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Bioprospecting of Arctic marine organisms

Employing bioassay-, chemistry-, and metabolomics-guided isolation

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Elisabeth Klungerbo Olsen

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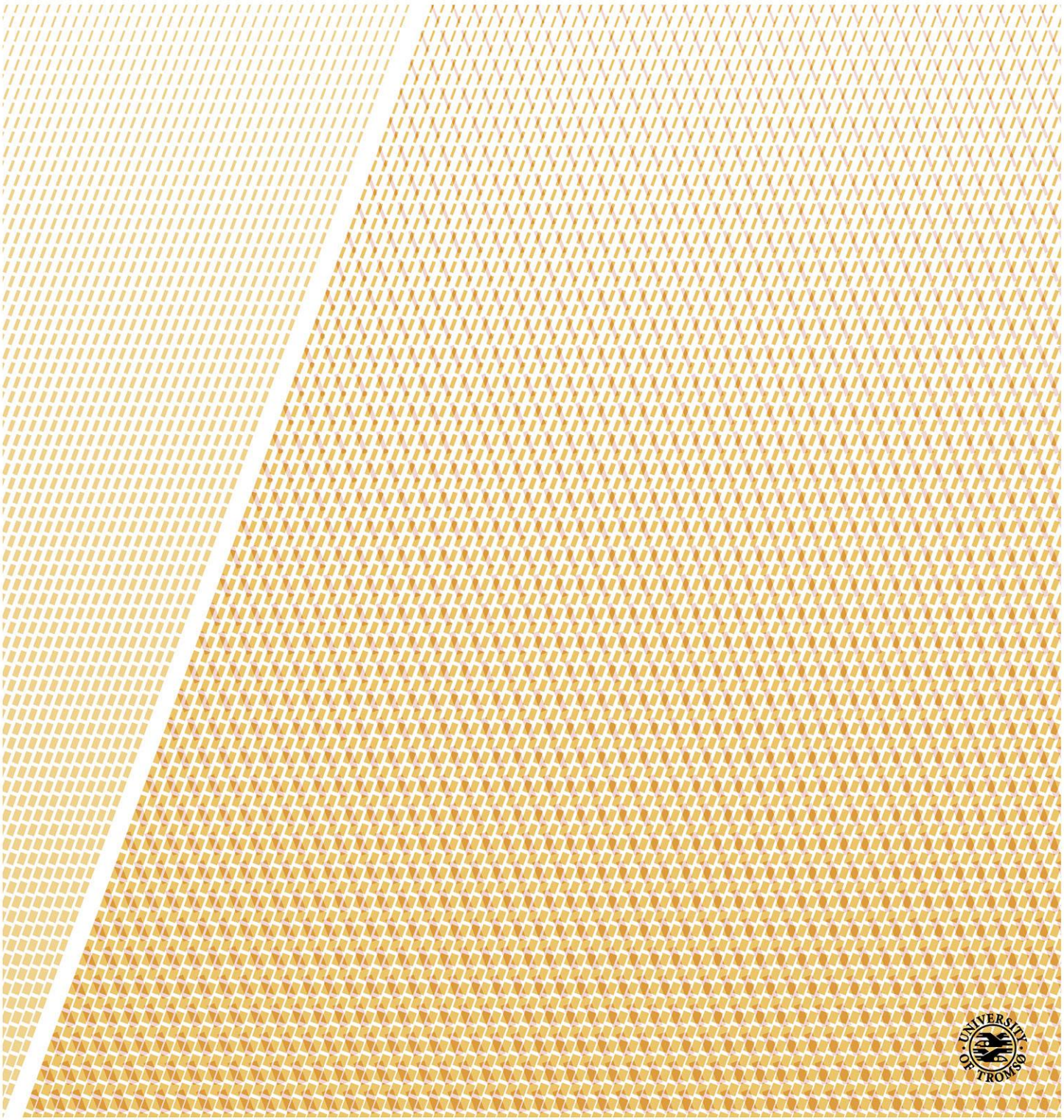

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Summary

The marine environment inhabits a large diversity of organisms that produce natural products as a strategy for survival. Many marine natural products are found to display different bioactivities and some of them have reached the market as drugs or dietary supplements. Comprehending the great potential of bioactive marine natural products motivates for the continuing bioprospecting of organisms from the oceans.

In this thesis the aim was to isolate bioactive marine natural products. Three different approaches were studied, namely bioassay-, chemistry- and metabolomics-guided isolation. The bioassay-guided isolation approach yielded an antioxidative bromophenol isolated from the alga *Vertebrata lanosa*, which was active in both biochemical and cellular assays. It was more potent than the known antioxidants luteolin and quercetin in both cellular assays and of quercetin in one of the assays.

The well-studied barettin was attributed an additional bioactivity as it, together with the structurally similar 8,9-dihydrobarettin, inhibited electric eel acetylcholine esterase (AChE). Inhibition by both barettins was in the range of the reported AChE inhibitors pulmonarin A and B and of the drug galanthamine. Two brominated indoles were isolated, using chemistry-guided isolation, from *Geodia barretti* based on their structural similarities with barettin and 8,9-dihydrobarettin. The brominated indoles were used as inspiration for synthesising a library of 22 structurally similar compounds. Synthetic compounds having a combination of a bromine substituent in a specific structural position and a positively charged amine were the most potent. However, the brominated indole was found not to be sufficient for AChE inhibition.

Metabolomics-guided isolation was used to study the differences in natural products produced by the closely related sponges *G. barretti* and *G. macandrewii*. Employing this bioprospecting approach a novel compound unique for *G.*

macandrewii was identified and it was suggested that metabolomics could be a valuable supplement to other bioprospecting approaches like the bioassay-guided isolation.

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Tromsø, July 2015.
Elisabeth Klungerbo Olsen

List of publications

The thesis is based on one published paper (I) and two manuscripts (II and III) referred to by their roman numbers in the text.

Paper I

Elisabeth K. Olsen, Espen Hansen, Johan Isaksson and Jeanette H. Andersen (2013)

Cellular Antioxidant Effect of Four Bromophenols from the Red Algae, *Vertebrata lanosa. Marine drugs*, **11** (8), 2769-2784

Paper II

Elisabeth K. Olsen, Espen Hansen, Lindon Moodie, Johan Isaksson, Kristina Sepčič, Marija Cergolj, Johan Svenson and Jeanette H. Andersen (2015)

Marine AChE inhibitors isolated from *Geodia barretti*: Natural compounds and their synthetic analogs (Manuscript)

Paper III

Elisabeth K. Olsen, Kine L. Søderholm, Johan Isakson, Jeanette H. Andersen and Espen Hansen (2015)

A Metabolomic Approach to Identify Novel Natural Products from Marine Sponges (Manuscript)

Abbreviations

1D	One-dimensional
2D	Two dimensional
AChE	Acetyl choline esterase
ADMET	Absorption, distribution, metabolism, excretion and toxicity
BI	Brominated indole
BP	Bromophenol
CAA	Cellular Antioxidant Activity
CLPAA	Cellular Lipid Peroxidation Antioxidant Activity
COSY	Correlated spectroscopy
CEM	Channel electron multiplier
CRM	Charged-residue model
Da	Dalton
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
ESI	Electrospray ionisation
FDA	Food and drug administration
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
HSQC	Heteronuclear single-quantum correlation spectroscopy
HTS	High throughput screening
IC_{50}	Inhibitory concentration at half maximum
IEM	Ion evaporation model
MCP	Microchannel plate
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NAT	N-Acyl-Taurine
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
NP	Natural product
ORAC	Oxygen Radical Absorbance Capacity
PAS	Perifer anionic site
RF	Radio frequency
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
Ro5	Lipinski's rule of five
RP-HPLC	Reversed phase HPLC
SAR	Structure-activity relationship
SPE	Solid phase extraction
ToF	Time of flight
UPLC	Ultra performance liquid chromatography
m/z	Mass-to-charge ratio

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1. Introduction

1.1 The marine environment

Oceans cover more than 70% of the earth's surface and display an incredible array of environmental factors and biological diversity. Living species are found throughout the extreme conditions of the marine environment, from temperatures in excess of 300 °C to seawater frozen to ice and regions depleted of oxygen.[1] Environmental abiotic factors such as pressure, sunlight, temperature and salinity directly affect the organisms' ability to exist in a habitat. Consequently, the biological distribution of a species is largely influenced by its capacity to adapt to environmental factors.[2] Oceanic depth, spanning from shallow coastal waters to Challenger Deep of the Mariana Trench at nearly 11,000 meters below sea-level, is an example of such an abiotic factor.[3] Pressure increases at a rate of about 1 atm for every ten meters in depth and is therefore extreme at the depths of the Mariana Trench. [4] Obligate barophilic bacteria found at the Challenger Deep have adapted to the high pressure and thus are able to occupy an ecological niche in this specific environment.[5]

Biological diversity, also known as biodiversity, is widely defined as the variety of life in a given ecological system.[6] The main contributors are the microbes, which by weight constitute up to 90% of all ocean biomass.[1] A map with the estimation of seafloor biomass divided according to size can be seen in Figure 1a. Marine biodiversity is unevenly distributed across the globe where the oceanic taxa (e.g. tunas and whales) tend to peak around Southeast Asia, while the coastal taxa (e.g. corals and coastal fishes) are more broadly located across the mid-latitude oceans (see Figure 1b). Different from biodiversity, the abundance of species appears to peak in temperate and colder waters.[1]

