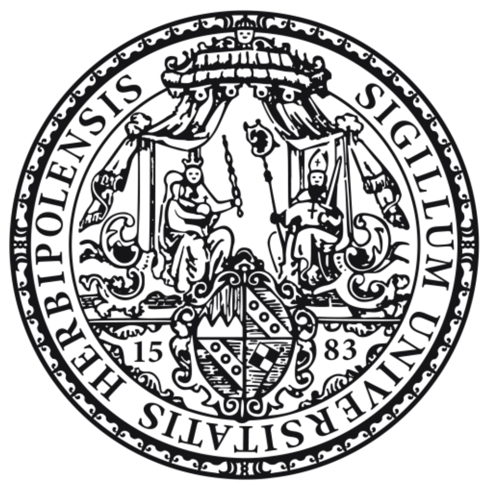
Antimicrobial, anti-protease and immunomodulatory activities of secondary metabolites from Caribbean sponges and their associated bacteria

Sekundärmetabolite mit antimikrobiellen, Protease-hemmenden und immunmodulatorischen Aktivitäten aus karibischen Schwämmen und assoziierten Bakterien



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AFFIDAVIT

I hereby declare that my thesis entitled “Antimicrobial, anti-protease and immunomodulatory activities of secondary metabolites from Caribbean sponges and their associated bacteria” is the result of my own work. I did not receive help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

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______________________________	______________________________
Place, date	Signature

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SUMMARY

Marine sponges and their associated bacteria have been proven to be a rich source of novel secondary metabolites with therapeutic usefulness in infection and autoimmunity. This Ph.D. project aimed to isolate bioactive secondary metabolites from the marine sponges *Amphimedon compressa*, *Aiolochroia crassa* and *Theonella swinhoei* as well as from bacteria associated with different Caribbean sponges, specifically actinomycetes and sphingomonads. In this study, amphitoxin was isolated from the crude methanol extract of the sponge *A. compressa* and it was found to have antibacterial and anti-parasitic activities. Amphitoxin showed protease inhibitory activity when tested against the mammalian protease cathepsin B and the parasitic proteases rhodesain and falcipain-2. Furthermore, miraziridine A was identified in the dichloromethane extract of the sponge *T. swinhoei* collected offshore Israel in the Red Sea. Miraziridine A, a natural peptide isolated previously from the marine sponge *Theonella* aff. *mirabilis*, is a potent cathepsin B inhibitor with an IC_{50} value of 1.4 μg/mL (2.1 μM).

Secondary metabolites from sponge-derived bacteria were also isolated and identified. A total of 79 strains belonging to 20 genera of the order Actinomycetales and seven strains belonging to two genera of the order Sphingomonadales were cultivated from 18 different Caribbean sponges and identified by 16S rRNA gene sequencing. Seven of these strains are likely to represent novel species. Crude extracts from selected strains were found to exhibit protease inhibition against cathepsins B and L, rhodesain, and falcipain-2 as well as immunomodulatory activities such as induction of cytokine release by human peripheral blood mononuclear cells. The isolates *Sphingobium* sp. CO105 and *Lapillicoccus* sp. BA53 were selected for cultivation, extraction and purification of bioactive metabolites based on initial bioactive screening results. The isoalloxazine isolumichrome was isolated from the strain *Sphingobium* sp. CO105 which inhibited the protease rhodesain with an IC_{50} of 0.2 μM. The strain *Lapillicoccus* sp. BA53 was found to produce p-aminosalicylic acid methyl ester, which showed activity against the proteases cathepsins B and L, falcipain-2 and rhodesain. These results highlight the significance of marine sponge-associated bacteria to produce bioactive secondary metabolites with therapeutic potential in the treatment of infectious diseases and disorders of the immune system.

ZUSAMMENFASSUNG

Marine Schwämme und damit assoziierte Bakterien stellen eine wertvolle Quelle für neuartige Sekundärmetabolite mit therapeutischer Bedeutung für Infektion und Autoimmunität dar. Ziel dieser Doktorarbeit war die Isolierung bioaktiver Sekundärmetabolite aus den marinen Schwämmen *Amphimedon compressa, Ailochroia crassa* und *Theonella swinhoei* sowie von Bakterien, die mit verschiedenen karibischen Schwämmen assoziiert sind, wie z. B. Actinomyceten und Sphingomonaden. Amphotoxin wurde in dieser Studie aus dem methanolhaltigen Rohextrakt des Schwammes *A. compressa* isoliert. Es konnte sowohl eine antibakterielle als auch antiparasitäre Aktivität nachgewiesen werden. Der Einfluss von Amphotoxin auf die humane Protease Cathepsin B und die parasitären Proteasen Rhodesain und Falcipain-2 wurde ebenfalls getestet und es zeigte sich eine inhibitorische Wirkung gegenüber diesen Proteasen. Darüber hinaus wurde aus dem Dichlormethanextrakt des Schwammes *T. swinhoei*, der aus dem Roten Meer in Israel gewonnen wurde, Miraziridin A isoliert. Dieses natürliche Peptid war bereits aus dem marinen Schwamm *Theonella* aff. *mirabilis* isoliert worden. Miraziridin A ist ein starker Cathepsin B Inhibitor, der IC_{50} Wert beträgt 1.4 mg/mL (2.1 μM).

Sekundärmetabolite von aus Schwämmen gewonnenen Bakterien wurden ebenfalls isoliert und identifiziert. Es konnten 79 Stämme, die zu 20 verschiedenen Gattungen der Ordnung Actinomycetales, sowie sieben Stämme, die zu zwei Gattungen der Ordnung Sphingomonadales gehören, isoliert werden. Diese Bakterienstämme wurden aus ingesamt 18 verschiedenen karibischen Schwämmen kultiviert und mit Hilfe der 16S rRNA Sequenzierung bestimmt. Sieben dieser Stämme stellen wahrscheinlich neue Arten dar. Rohextrakte ausgewählter Stämme zeigten eine Proteasehemmung gegen die Cathepsine B und L, Rhodesain, Falcipain-2 sowie immunmodulatorische Wirkungen wie z.B. die Induktion der Cytokinfreisetzung durch menschliche periphere mononukleäre Blutzellen. Die Isolate *Sphingobium* sp. CO105 und *Lapillicoccus* sp. BA53 wurden für die Kultivierung, Extraktion und Aufreinigung von bioaktiven Metaboliten aufgrund der ersten vielversprechenden bioaktiven Testergebnisse ausgewählt. Das Isoalloxazin Isolumichrom wurde aus dem Stamm *Sphingobium* sp. CO105 isoliert, welches die Protease Rhodesain mit einem IC_{50}-Wert von 0.2 μM inhibiert. Für den Stamm *Lapillicoccus* sp. BA53 konnte nachgewiesen werden, dass er p-Aminosalicylsäuremethylester produziert, der eine Aktivität gegen die Proteasen Cathepsin B und L, Falcipain-2 und Rhodesain zeigt. Diese Ergebnisse unterstreichen die Bedeutung mariner, Schwamm-assoziierter Bakterien, die bioaktive sekundäre Metabolite

mit therapeutischem Potential für die Behandlung von Infektionskrankheiten und Funktionsstörungen des Immunsystems produzieren.

1. Introduction

1.1. Infection and autoimmunity

Infection and autoimmunity represent serious problems concerning health care worldwide. These include infectious diseases e.g. malaria, trypanosomiasis and leishmaniasis, as well as autoimmune diseases such as rheumatoid arthritis. For example, the mosquito-borne infectious disease Malaria, caused by the parasite of the genus *Plasmodium*, is endemic in 109 countries and territories in tropical and subtropical zones (WHO, 2008). According to the World Health Organization millions of new cases are reported every year. An estimated 225 million cases of malaria led to nearly 968,000 deaths in 2009 (WHO, 2010b). Up to now, several treatments have been used to treat the malaria infection, such as chloroquine, sulfadoxine–pyrimethamine and mefloquine, treatments that have become ineffective in many countries due to the resistance developed by the parasite, especially in the treatment of the infection caused by the species *Plasmodium falciparum* (Marquino et al., 2003, Smithuis et al., 2004).

Another example of infectious diseases is sleeping sickness, which is a neglected tropical disease caused by infection with protozoan parasites belonging to the genus *Trypanosoma* (WHO, 2010a). Neglected tropical diseases afflict poor people of developing countries. Trypanosomiasis is a vector-borne disease and is usually fatal if left untreated. Among several species of the parasite, the species *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are responsible for the majority of the reported cases. *Trypanosoma brucei gambiense* is endemic in 24 countries of west and central Africa and is the cause for more than 90% of the reported cases of sleeping sickness. The species *Trypanosoma brucei rhodesiense* is endemic in 13 countries of eastern and southern Africa, representing less than 10% of reported cases (WHO, 2010a). According to the Weekly Epidemiological Record (WHO, 2006), the total number of cases in 2006 was estimated at 50,000 - 70,000. Neglected diseases cause massive, though hidden and silent, suffering, and are frequently lethal, albeit not in numbers comparable to the deaths caused by HIV/AIDS, tuberculosis or malaria (WHO, 2010a).

Rheumatoid arthritis (RA) is an autoimmune disease, more specifically a chronic inflammatory disease characterized by joint swelling, joint tenderness and destruction of synovial joints, leading to dramatic loss of life quality due to chronic pain as well as to premature mortality (Aletaha et al., 2010). Rheumatoid arthritis disorder affects millions of people around the world and is the result of the excessive production of TNF-α (tumor

necrosis factor alpha), IL-1 (interleukin-1) and IL-6 cytokines by activated T cells, and the stimulation of the production of immunoglobulin by B cells (Salliot et al., 2009). In the last decade, the availability of therapeutic agents for the treatment of RA has considerably increased. TNF blockers have been widely used in the treatment of RA, they have shown efficacy in reducing inflammation and joint destruction (Maini and Taylor, 2000, Kang et al., 2005). TNF blockers are derived from a recombinant TNF receptor; TNFRSF1B for etanercept, anti-TNFA monoclonal antibody for infliximab and adalimumab (Kang et al., 2005). They are able to inhibit the binding of TNFs to cell-surface TNF receptors. However TNF blockers have exhibited side effects and not all patients have shown a positive response to the treatment (Kang et al., 2005, Bathon et al., 2000). The use of the disease modifying antirheumatic drugs (DMARD), in particular the anchor DMARD methotrexate, has enhanced the success of RA management (Choi et al., 2002). There is currently no cure for rheumatoid arthritis and it would be ideal to develop and/or improve the treatments that can be applied to patients at a stage at which the evolution of joint destruction may still be prevented (Aletaha et al., 2010). The lack of effective treatments withing the currently available therapies leading to various undesirable effects, aggravates the problem and reveals the necessity of finding new therapeutic agents and/or improving the currently available treatments of infectious and autoimmune diseases.

1.2. Drug discovery approaches

Different strategies have been developed to find new treatments against infectious and autoimmune diseases. These are target-based screening, phenotypic screening, modification of natural substances and biology-based approaches (Swinney and Anthony, 2011) to name only a few. Despite the advances in developing drug candidates over the last decade, drug research remains a crucial challenge. Surprisingly, the number of drugs, in which new molecular entities (NMEs) were approved by the US Food and Drug Administration (FDA), decreased by 50%, when compared with the previous 5 years (Paul et al., 2010). The FDA approved 15 new molecular entities in 2010, which lags behind the 19 new molecular entities approved in 2009 and the 21 in 2008 (Hughes, 2009, Hughes, 2010, Mullard, 2011) (Figure 1). Furthermore, most of the therapeutic agents approved by the FDA in the last years are treatments against cancer and heart-related failure. This fact reflects the lack of research aiming to discover new alternative therapies against infectious diseases, among them such neglected diseases as, for example, the African trypanosomiasis and leishmaniasis.

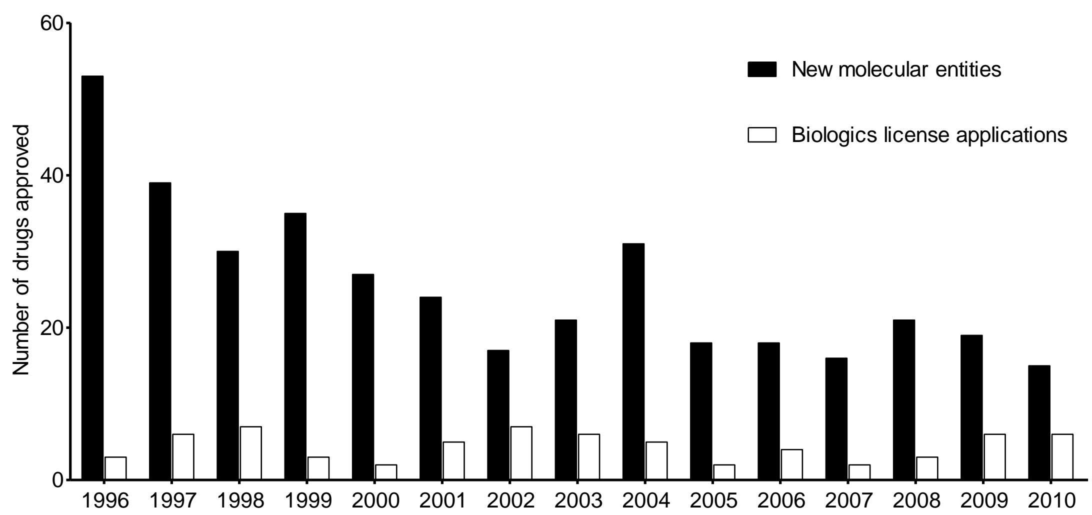


Figure 1. FDA drug approvals since 1996. New molecular entities and biologics license applications approved by the US Food and Drug Administration's (FDA's) Center for Drug Evaluation and Research. Modified from Mullard (2011)

1.2.1. Proteases as drug targets

In this study one of the approaches to discover drug candidates is the targeting of proteases, which are relevant drug targets in cancer, cardiovascular, inflammatory and infectious disease areas (Otto and Schirmeister, 1997, Turk, 2006). Proteases are enzymes that play essential functions in many signaling pathways, the development of certain types of cancer as well as in infectious diseases such as malaria and trypanosomiasis. Cathepsins B and L are human cysteine proteases involved in tumor metastasis (Calkins and Sloane, 1995). Falcipain-2 and rhodesain are proteases expressed by the parasites responsible for malaria and African trypanosomiasis. The papain-like and the main protease of the SARS coronavirus (SARS-CoV PL^{pro}, SARS-CoV M^{pro}) are enzymes essential for the replication of the severe acute respiratory syndrome (SARS) coronavirus (Ratia et al., 2006, Anand et al., 2003). Around 32 protease inhibitors are currently in clinical use and at least 9 are in development. Examples are ritonavir, an aspartic protease inhibitor of HIV-1 in clinical use since 1996 for the AIDS treatment, and boceprevir and telaprevir approved by the FDA in 2011 for the treatment of hepatitis C virus infection (Drag and Salvesen, 2010). Most of the protease inhibitors reported to date are synthetic molecules developed by structure-based design (Turk, 2006). Furthermore, protease inhibitors have also been found in natural sources. Miraziridine A (Figure 2), a pentapeptide inhibitor of cathepsins B and L, which was isolated from the marine sponge *Theonella mirabilis*, is one such example (Nakao et al., 2000). A family of aeruginosin inhibitors is active against human serine proteases and

was isolated from marine sponges and cyanobacterial waterblooms (Ersmark et al., 2008). Leupeptin was reported from actinomycetes by Hozumi et al. (1972).

Miraziridine A

(+)-Aeruginosin 298-A

Leupeptin Ac-LL

Figure 2. Examples of protease inhibitors isolated from marine sources

1.2.2. Search for immunomodulatory agents

A second relevant aspect in the drug discovery field is the search for immunomodulatory agents, such as stimulators of the cells of the immune system such as T cells and NKT cells, which play important roles in responses against microbial and tumor antigens. For example T cells, the mediators of cellular immunity, recognize the antigens of intracellular

microbes and destroy these microbes or the infected cells. T lymphocytes consist of functionally distinct populations, such as helper T lymphocytes, cytotoxic T lymphocytes, $CD4^+$ regulatory T cells and gamma delta T cells ($\gamma\delta$ T cells). Another example of immunomodulatory agents are lectins, which are glycoproteins that participate in numerous cellular processes, such as cell communication, host defense, fertilization and development (Rangel et al., 2011). NKT cells have been linked to microbial immunity, autoimmunity, allergy and cancer and, accordingly, they represent an important immunotherapeutic target with immense clinical potential (Pellicci et al., 2011). NKT cell-mediated regulation of immune responses has been demonstrated to influence a large number of disease states. In fact, some forms of human cancer have been correlated to a loss of NKT cells, whereas in animal models, stimulation of NKT cells leads to decreases in tumor size and growth (Savage et al., 2006). The substance KRN7000 (αGalCer), a synthetic analogue of the natural product agelasphin 9b (Figure 3) isolated originally from the sponge *Agelas mauritianus* (Natori et al., 1993), is an NKT cell stimulator and is widely used to study the behavior of NKT cells (Park et al., 2010). GSL-1a, a glycosphingolipid antigen from *Sphingomonas* bacteria that is structurally related to αGalCer, stimulates NKT cells and might be a good candidate for immunotherapy (Sullivan and Kronenberg, 2005). A variety of bacterial cells and their fractions have been used as immunoadjuvants for the host stimulation against cancer, infectious diseases, and other immunological disorders in both clinical and experimental situations (Azuma and Seya, 2001). Furthermore, immunoadjuvants are used in vaccination, as well as in immune therapy, i.e., the immunostimulatory CpG oligonucleotides (ODNs) induce maturation, differentiation and/or proliferation of multiple cell types (Klinman et al., 2010).

Agelasphing 9b

GSL-1a

Figure 3. Examples of NKT cell stimulator glycosphingolipids isolated from marine sources

1.3. Natural products as sources of therapeutic agents

Natural products from terrestrial sources such as plants (roots, latex, leaves, etc.) and microbial communities (fungi and bacteria) have yielded an exhaustive supply of

biologically active secondary metabolites including the plant related anti-tumor agents taxol and colchicine, and several antibiotics such as amphotericin B, tetracycline, and erythromycin (Demain, 1999, Dubois et al., 2003, Esquenazi et al., 2008). Before the rise of high-throughput screening (HTS), and the post-genomic era, more than 80% of drug substances were natural products or inspired by a natural compound (Harvey, 2008, Butler, 2008). Even though terrestrial environments have served as source of many drugs currently in clinical use, terrestrial environments have been exhaustively studied and, during the last years, the search for new therapeutic agents has been directed to other natural sources such as marine animals and their associated microorganisms.

Since the 1960's, more than 20,000 compounds have been discovered from marine sources (Hu et al., 2011). The organisms producing these marine natural products are divided into three major biological classes: microorganisms, algae and marine invertebrates. Between 1985 and 2008, approximately 75% of the compounds were isolated from marine invertebrates belonging to the phyla Porifera (sponges) and Coelenterate (coral) (Figure 4) (Hu et al., 2011).

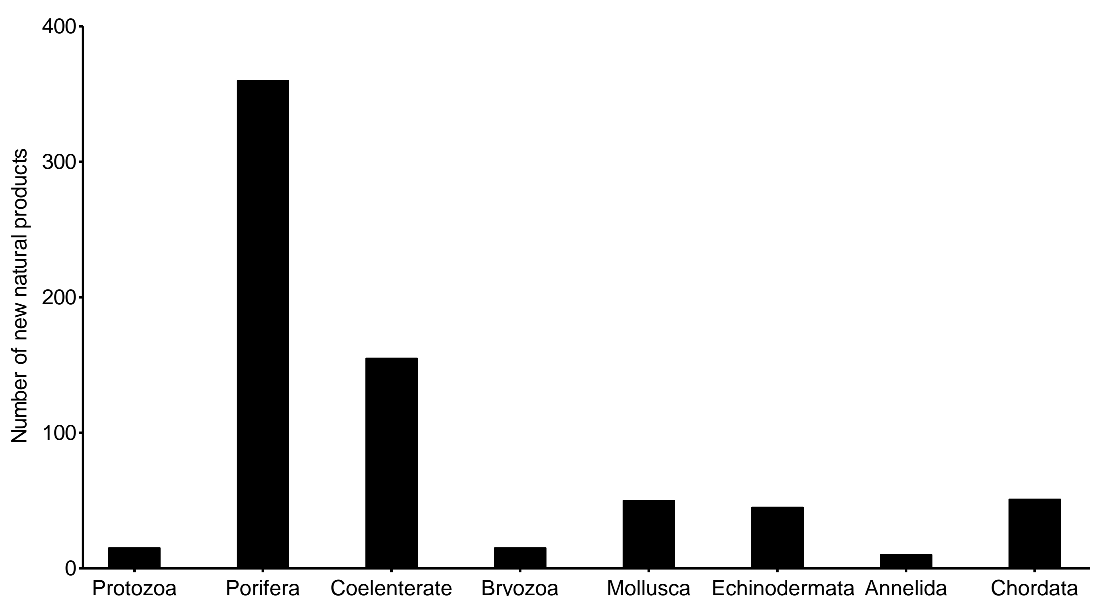


Figure 4. The number of novel compounds isolated from marine invertebrates between 1985 and 2008. Modified from Hu et al. (2011)

Currently there are four marine drugs approved by the FDA in the US Pharmacopeia, namely cytarabine, vidarabine, ziconotide and halaven (Mayer et al., 2010, Huyck et al., 2011) (Figure 5). Cytarabine is a synthetic pyrimidine nucleoside that was developed from

spongothymidine, a nucleoside originally isolated from the Caribbean sponge *Tethya crypta*, and is used in the treatment of acute lymphocytic leukemia (Molinski et al., 2009). Vidarabine, a purine nucleoside developed from the spongouridine, originally isolated from the Caribbean sponge *Tethya crypta*, is currently obtained from *Streptomyces antibioticus* (Shen et al., 2009), and is active against the herpes simplex encephalitis virus; it has been in clinical use for many years. Ziconotide is the equivalent of a naturally occurring 25-amino acid peptide, ν-conotoxin MVIIA, isolated from the venom of the fish-hunting marine snail *Conus magus* and is used in the management of severe chronic pain in patients with AIDS (Staats et al., 2004). The most recent marine metabolite approved by the FDA is halaven, a synthetic form of a chemotherapeutically active compound derived from the sponge *Halichondria okadai*, which is active in the treatment of advanced breast cancer (Huyck et al., 2011).

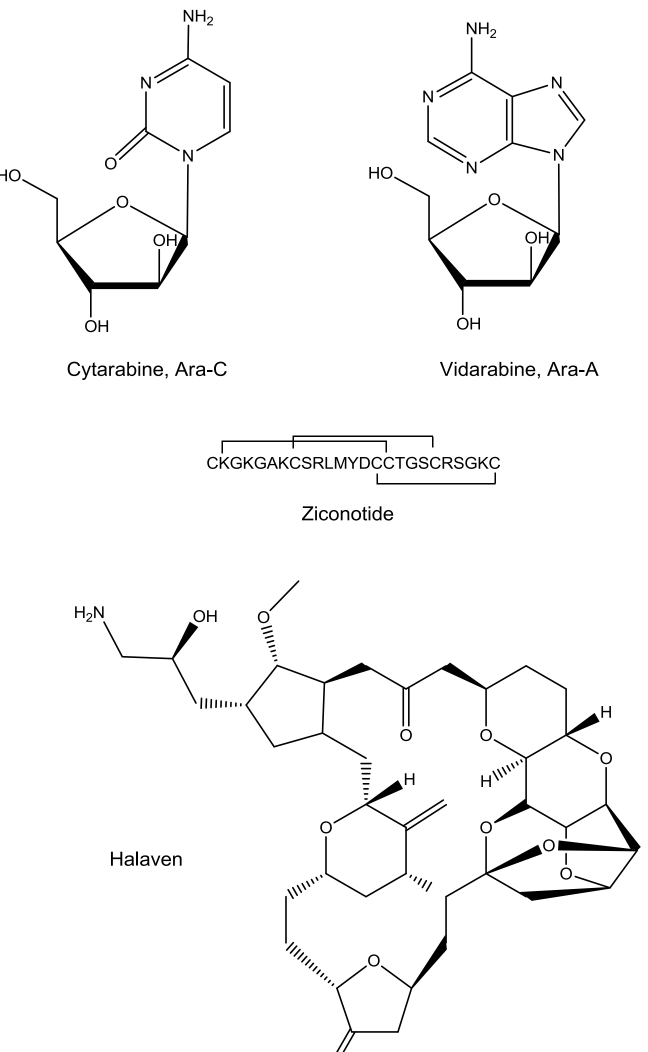


Figure 5. Marine natural products or derivatives approved for clinical use by the FDA

1.3.1. Marine sponges

Marine sponges (phylum *Porifera*) are among the oldest multicellular animals (Metazoans) (Li et al., 1998). The Porifera are divided into three classes: the Calcarea (calcareous sponges), the Hexactinellida (glass sponges) and the Demospongiae (demosponges). The latter is the most comprehensive and variable class covering about 95% of all sponge species (Brusca and Brusca, 1990). More than 6,000 species have been described for this phylum, classified in 25 orders, 127 families and 682 genera (Brusca and Brusca, 1990, Hooper and Van Soest, 2002). Marine sponges lack neurons or any other kind of nervous tissue. Instead, they have a unique bauplan, characterized by different reproductive modes as well as cellular totipotency and mobility balancing the lack of true tissues and organs (Brusca and Brusca, 1990). Sponges are highly variable in color and shape encompassing different morphotypes (i.e. tube, vase, rope, ball, encrusting) (Figure 6) that vary with exposure to currents and depth. Marine sponges are found mostly in tropical and subtropical benthic habitats, but also thrive in higher latitudes as well as in freshwater lakes and streams (Brusca and Brusca, 1990, Hooper and Van Soest, 2002). Marine sponges with an exceptionally rich chemistry have been the source of several bioactive secondary metabolites. To date, more than 5300 different products have been isolated from sponges and their associated microorganisms (Laport et al., 2009). Blunt et al. (2010) in a Natural Product Report review, described 287 new compounds from marine sponges isolated in 2009. Remarkable examples of bioactive secondary metabolites isolated from marine sponges are hemiasterlin (E7974) and discodermolide (Figure 7). Hemiasterlin (E7974), is a cytotoxic tripeptide originally isolated from the marine sponge *Hemiasterella minor*, currently in Phase I trial (Talpir et al., 1994). The polyketide natural product discodermolide, isolated from the marine sponge *Discodermia dissoluta* has potent cytotoxicity to human and murine cell lines (Kingston et al., 2011). Certain marine sponges have been recognized as potentially rich sources of various bioactive compounds. According to the MarinLit database, around 319 compounds have been reported from the genus *Xestospongia*, 244 compounds from the genus *Theonella*, 222 compounds from the genus *Halicondria* and 118 metabolites have been reported from the genus *Aplysina* among other sponges. Of special interest to this project are the species *Amphimedon compressa*, *Aiolochroia crassa* and *Theonella swinhoei* for the protease inhibitory activity of their crude extracts observed in previous studies (Degel, 2006).

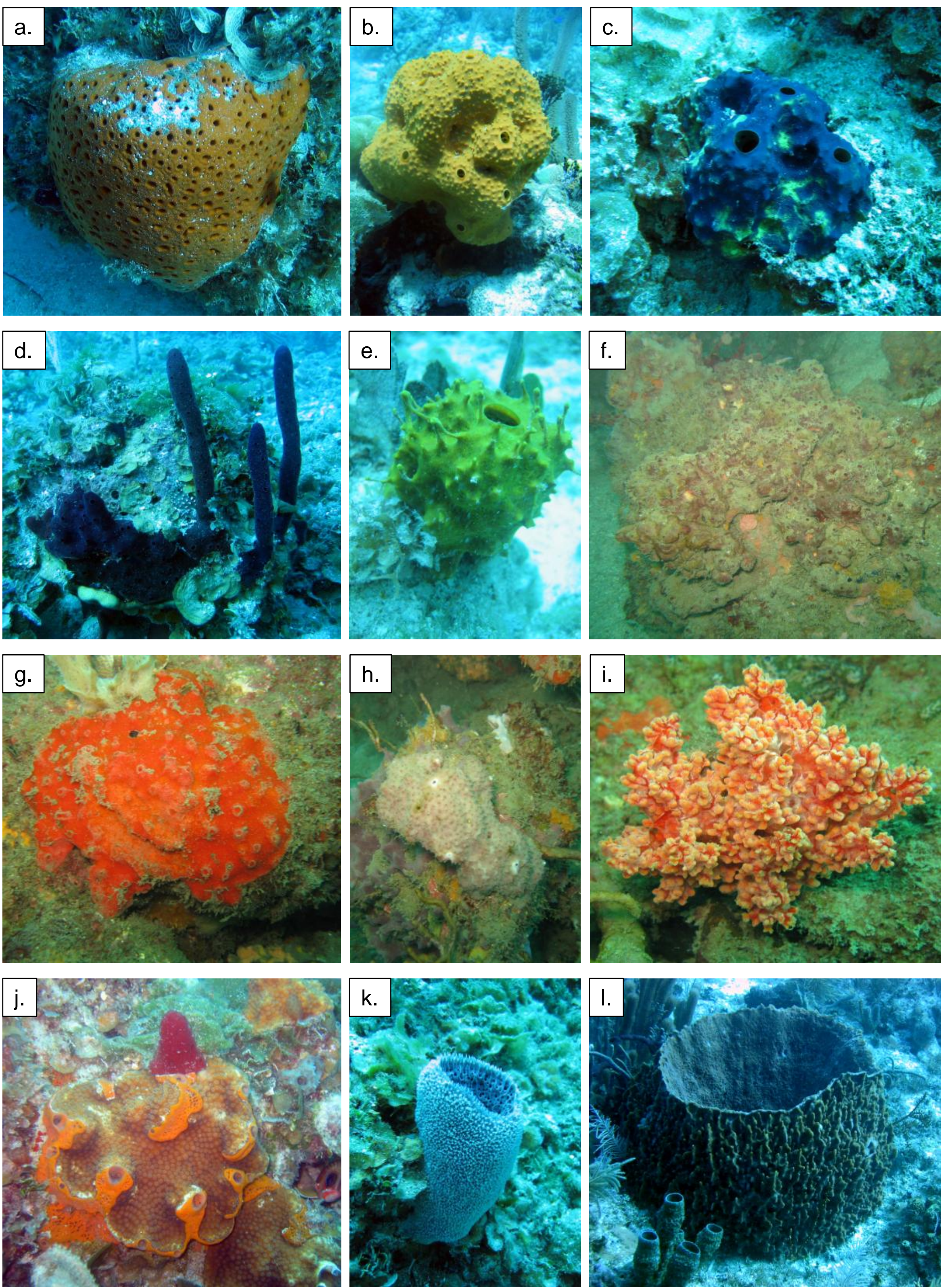


Figure 6. Demosponges: a. *Agelas clathrodes*, b and c two differently colored morphotypes of the sponge *Aiolochroia crassa,* d. *Amphimedon compressa*, e. *Aplysina fistularis*, f. *Discodermia dissoluta,* g. *Dragmacidon reticulata*, h. *Ircinia felix*, i. *Monanchora arbuscula*, j. *Mycale laevis*, k. *Niphates digitalis* and l. *Xestospongia muta*. Sponges a - e and j - l were photographed by Dr. Hilde Angermeier from the University of Würzburg and sponges f - i by Prof. Sven Zea from INVEMAR, Colombia.

