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„Combination of classical bioassay-guided and advanced genomics-driven discovery of novel natural products in Actinomycetes“

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Abbrevations

HPLC ..... high performance liquid chromatography

MS ..... mass spectrometry

NMR ..... nuclear magnetic resonance spectrometry

ELSD ..... evaporate light scattering detector

WT ..... wild-type

KN ..... knock-out

DNA ..... desoxyribonucleic acid

gDNA ..... genomic DNA

pDNA ..... plasmid DNA

BGC ..... biosynthetic gene cluster

SM ..... secondary metabolite

PKS ..... polyketide synthase

NRPS ..... non-ribosomal peptide synthetase

PCR ..... polymerase chain reaction

kb ..... kilobases

Mb ..... megabases

HF ..... high fidelity

ACN ..... acetonitrile

ddH_2O ..... double distilled water

rpm ..... revolutions per minute

BLAST ..... basic local alignment search tool

antiSMASH ... antibiotics and secondary metabolites analysis shell

Chapter 1

Zusammenfassung

In diesem Projekt wurden zwei Actinomycetes-Stämme, *Streptomyces noursei* und *Amycolatopsis* sp. YIM10, ausgewählt, um vermeintlich neue Sekundärmetabolite zu isolieren. Zu diesem Zweck wurde eine Kombination verschiedener Methoden verwendet, unter Anwendung des klassischen Bioassay-geführten Ansatzes und Genome Mining.

Eine frühere Studie zeigt, dass zusätzlich zur Herstellung der antimykotischen Substanz Nystatin, *Streptomyces noursei* ATCC 11455 die Fähigkeit besitzt eine antibakterielle Verbindung, bei der es sich um ein neues Polyketid handeln könnte, aktiv gegen Gram+ Bakterien, zu produzieren [1]. In diesem Diplomprojekt wurden die Ergebnisse aus der Primärstudie reproduziert und versucht den für die Bioaktivität verantwortlichen Metaboliten (Antibiotikum X) aufzureinigen. Neun Fraktionen dieses Aufreinigungsprozesses wurden zur MS-Analyse geschickt, wobei das Vorhandensein zahlreicher Makrolide bestätigt wurde, was die Aufreinigung erschwerte. In den Fraktionen wurden zwei bereits registrierte Moleküle (YL 02107Q und CAS 37359-09-4) vermutet, aber auch zusätzlich mögliche neue Metabolite mit einer Verwandtschaft zum Antibiotikum YL 02107Q. Die Analyse der Genomsequenzierungsdaten ergab eine potentiell neue T1PKS, welche von Cluster 14 kodiert wird und für die Produktion von YL 02107Q und verwandten Verbindungen verantwortlich sein könnte. Um diese Hypothese zu beweisen, wurde eine insertionale Disruption von Cluster 14 durchgeführt. Die Analysen dieses Stammes zeigten, dass in Bioassay-Experimenten keine antimikrobielle Aktivität nachgewiesen werden konnte.

Im Fall von *Amycolatopsis* sp. YIM10 wurde eine heterologe Expression von Cluster 5, der für

eine vermeintlich neue NRPS kodiert, in zwei ausgewählten Wirtsorganismen, *Streptomyces coelicolor* M1154 und *Streptomyces albus* Del14, durchgeführt. Die Fermentation in verschiedenen Medien und die anschließende Analyse der Extrakte mittels analytischer HPLC wies auf keine neuen Moleküle hin. Basierend auf den Ergebnissen von antiSMASH wurden acht Gene, die für mögliche regulatorische Proteine kodieren, innerhalb von Cluster 5 gefunden, von denen drei (*gntR*, *asnR* und *luxR*) ausgewählt wurden, um überexprimiert zu werden. Der Überexpressionsversuch wurde mit *gntR* im *S. albus* Del14 Stamm, der Cluster 5 beherbergt, durchgeführt, aber es wurden keine neuen Metaboliten mittels MS Analyse nachgewiesen. Allerdings konnte im rekombinanten Stamm eine deutliche Erhöhung der Metabolitenproduktion, nach der Überexpression von *gntR*, festgestellt werden.

Chapter 2

Abstract

In this project, two Actinomycetes strains, *Streptomyces noursei* and *Amycolatopsis* sp. YIM10, were selected to isolate putative new secondary metabolites. For this purpose, a combination of different methods was used, by application of classical bioassay-guided approach and genome mining.

A previous study showed that, additional to the production the antifungal compound nystatin, *Streptomyces noursei* ATCC 11455 produces an antibacterial compound active against Gram+ bacteria, which might be a new polyketide [1]. During this diploma project, the results from this primary study were reproduced and an attempt was made to purify the metabolite responsible for the bioactivity (antibiotic X). Nine fractions from this purification process were sent to MS analysis, confirming the presence of numerous macrolides, which made the purification difficult. Among other compounds, two already registered molecules (YL 02107Q and CAS 37359-09-4) were assumed to be present in the fractions, along with potentially new metabolites, related to the antibiotic YL 02107Q. The analysis of genome sequencing data revealed a potentially new T1PKS encoded by cluster 14, which might be responsible for the production of YL 02107Q and related compounds. To prove this hypothesis, an insertional disruption of cluster 14 was performed. Analyses of the extract from this strain confirmed that no antimicrobial activity could be detected in bioassay experiments.

In the case of *Amycolatopsis* sp. YIM10, a heterologous expression of cluster 5, which encodes for a putative new NRPS, was performed in two selected host organisms, *Streptomyces coelicolor* M1154 and *Streptomyces albus* Del14. The fermentation in different media and

the subsequent analysis of the extracts by analytical HPLC did not yield any new compounds. Based on the results from antiSMASH, eight genes encoding putative regulatory proteins were localized within cluster 5, of which three (*gntR*, *asnR* and *luxR*) were selected for overexpression. The overexpression attempt was accomplished with *gntR* in the *S. albus* Del14 harboring cluster 5, but no new metabolites were detected by the MS analysis. Nevertheless an increase of metabolite production was detected in the recombinant strain after overexpression of *gntR*.

Chapter 3

Introduction

3.1 History of antibiotic discovery

Antimicrobial substances are one of the most triumphant chemotherapeutic agents in the evolution of medicine [2]. The treatment of infections and cancer therapy have been revolutionized by the discovery of these drugs [3, 4].

Even before the peak of antibiotic discovery in the 1950s, antibiotic traces of for example tetracycline have been found in ancient human remnants, indicating the addition of antibiotic containing supplements in diet [2]. Nevertheless, after the discovery of salvarsan by Paul Ehrlich in 1910 and penicillin by Alexander Fleming in 1928, antibiotic exploration increased more and more leading to the antibiotic era and to the golden age (1940-1960) where most nowadays used antibacterial substances were discovered [3, 5, 6]. After that time, infectious diseases were assumed to be defeated and the research of antimicrobial substances decreased [5]. The decreasing chance of finding new substances, because of the rediscovery of already known compounds, as well as the declining profit led many pharmaceutical companies to abandon research in this area [7]. These circumstances lead to a gap in the development of new antibiotics and only a few new substance classes where identified since that time [7, 8]. This timeline is depicted in Figure 3.1.

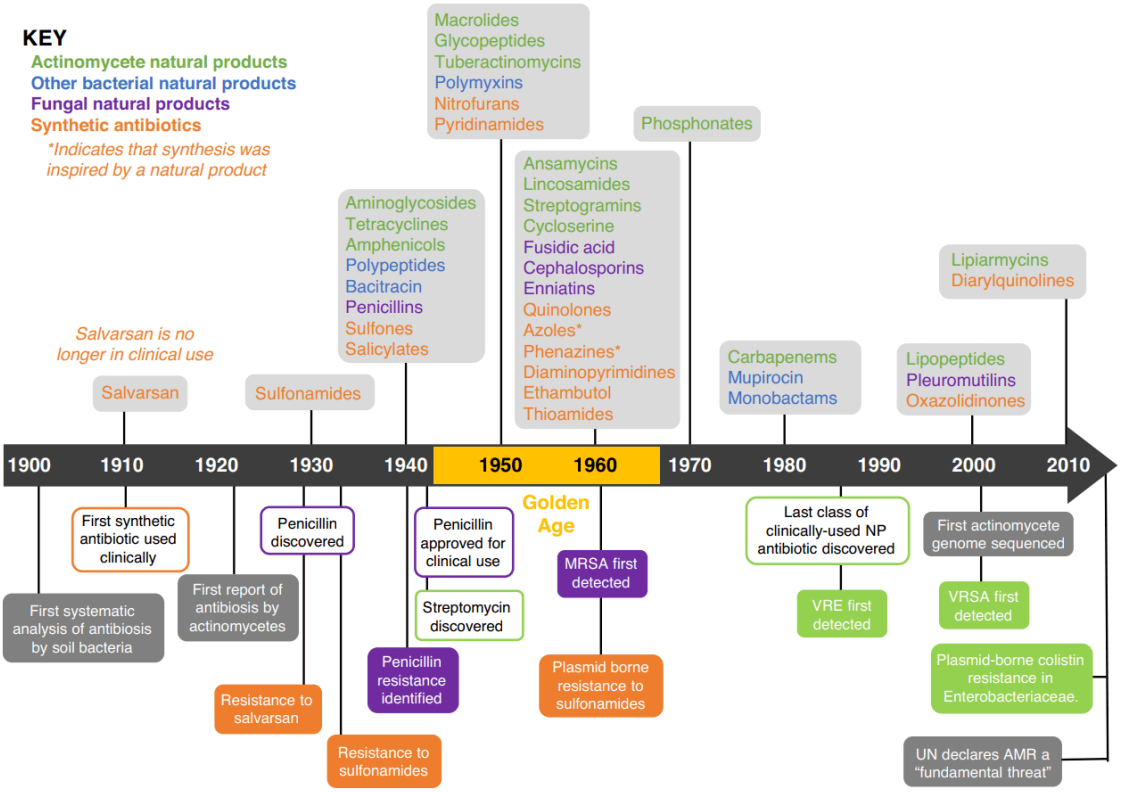


Figure 3.1: Timeline of antibiotic discovery from 1900 to 2010 including the golden age of antibiotic discovery (1940-1960) where most currently used antibiotics were detected [3].

Over the past years, the invention progress regarding the antibiotic discovery has been optimized and modified which led to a renewed increase in the interest of NP research [7, 9]. In the golden age, natural product (NP) discovery was merely done by cultivating different bacterial or fungal organisms under laboratory conditions and screening their produced metabolites for any antibiotic activity, which lead to the discovery of the most successful medicines [10]. Scaffolds of these found structures were also used for further chemical modifications to create new substances or enhance their drug-like properties [9, 11]. Even nowadays, modification of already established products is thought to be a win-bringing change to overcome the rising antibiotic resistance [12]. In the late 90s, another approach, the high-throughput screening, was followed as an alternative to common methods [12, 13]. Moreover, after the sequencing of the first genomes of two Actinomycetes strains at the beginning of the 21st century, a new era was established, giving new information about genome sequences and the possible production of novel secondary metabolites with yet unknown potential [10, 14].

3.2 Antibiotic resistance - the need for new substances

Selman Waksman defined an antibiotic as an effective tool, usually a small molecule produced by a microorganism, that can inhibit the growth of microbes by different interactions and using therapeutic concentrations, treat infections without being toxic to the user [3, 5]. These molecules are products of the secondary metabolism of bacteria and fungi as they are not essential for the survival of the organism [15, 16]. During the golden age of antibiotic discovery, many antibiotics were successfully introduced to medical practice, which led to the belief in the victory over the infectious diseases [5, 17]. However, resistances were detected very soon after the first antibacterial substances were administered and an antibacterial resistance crisis gradually developed [17].

Resistance rates rose over the last 2 decades and infectious diseases again became a human health threat, as according to the study of the European Centre for Disease Prevention and Control, it is predicted that about 33000 people die every year as a result of antibiotic-resistant infections [5, 18, 19]. Reasons for that enhancement are not just the increase of antibiotic prescriptions, but also the incorrect therapy times, prophylactic drug intake, false self-medication in form of antibiotic therapies or even the extensive use of antibiotic substances in the agricultural industry [3, 5, 20, 21]. Nevertheless, despite the apparent need of new antibiotics, only six new anti-infectives were discovered and marketed since 2003, even though, there already occurred resistances to every receivable antibiotic substance published by now [7, 14, 22].

These resistances can be either intrinsic, based on morphological characteristics of individual species or the absence of susceptible targets for a specific antibiotic, can be the caused by mutations, or be the result of the exchange of genetic information via horizontal transfer of resistance genes [23].

The first hallmark in the emergence of resistances was the penicillin resistance which was followed by tetracycline resistances, macrolide resistances and many more, which is visualized in Figure 3.2 [17, 20].

Penicillin targets the cell wall of bacteria, and the resistant bacteria produce β-lactamases which function as amidases that can catalyze a cleavage of the β-lactam ring and in this way deactivate the antibiotic [23, 24]. Another example of this hydrolytic resistance mechanism is the production of esterases to catalyze the cleavage of the macrolactone ring of macrolide

antibiotics such as erythromycin, whereas resistance against tetracycline on the other hand is accomplished via oxidation by a flavin-dependant monooxygenase (TetX) which leads to the disruption of the Mg^{2+} binding site which is needed for antibiotic activity [24, 25]. Nevertheless the tetracycline resistance also occurs via efflux pumps or ribosomal protection, which are the most frequently preferred mechanisms [25, 26].

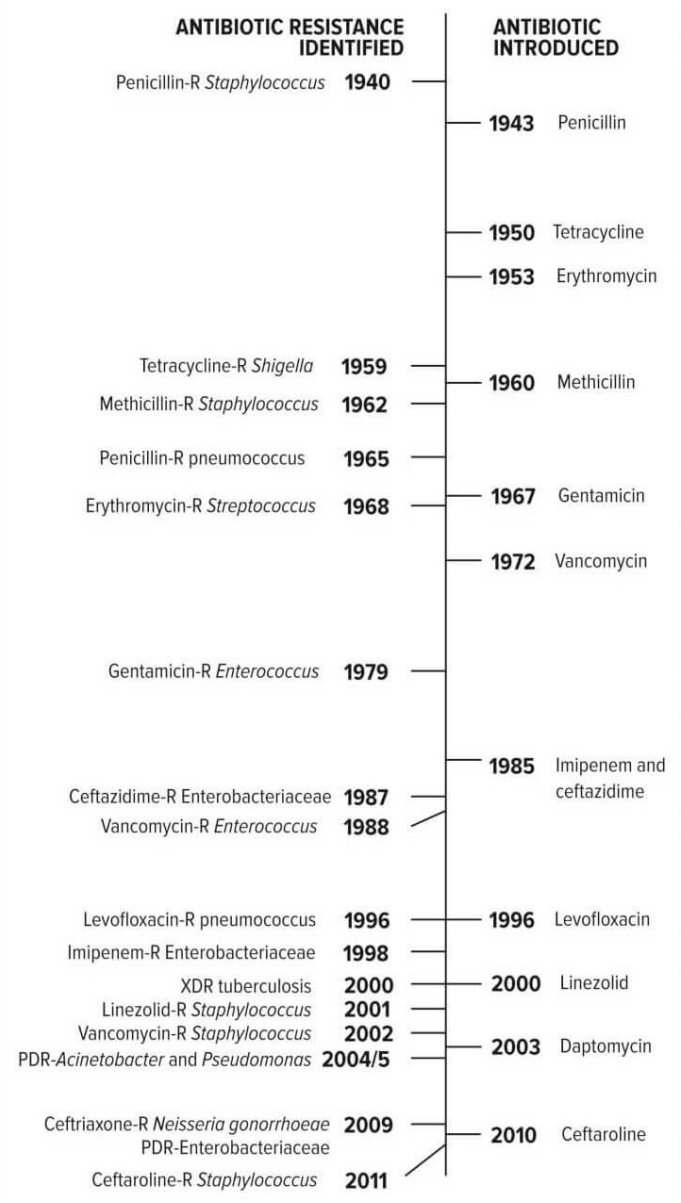


Figure 3.2: Timeline of the development of antibiotic resistances in various bacteria as related to the corresponding antibiotic substances. Data was collected from medicinal reports and early reports in literature. Penicillin was already used to a limited extent before 1943 before it was commercialized. R = resistant; XDR = extensively drug-resistant; PDR = pan-drug-resistant [17].

The World Health Organization (WHO) already tried to strengthen awareness of this situation by publishing a worldwide plan on antimicrobial resistance in May 2015 containing various points for the prevention of the spreading of antimicrobial resistance [27, 28]. The effects of not counteracting the increase in resistances will be that therapy with first line antibiotics will become more challenging which will be resulting in the use of more toxic drugs, longer hospitalization times and higher mortality rates [21, 27]. Accordingly, the need for the development of new antibiotic drugs is urgent [29, 30].

3.3 Actinomycete bacteria as antibiotic producers

Actinomycetes are filamentous bacteria with high potential for producing antibiotically active secondary metabolites, as was already postulated by Selman Waksman in the late 1930s [3]. Waksman discovered the first non-toxic antibiotic substance streptomycin produced by an Actinomycete, *Streptomyces griseus*, in 1944, followed by many more Actinomycetes-derived substances [4, 5, 6]. Today, more than 50 % of all clinically used antibiotics are derived from this bacterial phylum [7]. Some examples can be seen in Figure 3.3.

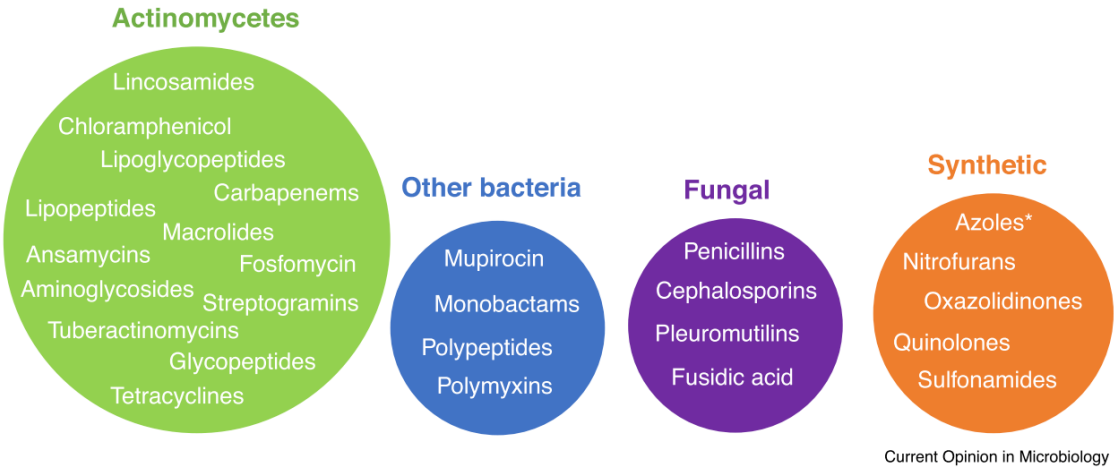


Figure 3.3: Demonstration of antibiotics derived from microorganisms and synthetically produced agents. As seen, most of the discovered antibiotics were produced by the phylum of the Actinomycetes [3].

With the possibility of the genome sequencing, it was discovered that there are many more secondary metabolites in this phylum to be explored, as each Actinomycete bacterium has the

genetic ability of producing up to 50 secondary metabolites [9, 31]. Together with the detection many, until then unknown, gene clusters responsible for secondary metabolite biosynthesis, their ability of producing new chemical substances is much higher than previously expected [31]. Within this, the genus of the *Streptomyces* is the most promising and its genetic resources have not yet been sufficiently exploited [3, 32]. The large, GC rich chromosome which harbors hundreds of genes involved in secondary metabolite production, is one important commonality between all the *Streptomyces* species [33]. All in all, every single *Streptomyces* species has the ability of producing approximately more than 30 secondary metabolites and strains of the same species are even able to produce a variety of different compounds [34, 35]. Hence, this species has a great potential for the discovery of new bioactive compounds, although most of them are not produced under laboratory conditions [34]. These products are specified by the so called "silent" gene clusters, which are further defined in 3.6.1 [36].

3.4 Biosynthesis of secondary metabolites

The biosynthesis of secondary metabolites is, in comparison to the one of primary metabolites, not necessarily relevant for the survival of an organism [15]. However, secondary metabolites, as for example antibiotics, antifungals, anticancer agents or many other medically important compounds, are utilized for communication between different cells, alteration of physiology or even as biological warfare strategy in case of nutrient limitation [37, 38]. This production can also be dependent on growth conditions of different species, or in case of the *Streptomyces* genus, even on the state of the life cycle of these organisms in different cultivation conditions [39, 40]. Within this species, the formation of reproductive aerial mycelium co-insides with initiation of the antibiotic production in solid cultures, whereas in liquid cultures, this production is attributed to the stationary phase of growth [40, 41, 42].

3.4.1 Biosynthetic gene clusters

Natural products, especially the ones derived from microorganisms, are produced by metabolic pathways encoded by genes which are co-localized in the chromosomes of the producers [39, 43]. These so called "gene clusters" consist of several genes responsible for the production of the antibiotic scaffold as well as for the modification of this scaffold, the regulation of

the corresponding genes, the transport of the biosynthesized molecule, as well as resistance genes [44, 45]. Regulatory genes are responsible for the control of the genes' transcription and therefore for the regulation of the secondary metabolite production [44]. Metabolites are transported out of the bacterial cells through "efflux pumps", which are also encoded within the corresponding gene cluster, as well as genes for resistance as a protection mechanisms of the organism against its own product [44, 45]. An example of this protective mechanism can be found within *Saccharopolyspora erythtraea*, which produces a methylase that methylates the ribosome, target of erythromycin, making the organism resistant to this antibiotic [46]. A representation of the organization of a biosynthetic gene cluster is shown in Figure 3.4, while the most common modifications of the molecule scaffolds are presented in Figure 3.5.

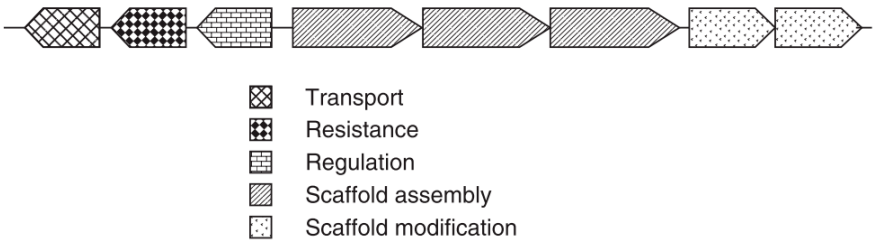


Figure 3.4: Organization of a biosynthetic gene cluster used for antibiotic production including genes for scaffold building, modifications, regulation, transport and resistance [44].

