

**Novel anti-infective secondary metabolites and biosynthetic
gene clusters from actinomycetes associated
with marine sponges**

**Neue anti-infektive Sekundärmetabolite und biosynthetische
Gencluster aus mit marinen Schwämmen
assoziierten Actinomyceten**

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submitted by

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ERKLÄRUNG

gemäß § 4 Abs. 3 Ziff. 3, 5 und 8
der Promotionsordnung der Fakultät für Biologie der
Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich ehrenwörtlich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben. Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen. Des Weiteren erkläre ich, dass ich früher weder akademische Grade erworben habe, noch zu erwerben versucht habe.

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(Sheila Marie Pimentel Elardo)

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FOR MY FAMILY

Summary

Marine sponges (Porifera) harbor diverse microbial communities within their mesohyl, among them representatives of the phylum *Actinobacteria*, commonly known as actinomycetes. Actinomycetes are prolific producers of pharmacologically important compounds and are responsible for producing the majority of antibiotics. The main aim of this Ph.D. study was to investigate the metabolic potential of the sponge-associated actinomycetes to produce novel anti-infective agents.

The first aim was to cultivate actinomycetes derived from different marine sponges. 16S rDNA sequencing revealed that the strains belonged to diverse actinomycete genera such as *Gordonia*, *Isoptericola*, *Micromonospora*, *Nocardiopsis*, *Saccharopolyspora* and *Streptomyces*. Phylogenetic analyses and polyphasic characterization further revealed that two of these strains represent new species, namely *Saccharopolyspora cebuensis* strain SPE10-1^T (Pimentel-Elardo et al. 2008a) and *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008b).

Furthermore, secondary metabolite production of the actinomycete strains was investigated. The metabolites were isolated using a bioassay-guided purification scheme followed by structure elucidation using spectroscopic methods and subjected to an elaborate anti-infective screening panel. Several interesting compounds were isolated namely, the novel polyketides cebulactam A1 and A2 (Pimentel-Elardo et al. 2008c), a family of tetromycin compounds including novel derivatives, cyclodepsipeptide valinomycin, indolocarbazole staurosporine, diketopiperazine cycloisoleucylprolyl and butenolide. These compounds exhibited significant anti-parasitic as well as protease inhibitory activities.

The third aim of this Ph.D. study was to identify biosynthetic gene clusters encoding for nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) present in the actinomycete strains. Genomic library construction and sequencing revealed insights into the metabolic potential and biosynthetic pathways of selected strains. An interesting NRPS system detected in *Streptomyces* sp. strain Aer003 was found to be widely distributed in several sponge species, in an ascidian and in seawater and is postulated to encode for a large peptide molecule. Sequencing of the PKS gene cluster of *Saccharopolyspora cebuensis* strain SPE10-1^T allowed the prediction of the cebulactam biosynthetic pathway which utilizes 3-amino-5-hydroxybenzoic acid as the starter unit

followed by successive condensation steps involving methylmalonyl extender units and auxiliary domains responsible for the polyketide assembly.

In conclusion, this Ph.D. study has shown that diverse actinomycete genera are associated with marine sponges. The strains, two of them novel species, produced diverse chemical structures with interesting anti-infective properties. Lastly, the presence of biosynthetic gene clusters identified in this study substantiates the biosynthetic potential of actinomycetes to produce exploitable natural products and hopefully provides a sustainable supply of anti-infective compounds.

Zusammenfassung

Zahlreiche marine Schwämme (Phylum: Porifera) beherbergen eine phylogenetisch diverse mikrobielle Gemeinschaft in der Mesohyl-Matrix, darunter auch viele Vertreter des bakteriellen Phylums *Actinobacteria*, die umgangssprachlich als Actinomyceten bekannt sind. Actinomyceten sind wichtige Produzenten vieler Antibiotika und von weiteren pharmazeutisch relevanten Substanzen. Das Hauptziel dieser Promotionsarbeit war die Untersuchung des Potentials Schwamm-assoziiierter Actinomyceten zur Produktion neuer Infektions-hemmender Substanzen.

Ein erstes Ziel dieser Doktorarbeit war die Kultivierung von Actinomyceten aus verschiedenen marinen Schwammarten. Die Sequenzierung der respektiven 16S rRNA Gene zeigte eine phylogenetische Zugehörigkeit der Isolate zu verschiedenen Actinomyceten-Familien, wie *Gordonia*, *Isoptericola*, *Micromonospora*, *Nocardiopsis*, *Saccharopolyspora* und *Streptomyces*. Durch phylogenetische Analysen und umfangreiche taxonomische Charakterisierungen konnten zwei neue Actinomyceten-Arten, *Saccharopolyspora cebuensis* strain SPE10-1^T (Pimentel-Elardo et al. 2008a) und *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008b) beschrieben werden.

Des Weiteren sollten die Actinomyceten-Isolate auf die Produktion von Sekundär-Metaboliten hin untersucht werden. Die Substanzen wurden „bioassay-guided“ aufgereinigt und isoliert sowie deren Struktur mittels spektroskopischer Methoden aufgeklärt. Anschließend wurden die Substanzen ausführlichen Screening-Methoden unterzogen, um sie auf anti-infektive Wirkungen hin zu untersuchen. Zahlreiche interessante Verbindungen konnten so isoliert werden, u. a. die neuen Polyketide Cebulactam A1 und A2 (Pimentel-Elardo et al. 2008c); eine Familie von Tetracyclin-Substanzen inklusive neuartiger Derivate; das Cyclodepsipeptid Valinomycin, Indolocarbazole Staurosporine, Diketopiperazine Cycloisoleucylprolyl und Butenolide. Die Verbindungen zeigten signifikante anti-parasitische und Protease-hemmende Aktivitäten.

Das dritte Ziel dieser Arbeit war es, die für nicht-ribosomale Peptidsynthetasen (NRPS) und Polyketidsynthetasen (PKS) kodierenden, biosynthetischen Gen-Cluster in den Actinomyceten-Isolaten zu identifizieren. Die Konstruktion von Genbanken sowie die Sequenzierung ausgewählter Cosmidklone lieferte erste Einblicke in das Stoffwechsel- und Biosynthesepotential ausgewählter Isolate. Beispielsweise konnte ein interessantes NRPS-System in *Streptomyces* sp. Stamm Aer003 identifiziert werden, welches in

verschiedenen Schwammarten, einer Ascidienart sowie im Meerwasser gefunden wurde. Die Sequenzierung eines PKS-Genclusters aus *Saccharopolyspora cebuensis* strain SPE 10-1^T ermöglicht die Voraussage des Cebulactam-Biosynthesewegs in dem 3-Amino-5-Hydroxybenzoesäure als Ausgangsprodukt dient, welches durch sukzessive Kondensationsschritte sowie Verlängerungen durch Methylmalonyl- und Zusatzdomänen zum endgültigen Polyketid führen.

Zusammenfassend konnte in dieser Promotionsarbeit gezeigt werden, dass marine Schwämme mit diversen Vertretern aus verschiedenen Familien der Actinomyceten assoziiert sind. Die Bakterienisolate, von denen zwei neue Arten repräsentieren, produzierten mehrere chemische Substanzen mit interessanten anti-infektiven Eigenschaften. Des Weiteren konnte mit dieser Arbeit durch die Identifizierung von Biosynthese-Genclustern das Potential von Actinomyceten zur Produktion verwertbarer bioaktiver Substanzen bekräftigt und somit ein Beitrag zur Entdeckung neuer anti-infektiver Substanzen erbracht werden.

Chapter 1

Introduction

1.1 Marine sponges (Porifera)

1.1.1 Biology and microbial diversity

Marine sponges (Porifera) are ancient metazoans dating back to the Precambrian era (Li et al. 1998). The phylum Porifera is divided into three classes: *Hexactinellida* (glass sponges), *Calcarea* (calcareous sponges) and *Demospongiae* (demosponges), with the last group containing an estimated 85% of the formally described living species (Hooper and van Soest 2002). These sessile invertebrates inhabit a wide variety of marine and freshwater systems and are found throughout tropical, temperate and polar regions (Hooper and van Soest 2002). The growth habits of sponges encompass various shapes, colors and sizes (Fig.1.1) (Brusca and Brusca 1990). Furthermore, sponges are comprised of a basic body plan (Bergquist 1978; Simpson 1984; Brusca and Brusca 1990). The outer surface, pinacoderm consists of pores called ostia which extend along interior canals that permeate through the sponge. Specialized flagellated cells called choanocytes form a series of choanoderm chambers where the choanocytes beat to pump water in through the ostia as well as through the aquiferous systems within the sponge body. These flagellated choanocytes also filter out food particles from the surrounding water, including bacteria and microalgae which are then transferred to the mesohyl and digested by archaeocytes through phagocytosis. After water is filtered through the choanocytes, it is then expelled from the sponge via the osculum rendering the effluent essentially sterile. Sponges are capable of turning over large volumes of water through its aquiferous canal system equaling to several thousands of liters for a 1-kg sponge per day (Vogel 1977).

Dense communities of microorganisms are present in the mesohyl of most sponges (Vacelet and Donadey 1977; Wilkinson 1978; Friedrich et al. 1999). These types of sponges have been termed 'bacteriosponges' or 'high-microbial-abundance sponges' (Vacelet and Donadey 1977; Hentschel et al. 2003) where bacterial densities may reach up to 10^8 - 10^{10} bacteria per gram of sponge wet weight (Hentschel et al. 2006), exceeding that of seawater by several orders of magnitude (Friedrich et al. 2001) and accounting to up to 40% of the sponge biomass (Vacelet and Donadey 1977). On the other hand, the mesohyl of other sponges that co-exist in the same habitat are essentially devoid of microorganisms, hence these are termed as 'low-microbial-abundance sponges' with 10^5 -

10^6 bacteria per gram of sponge wet weight which are within the range of natural seawater (Hentschel et al. 2006) (Fig. 1.2).

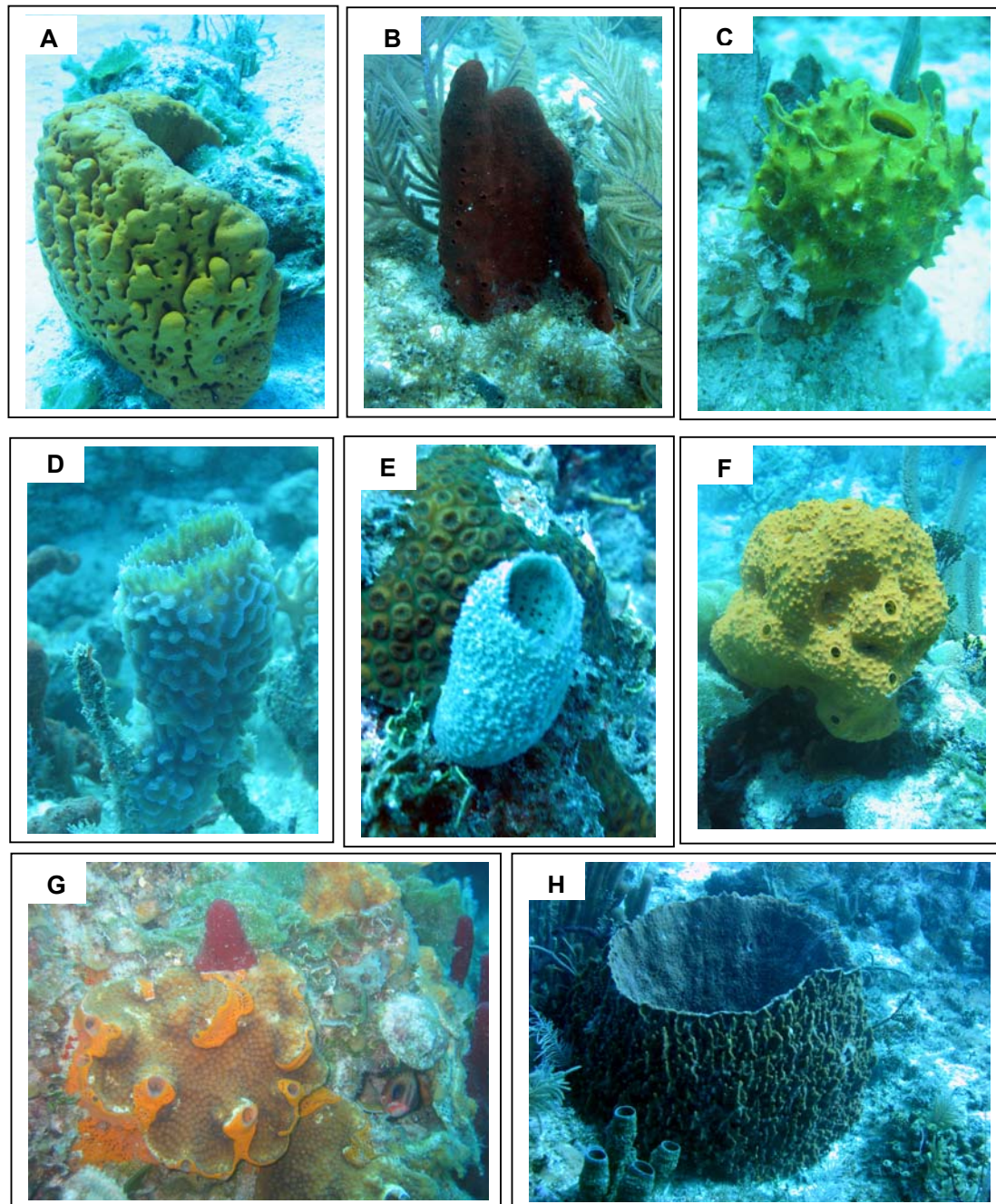


Fig. 1.1 Demosponges: (A): *Agelas clathrodes*; (B): *Amphimedon compressa*; (C): *Aplysina fistularis*; (D): *Callyspongia plicifera*; (E): *Niphates digitalis*; (F): *Pseudoceratina crassa*; (G): *Mycale laxissima*; (H): *Xestospongia muta* (underwater photography by H. Angermeier, University of Würzburg)

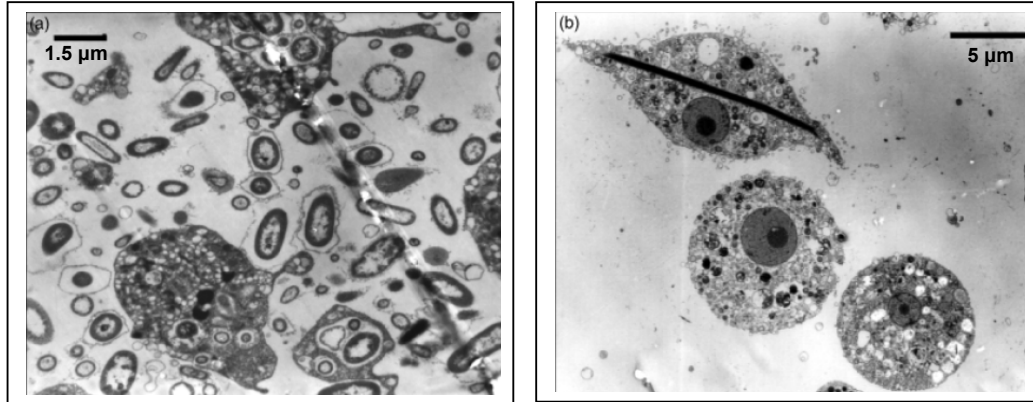


Fig. 1.2 Transmission electron micrographs of (A) *Xestospongia muta* (high-microbial-abundance sponge) and (B) *Callyspongia vaginalis* (low-microbial-abundance sponge) (Hentschel et al. 2006)

The associations between sponges and microorganisms are maintained such that microbial symbionts are passed on from the parent sponge through reproductive stages through vertical transmission (Usher et al. 2001; Ereskovsky et al. 2005; Schmitt et al. 2007; Schmitt et al. 2008a). These symbionts are stable populations which are phylogenetically distinct from transient bacteria and which are filtered out of the surrounding seawater during the feeding process of the sponge. Symbiotic functions that have been attributed include nutrient acquisition, sponge skeleton stabilization, metabolic waste processing and metabolite production (Wilkinson 1992; Unson et al. 1994; Bewley et al. 1996; Schmidt et al. 2000; Hentschel et al. 2006).

Furthermore, the microbial communities of sponges have been documented by electron microscopy studies indicating high morphological diversity as well as the presence of unusual microorganisms (Vacelet and Donadey 1977; Wilkinson 1978). Several studies have been reported on the diversity of the microbial consortia of sponges using cultivation-dependent approaches (Santavy et al. 1990; Webster and Hill 2001). With the advent of molecular tools such as 16S rRNA gene library construction, fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE), it is now possible to gain insights into the complex microbial consortia of sponges, including those that have eluded cultivation efforts (Head et al. 1998; Juretschko et al. 1998; Schmid et al. 2000; Webster et al. 2001; Hentschel et al. 2002; Olson and McCarthy 2005; Hill et al. 2006).

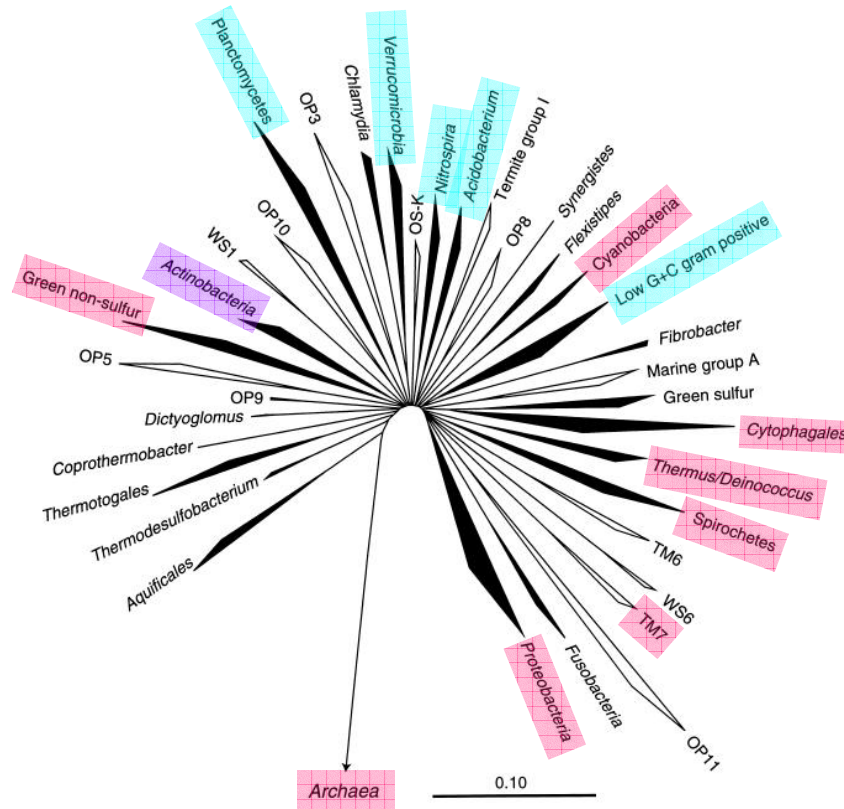


Fig. 1.3 Phylogenetic tree of prokaryotes indicating microbial groups recovered from marine sponges (highlighted in colored boxes) by cultivation-independent (pink boxes) and cultivation-dependent approaches (blue boxes). Members of Actinobacteria have been recovered using both approaches (violet box). Modified from Hugenholtz et al. (1998) and Schmitt et al. (2008b)

For instance, 16S rRNA gene library construction using universal bacterial PCR primers revealed a uniform, yet phylogenetically complex microbial population in sponges from different oceans (Hentschel et al. 2002; Hentschel et al. 2006). These 16S rRNA gene phylotypes are affiliated with the phyla *Chloroflexi*, *Actinobacteria*, *Proteobacteria*, *Nitrospira*, *Cyanobacteria*, and *Bacteroidetes*. A novel eubacterial candidate phylum *Poribacteria* was also discovered in Verongid sponges (Fieseler et al. 2004). In addition, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia* have also been isolated in pure culture from marine sponges (Burja and Hill 2001; Hentschel et al. 2001; Webster and Hill 2001; Olson et al. 2002; Pimentel-Elardo et al. 2003; Dieckmann et al. 2005; Kim et al. 2005; Montalvo et al.

2005; Enticknap et al. 2006; Kim and Fuerst 2006; Scheuermayer et al. 2006; Zhang et al. 2006; Jiang et al. 2007; Sertan-de Guzman et al. 2007). In contrast, freshwater sponge species have much lower bacterial diversity and abundance and only representatives of *Actinobacteria*, *Chloroflexi*, *Alpha-* and *Betaproteobacteria* were recovered (Gernert et al. 2005). Archaeal members have been detected from the phylum *Crenarchaeota* such as the well-studied sponge archaeon "*Candidatus Cenarchaeum symbiosum*" (Preston et al. 1996; Taylor et al. 2007). Fungal strains have also been recovered in several sponges such as *Penicillium* and *Aspergillus*, which are ubiquitous in terrestrial habitats (Höller et al. 2000).

The identification of *Actinobacteria* in marine sponges is interesting as members of this group are known to be prolific producers of pharmacologically important natural products (Munro et al. 1999; Fiedler et al. 2005; Fenical and Jensen 2006; Newman and Hill 2006). For example, the presence of *Actinobacteria* was detected in the sponge *Rhopaloeides odorabile* by culture-independent methods such as FISH and culture-dependent approaches (Webster et al. 2001). Furthermore, sponge-specific actinobacterial clusters were also recovered from *Theonella swinhoei* and *Aplysina aerophoba* (Hentschel et al. 2002; Montalvo et al. 2005). *Actinobacteria* exclusively within the sub-class *Acidimicrobidae* were shown to be the major components of the bacterial community of two *Xestospongia* species, namely *X. muta* and *X. testudinari* (Montalvo et al. 2005). Initial culturing attempts of the sponges also yielded different genera such as *Gordonia*, *Micrococcus* and *Brachybacterium*. Furthermore, over a hundred of actinomycete strains were cultivated from the marine sponge *Hymeniacidon perleve* and by phylogenetic analysis of the 16S rDNA gene sequences, these isolates belonged to the genera *Actinoalloteichus*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus* and *Streptomyces*, with the latter accounting for 74% of the isolates (Zhang et al. 2006). Using the same culture-dependent approach, several actinomycetes were isolated from the sponge *Haliclona* sp., such as *Streptomyces*, *Nocardiopsis*, *Micromonospora* and *Verrucosispora* (Jiang et al. 2007). Interestingly, marine actinomycetes related to the *Salinispora* group previously reported only from marine sediments were isolated from the Great Barrier Reef marine sponge *Pseudoceratina clavata* (Kim et al. 2005). *Salinispora*, the first seawater-requiring marine actinomycete discovered, produce secondary metabolites in a species-specific pattern (Jensen et al. 2007). Interestingly, genome sequencing revealed that *Salinispora tropica* dedicates a large percentage of its genome to natural product assembly, greater than previous *Streptomyces* species and other actinomycetes (Udwary et al. 2007).