Hemiasterlin (E7974)

Discodermolide

Figure 7. Examples of bioactive compounds isolated from marine sponges

Amphimedon compressa is a marine sponge (phylum Porifera, class Demospongiae) abundant in Florida, the Bahamas and the Caribbean (Angermeier, 2011), known to produce useful natural products, such as cytotoxins and antifouling agents (Jeanteur et al., 2006). *A. compressa* belongs to the family Haliclonidae, whose color varies from brown with green shades to red or purple-brown (Albrizio et al., 1995). At least seventeen secondary metabolites have been reported from the sponge *Amphimedon compressa* such as 2-hydroxydocosanoic acid, 2-hydroxytricosanoic acid (Carballeira and Lopez, 1989) (Figure 8), 17-tricosenal, 21-octacosenoic acid, 19-pentacosenal, 19-hexacosenal, 16-tricosenoic acid, 18-tricosenoic acid, 16-pentacosenoic acid, 18-pentacosenoic acid, 19-pentacosenoic acid, 20-hepacosenoic acid (Carballeira et al., 1992), methyl 2-methoxyhexadecanoate, 8,8'-dienecyclostellettamine (Carballeira et al., 1998), 8,8'-dienecyclostellettamine (Xu et al., 2007), amphitoxin (Albrizio et al., 1995) and amphiceramides A and B (Costantino et al., 2009). Amphitoxin, for example, showed a broad spectrum of biological activities including antimicrobial and anti-feedant activities (Jeanteur et al., 2006).

methyl 2-methoxyhexadecanoate

8,8'-dienecyclostellettamine

Amphitoxin

Amphiceramide A, R: -H
Amphiceramide B, R: -NHAc

Figure 8. Examples of secondary metabolites isolated from the sponge *A. compressa*

Aiolochroia crassa is a marine sponge of the order Verongida, which is massive, with lobate or, more rarely, ramose processes, yellow to violet in color (Ciminiello et al., 1995). *Aiolochroia crassa* is characterized chemically by a series of secondary brominated metabolites, biogenetically arising from bromotyrosines (Albrizio et al., 1994). Around twenty compounds have been isolated from the sponge *Aiolochroia crassa* (Figure 9) including N-methyl-aerophobin-2, aerophobin-1, aerophobin-2, purealidin L, isofistularin-3 (Assmann et al., 1998), araplysillin III, hexadellin C (Hamann et al., 1999). Fistularin-3 was active in vitro against *Mycobacterium tuberculosis* (Hamann et al., 1999). A recent study of the structural and physico-chemical properties of three-dimensional skeletal scaffold of the marine sponge *Aiolochroia crassa*, showed that these fibrous scaffolds have a multilayered design and are made of chitin (Ehrlich et al., 2010). Natural polymers like chitin are widely used in the biomedical field because of their high biocompatibility and the enriched functionalities being capable of integrating well with a variety of ligands (Rejinold et al., 2011).

N-methyl-aerophobin-2

araplysillin III

isofistularin-3

Figure 9. Examples of secondary metabolites isolated from the sponge *A. crassa*

The marine sponge *Theonella swinhoei*, a marine sponge of the order Lithistida is typically found in deeper waters and caves of tropical oceans. *T. swinhoei* has a structurally massive and rigid morphology. Its skeleton consists of fused or interlocked spicules, called desmas (Jeanteur et al., 2006). The sponge *Theonella swinhoei* has shown to be a source of anti-protease and anti-HIV secondary metabolites (Fusetani et al., 1999, Plaza et al., 2010). The marine sponge *Theonella* aff. *mirabilis*, has been reported to contain the protease inhibitor miraziridine A (Nakao et al., 2000) and the papuamides A and B (Figure 10) with anti-HIV properties (Ford et al., 1999). Miraziridine A is a secondary metabolite of particular interest due to its three structural elements (i) (*2R,3R*)-aziridine-2,3-dicarboxylic acid, (ii) (*3S,4S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine) and (iii) (E)-(*S*)-4-amino-7-guanidino-hept-2-enoic acid (vinylogous arginine residue), which are responsible for the inhibition of three different classes of proteases, such as serine (e.g. trypsin), cysteine (e.g. cathepsins B and L) and aspartyl proteases (e.g. pepsin) (Schaschke, 2004). The sponge *Theonella swinhoei* has been found to contain antifungals including cyclolithistide A, theonegramides and theopalauamide, as well as paltolides and cytotoxic polytheonamides (Schaschke, 2004).

papuamide A

cyclolithistide A

Figure 10. Examples of secondary metabolites isolated from the sponge *T. swinhoei*

1.3.1.1. Microbial diversity of marine sponges

Many marine sponges are associated with dense and phylogenetically diverse microbial consortia including bacteria, archaea and single-celled eukaryotes (fungi and microalgae), that can account for nearly half of the animal's biomass (Hentschel et al., 2006, Taylor et al., 2007). Sponges are filter-feeders capable of processing enormous volumes of seawater, providing a rich source of microorganisms. Two different sponge types in respect of their association with bacteria have been called "high-microbial-

abundance" (HMA) and "low-microbial-abundance" (LMA) sponges (Figure 11) (Hentschel et al., 2003). Bacterial populations in HMA are in the range of 6.4×10^8 – 1.5×10^9 bacteria g^{-1} (Vacelet and Donadey, 1977, Hentschel et al., 2006). So far, more than 25 bacterial phyla have been reported from sponges (Webster and Taylor, 2011, Schmitt et al., 2011) (Figure 12). The dominant bacterial taxa in marine sponges are *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria* and *Cyanobacteria* (Fieseler et al., 2004, Scheuermayer et al., 2006, Taylor et al., 2007).

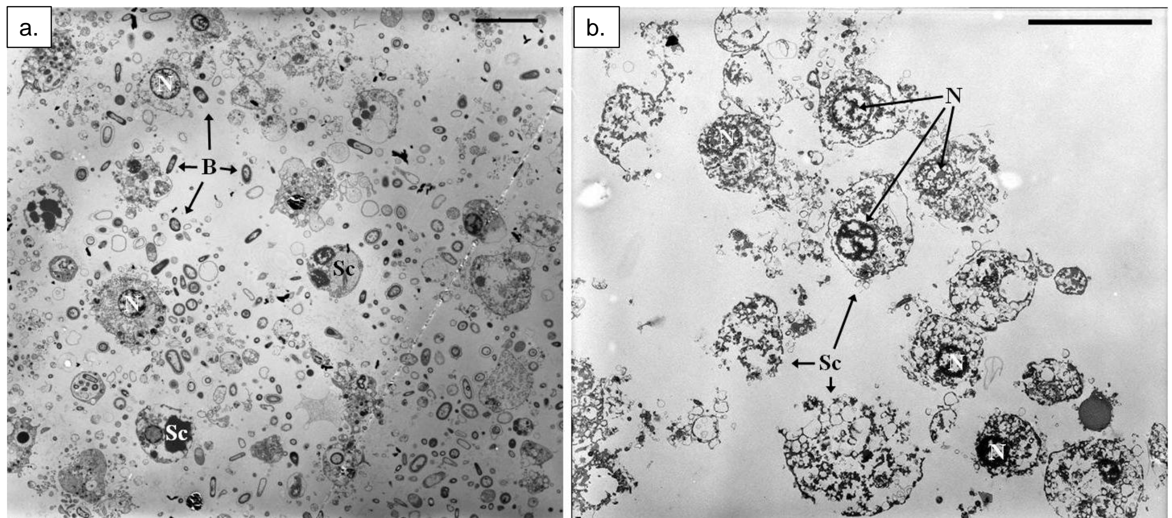


Figure 11. Transmission electron micrographs of sponge tissue. a. sponge *Xestospongia muta* (HMA) and b. sponge *Amphimedon compressa* (LMA). The scale bars represent 5 μm each. Abbreviations: N, nucleus; Sc, sponge cell; B, bacteria (Angermeier, 2011)

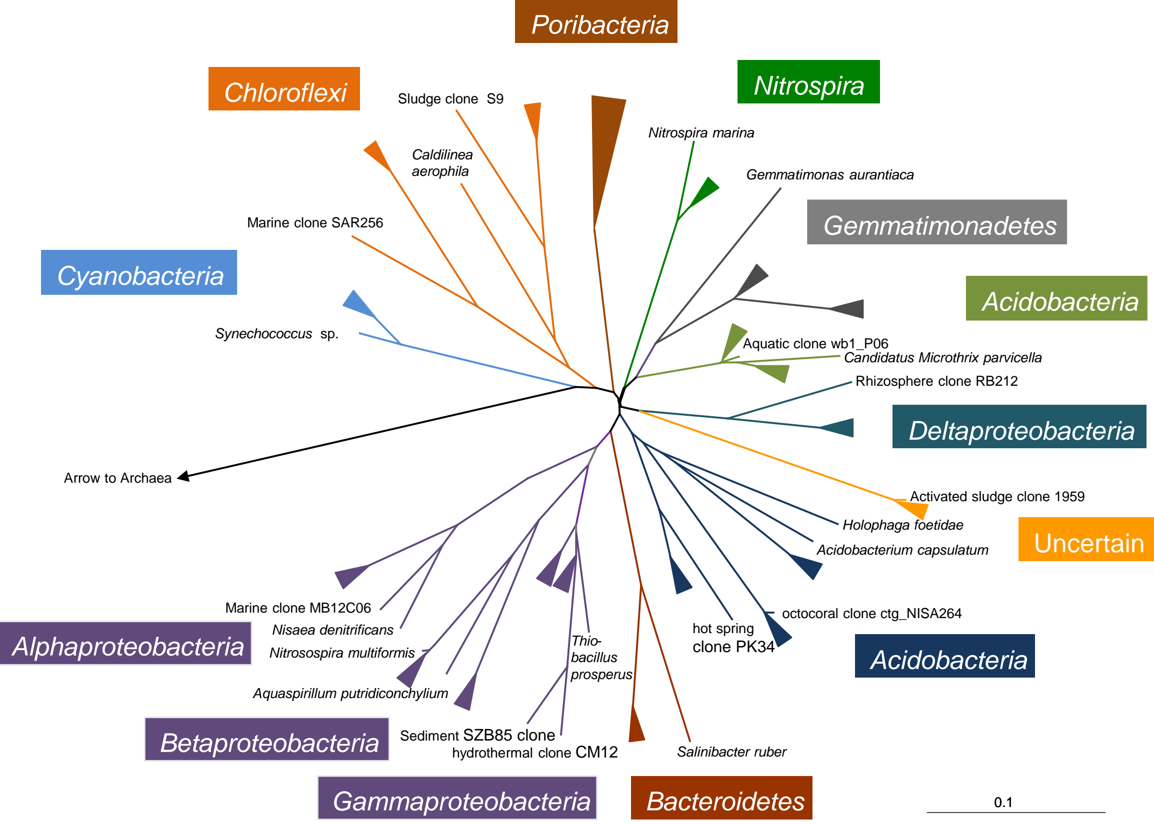


Figure 12. Phylogenetic distribution of sponge-associated bacteria. Modified from Schmitt and Hentschel (2008)

Marine microorganisms are well-known for being capable of producing bioactive secondary metabolites. Between 1985 and 2008, around 850 compounds were isolated from marine microorganisms including bacteria, fungi and phytoplankton (Hu et al., 2011) (Figure 13). A wide range of chemical and functional diversity has been observed among bioactive compounds such as polyketides, alkaloids, fatty acids, peptides and terpenes (Thomas et al., 2010). Most of the compounds isolated from marine microorganisms have shown biological properties such as antimicrobial, antitumor and anticancer activities. The phylum *Actinobacteria* dominates in the production of therapeutic compounds followed by *Proteobacteria*. Among fungi, members of the *Ascomycota* are predominant producers of bioactive molecules and members of *Deuteromycota* are also a potential group for exhibiting bioactivity (Thomas et al., 2010).

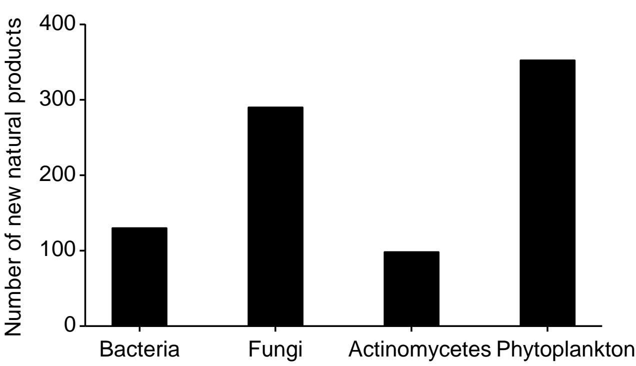


Figure 13. The number of novel compounds isolated from marine microorganisms between 1985 and 2008. Modified from Hu et al. (2011)

Of special interest to this PhD project are the members of the phylum *Actinobacteria* (Figure 14) which have been identified in sponges both by cultivation and cultivation-independent approaches (Webster et al., 2001, Hentschel et al., 2002, Kim et al., 2005, Montalvo et al., 2005, Jiang et al., 2008, Pimentel-Elardo et al., 2010, Abdelmohsen et al., 2010, Tabares et al., 2011b). Several genera have been described for this order, namely *Streptomyces*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Arthrobacter*, and *Microbacterium*. Actinomycetes have been described as potential sources of secondary metabolites. For example, the cebulactams A1 and A2 have been reported from the first obligate marine strain of the genus *Saccharopolyspora* (Pimentel-Elardo et al., 2008). *Streptomyces* has been shown to be the most prolific producer of bioactive metabolites accounting for the majority of the reported metabolites with antibiotic properties (Dharmaraj, 2010). Around 80% of all microbial-derived secondary metabolites are produced by members of the order *Actinomycetales* with the genus *Streptomyces* accounting for approximately 50%. For instance, four new tetromycin derivatives with antitrypanosomal and protease inhibitory activities have been isolated from the strain *Streptomyces axinellae*, which has been cultivated from the Mediterranean sponge *Axinella polypoides* (Pimentel-Elardo et al., 2011). *Actinobacteria*, and specifically members of the order actinomycetes, are, in fact, quite abundant in the marine environment (Stach and Bull, 2005). A number of obligately marine actinomycete species and genera have been described which indicates true forms of adaptation to the marine environment rather than simply being washed into the ocean as spores from terrestrial soils. The actinomycetes are of particular relevance due to their unmatched capacity to produce novel, and bioactive secondary metabolites. About 7,000 compounds have been isolated from this bacterial taxon alone (Jensen et al., 2005). The anticancer compounds salinosporamide and sporolide from the actinomycete *Salinispora tropica* (Fenical et al.,

2009, Buchanan et al., 2005), as well as the antitumor antibiotic marinomycin from the obligately marine genus *Marinispora* (Kwon et al., 2006), are examples of metabolites from marine actinomycetes (Figure 15).

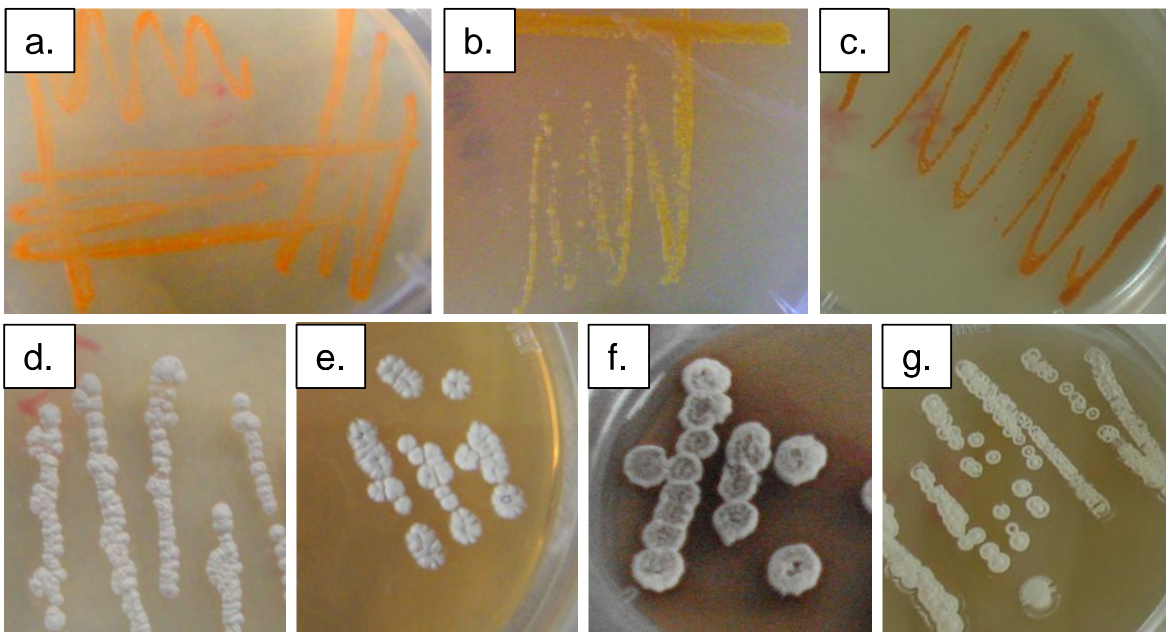


Figure 14. Various morphologies of actinomycete strains. a. *Gordonia terrae*, b. *Lapillicoccus* sp., c. *Micromonospora coxensis*, d. *Saccharopolyspora shandongensis*, e. *Streptomyces chartreusis*, f. *Streptomyces* sp. and g. *Streptomyces* sp. Photos: P. Tabares

Figure 15. Examples of bioactive secondary metabolites isolated from actinomycete strains

Other interesting taxa associated with marine sponges are bacteria of the order *Sphingomonadales*, which are yellow-pigmented, Gram-negative, rod-shaped bacteria that contain glycosphingolipids (GSLs) in their cell envelope and were first described by Yabuuchi et al. (1990). Glycosphingolipids are a class of compounds that have been shown to be potent stimulators of natural killer T cells (Kinjo et al., 2005, Sriram et al., 2005, Mattner et al., 2005). Long et al (2007) reported the synthesis and evaluation of stimulatory properties of the GSL-1 to GSL-4 series of glycosphingolipids isolated from the Sphingomonadaceae family. GSL-1 was found to be a potent NKT cell stimulator. GSL-4 (Figure 16), a metabolite isolated from a *Sphingomonas* strain has been previously found to have NKT cell stimulatory properties (Sriram et al., 2005). Interestingly, Laroche et al. (2007) suggested that glycolipids of the marine sponge *Plakortis simplex* are produced by microbial symbionts rather than by the sponge itself. Sphingomonads also produce other types of secondary metabolites, for example the diketopiperazine glionitrin B, was reported to be produced using a microbial co-culture of the bacterium *Sphingomonas* sp. KMK-001 and the fungus *Aspergillus fumigatus* KMC-901 (Park et al., 2011).

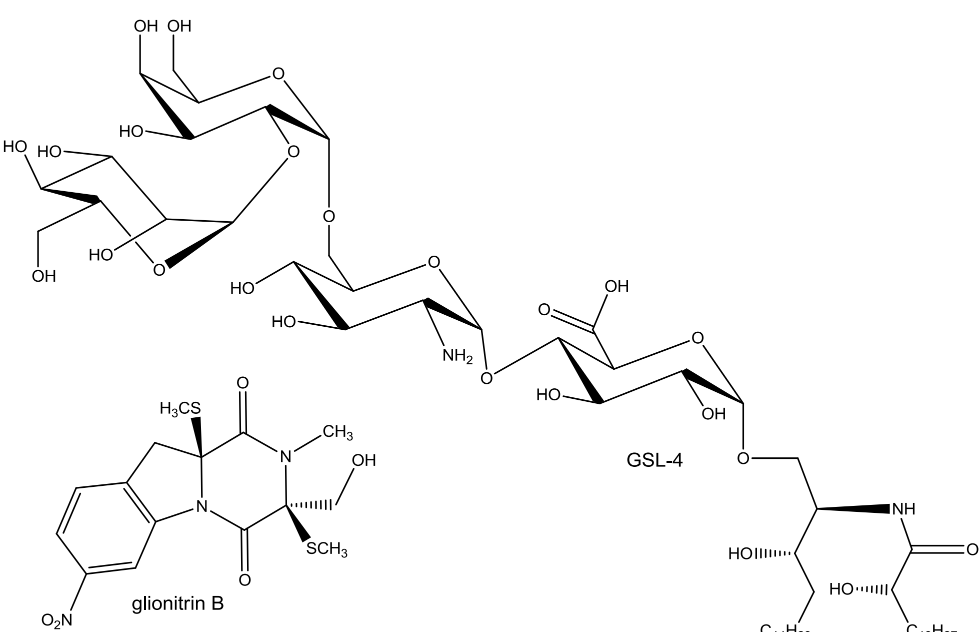


Figure 16. Examples of secondary metabolites isolated from *Sphingomonas* strains

Other interesting taxa of marine organisms associated with marine sponges that have a pharmacological significance are *Cyanobacteria* e.g. *Lyngbya*, *Oscillatoria*, *Symploca*, *Calothrix*, *Leptolyngbya*, *Dichothrix*, *Geitlerinema*, *Schizothrix*, *Aphanothece*, *Blennothrix*

and *Synechocystis* (Nagarajan et al., 2011). *Cyanobacteria* are known to produce diverse structural classes of metabolites. Malyngamide H (Figure 17), is an ichthyotoxic amide isolated from the marine Cyanobacterium *Lyngbya majuscula* (Orjala et al., 1995). Isomalyngamide A, a fatty acid amide isolated from the Taiwanese *Lyngbya majuscula*, was found to have therapeutic potential against tumor cell migration (Chang et al., 2011). It is known, for example, that the occurrence of scytonemins, which are metabolites composed of either an aminocyclohexenone or an aminocyclohexenimine ring, containing amino acid or amino alcohol substituents, is restricted to cyanobacteria or cyanobacterial lichens (Klisch and Hader, 2008). Scytonemins are interesting due to their pharmacological potential as modulator of cell cycle control and inflammation (Stevenson et al., 2002).

Isomalyngamide A

Malyngamide H

Figure 17. Examples of bioactive compounds from marine Cyanobacteria

In recent years, the attention of scientific researchers has been also directed toward the production of secondary metabolites by marine fungi, which have been shown to possess an interesting and unique chemistry, as well as biological properties (Rateb and Ebel, 2011). According to Rateb et al. (2011) more than 1,000 compounds have been isolated from marine fungi. For instance, fourteen anthracenedione derivatives (Figure 18) were separated from the secondary metabolites of the mangrove endophytic fungus *Halorosellinia* sp. and exhibited potent anticancer activity (Zhang et al., 2010).

anthracenedione derivatives

Figure 18. Examples of bioactive compounds from marine fungi

Thus, marine sponges and their associated microorganisms are sources of bioactive secondary metabolites, as yet underexplored. The focus of this PhD thesis was directed towards the isolation of bioactive secondary metabolites from the sponges *A. compressa*, *A. crassa* and T. *swinhoei*, as well as from putatively novel actinomycete and sphingomonad isolates associated with different Caribbean sponges. The discovery of novel bacterial communities from sponges increases the likelihood of finding innovative chemical compounds with new moieties and biological activities relevant to develop alternative therapeutic agents against different diseases.

2. Aims

The aim of this PhD thesis was to identify bioactive secondary metabolites in marine sponges and their associated bacteria. By following a bioactivity-guided scheme, crude extracts, fractions and pure compounds were tested for their antimicrobial and anti-protease activities, as well as for their immunomodulatory responses. The project is divided into two parts as follows:

2.1. Searching for bioactive secondary metabolites from marine sponges previously shown to exhibit anti-protease activity. Following the preparation of organic crude extracts and their testing for bioactivity, the structure of the bioactive compounds was elucidated by means of chromatographic, spectroscopic and spectrometric techniques.

2.2. Searching for bioactive secondary metabolites from bacteria associated with Caribbean marine sponges. This project involved the establishment of strain collections, the screening of organic extracts for bioactivities, the molecular phylogenetic identification of novel isolates and the elucidation of the structure of the bioactive compounds by using chromatographic, spectroscopic and spectrometric techniques.

3. Materials and Methods

This thesis was conducted in the research laboratories of Prof. Ute Hentschel (Dept. of Botany II, Julius-von-Sachs Institute for Biological Sciences), Prof. Thomas Hünig (Institute for Virology and Immunobiology), and Prof. Tanja Schirmeister (Institute of Pharmacy and Food Chemistry). Experimental procedures of this PhD project were performed by myself-unless stated otherwise. Molecular biology and microbiology procedures, as well as the preparation of crude extracts from bacterial biomass were performed in Prof. U. Hentschel's laboratory, immunology methods were performed in Prof. T. Hünig's laboratory with the guidance of the doctoral candidate Paula Römer and the technical assistance of Susanne Berr. Preparation of crude extracts from sponge biomass, fractionation of all crude extracts and structure elucidation of metabolites were conducted in Prof. T. Schirmeister´s laboratory. Protease inhibitory assays were performed by the technical assistant Cornelia Heindl in Prof. Schirmeister's group. Moreover, antimicrobial bioactivity testing including antibacterial, biofilm inhibition, antifungal, antileishmanial and antitrypanosomal assays, as well as cytotoxicity assays were performed by the SFB 630 collaboration partners: TP Z1 (Dr. Tobias Ölschläger, PD Dr. August Stich and Prof. Lorenz Meinel, U. Würzburg).

3.1. Collection and organic extract preparation from sponge biomass

3.1.1. Sponge collection

The marine sponges *Amphimedon compressa* and *Aiolochroia crassa* were collected by the SCUBA diver Dr. Hilde Angermeier (Würzburg University) at depths of 3-20 m in the Bahamas in May 2008 (GPS: 26° 27' 3.25" N, 77° 54' 14.59" W). The marine sponge *Theonella swinhoei* was collected by the SCUBA diver Prof. Micha Ilan (Tel Aviv University) in the Red Sea in Eilat (Israel) at a depth of 3 m in December 2004 (GPS: 29º30' 07'' N; 34º55' 02'' W). Sponge material was frozen and transported to the laboratory and subsequently stored at -80 ºC.

3.1.2. Organic extract preparation

30 g of biomass of the sponges *Amphimedon compressa, Aiolochroia crassa* and *Theonella swinhoei* were lyophilized with a Christ ALPHA II-12 freeze dryer. 10 g of the dried biomass from each sponge were extracted successively with cyclohexane, dichloromethane and methanol (1 L of each solvent) to obtain milligram amounts of the

three different crude extracts per sponge. Obtained crude extracts were submitted to bioactivity testing.

3.1.3. Fractionation of crude extracts

LC-MS experiments were performed on an Agilent 1100 LC/MSD trap with a HPLC system 1100, Agilent, using a Phenomenex Jupiter 4 μ Proteo 90A RP C18 column (4.6 x 150 mm) or using the ACQUITY UPLC®/Q-TOF System (Column: BEH C18, 1.7 μm, 2.1 x 50 mm). Column chromatography was performed on Sephadex HL-20. Solvents used for extraction and column chromatography were glass-distilled prior to use; the solvents used for LC-MS were HPLC grade. ^{1}H- and ^{13}C-NMR spectra were obtained on Bruker Avance 400 MHz or Bruker DMX 600 MHz spectrometers, in MeOD; δ in ppm.

The crude cyclohexane extract of the sponge *A. compressa* (Ah) was fractionated by using normal phase flash column chromatography, using a gradient system with hexane and ethyl acetate. Milligram amounts of fractions Ah1, Ah2, Ah3, Ah4 and Ah5 were collected and submitted for bioactivity testing.

The crude methanol extract from *A. compressa* (Am) was fractionated using column chromatography to obtain three fractions (Am1, Am2 and Am3). Fractions Am1 and Am2 were combined and given the name Am1. After preparative HPLC analysis of the sub-fraction Am1, seven sub-fractions (Am1a to Am1g) were collected and submitted to bioactivity testing.

Prepared extracts from the sponge *T. swinhoei* were subjected to reverse phase LC-MS, using H_2O (0.1% formic acid)/MeCN (0.1% formic acid) gradient (60% H_2O for 5 min, 60-5% H_2O in 20 min and 5% H_2O for 15 min), and the peaks were detected at 254 nm. Crude dichloromethane extract (Td) containing the $[M+H]^+$ peak for miraziridine A was subjected to column chromatography on sephadex (eluent: methanol) to generate three sub-fractions (Td-1, Td-2 and Td-3). The three sub-fractions obtained were subsequently analyzed by LC-MS; Td-3 contained the $[M+H]^+$ peak for miraziridine A. Furthermore, Td-3 and synthetic miraziridine A, synthesized according to Schaschke (2004), were subjected to LC–MS, H_2O/MeCN gradient (100% H_2O for 3 min, 100-0% for 32 min, 0-100% for 10 min), and detection at 220 and 254 nm.

3.2. Cultivation and identification of bacteria associated with marine sponges, and bioactivity-guided fractionation of crude extracts from bacterial isolates

3.2.1. Sponge collection

The first collection, consisting of the first group of sponges (*Aplysina fistularis*, *Plakortis* sp.*, Amphimedon compressa*, *Aiolochroia crassa*, *Agelas clathrodes*, *Agelas cerebrum*, *Ircinia felix*, *Scopalina ruetzleri*, *Erylus formosus*, *Chondrilla nucula* and *Aplysina archeri*) was obtained by SCUBA diver Dr. Hilde Angermeier, at depths of 3-20 m in the Bahamas in May 2008 (GPS: 26° 27' 3.25" N, 77° 54' 14.59" W). The second collection, consisting of sediments, seawater, and the second group of sponges (*Agelas clathrodes*, *Aplysina insularis*, *Agelas tubulata*, *Biemna cribaria*, *Aiolochroia crassa*, *Discodermia dissoluta*, *Scopalina ruetzleri*, *Dragmacidon reticulata*, *Ircinia felix*, *Monanchora arbuscula* and *Plakinastrella onkodes*) was collected by SCUBA diver Sven Zea (Institute of Marine and Coastal Research "José Benito Vives de Andreis", INVEMAR) at depths of 11-23 m in El Morro, Santa Marta Bay, Colombia (GPS: 11° 14' 59.04" N, 74° 13' 44.47" W) in December 2008. The sponges were transferred to plastic bags containing seawater and immediately transported to the laboratory. The sponge specimens were rinsed in sterile seawater, cut into pieces of ca. 1 cm^3, and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. The supernatant was diluted in ten-fold series (10^{-1}, 10^{-2}, 10^{-3}) and subsequently plated out on agar plates. The same protocol was repeated for sediment and seawater samples. Preparation of sponge homogenates and cultivation and isolation of Colombian bacterial isolates were done by myself in a research stay in INVEMAR and in the Universidad Tecnológica de Pereira, Colombia.

