# Searching for immunomodulatory compounds from Icelandic marine invertebrates

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# Leit að efnum með ónæmisbælandi áhrif úr íslenskum sjávarhryggleysingjum

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# Ágrip

Fjölbreytileg annars stigs lífefni er að finna í sjávarhryggleysingjum, en fáar rannsóknir hafa verið gerðar á náttúruefnum úr íslenskum sjávarhryggleysingjum. Hafsvæðið umhverfis Ísland er einstakt hvað varðar hitastig og jarðhitavirkni, sem hefur valdið því sjávarlífverur sem þar lifa gætu hafa þróað einstakar sameindir sem þær nota til að lifa af. Sjávarhryggleysingjar eru því tilvalin uppspretta fyrir leit að nýjum lífvirkum efnum. Markmið þessarar rannsóknar var að einangra og skilgreina ný efnasambönd með bólguhamlandi virkni úr sjávarhryggleysingjum sem safnað var við Íslandsstrendur.

Skimað var eftir bólguhamlandi virkni í sjö úrdráttum úr íslenskum sjávarhryggleysingjum í angafrumulíkani. Lífvirkir útdrættir voru þáttaðir með ýmsum skiljunaraðferðum og lífvirknileidd einangrun notuð til að einangra lífvirk efni. Fimm þekktar fjölómettaðar fitusýrur sem voru einangraðar úr óskilgreindum sjávarsvömpum og nokkrir fitusæknir þættir sem fengust úr sjávarsvampinum *Halichondria sitiens* höfðu ónæmishamlandi virkni sem fólst í því að þau minnkuðu seytun angafrumna á bólguhvetjandi boðefninu IL-12p40 og bólguhamlandi boðefninu IL-10. Samræktun angafrumna, sem höfðu verið ræstar í návist valinna þátta, með ósamgena $CD4^+$ T frumum leiddi til minni seytunar T frumna á IFN-$\gamma$, sem bendir til minni Th1 sérhæfingar frumnanna. Auk þess var nýr glýseról ester með fitusýrum skipuðum metýl hóp einangraður en hann hafði ekki bólguhamlandi virkni.

Úrdrættir úr sjávarsvampinum *Geodia barretti* og mosadýrinu *Flustra foliacea* voru greindir með UPLC-qTOF-MS og fundust bæði þekkt og ný efni sem voru skilgreind með því að bera saman sameinda gröf og massaróf við gögn úr vísindagreinum og gagnabönkum. Nítján nýir og sextán þekktir alkalóíðar voru einangraðir með ýmsum vökvaskiljunaraðferðum. Efnabyggingar nýju alkalóíðanna voru ákvarðaðar með kjarnsegulgreiningu, þar á meðal $^1$H NMR, $^{13}$C NMR, $^1$H-$^1$H COSY, HSQC, HMBC, NOESY, og massagreiningu (UPLC-qTOF-MS og HRESIMS). Rúmfræðileg greining (e. stereochemistry) var ákvörðuð með kjarnsegulgreiningu, snúningi ljóss, hringsnúningi ljóss (e. circular dicroism) greiningu og notkun á Marfey aðferð þar sem notast var við nýtt hvarfefni, L-$N^{\alpha}$-(1-fluoro-2,4-dinitrophenyl)tryptophanamide (L-FDTA). Nákvæm rúmfræðileg greining (e. absolute stereochemistry) sumra áður þekktra efnanna var auk þess

endurákvörðuð. Skimað var eftir bólguhamlandi virkni í öllum einangruðu efnunum. Tólf efni drógu úr seytun angafrumna á IL-12p40 og tvö efni juku seytun þeirra á IL-10. Þrjár sameindir voru valdar til frekari rannsókna, m.a. á skammtaháðri virkni þeirra og getu angafrumna meðhöndlaðra með þeim til að sérhæfa samræktaðar T frumur Th1 frumur eða T bælifrumur. Niðurstöðurnar sýna að *G. barretti* og *F. foliacea* innihalda margskonar alkalóíða, sem sumir hverjir hafa bólguhamlandi virkni sem hægt væri að þróa til að meðhöndla bólgusjúkdóma.

Niðurstöður verkefnisins sýna að sjávarhryggleysingar úr hafinu í kringum Ísland innihalda fjölda annarra stigs lífefna sem hafa bólguhamlandi virkni. Þær sýna einnig að hafsvæðið umhverfis Ísland getur verið góð uppspretta fyrir lífvirk efni sem hægt væri að þróa sem lyfjasprota til að meðhöndla bólgusjúkdóma.

**Lykilorð:**

Náttúrefni úr sjó, sjávarhryggleysingjar, ónæmishamlandi, bólgueyðandi, Ísland, alkalóíðar, fituefni

# Abstract

A substantial diversity of secondary metabolites from marine invertebrates has been described; however, only a few studies have investigated secondary metabolites of marine invertebrates collected in Icelandic waters. The unique marine environment around Iceland, in terms of temperature and underwater geothermal activity, has forced the living marine organisms to survive in the surroundings by developing unique biomolecules. The marine invertebrates are therefore an excellent starting point for searching for new bioactive compounds. The aim of this thesis was to isolate and identify new compounds, and known, with immunomodulatory activity from marine invertebrates collected in Icelandic waters.

Seven crude extracts from Icelandic marine sponges were screened for immunomodulatory activities using an *in vitro* dendritic cell (DC) model. The bioactive extracts were then fractionated by column chromatographic technique with the help of bioassay-guided isolation approach to isolate the active constituents. Five pure known polyunsaturated fatty acids (PUFAs) from unidentified marine sponges and several lipophilic fractions from *Halichondria sitiens* were obtained and they showed immunomodulatory activity, demonstrated by their ability to reduce DC secretion of the pro-inflammatory cytokine IL-12p40 and the anti-inflammatory cytokine IL-10. Maturing DCs in the presence of selected fractions before co-culturing them with allogeneic $CD4^+$ T cells mainly led to a decrease in T cell secretion of IFN-$\gamma$, indicating a reduction in Th1 immune response. In addition, one new glycerol ester featuring methyl branched fatty acid chain was obtained but it did not have immunomodulatory activity using the DC model.

Crude extracts from the marine sponge *Geodia barretti* and the bryozoan *Flustra foliacea* were chemically profiled by UPLC-qTOF-MS. Both previously known and unknown compounds were characterized by comparing the obtained molecular (precursor) ions and fragmentation patterns from obtained MS/MS data with data from the literature and databases. The crude extracts were subjected to various chromatographic techniques that yielded nineteen new alkaloids and sixteen known alkaloids. The structures of the isolated new compounds were elucidated by detailed spectroscopic analysis, including $^{1}$H NMR, $^{13}$C NMR, $^{1}$H-$^{1}$H COSY, HSQC, HMBC, NOESY, $^{1}$H-$^{15}$N HSQC, $^{1}$H-$^{15}$N HMBC, and HRESIMS. The stereochemistry was assigned by combination of

NMR spectroscopic data, optical rotation, ECD analysis and application of a Marfey's-type method employing the new reagent L-$N^{\alpha}$-(1-fluoro-2,4-dinitrophenyl)tryptophanamide (L-FDTA). The absolute stereochemistry of one known compound was re-evaluated. All the isolated compounds were screened for immunomodulatory activity using the DC model. Twelve of the compounds decreased DC secretion of IL-12p40 and two of them increased secretion of IL-10. Three of the compounds were chosen for further investigation of dose-dependency of their activity and subsequently their ability to induce a Th1 or a Treg immune response in T cells co-cultured with DCs previously incubated with the compounds. Overall, *G. barretti* and *F. foliacea* contain a variety of alkaloids, several of which have anti-inflammatory activity, some of them warranting further investigation into their potential as a candidate for relieving inflammatory diseases.

The results demonstrate that marine invertebrates collected from the marine environment around Iceland contain a number of secondary metabolites some of which have immunomodulatory effects. These studies indicate that the marine environment around Iceland may be a plentiful source of biological compounds with the potential of becoming a drug lead for treatment of inflammatory related diseases.

**Keywords:**

Marine natural products, marine invertebrates, immunomodulatory, anti-inflammatory, Iceland, alkaloids, lipids

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# Contents

**Ágrip** ..... **i**
**Abstract** ..... **iii**
**Acknowledgements** ..... **v**
**Contents** ..... **vii**
**List of abbreviations** ..... **ix**
**List of figures** ..... **xi**
**List of tables** ..... **xiii**
**List of original papers** ..... **xiv**
**Declaration of contribution** ..... **xv**
**1 Introduction** ..... **1**
1.1 Marine natural products and marine-derived drugs ..... 2
1.1.1 Marine natural products ..... 2
1.1.2 Marine-derived drugs ..... 3
1.2 Icelandic marine invertebrates ..... 7
1.2.1 Marine invertebrate ..... 7
1.2.2 Icelandic marine environment ..... 9
1.2.3 The sponge *Halichondria sitiens* ..... 10
1.2.4 The sponge *Geodia barretti* ..... 11
1.2.5 The bryozoan *Flustra foliacea* ..... 13
1.3 Immunomodulation ..... 18
1.4 Immunomodulatory/anti-inflammatory activity of compounds from marine invertebrates ..... 21
1.4.1 Evaluation of anti-inflammatory activity ..... 21
1.4.2 Anti-inflammatory agents in clinical trials ..... 21
1.4.3 Anti-inflammatory compounds ..... 22
1.5 Isolation methods ..... 27
1.5.1 Bioassay-guided isolation ..... 27
1.5.2 UPLC-qTOF-MS-guided isolation ..... 28
**2 Aims** ..... **31**
**3 Materials and methods** ..... **33**
3.1 Sample collection and identification ..... 33
3.2 Extraction of marine invertebrates ..... 33
3.3 UPLC-qTOF-MS analysis ..... 35
3.4 Separation and purification of pure compounds ..... 35

3.5 Structure elucidation ..... 36
3.5.1 NMR spectroscopy ..... 36
3.5.2 High resolution mass spectrometry ..... 36
3.5.3 Electronic circular dichroism (ECD) ..... 36
3.5.4 Marfey´s method ..... 37
3.5.5 Synthetic methods ..... 37
3.6 Immunomodulatory activity assay ..... 37
3.6.1 Screening of activity of marine invertebrates ..... 37
3.6.2 Maturation and activation of DCs ..... 38
3.6.3 Co-culture of DCs and allogeneic $CD4^+$ T cells ..... 39
3.6.4 Determination of cytokine concentration by ELISA ..... 39
3.6.5 Statistical analysis ..... 39

**4 Results ..... 41**
4.1 Bioassay-guided isolation ..... 41
4.2 *Halichondria sitiens* ..... 45
4.3 *Geodia barretti* ..... 48
4.4 *Flustra foliacea* ..... 52

**5 Discussion ..... 55**
5.1 Bioassay-guided isolation ..... 55
5.2 *Halichondria sitiens* ..... 56
5.3 *Geodia barretti* ..... 58
5.4 *Flustra foliacea* ..... 61

**6 Conclusions and future aspects ..... 65**
**References ..... 67**
**Paper I ..... 89**
**Paper II ..... 91**
**Paper III ..... 93**

# List of abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| AChE | Acetyl cholinesterase |
| nAChR | Nicotinic acetylcholine receptor |
| ACN | Acetonitrile |
| AHL | *N*-acyl-homoserine lactone |
| APC | Antigen presenting cell |
| BChE | Butyrylcholinesterase |
| BuOH | n-Butanol |
| CASE | Computer-assisted structure elucidation |
| CD86 | Cluster of differentiation 86 |
| $CHCl_3$ | Chloroform |
| CLR | C-type lectin receptor |
| COSY | Correlation spectroscopy |
| COX-2 | Cyclooxygenase-2 |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| DCM | Dichloromethane |
| DHA | Docosahexaenoic acid |
| DMSO | Dimethyl sulfoxide |
| DKP | Diketopiperazine |
| ECD | Electronic circular dichroism |
| EtOAc | Ethyl acetate |
| ESI | Electrospray ionization |
| EPA | Eicosapentaenoic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FDA | Food and Drug Administration |
| FDAA | *N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide |
| L -FDTA | L-$N^{\alpha}$-(1-fluoro-2,4-dinitrophenyl)tryptophanamide |
| GM-SCF | Granulocyte-macrophage colony-stimulating factor |
| HLA-DR | Human leukocyte antigen – DR isotype |
| HMBC | Heteronuclear multiple bond correlation |
| HPA | Heneicosapentaenoic acid |
| HPLC | High-performance liquid chromatography |
| HR-ESI-MS | High-resolution electrospray ionization mass spectrometry |
| HSQC | Heteronuclear single quantum correlation |

| | |
|---|---|
| $IC_{50}$ | Half maximal inhibitory concentration |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxygen synthase |
| IR | Infrared spectroscopy |
| LPS | Lipopolysaccharide |
| MACS | Magnetic-activated cell sorting |
| MeOH | Methanol |
| MNP | Marine natural product |
| NF-κB | Nuclear factor kappa light chain enhancer of activated B cells |
| NLR | NOD-like receptor |
| NME | New molecular entity |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| NOESY | Nuclear Overhauser effect spectroscopy |
| NP | Natural product |
| NRPS | Non-ribosomal peptide synthetase |
| PAMP | Pathogen-associated molecular pattern |
| PGs | Prostaglandins |
| PHVD | Prevention of heart and vascular disease |
| PLA2 | Phospholipase A2 |
| PN/NT | Protection of neurons/neurotoxicity |
| PRR | Pattern-recognition receptor |
| PUFA | Polyunsaturated fatty acid |
| RP | Reversed-phase |
| RPMI | Roswell Park Memorial Institute |
| SAR | Structure–activity relationship |
| SDA | Stearidonic acid |
| SEM | Standard error of the mean |
| SPE | Solid phase extraction |
| TLC | Thin-layer chromatography |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor alpha |
| UPLC-qTOF -MS/MS | Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry |
| UV | Ultraviolet |

# List of figures

**Figure 1.** The fraction of annually approved natural product new molecular entities (NMEs) of all new FDA-approved drugs by source/10 years. ..... 1

**Figure 2.** Timeline continuum for the development of marine-derived drugs. 3

**Figure 3.** Pie chart illustrating the original collected sources of marine natural product derived or inspired agents currently as approved drugs or in clinical trials. ..... 6

**Figure 4.** Number and proportion of bioactive compounds from marine organisms. ..... 8

**Figure 5.** A map showing the surface circulation of the ocean around Iceland. ..... 10

**Figure 6.** Photos of *Halichondria sitiens*, *Geodia barretti*, and *Flustra foliacea*. ..... 11

**Figure 7.** Chemical structure of compounds identified from *G. barretti*. ..... 12

**Figure 8.** Chemical structures of terpenoids identified in *F. foliacea*. ..... 15

**Figure 9.** Chemical structure of alkaloids isolated from *F. foliacea*. ..... 16

**Figure 10.** Dendritic cells and induction of Th cell subtypes in the immune system. ..... 19

**Figure 11.** A dereplication workflow for combinatorial approach procedures for isolation of new bioactive NP compounds. ..... 29

**Figure 12.** Flow chart of extraction and modified Kupchan partitioning method for sponges (A) and bryozoan (B). ..... 34

**Figure 13.** Schematic overview of screening fractions from marine sponges using a DC model. ..... 38

**Figure 14.** The effects of the fractions from marine sponges on DC secretion of IL-12p40 and IL-10. ..... 41

**Figure 15.** Extraction and fractionation flowchart of the five marine different species named SP1-SP5. ..... 42

**Figure 16.** The effects of the fractions and pure compounds from SP1-5 and -7 on DC secretion of IL-12p40, IL-10 and IL-6. ..... 43

**Figure 17.** Chemical structure of the isolated compounds from the sponge *H. sitiens*. ..... 44

**Figure 18.** Schematic overview of the fractionation process of *H. sitiens*. ... 45

**Figure 19.** The effects of bioactive *H. sitiens* fractions on cytokine secretion by DCs. ..... 46

**Figure 20.** Key HMBC (H→C) and $^{1}$H-$^{1}$H COSY (**—**) correlations of geobarrettin D (**9**). ..... 50

**Figure 21.** The effects of geobarrettin D (**9**) on DC secretion of IL-12p40 and IL-10. ..... 52

# List of tables

**Table 1.** A list of FDA-approved marine pharmaceuticals. ..... 5

**Table 2.** Selected compounds isolated from the sponge *G. barretti*..... 13

**Table 3.** Compounds from the marine bryozoan *F. foliacea*..... 14

**Table 4.** Marine invertebrate-derived anti-inflammatory agents in clinical and preclinical trials. ..... 23

**Table 5.** Selected compounds with anti-inflammatory activities from marine invertebrates. ..... 24

**Table 6.** NMR spectroscopic data for geobarrettin D (**9**) ..... 51

# List of original papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. Xiaxia Di, Jon T. Oskarsson, Sesselja Omarsdottir, Jona Freysdottir, Ingibjorg Hardardottir. Lipophilic fractions from the marine sponge *Halichondria sitiens* decrease secretion of pro-inflammatory cytokines by dendritic cells and decrease their ability to induce a Th1 type response by allogeneic $CD4^+$ T cells. Pharmaceutical Biology 2017; 55: 2116-2122.

II. Xiaxia Di, Caroline Rouger, Ingibjorg Hardardottir, Jona Freysdottir, Tadeusz F. Molinski, Deniz Tasdemir, Sesselja Omarsdottir. 6-Bromoindole derivatives from the marine sponge *Geodia barretti*: Isolation and anti-inflammatory activity. Marine Drugs 2018; 16(11): 437.

III. Xiaxia Di, Shuqi. Wang, Jon T. Oskarsson, Caroline Rouger, Deniz Tasdemir, Ingibjorg Hardardottir, Jona Freysdottir, Tadeusz F. Molinski, Sesselja Omarsdottir. Isolation, structure elucidation and anti-inflammatory activity of new alkaloids from Bryozoan *Flustra foliacea*. Manuscript in preparation.

# Declaration of contribution

With the supervision of Prof. Sesselja Omarsdottir, Jona Freysdottir, and Ingibjorg Hardardottir, I participated in designing the hypothesis of this project and interpretion of the data. I performed most the laboratory work and analysed most of the data. The contributions of the respective authors are listed as follows:

Paper I: In this paper, I performed the extraction, bioassay-guided isolation, and structure elucidation of fractions and compounds. Jon Thorir Oskarsson contributed to screening of the crude extracts and analysis of the immunomodulatory effects of the fractions and compounds. Some of the results were presented in his MSc thesis in Medical Life Sciences at the Faculty of Medicine, University of Iceland in 2017. Figures 18-19 are also presented in his thesis. I wrote the draft, took part in editing it and wrote the final version. Jon Thorir Oskarsson, Sesselja Omarsdottir, Jona Freysdottir, and Ingibjorg Hardardottir all contributed to the writing of the manuscript.

Paper II: I performed the isolation and structure elucidation of the new compounds and conducted data analysis. I carried out the immunomodulatory assay experiments with the assistance of Nathalie Krieglstein and analyzed and interpreted the data with the supervision of Jona Freysdottir and Ingibjorg Hardardottir. Finnur Freyr Eiriksson and Margret Thorsteinsdottir ran UPLC-qTOF-MS experiments. Deniz Tasdemir and Caroline Rouger ran NMR experiments for the new compounds and assisted in interpretation of the chemical data. Tadeusz F. Molinski performed the stereochemistry elucidation experiments for the new compound. I wrote the manuscript and all the other authors contributed to revising the paper.

Paper III: I performed the data analysis, isolation and structure elucidation of the new compounds. Jon Thorir Oskarsson and I conducted the immunomodulatory experiments and analyzed the data. Shuqi Wang, Deniz Tasdemir and Caroline Rouger ran NMR experiments and assisted in interpretation of the chemical data. Eydis Einarsdottir and Margret Thorsteinsdottir ran UPLC-qTOF-MS experiments. Tadeusz F. Molinski assisted in interpretation of the chemical data. I wrote the manuscript and all the authors contributed to revising the paper.

# 1 Introduction

Over the last decades, many naturally occurring compounds have drawn the attention of chemists and pharmacists due to their promising applicability as potential therapeutic agents for a variety of diseases. More than one third of drugs in use now are of nature origin (Figure 1), including antibiotics, anticancer, and immunosuppressive drugs [1, 2].

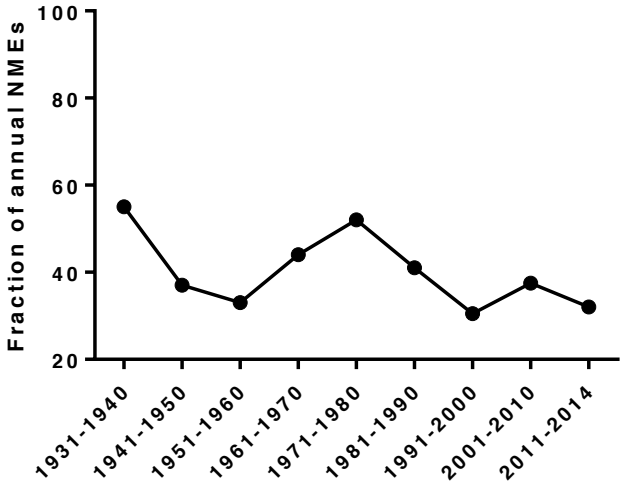



**Figure 1.** The fraction of annually approved natural product new molecular entities (NMEs) of all new FDA-approved drugs by source/10 years.
Based on [2].

Natural products (NPs) play a remarkable role in the drug development and have inspired drug discovery and therapy as observed when studying the history of medicine. Alexander Fleming, Ernst B. Chain, and Howard Florey received the Nobel Prize in Physiology or Medicine for the discovery of penicillin in 1945 and Selman A. Waksman in 1952 for the discovery of streptomycin. These great achievements mark the start of the Golden Age of NP drug discovery [3]. However, in the past 20 years, many pharmaceutical companies and researchers have transferred their interests from the NP discovery projects into other drug discovery, in part because of the advances in both high throughput screening and combinatorial synthesis and high drop of natural based-drug in clinical trials [4].

However, in 2015, the Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshi Omura for discovery of the microbial natural

product avermectins, and to Youyou Tu for discovery of the plant-derived anti-parasite natural product artemisinin. This award has surely encouraged the natural product community and inspired chemists and pharmacists to continue the search for natural product drugs. With the current advances in “smart screening” methods, robotic separation, high resolution techniques for structural analysis, fundamental understanding of natural product biosynthesis, metabolic engineering, the age of natural products drug discovery seems to be back [3, 4].

## 1.1 Marine natural products and marine-derived drugs

### 1.1.1 Marine natural products

Although a high number of new bioactive NPs from terrestrial plants, fungi, or microorganisms have been labeled as good candidates for potential new drug leads, only a few of those have successfully reached the end of the drug discovery road [5]. Moreover, many forms of cancer, microbiota infections, inflammatory diseases, and neurodegenerative diseases are still difficult to treat successfully. Therefore, it is not surprising that NPs from marine organisms are attracting increasing interests, due to the relatively unexplored biodiversity in the ocean compared to terrestrial environments, in order to find structurally diverse and biologically active compounds [6].

A statistical analysis by Kong in 2010 showed that marine natural products (MNPs) have greater chemical novelties and structurally unique bioactivities compared to their terrestrial counterparts [7], and many of them have advanced to be used in biological assays and pharmaceuticals. The marine organisms can survive in quite special environment, with stringent light availability, low oxygen levels, harsh water movement, high or low pH levels, density, high salinity and pressure, and fighting with competitors for the limited resources. Those extreme environmental conditions often affect the growth and survival of marine organisms, as well as productivity of their unique chemical structures of primary and secondary metabolites. A varied assembly of halogenated compounds, for example, can be found in marine organisms, including indoles, peptides, terpenoids, and hydrocarbons. However, the utilization of halogens in terrestrial secondary metabolism is, by comparison, a rare process observed in only a few microorganisms [8, 9].

A timeline for the development of MNPs chemistry is shown in Figure 2. MNPs chemistry started in 1951 when Bergmann and Feeny reported the isolation of the unusual nucleosides spongouridin and spongothymidin from the sponge *Cryptotethya crypta* [10-12], which served as lead structures for the

marketed anticancer drug Ara-C and antiviral drug Ara-A. In 1967, a small symposium was held in Rhode Island, USA, with the aspiring title “Drugs from the Sea”. It was the first academic meeting about searching for drugs from MNPs. However, the utilization of marine organisms as potential structures for drugs started seriously during 1960 to 1980 [13-15]. With improvement of techniques for separation and for structure elucidation of the molecules, as well as for chemical synthesis, there has been an increase in the number of bioactive NPs characterized from marine organisms since 1980. The marine natural product chemists and pharmacists focused on investigation of anti-inflammatory, anticancer, antiviral, and immunomodulatory drugs, and ion channel effectors. Since 1990, advances in technologies has facilitated the discovery of marine-derived drugs, including sampling strategies, nanoscale NMR for structure determination, total chemical synthesis, biosynthesis and genetic engineering methods [6, 16-18]. To date, more than 28,000 compounds [19, 20] from marine organisms have been reported and published in approximately 32,000 publications on the subject of MNPs, which cover structures, bioassay studies, ecological studies, syntheses, reviews, etc. Since 2008, more than 1000 new compounds have been discovered each year; thus, the expectation is that many more drugs will be discovered from MNPs in the near future [21].

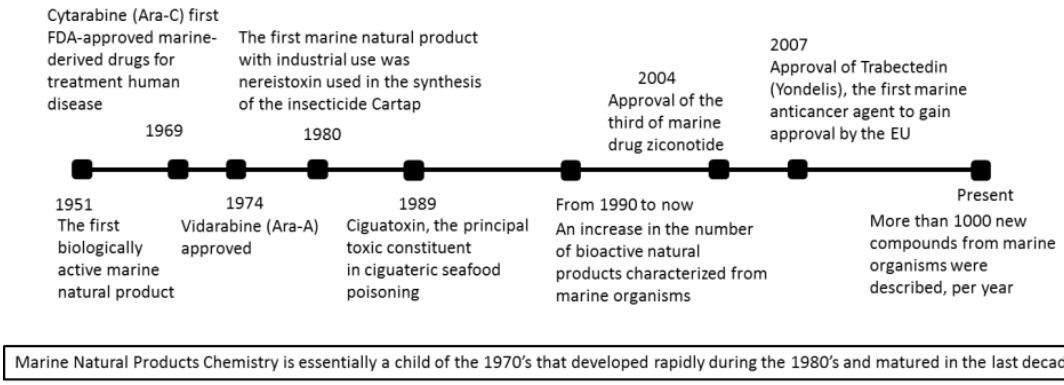



**Figure 2.** Timeline continuum for the development of marine-derived drugs.
Based on data from [12, 22, 23].

### 1.1.2 Marine-derived drugs

Until now, thirty marine compounds in total have now entered the clinical trials pipeline for drug development, of which seven have been approved by the Food and Drug Administration (FDA-approved) (Table 1), and the remaining 23 are in different phases of clinical trials [24]. In addition, a large number of marine chemicals are in the pre-clinical pipeline.

Of the seven marketed marine drugs (four anticancer, one antiviral, one pain control, and one for hypertriglyceridemia), two are the actual chemical structure as isolated from nature, and five others are synthetic or semi-synthetic agents inspired by natural compound. Table 1 details the origin of these products, and reveals that sponges are the main source of most of these marketed drugs.

