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Assessment of the antibiotic production potential of marine-derived actinomycetes via bio-activity screening and targeted genetic analysis

Thesis for the degree of Philosophiae Doctor

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Kerstin Engelhardt

ABSTRACT

Drug discovery from marine organisms is gaining momentum and research focusing on marine actinomycete diversity has yielded numerous novel secondary metabolites with unique chemical structures.

This study was based on targeted analyses of marine actinobacteria selected from a library of ca. 10.000 isolates cultivated from sediment- and sponge-samples in the Trondheim fjord, Norway. The two small sub-collections used in this study consisted of 35 shallow-water sediment-derived actinomycetes ("sub-library I") and 27 deep-water sediment- and sponge-derived actinobacterial isolates ("sub-library II"). The isolates in these collections were analyzed for: (i) the influence of seawater on growth; (ii) their phylogenetic diversity based on molecular taxonomy, and (iii) their antibiotic production potential.

The presence of seawater had different effects on the growth of the studied isolates. A preference for seawater or an obligatory requirement indicated the presence of indigenous marine actinomycetes in sub-library I and was used as a criterion for selection of isolates in sub-library II. The preliminary classification of isolates was performed by 16S rRNA gene sequence analysis which revealed significant biodiversity. Representatives of 11 different actinomycete genera were identified in sub-library I and members of 8 genera confirmed in sub-library II. The results of a PCR screening for polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes demonstrated the genetic potential to produce secondary metabolites with polyketide and/or non-ribosomal peptide backbones for the majority of isolates in both sub-libraries.

Whole-cell based antimicrobial assays involving microtitre cultivations and multiple fermentation media were performed using isolates of sub-library II with the objective to find producers of new antibacterial or antifungal compounds. Extracts with antibacterial or antifungal activity were identified for more than 50% of the isolates subjected to this

screening. Nine isolates produced compounds with activity against multi-resistant bacterial and/or fungal strains.

Fermentation extracts of isolate *Nocardiopsis* sp. TFS65-07 from sub-library II displayed high activity against vancomycin-resistant *Enterococcus faecium*. The active antibacterial compound was purified from fermentation extracts of TFS65-07 and structure elucidation identified it as a new member of the thiopeptide antibiotic family. The gene cluster for biosynthesis of the new thiopeptide, TP-1161, was identified by mining a draft genome sequence obtained for the producing organism. The proposed identity of the cluster was confirmed by gene inactivation experiments. Bioinformatics analyses of genes constituting the cluster and their products allowed proposition of the biosynthetic pathway for TP-1161. A cosmid containing the TP-1161 cluster (*tpa*) was isolated from a genomic cosmid library constructed for TFS65-07 and used for further studies of *tpa* gene functions. For heterologous expression, the cosmid was modified inserting the *Φ*C31 integration function and a selective marker, and successfully introduced into the genome of *Streptomyces coelicol*or M512. Heterologous production of TP-1161, however, could not be detected in M512. Further studies involving e.g. alternative hosts could pave the way for a systematic functional analysis of all genes involved in the TP-1161 biosynthesis.

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PAPER II

Engelhardt, K., K. F. Degnes, M. Kemmler, H. Bredholt, E. Fjærvik, G. Klinkenberg, H. Sletta, T. E. Ellingsen, S. B. Zotchev. 2010. Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. *Applied and Environmental Microbiology* **76**: 4969-4976.

PAPER III

Engelhardt, K., K. F. Degnes, S. B. Zotchev. 2010. Isolation and characterization of the biosynthetic gene cluster for thiopeptide antibiotic TP-1161. (Submitted to *Applied and Environmental Microbiology*).

ABBREVIATIONS

3D	Three-dimensional
aa	aminoacid(s)
aa-tRNA	aminoacyl tRNA
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
bp	Base pair(s)
C	Cytosine
DAD	Diode array detector
dha	Dehydroalanine
dhb	Dehydrobutyrine
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
ESI	Electrospray ionization
FMN	Flavin mononucleotide
G	Guanine
GC	Gas chromatography
GST	Genome sequence tag
GTP	Guanosine triphosphate
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
HTS	High-throughput screening
kb	Kilo base pairs
LC-MS	Liquid chromatography – mass spectrometry
m	meter(s)
Mb	Mega base pairs
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
nt	Nucleotide(s)
orf	open reading frame
OSMAC	One strain many compounds
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PTC	Peptidyl transferase center
QSAR/QSPR	Quantitative structure-activity/property relationships

ABBREVIATIONS

RDP	Ribosomal database project
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
TOF	Time-of-flight
tRNA	Transfer RNA
USD	United states dollar
UV	Ultraviolet
VRE	Vancomycin-resistant *Enterococcus*

1 INTRODUCTION

1.1 Antibiotics – a brief historic overview

The modern era of antibiotic chemotherapy started with introduction of penicillin into clinical practice in the 1940s (65) for treatment of infectious diseases in humans. Penicillin was first discovered in 1928 as a secondary metabolite derived from the common mold *Penicllium notatum*. The discovery of gramicidin in 1939 (29) - the first bactericidal agent isolated from a soil-derived bacterium (*Bacillus brevi*s) - initiated the systematic isolation and screening of soil bacteria and fungi for new antibiotics. The discovery of streptomycin in 1943 (94) put bacteria belonging to the order *Actinomycetales* – the actinomycetes – in the limelight of antibiotic drug discovery. Streptomycin was the first compound of a plethora of bioactive secondary metabolites derived from members of the genus *Streptomyces*, which would become established as the most prolific bacterial antibiotic producers delivering the majority of antibiotic drugs still in use today (11).

In the course of the 1940s and 1950s, most of the today known antibiotic classes, including the β-lactams, tetracyclines, aminoglycosides and macrolides, were discovered and introduced into the clinic (**Figure 1.1**) (61). With the discovery of the streptogramins and quinolones in 1962 this “golden era” of antibiotic drug discovery from microbes came to a halt. While the number of new compounds still increased, due to production of analogs by chemical derivatization of already known molecules, no genuinely new antibiotic class with novel mode of action was licensed during the following three decades (1970-2000) (11). It was only in 2000-2003 that two molecules belonging to novel antibiotic classes were launched, the chemically synthesized linezolid (oxazolidinone) and the bacterial secondary metabolite daptomycin (cyclic lipopeptide) (85).

Figure 1.1. An arms race: the introduction of new antibiotic classes and emergence of bacterial resistance (adapted from 36, 81).

While the original definition of antibiotics by the soil microbiologist Selman Waksman, the discoverer of streptomycin, was strictly limited to microorganism-derived low molecular metabolites inhibiting or destroying other microorganisms, contemporary use of the term also includes agents derived from other natural sources or compounds of synthetic or semi-synthetic origin (60).

1.2 Antibiotic resistance and the urgent need for new drugs

The results of a clinical study of 122 consecutive patients with systemic *Staphylococcus aureus* infection published in 1941 - just before the introduction of penicillin – documented 82% mortality, and of the 40 patients who were older than 40 years only one recovered (102). The introduction of antibiotic therapy has had a tremendous impact on human life expectancy and quality. Bacteria-caused infections such as pneumonia, typhus, plague, cholera, syphilis and tuberculosis, once being among the leading causes of death around the world, could now effectively be treated with the new drugs available.

With widespread and often unwarranted use of antibiotics a new threat began to arise in form of the emergence and spread of bacteria resistant to commonly used antibiotics (**Figure 1.1**). While antibiotic resistance determinants are a naturally occurring biological phenomenon, especially in antibiotic producers where self-resistance mechanisms usually pre-exist coupled to antibiotic biosynthesis pathways, antibiotic use favors the spread of resistance mechanisms throughout and across bacterial populations (46). Inadequate use, especially the widespread, mostly non-therapeutic application of antibiotics (as growth promotants or for prophylactic purposes) in intensive animal husbandry, is likely to have had a considerable impact on the pace of resistance development (46).

Antibiotics exert selective pressure on a microbial population, allowing the survival of only those cells, which have or have acquired resistance to them. The first line of defense is the bacterial cell envelope which, especially in Gram-negative bacteria

possessing an additional outer membrane, can provide a physical barrier preventing antibiotic molecules from entering the cell (116). Other bacterial resistance mechanisms can include active efflux or decreased uptake of antibiotics, enzymatic inactivation of the antibiotic molecule, modification of the cellular target (e.g. by mutation or enzyme-mediated decoration), overproduction of the target or bypass of the targeted metabolic pathway (101). Resistance determinants can be readily acquired by horizontal transfer of mobile genetic elements such as plasmids, bacteriophages, transposons, gene cassettes and integrons through transduction, transformation and conjugation processes (9).

However, back in the golden era of antibiotic discovery, the advent of penicillin-resistant *Staphylococcus aureus* strains only a few years after introduction of penicillin (103) was met with little concern due to the steady supply of new drugs and the development of a plethora of improved analogs of already known molecules. But also the introduction of semi-synthetic analogs such as the penicillin-derived methicillin in the 1960s, which had been developed to confront β-lactamase producing resistant pathogens, was soon followed by the emergence of methicillin-resistant staphylococci (**Figure 1.1**) (50). Today, a host of "bad bugs" including methicillin-resistant *Staph. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), and fluoroquinolone-resistant *Pseudomonas aeruginosa* species (16) have taken over hospital and community settings and with emerging pandrug-resistant bacteria (resistant to all available antibiotics) therapeutic options are often set back to pre-antibiotic times with no effective therapy available (70).

Although antibiotic resistance has been a problem ever since their introduction to human therapy, it is the increase in number, diversity and range of resistant microbes that causes the precarious current situation. The spectrum of human pathogens has dramatically changed and previous commensals like *Staph. aureus*, enterococci and *Escherichia coli* have become potent pathogens through acquisition of multiple antibiotic resistances and simultaneous increase in virulence (2). A whole new generation of opportunistic pathogens such as pandrug-resistant Gram-negative strains of *Acinetobacter baumannii, P.aeruginosa, Stenotrophomonas maltophilia* and

Burkholderia cepacia are now threatening an ever growing population of susceptible patients with compromised immune systems (116).

Despite the alarming development, the list of new antibacterials with novel modes of action is short. Since 2006, only one new antibacterial agent (doripenem) has been approved for therapy in USA and the number of potential candidates in clinical trials, with only three compounds in advanced development, is very meager (16).

1.2.1 Sources for new drugs

Modern drug discovery is based on three major sources: natural products, semi-synthetic derivatives of natural products and synthetic compounds derived from combinatorial chemistry libraries. The vast majority of clinically relevant antibiotics in use today are natural products or compounds derived thereof. Over the past 20 years, 60% of the 877 drugs that have reached the market were natural products (61). Despite these numbers, most major pharmaceutical companies have diverted their drug discovery programs away from natural product-based research. The availability of high-throughput-screening (HTS) technology in form of automation and robotization in the 1990s in addition to high expectations from identifying new molecular targets from the rising number of bacterial genome sequences, caused a shift away from natural product-based research efforts in favor of establishing HTSs of combinatorial libraries of synthetic compounds (85).

Developed as an alternative to the time- and resource-heavy process of empirical natural product-based drug discovery, *in silico* (computational) approaches have become an emerging trend in lead identification and optimization. *In silico* approaches are based on virtual screening of virtual or existing chemical structure libraries to identify potential drug leads which are then chemically synthesized and subjected to a biological screening (52).