3.2.2. Bacterial isolation

Six different media were used for the isolation of *Actinobacteria*, such as M1 (1 L artificial sea water (ASW) containing 10 g starch, 4 g yeast extract, 2 g peptone, 18 g Bacto Agar and 1 L ASW), M2 (1 L ASW was made up of 6 mL of 100% glycerol, 1 g arginine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ and 18 g Bacto Agar) (Mincer et al., 2002), ISP medium 2 (1 L ASW containing 4 g yeast extract, 10 g malt extract, 4 g dextrose and 18 g Bacto Agar) (Shirling and Gottlieb, 1966), M7 (1 L ASW containing 2 g peptone, 0.1 g asparagine, 4 g sodium propionate, K_2HPO_4, 0.1 g $MgSO_4$, 0.0001 g $FeSO_4$, 5 g glycerol, 20 g NaCl and 18 g Bacto Agar) (Webster et al., 2001), NaST21Cx (solution A: 750 mL of artificial seawater containing 1 g K_2HPO_4 and 10 g Bacto Agar and solution B: 250 mL artificial seawater containing 1 g KNO_3, 1 g $MgSO_4$, 1 g $CaCl_2 \cdot 2H_2O$, 0.2 g $FeCl_3$, and 0.1 g $MnSO_4 \cdot 7H_2O$) (Magarvey et al., 2004) and Oligotropic medium (1 L ASW containing 0.5 g

tryptone, 0.1 g sodium glycerophosphate, 0.05 g yeast extract and 12 g Bacto Agar) (Santavy et al., 1990). Heat shock (95 ºC for 10 min) and incubation with 1.5% phenol at 30 ºC for 30 min were applied to the sponge homogenates to select for spore-forming actinomycetes and rare actinomycete genera. All plates were incubated and observed for growth of colonies for 6-8 weeks. All media were supplemented with the following antibiotics: cycloheximide (100 μg/mL), nystatin and nalidixic acid (25 μg/mL) to inhibit the growth of fungi and Gram-negative bacteria. Sphingomonads were isolated on M1, ISP medium 2 and Oligotropic medium. The isolates from the Bahamas are abbreviated as "BA" and those from Colombia as "CO."

3.2.3. Molecular identification and phylogenetic analysis

The 16S rRNA genes from all Bahamian sponge isolates were amplified, cloned and sequenced according to Hentschel et al. (2001) using the universal primers 27F and 1492R (Lane, 1991). Isolates from the Colombian sponges were first sorted into groups according to their morphological characteristics. Restriction length fragment polymorphism (RFLP) analysis was then performed on all isolates to reduce strain replication. Following 16S rRNA gene amplification using the universal primers 27F and 1492R (Lane, 1991) and 341F and 907R (Muyzer et al., 1993), the PCR products were digested with the restriction enzymes *Hae*III and *Msp*I for 2 h. One to two isolates from each RFLP group were selected for 16S rRNA gene sequencing.

Chimeric sequences were identified using the Pintail program (Ashelford et al., 2005). Sequence alignment and phylogenetic analysis were performed using the ARB software package (Ludwig et al., 2004). The genus-level affiliation of the isolates was validated using the Ribosomal Database Project Classifier (Wang et al., 2007). Tree construction was conducted using neighbor-joining algorithm (Jukes-Cantor correction) with bootstrap values based on 1000 replications. The 16S rRNA gene sequences of the putatively novel isolates were deposited in GenBank under the accession numbers indicated in parentheses: BA21 (HM005239), BA53 (HM005240), CO58 (HM005241), CO104 (HM005242), CO105 (HM005243), CO132 (HM005244), CO155 (HM005245). All other 16S rRNA gene sequences (Table 5 and Table 6) were deposited in GenBank under the accession numbers: HQ398366-HQ398418.

3.2.4. Organic extract preparation of selected isolates

Sixteen strains were selected based on phylogenetic novelty (4 actinomycetes, 3 sphingomonads) or their affiliation to clades for which secondary metabolites had not yet

been reported (9 strains). The isolates were cultured in 100 mL Erlenmeyer flask*s* each containing 20 mL liquid M1 medium. The cultures were grown for 4-21 days at 30°C while shaking at 150 rpm and three mL culture aliquots were taken at different points in time (day 4, 7, 14, 21). 1.5 mL methanol was added to each culture aliquot for cell lysis and shaking was continued at 150 rpm for two hours at room temperature (E. Bühler shaker, SM 30). The broths were centrifuged in 15 mL-Falcon tubes at 5000 rpm for 15 min at room temperature (Megafuge 1.0R, Heraeus) and the supernatants were dried under reduced pressure using a rotary evaporator (Heidolph, Germany). Media control, using non-inoculated M1, was extracted using the same method as above.

A subset of eleven strains was also grown on M1 agar plates (three square plates per strain with approximately 120 mm diameter each) at 30 ºC for 7-10 days. Biomass was removed from the plates together with the agar by cutting it into small pieces and was macerated overnight with a sufficient volume of ethyl acetate to submerge the agar pieces. Extraction with ethyl acetate of the macerated agar was repeated, and the macerations were subsequently filtered by gravity using a Whatman filter paper No. 1. The filtrates were combined and dried under reduced pressure by rotary evaporation. Media control, using uninoculated M1 agar, was extracted using the same method as above.

3.2.5. Organic extract preparation of isolates *Lapillicoccus* sp. BA53, *Sphingobium* sp. CO132 and *Sphingobium* sp. CO105

Based on phylogenetic novelty and biological activity, the Bahamian strain *Lapillicoccus* sp. BA53 and the Colombian strains *Sphingomonas* sp. CO105 and *Sphingobium* sp. CO132 were selected for cultivation, extraction and further fractionation (Table 1). Strains *Lapillicoccus* sp. BA53 and *Sphingobium* sp. CO105 were grown on M1 agar plates (square plates with approximately 120 mm diameter each) at 30 ºC for 7-10 days. The biomass was removed from the plates together with the agar by cutting it into small pieces and was transferred to 1 L Erlenmeyer flasks and macerated overnight with 500 mL ethyl acetate (per flask) to submerge the agar pieces. Extraction with ethyl acetate of the macerated agar was repeated and the macerations were subsequently filtered by gravity using a Whatman filter paper No. 1. The filtrates were combined and dried by rotary evaporation. Strains *Lapillicoccus* sp. BA53 and *Sphingobium* sp. CO132 were also cultured in thirteen and nineteen L liquid M1 medium, respectively. The cultures were grown for 7-10 days (depending on their growth rate) at 30 °C while shaking at 150 rpm. Amberlite XAD-16 resin (Sigma, Germany) was added to the cultures (30 g/L) and

shaking was continued at 150 rpm for two hours at room temperature (E. Bühler, SM 30). The broths were filtered and secondary metabolites were eluted from the amberlite XAD-16 using acetone and methanol successively. The resulting crude extracts were dried under reduced pressure using a rotary evaporator (Heidolph, Germany) to obtain around 400 - 1500 mg each (Table 1).

Table 1. Preparation of crude extracts from active and novel strains

Strain	Activity	Cultivation (M1 medium)	Crude extract (mg)
Actinomycetes			
Lapillicoccus sp. BA53	Induction of IFN-γ and IL-2 release	300 agar plates for 10 days	644
		13 liter medium for 7 days	1000
Sphingomonads			
Sphingobium sp. CO105	PBMC proliferation and induction of IFN-γ, IL-2 and IL-10 release	200 agar plates for 10 days	423
Sphingobium sp. CO132	Inhibition of rhodesain, IFN-γ, IL-2 and IL-10 release	19 liter medium for 10 days	1500

3.2.6. Bioactivity-guided fractionation of extracts from isolates *Lapillicoccus* sp. BA53 and *Sphingobium* sp. CO105

3.2.6.1. Strain *Lapillicoccus* sp. BA53

600 mg of the dried crude extract obtained from the solid culture of strain *Lapillicoccus* sp. BA53 were subjected to preparative RP HPLC (Agilent, Prostar), using H_2O/MeOH gradient (90-0% H_2O in 40 min, 0-100% H_2O in 0.5 min, 100-90% H_2O in 10 min and 90% H_2O for 10 min), to yield 44 fractions named BA53-1 to 44. The 44 fractions thus obtained were combined according to their thin-layer chromatography (TLC) profiles (254 and 366 nm) with CH_3Cl/MeOH (45:5, v/v) to provide 11 fractions named BA53a to k, which were tested for their anti-protease and immunomodulatory activities, as described in detail in section 3.3. The fraction BA53h was further partitioned by preparative RP HPLC using H_2O/MeOH gradient (50-0% H_2O in 14 min, 100% MeOH for 3 min, 0-50% H_2O in 8 min

and 50% H_2O for 2 min) to obtain BA53h-1 and 2, which were further tested for their protease inhibitory activity. The fraction BA53h-1 and 2 were further partitioned by preparative RP HPLC and the resulting sub-fractions were tested for their protease inhibitory activity. According to the TLC and HPLC profiles, active sub-fraction BA53h2-12 (2 mg) was a nearly pure compound.

1000 mg of the dried crude extract obtained from the liquid culture of strain *Lapillicoccus* sp. BA53 were subjected to preparative RP HPLC (Agilent, Prostar), using H_2O/MeOH gradient (flow: 7 mL/min, 90-0% H_2O in 40 min, 0-100% H_2O in 0.5 min, 100-90% H_2O in 4.5 min and 90% H_2O for 10 min), to yield 40 fractions named BA53L1 to 40, which were submitted to anti-protease bioactivity testing. The active fractions BA53L30, BA53L33, BA53L34, BA53L36 and BA53L37 were subjected to semi-preparative RP HPLC (Agilent 1100), performed using H_2O + 0.05% TFA and CH_3CN as the solvents and the following gradient: flow 2.5 mL/min, 90-0% H_2O + 0.05% TFA in 20 min, 100% CH_3CN for 4 min, 0-50% H_2O + 0.05% TFA in 4 min and 50-90% H_2O + 0.05% TFA in 2 min. The fractions and nearly pure compounds thus obtained were submitted to bioactivity testing and NMR and MS analysis.

3.2.6.2. Strain *Sphingobium* sp. CO105

400 mg of the dried crude extract obtained from strain *Sphingobium* sp. CO105 were subjected to preparative RP HPLC (Agilent, Prostar), using H_2O/MeOH gradient (90-0% H_2O in 30 min, 100% MeOH for 5 min, 0-90% H_2O in 0.5 min and 90% H_2O for 14.5 min), to yield sixteen fractions named CO105-1 to 34. The fraction named CO105-32, active against the protease rhodesain, was further partitioned by preparative RP HPLC using H_2O/MeOH gradient (90-0% H_2O in 50 min, 100%, 0-100% H_2O in 0.5 min, 100-90% H_2O in 4.5 min and 90% H_2O for 10 min) to obtain nine fractions named CO105-32-1 to 9, which were further tested for their anti-protease activity. Fraction CO105-32-9 was active against the proteases cathepsins B and L, falcipain-2 and rhodesain. Fraction CO105-32-9 was subjected to preparative RP HPLC using H_2O/MeOH gradient (50-0% H_2O in 10 min, 100% MeOH for 10 min, 0-50% H_2O in 2 min, and 50% H_2O for 10 min) to obtain 3 fractions. Further bioactivity testing showed that fraction CO105-32-9-3 inhibited the proteases cathepsins B and L, falcipain-2 and rhodesain. The nearly pure bioactive compound CO105-32-9-3 was subjected to NMR and MS analysis.

Moreover, the immunomodulatory active fraction CO105-23 obtained as described above was partitioned by semi-preparative RP-HPLC using H_2O/MeOH gradient (50-0% H_2O in

14 min, 100% MeOH for 3 min, 0-50% H_2O in 8 min and 50% H_2O for 2 min) to obtain twenty fractions named BA105-23-1 to 20 and further tested for their immunomodulatory activity and subjected to LC-MS analysis.

3.3. Bioactivity screening

3.3.1. Antimicrobial assay – Disk diffusion

The disk diffusion assay was performed using the following microorganisms: *Staphylococcus aureus* NCTC 8325, *Pseudomonas aeruginosa, Enterococcus faecalis* JH212, *Escherichia coli* 536 and *Candida albicans*. 100 μL crude extracts (10 mg/mL) were added to sterile 13 mm disks three times, and were allowed to dry between each addition. Previously prepared LB (for bacterial pathogens) and YPD (for *C. albicans*) agar plates were uniformly inoculated with a lawn of the test bacterial strains on the agar surface. The disks were then pressed lightly on the agar surface and incubated at 37 °C overnight. Zones of inhibition were noted by measuring the diameter (mm) of the clearing zones around the disks.

3.3.2. Antibacterial assay

Bacterial (*Staphylococcus aureus* NCTC 8325, *Staphylococcus epidermidis* RP62A, *Enterococcus faecalis* JH212, *Enterococcus faecium* 6413, *Escherichia coli* 536, *Pseudomonas aeruginosa, Yersinia pseudotuberculosis* 252 01A and *Yersinia pestis* KUMA) strains were cultivated overnight at 37 °C (30 °C for *Yersinia*) in Luria-Bertani medium (per liter: 5 g NaCl, 5 g yeast extract and 10 g tryptone) in a shaking incubator. The culture was diluted 1:100 in Müller-Hinton broth (23 g per liter; Fluka) the following day, and again cultivated until the cells reached the exponential growth phase. Approximately 1 x 10^5 cells/mL were incubated with various concentrations of the compounds to make a final volume of 200 μL in a 96-well plate at 37 °C for 18 h (at 30 °C for 48 h for *Yersinia*). The final concentration of DMSO was 0.8% in each well. After incubation, the optical density of the cultures was determined at 550 nm wavelength using an ELISA microplate reader with respect to the control without bacteria or fungi. The lowest concentration of the compound at which no bacterial or fungal growth is detectable was determined as the minimal inhibitory concentration (MIC).

3.3.3. Biofilm inhibition assay

Bacterial (*Staphylococcus epidermidis* RP62A) strain was cultivated overnight at 37 °C in in Trypticase-soy-broth (TBS) in a shaking incubator. The culture was diluted 1:200 in

TBS and 100 μL of bacterial culture were transferred into wells of 96-well plates. 100 μL of various concentrations of each crude extract were transferred to the test bacteria in the 96 well-plates (five replicates per compound). Plates were incubated for 24 h at 37 °C and 220 rpm. After incubation, the optical density of the cultures was determined at 550 nm wavelength using an ELISA microplate reader with respect to the control without bacteria. The bacterial suspension was carefully removed from the wells and washed three times with PBS. The adhering biofilm-forming bacteria in the wells were dried at 60 ˚C and stained with a saturated crystal violet solution for 5 min. Excessive stain was removed by extensive washing under running water, and the plate was dried at room temperature. The optical density of the cultures was determined at 490 nm wavelength.

3.3.4. Antifungal assay

A colony of *Candida albicans* 5314 (ATCC 90028) was re-suspended in 2 mL of 0.9% NaCl. Four microliters of this suspension were added to 2 mL of HR medium. This medium was prepared by adding 14.67 g HR Medium in 450 mL H_2O, followed by the addition of 1.0 g $NaHCO_3$ in 50 mL H_2O and subsequent incorporation of the following solutions: 420 mL of sodium phosphate (35.60 g $Na_2HPO_4.2H_2O$ per liter) and 80 mL of potassium dihydrogen phosphate (27.22 g KH_2PO_4 per liter, pH 7.2). The test compounds were diluted in various concentrations in 100 μL of medium in a 96-well microplate with final DMSO concentration of 0.4%. One hundred microliters of the *Candida* suspension were added to each well, followed by incubation at 37 °C for 48 h. Optical density was measured at 530 nm with respect to a control well without cells. The minimal concentration of the compound at which no growth is detectable was considered as the MIC value.

3.3.5. Antileishmanial assay

Leishmania major promastigotes were seeded at a cell density of 1 x 10^7 cells/mL into 96-well plates in complete medium (RPMI with $NaHCO_3$, 10% FCS, 2 mM glutamine, 10 mM Hepes pH 7.2, 100 U/mL penicillin, 50 μg/mL gentamicin, 50 μM 2-mercaptoethanol) without phenol red (200 μL), in the absence or presence of different concentrations of the sample. These were then incubated for 24 h at 26 °C, 5% CO_2, and 95% humidity. Following the addition of 20 μL of Alamar Blue, the plates were incubated again and the optical densities (ODs) measured 24 and 48 h later with a Multiskan Ascent enzyme-linked immunosorbent assay (ELISA) reader (Thermo Electron Corporation, Dreieich, Germany) using a test wavelength of 540 nm and a reference wavelength of 630 nm.

Absorbance in the absence of compounds was set at 100% of the growth. Amphotericin B was used as a reference compound and positive control. The effects of cell density, incubation time and the concentration of DMSO were examined in control experiments. The final concentration of DMSO in the medium never exceeded 1% (v/v) and had no effect on the proliferation of extracellular or intracellular parasites (Ponte-Sucre et al., 2006).

3.3.6. Antitrypanosomal assay

Trypomastigote forms of *Trypanosoma brucei brucei* laboratory strain TC 221 were cultured in Complete Baltz medium (80 mL Baltz Medium Basic Solution, 0.8 mL 2 mercaptoethanol stock solution (20 mM), 0.8 mL penicillin/streptomycin (10,000 U/mL), 16 mL FCS (deactivated for 30 min at 56 °C)). Baltz Medium Basic Solution is composed of the following: 500 mL MEM with Earle's salts and L-glutamine, 3 g Hepes, 0.5 g monohydrate glucose, 0.110 g sodium pyruvate, 0.007 g hypoxanthine, 0.002 g thymidine, 0.0107 g adenosine, 0.0141 g bathocuproine disulfonic acid disodium salt, 0.146 g glutamine, 5 mL sterile non-essential amino acid concentrate (100x, pH 7.5) (Baltz et al., 1985). A defined number of parasites (104 trypanosomes per mL) were exposed in test chambers of 96-well plates to various concentrations of the test samples (previously dissolved in DMSO) to make a final volume of 200 μL in duplicate. Positive (trypanosomes in culture medium) and negative controls (test samples without trypanosomes) were run simultaneously with each plate. The plates were then incubated at 37 °C in an atmosphere of 5% CO_2 for a total time period of 72 h. After 24 h, 20 μL of Alamar Blue was added. The activity of the test samples was measured by light absorption using MR 700 microplate reader at a wavelength of 550 nm with a reference wave length of 630 nm. The first reading was done at 48 h and subsequently at 72 h. The effect of test substances was quantified in IC_{50} values by linear interpolation of three independent measurements (Huber and Koella, 1993, Raz et al., 1997).

3.3.7. Cytotoxicity assays

J774.1 macrophages were cultured in complete medium (RPMI with $NaHCO_3$, 10% FCS, 2 mM glutamine, 10 mM Hepes pH 7.2, 100 U/mL penicillin, 50 μg/mL gentamicin, 50 μM 2-mercaptoethanol) without phenol red in the absence or presence of different concentrations of the samples at a cell density of 1 x 10^5 cells/mL (200 μL) for 24 h at 37 °C, 5% CO_2, and 95% humidity. Following the addition of 20 μL of Alamar Blue, the plates were incubated and the ODs measured at 24, 48 and 72 h later. The same Alamar blue assay as previously described for *Leishmania* followed. Kidney epithelial 293T cells were

also tested in the same manner as the macrophages but using complete DMEM medium (4.5 g/L solution of DMEM high glucose solution with sodium pyruvate but without L-glutamine, FBS superior at final concentration of 20%, 200 mM L-glutamine 100x) and a cell density of 2 x 10^4 cells/mL.

3.3.8. Protease inhibition assays

Cathepsins B and L and rhodesain protease inhibition assays were performed, according to Vicik et al. (2006a, 2006b). Briefly, assays were done at 25 °C in a 20 mM Tris-HCl buffer pH 6.0 (cathepsins), or in an 50 mM acetate buffer pH 5.5 (rhodesain), with a total volume of 200 μL. The final substrate concentration was 6.25 and 100.0 μM for cathepsins B and L and 10.0 μM for rhodesain. The final enzyme concentration was 53 ng/mL for cathepsins B and L, and 41 nM for rhodesain. The falcipain-2 inhibition assay was performed as previously described by Breuning et al. (2010). The enzymes were incubated with crude extracts for 5 min prior to substrate addition in a total volume of 200 μL. The following buffer was used: 50 mM acetate, pH 5.5 supplemented with 5 mM 1,4-dithiothreitol (DTT) and 5 mM EDTA. Substrate (Cbz-Phe-Arg-AMC for all four enzymes) and inhibitor stock solutions were prepared in 10% final concentration dimethyl sulfoxide (DMSO) and were diluted with assay buffer. Crude extracts were tested in duplicate at a final concentration of 20 μg/mL. Protease inhibition assays were carried out on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 365 nm, emission 460 nm).

The fluorometric SARS-CoV M^{pro} and Pl^{pro} protease inhibition assays were performed according to Kaeppler et al. (2005). Briefly, assays were performed at 25 °C in a 20 mM Tris-HCl buffer pH 7.5 in a total volume of 200 μL. The final substrate (H_2N-Abz-Ser-Val-Thr-Leu-Gln-Ser-Gly-(NO_2)Tyr-Arg-(MTS)-TFA-salt for M^{pro} and Z-Arg-Leu-Arg-Gly-Gly-AMC-acetate salt for Pl^{pro}) concentration for inhibition assays was 50 μM, and the final enzyme concentration was 4.25 μg/mL. Crude extracts were tested in duplicate at a final concentration of 20 μg/mL. Assays were carried out at 325 nm (excitation) and 425 nm (emission) for SARS-CoV M^{pro} and at 365 (excitation) and 425 nm (emission) for SARS-CoV PL^{pro}.

3.3.9. Immunomodulatory assays

Peripheral blood mononuclear cells (PBMC) from healthy donors were prepared as a byproduct of platelet concentrates obtained with leukoreduction system chambers. The

cell concentrate was then diluted in versene (ethylenediaminetetraacetic acid, EDTA). PBMCs were isolated from this preparation using density gradient centrifugation with Lymphocyte Separation Medium (PAA Laboratories GmbH, Pasching, Austria) and washed with ice-cold balanced salt solution (BSS)/0.2% bovine serum albumin (BSA). Cells were counted and cultured in Roswell Park Memorial Institute (RPMI) medium 1640 plus L-glutamine, supplemented with 50 mM mercaptoethanol, MEM non-essential amino acids (100X), 1 mM sodium pyruvate (Gibco BRL, Gaithersburg, MD, USA), 100 U/mL penicillin (Grünenthal GmbH, Aachen, Germany) and 100 U/mL streptomycin sulfate (Riemser Arzeimittel, Greifswald, Germany), 10 mM (2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (AppliChem GmbH, Darmstadt, Germany) and 10% AB-positive heat-deactivated (30 min at 56 °C) human serum (PAA Laboratories GmbH, Pasching, Austria). Cells were stimulated in triplicate using 96-well cell culture plates (Greiner Bio-One, Kremsmünster, Austria) (2×10^5 cells in 200 μL per well) in a humidified incubator at 37 °C with 5% CO_2. To increase sensitivity of T cells to stimulation, PBMC were pre-cultured for 48 hours at 1.5×10^7 cells in 1.5 mL medium in 24 well plates (Römer et al., 2011). Supernatants were collected after 24 h and stored at -20 °C. A panel of cytokines (TNF, IFN-γ, IL-2 and IL-10) was measured by Cytometric Bead Array (CBA) (BD Biosciences, San Jose, CA, USA), using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) following the manufacturer’s instructions. Results were analyzed using FCAP Array software (Soft Flow, Inc., USA). Forty-eight hours after the stimulation, cell proliferation was measured as radioactivity incorporated into DNA from tritiated thymidine during a 24 hour pulse, using a liquid scintillation counter (PerkinElmer, Waltham, MA, USA).

4. Results

4.1. Bioactivity screening of crude extracts from sponge biomass

4.1.1. *Amphimedon compressa* and *Aiolochroia crassa*

4.1.1.1. Antimicrobial activity

Crude methanol, dichloromethane and cyclohexane extracts from the sponges *A. compressa* (Am, Ad and Ah) and *A. crassa* (Pm, Pd and Ph), as well as the fractions from the crude cyclohexane extract (Ah1 - Ah6) from the sponge *A. compressa* (Figure 19), were tested against different bacteria, parasites and fungi, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Yersinia pestis, Leishmania major*, *Trypanosoma brucei brucei* and *Candida albicans.* The cytotoxicity was tested by using macrophages and kidney epithelial cells. Only antitrypanosomal activity was detected. The following fractions exhibited activity against the parasite *Trypanosoma brucei brucei* (Table 2): cyclohexane sub-fractions Ah2, Ah3 and Ah5, from the sponge *A. compressa,* the crude dichloromethane extract Ad from *A. crassa*, and the methanol crude extracts Am and Pm from the sponges *A. crassa* and *A. compressa.*

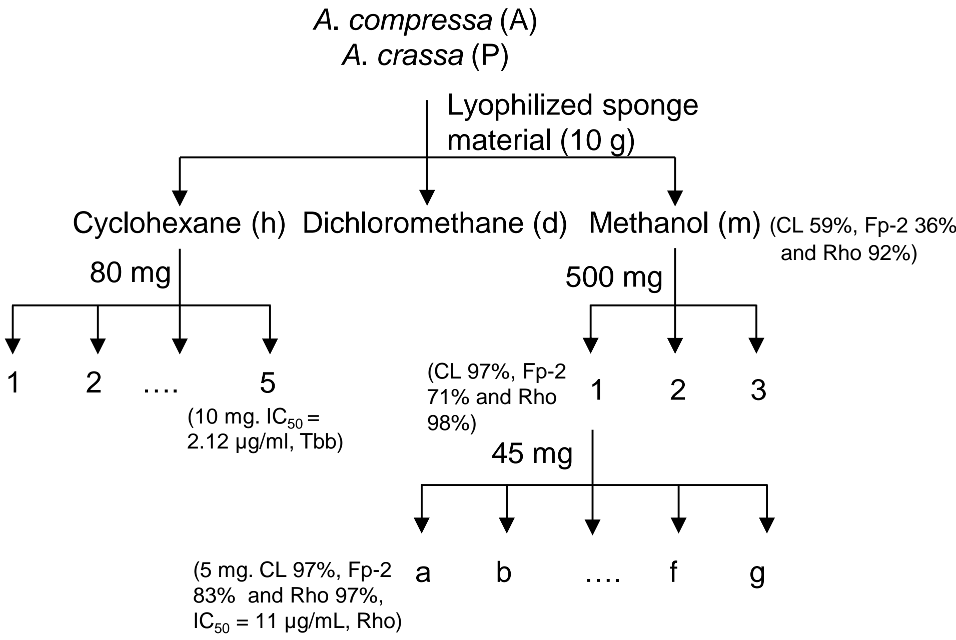


Figure 19. Fractionation of crude extracts from sponges *A. compressa* and *A. crassa*. Only crude extracts Ah and Am from *A. crassa* were further fractionated. CL: cathepsin L, Fp-2: falcipain-2, Rho: rhodesain and Tbb: *Trypanosoma brucei brucei*.

Table 2. Antitrypanosomal activity of crude extracts from sponges *A. compressa* and *A. crassa*

Sponge	Code	Description	48 h IC_{50} (μg/mL)	72 h IC_{50} (μg/mL)
Amphimedon compressa	Ah	crude hexane extract	ND	ND
	Ah1	hexane fraction 1	ND	ND
	Ah2	hexane fraction 2	16.64 ± 0,42	> 29 ± n.d.
	Ah3	hexane fraction 3	7.29 ± 2,73	> 10 ± n.d.
	Ah4	hexane fraction 4	ND	ND
	Ah5	hexane fraction 5	2.12 ± 0,34	2.78 ± 0,09
	Ad	crude dichloromethane extract	ND	ND
	Am	methanol crude extract	6.02 ± 2,27	>10 ± n.d.
Aiolochroia crassa	Pd	dichloromethane crude extract	3.95 ± 1	> 18 ± n.d.
	Pm	methanol crude extract	16.73 ± 21.18	> 27± n.d.

ND: Not detected.

4.1.1.2. Anti-protease activity

Six crude extracts from the sponges *A. compressa* and *A. crassa*, six sub-fractions from the crude cyclohexane extract and four sub-fractions from the crude methanol extract from *A. compressa*, were tested for their anti-protease activities (Table 3).

Table 3. Protease inhibitory activities of crude extracts from sponge biomass

Sponge	Code	Description	% Protease inhibition			
			Cathepsin B	Cathepsin L	Falcipain -2	Rhodesain
Amphimedon compressa	Ah	crude hexane extract	ND	ND	25	ND
	Ad	crude dichloromethane extract	12	24	37	12
	Am	crude methanol extract	10	59	36	92
	Am1	sub-fraction 1 from Am	*	97	71	98
	Am1a	Amphitoxin	*	97	83	97
	Am2	sub-fraction 2 from Am	*	91	48	95
	Am3	sub-fraction 3 from Am	*	26	13	21
Aiolochroia crassa	Ph	crude hexane extract	15	ND	20	ND
	Pd	crude dichloromethane extract	17	ND	ND	ND
	Pm	crude methanol extract	10	20	ND	ND

ND: Not detected. * Since Am did not exhibit activity against cathepsin B, SARS-CoV M^{pro} and SARS-CoV Pl^{pro}, the sub-fractions Am1, Am2, Am3 and Am1a were not tested against them. Crude extracts and fractions were tested at a final concentration of 20 μg/mL. The mean value of two assays is shown, standard deviation < 10%.

The crude methanol extract of *A. compressa* effectively inhibited rhodesain and, to a lesser degree, cathepsin L and falcipain-2. Am was fractionated using column chromatography to obtain three fractions; the first fraction, Am1, was active against rhodesain, cathepsin L and falcipain-2. After preparative HPLC analysis of the sub-fraction Am1, seven sub-fractions (Am1a - Am1g) were collected, and according to LC-MS analysis, it was noted that the first fraction (Am1a) corresponded to a nearly pure compound.

