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Screening of Marine Natural Products and Their Synthetic Derivatives for Antimicrobial and Antiproliferative Properties

Sofia Isabel Gonçalves Hernâni Martins Montalvão

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2 at Viikki Infocenter Korona (Viikinkaari 11) on February 26th, at 12 o’clock noon.

Helsinki 2016
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Abstract

Marine environment is prolific in organisms with unique properties. Seas and oceans contain a wide diversity of species with biologically active metabolites that represent a valuable source with great potential for the development of novel therapeutic agents. This dissertation is focused on the biological study of synthetic compounds based on marine scaffolds as well as on marine natural product extracts originating from the Aegean Sea. Furthermore, it offers an introduction on the importance of marine natural products in the search of new bioactive compounds, the use of natural products as scaffolds for the synthesis of new drugs, and a general overview on bioactivity screening and the current status of marine-derived bioactive compounds as therapeutic agents.

The potential of oroidin and clathrodin as parent structures for synthesis of novel compounds was explored. Antimicrobial and antiproliferative studies were conducted and it was concluded that 4-phenyl-2-aminoimidazoles 6g(I) and 6h(I) showed the best antimicrobial effect against Gram-positive bacteria (Enterococcus faecalis and Staphylococcus aureus), while compound 6j(I) showed the most interesting IC$_{50}$ in antiproliferative studies. Compound 7(II), a synthetic derivative of 2-aminobenzothiazole, showed IC$_{50}$ of 16 μM and 71 μM against a cancer cell line and a normal cell line, respectively. The selectivity index showed selectivity towards cancerous cells. In addition, okadaic acid was used as inspiration for the synthesis of crown ether acyl compounds. Compound (1,4,7,10,13,16-hexaoxacyclooctadecan-2-yl)methyl 3-(pyren-1-yl)propanoate) 1o(III) was found to be the most active in antimicrobial studies against Gram-positive Staphylococcus aureus with a MIC$_{50}$ of 7.2 μM.

The importance of bioprospecting the rich marine biodiversity in the Aegean Sea was also studied in this thesis. Biological activities of extracts from cyanobacteria, micro- and macroalgae were evaluated, and microalgae
extracts (*Amphora cf capitellata* and *Nitzschia communis*) showed the most interesting antimicrobial results against *Staphylococcus aureus* and fungus *Candida albicans*.

The results of the biological studies conducted in this thesis demonstrated antimicrobial and antiproliferative activity of several marine natural products and their synthetic derivatives. Further studies and structural optimization should be done to fully explore their potential for the development of therapeutic agents.
Resumo

O ambiente marinho é promissor na variedade de organismos com propriedades únicas. Dos mares e oceanos, existe uma diversidade imensa de metabólitos bioactivos que representam uma fonte de enorme valor e com grande potencial para o desenvolvimento de novos agentes terapêuticos. Esta tese mostra o estudo biológico de compostos sintéticos com base em “scaffolds” marinhos e extractos do Mar Egeu. Além disso, esta tese oferece uma introdução sobre a importância de produtos naturais marinhos e a procura de novos compostos bioactivos, no uso de produtos naturais como “scaffolds” para a síntese de novos fármacos e uma visão geral sobre avaliação da capacidade biológica de compostos marinhos e o status de compostos bioactivos como agentes terapêuticos.

O potential do composto clathrodin como modelo para a síntese de novos compostos foi explorado. Estudos antimicrobianos e antiproliferativos foram realizados e concluiu-se que 4-phenyl-2-aminoimidazoles 6g(I) e 6h(I) teve os melhores resultados antimicrobianos quando testados contra bactérias Gram-positivas (Enterococcus faecalis and Staphylococcus aureus) enquanto que o composto 6j(I) mostrou o IC₅₀ mais interessante nos estudos antiproliferativos. O composto 7(II), um derivado sintético de 2-aminobenzotiazole mostrou IC₅₀ de 16 μM e 71 μM quando testado contra uma linha celular cancerígena e uma linha celular não-cancerígena, respectivamente. O índice de selectividade mostrou uma seleção para células cancerígenas. O ácido ocadácio foi usado como inspiração para a síntese de compostos macrocíclicos. Composto (1,4,7,10,13,16-hexaoxacyclooctadecan-2-yl)methyl 3-(pyren-1-yl)propanoate) 1o(III) foi o mais activo em estudos antimicrobianos quando testado contra a bactéria Gram-positiva Staphylococcus aureus, com MIC₅₀ igual a 7.2 μM.

A importância de bioprospecção da enorme biodiversidade marinha no Mar Egeu, foi também estudado nesta tese. Estudos biológicos foram feitos em
extratos de cyanobacteria, micro- e macroalgas e as microalgas marinhas (Amphora cf capitellata e Nitzschia communis) mostraram a melhor actividade antimicrobial, quando testados contra a bacteria Staphylococcus aureus e o fungo Candida albicans.

Os resultados preliminares de estudos biológicos em compostos marinhos, presentes nesta tese, mostraram o potencial destes em terapias antimicrobianas e anticancerígenas. Num futuro próximo, uma optimização na estrutura química destes compostos poderá ser realizada, para explorar todo o potencial como agentes terapêuticos.
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Helsinki, 10 February 2016
List of original publications (I, II, III and IV)

This thesis is based on the following publications and manuscripts:

† These authors contributed equally to this work.


III  Martin Febles†, Sofia Montalvão†, Guillermo D. Crespín, Martin Norte, José M. Padrón, Päivi Tammela, José J. Férnandez, António H. Daranas. Synthesis and Biological Evaluation of Crown Ethers Acyl Derivatives (manuscript)
† These authors contributed equally to this work.


The publications are referred to in the text by their Roman numerals. The articles are reprinted with permission from the publishers. The supporting information of the original publications is not included in this thesis. The material is available from the author or online.
Author’s contribution

I Sofia Montalvão participated in the design of the study and executed the antimicrobial testing and collaborated with the other authors in writing the manuscript. Sofia Montalvão and Nace Zidar contributed equally.

II Sofia Montalvão participated in the design of the study and experimental work. She analyzed the results and wrote the manuscript in collaboration with the other authors.

III Sofia Montalvão participated in the design of the study, in the experimental work, in interpreting the results and writing the manuscript. Sofia Montalvão and Martín Febles contributed equally.

IV Sofia Montalvão participated in the design of the study and executed part of the experimental work. She analyzed the results and wrote the manuscript in collaboration with the other authors.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming Unit</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DSP</td>
<td>Diarrhetic Shellfish Poisoning</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HRS</td>
<td>High-Resolution Screening</td>
</tr>
<tr>
<td>HTS</td>
<td>High-Throughput Screening</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory Concentration</td>
</tr>
<tr>
<td>INDA</td>
<td>Investigational New Drug Application</td>
</tr>
<tr>
<td>kDA</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
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<td>MAX</td>
<td>Maximum</td>
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<td>MHA</td>
<td>Mueller-Hinton Agar</td>
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<tr>
<td>MHB</td>
<td>Mueller-Hinton Broth</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MIN</td>
<td>Minimum</td>
</tr>
<tr>
<td>MNP</td>
<td>Marine Natural Product</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
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<td>NP</td>
<td>Natural Product</td>
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<tr>
<td>NRPS</td>
<td>Nonribosomal Peptide Synthetase</td>
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<tr>
<td>OA</td>
<td>Okadaic Acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>P.C.</td>
<td>Positive Control</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PIA</td>
<td>Pyrrole-imidazole Alkaloid</td>
</tr>
<tr>
<td>PMX</td>
<td>Polymyxin B Sulphate</td>
</tr>
<tr>
<td>PS</td>
<td>Polysaccharides</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>S/B</td>
<td>Signal-to-background</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise</td>
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<td>SAR</td>
<td>Structure-activity Relationship</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SDA</td>
<td>Sabourad Dextrose Agar</td>
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<tr>
<td>SI</td>
<td>Selectivity Index</td>
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<tr>
<td>TI</td>
<td>Therapeutic Index</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$Z'$</td>
<td>Z-factor</td>
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1. Introduction

Yuri Gagarin once said, “The Earth is blue. How wonderful. It is amazing.” These were the words of the first human astronaut to travel into space and orbit our planet. Approximately three quarters, i.e. 77%, of planet Earth is occupied by water and because of that, some scientists believe that the word “Earth” should have been replaced by “Water”. As a consequence of the amount of water on the planet, the marine biodiversity is enormous – from shallow coastal waters to deep oceans - and is being considered a promising source of unique secondary metabolites for research and drug discovery [1].

Secondary metabolites – compounds not directly involved in the normal growth, development or reproduction [2], and with a molecular weight (MW) below 2 kDa – are normally produced as an answer to ecological pressures, such as competition for space, deterrence of predation, reproduction, among others [3]. Bergmann et al. reported the first discovery of a biologically active marine natural compound in the late 1950s – arabino and ribo-pentosyl nucleosides extracted from Cryptotethia crypta sponge, which was the first demonstration that naturally occurring nucleosides could contain sugars other than ribose and desoxyribose [4]–[6]. Chemical synthesis allowed the development of two derivatives, cytarabine and vidarabine, nucleosides with significant anticancer and antiviral activities [7]. However, only after the discovery of several prostaglandins, isolated from octocoral Plexaura homomalla [8], interest in marine-related research has increased.

Due to their broad panel of biological activities, such as their antibacterial, antifungal, anticancer, antiviral, anti-inflammatory, anticoagulant, antiprotozoal, antituberculosis, antidiabetic, antimalarial and antioxidant effects [9]–[12], marine natural products (MNPs) are remarkably interesting, high-value ingredients for applications not only in the pharmaceutical industry, but also in the cosmetic, nutraceutical and agrochemical industries [13]–[15]. The following review provides a comprehensive overview of the importance of marine compounds with a focus on the pharmaceutical field.
2. Review of literature

2.1. Sources of MNPs: overview of the marine-derived sources used in the search of new bioactive compounds

The extraordinary biological, biochemical and biosynthetic potential of marine organisms show that oceans and seas are promising hotspots in the research of secondary metabolites [9], [16]–[21]. Comparing the taxonomic levels, 76 phyla are recognized by the “Catalogue of Life” (online database of the world’s known species [22]), of which 60 include marine representatives, while 40 phyla are represented by terrestrial ones. Such a high number of representatives show great biological diversity, corroborating the fact that oceans and seas are the greatest source for the discovery of new therapeutic agents [23].

From mid-1980s until 2012, there has been an outstanding increase in discovered MNPs, mainly due to several advances in technology, and more than 22 000 compounds have been isolated from marine organisms (Figure 1).

Figure 2 shows data on marine-derived sources used in the discovery of new bioactive compounds from 2010 to 2013. More than 30% of new bioactive
compounds reported from marine sources are derived from microorganisms such as cyanobacteria and microalgae.

