UNIVERSITY OF TROMSØ UIT

FACULTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
NORWEGIAN COLLEGE OF FISHERY SCIENCE

Antimicrobial natural products from Arctic and sub-Arctic marine invertebrates

Margey Tadesse

A dissertation for the degree of
Philosophiae Doctor

Summer 2010

Abstract

Infectious diseases are a leading cause of death world-wide and there is a growing need for new anti-infective agents to combat multi-resistant strains of bacteria and fungi. Marine natural products are promising sources of novel antimicrobial compounds. In the present thesis, an investigation into the antimicrobial metabolites of Arctic and sub-Arctic marine invertebrate species is presented. Extracts of seven ascidian species, six sponge species, a soft-alcyonid coral and a bryozoan species, were screened for their antimicrobial activities. The extracts were pre-fractionated by solid phase extraction (SPE) and purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Active metabolites were characterized by electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) techniques.

The antibacterial tyrosine-derived guanidines, 3-dihydroxy-tubastrine and tubastrine, have been isolated from the ascidian *Dendrodoa aggregata*. This is the first report on the isolation of active metabolites from *D. aggregata*. 3-dihydroxy-tubastrine has previously been isolated from the Australian sponge species *Spongosorites* sp. The compound was present in high concentrations in extracts of the ascidian and could serve as a chemotaxonomic marker for the species.

Extracts of the ascidian *Synoicum pulmonaria* displayed the highest antimicrobial activities in our assays. Bio-guided fractionation of the extract, revealed the presence of three novel compounds, named synoxazolidinones A, B and C. The structures of the compounds were elucidated by spectroscopic methods including 1D and 2D NMR techniques, and analysis of mass spectrometric data. The absolute configuration of the compounds was also established

by computational methods. The synoxazolidinones contain a unique 4-oxazolidinone core rarely encountered in natural products. Biogenetically, the compounds appear to be derived from arginine and tyrosine. This is the first report on the chemistry of *S. pulmonaria*. Synoxazolidinones also displayed anticancer activities and provide novel chemical scaffolds for structure-activity relationship studies which are currently being carried out.

The dibrominated tryptophan-derived metabolite, eusynstyelamide B, and three new derivatives, eusynstyelamides D, E and F, have been isolated from the bryozoan *Tegella cf. spitzbergensis*. The structures of the compounds were elucidated by mass spectrometry and, 1D and 2D NMR techniques. All four compounds displayed potent antibacterial activities in our assays. This is the first report of bioactive metabolites from *T. spitzbergensis*. Eusynstyelamide B has previously been isolated from the Australian ascidian *Eusynstyela latericius*. The presence of the same metabolites in different organisms and environments, suggests biosynthesis by symbiotic microorganisms.

In addition, this thesis provides background information on natural product research and current antimicrobial investigations of marine invertebrate species. The potential of Arctic and sub-Arctic marine invertebrates as sources of structurally novel, bioactive metabolites is demonstrated.

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Acknowledgements

The present work was carried out at the Norwegian College of Fishery Science (NFH), University of Tromsø, and partly at the Marine Biodiscovery Centre, University of Aberdeen, during the period from June 2006 to June 2010. Financial support was obtained from the University of Tromsø. The research period at the University of Aberdeen was funded by FUGE, MABCENT and NFH.

First of all, I would like to thank my supervisor Assoc. Prof. Tor Haug, and co-supervisors Assoc. Prof. Morten B. Strøm, Prof. Klara Stensvåg and Prof. Olaf B. Styrvold. Olaf, thank you for securing the funds for this project and all your good thoughts. Klara, your dedication and hard work keeps our group going. Morten, your excellence and high standards always improve the end result. Your drive and enthusiasm for your work, which give you many sleepless nights, are an inspiration. Last but not least, many thanks go to Tor. It has been my absolute privilege and honor to be your first PhD student. Thank you for your patience and generosity, and for teaching me to venture into new and unfamiliar territories, as you do, because the work requires it. You are my hero.

I would also like to express my gratitude to Veronika Tørfoss, who was involved in the early stages of this work. You are a gifted scientist and I enjoyed working with you. I would further like to thank Johan Svenson for his many contributions to this project. Espen Hansen and Jeanette H. Andersen are thanked for their collaboration during these last four years. All of my co-authors are acknowledged for their contributions. I am also grateful to Terkel Hansen and Morten Engqvist for their comments on this thesis.

To Prof. Marcel Jaspars, thank you for the opportunity to benefit from your expertise and for being so nice. Jioji, thank you for allowing me to follow you around the lab and putting up with all my silly questions. Thank you to all the members of the Marine Biodiscovery Centre, who have showed me how to run the NMR instrument, process the data and made my stay enjoyable.

My gratitude and appreciation are also extended to my colleagues at old IMAB. I would especially like to thank Chun and Hans-Matti, who are such wonderful people, for helping me with the practical issues of my life in Tromsø. Thank you for helping me move here and there, storing my possessions while I was abroad, helping me assemble furniture and even reading my thesis. I'm in your debt.

I would also like to thank my friends in Tromsø and those in Oslo; thank you for always finding time for me during the odd weekends and holidays. I appreciate it. And the old Aussie crowd, I always enjoy meeting up when possible.

Finally, my gratitude goes to all my family for their eternal support and love, to my mummy, Martha, for being a steady rock who's numerous phone calls are appreciated, and my dearest aunt, Hawi, who was and always will be, my beacon of light through stormy seas. I love you.

Tromsø, July 2010.

Margey Tadesse

Abbreviations

ACN	Acetonitrile
AIDS	Acquired immune deficiency syndrome
CD	Circular dichroism
C. glutamicum	*Corynebacterium glutamicum*
^{13}CNMR	^{13}Carbon nuclear magnetic resonance
E. coli	*Escherichia coli*
ESI-MS	Electrospray ionization mass spectrometry
EST	Expressed sequence tag
ET-743	Ecteinascidin-743
EXSIDE	Excitation-sculptured indirect-detection experiment
gCOSY	Gradient correlation spectrosocopy
gHMBC	Gradient heteronuclear multiple bond correlation
gHSQC	Gradient heteronuclear single quantum correlation
HIV	Human deficiency syndrome
^{1}HNMR	Hydrogen nuclear magnetic resonance
HR-MS	High resolution mass spectrometry
L. anguillarum	*Listonella anguillarum*
LC-MS	Liquid chromatography mass spectrometry
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus areus*
m/z	Mass-to-charge
1D/2D NMR	One dimensional/two dimensional nuclear magnetic resonance
NOE	Nuclear Overhauser enhancement/effect
P. aeroginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
VRE	Vancomycin resistant enterococci
VRSA	Vancomycin resistant *Staphylococcus areus*
WHO	World Health Organization

List of papers

I. **Margey Tadesse, Bjørn Gulliksen, Morten B. strøm, Olaf B. Styrvold, Tor Haug. 2008.** Screening for antibacterial and antifungal activities in marine benthic invertebrates from northern Norway. *Journal of Invertebrate Pathology*, *99*, 286-293.

II. **Margey Tadesse, Veronika Tørfoss, Morten B. Strøm, Espen Hansen, Jeanette Hammer Andersen, Klara Stensvåg, Tor Haug. 2010.** Isolation and biological activity of (*E*)-1-(4-hydroxystyryl)guanidine from the sub-Arctic ascidian, *Dendrodoa aggregata*. *Biochemical Systematics and Ecology*. *In Press*.

III. **Margey Tadesse, Morten B. Strøm, Johan Svenson, Marcel Jaspars, Bruce F. Milne, Veronika Tørfoss, Jeanette H. Andersen, Espen Hansen, Klara Stensvåg and Tor Haug.** Synoxazolidinones A, B, and C; novel bioactive alkaloids from the ascidian *Synoicum pulmonaria*. Manuscript submitted July 2010.

IV. **Margey Tadesse, Jioji N. Tabudravu, Marcel Jaspars, Morten B. Strøm, Espen Hansen, Jeanette H. Andersen and Tor Haug.** The antibacterial eusynstelamides B, D, E and F, from the Arctic bryozoan *Tegella cf. spitzbergensis.* Manuscript submitted July 2010.

1. Introduction

1.1 Antibiotics

The emergence and re-emergence of resistant bacteria is one of the major challenges facing the pharmaceutical industry today. Even though the extent and speed of emergence of bacterial resistance to antimicrobial agents vary with different types of drugs, resistance has so far developed to all known antimicrobial drugs[1]. The prevalence of infectious diseases caused by antimicrobial resistant human pathogens is rapidly increasing. This includes the worldwide emergence of multidrug-resistant *Mycobacterium tuberculosis*[2 3]. Other examples of microbial resistance to conventional antibiotics include vancomycin resistance in *Staphylococcus aureus* (VRSA) and enterococci (VRE)[4], resistance to beta-lactam antibiotics, such as the cephalosporins in the gram-negative bacilli *Pseudomonas aeruginosa* and *Escherichia coli*[5], and penicillin resistance (often multidrug-resistant) in pneumococci[6]. Resistance has also spread to a variety of non-bacterial pathogens, such as viruses, fungi and parasites. The development of resistance to antifungal agents by opportunistic fungal pathogens such as *Candida albicans*[7] and *Saccharomyces cerevisiae*[8] which can cause life-threatening systemic infections in immunocompromised individuals such as HIV and cancer patients, is on the rise[9].

Resistance develops by genetic mutations[10] or by the acquisition of exogenous genetic material[11-14]. Chemical modification of known antibiotics has been the most frequently employed method to address the problem of resistance. For example derivation of the basic nuclear structure of the penicillins, 6-amino-penicillinic acid, yielded compounds with activity against gram-negative bacilli (ampicillin, amoxicillin, carbenicillin, ticarcillin,

mezlocillin, azlocillin, piperacillin, and a variety of other "broadspectrum" penicillins), and β-lactams with activity against β-lactamase producing *S. aureus* and coagulase negative staphylococci (methicillin and a variety of other antistaphylococcal penicillins)[15]. Modifications of the cephalosporin molecule have resulted in alterations in its *in vitro* spectrum of activity, its resistance to β-lactamases, and its pharmacokinetic properties[16]. Nonetheless, as each new analogue has been introduced over the years, it has ultimately been succeeded by the emergence of resistant organisms[17]. One of the major contributing factors to this is the abuse and overuse of new antibiotics. This is a practice that needs to be addressed[18]. The picture is further complicated by the fact that the speed of discovery and development of new antimicrobial drugs active against multidrug-resistant organisms have slowed down considerably, although billions of dollars are annually invested in this research area[19]. There are several reasons for this apparently contradictory situation, including the even greater costs of bringing a new antibiotic from discovery to the market. This is currently estimated at between $100 million and $350 million in the United States alone[1, 20]. Another reason is the limited revenue from sales expected by pharmaceutical companies due to the short duration of treatment with antibiotics relative to other drugs, such as cholesterol and hypertension agents, which are consumed for prolonged periods and relieve symptoms rather than provide a cure[21]. Only two of the few antibiotics introduced in the last 20 years, the oxazolidinones (which inhibit bacterial protein synthesis) and cationic peptides (which permeabilize bacterial membranes), have unconventional modes of action[22].