The sub-fraction Am1a from *A. compressa* exhibited 97% inhibition of the proteases cathepsin L and rhodesain, and 71% inhibition of falcipain-2 at 20 μg/mL. Am1a showed an IC_{50} value of 11.1 μg/mL when tested against rhodesain. Furthermore, antimicrobial screening showed that Am1a was active against *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Staphylococcus epidermidis*, *Enterocococcus faecalis*, *Enterococcus faecium* 6413, *Yersinia pseudotuberculosis*, *Trypanosoma brucei brucei* and *Leishmania* (Table 4). The cytotoxicity of sample Am1a was tested using human kidney epithelial cells and fibroblast cells, observing that Am1a is toxic to kidney epithelial cells with an IC_{50} value of 2,62 μg/mL (SI = 0.44). The selectivity index calculated for *Trypanosoma brucei brucei* suggested that Am1a contains a highly toxic compound, which should not be considered a drug candidate.

Table 4. Antimicrobial activity of fraction Am1a

Organism or cell type	Activity
Antimicrobial activity	
Yersinia pseudotuberculosis	MIC: 8 µg/mL
Yersinia pestis	MIC: 2 µg/mL
Staphylococcus epidermidis RP62A	MIC: 8 µg/mL
Enterocococcus faecalis JH 212	MIC: 8 µg/mL
Enterococcus faecium 6413	MIC: 8 µg/mL
Yersinia pseudotuberculosis	MIC: 8 µg/mL
Trypanosoma brucei brucei	IC_{50} (48 h): 6.02 ± 2.27 µg/mL
Anti-leishmanial activity	
J774.1 Macrophages (Mouse)	IC_{50} : 3.3 µg/mL
Leishmania major	IC_{50}: 3.75 µg/mL

MIC: Minimum inhibitory concentration, IC_{50}: half maximal inhibitory concentration

The ^{1}H-NMR data suggested that the active compound in Am1a was amphitoxin (Figure 20), a high molecular weight metabolite, containing 3-alkyl- and 3-alkenyl-pyridinium units, previously described in *A. compressa* (Albrizio et al., 1995, Thompson et al., 2010). In this study, NMR data showed signals in the aromatic region at δ(H) 8.8-8.9, 8.3-8.5 and 8, which are an evidence of the 3-substituted pyridinium ring in the molecule. The signals at δ(H) 5.59 and 5.70 showed the presence of an olefinic moiety and signals at 2.87, 3.67 and 4.61 suggested the presence of CH_2 groups attached to the pyridine ring (Suppl. Fig. S3). The signals between δ(H) 1.2 and 2.2 evidenced CH_2 groups located further away from the pyridinium moiety. Sub-fraction Am1a was subsequently analyzed by reversed phase LC-MS, only one peak at a retention time of 20 min was observed, detected at 254 nm (Suppl. Fig. S1). Several ion peaks in the range of 298-3923 m/z were observed, confirming the high molecular weight of the compound in Am1a (Suppl. Fig. S2). Based on the above observations, it was concluded that the protease inhibitor present in Am1a corresponded to amphitoxin.

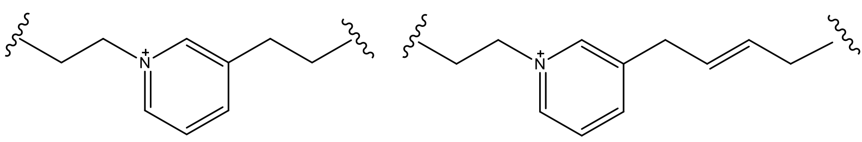


Figure 20. Amphitoxin

4.1.1.3. Immunomodulatory activity

Human PBMC were stimulated with crude extracts from the sponges *A. compressa* (Am) and *A. crassa* (Pm) in the presence and the absence of the T cell activating agent OKT3. After 24 h the cytokine concentration was measured in the culture supernatants, and after 48 hours, cell proliferation was determined by incorporation of tritiated thymidine. The crude extracts Am and Pm did not stimulate PBMC proliferation (Figure 21a) while they effectively suppressed the PBMC response to the monoclonal antibody OKT3 (Figure 21b). The fractions Am and Pm at a final concentration of 10 μg/mL exhibited 97% and 45% inhibition of PBMC response to OKT3 respectively.

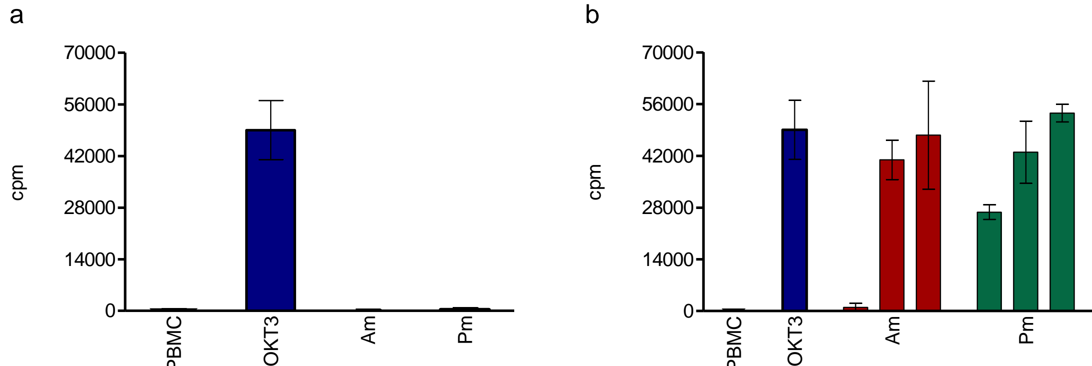


Figure 21. PBMC stimulation with crude extract Pm from the sponge *A. crassa* in the presence (a) and the absence (b) of OKT3.

When PBMC were stimulated with the crude extract Pm, the induction of IL-2, IL-10 and TNF cytokines was observed, indicating the proliferative activity of Pm. In the suppression experiment, inhibition of IL-2 and IL-10 was not observed; instead, IL-2 and IL-10 production by PBMC was higher than their production by PBMC in the positive control, where cells were stimulated only with OKT3 (Figure 22). This fact again indicates the mitogenic activity of the crude methanol extract Pm. Moreover, sample Pm inhibited TNF and IFN-γ release, suggesting the suppression of the T cell response to OKT3. In consequence, extract Pm may contain immunomodulatory metabolite(s). When PBMC were stimulated with crude extract Am, no cytokine release or inhibition was observed.

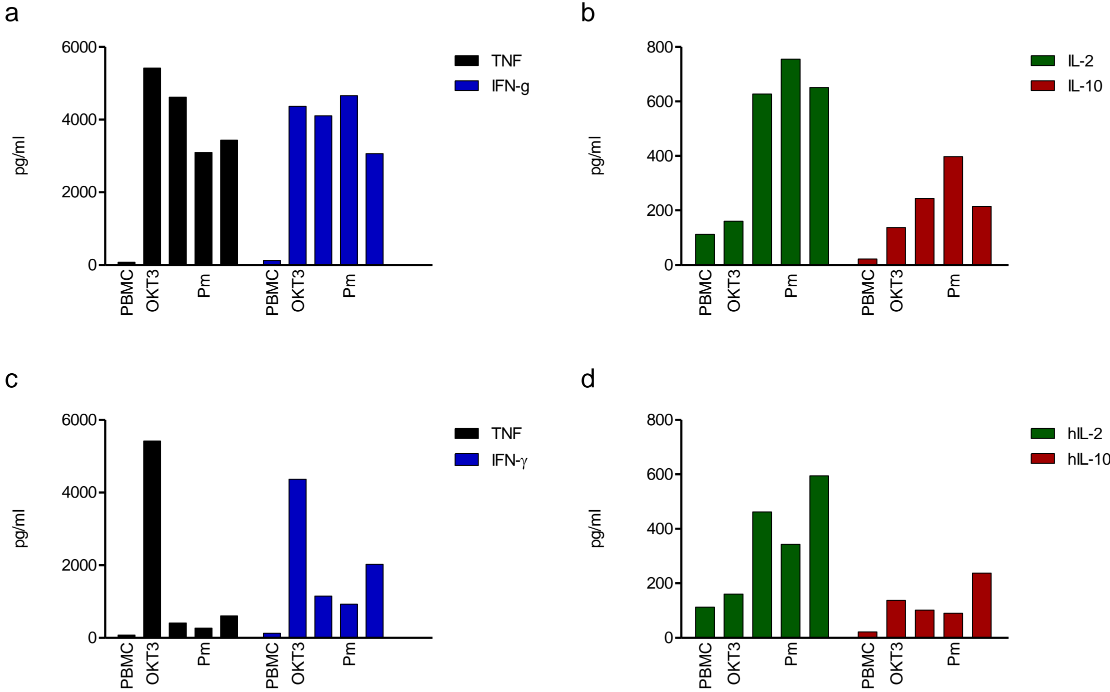


Figure 22. Cytokine responses of pre-cultured human PBMC to crude methanol extracts from *A. crassa* (Am) in the presence (a and b) and absence (c and d) of OKT3. Samples were tested in triplicate at three different concentrations (25, 2.5 and 0.25 μg/mL)

4.1.2. *Theonella swinhoei*

The sponge *T. swinhoei* was collected offshore Israel in the Red Sea, and extracts were prepared through sequential extraction of the freeze-dried sponge with cyclohexane, dichloromethane and methanol (Figure 23). LC-MS analysis of the three preparations showed that the crude dichloromethane extract (Td) yielded an ion peak at m/z 670.1 $[M+H]^+$, which indicated the presence of miraziridine A (Figure 24). The Td extract was further partitioned using sephadex HL-20 to obtain three sub-fractions (Td-1, -2, -3) which were subsequently analyzed by LC-MS, observing that the sub-fraction Td-3 contained the pentapeptide (Suppl. Fig. S4 and S5). When comparing the mass spectra of miraziridine A synthesized according to Schaschke (2004), and of Td-3, a molecular ion peak at m/z 670.1 $[M+H]^+$ was observed in both the solution of synthetic miraziridine A and in the sub-fraction Td-3. Co-elution experiments were pursued observing only one peak at a retention time of 16.2 min. Additionally, a fluorometric assay was performed to test the cathepsin L inhibition by the crude extract Td-3 at a concentration of 100 μg/mL, showing 60% inhibition.

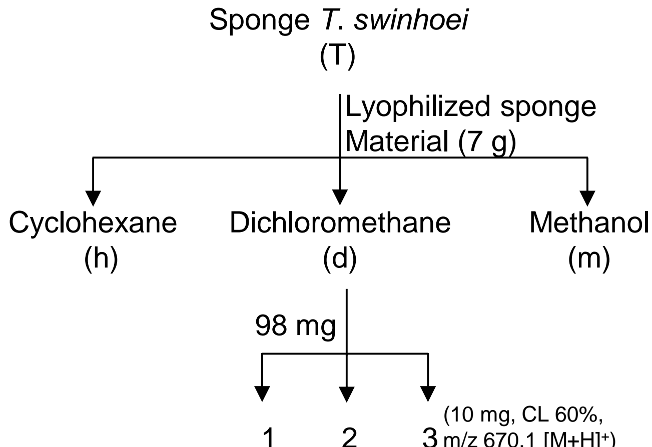


Figure 23. Fractionation of crude dichloromethane extract from sponge *T. swinhoei*

Figure 24. Miraziridine A

4.2. Cultivation and identification of bacterial isolates

4.2.1. Actinomycete and sphingomonad isolates

Out of 279 strains cultivated from marine sponges, seawater and marine sediments, a total of 79 isolates were found to belong to the order *Actinomycetales* based on their 16S rRNA gene sequences. From the Bahamian sponge collection, 21 actinomycete isolates were identified. From the sponge collection in Colombia, 136 strains were cultivated and classified according to their morphology and restriction fragment patterns. Forty-eight strains were differentiated into nine groups. One or two isolates from each group with the same restriction fragment patterns were then selected for subsequent sequencing of their 16S rRNA genes, including the remaining 88 strains that did not match to any group. Five groups were found to belong to *Microbacterium*, *Rhodococcus*, *Micrococcus*, *Curtobacterium*, and *Citromicrobium* genera, one group to *Sphingobium*, and the remaining three to the class Alphaproteobacteria. From the remaining 88 isolates, 29 isolates were found to belong to the order *Actinomycetales* and three isolates to the order *Sphingomonadales* (Table 5 and Table 6).

Table 5. 16S rRNA gene based phylogenetic affiliation of actinomycete isolates

Nr	Isolate code	Source	Closest relative by BLAST (Accesion number)	Sequence similarity (%)	Sequence length (bp)
1	BA1	*Plakortis* sp.	*Knoellia subterranea* AJ294413	98.2	1451
2	BA2	*Aplysina fistularis*	*Knoellia subterranea* AJ294413	98.2	1352
3	BA3	*Aplysina fistularis*	*Knoellia subterranea* AJ294413	98.1	1490
4	BA4	*Plakortis* sp.	*Knoellia subterranea* AJ294413	98.2	1519
5	BA5	*Plakortis* sp.	*Kocuria palustris* Y16263	99.5	1509
6	BA11	*Aplysina fistularis*	*Streptomyces thermocarboxydus* U94490	99.8	1492
7	BA17	*Scopalina ruetzleri*	*Salinispora arenicola* CP000850	99.9	1479
8	BA18	*Scopalina ruetzleri*	*Salinispora tropica* AY371895	99.9	1479
9	BA19	*Aplysina fistularis*	*Salinispora arenicola* AY371897	99.9	1481
10	BA21	*Aplysina fistularis*	*Nocardioides hwasunensis* AM295258	97.7	1485
11	BA22	*Scopalina ruetzleri*	*Agrococcus jenensis* X92492	99.3	1488
12	BA30	*Agelas clathrodes*	*Arthrobacter oxidans* X83408	98.6	1488
13	BA32	*Aplysina fistularis*	*Nocardioides oleivorans* AJ698724	98.2	1479
14	BA33	*Aplysina fistularis*	*Sanguibacter antarticus* EF211071	99.3	1490
15	BA34	*Erylus formosus*	*Arthrobacter oxydans* X83408	99.9	1489
16	BA43	*Agelas clathrodes*	*Arthrobacter oxydans* X83408	98.7	1489
17	BA45	*Scopalina ruetzleri*	*Microbacterium aurum* Y17229	98.6	1487
18	BA46	*Scopalina ruetzleri*	*Microbacterium schleiferi* Y17237	98.5	1486
19	BA47	*Scopalina ruetzleri*	*Microbacterium aurum* Y17229	98.6	1486
20	BA51	*Ircinia felix*	*Arthrobacter oxydans* X83408	98.6	1590
21	BA53	*Scopalina ruetzleri*	*Lapillicoccus jejuensis* AM398397	96.3	1498

Nr	Isolate code	Source	Closest relative by BLAST (Accesion number)	Sequence similarity (%)	Sequence length (bp)
22	C056	*Dragmacidon reticulata*	Rhodococcus corynebacterioides X80615	99.5	1304
23	CO63	*Discodermia dissoluta*	*Mycobacterium farcinogenes* AY457084	99.4	1406
24	CO74	*Scopalina ruetzleri*	*Micromonospora coxensis* AB241455	99.3	1275
25	CO75	*Scopalina ruetzleri*	*Microbacterium trichotecenolyticum* EU714362	99.8	1408
26	CO81	*Dragmacidon reticulata*	*Microbacterium lacticum* EU714364	98.8	1388
27	CO84	*Aplysina insularis*	*Streptomyces* sp. CNR940 PL04 DQ448741	99.7	1391
28	CO86	*Aplysina insularis*	*Saccharopolyspora shandongensis* EF104116	100	714
29	CO93	Marine sediments	*Micromonospora coxensis* AB241455	99.9	1357
30	CO99	*Aplysina insularis*	*Dietzia maris* X81920	98.5	1385
31	CO100	*Agelas clathrodes*	*Gordonia terrae* X79286	99.5	1416
32	CO104	*Scopalina ruetzleri*	*Microbacterium hominis* AB004727	98.0	1301
33	CO106	*Plakinastrella onkodes*	*Microbacterium hominis* AB004727	97.8	1387
34	CO109	*Agelas clathrodes*	*Cellulomonas hominis* X82598	99.1	1272
35	CO113	Marine sediments	*Gordonia nitida* AF148947	99.4	1409
36	CO116	*Scopalina ruetzleri*	*Rhodococcus rhodochrous* X79288	99.3	1395
37	CO121	*Discodermia dissoluta*	*Microbacterium chocolatum* AM181503	99.7	1410
38	CO128	*Plakinastrella onkodes*	*Microbacterium trichotecenolyticum* EU714362	99.8	1397
39	CO129	*Scopalina ruetzleri*	*Rhodococcus equi* X80613	99.7	1399
40	CO133	*Discodermia dissoluta*	*Gordonia terrae* X79286	99.6	1418
41	CO135	*Discodermia dissoluta*	*Mycobacterium vanbaalenii* X84977	98.9	1379
42	CO141	*Plakinastrella onkodes*	*Cellulosimicrobium cellulans* X83809	99.3	1315
43	CO143	Natural sea water	*Citromicrobium bathyomarinum* Y16267	98.8	1355
44	CO146	*Plakinastrella onkodes*	*Microbacterium paraoxydans* EU714373	99.9	1403
45	CO147	*Discodermia dissoluta*	*Curtobacterium citreum* X77436	99.7	1377
46	CO155	Marine sediments	*Rhodococcus rhodochrous* AB183422	97.4	1361
47	CO161	*Scopalina ruetzleri*	*Micromonospora coxensis* AB241455	99.4	1386
48	CO164	Marine sediments	*Micromonospora coxensis* AB241455	99.3	1372
49	CO165	*Scopalina ruetzleri*	*Micromonospora aurantiaca* AJ245712	99.7	1391
50	CO168	*Discodermia dissoluta*	*Gordonia terrae* X81922	99.2	1310
51	CO173	*Aplysina insularis*	*Saccharopolyspora shandongensis* EF104116	99.6	1392
52	CO174	*Aplysina insularis*	*Streptomyces* sp. CNR940 PL04 DQ448741	99.7	1451
53	CO177	*Monanchora arbuscula*	*Mycobacterium mageritense* AY457076	99.9	1391
54	CO178	*Aplysina insularis*	*Streptomyces chartreusis* 7106043	100	1393
55	CO181	*Discodermia dissoluta*	*Curtobacterium citreum* X77436	99.6	1387
56	CO183	*Scopalina ruetzleri*	*Mycobacterium duvalii* U94745	99.8	1308

Table 6. 16S rRNA gene based phylogenetic affiliation of sphingomonad isolates

Nr.	**Isolate code**	**Source**	**Closest relative by BLAST (Accession number)**	**Sequence similarity (%)**	**Sequence length (bp)**
1	CO58	*Dragmacidon reticulata*	*Sphingomonas mucosissima* AM229669	98.4	1363
2	CO105	*Discodermia dissoluta*	*Sphingobium lactosutens* EU675846	98.7	1353
3	CO132	*Monanchora arbuscula*	*Sphingobium abikonensis* AB021416	98.4	1349
4	CO180	Natural sea water	*Sphingobium abikonensis* AB021416	98.4	1352

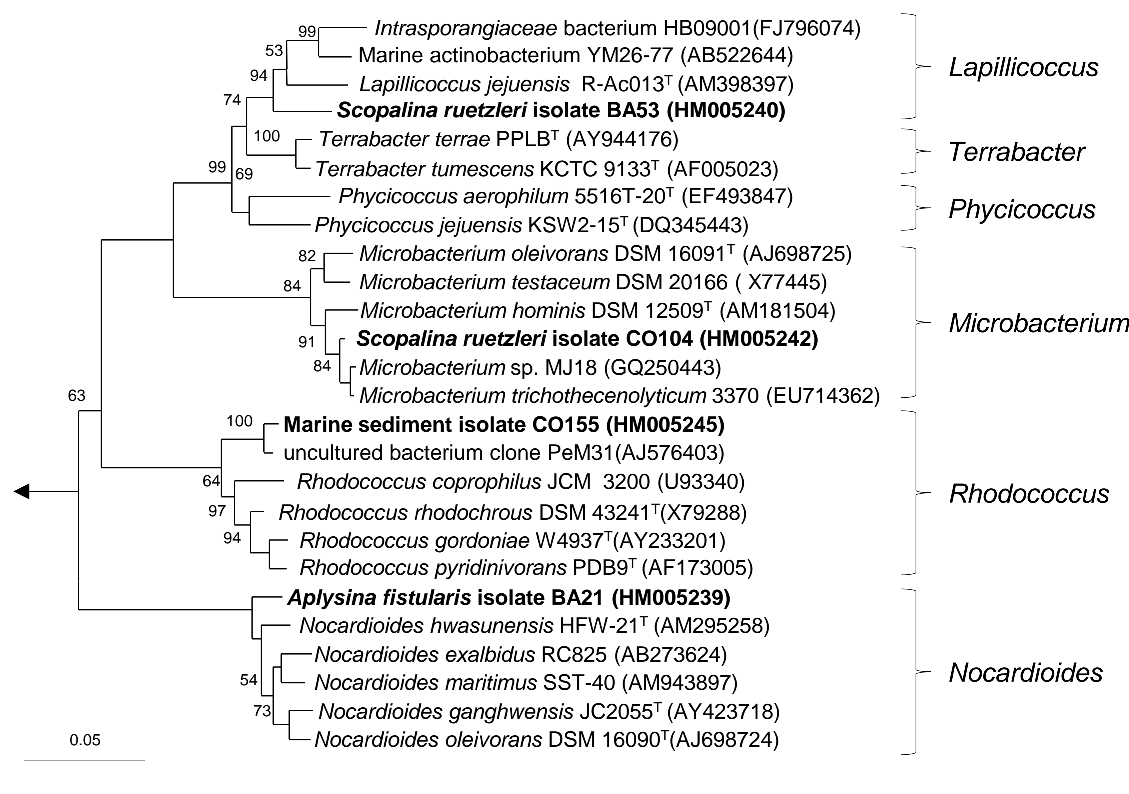
Remarkably, four actinomycete strains exhibited less than 98.1% 16S rRNA gene sequence similarities to validly described species. These low similarity values suggest that the strains belong to novel taxa (Stackebrandt and Ebers, 2006). Phylogenetic analysis (Figure 25a) revealed that these strains belong to the order *Actinomycetales* under the following genera: *Lapillicoccus, Microbacterium, Rhodococcus* and *Nocardioides*.

The highest number of actinomycete isolates was recovered from *Scopalina ruetzleri* (20 isolates), followed by *Aplysina insularis* (12), *Discodermia dissoluta* (12), *Aplysina fistularis* (7), *Dragmacidon reticulata* (5), *Agelas clathrodes* (5), *Plakinastrella onkodes* (4), *Ircinia felix* (2), *Plakortis* sp. (3), *Monanchora arbuscula* (1), *Aiolochroia crassa* (1) and *Erylus formosus* (1). Actinomycetes were not recovered from the remaining six sponges. Additionally, five isolates were recovered from marine sediments and one isolate was recovered from natural seawater.

In terms of actinomycete diversity, the 79 cultivated strains are represented by 20 different genera, namely *Microbacterium* (21 isolates), *Rhodococcus* (10), *Streptomyces* (5), *Mycobacterium* (6), *Micromonospora* (5), *Knoellia* (4), *Gordonia* (4), *Curtobacterium* (4), *Arthrobacter* (4) *Salinispora* (3), *Saccharopolyspora* (2), *Nocardioides* (2), *Citromicrobium* (2), *Sanguibacter* (1), *Lapillicoccus* (1), *Kocuria* (1), *Dietzia* (1), *Cellulosimicrobium* (1), *Cellulomonas* (1), and *Agrococcus* (1).

From the second sponge collection in Colombia, seven isolates were identified belonging to the order *Sphingomonadales* and the genera *Sphingobium* and *Sphingomonas*. One isolate belonging to the genus *Sphingomonas*, as well as two *Sphingobium* strains were recovered from the sponge *Dragmacidon reticulata*. The remaining 4 sphingomonad isolates were found to belong to the genus S*phingobium,* two of them were recovered from natural sea water and the remaining two from the sponges *Monanchora arbuscula* and *Ircinia felix*. Nearly complete 16S rRNA gene information is provided for four isolates (Table 6). The low 16S rRNA gene sequence similarities (<98.5%) of two of these strains also suggest the taxonomic novelty at the species-level (Figure 25b).

a



b

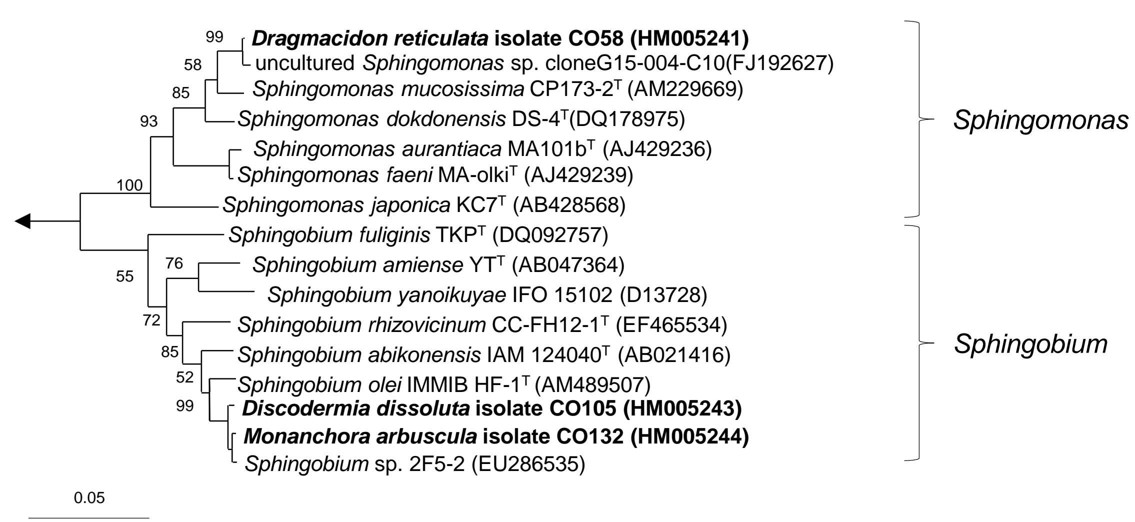


Figure 25. Neighbor-joining trees of isolates and representative species of the order (a) *Actinomycetales* and (b) *Sphingomonadales* based on nearly complete 16S rRNA gene sequences. Numbers at the nodes indicate the levels of the bootstrap support based on 1,000 resampled data sets. Only values greater than 50% are shown. The arrow points to the outgroup consisting of four species belonging to *Methanosarcinaceae.* The scale bar indicates 0.05 substitution per nucleotide position.

4.3. Bioactivity screening of crude extracts from bacterial isolates

4.3.1. Antimicrobial activity

According to taxonomic novelty and literature reports, 16 isolates were selected for bioactivity screening and grown using liquid and solid M1 medium. Broths and crude extracts from cultures were tested against gram negative and gram positive bacteria, as well as fungi, by performing the disk diffusion bioassay. *S. arenicola* strain BA17 exhibited activity against *S. aureus* and *C. albicans,* whereas the isolate *M. coxensis* strain CO74 showed an inhibitory effect on *S. aureus* growth (Table 7).

Table 7. Antimicrobial activity of crude extracts from bacterial isolates (disk diffusion assay)

Isolate	Microorganism	Zone of inhibition (mm)
S. arenicola strain BA17	*S. aureus*	10
	C. albicans	25
M. coxensis strain CO74	*S. aureus*	10

Furthermore, antimicrobial assays performed in cooperation with the TP Z1 project (SFB 630), has shown that crude extracts from *S. arenicola* strain BA17, *A. oxydans* strain BA34 and *Lapillicoccus* sp. BA53 are bioactive. The isolate *S. arenicola* strain BA17 was active against *S. aureus* and *C. albicans* and the *M. coxensis* strain CO74 was effective in inhibiting the growth of *S. aureus.* In terms of biofilm inhibitory activity, the isolate *S. arenicola* strain BA17 showed 35% biofilm inhibition, and isolates *A. oxydans* strain BA34 and *Lapillicoccus* sp. BA53 showed 30% biofilm inhibition. These results are not relevant; nevertheless, the biofilm inhibition observed in the assay is an indicator of a weak antibacterial activity. A compound is assumed to possess biofilm inhibiting activity, if sub-inhibitory concentrations (i.e. below the minimal inhibitory concentration) inhibit the biofilm formation without compromising cell growth.

4.3.2. Protease inhibitory activity

Crude extracts of 16 isolates cultivated in liquid M1 medium and obtained at different points in time (4, 7, 14 and 21 days), were tested for their activities against the proteases cathepsins B and L, falcipain-2, rhodesain, SARS-CoV PL^{pro}, and SARS-CoV M^{pro} (Table 8). Among the crude extracts tested, eight isolates exhibited anti-protease activity.

Extracts were considered active when, at a concentration of 20 μg/mL, inhibition of at least 40% was observed in the assays. The strains *Nocardioides* sp. BA21, *Saccharopolyspora shandongensis* strain CO86 and *Sphingobium* sp. CO132 inhibited rhodesain. The isolates *Agrococcus jenensis* strain BA22 and *Sphingobium* sp. CO180 inhibited cathepsin B and falcipain-2. *Micromonospora coxensis* strain CO74 was active only against falcipain-2 and *Rhodococcus* sp. CO155 was active only against cathepsin L. Extracts of *Micromonospora coxensis* strain CO164 were most active, inhibiting all four proteases tested. No inhibition of the SARS-CoV proteases was observed, while the rest of the crude extracts did not exhibit any protease inhibitory activity. In fluorimetric assays the enzyme activity is measured by the hydrolysis rate of a fluorogenic or chromogenic substrate (Ludewig et al., 2010). This means that the substrate and inhibitor compete for the enzyme's active site (Ludewig et al., 2010).

