Antimicrobial Activities from Plant Cell Cultures and Marine Sponge-Associated Actinomycetes

Antimikrobielle Aktivitäten aus Pflanzenzellkulturen und marinen Schwamm-assoziierten Actinomyceten

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Affidavit

I hereby declare that my thesis entitled “Antimicrobial activities from plant cell cultures and marine sponge-associated actinomycetes” is the result of my own work. I did not receive any help or support from commercial consultants or others. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I verify that this thesis has not been submitted as part of another examination process, neither in identical nor in similar form.

Würzburg, September 2010 Usama Ramadan Abdelmohsen

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Dedication

This dissertation is dedicated to

My Family

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Summary

This thesis is divided into three parts with the main goal allocating novel antimicrobial compounds that could be used as future antibiotics.

The first part aimed to evaluate the potential of plant suspension cultures for the production of antimicrobial proteins. The extracellular, intracellular and cell wall bound fractions of seven heterotrophic and photomixotrophic plant cell suspension cultures treated with nine different elicitors were tested for the elicitor dependent production of antimicrobial proteins. Bioactivities were tested against a selected panel of human isolates including Gram-positive and Gram-negative bacteria as well as fungi using the disc diffusion assay. The intracellular fractions of elicited cell cultures were more active than extracellular fractions while the cell wall bound fractions showed lowest activities. Among the 21 fractions tested, the intracellular fraction of *Lavendula angustifolia* elicited with DC3000 was most active against *Candida maltosa*. The second most active fraction was the intracellular fraction of *Arabidopsis thaliana* elicited with salicylic acid which was moreover active against all test strains. The antimicrobial activity of elicited *Arabidopsis thaliana* cell cultures was tested by bioautography to locate the antimicrobial proteins in the crude extract. The intracellular fraction of photomixotrophic *Arabidopsis thaliana* cells elicited with salicylic acid was selected for further gel filtration chromatography on S-200 column leading to the purification of one 19 kDa antimicrobially active protein, designated, AtAMP. Our findings suggest that elicited plant cell cultures may present a new promising alternative source of antimicrobial proteins.

The second part comprises the isolation of actinomycetes associated with marine sponges and testing the bioactivities of new species for further investigations. Actinobacterial communities of eleven taxonomically different sponges that had been collected from offshore Ras Mohamed (Egypt) and from Rovinj (Croatia) were investigated by a culture-based approach using different standard media for isolation of actinomycetes and media enriched with aqueous sponge extract to target rare and new actinomycete species. Phylogenetic characterization of 52 representative isolates out of 90 based on almost complete sequences of genes encoding 16S rRNA supported their assignment to 18 different actinomycete genera. Altogether 14 putatively new species were identified based on sequence similarity values below 98.2% to other strains in the NCBI database. The use of M1 agar amended with aqueous sponge extract yielded a putative new genus related to *Rubrobacter* which highlighting the need for innovative cultivation protocols. Biological activity testing showed that five isolates were active against Gram-positives only, one isolate was active against *Candida albicans* only and one isolate showed activity against both groups of pathogens.

Moreover, the antiparasistic activity was documented for four isolates. These results showed a high diversity of actinomycetes associated with marine sponges as well as highlighted their potential to produce anti-infective agents.

The third part of the thesis focused on the isolation and structure elucidation of new bioactive compounds. *Streptomyces* strain RV15 recovered from sponge *Dysidea tupha*, was selected for further chemical analysis by virtue of the fact that it exhibited the greatest antimicrobial potential against *Staphylococcus aureus* as well as *Candida albicans* among the all tested strains. Moreover, members of the genus *Streptomyces* are well known as prolific producers of interesting pharmacologically active metabolites. Chemical analysis of the methanolic crude extract using different chromatographic tools yielded four new compounds. The structures of the new compounds were spectroscopically elucidated to be four new cyclic peptides, namely, cyclodysidins A-D. Their bioactivity was tested against different proteases, bacteria and Candida as well as tumor cell lines. The compounds did not show any significant activities at this point.

Zusammenfassung

Die hier vorliegende Dissertation ist in drei Kapitel gegliedert und hatte die Bereitstellung neuer antimikrobieller Substanzen, die zukünftig als Antibiotika genutzt werden könnten, zum Hauptziel.

Das erste Kapitel befasst sich mit dem Potenzial von Pflanzen zur Produktion von Proteinen mit antimikrobieller Wirkung. Pflanzenzellkulturen wurden mit neun verschiedenen Induktoren stimuliert und anschließend auf die Produktion von Proteinen mit antimikrobieller Wirkung hin untersucht. Dafür wurden die extra-, intrazellulären sowie die membrangebundenen Proteinfraktionen von sieben heterotrophen und photomixotrophen Pflanzenzellkulturen extrahiert. Mittels Diffusionstests wurden die Wirkung der Proteine gegen eine Sammlung menschlicher Pathogene inklusive Gram-positiver und Gram-negativer Bakterien, sowie Pilze getestet. Die intrazellulären Fraktionen zeigten dabei höhere Aktivitäten als die extrazellulären, wohingegen die membrangebundenen Proteine die geringsten Aktivitäten aufwiesen. Von den insgesamt 21 getesteten Proteinfraktionen wies die mit DC3000 induzierte intrazelluläre Fraktion von *Lavendula angustifolia* die größte Wirkung gegen *Candida maltosa* auf. Die mit Salicylsäure induzierte intrazelluläre Proteinfraktion von *Arabidopsis thaliana* zeigte eine Hemmung aller getesteten pathogenen Stämme. Die antimikrobielle Aktivität der induzierten *Arabidopsis thaliana*-Zellkultur wurde mittels Bioautography weiter untersucht, um das wirksame Protein im Gesamt-(Roh-) extrakt einzugrenzen. Die intrazelluläre Fraktion der photomixotrophen *Arabidopsis thaliana*-Zellkultur wurde ausgewählt, um ein 19 kDa Protein mit antimikrobieller Wirkung, genannt AtAMP, mittels Gelfitrationschromatography über eine S-200 Säule aufzureinigen. Unsere Ergebnisse weisen darauf hin, dass induzierte Pflanzenzellkulturen zukünftig als aussichtsreiche alternative Quelle für antimikrobiell wirksame Proteine herangezogen werden können.

Der zweite Teil dieser Dissertation beinhaltet die Isolation von mit marinen Schwämmen assoziierten Actinomyceten und deren Testung auf Bioaktivität. Aus 11 taxonomisch verschiedenen, an den Küsten von Ras Mohamed (Ägypten) und Rovinj (Kroatien) gesammelten Schwammspezies, wurden Actinobakterien auf verschiedenen Standardmedien kultiviert. Um seltene, neue Stämme zu isolieren, wurden diese Medien mit wässrigen Schwammextrakten angereichert. Die auf der 16S rRNA-Gensequenz basierenden phylogenetischen Charakterisierung von 52 der insgesamt 90 Isolate, zeigte die Zugehörigkeit zu 18 verschiedenen Actinomyceten-Gattungen. Die 16S rRNA-Gene von 14

Isolaten zeigten Homologien von weniger als 98,2% zu denen anderer in Datenbanken abgelegten Bakterien und stellen somit vermutlich neue Arten dar. Die Verwendung von mit Schwammextrakt angereichertem M1-Agar resultierte in der Kultivierung einer mutmaßlich neuen, mit *Rubrobacter* verwandten Gattung und bestätigt die Notwendigkeit der Entwicklung neuer innovativer Kultivierungsprotokolle. Aktivitätstests von fünf Isolaten zeigten deren hemmende Wirkung nur gegen Gram-positive Bakterien, ein Isolat zeigte Aktivität nur gegen *Candida albicans* und ein Isolat war wirksam gegen beide genannten Pathogengruppen. Desweiteren konnten antiparasitäre Wirkungen von vier Isolaten dokumentiert werden. Die hier beschriebenen Ergebnisse zeigen die große Diversität von mit Schwämmen assoziierten Actinomyceten und deren Potential Antiinfektiva zu produzieren.

Der dritte Teil dieser Arbeit fokussierte sich auf die Isolation und Strukturaufklärung neuer bioaktiver Substanzen. *Streptomyceten* sind bekannt für die Produktion von interessanten, pharmakologisch aktiven Metaboliten. Der aus dem Schwamm *Dysidea tupha* isolierte Stamm *Streptomyces* RV 15 zeigte eine hohe Aktivität gegen *Staphylococcus aureus* und *C. albicans* und wurde deshalb für nähere Untersuchungen ausgewählt. Die chemische Analyse des Methanol-Rohextrakts unter der Verwendung verschiedener Chromatographie-Verfahren resultierte in der Isolation von vier Substanzen. Die spektroskopische Analyse zeigte, dass diese neuen Substanzen zyklische Peptidstrukturen aufweisen und wurden daraufhin als Cyclodysidin A-D benannt. Die Bioaktivitäten dieser Substanzen wurden gegen verschiedene Proteasen, Bakterien und Candida sowie gegen verschiedene Tumorzelllinien getestet. Bis zum jetzigen Zeitpunkt zeigte keine der getesteten Peptide eine aussagekräftige Wirkung.

Chapter 1

General introduction

1.1 Infectious diseases

Infectious disease is a clinically evident illness resulting from the presence of pathogenic viruses, bacteria, fungi, protozoa and parasites. Infectious diseases caused by viruses (human immunodeficiency virus, hepatitis C), parasites (malaria, trypanosomiasis and leishmaniasis) and bacteria (tuberculosis and cholera) are the world's biggest killers of children and young adults (Tibayrenc 2007). Perhaps it is not surprising to expect this crisis in developed countries but what may be remarkable is that mortality rates from infectious disease are actually growing in developing countries (Woolhouse 2008). The continuous misuse of antibiotics has resulted in multi-resistant bacterial strains all over the world such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) which are the most commonly encountered as multiple drug resistant organisms in patients residing in non-hospital healthcare facilities. At the time when few classes of antimycotic drugs are available for treatment of fungal infections, there is an increase in fungal infections (known and emerging) particularly in immunocompromised patients as in patients with AIDS, cancer chemotherapy or organ transplantation. The increased use of antifungal drugs has resulted in the development of resistance to these few available drugs. In addition to the bacterial and fungal resistance, parasitic infections resulted in severe complications and high mortality rates worldwide. Malaria is considered to be the most dangerous parasitic disease causing nearly one million deaths annually, mostly among African children (Zacarias and Andersson 2010). African trypanosomiasis (sleeping sickness), leishmaniasis and Chagas disease are also caused by parasitic protozoa. It is estimated that these three diseases are responsible for more than 110,000 deaths every year (Brun *et al.* 2010; Clayton 2010). The lack of long-term protection by vaccination makes the treatment difficult and frequently accompanied by severe side effects (D'Alessandro 2009). Generally, two major groups of antimicrobial agents were used in the treatment of infectious diseases. The first group include antibiotics which are natural compounds produced by certain groups of microorganisms and the second are the chemotherapeutic agents which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, wherein a compound produced by the microbe is then chemically modified to obtain desired properties or to avoid toxic effects of the parent drug. The first trial of antimicrobial chemotherapy began with the discovery of penicillin by Fleming (1929) and injection into experimental animals, where it was found not only active against infections but also to possess low toxicity for the host. Few years later, Domagk (1935) discovered the synthetic

antimicrobial agents, sulfonamides, with a broad antimicrobial activity spectrum. This discovery directed researchers towards searching for new antimicrobial agents of low toxicity to animals that might be useful in the treatment of infectious disease. The discovery of chloramphenicol, streptomycin, gentamicin, tetracycline and other antibiotics spurred the industry to develop large research and development programs around natural product discovery. The increase in the current problems of growing resistance of numerous pathogens together with the new emerging infectious diseases as well as the toxic effects of some of the currently used drugs necessitates the searching for new anti-infective drugs.

1.2 Drug discovery from nature

Historically, nature was the source for the majority of the drugs in use. Natural products have been successfully used for the treatment of various diseases through many centuries. Natural products are secondary metabolites of organisms such as animals, plants and microbes. Secondary metabolites are not directly involved in the normal growth, development or reproduction of organisms and their absence does not result in immediate death but rather in long-term impairment of the organism's survivability or perhaps in no significant effects at all. They might also be interpreted to be signal molecules or defense mechanisms against competitors, herbivores or pathogens. These characteristics make them lead compounds for development as drugs for oncology and infectious diseases wherein death or injury to populations of cells is desired. Although different exciting methodologies such as genomics, proteomics, combinatorial chemistry, DNA shuffling, combinatorial biosynthesis and bioinformatics have been discovered, screening of natural products is still one of the successful ways to allocate new drugs. Traditionally, dried herbs have been utilized for millennia either by decoction in water to make a tea or by infusion to treat systemic bacterial and fungal infections as well as directly on the skin to treat local infections. Garlic (*Allium sativum*) is a natural antibiotic herb that has been used since the days of the Egyptians and Babylonians to alleviate many infectious diseases. Crushed garlic extract is considered to be the most potent antibacterial agent listed by the British Journal of Biomedical Science (Gonzalez-Fandos *et al.* 1994). Golden Seal (*Hydrastis canadensis*) is a natural antibiotic herb traditionally used by Native Americans either internally or externally to cure mucous membrane, bladder as well as fungal infections. Golden Seal is available as a tincture, nutritional supplement and cream. Pharmaceutical companies developed different pharmaceutical formulations using plant extracts but with the advancement of drug research in the mid-twentieth century, semi-pure forms of compounds have been more in use. Natural products or their semi-synthetic derivatives have been developed for clinical use to treat human diseases in almost all therapeutic areas (Newman and Cragg 2007). Large number of terrestrial and marine natural products was used for treatment of bacterial and fungal

infections as well as various parasitic infections (**Table 1.1**). At least half of the antibiotics and antitumor agents approved by the Food and Drug Administration (FDA) have been natural products, derivatives of natural products or synthetic compounds inspired by natural product chemistry (Zhang 2005).

Table 1.1 Selected natural products with promising antibiotic activities.

Compound name	Source	Class	Activity	Reference
Allicin	*Allium sativum*	Alkaloid	Antibacterial	(Uchida *et al.* 1975)
Aspoquinolones A-D	*Aspergillus nidulans*	Alkaloid	Anticancer	(Scherlach and Hertweck 2006)
Parthenolide	*Tanacetum parthenium*	Sesquiterpene lactone	Antileishmanial	(Tiuman *et al.* 2005)
Berberine Jatrorrhizine	*Tinospora cordifolia*	Alkaloid	Antibacterial	(Chintalwar *et al.* 2003)
Biflorin	*Eremophila neglecta*	Naphthoquinone	Antibacterial	(Ndi *et al.* 2007)
Belamide A	*Symploca* sp.	Tetrapeptide	Anticancer	(SimmonSa *et al.* 2006)
Camalexin	*Arabidopsis thaliana*	Indole	Antifungal	(Sellam *et al.* 2007)
Cyanosporasides A & B	*Salinispora pacifica*	Sordarins	Anticancer	(Oh *et al.* 2006)
GE81112	*Streptomyces* sp.	Tetrapeptide	Antibacterial	(Brandi *et al.* 2006)
Lariatins A & B	*Rhodococcus* sp.	Cyclic peptide	Antibacterial	(Iwatsuki *et al.* 2007)
Manzamine A	*Haliclona* sp.	Alkaloid	Antimalarial	(Ang *et al.* 2001)
Marinomycins A-D	*Marinispora* sp.	Macrolide	Antibacterial Anticancer	(Kwon *et al.* 2006)
Moriniafungin	*Morinia pestalozzioides*	Sordarins	Antifungal	(Basilio *et al.* 2006)
Morin	*Pisidium guajava*	Flavonoid	Antibacterial	(Arima and Danno 2002)
Platensimycin	*Streptomyces platensis*	Ketolide	Antibacterial	(Wang *et al.* 2006)
Plectasin	*Pseudoplectania nigrella*	Linear peptide	Antibacterial	(Mygind *et al.* 2005)
Phomallenic acids A-C	*Phoma* sp.	Acetylenic allene	Antibacterial	(Ondeyka *et al.* 2006)
Rosmarinic acid	*Ocimum basilicum*	Polyphenolic	Antifungal	(Bais *et al.* 2002)
Bruceine A	*Brucea javanica*	Quassinoid	Antitrypanosomal	(Bawm *et al.* 2008)
Scopoletin	*Nicotiana tabacum*	Coumarin	Antifungal Antibacterial	(Goy *et al.* 1993)

Sorbicillactones A & B	*Penicillium chrysogenum*	Sorbicillinoid	Anticancer	(Bringmann *et al.* 2005)
Stellettazole B	*Stelletta* sp.	Alkaloid	Antibacterial	(Matsunaga *et al.* 1999)
Spongistatin	*Hyrtios erecta*	Macrocyclic lactone	Antifungal	(Ovechkina *et al.* 1999)
α-tomatine	*Lycopersicon esculentum*	Saponin	Antifungal	(Ito *et al.* 2007)
Topsentiasterol sulfates	*Topsentia* sp.	Sterol	Antibacterial Antifungal	(Fusetani *et al.* 1994)
Taxol, baccatin III	*Taxus baccata*	Terpenoid	Anticancer	(Cusido *et al.* 1999)
Withaferin A	*Withania somnifera*	Hydroquinone	Antibacterial	(Ciddi 2010)

1.2.1 Drug discovery from terrestrial plants

Several options are available for the production of pharmaceutically active natural products including natural harvesting, total chemical synthesis, semisynthesis from natural precursors and expression of biosynthesis pathways in microbial systems (Bourgaud *et al.* 2001). The first source for natural products are the terrestrial organisms. Plants have always been the main source of medicinal ingredients, providing a wide range of valuable biologically active substances. Among the 250,000 flowering plants in the world (Thorne 2000), more than 50,000 have been used for medicinal purposes (Schippmann *et al.* 2002). Plant cell cultures have been used as alternative biotechnological source for production of numerous commercially important compounds such as medicinal compounds, flavors, fragrances and colorants (Kolewe *et al.* 2008). This technology provided continuous and reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be easily extracted and purified (Mulabagal and Tsay 2004; Georgiev *et al.* 2009). In some cases due to the complexity of the chemical nature of molecule and/or the lack of knowledge about its complete biosynthetic pathway, plant cell culture technology has been successful alternative to complex molecules such as taxol (Paclitaxel). Paclitaxel is an anticancer drug against various types of cancer including breast, ovarian and lung cancer. Initially, it was obtained by extraction from the bark of *Taxus* sp. and currently derived mainly by biotechnological production through Phyton Biotech (USA) to supply Bristol-Myers Squibb paclitaxel for its taxol formulation (Zhong 2002; Tabata 2006; Frense 2007). The protoberberine alkaloids, berberine and jatrorrhizine isolated from *Tinospora* species are of considerable value due to their antimicrobial, anti-inflammatory, antileukemic as well as antimalarial activities (Kuo *et al.* 2004; Yu *et al.* 2005). They are currently produced by means of cell cultures though Mitsui Petrochemical Industries (Japan).

1.2.2 Drug discovery from marine animals

The second important source for natural products discovery is the marine environment. Whereas the terrestrial environment has been screened for sources of new drugs for thousands of years, until recently, the marine environment was to a large extent not accessible for such investigations. Ocean covers two-thirds of the earth and have remained an unexplored valuable source of novel natural pharmaceutical compounds (Molinski *et al.* 2009). Marine natural products are often very different in their chemical nature from their terrestrial counterparts. Therefore, marine-derived pharmaceuticals in many cases provide completely novel pharmacological modes of actions. Marine natural products played a crucial role in biomedical research and drug development, either directly as drugs or as lead structures for chemical drug synthesis. Dozens of marine-derived natural products are in clinical or pre-clinical trials for treating various diseases. Ziconotide is a synthetic form of ω-conotoxin MVIIA which has been isolated from the venom of marine snail *Conus magus*. It became the first marine-derived drug approved by the FDA under the trade name Prialt for the management of severe and chronic pain caused by various diseases (Bowersox and Luther 1998; Terlau and Olivera 2004). The anticancer cyclic tridecapeptide*,* Kahalalide F has been isolated from Sacoglossan mollusk *Elysia rufescens* as well as the green alga *Bryopsis* sp. and is currently in Phase II clinical trials against solid tumors including melanoma and hepatocellular carcinoma (Hamann and Scheuer 1993; Goetz *et al.* 1999). (+)-Discodermolide has been isolated from the deep-water sponge *Discodermia dissoluta* and showed immunosuppressant activity and *in vivo* activity against HCT-116 colorectal cancer and is in Phase I clinical trials (Hung *et al.* 1994; Honore *et al.* 2004). Bryostatin is a macrocylic lactone that has been purified from the bryozoan *Bugula neritina* (Berkow *et al.* 1993; Mutter and Wills 2000). It demonstrated potent *in vitro* activity against the P388 lymphocytic leukaemia cell line with an ED50 of 0.89 µg/ml and is currently in phase II. Although few representatives from sponges are approved as drugs, hundreds of new compounds with interesting pharmacological activities are discovered from sponges every year. Several sponge-derived compounds are already in clinical trials as agents against cancer, microbial infections, inflammation and other diseases. However, in many cases drug development is severely hampered by the limited supply of the respective compounds, as they are often present only in minute amounts in the sponge tissue.

1.2.3 Drug discovery from marine microorganisms

Microorganisms are the source of various medicaments for bacterial and fungal infections (e.g., penicillin, erythromycin, streptomycin, tetracycline, vancomycin, amphotericin B), cancer (e.g., daunorubicin, doxorubicin, mitomycin, bleomycin), transplant rejection (e.g.,

cyclosporin, FK-506, rapamycin) and high cholesterol (e.g., lovastatin and mevastatin). Marine bacteria have recently taken the limelight as potential sources of novel chemical structures with promising pharmacological activities that could be used as drug leads. Among marine bacteria, actinomycetes are prolific producers of clinically interesting compounds with various biological activities (Fenical and Jensen 2006; Solanki *et al.* 2008; Olano *et al.* 2009; Asolkar *et al.* 2010; Kekuda *et al.* 2010; Orhan *et al.* 2010). Examples of the recently discovered drugs from actinomycetes are the marinomycins A–D which are a novel class of polyketides that have been discovered from the marine actinomycete *Marinispora* with significant anti-bacterial activities against Gram-positive strains including the drug-resistant bacterial pathogens MRSA and VRE. They also exhibited potent and highly selective activity in the National Cancer Institute's (NCI) 60 tumor cell line panel with six of the eight melanoma cell lines, especially SK-MEL-5, being very sensitive to marinomycin A (Kwon *et al.* 2006). Salinosporamide A is another example that has been isolated from the new genus *Salinispora* (Feling et al. 2003). Salinosporamide A has completed Phase I clinical trials for multiple myeloma under the sponsorship of Nereus Pharmaceuticals in La Jolla, California (Fenical *et al.* 2009).

1.3 Problems in natural products research

Despite the medical and commercial success of natural products as drugs, the investment in the discovery and the commercialization of new natural products has significantly decreased since it reached its peak in the mid to late twentieth century. They are some problems behind this decline such as the decrease in the discovery of new classes of natural compounds with interesting new activities and the reisolation of known natural products. New strategies are required for natural products-based drug discovery that might increase chemical diversity and reduce redundancy. The output can be increased by spending more time to select the appropriate strains and extracts. In order to focus on the extracts of most interest as quickly as possible and to avoid repeatedly isolating known compounds, efficient dereplication process is required. Dereplication strategies can be done at early stages using analytical techniques and database searching either at the level of the microorganism or the chemical extracts. Each culture collection is likely to have a high number of duplicate strains. Thus, it is important to dereplicate the culture collection at the beginning taking in consideration that strains of the same subspecies may produce different compounds. By the aid of molecular methods such as restriction fragment length polymorphism (RFLP), morphogenic groups can be separated further to differentiate strains up to the subspecies level (Vermis *et al.* 2002).

The most effective selection method from the metabolic aspect is the chemical profile analysis using high throughput tools such as high-performance liquid chromatography

coupled with detectors such as photodiode array detection (HPLC-DAD) or evaporative light scattering detection (HPLC-ELSD) or by the aid of classical methods such as thin-layer chromatography (TLC) as well. Identical HPLC retention time or TLC Rf values are not enough to decide if two compounds are exactly the same or not but different values definitely indicate that they are different. If the extract contains a reported compound which is commercially available, the sample and the authentic material should be co-chromatographed for TLC and HPLC. If a reference standard is not available, MS data is enough to decide if the compounds are the same or not. A powerful and very sensitive method in identification of new natural products in the crude extracts is to couple the mass spectrometry (MS) to liquid chromatography (LC). Comparison of the extract chromatography to databases such as Antibase database or the Dictionary of Natural Compounds as well as published literature will provide valuable information to determine whether an extract and/or activity is novel or not. The coupling of high performance liquid chromatography with nuclear magnetic resonance spectroscopy (LC-NMR) is one of the recent and powerful methods for the separation and identification of known and unknown compounds in crude extracts (Bobzin *et al.* 2000; Provera *et al.* 2010). Although LC-NMR has lower sensitivity than LC-MS, it is very useful in the chemical screening and dereplication of crude extracts especially if the data from LC-MS are incomplete or do not allow confident identification of the active component of an extract.

The second reason behind the decline is the difficulty to have reliable sources to get significant quantities of relatively pure compounds for chemical and biological studies as well as for commercial production after finishing the clinical phases. Since the last two decades there have been considerable efforts made in the use of plant cell cultures in bioproduction, biotransformation and biosynthetic studies. Different classes of natural products such as alkaloids, flavonoids, terpenes, steroids, glycosides and proteins have been produced using plant-cell-culture technology (Smetanska 2008). The production of secondary products from the plant cell culture is less expensive than extraction of the whole plant grown under natural conditions and also less expensive than the chemical synthesis of the product of interest. Plant cell cultures have the potential for commercial production of pharmaceuticals if appropriate cell lines with suitable genetic, biochemical, and physiological characteristics have been selected. Plants and plant cells *in vitro* are capable to show physiological and morphological responses to microbial, physical and chemical factors which are known as elicitors. Thus, the use of various biotic and abiotic elicitors could be useful strategy to stimulate secondary metabolites production in plant cell cultures. An example of a high-value drug that is commercially produced by plant cell cultures is the paclitaxel which is an anti-cancer drug originally extracted from the bark of 50-year-old Pacific yew trees, *Taxus brevifolia* (Tabata 2006). The other reliable source for production of natural products at

commercial level is the microbes. Although not a new technology, microbial fermentation continues to evolve and is now the preferred production method for large number of therapeutics especially the antibiotics, offering an optimal economic route which allows pharmaceutical companies to shorten production processes and time to market.

The third reason behind the decrease in development of natural products is the time and expense for the production of compounds for clinical and commercial use. For example, paclitaxel, took over 20 years from structural determination and reporting until FDA approval. These reasons have moved the pharmaceutical drug discovery programs away from natural products in favor of synthetic approaches. However, the abundance of synthetic compounds with similar chemical functional groups and, therefore, limited chemical diversity has renewed interest in nature as a good resource for finding new fascinating leads to be applied to design the next generation of drugs.

1.4 Aims

The increase in the current problems of resistance of numerous pathogens together with the new emerging infectious diseases as well as the adverse effects of some of the currently used drugs necessitate the searching for new anti-infective drugs. Natural products have provided considerable value to drug market over the past half century. In particular, the therapeutic areas of infectious diseases and oncology have benefited from various drug classes derived from natural product sources. The main goal of this Ph.D. study is to allocate novel anti-infective compounds from terrestrial sources represented by plant cell cultures and marine sources represented by sponge-associated actinomycetes.

The first aim of the study (Chapter 2) was to evaluate the potential of heterotrophic and photomixotrophic plant suspension cultures for the production of antimicrobial proteins and/or peptides against a selected panel which includes human Gram-positive and Gram-negative bacterial pathogens as well as human Candida. This was followed by purification and characterization of an antimicrobial protein from an active fraction.

The second aim of the study (Chapter 3) was to isolate the bioactive novel actinomycete species associated with marine sponges collected from Egypt and Croatia by cultivation. Strains were identified based on 16S rRNA gene sequence analysis and their bioactivity was tested against a panel of clinically relevant, Gram-positive and Gram-negative bacterial pathogens. Moreover, human fungal pathogen as well as human parasites was also tested.

The third aim of the study (Chapter 4) was the isolation, identification, and structural elucidation of new and preferably biologically active secondary metabolites from marine sponge-associated actinomycetes. Bioactive and taxonomically novel strains were fermented at optimized conditions and the crude extracts were subjected to various chromatographic methods in order to obtain pure compounds. The compounds were then identified using different spectroscopic tools and the biological activity was evaluated for the pure compounds.

General introduction about the infectious diseases and natural product research was presented in Chapter 1.

Finally, a general discussion of new antimicrobials from plant cell cultures and marine sponge-associated actinomycetes as well as future perspectives was provided in Chapter 5.

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Chapter 2
Synthesis of distinctly different sets of antimicrobial activities by elicited plant cell suspension cultures

2.1 Introduction

2.1.1 Plant cell culture

Plant cell cultures have emerged as an important alternative source to whole plants in many fields of research (Karuppusamy 2009; Ratner 2010). Unlike field-grown plants, plant cell lines are cultivated independently from climate, soil, season, day length and weather conditions. Furthermore, there is no risk of contamination with mycotoxins, herbicides or pesticides. Perhaps the most striking advantage of secondary metabolite production via plant cell lines versus whole plants is the simpler procedure for isolation and purification of compounds, especially when the metabolites are secreted into the culture medium (Caretto *et al.* 2010). Among various approaches for in vitro cultivation of plant cells, research efforts have been focused on suspension cell cultures as they are the most amenable to GMP procedures and as they can be easily cultivated in large scale for industrial and/or pharmaceutical purposes (Hellwig *et al.* 2004; Shinde *et al.* 2010). It has already been shown that plant cell cultures possess antimicrobial activities against a wide spectrum of bacteria and fungi (Sokmen *et al.* 1999). Furthermore, many pharmacologically active and commercially interesting secondary metabolites such as berberine, codeine, diosgenin, ginsenosides, morphine, scopolamine, taxol, vinblastine and vincristine have been produced by plant cell cultures (Vanisree *et al.* 2004; Bøezinová *et al.* 2007; Hernandez-Vazquez *et al.* 2010). Moreover, in vitro cell cultures yielded multimeric proteins as well as glycoproteins (Marden *et al.* 1997) and this further underscores their significance.

Elicitors are compounds which are capable of stimulating any type of plant defense such as the induction of phytoalexin biosynthesis (Zhao *et al.* 2010). The treatment with elicitors has gained special interest in many biotechnological fields to enhance the production of secondary metabolites. So far, elicitors have been categorized according to their nature into two distinct groups: abiotic elicitors (light intensity, temperature and chemicals) (Chong *et al.* 2005; van Hulten *et al.* 2006) and biotic elicitors (bacteria, fungi and insects or their derivatives) (Solano *et al.* 2008; Ramos-Solano *et al.* 2010). The use of biotic elicitors as compared to abiotic elicitors for the enhanced production of metabolites from plant cell cultures (Zhao *et al.* 2010) is promising and could have important outcome for industry. Upon elicitation of plant cells, one or more signal transduction pathways are invoked by ligand

receptor interactions that lead to the activation of a set of defense-related genes (Nurnberger 1999; Abramovitch *et al.* 2006; Boller and He 2009). In cell culture systems, low elicitor concentrations and short incubation times are sufficient to provoke cellular reactions because there are no cuticles or thick walls which could hinder elicitor perception (Roitsch and Sinha 2002; Basu and Goyal 2009). Treatment of plant cells with elicitors such as chitosan, methyljasmonate and salicylic acid induces substantial modulations directed at establishing plant defense reactions and in many cases, resulted in enhanced production of secondary metabolites (Namdeo 2007; Baldi *et al.* 2009; Ciddi 2010). The diversity of biochemical pathways responding to elicitor treatment may reflect a switch from primary metabolism to primed secondary metabolism leading to the production of defense proteins and compounds (Chivasa *et al.* 2006; Allwood *et al.* 2010).

2.1.2 Antimicrobial proteins from plants

Plants have developed specific and nonspecific mechanisms of defense that can provide long-term protection against a wide array of pathogens. There are at least two main mechanisms of pathogen resistance in plants, the first being represented by the compounds synthesized during the plant normal development (constitutive resistance) and the second mechanism being activated only after contact with the pathogen (induced resistance) (Dangl and Jones 2001; Ahuja *et al.* 2010). The induced resistance can occur in a localized or systemic way. The localized resistance can only be detected immediately at host-pathogen interface, followed by a fast collapse and cell death, a reaction named hypersensitivity response (Lam *et al.* 2001). Systemic acquired resistance (SAR) is induced by prior challenge with a pathogen or the application of an elicitor (Jones and Dangl 2006; Gilbert and Parker 2010). Once activated, the SAR pathway can confer resistance to a number of pathogens. SAR has been studied extensively in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Kurusu et al. 2010). It involves the fast production of reactive oxygen species (ROS) which have a direct antimicrobial effect (Yoshioka *et al.* 2009), alterations in the cell wall constitution leading to reinforcement of cell walls (Lake and Wade 2009), increase of endogenously synthesized salicylic acid and the enhanced production of pathogenesis-related (PR) proteins (Alvarez 2000; Jha *et al.* 2009), activation and synthesis of defense peptides and proteins (Castro and Fontes 2005; Gomi 2009) and the induced accumulation of antimicrobial low molecular weight secondary metabolites known as phytoalexins (Gilbert and Parker 2010). Plants produce over 100,000 small-molecules (Dixon 2001; Lewis and Ausubel 2006), peptides or proteins with potentially inhibitory effects on the growth and development of other organisms (Fritig *et al.* 1998; Park *et al.* 2010).

The widespread resistance of microbes to commonly used antibiotics has increased interest in alternatives such as bacterial interference therapy (probiotics), bacteriophage therapy and in particular, antimicrobial peptides (Bolton *et al.* 2008; Coutinho *et al.* 2008; Labrie *et al.* 2010). Antimicrobial peptides offer the advantages of selective inhibition and low risk of resistance development (Pereira 2006). In recent years, many constitutive and inducible antimicrobial peptides have been characterized in animals, insects and plants and these have been recognized as important components of the innate defense system (Reddy *et al.* 2004; Kido *et al.* 2010). Several antibacterial and antifungal proteins have been isolated from plants (**Table 2.1**) and most of them were derived from seeds (Flores *et al.* 2002; Wang and Ng 2007; Hammami *et al.* 2009b; Pelegrini *et al.* 2009; Park *et al.* 2010). Among them are enzymes such as glucanases (Leelasuphakul *et al.* 2006) and chitinases (Ye and Ng 2005), thaumatin-like proteins (Liu *et al.* 2010) as well as several kinds of basic cysteine-rich peptides and other less-known low molecular weight proteins.

Table 2.1 Antimicrobial proteins from plants.

Antimicrobial protein	Source	Class	Activity	Reference
An 1	*Atriplex nummularia* seeds	Thionin	Antibacterial	(Last and Llewellyn 1997)
AP1	*Solanum tuberosum* leaves	Defensins	Antifungal	(Feng et al. 2003)
DmAMP1	*Dahlia merckii* seeds	Defensins	Antifungal	(Thevissen et al. 2003)
FaAMP1 Fa AMP2	*Fagopyrum esculentum Moench* seeds	Defensins	Antibacterial Antifungal	(Fujimura et al. 2003)
TuAMP 1 TuAMP 2	*Tulipa gesneriana* bulbs	Thionin- like	Antibacterial Antifungal	(Fujimura et al. 2004)
WJAMP1	*Wasabia japonica* leaves	Havein-like protein	Antibacterial Antifungal	(Kiba et al. 2003)
LJAMP1	*Leonurus japonicas seeds*	Napin-like storage protein	Antifungal	(Yang *et al.* 2007)
Pr 1	*pumpkin rind*	Thaumatin-like protein	Antifungal	(Park *et al.* 2010)
HaAP 10	*helianthus annus* seeds	lipid-transfer proteins	Antifungal	(Regente and de la Canal 2000)
PaAMP1	*Phytolacca americana* seeds	Cystein-rich	Antibacterial Antifungal	(Liu *et al.* 2000)

The main groups of antimicrobial peptides that found in plants are thionins, defensins and lipid transfer proteins (Broekaert *et al.* 1995; Carvalho *et al.* 2006; Franco *et al.* 2006; Jha and Chattoo 2010). A database named PhytAMP (http://phytamp.pfba-lab-tun.org) has been established for the different classes of antimicrobial peptides from plants (Hammami *et al.* 2009a). These peptides differentially inhibit a wide variety of phytopathogenic bacteria and fungi. Various plant antimicrobial peptides such a DmAMP1 from *Dahlia merckii* seeds, HsAFP1 from *Heuchera sanguinea* seeds and RsAFP2 from *Raphanus sativus* seeds showed potent activity against the human pathogen *Candida albicans* at micromolar concentrations (Portieles *et al.* 2006). This highlights the potential of plant antimicrobial peptides as new candidates for drug development.