There is a need to target screening more broadly to ensure that rare activities of unanticipated mode-of action are not missed[23] and to concentrate on the discovery of novel structural scaffolds to minimize the problem of resistance[24].

1.2 Natural products

Humans have always relied on nature for their supplies of medicine. Plants have been used for thousands of years for different remedies in traditional medicine systems of many cultures in places such as Africa, the Americas[25-27] and Asia[28]. Records show that the ancient Egyptians have been using myrrh for the local treatment of wounds as early as 2500 B.C. and papyri dating back to 2000 B.C. record the use of honey for the same purpose[29]. Records from Mesopotamia, written on clay tablets dating from 2600 B.C., describe the use of oils from plants such as cedars, cypress and poppy juice for the treatment of coughs, colds, parasitic infections and inflammation[30]. Records from 1100 B.C. show the use of herbal medicine in China[28] and documentation from about 1000 B.C. describes the Indian Ayurvedic system, which formed the basis for Tibetan medicine[31]. Hippocrates mentioned the wound healing properties of myrrh in 400 B.C.[29] and writings by the Greek philosopher and natural scientist, Theophrastus (~ 300 B.C.) portray how the Greeks dealt with the medicinal qualities of herbs. The Arabs preserved much of the Greco-Roman expertise in the Middle Ages in works such as *Canon Medicinae* by the Persian pharmacist, physician, philosopher and poet Avicenna[30]. The modern era of natural products in medicine is regarded to have began with the isolation of the first commercial pure natural product, morphine, by E. Merck in 1826 from opium produced by seed pods of the poppy, *Papaver somniferum*[32].

Data collected from 1959 to 1980, indicated that 25% of prescribed drugs in the United States were derived from plant extracts. Currently, at least 119 chemical substances, derived from 90 plant species, are important drugs used in many countries[33, 34]. The discovery of penicillin from fungal strains of *Penicillium notatum* in 1928[17] started an era of massive screening projects of microorganisms. Approximately 80% of drugs are either natural products or

derivatives of natural products[21], including some well known examples such as the antibiotic streptomycin from the fungi *Streptomyces griseus*, the antimalarial artemisinin from the wormwood, *Artemisia annua*, and taxol from the bark of the yew tree, *Taxus brevifolia*[30].

Between 2005 and 2007, 13 natural product derived drugs were approved in the United States. Five of these, the anticancer peptides exanatide and ziconotide, and the small molecules ixabepilone (anticancer agent), retapamulin (antibiotic) and trabectedin (anticancer compound), represent the first members of new classes of drugs[35, 36]. Just in the case of polyketides, high-throughput screenings have led to more than 20 commercial drugs with a "hit rate" of 0.3% for natural products compared to the <0.001% hit rate of synthetic libraries[37].

1.2.1 Natural product drugs: Challenges and prospects

Despite the successful record of natural products in drug discovery, many pharmaceutical firms have eliminated their natural product research in the last decade[21]. Even though more than 100 natural product based drugs are currently in clinical trials, this represents a drop of about 30% between 2001 and 2008[35]. Companies involved in drug discovery are under tremendous pressure to hit the target very quickly and profitably when it comes to new drugs. The inherent problems of natural product discovery such as the slow identification process due to the often complex nature of natural products with numerous oxygen-containing substituents and a number of stereocenters[38]. Furthermore, reliable access and supply are a problem, as well as intellectual property concerns of local authorities[39]. All these factors lead drug companies to prefer screening pure compounds from synthetic libraries[21].

Seasonal or environmental variations in the chemical composition of living organisms can cause problems with initial detection of active compounds and subsequent re-purification and repetition of assays. The initial concentration of active metabolites may be too low to be effectively detected by high-throughput screenings and assays can be plagued by poor solubility, by fluorescent or coloured contaminants or by the key compound being unstable in the extract mixture[21]. Synergistic effects can also occur where activity is lost upon separation of the constituents[40]. Dereplication procedures involving completion of structural characterization in order to determine whether the molecule is already known, are often time consuming.

A number of strategies are currently in use to address the challenges of drug discovery from natural products. One of these is so-called “smart screening”, involving the use of strains of microorganisms for antimicrobial testing which are resistant to common antibiotics, thereby lowering the chances of rediscovery of known metabolites[41]. Another strategy is molecular target specific screening[42, 43]. Further evolution in metagenomics is expected to access a number of biosynthetic products[44]. Advances in analytical methods are also being applied to high-throughput screenings in order to facilitate dereplication based on various hyphenated techniques. Each of these methods have their own advantages or drawbacks in sensitivity, resolution, time, sample size and efficiency in searching most of the commercial databases (AntiBase[45], Dictionary of Natural Products[46], MarinLit[47] and SciFinder Scholar or CAPlus). These techniques include LC/MS[48, 49], LC/MS/MS[50-52] and various semi-atuomated systems involving HPLC/NMR[53-55], often with cryo-probe NMR requiring only microgram samples[56, 57]. An example of HPLC-NMR is a semi-automated system where substances from HPLC are captured by solid-phase-extraction cartridges and eluted into an NMR cryoprobe (HPLC-SPE-NMR)[58]. Research is also being carried out into the development of nano-NMR[59].

Traditionally, soil bacteria (mainly actinomyecetes), fungi and higher plants, have been the main sources of natural products[22, 60, 61]. With estimates of numbers of living species ranging from 2-100 million[21], where the marine environment comprises approximately half of the total global biodiversity[62], it is obvious that the natural world represents a great wealth of resources. Unique structures are being discovered in organisms living under extreme conditions[63, 64] such as the great sea depths (piezophiles). One example is abyssomicin, a polycyclic antibiotic from an ocean floor sediment bacterium[65] and haloduracin, a lanthionine containing peptide antibiotic from bacteria living at an extreme pH of >9.0 (alkaliphile)[66]. The diversity of chemistry encountered in bioactive natural products arising from factors such as multiple chiral centres, heterocyclic substituents, and polycyclic structures, is by no means rivaled by combinatorial libraries[67]. Even though most natural product drugs do not comply with Lipinski's Rule-of-Five for orally available compounds due to their higher molecular weights, more rotable bonds and more stereogenic centres, they retain relatively low log P values and are generally more readily absorbed than synthetic drugs[68]. In the years to come, the prevailing relevance of natural products as sources of new drugs can only become more apparent.

1.3 The marine environment

Life on earth evolved in the seas billions of years ago and the greatest biodiversity of life is found in the oceans[69]. Out of the 36 phyla of life 34 are represented in the marine environment and 21 of these are exclusively marine. The oceans cover more than 70% of the earth's surface and contain more than 300 000 described species of plants and animals[70]. It is estimated that this is a small percentage of the total number of species[71]. Marine plants and animals have adapted to divers habitats ranging from tropical, shallow-water coral reefs to temperate areas and sub-zero, deep-ocean trenches with high-pressure and no light[62]. These vast differences in the ecosystems of the marine environment, have resulted in the production of structurally novel, biologically active secondary metabolites unknown from terrestrial sources[72]. The evolution of these metabolites has been driven by various ecological pressures including competition for space, biofouling of surfaces, predation and successful reproduction over distances[70]. While rare in terrestrial metabolites, marine natural products are often halogenated due to the abundance of bromide and chloride ions in seawater. It is noteworthy that bromine is the most commonly found halogen in marine compounds, even though its concentration in seawater is lower than that of chlorine[73].

Bacteria occur in seawater at concentrations of approximately one million cells per milliliter. Marine plants and animals are constantly exposed to high concentrations of bacteria, many of which are opportunistically pathogenic and readily attach when provided an appropriate surface. Bacteria associated with the surfaces, tissues, and internal spaces of marine invertebrates and animals, experience divers microenvironments and therefore have tremendous potential as a source of novel secondary metabolites[72].

1.4 Marine natural products

Little is known of the history of the use of marine sources in traditional medicines, but it has been reported that marine algae have been used in Chinese folk medicine for more than two thousand years[74]. Documentation from around 40-90 A.D. shows the use of marine invertebrates and fishes for the treatment of various diseases and ailments such as toothaches, ulcers and boils by the Greeks[75]. The ancient Phoenicians employed secretions from mollusks to produce purple dyes for cloth and seaweed have long been used as fertilizers[76]. Even though there are reports from the late 1940's of bioactivity from marine sources[77], the first notable discoveries were of the nucleosides spongothymidine in 1950[78, 79] and spongouridine in 1955[80] from the Caribbean sponge *Crypthitheca crypta*. The compounds were found to possess antiviral activity and synthetic analogue studies led to the development of the clinically relevant anticancer agent Ara-C approximately 15 years later[81], along with the antiviral compound Ara-A[30, 76, 82].

Systematic investigation of the marine environment as a source for novel bioactive natural products began in earnest in the mid -1970's[76], as improvements in scuba and submersible technologies made physical access to the oceans possible[69, 82]. The field of marine natural products is now 40 years old and Blunt *et al*. report that more than 15 000 marine natural products have been isolated in the period from 1965 to 2005[83]. The number of reported marine natural products in 2007 was 17 000[84] and with reports of 1065 new compounds in 2008[85], the number currently could be approaching 20 000. The compounds so far isolated from marine invertebrates often have no comparable equivalents in terrestrial sources[82].