1.2 Secondary metabolites

1.2.1 Natural products from marine sponges

Marine organisms produce novel secondary metabolites with a wide array of biological and pharmacological activities. Out of the 194,000 natural compounds listed in the first volume of the 2006 Chapman and Hall Dictionary of Natural Products, approximately 18,000 are derived from marine sources (Singh and Pelaez 2008). The number of marine natural products continues to rise with sponges as the most prolific producers of novel compounds, with more than 200 new metabolites reported annually (Blunt et al. 2003; Blunt et al. 2004; Blunt et al. 2005; Blunt et al. 2006; Blunt et al. 2007; Taylor et al. 2007; Blunt et al. 2008). These compounds have shown promise in treating inflammation, cancer as well as microbial and viral infections (Newman and Cragg 2004b; Fenical 2006; Newman 2008). The wealth of the bioactive compounds isolated especially from soft-bodied, sessile marine organisms such as sponges that lack morphological defense structures stresses the ecological importance of the compounds for these invertebrates (Thoms et al. 2004; Gross and König 2006; Paul et al. 2006; Thoms et al. 2006). Marine sponges accumulate structurally diverse secondary metabolites which have been found to be deterrent to potential fish predators (Pawlik et al. 1995), exhibit anti-fouling activity (Martin and Uriz 1993) and prevent the growth of competing invertebrates (Porter and Targett 1988; Paul et al. 2006).

The majority of the natural products currently in clinical and pre-clinical trials are produced by invertebrates, including marine sponges (Proksch et al. 2002; Haefner 2003; Newman and Cragg 2004a; Fenical 2006). One example is discodermolide, a polyhydroxylated lactone isolated from the sponge *Discodermia dissoluta* (Gunasekera et al. 1991) which is now under phase I clinical trial for pancreatic cancer. The compound KRN7000 from the sponge *Agelas mauritianus* (Kikuchi et al. 2001) and HTI-286, a hemiasterlin derivative from the South African sponge *Hemiasterella minor*, are anticancer drugs under phase II clinical trial (Newman and Cragg 2004b). The contignasterol derivative, IPL-576,092 isolated from the sponge *Petrosia contignata* (Coulson and O'Donnell 2000) is under phase II clinical trial for oral asthma therapy.

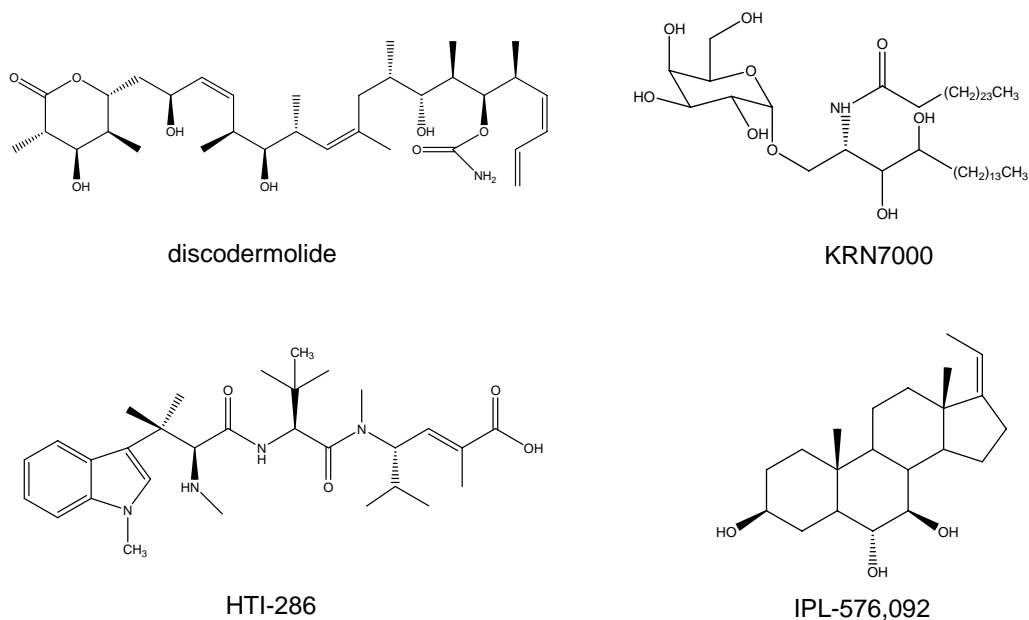


Fig. 1.4 Examples of natural products from sponges in clinical trials

Furthermore, there are several antitumour compounds from marine sponges in preclinical status (Newman and Cragg 2004a) such as laulimalide and isolaulimalide from the Pacific Ocean sponge *Cacospongia mycofijiensis* (Mooberry et al. 1999); peloruside A from the New Zealand sponge *Mycale hentscheli* (West et al. 2000); salicylialimides A and B from the Western Australian sponge *Haliclona* sp. (Erickson et al. 1987); and the variolins from the Antarctic sponge *Kirckpatrickia variolosa* (Perry et al. 1994; Trimurtulu et al. 1994).

Despite the large number of novel bioactive metabolites reported from marine sponges over the years, not a single compound has been approved as a drug, whether isolated or from total synthesis. A couple of exceptions are the nucleoside analogues Ara-A and Ara-C, now commercialized as antiviral and anticancer agents but these were not directly isolated from sponges but are synthetic derivatives of compounds from the Caribbean sponge *Cryptotethia crypta* (Bergmann and Feeny 1951). A major problem to the ultimate development of these compounds undergoing clinical or pre-clinical trials is the problem of supply. The concentrations of these bioactive compounds in marine invertebrates are minute, accounting typically less than 10⁻⁶ % of the wet weight (Proksch et al. 2002). This already causes considerable difficulties in clinical studies where gram

quantities of compounds are required. Such is the case of the potent antitumor halichondrins originally isolated from the Japanese sponge *Halichondria okadai* (Hirata and Uemura 1986). Although these macrolides have also been subsequently isolated from a number of other sponges such as *Axinella* sp. from the Western Pacific (Pettit et al. 1991), *Phakellia carteri* from the Eastern Indian Ocean (Pettit et al. 1993), and from *Lissondendoryx* sp. off the East Coast of South Island, New Zealand (Munro et al. 1999), still the demand for clinical trials and annual requirement as a commercial drug is not sufficient (Hart et al. 2000). The synthetic analogue of halichondrin B, E7389 having the same biological properties circumvented the supply problem and is now under phase II clinical trial (Choi et al. 2003; Sipkema et al. 2005).

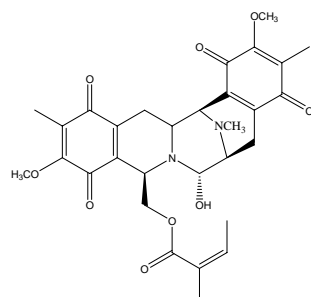
1.2.2 Metabolites from associated microorganisms

The majority of secondary metabolites that have been isolated from marine sponges are hypothesized to be produced by bacterial symbionts (Piel 2004). This speculation was based on the striking structural similarities with bacterial natural products (Table 1.1) belonging to substance classes that are typical for these microorganisms such as polyketides and nonribosomal peptides.

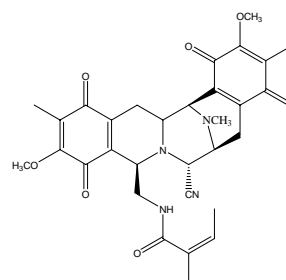
Table 1.1 Natural products from marine sponges that resemble bacterial metabolites (Piel 2004)

Sponge metabolite	Sponge Source	Bacterial metabolite	Bacterial Source
Arenastatin A	<i>Dysidea arenaria</i>	Cryptophycin	<i>Nostoc</i> sp.
Discodermide	<i>Discodermia dissoluta</i>	Alteramide A	<i>Alteromonas</i> sp.
		Ikarugamycin	<i>Streptomyces</i> sp.
Jaspamide	<i>Jaspis</i> spp.	Chondramide D	<i>Chondromyces crocatus</i>
Keramamide A	<i>Theonella</i> sp.	Ferintoic acid A	<i>Microcystis aeruginosa</i>
Mimosamycin	<i>Petrosia</i> sp.	Mimosamycin	<i>Streptomyces lavendulae</i>
Motuporin	<i>Theonella swinhoei</i>	Nodularin	<i>Nodularia spumigena</i>
Renieramycin E	<i>Reniera</i> sp.	Safracin B	<i>Pseudomonas fluorescens</i>
		Saframycin A	<i>Streptomyces lavendulae</i>
		Saframycin Mx1	<i>Myxococcus xanthus</i>
Salicylhalamide A	<i>Haliclona</i> sp.	Apicularen A	<i>Chondromyces</i> sp.
Swinholide A	<i>Theonella swinhoei</i>	Tolytoxin	<i>Tolypothrix</i> sp.
Misakinolide A	<i>Theonella</i> sp.	Scytophycin C	<i>Scytonema</i> sp.

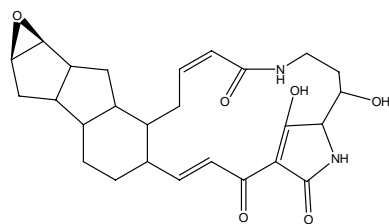
Despite structural complexity, the majority of these compounds exhibits only slight differences in the substitution patterns. Examples are renieramycin from *Reniera* sp. sponge (He and Faulkner 1989) and saframycin A from *Streptomyces lavendulae* (Arai et al. 1977), discodermide from the marine sponge *Discodermia dissoluta* (Gunasekera et al. 1980; Gunasekera et al. 1991) and ikarugamycin from *Streptomyces* sp. (Ito and Hirata 1972).



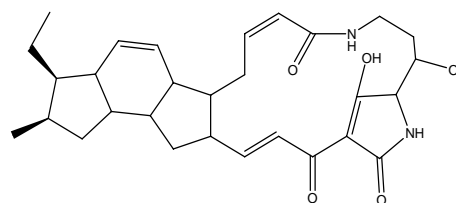
renieramycin E from
sponge *Reniera* sp.



saframycin A from
Streptomyces lavendulae



discodermide from sponge
Discodermia dissoluta



ikarugamycin from
Streptomyces sp.

Fig. 1.5 Examples of sponge metabolites structurally similar to bacterial metabolites

Secondary metabolites found in the tropical sponges *Dysidea herbacea* and *Theonella swinhoei* provide additional evidence to the involvement of microorganisms in natural product synthesis (Proksch et al. 2002). The Great Barrier reef sponge *Dysidea herbacea* was found to contain the sesquiterpenes spirodysin and herbadysidolide as well as the chlorinated amino acid derivative (Unson and Faulkner 1993). These compounds were also found in their cyanobacterial symbiont *Oscillatoria spongelliae* (Berthold et al. 1982) after disruption of the sponge tissue and separation of the cyanobacterial cells. The sponge *Theonella swinhoei* collected from the Philippines and Micronesia produces the cyclic peptide theopalumide and the macrolide swinholide and these compounds were

detected in cellular fractions containing filamentous bacteria as well as unicellular bacteria, respectively (Bewley et al. 1996; Piel 2004). Using 16S rDNA sequencing, the theopalauamide-containing symbiont was assigned as a novel δ -proteobacterium "*Candidatus Entotheonella palauensis*" (Schmidt et al. 2000).

1.2.3 Metabolites from marine actinomycetes

The class *Actinobacteria*, specifically bacteria belonging to the order *Actinomycetales*, accounts for approximately 7000 of compounds reported in the Dictionary of Natural Products (Jensen et al. 2005). Actinomycetes are responsible for the production of about half of the discovered bioactive secondary metabolites such as antibiotics. Remarkably, the genus *Streptomyces* accounts for 80% of actinomycete natural products reported (Berdy 2005), mostly producing phenazone and lactone amide type of compounds with antibiotic properties (Kelecom 2002). Although the majority of the actinomycetes were previously isolated from terrestrial sources, these bacteria have also been recovered from marine sediments, and even from deepest ocean trenches (Grein and Meyers 1958; Mincer et al. 2002; Maldonado et al. 2005b; Fenical and Jensen 2006; Pathom-Aree et al. 2006; Bredholdt et al. 2007). The isolation of these marine actinomycetes could be accounted for by the cultivation of spores that may have originated from soil-inhabiting strains that were washed into the marine environment. The first marine actinomycete described taxonomically was *Rhodococcus marinonascens* (Helmke and Weyland 1984) and quite recently, new genera have been described including *Salinispora*, the first obligate marine actinomycete isolated from ocean sediments (Han et al. 2003; Yi et al. 2004; Maldonado et al. 2005a). Furthermore, Mincer et al. (2005) demonstrated that *Salinispora* strains are actively growing in sediment samples indicating that these bacteria are metabolically active in the natural marine environment, addressing the question whether actinomycetes only exist as dormant spores. The discovery of numerous new marine actinomycete taxa, their demonstrated metabolic activity, and their ability to form stable populations in different habitats clearly illustrate that indigenous marine actinomycetes exist in the oceans (Lam 2006).

Numerous novel secondary metabolites have been isolated from marine actinomycetes, although exploitation of this group is still at its infancy. Example is the novel β -lactone- γ -lactam, salinisporamide A isolated from *Salinispora tropica* now undergoing phase I clinical trial as proteasome inhibitor for treating multiple myeloma cells (Feling et al. 2003; Chauhan et al. 2005). Another example is abyssomicin C, a novel polyketide antibiotic produced by the marine *Verrucosipora* strain (Riedlinger et al. 2004), is a potent inhibitor

of para-aminobenzoic acid biosynthesis inhibiting folic acid biosynthesis at an earlier stage compared to the well-known sulfa drugs (Bister et al. 2004). Diazepinomicin, produced by a *Micromonospora* strain, is another example (Charan et al. 2004). This unique farnesylated dibenzodiazepinone is now under preclinical development as an anticancer agent.

Table 1.2 Novel metabolites produced by marine actinomycetes during the period 2003-2005 (Lam 2006)

Compound	Source	Activity
Abyssomicins	<i>Verrucosispora</i>	Antibacterial
Aureoverticillilactam	<i>Streptomyces aureoverticillatus</i>	Anticancer
Bonactin	<i>Streptomyces</i> sp.	Antimicrobial
Caprolactones	<i>Streptomyces</i> sp.	Anticancer
Chandrananimycins	<i>Actinomadura</i> sp.	Antimicrobial; anticancer
Chinikomycins	<i>Streptomyces</i> sp.	Anticancer
Diazepinomicin	<i>Micromonospora</i> sp.	Antibacterial; anticancer; antiinflammatory
3,6-disubstituted indoles	<i>Streptomyces</i> sp.	Anticancer
Frigocyclinone	<i>Streptomyces griseus</i>	Antibacterial
Glaciapyrroles	<i>Streptomyces</i> sp.	Antibacterial
Gutingimycin	<i>Streptomyces</i> sp.	Antibacterial
Helquinoline	<i>Janibacter limosus</i>	Antibacterial
Himalomycins	<i>Streptomyces</i> sp.	Antibacterial
IB-00208	<i>Actinomadura</i> sp.	Anticancer
Komodoquinone A	<i>Streptomyces</i> sp.	Neuritogenic
Lajollamycin	<i>Streptomyces nodosus</i>	Antibacterial
Marinomycins	<i>Marinispora</i> sp.	Antibacterial; anticancer
Mechercharmucins	<i>Thermoactinomyces</i> sp.	Anticancer
Salinosporamide A	<i>Salinispora tropica</i>	Anticancer
Sporolides	<i>Salinispora tropica</i>	Unknown biological activity
Trioxacarcins	<i>Streptomyces</i> sp.	Antibacterial; anticancer; antimalarial

1.3 Biosynthetic gene clusters

A growing number of novel, bioactive secondary metabolites produced by marine invertebrates as well as marine bacteria appear to be structurally biosynthesized by polyketide synthases (PKS) and/or nonribosomal peptide synthetases (NRPS). These

enzymes are organized in a modular fashion utilizing specific domains to sequentially catalyze the condensation of simple carboxylic acids for PKS systems or amino acid building blocks for NRPS systems into a growing chain. Each module is a cluster responsible for chain elongation through the recognition, activation and incorporation of specific substrates and structural diversity is introduced through various combinations of the integral and auxiliary domains (Schwarzer and Marahiel 2001; Salomon et al. 2004).

Nonribosomally produced peptides exhibit a remarkable spectrum of biological and pharmacological activities such as the antibiotics vancomycin and penicillin, immunosuppressive agent cyclosporine and the antitumor compound bleomycin. Actinomycetes are known to produce these pharmacologically important peptides. PCR screening efforts for genes associated with secondary metabolism such as NRPS have been used to evaluate the biosynthetic potential of actinomycetes (Ayuso-Sacido and Genilloud 2005; Ayuso et al. 2005). PCR primers tested on a large collection of 210 reference strains encompassing major families and genera in actinomycetes revealed the wide distribution of these genes in *Streptomyces* species as well as in other minor lineages where in some cases only a few compounds have been isolated. The NRPS systems occur more frequently in strains of the main antibiotic producers from the families *Micromonosporaceae*, *Pseudonocardiaceae*, and *Actinosynnemataceae*. Furthermore, NRPS genes were also detected by PCR amplification in cultivated actinobacteria from the South China Sea sponge *Haliclona* sp. belonging to the genera *Streptomyces*, *Nocardiopsis*, *Micromonospora* and *Verrucosispora* (Jiang et al. 2007). Biosynthetic genes have also been recovered from marine invertebrates. However, in another study by Kennedy et al. (2008), NRPS genes were not detected from the microbial metagenome of the sponge *Haliclona simulans*. A remarkable discovery on biosynthetic pathways for investigating marine invertebrate-bacteria associations is the isolation of the peptides patellamide A and C (Schmidt 2005; Schmidt et al. 2005). These cyclic peptides were thought to be synthesized by the cyanobacterial symbiont *Prochloron didemni* of the ascidian *Lissoclinum patella*. Schmidt et al. identified these NRPS genes and confirmed their function by heterologous expression of the whole pathway. This work represents the potential of functional expression of a marine natural product pathway from an obligate symbiont, thus alleviating the problem of supply by genetic manipulation.

Polyketides are classified as aromatic, polyenes and polyethers including macrolides (Rawlings 1997). The PKSs are responsible for the synthesis of several pharmacologically important bacterial polyketides such as antibiotics (i.e. erythromycin, tetracycline), antitumor, immunosuppressive and cholesterol-lowering agents.

Remarkably, a number of important natural products isolated from sponges such as discodermolide, laulimalide and peloruside are of polyketide biosynthetic origin (Fortman and Sherman 2005). Piel et al. (2004) made a milestone discovery on polyketide genes from a marine sponge confirming the long-suspected role of symbiotic bacteria in the production of natural products such as polyketides. Using metagenomics, production of the antitumor onnamide was studied in the marine sponge *Theonella swinhoei* by first investigating the production of pederin subsequently linked to a beetle symbiont closely related to *Pseudomonas aeruginosa* (Piel 2002). The sponge metagenome strongly indicated bacterial origin for the genome fragment corresponding to the entire onnamide polyketide region. PKS systems have also been studied in other sponges by metagenomics. PCR amplification of ketosynthase domains from the microbial consortia of the Caribbean sponge *Discodermia dissoluta* revealed great diversity and a novel group of sponge-specific KS domains (Schirmer et al. 2005). The most abundant modular PKS appeared to be encoded by a bacterial symbiont that made up <1% of the sponge community. Fieseler et al. (2007) systematically investigated 20 demosponge species in different oceans and found that the sponge microbial metagenomes were dominated by an evolutionary distinct and highly sponge-specific group of polyketide synthases. In another study by Kim and Fuerst (2006), direct amplification of KS domains in the Great Barrier reef sponge *Pseudoceratina clavata* using fosmid library construction yielded KS domains falling into a sponge-specific cluster. Quite interestingly, cultivated bacteria from the sponge were also screened for KS domains and these were detected in representatives of *Actinobacteria*, *Alphaproteobacteria* and *Firmicutes*. This is not surprising since members of the *Actinobacteria*, in particular are known to produce important natural products with polyketide biosynthetic origin. An example is the rifamycin polyketide, known to be produced by the soil actinomycete *Amycolatopsis mediterranei* (August et al. 1998) was also found to be produced by the marine actinomycete *Salinispora* sp. isolated from the sponge *Pseudoceratina clavata*. Phylogenetic analysis of the KS gene sequences revealed that these are closely related to rifamycin B synthase of *A. mediterranei* (Kim et al. 2006). Additional examples of PKS gene clusters identified from marine actinomycetes are enterocin PKS from *Streptomyces maritimus* (Piel et al. 2000) and the aromatic polyketide griseorhodin from *Streptomyces* sp. (Li and Piel 2002).

Marine bacterial gene clusters that have been so far identified have demonstrated that while PKS and NRPS biosynthetic routes are conserved between terrestrial and marine systems, interestingly, there are additional novel catalytic enzymes responsible for the unique functional groups found solely in marine natural products (Piel et al. 2000; Chang et al. 2002; Li and Piel 2002; Salomon et al. 2004). Furthermore, recent investigations

focusing on marine actinomycetes have yielded numerous novel biologically active compounds, and more importantly, a surprisingly much more improved rate of discovery of new compounds in contrast to their terrestrial counterparts (Bernan et al. 1997). Thus, given the promising potential of marine actinomycetes, different strategies (Fig.1.6) were employed in this Ph.D. study to fully exploit the capacity of actinomycetes, specifically those associated with marine sponges, to produce bioactive secondary metabolites.