Commonly used computational approaches in drug design and development include (i) ligand-based design (pharmacophore modeling and 3D similarity searches), (ii) structure (target)-based design ("docking") and (iii) quantitative structure-activity/property relationships (QSAR/QSPR) for e.g. computational predictive toxicology (52). Both, ligand-based screening and structure (target-) based design are based on previous knowledge about active ligands and/or three-dimensional structure information of target receptors (e.g. x-ray crystallographic or NMR data) (52). In the process of "docking", libraries of potential ligands are generated *in silico*, selected for optimal positioning in the receptor binding site and scored for potential activity. Top-scoring compounds can then be synthesized and evaluated by experimental testing (51). Successful examples of this approach include the discovery of novel DNA gyrase inhibitors using 3D structural information of the targeted ATP binding site (15). While target-based approaches are based on the principle of complementarity, ligand-based screening approaches rely on similarity (69). Ligand-based virtual screenings rank potential novel ligands based on 3D similarity searches or on pharmacophore pattern matching (87).

1.2.2 Natural products vs. chemical synthesis

Payne et al. described in a review from 2007 the outcome of a seven-year antibacterial drug discovery program run at GlaxoSmithKline. The program consisted of a genomics-derived, target-based screening of synthetic compound libraries aiming at finding new classes of antibiotics with preferably novel modes of action (89). In the course of the screening more than 300 genes representing potential new antibacterial targets were evaluated and promising candidates investigated in 70 HTS campaigns. The outcome of the biochemical screenings of individual bacterial targets as well as additionally set-up whole-cell antibacterial screenings were described as "disappointing" with "unsustainably low" success rates (89). The authors concluded that screening approaches based on synthetic libraries and isolated biological targets are inadequate for the discovery of novel antibacterial leads (89).

Natural products have several advantages over compounds derived from combinatorial synthesis approaches. While synthetic molecules can be rapidly generated by combinatorial chemistry from sets of smaller chemical entities, their structural diversity is limited, often due to lack of chiral centers, aromatic rings, oxygen-containing substituents and structural rigidity (31). The diversity of natural products on the other hand can provide physicochemical properties such as specific interactions with often multiple biological targets that are hardly found in molecules derived from combinatorial synthesis (84). As metabolic products of living organisms, natural product-derived antibiotics have the genuine advantage of having evolved to be active in target cells *in vivo*, abandoning a major obstacle of target-based approaches – the conversion of *in vitro* hits into whole-cell active leads (6).

However, natural product-based drug discovery is a complex, time-consuming and expensive endeavor. Compared to drugs for long-term treatment of chronic illnesses, antibiotics (especially antibacterials) do not present commercial incentives to the pharmaceutical industry (84). It is estimated that the entire process from the discovery of a new drug to the development, pre-clinical research to clinical trials takes on average more than a decade with costs exceeding 800 million USD (28). High investments, short therapy times and the limited lifespan of antibiotics due to emerging resistances have led to decreased or abandoned antibacterial drug discovery efforts in most of the major pharmaceutical companies, leaving the field mainly to academia and smaller biotechnology firms (84).

1.3 Actinomycetes – major antibiotic producers

Traditionally, it has been mainly terrestrial bacteria and predominantly Gram-positive bacteria of the order *Actinomycetales*, the actinomycetes, that have been the focus of extensive screening efforts for new bioactive natural products. Actinomycetes have been the source for numerous important therapeutic drugs including antibacterials, antifungals, neostatics, antiparasitics and immunosuppressants and represent undoubtedly the most prolific antibiotic producers. 45% of all known bioactive natural

products are produced by actinomycetes (11), including more than 50% of all microbial antibiotics discovered so far. Members of the genus *Streptomyces* account for 70-80% (11) of the about 3000 currently characterized actinomycete antibiotics (111). However, the occurrence of these bacteria is by far not limited to soil environments. Actinomycetes are ubiquitous organisms with wide physiological and morphological diversity and have been isolated from all kinds of terrestrial and aqueous habitats where they can exist as free-living bacteria as well as pathogens (96) or in symbiotic associations with plants (10) and insects (25, 92) or as endophytes (7).

The development of specific enrichment and cultivation techniques as well as logistical and technological advances (e.g. unmanned submersibles) have extended sampling ranges and studies of actinomycetes to previously inaccessible locations such as the deep-sea ocean environments (**Table 1.1**) (88). It is also interesting to note that despite extensive screening of terrestrial isolates, soil samples have still only been taken from a minute fraction of the surface of the entire globe (6). Consequently, the taxonomic diversity even within the well-studied group of terrestrial actinomycetes is likely to be far from exhausted (111).

1.3.1 Marine actinomycetes – a promising source for new drug discovery

Investigations of marine micro- and macroorganisms such as mollusks, bryozoans, sponges and bacteria have yielded numerous pharmacologically interesting compounds during the last years, especially in the group of anticancer drugs (79). The first “drugs from the deep” included ziconotide, a peptide isolated from a tropical cone snail and launched in 2004 as “Prialt” for treatment of chronic pain, and the antitumor compound trabectedin originating from a tropical sea-squirt and approved by the EU in 2007 (79). Marine macroorganisms clearly represent a rich source for novel bioactive molecules (13), however the development of new pharmaceuticals relies on a sustainable supply with sufficient quantities of potential drug leads. Low abundance of the natural producer and/or low concentrations of the molecule of interest (as it is usually the case for marine macroorganisms) are therefore problematic. Bacteria, on the other hand, are a much

more amenable source for drug discovery and upscaled biotechnological production of interesting compounds (41). Furthermore, it has been demonstrated that many compounds which had been originally derived from marine macroorganisms were in reality produced by their bacterial symbionts (79).

The most productive marine bacterial phyla are represented by members of the Cyanobacteria and Actinobacteria; contributing 220 and 256 compounds, respectively, to the 659 marine bacterial compounds described over a 10 years period up to 2008 (114). As a response to increasing re-discovery rates of already known actinomycetes and their antibiotics in soil-derived libraries, isolation efforts were increasingly focused on actinobacterial diversity in the marine environment (66, 75). Although the oceans represent a largely untapped resource for biodiversity, the cultivation of marine-derived bacteria still represents a considerable challenge. While it is estimated that less than 0.3% of soil bacteria can be cultivated in common media, this fraction drops to 0.00001% for water-associated microorganisms (3).

Actinomycetes have been isolated from the most diverse marine habitats ranging from the sea-surface microlayer (39), over shallow-water sediments (17, 46) to extreme environments such as deep sea sediments (88) and hydrothermal vents (106) (**Table 1.1**). They have been found associated with marine micro- and macrofauna, marine invertebrates like sponges and vertebrates including fishes (109). The origin of marine populations of actinomycetes had long been disputed. Terrestrial isolates are known to produce resistant spores which can be washed into the seas where they would stay viable for years, and thus it had been suggested that marine actinomycete isolates are a mere result of terrestrial contamination (38).

Table 1.1. Examples of "rare" marine actinomycete genera identified by culture and molecular techniques in different marine habitats (adapted from 109).

Actinomycete genus	Species affiliation	Source
Actinomadura	*A. formosans*, *A. fulvescens*	Japan Trench, Canary Basin, fjord site. Sub-tropical sediment
Actinosynnema	*Actinosynnema* sp. IM-1402	Deep sea sediment 3800 m
Brachybacterium	*B. arcticum*	Barcelona neuston
Dietzia	*D. maris*	Japan Trench, Canary Basin, fjord site. Deep sea sediment 3800 m. Barcelona neuston
Frigoribacterium	*Frigoribacterium* sp. 301	Deep sea sediment 3800 m
Geodermatophilus	*Geodermatophilus* sp. BC509, IM-1092	Deep sea sediment 3800 m
Kineococcus-like	*Kineococcus*-like AS2978	Deep sea sediment 3800 m
Kitasatospora	*Kitasatospora* sp. IM-6832	Deep sea sediment 3800 m
Microbacterium	*M. kitamiense*, *M. esteraromaticum*	Japan Trench, Canary Basin, fjord site. Barcelona neuston. Wadden Sea aggregate
Nocardioides	*Nocardioides* sp. V4.BO.15, *N. jensenii*	Deep sea sediment 3800 m. Barcelona neuston
Nocardiopsis	*N. dassonvillei*	Ovaries of Pufferfish, Bohai Sea of China
Pseudonocardia	*P. alaniniphila*, *P. aurantiaca*, *P. alnii*	Deep sea sediment 3800 m
Rhodococcus	*R. fascians*, *R. koreensis*, *R. opacus*, *R. ruber*, *R. tsukamurensis*, *R. zopfii*	Deep sea sediment 3800 m, Pelagic clay
Salinispora	*S. arenicola*, *S. tropica*	Sub-tropical sediment
Serinicoccus	*S. marinus*	Sea water East Sea, Korea
Tsukamurella	*T. inchonensis*	Deep sea sediment 3800 m
Turicella	*T. otitidis*	Deep sea sediment 3800 m
Verrucosispora	*Verrucosispora* sp. AB-18-032, IM-6907	Japan Trench, Canary Basin and fjord site
Williamsia	*W. maris*, *W. marianensis*	Japan Trench, Canary Basin and fjord site

Numerous isolations and characterization of marine-derived actinomycetes, however, have proved that indigenous marine actinobacterial species indeed exist, and new genera or groups of marine actinomycetes have been described for more than six different families within the order *Actinomycetales* (33). The first marine actinomycete, *Rhodococcus marinonascens*, was described in 1984 (44) and in 2005 the first seawater-dependent marine actinomycete, *Salinispora*, was reported (67). It has been shown that marine actinomycete species are physiologically and phylogenetically distinct from their terrestrial relatives, they can possess specific adaptations to the ocean environment (e.g. to high salinity and pressure) and were found to represent a rich source for novel, chemically diverse bioactive secondary metabolites with potential applications in antimicrobial and anticancer therapy (18, 19, 49, 68, 109). The salinisporamides (32, 93, 115), abyssomicins (94), proximicins (34) and marinomycins (58) are amongst a variety of new marine-actinomycete derived products with significant biological activity and potential applications as anticancer and antibacterial drugs (see **Table 1.2**).

Table 1.2. Novel metabolites produced by marine actinomycetes discovered during the period 2003–2005 ((adapted from 59)).

Compound	Actinomycete producer	Biological activity
Abyssomicins	*Verrucosispora* sp.	Antibacterial
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal
Caprolactones	*Streptomyces* sp.	Anticancer
Chandrananimycins	*Actinomadura* sp.	Antialgal; antibacterial; anticancer; antifungal
Chinikomycins	*Streptomyces* sp.	Anticancer
Chloro-dihydroquinones	*Novel actinomycete*	Antibacterial; anticancer
Diazepinomicin (ECO-4601)	*Micromonosproa* sp.	Antibacterial; anticancer; anti-inflammatory
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutingimycin	*Streptomyces* sp.	Antibacterial
Helquinoline	*Janibacter limosus*	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
IB-00208	*Actinomadura* sp.	Anticancer
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	'*Marinispora*'	Antibacterial; anticancer
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer
MKN-349A	*Nocardiopsis* sp.	Unknown biological activity
Salinosporamide A (NPI-0052)	*Salinispora tropica*	Anticancer
Sporolides	*Salinispora tropica*	Unknown biological activity
Trioxacarcins	*Streptomyces* sp.	Antibacterial; anticancer; antimalarial

1.3.2 Bioprospecting of marine actinomycetes for new antibiotics

Prospecting the natural biodiversity – bioprospecting - aims to discover new natural products, genes or proteins with biomedical or biotechnological application potential. Nearly all antibiotics have been discovered in empirical approaches using simple antagonistic assays against different bacteria by assessing the inhibition of growth of different target organisms by whole broths or extracts of microbial fermentations (101). Despite major technical advances in instrumentation and automation, activity-based drug discovery is essentially very similar to the screening approaches used by Fleming and others in the very beginning of microbial drug discovery (101). A scheme showing bioprospecting for antibiotics in marine-derived actinomycete bacteria is outlined in **Figure 1.2**.

