.25
3′	67.06	75.45	72.9	72.47	72.94	74.73
4′	36.69	34.25	32.1	30.38	32.47	33.51
5′	24.49	25.78	24.3	124.37	24.66	25.89
6′	28.70*	30.58	26.3	132.34	28.63*	28.08
7′	28.70*	30.25*	129.9	26.80	28.71*	130.29
8′	31.28*	32.86*	129.3	28.73	28.84*	131.46
9′	22.10*	23.59*	26.6	26.80	28.98*	28.22
10′	13.94*	14.32*	31.3	31.17*	31.22*	30.83
11′			21.7	22.09*	22.11*	30.08
12′			13.8	13.94*	13.96*	32.99*
13′						23.73*
14′						14.46*
1′′	171.62	174.27	170.6	171.65	172.56	177.07
2′′	38.81	39.88	40.4	38.79	40.43	42.31
3′′	69.97	72.60	71.0	70.73	71.39	73.59
4′′	33.36	34.96	33.7	33.29	33.61	35.40
5′′	24.97	26.11	24.7	24.54	24.11	26.33
6′′	28.70*	30.30	28.6	28.37	28.99*	30.56
7′′	28.70*	30.18*	28.8	28.55	29.07*	30.36
8′′	31.19*	32.82*	31.2	31.19*	31.30*	32.95*
9′′	22.10*	23.58*	22.1	22.08*	22.10*	23.73*
10′′	13.94*	14.32*	14.0	13.93*	13.95*	14.45*

* Carbon resonances are not chain specifically assigned due to near identical shifts

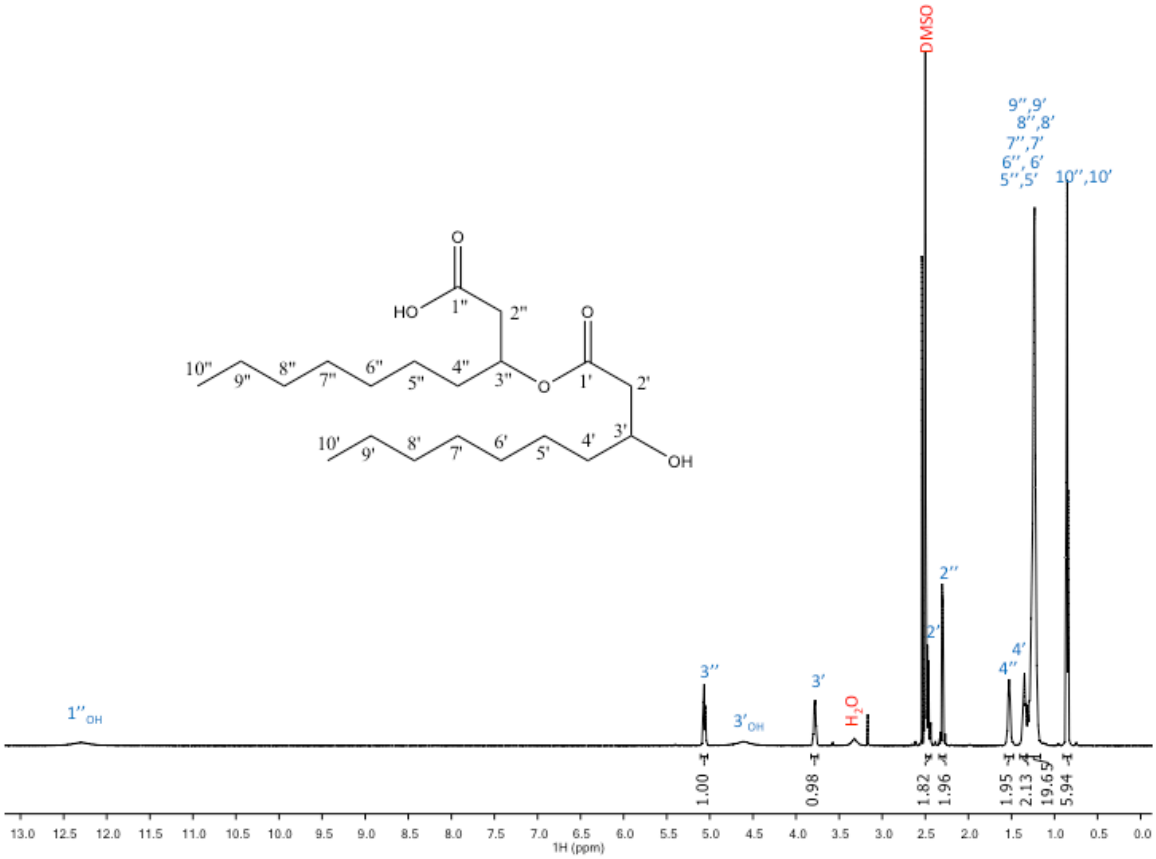


Figure S4. 1D proton of **1** in DMSO-d6, T=298 K.

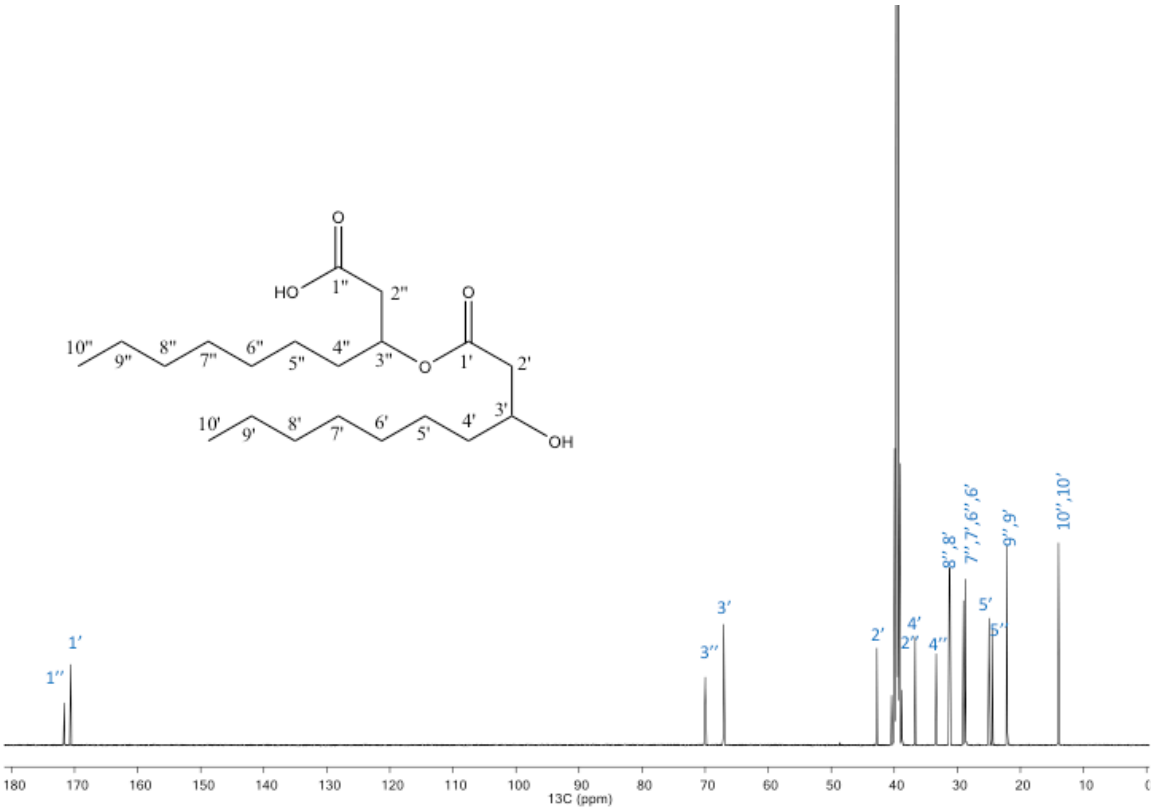


Figure S5. 1D carbon of **1** in DMSO-d6, T=298 K.

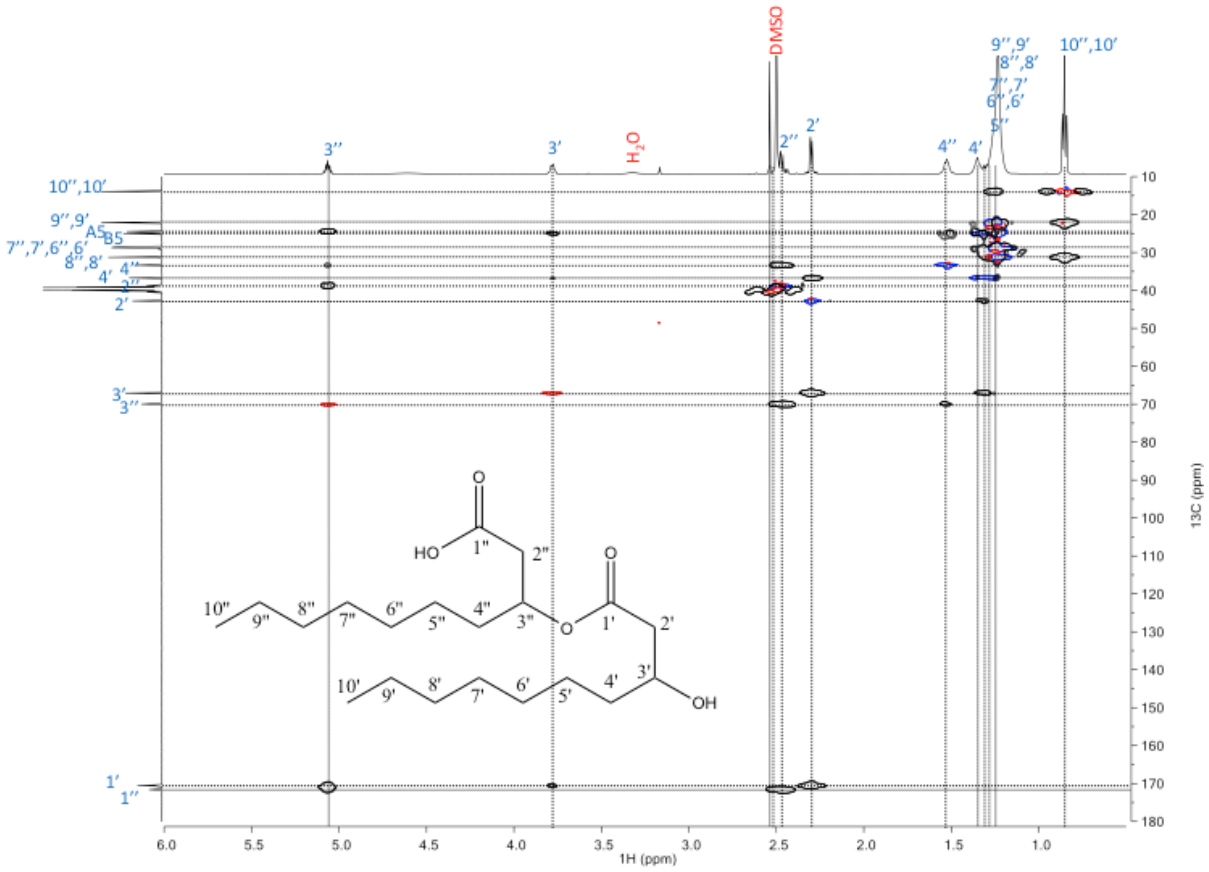


Figure S6. 2D superimposed ^{13}C-HSQC and HMBC of **1** in DMSO-d$_6$, T=298 K.

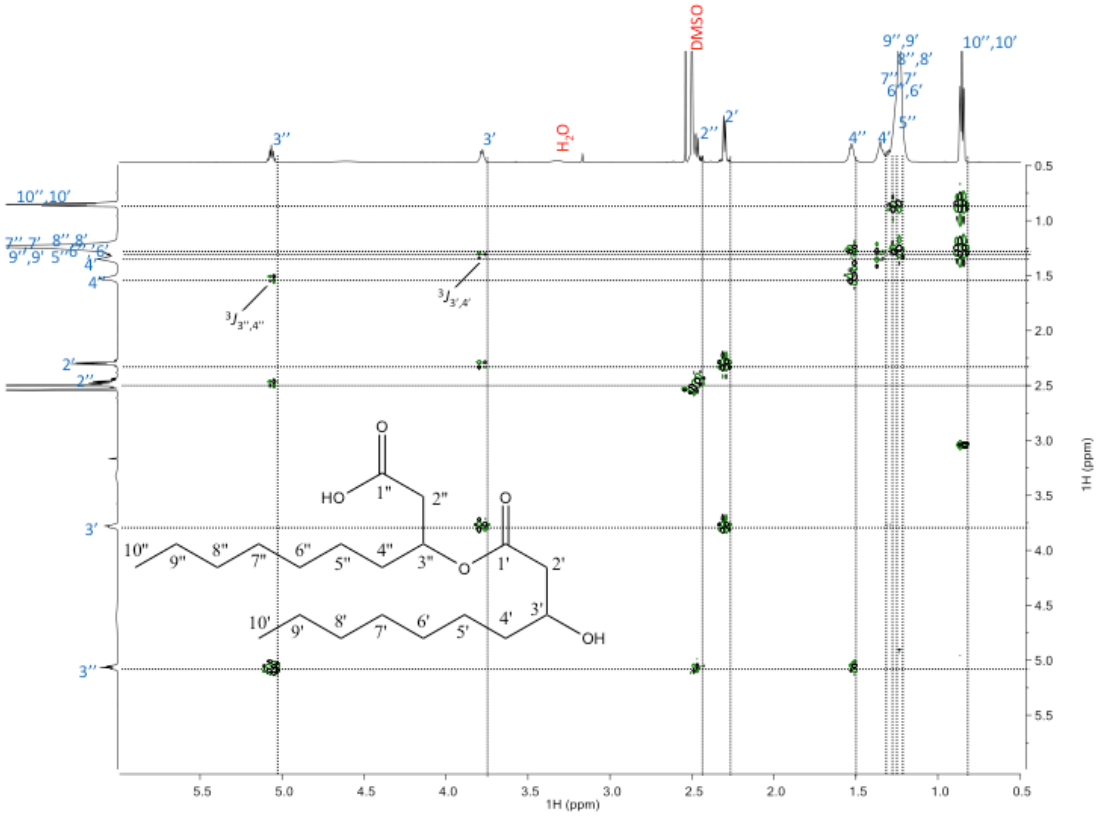


Figure S7. 2D DQF-COSY of **1** in DMSO-d$_6$, T=298 K.

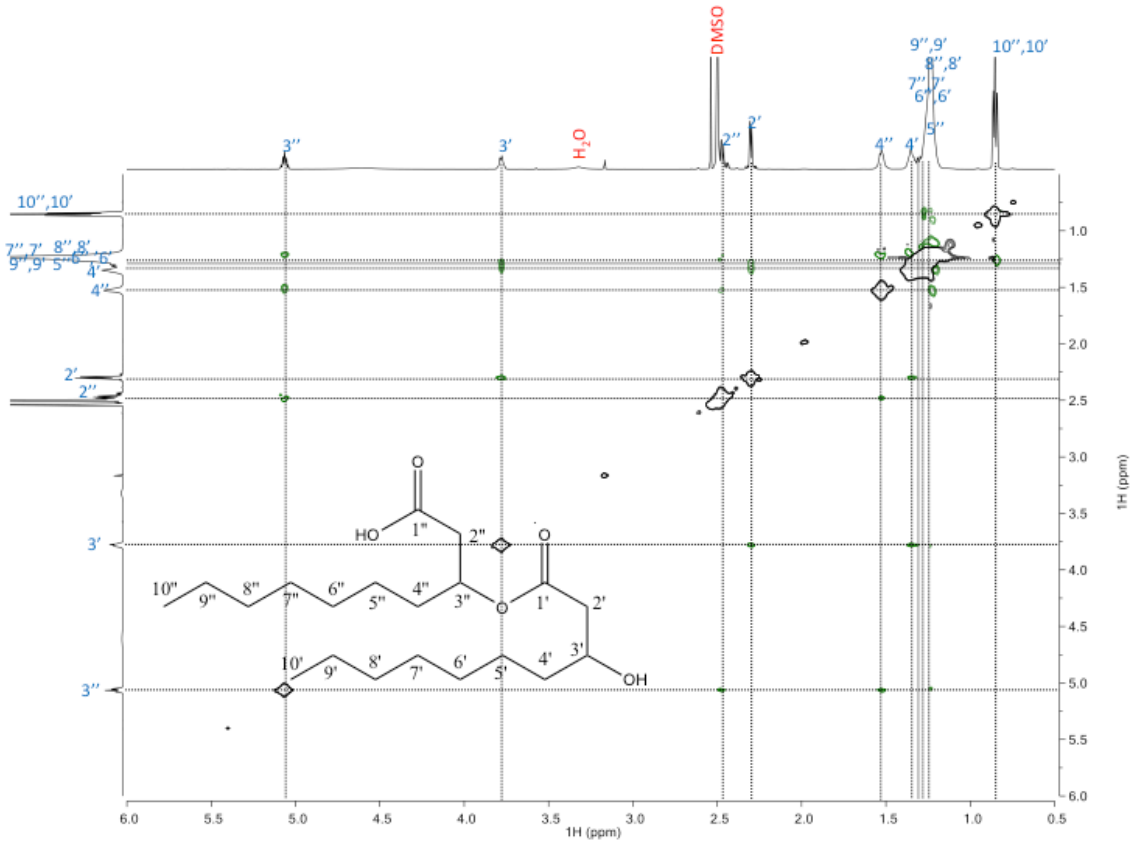


Figure S8. 2D ROESY (300 ms) of **1** in DMSO-d_6, T=298 K.

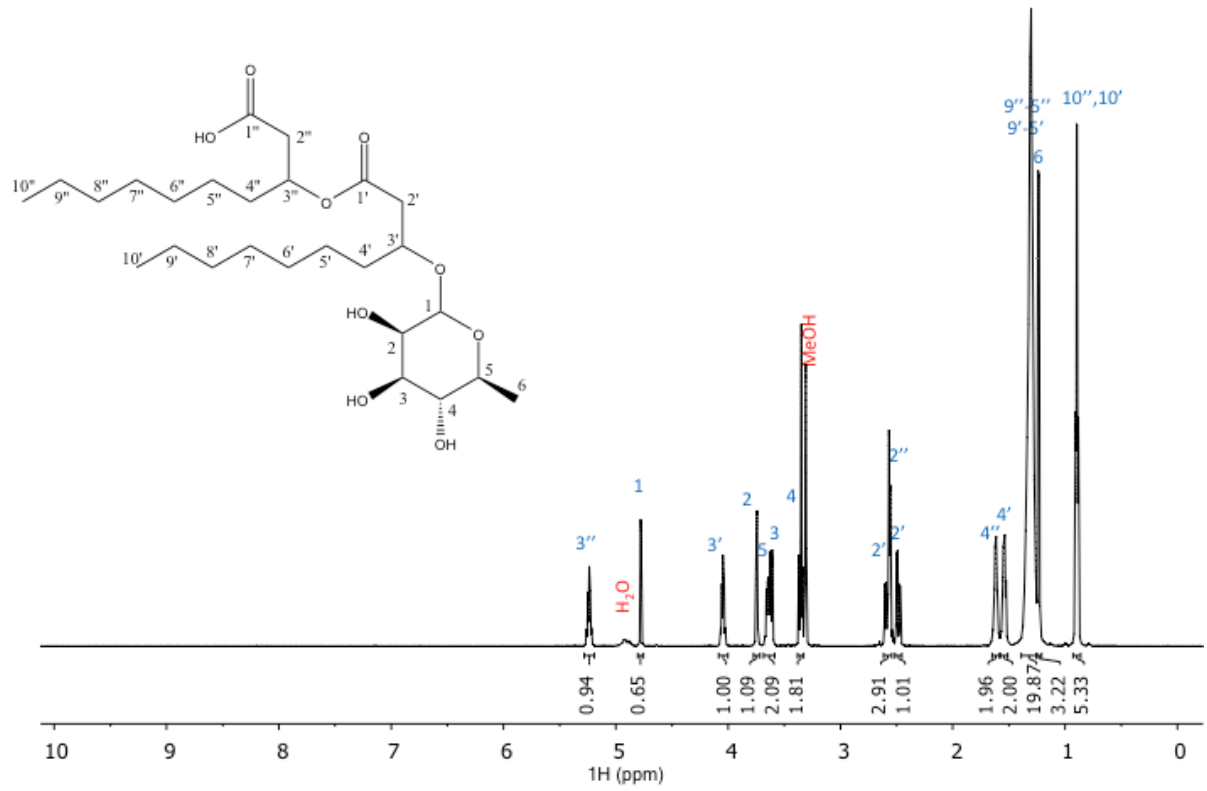


Figure S9. 1D proton of **2** in methanol-d_4, T=298 K.

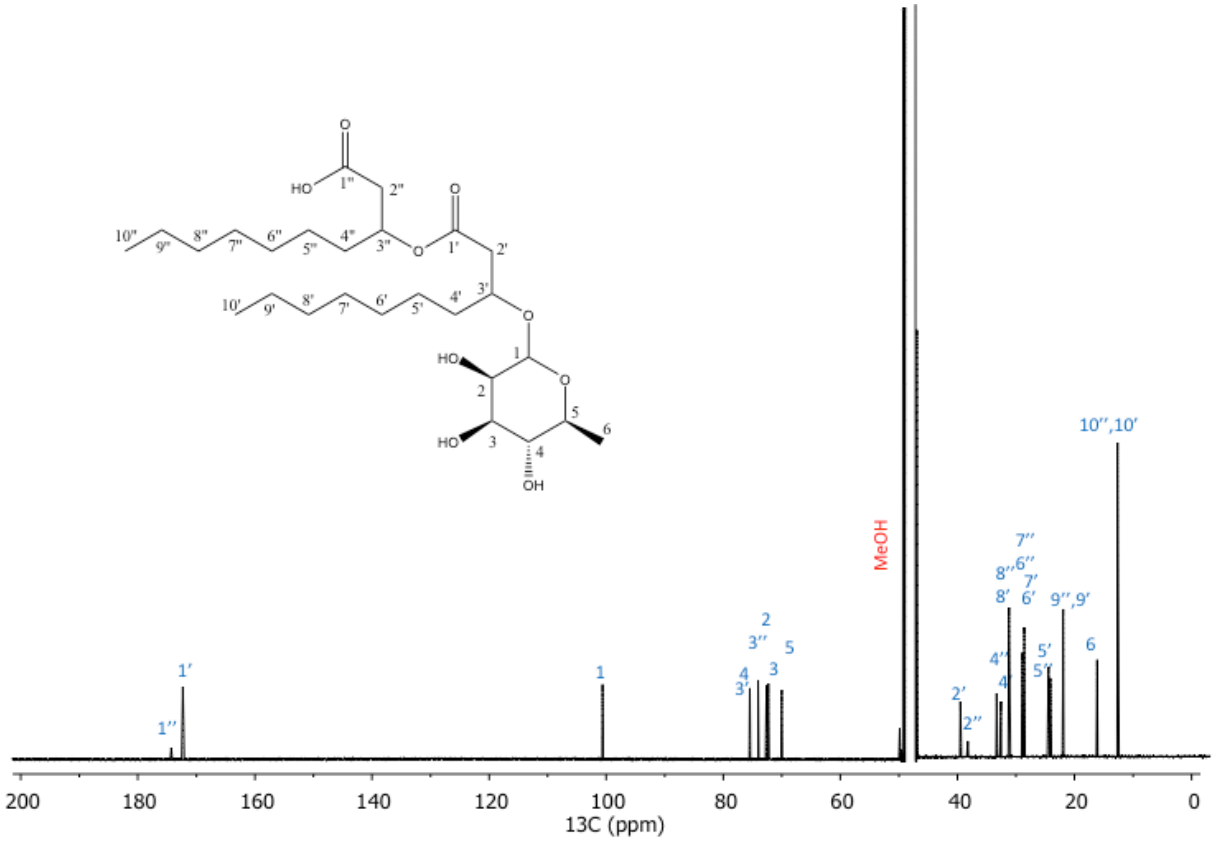


Figure S10. 1D carbon of **2** in methanol-d4, T=298 K.

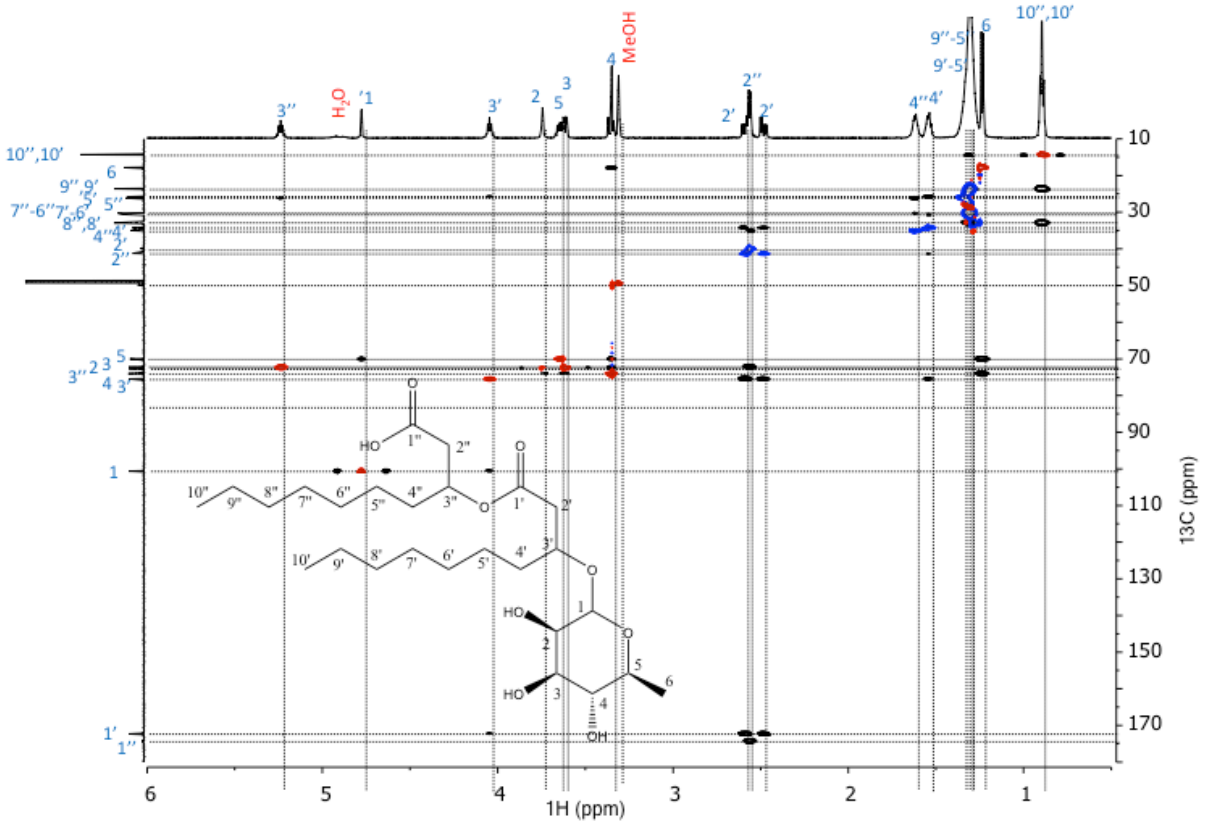


Figure S11. 2D superimposed ^{13}C-HSQC and HMBC of **2** in methanol-d4, T=298 K.

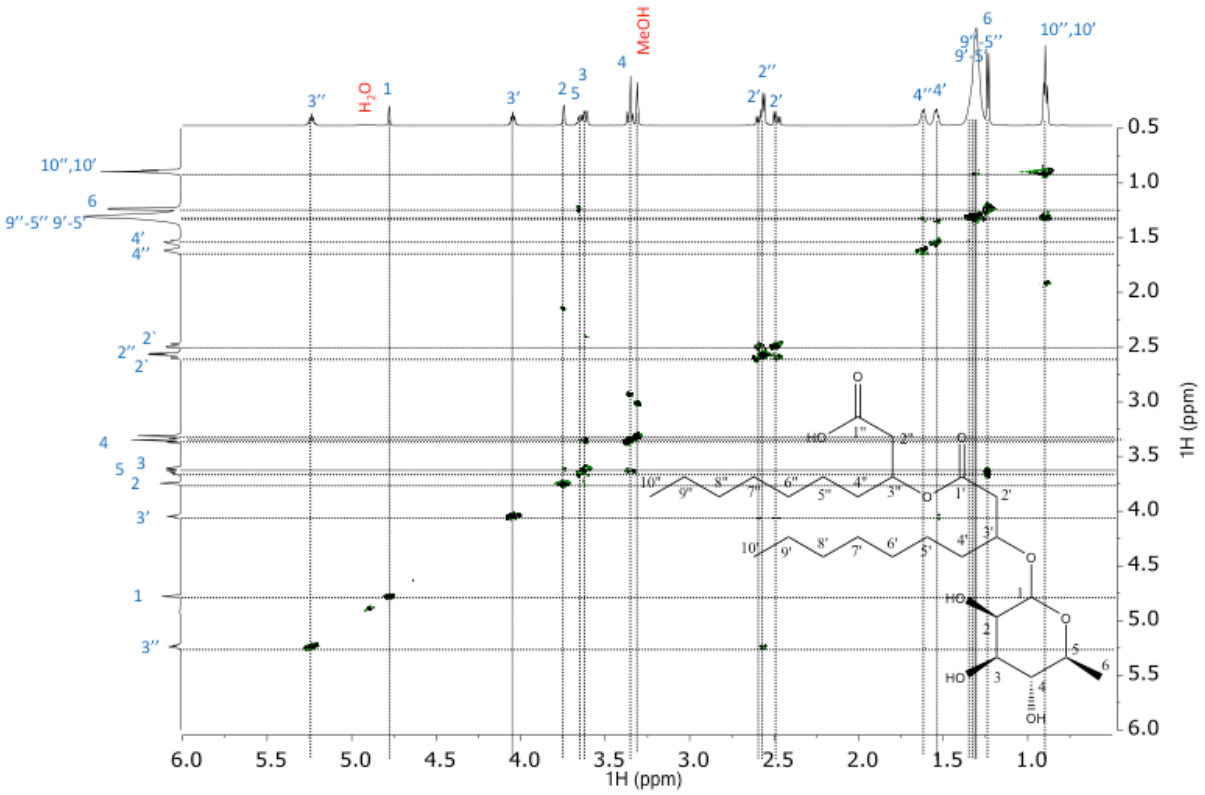


Figure S12. 2D DQF-COSY of **2** in methanol-d_4, T=298 K.

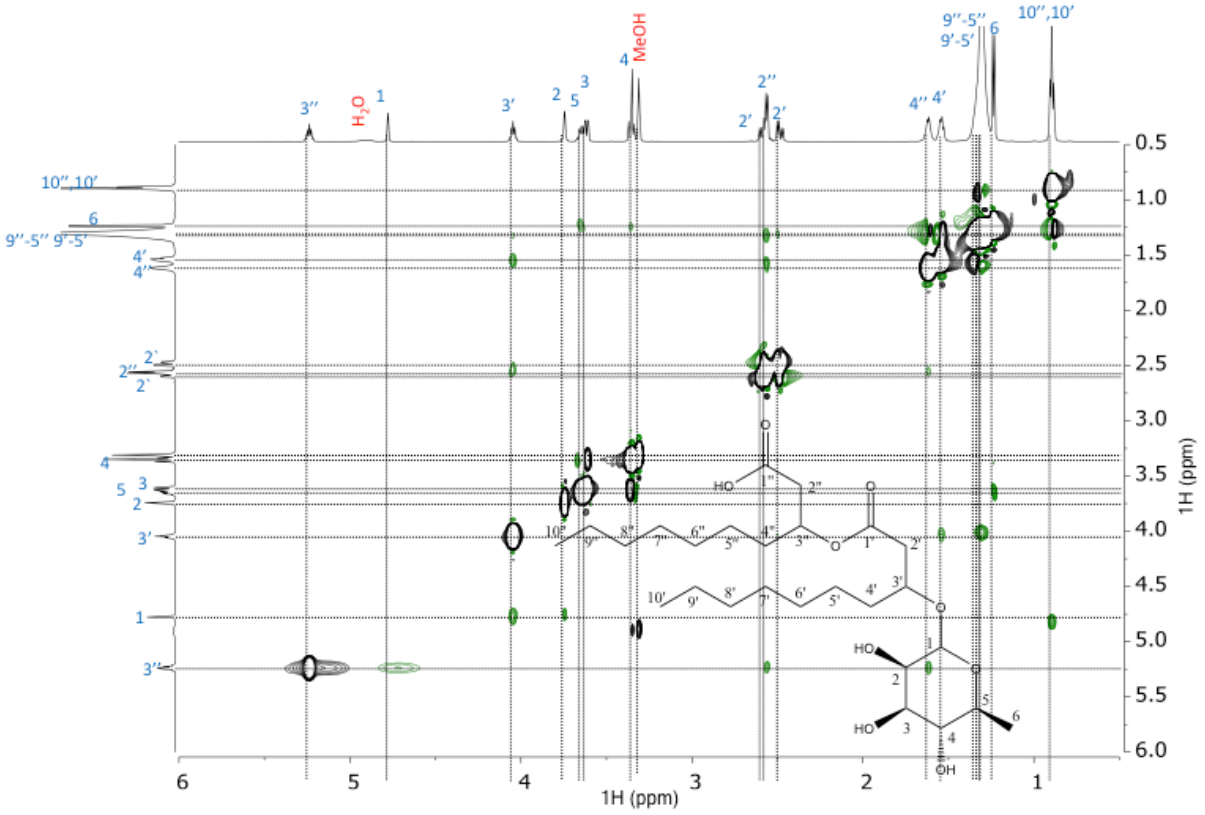


Figure S13. 2D ROESY (300 ms) of **2** in methanol-d_4, T=298 K.