MNPs have the stronghold in anticancer drug discovery, as indicated by the four approved anticancer drugs and nine potentials in clinical development. An early finding in the marine drug discovery field was cytarabine or Ara-C (Cytosar-U®), a synthetic analogue of a C-nucleoside from the Caribbean sponge, *Cryptothethya crypta*, approved in 1969 and still in use today to treat leukemia [25]. In 2007, the anticancer agent approved by EU, trabectedin (Yondelis®), from the tunicate, *Ecteinascidia turbinata*, was approved for the treatment of soft tissue sarcomas and ovarian cancer [26]. Commercialized by Spanish company Pharmamar as Yondelis® was severely hindered by low production of trabecetidin by the tunicate, however, the problem was solved by semi-synthesis from the microbial NP, cyanosafracin B, a fermentation product of the bacterium *Pseudomonas fluorescens*. In 2010, the third marine anticancer drug was approved, Eribulin (Halaven®), the most complex synthetic drug ever made, for the treatment of breast cancer [27]. It is a fully synthetic macrocyclic ketone analogue of the MNP halichondrin B, a potent naturally occurring mitotic inhibitor with a unique mechanism of action found in the sponge *Halichondria okadai* [28]. A recently approved anticancer marine-derived compound is brentuximab vedotin (Adcetris®), used for the treatment of Hodgkin's disease and malignant lymphoma [29].

MNPs have also been used as antiviral drugs. Vidarabine or Ara-A (Vira-A), a synthetic analogue of spongouridine with improved antiviral activity, was originally isolated in 1951 from the sponge *Cryptothethya crypta* [25], but recently it was found to be produced by the bacterium *Streptomyces antibioticus*. Ziconotide (Prialt®) is a synthetic calcium-channel binding conotoxin from the cone snail, *Conus magus*, which has been shown to be effective in patients suffering with severe chronic pain who either do not respond to or cannot tolerate other drugs [30]. Omega-3-acid ethyl ester (Lovaza®) approved in 2004 is used to reduce severe elevations of triglycerides associated with conditions such as obesity, insulin resistance, diabetes mellitus and other conditions that contribute to the risk of atherosclerosis or hardening of the arteries responsible for coronary artery disease [31].

**Table 1.** A list of FDA-approved marine pharmaceuticals.
Derived from [24].

| **Compound name** | **Trademark, Approved year** | **Organism** | **Chemical class** | **Molecular Target** | **Disease area** |
|---|---|---|---|---|---|
| Cytarabine, (Ara-C) | Cytosar-U®, 1969 | Sponge | Nucleoside | DNA polymerase | Cancer: Leukemia |
| Vidarabine, (Ara-A) | Vira-A®, 1976 | Sponge | Nucleoside | Viral DNA polymerase | Antiviral: Herpes Simplex Virus |
| Omega-3-acid ethyl esters | Lovaza®, 2004 | Fish | Omega-3 fatty acids | Triglyceride-synthesizing enzymes | Hypertriglyceridemia |
| Ziconotide | Prialt®, 2004 | Cone snail | Peptide | N-Type Ca channel | Pain: Severe Chronic Pain |
| Eribulin Mesylate. (E7389) | Halaven®, 2010 | Sponge | Macrolide | Microtubules | Cancer: Metastatic Breast Cancer |
| Brentuximab vedotin (SGN-35) | Adcetris®, 2011 | Mollusk/ cyanobacterium | Peptide | CD30 & microtubules | Cancer: Anaplastic large T-cell systemic malignant lymphoma, Hodgkin's disease |
| Trabectedin (ET-743) | Yondelis®, 2007 | Tunicate | Alkaloid | Minor groove of DNA | Cancer: Soft Tissue Sarcoma and Ovarian Cancer |

As mentioned above, 23 original marine compounds are in phase I to phase III clinical trials. Thereof, 19 are for different type of cancer treatment, one for chronic pain, one for Alzheimer's (along with cancer), and one for neurovascular diseases [24]. Figure 3 summarizes the sources of organisms from which compounds that have yielded to become FDA-approved drugs or are in clinical trials, and reveals that marine invertebrates (mollusks, sponges, and tunicates) are the largest collected source of these substances. However, further studies have revealed that 80% of those substances are originated from bacteria and cyanobacteria and not the invertebrates themselves [32] [33].

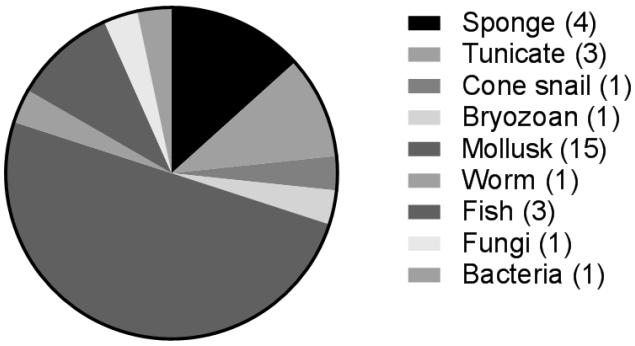



**Figure 3.** Pie chart illustrating the original collected sources of marine natural product derived or inspired agents currently as approved drugs or in clinical trials.

Gerwick & Moore [32] revealed that the ratio of seven clinically useful and approved drugs from 28,175 discovered molecular entities, is approximately 1.2- to 2.5-fold better than the industry average (1 in 5,000 to 10,000 tested compounds). Thus, the marine derived compounds are promising, and more and more marine-derived drugs are expected to reach the market soon [34].

In addition to pharmaceutical applications, many of MNPs may be of great interest as cosmeceuticals and nutraceuticals because of the beneficial effects on human health. These MNPs often have drug-like properties and contain active ingredients, such as vitamins, enzymes, antioxidants and essential oils which can be used as natural additives in foods, as nutritional supplements. They can also be used as industrial and biochemical tools. The first industrial use of the MNP nereistoxin isolated from the polychaeta worm *Lumbrinereis* sp. was for the synthesis of the insecticide Cartap (Padan®) in controlling insect plagues in orange plantation and other cultures such as rice and sugarcane fields. Palytoxin, originally isolated from the tropical soft coral *Palythoa sp.* collected in Hawaii, is toxic to all mammal cells, but could be a powerful tool to study cellular ion transport mechanisms [35].

## 1.2 Icelandic marine invertebrates

### 1.2.1 Marine invertebrate

A significant portion of the Earth´s biodiversity is comprised of marine species, which are approximately 25% of the total number of species on Earth [36]. The oceans cover 71% of the earth's surface, yet 95% of the marine species are considered to remain unknown [36]. Marine invertebrates account for over 89% of all extant marine animals, comprising of over 174,600 species [37]. Most of them make up a subphylum of one of over 30 known animal phyla, including Porifera (sponges), Echinodemata (sea stars, urchins, sand dollars, and sea cucumbers), Cnidarian (jellyfish, sea anemones, and corals), Chordata (tunicates and lancelets), Mollusca (shellfish, squid, nudibranchs, and sea snails), Arthropoda (insects), and Bryozoa (moss animals).

Analysis of the database of marine compounds from 1985 to 2012 by Hu [21] revealed that marine invertebrates produced most of the new bioactive compounds (Figure 4). However, it now becomes clear that the real producers of many of the bioactive natural products are not the organisms themselves, but symbiotic microorganisms [33]. Around 57% of the total bioactive compounds were from Porifera (mostly sponges) and Cnidaria (mostly corals), even though the proportion of bioactive compounds is not high. A substantial diversity of secondary metabolites was found from marine invertebrates, including alkaloids, steroids, terpenoids, aliphatic hydrocarbons, amino acids and peptides. These marine-derived NPs showed anticancer, antimicrobial, antifouling, antioxidant, anti-inflammatory, protection of neurotoxicity, wound healing, immunomodulating, antihypertensive, and other biological properties (Figure 4) according to the data reviewed by Blunt et al. from Natural Product Reports of 2010 to 2017 [20, 38-44]. As mentioned above, marine invertebrates (mainly sponges, tunicates, bryozoans and molluscs) (Figure 3) provided the majority of the MNPs involved in clinical or preclinical trials.

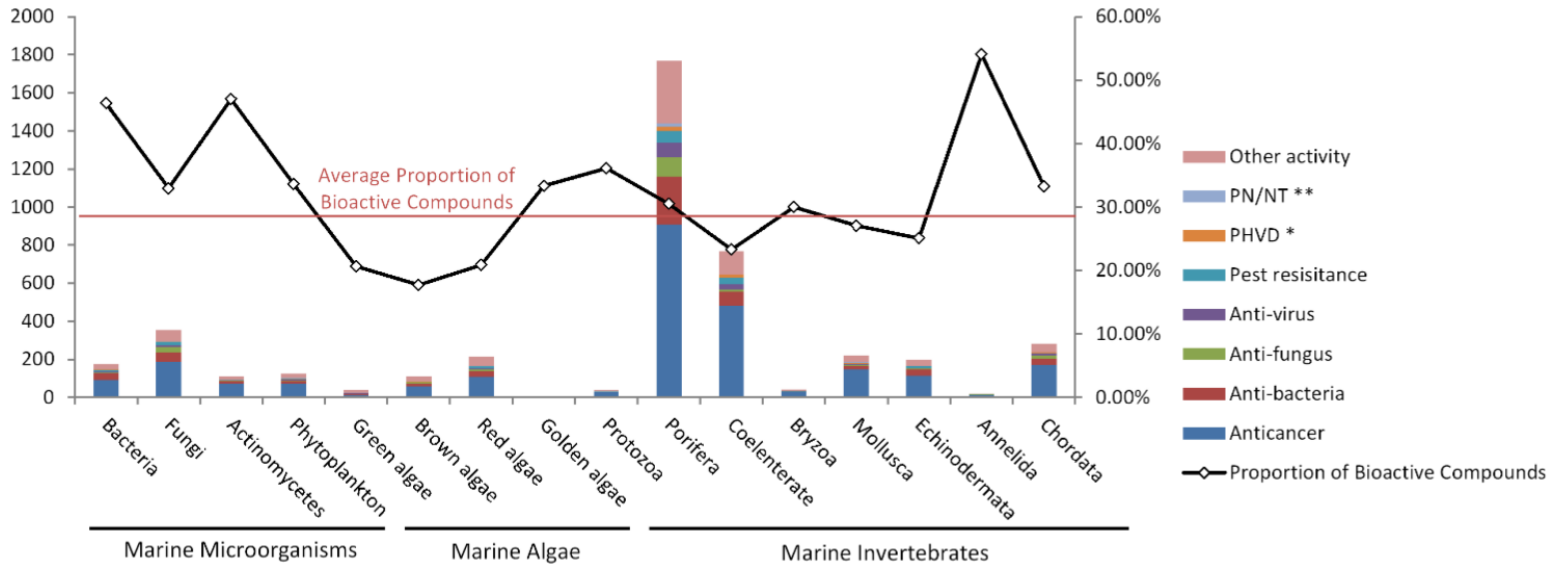



**Figure 4.** Number and proportion of bioactive compounds from marine organisms.

** PN/NT: Protection of neurons/neurotoxicity, * PHVD: Prevention of heart and vascular disease [21].

### 1.2.2 Icelandic marine environment

Iceland has a unique geology and geographical position in the midst of the North Atlantic Ocean. The physical oceanographic characters in the Southern and Western parts of the Icelandic marine ecosystem differ from those in the Northern and Eastern areas (Figure 5). In Figure 5, different kind of arrows represent different currents where black arrows around Iceland indicate the circulation of warm and saline Atlantic water; thin black arrows represent the Coastal Current; dark arrows around Greenland indicate cold low salinity polar water of the East Greenland Current; and light grey arrows represent Arctic water of the Iceland Sea including the East Icelandic Current. The Southern and Western areas are more or less continuously bathed by warm and saline Atlantic water while the Northern and Eastern areas are more variable and influenced by Atlantic, Arctic and even Polar water masses, and subject to larger inter-annual variation [45, 46]. The relative strengths and locations of these ocean currents and hence of the associated water masses and warm/cold water fronts, and ocean temperatures and salinities have exhibited large variations, which in turn, may have affected the distribution of many marine organisms. There are two projects, BIOICE and IceAGE, that have been studying the marine invertebrates from Icelandic waters, both of which help to understand the diversity of marine invertebrates. In the BIOICE project, almost 2,000 species of benthic invertebrate have been collected and identified. Of them, 41 new species have now been described and over 1,000 species were unknown previously in Icelandic waters [47].

Several submarine hydrothermal vents have been found in Icelandic waters, locating on the Reykjanes Ridge (250–350 m), near the island of Kolbeinsey (Jan Mayen Ridge) (100 m), and east of the island Grimsey (400 m) [47]. Promising organisms producing structurally unusual compounds may exist in extreme environments, such as at great sea depths, in thermal vents, or in salt lakes. For example, the alkaliphile *Bacillus halodurans* C-125, which produces the peptide haloduracin, could grow at an extreme pH of >9.0 [48]. The development of potential therapeutics of other peptide, lantibiotic, is blocked by its instability at neutral pH or above, whereas haloduracin can survive at pH ranges well above that of human serum, providing a basis for development of new lantibiotics with drug potential [49].

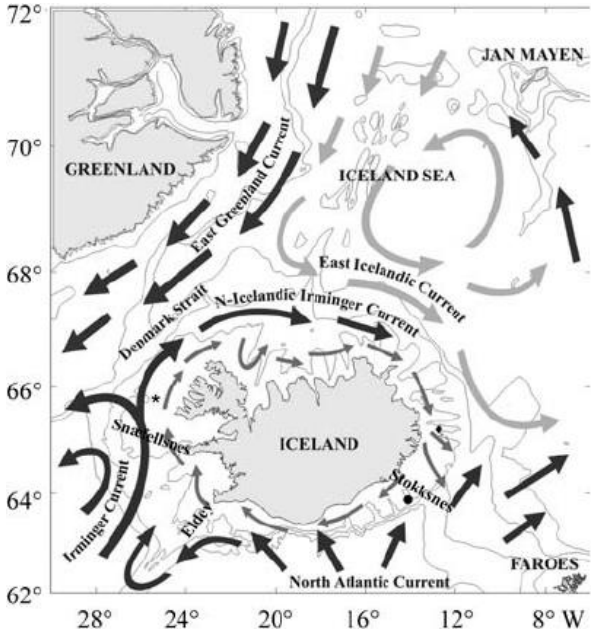



**Figure 5.** A map showing the surface circulation of the ocean around Iceland. Derived from [46].

The distinctive geology and variation of oceanographic character of Icelandic waters associated with geothermal active sites result in the formation of unusual marine environment surrounding Iceland, which could be expected to produce diverse chemical metabolites by marine organisms living there in order to survive. Collectively, the secondary metabolites of the marine organisms from Icelandic waters are worth to study by chemists, pharmacists, and biologists.

### 1.2.3 The sponge *Halichondria sitiens*

*Halichondria (Eumastia) sitiens* (Schmidt, 1870) (Figure 6), belong to genus *Halichondria*, phylum Porifera, is a Northern deep water species and distributed in the ocean around Norway, Northern Ireland, Iceland, Greenland, and Canada.

This genus *Halichondria* became important through the discovery of halichondrin B as mentioned in Chapter 1.1.1, a potent naturally occurring large polyether macrolide with anticancer activity with a unique mechanism of action. In 1998, Dr. Yoshito Kishi developed a completely synthetic method for producing halichondrin B and discovered that its cytotoxicity was due to the macrocyclic lactone C1–C38 moiety [50]. Afterwards the following analogue drug, Eribulin (Halaven®), with similar anticancer activity but more stability, was synthesized [50]. Moreover, other sponges in the genus *Halichondria* have been reported to contain various natural compounds and some of these compounds

possess antibacterial, cytotoxic, antifungal, and antimicrobial properties [51-55]. However, there are no records in the literature of secondary metabolites isolated from *H. sitiens*.

**Figure 6.** Photos of *Halichondria sitiens*, *Geodia barretti*, and *Flustra foliacea*. Those photos were taken by Eydis Einarsdottir, Kåre Telnes, and Dirk Schories, respectively.

The sponge *H. sitiens* was collected at a depth of approximately 10 m from the rock of Kolbeinsey, which formed during a volcanic eruption about 10,000 years ago in the northernmost point of Iceland. It is subject to rapid wave erosion and is expected to disappear in the near future, probably around the year 2020, based upon current rates of erosion. There is also a hydrothermal vent located around Kolbeinsey at the depth of approximately 100 m. The location of collection place of this sponge made it an interesting candidate to investigate with regard to secondary metabolites and biological activity.

### 1.2.4 The sponge *Geodia barretti*

*Geodia barretti* Bowerbank (family Geodiidae, class Demospongiae, order Astrophorida) is an abundant cold water sponge with a wide geographic distribution, including the Icelandic waters. Specimens of *G. barretti* are irregular in shape, and they can be up to 80 cm in diameter and weigh up to 80 kg. It is usually a large white boreal sponge with a generally smooth surface, which made it an interesting candidate for searching for antifouling compounds. Isolation of three structurally antifouling brominated alkaloids have been performed, i.e. barettin [56-58], 8,9-dihydrobarettin [59, 60], and bromobenzisoxazolone barettin [61] (Figure 7). Two 6-bromoindole derivatives, i.e. 2-(6-Bromo-1*H*-indol-3-yl)-2-hydroxy-*N*,*N*,*N*-trimethylethanaminium [62], and 6-bromoconicamin [62], and three N-methylated nucleosides, i.e. 3-methylcytidine, 3-methyl-2´-deoxycytidine, and 3-methyl-2´-deoxyuridine [63], were obtained as well. Moreover, cyclic peptides, fatty acids, amino acids, and sterols were found in this species [64, 65].

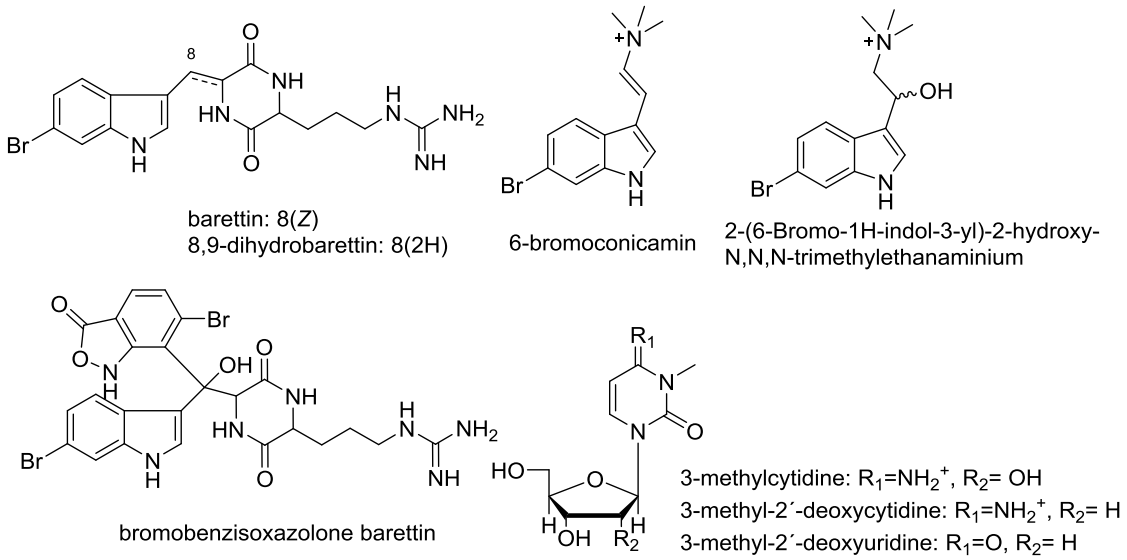



**Figure 7.** Chemical structure of compounds identified from *G. barretti*.

The chemical structures of barettins isolated from *G. barretti* were determined to be a cyclic ring dipeptide as a condensation product of 6-bromo-8-en-tryptophan and arginine residues in a “head-to-tail” fashion. These barettins belong to a group of compounds called diketopiperazines (DKPs). In terms of drug discovery and development, DKPs are a valuable source of bioactive compounds that have resulted in a number of approved drugs mentioned previously. Barettin, also named as cyclo [(6-bromo-8-en-tryptophan)arginine], is the most abundant compound in this species and has, in addition to 8,9-dihydrobarettin, attracted research interest because of the potent biological activity (Table 2). Both of them have been shown to be antifouling agents, acetyl cholinesterase (AChE) inhibitors, and selective serotonin ligands (5-HT). Barettin could inhibit the production of the inflammatory cytokines TNF$\alpha$ and Interleukin (IL)-1β by lipopolysaccharides (LPS) stimulated human acute monocytic leukemia cell line (THP-1), indicating an anti-inflammatory potency [66]. Further study showed that it could reduce the secretion of monocyte chemotactic protein-1 and IL-10 from LPS-stimulated monocytes [67]. It was also active in biochemical assays, where it reduced the level of iron and protected oxygen radical absorbance capacity, as well as in the cellular lipid peroxidation antioxidant assay by reducing lipid peroxidation in the HepG2 cells [66]. Thus, the properties of anti-inflammatory and antioxidant make barettin to be a potential drug lead for the prevention of the development of atherosclerosis. Considering above results, searching for more barettins from

this species and investigation of their structure-activity relationship was interesting.

**Table 2.** Selected compounds isolated from the sponge *G. barretti*.

| Compounds | Bioassay | | | | | | Ref |
|---|---|---|---|---|---|---|---|
| | Anti-fouling | Antioxidant | Anti-inflammatory | AChE inhibitor | Selective serotonin ligand | Contractile activity | |
| Barettin | + | + | + | + | + | N | [60, 62, 66-68] |
| 8,9-Dihydrobarettin | + | N | N | + | + | N | [60, 62, 68] |
| Bromobenzisoxazolone barettin | + | N | N | N | N | N | [61] |
| 6-Bromoconicamin | N | N | N | + | N | N | [62] |
| 2-(6-Bromo-1*H*-indol-3-yl)-2-hydroxy-*N*,*N*,*N*-trimethyl ethanaminium | N | N | N | - | N | N | [62] |
| 3-Methylcytidine | N | N | N | N | N | + | [63] |
| 3-Methyl-2´-deoxycytidine | N | N | N | N | N | + | [63] |
| 3-Methyl-2´-deoxyuridine | N | N | N | N | N | + | [63] |

+ bioactive; - inactive; N not tested

The animal material was collected west of Iceland (65°27.6′ N- 30°46.6′ W) at 388 m depth by the Icelandic Marine Institute in September 2010.

### 1.2.5 The bryozoan *Flustra foliacea*

Bryozoans, also known as seamats or sea mosses, are found in both freshwater and marine environment. Yet bryozoans produce a remarkable variety of chemical compounds, some of which may find uses in medicine. Bryostatin 1, obtained from the bryozoan *Bugula neritina*, and its structurally simpler synthetic analogues, are currently under serious testing as anticancer drugs [69]. Although more than 8,000 species of marine bryozoans are known, only 1% of NPs characterized so far are derived from Bryozoa [70].

*Flustra foliacea* (Linnaeus, 1758), which is often mistaken for a seaweed, but is actually a colony of animals, is abundant and with wide distribution in the North Atlantic Ocean. It is the most commonly studied bryozoan in terms of NP research, both with respect to the number of individual metabolites described and the number of biological studies.

A variety of secondary metabolites have been described from *F. foliacea* in a significant volume of literature (Table 3), including an allelochemical mixture of benzaldehydes, thirteen terpenoids (**1**-**13**) (Figure 8), six 6-bromoindole alkaloids with isoprenyl substituents at various positions (**14**-**20**), and twenty-eight unique brominated pyrrolo[2,3-b]indole alkaloids with isoprenyl substituents at various positions (**21**-**48**) (Figure 9).

**Table 3.** Compounds from the marine bryozoan *F. foliacea*.

| Chemical Class | Compound name | Activities | Ref |
|---|---|---|---|
| Terpenoids | Citral (**1**) | N | [71] |
| | Geranial (**2**) | N | |
| | Nerol (**3**) | N | |
| | Citronellol (**4**) | N | |
| | Geraniol (**5**) | N | |
| | Citronellal (**6**) | N | [72] |
| | 6-Methylhept-5-en--2-one (**7**) | N | [72] |
| | Linalool (**8**) | N | [73] |
| | 3-(4-methylpent-3-en-1-yl)furan (**9**) | N | [73] |
| | Rosefuran (**10**) | N | [72] |
| | Rosefuran epoxide (**11**) | N | [73] |
| | 4,6-dimethyl-5--(3-methylbut-2-en-1-yl)-6-(4-methylpent-3-en-1-yl)cyclohexa-1,3-diene-1-- carbaldehyde (**12**) | N | [73] |
| | 4,6-bis(4-methylpent-3-en-1-yl)-6-methylcyclohexa-1,3-diene-1-carbaldehyde (**13**) | Weak antibiotic&<br>Weak AChE inhibition | [72, 74, 75] |
| 6-Bromoindole alkaloids | Brominated quinoline (**14**) | N | [76] |
| | 6-bromo-$N_b$-methyl-$N_b$-formyltryptamine (**15**) | N | [77] |
| | 6-bromo-2-(1,1-dimethylprop-2-en-1-yl)-1*H*-indole-3-carbaldehyde (**16**) | Weak antibiotic<br>Weak AChE inhibition | [74, 75, 78] |
| | *N*-(2-(6-bromo-2-(1,1-dimethylprop-2-en-1-yl)-1*H*-indol-3-yl)ethyl)-*N*-methyl-methansulfonamide (**17**) | Antibiotic<br>Weak AChE inhibition | [74, 75, 78] |
| | Flustrabromine (**18**) | Antibiotic<br>Weak AChE inhibition | [74, 75, 79] |
| | Deformylflustrabromine (**19**) | Moderate cytotoxic<br>Moderate antibiotic<br>Strong AChE inhibition | [75, 78, 80-84] |
| | Deformylflustrabromine B (**20**) | Moderate antibiotic<br>Weak AChE inhibition | [74, 75, 85] |
| Brominated pyrrolo[2,3-b]indole alkaloids | Flustramide A (**21**) | N | [77] |
| | Flustramide B (**22**) | N | [86] |
| | Flustrarine B (**23**) | N | [86] |
| | Flustraminol A (**24**) | N | [87] |
| | Flustraminol B (**25**) | N | [87] |
| | Flustramine A (**26**) | Muscle-relaxation<br>Weak cytotoxic<br>Weak antibiotic<br>Weak AChE inhibition | [74, 75, 80, 88-91] |
| | Flustramine B (**27**) | Muscle-relaxation | [88-90] |
| | Flustramine C (**28**) | Weak antibiotic | [74, 78, 87] |

| | | | |
|---|---|---|---|
| | Flustramine D (**29**) | Weak Antibiotic<br>Weak AChE inhibition<br>Cytotoxic<br>Blocking<br>AHL biosensor | [74, 80, 92] |
| | Flustramine E (**30**) | Antibiotic | [93] |
| | Flustramine F (**31**) | - | [94] |
| | Flustramine G (**32**) | - | |
| | Flustramine H (**33**) | - | |
| | Flustramine I (**34**) | - | |
| | Flustramine J (**35**) | - | |
| | Flustramine K (**36**) | - | |
| | Flustramine L (**37**) | - | |
| | Flustramine M (**38**) | - | |
| | Flustramine N (**39**) | - | |
| | Flustramine O (**40**) | - | |
| | Flustramine P (**41**) | - | |
| | Flustramine B N-Oxide (**42**) | - | [92] |
| | Dihydroflustramine C N-Oxide (**43**) | - | [92, 95] |
| | Debromoflustramine B (**44**) | Weak AChE inhibition<br>Strong BChE inhibition | [93, 96] |
| | Dihydroflustramine C (**45**) | Weak cntibiotic<br>Weak Cytotoxic<br>Blocking<br>AHL biosensor | [74, 78, 80, 92, 97] |
| | Flustramine D N-Oxide (**46**) | N | [92] |
| | Isoflustramine D (**47**) | N | [92] |
| | (3a*R*,8a*S*)-6-bromo-3a-((2*E*)-3,7-dimethylocta-2,6-dien-1-yl)-1,2,3,3a,8,8a-hexahydropyrrolol(2,3-b)-indol-7-ol (**48**) | Weak antibiotic | [74, 78] |
| | - inactive; N not tested | | |