Table 8. Anti-protease activities of crude extracts from actinomycete and sphingomonad strains grown on M1 medium and extracted at different points in time (4 days^{A}, 7 days^{B}, 14 days^{C} and 21 days^{D}). Samples were tested in duplicate at a concentration of 20 μg/mL

Strain	% Protease inhibition			
	Cathepsin B	Cathepsin L	Falcipain-2	Rhodesain
Actinomycetes				
Nocardioides sp. BA21*	ND	ND	ND	40 ± 1^{C}
Agrococcus jenensis strain BA22	41 ± 1^{D}	ND	44 ± 2^{A}, 40 ± 4^{C}	ND
Micromonospora coxensis strain CO74	ND	ND	42 ± 2^{D}	ND
Saccharopolyspora shandongensis strain CO86	ND	ND	ND	52 ± 1^{B}
Rhodococcus sp. CO155*	ND	44 ± 4^{B}	ND	ND
Micromonospora coxensis strain CO164	45 ± 3^{A}	43 ± 2^{B}	41 ± 2^{B}	46 ± 3^{A}, 57 ± 5^{D}
Sphingomonads				
Sphingobium sp. CO132*	ND	ND	ND	53 ± 3^{B}
Sphingobium sp. CO180*	49 ± 5^{A}	ND	45 ± 1^{B}	ND

* Putatively novel species; ND: Not detected

4.3.3. Immunomodulatory activity

When culturing PBMC in the presence of crude extracts (Figure 26), the isolates *Agrococcus jenensis* strain BA22, *Arthrobacter oxidans* strain BA30 and *Arthrobacter oxydans* strain BA34 were effective in inducing TNF and IL-10 release (Figure 26a and d), while strains *Lapillicoccus* sp. BA53 and *Micromonospora coxensis* strain CO164

induced IFN-γ, IL-2 and IL-10 (Figure 26b - d). The isolate *Saccharopolyspora shandongensis* strain CO86 effectively induced the release of IL-2 and IL-10 (Figure 26c and d) and *Sphingobium sp*. CO132 induced the release of TNF, IFN-γ, IL-2 and IL-10 (Figure 26a - d). The remaining crude extracts from liquid and solid cultures from bacterial isolates did not induce cytokine release in PBMC culture supernatants.

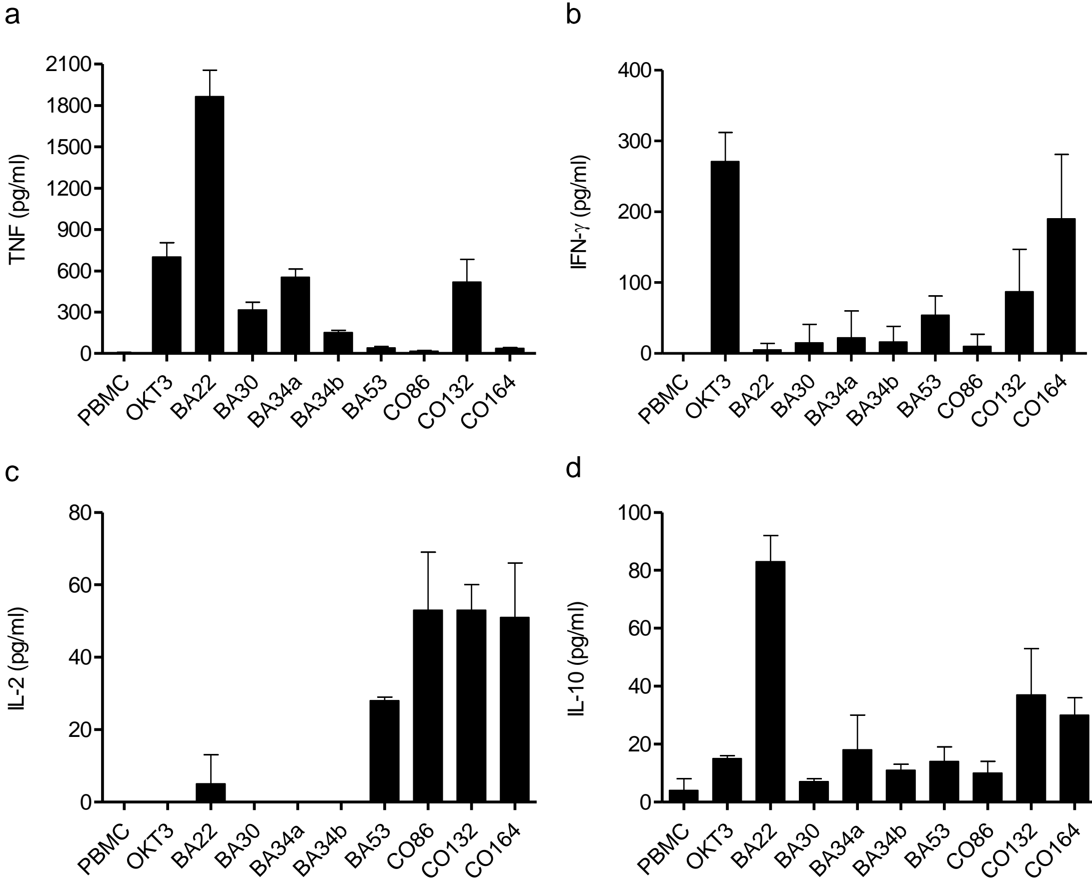


Figure 26. Cytokine responses of pre-cultured human peripheral blood mononuclear cells to crude extracts from bacterial isolates. a. TNF b. IFN-γ, c. IL-2 and d. IL-10 Crude extracts were tested in triplicate at 3 different concentrations (preparations from liquid cultures: 25 μg/mL, 2.5 μg/mL and 0.25 μg/mL, from solid cultures: 10 μg/mL, 1 μg/mL and 0.1 μg/mL) and the most active one is shown (25 μg/mL)

With regard to the induction of proliferation, the crude extract prepared from the solid culture of strain *Sphingobium* sp. CO105, exhibited the strongest mitogenic activity, reaching 14% of the positive control (OKT3, activating all T cells) (Figure 27). When the strain *Sphingobium* sp. CO105 was tested for its capacity to induce cytokine release, a weak induction of IL-2 was observed. The remainder of the samples did not stimulate PBMC proliferation.

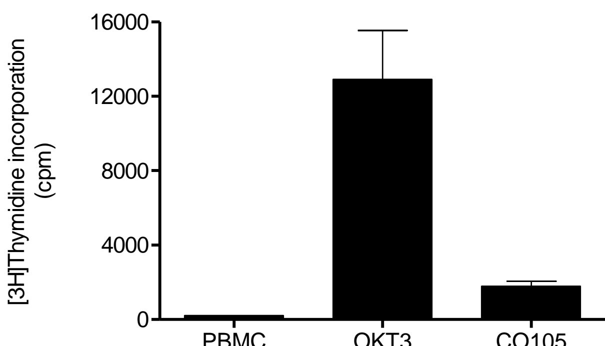


Figure 27. PBMC proliferation in response to stimulation with the crude extract from strain *Sphingobium* sp. CO105. The crude extract was tested in triplicate at a concentration of 65 ng/mL

4.3.4. Secondary metabolites from bioactive strains *Lapillicoccus* sp. BA53 and *Sphingobium* sp. CO105

4.3.4.1. Strain *Sphingobium* sp. CO105

Compound CO105-32-9-3. Bioactivity-guided fractionation of the active crude extract obtained from the putatively novel sphingomonad isolate *Sphingobium* sp. CO105 (Figure 28), yielded 1.5 mg of one nearly pure bioactive metabolite. The compound named CO105-32-9-3 was found to inhibit the proteases cathepsins B and L, falcipain-2 and rhodesain (Table 9).

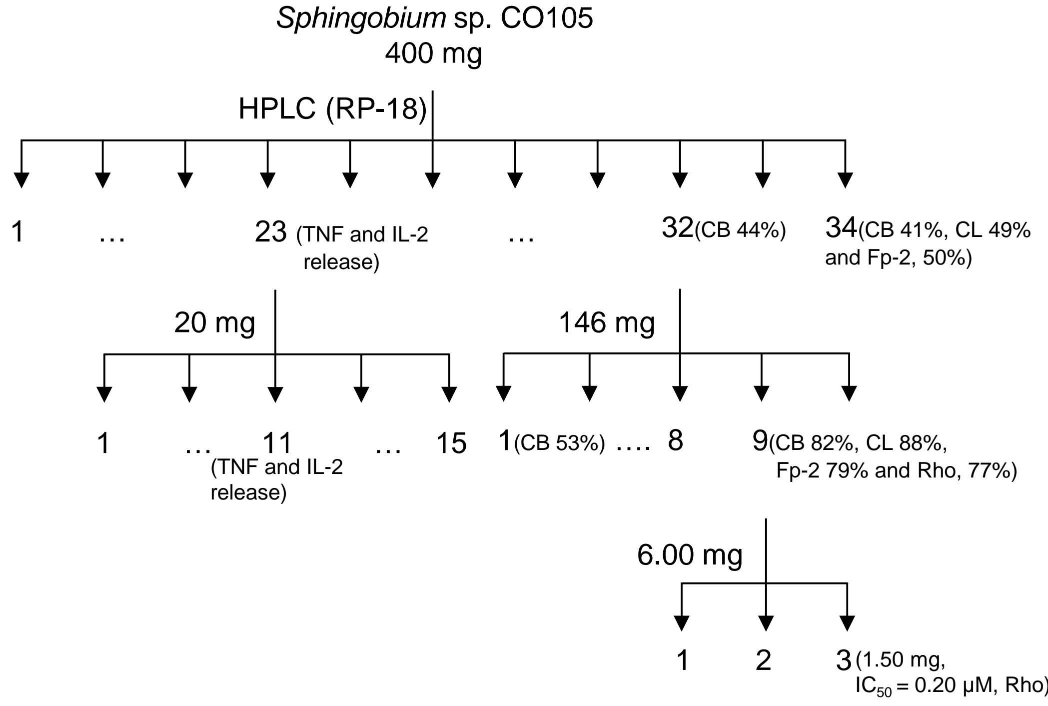


Figure 28. Bioactivity-guided fractionation of strain *Sphingobium* sp. CO105. Samples were tested in duplicate at a concentration of 20 μg/mL

Table 9. Anti-protease activities of compound 105-32-9-3 isolated from the actinomycete isolate *Sphingobium* sp. CO105. Sample was tested in duplicate at a concentration of 20 μg/mL

Sample	% Protease inhibition			
	Cathepsin B	Cathepsin L	Falcipain-2	Rhodesain
***Sphingobium* sp. CO105**				
Compound 105-32-9-3	59 ± 5	58 ± 7	50 ± 8	54 ± 5

The high resolution mass spectrum of compound 105-32-9-3 indicated the molecular formula $C_{12}H_{10}N_4O_2$ (Rt = 2.02 min, m/z 243.088 $[M+H]^+$). The ^{1}H-NMR spectrum of CO105-32-9-3 showed signals in the aromatic region at δ(H) 7.9 and 7.7 ppm and in the high field region at δ(H) 2.52 and 2.54 ppm. The COSY spectrum of CO105-32-9-3 showed a correlation between the proton at δ(H) 7.9 and the proton at δ(H) 2.52, as well as a correlation between the proton at δ(H) 7.7 and the proton at δ(H) 2.54. The comparison of the NMR and MS spectral data with the MarinLit database revealed that the compound CO105-32-9-3 corresponded to the known metabolite isolumichrome (Figure 29) (Li et al., 2004).

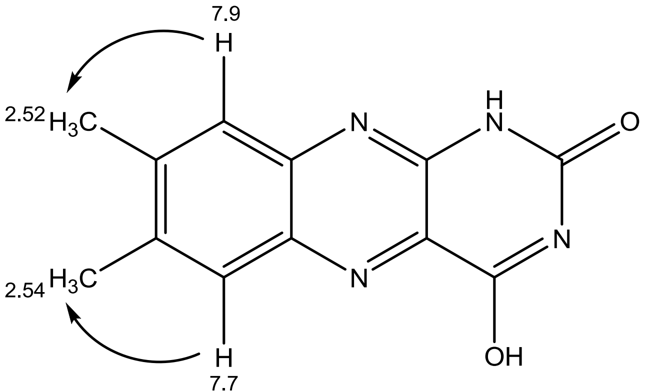


Figure 29. Isolumichrome isolated from the solid culture of strain *Sphingobium* sp. CO105. The ^{1}H chemical shifts (ppm) of protons and COSY correlations are shown

Compound CO105-23-11. When sub-fractions from strain *Sphingobium* sp. CO105 were tested for their immunomodulatory activity, sample CO105-23-11 was effective in inducing the release of TNF and, to a lesser degree, IL-10 cytokines (Figure 30). Chromatographic profile of CO105-23-11 showed this sample as a nearly pure compound

and ESI-MS analysis showed an ion peak at 2.4 min, m/z 511.2 $[M+H]^+$ (Figure 31a and b). The chemical structure of the immunomodulatory compound contained in fraction CO105-23-11 has yet to be elucidated.

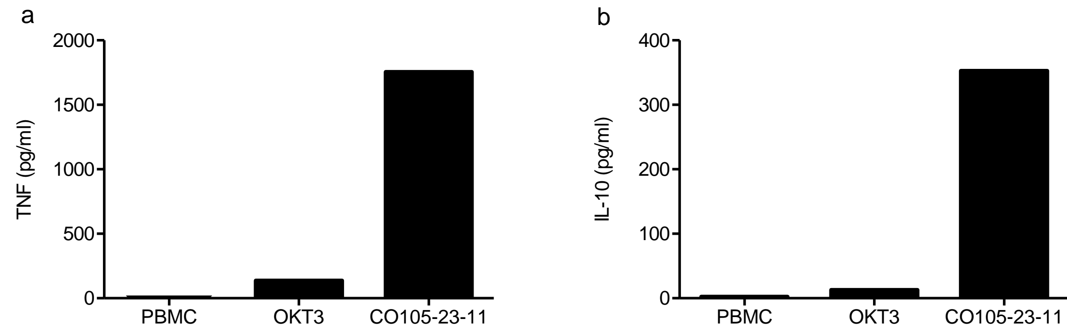
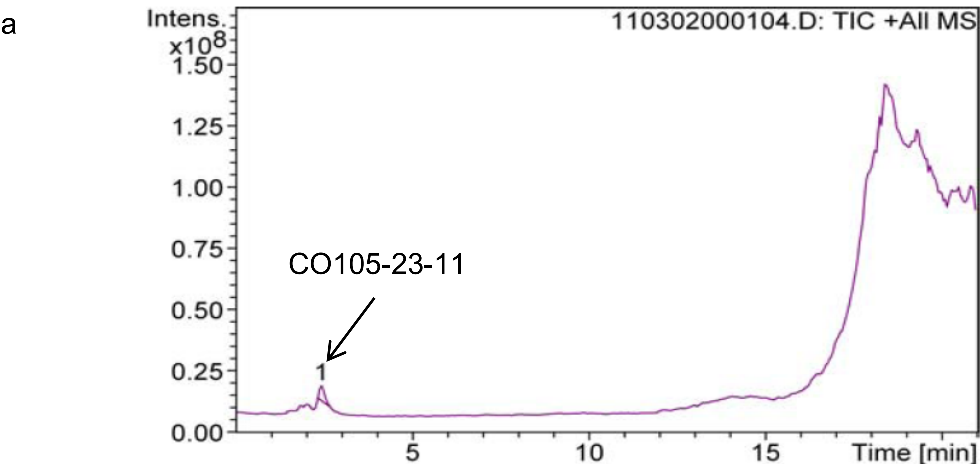


Figure 30. Cytokine responses of pre-cultured human peripheral blood mononuclear cells to the nearly pure compound CO105-23-11. a. TNF b. IL-10. The sample CO105-23-11 was tested in triplicate at 25 μg/mL



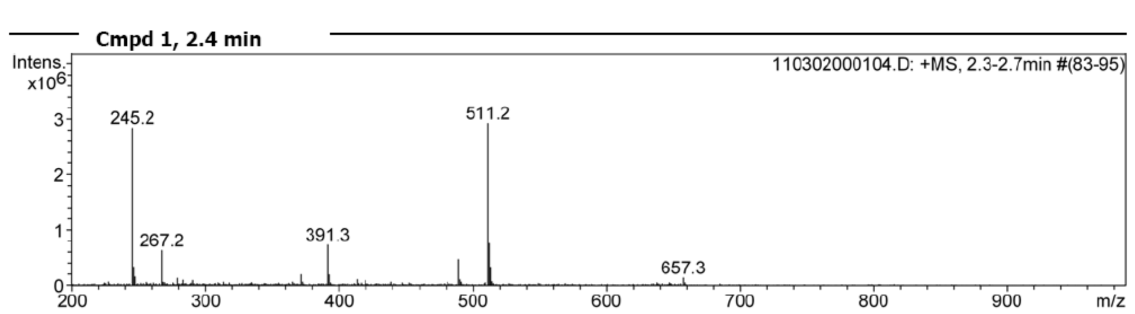


Figure 31. LC-MS profile of compound CO105-23-11. a. Total ion current (TIC) chromatogram. b. ESI-MS spectrum of the peak eluting at 2.4 min

4.3.4.2. Strain *Lapillicoccus* sp. BA53

Solid culture. Bioactivity-guided fractionation of the active crude extract obtained from the solid culture of the putatively novel acinomycete *Lapillicoccus* sp. BA53, yielded one nearly pure bioactive metabolite (Figure 32). The strain *Lapillicoccus* sp. BA53 was found to produce a metabolite named BA53H2-12, which inhibited the proteases cathepsins B and L, falcipain-2 and rhodesain (Table 10).

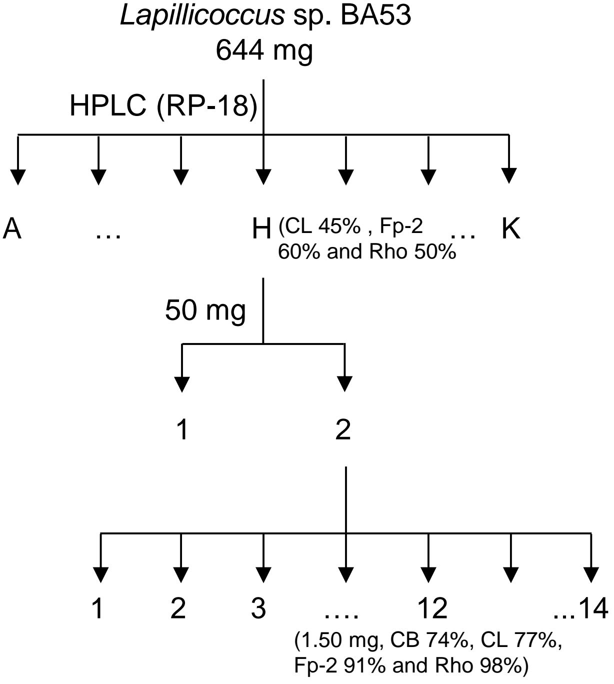


Figure 32. Bioactivity-guided fractionation of crude extract from solid culture of *Lapillicoccus* sp. BA53. Samples were tested in duplicate at a concentration of 20 µg/mL

Table 10. Anti-protease activities of compound isolated from the actinomycete isolate *Lapillicoccus* sp. BA53. The sample was tested in duplicate at a concentration of 20 µg/mL

Sample	% Protease inhibition			
	Cathepsin B	Cathepsin L	Falcipain-2	Rhodesain
***Lapillicoccus* sp. BA53**				
Compound BA53H2-12	74 ± 2	77 ± 2	91 ± 3	78 ± 2

The molecular formula for compound BA53H2-12 was established using high-resolution mass spectrometry as $C_8H_9NO_3$ (Rt = 3.18 min, m/z 268.067 $[M+H]^+$). The combination of NMR and MS data, and comparison of the spectral data with the MarinLit database (2010), showed that the protease inhibitor isolated from the strain *Lapillicoccus* sp. BA53 is the previously reported compound p-aminosalicylic acid methyl ester (Celmer et al., 1979) (Figure 33).

Figure 33. Compound BA53H2-12. p-Aminosalicylic acid methyl ester isolated from the solid culture of strain *Lapillicoccus* sp. BA53

Liquid culture. The crude extract obtained from the liquid culture of the isolate *Lapillicoccus* sp. BA53, was fractionated in several steps by RP HPLC (Figure 34). The six sub-fractions thus obtained (53L30-7, 53L30-9, 53L33-2, 53L34-5, 53L36-6 and 53L37-7, around 2 mg from each fraction) were tested for their protease inhibitory activity. Fractions from liquid culture of strain BA53 did not exhibit protease inhibitory activity.

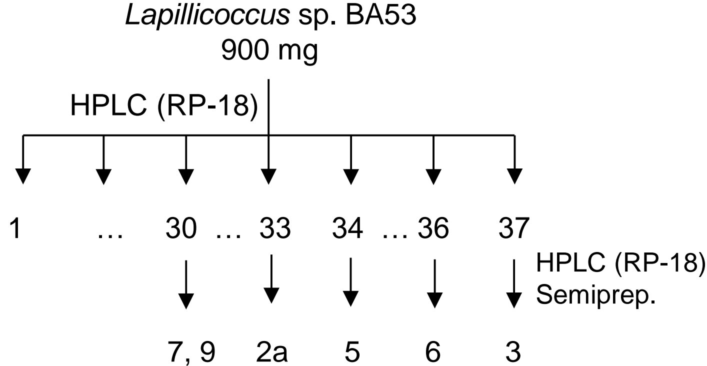


Figure 34. Fractionation of crude extract from the liquid culture of strain *Lapillicoccus* sp. BA53

The molecular formulas for eleven compounds contained in the six fractions obtained from strain *Lapillicoccus* sp. BA53 were established using high-resolution mass spectrometry as follows (Suppl. Fig. 6 and 7): 53L30-7 = $C_{14}H_{16}N_2O_2$ (Rt = 1.87 min, m/z 245.129 $[M+H]^+$); 53L30-9a = $C_{13}H_{16}N_2O_3$ (Rt = 2.11 min, m/z 249.122 $[M+H]^+$); 53L30-9b = $C_{25}H_{17}N_3$ (Rt = 3.12 min, m/z 360.150 $[M+H]^+$); 53L33-2a = C_9H_7NO (Rt = 1.28 min, m/z 146.060 $[M+H]^+$); 53L33-2b = $C_{17}H_{26}N_4O_3$ (Rt = 1.37, m/z 335.208 $[M+H]^+$); 53L33-2c = $C_{11}H_{12}N_2O$ (Rt = 1.98 min, m/z 189.103 $[M+H]^+$); 53L34-5 = $C_{16}H_{24}N_4O_3$ (Rt = 3.26 min, m/z 321.197 $[M+H]^+$), 53L36-6 = $C_{21}H_{26}N_2$ (Rt = 3.65, m/z 307.218 $[M+H]^+$), 53L37-7a = $C_{16}H_{35}NO_2$ (Rt =1.85, m/z 189.109 $[M-H]^-$), 53L37-7b = $C_{14}H_{18}N_2O_2$ (Rt = 2.07, m/z 247.145 $[M+H]^+$) and 53L37-7c = $C_9H_{18}O_4$ (Rt = 3.87, m/z 274.275 $[M+H]^+$).

Furthermore, the combination of NMR (1D and 2D) and comparison of the spectral data with the MarinLit database showed that six of the eleven compounds, were previously reported from marine sources (Table 11). The stereo-chemical properties of these compounds were not determined. The molecular formulas of the compounds 53L33-2b, 53L36-6, 53L37-7a and 53L37-7b did not have any matches in the MarinLit database, suggesting that these compounds may be novel secondary metabolites.

Table 11. Secondary metabolites identified in the actinomycete strain *Lapillicoccus* sp. BA53

Sample	Identified compound	Name	Reference	Source
53L30-7	$C_{14}H_{16}N_2O_2$, MW: 244.29	Benzyldiketopiperazine	Debitus et al. 1998 J. Mar. Biotechnol 6(3), 136-141	Arctic ice bacterium, marine *Pseudomonas* sp. from sponge *Suberea creba* and the fungi *Tyridiomyces formicarum*
53L30-9a	$C_{13}H_{16}N_2O_3$, MW: 248.278	SA4-3	Tanaka et al., 1987 Jpn. Kokai, Tokkyo Koho JP, Appl. 86/27,437	*Streptomyces actamyceticus* ms4-3
53L30-9b	$C_{25}H_{17}N_3$, MW: 359.423	Bis(3-indolyl)-3H-indolylidenmethan	Budzikiewicz H. et al. 1972 Tetrahedron Lett. 36, 3807-3810	*Saccharomyces cerevisiae*

53L33-2a	C_9H_7NO, MW: 145.158	Indole-3-carbaldehyde	Palermo et al. (1992) Tetrahedron Lett., 33, 3097-100	Red alga *Chondria* sp.; marine *Alteromonas luteoviolaceum*, marine *Janibacter limosus*, bacterium Bio232
53L34-5	$C_{16}H_{24}N_4O_3$, MW: 320.387	Arphamenine A	Umezawa et al. 1984 J. Antibiot. 36, 1572- 1575	*Chromobacterium violaceum* bmg 361-cf4
53L37-7	$C_{14}H_{18}N_2O_2$, MW: 246,305	Cyclo(L-Val-L-Phe)	Pickenhagen et al. 1975 Helv. Chim. Acta 58, 1078-86	Cocoa

5. Discussion

5.1. Secondary metabolites from the sponges *Amphimedon compressa*, *Aiolochroia crassa* and *Theonella swinhoei*

Marine sponges have been proven to be a rich source of secondary metabolites exhibiting a huge diversity of biological activities, including antimicrobial, antitumor and immunomodulatory activities. The motivation to investigate the potential of sponges *Amphimedon compressa*, *Aiolochroia crassa* and *Theonella swinhoei* as producers of bioactive secondary metabolites was based on a previous study were a group of 18 species of marine sponges were tested for their protease inhibitory activities. Among the investigated sponges, crude extracts from *Amphimedon compressa*, *Aiolochroia crassa* and *Theonella swinhoei* showed the highest biological potential. This fact, added to the few reports of protease inhibitors from marine organisms (see introduction), indicated that searching for protease inhibitors in these three marine sponges is an interesting approach to explore.

Furthermore, marine-derived secondary metabolites have inspired the development of many drugs currently in use for the treatments of a broad number of human diseases. A good example is the agelasphin 9b story. This sponge-derived immunomodulator has served as an inspiration compound to synthesize KRN7000 (αGalCer) (Natori et al., 1993). KRN7000 has been shown to be a potent NKT cell stimulator, and has been used to combat cancer, inflammation and infection (Godfrey and Kronenberg, 2004). Additional work has shown that αGalCer exhibits adjuvant properties that can be used for vaccine development (Fujii et al., 2003). The low solubility of αGalCer in water has complicated the in vivo applications to evaluate its potential as immunomodulator. In 2007, αgalcelMPEG has been synthesized by Ebensen et al., which is a derivative of the substance KRN7000 and has shown to have improved immunomodulatory properties at a lower concentration. Synthetic αGalCerMPEG exhibited in vitro stimulatory properties on immune cells, such as dendritic cells and splenocytes and strong adjuvant properties in vivo, which makes it suitable for use in vaccination (Ebensen et al., 2007). This is one example of what nature offers us; and one of my motivations to investigate marine environments, as laid out in this PhD thesis.

Theonella swinhoei

This study showed that the sponge *T. swinhoei* from the Red Sea represents an alternative source of the aziridinylpeptide miraziridine A to the previously identified *T. mirabilis* from Japan (Tabares et al., 2011a). Moreover, the results here presented confirmed that miraziridine A is produced by marine sponges from different geographic locations. *Theonella* species (order Lithistida, Demospongiae) have been shown to be a source of several bioactive secondary metabolites. *Theonella swinhoei* has been studied by Piel et al. (2005) for being associated to several microbial symbionts, which are suggested to be the true producers of many secondary metabolites. *T. swinhoei* has been reported for producing the antitumor polyketides onnamides and theopederins (Sakemi et al., 1988, Fusetani et al., 1992). In 2004 the antitumor polyketide biosynthesis by a bacterial symbiont of *T. swinhoei* was reported by Piel et al. In this study, miraziridine A was identified in the dichloromethane extract of the sponge *T. swinhoei* collected offshore Israel in the Red Sea. Miraziridine A, a natural peptide isolated previously from the marine sponge *Theonella* aff. *mirabilis*, is a potent cathepsin B inhibitor with an IC_{50} value of 2.1 μM (Nakao et al., 2000).

Miraziridine A has already served as a model for synthetizing protease inhibitors (Konno et al., 2007). Interestingly, protease inhibitors have been reported from microbial sources, as well; for example, (*2S,3S*)-aziridine-2,3-dicarboxylic acid (Naganawa et al., 1975) and leupeptin (Hozumi et al., 1972) have been reported from actinomycete strains, and circinamide from cyanobacteria isolates (Shin et al., 1997). (*2S,3S*)-Aziridine-2,3-dicarboxylic acid and circinamide contain an aziridine moiety, to which also the inhibitory activity of miraziridine A is mainly attributed (Konno et al., 2007). Moreover, the actinomycete *Kibdelosporangium* sp. was found to produce the aziridine-containing metabolite azinomycin A (Ogasawara and Liu, 2009). Actinomycetes and cyanobacteria constitute part of the microbial consortia present in marine sponges that can account for nearly half of the sponge's biomass (Hentschel et al., 2006, Taylor et al., 2007). This fact suggests that protease inhibitors such as miraziridine A can be the result of the symbiotic interactions between microbes and sponge.

Furthermore, protozoa, e.g. plasmodia, express a broad spectrum of proteases essential for the survival of the parasite (Breuning et al., 2010) and it is known that cysteine and aspartic acid protease inhibitors are synergistic against plasmodia (Semenov et al., 1998). It will be interesting to re-synthesize miraziridine A and perform further bioactivity testing in the future, where miraziridine A would be tested alone or in combination with

aspartic acid inhibitors. It will also be of scientific interest to synthesize miraziridine A-derived molecules. For instance, the shortening of the molecule by combining only two of the three protease-inhibiting building blocks (aziridine and statine, aziridine and vinylogous arginine, or vinylogous arginine and statine) will represent an alternative to produce potent protease inhibitors.

Amphimedon compressa

In this study, amphitoxin was isolated from the sponge *Amphimedon compressa* (order Haplosclerida) and was shown for the first time to have protease inhibitory activities. Alkylpyridinium compounds from marine sponges have also shown cytotoxic, ichthyotoxic, antibacterial, enzyme-inhibitory and anti-fouling properties (Jeanteur et al., 2006, Albrizio et al., 1995). The reports about the toxicity of amphitoxin in the respective literature, and the cytotoxicity analysis here presented, have shown amphitoxin to be a potent toxic agent. This fact leads me to consider the usefulness of amphitoxin and alkylpyridinium salts as materials with biotechnological potential instead of as an alternative for therapeutic agents for the treatment of human diseases. Several alkylpyridinium salts derived from sponges of the order Haplosclerida have been shown to have anti-fouling properties (Jeanteur et al., 2006). According to the previous observations, I consider it important to evaluate the potential of amphitoxin for developing anti-fouling paints for ships and offshore constructions, to prevent the growth of barnacles, algae, and marine organisms.