A B

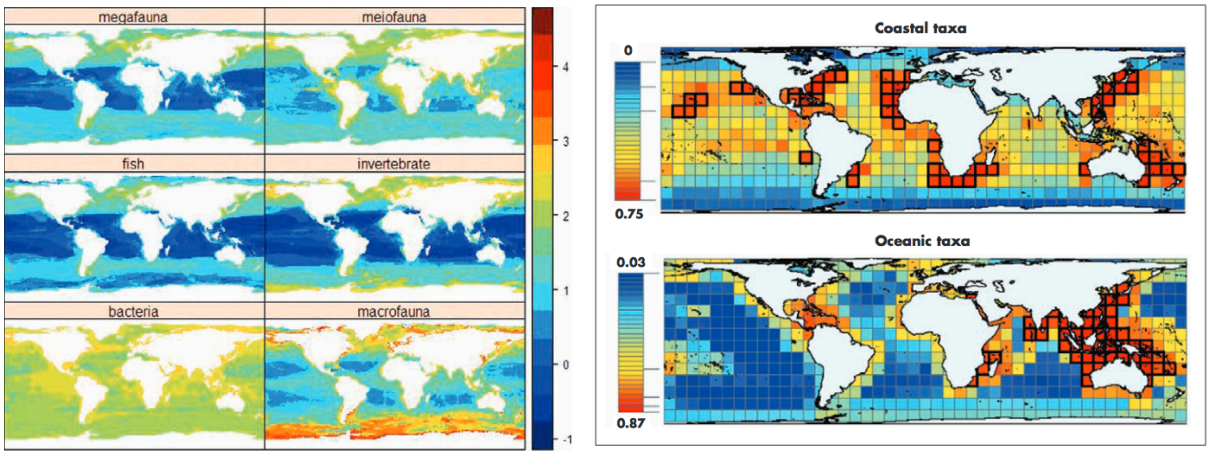


Figure 1. A: A map of estimated seafloor biomass (Log_{10} biomass (mg carbon m^{-2})), distributed according to size. B: Distribution of coastal and oceanic biodiversity, where red indicates area of high diversity. Horizontal tick marks to the right of the key indicates quartiles of diversity. Both pictures are reprinted from the first census of marine life.[1]

1.1.1 Natural products from the ocean

Not only does the marine environment display an impressive biological diversity, the chemical diversity is also remarkable. This chemical diversity makes the oceans an essential and sought after source of bioactive natural products (NPs).[7] Such compounds are often secondary metabolites produced by marine organisms in addition to their primary metabolites. Whilst the latter are essential for the growth, biosynthesis and development of living cells, secondary metabolites are thought to play a more subtle role. Absence of secondary metabolites are believed not to lead to an instant death of the organism, however may impair its long-term survival.[8,9] The distinction between primary and secondary metabolites is not always clear, and in this thesis the term NPs will be used for compounds isolated from marine organisms.[10]

It is believed that protection of the organism is a beneficial function of NP biosynthesis. The production of NPs can act as a form of chemical protection for marine organisms that are sessile, slow moving, lack defence structures such as spines or a protective shell. These compounds may paralyze prey or have a detrimental alleopathic effect against predators and microbial intruders.[11,12] It has been speculated that the majority of NPs isolated from marine invertebrates have microbial origin.[12] For instance symbiotic

microorganisms, such as archaea, bacteria, cyanobacteria, and microalgae, living on sponges may be responsible for the biosynthesis of defensive NPs rather than the sponge itself.[11,13]

Bioprospecting is the process of collecting and investigating biological material with the aim of finding bioactive substances for commercialisation.[14,15] This systematic investigation of novel biologically active NPs from the ocean started with the discovery of spongothymidine and spongouridine from the sponge *Tethya crypta* in the 1950s. The identification of these two compounds led to the development of anticancer and antiviral drugs.[16-20] Following this discovery the investigation of bioactive marine NPs has increased, and so has the number of novel marine compounds discovered.[11,21] Initially the reports of such NPs were scarce and the compounds were mainly isolated from easily accessible organisms like macro algae. Organisms living close to the shore were collected by hand using snorkel or simple scuba systems, whereas access to deeper waters was limited. The development of reliable scuba diving techniques made depths of 3-40 meter routinely achievable. In addition, deep-water collections became available through dredging, trawling and remotely operated vehicles.[22] As a consequence, greater depths could be investigated and an increasing number of novel marine NPs are reported every year. The marine environment has demonstrated to be a rich source of bioactive compounds; 382 articles published between 2011 and 2012 resulted in an 8% increase of marine NPs reported. This corresponded to 1241 new chemical entities.[23] Several of these compounds belong to novel chemical classes not found in terrestrial sources, whereas some display novel mechanisms of action compared to terrestrial compounds.[7,22,24-26] In addition, marine NPs need to be highly potent to exert an effect in nature as they are rapidly diluted in the seawater when released by an organism.[12] These features, and the great biodiversity of marine environments, have made the oceans an attractive source for drug discovery.[11]

Marine NPs have been applied in a range of different areas such as pharmaceuticals, agrochemicals, industrial chemicals, construction materials, crops, cosmetics, food and flavorings.[15] The process from finding a bioactive compound until releasing it as a drug to a market is time consuming and expensive, taking up to 14 years and costing up to 900 million USD.[17,27] Even though drug discovery and development is a long and

challenging process some NPs are successfully developed into drugs. Marine bioprospecting has so far resulted in eight marine NPs, or their derivatives, being approved as drugs on the market (Table 1).[7,16,28,29] The global market of marine-derived drugs is estimated to reach $8.6 billion by 2016.[28]

Substance	Brand name	Therapeutic area	Originate from
Cytarabine	Cytosar-U® Depocyt®, DepoCyte®	Anticancer	Sponge *Tethya rypta*
Vidarabine *	Vira-A®	Antiviral	Sponge *Tethya rypta*
Ziconotide	Prialt®	Severe chronic pain	Cone snail *Conus magus*
Trabectedin	Yondelis®	Anticancer	Tunicate *Ecteinascidia turbinate*,
Eribulin mesylate	Halaven®	Metastatic breast cancer	Marine sponge Halichondria okadai
Omega-3-acid ethyl esters	Lovaza®	Hypertriglyceridemia	Fish
Brentuximab vedotin	Adcetris®	Anticancer agent	Sea hare *Dolabella auricularia*
Iota-carrageenan	Carragelose®	Over the counter antiviral agent	Red alge *Eucheuma/Cnondus*

Table 1. An overview over marine-derived drugs that have reached the market. Vidarabine is now discontinued.[7,25,26,28]

From a bioprospecting perspective, the drug-discovery pipeline ranges from collection of biomass to the release of a drug to the market, and is illustrated in Figure 2. The biomass is extracted and tested for bioactive compounds (hits) in a series of screens. These hits, commonly NPs present in small amounts, can be isolated and their structures elucidated. By synthesising a library of structurally similar compounds based on the "hit" molecule a lead (or several leads) can be identified. In addition, the structural requirements for bioactivity can be determined. Leads can then undergo preclinical trials for the establishment of efficacy and toxicity in animal models. If successful, the molecule is advanced to human clinical trials. A lead that enters the clinical part of the pipeline is then referred to as a drug. The clinical trials are composed of three stages; phases I-III. Phase I trials are performed to establish if the drug is safe to use for humans, which dosage it can be used in without causing adverse side-effects and its metabolic

profile and bioavailability. The initial part of phase I studies are done on human volunteers and if the drug is considered safe it is tried out on a small patient population (commonly a total of 20-80 people). Phase II trials are investigations of effectiveness and safety of the drug on an increased patient population (generally no more than 100-200 patients). Effective drugs are then subjected to phase III trials. In phase III trials the drug is compared with current standard treatment(s) for the same condition in a large trial. The size of the patient population would vary between different treatments, however it must be high enough to obtain a precise estimate of the treatment response.[30] To become a commercially available drug, the lead has to be successful in both the preclinical and clinical trials. The probability of a lead becoming a drug is small; only one in approximately 50 preclinical leads will reach the market.[17] When a drug has reached the market it is continued to be under surveillance, a step known as phase IV or post-marketing. This involves monitoring for adverse effects and other large-scale and long-term studies of morbidity and mortality.[30]

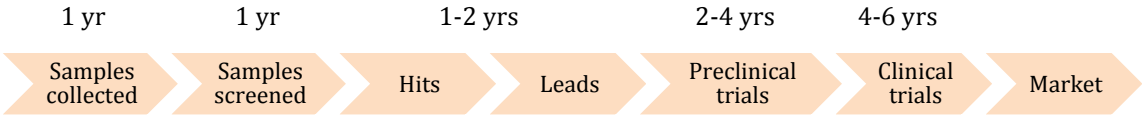


Figure 2. An illustration of a drug discovery and development pipeline, modified from Hunt and co-workers.[17]

1.2 The marine bioprospecting pipeline

The first step of marine bioprospecting is the collection of biomass, which is generally sampled in two stages: an initial collection and a secondary collection. During the initial collection small samples from a number of species are gathered. The organism should be collected in an amount that allows for isolation, structure elucidation and bioactivity profiling of NPs, whilst not impacting the natural population. Limited supply of material is a challenge within marine bioprospecting.[31,32] However, the sample size required has decreased considerably over the last 30 years. Sample sizes of 10 kg (wet weight) were reported in the 1970s, while 0.5 to 1 kg (wet weight) samples are now more typical.[33,34] Advances in technology and improvements in analytical techniques make it possible to detect, isolate and identify milligram and in some cases microgram amounts of NPs.[14,17] It is common that more of the NP is required for further investigation. This can be achieved by total synthesis or, if synthesis proves to be difficult, a secondary

collection(s). The secondary collection may be larger than the initial ones to ensure enough material for further investigation of the active compound.[17,34]

The screening efficiency may also be a challenge in bioprospecting. The development of automated High throughput screening (HTS) was a major contributor for increasing the output of discovered bioactive compounds. Automation of process like pipetting, assay reading and dispensing has reduced labour hours and increased efficiency.[35] The screening throughput efficiency has increased with the application of new technology. Improved analytical techniques have reduced the size of test volumes.