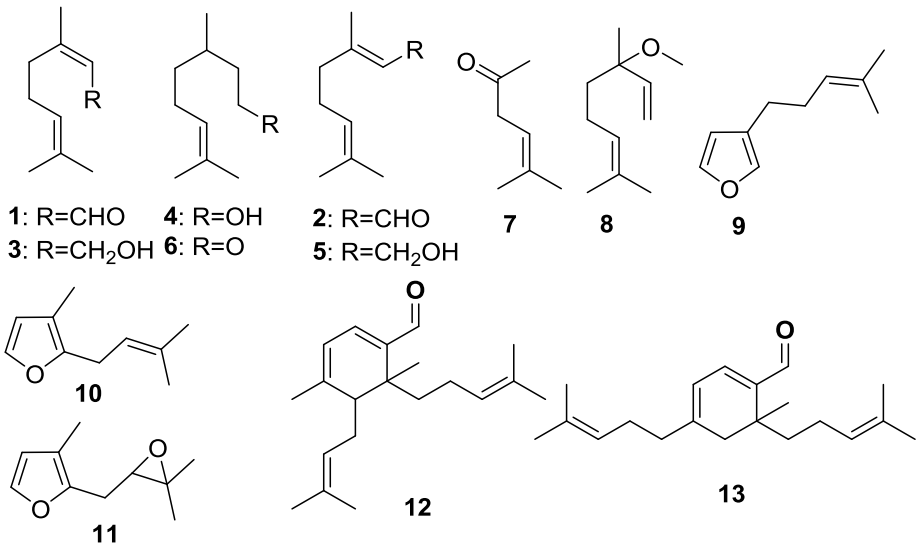



**Figure 8.** Chemical structures of terpenoids identified in *F. foliacea*.

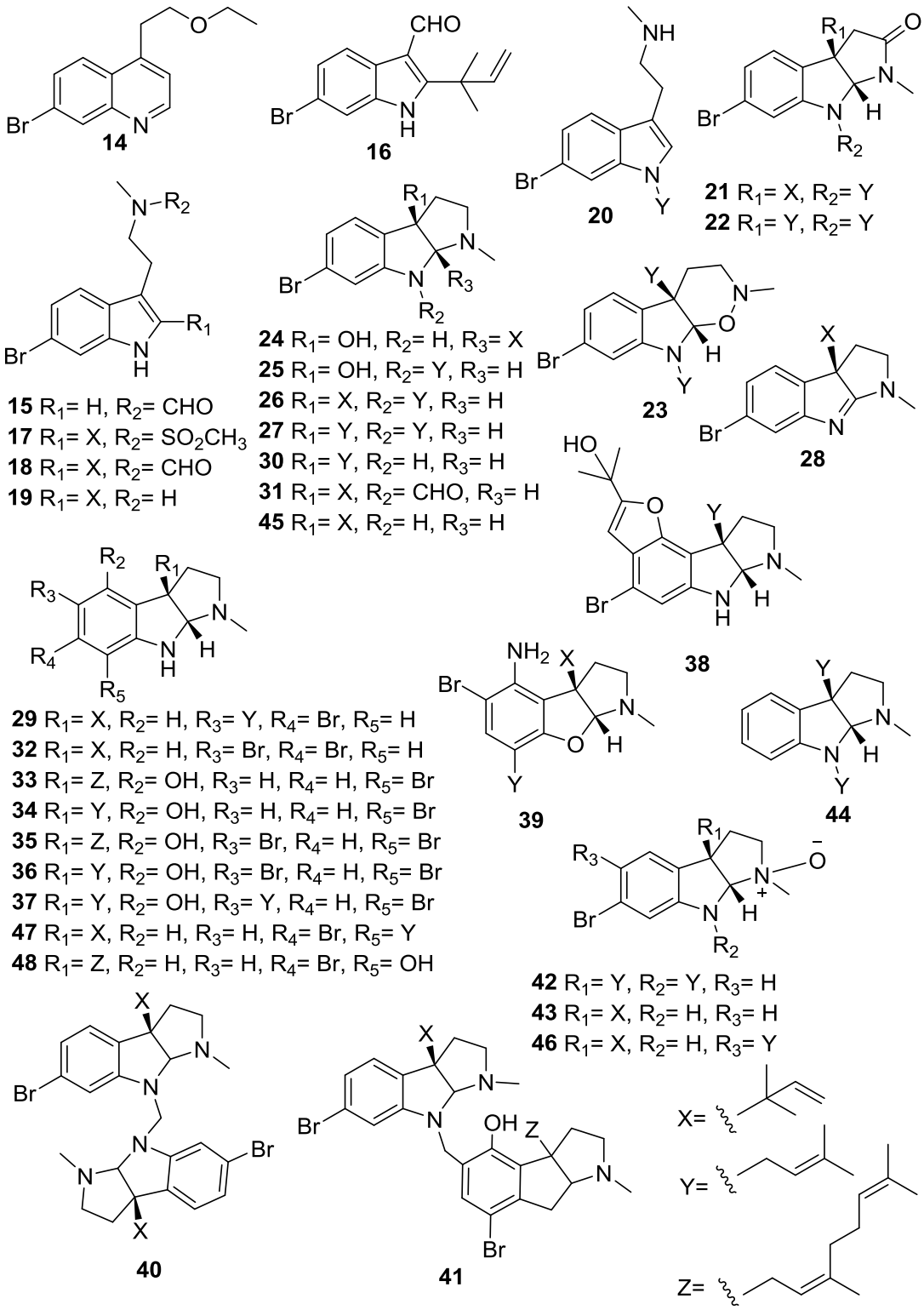



**Figure 9.** Chemical structure of alkaloids isolated from *F. foliacea*.

The functions of the terpenoids in *F. foliacea* are not known, but they were reported as chemical signaling molecules in terrestrial organisms. The most studied biological function of the 6-bromoindoles and brominated pyrrolo[2,3-b]indole alkaloids were antibiotic activity and inhibition of AChE (Table 3) due to the similar structure of physostigmine [88]. A series of alkaloids from *F. foliacea* exhibited moderate or weak antibiotic activity against bacterial strains, including *Paenibacillus pabuli*, *Roseobacer sp.*, *Sulfitobacter sp.*, *Psychroserpens sp.* and non-marine bacteria *Escherichia coli* and *Bacillus megaterium* [74, 93]. The most potent AChE inhibitor is deformylflustrabromine (**19**) and it is perhaps the first selective positive allosteric modulator of $\alpha$4β2 neuronal nicotinic ACh receptors (nAChR), failing to potentiate the actions of ACh at $\alpha$3β2, $\alpha$3β4, $\alpha$4β4, and $\alpha$7 nAChRs [98]. Flustramine D (**29**) and dihydroflustramine C (**45**) are able to block *N*-acyl-homoserine lactones (AHL)-based biosensor and may serve as lead structures for the development of anti-infective agents. Moreover, few compounds showed butyrylcholinesterase (BChE) inhibition, cytotoxic activity and had the effect of muscle relaxation. Considering the potential activities and the complex chemical structure of pyrrolo[2,3-b]indole alkaloids, more and more studies are conducted on the synthesis of these alkaloids and analogues [99-103].

Holst et al. found that the samples of *F. foliacea* collected from the North Sea yielded flustramine E (**30**), whereas Canadian samples have not been found to contain it, but contain flustramine D (**29**) [93]. In addition, different ratios of the two main alkaloids flustramine A (**26**) and B (**27**) were detected in the samples collected in different sites from the North Sea [93]. Therefore, *F. foliacea* from different geographical sites produces different metabolites, i.e. *F. foliacea* colonies collected in close sites give similar metabolite profiles, while distant colonies produced entirely different metabolite profiles, with different dominant and minor metabolites [70, 71, 73]. The reason could be different subspecies or bacterial symbionts, which are responsible for the production of some of the metabolites, and different environment (pressure, light, temperature).

Analysis of the published literature related to those metabolites isolated from *F. foliacea* has revealed that many of these studies have only reported the isolation, structure elucidation, and synthesis of those metabolites, without examining their potential activities (Table 3). Bromination of many natural products has the potential to increase biological activity significantly [104]. Thus, there has been considerable interest in examining the biological activity of the series of brominated pyrrolo[2,3-b]indole alkaloids to find lead compounds for the discovery of new drugs. Collectively, although a large number of compounds have been isolated from *F. foliacea*, the study of secondary metabolites from the

animal material in Icelandic waters collected in February 2015, near the west coast (64°10´N, 22°22´W) (depth 13 m), is still needed.

## 1.3 Immunomodulation

The immune system is spread throughout the body and is collection of cells, tissues and molecules aimed to protect the body against pathogens. It is activated by inflammatory inducers that indicate the presence of pathogens or tissue damage. In humans, the immune system can be classified into innate and adaptive immune system on the basis of the speed and specificity of the immune reactions, although the two systems are highly integrated. Innate immunity uses a variety of effector mechanisms to detect infections and eliminate pathogens, including the physical, chemical and cellular elements of the immune system, such as the actions of neutrophils, monocytes, macrophages, complement, and cytokines. Cells of the innate system use pattern-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and produce mediators (cytokines, chemokines, and other mediators) that induce inflammation and attract more immune cells into the infection sites [105].

As part of our innate immune system, inflammation is one of the first responses to infection and injury. The resident cells at the site of infection or injury respond immediately and cause inflammation. Inflammation is the body's protective response to combat all types of dangers. This process often causes temporary discomfort, resulting in the five signs of inflammation, i.e. redness, heat, swelling, pain and loss of function. The process of inflammation generally includes the entrance of effector molecules and cells from blood to sites of infection, the clearance and removal of the injurious stimuli, such as invading microorganisms and damaged cells, as well as the initiation of the repair of injured tissue. Too little inflammation can lead to progressive tissue destruction by the harmful stimulus and can compromise the survival of the organism. In contrast, excessive inflammation or lack of resolution of inflammation can lead to chronic inflammation, which is involved in the pathogenesis of many prevalent diseases, such as cardiovascular diseases, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and even cancer [106-108]. Inflammation is, therefore, normally balanced with anti-inflammation processes by the body.

Dendritic cells (DCs) are the most effective antigen presenting cells (APCs) and play a critical role in innate, but also in regulation of adaptive, immune responses. DCs are distributed throughout the body, including the mucosal tissues, where they are found below the epithelial cell barrier in an inactive stage

as immature (im)DCs. DCs are activated by microbial or danger stimuli through PRRs, such as the toll-like receptor (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and others (Figure 10). These receptors can sense not only external infectious and environmental antigens but are also capable of responding to internal danger signals and molecules generated during tissue injury or malfunctioning of any of the other processes in the body. Upon uptake of PAMPs and DAMPs via the PRRs, imDCs activate and become mature (m)DCs and migrate to the lymph nodes to present antigens to the T cells, and initiate an adaptive immune response. Three kinds of signals are involved in activation of naïve T cells by DCs, including the interaction of a specific peptide: MHC complex with the T cell receptor, co-stimulatory signals that promote the survival and expansion of the T cells, and cytokines that direct T cell differentiation of naïve $CD4^+$ T cells into functionally distinct T helper (Th) cell subtypes [109-111].

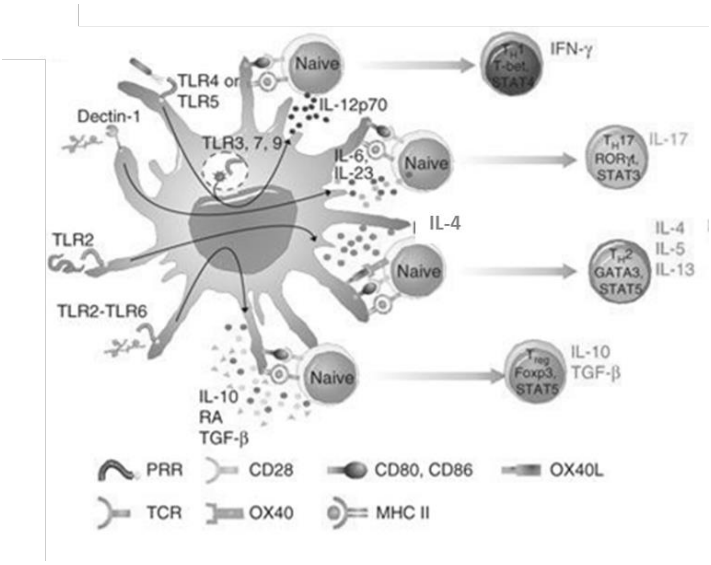



**Figure 10.** Dendritic cells and induction of Th cell subtypes in the immune system. Modified from [112].

Activated DCs produce pro-inflammatory cytokine IL-12, which preferentially directs the differentiation of naïve $CD4^+$ T cells into the Th1 type [113]. Th1 cells are induced in response to viral, protozoan and intracellular bacterial infections and secrete cytokine Interferon (IFN)-γ. The release of IFN-γ can activate macrophages to kill ingested pathogens either by production of nitric oxide (NO) and superoxide ($O_2^-$) or by other killing mechanisms. Furthermore, Th1 cells play a protective role against viral infection though the antiviral effect of IFN-γ and the

activation of $CD8^+$ cytotoxic T lymphocytes that kill virus-infected cells [114, 115]. Th1 dominated responses are responsible for different types of immunopathological reactions, including some inflammatory disorders, acute allograft rejection, autoimmune disorders, contact dermatitis, etc. [116].

Th2 cells arise from naïve T cells when exposed to antigens in the presence of anti-inflammatory cytokine IL-4 and play a central role in protective immunity to parasites through the production of IL-4, IL-5, and IL-13. IL-13 induces epithelial cell repair and mucus secretion which accelerates loss of parasite and increases mucosal smooth muscle [117]. IL-4 and IL-5 can activate mast cells, eosinophils, and basophils and drive antibody isotype class-switching to IgE in B cells. IL-4 and IL-13 produced by Th2 cells result in the differentiation of alternatively activated macrophages which promote tissue repair [118]. Th2-type responses are involved in allergic disorders, transplantation tolerance, chronic graft versus host disease, and atopic disorders [116].

The subset of Th17 cells can be induced by the pro-inflammatory cytokines IL-6, IL-23, and IL-1β by DCs. Th17 cells secrete the cytokines IL-17 and IL-22 and are now recognized as key inflammatory T cells that play a pathogenic role in many autoimmune diseases. However, these cells also play a major protective role in immunity against extracellular fungi and bacteria, by secreting chemokines which recruit and activate neutrophils to the site of infection. IL-17 and IL-22 also induce the production of antimicrobial peptides which can kill bacteria directly [119, 120].

Induced Tregs (iTregs) are derived from naïve $CD4^+$ T in the presence of cytokines IL-10 and/or TGF-$\beta$ cells in the periphery. They exert their role by suppression rather than activation through secretion of the same cytokines that are responsible for their induction. The anti-inflammatory cytokine IL-10 can inhibit the expression of MHC molecules and co-stimulatory molecules by APCs and TGF-$\beta$ can inhibit T cell proliferation [121, 122]. Another group of Tregs, natural (n)Tregs, are derived from thymus and their major function is to regulate immune responses to self-antigens and thereby prevent the development of autoimmunity [119, 123]. Tregs also dampen effector T cell responses to pathogens to limit infection-induced immunopathology. Thus, Tregs represent a powerful therapeutic tool for autoimmune disease, inflammation and anti-tumor treatment [119, 123].

Immunomodulation is a very broad term which refers to any changes in the immune response and may involve induction, expression, amplification or inhibition of any part or phase in the immune response. Many agents of natural origin and NP-derived molecules possess stimulatory, suppressive and

regulatory activity, which has led to therapeutics, such as immunostimulators, immunosuppressants, immunoadjuvants for vaccine, etc. There are various medicines used for controlling and suppressing inflammation; such as steroids, non-steroidal anti-inflammatory drugs, and immunosuppressants [124].

## 1.4 Immunomodulatory/anti-inflammatory activity of compounds from marine invertebrates

### 1.4.1 Evaluation of anti-inflammatory activity

A vast range of NPs, like sesquiterpenoids, diterpenoids, alkaloids, macrolides, peptides, steroids, fatty acids, polysaccharides, proteins, and other chemical compounds from marine invertebrates have been isolated and found to possess anti-inflammatory activities [20, 125-132]. Also, various experimental methods *in vitro* and *in vivo* to detect anti-inflammatory activity have been reported and some of them are used for screening natural compounds.

Many of these assays *in vitro* are focused on evaluating inhibition of reactive oxygen species NO, oxidative enzymes (inducible nitric oxygen synthase (iNOS), cyclooxygenase (COX)-2, 5-lipoxygenase), cytokines (IFN-$\gamma$, TNF$\alpha$, interleukins), immunoglobulin secretion, histamine release, cellular co-receptor expression, lymphocyte expression, phagocytosis assays using macrophages or neutrophils, and so on [127-129, 131]. Currently, the most widely used *in vitro* application to evaluate anti-inflammatory activity is measuring the expression of iNOS and COX-2 and the secretion of cytokines in inflammatory cell lines (such as the macrophage cell line RAW264.7, microglial cell line BV2 or THP-1 cells) induced by LPS.

*In vivo* preclinical trials for anti-inflammatory activity are usually performed in experimental models using mice or rats. Several irritants and antigens are used to stimulate the immune system in these animal models and then the immune responses are investigated by various methods. Commonly used animal models are paw edema, ear edema, and inflammation induced in lungs, colon and peritoneum [127, 128, 133].

### 1.4.2 Anti-inflammatory agents in clinical trials

A vast amount of NPs possessing anti-inflammatory potency *in vitro* and *in vivo* have been isolated; however, no marine-derived anti-inflammatory agent is currently on the market. Although they exhibit higher target potency, many compounds have not reached clinical phase study. Effective *in vitro* but

inactivate *in vivo* high toxicity, low stability, short half-life, and/or low oral bioavailability can be the reason for the failure. In addition, some of these compounds are only available in very small quantities. Table 4 summarizes the compounds from marine invertebrates reported to be in preclinical or clinical development and the compounds discontinued from development. Manoalide is the first marine NP reported as a phospholipase (PL)A2 inhibitor and is one of the most widely investigated marine PLA2 antagonists. It suppresses the synthesis of inflammatory lipids like prostaglandins (PGs), leukotrienes, and platelet activating factor *in vitro* [134]. It has reached phase II clinical trials as a topical anti-inflammatory drug. Due to disappointing results in treatment of psoriatic patients caused by insufficient bioavailability, the clinical development of manoalide was ceased [135]. However, some researchers have synthesized a series of new analogues of manoalide for use as anti-inflammatory agents, which are now in preclinical testing [135, 136].

### 1.4.3 Anti-inflammatory compounds

As was mentioned above, marine invertebrates have produced chemically diverse compounds with confirmed preclinical anti-inflammatory effects. The evaluation methods of anti-inflammatory activity are usually evaluating the inhibitory action on iNOS and COX-2 expression in macrophages and testing $O_2^-$ generation, and elastase release in neutrophils [128, 129]. The molecular mechanisms of action of several MNPs have been reported in detail. Many of these marine compounds block the COX-2 expression to inhibit the inflammatory response and reduce the production of inflammatory PGs. In addition to the COX pathway, some MNPs act to inhibit nuclear factor kappa light chain enhancer of activated B cells (NF-κB) inflammatory pathways [128, 129]. Table 5 presents the preclinical pharmacological research on the anti-inflammatory activity of few selected marine NPs.

**Table 4.** Marine invertebrate-derived anti-inflammatory agents in clinical and preclinical trials.

| Source | Compound | Chemistry | Molecule mechanism of action | Clinical trials/status | Ref |
|---|---|---|---|---|---|
| Sponge *Petrosia contignata* | IZP-94005 | Steroid | Inhibiting histamine release | Preclinical test for anti-asthmatic effects /ceased | [137] |
| Synthetic analogue of Contignasterol | IPL576092 | Steroid | Suppressing leukocyte | Phase II for anti-asthmatic effects/ceased | [138] |
| Sponge *Luffariella variabilis* | Manoalide | Nonsteroidal Sesterterpenoid | PLA2 inhibitor | Phase II for anti-inflammatory effects/ceased | [134] |
| Analogue of manoalide | AGN 190383 | 5-hydroxy-2(5*H*)-furanone ring analogue | PLA2 inhibitor | Preclinical test for anti-inflammatory effects/ceased | [136] |
| Sponge *Cacospongia mollior* | Scalaradial | Terpenoid | PLA2 inhibitor | Preclinical test for anti-inflammatory effects/No report yet | [139] |
| Soft coral *Pseudopterogorgia elisabethae*[a] | Pseudopterosin A | Diterpene glycoside | PLA2 inhibitor | Preclinical test for atopic dermatitis/Ceased<br>As an ingredient in a marketed cosmetic skin care product called Resilience® | [140, 141] |
| Methyl ether derivative of pseudopterosin A | Methoperosin | Diterpene glycoside | PLA2 inhibitor | Phase I/II for wound healing/Ceased | [34] |
| Synthetic derivative of anabaseine from marine worm in phylum Nemertea | GTS-21 or DMXBA | Alkaloid | Inhibiting production of pro-inflammatory cytokine through stimulating α7 nicotinic choline receptors | Clinical trial for anti-inflammatory effects<br>Phase I/II for attention-deficit hyperactivity disorder/No report<br>Phase II for schizophrenia<br>Phase II for Alzheimer's disease/Ceased | [142-144] |

*a.*Later study revealed it originated from symbiotic dinoflagellate *Symbiodinium sp.*[139]

**Table 5.** Selected compounds with anti-inflammatory activities from marine invertebrates.

| Chemistry | Species | Compound | Molecular mechanism of action | Ref |
|---|---|---|---|---|
| Alkaloid | Mollusc *Dicathais orbita* | 6-Bromoisatin | Inhibiting NO, TNFα, NF-κB in LPS-stimulated RAW264.7 mouse macrophage | [145] |
| Alkaloids | *Ascidian Aplidium* | Ascidiathiazones A and B | In vitro inhibition of $O_2^-$ production in PMA-stimulated human neutrophils; In vivo inhibition of $O_2^-$ production by peritoneal neutrophils in a murine model of gout | [146] |
| Sesquiterpenoid | Coral *Rumphella antipathies* | Rumphellaone C | Inhibition of $O_2^-$ anion generation and elastase release in fMLP/CB-activated human neutrophils | [147] |
| Diterpenes | Soft coral *Lobophytum crassum* | Lobocrasols A & B | TNFα-induced nuclear NF-κB transcriptional activation in HepG2 cells | [148] |
| Diterpenes | Coral *Briareum excavatum* | Excavatolides B, F & K | Inhibition TPA-induced vascular permeability and edema in skin by inhibition of COX-2, iNOS, and matrix metalloproteinases-9 expression; inhibition of IL-6 expression of LPS-activated mouse DCs | [149] |
| Triterpenoid | Soft coral *Capnella imbricate* | Capnellene | In vitro and in vivo inhibition of microglia activation by inhibition of iNOS and COX-2 | [150] |
| Polyketide | Sponge *Callyspongia sp.* | 14,15-Dihydrosiphonodiol | DC activation and activation of Th1/Th2 cell polarization or IL-10 producing T cells | [151] |
| Steroid | Sponge *Theonella swinhoei* | Solomonsterol A | Reduction in arthritic score in anti-type II collagen antibody-induced arthritis mice model | [152] |
| Sterol | Sponge *Xestospongia bergquisita* | Xestobergsterols A and B | Inhibitor of IgE-mediated histamine release from activated mast cells | [153] |
| Peptide | Sponge *Haliclona, sp.* | Halipeptins A-D | Reduction of carrageenan-induced paw edema in mice | [154-156] |
| Peptide | Sponge *Theonella swinhoei* | Perthamide C | Induction of carrageenan-induced paw edema; Inhibition of TNFα and IL-8 release in primary human keratinocyte cell lines *in vitro* | [157] |
| Glycolipid | Sponge *Terpios* sp | Terpioside B | Inhibition of iNOS expression in murine J774 cells | [158] |

Alkaloids are a group of naturally occurring organic amine and cyclic compounds having nitrogen in the ring, produced by various organisms, including plants, microorganisms, and marine organisms. Classifications of alkaloids are based on similarities of the carbon skeleton, such as pyrrolidines, pyridines, tropanes, pyrrolizidines, isoquinolines, indoles, and quinolines. Various classes of alkaloids from marine invertebrates have been reported to have anti-inflammatory activity. 6-Bromoisatin, an oxindole alkaloid from a marine mollusk, has anti-inflammatory activity, based on the inhibition of NO and TNF$\alpha$ in LPS-stimulated RAW264.7 macrophages and PGE2 in calcium ionophore-stimulated 3T3 CCL-92 fibroblasts [145]. The quinolinequinone alkaloids ascidiathiazones A and B, containing rare 1,1-dioxo-1,4-thiazine rings, were isolated from Ascidian *Aplidium* sp. that affected $O_2^-$ production by human neutrophils *in vitro*, as well as *ex vivo*, suggesting that these two compounds might become "potential anti-inflammatory pharmaceutical" leads [146]. Indole alkaloids are abundant in marine invertebrates and some have anti-inflammatory potentials, such as aplysinopsintype compound from sponge *Hyrtios erecta* [159], lepadiformines A and B from ascidian [160] and conicamin from tunicate [161]. The structure-activity relationship study of some indole derivatives have been conducted and suggested that the indole moiety contributed to the potency of anti-inflammation and other pharmaceutical core. Some researchers have suggested that halogenation of alkaloids seems beneficial in order to increase the anti-inflammatory activity [162, 163].

Terpenoids are one of the most abundant and most well studied group of NP compounds. Terpenoids, deriving from five-carbon isoprene units assembled and modified in thousands of ways, can be classified into hemi, mono-, sesqui-, di-, sester-, tri-, tetra or polyterpeonoids on the basis of the number of isoprene C5 units. Terpenoids are often present in soft corals as secondary metabolites and some of them have been shown to exert anti-inflammatory activities [164]. Few terpenoids and their derivatives have reached clinical trials and serve as templates for the design of new analogues for anti-inflammatory agents (Table 4). A series of new briarane-type diterpenoids, for example excavatolide B, isolated from soft coral *Briareum excavatum*, inhibited COX-2, iNOS and matrix metalloproteinases-9 production in 12-*O*-tetradecanoylphorbol-13-acetate induced vascular permeability and edema in skin and inhibited secretion of TNF$\alpha$ and IL-6 in LPS-activated mouse bone marrow-derived DCs [149, 164]. The sesquiterpenoid rumphellaone C, containing unprecedented γ-lactone moieties, significantly inhibited superoxide anion and elastase generation from human neutrophils *in vitro* [147]. The discovery of chemically novel structures of those active compounds gives a new clue to design new drug lead.

Marine invertebrates, especially the most primitive animals, such as sponges and gorgonians, produce a variety of lipids. These lipids include a great number of various fatty acids, such as saturated, unsaturated, branched, halogenated etc. Long-chain n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been demonstrated to reduce inflammation in both *in vitro* and *in vivo* studies [165]. Moreover, glycolipids attract increasing interest regarding their promising biological activities. The glycolipid terpioside B isolated from the marine sponge *Terpios* sp., containing two fucose residues in the furanose form in its pentasaccharide chain, inhibited NO release in murine J774 cells [158].

Steroids are used to treat a variety of inflammatory diseases by decreasing inflammation and suppressing various pathways of the immune system. Marine invertebrates, especially sponges, are a rich source of steroids with anti-inflammatory activity. Contignasterol, the first described steroid from a marine sponge with anti-inflammatory activity, is a highly oxygenated sterol with an unusual side chain [137]. It could inhibit histamine release in anti-IgE activated rat peritoneal cells; thus, acting as a strong antihistamine agent. The synthetic analogue of contignasterol had gone through a phase II clinical trial but unfortunately it was withdrawn from further clinical trials.