Thionins represent a family of low molecular weight (5 kDa) basic peptides (pI >8) which are rich in basic and sulfur-containing residues such as arginine, lysine and cysteine. Their diverse members have high sequential and structural similarities besides presenting potent inhibitory effects against wide range of bacteria and fungi. They were isolated from members of the *Viscaceae* family such as phoratoxins C-F from *Phoradendron tomentosum* which exhibited highly selective cytotoxicity to human breast cancer cells (Johansson *et al.* 2003). Another toxic protein was isolated from *Pyrularia pubera* seeds (Vernon *et al.* 1985). The *Pyrularia* thionin represents four disulfide bridges like cereal thionins and shares eight consecutive amino acid residues identical to purothionins. Susceptibility against thionins was demonstrated in phytopathogenic bacteria such as *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Corynebacterium* as well as in phytopathogenic fungi such as *Thielaviopsis paradoxa* and *Drechslera teres*. Cytotoxic effects on numerous cell lines such as mouse fibroblasts L929, HeLa cells and monkey cells CV10 have been reported (Castro and Fontes 2005). It was also observed that viscotoxins exert strong immunomodulatory effects (Tabiasco *et al.* 2002). Thionins inhibit protein synthesis through direct interaction with mRNA or at initial translation level (Brummer *et al.* 1994). Different thionins were found in seeds such as viscotoxins, purothonins and hordothionins which could also work as storage proteins, especially as sources of sulphur (Schrader-Fischer and Apel 1993). Some members of thionins such as α-hordothionin were already used to confer resistance to transgenic plants against phytopathogenic fungi. The expression of α-hordothionin gene under control of CaMV35S promoter led to increase the resistance against *Pseudomonas syringae* (Carmona et al. 1993). Another example is viscotoxin expression in *Arabidopsis thaliana* conferring resistance to *Plamodiophora brassicae* (Holtorf *et al.* 1998).

Defensins are cystein-rich peptides with a characteristic antiparallel *β* sheet folded infrastructure. *α* and *β* defensins share similar 3D structures but differ in the pairing of

cysteines in the disulfide bonds (Zasloff 2002). Plant defensins are quite diverse in their amino acid composition with only eight structure stabilizing cysteines appear to be conserved among all plant defensins (Allen *et al.* 2008). They are small (45–54 amino acids), highly basic cysteine-rich peptides that are apparently ubiquitous throughout the plant kingdom. They have been isolated from different plant species such as wheat, barley, sorghum, radishes and other species of *Brassicaceae* (Montesinos 2007). Sequences of more than 80 different plant defensin genes from different plant species are available and at least 13 putative plant defensin genes are only derived from *Arabidopsis thaliana* (Thomma et al. 2002). Many plant defensins showed a potent fungicidal action and occasional antibacterial activity (Segura *et al.* 1998). Interestingly, they are nontoxic to either mammalian or plant cells, indicating their role in the defense process (Thevissen *et al.* 1999). The exact mode of action of plant defensins is yet unclear, and most molecular components and targets involved in signaling remain unknown. There are different theories describing the mode of antimicrobial activity of plant defensins. One postulates the formation of multimeric pores within microbial membranes. After initial electrostatic binding of these positively charged peptides to negatively charged phospholipids on the target cell surface of the microbe, they enter into the excited cell membrane and most likely form ion-permeable channels in a voltage-dependent way (Wimley *et al.* 1994; Maget-Dana and Ptak 1997). The plant defensins DmAMP1 and RsAFP2 are examples of potent antifungal proteins exhibiting their action against *Fusarium culmorum* via induction of an array of relatively rapid responses in fungal cells accompanied with increased K^+ efflux and Ca^{2+} uptake leading to changes in membrane potential and membrane-permeabilization (Thevissen *et al.* 1999). Defensins were heterologously expressed in diverse hosts such as bacteria, yeasts, fungi and plants (Padovan *et al.* 2010). The constitutive expression of a novel alfalfa defensin gene in potato provides high levels of field resistance against *Dahliae*, the causal agent of the “early dying disease” of potato (Gao *et al.* 2000). Interestingly, some members within the plant defensin family do not exhibit antimicrobial activity but display other biological activities such as proteinase (Wijaya *et al.* 2000) and α-amylase (Zhang *et al.* 1997) inhibitory activities, which may also contribute to their role in plant defense.

Lipid transfer proteins (LTPs) facilitate movement of lipid from its biosynthesis site, especially the endoplasmic reticulum to other organelles like chloroplasts and mitochondria in membranes formation (Kader 1996). Several LTPs were purified from various plant species (Castro *et al.* 2003; del Carmen Ramirez-Medeles *et al.* 2003). The plant LTPs are divided into two subfamilies with relative molecular masses of 9 KDa (LTP1s) and 7 KDa (LTP2s). The plant LTPs consist mainly of α-helix segments (40% of secondary structures total) connected by disulfide bridges (Samuel *et al.* 2002). Various functions have been attributed

to this family of proteins such as its participation in cuticle formation and embryogenesis (Sterk *et al.* 1991), establishing symbiotic relationships (Krause *et al.* 1994), plant adaptation to various environmental conditions, like temperature, humidity and salinity (Torres-Schumann *et al.* 1992) and finally in the defense against pathogens (Castro and Fontes 2005). According to Garcia-Olmedo (1995), plant LTPs should be considered as important peptides involved in plant defense against pathogens, as they present adequate inhibitory activities, suitable distribution and concentration in plant tissues and a rapid increase in their gene expression levels soon after infection. Molina (1997) has shown that Barley LTP2 expression in tobacco and *Arabidopsis* transgenic plants promoted a steep reduction of necrotic effects resulted from *Pseudomonas* infection, confirming the involvement of LTPS in plant defense mechanisms against microbial pathogens. Ge and collaborators (2003) studied the antifungal and antibacterial properties of LTP110, a lipid transfer protein isolated from rice. After cloning and expression, the protein was purified and tested *in vitro* against rice pathogens, *Pyricularia oryzae* and *Xanthomonas oryzae*. LTP110 was able to inhibit the germination of *P. oryzae* spores with slight effect on the growth of *X. oryzae*.

2.1.3 Aim of the work

The aim of this chapter was to evaluate the potential of heterotrophic and photomixotrophic plant suspension cultures for the production of antimicrobial proteins and/or peptides against a selected panel which includes human Gram-positive and Gram-negative bacterial pathogens as well as human Candida. This was followed by purification and characterization of an antimicrobial protein from an active fraction.

2.2 Materials and Methods

2.2.1 Maintenance of plant cell cultures

Seven plant cell cultures were used in the study, six of them heterotrophic (*Agrostis tenuis*, *Baphicanthus cusi*, *Daucus carota*, *Lavendula angustifolia*, *Nicotiana tabacum petit havanna* and *Rauvolfia verticillata*) and one being a photomixotrophic (*Arabidopsis thaliana*). Cell suspension cultures were maintained in 300 ml Erlenmeyer flasks on a rotary shaker (130 rpm) in the light at 26 ºC (heterotrophic) or 22 ºC (photomixotrophic). Photomixotrophic cultures were grown on B5 medium (Gamborg *et al.* 1968) supplemented with 20 g sucrose and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) while the heterotrophic ones were grown on LS medium (Linsmaier and Skoog 1964) supplemented with 30 g sucrose and 10 µM 2,4-D naphthalene acetic acid (NAA). The pH of the media was adjusted to 5.8 before autoclaving. Cells were sub-cultured every 7 d (heterotrophic and photomixotrophic) using a 1:3 dilution of inoculum to fresh medium.

2.2.2 Treatment with elicitors

Cell cultures were elicited after 2 days incubation and elicitors were prepared as previously described (de Sa *et al.* 1992; Bourgaud *et al.* 1999; Frankfater *et al.* 2009; Roat and Ramawat 2009). Fungal elicitors were prepared by boiling a suspension of *Alternaria brassiciola* conidia (for Alt) with spore concentration of 5 x 10^5 spores/ml or 0.1% (w/v) mycelia of the fungus *Fusarium oxysporium lycopersici* (for Fol) for 10 min at 100 ^oC and stored at -20 ^oC. Bacterial elicitors were prepared by boiling a suspension of the virulent strain of *Pseudomonas syringae* (for DC3000) or avirulent strain of *Pseudomonas syringae* (for RPM1), each of OD_{600} = 0.2 for 10 min at 100 ^oC and stored at -20 ^oC. Acetylsalicylic acid (ASA) and salicylic acid (SA) were prepared by dissolving in H_2O_d and pH was adjusted to 7.2 with 5 M NaOH then microfiltration. Chitosan was prepared by dissolving chitosan in 1% acetic acid containing 0.1% sodium nitrite, neutralization with 1 M NaOH and dialyzing for 18 h against H_2O_d. Methyljasmonate (MeJ) was prepared by dissolving in 50% methanol. Polygalacturonic acid (PGA) was prepared by dissolving polygalacturonic acid in 10 mM sterile NaOH and dialyzed for 18 h at 4 ^oC against H_2O_d. The final concentrations of elicitors were listed in **Table 2.2**. For control treatments, only solvent was added to the cultures. All cultures were collected after a 2 days incubation period in the presence of elicitor.

Table 2.2 Final concentrations of elicitors in cell cultures.

Elicitor	Nature	Final Conc.
Alt	Fungal preparation (*A. brassicicola*)	100 µg/ml
Acetylsalicylic acid	Analogue to salicylic acid	1 mM
Chitosan	Microbial cell wall preparation	0.01%
DC3000	Bacterial preparation (virulent strain of *P. syringae*)	100 µg/ml
Fol	Fungal preparation (*F. oxysporium lycopersici*)	100 µg/ml
Methyljasmonate	Plant hormone	50 µM
Polygalacturonic acid	Plant cell wall preparation	0.01%
RPM1	Bacterial preparation (avirulent strain of *P. syringae*)	100 µg/ml
Salicylic acid	Plant hormone	1 mM

2.2.3 Preparation of fractions for bioactivity testing

The elicited plant cell cultures were collected by filtration through two layers of cheesecloth and the supernatant was collected as fraction A. Cells were immediately frozen in liquid nitrogen. Five grams of cells were ground under liquid nitrogen and powder was suspended in 15 ml of extraction buffer A (15 mM Na_2HPO_4, 15 mM NaH_2PO_4, 2 mM PMSF and 1 mM benzamidine hydrochloride, pH = 7) and stirred for 3 h at 4 ^oC. After centrifugation at 5000 rpm for 10 min, the supernatant was collected as fraction B. The pellet was resuspended in 15 ml extraction buffer B (15 mM Na_2HPO_4, 15 mM NaH_2PO_4, 1 M NaCl, 15 mM EDTA, 2 mM PMSF and 1 mM benzamidine hydrochloride, pH = 7) and stirred overnight at 4 ^oC. After centrifugation at 5000 rpm for 10 min, the pellet was discarded and the supernatant was collected as fraction C. The fractions were centrifuged at 16.000 rpm for 20 min and the pellets were dissolved in 500 µl buffer A to be used as extracellular (fraction A), intracellular (fraction B) and cell wall bound fractions (fraction C), respectively. The fractions were dialyzed using dialysis tube of cut off = 14 KDa, overnight against distilled water with 3x change of water during dialysis. The dialyzed fractions were stored at 4 ^oC for bioactivity testing.

2.2.4 Antimicrobial activity screening of extracellular, intracellular and cell wall bound fractions

The *in vitro* antimicrobial activity testing was performed using the standard disc diffusion assay (Inderlied and Salfinger 1995) against panel of human isolates included bacteria (*Bacillus subtilis* ATCC 6059, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus ATCC 6538*) and yeast (*Candida maltosa* SBUG 700). 100 µl of the overnight cultures (bacteria in LB medium and yeast in YPD medium) with an optical density of OD_{600} = 0.2 was spread on 100 mm plates containing solid LB or YPD agar medium. Sterile filter paper discs with a diameter of 6 mm were impregnated with testing solution (20 µl/disc), dried (3 times impregnation and drying) and placed on the agar plates inoculated with the target microorganism. The plates were inverted and incubated for 24 h at 37 ^oC (for bacteria) or 30 ^oC (for yeast) and the activity was quantitatively assessed by inhibition zone diameter (in mm) measurement. The result is an average of two readings (2 discs used for each sample) and each experiment was repeated 3 times. Chloramphenicol 25 µg/ml (as antibacterial) and nystatin 25 µg/ml (as antifungal) were used as positive controls and buffer A was used as a negative control.

2.2.5 Purification of antimicrobial proteins from *Arabidopsis thaliana* cell culture

Thirty grams of collected *A. thaliana* cells elicited with salicylic acid were ground under liquid nitrogen and powder was suspended in 60 ml of extraction buffer A (15 mM Na_2HPO_4, 15 mM NaH_2PO_4, 2 mM PMSF and 1 mM benzamidine hydrochloride, pH = 7) then stirred overnight at 4 ^oC. After centrifugation at 5000 rpm for 10 min, the pellet was discarded, the supernatant was centrifuged at 16.000 rpm for 20 min and the pellet (crude protein extract) was redissolved in 3 ml of 8 M urea for gel filtration. Crude protein extract was loaded onto a HiPrep Sephacryl S-200 HR column (Amersham Biosciences) connected to a peristaltic pump (Amersham Pharmacia Biotech) and previously equilibrated with 50 mM phosphate buffer for 30 min (2 ml/min). Elution was carried out using a gradient of 0-1 M NaCl in 50 mM phosphate buffer and about 100 fractions (each 1.5 ml) were collected at room temperature (RT) using a flow rate 1 ml/min. The peak fractions were analyzed for protein content using a UV spectrophotometer (Kontron Uvikon 860, Pharmacia) as well as for antimicrobial activity using the disc diffusion assay. The active fractions were pooled and concentrated by freeze drying. The combined freeze dried fractions were dissolved in 3 ml of 8 M urea and reloaded on Sephacryl S-200 column for further purification as previously described. The purity of different active fractions was tested using 12% SDS-PAGE and gels were silver stained. All purification steps were performed at RT.

2.2.6 Protein analysis

Protein concentrations in extracellular, intracellular and cell wall bound fractions were estimated according to Bradford (1976) using bovine serum albumin (BSA) as a standard. One hundred microliters of sample was mixed with 900 µl of Bradford reagent and incubated at RT for 10 min. The OD was measured at 578 nm and standard was made with 0-10 µg of BSA.

2.2.7 SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out according to Laemmli (1970). The acrylamide gel cast was set up by laying two spacers sandwiched between two rectangular glass plates. A separating gel (12%) was pipetted into the space. Upon polymerization of the separating gel, a comb was inserted in the sandwich plates. The stacking gel (5%) was filled on top of the separating gel and allowed to solidify. Protein samples were mixed with sample buffer (10:1) and were boiled at 95 ^{o}C for 5 min. The sample was loaded into the gel along with the unstained protein molecular weight standard (Fermentas). In the presence of electrophoresis running buffer, electrophoresis was performed with constant current 35 mA until the blue dye front reached the bottom.

2.2.8 Gel staining

For Coomassie Blue staining, the gels were immersed in 100 ml of Coomassie Blue staining solution (Merril 1990) overnight with continuous shaking. After removal of the remaining of staining solution, the gels were destained using destaining solution with changing of the solution until clearance of protein bands. Silver staining was performed according to Merril (1990) by fixation of the gel in 50% methanol supplied with 1% acetic acid for 20 min, washing in methanol for 10 min then in water for 10 min. After incubation with 150 ml 0.02% sodium thiosulfate for 1 min, the gels were rinsed with water 3 times. The gels were then submerged in 150 ml silver nitrate solution (0.1%) containing 0.08% formaldehyde for 20 min at 4 ^{o}C. After rinsing with water, developing was stopped using 5% acetic acid and final rinsing with water for 5 min. The gels were dried at 65 ^{o}C under vacuum.

2.2.9 Bioautography

Bioautography was performed according to the modified method by Vigers *et al* (1991). After SDS-PAGE, the gel was incubated with shaking in 100 ml of 1% (v/v) Triton X-100 for 20 min at room temperature and washed with water for 10 min then incubated in 100 ml of 20% LB broth medium with gentle shaking for 30 min at RT. The gel was placed in a 100 mm-

diameter Petri dish and 20 ml of slightly warm 20% LB medium containing 1.5% (w/v) agar inoculated with a final concentration of 4-6 x 10^5 per milliliter of bacterial or fungal cells was poured over the gel and allowed to solidify. The plate was incubated overnight at 30 ^oC (fungi) or 37 ^oC (bacteria) and the location of antimicrobial activity on the gel was detected as a clear band of growth inhibition against a background of microbial growth. For molecular weight determination of the targeted protein(s), an unstained protein molecular weight marker was run in one lane of the gel, and in a second lane, 50 µl of sample was run simultaneously. After electrophoresis, these two lanes were cut and visualized using silver staining. By comparing the stained part of the gel with the incubated gel, the molecular weight of the desired protein(s) which was responsible for the antimicrobial activity could be estimated.

2.3 Results and Discussion

2.3.1 Antimicrobial activities from plant cell cultures

In this Ph.D. study, heterotrophic and photomixotrophic plant cell cultures were investigated with respect to the induction of antimicrobial proteins upon challenge by different elicitors. Heterotrophic and photomixotrophic cultures showed a higher antimicrobial potential than the photoautotrophic cultures (**Table 2.3**) which have been studied by Ali (2007). Generally, intracellular fractions of elicited cell cultures were more active than extracellular fractions while the cell wall bound fractions showed low activities (**I-III**, **Annex**). Among the 21 fractions tested, the intracellular fraction of *L. angustifolia* elicited with DC3000 was most active against *C. maltosa*. The second active fraction was the intracellular fraction of *A. thaliana* elicited with salicylic acid which was active against all test strains with all stimuli with the highest activity in response to SA. *N. tabacum petit havanna* showed broad spectrum activity against all test pathogens upon elicitation with different elicitors but the activity was not as strong as *A. thaliana*. *R. verticillata* exhibited only antifungal activities with all elicitors. *D. carota* was active only against Gram-negative bacteria, while all elicitors except chitosan stimulated *B. cusi* to produce activity against Gram-positive bacteria. *A. tenuis* showed activities against both Gram-negative bacteria and *C. maltose* in response to all elicitors except MeJ which did not produce any detectable activity. The controls of the three fractions of the all cultures were performed simultaneously replacing the elicitor with sterile water and showed nearly no activity against all tester isolates and also the protein content for all fractions was low.

Table 2.3 Antimicrobial activity results of elicited plant cell cultures.

Cell line	Gram-positive bacteria	Gram-negative bacteria	Fungi
Agrostis tenuis	-	+	+
Arabidopsis thaliana	+	+	+
Baphicanthus cusi	+	-	-
Chenopodium rubrum	-	-	-
Daucus carota	-	+	-
Eschscholtzia californica (AST)	+	-	-
Eschscholtzia californica (ECO)	+	-	-
Gypsophila arabia	+	+	+
Lavendula angustifolia	-	-	+

Lycopersicon esculentum (autotrophic)	-	-	-
Lycopersicon esculentum (heterotrophic)	-	+	+
Nicotiana tabacum (BY2)	+	+	+
Nicotiana pulmbaginifolia	+	+	+
Nicotiana tabacum petit havanna	+	+	+
Petroselium crispum	+	-	
Rauvolfia verticillata	-	-	+

+: Active, -: Non active

Nine preparations of elicitors under three different categories and with diverse chemical natures were used. The first type of elicitors represents different fungal components and includes chitosan (polysaccharide formed by the action of plant enzymes on microbial cell walls), fungal spores such as Alt elicitor (fungal spore suspension of boiled spores of *Alternaria brassicicola*) and suspension of the whole fungus such as Fol elicitor (mycelial suspension of *Fusarium oxysporium lycopersici*). The second type of elicitors represents different bacterial components which includes DC3000 (boiled bacterial suspension of virulent strain of *Pseudomonas syringae*) and RPM1 (boiled bacterial suspension of avirulent strain of *Pseudomonas syringae*). The last type of elicitors represents different plant components including plant response-signaling compounds such as methyljasmonate, salicylic acid or their synthetic analogues such as acetylsalicylic acid (analogue to salicylic acid) and plant preparations such as polygalacturonic acid. Elicitation by the DC3000 and salicylic acid resulted in pronounced increases in the antimicrobial potential of heterotrophic and mixotrophic cell cultures. The bacterial (RPM1, DC3000) and fungal (Alt, Fol) elicitors were more effective than other elicitors of non microbial origin with respect to antimicrobial activity induction. The responses of the cell cultures to MeJ, PGA and chitosan were rather weak. Several studies have been done regarding the effect of elicitors on the induction of defense secondary metabolism and showed that application of elicitors to plants or cultured plant tissues resulted in increase the formation of secondary metabolites in addition to defense-related proteins (Zulak *et al.* 2009). This highlights the use of elicitors to induce plant defense reactions and production of antimicrobially active compounds that have not been isolated from untreated cells.

The antimicrobial activities of elicited *A. thaliana* cell cultures were selected for further characterization using bioautography by virtue of the fact that this culture exhibited broad

spectrum antimicrobial activity and it is the only plant with a sequenced genome in our screening analysis. The active fraction was detected as a clear inhibition zone on the gel after SDS-PAGE (**Figure 2.1**). The molecular weight of antimicrobial protein(s) was estimated to be around 19 KDa. We thus confirmed that the antimicrobial activity of an extract from an elicited *A. thaliana* cell culture is due to an antimicrobial protein(s). These results highlight the efficiency of bioautography as a technique for screening antimicrobial agents in complex matrices and mixtures.

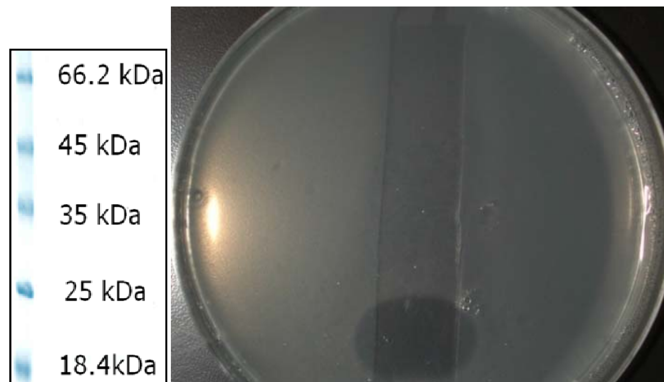

Figure 2.1 Bioautography of the intracellular fraction of *A. thaliana* cell culture elicited with salicylic acid against *C. maltosa*.

2.3.2 Optimization of antimicrobial proteins production from *Arabidopsis thaliana* cell culture

The rationale for choosing the *Arabidopsis thaliana* cell culture and salicylic acid as an elicitor were the highly promising results against the tester strains. One of the key limiting steps is the elicitor concentration which can control the production of pathogenesis related proteins and may affect the biosynthesis of some secondary metabolites. A high dosage of elicitor was reported to induce a hypersensitive response leading to cell death, whereas an optimum level was required for induction (Namdeo 2007). *A. thaliana* cell suspension culture was treated with five different concentrations of salicylic acid (0.1 mM, 0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM) to determine the optimum concentration required to produce high concentration of antimicrobial proteins and reduce other unwanted proteins. In this regard, two day-old cell cultures were used and the plant cell suspension cultures were exposed to the elicitor for another two days with the same nutrient composition of the medium. Among the five concentrations used (**Table 2.4**), one millimolar produced the highest titer of antimicrobial proteins against the tester isolates. On the other hand, salicylic acid at concentration 2.0 mM increased the protein content with decreasing the antimicrobial potency of the cell cultures against most of the pathogens which is not preferred and makes the purification process more difficult.

Table 2.4 Treatment with salicylic acid at different concentrations.

SA Conc. (mM) / Pathogen	Inhibition zone diameter (mm)					Protein content (µg/µl)
	B. subtilis	*C. maltosa*	*E. coli*	*P. aeruginosa*	*S. aureus*	
0.1	7	7	8	9	9	0.4
0.25	8	9	9	11	8	0.6
0.5	12	9	8	10	6	0.7
1.0	12	11	10	11	9	0.7
2.0	10	9	8	10	8	0.9

Furthermore, two periods of incubation, two days and four days were investigated. The use of two days incubation period increases the antimicrobial activity of the *Arabidopsis* cell culture and also the concentration of antimicrobial proteins (**Table 2.5**). While, in the case of four days incubation period the antimicrobial potency was decreased against most of the pathogenic bacteria and fungi with significant increase in the concentration of other non-microbial proteins.

Cell cultures of various ages such as two days, five days and seven days. Among them, two-day old cell culture produced the highest antimicrobial activity while the use of five day and seven day-old cell cultures gave low antimicrobial activities (**Table 2.5**). It therefore, concluded that the age of subculture plays an important role in production of bioactive compounds by elicitation process.

Table 2.5 Change in incubation period and duration of exposure to salicylic acid.

Incubation period / Pathogen	Inhibition zone diameter (mm)					Protein content (µg/µl)
	B. subtilis	*C. maltosa*	*E. coli*	*P. aeruginosa*	*S. aureus*	
2d,2d	12	13	10	10	11	0.6
2d,4d	8	7	0	8	9	0.9
5d,2d	0	9	8	7	8	0.6
5d,4d	0	7	0	0	7	0.4
7d,2d	0	9	0	8	7	0.5
7d,4d	0	7	0	0	0	0.7

2.3.3 Purification and characterization of the antimicrobial protein from *Arabidopsis thaliana* cell culture

The bioassay guided purification was performed using gel filtration with Sephacryl HR column. This column was selected because it has a cross-linked copolymer of allyl dextran and N,N-methylenebisacrylamide which gives the matrix good rigidity and chemical stability results in good resolution power for small peptides and proteins. Further purification was conducted by re-chromatography on the same column. This antimicrobial protein, designated AtAMP (*Arabidopsis thaliana* antimicrobial protein), was purified to homogeneity as determined by SDS-PAGE using silver staining and Coomassie blue staining (**Figure 2.2**). The molecular mass of AtAMP was estimated to be approximately 19 kDa, while calibrated with polypeptide markers on SDS-PAGE (**Figure 2.2**).

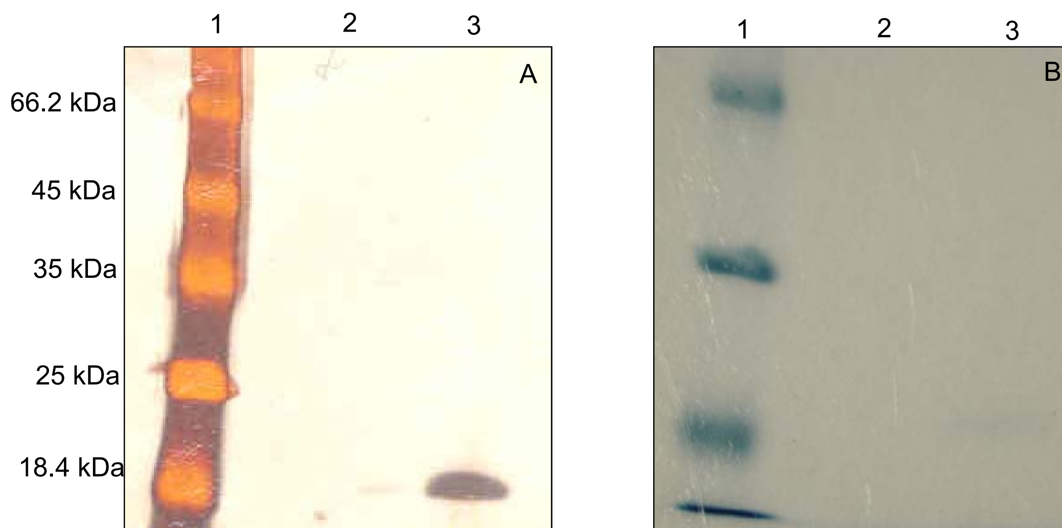

Figure 2.2 SDS-PAGE of AtAMP after (**A**) Silver staining (**B**) Coomassie blue staining. Lane 1: protein molecular weight marker, lane 3: purified protein.

The antimicrobial activity of AtAMP was tested *in* vitro using the disc diffusion assay against an array of human bacteria including Gram-positive and Gram-negative as well as one human Candida (**Table 2.6**). The protein showed potent activity against *Candida maltosa* and Gram-negative bacteria, while the activity against Gram-positive bacteria was low.

Table 2.6 Antimicrobial activity profile of AtAMP.

Pathogen	Inhibition zone diameter (mm)
B. subtilis	8
C. maltosa	14
E. coli	12
P. aeruginosa	11
S. aureus	7

The simple procedure of extraction and purification from plant cell cultures rather than intact plants makes the production of antimicrobial proteins more easy and efficient. To our knowledge this the first report describing antimicrobial proteins from plant cell cultures. This highlights the potential of plant antimicrobial peptides as promising molecules for drug development.

2.3.4 Conclusion and Outlook

Elicited heterotrophic and photomixotrophic plant cell suspension cultures showed diverse inhibitory activities against human pathogenic bacteria and fungi. One putatively 19 kDa antimicrobially active protein, named AtAMP, was purified from *Arabidopsis thaliana* cell culture elicited with 1.0 mM salicylic acid using sephacryl S-200 gel filtration chromatography. Future work will include further characterization of this protein and heterologous expression in transgenic tobacco plants. Our results show that plant cell cultures can be promising sources of novel peptide antibiotics.

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Chapter 3

Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated actinomycetes

3.1 Introduction

3.1.1 Sponge microbiology

Sponges (phylum *Porifera*) are one of the oldest, evolutionarily ancient multicellular animals dating back to Precambrian times (Li *et al.* 1998). The phylum *Porifera* consists of three major classes, namely, the Hexactinellida (glass sponges), Calcarea (calcareous sponges) and Demospongiae (demosponges), with the last group representing 85% of all living sponges (Wörheide *et al.* 2005). More than 6,000 species have been described so far and it is estimated that this is only half the number of species still to be discovered (Hooper *et al.* 2002). Sponges populate tropical reefs in great abundance but also the polar latitudes as well as fresh water lakes and rivers (Belarbi el *et al.* 2003). They are sedentary filter-feeders capable of pumping thousands of liters of water per day (Hooper *et al.* 2002). Microorganisms and other food particles removed from the flowing water by phagocytosis, are transported into the mesohyl interior and are then digested by amoeboid archaeocytes that move freely through the extracellular matrix of the sponge. Sponges feed unselectively on particles up to 50 µm in size which is about the maximum size that the pores allow (Pile 1996; Ribes *et al.* 1999). Microorganisms that can resist phagocytosis can successfully populate sponges (Wilkinson 1987). Sponges have developed intimate contact with diverse microorganisms such as viruses, bacteria, archaea, fungi, protozoa and single-celled algae and the nature of the sponge-microbe interaction is manifold (Vacelet J 1977; Lafi *et al.* 2005; Hentschel *et al.* 2006). Although the proportion of sponge-associated bacteria can vary dramatically from sponge to sponge, bacteria can occupy up to 40-60% of the sponge volume, being densely extracellulary in their mesohyl matrix (**Figure 3.1**). The type of sponges classified as “high-microbial abundance sponges” (HMA) harbor uniformly distributed cells with bacterial concentrations in the range of 10^8-10^{10} cells g^{-1} sponge, two to four orders of magnitude higher than what is typically found in seawater. The other group of sponges with a mesohyl essentially free of bacterial cells were classified as “low-microbial abundance sponges” (LMA) with 10^5-10^6 cells g^{-1} sponge which is equivalent to that of natural seawater (Hentschel *et al.* 2006).

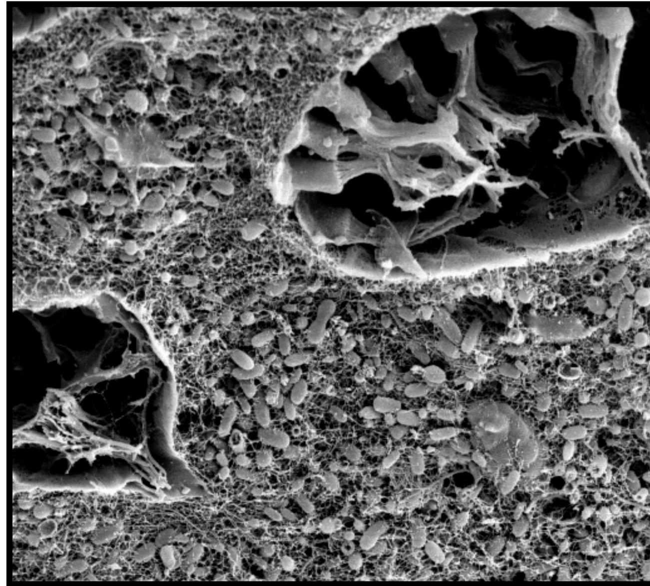


Figure 3.1 Scanning electron micrograph of *Aplysina aerophoba* showing the microorganism-filled mesohyl matrix (Hentschel *et al.* 2003).

The novel cultivation-independent techniques, including 16S rDNA sequencing, denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH) provided new insights on microbial communities in sponges (Lee *et al.* 2001; Imhoff and Stöhr 2003; Hill 2004; Hentschel *et al.* 2006; Olson and Kellogg 2010). At least 18 different prokaryotic phyla (Taylor *et al.* 2007) and one new candidate phylum *Poribacteria* have been so far discovered from marine sponges; with the most common phyla frequently recovered, being the *Acidobacteria*, *Chloroflexi* and *Proteobacteria* (**Figure 3.2**) (Fieseler *et al.* 2004; Scheuermayer *et al.* 2006; Taylor *et al.* 2007). Phyla such as *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia* have been isolated from marine sponges (Head *et al.* 1998; Schmidt *et al.* 2000; Friedrich *et al.* 2001; Montalvo *et al.* 2005; Jiang *et al.* 2007). Several diversity studies on sponge microbial communities have shown that actinomycetes are a major component of these communities (Webster *et al.* 2001; Sun *et al.* 2010).