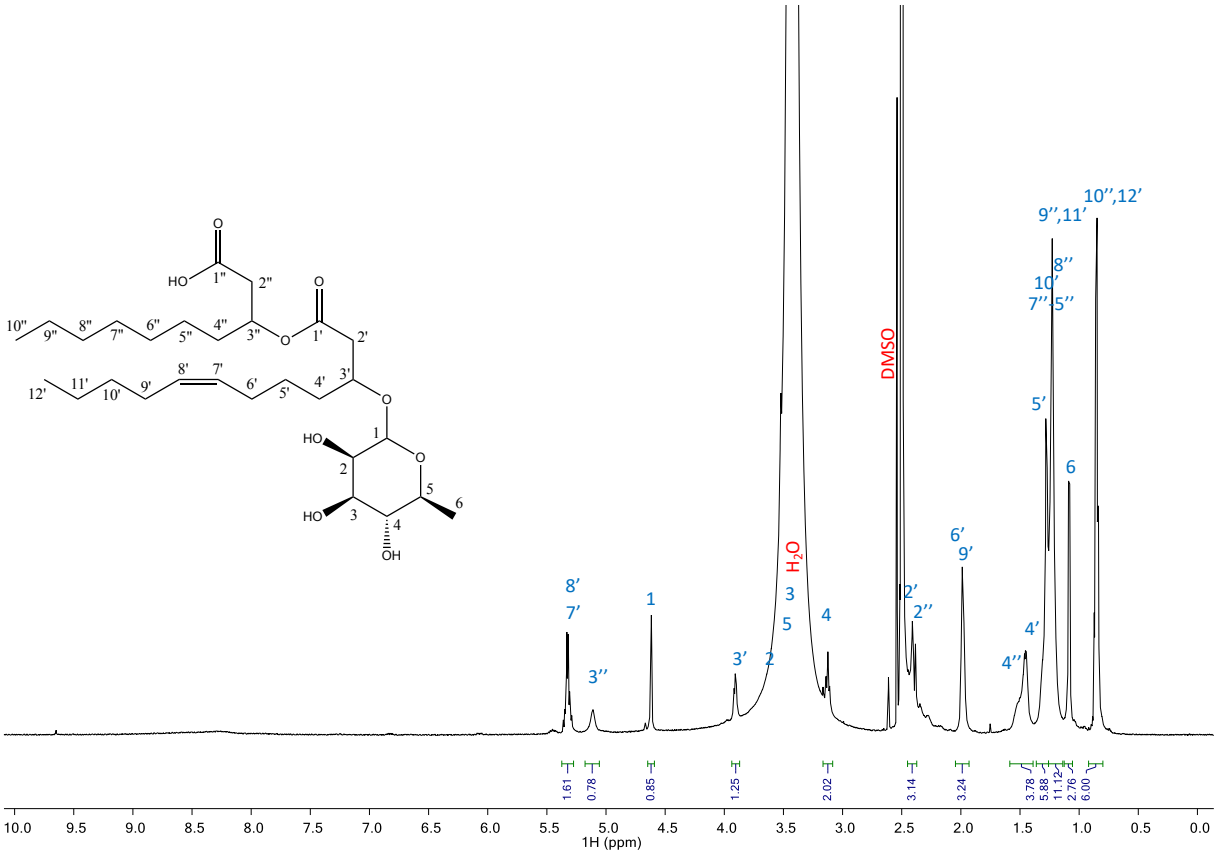


Figure S14. 1D proton of **3** in DMSO-d_6, T=298 K.

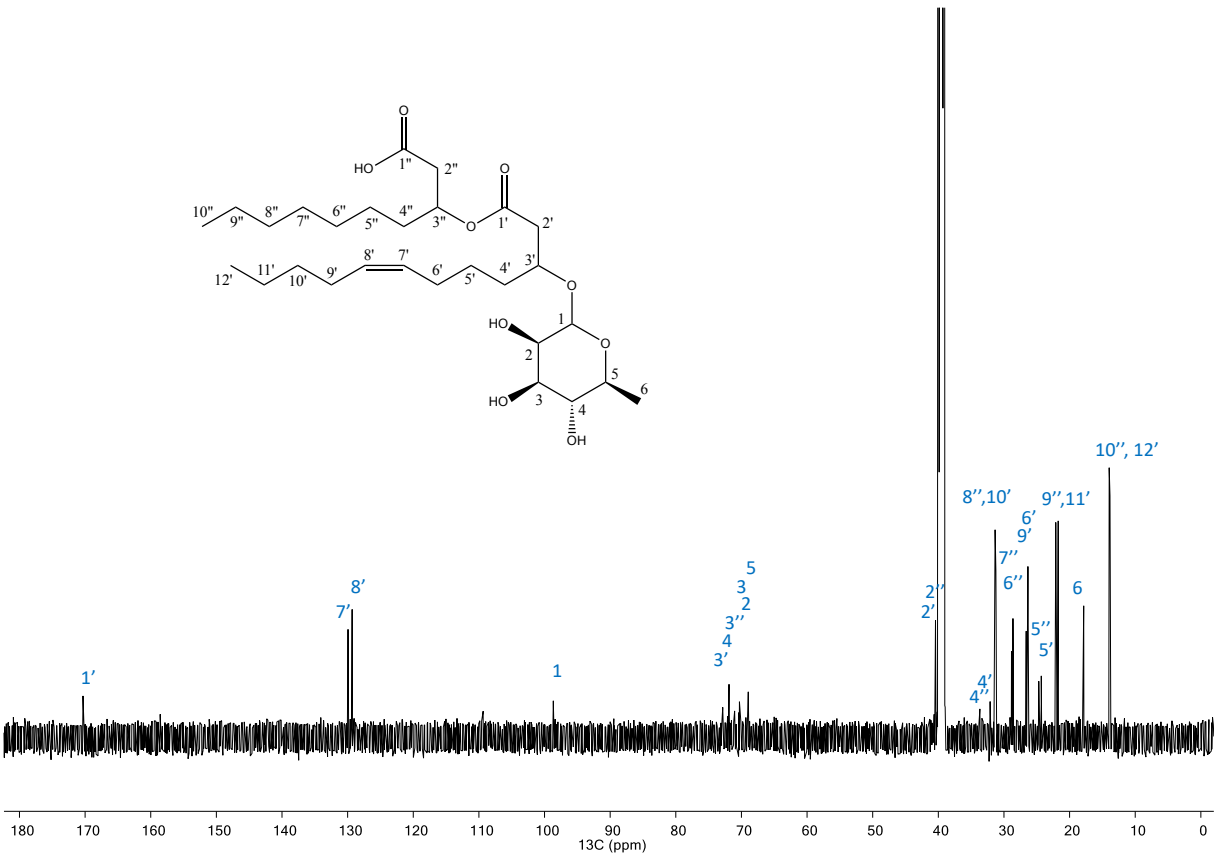


Figure S15. 1D carbon of **3** in DMSO-d_6, T=298 K.

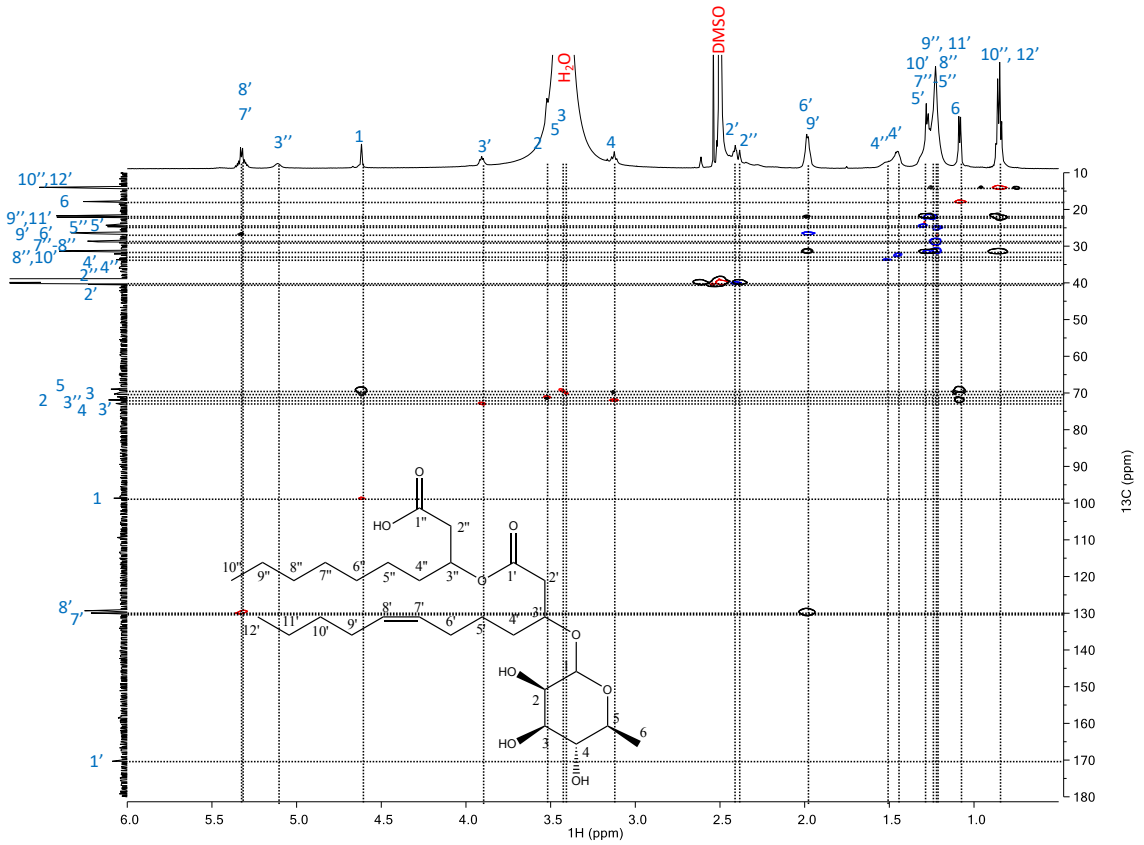


Figure S16. 2D superimposed ^{13}C-HSQC and HMBC of **3** in DMSO-d$_6$, T=298 K.

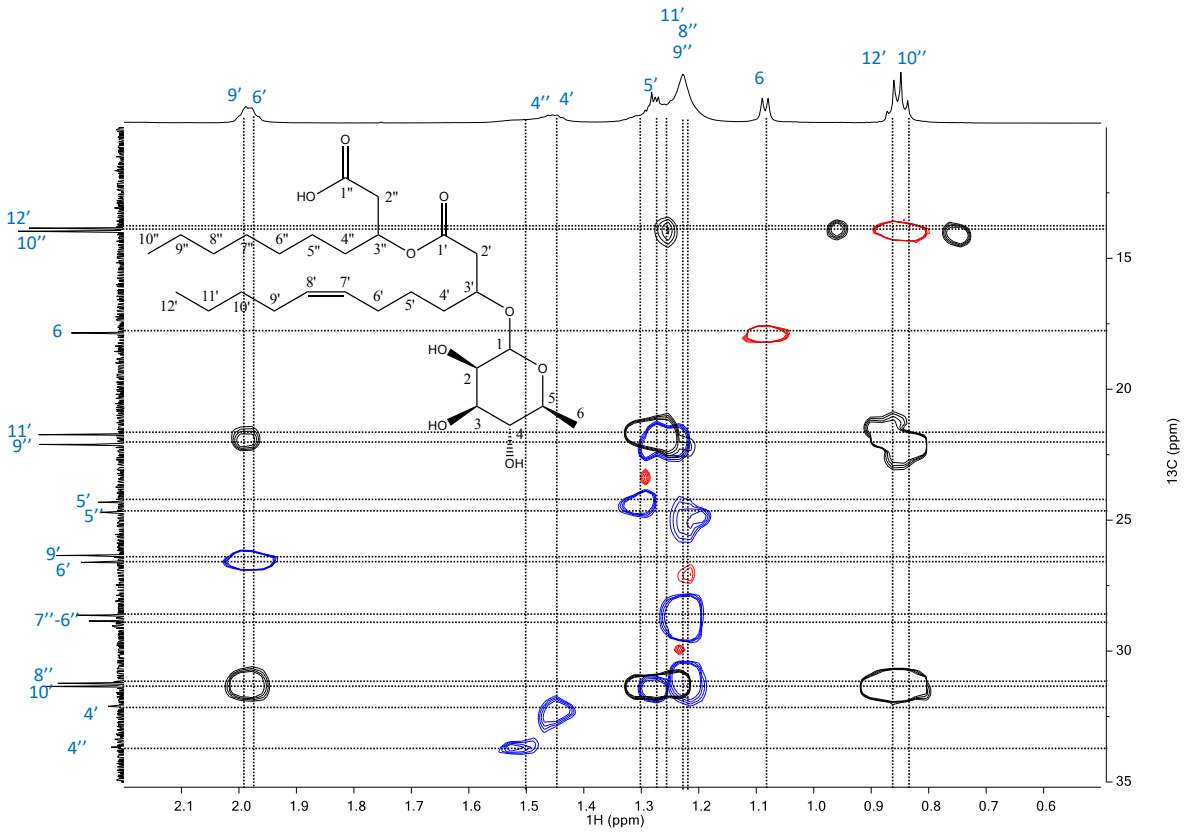


Figure S17. Blown up aliphatic region of superimposed 2D ^{13}C-HSQC and HMBC of **3** in DMSO-d$_6$, T=298 K.

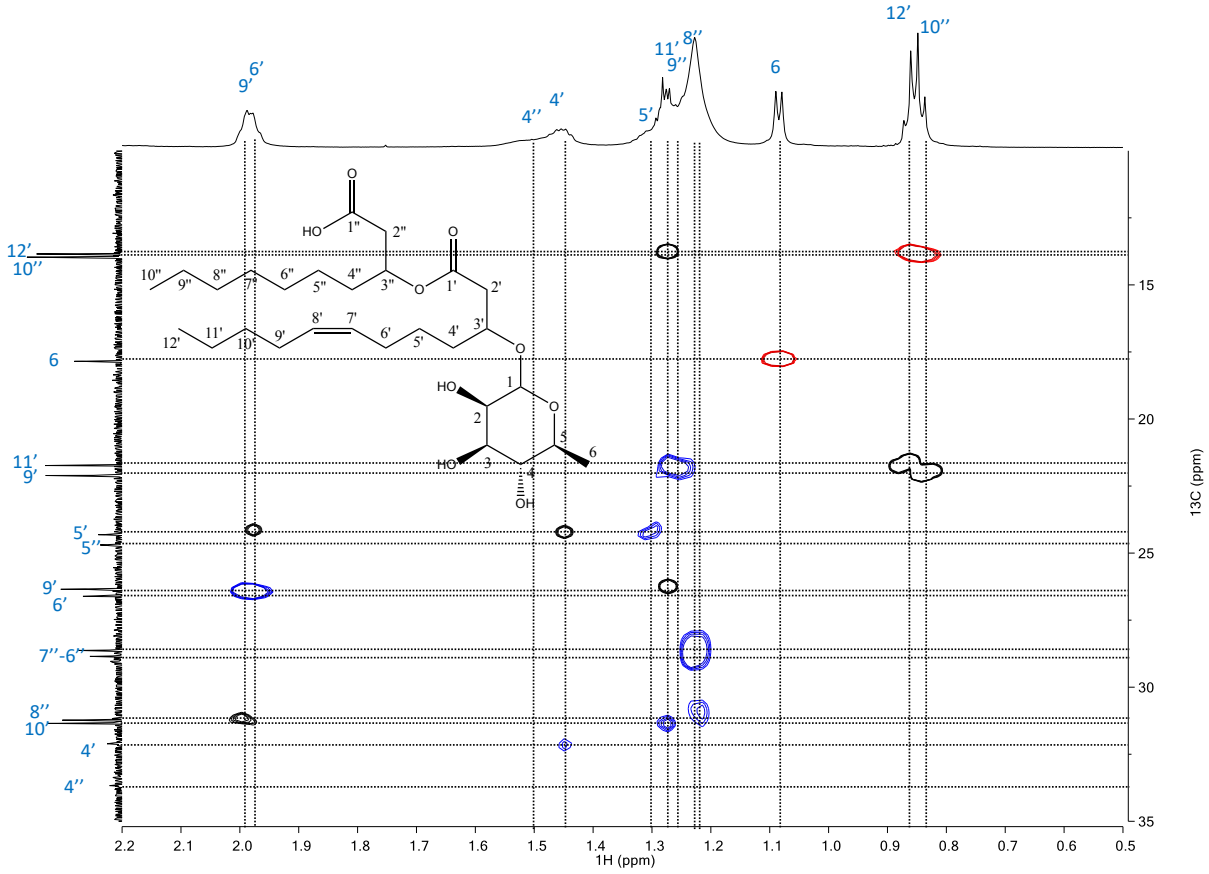


Figure S18. Blown up aliphatic region of superimposed 2D ^{13}C-HSQC and H2BC of **3** in DMSO-d$_6$, T=298 K.

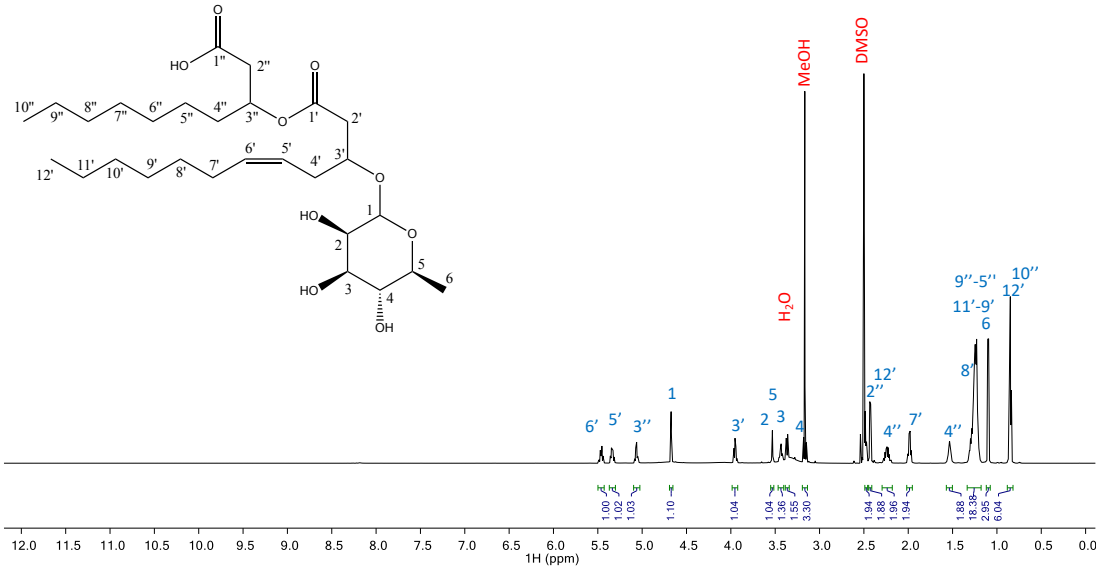


Figure S19. 1D proton of **4** in DMSO-d$_6$, T=298 K.

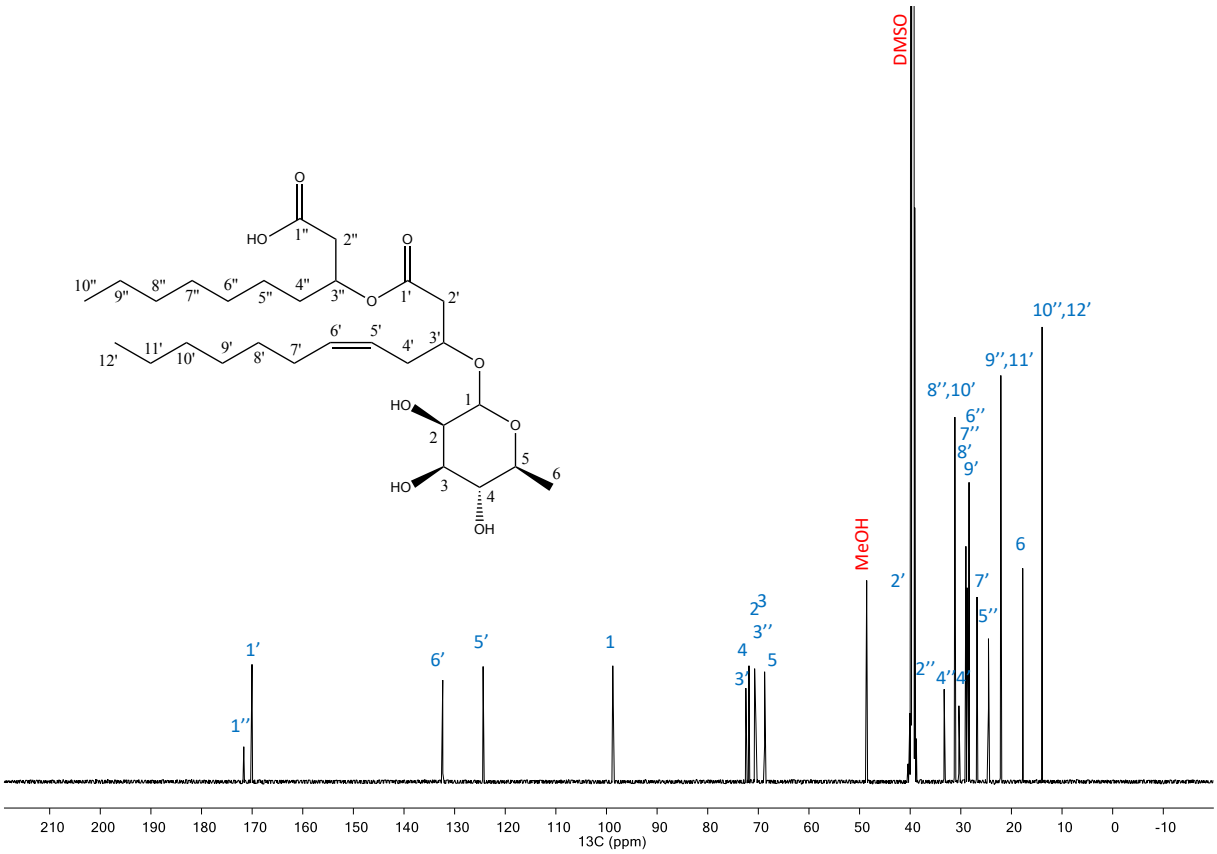


Figure S20. 1D carbon of **4** in DMSO-d_6, T=298 K.

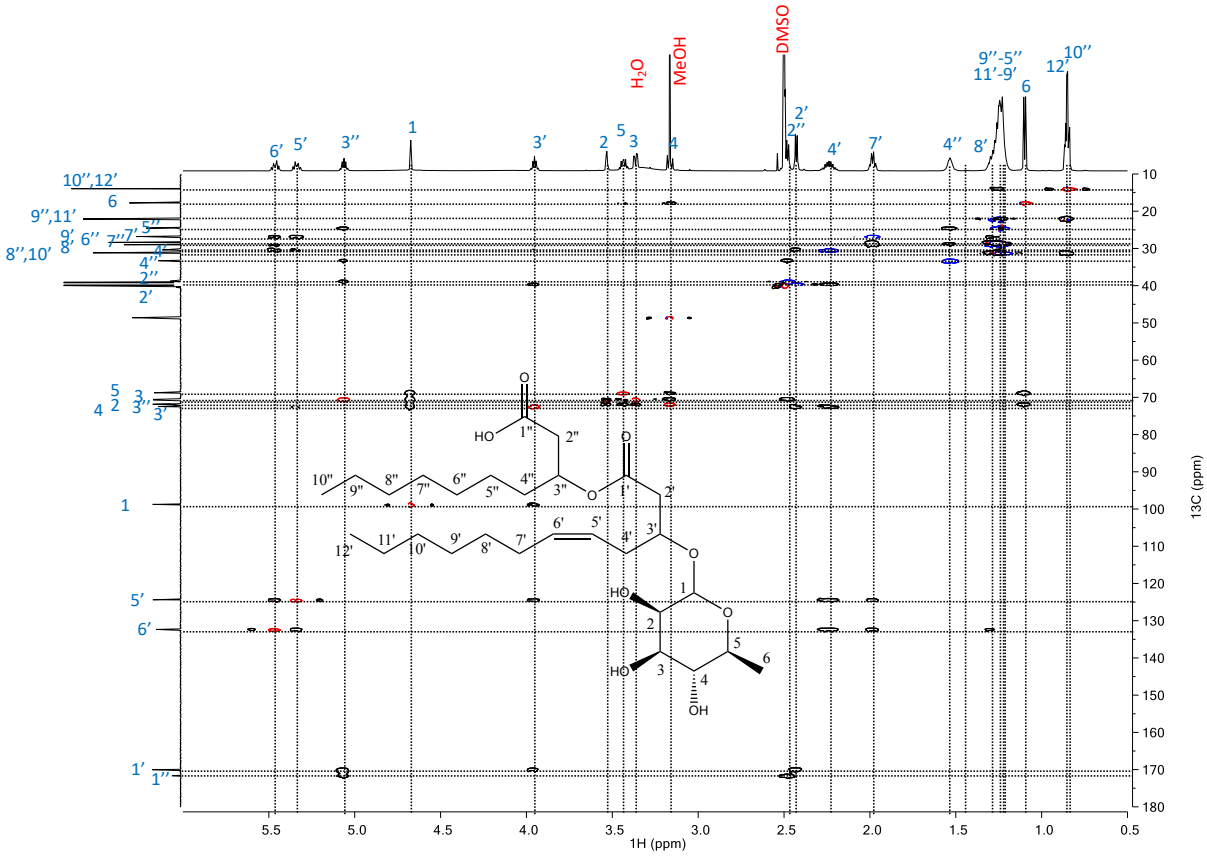


Figure S21. 2D superimposed ^{13}C-HSQC and HMBC of **4** in DMSO-d_6, T=298 K.

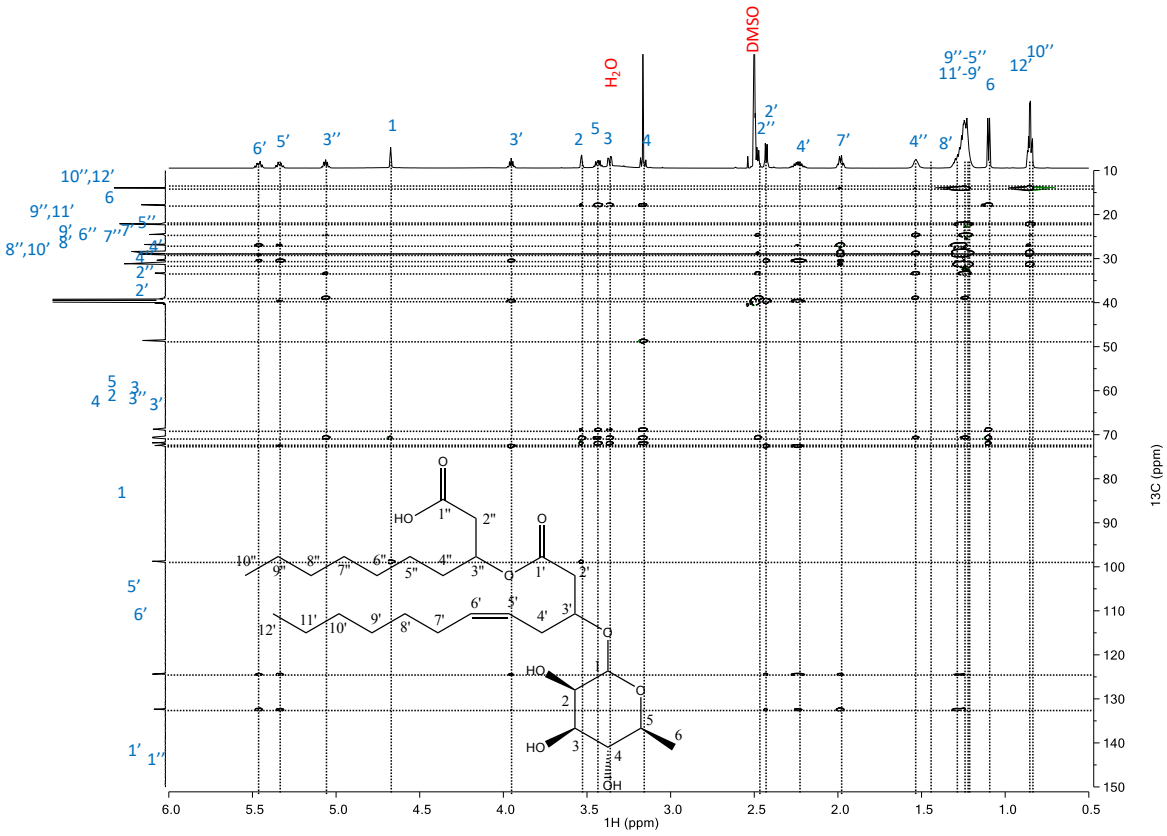


Figure S22. 2D ^{13}C-HSQCTOCSY of **4** in DMSO-d$_6$, T=298 K.

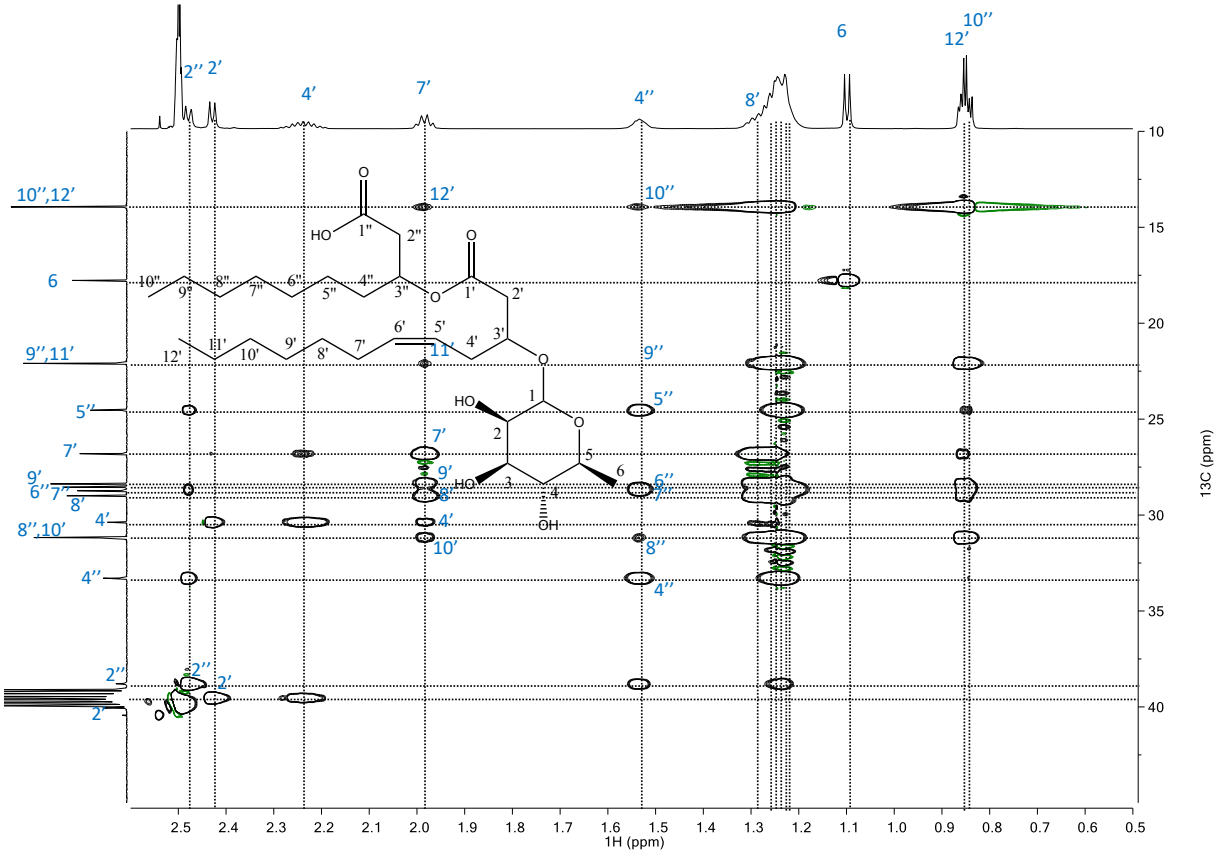


Figure S23. Blown up aliphatic region of the 2D ^{13}C-HSQCTOCSY of **4** in DMSO-d$_6$, T=298 K.

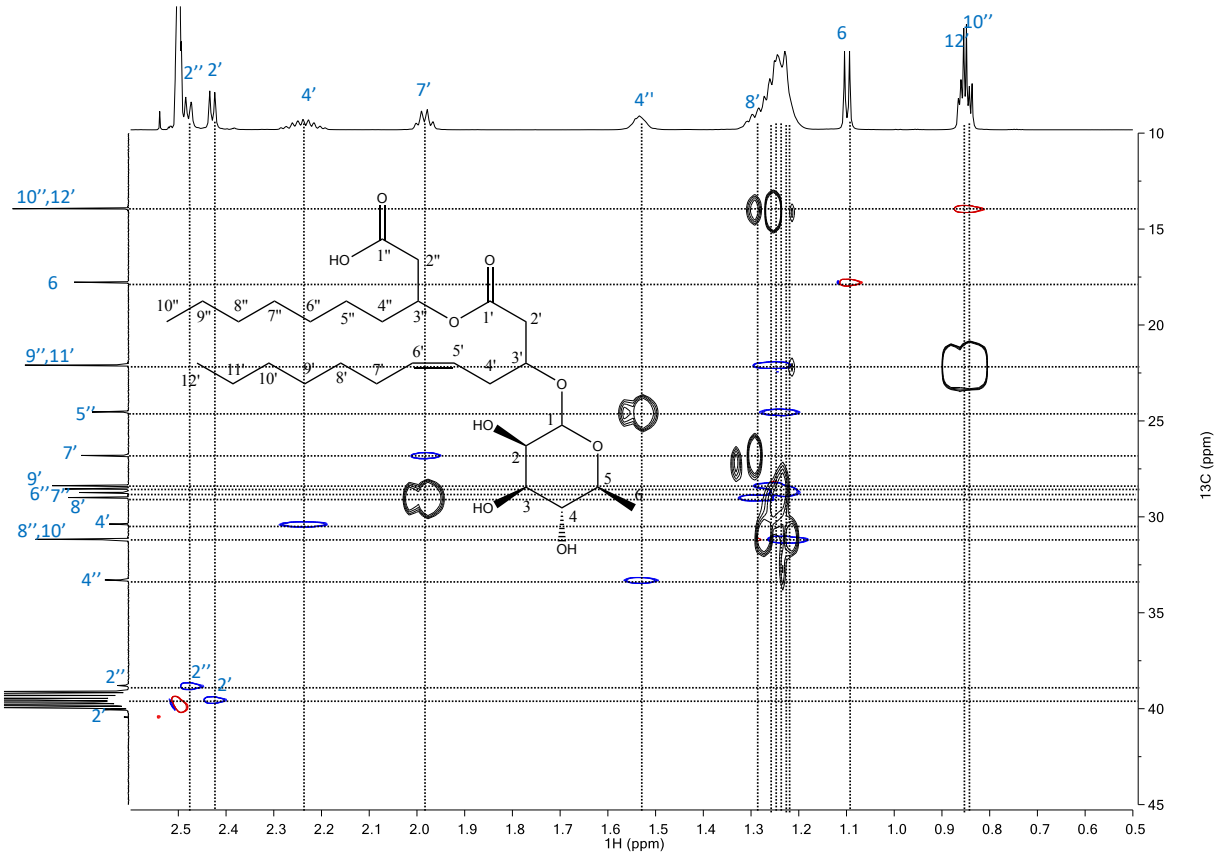


Figure S24. Blown up aliphatic region of superimposed 2D ^{13}C-HSQC and H2BC of **4** in DMSO-d$_6$, T=298 K.