Marine-derived peptides may have modified structures in the backbone or side chain structure compared to peptides in humans because of the aggressive demands of the marine environment; hence, they are suitable as scaffolds for drug design and provide stability against enzymes and thermal conditions [166]. The majority of marine peptides are derived from sponges, mollusks and ascidians. Even though there are no anti-inflammatory peptides from marine invertebrates reaching clinical trials, there are several peptides in preclinical research showing very interesting results. Halipeptins, isolated from the marine sponge *Haliclona* sp., are made up of a peptidic portion, possessing conventional alanine residues and unusual residues, assembled in a 17-membered macrolactone ring [154, 155]. Among these halipeptins, halipeptin A exhibited potent anti-inflammatory effects both *in vitro* and *in vivo*, causing a 60% reduction of mouse paw edema at a very low dose of 300 μg/kg, even was 40-130 times more potent than the classical anti-inflammatory drugs naproxene and indomethacin without the typical side effects [154-156].

Marine-derived polysaccharides, such as fucoidan, chitosan, chitin, and alginate, exert anti-inflammatory properties [167]. The main research interest has focused on polysaccharides, both sulfated and non-sulfated, derived from marine micro- and macro-algae. To date, few polysaccharides from marine invertebrates have been found with anti-inflammatory potential [168].

Finally, some polyketides, proteins and other classes of compounds have been isolated from marine invertebrates and are found to manifest an anti-inflammatory action, although not covered in this thesis [128, 129]. An increasing number of review papers related to the search words ‘anti-inflammatory’ and ‘marine invertebrates’ have been published recently, reflecting the rapid development of MNPs.

Overall, marine invertebrates provide an abundant source for NPs. These compounds not only possess chemically novel structures but some also have anti-inflammatory activity. Ongoing pre-clinical experiments and clinical trials should be continued to provide scientifically based data of effectiveness to reduce inflammation and promote wellness; thus, enhancing the marine pharmaceutical clinical pipeline. There are still some compounds in the deep, dark ocean waiting to be discovered.

## 1.5 Isolation methods

### 1.5.1 Bioassay-guided isolation

A few different approaches to isolate and search for drug leads from nature are employed, i.e. random selection followed by chemical isolation (isolate and then test), follow-up of biological activity reports of crude extracts (test and then isolate), and follow-up of ethnomedical (traditional medicine) use of plants. Although some drugs have been found, these approaches are always attached with problems, such as being time consuming, costly, it may be laborious to characterize the active components from the extracts, and there is a risk of rediscovery of various known compounds and losing the minor components.

Bioassay-guided isolation is a typical protocol to target bioactive pure compounds of natural origin, making the rapid localization of fractions containing bioactive compounds possible. Prior to the bioactivity screening the samples are pre-fractionated based on differences in their physical and chemical properties, in order to reduce the chemical complexity of the samples, as well as decreasing the content of nonselective compounds and inorganic salts [169]. Any fraction with strong bioactivity is separated and purified in a series of subsequent fractionation steps using various chromatographic techniques, and the bioactivity is traced by screening all fractions in the relevant bioassay.

However, this type of approach often leads to rediscovery of known bioactive compounds or discovery of compounds present in such minute quantities that they cannot be isolated. Potentially interesting compounds may be missed because they are not active in the selected biological assays but they might be

active in other bioassays. Also, biological effects of crude extracts are sometimes obtained because of multi-component synergism, which is lost when the NPs have been purified [170, 171].

### 1.5.2 UPLC-qTOF-MS-guided isolation

To decrease the high possibility of rediscovery of known bioactive compounds, an effective approach in finding novel chemical components and tracing components in a complex mixture of NPs (often called dereplication) using mass spectrometry (MS) combined with liquid chromatography (LC), ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS) was used for this purpose [171, 172]. The metabolites can be analyzed using different techniques, where LC-MS and nuclear magnetic resonance (NMR) spectroscopy are considered to be the most universal approaches. The MS provides high sensitivity and resolution, while NMR will give qualitative and quantitative information of the molecules [173, 174]. The data provided by this approach, in combination with chemical agent databases that contain chemical and physical information (including chemical structure, molecular formula, molecular weight, bioactivity and taxonomy), e.g. SciFinder, ChemSpider, MarinLit [175], can help to identify compounds rapidly as previously known or new chemical entities. If the physical and chemical data of a compound do not correlate with any known compounds, theoretically it is supposed to be a new compound that can be further investigated for bioactivity and structure elucidation. Thus, UPLC-qTOF-MS-guided isolation provides precursor ion and fragment ions to identify the structural similarities of possible analogues, which can be used in the structural elucidation and increase the efficiency for NP discovery [176, 177].

A combinatorial approach (Figure 11) is commonly performed, wherein extracts or fractions are tested for bioassay, and if the “interesting” activity is detected, then bioassay-guided approaches are used. On the other hand, profiling of the material by UPLC-qTOF-MS for unique chemical constituents can be followed by their isolation to rapidly remove undesired constituents and broad evaluation in diverse biological assays. In this way, the targeted compounds could be only bioactive and novel compounds in finial [178, 179].

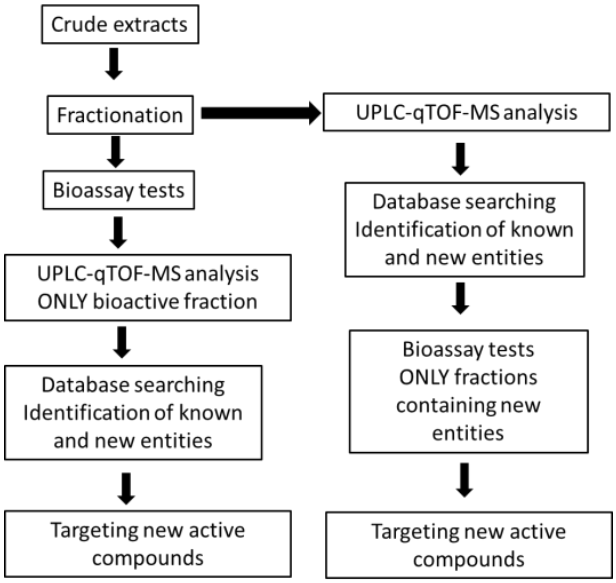



**Figure 11.** A dereplication workflow for combinatorial approach procedures for isolation of new bioactive NP compounds.

# 2 Aims

The main objective of this project was to isolate and identify novel pure compounds from marine invertebrates collected from the marine environment around Iceland that have anti-inflammatory properties and are, therefore, either likely to prevent and/or slow the progress of inflammation.

The aims can be specified as follows:

1. To extract, fractionate and isolate compounds from Icelandic marine invertebrates
2. To screen the crude extracts for bioactivity
3. To elucidate the structures of the isolated compounds using 1D and 2D NMR spectroscopy, together with high resolution mass spectrometry
4. To determine the stereochemistry of the new compounds and re-evaluate the stereochemistry of known compounds by nuclear Overhauser effect spectroscopy (NOESY), electronic circular dichroism (ECD) spectra, optical rotation, and Marfey′s method
5. To evaluate the immunomodulatory activity of the isolated compounds by determining their effects on DCs and the ability of the DCs to activate and differentiate $CD4^+$ T cells

# 3 Materials and methods

## 3.1 Sample collection and identification

The three marine invertebrates, *H. sitiens*, *G. barretti* and *F. foliacea*, were collected from the rocks of Kolbeinsey (depth of 10 m), north of Iceland in July 2015 by scuba diving, west of Iceland (65°27.6′ N- 30°46.6′ W) at 388 m depth in September 2010, near the west coast of Iceland (64°10′ N- 22°22′ W) (depth 13 m) in February 2015, respectively. The animal material was identified by Dr. Hans Tore Rapp, University of Bergen (Norway). Three voucher specimens of each species (300512#2, A12-2010-688, FF2015) were deposited at the Department of Natural Products Chemistry, Faculty of Pharmaceutical Sciences, University of Iceland.

## 3.2 Extraction of marine invertebrates

The wet collected invertebrates were immediately frozen and stored at -20°C. The frozen sponges, including *H. sitiens*, *G. barretti* and the screening unidentified sponges, were lyophilized, followed by extraction with methanol (MeOH)/dichloromethane (DCM) (*v*:*v*, 1:1) (3 times, each time for 24 h) at room temperature (RT). The combined extracts were filtrated and concentrated under reduced pressure to obtain crude extracts and stored at -18°C before next step. The crude extracts were fractionated into five fractions using solvent partitioning method (a modified Kupchan method) [180, 181]. The extracts from sponges were dissolved in 90% aqueous MeOH and partitioned against n-hexane (A fraction). The water content of the hydromethanolic phase was adjusted to 20% (v/v) and then to 40% (v/v) and the solutions were partitioned against chloroform ($CHCl_3$) twice (B and C fraction). The hydromethanolic phase was concentrated using a rotary evaporator to remove MeOH and the remaining water extract was partitioned against n-butanol (BuOH) to obtain organic layer (D fraction) and water layer (E fraction) (Figure 12A).

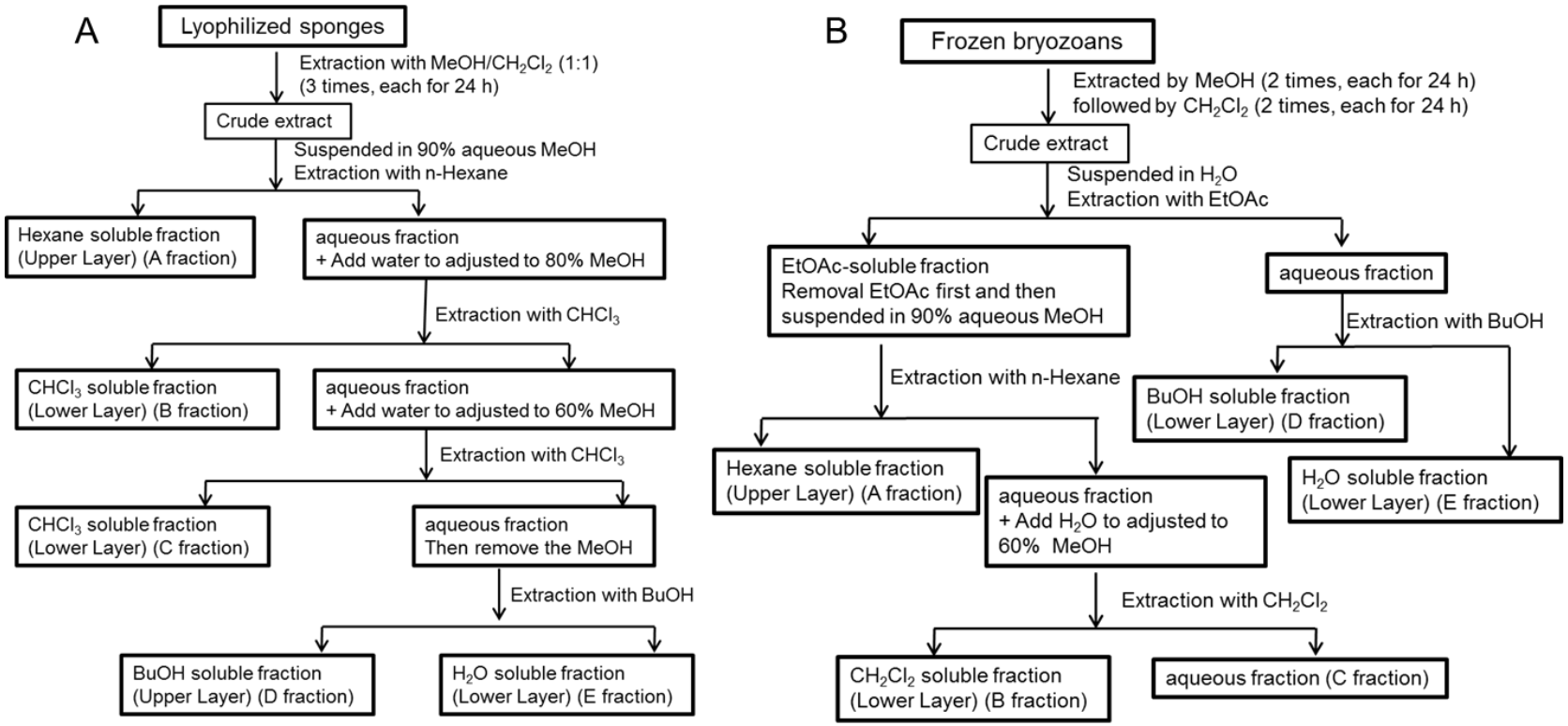



**Figure 12.** Flow chart of extraction and modified Kupchan partitioning method [180, 181] for sponges (A) and bryozoan (B).

The frozen wet materials of *F. foliacea* were extracted with MeOH (2 times, each time for 24 h) followed by $CH_2Cl_2$ (2 times, each time for 24 h). The combined extracts were concentrated and then fractionated into five fractions using modified partitioning Kupchan method. The crude extract was suspended in $H_2O$, and partitioned successively with ethyl acetate (EtOAc). After removal of EtOAc, the EtOAc-soluble fraction was suspended in 90% MeOH, followed by partitioning against hexane (A fraction). The 90% MeOH soluble fraction was adjusted to 60% MeOH and then extracted with DCM to obtain B (DCM layer) and C fraction (60% MeOH layer). The $H_2O$ fraction obtained from previous step was partitioned with BuOH to produce D (BuOH layer) and E fractions ($H_2O$ layer) (Figure 12B).

## 3.3 UPLC-qTOF-MS analysis

Each fraction was dissolved in MeOH in the concentration of 1 mg/mL and was analyzed before isolation work commenced by UPLC-qTOF-MS using a Waters ACQUITY UPLC system (Waters, Milford, MA, USA) coupled to a Waters Synapt G1 mass spectrometer equipped with an electrospray ionization (ESI) probe (Waters, Wilmslow, UK). The chromatographic column used was an ACQUITY UPLC BEH $C_{18}$ (2.1 mm × 100 mm, 1.7 µm) (Waters), which was maintained at 40 °C. The gradient system mobile phase consisted of solvent A: Milli-Q $H_2O$ in 0.1% formic acid and solvent B: Acetonitrile (ACN) in 0.1% formic acid, at a flow rate of 0.45 mL/min. The injection volume of 5 µL was followed by a linear gradient starting at 95% mobile phase A in 1.0 min up to 100% of mobile phase B in 14.0 min. The obtained data were processed and analyzed by MassLynx to obtain the information of molecular weight, molecular formula and possible fragmentation of existing compounds and then compared the data with ChemSpider, SciFinder Scholar database (Chemical Abstracts Service, Columbus, OH, USA).

## 3.4 Separation and purification of pure compounds

The selected B and/or C fraction was subjected to different chromatographic techniques to separate the fractions into sub-fractions, including solid phase extraction (SPE) Silica gel CC eluting with different portions of DCM-MeOH, from 100:0, 100:1, 50:1, 25:1, 10:1, 5:1, 1:1 to 0:100, Sephadex LH-20 (25–100 µm, Pharmacia Biotek, Denmark) in MeOH or MeOH/$H_2O$ (*v*:*v* 90:10), $C_{18}$ reversed-phase silica gel (LiChroprep RP-18, 40–63 µm, Merck Inc., Darmstadt, Germany) eluting with MeOH/$H_2O$. Thin-layer chromatography (TLC) was used to monitor fractions to combine fractions which were shown to be similar. The selected sub-fractions were performed on preparative HPLC

with a Dionex 3000 HPLC system equipped with a G1310A isopump, a G1322A degasser, a G1314A VWD detector, and a Phenomenex Luna C18 (2) column (250 × 21.2 mm, 5 μm) and purified by semi-prep HPLC with a Phenomenex Gemini-NX C18 column (250 × 4.6 mm, 5 μm) to produce purity compound. Every fraction from *H. sitiens* was tested for immunomodulatory activity using a DC model in the concentration of 10 μg/mL and the bioactive fractions were chosen for next separation.

## 3.5 Structure elucidation

### 3.5.1 NMR spectroscopy

1D NMR, correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC) spectra and NOESY were recorded on a Bruker AM-400 spectrometer (proton frequency 400.13 MHz and carbon frequency 100.61 MHz, respectively) using TMS as an internal standard or a Bruker Avance 600 spectrometer (proton frequency 600.13 MHz and carbon frequency 150.61 MHz, respectively). Processing and assignment of the spectra was performed using the software MestReNova. Chemical shifts were reported with reference to the respective residual solvent peaks i.e. $\delta_H$ 7.27 and $\delta_C$ 77.0 for $CDCl_3$; $\delta_H$ 3.31 and $\delta_C$ 49.0 for $CD_3OD$; $\delta_H$ 2.50 and $\delta_C$ 39.5 for dimethyl sulfoxide (DMSO)-$d_6$.

### 3.5.2 High resolution mass spectrometry

High-resolution electrospray ionization mass spectrometry (HRESIMS) of geobarrettin D and flustramine T were measured on a micrOTOF II mass spectrometer (Bruker) in positive mode. The $MS^2$ spectrum of geobarrettin D and flustramine T was measured on Acquity UPLC I-Class System coupled to the Xevo G2-XS QTof Mass Spectrometer (Waters, Massachusetts, USA). An Acquity UPLC HSS T3 column (High Strength Silica $C_{18}$, 1.8 μm, 2.1 x 100 mm, Waters, 60°C) was used. A binary mobile phase system (A: 0.1% formic acid in milliQ-$H_2O$ and B: 0.1% formic acid in ACN) was pumped at a flow rate of 0.6 mL/min at the following gradient: 5% to 100% B in 10.0 min followed by washing and reconditioning of the column. The MS and $MS^2$ spectrum was recorded with the following conditions: capillary voltage: 0.8 kV, cone voltage: 40 V, source temperature: 150°C, cone gas flow: 50 L/h, desolvation gas flow: 1000 L/h, collision energy: 30 eV. MassLynx was used for data acquisition.

### 3.5.3 Electronic circular dichroism (ECD)

For some of the new compounds, the absolute configuration of the stereogenic center was assigned by comparison of the Cotton effects at the

specific wavelength in the ECD spectra with known model compounds. The ECD spectrum of the compound to be analyzed was measured on a Chirascan spectrophotometer (Applied Photophysics Ltd, Surrey, UK).

### 3.5.4 Marfey´s method

This method was performed to determine the absolute stereochemistry of barettins from *G. barretti.* It employed four steps. Firstly, the natural compound was subjected to acid hydrolysis (6 M HCl) to release amino acid residues. Secondly, the amino acid residues were derivatized with the new chiral Marfey's reagents, L-$N^{\alpha}$-(1-fluoro-2,4-dinitrophenyl)tryptophanamide (L-FDTA) under alkaline conditions (1 M $NaHCO_3$). Then the derivatized amino acids were analyzed via HPLC-UV using RP-C18 column, and their retention times compared with those of derivatized L and D amino acid standards. In addition, the natural product was hydrogenated (MeOH, 20 equiv. $Et_3N$, 10% Pd-C, $H_2$) to give debromo epimers and then repeated on acid hydrolysis and derivatization with L-FDTA as before.

### 3.5.5 Synthetic methods

The structure of one new compound, flustramine S, from *F. foliacea* could not be elucidated by NMR and MS data only. Two possible candidates were synthesized and then the physical and chemical data of the natural compound and the synthetic ones were compared to determine the structure. The detail is shown in paper III.

## 3.6 Immunomodulatory activity assay

### 3.6.1 Screening of activity of marine invertebrates

A library of DCM/MeOH extracts from marine sponges was screened for potential anti-inflammatory effects using the DC model as described in below. The screening process is illustrated in detail in Figure 13. For all the screening specimens, the extracts of the sponge tissues were subjected to solvent partitioning to obtain A, B, C, D and E fractions. Fractions A, B, C and D were dissolved in DMSO to obtain stock solutions at 20 mg/mL. The stock solutions were diluted further and subjected to the first round of screening to identify potentially active fractions. The bioactive fractions were subjected to various sizes of column chromatography to give few sub-fractions that were also screened using a DC model. All active sub-fractions underwent further separation until pure compounds were identified. After obtaining the pure compounds, the final screening was conducted to find a target compound.

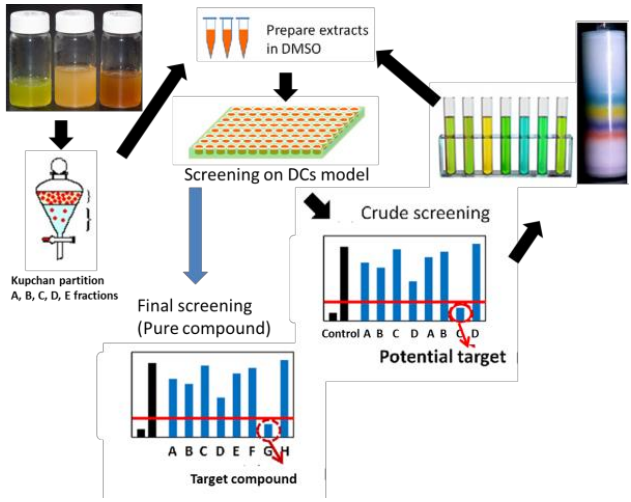



**Figure 13.** Schematic overview of screening fractions from marine sponges using a DC model.

### 3.6.2 Maturation and activation of DCs

Mononuclear cells were isolated from buffy coat obtained from the Icelandic Blood Bank (ethical approval #: 06-068) using Histopaque medium and density gradient centrifugation. The mononuclear cells were washed and $CD14^+$ monocytes purified by magnetic-activated cell sorting (MACS) using CD14 Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The $CD14^+$ monocytes were seeded into 48-well cell culture plates (Nunc, Roskilde, Denmark) at a concentration of $1.0 \times 10^6$ cells/mL in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal cal serum and 1% penicillin and streptomycin (all from Gibco, Life Technology, Paisley, UK) at 37 °C and 5% $CO_2$ for 7 days. Differentiation into DCs was induced by adding 25 ng/mL of IL-4 and 50 ng/mL of Granulocyte-macrophage colony-stimulating factor (GM-SCF) (both from R&D Systems, Bio-Techne, Abingdon, UK) into the cultures, with fresh medium and cytokines added at day 3. On day 7, the DCs were harvested and matured and activated by culturing them in 48-well culture plates at $2.5 \times 10^5$ cells/mL for 24 h with IL-1β at 10 ng/mL, TNFα at 50 ng/mL (both from R&D Systems) and LPS at 500 ng/mL (Sigma-Aldrich, Munich, Germany). Fractions and pure compounds were dissolved in DMSO at a concentration of 10 μg/mL and added to the DCs at the same time as the cytokines and LPS. The final concentration of DMSO in the cultures was 0.002% and the same concentration of DMSO was used as solvent control. After 24 h, the mature and activated DCs were harvested and the supernatant was collected and stored at -80°C for cytokine measurement by Enzyme-linked immunosorbent assay (ELISA). The cells

were collected and stained for flow cytometry analysis to determine the percentage of cells expressing certain surface molecules cluster of differentiation 86 (CD86) and human leukocyte antigen DR isotype (HLA-DR). Cell viability was determined by counting the cells following staining with trypan blue and calculating the percentage of live cells. In some experiments the DCs were co-cultured with allogeneic $CD4^+$ T cells.

### 3.6.3 Co-culture of DCs and allogeneic $CD4^+$ T cells

$CD4^+$ T cells were obtained from mononuclear cells using CD4 Microbeads (Miltenyi Biotec) following the same procedure as for the isolation of $CD14^+$ monocytes described above. DCs matured in the presence/absence of fraction/pure compound at 10 μg/mL were harvested and then seeded in 96-well round bottomed tissue culture plates (Nunc) at $2 \times 10^5$ cells/mL with freshly isolated allogeneic $CD4^+$ T cells at $2 \times 10^6$ cells/mL (DC:T cells ratio at 1:10) for 6 days. Then the cells harvested and the supernatant was collected and stored at -80°C for cytokine measurement by ELISA.

### 3.6.4 Determination of cytokine concentration by ELISA

The concentrations of IL-12p40 and IL-10 in culture supernatants from DCs and of IFN-γ, IL-17 and IL-10 in supernatants from co-cultures of DCs and allogeneic $CD4^+$ T cells were measured by sandwich ELISA using DuoSets from R&D Systems according to the manufacturer´s protocol. The results were given as secretion index (SI), which is the concentration of cytokine in culture supernatant from DCs treated with fraction/pure compound (alone or in co-culture with allogeneic T cells) divided by the concentration of cytokine in culture supernatant from DCs treated with solvent control (alone or in co-culture with allogeneic T cells).

### 3.6.5 Statistical analysis

Data are presented as the mean values ± standard error of the mean (SEM). As the data were not normally distributed, Mann-Whitney U test or Kruskal Wallis one-way ANOVA with Tukey's post-hoc test were used to determine statistical differences between treatments (SigmaStat 3.1, Systat Software, USA) and $p<0.05$ considered statistically significant.

# 4 Results

## 4.1 Bioassay-guided isolation

Unpublished data

In this study, a library of DCM/MeOH extracts from marine sponges collected in the Icelandic waters was screened for potential anti-inflammatory effects using human DC model. In the first round of screening, fractions A, B, C, and D obtained from crude extracts of six different marine sponges (SP1-5 and SP7) were screened, with the results shown in Figure 14. DCs matured in the presence of the A, B, and C fractions from SP1 and SP5, fractions B and C from SP2 and fraction B from SP3 and SP4 secreted less IL-12p40 than DCs matured in the absence of fractions. In contrast, DCs matured in the presence of fractions B and C from SP7 secreted more IL-12p40 than DCs matured in the absence of fractions. Similar effects were observed for IL-10 secretion by DCs matured in the presence of those fractions. Fractions from SP5 were the most potent in inhibiting DC production of IL-12p40 and IL-10, but this was probably due to their toxicity. Therefore, SP5 was omitted from further isolation and the remaining five species were chosen for further bioassay-guided isolation as shown in detail in Figure 15.

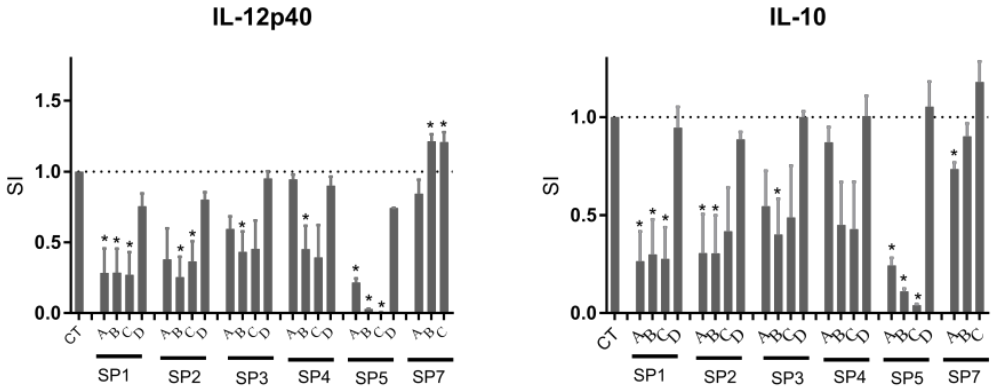



**Figure 14.** The effects of the fractions from marine sponges on DC secretion of IL-12p40 and IL-10.

DCs were matured in the presence of fractions A, B, C and D obtained from extracts of the marine species SP1-5 and SP7 at 10 µg/mL or with solvent only (control, CT). Cytokine concentration in the supernatant was determined by ELISA. Results are shown as secretion index (SI) calculated as concentration of cytokines in supernatant from DCs matured with fractions divided by concentration of cytokines in supernatants from DCs matured without fractions. Data are shown as mean + SEM, n = 2, *$p$<0.05.