Apart from the aforementioned Ara-A and Ara-C, only two marine natural product derived drugs have successfully reached the market as thereapetic drugs, as of mid 2009[86]. Ziconotide (Prialt) is the synthetic equivalent of a peptide first purified in 1984 from the venom of a marine mollusk *Conus geographus*[87]. The drug was approved in the United States in 2004 and in the European Union in 2005 for the management of severe chronic pain [88]. Trabectedin (Yondelis; ET-743), a tetrahydroisoquinolone alkaloid characterized at the end of the 1980's[89], is a synthetic antineoplastic agent derived from the Caribbean tunicate *Ecteinascidia turbinate* and is currently used in Europe for treatment of advanced soft tissue sarcoma and ovarian cancer[90]. Approximately 13 other compounds are currently undergoing various stages of clinical trials[86]. The anti-inflammatory pseudopterosins, isolated from the Caribbean gorgonian *Pseudopterogorgia elisabethae*[91, 92] are used as constituents of Estee Lauders anti-wrinkle cream, Resilience, and are a good example of commercialized human use of marine natural products[82]. Considering the short history of marine drug discovery and the odds of a natural compound reaching clinical use being estimated at 1 in 4,000-10,000[69], coupled with the fact that HPLC, one of the essentials of modern isolation methods, was not available until the late 1970's[82], it is understandable that the field has evolved slowly. Discovery of compounds has been relatively simple, but acquiring the large amounts of sample necessary for development, was and still is, a major challenge.

There are several methods which can be applied to ensure adequate supply of a bioactive compound from a marine source. These include collection, aquaculture, tissue culture, chemical synthesis, symbiont culture and molecular biological approaches[69]. Ecologically sustainable large-scale collection is often difficult to achieve. Aquaculture and cell culture have been successfully attempted as in the case of halicondrin B[69, 82], an anti-tumour macrolide originally isolated from the Japanese sponge *Halichondria okadai*[93, 94]. However, it

was ultimately total synthesis of the compound[95] which led to the development of the drug E7389[82], which is currently in Phase III clinical trials for breast cancer and Phase II trials for prostate cancer in the US and EU, as well as Phase II trials in the EU for sarcoma[86]. Due to the structural similarities of many invertebrate compounds to microbial metabolites, it is suspected that many of these compounds actually originate from symbiotic microorganisms, as in the case of many peptides[96]. Actual proof of such production was first demonstrated by Hamann and co-workers in 2003, when they showed that the manzamines, a family of alkaloids originally isolated from an Indonesian sponge *Acanthostrongylophora* sp. with activity against tuberculosis, HIV and AIDS opportunistic fungal infections[97], were actually produced by the culturable symbiotic microbe *Micromonospora* sp.[98] Many researchers are looking at developments in the field of molecular biology for genetic control of biosynthesis, either from an invertebrate or its symbiont, thus providing solutions for the supply problem as well as yielding novel compounds[21, 69, 82].

The list of marine pharmaceuticals currently in clinical trials does not include new antibiotics[86] and clearly reflects the focus of past funding, which has been on the development of new anticancer agents[82]. However, many researchers are directing their efforts at investigating the marine environment for the presence of potential antibiotics, antimalarial agents, anti-tuberculosis drugs and cures for other infective diseases[70, 97, 99-101]. Recently, a combinatorial library of 3828 compounds based on the core structure of the psammaplins, symmetrical bromotyrosine-derived disulfide dimers originally isolated from the *Psammaplysilla* sponge[102, 103], resulted in six compounds with activities against methicillin and vancomycin resistant strains of *S. areus* at a minimum inhibitory concentration (MIC) of less than 1 μg/ml[104].

Cold-water marine organisms or psychrophiles are found in waters with temperatures ranging from -2 to +4 °C, including the polar deep sea, the temperate and tropical deep-sea where the temperature is nearly constant 4 °C, Antarctica, Canadian Maritimes, the Northern Sea of Japan and the North Sea above 60 °N latitude[84]. Diverse and highly bioactive compounds have been isolated from these sources including mixirins A-C (cyclic lipopeptides isolated from a *Bacillus* sp. near the North Pole with potent anticancer activity)[105]. The structurally unique acyclic, dimeric 3-alkyl pyridine alkaloid, viscosaline, was isolated from the Arctic sponge *Haliclona viscosa*[106] and has a likely feeding deterrent activity in the organism[107]. The majority of the marine natural products currently being reported originate from warm climates such as the Caribbean, the China Sea, the Indian Ocean, Japan and the Western Pacific[108]. Treasures of the Polar regions remain largely unexplored.

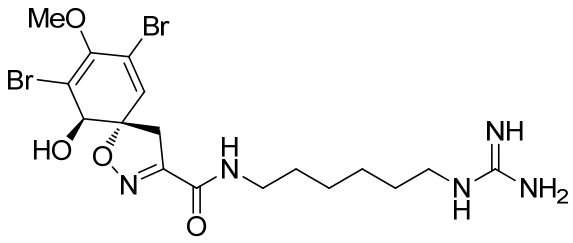
1.5 Marine alkaloids

Over 20 structural classes of antibiotics have so far been discovered through natural product screening. Among these are the chloramphenicols, tetracyclines, macrolides, polyenes, glycopeptides, lincosamides, cycloserines, streptolydigins, coumarins (including novobiocin), rifamycins, cephalosporins, glycolipids, polyoxins, phosphonates, elfamycins, cephamycins, monobactams, carbapenems, and lipopeptides[17]. Alkaloids are nitrogen-containing compounds and it has been estimated that approximately 40% of all marine natural products which have been reported since 1965, contain nitrogen[108]. Alkaloids also occur in plants, microorganisms and animals. These metabolites display significant biological activity and are often useful as drugs or biological probes for physiological studies[109]. A number of marine alkaloids have exhibited antimicrobial activity[85, 108, 110-112].

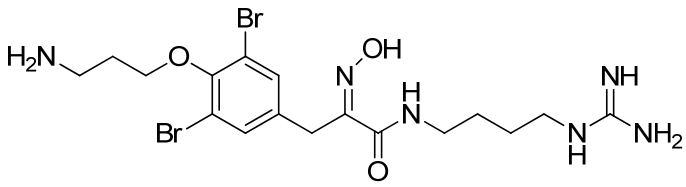
1.5.1 Marine bromotyrosine guanidines

A large number of the reported marine tyrosine guanidines, have been brominated compounds. Bromotyrosine metabolites have been steadily reported since the end of the 1960's. A wide range of bioactivities have been associated with these compounds including antimicrobial, antifouling, antiviral and anticancer activities[113]. Generally, the isolation of guanidine alkaloids from complex mixtures is difficult due to their basic nature and high polarity[114]. Examples of bromotryrosine guanidine derivatives isolated from marine organisms, presented in Figure 1, include aplysinamisine II, an antimicrobial and cytotoxic alkaloid isolated from the sponge *Aplysina cauliformis*[115]. An unnamed enzyme inhibitor was isolated from the sponge *Oceanapia* sp[116]. Ianthelline is an antimicrobial alkaloid isolated

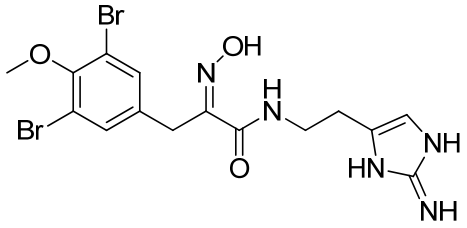
from the sponge *Ianthella ardis*[117]. Purealidin O is an amidase inhibitor, also isolated from the sponge *Oceanapia* sp[118].



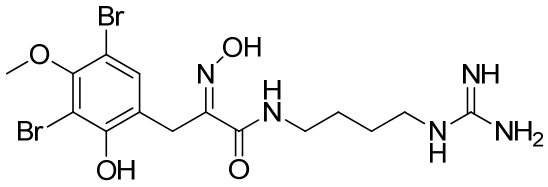
Aplysinamisine II



Oceanapia metabolite



Ianthelline



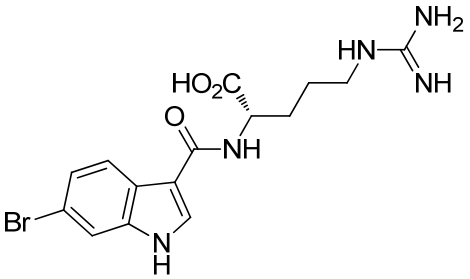
Purealidin O

Figure 1. Structures of aplysinamisine II, an *Oceanapia* metabolite, ianthelline and purealidin O.

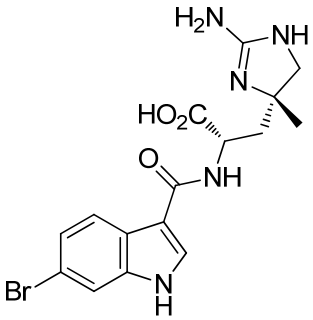
1.5.2 Marine bromo-indole guanidines

A number of marine, bromo-indole guanidines, derived from the amino acids tryptophan and arginine, have been reported[114]. These include the cytotoxic N-(6-bromo-1H-indolyl-3-carbonyl)-$_{L}$-arginine and N-(6-bromo-1H-indolyl-3-carbonyl)-$_{L}$-enduracididine (containing the rare amino acid enduracididine) (Figure 2), from the ascidian *Leptoclinides dubius*[119].

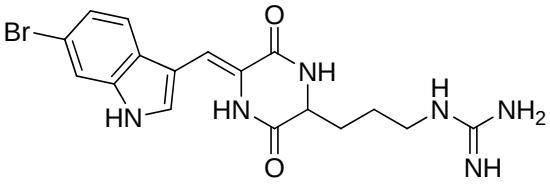
Barettin[120, 121] and dihydrobarettin[121] (Figure 2), are antifouling agents isolated from the sponge *Geodia baretti*.



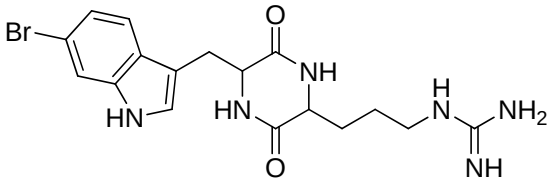
N-(6-bromo-1H-indolyl-3-carbonyl)-$_L$-arginine



N-(6-bromo-1H-indolyl-3-carbonyl)-$_L$-enduracididine



Barettin



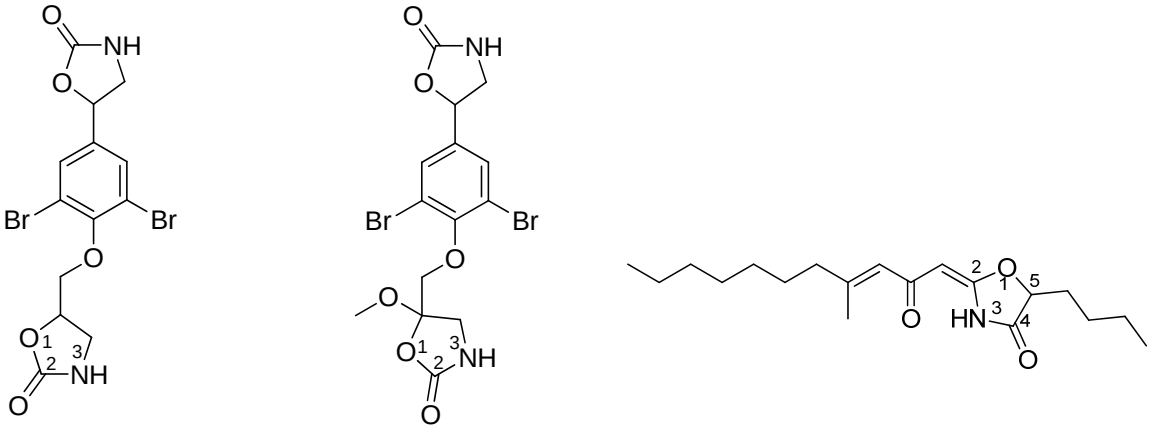
Dihydrobarettin

Figure 2. Structures of N-(6-bromo-1H-indolyl-3-carbonyl)-$_L$-arginine, N-(6-bromo-1H-indolyl-3-carbonyl)-$_L$-enduracididine, barettin and dihydrobarettin.