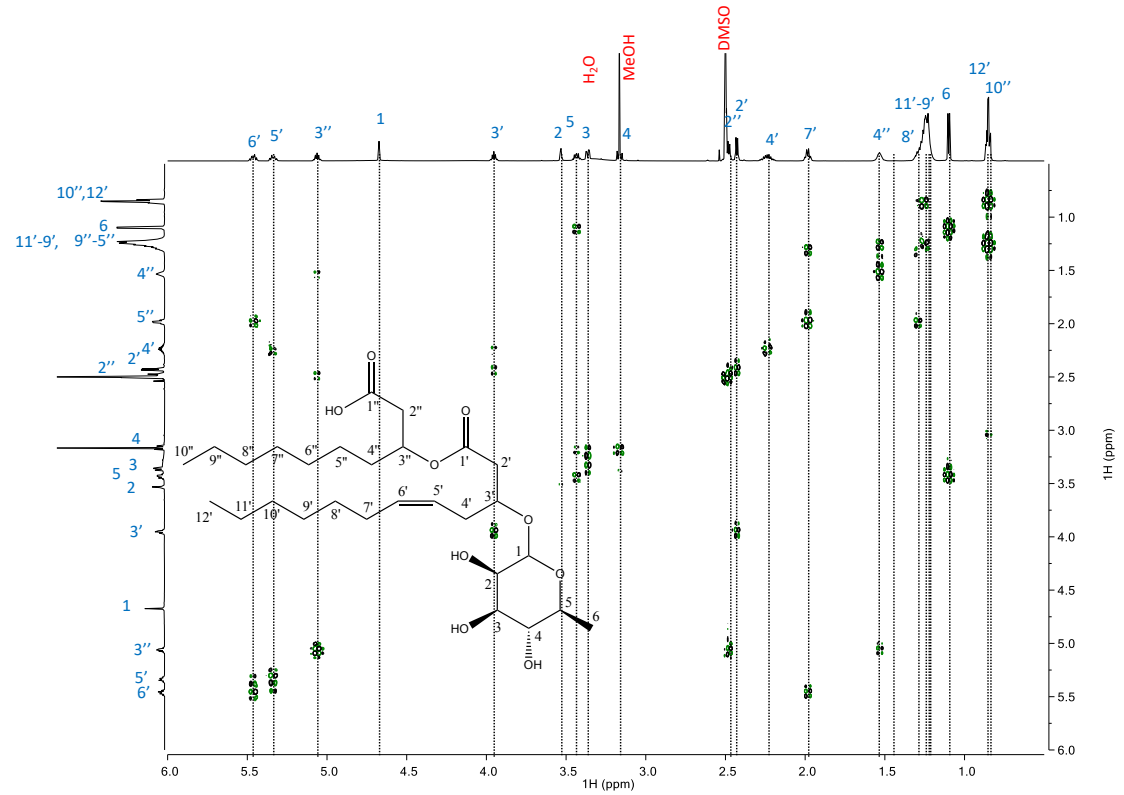


Figure S25. 2D DQF-COSY of **4** in DMSO-d$_6$, T=298 K.

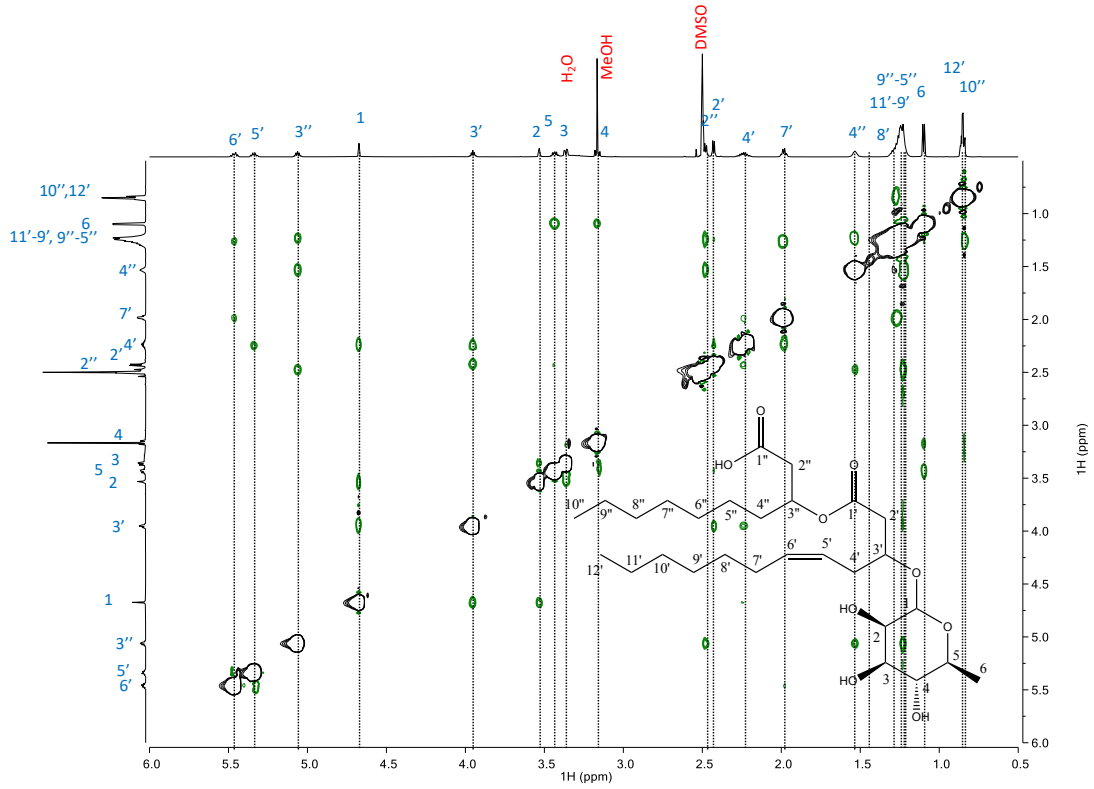


Figure S26. 2D ROESY (300 ms) of **4** in DMSO-d$_6$, T=298 K.

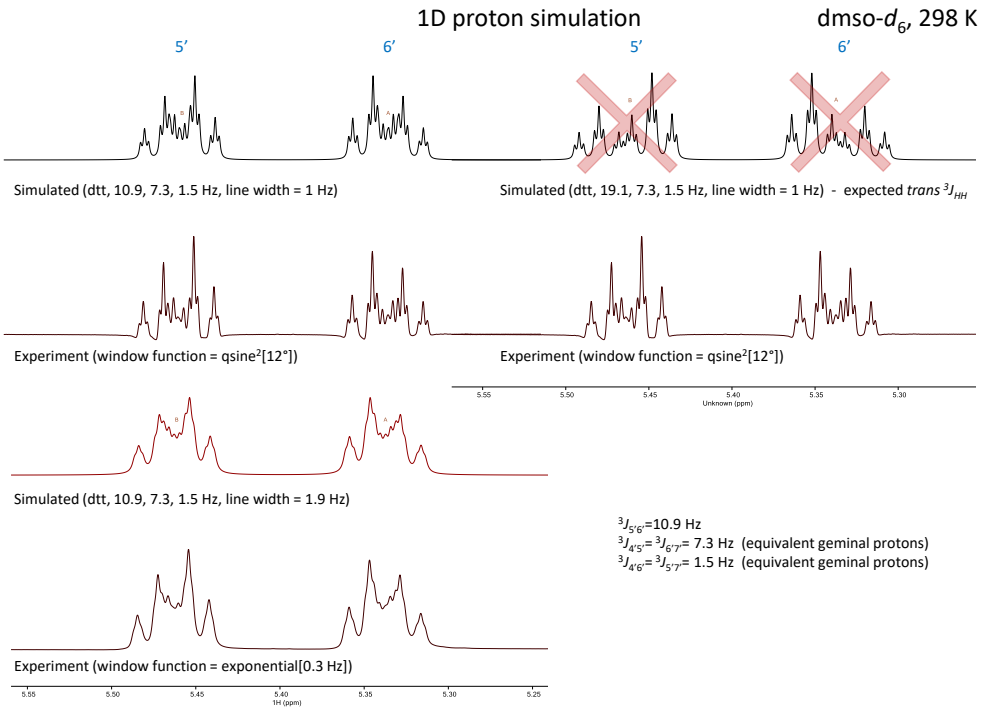
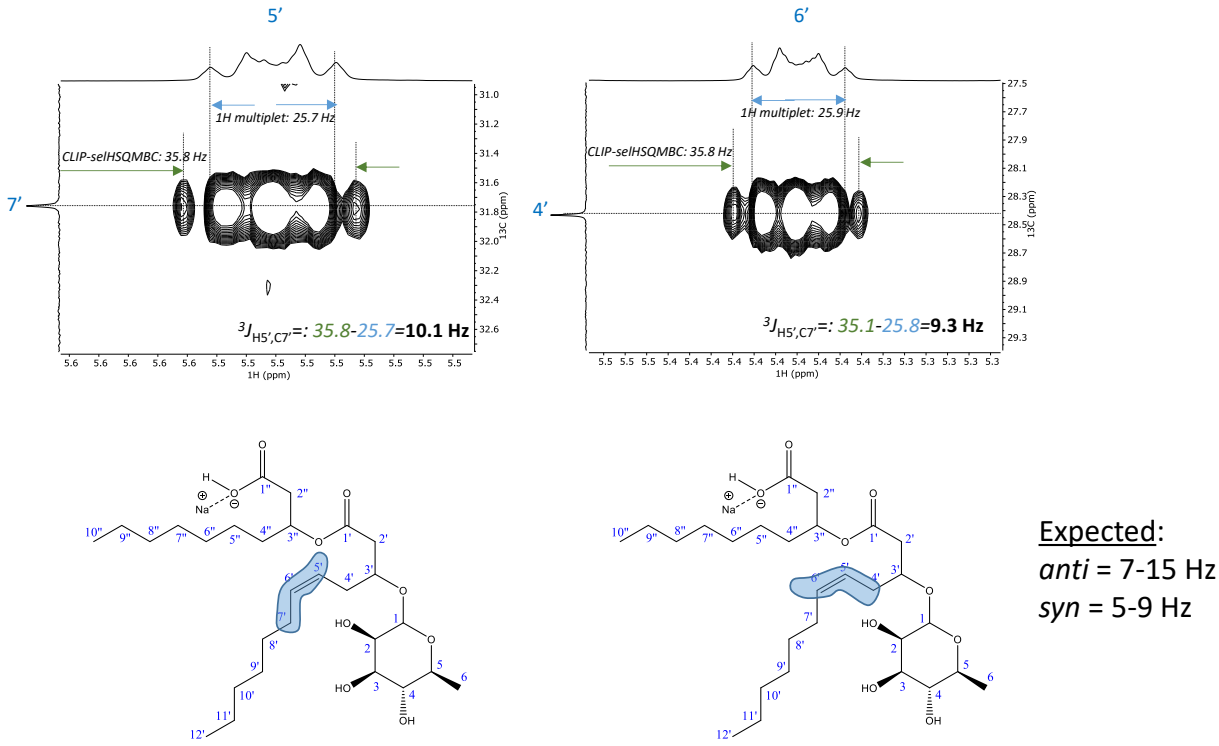
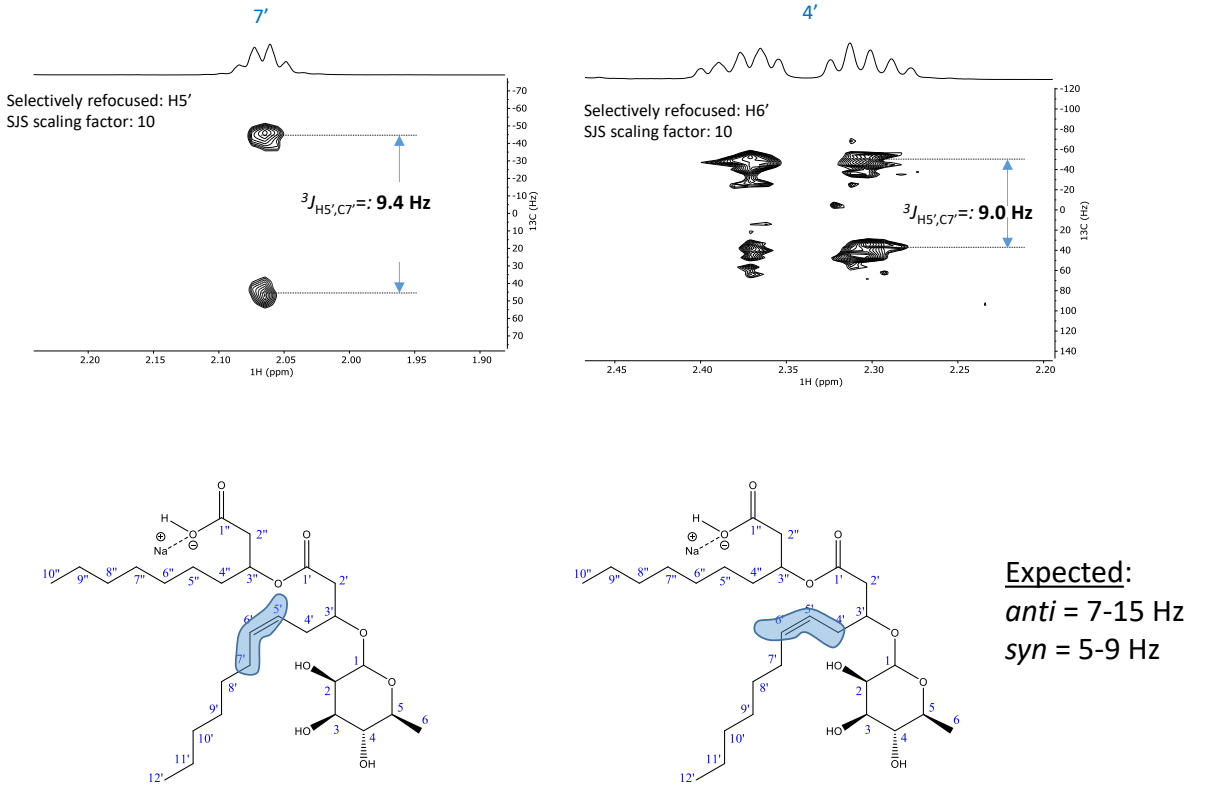


Figure S27. Comparison between experimental and simulated multiplets of the olefinic protons of **4**. The fit was made using a qsine^2(12°) window function (upper panel), and then compared to the raw multiplets (lower panel). The best fit was found to be a dtt, 10.9, 7.3, 1.5 Hz multiplet, which indicates a *cis* configuration (expected $^3J_{5'6'}$ ~ 11 Hz). The right panel shows the simulated peak for the expected *trans* coupling ~19 Hz with shows a poor fit.



Expected:
anti = 7-15 Hz
syn = 5-9 Hz

Figure S28. The $^3J_{CH}$ couplings of the olefinic protons are estimated using a selective CLIP-HSQMBC experiment. Values of 9.3 and 10.1 Hz is found for $^3J_{H6'C4'}$ and $^3J_{H5'C7'}$ respectively, suggesting anti configurations of the C and H.



Expected:
anti = 7-15 Hz
syn = 5-9 Hz

Figure S29. *The* $^3J_{CH}$ couplings of the olefinic protons are estimated using a SJS-HSQC experiment (j-resolved in f1). Values of 9.0 and 9.4 Hz is found for $^3J_{H6'C4'}$ and $^3J_{H5'C7'}$ respectively, suggesting anti configurations of the C and H.

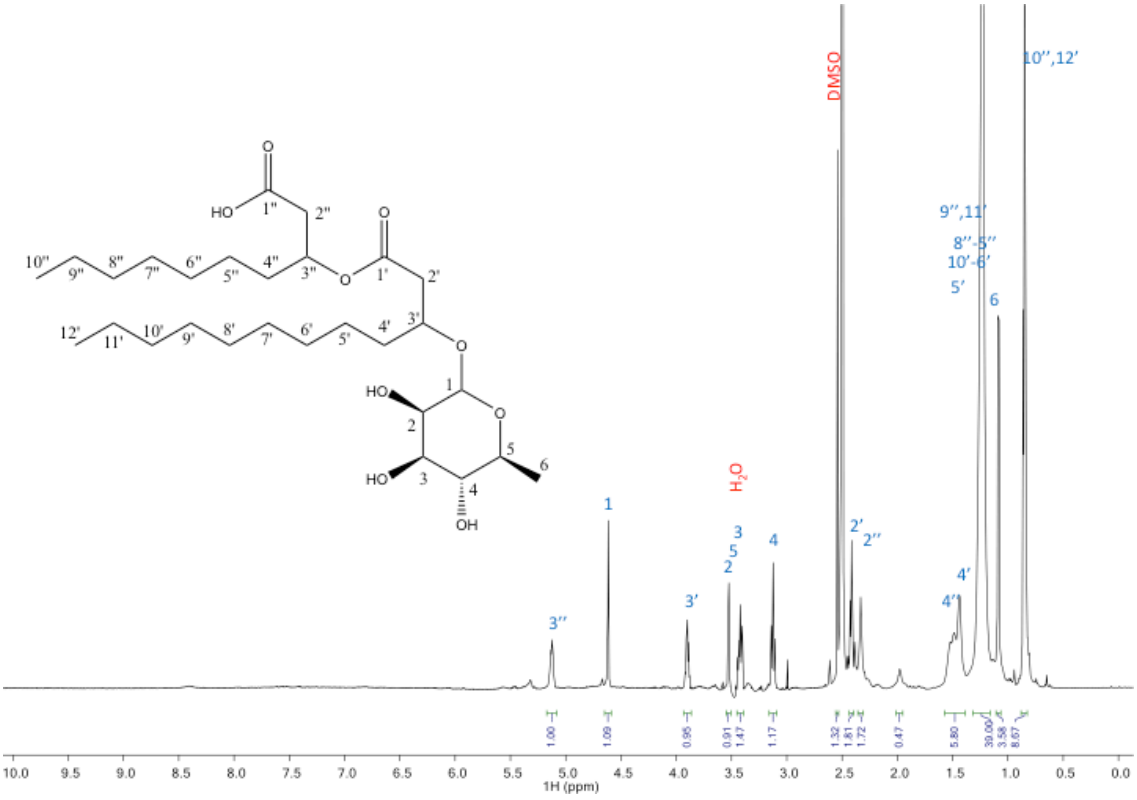


Figure S30. 1D proton of **5** in DMSO-d_6, T=298 K.

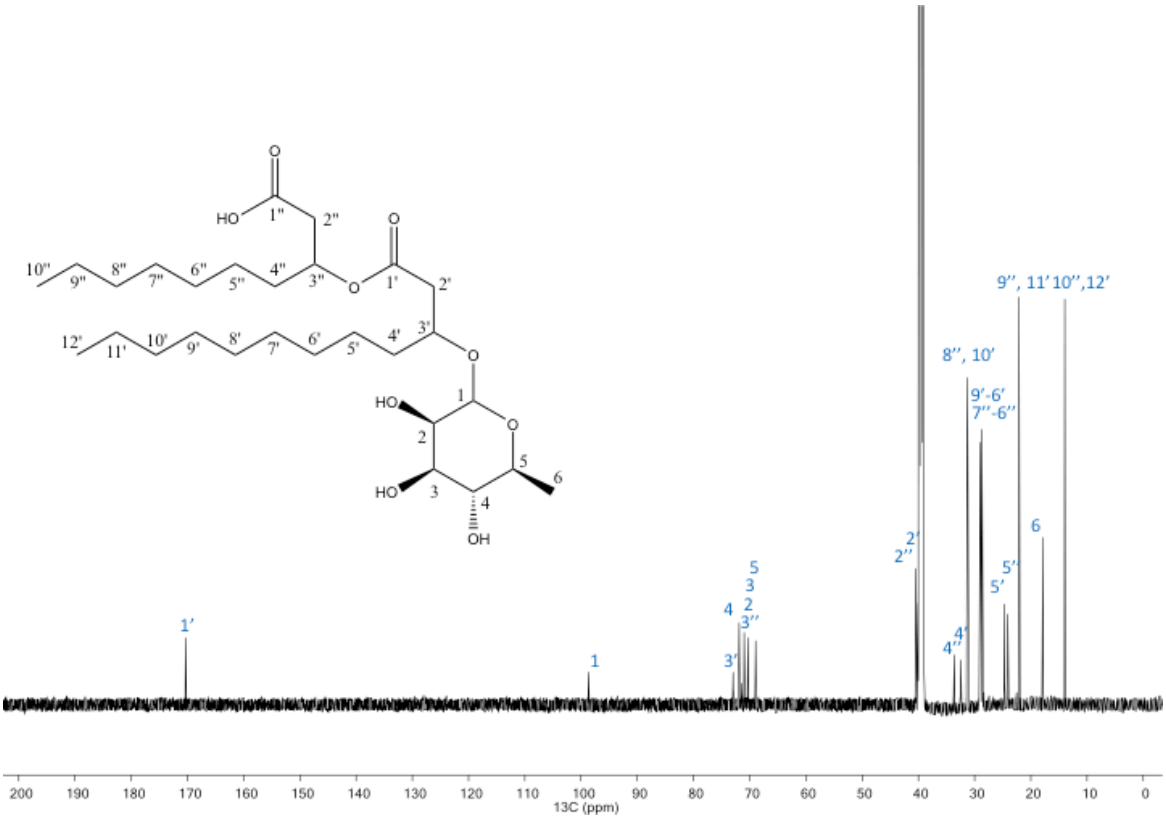


Figure S31. 1D carbon of **5** in DMSO-d_6, T=298 K.

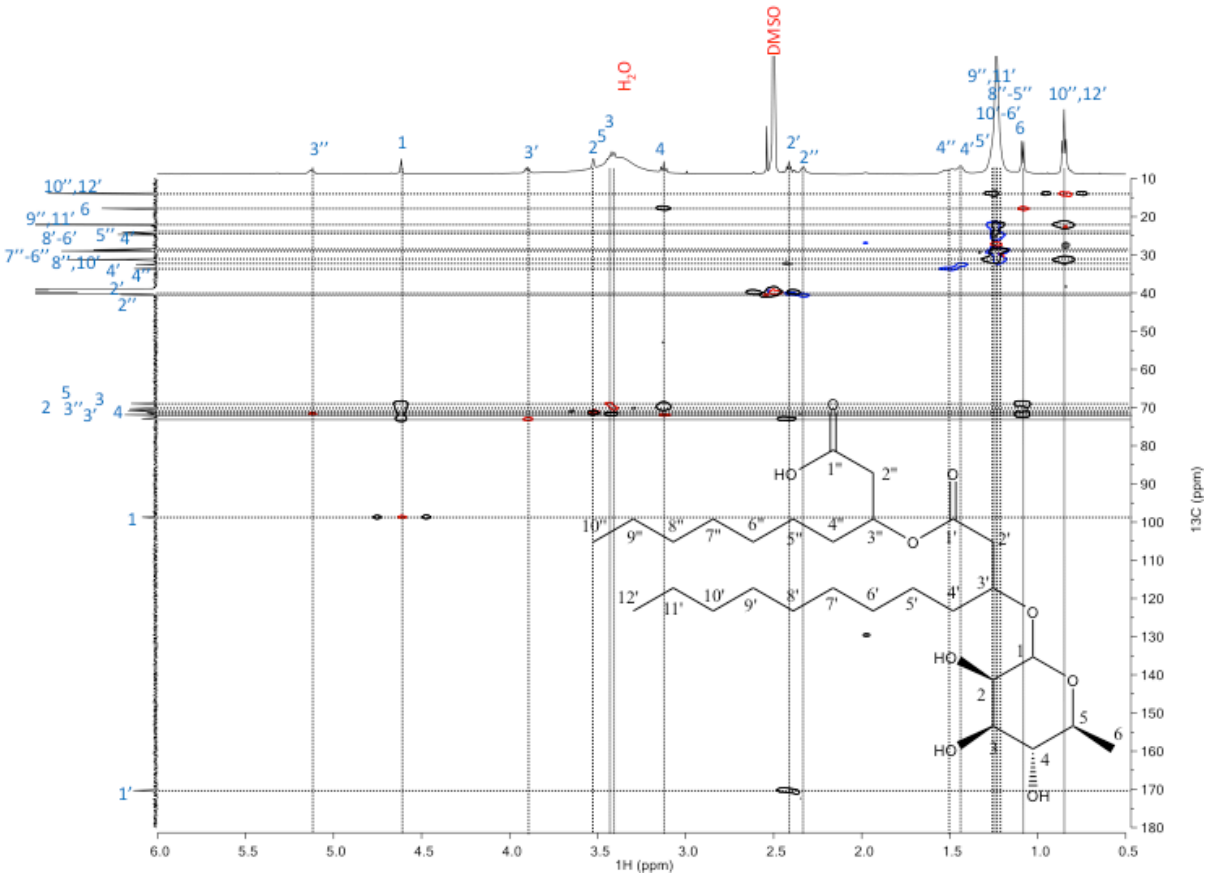


Figure S32. 2D superimposed ^{13}C-HSQC and HMBC of **5** in DMSO-d$_6$, T=298 K.

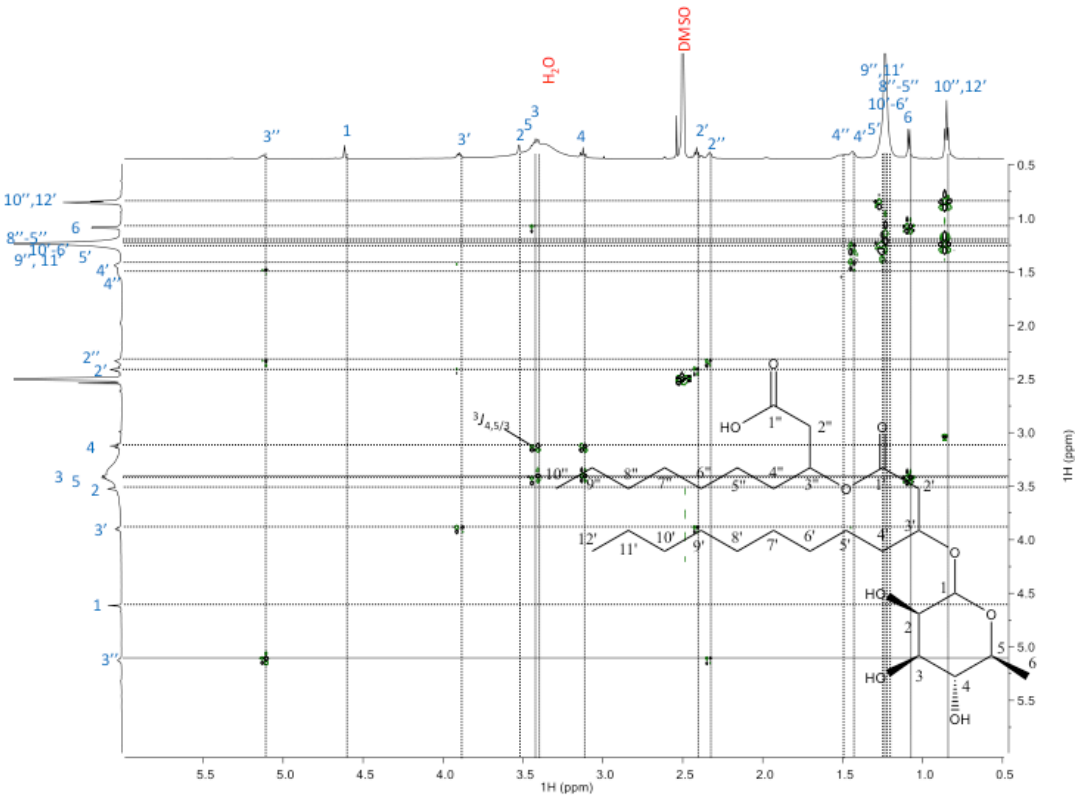


Figure S33. 2D DQF-COSY of **5** in DMSO-d$_6$, T=298 K.

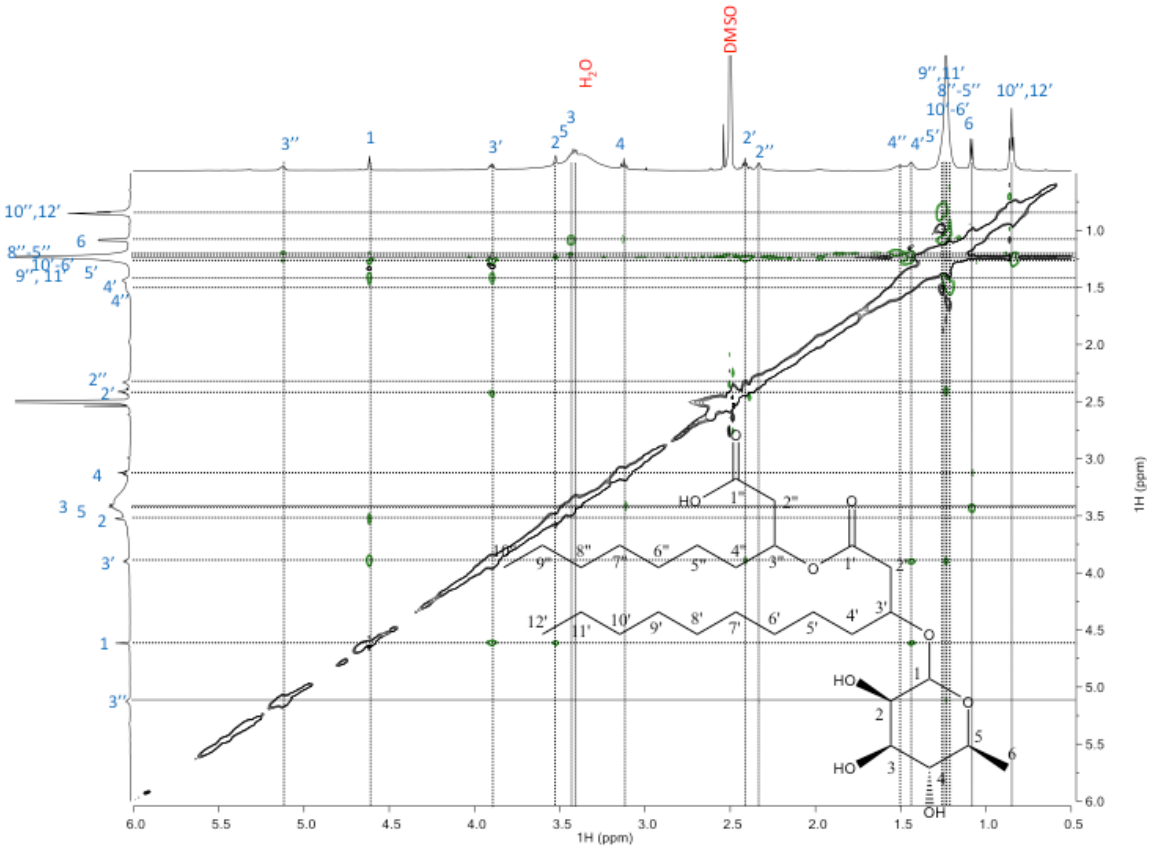


Figure S34. 2D ROESY (300 ms) of **5** in DMSO-d_6, T=298 K.

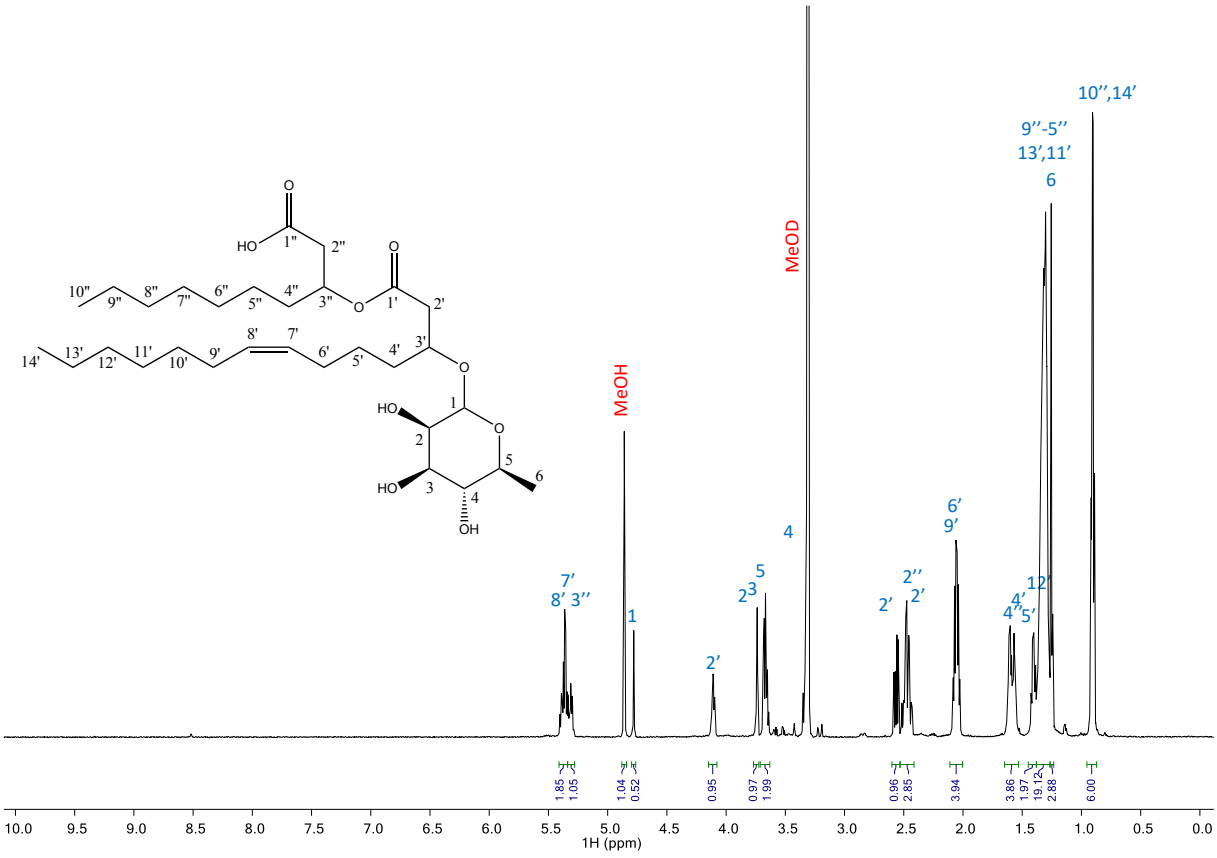


Figure S35. 1D proton of **6** in methanol-d_4, T=298 K.