The isolation of bioactive compounds can be achieved using several approaches.[36] In this thesis, we have explored three different strategies in which isolation has been guided by either bioactivity, chemical properties or metabolomics (Figure 3). Although these three methods share common features, the order of their application varies. In the following sections a general pipeline will be used to illustrate the three bioprospecting approaches, followed by a demonstration of the shared processes; partitioning, prefractionation, dereplication, isolation, structure elucidation and bioactivity profiling.

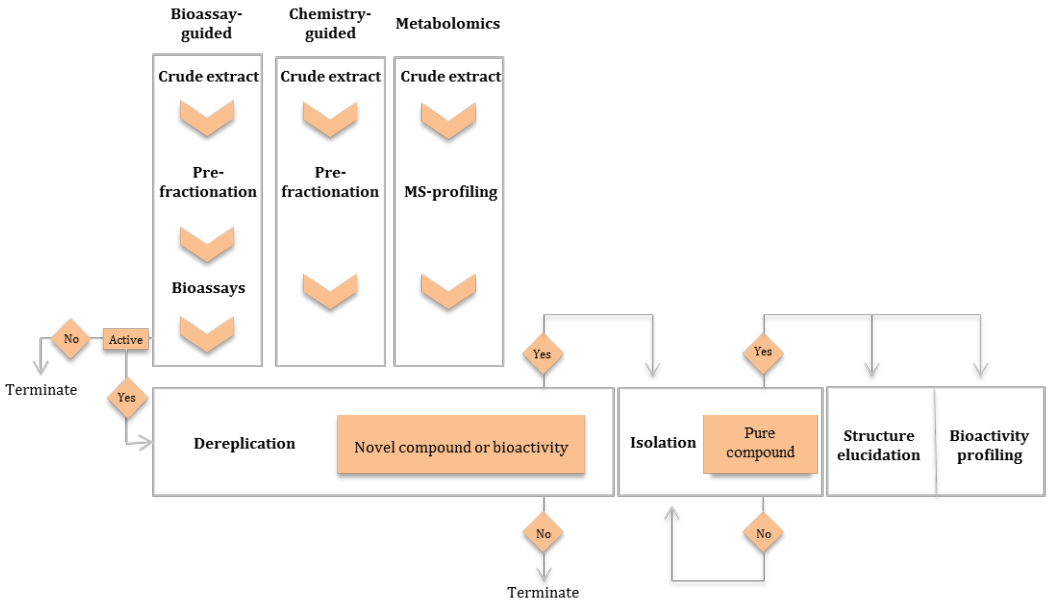


Figure 3: An illustration of a bioassay-, chemistry- and metabolomics-guided bioprospecting pipeline.

1.2.1 Bioassay-guided isolation

In this approach a bioassay an *in vitro* or *in vivo* system is used to detect the biological activity of an extract or a pure NP. Bioactivity testing is incorporated into the isolation process at an early stage in order to guide the isolation of bioactive compounds through the bioprospecting pipeline.[35,37,38] The extract is commonly subjected to several different bioassays to get an indication of the bioactivity profile, and isolation is initiated if the extract displays activity in one or several of the assays.

1.2.2 Chemistry-guided isolation

When using the chemistry-guided isolation approach, the aim is to find NPs with a novel chemical structures. This approach can also be used to search for NPs with a functional motif similar to known bioactive compounds. Isolation is initiated based on interesting chemical properties, where one example is halogenation.[36] NPs that are halogenated are often easy to identify in an extract due to their characteristic isotope patterns visible by mass spectrometry (MS). In addition, halogenated NPs from marine organisms are interesting since several of them are bioactive.[39-42]

1.2.3 Metabolomics-guided isolation

Metabolomics is a technology used for identification and quantification of small molecule (<1500 Da) metabolites produced by an organism. A metabolome is all of the small molecule metabolites or chemicals that can be found in a cell, tissue, organ or organism. Metabolomics can be used for quantitative and qualitative analysis of the whole metabolome present in an organism at a specific time and under a specific influence.[9,43-47] Several technologies are available for analysing a metabolome, where MS and nuclear magnetic resonance (NMR) are considered to be the most universal.[47] MS is highly sensitive and has the ability to detect compounds of a wide range of molecular weights.[46,47] NMR can be used to quantify compounds in a mixture or reveal the distinctive structures of co-eluting compounds which are not discernable using MS techniques.[9,47]

1.2.4 Shared processes; partitioning, prefractionation, dereplication, isolation, structure elucidation and bioactivity profiling

Partitioning

Crude extracts are often complex mixtures of diverse NPs, some not ideal for pharmaceutical purposes. Highly lipophilic compounds (e.g. lipids and fatty acids) may be difficult to handle. Additionally these compounds are not desirable for oral administration since a high lipophilicity will decrease the transport across intestinal epithelial cells and in the blood stream.[48] Solvent partitioning distributes solutes between two immiscible liquid phases and may be used to remove unwanted material like highly lipophilic or highly hydrophilic compounds (e.g. sugars and salts) into a hydrophobic or hydrophilic solvent respectively.[36] In addition, a series of solvents with increasing polarity can be used to divide the extract into fractions with compounds of similar preferential solubility.[36] Removal of highly lipophilic and/or hydrophilic compounds simplifies complex extract and aides the subsequent steps.

Prefractionation

The prefractionation step involves the separation of a crude extract into fractions, each with reduced complexity, before bioactivity screening. It is most applicable for the bioassay-guided isolation although it may also be used for chemistry-guided isolation. In metabolomics the aim is to analyse the whole metabolome and hence prefractionation is not desirable. Prefractionation can be achieved using a number of methods including column chromatography e.g. flash chromatography, solid phase extraction (SPE) and high-performance liquid chromatography (HPLC). One advantage of applying prefractionation to the bioassay-guided isolation is that the compounds in each fraction are tested at a higher concentration than if the whole extract was screened.[36,49] The reduced complexity may also decrease the possibility of some compounds masking the bioactivity of others and/or reduce the possibility of antagonism or synergism between compounds in an extract.[46]

Dereplication

Dereplication is the process of identifying known compounds in extracts or partially purified mixtures. It is an essential step and is common for all three bioprospecting approaches. This process helps to minimise the risk of isolating compounds already

identified and described in the literature.[35,46] High-resolution MS (HR-MS) is a highly sensitive technique for identifying compounds from semi-purified mixtures.[36] However, a limitation with HR-MS is that it only detects compounds that are ionised, in either the positive and/or negative mode. The elemental composition of a compound can be calculated from its accurate mass and isotopic pattern. The obtained data can be used to search for similar compounds e.g. in the online database Dictionary of Marine Natural Products. Compounds that would be considered for isolation are either novel, or they are known but with potential novel bioactivity.

Isolation of pure compounds

Isolation is performed to separate one compound from others in an extract, and thus allow for structure elucidation as well as bioactivity testing of the isolated compound. Chromatographic techniques distribute extract components between two phases, a stationary phase and a mobile phase. HPLC involves a solid stationary phase where the separation depends on a compounds' affinity between this phase and the mobile phase. The forces governing retention of a compound on a solid phase may arise from hydrogen bonding and/or Van der Waals or dipole-dipole interactions, where a longer retention time is indicative of a stronger interaction. For marine NP isolation a common approach is reversed phase HPLC (RP-HPLC) with a C_{18} (Octadecyl) stationary phase. RP-HPLC requires a hydrophobic mobile phase for eluting hydrophobic compounds since these are retained longer on the stationary phase.[36] Commonly, a gradient with an increasing amount of hydrophobic solvent is used for the isolation of NPs in RP-HPLC. A limitation to this approach is that each purification process is typically run on a small scale. [36]

Mass guided HPLC uses a mass spectrometer to assist isolation. The fraction collector is automatically triggered by the MS when a threshold intensity of the target mass is reached. To accomplish this, the flow from the column is split such that 99% proceeds to the fraction collector and 1% to the MS. Advantages for using such a system include increased reproducibility and precision. An illustration of a chromatogram for mass guided isolation is presented in Figure 4.

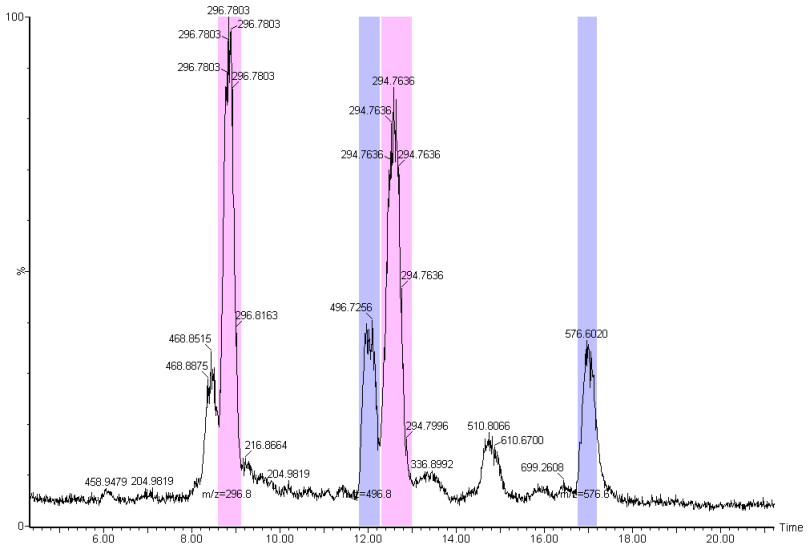


Figure 4: A total ion chromatogram displaying mass guided isolation of bromophenols from an extract of *V. lanosa* (Paper I).