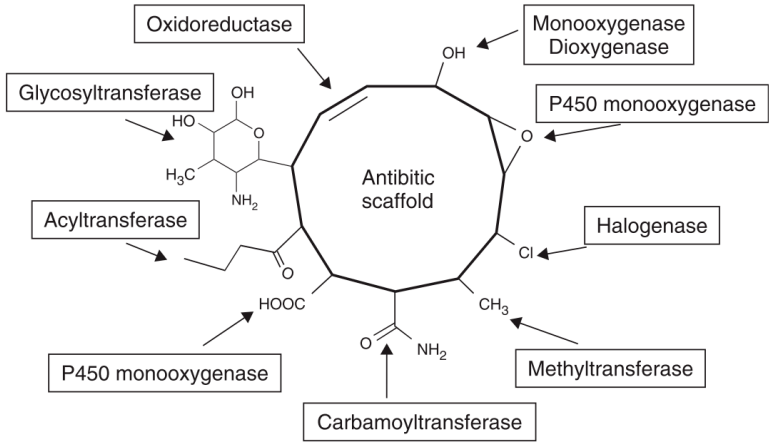


Figure 3.5: Most common possibilities of modification of an antibiotic scaffold [44].

The first step in the biosynthesis of a secondary metabolite is the building of a scaffold using different pathways as for example the polyketide synthase (PKS) for producing polyketide antibiotics, or the non-ribosomal peptide synthetase (NRPS) for creating peptide antibiotics [44].

3.4.2 Polyketide synthases

A particularly large group of secondary metabolites, produced by various microorganisms, which are used for treating numerous diseases, can be found within the class of polyketides [47]. Polyketides are formed by polyketide synthases (PKS), whereby there are different types to be distinguished such as PKS I, II or III, which are either multimodular (PKS I) or iterative [47, 48, 49]. These enzymes and their differences including functions and structures are described in the chapters 3.4.2.1 and 3.4.2.2.

3.4.2.1 Modular PKS I

The PKS type I is a modular polyketide synthase, featuring a multi-domain architecture, consisting of 3 core domains which include the acyltransferase (AT), the ketosynthase (KS) and the acyl carrier protein (ACP) [48, 50, 51, 52]. Molecules of acyl-CoAs are selected and loaded by AT onto the activated ACP, preparing the newly loaded building block and the already created polyketide chain to be condensed via the decarboxylative Claisen-type condensation by KS [48, 49, 50]. The number of modules in a modular PKS reflects the number of condensation reactions required by the synthase to build the desired polyketide, which is terminated by a thioesterase (TE) as the last domain [53, 54]. These essential steps in the biosynthesis of the polyketide backbone are presented in Figure 3.6.

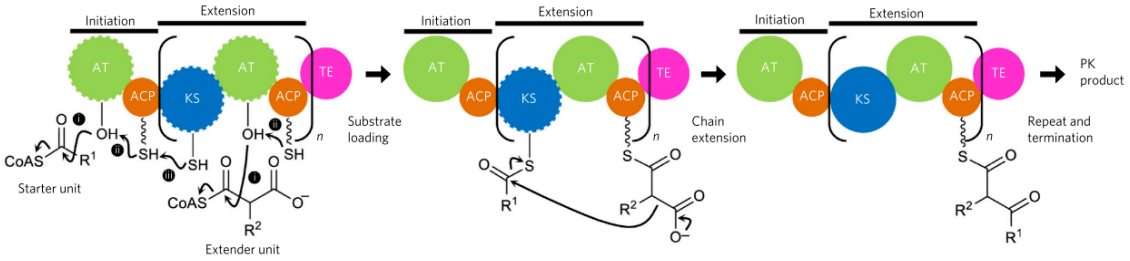


Figure 3.6: Basic steps in the biosynthesis of a polyketide backbone including the use of the acyltransferase (AT), the ketosynthase (KS) and the acyl carrier protein (ACP). The completed structure is released from the PKS via TE as a last domain [50].

Additionally, there are enzymes for the β-keto-processing as ketoreductases (KR), dehydratases (DH) and enoyl reductases (ER), which are optional domains for the modification of the produced backbone before continuing the elongation of the polyketide chain for another round,

positioned between AT and ACP [48, 50, 54, 55].

One of the best studied examples of antibiotics produced by a type I PKS is erythromycin, which is presented in Figure 3.7 [54].

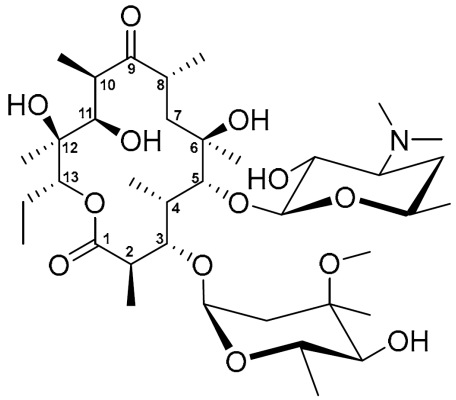


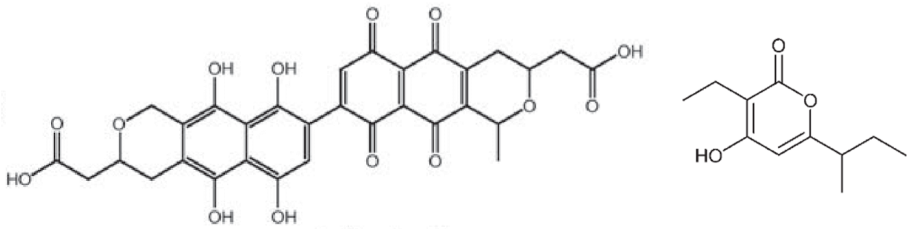
Figure 3.7: Chemical structure of the macrolide antibiotic erythromycin A produced by a type I modular PKS [48].

The PKS which is responsible for the production of erythromycin or in particular the parent macrolactone 6-deoxyerythronolide B (1, 6-dEB), 6-deoxyerythronolide B synthase (DEBS), consists of three multifunctional proteins (DEBS 1-3), which are composed of two modules each [56, 57]. In general, the biosynthesis of erythromycin can be divided into two steps, the production of the core polyketide via sequential condensation of methylmalonyl CoA building blocks catalyzed by the PKS and the modification of the core by the tailoring enzymes such as regiospecific hydroxylases, methyltransferases and glycosyltransferases [58]. The initiation of the biosynthesis is succeeded by loading a propyonyl primer onto the first module b< an AT-domain [57]. Stepwise after the finished core molecule 6-deoxyerythronolide B is produced, it will be hydroxylated using a cytochrome P450 hydroxylase, L-mycarose will be added to the C-3 hydroxyl group and after that, D-desosamine will be attached to the C-5 hydroxyl group, both reactions catalyzed using specific glycosyltransferases [58]. Finally, the molecule is methylated and hydroxylated, using an O-methyltransferase and a cytochrome P450 hydroxylase, to give the final erythromycin A [58].

3.4.2.2 PKS II and PKS III systems

In comparison to type I PKS, each catalytic unit of a type II PKS is represented by its own protein, which are used for building of aromatic polyketide, as for example the anticancer agent doxorubicin or the antibiotic agent actinorhodin [49, 51, 54, 59, 60]. These so called minimal PKSs consist of a KS_α and a KS_β, which are responsible for the exclusive elongation of manoyl-CoA molecules, and an ACP [48, 61]. After the elongation process is finished, the produced molecule will be cyclized, folded and modified if needed [48, 61, 62].

The last group of PKSs, the type III PKS, consists of simple homodimers with one catalytic center were elongation via condensation reaction takes place [63, 64]. This PKS is able to produce a wide range of various compounds of simple composition [65]. Moreover, products of the bacterial type III PKS may be used, for example, as precursors for antibiotics, lipids that serve as protection against antibiotics or pigments for UV protection [64]. Examples of a type II and type III PKS products are given in Figure 3.8.



(a) Actinorhodin (b) Germicidin

Figure 3.8: Examples of products of a type II PKS, actinorhodin (a), and of a type III PKS, germicidin (b) [51, 66].

3.4.3 Non-ribosomal Peptide Synthetases

Contrary to the common assumption that peptides are synthesized via the ribosome, non-ribosomal peptides are produced via large multi-functional enzymes, so-called non-ribosomal peptide synthetases (NRPSs), without needing the ribosome, in bacteria and fungi [67, 68, 69]. These enzymes are responsible for the production of a large variety of secondary metabolites with different functional applications such as antibiotics, antimycotics, immunosuppressive drugs or antitumor agents [69, 70, 71]. A selection of these substances is presented in Figure 3.9.

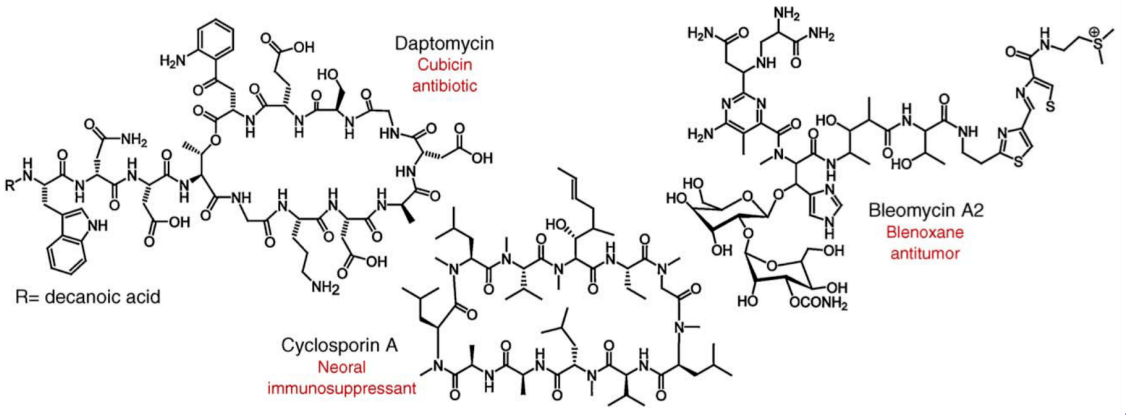


Figure 3.9: Presentation of different secondary metabolites which are clinically used and produced by NRPSs: daptomycin, a last resort antibiotic; bleomycin A2, a tumor therapeutic agent; cyclosporin A, an immunosuppressive drug [69].

Generally, NRPSs are composed of different modules of peptide synthetases, each responsible for another step in synthesizing a polypeptide chain, whereas there are three mandatory enzymes domains in each module that are essential for a catalytic cycle [67, 68]. The cycle starts with the activation of previously selected amino acids by adenylation domains (A), which is an ATP-dependent process [50]. The activated amino acids will be transferred to the peptidyl carrier protein (PCP), where it can be modified via N-methyltansferases, and then will be condensed with another amino acid, forming a polypeptide chain, catalyzed by condensation domains (C) [50, 69]. At the end of the biosynthesis, the thioesterase (TE domain), located after the PCP domain of the last module, releases the polypeptide chain via hydrolysis, often leading to the formation of a macrocyclic structure [68, 69]. The whole process is shown in Figure 3.10.

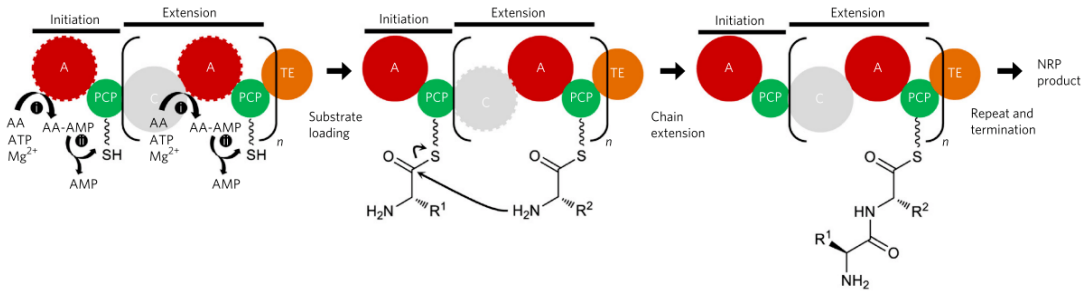


Figure 3.10: Illustration of the NRPS modules synthesizing a non-ribosomal peptide, including the use of adenylation (A) domains, peptidyl carrier protein (PCP) domains, condensation (C) domains and thioesterase (TE) domains [50].

It also has to be considered that there can be further modifications after the elongation of the polypeptide chain as for example by epimerization through E domains or also by oxidation (Ox domains) or hetero-cyclization (HC domains) [50].

Great variety of compounds generated by the NRPS systems is also accomplished by the use of non-proteinogenic amino acids, fatty acids or even α-hydroxy acids and many other molecules additionally to the 20 proteinogenic amino acids [69]. Furthermore, due to the reflecting similarity of the NRPS to the PKS, naturally derived hybrid products can be produced, like rapamycin, combining both biosynthetic pathways [50].

3.5 Regulation of secondary metabolism in *Streptomyces*

The regulation of the synthesis of secondary metabolites in *Streptomyces* was well investigated within the *Streptomyces coelicolor* model organism resulting in the awareness, that antibiotic production is a highly regulated and controlled process [40].Research on SM biosynthesis in this bacterium revealed the involvement of different global and pathway-specific regulators [40, 46, 72]. Moreover, there are a lot of environmental factors that can influence secondary metabolite production, such as nutrition and available carbon sources, small molecule concentrations or physiological stress [40, 46, 72]. However, in this chapter it will be focused on the regulatory specific influence on antibiotics production.

3.5.1 Regulation via gene cluster encoded regulators

Cloning of genes responsible for the antibiotic production in *S. coelicolor* showed that for the production of various compounds, regulator genes as for example those encoding pathway-specific activators were required. These regulatory gene in turn are controlled by pleiotropic regulatory genes such as *bldA* and *adpA* in *S. coelicolor* [72, 73, 74]. In case of the pathway-specific regulators, there can be activators, which show a positive effect on gene transcription, or repressors, which cause a negative effect on gene transcription[45, 75].

Either the stabilization of the initiation complex between the RNA polymerase and the promoter or the speeding up of the building of the open complex during transcription, are the most common operating modes of simple activators [33, 76]. Whereas in case of repressors, the mode

of action is accomplished either by preventing the initiation or elongation of transcription, or by competing for binding with an activator [33, 75]. The outcomes of the regulator functioning in gene cluster expression are shown in Figure 3.11.

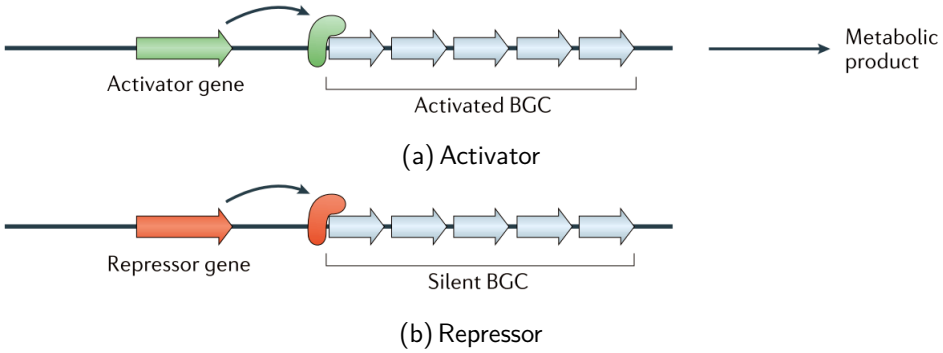


Figure 3.11: Illustration of the functions of an activator (a) in gene transcription resulting in secondary metabolite production, and a repressor (b), preventing secondary metabolite production (modified from [59]).

Many transcriptional activators within the *Streptomyces* species belong to the SARP (*Streptomyces* antibiotic regulatory protein) family, which contain a winged helix-turn-helix structure and recognize regulatory regions of their target genes, resulting in the activation of secondary metabolite production by the corresponding clusters [40, 41]. These proteins of the SARP family are only found in Actinomycetes, and are known to be responsible for the regulation of the biosynthesis of different secondary metabolites, such as polyketides, ribosomal peptides, non-ribosomal peptides and many others [59, 77].

The other main group of transcriptional activators in *Streptomyces* resembles the LAL-family which stands for large ATP-binding regulators of the LuxR family, originally identified in proteobacteria [40, 77]. Within the *Streptomyces* species, they mostly have been found in gene clusters encoding for PKS and hybrid PKS/NRPS systems for secondary metabolites biosynthesis [77]. The structure of the regulators belonging to this group is characterized by the presence of an ATP binding side at the N-terminal end and a helix-turn-helix motif at the C-terminus, which is responsible for the DNA binding [77, 78]. Members of the LAL family can be found for example in *Streptomyces venezuelae* within the cluster responsible for the production of pikromycin, in *Streptomyces hygroscopicus* regulating the production of rapamycin or in *S. noursei* where three LAL-regulators can be found within the cluster encoding for the production of nystatin [41, 78].

3.6 Genome mining - an effective tool for the discovery of new NPs

The concept of genome mining has provided a new aspect in the discovery of secondary metabolites by identifying the genes required for secondary metabolism, which has led to the discovery of an extraordinary potential in compound production of many species [79].

The first step in the genome mining process is the use of bioinformatic tools for the identification of previously undesignated compounds via interspecies comparison of conserved genome sequences [37, 80]. An important tool for enabling this is antiSMASH (antibiotics and secondary metabolites analysis shell), which is an online tool for the identification of biosynthetic gene clusters (BGCs) in the genomes of different species [14, 81]. In addition, this tool is linked to a database that provides access to many freely accessible genome sequences [81]. This prediction of the biosynthetic gene clusters is followed by experiments aimed at controlling the production of the compound in question and the identification of its structure [80]. The whole process of the genome mining is depicted in Figure 3.12.

One of the starting points for the genome mining is to choose an organism with available genome sequencing data which then can be further analyzed via bioinformatic tools to identify potential new BGCs [59, 80]. Another powerful tool of the genome mining is the molecular biology, which can be used for the activation of silent BGCs or for the improvement of the production of a target molecule. For example by applying techniques for the overexpression of an activator or for the inactivation of a repressor, it is possible to promote the expression of the BGC of interest [37, 45, 59]. Moreover, one of the broadly used analytical techniques is the comparison of metabolite profiles of a control strain with a non-activated BGC and the same strain after molecular-genetic modification [59]. This can be achieved for example by comparing chromatograms after performing high performance liquid chromatography (HPLC), measuring the absorbance in different wavelengths [59].

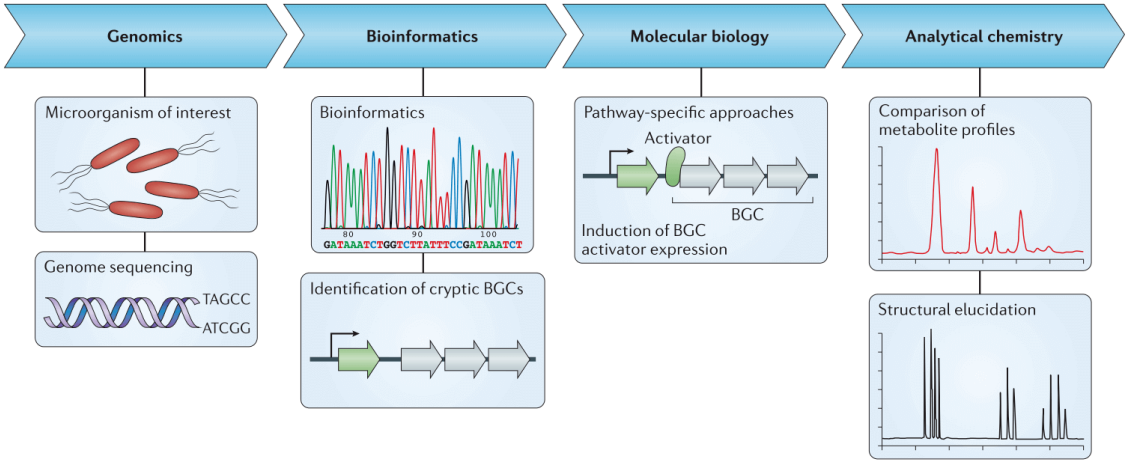


Figure 3.12: Illustration of the process of genome mining, including collecting the data of the organism of interest, the identification of the biosynthetic gene cluster using bioinformatic tools, controlling production of the compound through the use of molecular biology and the determination of the structure (modified from [59]).

This approach has been used since the uncovering of the fact, that *S. coelicolor* can produce much more secondary metabolites than originally expected, by David Hopwood and coworkers using whole genome sequencing [80]. The hope in finding new, clinically useful compounds using these until now unknown secondary metabolite biosynthesis gene clusters, also found within many more Actinomycetes, has been raised by that discovery [60].

3.6.1 Silent gene clusters

Progress in genome sequencing, in particular the use of genome mining, has shown that there is an unprecedented number of biosynthetic gene clusters in a wide variety of organisms that are not active under laboratory conditions or that yield very small quantities of the encoded products [82]. These "silent" or "cryptic" gene clusters offer a tremendous potential to produce new secondary metabolites and thus potentially new medically effective substances such as antibiotics or other chemotherapeutic agents [83]. It was shown, that the number of such silent gene clusters can be up to 5 - 10 times higher than the one of active clusters [84, 85].

Especially in case of the *Streptomyces* species, it was found that each strain is capable of producing more than 20 SMs, of which only a small number are actually biosynthesized under laboratory conditions [86, 87]. Therefore, activating those cryptic genes is of great interest for

natural product discovery hoping to induce production of new compounds [84, 87, 88].

There are a number of ways to enable the activation of these gene clusters, of which some selected are described in the next chapter [36].

3.7 Strategies of an optimization of NPs production

Due to the fact that most BGCs in microorganisms are silent or expressed on a very low level, the activation of these biosynthetic pathways is a major interest both for industry and for educational institutions [88]. The changing of cultivation parameters or the modification of genetic information are the two main approaches of activating gene clusters, of which some are presented in the next chapters [82].

3.7.1 Manipulation of growth conditions

The modification of easily controllable culture parameters such as media composition, oxygen supply, culture vessels, pH values, easy accessibility of carbon and nitrogen sources, the addition of enzyme inhibitors and many more, is applied in the so-called OSMAC, one strain many compounds, approach [89, 90, 91]. This method serves as an approach to increase the production of secondary metabolites and as an alternative to high-throughput screening done by industry [89, 92]. It has been proven that even the smallest change in the cultivation parameters can lead to changes in many aspects of the production of secondary metabolites and can even completely alter the metabolic profile of numerous organisms and change the quantity and variation of their fermentation products [89, 90].

Examples for this effect of using the OSMAC approach for triggering secondary metabolite production are the production of ectoine and 5-hydroxyectoine by *S. coelicolor* using high salt concentrations and/or high temperature during fermentation [93]. Another application for this approach is the treatment of the bacterial cells with limiting growth conditions and/or the shock of the bacteria by an acidic pH, resulting in the production of methylenomycin [93]. Even some simple changes in growth media constitution, such as the replacement of tap water by distilled water, during cultivation of *Paraphaeosphaeria quadriseptata*, lead to the isolation and identification of six new secondary metabolites [94].