Many different methods to increase the efficiency of each of the described steps have been developed. New cultivation techniques have improved culturability and isolation of previously undescribed bacterial species in pure culture. Examples include high-throughput cultivation under high dilution-to-extinction culturing conditions for recovery of slow-growing bacterial strains (23) or microencapsulation of single cells combined with flow cytometry (119). To increase the efficiency of fermentation extraction and to stabilize produced secondary metabolites, cultivation can be performed with resins and early mass spectral data and structural information - crucial for dereplication and identification of potentially novel compounds - can be obtained using liquid chromatography (LC) coupled with MS, UV and NMR analyses (78).

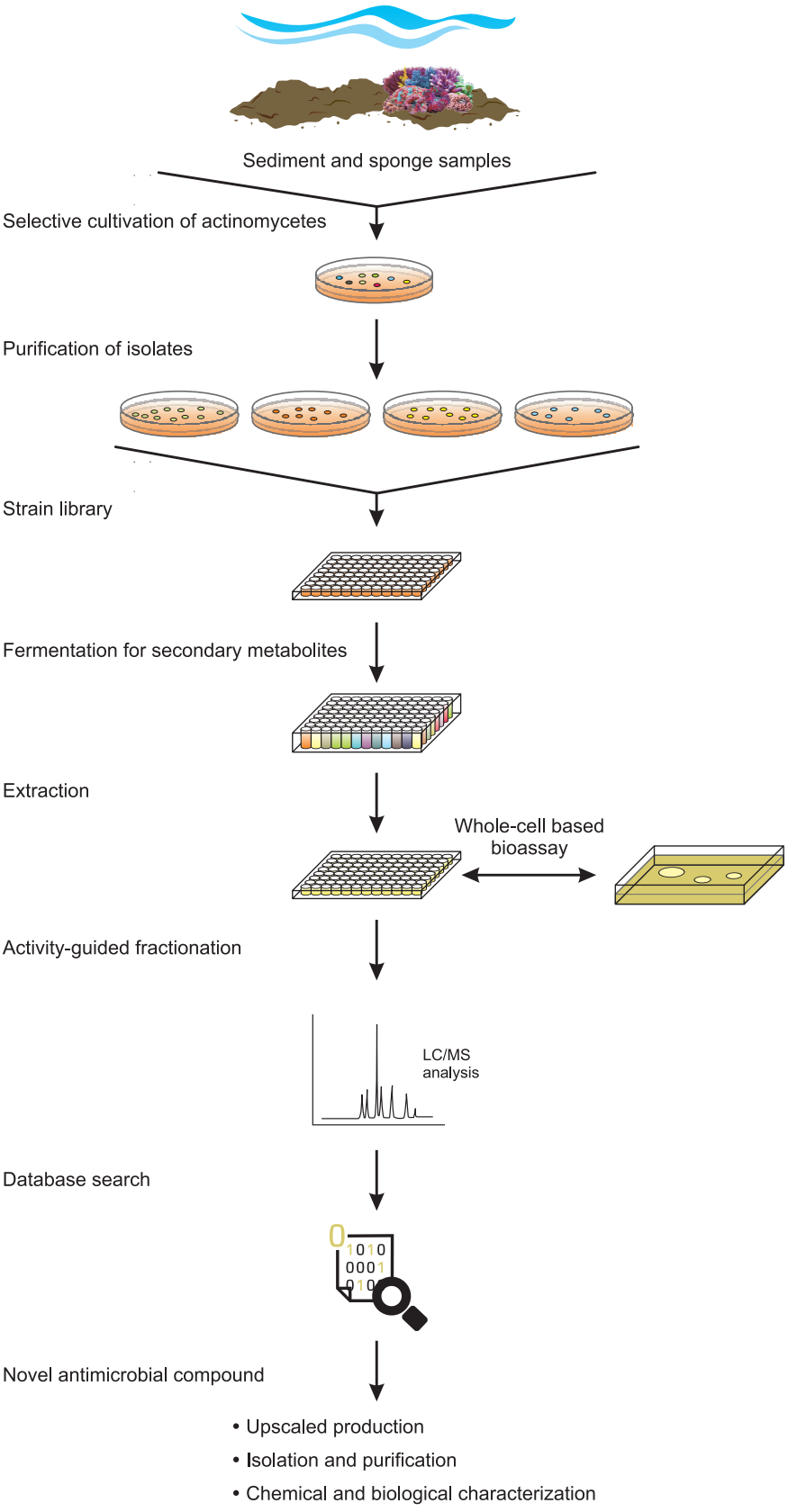

Figure 1.2. Bioprospecting of marine actinomycetes for new antimicrobials.

1.4 The influence of microbial genomics on drug discovery

The development of new sequencing technologies (73) has led to plummeting sequencing costs and bacterial genome sequencing and mining is becoming a common tool in drug discovery. More than 1000 completed bacterial genome sequences are now available at the NCBI Genome Database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) and advances in understanding microbial genetics and processes involved in secondary metabolite biosynthesis have made it possible to identify and analyze numerous gene clusters for biosynthesis of new bioactive compounds, including antibiotics.

Bacteria with larger genomes have turned out to be an especially rich source for secondary metabolite pathways. A comparison of 115 completed prokaryotic genome sequences revealed a disproportionate increase in genes for secondary metabolism and regulation in large genomes compared to medium- and small-sized genomes (56) and this trend is especially obvious in the actinomycetes. Genome sequencing efforts have yielded to date more than 70 completed and annotated actinobacterial genome sequences, the majority of which represent members of the *Actinomycetales* and it has been shown that the most prolific secondary metabolite producers generally have rather large genomes (>5 Mb) (82). Members of families such as the *Streptomycetaceae, Micromonosporaceae* and *Pseudonocardiaceae* often possess more than 20 gene clusters for secondary metabolite biosynthesis yielding a diverse array of natural products including polyketides, non-ribosomal peptides, bacteriocins and lantibiotics (82).

Table 1.3. Actinomycete species with completed genome sequences and predicted number of secondary metabolite gene clusters (82).

Actinomycete species	Genome size (chromosome)	Number of predicted clusters	Number of orphan pathways
Streptomyces coelicolor A3(2)	8667507	29	14
Streptomyces avermitilis MA-4680	9025608	37	24
Streptomyces griseus IFO 13350	8546929	36	27
Saccharopolyspora erythraea NRRL2338	8212805	27	24
Salinispora tropica CNB-440	5183331	19	14
Salinispora arenicola CNS-205	5786361	30	25

The complete 9 Mb genome of *Streptomyces avermitilis* was published in 2003 (47). The strain was known for production of the polyketide macrolides avermectin and oligomycin, and the genome sequence revealed a total of 37 secondary metabolite gene clusters, including 13 PKS (polyketide synthase), 8 NRPS (non-ribosomal synthetase), various terpenoid, siderophores and bacteriocin encoding clusters (**Table 1.3**). Summed up in size, these clusters represent almost 7% of the genome of *S. avermitilis* (82). The genomes of two marine actinomycete species, *Salinispora tropica* CNB-440 and *Salinispora arenicola* CNS-205, though smaller in size than that of *S. avermitilis*, contain an even higher percentage of genes involved in secondary metabolite biosynthesis. Almost 9% of the *Salinispora tropica* genome consists of secondary metabolite encoding clusters, while *Salinispora arenicola* devotes more than every tenth gene to secondary metabolite biosynthesis (82).

1.4.1 Genome mining and orphan pathways for secondary metabolite biosynthesis

Considering the wealth of available genome sequences, mining microbial genomes is becoming a powerful tool in drug discovery. Systematic identification of gene clusters for natural product biosynthesis using bioinformatics tools (e.g. homology-driven searches for conserved protein motifs) has for many bacteria revealed a massive gap

between the number of predicted biosynthetic gene clusters and known products (**Table 1.3**). So-called "orphan" pathways, for which no biosynthetic product has been reported, represent a rich source for drug discovery and different strategies can be applied to identify the metabolic products of cryptic gene clusters. In the genomisotopic approach, precursor amino acids, which have been predicted to be specifically incorporated into the final product based on genomic information, are isotopically labeled and tracked using NMR techniques (21). A second method based on gene disruption experiments followed by a comparison of the secondary metabolite spectra of mutant and wild type strains, can reveal the encoded product and has the simultaneous advantage of delivering a functional proof for the corresponding gene cluster (21). The comparative metabolite profiling strategy can be equally well combined with heterologous expression approaches, where metabolite spectra of the parental host are compared to those of recombinants (21).

How genome mining for new natural products can be successfully applied even in the absence of complete genome sequence information was demonstrated by Ecopia Biosciences with the discovery and isolation of the novel antifungal ECO-02301 from *Streptomyces aizunensis* NRRL B-11277 (71). For multimodular biosynthetic systems such as PKSs and NRPSs, the order of biosynthetic steps can often be directly derived from the gene organization in the biosynthetic gene cluster. Shot gun DNA sequencing of *S. aizunensis* was used to identify genome sequence tags (GSTs) predicted to be involved in the biosynthesis of secondary metabolites. Selected tags were used as probes to isolate cosmids from a genomic library and a gene cluster of 35 open reading frames predicted to include a large type I PKS was isolated and sequenced. Information on chemical novelty, approximate mass and UV absorbance could be derived from the predicted structure of the final product. In previous fermentation studies, *S. aizunensis* had only been reported to produce bicyclomycin. To obtain production of ECO-02301, the strain was grown in nearly 50 different media followed by fermentation extract analysis using LC-MS. Activity-guided fractionation identified several antifungal fractions with predicted masses and UV absorbance and finally led to isolation of the novel compound (71).

1.4.2 Metagenomics

Obtaining genome sequence information in most cases still requires cultivable microorganisms in pure culture. However, only a minute fraction of the overall bacterial diversity has been cultivated under laboratory conditions (3). Metagenomics involves the creation of libraries of DNA directly isolated from environmental sources (soil, seawater etc.) followed either by sequence or expression analysis in suitable heterologous hosts (98). Culture-independent approaches can access the true microbial diversity by analyzing the collective gene pool of all organisms present in a particular sample. However, to target entire functional operons, metagenomic approaches rely heavily on large capacity cloning vectors like cosmids or BACs and suitable hosts for heterologous expression of the recombinant products as well as suitable protocols to introduce DNA into heterologous hosts (37).

Cloning and expression studies focused on the soil metagenome have allowed isolation of a number of small bioactive molecules including violacein, indirubin and the turbomycins (63 and references cited therein). Using *E. coli* as host for both cloning and expression can considerably limit the number of possible hits (see also 1.4.4) (26), therefore the transfer of libraries to a variety of surrogate hosts can be advantageous. Craig et al. chose a rather unusual expression host, *Ralstonia metallidurans*, for screening a soil DNA library for the production of pigments and antibiotics (24). They succeeded in isolating two novel metabolites and further studies indicated that these compounds were produced as a result of interactions between host enzymes and the heterologously expressed metabolites (24).

1.4.3 OSMAC – One Strain MAny Compounds

OSMAC is based on the fact that microorganisms often have the biosynthetic potential to produce many different compounds in dependence on changing environmental conditions. The approach relies on rather random variation of cultivation conditions to trigger the production of multiple secondary metabolites by a single organism (14).

Simple variations of media composition, pH value, aeration, cultivation temperature, culture vessel or the addition of enzyme inhibitors can lead to dramatic differences in secondary metabolite profiles. Bode et al. were able to detect over 100 compounds representing more than 25 structural classes when using this approach with only six microorganisms including four *Streptomyces* strains and two fungi (14). Extensions of the OSMAC approach, e.g. for triggering expression of cryptic secondary metabolite clusters, are practically unlimited. Examples range from addition of cytotoxic compounds to fermentation media (22) to mixed fermentations of bacteria and/or fungi leading to the discovery of a range of novel bioactive metabolites (90).