Structure elucidation

The carbon scaffold and the identity and position of an organic NP can be detected using different methods. HR-MS, NMR, ultraviolet/visible spectroscopy and infrared absorption spectroscopy are extensively exploited for structure elucidation. Commonly, two or more of these techniques are used in combination to give the final structure.

HR-MS analysis

MS is an analytical tool that aims to identify a compound from the molecular or atomic masses of its constituents. This technique generates molecular ions that can be separated based upon their *mass-to-charge ratio (m/z)*. Information about molecular mass, relative abundance and isotopic patterns can be obtained from the MS data and the data are used to calculate a molecular formula and the level of unsaturation.[50,51] A mass spectrometer consists of an ion source, a mass analyser and a detector.[50] In this thesis an ultra performance liquid chromatography time of flight (UPLC-ToF) system with electrospray ionisation (ESI) and a microchannel plate (MCP) detector was used. The UPLC module allows separation of a complex mixture before the eluting compounds enter the ion source, and hence increasing the sensitivity. ESI is a commonly used ionization technique that is effective for most samples. Electrical energy is used to form and transfer ions from a solution into the gaseous phase before they are subjected to MS

analysis.[52] Ion formation in ESI involves three steps: (1) creation of an electrically charged spray (2) dramatic reduction in droplet size and (3) liberation of fully desolvated ions. There are two theories that rationalise the ion formation from charged droplets in ESI. The original theory, the charged-residue model (CRM), assumes that droplets successively lose solvent molecules until the complete desolvation of the ions. While the newer ion evaporation model (IEM) describes the formation of desolvated ions as the direct evaporation from the surface of highly charged microdroplets.[50] In the ToF analyser, ions with different *m/z* are separated during their flight along a field-free drift path of known length. Ions will arrive at the detector at different times, the lighter ones earlier than the heavier ones. The MCP detector is an ion-counting detector consisting of several channel electron multipliers (CEMs) in a cluster. A cascade of secondary electrons, resulting from energetic ions hitting the surface of a CEM, will amplify the signal reaching the detector.[50] The resulting ions are plotted as *m/z* versus relative abundance (%).[51] MS is a technique with high sensitivity, however it only detects compounds that can be ionised.

NMR spectroscopy

NMR spectroscopy is a tool to study the composition, structure and bonding of compounds using wavelengths in the radio wave area.[53] The NMR experiment utilises atomic nuclei that have magnetic properties. Commonly studied nuclei have spin quantum numbers of 1/2 like ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P. [54] When a sample tube is placed in a magnet, which produces a homogenous magnetic field, the nuclei will have a spin orientation either parallel (+1/2) or antiparallel (-1/2) to the field. Subjecting the sample to a radio frequency pulse will rotate the net magnetization away from the Z-axis where it produces a detectable NMR-signal before it returns to equilibrium.[54,55]

The proton (^{1}H) and carbon (^{13}C) spectra provide information about chemical shifts, integrals, splitting pattern and coupling constants. Chemical shifts and integrals are used to deduce types of nuclei and how many are present, while splitting and coupling constants indicate which nuclei are connected to each other. One example of a characteristic coupling constant is the $^{3}J_{HH}$ = ~12-18 Hz observed for a trans double bond.[54,55] This is illustrated by the proton spectrum of 6-bromoconicamin, having this three-bond proton-proton coupling. (see Figure 5).

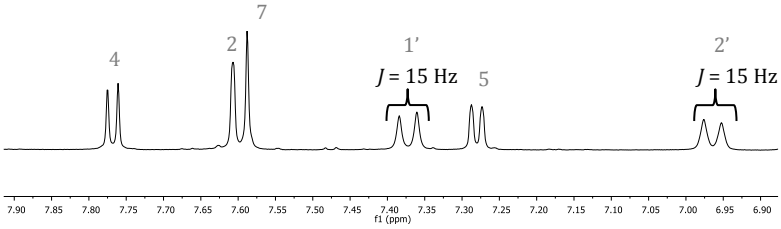


Figure 5. ^{1}H NMR spectrum of 6-bromoconicamin, presented in Paper II, where the trans couplings are highlighted.

The one-dimensional (1D) techniques of ^{1}H and ^{13}C can be supplemented with two-dimensional (2D) NMR approaches to establish nuclei connectivity in a molecule. Commonly used experiments are correlated spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC). COSY displays proton-proton spin couplings and is used to map the proton network of the molecule. HSQC correlations indicate the direct attachment of protons to a given carbon and HMBC shows long range "through bond" couplings between carbons and protons (2-3 bonds typically). The latter experiment is particularly useful for determining the positions of carbons that are not protonated. Examples of NMR experiments that utilise "through space" interactions are nuclear Overhauser effect spectroscopy (NOSEY) and rotating frame nuclear Overhauser effect spectroscopy (ROSEY). These two experiments can provide information about the spatial relationship between protons at close proximity (2-5 Å), which can be valuable when studying the three dimensional conformation of a molecule.[51]

Bioactivity profile of isolated compounds

Upon isolation, the bioactivity profile of a compound can be determined using different bioassays. Bioassays can be either biochemical or cell-based. Biochemical assays provide a way of examining a particular bioactivity without interference from other cellular processes. However, activities in such reconstituted *in vitro* assays do not always translate to the same activity in a cellular context. Reasons for a lower activity in the

cell-based assays may be due to poor membrane permeability, off-target effects and cytotoxicity. The cell-based assays resemble *in vivo* conditions better than biochemical assays. Cell-based assays select compounds that can be transported through cellular membranes either passively or actively, and interactions with cellular components are taken into account.[38] For an isolated compound, the aim is to get a general bioactivity profile, and, in this perspective, a combination of cellular and biochemical assays are favourable. The biochemical assays can be run faster while cellular assays can be used to discover compounds that are more interesting for an *in vivo* setting. A screening panel could look like the one presented in Table 2.

When the bioactivity profile has been established, the compound's efficiency is determined. Here concentration-ranges are used to find the minimum inhibitory concentration (MIC) or lowest concentration resulting in a 50% inhibition (IC_{50}). The compound's bioactivity should be compared with those of previously reported compounds to gauge its potential.

Bioactivity	**Assay**	**Type of assay**
Antioxidant	ORAC	Biochemical
	CAA	Cellular
	CLPAA	Cellular
Anticancer	Cell viability	Cellular
	Kinases	Biochemical
Antibacterial	MIC	Cellular
	Inhibition of biofilm formation	
Immune-modulatory	Immune-stimulatory (TNF-α)	Cellular
	Anti-inflammatory (TNF-α)	
Diabetes	PTP-1B	Biochemical

Table 2: An example of a screening panel.

1.2.5 Chemical synthesis in marine bioprospecting

Chemical synthesis can be used in marine bioprospecting for different purposes. One aim is to increase the chemical diversity of isolated NPs. By increasing the chemical diversity it may be possible to decrease the compounds' toxicity and/or increase potency and selectivity. In such an approach, bioactive NPs are used as leads for synthesizing an analogue series where the functional groups are varied systematically. Testing the bioactivity of the synthesised library may deduce structure-activity relationships (SAR). This allows the evolution of a lead compound into a molecule that exhibits an optimised biological profile.

A second motive for chemical synthesis in marine drug discovery is to address when the isolated compound is in limited supply. This can be due to the organism being scarce and/or the NP being present in trace amounts. An example of a NP being present in a small abundance, and used as a lead for SAR studies, is the antitumor peptide dolastatin 10. The initial identification of dolastatin 10 took over 10 years and required a collection of almost 2 tons of the sea hare *Dolabella auricularia* to isolate the first 1 mg.[36,56,57] SAR studies and synthetic drug design resulted in the tetrapeptide analogue TZT-1027, a compound with a lower toxicity than the parent compound although having maintained a potent antitumor activity.[56]

2. Aim of thesis

The overall aim of the thesis was to isolate and characterise bioactive NPs from marine organisms. Compounds of interest were either novel and/or had novel bioactivities.

The main objectives of this thesis are summarised below:

- Identify compounds in crude extracts either based on bioactivity, structural features or difference in NPs between two extracts
- Dereplicate the crude extract to identify target compound(s)
- Isolate and structure elucidate the target compound(s)
- Confirm the initial bioactivity and study the bioactivity profile of the isolated target compound(s)
- Prepare a synthetic library based on isolated target compound(s) to explore SAR

3. Summary of papers

Paper I

Cellular Antioxidant Effect of Four Bromophenols from the Red Algae, *Vertebrata lanosa.*

Elisabeth K. Olsen, Espen Hansen, Johan Isaksson and Jeanette H. Andersen

The antioxidant activity of bromophenols are commonly determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. This is a biochemical assay that does not take cell permeability into account. In this study the cellular antioxidant activity was reported for bromophenols for the first time. Four bromophenols were isolated from the intertidal alga *Vertebrata lanosa*, where one of them displayed antioxidant activity both in biochemical and cellular assays. Being active in the Cellular Antioxidant Activity (CAA) and Cellular Lipid Peroxidation Antioxidant Activity (CLPAA) assays, the compound demonstrated the possibility to act as an intracellular antioxidant in addition to prevent lipid peroxidation within cell membranes. Compared to the known antioxidants luteolin and quercetin, it proved to have a better antioxidant effect than the former in both the CAA and CLPAA assays and of latter in the CLPAA assay.