3.7.2 Modification of transcriptional regulator expression (activation, inactivation)

Another approach to the overproduction of secondary metabolites is the modification of regulator expression [45]. This includes the activation or induction of regulators with a positive impact on the production of secondary metabolites via overexpression, or the repression or rather the deletion of those which repress the production of potentially active compounds [45]. There can either be manipulation of global, pleiotropic regulators, which are responsible for the activation of different pathway specific regulators and therefore for the regulation of more than one metabolic pathway, or direct manipulation of pathway specific regulators to increase the production of secondary metabolites [95]. This chapter is focused on the latter. An illustration of the activation and inactivation of pathway specific regulators, activator and repressor, can be seen in Figure 3.13.

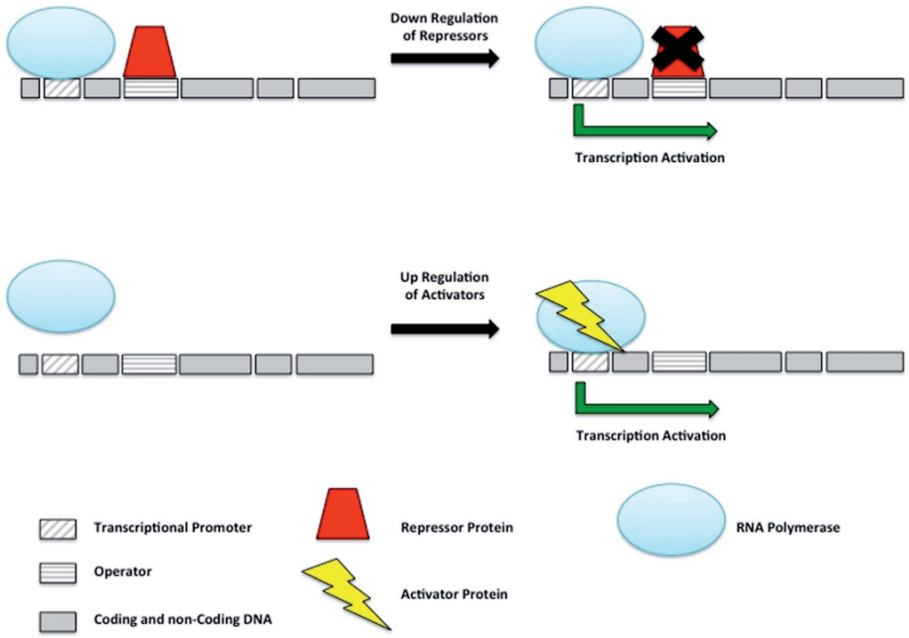


Figure 3.13: Illustration of the process of the down-regulation of a repressor to enhance metabolite production, and of the overexpression of an activator to accomplish overproduction of the secondary metabolite in question [75].

The function of a repressor is to bind to an operator and thus prevent the RNA polymerase from binding to the DNA strand and consequently inhibit the transcription of this DNA fragment. Hence, by inactivating these repressor genes, there is an increase in the transcription of the

previously repressed genes and thus an increase in the production of secondary metabolites [75]. The inactivation of a transcriptional regulator belonging to the LysR family in *S. lividans* for example, led to the overproduction of the antibiotic actinorhodin [45].

Another often used strategy for the enhancement of secondary metabolite production via modification of regulatory genes is the overexpression of pathway specific positive regulators to activate silent clusters [91]. One of the best examples is the overexpression of the genes *actII-orf4* and *redD*, which belong to the SARP regulator family and whose modification in *S. coelicolor* leads to increased production of actinorhodin and undecylprodigiosin [45].

3.7.3 Heterologous expression

Another way of activating silent gene clusters, to enhance production yields or to enable the production of SMs whose native producers cannot be cultivated, is the heterologous expression of whole gene clusters in defined host organisms [96, 97]. Generally, the process of the heterologous expression can be divided into the isolation of the DNA of interest, the insertion of this DNA into a suitable vector for mobilisation of DNA into the host organism, the selection of a suitable heterologous host, the possible genetic manipulation, the maintenance in the heterologous host and the optimization of fermentation processes to ensure maximum production yields [98]. The whole process showing the different steps is depicted in Figure 3.14.

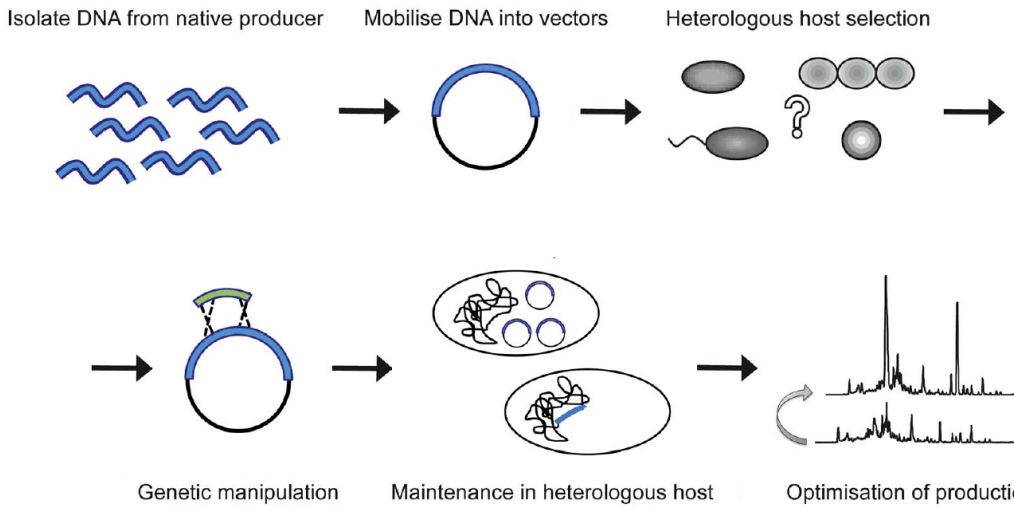


Figure 3.14: Illustration of a basic workflow of the heterologous expression of biosynthetic gene clusters in suitable host organisms for the enhancement of secondary metabolite production (modified from [98]).

The choice of the suitable host organism is crucial for the heterologous expression experiments, as they have to be culturable, need to be easily accessible to genetic modifications if needed, as well as contain the cellular machinery required for the expression of the heterologous genes [98, 99]. *Streptomyces* strains as for example the well-studied species *S. coelicolor* and *S. lividans* are often used as host organisms, even though these bacteria often produce their own metabolites, which can lead to a competition for building blocks in molecule synthesis and therefore to an unintended influence on the introduced heterologous pathway [100]. Dedicated hosts have been created to eliminate the background influence of other biosynthetic pathways, such as those for the production of antibiotics, as shown by the example of the increased production of the NRP antibiotic daptomycin in a *S. lividans* host, where the native production pathway of actinorhodin was eliminated and the production medium was optimized [101]. In addition, genetic modification, such as the replacement of promoters, can also increase the production rate of heterologous biosynthetic products, especially in host organisms that are genetically different from the native producers [98].

The last step of a successful heterologous expression, except the optimization of fermentation parameters, is the maintenance of the inserted genetic information which is basically performed either via integration into the hosts genome or the stable maintenance of the compatible plasmid within the host [98].

Chapter 4

Aim of the work

As already mentioned, growing resistance rates are progressively leading to an increase in infectious diseases [5, 18]. Therefore, new antibiotics are needed more than ever to counteract this trend [30].

Two different Actinomycetes strains were chosen to be investigated in this project, *Streptomyces noursei* wild-type strain and *Amycolatopsis* sp. YIM10. The aim of this work was the discovery of new secondary metabolites by a classical bioassay-guided fractionation and by using advanced genomics-driven methods within these two Actinomycetes species.

In the case of *S. noursei*, it was already known from a previous publication [1] that this species produces a putative new antibiotic substance under certain conditions. This compound was planned to be isolated, purified by bioassay-guided fractionation and the structure to be determined. Furthermore, the aim was to identify the gene cluster responsible for this and thus to explore the possible synthesis pathway with the help of genome mining and the resulting construction of a knock-out mutant.

For the *Amycolatopsis* sp. YIM10 we were interested in the heterologous expression and additionally the overexpression of cluster 5 via upregulation of identified regulator genes for the increase of secondary metabolite production. These genes were identified with the help of bioinformatic tools. Produced mutant strains were meant to be fermented, cultures extracted and extracts analyzed via bioassays and analytical HPLC.

Chapter 5

Materials

5.1 Organisms & vectors

Table 5.1: Plasmids used in this work.

Plasmid	Properties	Source/reference
pSOK201	pSG5 and ColE1 replicons, oriT and Am^R	[1]
pSOK201_KN	pSOK201 with an internal fragment of cluster 14 from *S. noursei* ATCC 11455 (inactivation of cluster 14); Am^R	This work
pCLY10	*E. coli*/yeast/*Streptomyces* shuttle vector; Am^R	[102]
pJP_Cl5	Vector for heterologous expression of cluster 5 from *Amycolatopsis* sp. YIM10, based on pCLY10, Am^R	This work
pUWLoriT	*E. coli*/*Streptomyces* shuttle vector with ermE promoter, Amp^R, $Thio^R$	[103]
puWLoriT_gntR	Overexpression of *gntR* regulator gene, cluster 5 from *Amycolatopsis* sp. YIM10, based on pUWLoriT, Amp^R, $Thio^R$	This work
puWLoriT_asnR	Overexpression of *asnR* regulator gene, cluster 5 from *Amycolatopsis* sp. YIM10, based on pUWLoriT, Amp^R, $Thio^R$	This work
puWLoriT_luxR	Overexpression of *luxR* regulator gene, cluster 5 from *Amycolatopsis* sp. YIM10, based on pUWLoriT, Amp^R, $Thio^R$	This work

Table 5.2: Strains used in this work.

Strain	Properties	Source/reference
Escherichia coli DH5α	Subcloning host	Agilent Technologies
Escherichia coli XL1-Blue MR	Subcloning host	Agilent Technologies
Escherichia coli ET12567	With pUZ8002 vector for pDNA conjugative interspecies transfer	[104]
Streptomyces noursei ATCC 11455	Wild-type (WT), nystatin producer	ATCC®
Streptomyces albus Del14	*S. albus* with 15 deleted BGCs	[105]
Streptomyces coelicolor M1154	*S. coelicolor* M145 with 4 deleted BGCs	[60]

5.2 Media

All media were autoclaved at 121 °C for 20 minutes to ensure sterilization and if needed, the pH has been adjusted before.

5.2.1 Media for pre-cultures

2YT (1 L)

Bacto Tryptone	16 g
Bacto Yeast Extract	10 g
NaCl	5 g

TSB (1 L)

Tryptic Soy Broth	30 g

5.2.2 Fermentation media

MP1 (1 L)

Glucose	40 g	
Yeast Extract	1.5 g	
NH_4NO_3	2.5 g	
$MgSO_4$ x $7H_2O$	0.5 g	
KH_2PO_4	0.5 g	
$CaCO_3$	3 g	
ddH_2O	up to 1000 ml	

TSB (1 L)

Tryptic Soy Broth	30 g	
ddH_2O	up to 1000 ml	

SM17 (1 L)

Glucose	2 g	
Glycerol	40 g	
Soluble Starch	2 g	
Soy Flour	5 g	
Peptone	5 g	
Yeast Extract	5 g	
NaCl	5 g	
$CaCO_3$	2 g	
Tap Water	up to 1000 ml	

5288 (1 L)

Glycerol	15 g	pH 6.8
Soy Meal	10 g	
NaCl	5 g	
$CaCO_3$	1 g	
$CoCl_2$ x $7H_2O$	1 mg	
ddH_2O	up to 1000 ml	

5333 (1 L)

Yeast Extract	4 g	pH 7.8
Soluble Starch	15 g	
K_2HPO_4	1 g	
$MgSO_4$ x $7H_2O$	0.5 g	
ddH_2O	up to 1000 ml	

YL (1 L)

Glucose	20 g	pH 7.0
Potato Starch	20 g	
Yeast Extract	5 g	
Polypeptone	5 g	
$CaCO_3$	4 g	
ddH_2O	up to 1000 ml	

PM4-1 (1 L)

Glucose	15 g
Soy Meal	15 g
Corn Steep Solids	5 g
$CaCO_3$	2 g
TMS 1 Stock Solution	6 ml
ddH_2O	up to 1000 ml

MYM (1 L)

Maltose	4 g	pH 7.3
Yeast Extract	4 g	
Malt Extract	10 g	
Tap Water	500 ml	
ddH_2O	up to 500 ml	
R2 Trace Elements	2 ml	

DNPM (1 L)

Dextrin	4 g	pH 6.8
Soytone	0.75 g	
Baking Yeast	0.5 g	
MOPS	2.1 g	
ddH_2O	up to 1000 ml	

SG (1 L)

Glucose	20 g	pH 7.2
Peptone	10 g	
$CaCO_3$	2 g	
$CoCl_2$	1 mg	
ddH_2O	up to 1000 ml	

R5 medium (1 L)

Sucrose	103 g
Glucose	10 g
Yeast Extract	5 g
Difco Casamino Acids	5 mg
$MgCl_2$ x $6H_2O$	10.12 ml
K_2SO_4	0.25 g
TES	5.73 g
ddH_2O	up to 1000 ml
Autoclave and add:	
$CaCl_2$ x $2H_2O$ (5 M)	0.4 ml
KH_2PO_4 (0.5 %)	1 ml
L-proline (20 %)	1.5 ml
NaOH (1 M)	0.7 ml
Trace elements as for R2YE	2 ml

R3 medium (1 L)

Glucose	10 g
Yeast Extract	5 g
Difco Casamino Acids	0.1 mg
$MgCl_2$ x $6H_2O$	10 ml
K_2SO_4	0.2 g
TES	5.6 g
ddH_2O	up to 1000 ml
Autoclave and add:	
$CaCl_2$ x $2H_2O$ (5 M)	0.4 ml
KH_2PO_4 (0.5 %)	1 ml
L-proline (20 %)	1.5 ml
NaOH (1 M)	0.7 ml
Trace elements as for R2YE	2 ml

GYM medium (1 L)

Glucose	4 g	pH 7.2
Yeast Extract	4 g	
Malt Extract	10 g	
Peptone	1 g	
NaCl	2 g	

5.2.3 Media for conjugation

2YT (1 L)

Bacto Tryptone	16 g
Bacto Yeast Extract	10 g
NaCl	5 g
ddH_2O	up to 1000 ml

SFM (1 L)

Soy Flour	20 g
Mannitol	20 g
Agar	20 g
ddH_2O	up to 1000 ml

10 ml/L 1 M $MgCl_2$ solution was added to SFM medium after autoclaving if used for conjugation.

5.2.4 Media for bioassays

LA (1 L)

Bacto Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar	15 g
ddH_2O	up to 1000 ml

YPD (1 L)

Bacto Peptone	20 g
Bacto Yeast Extract	10 g
D(+)-Glucose	20 g
Agar	15 g
ddH_2O	up to 1000 ml

PDA (1 L)

PDA Powder	39 g
ddH_2O	up to 1000 ml

5.3 Stock solutions

For the selection of gene modified recombinant strains from *E. coli* or Actinomycetes, antibiotics were used, which are listed in table 4. After preparation of the stock solutions, all antibiotics except for chloramphenicol and thiostrepton were filtrated via Syringe filters ROTILABO® PTFE, 0.20 µm by Roth®. Chloramphenicol and thiostrepton were used directly after dissolving without any filtration step. Antibiotic stocks were stored at −20 °C.

Table 5.3: Antibiotic stock solutions including the used concentrations and the solvents in which they were dissolved.

Antibiotic	**Stock concentration [mg/ml]**	**Used concentration *E.coli* [µl/ml]**	**Used concentration *Streptomyces* [µl/ml]**	**Solvent**
Ampicillin (Amp)	100	100	-	ddH_2O
Apramycin (Am)	100	100	50	ddH_2O
Chloramphenicol (Cml)	25	25	-	Ethanol abs.
Kanamycin (Kan)	25	25	-	ddH_2O
Nalidixic acid (Nal)	30	-	30	0.1 M NaOH
Thiostrepton (Thio)	30	30	30	DMSO

In this work, additional stock solutions were used for diverse experiments. Their compositions can be found below.

1 M $MgCl_2$ (1 L)

$MgCl_2$ x $6H_2O$	95.2 g
ddH_2O	up to 1000 ml

50 mM $CaCl_2$ (1 L)

$CaCl_2$ x $2H_2O$	7.35 g
ddH_2O	up to 1000 ml

20% Glycerol (1 L)

Glycerol	200 ml
ddH_2O	up to 1000 ml

Trace elements as for R2YE (1 L)

$ZnCl_2$	40 mg
$FeCl_3 \times 6H_2O$	200 mg
$CuCl_2 \times 2H_2O$	10 mg
$MnCl_2 \times 4H_2O$	10 mg
$Na_2B_4O_7 \times 10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	10 mg
ddH_2O	up to 1000 ml

TMS1 (1 L)

$FeSO_4 \times 7H_2O$	5000 mg
$CuSO_4 \times 5H_2O$	390 mg
$ZnSO_4 \times 7H_2O$	440 mg
$MnSO_4 \times H_2O$	150 mg
$Na_2MoO_4 \times 2H_2O$	10 mg
$CoCl_2 \times 6H_2O$	20 mg
HCl	50 ml

0.8% Agarose gel + GelRed®

Agarose	1.6 g
TBE buffer (1x)	up to 200 ml
GelRed®	10 µl

The agarose was dissolved in TBE buffer while heating in the microwave until the solution was totally clear. Following, the GelRed® Nucleic Acid Gel Stain was added. The fluid agarose gel solution was stored at 60 °C until used for gel electrophoresis.

Chapter 6

Methods

6.1 Gene modification methods

6.1.1 Bioinformatic tools

For this work, several bioinformatic tools were used to develop hypotheses, methods and to visualize different steps in gene modification techniques. These used methods are listed below in 6.1.1.1 and 6.1.1.2.

6.1.1.1 Genome analysis

This work was based on the results of genome sequencing data analysis which was done with the help of the online based open source program antiSMASH 5.0. This program is able to scan provided genome sequencing data on the presence of putative BGCs.The identification of the DNA region, which may carry potential genes which are clustered, is possible through the comparison of the genes in analyzed organisms with already known genes from a public database and prediction of their homology. In this way antiSMASH can not only give an overview about the BGC type and size, but also about the possible function of each gene. Based on that information, primers were designed for the amplification of regulatory genes from *Amycolatopsis* sp. YIM10 genome and for the construction of insertional knock-out construct in *Streptomyces noursei*(6.1.1.2).

6.1.1.2 Primer design

Forward and reversed primers were required for DNA amplification via Polymerase Chain Reaction (PCR). For that purpose, Clone Manager 6 , version 6.00 was used. To allow the cloning from the DNA fragments of interest into the target vector with the help of endonucleases, an additional modification during the primer design was required. Therefore, the addition of base pairs to the primer sequence as detection sequences for the restriction enzymes was performed. The designed primers for *S. noursei* are shown in Table 6.1. The primers for the *Amycolatopsis* sp. YIM10 strain are shown in Table 6.2.

Table 6.1: Primers designed for PCR of *S. noursei*. KN_fwd and KN_rev were designed for building a knock-out mutant (Figure 7.3). Snou_DT_fwd and Snou_DT_rev were designed for analyzing purpose of the mutant strain *S. noursei*/pSOK201_KN_SnC14. Sites for restriction enzymes were added as shown for further restriction steps.

Name	Sequence of the primer	Restriction enzyme
Snou_DT_fwd	5'-TCCGGTGTTCCTGGAGATCA-3'	*EcoR*I
Snou_DT_rev	5'-ATCACATGCGCGTTGGTTCC-3'	*Hind*III
KN_fwd	5'-ACGTCGAATTCACGTCTCCGACCGCGACCAA-3'	*EcoR*I
KN_rev	5'-CGACTAAGCTTGCAGGCCAGGTGCAGTGTCA-3'	*Hind*III

Table 6.2: Primers designed for *Amycolatopsis* sp. YIM10. Primers were used for upregulation of the given regulator genes *asnR*, *gntR* and *luxR*. Sites for restriction enzymes were added as shown for further restriction steps.

Name	Sequence of the primer	Restriction enzyme
asnR_AmC5_*EcoR*I	5'-GAGCGAATTCAGCGGTCCAGAAAGGACAG-3'	*EcoR*I
asnR_AmC5_*Hind*III	5'-GCGCCAAGCTTCCTGGTGGACATGGAACTC-3'	*Hind*III
gntR_AmC5_*EcoR*I	5'-GAGCGAATTCTGAGGATACCGGCAATCCC-3'	*EcoR*I
gntR_AmC5_*Hind*III	5'-GCGCCAAGCTTAGGTCCAGCCGGGGGAATG-3'	*Hind*III
luxR_AmC5_*EcoR*I	5'-GAGCGAATTCCACCCGACACCCTCTAAAG-3'	*EcoR*I
luxR_AmC5_*Hind*III	5'-GCGCCAAGCTTACCTTGGTATCGGTCAATG-3'	*Hind*III

6.1.2 *Streptomyces noursei* genomic DNA isolation

For the isolation of the genomic DNA (gDNA) of *S. noursei*, a Dneasy Kit from QIAGEN was used. Steps described in the manual were followed except for the step 3.

The pellet was resuspended by pipetting in 180 µl lysis Buffer (20 mM Tris-HCl, 10mM EDTA, 1.2 % triton X100, 15 mg/ml lysozyme; pH 8). The lysozyme was added just before the experiment via mixing the suitable amount of lysozyme and lysis Buffer by vortexing. After adding the lysozyme solution, the sample was incubated for 15 minutes at 37 °C and inverted every 5 minutes. The isolated gDNA was stored at −20 °C for later use.

6.1.3 Plasmid isolation

During the work with the *S. noursei* strain, the plasmid DNA (pDNA) of different *E. coli* strains was isolated with the Wizard® Pus SV Minipreps DNA Purification System from Promega, following the protocol provided by the manufacturer. Whereas for the work with the *Amycolatopsis* sp. YIM10 strain, the Monarch® Plasmid Miniprep Kit was used, also following the manufacturers protocol. The isolated plasmids were stored at −20 °C for later use.

6.1.4 PCR

To multiply the target gDNA fragment a polymerase chain reaction was performed. For this purpose, all ingredients shown in Table 6.3 were mixed together in a PCR tube and the reaction was carried out in a PCR cycler (Mastercycler® nexus 2 by Eppendorf®) which was programmed with the conditions responding to the different primers given in Table 6.4.

Table 6.3: Setup for PCR using Q5-Polymerase for 25 µl reaction.

Component	Volume [µl]
5X Q5 Reaction Buffer	5
10 mM dNTPs	0.5
10 µM Forward Primer	1.25
10 µM Reverse Primer	1.25
Template DNA 1:10	Variable
High-Fidelity DNA Polymerase	0.25
5x Q5 High GC Enhancer	5
Nuclease-Free Water	up to 25

Table 6.4: Reaction conditions for PCR used for*Streptomyces noursei* (a) and for the amplification of regulator genes from *Amycolatopsis* sp. YIM10 (b).