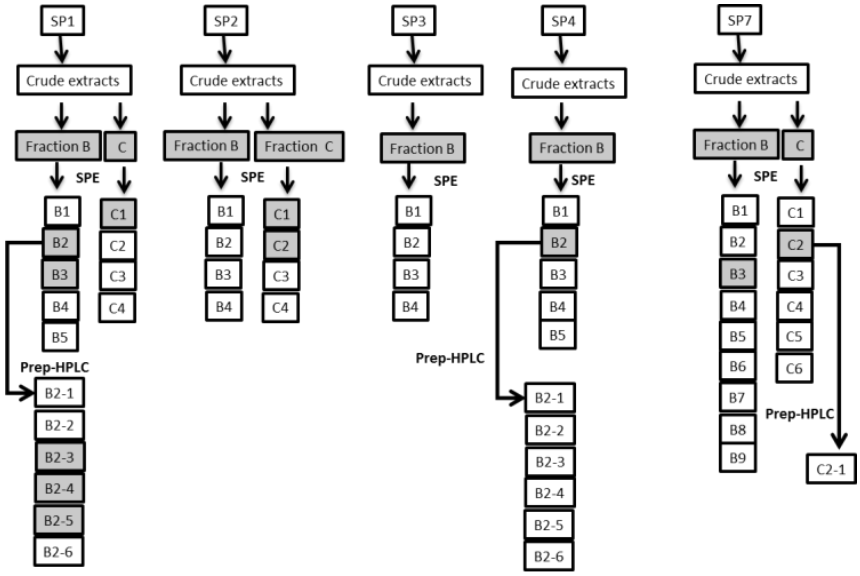



**Figure 15.** Extraction and fractionation flowchart of the five marine different species named SP1-SP5.

Grey color indicates that the fractions/subfractions showed immunomodulatory activity in the DC model.

Some sub-fractions led to inhibition of cytokine secretion; however, several of them were abandoned because of potential toxicity, limited amount available (e.g. SP2C2), or they contained polar constituents which turned out to be difficult to separate with the chromatography methods applied (e.g. SP1C1, SP2C1).

The SP1B fraction was fractioned into several sub-fractions (SP1B1-5). SP1B2 and SP1B3 showed anti-inflammatory effects in the DC model (Figure 16). They were further analyzed by HPLC and found to contain the same main compounds and, therefore, only one of them, SP1B2, was chosen for further fractionation. Fractionation of SP1B2 resulted in three pure compounds SP1B2-3, SP1B-4 and SP1B-5 and three fractions SP1B2-1, SP1B2-2 and SP1B2-6 (Figure 15). The pure compounds SP1B2-3, SP1B2-4, and SP1B2-5 reduced DC secretions of IL-12p40 and SP1B2-3, SP1B2-4 and SP1B2-5 decreased DC secretion of IL-10 (Figure 16). The pure compounds were elucidated by $^{1}$H NMR and $^{13}$C NMR to be PUFAs, i.e. stearidonic acid (SDA) (SP1B2-3) as the NMR and MS data matched that previously published [182], EPA (SP1B2-4) [183] and DHA (SP1B2-5) (Figure 17).

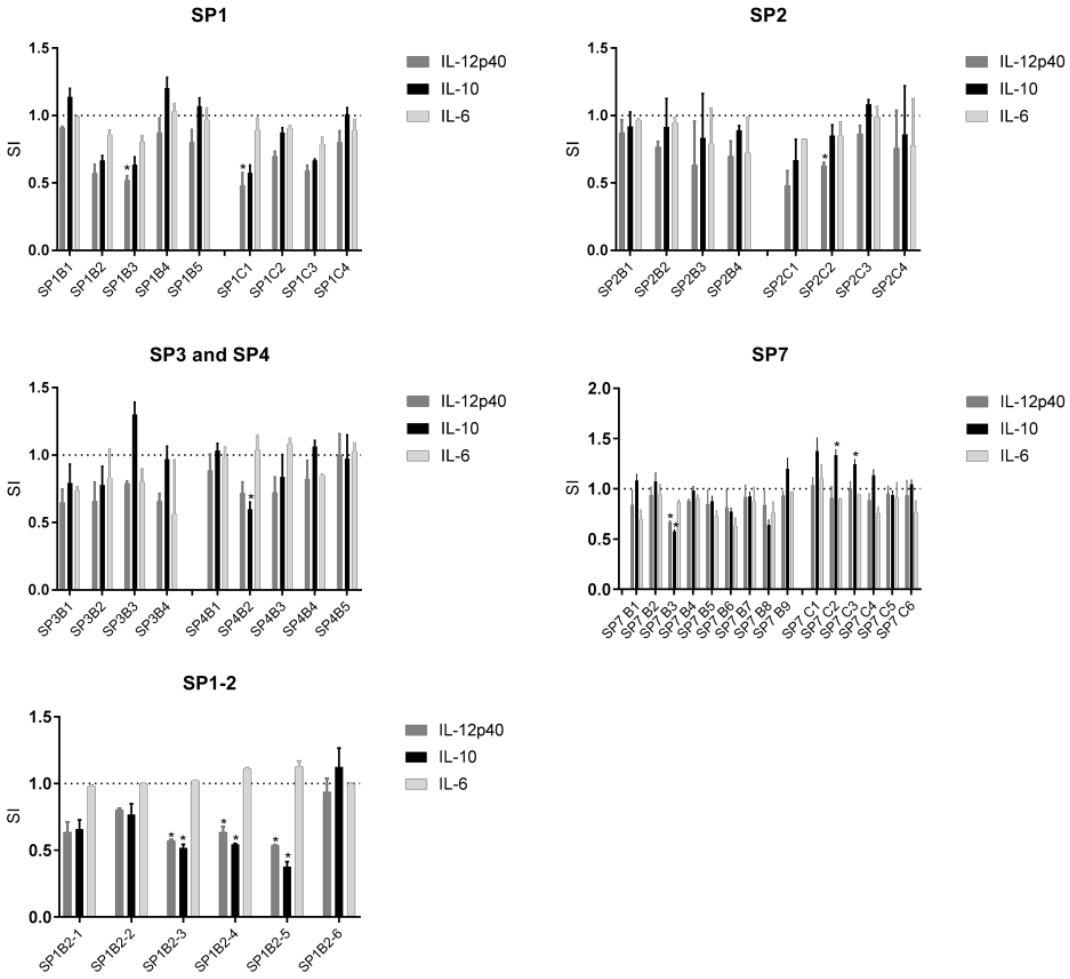



**Figure 16.** The effects of the fractions and pure compounds from SP1-5 and -7 on DC secretion of IL-12p40, IL-10 and IL-6.

DCs were matured in the presence of sub-fractions from marine species SP1-4 and SP7 at 10 µg/mL or with solvent only (control). Cytokine concentration in the supernatant was determined by ELISA. Results are shown as secretion index (SI) calculated as concentration of cytokines in supernatant from DCs matured with fractions divided by concentration of cytokines in supernatants from DCs matured without fractions. Data are shown as mean + SEM, n = 2-7, *$p$<0.05.

The SP4B fraction was fractioned into five sub-fractions (SP4B1-5) (Figure 15). SP4B2 led to reduced IL-10 secretion and had a tendency towards reducing IL-12p40 secretion by DCs (Figure 16). The SP4B2 sub-fraction was subjected to Prep-HPLC, which resulted in four pure compounds, which were determined to be EPA (SP4B2-2), DHA (SP4B2-3), heneicosapentaenoic acid (HPA) (SP4B2-4) [184] and arachidonic acid (AA) (SP4B2-5) [185] (Figure 17).

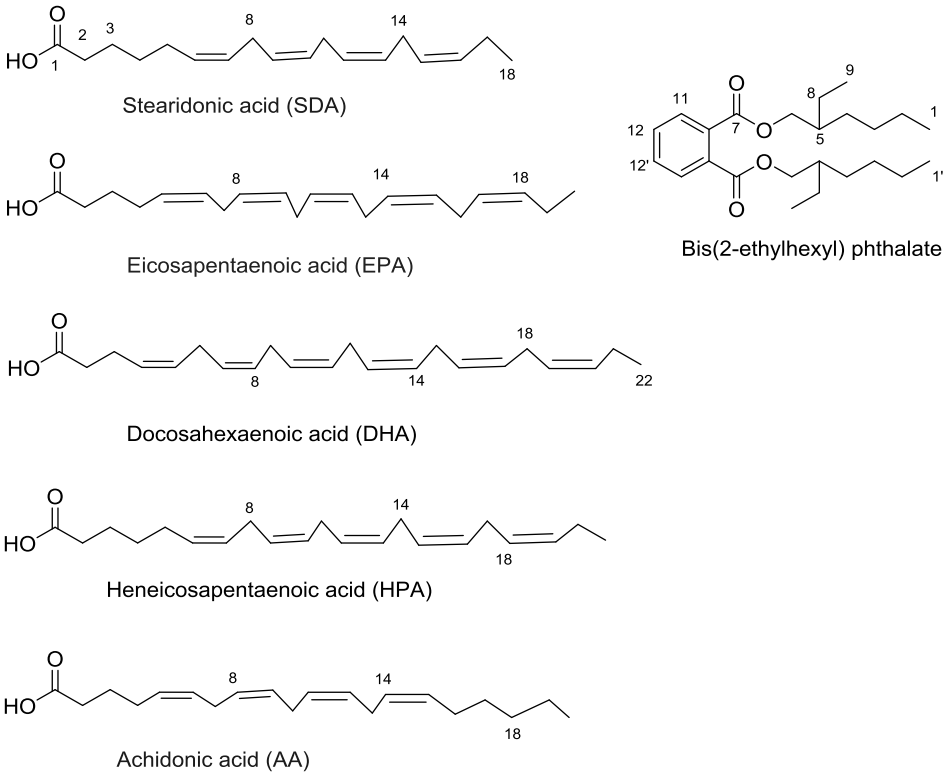



**Figure 17.** Chemical structure of the isolated compounds from the sponge *H. sitiens*.

The SP7B fraction was fractioned into nine sub-fractions (SP7B1-9) (Figure 15). Sub-fraction SP7B3 decreased DC secretion of both IL-12p40 and IL-10 (Figure 16). The SP7B3 contained DHA as shown by HPLC chromatography and TLC staining plate. The SP7C2 increased IL-10 secretion without affecting IL-12p40 secretion by the DCs (Figure 16). The separation of this sub-fraction via Prep-HPLC showed that the main compound in SP7C2 (SP7C2-1) was bis(2-ethylhexyl) phthalate [186]. Bis(2-ethylhexyl) phthalate is a plasticizer and its presence in the SP7 sponge is probably caused by the accumulation of plastic pollution.

As all the pure compounds mentioned above were either PUFAs with previously published anti-inflammatory activities [187, 188] or a plasticizer, further analyses were not conducted.

## 4.2 *Halichondria sitiens*

Paper I and unpublished data

Crude DCM/MeOH extracts of *H. sitiens* were fractionated using modified Kupchan partitioning [180, 181] to obtain fractions A-E. Figure 18 shows the schematic overview of the fractionation process.

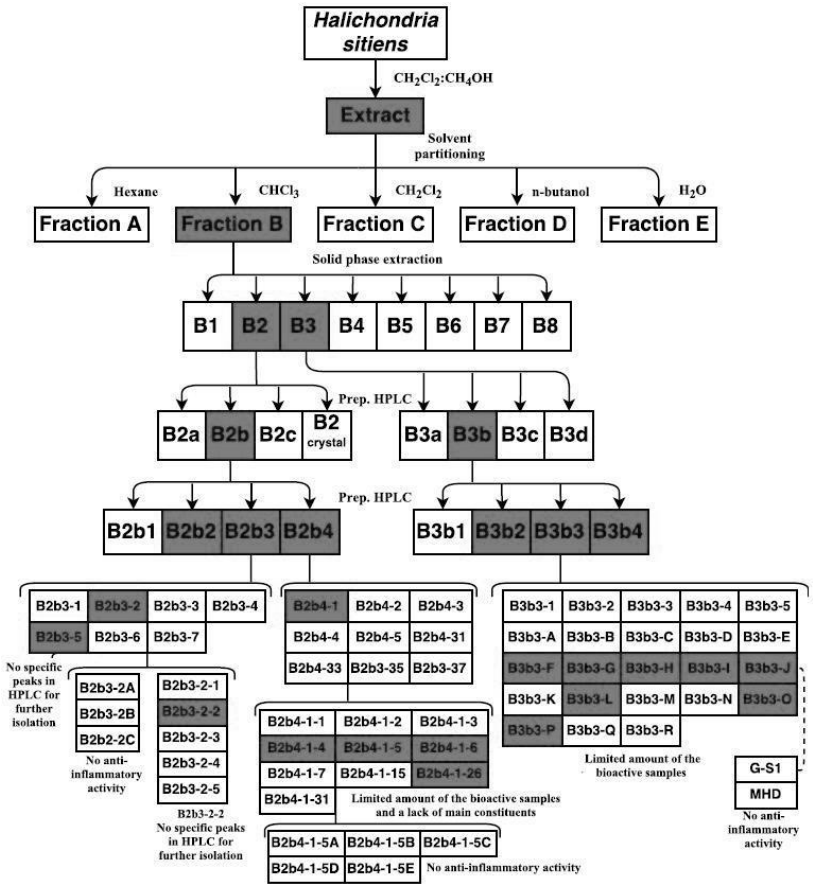
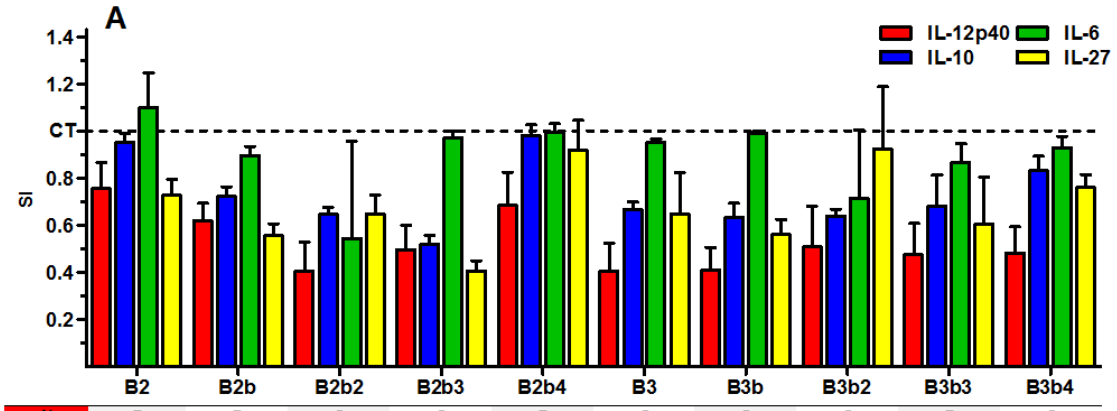


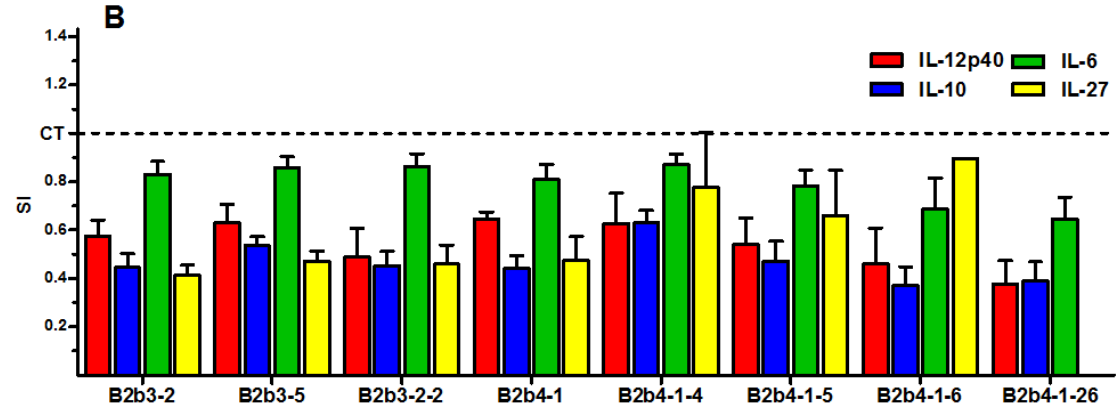

**Figure 18.** Schematic overview of the fractionation process of *H. sitiens*.
At each step in the isolation, the fractions were screened for anti-inflammatory activity and bioactive fractions (colored grey) were selected for further fractionation by various chromatographic techniques, using bioassay-guided isolation. Figure obtained from the master thesis of Jon Thorir Oskarsson.

The effects of the B2 fractions on DC secretion of IL-6 were less pronounced than their effects on IL-12p40 and IL-10 secretion (Figure 19A). Most of the sub-fractions within the B2b fractionation lineage led to around 50% decrease in IL-12p40 secretion by the DCs and decreased IL-10 secretion to similar levels or had no effect on IL-10 production (Figure 19). All sub-fractionation steps were discontinued at some point because of one or more of the following reasons: There was no main constituent in the fraction

(no specific peak in HPLC) (B2b3-5 and B2b3-2-2), there was limited amount of the promising fraction (B2b4-1-4, B2b4-1-6 and B2b4-1-26), the fraction contained a number of fatty acids and glycerides (B2b4-1-26) or the bioactivity was lost after further separation (B2b4-1-5).




| | B2 | B2b | B2b2 | B2b3 | B2b4 | B3 | B3b | B3b2 | B3b3 | B3b4 |
|---|---|---|---|---|---|---|---|---|---|---|
| N | 7 | 7 | 5 | 5 | 7 | 5 | 5 | 5 | 7 | 5 |
| Stat.diff. | * | *** | ** | ** | * | ** | ** | * | *** | ** |
| CT (ng/ml) | 57 ± 13 | 57 ± 13 | 57 ± 13 | 57 ± 13 | 57 ± 13 | 57 ± 19 | 57 ± 19 | 57 ± 19 | 77 ± 18 | 57 ± 19 |
| N | 7 | 7 | 5 | 5 | 7 | 5 | 5 | 5 | 5 | 5 |
| Stat.diff. | ns | *** | ** | ** | ns | ** | ** | ** | * | ns |
| CT (ng/ml) | 5.0 ± 0.99 | 5.0 ± 0.99 | 5.0 ± 0.99 | 5.0 ± 0.99 | 5.0 ± 0.99 | 5.1 ± 1.4 | 5.1 ± 1.4 | 5.1 ± 1.4 | 5.1 ± 1.4 | 5.1 ± 1.4 |
| N | 4 | 4 | 2 | 2 | 4 | 2 | 2 | 2 | 4 | 2 |
| Stat.diff. | ns | * | - | - | ns | - | - | - | ns | - |
| CT (ng/ml) | 27 ± 11 | 27 ± 11 | 45 ± 3.1 | 45 ± 3.1 | 27 ± 11 | 45 ± 3 | 45 ± 3 | 45 ± 3 | 34 ± 6.9 | 45 ± 3.1 |
| N | 4 | 4 | 3 | 3 | 5 | 3 | 3 | 3 | 5 | 3 |
| Stat.diff. | * | * | ns | ns | ns | ns | ns | ns | ns | ns |
| CT (ng/ml) | 8.5 ± 0.4 | 8.5 ± 0.4 | 10 ± 5.0 | 10 ± 5.0 | 6.9 ± 3.5 | 10 ± 5.0 | 10 ± 5.0 | 10 ± 5.0 | 6.5 ± 3.7 | 10.4 ± 5.0 |




| | B2b3-2 | B2b3-5 | B2b3-2-2 | B2b4-1 | B2b4-1-4 | B2b4-1-5 | B2b4-1-6 | B2b4-1-26 |
|---|---|---|---|---|---|---|---|---|
| N | 5 | 5 | 3 | 5 | 5 | 5 | 3 | 4 |
| Stat.diff. | ** | ** | ns | ** | ** | ** | * | * |
| CT (ng/ml) | 81 ± 12 | 81 ± 12 | 108 ± 12 | 77 ± 14 | 81 ± 12 | 81 ± 12 | 108 ± 13 | 108 ± 13 |
| N | 5 | 5 | 3 | 5 | 5 | 5 | 3 | 4 |
| Stat.diff. | ** | ** | ns | ** | ** | ** | ns | * |
| CT (ng/ml) | 6.1 ± 0.99 | 6.1 ± 0.99 | 6.3 ± 1.1 | 6.4 ± 0.77 | 6.1 ± 0.99 | 6.1 ± 0.99 | 3.4 ± 0.69 | 3.4 ± 0.69 |
| N | 5 | 5 | 3 | 5 | 5 | 5 | 3 | 4 |
| Stat.diff. | ** | ** | ns | ** | ** | ** | ns | * |
| CT (ng/ml) | 36 ± 5.5 | 36 ± 5.5 | 40 ± 7.8 | 30 ± 9.2 | 36 ± 5.5 | 36 ± 5.5 | 27 ± 3.9 | 27 ± 3.9 |
| N | 3 | 3 | 3 | 5 | 3 | 3 | 1 | 0 |
| Stat.diff. | ns | ns | ns | ** | ns | ns | - | - |
| CT (ng/ml) | 0.42 ± 0.13 | 0.42 ± 0.13 | 0.4 ± 0.09 | 0.88 ± 0.34 | 0.42 ± 0.13 | 0.42 ± 0.13 | 0.55 ± 0.30 | 0.55 ± 0.30 |

**Figure 19.** The effects of bioactive *H. sitiens* fractions on cytokine secretion by DCs. DCs were matured and activated in the presence or absence of fractions from *H. sitiens* of the first four fractionation steps (A) and the subsequent B2 (B) fractions at 10 μg/mL for 24 h. The levels of IL-12p40 (red), IL-10 (blue), IL-6 (green) and IL-27 (yellow) in the supernatant were determined by ELISA. The results are expressed

as the ratio of cytokine levels in the supernatant of mDCs cultured with *H. sitiens* fractions (T) to cytokine levels in the supernatant of mDCs cultured without fractions (CT) (SI = T / CT). The dotted line indicates that the ratio for the controls is one. The tables below the figures show the number of blood donors; statistical difference between cytokine secretion by cells incubated with the respective fraction and that by cells incubated without fractions; and absolute cytokine levels of corresponding controls (pg/ml). Difference between groups were evaluated using Mann-Whitney U test and determined significant if $p<0.05$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = non-significant. Figure obtained from the master thesis of Jon Thorir Oskarsson.

Fractions of the B3 lineage were, in general, more effective inhibitors of IL-12p40 production than fractions of the B2 lineage but showed less tendency to inhibit IL-10 secretion by DCs (Figure 19A). All B3b3 sub-fractions decreased IL-6 secretion by 10-20% (Figure 3B in paper I). Fractions B3b3F-P led to around 10-60% decrease in the secretion of IL-12p40, with fractions B3b3F-H, B3b3J and B3b3L, having the most pronounced effects. Of all the fractions, only two, B3b3O and B3b3P, enhanced production of IL-10 and two fractions, B3b3F and B3b3J, decreased IL-10 secretion by the DCs (Figure 3C in paper I).

In addition to analyzing the effects of the fractions from *H. sitiens* on DC secretion of cytokines, their effects on the capacity of DCs to activate T cells was investigated by evaluating cytokine production and expression of activation markers by T cells co-cultured with DCs matured in the presence or absence of fractions. The B3b3-F and J fractions were chosen because of their promising effects on cytokine production by DCs and the B3b3P was used for comparison as it showed similar effects in the DC model but to a lesser degree. Fractions B3b3F, B3b3J and B3b3P decreased the ability of DCs to induce T cell secretion of IFN-$\gamma$ (Figure 4 in paper I). The concentration of IL-10 was also decreased in co-cultures of T cells and DCs matured in the presence of B3b3F and B3b3J (Figure 4 in paper I). As IL-10 is produced by both DCs and T cells it cannot be determined whether the IL-10 in the co-cultures was produced by the DCs or the T cells. Chemical screening of the active fractions revealed the presence of glycerides (glycerol esters and glycerol ethers) along with long chain saturated and unsaturated fatty acids and some minor unknown constituents. Most of the fractions had a number of chemical constituents and no major ones, as demonstrated by the number of peaks on the HPLC chromatograms and mass spectra. As the amount of material available was limited, it was not feasible for further isolation. One new glyceride, 2,3-dihydroxypropyl 2-methylhexadecanoate, was isolated from fraction B3b3M and two known compounds, monoheptadecanoin and

3-[(1-methoxyhexadecyl)oxy]propane-1,2-diol, were identified in fraction B3b3J, but none of them had immunomodulatory activity (Figure 1 in paper I).The effects of the fractions from *H. sitiens* on the maturation of the DCs was also determined by evaluating their effects on expression of the co-stimulatory molecule CD86 and the antigen presenting molecule HLA-DR. None of the B2 or B3 fractions affected expression levels or the percentage of cells positive for either CD86 or HLA-DR (data not shown). The viability of DCs cultured with fractions from *H. sitiens* was assessed by trypan blue viability assay and showed no cytotoxic effects of any of the fractions (data not shown). Fraction B3b3 induced morphological changes in the DCs, characterized by extreme elongation of the dendrites and cell clustering (Figure 5 in paper I). Some of the B3b3 sub-fractions had similar, although less pronounced, effects on the morphology of the DCs.

## 4.3 *Geodia barretti*

Paper II and unpublished data

In this study, the raw extract of *Geodia barretti* was comprehensively profiled by UPLC-qTOF-MS method and the data were processed by MassLynx, followed by analysis with the molecular formula and fragmentation information to compare data from ChemSpider and SciFinder databases. The bromoindole derivatives from the marine sponge *G. barretti* were tracked by UPLC-qTOF-MS to guide the subsequent isolation procedure. The results show that the extracts of *G. barretti* are rich in brominated alkaloids, including compounds with molecular weights not previously reported.

The study yielded three new alkaloids, named geobarrettin A-C (**1**-**3**) and four known ones, i.e. barettin (**4**) [56], 8,9-dihydrobarettin (**5**) [60], 6-bromoconicamin (**6**) [189], and L-6-bomohypaphorine (**7**) [190] (Figure 1 in paper II).

The stereochemistry was assigned by NMR spectroscopic data, ECD analysis and Marfey's method. Hydrogenolysis of geobarrettin A (Scheme 1 in paper II) gave debrominated derivate debromodihydrogeobarrettin A with a CD spectrum which was identical with that of (*R*)-3-propyldioxindole, indicating the C-3 configuration is assigned as *R*. After Marfey´s analysis, D- and L-Arg-DTA derivatives were observed with integral values of 19:81 suggesting natural geobarrettin A is partially racemic at C-12. The absolute stereochemistry of barettin was re-evaluated using Marfey´s method. It showed that barettin was partially racemic as well, but mainly 12*S*, due to the presence of D- and L-Arg-DTA in a ratio of 19:81 on HPLC chromatography.

The isolated compounds were screened for immunomodulatory activity using the DC model. Of the new compounds, geobarrettin B (**2**) decreased DC secretion of IL-12p40 without affecting production of IL-10 and geobarrettin C (**3**) decreased DC secretion of IL-12p40 and concomitantly increased their IL-10 production (Figure 4 in paper II). Maturing DCs in the presence of geobarrettin B (**2**) or geobarrettin C (**3**) before co-culturing them with allogeneic $CD4^+$ T cells led to a decrease in T cell secretion of IFN-$\gamma$ (Figure 6 in paper II), indicating a reduction in Th1 differentiation of the T cells. The known compound barettin (**4**) decreased DC secretion of both IL-12p40 and IL-10 and showed the most potent activity of the compounds tested with $IC_{50}$ being 11.80 μM for IL-10 and 21.04 μM for IL-12p40 (Figure 5 in paper II). However, maturing DCs in the presence of barettin (**4**) did not affect their ability to induce T cell secretion of either IFN-$\gamma$ or IL-17, but led to reduced secretion of IL-10 (Figure 6 in paper II), i.e. had no effect on Th1 or Th17 polarization.

In addition to the compounds described above, one novel 6-bromoindole alkaloid, named as geobarrettin D (**9**), was obtained (Figure 20).