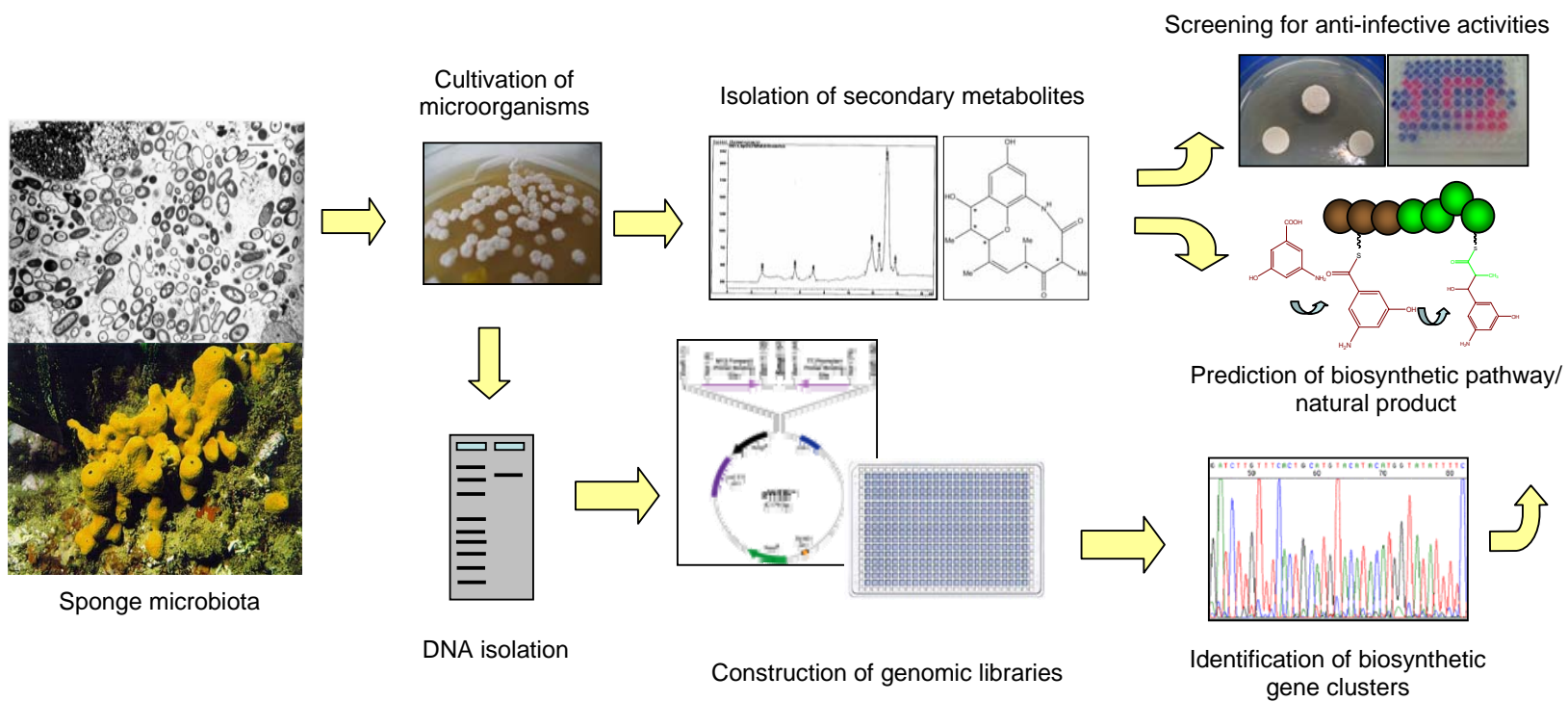


Fig. 1.6 Strategies to access the potential of sponge-associated microorganisms to produce anti-infective metabolites

1.4 Aims

Marine sponges, particularly 'high-microbial-abundance sponges' are known to harbour huge amounts of microorganisms which include members of the order *Actinomycetales*. Actinomycetes are known as prolific producers of pharmacologically important compounds such as antibiotics. The main aim of this Ph.D. study was to investigate the potential of actinomycetes associated with marine sponges to produce novel anti-infective agents.

The first aim of this Ph.D. study (Chapter 2) was to cultivate actinomycetes derived from different marine sponges with a particular focus on strains belonging to novel taxa. The strains were identified by 16S rRNA gene sequence analysis and further characterized using polyphasic techniques.

The second aim of this Ph.D. study (Chapter 3) was to investigate the secondary metabolites produced by the sponge-associated actinomycetes. The metabolites were isolated using a bioassay-guided purification scheme followed by structure elucidation using spectroscopic methods. These compounds were subjected to an elaborate anti-infective screening panel to determine various activities, namely antibacterial, antifungal, antiparasitic, cytotoxic as well as inhibition against different cysteine proteases.

The third aim of this Ph.D. study (Chapter 4) was to identify biosynthetic gene clusters encoding for nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) present in the actinomycete strains. Genomic library construction and sequencing were carried out in order to provide insights into the metabolic potential and biosynthetic pathways of selected strains.

Finally, a general discussion (Chapter 5) of the association of actinomycetes with marine sponges and their biosynthetic potential as well as future perspectives is provided.

1.5 References

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Chapter 2

Isolation and identification of sponge-associated actinomycetes

2.1 Materials and methods

2.1.1 Sponge collection and cultivation

The sponge *Haliclona* sp. was collected by SCUBA diving off Maribago waters (10° 17' 0.97" N, 124° 00' 01.8" E), Cebu, Philippines in February 2003. *Axinella polypoides* was collected offshore Banyuls-sur-mer, France (42° 29' N 03° 08' E) in May 2003. The sponges *Aplysina aerophoba*, *Chondrosia reniformis*, *Dysidea avara*, *Tedania* sp. and *Tethya* sp. were also collected by SCUBA diving offshore Rovinj, Croatia (45° 05' N, 13° 38' E) in May 2006.

Sponge tissues were excised from the center of individual sponge samples (ca. thumb-sized pieces) using flame-sterilized scalpel. The tissues (2-3 pieces per sponge sample) were rinsed with sterile natural or artificial seawater (ASW) (Lyman and Fleming 1940) and homogenized in seawater. The homogenates were diluted (10^{-1} to 10^{-3}) in seawater and plated out on different actinomycete-selective media: M1 (Mincer et al. 2002), M2 (Mincer et al. 2002), ISP medium 2 (Shirling and Gottlieb 1966), M7 (Webster et al. 2001) and NaSt21Cx (Magarvey et al. 2004). Media formulations are provided in detail in the Annex section. These media were supplemented with the following antibiotics: cycloheximide (100 µg/ml), nystatin (25 µg/ml) and nalidixic acid (25 µg/ml). Undiluted sponge homogenates (in duplicates) were heated at 90°C for 15 min to enrich for spore-forming actinomycetes and likewise plated out. The plates were incubated at 30°C for about 6-8 weeks and inspected regularly for growth. Colonies were picked up and transferred to fresh media for pure culture isolation and glycerol stocks were prepared for long-term storage at -80°C.

2.1.2 Cloning, sequencing and phylogenetic analysis

DNA was extracted from growing cultures either by boiling cells at 95°C for 10 min or using the FastDNA[®] spin kit for soil (Q-Biogene) following manufacturer's instructions. The broth cultures were centrifuged at 8000 rpm for 5 min and the supernatant was discarded. The resulting pellet was resuspended in 978 µl sodium phosphate buffer and 122 µl MT buffer. The solution was transferred to lysing matrix tubes and these were 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. The supernatant was transferred

to a clean tube and 250 μ l of PPS reagent was added. The solution was mixed by manually shaking the tube ten times. Centrifugation at 13000 rpm for 5 min followed to pellet the precipitate. The resulting supernatant was transferred to a clean 2-ml microfuge tube. One milliliter of binding matrix suspension was added to the supernatant and the mixture was mixed for 2 min to allow binding of the DNA to the matrix. The tube was placed in a rack and allowed to stand for 3 min to allow settling of the silica matrix. Five hundred microliters of the supernatant was discarded while 600 μ l was transferred to a spin filter provided by the kit. This was then centrifuged at 13000 rpm for 1 min. The catch tube was emptied and the remaining supernatant was added to the spin filter. Centrifugation at 13000 rpm for 1 min was repeated. To the spin filter, 500 μ l of SEWS-M was added which was followed by the same centrifugation step. 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. Fifty microliters of DES (DNAse/pyrogen-free) water was then added and the filter matrix was gently stirred using a pipette tip to resuspend the silica and to efficiently elute the DNA. Centrifugation at 13000 rpm for 1 min followed to transfer the eluted DNA to the catch tube. The resulting DNA extract was stored at -20°C .

PCR amplification using the specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-A-19 (5'-CCGTACTCCCCAGGCGG GG-3') targeting the class *Actinobacteria* was performed to initially screen the isolates (Stach et al. 2003). This was used to minimize bias in selecting isolates based solely on morphological characteristics typical for actinomycetes. Amplification of the 16S rRNA gene was then performed using the universal primers 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1492r (5'-TACGGCTACCTTGTTACGACTT-3') corresponding to *E. coli* numbering (Lane 1991). A standard PCR reaction mix (50 μ l) was prepared which consisted of the following: 10x reaction buffer, 5 μ l; Q solution, 10 μ l; 25 mM MgCl_2 , 2 μ l; 10 mM dNTPs, 1 μ l; 100 μ M of each primer, 1 μ l; 5 U/ μ l Taq polymerase, 0.25 μ l; H_2O , 28.75 μ l; DNA template, 1 μ l. PCR conditions were as follows: initial denaturation (2 min at 95°C) followed by 30 cycles of denaturation (1 min at 95°C), primer annealing (1 min at 56°C for 27f/1492r and 60°C for S-C-Act-0235-a-S-20/S-C-Act-0878-A-19), primer extension (1.5 min at 72°C) and a final extension step (10 min at 72°C). PCR products were purified using the QIAquick PCR purification kit (Qiagen). 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 μ l) 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, added with 30 μ l of buffer EB 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.

Ligation of the purified PCR products and transformation in competent *E. coli* XL1-Blue cells were subsequently performed. The following were mixed on ice: 2x T4 DNA ligase buffer, 5 μ l; 50 ng/ μ l pGEM-Teasy vector (Promega), 1 μ l; 3 U/ μ l T4 DNA ligase, 1 μ l; PCR product, 4 μ l. The ligation solution was incubated overnight at 4°C for maximum number of transformants. For transformation, 2-3 μ l of the ligation solution was added to 50 μ l of the competent *E. coli* cells. The resulting mixture was transferred to a UV-sterilized electroporation cuvette. Electroporation was applied using an Easyject Prima Electroporator (Equibio) at 2500V. The solution was transferred to a clean 2-ml microfuge tube, added with 1 ml of SOC medium with mixing to resuspend the pellet and incubated with shaking at 37°C for at least 3 hours. After incubation, 20% and 80% of the cells were plated out on LB/amp/IPTG/X-gal agar. The plates were incubated at 37°C overnight and successful transformants were observed using the blue-white colony screening. The plasmid DNA was then isolated using a standard miniprep protocol. Single, white colonies were picked up, inoculated in 2 ml of LB/amp broth and incubated with shaking at 37°C overnight. Following incubation, the culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 150 μ l of buffer P1. Addition of 150 μ l of buffer P2 with standing for 5 min at room temperature followed by the addition of 150 μ l of buffer P3 on ice for 5 min were subsequently done. The solution was mixed by gentle flicking of the tube after each addition of the buffer. The resulting mixture was centrifuged at 13000 rpm for 10 min. The supernatant was transferred to clean 1.5-ml microfuge tube and the centrifugation step was repeated. The supernatant was again transferred to clean tube, added with 0.7 volume of isopropanol and mixed by gently flicking the tube. This was then centrifuged at 13000 rpm for 15 min. The resulting pellet containing the plasmid DNA was washed with 70% ethanol and allowed to air-dry. Sterile water (50 μ l) was added to resuspend the pellet and the plasmid DNA was stored in -20°C until use. To verify the correct clones, the plasmid DNA was digested with a restriction endonuclease. Restriction digestion with *EcoRI* (New England Biolabs) was done by mixing the following: 10x *EcoRI* buffer, 2 μ l; water, 12 μ l; 20,000 U/ml *EcoRI*, 1 μ l; plasmid DNA, 5 μ l. The reaction mix was

incubated at 37°C for 2-3 hours and the restriction patterns were analyzed by agarose gel electrophoresis.

For amplification of the pGEM-T easy insert, sequencing PCR was done using the primers SP6 (5'- ATTTAGGTGACACTATAG-3') and T7 (5'- GTAATACGACTCACTATAGGG-3') and the BigDye[®] terminator cycle sequencing kit (Applied Biosystems). The PCR reaction mix was composed of the following: 5x BigDye[®] terminator v1.1 sequencing buffer, 2 µl; BigDye[®] terminator v1.1 premix, 2 µl; plasmid DNA, 2 µl; 25 µM of primer, 1 µl; sterile water, 3 µl. The PCR conditions were as follows: initial denaturation (2 min at 96°C), 25 cycles of denaturation (30 s at 96°C), primer annealing (15 s at 45°C), primer extension (4 min at 72°C) and a final extension step (10 min at 60°C). DNA sequencing was performed using an ABI 377XL automated sequencer (Applied Biosystems). Sequences were assembled using the ContigExpress tool in Vector NTI suite 6.0 (InforMax, Inc) and subsequently aligned using Clustal X. Phylogenetic analysis was done using the ARB software (Strunk and Ludwig 1997).

2.1.3 Morphological, physiological and biochemical characterization of selected strains

The strains were cultivated in liquid medium and the bacterial biomass was subsequently fixed with 2.5% glutaraldehyde and washed with 50 mM cacodylate buffer. The samples were fixed in 2% osmium tetroxide, washed with water five times, followed by dehydration with a series of ethanol solutions (50%, 70%, 90% and 100%) and incubation in propylene oxide. This was followed by overnight incubation in propylene oxide/glycidether (Epon 812, Roth) and polymerization of the resin for three days at 60°C. The samples were sectioned using an ultramicrotome (OM U3, C. Reichert) and contrasted with 1% uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 electron microscope operating at 80V.

For scanning electron microscopy, colonies grown on agar plates were stanced out using a sterile cork borer with surrounding material. These were then fixed in 6.25% glutaraldehyde, washed five times with Sørensen-phosphate buffer pH 7.4 and dehydrated with increasing concentrations of acetone (30%, 50%, 75%, 90% and 100%). After critical-point drying and platinum coating of the dried material, colonies were examined with a Zeiss DSM 962 scanning electron microscope.

Cultural characteristics of the strains such as mycelial morphology and production of diffusible pigments were observed on a number of standard International *Streptomyces* Project (ISP) agar media, namely: yeast-malt extract (ISP 2), oatmeal (ISP 3), inorganic salts-starch (ISP 4), glycerol-asparagine (ISP 5) and peptone-yeast extract-iron (ISP 6) and tyrosine (ISP 7) (Shirling and Gottlieb 1966). Growth requirement for seawater and salt tolerance were tested using various amounts of NaCl (0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5% and 15.0%) as well as artificial seawater (25%, 50%, 75% and 100%) in appropriate media. Optimum temperature for growth was also determined by growing the strains in different temperature conditions (4, 10, 15, 20, 25, 30, 37, 45 and 55 °C). Sensitivity to different antibiotics (100 µg/ml) such as ampicillin, chloramphenicol, gentamicin, kanamycin, lincomycin, oxacillin, penicillin, rifampin, streptomycin, tetracycline and vancomycin was also determined by observing the growth as turbidity in liquid medium supplemented with the antibiotics after incubation at 30°C for 7-10 days. Oxygen requirement for growth was also determined by incubating the agar plates in an anaerobic jar. For phenotypic characterization, API kits (Biomerieux) and Biolog (Biolog Inc.) plates were used following manufacturer's instructions. API CH system and Biolog SF-P2 plates were used to evaluate the utilization of different carbon sources while enzymatic activities were detected using the API Zym kit. The cells were harvested by aseptically scraping the spores and mycelial fragments on the agar media and suspended either in artificial seawater for use in the API kits or in 0.2% phytigel (Sigma) for Biolog SF-P2. Appropriate reagents supplied together with the kit were added to individual cupules following manufacturer's instructions and positive reactions were noted. For the carbon utilization tests, a positive reaction was noted as turbidity on the cupules or wells after incubation at 30°C for 7-10 days. Furthermore, the ability to degrade macromolecules was determined as clearing zones around colonies growing on agar media containing adenine, casein, chitin, hypoxanthine and tyrosine (Korn-Wendisch et al. 1989).

Diagnostic cell wall components, G+C content of the genomic DNA as well as DNA-DNA relatedness of the strains were determined and performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Established procedures were used to determine the diagnostic isomers of the diaminopimelic acid (A₂pm) and the predominant sugars of the whole organism (Staneck and Roberts 1974). A quinone analysis was carried out as described by Kroppenstedt (1985). The presence of mycolic acids was investigated following the procedure of Minnikin *et al.* (1975). The polar lipids were extracted and analyzed following the integrated procedure of Minnikin *et al.* (1984). The composition of the fatty acid pattern was determined by gas chromatography using MIDI

software. The DNA base composition of genomic DNA was determined by HPLC (Tamaoka and Komagata 1984; Mesbah et al. 1989).

2.2 Results

2.2.1 Strain description of new actinomycete species

2.2.1.1 *Saccharopolyspora cebuensis* sp. strain SPE 10-1^T (Pimentel-Elardo et al. 2008b)

Strain SPE 10-1^T was isolated from the marine sponge *Haliclona* sp. (Fig. 2.1A) collected from Cebu, Philippines and was cultivated on M1 agar, a medium specifically designed for the selective isolation of marine actinomycetes (Mincer et al. 2002).

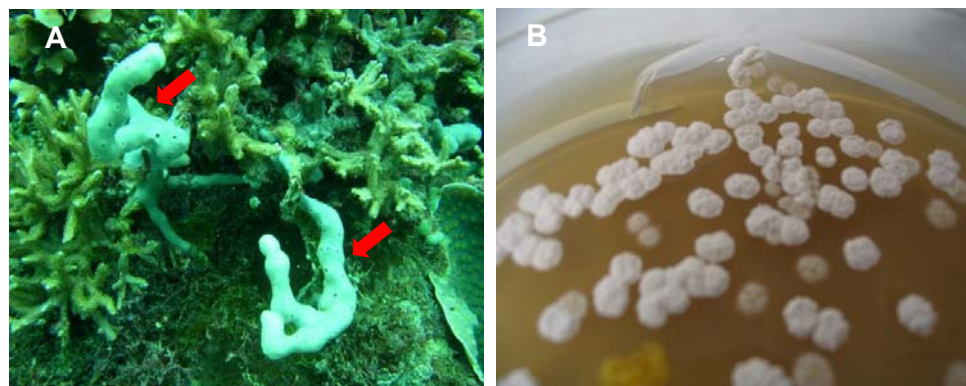


Fig. 2.1 (A) marine sponge *Haliclona* sp. (underwater photography by J. Apurado, University of San Carlos); (B) Strain SPE 10-1^T colonies on M1 agar

An almost complete 16S rDNA sequence (1483 nucleotides) was generated for the strain and compared to the validly described species of the genus *Saccharopolyspora* as its closest neighbors as well as representative genera from the family *Pseudonocardiaceae* (Fig. 2.2). Phylogenetic analysis revealed that the strain SPE 10-1^T has highest sequence similarity (96%) with *Saccharopolyspora gregorii* and 93-95% similarity with all other species of the genus *Saccharopolyspora*.

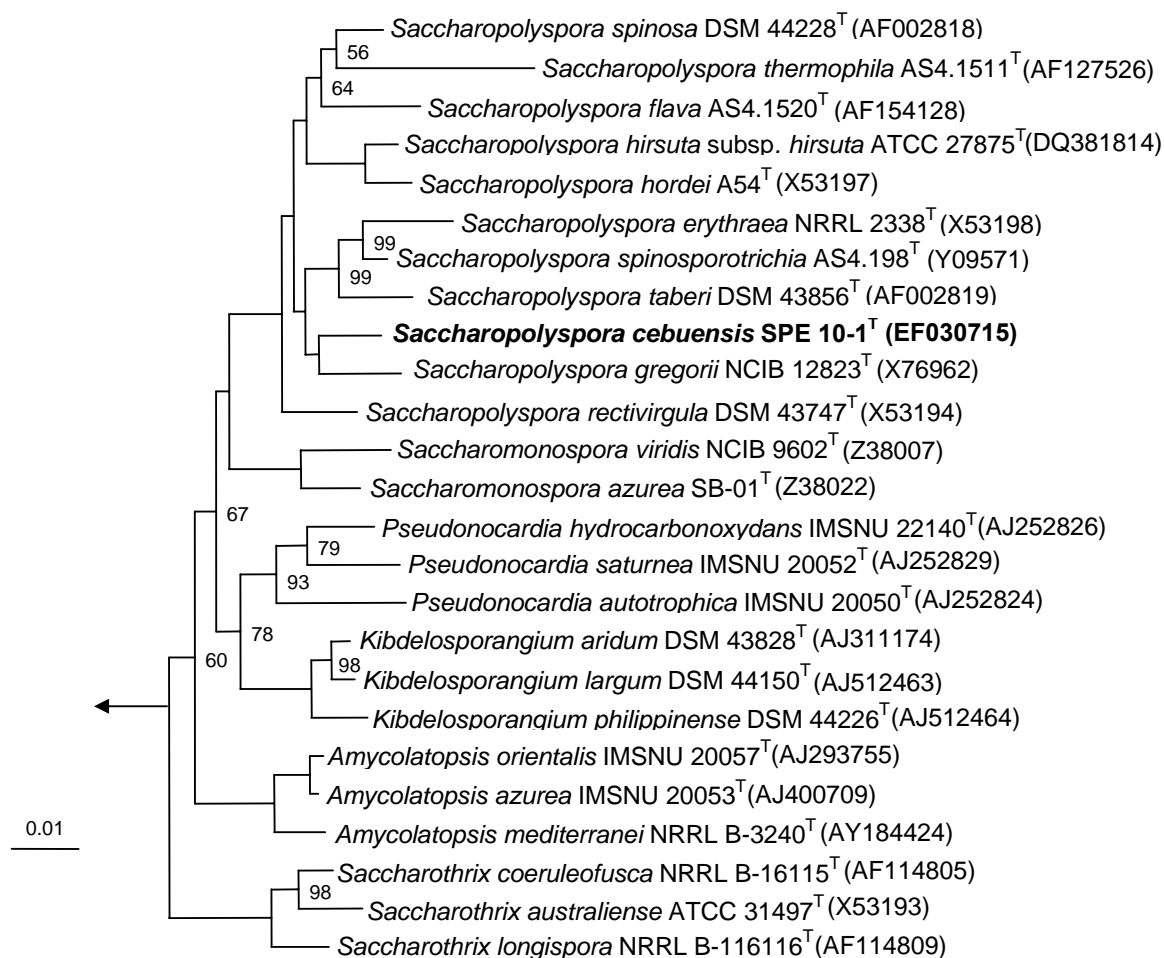


Fig. 2.2 Neighbor-joining tree based on nearly complete 16S rDNA sequences of strain SPE 10-1^T and representative strains of the family *Pseudonocardiaceae* and related taxa. *Escherichia coli* (DQ360844) was used as outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 100 resampled data sets; only values greater than 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

Strain SPE 10-1^T exhibited morphological properties characteristic of *Saccharopolyspora*, forming extensively branched substrate mycelia which fragments into rod-shaped elements (Fig. 2.3A). Scanning electron microscopy showed hyphae bearing short chains of spores as well as single spore cells (Fig. 2.3B). The spores were round to oval and the surface was smooth. Light microscopy of colonies confirmed the presence of spores in aerial mycelia.