Microalgae, one of the first photosynthetic microorganisms have been highlighted as the new source of marine bioactive compounds due to their interesting biological activities [24]. Their enormous biodiversity results from the capability to adapt to different environments, some of them extreme. To tolerate extreme adverse environments, microalgae develop defense strategies, resulting in a high diversity of compounds from different metabolic pathways. As cyanobacteria, some species of microalgae can produce value-added compounds such as antioxidants, polyunsaturated fatty acids (PUFAs), polysaccharides and sterols [25]. Other microalgae also produce high quantities of hydrocarbons – convertible into biodiesel or hydrogen used as alternative energy sources. In structure and function, microalgae can be prokaryotic or eukaryotic: the prokaryotic ones belong to the Cyanophyta.

Figure 2: Overview of marine natural products in the discovery of novel bioactive compounds, from 2010 to 2013 (Adapted from: [179]).
group (cyanobacteria)\(^1\); the eukaryotic ones are included mainly in Bacilariophyceae (diatoms), Dinophyceae (dinoflagellates), Prymnesiophyceae (coccolithophores), Cryptophyceae (cryptomonas), Prasinophyceae and Chlorophyceae. It is estimated that until now, around 30,000 microalgae have been studied and analyzed [26]. Chlorella sp. are eukaryotic green unicellular microalgae from the Chlorophyta group that has been extensively studied due to antitumoral, antibacterial, anticoagulant and antioxidant effects [27], [28]. These microalgae, reported to possess antimicrobial activity originally in 1945 [29], are rich in chlorophyll, polysaccharides, vitamins, minerals and essential amino acids [30]. Other species widely studied due to their tolerance of extreme habitat conditions and physiological aspects are green unicellular halotolerant microalgae that belong to the Chlorophyceae group: Dunaliella sp. These microalgae contain carotenoids, glycerol, lipids, enzymes and vitamins [31], and are known for their antioxidant, antibacterial and analgesic properties [32].

Cyanobacteria (or blue-green algae) are prokaryotic organisms and studies confirm that these organisms have existed approximately for 3.5 billion years [33], [34]. They are considered one of the main organisms responsible for the creation of the current Earth’s atmosphere [35]. All cyanobacteria are photoautotrophs, but there are some groups that can show also heterotrophic metabolism, described as mixotrophics. Endowed with the capacity to adapt to a wide range of environmental conditions, cyanobacteria colonize terrestrial, aquatic and extreme ecosystems. However, the majority of these organisms live in water. They have been studied broadly due to their capacity of producing bioactive compounds, such as toxins (microcystins), siderophores and antibiotics. The use of cyanobacteria in medicine and pharmacology dates back to 1500 B.C., when strains of Nostoc were used to treat gout, fistula and several forms of cancer [36]. Between 1950 and 1970, studies on prospecting, isolation and characterization of cyanobacteria started to be carried out regularly [37], [38]. After 1990, research on these

\(^1\) In some studies, cyanobacteria are considered members of the Bacteria domain.
prokaryotics received more emphasis and the pioneering work led by Richard Moore and William Gerwick showed the value of cyanobacteria for biomedical research [20]. The interest in studying compounds produced by marine cyanobacteria is also related to the fact that some of the isolated compounds show enormous structural variety, making them interesting as scaffolds for the synthesis of new drugs. Compounds derived from filamentous genera such as *Lyngbya, Leptolyngbya* and *Symploca* have shown anticancer activity [39]–[41]. In a review conducted by Tan [42], in which 128 compounds isolated from marine cyanobacteria were studied, more than 35% of the compounds showed activity in tumoral cell lines and 10% of compounds exhibited activity in normal cell lines (non-tumoral). Some of the compounds showed antimicrobial and anti-inflammatory activities. Table 1 shows examples of bioactive compounds from marine cyanobacteria, their chemical class, species (source) and biological activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical class</th>
<th>Source</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambiguine</td>
<td>Alkaloid</td>
<td><em>Fischerella ambigua</em></td>
<td>Antimicrobial</td>
<td>[43], [44]</td>
</tr>
<tr>
<td>Aulosirazole</td>
<td>Aromatic</td>
<td><em>Aulosira fertilissima</em></td>
<td>Anticancer</td>
<td>[45]</td>
</tr>
<tr>
<td>Cryptophycin</td>
<td>Lipopeptide</td>
<td><em>Nostoc sp.</em></td>
<td>Cytotoxic</td>
<td>[46], [47]</td>
</tr>
<tr>
<td>Didemnin</td>
<td>Lipopeptide</td>
<td><em>Synechocystis tridemni</em></td>
<td>Anticancer, antiviral</td>
<td>[48]</td>
</tr>
<tr>
<td>Dolastatin</td>
<td>Lipopeptide</td>
<td><em>Lyngbya sp.</em></td>
<td>Anticancer</td>
<td>[49]</td>
</tr>
<tr>
<td>Hapalindole</td>
<td>Alkaloid</td>
<td><em>Fischerella sp.</em></td>
<td>Cytotoxic, antibacterial</td>
<td>[44]</td>
</tr>
<tr>
<td>Hassallidin</td>
<td>Nonribosomal peptide synthetase (NRPS)</td>
<td><em>Anabaena sp.</em></td>
<td>Antifungal</td>
<td>[50]</td>
</tr>
</tbody>
</table>
of specific pigments into three main groups: Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae). The color of green algae is caused by the presence of green pigments called chlorophylls (a and b). The presence of fucoxanthin, chlorophyll (a and c) gives Phaeophyta a greenish brown color. The red color of Rhodophyta is attributed to the presence of phycobilins (phycoerythrin and phycocyanin). Several species of macroalgae have the capacity to produce a diverse array of secondary metabolites of potential medicinal values as antimicrobial [51]–[53] and antiproliferative agents [54]. Vairappan et al. showed antibacterial activities of eight halogenated compounds, isolated from five species of red algae Laurencia sp., against a range of Gram-positive bacteria, including antibiotic-resistant bacteria [55].

From the red alga Odonthalia corymbifera, bromophenol compounds have been isolated and synthesized, and antimicrobial activity tested against Gram-positive and Gram-negative bacteria and fungi [56]. The most active isolated compound was 2,2′,3,3′-tetrabromo-4,4′,5,5′-tetrahydroxydiphenylmethane, displaying activity against Candida albicans, Aspergillus fumigatus and Trichophyton sp. Two synthetic analogs from the same alga showed potent antibacterial effect against Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris, Micrococcus luteus and Salmonella typhimurium.

The antibacterial activity of two species of brown algae Ecklonia (Ecklonia kurome and Ecklonia stolonifera) have been evaluated against S. aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus cereus [57] and significant activity was observed against all bacteria except Gram-negative E. coli.

Another important marine resource are sponges (Porifera) – sessile aquatic animals that live in rivers or deep oceanic waters. These organisms constitute a mark in the evolutionary history of planet Earth, being one of the oldest existing lineages of metazoans. It is estimated that sponges appeared in Neoproterozoic Era more than 635 million years ago [58]. Recent studies indicate that Porifera played an important role in the oxygenation of the
Since sponges are benthic (fixed to the seabed during adult life) and with a simple morphology, they were able to develop, among others, an adaptive chemical strategy as a way of defense and competition. Many of the secondary metabolites from sponges present novel chemical structures, showing interesting biological activities [60]–[62]. Antimicrobial studies of secondary metabolites isolated from *Lotrochota purpurea* – i.e. halogenated alkaloids purpuroines A-J – have been reported to demonstrate inhibitory activity against fungi and bacteria [63]. The extract of an Indonesian marine sponge, *Haliclona* sp., have been shown to display potential cytotoxic activity against human solid cancer cell lines [MCF-7 (breast), LNCap (prostate), Caco-2 (colon) and HCT-15 (colon) cells] [64]. Among approved MNPs derived from sponges (Sub-chapter 2.5.1), there are some that have recently entered into clinical trials for cancer treatment, such as the synthetic tripeptide hemiasterlin, first identified from the marine sponge *Cymbastela* sp. [65].

**2.1.1. Main classes of marine bioactive compounds**

The following sub-chapters describe the most relevant chemical classes of marine natural compounds (terpenes, peptides, alkaloids, polyketides and polysaccharides) followed by a brief description on their properties. In Table 2, some selected examples of bioactive MNP structures that belong to these classes are presented.

**Table 2:** Selected examples of bioactive MNP structures belonging to the following chemical classes: terpenes, peptides, alkaloids, polyketides and polysaccharides.

<table>
<thead>
<tr>
<th>Chemical Classes</th>
<th>Structure and Compound name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terpenes</strong></td>
<td><img src="image" alt="Secomanoalide" /></td>
<td>[66]</td>
</tr>
</tbody>
</table>
Peptides

Didemnin B

[67]

Alkaloids

Nagelamide J

[68]

Polyketides

Spongistatin 1

[69], [70]
2.1.1.1. Terpenes

This class is considered one of the most diverse classes of metabolites, in their structure and number [73]. Terpenes are derived from a five-carbon isoprene structure and, depending on the combination of units, can be grouped into different biogenetic classes such as monoterpenes (C$_{10}$), sesquiterpenes (C$_{15}$), diterpenes (C$_{20}$), sesterpenes (C$_{25}$), triterpenes (C$_{30}$, steroids) and tetraterpenes (C$_{40}$, carotenoids). They can have several functional groups, e.g., isonitrile, dichloroimine, halogenated, isocyanate, among others. Several marine organisms produce terpenes, competing for space and reproduction – consequently, these compounds show bioactivities such as antibacterial and cytotoxic properties. For example, sesquiterpenes isolated from two seaweeds (Laurencia obtusa and Laurencia microcladia), collected from the North Aegean Sea, showed cytotoxic activity against five human tumor cell lines [74]. Scheuer et al. found that three metabolites belonging to sesterpene class, in which manoalide is the parent compound, displayed antibacterial activity against Gram-positive bacteria, i.e. Staphylococcus aureus and Bacillus subtilis [66].

2.1.1.2. Peptides

There has been an increasing interest in this class, since it offers a great potential for functional food and medical applications [75]. Bioactive peptides are specific protein fragments that act as sources of amino acids and
nitrogen, and have numerous potential physiological functions within the body [76], [77]. The sizes of bioactive peptides range from 2 to 20 amino acid residues in length and are encrypted within the sequence of parent protein. These peptides are inactive or latent in the parent, but are released in an active form by enzymatic hydrolysis.

Marine bioactive peptides have shown diverse biological activities. For example, the depsipeptide didemnin B, first isolated from Caribbean tunicate *Trididemnum solidum* show significant antitumor activity and antiproliferative activity against human prostatic cancer cell lines [67]. Didemnin B is also the first MNP evaluated in human clinical trials. Matsunaga et al. [78] reported that a peptide discodermin A, isolated from marine sponge *Discodermia kiiensis*, showed antimicrobial activity against a range of Gram-positive and Gram-negative bacteria and fungi. A well-known compound belonging to this class is ziconotide, originally derived from a tropical marine cone snail and commercialized under the trade name Prialt® for the treatment of chronic pain in spinal cord injury (see Sub-chapter 2.5.1 for further details).