Step	Temperature	Time
Initial Denaturation	98 °C	5 minutes[a], 3 minutes[b]
25 Cycles	98 °C *50-72 °C 72 °C	10 seconds[a], 30 seconds[b] 30 seconds 20-30 seconds/kb
Final Extension	72 °C	7 minutes
Hold	4-10 °C	

Table 6.5: Sizes of gene fragments multiplied by the different primers according to their annealing temperature. The annealing temperature was predicted via Clone Manager 6.00. For the detection of the recombinant *S. noursei* detection primer (Snou_DT_fwd and Snou_DT_rev) were used for the recombinant strain (b) as well as for the wild-type strain phenotype (a) for comparative analysis.

Name of primer	Size of expected product	Annealing temperature
KN_fwd, KN_rev	1485 kb	63 °C
Snou_DT_fwd, Snou_DT_rev	3320 kb[a], 8113 kb[b]	61 °C
AsnR_AmC5_*EcoR*I, AsnR_AmC5_*Hind*III	465 kb	58 °C
GntR_AmC5_*EcoR*I, GntR_AmC5_*Hind*III	965 kb	60 °C
LuxR_AmC5_*EcoR*I, LuxR_AmC5_*Hind*III	822 kb	57 °C

6.1.5 Restriction

For the ligation cloning steps and validation of final plasmids, restriction enzymes were used (New England Biolabs GmbH). The restriction reaction mix for the DNA restriction was prepared following the protocol of the manufacturer. The volume of the mix was depending on further experiments which have been done with a target fragment. If a with restriction enzymes treated fragment should be used for further ligation reaction, then a 50 µl mix (Table 6.6) was prepared in order to get a higher concentration of a DNA insert. If the restriction enzymes were applied for the analytical analysis of the newly constructed vectors in this work, then a 15 µl mix was prepared (Table 6.7). The reactions for both purposes were incubated for 30 minutes at a temperature of 37 °C.

Table 6.6: Restriction protocol for a 50 µl mix.

Component	Volume [µl]
DNA after isolation	15-30
Cutsmart buffer 10x	5
*EcoR*I HF	1
*Hind*III HF	1
ddH_2O	Up to 50

Table 6.7: Analytical restriction protocol for analyzing the success of the insertion of the insert.

Component	Volume [µl]
pDNA	4
Cutsmart buffer 10x	1.5
*EcoR*I HF	1
*Hind*III HF	1
ddH_2O	Up to 15

6.1.6 Gel electrophoresis

Gel electrophoresis was used for all intermediate steps in experiments with DNA, in order to determine the DNA quality and estimate the DNA quantity. Therefore, part of the product solution was mixed with purple gel loading dye (6x) from Thermo Scientific™. The samples were loaded onto an 0.8 % agarose gel (0,8 g agarose from Sigma, up to 100 ml 1x TBE buffer (12.1 g Tris base, 6.18 g Boric acid, 0.74 g EDTA (disodium salt), up to 1 l with ddH_2O)) which was previously mixed with GelRed® for visualizing the DNA under UV light. To estimate the size of DNA products, 1 µl of GeneRuler 1 kb DNA ladder (Thermo Scientific™) was used as a reference. The DNA separation was performed at 100 Voltage for approximately 40 minutes and after that detected with UV light in the GelDoc (ChemiDoc™ Touch Imaging System by Bio-Rad).

6.1.7 Gel DNA recovery

For some cloning steps the DNA fragments were separated on an agarose gel, cut out and the DNA was recovered with the ZymocleanTM Gel DNA Gel Recovery Kit from ZYMO RESEARCH. The pieces of gel were stored at −20 °C, if not used directly for gel recovery after the cut out.

6.1.8 DNA clean and concentrator

For the purification of the insert DNA fragments after the treatment with endonucleases or for the increase of the concentration of needed DNA, a DNA Clean & ConcentratorTM-5 from ZYMO RESEARCH was used, according to the manufacturers protocol, in association with *S. noursei*. For the *Amycolatopsis* sp. YIM10 strain, Monarch® PCR & DNA Cleanup Kit (5 µg) was used.

6.1.9 Ligation

To produce a pSOK201_KN_SnC14 vector for the deactivation of a gene in the cluster 14 in the *S. noursei* wild-type, and to insert the genes (*asnR*, *gntR* and *luxR*) encoding for regulatory proteins of *Amycolatopsis* sp. YIM10 into the pUWLoriT vector the enzyme T4 DNA ligase (New England Biolabs GmbH) was applied. Table 6.8 represents the composition of a standard mix which was used to ligate two fragments (an insert and a vector) in this work.

Table 6.8: Ligation protocol

Component	Volume [µl]
Vector	1
Insert	7
T4 ligation buffer 10x	2
T4 DNA Ligase	1
ddH_2O	Up to 20

The components were all pipetted together and the tube was incubated at room temperature for 30 minutes.

6.1.10 Transformation of exogenous DNA

Transformation of the recombinant pDNA into the different bacterial hosts was used in this work. To transfer the pDNA into the different *E. coli* strains, the chemical competent cells were prepared using the $CaCl_2$ method.

6.1.10.1 Transformation of *E. coli* competent cells

For this work, the competent *E. coli* strains XLBlue, ET12567 and DH5α‘ were produced.

The *E. coli* were shaken overnight at 37 °C in 3 ml LB medium. 0.5 ml of the overnight culture was added into 50 ml LB medium in a 200 ml flask and the growth of the cells was continued at 37 °C. The culture was chilled on ice when the OD600 reached 0.4 - 0.5. Afterwards the culture was transferred into a sterile centrifuge tube. The cells were collected by centrifugation at 6000 rpm at 4 °C for 8 minutes and the supernatant was discarded.

The cells were resuspended in 20 ml of ice cold 50 mM $CaCl_2$ and incubated on ice for 20 minutes. After that time, the cells were again collected by centrifugation (6000 rpm at 4 °C for 8 minutes).

The cells were resuspended in 2.5 ml of ice cold 50 mM $CaCl_2$ containing 10% glycerol for storing the stock for a long period at −80 °C. The 100 µl aliquots of the competent cells were split up in tubes and frozen in liquid nitrogen for later use.

The competent *E.coli* cells were thawed on ice. After that, 20 µl of the ligation reaction or 2 µl of a pDNA was added to the cells and the tubes were incubated on ice for 30 minutes. A heat shock at 42 °C was done for 1 minute and was followed by 5 minutes incubation on ice. 900 µl LB medium was added to the cell suspensions and the tubes were incubated for 45 minutes at 37 °C in the shaker. Afterwards the cells were plated on LA media containing suitable antibiotics for selection and incubated over night at 37 °C.

6.1.11 Conjugation

To transfer the pDNA to different *Streptomyces* hosts, an intergeneric conjugative transfer was applied. For this purpose, a conjugal donor strain *E. coli* ET12567 was used which carried a non-transmissible vector pUZ8002 with a, for conjugation required mobilisation system [72]. As a starting point for conjugative pDNA transfer was the transformation of a test vector into the *E. coli* ET12567/pUZ8002 strain (see for details 6.1.10.1).

Four colonies of the gotten transformants were chosen, plated on LA agar containing 3 selective markers (chloramphenicol and kanamycin resistance originate from the helper plasmid pUZ8002) and were incubated at 37 °C overnight. On the next day *E. coli* ET12567 cells were

swiped off the plate and dissolved in 500 µl of 2xYT medium.

50 µl spore suspension of the used *Streptomyces* host organism was mixed with 350 µl of 2xYT medium and incubated at 50 °C for 5 minutes. Following, the reaction was cooled down at room temperature for 5 minutes.

Afterwards the spores were mixed with 100 µl *E. coli* ET12567 cell suspension and the mixture was centrifuged at 5000 rpm for 1 minute.

300 µl supernatant was removed from the tube and the pellet was redissolved in the remaining liquid. This suspension was spread out onto SFM medium, containing 1 M $MgCl_2$ solution, without selective marker.

After 18-20 hours of incubation at 28 °C, the plates were coated with 1 ml sterile ddH_2O containing 30 µl nalidixic acid and the suitable antibiotic for second selection. After drying, the plates were incubated for another 2-3 days.

If bacterial growth in form of colonies could be observed, those colonies were picked and cultivated on fresh SFM plates containing nalidixic acid and the respective antibiotic and were again incubated at 28 °C for a few days.

If sporulating colonies could be observed, those were plated onto fresh SFM plates containing respective antibiotics for preparation of spore suspensions. After sporulation of these cultures, spore suspensions were prepared for storage at −80 °C as described in 6.2.1.

6.2 Glycerol stocks

6.2.1 Glycerol stocks from spore forming organisms

For preparing glycerol stocks of spore forming organisms, these bacteria had to be grown on respective solid media. For the *S. albus* and *S. coelicolor* strains this media was SFM. *S. noursei* was incubated on ISP2 agar plates. For all recombinant strains there also had to be some selective antibiotic apparent in the media to ensure the keeping of the transformed construct. All *Streptomyces* strains were incubated at 28 °C. After sporulation started, 5 ml of 20 % glycerol was added to the bacteria grown on the agar. The bacteria were scratched from the agar with the help of a pipette and the resulting suspension was filtrated through

cotton in a sterile syringe in order to separate the mycelium from the spores. Afterwards the spore suspension was aliquoted into cryopreservation tubes and stored at −80 °C.

6.2.2 Glycerol stocks from not spore forming organisms

Not spore forming organisms, as the bacterial test organisms which were used for the bioactivity testing, were grown in 15 ml selective media responding to the media shown in Table 6.9. After one night of incubation at the temperature shown in Table 6.9, the grown bacterial suspension was centrifuged, the medium was removed and the resulting pellet was resuspended in 15 ml of 20 % glycerol. These stocks were divided into cryopreservation tubes of 1 ml each and were stored at −80 °C. For recombinant *E. coli* strains which were used for subcloning steps and *E. coli* ET12567/pUZ8002, the same method was used but in a lower volume, to prepare stocks for later use under selection with suitable antibiotics.

Table 6.9: Growing parameters for the different bioassay test organisms used in this work, including the media and the incubation temperatures.

Strain	Temperature	Medium
Escherichia coli DH5αF	37 °C	LB
Staphylococcus carnosus DSMZ 20501	37 °C	LB
Enterococcus mundti DSMZ 4840	37 °C	LB
Pseudomonas putida KT 2440	28 °C	LB
Kocuria rhizophila DSMZ 348	28 °C	LB
Micrococcus luteus DSMZ 1790	28 °C	LB
Bacillus subtilis DSMZ 10	28 °C	LB
Erwinia persicina DSMZ 19328	28 °C	LB
Sacharomyces cerevisiae	28 °C	YPD
Aspergillus niger	Room temperature	PDA
Fusarium graminearum	Room temperature	PDA

The two fungi strains were also produced under standard conditions in the lab by a member of the working group of pharmaceutical biotechnology. Aliquots were provided by Mag. Dr. Martina Oberhofer.

6.3 Fermentation of *Streptomyces* strains

For all strains, pre-cultures were prepared. 100 µl of spore suspension from the used strain were cultivated over night at 28 °C in 25 ml of TSB-medium in 250 ml baffled flasks. Glass beads were added to avoid the aggregate formation in the flasks. If a recombinant strain was used, the appropriate antibiotic was added for selection.

For *S. noursei* the 2xYT medium was additionally tested as a pre-culture medium.

For the production of SMs, as for example antibiotics, fermentation was needed. For that reason, 5 ml of the pre-culture was added to 100 ml of respective media. Different media were tested for all the used strains in order to see differences in the SM production. The media and fermentation parameters for the *S. noursei* strains are shown in Table 6.10 and the ones for the heterologous expression of cluster 5 of *Amycolatopsis* sp. YIM10 in different host organisms are presented in Table 6.11.

Table 6.10: Media and fermentation parameters for the *Streptomyces noursei* strains.

Media	Temperature	Time
MP1	28 °C	5 days 7 days 14 days
SM17	28 °C	7 days
TSB	28 °C	7 days
5288	28 °C	7 days
5333	28 °C	7 days
YL	28 °C	7 days

Table 6.11: Media and fermentation parameters for the growth of recombinant strains of *Streptomyces albus* Del14 and *Streptomyces coelicolor* M1154 for the optimization of heterologous expression from putative new NRP from *Amycolatopsis* sp. YIM10.

Media	Temperature	Time
SM17	28 °C	7 days
PM4-1	28 °C	7 days
MYM	28 °C	7 days
DNPM	28 °C	7 days
SG	28 °C	7 days

6.4 Secondary metabolites extraction

6.4.1 Extraction of antibiotic X

Fermented bacterial broth was centrifuged to separate the pellet from the supernatant. The supernatant was then extracted twice with ethyl acetate in a 1:1 ratio for 1 hour on the shaker at 28 °C. Organic phases from two extracts were combined and evaporated at 40 °C in a rotavapor to remove the solvent. The pellet was dissolved in 2 - 3 mll methanol, depending on the original amount of fermentation culture. For the low scale, the pellet was also extracted separately in order to find differences in bioactivity of the extracts from the MP1 fermentation. Afterwards, only the supernatant was used. The methanolic extracts were always tested for their bioactivity in bioassays (6.6.1).

6.4.2 Extraction of Cl5 compound from *Amycolatopsis* sp. YIM10

The fermentation of the strains used for the heterologous expression of cluster 5 of *Amycolatopsis* sp. YIM10, were fermented in different media. The extraction protocols of fermentations in the different fermentation media are described below.

6.4.2.1 Extraction of fermentations in PM4-1 & SM17 media

25 ml per culture were taken and put into the freeze dryer in order to remove the water. After a few days, the crude residue was extracted with 25 ml MeOH on the shaker at 28 °C for 3 hours. After that, the solvent was evaporated and the pellet was dissolved in 500 µl MeOH. The bioactivity was determined in bioassays (6.6.1).

6.4.2.2 Extraction of fermentations in MYM, DNPM and SG media

The fermentation cultures were centrifuged so the supernatant could be separated from the pellet. The pellet was extracted once with 25 ml methanol at 28 °C for 1 hour on the shaker and then centrifuged. The organic phase was collected and evaporated. The supernatant was extracted once with 20 ml ethyl acetate for 1 hour at 28 °C on the shaker. The organic phase was collected, and the rest was extracted another two times with 20 ml ethyl acetate, but instead of shaking for one hour in the shaker, it was mixed with a vortex for at least 30 seconds. The organic phases were collected and evaporated. The residues were dissolved in 2.5 ml methanol. The extracts were tested for their bioactivity (6.6.1).

6.5 Upscale of antibiotic X from *Streptomyces noursei*

For the upscale of the production of antibiotic X from *S.noursei*, several litres of fermentation were required. The bacteria were fermented in MP1 medium. Different attempts of purifying the antibiotic X were approached, which are described below (6.5.2 and 6.5.3).

6.5.1 Extraction of antibiotic X

The first step in the extraction process was the separation of the pellet from the supernatant by filtration of the culture broth. If this was not sufficient enough, the culture was additionally centrifuged. To reduce the volume of the broth for extraction with organic solvent, freeze drying was performed. As a next step, the concentrated samples were extracted with 100 % ethyl acetate. The extraction steps were the same as in the low scale (6.4.1) except for the fact that the culture was extracted a third time to ensure maximal yield.

6.5.2 Pre-purification of antibiotic X by Silica gel

For purification of the compounds, column chromatography was performed. Therefore, cotton was first placed into a glass column followed by 0.5 cm of sea sand and 3 g Silica gel 60 (0.063 - 0.200 mm) by Merck per 100 mg extract. On top of the silica layer, a second layer of sand was added to avoid the silica to swirl up. After wetting the silica with a part of the first solvent, the methanolic extract was added on top of the column and got completely soaked into the silica before adding the first solvent again to start the purification process. Following, the column got rinsed by the solutions one after another and the different fractions were collected in round bottom flasks. The solutions used for purification can be found in Table 6.12. The solvents of these fractions were evaporated by a rotavapor, the residue was dissolved in 2 ml methanol and the different fractions were again tested for their bioactivity against *Micrococcus luteus*.

Table 6.12: Solvents and concentrations used for pre-purifying extracts of *Streptomyces noursei* via Silica gel chromatography.

Fraction	Solvent	Concentration
1	$CHCl_3$	100%
2	$CHCl_3$: MeOH	98 : 2
3	$CHCl_3$: MeOH	95 : 5
4	$CHCl_3$: MeOH	90 : 10
5	$CHCl_3$: MeOH	80 : 20
6	$CHCl_3$: MeOH	50 : 50
7	MeOH	100%

6.5.3 Preparative HPLC for antibiotic X isolation

Preparative HPLC was used for purification of the extract of *S. noursei* wild-type and the recombinant strain *S. noursei*/pSOK_KN_SnC14. Some fractions of *S. noursei* wild-type were merged and separated via preparative HPLC again for further analyzation (6.5.3.3).

6.5.3.1 Testing of the method

Before starting to purify the antibiotic X via preparative HPLC, the method, which can be found in Table 6.13, was tested. The column used was Shim-pack GIS 10 µm C18, LC Column 250 x 20 mm.

Table 6.13: Method used for testing of the method for preparative HPLC for purification of extracts achieved from cultivation of *Streptomyces noursei*. Shim-pack GIS 10 µm C18, LC Column 250 x 20 mm was used. Fractions were collected as noted in the table.

Flow rate	Minutes	Solvent B concentration [%]	Minutes collected	Volume collected
10 ml/min	0	5	0.33 - 40 min	50 ml
	40	95		
	45	95		
	45	stop		

6.5.3.2 Preparative HPLC of pre-purified extracts after Silica column chromatography

The extracts used for these methods are described in section II. The methods are given in the table below (Table 6.14). Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used for these methods.

Table 6.14: Method used for separating pre-purified fractions after column chromatography (6.5.2). The different collecting times resulted due to experience of the elution times of antibiotic X. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used.

Flow rate	Minutes	Solvent B concentration [%]	Minutes collected	Volume collected
10 ml/min	0	5	15 - 30 min / 17 - 27 min	10 ml
	40	95		
	45	5		
	45	stop		

6.5.3.3 Preparative HPLC of already separated fractions

To maximise the separation of the different compounds in the extracts achieved after the first preparative HPLC run, a second run was conducted. The samples were therefore divided into two mixtures of fractions which are described in (section III). For these two new mixed samples, two different methods were established based on their original retention times. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used for both experiments.

Table 6.15: Method used for sample A (Table 7.4) after first preparative HPLC run. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used. Every fraction was collected and tested in bioassays as well as analyzed in analytical HPLC.

Flow rate	Minutes	Solvent B concentration [%]	Minutes collected	Volume collected
10 ml/min	17	65	0.33 - 17 min	10 ml
	22	35		
	22	stop		

Table 6.16: Method used for sample B (Table 7.4) after first preparative HPLC run. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used. Every fraction was collected and tested in bioassays as well as analyzed in analytical HPLC.

Flow rate	Minutes	Solvent B concentration [%]	Minutes collected	Volume collected
10 ml/min	17	6	0.33 - 17 min	10 ml
	22	54		
	22	stop		

6.5.3.4 Preparative HPLC of an extract from *Streptomyces noursei* knock-out

An extract of *S. noursei*/pSOK_KN_SnC14 was also separated to compare the achieved fractions via preparative HPLC. The fractions were tested in bioassays and analyzed in analytical HPLC. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used to ensure the same conditions.

Table 6.17: Method used an extract of the *Streptomyces noursei* knock-out strain using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex®.

Flow rate	Minutes	Solvent B concentration [%]	Minutes collected	Volume collected
10 ml/min	0	5	15 - 30 min	10 ml
	40	95		
	45	5		
	45	stop		

6.6 Analytical determination of extracted secondary metabolites

For exploring and identifying different or newly produced secondary metabolites in extracts, different analyzing techniques were used which are described below.

6.6.1 Bioactivity tests via disk diffusion assays

To test if obtained extracts contain antibiotic compounds, a bioactivity test via disc diffusion method was carried out. For this purpose, 150 µl of the glycerol stock solutions of different strains were plated on suitable agar plates (Table 6.18) and were spread with a sterile spatula until the plate was dry. To prepare the discs for this disc diffusion assay, the extract was applied onto the filter discs (Oxoid™ Antimicrobial susceptibility test discs, 6 mm in diameter) and air-dried under sterile conditions. Depending on the concentration of the extract, 15 µl or 30 µl of extract were tested on antimicrobial properties. After a drying time of 15 - 20 minutes the discs were carefully placed on the agar plates. Additionally, in all experiments, methanol was used as negative control.

Table 6.18: Used strains for bioassays, including their suitable growing media and the optimal growth temperature.

Strain	Temperature	Medium
Escherichia coli DH5αF	37 °C	LA
Staphylococcus carnosus DSMZ 20501	37 °C	LA
Enterococcus mundti DSMZ 4840	37 °C	LA
Pseudomonas putida KT 2440	28 °C	LA
Kocuria rhizophila DSMZ 348	28 °C	LA
Micrococcus luteus DSMZ 1790	28 °C	LA
Bacillus subtilis DSMZ 10	28 °C	LA
Erwinia persicina DSMZ 19328	28 °C	LA
Sacharomyces cerevisiae	28 °C	YPD Agar
Aspergillus niger	Room temperature	PDA
Fusarium graminearum	Room temperature	PDA

All bacterial plates were incubated overnight at given temperature. All fungi needed longer to grow. The fungi strains were used just once for testing the bioactivity of the produced extract of antibiotic X produced by *S. noursei*. After that, only the bacterial strains were used for the bioactivity testing.

6.6.2 Analytical HPLC

The extracts of both projects were tested in analytical HPLC in order to find some new peaks. Especially the comparison of extracts achieved from fermentation of mutant strains to wild-type strains or strains with an empty vector inside were very promising. A method was designed with the help of the program Lab Solutions Lite Version 5.97 , a batch was installed before every run and the right solvents were chosen. Standardized methods were used, considering the requirements of the different columns, as described in Table 6.19. The used columns were the Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex®, and the DIONEX Acclaim® 120 C18, 4.6 x 150 mm 5 µm by Thermo Fisher Scientific®. The solvents used for analytical HPLC were ddH_2O (Solvent A) and ACN (Solvent B).

Table 6.19: Method used for analytical HPLC using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® (a) and DIONEX Acclaim® 120 C18, 4.6 x 150 mm 5 µm by Thermo Fisher Scientific® (b).

Flow rate	Minutes	Solvent A [%]	Solvent B [%]
1 ml/min[a], 0.5 ml/min[b]	0	95	5
	45	5	95
	55	95	5
	65	Stop	Stop

6.6.3 Optimization of purification of antibiotic X using isocratic methods

For the separation of the different derivatives of the extract of antibiotic X, different HPLC methods were tested. These isocratic methods, which were both adjusted for the Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex®, are presented below in Table 6.20 and Table 6.21.

