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# Isolation and Characterisation of Bioactive Secondary Metabolites from Arctic, Marine Organisms

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*A dissertation for the degree of Philosophiae Doctor – December 2014*







## Summary

The list of achievements in developing pharmaceuticals and other useful products originating from Nature is long, and it continues to extend. Marine bioprospecting alone has generated three novel drugs in the last four years. The continued success motivates further bioprospecting efforts in the search for utilisable products from natural sources.

In this thesis, extracts of Arctic marine organisms were screened for bioactivity. Bioactive extracts were dereplicated using high-resolution mass spectrometry (HR-MS) in an attempt to identify the component(s) responsible for the observed bioactivity and later isolate them using mass guided fractionation. This approach led to the isolation of ianthelline, a previously reported bromotyrosine-derived compound from the sponge *Stryphnus fortis* as well as two novel, highly modified dipeptides, breitfussin A and B, from the hydrozoa *Thuiaria breitfussi*.

Even though the structure of ianthelline was reported in 1986 by Litaudon et al. very limited bioactivity data was available. The potential of ianthelline as an anticancer agent was explored in paper I. Ianthelline inhibited cellular growth in a dose- and time dependent manner by several mechanisms, including inhibition of mitotic spindle formation and inhibition of protein kinase activity. In paper II, ianthelline was investigated for antibiofouling activity. The compound was found to inhibit all the major stages of the biofouling process, with the main effect being inhibition of marine bacterial growth and the settlement of barnacles.

By dereplication, breitfussin A and B were suspected to be novel compounds, with proton poor molecular compositions of  $C_{16}H_{11}N_3O_2BrI$  and  $C_{16}H_{11}N_3O_2Br_2$ , respectively. The high ratio of heavy atoms to protons, in addition to low isolation yields, complicated the structure elucidation of the two compounds. In the end, their structures could be elucidated using a combination of HR-MS analysis, nuclear magnetic resonance (NMR) spectroscopy, computer-assisted structure elucidation (CASE)- and density functional theory (DFT) calculations as well as atomic force microscopy (AFM) imaging. This represents the first example of AFM as a tool used for structure elucidation of a novel, natural product.

## Acknowledgements

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Tromsø, October 2014.

Kine Østnes Hanssen

## List of publications

This thesis is based on the following publications. They are referred to by their roman numerals in the text.

### Paper I

K. Ø. Hanssen, J. H. Andersen, T. Stiberg, R. A. Engh, J. Svenson, A.-M Genevière, E. Hansen (2012) Antitumoral and Mechanistic Studies of Ianthelline Isolated from the Arctic Sponge *Stryphnus fortis*. *Anticancer research*, **32**, 4287 – 4297.

### Paper II

K. Ø. Hanssen, G. Cervin, R. Trepos, J. Petitbois, T. Haug, E. Hansen, J. H. Andersen, H. Pavia, C. Hellio, J. Svenson (2014) The Bromotyrosine Derivative Ianthelline Isolated from the Arctic Marine Sponge *Stryphnus fortis* Inhibits Marine Micro- and Macrobiofouling. *Marine Biotechnology*, 10.1007/s10126-014-9583-y.

### Paper III

K. Ø. Hanssen, B. Schuler, A. J. Williams, T. B. Demissie, E. Hansen, J. H. Andersen, J. Svenson, K. Blinov, M. Repisky, F. Mohn, G. Meyer, J.-S. Svendsen, K. Ruud, M. Elyashberg, L. Gross, M. Jaspars, J. Isaksson (2012) A combined Atomic Force Microscopy and Computational Approach for the Structural Elucidation of Breitfussin A and B: Highly Modified Halogenated Dipeptides from *Thuiaria breitfussi*. *Angewandte Chemie International Edition*, **51**, 12238-12241.

## Abbreviations

1D	One-dimensional
2D	Two-dimensional
AFM	Atomic force microscopy
CASE	Computer-assisted structure elucidation
C <sub>18</sub>	Octadecyl
CoA	Coenzyme A
COSY	Correlation spectroscopy
Da	Dalton
DFT	Density functional theory
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethylsulfoxide
ESI	Electrospray ionisation
FDA	Food and drug administration
HMBC	Heteronuclear multiple-bond correlation
HMQC	Heteronuclear multiple-quantum coherence
HPLC	High performance liquid chromatography
HR-MS	High resolution mass spectrometry
HTS	High throughput screening
IC <sub>50</sub>	Half maximal inhibitory concentration
IPP	Isopentenyl diphosphate
LC	Liquid chromatography
LogP	Octanol-water partition coefficient
MIC	Minimum inhibitory concentration
MabCent	Centre for research-based innovation on marine bioactivities and drug discovery
Marbank	The Norwegian national marine biobank
Marbio	The Norwegian national screening platform
Mw	Molecular weight
MS	Mass spectrometer
NFH	Norwegian college of fishery science
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
NRPS	Nonribosomal peptide synthetases
PKB $\alpha$	Protein kinase B $\alpha$
PKB $\beta$	Protein kinase B $\beta$
PKS	Polyketide synthases
Prep-HPLC	Preparative high performance liquid chromatography
Rf	Radio frequency
RP	Reversed phase
RO5	Lipinski's rule of five
SCUBA	Self-contained underwater breathing apparatus
SGK1	Serum and glucocorticoid-induced kinase 1
TAK1	Transforming growth factor beta activated kinase 1
ToF	Time-of-Flight
UHPLC	Ultra-high performance liquid chromatography
UiT	UiT the Arctic university of Norway
UV	Ultra violet

## TABLE OF CONTENTS

<b>Summary</b> .....	<b>i</b>
<b>Acknowledgements</b> .....	<b>ii</b>
<b>List of publications</b> .....	<b>iii</b>
<b>Abbreviations</b> .....	<b>iv</b>
<b>1. Introduction</b> .....	<b>1</b>
1.1 <i>Bioprospecting</i> .....	1
1.1.1 <i>The marine environment</i> .....	1
1.1.2 <i>Natural products: Primary- and secondary metabolites</i> .....	2
1.1.3 <i>Secondary metabolites: Natural functions</i> .....	4
1.1.4 <i>Secondary metabolites: Biosynthesis</i> .....	4
1.1.5 <i>Secondary metabolites: Chemical properties</i> .....	7
1.1.6 <i>Marine secondary metabolites as pharmaceuticals</i> .....	10
1.1.7 <i>Marine secondary metabolites as antifouling agents</i> .....	13
1.2 <i>The bioprospecting pipeline</i> .....	16
1.2.1 <i>Bioassay-guided isolation exemplified by the workflow at MabCent</i> .....	16
1.2.2 <i>Prefractionation</i> .....	16
1.2.3 <i>Bioassays and high throughput screening</i> .....	17
1.2.4 <i>Dereplication</i> .....	20
1.2.5 <i>Isolation</i> .....	21
1.2.6 <i>Structure elucidation</i> .....	23
1.2.7 <i>Bioactivity profile of isolated compounds</i> .....	28
1.2.8 <i>Commercialisation of natural products</i> .....	29
<b>2. Aim of the thesis</b> .....	<b>31</b>
<b>3. Summary of papers</b> .....	<b>32</b>
<b>4. General discussion</b> .....	<b>35</b>
4.1 <i>Sample supply</i> .....	35
4.2 <i>Macroorganism associated microorganisms</i> .....	37
4.3 <i>Hit rate</i> .....	39
4.4 <i>Crude extract preparation</i> .....	40
4.5 <i>Bioactivity detection in prefractionated crude extracts</i> .....	40
4.6 <i>Dereplication of bioactive organic extract of <i>S. fortis</i> and <i>T. breitfussi</i></i> .....	41
4.7 <i>Preparation of samples for mass guided prep-HPLC isolation</i> .....	42
4.8 <i>Isolation of ianthelline and the breitfussins</i> .....	43
4.9 <i>Structure elucidation</i> .....	46
4.10 <i>Bioactivity profiling of ianthelline</i> .....	48
4.11 <i>Further work</i> .....	51
<b>5. Concluding remarks</b> .....	<b>52</b>
<b>6. References</b> .....	<b>53</b>

## Paper I, II and III

# 1. INTRODUCTION

## 1.1 Bioprospecting

Bioprospecting (or biodiversity prospecting) is the systematic search for- and utilisation of applicable products in Nature with the purpose of developing commercial products.<sup>1,2</sup> The products found through bioprospecting can be categorised in the following way: chemicals (e.g. for drug development, agrochemicals and cosmetics), genes (recombinant pharmaceutical proteins, enzymes and agricultural biotechnology) and designs (e.g. architecture and mechanical engineering).<sup>1</sup> In contrast to the direct value obtained from harvesting and consuming natural resources, such as timber harvesting, mining and oil extraction, bioprospecting is a value-added approach, where the natural product often holds little value in itself, it is their properties which makes them economically interesting.<sup>3,4</sup> When bioprospecting is conducted as basic- rather than applied research, it is popularly termed biodiscovery. The border between bioprospecting and biodiscovery is not a clean cut, as biodiscovery may lead to the commercialisation of products and bioprospecting efforts often simply result in expanded knowledge about natural diversity.<sup>5</sup> Amongst the three main habitats (terrestrial, freshwater, and marine), terrestrial plants have been by far most extensively examined for commercially interesting chemistry.<sup>1,6</sup> This is mainly due to the fact that terrestrial resources traditionally have been far more easily accessed compared with aqueous organisms.

### 1.1.1 The marine environment

The ocean covers 71% of the earth's surface and constitute more than 90% of the habitable space on our planet.<sup>7</sup> It holds an estimated 50 – 80% of all life on earth under its surface and is home to 32 out of 33 known animal phyla, where 15 are exclusively marine.<sup>8,9</sup> The habitats found in the oceans all have one thing in common: the presence of seawater. Apart from this, the living conditions for marine species can vary greatly, both between ecosystems and within a single ecosystem,<sup>7</sup> Among the variable factors are temperature, light- and nutrient



availability, salinity, dissolved gasses, acidity, turbulence and pressure.<sup>7,10,11</sup> In addition to this, most marine organisms live in complex ecosystems, where species diversity is high. Within these densely populated areas, also known as biodiversity “hot spots”, competition for nutrition and space is high, and the threats of being overgrown by biofouling organisms, or attacked by predators and pathogens, are constant.<sup>12</sup> Many organisms, including marine sponges and corals, found within these ecosystems are sessile or slow moving and cannot escape when they are attacked by predators. Nor can they rely on a complex immune system when challenged by bacteria, fungi or viruses. Not surprisingly, these variable and challenging conditions have facilitated the evolution of rich biodiversity, with species employing diverse survival strategies, including an arsenal of highly potent chemicals to defend themselves against external threats.

The oldest written record of the utilisation of natural products for their medicinal properties is the Egyptian Ebers Papyrus, which dates back to 2900 BC.<sup>13</sup> This papyrus documents the effect of over 700 plant-based drugs.<sup>14</sup> Historically, terrestrial plants was the main source of bioactive compounds used for medical purposes, as the sessile species living below the littoral zone at large were unavailable and therefore left unexplored. Following technological advantages in the last few decades, including self-contained underwater breathing apparatus (SCUBA) diving and remotely operated deep sea vessels, the oceans could be accessed to a much wider extent than previously, making sampling of the previously inaccessible organisms possible.<sup>13</sup>

### **1.1.2 Natural products: Primary- and secondary metabolites**

All organisms need to biosynthesise a large quantity of organic compounds into functional end products in order to live, grow and reproduce.<sup>15</sup> Natural products are any substances produced by living organisms, i.e. animals, plants or microorganisms.<sup>16</sup>

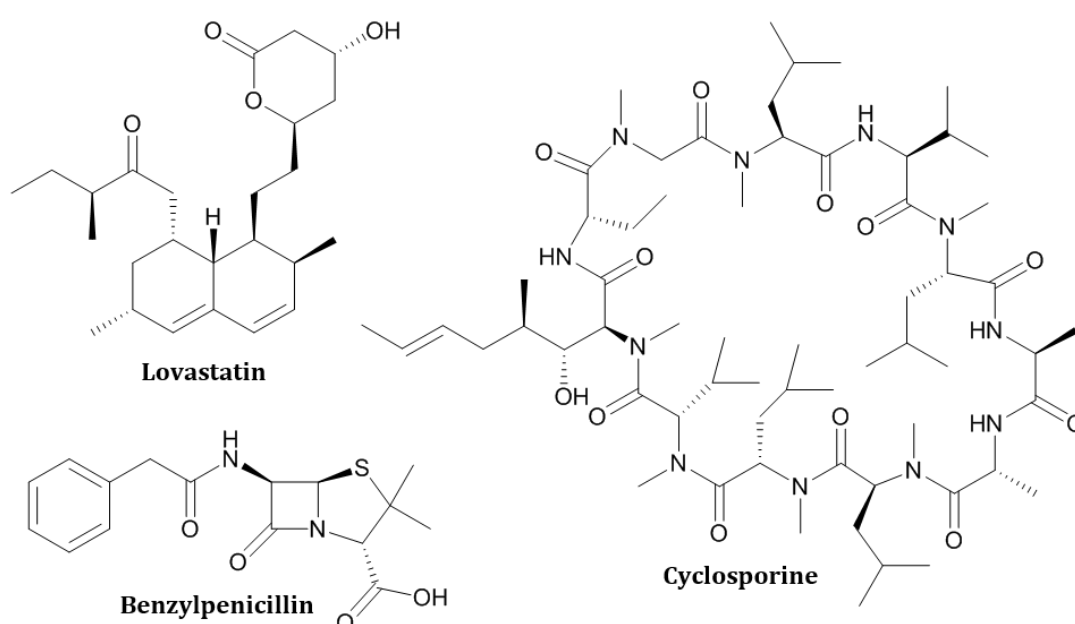
#### Primary metabolites

Some natural products represent the fundamental units of all living matter and are found (with variations) in all living organisms. These include common

carbohydrates, proteins, fats, and nucleic acids. Because of their indispensability and their extensive distribution, they are termed primary metabolites.<sup>17</sup>

### Secondary metabolites

Other products are not biosynthesised by general metabolic pathways and have a more restricted taxonomic distribution, often limited to a specific species or genus.<sup>18</sup> Marked differences in expression can even be found within a single species experiencing different environmental stress.<sup>19,20</sup> The chemical structures of some selected secondary metabolites used as drugs, can be seen in figure 1.



**Figure 1 | Chemical structures of selected secondary metabolites used as pharmaceuticals.**

Lovastatin was originally isolated from the fungus *Aspergillus terreus*.<sup>21</sup> It lowers serum cholesterol levels by competitive inhibition of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase,<sup>22</sup> a key enzyme in the cholesterol production pathway.<sup>23</sup> Cyclosporine, initially isolated from the fungus *Tolypocladium inflatum*, is used as an immunosuppressant.<sup>24</sup> Benzylpenicillin is an antibiotic agent, and was the first penicillin isolated from the fungus *Penicillium chrysogenum* (previously known as *Penicillium notatum*) by Sir Alexander Fleming in 1928.<sup>25</sup>

The natural function of secondary metabolites commonly do not affect the immediate survival of the producing organism, and they are not essential for growth, respiration, storage and reproduction. They rather influence long-term survival by affecting the organism's interactions with its surrounding environment. Because of this, they are termed secondary metabolites.