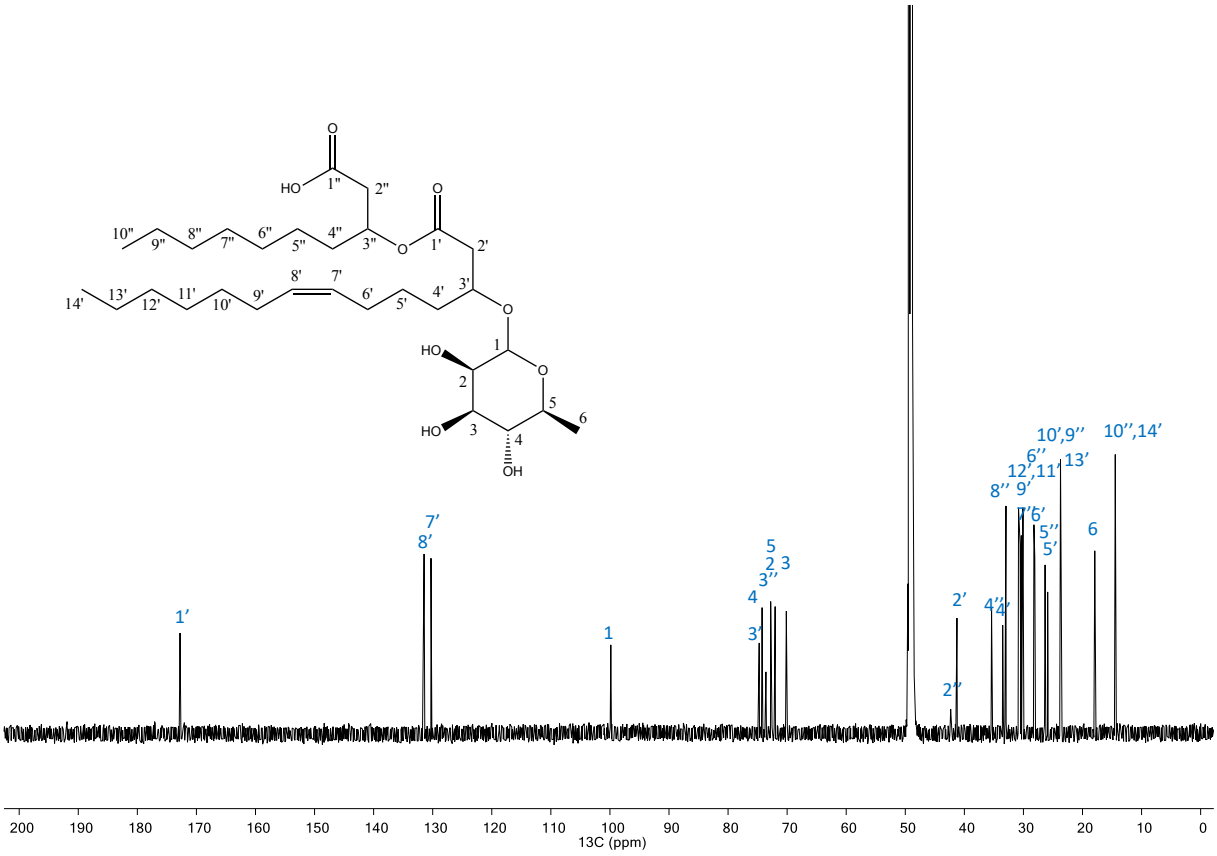


Figure S36. 1D carbon of **6** in methanol-d4, T=298 K.

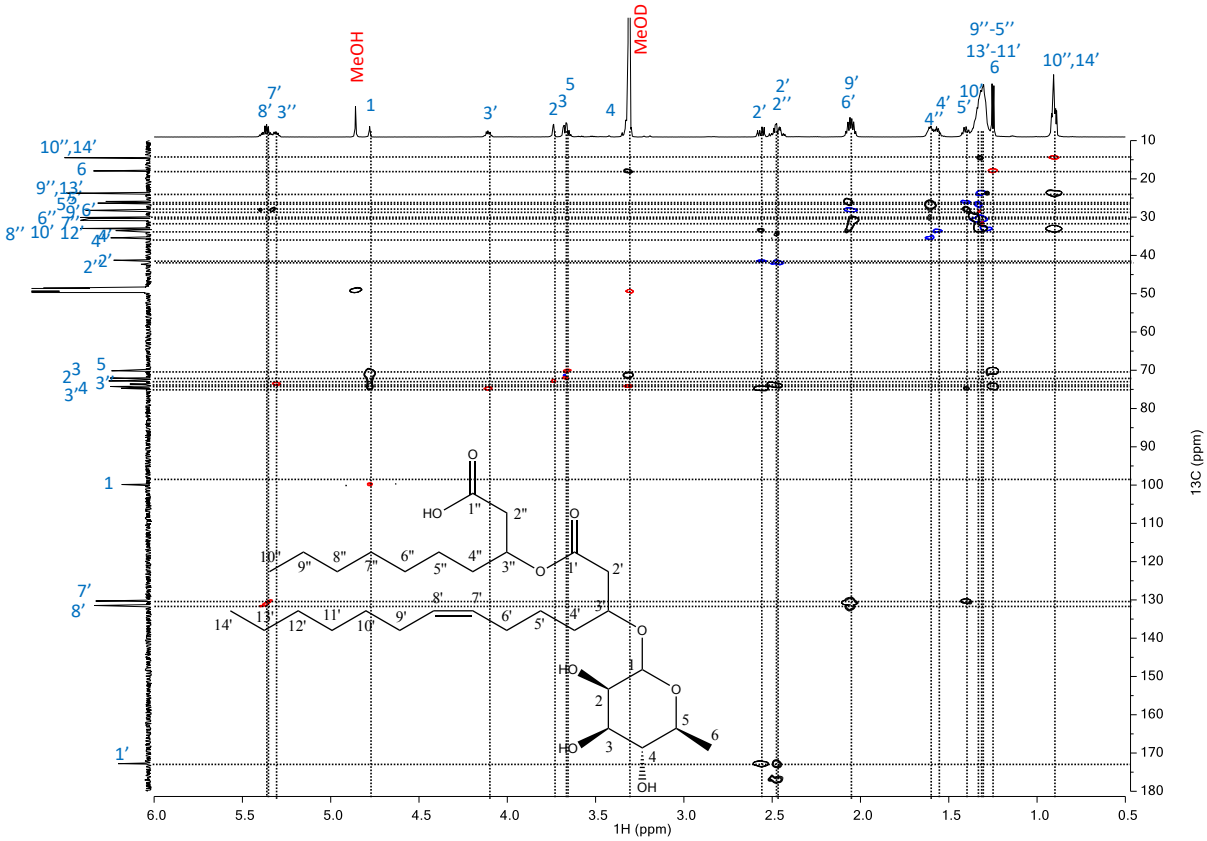


Figure S37. 2D superimposed ^{13}C-HSQC and HMBC of **6** in methanol-d4, T=298 K.

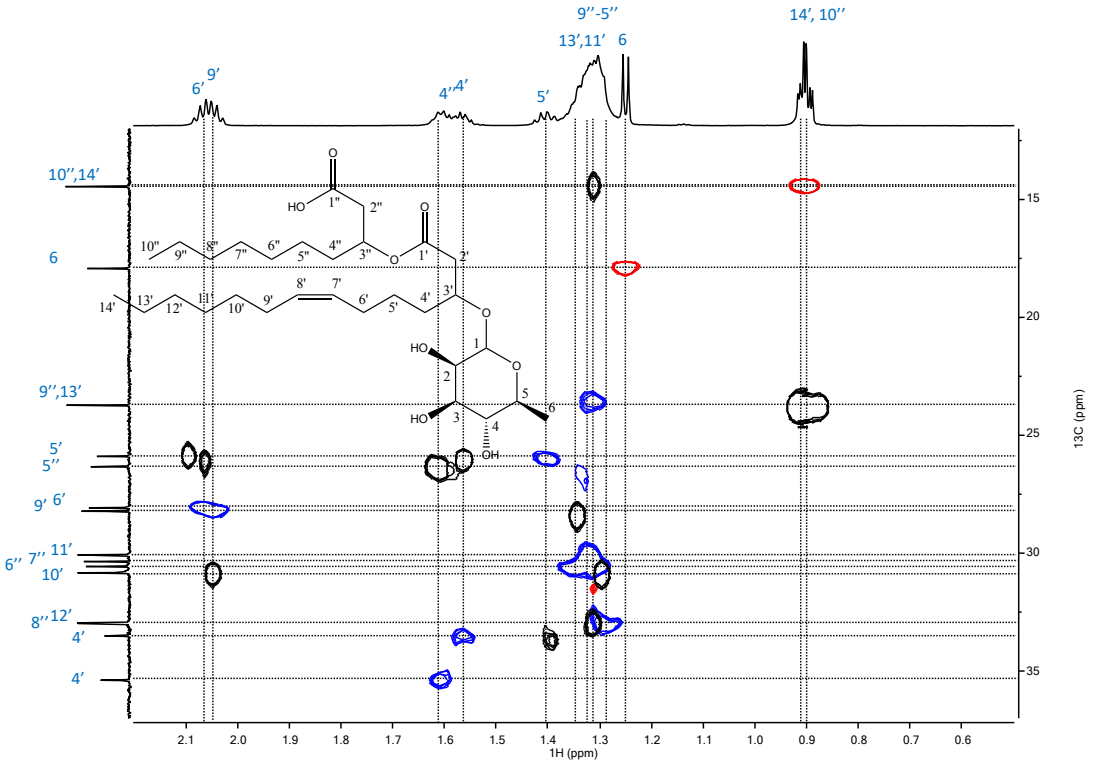


Figure S38. Blown up aliphatic region of superimposed 2D ^{13}C-HSQC and H2BC of **6** in methanol-d$_4$, T=298 K.

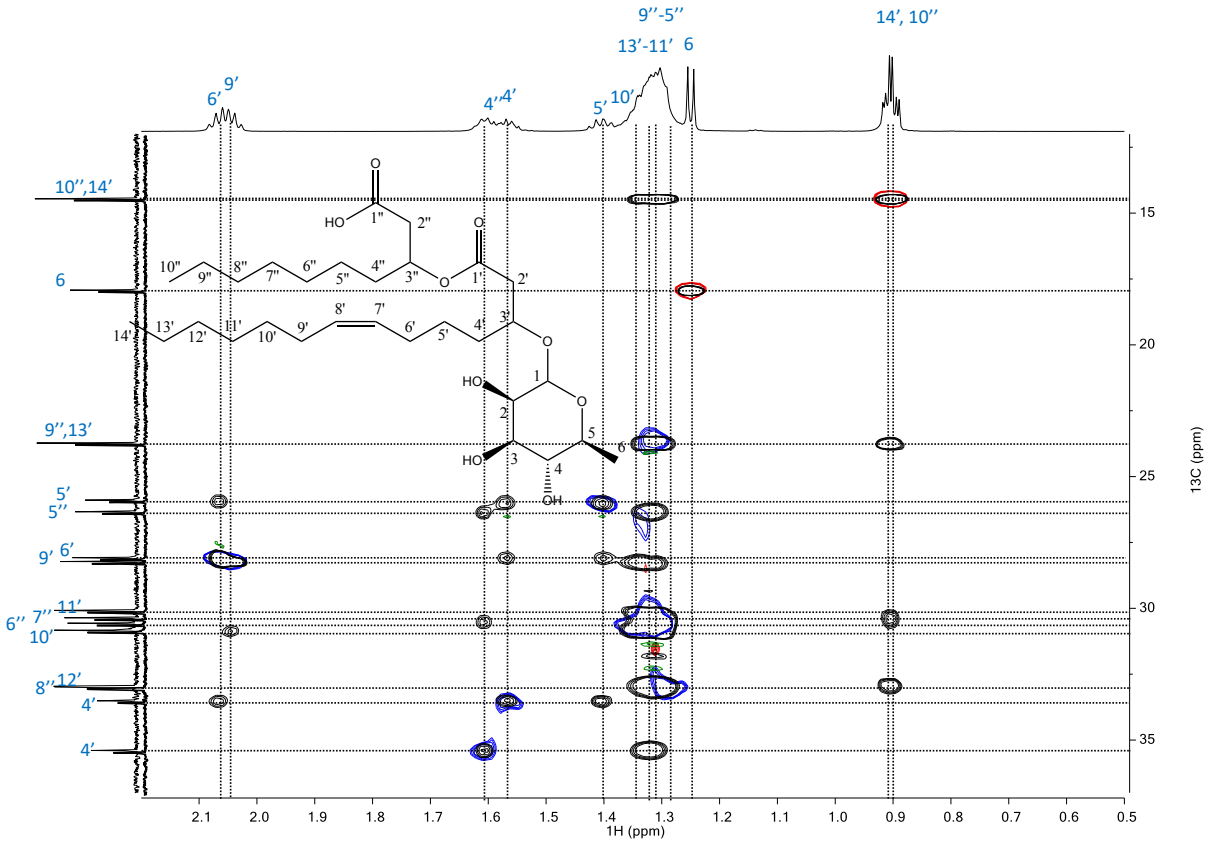


Figure S39. Blown up aliphatic region of superimposed 2D ^{13}C-HSQC and HSQC-TOCSY of **6** in methanol-d$_4$, T=298 K.

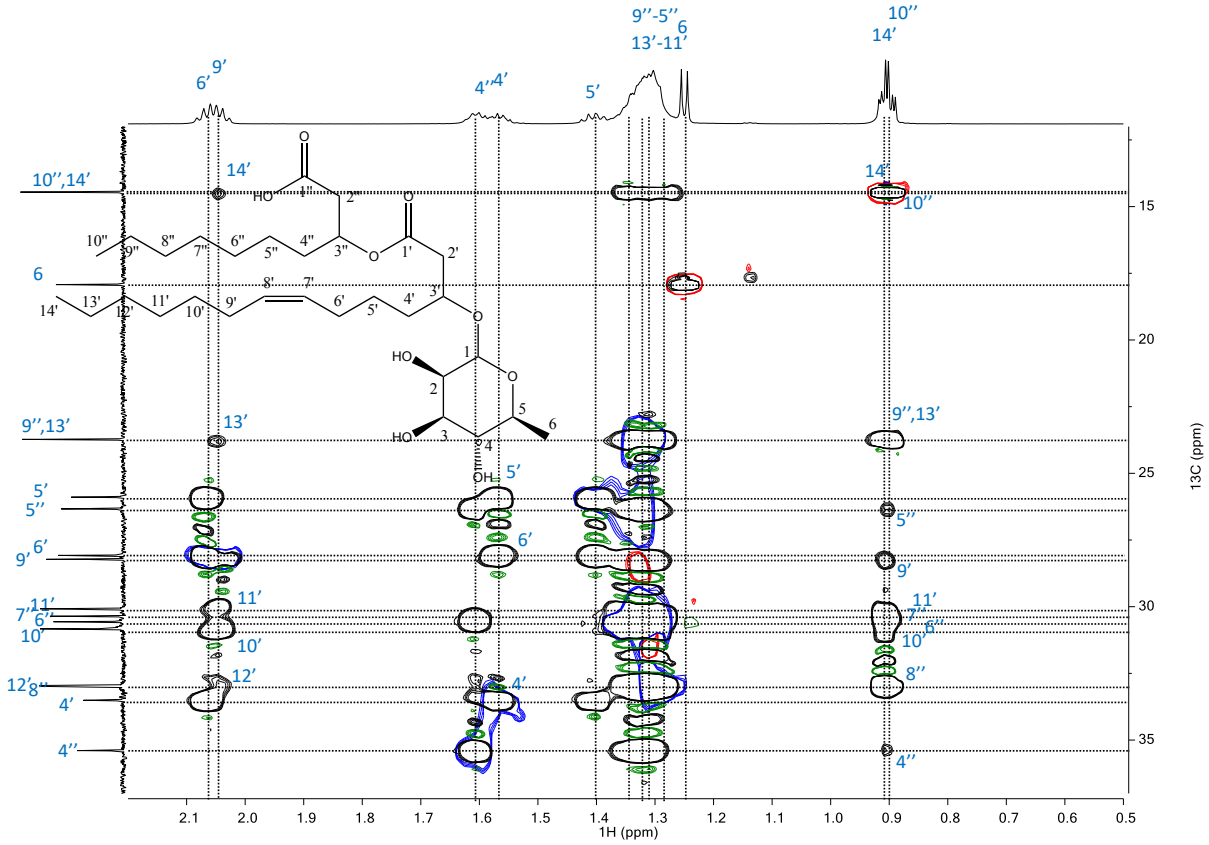


Figure S40. 2D ^{13}C-HSQC and HSQC-TOCSY at noise level of **6** in methanol-d$_4$, T=298 K.

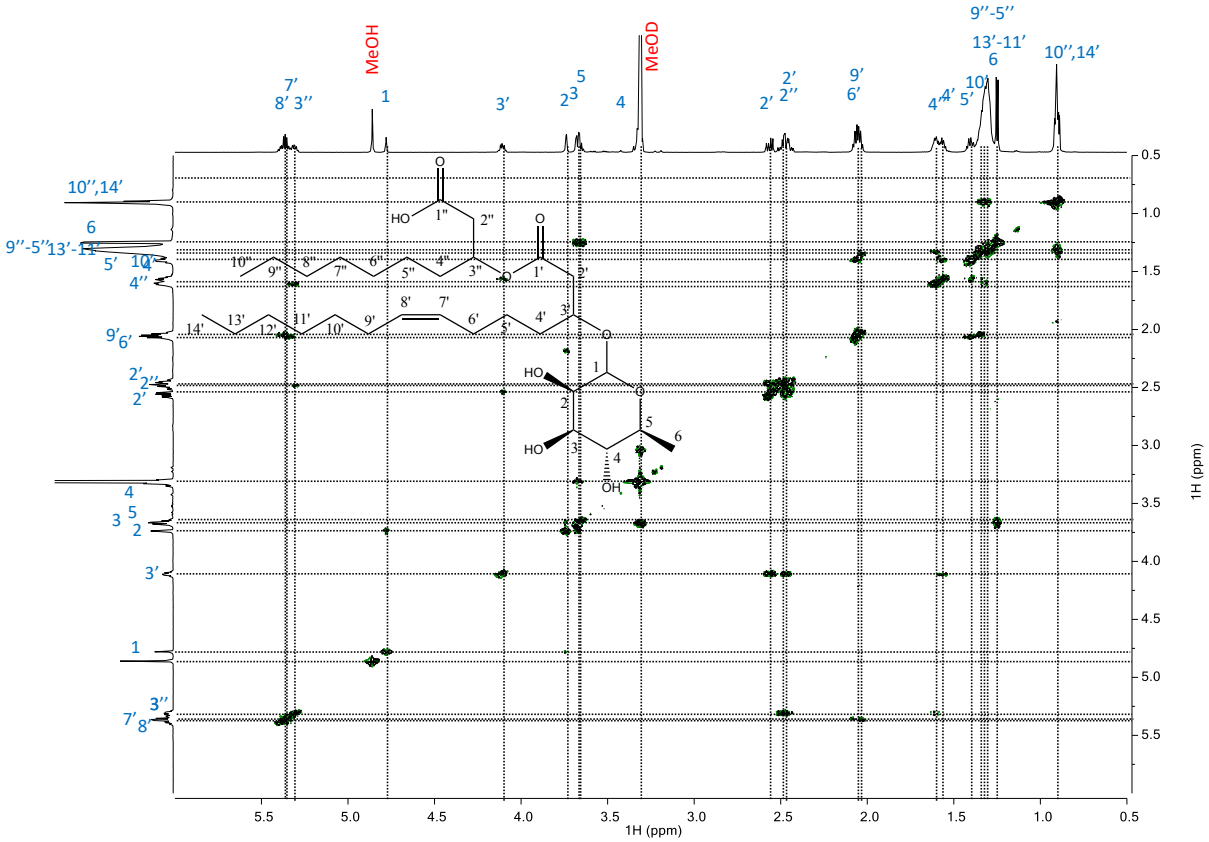


Figure S41. 2D DQF-COSY of **6** in methanol-d$_4$, T=298 K.

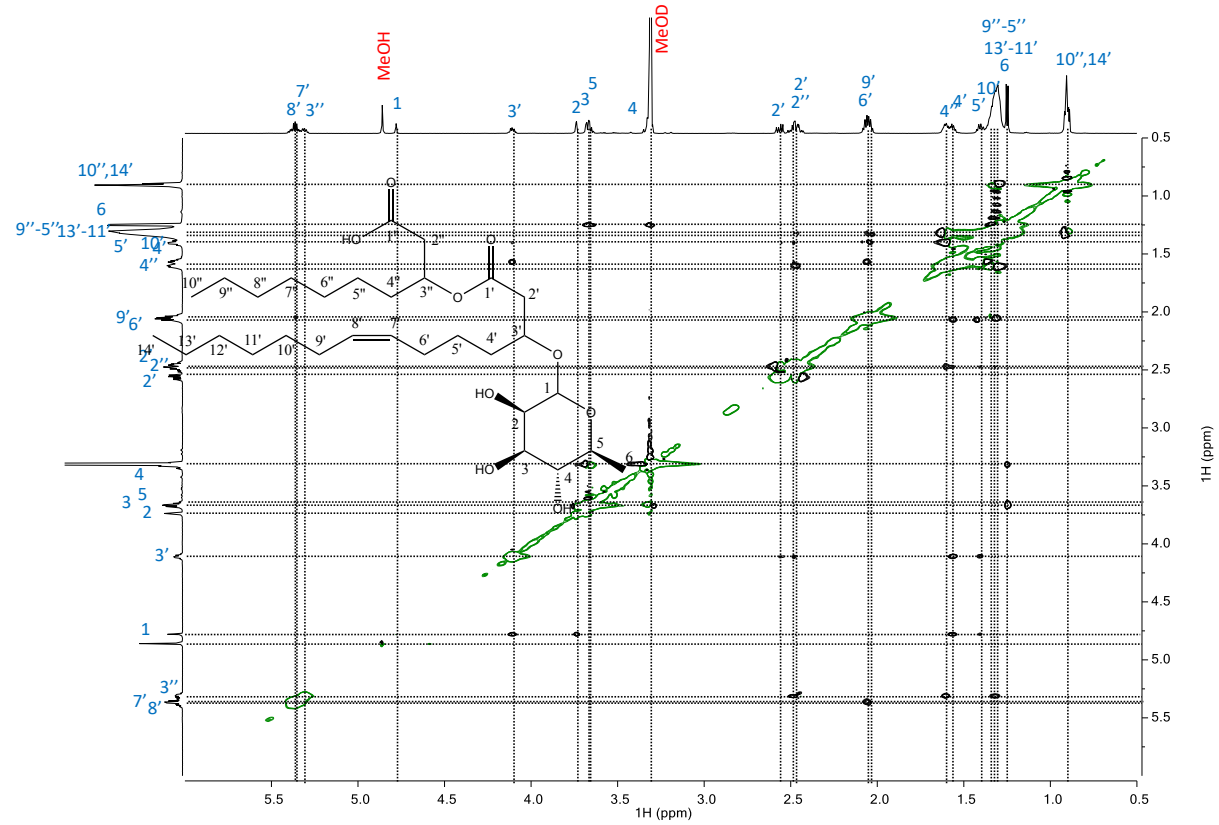


Figure S42. 2D ROESY (300 ms) of **6** in methanol-d4, T=298 K.

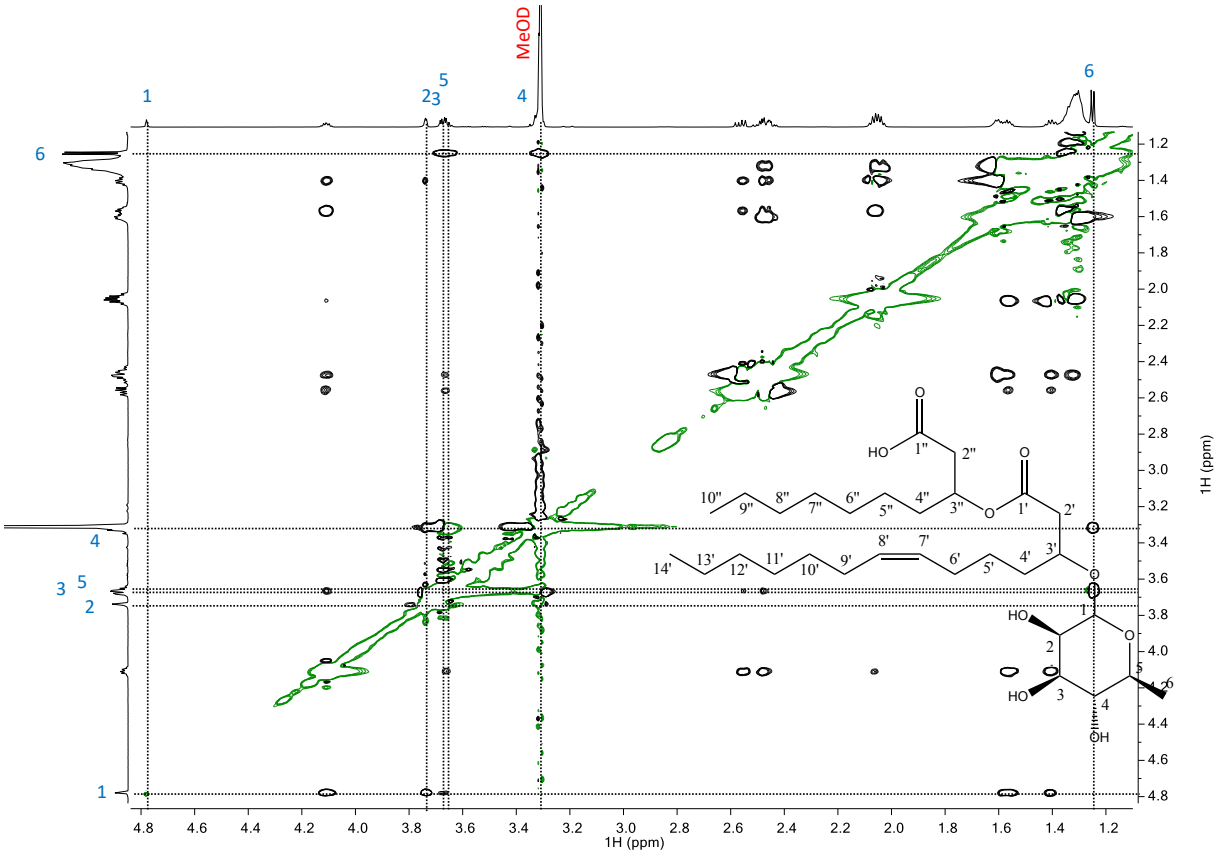


Figure S43. Blown up cyclic region of 2D ROESY (300 ms) of **6** in methanol-d4, T=298 K.

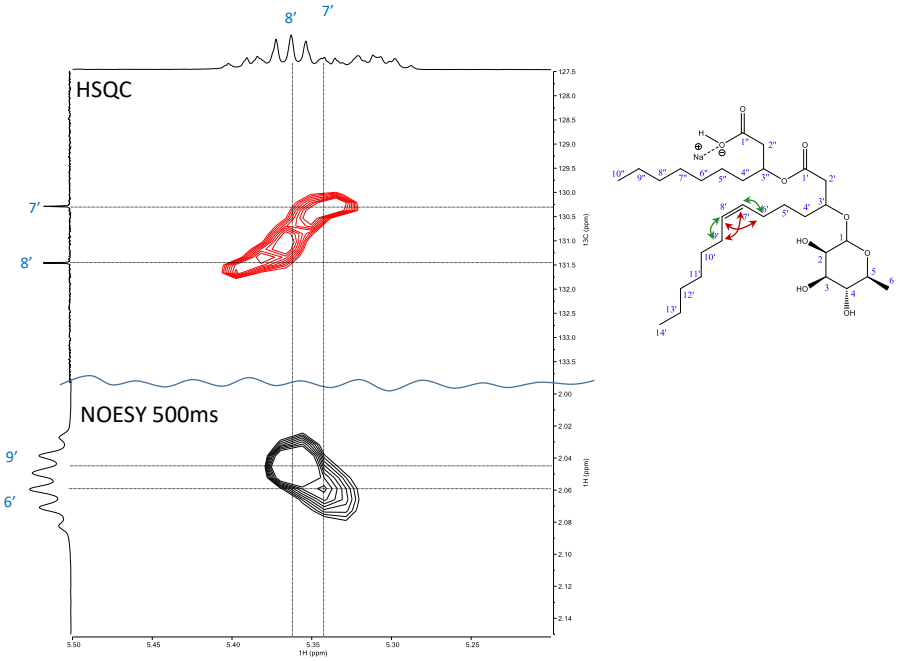


Figure S44. Expansion of the nearly overlapped olefinic protons in a 2D NOESY (500 ms) of **3** in methanol-d_4, T=298 K. The absence of NOE6′,8′ and NOE9′,7′ follows the *cis*-pattern observed for 4, which could be reliably determined to be in *cis* configuration.

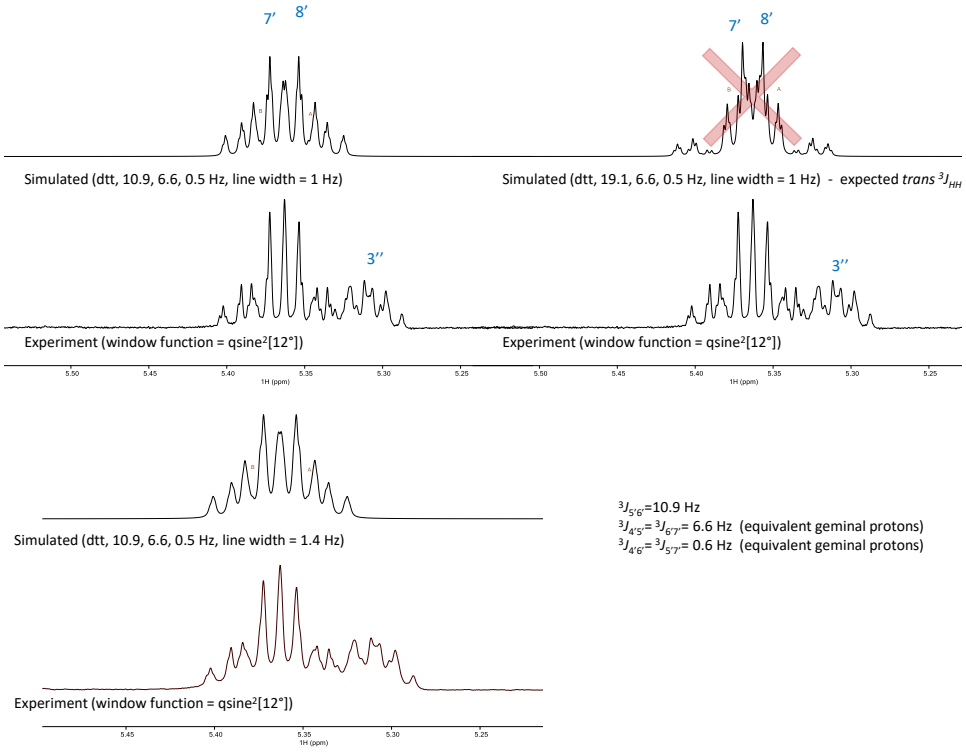


Figure S45. Comparison between experimental and simulated multiplets of the nearly overlapped olefinic protons of **3**. The fit was made using a qsine²(12°) window function (upper panel), and then compared to the raw multiplets (lower panel). The best fit was found to be a dtt, 10.9, 6.6, 0.6 Hz multiplet, which indicates a cis configuration (expected $^3J_{7'8'}$ ~ 11 Hz). The right panel shows the simulated peak for the expected trans coupling ~19 Hz with shows a poor fit.

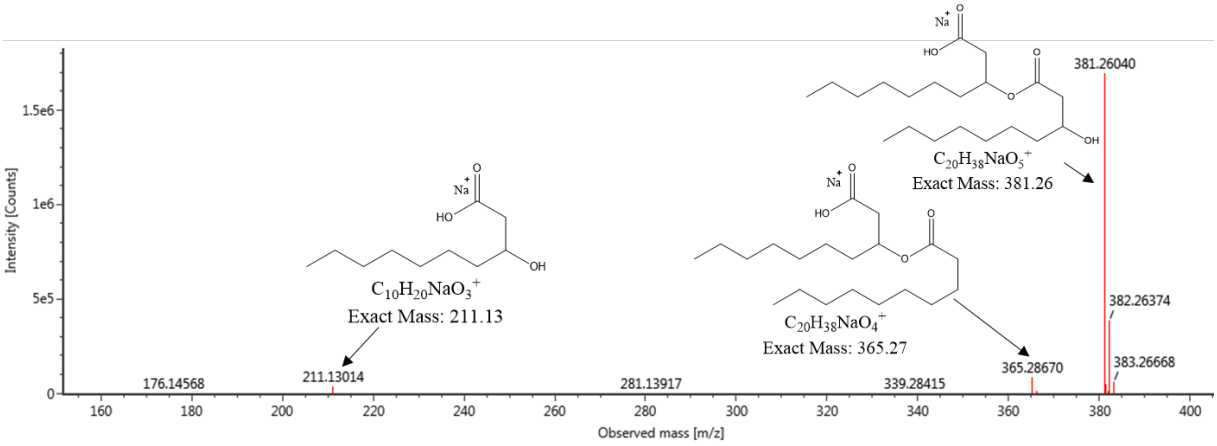


Figure S46. MS/MS spectrum of **1** [M + Na] $^{+}$. Ion mode ESI+.

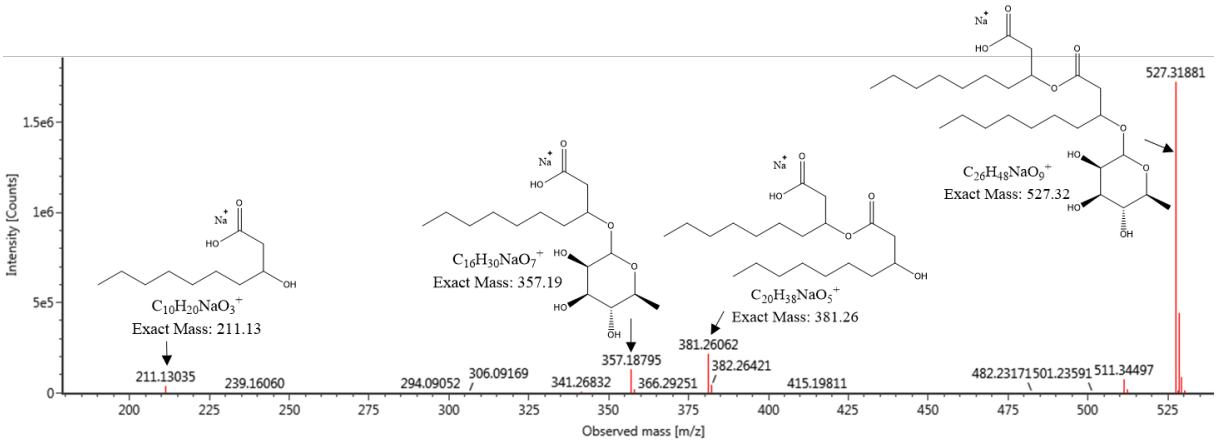


Figure S47. MS/MS spectrum of **2** [M + Na] $^{+}$. Ion mode ESI+.

