Sugars in early and late polyketide biosynthesis:

Functional studies of *rifL, rifK* and *rifM* in rifamycin biosynthesis

Towards the characterisation of a PKS gene cluster from *Streptomyces* sp. GW2/5831, encoding the biosynthesis of the polycyclic xanthone IB-00208

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für meine Eltern und für Meikel

„Überall geht ein früheres Ahnen dem späteren Wissen voraus."
Alexander von Humboldt (1769-1859)

Table of contents

List of abbreviations

A	Adenine
ACP	Acyl carrier protein
ATP	Adenosine tri phosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degree centigrade
CoA	Coenzyme A
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylen diamine tetra acetic acid
e.g.	Latin ‘exempli gratia’; for example
et al.	Latin ‘et alii’; and others
EtOH	Ethanol
FPLC	Fast protein liquid chromatography
g	G-force
g	Gram
G	Guanine
GC	Gas chromatography
h	Hour
HPLC	High performance liquid chromatography
kb	Kilo base pairs
KS	β-ketoacylsynthase, ketosynthase
λ	Wavelength [nm]
l	Liter
LB	Luria Bertani medium
M	Molar [Mol/l]
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
MCS	Multiple cloning site
mg	10^{-3} gram
ml	10^{-3} liter
mM	10^{-3} molar
min	Minute(s)
MS	Mass spectroscopy
m/v	Mass-to-volume ratio
m/z	Mass-to-charge ratio
µg	10^{-6} gram
µl	10^{-6} liter
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
ng	10^{-9} gram
nm	10^{-9} meter

NMR	Nuclear magnetic resonance
OD_{600}	Optical density at λ = 600 nm
PCR	Polymerase chain reaction
pH	Potentia hydrogenii
PKS	Polyketide synthase
ppm	Parts per million
rif	Gene from rifamycin cluster
Rif	Protein from rifamycin cluster
RNA	Ribonucleic acid
rpm	Rounds per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Second (s)
sp.	Species
t/min	Time in minutes
T	Thymine
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TLC	Thin layer chromatography
U	Unit
UV	Ultra violet
V	Volt
VIS	Visible

1 Summary

The first section of this work comprises investigations concerning the initial steps in rifamycin biosynthesis. The amino sugar kanosamine was described to represent the most likely source of the nitrogen atom in the aminoshikimate pathway and is putatively synthesised by the catalytic activity of the enzymes RifL, RifK and RifM. Furthermore, in earlier studies kanosamine was even converted to kanosamine 6-phosphate by the rifamycin biosynthetic enzyme RifN. Therefore, evidence but no proof for the occurrence of kanosamine at an early stage in rifamycin biosynthesis was provided before the beginning of the present work. In this work functional studies of the gene products of *rifL, rifK* and *rifM* from the rifamycin producer *Amycolatopsis mediterranei* S699 were carried out using the plasmid pHGF7604 for the heterologous expression of the *rifG-N* genes in *Streptomyces lividans* TK24. Kanosamine was supposed to be synthesised by RifL, RifK and RifM during the RifL assay, employing a cell-free extract of *Streptomyces lividans* pHGF7604. These studies were based on former results concerning the RifL assay and aimed at the analytical detection of the putatively synthesised intermediate kanosamine en route to the known ansamycin precursor AHBA. Therefore, the RifL assay system was optimised, protein purification of cell-free extracts from *Streptomyces lividans* pHGF7604 was performed and analytical methods for kanosamine detection were developed within the scope of this study. The amino sugar was not detected in any RifL incubation sample, whereas concurrently no RT-PCR product of RifL could be verified. However, actual proof for an involvement of *rifL, rifK* and *rifM* in the postulated biosynthetic pathway of rifamycin could not be demonstrated under the terms of this study.

The second section of this work is directed towards the characterisation of the gene cluster encoding the biosynthesis of the polycyclic xanthone IB-00208 in *Streptomyces* sp. GW2/5831. As polyketides represent an important source of nature-derived pharmaceuticals, their exploration provides promising opportunities for the discovery of new biogenic agents. Moreover, the acquirement of insights on the genetic level provides important information for genetic engineering that can lead to novel hybrid compounds with improved therapeutic values. In polyketide gene clusters “core” polyketide synthase (PKS) genes are encountered along with “post” PKS genes encoding modifying enzymes like oxygenases, methyl- and glycosyltransferases. In particular, glycosylation reactions that take place during post-PKS tailoring processes are relevant for genetic engineering. Especially genes encoding glycosyltransferases that catalyse (deoxy-) sugar attachments to the matured molecule are

focussed on. The information obtained in this part of the work should provide a prerequisite for a prospective genetic engineering of the IB-00208 and griseorhodin A biosynthetic pathways. A glycosylated griseorhodin A molecule is expected to feature improved pharmacokinetic properties. As both compounds share the same polyaromatic core skeleton, it can be envisioned that griseorhodin A will be accepted as an aglycon-substrate by the glycosylating enzyme from the IB-00208 cluster. To isolate the IB-00208 cluster, a genomic library was established from DNA of *Streptomyces* sp. GW2/5831, and screening was performed employing ketosynthase (KS) primers. Sequencing of the positive cosmid clones and subsequent BLAST search allowed the deduction of a putative cluster fragment containing protein coding regions characteristic of this kind of PKS clusters, and genes possibly involved in the biosynthesis of IB-00208 were identified. The library was constructed using a shuttle vector that enabled the heterologous expression of the cosmid sequences in *Streptomyces albus*. This study sets the stage for further investigations of the IB-00208 biosynthetic gene cluster from *Streptomyces* sp. GW2/5831.

Both parts of the study are in the field of actinomycete genetics in drug development, representing different types of PKS systems. The genetics of streptomycetes is of particular importance, as it opens up many possibilities in the fields of biotechnology and pharmacy. Therefore many sequencing projects contribute to an enlarged knowledge on biosynthetic gene clusters of therapeutic agents that are of substantial interest.

2 Introduction

Secondary metabolites are natural products originating from micro- and macroorganisms. They are widespread in nature and often exhibit potent physiological activities. The pharmacological properties of biogenic agents are beneficial for example in the treatment of infectious diseases or cancer.[1] Natural products are generally employed as putative drug candidates themselves or serve as drug leads in pharmaceutical research.[2] In contrast to the basic primary metabolism, the secondary metabolism is not absolutely essential for the survival of organisms. These compounds are more likely to inherit ecological functions regarding defence against predators or diseases, reproductive advantages, improved intraspecies communication or interspecies competition.[3] The secondary metabolism is usually induced at a certain developmental stage of an organism or by environmental conditions. Mediation of mutualistic interactions between higher organisms and producing microorganisms is frequently encountered (e.g., the pederin-producing bacterium, related to *Pseudomonas aeruginosa*, that is associated with the beetle *Paederus fuscipes*[4]). Natural products occur in a wide spectrum of chemical product classes (families) including alkaloids, glycosides, polyketides, terpenoids among others. Polyketides represent a prominent group of nature-derived drugs, prevalently antibiotics that are mainly produced by actinomycetes, in particular *Streptomyces* strains.

2.1 Streptomycetes as polyketide factories

The genus *Streptomyces* comprises Gram-positive, obligate aerobic bacteria exhibiting a high content of cytosine and guanine in their DNA.[5] Taxonomically, *Streptomyces* species reside in the diverse group of actinomycetes. Due to their growth as a vegetative hyphal mass that creates a filamentous mycelium, enabling them to easily spread through soil, they were formerly designated as “ray fungi”. Streptomycetes are ubiquitous soil bacteria, likely to be present in aquatic habitats as well,[6] and many of them are able to colonise plant rhizospheres.[7] They are important decomposers utilising extracellular hydrolytic enzymes. Their morphological differentiation is characterised by a life cycle consisting of the germination of vegetative mycelium from a single spore, followed by growth of aerial hyphae. Septation of these hyphae then produces unigenomic spore compartments that can commence a new cycle. The conidiospores represent a semi-dormant stage in the life cycle that can hold on for decades. It is reported by Morita that viable *Streptomyces* cultures were recovered from 70 year old soil samples.[8] Streptomycetes are still an outstanding source of therapeutically important agents, even though they have been known for a long time. As a consequence, they

are invaluable in the medical field, providing approximately half of all known antibiotics.[5] Many drugs for the treatment of cancer are *Streptomyces*-derived as well.[9] Furthermore, these compounds are utilised in agriculture as growth promoters, agents for crop protection, antiparasitic agents and herbicides.[10]

The aromatic polyketide actinorhodin **1** (figure 2.1), produced by *Streptomyces coelicolor,* provides one of the most thoroughly studied examples of all *Streptomyces* antibiotics.[11] The production of antibiotics in Streptomycetes is generally growth phase-dependent. The onset of growth in liquid cultures takes place at the beginning of the stationary phase, whereas on solid media it coincides with morphological differentiation.

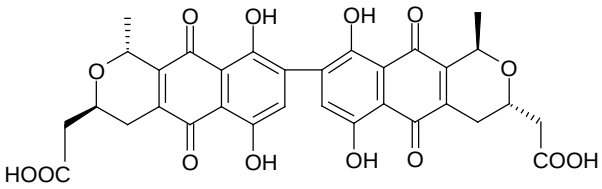


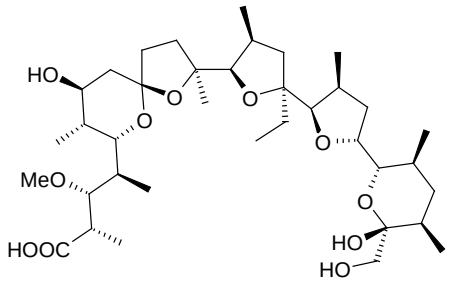
Figure 2.1: Chemical structure of actinorhodin **1** from *Streptomyces coelicolor* with antibacterial activity.

As microorganisms developed resistance against antibiotics, and multidrug-resistant pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) appeared, the search of new agents and modified compounds was greatly enforced. Therefore the development of analytical methods in chemistry and molecular biology led to improved screening techniques (counter screening), optimised fermentation procedures and target-based modifications on producing strains. For example, Lang et al. described a dereplication methodology for rapid small-scale investigation of natural products extracts.[12]

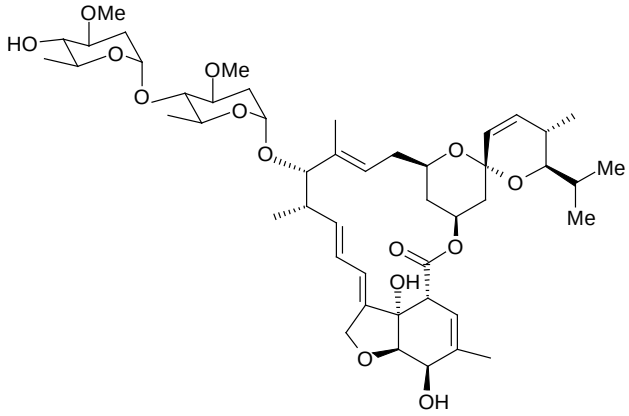
Many important *Streptomyces*-derived antibiotics belong to the functionally and structurally diverse group of polyketides sharing considerable pharmaceutical properties. They especially serve as lead structures for the synthesis of improved pharmacological compounds. A small selection of polyketide structures and their natural producers is displayed in figure 2.2. Polyketides inherit a wide spectrum of pharmaceutically relevant activities, including antibacterial (monensin **2**, tetracycline **6**, rifamycin **14**), antifungal (amphotericin **9**), antiparasitic (avermectin **3**), antitumour (doxorubicin **4,** rubromycin **5**, hedamycin **8**, bleomycin **10**) and immunosuppressant (tacrolimus **7**, rapamycin **11**) agents. Polyketides are a

prominent class of natural products often synthesised by soil bacteria like *Streptomyces* species, but also by other bacteria, fungi, marine organisms or plants.

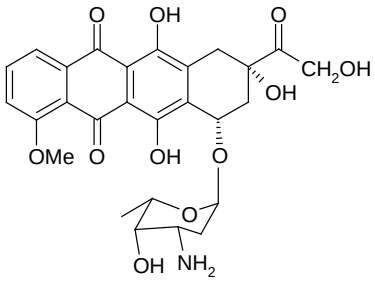
The mode of action of a polyketide can, for example, be due to the inhibition of bacterial protein biosynthesis by disruption of t-RNA binding to the ribosome 30S subunit (tetracycline **6**).[13, 14] Furthermore, the reaction can be based on a DNA alkylation process (hedamycin **8**)[15] that causes the inhibition of tissue growth, or nuclear or mitochondrial DNA strands can be ruptured by DNA intercalating agents like doxorubicin **4**, resulting in a cytostatic effect.[16]



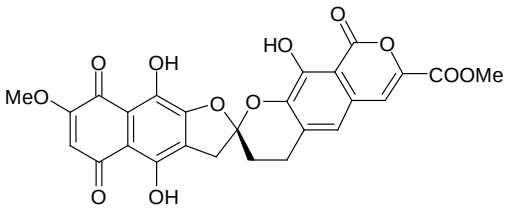
Monensin A **2** from *Streptomyces cinnamonensis*
Antibacterial activity



Avermectin B **3** from *Streptomyces avermitilis*
Antiparasitic activity

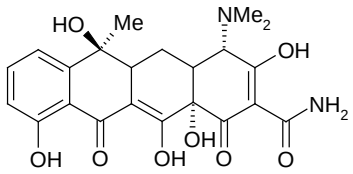


Doxorubicin **4** from *Streptomyces peucetius*
Antitumour activity

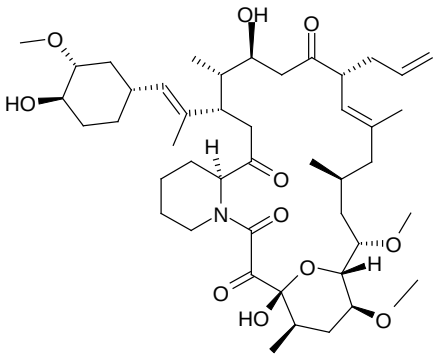


γ-rubromycin **5** from *Streptomyces collinus*
Antitumour activity

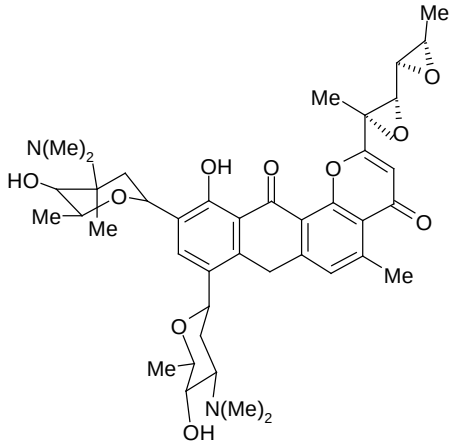
Figure 2.2: Chemical structures of a small selection of polyketides, their natural producers and pharmaceutical properties.



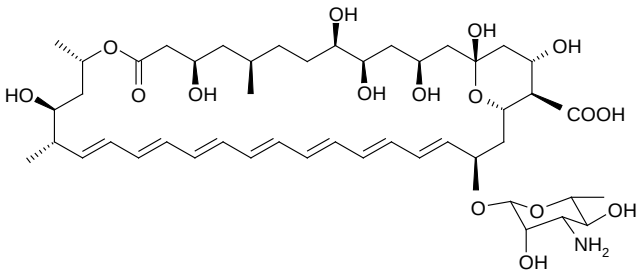
Tetracycline **6** from *Streptomyces aureofaciens*
Antibacterial activity



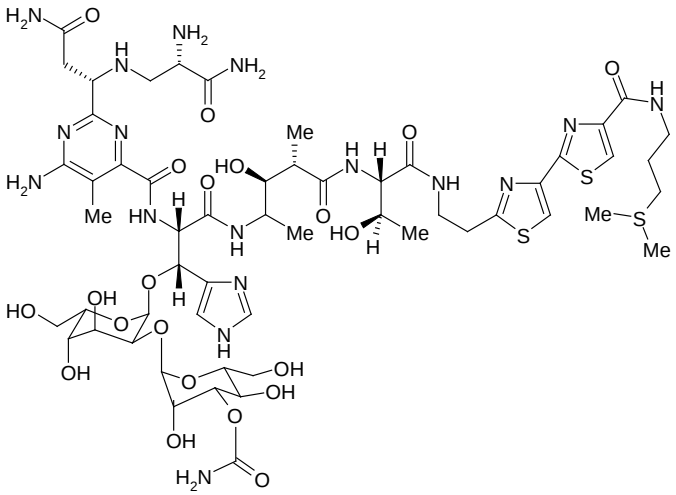
Tacrolimus **7** from *Streptomyces tsukubaensis*
Immunosuppressant



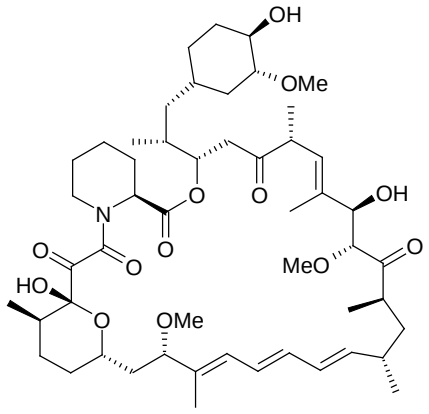
Hedamycin **8** from *Streptomyces griseoruber*
Antitumour and antimicrobial activity



Amphotericin B **9** from *Streptomyces nodosum*
Antifungal activity



Bleomycin **10** from *Streptomyces verticillus*
Antitumour activity



Rapamycin **11** from *Streptomyces hygroscopicus*
Immunosuppressant

Figure 2.2 continued: Chemical structures of a small selection of polyketides, their natural producers and pharmaceutical properties.

2.2 Biosynthesis of polyketides

Polyketide core structures are synthesised by polyketide synthases (PKSs) that are enzymes related to fatty acid synthases (FASs).[17] A wide variety of post-PKS tailoring enzymes act on the polyketide backbone, yielding structural diversity of the compounds (2.2.2). The abundance of polyketides can even be further enlarged by genetic engineering of different biosynthetic pathways (2.2.3).

2.2.1 Polyketide synthases

Different types of polyketides show different architectures of their biosynthetic enzymes, accompanied by distinct PKS programming. Based on their architecture, PKSs were initially classified into three families (type I, type II, type III PKS).[18] Type I PKSs were described to work in a modular way, whereas type II PKSs were reported to act in an iterative fashion and exclusively in bacteria. Ketosynthases (KSs) of type III were classified as iterative chalcone synthases from plants. However, increasing evidence shows that there are many exceptions to the original classification. Iterative usage of a type I PKS was reported by a number of groups, e.g. Bechthold and coworkers.[19] Furthermore, the iterative usage of single modules within a modular type I PKS was discovered ("stuttering").[20-22] Moreover, iteratively acting distant acyltransferases (ATs) were described that load the extender units *in trans* to AT-less type I PKSs, the so-called *trans*-AT PKSs.[23, 24] Later works showed that *trans*-AT PKSs evolved in a completely different way than *cis*-AT systems.[25] In the work of Kwon et al. a novel type II PKS is described that works non-iteratively and lacks an ACP.[26] The presence of (plant-like) PKSs of type III in *Streptomyces* strains was also discovered. For example, a bioinformatic approach revealed the presence of three type III PKSs in *Streptomyces coelicolor*.[27] It has thus become evident that transitional stages between all of the PKS classes can occur.[28] Even a type I/type III polyketide synthase hybrid pathway is described for the biosynthesis of the structurally diverse ansa compound kendomycin produced by *Streptomyces violaceoruber*.[29] However, despite the unambiguous occurrence of exceptions, the type I, II and III paradigms are well appreciated as a rough basis in polyketide biosynthesis research.[18] Therefore, I will now describe the general modes of operation of different PKS systems to this classification. All types of PKSs share sequential decarboxylative condensation of the acyl CoA precursors and C-C bond formation catalysed by the ketoacyl synthase (KS) domains (in type I PKSs) or -subunits (in type II and III PKSs).

Type I PKSs are multifunctional enzymes that are organised in modules, each harbouring a set of different domains that mostly work non-iteratively. Each domain catalyses a different step in the biosynthesis of the polyketide, whereas one cycle of polyketide chain elongation is catalysed by one module. The giant PKS protein consists of a certain number of chain-building modules that synthesise (PKS) and modify (reductive cycle, figure 2.3) the carbon chain. The different domains are ketosynthase- (KS), acyl carrier protein- (ACP), acyl transferase (AT)-, dehydratase- (DH) or ketoreductase- (KR) domains, except for the *trans*-AT PKSs that lack integrated AT domains.[25] The keto groups resulting from the condensation process may remain intact or can be modified or removed by a reductive cycle that consists of three steps (figure 2.3). In the first step, the keto group is reduced to a hydroxyl group by a KR. Adjacent dehydration is catalysed by a DH that leads to the introduction of a double bond in the second step. In the final ER-reaction a fully saturated carbon is generated.[30] The operation of the reductive cycle can be stopped at any stage in PKS biosynthesis, leading to coexistence of keto- and hydroxyl groups as well as double or saturated C-C bonds. An N-terminal initiation module directs the loading of the starter unit, whereas the molecule release is induced by an ending domain, containing a thioesterase (TE). The TE-domain catalyses the hydrolytic cleavage of the synthesised polyketide from the PKS. Type I PKSs are involved in the biosynthesis of complex polyketides such as the ansamycins (rifamycin **14**), polyenes (amphotericin **9**), macrolides (erythromycin **12**) or polyethers (monensin A **2**). The organisation of a type I PKS is displayed in figure 2.4, a.

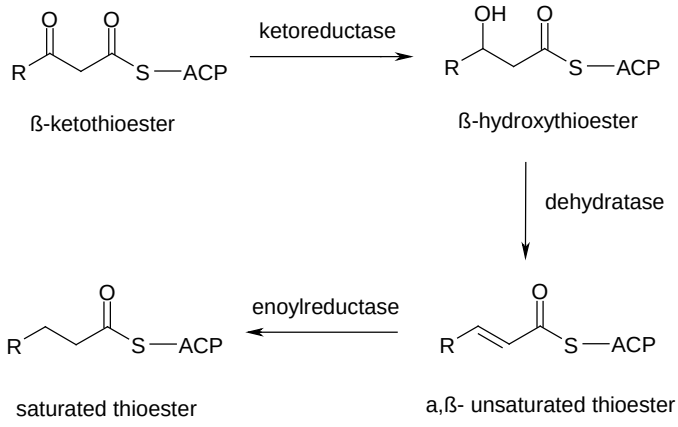


Figure 2.3: Reaction steps of the reductive cycle during polyketide biosynthesis in type I PKSs.[31]

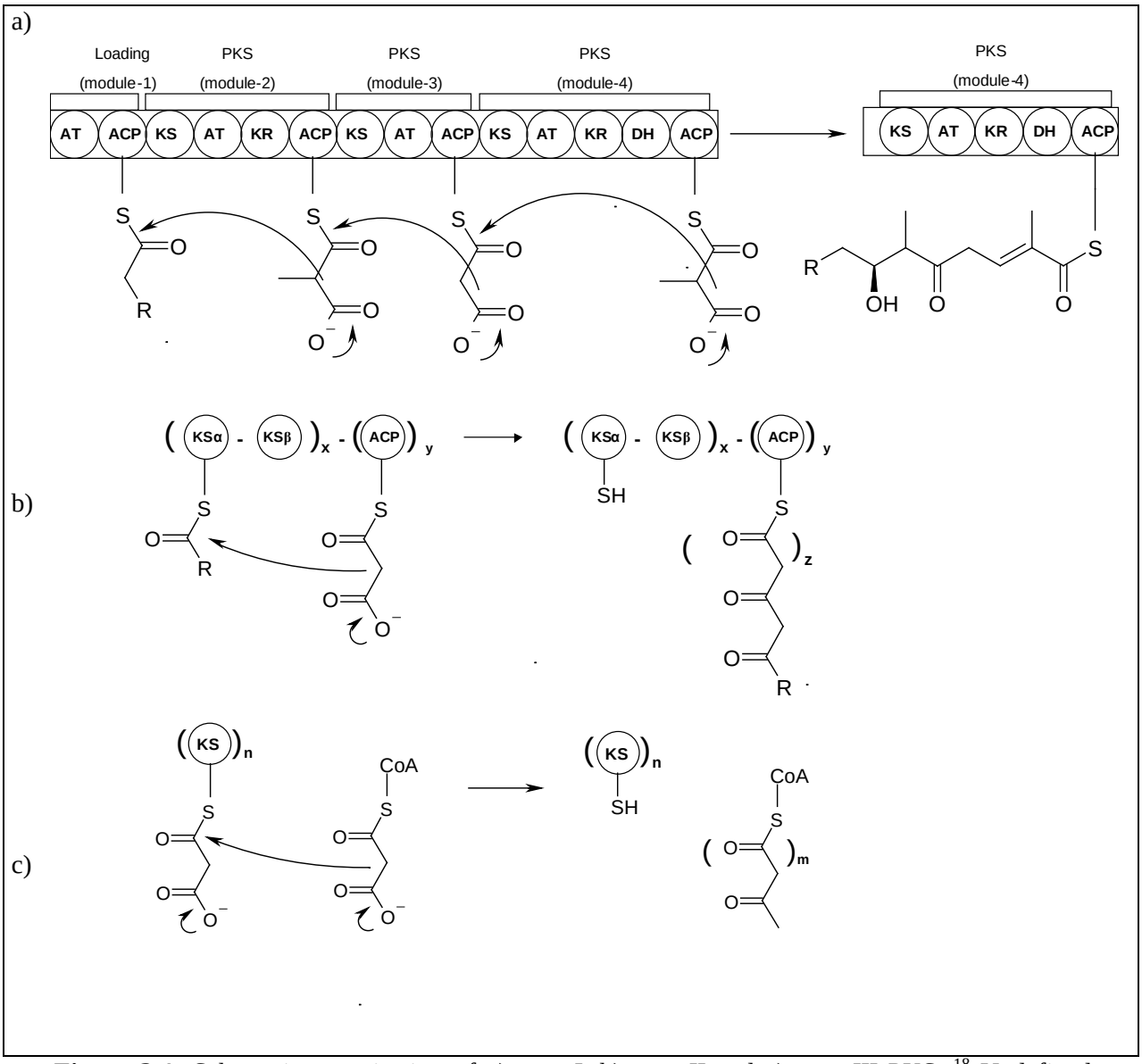


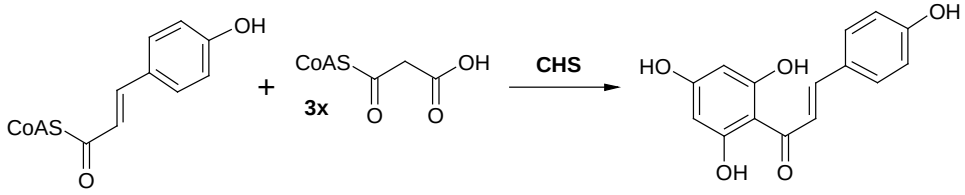
Figure 2.4: Schematic organisation of a) type I, b) type II and c) type III PKSs.[18] Undefined number of iterative rounds is indicated by lower-case letters.

Type II PKSs are multienzyme complexes carrying a single set of iteratively working proteins (figure 2.4, b). The enzymatic instrumentation of type II PKSs is based on a minimal PKS[32] consisting of the tandem pair termed ketosynthase (KS or KS_α) / chain length factor (CLF or KS_β), that are both beta-ketoacyl synthases, and an acyl carrier protein (ACP). The KS_β subunit is closely associated with the KS_α, thus generating a channel between the two proteins. When amino acids at four key positions in the KS_β are changed, the channel size is altered. This mechanism is the controlling factor for chain length determination.[33, 34] KS_α and KS_β catalyse the concerted condensation of acyl-thioesters to form a carbon skeleton. The ACP shuttles acyl components between the active sites of the minimal PKS, and it stabilises the nascent polyketide intermediates. The ACP unit tethers the growing chain that is extended by two carbon atoms with any further condensation round.[35, 36] The building blocks used for

extension are for example the activated derivatives of acetic acid and malonic acid, acetyl-CoA and malonyl-CoA. The acetyl-CoA starter unit is not directly loaded onto the PKS. It is derived from a previously loaded malonyl-CoA molecule by loss of carbon dioxide.[37] As only one KS pair is present in this type II PKSs, iterative rounds are necessary to produce a carbon chain. Additional PKS subunits like ketoreductases (KRs), cyclases (CYCs) and aromatases (AROs) direct the processes that convert the elongated poly-*β*-ketoacyl chain to the polyketide intermediate that is subsequently modified by tailoring enzymes (2.2.2). Cyclases and related subunits function as "chaperone-like" enzymes ensuring the proper assembly of one single product from the highly instable poly-*β*-ketoacyl chain.[38] Type II PKSs are responsible for the biosynthesis of aromatic polyketides like actinorhodin **1** (figure 2.1), doxorubicin **4**, rubromycin **5**, tetracycline **6** (figure 2.2), tetracenomycin **13** (figure 2.6) or griseorhodin A **17** (figure 3.1).[39] The basic mechanism of type II polyketide biosynthesis is displayed in figure 2.4, b.

A wide variety of natural products is synthesised by type III PKSs (figure 2.4, c), especially pigmented compounds like flavonoids occurring in flowers or fruits. These polyketides, such as chalcones, stilbenes or resveratrols, exhibit biological activities. For example, chalcones act as free radical scavengers (antioxidants) and stilbene phytoalexins are involved in plant pathogen defence.[40] Resveratrols, occurring for example in grapes, are described to show cancer chemopreventive properties.[41] Chalcones represent a type III polyketide scaffold, and the naringenin chalcone synthase (CHS) was identified as one of many enzymes that catalyse the flavonoid biosynthetic pathway.[42] In figure 2.5 the biosynthesis of the flavonoid precursor naringenin chalcone is displayed. Type III PKSs represent the structurally simplest members of the three PKS types and, mechanistically, they do not use ACP.[43] The monomer subunits of type III PKSs work independently, each capable of performing all reaction steps. The assembly of this class of natural products is governed by the simple homodimer KS (III) protein that performs a series of decarboxylation, condensation and cyclisation reactions. The active site uses free CoA-linked substrates. Type I and II PKSs use acyl carrier protein (ACP) for the activation of the acyl CoA substrates, and for the channelling of the growing polyketide intermediates. Type III PKSs are independent from an ACP function, as they directly act on the acyl CoA substrates. The iteratively working homodimeric KS protein is encoded in a single open reading frame and exhibits a small size (80-90 kDa). Therefore type III PKSs are ideal targets for protein engineering, and one focus in type III polyketide research is placed on rational engineering of their active sides by changing amino acid

residues to generate and modify diverse compounds. For example, three of the functionally divergent type III polyketide synthases, chalcone synthase[43], octaketide synthase[44] (OKS) and pentaketide chromone synthase[45] (PCS), have been characterised and engineered. Products of type III PKSs can also be incorporated into more complex natural products like the glycopeptide antibiotic vancomycin.[46]



p-coumaroyl-CoA malonyl-CoA naringenin chalcone

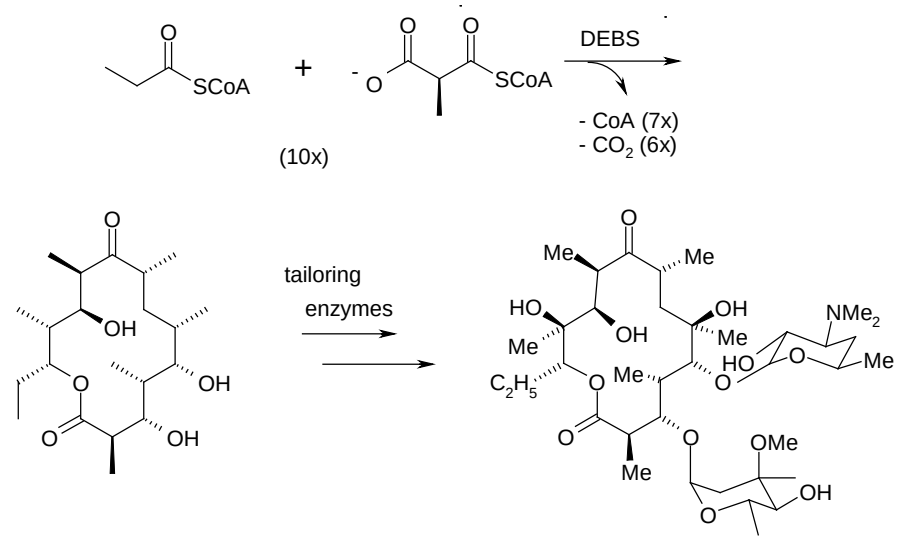
Figure 2.5: Biosynthesis of the flavonoid precursor naringenin chalcone via the type III PKS chalcone synthase (CHS).[42]

2.2.2 Post-PKS tailoring

The nascent polyketide molecule synthesised by the PKS can be further modified due to the activity of tailoring enzymes like monooxygenases, methyltransferases and glycosyltransferases that act on the natural product.[1] Thus the post-PKS tailoring processes have a huge impact on the overall structure of a polyketide, accompanied by biasing its pharmaceutical properties, for example antibiotic activity.[2] Particularly, glycosyltransferases play a very important role providing polyketides with sugar moieties.

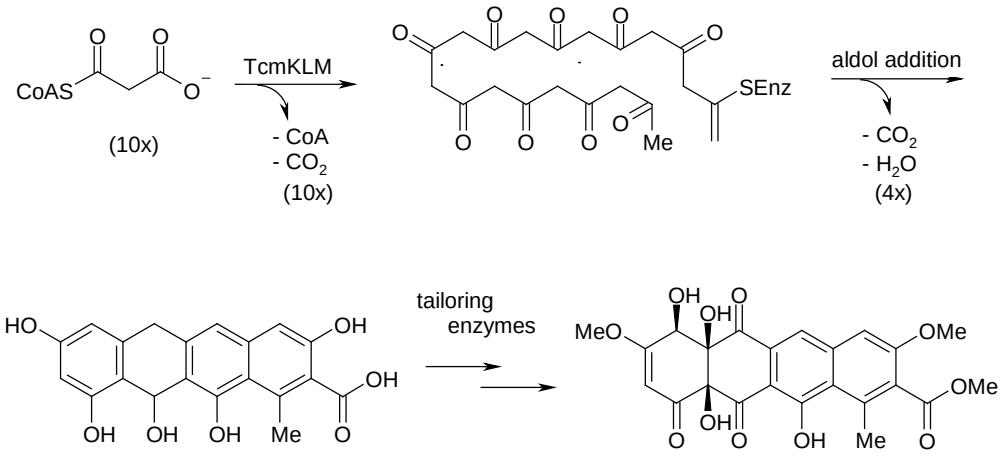
Sugar components, especially 6-deoxyhexoses, play a major role in natural product activity by changing the pharmacokinetic properties of the aglycons and serve as recognition sites for reaction mechanisms.[47] Many therapeutically important compounds like avermectin **3**, doxorubicin **4** or erythromycin **12** contain sugar moieties attached to the aglycone core. The attachment is catalysed by the activity of glycosyltransferases (GTs) that are usually located inside the biosynthetic gene clusters of the compounds. To a certain extent, some glycosyltransferases exhibit a "relaxed substrate specificity"[48] regarding the structure of the appended glycosyl unit of the aglycone target and the attachment site on the target core.[49, 50] These facts are of avail in genetic engineering (2.2.3). A brief insight into the results of tailoring enzyme activity on a polyketide core is shown in figure 2.6 instancing erythromycin A **12** and tetracenomycin **13** biosynthesis.

a)



Erythromycin A **12**

b)



Tetracenomycin **13**

Figure 2.6: Summarised biosynthetic scheme of a) erythromycin A, involving 6-deoxyerythronolide B synthase (DEBS) and b) tetracenomycin.[18, 51]

2.2.3 Engineered biosynthesis of "unnatural natural products"

In the past, therapeutic agents were solely produced by the isolation of natural products from different environmental niches, by chemical synthesis or semi synthesis. Since the mid-eighties a novel technique, engineered biosynthesis, opened up many possibilities in generating new pharmaceuticals.