1.4.4 Heterologous production of natural products

Efficient isolation, characterization and modification of natural products require sufficient quantities of the molecule of interest. Therefore, heterologous production of natural products can be an alternative to lengthy fermentation optimization involving the natural producer or costly total synthesis approaches (37). Furthermore, heterologous expression can be used for functional confirmation of biosynthetic gene clusters and as a platform for combinatorial biosynthesis. It can also be applied to trigger expression and analyze the products of cryptic gene clusters (39).

A number of challenges are connected to the production of natural products in heterologous hosts. As already mentioned, moving entire biosynthetic gene clusters from the natural producer to the heterologous hosts requires suitable large-capacity vectors, as well as established gene transfer protocols (37). The choice of the heterologous host is crucial and many factors can determine if expression is successfully achieved at satisfactory levels. These factors include growth characteristics of the heterologous host, precursor availability, codon usage, efficiency of transcription/translation and posttranslational modifications of pathway-specific enzymes (37). For easy detection of expressed compounds, a host background low in secondary metabolites production is desirable. Strains closely related to the native producer are often likely to provide most of the desired features (37).

Successful examples of heterologous expression of a range of natural products are compiled in **Table 1.4**, where expression of entire biosynthetic gene clusters has been achieved based on integrative or replicative cosmid vectors, combinations thereof, or modified BAC vectors equipped with mobilization functions to allow intergeneric transfer between *E. coli* and *Streptomyces* strains (37).

Table 1.4. Antibiotics successfully produced in heterologous hosts by introducing entire biosynthetic gene clusters (adapted from 37).

Compound	Class	Native producer	Size[a]	Vector used[b]	Host strain	Yield[c]
Actinorhodin	PKS II	*S. coelicolor* A3(2)	~32.5	pIJ922$_{rep}$	*S. parvulus*	100
Blasticidin S	Peptidyl nucleoside	*S. griseochromo-genes*	20	pOJ446$_{rep}$	*S. lividans* TK24	100
Cinnamycin	Lantibiotic	*S. cinnamoneus*	17.1	pOJ436$_{int}$	*S. lividans* 1326	NR
Clorobiocin	Amino-coumarin	*S. roseochromo-genes* var. *oscitans* DS 12976	34	SuperCos1$_{int}$	*S. coelicolor* M512	104
Daptomycin	NRPS	*S. roseosporus*	128	pStreptoBACV$_{int}$	*S. lividans* TK64	2
Epothilone	PKS-NRPS	*Sorangium cellulosum* SMP44	56	SCP2$_{rep}$ pSET152$_{int}$	*S. coelicolor* CH999	>250
Patellamides	Peptide	*Prochloron sp.*	<20	pPAC-S1, pPAC-S2	*E. coli* DH10B	NR[d]
Soraphen A	PKSI	*Sorangium cellulosum* Soce26	67.5	pTBK$_{int}$ pTBBH$_{int}$ pTUE$_{rep}$	*S. lividans* ZX7	<0.2
Thiocoraline	NRPS	*Micromonospora* sp. ML1	53	pARP$_{int}$	*S. lividans* TK24, *S. albus* J1274	NR

[a] size in kb; [b] replicative ($_{rep}$) or integrative ($_{int}$); [c] % of wildtype production; [d] uncultured native producer (only heterologous production reported), NR: not reported

Due to its excellent growth characteristics and genetic amenability, *E. coli* would be the first choice candidate for heterologous production of natural products. However, the expression of antibiotic biosynthesis clusters, especially of PKS- and NRPS-based systems, is met with a number of challenges in *E. coli* such as largely different GC-contents, promoter recognition problems, precursor availability (e.g. methylmalonyl-CoA) and essential posttranslational modifications (e.g. activation of PKS and NRPS

through phosphopantetheinylation) (37). To tackle the obstacles posed by *E. coli* hosts, engineered *E. coli* strains have been developed with plasmid-based copies of tRNA genes to overcome codon bias problems via efficient translation of rare codons. To ensure posttranslational activation of PKS and NRPS systems, strains carrying a chromosomally integrated phosphopantetheinyl transferase gene derived from *Bacillus subtilis* (110) have been constructed.

For expression of actinomycete-derived secondary metabolite gene clusters, *Streptomyces* strains including the model organisms *S. lividans* and *S. coelicolor,* but also *S. albus,* are still considered the most robust hosts (37). The industrial avermectin producer *S. avermitilis* has recently been engineered for heterologous production of secondary metabolites by genome minimization (55). The deletion of non-essential genes (reducing the 9.02 Mb linear chromosome by more than 1.4 Mb) yielded a host devoid of any major endogenous secondary metabolites. The suitability of this strain for high-level heterologous production of natural products was demonstrated by successful expression of exogenous antibiotic biosynthetic gene clusters including those for biosynthesis of streptomycin and cephamycin C (55).

1.5 Peptide-based antibiotics and biosynthetic mechanisms

Antimicrobial peptides are ubiquitous in nature and are produced by all kinds of organisms ranging from bacteria and fungi to plants and higher eukaryotes. They comprise a variety of structural classes including established antibiotic classes such as the β-lactams, glycopeptides and lipopeptides as well as lantibiotics (lanthionine-containing antibiotics) and other bacteriocins. The bacterial peptide-based antibiotics vancomycin and teicoplanin have been in use for treatment of Gram-positive bacterial infections for decades (108) and the lantibiotic nisin is widely used as a food preservative (40).

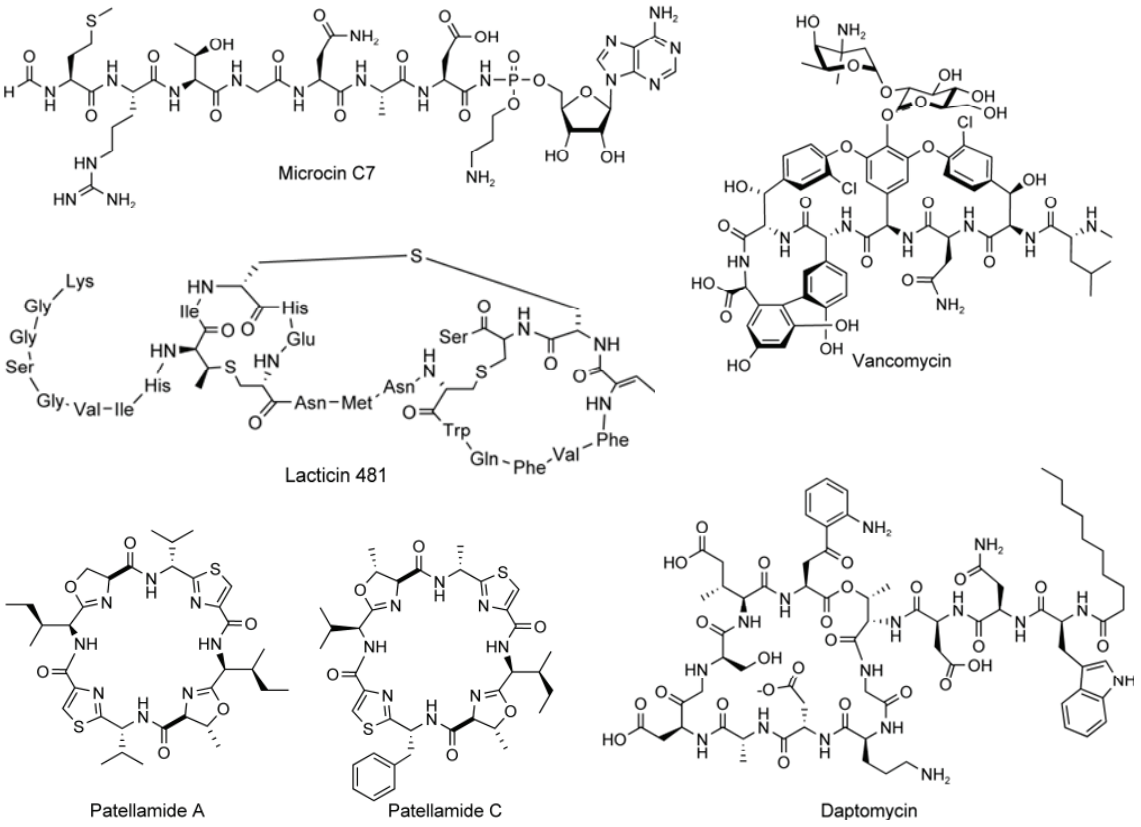

Figure 1.3. Examples of peptide-based antibiotics (ribosomally and non-ribosomally synthesized) (adapted from 83).

The synthesis of peptide-based antibiotics in bacteria is generally mediated in two different ways: ribosomally and non-ribosomally. Non-ribosomal synthesis is accomplished by non-ribosomal peptide synthetases (NRPS) and the products of these multimodular assembly lines can undergo further enzymatic modifications yielding e.g. glyco- and lipopeptides like vancomycin and daptomycin (**Figure 1.3**) (83).

Common to all ribosomally synthesized peptide antibiotics is that they are synthesized as (inactive) precursors from chromosomally encoded precursor peptide genes. Therefore, the composition of these peptides is limited to the 20 proteinogenic amino acids (plus selenocysteine), as opposed to NRPS-mediated biosynthesis where more than 200 non-proteinogenic amino acids can be used as building blocks (83). The native precursor peptides commonly undergo a series of posttranslational tailoring reactions introducing conformational rigidity and proteolytic stability. Examples of such tailored ribosomal peptides include the microcins, lantibiotics and patellamides (**Figure 1.3**). Common modifications in these molecules include dehydration of amino acids, exemplified by formation of dehydroalanine and dehydrobutyrine groups present in lantibiotics, and heterocyclization reactions, yielding e.g. the 5-membered aromatic heterocycles in microcins and patellamides (cyanobactins) (83).

1.5.1 Thiopeptide antibiotics

In another class of ribosomally synthesized peptide antibiotics, the thiazolyl peptides (thiopeptides), combinations of tailoring reactions from lantibiotic, microcin and cyanobactin biosynthesis can be found. Thiopeptide antibiotics have been isolated predominantly from different *Streptomyces* strains, but also from a range of other Gram-positive bacteria including *Micrococcus* and *Bacillus* (5).The defining feature of this rather large antibiotic class is the overall macrocyclic structure, which is centered by a characteristic 6-membered nitrogen-containing heterocycle (**Figure 1.4**) (5). According to the oxidation state of this heterocycle, five different subclasses (series *a*-*e*) are distinguished comprising in total more than 80 distinct structural entities. Amino acids in thiopeptides are usually heavily modified to multiple thiazole and (methyl)oxazole

groups as well as dehydro amino acids in form of dehydroalanine (dha) and dehydrobutyrine (dhb) (5).