Paper II

Marine AChE Inhibitors Isolated from *Geodia barretti*: Natural Compounds and Their Synthetic Analogs

Elisabeth K. Olsen, Espen Hansen, Lindon Moodie, Johan Isaksson, Kristina Sepčič, Marija Cergolj, Johan Svenson and Jeanette H. Andersen

Acetylcholine esterase (AChE) is an enzyme present in several organisms, both vertebrate and invertebrate. It is involved in the termination of nerve impulses by the hydrolysis of acetylcholine, and inhibition of the enzyme is used to alleviate the symptoms of dementia and Alzheimer's disease and glaucoma. In this study barettin, 8,9-dihydrobarettin, and two brominated indoles were isolated from the deep-water sponge *Geodia barretti* and tested for electric eel AChE inhibition. 8,9-dihydrobarettin was most potent followed by barettin, the activity of both compounds were comparable to the reported AChE inhibitors pulmonarin A and B and the drug galanthamine. One of the isolated brominated indoles displayed a weak inhibition of AChE while the other was inactive. To explore the role of the brominated indole on AChE inhibition, a library of 22 structurally similar compounds were synthesised. Of the synthetic library the brominated indoles having a combination of bromine in a specific position and a positively charged amine were the most potent. However, none of the synthetic compounds were as active as the two barettins. Based on these results it was suggested that the brominated indole system was not sufficient for AChE inhibition. Rather, the larger barettin and 8,9-dihydrobarettin are likely to participate in additional bindings beneficial for the inhibition of AChE.

Paper III

A Metabolomic Approach to Identify Novel Natural Products from Marine Sponges

Elisabeth K. Olsen, Kine L. Søderholm, Johan Isakson, Jeanette H. Andersen and Espen Hansen

In this study metabolomics was used to identify a novel natural product, and to examine differences in natural products between the closely related sponges *Geodia barretti* and *G. macandrewii* collected at different locations along the coast of Norway. The organic extracts were analysed using high-resolution MS and post acqusition of the data revealed one compound unique for the *G. macandrewii* sample. Another difference between the two sponges was that the *G. barretti* samples contained more barettin and 8,9-dihydrobarettin than *G. macandrewii*. Samples of the same species collected at different locations contained similar amounts of both barettin and 8,9-dihydrobarettin. These results demonstrated that metabolomics can be used to identify a novel natural product and suggest that this approach can be used as a supplement to the bioassay-guided isolation that is commonly used in marine bioprospecting.

4. General discussion

NPs isolated from marine organisms display a range of bioactivities that can be exploited in commercial products. Possible applications span from industrial products like antifouling paints to food additives such as alginate used as a thickening agent.[58,59] Additionally, extensive research is done to evaluate the pharmaceutical potential of marine NPs.[11,12,16,22] Employing marine bioprospecting is influenced by cost, time, novelty, scale-up and intellectual property.[60] In this thesis bioassay-, chemistry- and metabolomics-guided isolation was used in marine bioprospecting, and the results are presented in Papers I-III, respectively.

Comparisons of the three methods demonstrated that they are all valuable in identifying interesting marine NPs. Regardless of the method used, it should be stressed that dereplication is essential to avoid isolating known compounds. Bioassay-guided isolation proved the most advantageous strategy for finding compounds with a pre-defined bioactivity as bioactivity was the parameter used to select the target compounds. The compounds isolated using the chemistry-guided or metabolomics approaches were not necessarily bioactive in the assays initially examined. Since NPs are believed to bind to specific receptors as a strategy for an organism's survival, it is probable that they are bioactive. [61] The isolated NPs should be stored so they can be screened for additional bioactivities at a later stage.

The marine NPs isolated and presented in Papers I-III, represent different chemical classes and bioactivity profiles. Isolation of bromophenols (BPs) are reported in Paper I, diketopiperazines (DKP) and brominated indoles (BIs) in Paper II and a N-Acyl-Taurine (NAT) in Paper III.

4.1 Variations in structure and bioactivity profile of the isolated natural products

4.1.1 Paper I: Isolation of antioxidant bromophenols from Vertebrata lanosa

The antioxidants presented in Paper I were isolated from the macro-algae *Vertebrata lanosa*. Antioxidants can be defined as "substances which counteract free radicals and prevent the damage caused by them".[62] Being an intertidal organism, *V. lanosa* is exposed to stress that can result in an imbalance between production of free radicals and their removal.[63-66] Even though oxidative metabolism and radical formation is a normal biological process in aerobes, a state of oxidative stress can occur if more free radicals are produced than removed.[67-69] Oxidative stress can damage molecules like DNA, proteins, lipids or carbohydrates and is believed to be a contributing factor to several medical conditions like cancer, diabetes, cardiovascular and autoimmune diseases and neurodegenerative disorders.[67,70,71] Antioxidants are more commonly used as dietary supplements than drugs, which may be due to their general activity rather than affecting a specific receptor.[62]

The algal antioxidant defence system consists of several different compounds classes, where one group is the BPs.[72-77] Compound **2** was the most potent antioxidant in both the biochemical and cellular assays. The BPs isolated from *V. lanosa* were all structurally similar and are shown in Figure 6. Several marine organisms are found to biosynthesise a set of structurally similar NPs (see references in [23]). These compounds have different substituents, however are all based on a common structure. Producing molecular diversity from a common molecular skeleton is a known survival strategy as it can lead to a broad range of bioactivities beneficial to the organism. In addition to being antioxidants, BPs are known to exert cytotoxic and antimicrobial activities.[78]

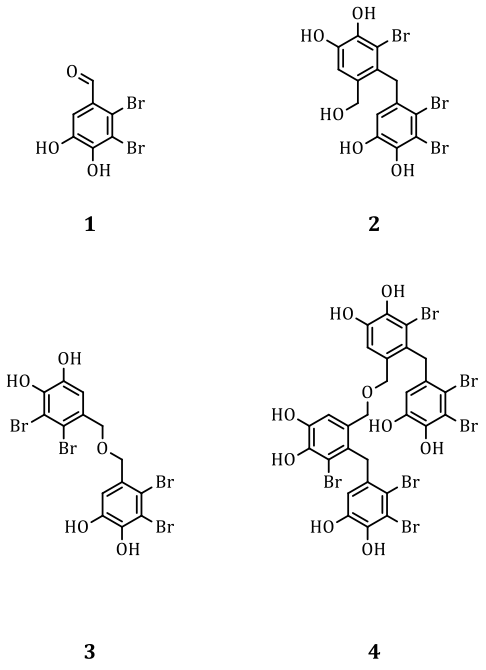


Figure 6. Molecular structures of compounds **1**-**4** presented in Paper I

As seen in Figure 6 compound **4** is structurally similar to the other BPs isolated from *V. lanosa*, however it proved to be an artefact of the isolation process rather than naturally occurring in the alga.[79] Artefacts may occur during several steps of the bioprospecting pipeline, although the extraction and isolation processes are most prone. The formation of artefacts has its consequences, compounds can be falsely reported as novel natural products and the isolation results can be difficult to reproduce. There is also a possibility that an inactive artefact is produced from a bioactive precursor.[80] The dimerization of **2** to yield **4** reduced the concentration of the former, which might result in a lower bioactivity. Additionally, the ratio between **2** and **4** will not be constant, rather it will depend on stability in an aqueous environment.[79]

It is important to be aware of artefact formation during marine bioprospecting, and following general guidelines may limit the frequency of its occurrence. Solvents and acid/bases are known to introduce artefacts and therefore they should be used with awareness. Extracts ought to be stored in a cold and dark place, and when solvents are removed in *in vacuo* the temperature should not exceed 40°C. A sample of the original material should be stored so if an artefact is suspected the original material can be used as a reference.[81]

4.1.2 Paper II: isolation of brominated indoles from Geodia barretti acting as acetyl choline inhibitors

Compounds isolated from *Geodia barretti*, reported in paper II, were structurally similar (Figure 7). Compounds **3** and **4** are 6-brominated indoles only differing at the 1' and 2' positions and can be viewed as truncated analogues of barettin and 8,9-dihydrobarettin. Upon structural consideration, it can be speculated that **4** is the hydration product of **3**, or alternatively, that **3** is the dehydration product of **4**. It is suspected that these compounds arise naturally from a biosynthetic pathway rather than as artefacts from the isolation process. This is supported by the fact that both **3** and **4** also were observed in *G. barretti* samples reported in Paper III. Additionally, the concentrations of both **3** and **4** varied between *G. barretti* samples collected at different locations, if one compound were to be formed by the other it would be more likely to observe a difference in ratio of **3**:**4** between the samples. The structural difference between the two DKPs, barettin and 8,9-dihydrobarettin, is that the latter lacks the double bond between carbons 8 and 9. Both NPs have been reported in the literature previously and hence one is not formed by the other during the extraction or isolation process.[82]

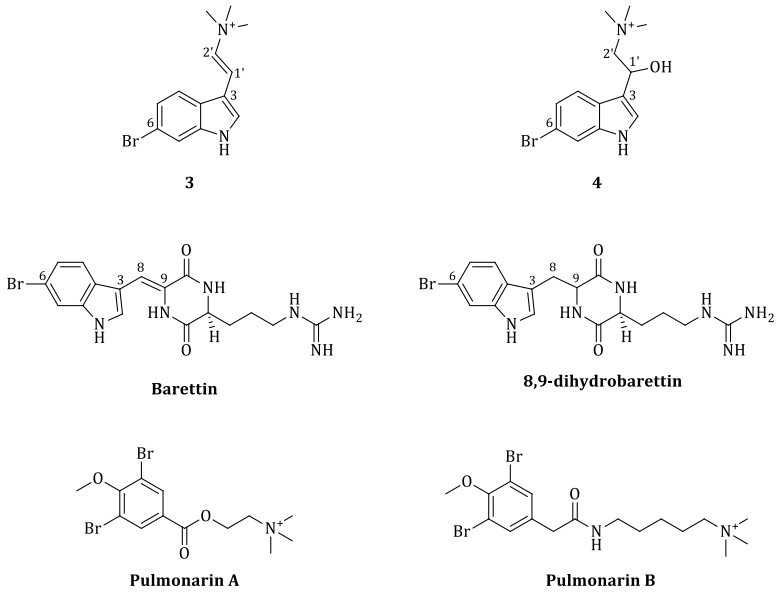


Figure 7. The four isolated compounds presented in Paper II. Pulmonarin A and B are included for structural comparison.

