Biosynthesis of liponucleoside antibiotics in *Streptomyces*: Molecular and biochemical investigations of the caprazamycin and the liposidomycin gene cluster

Biosynthese von Liponukleosid-Antibiotika in Streptomyceten: Molekularbiologische und biochemische Untersuchungen der Gencluster des Caprazamycins und Liposidomycins

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PUBLICATIONS, PATENTS AND PRESENTATIONS

1. Publications

L. Kaysser, E. Wemakor, S. Siebenberg, J. Salas, J.K. Sohng, B. Kammerer and B. Gust “Caprazamycin Biosynthesis: Formation and Attachment of the Deoxysugar Moiety and Assembly of the Gene Cluster.” *Applied and Environmental Microbiology* (2010), in press.

L. Kaysser, K. Eitel, T. Tanino, S. Siebenberg, A. Matsuda, S. Ichikawa, and B. Gust “A New Arylsulfate Sulfotransferase Involved in Liponucleoside Antibiotic Biosynthesis in Streptomycetes.“ *Journal of Biological Chemistry* (2010), Published online Feb 15.

L. Kaysser, S. Siebenberg, B. Kammerer and B. Gust “Analysis of the Liposidomycin Gene Cluster Leads to the Identification of New Caprazamycin Derivatives.” *ChemBioChem* (2010), 11(2):191-196.

L. Kaysser, L. Lutsch, S. Siebenberg, E. Wemakor, B. Kammerer and B. Gust, „Identification and Manipulation of the Caprazamycin Gene Cluster Leads to New Simplified Liponucleoside Antibiotics and Gives Insights into the Biosynthetic Pathway.” *Journal of Biological Chemistry* (2009), 284(22):14987-96.

2. Patents

B. Gust, L. Kaysser, K. Eitel „Die Sulfotransferase Cpz4, ein Biokatalysator zur Herstellung sulfatisierter Liponukleosid-Antibiotika“. *DE 10 2010 005 273.6*, Deutsches Patent- und Markenamt (2010).

B. Gust, L. Kaysser „Ein neues Caprazamycin Biosynthesegencluster“. *DE 10 2009 014 432.3*, Deutsches Patent- und Markenamt (2009).

3. Oral presentations

“Biosynthesis of the liponucleoside-type antibiotic caprazamycin“. B. Gust, L. Kaysser, L. Lutsch, S. Siebenberg, E. Wemakor, B. Kammerer, J.A. Salas, J.K. Sohng. *Biology of Streptomycetes*, Münster, 2009.

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"Identification and Heterologous Expression of the Caprazamycin Gene Cluster". Leonard Kaysser, Stefanie Siebenberg, Bernd Kammerer, Lutz Heide and Bertolt Gust. *IBS 2008, 13th International Biotechnology Symposium*, IUPAC, Dalian, China 2008.

„A Gene Cluster for the Biosynthesis of Caprazamycins in *Streptomyces sp.* MK730". Leonard Kaysser, Stefanie Siebenberg, Jose Salas, Bernd Kammerer, Lutz Heide and Bertolt Gust. *International Workshop of the VAAM-Section Biology of Natural Products Producing Bacteria*, Berlin, 2008.

4. Poster presentations

„Investigations in Caprazamycin Biosynthesis". L. Kaysser, E. Wemakor, K. Eitel, S. Siebenberg, J. Salas, J:K: Sohng and B. Gust. *Biology of Streptomycetes*, Münster, 2009.

„Insights into the Biosynthesis of Caprazamycins, Structurally Unique Translocase I Inhibitors". L. Kaysser, L. Lutsch, S. Siebenberg, E. Wemakor, J. Salas, J.K. Sohng, L. Heide and B. Gust. *21. Irseer Naturstofftage der DECHEMA e.V.*, Irsee, 2009.

"Identification and Heterologous Expression of the Caprazamycin Gene Cluster". Leonard Kaysser, Stefanie Siebenberg, Bernd Kammerer, Lutz Heide and Bertolt Gust. *IBS 2008,13th International Biotechnology Symposium*, IUPAC, Dalian, China 2008.

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"Investigations in the Methyl- and Acyl-transfer Reactions during Caprazamycin Biosynthesis". L. Lutsch, L. Kaysser, S. Siebenberg, B. Kammerer, L. Heide and B. Gust. *International Workshop of the VAAM-Section Biology of Natural Products Producing Bacteria*, Berlin, 2008.

"Identification and structural Elucidation of secondary metabolites from *Streptomyces sp.* MK730-62F by means of LC-DAD-MS and in-source LC-MS^3 coupling". S. Siebenberg, L. Kaysser, L. Heide, B. Gust, B. Kammerer. *analytica Conference 2008, 21. Internationale Leitmesse für instrumentelle Analytik*, Labortechnik und Biotechnologie, München, 2008.