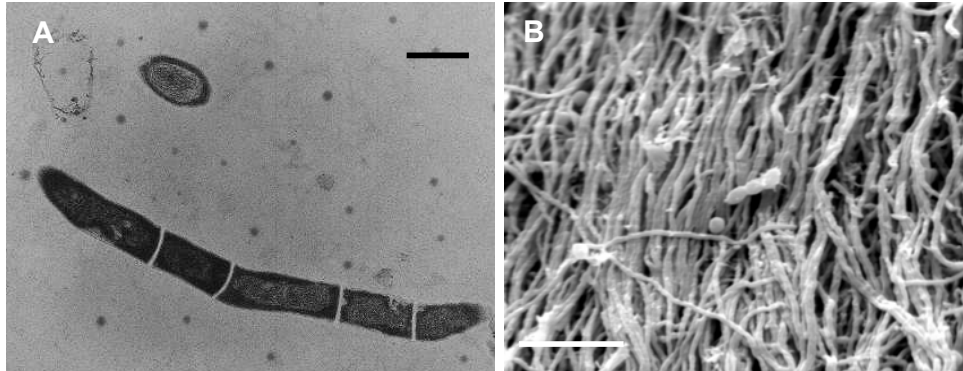


Fig. 2.3 Hyphae and spores of strain SPE 10-1^T grown in M1 broth (A) and on M1 agar (B) at 30°C for 7 days; (A), transmission electron microscopy, bar = 0.5 μm ; (B), scanning electron microscopy, bar = 5 μm

Furthermore, strain SPE 10-1^T was able to grow in ISP medium 2 with ASW as well as in Zobell marine medium (Oppenheimer and Zobell 1952). Brown diffusible pigment was also observed. Growth was observed at temperatures from 15 to 37°C, with optimal growth at 25 to 30°C. Colonies displayed chalky-white mycelia with brownish soluble pigment on M1 agar. Cultures of the strain SPE 10-1^T grown in M1 broth for 7-14 days appeared yellowish-brown to brown in color. Strain SPE 10-1^T did not grow in ISP medium 2 without ASW. M1 media supplied with different amounts of ASW or NaCl were used to test for requirement for seawater and salt tolerance. Growth was possible in regular strength M1 (100% ASW) and in M1 containing 75, 50 and 25% but not 0% ASW. Growth was also possible when regular strength ASW was replaced with 12.5, 10, 7.5 or 5% NaCl in distilled water. Growth was poor in M1 with 2.5% NaCl and growth was not observed without NaCl or with 15% NaCl. Furthermore, strain SPE 10-1^T was able to grow in M1 liquid medium supplemented with antibiotics (100 $\mu\text{g/ml}$) gentamicin and kanamycin but not with rifampin, penicillin, streptomycin, lincomycin, vancomycin, oxacillin, chloramphenicol, ampicillin and tetracycline. No growth was observed on M1

agar plates incubated in an anaerobic jar. Using the API CH system, the strain was able to utilize a variety of organic compounds such as glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, N-acetylglucosamine, amygdalin, esculin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-raffinose, amidon, glycogen, gentibiose, D-fucose, D-arabitol, and potassium gluconate as sole carbon source. Using the API Zym system, the following enzymes were tested positive: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase, -glucosidase, N-acetyl- -glucosaminidase and -mannosidase. The strain is able to degrade tyrosine but not adenine, casein, chitin and hypoxanthine. Furthermore, strain SPE 10-1^T stained positive by Gram-staining and was positive for catalase following standard reaction to hydrogen peroxide. Strain SPE 10-1^T tested negative for oxidase activity and for reduction of nitrate to nitrite. The organism can be distinguished from the other type strains of the validly described species of *Saccharopolyspora* by using a combination of phenotypic properties (Table 2.1).

The strain contained *meso*-A₂pm as the wall diamino acid, the diagnostic sugars arabinose and galactose were present but glucose and ribose were found in addition. A menaquinone with a tetra-hydrogenated-isoprenoid side chain of nine units MK-9 (H4) was the principal isoprenoid quinone. Small amounts of MK-8(H4) and MK-10(H4) were found in addition. Mycolic acids were not detected. The phospholipid pattern was composed of phosphatidyl-choline, phosphatidyl-ethanol amine, phosphatidyl-methylethanolamine, diphosphatidyl-glycerol, phosphatidyl-glycerol and phosphatidyl-inositol. Two unknown glycolipids were found in addition. The fatty acid pattern was mainly composed of terminally branched iso- and anteiso-fatty acids but small amounts of diagnostic 10-methyl-branched fatty acids were found in addition while 2-hydroxy fatty acids were missing. The DNA G+C content was 72.6 mol%.

Table 2.1 Selected physiological properties of *S. cebuensis* sp. nov. SPE 10-1^T in comparison to validly described *Saccharopolyspora* type strains

Strains: 1, *S. cebuensis* SPE 10-1^T; 2, *S. gregorii* (DSM 44324^T), 3, *S. spinosporotrichia* (DSM 44350^T), 4, *S. spinosa* (DSM 44228^T); 5, *S. erythraea* (DSM 40517^T); 6, *S. hirsuta* (DSM 43463^T); 7, *S. hordei* (DSM 44065^T); 8, *S. rectivirgula* (DSM 43747^T); 9, *S. flava* (AS4.1520^T); 10, *S. thermophila* (AS4.1511^T); 11, *S. taberi* (DSM 43856^T). Data for the reference strains other than *S. cebuensis* sp. nov. SPE 10-1^T were taken from Lu *et al.* (2001) except for the data on hypoxanthine, G+C content and D-mannitol utilization which were taken from Goodfellow *et al.* (1989), Labeda (1987), Lacey & Goodfellow (Lacey and Goodfellow 1975), Mertz & Yao (1990) and Zhou *et al.* (1998). Abbreviations: Bf, buff; Br, brown; C, colorless; G, grey; O, orange; P, pink; R, red; W, white; Y, yellow; +, positive; -, negative; n/a, no aerial mycelium; nd, not determined. All *Saccharopolyspora* strains were positive for utilization of D-fructose, glycerol and D-mannose as sole carbon source.

^a: The temperature data from Lu *et al.* (2001) on the type strains differ slightly from those of Korn-Wendisch *et al.* (1989) who reported a temperature range of 20-50°C for *S. hirsuta* and 37-60°C for *S. rectivirgula* species.

^b: *S. cebuensis* SPE 10-1^T is the only known isolate with a strict requirement for salt.

^c: Note that the ISP medium 2 was supplemented with ASW and that mycelia color may vary depending on media composition.

Characteristics	<i>Saccharopolyspora</i> reference strains										
	1	2	3	4	5	6	7	8	9	10	11
Carbon source utilization											
L-Arabinose	+	+	-	+	+	-	+	-	-	-	-
D-Galactose	+	+	+	-	+	+	+	+	+	+	+
D-Lactose	+	-	-	-	-	+	+	+	+	+	+
D-Maltose	+	+	+	-	+	+	+	+	+	+	+
D-Mannitol	-	+	+	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	-	+	+	+	+	+	+	+
L-Rhamnose	+	+	+	-	+	+	+	+	+	+	+
Sucrose	+	+	+	-	+	+	+	+	+	+	+
D-Xylose	+	+	+	-	+	+	+	+	+	-	+
Temp. range (°C)	15-37	10-35	28-37	15-37	20-42	25-50 ^a	20-60	37-63 ^a	28-37	45-55	20-45
Nitrate reduction	-	-	-	+	+	-	-	+	+	-	+
NaCl tolerance (%)	2.5-12.5 ^b	13	<3	<11	<5	<7	<13	<10	7	7	7
Degradation ability											
Adenine	-	-	-	-	+	+	+	-	+	+	+
Casein	-	+	+	-	-	+	+	-	-	-	+
Chitin	-	-	-	-	+	-	+	-	-	-	+
Hypoxanthine	-	+	+	+	+	+	+	+	+	-	+
Tyrosine	+	+	-	+	+	+	+	+	-	+	+
Color of											
Aerial mycelia	W ^c	W-Y	W-G	W-P	P-BrG-W	W	W-Y	W-lightP	W	W	n/a
Substrate mycelia	W ^c	C-Bf	Br-R	G-OY-Br	OY-RBr	C-Bf	C-Bf	Y-O	Y	C-Bf	C-Y
G+C content (mol %)	72.6	74.0	70.4	nd	71.1	71.5	72.0	70.4	67.0	73.1	70.8

2.2.1.2 *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008a)

Strain Pol001^T was isolated from the marine sponge *Axinella polypoides* (Fig. 2.4A) collected from Banyuls-sur-mer, France (Scheuermayer 2006).

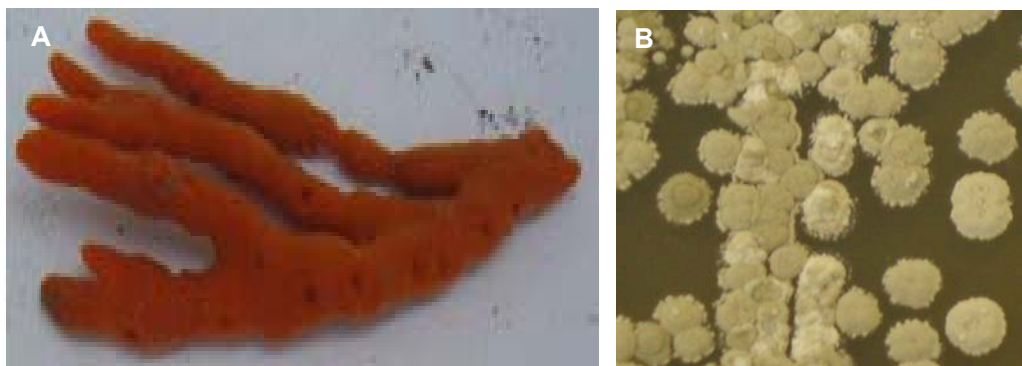


Fig. 2.4 (A) marine sponge *Axinella polypoides* (photography by M. Scheuermayer, University of Würzburg); (B) Strain Pol001^T colonies on ISP medium 2

An almost complete 16S rDNA sequence (1422 nucleotides) was generated for the strain and compared to the validly described species of the genus *Streptomyces* as its closest neighbors. Phylogenetic analysis revealed that the strain Pol001^T exhibits closest sequence similarities with the following: *Streptomyces sclerotialis* DSM 46032^T (97.61%), *Streptomyces rimosus* subsp. *rimosus* DSM 40260^T (97.47%), *Streptomyces niger* DSM 43049^T (97.20%) and *Streptomyces olivaceiscleroticus* DSM 40595^T (97.20%).

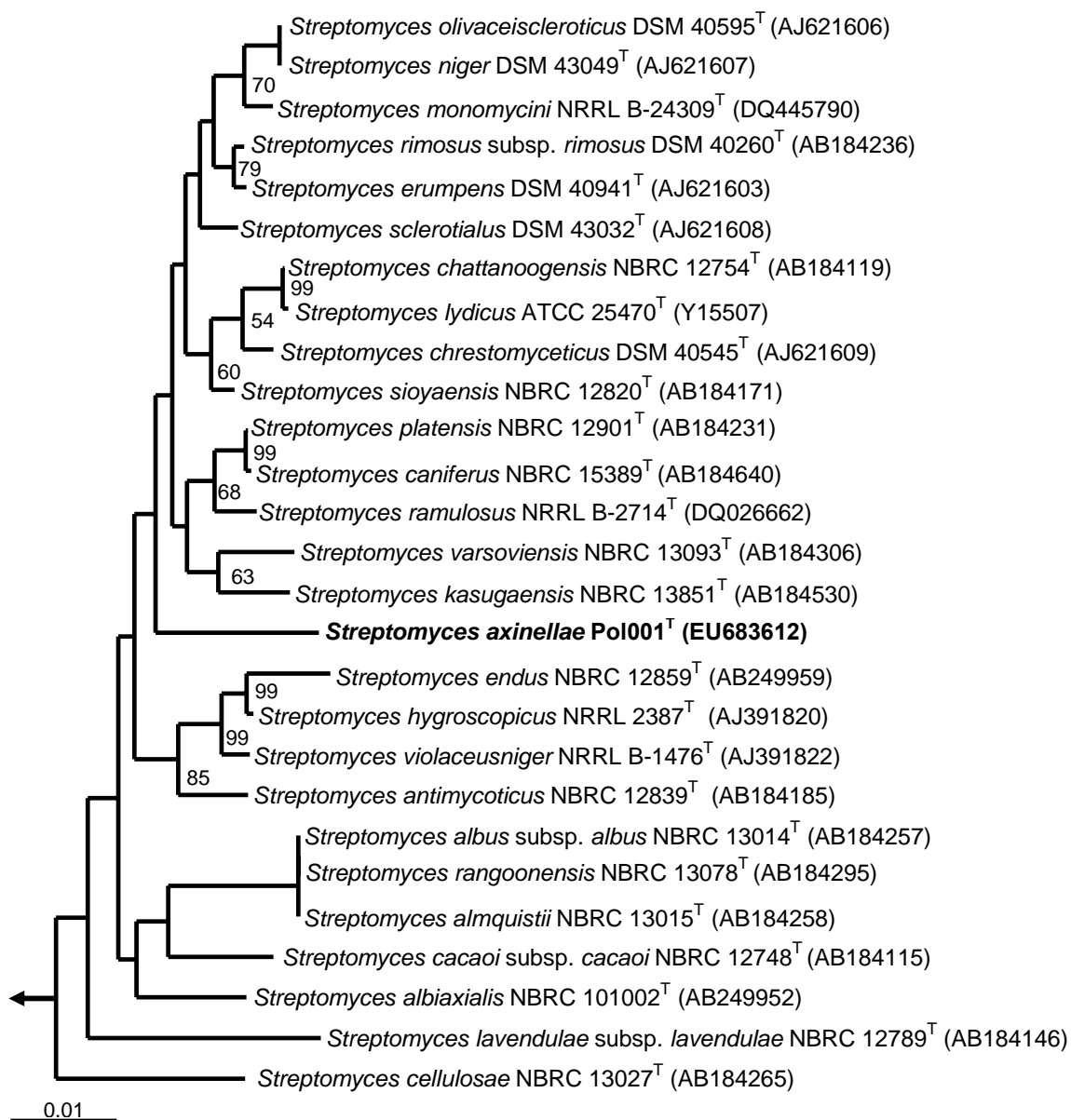


Fig. 2.5 Neighbor-joining tree of strain Pol001^T and representative species of the genus *Streptomyces* based on nearly complete 16S rDNA sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. Arrow points to outgroup, *Salinispora tropica* (AY040618). The scale bar indicates 0.01 substitutions per nucleotide position.

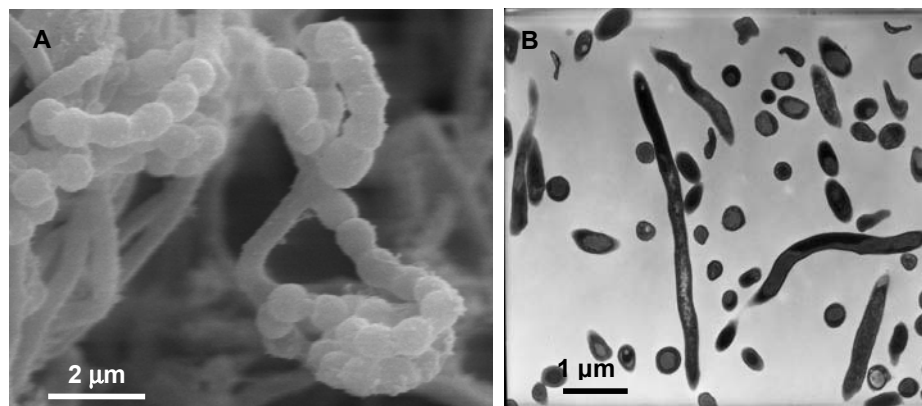


Fig. 2.6 (A) Scanning electron micrograph and (B) transmission electron micrograph of spores and hyphae of strain Pol001^T grown in ISP 2 media

Strain Pol001^T grew well on a variety of standard International *Streptomyces* Project (ISP) agar media after incubation at 30°C for 21 days: yeast-malt extract (ISP 2), oatmeal (ISP 3) and peptone-yeast extract-iron (ISP 6). A diffusible pigment was observed only on tyrosine agar (ISP 7) (Table 2.2). Extensively branched grey aerial and white substrate mycelia were abundant on ISP 2 medium (Fig. 2.4B). At 21 days, the strain produced spiral chains of spores with a smooth surface, elliptical shape and 0.8-0.9 μm in length (Fig. 2.6A). Furthermore, transmission electron microscopy revealed non-fragmenting hyphae (Fig. 2.6B). Strain Pol001^T was able to grow at 20-37°C, with optimum growth at 30°C. Growth was possible in 0, 2.5, 5, 7.5 % NaCl but not in 10, 12.5, and 15 % NaCl, with optimal growth at 0-2.5 % NaCl. Growth was also possible in ISP 2 medium with 25, 50, 75 and 100% ASW. Furthermore, strain Pol001^T was able to grow in medium supplemented with the antibiotics (100 μg/ml) ampicillin, chloramphenicol, nalidixic acid, penicillin and rifampicin but not with erythromycin, gentamicin, kanamycin and vancomycin.

Table 2.2 Cultural characteristics of strain Pol001^T on various agar media

Medium	Growth	Diffusible pigment	Aerial mycelium	Substrate mycelium
Yeast-malt extract (ISP 2)	Abundant	None	Grey	White
Oatmeal (ISP 3)	Abundant	None	Dark Brown	Light Green
Inorganic salts-starch (ISP 4)	Few	None	Yellow Orange	White
Glycerol-asparagine (ISP 5)	Moderate	None	White	Light Yellow
Peptone-yeast extract-iron (ISP 6)	Abundant	None	Light Pink	White
Tyrosine (ISP 7)	Moderate	Red	Light Pink	Light Green
Czapek	Abundant	None	Grey to Black	Light Green
LB agar	Abundant	None	White	White

Physiological tests indicated that strain Pol001^T is able to utilize a variety of organic compounds as carbon sources such as N-acetyl-β-D-mannosamine, N-acetyl-D-glucosamine, N-acetyl-L-glutamic acid, L-alaninamide, L-alanine, L-alanyl-glycine, D-arabitol, D-cellobiose, dextrin, D-fructose, D-galactose, gentiobiose, D-gluconic acid, α-D-glucose, L-glutamic acid, glycerol, DL-α-glycerol phosphate, α-D-lactose, L-malic acid, D-mannitol, D-mannose, propionic acid, L-rhamnose, D-ribose, L-serine, Tween 40, Tween 80, D-xylose but not acetic acid, adenosine, adenosine-5'-monophosphate, D-alanine, amygdalin, L-arabinose, arbutin, L-asparagine, 2,3-butanediol, α-cyclodextrin, β-cyclodextrin, 2'-deoxyadenosine, D-fructose-6-phosphate, L-fucose, D-galacturonic acid, α-D-glucose-1-phosphate, D-glucose-6-phosphate, glycogen, glycy-L-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, inosine, m-inositol, inulin, α-ketoglutaric acid, α-ketovaleric acid, lactamide, L-lactic acid, D-lactic acid methyl ester, lactulose, D-malic acid, maltose, maltotriose, mannan, D-melezitose, D-melibiose, α-methyl-D-galactoside, β-methyl-D-galactoside, 3-methyl-D-glucose, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-psicose, putrescine, L-pyroglutamic acid, pyruvic acid, pyruvic acid methyl ester, D-raffinose, salicin, sedoheptulosan, L-serine, D-sorbitol, stachyose, succinamic acid, succinic acid, succinic acid mono-methyl ester, sucrose, D-tagatose, thymidine, thymidine-5'-monophosphate, D-trehalose, turanose, uridine, uridine-5'-monophosphate and xylitol. Furthermore, the strain is positive for gelatin liquefaction but negative for melanin production, starch hydrolysis, nitrate reduction and hydrogen sulfide production. It is capable of degrading casein, but not adenine, chitin or hypoxanthine.

Levels of DNA-DNA relatedness between strain Pol001^T and four closely related *Streptomyces* species were as follows (average of two values): 26.8% (*S. sclerotialis* DSM 46032^T), 16.9% (*S. olivaceiscleroticus* DSM 40595^T), 8.75% (*S. niger* DSM 43049^T) and 8.65% (*S. rimosus* subsp. *rimosus* DSM 40260^T).

Strain Pol001^T contained LL-diaminopimelic acid in the cell wall. Analysis of the whole-cell sugar composition revealed the presence of glucose and ribose as well as traces of mannose. A menaquinone with a hexahydrogenated-isoprenoid side chain of nine units MK-9 (H₆) were found as the principal isoprenoid quinone. Two additional quinones with nine isoprene units [MK-9 (H₄, H₈)] were also found. Phospholipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, some unidentified phospholipids, phosphoglycolipids, glycolipids and an aminolipid. Fatty acid pattern consisted of iso-C_{16:0} (30.78%), anteiso-C_{15:0} (17.77%),

iso-C_{15:0} (12.03%), anteiso-C_{17:0} (9.80%), iso-C_{16:1} (6.92%), iso-C_{14:0} (5.77%) and iso-C_{17:1} (4.58%). The DNA G+C content of strain Pol001^T was 71.0 mol%.