### **1.1.3 Secondary metabolites: Natural functions**

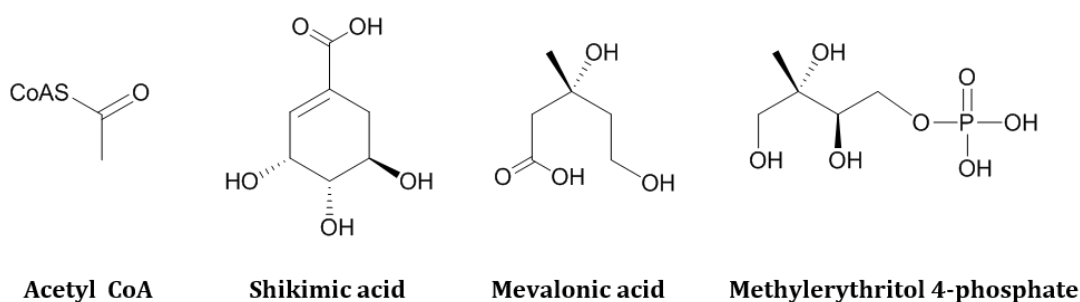
While the primary metabolites have essential structural or metabolic functions that are at large evident, the function of secondary metabolites are not always obvious. In fact, it was previously widely perceived that they were biologically insignificant and served no particular function.<sup>26</sup> This perception is no longer valid, and secondary metabolites are now recognised as an important contributor for an organism's interaction with and response to its surroundings.<sup>27</sup> In the vast majority of cases, the functions of each compound and their benefits to the organism are not precisely understood, but their molecular modes of action are being increasingly elucidated<sup>28</sup>. The natural activities include protecting the organism against biofoulers<sup>29,30</sup>, pathogens such as bacteria, fungi and viruses,<sup>31</sup> predators,<sup>32,33</sup> and against abiotic stress (Ultra violet (UV)-protectors, heavy metals, drought),<sup>34</sup> to increase virulence,<sup>35</sup> as well as improving competitiveness for space,<sup>36,37</sup> or increasing the chances to reproduce.<sup>38</sup>

### **1.1.4 Secondary metabolites: Biosynthesis**

Biosynthesis of secondary metabolites is initiated by a variety of environmental triggers. As many of these external factors are constantly changing, so is the expression of secondary metabolites under specific seasons, stress, nutrient availability, and developmental stages of the organism.<sup>39</sup> The triggering factors are commonly referred to as elicitors and can be of abiotic- (e.g. high or low temperatures, pressure, light availability, drought, salinity and UV-stress)<sup>34</sup> or biotic (fungi, yeast, bacteria, predation)<sup>40</sup> origin. In general, the biotic triggers are chemicals or biofactors interacting with plasma- or endomembrane receptors. Elicitor signal transduction leads to biosynthesis- or activation of transcription factors. These in turn regulate the expression of genes coding for the metabolic pathway enzymes needed for secondary metabolite biosynthesis.<sup>41,42</sup> The building blocks used for the biosynthesis of secondary metabolite are derived from the primary metabolism. Despite the vast chemical diversity of secondary metabolites, most are derived from intermediates of, or end products from, only four important primary metabolic pathways.<sup>17</sup> An overview of the pathways can be seen in table 1. The chemical structures of key pathway intermediates are shown in figure 2.

**Table 1 | Overview of four primary metabolic pathways important for provision of building blocks for secondary metabolism.**

Pathway	Key intermediate	End product examples	Species
Acetate	Acetyl CoA	Fatty acids and polyketides	Eukaryotes, bacteria and plants
Shikimate <sup>43</sup>	Shikimic acid	Aromatic amino acids and phenylpropanoids <sup>44</sup>	Bacteria and plants
Mevalonate <sup>45</sup>	Mevalonic acid	DMAPP and IPP, which forms the basis for terpenoids and steroids <sup>46</sup>	Higher eukaryotes and some bacteria <sup>47</sup>
Methylerythritol phosphate <sup>46</sup>	Methylerythritol 4-phosphate		Bacteria and plants <sup>47</sup>

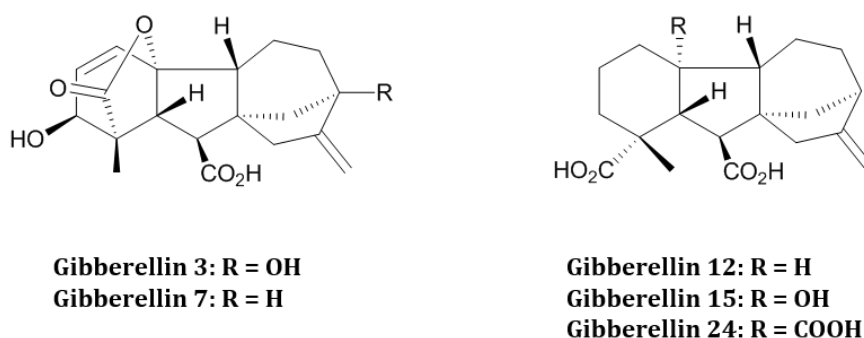


**Figure 2 | Chemical structures of key building blocks acetyl CoA, shikimic acid, mevalonic acid and methylerythritol phosphate.**

The acetate pathway has a coenzyme linked key intermediate, acetyl coenzyme A (CoA), from which the acetyl group can be conveyed into primary- or secondary metabolic pathways. The mevalonate and the methylerythritol phosphate (also known as the deoxyxylulose 5-phosphate pathway and the non-mevalonate pathway<sup>48</sup>), both lead to the production of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), by way of different key intermediates. Secondary metabolites may also be derived from a combination of products originating from different primary metabolic pathways or products resulting from modification of these. The backbones of the secondary metabolites are formed by condensation of the smaller building blocks. The resulting molecular framework

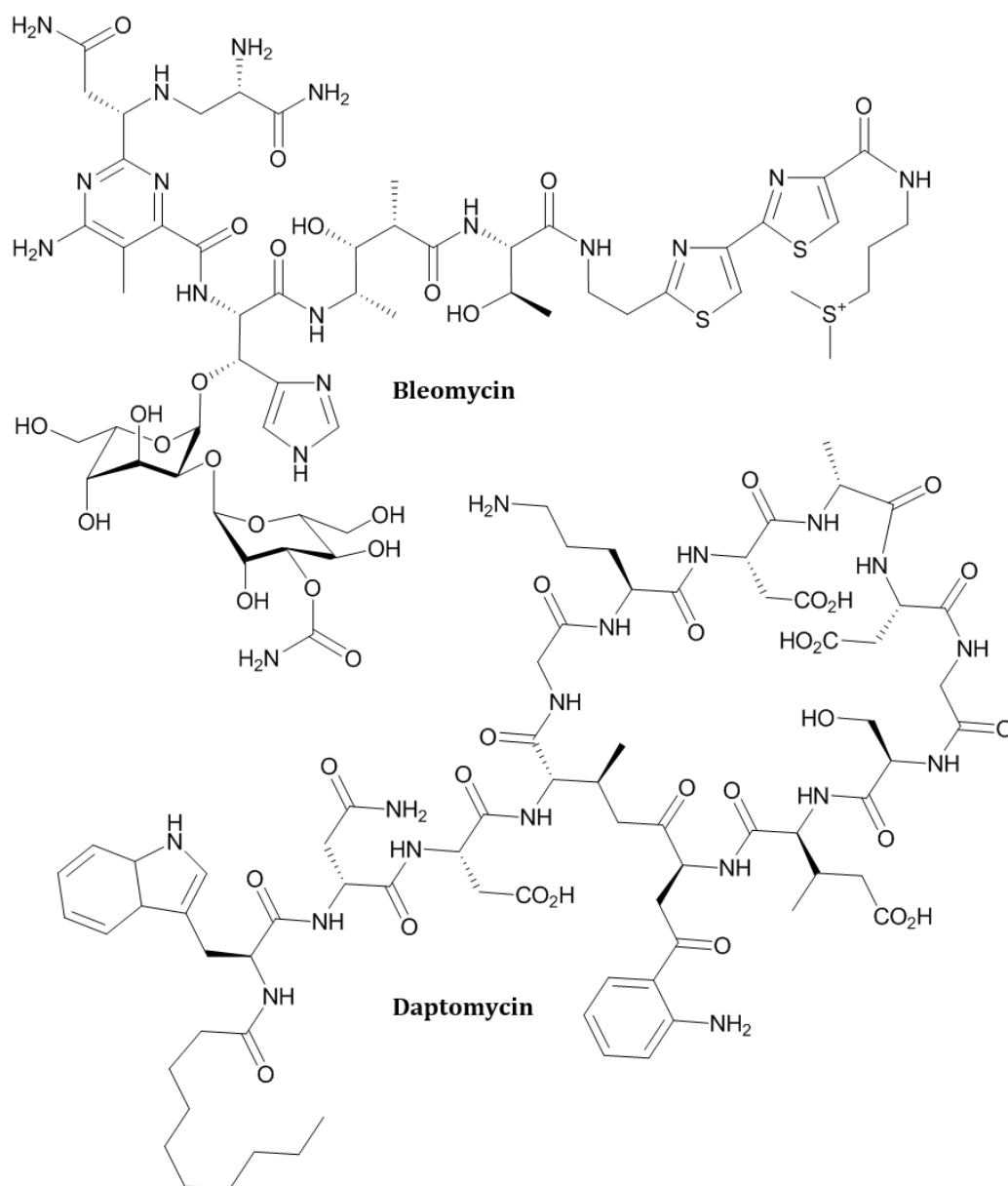
is subsequently modified by numerous enzyme-catalysed reactions, such as cyclisation, elimination, rearrangement, reduction, oxidation, methylation, and halogenation.<sup>49</sup>

This final modification of the chemical scaffolds contributes to the high degree of chemical diversity among the secondary metabolites. As opposed to primary metabolic pathways, which generally produce one (or a selected few) end product(s), secondary metabolic pathways often produce several.<sup>50</sup> This production of many similar end products is believed to be a strategy for increasing the chemical diversity and thereby increasing the chances of protein interaction.<sup>51</sup> For example, the biosynthesis of the gibberellins leads to the production of 136 closely related compounds, exemplified in figure 3.<sup>52</sup>



**Figure 3 | Chemical structures selected gibberellins.**<sup>53</sup> Gibberellin 3 and 7 have a four ring chemical scaffold and gibberellin 12, 15 and 25 display a simpler three ring chemical scaffold.

In addition, bacteria and fungi use large multifunctional enzymes, termed nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) in their biosynthesis of secondary metabolites. NRPSs produces nonribosomal peptides with broad biological activity. Many medically relevant secondary metabolites are produced by NRPSs, including  $\beta$ -lactam antibiotics and cyclosporine<sup>54</sup> (figure 1) and the lipopeptide antibiotic daptomycin<sup>55</sup> (figure 4). PKS produced secondary metabolites have also resulted in several marketed pharmaceuticals, including lovastatin (figure 1). In addition, some secondary metabolites are produced by a combination of NRPS and PKS activities<sup>56</sup> including the gibberellins<sup>57</sup> (figure 3) and the antitumour drug bleomycin<sup>58</sup> (figure 4).



**Figure 4 | Chemical structures of secondary metabolites daptomycin and bleomycin.** Daptomycin is biosynthesised in *Streptomyces roseosporus* by a NRPS.<sup>55</sup> Bleomycin is biosynthesised in *Streptomyces verticillus* by a combination of a NRPS and PKS.<sup>59</sup>

### 1.1.5 Secondary metabolites: Chemical properties

Secondary metabolites have historically been an important source of compounds with properties useful to humans, and the applications for which they are utilised go far beyond the activities they are produced to exert.<sup>60</sup> The beneficial properties include use as food, fragrances, pigments, insecticides, cosmeceuticals, nutraceuticals and pharmaceuticals.<sup>61-63</sup> The chemodiversity found in Nature has played an essential role within the field of drug discovery and development.<sup>64</sup> In



fact, approximately 60% of the pharmaceuticals marketed today are natural product-based drugs.<sup>65,66</sup>

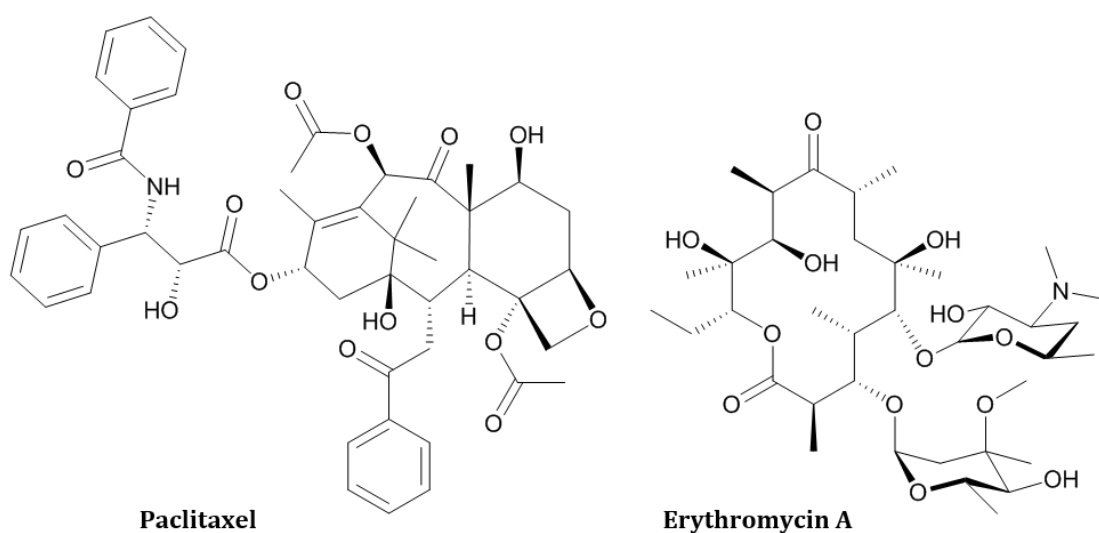
When compared to synthetic screening libraries, natural products are known to possess several chemical properties favouring them as lead structures for drug development.<sup>67</sup> This is evident even when comparing secondary metabolites to huge sample sets of compounds produced by combinatorial syntheses (combinatorial compounds), produced with the purpose of generating synthetic compound libraries with high chemical diversity.<sup>68</sup> Natural products in general have higher molecular weight (Mw), a higher number of chiral centres, ring structures, heavy atoms, hydrogen-bond donors and – acceptors, lower number of rotatable bonds, and are less lipophilic and more unsaturated.<sup>67,69,70</sup> Taken together, these differences make natural products more structurally diverse, - complex, and rigid than combinatorial compounds. It is proven that a higher number of chiral centres, decreased molecular flexibility and increased molecular size generally results in compounds with increased receptor specificity and potency.<sup>67</sup>

The Lipinski “rule-of-five” (RO5) was generated through analysis of orally active drug candidates that reached phase II clinical trials, and highlights possible bioavailability problems if more than one rule is broken.<sup>71</sup> The RO5 states that a compound is less drug-like, if it displays properties that violates more than one of the following physicochemical parameters:

- Less than five hydrogen bond donors
- Less than ten hydrogen bond acceptors
- Mw less than 500 Daltons (Da)
- A octanol-water partition coefficient (logP) of less than five

When examining 126 140<sup>72</sup> and 3287<sup>67</sup> secondary metabolites for Lipinski properties, it was found that about 80% of all displayed less than two violations of the RO5. Even so, it was also highlighted by Lipinski that most of the orally available drugs that violated more than one Lipinski rule belonged to the four therapeutic classes: antibiotics, antifungals, vitamins and cardiac glycosides. All

these classes are dominated by secondary metabolites. Lipinski suggested that the compounds in these classes display structural features allowing them to act as substrates for naturally occurring transporters.<sup>71</sup> It is believed that secondary metabolites are more subjected to active transportation than synthetic products, as biosynthetic pathways have universal features. An exogenous natural product would therefore be more similar to an endogenous ligand than a foreign synthetic compound, and thus have a higher probability of being absorbed actively by transporter protein found in for example the gut wall.<sup>73</sup> Figure 5 shows the molecular structure of two secondary metabolites used as drugs, where both violates two Lipinski rules, and one is orally available.