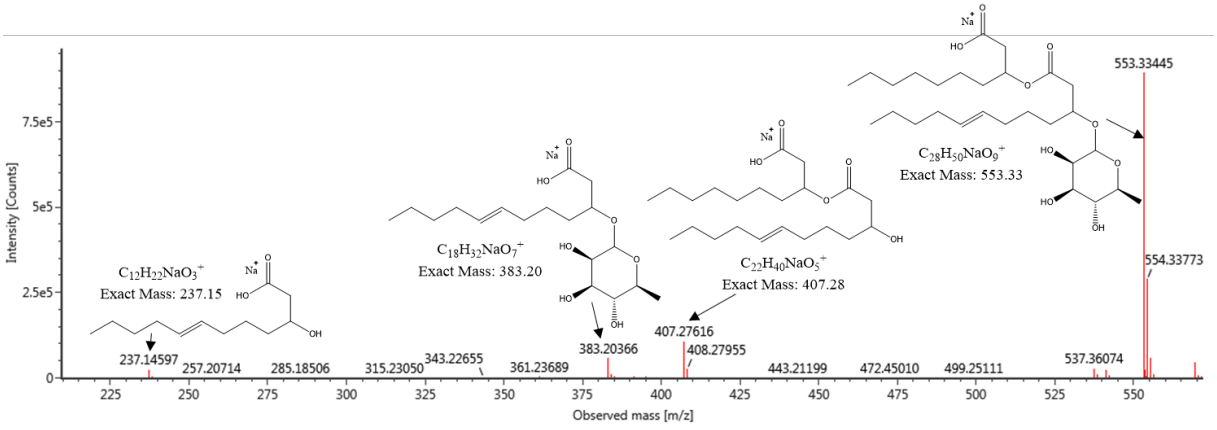


Figure S48. MS/MS spectrum of **3** [M + Na] $^{+}$. Ion mode ESI+.

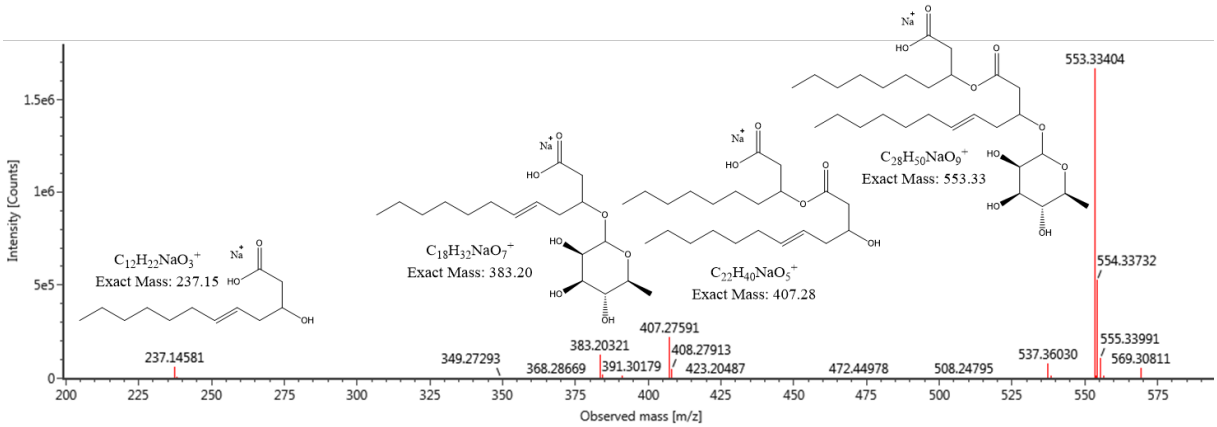


Figure S49. MS/MS spectrum of **4** [M + Na] $^{+}$. Ion mode ESI+.

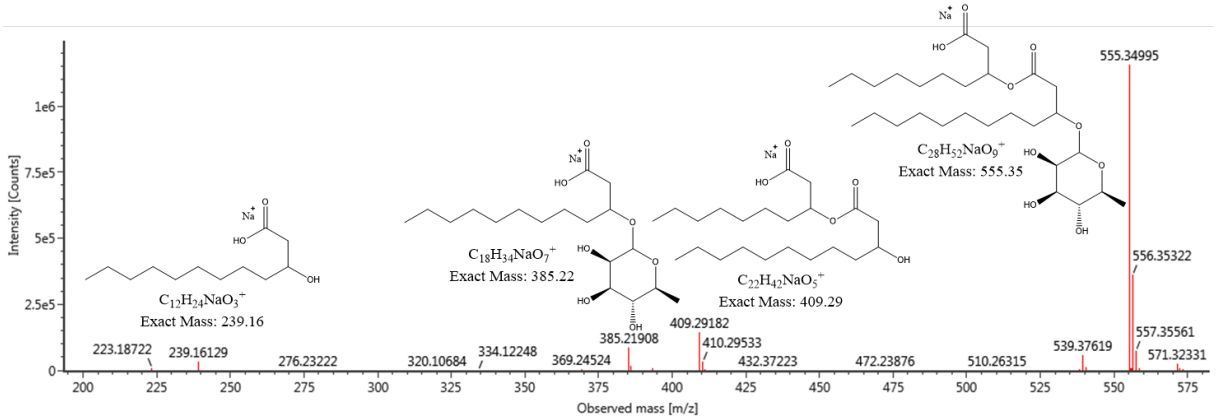


Figure S50. MS/MS spectrum of **5** [M + Na] $^{+}$. Ion mode ESI+.

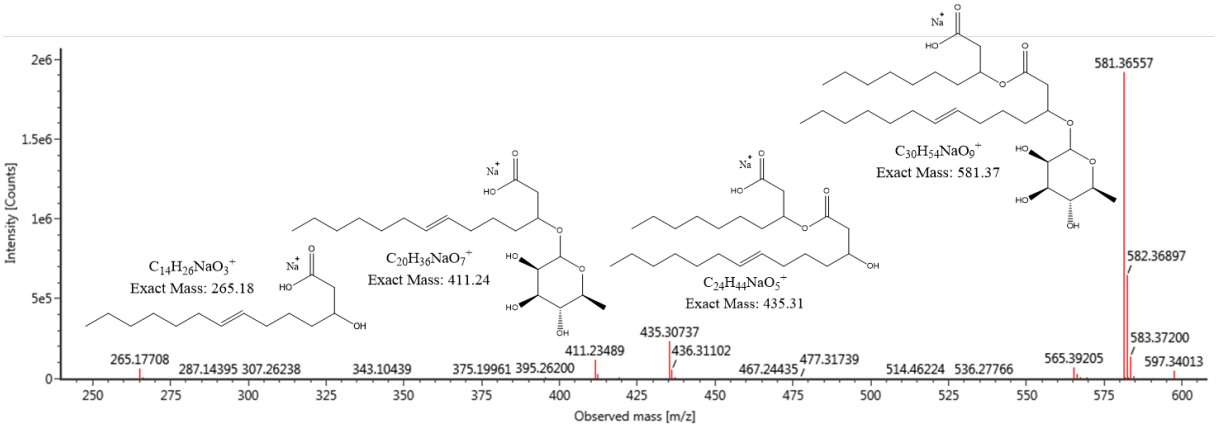


Figure S51. MS/MS spectrum of **6** [M + Na] $^{+}$. Ion mode ESI+.

Paper 2

molecules

MDPI

Article

Two Novel Lyso-Ornithine Lipids Isolated from an Arctic Marine *Lacinutrix* sp. Bacterium

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Abstract: The *Lacinutrix* genus was discovered in 2005 and includes 12 Gram-negative bacterial species. To the best of our knowledge, the secondary metabolite production potential of this genus has not been explored before, and examination of *Lacinutrix* species may reveal novel chemistry. As part of a screening project of Arctic marine bacteria, the *Lacinutrix* sp. strain M09B143 was cultivated, extracted, fractionated and tested for antibacterial and cytotoxic activities. One fraction had antibacterial activity and was subjected to mass spectrometry analysis, which revealed two compounds with elemental composition that did not match any known compounds in databases. This resulted in the identification and isolation of two novel isobranched lyso-ornithine lipids, whose structures were elucidated by mass spectrometry and NMR spectroscopy. Lyso-ornithine lipids consist of a 3-hydroxy fatty acid linked to the alpha amino group of an ornithine amino acid through an amide bond. The fatty acid chains were determined to be iso-C15:0 (**1**) and iso-C16:0 (**2**). Compound **1** was active against the Gram-positive *S. agalactiae*, while **2** showed cytotoxic activity against A2058 human melanoma cells.

Keywords: marine bacteria; lipoamino acid; secondary metabolites; amphiphilic compounds; antibacterial; cytotoxic; anti-cancer

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1. Introduction

Bacteria are the producers of many secondary metabolites that have been developed into drugs, including the tetracycline and aminoglycoside classes of antibiotics [1,2], that has paved the way for better health for millions of people around the world. Most of the bacterial secondary metabolites have been isolated from terrestrial organisms [3], suggesting that the chemical diversity of natural products can be expanded by investigating bacteria from other habitats.

The Arctic marine environment is home to numerous microorganisms thriving in cold water under the stark seasonal changes from midnight sun to polar darkness. Compared to terrestrial microorganisms, the bacteria living under these conditions must be adapted to cold saline water. It is therefore believed that these bacterial species have specialized metabolic systems tailored for survival in this niche environment. Today there are several marketed drugs originating from the marine environment [4]. While most of them were isolated from invertebrates, the true producers of many of these secondary metabolites are now known to be symbiotic bacteria, showing that marine bacteria is a promising source of new bioactive secondary metabolites [5,6]. To increase the likelihood of discovering novel bioactive compounds, one strategy is to search in underexplored places and sources. As

the Arctic water is less investigated than warmer waters and terrestrial environments, it represents a potential source for the discovery of novel bioactive bacterial compounds.

The *Lacinutrix* genus belongs to the family *Flavobacteriaceae*, which is the largest family in the Bacteroidetes phylum [7]. The genus consists of Gram-negative marine bacteria that have been isolated from both cold polar waters and warm waters. This genus was first described in 2005 by Bowman and Nichols, when *L. copepodicola* was isolated from an Antarctic marine calanoid copepod [8]. Today the genus includes 12 marine species, five isolated from polar waters and seven from warm waters. In addition to *L. copepodicola*, the polar species includes *L. mariniflava*, *L. algicola* [9] and *L. jangbogonensis* isolated from the Antarctic [10], and *L. himadriensis* isolated from the Arctic [11]. Species isolated from warm waters include *L. iliipiscaria* and *L. gracilariae* isolated from China [12–14], *L. cladophorae* and *L. chionocetis* from Japan [13,15], *L. venerupis* from Spain [16] and *L. undariae* and *L. salivirga* isolated from South Korea [17,18]. To date, the studies of *Lacinutrix* sp. have mainly focused on describing novel species; analyzing their genomic and cellular fatty acid content [10,16,19], while their ability to produce secondary metabolites has not yet been assessed.

As part of the current study, two new lyso-ornithine lipids were isolated and characterized. Lyso-ornithine lipids are known to be precursors of ornithine lipids, which are the most common lipoamino acids found in the bacterial membrane. Ornithine lipids are widely distributed in Gram-negative bacteria, but are also present in Gram-positive bacteria. The biosynthesis of ornithine lipids occurs in two steps, where the first step is the formation of lyso-ornithine-lipids from ornithine and 3-hydroxy fatty acyl-acyl carrier protein. Ornithine lipids are formed in the next step by the transfer of an acyl group from fatty acyl-acyl carrier protein to lyso-ornithine [20–22].

In the present work, the Arctic marine *Lacinutrix* sp. strain M09B143 was isolated from a *Halichondria* sp. sponge collected in the Barents Sea. The potential of the bacterium to produce bioactive metabolites was evaluated. It was cultivated and the secreted metabolites were extracted from the fermentation broth. The extract was fractionated into six fractions that were tested for antibacterial and cytotoxic activity. Fraction 5 was active against Gram-positive bacteria and was therefore selected for further chemical analysis. This resulted in the isolation and identification of two novel iso-branched lyso-ornithine lipids that were tested for antibacterial and cytotoxic activities.

2. Results

2.1. Isolation and Identification

Lacinutrix sp. strain M09B143 was isolated from a *Halichondria* sp. sponge collected in the Barents Sea. It was identified as a *Lacinutrix* sp. using 16S rRNA sequencing and Basic Local Alignment Search Tool (BLAST) searches against reference sequences in GenBank. The 16S rRNA gene sequence analysis confirmed that M09B143 was affiliated with the genus *Lacinutrix*, a member of the family *Flavobacteriaceae* and phylum Bacteroidetes, corresponding to the information provided by the Norwegian Marine Biobank Marbank. The bacterium clustered separately on its own branch with *L. algicola* (NR_043592), and sister taxon for this branch was *L. mariniflava* (NR_043592). *L. algicola* and *L. mariniflava* are both isolated from a red alga of the family *Gigartinaceae* [9]. Figure 1 shows the results from the phylogenetic analysis using PhyML. The phylogenetic analysis was also run using the MrBayes 3.2.6 plug-in in Geneious, and the results of this analysis are shown in Supplementary Information Figure S1. There were some differences between the Bayesian Inference tree and the Maximum Likelihood tree, caused by different placement of non-supported nodes in ML and Bayesian analyses and especially the polytomy at one basal node in the tree from MrBayes. The clade consisting of *Lacinutrix* M09B143, *L. algicola*, *L. mariniflava* and *L. jangbogonensis* was statistically supported and topologically similar using both methods.

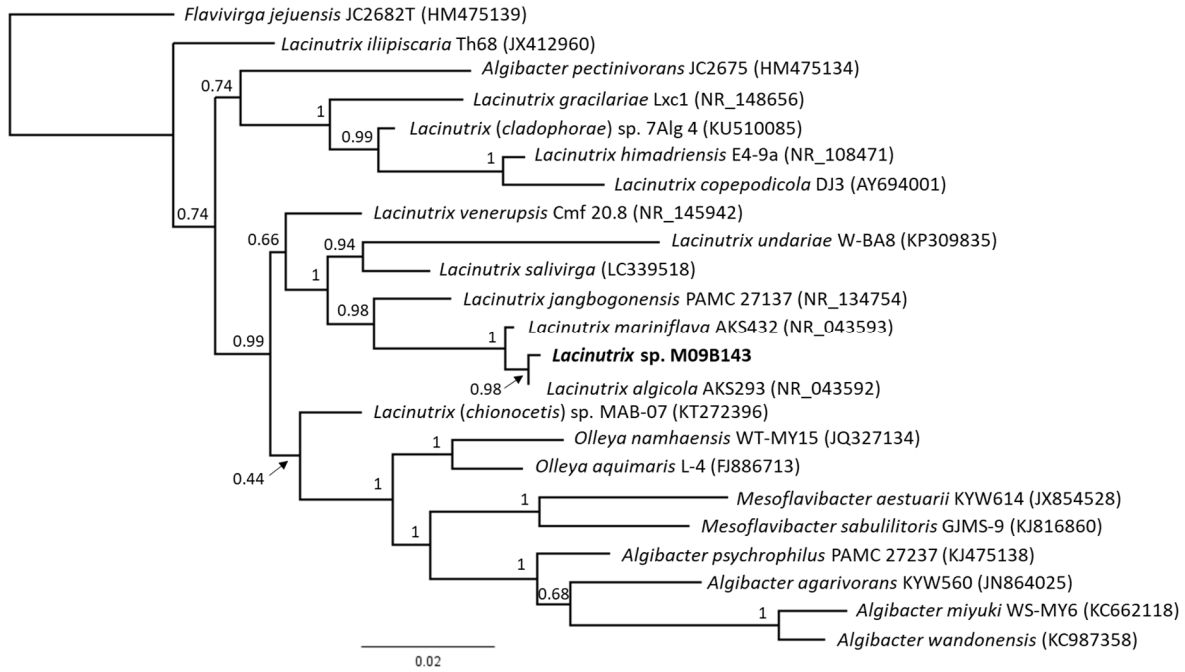


Figure 1. Maximum likelihood tree based on 16S rRNA gene sequences and showing the phylogenetic placement of the strain M09B143 (in bold) within Bacteroidetes. The tree was rooted with *Flavivirga jejuensis* as the outgroup. Branch support is given as aLRT values.

2.2. Bioactivity of Fractionated Extract

The M09B143 strain was fermented in 2 × 200 mL M19 medium in 1 L flasks. Secondary metabolites excreted into the medium were extracted with Diaion® HP20 resin and eluted with methanol. The bacterial extract was fractionated into six fractions by flash column chromatography and the fractions were tested for antibacterial and cytotoxic activities at 50 µg/mL. Only flash fraction 5, eluting at 100% methanol was active. It was active against the Gram-positive bacteria *Streptococcus agalactiae*, *Enterococcus faecalis* and *Staphylococcus aureus* (Figure 2). The activity appeared to be most potent against *S. agalactiae*, followed by *E. faecalis*. The six fractions were not active against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, or against the A2058 human melanoma cells (Figure S2).

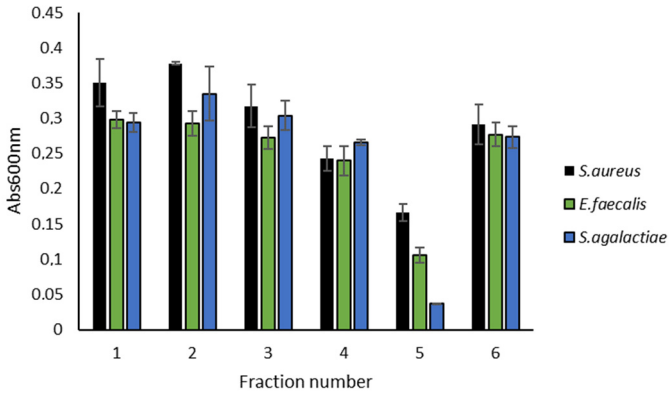


Figure 2. Antibacterial effect of flash fractions 1–6 from M09B143 extract against Gram-positive bacteria tested at 50 µg/mL in a growth inhibition assay (two technical replicates). Fraction 5 was active and was selected for further analysis with UHPLC-HR-MS to identify the compound(s) responsible for the observed activity.

2.3. Dereplication

Based on the observed antibacterial activity, fraction 5 was subjected to UHPLC-HR-MS analysis. The resulting data were compared to the equivalent data recorded for the inactive fractions 4 and 6 to identify compounds that were exclusively present, or present in higher amounts in fraction 5. The dereplication led to the identification of two compounds, **1**, with elemental composition $C_{20}H_{40}N_2O_4$ and **2**, with elemental composition $C_{21}H_{42}N_2O_4$. Compound **1** was the major peak, and **2** was among the most prominent peaks in the MS chromatogram of fraction 5 (Figure S3). Both compounds were only present in very small amounts in the inactive fractions 4 and 6. All other major peaks in the UHPLC-HR-MS chromatogram of fraction 5 were determined to be either media components, or compounds present in comparable amounts in the inactive fractions 4 and 6. Consequently, **1** and **2** were suspected to be responsible for the observed bioactivity of fraction 5. Fragmentation patterns in the UHPLC-HR-MS analysis indicated that they were lipoamino acids, and from their elemental composition and relatively similar retention time, it was assumed that the two compounds differed from each other with a methylene group in the lipid chain. Searches in relevant databases, such as ChemSpider, did not provide any hits that matched the two compounds. Moreover, the dereplication analysis revealed that **1** eluted in three peaks and **2** as two peaks. This indicated that different isomers of both compounds were produced by the bacterium (Figure S4). The three peaks recorded for sample **1** all had the same elemental composition, and the two peaks for sample **2** had the same elemental composition. Fragmentation patterns from MS/MS on the UHPLC-HR-MS were also identical for the different peaks. This strongly indicates that **1** was a mixture of three stereoisomers and that **2** was a mixture of two stereoisomers.

2.4. Isolation of Compound *1* and *2*

For purification of the two compounds, upscale cultivation of *Lacinutrix* sp. M09B143 and isolation were performed in two rounds using a preparative HPLC-MS system. The strain was fermented in 64 × 250 mL in round one, which resulted in 25.0 g of dry extract. Fractionation of the extract yielded 515.0 mg of fraction 5. Extensive efforts were put into separating the isomeric variants of each compound from each other. However, due to the lower chromatographic resolution of the preparative column, it was not possible to do so. Therefore, the three variants of **1** were isolated and further processed together, and so were the two variants of **2**. In the text below, compound **1** refers to the sample containing the three variants of **1**, and compound **2** refers to the sample containing the two variants of **2**.

The first isolation step of the two compounds in round one yielded 8.0 mg of **1** and 5.0 mg of **2**. After the second purification step, the yield of **1** was 1.5 mg and 0.6 mg of **2**.

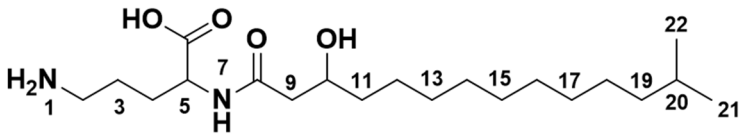
Fermentation and isolation in round two included 56 × 400 mL cultures, which resulted in 28.02 g of dry extract that was fractionated and yielded 1021.2 mg of fraction 5. First purification step of the two compounds with preparative HPLC-MS gave 26.8 mg of **1** and 23.2 mg of **2**. Compound **2** was subjected to a second purification step, resulting in 4.9 mg of **2**.

The two compounds were isolated as light brown waxes; total yield was 28.3 mg of **1** and 5.5 mg of **2**. The purity of the isolated compounds was checked using UHPLC-HR-MS. This revealed that **1** and **2** were completely separated from each other and that the samples only contained minor impurities.

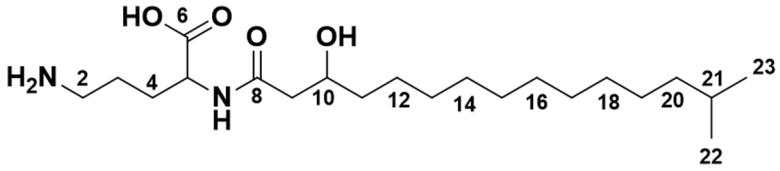
2.5. Structure Elucidation

The structures of **1** and **2** (Figure 3) were elucidated using 1D (^{1}H and ^{13}C, Table 1) and 2D (HSQC, HMBC, HSQC-TOCSY and COSY, COSY only recorded for **2**). NMR experiments in methanol-d_3 and UHPLC-HR-MS analysis. The compounds were determined to consist of a polar ornithine head group linked to a mono-hydroxylated 15:0 (**1**)/16:0 (**2**) iso-fatty acid through an amide bond. The structures of the individual variants of **1** and **2** could not be determined individually, but the presence of two stereoisomers in the

5-position could be observed as two near isochronous C-5 resonances and an unresolvable H-5 multiplet pattern.



1



2

Figure 3. Structures of lyso-ornithine lipids isolated from *Lacinutrix* sp. (**1**): $C_{20}H_{40}N_2O_4$, (**2**): $C_{21}H_{42}N_2O_4$.

Table 1. ^{1}H and ^{13}C assignments for **1** and **2**.

	(1)		**(2)**	
position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2	40.2, CH_2	2.95, t (7.3)	40.2, CH_2	2.95, t (7.3)
3	24.6, CH_2	1.71, dtd (17.1, 9.5, 8.5, 4.2)	24.6, CH_2	1.77–1.64, m [e]
4	30.9, CH_2	1.91, ddd (10.0, 8.4, 4.8)	30.9, CH_2	1.90, m
5	54.8, C	4.28, dq (9.9, 3.9, 2.6)	54.8, CH	4.28, d (5.4)
6	178.0, C	-	178.0, C	-
7	-	7.63, d (8.0)	-	7.62, d (8.0)
8	173.7, C	-	173,7, C	-
9a	45.0, CH_2	2.39, dd (14.3, 3.9)	45.0, CH_2	2.39, dd (14.4, 4.0)
9b		2.30, dd (14.4, 9.2)		2.30, dd (14.4, 9.2)
10	69.9, CH	3.95, ddt (8.9, 5.8, 3.1)	69.9, CH	3.95, m
11	38.4, CH_2	1.49, m [b]	38.4, CH_2	1.52, m
12	26.6, CH_2	1.35, m [c]	26.6, CH_2	1.48, dq (7.1, 4.4, 3.9)
13	30.7–30.6, CH_2 [a]	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
14	30.7–30.6, CH_2 [a]	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
15	30.7–30.6, CH_2 [a]	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
16	30.7–30.6, CH_2 [a]	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
17	30.7–30.6, CH_2 [a]	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
18	28.4, CH_2	1.40–1.22, m [c]	30.7–30.6, CH_2 [d]	1.40–1.22, m [f]
19	40.1, CH_2	1.16, qd (7.5, 4.2)	28.4, CH_2	1.40–1.22, m [f]
20	29.0, CH	1.52, m [b]	40.1, CH_2	1.17, q (7.1)
21	22.9, CH_3	0.86, dd (10.9, 6.7)	29.0, CH	1.77–1.64, m [e]
22			23.6, CH_3	0.87, d (6.8)
23	-	-		

[a–f] Signals are overlapping.

The molecular formula of **1** was calculated to be $C_{20}H_{40}N_2O_4$ (*m*/*z* 373.3055, $[M + H]^+$, calcd 373.3066) by HRESIMS, corresponding to two degrees of unsaturation. The ornithine substructure (atoms 1 to 7) of **1** was assembled through correlations found in the HMBC spectrum (Figures 4 and S5). Deshielding of carbon atom CH_2-2 (δ_C 40.2) places the NH_2 group at the delta carbon of the amino acid. The carbonyl group was determined to be located at C-6 (δ_C 178.0). The fatty acid chain was found to be linked to the polar head group through an amide bond between NH-7 (δ_H 7.63) and C-8 (δ_C 173.3) based

on a HMBC correlation between the two. Furthermore, carbon atoms C-9 to C-13, and C-17 to C-23 were linked through HSQC-TOCSY experiments (Figures 4 and S6), where the C-13 to C-17 resonances overlap in both dimensions. A hydroxy group was placed at carbon atom CH-10 (δ_C 69.9) based on HSQC data (Figure S5) and the deshielded shift value of the carbon atom. In agreement with previously reported data for similar compounds [23,24], the central methines (CH_2-13 to CH_2-17) could not be individually assigned due to complete signal overlap (Figures S7 and S8). The two equivalent CH_3 groups (CH_3-21 and CH_3-22) of the iso-terminal of the fatty acid were assigned based on ^{1}H and HMBC spectrum analysis, and were furthermore linked to a -CH-CH_2-CH_2- fragment (CH-20 (δ_C 29.0), CH_2-19 (δ_C 40.1) and CH_2-18 (δ_C 28.4)) through HMBC and HSQC-TOCSY correlations. Consequently, the structure of **1** was assigned as 5-amino-2-(3-hydroxy-13-methyltetradecanamido) pentanoic acid.

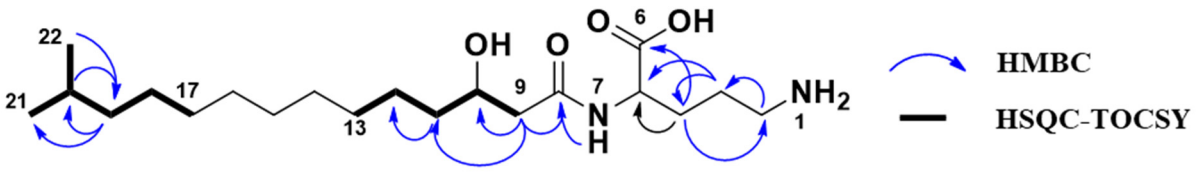


Figure 4. Selected 2D NMR correlations obtained for **1**.

Through HRESIMS analysis, **2** was determined to have a molecular formula of $C_{21}H_{42}N_2O_4$ (*m*/*z* 387.3212 $[M + H]^+$, calcd 387.3223). The structure of **2** (Figure 3) was assigned by analyzing the data from ^{1}H, ^{13}C, HSQC, HMBC, HSQC-TOCSY and COSY NMR experiments (Figures S9–S13). The structure of **2** was unambiguously assigned in a similar manner as described above for **1** and was found to have an extension of the fatty acid chain by a CH_2-group compared to **1** and was consequently assigned as 5-amino-2-(3-hydroxy-14-methylpentadecanamido) pentanoic acid.

2.6. Bioactivity Testing of Isolated Compounds

2.6.1. Antibacterial Assay

The two lyso-ornithine lipids were tested for antibacterial activity against the Gram-positive bacteria *S. agalactiae*, *E. faecalis* and *S. aureus*, and against the Gram-negative bacteria *E. coli* and *P. aeruginosa* in a growth inhibition assay in three biological replicates, each containing three technical replicates. The compounds were tested at 10, 50, 100 and 150 µM. As shown in Figure 5, **1** was active against *S. agalactiae*, while **2** showed no activity. A dose-response curve was observed for **1**, with minimum inhibitory concentration between 100 and 150 µM. Compound **1** also had modest effect against *E. faecalis* and *S. aureus* at the highest concentrations, but visible growth was observed in the wells at all concentrations, so complete growth inhibition was not achieved (Figure S14). Neither of the compounds were active against the Gram-negative bacteria (Figure S15).

2.6.2. Cytotoxic Effect of Isolated Lyso-Ornithine Lipids

The cytotoxicity of the two lyso-ornithine lipids was evaluated against human melanoma cell line A2058 and the non-malignant lung fibroblasts MRC-5 cell line at the concentrations 10, 25, 50, 100 and 150 µM. Some cytotoxic activity against the A2058 cell line was observed for **2**, with 23% cell survival at 50 µM, and ~0% cell survival at 100 and 150 µM (Figure 6). Compound **1** showed no activity against A2058 cells. Neither of the compounds were active against MRC-5 cells (Figure S16). The compounds were tested in three biological replicates with at least eight technical replicates in total.

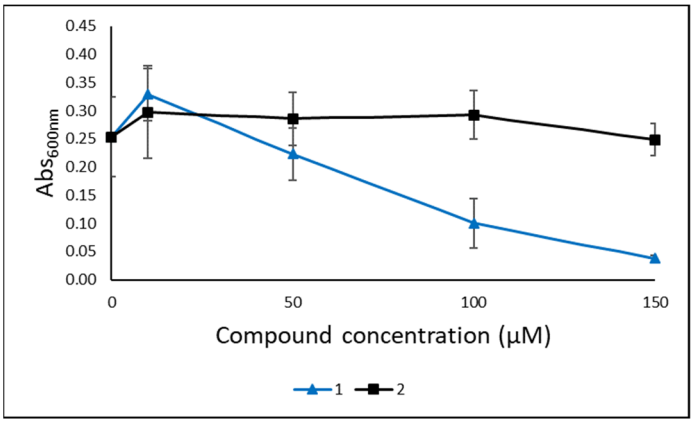


Figure 5. Antibacterial activity of **1** and **2** tested in a growth inhibition assay against the Gram-positive *S. agalactiae*. The assay was performed in three independent experiments, each with three technical replicates.

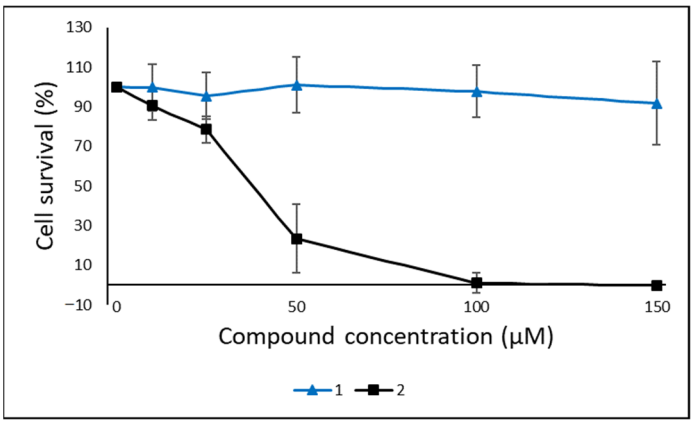


Figure 6. Cytotoxic activity of **1** and **2** against A2058 human melanoma cells. The compounds were tested in three experiments with at least eight technical replicates in total.

3. Discussion

The antibacterial activity of a fractionated extract from the Arctic marine bacterium *Lacinutrix* sp. led to the identification of two novel lyso-ornithine lipids, **1** and **2**.

Lyso-ornithine lipids are amphiphilic due to their nonpolar fatty acid chain and their polar amino acid head group. Previous studies from our group have identified amphiphilic compounds with antibacterial and cytotoxic activities [23,25]. This includes Lipid 430, with similar structure as the lyso-ornithine lipids. Lipid 430 and **2** have the same iso-branched fatty acid chain, they differ at the head group where Lipid 430 has two serine amino acids whereas **2** has one ornithine amino acid. Lipid 430 was active against the Gram-positive bacterium *S. agalactiae* and against A2058 human melanoma cells. In addition, lipoamino acids are reported to have various bioactivities, such as antibacterial, insecticidal, hemolytic, coagulant and macrophage activity [26–28]. Hence, it was likely that the two isolated compounds would be bioactive. After isolation, the two compounds were tested for antibacterial and cytotoxic activities. Compound **1** had some effect against Gram-positive bacteria, particularly *S. agalactiae*, and **2** was moderately cytotoxic to A2058 human melanoma cells. Considering the similarities in the structures of **1** and **2**, this discrepancy in bioactivity was unanticipated. As the compounds are mixtures of isomers, this could be a factor for the discrepancy in activity. However, based on our data, the isomers have the same iso-branched fatty acid linked to an ornithine head group, therefore, the differences in observed bioactivity are most likely due to the different length of the fatty

acid chain. The length of the fatty acid chain is known to affect the bioactivity of amphiphilic compounds. For example, Nashida et al. (2018) [29] synthesized mannosylerythritol lipids with various lipid chain length with different antibacterial activity. A study from Tareq et al. (2019) [30] also shows how small differences in the fatty acid chain can affect the bioactivity of amphiphilic compound. They isolated two gageostatins that showed differences in activity against various bacteria and fungi. The only difference between the two isolated gageostatins was a CH_2 in the lipid chain, similar to the differences between **1** and **2** in the present study.