Table 6.20: Isocratic method 1 for analytical HPLC using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® for separation of antibiotic X derivatives using acetonitrile, methanol, THF and ddH_2O, in the given concentrations, as solvents.

Flow rate	Minutes	ACN	MeOH	THF	ddH_2O
1 ml/min	40	20%	30%	10%	40%

Table 6.21: Isocratic method 2 for analytical HPLC using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® for separation of antibiotic X derivatives using acetonitrile, methanol, ammonium acetate and ddH_2O, in the given concentrations, as solvents.

Flow rate	Minutes	ACN	MeOH	NH_4-Acetate	ddH_2O
1 ml/min	40	45%	10%	10%	35%

6.6.4 Mass spectrometry

Mass spectrometry was done at the Mass Spectrometry Center of the University of Vienna at the institute for Analytical Chemistry, by Mag. Dr. Martin Zehl.

Chapter 7

Results

7.1 Antibiotic X

7.1.1 Proof of principle: testing of bioactive compounds in extracts produced by the *Streptomyces noursei*

To test the reproducibility of the results for production of antibiotic X by *Streptomyces noursei* wild-type, which was demonstrated earlier [1], the *Streptomyces noursei* strain was cultivated in MP1 medium for 5, 7 and 14 days, extracted as described in 6.4.1, and the bioactivity of the extracts was tested against the microorganisms shown in Table 6.18. The two fungi tested, were only used once for the extracts of the 5 days culture.

The extracts from the 7 and 14 days cultures had an additional activity against *Saccharomyces cerevisiae* compared to the one from 5 days (Table 7.1). Comparing the extracts of a fermentation culture from 14 days to the one from 7 days, no differences were detectable. The results of this activity testing can be seen in Table 7.1. After testing the different fermentation times, 7 days of fermentation was chosen as a standard method for all following fermentations.

Table 7.1: Bioactivity of extracts after cultivation of *Streptomyces noursei* in MP1 medium in different time ranges; + = activity shown in the bioassay; - = no activity shown in the bioassay; nt = not tested.

Strain	Gram	Bioactivity		
		5 days	7 days	14 days
Escherichia coli DH5α	-	-	-	-
Staphylococcus carnosus DSMZ 20501	+	+	+	+
Enterococcus mundti DSMZ 4840	+	-	-	-
Pseudomonas putida KT 2440	-	-	-	-
Kocuria rhizophila DSMZ 348	+	+	+	+
Micrococcus luteus DSMZ 1790	+	+	+	+
Bacillus subtilis DSMZ 10	+	+	+	+
Erwinia persicina DSMZ 19328	-	-	-	-
Sacharomyces cerevisiae	yeast	-	+	+
Aspergillus niger	fungi	-	nt	nt
Fusarium graminearum	fungi	+	nt	nt

7.1.2 *In silico* genome analysis of *Streptomyces noursei* wild-type

Based on the genome sequencing data of *S. noursei* (NCBI accession number NZ_CP011533) and the results from the antiSMASH 5.0 analysis, it was shown that the genome from this bacterium is approximately 9.82 Mb in size and encodes at least 38 biosynthetic gene clusters (Figure 7.1). Figure 7.1 shows all the gene clusters contained in the genome associated to the most similar looking clusters known giving the percentage of similarity.

Region	Type	From	To	Most similar known cluster		Similarity
Region 1	lassopeptide	117,423	139,067	A54145	NRPS	5%
Region 2	terpene	223,236	243,282	Tylactone	polyketide	6%
Region 3	T1PKS	350,589	409,828	Simocyclinone	t1pks+t2pks-saccharide-other	8%
Region 4	terpene	584,233	603,773			
Region 5	terpene	688,481	708,120			
Region 6	T1PKS	769,835	911,487	Nystatin	t1pks-saccharide	100%
Region 7	terpene	1,065,461	1,085,454			
Region 8	butyrolactone	1,244,054	1,254,532	Merochlorin	t3pks-terpene	4%
Region 9	NRPS-like , butyrolactone	1,487,461	1,530,351	Sch47554 / Sch47555	t2pks	10%
Region 10	NRPS-like	1,574,713	1,616,131	Abyssomicin	t1pks	10%
Region 11	T1PKS	1,644,557	1,690,956	Actinomycin	NRPS	10%
Region 12	terpene	1,958,530	1,984,265	Hopene	terpene	61%
Region 13	lassopeptide	2,157,118	2,178,457	Echosides	NRPS	11%
Region 14	T1PKS	2,214,299	2,292,209	Erythromycin	t1pks-saccharide	56%
Region 15	bacteriocin	2,670,601	2,680,390			
Region 16	T3PKS	2,743,304	2,782,651	Naringenin	terpene	100%
Region 17	betalactone , NRPS	2,853,457	2,914,351	Ulleungmycin	NRPS	80%
Region 18	siderophore	2,922,576	2,935,753	Ficellomycin	NRPS	3%
Region 19	CDPS	3,258,238	3,278,957	Albonoursin	other	83%
Region 20	NRPS-like	3,884,958	3,928,411			
Region 21	linaridin	4,361,283	4,382,173	Legonaridin	other	55%
Region 22	thiopeptide , bacteriocin	4,401,731	4,438,354	Radamycin / globimycin	ripp	94%
Region 23	terpene	4,524,960	4,544,791	Geosmin	terpene	100%
Region 24	lanthipeptide	6,032,920	6,057,223	Guadinomine	nrps-t1pks	7%
Region 25	T1PKS	6,511,872	6,553,402	Collismycin A	nrps-t1pks	18%
Region 26	ectoine	6,984,258	6,994,662	Ectoine	other	100%
Region 27	siderophore	7,075,833	7,087,638	Desferrioxamine	other	100%
Region 28	lanthipeptide	7,331,492	7,355,389	Streptomycin	saccharide	10%
Region 29	T2PKS	7,849,563	7,922,078	Spore pigment	t2pks	83%
Region 30	bacteriocin	7,950,154	7,960,369	Conglobatin	NRPS	10%
Region 31	transAT-PKS , NRPS , NRPS-like	8,267,262	8,375,326	Cycloheximide / actiphenol	transatpks	50%
Region 32	NRPS	8,386,871	8,461,308	Actinomycin	NRPS	10%
Region 33	terpene	8,911,916	8,931,024	Meoabyssomicin / abyssomicin	polyketide	6%
Region 34	NRPS , T1PKS , T3PKS , terpene	9,016,768	9,100,074	Xiamycin	terpene	9%
Region 35	NRPS , lanthipeptide	9,139,090	9,194,236	S56-p1	NRPS	17%
Region 36	NRPS-like	9,200,679	9,242,057	Tiacumicin B	t1pks	9%
Region 37	T1PKS , NRPS-like , terpene	9,247,044	9,361,469	Natamycin	t1pks	68%
Region 38	lassopeptide	9,676,601	9,699,082	A54145	NRPS	5%

Figure 7.1: *Streptomyces noursei* genome analysis via antiSMASH 5.0 including predictions of the possible type of the biosynthetic gene clusters and detections of their similarity with the known clusters of the databases.

After the manual evaluation of the resulting data from antiSMASH 5.0 analysis, it was decided to work with cluster 14 which was predicted to encode for a type 1 polyketide synthase (T1PKS) and showed 56% of similarity to an already known cluster which is responsible for the production of the macrolide antibiotic erythromycin (personal communication with Univ.-Prof. Dr. Sergey B. Zotchev). Figure 7.2 represents the gene arrangement within the cluster 14.

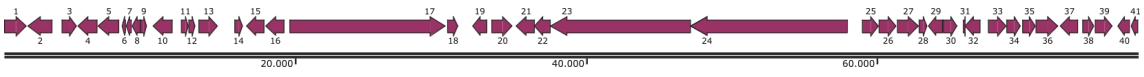


Figure 7.2: Overview of the gene compositions of cluster 14 in *Streptomyces noursei*.

Based on the hypothesis that cluster 14 encodes for antibiotic X, this cluster was chosen to be inactivated via insertional disruption. For that reason, gene number 17 was selected as the

homolog protein of this gene encodes for modules 1 and 2 of the PKS which is responsible for the first steps in the macrolide synthesis. Therefore, it was assumed, that the interruption of this gene will prevent the biosynthesis of antibiotic X.

7.1.3 Cluster 14 T1PKS inactivation

To determine which gene cluster is responsible for the biosynthesis of antibiotic X from the *S. noursei* wild-type, the knock-out mutant *S. noursei*/pSOK201_KN_SnC14 was constructed. The gene cluster potentially responsible for the production of antibiotic X was disrupted via insertion of the vector into gene 17. This would allow to observe differences in antibiotic production between the recombinant and the wild-type strain of *S. noursei* with the help of antibiotic profiling.

7.1.3.1 Construction of the knock-out plasmid pSOK201_KN_SnC14

Primers for the amplification of a fragment of gene 17 were designed as described in 6.1.1.2. PCR has been done using the primers KN_fwd and KN_rev (Table 6.1) and template gDNA of the *S. noursei* wild-type (6.1.2). The PCR product 'gene17' was separated on agarose gel (6.1.6) and the band marked in Figure 7.4 (a) was extracted (6.1.7). Furthermore, the plasmid pSOK201 was transformed into *E. coli* DH5α and isolated (6.1.3).

The PCR product 'gene17', as well as the pSOK201 plasmid, were digested using the restriction enzymes *EcoR*I and *Hind*III as both DNA molecules contain binding sites for these restriction endonucleases. The digested pSOK201 was separated via gel electrophoresis (6.1.6) and the marked band was isolated from the gel (6.1.7). The used part of the plasmid, which was selected, had a size of approximately 3.0 kb and is shown in Figure 7.4 (b) In case of the digestion of the 'gene17', the product was subsequently purified using a DNA Clean & Concentrator TM-5 from ZYMO RESEARCH clean and concentrator (6.1.8). Next, the ligation of purified DNA fragments was performed as noted in Table 6.8. All the construction steps are depicted in Figure 7.3.

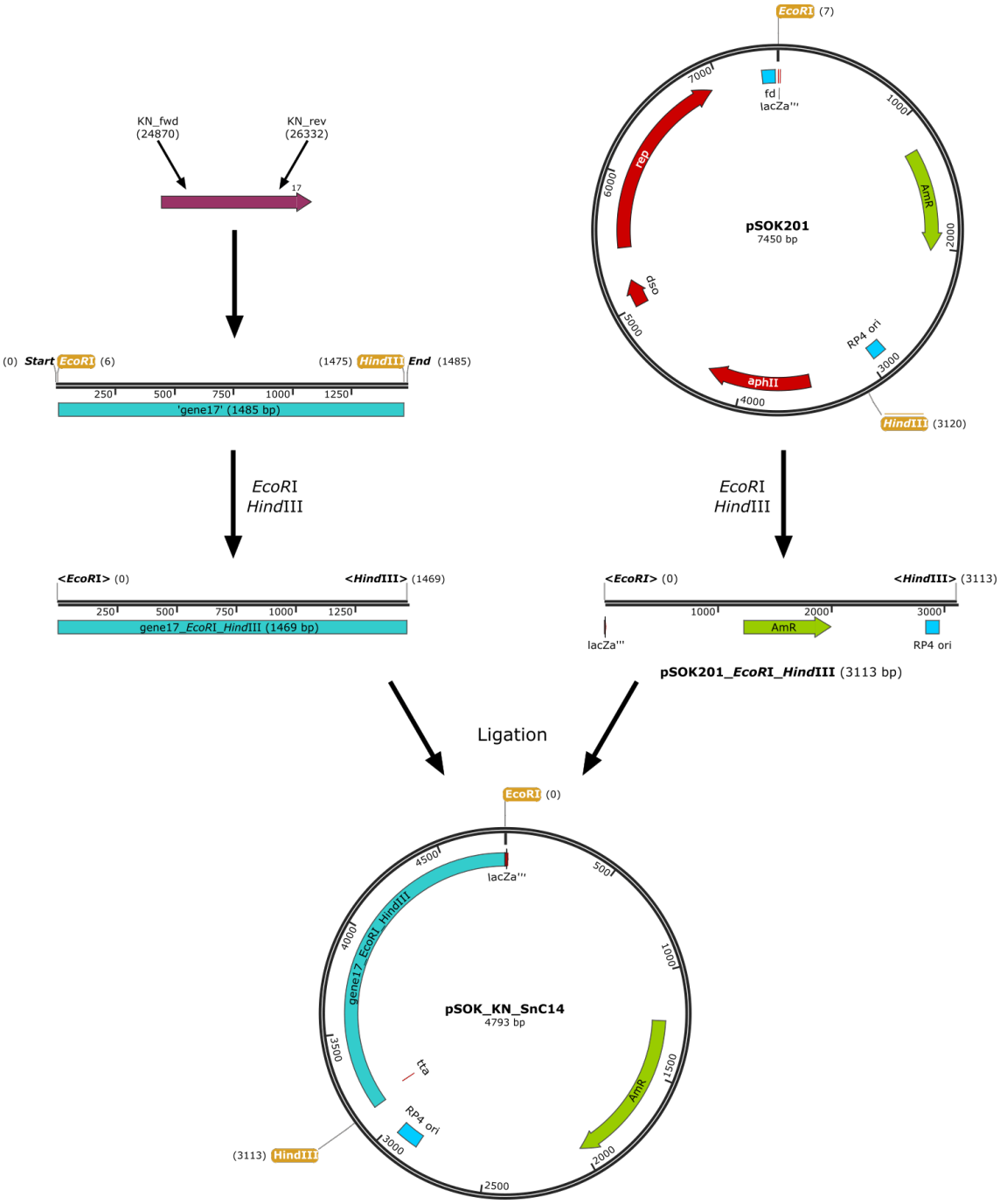
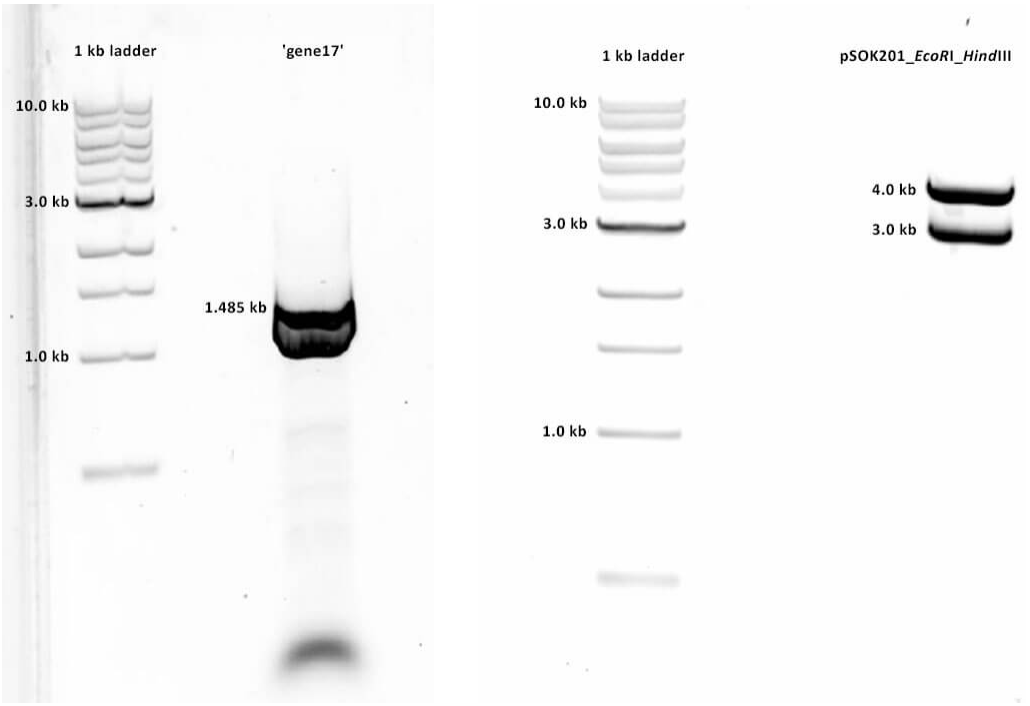


Figure 7.3: Scheme of the generation of the knock-out plasmid pSOK201_KN_SnC14 from the PCR product 'gene17' and the part of pSOK201 to the point of ligation of the isolated restriction fragments forming the ligation product pSOK201_KN_SnC14; rep = replication site; AmR = apramycin resistance gene; RP4 ori = origin of plasmid transfer via conjugation.

As presented in Figure 7.3, the ligation product pSOK_KN_SnC14 inherits a selective marker gene for apramycin, an origin for conjugative transfer and the cassette for insertional deactivation of gene 17 in cluster 14. The apramycin resistance gene is obligatory for the following conjugation step, as without any selective marker, it would not have been possible to differentiate between a mutant and a wild-type colony. Ligation was followed by transformation (6.1.10.1) into *E. coli* DH5α cells.



(a) Gel electrophoresis of 'gene17' (b) Gel electrophoresis of pSOK201_*EcoR*I_*Hind*III

Figure 7.4: (a) shows the separation via gel electrophoresis of 'gene17' after PCR.The marked band at 1.485 kb was isolated and used for restriction. (b) shows the separation via gel electrophoresis of the digested pSOK201 plasmid (pSOK201_*EcoR*I_*Hind*III). The marked band at 3.0 kb was isolated and used for ligation afterwards.

7.1.3.2 Analytical restriction

Analytical restriction (Table 6.7) was performed to determine whether the plasmid formed during ligation contains the desired parts gene17_*EcoR*I_*Hind*III and pSOK201_*EcoR*I_*Hind*III. Therefore, transformed *E. coli* DH5α colonies were grown in LB medium containing apramycin and their pDNA was isolated (6.1.3). Restriction was done following the protocol given in Table 6.6. Afterwards, gel electrophoresis (6.1.6) was done. The result can be seen in Figure 7.5.

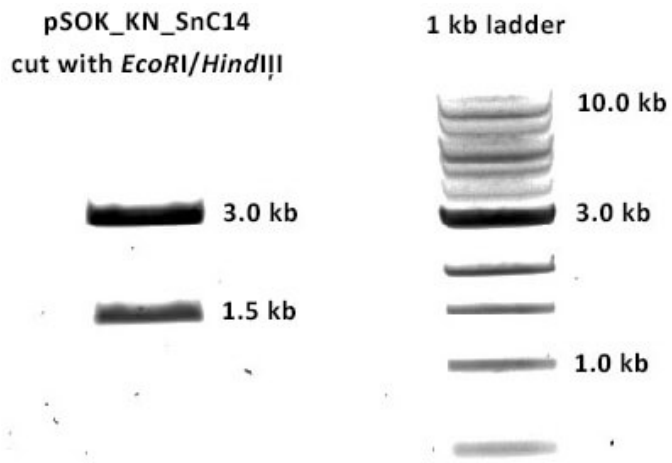


Figure 7.5: Separation via gel electrophoresis of the fragments produced via analytical restriction of pSOK201_KN_SnC14. The marked bands reflect the sizes of the used pSOK201_*EcoR*I_*Hind*III fragment (3.0 kb) and the gene17_*EcoR*I_*Hind*III fragment (1.5 kb).

Both bands shown in Figure 7.5 correlate with the sizes of the gene17_*EcoR*I_*Hind*III and the pSOK201_*EcoR*I_*Hind*III which were used for ligation. Hence, one can assume, that preparation of the knock-out plasmid was successful and conjugation (6.1.11) could be done as a following and last step of making a knock-out mutant, where the new constructed vector pSOK201_KN_SnC14 was transferred into the *S. noursei* wild-type strain, resulting the recombinant strain *S. noursei*/pSOK201_KN_SnC14.

7.1.3.3 Verification of *Streptomyces noursei*/pSOK201_KN_SnC14

The determination for insertion of the construct pSOK201_KN_SnC14 into gene 17 of cluster 14 was done using PCR. Here, primers were designed which bind outside of the DNA region targeted for insertion in *S. noursei* (Snou_DT_fwd and Snou_DT_rev shown in Table 6.1). As a result, it was expected that the PCR product using template gDNA of the recombinant strain will be around 8.0 kb, whereby the PCR product of the wild-type gDNA was expected to be around 3.0 kb in size.

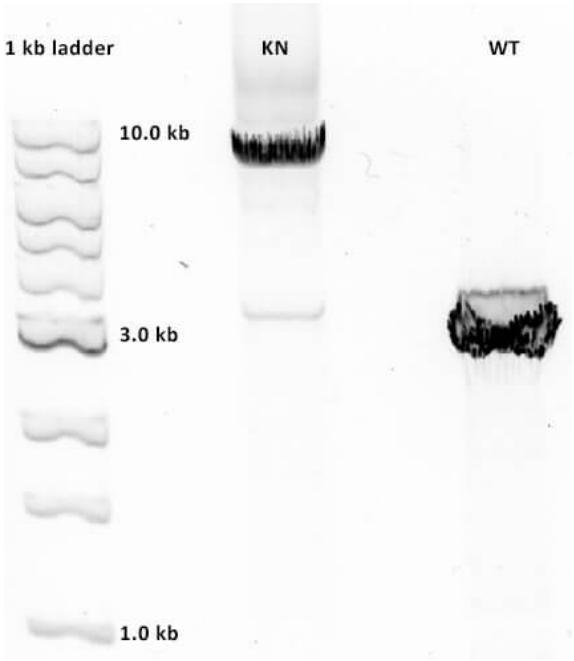


Figure 7.6: Gel electrophoresis of the PCR products after PCR using detection primers. KN = PCR product using gDNA of the *Streptomyces noursei*/pSOK201_KN_SnC14; WT = PCR product using gDNA of the *Streptomyces noursei* wild-type.

On the gel presented in Figure 7.6, it can be seen that the fragment amplified from the gDNA of the knock-out mutant *S. noursei*/pSOK201_KN_SnC14 is clearly larger (8.0 kb) than the one amplified from the gDNA of *S. noursei* wild-type (3.5 kb). The difference detected in size approximately corresponds to the size of the inserted pSOK201_KN_SnC14 construct, indicating that the insertion was performed at the expected position.

The first evidence that the product of cluster 14 is the antibiotic X was observed by performing of a bioassay (6.6.1) against *M. luteus* after fermentation of both *S. noursei* strains (wild-type and disruption mutant) in MP1 medium for 7 days under standard conditions and extraction as noted in 6.4.1.