Engineered biosynthesis utilises nature's machinery for the genetic manipulation and combination of biosynthetic pathways. This technique was first demonstrated by Hopwood[52] and coworkers who successfully introduced actinorhodin genes from *Streptomyces coelicolor*

into the medermycin producer *Streptomyces* sp. AM-7161, yielding the new compound mederrhodin A that carried an additional OH-group as compared to actinorhodin. This combination of genes from two or more pathways is called combinatorial biosynthesis. Genetic engineering has become a powerful tool in recent years, leading to the construction of hybrid compounds that feature improved drug actions.[53] Moreover, by generating new effective agents, genetic engineering might represent an answer to overcoming the resistance of microorganisms against antibiotics. Due to the fact that sugars are crucial for the effectivity of many pharmaceutical agents they represent an interesting target for genetic engineering that is aimed at the biosynthesis of new glycosylated natural products. As a precondition, the corresponding gene clusters and their functionalities must be investigated and described. Genes encoding GTs and deoxysugar biosynthesis represent an important biotechnological tool for altering glycosylation reactions in combinatorial biosyntheses of medically important compounds. Natural products are of high complexity, making chemical modifications as well as their total synthesis difficult and non-economic. Therefore the biological approach lends itself to generating new versions of therapeutical agents. The key benefit of known natural core skeletons is their validation by evolution. One approach to genetic engineering can be the modification of the glycosylation pattern. The first recombination experiment involving a GT was reported by Solenberg et al. where a hybrid glycopeptide was synthesised by using a cloned GT from the vancomycin producer *Amycolatopsis orientalis* expressed in *Streptomyces toyocaensis*.[54] A further study describes the production of several novel glycosylated tetracenomycins. The hybrid compounds were synthesised by engineered biosynthesis of the *elm* gene encoding a GT from the elloramycin producer *Streptomyces olivaceus* Tü2352 that was transformed into the urdamycin producer *Streptomyces fradiae* Tü2717 and the mithramycin producing strain *Streptomyces argillaceus* ATCC12956.[55]

2.3 Sugar moieties in early and late polyketide biosynthesis

2.3.1 Rifamycins

Rifamycins were first isolated from the actinomycete *Amycolatopsis mediterranei* by Sensi and coworkers.[56, 57] The compounds of this family are potent antibacterial agents, inhibiting RNA polymerase and thus DNA transcription.[58] Rifamycin B **14** (figure 2.7) and derivatives like rifampicin represent the most important drugs in the treatment of tuberculosis and leprosy. Rifamycins (A, B, C, D, E, S, SV, W) belong to the family of ansamycins that are macro cyclic lactam antibiotics.[59] Their characteristic structure consists of an aromatic (or

quinoid) core bridged by an aliphatic lactam handle (handle = ansa, lat.). All ansamycins, including rifamycins, share the same core structure that is introduced by the key intermediate 3-amino-5-hydroxybenzoic acid (AHBA, figure 2.8) of their amino shikimate biosynthetic pathway.[60, 61] The core skeleton of rifamycin is synthesized by a type I PKS, whereas AHBA represents the starter unit.[62]

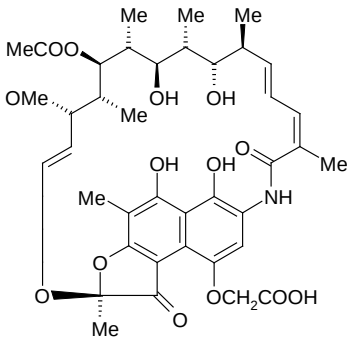


Figure 2.7: Chemical structure of rifamycin B **14**, from *Amycolatopsis mediterranei* S699 with antibacterial activity.

2.3.2 Functional studies of *rifL, rifK* and *rifM* in early rifamycin biosynthesis

As described by Guo and Frost[63], the origin of the amino shikimate pathways nitrogen atom in rifamycin biosynthesis is most likely represented by the amino sugar kanosamine (3-amino-3-desoxy-D-glucose). For this reason, the biosynthesis of kanosamine was then focussed on. Guo and Frost postulated that the introductory step in AHBA biosynthesis is the conversion of the substrate UDP-glucose to 3-keto-UDP-glucose by means of *rifL*, which codes for the oxidoreductase (UDP-3-keto-D-glucose dehydrogenase) RifL.[64] A subsequent RifK (UDP-3-keto-D-glucose transaminase)-catalysed amino transferase reaction in the presence of L-glutamine was supposed to yield UDP-kanosamine, which would be finally released as kanosamine after separation from uridine diphosphate by RifM (UDP-kanosamine phosphatase). There are indications that RifK may have two enzymatic activities: The dehydratase activity involved in aromatisation of 5-deoxy-5-amino-3-dehydroshikimate[65] and an aminotransferase activity introducing the nitrogen into a carbohydrate precursor of aminoDAHP.[66] The presence of two *rifK* homologues in the *asm* gene cluster is consistent with this notion.[67] The reaction scheme is displayed in figure 2.8.

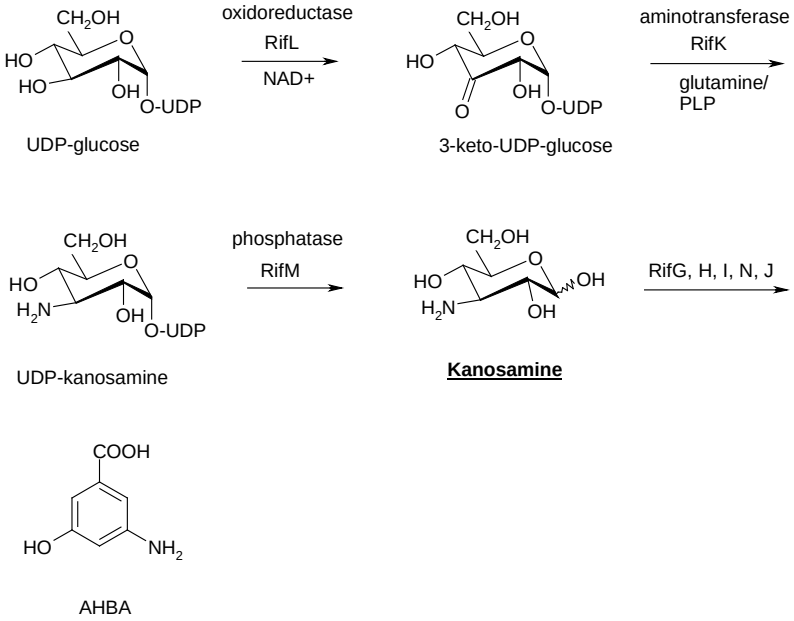


Figure 2.8: Proposed introductory steps in AHBA biosynthetic pathway yielding the intermediate kanosamine.[63]

Guo and Frost detected kanosamine by electrospray mass spectrometry under reaction conditions including UDP-6,6-[$^2H_2$]-glucose, NAD^+, glutamine, a cell-free extract from *Amycolatopsis mediterranei* and pH 6.8.[63] Interestingly, in their study no additional supplements like PLP or magnesium were required for kanosamine formation. Their work was inspired by experiments carried out by Umezawa[68], where a cell-free extract of the natural kanosamine producer *Bacillus pumilus*[69] (formerly known as *Bacillus aminoglucosidicus*) was incubated with UDP-^{14}C-D-glucose, NAD^+ and glutamine yielding 3-amino-3-deoxy-D-glucose (kanosamine). Furthermore, it was shown by Arakawa et al.[70] that the RifN protein (kanosamine kinase) specifically converts kanosamine into kanosamine-6-phosphate. In the work of Guo, Frost and Arakawa a cell-free extract of the natural rifamycin producer *Amycolatopsis mediterranei* was used.

The gene cluster encoding zwittermicin A biosynthesis in *Bacillus cereus* UW85 was isolated in a study by Thomas and coworkers.[71] Interestingly, it was proposed that this cluster also includes genes encoding enzymes involved in kanosamine biosynthesis. The deduction from the gene sequences led to a putative kanosamine biosynthetic pathway that is analogous to the mechanism proposed by Guo and Frost.[63]

A preliminary work by Boettcher[72] showed that the RifL oxidoreductase activity was detectable in cell-free extracts of *Streptomyces lividans* TK24 pHGF7604. Furthermore, RifL oxidoreductase activity was not present in cell-free extracts of *Streptomyces lividans* TK24

that contained the plasmid pHGF7607 (carrying a mutated *rifL* gene) or pHGF7608 (carrying a mutated *rifM* gene), respectively. Therefore, a specific RifL reaction as well as a co-operative mechanism among the gene products of at least *rifL* and *rifM* could be assumed. These findings formed the basis of the first section of this work. The putative multienzyme complex formed by RifL, RifK and RifM could be further investigated by utilisation of mutants harbouring either a point mutated *rifK** gene or a *rifK* deletion mutant, respectively.

Functional proof of *rifL*, *rifK* and *rifM* in kanosamine biosynthesis is still missing. Although several indications for the involvement of kanosamine in AHBA formation existed, the amino sugar had not been verified as an intermediate (formed by RifL, RifK and RifM) of the rifamycin pathway before the beginning of this work.

2.3.3 Pentangular aromatic polyketides

Pentangular aromatic polyketides are a group of biosynthetically related, albeit structurally diverse compounds from actinomycetes. Members of this group are the benastatins (apoptosis inducing), pradimicins (antifungal), fredericamycins (topoisomerase inhibitor) and members of the griseorhodin/ rubromycin (human telomerase inhibitor, HIV reverse transcriptase inhibitor) family (figure 2.9). Different pentangular polyketides are naturally synthesised from the shared precursor collinone by utilisation of different post-PKS tailoring enzymes. Another compound that is structurally related to collinone is the polycyclic xanthone IB-00208 (2.3.4, figure 2.9). The biosynthetic family was termed “pentangular polyketides” by Piel and Hertweck.[73] They identified an unusual type of C-19 ketoreductase (BenL in the benastatin cluster) that occurs in all pentangular polyketide pathways. Furthermore, gene inactivation experiments revealed collinone as common intermediate of the rubromycin and griseorhodin pathways.[74] The members of the griseorhodin/ rubromycin family are extensively modified aromatic polyketides that possess a unique spiroketal pharmacophore crucial for the antitumour (telomerase inhibition) activity.[75] The spiroketal moiety is generated in a highly complex oxidative tailoring process.[76] This includes the cleavage of four carbon-carbon bonds in a pentangular polyketide precursor followed by an epoxidation event. Concurrent, a large number of oxidoreductases is encoded in the griseorhodin A PKS system.

2.3.4 IB-00208 and post-PKS tailoring in late polyketide biosynthesis

The polycyclic xanthone IB-00208 displays antibiotic activity against Gram-positive organisms and antitumour activity against mouse leukaemia, human lung and colon

carcinoma and human melanoma cell lines.[77] Another member of the family of polycyclic xanthones is the aromatic polyketide lysolipin that is a potent antimicrobial and antitumour agent that was characterised on the genetic level.[78] Other kinds of antibacterial and antitumour compounds structurally related to IB-00208 are cervinomycins and citreamicins.[79, 80] IB-00208 was first isolated from a marine-derived *Actinomadura* sp. strain by Malet-Cascon and co-workers.[81] The polycyclic xanthone structure is glycosylated. The appended sugar is a trimethyldeoxypyranose hexose introduced by a GT during post-PKS tailoring processes. Moreover, IB-00208 exhibits oxygen units at positions C-2 and C-9. This represents another similarity to the highly oxidised griseorhodin A and other members of the rubromycin family (besides the similar intermediate), albeit a spiroketal part is not present in IB-00208.

Genetic engineering between different pentangular post-PKS biosynthetic routes seems to be a promising method for generating pharmaceuticals tailored to particular needs.

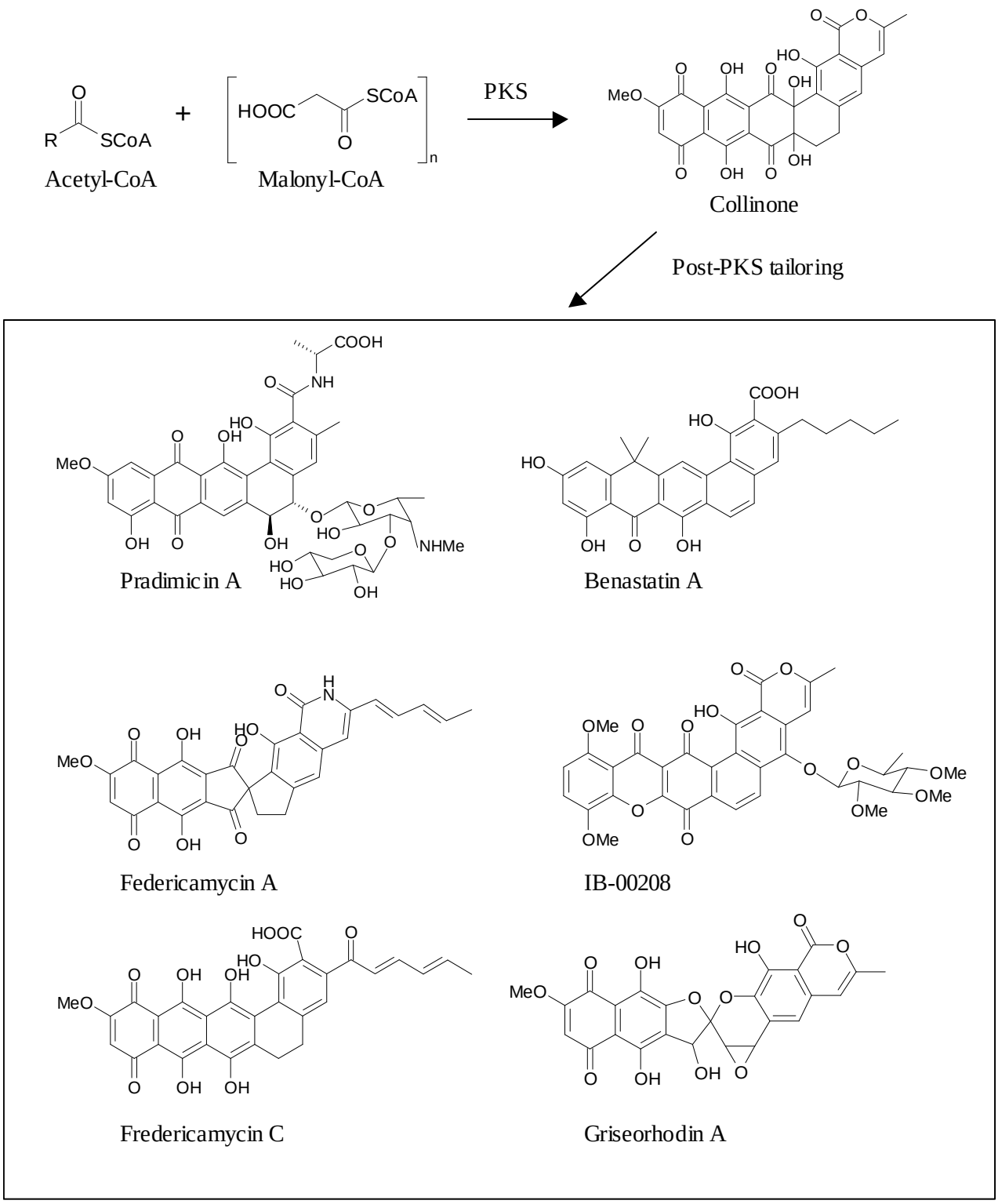


Figure 2.9: Abundance of pentangular aromatic polyketides due to diverse tailoring processes of a common pathway intermediate.

3 Goals of the present study

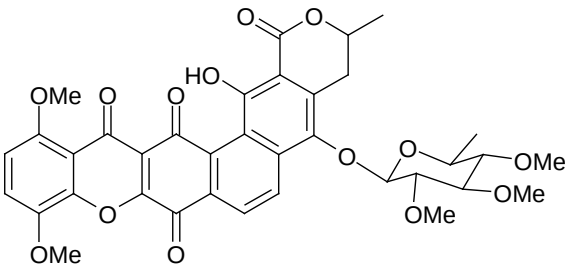
The present study comprises two parts that display sugars involved in early and late polyketide biosynthesis.

Goal of the work presented in chapter 4.1 was to provide a better understanding of the introductory steps in rifamycin biosynthesis, specifically concerning the formation of the amino sugar intermediate kanosamine that is putatively encoded by the genes *rifL*, *rifK* and *rifM*.[64] Functional studies of *rifL*, *rifK* and *rifM* from rifamycin B (**14)** producer *Amycolatopsis mediterranei* S699 in a heterologous host should verify their involvement in kanosamine formation. The analytical detection of kanosamine should be carried out after an enzymatic catalysis by means of RifL, RifK and RifM from a cell-free extract of *Streptomyces lividans* pHGF7604 in the RifL assay system. The enzyme assay should be performed by the use of a cell-free extract from *Streptomyces lividans* pHGF7604, harbouring all genes required for AHBA biosynthesis, the starter unit UDP-glucose and all necessary cofactors (5.6.6). The RifL assay was previously described to monitor the oxidoreductase reaction catalysed by RifL.[72] Moreover, the existence of a multienzyme complex putatively formed by RifL, RifK and RifM should be verified. Therefore, cell-free extracts of the *rifK* mutants *Streptomyces lividans* pHGF*rifL*, *K**, *M* and *Streptomyces lividans* pHGF*rifL*, *M* should be applied to the assay system. As a consequence, kanosamine should not be formed due to the pathway interruption caused by a non-functional or lacking *rifK* gene. The oxidoreductase RifL was supposed to show activity when associated with inactive RifK*, whereas the absence of RifK would inhibit RifL activity.

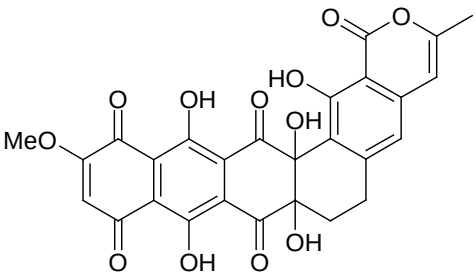
Focus of the second part of this work is the isolation of the polyketide gene cluster encoding the biosynthesis of IB-00208 **15** in *Streptomyces* sp. GW2/5831 (4.2). The strain *Streptomyces* sp. GW2/5831 was isolated from a soil sample by co-workers of Prof. Laatsch.[82] The bacterial cells exhibit a red colour when cultivated on solid medium and in liquid culture due to the presence of the polyketide. Other compounds produced by *Streptomyces* sp. GW2/5831 are not known. The structure of IB-00208 contains a deoxysugar moiety putatively introduced to the polyketide core structure by a glycosyltransferase during the tailoring processes in late polyketide biosynthesis. IB-00208 is reminiscent of the polyaromatic tridecaketide collinone **16**, a precursor of pentangular polyketides like, for example, griseorhodin A **17** (figure 3.1).[83] It was therefore assumed that IB-00208 might be synthesised by a type II PKS as well. Griseorhodin A (a member of the rubromycin family) is a telomerase inhibitor and therefore a

potential anti tumour agent. Due to its insolubility in most solvents, severe restrictions exist on the utilisation as a drug, whereas structure modification could lead to improved pharmacokinetics. A promising approach could be the introduction of a deoxysugar moiety into griseorhodin A structure. The tailoring steps in griseorhodin biosynthesis could thus be modified by adding the glycosyltransferase gene from *Streptomyces* sp. GW2/5831. A hybrid biosynthetic pathway could therefore lead to a glycosylated griseorhodin A compound, exhibiting improved pharmacokinetic properties. These properties comprise the liberation, absorption (resorption), distribution, metabolism and excretion of therapeutic agents (LADME principle).

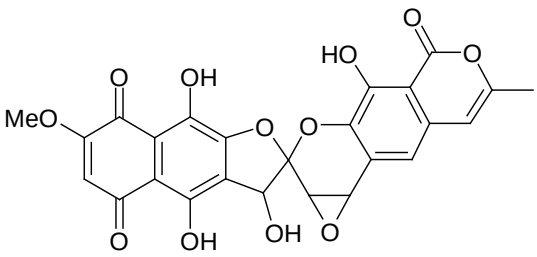
In Streptomycetes all genes required for the biosynthesis of a particular antibiotic usually occur in a single cluster accompanied by one or more antibiotic self-resistance genes.[84] Type II PKS biosynthetic gene clusters generally consist of 15 to 40 open reading frames, holding coding capacities between 20 to 60 kb. Therefore it is promising to establish a genomic library of a producing organism using lambda phage particles that can be packaged with 40 kb DNA strands as the maximum size. As the average insert size of the cosmids in the library is 35 kb (4.2), it might be possible to encounter a large portion or even the complete IB-00208 cluster located on one of the positive cosmids. The strategy for detecting the IB-00208 cluster from *Streptomyces* sp. GW2/5831 relied on earlier work on griseorhodin A biosynthesis, in which degenerate PKS primers (deduced from highly conserved motifs of ketosynthase domains) were utilised as probes to identify the PKS genes encoding griseorhodin A biosynthesis.[83] Therefore a genomic cosmid library should be established and screened for the ketosynthase domain. Sequence alignments of the positive cosmid clones should reveal similarities to other type II PKS systems in the data base, and a putative genetic organisation of the cluster should be deduced. Another goal was the heterologous expression of genomic fragments from the IB-00208 cluster, harbouring the ketosynthase domain, in *Streptomyces albus* that should help to verify the cluster by functional proof.



IB-00208 **15**



Collinone **16**



Griseorhodin A **17**

Figure 3.1: Chemical structures of IB-00208, collinone and griseorhodin A.

4 Results and discussion

4.1 Investigating the initial steps in rifamycin biosynthesis

The initial steps in rifamycin biosynthesis were proposed to be catalysed by the enzymes RifL, RifK and RifM yielding the amino sugar kanosamine (figure 2.8).[63] In this study, it was intended to reveal their biosynthetic function in precursor supply by detecting kanosamine synthesised in a cell-free system (RifL assay, 5.6.6).

The procedure to assay the RifL-oxidoreductase was established in our lab by Boettcher within the scope of her study on AHBA biosynthesis.[72] In the former study, the over-expression of the relevant enzymes RifL, RifK and RifM in *E. coli* was not successful as it resulted in inactive protein. In general, the expression of *Streptomyces* genes in *E. coli* can be difficult because of different codon usage in these strains. Furthermore, incorrect protein folding or the formation of insoluble inclusion bodies can result in inactive proteins. As attempts to perform an over-expression of RifL, RifK and RifM in *Streptomyces lividans* had been unsuccessful in the study of Boettcher, the *rif* genes could only be constitutively expressed. Therefore, *Streptomyces lividans* TK24 was used by Boettcher as a heterologous host to the shuttle vector pHGF7604 (figure 4.1) harbouring all eight rifamycin biosynthetic genes *rifG-N* required for AHBA biosynthesis.[66]

In the present study, analytical experiments were developed and carried out in order to detect the early stage intermediate kanosamine in RifL incubations. The assays were performed employing cell-free extracts from *Streptomyces lividans* (TK24) pHGF7604 that was kindly provided by Boettcher. The assays described in 4.1.9 were carried out using cell-free extracts from *Streptomyces lividans* TK23 harbouring different plasmids respectively (figure 4.16, table 5.8). These experiments were performed in order to access only the relevant genes for kanosamine formation instead of the complete equipment for AHBA biosynthesis. The plasmids and recombinant strains employed in 4.1.9 were kindly constructed by Kaulard.[85]

In this study, the RifL assay was reproduced and carried out using minimal and standard conditions (4.1.1), different nitrogen sources (4.1.2), different hydride acceptors (4.1.3) and different purification grades of the cell-free extracts respectively (4.1.4). Protein analysis of the cell-free extracts was performed using SDS-PAGE and MALDI-TOF-MS (4.1.5). Means to detect kanosamine comprised TLC, HPLC and GC-MS measurements (4.1.6). Furthermore,

the RifL reaction was monitored via ^{1}H-NMR (4.1.7). RT-PCR was carried out in order to verify the presence of the required gene products (4.1.8). A putative enzyme complex between RifL, RifK and RifM was studied (4.1.9) and the RifK and RifM reactions were investigated separately (4.1.10).

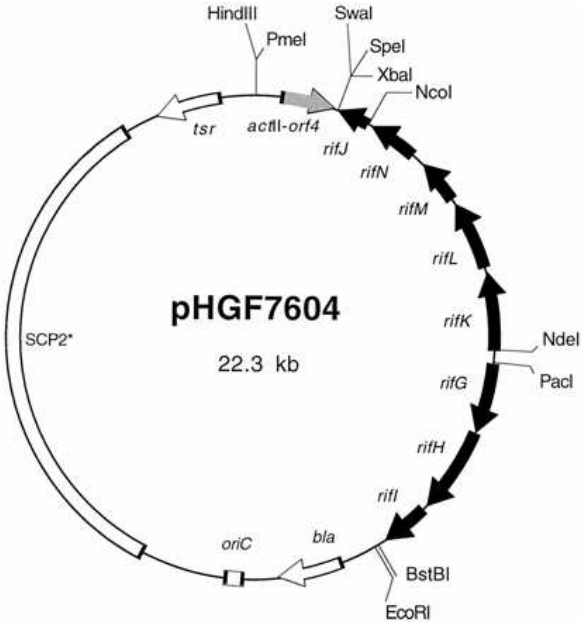


Figure 4.1: Plasmid pHGF7604, map taken from Yu et al. (2001). Shuttle vector, ColE1-origin, Amp^{r} (selection in *E. coli*); SCP2*-origin, Thio^{r} (selection in *Streptomyces*), genes *rifG-N* from the rifamycin biosynthetic gene cluster of *Amycolatopsis mediterranei* S699, under control of the *act*II-orf4 promoter.

4.1.1 The RifL-oxidoreductase assay

Reproduction of previous experiments

Preliminary studies concerning the NAD^{+}-dependent oxidoreductase RifL were described to result in the specific conversion of UDP-glucose by RifL in the presence of the hydride acceptor NAD^{+}.[72] At first the earlier assays were reproduced as a precondition to the analytical detection of the putative assay product kanosamine. The RifL oxidoreductase enzyme assay was conducted as described in 5.6.6. Initially, no RifL enzyme activity was encountered. This was overcome by verification of the procedure with regard to all assay parameters that were double checked. To verify the cultivation period of seven days for *Streptomyces lividans* pHGF7604, a growth kinetics was conducted as described in 5.4.7. The corresponding culture mycelia were harvested after different cultivation periods between one day to seven days. Each cell pellet was subjected to the production of a cell-free extract that was applied to the RifL assay. This experiment was the crucial step, revealing NAD^{+}

consumption in a cell-free extract obtained after a five-days cultivation (table 4.1, figure 4.3) instead of after a seven-days cultivation that had been recommended before. All other assay conditions were applied as described before, and it was then possible to carry out the assay, yielding reproducible data relating to NADH accumulation.

Table 4.1 Growth kinetics of *Streptomyces lividans* (TK24) pHGF7604 and RifL assay results of the corresponding cell-free extracts prepared from mycelia cultivated between one and seven days

Cultivation time [h]	Weight [g]	Colour of mycelium and R5-medium	UV signal (340 nm) in RifL assay
24	1,6	Yellow	-
48	1,9	Yellow	-
72	3,3	Yellow	-
96	3,9	Yellow	-
120	4,2	Blue	+
144	4,8	Blue	-
168	4,8	Blue	-

Figure 4.2: Liquid culture of *Streptomyces lividans* pHGF7604 in R5 medium, showing blue colour after 120 h cultivation time (table 4.1).

The emerging blue colour of the cultivation medium was attributed to the *act*II-orf4 promoter activity located on plasmid pHGF7604 to control gene transcription (figure 4.2). The *act*II-orf4 regulatory gene originates from the gene cluster of *Streptomyces coelicolor* encoding the biosynthesis of the red-blue coloured antibiotic actinorhodin.[5] The formation of this antibiotic, that is physiologically produced at very low amounts in *Streptomyces lividans*, can be highly increased if actinorhodin activation genes are cloned into *Streptomyces lividans*. This indicates that ActII-ORF4 should also regulate the downstream genes on pHGF7604 properly.[72]

Negative control reactions

To provide the test system with a negative control, the unmodified *Streptomyces lividans* TK 24 strain was utilised and the corresponding preparation of a cell-free extract was performed as described before. In RifL assay measurements that were carried out employing the unmodified *Streptomyces lividans* TK 24 cell-free extract no UV signal at 340 nm occurred and therefore it was assumed that no NADH production took place (figure 4.3). This was considered to putatively correlate with the absence of RifL in the cell-free extract of the unmodified strain. Further control reactions were carried out omitting UDP-glucose, NAD^+ or the cell-free enzyme solution, respectively (figure 4.3). Furthermore, denatured enzyme that was obtained after heating at 95 °C for 10 min was used as negative control (figure 4.3). All of these control measurements showed no deviation from a negative reaction. These observations formed the new basis of RifL enzyme assay, providing evidence that the predicted RifL-catalysed oxidoreductase reaction takes place.

Standard assay conditions

In order to provide all cofactors and reaction conditions required for kanosamine biosynthesis, the following substances were included in the standard RifL enzyme essay (5.6.6): pyridoxalphosphate (PLP) serves as cosubstrate for the RifK aminotransferase. In crystal structure experiments RifK was shown to be tightly bound to its cofactor PLP.[86] The gene product RifK is known to belong to a family of PLP-dependent aminotransferases preferentially used by streptomycetes in secondary metabolic pathways for the formation of amino sugars, aminocyclitols and other amino compounds.[87] Due to a typical PLP-binding motif with a conserved aspartate (Asp-159) and the active site lysine (Lys-188) it is presumed that the cofactor binds as a Schiff's base.[65] As RifM has considerable similarity to the CBBY family of phosphoglycolate phosphatases, the RifM-catalysed reaction was supposed to require magnesium chloride as reported for other CBBY phosphatases.[66, 88] Moreover, magnesium chloride was found to enhance RifL activity (Arakawa, personal communication). The incubation also required the supply of a nitrogen source for the introduction into the kanosamine ring (figure 2.8). As reported by Guo and Frost, glutamine was supposed to be the appropriate nitrogen donor.[63]

Minimal assay conditions

As described above, the standard enzyme assay included components required for RifL activity and additionally contained putative supplements for RifK and RifM reactions. In

order to determine the RifL-catalysed reaction only in the presence of its substrate and cofactor, a minimal assay was carried out containing cell-free extract of *Streptomyces lividans* pHGF7604, UDP-glucose and NAD^+. The final absorbance rate was almost identical after 20 min regardless of whether a minimal or a complete assay was performed (figure 4.3) showing that glutamine, magnesium chloride and pyridoxal phosphate do not show a visual influence on the measurement. In later experiments it was observed that the UV signal was slightly higher when any nitrogen source was omitted in the assay system (figure 4.5).

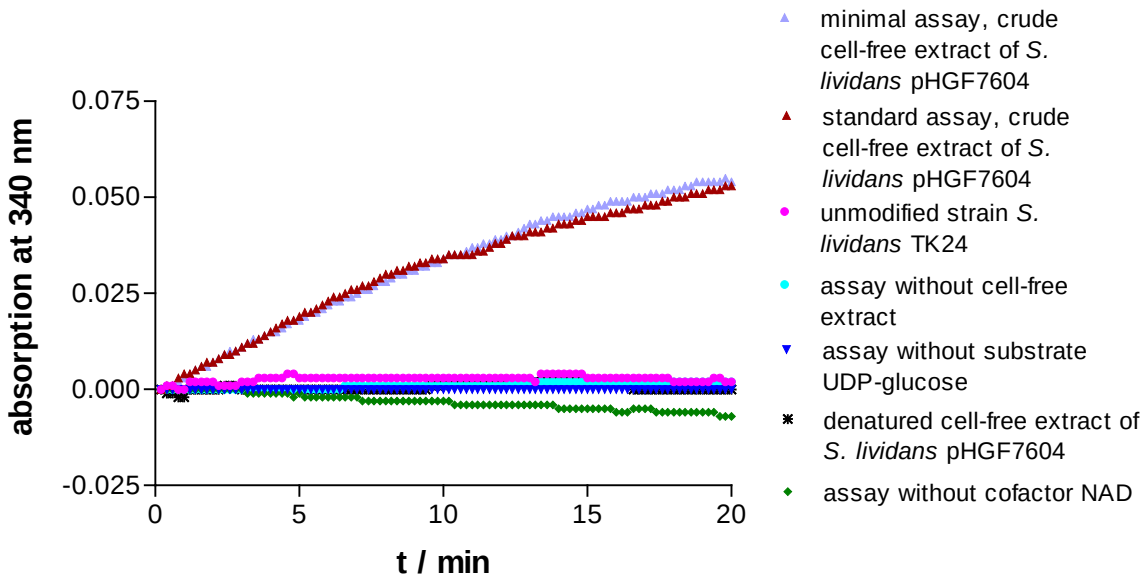


Figure 4.3: RifL assay utilising a cell-free extract from *Streptomyces lividans* pHGF7604.

The RifL assay results of positive reactions and negative controls are displayed in figure 4.3. The RifL reaction is dependent on a native cell-free extract, UDP-glucose and NAD^+. The minimal assay was required to determine the influence of the RifK and RifM cofactors on the RifL reaction (4.1.2). The minimal assay showed no difference from a standard assay assuming no adverse effect on RifL reaction. Compared to the minimal and standard assays, all negative controls do not exhibit any activities related to NADH accumulation in the RifL assay. The negative control employing the unmodified strain verifies that the reaction is putatively due to the activity of a recombinant enzyme originating from the plasmid pHGF7604. The only NAD^+ consuming enzyme expressed from genes encoded on the plasmid is the oxidoreductase RifL.

Reaction time

It was discovered by Umezawa et al. that kanosamine was formed within 30 min when UDP-glucose was incubated with a cell-free extract of *Bacillus pumilus* (formerly: *Bacillus*

aminoglucosidicus), glutamine and NAD^+ in phosphate buffer.[68] In a study by Lee it was reported that UDP-kanosamine can be detected via HPLC after the incubation of NapL (homologous to RifL) and NapK (homologous to RifK) with 4 mM UDP-glucose, 4 mM NADP and 3 mM glutamine for 12 hours at 37 °C and pH 7.4.[89] Lee observed that NapL activity (absorbance rate) was ten-fold higher when NapL was incubated together with NapK. NapL and NapK are enzymes from the geldanamycin producer *Streptomyces hygroscopicus* that correspond to RifL and RifK from the rifamycin producer *Amycolatopsis mediterranei*. On the one hand, the first two biosynthetic steps catalysed by RifL and RifK can be assumed to take place within 12 hours as well, because the absorbance rate of NapL (0.03) was comparable to RifL (0.05) in this study both determined after 20 min incubation time. On the other hand, although the Rif and Nap proteins are homologous enzymes, they are functionally not completely identical. For example, NADP serves as hydride acceptor in NapL/ NapK incubation as described above, whereas it is not accepted in the RifL reaction (4.1.3.2). Moreover, there was no PLP needed to accomplish UDP-kanosamine formation, although RifK is described to be a PLP-dependent aminotransferase.[65]

The RifL assays were directly subjected to HPLC, and additional assay duplicates were prepared and were stored at 37 °C for 1, 2, 5, 12 and 24 h for kanosamine formation before they were proceeded to analysis.

4.1.2 Impact of different nitrogen sources on the RifL assay

For the introduction of the nitrogen atom into the amino sugar ring, glutamine was supposed to be the appropriate source (figure 2.8).[63, 68] To test the influence of different nitrogen sources on NADH accumulation during the UV assay, glutamine, glutamic acid, asparagine, aspartic acid and ammonium chloride were tested separately. As reported by Arakawa et al., the rate of NADH formation increases when glutamine is added to the RifL assay as nitrogen source. Asparagine and ammonia were less effective in his study, whereas glutamate and aspartate were inactive.[70] Lee reported that NapL activity was increased in the presence of glutamine. In this study, all tested nitrogen sources yielded the same absorption rate for NADH production (figure 4.4).