The ability of the isolated compounds presented in Paper II to inhibit acetylcholine esterase (AChE) was predicted from their structures. Compounds **3** and **4** had the same positively charged quaternary ammonium group as the reported AChE inhibitors pulmonarin A and B (Figure 7), and was therefore subjected to the AChE inhibition assay.[83] The guanidyl group of barettin and 8,9-dihydrobarettin is positively charged at physiological pH and these compounds were therefore also examined for AChE inhibition. 8,9-dihydrobarettin displayed the highest AChE inhibiton followed by barettin and **3**, while **4** was inactive. The combination of barettin and 8,9-dihydrobarettin has previously displayed increased antifouling activity resulting from a synergistic effect between the two compounds. Hence, it would be interesting to see if this effect could be also observed for their AChE inhibition.[84]

The AChE inhibition by barettin and 8,9-dihydrobarettin is pharmaceutically interesting since both compounds have IC_{50} values similar to other marine AChE inhibitors and the FDA approved drug galanthamine.[83,85,86] Barettin displays a range of bioactivities that can be exploited separately, such as developing it towards an anti-inflammatory agent. Alternatively, the combined bioactivities of barettin could be utilised for the treatment of conditions such as atherosclerosis and Alzheimer's disease. Atherosclerotic compounds seem to benefit from having combined antioxidant and anti-inflammatory properties. Meanwhile for Alzheimer's disease, the combination of 5-HT_4 antagonism, antioxidant activity and AChE inhibition can be beneficial.[87-89]

The BIs isolated from *G. barretti* were screened in the same bioassays as the BPs reported in Paper I, revealing that the BIs and BPs have different bioactivity profiles. The biosynthesis of diverse NPs most likely reflects the habitat where the different organisms live. Being a deep-water sponge *G. barretti*, is not exposed to tidal variations, and the resulting environmental stress, like *V. lanosa*.

4.1.3 Paper III: isolation of a novel natural product from Geodia macandrewii

Compound **1**, reported in Paper III, was identified as a N-Acyl-Taurine (NAT) and inhibited melanoma and normal lung fibroblasts in a µM range. It shares structural similarities with other NATs (Figure 8), that are reported to inhibit proliferation of a prostate cancer and breast cell line.[90] Based on that all the NATs presented in Figure 8 inhibited several cell lines, it was assumed that the NATs do not exert a specific inhibitory activity.

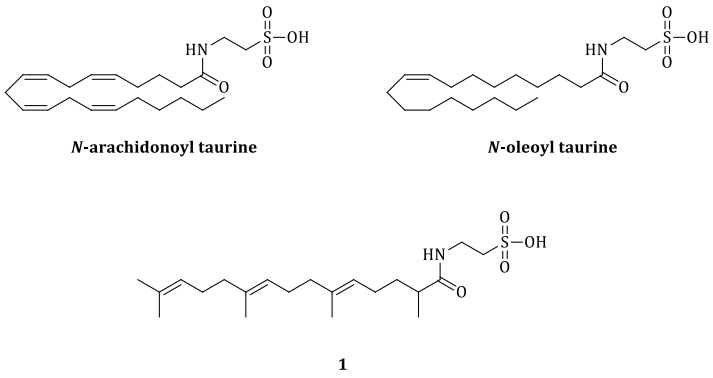


Figure 8. Molecular structures compound **1** reported in Paper III and two bioactive *N*-acyl taurines reported in the literature.

4.1.4 Occurrence of different natural products in two closely related marine organisms

G. barretti is a white sponge with a generally smooth surface.[91] The sponge surface is generally free from attached organisms, which has been associated with its biosynthesis of the antifouling NPs barettin and 8,9-dihydrobarettin.[82,84] *G. macandrewii* is also a member of the *Geodiidae* family. As they are closely related to each other, the sponges could share similar biosynthetic machinery and therefore produce structurally similar NPs. Barettin has been isolated from both *G. macandrewii* and *G. barretti*, and they both have generally fouling free surfaces (see Figure 9). However, as presented in Paper III, *G. barretti* produces larger amounts of barettin compared to *G. macandrewii*.

Compounds **3** and **4**, presented in paper II, were found in all six samples (four *G. barretti* and two *G. macandrewii* samples). These two BIs showed species variations since *G. barretti* contained a larger amount of both compounds than *G. macandrewii*. Additionally, the two BIs seem to display geographical variations since *G. barretti* samples collected from the southern-most locations had the highest concentration of both compounds.

A B

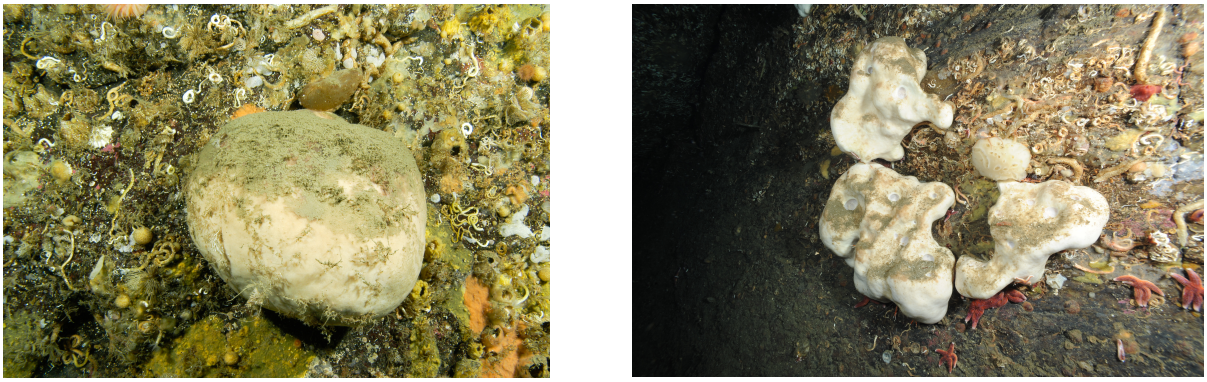


Figure 9. Picures of (A) *G. barretti* (Photo: Sten-R Birkely, Marbank) and (B) *G. macandrewii* (Photo: Bjørn Gulliksen).

G. macandrewii was found to produce one unique compound, not found in *G. barretti* and not previously reported in the literature. The compound was tested in several bioassays and was found active against the melanoma and normal lung fibroblasts cell lines. The difference in NPs produced demonstrates that, even though *G. barretti* and *G. macandrewii* are closely related organisms, they have different metabolite profiles. This can be rationalised by the fact that they live in slightly different habitats, have diverse predators or dissimilar associated microbial populations. These factors could also explain why some samples of *G. barretti* contained more of the two BIs than others. Differences in sponge biosynthesis of NPs are known to vary with location and season. Additionally the production of NPs may be induced by external influences, e.g. pressure, wounding, defence against infection and/or overgrowth by other organisms.[92-99]

4.2 The supply issue: providing sufficient amounts of marine natural products

A steady supply of marine NPs is essential for their development into commercial products. The amounts of compound necessary for the different steps of the drug discovery pipeline vary. For the structure elucidation and initial bioactivity screening a couple of milligrams can be sufficient. The amount of NP required for preclinical and clinical trials would be in the gram-range, although this depends on the compound's potency and application area. If a NP is developed into a clinically available drug, it can be required on a tonne scale.[17]

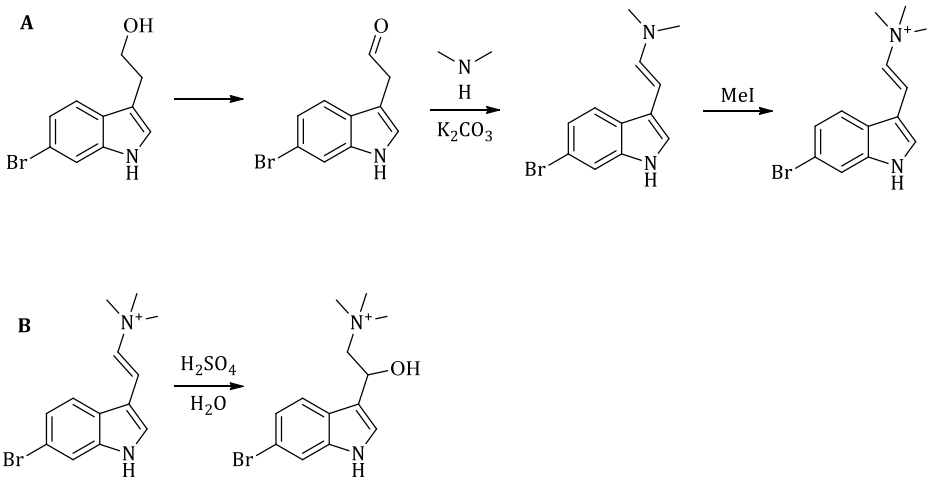
The supply issue is a recurrent challenge in marine bioprospecting as organisms may not be readily accessible and/or the amount of bioactive NPs in the organism is scarce. Wild harvesting of organisms is employed for the initial collection, however it is not always suitable for larger subsequent collections which may cause over-harvesting and, in the worst case, extinction of the organism. Today there are a number of different methods to ensure a steady supply of bioactive marine NPs. It has been stated that there is no generic recipe for success and no straightforward prediction of a given process. Rather the method should be evaluated for its cost-efficient, sustainable and reliable supply of NPs in the time-scale of drug development and production. [100]