**Figure 5 | The chemical structures of secondary metabolite drugs paclitaxel and erythromycin A, both of which violate two Lipinski rules.** Paclitaxel is a mitotic inhibitor used in cancer chemotherapy.<sup>74</sup> With a Mw of 853 Da, a LogP of 4.49, four hydrogen bond donors and 15 hydrogen bond acceptors, it violates two of the Lipinski rules. Paclitaxel is not orally bioavailable, as it cannot passively diffuse across lipid membranes, as well as being substrate to efflux transporters like the P-glycoprotein.<sup>75</sup> The macrolide class antibiotic erythromycin A also violates two of the Lipinski rules (Mw = 733.5 Da, hydrogen bond acceptors = 14), nevertheless, it has an oral bioavailability varying from 20 – 40%.<sup>76</sup> Erythromycin A is actively transported across lipid membranes by at least two transporters belonging to the oligopeptide transporter family.<sup>77</sup>

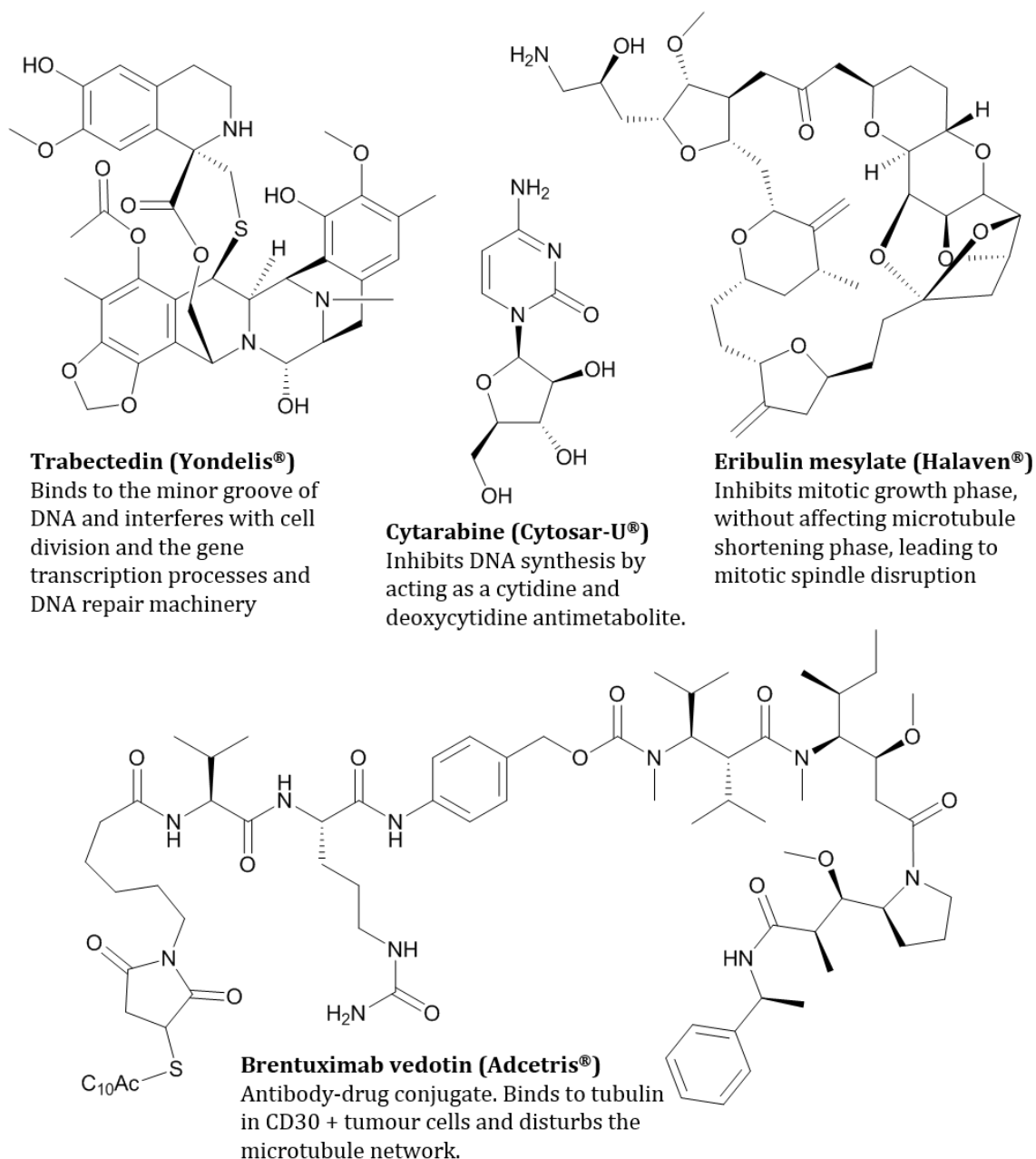
Carrier-mediated cellular uptake of pharmaceuticals is now believed to be more common than previously presumed.<sup>77</sup> It has been suggested that the R05 cannot be used when determining the drug-likeness of secondary metabolites as the rules

are based upon passive absorption through lipid membranes, and are thus no longer applicable for drugs that are actively transported across membranes.<sup>78</sup>

### **1.1.6 Marine secondary metabolites as pharmaceuticals**

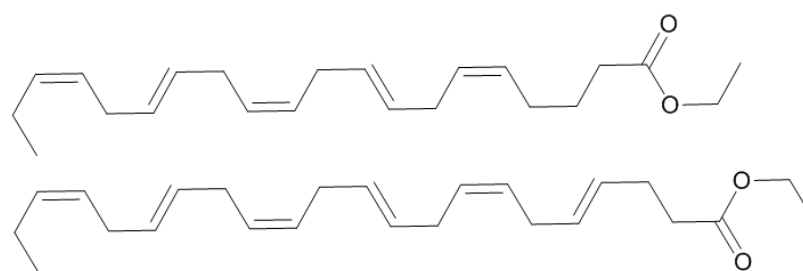
In the past decades, marine life forms have been the origin of a remarkable number of novel secondary metabolites. In fact, since the 1960s, more than 20,000 marine secondary metabolites have been characterised.<sup>79</sup> They have been isolated from macroorganisms like sponges, corals and other invertebrates, as well as from algae and microorganisms.<sup>79</sup> However, it is now becoming increasingly evident that the majority of compounds are actually produced by microorganisms associated with the collected macroorganisms.<sup>80,81</sup> The number of isolated compounds from marine sources has increased steadily, from an annual number of approximately 20 in 1984 (the total number of all novel natural products reported in 1985 was 3500<sup>39</sup>) to an annual number of more than 1000 in 2010.<sup>82</sup> As opposed to the terrestrial environment, where plants are considerably richer in secondary metabolites, marine invertebrates and bacteria have yielded substantially more bioactive natural products than marine plants.<sup>83</sup>

The total number of approved drugs from the marine environment is steadily increasing, from four in 2010<sup>84</sup> to seven in 2014.<sup>85</sup> The first U.S. Food and Drug Administration (FDA) approved marine derived drug reached the market in 1969. This was the anticancer pyrimidine analogue cytarabine (Cytosar-U<sup>®</sup>), isolated from the Caribbean sponge *Cryptotheca crypta*, which acts by interfering with DNA synthesis.<sup>63</sup> Since then, six more marine natural products have been approved as drugs (one of which is only registered in the European Union), including the analgesic cone snail derived peptide ziconotide (Prialt<sup>®</sup>), and the anticancer sponge derived macrolide eribulin mesylate (Halaven<sup>®</sup>), and four more with anticancer, antiviral and antihypertriglyceridemia activities.<sup>85</sup> The chemical structure, generic name, and pharmaceutical applications of the marketed marine secondary metabolites are shown in figure 6A and 6B.

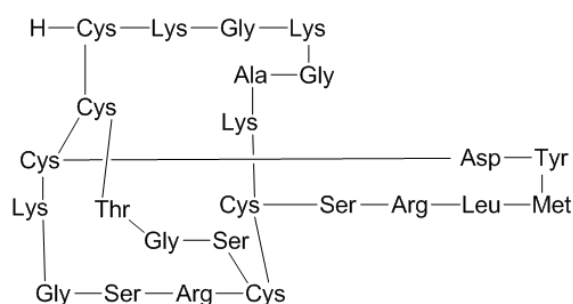


**Figure 6A | The marketed anticancer marine derived drugs.** The chemical structures and modes of action of the four marketed anticancer drugs Yondelis®,<sup>86</sup> Halaven®,<sup>87</sup> Adcetris®,<sup>88</sup> and Cytosar-U®.<sup>89</sup> In Adcetris®, the marine derived secondary metabolite is covalently attached to cAC10, a monoclonal antibody directed against CD30, a cell membrane protein used as a tumour marker.<sup>90</sup>

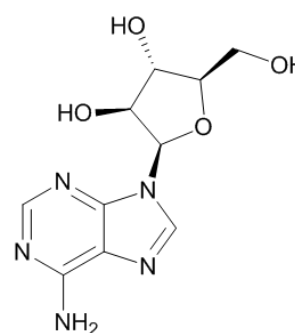
Out of these seven approved drugs, five have chemical structures optimised by synthesis, and are thus said to be synthetic- (analogue produced solely by chemical synthesis) or semisynthetic (using the natural product or a natural precursor as starting material) derivatives of the secondary metabolites.<sup>91</sup>



**Eicosapentaenoic acid and docosahexaenoic acid (Lovaza®)**  
 Reduces synthesis of triglycerides and increases peroxisomal  $\beta$ -oxidation of fatty acids in the liver



**Ziconotide (Prialt®)**  
 Acts as a selective N-type voltage gated calcium channel blocker



**Vidarabine (Vira-A®)**  
 Interferes with viral DNA synthesis by inhibiting viral DNA polymerase

**Figure 6B | The marketed marine derived drugs with other activities than against cancer.** Three marine derived compounds with activities other than against cancer are currently marketed. Lovaza® is antihyperglycemic,<sup>92</sup> Prialt® acts against chronic neurological pain<sup>93</sup> and Vira-A® is an antiviral.<sup>94</sup>

Another 23 marine derived compounds are currently part of the clinical pipeline.<sup>85</sup> Out of these, 21 are being examined for potential use as anticancer agents. Cancer is an area where natural products have played a particularly important role.<sup>95-97</sup> The remaining two compounds, tetrodotoxin and DMXBA, are being evaluated for analgesic properties against chronic pain and for CNS diseases like schizophrenia and Alzheimer's disease, respectively. In addition, a substantial number of compounds are being examined for clinical potential and are thus a part of the preclinical pipeline.<sup>98</sup> Their bioactivities include antibacterial, antidiabetic, antifungal, antiinflammatory, antiprotozoal, antituberculosis, and antiviral, in addition to activities affecting the nervous system.<sup>98</sup>

### **1.1.7 Marine secondary metabolites as antifouling agents**

Biofouling is commonly defined as unwanted attachment and subsequent overgrowth of submerged man-made surfaces.<sup>99,100</sup> The biomass of the settled organisms causes substantial economic losses for a wide range of economic sectors,<sup>101</sup> including the offshore petroleum industry, shipping, fishing and for other installations found in water, for example drinking water systems and power plants.<sup>102</sup> The dynamic biofouling cascade is complex and can be divided into two phases: microfouling and macrofouling.<sup>101</sup>

#### Microfouling

The microfouling process is often initiated by the attachment of dissolved organic matter to the submerged surface, which forms a conditioning film.<sup>103,104</sup> This is a process known as molecular fouling<sup>105</sup> or biochemical conditioning,<sup>100</sup> and begins within seconds after the object is submerged. This is quickly followed by the attachment of fouling microorganisms, including marine bacteria, fungi, diatoms, protozoans and algal spores.<sup>99</sup> These sequences of events lead to the formation of a biofilm on the submerged surface.

#### Macrofouling

Macrofouling refers to settlement of macroorganisms onto the growing layer of microfoulers.<sup>106</sup> The macrofouling organisms are divided into soft- and hard foulers. Soft foulers are shell free, and include visible algae and invertebrates, while hard foulers are shelled invertebrates, like barnacles, tube worms and bivalves.<sup>107,108</sup> Though the attachment and growth of all fouling organisms is unwanted on underwater man-made surfaces, the hard macrofoulers cause particular damage due to their calcium carbonate skeletal structures, which makes them difficult to remove. In addition, their presence on ship hulls result in increased friction between the ship and its surrounding water, causing increased drag and rising fuel expenses.

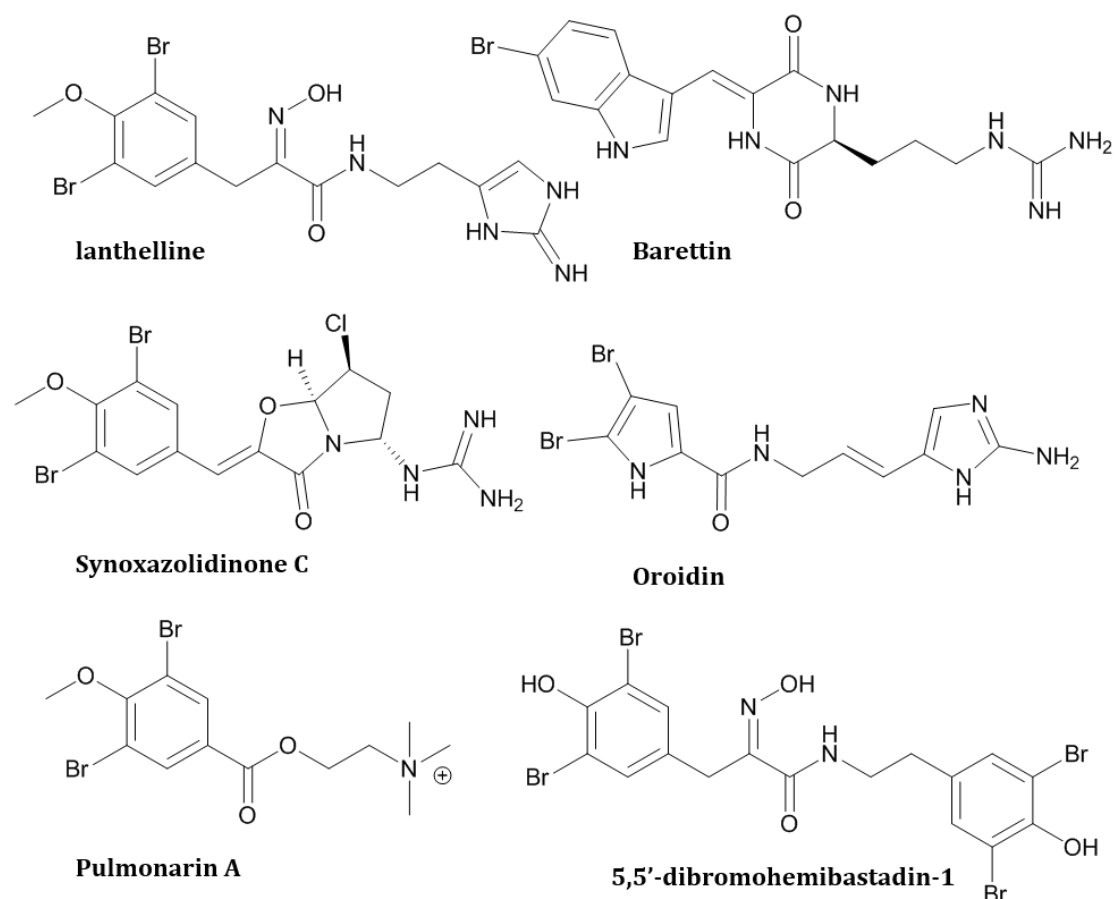
#### Antifouling

Antifouling is the process of preventing or interfering with the biofouling cascade, or the removal of an already established coat of organisms from a submerged



object.<sup>109</sup> Until recently, most marine installations were protected from biofouling by a coat of antifouling paint, where organotin compounds, like tributyltin and tributyltin oxide, were the active ingredients.<sup>110</sup> However, due to severe adverse effects on both target and non-target organisms (including mammals),<sup>111,112</sup> a ban of their use was implemented in 2008 by the International Maritime Organization.<sup>113</sup> This ban led to an urgent need for new, environmentally friendly antifouling agents. The ideal replacement would be an environmentally neutral coating with a broad spectrum of activity inhibiting the settlement of biofouling organisms without any side effects on the biofouling organisms, or other, non-target organisms.<sup>114</sup> Several ways of inhibiting biofouling in an environmentally friendly fashion have been proposed. These include antifouling surface topographies inspired by the natural antifouling surfaces of mollusk shells, fish and mammals,<sup>108</sup> surfaces with self-renewing properties,<sup>115</sup> as well as chemical antifouling strategies.

In analogy to submerged man-made surfaces, marine organisms face a constant threat of being overgrown by biofouling organisms. Despite this, many marine organisms have a clean surface, free of settling organisms. This has motivated the identification and extraction of natural antifouling agents from the chemical defence systems from marine organisms.<sup>116</sup> In particular, the sessile and filter feeding marine sponges have proven to be a rich source of secondary metabolites with antifouling properties.<sup>116</sup> In figure 7, the molecular structures of some selected sponge derived antifouling compounds are shown.



**Figure 7 | Selected marine derived compounds with antifouling activity.** The antifouling activities of ianthelline were investigated as part of this thesis<sup>117</sup> and compared with the activities of the known antifouler baretin, as a reference for the initial microfouling process.<sup>118</sup> Oroidin, isolated from sponges of the *Agelasidae* family, inhibits the attachment and colonisation of fouling bacteria<sup>119</sup> and inhibits the settlement of *Balanus amphitrite* larva.<sup>120</sup> In addition, it has served as a template for the creation of a 50-compound library of synthetic analogues<sup>121</sup>. 5,5'-dibromohemibastadin-1 inhibits the settlement of *Balanus improvisus*.<sup>122</sup> Synoxazolidinone C and pulmonarin A inhibit both adhesion and growth of several marine bacteria and microalgae.<sup>123</sup> In addition, synoxazolidinone C inhibits the settlement of *B. improvisus* larva<sup>123</sup>.