### 2.1.1.3. Alkaloids

Alkaloids are well known by their wide spectrum of biological activities [79], [80]. Pelletier et al. defined this class as “cyclic organic compounds containing nitrogen in a negative oxidation state which is of limited distribution among living organisms” [81]. They can be divided into seven subclasses: indole alkaloids, pyrrole alkaloids, pyridoacrine alkaloids, isoquinoline alkaloids, gadinine alkaloids, aminoimidazole alkaloids and sterol alkaloids [82]. Pyrrole alkaloids, especially bromopyrroles, are well known for being present in sponges, e.g. *Agelas, Axinella, Acanthella, Hymeniacidon* and *Pseudoaxinyssa*. Oroidin is a secondary metabolite from this subclass that exists in more abundance, being also the first one to be isolated² [83], and it is

---

² A revised structure was established in 1973 and proven by total synthesis in 1986 [177].
considered to be the biogenetic precursor of all the other bromopyrrole alkaloids. Nagelamide J, isolated from sponge *Agelas* sp. and the first bromopyrrole alkaloid possessing a cyclopentane ring fused to an aminoimidazole ring, showed antimicrobial activity against *Staphylococcus aureus* and yeast [68]. Nagelamide A, isolated as well from the same family, inhibited not only the growth of two Gram-positive bacteria but also Gram-negative *Escherichia coli* [84].

Tunicate-derived trabectedin, a secondary metabolite also belonging to the alkaloid class, is one of the marine-derived drugs that has passed cancer clinical trials [85]. More than 40 years after its discovery, it became the first marine anticancer drug to be approved in the European Union.

2.1.1.4. **Polyketides**

Polyketides are low molecular weight compounds assembled via sequential condensations of small carboxylic acids. Their sub-classes comprise polyethers, polyenes, polyphenols, macrolides and polyols, being mainly derived from the simplest (or one of the simplest) building blocks available in nature, i.e. acetic acid. From a pharmaceutical point of view, polyketides are an important source of novel drugs. Eribulin, a synthetic derivative of halichondrin B (natural polyketide) is one example of marine-derived anticancer drugs, approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA). Spongistatin 1, a macrolide initially isolated from sponges *Spirastrella spinispirulifera* and *Hyrtios* [69], [70], display significant growth inhibition against a wide variety of cancer cell lines [86]. Macrolides (+)-brefeldin A, (+)-brefeldin C and 7-oxobrefeldin A, isolated from marine-derived fungus *Penicillium* sp. PSU-F44, have been described to show antimicrobial activity [87].

2.1.1.5. **Polysaccharides**
The majority of polysaccharides (PS) produced by marine organisms are heteropolysaccharides, i.e. complex carbohydrates composed of repeating units of several types of monosaccharides connected by glycosidic bonds. Mainly micro- and macroalgae contain PS, such as agar, alginates, agarose, carrageenans and fucoidans. There are several applications for PS, such as drug or nutraceutical carriers in pharmaceutical industry, thickeners and gelling agents in food industry, in soil and water treatments [88]. Review conducted by Raposo et al. [72], showed that crude PS and their derivatives can show anticoagulant, antitumor and anticancer activities and can also be potent antibiotic, antioxidant and anti-inflammatory agents. For example, carrageenan, a family of PS obtained by extraction from certain species of red seadweeds (Rhodophyta), have shown potential antiviral properties – Buck and coworkers demonstrated that by testing it against a range of sexually transmitted human papillomaviruses (HPVs) [71].

2.2. MNPs as scaffolds

Nowadays, drug discovery is based on three major sources: NPs (natural products), semi-synthetic derivatives of NPs and synthetic compounds derived from combinatorial chemistry [89]. NPs can provide physicochemical properties such as specific interactions with multiple biological targets (barely found in molecules derived from combinatorial synthesis) and contain more chiral centers and a larger number of rings. However, the process from discovery to commercialization is complex, time-consuming and expensive, causing pharmaceutical industries to direct their drug discovery programs from NP-based research into synthetic pathways [90]. Since NPs generally have poor pharmacokinetics (but excellent bioactivity), using NPs as scaffolds for synthesis of new compounds can be pursued to improve the properties. Development of novel compounds by chemical synthesis includes modification/removal of functional groups and introduction of novel groups and stereocenters into the molecule. Sometimes, more radical changes in the scaffold are possible. One thing to keep in mind in the synthesis of new
compounds using MNPs as scaffolds is that marine compounds are typically very hydrophobic and this may become a problem further on in drug development [91].

An example of a structurally complex MNP that was used as scaffold for the design of new compounds is halichondrin B (Fig. 3), originally isolated from a Japanese marine sponge and firstly reported by Uemura et al. in 1985 [92], [93]. This compound had shown promising in vitro and in vivo anticancer activity and in 1992, total synthesis of this metabolite was reported [94]. In collaboration with Eisai Research Institute (Tokyo, Japan) work on the SAR of halichondrin B, a new synthetic analog was created – eribulin mesylate. Eribulin is commercialized under the trade name Halaven® for the treatment of metastatic breast cancer. Nowadays, this synthetic drug is also being studied for the treatment of other solid malignancies, e.g. advanced soft tissue sarcoma not responsive to radiotherapy and in patients who have not responded to hormone therapy.

![Chemical structures](image)

**Figure 3:** Chemical structure of halichondrin B (top): the part of the molecule to make eribulin mesylate is shown in blue. Chemical structure of eribulin mesylate (bottom, left). Japanese sponge *Halichondria okadai* (bottom, right).
Another example of a lead compound used for the synthesis of new derivatives is bryostatin 1. This macrocycle, isolated from a bryozoan (aquatic invertebrate animal) - *Bulgula neritina* [95] - is known to present remarkable *in vitro* and *in vivo* activities related to cancer treatment, i.e., restoration of apoptotic function, reversal of multidrug resistance, among others [96]. Since economic and environmental factors limited further development of this source organism and its production, new functional derivatives (known as the bryologs) were made. These analogs exhibited greater *in vitro* and *in vivo* potency than the parent compound and have been tuned for clinical trials [96].

2.3. Extraction of bioactive compounds: overview of sample preparation and extraction conditions

Sample preparation and the following extraction are two of the most critical steps in the isolation of marine compounds, necessary for extracting the desired chemical components from the material for further separation and characterization. Firstly, marine organisms are collected from the selected environment. However, intrinsic (as genetic) and extrinsic (as environment) collection method, sampling (life cycle, season, day/night and preservation) should be taken into account. The most serious risks that can occur when sampling all type of organisms are contamination and cross-contamination. Once samples are collected, they should be quickly frozen with dry ice and stored at -20 °C until processing in the shortest gap of time possible [97]. When in a laboratory, compounds should be isolated from marine organisms, following a procedure with several steps. Before starting any screening process, some objectives should be kept in mind when preparing extracts (crude or fractions) [98]:

- steps should be taken to provide chemical stability of compounds in the extract;
- efforts need to be made to minimize material losses;
- sample preparation costs need to be minimized.
Choosing a method of extraction is dependent on the bioactive compound(s) to be isolated and features of the organism(s). Since the bioactive compounds of interest can be unknown, known or structurally similar to a group of known compounds, the extraction protocol should be clear and precise. Also, the success of an extraction process is affected by the content of bioactive compounds in the marine organisms: for example, algal protein content is higher in red macroalgae than in brown macroalgae. Thus, extraction of algal proteins is influenced by the chemical composition, morphological and structural characteristics, and content [99].

Generally, the extraction of bioactive compounds consists in the use of solvents with different polarities. The main advantages of using this type of extraction compared with other methodologies (for example, supercritical fluid extraction, ultrasound-assisted extraction) are low processing costs and ease of operation; disadvantages focus on the low selectivity and extraction efficiency, solvent residuals and environmental pollution [100]. Table 3 reports examples of bioactive compounds and the solvents commonly used for their extraction.

<table>
<thead>
<tr>
<th>Type of bioactive compound</th>
<th>Examples of bioactive compounds</th>
<th>Solvents used (commonly)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polar organic compounds</strong></td>
<td>Alkaloids Aminoacids Polyhydroxysteroids Polyketides Saponins Shikimates Sugars</td>
<td>n-butanol Methanol Ethanol Ethyl acetate Water Chloroform Acetone</td>
</tr>
<tr>
<td><strong>Medium-polarity compounds</strong></td>
<td>Peptides</td>
<td>Dichloromethane Methanol Carbon tetrachloride</td>
</tr>
<tr>
<td><strong>Low-polarity compounds</strong></td>
<td>Fatty acids Hydrocarbons Fatty acids</td>
<td>Carbon tetrachloride Hexane</td>
</tr>
</tbody>
</table>
Extraction by solvents can follow the principle of either liquid-liquid or solid-liquid extractions. Kupchan’s extraction method [102] is probably the most popular liquid-liquid fractionation scheme. This method separates compounds based on their relative solubility in two different immiscible liquids. The selective partitioning of components of interest into one of the immiscible phases results from the choice of the most adequate extraction solvent. However, during the recent years, solid-liquid extractions have emerged as an alternative. There the material is placed in contact with a solvent, which diffuses into the cells, solubilizing the metabolites. After that, the metabolites are diffused out of the cells into the solvent. This extraction method has been commonly used for obtaining marine compounds, using a marine organism directly as a solid matrix [99].

2.4. Importance and approaches for screening bioactive compounds: general overview of bioactive screening

Due to the great biodiversity in marine environment, the use of appropriate methodologies for biological screening of marine sources is of great importance. Basically, the selection of bioactivity screening assay depends mostly upon the target disease as well as the available information about the target marine source/organism. For example, if a marine source has some pharmacological history of use for a specific disease, we would rationally use a particular bioassay, which can show the known therapeutic activity, in order to isolate the active compound, which is accountable for that bioactivity.

Whichever assay format is selected, the following factors need to be considered [103]:

- Tolerance to several impurities available in crude extracts;
- Sensitivity and capability to detect the presence of potentially active substances present in low concentrations (limit of 0.0001% of an active compound, based on the dry weight of extract);
- Reproducibility and reliability;
• High throughput;
• Tolerance to DMSO (commonly used solvent for solubilization of samples).

During the primary biological screening, multiple samples are screened to evaluate whether any desired bioactivity is present. These types of assays are capable of providing results fast, not always quantitative, but economic. If a positive hit is observed in the primary screening, follow-up assays should be performed in order to eliminate false positives. Generally, a hit rate of ≤1% is considered reasonable from a primary screening for progressing to follow-up studies [104].