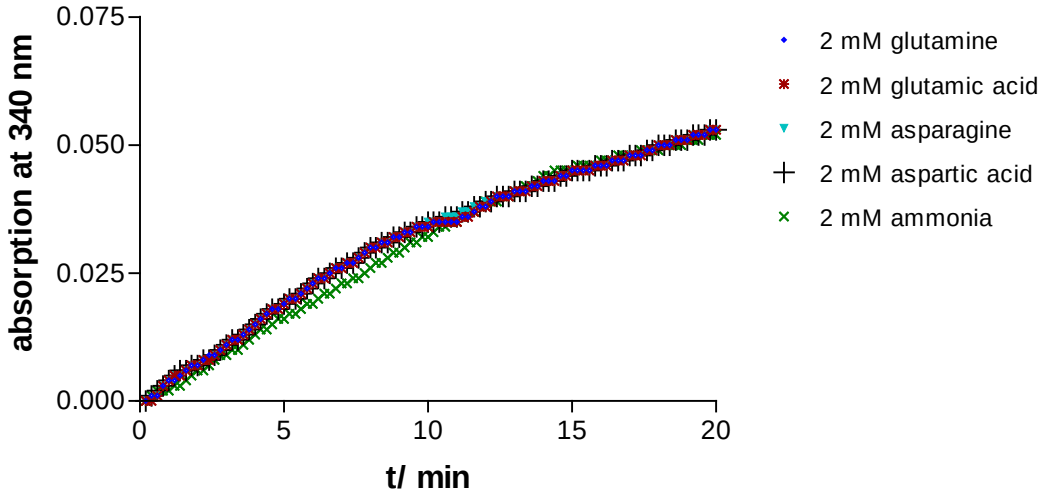


Figure 4.4: Different nitrogen sources applied to the RifL standard assay.

In another UV incubation series, any nitrogen source was omitted from the assay system in order to determine the influence of nitrogen absence on the RifL reaction. The RifL assays without a nitrogen source were carried out employing crude cell-free extract or 60% ammonium sulfate-precipitated cell-free extract from *Streptomyces lividans* pHGF7604 respectively. In comparison to standard assays using crude or precipitated cell-free extract, the absorption rate was increased if any nitrogen source was omitted in the incubation reaction (figure 4.5).

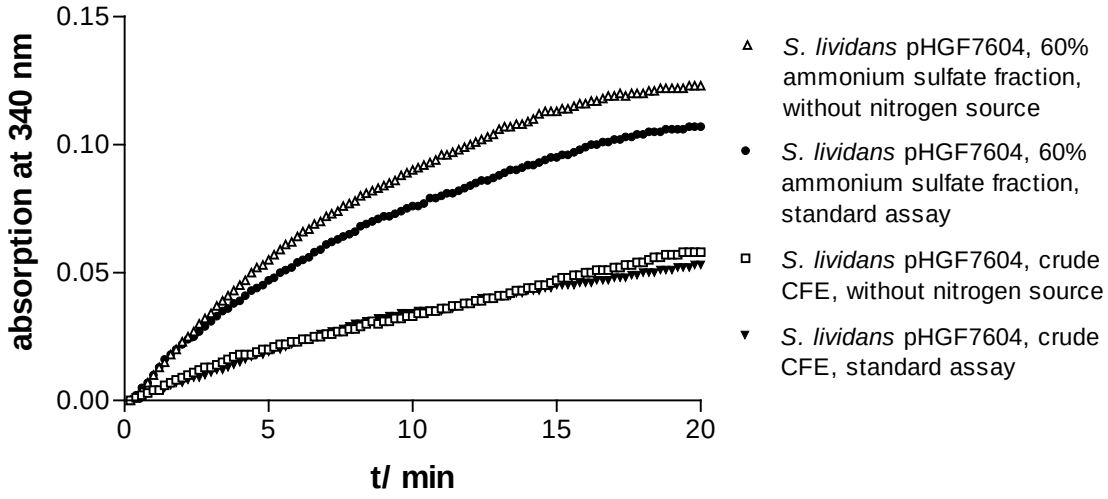


Figure 4.5: The effect of nitrogen absence in the RifL assay.

4.1.3 Application of different hydride acceptors in the RifL assay

4.1.3.1 Acetylpyridine adenine dinucleotide (APAD)

Acetylpyridine adenine dinucleotide (figure 4.6) is a NAD^+ analogue with higher oxidation potential than NAD^+. It can substitute for NAD^+ as a hydrogen-accepting cofactor in dehydrogenase reactions.[90] APAD is known to shift the equilibrium of enzymatic reactions. It was therefore used to enhance RifL catalytic activity. In fact, APAD yielded a higher conversion rate compared to NAD^+ in the RifL assay (figure 4.7). A prominent increase of the absorption rate occurred when using FPLC-purified protein solution (4.1.4) which showed a 16-fold higher absorption rate than previously obtained with the crude cell-free extract employing NAD^+ (figure 4.9). This experiment was carried out in order to increase substrate conversion accompanied by a putatively higher product yield in the RifL assay.

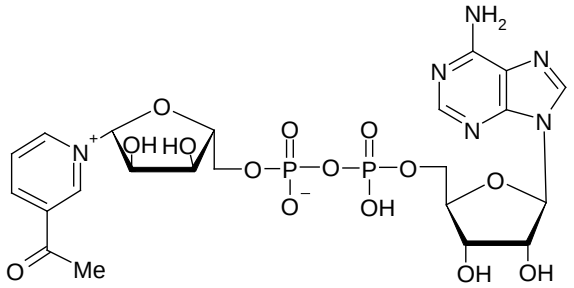


Figure 4.6: Chemical structure of acetylpyridine adenine dinucleotide (APAD).

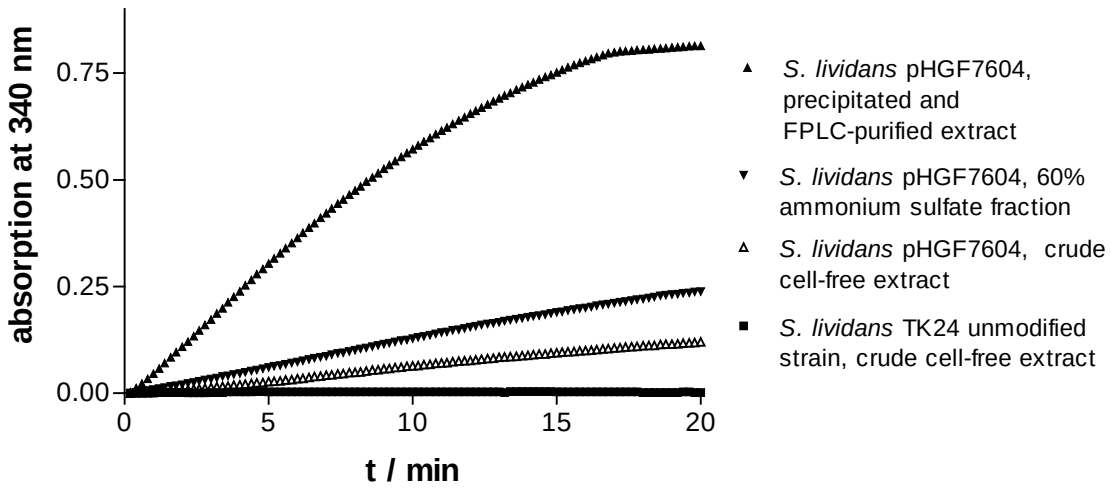


Figure 4.7: RifL assay utilising APAD as a hydride acceptor instead of NAD^+.

4.1.3.2 Nicotine amide adenine dinucleotide phosphate (NADP)

NADP was applied to the RifL assay instead of NAD^+ and compared to a standard assay using NAD^+ (figure 4.8). In the incubation including NADP as a hydride acceptor no increase

in absorbance rate was monitored, and the standard assay exhibited the NAD^+ consumption as described before (4.1.1). This experiment revealed that NADP is not involved in the RifL reaction, whereas Lee reported that NADP was required for UDP-kanosamine formation.[89] In contrary to Lee's observation that absorbance rate doubled when using NADP instead of NAD^+, in the RifL assay NADP did not function as hydride acceptor.

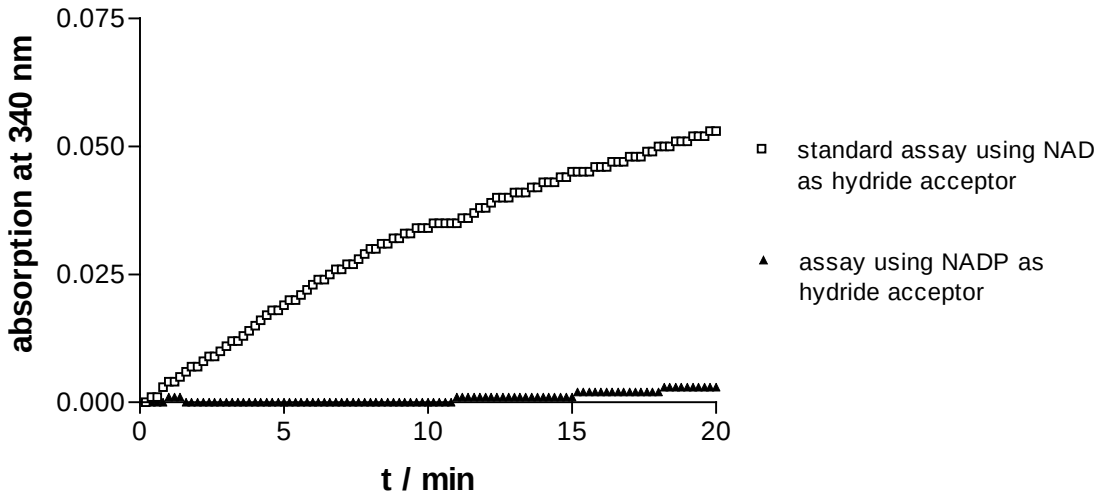


Figure 4.8: RifL assay utilising NADP as a hydride acceptor instead of NAD^+.

4.1.4 Purification of cell-free extracts from *Streptomyces lividans* pHGF7604

In the experiments conducted so far, the *rif* genes were constitutively expressed in the heterologous host, and therefore expected to be present in very low amounts in the cell-free extracts. Therefore protein purification was performed by means of ammonium sulfate precipitation (4.1.4.1) and FPLC (4.1.4.2 and 4.1.4.3) in order to increase the protein content in the cell-free extract.

4.1.4.1 Ammonium sulfate precipitation for the purification of RifL

Purification of the crude cell-free extract of *Streptomyces lividans* pHGF7604 was accomplished by ammonium sulfate precipitation followed by using Sephadex® columns for desalting procedure. From 8 g of wet cell mass, 8 mg total protein were obtained by 60% ammonium sulfate precipitation. The active protein, detected by the UV assay indicating RifL oxidoreductase reaction, was located in the 60% ammonium sulfate fraction. All other fractions tested between 10 and 90% ammonium sulfate did not exhibit activity. A spectrophotometric enzyme assay of 100 µg enriched protein yielded a doubled absorption

rate for NADH production, compared with 100 µg of total protein from the crude extract (figure 4.9).

4.1.4.2 Fast Protein Liquid Chromatography (FPLC) of precipitated extracts using DEAE-Sepharose

Advanced purification of previously pre-purified extracts (4.1.4.1) was performed by means of FPLC using DEAE-Sepharose. The separated fractions consisting of 10 ml were concentrated up to 1.5 ml each by using Amicon® Ultra-15 centrifugal filter devices. The concentrated protein fractions were each applied to the RifL assay. One active fraction was detected that showed a higher absorption rate after 20 min compared to the 60% ammonium sulfate fraction. The active fraction comprised approximately 1.5 mg protein. The FPLC-chromatogram is shown in the appendix (6.1).

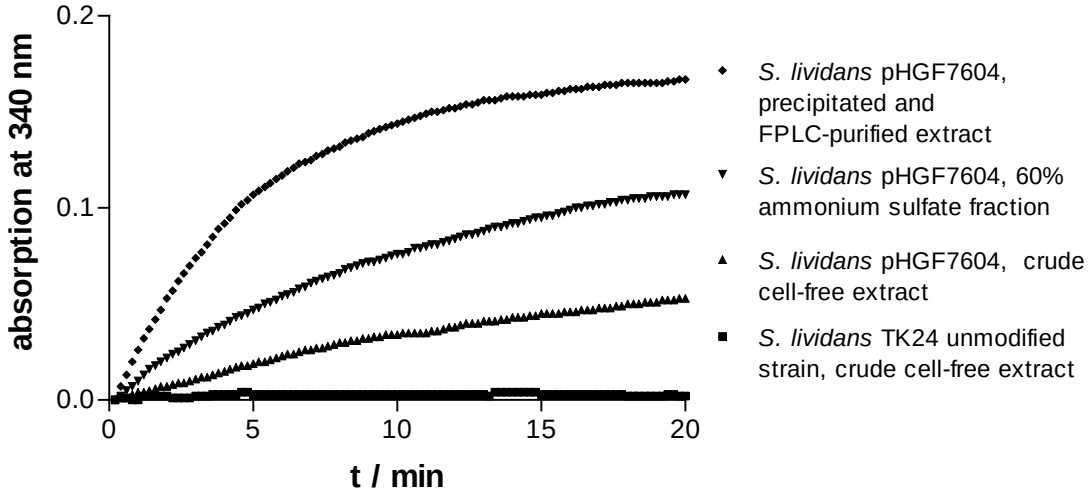


Figure 4.9: RifL assay utilising different purification grades of *Streptomyces lividans* pHGF7604 cell-free extracts.

The use of cell-free extracts with higher purification grades resulted in higher absorption rates in the RifL assay compared to that with lower purity or the crude extract respectively. These findings are summarised in figure 4.9, where the negative control is also included. Therefore it was assumed that the target enzyme RifL had been purified or at least enriched.

4.1.4.3 Intermediate protein purification and gel filtration of *Streptomyces lividans* pHGF7604 cell-free extract by means of FPLC

After the protein capture step (4.1.4.2), intermediate purification of the active protein fraction was attempted by hydrophobic interaction chromatography (HIC). The intention of this

purification step was to prepare the protein for gel filtration. This procedure yielded an inactive protein (data not shown). The loss of enzyme activity after FPLC-HIC purification might be due to long room temperature exposure, since it was not possible to cool the equipment. This was nevertheless followed by gel filtration on a Superdex© column in order to at least determine the protein sizes. No proper separation could be achieved (data not shown). For the determination of the protein sizes SDS-PAGE (4.1.5.1) and MALDI-TOF analysis were therefore carried out (4.1.5.2).

4.1.5 Size determination of proteins from *Streptomyces lividans* pHGF7604

4.1.5.1 SDS-PAGE

A protein gel (SDS-PAGE) was employed to analyse a precipitated cell-free extract of *Streptomyces lividans* pHGF7604 (4.10, lane 4) as well as three different FPLC-purified fractions (4.10, lanes 1-3) thereof. The extracts applied to lane 2 and lane 4 showed activity in the RifL assay, whereas the extracts applied to lane 1 and 3 were inactive but flanked the active fraction in FPLC. The following protein sizes were expected: RifL exhibits a size of 40 kDa, RifK comprises 43 kDa in size and RifM resembles a 26 kDa protein.[91] It was shown on the gel that a ~25 kDa protein was enriched in the active cell-free extract fraction from *Streptomyces lividans* pHGF7604 after FPLC purification (figure 4.10, lane 2). As the size determination is not precise, this band resides in the range of the 26 kDa RifM phosphatase. This was not expected, because the RifL protein (~40 kDa) was the targeted molecule as it was monitored with the enzyme assay system. An approximately 40 kDa protein was also enriched, but in much lower amounts than the ~25 kDa protein. Maybe this is evidence for the existence of a multi enzyme complex that was reported earlier for RifL and RifK (4.1.9).

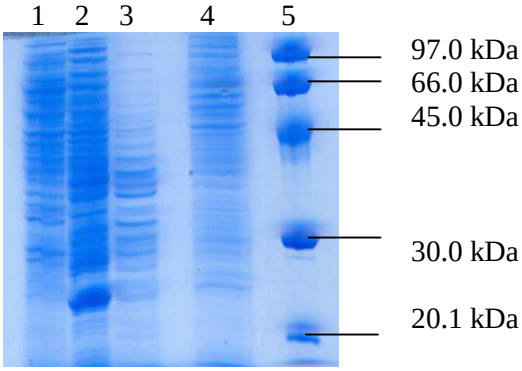


Figure 4.10: SDS-PAGE, all protein solutions were obtained from a cell-free extract of *Streptomyces lividans* (TK24) pHGF7604. Lane 1: FPLC purified protein fraction 1 showing no RifL activity, lane 2: FPLC purified protein fraction 2 showing high RifL activity, lane 3: FPLC purified protein fraction 3 showing no RifL activity, lane 4: 60% ammonium sulfate fraction, lane 5: molecular weight marker.

4.1.5.2 MALDI-TOF-MS

The purified cell-free extract (4.1.4.2) from a liquid culture (5.4.2.2) of *Streptomyces lividans* pHGF7604 was prepared and applied to MALDI-TOF-MS analysis (5.7.4.3). The mass range comprised 0.2 to 100 kDa. The ions were detected in the range between 0.5 and 2.2 kDa, whereas Rif-proteins exhibit sizes of 40 kDa (RifL), 43 kDa (RifK) and 26 kDa (RifM).[91] Therefore the presence of the proteins RifL, RifK and RifM in the cell-free extract of *Streptomyces lividans* pHGF7604 could not be confirmed. For the mass spectrum see appendix.

4.1.6 Detection of kanosamine

RifL, RifK and RifM were supposed to catalyse the enzymatic steps that would finally release kanosamine. Previous experiments indicated that the RifL-catalysed reaction takes place, and subsequent RifK and RifM catalysis was assumed (4.1.1). It was therefore now attempted to provide evidence for kanosamine to be an intermediate in early stage AHBA biosynthesis. As the formation of kanosamine was to be elucidated in incubation samples, TLC and HPLC methods for the detection of the amino sugar were developed. Furthermore, mass spectrometry and NMR analysis were carried out to check for the presence of kanosamine.

4.1.6.1 TLC for kanosamine detection

The reference kanosamine was detected by TLC at amounts of up to 20 µg (figure 4.11). However this sample showed 40% impurity determined in the laboratory of H. Floss. The detection limit of the pure compound was assumed to be much lower. The structure isomer 2-amino-2-desoxy-D-glucose (glucosamine) was tentatively used as reference substance because it was available in unlimited amounts. The detection limit of glucosamine was determined to be 0.2 µg, although this method is maybe still not sensitive enough for very low amounts of the analyte that might be encountered in the enzyme assay. Furthermore, since the components of the RifL assay solution interfered with the detection on a TLC, this method was not applicable to the samples generated in this study (data not shown).

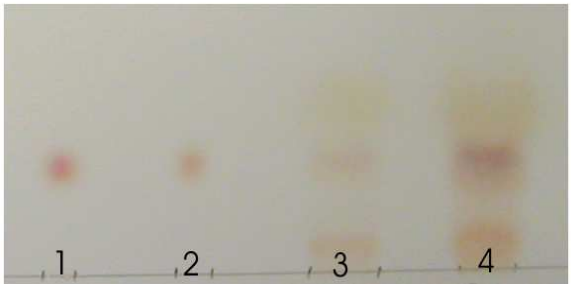


Figure 4.11: TLC of kanosamine and glucosamine. Lane 1: glucosamine 1 µg, lane 2: glucosamine 0,5 µg, lane 3: kanosamine 20 µg, lane 4: kanosamine 50 µg.

4.1.6.2 HPLC for kanosamine detection

A derivatization of the kanosamine reference, and in parallel glucosamine, was attempted using 9-fluorenylmethoxycarbonyl chloride (Fmoc-chloride) according to the method described by Kirschbaum et al.[92] However, no major compound was detected. Due to many by-products the reaction was unspecific, and the method was therefore regarded as unsuitable (data not shown).

The kanosamine reference was successfully detected with high-performance anion-exchange (HPAE)- chromatography with pulsed amperometric detection (PAD) like described in 5.7.2. Kanosamine exhibits a retention time of 10 min (figure 4.12). Interestingly, the same method was recently reported to be highly recommended for sensitive and direct kanosamine detection within the scope of impurity control for the antibacterial agent tobramycin.[93]

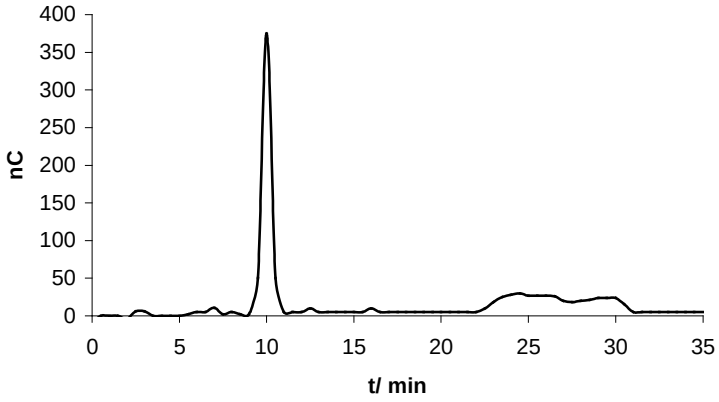


Figure 4.12: HPLC chromatogram of 3.8 µg kanosamine using pulsed amperometric detection (PAD).

The detection limit of the kanosamine reference (60%) was determined at 1.1 nM (= 0.2 µg), causing a PAD response of 16 nano Coulomb (nC). In HPLC experiments, the proposed

compound kanosamine that is putatively formed by the gene products of *rifL*, *rifK* and *rifM* could not be detected in any RifL incubation sample. However the assay components interfered with a proper HPLC detection showing many peaks in the chromatogram (data not shown). All samples were therefore subjected to GC-MS analysis (4.1.6.3), because this was supposed to provide more reliable results.

4.1.6.3 GC-MS measurements for kanosamine detection

Samples for GC-MS analysis were prepared according to the procedure described in 5.6.6, including measurement of absorbance change at 340 nm for 20 minutes. Prior to further analysis, the samples were stored at 37 °C for 24 hours in order to allow sufficient time for the formation of the putative compound. The reaction was then stopped by addition of 500 µl methanol. Variables in the RifL assay comprised the use of different cofactors, nitrogen sources and protein purification grades. For cofactor supply, either 4 mM NAD^+ or 0.8 mM APAD was utilised. The following nitrogen sources (2 mM each) were employed individually to investigate kanosamine formation: glutamine, glutamic acid, asparagine, aspartic acid. The enzyme solution was represented by a crude cell-free extract, a fraction precipitated in 60% ammonium sulfate or a purified FPLC fraction. Only a single parameter was varied in each assay sample.

It was required to stabilise the analyte using trimethylsilyl (TMS) derivatization in order to prevent degradation during GC-MS chromatography. The reference kanosamine was detected as trimethylsilyl ether exhibiting a molecular mass of 553.36 *m/z* (see appendix). However, in silylated assay samples no compound with a corresponding molecular mass was detected.

4.1.7 ^{1}H-NMR experiments of RifL-incubation samples

To gain further insights into the procedures of the RifL assay ^{1}H-NMR measurements were carried out. For ^{1}H-NMR experiments RifL incubation reactions were pipetted as listed in 5.6.6, whereas the reagents were dissolved in deuterium oxide and immediately placed in the NMR tube for measurement. A duplicate sample was always measured by UV spectroscopy for comparison and verification of “active” protein.

A signal emerging at 5.5 ppm (figure 4.13) was observed. This was attributed to a background epimerase reaction (figure 4.14) by G. Dräger on the basis of his earlier works. Therefore, an incubation was set up according to the RifL assay except for containing UDP-glucose-

epimerase instead of a cell-free extract. In fact, the same signal at 5.5 ppm was observed as described before, and no signal apart from that occurred. Blank tests employing either a cell-free extract from the unmodified strain *Streptomyces lividans* TK24 or omitting any protein showed no signal at 5.5 ppm. The cell-free extract from the unmodified strain *Streptomyces lividans* TK23 was analysed as well as a cell-free extract from *Streptomyces lividans* TK23 pHGF*rifK*, *L*, *M*. Both exhibited the signal at 5.5 ppm. Also tested was the incubation with UDP-galactose instead of UDP-glucose that yielded the same signal as well.

It is noted by Oguiza et al. that *Streptomyces lividans* harbours a *galE* gene encoding a protein that shares a high degree of identity to known UDP-galactose-4-epimerases from gram-positive microorganisms.[94] These proteins catalyse the interconversion of UDP-galactose and UDP-glucose.[95] As the GalE protein has a size of ~34 kDa,[96] it is unlikely that the enriched unknown ~25 kDa protein shown in SDS-PAGE (4.10) represents GalE. But there are other bands visible that are located within the range of 34 kDa.

These results suggest that NAD^+ consumption in the RifL assay was only based on this putative epimerase reaction. Epimerases and oxidoreductases are both NAD^+- consuming enzymes, and in the RifL assay only NADH accumulation is monitored. Besides this fact there is no further indication for the proposed RifL reaction. There is no other NAD^+-consuming enzyme encoded on the plasmid pHGF7604.

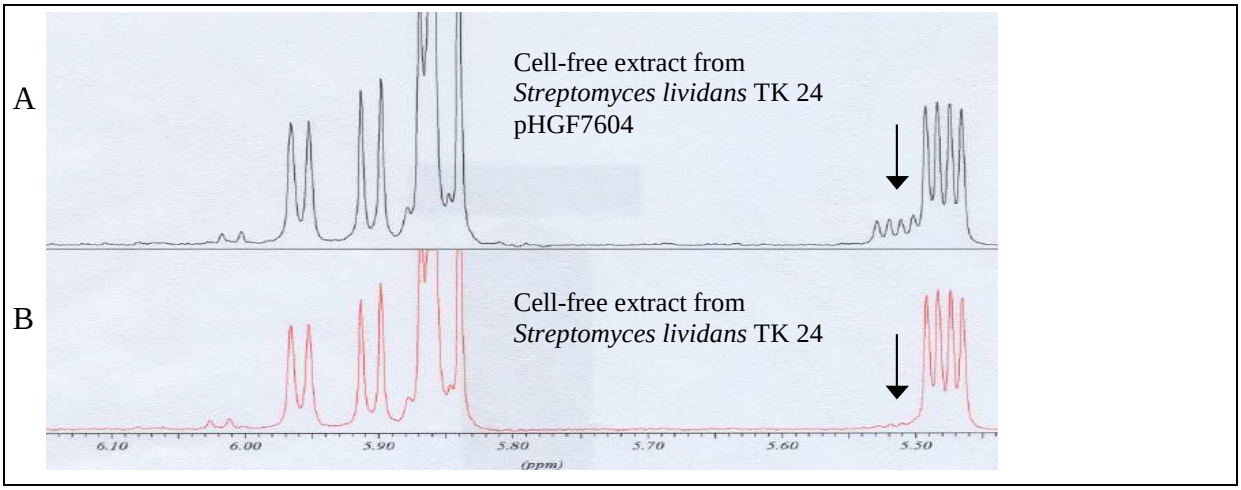


Figure 4.13: ^{1}H-NMR spectra of cell-free extracts from *Streptomyces lividans* TK24 pHGF7604 (A) and *Streptomyces lividans* TK24 (B), arrows indicate the ^{1}H chemical shift at 5.50-5.54 ppm (A) and the absent signal (B).

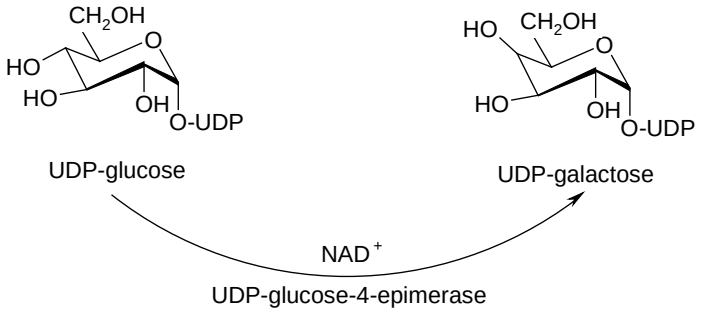


Figure 4.14: Epimerisation reaction of UDP-glucose via UDP-glucose-4-epimerase.

The enzyme measurement designated as RifL assay might actually not show RifL activity. The reaction is probably caused by an epimerase. The use of an epimerase inhibitor, for example 8-anilino-1-naphthalenesulfonic acid, could therefore be applied.[97] Due to the fact that only 15 to 30% of UDP-glucose are converted to UDP-galactose there was still a sufficient amount of UDP-glucose available for the actual RifL reaction (G. Dräger, personal communication). However, kanosamine was not detected in any incubation sample. Therefore, doubts concerning the presence of RifL in cell-free extracts of *Streptomyces lividans* pHGF7604 should be clarified via RT-PCR (4.1.8).

4.1.8 RT-PCR of cDNA from *Streptomyces lividans* pHGF7604

In order to clarify the presence of the *rifL* gene product (RifL) in the recombinant strain *Streptomyces lividans* pHGF7604, RT-PCR was carried out. Approximately 50 µg of total RNA were isolated (5.5.6.1) from 65 mg cell material of a liquid culture of *Streptomyces lividans* pHGF7604 (5.4.2.2). The remaining DNA was degraded using DNAse (RQ1DNAse, Promega) according to the manufacturer's instructions. Total RNA, and in parallel a control RNA supplied in the cDNA synthesis Kit (Fermentas), was employed in cDNA synthesis (5.5.6.2). Successful synthesis of cDNA from both, sample and control, was verified on an agarose gel. Reverse transcriptase (RT)-PCR was performed using sample copy DNA and the specific primer pair "RT-PCR-Oxido", and RT-PCR was conducted as described in 5.5.6.3. The plasmid *rifKLM* was used as a positive control, respectively, that yielded the expected PCR product of approximately 800 bp, whereas the cDNA from *Streptomyces lividans* pHGF7604 resulted in no PCR product (figure 4.15). Therefore, in all probability, no transcription of the cloned gene *rifL* took place under the terms of this study.

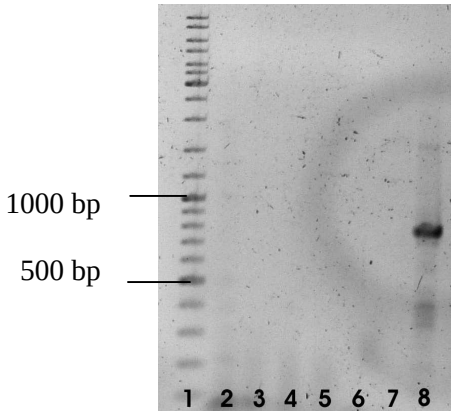


Figure 4.15: RT-PCR on an agarose gel. Lane 1: DNA Ladder Mix; lane 2: sample cDNA; lane 3: sample cDNA; lane 4: sample cDNA; lane 5: sample cDNA; lane 6: sample cDNA; lane 7: negative control reaction using no template DNA; lane 8: positive control reaction using *rifKLM* as template DNA.

4.1.9 Studies of a putative functional interaction between RifL, RifK and RifM

In parallel, other plasmids were constructed during the work of Kaulard[85] that were intended for the study of a putative enzyme complex. It should be demonstrated in the present work that the three enzymes RifL, RifK and RifM exhibit functional interaction and therefore pass their products among each other. It was shown by Guo and Frost that RifL and RifK form a complex.[63] Moreover, RifL and RifM deletion mutants show no UV reaction in RifL assay,[72] and the RifK homologue NapK is co-eluted if only the RifL homologue NapL is His-tagged.[89]

This part of the study was carried out to access only the relevant genes for kanosamine formation. Therefore *rifL*, *rifK* and *rifM* were cloned in a Streptomyces shuttle vector, and transformed into *Streptomyces lividans* host cells.[85] However, the introduction of the plasmids into *Streptomyces lividans* TK24 that was used for pHGF7604 was not possible, and therefore the TK23 derivative of *Streptomyces lividans* was used. The strains *Streptomyces lividans* TK23 and *Streptomyces lividans* TK24 are mutants of *Streptomyces lividans* 1326 that should only differ in their antibiotic resistance.[5] For comparison with my earlier experiments the plasmid pHGF7604 and the empty plasmid pHGF were also transformed into *Streptomyces lividans* TK23 and were applied to the RifL assay (4.16). In parallel, two other constructs, pHGF*rifL*, *K**, *M* and pHGF*rifL*, *M*, were cloned to analyse the putative multi enzyme complex formed by RifL, RifK and RifM. These constructs were designed in order to explore the influence of RifK aminotransferase on kanosamine production and on a functional interaction of *rifL*, *rifK* and *rifM* gene products respectively. Whereas the *rifK* gene carries a point mutation in pHGF*rifL*, *K**, *M*, it is deleted on the plasmid pHGF*rifL*, *M*. In the proposed

pathway kanosamine will not be formed in the absence of *rifK* or if *rifK* is mutated (*rifK**). Enzymatic activity of RifL should not be influenced by the point mutation found in pHGF*rifL, K**, *M* in which RifK* is assumed to have no altered conformation, and is therefore available for complex formation. A conversion of 3-keto-UDP-glucose to kanosamine ought to be impossible, and heterologous expression of pHGF*rifL, M* should impede complex formation and thus RifL activity. Site-directed mutagenesis to construct pHGF*rifL, K**, *M* was carried out by replacing the codon for lysine with the codon for aspartic acid at position 188 of RifK by change of two nucleotides. As all PLP-requiring enzymes share this conserved region as an active site for cofactor-binding, catalysis of RifK reactions should be blocked in the *rifK** mutant. The gene products should be expressed and biosynthetic analysis was intended in the present work. As a precondition, a cell-free extract from the unmodified strain *Streptomyces lividans* TK23 was tested in *rifL* assay (figure 4.16). This resulted in showing a high NAD^+ consumption, unlike what was observed for *Streptomyces lividans* TK24 unmodified strain. Therefore, the general expression of pHGF7604 and in particular in *Streptomyces lividans* TK23 was challenged. Nevertheless, the samples were checked for the presence of kanosamine via GC-MS, and kanosamine was not detected. Purification by means of 60% ammonium sulfate fractionation did not result in a higher absorption rate in the RifL assay compared to the crude cell-free extract. Both showed an absorption rate of 0.06 after 20 min.

The *rifL*, *rifK* and *rifM* genes are absolutely essential for the biosynthesis of AHBA.[65] However they are not related to any shikimate pathway enzymes, whereas the other *rif* genes resemble their counterparts in shikimate pathway. Different AHBA gene clusters were compared, and it was observed that the arrangement of the *rifG-N* homologues involved in AHBA biosynthesis relative to *rifK* and its homologues is not uniform in all studied gene clusters. A variation of the relative arrangement of *rifK* (and homologues) to the other AHBA biosynthetic genes exists, although a conserved close association between *rifK*, *rifL* and *rifM* homologues occurs in all analyses. The linked arrangement of *rifK*, *rifL* and *rifM* in one operon is highly conserved in all analysed clusters. The *rifL* gene encodes a 359-amino acid protein (40 kDa) with its initiation codon located 73 bases downstream of the *rifK* gene. The *rifM* gene encodes a 232-amino acid protein (26 kDa), and is located 14 bases downstream of *rifL*. The *rifK* gene encodes a 388-amino acid protein (43 kDa).[91] Therefore a functional interaction of the corresponding proteins was assumed, and the existence of a multienzyme complex between RifL, RifK and RifM was hypothesised.[98] In an enzyme complex the

involved proteins exhibit coordinated catalytic activity. The channelling of substrates via multienzyme complexes has many advantages over the free diffusion of intermediates. For instance the transit time between active sites is reduced,[99] and labile intermediates (like 3-keto-UDP-glucose in this work) are protected from decomposition.[100]

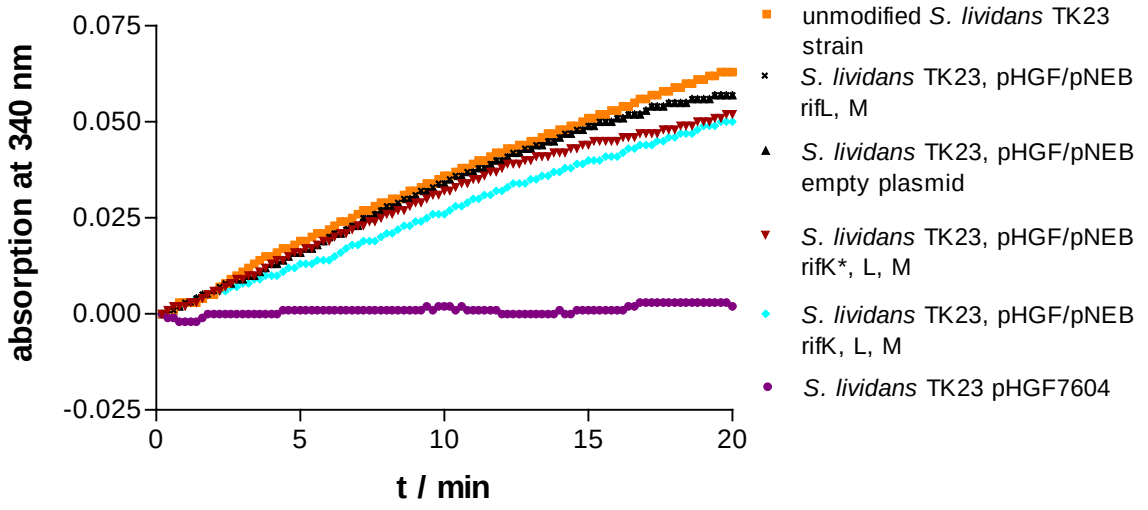


Figure 4.16: RifL assay using crude cell-free extracts of *Streptomyces lividans* (TK23) harbouring different plasmids.