One potential target for an antifouling compound is phenoloxidase. This enzyme is highly involved in the initial settling of several macrofoulers, including bivalves, barnacles and bryozoans.<sup>124,125</sup> It catalyses the oxidation of o-quinones from phenols via catechols, a vital part of the polymerisation of proteins used in the attachment process, resulting in formation of byssus threads, anchoring the fouling organisms to the surface.<sup>126</sup>

## 1.2 The bioprospecting pipeline

### 1.2.1 Bioassay-guided isolation exemplified by the workflow at MabCent

The application of bioassays to follow the presence of bioactive compound(s) through an isolation process is called bioassay-guided isolation.<sup>127</sup> Figure 8 illustrates the standard workflow from crude extract to pure compound performed at MabCent.<sup>128</sup> The chain of events is initiated by biological screening of prefractionated crude extracts in various bioassays, including cell based anticancer-, antibacterial- and antiinflammatory assays, as well as target based assays for kinase inhibition. A positive bioactivity result nominates the sample for dereplication by ultra-high performance liquid chromatography (UHPLC)-HR-MS analysis. If the sample contains a suspected novel bioactive compound, or a previously reported compound with a novel bioactivity, the compound will be isolated using mass guided preparative high performance liquid chromatography (prep-HPLC) fractionation. After isolation, the purity of the compound is examined by UHPLC-HR-MS- and/or NMR analysis. This workflow resembles work conducted at other bioprospecting laboratories, though several variations of the approach exist.<sup>68</sup>

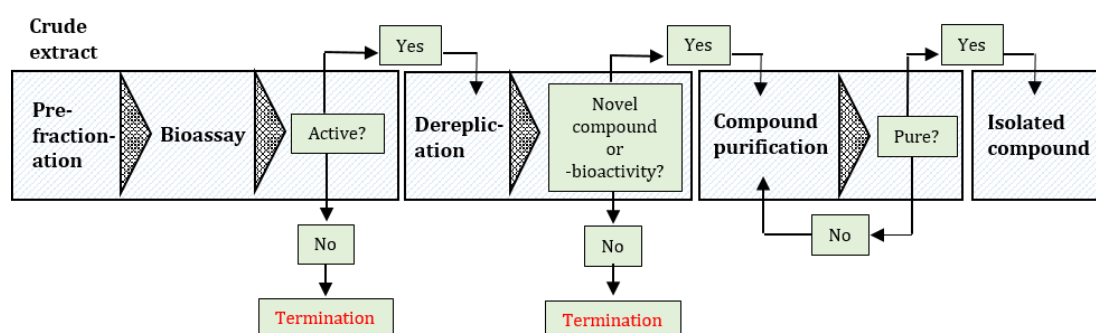


Figure 8 | Schematic illustration of the bioassay-guided isolation workflow at MabCent.<sup>128</sup>

### 1.2.2 Prefractionation

Crude extracts are complex mixtures, and are often composed of hundreds of different constituents. Fractionation of crude extracts prior to further investigation is known as prefractionation. Though not essential for the output of novel compounds in bioprospecting, it has been shown to increase effectiveness of the bioactivity screening. Prefractionation increases the chances of detecting

bioactivity of the actual secondary metabolite in the extracts via removal of salts, sugars and lipids. The increased hit rate can also be accounted for by a decreased risk for masking the secondary metabolite activity by other interfering compounds, as fractions of crude extracts have reduced complexity.<sup>129</sup> The most widely employed prefractionation technique is liquid chromatography (LC), including high performance liquid chromatography (HPLC) or flash chromatography, although several other techniques may be employed, including liquid-liquid partitioning. Prefractionation may result in any number of fractions ranging from a few to several hundred.<sup>127</sup> In LC, the dissolved sample is loaded onto a column where sample components are separated due to their distribution between a solid stationary phase with a large surface area and an applied liquid mobile phase. During the sample run, the elution strength of the mobile phase is increased, resulting in a gradual elution of the applied compounds. At MabCent, prefractionation of the crude extracts is carried out by flash-chromatography, using 1 g of the crude extract resulting in eight fractions. A polymeric HP-20SS absorbent media is used as stationary phase. The amount eluting in each fraction varies between fraction number and is dependent on the natural composition of the crude extracts. The fractions are dried and redissolved to a concentration of 40 or 80 mg/mL in dimethyl sulfoxide (DMSO), dependent on the amount of eluting material.

### **1.2.3 Bioassays and high throughput screening**

A bioassay is any *in vitro* or *in vivo* system used to detect the presence of a biologically active constituent in a sample.

The bioassays can be divided into two groups:

- Target-based assays: These assays measure the effect of compounds on a single, defined target.<sup>130</sup> The targets are typically proteins, with key roles in in disease pathogenesis.<sup>131</sup> Examples of targets are G protein coupled receptors and kinases.<sup>132</sup>
- Phenotypic assays: In these assays, cells, tissues or whole living organisms are used to detect an activity. The aim is to discover a desired effect on the

selected system, independent of any defined target, and does therefore not require any prior knowledge to the pathophysiology of the disease.

In natural product drug discovery, the bioassays are utilised at different stages of the drug discovery process. In the initial phase of the bioprospecting pipeline, bioassays are conducted in a high-throughput manner to detect bioactivity in crude extracts or fractions. Usually a combination of bioassays is used. It is estimated that the screening of five million extracts will generate 1000 hits, from which ten leads will be generated. Out of these, five compounds will enter clinical trials and in the end, one will become a marketed drug.<sup>133</sup> The initial bioactivity screening is followed by a more careful examination of the bioactive crude extract or fraction, in which a positive result nominates the sample for dereplication. Finally, the bioassays can be used to elucidate the bioactivity profiles of isolated compounds.<sup>134</sup>

#### High throughput screening for detection of bioactivity in crude extracts or fractions

High throughput screening (HTS) is the process of assaying huge numbers of crude extracts or fractions against selected targets in a relatively short amount of time.<sup>135</sup> In order to conduct bioactivity screening in a high throughput manner, validated drug targets and assays suitable for detecting the bioactivity of a compound or an extract need to be developed. In addition to the bioassay targets, the necessary equipment, like microtiter plates and laboratory automation techniques, are needed to make HTS executable.<sup>136</sup> Development of the HTS technology started in the 1950s as a way of screening for bioactivity in samples originating from microorganisms.<sup>134</sup> With the development of combinatorial synthesis in the beginning of 1990s, efforts towards detecting bioactive natural products declined. From the end of the 1980s and through the 1990s, HTS analysis mainly evolved around screening for bioactivity in small molecule libraries generated from combinatorial chemistry. This failed to increase the output of new pharmaceuticals though,<sup>65</sup> and from the beginning of the 21st century, HTS of crude extracts or fractions have regained much of its popularity. The number of biological targets available for testing has increased dramatically since the

1950s.<sup>129</sup> As the number of available targets increased, the possibility of testing “old” crude extracts, fractions or isolated compounds for new activities opened up.<sup>137</sup>

### The high throughput screening bioassays

In the systematic attempt to identify bioactive crude extracts or fractions, selected biochemical and/or phenotypical targets are assayed in a HTS manner as part of the bioassay-guided isolation process. The bioassays constituting a HTS program are chosen on the basis of the research area of interest for the individual bioprospecting laboratories. A HTS program may consist of bioassays devoted to detect bioactivity within one area of interest, for example anticancer agents. For this purpose, cell based anticancer assays, as well as kinase- and caspase inhibition assays may be used. It may also consist of bioassays for detecting a variety of activities towards a range of diseases or interest areas. At MabCent, the HTS program consists of assays able to detect anticancer-, antibacterial-, immunostimulatory- and immunosuppressive effects.<sup>128</sup>

Analysis of the origin of new FDA approved drugs between 1999 and 2008 suggest that phenotypic screening strategies have been more productive than target-based approaches in drug discovery.<sup>138</sup> It has been shown that utilisation of phenotypic assays early in the screening cascade generates hits of higher quality, as opposed to target based screening.<sup>132</sup> This is because many other factors, in addition to compound-target interactions come into play when a compound is to be used as a drug. Examples of these are membrane permeability, unspecific protein binding and metabolism.<sup>135</sup>

In HTS of crude extracts or fractions, the assays need to detect desired bioactivity properties of constituents of complex samples. The assays are designed to possess a high capacity and deliver rapid answers at relatively low cost. In addition, they should be convenient, reliable, sensitive and require little material.<sup>134</sup> HTS is typically performed at a single concentration, and a positive hit is followed by additional testing to estimate potency and target- or phenotypic specificity. Additional testing also aims at eliminating false positives caused by nonspecific



activities of constituents of the assayed crude extracts or fractions.<sup>139</sup> These nonspecific interactions are not unusual and they are often seen when one or more constituents are present at a high concentration. As an example, when cell lines are screened against samples with a high fatty acid concentration these might cause necrotic cell death by acting on the cell wall in a detergent like manner, thus giving rise to a nonspecific bioactivity.<sup>129</sup> These sample constituents are often called nuisance compounds. One possible way of revealing the nonspecific interactions is by a titration-based approach where the desired result is a dose-response relationship.<sup>140</sup> At MabCent, a three to four dilution curve is created for each active fraction, and those producing a dose-dependent result, or are active down to the lowest concentration point, will be further processed.

#### **1.2.4 Dereplication**

Dereplication is the rapid identification of known compounds in bioactive crude extracts or fractions. The process differentiates the bioactive extracts or fractions containing nuisance compounds or known secondary metabolites, from those containing secondary metabolites with novel chemistry and/or novel bioactivity<sup>141</sup> prior to compound isolation. The dereplication process aims to make efficient use of often limited resources, as it prevents compound reisolation and reidentification. The strategies used in bioactive sample dereplication are many, and often include species and taxonomic information, bioassay results, as well as analytical data obtained from various chromatographic and spectroscopic techniques.<sup>142,143</sup>

HR-MS has become the analytical tool of choice in dereplication owing to its speed, dynamic signal range, sensitivity and the ability to interface with chromatographic separation methods.<sup>144-146</sup> The utilisation of an HPLC/UHPLC-HR-MS system is a powerful dereplication approach, which have been growing in popularity ever since the introduction of electrospray ionisation (ESI) at the end of the 20<sup>th</sup> century.<sup>147</sup> ESI facilitates the transfer of analyte molecules from an uncharged liquid phase species to gas phase ions, hence making the hyphenation of a mass spectrometer to an LC systems technically feasible.<sup>148</sup> HR-MS acquired Mw and isotopic patterns can be used to calculate the elemental composition of individual

sample compounds. The high separation efficiency of UHPLC combined with the acquisition of high resolution mass data from the HR-MS permits characterisation of individual components of samples with complex matrixes. These properties makes this strategy of analysis ideal for dereplication of bioactive crude extracts or fractions.<sup>146</sup> Elemental compositions, bioactivity profiles and taxonomical information of the samples can be used to search internal or commercial databases. These databases offer a way of relatively straightforward comparison of obtained data with the huge amounts of available compound data. Several databases exists to facilitate these processes, including:

- MARINLIT (<http://pubs.rsc.org/marinlit/>)
- Marine Natural Product Database (<http://naturalprod.ucsd.edu/>)
- Chempider (<http://www.chemspider.com/>)

### **1.2.5 Isolation**

In natural product drug discovery, isolation is an essential step in the identification of new chemical entities. A purified compound allows for chemical characterisation, as well as confirmation and further evaluation of its bioactivity.<sup>149</sup> When the desired compound is present as the major metabolite in the extract, isolation can be rapid. This is typically not the case though, as the target compound often exist in trace quantities<sup>150</sup> in a matrix of dozens of other constituents. The isolation of secondary metabolites from a crude extract is generally a time consuming process, and is known to be one of the bottlenecks in natural product drug discovery.<sup>68</sup>

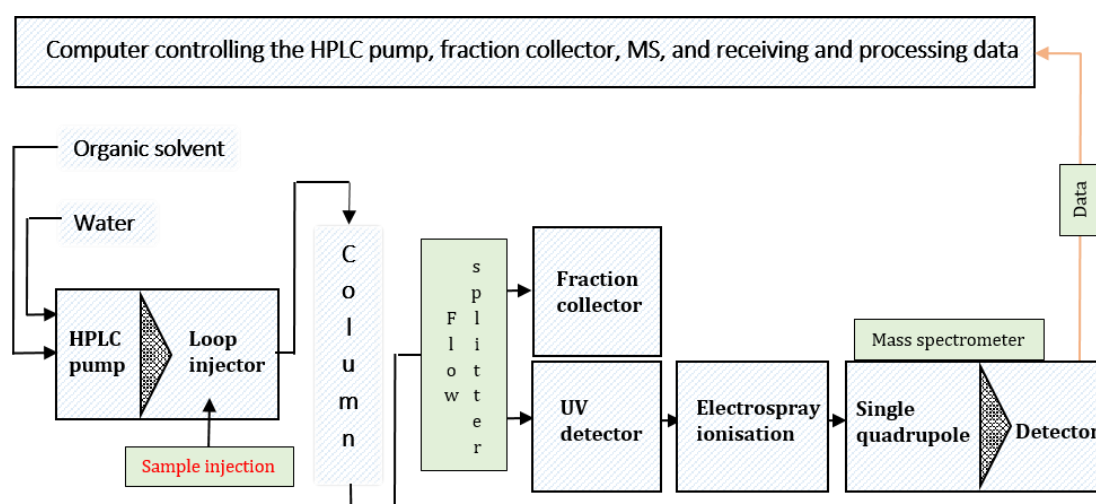
#### Preparative high performance liquid chromatography

Several chromatographic techniques can be utilised for separation and purification of biologically active molecules from complex matrixes.<sup>151</sup> Amongst the available chromatographic techniques, Prep-HPLC has emerged as the method of choice for secondary metabolite isolation.<sup>149,152</sup> HPLC is the most versatile and robust technique for secondary metabolite isolation and offers high resolving power and can be scaled up as well as automated.<sup>153</sup> The term “preparative” refers to a chromatographic analysis, where the objective is to collect a valuable product after it is separated from the other sample constituents.

A wide range of prep-HPLC columns are available, including normal phase-, reversed phase (RP)-, size exclusion- and ion exchange columns. The surface modification of the columns packing material determines which retaining interactions will occur between the sample analytes and the stationary phase. In the isolation of secondary metabolites, RP columns are most frequently utilised, as most drug-like compounds can be purified using RP-HPLC.<sup>154</sup> Amongst the available RP column packing material surface modifications, octadecyl (C<sub>18</sub>) bonded silica is most widely used. In addition, a wide range of other RP column packing material surface modifications exist, like phenylhexyl, fluorophenyl and dihydroxypropane.<sup>149</sup> The isolation process is often initiated by a phase of trial and error, where various HPLC columns and elution gradients are tested for their ability to separate the desired compound from the rest of the sample matrix.

#### Mass guided preparative high performance liquid chromatography fractionation

Using mass spectrometry (MS) to trigger collection of the compounds eluting from an HPLC column, is known as mass guided fractionation. Mass guided prep-HPLC fractionation is a powerful tool, as it combines the high separation efficiency of the HPLC column with the convenience of triggering the fraction collector at the presence of defined masses. This allows for the collection of narrow fractions as well as a correction for drifts in the retention times found between the individual injections. At MabCent, mass guided fractionation is used to isolate secondary metabolites from bioactive crude extracts (Figure 9).



**Figure 9 | Schematic illustration of the prep-HPLC-UV-ESI-single quadrupole MS system used for compound isolation at MabCent.**

Several steps of purification is commonly necessary to obtain the compound in sufficient purities and amounts to allow for structure elucidation and further bioactivity confirmation and profiling.