The two isolated lyso-ornithine lipids showed no activity against the Gram-negative bacteria. This is likely due to the lipopolysaccharide on the outer membrane of Gram-negative bacteria, making it harder for the compounds to access the membrane, as the bioactivity of amphiphilic compounds is commonly due to membrane interactions. Tahara et al. (1977) [31] reported a lyso-ornithine lipid with the same molecular formula as **2**, but with an unbranched fatty acid chain instead of an iso-branched chain, that killed the Gram-negative *E. coli* and *P. aeruginosa* in liquid cultures at 360 μg/mL and 480 μg/mL, respectively. These concentrations are 6-9 times higher than the maximum concentration used in our study, and much higher compared to minimum inhibitory concentrations of marketed antibiotics [32], indicating a fairly weak activity against Gram-negative bacteria.

As lyso-ornithine lipids are precursors for ornithine lipids, it was possible that the extract could contain ornithine lipids. The UHPLC-HR-MS data were therefore specifically checked for the presence of such compounds, but no signals that matched the mass and elemental composition of potential ornithine lipids were detected, indicating that no ornithine lipids were produced. This could be due to the growth conditions used in this study, as the membrane lipid composition can be changed as part of the regulation of membrane fluidity. The amount of iso-branched lipids and lipoamino acids in the membrane is affected by temperature and cultivation conditions [33–36]. Some bacteria produce lipoamino acids only under limiting phosphate conditions, while others produce them regularly [37–40].

In the present study we found that lyso-ornithine lipids have some antibacterial and cytotoxic activities. Previous bioactivity studies of lyso-ornithine lipids are limited. In addition to the mentioned study of Tahara et al. (1977), they include a study by Williams et al. (2019) [41], where a lyso-ornithine lipid with good surface activity was described. Surface activity is a feature possessed by surfactants, which are compounds with amphiphilic nature. Biosurfactants (surfactants produced by microorganisms) have the potential to replace chemical surfactants within industrial applications such as remediation of heavy metal and hydrocarbon-contaminated sites, soil washing technology and in cosmetics. In addition, they are known to have various bioactivity properties. These properties include cytotoxicity and antibacterial activity, and are due to their interaction with membranes of target cells, affecting the integrity and stability of the membranes [42–45]. From this, it is likely that the activity of **1** and **2** is a result of the two compounds interacting with the membranes of the bacteria and the human melanoma cells.

The approach used in this study, investigating underexplored Artic marine bacteria for the production of novel compounds resulted in the characterization of two compounds not described before, showing the potential of Arctic marine bacteria as a source for novel compounds. Bioassay-guided isolation was used to identify the two compounds, as the selection of fractions for further analysis was based on the observed activity in the bioassays. The use of phenotypic bioassays resulted in the isolation of two active compounds with unspecific mode of action. The activity places them outside the potency level needed to be considered relevant for further development toward becoming commercially available pharmaceuticals. Despite of being widely studied, with a few exceptions, the use of biosurfactants within the pharmaceutical industry is today limited. Regarding replacing biosurfactants with chemical surfactants, biosurfactants are today used in cosmetics and in food, but in other industrial applications such as bioremediation and antifouling, the

research is still at laboratorial level [46,47]. However, as the research continues, that may change one day.

4. Materials and Methods

4.1. Sampling and Identification of Lacinutrix sp.

The strain was isolated from a *Halichondria* sp. sponge in the Barents Sea at 74°22′12″ N and 19°11′54.2652 E, in January 2009. Glycerol stocks of the bacterium were prepared and provided by Marbank. The bacterial glycerol was plated onto FMAP agar (15 g Difco Marine Broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA), 5 g peptone from casein, enzymatic digest (Sigma, St. Louis, MS, USA), 15 g/L agar, 700 mL ddH_2O, and 300 mL filtrated sea water), and incubated at 10 °C until sufficient growth. The characterization of the bacterial strains was done by sequencing of the 16S rRNA gene through colony PCR and Sanger sequencing as described previously [48]. The primer set used for gene amplification was the 27F primer (forward primer; 5′-AGAGTTTGATCMTGGCTCAG) and the 1429R primer (reverse primer; 5′-TACCTTGTTACGACTT), both from Sigma. The PCR product was sequenced at the University Hospital of North Norway (Tromsø, Norway). The forward and reverse sequences obtained were assembled using the Geneious Prime® 2021.0.3 software (https://www.geneious.com/) (accessed on 2 July 2021), with the built-in Geneious assembler (sequences trimmed using a 0.05 error probability limit). The *Lacinutrix* M09B143 16S rRNA sequence was deposited in Genbank with the following accession number MZ414169. Reference sequences for the phylogenetic analysis were obtained from Genbank and were selected among top BLAST results of the M09B143 sequence and from recent phylogenetic studies on *Lacinutrix* sp. strains (Supplementary Table S1). The multiple sequence alignment of 23 sequences (including the outgroup *Flavivirga jejuensis*) was conducted using the multiple sequence alignment plug-in Clustal Omega 1.2.2 [49] in Geneious, using the default settings. The alignment was manually adjusted, resulting in a final alignment of 1413 bp length.

Phylogenetic analysis was conducted using the online version of PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) (accessed on 2 July 2021) [50], and Smart Model Selection [51] was used to select the appropriate substitution model, using the Akaike Information Criterion (AIC) as selection criterion and aBayes for branch support. This suggested the following model to be most appropriate for the dataset: GTR + G + I. The tree was rooted with *F. jejuensis*, branch support is given as aLRT (approximate likelihood ratio test) values. In addition, a phylogenetic analysis was conducted on the same alignment, using the MrBayes 3.2.6 [52] plug-in in Geneious. The analysis was run with the GTR substitution model and rate variation gamma, chain length 1,100,000, subsampling frequency 200 and burn-in length 550,000. The resulting consensus tree was built using default settings.

4.2. Fermentation

The M09B143 *Lacinutrix* strain was cultivated in 250 and 400 mL M19 medium in 1 L Erlenmeyer flasks at 10 °C with 140 rpm shaking for 2–3 week until sufficient growth. M19 medium was prepared of 1 L Milli-Q water (Merck Millipore), 20 g D-Mannitol (63560), 20 g Peptone (82303) and 20 g Sea Salt (S9883), all from Sigma-Aldrich. Diaion® HP-20 resin beads (13607, Supelco Analytica) activated in methanol (34860, Sigma-Aldrich) for 20 min and washed with Milli-Q water were added to the cultures to extract compounds secreted into the medium. After 3–4 days the resin was separated from the cultures by filtrating the cultures under vacuum using a mesh cheesecloth (1057, Dansk Hjemmeproduktion, Ejstrupholm, Danmark). Resin collected on the cheesecloth were washed with 100 mL Milli-Q water and compounds adsorbed to the resin was eluted with methanol. The elution was done twice at 140 rpm for 1 h in 150 mL methanol per 40 g resin. The extract was vacuum filtered through Whatman Ø 90 mm No. 3 filter (Whatman plc), dried under reduced pressure at 40 °C and stored at −20 °C.

4.3. Flash Fractionation, Bioactivity Testing of Flash Fractions, and Dereplication

Extract of M09B143 was dissolved in 90% methanol before Diaion® HP20 resin was added and the sample was dried under pressure at 40 °C. For each sample, 2 g of extract, 2 g of resin and 8 mL methanol were used. Flash column (Biotage® SNAP Ultra, Biotage, Uppsala, Sweden) was prepared with 6.5 g resin activated in methanol for 20 min before rinsing with Milli-Q water. The resin was loaded in the column and equilibrated with 5% methanol before the extract sample was loaded on top of the column. Fractionation was performed with a Biotage SP4™ system using first a step-wise gradient from 5–100% methanol over 36 min (the steps were 5, 25, 50 and 75% methanol, 6 min each, and 100% methanol for 12 min). Then a gradient with methanol:acetone (34850, Sigma-Aldrich) for 4 min and 100% acetone for 12 min was used. The flow rate was 12 mL/min, resulting in 27 sub fractions with 24 mL in each tube. Sub fraction 1–3, 4–6, 7–9, 10–12, 13–15 and 16–27 were pooled together to a total of six flash fractions and dried under pressure at 40 °C.

4.4. Dereplication

The samples were analyzed with ESI+ and ESI- ionization mode on a UPLC-QToF-MS for dereplication. The system (all from Waters) consisted of an Acquity UPLC I-class coupled to a PDA detector and a Vion IMS QToF. An Acquity C18 UPLC column (1.7 µm, 2.1 mm × 100 mm) was used for the separation. Milli-Q water was used for mobile phase A and acetonitrile (HiPerSolv, VWR) for mobile phase B, both containing 0.1% formic acid (*v*/*v*) (33015, Sigma). A 12-min gradient increasing from 10% to 90% acetonitrile with flow rate 0.45 mL/min was used. UNIFI 1.9 (Waters) was used to process the data.

*4.5. Purification of **1** and **2***

The compounds were purified in two different isolation rounds.

4.5.1. Purification Round One

A preparative HPLC-system (Waters) with a 600 HPLC pump, a 2996 photo diode array detector, a 3100 mass spectrometer and a 2767 sample manager was used to isolate the two compounds. MassLynx version 4.1 was used to control the system. The mobile phases consisted of A; Milli-Q water and B; acetonitrile (Prepsolv®, Merck), both containing 0.1% formic acid (*v*/*v*), and flow rate was set to 6 mL/min. Atlantis Prep dC18 column (10 µm, 10 mm × 250 mm) (Waters) was used for the initial separation of the two compounds with gradient 10–88% acetonitrile over 13 min. XSelect CSH Prep Fluoro-Phenyl column (5 µm, 10 mm × 250 mm) (Waters) was used for final purification of **1**, gradient 10–76% acetonitrile over 10 min. For the final purification of **2**, XSelect CSH Phenyl-Hexyl prep column (5 µm, 10 mm × 250 mm) (Waters) was used with gradient 10–54% acetonitrile over 11 min.

4.5.2. Purification Round Two

The initial purification of the compounds in the second round was performed with the same preparative HPLC-system described in the previous section, and the same mobile phases and flow rate. A SunFire C18 OBD column (5 µm, 10 mm × 250 mm) with gradient 50–85% acetonitrile over 10 min was used. A second purification step was performed with **2** on a preparative HPLC-system consisting of Acquity Arc Sample Manager FTN-R, Acquity Arc Quaternary Solvent Manager-R, Acquity Arc Column manager, Acquity QDa Detector and Photodiode Array Detector 2998. Masslynx software was used to control the system. An Atlantis T3, C18 column (3 µm, 3 mm × 150 mm) was used. Flow rate was set to 1.5 mL/min, with gradient 35–55% acetonitrile over 12.5 min.

4.6. Antibacterial Activity

Antibacterial activity screening of the fractions and isolated compounds was performed in a growth inhibition assay against the Gram-positive bacteria *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29122), and *S. agalactiae* (ATCC 12386), and the Gram-negative bac-

teria *E. coli* (ATCC 259233) and *P. aeruginosa* (ATCC 27853). Flash fractions in the primary screening were dissolved in Milli-Q water with 1% dimethyl sulfoxide (DMSO, D4540, Sigma-Aldrich) to 1 mg/mL, further diluted with Milli-Q water and tested in duplicates at final concentration 50 μg/mL. The isolated compounds were dissolved in DMSO to 20 mM. They were further diluted in Milli-Q water and added to the wells at the final concentrations 10, 50, 100, and 150 μM. The assay was performed as previously described by Kristoffersen et al. (2018) [25]. In total, three biological experiments were performed, with three replicates in each experiment.

4.7. Cytotoxic Activity Assay

The cytotoxicity of the fractions in the preliminary screening and of 1 and 2 was tested in an MTS in vitro cell proliferation assay. The fractions and compounds were tested against human melanoma A2058 cells (ATCC, CRL-1147TM). The isolated compounds were in addition tested against normal lung fibroblasts MRC-5 cells (ATCC CCL-171TM). The flash fractions were dissolved in Milli-Q water with 1% DMSO to 1 mg/mL and further diluted in Roswell Park Memorial Institute cell media (FG1383, Merck) with 10% fetal bovine serum (S0115, Biochrom) and tested at 50 μg/mL in three replicates.

Compounds **1** and **2** were dissolved in DMSO to 20 mM, and further diluted in Roswell Park Memorial Institute cell media with 10% fetal bovine serum and tested at the concentrations 10, 25, 50, 100 and 150 μM. One biological experiment with three replicates (test concentration 25 μM was not used here), and two biological experiments with four replicates each were performed. The bioassay was performed as previously described by Kristoffersen et al. (2018) [25].

4.8. NMR Spectroscopy

The structures of **1** and **2** were established by 1D and 2D NMR experiments. NMR spectra were acquired in methanol-d_3 (CD_3OH) and 298 K in a 3 mm shigemi tube on a Bruker Avance III HD spectrometer operating at 600 MHz for protons, equipped with an inverse TCI cryo-probe enhanced for ^{1}H, ^{13}C, and ^{2}H.

5. Conclusions

Lacinutrix sp. was evaluated for its production of bioactive molecules. This resulted in the isolation and characterizing of two novel lyso-ornithine lipids. The bioactive profiling revealed that **1** had some antibacterial activity against the Gram-positive bacterium *S. agalactiae*, with minimum inhibitory concentration between 100 and 150 μM, and that **2** had moderate cytotoxic activity against human melanoma A2058 cells with 23% cell survival at 50 μM, and ~0% cell survival at 100 μM. The length of their lipid chain seemed to affect their activity as (considering the 2-dimentional structure of the two compounds) they only differed with one methylene group in the lipid chain, but showed activity in different bioassays. Should the two compounds be more potent in other bioassays, further studies to determine the structure of the isomers can be performed. This is to our knowledge the first time bioactive molecules have been reported from *Lacinutrix* sp., and the first data describing lyso-ornithine lipids with cytotoxic activity, and with antibacterial activity against Gram-positive bacteria. This shows that exploration of the secondary metabolite content of underexplored bacteria is a viable strategy to discover novel molecules.

Supplementary Materials: The following are available online, Figure S1: Bayesian Inference tree based on 16S rRNA gene sequence similarity. Figure S2: Fractions 1-6 tested against Gram-negative bacteria and human melanoma A2058 cells. Figure S3: UHPLC-HR-MS base peak intensity chromatogram of flash fraction 5. Figure S4: Extracted UHPLC-HR-MS mass chromatogram of **1** and **2.** Figure S5: HSQC + HMBC (600 MHz, CD_3OH) spectrum of **1.** Figure S6: HSQC-TOCSY (600 MHz, CD_3OH) spectrum of **1**. Figure S7: ^{1}H NMR (600 MHz, CD_3OH) spectrum of **1**. Figure S8: ^{13}C (151 MHz, CD_3OH) spectrum of **1**. Figure S9: ^{1}H NMR (600 MHz, CD_3OH) spectrum of **2**. Figure S10: ^{13}C (151 MHz, CD_3OH) spectrum of **2**. Figure S11: HSQC + HMBC (600 MHz, CD_3OH) spectrum of **2**. Figure S12: HSQC-TOCSY (600 MHz, CD_3OH) spectrum of **2**. Figure S13: COSY (600 MHz, CD_3OH)

spectrum of **2**. Figure S14: Antibacterial activity of **1** and **2** against Gram-positive bacteria. Figure S15: Antibacterial activity of **1** and **2** against Gram-negative bacteria. Figure S16: Cytotoxic activity of **1** and **2** against non-malignant lung fibroblast cell line MRC-5. Table S1: 16S rRNA sequences used in the phylogenetic analysis of *Lacinutrix* M09B143.

Author Contributions: Conceptualization, V.K., J.H.A., E.H.H. and T.R.; methodology, V.K. and M.J.; validation, V.K., M.J., J.H.A., K.Ø.H. and E.H.H.; formal analysis and investigation, J.I., K.Ø.H., M.J., V.K. and H.R.J.; resources and funding acquisition, J.H.A.; data curation and writing—original draft preparation, and visualization V.K., M.J. and K.Ø.H.; writing—review and editing and supervision, J.H.A. and E.H.H.; project administration, V.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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Supplementary Materials

Two Novel Lyso-Ornithine Lipids Isolated from an Arctic Marine *Lacinutrix* sp. Bacterium

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Table S1. The names, accession numbers and lengths (bp) of all 16S rRNA sequences used in the phylogenetic analysis of *Lacinutrix* M09B143. All sequences were acquired from Genbank.

Name/Information	Acc.nr	Length (bp)
Algibacter agarivorans strain KYW560 16S ribosomal RNA gene	JN864025	1452
Algibacter miyuki strain WS-MY6 from South Korea 16S ribosomal RNA gene	KC662118	1441
Algibacter pectinivorans strain JC2675 from South Korea 16S ribosomal RNA gene	HM475134	1442
Algibacter psychrophilus strain PAMC 27237 16S ribosomal RNA gene	KJ475138	1510
Algibacter wandonensis 16S ribosomal RNA gene	KC987358	1443
Flavirhabdus (now *Lacinutrix*) *iliipiscaria* strain Th68 16S ribosomal RNA gene	JX412960	1486
Flavivirga jejuensis strain JC2682 from South Korea 16S ribosomal RNA gene, partial sequence (outgroup)	HM475139	1439
Lacinutrix (*chionocetis*) sp. MAB-07 16S ribosomal RNA gene	KT272396	1421
Lacinutrix (*cladophorae*) sp. 7Alg 4 16S ribosomal RNA gene	KU510085	1478
Lacinutrix algicola strain AKS293 16S ribosomal RNA	NR_043592	1496
Lacinutrix copepodicola strain DJ3 16S ribosomal RNA gene	AY694001	1364
Lacinutrix gracilariae strain Lxc1 16S ribosomal RNA	NR_148656	1444
Lacinutrix himadriensis strain E4-9a 16S ribosomal RNA	NR_108471	1488
Lacinutrix jangbogonensis strain PAMC 27137 16S ribosomal RNA	NR_134754	1443
Lacinutrix mariniflava strain AKS432 16S ribosomal RNA	NR_043593	1454
Lacinutrix salivirga gene for 16S ribosomal RNA	LC339518	1460
Lacinutrix sp. strain M09B143 16S ribosomal RNA gene	Must submit	Must submit
Lacinutrix undariae strain W-BA8 16S ribosomal RNA gene	KP309835	1442
Lacinutrix venerupis strain Cmf 20.8 16S ribosomal RNA	NR_145942	1337
Mesoflavibacter aestuarii strain KYW614 16S ribosomal RNA gene	JX854528	1443
Mesoflavibacter sabulilitoris strain GJMS-9 16S ribosomal RNA gene	KJ816860	1446
Olleya aquimaris strain L-4 16S ribosomal RNA gene	FJ886713	1443
Olleya namhaensis strain WT-MY15 16S ribosomal RNA gene	JQ327134	1441

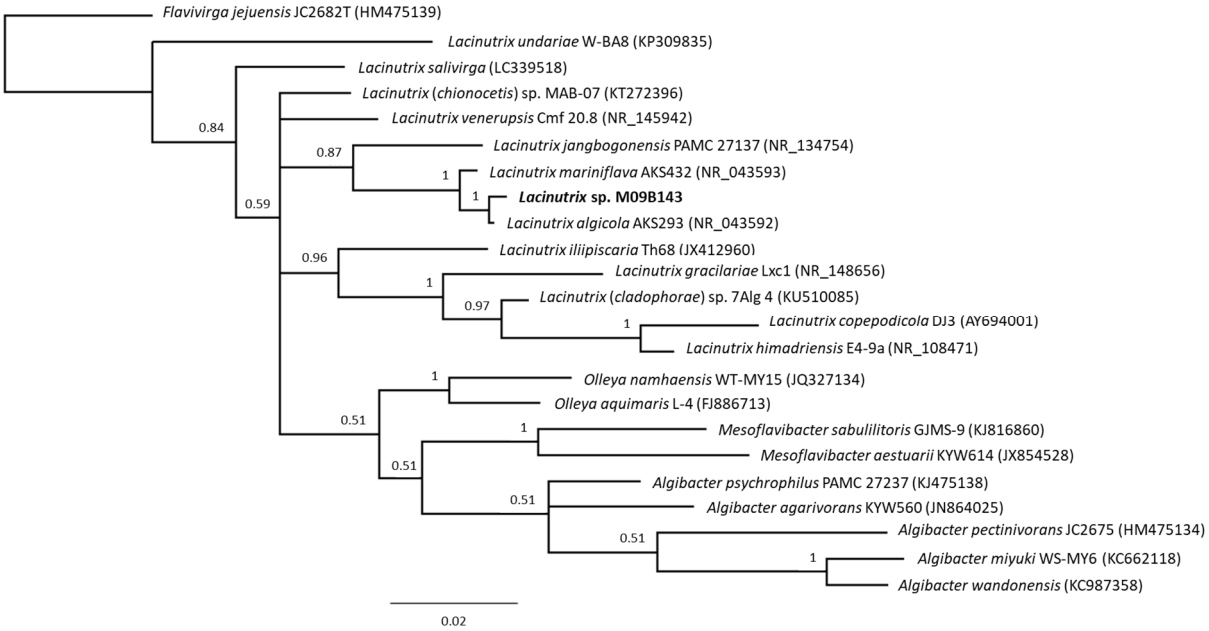

Figure S1. Bayesian Inference tree based on 16S rRNA gene sequence similarity and showing the phylogenetic placement of the isolate M09B143 (in bold) within Bacteroidetes. The tree was rooted with *Flavivirga jejuensis* as the outgroup. Branch support is given Bayesian posterior probability.

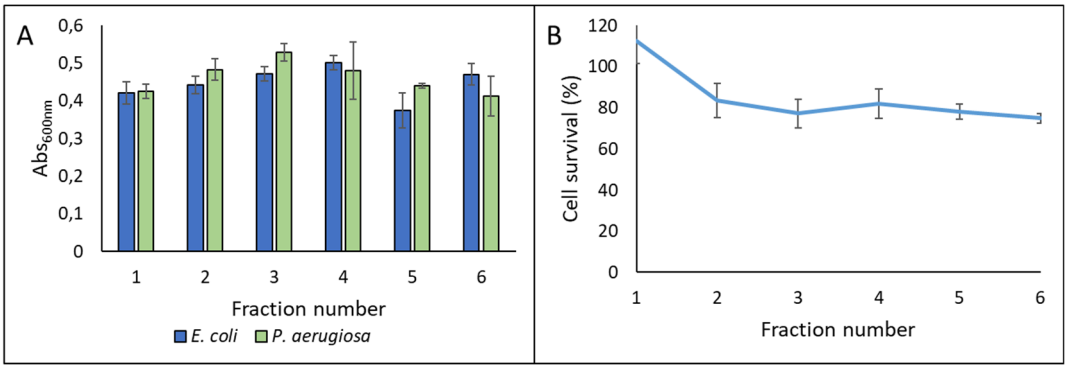

Figure S2. Fractions 1-6 showed no activity against the Gram-negative bacteria *E. coli* and *P. aeruginosa* in the growth inhibition assay, results shown in **A**. There was visible growth in all wells, and the OD values were 0.37 or higher. In comparison, the OD value of fraction 5 was 0.05 when it was active against *S. agalactiae* (Figure 2). The assay was performed in duplicates. Fractions 1-6 showed no activity against human melanoma A2058 cells, results shown in **B**. Cell survival was 75 % or higher for the fractions. The assay was performed with three technical replicates.

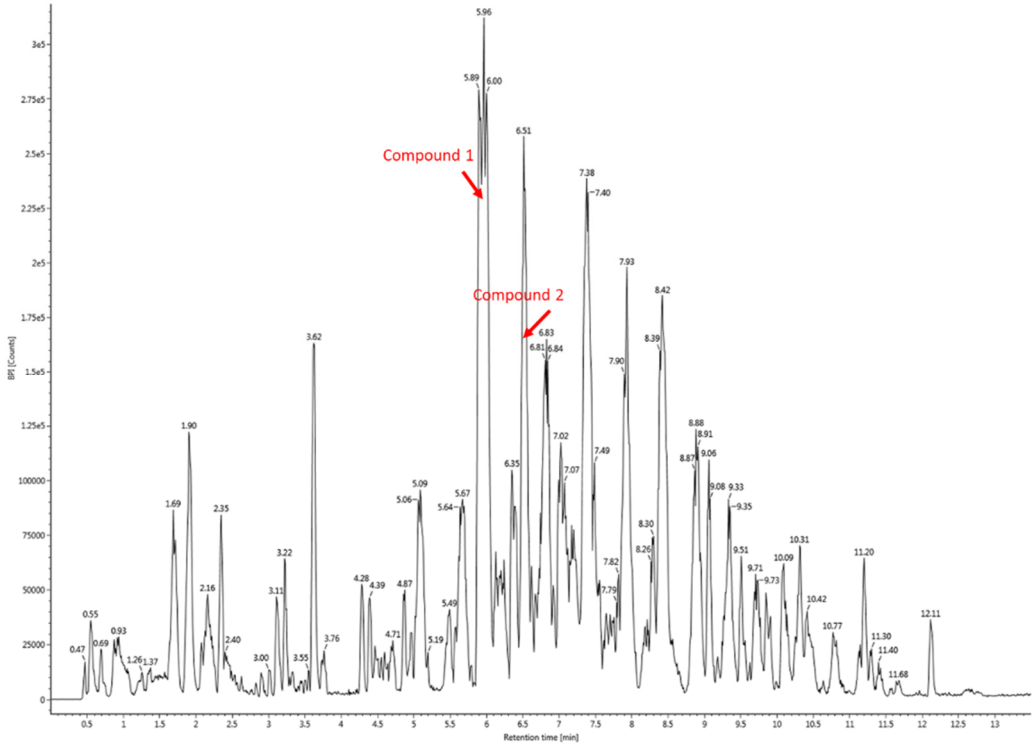

Figure S3. UHPLC-HR-MS base peak intensity chromatogram of fraction 5 of *Lacinutrix* sp., where **1** is the major peak, and **2** among the highest peaks.

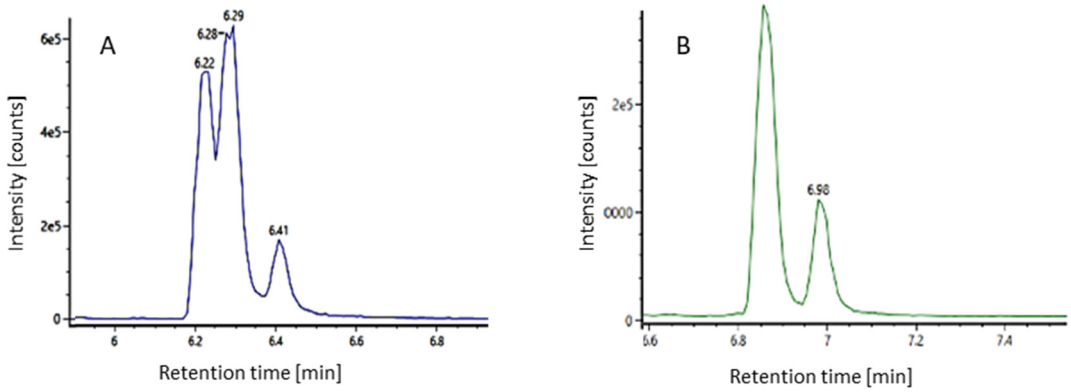

Figure S4. Extracted UHPLC-HR-MS mass chromatogram of **1** shown in **A**, and **2** shown in **B**. Possible isomers were observed, as **1** eluted in three peaks and **2** eluted in two peaks.

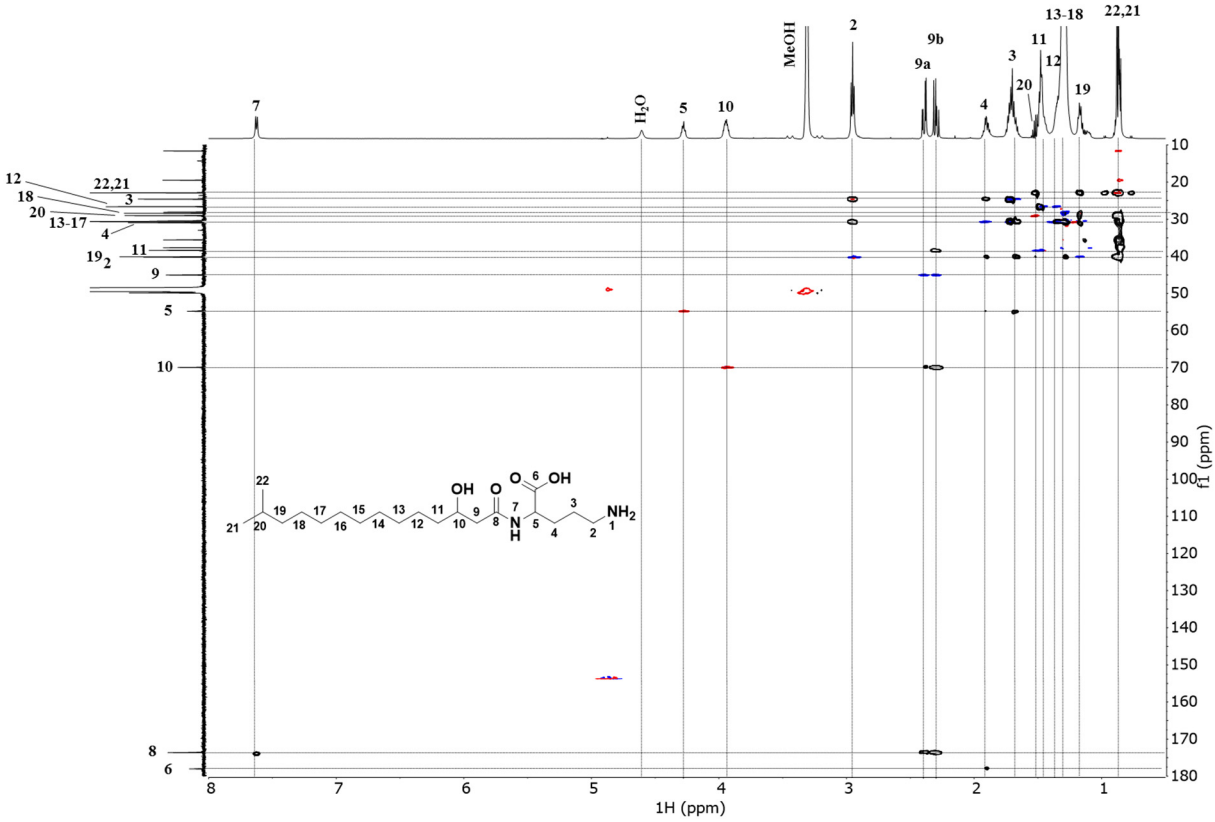

Figure S5. HSQC + HMBC (600 MHz, CD_3OH) spectrum of **1**.

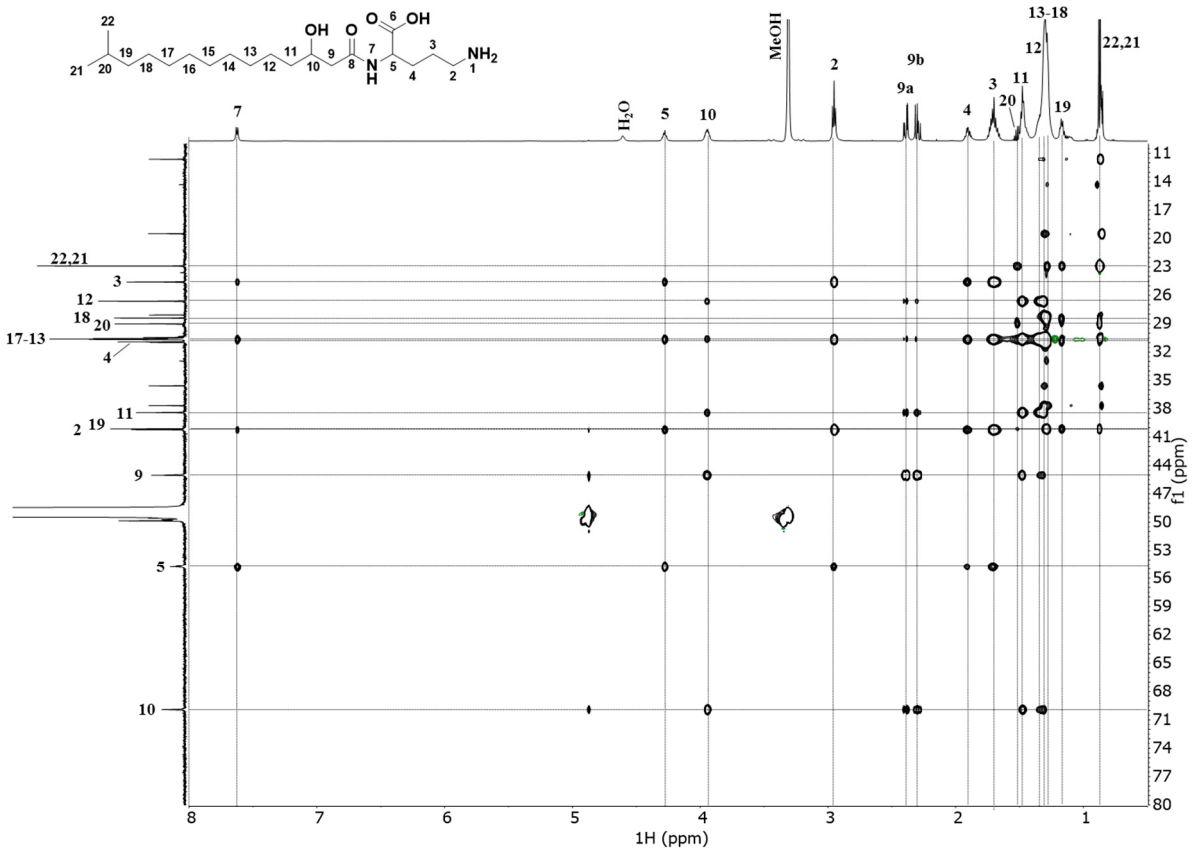

Figure S6. HSQC-TOCSY (600 MHz, CD_3OH) spectrum of **1**.