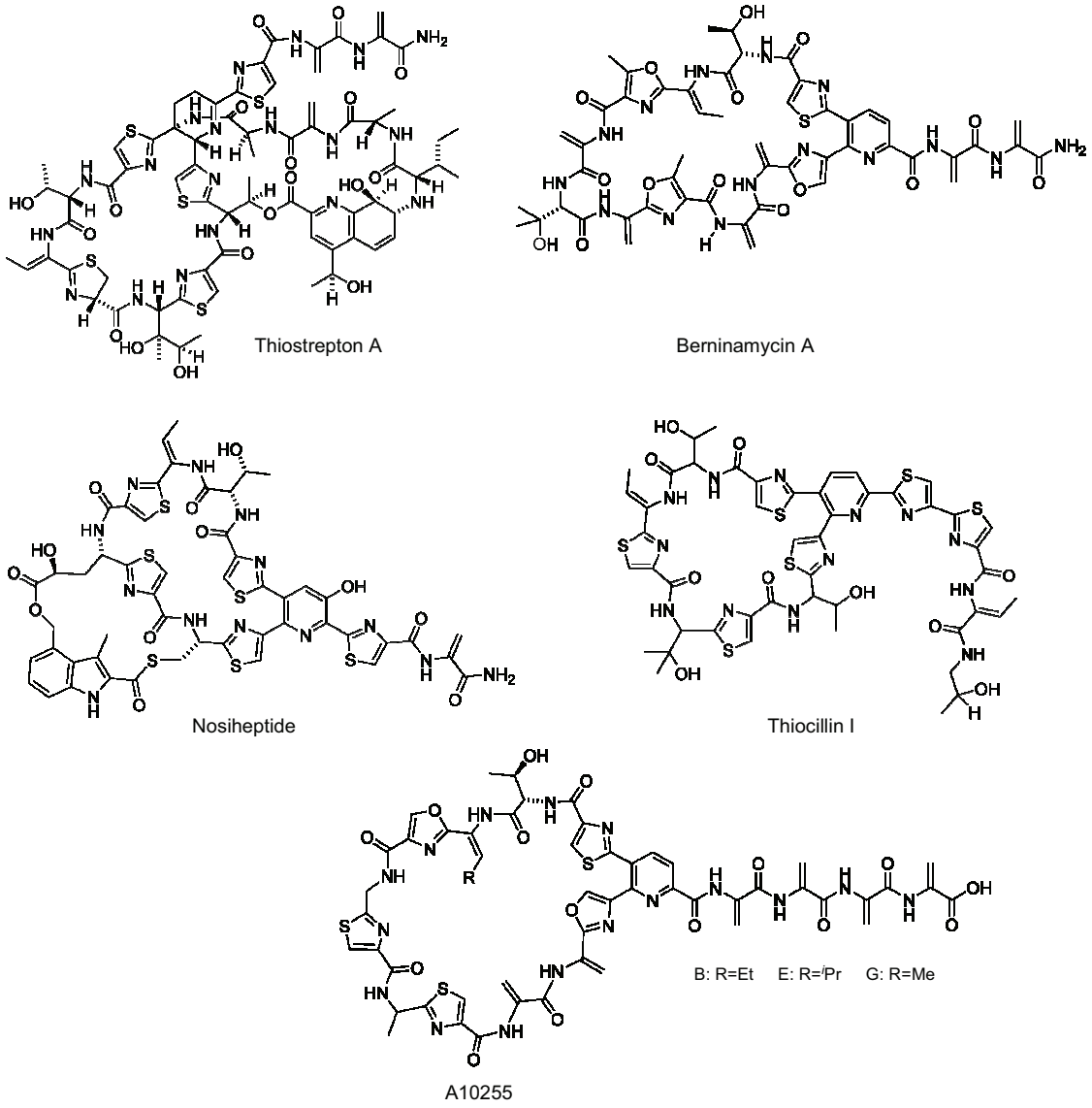

Figure 1.4. Examples of thiopeptide antibiotics: thiostrepton A (series *b*), berninamycin A, thiocillin I and A10255 factors (series *d*) and nosiheptide (series *e*) (adapted from 5).

Micrococcin, isolated in 1948 from a *Micrococcus* strain was the first thiopeptide to be discovered (104). As series *d* thiopeptide it is a representative of the by far largest series with more than 50 different compounds including other well-known members like the thiocillins and the amythiamicins. All series *d* compounds are characterized by a tri-

substituted pyridine domain, a single peptide macrocycle and a side chain consisting of multiple dehydrated residues (5). Series *a* and *b* molecules such as thiostrepton and siomycin are defined by quinaldic acid-containing bicyclic frameworks and central piperidine or dehydropiperidine heterocycles. A tetra-substituted piperidine domain is the defining feature of series *e* thiopeptides (e.g. nosiheptide, nocathiacin) and like series *a*/*b* compounds they usually have a backbone consisting of at least two peptide macrocycles (5).

The biosynthetic route which leads from plain ribosomally synthesized precursor peptides to the tailored molecules has been unraveled only recently (53, 64, 80, 113). The thiopeptide backbone derived exclusively of proteinogenic amino acids is transformed in a series of conserved posttranslational modifications through the action of a set of unique enzymes, most of which have distant homologs involved in the biosynthesis of lantibiotics and cyanobactins. Cyclodehydratases in combination with dehydrogenases mediate cyclization of cysteine, serine and threonine side chains against the preceding carbonyl group and oxidize the resulting thiazoline, oxazoline and methyl-oxazoline products to aromatic thiazoles and (methyl)oxazoles (**Figure 1.5A**) (83). Dehydration of serine and threonine residues through the action of dehydratases similar to those involved in lantibiotic biosynthesis yields dha and dhb, respectively (**Figure 1.5B**).

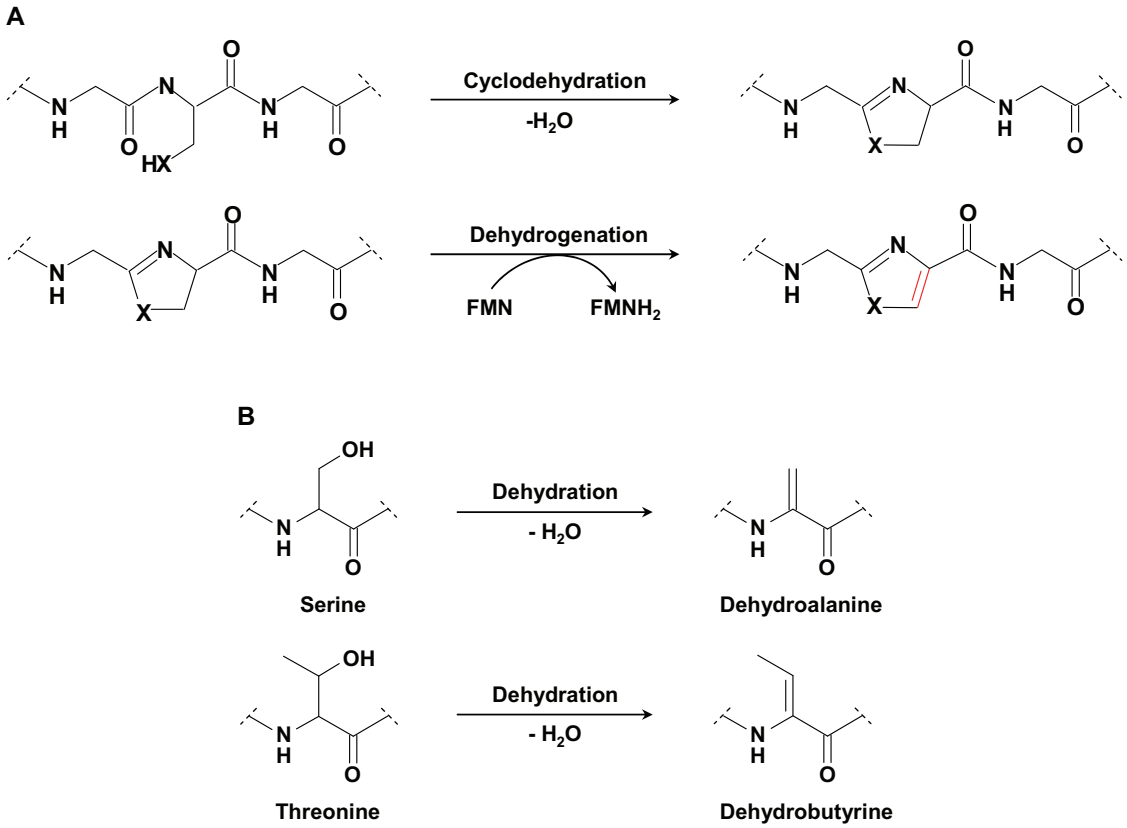

Figure 1.5. Typical posttranslational modifications during thiopeptide maturation. A) Two-step formation of aromatic heterocycles by cyclodehydration and FMN-dependent dehydrogenation. B) Formation of dehydroalanine and dehydrobutyrine from serine and threonine residues.

The exact mechanisms and enzymes that mediate the formation of the characteristic central 6-membered heterocycle remain to be verified, but feeding studies have shown that it arises from two dehydroalanine groups (derived from serine) and a neighboring cysteine carboxyl group (76). Cyclization of these residues could then yield a dehydropiperidine intermediate which is suggested to be the common basis for thiopeptides in all groups (118). Dehydration of dehydropiperidine would then give rise to the pyridine series, and further hydroxylation would yield the hydroxypiperidine thiopeptides. Piperidine rings can be obtained through reductive conversion from dehydropiperidine (118).

1.5.2 Thiopeptides – biological activities

Inhibition of bacterial protein synthesis is a very common mode of action employed by the majority of existing antibacterial classes (84). Aminoglycosides and tetracyclins, for example, bind to the decoding site (A-site) within the bacterial 16S rRNA of the 30S ribosomal subunit and interfere with translational fidelity by causing misincorporation of amino acids. Macrolide antibiotics, streptogramins, chloramphenicol and the oxazolidinones all bind to the 23S rRNA at the peptidyl-transferase center (PTC) and inhibit peptide bond formation (45).

Also thiopeptides act on the bacterial ribosome inhibiting the translational machinery. Molecular targets include both ribosomal RNA (rRNA) and ribosomal proteins at the so-called GTPase associated center on the 50S ribosomal subunit (8). The binding sites of thiopeptides were shown to be distinct from those exploited by other translational inhibitors and as a result no cross-resistance to existing antibiotics has been found so far (8). Thiopeptides most commonly inhibit processes involved in peptide elongation and two different mechanisms are employed to achieve this inhibition: i) interference with translocation through direct binding to a complex of 23S rRNA and ribosomal protein L11 (5, 42) and ii) binding to elongation factor Tu (EF-Tu) (80).

Both, thiostrepton and micrococcin bind to the L11-binding region on the 23S rRNA at overlapping sites but with different effects (8). The GTP-bound form of elongation factor G (EF-G) promotes translocation of the ribosome along the mRNA by one codon (20). Thiostrepton stabilizes a ribosome conformation which is incompatible with EF-G·GTP binding and thus inhibits GTP hydrolysis. Binding of micrococcin to the same region promotes GTP hydrolysis and induces a more rapid dissociation of EF-G·GDP from the ribosome (20).

A second mechanism in form of direct binding to EF-Tu rather than the ribosome is employed by monocyclic thiopeptides including GE2270 and the thiomuracins. The GTP-bound form of EF-Tu mediates recognition and transport of aminoacyl tRNAs to

the mRNA codon in the A-site of the bacterial ribosome (5). Binding to EF-Tu or EF-Tu·GTP blocks the binding site for aminoacyl(aa)-tRNAs and prevents the formation of an EF-Tu·GTP·aa-tRNA ternary complex. Consequently, tRNAs cannot be delivered to the ribosome and the elongation cycle is interrupted (4).

The biological activity of thiopeptides is not limited to their antibacterial properties. Anti-cancer, antimalarial and immunosuppressive effects of thiopeptide antibiotics have been described. Thiostrepton and siomycin induce apoptosis in neuroblastoma, leukemia and liver cancer cells by inhibiting the activity of transcription factor FoxM1, a key positive regulator of the cell cycle. In addition to inhibition of its transcriptional activity, they also cause downregulation of FoxM1 expression (12, 57).

Growth inhibition of the malaria parasite *Plasmodium falciparum* has been reported for thiostrepton and micrococcin. High similarity between bacterial 70S ribosomes and the mitochondrial or apicoplast ribosomes of the eukaryotic parasite are suggested as basis for this activity (72, 95) and new semisynthetic derivatives of thiostrepton have been developed which even employ a dual mode of action by targeting both the apicoplast ribosomes and the 20S proteasome in parallel (99). Immunomodulating effects by suppression of B- and T-cells have been reported for siomycin, thus indicating a potential for development of this compound for treatment of antibody-mediated diseases (107).