Table 2.3 Selected physiological properties that separate strain Pol001^T from closely related *Streptomyces* species

Strains: 1, Pol001^T; 2, *S. sclerotialis* DSM 46032^T; 3, *S. rimosus* subsp. *rimosus* DSM 40260^T; 4, *S. niger* DSM 43049^T; 5, *S. olivaceiscleroticus* DSM 40595^T. Color of mycelium, reverse side of colony, production of diffusible and melanoid pigments were compared using growth on ISP 2 medium. Smooth spore surface, absence of melanoid pigment and utilization of glucose and fructose as carbon sources were observed for all strains. Data for reference strains were taken from Shirling & Gottlieb (Shirling and Gottlieb 1968a; Shirling and Gottlieb 1968b; Shirling and Gottlieb 1972).

Characteristic	1	2	3	4	5
Spore-chain morphology	SP	SP	SP, RA	SP	SP
Color of aerial mycelium	GW	LYR	R or W	YG	BG
Reverse-side of colony	W	YB	GY	DB	GY
Production of diffusible pigment	-	-	-	+	+
Utilization of					
L-Arabinose	-	+	+	+	+
Inositol	-	+	+	+	+
Mannitol	+	+	+	+	+
Raffinose	-	+	+	+	+
Rhamnose	+	+	-	+	+
Sucrose	-	+	-	+	+
Xylose	+	+	d	+	+

Abbreviations: SP, *Spirales*; RA, *Retinaculiaperti*; G, grey; W, white; Y, yellow; R, red; B, brown; L, light; D, dark; +, positive; -, negative; d, doubtful.

2.2.2 Isolation of other actinomycete strains

Nineteen additional strains cultivated from various marine sponges exhibiting typical actinomycete morphology (Fig. 2.7) were identified by 16S rDNA sequencing (Table 2.4). These strains were found to cluster together with known species of different actinomycete genera such as *Streptomyces*, *Isoptericola*, *Micromonospora*, *Gordonia*, *Nocardiopsis* and *Saccharopolyspora* (Fig. 2.8). Furthermore, the strains account to about 60% of the total number of isolates positively identified from the strain collection, the majority of which were originally cultivated from M1 and ISP 2 media after 2-3 weeks of incubation at 30°C.

Table 2.4 16S rDNA phylogenetic affiliation of actinomycete strains

Isolate code	16S rDNA closest relative (% homology)	Source sponge
Aer003*	<i>Streptomyces bingchengensis</i> 226541 (99.5)	<i>Aplysina aerophoba</i>
A188*	Actinomycetales bacterium XJSS-18 (100.0)	<i>Aplysina aerophoba</i>
Cr03	<i>Nocardiopsis</i> sp. 20052 (99.7)	<i>Chondrosia reniformis</i>
Da02	<i>Streptomyces</i> sp. CNS-774_SD06 (99.9)	<i>Dysidea avara</i>
T02	<i>Streptomyces</i> sp. CNS-774_SD06 (99.9)	<i>Tethya</i> sp.
T03	<i>Streptomyces</i> sp. MP47-91 (99.7)	<i>Tethya</i> sp.
4-3	<i>Micromonospora</i> sp. HBUM84229 (99.7)	unidentified sponge
8-6	<i>Saccharopolyspora gregorii</i> (96.0) *	unidentified sponge
9-1	<i>Streptomyces</i> sp. C12 (99.0) *	unidentified sponge
9-3	<i>Gordonia terrae</i> AIST-1 (99.8)	unidentified sponge
10**	<i>Streptomyces albogriseolus</i> NBRC 3709 (99.9)	<i>Aplysina aerophoba</i>
10-11	<i>Nocardiopsis</i> sp. M048 (97.0) *	unidentified sponge
11**	<i>Streptomyces spinoverrucosus</i> 174464 (99.8)	<i>Tedania</i> sp.
11-2	<i>Streptomyces</i> sp. 3194 (99.0) *	unidentified sponge
11-11	<i>Isoptericola</i> sp. TUT1258 (98.3)	unidentified sponge
17**	<i>Streptomyces variabilis</i> strain 173733 (99.9)	<i>Aplysina aerophoba</i>
22**	<i>Streptomyces</i> sp. VTT E-042674 (100.0)	<i>Aplysina aerophoba</i>
27**	<i>Streptomycetaceae</i> bacterium WBF21 (99.6)	<i>Aplysina aerophoba</i>
34**	<i>Streptomyces</i> sp. VTT E-042674 (99.9)	<i>Axinella polypoides</i>

* Strains from previous study (Scheuermayer 2006).

** Strains isolated by S. Kozitska (AG Hentschel, University of Würzburg).

* Based on partial 16S rDNA sequences.

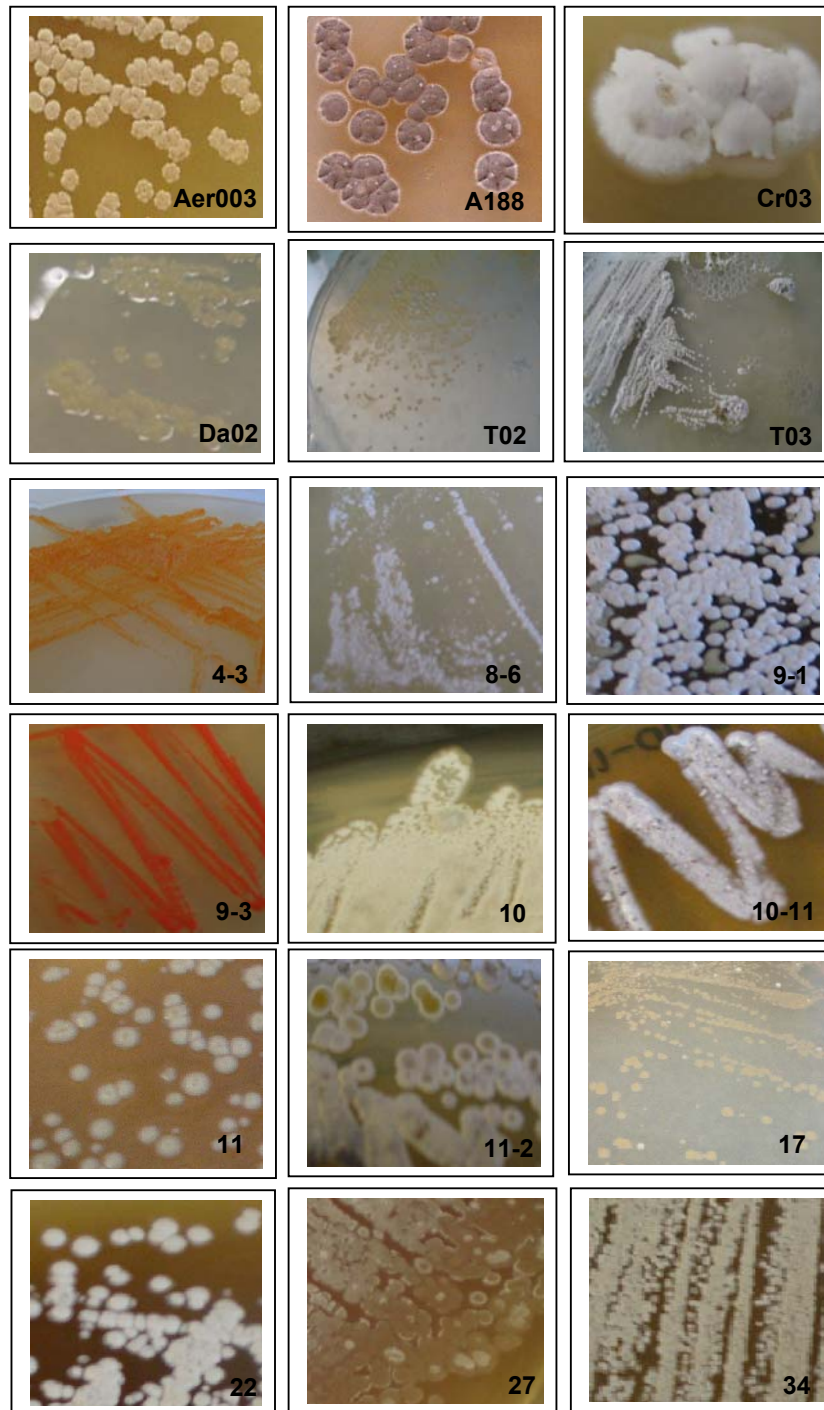


Fig. 2.7 Various colony morphologies of actinomycete strains grown on M1 and ISP 2 media at 30°C for 7-14 days

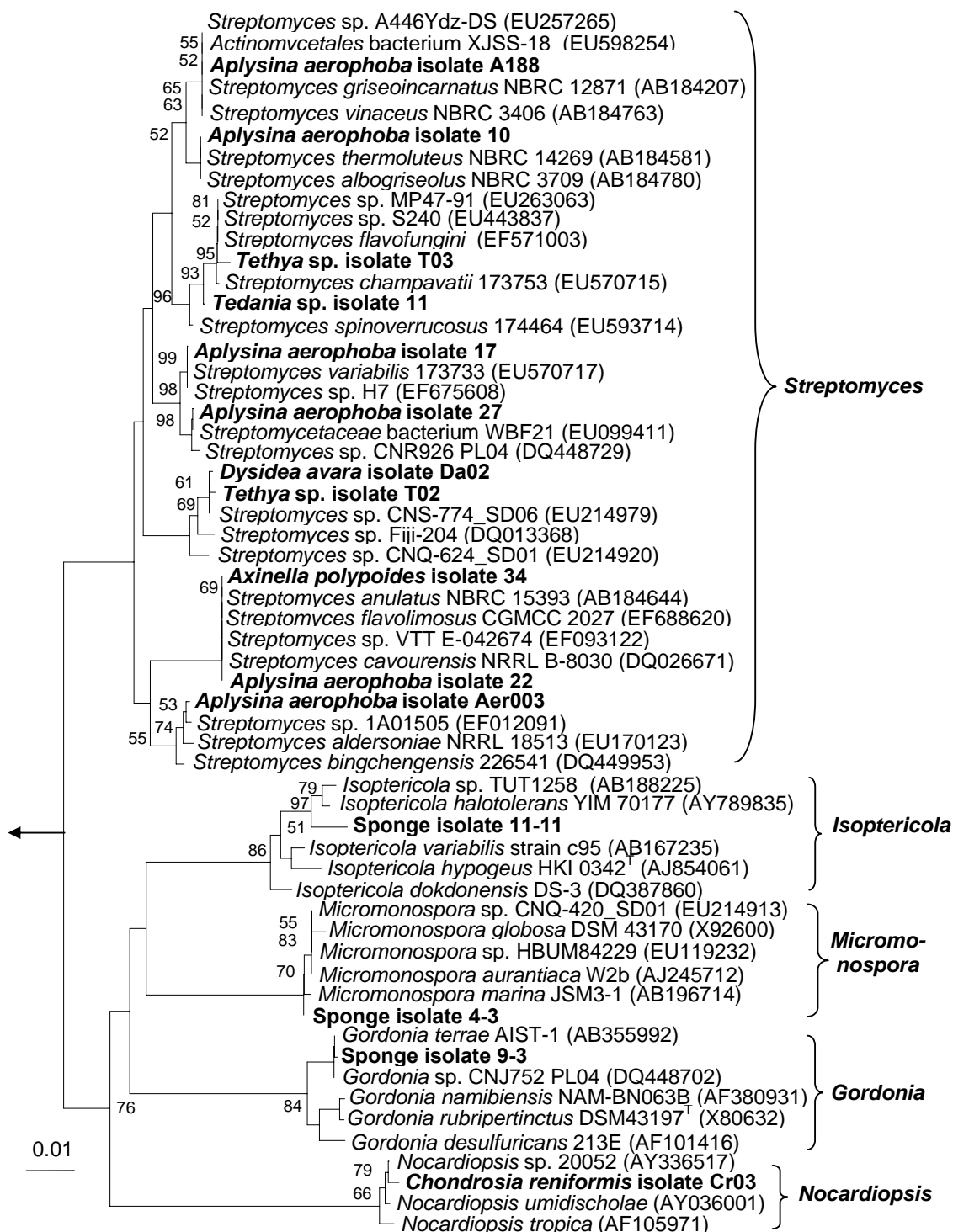


Fig. 2.8 Neighbor-joining tree based on nearly complete 16S rDNA sequences (>1400 bp) of the different sponge isolates. *Escherichia coli* (DQ360844) was used as outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 100 resampled data sets; only values greater than 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

2.3 Discussion

The order *Actinomycetales* is composed of approximately 80 genera, mostly from terrestrial soils (Magarvey et al. 2004). The order encompasses bacteria that are diverse with respect to morphology and biochemistry as well as having G+C-rich DNA. Members of this taxon are of significant interest primarily because these bacteria have consistently produced a considerable amount of bioactive metabolites of pharmacological and biomedical importance such as the antibiotics, erythromycin and streptomycin. As the search for producers of novel compounds continues, the rate of finding the same compounds from existing terrestrial actinomycete genera has increased (Kelecom 2002; Fenical and Jensen 2006) while the chance of finding new bioactive molecules from screening of large actinomycete libraries has decreased (Busti et al. 2006). Hence, efforts are now directed at exploiting new potential sources such as the marine environment. A number of reports have been published describing the isolation of novel actinomycete genera from marine sediments and invertebrates and these actinomycetes have been found to exhibit diverse biological activities, suggesting that the marine environment is an interesting source for bioprospecting (Fiedler et al. 2005; Jensen et al. 2005a; Jensen et al. 2005b; Kim et al. 2005; Montalvo et al. 2005; Fenical and Jensen 2006; Lam 2006).

The aim of this Ph.D. study was to isolate novel actinomycete taxa from marine sponges using selective isolation procedures and polyphasic characterization of selected strains. The ability to order prokaryotic taxa hierarchically has been improved by high quality 16S rDNA sequence analyses (Stackebrandt et al. 2002). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker. It has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of <97% represents a new species (Janda and Abbott 2007). Based on 16S rDNA phylogenetic analysis of all the isolated strains in this study, it was found that 2 out of the 19 identified strains are new actinomycete species belonging to the genera *Saccharopolyspora* and *Streptomyces*.

Strain SPE 10-1^T isolated from the sponge *Haliclona* sp. was the first strain identified by 16S rDNA sequencing belonging to the genus *Saccharopolyspora*. This genus was first described by Lacey & Goodfellow from sugar-cane bagasse (1975) and at present comprises ten validly described species: *Saccharopolyspora hirsuta* (Korn-Wendisch et al. 1989), *Saccharopolyspora erythraea* (Labeda 1987), *Saccharopolyspora taberi* (Korn-Wendisch et al. 1989), *Saccharopolyspora gregorii* (Goodfellow et al. 1989),

Saccharopolyspora hordei (Goodfellow et al. 1989), *Saccharopolyspora rectivirgula* (Korn-Wendisch et al. 1989), *Saccharopolyspora spinosa* (Mertz and Yao 1990), *Saccharopolyspora spinosporotichia* (Zhou et al. 1998), *Saccharopolyspora flava* (Lu et al. 2001) and *Saccharopolyspora thermophila* (Lu et al. 2001). Members of this genus are aerobic, Gram-positive, non-acid fast organisms with substrate hyphae that either fragments into rod-shaped elements, do not fragment or are transformed partially into chains of spores (Korn-Wendisch et al. 1989). They lack mycolic acid but contain meso-diaminopimelic acid, arabinose and galactose in the cell wall and predominant amounts of tetra-hydrogenated menaquinones with nine isoprene units. DNA base composition falls within the range of 67-74 mol% G+C for the type strains (Embley et al. 1987; Goodfellow et al. 1989; Korn-Wendisch et al. 1989). Strain SPE10-1^T exhibited morphological and biochemical characteristics consistent with members of the genus *Saccharopolyspora* but can be distinguished from the other type strains of this genus based on a combination of phenotypic properties. The low 16S rDNA sequence similarities of the strain with validly described species of *Saccharopolyspora* further suggest that the strain constitutes a novel species of this genus. Interestingly, the strain is only able to grow in medium with sodium chloride or ASW indicating a strict requirement for salt and hence, suggests that it is an obligate marine bacterium. Taken together, the phenotypic and genotypic data obtained in this study clearly show that strain SPE 10-1^T represents a novel and obligate marine species within the genus *Saccharopolyspora*. Thus, this type strain was assigned the species name as *S. cebuensis* (se.bu.en'sis N.L. fem. adj. *cebuensis*) pertaining to the province of Cebu in the Philippines, where the type strain was collected. It has been recently considered as a validly described species by the *International Journal of Systematic and Evolutionary Microbiology* (Pimentel-Elardo et al. 2008b) and is deposited at two public culture collections (=DSM 45019^T, =CIP 109355^T). It is also interesting that a similar strain 8-6 cultivated in this Ph.D. study from another sponge sample but from the same collection site also exhibited high sequence similarity (based on partial 16S rDNA sequence) with *S. gregorii* (96.0%), the closest relative of *S. cebuensis* suggesting that SPE 10-1 and 8-6 could possibly be the same strains. However, complete 16S rDNA sequencing and phenotypic characterization of the strain 8-6 must be done in order to confirm whether the same strain has been in fact, re-isolated from different sponge species.

The second strain Pol001^T isolated from the sponge *Axinella polypoides* was found by 16S rDNA sequencing to belong to the genus *Streptomyces*. The genus *Streptomyces* was first proposed by Waksman and Henrici (1943) for aerobic, spore-forming actinomycetes. These Gram-positive bacteria have distinct features such as extensive

branching substrate and aerial mycelium, high DNA G+C content (69-78 mol %), presence of LL -diaminopimelic acid and the absence of characteristic sugars in the cell wall (Anderson and Wellington 2001). The strain Pol001^T exhibited morphological, biochemical and chemotaxonomic characteristics consistent with the genus *Streptomyces*. There are currently more than 500 validly described species and subspecies under *Streptomyces*, making this genus to contain the largest number of species in the *Bacteria* domain (Hain et al. 1997). It is not surprising therefore that the 16S rDNA sequence similarity of the strain Pol001^T against reference *Streptomyces* strains was slightly above the 97% cut-off for species delineation (highest similarity value of 97.6%) considering the complexity of *Streptomyces* taxonomy. The 16S rDNA sequence similarity values still suggest that the strain Pol001^T can be considered a new *Streptomyces* taxon and this is also supported by the distinct phyletic line formed by the strain as seen in the neighbor-joining tree shown in Fig. 2.5. To further support this claim, DNA-DNA hybridization was carried out against the four reference *Streptomyces* strains with which strain Pol001^T exhibited the highest sequence similarities. The hypothesis for the species concept in the genus *Streptomyces* is that strains of the same species have DNA relatedness >70% (with a T_m of <5°C) (Wayne et al. 1987; Labeda 1992; Anderson and Wellington 2001). Remarkably, the strain Pol001^T showed very low DNA-DNA relatedness values (highest value at 26.8%), hence further confirming that the strain is indeed a novel taxon of the genus *Streptomyces*. Thus, the type strain was assigned the species name *Streptomyces axinellae* (a.xi.nel'la.e. N.L. gen. n. *axinellae*) pertaining to the marine sponge *Axinella polypoides*, from which the strain was originally isolated. The strain has likewise been recently considered as a validly described species by the *International Journal of Systematic and Evolutionary Microbiology* (Pimentel-Elardo et al. 2008a) and is currently deposited in two internationally recognized culture collections (= DSM 41948^T, = CIP 109838^T).

The remainder of the strains isolated from various marine sponges were found by 16S rDNA sequencing belonging to different actinomycete genera and exhibiting sequence similarities between 98-100% with known species, with the majority belonging to the genus *Streptomyces*. This is not surprising since *Streptomyces* bacteria are easy to cultivate and in fact, generally dominate several strain collections (Fiedler et al. 2005; Maldonado et al. 2005; Busti et al. 2006; Bredholdt et al. 2007). Several studies have also shown that diverse actinomycetes are associated with marine sponges using cultivation-dependent and cultivation-independent approaches (Webster and Hill 2001; Webster et al. 2001; Hentschel et al. 2002; Kim et al. 2005; Montalvo et al. 2005; Zhang et al. 2006; Jiang et al. 2007; Xin et al. 2008). Interestingly, some representatives of the class

Actinobacteria have been found belonging to monophyletic, sponge-specific sequence clusters using 16S rRNA gene-based techniques (Hentschel et al. 2002). However, none of the strains cultivated in this Ph.D. study overlapped with the sponge-specific actinobacterial clusters. These results suggest that the strains are transient bacteria that have been taken up by the host sponge through its aquiferous canal at the time that the sponge samples were collected. It is also highly likely that these isolates are present in the surrounding seawater or sediments since the majority of the strains isolated in this study showed very high sequence similarities with those previously derived from marine sediments. For example, isolates Da02 and T02 cultivated from two different Mediterranean sponges *Dysidea avara* and *Tethya* sp., respectively showed 100% 16S rDNA sequence similarity against each other suggesting that these are the same strains and nearly identical sequences (99.9%) with *Streptomyces* sp. strain CNS-774_SD_06, previously isolated from the marine sediment off the coast of California. Remarkably, several studies have also shown that marine actinomycetes are in fact, abundant in various ocean sediments (Zobell and Upham 1944; Grein and Meyers 1958; Mincer et al. 2002; Fiedler et al. 2005; Jensen et al. 2005a; Maldonado et al. 2005; Fenical and Jensen 2006; Pathom-Aree et al. 2006; Bredholdt et al. 2007).

Thus, the results provided here illustrate further that indeed diverse genera, including taxonomically novel actinomycetes are associated with marine sponges. However, the type of association of actinomycetes with marine sponges and their functions were not the main aims of this Ph.D. study but rather, their potential to produce bioactive secondary metabolites which shall be discussed in the succeeding chapters.