So far the data show that *Streptomyces lividans* TK23 cannot be used as host strain to the plasmid pHGF7604 because of its residual activity.

It was shown by SDS-PAGE in the work of Kaulard that even a crude cell-free extract of the unmodified host strain *Streptomyces lividans* TK23 contains a highly concentrated ~25 kDa protein.[85] This is consistent with the finding in 4.1.5.1 where an ~25 kDa protein was enriched in a purified cell-free extract of *Streptomyces lividans* pHGF7604 that showed high activity in RifL assay. As displayed in figure 4.16, a crude cell-free extract of *Streptomyces lividans* TK23 exhibits a considerable activity in RifL assay. This provides evidence for the observed activity not being related to a recombinant gene product encoded on plasmid pHGF7604, but it might be due to a native protein from *Streptomyces lividans* that is constitutively expressed at a higher level in TK23 than in TK24 derivative.

The outcome of the chapters 4.1.8 and 4.1.9 suggested that it would not be promising to proceed this topic via the current strategy. Further experiments conducted on AHBA enzymes are listed below despite they are of no interest after the findings reported before.

4.1.10 The steps catalysed by RifK and RifM

As *Streptomyces lividans* pHGF7604 was assumed to be equipped with the genetic tools to synthesize AHBA, there was no tight control of the enzyme assay in order to stop the reaction at the stage of kanosamine or, on the other hand, to monitor the actual state during RifL assay. Therefore the remaining RifK- and RifM- catalysed reactions were originally intended to be investigated individually.

4.1.10.1 Aminotransferase reaction putatively catalysed by RifK

In a study by Lucher et al., the keto acid product derived from the amino donor glutamine was characterized as 2-oxoglutaramate, indicating that the α-amino group of glutamine participates in the transamination reaction.[101] For this reason, 2-oxoglutaramate was synthesised as described in 5.8 for employment as the predicted amino acceptor deriving from glutamine. The unstable intermediate 3-keto-UDP-glucose was not available for an individual RifK reaction. Therefore the reversed reaction should have been carried out employing UDP-kanosamine and the putative amino acceptor 2-oxoglutaramate. However, precipitation occurred in the UV cuvette that was attributed to 2-oxoglutaramate that would probably interfere with any substantial reaction.

4.1.10.2 Phosphatase reaction putatively catalysed by RifM

RifM has considerable similarity to the CBBY family of phosphatases.[66] UDP-kanosamine was incubated with a cell-free extract of *Streptomyces lividans* pHGF7604 in the presence of magnesium chloride, but the conversion of UDP-kanosamine to kanosamine was not confirmed in HPLC measurements of the present work correlating with the absence of *rif* genes (4.1.8) in the cell-free extracts (data not shown).

Conclusion

In a study by Yu et al.[66] the genes *rifG-N* were heterologous expressed in *Streptomyces coelicolor* YU105 that is unable to produce AHBA or AHBA-derived ansamycins. This resulted in the production of AHBA. Therefore it was assumed that AHBA biosynthesis must be attributed to the *rifG-N* genes. In the present study, however, the early stage intermediate kanosamine was not detected in RifL incubation samples using cell-free extracts of *Streptomyces lividans* pHGF7604 harbouring the *rifG-N* genes. The *rifG-N* genes, in particular *rifL*, were putatively not expressed under the terms of the present study. It might be the case that *Streptomyces lividans* does not provide the genetic equipment for a proper

expression of the *rif* genes. Unfortunately, no further insights into the early steps of rifamycin biosynthesis could be gathered during this part of the work. To date the mechanism of nitrogen introduction in early stage AHBA biosynthesis via kanosamine is neither verified nor another mechanism is proposed in further studies. Therefore this mechanism still remains unclear.

4.2 Isolation of a polyketide gene cluster from *Streptomyces* sp. GW2/5831

This section of the present study was directed towards the isolation and investigation of the gene cluster encoding the biosynthesis of the polycyclic xanthone IB-00208 in *Streptomyces* sp. GW2/5831, a polyketide to which sugar units are attached in the post-PKS stage. The approach is presented in the following steps.

4.2.1 Isolation of genomic DNA from *Streptomyces* sp. GW2/5831

Genomic DNA from *Streptomyces* sp. GW2/5831 was isolated (5.5.1, figure 4.17) for the use in subsequent PCRs (5.5.5.1, 4.2.2) and for the production of a genomic library (5.5.10, 4.2.4).

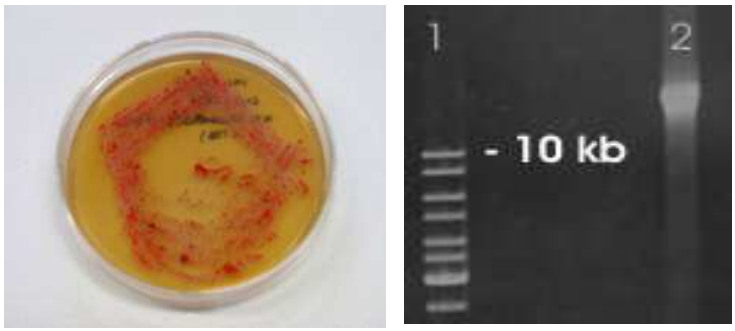


Figure 4.17: *Streptomyces* sp. GW2/5831 on M2 agar (left) and agarose gel of isolated high molecular weight genomic DNA (right, lane 1: DNA ladder Mix, lane 2: genomic DNA).

4.2.2 Amplification, cloning and sequencing of a gene fragment encoding a ketosynthase in genomic DNA of *Streptomyces* sp. GW2/5831

The genomic DNA (4.2.1) was subjected to PCR employing the degenerate ketosynthase (KS) primers KS2F, KS2R, KS4F and KS1R (table 5.11, figure 4.20).[83] These primers were designed from KS_{α} motifs conserved in type II PKSs of pentangular polyketide pathways. The degenerate PCR primers were utilised for the amplification of the KS in the type II PKS gene cluster of the pentangular polyketide IB-00208. Since a ketosynthase is the most conserved component of a PKS, it represented the most suitable target for the detection of a previously not sequenced type II PKS cluster in a producing organism.[102] The detection of the ketosynthase in the biosynthetic gene cluster of IB-00208 was used as a tool for accessing adjacent regions of the PKS that encode tailoring enzymes like glycosyltransferases. A

glycosyltransferase was expected to direct the attachment of the sugar moiety to the core structure of IB-00208 (figure 4.18).

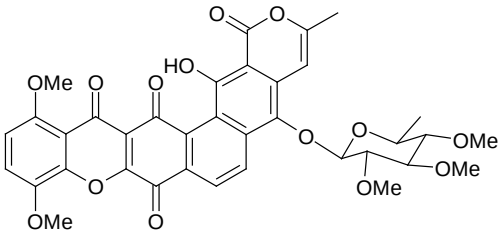


Figure 4.18: Chemical structure of the polycyclic xanthone IB-00208.

```
   1 gtgagccgac gcgccgtcat cacggggatc ggagccgtcg ctcccggcgg catcggacgg
  61 gacgcgtact gggacctgct cgcgcacggc cgtacggcca cccggcggat cagccggttc
 121 gacgcgtcgg cgttccgctc gcaggtcgcc gccgaatgcg acttcgaccc ggtcgccgcg
 181 gggctgagcc cgcaggaagt gcgccggatg gaccgggccg cccagttcgc ggtggtcgcg
 241 gcccaggagg cgatcgagga cagcggcatc gacgtcggcg cgggcgaccc ggggcggacc
 301 gcggtcagca tcggcagcgc ggtcggctgc accaccggcc tggaggacga gtacgcggtc
 361 ctctcggacg gcggccggaa ctggctggtc gaccacacct acggggtccc gcacctgtac
 421 ggctacatgg tccccagcac gctggcggcc gaagtggcct ggcgggcgcg ggcggagggc
 481 ccggtggcac tgatctcgac cggctgcacc tccggtctcg acgcgatcgc ccacggcgtg
 541 cagctcatcg aggagggcgc cgccgacatc gtcctggcgg gcgccaccga cgccccgctg
 601 tcccccatca cctccgcctg cttcgacgcg atcaaggcga cgacgcccaa cgcggaccag
 661 gacccgggca gcgcctcccg ccccttcgac ggccgccgcg acggcttcgt gctgggcgag
 721 ggctcggcgg tcatggtgat cgaggaggcc ggggcggccc gccgccgggg ggccaccgtc
 781 tacgccgagg tggccggttt cgccaaccgc tccaacgcct tccacatgac cgggctcaag
 841 cccgacgggc gggagatggc cgaggccatc cgggtcgccc tggaccgcgc gcggctcgac
 901 cccggcgcga tcgaccacgt caacgcgcac ggctccggca ccaagcagaa cgaccggcac
 961 gagacggccg ccttcaagcg gtccctgggg cagcgcgccc acgagatccc ggtcagctcc
1021 atcaagtcga tgatcgggca ctccctgggg gcgatcggct ccctggaggt ggccgcctgc
1081 gccctggcgc tgcgccacca ggtggtgccg cccacggcga acctcacggt ccccgacccc
1141 cagtgcgacc tggactacgt cccggtcacc gcccgggagc accgcatgga ccacgtcctc
1201 agcgtcggca gcggattcgg cggcttccag agcgccatga tcctcagccg gcccggagcc
1261 cgtaaggagg cggtcgcgtg a
```

Figure 4.19: Primer binding sites (bold and underlined) of the ketosynthase primer pair KS2F/ KS2R inside the griseorhodin A gene cluster, indicating the gene sequence encoding the KS_{α} subunit (*grhA*) of griseorhodin A in the genome of the producing strain *Streptomyces* JP95.[83]

The degenerate primer pair KS2F/ KS2R (figure 4.19) amplified a clear PCR product of approximately 900 bp (expected: 880 bp) in size (5.5.5.1, figure 4.20: lane 2), whereas the other two degenerate primer pairs KS4F/ KS1R and KS4F/ KS2R yielded several unspecific bands (figure 4.20, lanes 3 and 4). The PCR product (figure 4.20: lane 2) was cloned,

sequenced and used to design specific primers (4.2.3) to bind in a homologous fashion to the genomic region encoding the ketosynthase in the targeted PKS gene cluster of *Streptomyces* sp. GW2/5831. Therefore, the amplified and recovered PCR product was cloned into pBluescript (5.5.8), and heat shock transformation was carried out using *E. coli* XL1-Blue chemically competent cells (5.5.9.1, 5.5.9.2). Plasmid DNA was isolated (5.5.2) from 14 clones that indicated the insertion of foreign DNA by showing a white colour in "blue-white" screening (5.5.9.6). Subsequent cleavage of the samples with the endonuclease *Rsa*I (5.5.7.1) resulted in five different restriction patterns, and the corresponding plasmids were subjected to sequence analysis using the T7 standard primer-binding site of pBluescript. The obtained sequences were analysed by homology search using BLAST X. The results are listed in table 4.1.

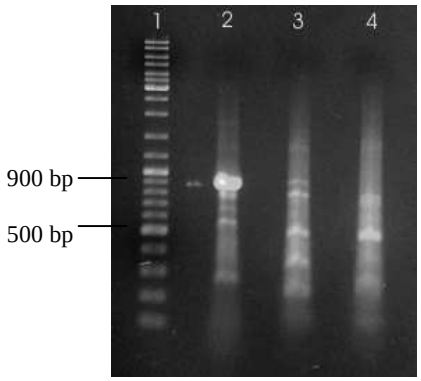


Figure 4.20: Agarose gel of PCRs of degenerate primers with genomic DNA of *Streptomyces* sp. GW2/5831. Lane 1: DNA Ladder Mix; lane 2: KS2F/ KS2R; lane 3: KS4F/ KS1R; lane 4: KS4F/ KS2R.

Table 4.1 Deduced functions of BLAST X data base search results

Sample name	Significant sequence alignment		Identity (%)	Length (bp)	Protein accession number (GenBank)
		origin			
pBES1	KS_{α} subunit (RubA)	*Streptomyces collinus* DSM2012	70	428	AF293355
pBES2	S-adenosylmethionine synthetase (MetK)	*Thermobifida fusca* YX	86	397	CP000088
pBES3	KS_{α} subunit (RubA)	*Streptomyces collinus* DSM2012	70	428	AF293355
pBES4	putative uncharacterised protein	*Streptomyces avermitilis* MA-4680	57	275	BA000030
pBES5	KS_{α} subunit (RubA)	*Streptomyces collinus* DSM2012	70	428	AF293355

The sequence alignment of the cloned PCR product (figure 4.20, lane 2) showed a high similarity to the rubromycin *rubA* sequence, exhibiting an identity of 70% to the KS_α subunit. This result is represented in the samples pBES1, pBES3 and pBES5. The query lengths comprised 701 bp (pBES1), 710 bp (pBES3) and 602 bp (pBES5), whereas the region of sequence homology spanned 428 bp in all of the three samples. Since the KS of a pentangular type II PKS system from a *Streptomyces* strain was the targeted sequence and the KS_α subunit of the pentangular type II PKS rubromycin gene cluster from *Streptomyces collinus* exhibited a significant similarity, these data strongly suggest that the analysed sequence belongs to the IB-00208 gene cluster. The sequence of pBES2 encodes S-adenosylmethionine synthetase from *Thermobifida fusca*. Taxonomically, *Thermobifida fusca* and *Streptomyces* strains are in the order of Actinomycetales. The sequence encoded on pBES4 produced a significant alignment with a putative uncharacterised protein from *Streptomyces avermitilis*. As only 3 samples out of five contained the targeted KS-fragment, it was shown that degenerate primers can cause false positive detections. Therefore a specific primer pair was required for the library screening (4.2.3).

4.2.3 Design of specific primers

Specific primers were designed based on the sequenced PCR product between positions 45 bp and 734 bp of plasmid pBES1. Thus, the specific primer pair BES1 should amplify a PCR product with a size of 691 bp (figure 4.21).

```
  1 ggccccctcg aggtcgacgg tatcgataag cttgattccg atcatcgact tgatggagct
 61 gaccggcacc tcgtaggccc gctgcccgag gctgcgcttg aacgcggccg tctcgtgccg
121 gtcgttctgc ttggtgcccg acccgtgcgc gttgatgtag tcgatgtcgt ccggccccag
181 cctcgcctgc cgcatggcga ccgtgatggc ctcggccatc tcgcggccgt cgggcttcag
241 cccggtcatg tggtaggcgt tgctgcgccc ggcgaccccg gccacctcgg cgtacacgtg
301 ggcgtcgcgg cggcgcgccg ccgacgcctc ctccagcaca acacggccga gccctcgccg
361 aggacgaacc cgtcccggtc gcggtcgaac ggccgcgagg cgtgcgcggg gtcgtcgttg
421 ttgggggacg tcgccttgat cgcgtcgaag caggccgagg tgatcggcga gatcggcgcg
481 tcggtggccc cggcgatcac cacgtccgcg gcgccctccc ggatgagctg ggcgccgtgc
541 gcgacggcgt ccagcccgga ggtgcagccg gtggagatca gcgccaccgg cccctcggcg
601 ccgacccgcc aggccacctc gaccgccagc gtgctgggca ccatgtagcc gtacagctgc
661 ggcaccccgt actcgtggtc gaccagccac tgccggccgc cgtcgctgag gacgatgtac
721 tcctcctcca gcccatcgt gcagccgacc gcgctgccga ggctgacgcc gacgcggtcc
781 cggtcgcagc
```

Figure 4.21: Primer binding sites (bold and underlined) of the specific primer pair BES1 in the ketosynthase region of *Streptomyces* sp. GW2/5831 DNA sequence.

The specific primer pair was used in a PCR with genomic DNA from *Streptomyces* sp. GW2/5831, yielding an amplified product of approximately 700 bp (figure 4.22). Based on this result, the BES1 primer pair was then used for screening a *Streptomyces* sp. GW2/5831 library for the ketosynthase of interest (4.2.5).

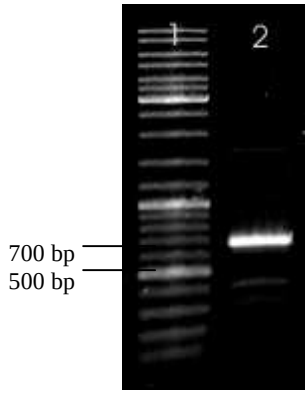


Figure 4.22: Agarose gel of PCR of specific primer pair BES1 with genomic DNA. Lane 1: DNA Ladder Mix; lane 2: ~700 bp PCR product.

4.2.4 Production of a genomic library from *Streptomyces* sp. GW2/5831

A genomic cosmid library was constructed as described in 5.5.10, using genomic DNA from *Streptomyces* sp. GW2/5831 (4.2.1) and the pAY1 shuttle vector. Both are displayed in figure 4.23, visualised on agarose gels. PAY1 was constructed based on the stable integrative plasmid pSET152 containing the *oriT* region for conjugative transfer, the ΦC31 attachment site and the *cos* site that was inserted from the plasmid pWEB.[83] Shuttle vectors are employed to enable intergeneric transfer between different bacterial strains. In this case we hoped that this technique allows gene expression of a cosmid from the library in a heterologous host.

The sheared, non blunt-ended, genomic DNA was recovered from a low-melting point agarose gel with the appropriate size of ~ 40 kb (figure 4.23). For the ligation into pAY1 it was required to be blunt-ended, because the vector was restricted with *Eco*RV yielding blunt ends. Therefore the sticky ends of the DNA fragments were filled with deoxynucleotides during the blunt ending reaction (5.5.10.1). The pAY1 vector DNA was prepared as described in 5.5.10.2.

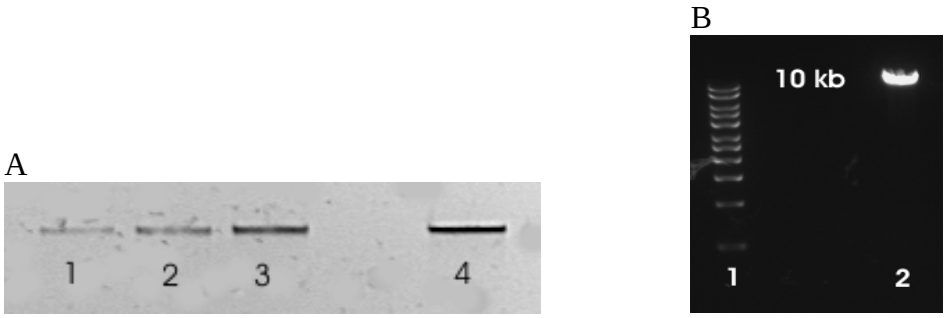


Figure 4.23: Quantitative analysis of genomic DNA during library production (left), pAY1 vector DNA after purification (right). A: DNA before blunt ending reaction: lane 1: 0.5µl; lane 2: 1 µl; lane 3: 2 µl; lane 4: 20 ng of Fosmid Control DNA (~36 kb); B: Lane 1: DNA Ladder Mix, lane 2: 0.5 µl pAY1 vector DNA (~10 kb) after purification (5.5.10.2).

A ligation reaction was carried out as described in 5.5.10.3 using the blunt ended genomic DNA and the pAY1 vector (figure 4.23). The transfection of *E. coli* EPI300™ $T1^R$ plating strain with the plasmid-packaged lambda phages was performed according to the manufacturers instructions.

The titer of the transfected cells comprised about 4,800 colony forming units. This was about 3.5-fold larger than the number of clones required to cover the approximately 40 kb sequence of the *Streptomyces* sp. GW2/5831 PKS gene cluster (~ 1400). The amount was calculated according to the formula presented in chapter 5.5.10.4. Actually, 2016 random cosmid clones were preserved in 21 microtiter plates (5.5.10.5, figure 4.24) representing the genomic DNA library. Each cosmid harboured a ~ 40 kb genomic DNA fragment of *Streptomyces* sp. GW2/5831.

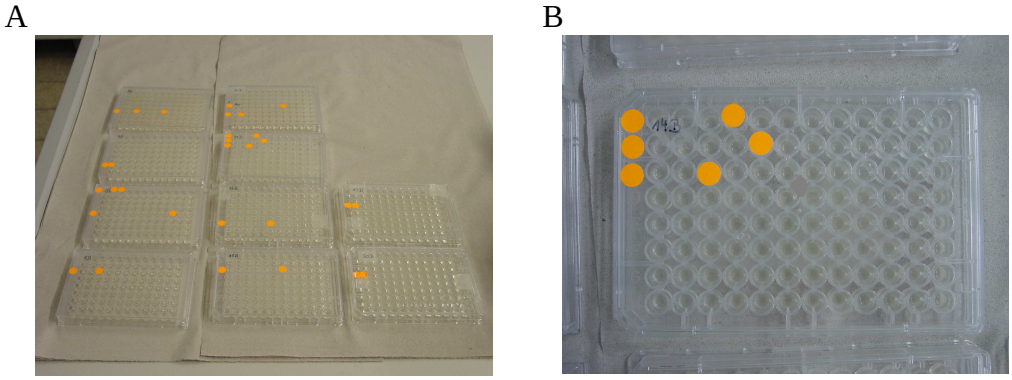


Figure 4.24: Microtiter plates from the library. A: Micro titer plates containing positive clones (4.2.5) that are indicated by an orange dot on the corresponding row (A-H) and single well (1-12); B: Micro titer plate number 14, holding positive clones on locations A4, B5 and C3.

4.2.5 Library screening for polyketide synthase gene fragments

To isolate the gene cluster responsible for IB-00208 biosynthesis, a PCR based screening of the gene library from *Streptomyces* sp. GW2/5831 was performed. This approach was conducted using the KS-specific primer pair BES1 (see also 4.2.3).

To save time and minimize reagent amount, "row-pools" were established by combining aliquots of each well (12) in a row into one eppendorf tube (5.5.10.5). Thus eight "row pools" resulted from each microtiter plate. The "row-pools" were then screened for the PCR product (5.5.10.6). After the detection of a positive pool (figure 4.25 A), all the 12 wells of the corresponding row were screened individually (figure 4.25 B). The positive clones identified in this manner are listed in table 4.2.

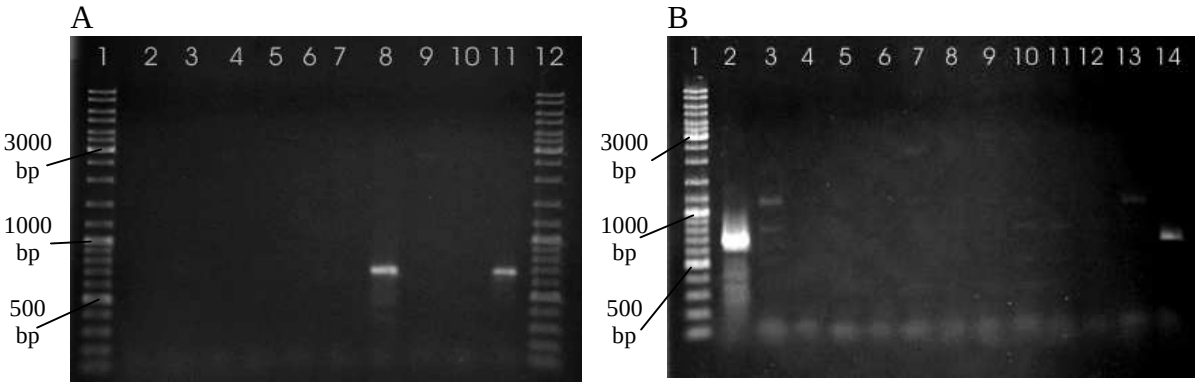


Figure 4.25: Agarose gels of PCRs from screening procedure. A: PCR from the pooled rows A-H of microtiter plate four, lane 1: DNA Ladder Mix; lane 2: pool A; lane 3: pool B; lane 4: pool C; lane 5: pool D; lane 6: pool E; lane 7: pool F; lane 8: pool G; lane 9: pool H; lane 10: negative control; lane 11: positive control; lane 12: DNA Ladder Mix. B: PCR from 12 single wells in row G of microtiter plate four, lane 1: DNA Ladder Mix; lane 2: well 1; lane 3: well 2; lane 4: well 3; lane 5: well 4; lane 6: well 5; lane 7: well 6; lane 8: well 7; lane 9: well 8; lane 10: well 9; lane 11: well 10; lane 12: well 11; lane 13: well 12; lane 14: positive control.

The screening of the genomic library from *Streptomyces* sp. GW2/5831 with the KS-specific primer pair BES1, conducted as described in 5.5.5.3, yielded 16 positive cosmid clones that are listed in table 4.2. The corresponding PCRs were double-checked and are shown on an agarose gel (figure 4.26). This confirmed the location of the sixteen positive cosmid clones in the library.

Table 4.2 Location of positive cosmid clones in the library exhibiting a BES1 PCR product (~700 bp)

Cosmid number	Coordinates		
	Microtiter plate	Row (A-H)	Number (1-12)
1	1	C	3
2	3	A	2
3	3	A	3
4	3	E	10
5	4	G	1
6	9	F	3
7	9	F	7
8	10	E	8
9	10	G	2
10	14	A	4
11	14	B	5
12	14	C	3
13	17	G	6
14	18	C	7
15	19	D	1
16	20	D	1

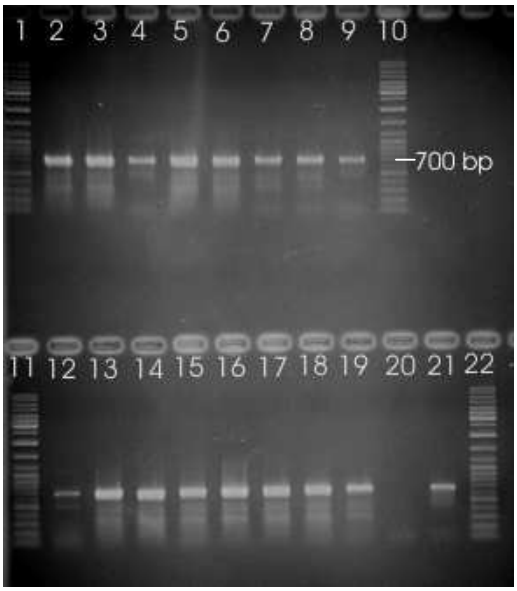


Figure 4.26: Agarose gel of all positive cosmid clones after amplification of the ~700 bp PCR product. Lane 1: DNA Ladder Mix; lane 2: cosmid 1; lane 3: cosmid 2; lane 4: cosmid 3; lane 5: cosmid 4; lane 6: cosmid 5; lane 7: cosmid 6; lane 8: cosmid 7; lane 9: cosmid 8; lane 10: DNA Ladder Mix; lane 11: DNA Ladder Mix; lane 12: cosmid 9; lane 13: cosmid 10; lane 14: cosmid 11; lane 15: cosmid 12; lane 16: cosmid 13; lane 17: cosmid 14; lane 18: cosmid 15; lane 19: cosmid 16; lane 20: negative control; lane 21: positive control; lane 22: DNA Ladder Mix.

4.2.6 Restriction mapping and spot-sequencing of the positive cosmid clones

The obtained positive cosmid clones (4.2.5) were further analysed for discovery of putative identical clones. Therefore cosmid DNA was isolated from an overnight culture of every

positive clone. Subsequent single restriction analysis was carried out using *Bam*HI endonuclease. A distinct restriction pattern was obtained for each of the 16 positive clones that showed that the clones contained different sequences (figure 4.27).

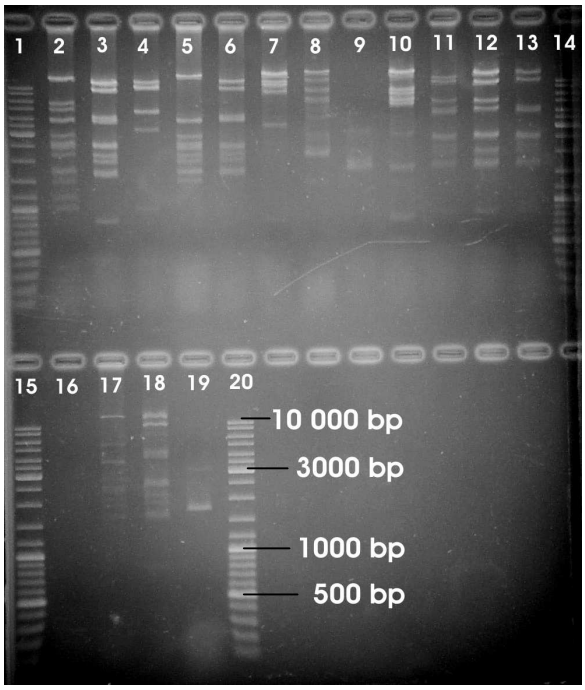


Figure 4.27: Restriction analysis of positive cosmid clones with *Bam*HI. Lane 1: DNA Ladder Mix; lane 2: cosmid 1; lane 3: cosmid 2; lane 4: cosmid 3; lane 5: cosmid 4; lane 6: cosmid 5; lane 7: cosmid 6; lane 8: cosmid 7; lane 9: cosmid 8; lane 10: cosmid 9; lane 11: cosmid 10; lane 12: cosmid 11; lane 13: cosmid 12; lane 14: DNA Ladder Mix; lane 15: DNA Ladder Mix; lane 16: cosmid 13; lane 17: cosmid 14; lane 18: cosmid 15; lane 19: cosmid 16; lane 20: DNA Ladder Mix.

In order to gain insight into the cosmid content, spot-sequencing primers were designed employing the upstream and downstream parts of the *Eco*RV restriction site of the pAY1 vector (figure 4.28). As the genomic DNA fragments of *Streptomyces* sp. GW2/5831 were cloned at the *Eco*RV restriction site of pAY1 vector, it was thus possible to end-sequence each positive construct.

```
4100 agcacgccat agtgactggc gatgctgtcg gaatggacga ta_tcgaattc
                                                  EcoRV

4151 gtaatcatgt catagctgtt tcctgtgtga aattgttatc cgctcacaat
```

Figure 4.28: Section around *Eco*RV cleavage site (4142 bp, underlined) from pAY1 vector sequence (4100-4200bp), primer binding sites (AY1ES1 forward, AY1ES1 reverse) are bold and underlined.

All positive cosmids were subsequently end-sequenced to obtain insights into the outer parts of the cloned fragments. On the basis of these results, it was then decided which cosmids were of further interest.

The vector primers AY1ES1 forward and AY1ES1 reverse were used for spot-sequencing of the 16 positive cosmids (pAYES 1-16). The sequences were then analysed by BLAST X, which retrieved homologies to proteins that are presented in table 4.3.

Table 4.3 Sequence analysis of insert ends from all positive cosmids

Insert designation	Co-ordinates[1]	Sequence length[2] (bp)	Homology[3] (proposed function)	Gene region[4] (bp)	Protein region (aa)[5]	Protein length (aa)	Identity[6] (%)
pAYES 1 forward	1C3	178	ATP-binding cassette (ABC)-type drug export system [*Streptomyces avermitilis*][103]	40-170	4-49	258	56
			ABC-type drug transport system (PdmR2, pradimicin cluster)[*Actinomadura hibisca*][104, 105]	50-160	7-44	259	57
pAYES 1 reverse	1C3	373	UDP-glucose 4-epimerase (GalE) [*Saccharopolyspora erythraea*][106]	40-372	111-221	355	60
pAYES 2 forward	3A2	103	no significant similarity	-	-	-	-
pAYES 2 reverse	3A2	700	oxidoreductase [*Mycobacterium ulcerans*][107]	21-677	1-228	292	36
pAYES 3 forward	3A3	557	putative secreted protein [*Streptomyces coelicolor*][108]	5-127	35-76	318	45
			conserved hypo-thetical regulatory protein [*Mycobacterium ulcerans*][107]	101-184	267-294	301	60
pAYES 3 reverse	3A3	814	oxidoreductase, short-chain dehydrogenase/ reductase (SDR) family [*Arthrobacter aurescens*][109]	70-807	1-259	264	48

[1] micro titer plate (number) row (letter) column (number)
[2] of the read from vector to insert
[3] highest sequence homology on protein level, additional hits are only listed in case of importance or if related to different gene regions
[4] first and last base pair position of insert indicating homology of fragment sequence and data base element
[5] first and last amino acid position indicating homology of fragment sequence and homologous protein
[6] percentage of sequence homology

			3-oxo-acyl-reductase (acyl-carrier-protein) (FabG) [*Saccharopolyspora erythraea*][106]	70-813	1-265	268	43
pAYES 4 forward	3E10	390	no significant similarity	-	-	-	-
pAYES 4 reverse	3E10	579	putative FAD-binding monooxygenase [*Bradyrhizobium* sp.][110]	227-568	4-122	562	43
pAYES 5 forward	4G1	571	FAD-dependent oxidoreductase [*Myxococcus xanthus*][111]	1-180	297-356	385	41
pAYES 5 reverse	4G1	362	ABC-transporter related precursor [*Frankia* sp.][112]	44-361	753-862	1302	36
pAYES 6 forward	9F3	542	sodium/hydrogen exchanger [*Stigmatella aurantiaca*][113]	162-341	16-86	677	50
pAYES 6 reverse	9F3	646	putative dehydrogenase (LlpS, lysolipin cluster) [*Streptomyces tendae*][78]	2-571	93-281	287	36
pAYES 7 forward	9F7	-	poor DNA quality	-	-	-	-
pAYES 7 reverse	9F7	1031	oriT-binding protein (TraJ) [uncultured bacterium][114]	67-381	19-123	123	75
			aminoglycoside-acetyltransferase (AacC4) [*E. coli*][115]	818-1030	188-258	258	94
pAYES 8 forward	10E8	-	poor DNA quality	-	-	-	-
pAYES 8 reverse	10E8	-	poor DNA quality	-	-	-	-
pAYES 9 forward	10G2	816	hypothetical protein [*Oryza sativa*][116]	605-796	171-242	282	34
pAYES 9 reverse	10G2	469	putative ATP-dependent RNA helicase [*Streptomyces avermitilis*][117]	71-469	557-687	877	62
pAYES 10 forward	14A4	832	putative integral membrane protein (MMPL) [*Salinispora arenicola*][118, 119]	3-359	590-708	714	67
pAYES 10 reverse	14A4	506	no significant similarity	-	-	-	-
pAYES 11 forward	14B5	810	hypothetical protein [*Oryza sativa*][120]	88-513	76-221	339	30
			putative serine/ threonine protein	439-786	408-523	576	27

			kinase [*Streptomyces coelicolor*][108]				
pAYES 11 reverse	14B5	516	putative cyclase (PdmL, pradimicin cluster) [*Actinomadura hibisca*][104, 105]	222-515	2-99	147	69
			putative polyketide cyclase [*Streptomyces griseus*][121]	234-515	1-94	141	62
			putative cyclase (GrhS, griseorhodin cluster) [*Streptomyces* sp. JP95][83]	255-515	10-96	144	62
			polyketide synthase [*Frankia alni*][112]	237-515	3-93	172	59
			putative cyclase (RubE, similar to PdmL in pradimicin cluster) [*Streptomyces collinus*][122]	11-133	70-110	123	56
pAYES 12 forward	14C3	-	poor DNA quality	-	-	-	-
pAYES 12 reverse	14C3	-	poor DNA quality	-	-	-	-
pAYES 13 forward	17G6	471	no significant similarity	-	-	-	-
pAYES 13 reverse	17G6	742	FAD-dependent oxidoreductase [*Myxococcus xanthus*][111]	1-699	133-356	385	40
pAYES 14 forward	18C7	778	oxygenase (GrhM) [*Streptomyces* sp. JP95][83]	254-577	5-112	150	47
			putative monooxygenase (fredericamycin cluster) [*Streptomyces griseus*][121]	239-568	2-110	149	45
			oxygenase (PdmE, pradimicin cluster) [*Actinomadura hibisca*][104, 105]	263-583	8-114	153	42
			RubQ (rubromycin cluster, similar to ORF5 in pradimicin cluster) [*Streptomyces collinus*][122]	251-577	3-110	148	47
pAYES 14 reverse	18C7	-	poor DNA quality	-	-	-	-
pAYES 15 forward	19D1	584	hemerythrin cation-binding domain protein (HHE) [*Mycobacterium* sp.][118]	33-584	351-533	747	30

pAYES 15 reverse	19D1	501	6-aminohexanoate cyclic dimer hydrolase [*Mycobacterium smegmatis*][123]	75-194	525-565	1075	39
pAYES 16 forward	20D1	-	poor DNA quality	-	-	-	-
pAYES 16 reverse	20D1	999	aminoglycoside-acetyltransferase (AacC4), apramycin resistance gene [*E. coli*][115]	492-998	90-258	258	86

Homologies to gene products from type II PKS systems occur frequently within the sequenced DNA fragments. Several of them (pAYES 1 forward, pAYES 11 reverse, pAYES 14 forward) belong to the biosynthetically related pradimicin-type pentangular polyketides like the fredericamycins and members of the griseorhodin/ rubromycin family.[73] Furthermore, oxidoreductases that often occur as tailoring enzymes in polyketide biosynthesis were discovered on the fragments pAYES 2 reverse, pAYES 3 reverse, pAYES 5 forward and pAYES 13 reverse. Therefore it is most likely that at least parts of the targeted IB-00208 gene cluster are encountered on the positive cosmid clones.