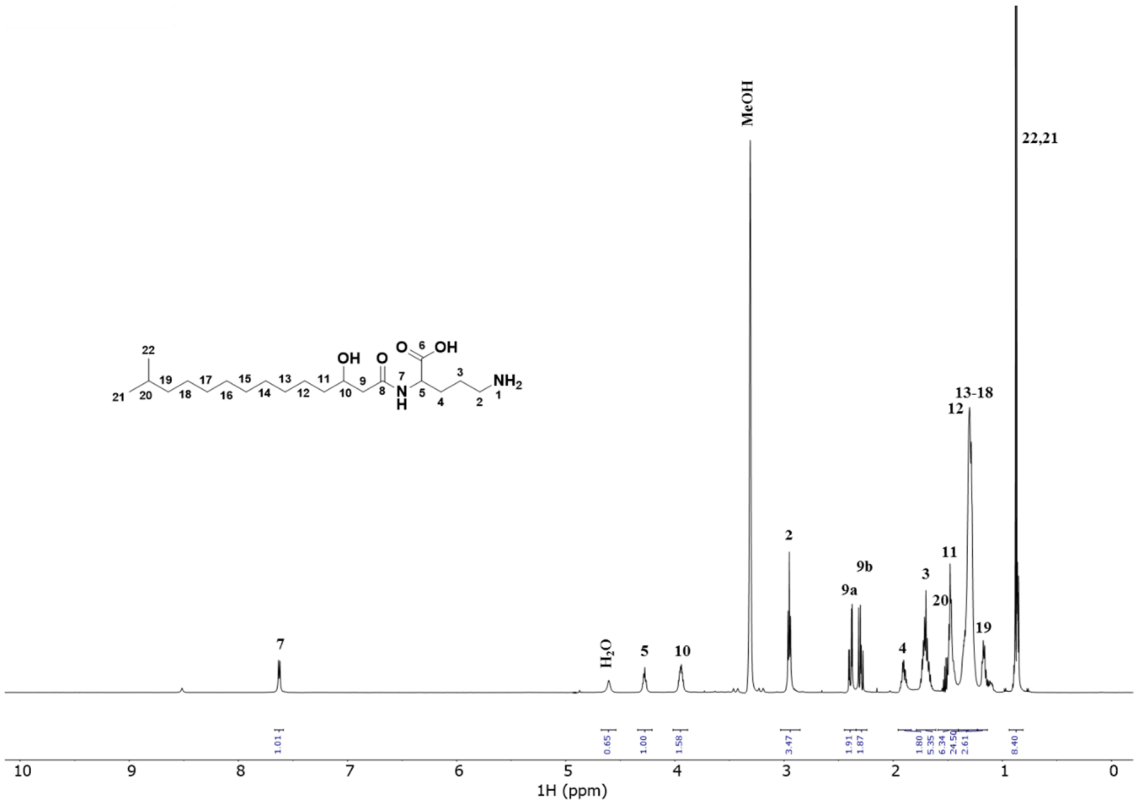

Figure S7. ^{1}H NMR (600 MHz, CD_3OH) spectrum of **1**.

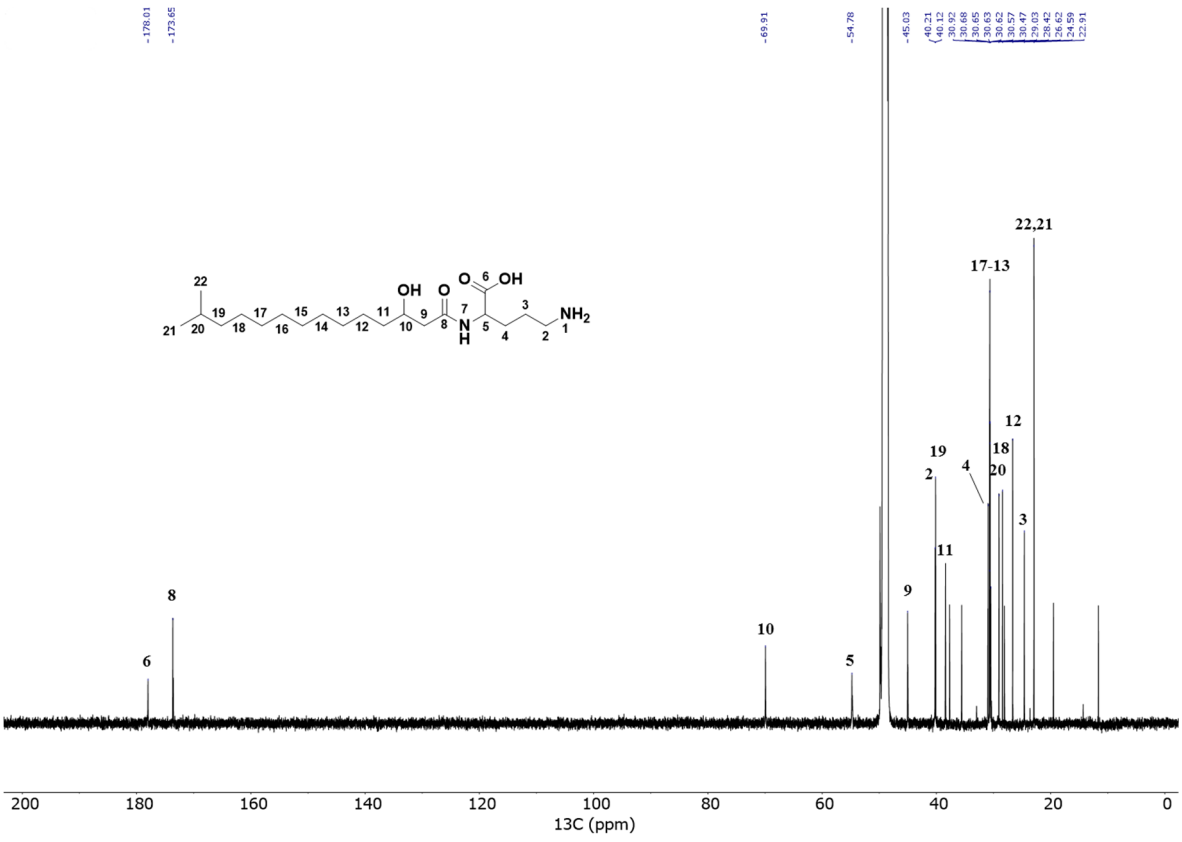

Figure S8. ^{13}C (151 MHz, CD_3OH) spectrum of **1**.

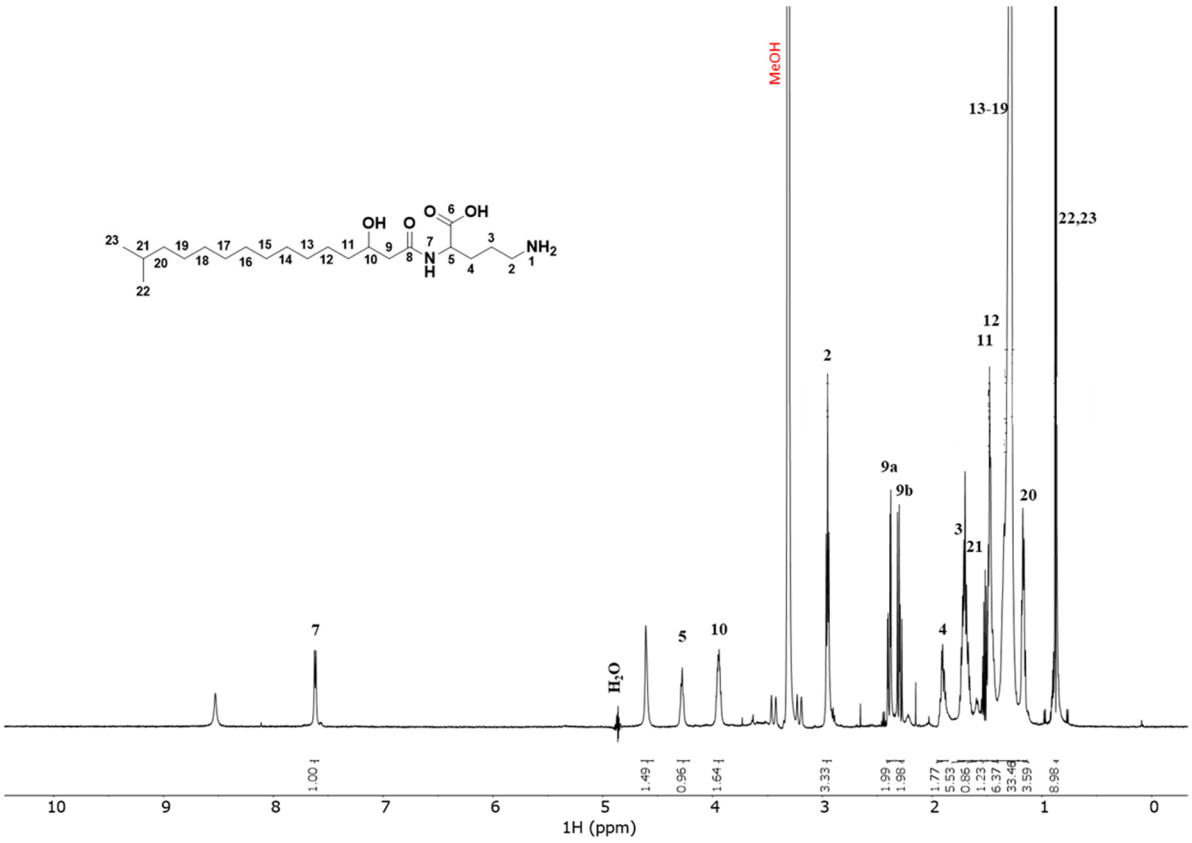

Figure S9. ^{1}H NMR (600 MHz, CD_3OH) spectrum of **2**.

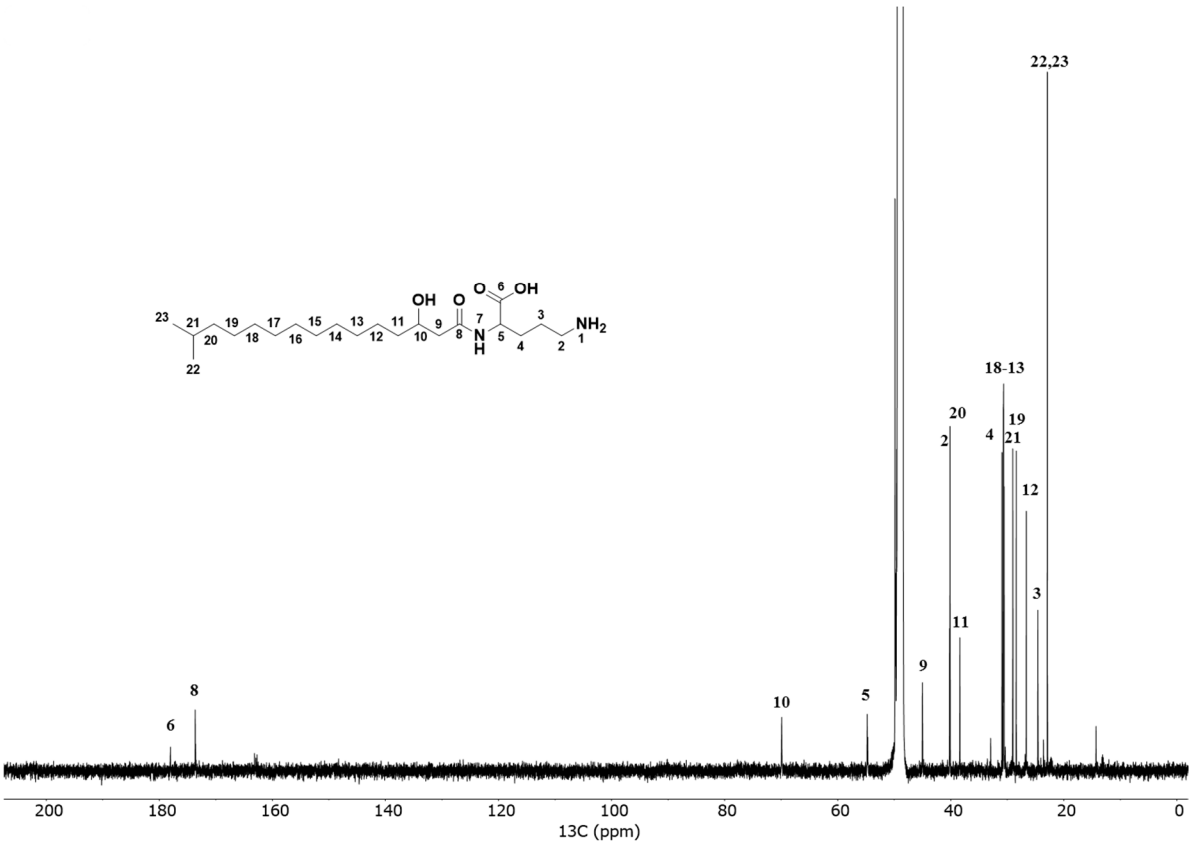

Figure S10. ^{13}C (151 MHz, CD_3OH) spectrum of **2**.

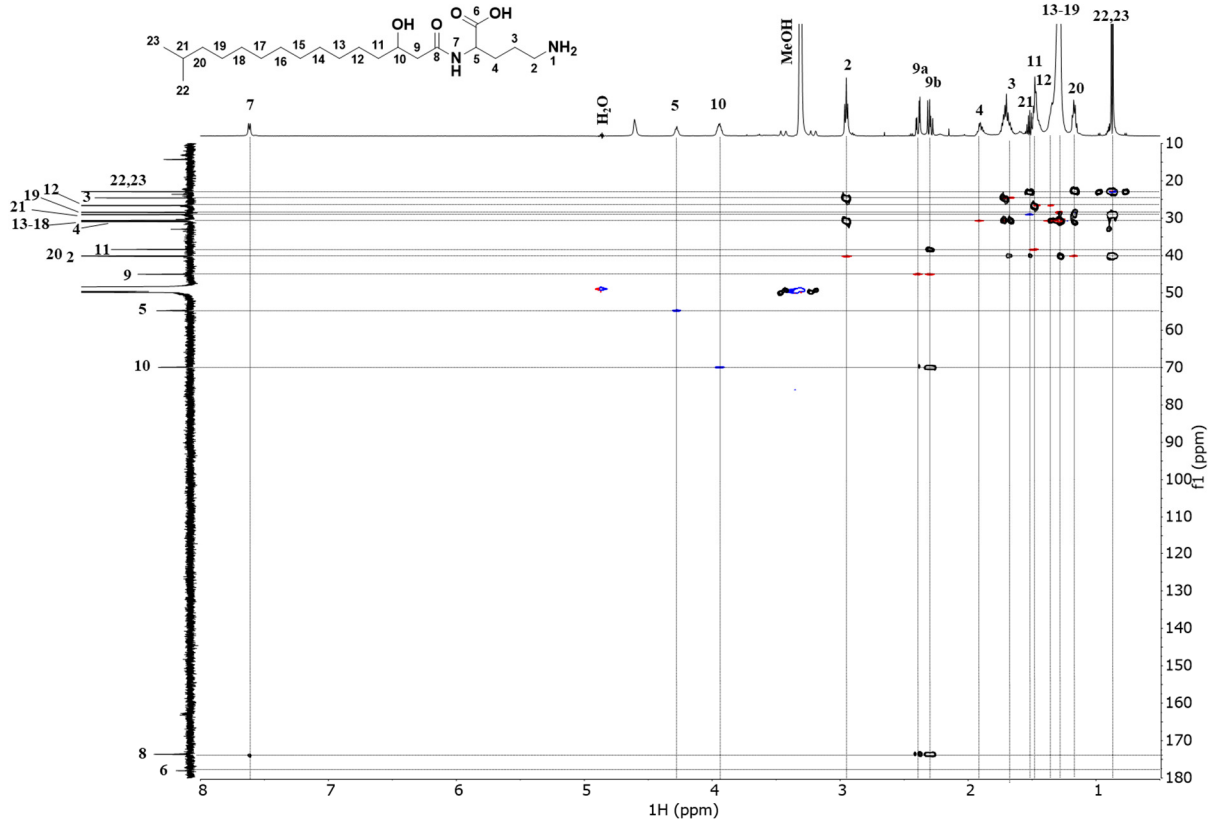

Figure S11. HSQC + HMBC (600 MHz, CD_3OH) spectrum of **2**.

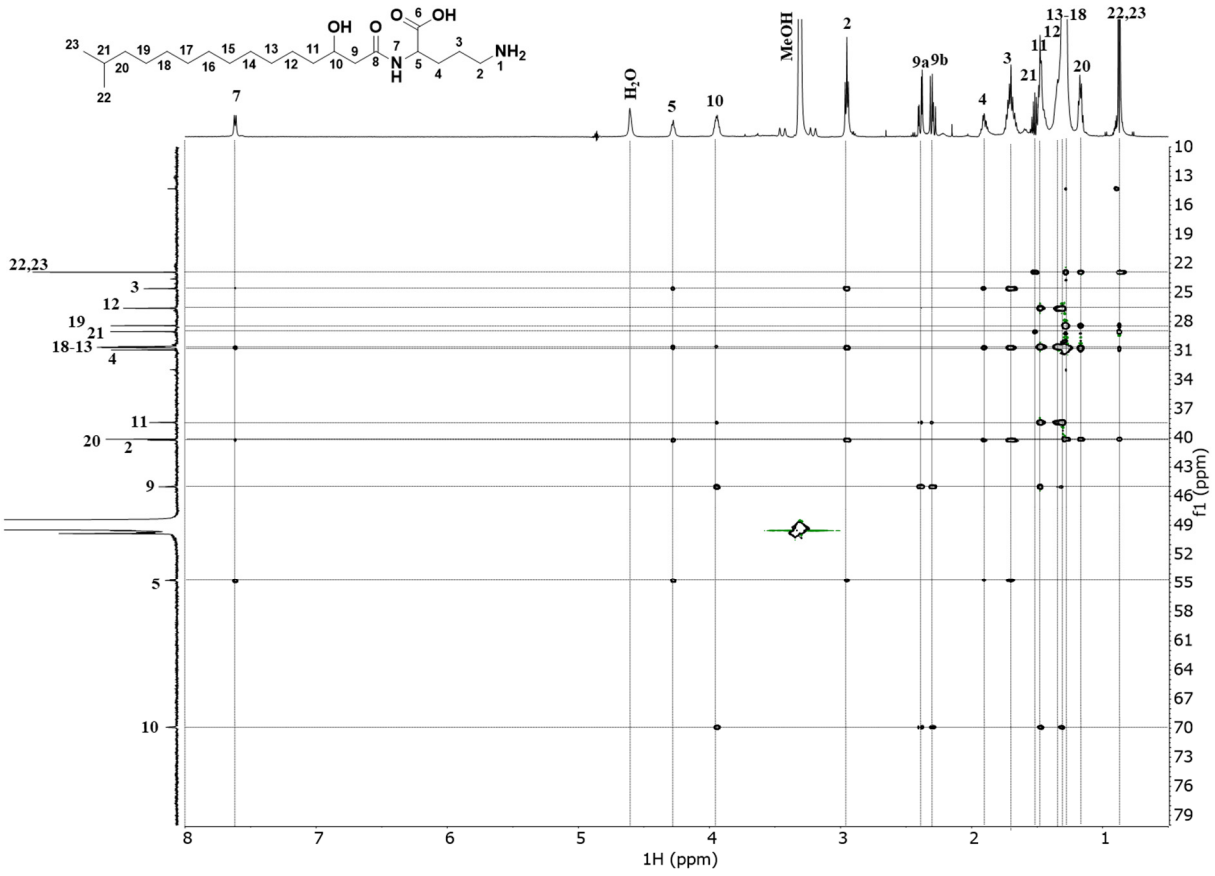

Figure S12. HSQC-TOCSY (600 MHz, CD_3OH) spectrum of **2**.

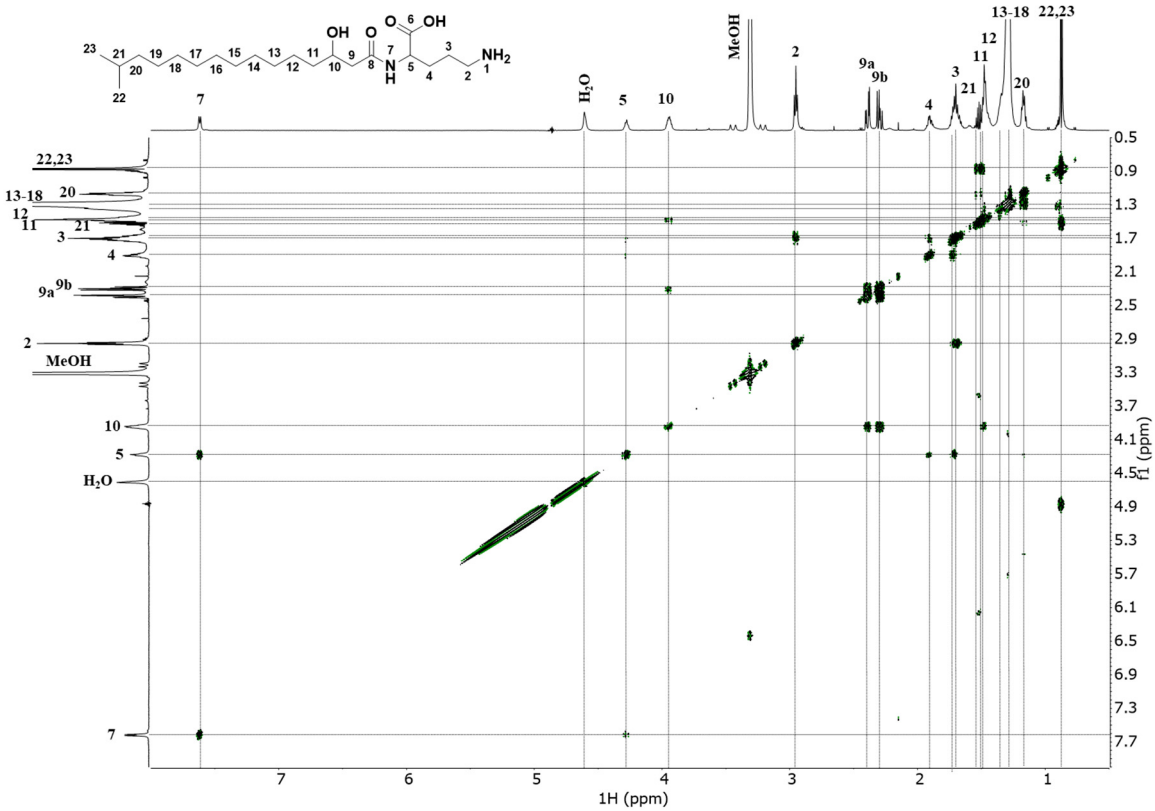

Figure S13. COSY (600 MHz, CD_3OH) spectrum of **2.**

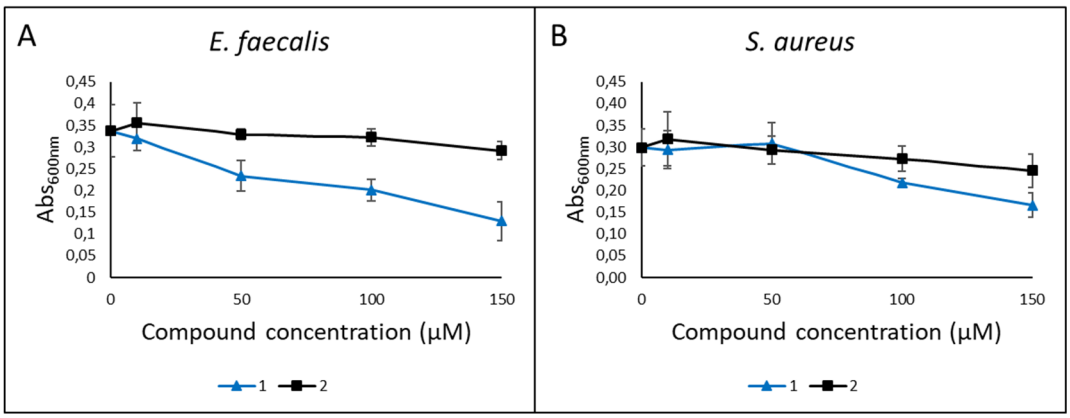

Figure S14. Antibacterial activity of **1** and **2** was tested in a growth inhibition assay. The results are shown for the Gram-positive bacteria *E. faecalis* in A and *S. aureus* in B. The assay was performed in three biological experiments with three technical replicates each.

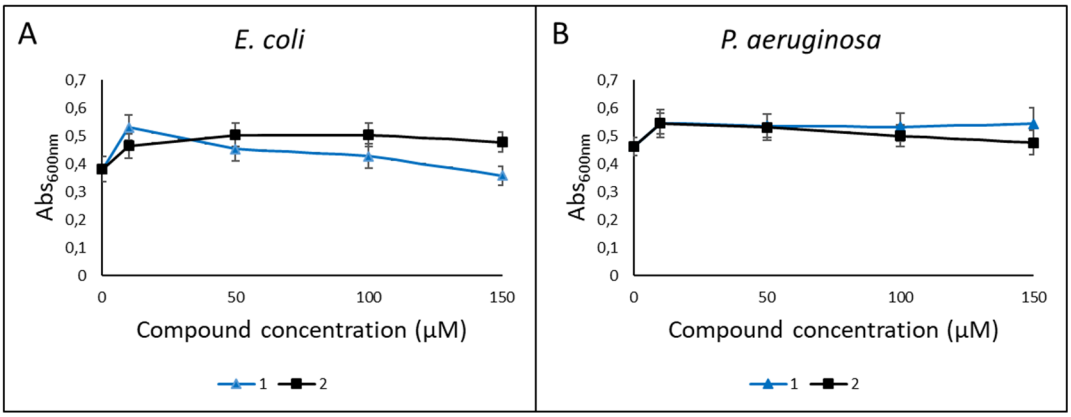

Figure S15. Antibacterial activity of **1** and **2** was tested in a growth inhibition assay. The results are shown for the Gram-negative bacteria *E. coli* in A and *P. aeruginosa* in B. The assay was performed in three biological experiments with three technical replicates each.

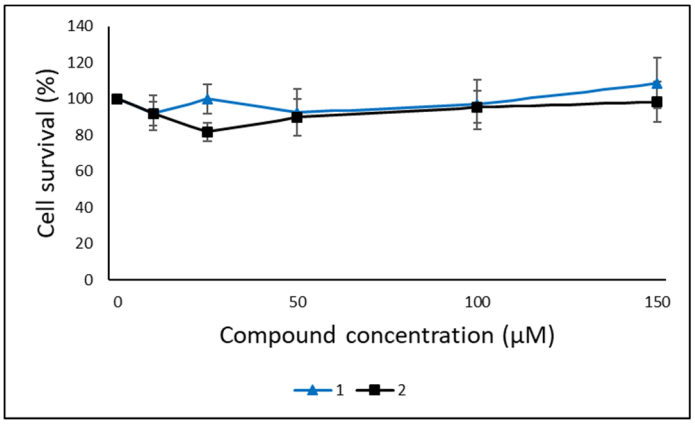

Figure S16. Cytotoxic activity of **1** and **2** was tested against non-malignant lung fibroblasts MRC-5 cells in a viability assay at 10, 25, 50, 100 and 150µM. Three experiments were conducted, one with three replicates (test concentration 25 µM was not used in this setup) and two with four replicates.

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Chlovalicin B, a Chlorinated Sesquiterpene Isolated from the Arctic Marine Mushroom *Digitatispora marina*

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Abstract: As part of our search for bioactive metabolites from understudied marine microorganisms, the new chlorinated metabolite chlovalicin B (**1**) was isolated from liquid cultures of the Arctic marine basidiomycete *Digitatispora marina*, collected and isolated from driftwood found at Vannøya, Norway. The structure of the novel compound was elucidated by spectroscopic methods including 1D and 2D NMR and analysis of HRMS data, revealing that **1** shares its molecular scaffold with a previously isolated compound, chlovalicin. The compound was evaluated for its antibacterial activities against a panel of five bacteria, ability to inhibit bacterial biofilm formation, antifungal activity against *Candida albicans* and for cytotoxic activities against malignant and non-malignant human cell lines. Compound **1** displayed weak cytotoxic activity against the human melanoma cell line A2058 (~50% survival at 50 μM), otherwise no activity was detected. This is the first reported isolated compound from the marine mushroom *sensu stricto D. marina*.

Keywords: *Digitatispora marina*; marine fungus *sensu stricto*; Basidiomycota; bioprospecting; chlorinated secondary metabolite; natural products

1. Introduction

Fungi isolated from the marine environment have proven to be a promising source of novel bioactive compounds [1]. Still, marine derived fungi are under-explored compared to their terrestrial counterparts [1,2], and the studies of marine fungi have mainly been focused on just a few genera, that is; *Penicillium, Aspergillus*, and in part *Fusarium* and *Cladosporium [1]*. The *Digitatispora* genus was first described by Gaston Doguet in 1962. It consists of two spesies: *Digitatispora marina* Douget and *Digitatispora lignicola* E.B.G. Jones, which both grow on and decay marine-submerged wood. The genus, which is part of the Atheliaceae family and Basidiomycota phylum, has been included in a number of phylogenetic studies, and has been placed in different orders and families. In a recent study by Sulistyo and co-authors, *Digitatispora* was placed in the order Agaricales with strong support both from bootstrap and posterior probability (BS/PP = 98/1.00) [3]. In Index Fungorum, *Digitatispora marina* is placed in the family Niaceae (order Agaricales, accessed March 2021), while in MycoBank *Digitatispora* is systematically placed in Atheliaceae (order Atheliales, accessed March 2021).

In a survey from 2014, Rämä and co-workers identified 28 different species of marine fungi, out of which *Digitatispora marina* was the only basidiomycete [4]. Tibell and co-authors (2020) performed a survey on marine fungi from the Baltic Sea, revealing that only two of the 77 recorded species belonged in Basidiomycota, one of which was *Digitatispora marina* [5]. In 2015, only 21 of the 1,112 identified species of marine fungi were Basidiomycota, as opposed to the Ascomycota which contributed to 805 species [6], indicating that Basidiomycota are less widespread in marine habitats. The distribution of the marine fungus has been studied, but its biosynthetic potential has not yet been assessed. The current article provides new and valuable information regarding the biosynthetic potential of the marine genus *Digitatispora*, and of marine Basidiomycota in general.

As part of our ongoing search for novel bioactive metabolites from under-explored Arctic marine fungi, *Digitatispora marina* was chosen for up-scaled cultivation for the isolation of metabolites. The up-scaled culture was extracted and fractionated, and the fractions were analyzed using UHPLC-ESI-HRMS. This led to the identification of a chlorinated compound. When using the elemental composition of this compound as input in compound database searches, no likely hits were found, and the compound was therefore presumed to be novel. After compound isolation and structure elucidation, the compound was determined to be a new chlorinated chlovalicin variant, chlovalicin B (**1**). Compound **1** shares its molecular scaffold with the previously isolated compound chlovalicin [7]. The structure of **1** differs from that of chlovalicin by having the methoxy group in the C3 position of the cyclohexane ring replaced by a hydroxyl group. To the authors' knowledge, this is the first publication of a compound isolated from the genus *Digitatispora* and the first isolation of a chlovalicin variant from a basidiomycete. Herein, the cultivation of *D. marina*, extraction, isolation and structure elucidation of **1** is described along with the evaluation of its antimicrobial, cytotoxic and anti-inflammatory properties.

2. Results and Discussion

The *D. marina* fungus was isolated from driftwood of the *Betula* sp. (Figure S1) collected at Vannøya, Norway, in 2010 [4]. As part of a routine screening campaign of marine fungi, the *D. marina* isolate was cultivated under different cultivation schemes, extracted and fractionated into six fractions using RP-flash chromatography. The fractions were assayed for bioactivity and different fractions from several different cultivation schemes were bioactive (cytotoxicity and/or antibacterial). The capability of *D. marina* to produce bioactive metabolites has not been previously examined. This, coupled with the observed bioactivity in our routine screening campaign, nominated this fungus for further examination.

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A large-scale cultivation of the fungus was initiated to obtain sufficient extract amount for compound isolation. The fungus was cultivated in several rounds using a liquid malt extract medium, yielding a total of 30 L fermentation broth. This media was selected for scale-up as the fungus grew well in this media during the initial cultivation. The metabolites were harvested from the fermentation broth using Diaion® HP20 resin and extracted with methanol, resulting in 25.1 g extract. Aliquots of the fungal extract were repeatedly fractionated into six fractions using the same RP flash chromatography protocol as in the production of fractions for the routine screening mentioned above. In order to generate sufficient amounts for compound isolation of each fraction, the different flash fractions were pooled and dried together. The fractions were analyzed using UHPLC-ESI-HRMS in an attempt to identify novel compounds. In flash fractionation five (eluting at 100% methanol, yield 244.3 mg) compound **1** was identified. HRESIMS analysis revealed that the molecular ion cluster of **1** displayed the distinctive isotopic pattern of a monochlorinated compound ($[M+Na]^+$ = *m*/*z* 341.1132 and *m*/*z* 343.1103 in a 1:0.33 ratio. The molecular formula was established as $C_{15}H_{23}O_5Cl$ by UHPLC-ESI-HRMS ($[M+Na]^+$ = *m*/*z* 341.1132). The low-collision and high-collision energy mass spectra of **1** can be seen in Supplementary Figure S2. The elemental composition was used as input in various database searches (e.g. Dictionary of Natural Products and ChemSpider) yielding no plausible hits. The compound was therefore suspected to be novel, and it was nominated for isolation. The compound was isolated from the pooled flash fraction five using mass guided preparative HPLC fractionation, yielding 0.6 mg of **1**.

Compound **1** (1-(chloromethyl)-1,2,3-trihydroxy-2-(1′-methyl-2′-(5′-methylbut-4′-en)oxiran-1′-yl) cyclohexan-4-one) was isolated as a brown powder and its structure was elucidated by high resolution MS and NMR (Figure 1). The UV λmax of **1** was 221.60 nm. The *m*/*z* value of 341.1132 ($[M+Na]^{+)}$ suggested a molecular formula of $C_{15}H_{23}ClO_5$ with four degrees of unsaturation. A set of 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, HSQC, HMBC and H2BC) NMR experiments were acquired to elucidate the structure (Figures S3-S8 in the Supplementary Information).

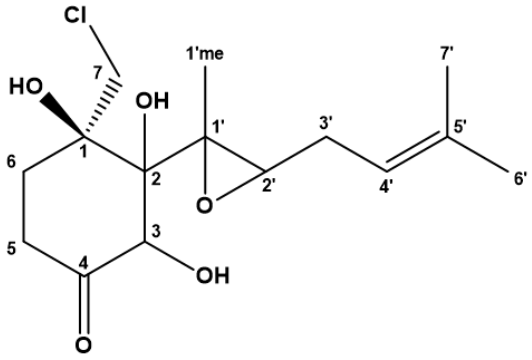

Figure 1: The structure of chlovalicin B (**1**).

The ^1H spectrum displayed all the expected 23 protons and all 15 carbons were detected in the ^{13}C spectrum. HSQC allowed the identification of three methyl- and four methylene groups, one methine proton (5.22 PPM) as well as two aliphatic CH groups with deshielded chemical shifts (4.83 and 2.79 PPM). The three remaining protons were attributed to hydroxyl protons (4.27, 4.45 and 5.79 PPM). Four quaternary carbons (75.3, 81.7, 60.4 and 133.9 PPM) and one ketone carbon remained (209.6 PPM). COSY, HMBC and H2BC displayed sufficient correlations to unambiguously connect the observed fragments into **1**. The observed correlations are summarized in Table 1 and Figure 2.