Secondary evaluation/confirmatory assays involve more exhaustive and comprehensive testing of active compounds. As a part of this process, consideration should be given to the properties of each compound, such as, if there is a SAR between the molecular structure and biological activity of the compounds.

2.5. Marine drug discovery and development

MNP discovery is a complex and multidisciplinary effort requiring work and interaction between marine biologists, chemists, microbiologists and pharmacologists. As it is known, the marine realm contains a rich variety of organisms, many of them under-investigated. Bioprospecting is a tool for biodiversity conservation, defined as the collection of biological material and analysis of its properties and/or its molecular, biochemical or genetic content for the purpose of developing a commercial product [105]. However, attention must be paid to ethical issues and conservation policies, since the loss of biodiversity is often associated with overexploitation and habitat degradation. In the last decades, discovery of new MNPs has exponentially increased, but the number of marine-derived molecules that are commercialized is still very low – eight approved drugs by FDA and/or EMA. Despite that, more than 20
marine products are in clinical trials and more than 1000 are in pre-clinical trials [106]–[108]. The process of R&D of new drugs is neither easy nor always successful; it is statistically shown [109] that of 5000 compounds that are discovered, only 5 progress to clinical studies and only 1 leads to an approved and commercialized drug (Fig. 4).

A clinical trial is, *per se*, the testing carried out on human beings for determining its value for treatment or for prevention of diseases, involving a number of parameters. These parameters [110] include: the patient population to be studied (patients should meet specific criteria to ensure that they are as similar as possible to each other so that the results of treatments effect can be associated as much as possible with the drug treatment), use of controls [placebo (e.g., medically ineffectual treatment) or standard treatment (e.g., in wide use and considered effective at the time that the trial is designed)], endpoints (existence of a primary endpoint that usually assesses the treatment efficacy) and methods by which the trial will be conducted (randomly allocated to receive one or other of the alternative treatments being studied and/or partitioned by a factor other than the treatment, to ensure that equal number of patients with a characteristic thought to response to the intervention will be allocated to each comparison group).
Between Phase I and III, the potential of failing and withdrawals of drug development is high, mainly due to lack of efficacy and drug toxicity [111]. Upon authorization by the EMA/FDA, the therapies that have proven safety, efficacy and quality in the clinical trials may be made available to the general population. Still, EMA/FDA requires continued evaluation after release to evaluate safety signs that can affect the benefit-risk ratio [112], [113].
2.5.1. Commercial uses and applications of MNPs

A large number of bioactive compounds found in marine organisms have proceeded into clinical trials and until 2014, eight FDA or EMA approved drugs originating from marine microbes are currently on the market [114]. For example, companies such as PharmaMar (Spain), AquaPharm Biodiscovery Ltd. (United Kingdom) or Nereus Pharmaceutical (United States of America) have several compounds in clinical trials, preclinical trials or even on the market. However, until now, only three compounds (Prialt®, Yondelis® and Carragelose®) have become drugs without modification of the original natural molecule, i.e. without any type of synthesis and optimization in the structure. Selected marine bioactive compounds in trials or commercialized from 2004 until now are highlighted in Table 4.

Figure 5: Different stages of clinical trials (Adapted from: [180]). In Phase IV, active post-marketing surveillance of drug side effects is essential.
Cytarabine and vidarabine were the first marine-derived drugs approved by the FDA, as anticancer (1969) and antiviral (1976) drugs [115], respectively. **Cytarabine (Cytosar-U®)** is used for the treatment of two types of leukemia (myeloid and meningeal) and non-Hodgkin’s lymphoma. However, this drug has a short plasma half-life, low stability and limited bioavailability. **Vidarabine (Vira-A®)**, has been used as an antiviral drug for the treatment of keratoconjunctivitis, an epithelial keratitis caused by the herpes simplex virus, but it has been discontinued by FDA in the US market because of its side effects such as fever, sore or other signs of infection. Another compound included in the same group as cytarabine and vidarabine based on their mechanism of action (DNA interaction), is **trabectedin (Yondelis®)**. PharmaMar markets Yondelis® in Europe and Japan and new clinical trials have been carried out for breast and prostate cancer. The active ingredient was originally isolated from a Caribbean sea squirt called *Ecteinascidia turbinata* and it shows potent antitumor [116], [117] and antiproliferative [118] activities towards cancer cells.

**Prialt®,** a ziconotide (synthetic derivative of ω-Conotoxin MVIIA) is being used for the treatment of chronic pain in the spinal medulla [11]. This compound was isolated from the marine snail *Conus magnus* in 1979. However, the complete synthesis was only finalized in 1987 [11]. Recently, a synthetic derivative of halichondrin B, **eribulin mesylate (Halaven®)** gained FDA’s approval for the treatment of metastatic breast cancer (Sub-chapter 2.2).

An Austrian company, Marinomed Biotechnologie GmbH, has developed an antiviral nasal spray containing **lota-carrageenan (Carragelose®)**. This copolymer derives from carrageenan, high molecular weight sulphated polysaccharide extracted from red edible seaweeds, such as *Rhodphyceae* sp. This substance is clinically effective against early symptoms of the cold and is marketed as an OTC (over-the-counter) product.
Lovaza®, an anti-hypertriglyceridemia drug, is commercialized by GlaxoSmithKline and consists of a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The approach to this drug discovery was based on studies stating that some ethnic populations, such as Native Alaskans, had much lower mortality rates from cardiovascular diseases, assumingly due to their diets that were high in polyunsaturated fatty acids [119]. Other studies confirmed also that ingesting omega-3 fatty acids was associated with a reduced rate of premature cardiovascular deaths [120], [121].

The latest marine-derived drug to enter the market until now was brentuximab vedotin (Adcetris™), consisting of an antibody brentuximab and monomethyl auristatin (vedotin), a synthetic analog of dolastatin 10. Dolastatin 10 is a compound originally isolated from the marine mollusk Dolabella auricularia [122]. Of the antibody-drug conjugate, the chimeric monoclonal antibody brentuximab targets the CD30 antigen on the surface of malignant cells, leading to the uptake of vedotin (an antimitotic agent) into the targeted cells. Brentuximab vedotin is used for treating Hodgkin’s lymphoma and systemic anaplastic large-cell lymphoma.
Table 4: Marine-derived compounds in drug discovery and development (Adapted from: [123]). NP = natural product.

<table>
<thead>
<tr>
<th>Product</th>
<th>NP or derivative</th>
<th>Source organism</th>
<th>Chemical class</th>
<th>Company</th>
<th>Therapeutic area</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compounds targeting ion channels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ziconotide (Prialt®)</td>
<td>NP</td>
<td>ω-Conotoxin/ marine cone snail <em>Conus magus</em></td>
<td>Peptides</td>
<td>Elan Corporation (Dublin, Ireland)</td>
<td>Chronic (neurophatic) pain</td>
<td>FDA/EMA approved</td>
</tr>
<tr>
<td>DMXBA (GTS-21)</td>
<td>NP derivative</td>
<td>Anabeseine/worm <em>Paranemertes peregrina</em></td>
<td>Pyridines and derivatives</td>
<td>Comentis (San Francisco, CA, USA)</td>
<td>Alzheimer’s disease and schizophrenia</td>
<td>Phase II</td>
</tr>
<tr>
<td><strong>Compounds targeting enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bryiostatin-1</td>
<td>NP</td>
<td>Bryozoan <em>Bugula neritina</em></td>
<td>Polyketides</td>
<td>NCI (Bethesda, MD, USA)</td>
<td>Cancer and Alzheimer’s disease</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Eribulin mesylate (Halaven®)</td>
<td>NP derivative</td>
<td>Halichondrin B/sponge <em>Halichondria okadai</em></td>
<td>Furopyrans</td>
<td>Eisai (Tokyo, Japan)</td>
<td>Cancer</td>
<td>FDA/EMA approved</td>
</tr>
<tr>
<td><strong>Microtubule-interfering agents</strong></td>
<td></td>
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</tr>
<tr>
<td>Bretuximab vedotin (SGN-35) (Adcetris™)</td>
<td>NP derivative</td>
<td>Dolastatin 10/ sea hare <em>Dolabella auricularia</em></td>
<td>Carboxylic acids and derivatives</td>
<td>Seattle Genetics (Bothell, WA, USA); Takeda GRDC (Osaka, Japan)</td>
<td>Cancer</td>
<td>FDA/EMA approved</td>
</tr>
<tr>
<td>Tasidotin (ILX-651)</td>
<td>NP derivative</td>
<td>Dolastatin 15/ sea hare <em>Dolabella auricularia</em></td>
<td>Peptides</td>
<td>Genzyme Corporation (Cambridge, MA, USA)</td>
<td>Cancer</td>
<td>Discontinued</td>
</tr>
<tr>
<td>Discodermolide</td>
<td>NP</td>
<td>Sponge <em>Discoderma dissouta</em></td>
<td>Polyketides</td>
<td>Novartis (Basel, Switzerland); Harbor Branch (Fort Pierce, FL, USA)</td>
<td>Cancer</td>
<td>Discontinued</td>
</tr>
<tr>
<td><strong>HTI-286</strong></td>
<td>NP derivative</td>
<td>Hemiasterlin/sponge <em>Hemiastrella minor</em></td>
<td>Tripeptides</td>
<td>former Wyeth (Philadelphia, PA, USA)</td>
<td>Cancer</td>
<td>Discontinued</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
</tbody>
</table>

**DNA-interactive agents**

<table>
<thead>
<tr>
<th><strong>Trabectedin (Yondelis®)</strong></th>
<th>NP derivative</th>
<th>Ecteinascidin 743/tunicate <em>Ecteinascidia turbinata</em></th>
<th>Benzene and substituted derivatives</th>
<th>PharmaMar (Colmenar Viejo, Madrid, Spain)</th>
<th>Cancer</th>
<th>EMA approved</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Cytarabine (Cytosar-U®; Depocyt®)</strong></th>
<th>NP derivative</th>
<th>Spongouridine/sponge <em>Cryptotheca cypta</em></th>
<th>Pyrimidine nucleosides</th>
<th>Bedford (Bedford, OH, USA); Enzon (Piscataway, NJ, USA)</th>
<th>Cancer</th>
<th>FDA/EMA approved</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Vidarabine (Vira-A®)</strong></th>
<th>NP derivative</th>
<th>Spongouridine/sponge <em>Cryptotheca cypta</em></th>
<th>Purine nucleosides</th>
<th>King Pharma (Tenaflly, NJ, USA)</th>
<th>Antiviral</th>
<th>FDA/EMA approved but discontinued in USA</th>
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</thead>
</table>