4.2.7 Heterologous expression of the positive cosmid sequences

To reveal the function of the positive cosmid sequences (table 4.2), heterologous expression was carried out using *Streptomyces albus* as host strain. Intergeneric transfer of the cosmid DNA was accomplished as described in 5.5.11. The inserted cosmids and the corresponding designation of the conjugated strains are listed in table 4.4.

Biosynthetic gene clusters that code for aromatic polyketides usually comprise 15 to 40 open reading frames, holding capacities between 20 to 60 kb. For IB-00208 we expected a size of approximately 40 kb. Since the sizes of the inserts in the generated cosmid gene library reach approximately 40 kb, it is theoretically possible to encounter the whole IB-00208 gene cluster in one of the 16 positive clones. A heterologous expression could therefore be accomplished by the transfer of a single cosmid. Ideally, successful transfer of the complete gene cluster would be accompanied by the production of the compound. Due to the intense red colour of the xanthone, a producing recombinant may be identified visually after the heterologous expression in *Streptomyces albus* host strain via conjugal transfer. Therefore, any deviation from a white mycelium was monitored. On the other hand, it could be possible that the heterologous expression of only parts of the cluster, dependent on the cosmid content, yields

another secondary metabolite, even previously unknown. For example collinone, an intermediate from the griseorhodin pathway, is produced by a knockout mutant lacking the oxygenase GrhO5.[73]

The transfer of genetic information between *E. coli* and *Streptomyces* can be accomplished by intergeneric conjugation.[5] This method is advantageous because it is still easier to manipulate *E. coli* rather than *Streptomyces* DNA. The mating system consisted of the non-methylating/ mobilisation-proficient *E. coli* ET12567/pUZ8002 donor strain [124] and *Streptomyces albus* J1074 host strain. The donor strain was transformed with the positive pAY1 cosmid clone DNA. The vector pAY1 possesses an origin of transfer (*oriT*) sequence and contains an integrase function of the actinomycete phage ΦC31 where the site-specific integration of the foreign DNA into the host genome takes place.[125] A successful transfer of cosmid DNA can be verified by selection on the apramycin resistance gene marker (*aacC4*). The overlay with nalidixic acid inhibits the bacterial growth of the donor strain during incubation after the conjugation event. Streptomyces exconjugants, that usually appeared after five days of cultivation, were repeatedly transferred onto fresh agar plates containing different media in order to remove remaining *E. coli* cells. Use of different solid and liquid media enhanced the possibility of finding a putative new secondary metabolite produced by one of the recombinant *Streptomyces albus* strains SE01-SE16. This method is described as "one strain many compounds"- (OSMAC) principle.[126] According to this, one strain is enabled to produce different compounds depending on the provided media components.

Table 4.4 Conjugated *Streptomyces albus* strains carrying cloned fragments from *Streptomyces* sp. GW2/5831 (for cosmid numbers see table 4.2.)

Strain designation	Conjugated fragment
Streptomyces albus SE01	Cosmid 01
Streptomyces albus SE02	Cosmid 02
Streptomyces albus SE03	Cosmid 03
Streptomyces albus SE04	Cosmid 04
Streptomyces albus SE05	Cosmid 05
Streptomyces albus SE06	Cosmid 06
Streptomyces albus SE07	Cosmid 07
Streptomyces albus SE08	Cosmid 08
Streptomyces albus SE09	Cosmid 09
Streptomyces albus SE10	Cosmid 10
Streptomyces albus SE11	Cosmid 11
Streptomyces albus SE12	Cosmid 12
Streptomyces albus SE13	Cosmid 13
Streptomyces albus SE14	Cosmid 14
Streptomyces albus SE15	Cosmid 15
Streptomyces albus SE16	Cosmid 16

All sixteen conjugated *Streptomyces albus* strains (SE01-SE16) are represented in figure 4.29. They were grown on solid M2 medium which is known as the production medium for IB-00208 in *Streptomyces* sp. GW2/5831.

Figure 4.29 : Conjugated *Streptomyces albus* strains (SE01-SE16) on M2 medium.

Fifteen of the strains show no visual deviation from the wild type of *Streptomyces albus*. In contrast, the characteristic white colour of the spores was repeatedly not exhibited by *Streptomyces albus* SE08, which might be due to the lack of sporulation. The morphology of all conjugated *Streptomyces albus* (SE01-16) strains cultured on other media did not differ from appearance in figure 4.29. Utilised agar plates consisted of LB- (figure 4.30), MS- and 2CM medium (without illustration). For the cultivation in liquid media, LB-, TSB- and meat extract medium were employed (without illustration). Meat extract medium is known as production medium for IB-00208 in liquid cultures of *Streptomyces* sp. GW2/5831 (Prof. H. Laatsch, personal communication). No variation in appearance from unmodified *Streptomyces albus* was detected. The absence of any coloured compound indicated that IB-00208 is not produced by one or more of the conjugated strains. None of the cosmids harbours the entire IB-00208 biosynthetic gene cluster. To obtain verification of received apramycin resistance of the host strains, the conjugated strains were spread on LB medium containing apramycin (figure 4.30). The strains SE02, SE07, SE10, SE12 and SE16 do not grow on apramycin agar plates, whereas all other conjugated strains exhibit apramycin resistance.

Figure 4.30: Conjugated *Streptomyces albus* strains (SE01-SE16) on LB medium including apramycin.

The growth of conjugated strains on and in apramycin containing media does not necessarily mean that the complete cosmid DNA, including apramycin resistance gene cassette, is integrated into the genome of the host strain. Resistance may occur spontaneously. To unequivocally proof the integration of the cosmid DNA into the genome of *Streptomyces albus*, genomic DNA was therefore isolated from conjugated strains and employed in a PCR with BES1 primer pair under the same conditions as applied in the library screening. The PCRs are visualised on an agarose gel (figure 4.31). Amplification of the known ~700 bp PCR product documents the presence of the cloned fragment in the heterologous host.

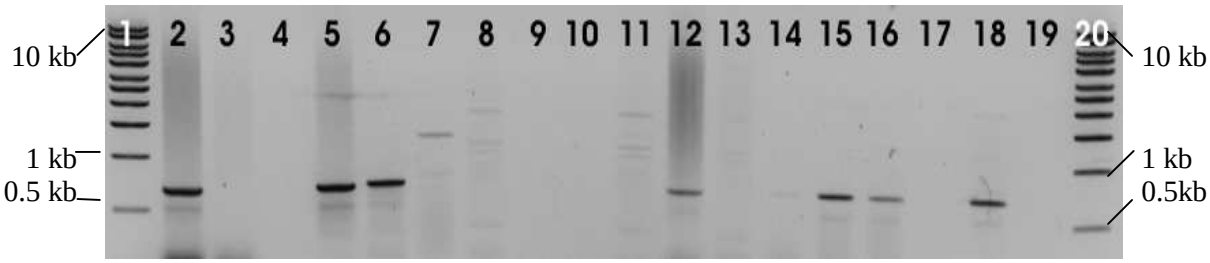


Figure 4.31: Visualised PCR of genomic DNA from conjugated *Streptomyces albus* strains (SE01-SE16) with BES1 primers. Lane 1: 1 kb ladder; lane 2: SE01; lane 3: SE02; lane 4: SE03; lane 5: SE04; lane 6: SE05; lane 7: SE06; lane 8: SE07; lane 9: SE08; lane 10: SE09; lane 11: SE10; lane 12: SE11; lane 13: SE12; lane 14: SE13; lane 15: SE14; lane 16: SE15; lane 17: SE16; lane 18: positive control (genomic DNA from *Streptomyces* sp. GW2/5831); lane 19: negative control (without any template DNA); lane 20: 1 kb ladder.

Integration was proven for seven strains (SE01, SE04, SE05, SE11, SE13, SE14 and SE15), although the PCR product of SE13 is amplified to a lesser extent than all others. As no coloured compound was visually detected in these confirmed conjugated *Streptomyces albus* strains SE01, SE04, SE05, SE11, SE13, SE14 and SE15, the entire IB-00208 gene cluster is not located on one of these cosmids. The remaining nine strains had not integrated the cloned

fragment into their genome. Thus the colour of the mycelia is not significant, and can therefore not be appraised. The strains SE02, SE07, SE10, SE12 and SE16 neither grow on apramycin nor do they show a PCR product. The strains SE03, SE06, SE08 and SE09 grow on apramycin, although their genomic DNA does not contain the corresponding cosmid sequence. Further attempts could generate exconjugants of SE02, SE03, SE06, SE07, SE08, SE09, SE10, SE12 and SE16.

The lack of any visible compound might be due to the heterologous expression of the incomplete IB-00208 biosynthetic gene cluster in the so far conjugated strains. Analytical investigations could lead to the detection of a putative, uncoloured pathway intermediate that might be produced by one of the conjugated *Streptomyces albus* strains containing cluster fragments.

4.2.8 Subcloning and sequencing of selected cosmid clones

Important sequences might be hidden in regions of the cosmids that were not sequence-analysed so far. To gain more insight into the sequence content of the cloned fragments, another approach was required. Therefore, subcloning technique was performed.

4.2.8.1 Subcloning of selected cosmid clones

To obtain more sequence information about the cosmid DNA samples, two out of the sixteen positive cosmid clones were selected and digested into smaller fragments. Aside from the results of restriction analysis (4.2.6) and heterologous expression (4.2.7), the information in table 4.3 was consulted for sample selection. To obtain as much sequence data as possible, the maximum cluster coverage was aspired. It was putatively represented by the cosmids 11 and 14.

For restriction of the cosmid DNA three different type II endonucleases (table 4.5) were tested. *Not*I is an eight base cutter, whereas *Sac*I and *Pst*I are six base cutters. For cosmid 11, the *Not*I restriction pattern was the most suitable which exhibited the best band separation and yielded 12 fragments of reasonable size for cloning (table 4.6). The endonuclease *Sac*I digested cosmid 14 into 14 fragments (table 4.6). *Pst*I did not generate a useful restriction pattern. The fragments were recovered from the gel via gel extraction (5.5.4.2). The vector pBluescript was restricted with *Not*I for cosmid 11 and *Sac*I for cosmid 14. After the ligation reaction of the recovered fragments with pBluescript, the DNA was introduced into *E. coli*

XL-1 Blue electrocompetent cells. Plasmid DNA was isolated from overnight cultures of white transformants (5.5.9.6), and restriction was carried out. The sizes of vector and fragments were verified (table 4.6, figures 4.32 and 4.33). As fragment pBSSE1105 (lane 6, figure 4.32) and pBluescript both exhibit a size of about 3000 bp, the plasmid DNA was restricted with two endonucleases. Cleavage of pBSSE1105 was therefore carried out with *Not*I and *Sca*I simultaneously, generating three fragments. The fragment pBSSE1105 is represented in the 3000 bp band, whereas pBluescript was restricted by *Sca*I yielding one band at ~1000 bp and a further band at ~ 2000 bp, respectively. A double digestion with *Sca*I was also required for fragment pBSSE1406 (lane 8, figure 4.33) that resulted in a 3000 bp fragment derived from pBSSE1406, and pBluescript was cleaved as described above.

The subclones were subjected to sequence analysis. Sequencing of the obtained fragments, based on the standard T3 and T7 primers, provided more evidence for putative protein coding regions involved in IB-00208 biosynthesis (see table 4.7).

Table 4.5 Sequences, cleavage sites and evolving overhang of utilised endonucleases

Endonuclease	Sequence (5'-3')	Cleavage site (5'-3')	Overhang (5'-3')
*Not*I	GCGGCCGC	GC GGCCGC CGCCGG CG	5'- GGCC
*Sac*I	GAGCTC	GAGCT C C TCGAG	AGCT-3'
*Pst*I	CTGCAG	CTGCA G G ACGTC	TGCA-3'

Table 4.6 Fragments and sizes of cosmids 11 and 14

Sample designation	Fragment size (bp)	Sample designation	Fragment size (bp)
pBSSE1101	14.000	pBSSE1402	10.000
pBSSE1102	10.000	pBSSE1403	8.000
pBSSE1103	6.000	pBSSE1404	6.000
pBSSE1104	5.000	pBSSE1405	3.800
pBSSE1105	3.000	pBSSE1406	3.000
pBSSE1106	1.800	pBSSE1407	2.500
pBSSE1107	1.400	pBSSE1408	1.800
pBSSE1108	1.100	pBSSE1409	1.600
pBSSE1109	700	pBSSE1410	1.300
pBSSE1110	600	pBSSE1411	1.200
pBSSE1111	550	pBSSE1412	800
pBSSE1112	< 500	pBSSE1413	650
pBSSE1401	12.500	pBSSE1414	550

The sizes of the large fragments were determined by means of the 1 kb extension ladder (figure 4.32, lane 1 and 4.33, lane 1).

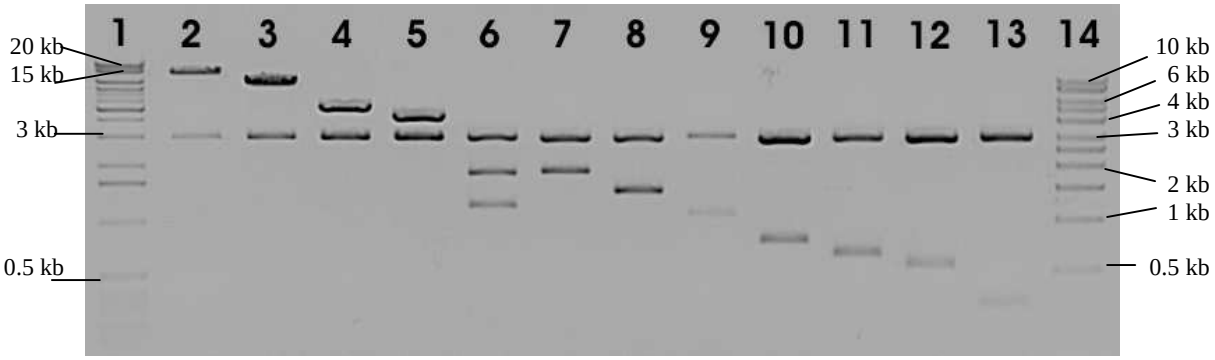


Figure 4.32: Subclones of cosmid 11 after plasmid DNA isolation and restriction cleavage with *Not*I, the plasmid DNA in lane 6 was additionally cleaved with *Sca*I. Lane 1: 1 kb extension ladder; lane 2: fragment 1101; lane 3: fragment 1102; lane 4: fragment 1103; lane 5: fragment 1104; lane 6: fragment 1105; lane 7: fragment 1106; lane 8: fragment 1107; lane 9: fragment 1108; lane 10: fragment 1109; lane 11: fragment 1110; lane 12: fragment 1111; lane13: fragment 1112; lane 14: 1 kb ladder.

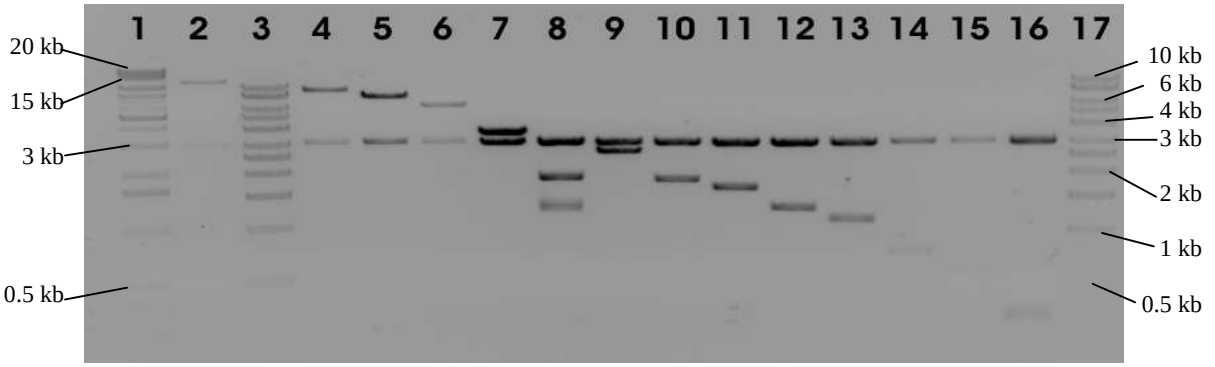


Figure 4.33: Subclones of cosmid 14 after plasmid DNA isolation and restriction cleavage with *Sac*I, the plasmid DNA in lane 8 was additionally cleaved with *Sca*I. Lane 1: 1 kb extension ladder; lane 2: fragment 1401; lane 3: 1 kb ladder; lane 4: fragment 1402; lane 5: fragment 1403; lane 6: fragment 1404; lane 7: fragment 1405; lane 8: fragment 1406; lane 9: fragment 1407; lane 10: fragment 1408; lane 11: fragment 1409; lane 12: fragment 1410; lane13: fragment 1411; lane 14: fragment 1412; lane 15: fragment 1413; lane 16: fragment 1414; lane 17: 1 kb ladder.

4.2.8.2 Sequence analysis of subcloned fragments

The subclones were then subjected to DNA sequencing (5.5.12.3), and the results of the BLASTX queries (5.5.12.4) are listed in table 4.7.

Table 4.7 Results of BLAST analysis of sequenced subclone-fragments (cosmids 11, 14)

Sample designation	Sequencing direction	Read length (bp)	Proposed function (homolog)	Origin	Region of homology (bp)	Identity (%)	Seq.length (homolog) (aa)	Region of homology (aa)	Accession number
pBSSE1101	T3	866	KS_{β} subunit (RubB)	*Streptomyces collinus*	204-4	59	422	24-90	AF293355
			KS_{α} subunit (RubA)	*Streptomyces collinus*	845-264	79	428	224-417	AF293355
pBSSE1101	T7	1027	serine/threonine protein kinase	*Streptomyces coelicolor*	680-501	37	576	462-523	NP_628048
pBSSE1102	T3	395	high-affinity branched-chain amino acid transport protein (ABC superfamily, ATP-binding)	*Frankia alni* ACN14a	77-388	51	256	75-181	YP_715216
pBSSE1102	T7	703	ABC transporter, transmembran region, type 1 precursor	*Nocardioides* sp.	651-367/343-113	53/42	1081	434-528/557-633	YP_922154
pBSSE1103	T3	734	transcriptional regulator (repressor) TetR family protein	*Streptomyces avermitilis*	351-665	34	242	42-146	NP_822557
pBSSE1103	T7	525	hypothetical protein	*Stigmatella aurantiaca* DW4/3-1	518-207	54	499	299-398	ZP_01460657
pBSSE1104	T3	760	putative ABC transporter, drug export system	*Streptomyces avermitilis*	740-135	44	258	55-256	NP_827888
pBSSE1104	T7	714	KS_{β} subunit (RubB)	*Streptomyces collinus*	86-712	67	422	88-302	AF293355
pBSSE1105	T3	695	putative secreted protein	*Streptomyces coelicolor*	18-692	38	342	97-324	NP_630686
pBSSE1105	T7	946	putative ABC transporter (CydCD)	*Streptomyces coelicolor*	145-696	49	1172	942-1139	NP_628131
pBSSE1106	T3	925	sodium/hydrogen exchanger	*Stigmatella aurantiaca*	584-796	50	677	16-86	EAU68588

pBSSE1106	T7	945	sodium/hydrogen exchanger	DW4/3-1 *Stigmatella aurantiaca*	708-166	41	677	208-388	EAU68588
pBSSE1107	T3	816	putative ABC transporter ATP-binding protein	DW4/3-*Nocardia farcinica*	261-815	35	926	5-184	YP_119573
pBSSE1107	T7	873	putative ABC transporter ATP-binding protein	*Nocardia farcinica*	426-797	42	926	1-126	YP_119573
pBSSE1108	T3	907	high-affinity branched-chain amino acid transport protein (LivG) (ABC superfamily, ATP-binding)	*Frankia alni*	850-563	58	272	12-107	YP_715217
pBSSE1108	T7	982	high-affinity branched-chain amino acid transport protein (ABC superfamily, ATP-binding)	*Frankia alni*	240-944	51	272	10-246	YP_715217
pBSSE1109	T3	1036	putative branched-chain amino acid transport system	*Frankia alni*	747-25	26	401	12-262	YP_713343
pBSSE1109	T7	1041	putative branched-chain amino acid transport system	*Frankia alni*	40-831	27	401	17-269	YP_713343
pBSSE1110	T3	648	polyketide synthase modules and related proteins	*Burkholderia mallei* GB8 4	431-291	42	1516	1150-1198	ZP_00439137
pBSSE1110	T7	804	polyketide synthase modules and related proteins	*Burkholderia mallei* GB8 4	238-444	41	1516	1150-1205	ZP_00439137
pBSSE1111	T3	1005	putative ABC transporter ATP-binding protein	*Frankia alni*	495-1	44	937	354-534	YP_713339

pBSSE1111	T7	1020	putative ABC transporter ATP-binding protein	*Frankia alni*	78-638	42	937	354-540	YP_713339
pBSSE1112	T3	1065	putative ABC transporter ATP-binding protein	*Frankia alni*	290-111	48	937	536-595	YP_713339
pBSSE1112	T7	-	-	-	-	-	-	-	-
pBSSE1401	T3	742	SARP family pathway specific regulatory protein	*Streptomyces griseus*	321-13	36	280	1-105	YP_001827897
			putative monooxygenase	*Streptomyces griseus*	731-438	46	246	29-127	AAQ08922
pBSSE1401	T7	595	putative *O*-methyltransferase	*Streptomyces griseus*	98-589	52	353	157-322	AAQ08925
pBSSE1402	T3	1010	conjugal transfer protein TraJ	Birmingham IncP-alpha plasmid	763-419	98	123	9-123	YP_001687709
pBSSE1402	T7	761	putative 3-oxoacyl-ACP reductase	*Streptomyces tendae*	472-86	51	245	45-173	CAM34370
pBSSE1403	T3	1010	hypothetical protein	*E. coli*	873-508	95	123	2-123	NP_040360
pBSSE1403	T7	984	PadR-like family transcriptional regulator	*Streptomyces avermitilis* MA-4680	101-667	44	215	26-212	NP_827886
			daunorubicin resistance ABC transporter ATPase subunit	*Alkaliphilus metalliredigens* QYMF	861-980	57	323	11-50	YP_001321687
pBSSE1404	T3	983	putative 3-oxoacyl-ACP reductase	*Streptomyces tendae*	11-199	51	250	183-248	CAM34340
			Antibiotic biosynthesis monooxygenase (BenJ)	*Streptomyces* sp. A2991200	536-769	38	115	13-90	CAM58802
pBSSE1404	T7	992	uncharacterised protein	*Streptomyces fradiae*	989-303	36	345	115-343	P20187
pBSSE1405	T3	181	no hits found	-	-	-	-	-	-
pBSSE1405	T7	923	KS_{α} subunit (RubA)	*Streptomyces collinus*	396-922	67	428	6-181	AF293355
			putative cyclase (RubE)	*Streptomyces*	93-377	61	143	45-140	AAG03066

				collinus					
pBSSE1406	T3	853	short-chain dehydrogenase/reductase (SDR)	*Frankia* sp. EAN1pec	814-752	66	312	13-33	YP_001511055
pBSSE1406	T7	639	hypothetical protein (Pd2U) angucycline cluster	*Streptomyces* sp. WP 4669	173-550	50	311	71-195	AAO65359
pBSSE1407	T3	892	FAD-dependent oxidoreductase	*Burkholderia* sp. 383	830-6	40	566	257-537	YP_366401
pBSSE1407	T7	953	short-chain dehydrogenase/reductase (SDR)	*Frankia* sp. EAN1pec	342-812	40	246	36-185	YP_001507428
pBSSE1408	T3	931	*O*-methyltransferase	*Streptomyces griseus*	382-2	51	353	21-147	AAQ08925
			dehydrogenase (LlpS)	*Streptomyces tendae*	931-551	47	293	161-287	CAM34368
pBSSE1408	T7	917	putative dehydrogenase (LlpS)	*Streptomyces tendae*	417-872	51	293	13-164	CAM34368
			3-oxoacyl-ACP reductase	*Streptomyces tendae*	86-307	58	245	172-245	CAM34370
pBSSE1409	T3	928	putative methyltransferase (GrhL)	*Streptomyces* sp. JP95	77-772	42	343	65-288	AF509565
pBSSE1409	T7	662	hypothetical protein	*Streptomyces pristinaespiralis* ATCC 25486	253-612	33	244	117-241	ZP_03191407
pBSSE1410	T3	899	putative polyketide cyclase (RubE)	*Streptomyces collinus*	115-2	60	143	1-38	AAG03066
			putative polyketide cyclase (RubD)	*Streptomyces collinus*	545-222	69	123	1-108	AAG03065
pBSSE1410	T7	890	flavin reductase domain-containing protein	*Frankia* sp. EAN1pec	541-101	46	170	6-150	YP_001510489
pBSSE1411	T3	1067	putative 3-oxoacyl-ACP reductase (GrhO10)	*Streptomyces* sp. JP95	504-4	45	249	1-167	AF509565
pBSSE1411	T7	948	SARP-family regulatory	*Streptomyces*	104-499	41	280	119-250	YP_001827897

			protein	*griseus*					
pBSSE1412	T3	-	-	-	-	-	-	-	-
pBSSE1412	T7	993	apramycin resistance gene	Cloning vector pSET152	856-98	99	258	6-258	CAC93947
pBSSE1413	T3	-	-	-	-	-	-	-	-
pBSSE1413	T7	965	putative monooxygenase, momA-like	*Streptomyces cinnamonensis*	385-696	30	206	30-152	CAL34085
pBSSE1414	T3	-	-	-	-	-	-	-	-
pBSSE1414	T7	962	LacZ alpha peptide	Cloning vector pYESW29	596-1066	100	191	28-184	ABI35978

The clones utilised for subcloning are located at the library coordinates 14B5 (cosmid 11) and 18C7 (cosmid 14).

4.2.8.3 Appraisal of subcloning results

The obtained sequence data (table 4.7) were analysed to identify open reading frames (5.5.12.5). The predictions of these protein coding regions are listed in table 4.8. The putative organisation of the identified open reading frames (ORFs) within the genome of *Streptomyces* sp. GW2/5831 in the region of the IB-00208 biosynthetic gene cluster is presented in figure 4.34. It was not possible to arrange all sequence information from table 4.7 inside the cluster scheme. In cosmid 11 the content of sequenced DNA comprises 13.6 kb, covering one third of the insert. Subcloning of cosmid 14 yielded 19.5 kb of sequenced DNA that corresponds to one half of the insert size. Sequencing of 33.1 kb of genomic DNA from *Streptomyces* sp. GW2/5831 revealed 20 open reading frames encoding PKS-, -post PKS tailoring, -regulatory, -resistance and transport proteins as well as proteins with unassigned functions.

Table 4.8: Proposed functions of ORFs putatively involved in the biosynthesis of IB-00208

ORF	Amino Acids	Proposed Protein Function	Sequence Similarity (Protein, Origin)	Identity	Protein Accession Number
ORF01	422	KS_β	RubB, *Streptomyces collinus*	67%	AAG03068
ORF02	428	KS_α	RubA, *Streptomyces collinus*	79%	AAG03067
ORF03	143	rubromycin biosynthesis-related cyclase I	RubE, *Streptomyces collinus*	69%	AAG03065
ORF04	143	rubromycin biosynthesis-related cyclase II	RubD, *Streptomyces collinus*	60%	AAQ08915
ORF05	170	Flavin reductase domain-containing protein	Franean1_6241, *Frankia* sp. EAN1pec	46%	YP001510489
ORF06	311	Hypothetical protein from angucycline type II cluster	Pd2U, *Streptomyces* sp. WP 4669	50%	AAO65359
ORF07	312	short-chain dehydrogenase/reductase (SDR)	Franean1_6815, *Frankia* sp. EAN1pec	66%	YP001511055
ORF08	246	short-chain dehydrogenase/reductase (SDR)	FabG, *Frankia* sp. EAN1pec	40%	YP001507428
ORF09	566	FAD-dependent oxidoreductase	Bcep18194 _C6708, *Burkholderia* sp. 383	40%	YP366401
ORF10	345	Unknown	Uncharacterised protein, *Streptomyces fradiae*	36%	P20187
ORF11	115	Antibiotic biosynthesis monooxygenase	BenJ, *Streptomyces* sp. A2991200	38%	CAM58802.1
ORF12	249	3-oxoacyl-ACP reductase	GrhO10, *Streptomyces* sp. JP95	45%	AAM33668
ORF13	280	Regulatory protein	SARP family pathway specific regulatory	41%	YP001827897

			protein, *Streptomyces griseus*		
ORF14	246	Monooxygenase	FdmL, *Streptomyces griseus*	46%	AAQ08922
ORF15	353	*O*-methyltransferase	FdmN, *Streptomyces griseus*	52%	AAQ08925
ORF16	293	Dehydrogenase	LlpS, *Streptomyces tendae*	47%	CAM34368.1
ORF17	245	3-oxoacyl-ACP reductase	LlpZIII, *Streptomyces tendae*	58%	CAM34370.1
ORF18	323	Resistance protein	daunorubicin resistance ABC-transporter ATPase subunit, *Alkaliphilus metalliredigens*	57%	YP_001321687
ORF19	215	Transcriptional regulator	PadR, *Streptomyces avermitilis* MA-4680	43%	BAC74421
ORF20	206	Monooxygenase	MomA, *Streptomyces cinnamonensis*	30%	CAL34085

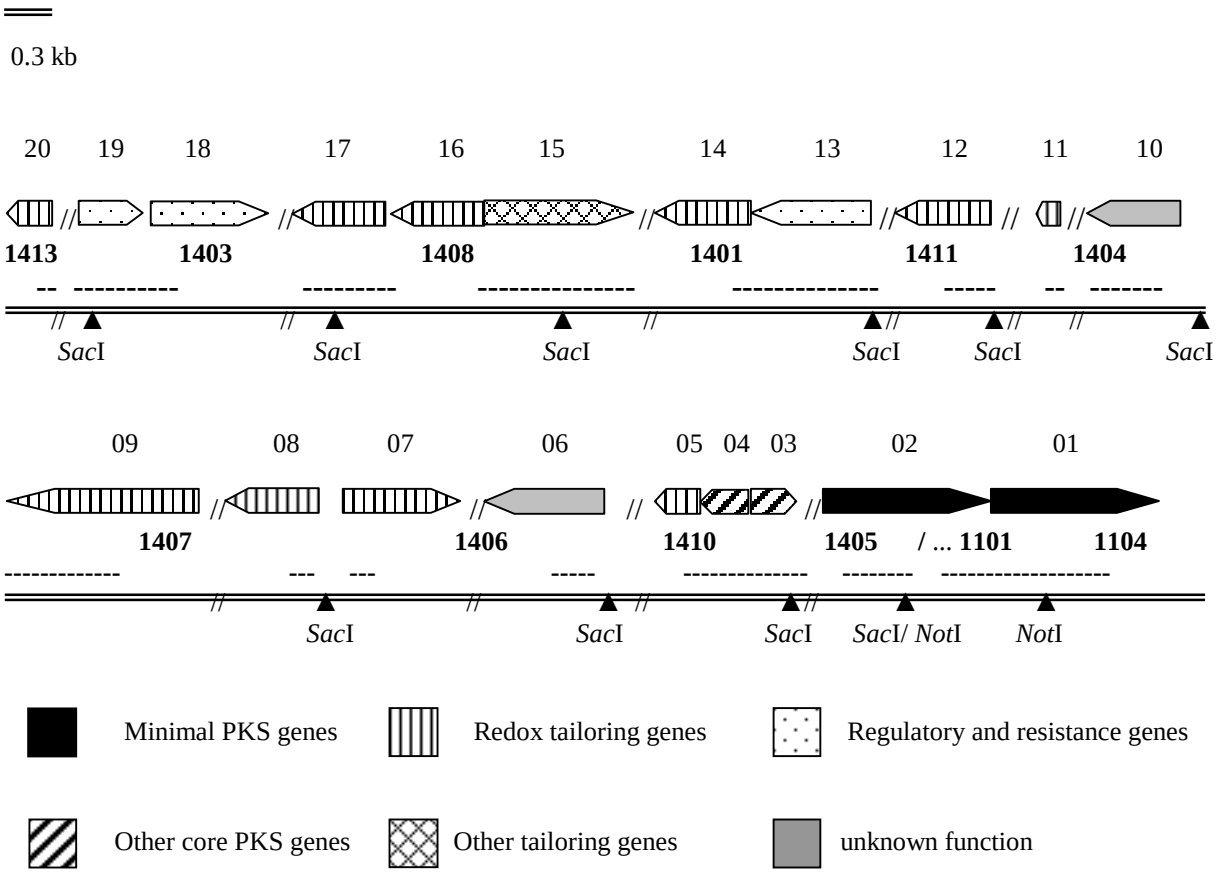


Figure 4.34: Genetic organisation of ORFs in the putative IB-00208 gene cluster deduced from the sequences of the cosmids 11 and 14. Each arrow represents the direction of transcription of an ORF (1-20), proposed functions are summarized in table 4.8. Restriction sites (*Not*I/ *Sac*I) of the cosmid DNA are indicated by black triangles (▲), homologous regions are labelled by a black dashed line (----), gaps are marked with (//), fragment numbers are shown in bold, and the border between cosmids 11 and 14 is indicated by (/...).