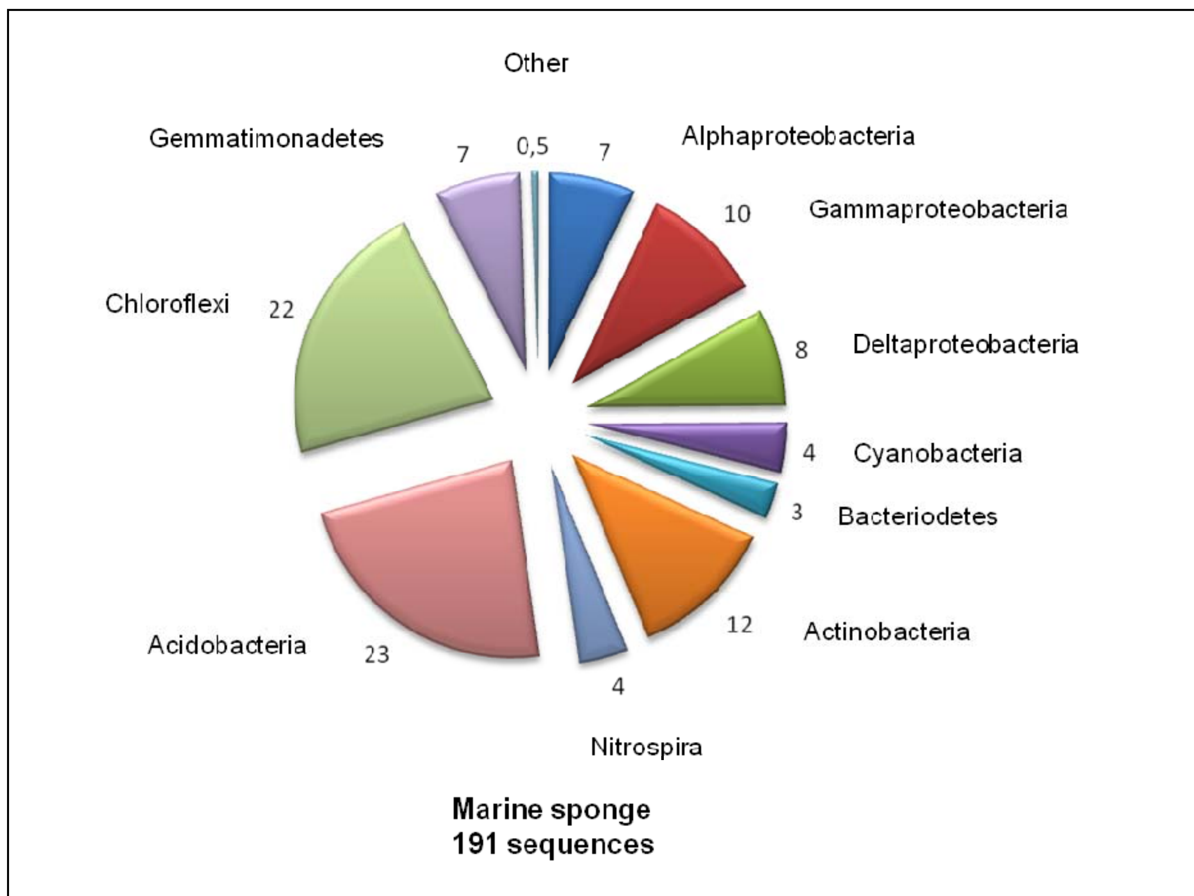


Figure 3.2 16S rRNA gene sequences derived from marine sponges (Hentschel *et al.* 2002).

Archaeal members (Preston *et al.* 1996; Margot *et al.* 2002) as well as fungi belonging to genera *Acremonium*, *Aspergillus*, *Arthrium*, *Coniothyrium*, *Fusarium*, *Mucor*, *Penicillium* and *Trichoderma* have also been reported from different marine sponges (Höller *et al.* 2000; Thakur and Muller 2004; Paz *et al.* 2010). The bacterial diversity and abundance from freshwater sponges was much lower and only *Actinobacteria*, *Chloroflexi*, *Alpha-* and *Betaproteobacteria* have been recovered from 16S rRNA gene library constructed from the freshwater sponge *Spongilla lacustris* (Gernert *et al.* 2005). Microbial distribution within sponge follows a general pattern with the photosynthetically active microorganisms such as *cyanobacteria* and eukaryotic algae, being located in the outer light exposed layers (Wilkinson 1992) while, heterotrophic and possibly autotrophic bacteria inhabit the inner core (Hentschel *et al.* 2003).

The interaction between sponges and microbes is highly diverse, ranging from mutualism to commensalism to exploitative (parasitism/pathogenesis) interactions (Taylor *et al.* 2007). Many potential benefits have been attributed to the associated microorganisms such as nutrient acquisition (Wilkinson 1992; Vacelet *et al.* 1995), stabilization of the sponge skeleton (Rutzler 1985), processing of metabolic waste (Bewley *et al.* 1996; Beer and Ilan 1998), protection from UV light (Shick and Dunlap 2002) and chemical defense (Proksch 1994;

Unson *et al.* 1994; Schmidt *et al.* 2000). However, until details of the functional relationships of sponges and their microbial associates can be estimated, it is difficult to anticipate the true nature of the association. Recent studies with the aid of molecular approaches have shown that bacterial communities are consistently associated with a particular species (Webster *et al.* 2004). Substantial variability exists in microbial communities from different sponge species, and therefore, some bacteria are thought to be host-specific (Taylor *et al.* 2004). Sponge-specific actinobacterial clusters have been recovered from sponges *Aplysina aerophoba* and *Theonella swinhoei* (Hentschel *et al.* 2002; Montalvo *et al.* 2005). *Theonella swinhoei* showed a specific association species of δ-*proteobacteria* (Schmidt *et al.* 2000). A species of α-*proteobacteria* is dominant in sponge *Rhopaloeides odorabile* over various habitats and it has not been detected from seawater, which strongly suggests that the symbionts are species specific (Webster *et al.* 2001). The new group of bacteria "*Poribacteria*," has been identified in several sponges and proved to be sponge-specific using molecular approaches (Fieseler *et al.* 2004).

3.1.2 Sponge-derived compounds of likely microbial origin

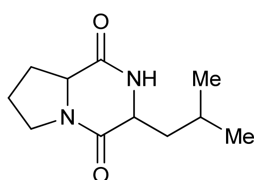
Because sponges cannot move and lack physical defences, they are highly susceptible to marine predators such as fish, turtles, and invertebrates. Thus, it is not surprising that sponges have developed a wide suite of defensive chemicals to deter predators (Thomas *et al.* 2010). They also use their defensive chemicals to keep the offspring of small plants and animals (fouling organisms) from settling onto their outer surfaces (Mol *et al.* 2009; Hertiani *et al.* 2010). These sessile animals are a prolific source of a huge diversity of secondary metabolites that has been discovered over the past 50 years (Faulkner 2002; Blunt *et al.* 2005; Laport *et al.* 2009; Hertiani *et al.* 2010; Proksch *et al.* 2010). The bioactive compounds are very diverse in both structure and bioactivity. It is often unclear whether the compounds of interest are produced by the sponges or their microbial associates (Osinga *et al.* 2001). Many bioactive natural products from marine invertebrates have striking similarities to metabolites of their associated microorganisms especially bacteria (**Table 3.1**) (Unson *et al.* 1994; Flowers *et al.* 1998; Proksch *et al.* 2002; Thiel and Imhoff 2003; Piel 2004).

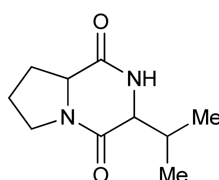
Table 3.1 Structurally-related microbial metabolites with sponge metabolites (Piel 2004).

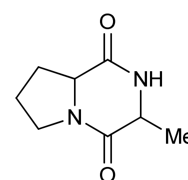
Sponge metabolite	Source	Bacterial metabolite	Source
Andrimid	*Hyatella* sp.	Andrimid	*Vibrio* sp.
Arenastatin A	*Dysidea arenaria*	Cryptophycin 1	*Nostoc* sp.
Diketopiperazines	*Tedania ignis*	Diketopiperazines	*Micrococcus* sp.

Jaspamide	*Jaspis* sp.	Chondramide	*Chondromyces crocatus*
Mimosamycin	*Petrosia* sp.	Mimosamycin	*Streptomyces lavendulae*
Manzamine	*Acanthostrongylophora* sp.	Manzamine	*Micromonospora* sp.
Okadaic acid	*Halichondria okadai*	Okadaic acid	*Prorocentrum lima*
Salicylihalamide	*Haliclona* sp.	Apicularen A	*Chondromyces* sp.
Swinholide A	*T. swinhoei*	Tolytoxin	*Tolypothrix* sp.

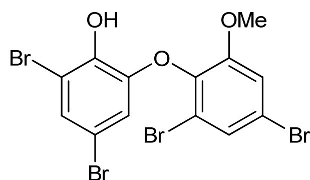
In most cases development and production of sponge-derived drugs is hindered by environmental concerns and technical problems associated with harvesting large amounts of sponges. The presence of possibly producing microbial symbionts is therefore especially intriguing, as a sustainable source of sponge-derived drug candidates could be generated by establishing a symbiont culture or by transferring its biosynthetic genes into culturable bacteria. For example, the sponge *Tedania ignis* produces diketopiperazines which are also produced by an associated bacterium *Micrococcus* sp. (Stierle *et al.* 1988). Another bacterium *Vibrio* sp. produces brominated biphenyl ethers formerly ascribed to the host sponge *Dysidea* sp. (Elyakov *et al.* 1991). A dinoflagellate *Prorocentrum lima* produces okadaic acid, first isolated from the host sponge *Halichondria okadai* (Kobayashi and Ishibashi 1993). A *Vibrio* sp. produces peptide, andrimid that was purified from the sponge *Hyatella* sp. extract (Oclarit *et al.* 1994). Manzamine alkaloids, the promising leads for extended preclinical assessment against malaria, tuberculosis and HIV, have been previously isolated from sponge *Acanthostrongylophora* sp. and have also been isolated from the associated microorganism *Micromonospora* sp. (Hill *et al.* 2005). Thus, the microbial association that occurs on or in sponges could be of great interest to look for potential uses as new sources of bioactive compounds, in particular as a solution of the supply problem of most of pharmaceutical compounds produced by reef's invertebrates.



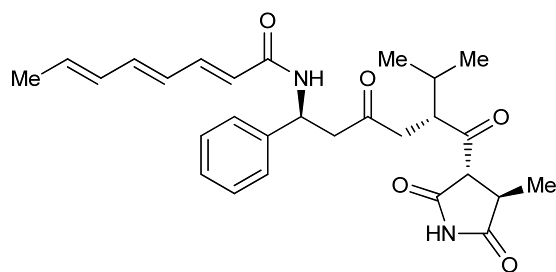




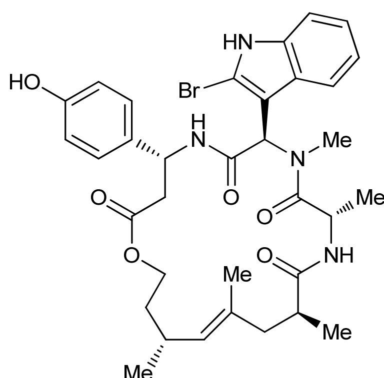
Diketopiperazines



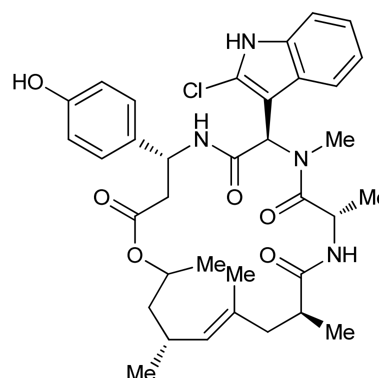
Brominated biphenyl ether



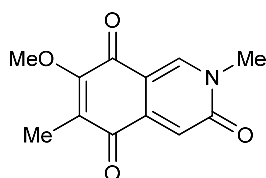
Andrimid



Jaspamide



Chondramide



Mimosamycin

3.1.3 Actinomycetes

The phylum *Actinobacteria* represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria (Ventura *et al.* 2007). The best known of these is the subclass *Actinobacteridae*, which includes the order *Actinomycetales* whose members are commonly referred to as actinomycetes. Thirty nine families have been recognized with the most common ones being *Actinomycetaceae*, *Actinoplanaceae*, *Dermatophilaceae*, *Frankiaceae*, *Mycobacteriaceae*, *Micromonosporaceae*, *Nocardiaceae*, and *Streptomycetaceae* comprising altogether 147 genera (Garrity *et al.* 2004). Based on the 16S rRNA gene classification system, these genera have been grouped in ten suborders: *Actinomycineae*, *Corynebacterineae*, *Frankineae*, *Glycomycineae*, *Micrococineae*, *Micromonosporineae*, *Propionibacterineae*,

Pseudonocardineae, *Streptomycineae*, *Streptosporangineae* and a large number of actinomycetes are still remains to be grouped (Nisbet and Fox 1991). Actinomycetes are a diverse group of aerobic Gram-positive microorganisms with high G+C content in their DNA. They are characterized by their diverse shapes and colors, formation of a network of aerial and substrate hyphae as well as production of melanoid pigments of various colors (*i.e.*, greenish brown, brownish black or distinct brown) on the medium. The composition of cell wall in actinomycetes varies greatly among different groups and is of considerable taxonomic significance based on the composition and structure of peptidoglycan. Actinomycetes are metabolically and morphologically more complex than other bacteria. Metabolically, they are prolific producers of a vast array of secondary metabolites. Complex morphological development between their genera is apparently phenotypically related to secondary metabolism (Conn *et al.* 2008). Although most of actinobacteria are of medical or economical significance, serious human diseases could be caused by some members of actinobacteria such as *Mycobacterium*, *Corynebacterium* and *Nocardia* (Hall 2008).

Actinomycete diversity

Actinobacteria are widespread in nature and have been recovered from wide variety of sources, where they can be as saprophytes, symbionts, parasites or even pathogens (Shirokikh and Merzaeva 2005; Ward and Bora 2006). Actinomycetes populations have been identified as one of the major groups of the soil microbial community (Loqman *et al.* 2009; Jayabarath *et al.* 2010; Lin *et al.* 2010), where they play a crucial role in the recycling of refractory biomaterials by decomposing complex mixtures of polymers in dead plant, animal and fungal materials producing several volatile sesquiterpene substances namely, geosmins, responsible for the characteristic “wet earthy odor”. In a study carried out by Moncheva *et al* (2002), 47 actinomycete strains belonged to the genera *Actinomadura*, *Kitasatospora*, and *Streptomyces*, have been isolated from soil. Actinobacteria have also developed intimate associations with plants and colonize their inner tissues. The actinomycete bacteria that reside in the tissue of living plants and do not visibly harm the plants are known as endophytic actinobacteria. Endophytic actinobacteria have attracted attention, with increasing reports of isolates from a range of plant types, including crop plants, cereals, such as wheat, rice, potato, carrots, tomato and citrus (Chen *et al.* 2009; Qin *et al.* 2009) as well as from medicinal plants (Zin *et al.* 2007; Qin *et al.* 2008). The culturable endophytic actinobacteria from various plants have been found to fall within a narrow species distribution, with *Streptomyces* as the most predominant genus and the rest belonging to genera *Actinosynnema*, *Actinomadura*, *Microbispora*, *Micromonospora*, *Streptosporangium*, *Nocardia* and *Nocardioides* (Coombs and Franco 2003; Hasegawa *et al.* 2006). From the healthy shoots and roots of *Aquilaria crassna Pierre*, 10 endophytic actinomycete strains

have been recovered (Nimnoi *et al.* 2010). Phylogenetic analysis based on 16S rDNA sequencing of those isolates showed that they belong to genera: *Actinomadura*, *Nocardia*, *Nonomuraea*, *Pseudonocardia* and *Streptomyces*. Actinomycetes have also been isolated from niche habitats such as caves, pristine forests, high salt environments and endophytic niches (Seong *et al.* 2001; Groth *et al.* 2002; Ningthoujam *et al.* 2009). Wang *et al*. (1999) investigated the actinomycete diversity in the rainforests of Singapore. Thirty six actinomycete genera were recovered, among them *Streptomyces*, *Micromonospora*, *Actinoplanes*, *Actinomadura*, *Nonomuria*, *Nocardia* and *Streptosporangium* were the most abundant. They have been also found in sewage sludge compost (Vaz-Moreira *et al.* 2008). Actinomycetes belong to genera *Actinomyces*, *Amycolatopsis*, *Corynebacterium*, *Streptomyces*, *Nocardia* and *mycobacterium* have been isolated from various clinical human specimens (Sarkonen *et al.* 2001; Huang *et al.* 2004; Wauters *et al.* 2005).

It has long been known that actinomycetes can be cultured from marine samples (Grein and Meyers 1958), yet it was not clear if these common soil bacteria should be considered terrestrial “contaminants” or a true component of the marine microbial community (Goodfellow and Haynes 1984). Early evidence for the existence of indigenous marine actinomycete populations came from the recovery of strains from deep-sea sediments (Weyland 1969), the description of the first marine species, *Rhodococcus marinonascene* (Helmke and Weyland 1984), the metabolic activity of some strains in marine sediments (Moran *et al.* 1995) and the isolation of obligate marine strains (Jensen *et al.* 1991; Moran *et al.* 1995). The first marine actinomycete taxon, *Salinispora*, was described in 2005 (Maldonado *et al.* 2005a). Actinomycetes have been cultivated from seawater (Agogue *et al.* 2005), marine snow (Alldredge and Silver 1988) and marine sediments (Jensen *et al.* 2005; Xiao *et al.* 2010), including deep-sea sediments (Stach *et al.* 2003). Actinomycetes are ubiquitous in marine sediments, but at lower abundances than in soil (Fiedler *et al.* 2005; Fenical and Jensen 2006; Bredholdt *et al.* 2007a; Maldonado *et al.* 2009). Members from families *Micromonosporaceae*, *Nocardiaceae*, *Pseudonocardiaceae*, *Streptomycetaceae* and *Thermomonosporaceae* have been recovered by Gontang *et al* (2007) from marine sediments collected in the Republic of Palau. Actinomycetes have been found in symbiosis with different marine invertebrates such as sponges, corals, tunicates and jellyfish (Han *et al.* 2003; Inagaki *et al.* 2003; Charan *et al.* 2004; Lampert *et al.* 2008), with the majority have been isolated from sponges (Hentschel *et al.* 2002; Kim and Fuerst 2006; Wang 2006; Zhang *et al.* 2008; Selvin *et al.* 2010). At least 28 actinobacterial genera have been reported from sponges (Taylor *et al.* 2007). Montalvo *et al.* (2005) showed that actinobacteria are major component of the microbial communities of *Xestospongia muta* and *X. testudinaria*.

Both molecular studies and culture-based methods revealed the existence of extensive, uncharacterized marine actinobacterial diversity in comparison to terrestrial ones (Sun *et al.* 2010). The difference observed in almost all cases between cultured and culture-independent marine actinobacterial diversity could be discussed in a number of ways. It is known that the majority of the recovered actinomycetes belong to the suborders *Micromonosporineae* and *Streptomycineae*, thus, it is possible that these bacteria could occur in the marine environment as spores which are not easily detected due to relatively poor DNA extraction procedures when culture-independent approaches are used. It is also could be that these actinomycetes occur at low abundances which could not be detected by most of culture-independent approaches (Mincer *et al.* 2005). Nevertheless, all of these studies revealed considerable actinobacterial diversity in marine samples, evidence that actinomycetes are capable of growing in the marine environment.

Members of actinobacteria have been cultivated from diverse marine samples when selective isolation protocols are applied with recent efforts leading to the description of new genera. Taxonomically novel isolates have been discovered from ocean sediments from four different locations, displayed an obligate requirement of seawater for growth. Phylogenetic characterization of representative isolates based on almost complete sequences of genes encoding 16S rRNA yielded a monophyletic clade within the family *Micromonosporaceae* and suggested novelty at the genus level with the name proposed name, *Salinispora* gen. nov. Different species have been recognized from the new genus such as *Salinispora arenicola*, *Salinispora tropica* and *Salinispora pacifica* which have been recovered from ocean sediments (Maldonado *et al.* 2005a) as well as from a sponge (Kim *et al.* 2005). A new actinomycete strain containing L-ornithine as the diagnostic amino acid has been isolated from a sea water sample from the East Sea, Korea. A phylogenetic analysis based on 16S rRNA gene sequences showed that this strain represents a phyletic line within the suborder *Micrococcineae*, adjacent to the genus *Ornithinimicrobium* with a sequence similarity of 94.1-94.3% to other published strains within this genus. Physiological, biochemical and chemotaxonomical evidences clearly separated the marine isolate from other members of the suborder *Micrococcineae* and proposed the affiliation to a novel genus and species, for which the name *Serinicoccus marinus* gen. nov., sp. nov. has been proposed (Yi *et al.* 2004). Another example of newly described taxa is *Salinibacterium amurskyense* gen. nov., sp. nov., the new actinomycete strain that has been isolated from sea-water samples and represented a distinctive taxon in the family *Microbacteriaceae* (Han *et al.* 2003). The marine environment still unexplored source of new species and sometimes new genera of actinomycetes that could be exploited in the biotechnological, medicinal, and agricultural industries. In addition to the new genera that have been discovered from marine

environment, several obligate marine species have also been isolated from such as *Streptomyces axinellae* sp. nov. (Pimentel-Elardo *et al.* 2009) and *Saccharopolyspora cebuensis* sp. nov. (Pimentel-Elardo *et al.* 2008b).

Marine actinomycetes as a source of new bioactivities

Marine environments consist of taxonomically diverse microbial groups which exhibit unique physiological and structural characteristics enabling them to survive in the extremes of pressure, salinity and temperature, with the potential production of interesting pharmacological activities not observed in terrestrial microorganisms. Actinomycetes account for approximately half of the bioactive secondary metabolites that have been discovered among associated bacteria yet (**Figure 3.3**) (Lam 2006; Thomas *et al.* 2010). These metabolites exhibited diverse activities such as antibacterial, antifungal, pesticidal, herbicidal, immunomodulatory, anti-inflammatory and anticancer activities (Newman and Cragg 2007; Olano *et al.* 2009; Engelhardt *et al.* 2010; Pimentel-Elardo *et al.* 2010). The activities represented by diverse classes of natural products such as polyketides, alkaloids, fatty acids, peptides and terpenes (Boonlarppradab *et al.* 2008; Oh *et al.* 2008; Asolkar *et al.* 2010).

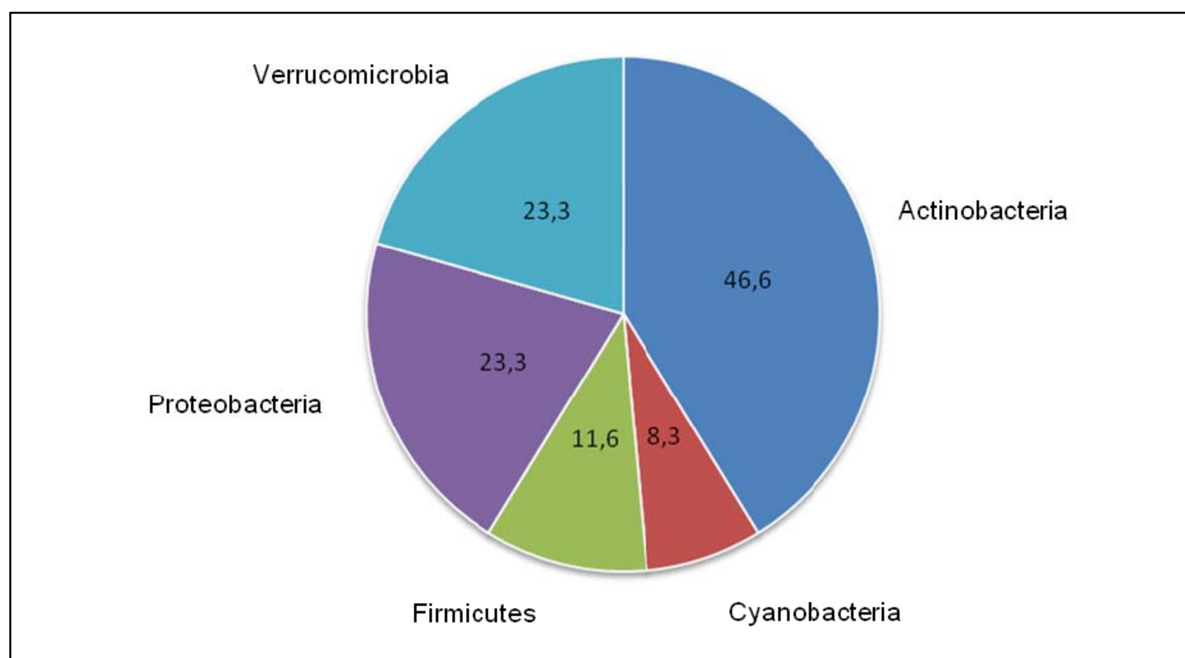
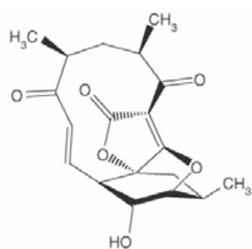
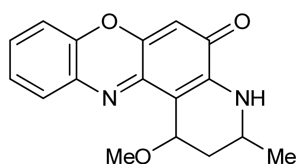


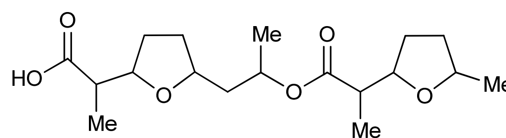
Figure 3.3 Percentage distribution of compounds produced by associated bacteria (Thomas *et al.* 2010).

Resistance of pathogenic bacteria and fungi toward antibiotics is certainly increasing; meanwhile the rate of discovery and development of new and effective antibiotic compounds is declining. Thus, the discovery of novel anti-infective agents has become a pressing need. Among the clinically used antibiotics, over two thirds have been discovered from microbial

sources or are the semi-synthetic derivatives of microbial products (Newman 2008; Rahman *et al.* 2010) and about three quarters have been derived only from actinomycetes. Abyssomicin C, is a novel polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain (Riedlinger *et al.* 2004). It is a potent inhibitor of para-aminobenzoic acid biosynthesis therefore, inhibits the folic acid biosynthesis at an earlier stage than the well-known synthetic sulfa drugs (Rath *et al.* 2005). Abyssomicin C exhibited potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant and vancomycin-resistant *Staphylococcus aureus*. Abyssomicin C has the potential to be developed as antibacterial agent against drug-resistant pathogens and is now in clinical trials. Three novel phenoxazin-derived antibiotics, chandrananimycins A, B and C have been isolated from the culture broth of a marine *Actinomadura* sp. isolate M045, exhibited high antibacterial and antifungal activities (Maskey *et al.* 2003). The new macrotetrolide antibiotic, bonactin has been purified from the liquid culture of a marine *Streptomyces* sp. BD21-2. Bonactin displayed antibacterial activity against both Gram-positive and Gram-negative bacteria as well as antifungal activity (Schumacher *et al.* 2003).



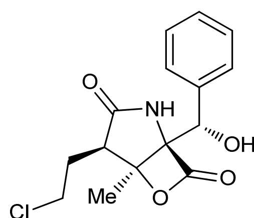




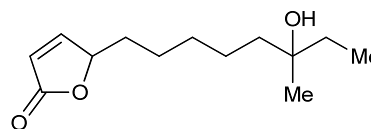
Abyssomicin C Chandrananimycin Bonactin

The antiparasitic activity from marine environment has been reported especially from sponges with few representatives have been characterized from actinomycetes (Gademann and Kobylinska 2009; Orhan *et al.* 2010). Salinosporamide A has been isolated from the marine *Salinispora tropica* and is known to be potent inhibitor of human multiple myeloma, has been found to be highly potent inhibitor of the human malaria parasite both in vitro (IC50 = 11.4 nM, *Plasmodium falciparum*) and in vivo (ca. 90 % reduction in parasitemia at 130 µg/kg in mice, *P. yoelii*) (Chauhan *et al.* 2005; Prudhomme *et al.* 2008). The lactone-derived compound, butenolide has been isolated from *Streptomyces* sp. cultivated from sponge *Tethya* sp. as well as from marine sediment-derived *Streptomyces* sp. strain M027750 (Cho *et al.* 2001). Butenolide exhibited antitrypanosomal activity (IC50 0.022 µM) (Pimentel-Elardo *et al.* 2010). The cyclic depsipeptide, valinomycin has been purified from *Streptomyces* sp. recovered from sponge *Aplysina aerophoba* as well as from previous terrestrial actinomycetes (Brockmann and Schmidt-Kastner 1955). Valinomycin exhibited significant

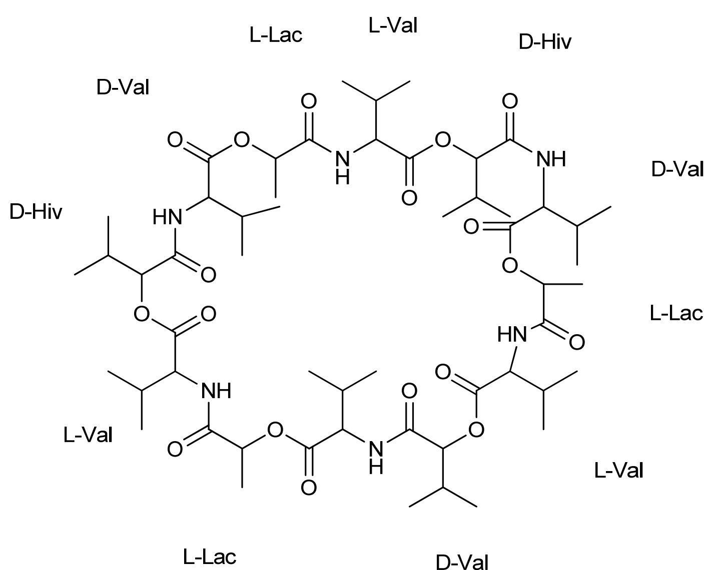
inhibitory activities against the parasites *Leishmania major* (IC50 < 0.11 μM) and *Trypanosoma brucei* (IC50 0.0032 μM) (Pimentel-Elardo *et al.* 2010).



Salinosporamide A



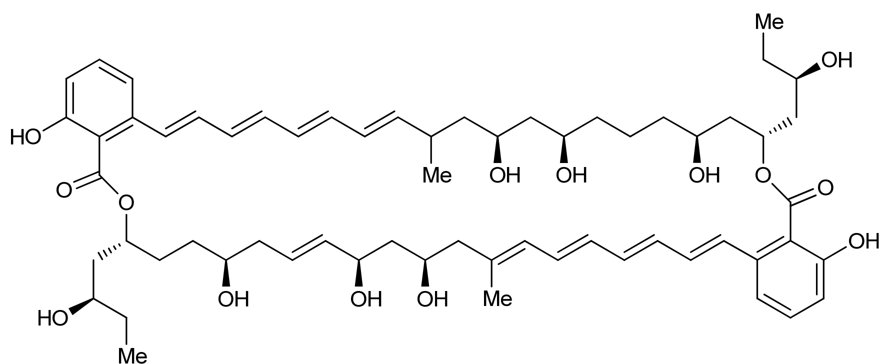
Butenolide



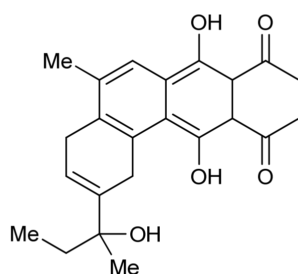
Valinomycin

A great number of known antitumor compounds are natural products or their derivatives, many of them produced by marine microorganisms (Fiedler *et al.* 2008). In particular, actinomycetes are the producers of a large number of secondary metabolites with antitumor properties (Olano *et al.* 2009). These antitumor compounds belong to several structural classes such as anthracyclines, enediynes, indolocarbazoles, isoprenoides, macrolides, non-ribosomal peptides and others (Fiedler *et al.* 2008; Salas and Mendez 2009). Marinomycins, novel class of polyketides which contain unusual macrodiolides composed of dimeric 2-hydroxy-6-alkenyl-benzoic acid lactones with conjugated tetraene-pentahydroxy polyketide chains, produced by marine *Marinispora* sp. CNQ-140. These compounds inhibit cancer cell proliferation with an average IC50 of 0.2-2.7 μM against the NCI's 60 cancer cell line panel (Kwon *et al.* 2006). Parimycin, an anthraquinone derivative produced by marine *Streptomyces* sp. isolate B8652 showed activity against human tumor cell lines of stomach

cancer (GXF 251L), lung cancer (H460, LXFA 629L, and LXFL 529L), breast cancer (MCF-7 and MAXF 401NL), melanomas (MEXF 462NL and MEXF 514L) with IC70 values ranging from 0.9 to 6.7 µg/ml (Maskey *et al.* 2002).



Marinomycin



Parimycin

3.1.4 Aim of the work

Actinomycetes are prolific producers of secondary metabolites and preferential targets in the search for new bioactive natural products. The aim of this work was to isolate the bioactive novel actinomycete species associated with marine sponges collected from Egypt and Croatia by cultivation. Strains were identified based on 16S rRNA gene sequence analysis and their bioactivity was tested against a panel of clinically relevant, Gram-positive and Gram-negative bacterial pathogens. Moreover, human fungal pathogen as well as human parasites was also tested.

3.2 Materials and Methods

3.2.1 Sponge collection

The first group of sponges (*Aplysina aerophoba*, *Dysidea avara*, *D. tupha*, *Hemimycale culumella*, *Ircinia fasciculata*) was collected by SCUBA diving at depths of 3-20 m in the Mediterranean Sea (Rovinj, Croatia, (GPS: 27˚47.655 N; 34˚12.904 W) in August 2008. Taxonomic identification was performed by W.E.G. Müller and I. Müller (University of Mainz, Germany). The second group (*Amphimedon* sp., *Callyspongia* sp., *C.* aff. *implexa*, *Hyrtios erecta*, *Negombata magnifica, Spheciospongia vagabunda*) was collected at a depth of 10 m in the Red Sea (Ras Mohamed, Sinai, Egypt; (GPS: 27˚47.655 N; 34˚12.904 W) in August 2006. *Amphimedon* sp. and *Hyrtios erecta* were identified by M. Kelly (National Institute of Water and Atmospheric Research (NIWA) Auckland, New Zealand) and the remaining sponges by R.W.M. van Soest (University of Amsterdam, Netherlands). Sponges were transferred to plastic bags containing seawater and transported to the laboratory. 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.

3.2.2 Isolation of sponge-associated actinomycetes

Eight different media [M1 (Mincer *et al.* 2002), ISP medium 2 (Shirling and Gottlieb 1966), Oligotrophic medium (OLIGO) (Olson *et al.* 2000), M1 plus (Webster *et al.* 2001), Actinomycete Isolation Agar (AIA) (Kurup *et al.* 1975), Marine Agar (MA) (Weiner *et al.* 1985), Glycerol Asparagine Agar (GAA) (Lechevalier and Lechevalier 1975) and R2A Agar (Reasoner and Geldreich 1985)] were used for the isolation of actinobacteria. All media were supplemented with 0.2 µm pore size filtered cycloheximide (100 µg/ml), nystatin (25 µg/ml) and nalidixic acid (25 µg/ml) to facilitate the isolation of slow-growing actinobacteria. Cyclohexamide and nystatin inhibit fungal growth, while nalidixic acid inhibits many fast-growing Gram-negative bacteria (Webster *et al.* 2001). All media contained Difco Bacto agar (18 g/L) and were prepared in 1 L artificial sea water (Lyman and Fleming 1940). To promote the growth of selected sponge-associated actinobacteria, 1% “aqueous sponge extract” was added to the autoclaved medium (M1 plus). Aqueous sponge extract was prepared by grinding 20 g of sponge biomass in a mortar containing 20 ml of sterile seawater followed by centrifugation (5000 rpm, 10 min) and sterilized by filtration through a 0.2 µm pore size filter. The freshly prepared supernatant served as aqueous sponge extract. The inoculated plates were incubated at 30 °C for 6-8 weeks. Distinct colony morphotypes were picked and re-streaked until visually free of contaminants. The strains were selected by the morphological differences based on visible examination of the growth characteristics (tough leathery

texture, dry or folded appearance, aerial mycelium, substrate mycelium and diffusible pigments). Pure isolates were inoculated into liquid media (M1 or the medium on which colonies were initially isolated). The isolates were maintained on plates for short-term storage and long-term strain collections were set up in medium supplemented with 30% glycerol at −80 °C. The isolates from Egypt are abbreviated as “EG” and from Rovinj as “RV”.