1.5.3 Thiopeptide resistance

Different mechanisms exist in thiopeptide producing bacteria which ensure resistance to the toxic action of self-produced or exogenous compounds. A common self-resistance mechanism consists of methylation of the 23S rRNA at the A1067 2′-hydroxyl group through action of chromosomally encoded methyltransferases. Methylation at this key position prevents the binding of thiopeptide compounds to the ribosome (105). Two residues, A1067 and A1095 (*E. coli* numbering), of the 23S rRNA are crucial for thiopeptide recognition and mutations at these positions lead to reduced binding affinity

and confer thiopeptide resistance (8). Resistance-conferring mutations in ribosomal protein L11 can be caused by single amino acid substitutions (especially at key residue P25). Furthermore, mutants deficient in L11 or with truncated versions of L11 lacking the N-terminal ligand-binding domain, have been described (8 and references cited therein).

Also in EF-Tu-inhibitor producing bacteria, the presence of mutations in the amino acid sequence of the target protein can confer resistance. The producer of EF-Tu inhibitor GE2270A, *Planobispora rosea,* has a single *tuf* gene encoding an EF-Tu protein completely resistant to the inhibitory action of GE2270A (77). *Streptomyces* spp. producing EF-Tu targeting antibiotics can have up to three gene variants encoding sensitive and resistant forms of otherwise functional EF-Tu proteins which can be expressed in a growth-phase dependent manner and at least partly confer self-resistance (86).

1.5.4 Thiopeptide engineering

Since the biosynthetic origin of thiopeptides was only elucidated recently, engineering and structure-activity relationship studies of thiopeptide antibiotics are still at their very beginning. The aim of these studies is mostly to create novel thiopeptide molecules with improved solubility while still retaining biological activity. Using semi-synthesis from nocathiacin I, Xu et al. were able to obtain nocathiacin acid as a more versatile intermediate for chemical creation of more soluble amide analogs (117). Since the chemical conversion process was tedious and involved toxic reagents, an *in situ* production of nocathiacin acid was more desirable. Wei et al. were able to achieve *in situ* bioconversion of nocathiacin I in *Amycolatopsis fastidiosa* LCB1001. By adding $CuCl_2$ to fermentation cultures, the activity of enzymes involved in hydrolysis of the C-terminal dehydroalanine tail could be modified yielding the desired nocathiacin acid (112).

Two recent studies describe the generation and determination of antimicrobial activities of a range of thiocillin variants (1, 17). The thiocillin biosynthetic gene cluster from *Bacillus cereus* ATCC 14579 contains four tandem genes, *tclE-H*, each of them encoding identical copies of the thiocillin precursor peptide. The observation that thiocillin production could be rescued in a non-producing mutant where all four genes had been deleted *via* introduction of a single plasmid-based copy of one precursor peptide gene was used as basis for structure-activity studies (1). Precursor peptide variants were created using site-directed mutagenesis exchanging in total 14 amino acids at five different positions in the original precursor. The introduction of these variants into the *B. cereus* Δ*tclE-H* mutant gave rise to 65 novel thiocillin variants (1). However, while antibiotic activity of conservative variants was maintained, variants where additional charges had been introduced were inactive (1). A second round of mutations consisted of a complete alanine scan of all 14 residues of the thiocillin precursor and a serine scan of the six cysteine residues which are present as thiazoles in the matured thiocillins (17). It was shown that substitutions of the two serine residues building the pyridine core of thiocillin resulted in complete loss of thiocillin or any derivatives. Substitutions affecting heterocycle formation in the thiocillin macrocycle resulted in derivatives with dramatically reduced activity. Even the disruption of a single thiazole in the thiocillin macrocycle significantly diminished or abolished activity of the resulting product indicating the importance of global conformational rigidity of the thiopeptide macrocycle for biological activity. Also substitution in regions proposed to be involved in binding to ribosomal protein L11 led to drastically reduced activity (17).

2 AIMS OF THE STUDY

The aim of this study was to analyze marine actinomycete bacteria from the Trondheim fjord for their antibiotic production potential. The analyses were to be focused on indigenous marine isolates with high potential to produce bioactive secondary metabolites.

Many biologically active secondary metabolites, including antibiotics, are synthesized by actinomycete bacteria through polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) pathways. A PCR screening for PKS and NRPS genes was used as a tool to estimate the genetic potential of isolates to produce bioactive compounds.

Since the expression of gene clusters for secondary metabolites is dependent on specific, usually unknown environmental triggers, fermentation studies including a wide range of cultivation media should be used to identify conditions favorable for synthesis of antimicrobial compounds. Isolates with good antibacterial and/or antifungal activity would then be chosen based on the results of this screening for further studies.

These studies would involve identification of the active compounds and cloning of the corresponding biosynthetic gene clusters, followed by functional analyses and, if feasible, biosynthetic engineering.

3 SUMMARY OF RESULTS AND DISCUSSION

3.1 Isolation of actinomycetes from fjord sediments and sponges

Analyses performed in this study were based on two different actinomycete collections selected from isolate libraries which were established from three different sampling sources in the Trondheim fjord, Norway. In the three independent isolations, actinomycetes were recovered from (i) shallow water sediments (4.5-28 m depth), (ii) sediments from deeper fjord regions (10-547 m depth) and (iii) from different sponge species recovered from 60 and 120 m depth.

Different enrichment techniques in combination with selective agar media supporting selection and growth of actinomycete bacteria were applied. Sediment samples were generally pre-treated by desiccation, which favored the growth of spore-forming bacteria. Other methods were applied to permit growth of only filamentous actinomycetes (the filter-technique (91)), or of only certain actinomycete genera, e.g. by applying different kinds of sample irradiation (18, 39, 64) or differential centrifugation (43). For cultivation, rich media, often with complex carbon sources such as chitin to exploit enzymatic capabilities of actinomycetes enabling them to use a variety of substrates, as well as nutrient-poor, habitat-specific media (e.g. sediment- and sponge-extract agar) were used. Cultivation media were generally supplemented with different antibiotics including nalidixic acid, to prevent the growth of Gram-negative bacteria, and nystatin or cycloheximide to prevent fungal growth.

Using different combinations of these methods, a diverse collection of ca. 10.000 actinomycete isolates was established. Paper I describes further studies on 35 shallow-water sediment-derived isolates, while Paper II is based on analysis of 27 deeper sediment and sponge-derived actinomycetes.

3.2 Influence of seawater on growth of marine-derived isolates

The growth of actinomycete isolates on seawater-containing agar media was compared to growth on seawater-deficient media (Papers I and II). It was observed that marine-derived isolates were differently affected by the presence of seawater in the growth medium and growth phenotypes ranged from obligatory dependence on seawater (no growth in its absence) to varying degrees of preference for seawater (smaller colony size, later onset of sporulation etc. in the absence of seawater) to opposite effects (no growth in the presence of seawater). A positive influence of seawater on growth indicated that the corresponding isolates could represent indigenous marine bacteria which have adapted to the marine environment in the course of evolution (48). For Paper II, seawater dependency or preference was used as a criterion to select a collection of 27 marine actinomycetes for further studies.

3.3 Assessing biodiversity and antibiotic production potential of actinomycete isolates

Molecular taxonomy based on amplification and sequencing of 16S rRNA genes was used as a tool to assess the biodiversity of selected sub-collections of actinomycete isolates (Papers I and II). 16S rRNA gene sequence analysis provided information about similarity to published sequences of other actinomycetes, and the preliminary taxonomic information was used to assess diversity and potential novelty of the selected isolates. The preliminary identification of genus and species of isolates can be very useful as it can aid in dereplication of strain collections and is often crucial for further analyses such as fermentation studies and genetics.

A phylogenetic tree based on the 16S rRNA gene sequence data for the isolates described in Paper I revealed high biodiversity of actinomycetes in this collection containing representatives of 11 different actinomycete genera, two of which (*Knoellia* and *Glycomyces*) had not been reported among marine-derived isolates by then. Based on the topology of the derived phylogenetic tree, it could be assumed that potentially

new species for 10 of the 11 genera were represented in the collection. Preliminary classification of the 27 isolates in Paper II revealed representatives of eight different actinomycete genera with the majority of isolates typed as *Nocardiopsis*, *Micromonospora* and *Streptomyces* spp. Many of the isolates showed closest 16S rRNA gene sequence similarity to marine-derived actinomycetes.

Apart from the phylogenetic analysis, the potential of isolates in both collections to produce secondary metabolites was assessed. A PCR screening for genes associated with the biosynthesis of polyketide and peptide-based antibiotics was performed. Three sets of degenerate primers were employed to specifically target genes encoding ketosynthase (KS) domains of modular type I and iterative type II polyketide synthases (PKSs) as well as adenylation domains of non-ribosomal peptide synthetases (NRPSs). In both collections, the overall majority of isolates yielded PCR products for at least one of the biosynthetic systems, and for 30% (Paper I) up to 55 % (Paper II) of isolates amplification products for all three target genes could be obtained. This demonstrated the genetic potential of most of the selected isolates for biosynthesis of secondary metabolites with polyketide and/or peptide backbone.

Combined with sequence and bioinformatics analyses of the amplified gene fragments, this approach can be a powerful tool to predict the nature of encoded compounds and to select strains with the highest potential to produce novel secondary metabolites for fermentation studies. Furthermore, amplified gene fragments can also be used for identification and cloning of the corresponding biosynthetic gene clusters by using them as probes in the screening of genomic libraries.

3.4 Screening of 27 marine actinomycetes for production of antibacterial and antifungal compounds

Twenty-seven selected isolates displaying a preference for or dependence on seawater for growth were analyzed for the production of antibacterial and antifungal compounds (Paper II). Fermentations of isolates for secondary metabolite production were

performed in 96-well microtitre plates with 800 µl culture volume and using up to 14 different media. It has been shown that conditions in micro-cultures (≤ 3 ml) with respect to oxygen transfer rates, biomass yield and secondary metabolite production compare well or exceed those achieved in larger scale shake-flask cultivations (74, 100). Using this cultivation system, all steps in the screening procedure, such as fermentation, extraction of fermentation cultures, storage of extracts and bioassays, could be performed efficiently in 96-well format. Crude extracts of the entire fermentation cultures were prepared with DMSO and analyzed for antibacterial/antifungal activity in whole-cell-based growth inhibition assays against *Micrococcus luteus* and *Candida albicans.* Fifteen out of 27 isolates subjected to this screen produced compounds inhibiting the growth of at least one of the indicator strains.

Those extracts displaying the strongest antagonistic activity (nine of 15 active extracts) were subsequently analyzed for activity against multi-resistant clinical isolates of *Enterococcus faecium* and *C. albicans*. Eight out of the nine extracts tested also inhibited the growth of the multi-resistant test strains. Extracts of isolate TSF65-07, preliminary typed as *Nocardiopsis* sp. based on analysis of the 16S rRNA gene sequence, caused potent growth inhibition of vancomycin-resistant *E. faecium* while displaying no activity against *C. albicans*. Hence, isolate TFS65-07 was chosen for further studies aiming at identification of the active antibacterial compound.

Although the fermentation screening only included few variables in form of different media, seawater content and cultivation periods, more than 50% of the subjected isolates produced antibacterial/antifungal compounds. It seems likely that through further variation of cultivation conditions (e.g. more media/different pH, additives, cultivation temperature) conditions for expression of secondary metabolite gene clusters in the remaining isolates could be triggered as well (14).