Polyketide core genes

The backbone of the polyketide structure is synthesised due to catalytic activity of a minimal PKS (KS_α, KS_β, ACP). Genes belonging to the minimal PKS of the putative IB-00208 gene cluster are encountered within the sequenced fragments of the subclones. Parts of the KS_α subunit are encoded on the fragments "pBSSE1405 T7" (6-156 aa) and "pBSSE1101 T3" (224-417 aa) that together represent ORF02. The query sequence of ORF02 shows a high identity (79%) to KS_α subunits of other type II PKS systems from pentangular[73] polyketides like rubromycin from *Streptomyces collinus* (RubA). The fragment "pBSSE1101 T3" contains an acyltransferase GHSxG motif around Ser350 that is typical for a KS_α. Furthermore, "pBSSE1104 T7" and "pBSSE1110 T3" cover homologies to polyketide synthases and related proteins. The sequence on "pBSSE1104 T7", designated as ORF01, encodes a part of the KS_β subunit similar to RubB (88-302 aa) in the rubromycin biosynthetic gene cluster. The sequence on "pBSSE1110 T3" is similar to polyketide modules and related proteins. Therefore it may harbour the so far undetected ACP component of the minimal PKS. The KS_β-encoding fragment "pBSSE1104" exhibits a size of approximately 5 kb, whereas the fragment "pBSSE1101 T3", that is connected to "pBSSE1104" in the upstream direction, comprises approximately 14 kb. The resulting distance between the KS subunits and ACP would suggest a disconnected minimal PKS like described before for the griseorhodin and daunorubicin clusters (see regulatory and resistance genes). On the other hand it could also be the case that the ACP component follows downstream the KS_β on the unsequenced region of "pBSSE1104 T7". The formation of a polyketide ring system is referred to catalytic activity of at least two cyclases.[83] Two cyclases were identified on the subcloned fragments "pBSSE1410 T3" and "pBSSE1405 T7" holding sequence homologies to genes involved in the biosynthesis of pentangular polyketides. The sequence encoded on "pBSSE1410 T3" shows a high identity to the RubD (60%) and RubE (69%) putative polyketide cyclases (ORF04, ORF03) from the rubromycin cluster. A part of ORF3 (RubE, 45-140) is located on "pBSSE1405 T7".

PKS tailoring genes

The tailoring genes that are detected inside the analysed sequences encode proteins involved in electron transfer reactions, except for one that putatively directs a methyltransferase reaction. As IB-00208 contains many oxygenated residues, enzymes that catalyse redox processes are expected. Parts of the fragments "pBSSE1411 T3" (1-167 aa) and "pBSSE1404 T3" (183-249 aa) contain a sequence homologous to GrhO10 that has the proposed function

of a 3-oxoacyl-ACP reductase (ORF12). Interestingly, GrhO10 is described to code for an unusual type of ketoreductase of the SDR family that is found in gene clusters of pentangular polyketide pathways.[73] The ORF17 that is spread between "pBSSE1402 T7" (38-172 aa) and "pBSSE1408 T7" (173-245 aa) exhibits similarity to the lysolipin LlpZIII 3-oxoacyl-ACP reductase from *Streptomyces tendae*. Another similarity to the lysolipin cluster is detected in ORF16 that is functionally related to the dehydrogenase LlpS on the protein level. The sequences on "pBSSE1406 T3" and "pBSSE1407 T7" produce alignments to proteins of the family of short-chain dehydrogenases/ reductases (SDRs) and are not further characterised (ORF07, ORF08). As monooxygenases transfer oxygen atoms to a substrate they are expected in the biosynthetic pathway of IB-00208. The ORF14 encodes a putative monooxygenase from *Streptomyces griseus* known as the producer of fredericamycin. Two other monooxygenases from *Streptomyces* strains are detected in ORF11 ("pBSSE1404 T3") and ORF20 ("pBSSE1413"). ORF11 is similar to the BenJ antibiotic biosynthesis monooxygenase from the benastatin cluster, whereas ORF20 represents a MomA-like monooxygenase that is not further characterised. These oxygenases may play roles in common post-PKS tailoring steps.[121] The transfer of methyl groups from the ubiquitous *S*-adenosyl-L-methionine (SAM) to nitrogen, oxygen or carbon atoms is frequently employed in bacteria, and is well known in Streptomycetes. As IB-00208 contains five methoxy groups, the presence of *O*-methyltransferases was expected. The sequences "pBSSE1401 T7" (157-329 aa) and "pBSSE1408 T3" (21-147 aa) feature homology to the FdmN *O*-methyltransferase (ORF15) from the fredericamycin cluster.

Regulatory and resistance genes

The sequence of "pBSSE1403 T7" is similar to a PadR transcriptional regulator (ORF19). The sequence encoded in ORF13 belongs to the *Streptomyces* antibiotic regulatory protein (SARP) family.[127] Proteins of this family bind to promoter regions to activate the transcription of downstream genes. They are part of many PKS systems, and they often contain a TTA codon involved in regulation. A TTA codon is present in the sequence of "pBSSE1411 T7" (ORF13). The gene encoded in ORF18 belongs to the branched-chain amino acid transport protein family within the large super family of ATP-binding cassette (ABC) transporter proteins.[112] They are known to be responsible for the translocation of a variety of compounds across biological membranes. ABC transporters are involved in the extrusion of noxious substances, ranging from small ions to macromolecules. They therefore may be responsible for antibiotic resistance (drug transporter) or fulfil functions in primary metabolism. The

protein function of the sequence encoded on "pBSSE1403 T7" (ORF18) is annotated as a daunorubicin resistance ABC transporter ATPase component in the database. Many ABC transporters are detected on cosmid 11 that might contain mainly DNA beyond the cluster.

Proteins with unknown functions

Proteins with unknown functions (ORF06, ORF10) are similar to uncharacterised proteins from Streptomycetes.

Outlook and prospects for future research

The sequencing results of the cosmid DNA fragments from two of the positive clones from the library strongly suggest an involvement of the corresponding gene products in the biosynthesis of IB-00208. Although the presence of polyketide core genes, tailoring genes, regulation and resistance genes provides an indication of finding the demanded cluster, one must keep in mind that further experiments, like gene inactivation, are still necessary for proof of the actual function of the isolated DNA fragments.

As no genes coding for sugar biosynthesis or sugar attachment were identified so far, the remaining DNA regions of the subclones should be analysed. Furthermore, screening of the cosmids should be attempted by chromosome walking.

Another approach for generating a glycosylated compound could be the usage of biosynthetic gene cassettes for the heterologous production of several deoxyhexoses.[128] Their transfer to polyketide aglycones could then be accomplished by utilising glycosylation engineering to replace the natural substituents.[129-131]

A new candidate, IB-00208, is here described that belongs to the rare and barely investigated class of pentangular polyketides. IB-00208 carries an unusually high number of methyl groups. This class of pentangular polyketides includes compounds that exhibit unusual spiroketal moieties resulting from the cleavage of C-C bonds, like, for example, the heavily oxidised griseorhodin A.[76] Members of this class share the pathway precursor collinone. Therefore it can be envisioned that either griseorhodin A can be glycosylated by a GT from the IB-00208 cluster or it can be considered possible that the griseorhodin tailoring genes[76] can be used to convert IB-00208 into a hybrid polyketide carrying the spiroketal pharmacophore.

In conclusion, the findings on the genetic level in this work so far correlate very well with the chemical structure of the molecule, and represent a basis for further investigations and modifications of the IB-00208 biosynthetic gene cluster from *Streptomyces* sp. GW2/5831. These insights contribute to polyketide research, and can be applied to tailored biosynthesis.

5 Materials and methods (experimental procedures)

5.1 Chemicals, laboratory equipment and other materials

5.1.1 Chemicals

Table 5.1 Chemicals utilised in experiments of the present work

Chemical	Source
Acetonitrile	SDS GmbH, 76437 Rastatt
Acetylpyridine adenine dinucleotide (APAD)	Sigma-Aldrich, 82024 Taufkirchen
Acrylamide	Carl Roth GmbH, 76185 Karlsruhe
Agar	Merck-Eurolab, 64295 Darmstadt
Agarose, NEEO ultra	Carl Roth GmbH, 76185 Karlsruhe
Agarose, low melt	Carl Roth GmbH, 76185 Karlsruhe
L-Amino acid oxidase	Sigma-Aldrich, 82024 Taufkirchen
Ammonia, liquid	Merck-Eurolab, 64295 Darmstadt
Ammonium chloride	Merck-Eurolab, 64295 Darmstadt
Ammonium molybdate heptahydrate	Merck-Eurolab, 64295 Darmstadt
Ammonium peroxodisulfate (APS)	Merck-Eurolab, 64295 Darmstadt
Ammonium sulfate	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Ampicillin	Merck-Eurolab, 64295 Darmstadt
Apramycin	Sigma-Aldrich, 82024 Taufkirchen
Asparagine	Merck-Eurolab, 64295 Darmstadt
Aspartic acid	Merck-Eurolab, 64295 Darmstadt
Boric acid	Merck-Eurolab, 64295 Darmstadt
Bradford Reagent	Sigma-Aldrich, 82024 Taufkirchen
Bromophenol Blue	Sigma-Aldrich, 82024 Taufkirchen
Calcium chloride dihydrate	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Calcium carbonate	Merck-Eurolab, 64295 Darmstadt
Casaminoacids	Life Technologies (Gibco BRL), 76131 Karlsruhe
Catalase	Sigma-Aldrich, 82024 Taufkirchen
Chloramphenicol	Sigma-Aldrich, 82024 Taufkirchen
Chloroform	SDS GmbH, 76437 Rastatt
Coomassie® Brilliant Blue G250	Sigma-Aldrich, 82024 Taufkirchen
Copper(II) chloride dihydrate	Sigma-Aldrich, 82024 Taufkirchen
Deoxynucleoside triphosphates (dNTP's) 10 mM set of dATP, dCTP, dGTP, dTTP	Invitrogen GmbH, 76131 Karlsruhe
Deuterium oxide	Deutero, 56288 Kastellaun
Diethylaminoethyl-Sepharose® FF (DEAE)	GE Healthcare Bio-Sciences AB Uppsala, Sweden
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH, 76185 Karlsruhe
Dithiothreitol (DTT)	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Ethanol	SDS GmbH, 76437 Rastatt
Ethidium bromide	Carl Roth GmbH, 76185 Karlsruhe
Ethidium bromide solution (1%)	Merck-Eurolab, 64295 Darmstadt
Ethyl acetate	SDS GmbH, 76437 Rastatt
Ethylenediamine-tetraacetic acid (EDTA)	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Glacial acetic acid (99%)	Merck-Eurolab, 64295 Darmstadt
Glucose	Merck-Eurolab, 64295 Darmstadt
Glutamic acid	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Glutamine	Sigma-Aldrich, 82024 Taufkirchen
Glycerol	Merck-Eurolab, 64295 Darmstadt
Glycine	Carl Roth GmbH, 76185 Karlsruhe

Hydrazine	Merck-Eurolab, 64295 Darmstadt
Instant Ocean®	Aquarium Systems, 57400 Sarrebourg, France
IPTG (Isopropyl-ß-D-thiogalactopyranoside)	Carl Roth GmbH, 76185 Karlsruhe
Iron(III) chloride hexahydrate	Merck-Eurolab, 64295 Darmstadt
Iron(II) sulfate heptahydrate	Sigma-Aldrich, 82024 Taufkirchen
Kanamycin	Sigma-Aldrich, 82024 Taufkirchen
Kanosamine	Prof. Floss, Seattle, WA 98195, USA
LMW-SDS Marker Kit	GE Healthcare Bio-Sciences, 80807 München
Lysozyme	Merck-Eurolab, 64295 Darmstadt
Magnesium chloride hexahydrate	Merck-Eurolab, 64295 Darmstadt
Magnesium sulfate heptahydrate	Merck-Eurolab, 64295 Darmstadt
Malt extract	Merck-Eurolab, 64295 Darmstadt
Manganese chloride tetrahydrate	Merck-Eurolab, 64295 Darmstadt
Mannitol	Sigma-Aldrich, 82024 Taufkirchen
Meat extract	Carl Roth GmbH, 76185 Karlsruhe
Mercaptoethanol	Serva, 69115 Heidelberg
Methanol	SDS GmbH, 76437 Rastatt
MOPS (3-morpholinopropanesulfonic acid)	Sigma-Aldrich, 82024 Taufkirchen
Nalidixic acid	Sigma-Aldrich, 82024 Taufkirchen
Nicotinamide adenine dinucleotide (ß- NAD^+)	Sigma-Aldrich, 82024 Taufkirchen
Ninhydrine	Merck-Eurolab, 64295 Darmstadt
Nitrogen	Praxair Industriegase, 56659 Burgbrohl
Nitrogen, liquid	Institut für Strahlen- und Kernphysik, 53115 Bonn
Orange G	Sigma-Aldrich, 82024 Taufkirchen
Orcinol	Sigma-Aldrich, 82024 Taufkirchen
Oxygen (99,5%)	Praxair Industriegase, 56659 Burgbrohl
Peptone	Carl Roth GmbH, 76185 Karlsruhe
Phenol-chloroform-isoamyl alcohol mixture (25:24:1)	Carl Roth GmbH, 76185 Karlsruhe
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich, 82024 Taufkirchen
Potassium acetate (KOAc)	Carl Roth GmbH, 76185 Karlsruhe
Potassium chloride	Carl Roth GmbH, 76185 Karlsruhe
Potassium dihydrogen phosphate	Merck-Eurolab, 64295 Darmstadt
di-Potassium hydrogen phosphate	Merck-Eurolab, 64295 Darmstadt
Potassium sulfate	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
L-Proline	Merck-Eurolab, 64295 Darmstadt
2-Propanol	Merck-Eurolab, 64295 Darmstadt
Pyridine	Merck-Eurolab, 64295 Darmstadt
Pyridoxal 5`-phosphat (PLP)	Sigma-Aldrich, 82024 Taufkirchen
Sodium acetate	Carl Roth GmbH, 76185 Karlsruhe
Sodium chloride	Merck-Eurolab, 64295 Darmstadt
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH, 76185 Karlsruhe
Sodium hydroxide	Merck-Eurolab, 64295 Darmstadt
Sodium hydroxide solution 50%	Sigma-Aldrich, 82024 Taufkirchen
Sodium tetraborate	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Soytone	Difco Laboratories, Detroit, MI 48288, USA
Starch from potato, soluble	Sigma-Aldrich, 82024 Taufkirchen
Sucrose	Merck-Eurolab, 64295 Darmstadt
TES-buffer (*N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid)	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Tetramethylethylenediamine (TEMED)	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Thiostrepton	Sigma-Aldrich, 82024 Taufkirchen
Trifluoroacetic acid (TFA)	Merck-Eurolab, 64295 Darmstadt
Tris-Base (Tris-[hydroxymethyl]-aminomethane)	Merck-Eurolab, 64295 Darmstadt
Tryptone	Carl Roth GmbH, 76185 Karlsruhe
UDP-galactose	Fluka/ Sigma-Aldrich, 82024 Taufkirchen

UDP-glucose	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
UDP-glucose epimerase	Calbiochem (Merck KgaA), 64293 Darmstadt
UDP-kanosamine	Prof. Floss, Seattle, WA 98195, USA
X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside)	Life Technologies (Gibco BRL), 76131 Karlsruhe
Xylene Cyanol FF	Sigma-Aldrich, 82024 Taufkirchen
Yeast Extract (Select®)	Life Technologies (Gibco BRL), 76131 Karlsruhe
Zinc(II) chloride	Fluka/ Sigma-Aldrich, 82024 Taufkirchen
Zinc(II) sulfate heptahydrate	Sigma-Aldrich, 82024 Taufkirchen

5.1.2 Molecular biological kits, enzymes and molecular weight markers

Table 5.2 Reagent sets used in the present work

Name	Manufacturer
Epicentre® CopyControl™ Fosmid Library Production Kit CCFOS110	Epicentre Biotechnologies, Madison, WI 53713, USA
First Strand cDNA Synthesis Kit	Fermentas GmbH, 68789 St. Leon-Roth
QIAquick Gel Extraction Kit	Qiagen GmbH, 40724 Hilden
RNeasy® Mini Kit	Qiagen GmbH, 40724 Hilden

Table 5.3 Enzymes employed in molecular biological experiments

Enzyme	Manufacturer
Antarctic phosphatase	New England Biolabs, 65926 Frankfurt
Bovine serum albumin (BSA)	New England Biolabs, 65926 Frankfurt
DNase (RNase-free)	Promega, 68199 Mannheim
GoTaq® DNA Polymerase (5u/µl)	Promega, 68199 Mannheim
RNAse A	Invitrogen GmbH, 76131 Karlsruhe
T4 DNA Ligase	New England Biolabs, 65926 Frankfurt
Restriction endonucleases	
*Bam*HI	New England Biolabs, 65926 Frankfurt
*Eco*RV	New England Biolabs, 65926 Frankfurt
*Not*I	New England Biolabs, 65926 Frankfurt
*Rsa*I	Jena Bioscience GmbH, 07749 Jena
*Sac*I	New England Biolabs, 65926 Frankfurt
*Sca*I	Jena Bioscience GmbH, 07749 Jena

Enzymes included in molecular biological kits (5.1.2) are not listed here.

Table 5.4 Molecular weight markers for gel electrophoresis

Name	Source
1 Kb DNA Ladder	Invitrogen GmbH, 76131 Karlsruhe
1 Kb DNA Extension Ladder	Invitrogen GmbH, 76131 Karlsruhe
GeneRuler™ DNA Ladder Mix	Fermentas, 68789 St-Leon-Rot

5.1.3 Laboratory equipment and other materials

Table 5.5 Laboratory equipment

Facility	Manufacturer
Analytical balances	Mettler, 35353 Gießen; Sartorius, 37075 Göttingen
Analytical column Carbopac™ PA-10	Dionex, Sunnyvale, CA 94085, USA
Centrifuges 5804R, 5417R	Eppendorf, 22339 Hamburg
Centrifuge Rotina 35R	Hettich, 78532 Tuttlingen
Clean bench Laminar Airflow BSB 4A	Gelaire Flow Laboratories, 53340 Meckenheim
Clean bench Kojair Biowizard Class II	Kojair, 35700 Vilppula, Finland
Digital camera DC 90	Kodak, 70327 Stuttgart
FPLC fraction collector Frac-900	GE Healthcare Bio-Sciences AB Uppsala, Sweden
FPLC pump Äkta™ P-920	GE Healthcare Bio-Sciences AB Uppsala, Sweden
FPLC UV monitor UPC-900	GE Healthcare Bio-Sciences AB Uppsala, Sweden
Gel documentation Syngene gene genius Bio imaging system 3088S Imager	Frederick, MD 21704, USA
Gel electrophoresis systems	Biometra, 37079 Göttingen; Bio-Rad, Hercules, CA 94547, USA
HPLC detector (ED-40)	Dionex, Sunnyvale, CA 94085, USA
HPLC pump (GP-40)	Dionex, Sunnyvale, CA 94085, USA
Microbiological incubators	WTB Binder GmbH, 78532 Tuttlingen; Heraeus 63450 Hanau
Microbiological incubator shaker Certomat BS-1	Sartorius, 37075 Göttingen
Microbiological incubator shaker HT Infors	Infors AG, CH-4103 Bottmingen, Schweiz
MicroPulser™ Electroporator	Bio-Rad, Hercules, CA 94547, USA
Milli-Q® Water System	Millipore, 65760 Eschborn
Mixer uzusio VTX-3000L	LMS Laboratory and Medical Supplies, 78086 Brigachtal
NMR Bruker Avance 300 DPX	Bruker, 76287 Rheinstetten
NMR Bruker DPX 500	Bruker, 76287 Rheinstetten
PCR thermo cycler T3	Biometra, 37079 Göttingen
PCR thermo cycler TGradient	Biometra, 37079 Göttingen
PH meter 766 Calimatic	Knick GmbH, 14163 Berlin
Polyacrylamide gel electrophoresis Maxigel system	Biometra, 37079 Göttingen
Spectral photometer Biomate3	Thermo Electron/ Thermo Fisher Scientific, Waltham, MA 02454, USA
Spectral photometer Uvikon 860	Kontron, 85386 Eching- München
Steam autoclave V65	Systec GmbH, 35435 Wettenberg
Steam autoclave Varioklav	H+P, 85764 Oberschleißheim
Thermomix comfort	Eppendorf, 22339 Hamburg
Ultra-low temperature freezer VIP series –86 °C	Sanyo, Bensenville, IL 60106, USA
Ultrasonic disintegrator Sonifier model 250	Branson, Danbury, Connecticut, USA
Ultraviolet crosslinker CL-1000 UVP	UVP Inc., Upland, CA 91786, USA
Vacuum concentrator Speed Vac Plus SC110A	Thermo Electron/ Savant Waltham, MA 02454, USA
Vacuum concentrator 5301	Eppendorf, 22339 Hamburg
Water bath GFL 1083	GmbH für Labortechnik, 30938 Burgwedel

Table 5.6 Expendable items and other materials

Material	Vendor
Amicon Ultra 15 centrifugal filter units	Millipore, Bedford, MA 01730, USA
Amprep Octa decyl mini columns	GE Healthcare Bio-Sciences AB Uppsala, Sweden
Falcon™ tubes for centrifugation	BD Biosciences, Fa. Faust, 53340 Meckenheim
Membrane filters 0,2 µm	Schleicher und Schüll, 37586 Dassel
Nalgene® cryogenic vials	via Fa. Faust, 53340 Meckenheim
Parafilm®	(American National Can) via Fa. Brand 46562 Voerde-Friedrichsfeld
Petri dishes	Greiner Bio-One GmbH, 45326 Essen
Pipet tips	Sarstedt, 51582 Nümbrecht
Reaction vessels (0,2 ml, 0,5 ml, 1,5 ml, 2,0 ml)	Fa. Eppendorf, 22339 Hamburg
Sephadex™ G-25 solid phase extraction columns (PD-10)	GE Healthcare Bio-Sciences AB Uppsala, Sweden
TLC silica gel (Merck 60 F^{254})	Merck, 64295 Darmstadt
UV cuvettes	Ratiolab GmbH, 63303 Dreieich-Buchschlag

5.1.4 Sterilisation of solutions and equipment

Sterilisation of media, buffers and glassware was carried out using a steam autoclave (normally at 121 °C, 20 min). Temperature-sensitive solutions were sterilised with Millipore® filters exhibiting a pore size of 0.22 µm.

5.1.5 Preparation of Millipore® water

Deionised water obtained from a Milli-Q® Water System was used for all applications. For molecular biology techniques this water was additionally autoclaved.

5.1.6 Clean room conditions

Tasks that required clean room conditions were conducted under a class II laminar airflow cabinet.

5.2 Biological material, plasmids and oligonucleotides

Table 5.7 Bacterial strains used in biosynthetic studies

Name	Genotype	Function	Source
Streptomyces lividans TK24	str-6, $SLP2^-$, $SLP3^-$	Host strain for heterologous expression or negative control	HKI Jena
Streptomyces lividans TK23	spc-1, $SLP2^-$, $SLP3^-$	Host strain for heterologous expression or negative control	HKI Jena

Table 5.8 Recombinant strains for biosynthetic studies

Host strain	Inserted plasmid
Streptomyces lividans TK24	pHGF7604
Streptomyces lividans TK23	pHGF7604
Streptomyces lividans TK23	pHGF/pNEB *rifK, L, M*
Streptomyces lividans TK23	pHGF/pNEB *rifK*, L, M*
Streptomyces lividans TK23	pHGF/pNEB *rifL, M*
Streptomyces lividans TK23	pHGF empty plasmid (1,48 kb), without *rif*-genes, as a negative control

Table 5.9 Bacterial strains used in PKS project

Name	Genotype	Function	Reference or source
Streptomyces sp. GW2/5831	-	Donor strain for gene library	Prof. Laatsch, Göttingen
Streptomyces albus J1074	-	Host strain for heterologous expression after conjugal transfer	Waksman and Henrici (1943)[132]
E. coli EPI300® T1^R	F $^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80d*lac*ZΔM15Δ *lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *galU galK* λ$^-$ *rpsL nupG trfA tonA dhfr*	Host strain for DNA fragments of gene library	Epicentre, Madison, WI 53713, USA
E. coli ET12567/pUZ8002	*dam*$^-$, *dcm*$^-$, *hsdS*$^-$, *cat, tet*, Cm^r, Kan^r	Non-methylating plasmid donor strain for intergeneric conjugation	Flett et al. (1997)[133] MacNeil et al. (1992)[124]
E. coli XL1 blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacl$_q$ZΔM15* Tn*10* (Tet$_r$)]	Host strain for subcloning of DNA fragments	Stratagene, La Jolla, CA 92037, USA

Table 5.10 Plasmids applied in this work

Vector	Biological property	Size	Reference or source
	Plasmids used in the kanosamine project		
pHGF7604 *rifG-N*	Shuttle vector, ColE1-origin, Amp^r (selection in *E. coli*); SCP2*-origin, Thio^r (selection in *Streptomyces*), genes *rifG-N* under actII-orf4 promoter	22.3 kb	Yu et al. (2001)[66]
pHGF/pNEB *rifK, L, M*	Shuttle vector, ColE1-origin, Amp^r (selection in *E. coli*); SCP2*-origin, Thio^r (selection in *Streptomyces*), genes *rifK,L,M* under actII-orf4 promoter	19.2 kb	Kaulard (2006)[85]
pHGF/pNEB *rifL, M*	Shuttle vector, ColE1-origin, Amp^r (selection in *E. coli*); SCP2*-origin, Thio^r (selection in	17.8 kb	Kaulard (2006)[85]

	Streptomyces), genes *rifL,M* under actII-orf4 promoter		
pHGF/pNEB *rifK*, L, M*	Shuttle vector, ColE1-origin, Amp^r (selection in *E. coli*); $SCP2^*$-origin, $Thio^r$ (selection in *Streptomyces*), genes *rifL,M* under actII-orf4 promoter, *rifK** point mutated	19.2 kb	Kaulard (2006)[85]
pHGF empty plasmid (1,48 kb)	1480 bp fragment of promoter region of pNEB193C in pHGF7604 without *rif* gene cassette	16 kb	Kaulard (2006)[85]
Plasmids used for the characterisation of the IB-00208 gene cluster			
pAY1	shuttle vector, $Apra^r$, *oriT*	9.4 kb	Li and Piel (2002)[83]
pBluescript II SK(+)	cloning vector Amp^r, *lacZ*	3 kb	Stratagene, La Jolla, CA 92037, USA
ET12567/pUZ8002	helper plasmid, Cm^r/Kan^r		Flett et al. (1997)[133]

Table 5.11 PCR primer

Primer name	DNA- sequence (5`- 3`)	Melting point T (°C)	Number of base pairs	Reference
Primer pairs for amplification of oxidoreductase gene *rifL*				
Oxidofw (degenerate)	GCG GTG GTC GGG CTC GGS TGG G	80,0	22	Boettcher (2003)[72]
Oxidorv (degenerate)	GGT TSG GGC TGA AGC CGA AGG TGC	80,0	24	Boettcher (2003)[72]
RT-PCR-Oxido_for (homologous)	GCG GTC GTC GGG CTC GGC TGG G	73,3	22	Yu et al. (2001)[66] NCBI- accession-no. AAS07754
RT-PCR-Oxido_rev (homologous)	GGT TGG GGC TGA AGC CGA AGG TGC	69,6	24	Yu et al. (2001)[66] NCBI- accession-no. AAS07754
Primer pairs for amplification of a PKS fragment in analysis of the cosmid gene library				
KS2F	YGS YGC SAR TGC GAC TTC G	51	19	Li and Piel (2002)[83] GenBank-accession no. AF509565
KS2R	CCG AYC ATS GAC TTG ATG GAG C	53	22	Li and Piel (2002)[83] GenBank-accession no. AF509565
KS4F	GAG GCS VTC GMS GAC AGC GG	57	20	Li and Piel (2002)[83] GenBank-accession no. AF509565

KS1R	AGT CSA GGT CRC AYT CSG GGT C	55	22	Li and Piel (2002)[83] GenBank-accession no. AF509565
BES1 T7 1F 2F (homologous)	TCG ACT TGA TGG AGC TGA CC	60.2	20	this work
BES1 T7 1F 2R (homologous)	GGG CTG GAG GAG GAG TAC AT	59.4	20	this work
Primer pair for spot-sequencing of the positive cosmid clones				
AY1ES1 Forward	AGC ACG CCA TAG TGA CTG GC	(51)	20	this work
AY1ES1 Reverse	TTT CAC ACA GGA AAC AGC TA	(43)	20	this work

The following letter code was used to indicate base variables in degenerate primer sequences: Y=C,T/ S=C,G/ R=A,G/ M=A,C/ V=G,A,C

Primers for *rifL* studies were supplied by MWG Biotech (Eggenstein) in a lyophilised form, and were redissolved in TE buffer yielding a concentration of 20 µM/L.

Primers for PKS studies were supplied by Invitrogen™ (Karlsruhe) in a lyophilised form, and were redissolved in TE buffer yielding a concentration of 50 µM/L.

5.3 Buffers and solutions

Table 5.12 Buffers for enzyme purification

Buffer	**Composition**	**Ionic strength/ concentration (m/v)**	**pH- value**
Lysis buffer *(Gomori, 1955)*	Potassium phosphate buffer	0,1 M	pH 7,5
FPLC- buffer A	Tris- HCl	25 mM	pH 7,4
	EDTA	1 mM	
	DTT	2 mM	
	in Glycerol	10%	
FPLC- buffer B	Tris- HCl	25 mM	pH 7,4
	EDTA	1 mM	
	DTT	2 mM	
	NaCl	1 M	
	in Glycerol	10%	

Table 5.13 Buffers and solutions for SDS-PAGE and staining of PA matrix

Name	Composition		Comment
denaturing loading buffer (5x)	Glycerol 87 % Mercaptoethanol SDS Bromophenol blue 1% (m/ v) Tris-HCl (1,9 M; pH 6,8)	5,75 g 2,5 ml 9,0 g 0,1 ml 1,65 ml	Ingredients were mixed and heated until completely dissolved, stored at 4-8 °C, warmed up to room temperature before use
APS-solution 20 %	Ammonium peroxodisulfate H_2O	2,0 g ad 10 ml	Store at –20 °C
Stacking gel buffer	SDS Tris-HCl	0,4 % (m/v) 0,5 M	pH 6,8
Resolving gel buffer	SDS Tris-HCl	0,4 % (m/v) 1,5 M	pH 8,8
Electrophoresis buffer (5x)	Glycine Tris-HCl SDS	0,96 M 0,125 M 0,5 % (m/ v)	pH 8,5
Coomassie staining solution	Coomassie Brilliant blue G250 Glacial acetic acid (100%) Methanol	1 g/ l 75 ml/ l 200 ml/ l	
Colour stripping solution	Glacial acetic acid (100%) Methanol	75 ml/ l 200 ml/ l	
Stacking gel matrix 4 %	Stacking gel buffer Acrylamide solution 40% H_2O APS (20%) TEMED	2,5 ml 1,0 ml 6,4 ml 75 µl 15 µl	Components 1-3 were mixed and degassed, after adding of APS and TEMED directly poured into glass chamber
Resolving gel matrix 13,5 %	Resolving gel buffer Acrylamide solution 40% H_2O APS (20%) TEMED	10 ml 13,4 ml 16,4 ml 200 µl 25 µl	Prepared as above, covered with a layer of butanol, polymerised over night
Low molecular weight marker	Phosphorylase B (97,4 kDa) BSA (66,2 kDa) Ovalbumin (42,7 kDa) Carbohydrase (31 kDa) Soy bean trypsin inhibitor (21,5 kDa) Lysozyme (14,4 kDa)		

Table 5.14 Buffers for MALDI-TOF-MS sample preparation

Name	Composition
Buffer A	TFA 0.1%
Buffer B	Acetonitrile 90%, TFA 0.085%

Table 5.15 Buffers for DNA, RNA and plasmid DNA isolation

Name	Composition
Buffer P1	50 mM Tris-HCl (pH 8) 10 mM EDTA 100 µg/ml RNAse A (heated at 99 °C for 1 min prior to addition to buffer to remove DNAse activity)
Buffer P2	200 mM NaOH 1% SDS
Buffer P3	3 M KOAc (pH 5.5)
STE buffer	300 mM Sucrose 25 mM Tris-HCl (pH 8) 25 mM EDTA (pH 8) Lysozyme 5 mg/ml
TE buffer	10 mM Tris-HCl (pH 8) 1 mM EDTA (pH 8)

Table 5.16 Buffers for native agarose gel electrophoresis

Name	Composition		Preparation
Loading buffer (6x)	Bromophenol Blue	0.05%	
	Xylene cyanol	0.05%	
	Orange G	0.05%	
	Glycerol	30%	
TAE buffer (50x), pH 8.5	Tris-HCl	242 g	
	Glacial acetic acid	57.1 ml	
	EDTA (0.5 M)	100 ml	
TBE buffer (10x), pH 8.0	Tris	0.90 M	pH adjustment with HCl
	Boric acid	0.90 M	
	EDTA (pH 8)	0.02 M	

Table 5.17 Solutions for preparation of competent cells by chemical method

Name	Composition	
TFB I	Potassium acetate	30 mM
	Calcium chloride	10 mM
	Potassium chloride	100 mM
	Manganese chloride	50 mM
	Glycerol	15%
TFB II	MOPS (Sigma)	10 mM
	Calcium chloride	75 mM
	Potassium chloride	10 mM
	Glycerol	15%

5.4 Microbiological methods

5.4.1 Culture media and antibiotics

Table 5.18 Media for cultivation and preservation of bacteria

Medium	Composition		Annotation/remark/comment
R5 medium (Thompson et al., 1980) modified	K_2SO_4	0,25 g	
	$MgCl_2$ x 6 H_2O	10,12 g	
	Glucose	10,00 g	
	Casaminoacids	0,10 g	
	Trace element solution	2 ml	
	Yeast extract	5,00 g	
	TES buffer	5,73 g	
	KH_2PO_4 (0,5%)	10,0 ml	
	$CaCl_2$ x 2 H_2O (5 M)[1]	4,0 ml	
	L-Proline (20%)[2]	15,0 ml	
	NaOH (1 M)[3]	7,0 ml	[1, 2, 3)] Added after autoclaving
Trace element solution	$ZnCl_2$	40 mg	
	$FeCl_3$ x 6 H_2O	200 mg	
	$CuCl_2$ x 2 H_2O	10 mg	
	$MnCl_2$ x 4 H_2O	10 mg	
	NaB_4O_7 x 10 H_2O	10 mg	
	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	10 mg	
M2 medium (=YMG)	Yeast extract	4,0 g	pH 7.8
	Malt extract	10,0 g	
	Glucose	4,0 g	
	Agar	16,0 g	
Stock culture medium for *Streptomyces*	Glycerol	15% (m/v)	
Stock culture medium for *E. coli*	Glycerol	30% (m/v)	
Meat extract medium	Glucose	10 g	pH 7.8
	Peptone from meat	2 g	
	Yeast extract	1 g	
	Meat extract	1 g	
	Instant Ocean®	7.5 g	
TSB medium	Tryptic Soy Broth	30 g	
MS medium (Hobbs et al., 1989)	Mannitol	20 g	autoclave twice at 115 °C for 15 min with gentle shaking between the two runs
	Soya flour	20 g	
2CM medium (Yuan, 1983)	NaCl	1 g	
	K_2HPO_4	1 g	
	Potato starch, water-soluble	10 g	
	Salt solution	1 ml	
	Mg_2SO_4 x 7 H_2O	2 g	
	Tryptone	2 g	
	$CaCO_3$	2 g	
	$(NH_4)_2SO_4$	2 g	
	Agar	22 g	

Salt solution	$FeSO_4$ x 7 H_2O	1 g	
	$MgCl_2$ x 6 H_2O	1 g	
	$ZnSO_4$ x 7 H_2O	1 g	
DSG medium	Yeast extract	10 g	
(Kieser et al., 2000)	Casaminoacids	10 g	
	$CaCl_2$ (5M)	2 ml	
LB medium	NaCl	10 g	pH 7
(Bertani, 1951)	Tryptone	10 g	
	Yeast extract	5 g	

Quantities are listed for the preparation of 1 liter solutions

Table 5.19 Antibiotics for the counterselection of recombinant strains

Antibiotic	Concentration Medium (µg/ ml)	Concentration Stock solution (mg/ ml)	solvent
Ampicillin	100	100	water
Apramycin	50	50	water
Chloramphenicol	25	25	ethanol
Kanamycin	25	25	water
Nalidixic acid	-	1	water
Thiostrepton	20	50	DMSO

5.4.2 Cultivation and long term storage of *Streptomyces lividans* strains

Streptomyces lividans strains were used for biosynthetic studies of RifL oxidoreductase.