Aiolochroia crassa

The crude extracts from the sponge *A. crassa* exhibited antitrypanosomal and immunomodulatory activities. Few reports of secondary metabolites from the sponge *A. crassa* (order Verongida) have been found e.g. aerophobins, purealidin L and isofistularin (Assmann et al., 1998) (see introduction). *A. crassa* is considered to be a sponge with high microbial abundance (Weisz et al., 2008); consequently, a higher diversity of bioactive metabolites from this sponge might have to be expected in the literature. The order Verongida is well known for producing bromotyrosine-derived alkaloids, which have shown biological properties (Ciminiello et al., 1994, Gao et al., 1999). Considering the few reports of secondary metabolites from the sponge *A. crassa* as well as the bioactivities of its crude extracts here presented, I consider it interesting in future investigations to search for novel and bioactive brominated alkaloids, in this sponge species. Additionally, further bioactivity-guided fractionation of the active crude extracts from *A. crassa* which

was beyond the scope of this PhD thesis should be carried out in order to complete the elucidation of the structure of the bioactive metabolite(s).

5.2. Isolation and identification of sponge-associated actinomycetes and sphingomonads

Isolates from the order Actinomycetales

The class *Actinobacteria*, belonging to the order Actinomycetales, represent Gram-positive bacteria that are diverse with respect to morphology and biochemistry, as well as G+C-rich DNA content. According to Zhi et al. (2009), 219 genera (classified in 48 families) have been so far accommodated in the class *Actinobacteria*. In this study, four actinomycete isolates were shown to be putatively novel species. While the phenotypic and genotypic characterization of novel isolates were not the major aim of this project, it is, however, worthwhile to pursue them in further research.

79 actinomycete isolates, represented by 20 genera, were cultivated from 18 different species of Caribbean marine sponges (Tabares et al., 2011b). To date this is the only report where such a number of species have been studied for cultivation of *Actinobacteria*. Webster et al. (2001) studied the phylogenetic diversity of bacteria associated with the sponge *Rhopaloeides odorabile* where the importance of using different media and culture conditions for the cultivation of actinomycetes was shown. Zhang et al. (2006) published the cultivation of 106 actinomycete strains from the sponge *Hymeniacidon perlevis* belonging to seven genera. Zhang et al. (2008a) made a comparative study on the actinomycete diversity in five marine sponge species from the yellow sea in China where a total of 181 actinobacterial strains belonging to seven genera were cultivated. More recently, Abdelmohsen et al. (2010) reported the phylogenetic characterization of 90 actinomycetes that were isolated from 11 different species of marine sponges from offshore Ras Mohamed (Egypt) and from Rovinj (Croatia). In addition, among the six cultivation media used to isolate actinomycetes in this project, M1 and oligotropic media exhibited the highest recovery of isolates. M1 medium has been previously reported to be an effective cultivation medium for isolation of actinomycetes. Nevertheless, the use of different media as well as different shaking and temperature conditions might lead to an increase in the number of rare and novel actinomycete bacteria. The cultivation of novel actinomycete strains represents a good strategy for isolation of new secondary metabolites with novel skeletons and reduces the efforts of re-isolating known compounds.

The highest number of actinomycete isolates was recovered from the sponge *Scopalina ruetzleri* including the novel and obligate marine strain *Lapillicoccus* sp. BA53. No reports of secondary metabolites from the sponge *Scopalina ruetzleri* were found in the literature. These results showed *Scopalina ruetzleri* as a significant source of actinomycetes, and as such, is presumably also a source of bioactive secondary metabolites. It will be interesting in future studies, to search for novel and rare actinomycete isolates in the sponge *Scopalina ruetzleri*, and the use of different cultivation approaches in doing so is highly recommended.

Several recent publications have shown that *Streptomyces*, *Micromonospora*, and *Rhodococcus* are among the dominant genera commonly isolated from marine sponges (Abdelmohsen et al. 2010; Schneemann et al. 2010; Sun et al. 2010; Zhang et al. 2008). The actinomycetes isolated in this study displayed considerable diversity; *Microbacterium* (27%) was the most dominant strain followed by *Rhodococcus* (12%) and *Streptomyces* (9%). Nevertheless, this study also disclosed the isolation of rare *Actinobacteria* genera, namely *Cellulosimicrobium*, *Citromicrobium*, *Sanguibacter*, and *Lapillicoccus*. Furthermore, this is the first report on the cultivation of the genera *Citromicrobium*, *Sanguibacter*, and *Lapillicoccus* from marine sponges and of the genus *Lapillicoccus* from the marine environment in general. Interestingly, the strain *Lapillicoccus* sp. BA53 was found to be an obligate marine actinomycete. This is also the first report on the isolation of actinomycetes from the sponges *Agelas cerebrum*, *Agelas tubulata*, *Amphimedon compressa*, *Aplysina archeri*, *Biemna cribaria* and *Chondrilla nucula*. Based on 16S rRNA phylogenetic analyses of actinomycete strains as part of this study, 4 out of the 79 identified isolates are new actinomycete species belonging to the genera *Lapillicoccus, Microbacterium, Nocardioides* and *Rhodococcus*.

The strain *Lapillicoccus* sp. BA53, showed a strict requirement for salt and is the first obligate marine bacterium of the genus *Lapillicoccus*. The genus *Lapillicoccus* was first described by Lee and Lee (2007), reporting the isolation of the species *Lapillicoccus jejuensis* from a small stone collected in Jeju, in the Republic of Korea. *Lapillicoccus* belongs to the family Intrasporangiaceae (Lee and Lee, 2007), which contains 19 genera with validly published names. Moreover, strain *Lapillicoccus* sp. BA53 is of special interest due to its protease and immunomodulatory properties. The use of different cultivation and extraction techniques might lead to the isolation of novel and bioactive compounds from the novel *Lapillicoccus* species here presented.

The genus *Microbacterium* belonging to the family Microbacteriaceae was the dominant genus of this study. The Microbacteriaceae family accommodates Gram-positive bacteria with high content of G+C DNA and with the group-B-type peptidoglycan (Park et al., 1993). Around 73 species have validly published names, including the marine-derived species *M. flavum*, *M. lacus*, *M. marinilacus* and *M. sediminicola*. With regard to the production of bioactive secondary metabolites from the genus *Microbacterium*, four glycoglycerolipids from a marine sponge-associated *Microbacterium* species have been reported by Wicke et al. (2000); these showed surfactant properties and antitumor activity.

The genus *Rhodococcus*, belonging to the Nocardiaceae family, has shown a broad catabolic diversity and array of unique enzymatic capabilities, and has gained interest from scientific researchers in the last decades due to its commercial potential (Bell et al., 1998, van der Geize and Dijkhuizen, 2004). For example, species of the genus *Rhodococcus* are able to degrade hydrophobic natural compounds and xenobiotics, such as polychlorinated biphenyls (PCBs). They have, moreover, been shown as good candidates for the industrial production of steroid compounds with pharmacological potential (Fernandes et al., 2003, van der Geize and Dijkhuizen, 2004). According to the MarinLit database, around 29 secondary metabolites have been isolated from the genus *Rhodococcus*. Considering the interesting background of the genus *Rhodococcus*, it could be worthwhile to use different approaches to produce bioactive and novel secondary metabolites from the novel strain *Rhodococcus* sp. CO155. For example, the cultivation of the novel strain *Rhodococcus* sp. CO155 under different conditions as well as the preparation of crude extracts using different extraction methods might increase the likelihood of obtaining novel chemical entities.

Isolates from the order Sphingomonadales

Sphingomonads are of potential importance in the ecology of a range of marine habitats (Cavicchioli et al., 1999); they can act as coral pathogens (Richardson et al., 1998), hosts for temperate phage (Jiang et al., 1998) and hydrocarbon degraders (Gilewicz et al., 1997). Sphingomonads are also well known for their biotechnological applications in the degradation, bioremediation and wastewater treatment of xenobiotic pollutants (Fredrickson et al., 1995, Zipper et al., 1996), and also for their biosynthetic production of extracellular polymers (Denner et al., 2001). Seven isolates were found to belong to the order *Sphingomonadales* in this PhD thesis, and four of them represent putatively novel species (three *Sphingomonas* and one *Sphingobium*). This is the first report on the

cultivation of the genus *Sphingobium* from the marine environment in general. The pharmacological activities of sphingomonad isolates were also tested as part of this study, which will be discussed in the following sections; however, the evaluation of the biotechnological potential of sphingomonad isolates was not included in this project's aims. It would therefore be interesting to evaluate the role of the new strains shown here as pollutant degraders, but also as a source of polymers with industrial potential. Moreover, further phenotypic and genotypic characterization is required to confirm the taxonomic affiliation of these strains.

5.3. Bioactivity screening of sponge-derived actinomycetes and sphingomonads

Protease inhibitory activity

The protease inhibitory assays here described involved a fluorometric enzymatic assay. This is an effective and rapid method to pursue the identification of enzyme inhibitory substances, and is therefore useful to determine kinetic data of protease inhibitors (Birdsall et al., 1983, Turk, 2006, Ludewig et al., 2010). The crude extracts of sixteen isolates cultivated in M1 medium and extracted at four different points in time were tested for their anti-protease activities. This involved testing against the human cysteine proteases, cathepsin B and L, and against parasitic proteases falcipain-2 (*Plasmodium falciparum*) and rhodesain (*Trypanosoma brucei rhodesiense*). Cathepsins B and L are proteases ubiquitously expressed in human tissues; they play a role in a large number of important physiological processes in the organism, including MHC-II-mediated antigen presentation, bone remodeling and keratinocyte differentiation (Turk et al., 2011, Turk et al., 2002, Vasiljeva et al., 2007). Furthermore, cathepsins B and L are involved in tumor progression and invasion either by direct degradation of the extracellular matrix or by activation of other proteases, such as the urokinase-type plasminogen activator (Turk et al., 2000). Falcipain-2 is a protease essential to the nutrition of the *Plasmodium falciparum* (malaria), which hydrolyzes erythrocyte hemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis (Rosenthal et al., 1988). Rhodesain is involved in the degradation of parasitic proteins as well as in intracellularly transported host proteins in both the insect and the mammalian host (Caffrey et al., 2001). Additionally, crude extracts were tested for their capacity to inhibit the papain-like (SARS-CoV PL^{pro}), and the main protease (SARS-CoV M^{pro}) of the SARS coronavirus. These enzymes are essential for the replication of the severe acute respiratory syndrome (SARS) coronavirus (Anand et al., 2003, Ratia et al., 2006). Cathepsin B and L, rhodesain and falcipain-2 enzymes belong to the cathepsin L subfamily of cysteine proteases (clan CA, family C1; CAC1). SARS-CoV PL^{pro} also belongs to the clan of

cysteine proteases CA, but is affiliated with the family C16, which contains polyprotein endopeptidases from coronaviruses. The protease SARS-CoV M^{pro} belongs to the clan PA (family 30) with a catalytic type of mixed cysteine, serine and threonine (Rawlings et al., 2010).

The crude extracts of eight isolates showed certain specificity to the clan CA (family C1; CAC1) group of proteases, whereas no protease inhibition was observed against viral proteases. Six actinomycete and two sphingomonad isolates are shown as potential sources of protease inhibitors. The inhibition of rhodesain, to which the substrate has a particularly high affinity, by extracts of *Nocardioides* sp. BA21, *Saccharopolyspora shandongensis* CO86, *Micromonospora coxensis* strain CO164 and *Sphingobium* sp. CO132 is, thus remarkable. Interestingly, the putatively novel strains *Nocardioides* sp. BA21, *Sphingobium* sp. CO132 and *Rhodococcus* sp. CO155 exhibited protease inhibitory acivity. According to previous reports novel actinomycete species will produce novel bioactive secondary metabolites (Pimentel-Elardo et al., 2008, Pimentel-Elardo et al., 2011, Kwon et al., 2006). Only few reports of actinomycete-derived protease inhibitors were found in the literature (see introduction). These results suggest that marine actinomycetes might be a potential source of protease inhibitors, offering a more productive route to the discovery of bioactive secondary metabolites.

Immunomodulatory activity

The protocol employed in this project for evaluating the PBMC response to sponge and bacteria-derived substances involved a new methodology developed by Römer et al (2011). This protocol includes the stimulation of high cell density pre-cultured PBMC instead of freshly prepared PBMC, in order to resemble the environment to which T cells are subjected in the lymph nodes, and thus become more reactive; providing a more reliable screening method for finding immunomodulatory agents. Römer et al. (2011) showed that freshly isolated PBMC fail to respond to the monoclonal antibody (mAb) TGN1412, which led to a massive cytokine storm in humans (Suntharalingam et al., 2006, Hünig, 2007). Stimulation of PBMC, after two days of pre-culturing at a 10-fold higher cell density than usually utilized for in vitro assays resulted in an increase of the response to the CD28 superagonist TGN1412, which is comparable to the T cell activating mAb OKT3 Römer et al. (2011). Moreover, responses to other T cell activating agents such as *Staphylococcus* enterotoxin B (SEB) and a combined tetanus/diphteria toxoid preparation were also enhanced when PBMC were pre-cultured for two days before stimulation (Römer et al., 2011).

When culturing PBMC in the presence of crude extracts, different patterns of cytokine release were observed. Even though this assay does not distinguish which cells are responsible for cytokine production, some assumptions can be made about their cellular source. This is facilitated by the fact that individual extracts induced rather distinct patterns of cytokine release. For example, *Agrococcus jenensis* strain BA22 was very active in inducing TNF and IL-10, suggesting that this preparation addresses monocytes. In contrast, *Micromonospora coxensis* strain CO164 was a potent inducer of IFN-γ, IL-2, and, to a lesser extent, IL-10. Since the main source of IFN-γ are T_H1 $CD4^+$ T cells and $CD8^+$ T cells, this preparation appears to have T cell activating properties. Furthermore, the induction of IL-2 but not of IFN-γ by *Saccharopolyspora shandongensis* strain CO86 suggests a further specificity for $CD4^+$ T cell activation. Isolates *Agrococcus jenensis* strain BA22, *Arthrobacter oxidans* strain BA30 and *Arthrobacter oxydans* strain BA34 were effective in inducing TNF and IL-10 release, cytokines induced mainly by monocytes and T cells (TNF: T_H1, IL-10: T_H2 and T reg). Strains *Lapillicoccus* sp. BA53 and *Micromonospora coxensis* strain CO164 induced IFN-γ, IL-2 and IL-10. IFN-γ and IL-2 can be released by T helper 1 cells and IL-10 by T helper 2 and regulatory T cells as well as monocytes. *Saccharopolyspora shandongensis* strain CO86 effectively induced the release of IL-2 and IL-10. IL-2 is mainly released by T_H1 cells and IL-10 is mostly produced by macrophages and T helper 2 and regulatory T cells. *Sphingobium* sp. CO132 induced the release of TNF, IFN-γ, IL-2 and IL-10, indicating a stimulatory effect on both T cells and monocytes.

PBMC contain monocytes and lymphocytes, the latter consisting of B cells, T cells (CD4+ T cells, CD8+ T cells, and regulatory T cells), as well as NKT cells. Cytokines are proteins which are secreted by the cells of innate and adaptive immunity, and which are involved in many cellular functions. In the present study, the following cytokines were analyzed which are key pro- and anti-inflammatory factors and which are characteristic of particular types of immune responses to pathogens: TNF, the principal mediator of the acute inflammatory response to Gram-negative infectious microbes, is responsible for many of the systemic complications of severe infections. IFN-γ is the principal macrophage-activating cytokine and serves critical functions in innate and in adaptive cell-mediated immunity. IL-2 is a cytokine responsible for T cell clonal expansion after antigen recognition. IL-10, a cytokine with anti-inflammatory properties, has a central role in infection by limiting the immune response to pathogens and by thus preventing damage to the host.

Additionally, It was observed that the *Agrococcus jenensis* strain BA22, *Arthrobacter oxidans* strain BA30 and *Micromonospora coxensis* strain BA164 were active only when crude extracts were obtained from solid cultures, while crude extracts from the *Arthrobacter oxydans* strain BA34, either from liquid or solid culture, showed an effect on the cytokine release by PBMC. Moreover, *Lapillicoccus* sp. CO53, *Saccharopolyspora shandongensis* strain CO86 and *Sphingobium* sp. CO132 were active only when crude extracts were obtained from liquid cultures.

5.4. Secondary metabolites from actinomycete and sphingomonad isolates

Isolumichrome

Bioactivity-guided fractionation of the active and putatively novel strains *Sphingobium* sp. CO105 and *Lapillicoccus* sp. CO53 was conducted using chromatographic, spectrometric and spectroscopic techniques. Strain *Sphingobium* sp. CO105 was found to produce the isoalloxazine isolumichrome (Figure 35), which was previously isolated from the Chinese marine sponge *Cinachyrella australiensis* by Li et al. in 2004. There are no reports of biological activity of isolumichrome in the literature. In this study, isolumichrome showed protease inhibitory activity with an IC_{50} value of 0.20 μM, when tested against the protease rhodesain. Isolumichrome was also active against the proteases cathepsins B and L as well as against falcipain-2. However, it will prove valuable to synthesize isolumichrome in the future, and to confirm the biological activity, as well as to produce new synthetic derivatives of isolumichrome in order to conduct further activity/structure relationship studies. In this project no new compounds were found, nevertheless it is recommended to establish strategies in order to isolated novel compounds from sphingomonad strains. An interesting approach would be to focus the search for novel compounds on certain groups of metabolites. For example, glycosphingolips are a group of compounds with immunomodulatory substances commonly produced by sphingomonad strains (see introduction). Directing efforts to the isolation of this kind of metabolites, would augment the likelihood of finding novel sphingomonad-derived molecules and it will also reduce the exhaustive effort of screening the whole crude extract.

Figure 35. Isolumichrome

Compound CO105-23-11

Strain *Sphingobium* sp. CO105 produced the immunomodulatory compound CO105-23-11, which induced TNF and IL-10 cytokine production and additional ESI-MS analysis showed an ion peak at m/z 511 $[M+H]^+$. Nevertheless, the elucidation of the structure of compound CO105-23-11 has yet to be pursued, as well as the evaluation of its mechanism of action, by means of performing in vitro assays with cells of the immune system to evaluate the responding cell types, the toxicity and apoptosis/necrosis. The study of the mechanism of action of bioactive compounds was not included in the major aims of this PhD project; it will be very interesting to perform further experiments to gain insight into the structure and mechanism of action of compound CO105-23-11, which might be a novel immunomodulatory substance.

p-Aminosalicylic acid methyl ester

Strain *Lapillicoccus* sp. CO53 (solid culture) was found to produce the antibiotic compound p-aminosalicylic acid methyl ester (Figure 36), isolated previously from the genus *Actinoplanes* and patented by Celmer et al. in 1979 (Pfizer Inc. New York). Interestingly, the synthetic analog p-aminosalicylic acid (PAS) is currently commercially available and is used in the treatment of tuberculosis and inflammatory bowel diseases (Daniel et al., 2004). In addition, PAS has served as starting material to synthesize a series of hydrophobic p-aminosalicylic acid derivatives which are active against the influenza A virus (Zhang et al., 2008b).

Figure 36. p-Aminosalicylic acid methyl ester

Secondary metabolites isolated from *Lapillicoccus* sp. BA53

Besides, the compounds benzyldiketopiperazine, SA4-3, bis(3-indolyl)-3H-indolylidenmethan, indole-3-carbaldehyde, arphamenine A and cyclo(L-Val-L-Phe) were found to be produced by strain *Lapillicoccus* sp. CO53 (liquid culture) (Figure 37). Compound SA4-3 isolated from the genus *Streptomyces*, possess antimicrobial activity against Gram-positive and Gram-negative bacteria (Toshiyuki, 1987). Indole-3-carbaldehyde has been previously isolated from the red alga *Chondria* sp., and in 2003 was reported by Shimizu et al. for its tyrosinase inhibitory activity. Arphamenine A is an aminopeptidase B inhibitor isolated from the marine bacterial strain *Chromobacterium violaceum* by Umezawa et al. in 1983. There are no bioactivity reports of the compounds benzyldiketopiperazine, bis(3-indolyl)-3H-indolylidenmethan and cyclo(L-Val-L-Phe). Even though compounds isolated from the liquid culture of strain *Lapillicoccus* sp. BA53 did not show protease inhibitory or immunomodulatory activities, it will be profitable for future projects to evaluate the biological activity, using different screening approaches, as well as to synthesize derivatives and evaluate their biological potential.

Bioactivity-guided fractionation has been the most successful strategy to isolate bioactive secondary metabolites from marine sources (Ebada et al., 2008). The bioactivity-guided scheme followed to pursue this project involved a wide spectrum of biological activities, which served to successfully detect the most promising bacterial isolates. For future research, I suggest that crude extracts and fractions obtained from bacterial isolates need to be monitored, using high resolution mass spectrometry in order to detect known compounds in an early stage, and thus direct the efforts of isolation and purification only to novel molecules.

benzyldiketopiperazine

SA4-3

arphamenine A

cyclo(L-Val-L-Phe)

indole-3-carbaldehyde

bis(3-indolyl)-3H-indolylidenmethan

Figure 37. Secondary metabolites isolated from strain *Lapillicoccus* sp. BA53

6. Conclusions

The cultivation of 79 strains belonging to 20 different *Actinomycetales* genera as well as seven strains belonging to two *Sphingomonadales* genera represents a considerable diversity of culturable bacteria in 18 Caribbean sponges. The isolation of rare actinomycete genera that have not previously been reported from marine organisms, as well as the identification of seven putatively novel actinomycete and sphingomonad species based on the phylogenetic analysis of their 16S rRNA gene sequence, interestingly adds to the microbial population associated with marine sponges. These results further prove that marine sponges still remain a relatively untapped resource for actinomycetes and sphingomonads.

Moreover, anti-protease activities against cathepsin B and L, rhodesain and falcipain-2, as well as immunomodulatory activities, specifically the induction of cytokine release by PBMC and induction of cell proliferation were found to be exhibited by the crude extracts from marine sponges and their associated actinomycete and sphingomonad isolates. These results show marine sponges and their associated bacteria as potential sources for developing protease inhibitors and immunomodulatory substances. Bioactivity-guided fractionation of crude extracts prepared from marine sponges and their associated actinomycetes and sphingomonads yielded two protease inhibitors and one immunomodulatory substance. Additionally, six known compounds were identified from strain *Lapillicoccus* sp. BA53. Even though all compounds found in this study are known secondary metabolites, future bioinformatics approaches can be used to evaluate the biological potential of the metabolites here presented. The results of this study highlight the therapeutic potential of these secondary metabolites; therefore, studies regarding the structure/activity relationship of synthetic derivatives of these compounds will indeed be an advantageous pursuit.

Finally, I would like to conclude by saying that this PhD thesis is an interdisciplinary project that merged biology and chemistry and highlights the potential of marine sponges and their associated bacteria to produce bioactive metabolites that might lead to the development of drug candidates against infectious and immune diseases.

7. Outlook

Further genotypic and phenotypic characterizations of the seven putatively novel isolates presented in this study should be done in order to confirm the phylogenetic affiliation of these strains as well as to describe in more detailed their morphological and biochemical characteristics. Moreover, different cultivation conditions of the sponge associated bacteria e.g. the use of different media, temperature, etc. will increase the diversity of the isolates, and in consequence will conduct to a higher production of bioactive secondary metabolites.

In order to further increase the number of novel secondary metabolites from marine sponges as well as from their associated actinomycetes and sphingomonads, various extraction methods (different resins: amberlite, RP-18, sephadex, etc) and solvents (acetone, ethyl acetate, methanol, etc) as well as different chromatographic techniques (column chromatography, semipreparative HPLC, etc) can be used, which might lead to a higher variety of biologically active compounds.

Another valuable strategy in natural product discovery, which can be employed in future studies, is the more directed search of bioactive compounds taking the prior knowledge of the secondary metabolite capacities of an organism into account. For instance, the search for glycosphingolipids from sphingomonads or the search for brominated alkaloids from sponges of the order Verongida might represent a more direct route to the discovery of novel chemical compounds.

Annex

I. Abbreviations and Acronyms

δ_H	chemical shift (ppm)
°C	degree Celsius
ASW	artificial seawater
BLAST	basic local alignment search tool
BSS/BSA	Balanced salt solution/bovine serum albumin
bp	base pair
ca.	approximately
CBA	cytometric bead array
^{13}C-NMR	carbon nuclear magnetic resonance
CB	cathepsin B
CL	cathepsin L
COSY	correlation spectroscopy
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EtOH	ethanol
FBS	fetal bovine serum
Fp-2	falcipain-2
g	gram
h	hour
H_2O_d	distilled water

H_2O_{dd}	double distilled water
^{1}H-NMR	proton nuclear magnetic resonance
HMA	high microbial abundance
HPLC	high performance liquid chromatography
Hz	hertz
IL	interleukin
IFN-γ	interferon-gamma
ISP	International *Streptomyces* Project
kb	kilobase
L	liter
LC	Liquid chromatography
LMA	low microbial abundance
M	molar
MeCN	acetonitrile
MeOD	deuterated methanol
MeOH	methanol
MSD	mass selective detector
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mM	millimolar
MS	mass spectrometry
MR	microplate reader
NMR	nuclear magnetic resonance
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reaction
ppm	parts per million
Q-TOF	Quadrupole time-of-flight mass spectrometer
RFLP	restriction fragment length polymorphism
Rho	rhodesain
rRNA	ribosomal RNA
RNA	ribonucleic acid
RP	reverse phase
RPMI	Roswell Park Memorial Institute
rpm	revolutions per minute
Rt	retention time
RT	room temperature
sp.	species
Tbb	*Trypanosoma brucei brucei*
TFA	trifluoroacetic acid
TNF	tumor necrosis factor
U	enzyme units
μg	microgram
μl	microliter
μM	micromolar
UPLC	Ultra performance liquid chromatography
v/v	volume concentration
w/v	weight per volume

II. Buffers, Solutions and Media

The following buffers, solutions and media were sterilized by autoclaving at 121 °C for 30 min. Antibiotics and other solutions, when specified, are added to the media at hand-warm temperature following sterilization. Media containing agar were aseptically poured into sterile disposable Petri dishes and allowed to solidify at room temperature.

Buffers and Solutions

Artificial seawater

NaCl	234.70 g
Na_2SO_4	39.20 g
$MgCl_2.6H_2O$	106.40 g
$CaCl_2$	11.00 g
$NaHCO_3$	1.92 g
KCl	6.64 g
$SrCl_2$	0.024 g
KBr	0.96 g
H_3BO_3	0.24 g
NaF	0.03 g
H_2O_{dd} ad	10.00 L

EDTA (ethylenediamine tetraacetic acid) (0.5 M, pH 8.0)

$Na_2EDTA.2H2O$	93.05 g
NaOH (10 M) ca.	25.00 mL
H_2O_{dd} ad	500 mL

The pH of the solution was adjusted to 8.0 with NaOH since EDTA does not begin to dissolve until the pH reaches 7.0. Water was added to make a final volume of 500 mL.

Gel-loading buffer (5x)

Bromphenol blue	25.0 mg
Xylene cyanol	25.0 mg
Ficoll (type 400)	1.5 g
H_2O_{dd} ad	10.0 mL

IPTG (Isopropyl-β-D-thiogalactopyranoside) (1 M)

IPTG	2.38 g
H_2O_{dd} ad	10.00 mL

Plasmid mini-prep buffers

Buffer P1

Tris-Cl (1 M, pH 7.5)	5.0 mL
EDTA (0.5 M, pH 8.0)	2.0 mL
RNAse A (10 mg/mL)	1.0 mL
H_2O_{dd} ad	100.0 mL

Buffer P2

NaOH (2 M)	5.0 mL
SDS (10%)	10.0 mL
H_2O_{dd} ad	100.0 mL

Buffer P3

Potassium acetate	29.4 g
Acetic acid ca.	11.5 mL
H_2O_{dd} ad	100.0 mL

The pH of the solution was adjusted to pH to 5.5 using glacial acetic acid, and water was added to make a final volume of 100 mL.

SDS (sodium dodecyl sulfate) solution (10% w/v)

SDS	10.0 g
H_2O_{dd} ad	100.0 mL

SDS was first dissolved in 80 mL water, and was heated to 50 °C to accelerate dissolution. The pH of the solution was adjusted to 7.0 by adding several drops of concentrated HCl, and water was added to make a final volume of 100 mL.

TAE buffer (5x)

Tris base	242.00 g
Sodium acetate.3H_2O	136.12 g
$Na_2EDTA \cdot 2H_2O$	19.00 g
H_2O_{dd} ad	1.00 L

The above reagents were dissolved in 700 mL water and the pH was adjusted to 7.2 with acetic acid. Water was added to make a final volume of 1 L.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (5% w/v)

X-gal	0.5 g
DMFA ad	10.0 mL

Media

ISP 2

Yeast extract	4.0 g
Malt extract	10.0 g
Glucos	4.0 g
Agar	18.0 g
H_2O_{dd} ad	1.0 L

LB (Luria-Bertani) agar

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Agar	18.0 g
H_2O_{dd} ad	1.0 L

LB/amp

LB agar	1.0 L
Ampicillin	1.0 mL

Filter-sterilized ampicillin (100 μg/mL) was added to LB agar after autoclaving.

LB/amp/IPTG/X-gal

LB agar	1.0 L
Ampicillin (100 mg/mL)	1.0 mL
IPTG (1 M)	0.4 mL
X-gal (5%)	1.6 mL

Filter-sterilized ampicillin (100 μg/mL), IPTG (0.5 mM) and X-gal (80 μg/mL) were added to LB agar after autoclaving.

M1

Soluble starch	10.0 g
Yeast extract	4.0 g
Peptone	2.0 g
Agar	18.0 g
Artificial seawater a	1.0 L

M2

Glycerin (100%)	6.0 mL
Arginine	1.0 g
K_2HPO_4	1.0 g
$MgSO_4$	0.5 g
Agar	18.0 g
Artificial seawater a	1.0 L

M7

Peptone	2.0 g
Asparagine	0.1 g
Sodium propionate	4.0 g
K_2HPO_4	1.0 g
$MgSO_4$	0.1 g
$FeSO_4$	0.001 g
Glycerol	5.0 g
NaCl	20.0 g
Agar	18.0 g
Artificial seawater ad	1.0 L

NaSt21Cx

Solution A	
K_2HPO_4	1.0 g
Agar	18.0 g
Artificial seawater	750.0 mL
Solution B	
KNO_3	1.0 g
$MgSO_4$	1.0 g
$CaCl_2.2H_2O$	1.0 g
$FeCl_3$	0.2 g
$MnSO_4.7H_2O$	0.1 g
Artificial seawater	250.0 mL

Solutions A and B were autoclaved separately and subsequently combined.