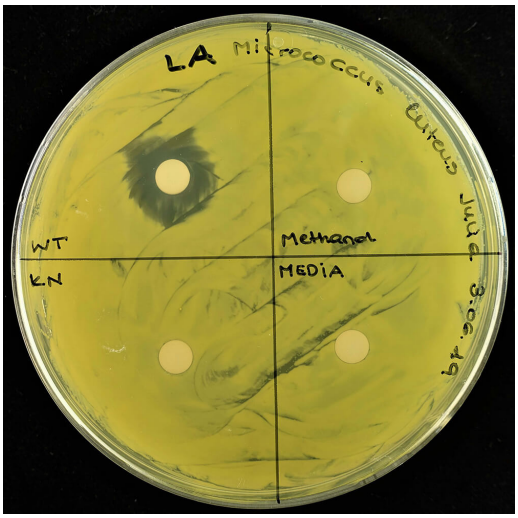


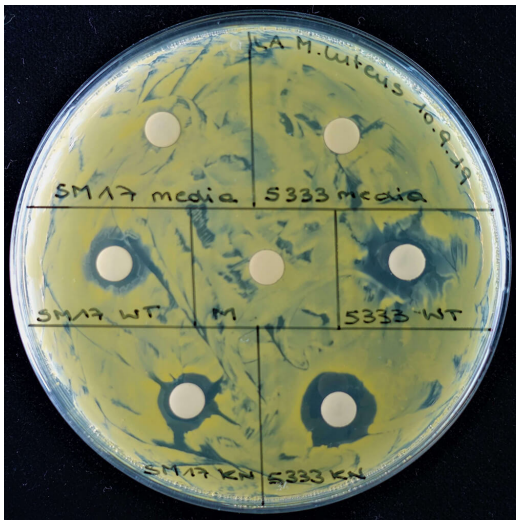
Figure 7.7: Bioassay of extracts of antibiotic X from *Streptomyces noursei* cultivated in MP1 medium, tested against *Micrococcus luteus*. WT = extract from *Streptomyces noursei* wild-type. KN = extract from *S. noursei*/pSOK201_KN_SnC14. MEDIA = media control of MP1 medium. Methanol = methanol blank.

The comparison of the extracts of *S. noursei* wild-type and *S. noursei*/pSOK_KN_SnC14 showed a definite lack of antibiotic activity in the extract of the recombinant strain, clearly suggesting that disruption of gene number 17 in the recombinant *S. noursei*/pSOK_KN_SnC14 was achieved and that cluster 14 is responsible for the production of antibiotic X.

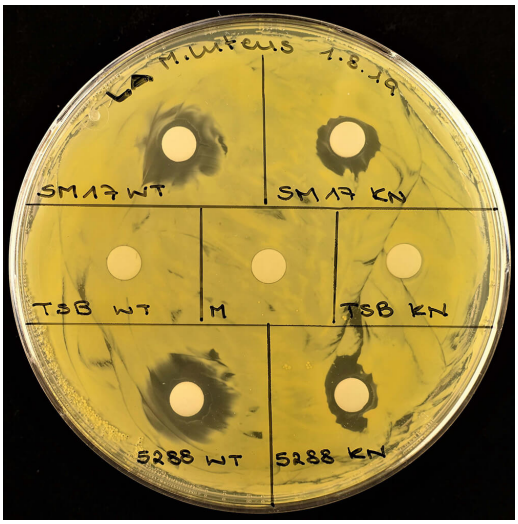
Nevertheless, the comparison of the *S. noursei* wild-type extract to the one produced by the knock-out mutant via analytical HPLC did not show any differences. Therefore, further investigations were needed in the form of preparative HPLC (6.5.3) and MS, including the wild-type and the mutant extracts, in order to isolate and identify antibiotic X.

7.1.3.4 Activity of extracts of *Streptomyces noursei* wild-type compared to knock-out mutant in different media

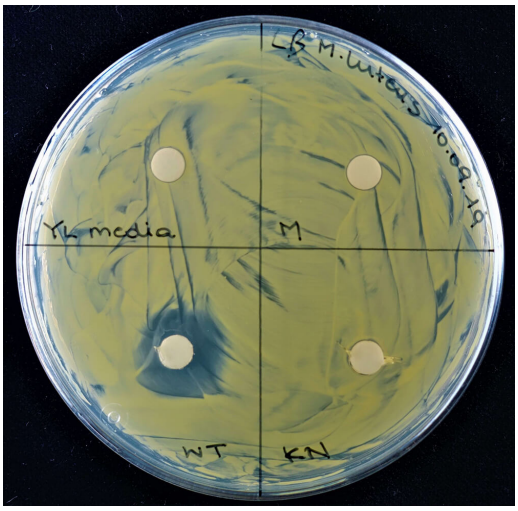
The *S. noursei* wild-type and the knock-out mutant were fermented in different media, extracted and tested for bioactivity against *M. luteus*. The results of these tests are shown in Figure 7.8. Tests have been carried out in order to get better peak resolution in HPLC to simplify the purification of antibiotic X.



(a) Extracts from cultivations in SM17 & 5333 media



(b) Extracts from cultivations in TSB, SM17 & 5288 media



(c) Extracts from cultivation in YL medium

Figure 7.8: Bioassays against *Micrococcus luteus* of extracts from fermentations of *Streptomyces noursei* wild-type (WT) and knock-out mutant (KN) in given media under standard conditions. M = methanol blank. SM17 media = media control of SM17. 5333 media = media control of 5333. YL media = media control of YL.

Based on the results of these bioactivity tests, only the YL medium was investigated further in the upscale additionally to the MP1 medium, as there was also a difference visible between the wild-type extract and the knock-out mutant extract. Fermentation in other media made comparison difficult as the production of other antibiotic substances, likely to be different to antibiotic X, could be observed. The yields of the extracts used in the bioassays are listed in

Table 11.1.

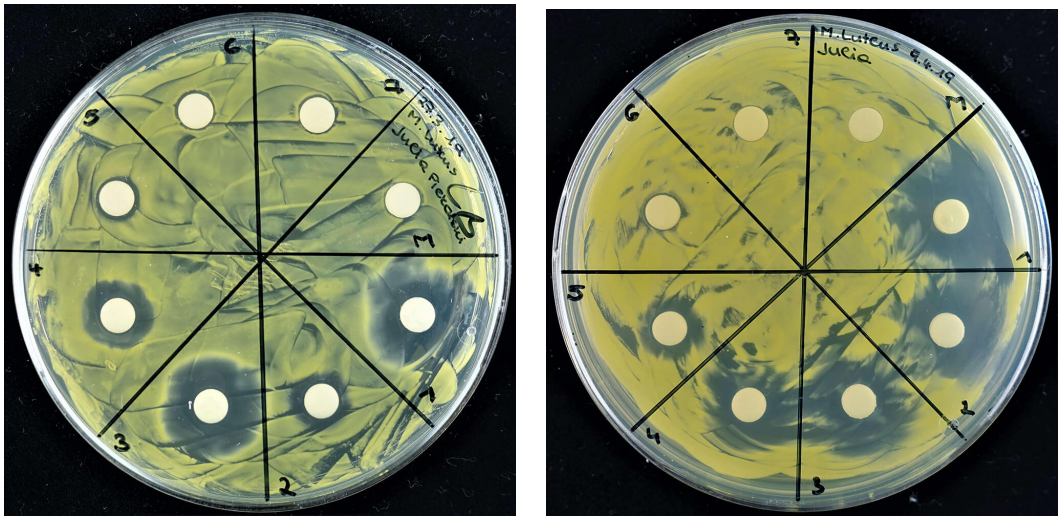
7.1.4 Purification and isolation of antibiotic X in upscale

7.1.4.1 Silica gel column chromatography

For pre-purification of the extracts of antibiotic X, silica gel column chromatography was performed as described in 6.5.2.

For fractionation A (Figure 7.9 (a)), 434.15 mg of crude extract were used for purification. This was extracted from 1 l fermentation culture in MP1 medium. As for fractionation B (Figure 7.9 (b)), 611.34 mg of crude extract was used which was gained from a MP1 fermentation culture of 1.5 l. The extracts were dissolved in 3 ml methanol each before applying them onto the column for separation.

The solvents in the different fractions were evaporated, the residues dissolved in 500 µl methanol and 15 µl were tested in bioassays for their bioactivity against *M. luteus*.



(a) Fractionation A (b) Fractionation B

Figure 7.9: Fractions after column silica chromatography tested for their bioactivity against *Micrococcus luteus*.

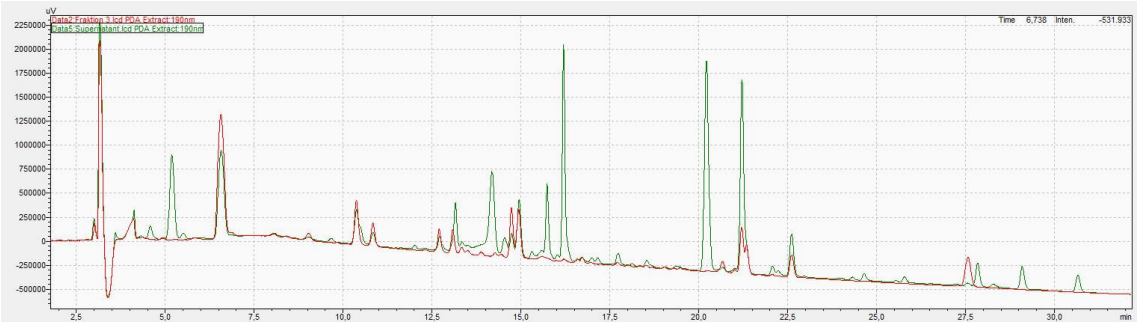
As presented in Figure 7.9, the separation could not be fully reproduced within fractionation B as the bioactivity of fraction 2 is strikingly different compared to the one of fractionation

A. Nevertheless, it can be seen, that the bioactivity of the fractions decreased significantly in correlation with the solvents used. The yields of the different fractions are listed in Table 7.2.

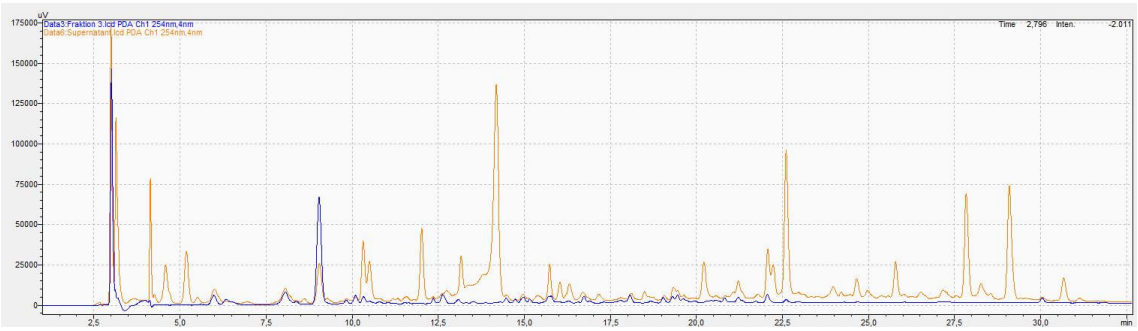
Table 7.2: Yields of the collected fractions 1 - 4 of fractionations A and B in mg.

Fractions	Fractionation A [mg]	Fractionation B [mg]
1	251.4	200.38
2	41.78	52.08
3	36.96	151.36
4	50.9	92.28

Performing analytical HPLC of the fractions after silica column chromatography led to the validation of the effect of the pre-purification using this method. This purification step was necessary to minimize the amount of additional substances present in the extracts to simplify separation afterwards. In Figure 7.10 (a) and (b) the differences between the initial extract of the supernatant to fraction 3 are presented.



(a) 190 nm



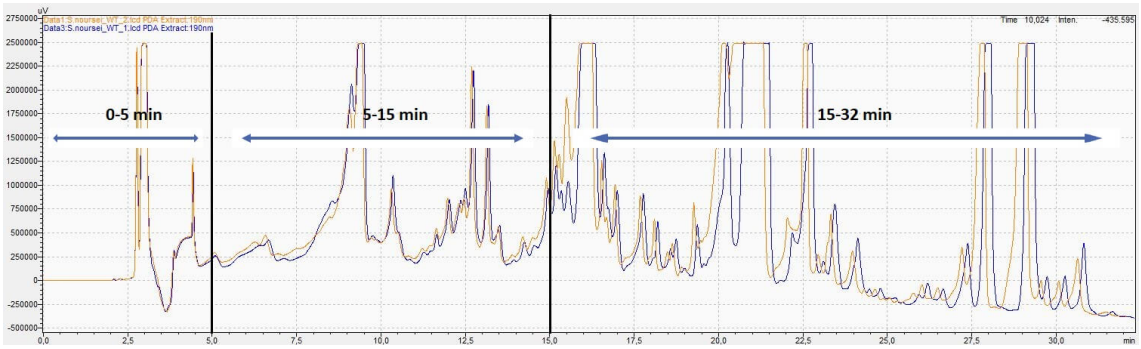
(b) 254 nm

Figure 7.10: Analytical HPLC chromatogram of the comparison of the Initial extract of the supernatant (green/yellow) and fraction 3 (red/blue) of fractionation A (Figure 7.9), at 190 nm (a) and 254 nm (b). Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® was used, including the according method (Table 6.19)

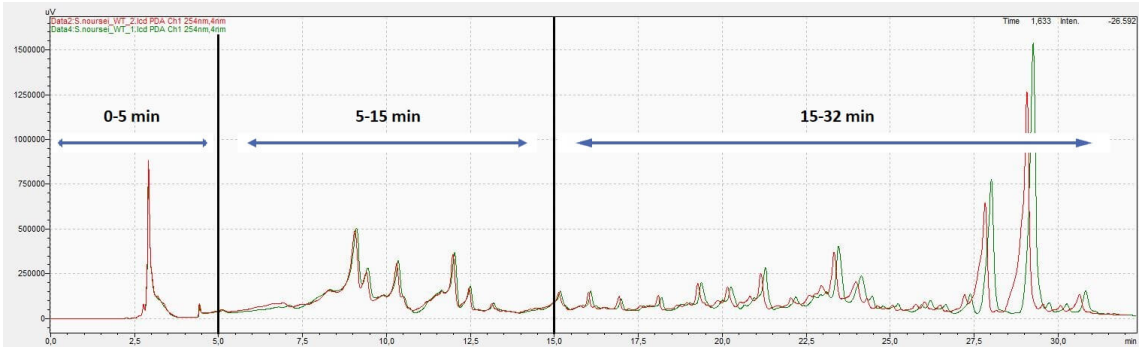
The presented spectra were obtained after analytical HPLC of the fractionated extract of fractionation A shown in Figure 7.9 (a). All in all, it can be seen, that the amount and the concentrations of additional products produced during fermentation, were minimized due to the fractionation via silica gel column chromatography. Later prepared fractionations and their corresponding fractions showed similar results (data not shown).

7.1.4.2 Micro fractionation using analytical HPLC

To identify the retention time and the correlating peak of antibiotic X, analytical HPLC was used to collect fractions in 3 different time ranges. The HPLC was run 2 times to increase the amount of antibiotic X. In total, 0.5 mg of crude extract was applied to the column for separation.



(a) 190 nm



(b) 254 nm

Figure 7.11: HPLC spectra of *Streptomyces noursei* wild-type extract unpurified at 190 nm (a) and 254 nm (b). Peaks according to the given fractions in minutes were collected in 2 runs of analytical HPLC as depicted in the illustrations above, using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® and suitable standard conditions (Table 6.19); Fractions were tested in bioassays.

Solvents of the fractions were evaporated, the residues dissolved in 100 µl methanol and these extracts tested for bioactivity against *M. luteus*. The result of the bioassay can be seen in Figure 7.12. The spectra of the chromatograms obtained while collecting the different fractions are shown in Figure 7.11. In accordance with these results, a method for preparative HPLC was established.

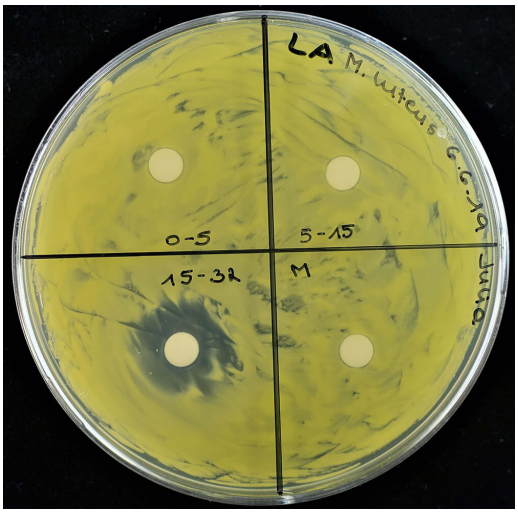


Figure 7.12: Collected fractions, obtained via micro fractionation with analytical HPLC, tested for their bioactivity against *Micrococcus luteus*. M = methanol blank; fractions in minutes of retention time.

7.1.4.3 Preparative HPLC

For purification using the preparative HPLC (6.5.3), various fractions of the ones pre-purified by silica gel column chromatography were used.

I Testing of the method

The method used for the testing is described in 6.5.3.1. The used extract was not purified beforehand as it should only lead to the determination of the best method for further experiments. The aim of this test was to reproduce the results of the micro fractionation to see if same results can be achieved via separation using preparative HPLC.

Fractions were collected for every 5 minutes, which led to a total of 8 fractions, of 50 ml each. The solvents of the fractions were evaporated, the residues were dissolved in 300 µl methanol and the extracts were tested in disc diffusion bioassays (Figure 7.13).

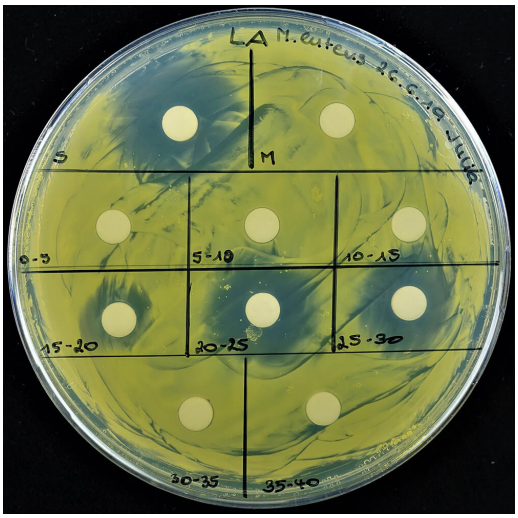


Figure 7.13: Bioassay of the fractions obtained after testing the method for preparative HPLC. Method and column used are described in 6.5.3.1. Fractions were collected every five minutes from 0 - 40 minutes. S = supernatant extract before injection into preparative HPLC; M = methanol blank.

Due to the results shown in Figure 7.13, the following preparative HPLC runs were done collecting only fractions from minute 15 - 30. Fractions were collected every minute, as it can be seen hereafter.

II Preparative HPLC of pre-purified extracts after column chromatography

Preparative HPLC was done as described in Table 6.14 using a pre-purified extract from the silica gel column chromatography of fractionation A (Figure 7.9 (a)). There, fraction 1 (251.4 mg dissolved in 3 ml methanol) was taken and separation was performed three times with an injection volume of 1 ml each.

The different fractions (shown in the chromatogram in Figure 7.14) were collected, the solvents evaporated, residues were dissolved in 1 ml methanol and the fractions were tested in bioassays (6.6.1) against *M. luteus*. The yields of the fractions are listed below in Table 7.3. While collecting the fractions, part of fraction 23 got mixed with fraction 24. The affected sample was marked with an "x".

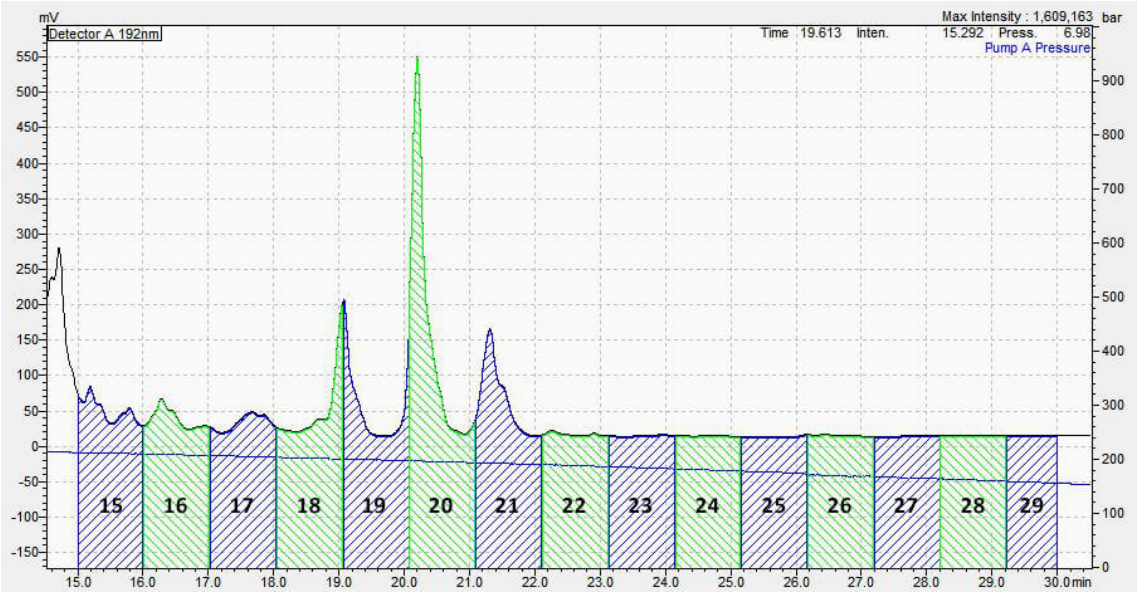
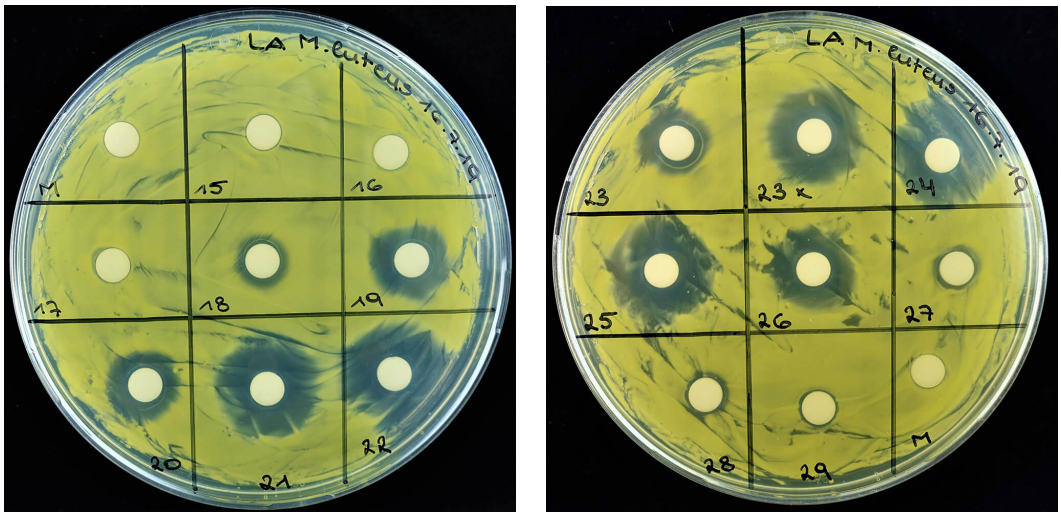


Figure 7.14: Illustration of collected fraction via preparative HPLC at 192 nm using method and column as described in Table 6.14.