4.2.1 Total synthesis and semi-synthesis to increase the amount of scarce marine natural products or to explore structure-activity relationship

Even though marine NPs often display substantial structural complexity, total synthesis is often reported shortly after they are discovered.[25] Total synthesis is probably the most common method for increasing the supply of marine NPs, however there are limitations regarding which compounds may be synthesised. Complex compounds with several stereocentres can be challenging to synthesise and additionally multistep and large-scale synthesis may not be economically favourable.[101] Despite these challenges, total synthesis has, in some cases, provided a viable route to large-scale production of bioactive marine NPs. An example is discodermolide, first isolated from the sponge *Discodermia dissolute*.[102,103]

A steady supply of the structurally simple BIs and DKPs from *G. barretti*, reported in Paper II, could be provided by total synthesis. Johnson and co-workers has reported the

synthesis of barettin and 8,9-dihydrobarettin, while no reports could be found for the synthesis of compounds **3** and **4**.[104] Following are suggestions to synthetic approaches of **3** and **4**, where the synthesis schemes are outlined in Schemes 1A and 1B respectively. Oxidation of 6-bromo-1H-Indole-3-ethanol, using Dess-Martin periodinane as an oxidizing agent, gives 6-bromo-3-indole acetaldehyde.[105] Treating the resulting aldehyde with K_2CO_3 and dimethylamine to yield the dimethylated amine followed by methylation using MeI is a plausible mechanism for the synthesis of **3**.[106] The hydration of compound **3** to **4** is not trivial but it could be envisioned that an acid catalysed addition of water may achieve this.



Scheme 1. Suggestions of a synthesis approaches to produce **3** (A) and **4** (B) in Paper II

Total synthesis is not exclusively used to increase the amount of a scarce NP. It is also useful for the production of chemical libraries for SAR studies. Such libraries commonly consist of structurally similar compounds based on the same core structure. By systematically varying different parts of the structure, followed by bioactivity profiling, analogues that are more active/less toxic can be identified. During the synthesis process a pharmacophore, the part of a molecule that is essential for the activity, should be identified. In some cases the identification of a pharmacophore can lead to the production of analogues that have an improved activity, however have a less complex structure than their NP lead compound. The discovery of eribulin, a more potent and structurally simpler analogue of the complex marine NP Halichondrin B is a commonly cited example.[22,25,107,108]

In Paper II a small SAR study, using the core structure of the two isolated BIs, is reported. Influence of the bromine position on AChE inhibition was explored using 4-, 5-, 6- and 7-bromoindoles as starting materials. The chosen synthetic route contains four steps starting from the BI and ending with the formation of a quaternary ammonium substituent in the 3-position. 6-bromoindole was initially used to optimise the reaction conditions required for each step. To increase the number of different compounds in the library a sample from every step of the synthesis was retained and tested. This allowed the SAR comparison of compounds that shared a high degree of structural similarity (e.g. nitrovinyl vs. nitroethyl in the 3-position) and compounds with a smaller degree of structural similarity (nitrovinyl vs. a quaternary ammonium group in the 3-position) (Figure 10).

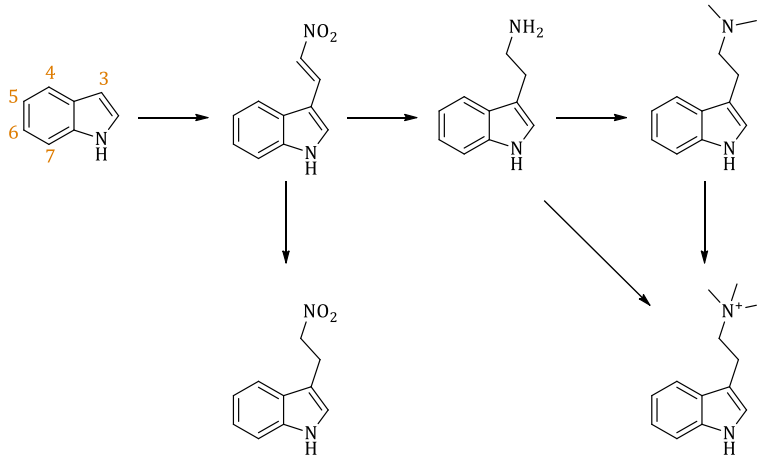


Figure 10. An illustration of the synthetic library presented in Paper II, the 3 to 7-positions are highlighted. Compounds were either non-brominated or monobrominated in the 4, 5, 6 or 7 position.

4.2.2 Semisynthesis; a combination of biosynthesis and chemical synthesis

Some marine NPs may be difficult to synthesise due to their complex structures, and such compounds can, in some cases, be made by semisynthesis. This is a combination of biosynthesis and chemical synthesis.[109] The technique can utilise the production of complex chemical structures from easily cultivated microorganisms. This approach is particularly useful when target compounds contain defined stereochemical features or if unstable or complex intermediates must be produced to generate the desired compound. Semisynthesis has played an important role in the industrial large-scale supply of drugs like trabectedin. The first step of the trabectedin semisynthesis involves

fermentation of the bacterium *P. fluorescens* to yield the precursor cyanosafracin B. Cyanosafracin B is then subjected to a chemical synthetic route to yield the final drug.[110]

4.2.3 Wild harvesting and aquaculture

For rapidly growing and abundant organisms, wild harvesting might yield sufficient amounts of a desired NP for pre-clinical experiments and commercial products. Aquaculture and fermentation are valuable alternatives. Aquaculture can be described as farming of aquatic organisms, and can be performed in-sea, in-lake or on-land. Factors that vary in nature, like food supply, temperature and light, are controlled under aquaculture conditions. Regulating and optimizing these factors can increase the production of NPs. Aquaculture has been utilised to increase the supply of several marine NPs like bryostatin 1 from the bryozoan *Bugula neritina*.[32,111,112]

The steady supply of compound **2** presented in Paper I can be ensured by wild harvest of *V. lanosa.* This macro alga is an epiphyte most frequently associated with the brown algae *Ascophyllum nodosum* (see Figure 11).[113] Furthermore, *A. nodosum* has been successfully harvested in large-scale both in Norway and abroad.[114,115] Since *V. lanosa* can be considered a byproduct when harvesting *A. nodosum* it would be environmentally friendly to exploit the harvest of a company that only utilises *A. nodosum.*

Figure 11. A picture of *V. lanosa* on its host *A. nodosum*. Photo: Robert Johansen, Marbank

In several cases the true producers of marine NPs have shown to be associated microorganisms. Bryostatin 1 is produced by colonies of *B. neritina* only when a particular form of the symbiotic microorganism, *C. endobugula sertula,* is present.[17] Dolastatin 10 was first isolated from the gastropod mollusc *Dolabella auricularia*.[57] It was later revealed that dolastatin 10 was originally produced by a marine cyanobacterium and accumulated by *D. auricularia* through its diet.[57,116] Before scaling up the production of marine NPs the producing organism should be identified. The fermentation of microorganisms producing NPs is an alternative to aquaculture of the host organism. Large-scale fermentation of the marine-sediment actinomycete strain *S. tropica* has been used to provide quantities of the cytotoxic compound salinosporamide A.[25] Sponges are often associated with microorganisms that produce NPs, and this may also be the situation for compound **1** (Paper III). If a microorganism does indeed produce the compound, fermentation would be the most optimal method for a steady supply of **1**.

A drawback of both aquaculture and fermentation is that the production of NPs may be difficult to reproduce. A large proportion of marine microorganisms cannot be cultured with current techniques. In addition, microbial cultures may display low growth rates and produce low concentrations of NPs.[17,21] A reason for the low reproducibility may be that a host-microbe interaction is essential for the production of specific NPs.[25] There are however various large EU projects focusing on improving cultivation techniques to overcome the challenges associated with growing marine bacteria. [117,118]

4.3 Evaluating the potential of the isolated natural products as products on the market

Related to the application of other antioxidants, compound **2** reported in Paper I could be of interest as a dietary supplement. Research has not established that antioxidant dietary supplements prevent diseases, according to the U.S National Center for Complementary and Integrative Health, however a diet rich in antioxidants e.g. vegetables and fruits is considered healthy.[119]

Barettin, 8,9-dihydrobarettin and the BIs isolated from *G. barretti*, presented in Paper II, share structural similarities. To determine the structural features beneficial for

inhibiting AChE, and to provide guidance for future AChE inhibitor development, a SAR study of 22 BIs was performed. The SAR study revealed that barettin and 8,9-dihydrobarettin were the most potent AChE inhibitors, and they may have potential as drugs against Alzheimer's disease.