Oligotropic

Tryptone	0.50 g
$C_3H_7Na_2O_6P$	0.1 g
Yeast extract	0.05 g
Agar	12 g
H_2O_d ad	1.0 L

SOC medium

Tryptone	20.0 g
Yeast extract	5.0 g
NaCl (1 M)	10.0 mL
KCl (1 M)	2.5 mL
$MgCl_2$ (1 M)	10.0 mL
$MgSO_4$ (1 M)	10.0 mL
Glucose (2 M)	10.0 mL
H_2O_d ad	1.0 L

Tryptone (2%), yeast extract (0.5%), NaCl (10 mM) and KCl (10 mM) were added to 970 mL distilled water prior to autoclaving. The following filter-sterilized solutions: $MgCl_2$ (10 mM), $MgSO_4$ (10 mM) and glucose (20 mM) were subsequently added to the medium after autoclaving.

YPD medium

Yeast extract	10.0 g
Peptone	20.0 g
Glucose	20.0 g
H_2O_{dd} ad	1.0 L

Zobell medium

Yeast extract	1.0 g
Peptone	5.0 g
Artificial seawater	750.0 mL
H_2O_{dd} ad	1.0 L

III. Cells

Cell type	Source
Human PBMC	Institute of Transfusion Medicine and Hemotherapy, University Clinic Würzburg
J774.1 murine macrophages	Institute for Molecular Infection Biology, University of Würzburg
Human kidney epithelial 293T cells	Institute for Molecular Infection Biology, University of Würzburg.

IV. Chemicals

Chemical name	Manufacturer
AB-positive human serum	PAA Laboratories GmbH
Acetic acid	AppliChem
Acetonitrile	Sigma
Agar, granulated	Difco
Agarose, ultrapure	Gibco
Ampicillin	AppliChem
Arginine	Sigma
Asparagine	Sigma
Boric acid (H_3BO_3)	AppliChem
5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	Sigma
Bromphenol blue	Merck
Calcium chloride ($CaCl_2$)	AppliChem
Cbz-Phe-Arg-AMC	Bachem
Chloroform	Roth
Cycloheximide	Sigma
Dipotassium hydrogen phosphate (K_2HPO_4)	Roth
Dimethylformamide	AppliChem
Dimethylsulfoxide	Sigma
Ethanol absolute (EtOH)	Merck
Ethanol denatured (EtOH)	Roth
Ethidium bromide (1% solution)	Roth
Ethyl acetate	Roth
Ethylenediamine tetraacetic acid dihydrate	Serva
Ferric chloride (FeCl3)	Roth
Ferrous sulfate heptahydrate (FeSO4 .7H2O)	Fluka
Gentamicin	Sigma
Glucose	AppliChem
Glutaraldehyde	Sigma

Chemical name	Manufacturer
Glycerin/Glycerol	Roth
Hepes	AppliChem GmbH
Hydrochloric acid (HCl)	AppliChem
H_2N-Abz-Ser-Val-Thr-Leu-Gln-Ser-Gly-(NO2)Tyr-Arg-(MTS)-TFA-salt	Radim Vicik, Würzburg, now in Switzerland
Isopropanol	Roth
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Sigma
Lymphocyte separation medium	PAA Laboratories GmbH
Malt extract	AppliChem
Magnesium chloride hexahydrate (MgCl2.6H2O)	AppliChem
Magnesium sulfate heptahydrate (MgSO4.7H2O)	AppliChem
Manganese chloride tetrahydrate (MnCl2. 4H2O)	AppliChem
Manganese sulfate (MnSO4.7H2O)	Roth
Methanol	Sigma, Roth
Nalidixic acid	Sigma
Nystatin	Sigma
Oxacillin	Sigma
Penicillin	Sigma
Peptone	Roth
Phenol	AppliChem
Potassium acetate (CH3CO2K)	Applichem
Potassium bromide (KBr)	AppliChem
Potassium chloride (KCl)	Fluka
Potassium nitrate (KNO3)	AppliChem
Propylene oxide	Roth
Rifampin	Sigma
Saccharose	Roth
Sodium acetate trihydrate	AppliChem

Chemical name	Manufacturer
Sodium bicarbonate (NaHCO3)	Merck
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	AppliChem
Sodium fluoride (NaF)	Fluka
Sodium glycerolphosphate (C3H7NaO6P)	Roth
Sodium hydroxide (NaOH)	AppliChem
Sodium propionate (C3H5NaO2)	AppliChem
Sodium sulfate (Na2SO4)	Merck
Sodium thiosulfate (Na2S2O3)	AppliChem
Starch	Roth
Streptomycin	Sigma
Strontium chloride (SrCl2)	Fluka
RPMI	Grünenthal GmbH
Tetracycline	Sigma
Trifluoroacetic acid (TFA)	Sigma
Tris (hydroxymethyl) aminomethane hydrochloride	Sigma
[3H]-thymidine	Hartmann Analytic
Tryptone	Roth
Tyrosine	Sigma
Vancomycin	Sigma
Xylene cyanol	AppliChem
XAD-16 resine	Sigma
Yeast extract	Gibco
Z-Arg-Leu-Arg-Gly-Gly-AMC-acetate salt	Bachem

V. Computer Programs

Software	Application	Reference
Align	sequence alignment and editing	Hepperle 2002
AntiMarin database	marine natural products database	University of Canterbury, New Zealand
ARB	phylogenetic tree construction	http://www.arb-home.de/
BLAST	sequence comparison/alignment	http://www.ncbi.nlm.nih.gov/BLAST/
ChemBioOffice 2008	illustration of chemical structures	Chem Office 2004
ClustalX	alignment of nucleotide and amino acid sequences	http://www-igbmc.u- strasbg.fr/ BioInfo /ClustalX/Top.htmL
Dictionary of Natural Products	online database of natural products	http://www.chemnetbase.com
FCAP Array software	flow cytometry data analysis and processing	Soft Flow, Inc., USA
MestReNova	NMR processing, analysis and reporting	http//mestrelab.com
Multi Analyst 1.1	documentation of agarose gel	BioRad
Phylip	phylogenetic tree construction	http://evolution.genetics.washington.ed u /phylip/getme.htmL
SciFinder Scholar	natural products access tool	http://www.cas.org/scifinder/scholar
Treeview	visualization of phylogenetic trees	http://taxonomy.zoology.gla.ac.uk/rod /treeview.htmL
Vector NTI Advance TM 10	ORF identification and sequence annotation	https://catalog.invitrogen.com/index.cf m?fuseaction=userGroup.downloadCe nter

VI. Enzymes and Kits

Name of Enzyme/ Kit	Manufacturer
pGEM-Teasy vector system	Promega
Cathepsin L (Paramecium tetraurelia)	Calbiochem
Cathepsin B (human liver)	Calbiochem
Falcipain 2 (Plasmodium falciparum)	Prof. P. Rosenthal, SF, USA/Prof. C. Kisker, Germany
Rhodesain (Trypanosoma rhodesiense)	Prof. J. McKerrow, Dr. C. Caffrey, SF, USA/ Prof. C. Kisker, Germany
Human Soluble Protein Flex Sets	BD Biosciences, San Jose, CA, USA
Human Soluble Protein Master Buffer	BD Biosciences, San Jose, CA, USA
QIAquick PCR purification kit	Qiagen
Restriction endonucleases and buffers	New England Biolabs
RNase	Roche
SARS-CoV Mpro and SARS-CoV Plpro	Prof. J. Ziebuhr, Germany/Prof. C. Kisker, Germany
Taq DNA polymerase and buffer	Qiagen
T4 DNA ligase and buffer	New England Biolabs
GeneRulerTM 1kb DNA ladder	Fermentas
GeneRulerTM 100bp DNA ladder	Fermentas

VII. Equipment and Supplies

Equipment/ Supplies	Manufacturer	Specifications
Autoclave	Fedegari	Tec 120, 9191E, FV 3.3
	H+P Labortechnik	Varioklav 500, 135S
Benchtop centrifuge	Hereaus Instruments	Biofuge Frasco
Distilling apparatus for H_2O_{dd}	GFL	Bi-Dest 2304
Disposable cuvette	Plastibrand	halbmikro 1,5 mL
Electroporator	EquiBio	Easyject PRIMA

Equipment/ Supplies	Manufacturer	Specifications
Electroporation cuvette	EquiBio	EPC 102
ELISA plate reader	Cary Eclipse fluorescence spectrophotometer	Varian
Filter disks	Becton Dickinson	-
Filter membranes	Millipore	Millex-GS 0.22 µm
Flow cytometer	BD Biosciences	LSR II
Gel documentation	BioRad	Gel Doc 2000
Gel electrophoresis chamber	BioRad	-
Heat block	Laboratory Devices	Digi-Block Jr.
HPLC	Agilent	Agilent 1100
	Agilent	ProStar
HPLC columns	Phenomenex	RP18
	Varian	RP18
Ice maker	Scotsman	AF-20
Incubator	Heraeus	Kelvitron®t
	Memmert	TV 40b
LC-MS	Agilent 1100	LC/MSD trap
MS	Synapt G2 HDMS	TOF-MS
MS	Bruker Daltonics	micrOTOF
Liquid scintillation counter	PerkinElmer	
Magnetic stirrer	Labinco	L32
Micropipettes	Microlab	MicroOne 0,5-10 µL
	Microlab	MicroOne 2-20 µL
	Microlab	MicroOne 20-200 µL
	Microlab	MicroOne 100-1000 µL
Microfuge tubes	Sarstedt	1,5 mL; 2,0 mL
	Abgene	0,5 mL Thermo tubes
	Abgene	0,2 mL Thermo Stripes

Equipment/ Supplies	Manufacturer	Specifications
Microplates	Nalgene	NunclonTM
Microwave	AEG	Micromat
	Privileg	8020
NMR	Bruker	Bruker DMX 600
	Bruker	Advance 400 MHz
PCR cycler	Biometra	T3-Thermocycler
Petri dishes, round	Greiner	-
Petri dishes, square	Nalgene	NunclonTM
96 well plates	Greiner Bio-one	Cell culture
14 well plates	Greiner Bio-one	Cell culture
pH Meter	WTW	MultiLine P4, SenTix 41
Pin replicator	Nalgen Nunc International	384 pin replicator
Quartz cuvette	Hellma	Suprasil
Refrigerator	Privileg	Superöko
Rotary evaporator	Heidolph	Laborota 4010
Sequencer	ABI Prism	ABI 377XL
Spectrophotometer	Pharmacia Biotech	Ultraspec 3000
	PeqLab	NanoDrop ND1000
Shakers	Braun	Certomat U
	Edmund Bühler	SM-30
	Eppendorf	Rotationsmischer 3300
	Infors	HT

VIII. Microorganisms

Microorganism	Application
Staphylococcus aureus NCTC 8325	Bioactivity testing
Staphylococcus epidermidis RP62A	Bioactivity testing
Enterococcus faecalis JH212	Bioactivity testing
Enterococcus faecium 6413	Bioactivity testing
Escherichia coli 536	Cloning
Escherichia coli XL1-Blue	Bioactivity testing
Escherichia coli EPI^{100}	Cloning
Pseudomonas aeruginosa	Bioactivity testing
Yersinia pseudotuberculosis 252 01A	Bioactivity testing
Yersinia pestis KUMA	Bioactivity testing
Candida albicans 5314 (ATCC 90028)	Bioactivity testing
Trypanosoma brucei brucei 221	Bioactivity testing
Leishmania major	Bioactivity testing

IX. Oligonucleotides

Oligonucleotide	Sequence 5' – 3'	Annealing (°C)	Reference	Specificity
27f	GAGTTTGATCCTGGCTCA	56	Lane 1991	bacterial 16S rRNA gene (universal)
1492r	TACGGCTACCTTGTTACGACTT	56	Lane 1991	bacterial 16S rRNA gene (universal)
341f	CCTACGGGAGGCAGCAG	59	Muyzer 1993	bacterial 16S rRNA gene (universal)
907r	CCGTCAATTCMTTTGAGTTT	52	Muyzer 1993	bacterial 16S rRNA gene (universal)
SP6	ATTTAGGTGACACTATAG	45	Promega	pGEM-T easy cloning vector
T7	GTAATACGACTCACTATAGGG	45	Promega	pGEM-T easy cloning vector

Supplementary Material

Figure S1. RP LC-MS profile of amphitoxin

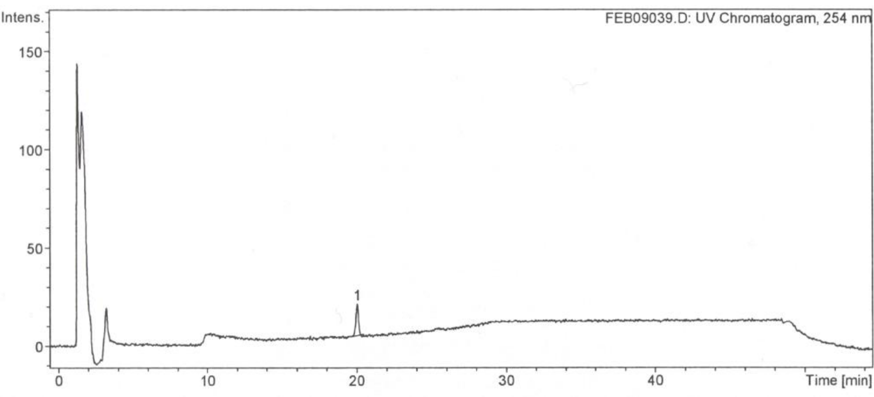


Figure S2. ESI-MS spectrum for amphitoxin

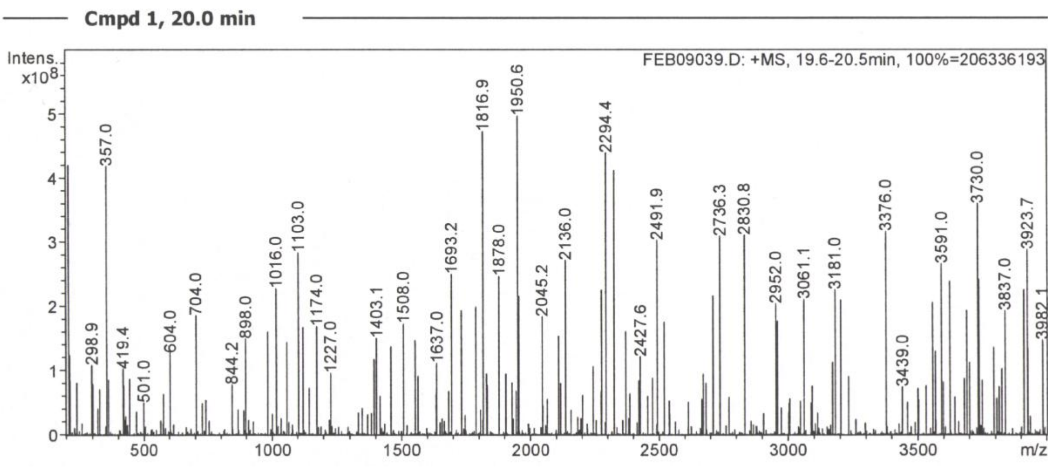


Figure S3. ^{1}H-NMR spectrum for amphitoxin in CD_3OD (400 MHz)

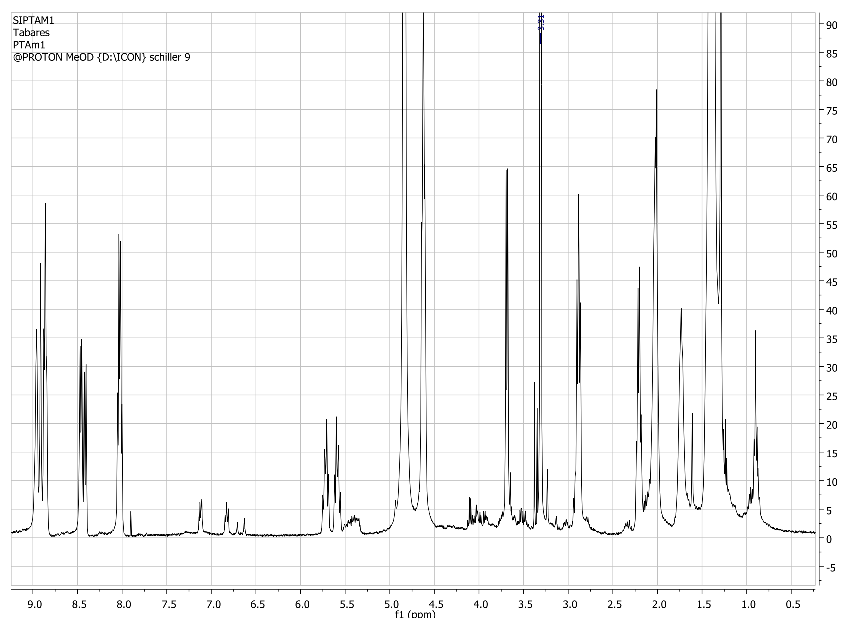


Figure S4. RP LC-MS profile of miraziridine A

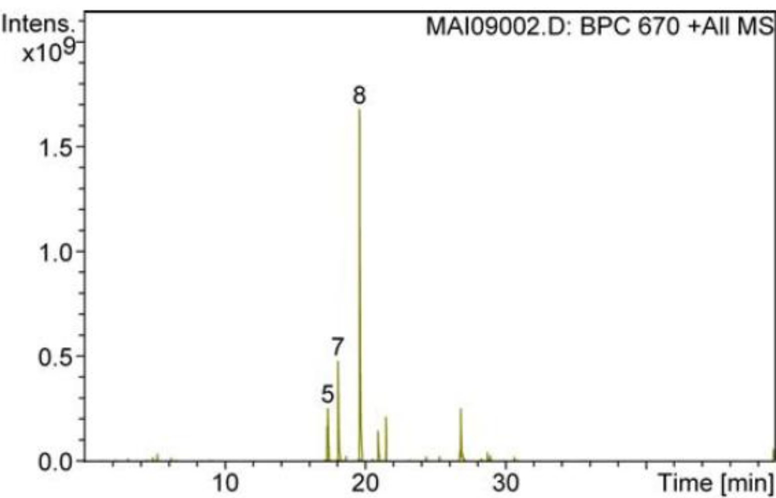


Figure S5. ESI-MS for miraziridine A

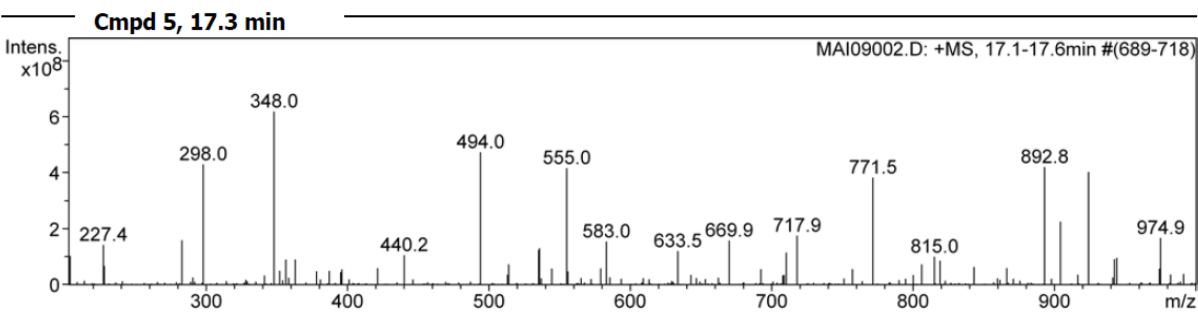
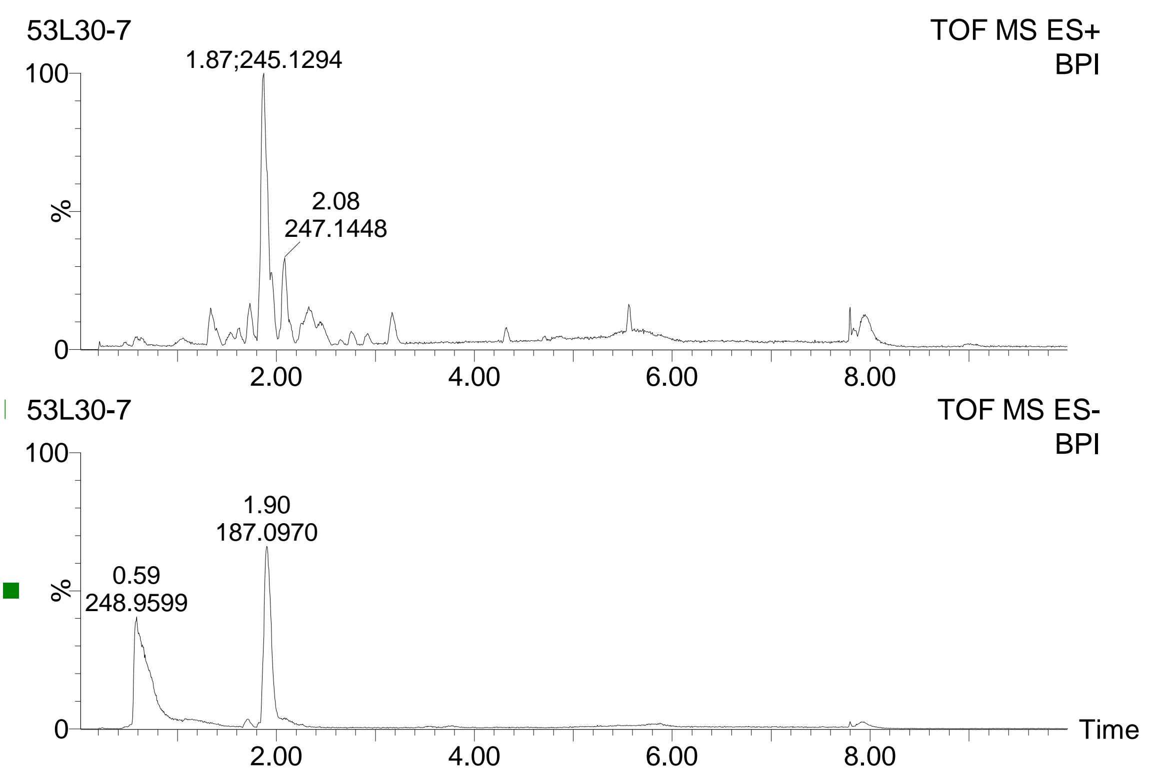
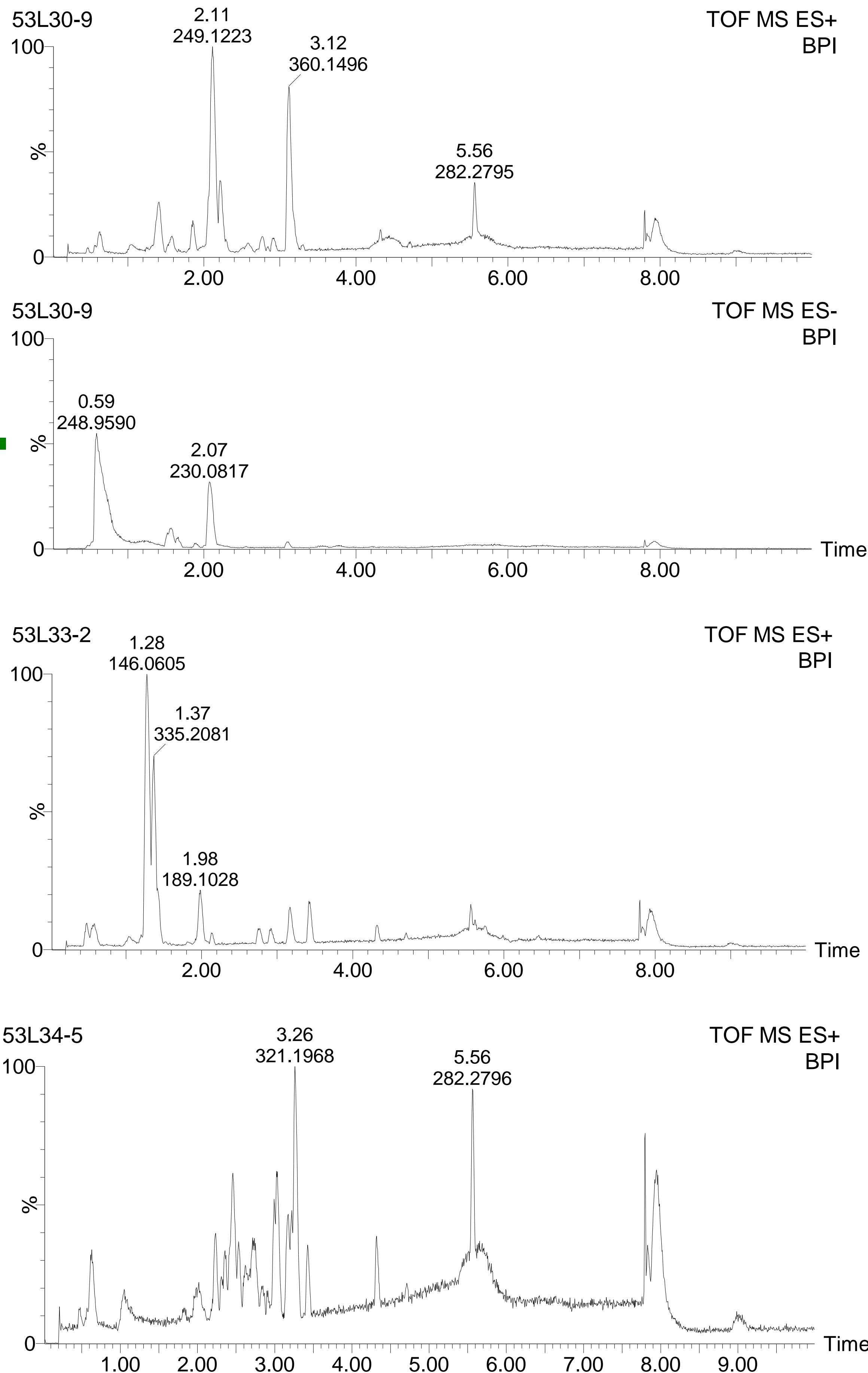
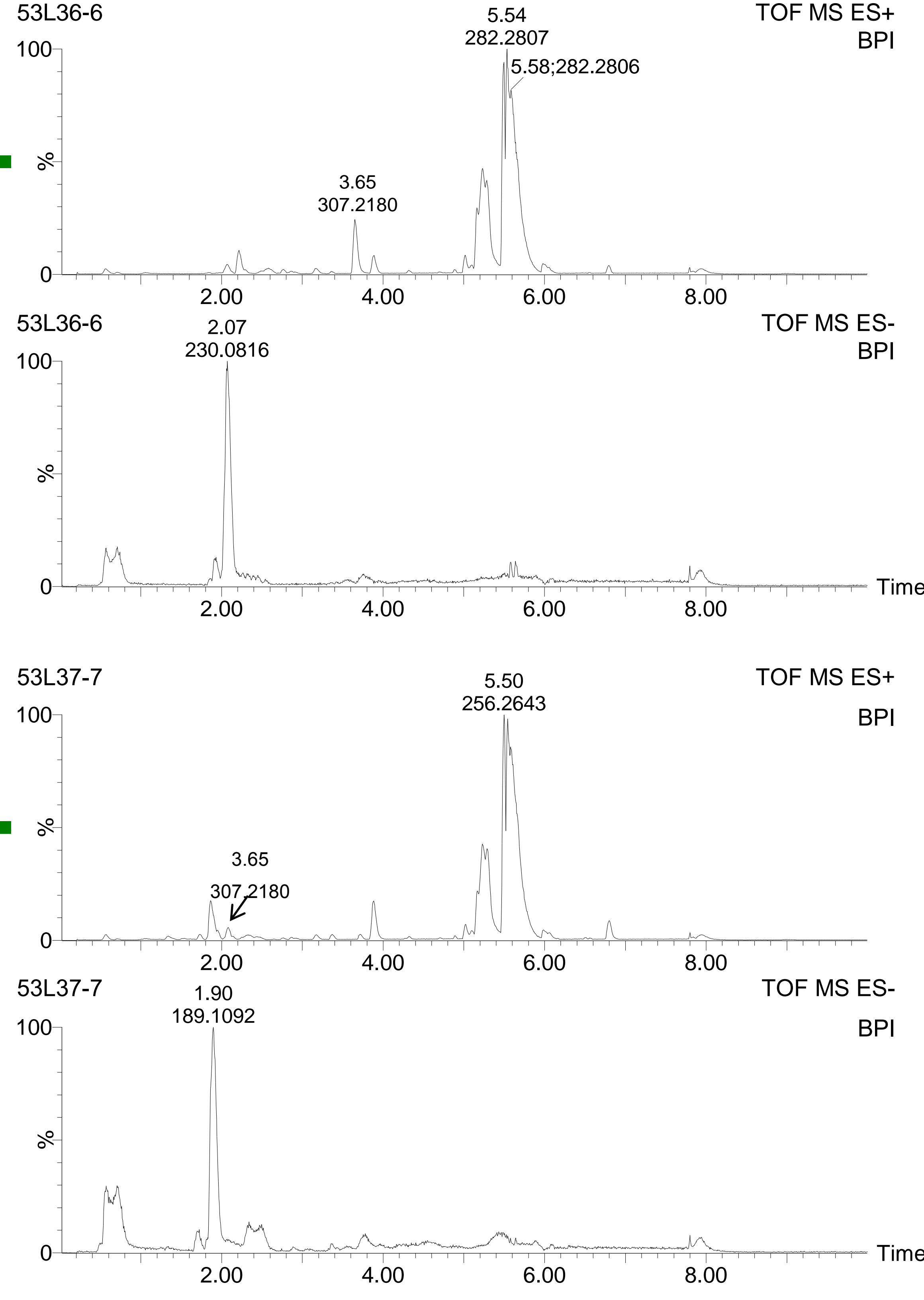


Figure S6. Chromatograms of fractions obtained from the crude extract from liquid culture of strain BA53