Table 7.3: Yields of the different fractions (min) of preparative HPLC Table 6.14 of the *Streptomyces noursei* wild-type extract pre-purified via silica gel column chromatography. Used amount of extract = 36,96 mg; x = fraction mixed up with fraction 24. Fractions marked with / could not be measured.

Fractions in minutes	Yield [mg]
18	/
19	4.38
20	4.48
21	4.94
22	0.08
23	/
23x	1.22
24	0.59
25	7.72
26	7.82



(a) Fractions 15-22 (b) Fractions 23-29

Figure 7.15: Bioassay of the fractions collected via preparative HPLC (Table 7.3) against *Micrococcus luteus*. Fractions are labeled with the minutes of retention time. Active fractions were used for further separation via preparative HPLC as seen below.

The fractions were sent to MS for further analysis (7.1.6) and 300 µl of the methanolic extracts were evaporated, residues dissolved in 50 µl water and tested for their antihelminthic activity at the Veterinary University of Vienna (7.1.7).

Preparative HPLC was also performed using a not pre-purified extract of the knock-out extract (Table 6.17) to compare the obtained spectra in analytical HPLC and the bioassay results. For this purpose, 27.9 mg of extract were used for preparative HPLC, making one run due to the concentration of the extract. The residues of the knock-out sample were dissolved in 200 µl methanol and tested for their bioactivity against *M. luteus*.

As expected, none of the fractions collected from the knock-out extract separated via preparative HPLC showed any activity against *M. luteus*. Moreover, fractions were sent to MS analysis to determine the existence of any macrolide antibiotics left in the fractions. There it could be ascertained, that macrolides were apparent only in traces (data not shown).

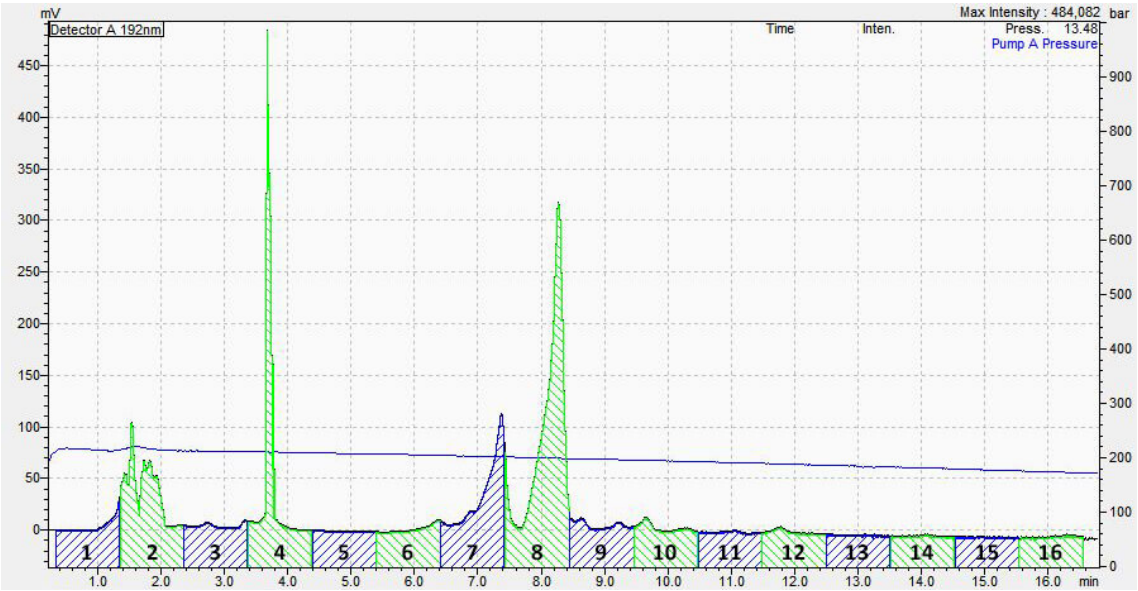
III Preparative HPLC of already separated fractions

The fractions obtained (Table 7.3) were mixed as shown in Table 7.4 and reprocessed by preparative HPLC using the methods described in 6.5.3.3. The aim was to better isolate the

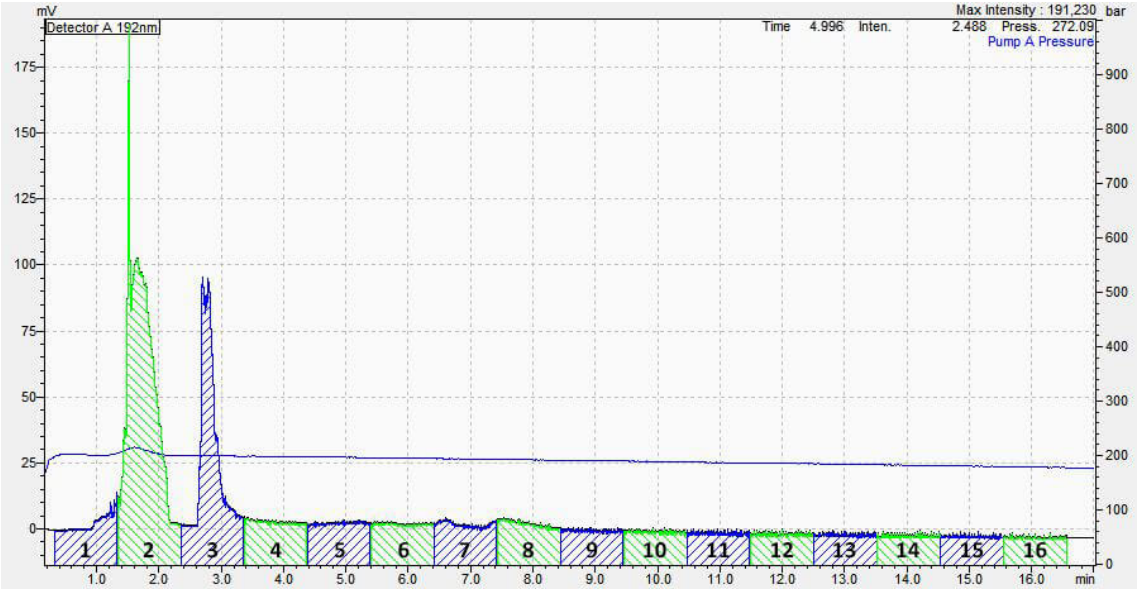
peaks in order to better separate the compounds present in the extracts and thus achieve a purer product. The collected subfractions were evaporated, and the residues dissolved in 500 µl methanol, and tested in bioassays. The active fractions can be seen in Figure 7.17.

Table 7.4: Sample constitution of two different preparative HPLC runs. Sample A consists of the shown fractions achieved in Table 7.3. Sample B consists of the shown fractions achieved in Table 7.3. Fractions were mixed and loaded onto the column doing preparative HPLC again for further separation. The different methods used for sample A and sample B are shown in 6.5.3.3. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 10 mm by Phenomenex® was used.

Sample A: included subfractions of Table 7.3	Sample B: included subfractions of Table 7.3
18	23
19	23x
20	24
21	25
22	26

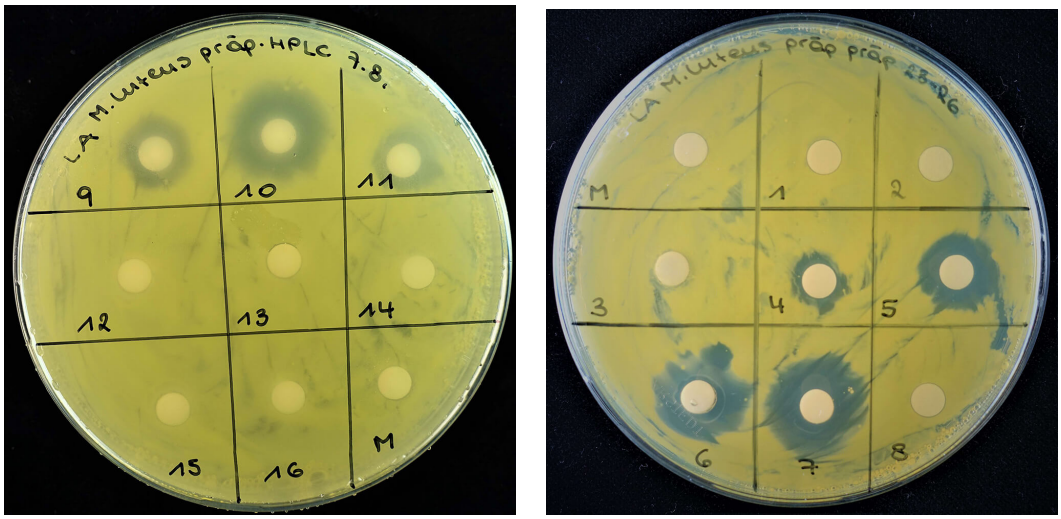


(a) Sample A



(b) Sample B

Figure 7.16: Preparative HPLC data of the fractionation of sample A and sample B described in III and Table 7.4. Method used for both runs and column used are described in Table 6.15 and Table 6.16. Samples tested were analyzed at 192 nm.



(a) subfractions of fractionation of sample A (Table 7.4)

(b) subfractions of fractionation of sample B (Table 7.4)

Figure 7.17: Bioassays of the subfractions in minutes, achieved via preparative HPLC of samples A and B (Table 7.4), tested against *Micrococcus luteus*. Fractionation of sample A (a) showed active fractions in minutes 9 - 11 whereas fractionation of sample B (b) showed active fractions from minutes 4 - 7. Preparative HPLC have been done using the according methods and column described in 6.5.3.3.

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The yields of the active fractions of sample A (Figure 7.17 (a)) could not be measured. Yields of the ones achieved of sample B are listed in Table 7.5.

Table 7.5: Yields of the subfractions achieved from fractionation of sample B (Figure 7.17) in mg.

Active subfraction of Figure 7.17 (b)	Yield [mg]
4	0.21
5	0.22
6	0.24
7	0.3

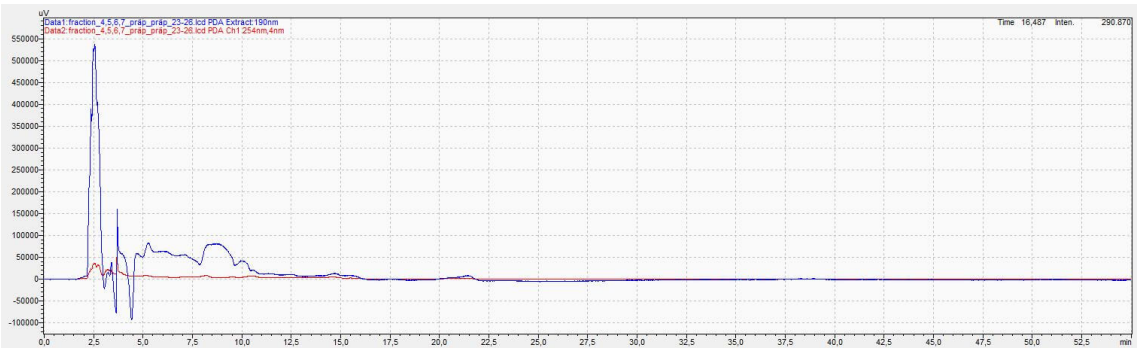
7.1.5 Optimization of purification of antibiotic X using isocratic methods in analytical HPLC

After preparative HPLC, analytical HPLC was performed to see if the purification process already ensured enough purity for NMR structure analysis. As this was not the case, isocratic

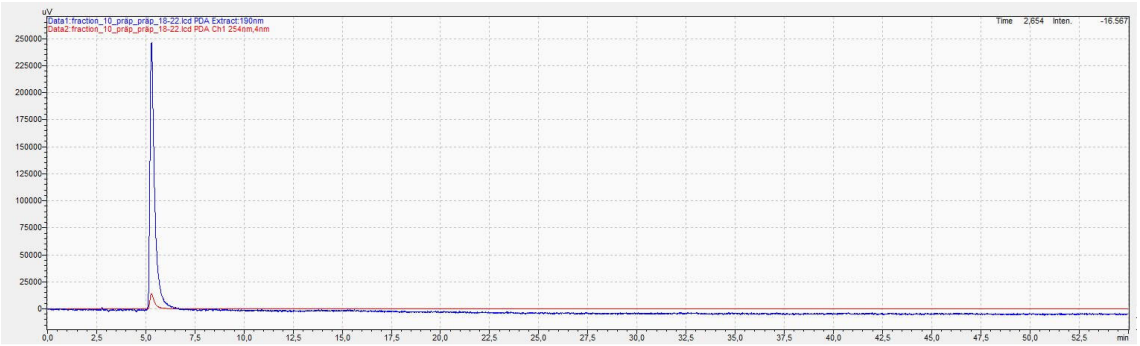
methods using analytical HPLC were tested in order to find a suitable method for separation of the different peaks.

Following the second preparative HPLC, the active fractions obtained by this (Figure 7.17) were used to perform further analysis regarding the purification of the peaks. Subfractions 4,5,6 and 7 of the fractionation of sample B (Table 7.5) were mixed before testing as for the fractionation of sample A only subfraction 10 (Figure 7.17) was used.

Method 1 was used as described in Table 6.20. Results can be seen below in Figure 7.18.



(a) Subfractions 4, 5, 6, and 7 mixed (Table 7.5); injection volume = 75 µl



(b) Subfraction 10 (Figure 7.17 (a)); injection volume = 150 µl

Figure 7.18: Subfractions 4,5,6 and 7 (Table 7.5) were mixed and tested via analytical HPLC (a) as well as Subfraction 10 (Figure 7.17 (a)) (b), using an isocratic method given in Table 6.20. Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® was used; BLUE = 190 nm; RED = 254 nm

As this isocratic method did not yield the desired outcome, method 2 (Table 6.21) was established and tested using only the mixture of samples 4, 5, 6, and 7. The result is shown below in Figure 7.19. Within this method micro fractionation was done collecting the different peaks presented in Figure 7.19. These peaks were tested for their bioactivity and sent to MS

analysis. Examinations showed that there was neither any bioactivity against *M. luteus* nor any macrolides left in the samples, which was determined by MS analysis (data not shown).

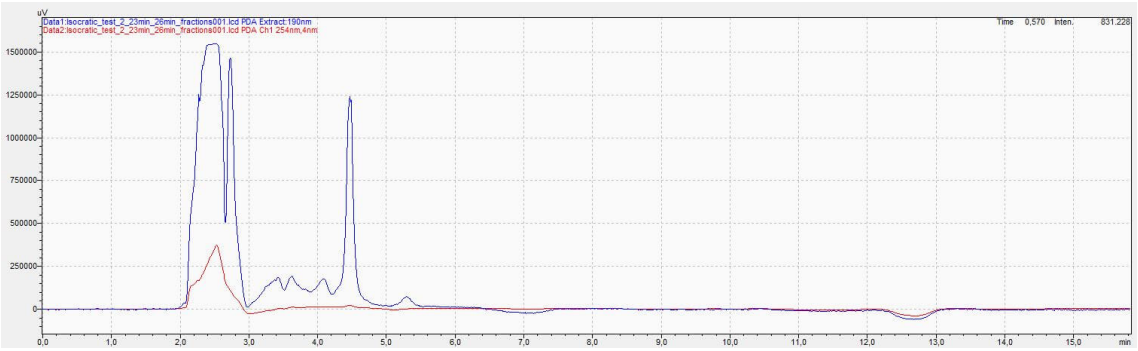


Figure 7.19: Subfractions 4,5,6 and 7 (Table 7.5) were mixed and tested via analytical HPLC using an isocratic method given in Table 6.21, using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex®; BLUE = 190 nm; RED = 254 nm; Injection volume = 70 µl; Micro fractionation was done additionally by collecting the seven peaks visible in the chromatogram.

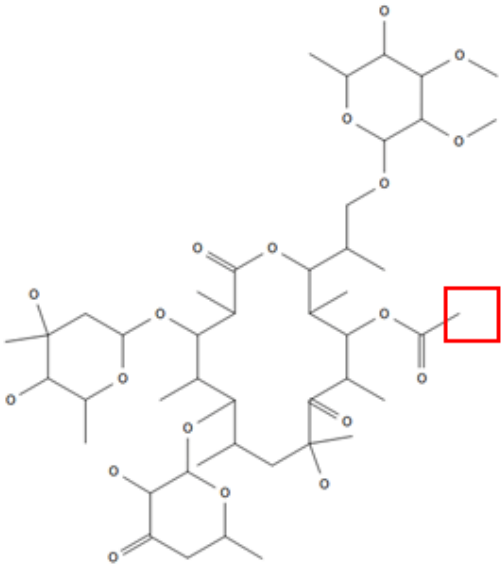
7.1.6 Mass Spectrometry

MS analysis was performed in order to obtain structural information on antibiotic X in the different fractions of the separation process. Positive ion mode base peak chromatograms in the range of m/z 100 - 1500 and 700 - 1500 were obtained (data not shown). Results revealed that not one, but more macrolide based compounds were present in the extracts and further separation was needed for final structure determination via NMR.

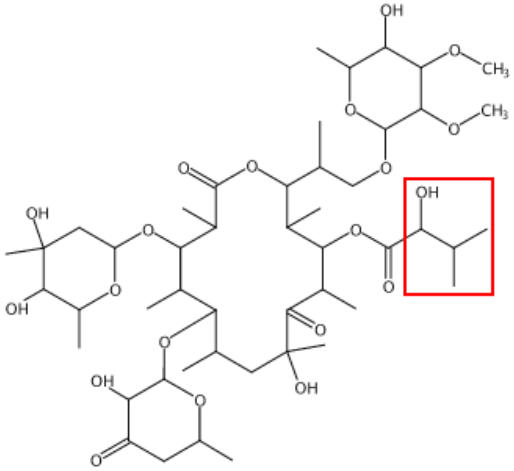
Nevertheless, using MS/MS analysis, compounds were identified in the fractions that have already been published, such as YL 02107Q and CAS 37359-09-4, but also some new structures were found. Hypothetical structures were suggested, sum formulas were estimated and possible appearances were noted. The results are listed in Table 7.6. The structures suggested via analyzing MS data of the two known products are listed below (Figure 7.20), as well as the possible structure of a derivative of YL 02107Q.

Table 7.6: Results of mass spectrometry of fractions 18 - 26 (Table 7.3) including the proposed sum formulas, the novelty of the structures found and information about the expected art of novelty of these substances. Results were provided by Dr. Martin Zehl.

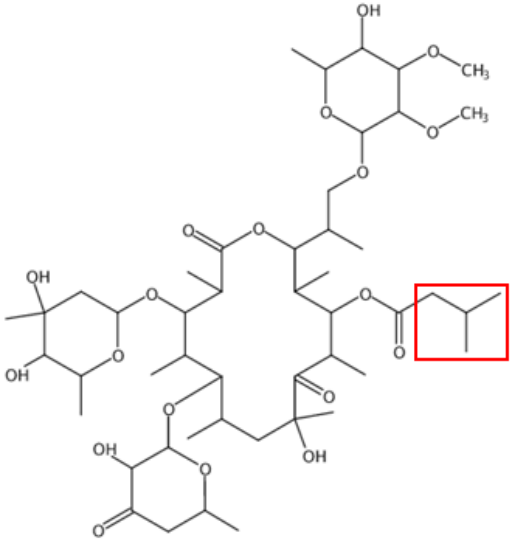
Proposed sum formula	Novelty of structure	Art of novelty
C45 H76 O19	Yes	YL 02107Q with acetyl instead of isovaleryl group (Figure 7.20 (a))
C48 H82 O20	CAS 37359-09-4	shown in Figure 7.20 (b)
C55 H88 O20 N2	Yes	Modification or substitution of the 4,6-dideoxy-hexopyranos-3-ulose
C55 H94 O23	Yes	
C48 H82 O19	Antibiotic YL 02107Q	shown in Figure 7.20 (c)
C55 H88 O19 N2	Yes	Modification or substitution of the 4,6-dideoxy-hexopyranos-3-ulose
C42 H74 O16	Yes	No 4,6-dideoxy-hexopyranos-3-ulose



(a) M = C45 H76 O19; new; YL 02107Q with acetyl instead of isovaleryl group



(b) M = C48 H82 O20; CAS 37359-09-4



(c) M= C48 H82 O19; Antibiotic YL 02107Q

Figure 7.20: Suggested structures based on analyses of the mass spectrometry data. The sum formulae given in the descriptions resemble the ones in Table 7.6, where further information is shown. The structural differences of the compounds are marked in red.

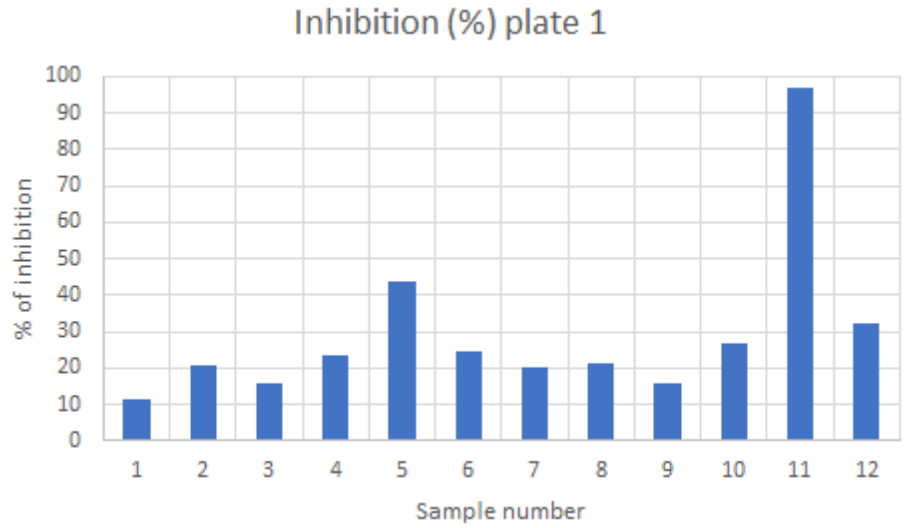
7.1.7 Testing for antihelminthic activity

As mentioned in section II, samples were sent to the Veterinary University of Vienna for testing of the possible antihelminthic activity of the extracts presented in Table 7.3. The samples obtained through fractionation process were examined by using a larval migration assay. The experiments were carried out with the third larvae of *Oesophagostomum dentatum* (*Nematoda*: *Strongylida*). Ivermectin and water were used as positive and negative control substances. Tests have been performed twice. In figure Figure 7.21 the results are shown, including the percentage of the inhibition of the migration of the larvae according to their treatment solution.