3.2.3 Molecular identification and phylogenetic analysis

16S rRNA gene amplification, cloning and sequencing were performed according to Hentschel *et al*. (Hentschel *et al.* 2001) using the universal primers 27F and 1492R (Lane 1991). Total genomic DNA was extracted from each strain by scrapping few colonies, suspending in sterile water and incubation at 95 °C for 10 min then centrifugation at 3000 rpm for 30 s or using FastDNA spin kit for soil (Q-Biogene) following manufacturer’s instructions. Three milliliters of well grown liquid cultures were centrifuged at 8000 rpm for 5 min and the supernatant was discarded. Pellet was resuspended in 978 µl sodium phosphate buffer and 122 µl MT buffer. The solution was transferred to lysing matrix tubes then processed using a FastPrep® instrument (Q-Biogene) for 30 s with speed set at 5.5. The resulting solution was centrifuged at 13000 rpm for 30 s and supernatant was transferred to a clean tube and 250 µl of PPS reagent was added. The solution was mixed by shaking the tube ten times with hands, centrifuged at 13000 rpm for 5 min. Supernatant was transferred to clean 2-ml microfuge tube. 1 ml of binding matrix suspension was added to the supernatant and the solution was mixed for 2 min to allow binding of the DNA to the matrix. The tube was allowed to stand for 3 min to allow settling of the silica. Five hundred microliters of the supernatant was discarded and 600 µl was transferred to a spin filter, centrifuged at 13000 rpm for 1 min and catch tube was emptied. The remaining supernatant was added to the spin filter and centrifuged at 13000 rpm for 1 min. 500 µl of SEWS-M was added, followed by centrifugation at 13000 rpm for 1 min. The flow-through was discarded and the spin filter was centrifuged at 13000 rpm for 2 min. The spin filter was removed, placed in a new catch tube and subsequently air-dried for 5 min at room temperature. 50 µl of DES (DNAse/pyrogen-free) water was added and the filter matrix was gently stirred using a pipette tip to resuspend the silica and for efficient elution of the DNA. After centrifugation at 13000 rpm for 1 min, eluted DNA was transferred to new catch tube and stored at -20 °C.

From the genomic DNA, nearly full-length 16S rRNA genes were amplified by polymerase chain reaction (PCR) using primers 27F (GAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCR mixture consisted of 5 µl of 10 X buffer (Mg^{2+} free), 2 µl of 2.5 M $MgCl_2$, 1 µl of dNTPs mixture, 10 µl of Q solution, 1 µl of each primer, 1 µl of template DNA and 0.25 µl of 5 U Taq DNA polymerase (Promega, USA) and 28.75 µl of

water in a final volume of 50 µl. PCR was performed in a thermal cycler (Biometra, Germany) using an initial denaturation at 95 ^{o}C for 2 min, followed by 30 cycles of denaturation at 95 ^{o}C for 1 min, annealing at 56 ^{o}C for 1 min followed by primer extension at 72 ^{o}C for 90 s and a final extension at 72 ^{o}C for 10 min. PCR amplification products were visualized by agarose gel electrophoresis.

PCR amplification products were purified using QIAquick gel extraction kit (Qiagen, Germany). Five volumes of buffer PB was added to one volume of PCR product and mixed using a pipette. The mixture was applied to a QIAquick column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same tube. Buffer PE (750 µl) was added to the column followed by centrifugation at 13000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional min at 13000 rpm. The column was then placed in a clean 1.5-ml microfuge tube, 30 µl of buffer EB was added and allowed to stand for 1 min. A final centrifugation step at 13000 rpm for 1 min was performed and the resulting purified PCR product was stored at -20 °C.

Purified PCR product were subsequently ligated into the pGEM-T Easy vector (Promega, USA) by mixing 5 µl (50 ng/µl) 2x T4 DNA ligase buffer, 1 µl (3 U/µl) pGEM-Teasy vector (Promega), 1 µl T4 DNA ligase and 4 µl PCR product on ice. The ligation reaction was incubated overnight at 4 °C. 2-3 µl of the ligation mixture was transformed into DH5α *Escherichia coli* Novablue cells. The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside)-IPTG (isopropyl-β-thiogalactopyranoside)-ampicillin-indicator plates by colour-based recombinant selection. Two clones were selected from each library and the plasmid DNA was purified using the QIAquick spin Miniprep kit (Qiagen, Germany). Few white colonies were inoculated in 2 ml of LB/amp broth and incubated with shaking at 37 °C overnight. After incubation, liquid culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. The pellet was then resuspended in 150 µl of buffer P1. 150 µl of buffer P2 was added and allowed to stand for 5 min at RT and subsequently 150 µl of buffer P3 was added on ice for 5 min. The solution was mixed by gentle shaking of the tube after each addition of the buffer. Suspension was centrifuged at 13000 rpm for 10 min; supernatant was transferred to clean 1.5-ml microfuge tube and centrifuged 13000 rpm for 10 min. The supernatant was again transferred to clean tube, mixed with 0.7 volume of isopropanol with gentle shaking, followed by centrifugation at 13000 rpm for 15 min. Pellet containing the plasmid DNA was washed with 70% ethanol and allowed to air-dry. Pellet was then resuspended in 50 µl sterile water and the plasmid DNA was stored in -20 °C. The correct inserts were checked by digestion with EcoRI restriction endonuclease (Promega, USA). Restriction digestion (New England Biolabs) was carried out by mixing 2 µl 10x EcoRI buffer, 12 µl water, 1 µl EcoRI (20,000 U/ml) and 5 µl plasmid DNA.

The reaction mix was incubated at 37 °C for 3 h and the restriction patterns were analyzed by agarose gel electrophoresis.

Sequencing was done using an ABI 377XL automated sequencer (Applied Biosystems) and almost complete 16S rRNA gene sequences were determined using sequencing primers SP6 (5′ GATTTAGGTGACACTATAG-3′), T7 (5′-TAATACGACTCACTATAGGG-3′) and the 16S rDNA-specific primers, 341F (5'- CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTAAGTTT-3') with the following PCR conditions: 96 °C for 2 min, 25 cycles of 96 °C for 30 s, 45 °C (SP6 and T7), 59 °C (341F) or 52 °C (907R) for 15 s, 60 °C for 4 min, with a final extension step of 60 °C for 10 min. Sequences were manually edited using Invitrogen's Vector NTI Advance 10 (InforMax, Inc) and Contigs of ~1,400-kb length were tested against all DNA sequences available in GenBank (www.ncbi.nml.nih.gov) using the BLASTn tool to identify the nearest neighbor to the amplified sequence. Chimeric sequences were identified by using the Pintail program (Ashelford *et al.* 2005). The genus-level affiliation of the sequences was validated using the Ribosomal Database Project Classifier (Wang *et al.* 2007). Sequence alignment and phylogenetic analysis were performed using the ARB software package (Ludwig *et al.* 2004). Tree construction was conducted using neighbour-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: EG4 (GU318354), EG7 (GU318355), EG33 (GU318356), EG36 (GU318357), EG37 (GU318358), EG45 (GU318359), EG47 (GU318360), EG49 (GU318361), EG62 (GU318362), EG69 (GU318363), RV15 (GU318364), RV113 (GU318365), RV13 (GU318366) and RV89 (GU318367).

3.2.4 Extract preparation and anti-infective activity screening

Fourteen strains selected based on phylogenetic novelty and six selected based on their affiliation to known metabolite-producers were cultured in 100 ml Erlenmeyer flasks containing 50 ml of 5 different production media (M1, ISP2, OLIGO, AIA and R2A) for each isolate. The liquid cultures were grown for 7-14 days depending on their growth rate at 30 °C while shaking at 150 rpm. An equal volume of methanol was added to the liquid cultures for cell lysis and shaking was continued (150 rpm, 1 h at RT; Shaker SM 30, E. Bühler). The broth was centrifuged in 50 ml falcon tubes (5000 rpm, 15 min at RT; Megafuge 1.0R, Heraeus) and the supernatant was stored at 4 °C for bioactivity testing.

Antibacterial and antifungal activities

The *in vitro* antimicrobial activity testing was carried out using the standard disc diffusion assay (Inderlied and Salfinger 1995) against pathogenic bacteria (*Staphylococcus aureus* strain 8325, *Enterococcus faecalis* strain JH212, *Escherichia coli* strain 536, *Pseudomonas aeruginosa* strain Nr. 3) and yeast (*Candida albicans* strain S314). The overnight broth cultures prepared freshly from plates for each assay by overnight growing the bacterial isolates in LB and yeast in YPD broth at 37 °C with shaking at 220 rpm (Innova 4300 incubator shaker). 100 µl of the overnight culture with an optical density of OD_{600} = 0.2 adjusted with LB or YPD using spectrophotometer (Ultrospec 3100 Pro) was spread on 100 mm-dry plates containing solid LB or YPD-agar medium, allowed to absorb in the agar for 10 min, then the plates dried and inverted at 37 °C for approximately 30 min until the bacterial overlay had dried. Sterile Whatman filter paper (Roth, Germany) discs with a diameter of 6 mm were impregnated with testing solution (20 µl/disc) and let to dry under the fume hood (3 times impregnating then drying). Loaded discs were placed on the agar plates inoculated with the target microorganism and gently taped each disc to ensure contact with the agar surface. The plates were inverted incubated for 24 h at 37 °C and the antimicrobial potential was quantitatively assessed as diameter of the inhibition zone (n = 2). Different antibiotics (chloramphenicol and gentamycin as antibacterial and nystatin as antifungal) were used as positive controls in the plates and 50% methanol was also used as a negative control.

Antileishmanial activity

Antileishmanial activity was tested following the method of Ponte-Sucre *et al.* (Ponte-Sucre *et al.* 2006). *Leishmania major* promastigotes were seeded at a cell density of 1 x 10^7 cells/ml into 96-well plates containing 200 µl Complete Medium without phenol red, in the presence or absence of the extracts. 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 measured after 24 and 48 h 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 extracts was set as 100% of growth. Amphotericin B was used as a reference compound and positive control. Each extract was assayed in duplicate from two independent experiments.

Antitrypanosomal activity

Antitrypanosomal activity was tested following the protocols of Huber and Koella (Huber and Koella 1993). Trypomastigote forms of *Trypanosoma brucei brucei* laboratory strain TC 221

were cultured in Complete Baltz medium (Baltz *et al.* 1985). A defined number of parasites (10^4 trypanosomes per ml) were exposed in test chambers of 96-well plates to various the test extracts (previously dissolved in DMSO) to make a final volume of 200 µl in duplicates. Positive (trypanosomes in culture medium) and negative controls (test substance 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 addition of 20 µl of Alamar Blue, the activity of extracts was measured by light absorption using MR 700 Microplate Reader at a wavelength of 550 nm with a reference wave length of 630 nm. First reading was done at 48 h and subsequently at 72 h. Each extract was assayed in duplicate from two independent experiments.

3.3 Results and Discussion

3.3.1 Diversity of sponge-associated actinomycetes

Homogenates from eleven taxonomically different marine sponges collected from Ras Mohamed (Egypt) and Rovinj (Croatia) were plated on a range of selective media for actinomycetes isolation. On the basis of characteristic colonial morphology, notably the dry folded appearance, the ability to form aerial hyphae and substrate mycelia as well as pigment production, organisms putatively identified as actinobacteria were selected and obtained as pure cultures. Phylogenetic analysis based on nearly complete 16S rRNA gene sequences revealed that 52 out of 90 putative actinobacterial cultivated strains were affiliated to 18 different genera (**Table 3.2**) after BLAST against the sequences in the NCBI GenBank database (Abdelmohsen *et al.* 2010b). Taxonomic ordering using Bergey's Manual of Systematic Bacteriology (Garrity *et al.* 2004) showed that these genera represent 14 different families and seven suborders under two subclasses (**Figure 3.4**).

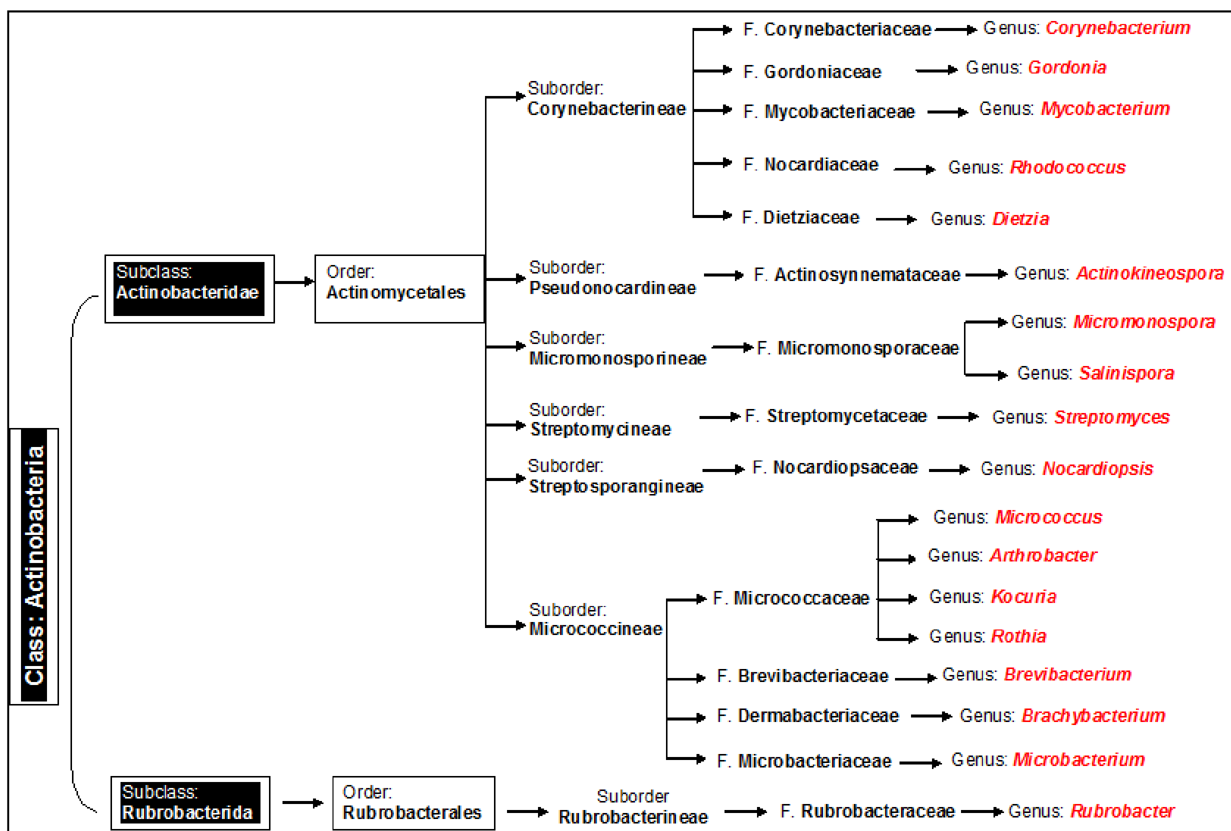


Figure 3.4 Taxonomic ordering of cultivated actinomycete strains.

The number of Actinobacteria isolated from the eleven sponge species was significantly different. *Callyspongia* aff. *implexa* yielded the highest number of isolates (22), followed by *Aplysina aerophoba* (16), *Spheciospongia vagabunda* (11), *Hemimycale culumella* (9), *Hyrtios erecta* (8), *Dysidea tupha* (6), *Callyspongia* sp. (6), *Dysidea avara* (4), *Amphimedon*

sp. (4), *Negombata magnifica* (4), while no actinomycete strains were cultivated from *Ircinia fasciculata* (**Figure 3.5**). It has been shown by different studies that actinomycetes are consistent, diverse and sometime major component of sponge-associated microbial communities (Hentschel *et al.* 2002; Sun *et al.* 2010). Zhang *et al*. (2006a) isolated 106 actinomycete strains representing seven genera from the sponge *Hymeniacidon perleve*. Another study described the diversity of actinobacteria isolated from the marine sponge *Iotrochota* sp. collected in the South China Sea, revealed 52 isolates belonged to three genera (Jiang *et al.* 2008).

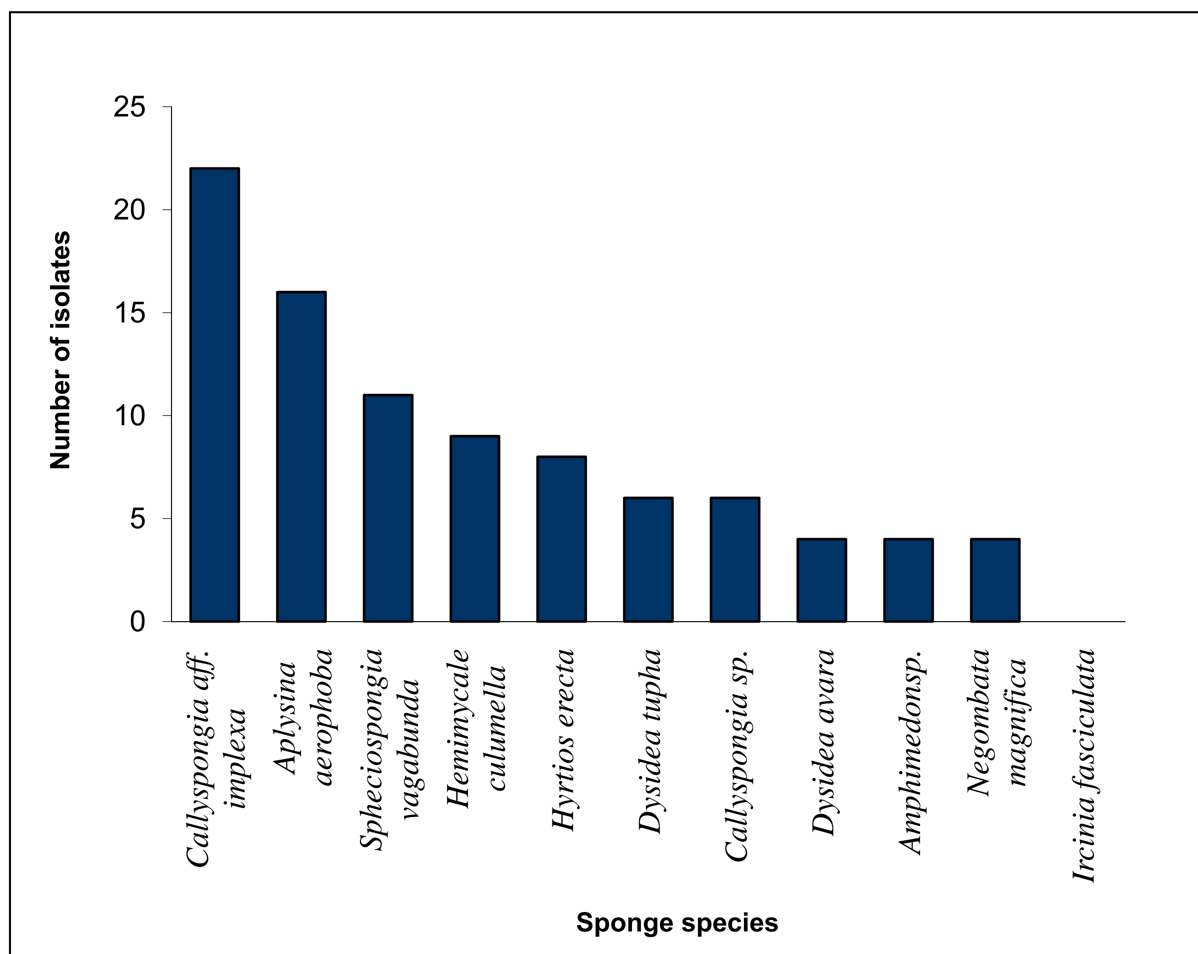


Figure 3.5 Number of actinomycete isolates per sponge species.

The actinomycetes isolated in this study displayed considerable diversity, which distributed under seven suborders with the highest number of isolates were found to be affiliated with the genus *Mycobacterium* (14.4%), followed by *Micrococcus* (13.3%), *Micromonospora* (12.2%), *Microbacterium* (12.2%), *Brevibacterium* (10%), *Kocuria* (6.6%), *Corynebacterium* (5.5%), *Rhodococcus* (5.5%), *Brachybacterium* (3.3%), *Rubrobacter* (2.2%), *Streptomyces* (2.2%), *Dietzia* (2.2%), *Salinispora* (2.2%), *Actinokineospora* (2.2%), *Gordonia* (2.2%), *Arthrobacter* (1.1%), *Nocardiopsis* (1.1%) and *Rothia* (1.1%) (**Figure 3.6**). Diverse genera have been isolated from marine sponges, specifically, *Actinoalloteichus*, *Brachybacterium*,

Brevibacterium, Curtobacterium, *Gordonia*, *Kocuria*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus*, *Salinispora* and *Streptomyces* which may therefore be common components thereof (Lee *et al.* 1998; Montalvo *et al.* 2005; Radwan *et al.* 2009). However, none of the strains isolated in our study showed sequence similarities with those previously reported from sponges, thus rendering the existence of sponge-specific actinomycete clades in the sense of Hentschel *et al*. (2002) unlikely. It has been reported that the genera belonging to *Micromonospora*, *Rhodococcus* and *Streptomyces* are the dominant actinobacterial genera in marine environments (Zhang *et al.* 2008). Representatives of all three taxa were isolated from the marine sponges studied here although other genera such as *Mycobacterium*, *Micrococcus, Microbacterium* were also represented by high numbers of isolates.

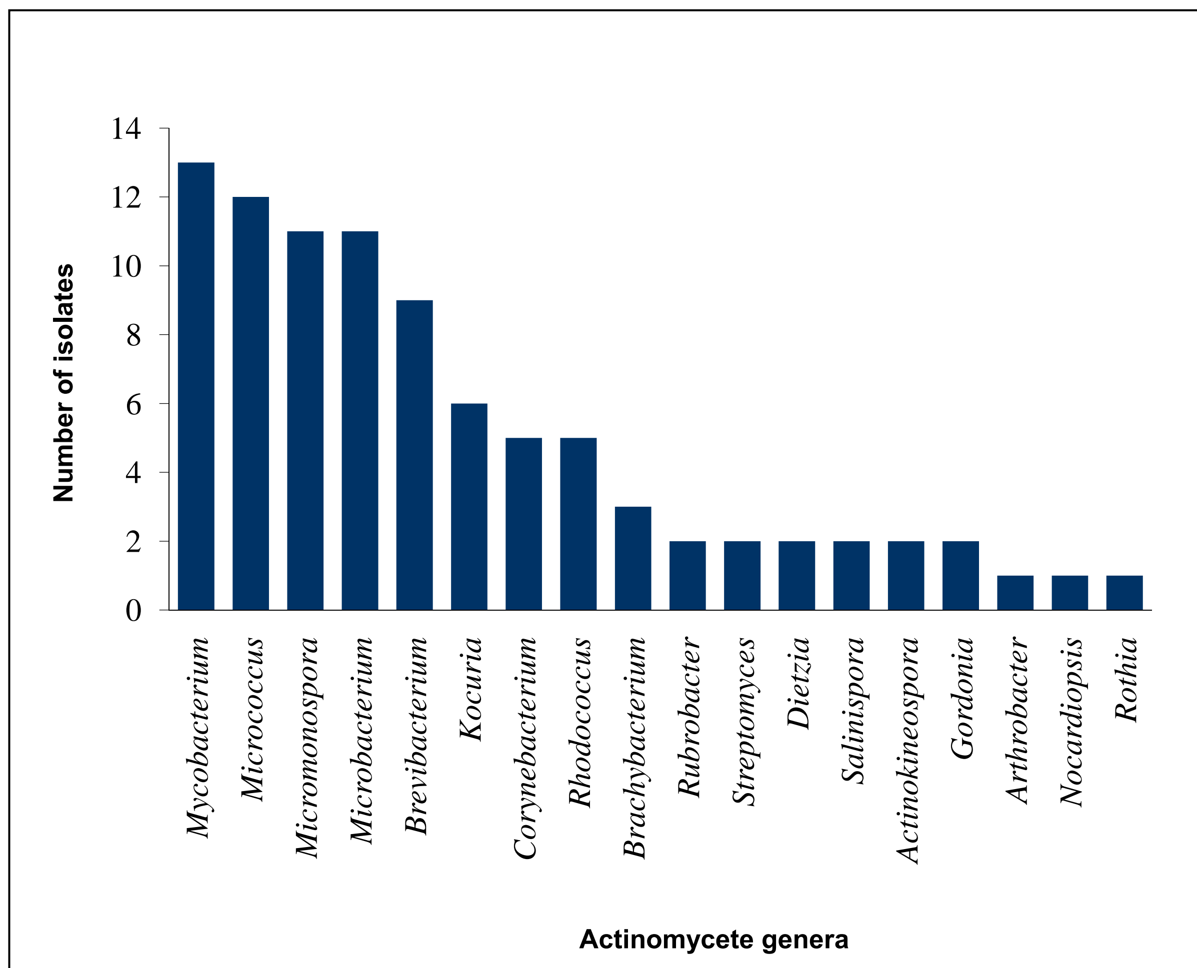


Figure 3.6 Number of actinomycete isolates per actinomycete genera.

Among the eight actinomycetes-selective media used in the study, M1 exhibited the highest recovery producing 27 isolates and MA recovered only two isolates (**Figure 3.7**). In terms of diversity, M1 also produced isolates belonging to the highest number of genera (13), followed by ISP medium 2 (11 genera), R2A (eight genera), AIA (six genera), M1 plus (five genera),

OLIGO (four genera), GAA (two genera) and the lowest diversity was observed with MA which recovered only one genus. Previous studies showed that only 0.1% of microorganisms from marine sponges were amenable to culture using standard cultivation approaches (Webster *et al.* 2001; Zhang *et al.* 2008). The diversity of recovered actinomycetes among the different media likely reflects the effect of media composition on the growth of specific strains as many marine bacterial species have unknown growth requirements and have not yet been cultured.

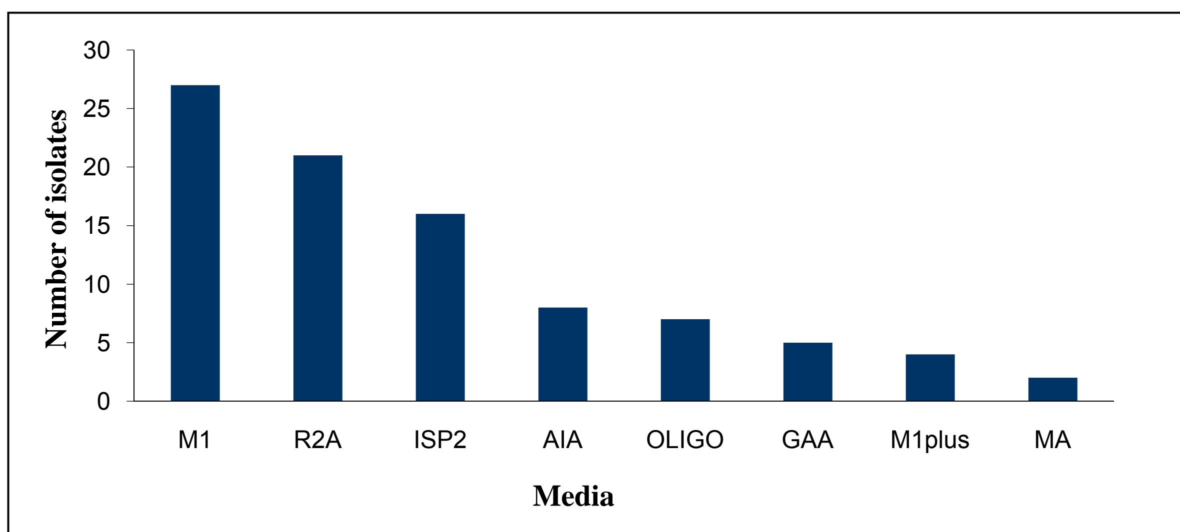


Figure 3.7 Number of actinomycete isolates per cultivation media.

Actinobacterial communities in sponges are diverse and the extent of diversity may vary between different sample collection regions, different sponge species and the isolation methods. In our study, six Red Sea sponges yielded 67 actinomycete isolates representing 13 genera. Red Sea sponges represent rich source for diverse bioactive compounds however, only few studies have been done to investigate the actinobacterial diversity of Red Sea sponges. One study has been recently carried out by Radwan *et al* (2009) to explore the microbial diversity of two Red Sea sponges, *Amphimedon* sp. and *Hyrtios erectus* using cultivation-dependent and cultivation-independent analyses. Focused cultivation on actinobacteria yielded 35 actinomycetes represented by four genera. Comparing our results with the previous study, we recovered a high diversity of culturable actinobacteria from Egyptian marine sponges. A broad study concerning diversity of actinobacteria associated with the marine sponge *Hymeniacidon perleve* from the South China Sea has been carried out by Sun et al (2010) using both culture-independent and culture-dependent methods. Eight actinobacterial genera (*Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Microlunatus*, *Mycobacterium*, *Nocardioides*, *Pseudonocardia* and *Streptomyces*) have been uncovered by the molecular method while, the cultivation techniques using seven media revealed six actinobacterial genera (*Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Salinispora* and

Streptomyces). Interestingly, only two genera (*Mycobacterium* and *Streptomyces*) have been detected by both approaches. Although, culture-based and molecular-based approaches provided very different views of actinobacterial diversity, both techniques are complementary and important in exploring the diversity of marine sponge-derived actinobacteria.

3.3.2 Phylogenetic analysis

Out of 90 isolates cultivated in this study, fourteen strains representing five suborders and belonged to eleven different genera, exhibited only 92.9-98.2% sequence similarities with the closest type strains (**IV**, **Annex**). The low sequence similarities suggest that these strains belong to novel actinomycete taxa which is further supported by phylogenetic analysis (Stackebrandt and Ebers 2006). These putatively novel isolates were affiliated to the genera *Rothia*, *Kocuria*, *Micrococcus*, *Brachybacterium*, *Brevibacterium* and *Microbacterium* (suborder *Micrococcineae*) (**Figure 3.8**). Pairwise comparison of the 16S rRNA gene sequences from the two *Microbacterium* isolates (EG4 and EG62 from *Callyspongia* sp. and *Amphimedon* sp., respectively) showed relatively high similarities (98.0 and 97.4%) to the type strains of the genus *Microbacterium*. However, the phylogenetic analysis indicated that they were diversely distributed within this genus and clustered singly in the phylogenetic tree, suggesting that isolates EG4 and EG62 might belong to new species. The high sequence similarity (99.8%) of the isolates with only three nucleotide differences suggests that they are the same strain although they were isolated from different sponge species. The other putatively novel isolates were affiliated with the following genera: *Mycobacterium* and *Rhodococcus* (suborder *Corynebacterineae)*, *Actinokineospora* (suborder *Pseudonocardineae*), *Streptomyces* (suborder *Streptomycineae*) and *Rubrobacter* (suborder *Rubrobacterineae*) (**Figure 3.9**). Comparison of the 16S rRNA gene sequences of this isolate US49 and the closest neighbours indicated that this strain was closely related to the type strain *Actinokineospora diospyrosa*. New actinomycete species have been cultivated from marine sponges in different studies. In a study carried by Tabares *et al* (2010), seventy nine actinomycetes belonging to 20 genera have been recovered from 18 different Caribbean sponge species, sediment and seawater; seven of these strains exhibited sequence similarity less than 98.1% to the validly described sequences in NCBI GenBank database, suggesting that these strains belong to putatively novel species. This highlight sponges potential as excellent source of actinobacteria especially the new species.

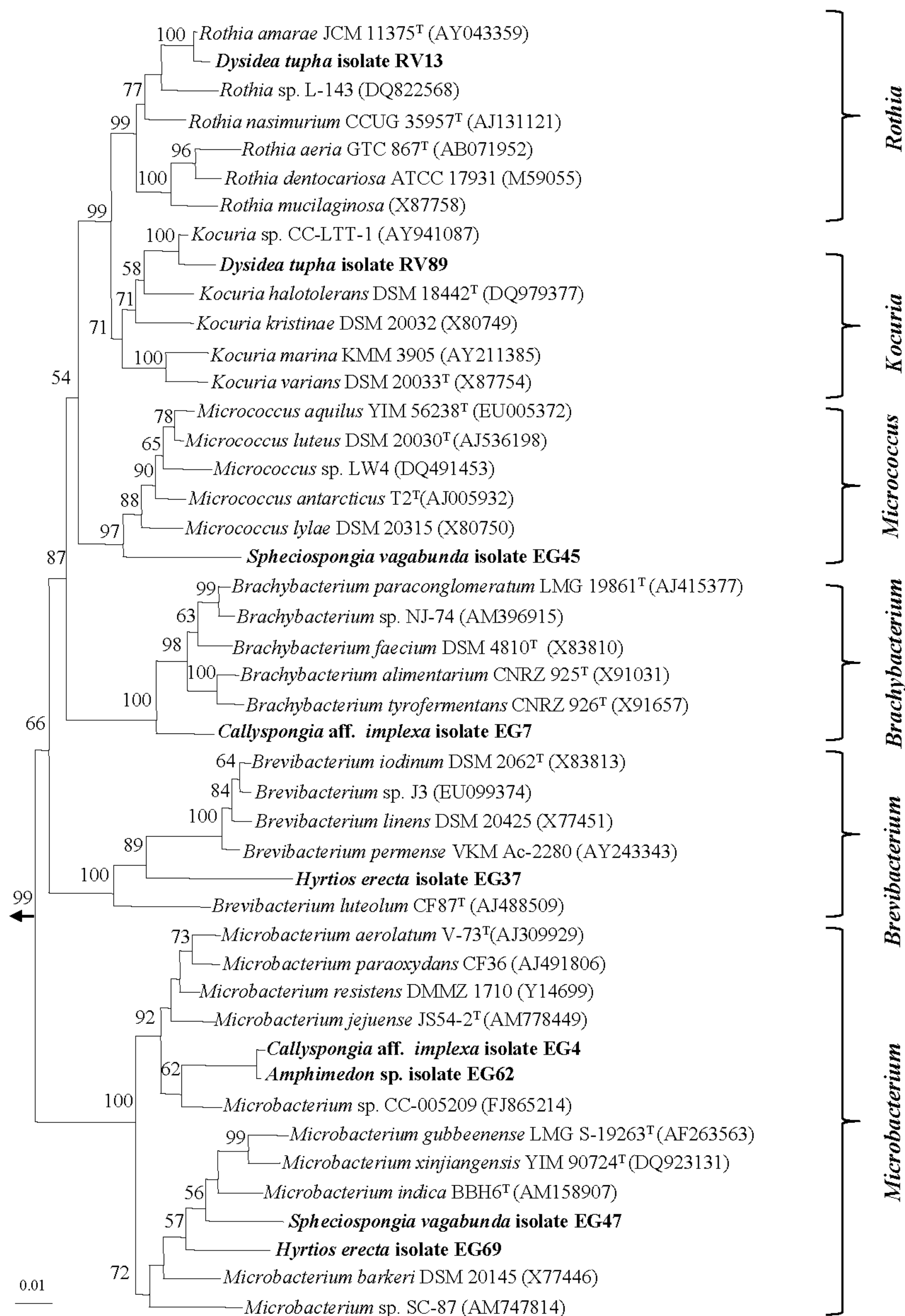


Figure 3.8 Neighbor-joining tree of the strains and representative species of the suborder *Micrococcineae* based on nearly complete 16S rRNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The arrow points to the outgroup consisting of five species belonging to *Enterobacteriaceae.* The scale bar indicates 0.01 substitution per nucleotide position.