**Oxidative stress inducers**

<table>
<thead>
<tr>
<th><strong>Plitidepsin (Aplidin®)</strong></th>
<th>NP derivative</th>
<th>Ascidian <em>Aplidium albicans</em></th>
<th>Carboxylic acids and derivatives</th>
<th>PharmaMar (Colmenar Viejo, Madrid, Spain)</th>
<th>Cancer</th>
<th>Phase II/III</th>
</tr>
</thead>
</table>

**Lysosomotropic compounds**

<table>
<thead>
<tr>
<th><strong>Kahalalide F</strong></th>
<th>NP derivative</th>
<th>Sea slug <em>Elysia rufescens</em></th>
<th>Cyclic depsipeptides</th>
<th>PharmaMar (Colmenar Viejo, Madrid, Spain); Hawai University (Honolulu, HI, USA)</th>
<th>Cancer</th>
<th>Discontinued</th>
</tr>
</thead>
</table>

**Immunostimulatory agents**

<table>
<thead>
<tr>
<th><strong>KRN-7000</strong></th>
<th>NP derivative</th>
<th>Agelasphins/sponge <em>Agelas mauritianus</em></th>
<th>α-galactosylceramides</th>
<th>Vrije Universiteit Medical Center (Amsterdam, Netherlands)</th>
<th>Cancer</th>
<th>Discontinued</th>
</tr>
</thead>
</table>

**Calcium binding protein antagonists**

<table>
<thead>
<tr>
<th><strong>Squalamine</strong></th>
<th>NP derivative</th>
<th>Dogfish shark <em>Squalus acantbias</em></th>
<th>Aminosteroids</th>
<th>former Genaera (Plymouth Meeting, PA, USA)</th>
<th>Cancer</th>
<th>Discontinued</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compounds with antiviral activity</td>
<td></td>
<td></td>
<td></td>
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<td>----------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Iota-carrageenan (Carragelose®)</td>
<td>NP</td>
<td>Iota-carrageenan/red algae <em>Eucheuma/Cnondus</em></td>
<td>Carbohydrates</td>
<td>Marinomed (Vienna, Austria); Boehringer Ingelheim (Ingelheim, Germany)</td>
<td>Antiviral</td>
<td>Over-the-counter drug (OTC)</td>
</tr>
<tr>
<td>Compounds with other or unknown mechanism of action</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPL-576092 and derivatives</td>
<td>NP derivative</td>
<td>Contignasterol/sponge <em>Petrosia contignata</em></td>
<td>Steroids</td>
<td>former Aventis (Strasbourg, France)</td>
<td>Anti-asthmatic</td>
<td>Discontinued</td>
</tr>
<tr>
<td>Omega-3-acid ethyl esters (Lovaza®)</td>
<td>NP derivative</td>
<td>Omega-3-fatty acids/fish</td>
<td>Esters</td>
<td>GlaxoSmithKline (Brentford, UK)</td>
<td>Hypertriglyceridemia</td>
<td>FDA/EMA approved</td>
</tr>
<tr>
<td>Iota-carrageenan (Carragelose®)</td>
<td>NP</td>
<td>Iota-carrageenan/red algae <em>Eucheuma/Cnondus</em></td>
<td>Carbohydrates</td>
<td>Marinomed (Vienna, Austria); Boehringer Ingelheim (Ingelheim, Germany)</td>
<td>Antiviral</td>
<td>Over-the-counter drug (OTC)</td>
</tr>
</tbody>
</table>
3. Aims of the study

The aim of this study was to evaluate, using in vitro assays, the biological activity of extracts and synthetic compounds derived from marine organisms. NPs, especially those from terrestrial plants and microbes, have been the most successful sources of potential drug leads. MNPs have only become a “hot topic” in the last decades, as a consequence of their proven potential as antimicrobial, anticancer, antiviral and anti-inflammatory agents, among other properties. The studies included in this thesis focused on the assessment of biological potential of marine-derived substances that can have a significant impact in the pharmacological field during the coming years.

The specific aims of this doctoral thesis were:

- to study the biological properties of synthetic derivatives designed by using marine alkaloids oroidin and clathrodin as parent structures (I, II);
- to determine the biological activities of synthetic crown acyl ether derivatives, developed by using okadaic acid structure as inspiration (III);
- to evaluate the bioactive potential of cyanobacteria, micro- and macroalgae extracts originating from the Aegean Sea (IV).
4. Material and Methods

Microbial strains and cancer cell lines used in this study are listed in sub-chapters 4.1 and 4.2. Types of biological assays used are listed below.

**Table 5:** Biological assays used in studies (I)-(IV).

<table>
<thead>
<tr>
<th>Biological assays</th>
<th>Methods</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-inflammatory</td>
<td>Cytokine analysis</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Flow cytometric analysis</td>
<td></td>
</tr>
<tr>
<td>Antifouling</td>
<td>Agar diffusion</td>
<td>IV</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>Agar diffusion</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Broth microdilution</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Antiproliferative/Cytotoxicity</td>
<td>ATP assay</td>
<td>I, IV</td>
</tr>
<tr>
<td></td>
<td>LDH assay</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>MTT assay</td>
<td>II, IV</td>
</tr>
<tr>
<td></td>
<td>Sulforhodamine B assay</td>
<td>III</td>
</tr>
<tr>
<td>Antiviral</td>
<td>HCV replicon supression¹</td>
<td>II</td>
</tr>
</tbody>
</table>

¹ An HCV replicon model [124] was used in this assay.

4.1. Microbial strains and growth conditions

Four human pathogenic strains were used in the antimicrobial study: two Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923), one Gram-negative bacterium (*Escherichia coli* ATCC 25922) and one fungus (*Candida albicans* ATCC 90028). Brief descriptions of these pathogens are shown in Table 6. All strains were obtained from Microbiologics Inc. (USA). Strains were stored long-term at -70 °C and used as needed. Bacterial strains were grown on MHA (Becton Dickinson, USA) and *C. albicans* on SDA (Becton Dickinson, USA) at 37 °C and 28 °C, respectively. All stock cultures were maintained at 4 °C.
Table 6: Characteristics of pathogenic bacteria and fungus used in this work [125].

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease</th>
<th>Main symptoms</th>
<th>Mode of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Urinary tract, wound and soft-tissue infections, endocarditis</td>
<td>Burning pain with urination, urinary urgency, fever, back/flank pain, night sweats, fatigue</td>
<td>Nosocomial and person-to-person transmission, transmission by food products</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Scalded skin syndrome, life-threatening diseases (e.g., pneumonia)</td>
<td>Nausea, vomiting, abdominal cramps, retching, prostration</td>
<td>Ingestion of food containing enterotoxins (specific toxin for cells of intestinal mucosa)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Urinary and biliary duct infections, gastroenteritis, pneumonia, meningitis, sepsis</td>
<td>Hemorrhagic colitis, diarrhea, dysentery, fever, abdominal pains</td>
<td>Ingestion of contaminated food/water, fecal-oral transmission, person-to-person transmission</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>Candidiasis</td>
<td>White coated tongue, body rash, painful urination and discharge, fever, general pain</td>
<td>Human own natural flora, external source (even if rare)</td>
</tr>
</tbody>
</table>
4.2. Cell lines and culture conditions

For antiproliferative and cytotoxicity studies, the following cell lines were used: A-375 (human malignant melanoma) and BALB/c 3T3 clone A31 (embryonic mouse fibroblast). A-375 was kindly provided by Prof. Marikki Laiho (University of Helsinki, FI) and BALB/c 3T3 clone A31 was obtained from the European Collection of Cell Cultures (ECACC, UK). Melanoma cells were grown in Glutamax DMEM with 4.5 g/L D-glucose (Gibco, USA), supplemented with 10% FBS (Gibco, USA), 100 IU/mL penicillin (Gibco, USA) and 100 μg/mL streptomycin (Gibco, USA). BALB/c 3T3 cell line was cultured in DMEM (Sigma-Aldrich, USA), supplemented with 5% FBS (Gibco, USA), 5% NCS (Gibco, USA), 292 μg/mL L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin.

Methods related to human breast epithelial (HBL-100), human cervical cancer (HeLa), alveolar carcinoma (SW1573) and colon adenocarcinoma (WiDr) cell lines are described in detail in Publication (III). To prostate cancer (LNCa, PC-3), breast cancer (MCF-7) and non-tumorigenic epithelial (MCF-10A) cell lines, methods are described in Publication (IV).

4.3. Reference compounds

Stock solutions of amphotericin B (Sigma-Aldrich, USA) and ciprofloxacin (MP Biomedicals, USA) were made in DMSO and milliQ water, respectively. The reference antibiotics were used for assay validation and as positive controls in the antimicrobial assays. Stock solutions of polymyxin B sulphate (PMX, Sigma-Aldrich, DK) were diluted in milliQ water and assays were validated. PMX was used as positive control in antiproliferative assays.

4.4. Marine test material

A set of marine natural products and marine-derived compounds were used in experiments and evaluated for their antimicrobial and antiproliferative
properties. In publications I, II and III, the following sets of marine-derived synthetic compounds were studied:

- (I): set of thirty-four novel analogs, using oroidin as scaffold;
- (II): set of fourteen new clathrodin-inspired 2-aminobenzothiazole and benzimidazole derivatives and their synthetic intermediates;
- (III): set of twenty-two macrocyclic compounds, employing secondary metabolite okadaic acid (OA) as inspiration.

In the last publication (Study (IV)), ninety-eight crude extracts (solvent used in extraction: ethanol) of cyanobacteria, micro- and macroalgae originating from the Aegean Sea were studied.

Methods related to the synthesis and chromatographic analyses of the compounds are described in detail in Publications (I), (II) and (III).

4.5. Screening for bioactivity

4.5.1. Antimicrobial screening

*Broth microdilution*

Broth microdilution method was used in all studies (I, II, III, IV). Together with agar dilution, it is the standard susceptibility testing method approved by EUCAST [126] and CLSI [127]. Briefly, the bacterial or fungal strain to be used in the assay was inoculated the day before on MHB or SDA, respectively, and grown for 16-24 h. For the experiment, CFU/mL was calculated and suspensions prepared, based on the absorbance values at 620 nm, previously calibrated against plate counts. Compounds were diluted into the assay media, and dispensed into clear 96-well microplates (Thermo Fisher Scientific, FI) and the bacterial/fungal suspension added to the plates. The assay plates also included the positive control antibiotics: amphotericin B for *C. albicans* and ciprofloxacin for bacterial strains (MICs are mentioned in the articles). The plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for *Candida*. Synthetic compounds and extracts were initially screened at the final concentration of 50 μM and 100 μg/mL, respectively.
Samples that demonstrated potent activity (growth inhibition >50%) were selected for confirmatory testing (dose-response) and MIC determination.