1.5.3 Marine oxazolidinones

Although rare in nature, a few oxazolidinones have nevertheless been reported from marine organisms. The most common structural motif is the 2-oxazolidinone configuration of the five-membered ring. A dibrominated phenolic derivative containing two 2-oxazolidinone groups (LL-PAA216), has been isolated from the sponge *Verongia lacunosa* collected off the coast of Puerto Rico[122] (Figure 3). The compound did not exhibit any significant antimicrobial activity. A derivative of the *Verongia* metabolite, with an O-methyl group attached to the 5C of one of the oxazolidinone-rings, has been reported from the Caribbean sponge *Aplysina insularis*[123] (Figure 3).

The antibacterial lipoxazolidinones A, B, and C are in addition to the compounds presented in the current study, the only other 4-oxazolidinones reported from nature. The metabolites were isolated from a marine actinomycete strain of the genus *Marinspora*, collected from a Guam marine sediment[124]. Lipoxazolidinone A is shown in figure 3.



LL-PAA216 *Aplysina* metabolite Lipoxazolidinone A

Figure 3. Structures of the marine oxazolidinones LL-PAA216, an *Aplysina* metabolite and lipoxazolidinone A.

2. Antimicrobial natural products from invertebrate species

2.1 Antimicrobial natural products from sponge species

Sponges are undoubtedly the most widely investigated organisms in marine natural product history. Allegedly, a new molecule has been isolated from sponges every two days within the last two decades[125]. All chemical classes are widely represented among the sponges. The vast chemodiversity of sponge species is probably due to their position in the Tree of Life, as the first examples of multicellular organisms. Sponges emerged approximately 500-550 million years ago and are exclusively aquatic. A sponge is a double cell-walled bag pierced with a very large number of small holes called ostioles and with an opening called the oscule. The cell membrane consists of many combinations of phospholipids and sterols, most of which are absent from the cell walls of other animals. The constant movement of the flagellated cells lining the inner wall, the chaonocytes, draws water, oxygen and nutrients into the sponge and pushes it out through the oscule. Between the outside of the sponge and the interior is the mesohyl, which consists of collagen fibres and various types of cells including the archaeocytes, which are able to turn into any other type of cell. The mesohyl also contains sclerocytes, which produce the mineral elements of the skeleton, called spicules[125].

Approximately 95% of sponge species belong to the subphylum Demospongiae (demosponges) and have skeletons consisting of silica and/or spongin fibres. The latter is a fibrous protein similar to keratin. Sponges live at all depths from shore level to the abyss. Sponges are permanently attached (sessile) and live in association with a number of organisms, which may be extra- and intracellular symbionts, commensals, parasites, or simply guests taking advantage of the shelter provided by the cavities of the sponge (crustaceans and nematodes). All demosponges contain extracellular bacteria, which sometimes can constitute

a biomass comparable or greater than that of the sponge itself. These symbiotic associations have considerable implications for attempts to establish the origin of metabolites extracted from sponges[126]. These compounds might be derived of the sponge itself, from one of the associated microorganisms or by some interaction between the sponge and the microorganism. In particular, associated bacteria are the origin of many substances involved in the defence of the sponge against predators, as in the case of the sesterterpenes produced by association between certain sponges and symbiotic zooxanthellae[125].

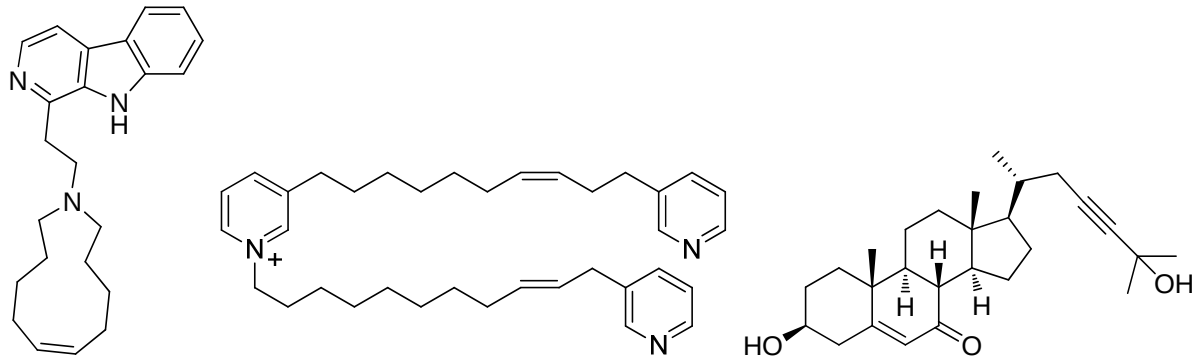
The great chemodiversity of metabolites from sponge species is equaled by the diversity of biological activity reported for the compounds; sponge metabolites have shown positive results in all the tests of biological activity which are in common use[125]. Table 1 lists some antimicrobial sponge metabolites isolated mainly in the last decade.

The structures of some sponge metabolites are shown in Figure 4. The manzamines are a group of polyheterocyclic alkaloids containing a β-carboline. These compounds, which are isolated from the sponge *Haliclona* sp., exhibit a number of bioactivities including antibacterial, antifungal, cytotoxic and antimalarial activities[125]. Manzamine C is shown in Figure 4[127]. Niphatoxin A is a cytotoxic tripyridine alkaloid isolated from the sponge *Niphates* sp.[128] Gelliusterol A-D are acetylenic sterols isolated from the sponge *Gellius* sp.[129]. The structure of Gelliusterol D is shown in Figure 4.

Table 1. Examples of antimicrobial natural products from sponge species isolated mainly in the last decade.

Compound	Region	Species	Chemistry
Membranolides C-D	Antarctica	*D.membranosa*	Diterpenes[130]
Polymastamide A	Norway	*P. boletiformis*	Steroid[131, 132]
Discorhabdin R	Antarctica	*Latrunculia* sp.	Alkaloid[133]
Caminosides B-D	Caribbean Sea	*C. sphaeroconia*	Glycolipids [134]
Spongosorites	Korea	*Spongosorites* sp.	Alkaloid[135]
Batzellaside A-C	Madagascar	*Batzella* sp	Alkaloid[136]
Dendridine A	Japan	*Dictyodendrilla* sp.	Alkaloid[137]
Halichonadin C	Japan	*Halichondria* sp	Sequiterpene[138]
Phenol	Zanzibar	*D. herbacea*	Polyketide [139]
Spongistatin	Indian Ocean	*H. erecta*	Polyketide[140]
Latrunculins	Red Sea	*N. magnifica*	Polyketide[141]
Nagelamide A	Australia/Japan	*Agelas* sp	Alkaloid[142]
Kalihinol Y and X	Philippinnes	*Acanthella* sp.	Diterpene[143]
Manoalide	Palau	*Luffariella* sp.	Sesterterpene[144]
Melophlin C	Guam	*M. sarassinorum*	Polyketide[145]
Ptilocaulis guanidine	USA	*P. spiculifer*	Alkaloid[146]
Cribrostatin 6	USA	*Cribrochalina* sp.	Alkaloid[147]
Purpuramine L	India	*P. purpurea*	Bromotyrosine alkaloid [148]
Germacrane	Thailand	*Axinyssa* sp.	Sesquiterpene[149]
Astroscleridae sterol/	Bahamas (from the deep sea)	*Astroscleridae* sp	Sterol sulfate[150]
Dysidea sterols	Australia	*D. arenaria*	Sterol[151]
Massadine	Japan	*S.* aff. *massa*	Alkaloid[152]
Naamine G	Indonesia	*L. chagosensis*	Alkaloid[153]
Utenospongin B	Morocco	*H. communis*	Diterpene[154]
Caminoside A	Canada	*C. sphaeroconia*	Glycolipid[155]
Zammamistatin	Japan	*P. purpurea*	Bromotyrosine[156]
Corticatic acids A-B	Japan	*P. corticata*	Polyacetylenic acid[157]
Swinehoeiamide A	Papua New Guinea	*T. swinhoei*	Polyketide[158]
Acetylenic acid	Japanese	*Oceanapia* sp	Fatty acid[159]
Dysidea ether	Micronesia	*Dysidea* sp.	Bromodiiphenyl ether[160]

Adapted from the series on marine pharmacology by Mayer *et al*.[161 162, 163 164]



Manzamine C Niphtaxin A Gelliusterol D

Figure 4. Structures of the sponge metabolites manzamine C, niphtaxin A and gelliusterol D.

2.2 Antimicrobial natural products from ascidian species

The class of ascidians (tunicates, phylum chordata) are sessile organisms, characterized by the presence of the notochord, a stiff rod which supports the body and is replaced in vertebrates by the spinal chord. Solitary ascidians are termed “simple ascidians” and colonial ascidians are described as “synascidians”. All ascidians are shaped like bags equipped with two siphons; an inhaling oral and exhaling excretion chamber. The ascidians live inside an external tunic (hence the name tunicates), which is rich in cellulose, and helps fix the animal to its substrate as well as providing shelter. These organisms are filter-feeders or suspension feeders; seawater charged with food particles enters through the oral siphon and circulates in the body through the gill. Some species filter several thousand times their own volume each day. Fixed ascidians can accommodate photosynthetic symbionts such as cyanobacteria. Ascidians are found in all the World’s seas and at all depths, but most species have been harvested within the upper 500 meters. Only 50 species have been found between 2000 and 5000 metres[165].