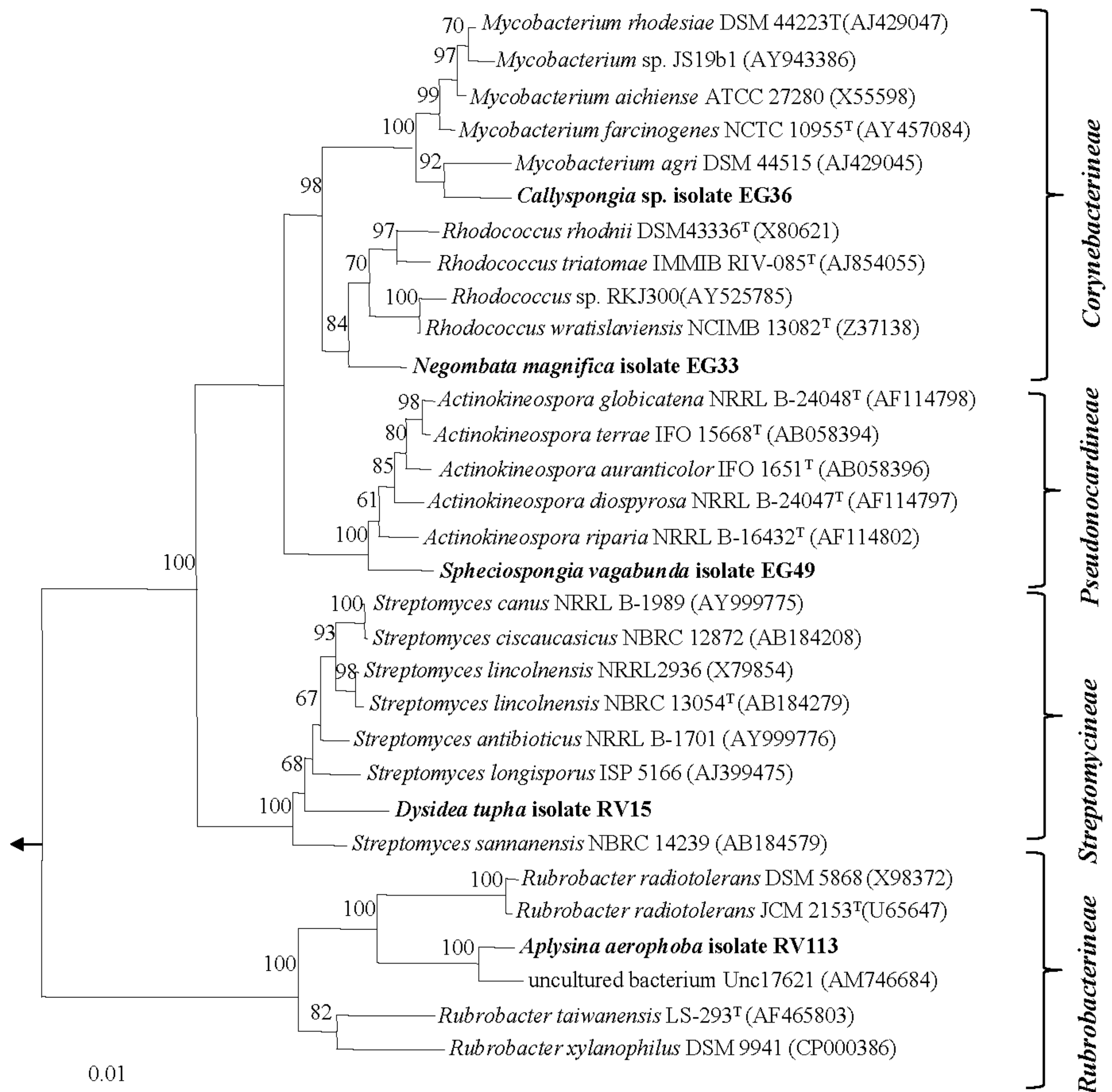


Figure 3.9 Neighbor-joining tree of the strains and representative species of the suborders *Corynebacterineae*, *Pseudonocardineae*, *Streptomycineae* and *Rubrobacterineae* based on nearly complete 16S rRNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The arrow points to the outgroup consisting of five species belonging to Enterobacteriaceae. The scale bar indicates 0.01 substitution per nucleotide position.

Eight media with different compositions were used for isolating new species and a dilution culture technique was used to isolate oligotrophic species which do not grow on nutrient-rich media. Given that the majority of microorganisms are not easily cultured using standard microbiological techniques (Rappe and Giovannoni 2003), the use of innovative isolation approach is critical for improving the recovery of actinobacteria (Webster *et al.* 2001). The use of M1 medium without added sponge extract produced the largest number of different colony morphotypes, while the addition of aqueous sponge extract to M1 medium resulted in an increase in the number of novel cultivated morphotypes but decrease in the total number of morphotypes isolated. This new approach yielded an isolate RV113 from sponge *Aplysina aerophoba* which exhibited 92.9% sequence similarities to type strains of genus *Rubrobacter* as well as to several uncultured clones (**Figure 3.9**). Phylogenetic analysis revealed that the isolate RV113 forms a distinct clade within the family *Rubrobacteraceae* in the phylogenetic tree, most closely related to *Rubrobacter radiotolerans* and the low sequence similarity values further suggest that RV113 possibly represent a novel genus within the family *Rubrobacteraceae*. This is the first report describing the isolation of genus *Rubrobacter* from marine sponges. Members of this genus are radiotolerant and slightly thermophilic with an optimum growth temperature of about 45 ^{o}C (Carreto *et al.* 1996). They have been isolated from terrestrial sources especially thermal environments and desert soils where they survive for long periods of time and grow during sporadic rainy periods (Chen *et al.* 2004). Recently, they have been described from seawater (Jensen and Lauro 2008). Very little research has been done on these organisms and little is known other than their taxonomic characterization (Suzuki *et al.* 1988). This may be interesting to go further within this new genus to know more about its members especially from the chemical point of view. These results show that variation from standard isolation protocols is a worthwhile procedure.

3.3.3 Anti-infective activity screening

A major goal of our study is to establish new sources of antibiotics and other bioactive compounds. It has been reported that the novel actinomycete taxa produce new leads with new bioactivities, making this targeted approach a more productive way to new discoveries (Fiedler *et al.* 2005; Pimentel-Elardo *et al.* 2008a). In order to avoid reisolation of already known compounds, the 14 putatively novel strains plus six additional ones based on their moderate relatedness to known metabolite producers from literature were selected for bioactivity against panel including clinically relevant, Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial pathogens, the human fungal pathogen *Candida albican*s as well as parasites (*Leishmania major*, *Trypanosoma brucei*). The antimicrobial activity was assessed by the disc diffusion assay as the diameter of inhibition zone while the antiparasitic activity

was measured in multi-well plates and is represented as the percentage of growth inhibition in comparison to a reference compound. Of the twenty strains tested, ten produced antimicrobially active metabolites inhibiting at least one tested pathogenic microorganism. Five isolates were capable of inhibiting the growth of Gram-positives only, one isolate was active against *C. albicans* only and one isolate showed activity against both groups of pathogens (**Table 3.2**). The inhibition was greatest with the *Streptomyces* sp. RV15 against *S. aureus*. Gram-negative bacteria were not susceptible to our isolates.

Table 3.2 Antimicrobial activities of sponge-derived actinomycetes.

Sponge isolate	Inhibition zone diameter (mm)		
	***S. aureus* 8325**	***E. faecalis* JH212**	***C. albicans* S314**
Actinokineospora sp. EG49*	0	0	12
Dietzia sp. EG67	13	0	0
Microbacterium sp. EG69*	10	9	0
Micromonospora sp. RV115	12	10	0
Rhodococcus sp. EG33*	12	8	0
Rubrobacter sp. RV113**	9	0	0
Streptomyces sp. RV15*	17	11	13

*: putatively new species; **: putatively new genus.

In terms of the antiparasitic activity, four isolates exhibited activity against *T. brucei* and two isolates showed activity against *L. major* (**Table 3.3**). The putatively novel *Actinokineospora* sp. EG49 showed highest antitrypanosomal potential at 48% growth inhibition. The inhibitory activity of these strains against a variety of pathogens suggested that these actinobacterial strains may be potential candidates for the production of bioactive compounds. Antiparasitic activities from marine-derived actinomycetes are reported only sporadically (Loiseau *et al.* 2002). The cyclic depsipeptide, valinomycin that has been purified from sponge-derived *Streptomyces* sp. strains 22, exhibited significant inhibitory activities against the parasites *L. major* (IC50 < 0.11 µM) and *T. brucei* (IC50 3.2 nM) (Pimentel-Elardo *et al.* 2010).

Table 3.3 Antiparasitic activities of sponge-derived actinomycetes.

Sponge isolate	% Growth inhibition	
	L. major	***T. brucei TC 221***
Actinokineospora sp. EG49*	24	48
Brevibacterium sp. EG10	0	30
Gordonia sp. EG50	36	28
Kocuria sp. RV89*	0	19

*: putatively new species.

Five putatively novel actinomycetes exhibited antibacterial and antifungal activities while two strains showed antiparasitic activity and one strain related to genus *Actinokineospora*, showed both antifungal and antiparasitic potential. Because crude extracts were tested for bioactivities, the chemical nature of the bioactive compound is currently unknown. Nevertheless, some genera, to which the novel isolates belong to, are well known and prolific metabolite producers. For example, the genus *Streptomyces* accounts for about 80% of all natural products recovered from actinomycetes to date (Bull and Stach 2007) and produced the of majority of the antibiotics available in the market (Watve *et al.* 2001). As one example, *Streptomyces sannanensis* produces new aminoglycoside antibiotics sannamycins A, B and C with potent activities against Gram-positive bacteria (Deushi *et al.* 1980). *Rhodococcus* species, of which one putatively novel and bioactive strain was isolated in this study, produced a number of commercially interesting and potentially useful products including various types of steroids and peptides (Bell *et al.* 1998). For example, two antimycobacterial cyclic peptides, lariatins A and B, were isolated from the culture broth of *Rhodococcus* sp. K01-B0171 (Iwatsuki *et al.* 2007). The genus *Microbacterium*, of which two putatively new species were isolated in this study, produced different glycolipids. For example, five anti-tumor glycoglycerolipids were obtained from *Microbacterium* sp. which was isolated from the sponge *Halichondria panicea* (Wicke *et al.* 2000). Antimicrobial activities have to our knowledge not been reported for the genera *Actinokineospora*, *Kocuria* and *Rubrobacter*, making them good candidates for further chemical analysis to identify the bioactive candidates.

3.3.4 Conclusion and Outlook

Considerable actinobacterial diversity was recovered from eleven marine sponges from two different geographical locations. Altogether 90 actinomycete isolates were affiliated with 18 different genera including 14 putatively novel species. One possibly new genus related to *Rubrobacter* was isolated on M1 agar amended with sponge extract. Antibacterial, antifungal, antitrypanosomal and antileishmanial activities were reported for 10 of the isolates including six putatively novel species. Marine sponges represent therefore a still largely untapped resource for novel actinomycete diversity as well as for new bioactivities.

Our future plans will include physiological, biochemical and chemotaxonomical characterization to validate the taxonomic position of the putatively novel actinomycete isolates cultivated in this study. The bioactive new strains will be chemically investigated using different chromatographic and spectroscopic techniques to identify and characterize the anti-infective metabolites.

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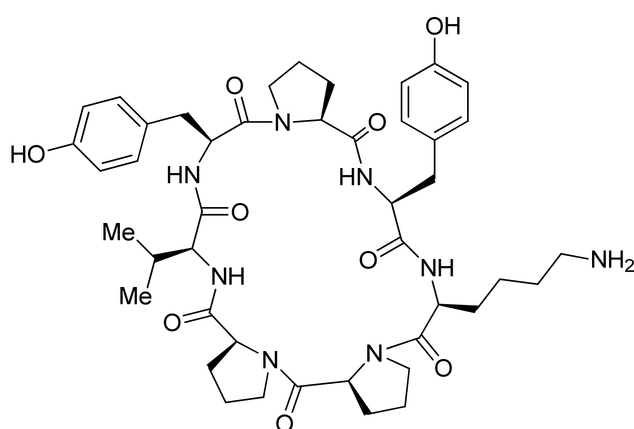
Chapter 4

New cyclodysidins from the new *Streptomyces* strain RV15

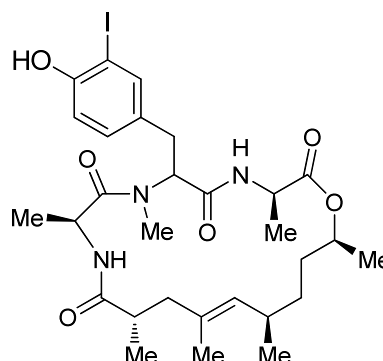
4.1 Introduction

4.1.1 Cyclic peptides from the marine environment

The marine environment continues to yield a diverse array of biologically active molecules, a remarkable number of which are peptides (Fenical 1993). Cyclic peptides are polypeptide chains whose amino and carboxyl termini are themselves linked together with a peptide bond that forms a circular chain. A number of cyclic peptides have been discovered in nature and they can range from just a few amino acids to hundreds in length. The increased discovery of cyclic peptides has enriched our understanding of their chemical behavior and the associated pharmacological activities. One property of cyclic peptides is that they tend to be extremely resistant to the process of digestion, enabling them to survive intact in the human digestive tract. This makes them very interesting candidates for drug development especially when oral delivery is required. Cyclic peptides have been isolated from different marine invertebrates especially sponges which produced large number of cyclic peptides and cyclodepsipeptides with unique structures and diverse biological activities (Vera *et al.* 2009; Andavan and Lemmens-Gruber 2010; Fusetani 2010). The cyclic heptapeptide, stylissamides A has been isolated from the Caribbean sponge *Stylissa caribica* (Schmidt *et al.* 2007). Another example is the callyaerin G which has been isolated from the Indonesian sponge *Callyspongia aerizusa* and was found to be cytotoxic towards the mouse lymphoma cell line (L5178Y) and HeLa cells with ED50(s) of 0.53 and 5.4 µg/ml, respectively (Ibrahim *et al.* 2008). Four new cytotoxic cyclodepsipeptides, geodiamolides, have been isolated from the sponge *Pseudaxinyssa* sp. with *in vitro* cytotoxicity against different cell lines (de Silvaa *et al.* 1990).

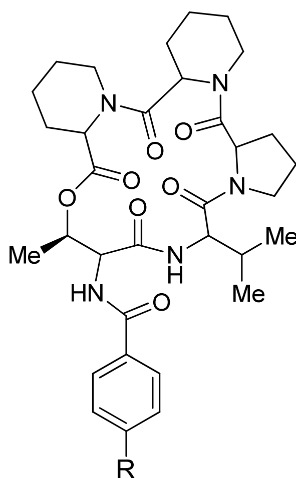


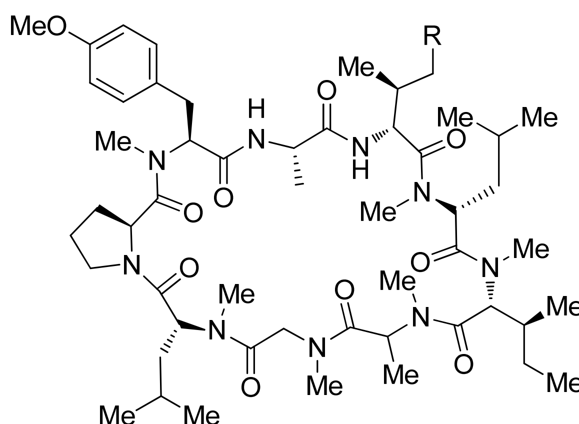
Stylissamide A



Geodiamolide A

Cyclic peptides have been also reported from various marine fungi (Cueto *et al.* 2000; Tan *et al.* 2003; Meyer *et al.* 2010) such as the petrosifungins which have been purified from *Penicillium brevicompactum,* isolated from the Mediterranean sponge *Petrosia ficiformis* (Bringmann et al. 2004). The cyclic peptides, clonostachysins A and B have been purified from a marine sponge-derived fungus *Clonostachys rogersoniana* and exhibited a selectively inhibitory effect against the dinoflagellate *Prorocentrum micans* at a concentration of 30 mM (Adachi *et al.* 2005).

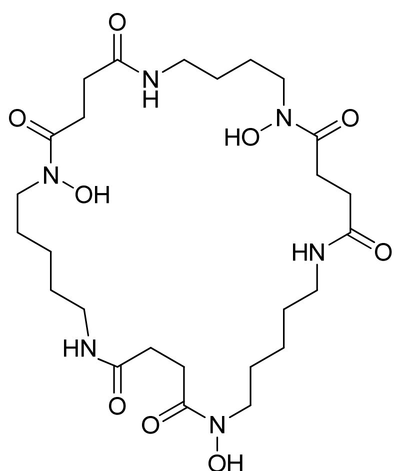




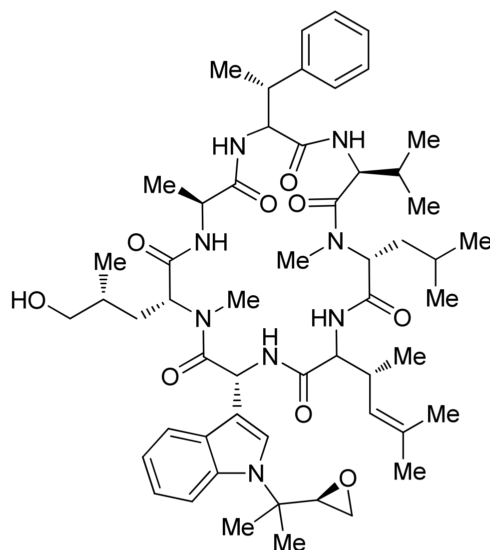
Petrosifungin A: R = H
Petrosifungin B: R = OH

Clonostachysin: A R = H
Clonostachysin: B R = Me

Cyclic peptides with diverse biological activities have been isolated from the marine bacteria such as cyanobacteria and members of the genus *Bacillus* (Luesch *et al.* 2001; Zhang *et al.* 2004; Plaza and Bewley 2006; Seto *et al.* 2007; Sivonen *et al.* 2010). Few representatives have been isolated from marine actinomycetes (Schultz *et al.* 2008). Cyclomarins are cyclic heptapeptides that have been isolated from the marine *Streptomyces* strain CNB-982 and exhibited antiviral and anti-inflammatory activities (Renner *et al.* 1999). Nocardamine is a dimeric cyclic peptide that was purified from a *Streptomyces* isolate which was recovered from an unidentified sponge (Lee *et al.* 2005). Nocardamine is related to the ferrichrome antibiotics and exhibited significant binding affinity to ferric ion displaying antibacterial activity against mycobacteria. The cyclic depsipeptide, valinomycin, was purified from *Streptomyces* sp. recovered from sponge *Aplysina aerophoba* and is known as potent antibiotic, exhibiting also good antiparasitic activities against *Leishmania major* and *Trypanosoma brucei* (Pimentel-Elardo *et al.* 2010).

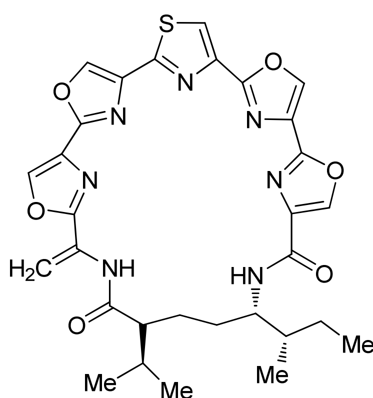


Nocardamine



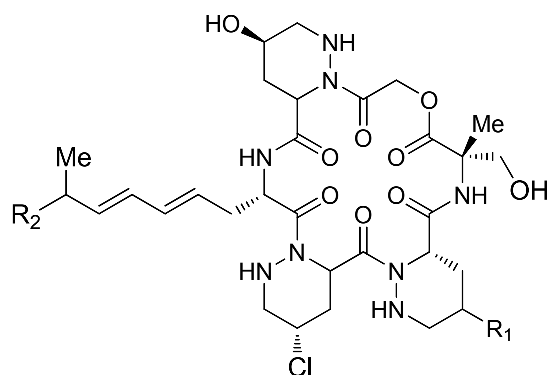
Cyclomarin

In addition to the anti-infective activity of cyclic peptides from marine actinomycetes, mechercharmycin A is an example of anticancer cyclic peptide which has been isolated from a marine-derived *Thermoactinomyces* sp. strain YM3-251 collected at Mecherchar in the Republic of Palau (North Pacific Ocean). Mechercharmycin A showed cytotoxic activities against human lung adenocarcinoma A549 and Jurkat leukemia cells with IC50 values of 0.04 µM (Kanoh *et al.* 2005).



Mechercharmycin A

Another example is the cyclic hexadepsipeptide, piperazimycin A which has been isolated from the fermentation broth of a *Streptomyces* sp. strain cultivated from marine sediments. Piperazimycin A exhibited potent in vitro cytotoxicity toward multiple tumor cell lines with a mean GI50 of 100 nM (Miller *et al.* 2007).

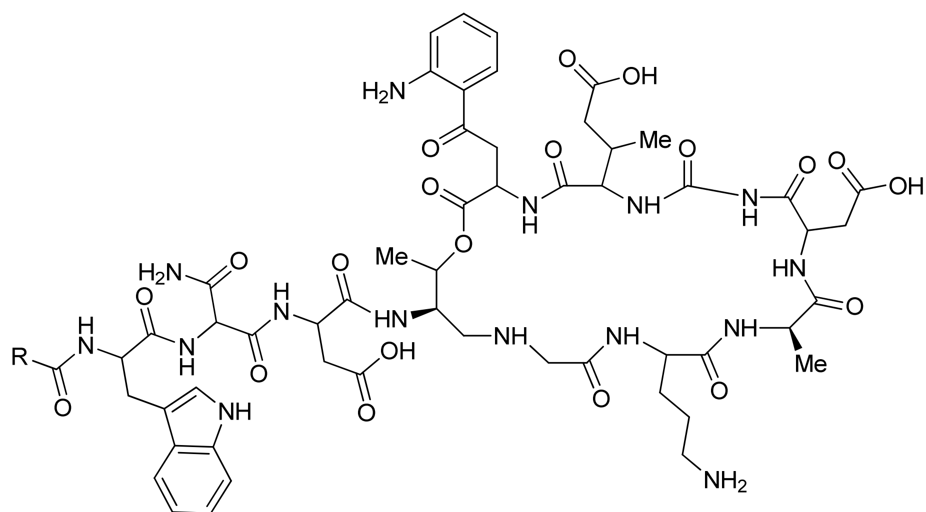


Piperazimycin A: R1 = OH, R2 = CH_3

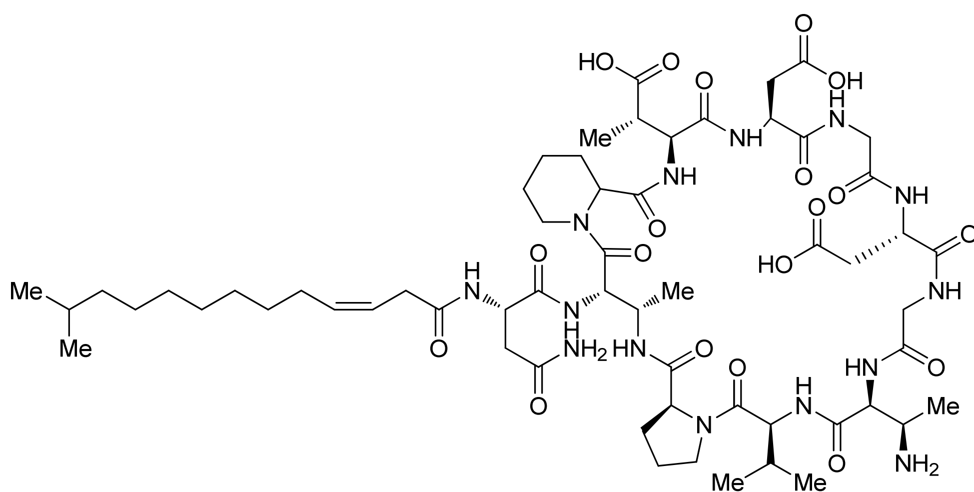
Piperazimycin B: R1 = H, R2 = CH_3

Piperazimycin C: R1 = OH, R2 = CH_2CH_3

Moreover, cyclic peptides could be modified in a different ways such as sulfation, glycosylation or lipidation. Among these combinations, cyclic lipopeptides (CLPs) have received considerable attention due to their interesting activities which vary from antimicrobial, cytotoxic, to surfactant activities (Nielsen *et al.* 2002; de Bruijn *et al.* 2007). CLPs consist of an acyl side chain linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. CLPs are diverse both structurally and in terms of their biological activity. The structural diversity is due to differences in the length and composition of the fatty acid tail as well as the variations in the number, type and configuration of the amino acids in the peptide part. *Streptomyces roseosporus* for example, produces daptomycin which is a cyclic lipopeptide consisting of 13 amino acids which includes three D-amino acid residues (D-asparagine, D-alanine, and D-serine) linked to decanoic acid (Huber *et al.* 1988). The high ratio of D- amino acids than the normal L-form confirm that these compounds have been biosynthesized to be more resistant to environmental conditions and also to perform special biological functions. Daptomycin was licensed by the FDA in the United States for use against skin and soft tissue infections in 2003 and for methicillin-resistant *S. aureus* (MRSA) infections of the bloodstream in 2006 (Diederen *et al.* 2006). Friulimicin B is a naturally occurring cyclic lipopeptide which was purified from the fermentation broth of actinomycete *Actinoplanes friuliensis*. Friulimicin B is highly active against a broad range of gram-positive bacteria including antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus*, enterococci and obligate anaerobes.



Daptomycin R = decanoyl



Friulimicin B

4.1.2 Aim of the work

The main goal of the present study was the isolation, identification, and structural elucidation of new and preferably biologically active secondary metabolites from marine sponge-associated actinomycetes. After our biological screening of 14 putatively novel actinomycete strains cultivated from marine sponges, six actinomycetes were found to produce metabolites capable of inhibiting growth of at least one pathogen. Bioactive and taxonomically novel strains were fermented at optimized conditions and the crude extracts were subjected to various chromatographic methods in order to obtain pure compounds. The compounds were then identified using different spectroscopic tools and the biological activity was evaluated for the pure compounds.

4.2 Materials and Methods

4.2.1 General procedure

Melting points were determined on a Reichert-Jung Thermovar hot-plate. IR spectra were measured on a Jasco FT/IR-410 spectrometer. UV spectra were recorded on Varian CARY-50-Conc-UV-visible spectrophotometer. Optical rotations were carried out on Perkin-Elmer 241MC polarimeter (25 °C, 10 cm cell). Accurate electrospray ionization mass spectra (ESI) were obtained by a Bruker Daltonics micrOTOF focus. Column chromatography was performed using silica gel 60 (0.032-0.064 mm, Merck), while pre-coated silica gel 60 F_{254} plates (Merck) were used for TLC analyses. ^{1}H NMR (400 MHz, 600 MHz) and ^{13}C NMR (151 MHz) spectra were recorded on Bruker Avance 400 and DMX 600 instruments. Proton-detected, heteronuclear correlations were measured using HSQC (optimized for ^{1}JHC = 145 Hz) and HMBC (optimized for ^{n}JHC = 8.3 Hz or ^{n}JHC = 4.0 Hz) pulse sequences. ROE effects were measured using a standard pulse sequence from the standard Bruker pulse program library. The samples were dissolved in deuterated solvents ($CDCl_3$, CD_3OD). Residual solvent signals of (CD3OD at 3.305 ppm,^{1}H, and 49.0 ppm,^{13}C), ($CDCl_3$ at 7.26 ppm and 77.0 ppm) were considered as internal reference signal for calibration. The observed chemical shift values (δ) were recorded in ppm and the coupling constants (*J*) in Hz. Preparative HPLC separation was performed on Jasco system (pump PU1580, gradient unit LG-980-025, degasser DG-2080-53, UV detector MD-2010Plus) on a Chromolith SemiPrep RP18e 10 x 100 mm (Merck) column, while the analytical detection was done on HPLC Jasco system (pump PU1580, gradient unit LG-980-025, degasser DG-2080-53 and UV detector MD-2010Plus) on a Chromolith RP-18e column (4.6 x 100 mm, Merck). ESI MS/MS spectra were performed using Orbitrap LTQ XL (Thermo Scientific). Five microliters of sample was loaded into a nanospray needle, voltage 5 kV, source temperature 275 °C, using MeOH supplemented with 0.1% HCOOH as solvent. For the recording of ESI MS/MS spectra the quadrupole was operated as a mass filter and used to select the desired precursor ion isotope cluster. During product ion analysis the resolving quadrupole was set to transmit *a* ± 2 *m*/*z* window around the precursor ion. Precursor ions were directed into a hexapole collision cell. The collision gas was helium and the collision energy was optimized for each analysis.

4.2.2 Fermentation of *Streptomyces* strain RV15

Sixteen 2 L Erlenmeyer flasks, each containing 1 L of ISP2 medium were inoculated with 0.5 ml of 7 days culture of *Streptomyces* RV15 and shaking at 150 rpm at 30 °C for ten days.

4.2.3 Purification and characterization of the cyclic peptides

After ten days of cultivation, sterilized XAD-16 resin (30 g L^{-1}) was added. Resin was collected 6 h later by filtration and eluted with acetone. The acetone was removed under reduced pressure, and the aqueous layer was extracted with ethyl acetate (3 x 500 ml). The combined extracts were concentrated under vacuum and stored at 4 °C for chromatographic fractionation. After fractionation of crude extract (10 g) via silica gel chromatography, the fraction (935 mg) eluted with 40% chloroform in methanol was further chromatographed on sephadex LH 20. The fraction (56 mg) eluted with 70% methanol in water, was enriched in cyclic peptides. Further purification was carried out on HPLC using an acetonitrile (ACN) and water solvent mixture complemented by 0.05% trifluoroacetic acid: (10% ACN:H_2O to 100% ACN over 20 min at flow rate of 10 ml/min), giving four pure compounds.

Cyclodysidin A: 2 mg of white amorphous powder (Rt 7.3 min); $[\alpha]^{20}$ D -19 (*c* 0.55, MeOH); UV (MeOH): λ_{max} (log ε) = 220 (5.75), 235 (12.0) nm; IR: 3445, 2924, 1730, 1638, 1553, 1463, 1238, 1160, 1095, 730, 667 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 4.1**; ESI-MS *m/z* $[M + H]^+$ 1006.5207 (calcd for $C_{45}H_{71}N_{11}O_{15}$, 1006.5209).

Cyclodysidin B: 1.5 mg of white amorphous powder (Rt 7.7 min); $[\alpha]^{20}$ D -15.3 (*c* 0.55, MeOH): UV (MeOH): λ_{max} (log ε) = 223 (3.82), 238 (13.13) nm; IR: 3451, 2935, 1740, 1633, 1549, 1455, 1243, 1165, 1089, 738, 661 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 4.2**; ESI-MS *m/z* $[M + H]^+$ 1020.5364 (calcd for $C_{46}H_{73}N_{11}O_{15}$, 1020.5366).

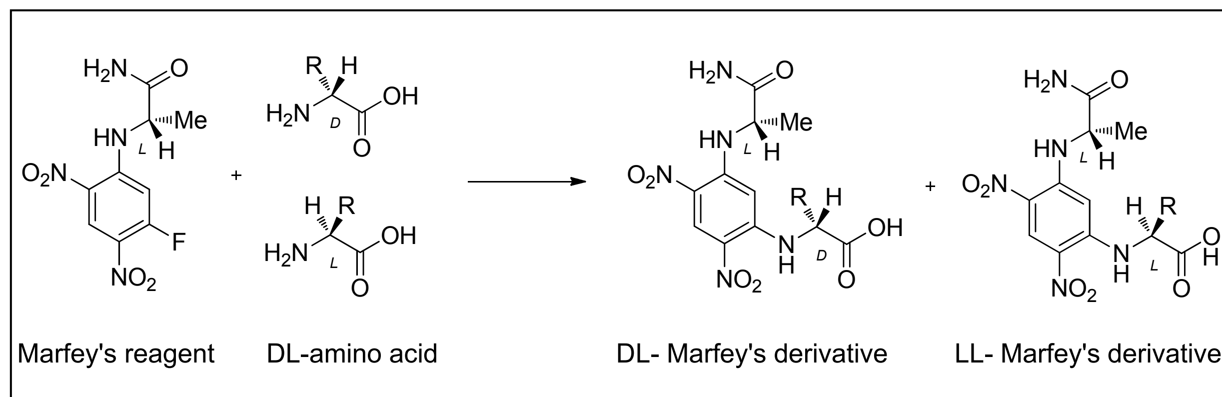
Cyclodysidin C: 2 mg of white amorphous powder (Rt 8.2 min); $[\alpha]^{20}$ D -22.5 (*c* 0.55, MeOH); UV (MeOH): λ_{max} (log ε) = 222 (4.3), 237 (14.2) nm; IR: 3440, 2920, 1735, 1629, 1560, 1450, 1235, 1158, 1090, 729, 665 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 4.3**; ESI-MS *m/z* $[M + H]^+$ 1034.5536 (calcd for $C_{47}H_{75}N_{11}O_{15}$, 1034.5522).

Cyclodysidin D: 1.7 mg of white amorphous powder (Rt 8.5 min); $[\alpha]^{20}$ D -27.7 (*c* 0.55, MeOH); UV (MeOH): λ_{max} (log ε) = 224 (6.55), 238 (13.63) nm; IR: 3442, 2928, 1735, 1632, 1557, 1460, 1238, 1163, 1088, 733, 662 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 4.4**; ESI-MS *m/z* $[M + H]^+$ 1062.5832 (calcd for $C_{49}H_{79}N_{11}O_{15}$, 1062.5835).

4.2.4 Marfey's derivatization

Since this method is important to identify the stereochemistry of our compounds, we will introduce it her. Marfey's reagent (MR) or 1-fluoro-2,4-dinitrophenyl-5-L-alanine amid (FDAA) was used to separate and determine enantiomeric amino acids in mixtures (Marfey 1984). It reacts in a stochiometric way with the amino group of the enantiomeric amino acid producing diastereoisomers that can be resolved via HPLC analysis (Kochhar and Christen 1989) in a

non chiral environment. The diastereoisomers derivatives are relatively stable if kept at 4 °C and protected from light. The detection of Marfey's derivatives is easy due to the new chromophore which strongly absorbs at 340 nm, allowing detection in the nanomolar range. D-diastereomer exhibited stronger intramolecular hydrogen bonding producing a more hydrophobic molecule which interact more strongly with the reversed phase of the column and thus have a stronger retention time and eluted later than an L-diastereomer (Bhushan and Bruckner 2004).



One milligram of each peptide was hydrolyzed by heating in a sealed vial with 6 M HCl (2 ml) at 110 °C for 24 h. The hydrolysate was cooled to RT, dried under vacuum and dissolved in 250 µl of water. Marfey's derivatization (Marfey 1984) was done by adding 100 µl of 1% of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amid) in acetone to 50 µl of acid hydrolysate followed by 20 µmol of $NaHCO_3$, the contents were mixed and incubated at 40 °C for 1 h with frequent mixing. After cooling, the reaction was stopped by adding 20 µmol HCl, followed by drying under vacuum and finally dissolving in 1 ml of MeOH-H_2O (1:1). Amino acid standards were individually derivatized in the same manner using 2.5 µmol of amino acid standard to react with 3.6 µmol FDAA to obtain the Marfey's derivative of amino acid. The obtained derivatives were filtered and analyzed by reversed phase HPLC using a linear gradient of CH_3CN in 0.1% (v/v) aqueous TFA (20- 70% CH3CN over 50 min), flow rate 1 ml min^{-1} and detection at 340 nm. After comparison of Retention time (Rt) of the hydrolysate derivatives with that of derivatized standards, absolute configuration of diastereoisomers was detected in each peptide hydrolysate. Retention times (min) of authentic amino acids were as follows: L-Thr (29.7), D-Thr (31.3), L-allo-Thr (29.8), L-Asp (27.6), D-Asp (32.8), L-Glu (28.9), D-Glu (30.3), L-Ser (23.5), D-Ser (28.3), L-Tyr (31.9), D-Tyr (33.7). A better resolution of the L-Thr and the L-allo-Thr derivatives was achieved using a linear gradient of CH_3CN in 0.1% (v/v) aqueous TFA (23- 35% CH3CN over 50 min): L-Thr (33.5), L-allo- Thr (37.8).

4.2.5 Bioactivity testing

The antibacterial, antifungal, cytotoxicity and protease inhibition assays were performed by the SFB 630 collaboration partners: TP Z1 (T. Öschläger, U. Würzburg) and TP A4 (T. Schirmeister, U. Würzburg), respectively. The anticancer activity against melanoma and ovarian tumor cell lines were carried out in the lab. of S. Mejerjohann (SFB 487, TP B3, U. Würzburg) and J. Wischhusen (Department of Gynaecology, School of Medicine U. Würzburg), respectively.