5.4.2.1 Cultivation of *Streptomyces lividans* on solid medium

For plating on YMG agar (table 5.18), 100 µl of a stock culture (5.4.2.3) were used. The selection of recombinant *Streptomyces lividans* strains was accomplished by the addition of thiostrepton in a concentration of 20 µg/ml medium. The petri dish, containing approximately 30 ml of medium, was incubated at 28 °C for 7 days.

5.4.2.2 Cultivation of *Streptomyces lividans* in nutrient solution

Bacterial growth in liquid culture was initiated by a slice of agar (1 cm^2) obtained in 5.4.2.1. A sterile 500 ml baffled flask was filled with 50 ml of R5 medium containing 20 µg/ml thiostrepton when required for the recombinant strains. The baffles provided sufficient

aeration of the culture broth. The flasks were covered with aluminium foil. The cells were harvested for expression studies after shaking at 180 rpm and 28 °C for 5 days (5.4.7).

Alternatively 500 µl of a stock solution from a permanent culture (5.4.2.3) were used to inoculate liquid cultures of *Streptomyces lividans* strains.

5.4.2.3 Long term storage of *Streptomyces lividans* strains

A liquid culture (5.4.2.2) was incubated for three days. Sedimentation was carried out at 5000 rpm and 4 °C for 15 minutes. Subsequent washing with 20 ml of a 15% glycerol solution was followed by sedimentation at 5000 rpm and 4 °C for 15 minutes and resuspension in a 10 ml glycerol solution. Aliquots of 1 ml were transferred to Nalgene® cryo tubes and stored at –80 °C. All steps were carried out under sterile conditions.

5.4.3 Cultivation of *Streptomyces* sp. GW2/5831

Streptomyces sp. GW2/5831 and *Streptomyces albus* J1074 were employed in polyketide studies.

5.4.3.1 Cultivation of *Streptomyces* sp. GW2/5831 for the production of the red-coloured polycyclic xanthone IB-00208

The fermentation protocol was kindly provided by F. Lissy from the work group of Prof. Laatsch in Göttingen.

An agar slant of *Streptomyces* sp. GW2/5831 was used for the inoculation of solid M2 (=YMG) medium, whereas meat extract medium was employed for the production of the red-coloured compound in liquid broth. The cultures were incubated for 18 days at 28 °C, and 95 rpm respectively.

5.4.3.2 Cultivation of *Streptomyces* sp. GW2/5831 for genomic DNA isolation

The bacterial strain was grown in tryptic soy broth (TSB) at 28 °C and 95 rpm for 18 days. For the isolation of genomic DNA the cells were harvested by centrifugation at 5000 rpm

under sterile conditions. Approximately 100 mg of pelleted bacterial cells were used for genomic DNA isolation (5.5.1). The excess of cell material was stored at –20 °C for later use.

5.4.4 Preparation of a spore suspension from *Streptomyces albus* for conjugation

Streptomyces albus was inoculated on a MS agar plate and incubated at 30 °C for 7 days. The mould was then transferred to 2 ml of 20% glycerol using a sterilised Q-tip. The concentration of the spore suspension was estimated by means of a dilution series that was obtained by plating of different amounts of spores.

5.4.5 Cultivation and long term storage of recombinant *Streptomyces albus* strains

5.4.5.1 Cultivation of conjugated *Streptomyces albus* strains for the isolation of genomic DNA

Conjugated *Streptomyces albus* strains were inoculated in 20 ml LB medium including 500 µl of a 20% Glycine solution. The flasks were each equipped with a metal coil. After 20-24 hours of cultivation at 30 °C and 200 rpm, 1 ml of liquid culture was used for genomic DNA isolation (5.5.1). The remaining culture broth was harvested by centrifugation at 5000 rpm under sterile conditions, and was stored at –20 °C for later use.

5.4.5.2 Cultivation of conjugated *Streptomyces albus* strains for monitoring of potential secondary metabolite producing recombinants

For the observation of a potential colour change of the liquid medium or agar plate due to secondary metabolite production, conjugated strains were inoculated in and on different media. Liquid cultures were set up in meat extract and LB nutrient broth, whereas agar plates were established using 2CM, MS, M2 and LB medium.

5.4.5.3 Cultivation of conjugated *Streptomyces albus* strains for selection of incoming cosmid

Conjugated *Streptomyces albus* strains were spread on LB agar plates containing 50 µg/ml apramycin.

5.4.5.4 Preservation of conjugated *Streptomyces albus* strains

Conjugated *Streptomyces albus* strains were grown on MS agar plates for 7 days at 30 °C. A sterile Q-tip was used to transfer the mould to a tube that contained 15% glycerol solution. These stock cultures were stored at –80 °C.

5.4.6 Cultivation and long term storage of recombinant *E. coli* strains

Recombinant *E. coli* strains were inoculated in 3 ml cultures of LB medium containing the appropriate antibiotic in the required concentration. Inoculation was carried out using a sterilised tooth pick that remained in the culture broth. These cultures were grown for 16 hours at 37 °C, and were then utilised for the preparation of plasmid DNA (5.5.2). Stock cultures were produced by mixing 750 µl of overnight culture with the same amount of 30% glycerol solution. These stock cultures were stored at –80 °C.

5.4.7 Growth kinetics of *Streptomyces lividans*

Cultures for protein expression studies were harvested at the end of the exponential growth phase. To determine the specific time of the end of exponential growth phase, a set of ten liquid cultures (5.4.2.2), representing different growth phases, was inoculated during ten consecutive days. On the eleventh day all cultures were subjected to cell-free extract production (5.6.1) and spectrophotometric measurement (5.6.6). Cell free extract production was accompanied by weight determination of the cell pellet. Therefore, bacterial growth was simultaneously documented by quantity. Measurement of the optical density of a cell suspension is not suitable in this case, because *Streptomyces* tend to aggregate in liquid cultures.

5.5 Molecular biological methods

5.5.1 Isolation of genomic DNA from gram positive bacteria

A S*treptomyces* culture consisting of 20 ml LB medium including 500 µl of a 20% glycine solution was incubated with shaking at 200 rpm and 37 °C. After 24 hours (for *Streptomyces* sp. GW2/5831 see chapter 5.4.3.2) 1 ml was removed from the flask and centrifugation was carried out in a 2 ml Eppendorf tube at 14000 rpm for 30 seconds. The supernatant was discarded, and the pellet was resuspended in 500 µl of STE buffer containing 2.5 mg lysozyme. This mixture was incubated at 37 °C and 200 rpm for 3 hours. After the addition of 500 µl of 10% SDS solution and 100 µl of 3 M sodium acetate pH 5.3 and vigorous mixing, incubation was continued for 45 minutes. The samples were then extracted three times with a

mixture of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous layer above the protein interphase was carefully transferred to 600 µl isopropyl alcohol and was vortexed heavily. High molecular weight DNA was recovered after centrifugation at 10000 rpm for 2 minutes. The pellet was rinsed with 500 µl of 70% ice cold ethanol, dried at room temperature and dissolved in 30 µl TE buffer (pH 8). The DNA quality (size and concentration) was determined on an agarose gel (5.5.4.1).

5.5.2 Isolation of plasmid and cosmid DNA

The isolation of plasmid or cosmid DNA from recombinant *E. coli* cells was carried out according to the method described by Birnboim and Doly with slight modifications.[134] The modified alkaline lysis procedure of the cells was performed as follows:

A 1.5 ml of *E. coli* overnight culture (5.4.6) was centrifuged in a 1.5 ml Eppendorf tube at 10000 rpm for 30 seconds. The harvested cell pellet was resuspended in 200 µl of buffer P1 (table 5.15). For alkaline lysis 200 µl of buffer P2 were added, and the tube was inverted several times. After a maximum of 2 minutes 200 µl of buffer P3 were added to the lysate, after which the tube was inverted several times. The resulting precipitate was removed from the solution by centrifugation at 13000 rpm for 3 minutes. The supernatant was transferred to a 1.5 ml Eppendorf tube filled with 500 µl of chloroform. After vigorous mixing the sample was centrifuged at 13000 rpm for 5 minutes. A 300 µl of the aqueous upper layer were removed, omitting the protein interphase, and were then intensely mixed with 350 µl of isopropyl alcohol. After centrifugation at 13000 rpm for 20 minutes the supernatant was discarded. The DNA pellet was rinsed with 500 µl of ice cold 70% ethanol. The supernatant was removed and the pellet was vacuum-dried and dissolved in 30 µl of MilliQ water.

5.5.3 Purification, concentration and quantification of DNA

5.5.3.1 Purification of vector DNA via phenol-chloroform extraction

The DNA was extracted twice with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1, v/v/v), followed by a single chloroform extraction. Between each step, centrifugation was carried out for 1 minute at 14000 rpm and room temperature. The supernatant was subjected to precipitation (5.5.3.2 to 5.5.3.4).

5.5.3.2 Purification and concentration of vector DNA by means of isopropyl alcohol precipitation

The aqueous DNA solution was mixed with 1 volume of isopropyl alcohol and 1/10 volume of 3 M sodium acetate (pH 5) that provided the required monovalent cations. After storage at –20 °C for 30 minutes, centrifugation was performed at 14000 rpm and 4 °C for 45 minutes. The obtained pellet was rinsed two times with ice cold 70% ethanol, and sedimented at 14000 rpm and 4 °C for 15 minutes. The vacuum-dried pellet was resuspended in 5 µl of purified water.

5.5.3.3 Purification and concentration of insert DNA for library production by means of ethanol precipitation

The aqueous DNA solution was gently mixed with 1/10 volume of 3 M sodium acetate (pH 7), and 2.5 volume of room temperature-equilibrated 100% ethanol was added. After standing for 10 minutes, centrifugation was carried out for 40 minutes at 14000 rpm and room temperature. A small white pellet was visible, and approximately 95% of the supernatant was carefully removed. Approximately 5 ml of ice cold 70% ethanol were slowly added along the rim of the vessel. Sedimentation was performed at 4 °C and 11000 rpm for 20 minutes. The last three steps were repeated, and at the end most of the supernatant was removed, leaving 50 µl of remaining liquid. 50 µl of elution buffer (Qiagen) were added to immediately dissolve the pellet. Residual ethanol was evaporated under the clean bench.

5.5.3.4 Purification and concentration of blunt ended insert DNA for library production by means of isopropyl alcohol precipitation

The aqueous DNA solution resulting from blunt ending reaction (5.5.10.1) was gently mixed with 1.7 volume of isopropyl alcohol and 1/4 volume of 3 M sodium acetate (pH 5). After a 30 min incubation at room temperature, the sample was centrifuged at 4 °C and 14000 rpm for 45 minutes. 95% of the supernatant was removed, avoiding contact with the inner surface of the tube. Approximately 500 µl of ice cold 70% ethanol were slowly added, and sedimentation was carried out for 5 minutes at 14000 rpm. The supernatant was discarded, leaving approximately 7 µl in the tube. After the addition of 7 µl elution buffer (Qiagen) the pellet was resuspended while heating at 50 °C in a water bath.

5.5.3.5 Quantification and purity of nucleic acids

The concentration and purity of DNA and RNA samples was determined by means of a spectrophotometer. Assuming a seam of 1 cm in the cuvette, the absorption value of 1.0 at a wavelength of 260 nm corresponds to a concentration of 40 µg/ml for single-stranded DNA or RNA, and to 50 µg/ml for double-stranded DNA. As proteins exhibit an absorption maximum at 280 nm, the scale of purity was obtained by use of the $A_{260\ nm}$ to $A_{280\ nm}$ ratio. This quotient was valid up to an absorption value of 2.0, whereas high quality nucleic acid solution was reflected by a quotient between 1.7 and 2.0.[135]

The DNA concentration was calculated according to the following formula:

[c] = A_{260} x dilution factor x 50 [mg/ ml]

5.5.4 Agarose gel electrophoresis

5.5.4.1 Native agarose gel electrophoresis

Due to the negatively charged phosphate groups in DNA molecules, DNA fragments can be separated in an electric field. According to their size, smaller DNA fragments migrate longer distances in agarose gel matrices than larger DNA molecules.

Agarose gel electrophoresis was carried out to check for the presence of DNA, to separate DNA fragments of different sizes and to determine their molecular weight, yield and purity. This method was additionally used to isolate particular bands for downstream applications (5.5.5.2, 5.5.10.1) and to visualize PCR products. The gels consisted of 1% agarose NEEO ultra in either 0.5 x TBE or 1 x TAE buffer. The respective buffer served as an electrophoresis buffer as well, and thus covered the gel in horizontal gel chambers during electrophoresis. The current inducing the electric field ranged between 80 V and 120 V, depending on the required separation. A loading dye was added to each sample in a ratio of 1:10 to visualize DNA migration on the gel. This dye contained a mixture of Xylene Cyanol FF (migrates at ~ 5000 bp), Bromophenol Blue (migrates at ~ 300 bp) and Orange G (migrates at ~ 50 bp). Glycerol was included in this loading dye to weight the sample solution down to the gel slots. 2-5 µl of a commercial size marker were applied to at least one outer lane. Specific size markers allowed simultaneous DNA quantification where required (5.5.10.1). The DNA was visualised by the addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and when exposed to ultraviolet light, it will fluorescent with an orange colour. Ethidium bromide was either directly included in the gel matrix to enable visualisation

under UV light in the gel documentation system or used for staining of the gel after electrophoresis. Therefore it was agitated in ethidium bromide solution (10 µg/ml) for 3-5 minutes. Non intercalated ethidium bromide was subsequently washed out with purified water while shaking for 5 minutes. Analysis was performed using a UV transilluminator and a digital camera.

5.5.4.2 DNA extraction from agarose gels

If required for downstream applications, the designated DNA fragment was excised from the agarose matrix after the separation on an agarose gel omitting ethidium bromide (5.5.4.3). For DNA recovery the QIAquick Gel Extraction Kit (Qiagen) was used according to the manufacturer's instruction manual.

5.5.4.3 Gel electrophoresis without ethidium bromide

To avoid DNA damage by exposure to UV light, electrophoresis was also performed without including ethidium bromide in the agarose solution. The DNA was loaded in a larger well of the agarose gel, with a ladder on each side. After electrophoresis, both sides including the ladder were cut off with a sharp scalpel, and were then stained with ethidium bromide solution for 15 minutes with shaking. A scalpel was used to mark the positions were the band of interest should be situated and then all gel parts were again reassembled. Out of the UV range, the desired fragments were excised from the gel according to the markings and then subjected to DNA gel extraction (5.5.4.2).

5.5.5 DNA amplification by the polymerase chain reaction (PCR)

The polymerase chain reaction is a rapid *in vitro* technique for the amplification of DNA fragments.[136] This enzymatic reaction can only be conducted if at least the sequence information of the flanked regions of the targeted DNA is known.

The sequence of the target region was taken as a template for deducing complementary oligonucleotides, which are referred to as primers in the further course of the study (table 5.11).

The PCRs were carried out in a thermo cycler T3 or TGradient for optimisation of cycle settings respectively. Optimisation of PCR conditions is indispensable for the efficient and reliable amplification of DNA fragments. Therefore the reaction mixture and the cycling protocol were varied with respect to the usage of DMSO for GC rich templates, $MgCl_2$

concentration and primer annealing temperature. The most suitable parameters to obtain a distinct product, without unspecific amplification, were used for analysis as listed below.

For each PCR experiment positive and negative controls were carried out. Genomic DNA of *Streptomyces* sp. GW2/5831 was used as template for the positive controls, whereas no DNA was included in negative controls.

5.5.5.1 PCR conditions for pre testing of degenerate ketosynthase primer pairs on genomic DNA from *Streptomyces* sp. GW2/5831

The following degenerate PCR primers were used for pre testing: KS2F, KS2R, KS4F and KS1R (table 5.11). The primer pairs were applied in the PCR of genomic DNA from *Streptomyces* sp. GW2/5831 as listed in table 5.20. Conserved motifs of KS_{α} proteins from pradimicin-type compound biosynthesis formed the basis of degenerate PCR primer construction.[83] The obtained PCR product was, subcloned in pBluescript II SK (+), sequenced and the sequences were then used for designing specific primers (5.5.5.2) for library screening.

Table 5.20 Degenerate primer pairs

Primer pair	Upstream primer	Downstream primer	Annealing temperature
1	KS2F	KS2R	46 °C
2	KS4F	KS1R	50 °C
3	KS4F	KS2R	48 °C

Table 5.21 PCR mixture

Reagent	Volume
GoTaq® reaction buffer (green)	10.0 µl
dNTP mix 10 mM	1.0 µl
DMSO	5.0 µl
Upstream primer	1.0 µl
Downstream primer	1.0 µl
GoTaq® DNA polymerase (5 units/µl)	0.5 µl
Millipore water	30.5 µl
Template (genomic DNA of *Streptomyces* sp. GW2/5831)	1.0 µl

The reaction buffer contained magnesium chloride in a final concentration of 1.5 mM in each PCR.

Table 5.22 Cycling protocol

Step	Temperature	Time
1. initial denaturation	97 °C	5 minutes
2. primer annealing	46 °C/ 50 °C/ 48 °C	1.5 minutes
3. extension	74 °C	1 minute
4. denaturation	95 °C	0.5 minutes
5. primer annealing	46 °C/ 50 °C/ 48 °C	1 minute
6. repeat steps 3-5	-	29 cycles
7. final extension	74 °C	10 minutes
8. Cooling	4 °C	indefinitely

5.5.5.2 Design of specific primers for the screening of the *Streptomyces* sp. GW2/5831 library

The yielded PCR product, amplified with degenerate primers (table 5.20), was visualised on a gel and purified via gel extraction by means of QIAquick® Gel Extraction Kit (Qiagen). The recovered DNA fragment, having 3'-A overhangs due to terminal transferase activity of *Taq* polymerase, was subcloned into pBluescript II SK(+) vector having 3'-T overhangs (5.5.8). Heat shock transformation (5.5.9.2) was carried out using chemical competent *E. coli* XL-1 Blue cells (5.5.9.1). Colonies were grown in overnight cultures, consisting of LB medium including ampicillin, and plasmid DNA was isolated (5.5.2). After restriction analysis with *Rsa*I, samples were subjected to sequence analysis (5.5.12). Sequence data were then used for designing specific primers that were required for the PCR screening of the genomic library. The primer design was done with OligoPerfect™ software from Invitrogen™.

5.5.5.3 PCR conditions for library screening and monitoring of cosmid integration into *Streptomyces albus* genome

To optimise PCR conditions, the annealing temperature of the specific primer pair was varied between 48.5 and 56.2 °C (in steps of 2 °C, and 1.7 °C respectively), whereas 54.5 °C emerged as suitable annealing temperature (table 5.24) resulting in one clear PCR product with a size of ~700 bp. The usage of DMSO did not show any improvement of the PCR, although generally recommended for GC-rich DNA. PCR was carried out in the presence of 1.5 mM magnesium chloride ($MgCl_2$). Additional $MgCl_2$ up to 3 and 4 mM showed no effect on product amplification. The genomic DNA from *Streptomyces* sp. GW2/5831 served as a positive control for each set of PCR. Negative controls were performed simultaneously excluding any template DNA in the corresponding PCR vessel.

Usage of the PCR program for GC-rich DNA matrices described in 5.5.6.3 resulted in highly unspecific amplification. Therefore the whole cell cycling program listed in table 5.24 was used for PCR mixtures (table 5.23).

For saving time and materials, row superpools were prepared for PCR. For that, aliquots of 20 µl culture from each of the 12 wells of an entire row of the microtiter plate were pipetted together in one eppendorf tube. In this way, one microtiter plate could be screened in a round of 8 PCRs instead of 96. Once a positive superpool identified, all the 12 wells of the corresponding row were screened separately.

Screening of the library was conducted via whole cell PCR of *E. coli*. Direct usage of *Streptomyces* cells in PCR is not possible, therefore genomic DNA had to be isolated (5.5.1).

Table 5.23 PCR mixture

Reagent	Volume
GoTaq® reaction buffer (green)	1.25 µl
dNTP mix 10 mM	0.125 µl
Bovine serum albumin (BSA) 100x	0.0625 µl
Upstream primer BES1 T7 1F 2F	1.25 µl
Downstream primer BES1 T7 1F 2R	1.25 µl
GoTaq® DNA polymerase (5 units/µl)	0.03125 µl
Millipore water	9.78125 µl
Template (pooled fractions or single wells of library, genomic DNA of *Streptomyces* sp. GW2/5831 or conjugated *Streptomyces albus* strains)	1.0 µl

Table 5.24 Cycling protocol

Step	Temperature	Time
1. cell lysis	95 °C	5 minutes
2. initial denaturation	95 °C	0.5 minutes
3. primer annealing	54.5 °C	1 minute
4. extension	72 °C	1 minute
5. repeat steps 2-4	-	34 cycles
6. final extension	72 °C	5 minutes
7. Cooling	4 °C	indefinitely

5.5.6 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a very sensitive technique intended for monitoring gene expression. To prove the presence of *rifL* mRNA transcript, total RNA was isolated from *Streptomyces lividans* pHGF7604 using the RNeasy® Mini Kit (Qiagen) according to the manual. First strand synthesis was performed by means of reverse transcriptase and random hexamer primer

according to the technical manual of First Strand cDNA Synthesis Kit (Fermentas). The obtained cDNA product was used as a template for PCR with the specific primer pair RT-PCR-Oxido_for and RT-PCR-Oxido_rev (table 5.11). The amplified PCR product was visualised under UV light after staining with ethidium bromide.

5.5.6.1 Isolation of total RNA from *Streptomyces lividans* pHGF7604

RNA was isolated using the method described by Chomczynski and Sacchi.[137] All steps were carried out under the clean bench in order to avoid contamination with RNA degrading enzymes (ribonucleases). Additionally, all instruments (e.g. pipettes, …) were treated with 0.1 M sodium hydroxide solution if possible and exposed to UV light for 30 minutes.

A 50 ml liquid culture (5.4.2.2) of *Streptomyces lividans* pHGF7604 was harvested at 11000 rpm and 4 °C for 5 minutes. Approximately 65 mg of the cell material were subjected to RNA isolation, according to the technical manual of the RNeasy® Mini Kit (Qiagen).

5.5.6.2 Protocol for cDNA-synthesis

The First Strand cDNA Synthesis Kit (Fermentas) was used for synthesis of complementary DNA.

Total RNA	6 µl
Random hexamer primer	1 µl
Deionised, RNase free water	ad 11 µl

were carefully mixed in a PCR vessel and centrifuged for 5 seconds. The mixture was heated at 70 °C in a thermo cycler for 5 minutes and cooled on ice.

5x reaction buffer	4 µl
Ribonuclease inhibitor	1 µl
10 mM dNTP mix	2 µl

were carefully added, centrifuged for 5 seconds and incubated at 25 °C in a thermo cycler for 5 minutes.

M- MuLV Reverse Transcriptase	2 µl

was included in the reaction mixture. The mixture was first incubated at 25 °C for 10 minutes, then at 37 °C for 60 minutes.

The reaction was stopped while heating at 70 °C for 10 minutes. The sample was cooled down on ice, quantified and applied to PCR amplification.

5.5.6.3 PCR conditions for RT-PCR

As a precondition to this experiment, a PCR was carried out using the degenerate primer pair "Oxidofw" and "Oxidorv" (table 5.11) and genomic DNA (5.5.1) from *Streptomyces lividans* pHGF7604 as DNA template. The PCR mixture was set up as listed in table 5.24, and the cycling protocol shown in 5.26 was applied. For increased specificity, the specific primer pair "RT-PCR-Oxido" (table 5.11) was employed in a PCR performed under the same conditions as described above. After successful amplification of the *rifL* fragment, the specific primer pair "RT-PCR-Oxido" was used for RT-PCR.

Table 5.25 PCR mixture for RT-PCR and corresponding pre test

Reagent	Volume
GoTaq® reaction buffer (green)	10.0 µl
dNTP mix 10 mM	1.0 µl
DMSO	5.0 µl
Upstream primer (20 µM)	1.0 µl
Downstream primer (20 µM)	1.0 µl
GoTaq® polymerase (5 units/µl)	0.2 µl
Millipore® water (sterilised)	30.8 µl
DNA template (obtained in 5.5.6.2)	1.0 µl

Table 5.26 Cycling protocol for GC-rich DNA

Step	Temperature	Time
1. Initial denaturation	97 °C	5 minutes
2. Primer annealing	65 °C	1.5 minutes
3. Extension	74 °C	1 minute
4. Denaturation	95 °C	0.5 minutes
5. Repeat steps 3-6		29 cycles
6. Primer annealing	65 °C	1 minutes
7. Final extension	74 °C	10 minutes
8. Cooling	4 °C	indefinitely

5.5.7 Enzymatic manipulation of DNA

5.5.7.1 DNA analysis by restriction

Endonucleases were used together with the supplied buffer from the manufacturer. Sequence specific cleavage was carried out utilising restriction endonucleases type II. Incubation of the DNA with the enzyme and appropriate buffer, including BSA, was conducted at 37 °C for 2 hours.

Standard protocol for digestion of DNA

Reaction:

DNA	0.5 µl
Endonuclease	0.5 µl
Buffer	1.0 µl
BSA (100x)	0.1 µl
Purified water	7.9 µl

5.5.7.2 Restriction cleavage for subcloning technique

For the subcloning of cosmid DNA fragments into pBluescript II SK (+) vector, both insert and vector DNA were digested with the same restriction enzyme. The reactions were carried out as listed below.

Standard protocols for restriction cleavage in subcloning work:

Reaction for insert DNA

Cosmid DNA	50.0 µl
Endonuclease	10.0 µl
Buffer	15.0 µl
BSA (100x)	1.25 µl
Purified water	23.75 µl

Incubation time at 37 °C was extended to 3 hours to ensure complete digestion. The restriction reaction was applied to a gel without ethidium bromide. The DNA fragments were excised separately, as far as possible, and were then recovered from the gel (5.5.4.2) for usage in ligation reactions (5.5.7.4).

Reaction for vector DNA:

Vector DNA	20.0 µl
Endonuclease	2.5 µl
Buffer	5.0 µl
BSA (10x)	5.0 µl
Purified water	17.5 µl

After 2 hours of incubation at 37 °C, inactivation of the enzyme was achieved by heating at 65 °C for 20 minutes. The DNA was then dephosphorylated (5.5.7.3).

5.5.7.3 Dephosphorylation of linearised pBluescript vector DNA

Antarctic phosphatase catalyses the removal of 5´ phosphate groups from DNA. Dephosphorylation was carried out incubating 50 µl of the vector restriction solution with 6.25 µl of antarctic phosphatase in an appropriate buffer at 37 °C. After 30 minutes another 1 µl of antarctic phosphatase was added, and incubation was continued for further 15 minutes. Dephosphorylation of pBluescript vector DNA was followed by purification via gel extraction (5.5.4.2). The vector was then used for ligation (5.5.7.4).

5.5.7.4 Ligation by means of T4 DNA ligase

During ligation, DNA ligase catalyses the formation of covalent phosphodiester bonds between adjacent nucleotides in duplex DNA strands. As a precondition, one nucleotide requires a 5'-phosphate group and the other a 3'-hydroxyl group. In this work, T4 DNA ligase (NEB) was used for all ligation reactions together with the supplied buffer.

Ligation reaction:

Insert DNA	7 µl
Vector DNA	1 µl
T4 ligase	1 µl
Ligase buffer	1 µl
	10 µl

The ligase reaction was incubated in a thermo block at 16 °C over-night. T4 DNA ligase was then inactivated by heating at 65 °C for 20 minutes.

Each time, between 2-5 µl of the ligation reaction were transformed either by electroporation or by heat shock, depending on the size of the introduced foreign DNA. Electroporation was suitable for large inserts like cosmids, whereas smaller plasmids were transferred by heat shock transformation.

5.5.8 Cloning of PCR fragments in a T-overhang vector

This method was performed as described by Marchuk et al.[138]

5.5.8.1 Preparation of a T-overhang vector

The vector pBluescript II SK(+) was digested with the endonuclease *Eco*RV yielding blunt ends. Therefore 10 µl of the vector plasmid preparation were incubated with 2.5 µl of the

restriction enzyme, including the appropriate buffer, BSA and purified water to a final volume of 50 µl. The mixture was incubated for 2 hours at 37 °C, followed by heat inactivation of the enzyme at 80 °C for 20 minutes. Addition of 3' T-overhangs was carried out during two hours of incubation at 70 °C with 0.5 µl *Taq* polymerase and dTTP's in a final concentration of 2 mM. The extraction and precipitation of the vector DNA from the reaction mixture was achieved as described below:

- 100 µl of chloroform were added and the solution was mixed thoroughly
- centrifugation was carried out at 13000 rpm for 5 minutes
- the upper layer was transferred to 70 µl of isopropyl alcohol, avoiding the protein interlayer
- after vigorous shaking, the solution was centrifuged at 4 °C and 13000 rpm for 20 minutes
- the supernatant was discarded, and the pellet was rinsed with 200 µl of ice cold 70% ethanol
- the pellet was vacuum-dried and dissolved in 10 µl of purified water

5.5.8.2 Ligation of unmodified PCR fragments into T-overhang vector

The TA cloning method benefits from the terminal transferase activity exhibited by *Taq* polymerase. This enzyme adds an A-overhang to each end of a PCR product. Therefore it is possible to perform a ligation of a PCR product directly into a vector provided with T-overhangs. Therefore the PCR product obtained in 5.5.5.1 could be directly cloned into the vector pBluescript II SK(+) that was prepared as described in 5.5.8.1.

5.5.9 Preparation of competent cells and their transformation

5.5.9.1 Preparation of *E. coli* XL1-Blue competent cells for heat shock transformation

Approximately 1 ml of an overnight-culture was added to 100 ml of fresh LB medium, and grown at 37 °C and 200 rpm to an OD_{600} between 0.4 and 0.5. Cells were harvested by centrifugation in 50 ml falcon tubes at 5000 rpm and 4 °C for 6 minutes. Pellets were dissolved in 12.5 ml TFB I solution (table 5.17), sedimented as above and redissolved in 2 ml TFB II solution (table 5.17). Aliquots of 100 µl were shock-frozen in liquid nitrogen, and stored at –80 °C.

5.5.9.2 Heat shock mediated transformation

Chemically competent cells obtained in 5.5.9.1 were mixed with 10 µl of a ligation reaction (5.5.7.4, 5.5.8.2) and stored on ice for 20 minutes. Heat shock was carried out for 1 minute at 42 °C. Subsequently, the mixture was incubated on ice, and 900 µl of LB medium were added. Incubation at 37 °C and 200 rpm for 1 hour was followed by centrifugation at 5000 rpm at room temperature for 10 seconds. Approximately 750 µl of the cell suspension were spread on LB agar plates containing Ampicillin (100 µg/ml) that were impregnated with 20 µl of X-gal (50 mg/ml) and 100 µl of IPTG (100 mM) at least 30 minutes prior to use. After incubation at 37 °C for 16 hours colonies were visible. Blue and white colonies were identified after at least 2 hours of storage at 4-8 °C. White colonies contained the DNA of interest ligated into the vector (5.5.9.6), and were therefore subjected to plasmid DNA isolation (5.5.2).

5.5.9.3 Preparation of *E. coli* ET12567/pUZ8002 competent cells for electroporation

Cells of *E. coli* ET12567/pUZ8002 were grown overnight at 37 °C for 16 hours in 5 ml LB medium containing 25 µg/ml chloramphenicol and 25 µg/ml kanamycin. On the next day, 1 ml of this pre-culture was used to inoculate 100 ml of fresh LB medium containing antibiotics. After reaching the OD_{600} of approximately 0.4, the cells were recovered by centrifugation at 4000 rpm and 4 °C for 5 minutes. After decanting the supernatant, the pellet was washed by gentle resuspension in 40 ml of ice-cold 10% glycerol. This was repeated with 30 ml, as well as 15 ml of ice-cold glycerol. After sedimentation in 15 ml glycerol, the supernatant was discarded after centrifugation and the cell pellet was resuspended in the residual glycerol. Aliquots of 50 µl were placed in tubes and treated with liquid nitrogen. The obtained electrocompetent cells were stored at –80 °C.

5.5.9.4 Preparation of *E. coli* XL-1 Blue competent cells for electroporation

A 5 ml overnight-culture was used to inoculate 200 ml of fresh LB medium, followed by incubation at 37 °C and 200 rpm to an OD_{600} between 0.4 and 0.6. All subsequent steps were carried out either on ice or at 4 °C. For centrifugation at 5000 rpm for 5 minutes, the culture broth was portioned into 50 ml falcon tubes. After resuspension in 25 ml of 10% glycerol and sedimentation, the cells were washed twice with 10 ml of 10% glycerol. Two tubes were combined and diluted with 5 ml of 10% glycerol to a volume of 25 ml. The next centrifugation steps were carried out as described above (5.5.9.3). The cell pellet was first

resuspended in 5 ml, then finally in 1 ml glycerol solution. The contents of all tubes were combined, and aliquots of 70 µl were shock-frozen in liquid nitrogen and stored at –80 °C.

5.5.9.5 Electroporation

Electroporation was carried out using a Bio-Rad MicroPulser® and electroporation cuvettes (2 mm). Electrocompetent cell suspensions were mixed with 2 µl of *oriT*-containing vector, including the desired DNA fragment. Electroporation was carried out (program settings: bacteria, Ec2), and 1 ml of ice-cold LB medium was immediately added to the shocked cells. This mixture was transferred to a tube and incubated with shaking for 1 hour at 37 °C. Approximately 500 µl were spread on LB agar plate with appropriate antibiotic selection. Subsequent incubation at 37 °C took place for 16 hours. The selection of recombinant clones by "blue-white" screening is referred to in chapter 5.5.9.2 and 5.5.9.6.

5.5.9.6 Identification of recombinant clones using "blue-white" screening

The vector pBluescript II SK(+), employed in primer design and subcloning, contains the *lacZ* gene with an internal multiple cloning site harbouring different restriction sites that can be cleaved with the corresponding endonucleases. The foreign DNA is then inserted within the *lacZ* gene in a ligation reaction, preventing the correct expression of ß-galactosidase that metabolises the modified galactose sugar X-gal after induction with isopropyl-ß-D-1-thiogalactopyranoside (IPTG). This reaction was then screened by examining the colour of the resulting colonies. The vector without an insert expressed a functional ß -galactosidase enzyme that converted its substrate X-gal to a bright blue-coloured product that is visible in colony clones. The disruption of the *lacZ* gene results in white colonies that were utilised for further studies. The insertion of a foreign DNA fragment can thus be monitored, but evidence for a specific fragment must be obtained otherwise. Therefore plasmid DNA of white clones was isolated after transferring to liquid medium, and restriction analysis was performed. The samples were then subjected to sequence analysis.