The mass spectrum of geobarrettin D (**9**) showed the highest peak m/z 399.0618/401.0602 in a 1:1 ratio, indicating the presence of bromine in the molecule and the molecular formula $C_{17}H_{16}{}^{79}BrN_6O$. The $^{13}C$ NMR spectra showed 20 carbons which could not match with the molecular formula from mass spectrum, including five methyls ($\delta_C$ 54.8 (×3), 32.1, and 36.2), one methylene ($\delta_C$ 69.3), one heteroatom-bound aliphatic methine ($\delta_C$ 45.4), four aromatic methines ($\delta_C$ 125.9, 120.8, 124.2 and 115.9), six quaternary aromatic carbons ($\delta_C$ 113.4, 125.2, 117.0, 139.1, 140.1, and 110.0), three quaternary heteroatom-bound $sp^2$ carbons ($\delta_C$ 155.0, 154.9 and 151.0) (Table 6). After amplifying the mass spectrum, another peak at *m/z* 458.1300/460.1281 was identified, indicating the molecular formula to be $C_{20}H_{25}{}^{79}BrN_7O$. This molecular formula could match the numbers of carbon signal in $^{13}C$ NMR. The highest peak at 399.0618/401.0602 was obtained by losing a *N,N,N*-trimethylethanaminium group. IR absorptions (3254, 1182, and 1131 $cm^{-1}$) implied the presence of OH and/or NH functionalities. The $^1H$ NMR in $D_2O/H_2O$ (10/90) showed an exchangeable NH proton at $\delta_H$ 10.60 (1H, d, *J* = 2.0 Hz, NH-1′), three aromatic resonances with AB-X pattern at $\delta_H$ 7.65 (1H, d, *J* = 8.5 Hz, H-4′), 7.24 (1H, dd, *J* = 8.5, 1.6 Hz, H-5′) and 7.60 (1H, d, *J* = 1.6 Hz, H-7′), suggesting the presence of a tri-substituted benzene ring, and an isolated aromatic proton at $\delta_H$ 7.57, which showed a weak coupling (*J* = 2.0 Hz) with the NH proton. The combined use of HMBC data (Table 6) led to the assignment of a 3-carbonyl-6-bromo-indol-3-yl residue. It can be supported by

the UV spectrum showing the characteristic indole chromophore with absorptions at 228, 261 and 287 nm. The $^{1}$H-$^{1}$H COSY correlations from H-2′′ ($\delta_H$ 6.05 (1H, t, *J* = 6.3 Hz)) to H-1′′ ($\delta_H$ 4.07 (1H, dd, *J* = 13.7, 5.9 Hz); 4.15 (1H, dd, *J* = 13.7, 6.8 Hz)) and HMBC correlations $CH_3$-*N* ($\delta_H$ 3.30 (9H, s))/C-1′′ ($\delta_C$ 69.3), H-1′′/C-2′′ ($\delta_C$ 45.4) and H-2′′/C-1′′ indicate the presence of 2,2-disubstituted *N*,*N*,*N*-trimethylethanaminium group. The connectivity of 3-carbonyl-6-bromo-indol-3-yl moiety and *N*,*N*,*N*-trimethylethanaminium group was supported by the HMBC correlations H-2′′/C-3′ ($\delta_C$ 113.4), H-2′′/C-2′ ($\delta_C$ 125.9), H-2′′/C-3a′ ($\delta_C$ 125.2), and H-1′′/C-3.

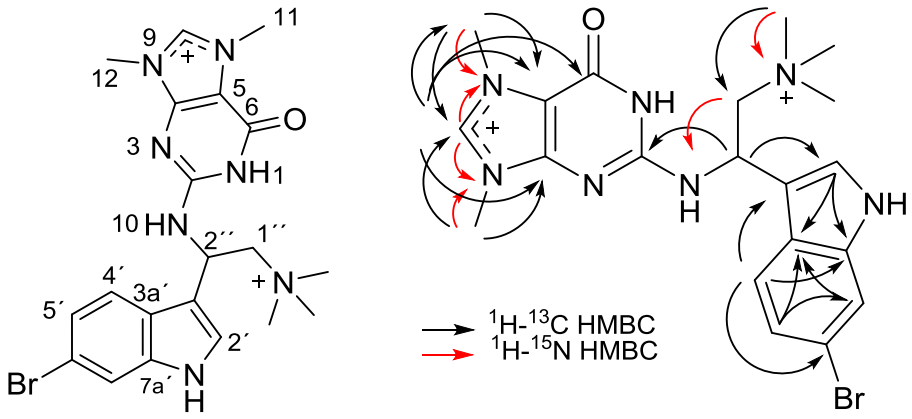



**Figure 20.** Key HMBC (H→C) and $^{1}$H-$^{1}$H COSY (**—**) correlations of geobarrettin D (**9**).

Considering the molecular formula $C_{17}H_{16}{}^{79}BrN_6O$ of geobarrettin D (**9**), in adjunction with the 3-substituted 6-bromoindole and 2,2-disubstituted *N*,*N*,*N*-trimethylethanaminium moieties, it was implied that there is an additional $C_7N_5H_8O$ moiety with 6 degrees of unsaturation connected to C-8. The HMBC cross-peak H-2′′/C-2 ($\delta_C$ 154.9) revealed that the $C_7N_5H_8O$ unit was connected to C-2′′ via nitrogen, which can explain the chemical shift of C-2′′ at $\delta_C$ 45.4. Analysis of the $^{13}$C NMR data, showed the seven remaining carbons to be five quaternary sp carbons with chemical shifts of $\delta_C$ 154.9, 155.0, 151.0, 140.1, 110.0 and two sp$^3$ carbons $\delta_C$ 32.1, 36.2 (Table 6). Only one proton signal $\delta_H$ 8.81 in $D_2O$/$H_2O$ solvent was observed using a variety of different NMR experiments ($^{1}$H, $^{1}$H−$^{15}$N HSQC, $^{1}$H-$^{15}$N HMBC in $D_2O$/$H_2O$) and deuterated solvents ($CD_3OD$, $D_2O$/$H_2O$ 10/90). The chemical shift of C-2′′ and the correlations decided the correlations from H-8 ($\delta_H$ 8.81 (1H, s)) to N-9 ($\delta_N$ 157.3), N-7 ($\delta_N$ 156.4), C-11 ($\delta_C$ 36.2), C-4 ($\delta_C$ 151.0), from $CH_3$-12 ($\delta_H$ 3.91 (3H, s)) to N-9, from $CH_3$-11 ($\delta_H$ 4.11 (3H, s)) to N-7 in $^{1}$H−$^{15}$N HMBC and $^{1}$H−$^{13}$C HMBC spectra established the 7,9-dimethyl-2-(*N*-amino)guaninium unit that connected to C-2′′ via N-10. Moreover, the NMR data are in good agreement with the compound 7,9-dimethyl-2-(*N*-methylamino)guaninium

chloride. However, significant structural ambiguity remained due to the large number of adjacent quaternary carbons and non-protonated nitrogen atoms, along with the lack of some observable NH signals. Thus, NMR experiments running in DMSO-$d_6$ for this compound is still needed, which is the next step for further study.

**Table 6.** NMR spectroscopic data for geobarrettin D (**9**)

| **No.** | $\delta_H$ [a] | $\delta_H$ [b] | $\delta_C$ [a] | $\delta_N$ [b] | $^1$H-$^{13}$C HMBC [a] | $^1$H-$^{15}$N HMBC [b] |
|---|---|---|---|---|---|---|
| N-1′ | | 10.60 (1H, d, 2.5) | | 131.3 | | |
| 2′ | 7.57 (1H, s) | 7.53 (1H, d, 2.5) | 125.9 | | C-1′′, 3′, 5′, 3a′, 7a′ | |
| 3′ | | | 113.4 | | | |
| 3a′ | | | 125.2 | | | |
| 4′ | 7.65 (1H, d, 8.5) | 7.12 (1H, d, 9.4) | 120.8 | | C-3′, 6′, 3a′, 7a′ | |
| 5′ | 7.24 (1H, dd, 8.5, 1.6) | 7.45 (1H, dd, 8.9, 1.9) | 124.2 | | C-7′, 3a′ | |
| 6′ | | | 117.0 | | | |
| 7′ | 7.60 (1H, d, 1.5) | 7.61 (1H, d, 1.9) | 115.9 | | C-6′, 5′, 3a′ | |
| 7a′ | - | - | 139.1 | | | |
| 2′′ | 6.05 (1H, t, 6.3) | 6.05 (1H, t, 5.9) | 45.4 | | C-1′′, 3′, 3a′, 2′, N-10 | |
| 1′′ | 4.07 (1H, dd, 13.7, 5.9)<br>4.15 (1H, dd, 13.7, 6.8) | 4.07 (2H, m) | 69.3 | | C-2′′, 3′, -N($CH_3$)$_3$ | N-10 |
| N-3′′ | | | | 47.9 | | |
| -N($CH_3$)$_3$ | 3.30 (9H, s) | 3.27 (9H, s) | 54.8 | | C-2′′, , 1′′ | N-3′′ |
| N-1 | | | | | | |
| 2 | | | 154.9 | | | |
| 3 | | | | | | |
| 4 | | | 151.0 | | | |
| 5 | | | 110.0 | | | |
| 6 | | | 155.0 | | | |
| N-7 | | | | 156.4 | | |
| 8 | 9.01 (1H, s) | 8.81 (1H, s) | 140.1 | | C-11, 4, 5 | N-7, 9 |
| N-9 | | | | 157.3 | | |
| N-10 | | | | 96.3 | | |
| 11 | 4.11 (3H, s) | 3.84 (3H, s) | 36.2 | | C-5, 8 | N-7 |
| 12 | 3.91 (3H, s) | 3.92 (3H, s) | 32.1 | | C-4, 8 | N-9 |

[a] Recorded in $CD_3OD$ at 600 MHz; [b] Recorded in $D_2O/H_2O$ (10/90) at 600 MHz.

To support the accurate structure of geobarrettin D (**9**), the HRESIMS spectrum and $MS^2$ spectrum were used to obtain the fragmentation but unfortunately the fragmental information cannot solve the problem. Thus, this compound was crystallized to give single crystals suitable for X-ray diffraction studies. However, initial tests of vapor diffusion with EtOH resulted in oils that were not suitable for X-ray analysis. Optimization of crystal growth by a limited set of solvent/anti-solvent (MeOH/diethyl ether, EtOH/diethyl ether, EtOAc/hexane, ACN/diethyl ether, and acetone/hexane) was eventually not successful for growing crystal, although the vapor diffusion, evaporation, and layering techniques were applied. Thus, the crystal of this compound could not be obtained.

Geobarrettin D (**9**) decreased DC secretion of IL-12p40 by 47%, without affecting secretion of IL-10 at 10 μg/mL (Figure 21), which suggests an overall anti-inflammatory activity of this compound.

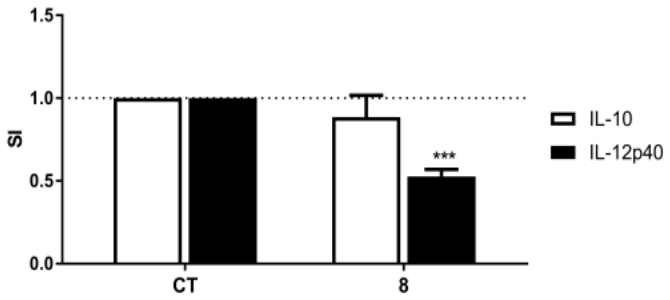



**Figure 21.** The effects of geobarrettin D (**9**) on DC secretion of IL-12p40 and IL-10. DCs were matured and activated in the absence (solvent control (CT)) or presence of geobarrettin D (**9**) at 10 μg/mL. The concentrations of IL-12p40 and IL-10 were determined by ELISA. The data are presented as SI, i.e. the concentration of each cytokine in the supernatant of cells in the presence of compounds divided by the concentration of the cytokine in the supernatant of cells in the presence of the solvent. The results are shown as mean + SEM, n = 3. Different from CT: ***$p$<0.001.

## 4.4 *Flustra foliacea*

Paper III and unpublished data

The combined MeOH and DCM extract of *Flustra foliacea* was subjected to UPLC-qTOF-MS analysis and the data processed by MassLynx. The results were submitted for database searching using the online ChemSpider database to obtain comprehensive chemical profiling, resulting in identification of both previously known as well as new compounds. The crude extract was worked-up by using a combination of silica gel, Sephadex LH-20 and

preparative RP-HPLC to provide fifteen new alkaloids (**1**-**15**) (Figure 1 in paper III), including thirteen new bromotryptamine-based alkaloids, flustramine Q-W (**1**-**7**) and flustraminol C-H (**8**-**13**) and two new imidazole alkaloids, flustraimidazole A-B (**14**-**15**). Their structures were established by detailed spectroscopic analysis, including $^{1}$H NMR, $^{13}$C NMR, $^{1}$H-$^{1}$H COSY, HSQC, HMBC, NOESY, HRESIMS, optical rotation and ECD spectra (Figures 1-4 in paper III).

In addition to the fifteen new alkaloids described, three alkaloids that have been reported in synthetic papers were identified but were isolated for the first time from natural sources. These three alkaloids are $N_a$-methyldeformylflustrabromine (**16**), (3a,8a)-6-bromo-1,8-dimethyl-3a-(2-methylbut-3-en-2-yl)-1,2,3,3a,8,8a-hexa-hydropyrrolo[2,3-*b*]indole (**17**) [91] and 6-bromo-*N*,*N*-dimethyltryptamine (**18**) [62]. Nine known compounds isolated from *F. foliacea*, namely 2-[6-bromo-1*H*-indol-3-yl]-*N*-methyl-ethanemine (**19**) [191], flustramine E (**20**) [78], dihydroflustramine C (**21**) [80], flustramine I (**22**) [94], flustramine B (**23**), flustramine A (**24**) [88], flustramine C (**25**) [88], 6-bromoindole-3-carbaldehyde (**26**) [192] and deformylflustrabromine B (**27**) [85] were identified on the basis of a comparison of their spectroscopic data with those reported in the literature.

The compounds were tested for their immunomodulatory activity using the DC model. Eight of the compounds decreased DC secretion of IL-12p40 (**1**, **3**, **5**, **13**, **16**, **18**, **26** and **27**) and two of them increased secretion of IL-10 (**4** and **14**) (Figure 5 in paper III). None of the active compounds affected viability of the DCs, at the concentration 10 µg/mL. Deformylflustrabromine B (**27**) showed the most potent anti-inflammatory effect ($IC_{50}$ 2.90 µM) (Figure 6 in paper III). These results show that *F. foliacea* contains a variety of brominated alkaloids, where several have anti-inflammatory activity, with the activity of the most potent one (**27**) warranting further investigation into its potential as a candidate for relieving inflammatory diseases.

# 5 Discussion

## 5.1 Bioassay-guided isolation

Bioassay-guided isolation procedures have been widely used for the isolation of bioactive compounds from plants and marine organisms. In this study, after screening over hundred extracts, several fractions from marine sponges were found as positive hit specimens with anti-inflammatory effects using the DC model. The bioassay-guided isolation procedure indicated that PUFAs were the main contributors of the anti-inflammatory effects. These results demonstrated, as has been illustrated previously, that bioassay-directed isolation can eliminate inactive compounds early in the process, thus avoiding unnecessary work in the search for active compounds. However, this approach can also, after a great deal of work, lead to rediscovery of known bioactive compounds, like the known PUFAs that were identified in this study. Thus, the bioassay-guided isolation approach can be very efficient but another dereplication-guided isolation approach may be needed intermittently to increase the effectiveness of the procedure in finding new active compounds.

In the present study, the isolated fatty acids SDA, EPA, and DHA decreased IL-12p40 and IL-10 secretion by DCs. The pro-inflammatory cytokine IL-12p40 is shared by the cytokines IL-12 and IL-23, which direct polarization of naïve T cells into Th1 or Th17 subtypes, respectively, phenotypes characterized by secretion of the pro-inflammatory cytokines IFN-$\gamma$ or IL-17, respectively [113, 116, 119, 120], whereas, the anti-inflammatory cytokine IL-10 is a major determinant of differentiation of naïve T cells into a T regulatory phenotype [115, 123]. Thus, the effects of SDA, EPA and DHA on cytokine secretion by DCs may suppress their potential to direct T cell maturation towards the Th1 and the Treg phenotypes. Several studies have reported anti-inflammatory effects of these fatty acids, especially that of EPA and DHA, in a variety of cell culture studies (neutrophils, macrophages, DCs, T lymphocytes, and endothelial cells), animal models, trials in healthy human volunteers and clinical trials in various patient groups [193-195]. The current study showed that PUFAs inhibited the IL-10 secretion, which suggests a possible pro-inflammatory activity, not anti-inflammatory. Interestingly, the result obtained in the present study is in agreement with a report on human monocyte-derived DC generated in the

presence of DHA [196]. Exposure to DHA inhibited the production of pro-inflammatory molecules, i.e. IL-6, TNFα, CCL4, and of the anti-inflammatory cytokine IL-10. EPA and AA displayed the same pattern but with reduced inhibitory effects.

It was not surprising to find in the present study that Icelandic marine sponges are a good source of omega-3 PUFAs as high proportions of EPA and DHA have been recorded in sponges from cold climates [197]. However, sponges are heterotrophic organisms and cannot synthesize all the fatty acids necessary to them. Thus, they may be derived from the primary producers being photosynthetic microalgae, heterotrophic protists and bacteria [197].

As mammals cannot synthesize essential PUFAs, they are considered essential nutrients. They are important for a number of functions, including cognitive and cardiovascular functions [198, 199]. As was mentioned in chapter 1.1, omega-3-acid ethyl esters (Lovaza®) has been on the market for treating hypertriglyceridemia and omega-3 fish oil is now one of the most commonly consumed dietary supplement [31, 200]. Although the presence of omega-3 PUFAs in Icelandic marine sponges may point out to it as a potential source of these fatty acids, they are not likely to be a source for large-scale production of essential PUFAs with commercial applications in the nutraceutical and pharmaceutical industries. One major obstacle is the limited availability of large quantities of sponge material. Recently, some studies have set up and used *in vitro* culture systems to culture sponge cells. The major advantage of cell cultures is the possibility to control the target metabolites by changing the cultivation conditions. However, to date, cell and primmorph cultures are not feasible at present for producing large amounts of biomass [201].

## 5.2 *Halichondria sitiens*

Extract from the sponge *H. sitiens* affected cytokine secretion by the DCs and was, therefore, fractioned further. This resulted in several lipophilic fractions that were shown to contain compounds that decreased DC secretion of the pro-inflammatory cytokines IL-12p40 and IL-6. IL-6 directs polarization of naïve T cells into a Th17 subtype, a phenotype characterized by secretion of the pro-inflammatory cytokine IL-17. Only a few of the fractions decreased secretion of the anti-inflammatory cytokine IL-10 and to a much less extent than that of IL-12p40. Therefore, the overall effects of these fractions were considered anti-inflammatory.

The lipophilic fractions obtained from *H. sitiens* contained many components, which required a number of fractionation steps to obtain pure compounds. Unfortunately, for many of the fractions this led to a lack of material available for further fractionation and isolation of pure compounds was, therefore, not possible. This is a common problem and one of the key challenges in isolation of natural products, especially when the fractions are as complex as observed for these lipophilic fractions. To solve this problem, raw material in sufficient quantity has to be obtained, which is not always possible. No pure immunomodulatory compounds were obtained from the *H. sitiens* extract due to this limitation. Logistical and ecological reasons limit the quantity of the sponge material that can be collected. In addition, the secondary metabolites in marine invertebrates are affected by the collection environment, including the collection site, the season and the climate. Hence, it would be essential to obtain enough initial sponge material to have the desired quantity as following collection of material might not display the same chemical profiling.

Three of the lipophilic fractions from *H. sitiens* were chosen for further examination of their effects on the potential of DCs to induce differentiation of T cells into different subtypes. The results revealed that DCs matured and activated in the presence of two of the fractions, B3b3F and B3b3J, reduced IFN-$\gamma$ secretion by co-cultured allogeneic $CD4^+$ T cells, with only a modest reduction in IL-10 secretion, thus decreasing the ability of the DCs to induce a Th1-type immune response. The decreased concentration of IL-10 in co-culture supernatants was unexpected as these fractions had either no effect or had increased IL-10 secretion by the DCs, when they were cultured without the T cells. Therefore, the reduced levels observed in the co-cultures were most likely the result of reduced secretion by the T cells but not the DCs. Whether this is the case could be determined using intracellular staining for IL-10 in T cells co-cultured with DCs matured in the presence or absence of the fractions. Despite the slight decrease in IL-10 secretion, the downregulation of IFN-$\gamma$ secretion by T cells co-cultured with DCs matured in the presence of the two fractions is strongly suggestive of inhibition of inflammatory Th1 response and subsequently that components in these two fractions may have the potential of being anti-inflammatory drug leads.

The bioactive fractions obtained from *H. sitiens* in the present study contained mostly glycerol esters and glycerol ethers featuring acyl and alkyl ether chains. A small number of long-chain glycerol esters (also called acyl glycerols) and ethers have been found in marine sponges, either in the phospholipid fraction or as free derivatives [202, 203]. The fatty acids linked to

glycerol moiety via ester or ether bonds in the glycerol esters and ethers can have different lengths of chains, an odd number of carbon atoms, and/or methyl-branched chains. In general, glycerol esters are more common than glycerol ethers, even though the ether bond is more stable than ester or amide bonds. The new glycerol ester isolated in the present study is a methyl-branched fatty acid linked to glycerol via an ester bond. Methyl branching may be introduced into fatty acids through the addition of carbon units from *S*-adenosyl methionine donor or by synthesis of an isoprenoid unit by biosynthesis pathway [204, 205].

Glycerol ethers and esters have been recognized as components of marine animal liver oils [206] and gut walls of a sea cucumber. However, in the case of the sea cucumber the glycerol ethers and esters may be attributed to gut microbes [207]. Glycerol ethers occur at relatively high abundance in marine sediments from hydrothermal vents [208] which might explain the presence of glycerides in *H. sitiens* collected in Icelandic waters. A variety of glycerol ethers have been reported from pure strains of bacteria and from diverse natural settings covering a large range of environmental conditions [209]. Although those glycerides have also been found in marine invertebrates [210, 211], their nonisoprenoid structure and short-chain carbon skeleton (14-20) supports a bacterial origin. Therefore, it would be interesting to study metabolites of the bacteria associated with *H. sitiens* to determine whether they or the sponge is the real producer.

## 5.3 *Geodia barretti*

Encouraged by the advantage of UPLC-qTOF-MS dereplication-guided strategy and the anti-inflammatory activity of the previously isolated compound barettin, the aim of this part of the study was to investigate the anti-inflammatory potential of brominated alkaloids from the marine sponge *G. barretti* using UPLC-MS-guided dereplication methodology. Four new 6-bromoindole derivatives, geobarrettin A-D, and four known alkaloids were obtained.

Several studies have used UPLC-MS-guided dereplication methodology to successfully drive separation of new compounds, including terpenoids [212] and steroids [213]. The application of UPLC-MS-guided dereplication methodology in the present study, providing molecular formula and fragmentation patterns of chemical structures of secondary metabolites, gave a hint to find hit compounds and allowed for a rapid separation of unknown compounds. The successful and efficient discovery of new bromoindole

derivatives in this study demonstrated this to be a powerful tool, and useful for the characterization and identification of different classes or types of natural products.

Geobarrettin A, one of the new compounds identified in the present study, features 6-bromo-3-hydroxy-oxindole skeleton and DKP-type cyclic dipeptide substructure. It is an oxidative analogue of barettin through oxidative cleavage between the C-2 and C-3 bond of the indole ring of barettin to produce the 3-hydroxy-oxindole moiety. 3-Substituted-3-hydroxy-oxindoles have been isolated from diverse sources ranging from terrestrial to marine origins. The brominated 3-hydroxy-oxindoles have been found in marine bryozoa *Amathia convoluta* [214, 215], however the current study is the first to demonstrate isolation of a brominated 3-hydroxy-oxindole from a marine sponge.

The stereochemistry of geobarrettin A and barettin was secured by Marfey's method, based on the determination of the amino acid composition in peptide hydrolysates, using the new chiral reagent L-FDTA. The general Marfey´s-type reagent *N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) is quite stable, neutral and apparently not easily racemized. However, it is not a good choice for determination of the stereochemistry of acidic, basic and hydroxyl amino acids, due to the poor resolution of the FDAA-derivatives of the L- and D-amino acids in the HPLC analysis [216, 217]. The corresponding L-FDTA derivatives of L- and D-amino acids are well-resolved [217, 218].

Application of this method to determine the absolute configuration of geobarrettin A showed that it is partially racemic at C-12. Barettin isolated in the present study was also partially racemic, as D- and L-Arg were in a ratio of 19:81 and L-Arg, therefore, predominant, which is consistent with partially racemic natural barettin in a sample obtained from Swedish waters [57]. The possibility that Arg have undergone partial racemization under the conditions of hydrolysis is ruled out by precedence; Arg essentially is stable to the conditions of hydrolysis.

DKPs are quite ubiquitous in Nature and have been found in marine invertebrates, bacteria, fungi and higher plants [219]. To date, more than 200 DKPs have been derived from marine organisms, particularly marine microorganisms. DKPs more often originate from the marine-derived fungi than from other marine organisms [220]. Most DKPs are formed using the biosynthetic pathway of non-ribosomal peptide synthetase (NRPS) [219, 221]. However, to the best of our knowledge, tryptophan biosynthesis begins with the shikimic acid pathway [222], which is lacking in metazoans [223, 224].

Therefore, it is possible that these metabolites are not produced by the sponges themselves.

A DKP molecule, cyclo(L-phenylalanyl-L-proline), suppressed the macrophage production of pro-inflammatory cytokines, NO, and ROS and inhibited host innate immune responses through the NF-κB pathway [225]. In addition, barettin has been shown to have anti-inflammatory effects by inhibiting macrophage secretion of IL-1β and TNFα [66]. The observations described above indicate that DKPs could be a source of potential immunomodulatory agents. The new compounds geobarrettin B and C decreased DC secretion of IL-12p40 and reduced IFN-γ secretion by co-cultured T cells, hence reduced Th1 responses and may, therefore, have the potential of becoming anti-inflammatory drug leads.

As mentioned above, barettin has previously been shown to have anti-inflammatory effects by inhibiting macrophage secretion of IL-1β and TNFα and reducing the production of IL-10, possibly via inhibition of kinases [66, 67]. However, when DCs were treated with barettin in the present study and then co-cultured with T cells, the effects of barettin were not anti-inflammatory as secretion of Th1 and Th17 cytokines was not affected and secretion of the anti-inflammatory cytokine IL-10 was reduced. In fact, these results suggest a pro-inflammatory effect of barettin. Had the immunomodulatory effects of barettin, in the present study, only been studied using DCs cultured by themselves, the conclusion would have been that it was immunosuppressive as it decreased DC secretion of both IL-12p40 and IL-10. But the effect of barettin to decrease DC secretion of IL-10 seems to be the determining factor in the effects of barettin on co-cultured T cells and overriding the downregulation of DC secretion of IL-12p40. These results demonstrate that the immunomodulatory effects of natural compounds need to be studied in more than one setting to gain insight into their real effects. The unexpected results might also be explained by the different sources of barettin used in the present and the previous studies. In the previous study, the synthetic barettin assigned as 12*S* was used, whereas in the present study the partially racemic barettin from a natural source was used.

Despite structural similarities of the series of 6-bromoindole derivatives with the DKP-type cyclic dipeptide unit, there were remarkable differences in their anti-inflammatory effects. Analysis of the SAR revealed that the anti-inflammatory activity of the 6-bromoindole derivatives may depend on the bromotryptophan nucleus and the side chain at C-3 position of the 6-bromoindole.

Collectively, the results from this part of the thesis indicate that *G. barretti* is a source of 6-bromoindoles with potential anti-inflammatory activities, which may become lead compounds for the treatment of inflammatory diseases in the future.

## 5.4 *Flustra foliacea*

Chemical investigation of the marine bryozoan *F. foliacea* resulted in isolation of thirteen new bromotryptamine-based alkaloids and two new imidazole alkaloids, together with twelve previously described compounds. It suggests that marine invertebrates collected in Icelandic waters can be a rich source of structurally diverse new compounds. The current research greatly extends the chemical space of brominated alkaloids and enriches our knowledge of secondary metabolites from *F. foliacea*.