4.3.1 Exploring the influence of brominated indoles' structural features on acetylcholine esterase inhibition

AChE's principal function is termination of the nerve impulse by rapid hydrolysis of acetylcholine in the synapse and neuromuscular junction. At the surface of AChE lies the peripheral anionic site (PAS), which binds acetylcholine as a first step of the catalytic pathway. Additionally the PAS binds quaternary inhibitors having structures that resemble acetylcholine.[120,121] The gorge of AChE containing the active site is displayed in Figure 12.

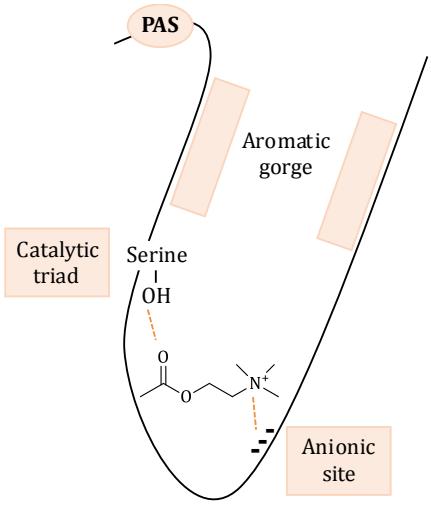


Figure 12. An illustration of the AChE gorge depicting the location of the active site, aromatic entry point of the cavity and PAS. The binding of acetylcholine to the active site is displayed.

The PAS contains an aromatic and an anionic site, where the former bind ligands by pi-stacking or π-interactions and the latter through ionic interactions or formation of hydrogen bonds.[121,122] In Paper II AChE inhibition of compounds having a different degree of ionisation, hydrogen bonding and hydrophobic groups were explored (Figure 13). The 9 series has a permanently positively charge. Whilst at physiological pH, also maintained in the AChE assay, the amine substituents of series 7 and 8 are likely to be

positively charged.[123] As displayed in Figure 13, the positively charged BIs containing a bromine substituent in the 4-position were found to be the most potent AChE inhibitors. The observation of series 7-9 being the most potent inhibitors was suspected as these can form ionic interactions with the PAS of the AChE enzyme. Only the BIs featuring a positively charged amine in combination with a bromine substituent in the 4-position displayed potent AChE inhibition, which indicate that a bromine substituent in this position is beneficial for binding.

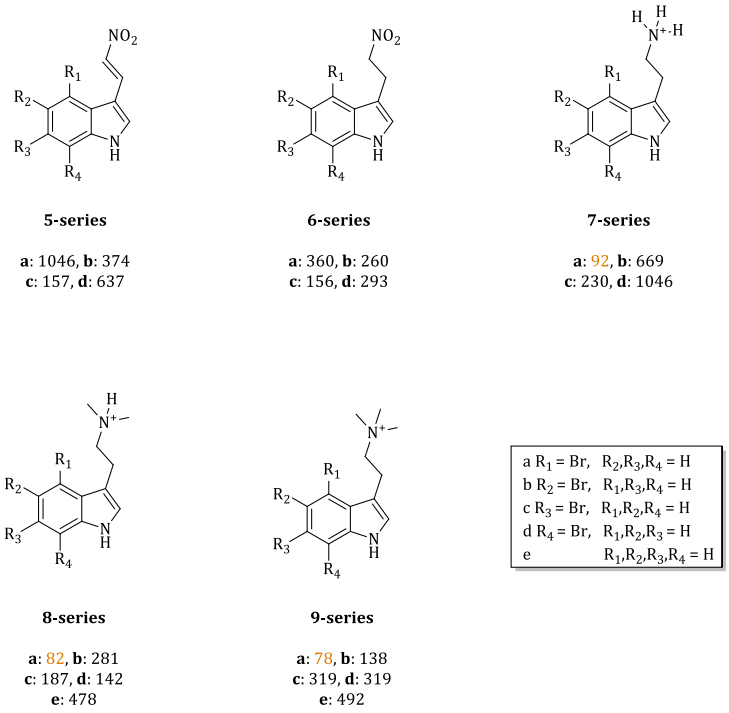


Figure 13: An illustration of the synthetic library and their IC_{50} values of AChE inhibitions are presented in μM. The most potent AChE inhibitors of the synthetic BIs are marked.

Halogenation is known to influence bioactivity and it is a valuable parameter to include in a SAR study. Halogens are bulky groups and can influence volumetric and conformational changes of a compound.[124,125] These changes may improve the compounds' way of occupying the binding site of a molecular target or strain the compound into a beneficial conformation.[125] Non-brominated analogues of the tertiary and quaternary ammonium BIs were synthesised (Figure 13). When comparing the IC_{50} values, the brominated analogues in series 8 and 9 are more potent AChE inhibitors than the non-brominated. The presence of a halogen atom in a molecule has on several

occasions been associated with increased potency. One example is barettin and its debrominated analogue, where the former has antifouling bioactivity and is able to decrease lipid peroxidation within cell membranes, while the latter is relatively inactive in the same assays.[126,127]

The SAR study of the BIs indicated that the brominated indole system alone was not sufficient for AChE inhibition. The structurally similar, barettin and 8,9-dihydrobarettin proved to be the most potent AChE inhibitors when compared with their simplified analogues. It was rationalised that the larger compounds are involved in additional interactions, which may be the reason why they are more potent AChE inhibitors than the synthetic BIs.

4.3.2 Possible follow-up studies for barettin and 8,9-dihydrobarettin; ADMET, bioavailability and drug-likeness properties

A compound intended as a drug should not only have to be potent, it also needs to reach the target site in the body, at sufficient concentration, and within a reasonable time. Hence, it is critical to explore a compound's absorption, distribution, metabolism, excretion and toxicity (ADMET) properties. The oral route is probably the most abundant and traditional way of delivering drugs. A balance of potency, selectivity and ADMET will ultimately determine the compound's potential as an oral agent.[128] Considering ADMET properties at an early stage of the bioprospecting pipeline is advantageous as it guides the process towards practically viable compounds that could have commercial potential.

Drugs have to be bioavailable for the body to utilise them, and bioavailability can be defined as the "rate and extent to which the active ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action".[62] The bioavailability of a compound after ingestion is largely influenced by its chemical structure.[62,129,130] Several molecular properties have been suggested to affect bioavailability, and different guidelines are developed to indicate the drug-likeness of a compound.[131] Veber and co-workers have presented a set of guidelines, while the rule of five (Ro5) presented by Lipinski has been commonly recognised by the pharmaceutical industry as a method for identifying drug-likeness of orally administered

compounds.[129,132-134] A limitation with the Ro5 is that it does not apply to NPs. It has been suggested that this is because NPs have evolved to use active transport or developed conformational features that are beneficial for passive transport.[133]

Barettin display the possibility to penetrate cells by being active in two cell-based, one antioxidant and one anti-inflammatory, assays.[87] 8,9-dihydrobarettin shares a high degree of structural similarity with barettin, and it is therefore probably that it also penetrate cell membranes. Subjecting 8,9-dihydrobarettin to cell-based bioassays would reveal this. However, even though cellular assays are more relevant for *in vivo* conditions than biochemical ones, additional studies of ADMET and bioavailability have to be performed to establish if the compounds have a future as drugs.

4.4 Future perspectives

Paper I: Compound **2** displayed antioxidant activity and the ability to penetrate cell membranes, and could therefore be an interesting hit for further exploration as a possible dietary supplement. If **2** is found to have beneficial ADMET properties a SAR study could explore how cellular antioxidant activity is affected by the BP's degree of hydroxylation and bromination.

Paper II: The results from this paper provide a good starting point for several follow-up studies. Barettin and 8,9-dihydrobarettin should be subjected to ADMET studies as a next step to consider their potential as drugs against Alzheimer's disease. The possibility of a barettin and 8,9-dihydrobarettin having a synergistic effect in AChE inhibition should also be investigated. Expanding the biological profiles of the isolated NPs and the synthetic library is an option. Additionally, more synthetic analogues could be synthesised in order to explore the BIs' SAR. Analogues that feature a permanent positive charge other than the quaternary amine functionality could be incorporated (e.g. a guanidine moiety). The substitution pattern of the indole phenyl ring could also be explored, both in terms of the functionality (e.g. –Cl, –F, –I, –OH, $–NO_2$, $–CH_3$) and the degree of substitution

Paper III: A novel compound was isolated from *G. macandrewii* using a metabolomics approach. In order to expand the biological profile of compound **7**, it could be submitted

to assays in which structurally analogous compounds have demonstrated activity. Metabolomics proved to be a successful approach for discovering new marine NPs and this method should be incorporated in future marine bioprospecting projects.

5. Concluding remarks

The work in this thesis demonstrates that bioassay-guided isolation is a suitable approach for the discovery of bioactive marine natural products. Employing the chemistry-guided approach yielded compounds that served as inspiration for a synthetic library and metabolomics provided a novel compound whose bioactivity profile should be further investigated.

Bioactive natural products are of interest and these are most efficiently discovered using the bioassay-guided isolation. This approach selects bioactive compounds early in the pipeline and should be combined with Veber's guidelines and ADMET properties to increase the possibility of yielding a potential commercial product. In addition, chemistry- and metabolomics- guided approaches are valuable for isolation of compounds with novel chemistry and compounds that are species specific, respectively.

Bioactive natural products derived from marine sources have resulted in a diverse array of commercial products. It is to our benefit that these molecules can also provide solutions to problems facing humanity. Given the rich biodiversity, yet unexplored nature of the ocean, this trend is expected to continue.

6. References

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