5.5.10 Construction of a gene library from *Streptomyces* sp. GW2/5831

5.5.10.1 Genomic DNA isolation and library construction

Genomic DNA from *Streptomyces* sp. GW2/5831 was isolated according to the previously described procedure (5.5.1). The highly concentrated high molecular weight DNA solution obtained in the isolation procedure was dissolved with 1000 µl Millipore® water. Approximately 700 µl (~ 2 µg) from this crude DNA solution were loaded onto a 0.9% (w/v)

low melting point agarose gel. After electrophoresis, fragments of approximately 40 kb in size were recovered from the gel by gel-digestion with GELase (Epicentre Biotechnologies), according to the manufacturer's protocol with following modifications: 1 U of GELase enzyme preparation was added to each 300 µl of melted agarose, followed by incubation at 45 °C for 2 h. After inactivating the enzyme at 70 °C for 10 min and removing any undigested agarose by centrifugation at 11.000 rpm for 30 min, the DNA was precipitated at room temperature with 2.5 volumes of 100% ethanol (5.5.3.3) and 1/10 volume of sodium acetate (pH 7). That was followed by centrifugation at 11.000 rpm for 40 min and two washing steps with 70 % Ethanol at 4 °C. The DNA was then dissolved in 50 µl Tris buffer (10 mM, pH 8.5). This size-selected genomic DNA was first blunt-ended for 45 min at room temperature (see below), the reaction being stopped by adding EDTA to a final concentration of 10 mM and by a subsequent 10 min inactivation step at 70 °C. The DNA was then precipitated with isopropyl alcohol (5.5.3.4) and 3 M sodium acetate (pH 5), and after a 30 min incubation step at room temperature, the nucleic acids were recovered after 45 min of centrifugation at 14.000 rpm and 4 °C. Approximately 50 ng of blunt-ended DNA eluted with 7 µl Tris buffer (10 mM, pH 8.5) were used in a 2 h ligation step (5.5.10.3) at room temperature with approximately 200 ng of pAY1 vector (5.5.10.2), using Fast-Link DNA ligase (Epicentre Biotechnologies). The DNA was then packaged in lambda phage and used to infect EPI300™ $T1^R$ *E. coli* cells according to the manufacturers protocol (CopyControl Fosmid Library Production Kit from Epicentre Biotechnologies). The infected EPI300™ $T1^R$ *E. coli* cells were spread on 25 LB agar plates containing apramycin, and incubated for 16 hours over night. Approximately 4800 clones were obtained, and the library was established comprising 2016 random clones (5.5.10.4). The clones were subjected to whole cell PCR screening as described in 5.5.10.6 and 5.5.5, using the specific primers “BES1 T7 1F 2F” and “BES1 T7 1F 2R”.

blunt ending reaction

End-repair buffer (10x)	8 µl
dNTP mix (2.5 mM)	8 µl
ATP (10 mM)	8 µl
DNA (~40 kb)	50 µl
End-repair enzyme mix (T4 DNA ligase + T4 polynucleotide kinase)	4 µl

5.5.10.2 Preparation of pAY1 cosmid vector DNA

Cosmid vector DNA was isolated from the *E. coli* DH5α host strain according to the plasmid isolation protocol (5.5.2). For the clean-up procedure, the vector DNA was loaded onto a gel and was recovered via gel extraction (5.5.4.2). Approximately 34 µl of the vector DNA were then restricted with 2 µl of the endonuclease *Eco*RV in appropriate buffer including BSA. The resulting blunt ended DNA was yielded after 3 hours of incubation at 37 °C. The removal of 5'phosphate groups was catalysed by antarctic phosphatase. This was performed during incubation of 20 µl of vector DNA with 2 µl antarctic phosphatase (see also 5.5.7.3) at 37 °C. After 30 minutes another 1 µl antarctic phosphatase was added, and incubation was continued for 30 minutes. The previous step was repeated. The enzyme was inactivated by heating at 65 °C for 5 minutes. The mixture was purified by phenol-chloroform extraction (5.5.3.1) and concentrated by precipitation (5.5.3.2). The vector DNA was quantified on an agarose gel by comparing with Fosmid Control DNA.

5.5.10.3 Ligation

The ligation reaction was set up as follows:

8.5 µl insert DNA (approximately 50 ng)
0.5 µl vector DNA (approximately 200 ng)
1.2 µl Fast-Link™ Ligation Buffer (10x)
1.0 µl ATP (10 mM)
1.0 µl Fast-Link™ DNA Ligase
12.2 µl

incubated at room temperature for 2 hours, heat-inactivated at 70 °C for 10 minutes.

5.5.10.4 Size of library for genome coverage

Evaluation of the correct number (N) of clones required for the coverage of the whole genome of *Streptomyces* sp. GW2/5831, for an average insert length of 40 kb, was achieved by the following formula:[139]

$$N = \ln (1 - P) / \ln (1 - f)$$

In this equation P (99.9% = 0.999) represents the probability of a genome fragment to occur in the library, whereas f (= 0.005) denominates the quotient of insert length to the genome size of 8 Mb for Streptomycetes.[5]

5.5.10.5 Clone picking and replication

The construction of the library was performed by inoculating individual clones into the wells of 21 microtiter plates, resulting in 2016 preserved clones. Therefore, each well was pre-filled with 100 µl of LB broth containing 50 µg/ml apramycin. The cosmid clones were transferred to the medium via sterilised tooth picks, and incubated over night at 37 °C. On the next day a copy of each microtiter plate was established using a transfer seal to inoculate a fresh pre-filled microtiter plate as described above. After replication 100 µl of 50% glycerol were added to the wells of the original plates by gentle mixing. The yielded 25% glycerol stock cultures that harboured one individual cosmid clone each were stored at –80 °C. The replicas were used to combine 20 µl aliquots of the wells 1 to 12 in each row, obtaining 8 pooled fractions from each microtiter plate consisting of 96 wells.

5.5.10.6 PCR based screening of the library

To obtain a general survey on the number and rough location of positive clones included in the library, whole cell PCR was carried out employing the pooled lines from replicates (5.5.10.5). Each microtiter plate was analysed by a set of ten PCRs comprising 8 pooled fractions as well as a positive and a negative control. After the detection of a positive pool, corresponding wells were screened separately. Hence thirteen PCRs were carried out, including twelve single well samples and a positive control. This resulted in the identification of the exact coordinates of single positive clones harbouring the DNA fragment of interest.

5.5.11 Intergeneric transfer of cosmid DNA between *E. coli* and *Streptomyces*

This technique was described by Flett et al.[133] The cosmid containing *oriT* was introduced into the competent cells of *E. coli* ET12567/pUZ8002 by electroporation (5.5.9.5). A single colony of the transformant was inoculated into 3 ml of LB medium containing 25 µg/ml chloramphenicol (for ET12567 selection), 25 µg/ml kanamycin (for pUZ8002 selection) and 50 µg/ml apramycin (for pAY1 selection). The culture was incubated for 16 hours at 30 °C and 200 rpm. On the next day 100 µl of the pre-culture were used to inoculate fresh LB medium including appropriate antibiotics. Cultivation was continued until an optical density of OD_{600} 0.4-0.6 was obtained. At the same time 100 µl of a *Streptomyces albus* spore

suspension (10^8) (5.4.4) were mixed with 500 µl of TES buffer (0.05 M TES, pH 8). The mixture was heat shocked for 10 minutes at 50 °C, and, after cooling down, 500 µl of DSG (double strength germination) medium were added. Incubation was continued for 1.5 hours at 37 °C.

Approximately 1 ml of *E. coli* cells was washed three times with fresh LB medium without any antibiotic, and mixed with the spores after germination. The mixture was shortly centrifuged, the supernatant was decanted, and the cells were plated on 2CM agar plates. After incubation at 30 °C for 15-16 hours, the overlay was carried out using 1 ml water containing 1 mg nalidixic acid and 1 mg apramycin to inhibit overgrowth of *E. coli* cells. Agar plates were continuously incubated at 30 °C for approximately 5-7 days until potential exconjugants matured. Exconjugants were then transferred to different solid and liquid media for colour monitoring (5.4.5.2).

5.5.12 DNA sequence analysis

5.5.12.1 Sequence analysis of large cosmid DNA via spot-sequencing

For evidence of PKS fragments and coverage of the gene cluster, the positive cosmids were end-sequenced with primers designed according to the pAY1 sequence adjacent to *Eco*RV cleavage site (AY1ES1 Forward and AY1ES1 Reverse) deduced as shown in 4.2.6 (figure 4.26). The end-sequences were analysed by conducting BLAST homology searches with Advanced BLAST X data base tool (5.5.12.4).

5.5.12.2 Sequence analysis of large cosmid DNA via subcloning technique

To obtain more sequence data, the DNA of two selected cosmid clones comprising approximately 50 kb was digested into 12-14 smaller fragments by restriction cleavage with *Not*I and *Sac*I endonucleases (5.5.7.2). The cloning vector pBluescript was treated with the equivalent endonuclease, and dephosphorylation was carried out using antarctic phosphatase (5.5.7.3). After purification of the vector DNA by means of gel extraction (5.5.4.2), each fragment was inserted into pBluescript via ligation reaction (5.5.7.4). The inserts were end-sequenced with T7 and T3 universal primers. Sequences were analyzed by BLAST homology searches.

5.5.12.3 DNA sequencing

The Sequence data were obtained from GATC Biotech AG service or 4baseLab. Samples were automatically sequenced on an ABI 3730*xl* DNA Analyzer (Applied Biosystems) using the dideoxy mediated chain termination method.[140] The employed dideoxy nucleotides were linked with four fluorescent dyes, each base type carrying a specific colour. Therefore the nucleotide sequences were determined by means of a fluorescence detector subsequent to sample separation via capillary electrophoresis in a polyacrylamide matrix.

5.5.12.4 Computer-assisted appraisal of obtained DNA sequences

The software programs BioEdit and Vector NTI® were used to attend DNA sequences. The raw data were subjected to the Advanced BLAST X homology search data base for nucleotide sequence alignment (http://www.ch.embnet.org/software/aBLAST.html). Advanced BLAST X program searches a protein database using a translated nucleotide query that provides information about putative gene products and their functions.[141] The computation was performed using the BLAST network service that utilises the NCBI (National Centre for Biotechnology Information) BLAST 2 software.

5.5.12.5 Determination of open reading frames (ORFs)

After the determination of the nucleotide sequence, the obtained data were analysed to identify open reading frames.[5] An ORF is a nucleotide sequence of substantial length between a start and a stop codon that could potentially code for a protein. Analysis of *Streptomyces* DNA results in a high Guanine and Cytosine content. In all likelihood the positions 1 and 3 of each triplet are occupied with a Guanine or Cytosine base (see table 5.27).[142] In *Streptomyces* G+C rich DNA it is encountered frequently that all of the six possible reading frames (both directions, position 1, 2 or 3 of a triplet possible as ORF start) produce large ORFs. For assured prediction of protein coding regions a FRAME analysis was performed using the FramePlot 4.0 program.[143] This program was developed on the basis of the FRAME program in which the G+C distribution across codons is determined, and the corresponding open reading frames are calculated with graphical representation.[142] Frame shift errors can be detected in the graph.

Table 5.27: Asymmetric G+C distribution across codons in 64 *Streptomyces* genes

Position in triplet	Average G+C content
Position 1	70%
Position 2	51%
Position 3	92%

5.6 Protein biochemical methods

5.6.1 Preparation of crude cell-free extracts from *Streptomyces* liquid cultures

All steps were carried out at 4 °C or on ice to sustain active protein conformation.

Liquid cultures (5.4.2.2) were harvested after five day cultivation (5.4.7) by centrifugation at 11000 rpm for 20 minutes. For that purpose, the culture broth was transferred to 50 ml falcon tubes. The resulting cell pellet was washed with chilled 0.1 M potassium phosphate buffer pH 7.5 (table 5.12) and centrifuged again. The supernatant was poured off, and the weight of the pellet was determined. Potassium phosphate buffer served as a lysis buffer, and was therefore added in a ratio of 1.5 ml per gram of cell pellet. To inhibit serine degrading enzymes the protease inhibitor PMSF was required in 1 mM final concentration. Cell disruption was performed by sonication with a micro tip (ultrasonic disintegrator Sonifier 250, Branson). Disintegration was directly stopped after the acoustic sound pattern was changed from a high pitched to a lower pitched frequency. At this stage the cells were lysed and thus proteins were released into the buffer. After centrifugation at 11000 rpm for 20 minutes the supernatant was used as a crude cell-free extract (CFE) for subsequent purification and analysis. The samples were stored at –20 °C, and retained activity for the following 7 days.

5.6.2 Protein purification by means of fractionated ammonium sulfate precipitation

All steps were carried out at 4 °C.

Ammonium sulfate exhibits high water solubility, even at low temperatures, and it has no adverse effect on protein activity. At sufficiently high ionic strength, proteins can be completely precipitated from the solution by hydrophobic interaction. This salting out procedure is a native precipitation, and thus conserves biological activity of the protein.

The cell-free extracts (5.6.1) were subjected to saturation degrees between 10 and 90% of ammonium sulfate. The cell-free extracts were carefully stirred in an ice water bath, while the appropriate amount of grinded salt was slowly added to the protein solution in small

portions.[144] Ammonium sulfate was added only after previous portions had been completely solved. An extensive foam formation was avoided. The protein solution was stirred for another 30 minutes after the salt addition was completed. Sedimentation was then carried out for 15 minutes at 11.000 rpm, and the pellet was resuspended in 3.5 ml of phosphate buffer. The protein solutions from the different fractions were subjected to the desalting treatment (5.6.3) and activity testing (5.6.6).

5.6.3 Removal of low molecular weight components from crude cell-free extracts (desalting procedure)

All steps were carried out at 4 °C.

Compounds with low molecular weight are present in crude cell-free extracts. As their precipitation can interfere with optical tests like UV-enzyme assays, these compounds were eliminated through size exclusion chromatography. Compounds (M < 1000) such as salts migrate through small matrix pores thus covering a longer distance through the column than large size fragments passing through large pores.

A solid phase extraction column, PD-10 Amersham®, filled with 5 ml Sephadex® G-25, was first conditioned with 25 ml potassium phosphate buffer. The enzyme solution was then added to the column, and the flow-through was discarded. Elution of the proteins was carried out with 3.5 ml of potassium phosphate buffer.
This desalting procedure was used after ammonium sulfate precipitation if necessary.

The column was flushed with 25 ml of 0.1 M sodium hydroxide for regeneration, as well as washed with deionized water to pH 7.

5.6.4 Concentration of pre-purified proteins from column eluents

Amicon® Ultra-15 centrifugal filter units were used for the concentration of pre-purified proteins from column eluents. The filtering membrane is made up of regenerated cellulose (Ultracel). The molecular weight cut-off (= MWCO) lies at 10 kD, therefore compounds with a molecular weight from 10 kD upwards are retained in the filtering cartridge.

5.6.5 Colorimetric quantitative analysis based on Bradford's method

The utilized Bradford-reagent contains the synthetic, amphoteric dye Coomassie® Brilliant Blue G250, ethanol and phosphoric acid. The assay is based on an absorbance shift in the dye when the previously red form of the Coomassie reagent changes into blue by the binding of protein. In acidic medium the dye is present in its red, cationic form showing an absorbance maximum of 465 nm. The amino acids of the proteins form a complex with the dye resulting in a shift of the absorbance maximum to 595 nm. This is accompanied by a blue coloration. The increase of the absorbance value at 595 nm correlates with protein quantity. Not all amino acids take part in complex formation in the same manner, particularly basic and aromatic amino acid residues are favoured. Therefore it is only possible to determine the total protein concentration. Since the transient dye-protein complex formation is time-dependent, it is important to measure all samples after the same reaction time.

The measurement in a spectral photometer at 595 nm took place five minutes after the addition of 900 µl of Bradford's reagent to 100 µl of sample solution. The sample solution contained 10 µl of cell-free extract and 90 µl of purified water. The reference cuvette was filled with water instead of the sample solution. A calibration curve was established for each measurement series. Therefore Bovine Serum Albumin (BSA), which was used as a standard protein was applied in concentrations between 1 and 40 µg per ml.

5.6.6 UV-spectroscopy

Photometric enzyme assay

Light absorption is often an important indicator for activity measurements of enzymes. Time-referenced change in light absorption represents the velocity of the catalyzed reaction, associated with the activity of the enzyme.

UV data were recorded on a two-beam spectral photometer (Uvikon 860, Kontron).

The intended purpose of this enzyme assay was to determine the oxidoreductase activity of the heterologous expressed RifL protein from *Amycolatopsis mediterranei* by the use of recombinant *Streptomyces lividans* strains.

Determination of RifL oxidoreductase activity

The course of the reaction was followed by measuring the consumption of the coenzyme nicotinamide adenine dinucleotide (NAD^+) over a period of 20 minutes. NAD^+ and its reduced form NADH deviate in their absorption maxima. The emerging reduced form NADH was monitored by the increase of absorption at a wavelength of 340 nm. The temperature of the photometric chamber was adjusted to 37 °C.

Table 5.28 Assay composition

constituent	amount		final concentration
	sample	reference	
UDP-Glucose 50 mM	120 µl	-	6 mM
NAD^+ 50 mM	80 µl	80 µl	4 mM
Glutamine 50 mM *	40 µl	40 µl	2 mM
Magnesium chloride 1 M**	10 µl	10 µl	10 mM
Pyridoxal phosphate 5 mM	2 µl	2 µl	0.01 mM
Cell-free extract	100 µg	100 µg	
Potassium phosphate buffer 0.1 M; pH 7.5 **	ad 1000 µl	ad 1000 µl	

Solutions were stored at –20 °C, except for * that was stored at 4-8 °C and ** at room temperature.

The assay components (as listed in table 5.28) were combined in the sample cuvette except for the substrate UDP-Glucose which was added later. At the same time the reference cuvette was prepared with the assay components as listed above. For volume adjustment 120 µl of additional buffer were filled in the reference mixture. Both solutions were stirred through cautious pipetting. The reaction was started after pre incubation at 37 °C for 5 minutes by the addition of UDP-glucose, and after 1 minute of stabilizing time the change in absorption at 340 nm was monitored.

A negative control reaction was set up in the same manner with the exception that the applied cell-free extract was obtained from an unaltered *Streptomyces lividans* strain.

5.6.7 Protein separation via polyacrylamide gel electrophoresis (PAGE)

All buffers and solutions are referred to in table 5.13 .

SDS disrupts the secondary, tertiary and quaternary structure of the analysed proteins in order to produce a linear polypeptide chain that is coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing disulfide bonds. After a uniform

charge had been applied to the proteins using the anionic detergent SDS, electrophoretic mobility depends primarily on size. Each SDS molecule has one negative charge. Under these conditions proteins migrate at a rate that reflects their molecular weight. In polyacrylamide gels larger molecules are restrained from migrating as fast as smaller molecules. The gel has the function of a molecular sieve.

The discontinuous gel system consisted of two types of matrix compositions that differed in polyacrylamide concentration as well as in pH value. The resolving gel solution was poured into the glass apparatus, and was covered by a layer of butanol in order to obtain a straight edge of the matrix. The organic solvent also prevents the polyacrylamide from oxygenation, and thus it speeds up the polymerisation that was completed after one hour at room temperature. The upper organic layer was discarded and the stacking gel solution was added. The sample-well forming comb was placed in the stacking gel phase and allowed to dry for 30 minutes. Application of a stacking gel is useful to obtain a high resolution due to isotachophoresis.

The protein samples were mixed with 0.2 parts per volume of the denaturing loading buffer and were then heated to 100 °C for 10 minutes. Due to the annealing of the SDS molecules to the protein surface, the derived poly anions mediate the migration through the polyacrylamide porous matrix when an electrical field is applied. After cooling on ice 20 µl of the sample solution were filled in the gel slot. The low molecular weight marker was solved in 100 µl of loading buffer, and was treated in the same manner as the sample solution. 5 µl of the marker were applied to the matrix in order to estimate the size of the sample proteins. Gels were developed until the sample reached the lower border of the matrix. This was indicated by the tracking dye Bromophenol Blue included in the loading buffer. Migration through the stacking gel takes place at a currency of 20 mA, whereas the resolving gel is passed through at 60 mA. For staining of the separated proteins, the resolving gel was gently mixed in Coomassie solution for at least one hour. The stacking gel was discarded. Visualization of the protein bands was possible after shaking in colour stripping solution over night, and thus removing unspecific staining of the gel matrix.

5.7 Analytical methods

5.7.1 Thin layer chromatography (TLC)

Thin layer chromatography was used to detect the amino sugar kanosamine. Aluminium sheets coated with silica gel 60 F_{254} were applied as the stationary phase. Chromatograms were developed with acetonitrile, ethyl acetate, water and glacial acetic acid in a ratio of 5:5:3:1 at room temperature.[145] Visualisation was accomplished using ninhydrine spraying reagent (1 g ninhydrine, 50 ml ethanol 96%, 10 ml glacial acetic acid) followed by heating at 100 °C for 5 minutes. Kanosamine caused red-coloured spots on a white plate. Alternatively an orcinol spraying reagent (0.2% orcinol, 20% sulfuric acid) was used indicating gray-blue spots of the amino sugar.

For optimization of chromatography conditions, other eluents such as propanol or pyridine were tested instead of acetonitrile. Every mobile phase mixture was also applied to cellulose-covered TLC sheets.

For the detection of 2-oxoglutaramate on silica gel plates the mobile phase consisted of methanol, chloroform and liquid ammonia (20%) in a ratio of 2:2:1.[146] The detection was also carried out using ninhydrine spraying reagent.

5.7.2 High performance liquid chromatography (HPLC)

HPLC was performed on a Dionex system consisting of a GP-40 pump and an ED-40 electrochemical detector. The anion exchange column Carbopac® PA-10 was used with an adequate pre column, and 18 mM sodium hydroxide represented the mobile phase. The resulting data were processed with Dionex PeakNet ® software. The high-pH anion-exchange (HPAE) chromatography coupled with pulsed amperometric detection (PAD) was described by Hardy and Townsend[147] and complemented by Dionex Technical Note 21[148] for the application presented here.

5.7.3 Fast Protein Liquid Chromatography (FPLC)

This method was used on the basis of a protein purification strategy described by Amersham Biosciences (2000, application note 18-1142-32 AA). Fast Protein Liquid Chromatography was carried out using the medium pressure system Äkta® FPLC for the purification of protein solutions. The chromatography column was filled with 20 ml of the anion exchange matrix

DEAE- Sepharose® FF. The chloride anion from the Tris-HCl-buffer solution represents the counter ion. The previously fractionated (5.6.2) and desalted (5.6.3) cell-free extracts were applied to the column, and separation was carried out according to the protocol given below (table 5.29). Buffer composition is listed in table 5.12. Fractions of 10 ml were collected by means of a fraction collector. Amicon® ultracentrifugation tubes were used to concentrate the protein fractions up to a volume of 1 ml each (5.6.4).

Quantitative analysis was conducted (5.6.5), and the purified protein fractions were applied to the enzyme assay (5.6.6).

Table 5.29 Purification protocol , DEAE- Sepharose® FF

Chromatography steps	Buffer A	Buffer B	Volume (ml)
1. Equilibration	100%	0%	40
2. Sample application			
3. Wash step 1	100%	0%	80
4. Gradient elution	100-50%	0-50%	400
	50%	50%	20
5. Wash step 2	0%	100%	40

5.7.4 Mass spectroscopy

5.7.4.1 LC-MS analysis

HPLC-MS measurements were performed using an Agilent 1100 Series HPLC with an diode array detector (DAD). Sample separation was accomplished by means of a Nucleodur 100 column (RP18, Macherey-Nagel) and gradient elution (from MeOH/H_2O 10/90 to MeOH/H_2O 100/0 in 20 minutes, MeOH 100% for 10 minutes, with added NH_4OAc, 2 mM as external standard). This system was coupled to an API 2000, Triple Quadrupole, MS/MS, Applied Biosystems/MDS Sciex with electro spray ionisation (ESI).

5.7.4.2 GC-MS analysis

GC-MS analysis was kindly carried out by Dr. Marc Lamshöft (INFU, Dortmund) on a Surveyor MSQ (Thermo Finnigan).

5.7.4.3 MALDI-TOF-MS analysis

MALDI-TOF-MS (Matrix assisted laser desorption ionisation time of flight mass spectrometry) can be used for molecular weight determination of large sized molecules like proteins. This application was described by Karas and Hillenkamp.[149] Fragmentation of the analyte was avoided by embedding in the organic matrix sinapic acid. Measurements were performed on a Voyager-DE STR 4337 (Applied Biosystems), which used an acceleration voltage of 25 kV. The recorded spectrum was set between 2000 and 100 000 Dalton.

This procedure was applied to cell-free extracts in order to detect the enzymes RifK, RifL and RifM. Sample preparation was therefore accomplished using an Amprep octadecyl mini column. That cartridge was first wetted with 5 ml isopropyl alcohol and then equilibrated with 5 ml of buffer A. The sample was diluted with 500 µl of buffer A before it was filled in the column. Elution of the proteins took place using a 5 ml mixture of buffer A and buffer B (1:1). Regeneration of the Amprep matrix was possible with 5 ml of a mixture of buffer A and B in a ratio of 1:9.

5.7.5 ^{1}H-NMR experiments

NMR spectra in biosynthetic studies were measured by Dr. Gerald Dräger (Institut für Organische Chemie, Hannover) on a Bruker DPX 500.
NMR experiments for the verification of 2-oxoglutaramate were recorded on a Bruker Avance 300 DPX (Institut für Pharmazeutische Biologie, Bonn).

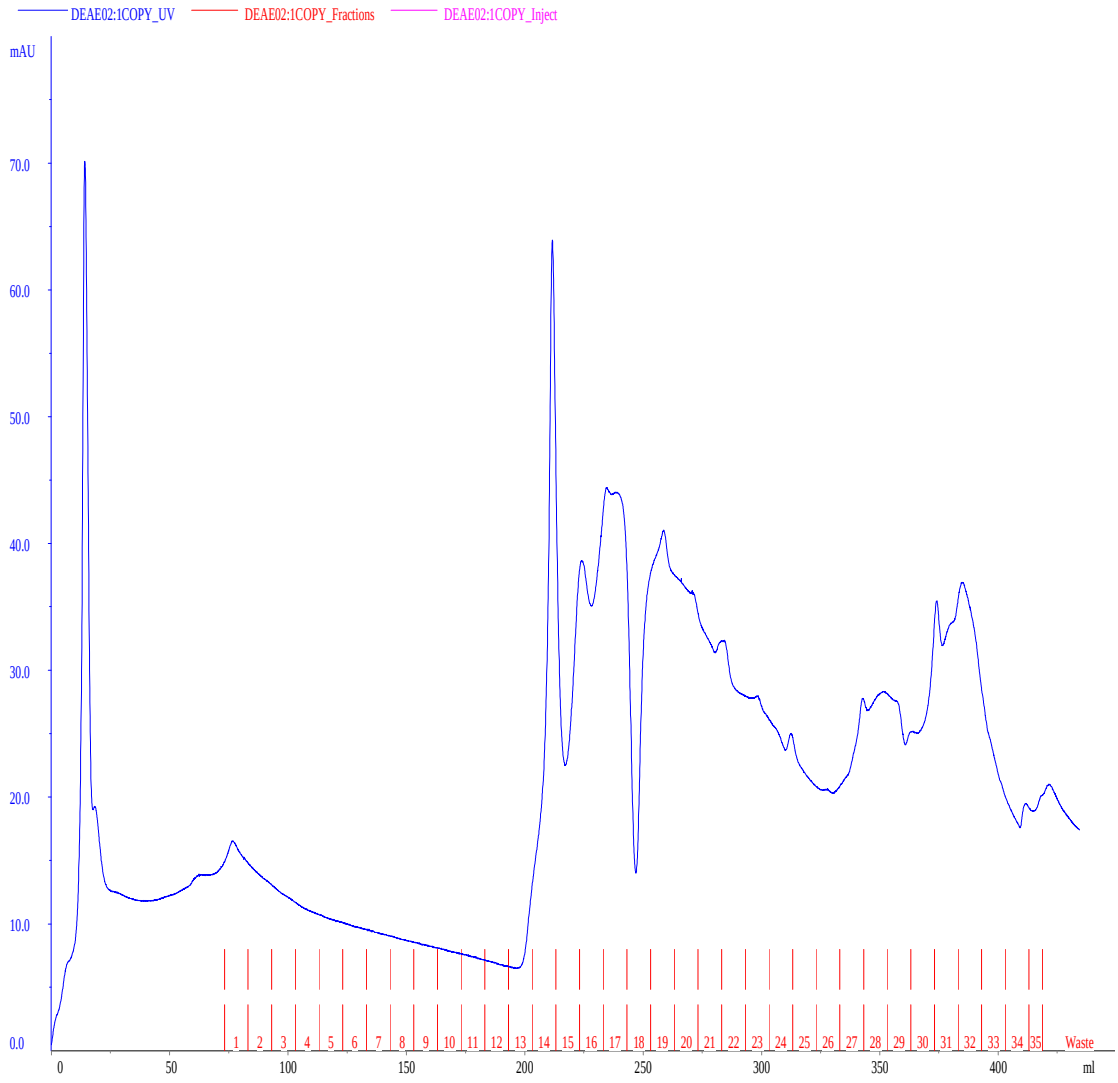
5.8 Synthesis of 2-oxoglutaramate

The substance 2-oxoglutaramate was employed as a nitrogen acceptor in an enzyme assay (5.6.6), and could not be purchased. Therefore it was synthesized according to the method described by Nissim et al.[150] The synthesis is based on the conversion of the L-amino acid L-glutamine by the L-amino acid oxidase from the eastern diamond rattle snake *Crotalus adamanteus*. The reaction took place for 10 h at 37 °C in 20 mM Tris-HCl buffer pH 8. As a side effect, the amino acid oxidase irreversibly transferred hydrogen to oxygen yielding hydrogen peroxide. Added catalase prevented the accumulation of hydrogen peroxide through its rapid degradation. The enzymes were removed from the solution using an Amicon® Ultra-15 centrifugal filter device (5.6.4), and the flow-through was applied to a PD-10 column (5.6.3) for buffer exchange.

The cyclization from 2-oxoglutaramate to 5-hydroxypyroglutamate occurs spontaneously in aqueous solutions. The formation of 5-hydroxypyroglutamate was verified via LC-MS analysis (5.7.4.1) and ^{1}H- NMR experiments (5.7.5).

6 Appendix

6.1 FPLC (DEAE-Sepharose®) of a cell-free extract from *Streptomyces lividans* pHGF7604 that was precipitated using 60% ammonium sulfate prior to FPLC.

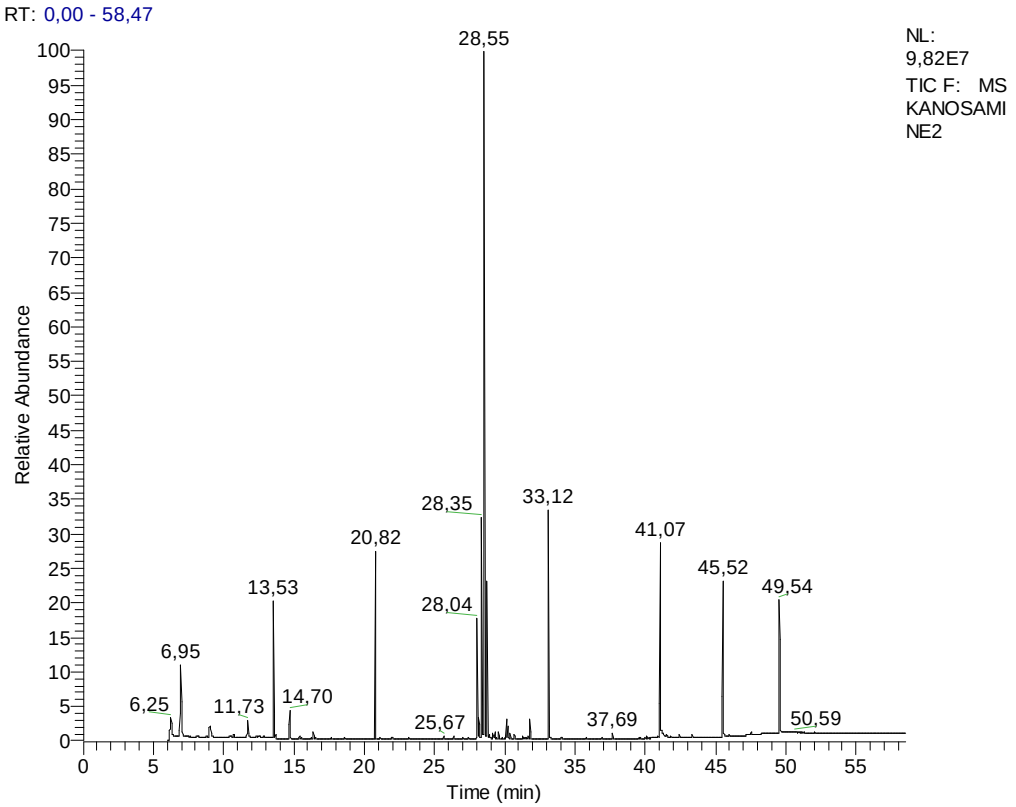


RifL assay runs 1-6 the following FPLC fractions were pooled:

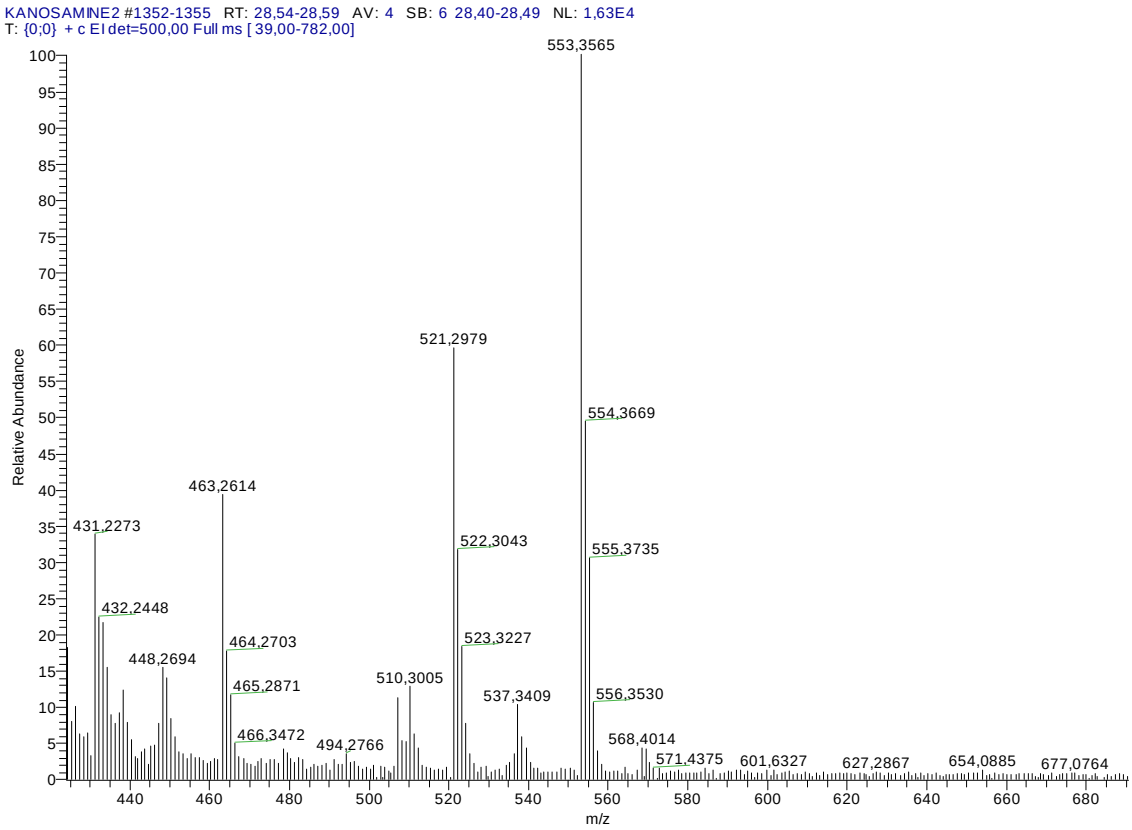
1) tested protein solution consisted of fraction 14 and 15
2) tested protein solution consisted of fraction 16 and 17
3) tested protein solution consisted of fraction 19 and 20
4) tested protein solution consisted of fraction 21-24
5) tested protein solution consisted of fraction 27-29
6) tested protein solution consisted of fraction 31-33

RifL activity was only detected in assay run 2) comprising protein fractions 16 and 17.

6.2 Total ion current (TIC) of a GC-MS run using kanosamine reference



6.3 Mass spectrum of a GC-MS run (see 6.2, TIC) using kanosamine reference- displayed retention time between 28,54 and 28,59 min



8 Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und ausschließlich mit Hilfe der angegebenen Quellen und Hilfsmittel angefertigt habe. Die Arbeit wurde bisher weder in Teilen noch im Ganzen als Prüfungsleistung zur Begutachtung an einer Hochschule vorgelegt. Die Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn ist mir bekannt.

Silke Engels
Bonn, den 20.07.2009

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