3.5 Identification, isolation and structure elucidation of the new thiopeptide antibiotic TP-1161

Based on the results from the extract screenings, those conditions that yielded the highest bioactivity were chosen for upscaled production of the antibacterial compound by TFS65-07 in shake flasks. Culture extracts were subjected to activity-guided fractionation and antibacterial fractions analyzed by LC-DAD-TOF. The UV absorption spectrum of these fractions revealed a UV absorption maximum at 250 nm, which correlated with a significant MS-peak. To obtain sufficient material for further characterization, upscaled production in both shake flasks and batch fermentors were performed. The antibacterial compound could be purified, and using LC-DAD-TOF analysis its accurate mass was determined to be between 1161.2619 and 1161.2665. A database search (Dictionary of Natural Products) for molecules with a similar accurate mass and UV absorption maximum did not yield any significant hits, suggesting the novelty of the identified compound. Thus, it was decided to solve the molecular structure of the potentially new antibacterial compound. HRMS and NMR experiments provided the molecular formula ($C_{50}H_{47}N_{15}O_{13}S_3$) and determination of the structure using NMR, HSQC and HMBC experiments revealed the compound to be a new member of the thiopeptide antibiotics (**Figure 3.1**). The compound was designated TP-1161.

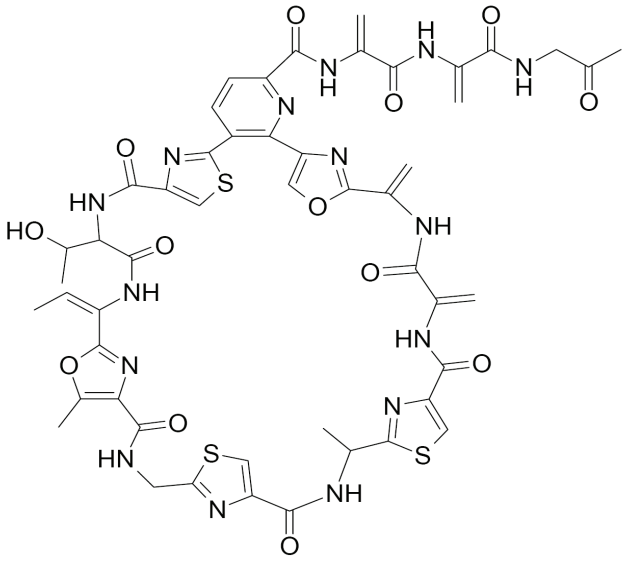

Figure 3.1. Molecular structure of the thiopeptide antibiotic TP-1161.

The TP-1161 structure is like other members of this antibiotic family defined by a macrocyclic peptide framework consisting of modified amino acid residues including multiple heterocycles and dehydrated amino acids, and a central 6-membered nitrogen-containing heterocycle (5). Structure and oxidation state of the central heterocyclic domain serve as basis for classifying thiopeptides into 5 different series (series *a*-*e*). The presence of a characteristic 2,3,6-tri-substituted pyridine domain in TP-1161 defined the molecule as a series *d* thiopeptide, the numerically largest series, which includes members such as the very first discovered thiopeptide micrococcin and other well-studied compounds like the thiocillins and GE2270 (5). Most closely related compounds in this series include berninamycin A and B, isolated from *Streptomyces bernensis*, and the A10255 complex produced by *S. gardnerii* NRRL 15537 based on the shared central oxazole−thiazole−pyridine-type *d* domain (**Figure 1.4**) (5).

3.6 Isolation of the TP-1161 biosynthetic gene cluster and pathway proposition

Early LC/GC and MS results indicated the isolated antibacterial compound to be of peptidic nature. Since most peptide antibiotics are known to be produced by NRPS systems, initial attempts to isolate the corresponding biosynthetic gene cluster were based on an NRPS-based screening approach. A genomic cosmid library of producer *Nocardiopsis* sp. TFS65-07 was constructed and 3072 cosmid clones screened using PCR-amplified gene fragments encoding NRPS adenylation domains as probes. However, identification of positive cosmid clones followed by partial DNA sequencing did not yield any putative gene cluster that could be linked to the preliminary data for the antibacterial peptide-based antibiotic from TFS65-07.

The structure determination, proving TP-1161 to be a member of the thiopeptide antibiotic family was followed by three almost simultaneously published reports elucidating the biosynthetic origin of thiopeptides (55, 68, 123). Overthrowing the by then widely accepted theory of NRPS-mediated thiopeptide biosynthesis, the reports documented that the highly modified peptides are generated instead by ribosomal

synthesis from chromosomally encoded precursor peptide genes and that all modifications, including heterocyclisation and dehydration of amino acids, are introduced posttranslationally by a range of conserved enzymes, some of them reminiscent to enzymes involved in the biosynthesis of other ribosomally encoded peptide antibiotics including lantibiotics and cyanobactins (62).

The new data prompted the change of strategy for identification of the TP-1161 gene cluster. A draft genome sequence of *Nocardiopsis* sp. TFS65-07 was obtained covering the about 6 MB genome in form of 373 contigs with an average size of 16 kb. Based on the information about conserved thiopeptide tailoring reactions, the TP-1161 molecular structure could be used to deduce the primary amino acid sequence constituting the unmodified TP-1161 structural peptide (**Figure 3.2**).

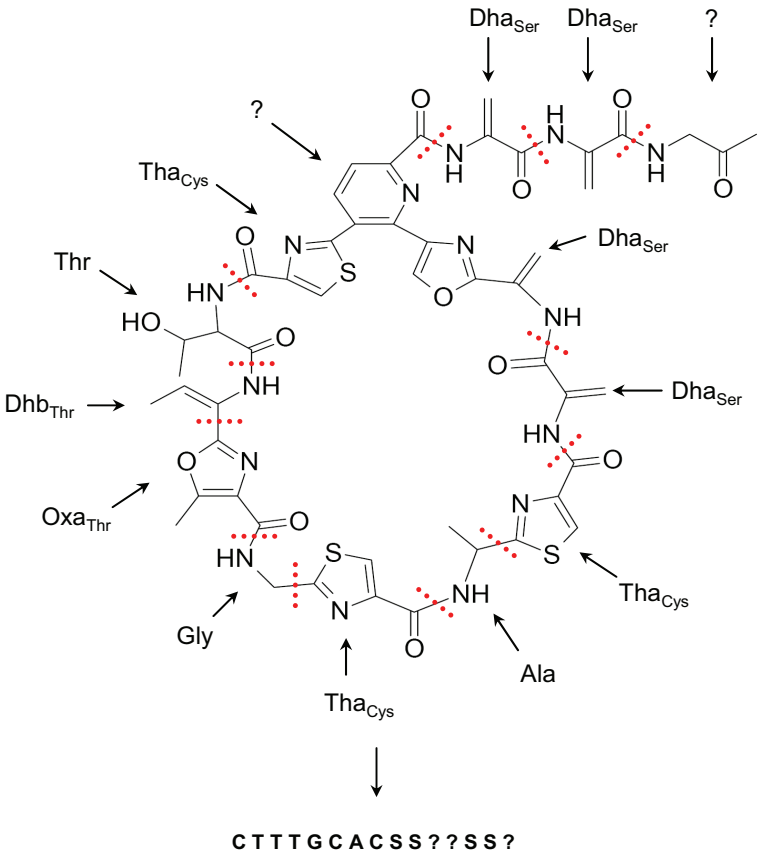

Figure 3.2. Prediction of TP-1161 precursor peptide sequence based on molecular structure of modified thiopeptide framework.

The draft genome was then scanned for the gene encoding the structural TP-1161 peptide. This approach led to identification of the TP-1161 biosynthetic gene cluster proposed to consist of 13 open reading frames encoding two thiopeptide precursors and 11 enzymes mediating posttranslational modifications and host resistance (**Figure 3.3**).

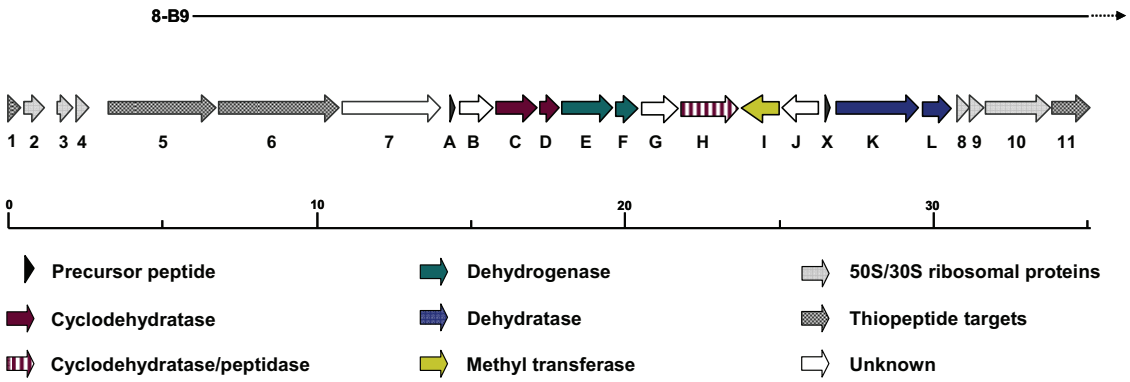

Figure 3.3. Organization of the TP-1161 biosynthetic gene cluster in *Nocardiopsis* sp. TFS65-07. Displayed are the proposed core biosynthetic genes (*tpa*) with deduced functions and surrounding regions.

Annotation of the genomic region using FramePlot and protein BLAST analyses, along with data from the literature allowed functional predictions for the genes proposed to constitute the TP-1161 biosynthetic pathway and surrounding regions (**Table 3.1**).

Table 3.1. Deduced functions of open reading frames in the TP1161 biosynthetic gene cluster and surrounding regions.

Gene designation	Protein[a]	Proposed function
orf1	142	50S ribosomal protein L11
orf2	225	50S ribosomal protein L1
orf3	175	50S ribosomal protein L10
orf4	129	50S ribosomal protein L7/L12
orf5	1155	DNA-directed RNA polymerase subunit β
orf6	1292	DNA-directed RNA polymerase subunit β'
orf9	1060	Unknown
tpaA	48	TP-1161 precursor peptide
tpaB	355	Unknown
tpaC	448	Cyclodehydratase
tpaD	200	Cyclodehydratase
tpaE	546	Thiazoline/oxazoline dehydrogenase
tpaF	233	Thiazoline/oxazoline dehydrogenase
tpaG	391	Unknown
tpaH	621	Cyclodehydratase/peptidase
tpaI	269	23S rRNA methyltransferase
tpaJ	386	Dehydrogenase
tpaX	48	Precursor peptide for unknown thiopeptide
tpaK	900	Serine/threonine dehydratase
tpaL	310	Serine/threonine dehydratase
orf8	123	Ribosomal protein S12P
orf9	156	Ribosomal protein S7P
orf10	703	Translation elongation factor EF-G
orf11	397	Translation elongation factor EF-Tu

[a]protein size in amino acids

Unusual for thiopeptide biosynthetic gene clusters, the TP-1161 cluster was found to encode a second thiopeptide precursor (TpaX) with 70% homology to the TP-1161 precursor TpaA. However, the posttranslationally modified product of TpaX could not be detected in fermentation extracts of TFS65-07.

SUMMARY OF RESULTS AND DISCUSSION

Based on the predicted functions of *tpa* genes in the cluster, a model for TP-1161 biosynthesis was proposed (**Figure 3.4**). In total 10 different enzymes are predicted to act on the TP-1161 precursor peptide, introducing posttranslational modifications in 13 out of 15 residues of the structural peptide and catalyzing macrocyclization and elimination of the leader peptide.

The predicted cyclodehydratases TpaC and TpaD in combination with the assigned dehydrogenases TpaE and TpaF mediate oxazole and thiazole formation in TP-1161 by (i) cyclization of serine, cysteine and threonine side chain onto their preceding carbonyl groups and (ii) oxidation of the resulting dehydroheterocycles to aromatic oxazoles and thiazoles. The lantibiotic dehydratase homolog TpaL is predicted to be involved in dehydration of serine and threonine residues yielding a single dehydrobutyrine as well as multiple dehydroalanine moieties in TP-1161.