Ascidians are one of the most widely studied marine organisms[112]. The majority of compounds isolated from ascidians are alkaloids[111] and nitrogen-containing cyclic peptides[165]. The nitrogen-containing derivatives are often associated with aromatic nuclei among the alkaloids (indole, carbazole, pyridoacridine, isoquinoline) and with heteroaromatic nuclei among the cyclic peptides (thiazole, thiazoline, oxazole, oxazoline). Most of these compounds possess antibiotic, anti-tumour, antiviral and immunosuppressive activities[165]. Most of the antimicrobial ascidian metabolites isolated in the last decade are listed in Table 2.

Some of the ascidian alkaloids have been found in other phyla of invertebrates suggesting that they might be of symbiotic bacterial origin. It is believed that most of the cytotoxic ascidian metabolites are involved in defense against predators and antifouling[166, 167].

Table 2. Antimicrobial natural products from tunicate species isolated in the last decade.

Compound	Region	Species	Chemistry
Halocidin	Korea	*H. aurantium*	Peptide[168]
Plicatamide	USA	*S. plicata*	Peptide[169]
Eudistomin X	Micronesia	*Eudistoma* sp	Alkaloid[170]
(2S, 3R)-2-aminododecan-3-ol	Brazil	*C. oblonga*	Polyketide[171]
Dicunthaurin	South Korea	*H. aurantium*	Peptide[172]
Eudistomins Y6	Korea	*Eudistoma sp*	β-carboline[173]
Tunichromes	USA	*A. nigra*	Dihydrodpa alkaloids[174]
Ciona peptide	Germany	*C. intestinalis*	Peptide[175]
Styela alkene	Korea	*S. clava*	Sulfated alkene[176]
Shishididemniols 1-2	Japan	Family Didemnidae	Lipids[177, 178]
Lissoclibadins 4-7	Indonesia	*L.* cf. *badium*	Polysulfur alkaloids[179]

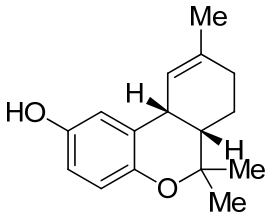
2.2.1 Metabolites from *Synoicum* species

Approximately 20 structures have been isolated from *Synoicum* species (*Synoicum pulmonaria* is shown in Figure 5). These have mainly been comprised of meroterpenes, macrolides and halogenated aromatic derivatives. *Synoicum* species consist only of colonial ascidians. The tunics of these species do not contain mineral spicules (skeletons) and several of the organisms are devoid of epibiosis[165]. However, microscopic analysis of *Synoicum adareanum* revealed a dense microbial community inside the tunicate[180].

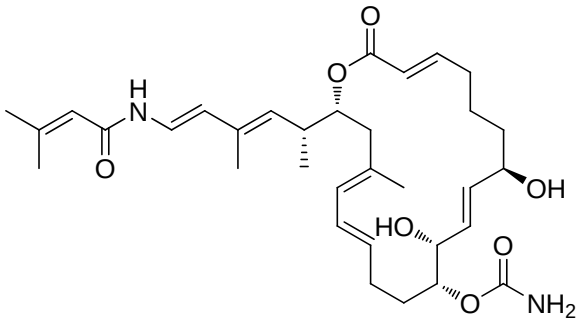
Epiconicol (Figure 6), a cytotoxic meroterpene which was the first marine derivative of tetrahydrocannabinol, was isolated from an Asutralian ascidian, *Synoicum castellatum*[181]. The highly cytotoxic nitrogen-containing macrolide, palmerolide A (Figure 6), was isolated from the Antarctic species *Synoicum adareanum*[182]. The rubrolides are a series of 15 halogenated

aromatic derivatives (without nitrogen) possessing strong antibacterial properties and moderate cytotoxicity[165]. Rubrolides have been isolated from a number of ascidians[183, 184]. Rubrolide O, isolated from a *Synoicum* n. sp. from New Zealand, also exhibited anti-inflammatory activites (Figure 6)[185].

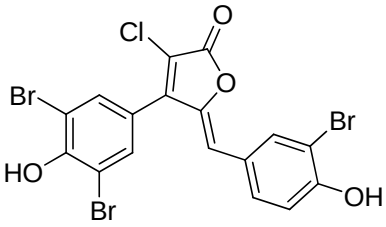
Figure 5. *Synoicum pulmonaria* collected from the coast of northern Norway (picture taken by Bjørn Gulliksen).



Epiconicol



Palmerolide A



Rubrolide O

Figure 6. Structures of the *Synoicum* metabolites epiconicol, palmerolide A and rubrolide O.

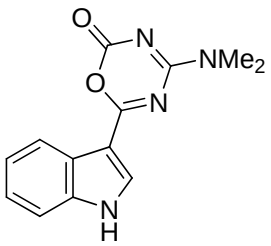
2.2.2 Metabolites from *Dendrodoa* species

Ascidians of the genus *Dendrodoa* (*Dendrodoa aggregata* is shown in Figure 7) belong to the family Stylidae, which have yielded classical indole and pyridoacridine alkaloids and antibacterial peptides. Approximately a dozen structures have been isolated from *Dendrodoa* species. The common *Dendrodoa grossularia* (also known as baked bean ascidian and gooseberry seasquirt) is the most studied *Dendrodoa* species[165].

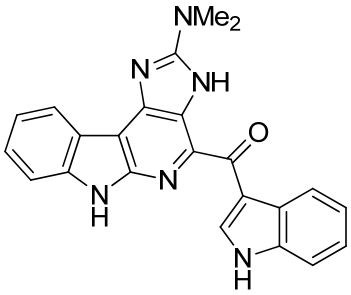
Several indolic alkaloids combining imidazolone have been isolated from *Dendrodoa grossularia* [186, 187]. Alboinone, contains a rare oxadiazinone, the first of its kind from nature (Figure 8)[188]. No biological activity has been reported for these metabolites. Grossularines 1 and 2 are two α-carboline alkaloids with moderate cytotoxic activity which have been isolated

from *Dendrodoa grossularia* (Figure 8)[189]. A cytotoxic, indolic derivative containing a thiadozole-1,2,4, named dendrodoine, has also been isolated from *Dendrodoa grossularia* (Figure 8)[190].

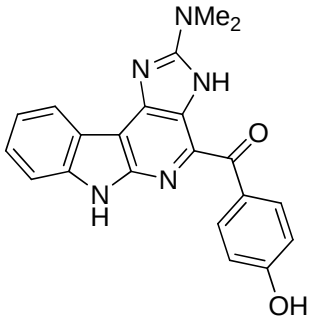
Figure 7. *Dendrodoa aggregata* collected from the coast of northern Norway (picture taken by Bjørn Gulliksen).



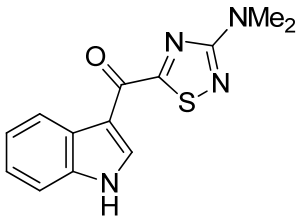
Alboinone



Grossularine 1



Grossularine 2



Dendrodoine

Figure 8. Structures of the *Dendrodoa* metabolites alboinone, grossularines 1 and 2, and dendrodoine.

2.3 Antimicrobial natural products from coral species

Soft corals, gorgonians or sea feathers, which belong to the phylum of Cnidaria (class Anthozoa), have been extensively studied and have produced the largest number of marine natural products besides sponge species[85, 108, 110-112]. These organisms are either solitary or colonial and are almost always fixed, with horny or calcareous skeletons. The class encompasses nearly 7 000 species, from which over 2 500 structures are known[125]. Most of the bioactive metabolites from coral species have exhibited cytotoxicity[191].

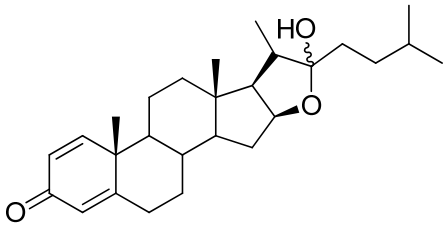
Approximately 90% of natural products from coral species have been terpenes, where the majority are diterpenes, followed by triterpenes and sesquiterpenes[111, 125]. This is reflected in

the list of antimicrobial compounds isolated from corals in the last decade (Table 3). Several hundred structures of prostanoids, a group of mainly cytotoxic terpenes which have been isolated from corals, appear to act as defense substances against predators[125]. Biosynthetic pathways for the production of prostanoids have been shown which appear to be specific to corals[192-194].

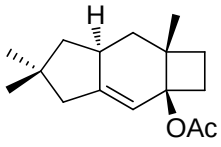
Table 3. Antimicrobial natural products from coral species isolated in the last decade.

Compound	Region	Species	Chemistry
Xeniolide I	Kenya	*X. novaebrittanniae*	Terpene[195]
Pseudopterosin X and Y	Bahamas	*P. elisabethae*	Diterpene[196]
A group of Lipids	Indian Ocean	*S. grandilobata*	Polyketide[197]
Erogorgiaenes	Puerto Rico	*P. elisabethae*	Diterpene[198]
Rumphellatin A	Taiwan	*R. antipathies*	Norsesquiterpenoid[199]
Caribenols 1-2	West Indies	*P. elisabethae*	Norditerpenes[200]
Robustolides A-B	Taiwan	*E. robusta*	Diterpene[201]
C-secosteroids	Argentina	*T. clavaria*	Steroids[202]

Eighty natural products have been isolated from *Alcyonium* sp. consisting of sesqui- and diterpenes, preylquinones, sterols[125] and nitric esters (these were the first known examples of natural nitrates)[203]. The moderately cytotoxic and antiviral steroid shown in Figure 9, was isolated from *Alcyonium gracillimum* along with six other steroids. 3-acetoxy-sterpurene (1) (Figure 9), a sesquiterpenoid with an unusual carbon skeleton was isolated from *Alcyonium acaule*[204]. No bioactivity was reported for this compound.



A. gracillium sterol



3-acetoxy-sterpurene

Figure 9. Structures of a sterol from *Alcyonium gracillimum* and 3-acetoxy-sterpurene isolated from *Alcyonium acaule*.

2.4 Antimicrobial natural products from bryozoan species

Over 8000 species of bryozoans (also known as sea mats or sea mosses) have been described. Bryozoans are sessile filter-feeders and food is collected via a circle of tentacles (the lophophores). The basic body plan consists of a polyp, protected by a calcareous box termed a zooid and many zooids are grouped together to form the colony. These colonies host whole communities of microorganisms and small invertebrates within their structures[205]. It has been shown that *Endobugula sertula*, a symbiont bacterium to the bryozoan *Bugula neritina*, is responsible for the production of the cytotoxic brysostatins, which cause the larvae to be unpalatable to predators. This is the first documentation of a symbiont providing defense for its host[206]. Figure 10 shows bryostatin 1[207].