Antibacterial activity

Antibacterial activity was tested against a panel including *Enterococcus faecalis* JH212, *Enterococcus faecium* 6413, *Staphylococcus aureus* NCTC 8325, *Staphylococcus epidermidis* RP62A. After 24 h incubation at 37 °C, broth cultures were diluted in Müller-Hinton broth (1:100) and cultivated again until the cells reached the exponential growth phase. 1 x 10^5 cells/ml were incubated in the presence of various concentrations of the tested compounds in DMSO to a final volume of 200 µl in a 96-well plate at 37 °C. The final concentration of DMSO was 0.8% in each well. After 18 h of incubation, the optical density of the cultures was determined at 550 nm using an ELISA microplate reader with respect to the control without bacteria. The lowest concentration of the compound that inhibits bacterial or fungal growth was defined as the minimal inhibitory concentration (MIC).

Antifungal activity

Antifungal activity testing was done by resuspending a colony of *Candida albicans* 5314 (ATCC 90028) in 2 ml of 0.9% NaCl. Four microliters of this suspension was transferred to 2 ml of HR medium. Various concentrations of the test compounds were diluted in 100 µl of medium in a 96-well microplate with final DMSO concentration of 0.4%. One hundred microliters of the Candida suspension was added to each well then incubated at 37 °C for 48 h. Optical density was measured at 530 nm with respect to a control well without Candida cells. The lowest concentration of the compound where no growth is detectable was used as the MIC.

Antiprotease activity

SARS M and PI protease inhibition assay

The fluorometric enzyme assays were carried out on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) with a microplate reader (excitation 325

nm, emission 425 nm) and using 96-well microplates (Nunc GmbH, Wiesbaden, Germany) were used. Assays were done at 25 °C in a 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 12.5% DMSO (final concentration) in a total volume of 200 μl. The 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] was used at final concentration of 50 μM, and the final enzyme concentration was 4.25 μg ml^{-1}. Inhibitors were used at 100 μM final concentration for preliminary screening. For determination of *K*m values, the substrate was used in concentrations between 50 and 300 μM. Values were corrected for the inner filter effect. Increase in fluorescence was determined over a period of 10 min for *K*m determination and 20 min for inhibition assays. Substrate and inhibitor stock solutions were prepared in DMSO after dilution in assay buffer. The *K*m value was calculated by nonlinear regression analyses using the program GraFit. All values are mean values from at least three independent assays (Kaeppler *et al.* 2005).

Cathepsin L and B protease inhibition assay

The fluorometric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 365 nm, emission 460 nm) and 96-well microplates. Assays were performed at 25 °C in a 20 mM Tris-HCl buffer (pH 6.0) containing 5 mM EDTA, 2.5 mM DTT, 200 mM NaCl and 0.005% Brij 35 in a total volume of 285 ml. Substrate (Cbz-Phe-Arg-AMC for both enzymes) and inhibitor stock solutions were prepared in DMSO (10% final concentration) after dilution with assay buffer; enzymes were held in buffer. The final substrate concentration for inhibition assays was between 10.0 and 81.0 mm; the final enzyme concentration was 53 ng ml^{-1} for CL (P. tetraurelia) and 58 pg ml^{-1} for CB (recombinant, human liver) (Vicik *et al.* 2006a). Inhibitors were tested at final concentration of 100 µM.

Rhodesain protease inhibition assay

The fluorometric enzyme assays were performed as described in Cathepsin assays using 50 mM acetate (pH 5.5) containing 5 mM EDTA, 5 mM DTT, 200 mM NaCl, and 0.005% Brij 35 in a total volume of 285 µl. Substrate (Cbz-Phe-Arg-AMC) and inhibitor stock solutions were prepared in DMSO (10% final concentration) and diluted with the assay buffer. The final substrate concentrations used in the inhibition assays ranged from 12.4 to 81.0 µM and the final enzyme concentration was 41 nM. Inhibitors were used at final concentration of 100 µM (Vicik *et al.* 2006b).

Cytotoxicity

J774.1 macrophages and kidney epithelial 293T cells were cultured in complete medium (DMEM medium for kidney epithelial cells) without phenol red in the absence or presence of increasing concentrations of the test compounds at a cell density of 1 x 10^5 cells/ml (2 x 10^4 cells/ml for kidney epithelial cells) 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 optical densities were measured at 24, 48 and 72 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.

Anticancer activity

Melanoma testing

Human melanoma cell lines A375 and Mel Juso were seeded at a density of 1x10^4 cells per well of a 12-well plate and were cultivated in DMEM containing 10% FCS, either in the presence or absence of the test compounds. Cells were harvested by trypsinization after 3, 5, and 8 days, pelleted, resolved in PBS and counted under the microscope.

Ovarian cell carcinoma testing

SKOV3 ovarian carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were seeded in microtiter plates 10000/well and 24 h later, different concentrations of test substances were added and serially diluted. The cells were incubated at 37 °C for 24 h. The medium was removed from the cells and cells were rinsed with PBS 100 µl/well. Fifty microliters of crystal violet was added with shaking for 20 min at room temperature. Stain was discarded and the excess of the crystal violet was washed with water till clearance. The plates were dried overnight and 200 µl methanol/well was added with for 20 min at room temperature. Absorbance was then measured by ELISA reader at 540 nm.

4.3 Results and Discussion

4.3.1 Isolation and structure elucidation of cyclodysidins

Among all actinomycete strains tested in our study against different bacteria, Candida and parasites, *Streptomyces* sp. RV15 gave the greatest inhibition against *Staphylococcus aureus* as well as against *Candida albicans*. This strain was isolated from sponge *Dysidea tupha*, collected from Rovinj (Croatia) in 2008. The strain was fermented in ISP2 medium and the secondary metabolites were adsorbed on XAD-16 resin. The resin was then eluted with acetone and the crude extract was fractionated via normal phase silica gel chromatography followed by gel filtration on Sephadex LH-20 to give the fraction enriched in peptides (**Figure 4.1**).

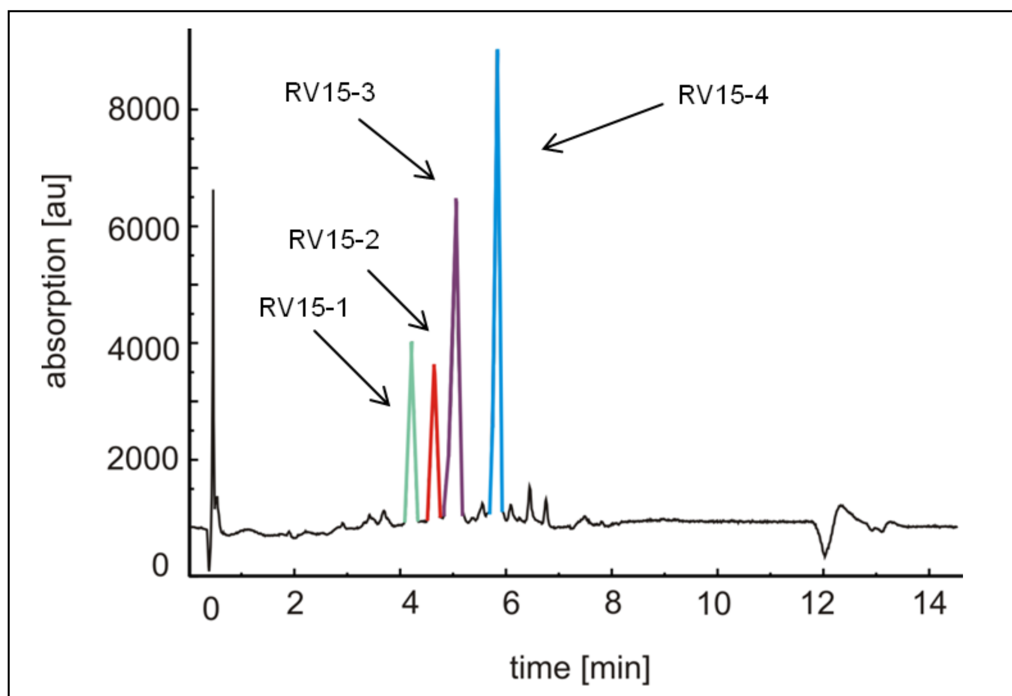


Figure 4.1 HPLC chromatogram of *Streptomyces* strain RV15 peptidic fraction at 220 nm.

Final purification was performed on reversed phase HPLC (Chromolith SemiPrep RP18e 10 x 100 mm (Merck) column, with the solvents (A) Water (0.05% trifluoroacetic acid) and (B) MeCN (0.05% trifluoroacetic acid) as the eluents, linear gradient, 0 min 10% B, 20 min 100% B, flow rate 10 ml min^{-1}) to afford four pure compounds, designated, cyclodysidins A-D. cyclodysidin A, showed molecular formula $C_{45}H_{71}N_{11}O_{15}$ through ESIMS analysis (measured, *m/z* 1006.5207, $[M+H]^+$) with 16 degrees of unsaturation. The presence of several doublets, and doublets of doublets at 4-5 ppm (**Figure 4.2**), attributed to α-protons of amino acids as well as the characteristic chemical shifts for the amide carbonyls at 170-180 ppm, suggesting a peptidic nature of the molecules. Moreover, the IR spectrum showed bands at 3381, 3260,

and 1668 cm^{-1}, confirming the presence of amide functionality. The compounds gave no colour with ninhydrin reagent but they gave purple colour upon hydrolysis with 6 M HCl, indicating a cyclic nature.

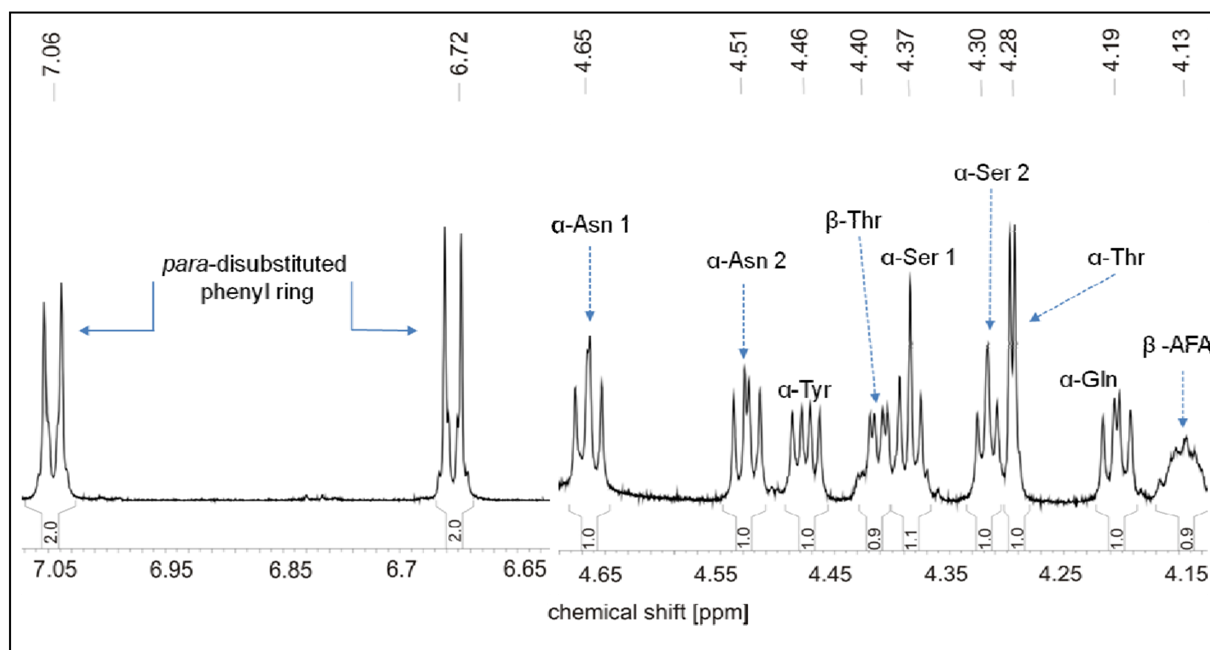


Figure 4.2 Characteristic regions of ^{1}H NMR spectrum of compound cyclodysidin A.

The ^{13}C NMR spectrum displayed eleven ester/amide-type carbonyls (δ 172.4, 172.9, 173.0, 173.7, 174.1, 174.3, 174.4, 174.5, 174.9, 175.1, 176.6), seven α-methine carbons (*δ* 52.3, 52.4, 55.6, 56.9, 57.2, 57.4, 60.0), two primary carbinols (*δ* 62.3, 62.4), a secondary carbinol (*δ* 67.3), tertiary carbinol (*δ* 157.3), aromatic signals for a di-substituted phenyl ring (131.3, 129.0, 157.3, and 116.4) and distinct signals at δ 23.6, 30.1-30.4, 35.7 and 14.4 consistent with a terminal hydrocarbon side chain supporting the presence of a lipopeptide metabolite. Analysis of COSY, HSQC, ROESY and HMBC data assigned 7 partial structures (**Table 4.1**): (2) asparagine (Asn), (1) glutamine (Gln), (2) serine (Ser), (1) tyrosine (Tyr) and (1) threonine (Thr). The COSY, HSQC and HMBC experiments also correlated deshielded methylene (H-2, δ 2.43), a deshielded methine (H-3, δ 4.13), contiguous methylene resonances (H4-5 to H11-12, δ 1.25-1.31, brs) and a terminal methyl proton (H-13, δ 0.88), delineating an amino fatty acid residue (*β*-AFA) accounted for *β*-aminotridecanoic acid. HMBC correlations from each α-proton (*β*-proton of *β*-aminotridecanoic acid) to the carbonyl carbon of the neighboring amino acid and to its own carbonyl carbon were detected. Lipidation of cyclic peptides via ester or amide bonds has been reported from a variety of bacterial genera including *Mycobacterium*, *Streptomyces*, *Pseudomonas* and *Bacillus* (Debono *et al.* 1988; Nielsen and Sorensen 2003; Zhang *et al.* 2004; Seto *et al.* 2007). Because only 10 of the calculated 11 degrees of unsaturation could be accounted for by the functionalities in the eight individual fragments, it became obvious that cyclodysidin A had a cyclic nature.

Table 4.1 CD_3OD NMR data of cyclodysidin A.

Position		^{13}C (ppm)	^{1}H (ppm)	COSY	HMBC	ROESY
Asn1						
	CO	173.7				
	α	52.4	4.65, dd (7.5,6.4)	*β*	CO, *β*, *γ*, Tyr-CO	Tyr- *β*
	β	37.1	2.76/2.72, m	*α*	CO, *α*, *γ*	
	γ	174.9				
Asn2						
	CO	174.3				
	α	52.3	4.51, dd (7.9,5.7)	*β*	CO, *β*, *γ*, Gln-CO	
	β	37.4	2.52/2.44, m	*α*	CO, *α*, *γ*	Gln-*α*
	γ	175.1				
Gln						
	CO	174.1				
	α	55.6	4.19, dd (8.3,5.8)	*β*	CO, *β*, *γ*, Ser2-CO	Asn2- *β*
	β	27.1	2.17/2.10, m	*α*, *γ*	CO, *α*, *γ*, *δ*	
	γ	32.9	2.47, m	*β*	*α*, *β*, *δ*	
	δ	176.6				
Ser1						
	CO	172.9				
	α	57.4	4.37, t (5.6)	*β*	CO, β, Asn1-CO	Asn1-*β*
	β	62.4	3.84, m	*α*	CO, α	Thr-β
Ser2						
	CO	173.0				
	α	57.2	4.31, t (5.3)	*β*	CO, *β*, *β*-AFA-CO	*β*-AFA-2
	β	62.3	3.92, m	*α*	CO, *α*	
Thr						
	CO	172.4				
	α	60.0	4.28, d (2.6)	*β*	CO, *β*, *γ*, Ser1-CO	
	β	67.3	4.40, m	*α*, *γ*	CO, *α*, *γ*	Ser1- *β*
	γ	20.4	1.16, d (6.5)	*β*	*α*, *β*	
Tyr						
	CO	174.4				
	α	56.9	4.46, dd (9.2,4.8)	*β*	CO, *β*, Asn2-CO, 1	
	β	36.4	3.14/2.85, dd (14.2,4.7)	*α*	CO, *α*, 1, 2, 6	Asn1-*α*
	Bz-*I*	129.0				
	Bz-*o*	131.3	7.06, d (8.5)	Bz-*m*	Bz-*I*, Bz-*m*, Bz-*p*	
	Bz-*m*	116.4	6.71, d (8.4)	Bz-*o*	Bz-*I*, Bz-*o*, Bz-*p*	
	Bz-*p*	157.3				
***β*-AFA**						
	CO	174.5				
	2	42.1	2.43, m	3	CO, 3, 4	Ser2-*α*
	3	48.8	4.14, m	2, 4	CO, 2, 4, Thr-CO	
	4	35.7	1.5, m	3, 5-12	2, 3	
	5-12	23.6, 30.1-30.4, 33.1	1.25-1.31, m	4, 13	4, 13	
	13	14.4	0.88, t (7.1)	5-12	5-12	

The sequential arrangement of amino acids was accomplished by interpretation of HMBC and ROESY data as well as fragmentation by CID MS/MS (**Figure 4.3**).

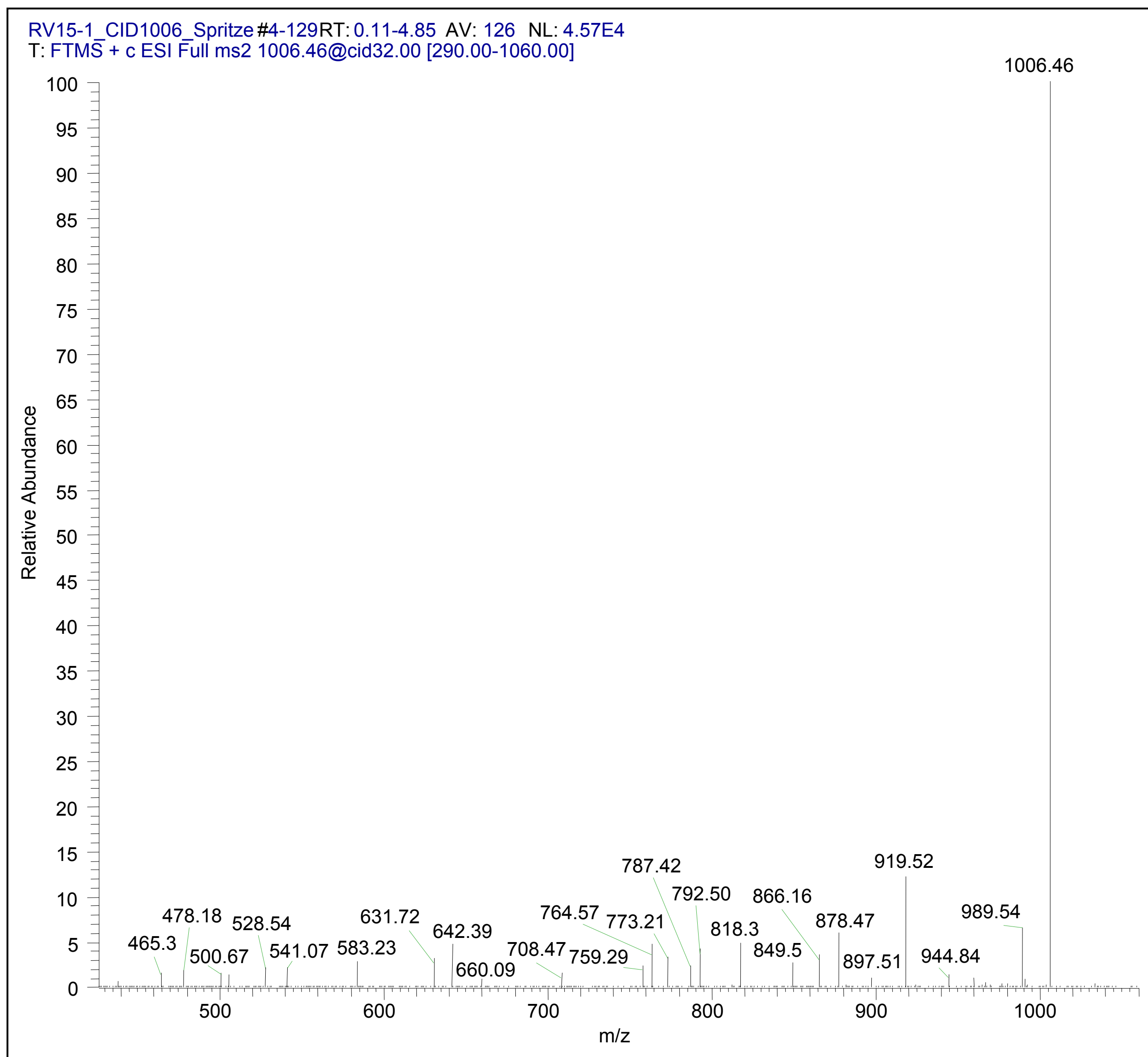


Figure 4.3 CID MS/MS mass spectrum of cyclodysidin A.

The complete sequence was confirmed on the basis of the results of CID MS/MS experiment. This technique employs collision-induced dissociation (CID) to fragment a precursor ion. After generation of a spectrum of ions by ESI, The ion of interest is then selectively allowed to progress into the collision cell, containing an inert gas typically argon or helium, which is allowed to collide with the selected ion. This induces fragmentation and the resulting ions are then analyzed as product ions. Sequence information derived from the mass differences between the b series ions and between the *y* series ions (**Table 4.2**). The structure of cyclodysidin A was thus confirmed as cyclo-(-AFA-Ser-Gln-Asn-Tyr-Asn-Ser-Thr).

Table 4.2 Annotated MS-fragments for cyclodysidin A.

Experimental [Da]	Predicted [Da]	Fragmentation	Sequence after first cleavage	Fragment after second cleavage
478.18	478.18	b/b4-1	NYNSTxSQ	NYNS
505.32	505.32	b/y4	QNYNSTxS	QNYN
583.23	583.24	b/z5+1	xSQNYNST	NYNST
642.39	642.38	b/y5-H_2O	YNSTxSQN	TxSQN
787.42	787.43	b/b6-H_2O	TxSQNYNS	TxSQNY
833.49	833.48	b6	TxSQNYNS	TxSQNY
849.53	849.46	b/a7-1	NYNSTxSQ	NYNSTxS
878.47	878.46	b/y7- H_2O	QNYNSTxS	NYNSTxS
897.52	897.49	b/c7+2	NYNSTxSQ	NYNSTxS
919.52	919.49	b/y7- H_2O	STxSQNYN	TxSQNYN
989.54	989.52	M- H_2O+1		
1006.46	1006.52	M		

Cyclodysidin B, exhibited molecular formula $C_{46}H_{73}N_{11}O_{15}$ by ESIMS analysis (measured, *m/z* 1020.5365, $[M+H]^+$) with 16 degrees of unsaturation. Cyclodysidin C, showed molecular formula $C_{47}H_{75}N_{11}O_{15}$ by ESIMS analysis (measured, *m/z* 1034.5517, $[M+H]^+$) with 16 degrees of unsaturation. Cyclodysidin D, had a molecular formula of $C_{49}H_{79}N_{11}O_{15}$ by ESIMS analysis (measured, *m/z* 1062.5834, $[M+H]^+$) with 16 degrees of unsaturation. The difference in molecular weight between all four compounds was only 14 or 28 mass units indicating a difference of CH_2 group. The compounds showed similar NMR spectra and the only difference was in the aliphatic part (δ 1.25-1.28 ppm) in the ^{1}H NMR spectrum corresponding to the difference in hydrocarbon of fatty acid part, indicating the same amino acid building blocks but different fatty acid. The UV spectrum of all compounds showed absorption λmax (MeOH) at 220 and 235 nm indicating the same chromophoric functionalities. Thus, the structures of all cyclic peptides were confirmed to be cyclo-(-AFA-Ser-Gln-Asn-Tyr-Asn-Ser-Thr-) by NMR data (**Figure 4.5**) and CID MS/MS experiment (**Figure 4.4**, **Table 4.6**). Comparison of the spectral data of the compounds with that in the database using the SciFinder Scholar tool and AntiMarin database revealed that these compounds are new and have never been isolated from other sources.

Table 4.3 CD_3OD NMR data cyclodysidin B.

Position		^{13}C (ppm)	^{1}H (ppm)	COSY	HMBC	ROESY
Asn1						
	CO	173.9				
	α	52.5	4.66, m	*β*	CO, *β*, *γ*, Tyr-CO	Tyr- *β*
	β	37.2	2.77/2.73, m	*α*	CO, *α*, *γ*	
	γ	175.0				
Asn2						
	CO	174.3				
	α	52.4	4.53, m	*β*	CO, *β*, *γ*, Gln-CO	
	β	37.5	2.53/2.45, m	*α*	CO, *α*, *γ*	Gln-*α*
	γ	175.2				
Gln						
	CO	174.2				
	α	55.9	4.21, m	*β*	CO, *β*, γ, Ser2-CO	Asn2-*β*
	β	27.2	2.16/2.10, m	*α*, *γ*	CO, *α*, *γ*, *δ*	
	γ	33.2	2.46, m	*β*	*α*, *β*, *δ*	
	δ	176.7				
Ser1						
	CO	173.0				
	α	57.5	4.38, t (5.6)	*β*	CO, *β*, Asn1-CO	Asn1-*β*
	β	62.7	3.86, m	*α*	CO, *α*	Thr-*β*
Ser2						
	CO	173.1				
	α	57.4	4.33, t (5.4)	*β*	CO, *β*, *β*-AFA-CO	*β*-AFA-2
	β	62.6	3.93, m	*α*	CO, *α*	
Thr						
	CO	172.4				
	α	60.3	4.29, d (2.5)	*β*	CO, *β*, *γ*, Ser1-CO	
	β	67.7	4.42, m	*α*, *γ*	CO, *α*, *γ*	Ser1- *β*
	γ	20.6	1.17, d (6.5)	*β*	*α*, *β*	
Tyr						
	CO	174.4				
	α	57.1	4.48, dd (9.3, 4.9)	*β*	CO, *β*, Asn2-CO, 1	
	β	36.7	3.15/2.86, dd (14.3,4.8)	*α*	CO, *α*, 1, 2, 6	Asn1-*α*
	Bz-*I*	129.2				
	Bz-*o*	131.5	7.08, d (8.5)	Bz-*m*	Bz-*I*, Bz-*m*, Bz-*p*	
	Bz-*m*	116.5	6.73, d (8.4)	Bz-*o*	Bz-*I*, Bz-*o*, Bz-*p*	
	Bz-*p*	157.5				
β-AFA						
	CO	174.5				
	2	42.4	2.44, m	3	CO, 3, 4	Ser2-*α*
	3	48.6	4.14, m	2, 4	CO, 2, 4, Thr-CO	
	4	35.9	1.5, m	3, 5-13	2, 3	
	5-13	23.9, 30.3-30.8, 33.3	1.26-1.32, m	4, 14	4, 14	
	14	14.6	0.89, t (7.2)	5-13	5-13	

Table 4.4 CD_3OD NMR data cyclodysidin C.

Position	^{13}C (ppm)	^{1}H (ppm)	COSY	HMBC	ROESY
Asn1					
CO	173.9				
α	52.5	4.67, dd (7.6,6.5)	*β*	CO, *β*, *γ*, Tyr-CO	Tyr- *β*
β	37.3	2.77/2.73, m	*α*	CO, *α*, *γ*	
γ	174.8				
Asn2					
CO	174.3				
α	52.4	4.54, dd (7.9,5.8)	*β*	CO, *β*, *γ*, Gln-CO	
β	37.6	2.53/2.44, m	*α*	CO, *α*, *γ*	Gln-*α*
γ	174.9				
Gln					
CO	174.2				
α	55.8	4.21, m	*β*	CO, *β*, *γ*, Ser2-CO	Asn2- *β*
β	27.0	2.16/2.10, m	*α*, *γ*	CO, *α*, *γ*, *δ*	
γ	32.9	2.46, m	*β*	*α*, *β*, *δ*	
δ	176.7				
Ser1					
CO	172.9				
α	57.0	4.38, m	*β*	CO, *β*, Asn1-CO	Asn1-*β*
β	62.7	3.85, m	*α*	CO, *α*	Thr-*β*
Ser2					
CO	173.0				
α	57.6	4.34, t (5.4)	*β*	CO, *β*, *β*-AFA-CO	*β*-AFA-2
β	62.6	3.93, m	*α*	CO, *α*	
Thr					
CO	172.3				
α	60.3	4.28, d (2.5)	*β*	CO, *β*, *γ*, Ser1-CO	
β	67.7	4.40, m	*α*, *γ*	CO, *α*, *γ*	Ser1- *β*
γ	20.6	1.17, d (6.6)	*β*	*α*, *β*	
Tyr					
CO	174.5				
α	57.0	4.48, dd (9.4,4.9)	*β*	CO, *β*, Asn2-CO, 1	
β	36.7	3.14/2.85, dd (14.3,4.8)	*α*	CO, *α*, 1, 2, 6	Asn1-*α*
Bz-*I*	129.2				
Bz-*o*	131.5	7.06, d (8.6)	Bz-*m*	Bz-*I*, Bz-*m*, Bz-*p*	
Bz-*m*	116.5	6.72, d (8.5)	Bz-*o*	Bz-*I*, Bz-*o*, Bz-*p*	
Bz-*p*	157.5				
β-AFA					
CO	174.6				
2	42.3	2.43, m	3	CO, 3, 4	Ser2-*α*
3	48.9	4.13, m	2, 4	CO, 2, 4, Thr-CO	
4	35.8	1.5, m	3, 5-11	2, 3	
5-14	23.9, 30.4-30.9, 33.2	1.25-1.31, m	4, 15	4, 15	
15	14.6	0.88, t (6.5)	5-14	5-14	

Table 4.5 CD_3OD NMR data cyclodysidin D.

Position		^{13}C (ppm)	^{1}H (ppm)	COSY	HMBC	ROESY
Asn1						
	CO	173.7				
	α	52.4	4.65, dd (7.5,6.5)	*β*	CO, *β*, *γ*, Tyr-CO	Tyr- *β*
	β	37.2	2.77/2.71, m	*α*	CO, *α*, *γ*	
	γ	175.0				
Asn2						
	CO	174.3				
	α	52.3	4.53, m	*β*	CO, *β*, *γ*, Gln-CO	
	β	37.3	2.52/2.44, m	*α*	CO, *α*, *γ*	Gln-*α*
	γ	175.2				
Gln						
	CO	174.1				
	α	55.9	4.21, dd (8.4,5.9)	*β*	CO, *β*, *γ*, Ser2-CO	Asn2- *β*
	β	27.4	2.18/2.10, m	*α*, *γ*	CO, *α*, *γ*, *δ*	
	γ	32.8	2.48, m	*β*	*α*, *β*, *δ*	
	δ	176.7				
Ser1						
	CO	172.9				
	α	57.4	4.37, m	*β*	CO, *β*, Asn1-CO	Asn1-*β*
	β	62.5	3.84, m	*α*	CO, *α*	Thr-*β*
Ser2						
	CO	173.1				
	α	57.3	4.33, m	*β*	CO, *β*, *β*-AFA-CO	*β*-AFA-2
	β	62.3	3.92, m	*α*	CO, *α*	
Thr						
	CO	172.3				
	α	60.1	4.28, d (2.5)	*β*	CO, *β*, *γ*, Ser1-CO	
	β	67.4	4.40, m	*α*, *γ*	CO, *α*, *γ*	Ser1- β
	γ	20.5	1.16, d (6.4)	*β*	α, β	
Tyr						
	CO	174.5				
	α	57.0	4.47, dd (9.3,4.9)	*β*	CO, *β*, Asn2-CO, 1	
	β	36.6	3.14/2.85, dd (14.3,4.8)	*α*	CO, *α*, 1, 2, 6	Asn1-*α*
	1	129.2				
	2,6	131.1	7.06, d (8.5)	Bz-*m*	Bz-*I*, Bz-*m*, Bz-*p*	
	3,5	116.4	6.72, d (8.4)	Bz-*o*	Bz-*I*, Bz-*o*, Bz-*p*	
	4	157.5				
β-AFA						
	CO	174.6				
	2	42.2	2.43, m	3	CO, 3, 4	Ser2-α
	3	48.6	4.14, m	2, 4	CO, 2, 4, Thr-CO	
	4	35.6	1.5, m	3, 5-11	2, 3	
	5-16	23.6, 30.4-30.8, 33.3	1.26-1.32, m	4, 17	4, 17	
	17	14.4	0.88, t (7.2)	5-16	5-16	

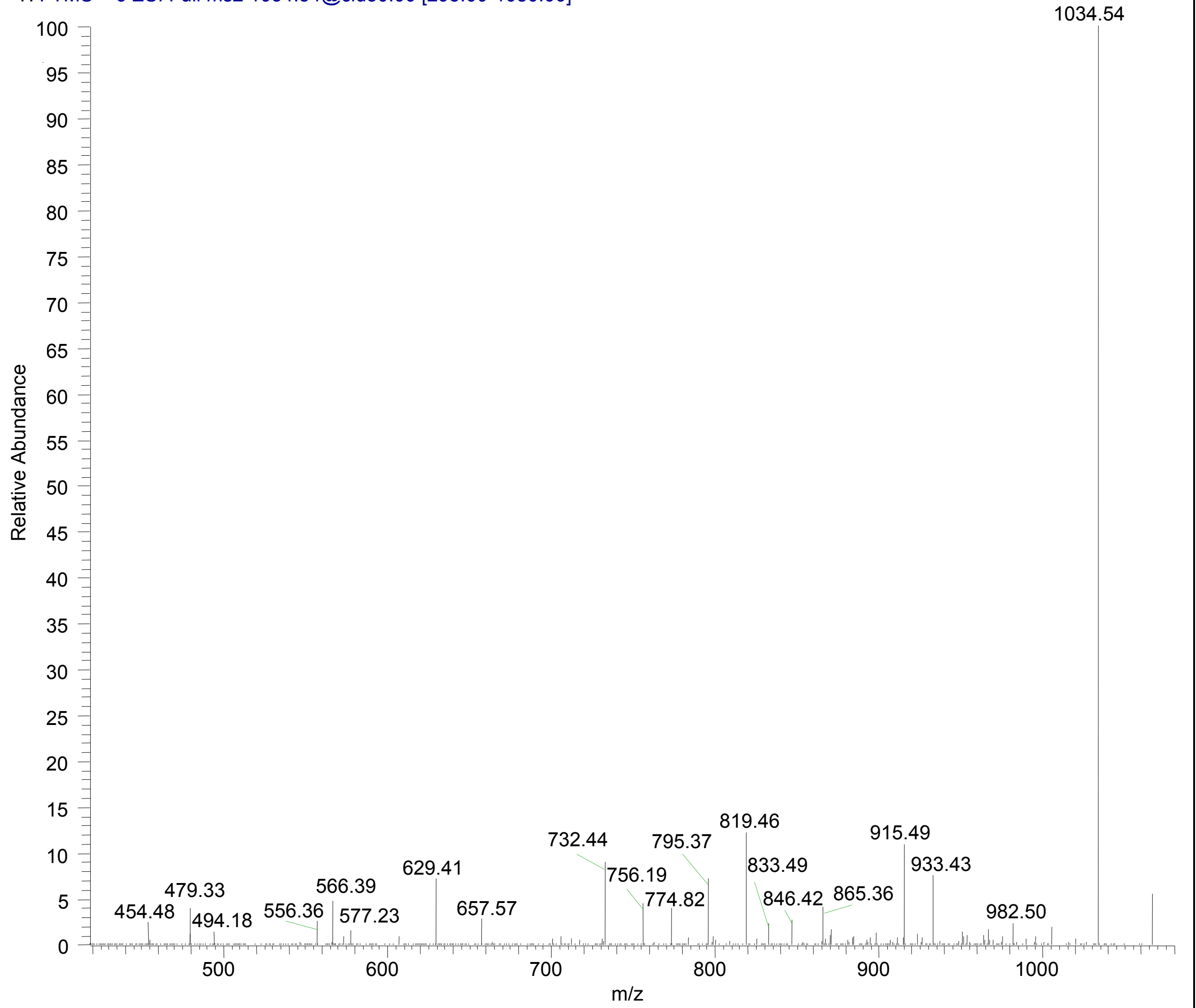


Figure 4.4 CID MS/MS mass spectrum of cyclodysidin C.

Table 4.6 Annotated MS-fragments for cyclodysidin C.