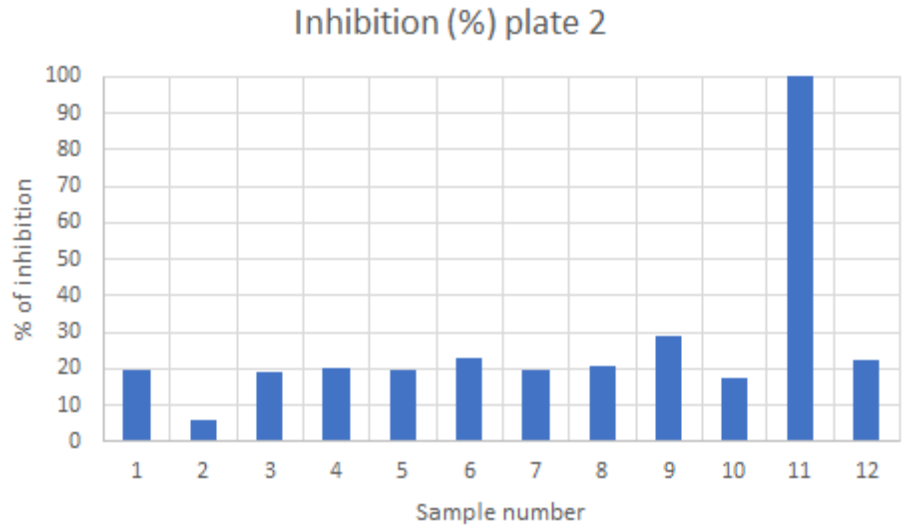
The samples used are listed below, together with their corresponding fractions and used concentrations.

Table 7.7: Sample numbering, in relation to the different bioactive fractions from preparative HPLC (Figure 7.15), including the used concentration and the applied volume for the larval migration assay.

Sample	**Fraction of preparative HPLC**	**Amount [mg/ml]**	**Used volume [µl]**
1	18	0.3	20
2	19	26.28	10
3	20	26.88	10
4	21	29.64	10
5	22	0.48	20
6	23	0.3	20
7	23x	7.32	20
8	24	3.54	20
9	25	46.32	10
10	26	46.92	10
Sample	**control**	**Amount [mg/ml]**	**Used volume [µl]**
11	Ivermectin		90
12	H_2O	0	90



(a) Plate 1



(b) Plate 2

Figure 7.21: Results of the migration assays done by the Veterinary University of Vienna. The percentage of inhibition according to the different samples can be seen in the graphs. Tests have been performed twice on 2 different plates. Tests were done under the same conditions.The results of plate 1 are presented in (a) and the results of plate 2 can be seen in (b). Sample numbers and further information are described in Table 7.7.

7.2 Heterologous expression of a putative new cluster of *Amycolatopsis* sp. YIM10 in different *Streptomyces* hosts

Cluster 5 of *Amycolatopsis* sp. YIM10 was used in the heterologous expression experiments, as it was suspected that this could lead to the production of previously unknown antibiotic substances. The reason for this assumption was the low similarity of the contained genes to already postulated clusters in the database. In addition, the cluster carries the genes for a drug transporter, which could indicate the production of an antibiotic substance.

The vector pCLY10_Cl5 of *Amycolatopsis* sp. YIM10 was conjugated into the two *Streptomyces* hosts *S. albus* Del14 and *S. coelicolor* M11455 for the heterologous expression of cluster 5. To investigate the possible production of new secondary metabolites by the recombinant strains, their fermentation cultures were prepared as noted in Table 6.11.

For cultivation in SM17 and PM4-1 medium, the recombinant strains and the *Streptomyces* hosts *S. albus* Del14 and *S. coelicolor* M11455 were cultivated for 7 days. The cultivation broth was freeze-dried before extraction (6.4.2.1). Extracts were analyzed by bioassays (6.6.1) as well as by analytical HPLC (6.6.2).

For cultivation in SG, DNPM and MYM media, strains of *S. albus* Del14 and *S. coelicolor* M11455 conjugated with the empty pCLY10 vector were used instead of the wild-type strains as a control. Extraction (6.4.2.2) was done separately for the supernatant and for the pellet, before analyzing the extracts in bioassays (6.6.1) and analytical HPLC (6.6.2).

None of the extracts obtained in the above-mentioned steps showed any significant results. Due to these outcomes, a method for the overexpression of three cluster-specific regulatory genes was established (7.3).

7.3 Expression of cluster 5 of *Amycolatopsis* sp. YIM10 in *Streptomyces* hosts along with transcriptional regulators

Cluster 5 of *Amycolatopsis* sp. YIM10 was analyzed by antiSMASH 5.0. Genes encoding regulators were identified and three of them were chosen for the overexpression together with cluster 5. The full list of the inspected genes can be found in Table 7.8.

Table 7.8: Results of the antiSMASH analysis of all 42 genes of cluster 5 of *Amycolatopsis* sp. YIM10. antiSMASH version 5.0 was used to erode homologous proteins including the corresponding microorganisms that produce them and the percentage of similarity of these genes. Genes modified by the different primers (Table 6.2): asnR_AmC5 = ctg2_3168; gntR_AmC5 = ctg2_3156; luxR_AmC5 = ctg2_3159.

gene number	start gene	end gene	homolog protein	protein source	identify
ctg2_3144	405	1	LysR family transcriptional regulator	*Amycolatopsis orientalis*	87%
ctg2_3145	794	435	LysR family transcriptional regulator	*Amycolatopsis orientalis*	92%
ctg2_3146	1519	914	TetR/AcrR family transcriptional regulator	*Streptomyces* sp. CB02923	74%
ctg2_3147	2127	1525	TetR family transcriptional regulator	*Streptomyces zhaozhouensis*	80%
ctg2_3148	2774	2151	MULTISPECIES: GNAT family N-acetyltransferase	*Pseudonocardia*	67%
ctg2_3149	3181	2924	hypothetical protein A4R43_07960	*Amycolatopsis albispora*	91%
ctg2_3150	4064	3366	hypothetical protein A4R43_07960	*Amycolatopsis albispora*	86%
ctg2_3151	6196	4553	alpha-galactosidase	*Streptomyces* sp. CNZ289	85%
ctg2_3152	7801	6221	glycosyl hydrolase 43 family protein	*Kibdelosporangium aridum*	82%
ctg2_3153	9351	7831	alpha-L-arabinofuranosidase	*Amycolatopsis albispora*	91%
ctg2_3154	10642	9395	cellulose-binding protein	*Actinophytocola xinjiangensis*	67%
ctg2_3155	11204	10647	DJ-1/PfpI/YhbO family deglycase/protease	*Saccharothrix saharensis*	90%

ctg2_3156	12175	11408	DNA-binding FadR family transcriptional regulator	*Saccharothrix carnea*	82%
ctg2_3157	13383	12283	hypothetical protein	*Saccharothrix* sp. ALI-22-I	73%
ctg2_3158	14486	13371	hypothetical protein	*Saccharothrix* sp. ALI-22-I	76%
ctg2_3159	15139	14471	response regulator transcription factor	*Saccharothrix* sp. ALI-22-I	92%
ctg2_3160	15549	15223	DUF4333 domain-containing protein	*Amycolatopsis saalfeldensis*	55%
ctg2_3161	16943	15546	tryptophanase	*Amycolatopsis nigrescens*	87%
ctg2_3162	18111	16945	sodium:proton antiporter	*Amycolatopsis xylanica*	68%
ctg2_3163	19468	18104	FAD-dependent oxidoreductase	*Amycolatopsis orientalis*	82%
ctg2_3164	21222	19465	amino acid adenylation domain-containing protein	*Amycolatopsis nigrescens*	74%
ctg2_3165	22000	21215	thioesterase	*Amycolatopsis nigrescens*	63%
ctg2_3166	23271	22285	oxidoreductase	*Streptomyces* sp. AcE210	91%
ctg2_3167	23746	23345	arsenate reductase ArsC	*Amycolatopsis albispora*	93%
ctg2_3168	24123	23803	helix-turn-helix transcriptional regulator	*Amycolatopsis albispora*	90%
ctg2_3169	25175	24120	ACR3 family arsenite efflux transporter	*Amycolatopsis albispora*	95%
ctg2_3170	25761	25276	DinB family protein	*Arthrobacter* sp. Hiyo1	81%
ctg2_3171	26529	25768	ABC transporter permease	*Amycolatopsis albispora*	89%

ctg2_3172	27329	26526	ATP-binding cassette domain-containing protein	*Streptosporangium roseum*	83%
ctg2_3173	28267	27632	TetR/AcrR family transcriptional regulator	*Amycolatopsis* sp. WAC 01376	86%
ctg2_3174	29821	28373	MFS transporter	*Amycolatopsis* sp. WAC 01416	88%
ctg2_3175	31011	29827	FAD-dependent oxidoreductase	*Amycolatopsis* sp. WAC 01376	83%
ctg2_3176	31773	31021	sugar nucleotide-binding protein	*Microtetraspora niveoalba*	82%
ctg2_3177	32269	31787	carboxymuconolactone decarboxylase family protein	*Saccharothrix deserti*	82%
ctg2_3178	33284	32391	RNA polymerase sigma-70 factor	*Microtetraspora niveoalba*	89%
ctg2_3179	34186	33362	type I methionyl aminopeptidase	*Microtetraspora niveoalba*	91%
ctg2_3180	36033	34189	PhoX family phosphatase	*Amycolatopsis marina*	81%
ctg2_3181	37544	36180	MFS transporter	*Streptomyces* sp. WAC 05977	86%
ctg2_3182	37995	37603	MerR family transcriptional regulator	*Amycolatopsis albispora*	93%
ctg2_3183	38939	38004	NADP-dependent oxidoreductase	*Streptomyces* sp. CB02959	84%
ctg2_3184	40153	39077	hypothetical protein	*Actinophytocola xanthii*	81%
ctg2_3185	40641	40081	TetR/AcrR family transcriptional regulator	*Streptomyces* sp. MP131-18	80%

After investigating cluster 5 via antiSMASH 5.0, primers for the overexpression of regulatory genes from cluster 5 of *Amycolatopsis* sp. YIM10 were designed by Dr. Olha Schneider as described in 6.1.1.2, and are listed in Table 6.2.

PCR was done (6.1.4) using the suitable parameters for the different primers (Table 6.2), gel electrophoresis (6.1.6) was performed and the DNA was extracted as described in 6.1.7.

PCR products were digested and purified before ligation with the previously digested puWLoriT plasmid vector. Ligation was followed by transformation into *E. coli* XL1-Blue cells. Thereafter, as a control step, the plasmid was isolated and analytical restriction (Table 6.7) was performed. As seen in Figure 7.22, the obtained fragments represented the parts ligated in the constructed plasmids, as expected.

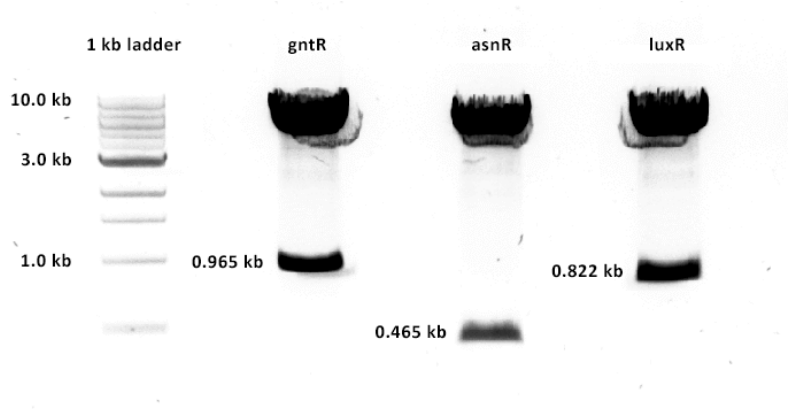


Figure 7.22: Analytical digestion of the constructed plasmids. 1 kb ladder was used as a marker; gntR = digestion product of the isolated puWLoriT_gntR with the expected size of 965 bps; asnR = digestion product of the isolated puWLoriT_asnR with the expected size of 465 bps; luxR = digestion product of the isolated puWLoriT_gntR with the expected size of 822 bps.

As a next step for the overexpression of cluster 5, conjugation was done, transferring the constructed plasmids into the host organisms *S. albus* Del14/pCLY10, *S. albus* Del14/pJPC5, *S. coelicolor* M11455/pCLY10 and *S. coelicolor* M11455/pJPC5. *S. albus* transconjugants were cultivated in SG, MYM and YL media, *S. coelicolor* transconjugants were grown in R3, R5 and GYM media. Strains were fermented for 7 days, extracted, and the metabolite profiles from the strains with overexpressed regulators and the control strains extracts were analyzed via analytical HPLC and the chromatograms were compared. An additional peak was supposed to be detected by analyzing the extracts of the test organisms, if the expression of cluster 5

in one of the heterologous expressed hosts was successful. From 3 expressed regulator genes, only the overexpression of the *gntR* gene in SG medium showed significant differences in HPLC spectra, presented in Figure 7.23.

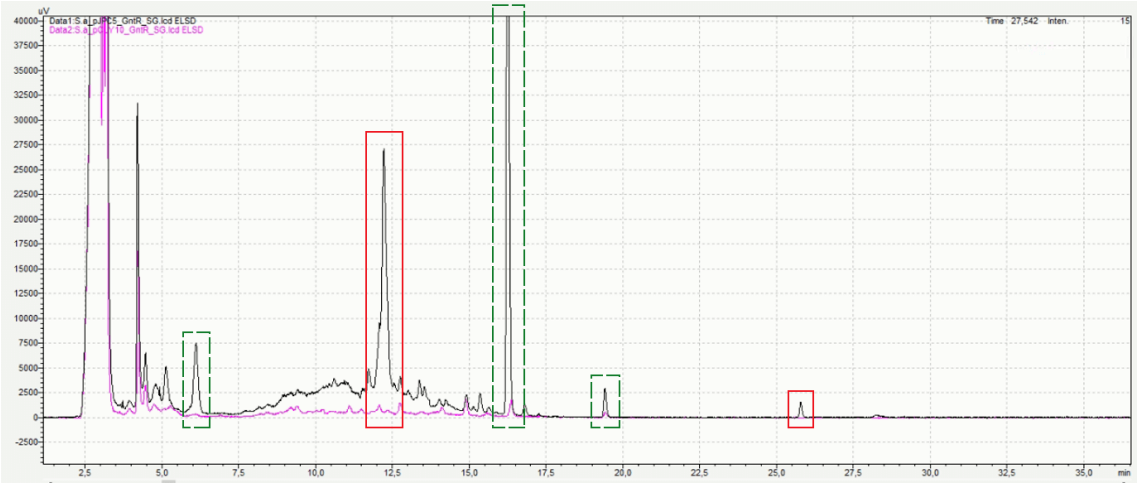


Figure 7.23: HPLC spectrum of ELSD analysis of analytical HPLC using Luna® 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm by Phenomenex® and the according method Table 6.19. Differences in concentration of the peaks are marked with dashed green boxes. Newly appeared substances are marked with red boxes. Violet = S.a._pcLY10_gntR; for *Streptomyces albus* with empty pCLY10 vector and overexpressed *gntR* gene, as negative control; Black = new construct with overexpressed cluster 5 of *Amycolatopsis* sp. YIM10 and overexpressed *gntR* gene in *Streptomyces albus* (*S.a.*_pJPC5_gntR). Both constructs cultivated in SG medium.

Finally, the extracts from *S. albus*/pCLY10_gntR and *S. albus*/pJPC5_gntR were analyzed via MS. Although it can be seen from the chromatogram above that the recombinant strain which contains the cluster 5 and overexpressed *gntR* gene showed the presence of more metabolites and higher concentrations of already produced metabolites, the MS analysis data provided the results that those are the same metabolites like in the control strain, but their production is distinctly increased in the test strain. Unfortunately, no potential new compounds could be detected via MS analysis.

Chapter 8

Discussion

Within this project, two approaches of finding new secondary metabolites, especially new antibiotics, were applied using two different strains, *S. noursei* and *Amycolatopsis* sp. YIM10. These two approaches, the classical bioassay-guided fractionation and the advanced genomics-driven method of heterologous expression for the activation of biosynthetic gene cluster provided mixed results, which prompt further investigations.

The application of classical bioassay-guided methods for the discovery of new natural products from *S. noursei*, based on information described in [1], led to the verification of the responsible gene cluster encoding for the production of antibiotic X. Furthermore, possible structures and sum formulas of unknown macrolides could be predicted due to MS analysis and the presence of two already published compounds, the antibiotic YL 02107Q and CAS 37359-09-4, could be determined. Nonetheless, there are still aspects that need to be considered.

Although it was planned to isolate the antibiotic X produced by *S. noursei*, this process could not be accomplished due to different possible reasons. First of all, the substance in question was not visible in HPLC spectra, which resulted in the fact, that even though preparation of a knock-out mutant was successful, no differences in the chromatograms between the wild-type extract and mutant extract could be detected. Anyway, bioassay-guided fractionation has been done several times to ensure the purification of the compounds to enable structure determination. Fractions were sent to MS analysis for further investigations which resulted in the confirmation of the presence of macrolides in the extracts, and in the prediction of possible structures and mass formulae. However, the compounds could not be analyzed by NMR due to

the low purity. The MS analysis confirmed the presence of possible derivatives of antibiotic X, which is probably also the reason why they are very difficult to separate as they are chemically just slightly different. As the patent on the antibiotic substance YL 02107Q found in the extracts already reported the absorption of this molecule, the fact that no peak at this point could be detected suggests that this substance is present in very low concentration, despite strong bioactivity.

The MS analysis of an extract obtained after cultivating the recombinant strain *S. noursei*/pSOK201_KN_SnC14, showed, that there were still some macrolides left, even though the gene responsible for the production had been knocked out. This fact can for example be explained by the excision of the inserted knock-out plasmid via recombination in the absence of antibiotic selection. The presences of some bacteria in the population lacking the inserted plasmid can be verified by the gel electrophoresis of PCR products of the recombinant strain compared to the one of the wild-type strain. Indeed, in Figure 7.6 it can be seen, that there is low intensity band at 3.0 kb displayed, which corresponds to the size of the PCR product of the wild-type strain. This genetic instability has already been observed in *Streptomyces* species in form of plasmid loss or rearrangement [106]. Nevertheless, one can say that the insertional disruption of cluster 14 was successful, because the produced macrolides in the knock-out mutant were only present in small traces, and therefore the responsible gene cluster of the production of antibiotic X could be identified and the biosynthetic pathway can be verified.

Additional to the verification of the involvement of cluster 14 in the biosynthesis of antibiotic X, the production of this macrolide antibiotic was meant to be optimized by testing different fermentation media. Once more, the wild-type strain was compared to the knock-out mutant. Extracts have been tested in bioassays against *M. luteus*, which showed significant differences in activity, depending on the media used (Figure 7.8). This results in the assumption of a nutrient dependent activation of other gene cluster resulting in the production of another antibiotic.

Fractions from preparative HPLC tested for antihelminthic activity at the Veterinary University of Vienna, did not show significant results. However, giving the data obtained, the concentrations of the different fractions might have been too low and additionally, concentrations used were very low, which could have led to negative results compared to the antihelminthic substance ivermectin.

As a next candidate for the discovery of new natural products, the newly isolated *Amycolatopsis* sp. YIM10 strain was chosen, which originates from the rare earth mine of Bayan Obo, Inner Mongolia in China. *In silico* analysis of the genome sequencing data showed that the genome from this organism consists of at least 35 BGCs. The product from cluster 5 was predicted by antiSMASH to be a peptide with non-ribosomal origin. The combination of genes within the gene cluster was not found in the databases as well as the homology of the gene products was also low to known proteins in the databases. Based on the results of the *in silico* analysis, it was suggested that cluster 5, a NRPS-like cluster, might encodes for a potential new compound. The fact that this cluster also carries genes which encode for a putative drug resistance-transporter might indicates that the product of cluster 5 could have an antibiotic function.

The advanced genomics-driven approach attempted to achieve the activation of cluster 5 of *Amycolatopsis* sp. YIM10 after heterologous expression in two *Streptomyces* hosts. However, this did not lead to the production of any new compounds. Therefore, it was tried to stimulate the expression of possible secondary metabolites by overexpressing 3 different regulatory genes in *Streptomyces* strains harboring cluster 5.

The introduction of overexpressed regulators into these hosts was only accomplished in the case of *gntR*. There can be various reasons for this, as the heterologous expression of genes is a very complex and uncertain process. The achieved transconjugants were fermented in different media and resulting extracts were analyzed by HPLC. Nevertheless, no new compounds were produced by those. But still, stimulation of some secondary metabolites biosynthesis could be observed in case of the heterologous expression of cluster 5 including overexpressed *gntR* when fermented in SG medium. This effect has already been described using another example, the heterologous expression of the novobiocin BGC in *S. coelicolor* and the subsequent overexpression of the regulator gene *novG*, where the overexpression also led to a significantly increased metabolite production rate [107].

Giving all these aspects in both approaches of isolating new secondary metabolites, there still is a lot that must be investigated further. Especially in case of the optimization of the fermentation process and of the separation of the different compounds present in the extracts.

Chapter 9

Outlook

As it can be seen, even though bacteria have long been used for the discovery of antibiotics, the possibilities of discovering new secondary metabolites through new approaches are far from exhausted. Especially with the help of today's bioinformatic tools and methods for the genetic modification of genomic DNA, there are still numerous approaches which can be pursued and bacteria that can be investigated.

With regard to this work, it can be said that, especially in the case of *Streptomyces noursei*, further investigations and considerations should be made regarding the isolation of the metabolites found, since despite the low concentrations of the included compounds, the bioactivity against gram+ bacteria is extremely high and the chance of discovering previously unknown substances is quite promising. In any case, production yields must be enhanced by for example increasing the fermentation volumes and an optimization of the separation process must be undertaken in order to ensure structure elucidation by NMR analysis.

The same applies to the advanced genomics driven approach. Here, too, further measures can be taken with regard to fractionation of the existing metabolites and structural elucidation of the individual compounds. In addition, cluster 5 of *Amycolatopsis* sp. YIM10 still contains numerous regulators, whose modifications could yield interesting outcomes.

Chapter 10

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Appendix

11.1 Yields

Table 11.1: Information about the extracts, received from cultivation of *Streptomyces noursei* in different media, used for bioassays in Figure 7.8.

Extract	Extraction volume [ml]	Yield [mg]	MeOH Dissolved [ml]	Used volume for bioassays [µl]
SM17 WT 1.08.2019	10	8.8	1	15
SM17 KN 1.08.2019	10	8.15	1	15
TSB WT 1.08.2019	10	3.68	1	15
TSB KN 1.08.2019	10	3.91	1	15
5288 WT 1.08.2019	10	12.06	1	15
5288 KN 1.08.2019	10	8.48	1	15
5333 WT 10.09.2019	100	8	10	30
5333 KN 10.09.2019	100	10	10	30
5333 media 10.09.2019	50	0	5	30
SM17 WT 10.09.2019	300	488	30	30
SM17 KN 1.08.2019	10	8.15	1	30
SM17 media 10.09.2019	50	13	5	30
YL WT 10.09.2019	100	64.66	10	30
YL KN 10.09.2019	100	55.78	10	30
YL media 10.09.2019	50	26.37	5	30

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