4.5.2. *In vitro* antiproliferative and cytotoxicity screening

The general procedures for routine subculturing and exposure of cells to compounds are found in publications (I) and (II). In Study (I), Huh-7 (human hepatocellular carcinoma) cell line was used for selectivity studies. In Study (II), two cell lines were used: A-375 (human malignant melanoma, epithelial, cancer) and BALB/c 3T3 clone 31 (embryonic mouse fibroblast, non-cancer). Melanoma is a cancer that involves the melanocytes (specialized pigment cells) in the basal layer of epidermis and is one of the most common and aggressive forms of skin cancer and related deaths. This “fatal black tumor” [128] displays a growth of incidence in most populations of Caucasian origin [129]; in Europe, the highest incidence rates of melanoma are reported in Scandinavian countries [130]. The main environmental factor, UV light exposure, can cause damage in keratinocytes and melanocytes synthesize melanin (protective pigment). If exposure to UV is extreme, keratinocyte population can die but melanocytes survive – due to efficient DNA damage repair response -, having the chance of developing mutations [131], [132]. BALB/c 3T3 non-cancerous cell line was used in publication II as a model for selectivity studies in comparison with melanoma cell line.

**ATP assay (I)**

Adenosine triphosphate (ATP) is indicative of metabolically active cells, as it is responsible for intracellular energy transfer [133]. Non-viable cells are not metabolically active, and therefore do not produce ATP. CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, USA) was used to study the effect of the most promising compounds on the metabolic activity of Huh-7 cells. This assay is based on luciferase reaction that produces light in the presence of ATP, and is thus used for measuring the intracellular ATP content. After the exposure of cells to compounds, cells were washed with phosphate buffered saline (PBS) and 50 μL of assay media and 50 μL of
CellTiter-Glo reagent were added. After plate shaking for 2 min and incubation at room temperature (rt) for 10 min, the luminescent signal was determined using a Varioskan Flash Plate reader (Thermo Fisher Scientific, FI). Cytotoxicity (%) was calculated by normalizing to vehicle-treated control values.

**MTT assay (II)**

MTT assay [134], [135], based on MTT dye [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], was used to estimate the cytotoxic effects of compounds. This assay is based on the conversion of MTT to formazan crystals by reduction, catalyzed by intracellular dehydrogenases and it is widely used for measuring in vitro cytotoxicity. Briefly, after the adhesion of cells to 96-well microplates (Thermo Fisher Scientific, FI) and treatment with compounds (duration of 24 h), 10 μL of 5 mg/mL MTT stock solution (Sigma-Aldrich, USA) in sterile PBS was added to the cells. Following 3 h incubation in a humidified atmosphere (37 ºC, 5% CO₂), the medium was removed and the blue formazan crystals trapped in the cells were dissolved in 200 μL of DMSO. The plates were agitated until formazan had completely dissolved. The absorbance at 550nm/655nm was measured with Multiskan® GO UV/Vis spectrophotometer (Thermo Fisher Scientific, FI). Cytotoxicity (%) was calculated by normalizing to vehicle-treated control values.

**LDH assay (II)**

Leakage of intracellular lactate dehydrogenase into culture medium can be measured by LDH leakage assay. The activity is measured by monitoring the rate at which the substrate (pyruvate) is reduced to lactate. Its evaluation is based on the decrease of membrane integrity to retain the enzyme in the cytoplasm when cells are exposed to toxic agents [136], [137]. The more damaged a cell membrane is, the more leakage of LDH occurs. The CytoTox-ONE-Homogeneous Integrity Assay Kit (Promega, USA) was used for measuring LDH leakage. After exposure of cells to compounds, 50 μL samples from culture supernatants were transferred into sterile, white and
clear-bottom 96-well plates (PerkinElmer Inc., USA). After 20-30 min of incubation at room temperature (rt) in dark, 50 μL of CytoTox-ONE reaction mixture was added to the plates and shaken for 10 min. The enzyme reaction was stopped with 25 μL CytoTox-ONE stop solution. After plate shaking for 10s, fluorescent signal was measured at a wavelength of 560 nm (excitation) and 655 nm (emission) with a Varioskan Flash Plate reader (Thermo Fisher Scientific, FI). The % of LDH leakage was calculated by normalizing to vehicle-treated control values.

4.6. Selectivity index

Selectivity index (SI), also known as therapeutic index (TI), is an important criterion that determines which compounds will advance further in laboratory and clinical studies. It is considered a quantitative relationship between their efficacy (pharmacology) and safety (toxicology). In Study (I), the selectivity was based on the effects between eukaryotic (IC\textsubscript{50}) and prokaryotic (MIC\textsubscript{50}) cells. In Study (II), the ratio between a normal cell line (IC\textsubscript{50}) and a cancer cell line (IC\textsubscript{50}) was used to measure this index.

4.7. Data analysis

All data presented are means ± SD. Data were analyzed using Microsoft Office Excel 2010 and the IC\textsubscript{50} values were determined using Origin Graphing and Analysis, version 8.6 (OriginLab, USA). All experiments were performed at least in triplicate.

4.8. Quality parameters

Three types of quality parameters were used for evaluating the quality of the results: Z’, S/B and S/N. The data quality of the assays can be estimated by the following formulas [138], [139]
\[ Z' = 1 - \frac{3\times \sigma_{\text{max}} + 3\times \sigma_{\text{min}}}{|\mu_{\text{max}} - \mu_{\text{min}}|} \]

\[
S/B = \frac{\mu_{\text{max}}}{\mu_{\text{min}}}
\]

\[
S/N = \frac{\mu_{\text{max}} - \mu_{\text{min}}}{\sqrt{\sigma_{\text{max}}^2 + \sigma_{\text{min}}^2}}
\]

where \( \mu_{\text{max}} \) and \( \sigma_{\text{max}} \) are the mean and standard deviation for the high reference control samples (maximum) and \( \mu_{\text{min}} \) and \( \sigma_{\text{min}} \) are the mean and standard deviation for the low reference control samples (i.e. background).

\( Z' \) factor is used as a screening quality assurance parameter and it is the most practical factor to use when evaluating assay quality. \( Z' \) close to 1 is considered an ideal assay and values between 0.5 and 1 are considered of good quality. In general, if \( Z' \) value is lower than 0.5, the assay is considered of low quality and should be repeated (for cell-based assays, threshold 0.3 or 0.4 is sometimes considered acceptable). The S/B (signal-to-background) and S/N (signal-to-noise) ratios should also be taken into account since they reflect the assay signal strength. The S/B ratio evaluates the strength of signal in comparison with background measurements (\( S/B \geq 5 \), good quality) and S/N ratio signifies the strength of signal in relation to the noise (high S/N desired if S/B is low) [140].
5. Summary of Results and Discussion

This chapter of the thesis summarizes the main results of the work. More details can be found in the original publications (I-IV).

Articles (I), (II) and (III) focus on biological studies of synthetic derivatives. The innovations and advances in marine research have contributed to the perception that MNPs can be considered as privileged structures or biologically validated starting points for the design of new compounds. Article (IV) shows results from a large-scale screening programme of bioprospecting of species from the Aegean Sea, where several crude extracts from cyanobacteria, micro- and macroalgae were evaluated for biological activities. Marine ecosystems and rarely explored places such as the Aegean Sea that contains a rich biodiversity of marine organisms can be particularly suited for bioprospecting, a process that aims to identify and isolate bioactive natural compounds.

5.1. Oroidin and clathrodin as parent structures for synthesis of new compounds (I), (II)

5.1.1. Antimicrobial studies (I), (II)

Clathrodin is a secondary metabolite isolated from Caribbean sponge Agelas sp. that belongs to a class of alkaloids, pyrrole-imidazole alkaloids (PIAs). These metabolites contain 2-aminoimidazole, pyrrole amide structures, and are rarely found in terrestrial flora and fauna. Oroidin, an achiral and monomeric product also used in Study (I), is generally considered the biogenetic precursor of other alkaloids from the same family. Within the PIA’s, oroidin and clathrodin (Figure 6) are believed to be one of the basic biosynthetic units for the construction of more complex and larger analogs. Oroidin, for example, due to its low molecular mass and simple structure, is believed to offer several possibilities for chemical optimization, with the introduction of additional side chains/functional groups.
Neither oroidin nor clathrodin has been intensively studied until now. However, both secondary metabolites have rekindled interest in synthetic chemistry due to their intricate structural complexity and spectrum of bioactivities, including antimicrobial [141], cytotoxic [142], antimuscarinic [143] and voltage-gated sodium channel blocking ability [144]–[146].

The antimicrobial activity and cytotoxic effects of thirty-four synthesized derivatives using marine alkaloids clathrodin 2a(I) and oroidin 2b(I) as scaffolds were evaluated in Study (I). The compounds belong to four different structural classes, i.e.:

- compounds 2a(I)-2d(I): 4-(3-aminoprop-1-en-1-yl)-2-aminoimidazoles;
- compounds 5a(I)-5c(I), 5f(I)-5l(I), 6a(I)-6l(I), 7(I) and 8(I): 4-phenyl-2-aminoimidazoles;
- compounds 10a(I)-10c(I) and 11a(I)-11c(I): 4-phenyl-4,5-dihydro-(N-methylamino)-imidazoles;
- compounds 15(I) and 16(I): 1-benzyl-4-phenyl-2-aminoimidazoles.

Clathrodin 2a(I) did not show any relevant activities at a concentration of 50 μM, against all the microbial strain in the primary screening. On the other hand, its analog, oroidin 2b(I), displayed promising activity against Gram-positive bacteria in the primary screening (Article (I), Figure 3) and because of that, new oroidin derivatives with indole and substituted indole rings were designed and prepared. Table 7 shows the chemical structures and antimicrobial dose-response data for 4-phenyl-2-aminoimidazoles 6g(I) and
6h(I) that were active (>80% inhibition of growth) in the primary screening. Both obtained the best results against Gram-positive bacteria; compound 6h(I) showed MIC$_{50}$ of 8.0 and 7.3 μM with *Enterococcus faecalis* and *Staphylococcus aureus*, respectively.

In a study conducted by Zidar et al., synthetic compounds belonging to the same structural class acted as state-dependent voltage-gated sodium channel modulators and have shown interesting activities with moderate isoform selectivities [147].

Hammami et al. have shown that oroidin display antifungal activity against *Alternaria solani*, a plant pathogen [148]. In a study conducted by Scala et al, two analogs of oroidin (dispacamide B and spongiacidin) have displayed antimalarian activity (IC$_{50}$ of 1.34 and 1.09 μg/mL, respectively) against a protozoa belonging to the genus *Plasmodium* [149]. In the same study, a dimeric derivative of the same parent compound (dibromopalaumine) has been identified as a trypanocidal agent, showing an IC$_{50}$ of 0.46 μg/mL [149].

In another recent article, oroidin and clathrodin were found to be weakly active when tested for apoptosis-inducing activities against the human hepatocellular carcinoma (HepG2) cell line; however, some of the indole-based analogs showed promising activities, with EC$_{50}$ values between 13 and 42 μM [150].