2.4 References

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Chapter 3

Secondary metabolites from sponge-associated actinomycetes

3.1 Materials and methods

3.1.1 Preliminary bioactivity testing by disk diffusion

The cultivated actinomycete strains were initially screened for bioactivity using a disk diffusion assay against the following test organisms: *Staphylococcus epidermidis* RP62A, *Staphylococcus aureus* NCTC 8325, *Pseudomonas aeruginosa*, *Enterococcus faecalis* JH212, *Escherichia coli* 536 and *Candida albicans*. Strains were grown in M1 culture broths (50 ml) as well as on agar plates (10 plates each) at 30°C for 7-14 days depending on the growth period of the strains. An equal volume of methanol was added to the liquid cultures to effectively lyse the cells and release intracellular metabolites. A volume of 300µl was impregnated on sterile 13-mm disks and the disks were air-dried. Previously prepared LB (for bacteria) and YPD (for *C. albicans*) plates were uniformly inoculated with a lawn of the test bacterial strains on the agar surface. The disks were then pressed lightly on the agar surface in equidistant positions and incubated at 37°C overnight. Zones of inhibition were noted by measuring the diameter (in mm) of the clearing zones around the disks. As for the strains grown on agar plates, the colonies growing on the agar surface together with the rest of the solid media were cut into small pieces and these were macerated separately in methanol and ethyl acetate overnight. The resulting extracts were filtered and then dried using a rotary evaporator (Heidolph, Germany), weighed and re-dissolved in their original extracting solvent to make a 20 mg/ml stock solution. A volume of 300µl was impregnated on sterile disks and the same process of disk diffusion assay described previously was followed.

3.1.2 Bioassay-guided isolation and purification

Strains that were found to produce bioactive metabolites from the preliminary antimicrobial assays were cultured on large scale (2-5 L) using the same media, growth conditions and cell lysis with methanol. Ethyl acetate was used as the extracting solvent and the resulting extracts were dried in vacuo using a rotary evaporator (Heidolph, Germany). The crude extracts were subjected to pre-fractionation with Diaion HP-20ss resin (Mitsubishi Chemical Corporation, Japan) using the following eluents: water (FW); 25% isopropanol: 75% water (F1); 50% isopropanol: 50% water (F2); 75% isopropanol: 25% water (F3); 100% methanol (F4), yielding five fractions with 15 ml each. These fractions were completely dried using a Savant speedvac concentrator (Thermo Scientific,

USA), re-dissolved and subjected to the disk diffusion assay at a dose of 200 µg/6-mm disk. HPLC analysis was performed on an Agilent 1100 series chromatography system (Agilent Technologies, USA) with a photodiode array detector to purify the active fractions. To determine the proper solvent gradient for purifying the fractions, analytical HPLC (Phenomenex Luna Analytical RP18e 4.6 x 250 mm) was first performed using H₂O (A) and CH₃OH (B) as the solvents and the following gradient: flow 1.0 ml/min; 0-5 min 10% B, 35-40 min 100% B. The solvent gradient was adjusted accordingly for improved resolution as well as separation of the peaks and the fractions were purified using semi-preparative HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm).

For *Saccharopolyspora cebuensis* strain SPE 10-1, 100 µl of the glycerol stock was inoculated in 3 x 100 ml of ISP 2 medium, incubated with shaking at 180 rpm at 30°C for five days. An aliquot of 50 ml of the inoculum was transferred to 6 x 750 ml of fresh ISP 2 medium and the cultures were incubated further under the same conditions for 7 days. After incubation, methanol was added to each culture (1:2) with shaking at 20°C overnight. The resulting mixtures were filtered and twice extracted with half-volume of ethyl acetate. The ethyl acetate layers were separated and dried by rotary evaporation. The same procedure was repeated for *Streptomyces* sp. strain A188 except that the strain was grown in 1.8 L of M1 medium.

The following *Streptomyces* sp. strains 11, 22, 34 and T03 were grown on 100 M1 agar plates and incubated at 30°C for seven days. Mycelial mass together with the agar were cut into small pieces and macerated overnight with sufficient volume of ethyl acetate. The resulting solution was filtered and maceration with ethyl acetate was repeated. Both filtrates were combined and subsequently dried. The same procedure was done for *Streptomyces axinellae* strain Pol001, except that the strain was grown on MS (Hobbs et al. 1989) agar.

3.1.3 Structure elucidation of compounds

¹H, ¹³C, HMBC, HSQC and COSY NMR spectra were recorded on a Varian Inova 500 or 600 MHz and Avance 400 MHz spectrometers. Accurate mass measurements were conducted on a Micromass Q-ToF Micro and microTOF mass spectrometers.

3.1.4 Anti-infective profiling of compounds

Pure compounds were dissolved in DMSO at 20mM concentration and were subjected to an anti-infective panel against bacteria (*Staphylococcus aureus* NCTC 8325,

Staphylococcus epidermidis RP62A, *Enterococcus faecalis* JH212, *Enterococcus faecium* 6413, *Escherichia coli* 536, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis* 252 01A and *Yersinia pestis* KUMA), fungi (*Candida albicans*) and parasites (*Trypanosoma brucei brucei* 221, *Leishmania major*). Cytotoxicity testing against J774.1 macrophages and 293T kidney epithelial cells was also done. Furthermore, inhibitions of selected proteases such as SARS-PL^{PRO} SARS-Coronavirus papain-like protease, SARS-M^{PRO} SARS-Coronavirus main protease, cathepsin B and L mammalian, rhodesain *Trypanosoma brucei rhodesiense* and falcipain-2 *Plasmodium falciparum* were tested. The anti-parasitic, cytotoxicity assays and protease inhibition tests as were performed by the SFB 630 collaboration partners: TP Z1 (T. Ölschläger and H. Bruhn, U. Würzburg) and TP A4 (T. Schirmeister, U. Würzburg), respectively.

3.1.4.1 Antimicrobial assays

Antibacterial assay

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

Antifungal assay

A colony of *Candida albicans* 5314 (ATCC 90028) was resuspended in 2 ml of 0.9% NaCl. Four microliters of this suspension was added to 2 ml of HR medium. This medium was prepared by adding 14.67 g HR Medium in 450 ml H₂O, followed by the addition of 1.0 g NaHCO₃ in 50 ml H₂O and subsequent incorporation of the following solutions: 420 ml of sodium phosphate (35.60 g Na₂HPO₄·2H₂O per liter) and 80 ml of potassium dihydrogen phosphate (27.22 g KH₂PO₄ per liter, pH 7.2). The test compounds were diluted in various concentrations in 100 μ l 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 followed by incubation at 37°C for 48 h. Optical density was measured at 530 nm with respect to a control well without cells. The minimal concentration of the compound where no growth is detectable was considered as the MIC value.

3.1.4.2 Anti-parasitic assays

Anti-Leishmania assay

Leishmania major promastigotes were seeded at a cell density of 1×10^7 cells/ml into 96-well plates in complete medium (RPMI with NaHCO_3 , 10% FCS, 2mM glutamine, 10 mM Hepes pH 7.2, 100 U/ml penicillin, 50 $\mu\text{g/ml}$ gentamicin, 50 μM 2-mercaptoethanol) without phenol red (200 μl), in the absence or presence of different concentrations of the compounds. These were then incubated for 24 h at 26°C, 5% CO_2 and 95% humidity. Following the addition of 20 μl of Alamar Blue, the plates were incubated again and the optical densities (ODs) measured 24 and 48 h later with an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan Ascent, Germany) using a test wavelength of 540 nm and a reference wavelength of 630 nm. Absorbance in the absence of compounds was set as 100% of growth. Amphotericin B was used as a reference compound and positive control. The effects of cell density, incubation time and the concentration of DMSO were examined in control experiments. The final concentration of DMSO in the medium never exceeded 1% vol/vol and had no effect on the proliferation of extracellular or intracellular parasites. For each experiment, each drug concentration was assayed in duplicate wells (Ponte-Sucre et al. 2006).

Anti-Trypanosoma assay

Trypomastigote forms of *Trypanosoma brucei brucei* laboratory strain TC 221 were cultured in complete Baltz medium [80 ml Baltz medium basic solution, 0.8 ml 2 mercaptoethanol stock solution (20 mM), 0.8 ml penicillin/streptomycin (10,000 U/ml), 16 ml FCS (inactivated for 30 min at 56°C). Baltz medium basic solution is composed of the following: 500 ml MEM with Earle's salts and L-glutamine, 3 g Hepes, 0.5 g monohydrate glucose, 0.110 g sodium pyruvate, 0.007 g hypoxanthine, 0.002 g thymidine, 0.0107 g adenosine, 0.0141 g bathocuproine disulfonic acid disodium salt, 0.146 g glutamine, 5 ml sterile non-essential amino acid concentrate (100x, pH 7.5) (Baltz et al. 1985). A defined number of parasites (10^4 trypanosomes per ml) were exposed in test chambers of 96-well plates to various concentrations of the test substances (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% CO₂ for a total time period of 72 h. After 24 h, 20 µl of Alamar Blue was added. The activity of the test substances was measured by light absorption using MR 700 Microplate Reader at a wavelength of 550 nm with a reference wavelength of 630 nm. The first reading was done at 48 h and subsequently at 72 h. The effect of the test substances was quantified in IC₅₀ values by linear interpolation of three independent measurements (Huber and Koella 1993; Raz et al. 1997).

3.1.4.3 Cytotoxicity assays

J774.1 macrophages were cultured in complete medium (RPMI with NaHCO₃, 10% FCS, 2mM glutamine, 10 mM Hepes pH 7.2, 100 U/ml penicillin, 50 µg/ml gentamicin, 50 µM 2-mercaptoethanol) without phenol red in the absence or presence of increasing concentrations of the compounds at a cell density of 1 x 10⁵ cells/ ml (200 µl) for 24 h at 37°C, 5% CO₂ and 95% humidity. Following the addition of 20 µl of Alamar Blue, the plates were incubated and the ODs measured at 24, 48 and 72 h. The same Alamar blue assay previously described for *Leishmania* was followed. Kidney epithelial 293T cells were also tested in the same manner as the macrophages but using complete DMEM medium (4.5 g/l solution of DMEM high glucose solution with sodium pyruvate but without L-glutamine, FBS superior at final concentration of 20%, 200mM L-glutamine 100x) and cell density (2 x 10⁴ cells/ ml).

3.1.4.4 Protease inhibition assays

SARS M^{pro} and P^{pro} protease inhibition assay

The fluorometric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 325 nm, emission 425 nm). For the inhibition assays, 96-well microplates (Nunc GmbH, Wiesbaden, Germany) were used. Assays were performed 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 final substrate (H₂N-Abz-Ser-Val-Thr-Leu-Gln-Ser-Gly-(NO₂)Tyr-Arg-(MTS)-TFA-salt for M^{pro} and Z-Arg-Leu-Arg-Gly-Gly-AMC-acetate salt for P^{pro}) concentration for inhibition assays was 50 µM, and the final enzyme concentration was 4.25 µg/mL. 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. Fluorescence increase was measured 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 and were diluted with assay buffer, and the enzyme was dissolved in buffer. The K_m value was calculated by nonlinear regression analyses using the program GraFit. All values were mean values from at least three independent assays (Kaepler et al. 2005).

Cathepsin L and B protease inhibition assay

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, 0.005% Brij 35 in a total volume of 285 μ L. Substrate (Cbz-Phe-Arg-AMC for both enzymes) and inhibitor stock solutions were prepared in DMSO (10% final concentration) and were diluted with assay buffer. The final substrate concentration for the inhibition assays was between 10.0 and 81.0 μ M. The final enzyme concentration was 53 ng/mL for cathepsin L (*P. tetraurelia*) and 58 μ g/mL for cathepsin B (recombinant, human liver) (Vicik et al. 2006a). Inhibitors were tested at 100 μ M (final concentration). 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).

Rhodesain protease inhibition assay

For inhibition of rhodesain, 96-well microplates were used. Assays were performed at 25°C in 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 μ L. 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 μ M and the final enzyme concentration was 41 nM. Inhibitors were used at a concentration of 100 μ M (Vicik et al. 2006b). The fluorometric enzyme assay was performed in a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 365 nm, emission 460 nm).

Falcipain protease inhibition assay

The enzyme assay with the cysteine protease Cbz-Phe-Arg-AMC (falcipain-2, 50 μ M) as substrate was performed. The enzyme was incubated with 100 μ M concentration of the compounds for 0, 15 or 30 min prior to substrate addition. Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed in 96-well plates in a total volume of 120 or 300 μ L ($n = 2-6$ independent assays) with standard deviations of <10%. The following buffer was used: 100 mM acetate, pH 5.5, 10 mM DTT. The Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) with a microplate reader (excitation 365 nm, emission 460 nm) was used to measure fluorescence.

3.2 Results

3.2.1 Isolation and characterization of metabolites from actinomycete strains

3.2.1.1 Novel macrolactam polyketides from *Saccharopolyspora cebuensis* strain SPE 10-1^T

The crude extract obtained from solvent partitioning with ethyl acetate was purified by preparative HPLC (Merck Chromolith SemiPrep RP18e 10 x 100 mm) using H₂O + 0.05% TFA (A) and CH₃CN + 0.05% TFA (B) as the solvents and the following gradient: flow 10 ml/min; 0 min 75% B, 10 min 75% B. Two novel, constitutionally identical macrolactams (Fig. 3.1) named as cebulactam A1 (5.1 mg; R_t = 2.5 min) and cebulactam A2 (4.1 mg; R_t = 4.3 min) were identified. Their constitutionally identical structures, each bearing a 6-membered cyclic ether as part of the macrocycle and their relative configurations were elucidated by MS methods and by 1D and 2D NMR techniques (Tables 3.1 and 3.2). HPLC-ESI-MS analysis revealed the molecular masses of both compounds to be identical (m/z 346.3 for [M+H]⁺), suggesting the two compounds to be structurally highly similar to each other. These assumptions were further corroborated by HR-ESIMS (TOF) measurements which clearly showed an identical molecular formula of C₁₉H₂₃NNaO₅ (m/z 368.1478 for [M+Na]⁺, calculated 368.1468) for cebulactams A1 and A2 (Gulder 2008; Pimentel-Elardo et al. 2008). The relative configurations of the stereocenters of cabulactams A1 and A2 were elucidated by NOESY NMR spectroscopy. This work was done in collaboration with T.A.M. Gulder (AG Bringmann, Institute of Organic Chemistry, University of Würzburg). The absolute configurations of both compounds have yet to be elucidated.

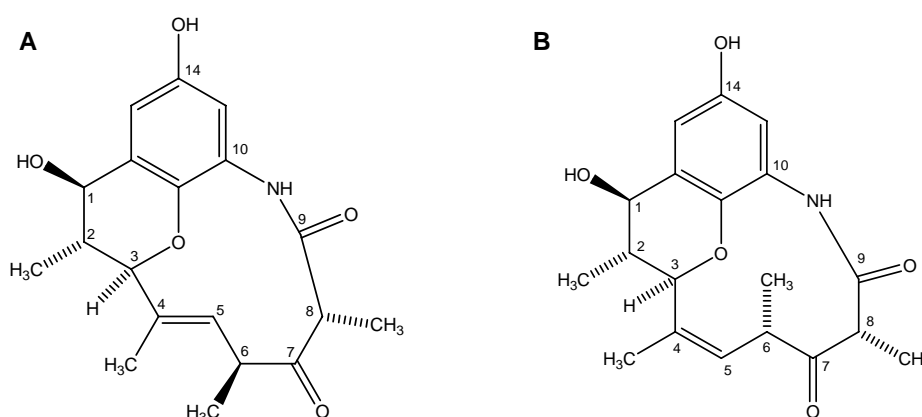


Figure 3.1 Cebulactam A1 (A) and cebulactam A2 (B) polyketides isolated from strain SPE 10-1^T

Table 3.1 NMR spectroscopic data of cebulactam A1 acetone-d₆
(¹H: 400 MHz; ¹³C: 100 MHz)

Position	δ_C	δ_H , mult (J in Hz)	COSY	HMBC
1	70.9	4.38, d (10.1)	2	2, 2-CH ₃ , 3, 11, 12, 13
2	42.4	1.82, m	2-CH ₃ , 1, 3	1, 2-CH ₃ , 3, 12
2-CH ₃	16.7	1.19, d (6.6)	2	1, 2, 3
3	87.4	4.27, d (8.5)	2	2, 2-CH ₃ , 4, 4-CH ₃ , 5, 11
4	140.5			
4-CH ₃	19.3	1.86, s	5	3, 4, 5, 6-CH ₃ , 7
5	124.7	4.98, d (9.8)	4-CH ₃ , 6	3, 4-CH ₃ , 6, 7
6	46.2	3.19, m	6-CH ₃ , 5	4, 5, 6-CH ₃ , 6, 7
6-CH ₃	17.8	1.00, d (6.3)	6	5, 6, 7
7	207.1			
8	48.2	3.43, q (6.8)	8-CH ₃	7, 8-CH ₃ , 9
8-CH ₃	16.6	1.21, d (6.8)	8	7, 8, 9
9	173.9			
10	131.0			
11	142.7			
12	140.9			
13	111.1	7.02, d (2.8)		1, 11, 14, 15
14	154.4			
15	114.0	6.65, d (2.8)		10, 11, 13, 14
NH		7.88, br		

*(Gulder 2008; Pimentel-Elardo et al. 2008)

Table 3.2 NMR spectroscopic data of cebulactam A2 in acetone-d₆
(¹H: 400 MHz; ¹³C: 100 MHz)

Position	δ_C	δ_H , mult (J in Hz)	COSY	HMBC
1	70.8	4.36, d (10.1)	2	2, 2-CH ₃ , 3, 11, 12, 13
2	46.2	1.51, m	2-CH ₃ , 1, 3	1, 2-CH ₃ , 3, 12
2-CH ₃	15.0	1.15, d (6.6)	2	1, 2, 3
3	86.2	4.24, d (10.0)	2	2, 2-CH ₃ , 4, 4-CH ₃ , 5, 11
4	145.4			
4-CH ₃	19.8	2.02, s	5	3, 4, 5
5	125.1	5.07, d (10.5)	4-CH ₃ , 6	3, 4-CH ₃ , 6, 7
6	48.3	3.34, m	6-CH ₃ , 5	4, 5, 6-CH ₃ , 6, 7
6-CH ₃	16.9	0.99, d (6.8)	6	5, 6, 7
7	205.2			
8	55.6	3.97, q (6.7)	8-CH ₃	7, 8-CH ₃ , 9
8-CH ₃	15.1	1.27, d (6.7)	8	7, 8, 9
9	173.3			
10	130.4			
11	139.5			
12	138.1			
13	107.3	6.76, d (2.8)		1, 11, 14, 15
14	153.4			
15	110.9	6.83, d (2.8)		10, 11, 13, 14
NH		8.61, br		

*(Gulder 2008; Pimentel-Elardo et al. 2008)

Isolation and structure elucidation of the following compounds (3.2.1.2 to 3.2.1.6) were performed at the Ireland Research Lab, Department of Medicinal Chemistry, University of Utah.

3.2.1.2 Novel tetromycin compounds from *Streptomyces axinellae* strain Pol001^T

The crude extract obtained from maceration of the mycelial mass with ethyl acetate was purified by preparative HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H₂O + 0.1% TFA (A) and CH₃CN (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-10 min 90% B, 11-15 min 100% B. The following compounds were isolated (Fig. 3.2): Pol001-1 (2.7 mg; R_t = 5.938 min); Pol001-3 (4.4 mg; R_t = 7.746 min); Pol001-4 (2.2 mg; R_t = 9.666 min); Pol001-5 (4.4 mg; R_t = 12.240 min); and Pol001-7 (2.1 mg; R_t = 17.468 min).

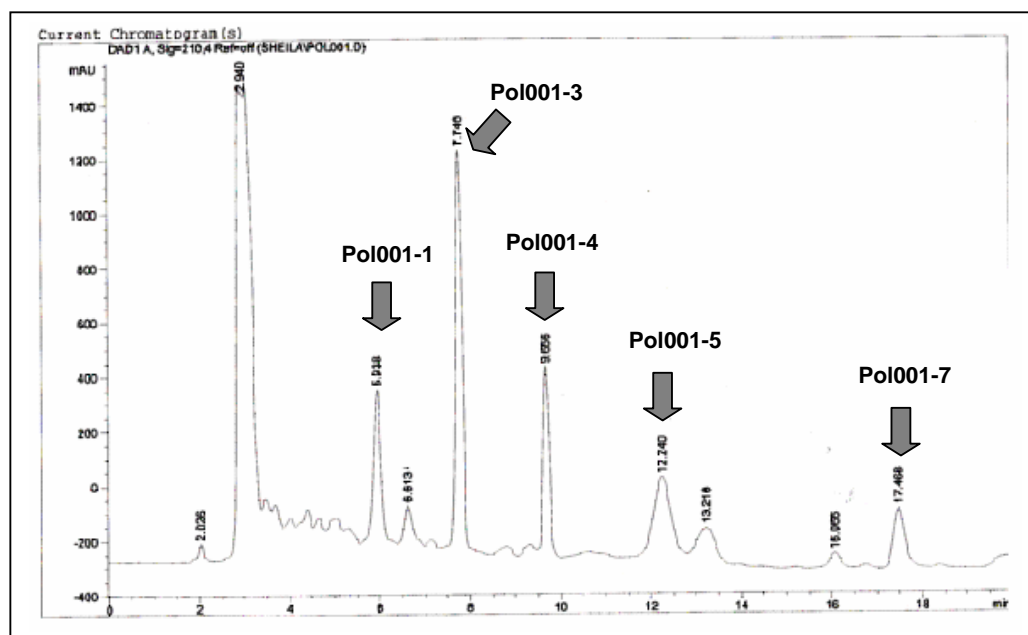


Figure 3.2 HPLC chromatogram of EtOAc extract from *Streptomyces axinellae* strain Pol001^T

Molecular formulas for each of the compounds were established using high-resolution mass spectrometry as follows: Pol001-1 = C₅₀H₆₅NO₁₃ (*m/z* 910.4388 for [M+Na]⁺, calculated 910.4354); Pol001-3 = C₅₀H₆₄O₁₄ (*m/z* 911.4232 for [M+H]⁺, calculated 911.4219); Pol001-4 = C₄₈H₆₀O₁₄ (*m/z* 861.4062 for [M+H]⁺, calculated 861.4062); Pol001-5 = C₄₉H₆₂O₁₄ (*m/z* 897.4066 for [M+Na]⁺, calculated 897.4037); and Pol001-7 = C₃₄H₄₆O₅ (*m/z* 557.3229 for [M+Na]⁺, calculated 557.3243).