Formation of the central pyridine domain by macrocyclization and elimination of the leader peptide is proposed to be achieved through concerted action of two enzymes, cyclodehydratase/peptidase TpaH and a second lantibiotic dehydratase homolog, TpaK. Two serine groups and the carboxyl group of an adjacent cysteine residues have been established as the origin of the central pyridine domain (76). Cycloaddition of these residues is proposed to require dehydration of the two serine residues prior to formation of a dehydropiperidine ring intermediate from which the pyridine moiety can be obtained after dehydration and elimination of the leader peptide (62 and references cited therein). Dehydration of two serine residues (residues 11 and 12 in the structural peptide) through action of TpaK dehydratase coupled with cyclodehydration of the N-terminal cysteine residue and cyclization could thus yield the pyridine domain in TP-1161. Bioinformatics analysis of TpaH revealed a C-terminal peptidase domain in addition to the YcaO-family domain commonly present in cyclodehydratases. In addition to its cyclodehydratase activity this indicated a possible role of TpaH in cleavage of the leader peptide during macrocyclization.

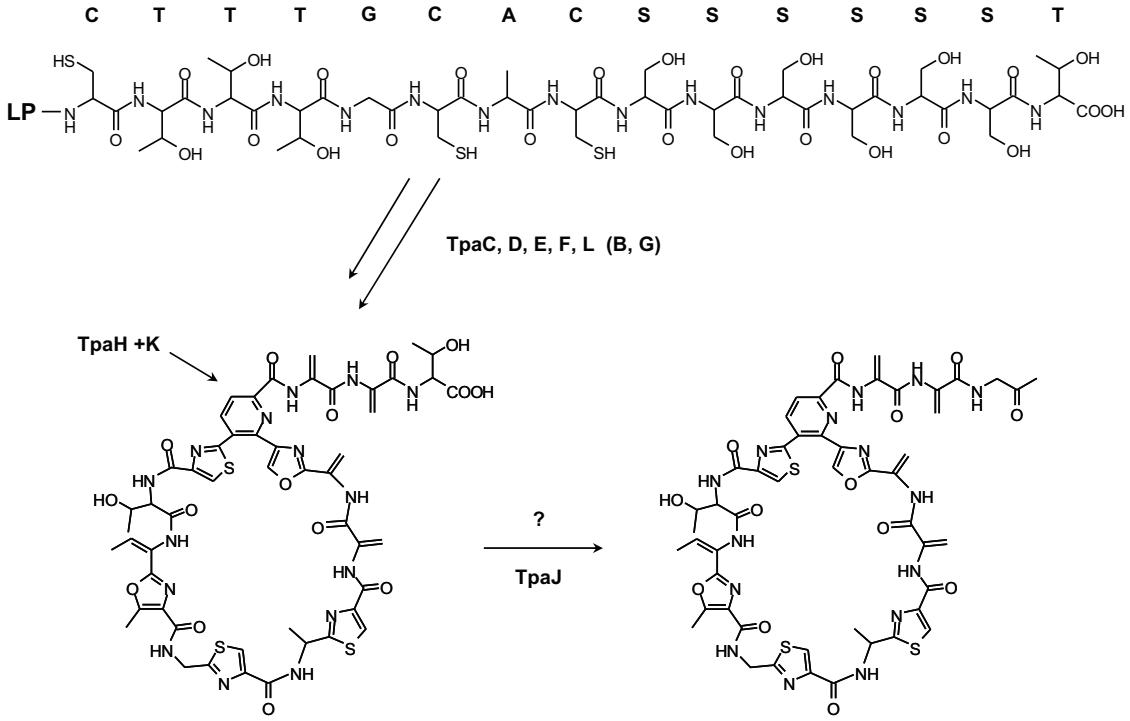

Figure 3.4. Proposed pathway for TP-1161 biosynthesis in TFS65-07. Displayed is the conversion of the unmodified linear precursor peptide (LP = leader peptide) to the final decorated thiopeptide macrocycle through action of the proposed TP-1161 modification enzymes (Tpa).

Also unusual for thiopeptides, the terminus of the mature TP-1161 molecule consists of an aminoacetone instead of typically present carboxamide or carboxyl groups. This posed the question of how the terminus of TP-1161 is generated during biosynthesis. The TP-1161 structural peptide contains a C-terminal threonine indicating that the aminoacetone group is derived from this amino acid. A suggested candidate for performing this tailoring reaction is TpaJ, assigned as iron-dependent dehydrogenase with similarity to 4-hydroxybutyrate dehydrogenases. The latter enzyme catalyzes the conversion of 4-hydroxybutanoic acid to succinate semialdehyde. High structural similarity between 4-hydroxybutanoic acid and threonine led to the hypothesis that aminoacetone can be obtained through oxidative action of TpaJ dehydrogenase, followed by spontaneous decarboxylation of the intermediate and enol-keto tautomerization.

3.7 Functional confirmation of the TP-1161 gene cluster

For functional studies of genes involved in TP-1161 biosynthesis in the producer TFS65-07, a gene transfer system in form of intergeneric conjugation from *E. coli* (54) was established using derivatives of the mobilizable suicide vector pK18mob (97). To verify involvement of the identified genetic locus in TP-1161 biosynthesis, the genes encoding the two putative cyclodehydratases, TpaC and TpaH, were inactivated. Two mutants were created, one obtained from gene disruption of *tpaC* and the other one from insertional inactivation of *tpaH*. Both mutants lost the ability to produce TP-1161, confirming a role of both enzymes in the biosynthesis of TP-1161. However, as described for other thiopeptides, disruptions of cyclodehydratase genes usually do not yield any identifiable derivatives, thus preventing any conclusions about functional assignments or the order of posttranslational modifications.

To investigate the putative role of TpaJ in tailoring the TP-1161 terminal threonine, an in-frame deletion mutant of *tpaJ* was created. According to the proposed hypothesis, the Δ*tpaJ* mutant should produce a TP-1161 derivative with a terminal threonine and thus produce a more soluble derivative due to the presence of the threonine carboxyl terminus. Although the successful in-frame deletion of *tpaJ* was confirmed by PCR and Southern blot analyses, neither TP-1161 nor any new derivative could be detected in fermentation extracts of the corresponding mutant.

Posttranslational tailoring of thiopeptides introduces structural constraints and provides hydrolytic stability to the final product (108). Since the order of tailoring reactions is not known, the inactivation of any of the modification enzyme-encoding genes could lead to either abolished production or the generation of intermediates or derivatives which are unstable and subject to rapid proteolytic degradation preventing detection.

3.8 Attempt to heterologously express the TP-1161 gene cluster in *Streptomyces coelicolor* M512

To establish a more versatile platform for systematic functional gene analysis and for production of TP-1161 derivatives by biosynthetic engineering, heterologous expression of the entire TP-1161 cluster was attempted in *S. coelicolor* M512. The latter is an engineered *S. coelicolor* strain deficient in production of actinorhodin (Act) and undecylprodigiosin (Red) (35), and thus can be used as a convenient host for heterologous production and simplified detection of secondary metabolites due to low host background (30). For site-specific integration of the gene cluster into the genome of M512, the SuperCos1-based cosmid 8-B9 carrying the entire TP-1161 cluster and surrounding regions was equipped with transfer and integration functions using λ-Red-mediated recombination (27, 30) and introduced into M512 by protoplast transformation. Recombinant clones were confirmed by Southern blot analysis and tested for TP-1161 production. Fermentation extracts of M512 integration mutants were prepared from a range of different media and conditions. However, TP-1161 could not be detected in any of the extracts, suggesting a lack of functional expression of the *tpa* cluster in M512.

To investigate if this was the case for all introduced *tpa* genes the recombinant strains were analyzed for growth on medium supplemented with thiostrepton. While the parental M512 was sensitive to 25 μg/ml thiostrepton, the recombinant strains displayed thiostrepton resistance indicating the presence of an active resistance determinant on the introduced cosmid. It is high likely that this determinant is represented by TpaI, a 23S rRNA methyltransferase which is highly similar (66% identity) to the rRNA methyltransferase from *S. azureus* conferring thiostrepton resistance (105). Therefore, it could be concluded that not all genes in the *tpa* cluster are silent in *S. coelicolor*.

Assuming that all genes necessary for TP-1161 biosynthesis are present on cosmid 8-B9, the lack of TP-1161 production in M512 could be due to the absence of specific regulatory protein(s) in this host that is (are) needed for successful and coordinated expression of the *tpa* genes.

No genes encoding potential regulators were identified in the *tpa* cluster or its surroundings and further studies are necessary to analyze the reasons for the lack of TP-1161 production in M512. These could include introduction of 8-B9 into alternative hosts such as *S. lividans* or *S. albus* followed by analyses of TP-1161 production and expression analysis of the *tpa* genes in the different host backgrounds.

4 CONCLUDING REMARKS AND PERSPECTIVES

Modern drug discovery still relies heavily on natural products, especially in the area of antimicrobials. Bacterial secondary metabolites represent the most important source for new antimicrobial compounds both in their native forms and as templates for synthetic modifications or *de novo* design. Members of the bacterial order *Actinomycetales* have delivered most of the antibiotics in clinical use today, and the focus on marine actinomycetes contributed new biodiversity and structurally unique bioactive compounds to drug development pipelines.

The work presented in this thesis indicates that Trondheim fjord environmental niches represent suitable and promising sources for bioprospecting of marine actinomycete bacteria. Isolates belonging to diverse and "rare" actinomycete genera could be isolated from sediment- and sponge samples. These included members of genera which had previously not been isolated from the marine environment, including potentially new species.

The marine actinomycetes from the Trondheim fjord were proven to be a rich source for antimicrobials. Genetic studies confirmed the potential of the majority of analyzed isolates to produce PKS- and NRPS-derived secondary metabolites. Fermentation studies could trigger production of antibacterial and/or antifungal compounds from more then 50% of the isolates and nine isolates produced compounds with antagonistic activity against multi-resistant bacterial and fungal strains. It is likely that through further variation of fermentation conditions the production of antimicrobial compounds could have been triggered in all isolates. This in turn means that it might be more feasible to restrict fermentation analyses to a few "rare" and promising (from the genetic point of view) isolates, and to simultaneously extend the variation of cultivation conditions for secondary metabolite production.

A new thiopeptide antibiotic, TP-1161, was isolated as a result of the presented fermentation studies. The biosynthetic gene cluster was identified by genome mining,

isolated and its involvement in the TP-1161 biosynthesis confirmed by gene inactivation studies. Due to their unique mode of action and excellent *in vitro* properties, i.e. antibacterial as well as properties relevant to other medical applications, there has been renewed interest in developing thiopeptide antibiotics for clinical use in humans. The elucidation of origin and mechanisms involved in thiopeptide biosynthesis has laid the foundation for genetic engineering and biosynthesis of thiopeptides with improved pharmacological properties.

A first step in engineering a more soluble TP-1161 derivative attempted in this study failed, and more functional studies on thiopeptide modification enzymes are necessary. Of special interest would be studies establishing the order and interdependency of posttranslational modifications during thiopeptide maturation. Although not successfully accomplished in *S. coelicolor* M512, heterologous expression would be the method of choice for functional gene analysis and TP-1161 engineering. Further studies could involve introduction of the TP-1161 cluster to alternative heterologous hosts and expression analysis of all genes involved in the biosynthesis. The results could provide the knowledge necessary for successful thiopeptide derivatization by genetic manipulation of both structural peptides and enzymes mediating their maturation.

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