Although there is much higher concentration of chloride than bromide in the oceans (bromide 65 mg/L; chloride 19,000 mg/L) [162], marine organisms can oxidize bromide more easily and incorporate it into organic compounds. From a survey of the literature, it is evident that extensive bromination can occur at all positions in the carbocyclic aromatic ring of indole alkaloids and some of them were found in marine invertebrates [162, 226]. Interestingly, bromination of the indoles isolated from *F. foliacea* generally occurs at C-6 of the indole ring and only a few of them occur at C-7.

Quinolinones are relatively common in Nature, whereas brominated quinolinones are rare [227]. The brominated quinolinone, flustramine Q, obtained from *F. foliacea* in the present study is a rearranged product from prenylated indole. A plausible biosynthesis pathway can be from the brominated alkaloid flustramine E [78] through methylation, 1,2-alkyl shift, oxidation and dehydration to forge the quinolinone skeleton. To the best of our knowledge, this is the first example of rearrangement leading to a brominated quinolinone.

Naturally occurring 1,4-thiazine-1,1-dioxide derivatives are relatively rare in Nature, but an increasing number of 1,4-thiazines have been found in marine invertebrates [228], including the compounds euthyroideone A-C from the marine bryozoan *Euthyroides episcopalis* [228]. A substructure search of the MarinLit database using this ring fragment identified less than 30 analogues. However, most of them are featuring the 1,1-dioxo-1,4-thiazine ring fused with the quinone unit. Flustramine R obtained in the present study is a 6-bromoindole featuring an independent 1,1-dioxo-1,4-thiazine ring. The biosynthesis of 1,4-thiazines has been studied in detail in some cases, and

the evidence suggests that they are mainly derived from cysteine or its derivatives [219]. We propose that the compound flustramine R is most likely biosynthesized from 6-bromo-*N*-methyltryptamine, which could undergo methylation, prenylation, oxidation by cysteine dioxygenase, decarboxylation and cyclization to form the 1,1-dioxo-1,4-thiazine ring [229].

In addition, flustramine S, as a rearranged benzoazetinone derivative, was also identified from *F. foliacea*. The monocyclic 2-azetidinone ring system is the common structural feature of a number of broad spectrum β-lactam antibiotics [230-232]. However, benzoazetinones, which are composed of β-lactam fused with a benzene ring, is an unusual class of natural products. To the best of our knowledge, only one benzoazetinone compound, 4-methoxybenzo[b]azet-2(1*H*)-one, has been found in plant [233] and flustramine S is the first example of a naturally occurring benzoazetinone alkaloid from marine invertebrate.

Bisindoles have created great interest due to their structural complexity and diverse pharmacological activities. The bisindole alkaloid obtained in the present study, flustramine T, shares the same back core with the compound dimethylisoborreverine without bromination in the indole rings. Some synthetic chemists have been working on the biomimetic total syntheses of dimethylisoborreverine, which undergoes a scalable and catalytic formal [3+2] and [4+2] cycloaddition [234-236]. Thus, flustramine T is likely to be biosynthesized via reaction with the Diels−Alder dimerization reaction.

To the best of our knowledge, oxazolidinum-containing indoles are also rare in Nature. The plant-derived picraline-type alkaloids alstiphyllanines B-G isolated from *Alstonia macrophylla* are featuring the 3-methyl-1,3-oxazolidinum ring [237, 238]. Flustramine U is the first compound featuring 3-methyl-1,3-oxazolidinum skeleton that has been obtained from an invertebrate.

Five highly oxygenated pyrrolo[2,3-b]indoles, flustraminol C-H, were obtained from *F. foliacea*. Oxygenation of the new compounds in this study only occurs within the prenyl moiety but the oxygenation generally occurs only in the carbocyclic aromatic ring of known indoline alkaloids from *F. foliacea* [94]. This difference might be due to the different collection sites of *F. foliacea* or the season in which the collection took place. The major enzymes responsible for the oxidation are monooxygenases, catalysing the oxidation of arenes to arene oxides or phenols and alkenes to alkene oxides [239].

There are no publications reporting anti-inflammatory activity of alkaloids from *F. foliacea*. Of all the 27 isolated compounds in this study, eight of them decreased DC secretion of IL-12p40 and two of them increased secretion of IL-10. Deformylflustrabromine B showed the most potent anti-inflammatory effect, warranting further investigation into its potential as a candidate for relieving inflammatory diseases. With regard to the complexity of their structures, it is hard to identify the skeleton part of the structure which contributes to the activity. However, after SAR analysis of these compounds, it can be suggested that the 6-bromoindole structure may be necessary for the activity but that modifications of the heterocycle, as described for the pyrrolo[2,3-b]indole and oxindole derivatives, lead to suppression of the anti-inflammatory activity. Unfortunately, whether the bromine atom could enhance the activity of those alkaloids is not clear from our study. Some studies have found that halogenation of secondary metabolites resulted in an increase in the biological activity which may provide an evolutionary advantage for the organism [163, 240].

The *F. foliacea* collected in Icelandic waters has different chemical composition from that of *F. foliacea* collected at the coast of Canada and Denmark [93]. The bryozoan collected in Icelandic waters contains not only prenylated 6-bromo-pyrrolo[2,3-b]indoles, but also high levels of highly oxygenated prenyl groups in the 6-bromo-pyrrolo[2,3-b]indoles. Two main alkaloids, flustramines A and B, were found in *F. foliacea* collected from the Danish coast [93] but these could only be detected at low levels in the *F. foliacea* collected in Icelandic waters. It is, therefore, likely that there is a strong relationship between the collection environment and the metabolites of the marine organisms. More data is needed to determine how the surrounding environment, including temperature, light, oxygen levels, water movement, pH levels, salinity and pressure affect production of secondary metabolites.

To date, MNPs isolated from marine invertebrates collected in Icelandic waters have been reported in less than 10 papers. The findings from this study highlight the unacknowledged value of marine organisms collected from Icelandic waters and extend the chemical space of glycerides and alkaloids. Collectively, although the Icelandic marine invertebrates contain various natural products, reports of isolated natural products from Icelandic marine invertebrates are limited and fewer than that of natural products from marine invertebrates collected from tropic regions. This is most likely due to the limited exploration of secondary metabolites in Icelandic marine invertebrates.

Moreover, a latitudinal hypothesis, proposing an inverse correlation between latitude and chemical defense strategies in marine invertebrates, has been put forth based on early chemical ecology studies and related geographical comparisons [241, 242]. The hypothesis pointed out that predation is higher in tropical than polar habitats, and that tropical sponge species are more diverse and produce higher chemical diversity than polar species. However, a study published by Becerro et al. found that tropical and temperate sponges have comparable deterrence, suggesting that chemical defenses from tropical and temperate sponges are equally strong [243]. While this study does not assess the effectiveness of metabolites isolated from temperate and tropical climates, it does give a hint that the invertebrates collected in the Icelandic waters can produce compounds with diverse chemical structures.

# 6 Conclusions and future aspects

The main objective of this project was to search for new compounds from Icelandic marine invertebrates that have anti-inflammatory properties.

In this study, a screening platform was successfully established to identify compounds with immunomodulatory effects from crude extracts of marine sponges. In the first and second parts of this study, a bioassay-guided isolation method was successfully exploited to identify pure PUFAs and lipophilic fractions, from marine sponges, which had immunomodulatory effects. However, the components of the lipophilic fractions could not be purified due to insufficient material being available. The lack of sufficient material highlights one of the limiting factors for separation and isolation of pure natural products. The identified known PUFAs indicate that the bioassay-guided isolation approach can be very efficient but it can result in identification of known compounds, which is also a limitation well known for this procedure.

In the later parts of this study, it was shown that *G. barretti* and *F. foliacea* collected from Icelandic waters contain new and previously known alkaloids, including DKP dipeptides and 6-bromoindoles, as well as brominated quinolinone, oxindoles, pyrrolo[2,3-b]indoles, and imidazoles. These results demonstrate that marine invertebrates collected in Icelandic waters could be a source of structurally diverse new compounds. Some of the isolated compounds showed immunomodulatory activity by affecting DC secretion of the cytokines IL-12p40 and IL-10 as well as the ability of the DCs to direct differentiation of co-cultured T cells to a Th1 phenotype. As some of the alkaloids had anti-inflammatory effects on the DCs they could be further explored to find out if they are lead compounds for inflammatory diseases.

In addition, the results provide an insight into the SAR for the 6-bromoindole derivatives. It highlights that the 6-bromoindole core is important for the anti-inflammatory activity. However, it did not give an insight into the substitution pattern required for the best biological potencies due to the complexity of their structure and the limited number of compounds. Thus, a series of compounds with different functional groups substituted in the 6-bromoindole skeleton could be designed, synthesized and optimized to study further the SAR.

Overall the study has increased the knowledge of secondary metabolites from Icelandic marine invertebrates and their anti-inflammatory effects. This study also indicates that the collecting site of the marine invertebrates influences their production of secondary metabolites. This invites many opportunities for further investigation of the effect of the environment on the secondary metabolic profile of Icelandic marine invertebrates.

Further studies could include investigation into the mode-of-action of the active 6-bromoindole derivatives from the present study, for example by examining their effects on intracellular signaling pathways using either western blotting or phosphoflow techniques. The effects of the bioactive compounds *in vivo* could then be studied using animal models for inflammation, such as the adjuvant-induced arthritis in rats [244] or antigen-induced inflammation in mice [245]. At last, considering the limited availability of the raw material, synthetic methods for the production of the bioactive compounds might be needed, so they can be studied in more detail, and it determined whether they are potential drug leads.

# References


1. Newman, D.J. and G.M. Cragg, *Natural products as sources of new drugs from 1981 to 2014.* J Nat Prod, 2016. **79**(3): p. 629-61.
2. Patridge, E., et al., *An analysis of FDA-approved drugs: natural products and their derivatives.* Drug Discov Today, 2016. **21**(2): p. 204-7.
3. Shen, B., *A new golden age of natural products drug discovery.* Cell, 2015. **163**(6): p. 1297-300.
4. Li, J.W. and J.C. Vederas, *Drug discovery and natural products: end of an era or an endless frontier?* Science, 2009. **325**(5937): p. 161-5.
5. Altmann, K.H., *Drugs from the oceans: Marine natural products as leads for drug discovery.* Chimia (Aarau), 2017. **71**(10): p. 646-52.
6. Montaser, R. and H. Luesch, *Marine natural products: a new wave of drugs?* Future Med Chem, 2011. **3**(12): p. 1475-89.
7. Kong, D.X., Y.Y. Jiang, and H.Y. Zhang, *Marine natural products as sources of novel scaffolds: achievement and concern.* Drug Discov Today, 2010. **15**(21-22): p. 884-6.
8. Fenical, W., *Natural products chemistry in the marine environment.* Science, 1982. **215**(4535): p. 923-8.
9. Butler, A. and J.N. Carter-Franklin, *The role of vanadium bromoperoxidase in the biosynthesis of halogenated marine natural products.* Nat Prod Rep, 2004. **21**(1): p. 180-8.
10. Bergmann, W. and R.J. Feeney, *Contributions to the study of marine products. XXXII. The nucleosides of sponges. I. J Org Chem,* 1951. **16**(6): p 981-7.
11. Bergmann, W. and R.J. Feeney, *The isolation of a new thymine pentoside from sponges.* J Am Chem Soc, 1950. **72**(6): p. 2809-2810.
12. Rangel, M. and M. Falkenberg, *An overview of the marine natural products in clinical trials and on the market.* J Coast Life Med, 2015. **3**(6): p. 421-8.
13. Burkholder, P.R., R.M. Pfister, and F.H. Leitz, *Production of a pyrrole antibiotic by a marine bacterium.* Appl Microbiol, 1966. **14**(4): p. 649-53.
14. Weinheimer, A.J. and R.L. Spraggins, *The occurrence of two new prostaglandin derivatives (15-epi-PGA2 and its acetate, methyl ester)*

*in the gorgonian Plexaura homomalla chemistry of coelenterates. XV.* Tetrahedron Lett, 1969(59): p. 5185-8.

15. Proksch, P., R.A. Edrada, and R. Ebel, *Drugs from the seas-current status and microbiological implications.* Appl Microbiol Biotechnol, 2002. **59**(2-3): p. 125-34.

16. Gordon, E.M., et al., *Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions.* J Med Chem, 1994. **37**(10): p. 1385-401.

17. Mehbub, M.F., et al., *Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives.* Mar Drugs, 2014. **12**(8): p. 4539-77.

18. Molinski, T.F., et al., *Drug development from marine natural products.* Nat Rev Drug Discov, 2009. **8**(1): p. 69-85.

19. *MarinLit Database*. July 2017; Available from: http://pubs.rsc.org/marinlit/.

20. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2018. **35**(1): p. 8-53.

21. Hu, Y., et al., *Statistical research on the bioactivity of new marine natural products discovered during the 28 years from 1985 to 2012.* Mar Drugs, 2015. **13**(1): p. 202-21.

22. Faulkner, D.J., *Highlights of marine natural products chemistry (1972-1999).* Nat Prod Rep, 2000. **17**(1): p. 1-6.

23. Newman, D.J. and G.M. Cragg, *Marine natural products and related compounds in clinical and advanced preclinical trials.* J Nat Prod, 2004. **67**(8): p. 1216-38.

24. Mayer, A.M.S. *Marine pharmaceuticals: the clinical pipeline*. March 2018; Available from: http://marinepharmacology.midwestern.edu/clinPipeline.htm.

25. Sagar, S., M. Kaur, and K.P. Minneman, *Antiviral lead compounds from marine sponges.* Mar Drugs, 2010. **8**(10): p. 2619-38.

26. Schoffski, P., et al., *Clinical impact of trabectedin (ecteinascidin-743) in advanced/metastatic soft tissue sarcoma.* Expert Opin Pharmacother, 2008. **9**(9): p. 1609-18.

27. Menis, J. and C. Twelves, *Eribulin (Halaven): a new, effective treatment for women with heavily pretreated metastatic breast cancer.* Breast Cancer (Dove Med Press), 2011. **3**: p. 101-11.

28. Hirata, Y. and D. Uemura, *Halichondrins-antitumor polyether macrolides from a marine sponge.* Pure Appl Chem, 1986. **58**(5): p. 701-710.

29. Katz, J., J.E. Janik, and A. Younes, *Brentuximab Vedotin (SGN-35).* Clin Cancer Res, 2011. **17**(20): p. 6428-36.

30. Klotz, U., *Ziconotide-a novel neuron-specific calcium channel blocker for the intrathecal treatment of severe chronic pain-a short review.* Int J Clin Pharmacol Ther, 2006. **44**(10): p. 478-83.

31. Schindler, A.E., *Dienogest in long-term treatment of endometriosis.* Int J Womens Health, 2011. **3**: p. 175-84.

32. Gerwick, W.H. and B.S. Moore, *Lessons from the past and charting the future of marine natural products drug discovery and chemical biology.* Chem Biol, 2012. **19**(1): p. 85-98.

33. Crawford, J.M. and J. Clardy, *Bacterial symbionts and natural products.* Chem Commun, 2011. **47**(27): p. 7559-66.

34. Mayer, A.M., et al., *The odyssey of marine pharmaceuticals: a current pipeline perspective.* Trends Pharmacol Sci, 2010. **31**(6): p. 255-65.

35. Ramos, V. and V. Vasconcelos, *Palytoxin and analogs: biological and ecological effects.* Mar Drugs, 2010. **8**(7): p. 2021-37.

36. Mora, C., et al., *How many species are there on Earth and in the ocean?* Plos Biology, 2011. **9**(8): p. e1001127.

37. WOMAS. *World Register of Marine Species*. June 2018; Available from: http://www.marinespecies.org.

38. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2011. **28**(2): p. 196-268.

39. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2012. **29**(2): p. 144-222.

40. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2013. **30**(2): p. 237-323.

41. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2014. **31**(2): p. 160-258.

42. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2015. **32**(2): p. 116-211.

43. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2016. **33**(3): p. 382-431.

44. Blunt, J.W., et al., *Marine natural products.* Nat Prod Rep, 2017. **34**(3): p. 235-94.

45. Astthorsson, O.S., A. Gislason, and S. Jonsson, *Climate variability and the Icelandic marine ecosystem.* Deep Sea Res Pt II, 2007. **54**(23-26): p. 2456-77.

46. Jonsson, S. and H. Valdimarsson, *The flow of Atlantic water to the North Icelandic Shelf and its relation to the drift of cod larvae.* ICES J Mar Sci, 2005. **62**(7): p. 1350-9.

47. Omarsdottir, S., et al., *Biodiversity of benthic invertebrates and bioprospecting in Icelandic waters.* Phytochem Rev, 2013. **12**(3): p. 517-29.

48. Cooper, L.E., et al., *Structure-activity relationship studies of the two-component lantibiotic haloduracin.* Chem Biol, 2008. **15**(10): p. 1035-45.

49. Oman, T.J. and W.A. van der Donk, *Insights into the mode of action of the two-peptide lantibiotic haloduracin.* ACS Chem Biol, 2009. **4**(10): p. 865-74.

50. Aicher, T.D., et al., *Total synthesis of halichondrin B and norhalichondrin B.* J Am Chem Soc, 1992. **114**(8): p. 3162-4.

51. Imbs, A.B. and S.A. Rodkina, *Isolation of 2-methyl branched unsaturated very long fatty acids from marine sponge Halichondria panicea and identification of them by GC-MS and NMR.* Chem Phys Lipids, 2004. **129**(2): p. 173-81.

52. Ishiyama, H., et al., *Halichonadin F and the Cu(I) complex of halichonadin C from the sponge Halichondria sp.* J Nat Prod, 2008. **71**(7): p. 1301-3.

53. Jin, Y., et al., *Halichondria sulfonic acid, a new HIV-1 inhibitory guanidino-sulfonic acid, and halistanol sulfate isolated from the marine sponge Halichondria rugosa Ridley & Dendy.* Nat Prod Res, 2006. **20**(12): p. 1129-35.

54. Li, H.Y., S. Matsunaga, and N. Fusetani, *Halicylindramides A-C, antifungal and cytotoxic depsipeptides from the marine sponge Halichondria cylindrata.* J Med Chem, 1995. **38**(2): p. 338-43.

55. Nagle, D.G., W.C. McClatchey, and W.H. Gerwick, *New glycosphingolipids from the marine sponge Halichondria panicea.* J Nat Prod, 1992. **55**(7): p. 1013-7.

56. Solter, S., et al., *Barettin, revisited?* Tetrahedron Lett, 2002. **43**(18): p. 3385-6.

57. Lidgren, G. and L. Bohlin, *Studies of Swedish marine organisms.7. A novel biologically-active indole alkaloid from the sponge Geodia barretti.* Tetrahedron Lett, 1986. **27**(28): p. 3283-4.

58. Johnson, A.L., et al., *Synthesis of barettin.* Tetrahedron, 2004. **60**(4): p. 961-5.

59. Kondo, K., et al., *Two new tryptophan-derived alkaloids from the Okinawan marine sponge Aplysina sp.* J Nat Prod, 1994. **57**(7): p. 1008-11.

60. Sjogren, M., et al., *Antifouling activity of brominated cyclopeptides from the marine sponge Geodia barretti.* J Nat Prod, 2004. **67**(3): p. 368-72.

61. Hedner, E., et al., *Antifouling activity of a dibrominated cyclopeptide from the marine sponge Geodia barretti.* J Nat Prod, 2008. **71**(3): p. 330-3.

62. Olsen, E.K., et al., *Marine AChE inhibitors isolated from Geodia barretti: natural compounds and their synthetic analogs.* Org Biomol Chem, 2016. **14**(5): p. 1629-40.

63. Lidgren, G., L. Bohlin, and C. Christophersen, *Studies of swedish marine organisms .10. Biologically-active compounds from the marine sponge Geodia barretti.* J Nat Prod, 1988. **51**(6): p. 1277-80.

64. Hougaard, L., et al., *The chemical-composition of species of Geodia, Isops and Stryphnus (Choristida, Demospongia, Porifera)-A comparative-study with some taxonomical implications.* Biochem Syst Ecol, 1991. **19**(3): p. 223-35.

65. Carstens, B.B., et al., *Isolation, characterization, and synthesis of the barrettides: disulfide-containing peptides from the marine sponge Geodia barretti.* J Nat Prod, 2015. **78**(8): p. 1886-93.

66. Lind, K.F., et al., *Antioxidant and anti-inflammatory activities of barettin.* Mar Drugs, 2013. **11**(7): p. 2655-66.

67. Lind, K.F., et al., *The immunomodulatory effects of barettin and involvement of the kinases CAMK1$\alpha$ and RIPK2.* Immunopharmacol Immunotoxicol, 2015. **37**(5): p. 458-64.

68. Hedner, E., et al., *Brominated cyclodipeptides from the marine sponge Geodia barretti as selective 5-HT ligands.* J Nat Prod, 2006. **69**(10): p. 1421-4.

69. Farokhzad, O.C., et al., *Effects of bryostatin 1, a novel anticancer agent, on intestinal transport and barrier function: role of protein kinase C.* Surgery, 1998. **124**(2): p. 380-6.

70. Sharp, J.H., M.K. Winson, and J.S. Porter, *Bryozoan metabolites: an ecological perspective.* Nat Prod Rep, 2007. **24**(4): p. 659-73.

71. Christophersen, C. and J.S. Carle, *Chemical signals from a marine bryozo.* Naturwissenschaften, 1978. **65**(8): p. 440-1.

72. Holst, P.B., et al., *A racemic diterpene from the marine bryozoan Flustra foliacea, natural product or artifact.* Acta Chem Scand, 1994. **48**(9): p. 765-8.

73. Peters, L., et al., *Variation of brominated indoles and terpenoids within single and different colonies of the marine bryozoan Flustra foliacea.* J Chem Ecol, 2004. **30**(6): p. 1165-81.

74. Peters, L., et al., *Secondary metabolites of Flustra foliacea and their influence on bacteria.* Appl Environ Microbiol, 2003. **69**(6): p. 3469-75.

75. Sala, F., et al., *Potentiation of human $\alpha4\beta2$ neuronal nicotinic receptors by a Flustra foliacea metabolite.* Neurosci Lett, 2005. **373**(2): p. 144-9.

76. Wulff, P., J.S. Carle, and C. Christophersen, *Marine alkaloids. 6. The first naturally-occurring bromo-substituted quinoline from Flustra foliacea*. Comp Biochem Physiol B, 1982. **71**(3): p. 525-6.

77. Wulff, P., J.S. Carle, and C. Christophersen, *Marine alkaloids. 5. Flustramide A and 6-bromo-$N_b$-methyl-$N_b$-formyltryptamine from the marine bryozoan Flustra foliacea.* Comp Biochem Physiol B, 1982. **71**(3): p. 523-4.

78. Peters, L., et al., *Four new bromotryptamine derivatives from the marine bryozoan Flustra foliacea.* J Nat Prod, 2002. **65**(11): p. 1633-7.

79. Wulff, P., J.S. Carle, and C. Christophersen, *Marine alkaloids .4. A formamide, flustrabromine, from the marine bryozoan Flustra foliacea.* J Chem Soc, Perkin Trans. 1, 1981(11): p. 2895-8.

80. Lysek, N., E. Rachor, and T. Lindel, *Isolation and structure elucidation of deformylflustrabromine from the North Sea bryozoan Flustra foliacea.* Z Naturforsch C, 2002. **57**(11-12): p. 1056-61.

81. Weltzin, M.M. and M.K. Schulte, *Desformylflustrabromine modulates $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor high- and low-sensitivity isoforms at allosteric clefts containing the $\beta2$ Subunit.* J Pharmacol Exp Ther, 2015. **354**(2): p. 184-94.

82. Weltzin, M.M. and M.K. Schulte, *Pharmacological characterization of the allosteric modulator desformylflustrabromine and its interaction with $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor orthosteric ligands.* J Pharmacol Exp Ther, 2010. **334**(3): p. 917-26.

83. Pandya, A. and J.L. Yakel, *Allosteric modulator desformylflustrabromine relieves the inhibition of $\alpha2\beta2$ and $\alpha4\beta2$ nicotinic acetylcholine receptors by $\beta$-Amyloid(1-42) peptide.* J Mol Neurosci, 2011. **45**(1): p. 42-7.

84. Bunders, C.A., et al., *Intercepting bacterial indole signaling with flustramine derivatives.* J Am Chem Soc, 2011. **133**(50): p. 20160-3.

85. Peters, L., et al., *Prenylated indole alkaloids from Flustra foliacea with subtype specific binding on NAChRs.* Planta Med, 2004. **70**(10): p. 883-6.

86. Keil, P., et al., *Marine alkaloids .11. Flustramide B and flustrarine B from the marine bryozoan Flustra foliacea. Synthesis of flustrarine B.* Acta Chem Scand B, 1986. **40**(7): p. 555-8.

87. Carle, J.S. and C. Christophersen, *Marine alkaloids .3. Bromo-substituted alkaloids from the marine bryozoan Flustra foliacea, flustramine C and flustraminol A and B.* J Org Chem, 1981. **46**(17): p. 3440-3.

88. Carle, J.S. and C. Christophersen, *Bromo-substituted physostigmine alkaloids from a marine bryozoa Flustra foliacea.* J Am Chem Soc, 1979. **101**(14): p. 4012-3.

89. Christophersen, C., *Secondary metabolites from marine bryozoans. A review.* Acta Chem Scand B, 1985. **39**(7): p. 517-29.

90. Sjoblom, T., L. Bohlin, and C. Christophersen, *Studies of Swedish marine organisms. II. Muscle-relaxant alkaloids from the marine bryozoan Flustra foliacea.* Acta Pharm Suec, 1983. **20**(6): p. 415-8.

91. Adla, S.K., et al., *Doubly prenylated tryptamines: cytotoxicity, antimicrobial activity and cyclisation to the marine natural product flustramine A.* Org Biomol Chem,,2013. **11**(36): p. 6119-30.

92. Laycock, M.V., et al., *New physostigmine related bromoalkaloids from the marine bryozoan Flustra foliacea.* Can J Chem, 1986. **64**(7): p. 1312-6.

93. Holst, P.B., et al., *Marine alkaloids. 15. Two alkaloids, flustramine E and debromoflustramine B, from the marine bryozoan Flustra foliacea.* J Nat Prod, 1994. **57**(7): p. 997-1000.

94. Rochfort, S.J., et al., *Further studies on the chemistry of the flustra alkaloids from the bryozoan Flustra foliacea.* J Nat Prod, 2009. **72**(10): p. 1773-81.

95. Holst, P.B., et al., *Marine alkaloids.16. Reversible conversion of flustramine B N-1-oxide to flustrarine B.* J Nat Prod, 1994. **57**(9): p. 1310-2.

96. Rivera-Becerril, E., et al., *Synthesis and biological evaluation of (-)- and (+)-debromoflustramine B and its analogues as selective butyrylcholinesterase inhibitors.* J Med Chem, 2008. **51**(17): p. 5271-84.

97. Wright, J.L.C., *A New antibiotic from the marine bryozoan Flustra foliacea.* J Nat Prod, 1984. **47**(5): p. 893-5.

98. German, N., et al., *Deconstruction of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor positive allosteric modulator desformylflustrabromine.* J Med Chem, 2011. **54**(20): p. 7259-67.

99. Bruncko, M., D. Crich, and R. Samy, *Chemistry of cyclic tautomers of tryptophan: formation of a quaternary center at C3a and total synthesis of the marine alkaloid (+)-ent-debromoflustramine B.* J Org Chem, 1994. **59**(19): p. 5543-9.

100. Kawasaki, T., et al., *Enantioselective total synthesis of (-)-flustramines A, B and (-)-flustramides A, B via domino olefination/isomerization/Claisen rearrangement sequence.* Chem Comm, 2006(4): p. 420-2.