### **1.2.6 Structure elucidation**

Elucidating the structure of secondary metabolites often involves the accumulation of data from numerous sources. A wide range of spectroscopic instrumentation, like UV/visible- and infrared absorption spectroscopy, NMR, and MS, currently form the backbone of modern structure analysis.<sup>68,129</sup>

#### High-resolution mass spectrometry

MS is an analytical technique that measures the mass-to-charge ratio of gas-phase ions. High-resolution mass analysers have the ability to separate ions with very similar masses.<sup>155</sup> The combination of high resolution, high mass accuracy and high sensitivity makes HR-MS a powerful tool for analysing complex samples like crude extracts or fraction. HR-MS measures exact masses with the typical resolution being tenth of a millimass unit (e.g 482.9079). HR-MS analysis results in a mass spectrum; a plot of the relative abundance of detected ions as a function of the mass-to-charge ratio. From the mass spectrum, the accurate mass and isotopic pattern of a compound can be extracted and subsequently used to calculate its elemental composition.<sup>156</sup> Some mass spectrometers can fractionate parent gas-phase ions into daughter ions, allowing for elemental composition calculations of smaller parts of the parent ion.<sup>157</sup> This information can be pieced together to generate structural information regarding the intact molecule. Without the necessity of initial isolation, this can save both time and money. This type of experiment is particularly useful for providing information concerning secondary metabolites and for amino-acid sequencing. There are many types of mass analysers, all separating the ions according to their mass-to-charge ratio by ways of various strategies. Two different high resolution mass analysers have been used as part of this thesis: Time-of-Flight (ToF) and orbitrap.

ToF-MS determines the mass-to-charge ratio via flight time measurements. Ions are accelerated by an electric field to a common kinetic energy, and travels

through a flight tube to the detector with velocities depending on their mass. Lighter ions will travel faster than the heavier ions. The ToF analyser corrects for small differences in initial energy and angle by a combination of linear drift paths and ion mirrors. From the measurement of the ion flight time, the mass of the ion can be calculated.<sup>155</sup>

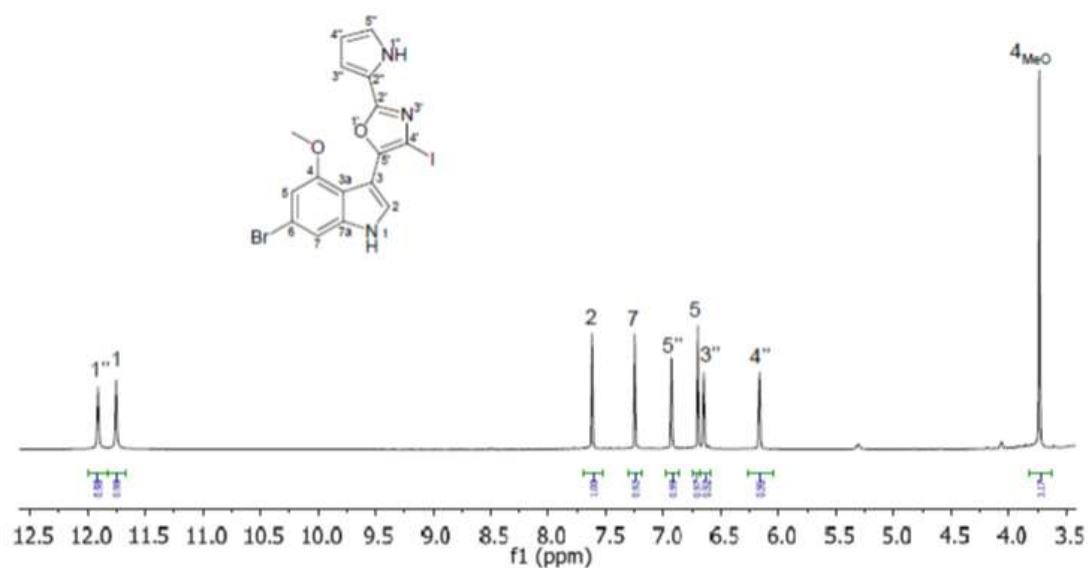
Orbitrap MS is based on the electrostatically orbital trapping of ions around a central, spindle shaped electrode. Ions are trapped because their electrostatic attraction to the inner electrode is balanced by centrifugal forces. Axial oscillation of ion rings are detected by their image current induced on an outer electrode, which is split in two symmetrically pick-up sensors connected to different amplifiers. The mass-to-charge ratio of different ions in the orbitrap can be detected from respective frequencies of oscillation after a Fourier transformation.<sup>144,158</sup>

### Nuclear magnetic resonance spectroscopy

Spectroscopy is the study of the interaction between electromagnetic radiation and matter. NMR spectroscopy utilises the physical phenomenon where a magnetic nucleus in a fixed external magnetic field absorbs and re-emit measurable electromagnetic radiation. The two most commonly examined nuclei are  $^1\text{H}$  and  $^{13}\text{C}$ . A parallel alignment of the previously randomly oriented nuclei will occur when they are subjected to an external magnetic field. The nuclei will align either with it or against the magnetic field, with the latter being the alignment requiring least energy. The difference in energy between the two spin states increase with increased strength of the external magnetic field. The sample is now applied irradiation energy in the radio frequency (rf) range. When the nuclei are exposed to electromagnetic radiation with a frequency matching its Larmour frequency, a nucleus in the lower-energy spin state will transition to the higher energy spin state. When the rf is switched off, the nuclei relaxes back to the lower energy state by re-emitting the absorbed rf energy. This emitted energy is of a particular resonance frequency, dependent upon the magnetic field and the magnetic properties of the isotope, and produces a measurable rf signal.<sup>159</sup> This signal, called the resonance frequency, creates means to recover structural

information about the analysed molecule. The phenomenon is known as the chemical shift, and is the most important characteristic of a nucleus in terms of NMR. The shift of an individual atom depends on its atomic properties, such as type of nucleus, its hybridisation state and the overall electronic environment surrounding the nucleus (bonds, conjugation network etc.).<sup>159</sup>

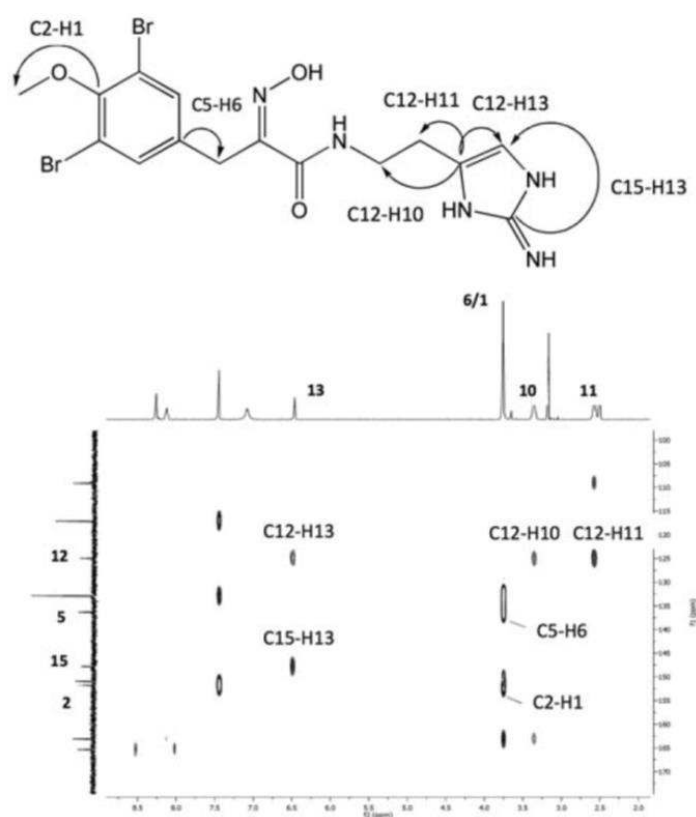
The measured transmitted rf frequencies are processed into an NMR spectrum. In an NMR spectrum, each atom of interest is associated with a peak characterised by a chemical shift, an intensity and one or more couplings associated with interacting nuclei. More than 1000 different NMR experiments have been developed to provide spectra delivering various information about the examined nuclei, and can be either one-dimensional (1D) or two-dimensional (2D).<sup>160</sup> 1D experiments are spectroscopic analysis of a single nucleus. The most commonly used NMR experiment is <sup>1</sup>H-NMR, providing information about chemical shifts, multiplet structures, homonuclear coupling constants and integrations of all protons present in the sample. The <sup>1</sup>H-NMR spectrum for breitfussin A can be seen in figure 10 as an example.



**Figure 10 | <sup>1</sup>H-NMR spectra obtained for the halogenated dipeptide breitfussin A obtained at 298 K in deuterated DMSO as part of this thesis.<sup>161</sup>**



When the  $^{13}\text{C}$  nucleus is examined using a 1D-NMR experiment, each peak in the resulting NMR spectrum identifies a carbon atom in a different environment within the molecule. Since nuclei themselves behave like small magnets, they can influence each other and change the energy of nearby nuclei as they resonate. This phenomenon is known as spin-spin coupling, and forms the basis for 2D-NMR experiments.<sup>159</sup> The most important type of interaction is scalar coupling which occurs between two nuclei mediated through chemical bonds, and can be seen up to three bonds away. The most common 2D-NMR experiments for structure elucidation include correlated spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC).<sup>68</sup> All the different 2D-NMR experiments are designed to ascertain a different type of physical information about the molecule being studied. Figure 11 shows the structure of ianthelline with arrows highlighting observed HMBC correlations as well as the obtained HMBC spectrum.

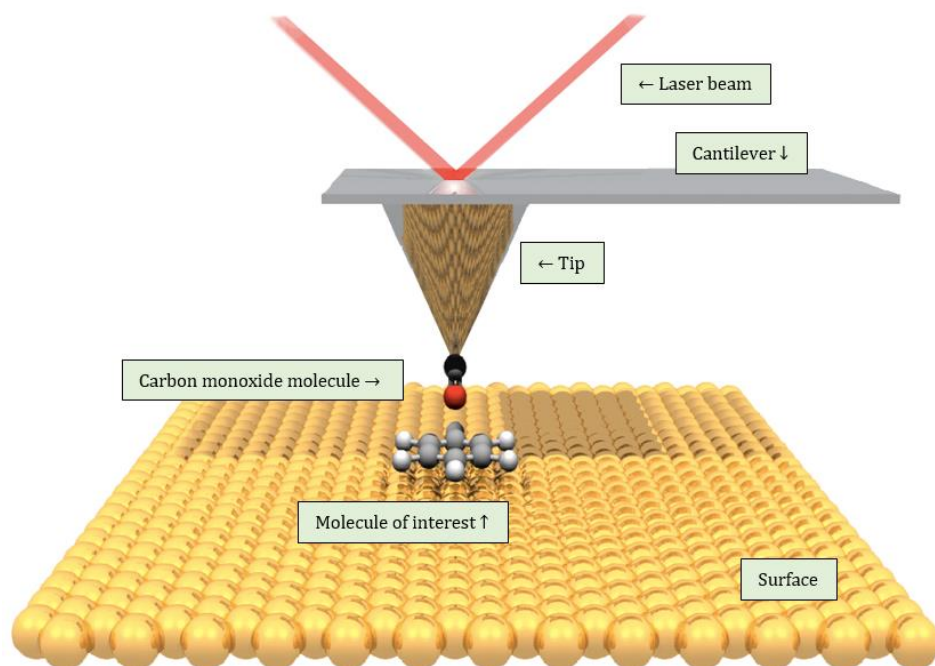


**Figure 11 | The molecular structure of ianthelline with arrows highlighting C-H correlations as well as the HMBC spectrum recorded as part of this thesis.<sup>162</sup>**

Homonuclear and heteronuclear couplings and spectra can be envisaged to represent the fingerprint of a structure, and information contained within a spectrum can provide sufficient information to elucidate a chemical structure. To elucidate the structure of complex compound a series of different spectra and other forms of analytical data are generally required.

### Atomic force microscopy

AFM of molecules is conducted by adsorbing the compound onto a flat surface and scanning the surface with a narrow probe. The interactions between the tip of the probe and the surface generate a measurable signal that can be transformed into an image of the surface investigated. AFM can be used to study surfaces at high resolution. To obtain images at atomic resolution, the tip can be modified with a carbon monoxide molecule to increase image resolution. The tip is attached to a cantilever, which places the tip in close proximity to- and moves it across the surface, where the molecule of interest is placed. When the tip is moved over the molecules, it senses the changes in the strength of the electronic force over the surface. This results in movement of the cantilever, which is detected by a laser beam allowing for computing of AFM images (Figure 12).



**Figure 12 | The basic principle behind AFM analysis.** An atomic force microscope probes a molecule adsorbed onto a surface, using a carbon monoxide molecule at the tip for sensitivity. Reprint from <https://newscenter.lbl.gov/2013/05/30/atom-by-atom/>.

The tip is not only able to detect the individual atoms, but also the forces representing the bonds formed by the electrons shared between them. In this way, AFM is able to create an image of the force gradient variations above the surface. AFM imaging has recently been used to create images of synthetic compounds with known structures.<sup>163</sup> In 2010, AFM aided for the first time in the structure determination of a secondary metabolite.<sup>164</sup> The structure of cephalandole A, isolated from an actinobacterium, was previously reported. The reported structure was found to be misassigned, and AFM could subsequently be used to correctly assign its molecular connectivity. In 2012, AFM was for the first time used in the structure elucidation of novel secondary metabolites, when it aided in the structure elucidation of the MabCent novels breitfussin A and B.<sup>161</sup>

#### Computer-assisted structure elucidation and density functional theory

CASE is a technique using software to generate all possible molecular structures consistent with a set of spectroscopic data.<sup>165</sup> CASE was first described in the late 1960s,<sup>166</sup> and has since been developed into a powerful tool meant to aid in the structure elucidation of compounds. The number of possible structures for a given molecular formula is finite, due to the number of isomers corresponding to it, but the number of possible structures is often too high to be of any real use. CASE uses algorithms to calculate possible molecular structures by using obtained spectroscopic data as input data. DFT is a computational quantum mechanical modelling method, which can be used as part of structure elucidation to investigate the electronic structure of molecules. DFT provides the ground state properties of a molecule, where the electron density plays a key role. DFT can be used to calculate a great variety of molecular properties, including molecular structures, and can thus aid in structure elucidation. The powerful combination of CASE and DFT with experimental data makes the elucidation of molecular structures easier.

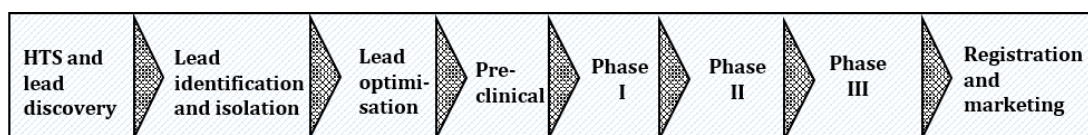
#### **1.2.7 Bioactivity profile of isolated compounds**

After isolation, the bioactivity profile of the purified compound is elucidated. The panel of bioactivities that an isolated compound is tested against will depend on its originally detected bioactivity and the availability of bioassays. This might

include testing a compound against a wide range of targets for different disease areas, or include assays meant to elucidate the mode of action for the compound for one specific disease area.

### 1.2.8 Commercialisation of natural products

The challenges and milestones that needs to be addressed and cleared before a bioactive compound with an elucidated structure and confirmed *in vitro* bioactivity can be made commercially available, are many. This is especially true for secondary metabolites with prospects of becoming pharmaceuticals. Any further work following lead identification and isolation was beyond the scope of this thesis, but can be briefly mentioned and include the events illustrated in figure 13.



**Figure 13 | The sequential steps of the drug discovery and development pipeline.** Figure adapted from<sup>167</sup> and<sup>168</sup>.

In lead optimisation, analogues of the lead compounds are produced and tested. In addition to potency, effectiveness and selectivity, a compound must be bioavailable as well as chemically- and metabolically stable. When the drug target is known, *in silico* modelling might be conducted to enable lead compound modulation in a more rational way.

In preclinical studies, the safety, efficacy and potential toxicity of the secondary metabolite, or one or a selected few of optimised analogues of the secondary metabolite, is tested in animal models. Satisfactory results take the compound through to phase I clinical trials, where it is tested in humans for the first time, to evaluate its safety. The efficient concentration range and toxicity of the compound will determine the dosage range. In addition, side effects are identified, as well as pharmacokinetic properties. In phase II clinical trials, the compound is tested for its efficiency in treating patient with the disease or the condition targeted by the medication. In the following step, phase III, blinded clinical trials are designed to

verify the benefit of the compound in a larger targeted patients population, compared to known treatments and placebo. The studies, normally conducted over several years, can generate the required data for marketing approval. Overall, the preclinical and clinical development is a time consuming and expensive process. In the last phase, marketing and safety monitoring (also termed phase IV), clinical trials may be conducted to meet requirements set by the approving authorities (e.g. the FDA), to test the drug with additional patient populations, or to test the drug for new indications.