Experimental [Da]	Predicted [Da]	Fragmentation	Sequence after first cleavage	Fragment after second cleavage
494.18	494.21	y4	NSTxSQNY	SQNY
556.36	556.37	b4	TxSQNYNS	TxSQ
629.41	629.39	b5	NSTxSQNY	NSTxS
732.44	732.43	b5	xSQNYNST	xSQNY
819.46	819.46	b6	NYNSTxSQ	NYNSTx
833.49	833.48	b6	TxSQNYNS	TxSQNY
846.42	846.47	b6	xSQNYNST	xSQNYN
915.49	915.49	b7-H2O	xSQNYNST	xSQNYNS
1034.54	1034.54	M		

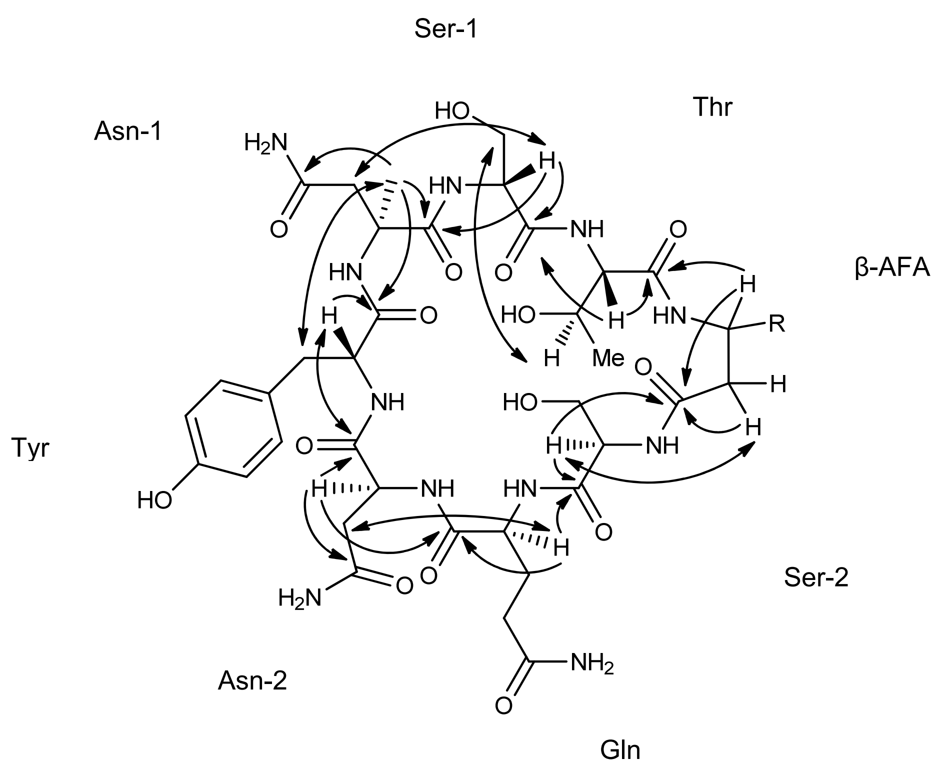


Figure 4.5 Selected NMR correlations of cyclodysidin A

Synthesis of (R) and (S) 3-amino tridecanoic acid

This work was done by A. Phillipe (AG Bringmann, Institute of Organic Chemistry, U. Würzburg). 3-amino fatty acid is a component of the new cyclic peptides, cyclodysidins. To determine the stereochemistry of this residue in all cyclic peptides, (*S*)- and (*R*)-3-amino fatty acids (1-4) were synthesized as authentic standards (**Figure 4.6**). A synthesis giving an easy access to the both eantiomers is required. The aliphatic -amino acids were obtained starting with the corresponding aliphatic aldehydes. The aldehydes were then condensed with maleic acid and ammonium acetat in refluxing ethanol (Rodionov reaction) to give the corresponding amino acids as insoluble crystals. The two enantiomers were afterwards separated on chiral HPLC.

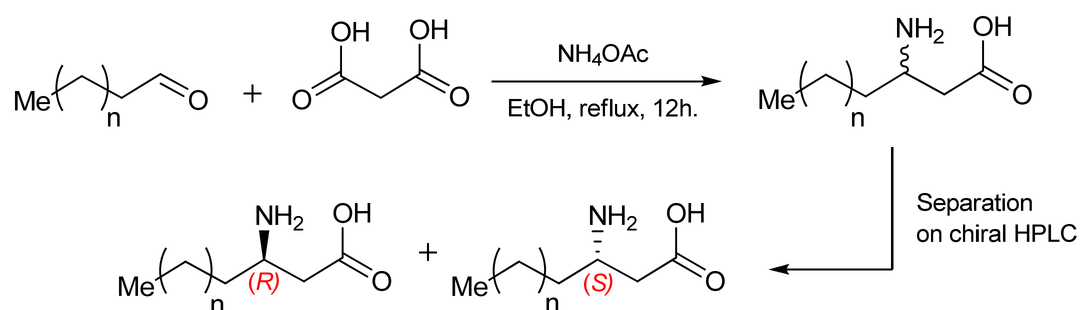


Figure 4.6 Synthesis plan of 3-amino fatty acid residue.

The absolute configuration of (*S*) and (*R*)-3-amino fatty acids was determined by comparison of the optical rotation of those compounds with the values of similar examples reported in the literature. Analysis of CD-spectra of the secondary amines with the help of computational method was helpful to confirm the absolute configuration of those compounds.

Chiral derivatization

Absolute configuration was determined by acid hydrolysis followed by chiral derivatization with Marfey's reagent. HPLC analysis of Marfey's derivatives in comparison to their analogues of authentic amino acids (**Table 4.7**) revealed that all of our cyclic peptides consist of L-asparagine, D-serine, L-glutamine, L-threonine, and D-tyrosine. Moreover, Marfey's analysis revealed that the second stereogenic center in threonine is R which is the normal form produced by microorganisms. The absolute configuration of *β*-AFA is currently in progress.

Table 4.7 Retention times of Marfey's derivatives of amino acids.

Amino acid	Rt [min]
D-Ser	28.3
L-Ser	23.5
D-Asp	32.8
L-Asp	27.6
D-Tyr	33.7
L-Tyr	31.9
D-Thr	31.3
L-Thr	29.7
L-allo Thr	29.9
D-Glu	30.3
L-Glu	28.9

Cyclodysidin A: R =

Cyclodysidin B: R =

Cyclodysidin C: R =

Cyclodysidin D: R =

Figure 4.7 Structures of cyclodysidins A-D.

4.3.2 Bioactivity of cyclodysidins

In spite the fact that the crude methanolic extract of the *Streptomyces* strain RV15 showed antibacterial activity against Gram positive bacteria and antifungal activities against *C. alibicans*, the four cyclic peptides did not exhibit any antimicrobial activities. The activity of cyclic peptides were tested against different proteases but no activity was detected (**Table 4.8**). They also did not show any cytotoxic against kidney epithelial cells and macrophages indicating that they are not cytotoxic to human cells. Diverse activities have been reported for the cyclic peptides such as antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antioxidant and anticancer activities (Dalsgaard *et al.* 2005; Steenbergen *et al.* 2005; Li *et al.* 2008; Yang *et al.* 2008; Barbosa *et al.* 2010; Hashizume *et al.* 2010; Ibrahim *et al.* 2010; Pimentel-Elardo *et al.* 2010; Tabbene *et al.* 2010).

Table 4.8 Antiprotease activity of cyclic peptides.

Sample	% Protease inhibition					
	Cath B	Cath L	Falcipain	Rhodesain	SARS M^{pro}	SARS pl^{pro}
Cyclodysidin A	6.4	7.4	ND	6.2	5.0	11.7
Cyclodysidin B	3.7	9.2	6.2	7.7	7.4	23.1
Cyclodysidin C	ND	11.1	11.3	13.4	6.3	13.4
Cyclodysidin D	8.9	20.6	18.4	19.5	14.8	15.8

ND: Not detected

Several cyclic peptides from different sources exhibited anticancer activities. Over the past decade, a great number of cyclic peptides with potent antitumor activity have been discovered from plants, animals, marine organisms and microorganisms (da Rocha *et al.* 2001; Schmidt *et al.* 2004; Simmons *et al.* 2005; Kinghorn *et al.* 2009; Olano *et al.* 2009; Skinner 2010). Our aim was not to test the anticancer activity of our compounds but because the anticancer activity from cyclic peptides is well known, cyclodysidins were tested against ovarian and melanoma cell lines but no activity was reported. There are still a lot of tumor cell lines to be tested but this will require the availability of more material from the cyclic peptides. Bioassay-guided fractionation was successfully used to isolate compounds with particular activity from crude extracts of several organisms (Han *et al.* 2007; Awad *et al.* 2009; Ene *et al.* 2009; Asolkar *et al.* 2010; Ibrahim *et al.* 2010; Peters *et al.* 2010; Sheeja *et al.* 2010). It is based on the chromatographic fractionation of the whole extract and

refractionation until pure biologically active compounds are isolated. Activity-directed fractionation of the methanolic extract of *Streptomyces* strain RV15 yielded four inactive compounds. The absence of activity might be explained in many ways. The change in chemical nature of the compounds due to harsh extraction conditions, bad storage conditions or compounds instability can lead to degradation or loss of essential parts of the molecule such as alcoholic or carboxylic functions, sugar or hydrocarbon part. It might be also due to the presence of other compounds in the extract which could be responsible for the antimicrobial activity and this consistent with our results due to the presence of other peaks to be identified. Another possibility might be the presence of other compounds in the extract which work synergistically with the cyclic peptides.

4.3.3 Conclusion and Outlook

Four new cyclic lipopeptides were isolated from marine *Streptomyces* RV15 strain which was recovered from sponge *Dysidea tupha* that was collected from Rovinj (Croatia, 2008). Their structures were elucidated using NMR, MS, and chiral derivatization with Marfey's Reagent. The new cyclic peptides have the sequence cyclo-(AFA-Ser-Gln-Asn-Tyr-Asn-Ser-Thr) based on NMR data and CID MS/MS. The difference between the four compounds was in the hydrocarbon side chain of the amino fatty acid part. The absolute configuration of amino acid building blocks was found to be L for all except tyrosine and serine had D-configuration. Moreover, Marfey's analysis revealed that the second stereogenic center in threonine is R which is the normal form produced by microorganisms. The absolute configuration of *β*-AFA is currently in progress. The cyclic peptides were tested against different proteases, bacteria, fungi and tumor cell lines but no activity was reported.

Our future directions will include synthesis of the cyclic peptides and test them against NCI 60 panel as well as further chemical investigation of *Streptomyces* RV15 strain to get the bioactive compounds.

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Chapter 5

General discussion and outlook

5.1 Plant cell cultures as sources of antimicrobial proteins

Antimicrobial resistances together with emerging and re-emerging infections are thought to be driven largely by socio-economic, environmental and ecological factors (Morens *et al.* 2004; Jones *et al.* 2008; Woolhouse 2008). This is happening at the time when the arsenal of antibiotics available to treat infectious diseases, is decreasing due to progressive increasing resistance of microbes to antimicrobial drugs, necessitating the looking for new sources of novel antibiotics. Nature was the main supply for most of discovered antibiotics and still many sources are unexplored. About 100,000 compounds of molecular weight less than 2500 have been characterized, half from microbes and the other half from plants (Zhang 2005). Natural product-derived drugs are still a major portion among the total number of anti-infective approved drugs (**Table 5.1**).

Table 5.1 Number of approved antibacterial drugs in the period from 1990 to 2002 (Newman *et al.* 2003).

Year	Number of antibacterial drugs	Natural product-derived	Synthetic
1990–1995	24	17	7
1996–2000	11	6	5
2001–2002	2	2	0

Plant suspension cultures have proven to be valuable experimental systems applicable in various aspects of defense response, secondary metabolite production and signal transduction (Baldi *et al.* 2008; Caretto *et al.* 2010). They have been used to produce pharmacologically and commercially interesting compounds (Vanisree *et al.* 2004; Hernandez-Vazquez *et al.* 2010). Treatments such as the use of biotic and abiotic elicitors were reported by several studies to enhance the production of secondary metabolites from plant cell cultures (Namdeo *et al.* 2002; Baldi *et al.* 2009; Ramos-Solano *et al.* 2010; Zhao *et al.* 2010). Bioactivity testing of different fractions from seven heterotrophic and photomixotrophic plant cell cultures elicited with nine elicitors revealed that the intracellular fraction of *L. angustifolia* elicited with DC3000 was the most active fraction against *Candida maltosa*. Furthermore, the bacterial and fungal elicitors were more effective than other elicitors of non microbial origin with respect to antimicrobial activity induction which was consistent with the previous studies (Broeckling *et al.* 2005; Naoumkina *et al.* 2008). The

antimicrobial activities from plant cell cultures were attributed to proteins rather than small molecular weight organic compounds. Antimicrobial proteins (AMPs) have been isolated from various multicellular plant and animal species as well as by some single-celled organisms (Zasloff 2002; Harvey 2008). AMPs help plants and animals to resist the infection by environmental microbes and represent evolutionarily conserved component of the innate immune response of animals (Boman 1995). Several antimicrobial proteins were purified from plants with the majority have been isolated from seeds (Hammami *et al.* 2009b; Pelegrini *et al.* 2009; Park *et al.* 2010). The majority of them are broad spectrum antibiotics with potent activities which demonstrate their potential as novel therapeutic agents (Brogden 2005; Hancock and Sahl 2006). Here, we reported plant cell suspension cultures as new sources for isolating AMPs after elicitation with different elicitors. One putatively 19 kDa antimicrobially active protein, named AtAMP, was purified from *A*. *thaliana* cell culture elicited with salicylic acid. Some antimicrobial proteins have been applied in the agriculture such as the expression of *α*-hordothionin gene under control of CaMV35S promoter to increase the resistance against *Pseudomonas syringae* (Carmona et al. 1993). The constitutive expression of a novel alfalfa defensin gene in potato to provide resistance against *Dahliae* (Gao *et al.* 2000). AMPs exhibited several advantages such as the potent activity against a broad range of microbes (Andra *et al.* 2001; Cole 2003; Toke 2005; Rivas *et al.* 2009), the lower probability of developing microbial resistance than observed with other anti-infective agents as well as the synergistic compatibility with other classes of antibiotics (Biragyn *et al.* 2002). These advantages make AMPs as attractive candidates for biopharmaceutical development. However, the development of AMPs as real antibiotics has been hindered, in large part, by the lack of a cost-effective means of commercial-scale production (Zasloff 2002). This study highlights plant cell cultures as alternative new sources for AMPs that could be possible candidates for future antibiotics taking the advantages of simpler procedure for isolation and purification of compounds from plant cell cultures as well as the possibility to scale up to commercial level. Future work will include further characterization of the purified protein, bioactivity testing and heterologously expressing it in transgenic plants.

5.2 Marine actinomycetes as sources of new anti-infectives

Marine sponges (Porifera) have attracted significant attention from various scientific disciplines, remarkably due to their potential to produce arsenal of diverse compounds with various biological activities (Dalisay *et al.* 2009; Laville *et al.* 2009; Andavan and Lemmens-Gruber 2010; Ebada *et al.* 2010; Fusetani 2010). Sponges are reservoirs of abundant, dense and morphologically diverse microbial communities (Hentschel *et al.* 2006). Recent studies have shown that some secondary metabolites with promising activities isolated from marine sponges were speculated to be biosynthesized by sponge-associated microbes rather than

the host itself (Kobayashi *et al.* 1993; Piel 2004; Kim and Fuerst 2006). In the case of production of compounds by a microbe, isolation and culture of the producing bacteria can provide with a reliable source for the bioactive compound of interest and open a wide range of possible testing and analyses. By the use of molecular approaches to investigate the diversity of sponge microbial communities, actinomycetes have been shown to be a major component of these communities (Webster *et al.* 2001; Hentschel *et al.* 2002; Schneemann *et al.* 2010). The cultivation efforts have recently revealed considerable actinomycete diversity from various marine samples (Mincer *et al.* 2002; Jensen *et al.* 2005; Maldonado *et al.* 2005b; Bredholdt *et al.* 2007b; Lampert *et al.* 2008; Hardoim *et al.* 2009; Sun *et al.* 2010). Both culture-independent studies and culture-based methods revealed the existence of high actinobacterial diversity in sponges (**Table 5.2**). In this Ph.D. study, 90 actinomycete isolates belong to 18 diverse genera were recovered from 11 taxonomically and geographically different marine sponges which represents high diversity in comparison to other studies (**Table 5.2**).

Table 5.2 Actinomycetes diversity in terrestrial and marine environments.

No of actinomycete isolates	No of genera recovered	Source	Reference
47	4	Soil	(Moncheva *et al.* 2002)
60	3	Soil	(Abbas 2006)
330	4	Plant	(Taechowisan *et al.* 2003)
10	5	Plant	(Nimnoi *et al.* 2010)
55	6	Plant	(Verma *et al.* 2009)
242	5	Plant	(Cao *et al.* 2004)
289	11	Marine sediment	(Jensen *et al.* 1991)
192	9	Marine sediment	(Vijayakumar *et al.* 2007)
600	4	Marine sediment	(Fiedler *et al.* 2005)
90	18	Sponge	(Abdelmohsen *et al.* 2010a)
79	20	sponge	(Tabares *et al.* 2010)
35	4	Sponge	(Radwan *et al.* 2009)
184	6	Sponge	(Sun *et al.* 2010)
106	7	Sponge	(Zhang *et al.* 2006b)
181	3	Sponge	(Zhang *et al.* 2008)
59	10	Sponge	(Xin *et al.* 2009)
19	6	Sponge	(Pimentel-Elardo 2009)
46	5	Sponge	(Schneemann *et al.* 2010)

Fourteen putatively novel species belong to 11 genera recovered from Mediterranean Sea and Red Sea sponges were identified by 16S rRNA gene analysis. Strains belonging to

Actinoalloteichus, *Brachybacterium*, *Brevibacterium, Curtobacterium*, *Gordonia*, *Kocuria*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus*, *Salinispora* and *Streptomyces* have been previously isolated from marine sponges (Montalvo *et al.* 2005; Taylor *et al.* 2007; Zhang *et al.* 2008; Radwan *et al.* 2009). However, none of the strains recovered in our study showed sequence similarities with those previously reported from sponges, thus raising the question if these strains are true sponge symbionts or they might be transient bacteria and/or just came from surrounding seawater.

Given that less than 1% of bacteria associated with sponges can be cultured hindering the access to this enormous reservoir of secondary metabolites producers. It could be argued that the traditional culturing strategies use conditions that are completely different from the normal growth habit of many microbes and are a major contributing factor to the failure to cultivate most microorganisms in pure culture. Thus, the use of appropriate isolation protocols could be efficient for improving the isolation of rare and new actinomycete species. It has been reported that the structure of actinobacterial community could be influenced by DNA extraction methods and the use of different media (Seong *et al.* 2001; Hardoim *et al.* 2009; Schneemann *et al.* 2010). In this study, DNA was extracted from actinomycetes using two different methods. The first method was done by heating the colonies with water and the second using FastDNA spin kit and both methods were successful in most of cases except in some cases where the colonies are hard, only the kit was able to extract DNA. Among the eight different media used in the study, M1 agar exhibited the highest recovery. Several studies have shown that M1 was effective medium for cultivating different genera of marine actinomycetes (Mincer *et al.* 2002; Pimentel-Elardo *et al.* 2008b; Solano *et al.* 2009). The selectivity of the isolation procedure may be influenced by pretreatment of the sample before plating and by the conditions under which the bacterial communities in suspensions of the sample are cultivated. Different pretreatments have been used in previous studies to enhance growing of actinomycetes or specific group of actinomycetes. Heat shock was used to reduce the numbers of Gram negative bacteria that growing and overrunning the plates (Takizawa *et al.* 1993). Pretreatment with UV irradiation and high frequency radiations have been shown to effectively stimulate the germination of spores of rare actinomycete genera (Bredholdt *et al.* 2007a). The centrifugation has been used to concentrate spores in seawater samples (Goodfellow and Williams 1983). In this study, cultivation media were supplemented with antibiotics cycloheximide and nystatin to inhibit fungal growth while, nalidixic acid was added to inhibit many fast-growing Gram-negative bacteria (Webster *et al.* 2001). Additives such as sodium carbonate has been utilized in the previous studies to enhance the growth of alkalophilic actinomycetes (Selianin *et al.* 2005). Media with low-nutrient composition (Santavy and Colwell 1990; Olson et al. 2000) have been used to enhance growth of

oligotrophic bacteria which are capable of producing trace concentrations of organic substances in the medium (Kuznetsov et al. 1979). The cellulose-based media have been used in order to isolate cellulose-decomposing bacteria (Magarvey *et al.* 2004). Furthermore, macromolecules such as casein, chitin, and humic acid have been used as carbon and nitrogen sources to enhance growth of rare actinomycetes (Cho *et al.* 1994). In this study, the addition of aqueous sponge extract to M1 medium resulted in the isolation of a putatively new genus with *Rubrobacter* as its closest relative. This shows that variation from standard protocols is a worthwhile procedure which is consistent with previous observations (Webster *et al.* 2001; Bredholt *et al.* 2008; Zhang *et al.* 2008).

Extracts of cultures of 20 selected actinomycetes out of 90 strains, isolated from different sponges, were first screened to detect their ability to produce bioactive substances. Screening tests were performed to detect antibacterial, antifungal activities as well as antiparasitic activities. Of these extracts, 10 (11%) obtained from different species belonging to ten genera showed interesting biological activities, confirming marine actinobacteria as a prolific source for active compounds. Out of the 10 actinomycete strains that were selected as producers of active secondary metabolites, 50% exhibited significant antibacterial and antifungal activities and 20% were active against parasites. Furthermore, one putatively novel strain related to the genus *Actinokineospora* showed both antifungal as well as antiparasitic potential. This great taxonomical variety of the producer microorganisms demonstrates that the production of active secondary metabolites is not restricted to particular genera or species.

The rate of discovery of new compounds from existing genera has decreased with increasing the rate of re-isolation of known compounds. It has been reported that new actinomycete taxa produce new bioactive compounds, making this targeted approach a more productive way to new discoveries (Feling *et al.* 2003; Kwon *et al.* 2006; Pimentel-Elardo *et al.* 2008a). This could be best exemplified by isolation of salinisporamide A and arenimycin from the new genus *Salinispora* (Feling *et al.* 2003; Asolkar *et al.* 2010) as well as the isolation of marinomycins A–D from the new genus *Marinispora* (Kwon et al. 2006). Thus, it is critical to pursue with new groups of microbes from unexplored habitats as sources of novel compounds. In order to avoid rediscovery of known secondary metabolites, new approach was used in this study to target new bioactive compounds (**Figure 5.1**).

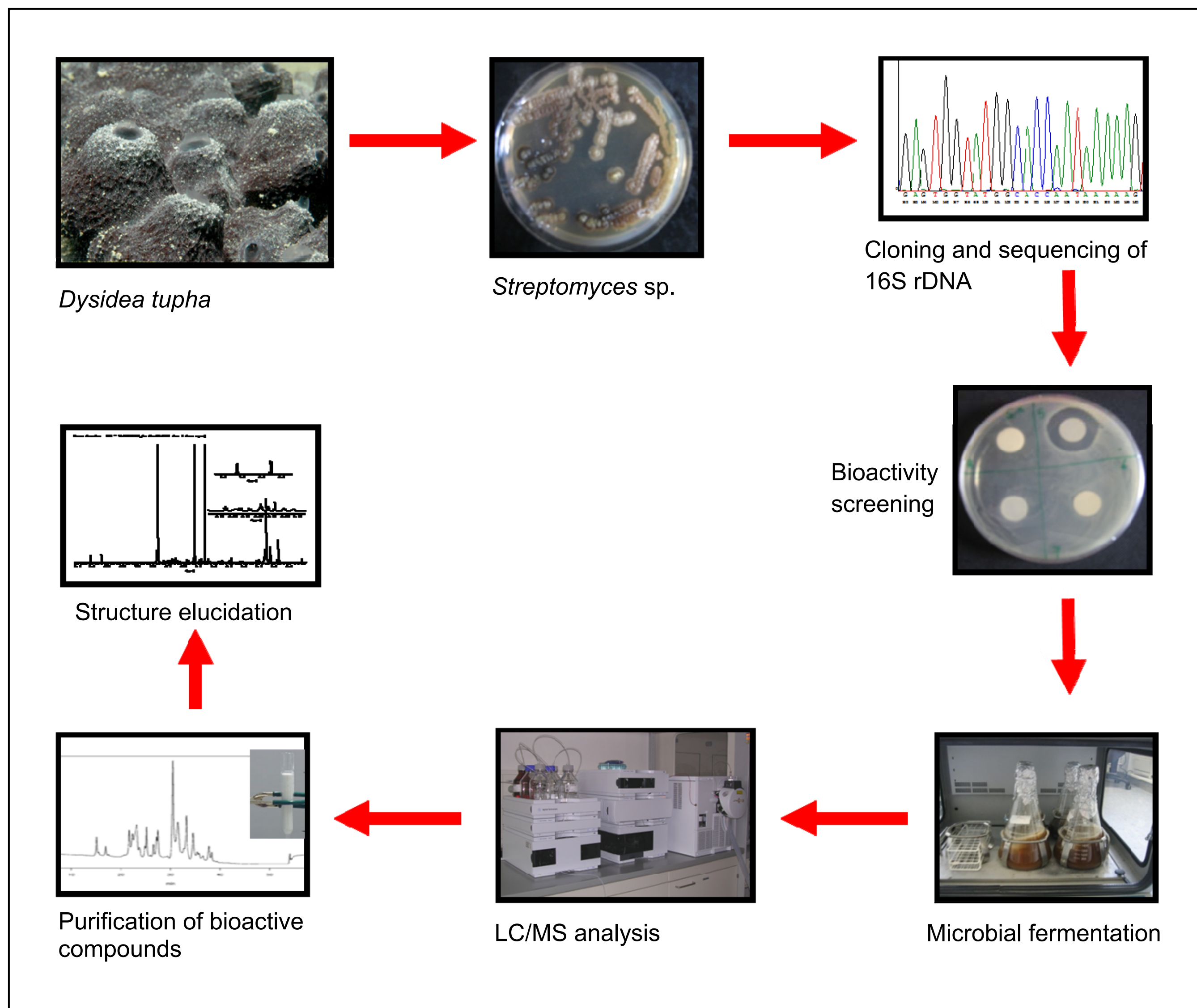

Figure 5.1 Protocol targeting new bioactive compounds.

In spite of the fact that strains belonging to unusual genera may provide a higher probability of finding novel bioactive molecules, the little knowledge about their physiology and appropriate cultivation might hinder the processing with those strains. Among the thousands of compounds that have been isolated from microbes, around 80% were produced by members of the order *Actinomycetales* (phylum *Actinobacteria*) with the genus *Streptomyces* accounting for approx 50% (Strohl 1997; Lazzarini *et al.* 2001). Numerous, useful compounds have been isolated from the genus *Streptomyces*, including anti-inflammatory, antiviral, antitumor, antifungal and antibacterial compounds (Pfefferle *et al.* 2000; Kelecom 2002; Berdy 2005; Hughes *et al.* 2008; Dharmaraj 2010). Despite their broad range of activities, the most studied secondary metabolites through the history were the antibiotics. It was reported that some microorganisms are capable of producing a variety of compounds belong to different chemical classes and are termed metabolically talented (Bode *et al.* 2002; Zhang 2005). One metabolically talented microorganism, *Streptomyces* sp. strain Go.40/10,

synthesized at least 30 different compounds, many of which are new compounds (Schiewe and Zeeck 1999).

The secondary metabolites produced by marine invertebrates as well as marine bacteria have been shown to be structurally biosynthesized by polyketide synthases (PKS) and/or nonribosomal peptide synthetases (NRPS). Many microbial strains have been found to have several gene clusters based on PCR screening but they do not readily express them when grown under standard growth conditions (Bentley *et al.* 2002). It is possible that these gene clusters are not expressed under standard growth conditions and require specific media or conditions or their constitutive expression require external signaling. Thus by using different fermentation conditions, the probability that each of its gene clusters will be expressed will be increased. Carbon source is important factor that could affect the secondary metabolites production and usually there are differences between the optimal carbon sources for growth and those that are appropriate for secondary metabolism. Glucose was the excellent carbon source for growth in most cases, but suppresses the production of a series of secondary metabolites such as actinomycin and cephalosporin (Aharonowitz and Demain 1978; Praveen *et al.* 2008). Glucose has also been reported to transiently repress erythromycin production in *Saccharopolyspora ervthraea*, although, it does not interfere with the production of aminoglycosides or chloramphenicol (Doull and Vining 1990). An excess supply of a readily assimilated nitrogen source might negatively stimulate secondary metabolites biosynthetic pathways, especially those that are biosynthetically related to lipid metabolism, notably the polyketide-derived metabolites. The addition of ammonium salts was capable of inhibiting the production of cephamycin and rifamycin (Aharonowitz 1980). Inorganic phosphate could suppress the biosynthesis of many secondary metabolites. Thus, the optimal phosphate concentration needed for production of secondary metabolites is generally lower than that required for growth (Gonzalez *et al.* 1990). However, the optimal concentration can vary drastically between strains as in case of avermectin biosynthesis in *Streptomyces avermitilis* which tolerates high concentrations of phosphate in the medium. The influence of amino acids on secondary metabolite production is very variable and can depend on the precursor or the natural inducer. The addition of L-valine increased the specific production of cyclosporin A by 62% in comparison to an unsupplemented control culture (Lee and Agathos 1989). Optimal temperatures for the production of secondary metabolites are in general lower than for growth but can vary considerably. For example, the optimal temperature for biosynthesis of cyclosporin by *Tolypocladium inflatum* 21 °C is optimal (Ly and Margaritis 2007), while 28 °C is best for nebramycin production by *Streptomyces tenebrarius* (Stark *et al.* 1971). Incubation time is also critical factor and is totally dependent on the growth characteristics of the microorganism and the culture

conditions. The addition of adsorbents such as XAD-16 resin to liquid cultures can enhance the concentration of secondary metabolites produced (Buchanan *et al.* 2005). The solid-phase fermentation might be a good approach to get novel compounds which allows the biosynthesis of new compounds that are mainly related to the sporulation process. Cell immobilization technology is one of the main trends in modern biotechnology and is employed mostly in secondary metabolites production. It provides many operational and economic advantages such as prolonged metabolic activity, reuse of the biocatalyst and the possibility to increase the cell concentration. In last few years, the production of antibiotics by immobilized microbial cells was successfully reported. Erythromycin and neomycin were produced by *Saccharopolyspora* and *Sterptomyces* cells, respectively, entrapped in calcium alginate beads (Srinivasulu *et al.* 2002; Hamedi *et al.* 2005). The antibiotic producing microorganisms *Streptomyces rimosus* PFIZER 18234-2 and *Amycolutopsis mediterranei* CBS 42575 were immobilized in glass wool for the production of oxytetracycline and rifamycins B, respectively (Farid *et al.* 1994). The identification of appropriate culture conditions for optimal production of bioactive metabolites was critical for facilitating the isolation and characterization of the bioactive molecules from the isolated actinomycete strains. In this Ph.D. study, five different media with different carbon and nitrogen sources were used to optimize the production of antibiotics based on activity testing by disc diffusion assay. ISP2 medium produced the maximal activity which was consistent with previous reports (Yang *et al.* 2010).

Bioassay-guided fractionation was used in several studies to monitor and direct the isolation of the compounds with corresponding activity from the crude extract (Ene *et al.* 2009; Asolkar *et al.* 2010; Peters *et al.* 2010). The putatively new bioactive *Streptomyces* RV15 strain cultivated from the sponge *Dysidea tupha* yielded four new cyclic peptides, namely, cyclodysidins after chromatographic and spectroscopic analysis. Although the methanolic crude extract of the strain showed potent antibacterial (against Gram positive bacteria) and antifungal (against *C. albicans*) activities, the bioassay-guided fractionation yielded pure compounds which did not exhibit such activities. They were also additionally tested against various proteases and two different tumor cell lines but no activities were detected. The lack of activity could be explained by the presence of other compounds which could be responsible for these activities and this is consistent with the presence of other peaks which to be identified. The other possibility is the synergism between the cyclic peptides and other compounds in the extract. Our future work will include further identification of other compounds in the crude extract since some peaks still unidentified. Intelligent screening approaches towards natural products discovery are required. One of the approaches is the screening of synergism between natural products and commercially used pharmaceuticals.

Some marketed drugs might be more effective at a reduced dosage if they taken with low dosages of other synergistic natural products which might fail to show potent activity alone. In addition to the high efficacy of the synergistic action, there is also decrease in the toxic effects of the synthetic drugs. Many marketed traditional medicines have demonstrated great efficacy and safety profiles during their long history. However, the activity was lost when attempts were done to purify a single molecule. Synergistic co-drugs from natural products will help existing drugs to be more effective to cure disease. Because it was reported by many studies that cyclic peptides exhibited anticancer activities against various tumor cell lines (Fenical *et al.* 2003; Simmons *et al.* 2005; Olano *et al.* 2009; Sivonen *et al.* 2010), we will pursue synthesizing the cyclic peptides to generate enough amounts for testing against National cancer Institute (NCI) 60 tumor cell line panel.