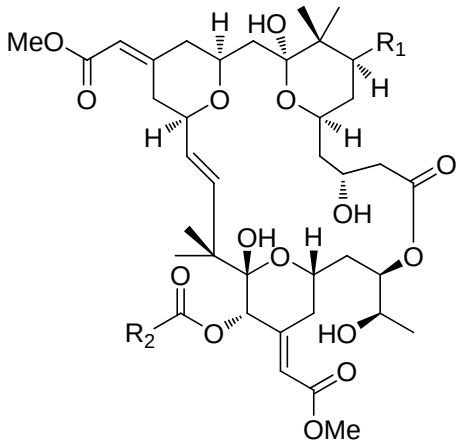


Figure 10. Structure of bryostatin 1 (R1 = $OCOCOCH_3$, R2 = $(CH)_4(CH_2)_2CH_3$).

Until 2006, only about 1% of reported marine natural products originate from bryozoan species (approximately 200 compounds)[112] and only 32 species have so far been investigated.[205]. The majority of the bioactivities associated with bryozoans have been cytotoxic in nature; in fact, bryozoan metabolites have, along with those isolated from sponge

species, been some of the most potent cytotoxins isolated from marine species[112]. Only a few of the reported bryozoan metabolites have shown antimicrobial activity (Table 4). Chemically, the bryozoan metabolites have mainly been comprised of polyketides and alkaloids[111, 165]. There have been no previous reports of metabolites from *Tegella* species.

There have been several discussions as to whether some bryozoan metabolites might be of microbial origin, as in the case of Flustramine E, isolated in Denmark from the bryozoan *Flustra folicea*[208]. The same compound has also been isolated from the Australian frog *Pseudophyrne coriacea*[209]. Different chemotypes of *B. neritina* were found at the same geographical location which harboured different strains of the symbiotic bacterium, *E. sertula*, known to produce the bryostatins[210].

Table 4. Antimicrobial natural products from bryozoan species isolated in the last 20 years.

Compound	Region	Species	Chemistry
Pterocellins C-F	New Zealand	*P. vesiculosa*	Alkaloid[211]
Amathaspiramides A, E/	New Zealand	*A. wilsoni*	Dibrominated alkaloid[212]
Alternatamides A-D	USA	*A. alternata*	Peptide[213]
Flustramine E	Denmark	*F.foliacea*	Indolealkaloid[214]
Biflustra quinine	Australia	*B. perfragilis*	Isoquinoline quinone[215]
Cribiceflina alkaloids	New Zealand	*C. cribaria*	β-carboline alkaloid[216]

3. Aims of the study

The growing prevalence of infectious diseases and increasing resistance development by pathogenic bacteria and fungi poses a serious threat to the public health. Marine natural products have tremendous potential to yield novel antibiotics which can be effective in fighting multi-resistant pathogens. The aim of the present study was to investigate the presence of antimicrobial compounds in Arctic and sub-Arctic marine invertebrates and to determine the structures of active metabolites. Sponge and ascidian species in particular have yielded a number of bioactive compounds and were deemed to be promising targets for investigation.

The main objectives of this study were to:

- Screen extracts of a number of marine invertebrates for antimicrobial activity.
- Purify and structurally elucidate active metabolites by mass spectrometric methods and 1D and 2D NMR techniques.

4. Summary of papers

Paper I

Margey Tadesse, Bjørn Gulliksen, Morten B. strøm, Olaf B. Styrvold, Tor Haug.

Screening for antibacterial and antifungal activities from northern Norway.

Benthic marine invertebrates collected from sub-Arctic regions of northern Norway, were found to be a promising source of novel bioactive compounds against human and fish pathogenic bacteria and fungi. Lyophilized material from seven species of ascidians, six sponges and one soft alcyonid coral were extracted with 60% acidified acetonitrile (ACN). After separation into an ACN-rich phase (ACN-extract) and an aqueous phase, and subsequent solid-phase extraction of the aqueous phase, fractions differing in polarity were obtained and screened for antibacterial and antifungal activities, along with the more lipophilic ACN-extracts. Antimicrobial activity was determined against two Gram-negative, two Grampositive bacteria, and two strains of fungi. Notably, all the invertebrate species in the study showed activity against all four strains of bacteria and the two strains of fungi. In general, the aqueous fractions displayed highest antimicrobial activity, and the most potent extracts were obtained from the colonial ascidian *Synoicum pulmonaria* which displayed activity against bacteria and fungi at a concentration of 0.02 mg/ml; the lowest concentration tested.

Paper II

Margey Tadesse, Veronika Tørfoss, Morten B. Strøm, Espen Hansen, Jeanette Hammer Andersen, Klara Stensvåg, Tor Haug.

Isolation and biological activity of (*E*)-1-(4-hydroxystyryl)guanidine from the sub-Arctic ascidian, *Dendrodoa aggregata*.

Bioguided-fractionation of an extract of the sub-Arctic ascidian, *Dendrodoa aggregata*, led to the isolation of the antibacterial (E)-1-(4-hydroxystyryl)guanidine (3-dihydroxy-tubastrine). The derivative, tubastrine, was also detected for the first time in *Dendrodoa aggregata*. The high content of 3-dihydroxy-tubastrine in *Dendrodoa aggregata* suggests that the compound could be a useful chemotaxonic marker for this species.

Paper III

Margey Tadesse, Morten B. Strøm, Johan Svenson, Marcel Jaspars, Bruce F. Milne, Veronika Tørfoss, Jeanette H. Andersen, Espen Hansen, Klara Stensvåg and Tor Haug.

Synoxazolidinones A, B, and C; novel bioactive alkaloids from the ascidian *Synoicum pulmonaria*.

Bioassay-guided fractionation of the sub-Arctic ascidian *Synoicum pulmonaria* collected off the Norwegian Coast, led to the isolation of a novel family of brominated guanidinium oxazolidinones named synoxazolidinones A-C. The backbone of the compounds contains a 4-oxazolidinone ring rarely seen in natural products. The structure of the compounds was determined by spectroscopic methods. The synoxazolidinones exhibited antibacterial, antifungal and anticancer activities.

Paper IV

Margey Tadesse, Jioji N. Tabudravu, Marcel Jaspars, Morten B. Strøm, Espen Hansen, Jeanette H. Andersen and Tor Haug.

The antibacterial eusynstelamides B, D, E and F, from the Arctic bryozoan *Tegella cf. spitzbergensis*.

The brominated tryptophan-derived metabolite eusynstyelamide B (**1**) and three new derivatives, eusynstyelamides D, E and F (**2**-**4**), were isolated from the Arctic bryozoan *Tegella cf. spitzbergensi*s. The structures were elucidated by spectroscopic methods including 1D and 2D NMR, and analysis of mass spectrometric data. Eusynstyelamide B (**1**) has previously been isolated from the Australian ascidian, *Eusynstyela latericius*. In contrast to the former study, antibacterial activity is here reported for **1** against a number of bacterial strains at concentrations as low as 6.25 μg/ml. Likewise, antibacterial activity is reported for **2**-**4**. Eusynstyelamides **1**-**4** also exhibited antifungal activity against *Candida albicans*, and activity against the human melanoma cell line A-2058 was detected for **2** and **3**. This is the first report of bioactive metabolites from the Arctic bryozoan *T. spitzbergensis*.

5. General discussion

5.1 Methodical considerations

Norway possesses a long coastline with an abundance of biotas which have barely been investigated for bioactive compounds. At the commencement of the present work, seven species of ascidians, six sponge species and one soft alcyonid coral collected from the coast of northern Norway were extracted and investigated for their antimicrobial properties (Paper I). Common and abundant marine invertebrates, which would be easy to recollect if more material was needed, were chosen for the study. The organisms were typically collected by hand or trawl and deposited in large containers filled with circulating seawater, where they remained for a few days. The organisms were then frozen at -80 °C and lyophilized before extraction. Taxonomic identification was carried out by Prof. Bjørn Gulliksen (Norwegian College of Fisheries Science, University of Tromsø, Tromsø, Norway) and Ninel Panteleeva (Murmansk Marine Biological Institute KSC RAS, Murmansk, Russia).

An extraction protocol using 60% acetonitrile (ACN) containing 0.1% triflouroacetic acid (TFA) as an extraction medium, was followed. This mixture has been proven effective at extracting both antibacterial peptides[217-219] and other compounds such as steroids[220] from a number of marine organisms. The extracts were incubated for 1-2 hours at -20 °C, resulting in two phases, an ACN-rich phase and a salt-rich water phase. The water phase was subsequently loaded on a solid phase extraction (SPE) cartridge and sub-fractionated with 10%, 40% and 80% ACN containing 0.05% TFA. Active compounds were isolated from these fractions by reverse-phase high performance chromatography (RP-HPLC), as described in Papers II-IV. The ACN extracts were typically loaded on filter syringes prior to HPLC to discard insoluble material. The bryozoan species referred to in Paper IV was obtained through

our collaboration with Marbio (the Norwegian national screening platform) as a dichloromethane:methanol (DCM:MeOH) (1:1) extract, which was pre-purified by SPE prior to HPLC.

An established antibacterial screening assay utilising a panel of human and fish pathogenic bacteria was used for testing[218]. The test bacteria consisted of two Gram positive bacteria, *Staphylococcus aureus* (ATTC 9144) and *Corynebacerium glutamicum* (ATTC 13032) (the latter being a particularly sensitive test bacterium)[217], and three Gram negative bacteria, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATTC 27853) and *Listonella anguillarum* (serotype O2, FT 1801, a fish pathogenic strain). An antifungal assay using two strains of pathogenic fungi, *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (a gift from Dr. Arne Tronsmo, The Norwegian University of Life Sciences, Ås, Norway) was also established. In paper I, an actively growing log phase culture of bacteria were diluted to an initial density of 5×10^3 cells per well and incubated with the test extracts for 72 hours. This was done in order to render the method sensitive to activity from low concentration compounds in the extracts. The test was conducted at 20 °C due to the inclusion of the marine bacterium *L. anguillarum* which does not grow at higher temperatures. Minimum inhibitory concentration (MIC) values of the pure compounds in papers II, III and IV were obtained using only human pathogenic bacteria with an initial bacterial concentration of 5×10^5 cells per well. The bacteria were incubated with the test compounds for 24 hours at 37 °C as a standard procedure.