Furthermore, the combination of NMR (1D and 2D), and comparison of the spectral data with the database using the SciFinder Scholar tool revealed that the compounds (Pol001-1, Pol001-5, Pol001-7) are identical to the published family of tetracycline compounds previously isolated from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989) (Fig. 3.3).

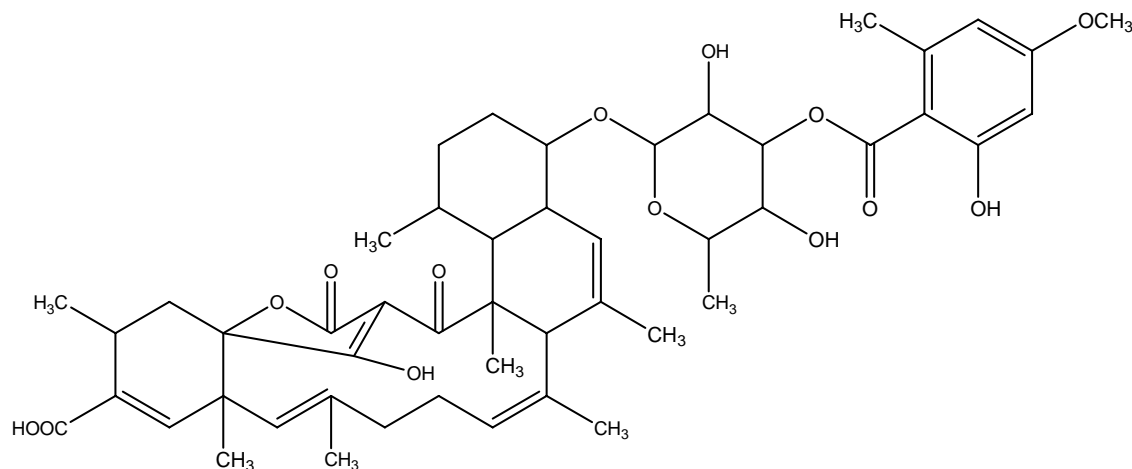


Fig. 3.3 Tetracycline from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989)

Interestingly, mass and NMR data of Pol001-3 and Pol001-4 suggest that these compounds are new derivatives of tetracycline. Partial structures (Fig. 3.4, 3.5, 3.6) were elucidated revealing a difference at position 10: hydroxyl function for Pol001-4 (Fig. 3.4) and a methoxy group for Pol001-3 (Fig. 3.5). Furthermore, the additional novelty of these structures is postulated to be found in the decalin ring of the tetracyclines (Fig. 3.3) with the difference in the number or position of the methyl groups. Complete elucidation of the structures was not yet possible due to some ambiguous chemical shift assignments in some positions. Nevertheless, preliminary structure analyses still suggest that *Streptomyces axinellae* strain Pol001^T produced novel tetracycline derivatives. Further NMR analysis should therefore be carried out in order to confirm and complete the structure elucidation of these interesting compounds. Stereochemical analysis is also worthwhile to pursue since relative or absolute configurations of the stereocenters in tetracyclines have not been reported for any of the compounds.

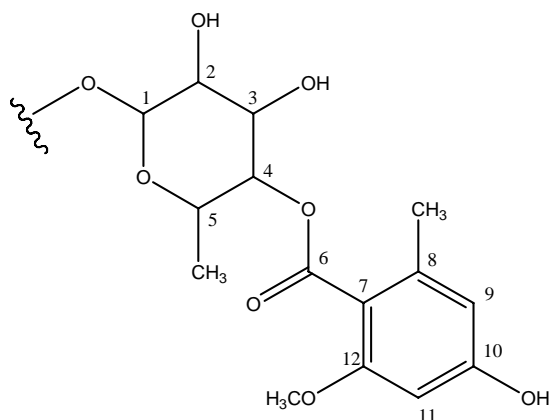


Figure 3.4 Pol001-4 partial structure A

Table 3.3 NMR spectroscopic data of Pol001-4 partial structure A in acetone- d_6 (^1H : 500 MHz; ^{13}C : 125 MHz)

Position	δ_{C}	δ_{H} , mult (J in Hz)	COSY	HMBC
1	102.0	4.80, d (7.9)	2	2
2	71.1	3.54, dd (7.9, 2.9)	1	3
3	68.8	4.38, t (2.9)	4	3
4	76.0	4.75, dd (10.0, 2.9)	3	4
5	66.4	4.16, dq (10.0, 6.3)	5-CH ₃	3, 5-CH ₃
5-CH ₃	17.5	1.25, d (6.3)	5	
6	170.2			4
7	105.3			11, 8-CH ₃
8	143.7			8-CH ₃
8-CH ₃	23.6	2.55, s		
9	110.8	6.37, d (2.1)		8-CH ₃
10	165.2			11
11	98.7	6.34, d (2.1)		
12	164.3			
12-OCH ₃	54.9	3.83, s		12

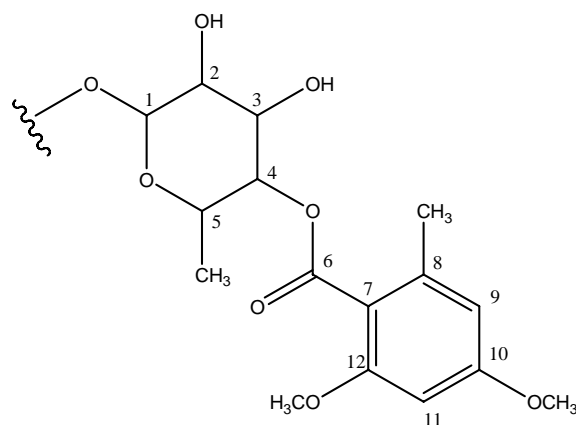


Figure 3.5 Pol001-3 partial structure A

Table 3.4 NMR spectroscopic data of Pol001-3 partial structure A in acetone- d_6 (^1H : 500 MHz; ^{13}C : 125 MHz)

Position	δ_{C}	δ_{H} , mult	COSY	HMBC
1	102.4	4.79, d	2	
2	71.4	3.54, dd	1	
3	69.5	4.34, t		5-CH ₃
4	75.0	4.69, dd	5	5-CH ₃
5	66.6	4.03, dq	4, 5-CH ₃	5-CH ₃
5-CH ₃	17.2	1.26, d	5	
6	170.0			
7	106.9			8-CH ₃ , 11
8	143.0			8-CH ₃
8-CH ₃	23.8	2.56, s		8
9	110.8	6.37, d		8-CH ₃ , 11
10	161.6			10-OCH ₃
10-OCH ₃	54.9	3.84, s		
11	96.0	6.46, d		9
12	158.0			12-OCH ₃
12-OCH ₃	55.4	3.83, s		

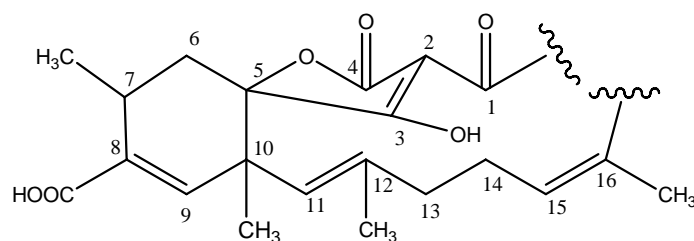


Figure 3.6 Pol001-4 and Pol001-3 partial structure B

Table 3.5 NMR spectroscopic data of partial structure B of Pol001-4 and Pol001-3 in acetone- d_6 (^1H : 500 MHz; ^{13}C : 125 MHz)

Position	Pol001-4		Pol001-3	
	δ_{C}	δ_{H} , mult	δ_{C}	δ_{H} , mult
1	201.0		200.0	
2	135.0		138.0	
3	206.0		205.0	
4	166.6		168.0	
5	81.8		84.6	
6	34.9	2.29, br; 1.77, br	33.6	2.26, br; 1.67, br
7	27.4	2.86, br	27.0	2.94, br
7- CH_3	20.2	1.31, d	20.9	1.32, d
8	120.9		119.8	
8-COOH	166.7		167.0	
9	138.8	6.67, s	147.6	6.93, s
10	68.8		68.0	
10- CH_3	22.6	1.51, s	23.3	1.48, s
11	122.0	6.15, s	122.1	6.18, s
12	124.6		116.5	
12- CH_3	22.4	1.49, s	24.8	1.47, s
13	24.6	2.23, br; 1.93, br	24.6	2.23, br; 1.92, br
14	24.8	2.26, br; 1.95, br	24.8	2.24, br; 1.94, br
15	130.0	5.06, d	130.5	5.14, d
16	135.0		134.9	
16- CH_3	13.1	1.38, s	12.4	1.35, s

3.2.1.3 Valinomycin from *Streptomyces* sp. strains 34 and 22

The crude ethyl extract obtained from maceration of the mycelial mass of *Streptomyces* sp. strain 34 with ethyl acetate was found to exhibit activities (zones of inhibition in mm, 600 µg/ 6-mm disk) against the following organisms: *Staphylococcus aureus* (20 mm), *Staphylococcus epidermidis* (23 mm), and *Escherichia coli* (8 mm). No activities were found against *Pseudomonas aeruginosa* and *Candida albicans*. The ethyl acetate extract was further pre-fractionated using Diaion HP-20ss resin. Out of the five fractions obtained (FW, F1, F2, F3, F4), secondary antibacterial assay against *Staphylococcus aureus* and *Escherichia coli* revealed that fractions F2 and F3 were found to exhibit activity (200 µg/ 6-mm disk) against *S. aureus* with zones of inhibition values of 9 and 8 mm, respectively. All other Diaion fractions were not active against *E. coli*. HPLC chromatograms of both fractions also revealed the same profile. Thus, fraction 11-F3 (20 mg) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H₂O (A) and CH₃OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 90% B, 11-15 min 100% B. The major peak, 34-F3-3 (8.4 mg; $R_t = 13.085$ min) (Fig. 3.7) yielded the cyclic peptide, valinomycin (Fig. 3.8; Table 3.6).

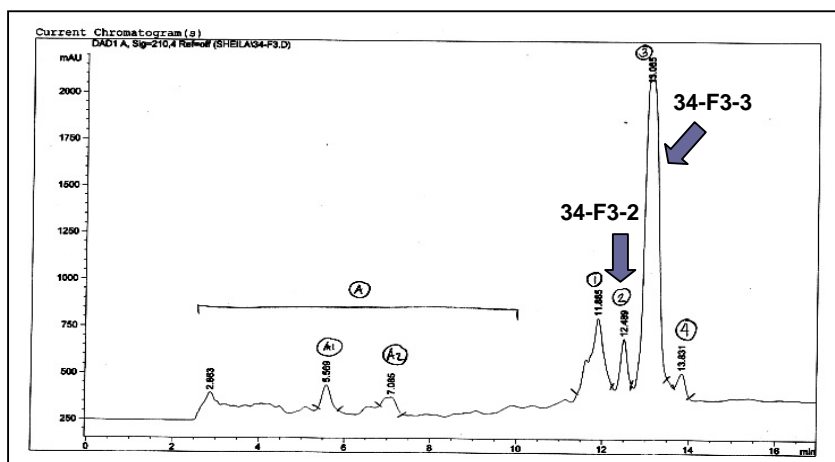


Fig. 3.7 HPLC chromatogram of fraction 34-F3 from *Streptomyces* sp. strain 34

High-resolution mass spectrometry of the purified compound established a molecular formula of C₅₄H₉₀N₆NaO₁₈ (m/z 1133.6385 for [M+Na]⁺, calculated 1133.6394). A combination of NMR (1D and 2D), MS-MS fragmentation and comparison of the spectral data with the database, Dictionary of Natural Products (www.chemnetbase.com) suggested that 34-F3-3 contains one α -hydroxyisovaleryl unit (Hiv), a lactoyl group (Lac), and two valines (Val), thus confirming the identity of the compound as valinomycin. However, the stereochemical properties of this compound were not determined.

Furthermore, the structure was also confirmed by comparison of the ^1H NMR and ^{13}C NMR data with published data (Brockmann and Schmidt-Kastner 1955; Heisey et al. 1988).

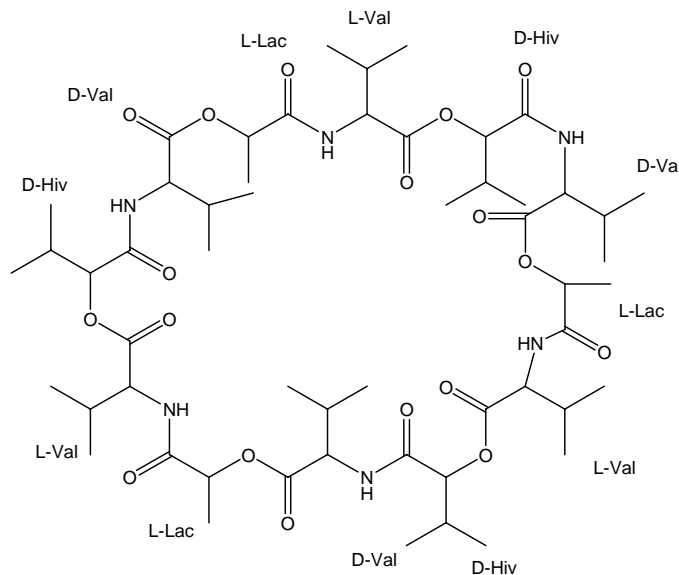


Fig. 3.8 Chemical structure of 34-F3-3 (valinomycin)

Table 3.6 NMR spectroscopic data of 34-F3-3 (valinomycin) in CDCl_3 (^1H : 500 MHz; ^{13}C : 125 MHz)

Unit	Position	δ_{C}	δ_{H} , mult	COSY	HMBC
Hiv	1-CO	171.0			2
	2-CH	78.8	5.02, d	3	4, 5
	3-CH	28.8	2.30, m	4, 5	4, 5
	4-CH ₃	22.9	0.85, d		
	5-CH ₃	22.8	0.86, d		
Val	6-CO	170.4			
	7-CH	59.1	4.10, t	8	9, 10
	8-CH	28.6	2.25, m	9, 10	9, 10
	9-CH ₃	19.5	0.96, d		
	10-CH ₃	16.8	0.97, d		
Lac	NH		7.85, d	7	
	11-CO	172.6			13
	12-CH	70.4	5.30, q	13	13
	13-CH ₃	17.2	1.46, d		12
	14-CO	171.9			15
Val	15-CH	60.5	4.00, t	16	17, 18
	16-CH	27.4	2.04, m	17, 18	17, 18
	17-CH ₃	19.2	0.98, d		
	18-CH ₃	18.4	0.99, d		
	NH		7.73, d	15	

This cyclodepsipeptide contains a three-repeat sequence of a tetradepsipeptide basic unit, D- α -hydroxyisovaleryl-D-valyl-L-lactoyl-L-valyl to form a symmetric 36-membered ring molecule. Interestingly, the same compound was isolated from *Streptomyces* sp. strain 22. The fractions in fact, obtained from this strain exhibited exactly the same chromatographic and NMR profiles suggesting that *Streptomyces* sp. strains 22 and 34 produced exactly the same metabolites when cultivated under the same conditions. This is not surprising since a close look at their 16S rDNA sequence revealed that they exhibited 99.93% sequence similarity (1409/1410) with only one nucleotide difference indicating that these are the same strains regardless of the fact that these were isolated from different sponge sources (*Axinella polypoides* for strain 34 and *Aplysina aerophoba* for strain 22).

A derivative of valinomycin was also detected in the fraction 34-F3-2 (1.5 mg; $R_t = 12.489$ min) (Fig.3.7) with a molecular formula of $C_{53}H_{88}N_6NaO_{18}$ (m/z 1119.6064 for $[M+Na]^+$, calculated 1119.6053). This could possibly be a new analog of valinomycin exhibiting a difference of a CH_2 unit but complete spectroscopic analysis should be done to confirm the structure of this potentially novel compound.

3.2.1.4 Staurosporine from *Streptomyces* sp. strain 11

Preliminary disk diffusion assay of the crude ethyl extract obtained from maceration of the mycelial mass of *Streptomyces* sp. strain 11 with ethyl acetate revealed the following activities (zones of inhibition in mm, 600 μ g/ 6-mm disk) against the following organisms: *Staphylococcus epidermidis* (8 mm), and *Candida albicans* (9 mm). No activities were found against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The ethyl acetate extract was further pre-fractionated using Diaion HP-20ss resin. For a bioassay-guided isolation, the five fractions obtained (FW, F1, F2, F3, F4) were subjected to secondary antibacterial assay. Fractions F2 and F3 were found to exhibit activity (200 μ g/ 6-mm disk) against *S. aureus* (7 mm) and no activity against *Escherichia coli*. 1H NMR of both fractions revealed the same profile. Fraction 11-F3 (13.9 mg) was then further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O + 0.1% TFA (A) and CH_3OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 70% B, 10 min 80% B, 20-25 min 100% B.

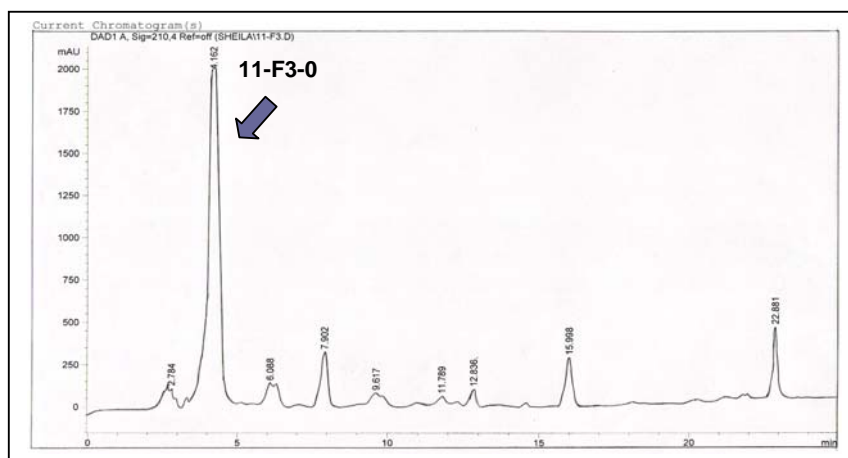


Fig. 3.9 HPLC chromatogram of fraction 11-F3 from *Streptomyces* sp. strain 11

The major peak, 11-F3-0 (1.4 mg; $R_t = 4.162$ min) (Fig. 3.9) yielded the indolocarbazole, staurosporine (Fig. 3.10). This was confirmed by mass spectrometry revealing the compound to have a molecular formula of $C_{28}H_{26}N_4O_3$ (m/z 467.2078 for $[M+H]^+$, calculated 467.2083) coupled with NMR (1D and 2D) analysis (Table 3.7) and comparison of the spectral data with the database, Dictionary of Natural Products (www.chemnetbase.com). Furthermore, the structure was also confirmed by comparison of the 1H NMR and ^{13}C NMR data with published data (Meksuriyen and Cordell 1988; Schupp et al. 1999). However, the stereochemical properties of this compound were not determined.

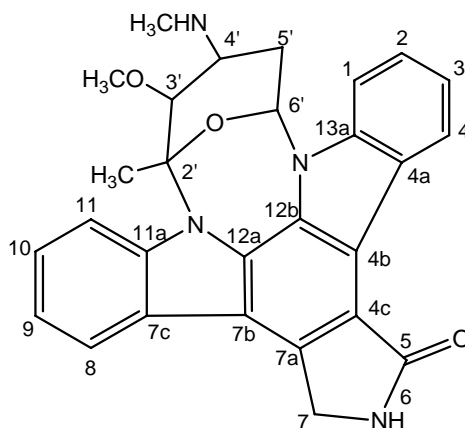


Fig. 3.10 Chemical structure of 11-F3-0 (staurosporine)

Table 3.7 NMR spectroscopic data of 11-F3-0 (staurosporine) in CD₃OD (¹H: 500 MHz; ¹³C: 125 MHz)

Position	δ_c	δ_H , (H, mult)	COSY	HMBC
1	109.3	7.16 (1H, d)	2	4
2	126.5	7.52 (1H, t)	3	4
3	126.6	7.41 (1H, t)	2	1
4	127.2	9.22 (1H, d)	3	1, 4a, 12b, 13a
4a	127.6			4
4b	115.8			4
4c	120.2			7A, 7B
5	175.1			7A, 7B
7	46.8	7A: 4.73 (1H) 7B: 4.44 (1H)		4b, 4c, 5, 7a
7a	133.8			7A, 7B
7b	115.8			
7c	124.5			11
8	122.7	7.81 (1H, d)	9	9
9	120.7	7.26 (1H, t)	10	
10	122.0	7.38 (1H, t)	9	11
11	113.4	7.96 (1H, d)	10	2'-CH ₃
11a	139.4			11
12a	131.4			7A, 7B
12b	125.9			6'
13a	137.7			4
2'	94.2			2'-CH ₃
3'	81.4	4.21 (1H, s)	4'	2', 2'-CH ₃ , 3', 4'
4'	55.9	3.90 (1H, dd)	3', 5'A, 5'B	3', 4'-NCH ₃
5'	28.8	5'A: 2.13 (1H) 5'B: 3.20 (1H)	4', 6'	6'
6'	82.0	6.44 (1H, dd)	5'A, 5'B	
2'-CH ₃	28.7	2.53 (3H, s)		2', 6', 11
3'-OCH ₃	60.6	2.14 (3H, s)		3'
4'-NCH ₃	31.3	2.78 (3H, s)		4'

3.2.1.5 Cycloisoleucylprolyl from *Streptomyces* sp. strain A188

Initial bioassay testing of the metabolites produced by *Streptomyces* sp. strain A188 after growing in M1 broth indicated activity against *Staphylococcus aureus* (300 μ l/ 13-mm disk) with a zone of inhibition of 26 mm. No activity was found against *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. The crude extract obtained from solvent partitioning with ethyl acetate was pre-fractionated with Diaion HP-20ss. The fraction F1 was found to exhibit antibacterial activities against *S. aureus* and *E. coli* with zones of inhibition (200 μ g/ 6-mm disk) of 1.0 and 2.2 mm, respectively. Thus, fraction A188-F1 (22 mg) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H₂O (A) and CH₃OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 15% B, 25 min 60% B, 30-35 min 100% B.