The importance of an imidazole ring in the chemical structure of a compound for antimicrobial activity was also found in this study. For example, compounds 10a(I)-c(I) and 11a(I)-c(I) did not show any antimicrobial activity; these compounds have in their chemical structure a reduced imidazole C = C bond. The incorporation of imidazole ring can result in exhibition of a wide
Table 7: Antimicrobial minimum inhibitory concentration activity (MIC$_{90}$ and MIC$_{50}$) of analogs 6g(I) and 6h(I) against *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 90028. All results are shown in μM.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Chemical structure</th>
<th><em>E. faecalis</em></th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC$_{90}$</td>
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<td>7.3</td>
</tr>
</tbody>
</table>
spectrum of biological activities [151]–[154]; its unique structural feature is valuable for derivatives to bind with receptors in biological systems.

In Study (II), clathrodin was used as an inspirational structure for the synthesis of fourteen new benzimidazole and 2-aminobenzothiazole analogs. Benzimidazole, a heterocyclic aromatic compound that consists of the fusion of benzene and imidazole, has been used as nucleus structure of various drugs and is known for its biological activities [155]–[157]. Benzothiazole is considered a privileged bicyclic ring system due to its potent and significant biological activities of pharmaceutical importance [158], [159]. A derivative of 2-aminobenzothiazole, compound 7(II), showed 56% growth inhibition against Gram-positive \textit{E. faecalis} in the primary screening. In dose-response assay, the inhibition did not reach MIC\textsubscript{90} level (even at the highest concentration tested), concluding that an optimization of the compound structure should be made in order to improve its properties.

5.1.2. Antiproliferative (I), (II) and antiviral studies (II)

In Study (I), the metabolic activity of Huh-7 hepatocytes after exposure to the most promising antimicrobial compounds was evaluated by intracellular ATP quantitation. This test is based on a luciferase reaction to measure the amount of ATP in cells, correlating directly with the number of cells and their viability because cells lose the ability to synthesize ATP directly after e.g. loss of membrane integrity or other cytotoxic event. Most of the derivatives that were active in antimicrobial assays, showed also an effect on eukaryotic cells. 4-phenyl-2-aminoimidazole 6j(I) showed the most interesting IC\textsubscript{50} (19.8 \(\mu\)M) while the compounds that had the best antibacterial results, i.e. 6g(I) and 6h(I), showed only moderate IC\textsubscript{50} of 31 and 21.2 \(\mu\)M, respectively, against eukaryotic cells.

In Study (II), the antiproliferative activities of compounds were studied against a human cancer cell line and a normal cell line: A-375 (melanoma) and
BALB/c 3T3 (fibroblast). MTT and LDH assays were used in the experiments. The hydrogen acceptor 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) is commonly used to estimate cell viability (and therefore cytotoxicity) in drug screening, while LDH is the most common cytotoxicity test used to measure the release of intracellular enzymes into culture medium [136].

Results from MTT primary studies with melanoma cell line showed that \( N^-((2\text{-}\text{aminobenzo[d]thiazol-6-yl})\text{methyl})\text{benzamide 4a(II) and (9H-fluoren-9-yl})\text{methyl [(2-aminobenzo[d]thiazol-6-yl})\text{methyl]carbamate 7(II), both derivatives of 2-aminobenzothiazole showed cytotoxicity above the threshold of 50%}. In confirmatory dose-response experiments, only for compound 7(II) the cytotoxic effect was clear, yielding an IC\(_{50}\) of 16 \( \mu \)M (Fig. 7a)). This compound also caused elevated intracellular enzyme leakage at the highest concentration tested (Fig. 7b).

In results using BALB/c 3T3 clone A31 as non-cancerous cell line for selectivity studies, the IC\(_{50}\) of compound 7(II) was higher (71 \( \mu \)M) than against the A-375 melanoma cell line. The effect in LDH leakage assay followed the same trend, where an increasing leakage was observed at concentrations higher than 75 \( \mu \)M (the effect of the positive control, PMX at 20 000 IU/mL was 2.1-fold increase).
When comparing both tests used for cytotoxicity assessment, MTT appears to be an earlier indicator of cytotoxicity compared with LDH test. Also, the most active compound not only in antiproliferative screening against melanoma and fibroblast cell lines, but as well in antimicrobial screening was compound 7\( \text{II} \), as stated previously. This compound is an intermediate and not a final amide, suggesting that the length of the linker chain is not optimal. When comparing with Study (I), where bulky 4-phenyl-2-aminoimidazoles were the most active, is possible to say that probably bulkier lipophilic groups than pyrrole are preferred on the right side of the molecule.

To study the antiviral properties of compounds 2b(II)-18(II) against hepatitis C virus (HCV) replication, a replicon cell line in which a HCV replicon is co-expressed in a hepatocyte derived cellular carcinoma (Huh-7) cell line with firefly luciferase (marker protein) was used. Luciferase expression was measured using a Firefly Luciferase Assay Kit (Promega, USA) and

**Figure 7:** a) Dose-response curves of compound 7\( \text{II} \) against human melanoma cell line A-375, by MTT assay. Results are expressed as percentage of cytotoxicity. b) LDH leakage in A-375 human melanoma cells and BALB/c 3T3 clone A31 fibroblast cells after compound 7\( \text{II} \) exposure. Results are expressed as percentage of intracellular lactate dehydrogenase leakage. Data are the means from three independent experiments.
Cytotoxicity effect was determined using a CellTiter GLO® Luminescent Cell Viability Assay Kit (Promega, USA). The methods are explained in detail in Article (II).

Compounds 4d(II) and 7(II) showed HCV replicon inhibition of 73% and 86%, respectively. However, when measuring the viability of cells after treatment with compounds, it was shown that both had cytotoxic effects on the HCV replicon host cells; therefore, the antiviral properties were not studied further.

5.1.3. Selectivity studies

To determine the selectivity of a compound between different cell types, a selectivity index (or therapeutic index) was used in both studies:

- Study (I): eukaryotic cells vs prokaryotic cells;
- Study (II): embryonic mouse fibroblast cell line (BALB/c 3T3 clone A31) vs human malignant melanoma cell line (A-375).

In drug discovery, this parameter is useful for selecting candidate compounds for further development or in the characterization of lead compounds.

The most active synthetic analogs from Study (I) in antimicrobial studies showed in general nonselectivity towards prokaryotic cells: the majority of SI values were under 5. However, 5-chloro-indole derivative 5i(I) and 4-phenyl-2-aminoimidazole derivative 7(I) displayed selectivity towards prokaryotic cells (i.e. S. aureus), with SI values higher than 8, close to minimum threshold to be selected for further testing [160]. With further structural optimisation of the analogs that showed higher antimicrobial selectivity against Gram-positive bacteria, these compounds could be useful for further development.

When estimating the SI of compound 7(II), results exhibited a moderate level of selectivity, around 4. In a study performed by Delgado et al. [161], two amiquinones structurally related to marine isoquinoline quinones, showed SI between 1.3 and 3.7 and were selected as promising lead compounds. According to Suffness et al. [162], the selectivity index for anticancer effects is
interesting even at values equal or greater than two. Therefore, \((9H\text{-}\text{fluoren-9-yl})\text{methyl } [(2\text{-}\text{aminobenzo[}\text{d}]\text{thiazol-6-yl})\text{methyl}]\text{carbamate} \ 7(\text{II})\) should be considered promising for further development towards new anticancer agents.

5.2. Okadaic acid as inspiration for macrocyclic synthetic compounds (III)

Following the line of Study (I) and (II), Study (III) originated from the preparation of compounds having protein-okadaic acid (OA) structure as an initial inspiration. OA binds adopting a structure of a macrocycle that interacts with the metal atom of the protein and a hydrophobic tail. Additionally, OA binds potassium ions – their presence seems to be a requirement for biological activity [163]–[165]. Aiming to find a simple way to synthesize molecules mimicking the properties of OA, crown ethers were used. Before any biological studies, computational methods showed promising results between the crown ether acyl derivatives created and the receptor protein.

Since Pedersen’s discovery of crown ethers in 1967 [166], the study of crown ethers have attracted attention thanks to their powerful noncovalent interaction properties, not only in chemistry but also in the fields of pharmacology [167], [168] and medical imaging [169], [170]. Crown ethers are macrocyclic compounds constituted by organic rings, normally with more than 12 atoms in the cycle, and having oxygen, nitrogen, sulphur or other heteroatoms. They are named as \(x\)-crown-\(y\) where \(x\) denotes the total number of atoms in the cyclic backbone and \(y\) denotes the number of oxygen atoms. The classic ones contain between 3 and 20 oxygen atoms, separated from each other by two or more carbon atoms. There are studies that show the use of crown ethers as systems to mimic biological processes based on molecular recognition, since these macrocycles behave very similarly to natural ionophores (such as gramicidin) and exhibit an exceptional versatility in selective binding to a variety of ions.
This capacity of molecular recognition and mimic behaviour enhances the application of crown ethers in the biomedical field [167].

![Figure 8: Examples of crown ethers, with increasing ring size (from left to right): 12-crown-4, 15-crown-5 and 18-crown-6 ether.](image)

The twenty-two synthesized compounds in Study (III), were macrocyclic, i.e., molecules with ring architecture of 12 or more atoms. Since macrocyclic compounds are more stable thermodynamically and kinetically, they have captured the attention of synthetic chemists for the development of new compounds. In this study, the antimicrobial and antiproliferative effects of the crown ether acyl derivatives, based on an 18-crown-6 moiety (Study (III), Figure 1), were determined.

5.2.1. Antimicrobial studies

Based on antimicrobial data, (1,4,7,10,13,16-hexaoxacyclooctadecan-2-yl)methyl 3-(pyren-1-yl)propanoate 10(III) was the most active against Gram-positive \textit{S. aureus} and fungus \textit{C. albicans}, presenting a full inhibition for both strains in the primary screening and MIC\textsubscript{50} of 7.2 μM and 39 μM, respectively (Figure 9 and Table 8). Against Gram-negative bacterium, no significant activity was shown, possibly due to the molecular composition and presence of an outer membrane. Only scarce data on antimicrobial properties of crown ether derivatives have been previously reported. However, Leevy et al. [171] showed that antibacterial activity against \textit{E. coli} is altered by the spacer length.
5.2.2. Antiproliferative studies

To determine the in vitro antiproliferative activity of the derivatives, the following human cell lines were used: human breast epithelial (HBL-100), human cervical cancer (HeLa), alveolar carcinoma (SW1573) and colon adenocarcinoma (WiDr). The determination of GI$_{50}$ (proliferation inhibition by 50%) values by using the SRB assay, showed that (1,4,7,10,13,16-hexaoxacyclooctadecan-2-yl)methyl anthracene-9-carboxylate 1p(III) displayed promising activity, with values in the range of 3.7-5.6 μM (Study (III), Table 1). However, in antimicrobial studies, this compound showed a moderate MIC$_{50}$ of 20 μM against S. aureus. Further, the most active compound against S. aureus ((1,4,7,10,13,16-hexaoxacyclooctadecan-2-yl)methyl 3-(pyren-1-yl)propanoate 1o(III)) showed only moderate GI$_{50}$ against all cancer cell lines.