## 2. AIM OF THE THESIS

The work included in this thesis has been performed as part of the ongoing work at MabCent. The overall aim was to search for novel bioactive natural products, as well as previously known compounds with novel bioactivities. The target activities are mainly pharmaceutically oriented, and include treatment of cancer, diabetes, inflammation and bacterial infections. In addition, related bioactivities such as inhibition of biofouling have been examined.

The main objectives of the present study were to:

- Identify bioactive crude extracts or fractions through primary bioactivity screening
- Dereplicate the bioactive crude extract or fraction in order to identify the bioactive component(s)
- Isolate the active metabolite(s)
- Elucidate the structures of the active metabolite(s)
- Confirm the initially observed bioactivity and conduct further thorough bioactivity elucidation
- Elucidate the mode of action of the isolated compound(s)

### 3. SUMMARY OF PAPERS

#### Paper I

Kine Ø. Hanssen, Jeanette H. Andersen, Trine Stiberg, Richard A. Engh, Johan Svenson, Anne-Marie Genevière, Espen Hansen.

#### Antitumoral and Mechanistic Studies of Ianthelline Isolated from the Arctic Sponge *Stryphnus fortis*.

*Anticancer research*, **32**, 2012, 4287 – 4297.

Cytotoxicity of ianthelline (Figure 14) was tested against eleven cell lines and the effects of ianthelline on key cell division events were studied in sea urchin embryos. Tyrosine kinase Abl, cAMP-dependent protein kinase A (PKA), protein-tyrosine phosphatase 1B, and a panel of 131 kinases was further tested for ianthelline sensitivity. The effect of ianthelline was further found to be time dependent, as it inhibited the human melanoma cancer cell line A2058 in a dose- and time dependent manner. Disturbed mitotic spindle formation was seen in sea urchin embryos exposed to ianthelline. In addition, pronuclear migration and cytokinesis were severely inhibited. No effect on DNA synthesis was detectable. Ianthelline did not significantly inhibit Abl, but did show weak dose-dependent inhibition of PKA and PTP1B. Ianthelline strongly inhibited (to residual activity  $\leq 10\%$ ) three out of 131 tested kinases, showing a Gini coefficient of 0.22 for the degree of kinase inhibition selectivity. These results demonstrate that ianthelline is a cytotoxic marine compound and it exerts its antiproliferative effect by several mechanisms that includes inhibition of mitotic spindle formation and inhibition of protein kinase activity.

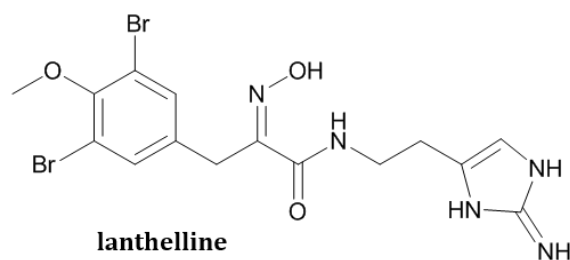


Figure 14 | The molecular structure of ianthelline.

## Paper II

Kine Ø. Hanssen, Gunnar Cervin, Rozenn Trepos, Julie Petitbois, Tor Haug, Espen Hansen, Jeanette H. Andersen, Henrik Pavia, Claire Hellio, Johan Svenson

### **The Bromotyrosine Derivative ianthelline Isolated from the Arctic Marine Sponge *Stryphnus fortis* Inhibits Marine Micro- and Macrobiofouling.**

*Marine Biotechnology*, 2014, 10.1007/s10126-014-9583-y.

The ability of ianthelline (Figure 14), a bromotyrosine derivative isolated from the Arctic marine sponge *Stryphnus fortis*, to inhibit marine biofouling was examined. Inhibition of the initial stages of the biofouling process was investigated by exposing ten strains of marine bacteria and eight microalgal strains to the compound. Both inhibition of surface adhesion and growth were investigated. The potential of ianthelline as an inhibitor of macrofouling was investigated by exposing settling cyprid larvae of *B. improvises* to the compound. In addition, ianthelline was further explored for inhibitory activity against the blue mussel (*Mytilus edulis*) phenoloxidase. Finally, ianthelline was tested for activity against seven terrestrial bacterial strains known to cause disease in human, and for antibiofilm activity against *S. epidermidis*.

Ianthelline was found to inhibit all the major stages of the fouling process, with the main activity being against marine bacteria (Minimum inhibitory concentration (MIC) as low as 0.1 µg/mL) and the settlement of barnacles (Half maximal inhibitory concentration (IC<sub>50</sub>) = 3 µg/mL). A lower activity was seen against microalgae and the blue mussel enzyme phenoloxidase. In addition, ianthelline was found to display activity against all human pathogenic bacteria strains tested in the study (MIC ≥ 2.5 µg/mL), both Gram positive and Gram negative strains.



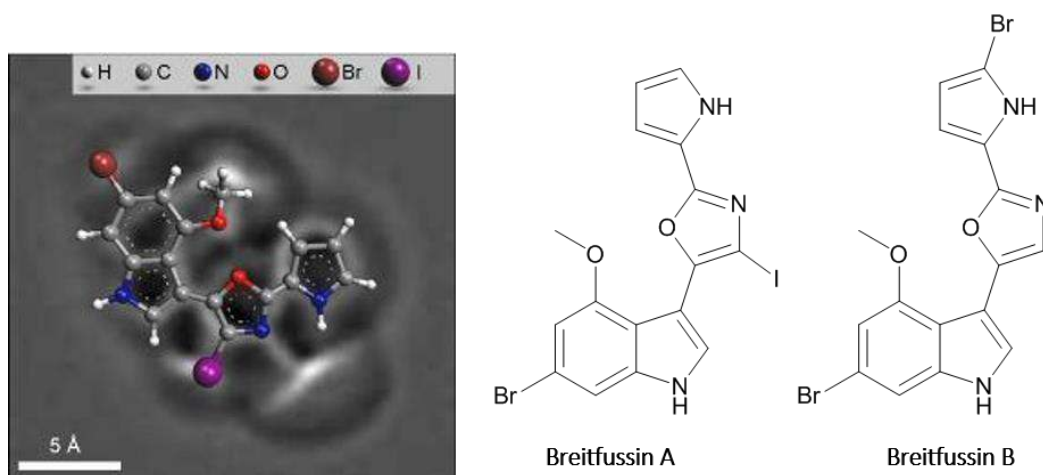
### Paper III

Kine Ø. Hanssen, Bruno Schuler, Antony J. Williams, Taye B. Demissie, Espen Hansen, Jeanette H. Andersen, Johan Svenson, Kirill Blinov, Michal Repisky, Fabian Mohn, Gerhard Meyer, John-Sigurd Svendsen, Kenneth Ruud, Mikhail Elyashberg, Leo Gross, Marcel Jaspars, Johan Isaksson

#### A combined Atomic Force Microscopy and Computational Approach for the Structural Elucidation of Breitfussin A and B: Highly Modified Halogenated Dipeptides from *Thuiaria breidfussi*.

*Angewandte Chemie International Edition*, **51**, 2012, 12238-12241.

Breitfussin A and B were isolated from the Arctic, marine hydrozoan *Thuiaria breidfussi*. Their structures were solved using a novel combination of AFM, CASE, and DFT calculations, as well as HR-MS and NMR. Visualisation by AFM determined all the connection points of the cyclic systems and the other substituents. The elucidated structure and the AFM image of breitfussin A can be seen in figure 15, as well as the molecular structures of breitfussin A and B.



**Figure 15 | The elucidated molecular structure of breitfussin A overlaid the AFM image of the same compound, as well as the molecular structures of breitfussin A and B.**

## 4. GENERAL DISCUSSION

As highlighted throughout the introduction, Nature is a rich source of bioactive secondary metabolites with diverse chemistry. To identify secondary metabolites with the potential of becoming commercially available products, research needs to be put into the initial stages of natural compound discovery: the bioprospecting pipeline. In this thesis, the established bioprospecting pipeline found at MabCent was used to discover two novel compounds where the full bioactivity profile remains to be elucidated (Breitfussin A and B, Paper III), as well as a previously reported compound with novel bioactivities (Ianthelline, Paper I and II).

### 4.1 Sample supply

Bioprospecting is dependent upon the provision of biological samples, and one of the major hurdles, especially in marine bioprospecting, is sample availability.<sup>169</sup> The samples of *S. fortis* and *T. breitfussi* used in this thesis were provided by the Norwegian national marine biobank (Marbank). Marbank undertakes several annual research cruises, collecting samples from Arctic- and sub-Arctic waters in Norway, with the goal of collecting at least one kilogram of each species for further processing. The collection is regulated, and conducted according to, among others regulations, the Norwegian marine resource act, the Norwegian nature diversity act, the law of the sea convention and the convention on biological diversity. Pictures of the collected specimens of *S. fortis* and *T. breitfussi* can be seen in figure 16 and an overview of collected amounts, collection site, amounts of isolated compounds as well as isolation yield can be seen in table 2.



**Figure 16 | Specimens of *S. fortis* and *T. breitfussi*.** Photo: Robert Johansen, Marbank.

**Table 2 | An overview of collection site, collected wet weight, weight of the isolated compound and isolation yield of lanthelline and the breitfussins, isolated from *S. fortis* and *T. breitfussi*.**

Organism	Collection site	Wet weight	Weight isolated compound	Isolation yield
<i>S. fortis</i>	Off the coast of Spitsbergen	2.015 kg	lanthelline: 210 mg	0.0104%
<i>T. breitfussi</i>	Off the coast of Bear Island	0.309 kg	Breitfussin A: 6.2 mg Breitfussin B: 4.0 mg	0.002% 0.0012%

Understandably, the total quantity of organic material that can be collected during a research cruise is limited, and a balanced relationship between the quantities of each collected sample and the total number of collected samples exists. Lanthelline was isolated in amounts that allowed for structural determination and thorough bioactivity profiling. The low quantity of collected *T. breitfussi* and low isolation yields of breitfussin A and B, left one wishing that more of the original sample had been collected.

Pure bioactive compounds need to be isolated in amounts allowing structure elucidation, bioactivity confirmation, and also preferably thorough biological characterisation. This task has proven to be a challenge for many bioactive marine derived secondary metabolites, and limited amounts of compounds available might severely delay, or even prevent, the development of commercial products. One example is the cytostatic sponge derived polyketide halichondrin B, where 1 tonne of sponge material was processed for isolating 300 mg of a mixture of two halichondrin analogues.<sup>170</sup> Another example is the ecteinascidins and their lead compound trabectedin (Molecular structure shown in figure 6A). Trabectedin, discovered in 1990 and now marketed as Yondelis®, gave a yield of 0.0001% when isolated from its natural source, the tunicate *Ecteinascidia turbinata*.<sup>171</sup> The tunicate could not be harvested in quantities sufficient for providing the amount of trabectedin needed for preclinical trials, delaying the bioactivity profiling of the compound. The final solution to the supply problem was solved by a semisynthetic process, where trabectedin is produced from cyanosafracin B.<sup>171</sup>

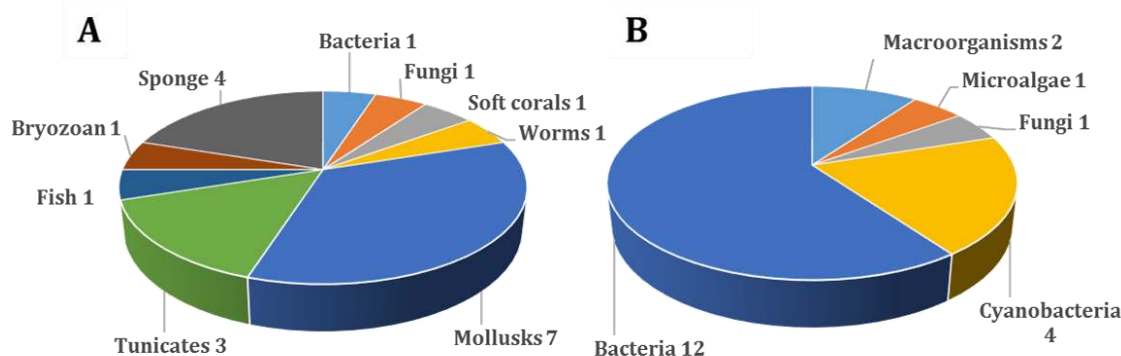
In order to overcome supply problems, most marketed drugs are being produced synthetically or semisynthetically (with some important exceptions, like morphine, which is extracted from the poppy *Papaver somniferum*). The synthesis of natural products is not necessarily straightforward, owing to the complexity of the compounds. The total synthesis of halichondrin B was developed, but could not provide sufficient amounts to put the compound to clinical trials. Hundreds of analogues of the compound were later produced. One of these, eribulin mesylate, is now approved as a pharmaceutical under the generic name Halaven<sup>®172</sup> (See figure 6A for chemical structure). The synthesis of ianthelline is already published,<sup>173</sup> and more compound could be made available independently of availability of *S. fortis*. The breitfussins are novel compounds, and *T. breitfussi* has thus to be recollected to allow for reisolation or a synthetic route towards breitfussin A and B has to be developed to obtain higher compound quantities.

#### **4.2 Macroorganism associated microorganisms**

Marine microorganisms are a prolific source of structurally diverse bioactive metabolites.<sup>174</sup> It is now becoming increasingly evident that for many of the isolated bioactive marine secondary metabolites, the collected macroorganism originally believed to be the producer of the secondary metabolite, simply was the host for or predator of the true producing microorganism. This is true for many of the marketed marine derived drugs, as well as for several of the marine derived compounds in the clinical pipeline. The pie charts in figure 17 illustrate the difference between collected organisms and suspected biosynthetic origin of selected marine derived secondary metabolites,<sup>91</sup> out of which 80% are suspected to be produced by bacteria or cyanobacteria.

This realisation came from the observation of identical marine secondary metabolites isolated from marine invertebrates and microorganisms with similar or even identical chemical structures. One example of such compounds is the antitumour agent dolastatin 10. It was originally isolated from the mollusc *Dolabella auricularia*, and later from the marine cyanobacterium *Symploca species* VP642.<sup>175</sup> A synthetic analogue of dolastatin 10, monomethyl auristatin E, is now part of the antibody-drug conjugate marketed as Adcetris<sup>®</sup> (Molecular structure

shown in figure 6A).<sup>176</sup> Another example is the protein kinase inhibitor staurosporine. As opposed to dolastatin 10, it was originally isolated from a microorganism, the bacterium *Streptomyces staurosporeus*,<sup>177</sup> and later from the tunicate *Eudistoma toeaensis*.<sup>178</sup>



**Figure 17 | Pie charts showing the collected source and predicted biosynthetic source of marine derived or inspired drugs and clinical trial agents.** Pie chart A shows the collected source of 20 marine secondary metabolites used as pharmaceuticals or is part of the clinical pipeline as of January 2012. Pie chart B shows the suspected biosynthetic origin of the same compounds. Figure made with inspiration from reference <sup>91</sup>.

Due to this, much focus is now devoted to the study and cultivation of marine microorganisms. An increased focus on fermentation of microorganisms as a source of organic material available for crude extract production and bioactivity screening might also help surpass the limitation of biological material needed a successful product development. This can result in both a higher number of screenable samples while also eliminating the supply problem. For example, the EU-funded PharmaSea project (<http://www.pharma-sea.eu/>), an international collaboration between bioprospecting laboratories all over the world, exclusively works with extracts produced from fermented bacteria in the search for novel antibiotics, antiinflammatory and antineurodegenerative compounds.

While fermentation of microorganisms undoubtable is a good strategy, one should not refrain from collecting macroorganisms like *S. fortis* and *T. breittfussi* for bioprospecting purposes. Many microorganisms are host-specific and not found in the surrounding seawater nor in any other habitats, and many of these

microorganisms have proven difficult to cultivate.<sup>179,180</sup> Microorganisms living in symbiosis with macroorganisms can be responsible for a large portion of the biomass of their host organism. For marine sponges, microorganisms comprise up to 40% of sponge volume. The collected microorganism biomass may therefore very well be substantial enough to enable secondary metabolite isolation. In addition, the microorganisms might only produce the desired compound under certain environmental or ecological conditions. The growth conditions triggering the production of bioactive secondary metabolites can be difficult to mimic when the microorganisms are fermented in the laboratories.<sup>181</sup>

*S. fortis* is a marine sponge and *T. breitfussi* a marine cnidarian. Both phyla are known to exist in symbiosis with microorganisms. It has been indicated that the microbial symbionts of marine sponges are producers by enzymes involved in the halogenation of secondary metabolites.<sup>182</sup> It is still not known whether ianthelline, or the wide range of other bromotyrosine derivative isolated from marine sponges are biosynthesised by bacterial symbionts, or as a collaboration between symbionts and host enzymes.<sup>183</sup> The true biosynthetic origin of the breitfussins also remains to be confirmed.