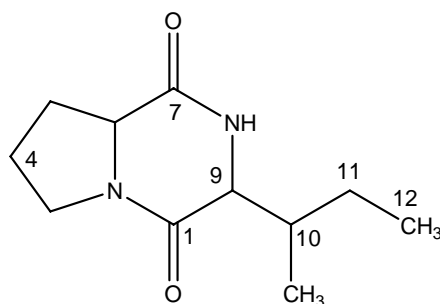


Fig. 3.11 Chemical structure of A188-F1-3 (cycloisoleucylprolyl)

The fraction A188-F1-3 obtained from HPLC yielded the diketopiperazine, cycloisoleucylprolyl (Fig 3.11) (2.2 mg; $R_t = 20.295$ min). Mass analysis revealed a molecular formula of $C_{11}H_{18}N_2NaO_2$ (m/z 233.1262 for $[M+Na]^+$, calculated 233.1266). The structure was confirmed by comparison of NMR (1D and 2D) analysis (Table 3.8) with published spectral data of the compound (Adamczeski et al. 1995; Fdhila et al. 2003) but the stereochemical properties of this compound were not further investigated.

Table 3.8 NMR spectroscopic data of A188-F1-3 (cycloisoleucylprolyl) in CD_3OD (1H : 500 MHz; ^{13}C : 125 MHz)

Position	δ_C	δ_H , (H, mult)	COSY	HMBC
1	167.3			11
3	46.4	3A: 3.50 (1H, m) 3B: 3.51 (1H, m)	4	
4	25.8	4A: 1.94 (1H, m) 4B: 2.00 (1H, m)	3	
5	29.1	5A: 2.02 (1H, m) 5B: 2.30 (1H, m)	5B, 6 5A, 6	3
6	60.3	4.26 (1H, t)	5A, 5B	
7	167.8			
9	54.6	4.13 (1H, m)		11
10	23.6	1.89 (1H, m)	10	11B, 10-CH ₃
11	39.4	11A: 1.91 (1H, m) 11B: 1.53 (1H, m)	11A	10-CH ₃ 9, 12
12	22.2	0.96 (3H, d)	11A	11B
10-CH ₃	23.3	0.97 (3H, d)	10	12

3.2.1.6 Butenolide from *Streptomyces* sp. strain T03

The crude ethyl extract obtained from maceration of the mycelial biomass of *Streptomyces* sp. strain T03 was further fractionated with Diaion HP-20ss resin. Fraction T03-F1 which was found to be active against *Staphylococcus aureus* with a zone of inhibition (200 µg/ 6-mm disk) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H₂O (A) and CH₃OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 40% B, 25 min 60% B, 15 min 70% B, 20 min 85% B, 21-26 min 100% B. The fraction T03-F1-2A yielded a compound (1.0 mg; R_t = 17.168 min) with a molecular formula of C₁₃H₂₂O₃Na (*m/z* 249.1447 for [M+Na]⁺, calculated 249.1467). NMR analysis (Table 3.9) confirmed the compound to be a butenolide (Fig. 3.12), exhibiting identical spectral data with published literature (Mukku et al. 2000; Cho et al. 2001). However, the stereochemical properties of this compound were not determined.

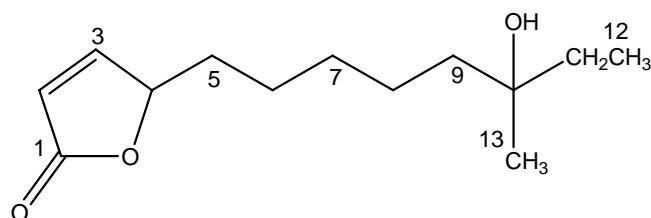


Fig. 3.12 Chemical structure of T03-F1-2A (butenolide)

Table 3.9 NMR spectroscopic data of T03-F1-2A (butenolide) in CD₃OD
(¹H: 500 MHz; ¹³C: 125 MHz)

Position	δ _C	δ _H , (H, mult)	COSY	HMBC
1	175.8			2
2	121.6	6.11 (1H, ddd)		3
3	159.6	7.71 (1H, ddd)	2, 4	2, 5A
4	85.6	5.13 (1H, m)	2, 5A	2, 3, 5A
5	34.9	5A: 1.81 (1H, m) 5B: 1.64 (1H, m)	4, 6	
6	25.8	1.45 (2H, m)	5A	
7	30.7	1.35 (2H, m)		5A, 6
8	24.3	1.34 (2H, m)		9
9	42.0	1.42 (2H, m)		11
10	72.2			11, 12, 13
11	41.1	1.46 (2H, q)	12	12, 13
12	8.5	0.89 (3H, t)		11
13	25.9	1.11 (3H, s)		11

3.2.2 Bioactivity profile of compounds

3.2.2.1 Antimicrobial activity

The compounds were subjected to a panel of antimicrobial tests to determine the minimum inhibitory concentration against various Gram-positive and Gram-negative bacteria as well as fungi (Table 3.10). Among the compounds tested, only four exhibited antibacterial activities as follows: the novel tetromycin derivative Pol001-3 exhibited MIC values of 100 μ M and 50 μ M against *S. aureus* and *S. epidermidis*, respectively; Pol001-4 exhibited slight activity with MIC value of 200 μ M against these two bacteria; Pol001-7 exhibited MIC of 200 and 100 μ M against *S. aureus*, *S. epidermidis* respectively; and staurosporine against *S. aureus* with MIC of 100 μ M. The novel derivative Pol001-4 was the only compound that exhibited slight activity against *E. faecalis* (MIC of 200 μ M). Valinomycin and staurosporine were the only compounds that exhibited significant anti-*Candida* activity with MIC values of 0.63 and 0.31 μ M, respectively.

Surprisingly, neither the compounds cebulactam A1 and cebulactam A2 nor the combination of these 2 compounds exhibited any antimicrobial activities, despite the fact that the crude ethyl acetate extract showed antibacterial activity from the preliminary screen. The other tetromycin compounds Pol001-1 and Pol001-5 as well as butenolide also did not show any activity. The compound cycloisoleucylprolyl was not tested due to the lack of material.

Table 3.10 Antimicrobial activity testing of compounds (Minimum inhibitory concentration, μ M)

Compound	Sa	Se	Efa	Efe	Ec	Pa	Yps	Ype	Ca
Cebulactam A1	>160	>160	>160	>160	>160	>160	>160	>160	>80
Cebulactam A2	>160	>160	>160	>160	>160	>160	>160	>160	>80
Cebulactam A1+A2	>160	>160	>160	>160	>160	>160	>160	>160	>80
Pol001-1	>200	>200	>200	nd	>200	>200	nd	nd	>80
Pol001-3	100	50	>200	nd	>200	>200	nd	nd	>80
Pol001-4	200	200	200	nd	>200	>200	nd	nd	>80
Pol001-5	>200	>200	>200	nd	>200	>200	nd	nd	>80
Pol001-7	200	100	>200	nd	>200	>200	nd	nd	>80
Valinomycin	>200	>200	>200	nd	>200	>200	nd	nd	0.63
Staurosporine	100	>200	>200	nd	>200	>200	nd	nd	0.31
Butenolide	>200	>200	>200	nd	>200	>200	nd	nd	>80

Legend:

Sa = *Staphylococcus aureus* NCTC 8325

Se = *Staphylococcus epidermidis* RP62A

Efa = *Enterococcus faecalis* JH212

Efe = *Enterococcus faecium* 6413

Ec = *Escherichia coli* 536

Pa = *Pseudomonas aeruginosa*

Yps = *Yersinia pseudotuberculosis*

Ype = *Yersinia pestis*

Ca = *Candida albicans*

nd = not determined

3.2.2.2 Anti-parasitic and cytotoxic activities

The compounds were further tested against the parasites *Leishmania major* and *Trypanosoma brucei* subsp. *brucei* as well as their cytotoxicities against the two cell lines, 293T kidney epithelial cells and J774.1 macrophages. All of the compounds, except for the cebulactams A1 and A2 exhibited significant anti-*Trypanosoma* activities exhibiting IC₅₀ values below 100 µM with valinomycin and staurosporine showing the lowest IC₅₀ values of 0.0032 and 0.022 µM, respectively. Only three compounds showed activity against the parasite *Leishmania major*: Pol001-4 (IC₅₀, 36.80 µM), valinomycin (IC₅₀, <0.11 µM) and staurosporine (IC₅₀, 5.30 µM). The majority of the compounds that showed significant anti-parasitic activities also showed cytotoxicities (<100 µM) with the exception of the compounds, Pol001-1 and butenolide (Table 3.11).

It is interesting to note that among the tetromycin compounds isolated in this Ph.D. study, the novel derivative Pol001-4 exhibited significant anti-*Leishmania* activity. The cebulactams A1 and A2 including the mixture of both substances did not exhibit any activities at all.

Table 3.11 Antiparasitic and cytotoxic activities (IC₅₀, µM) of compounds

Compound	LM	TB, 48 h	TB, 72 h	KE	JM
Cebulactam A1	>100	>100	>100	nd	>100
Cebulactam A2	>100	>100	>100	nd	>100
Cebulactam A1+A2	>100	>100	>100	nd	>100
Pol001-1	>100	29.30	31.69	123.38	>100
Pol001-3	>100	45.39	80.27	160.00	50.21
Pol001-4	36.80	26.90	30.35	33.38	25.72
Pol001-5	>100	35.85	41.61	58.58	27.54
Pol001-7	>100	30.87	34.22	71.77	20.20
Valinomycin	<0.11	0.0032	0.0036	11.24	<0.1
Staurosporine	5.30	0.022	0.035	1.30	<0.13
Butenolide	>100	31.77	33.08	>100	>100

*Activity not determined for cycloisoleucylprolyl due to lack of material.

Legend:

KE = 293T kidney epithelial cells
 JM = J774.1 macrophages
 LM = *Leishmania major*
 TB = *Trypanosoma brucei brucei* 221
 nd = not determined

3.2.2.3 Protease inhibition

Different cysteine proteases were tested to evaluate the ability of the compounds to inhibit these enzymes found in the parasites *Plasmodium falciparum* (falcipain-2) and *Trypanosoma brucei rhodesiense* (rhodesain), mammalian cells (cathepsin B and L), and SARS coronavirus (SARS-PL^{pro} and SARS-M^{pro}) (Table 3.12). A percent inhibition value above 70% at 100 μ M concentration was considered as significant protease inhibition activity (T. Schirmeister, personal communication). Among the compounds tested, the tetromycin family of compounds (Pol001 series) showed interesting activities. For example, inhibition of the malarial protease, falcipain-2 was exhibited by three of the tetromycin compounds namely, Pol001-4, Pol001-5 and Pol001-7 at 91.2, 92.9 and 81.4 % inhibition, respectively. Furthermore, only two compounds showed rhodesain inhibition: Pol001-4 (79.9%) and Pol001-5 (77.5%). Inhibition against the mammalian protease, cathepsin B, was found to be exhibited by the compounds Pol001-3 (70.6%), Pol001-5 (77.9%) and Pol001-7 (79.2%), while Pol001-5 was the only compound that inhibited cathepsin L at 72.7% and SARS-PI^{pro} at 73.4%. Interestingly, the novel tetromycin derivative Pol001-4 displayed specific inhibition against only the parasite proteases, falcipain-2 and rhodesain. On the other hand, the compounds Pol001-1, cebulactam A1 and A2, valinomycin and staurosporine did not show any inhibition against a single protease in these assays. The compound cycloisoleucylprolyl was not tested due to the lack of material.

Table 3.12 Percent inhibition values* (100 μ M) against different proteases

Compound	Falcipain -2	Rhodesain	SARS PI ^{pro}	SARS M ^{pro}	Cathepsin B	Cathepsin L
Cebulactam A1	51.7	8.3	3.8	5.5	5.8	7.5
Cebulactam A2	51.7	15.0	20.5	10.9	8.3	ni
Cebulactam A1+A2	53.7	17.2	9.4	3.3	7.8	ni
Pol001-1	nd	7.1	16.4	4.8	68.9	nd
Pol001-3	50.7	43.8	34.5	ni	70.6	50.3
Pol001-4	91.2	79.9	59.6	8.2	67.5	62.9
Pol001-5	92.9	77.5	73.4	9.5	77.9	72.7
Pol001-7	81.4	53.6	32.1	9.5	79.2	37.8
Valinomycin	nd	13.6	ni	ni	4.9	ni
Butenolide	nd	ni	2.6	ni	2.5	2.0

*Mean values of two independent assays; nd = not determined; ni = no inhibition

3.3 Discussion

The actinomycetes isolated in this study were found to produce diverse compounds with interesting chemistry and biological activities. The strains belonging to the new actinomycete taxa namely, *Saccharopolyspora cebuensis* strain SPE 10-1^T and *Streptomyces axinellae* strain Pol001^T produced the novel secondary metabolites, cebulactams and the tetromycin family of compounds including novel derivatives, respectively. These results clearly indicate that the cultivation of novel actinomycetes proves to be a good strategy for isolating new chemical entities and minimizes the exhaustive effort of re-isolating known compounds. The cebulactams A1 and A2 represent constitutionally identical macrolactams with the unique cyclic ether connecting the aromatic ring system to the highly functionalized, polyketide-derived carbon chain (Pimentel-Elardo et al. 2008). There are structurally related secondary metabolites that resemble these macrolactams, but are lacking the characteristic cyclic ether. Examples are the anti-oxidative compounds from *Pseudonocardia* Q-1047 isolated from a soil sample from the Zamami Islands, Okinawa Prefecture, Japan (Imai et al. 1989a; Imai et al. 1989b; Yazawa et al. 1990) and the ansamycin antibiotics geldanamycin (Rascher et al. 2003) and herbimycin (Rascher et al. 2005) isolated from *Streptomyces hygroscopicus*. Despite the fact that the cebulactam polyketides produced by *Saccharopolyspora cebuensis* sp. strain SPE10-1^T are structurally related to the ansamycin antibiotics and that the crude extract from which these polyketides were isolated showed preliminary activity, these compounds, still did not exhibit any bioactivities using the different anti-infective screening panel used in this study. The question of synergistic action of the two compounds has also been addressed since a cocktail of these substances also did not show any activity. In contrast to the other structurally related bioactive metabolites, the aromatic ring via the cyclic ether in the cebulactams might possibly be protected from oxidation, preventing the formation of the benzoquinone moiety which appears to be the putative bioactive part of the molecule. In this regard, conversion of the compounds to its oxidized form (i.e. benzophenonic) has been attempted (T.A.M Gulder, University of Würzburg) but the testing of the resulting oxidation products also did not yield bioactivities. Therefore, this semi-synthetic work warrants further investigation to improve the bioactivity potential of the compounds.

The compounds isolated from *Streptomyces axinellae* strain Pol001^T exhibited structural resemblance to tetromycins isolated from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989) with the exception of the novel derivatives Pol001-3 and Pol001-4. The compounds reported in this Japanese patent were shown to exhibit activity against

methicillin-resistant *Staphylococcus aureus*. However, the compounds are reported in this study to have a wider spectrum of activity that includes anti-parasitic and protease inhibition activities. Moreover, it is worthwhile to examine whether *Streptomyces axinellae* strain Pol001^T as well as *Saccharopolyspora cebuensis* sp. strain SPE10-1^T produce other compounds when cultivated under different conditions given the fact that these strains belong to novel actinomycete taxa and as shown in this PhD study, have a great potential of producing new secondary metabolites.

Additional interesting yet previously known compounds were also isolated in this Ph.D. study from the different actinomycete strains namely, valinomycin, staurosporine, cycloisoleucylprolyl and butenolide. Valinomycin was isolated from *Streptomyces* sp. strains 34 and 22 cultivated from the Mediterranean sponges *Axinella polypoides* and *Aplysina aerophoba*. This cyclodepsipeptide was originally recovered from various soil-derived actinomycetes: *Streptomyces fulvissimus* (Brockmann and Schmidt-Kastner 1955; Brown et al. 1962), *Streptomyces roseochromogenes* (Patterson and Wright 1970) and *Streptomyces griseus* var. *flexipartum* (Heisey et al. 1988). To date, this is the first report of valinomycin isolated from a marine organism. This cyclic depsipeptide is an oligomer similar to peptides but in which some of the amino acids are replaced by hydroxyl acids, so that amide and ester bonds are present along the chain. Furthermore, valinomycin is structurally similar to onchidin B isolated from a mollusc *Onchidium* sp. (Fernández et al. 1996) and montanastatin, an antineoplastic compound from a soil-derived *Streptomyces annulatus* (Pettit et al. 1999) possessing a cyclic structure with polar groups oriented toward the central cavity, whereas the rest of the molecule is relatively nonpolar. The structure behaves as an ionophore that modulates transport of ions such as potassium across biological membranes (Haynes et al. 1969). It is for this reason that several studies have reported on the chemical synthesis of valinomycin and its analogs (Fonina et al. 1971; Smith et al. 1975; Vinogradova et al. 1975; Kuisle et al. 1999). Valinomycin has also been reported to exhibit other biological activities such as insecticidal (Patterson and Wright 1970; Heisey et al. 1988), nematocidal (Patterson and Wright 1970) and antifungal (Park et al. 2008). In this Ph.D. study, valinomycin is found to exhibit not only antifungal activity but also anti-parasitic activities thus extending the pharmacological value of this known compound.

Another interesting compound isolated in this study is staurosporine from *Streptomyces* sp. strain 11 cultivated from the sponge *Tedania* sp. which apparently has been identified from various sources. This indolocarbazole alkaloid was first isolated from *Streptomyces staurosporeus* Awaya AM-2282 (Omura et al. 1977) and subsequently from various

terrestrial actinomycetes: *Streptomyces actuosus* (Morioka et al. 1985), *Streptomyces* sp. strain M-193 (Oka et al. 1986), *Streptomyces platensis* subsp. *malvinus* RK-1409 (Osada et al. 1992), *Streptomyces* sp. AB 1869R-359 (McAlpine et al. 1994) and *Streptomyces longisporoflavus* (Cai et al. 1995; Cai et al. 1996). Interestingly, staurosporine and its derivatives have been reported to be produced by the marine ascidian *Eudistoma toetalensis* and its predatory flatworm *Pseudoceros* sp. (Schupp et al. 1999; Schupp et al. 2002). Furthermore, staurosporine and its derivatives have aroused considerable interest as these compounds exhibit strong inhibitory activities against protein kinase C (Tamaoki et al. 1986) as well as inhibition of platelet aggregation (Oka et al. 1986), blocking of growth phases in cancer cells (Beltran et al. 1997) and reversal of multidrug resistance (Utz et al. 1998). Additionally, staurosporine was found in this Ph.D. study to exhibit significant inhibitory activity against the parasites *Leishmania major* and *Trypanosoma brucei* which has not been previously reported for this compound.

Another compound isolated in this Ph.D. study is cycloisoleucylprolyl from *Streptomyces* sp. strain A188 cultivated from the Mediterranean sponge *Aplysina aerophoba*. This diketopiperazine has also been previously isolated from the Caribbean sponge *Calyx* cf. *podatypa* (Adamczeski et al. 1995) and a structurally similar diketopiperazine from *Pseudomonas aeruginosa* isolated from an Antarctic sponge *Isodictya setifera* (Jayatilake et al. 1996). Diketopiperazines are the smallest peptides known, commonly biosynthesized from amino acids by different organisms and are considered to be secondary functional metabolites or side products of terminal peptide cleavage (Martins and Carvalho 2007). It is common to find diketopiperazines in fermentation mixtures as microorganisms are capable of protein hydrolysis and that a certain proportion of diketopiperazines present may arise during the fermentation to replace or supplement the quantity supplied in the medium (Mitscher et al. 1963). These compounds were in fact found to be produced by the majority of the strains cultivated in this study.

Furthermore, butenolide was isolated from *Streptomyces* sp. strain T03 from *Tethya* sp. sponge and exhibited significant activity against *Trypanosoma brucei*. This lactone-containing metabolite has also been previously isolated from a marine sediment-derived *Streptomyces* sp. strain M027750 (Cho et al. 2001). Butenolides are a family of α , -unsaturated lactones often encountered among fungi, bacteria and gorgonians (Rodriguez and Ramirez 1994; Braun et al. 1995; Smith et al. 2000). Their saturated analogues act as signaling substances in bacteria and enhance spore formation of *Streptomyces* sp. or induce metabolite formation (Mukku et al. 2000).

Bioassay data of the secondary metabolites isolated in this study revealed that the majority of the compounds exhibited significant anti-parasitic and protease inhibitory activities. The specificity of the various screening panels employed here provides further insights into the mechanisms of action against particular pathogens and underscores the anti-infective potential of the compounds. The parasites *Leishmania major* and *Trypanosoma brucei* are clinically important pathogens as these are known to cause leishmaniasis and the African trypanosomiasis, respectively (Fenwick 2006; Caffrey et al. 2007). Furthermore, cysteine proteases play pivotal roles in the growth, differentiation and pathogenicity of various pathogens (Otto and Schirmeister 1997; Mahmoudzadeh-Niknam and McKerrow 2004). Therefore, inhibition of cysteine proteases presents a promising strategy for combating infection. For example, the enzyme rhodesain in *Trypanosoma brucei rhodesiense* has been the target in developing new anti-trypanosomal drugs. Furthermore, proteases of *Plasmodium falciparum* parasites play important roles in the processes of host erythrocyte rupture, erythrocyte invasion and hemoglobin degradation (Rosenthal 2004). Treatment with cysteine protease falcipain inhibitors blocks hemoglobin hydrolysis and development of the parasite (Schulz et al. 2007). Additionally, the coronavirus SARS Co-V is another important pathogen causing the severe acute respiratory syndrome (SARS) which was responsible for the severe epidemic in 2002-2003 with more than 800 reported deaths worldwide (Peiris et al. 2003). Coronaviruses are plus-strand RNA viruses where the genome RNA is translated to produce two large replicase polyproteins that are autocatalytically cleaved by viral proteases (Kaeppeler et al. 2005). The coronavirus main protease M^{pro} is the key enzyme in the proteolytic process and is therefore also considered as an attractive target for new antiviral drugs against SARS and other coronavirus infections (Anand et al. 2003).

Therefore, the novelty of the chemical structures as well as the bioactivities exhibited by the different compounds isolated in this study clearly shows the potential of marine sponge-associated actinomycetes to produce pharmacologically important secondary metabolites. Expanding the anti-infective screening panel to include more clinically relevant pathogens and improving the bioactivity of the compounds by structure-activity studies are thus worthwhile pursuing.

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