Table 1: NMR spectroscopic data[a] of chlovalicin B (1) (600 Mhz, DMSO-d_6)

Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC[b]
1	75.3, C			7a, 6a, 6b
1_{OH}		5.79, s		
2	81.7, C			3, 7a, 2', 6a, 1'Me
2_{OH}		4.27, s		
3	75.9, CH	4.83, d (7.1)	3_{OH}	5b
3_{OH}		4.45, d (8.3)		
4	209.6, C			
5a	34.4, CH_2	2.64, td (13.9, 6.9)	6a, 6b	6a, 6b
5b		2.15, ddd (14.2, 5.1, 1.4)	5b	
6a	31.5, CH_2	2.09, ddd (13.4, 6.9, 1.6)	5a, 5b, 6a	7a, 7b, 5a, 5b
6b		1.91, td (13.5, 5.3)		
7a	51.9, CH_3	3.70, d (11.0)	7	
7b		3.63, d (11.0)		
1'Me	15.8, CH_3	1.48, s		
1'	60.4, C			2', 3'b, 1'Me
2'	55.2, CH	2.79, t (6.5)	3'a, 3'b	3'a, 3'b. 6', 7', 1'Me
3'a	26.7, CH_2	2.27, dt (14.6, 7.1)	2', 4'	2', 7'
3'b		2.19, m[c]		
4'	119.2, CH	5.21, t (7.4)	3'a, 3'b	
5'	133.9, C			3'a, 3'b, 6', 7'
6'	25.5, CH_3	1.70, s		4', 7'
7'	17.8, CH_3	1.63, s		4', 6'

[a] ^{1}H ^{1}D, ^{13}C ^{1}D, ^{1}H-COSY and ^{1}H, ^{13}C-HMBC

[b] ^{1}H ^{1}D, ^{13}C-HMBC correlations are from the proton(a) stated to the indicated carbon

[c] Overlapping and/or broadened peaks impending complete analysis

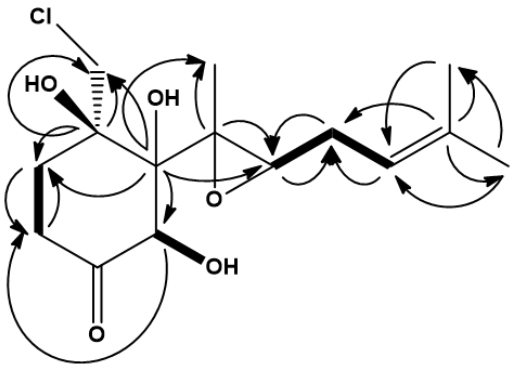

Figure 2: Selected COSY (bold) and HMBC (black arrows) correlations used to assemble the structure of chlovalicin B (**1**).

In more detail, long-range proton-carbon correlations between 6′, 7′ and 4′ plus a clear ROE between 4′ and 6′ established the methyl vinyl group. The spin system could be traced continuously through the molecule, the key correlations being the $^3J_{C2H2'}$, $^3J_{C2H1'Me}$, $^3J_{C2H2'}$ and $^3J_{C1'H3'}$ to cross the epoxide, and from there the ring system displayed all the

expected long-range correlations. The epoxide itself was indicated by both carbons being less deshielded by the oxygen (55.2 and 60.4 PPM) than had been expected for free hydroxyls (70-80 PPM). Furthermore, in epoxides you expect the one bond proton-carbon coupling to be around ~180 Hz, which is unusually high compared to the normally expected ~140 Hz for sp3 carbons next to oxygens. The $^{1}J_{CH2'}$ is estimated to ~177 Hz from the incomplete filtering of the one bond coupling in the HMBC spectra for **1**, this supporting an epoxide. The only remaining potential uncertainty was the position of the chlorine atom vis-à-vis the hydroxyl groups as the influence on the attached carbon chemical shifts are similar. The three free hydroxyls were observable (5.79, 4.45 and 4.27 PPM). The 3-OH (4.45 PPM) could be assigned to C3 through a $^{3}J_{H3HO3}$ COSY correlation, while the other two were connected to carbons not carrying any proton. The ROESY pattern is not entirely unambiguous since no conformation analysis has been conducted, but it is consistent with the assignment of 1-OH at 5.79 PPM and 2-OH at 4.27 PPM and the relative stereochemistry reported in the original chlovalicin publication [7]. ROEs are observed between H1'me and H3 as well as between 2-OH and H7b, indicating that the 2-OH and 3-OH are both on the same side of the ring, which is consistent with the absolute configuration reported for chlovalicin where both hydroxyls and the chlorinated methyl group are below the ring [8]. Furthermore, in carbon spectra that are sufficiently well resolved it can be possible to observe a small $^{37}/^{35}$Cl isotope shift of carbon resonances bound to chlorine [9]. A possible isotope shift of 1.2 Hz is indeed observed for C1 (Figure S4), but it cannot be excluded that the observed splitting is caused by slowly exchanging conformations since the line width is broadened and the shift slightly larger than the expected 0.5-0.9 Hz at 150 MHz carbon Larmor frequency. The shift is however conformation and temperature dependent, making it a viable explanation. Over all, the carbon chemical shifts of **1** are in excellent agreement with the published chemical shifts of chlovalicin [7] except for the C3, which is the carbon where **1** has a hydroxyl group instead of a methoxy group (Figure S4).

Compound **1** is structurally related to a group of compounds that have all been studied for their angiogenesis inhibiting properties, including fumagillin, ovalicin and chlovalicin [7,10-14]. In 1949, Elbe and Hanson isolated fumagillin from a culture of *Aspergillus fumigatus* [11,12], and since then, several compounds with relating chemical structures have been isolated from different fungal sources, all containing the cyclohexane ring and one or two epoxides (one epoxide when there is a chloride attached to the cyclohexane unit, as with chlovalicin and **1**). Most of these compounds have been studied for their ability to inhibit angiogenesis, and many have also been successfully synthesized [10]. Chlovalicin was isolated from the fermentation broth of a soil-derived *Sporothrix* sp. fungus, its structure elucidated and assayed for bioactivity in 1996 [7,13]. Compounds belonging to the fumagillin family have also been isolated from marine-derived fungi; chlovalicin from a marine-derived *Aspergillus niger* [15], and ligerin from a marine-derived *Penicillium* sp. [16], both containing chloride in their structures. It is not uncommon for marine organisms to incorporate halogens into their chemical structures [17], as chloride is present in large amounts both in seawater and the artificial sea salts used in the current study. Chlovalicin is similar to ovalicin, but substituted with a chlorinated methylene moiety at the C1 position of the cyclohexane ring, represented by an epoxide ring in ovalicin. In **1**, the methoxy group in the C3 position of the cyclohexane ring is replaced by a hydroxyl group. Chlovalicin was found to inhibit the growth of IL-6 dependent MH60 cells (IC_{50} = 7.5 μM) and B16 mouse melanoma cells (IC_{50} = 37 μM) [13]. Chlovalicin has also displayed inhibitory activity on osteoclastogenesis [18].

The bioactivity of **1** was broadly evaluated. The compound was tested for antibacterial activities against five bacterial strains, for the ability to inhibit biofilm formation by *S. epidermidis*, for antiproliferative activities against two human cell lines, one malignant and one non-malignant and for antifungal activity against *Candida albicans*. The compound did not show any activity against the bacterial strains at 100 μM (highest assayed concentration) or towards biofilm formation at a concentration of 50 μM (highest assayed concentration). The compound was also assessed for antifungal activity against *Candida albicans*,

and no activity was discovered at concentrations up to 100 μM (highest assayed concentration). The compound displayed weak activity against the human melanoma cell line A2058 at 50 μM (~50% cell survival, highest assayed concentration). Further testing against this cell line was not prioritized due to the high concentration needed and low quantities of isolated compound. No activity was observed against the human non-malignant lung fibroblast cell line MRC-5 at 50 μM of **1**. Chlovalicin has shown activity against a mouse melanoma cell line, B16, with IC_{50} = 37 μM [13], while displaying no or significantly weaker activity against other cell lines. This may indicate that the chlovalicins affect a common cellular target on A2058 and B16 (both melanoma cell lines).

We isolated 0.6 mg of chlovalicin B (**1**) from 30 L liquid culture of the marine fungus *D. marina*. This is the first report of isolated compounds from the *Digitatispora genus*, and the first fumagillin/ovalicin derivative isolated from a Basidiomycete. The current study adds on to the knowledge on cultivation of strictly marine fungi with the purpose of isolating novel compounds from these understudied organisms.

3. Materials and Methods

3.1. General Experimental Procedures

NMR spectra were acquired in DMSO-d_6 on a Bruker Avance III HD spectrometer (Bruker, Billerica, MA, USA) operating at 600 MHz for protons, equipped with an inverse TCI cryo probe enhanced for ^{1}H, ^{13}C, and ^{2}H. All NMR spectra were acquired at 298 K, in 3-mm solvent-matched Shigemi tubes using standard pulse programs for proton, carbon, HSQC, HMBC, COSY, and ROESY with gradient selection and adiabatic versions where applicable. $^{1}H/^{13}C$ chemical shifts were referenced to the residual solvent peak (DMSO-d_6: δ_H = 2.50, δ_C = 39.51). UHPLC-ESI-HRMS was performed using an Acquity I-class UPLC with an Acquity UPLC C18 column (1.7 μm, 2.1 mm x 100 mm), coupled to a Vion IMS QToF and a PDA detector (all from Waters). ESI+ ionization was used. The gradient extended over 12 minutes, increasing from 10% to 90% acetonitrile (LiChrosolv®, 1.00029, Supelco) with 0.1% formic acid (33015, Sigma-Aldrich) in Milli-Q® H_2O, with a flow rate of 0.45 mL/min. Waters UNIFI 1.8.2 Scientific Information System was used to process and analyze the data. The preparative-HPLC system consisted of a 600 HPLC pump, a 3100 mass spectrometer, a 2996 photo diode array detector and a 2767 sample manager (all from Waters). The system was controlled with MassLynx version 4.1.

3.2. Fungal Material and Cultivation Condition

The fungus was isolated from driftwood of the *Betula* sp. by Teppo Rämä, collected at Vannøya, Norway, in 2010 [4]. The fungus was identified as *Digitatispora marina* through morphological and sequencing studies by Rämä. Strain ITS sequence and LSU sequence are accessible from Genbank with the NCBI accession numbers KM272371 and KM272362, respectively. The fungus was stored in 20% glycerol solution at -80°C, as submerged pieces of agar with mycelium. It was grown and kept on malt agar with sea salts (4 g/L store bought malt extract (Moss Malt Extrakt, Jensen & Co AS), 40 g/L sea salts (S9883, Sigma-Aldrich), 15 g/L agar (A1296, Sigma-Aldrich) and Milli-Q® H_2O). Agar with fresh mycelium was used to inoculate the liquid culture, approximately ¼ to ½ agar plate per flask. For the isolation of compounds, the fungus was cultivated in liquid malt extract medium containing 4 g/L malt extract (Moss Malt Extrakt, Jensen & Co AS), 40 g/L sea salts (S9883, Sigma-Aldrich) and Milli-Q® H_2O. The fungus was cultivated over several rounds, in 250 mL media in 1000 mL culture flasks for 73-110 days at 13°C without shaking. The total volume of culture used to obtain **1** was 30 L.

3.3. Extraction and Isolation

After cultivation, the metabolites were extracted from the fermentation broth using Diaion® HP-20 resin (13607, Supelco), and extracted from the resin using methanol (20864, HPLC grade, VWR) in two rounds as described previously (Schneider et al., 2020). The cultures were incubated with the resin for 3-5 days before the extraction. The resin and fungal mycelium was separated from the liquid by vacuum filtration through a cheesecloth filter (Dansk hjemmeproduktion, Danmark). The extract was dried under reduced pressure at 40^{0}C, yielding an extract of 25.1 g. The extract was fractionated using RP flash chromatography (Biotage SP4™ system), with Diaion® HP-20SS resin as the stationary phase. The extract was dissolved in 90% methanol and fractionated (maximum 2 g extract per round of fractionation). An aliquot was combined with 2 g resin before removing the solvent under reduced pressure. The column was equilibrated using 5% methanol before the extract-column material was applied to the top of the pre-equilibrated column. The following stepwise elution method with a flow rate of 12 mL/min was used: methanol:water (5:95, 25:75, 50:50, 75:25. Six min per step) followed by methanol (100% over 12 min). The eluate was collected in six minute fractions, which subsequently were dried under reduced pressure at 40^{0}C. In preparation for the isolation of **1**, the eluent resulting in flash fraction five (samples eluting in the first six minutes of 100% methanol) from repeated rounds of flash fractionation were pooled and dried under vacuum yielding 244.3 mg sample.

Isolation of **1** from the flash fraction was performed using mass-guided preparative-HPLC. The first round of isolation of **1** was performed with a XSelect CSH Prep Fluoro-Phenyl column (5 μM, 10 mm × 250 mm, Waters) with a gradient of 10-100% acetonitrile over 15 minutes with a flow rate of 6 mL/minute. In order to remove additional impurities, a second isolation step was performed using a XSelect™ CSH™ phenyl hexyl prep column (5 μm, 10 x 250 mm, Waters), with a gradient of 10-100% acetonitrile over 15 minutes with a flow rate of 6 mL/minute, yielding 0.6 mg of **1**.

Chlovalicin B (1)

Brown powder. UV = (ACN) λmax 221.60 nm. ^{1}H and ^{13}C NMR data (see Table 1). HRMS *m/z* 341.1132 $[M+Na]^+$ (calculated for $C_{15}H_{24}O_5ClNa$ = 341.1132). The collision cross section (CCS) of the sodium adduct of **1** was 178.11 Å^2.

*4.5. Bioactivity testing of compound **1***

Compound **1** was tested in a variety of assays to broadly assess possible biological activities. The compound was tested for biofilm inhibition properties against a biofilm forming *Staphylococcus epidermidis* as previously described [19]. The compound was assayed at one concentration, 50 μM, using three technical replicates (n=3). The compounds ability to inhibit the growth of five bacterial strains was assessed, as previously described [19], at 100 μM using three technical replicates (n=3). The assayed strains were the following: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212) and *Streptococcus agalactiae* (ATCC 12386), all strains from LGC Standards (Teddington). Antifungal activity was assayed against *Candida albicans* at 50 μM, as described previously [20]. Potential anti-inflammatory activity was assayed at 50 μM in an ELISA based assay that monitors the tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) production of a human acute monocytic leukemia cell line (THP-1) in the presence of **1** was conducted, as previously described [21]. Lastly, **1** was assessed for its antiproliferative activities at 50 μM towards the human melanoma cell line A2058 and the human non-malignant lung fibroblast cell line MRC-5 as previously described [22].

4. Conclusions

As part of our ongoing search for novel compounds from under-studied marine fungi, chlovalicin B (**1**) was isolated from the liquid culture of an Arctic marine mushroom, *Digitatispora marina*. This represents the first compound isolated from the *Digitatispora* genus, and the first reported fumagillin/ovalicin-like compound isolated from a Basidiomycete. The current study adds on to the knowledge on the biosynthetic potential of marine fungi *sensu stricto*, especially obligate marine Basidiomycetes.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: *Digitatispora marina* in different growth conditions, Figure S2: Low-collision and high-collision energy mass spectra of chlovalicin B (**1**) in ESI+, Figure S3: ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**), Figure S4: ^{13}C (151 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**), Figure S5: HSQC + HMBC (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**), Figure S6: COSY (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**), Figure S7: H2BC (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**), Figure S8: ROESY (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

Author Contributions:. Conceptualization, M.J., T.R., J.H.A., E.H.H., K.Ø.H..; methodology, M.J., V.K., K.M.C., J.I.; validation, M.J., V.K., K.M.C., J.I. and K.Ø.H.; formal analysis, M.J. and K.Ø.H.; investigation, M.J., V.K., K.Ø.H., K.M.C.; resources, J.H.A.; data curation, M.J., K.Ø.H., K.M.C. and J.I.; writing—original draft preparation, M.J. and K.Ø.H.; writing—review and editing, M.J., K.Ø.H., E.H.H. and J.H.A.; visualization, M.J. and K.Ø.H.; supervision, J.H.A. and E.H.H.; project administration, M.J. and K.Ø.H.; funding acquisition, J.H.A.

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Data Availability Statement: The data are available within the article and in the Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds not are available from the authors.

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SUPPLEMENTARY MATERIAL

Chlovalicin B, a Chlorinated Sesquiterpene Isolated from the Arctic Marine Mushroom *Digitatispora marina*

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Abstract

As part of our search for bioactive metabolites from understudied marine microorganisms, the new chlorinated metabolite chlovalicin B (**1**) was isolated from liquid cultures of the Arctic marine basidiomycete *Digitatispora marina*, collected and isolated from driftwood found at Vannøya, Norway. The structure of the novel compound was elucidated by spectroscopic methods including 1D and 2D NMR and analysis of HRMS data, revealing that **1** shares its molecular scaffold with a previously isolated compound, chlovalicin. The compound was evaluated for its antibacterial activities against a panel of five bacteria, ability to inhibit bacterial biofilm formation, antifungal activity against *Candida albicans* and for cytotoxic activities against malignant and non-malignant human cell lines. Compound **1** displayed weak cytotoxic activity against the human melanoma cell line A2058 (~50% survival at 50 µM), otherwise no activity was detected. This is the first reported isolated compound from the marine mushroom *sensu stricto D. marina*.

Key words

Digitatispora marina; marine fungus *sensu stricto*; Basidiomycota; bioprospecting; chlorinated sec-ondary metabolite; natural products

Supplementary Material Table of Contents

Figure S1. *Digitatispora marina* in different growth conditions. 1) *D. marina* growing on driftwood of the *Betula* sp. Photo: Teppo Rämä, 2) *D. marina* grown in liquid culture in malt extract medium supplemented with sea salts. Photo: Marte Jenssen, 3) *D. marina* grown on corn meal agar (top) and malt extract agar (bottom). Photo: Marte Jenssen

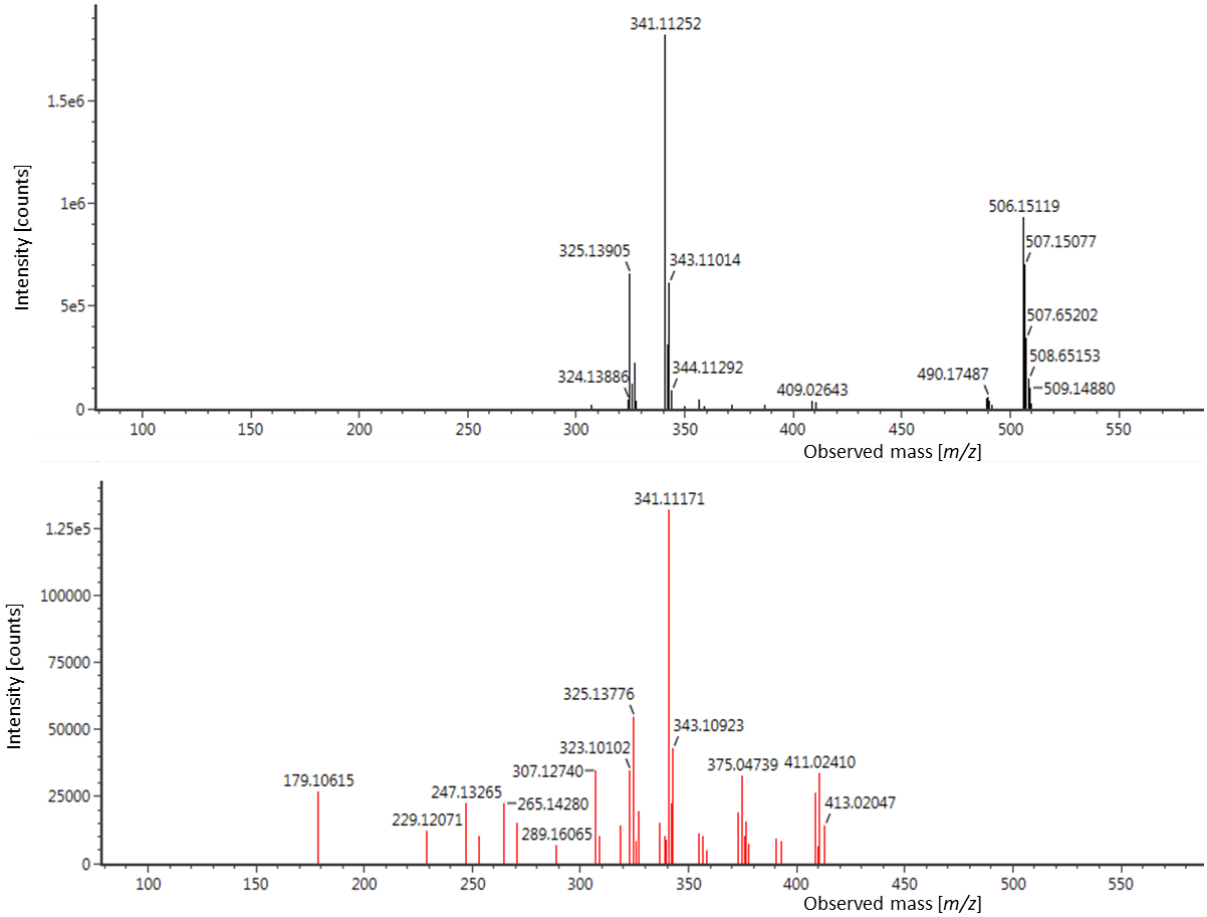

Figure S2. Low-collision (top) and high-collision (bottom) energy mass spectra of chlovalicin B (**1**) in ESI+

Figure S3. ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

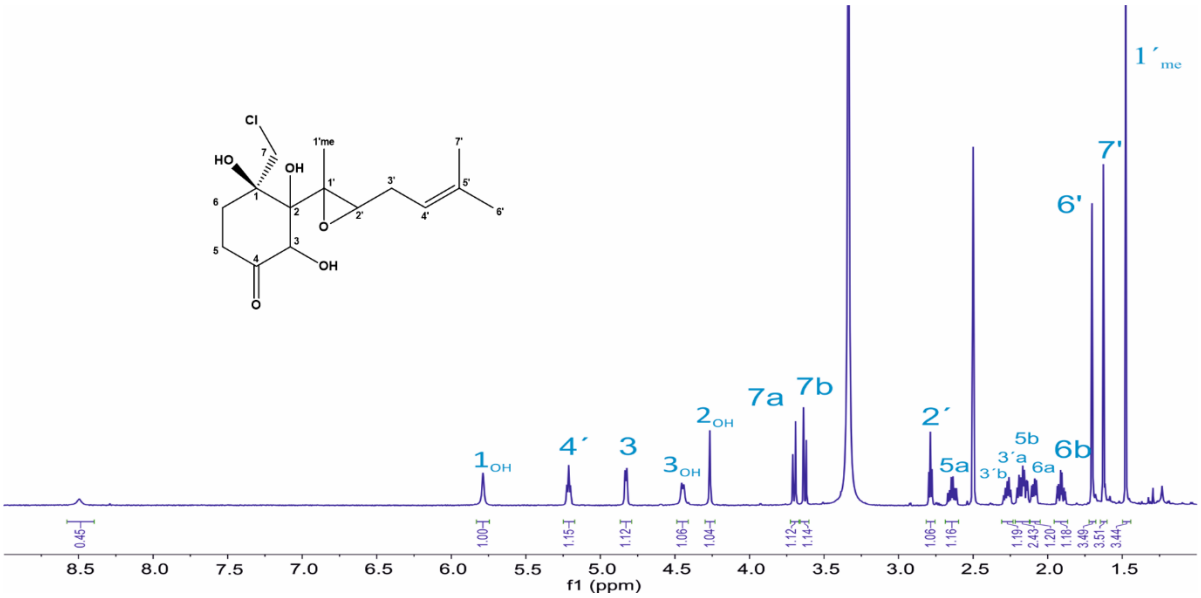

Figure S4. ^{13}C (151 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

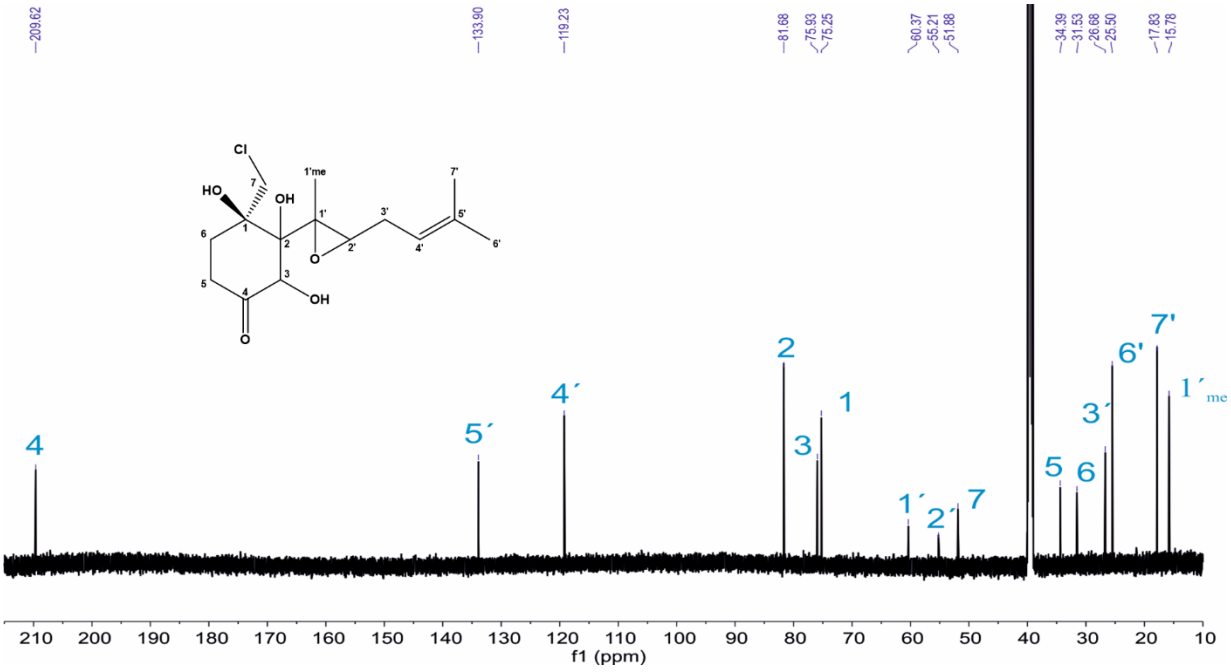

Figure S5. HSQC + HMBC (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

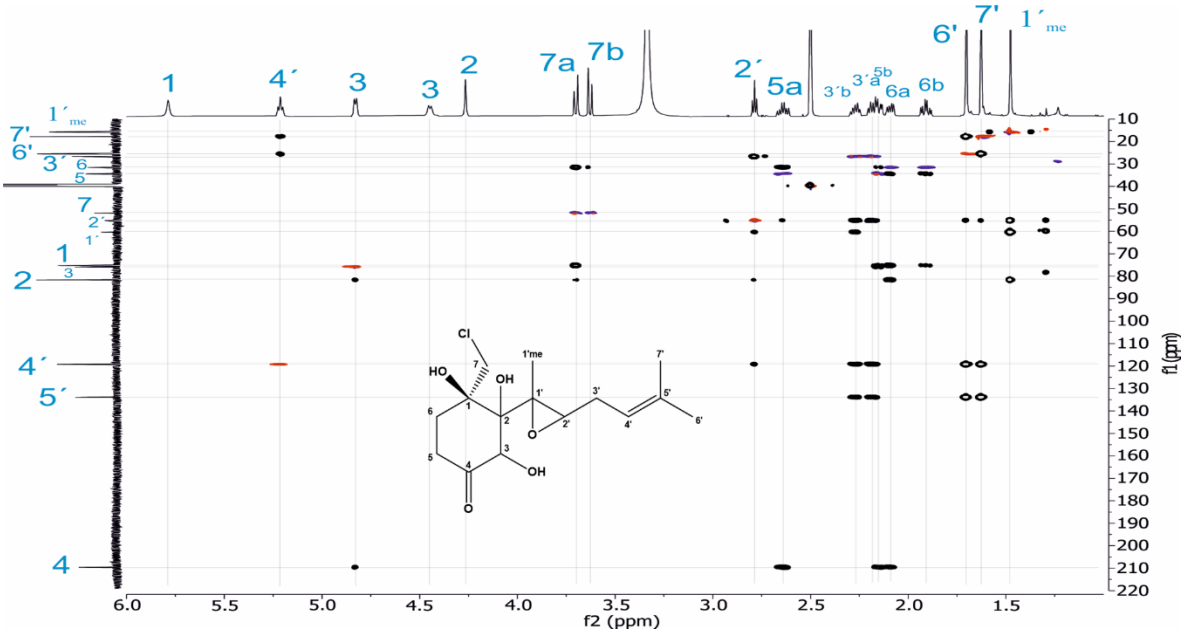

Figure S6. COSY (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

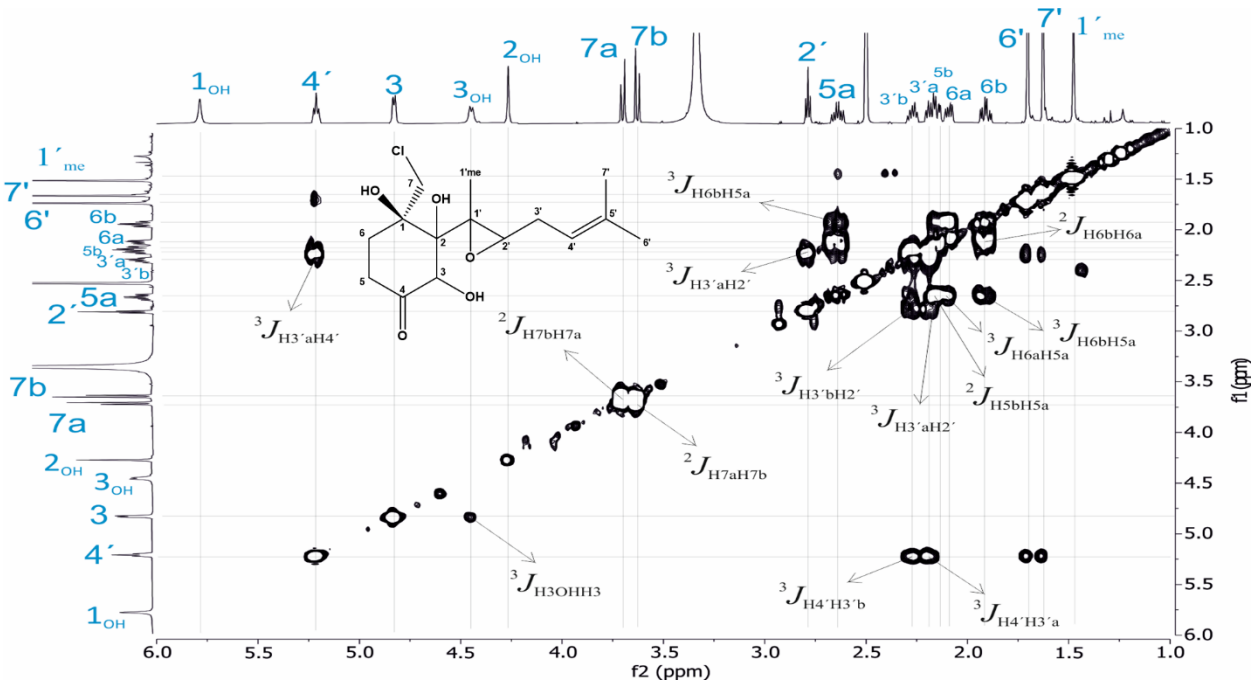

Figure S7. H2BC (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)

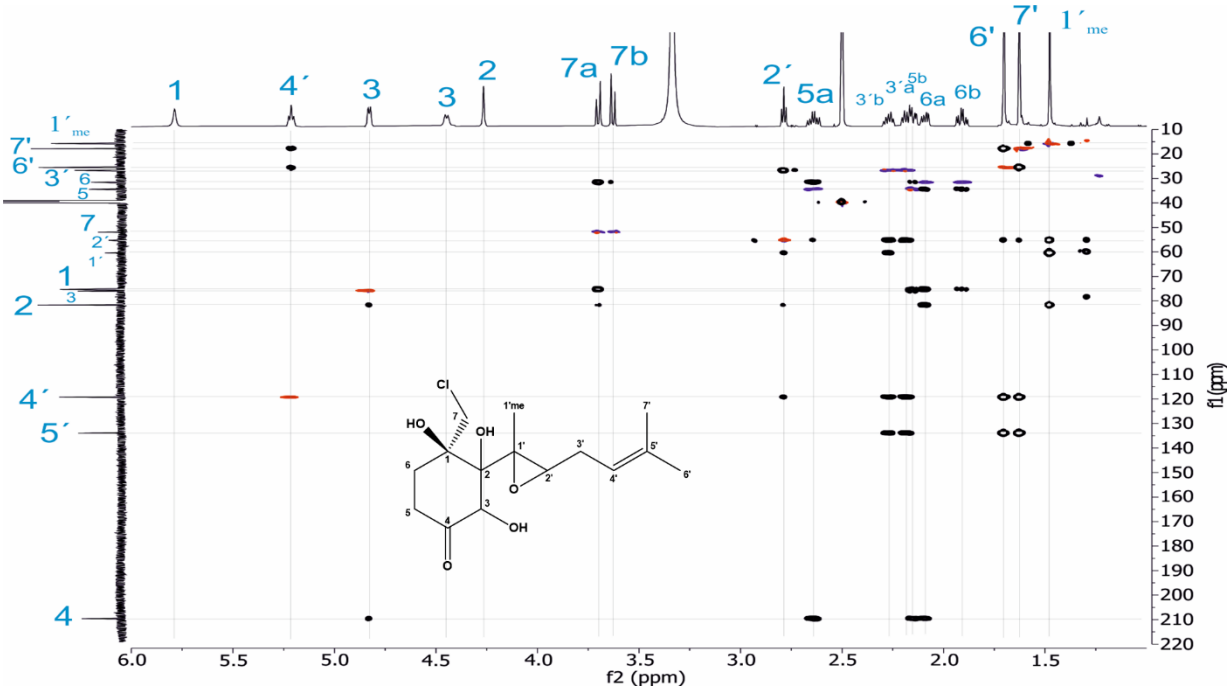

Figure S8. ROESY (600 MHz, DMSO-d_6) spectrum of chlovalicin B (**1**)