### **4.3 Hit rate**

As discussed in the introduction, it is estimated that screening of five million fractions results in 1000 hits, giving an overall hit rate of 0.02%. One goal of a screening library is to provide a high level of structural diversity in order to optimise the bioactivity screening and the identification of lead structures. This can be accomplished by sampling a wide variety of species, or by collecting in unexplored ecological niches. It is hypothesised that tapping into largely unexplored marine diversity has great potential for yielding active extracts at a hit rate much larger than previously reported. Schupp et al. reported an increased hit rate when samples were collected in the largely unexplored biodiversity at a depth of 50 – 150 m around Guam.<sup>184</sup> The collected samples were able to generate a hit rate of 42% in the initial bioactivity screening.<sup>184</sup> The authors argued that sampling in previously unexplored locations as well as previously unexplored species will lead to an increased bioactivity hit rate in initial screening. The Arctic

and Sub-Arctic environment is a largely unexplored habitat, and the collection of organisms here for bioprospecting can therefore easily be rationalised.

#### 4.4 Crude extract preparation

Both *S. fortis* and *T. breitfussi* were subjected to extraction, resulting in an aqueous and an organic extract for both organisms. In the bioactivity screening performed as part of the Norwegian national screening platform (Marbio), the aqueous extracts deliver a lower hit rate than the organic extracts. The aqueous extracts are prepared not only to become an additional fraction for bioactivity screening. It also clears the organic extract of inorganic salts and other unwanted compounds and can concentrate the active principal component in the extract.

#### 4.5 Bioactivity detection in prefractionated crude extracts

Both target based and phenotypic bioactivity screening assays were used to evaluate the bioactivity of the crude extracts of *S. fortis* and *T. breitfussi*, see table 3. The initial bioactivity screening for antibacterial activity consists of six bacteria strains, where two are Gram positive and four are Gram negative. All the tested bacteria strains are human pathogens. In addition, the antidiabetic type II target protein tyrosine phosphatase 1B and the *Herpes simplex* virus were part of the initial bioactivity screening. A positive result in the initial bioactivity screening nominated the active fraction(s) for further bioactivity testing.

**Table 3 | An overview of the bioactivity assays used as part of the bioactivity guided fractionation of fractions prepared from *S. fortis* and *T. breitfussi*.**

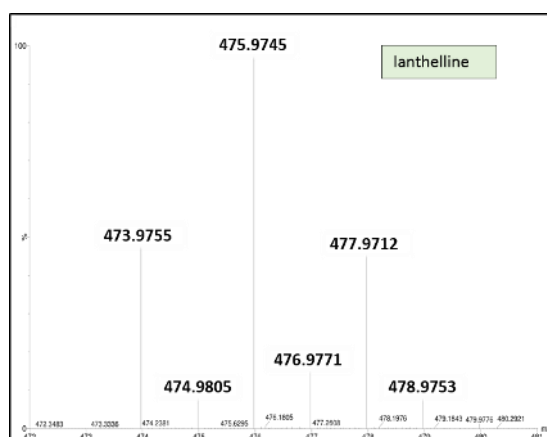
Assay	Type	Target
Anticancer	Phenotypical	Human melanoma cell line
	Target based	Kinase inhibition: PKA and Abl
Antibacterial	Phenotypical	<i>E. coli</i> , <i>S. aureus</i> , MRSA, <i>P. aeruginosa</i> , <i>E. faecalis</i> and <i>S. epidermidis</i>
Biofilm	Phenotypical	<i>S. epidermidis</i>
Immunostimulants	Phenotypical	TNF- $\alpha$ and IL-1 $\beta$
Anti-inflammatory	Phenotypical	TNF- $\alpha$ and IL-1 $\beta$

#### 4.6 Dereplication of bioactive organic extracts of *S. fortis* and *T. breitfussi*

The organic extracts of *S. fortis* and *T. breitfussi* were both subjected to dereplication by HR-MS analysis. Based on a combination of the results from the initial bioactivity screening and the dereplication, isolation of ianthelline and the breitfussins were initiated.

##### *Stryphnus fortis*

In the extract of *S. fortis*, the presence of one major peak was evident, eluting at approximately 35% aqueous acetonitrile from a C<sub>18</sub> column. The mass spectrum of the peak can be seen in figure 18 and revealed a compound with a dibrominated isotope pattern. The calculated protonated elemental composition was C<sub>15</sub>H<sub>18</sub>Br<sub>2</sub>N<sub>5</sub>O<sub>3</sub>, an elemental composition found to match ianthelline based on natural product database searches.



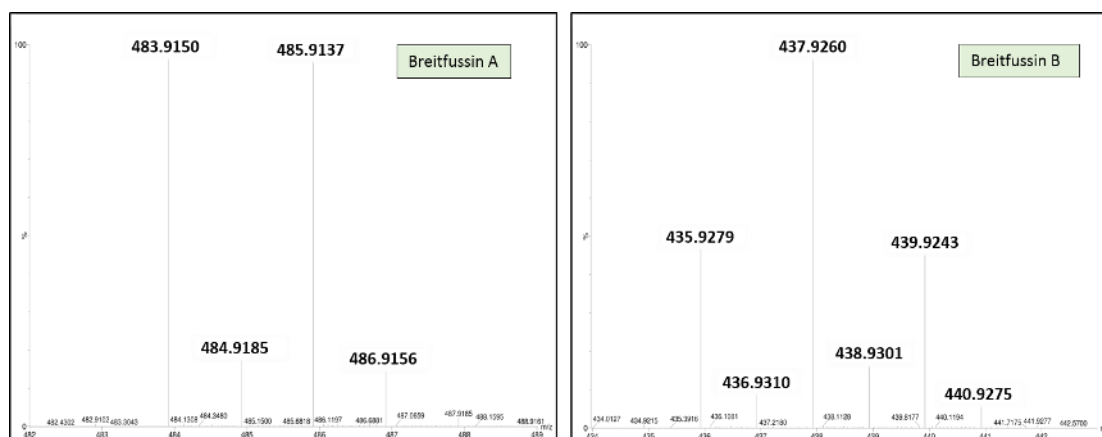
**Figure 18 | Isotope pattern of the dibrominated compound discovered in the organic extract of *S. fortis* later confirmed to be ianthelline.**

##### *Thuiaria breitfussi*

HR-MS analysis of the organic extract of *T. breitfussi* revealed the presence of several compounds with halogenated isotope patterns eluting at approximately 70% aqueous acetonitrile from a C<sub>18</sub> column. Breitfussin A and B were found as two of these peaks, and had monobrominated- and dibrominated isotope patterns with calculated protonated elemental compositions of C<sub>16</sub>H<sub>12</sub>N<sub>3</sub>O<sub>2</sub>BrI and C<sub>16</sub>H<sub>12</sub>N<sub>3</sub>O<sub>2</sub>Br<sub>2</sub>, respectively (Figure 19). As can be seen from the calculated protonated elemental compositions, the difference is that breitfussin A has one Br



and one I, while Breitfussin B is halogenated with two bromine atoms. It was therefore hypothesised that the two compounds shared a common molecular framework, and that the difference between the two might be as simple as the presence of a bromine- or an iodine atom in the same position. When searching the databases, the two compounds did not match any previously described compounds, and were thus believed to be novels.

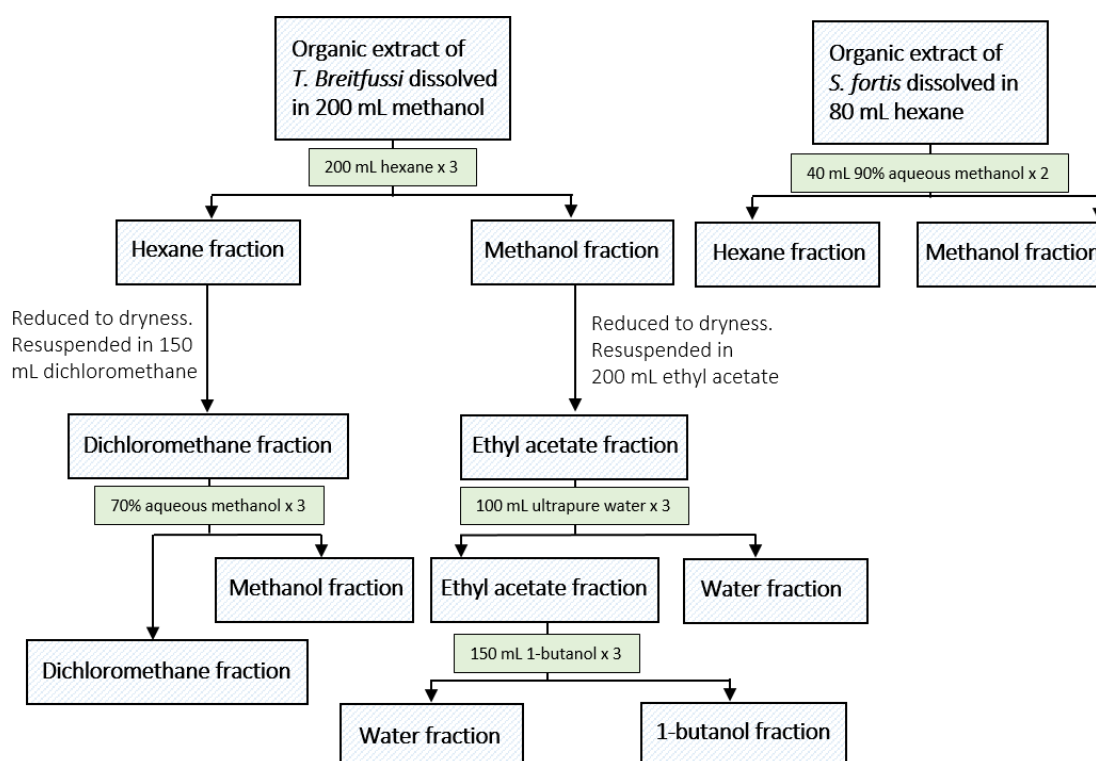


**Figure 19 | Isotope pattern for breitfussin A and B.** Breitfussin A has the characteristic monobrominated isotope pattern, and breitfussin B the characteristic dibrominated isotope pattern.

#### 4.7 Preparation of samples for mass guided prep-HPLC isolation

The organic extracts of *S. fortis* and *T. breitfussi* were prepared for mass guided prep-HPLC using liquid-liquid partitioning. The overview of the two partitioning strategies can be seen in figure 20. The sample preparation of *T. breitfussi* was quite labour intensive, using a series of two-phase mixtures in separation funnels to sort components by partitioning coefficients. HR-MS analysis was used to identify the fractions containing breitfussin A and B. In hindsight, flash chromatography might have been a more practical choice for sample preparation. Flash chromatography is less labour intensive, uses less organic solvent and eliminates the waiting time caused by formation of emulsions between two phases. Preparation of organic extracts prior to compound isolation on HPLC brings several advantages. It aims to remove impurities, and thereby generate a cleaner starting point for compound isolation. By removing impurities, the concentration of the desired compound is increased. With this, the capacity of the

HPLC column can be utilised better as a higher quantity of the interesting analyte can be injected onto it in every injection. This results a lower number of total injections needed as part of the compound isolation. In addition, it spares the columns for exposure to highly lipophilic compounds that might be difficult to remove.



**Figure 20 | The two different sample preparation liquid-liquid partitioning strategies used for the organic extract of *S. fortis* and *T. breitifussi*.**

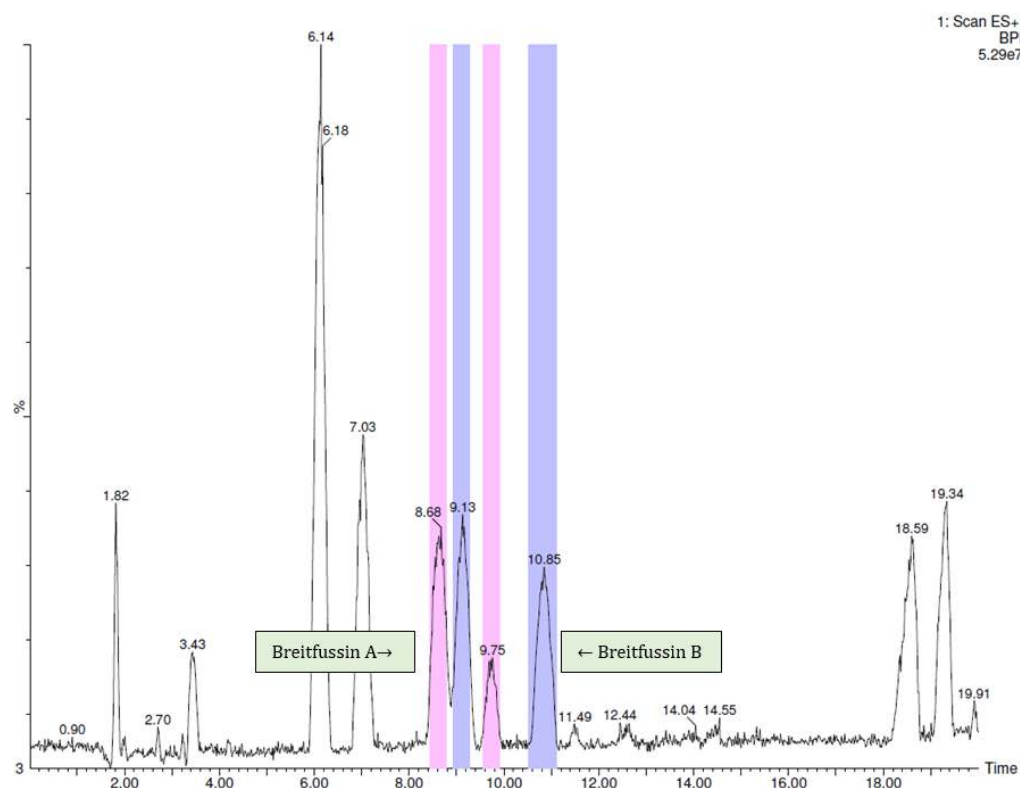
#### 4.8 Isolation of ianthelline and the breitifussins

Two important factors are commonly manipulated in order to isolate secondary metabolites from complex crude extracts by HPLC: The stationary phase of the HPLC column and the eluent gradient.

##### Selection of high performance liquid chromatography columns

When choosing columns for the purification of the breitifussins, a small volume of the organic *T. breitifussi* extract was injected onto HPLC columns with different surface chemistry. For every tested HPLC column, the ability of the column to separate breitifussin A and B from the impurities was evaluated. Based on these results, both breitifussins were initially isolated using an HPLC column with a

fluorophenyl stationary phase. A base peak intensity chromatogram from the initial isolation can be seen in figure 21.



**Figure 21 | Base peak intensity chromatograms from the initial isolation of breitfussin A and B from the organic extract of *T. breitfussi* separated over a RP fluorophenyl HPLC column using the masses of the breitfussins as fraction triggers.** Also visible in the chromatogram is the collection of isomers of breitfussin A and B. The collected breitfussin B peak was a mixture of two breitfussin isomers with equal masses, inseparable in the initial isolation step. They were later separated using a C<sub>18</sub> HPLC column.