Figure 9: Dose-response curves of compound 1o(III) against S. aureus (square) and C. albicans (triangle). Results were expressed as % of growth inhibition.
In a study conducted by Supek et al. [172], it was shown that the lipophilicity coefficient (logP) of 18-crown-6 ethers is considered an important molecular descriptor in determining biological potency. In Study (III), calculated logP values for four compounds showed values higher than 3 (Article (III), Table 1). However, the value was not directly correlated with the observed biological activity.

Nevertheless, the results obtained may enhance the understanding of biological effects of crown ether derivatives studied and thus provide information for synthetic optimization that can lead to novel antimicrobial and antiproliferative agents.
Table 8: Chemical structures of selected crown ether derivatives and their biological activities. For antimicrobial studies, ciprofloxacin (bacteria) and amphotericin B (C. albicans) were used as positive controls. For antiproliferative studies, standard anticancer drugs cisplatin and etoposide were used as positive controls. All results were tested in triplicate (except 1I(III), antiproliferative assay) and values are shown in μM.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Chemical structure (R=)</th>
<th>C. albicans</th>
<th>S. aureus</th>
<th>HBL-100 (breast)</th>
<th>HeLa (cervix)</th>
<th>SW1573 (lung)</th>
<th>WiDr (colon)</th>
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</thead>
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<td>n.d.</td>
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<td>24</td>
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<td>5.1</td>
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<td>5.0</td>
<td>3.7</td>
<td>3.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>

n.d.: not determined.
5.3. Bioprospecting focused on the Aegean Sea (IV)

While the Mediterranean Sea is considered a marine biodiversity hotspot [173], the Aegean Sea is still an untapped and mostly unstudied source of potentially bioactive species. Marine bioprospecting is, per se, a targeted and systematic search for components, bioactive compounds or genes within marine organisms. From the business perspective, the purpose of bioprospecting is to find compounds that may be included as components in products or processes. In this study, five different steps were employed, as illustrated below:

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Table 9: Number of active extracts (% inhibition > 50) in each phyla.

<table>
<thead>
<tr>
<th>Phyla</th>
<th>% extracts</th>
<th>Nr. active extracts</th>
</tr>
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<tbody>
<tr>
<td>Cyanobacteria</td>
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<td>0</td>
</tr>
<tr>
<td>Chlorophyta</td>
<td>27</td>
<td>3</td>
</tr>
</tbody>
</table>

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Figure 10: Procedures performed for the discovery of bioactive species. Cyanobacteria and microalgae species were cultivated in batch cultures.

A large-scale biological screening of 98 specimens collected from the Aegean Sea located between the mainlands of Greece and Turkey was conducted in Study (IV). Samples were evaluated for antimicrobial, anticancer, antifouling and anti-inflammatory properties altogether in 32 different assays. The crude extracts originated from organisms such as cyanobacteria, micro- and macroalgae. Cyanobacteria, micro- and macroalgae are known for their potential to produce biologically active components (described in detail in Chapter 2). The number of active extracts (threshold = 50%) per phyla is presented in Table 9.
5.3.1. Antimicrobial and antifouling studies

Extracts derived from microalgae showed inhibition of microbial growth higher than 50%: *Amphora cf capitellata* **10S014** and *Nitzschia communis* **10S152** fully inhibited fungus *Candida albicans* and bacterium *Staphylococcus aureus*, respectively, at a concentration of 100 μg/ml. Also, the first microalgae previously stated had an effect of inhibition against Gram-positive bacteria *Enterococcus faecalis* (68%) and *S. aureus* (83%).

In antifouling studies, 2 macroalgae species belonging to a same genus – *Cystoseira barbata* **10S022-1**(IV) and *Cystoseira crinita* **10S027**(IV) – showed moderate antifouling activity equal or higher than 57% against two marine bacteria, *Alcanivorax* sp. and *Pseudoalteromonas* sp.

These preliminary results summarize the importance of algal extracts, which may have potential as antimicrobial agents and should be considered to study further, to isolate and identify bioactive compounds from the extracts.

5.3.2. Antiproliferative studies

Two prostate cancer (LNCa, PC-3), a breast cancer (MCF-7) and non-tumorigenic epithelial (MCF-10A) cell lines were used in this work for evaluation of antiproliferative activity.

While extracts of macroalgae extracts *Dictyopteris membranacea* **10S039**(IV) and *Hypnea musciformis* (**10S049**(IV) and **10S053**(IV)) showed growth inhibition higher than 60% for all the cell lines at 50 μg/ml, *Cystoseira* sp. (**10S035**(IV), **10S037**(IV), **10S038**(IV)), microalgae *Nitzschia thermalis* **10S151**, and red algae *Laurencia papillosa* (**10157**(IV)) and **10S041**(IV)) only
showed significant cytotoxic effects against cancer cell lines (Study (IV), Fig. 1). It has been reported that *Nitzschia* sp. can induce leukemia cell death (apoptosis) [174] and that polysaccharides extracted from *D. membranacea* have been demonstrated to be effective antitumor agents (IC$_{50}$ of 9.83 μg/dL, HeLa cell line) [175]. Mhadhebi et al. [54] reported similar antiproliferative effects for aqueous extracts from *Cystoseira* sp., with different cancer and normal cell lines, exhibiting an IC$_{50}$ between 17.9 and 29.5 μg/mL against MCF-7.

These results suggest that the extracts that showed promising, selective results against cancer cell lines should be studied further.

### 5.3.3. Anti-inflammatory studies

Anti-inflammatory properties of the extracts were studied by using primary cell cultures and the following assays:

- MTT reduction assay: used for evaluating the effects on neuronal cells and astrocytes. Data are presented as percentage of survival/activation of treated versus untreated cells;
- MHC class-I and MHC class-II antigen expression: used for flow cytometric analysis, using a FACScan flow cytometer (Becton Dickinson, USA);
- Determination of cytokine concentration: supernatants obtained from microglial cell cultures were analyzed for TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-18 cytokines. Concentration was calculated via a standard curve of the corresponding recombinant cytokine.

Results summarized in Article (IV), Fig. 1 showed that four extracts belonging to macroalgae *Caulerpa racemosa*, *Chaetomorpha aerea*, *Cystoseira crinita* (reproductive) and *Dilophus fasciola*, exhibited the most promising anti-inflammatory activity for both astrocyte and Class-II microglial activation. It has been shown in similar studies that *C. racemosa* possess anti-inflammatory activity [176].
These results encourage the continuation of anti-inflammatory studies of these extracts, to define whether they might provide new agents for the treatment of inflammatory diseases.
6. Conclusions and Future Considerations

Marine natural products possess a tremendous chemical and pharmacological potential for drug discovery and development. Marine organisms have existed for more than 3.5 billion years. Throughout human history, they have been mainly seen as a source of food by the populations that were living near seas, rivers or lakes. However, with the advent of modern science, their significant research potential have been realized. Nowadays, marine organisms have been studied in several fields such as ecology, biology, chemistry and pharmacology.

The aim of this thesis was to investigate the biological activity, i.e. antimicrobial and antiproliferative activities of extracts and synthetic compounds derived from marine resources. Study (I) and (II) showed that alkaloids clathrodin and oroidin could be considered as important scaffolds for the design of new drug candidates. In Study (I), where pyrrole-2-aminoimidazole alkaloids clathrodin and oroidin, and synthetic analogs were prepared, it was possible to conclude that two indole-based derivatives showed promising results with MIC$_{50}$ between 7 $\mu$M and 8 $\mu$M against Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*). However, these same derivatives presented low IC$_{50}$, when tested against Huh-7 hepatocyte-derived cellular carcinoma cell line. Selectivity index, an important and widely used parameter, was also applied to evaluate the compounds as antimicrobial agents (eukaryotic vs prokaryotic cells). A 5-chloro-indole derivative and a 4-phenyl-2-aminoimidazole derivative displayed values higher than 8, showing a greater specificity for prokaryotic cells. In Study (II), where clathrodin was used as an inspirational structure for the synthesis of new benzimidazole and 2-aminobenzothiazole analogs, a derivative of 2-aminobenzothiazole was found to display the best activity with an IC$_{50}$ of 16 $\mu$M against an human melanoma cell line and IC$_{50}$ of 71 $\mu$M against a non-cancerous, embryonic mouse fibroblast cell line, showing a moderate selectivity towards cancerous cells (SI = 4).
Study (III) showed the importance of a marine natural product scaffold for the design of new molecules. In this case, okadaic acid was used as an inspiration for the synthesis of crown ether acyl derivatives. A macrocycle derivative demonstrated to be the most active with MIC$_{50}$ of 7.2 μM and 38.5 μM against bacteria *S. aureus* and fungus *C. albicans*.

Finally, Study (IV) showed the importance of marine bioprospecting in the Aegean Sea. Cyanobacteria, micro- and macroalgae specimens were collected and several bioassays were carried out. Extracts derived from a diatom, *Amphora cf capitellata*, showed antimicrobial effects at 100 μg/mL, i.e. full growth inhibition of *Candida albicans*, and inhibitions in the order of 68% and 83% of Gram-positive *E. faecalis* and *S. aureus*.

With the results of the existing studies, a lot of future work can be developed: optimizing the existing synthetic compound structures, fractionating the active extracts studied and performing new bioactivity assays and structure-activity relationship analyses.

### 6.1. Future research

The results of this thesis showed that some of the samples studied have potential, in the future, to become candidates for generating new pharmaceuticals, nutraceuticals or cosmeceuticals. As future research, a number of ideas can be considered:

- screening with more resistant strains: in the past decades, microbes, especially bacteria, have developed cross-resistance to a series of structurally and functionally unrelated drugs, making multidrug resistance one of the most alarming threats to human health. The combination of a marine-derived compound and other drugs could provide a novel insight to combat multidrug resistance;
- on-line combination of bioassays with chemical and structural characterization: although challenging, this strategy could allow for a
rapid and simultaneous screening, and identification of bioactive compounds with several biological activities (for example, the ones presented in Study (IV)) in matrices with no prior purification steps. However, it would require significant time and effort to establish an assay amenable for this type of samples;

- study of the mode of action: this task would be challenging as well, but critical for understanding the principles of the bioactive compound uptake, metabolism of a cell exposed to a compound and identifying the molecular target sites of the compound. The goal of this study would be to understand the action of compounds in its entirety.
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2007.


SOFIA MONTALVÃO
Screening of Marine Natural Products and Their Synthetic Derivatives for Antimicrobial and Antiproliferative Properties

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