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Annex

I. Screening of intracellular fractions for antimicrobial activities (Chapter 2)

Plant	Pathogen \ Elicitor	Alt	ASA	Chitosan	DC3000	Fol	MeJ	PGA	RPM1	SA
Agrostis tenuis	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	11	7	9	12	11	0	7	9	9
	E. coli	9	7	11	11	9	0	7	12	11
	P. aeruginosa	12	8	9	12	10	0	8	11	12
	S. aureus	0	0	0	0	0	0	0	0	0
Arabidopsis thaliana	*B. subtilis*	13	12	9	11	12	13	9	11	15
	C. maltosa	10	13	10	12	11	12	11	13	17
	E. coli	11	12	9	11	13	10	9	11	16
	P. aeruginosa	11	13	9	12	11	8	11	12	15
	S. aureus	9	11	10	9	13	9	12	11	14
Baphicanthus cusia	*B. subtilis*	10	11	7	14	9	9	12	10	12
	C. maltosa	0	7	0	7	0	7	0	0	7
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	7	7	0	0	0	0	0	0	0
	S. aureus	9	9	7	11	12	9	11	10	13
Daucus carota	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	0	0	0	7	0	0	0	7	7
	E. coli	11	11	8	12	9	10	7	10	12
	P. aeruginosa	9	10	7	10	11	9	8	9	10
	S. aureus	0	0	0	0	0	0	0	0	0
Lavendula angustifolia	*B. subtilis*	0	7	0	7	0	0	0	7	0
	C. maltosa	12	13	11	16	10	10	9	12	14
	E. coli	7	0	7	7	0	0	0	0	0
	P. aeruginosa	7	0	7	0	0	0	0	0	0
	S. aureus	0	0	0	0	0	0	0	0	0
Nicotiana tabacum petit havanna	*B. subtilis*	8	8	8	11	8	7	7	9	11
	C. maltosa	9	9	7	9	9	8	7	10	9
	E. coli	9	9	7	10	7	7	8	8	10
	P. aeruginosa	10	10	8	8	8	8	7	8	8
	S. aureus	9	9	9	8	8	7	7	7	9
verticillata	*B. subtilis*	7	0	0	7	0	0	0	7	7
	C. maltosa	11	10	11	12	12	11	9	11	13
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	0	0	0	0	0	0	0	0	0
	S. aureus	7	0	7	7	0	0	0	0	7

II. Screening of extracellular fractions for antimicrobial activities (Chapter 2)

pathogen	Elicitor	Alt	ASA	Chitosan	DC3000	Fol	MeJ	PGA	RPM1	SA
Agrostis tenuis	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	10	0	9	9	10	0	0	8	8
	E. coli	9	0	10	11	9	0	0	12	10
	P. aeruginosa	11	0	9	10	9	0	0	9	12
	S. aureus	0	0	0	0	0	0	0	0	0
Arabidopsis thaliana	*B. subtilis*	12	11	8	10	12	11	9	11	13
	C. maltosa	9	12	9	10	11	12	9	9	15
	E. coli	9	12	8	11	13	9	7	10	13
	P. aeruginosa	11	13	9	12	10	0	11	9	12
	S. aureus	7	10	9	9	13	9	8	11	12
Baphicanthus cusia	*B. subtilis*	9	11	0	13	8	9	11	10	11
	C. maltosa	0	7	0	7	0	7	0	0	7
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	7	8	0	0	0	0	8	0	8
	S. aureus	9	9	0	11	12	0	10	9	9
Daucus carota	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	0	0	0	0	0	0	0	0	0
	E. coli	10	11	0	11	8	9	0	10	11
	P. aeruginosa	8	10	0	9	10	9	0	7	8
	S. aureus	0	0	0	0	0	0	0	0	0
Lavendula angustifolia	*B. subtilis*	0	7	0	7	0	0	0	7	0
	C. maltosa	11	12	9	14	10	7	8	11	13
	E. coli	7	0	7	7	0	0	0	0	0
	P. aeruginosa	7	0	7	0	0	0	0	0	0
	S. aureus	0	0	0	0	0	0	0	0	0
Nicotiana tabacum petit havana	*B. subtilis*	7	8	0	10	8	8	0	8	10
	C. maltosa	8	8	0	9	9	8	0	9	9
	E. coli	9	9	0	9	7	7	0	8	9
	P. aeruginosa	8	7	0	8	8	8	0	8	8
	S. aureus	7	9	0	7	8	7	0	7	8
Rauvolfia verticillata	*B. subtilis*	0	0	0	7	0	0	0	0	0
	C. maltosa	10	9	8	11	10	8	7	9	12
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	0	0	0	0	0	0	0	0	0
	S. aureus	0	0	0	0	0	0	0	0	0

III. Screening of cell wall bound fractions for antimicrobial activities (Chapter 2)

Pathogen	Elicitor	Alt	ASA	Chitosan	DC3000	Fol	MeJ	PGA	RPM1	SA
Agrostis tenuis	*B. subtilis*	0	0	0	7	0	0	0	0	7
	C. maltosa	0	0	7	0	0	0	7	0	0
	E. coli	0	0	0	0	0	7	0	0	0
	P. aeruginosa	0	7	0	0	0	0	7	0	0
	S. aureus	0	0	7	0	0	7	0	0	0
Arabidopsis thaliana	*B. subtilis*	7	7	0	0	0	7	0	0	8
	C. maltosa	0	7	7	0	0	0	0	7	8
	E. coli	0	0	0	7	0	0	0	8	7
	P. aeruginosa	7	0	0	8	7	0	0	0	8
	S. aureus	0	0	0	0	7	0	0	0	0
Baphicanthus cusia	*B. subtilis*	0	0	7	0	0	0	0	0	0
	C. maltosa	0	7	0	7	0	0	0	0	7
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	7	7	0	0	0	0	0	0	0
	S. aureus	0	0	7	8	7	0	0	0	7
Daucus carota	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	0	0	0	7	0	0	0	7	7
	E. coli	0	7	0	8	0	0	7	0	0
	P. aeruginosa	7	8	7	7	0	0	0	0	7
	S. aureus	7	0	0	0	0	0	0	0	0
Lavendula angustifolia	*B. subtilis*	0	0	0	0	0	0	0	0	0
	C. maltosa	0	8	0	8	0	0	0	7	8
	E. coli	0	0	0	0	0	0	0	0	0
	P. aeruginosa	7	0	0	0	0	0	0	0	0
	S. aureus	0	0	0	0	0	0	0	0	0
Nicotiana tabacum petit havanna	*B. subtilis*	8	0	0	7	0	7	0	0	7
	C. maltosa	0	0	0	0	0	0	0	0	0
	E. coli	0	0	0	7	0	0	0	0	7
	P. aeruginosa	7	0	7	7	0	0	0	0	7
	S. aureus	0	0	0	0	0	0	0	0	0
Rauvolfia verticillata	*B. subtilis*	7	0	0	7	0	0	0	7	7
	C. maltosa	0	0	0	0	0	0	0	0	0
	E. coli	0	0	0	0	0	0	0	0	7
	P. aeruginosa	0	0	0	0	0	0	0	0	0
	S. aureus	7	0	0	7	0	0	0	0	7

0: no activity was detected

IV. 16S rRNA gene phylogenetic affiliation of cultivated strains (Chapter 3)

Isolate Code	Sequence Length (bp)	Closest relative by BLAST (Accession number)-Source	% Sequence Similarity
EG2	1483	*Brachybacterium conglomeratum* NCIB 9859 (X91030)-environmental sample	99.1
EG4	1482	*Microbacterium resistens* 3352 (EU714361)-environmental sample	98.0
EG6	1486	*Micrococcus luteus* CV44 (AJ717369)-environmental sample	99.3
EG7	1482	*Brachybacterium* sp. 511 (EU086801)-environmental sample	98.0
EG8	1484	*Corynebacterium ureicelerivorans* strain CNM462/06 (FJ392017)-environmental sample	98.7
EG10	1488	*Brevibacterium iodinum* DSM 2062T (X83813)- environmental sample	98.9
EG11	1486	*Microbacterium resistens* 3352 (EU714361)-environmental sample	98.2
EG12	1488	*Micrococcus luteus* CV44 (AJ717369)-environmental sample	99.5
EG14	1495	*Kocuria* sp. CNJ900 PL04 (DQ448710)-marine sediments	99.7
EG16	1483	*Mycobacterium* sp. CNJ823 PL04 (DQ448717)-marine sediments	99.9
EG18	1492	*Brevibacterium iodinum* DSM 2062T (X83813)-environmental sample	98.8
EG22	1488	*Microbacterium oxydans* B5 (DQ350825)-environmental sample	97.3
EG33	1379	*Rhodococcus opacus* B4(AB192962)-enviromental sample	98.0
EG34	1489	*Brevibacterium casei* 3Tg (AY468375)-environmental sample	98.4
EG36	1490	*Mycobacterium houstonense* ATCC 49403 (AY012579)-environmental sample	97.9
EG37	1486	*Brevibacterium epidermidis* ZJB-07021 (EU046495)-environmental sample	97.1
EG38	1498	*Kocuria* sp. CNJ900 PL04 (DQ448710)-marine sediments	99.9
EG40	1486	*Micrococcus* sp. B5W22-1 (EF114312)-environmental sample	99.3
EG43	1495	*Brevibacterium* sp. BBH7 (AM158906)-marine sediments	98.3
EG45	1487	*Micrococcus* sp. CNJ719 PL04 (DQ448712)-marine sediments	97.9
EG47	1489	*Microbacterium indicum* BBH9 (AM286267)-marine sediments	97.5
EG49	1482	*Actinokineospora diospyrosa* NRRLB-24047T (AF114797)-marine sediments	97.0
EG50	1485	*Gordonia terrae* AIST-1 (AB355992)-sea water	99.3
EG53	1501	*Arthrobacter sp.* VTT E-052904 (EF093123)-environmental sample	99.3
EG55	1328	*Salinispora pacifica* CNH732 RS00 (DQ224165)-sea water	99.6
EG56	1488	*Micrococcus* sp. TPR14 (EU373424)-environmental sample	99.0
EG62	1487	*Microbacterium oxydans* B5 (DQ350825)-environmental sample	97.4

Isolate Code	Sequence Length (bp)	Closest Relative by BLAST (Accession number)-Source	% Sequence Similarity
EG65	1497	*Brevibacterium aurantiacum* NCDO 739 (X76566)-environmental sample	99.0
EG67	1390	*Dietzia cinnamea* IMMIB RIV-399 (AJ920289)-environmental sample	99.4
EG69	1487	*Microbacterium indicum* BBH6 (AM158907)-marine sediments	97.3
RV13	1495	*Rothia* sp. L-143 (DQ822568)-environmental sample	97.0
RV15	1492	*Streptomyces lincolnensis* NRRL2936 (X79854)-marine sediments	98.0
RV28	1494	*Mycobacterium septicum* ATCC 700731 (AY457070)-environmental sample	99.1
RV43	1478	*Micromonospora* sp. HBUM84229 (EU119232)-environmental sample	99.8
RV45	1477	*Micromonospora* sp. HBUM84229 (EU119232)-environmental sample	99.9
RV63	1481	*Micromonospora* sp. RtII195 (EU427443)-environmental sample	99.7
RV71	1481	*Micromonospora matsumotoense* IMSNU 22003T (AF152109)-environmental sample	98.3
RV75	1491	*Kocuria palustris* isolate SCH0404 (AY881237)-marine sediment	99.6
RV76	1485	*Gordonia terrae* strain COE-O1 (AF467984)-environmental sample	99.8
RV77	1482	*Rhodococcus opacus* B-4 (AB192962)-environmental sample	98.4
RV83	1487	*Mycobacterium septicum* ATCC 700731 (AY457070)-environmental sample	99.3
RV89	1493	*Kocuria kristinae* DSM 20032 (X80749)-environmental sample	96.6
RV113	1512	*Rubrobacter radiotolerans* DSM 46359-T (X87134)-environmental sample	92.8
RV115	1481	*Micromonospora* sp. 215009 (FJ263418)-environmental sample	99.2
RV116	1484	*Micromonospora* sp. 215009 (FJ263418)-environmental sample	99.3
RV145	1481	*Corynebacterium* sp. 3301750 (AY581884)-enviromental sample	99.0
RV147	1484	*Corynebacterium* sp. 3301750 (AY581884)-enviromental sample	99.1
RV151	1502	*Mycobacterium gordonae* agha3 (AJ581472)-environmental sample	99.0
RV152	1493	*Mycobacterium gordonae* agha3 (AJ581472)- environmental sample	99.1
RV154	1493	*Kocuria* sp. Am 16 (AJ971854)-environmental sample	99.5
RV157	1483	*Rhodococcus opacus* B-4 (AB192962)-environmental sample	98.6
RV163	1345	*Nocardiopsis dassonvillei* HBUM174177 (FJ532466)-environmental sample	99.4

V. Abbreviations and Acronyms

δ	Chemical shift (ppm)
Alt	Boiled spores of *Alternaria brassiciola*
ASW	Artificial Sea Water
AtAMP	*Arabidopsis thaliana* Antimicrobial Protein
AMPs	Antimicrobial Peptides or Proteins
APS	Ammonium persulfate
ASA	Acetyl Salicylic Acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
B. subtilis	*Bacillus subtilis*
^{13}C NMR	Carbon Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
d	Doublet
dd	Doublet of doublet
DC3000	Boiled bacterial suspension of virulent strain of *Pseudomonas syringae*
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
E. Coli	*Escherichia coli*
EtOH	Ethanol
FDAA	1-Fluoro-2,4-Dinitrophenyl-5-L-Alanine Amide
Fol	Mycelial suspension of *Fusarium oxysporium lycopersici*
g	Gram
h	Hour
HMBC	Heteronuclear Multiple Bond Correlation
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
J	Coupling constant

V. Abbreviations and Acronyms

kDa	Kilo Dalton
µg	Microgram
µl	Microliters
M	Molar
mA	Milli Ampere
Mej	Methyljasmonate
min	Minute
ml	Milliliter
mM	Millimolar
m	Multiplet
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OD	Optical Density
ppm	Parts Per Million
PAGE	Polyacrylamide Gel Electrophoresis
PGA	Polygalcturonic acid
P. aeruginosa	*Pseudomonas aeruginosa*
R_t	Retention time
rpm	Revolution per minute
RPM1	Boiled bacterial suspension of avirulent strain of *Pseudomonas syringae*
RT	Room Temperature
s	Singlet
SA	Salicylic Acid
$strain^T$	Type strain
t	Triplet
UV	Ultraviolet
V/V	Volume/Volume
W/V	Weight/Volume

VI. Recipes for different preparations

Media were sterilized by autoclaving at 121 °C for 30 min. Components that are volatile, altered or damaged by heat or whose pH or concentration are critical were sterilized by filtration through a 0.22 µm filter and added to autoclaved media after cooling to hand warm temperature.

A- Growth media

AIA medium		
Asparagine		0.1 g
Ferrous sulfate		1.0 mg
Magnesium sulfate		100.0 mg
Potassium phosphate		0.5 g
Sodium propionate		4.0 g
Sodium casein		2.0 g
Agar		18.0 g
ASW	to	1.0 L

Baltz medium		
Adenosine		10.0 mg
Bathocuproine disulfonic acid		14.0 mg
Glucose		1.0 g
Glutamine		146.0 mg
HEPES		5.95 g
Hypoxanthine		2.0 mg
MEM		500.0 ml
Sodium pyruvate		220.0 mg
Thymidine		4.0 mg
H_2O_{dd}	to	1.1 L

After autoclaving, 10 ml sterile non-essential amino acid concentrate (100x, pH 7.5) was added.

B5 medium		
2,4-dichlorophenoxyacetic acid		994.5 µg
Gamborg B5		3.20 g
Sucrose		20.0 g
H_2O_{dd}	to	1.0 L

pH of the medium was adjusted to 5.7 before autoclaving.

Complete medium		
L- glutamine		0.3 g
Foetal calf serum		16.0 ml
HEPES		5.95 g
Gentamicin (50 µg/ml)		0.8 ml
2-mercaptoethanol		3.9 mg
Penicillin (100 U/ml)		0.8 ml
Sodium bicarbonate		0.35 g
H_2O_{dd}	to	1.0 L

Complete Baltz medium		
2-mercaptoethanol		3.9 mg
Baltz Medium Basic Solution		0.8 ml
Foetal calf serum		16.0 ml
Penicillin (10,000 U/ml)		0.8 ml
Streptomycin (10,000 U/ml)		0.8 ml
H_2O_{dd}	to	1.0 L

FCS (pH 7.2) was inactivated for 30 min at 56 °C before autoclaving while penicillin and streptomycin were added after autoclaving.

DMEM medium		
DMEM without glutamine		4.5 g
Foetal calf serum		20 g
L-glutamine		29.2 g
2-mercaptoethanol		3.9 mg
H_2O_{dd}	to	1.0 L

HR medium		
Disodium hydrogen phosphate		35.60 g
HR medium		14.67 g
Potassium dihydrogen phosphate		27.22 g
Sodium bicarbonate		1.00 g
H_2O_{dd}	to	1.0 L

GAA medium		
Glycerol		10.0 g
L-asparagine		1.0 g
Potassium phosphate		1.0 g
Agar		18.0 g
ASW	to	1.0 L

ISP 2 medium		
Dextrose		4.0 g
Malt extract		10.0 g
Yeast extract		4.0 g
Agar		18.0 g
ASW	to	1.0 L

LB medium		
Peptone		10.0 g
Sodium chloride		5.0 g
Yeast extract		5.0 g
Agar		18.0 g
H_2O_{dd}	to	1.0 L

LB/amp	
Ampicillin	100.0 µg
LB medium	1.0 L

Ampicillin was sterilized by filtration and then added to LB agar after autoclaving.

LB/amp/IPTG/X-gal	
Ampicillin	100 µg
IPTG	119.2 mg
X-gal	80 µg
LB agar	1.0 L

Ampicillin, IPTG and X-gal were sterilized by filtration and then added LB agar after autoclaving.

LS medium		
2,4-dichlorophenoxyacetic acid		221.0 µg
Linsmaier & Skoog medium		4.60 g
Naphthalene acetic acid		186.2 µg
Sucrose		30.0 g
H_2O_{dd}	to	1.0 L

pH of the medium was adjusted to 6.0 before autoclaving.

MA medium		
Ammonium carbonate		1.5 mg
Boric acid		22.0 mg
Calcium chloride		1.8 g
Ferric citrate		0.1 g
Magnesium chloride		5.9 g
Magnesium sulfate		3.2 g
Potassium bromide		80.0 mg
Potassium chloride		0.5 g
Sodium chloride		19.5 g
Sodium bicarbonate		0.16 g
Sodium fluoride		2.4 mg
Sodium phosphate		8.0 mg
Strontium chloride		34.0 mg
Sodium silicate		4.0 mg
Starch		0.5 g
Yeast extract		1.0 g
Agar		18.0 g
ASW	to	1.0 L

M1 medium		
Peptone		2.0 g
Soluble starch		10.0 g
Yeast extract		4.0 g
Agar		18.0 g
ASW	to	1.0 L

M1^{+} medium		
Peptone		2.0 g
Soluble starch		10.0 g
Yeast extract		4.0 g
Sponge extract (1%)		1.0 ml
Agar		18.0 g
ASW	to	1.0 L

Sponge extract was sterilized by filtration and then added to the medium after autoclaving.

OLIGO medium		
Sodium glycerophosphate		100.0 mg
Tryptone		0.5 g
Yeast extract		50.0 mg
Agar		18.0 g
ASW	to	1.0 L

R2A medium		
Casamino acids		0.5 g
Dextrose		0.5 g
Peptone		0.5 g
Potassium phosphate		0.3 g
Sodium pyruvate		1.0 mg
Starch		0.5 g
Yeast extract		0.5 g
Agar		18.0 g
ASW	to	1.0 L

SOC medium		
Glucose		12.3 g
Magnesium chloride		12.8 g
Magnesium sulfate		15.4 g
Potassium chloride		4.6 g
Tryptone		20.0 g
Sodium chloride		3.6 g
Yeast extract		5.0 g
H_2O_{dd}	to	1.0 L

Glucose, magnesium chloride and magnesium sulfate were sterilized by filtration and then added to the medium after autoclaving.

YPD medium		
Dextrose		20.0 g
Peptone		20.0 g
Yeast extract		10.0 g
Agar		18.0 g
H_2O_{dd}	to	1.0 L

B- Miscellaneous solutions and buffers

Artificial seawater		
Boric acid		0.26 g
Calcium chloride		11.0 g
Magnesium chloride		106.4 g
Potassium chloride		6.64 g
Potassium bromide		0.96 g
Sodium chloride		234.7 g
Sodium fluoride		30.0 mg
Sodium sulfate		39.2 g
Sodium bicarbonate		1.92 g
Strontium chloride		0.24 g
H_2O_{dd}	to	10.0 L

Bradford reagent		
Coomassie Brilliant Blue G-250		10.0 mg
Ethanol (95%)		5.0 ml
Phosphoric acid (85%)		10.0 ml
H_2O_{dd}	to	100.0 ml

Coomassie staining solution		
Acetic acid		9.0 ml
Coomassie blue R 250		250.0 mg
Methanol		45.5 ml
H_2O_{dd}	to	100.0 ml

Coomassie destaining solution		
Acetic acid		9.0 ml
Methanol		45.5 ml
H_2O_{dd}	to	100.0 ml

EDTA (0.5 M, pH 8.0)		
$Na_2EDTA.2H_2O$		93.05 g
H_2O_{dd}	to	500.0 ml

EDTA was dissolved in 450 ml water and the pH of the solution was adjusted to 8.0 with sodium hydroxide pellets. The water was then added to a final volume of 500 ml.

Extraction buffer A		
Benzamidine hydrochloride		156.0 mg
Disodium hydrogen phosphate		2.67 g
PMSF		348.0 mg
Sodium dihydrogen phosphate		2.07 g
H_2O_{dd}	to	1.0 L

Extraction buffer B		
Benzamidine hydrochloride		156.0 mg
Disodium hydrogen phoshate		2.67 g
EDTA		4.38 g
PMSF		348.0 mg
Sodium dihydrogen phosphate		2.07 g
Sodium chloride		0.88 g
H_2O_{dd}	to	1.0 L

IPTG (1 M)		
IPTG		2.38 g
H_2O_{dd}	to	10.0 ml

Plasmid mini-prep. buffers

Buffer P1	
EDTA (0.5 M, pH 8.0)	2.0 ml
RNAse A (10 mg/ml)	1.0 ml

Tris-Cl (1 M, pH 7.5)		5.0 ml
H_2O_{dd}	to	100.0 ml

Buffer P2		
Sodium hydroxide		0.8 g
SDS		1.0 g
H_2O_{dd}	to	100.0 ml

Buffer P3		
Potassium acetate		29.4 g
Glacial acetic acid		11.5 ml
H_2O_{dd}	to	100.0 ml

pH of the solution was adjusted to pH 5.5 using glacial acetic acid.

PMSF		
PMSF		1.57 g
Ethanol	to	100.0 ml

Sample buffer for SDS-PAGE		
Bromophenol blue		20.0 mg
Glycerin		40.0 g
SDS		10.0 g
EDTA (4 µM)		1.6 ml
Tris-HCl (0.5, pH 6.8)	to	50.0 ml

SDS running buffer		
Glycine		14.4 g
SDS		1.0 g
Tris		3.0 g
H_2O_{dd}	to	1.0 L

SDS solution (10% w/v)		
SDS		10.0 g
H_2O_{dd}	to	100.0 ml

SDS was dissolved in about 85 ml distilled water with warming at 50 °C. The pH of the solution was then adjusted to 7.0 using concentrated HCl and the water was added to obtain a final volume of 100 ml.

Separating gel solution (12%)	
30% Acrylamide/Bisacrylamide	4.0 ml
Ammonium persulfate (10%)	100.0 µl
SDS (10%)	100.0 µl
Tris (1.5 M, pH 8.8)	2.5 ml
TEMED	4.0 µl
H_2O_{dd}	3.3 ml

Stacking gel solution (5%)	
30% Acrylamide/Bisacrylamide	330.0 µl
Ammonium persulfate (10%)	20.0 µl
SDS (10%)	20.0 µl
Tris (1 M, pH 6.8)	250.0 µl
TEMED	2.0 µl
H_2O_{dd}	1.4 ml

Tris-Cl (1.5 M, pH 8.8)		
Tris		18.17 g
H_2O_{dd}	to	100.0 ml

Tris was dissolved in about 85 ml water. The pH of the solution was then adjusted to 8.8 by adding concentrated HCl. The water was then added to obtain a final volume of 100 ml.

TAE buffer (5x)		
$Na_2EDTA.2H_2O$		19.0 g
Sodium acetate.$3H_2O$		136.12 g
Tris base		242.0 g
H_2O_{dd}	to	1.0 L

The components were dissolved in about 900 ml water and the pH was adjusted to 7.2 with acetic acid. The water was then added to obtain a final volume of 1 L.

Urea (8 M)		
Urea		48.05 g
H_2O_{dd}	to	100.0 ml

X-gal (5% w/v)		
X-gal		0.5 g
Dimethylformamide	to	10.0 ml

VII. Chemicals

Chemical name	Manufacturer
Acetic acid	AppliChem
Acetonitrile	Sigma
Acetylsalicylic acid	Sigma
Acrylamide/Bisacrylamide 37.5:1	Roth
Agar, granulated	Difco
Agarose, ultrapure	Gibco
Ammonium sulfate	AppliChem
Ammoniun persulfate	Sigma
Ampicillin	AppliChem
Asparagine	Sigma
Bradford dye	BioRad
Benzamidine hydrochloride	Roth
Boric acid	AppliChem
Bovine serum albumin	BioRad
Bromphenol blue	Merck
Calcium carbonate	AppliChem
Calcium chloride	AppliChem
Chitosan	Roth
Chloramphenicol	Sigma
Chloroform	Roth
Coomassie R250	Pierce
Cycloheximide	Sigma
2,4-dichlorophenoxyacetic acid	Sigma-Aldrich
Dichlormethan	Sigma-Aldrich
Deuterated methanol	Sigma-Aldrich
Deuterated chloroform	Sigma-Aldrich
Dipotassium hydrogen phosphate	Roth
Disodium hydrogen phosphate	Sisma
Dimethylformamide	AppliChem
Dimethylsulfoxide	Sigma
Ethanol absolute	Merck
Ethanol denatured	Roth
Ethidium bromide (1% solution)	Roth
Ethyl acetate	Roth
Ethylenediamine tetraacetic acid dihydrate	Serva
Ferric ammonium citrate	AppliChem
Ferrous sulfate heptahydrate	Fluka
Formaldehyde	Roth
Gamborg B5 medium	Roth

Chemical name	Manufacturer
Gentamicin	Sigma
Glucose	AppliChem
Glutamine	Sigma
Glutaraldehyde	Sigma
Glycerin	Roth
Glycin	Roth
Hydrochloric acid	AppliChem
Isopropanol	Roth
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Sigma
LS medium	Roth
Malt extract	AppliChem
Magnesium chloride hexahydrate	AppliChem
Magnesium sulfate heptahydrate	AppliChem
Mannitol	AppliChem
Marfey's reagent	Fluka
Methanol	Sigma, Roth
Methyl jasmonate	Sigma-Aldrich
Naphthalene acetic acid	Sigma
Nalidixic acid	Sigma
Nystatin	Sigma
Peptone	Roth
Polygalacturonic acid	Sigma
Phenol	AppliChem
PMSF	Roth
Potassium bromide	AppliChem
Potassium chloride	Fluka
Potassium pyruvate	Roth
Potassium nitrate	AppliChem
Salicylic acid	Sigma
Serine	Sigma
Silver nitrate	Roth
Sodium acetate trihydrate	AppliChem
Sodium bicarbonate	Merck
Sodium carbonate	Merck
Sodium chloride	Roth
Sodium dihydrogen phosphate	Sigma
Sodium dodecyl sulfate	AppliChem
Sodium fluoride	Fluka
Sodium glycerophosphate	Roth
Sodium hydroxide	AppliChem

Chemical name	Manufacturer
Sodium propionate	AppliChem
Sodium sulfate	Merck
Sodium thiosulfate	AppliChem
Starch	Roth
Streptomycin	Sigma
Strontium chloride	Fluka
Sucrose	Roth
TEMED (N,N,N',N'-Tetramethylethylendiamin)	Roth
Threonine	Sigma
Trifluoroacetic acid	Sigma
Tris (hydroxymethyl) aminomethane hydrochloride	Sigma
Triton X-100	AppliChem
Tryptone	Roth
Tyrosine	Sigma
Urea	Roth
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Sigma
XAD-16 resin	sigma
Yeast extract	Gibco

VIII. Software

Software	Application
Align	Sequence alignment and editing
Antimarin Database	Marine natural products database
ARB	Construction of phylogenetic tree
Basic Local Alignment Search Tool (Blast)	Searching within the sequences stored in the database
ChemDraw	Drawing of chemical structure
ClustalX	Alignment of sequences
Multianalyist 1.1	Documentation of agarose and polyacrylamide gels
Topspin 21	Visualisation of spectral data
Treeview	Visualisation of phylogenetic tree
Vector NTI Advance™ 10	Opening and editing of sequences

IX. Enzymes and Kits

Enzyme/ Kit	Manufacturer
ABI Prism™ Big Dye ™ terminator cycle sequencing ready reaction kit	Applied Biosystems
FastDNA® spin kit for soil	Q-Biogene
Lysozyme	Sigma
Proteinase K	Sigma
pGEM-Teasy vector system	Promega
QIAquick PCR purification kit	Qiagen
REDTaq® ReadyMix™ PCR reaction mix	Sigma
Restriction endonucleases and buffers	New England Biolabs
RNase	Roche
Taq DNA polymerase and buffer	Qiagen
T4 DNA ligase and buffer	New England Biolabs
Molecular weight markers	
GeneRuler™ 1kb DNA ladder	Fermentas
GeneRuler™ 100bp DNA ladder	Fermentas
FastRuler™ DNA Ladder, high range	Fermentas
Stained protein molecular weight marker	Fermentas
Prestained protein molecular weight marker	Fermentas

X. Laboratory equipments

Equipment/ Supplies	Manufacturer
Autoclave (Tec 120, 9191E, FV 3.3)	Fedegari
(Varioklav 500, 135S)	H+P Labortechnik
Benchtop centrifuge (Biofuge Frasco)	Hereaus Instruments
Distilling apparatus for H_2O_{dd} (Bi-Dest 2304)	GFL
Disposable cuvettes (halbmikro 1,5 ml)	Plastibrand
ELISA plate reader (Multiskan Ascent)	Thermo Electron
Gel documentation (Gel Doc 2000)	BioRad
Gel electrophoresis chamber	BioRad
Heat block (Digi-Block Jr.)	Laboratory Devices

Equipment/ Supplies	Manufacturer
HPLC	JASCO
HPLC columns (Chromolith RP18)	Phenomenex
Ice maker (AF-20)	Scotsman
Incubator (Kelvitron®t)	Heraeus
(TV 40b)	Memmert
Magnetic stirrer (L32)	Labinco
Micropipettes (MicroOne 0,5-10 µl)	Microlab
Micropipettes(MicroOne 2.0-20 µl)	Microlab
(MicroOne 20-200 µl)	Microlab
(MicroOne 100-1000 µl)	Microlab
Microwave (Micromat)	AEG
(8020)	Privileg
MS (Micromass Q-TOF, MicroTOF)	Bruker Daltonik
NMR (400, 600 MHz)	Bruker
PCR cycler (T3-Thermocycler)	Biometra
pH Meter (MultiLine P4, SenTix 41)	WTW
Rotary evaporator (Laborota 4010)	Heidolph
Sequencer (ABI 377XL)	ABI Prism
Spectrophotometer (Ultraspec 3000)	Pharmacia Biotech
Speedvac concentrator (Savant)	Thermo Scientific
Shakers (Certomat U)	Braun
(SM-30)	Edmund Bühler
(Rotationsmischer 3300)	Eppendorf
(HT)	Infors

XI. Microorganisms

Microorganism	Application
Bacillus subtilis ATCC 6051	Bioactivity testing
Candida albicans 5314	Bioactivity testing
Candida maltosa SBUG 700	Bioactivity testing
Enterococcus faecalis JH212	Bioactivity testing
Enterococcus faecium 6413	Bioactivity testing
Escherichia coli ATCC 11229	Bioactivity testing
Escherichia coli 536	Cloning
Escherichia coli XL1-Blue	Bioactivity testing

Microorganism	Application
Leishmania major	Bioactivity testing
Pseudomonas aeruginosa Nr. 3	Bioactivity testing
Pseudomonas aeruginosa ATCC 22853	Bioactivity testing
Staphylococcus aureus NCTC 8325	Bioactivity testing
Staphylococcus aureus ATCC 6538	Bioactivity testing
Staphylococcus epidermidis RP62A	Bioactivity testing
Trypanosoma brucei brucei 221	Bioactivity testing
Yersinia pseudotuberculosis 252 01A	Bioactivity testing
Yersinia pestis KUMA	Bioactivity testing

XII. Oligonucleotide primers

Oligonucleotide	Sequence 5' – 3'	Annealing (°C)	Specificity
27f	GAGTTTGATCCTGGCTCA	56	Bacterial 16S rRNA gene (universal)
1492r	TACGGCTACCTTGTTACGACTT	56	Bacterial 16S rRNA gene (universal)
SP6	ATTTAGGTGACACTATAG	45	Sequencing of cloning vectors
T7	GTAATACGACTCACTATAGGG	45	Sequencing of cloning vectors

XIII. Plant cell cultures

Cell culture	Source
Agrostis tenuis	Prof. M. J. Müller (Würzburg, Germany)
Arabidopsis thaliana	Prof M. H. Zenk (München, Germany)
Baphicanthus cusi	Prof. M. J. Müller (Würzburg, Germany)
Daucus carota	Prof. M. J. Müller (Würzburg, Germany)
Lavendula angustifolia	Prof. M. J. Müller (Würzburg, Germany)
Nicotiana tabacum petit havanna	Prof. M. J. Müller (Würzburg, Germany)
Rauvolfia verticillata	Prof. M. J. Müller (Würzburg, Germany)

XIV. Special laboratory tools

Tool	Manufacturer
Cryotubes (1.5 ml)	Greiner Bio-One
Eppendorf tubes	Sarstedt
Falkon tubes (50 ml)	Cell star
Filter paper discs (5 mm)	Roth
96-well plates	Greiner Bio-One
Parafilm	Pechiney
PCR tubes (0.5 ml)	B. Braun
Pipette tips	Sarstedt
Petri Dishes (145/20 mm)	Greiner Bio-One
Petri Dishes (60/20 mm)	Greiner Bio-One
Syringe filters (0.2 µm)	Schleicher & Schuell
Serological pipettes	Sarstedt

Publications

1. **Abdelmohsen UR**, Pimentel-Elardo SM, Hanora A, Radwan M, Abou-El-Ela SH, Ahmed S and Hentschel U (2010). Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated actinomycetes. Marine Drugs *8*: 399-412.
2. **Abdelmohsen UR**, Ali W, Eom SH, Hentschel U and Roitsch T. Screening for antimicrobial activities from elicited plant cell suspension cultures. Plant Cell Tissue and Organ Culture (submitted, August 2010).
3. Angermeier H, Kamke J, **Abdelmohsen UR**, Krohne G, Pawlik JR, Lindquist NL and Hentschel U. The pathology of Sponge Orange Band disease affecting the Caribbean barrel sponge *Xestospongia muta*. FEMS Microbiology Ecology (submitted, June 2010).
4. Naseem M, Großkinsky D, **Abdelmohsen UR**, Plickert N, Engelke T, Griebel T, Zeier J, Strnad M, Pfeifhofer H, van der Graaff E, Simon U and Roitsch, T. Cytokinin-mediated resistance against *Pseudomonas syringae* in tobacco is independent of salicylic acid signalling and accompanied by increased antimicrobial phytoalexin synthesis. PLOS Pathogens (submitted, September 2010).

Symposia

10-13 Oct 2007, DPhG Jahrestagung, Institute of Pharmaceutical Biology, Erlangen, Germany. Poster presentation (Screening, purification and characterization of antimicrobial proteins from plant cell cultures).

10 Oct 2008, 36th Natural Products Meeting: Chemistry, Biology and Ecology, Institute of Organic Chemistry, Würzburg, Germany.

20-22 Nov 2008, 4th Joint Ph.D. Students Meeting, SFB 630, SFB 544 and SFB 766, Kloster Bronnbach, Germany.

26-27 Mar 2009, 4th International Symposium: Research evolution, Graduate School of Life Sciences, Würzburg, Germany.

15 May 2009, 37th Natural Products Meeting: Chemistry, Biology and Ecology, Institute of Organic Chemistry, Bayreuth, Germany.

19-21 Nov 2009, Ph.D. Students Meeting, SFB 630, Heidelberg, Germany. Poster presentation (Isolation, phylogenetic analysis and antimicrobial activity screening of marine sponge-associated actinomycetes).

Workshops

28th May 2009, Poster Design and Presentation, Graduate School of Life Sciences, Würzburg, Germany.

15th Jan 2010, Writing for Publication, Graduate School of Life Sciences, Würzburg, Germany.

12th Mar 2010, Giving Academic Talks, Graduate School of Life Sciences, Würzburg, Germany.

16th Apr 2010, 39 Natural Products Meeting: Chemistry, Biology and Ecology, Max-Planck Institute of Chemical Ecology, Jena, Germany.

27th Apr 2010, Literaturverwaltung mit EndNote, Universitätsbibliothek, Würzburg, Germany.

Curriculum Vitae

Personal data

Name	Usama Ramadan Abdelmohsen
Date of birth	12.07.1980
Place of birth	Minia, Egypt
Nationality	Egyptian
Status	Married

Academic career

10/2008-10/2010	Topic “Antimicrobial activities from marine sponge-associated actinomycetes”, Supervisor (Prof. Dr. Ute Hentschel, Botany II Department, Julius-Maximilians-University Würzburg, Germany)
04/2007-09/2008	Topic “Antimicrobial proteins from plant cell cultures”, Supervisor (Prof. Dr. Thomas Roitsch, Pharmaceutical Biology Department, Julius-Maximilians-University Würzburg, Germany)
09/2002–03/2007	Research assistant in Pharmacognosy Department, Faculty of Pharmacy, Minia University, Egypt
09/1997-05/2002	Bachelor of Pharmaceutical Sciences, Minia University, Egypt with the grade, excellent with degree of honour

Schooling

09/1986-05/1997	Primary, Preparatory and Secondary Schools, Minia, Egypt

Experiences

Academic experience	Five years teaching practical courses of Pharmacognosy to undergraduate students of the Faculty of Pharmacy, Minia University, Minia, Egypt
Pharmaceutical experience	300 hours training in Egyptian pharmacies

Würzburg, Usama Ramadan Abdelmohsen