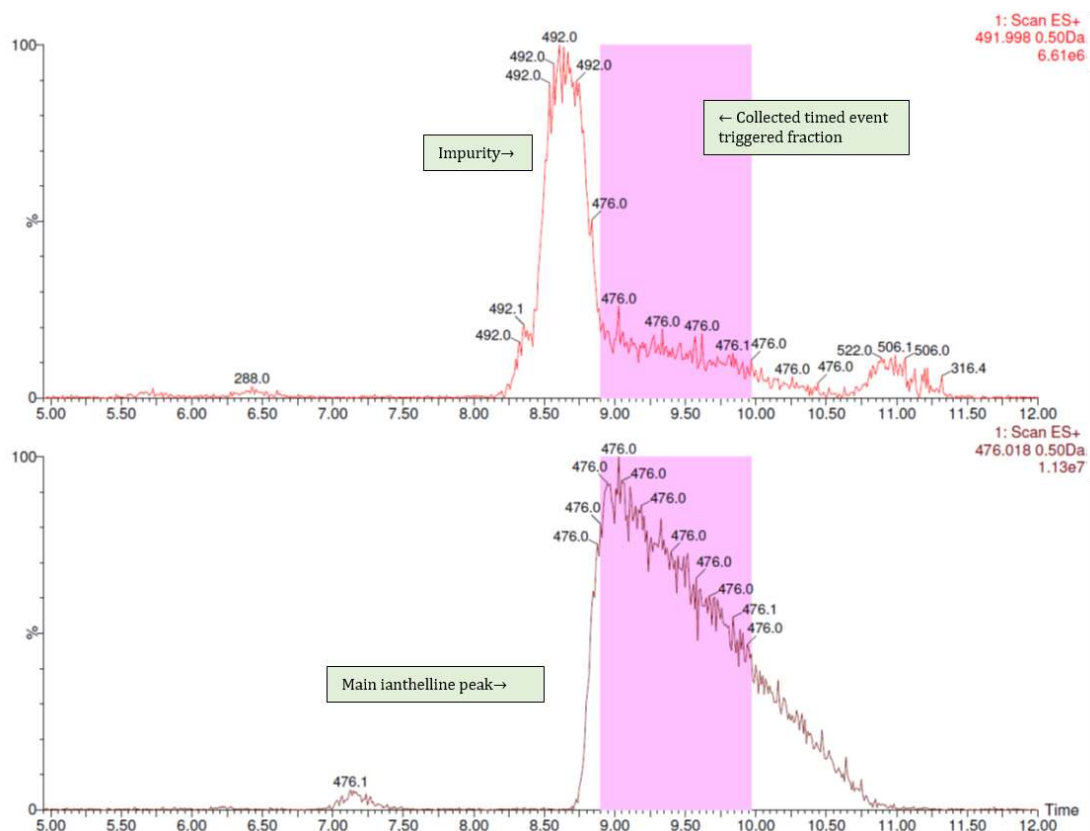
A single purification step is rarely sufficient for obtaining a pure active compound from a crude extract. Normally, when a compound of interest is subjected to a second HPLC purification step, columns with stationary phases different from the initial HPLC column are useful. While breitfussin A was further purified using the same fluorophenyl HPLC column with a slightly different elution gradient, breitfussin B was purified in a second step using a column with C<sub>18</sub> stationary phase. As a final purification step, both samples were run through a prep-HPLC column with a phenylhexyl stationary phase to remove remaining phospholipids.

### Optimisation of an elution gradient

To utilise the separation capacity of HPLC columns, a mobile phase gradient needs to be optimised for the isolation of the compounds of interest. When a RP-HPLC column is used, lipophilic sample constituents will interact with the hydrophobic surface of the stationary phase and an organic solvent is used to cause analyte elution. For both ianthelline and the breitfussins, elution gradients with increasing amounts of acetonitrile in water with 0.1% formic acid were used to separate the compounds of interest from the different sample components. In particular, the separation of breitfussin B and its closely eluting isomer required careful optimisation of the elution gradient.

### Mass- and timed-events triggering of fraction collection

Ianthelline was found in high quantities in the organic extract of *S. fortis*, and was easily collected using a C<sub>18</sub> column. However, one impurity could not be separated using different gradients. The ion chromatogram of the impurity and ianthelline can be seen in figure 22.

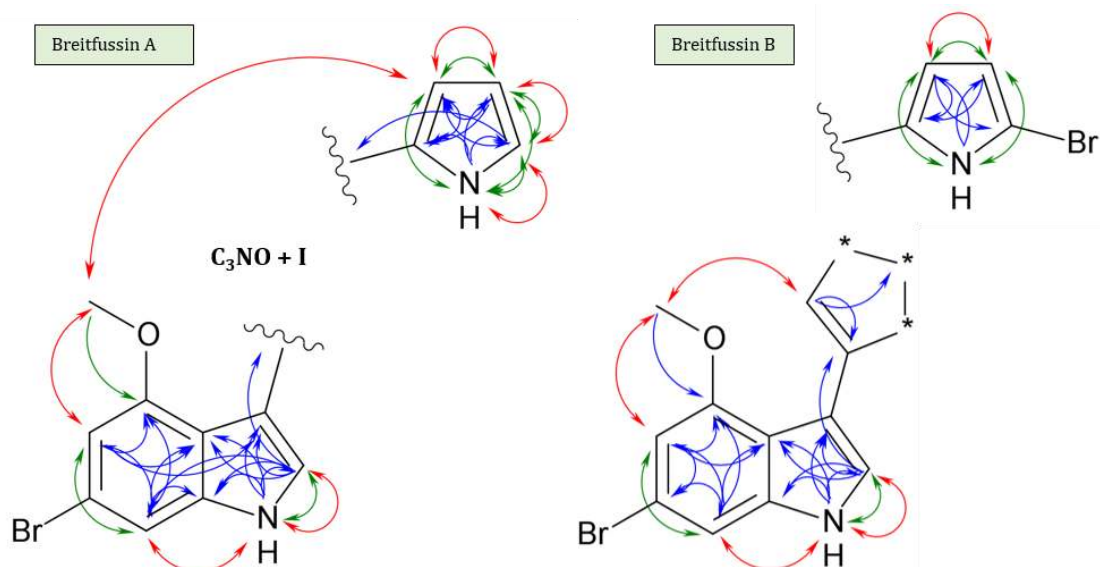


**Figure 22 | Ion chromatogram of ianthelline and the separated impurity.**

The impurity was finally eliminated from the collected fraction of the ianthelline peak by delaying the collection using timed events as opposed to triggering by mass. The small amount of ianthelline lost at the beginning of the peak was considered expandable, and the isolation resulted in 47.5 mg of pure end product from the initial isolation round.

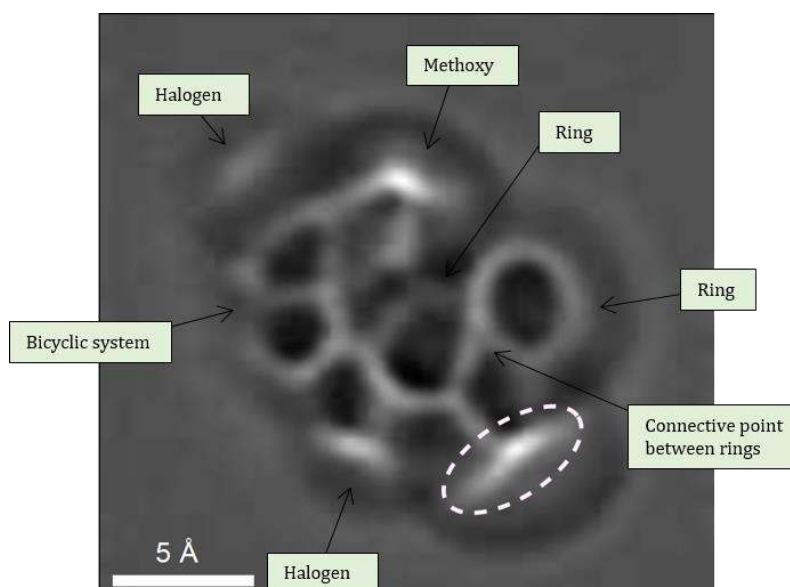
#### **4.9 Structure elucidation**

In natural product drug discovery, one of the major bottlenecks is structure elucidation. For ianthelline, a previously reported compound with published NMR data the structure confirmation was rapid, and could be done on the basis of HMBC and HSQC experiments, as well as HR-MS analysis. The structures of the novel breitfussins on the other hand, proved to be difficult to unambiguously elucidate using the readily available HR-MS and NMR experiments. Structure elucidation of unknown compounds using NMR relies heavily on the ability to detect interactions between neighbouring protons, and between protons and their neighbouring carbon atoms. The breitfussins are proton poor compounds, with a ratio of heavy atoms to protons of approximately 2:1. Because of this, structure determination with NMR was challenging, mostly due to a silent region in the centre of the compounds. Following NMR analysis, no unambiguous structure could be determined. On the basis of HR-MS analysis and 2D-NMR experiments, the fragments shown in figure 23 were determined to be the most likely substructures of the two compounds, although several other possible substructures could not be excluded, including tricyclic structural options.



**Figure 23 | A graphical representation of the NMR correlations found for breitfussin A and B : COSY (green), HMBC (blue) and ROESY (red) for the most likely substructures that could be puzzled together based on HR-MS and NMR analysis.**

The structures elucidation of the breitfussins had come to a complete halt before the opportunity of AFM imaging appeared. The breitfussins are planar compounds, and breitfussin A proved to be an excellent candidate for AFM imaging. The obtained AFM image of breitfussin A with highlighting of key observations can be seen in figure 24. The image revealed a bicyclic system that included a five-membered ring (the indole). In addition, it showed two connected rings and the connective point between the rings. The halogenations were visible as elongated spots, and a bulky methoxy group could also be seen. One part of the AFM image could not readily be explained by the structure of breitfussin A (circled in figure 24). This part of the image is believed to be an experimental artifact. It is believed to be a salt, an impurity from the solvent or an iodine atom that has disassociated from one molecule and that is now attached to this particular molecule. However, no absolute explanation for this observation is available. The AFM image excluded all tricyclic structural options and allowed for the focus of structural determination of the middle part of the breitfussins. CASE and DFT calculations of breitfussin A were conducted, both resulting in the structure now determined to be breitfussin A as the most likely structural alternative.



**Figure 24 | AFM image of breitfussin A with key observation highlighted.**

The combination of AFM imaging with the more traditional structure elucidation methods HR-MS, NMR and to a certain extent CASE and DFT, made the structure elucidation of breitfussin A and B possible. This represents a novel combination of structural elucidation techniques, and resulted in the opportunity to further investigate the bioactivity of the breitfussins as well as to potentially produce analogues of the compounds if desired.

#### **4.10 Bioactivity profiling of ianthelline**

The bioactivity of ianthelline was examined in paper I and II. The activities explored in the bioactivity profiling were detected in initial anticancer- and antibacterial screening assays of the crude organic extract of *S. fortis*. The results were followed up with phenotypic assays for further exploration of the bioactivity of ianthelline after isolation. Later, the mode of action of ianthelline was evaluated by using target based assays.

In paper I, the anticancer potential of ianthelline was explored. After isolation, the effect of ianthelline was elucidated by exposing one non-malignant and ten malignant cell lines to ranging compound concentrations. The result showed that ianthelline had a modest, dose-dependent activity against seven cell lines (including the non-malignant cell line). Furthermore, initial mode of action studies

revealed that the effect was time dependent, increasing with increasing exposure time. The combined results from the initial phenotypic bioassays indicated that ianthelline might interfere with a cellular process not common for all the exposed cell lines, and also that it affected an ongoing cellular process rather than causing rupture of the cell membrane, leading to death by necrosis.

These observations motivated the elucidation of the mode of action behind the observed bioactivity. Ianthelline was tested against a panel of 131 kinases at the international centre for kinase profiling in Dundee, as well as in two in-house kinase inhibition assays (kinases: Protein kinase A and Abl). Calculation of the Gini coefficient based on the results, showed that ianthelline inhibited the kinase panel with a modest selectivity. Despite the low selectivity, differences in kinase inhibition were observed. Three kinases were significantly inhibited: transforming growth factor beta activated kinase 1 (TAK1), serum and glucocorticoid-induced kinase 1 (SGK1) and protein kinase B $\beta$  (PKB $\beta$ ). All three kinases are commonly overexpressed or constitutively active in human cancers, including colon and breast cancer.<sup>185-187</sup> Another interesting observation was the apparent selectivity in the inhibition seen between the closely related kinases PKB $\beta$  and protein kinase B $\alpha$  (PKB $\alpha$ ).

Furthermore, fertilised sea urchin *P. lividus* eggs were used as test organism for examining the effect of ianthelline on cell cycle progression. Inhibition of cell division was found to be dose dependent. Against the same test organism, the effect of ianthelline on DNA synthesis, microtubule organisation and cytokinesis were also explored. No visible inhibition of DNA replication could be detected. Ianthelline was found to severely inhibit mitotic spindle formation, and thus also cytokinesis. Disruption of the highly dynamic mitotic-spindle microtubules is among the most successful targets for anticancer therapy, and is the cellular process targeted by paclitaxel.<sup>188</sup> The concentration of ianthelline, at which the mitotic-spindle dynamics are disturbed, is higher than the common low micro- to nanomolar bioactive concentrations of marketed anticancer drugs. However, ianthelline may be used as a scaffold production of analogues with structures optimised for microtubule and/or protein kinase interactions.



In paper II, the potential of ianthelline as an antibacterial drug, as well as an antifouling compound was elucidated. This was motivated by the initial antibacterial bioactivity screening results, as well as database searches revealing that compounds with chemical structures similar to ianthelline exerted antifouling activities.

The MIC of ianthelline was determined against seven human pathogenic bacteria. The ability to inhibit the biofilm formation of *S. epidermidis* was also studied. The lowest MIC value was found against Gram-positive *S. aureus*. Furthermore, the effect of ianthelline against the growth and adhesion of ten marine bacteria and eight microfouling microalgae was explored, to elucidate the potential of ianthelline as an inhibitor of microfouling. The growth of two strains of marine bacteria and one microalgal strain revealed MIC values as low as 0.1 µg/mL. Three bacteria strains and two microalgae strains had MIC values of 1 µg/mL. Growth of marine bacteria and microalgae was generally inhibited at lower concentrations than their adhesion, except for at the bacteria strain *Vibrio natriegens*, where adhesion was inhibited at a lower concentration. The potential of ianthelline as an antifouling agent was further explored by testing its ability to inhibit the settlement of *B. improvisus*.

Both antibacterial pharmaceuticals and antifouling agents should ideally exert no side-effects to human or any other exposed organism. When it comes to biofouling organisms, this is also true for the fouling organism which should ideally only have its attachment inhibited, and should not experience any other effects. The potential of ianthelline as an antifouling agent thus becomes even more evident when the results of paper I and II are compared. The active concentration range for the antifouling activities of ianthelline is substantially lower than active concentration range against the tested cell lines. A similar gap between active concentrations is also evident when comparing the activity against cell cycle progression of the sea urchin *P. lividus* (IC<sub>50</sub> value = 53 µM), while the settlement of *B. improvisus* cyprid was inhibited at significantly lower concentrations (IC<sub>50</sub> value of 3 µg/mL, corresponding to 6.3 µM). The gap between the active antifouling concentration and the concentrations at which ianthelline causes

damage to surrounding marine species thus appears to be significant. Further analysis needs to be conducted though, to elucidate the potential toxic effects of ianthelline to non-target organisms.

#### **4.11 Further work**

During this project, the bioactivity profile of the previously reported compound ianthelline was elucidated. Based on these results it would be interesting to peruse its potential as an inhibitor of the protein kinases TAK1, SGK1 and PKB $\beta$ . In addition, to further explore the potential of ianthelline as an agent selectively inhibiting PKB $\beta$  without affecting the closely related kinase PKB $\alpha$ . First and foremost though, the potential of ianthelline as an antifouling agent should be investigated. This could include further bioactivity and toxicity testing, as well as the production of analogues.

The breitfussins represent a novel chemical scaffold that may provide inspiration for the design of future bioactive compounds. Before that can be realised, the bioactivity profile of breitfussin A and B remains to be elucidated. This is currently under investigation.

## 5. Concluding remarks

The papers presented in this thesis are not collectively trying to answer one research problem, but are connected through a common research field: marine bioprospecting. The work conducted over the four years it took to complete this thesis has proven that the bioprospecting efforts put in at MabCent enables the discovery of both secondary metabolites with novel chemistry and bioactivity, as well as a previously reported compound which novel bioactivity.

Natural sources have yielded many important new pharmaceuticals and utilisable products for a wide range of applications in the past.<sup>189,190</sup> This can easily be explained, as evolution has favoured the tendency for organisms to produce chemically diverse compounds with structures optimised for specific target interactions. The previous success stories underscore the potential of secondary metabolites as a source of drugs and useful products, as well as emphasises the necessity of natural product research. Keeping in mind that the examination of only a small fraction of the biosphere for the potential content of bioactive secondary metabolites has resulted in 60% of all pharmaceuticals currently in use, the potential is enormous.

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# Paper I





# Paper II



# Paper III