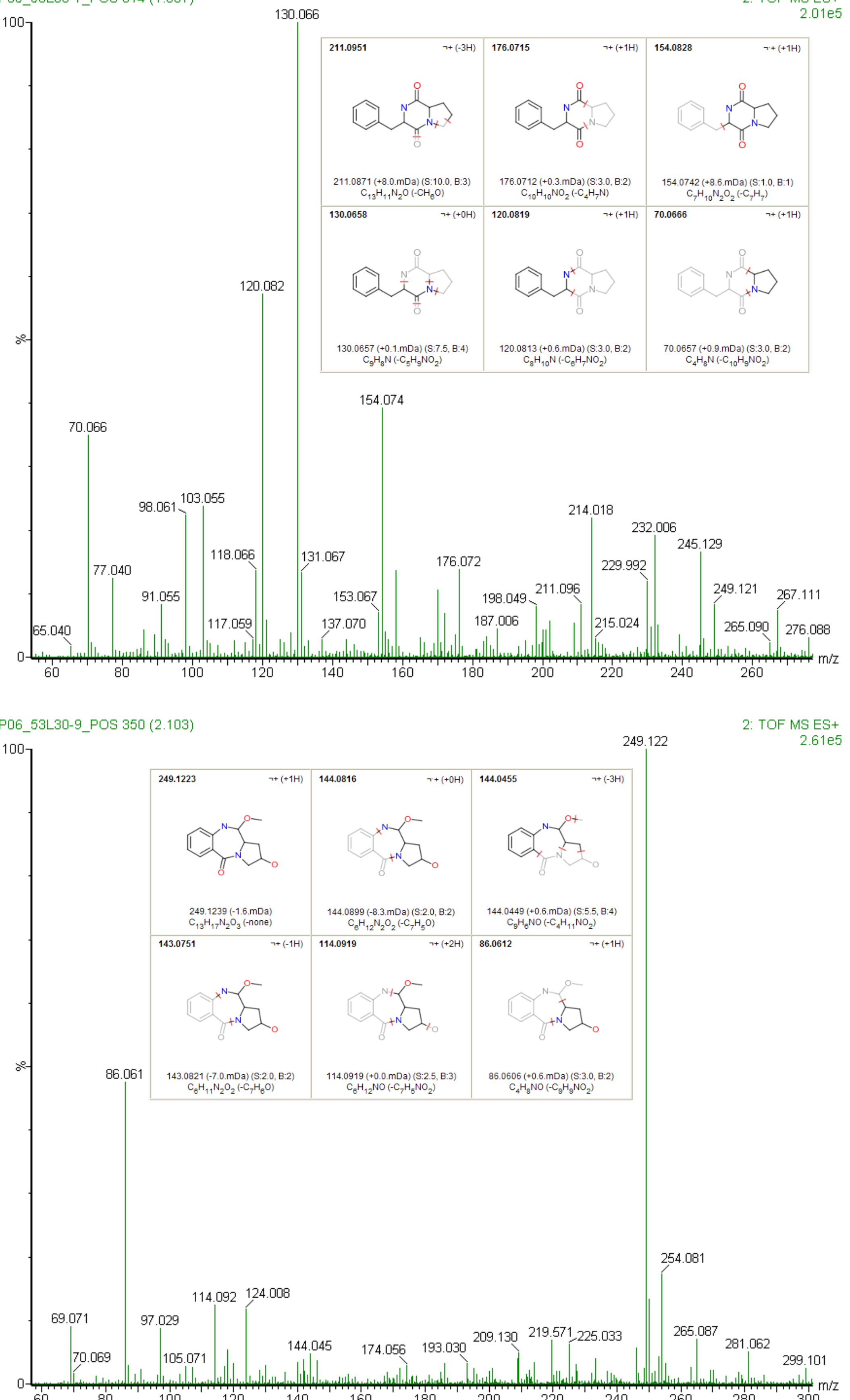


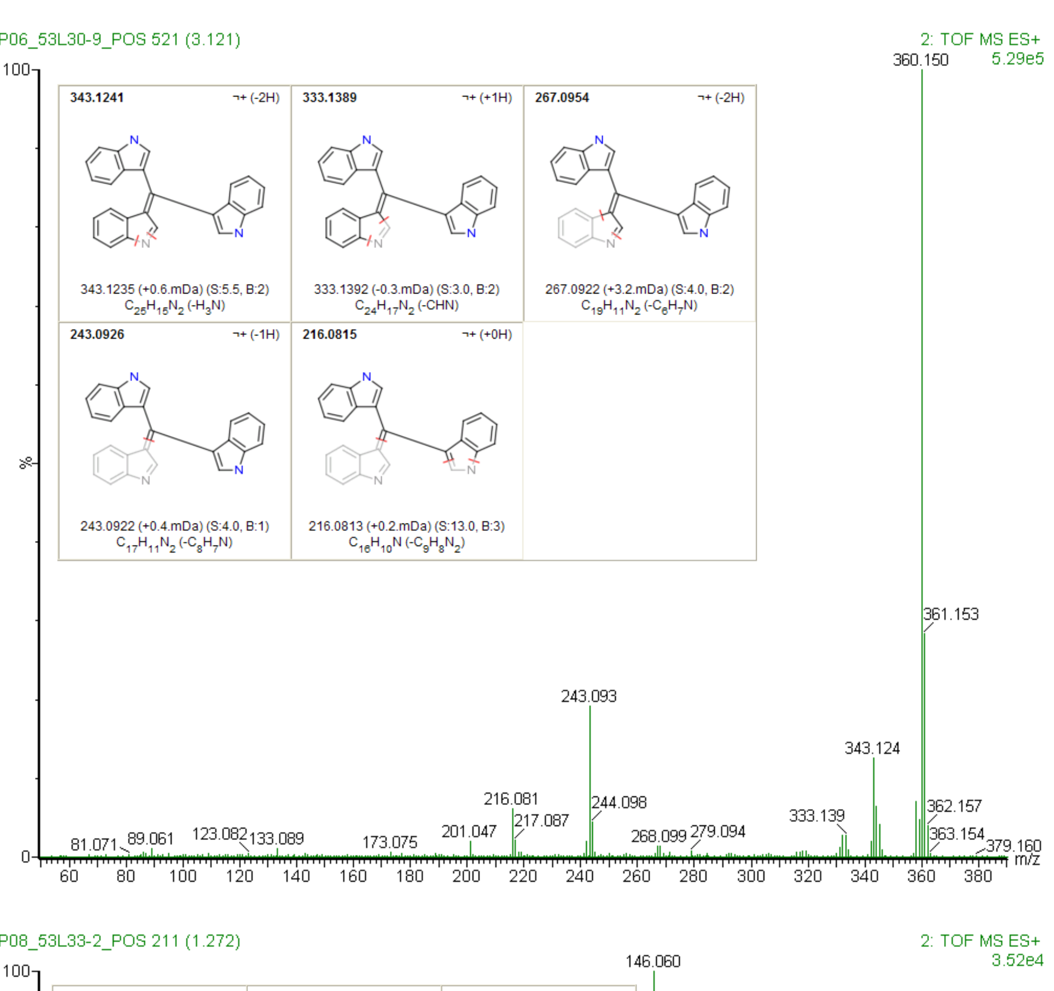

53L30-9
TOF MS ES+
BPI
2.11
249.1223
3.12
360.1496
5.56
282.2795
100
%
0
2.00
4.00
6.00
8.00
53L30-9
TOF MS ES-
BPI
0.59
248.9590
2.07
230.0817
Time
53L33-2
TOF MS ES+
BPI
1.28
146.0605
1.37
335.2081
1.98
189.1028
53L34-5
TOF MS ES+
BPI
3.26
321.1968
5.56
282.2796
1.00
3.00
5.00
7.00
9.00

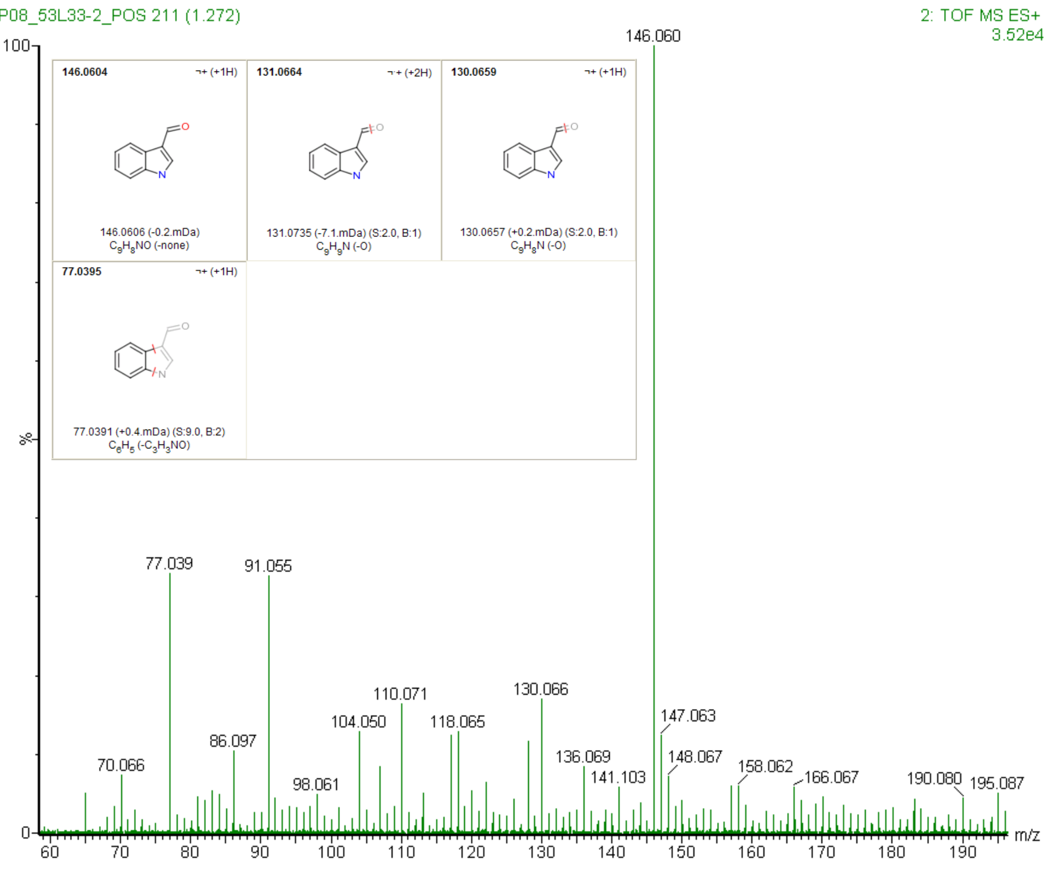


53L36-6
TOF MS ES+
BPI
5.54
282.2807
5.58;282.2806
3.65
307.2180
100
%
0
2.00
4.00
6.00
8.00
53L36-6
TOF MS ES-
BPI
2.07
230.0816
100
%
0
Time
2.00
4.00
6.00
8.00
53L37-7
TOF MS ES+
BPI
5.50
256.2643
3.65
307.2180
100
%
0
2.00
4.00
6.00
8.00
53L37-7
TOF MS ES-
BPI
1.90
189.1092
100
%
0
Time
2.00
4.00
6.00
8.00

Figure S7. Fragment analysis of compounds identified in the fractions obtained from crude extracts from the liquid culture of strain BA53.




P06_53L30-9_POS 521 (3.121)
2: TOF MS ES+
5.29e5
343.1241
333.1389
267.0954
343.1235 (+0.6.mDa) (S:5.5, B:2)
333.1392 (-0.3.mDa) (S:3.0, B:2)
267.0922 (+3.2.mDa) (S:4.0, B:2)
243.0926
216.0815
243.0922 (+0.4.mDa) (S:4.0, B:1)
216.0813 (+0.2.mDa) (S:13.0, B:3)
360.150
361.153
243.093
343.124
216.081
244.098
333.139
362.157
m/z


P08_53L33-2_POS 211 (1.272)
2: TOF MS ES+
3.52e4
146.0604
131.0664
130.0659
146.0606 (-0.2.mDa)
131.0735 (-7.1.mDa) (S:2.0, B:1)
130.0657 (+0.2.mDa) (S:2.0, B:1)
77.0395
77.0391 (+0.4.mDa) (S:9.0, B:2)
146.060
77.039
91.055
130.066
110.071
147.063
m/z

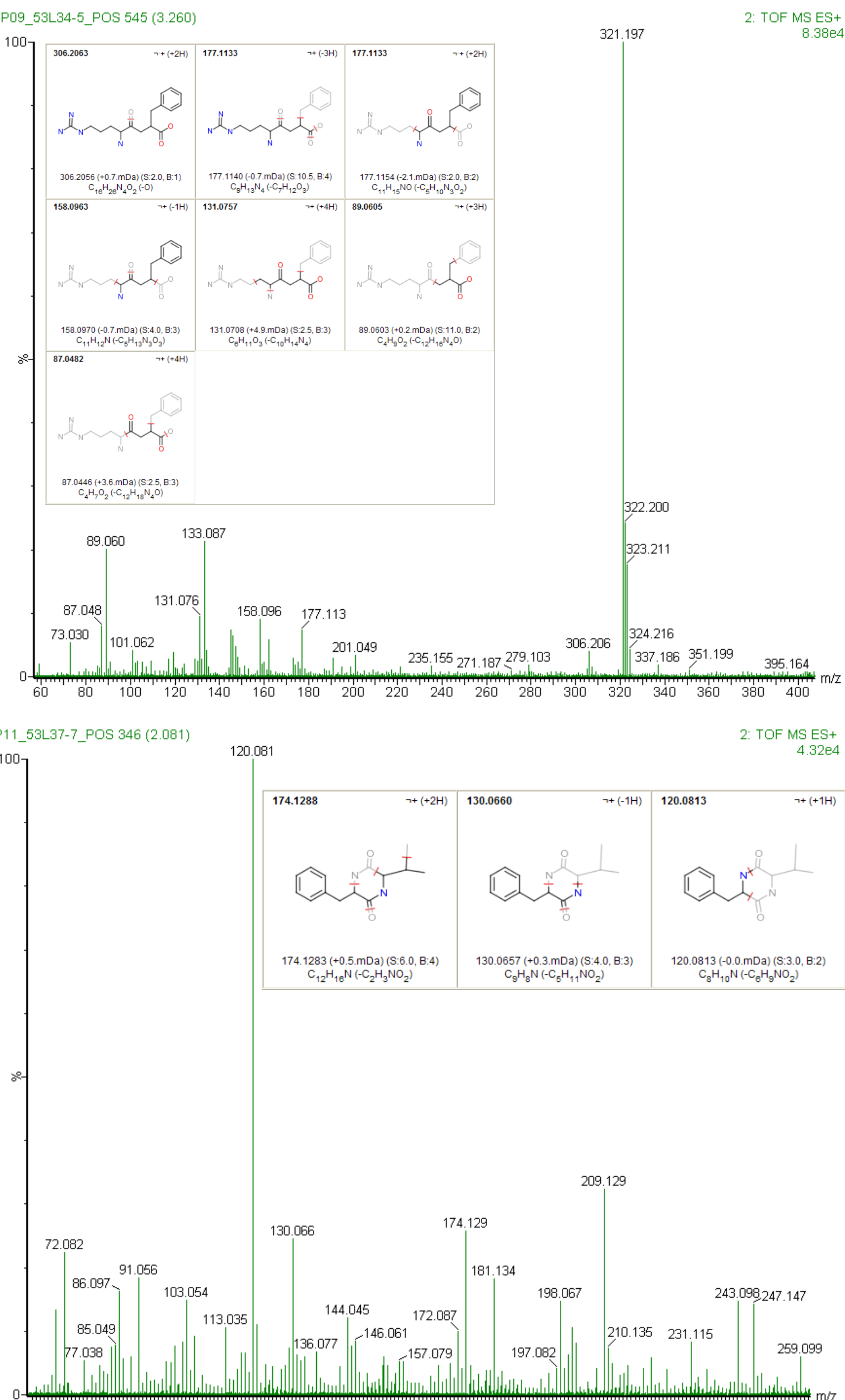

P09_53L34-5_POS 545 (3.260)
2: TOF MS ES+
8.38e4
321.197
306.2063
177.1133
158.0963
131.0757
89.0605
87.0482
306.2056 (+0.7.mDa) (S:2.0, B:1)
177.1140 (-0.7.mDa) (S:10.5, B:4)
177.1154 (-2.1.mDa) (S:2.0, B:2)
158.0970 (-0.7.mDa) (S:4.0, B:3)
131.0708 (+4.9.mDa) (S:2.5, B:3)
89.0603 (+0.2.mDa) (S:11.0, B:2)
87.0446 (+3.6.mDa) (S:2.5, B:3)
322.200
323.211
89.060
133.087
87.048
131.076
158.096
177.113
73.030
101.062
201.049
306.206
324.216
235.155
271.187
279.103
337.186
351.199
395.164
m/z
P11_53L37-7_POS 346 (2.081)
2: TOF MS ES+
4.32e4
120.081
174.1288
130.0660
120.0813
174.1283 (+0.5.mDa) (S:6.0, B:4)
130.0657 (+0.3.mDa) (S:4.0, B:3)
120.0813 (-0.0.mDa) (S:3.0, B:2)
209.129
72.082
130.066
174.129
91.056
181.134
86.097
103.054
198.067
243.098
247.147
144.045
172.087
113.035
85.049
210.135
231.115
146.061
136.077
259.099
77.038
197.082
157.079
m/z

Figure S8. Chromatographic profile for isolumichrome (detection at 254 nm)

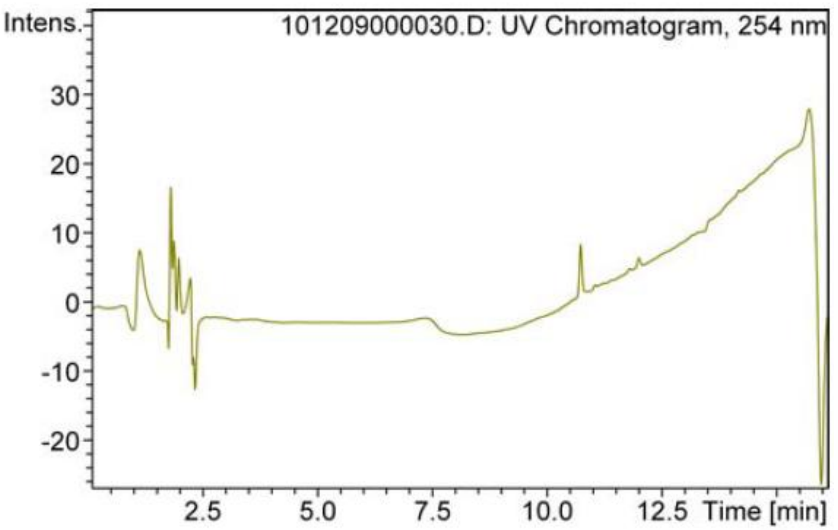


Figure S9. ESI-MS spectrum for isolumichrome.

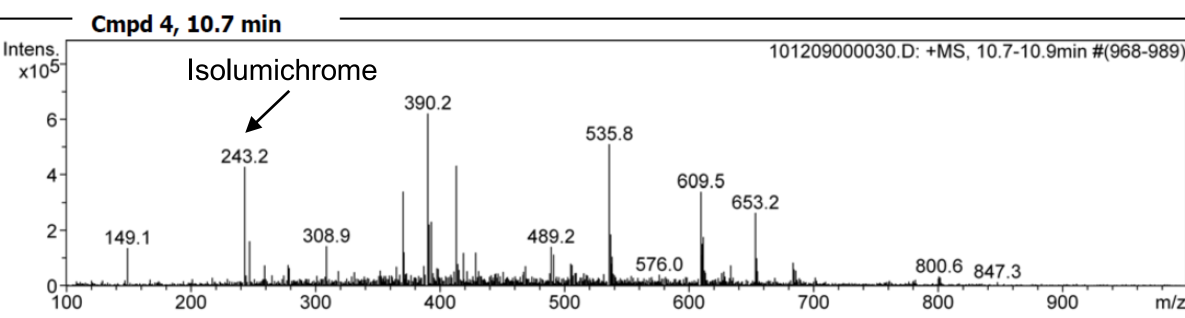


Figure S10. ^{1}H-NMR spectrum for isolumichrome in CD_3OD (600 MHz)

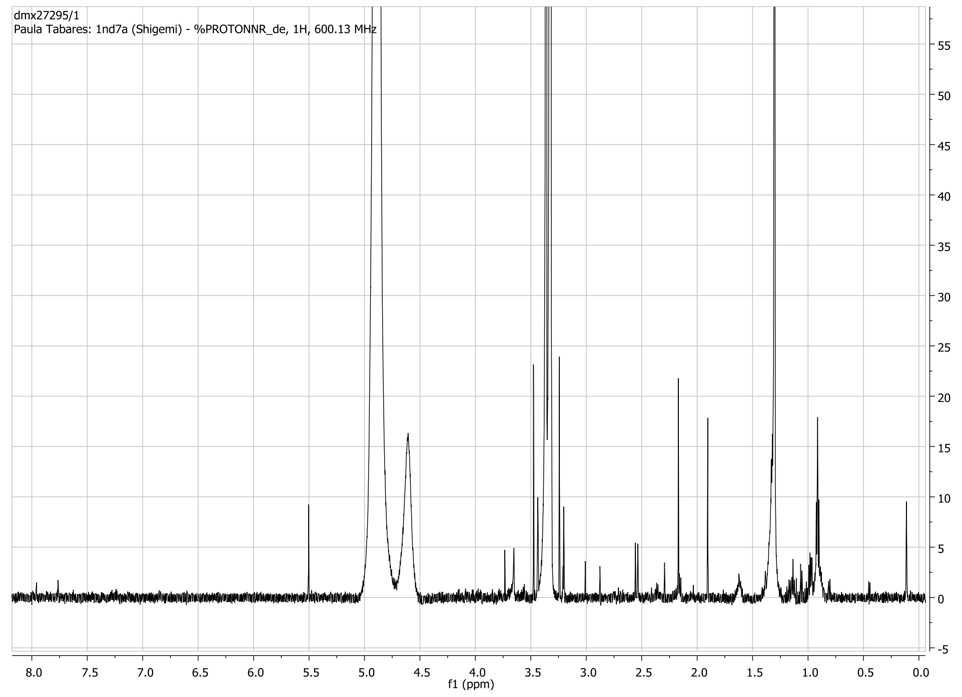
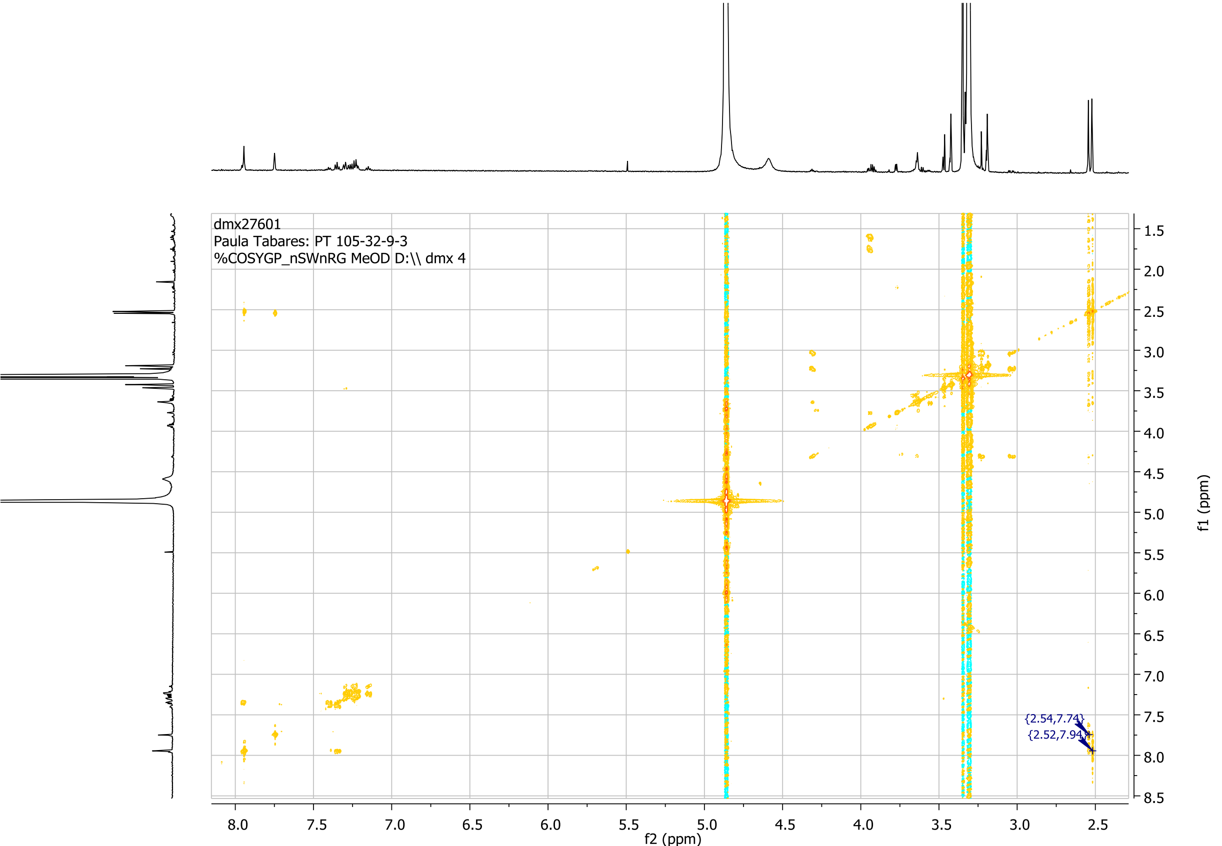


Figure S11. COSY spectrum of isolumichrome in CD_3OD (600 MHz)



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Publications

Publications derived from the PhD

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Publications prior to the PhD

Forero JE, Avila L, Taborda N, Tabares P, López A, Torres F, Quiñones W, Bucio MA, Mora-Pérez Y, Rugeles MT, Joseph-Nathan P & Echeverri F (2008) In vitro anti-influenza screening of several Euphorbiaceae species: Structure of a bioactive cyanoglucoside from *Codiaeum variegatum*. Phytochemistry 69: 2815-2819.

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Curriculum Vitae

Personal Information

Name:	Paula
Last name:	Tabares
Date of birth:	September, 22nd 1978
Place of birth	Pereira, Colombia
Nationality	Colombian
Status	Married

Academic Education

Oct 2007-present	PhD dissertation “Antimicrobial, anti-protease and immunomodulatory activities of secondary metabolites from Caribbean sponges and their associated bacteria”, University of Würzburg, Würzburg, Germany. GSLS advisory team: U. Hentschel, T. Hünig, T. Schirmeister.
Oct 2007-present	Member of the Graduate School of Life Sciences (GSLS), University of Würzburg. Würzburg, Germany.
Oct 2007-Dec 2010	Member of the Graduate College “Immunomodulation” GK 520, University of Würzburg. Würzburg, Germany.
2004	Attainment of the Industrial Chemist Degree, Universidad Tecnológica de Pereira. Pereira, Colombia.
2001	Attainment of the Chemistry Technologist degree, Universidad Tecnológica de Pereira. Pereira, Colombia.

Academic Awards

2007 Oral presentation in the IX Colombian Congress of Phytochemistry, Pereira, Colombia, second place.

Fellowships

2010 One year doctoral fellowship, Frauenbüro "Qualifizierungsprogramms für Wissenschaftlerinnen."

2007 Two years doctoral fellowship, DFG - Graduate College 520 "Immunomodulation."

Teaching Experience

2009-2011 Advisor of the PhD student Swarna Oli, Institute for Pharmacy and Food Chemistry, University of Würzburg, Germany.

Meetings and Symposia

2011 Sixth International GSLS Symposium 2011 - Bio Bang. Contribution: Oral presentation "Anti-protease and immunomodulatory activities of bacteria associated with Caribbean sponges." Würzburg, Germany, Oct. 19th – 20th.

2011 First Sponge Microbiology Symposium, poster presentation "Immunomodulatory, and anti-protease activities of bacteria associated with Caribbean sponges." Würzburg, Germany, March 21st – 22nd.

2010 Summer School of Immunology, poster presentation "immunomodulatory, anti-protease and antimicrobial activities of bacteria associated with Caribbean sponges." Capo Caccia, Sardinia, Italy, May 9th – 16th.

2009 Fourth Network Meeting of the Graduate School of Life Sciences of the University of Würzburg, Erlangen and Tübingen, poster presentation "Anti-protease, anti-infective and immunomodulatory activities of crude extracts from actinomycetes and sphingomonas isolates from Caribbean marine sponges." Kloster Schöntal, Germany, Nov. 15th to 17th.

2009 Second International Symposium of the Collaborative Research Center 630, Novel Agents against Infectious Diseases, poster presentation “Anti-protease and anti-infective activities of crude extracts from Actinobacteria and Sphingomonas isolates from Caribbean marine sponges.” Würzburg, Germany, Oct. 7th to 10th.

2009 Second European Congress of Immunology. Berlin, Germany, September 13th to 16th.

2009 Fourth International Symposium of the Graduate School of Life Science, Evolution - Life and Sciences: A journey through time. Würzburg, Germany, March 26th and 27th.

2008 Joint PhD students meeting of the collaborative research centers 544, 766 and 630, poster presentation “Searching for anti-protease and immunomodulatory compounds from Caribbean sponges and their associated actinomycetes.” Wertheim, Germany, Nov. 20th to 22th.

2008 Summer School organized by BiotecMarin, with the oral presentation “Novel anti-infective and immunomodulatory compounds from Caribbean sponges and their associated actinomycetes.” Rovinj, Croatia, August 18th to 22nd.

2008 Seventh Joint Meeting of AFERP, ASP, GA, PSE and SIF, Natural Products with Pharmaceutical, Neutraceutical, Cosmetic and Agrochemical Interest. Athens, Greece, August 3th to 8th.

2008 Annual retreat of the Graduate Schools for Life Sciences of the University of Würzburg, Erlangen and Tübingen, presenting the poster “Novel anti-infective and immunomodulatory compounds from Caribbean sponges and their associated actinomycetes.” Wildbad Rothenburg, Germany, July 6th to 8th.

2007 International Symposium of the SFB479, “Host-Pathogen Co-Evolution: a Tale of Struggle and Affection.” Würzburg, Germany, Nov. 05th and 6th.

Additional Training and Skills

2011 Project management in the biotech industry; Graduate School of Life Sciences; Würzburg; Germany

2011 Postdoc opportunities in Berlin workshop; Graduate School of Life Sciences; Berlin; Germany

2010 Writing for publication workshop; Graduate School of Life Sciences, GSLS, University of Würzburg.

2010 Poster design and presentation workshop; Graduate School of Life Sciences, GSLS, University of Würzburg.

2008 Catalogue and database research workshop. GSLS, University of Würzburg.

2008 Presenting academic talks workshop. GSLS, University of Würzburg.

2008 Academics English basics workshop. GSLS, University of Würzburg.

2008 Academics English advance workshop. GSLS, University of Würzburg.

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Place, date Signature