101. Kawasaki, T., et al., *Total synthesis of (+/-)-flustramines A and C, (+/-)-flustramide A, and (-)- and (+)-debromoflustramines A.* J Org Chem, 2008. **73**(15): p. 5959-64.

102. Morales-Rios, M.S., et al., *Conformational studies on indole alkaloids from Flustra foliacea by NMR and molecular modeling.* Magn Reson Chem, 2002. **40**(10): p. 677-82.

103. Sayed, K.A., et al., *Semisynthetic studies on the manzamine alkaloids.* J Nat Prod, 2008. **71**(3): p. 300-8.

104. Gul, W. and M.T. Hamann, *Indole alkaloid marine natural products: an established source of cancer drug leads with considerable promise for the control of parasitic, neurological and other diseases.* Life Sci, 2005. **78**(5): p. 442-53.

105. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

106. Libby, P., et al., *Inflammation in atherosclerosis: from pathophysiology to practice.* J Am Coll Cardiol, 2009. **54**(23): p. 2129-38.

107. Okamoto, R. and M. Watanabe, *Role of epithelial cells in the pathogenesis and treatment of inflammatory bowel disease.* J Gastroenterol, 2016. **51**(1): p. 11-21.

108. Crusz, S.M. and F.R. Balkwill, *Inflammation and cancer: advances and new agents.* Nat Rev Clin Oncol, 2015. **12**(10): p. 584-96.

109. Banchereau, J., et al., *Immunobiology of dendritic cells.* Annu Rev Immunol, 2000. **18**: p. 767-811.

110. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. **20**: p. 621-67.

111. Walsh, K.P. and K.H. Mills, *Dendritic cells and other innate determinants of T helper cell polarisation.* Trends Immunol, 2013. **34**(11): p. 521-30.

112. Pulendran, B., H. Tang, and S. Manicassamy, *Programming dendritic cells to induce $T_H2$ and tolerogenic responses.* Nat Immunol, 2010. **11**(8): p. 647-55.

113. Gee, K., et al., *The IL-12 family of cytokines in infection, inflammation and autoimmune disorders.* Inflamm Allergy Drug Targets, 2009. **8**(1): p. 40-52.

114. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.

115. Schmitt, N. and H. Ueno, *Regulation of human helper T cell subset differentiation by cytokines.* Curr Opin Immunol, 2015. **34**: p. 130-6.

116. Romagnani, S., *Th1 and Th2 in human diseases.* Clin Immunol Immunopathol, 1996. **80**(3 Pt 1): p. 225-35.

117. Allen, J.E. and R.M. Maizels, *Diversity and dialogue in immunity to helminths.* Nat Rev Immunol, 2011. **11**(6): p. 375-88.

118. Chen, F., et al., *An essential role for $T_H2$-type responses in limiting acute tissue damage during experimental helminth infection.* Nat Med, 2012. **18**(2): p. 260-6.

119. Mills, K.H., *Induction, function and regulation of IL-17-producing T cells.* Eur J Immunol, 2008. **38**(10): p. 2636-49.

120. Kumar, P., K. Chen, and J.K. Kolls, *Th17 cell based vaccines in mucosal immunity.* Curr Opin Immunol, 2013. **25**(3): p. 373-80.

121. Mittal, S.K. and P.A. Roche, *Suppression of antigen presentation by IL-10.* Curr Opin Immunol, 2015. **34**: p. 22-7.

122. Filippi, C.M., et al., *Transforming growth factor-$\beta$ suppresses the activation of $CD8^+$ T-cells when naive but promotes their survival and function once antigen experienced: a two-faced impact on autoimmunity.* Diabetes, 2008. **57**(10): p. 2684-92.

123. Workman, C.J., et al., *The development and function of regulatory T cells.* Cell Mol Life Sci, 2009. **66**(16): p. 2603-22.

124. Ghasemian, M., S. Owlia, and M.B. Owlia, *Review of anti-Inflammatory herbal medicines.* Adv Pharmacol Sci, 2016. **2016**: p. 9130979.

125. Keyzers, R.A. and M.T. Davies-Coleman, *Anti-inflammatory metabolites from marine sponges.* Chem Soc Rev, 2005. **34**(4): p. 355-65.

126. Cheung, R.C., et al., *Marine natural products with anti-inflammatory activity.* Appl Microbiol Biotechnol, 2016. **100**(4): p. 1645-66.

127. Ahmad, T.B., et al., *Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs.* J Ethnopharmacol, 2018. **210**: p. 156-178.

128. Mayer, A.M.S., et al., *Marine pharmacology in 2012-2013: marine compounds with antibacterial, antidiabetic, antifungal, anti-Inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action.* Mar Drugs, 2017. **15**(9): p. 273.

129. Mayer, A.M., et al., *Marine pharmacology in 2009-2011: marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action.* Mar Drugs, 2013. **11**(7): p. 2510-73.

130. Mayer, A.M., et al., *Marine pharmacology in 2005-6: marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action.* Biochim Biophys Acta, 2009. **1790**(5): p. 283-308.

131. Mayer, A.M.S., et al., *Marine pharmacology in 2007-8: marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action.* Comp Biochem Physiol C Toxicol Pharmacol, 2011. **153**(2): p. 191-222.

132. Senthilkumar, K. and S.K. Kim, *Marine invertebrate natural products for anti-inflammatory and chronic diseases.* Evid Based Complement Alternat Med, 2013. **2013**: p. 572859.

133. Sindhu, R.K., et al., *Various Animal Models for Preclinical Testing of Anti- Inflammatory Agents.* Int J Pharm Sci Res, 2017. **8**(4): p. 1550-7.

134. Defreitas, J.C., L.A. Blankemeier, and R.S. Jacobs, *In vitro inactivation of the neurotoxic action of $\beta$-bungarotoxin by the marine natural product, manoalide.* Experientia, 1984. **40**(8): p. 864-5.

135. Tibes, U. and W.G. Friebe, *Phospholipase A2 inhibitors in development.* Expert Opin Investig Drugs, 1997. **6**(3): p. 279-98.

136. De Vries, G.W., et al., *AGN 190383, a novel phospholipase inhibitor with topical anti-inflammatory activity.* Agents Actions, 1991. **34**(1-2): p. 70-2.

137. Takei, M., D.L. Burgoyne, and R.J. Andersen, *Effect of contignasterol on histamine release induced by anti-immunoglobulin E from rat peritoneal mast cells.* J Pharm Sci, 1994. **83**(9): p. 1234-5.

138. Kasserra, C.E., et al., *IPL576,092, a novel anti-inflammatory compound, inhibits leukocyte infiltration and changes in lung function in response to allergen challenge.* Pulm Pharmacol Ther, 2004. **17**(5): p. 309-18.

139. Marshall, L.A., et al., *Effects of scalaradial, a type-II phospholipase A2 inhibitor, on human neutrophil arachidonic acid mobilization and lipid mediator formation.* J Pharm Exp Ther, 1994. **268**(2): p. 709-717.

140. Look, S.A., et al., *The pseudopterosins: a new class of antiinflammatory and analgesic diterpene pentosides from the marine sea whip Pseudopterogorgia elisabethae (Octocorallia).* J Org Chem, 1986. **51**(26): p. 5140-5.

141. Look, S.A., et al., *The pseudopterosins: anti-inflammatory and analgesic natural products from the sea whip Pseudopterogorgia elisabethae.* Proc Natl Acad Sci U S A, 1986. **83**(17): p. 6238-40.

142. Hu, Y., et al., *Attenuation of collagen-induced Arthritis in rat by nicotinic $\alpha$7 receptor partial agonist GTS-21.* Biomed Res Int, 2014. **2014**: p. 325875.

143. Cai, B., et al., *Alpha7 cholinergic-agonist prevents systemic inflammation and improves survival during resuscitation.* J Cell Mol Med, 2009. **13**(9B): p. 3774-85.

144. Rosas-Ballina, M., et al., *The selective $\alpha$7 agonist GTS-21 attenuates cytokine production in human whole blood and human monocytes activated by ligands for TLR2, TLR3, TLR4, TLR9, and RAGE.* Mol Med, 2009. **15**(7-8): p. 195-202.

145. Ahmad, T.B., et al., *Anti-inflammatory activity and structure-activity relationships of brominated indoles from a marine mollusc.* Mar Drugs, 2017. **15**(5): p. 133.

146. Pearce, A.N., et al., *Anti-inflammatory thiazine alkaloids isolated from the New Zealand ascidian Aplidium sp.: inhibitors of the neutrophil respiratory burst in a model of gouty arthritis.* J Nat Prod, 2007. **70**(6): p. 936-40.

147. Chung, H.M., et al., *Rumphellaones B and C, new 4,5-seco-caryophyllane sesquiterpenoids from Rumphella antipathies.* Molecules, 2014. **19**(8): p. 12320-7.

148. Thao, N.P., et al., *New anti-inflammatory cembranoid diterpenoids from the Vietnamese soft coral Lobophytum crassum.* Bioorg Med Chem Lett, 2014. **24**(1): p. 228-32.

149. Wei, W.C., et al., *Topical application of marine briarane-type diterpenes effectively inhibits 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and dermatitis in murine skin.* J Biomed Sci, 2011. **18**: p. 94.

150. Jean, Y.H., et al., *Capnellene, a natural marine compound derived from soft coral, attenuates chronic constriction injury-induced neuropathic pain in rats.* Br J Pharmacol, 2009. **158**(3): p. 713-25.

151. Takei, M., et al., *Polyacetylenediols regulate the function of human monocyte-derived dendritic cells.* Int Immunopharmacol, 2010. **10**(8): p. 913-21.

152. Mencarelli, A., et al., *Solomonsterol A, a marine pregnane-X-receptor agonist, attenuates inflammation and immune dysfunction in a mouse model of arthritis.* Mar Drugs, 2013. **12**(1): p. 36-53.

153. Shoji, N., et al., *Two unique pentacyclic steroids with cis C/D Ring junction from Xestospongia bergquistia Fromont, Powerful Inhibitors of Histamine-Release.* J Org Chem, 1992. **57**(11): p. 2996-7.

154. Randazzo, A., et al., *Halipeptins A and B: two novel potent anti-inflammatory cyclic depsipeptides from the Vanuatu marine sponge Haliclona species.* J Am Chem Soc, 2001. **123**(44): p. 10870-6.

155. Nicolaou, K.C., et al., *Total synthesis and biological evaluation of halipeptins A and D and analogues.* J Am Chem Soc, 2006. **128**(13): p. 4460-70.

156. Yu, S.Y., et al., *Total synthesis of halipeptin A: a potent anti-inflammatory cyclic depsipeptide.* Angew Chem Int Ed, 2005. **44**(1): p. 135-8.

157. Vilasi, A., et al., *Differential in gel electrophoresis (DIGE) comparative proteomic analysis of macrophages cell cultures in response to perthamide C treatment.* Mar Drugs, 2013. **11**(4): p. 1288-99.

158. Costantino, V., et al., *Terpioside B, a difucosyl GSL from the marine sponge Terpios sp. is a potent inhibitor of NO release.* Bioorg Med Chem, 2010. **18**(14): p. 5310-5.

159. Aoki, S., et al., *Novel neuronal nitric oxide synthase (nNOS) selective inhibitor, aplysinopsin-type indole alkaloid, from marine sponge Hyrtios erecta.* Chem Pharm Bull, 2001. **49**(10): p. 1372-4.

160. Sauviat, M.P., et al., *Sensitivity of cardiac background inward rectifying $K^+$ outward current (IK1) to the alkaloids lepadiformines A, B, and C.* J Nat Prod, 2006. **69**(4): p. 558-62.

161. Aiello, A., et al., *Conicamin, a novel histamine antagonist from the mediterranean tunicate Aplidium conicum.* Bioorg Med Chem Lett, 2003. **13**(24): p. 4481-3.

162. Pauletti, P.M., et al., *Halogenated indole alkaloids from marine invertebrates.* Mar Drugs, 2010. **8**(5): p. 1526-49.

163. Wylie, B.L., et al., *Marine natural products as phospholipase A2 inhibitors.* Phospholipase A2, 1997. **24**: p. 146-52.

164. Wei, W.C., et al., *Anti-inflammatory activities of natural products isolated from soft corals of Taiwan between 2008 and 2012.* Mar Drugs, 2013. **11**(10): p. 4083-126.

165. de Roos, B., Y. Mavrommatis, and I.A. Brouwer, *Long-chain n-3 polyunsaturated fatty acids: new insights into mechanisms relating to inflammation and coronary heart disease.* Br J Pharmacol, 2009. **158**(2): p. 413-28.

166. Sable, R., P. Parajuli, and S. Jois, *Peptides, peptidomimetics, and polypeptides from marine sources: a wealth of natural sources for pharmaceutical applications.* Marine Drugs, 2017. **15**(4): p. 124.

167. Ruocco, N., et al., *Polysaccharides from the marine environment with pharmacological, cosmeceutical and nutraceutical potential.* Molecules, 2016. **21**(5): p. 551.

168. Belmiro, C.L., et al., *Unfractionated heparin and new heparin analogues from ascidians (chordate-tunicate) ameliorate colitis in rats.* J Biol Chem, 2009. **284**(17): p. 11267-78.

169. Camp, D., et al., *Drug-like properties: guiding principles for the design of natural product libraries.* J Nat Prod, 2012. **75**(1): p. 72-81.

170. Wagner, H., *Synergy research: approaching a new generation of phytopharmaceuticals.* Fitoterapia, 2011. **82**(1): p. 34-7.

171. Hubert, J., J.M. Nuzillard, and J.H. Renault, *Dereplication strategies in natural product research: how many tools and methodologies behind the same concept?* Phytochem Rev, 2017. **16**(1): p. 55-95.

172. Gaudencio, S.P. and F. Pereira, *Dereplication: racing to speed up the natural products discovery process.* Nat Prod Rep, 2015. **32**(6): p. 779-810.

173. Yuliana, N.D., et al., *Metabolomics for bioactivity assessment of natural products.* Phytother Res, 2011. **25**(2): p. 157-69.

174. Wang, W.G., et al., *LC-MS-guided isolation of penicilfuranone A: A new antifibrotic furancarboxylic acid from the plant endophytic fungus Penicillium sp. sh18.* J Nat Prod, 2016. **79**(1): p. 149-55.

175. Pence, H.E. and A. Williams, *ChemSpider: an online chemical information resource.* J Chem Educ, 2010. **87**(11): p. 1123-4.

176. Ahn, J., et al., *Dereplication-guided isolation of new phenylpropanoid-substituted diglycosides from Cistanche salsa and their inhibitory activity on NO production in macrophage.* Molecules, 2017. **22**(7): p. 1138.

177. de Medeiros, L.S., et al., *Dereplication-guided isolation of depsides thielavins S-T and lecanorins D-F from the endophytic fungus Setophoma sp.* Phytochem, 2015. **111**: p. 154-62.

178. O'Donnell, F., et al., *An investigation of bioactive phytochemicals in the leaves of Melicope vitiflora by electrospray ionisation ion trap mass spectrometry.* Anal Chim Acta, 2009. **634**(1): p. 115-20.

179. Pan, L., et al., *Bioactive flavaglines and other constituents isolated from Aglaia perviridis.* J Nat Prod, 2013. **76**(3): p. 394-404.

180. Kupchan, S.M., G. Tsou, and C.W. Sigel, *Datiscacin, a novel cytotoxic cucurbitacin 20-acetate from Datisca glomerata.* J Org Chem, 1973. **38**(7): p. 1420-1.

181. Vanwagenen, B.C., et al., *Ulosantoin, a potent insecticide from the sponge Ulosa ruetzleri.* J Org Chem, 1993. **58**(2): p. 335-7.

182. Alamsjah, M.A., et al., *Isolation and structure determination of algicidal compounds from Ulva fasciata.* Biosci Biotechnol Biochem, 2005. **69**(11): p. 2186-92.

183. Viala, J. and J. Sandri, *Total stereospecific synthesis of all cis-5,8,11,14,17-eicosapentaenoic Acid (EPA).* Tetrahedron Lett, 1992. **33**(34): p. 4897-4900.

184. Larsen, L.N., et al., *Heneicosapentaenoate (21:5n-3): its incorporation into lipids and its effects on arachidonic acid and eicosanoid synthesis.* Lipids, 1997. **32**(7): p. 707-14.

185. Fu, M., et al., *Chemical characterisation of three haemolytic compounds from the microalgal species Fibrocapsa japonica (Raphidophyceae).* Toxicon, 2004. **43**(4): p. 355-63.

186. Amade, P., M. Mallea, and N. Bouaicha, *Isolation, structural identification and biological activity of two metabolites produced by Penicillium olsonii Bainier and Sartory.* J Antibiot, 1994. **47**(2): p. 201-7.

187. Han, L.R., et al., *Immunomodulatory activity of docosahexenoic acid on RAW264.7 cells activation through GPR120-mediated signaling pathway.* J Agric Food Chem, 2018. **66**(4): p. 926-34.

188. Chapkin, R.S., et al., *Immunomodulatory effects of (n-3) fatty acids: putative link to inflammation and colon cancer.* J Nutr, 2007. **137**(1 Suppl): p. 200S-4S.

189. Takahashi, Y., et al., *Heteroaromatic alkaloids, nakijinamines, from a sponge Suberites sp.* Tetrahedron, 2012. **68**(41): p. 8545-50.

190. Kasheverov, I.E., et al., *6-Bromohypaphorine from marine Nudibranch mollusk Hermissenda crassicornis is an agonist of human $\alpha$7 nicotinic acetylcholine receptor.* Mar Drugs, 2015. **13**(3): p. 1255-66.

191. Penez, N., et al., *Antifouling properties of simple indole and purine alkaloids from the Mediterranean gorgonian Paramuricea clavata.* J Nat Prod, 2011. **74**(10): p. 2304-8.

192. OlguinUribe, G., et al., *6-Bromoindole-3-carbaldehyde, from an Acinetobacter sp. bacterium associated with the ascidian Stomozoa murrayi.* J Chem Ecol, 1997. **23**(11): p. 2507-21.

193. Calder, P.C., *Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance.* Biochim Biophys Acta, Mol Cell Biol Lipids, 2015. **1851**(4): p. 469-84.

194. Calder, P.C., *Omega-3 fatty acids and inflammatory processes: from molecules to man.* Biochem Soc Trans, 2017. **45**(5): p. 1105-15.

195. Calder, P.C., *Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology?* Br J Clin Pharmacol, 2013. **75**(3): p. 645-62.

196. Zapata-Gonzalez, F., et al., *Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly through PPAR$\gamma$: RXR heterodimers: comparison with other polyunsaturated fatty acids.* J Leukoc Biol, 2008. **84**(4): p. 1172-82.

197. Monroig, O., D.R. Tocher, and J.C. Navarro, *Biosynthesis of polyunsaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms.* Mar Drugs, 2013. **11**(10): p. 3998-4018.

198. Yagi, S., et al., *N-3 polyunsaturated fatty acids: promising nutrients for preventing cardiovascular disease.* J Atheroscler Thromb, 2017. **24**(10): p. 999-1010.

199. Appleton, K.M., et al., *Effects of n-3 long-chain polyunsaturated fatty acids on depressed mood: systematic review of published trials.* Am J Clin Nutr, 2006. **84**(6): p. 1308-16.

200. Koski, R.R., *Omega-3-acid ethyl esters (Lovaza) for severe hypertriglyceridemia.* Pharm Ther, 2008. **33**(5): p. 271-303.

201. Brummer, F. and M. Nickel, *Sustainable use of marine resources: cultivation of sponges.* Prog Mol Subcell Biol, 2003. **37**: p. 143-62.

202. Abou-Hussein, D.R. and D.T. Youssef, *Mirabolides A and B; new cytotoxic glycerides from the red sea sponge Theonella mirabilis.* Mar Drugs, 2016. **14**(8); p. 155.

203. Zhao, Q.C., et al., *New lysophosphatidylcholines and monoglycerides from the marine sponge Stelletta sp.* J Nat Prod, 2003. **66**(5): p. 725-8.

204. Lin, H., *S-Adenosylmethionine-dependent alkylation reactions: when are radical reactions used?* Bioorg Chem, 2011. **39**(5-6): p. 161-70.

205. Yu, C.M., et al., *An unusual fatty acid and its glyceride from the marine fungus Microsphaeropis olivacea.* Can J Chem, 1996. **74**(5): p. 730-5.

206. Hallgren, B. and S. Larsson, *The glyceryl ethers in the liver oils of elasmobranch fish.* J Lipid Res, 1962. **3**(1): p. 31-8.

207. Santos, V.L.C.S., D.S.M. Billett, and G.A. Wolff, *1-O-alkylglyceryl ether lipids of the gut walls and contents of an abyssal holothurian (Oneirophanta mutabilis).* J Braz Chem Soc, 2002. **13**(5): p. 653-7.

208. Bradley, A.S., et al., *Structural diversity of diether lipids in carbonate chimneys at the Lost City Hydrothermal Field.* Org Geochem, 2009. **40**(12): p. 1169-78.

209. Vincon-Laugier, A., et al., *Temperature-dependent alkyl glycerol ether lipid composition of mesophilic and thermophilic sulfate-reducing bacteria.* Front Microbiol, 2017. **8**: p. 1532.

210. Costantino, V., et al., *Glycolipids from sponges. 11. Isocrasserides, novel glycolipids with a five-membered cyclitol widely distributed in marine sponges.* J Nat Prod, 2002. **65**(6): p. 883-6.

211. Mansoor, T.A., et al., *New fatty acid derivatives from Homaxinelia sp., a marine sponge.* Lipids, 2005. **40**(9): p. 981-5.

212. Yang, J., et al., *UPLC-MS-ELSD-PDA as a powerful dereplication tool to facilitate compound identification from small-molecule natural product libraries.* J Nat Prod, 2014. **77**(4): p. 902-9.

213. Pan, L., et al., *Isolation, structure elucidation, and biological evaluation of 16,23-epoxycucurbitacin constituents from Eleaocarpus chinensis.* J Nat Prod, 2012. **75**(3): p. 444-52.

214. Kamano, Y., et al., *Convolutamydine A, a novel bioactive hydroxyoxindole alkaloid from marine bryozoan Amathia convoluta.* Tetrahedron Lett, 1995. **36**(16): p. 2783-4.

215. Zhang, H.P., et al., *Isolation and structure of convolutamydines B-D from marine bryozoan Amathiaconvoluta.* Tetrahedron, 1995. **51**(19): p. 5523-8.

216. Fujii, K., et al., *A nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: Combination of Marfey's method with mass spectrometry and its practical application.* Anal Chem, 1997. **69**(24): p. 5146-51.

217. Salib, M.N. and T.F. Molinski, *Cyclic hexapeptide dimers, Antatollamides A and B, from the ascidian Didemnum molle. A tryptophan-derived auxiliary for L- and D-amino acid assignments.* J Org Chem, 2017. **82**(19): p. 10181-7.

218. Bhushan, R. and H. Bruckner, *Marfey's reagent for chiral amino acid analysis: a review.* Amino Acids, 2004. **27**(3-4): p. 231-47.

219. Borthwick, A.D., *2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products.* Chem Rev, 2012. **112**(7): p. 3641-716.

220. Huang, R.M., et al., *An update on 2,5-diketopiperazines from marine organisms.* Mar Drugs, 2014. **12**(12): p. 6213-35.

221. Sioud, S., et al., *Targeted gene disruption of the cyclo (L-Phe, L-Pro) biosynthetic pathway in Streptomyces sp. US24 Strain.* J Biomed Biotechnol, 2007. **2007**(6): p. 91409.

222. Alkhalaf, L.M. and K.S. Ryan, *Biosynthetic manipulation of tryptophan in bacteria: pathways and mechanisms.* Chem Biol, 2015. **22**(3): p. 317-28.

223. Zucko, J., et al., *Global genome analysis of the shikimic acid pathway reveals greater gene loss in host-associated than in free-living bacteria.* BMC Genomics, 2010. **11**: p. 628.

224. Dias, M.V., et al., *Chorismate synthase: an attractive target for drug development against orphan diseases.* Curr Drug Targets, 2007. **8**(3): p. 437-44.

225. Kim, K., et al., *Cyclo(Phe-Pro) produced by the human pathogen Vibrio vulnificus inhibits host innate immune responses through the NF-kappaB pathway.* Infect Immun, 2015. **83**(3): p. 1150-61.

226. Netz, N. and T. Opatz, *Marine indole alkaloids.* Mar Drugs, 2015. **13**(8): p. 4814-914.

227. Michael, J.P., *Quinoline, quinazoline and acridone alkaloids.* Nat Prod Rep, 2001. **18**(5): p. 543-59.

228. Morris, B.D. and M.R. Prinsep, *Euthyroideones, novel brominated quinone methides from the bryozoan Euthyroides episcopalis.* J Org Chem, 1998. **63**(25): p. 9545-7.

229. Davis, R.A., et al., *Thiaplakortones A-D: antimalarial thiazine alkaloids from the Australian marine sponge Plakortis lita.* J Org Chem, 2013. **78**(19): p. 9608-13.

230. Singh, G.S., *Beta-lactams in the new millennium. Part-II: cephems, oxacephems, penams and sulbactam.* Mini Rev Med Chem, 2004. **4**(1): p. 93-109.

231. Singh, G.S., *Beta-lactams in the new millennium. Part-I: monobactams and carbapenems.* Mini Rev Med Chem, 2004. **4**(1): p. 69-92.

232. Mehta, P.D., N.P. Sengar, and A.K. Pathak, *2-Azetidinone-a new profile of various pharmacological activities.* Eur J Med Chem, 2010. **45**(12): p. 5541-60.

233. Bhatt, U.P., et al., *Two antidiabetic constituents from Roylea cinerea (D.Don) Baill.* Nat Prod Res, 2018. **32**(11): p. 1281-6.

234. Dethe, D.H., R.D. Erande, and A. Ranjan, *Biomimetic total syntheses of borreverine and flinderole alkaloids.* J Org Chem, 2013. **78**(20): p. 10106-20.

235. Tillequin, F., et al., *Biomimetic synthesis of borreverine and isoborreverine.* Chem Comm, 1978(19): p. 826-8.

236. Vallakati, R., et al., *Biomimetic synthesis and studies toward enantioselective synthesis of flindersial alkaloids.* Chirality, 2015. **27**(1): p. 14-17.

237. Hirasawa, Y., et al., *Alstiphyllanines A-D, indole alkaloids from Alstonia macrophylla.* J Nat Prod, 2009. **72**(2): p. 304-7.

238. Arai, H., et al., *Alstiphyllanines E-H, picraline and ajmaline-type alkaloids from Alstonia macrophylla inhibiting sodium glucose cotransporter.* Bioorg Med Chem, 2010. **18**(6): p. 2152-8.

239. Boyd, D.R., et al., *Synthesis, structure and stereochemistry of quinoline alkaloids from Choisya ternata.* Org Biomol Chem, 2007. **5**(18): p. 2983-91.

240. Neumann, C.S., D.G. Fujimori, and C.T. Walsh, *Halogenation strategies in natural product biosynthesis.* Chem Biol, 2008. **15**(2): p. 99-109.

241. Bakus, G.J., *Toxicity in holothurians: a geographical pattern.* Biotropica, 1974. **6**(4): p. 229-36.

242. Leal, M.C., et al., *Trends in the discovery of new marine natural products from invertebrates over the last two decades-where and what are we bioprospecting?* PLoS One, 2012. **7**(1): p. e30580.

243. Becerro, M.A., et al., *Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses.* Oecologia, 2003. **135**(1): p. 91-101.

244. Cascao, R., et al., *Effective treatment of rat adjuvant-induced arthritis by celastrol.* Autoimmun Rev, 2012. **11**(12): p. 856-62.

245. Tomasdottir, V., et al., *Murine antigen-induced inflammation-a model for studying induction, resolution and the adaptive phase of inflammation.* J Immunol Methods, 2014. **415**: p. 36-45.