Activity screenings against *S. aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), as well as anti-cancer assays, were conducted through our collaboration with Marbio. Active compounds were characterized

by high resolution mass spectrometry (HR-MS) and nuclear magnetic resonance (NMR) spectroscopy, as described in Papers II-IV.

5.2 Invertebrate species investigated in the present study

Seven ascidian species, *Synoicum pulmonaria*, *Ciona intestinalis*, *Dendrodoa aggregata*, *Styela rustica*, *Ascidia virginea*, *Corella parallelogramma* and *Halocynthia pyriformis*, were extracted and investigated for antimicrobial activity. Six sponge species were also extracted and screened for activity; *Geodia barretti*, *Haliclona* sp. 1, *Haliclona* sp. 2, *Haliclona rosea*, *Myxilla incrustans* and *Polymastia* sp. Extracts of the soft alcyonid coral *Alcyonium digitatum* and the bryozoan *Tegella cf. spitzbergensi*s, were also tested for activity.

5.2.1 Antimicrobial activity in *C. intestinalis*

The 40% and 80% SPE fractions of *C. intestinalis* displayed potent antimicrobial activity (Table 1 of Paper I.). HPLC and mass spectrometry were performed on both fractions, yielding two peptides with masses of 4.3 and 4.6 kDa in the 40% SPE fraction which co-eluted on the HPLC. Identification of the peptides through database searches was not successful, possibly due to the fact that certain peptides often possess extensive post-translational modifications including multiple monosulfide (lanthionine) bridges, disulfide-bridges, amino acid derivatives and keto amide residues at the N-terminus[40]. Tryptination of the peptides followed by ESI-MS/MS analysis also proved unsuccessful. Re-purification of the peptides from the same extract was not possible, probably due to a degradation of the sample which can occur for a number of reasons. Peptides often prove to be problematic to purify. One example is the tunichrome peptides, which were extremely difficult to isolate due

to their great sensitivity to air, alkaline conditions and the solid phases usually used in chromatography[221]. Further work on the identification of the peptides was abandoned.

Although *C. intestinalis* is a model organism, there are no reports so far of the isolation of antimicrobial peptides from this organism. However, a gene encoding for a putative antimicrobial peptide was recently identified in an expressed sequence tag (EST) database of *C. intestinalis*. A highly potent antimicrobial peptide was synthesized corresponding to the core cationic regions of the putative defense protein[175].

5.2.2 Antimicrobial activities in ascidian species

Fractions of *S. rustica* and *A. virginea* displayed good antimicrobial activity (Paper I). HPLC and activity screening was performed both on the 40% and 80% SPE fractions without resulting in the isolation of active metabolites. Only minor peaks were observed by HPLC signifying that the fractions contained compounds present at minute concentrations. Antimicrobial activity was not found in any of these HPLC fractions indicating that the initial activity might be due to a possible synergetic effect which is lost upon separation of the components[40, 66]. The fact that active substances have previously been isolated from these species could signify that the organisms collected for this study did not produce these metabolites due to environmental, seasonal or chemotype variations. It is also possible that the separation method employed in the present investigation was not amenable to the isolation of the metabolites potentially present in these organisms.

A very limited amount of sample of *C. parallelogramma* was available for extraction and it was not deemed feasible to extract enough material for structural elucidation. Fractions of *H.*

pyriformis possessed poor activity in our assays, thus no attempt at purification of compounds from the organism was carried out.

5.2.3 Antimicrobial activities in sponge and coral species

Bio-guided fractionation of the sponge *G. Baretti*, led to the isolation of the known, brominated antifouling compound barettin[222, 223] (Figure 2). The compound was detected in the 40% SPE fraction and identified by HR-MS which showed the isotope pattern and mass (*m/z* 419 $[M + H]^+$). The HPLC fraction containing the compound showed antibacterial activity against the Gram-negative bacterium *P. aeruginosa* and the Gram-postive bacterium *S. aureus*. However, no MIC studies were carried out and further isolation for NMR studies was not pursued due to dereplication.

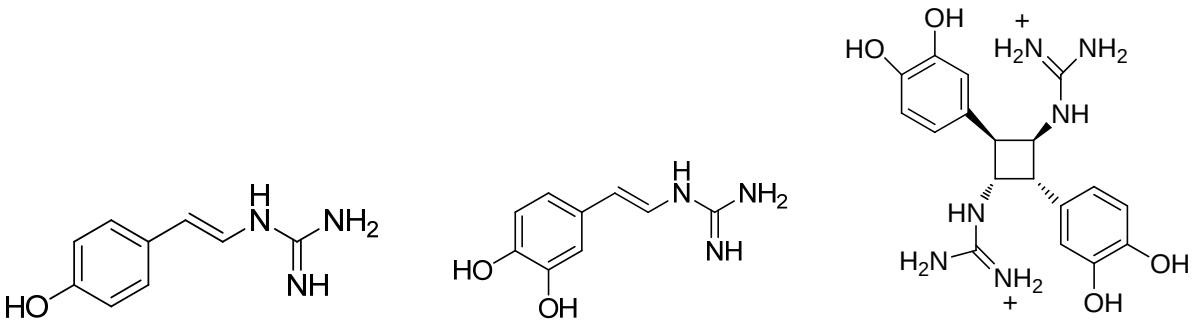
HPLC and activity screening in the present work did not result in the isolation of antimicrobial metabolites from the sponge species *Haliclona* sp. 2, *H. rosea*, *M. incrustans*, *Polymastia* sp. or the soft coral *A. digitatum*. Attempts at isolation of compounds from *Haliclona* sp 1 was not carried out due to the poor activity of the SPE fractions. The taxonomy of *Haliclona* sp 2 was later corrected to *Mycale* sp. and a Master project was carried out at our department attempting to isolate active metabolites from this species. However, it did not lead to the identification of new compounds[224].

5.2.4 3-dehydroxy-tubastrine and tubastrine from the ascidian *D. aggregata*

Bio-guided fractionation of the ascidian *D. aggregata* resulted in the isolation and characterization of the antimicrobial hydroxystyrylguanidine alkaloids, 3-dehydroxy-

tubastrine and tubastrine (Figure 11), as described in Paper II. The structure of 3-dehydroxy-tubastrine was elucidated by high resolution mass spectrometry and NMR techniques, while tubastrine was characterized by high resolution mass spectrometry. 3-dehydroxy-tubastrine, which had previously been isolated from the Australian sponge *Spongosorites* sp., displayed antibacterial activity against *S. areus*, *C. glutamicum* and MRSA at an MIC of 100 μg/ml. Antibacterial activity against *S. aureus* and *C. glutamicum* were observed for tubastine but MIC-values were not determined. This is the first report of active metabolites from *D. aggregata*. Due to its abundance in the organism, 3-dehydroxy-tubastrine can serve as a chemotaxonomic marker for the species. However, as noted in Paper II, there is a good probability of biogenesis by symbiotic microorganisms which should be considered.

Tubastrine has previously been isolated from a number of organisms and has exhibited various activities including antiviral properties, kinase inhibition, as well as functioning as a defense for the organisms against microbes and larvae (Paper II). A series of dimers of tubastrine, exhibiting anti-inflammatory activity, have been isolated from the ascidian *Aplidium orthium*[225]. One of these, orthidine E is shown in figure 11.

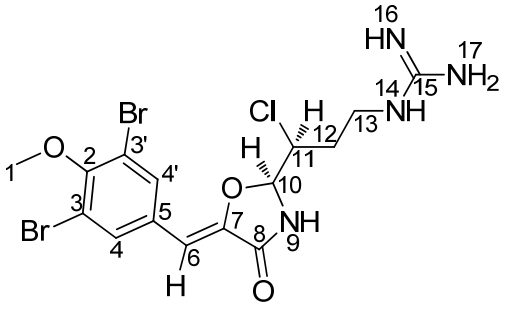


3-dehydroxy-tubastrine Tubastrine Orthidine E

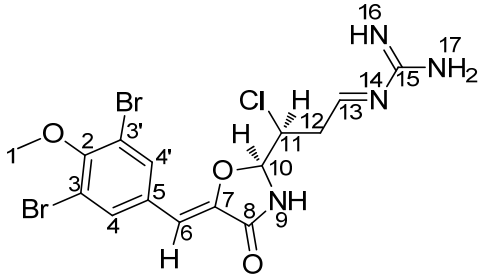
Figure 11. Structures of 3-dehydroxy-tubastrine, tubastrine and orthidine E.

5.2.5 Synoxazolidinones A, B, and C, novel oxazolidinones from *S. pulmonaria*

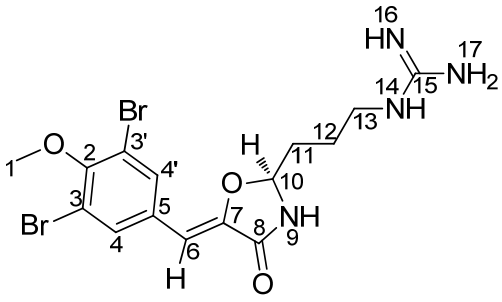
HPLC was performed on the ACN extract of *S. pulmonaria*, which was the most active fraction in the initial antimicrobial screening (Paper I). This extract was found to mainly consist of three distinct peaks in approximately a 1:7:2 ratio. The first peak yielded a compound with *m/z* of 460 ($C_{15}H_{18}Br_2N_4O_3$), the second and most abundant peak showed a *m/z* of 494 ($C_{15}H_{17}Br_2ClN_4O_3$) and a *m/z* of 492 ($C_{15}H_{15}Br_2ClN_4O_3$) was observed for the last compound. Tandem mass spectrometric analysis along with 1 and 2D NMR experiments discussed in Paper III, led to the structural elucidation of these compounds which were named synoxazolidinones A, B, and C (Figure 12). Synoxazolidinones A, B, and C contain a unique 4-oxazolidinone core. The absolute stereochemistry of the compounds was deduced by computational experiments, which are described in detail in the supporting information to Paper III. This needs to be confirmed by future synthesis of the synoxazolidinones.



Synoxazolidinone A (6*Z*, 10*S*, 11*S*)



Synoxazolidinone B (6*Z*, 10*S*, 11*S*)



Synoxazolidinone C (6*Z*, 10*S*)

Figure 12. Structures of the novel 4-oxazolidinones, synoxazolidinones A, B, and C.

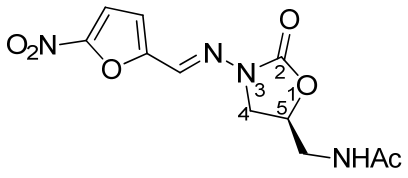
The synoxazolidinones represent a novel scaffold incorporating a rare 4-oxazolidinone core that adds to the chemodiversity of reported natural products. The compounds exhibited various antimicrobial and anti-cancer activities, some with MIC-values as low as 6.25 μg/ml (Paper III). Isolation of bioactive compounds has previously not been reported for *S. pulmonaria* species. Notably, the synoxazolidinones were not present in *S. pulmonaria* species collected off the coast of Spitsbergen (data not shown), a different location than the north Norwegian coast, where the organisms originally extracted were obtained. This is yet an example of environmental and/or seasonal variations in the chemical content of organisms.

The ^{1}HNMR and ^{13}CNMR spectra of the synoxazolidinones, contained relatively few proton and carbon signals. However, adequate gCOSY and gHMBC correlations were observed enabling determination of the structure of the compounds (Paper III). Tandem mass spectrometric fragments of synoxazolidinone A (shown in the supporting information to Paper III) also yielded a stepwise confirmation of the proposed structure of synoxazolidinone A.

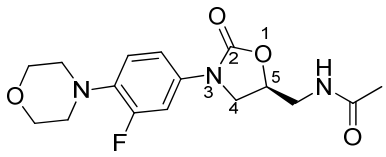
The configuration of the double bond between C-6 and C-7 was determined as *Z* due to the low coupling constants between H-6 and C-8 (^{3}J(C,H) <3 Hz). The assignment is based on the literature value for the ^{3}J(C,H) coupling constant for a cis-double bond (7.6 Hz), which is lower than the ^{3}J(C,H) (14.1 Hz) coupling constant for a trans-double bond (Paper III). An EXSIDE experiment, which directly measures ^{n}J(C,H) coupling constants by analysing correlations in the ^{13}C dimension, was used to measure the coupling constants (supporting information to paper III). Calculations based on CD experiments also supported a *Z*-configuration at this position (Paper III).

The structures of the synoxazolidinones suggest biogensis from tyrosine and arginine/agmatine: The guanidine group of arginine is conserved in the synoxazolidinones. A suggested biosynthetic route for the formation of the synoxazolidinones is presented in Paper III.

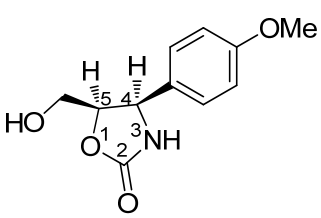
The first member of the oxazolidinone class of antibacterials was furazolidone, which was synthesized in the 1950's (Figure 13). DuPont Pharmaceuticals developed the oxazolidinone lead compounds DuP 105 and D 721 in the late 1980's but these were subsequently abandoned due to their toxic nature. Linzolid (Zyvox ^{TM}) is the most recent antibacterial oxazolidinone drug to be marketed (Figure 13). Eperzolid, an oxazolidinone drug similar to linzolid, was a back-up candidate which could not be developed due to large dosages and bone-marrow toxicity[226]. Apart from the marine oxazolidinones discussed previously, two oxazolidinones have been isolated from terrestrial sources; the cytokine modulator cytoxazone, isolated from a soil *Streptomyces* strain[227], and the mildly antimicrobial streptazoline, which was isolated from *Streptomyces viridochromogenes*[228] (Figure 13).



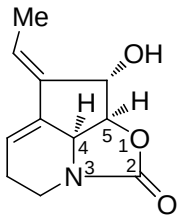
Furazolidone



Linezolid



Cytoxazone



Streptazoline

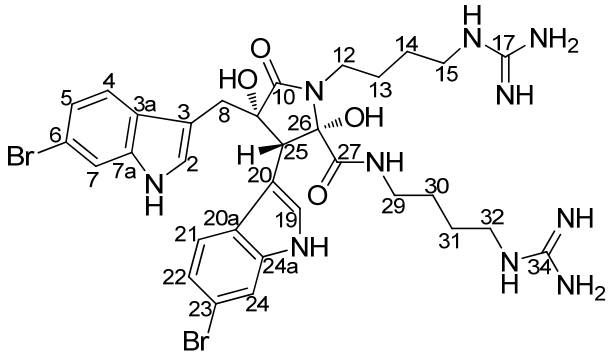
Figure 13. The oxazolidinones furazolidone, linezolid, cytoxazone and streptazoline.

Numerous derivatives of linezolid have been synthesized. Srivastava *et al*.[226] discuss the structure-activity relationships governing oxazolidinone development. An electron rich atom such as the nitrogen of the morpholine ring of linezolid, was found to improve the safety profile of the drug. The fluorine on the phenyl group was found to improve antibacterial activity. The N-aryl group at position N3 and an *S*-configuration at C5 of the oxazolidinone-ring, were requirements for antibacterial activity. The synoxazolidinones also contain an electron-rich methoxy group in the 4 position in addition to two bromines in the 3 and 5 positions of the phenyl ring (a di-flouro derivative of linezolid was found to be promising). Synoxazolidinones contain a unique 4-oxazolidinone moiety, providing an entirely novel scaffold for structure-activity relationship studies. These studies are currently being carried out in order to optimalise the structures of the synoxazolidinones for clinical use.

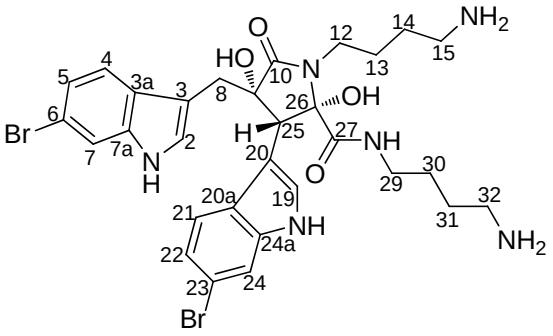
5.2.6 The antibacterial eusynstyelamides B, D, E and F, from *T. spitzbergensis*

An investigation into the chemistry of the Arctic bryozoan *Tegella cf. spitzbergensis*, resulted in the isolation and structural identification of the known dibrominated tryptophan-derived, guanidine dimer, eusynstyelamide B[229] (Figure 14). Three new derivatives, eusynstyelamides D, E and F, were also isolated and characterized (Figure 14). Eusynstyelamide B has previously been isolated from the Australian ascidian *Eusynstyela latericius*[229]. The structures of the eusynstyelamides were elucidated by mass spectrometric and, 1D and 2D NMR techniques. The carbon shift values were obtained through gHSQC and gHMBC experiments (Paper IV). Eusynstyelamide B was identified by comparison of the mass, isotope pattern and NMR shift values of the compound with those reported by Tapiolas *et al*[229]. Eusynstyelamides D, E and F, were characterized by their mass values, isotope pattern and comparison of their ^{1}HNMR and ^{13}CNMR shift values with those of eusynstyelamide B. Determination of the

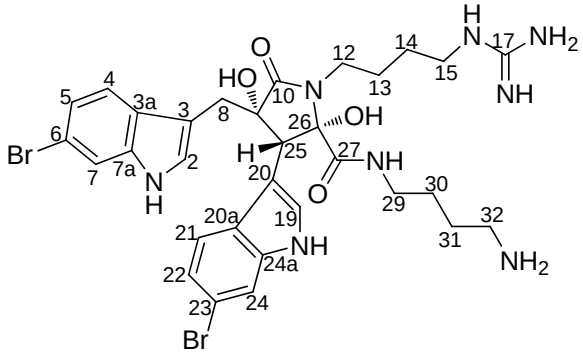
relative configuration of eusynstyelamide B based on NOE signals, is discussed in paper IV. The same relative configuration as eusynstyelamide B is proposed for eusynstyelamides D, E and F, based on the similarities of their NMR shift values to those of eusynstyelamide B.



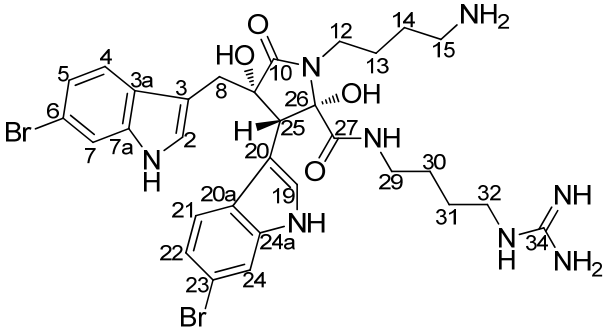
Eusynstyelamide B (9*R**, 25*S**, 26*R**)



Eusynstyelamide D (9*R**, 25*S**, 26*R**)



Eusynstyelamide E (9*R**, 25*S**, 26*R**)



Eusynstyelamide F (9*R**, 25*S**, 26*R**)

Figure 14. Structures of the antibacterial eusynstyelamides B, D, E and F from *T. spitzbergensis*.

All four compounds exhibited antibacterial activity. There was no apparent relationship between the loss of guanidine-groups at various locations in eusynstyelamides D, E and F, and their antibacterial properties, as all four compounds exhibited similar antibacterial activities (Paper IV). This is the first report of bioactive metabolites from a *Tegella* species. The fact that eusynstyelamide B was found in different geographical zones points to biosynthsis by symbiotic microorganisms.

Tapiolas *et al*.[229] attempted to determine the absolute configuration of eusynstyelamide B without success. In the future, an attempt could be made to solve the absolute configuration of the eusynstyelamides either through computational means or X-ray crystallography. Structure-activity relationship studies could also be carried out to identify structural elements essential for the antibacterial activity of eusynstyelamides B, D, E and F, and thereby synthesize more potent analogues.

5.3 Main conclusions

- Arctic and sub-Arctic marine invertebrates were found to be promising sources of novel antimicrobial metabolites.

- The antibacterial compounds 3-dihydroxy-tubastrine and tubastrine, have been isolated from the ascidian *Dendrodoa aggregata*. This is the first report on the isolation of bioactive metabolites from this species.

- The antimicrobial and anticancer compounds, synoxazolidinones A, B, and C, have been isolated from the ascidian *Synoicum pulmonaria* and provide novel scaffolds for structure-activity relationship studies. This is the first report of bioactive metabolites from *S. pulmonaria*.

- The antimicrobial eusynstyelamide B and three new derivatives, eusynstyelamides D, E and F, have been isolated from the bryozoan *Tegella cf. spitzbergensis*. This is the first report on the chemistry of *T. spitzbergensis*.

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ISBN xxx-xx-xxxx-xxx-x