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5. Grants & Awards

Best Oral Presentation Prize, 15th International Symposium on the Biology of Actinomycetes, Shanghai, 2009.

DAAD - Travel Grant 7.8.09 to Shanghai, China. Deutscher Akademischer Austauschdienst (DAAD), August 2009.

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ABBREVATIONS

°C	degree Celsius
µ	micro
aa	amino acids
aac(3)IV	apramycin resistance gene
aadA	spectinomycin/streptomycin resistance gene
Amp	ampicillin
Apra	apramycin
ASST	arylsulfate sulfotransferase
ATP	adenosine triphosphate
bla	ampicillin/carbenicillin resistance gene
bp	base pair
cccDNA	covalently closed circular DNA
CFU	colony forming unit
CID	collision induced dissociation
Cm	chloramphenicol
CoA	coenzyme A
CPZ	caprazamycin
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5´-triphosphate
dsDNA	double-stranded DNA
dTDP	deoxythymidine triphosphate
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetra-acetic acid
ESI	electrospray ionization
FAB	fast atom bombardment
FRT	FLP recognition target
h	hour
HCl	hydrochloric acid
HCOOH	formic acid
His_8	octahistidine
HMG	hydroxyl-methylglutarate
HPLC	high performance liquid chromatography
Hyg	hygromycin
hyg	hygromycin resistance gene
IPTG	isopropyl-*β*-thiogalactoside
k	kilo
KAc	potassium acetate
Kan	kanamycin
kb	kilo base pairs
k_{cat}	turnover rate
kDa	kilo Dalton
K_i	inhibition constant
K_m	Michaelis-Menten constant
L	litre
lacZα	gene portion for α-complementation of *β*-galactosidase

LC	liquid chromatography
loxP	Cre-recombinase recognition target
LPM	liposidomycin
M	molar
M.	*Mycobacterium*
m	milli
Mb	mega base pairs
MG	methylglutarate
min	minute
MS	mass spectrometry
MUS	methylumbelliferone sulfate
MW	molecular weight
m/z	mass-to-charge ratio
n	nano
NADP	nicotine amide adenine dinucleotide phosphate
NaOH	sodium hydroxide
neo	neomycin/kanamycin resistance gene
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriT	origin of transfer
p	pico
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phospho adenosine 5'-phosphosulfate
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLP	pyridoxal phosphate
PMSF	phenylmethylsulfonyl fluoride
pNS	*p*-nitrophenol sulfate
^{R}	resistant
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse phase
rpm	rounds per minute
s	second
S.	*Streptomyces*
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
TEMED	N,N,N´,N´-tetramethylethylenediamine
TES	N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid
Thio	thiostrepton
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-maleate	Tris-(hydroxymethyl)-aminomethane-maleate
U	unit
UDP	uridine diphosphate
UV	ultraviolet
V_{max}	maximal reaction velocity
WT	wild-type
×*g*	ground acceleration
X-gal	5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside

SUMMARY

Caprazamycins are potent anti-mycobacterial liponucleoside antibiotics isolated from *Streptomyces* sp. MK730-62F2 and belong to the translocase I inhibitor family. Their complex structure is derived from 5'-(*β*-*O*-aminoribosyl)-glycyluridine and comprises a unique *N*-methyl-diazepanone ring. The biosynthesis of these unusual compounds is unknown and only limited information is available on the formation of other translocase I inhibitors.

The first part of the presented thesis describes the identification, cloning and sequencing of the caprazamycin biosynthetic gene cluster, representing the first identified gene cluster of a translocase I inhibitor. Sequence analysis revealed the presence of 23 open reading frames putatively involved in export, resistance, regulation and biosynthesis of the caprazamycins. Heterologous expression of cosmid cpzLK09, containing the gene cluster, in *Streptomyces coelicolor* M512 led to the production of non-glycosylated bioactive caprazamycin derivatives. A set of gene deletions validated the boundaries of the cluster. In the following part of the thesis (carried out in collaboration with Diploma student Liane Lutsch) inactivation of *cpz20, cpz21* and *cpz25* resulted in the accumulation of novel simplified liponucleoside antibiotics which lack the 3-methylglutaryl moiety. Cpz21 was therefore assigned to act as an acyltransferase, responsible for the attachment of methylglutarate in caprazamycin biosynthesis. The co-enzyme A activated substrate would be generated from 3-methylglutaconate via dehydrogenation and activation catalyzed by Cpz25 and Cpz20, respectively.

The next part of the thesis is focused on the biosynthetic origin of the deoxysugar moiety of caprazamycins. On cosmid cpzLK09 which harbours the caprazamycin gene cluster the genes for the formation of dTDP-L-rhamnose could not be identified. However, co-expression of cpzLK09 in *S. coelicolor* M512 with plasmid pRHAM, containing all required genes for dTDP-L-rhamnose biosynthesis, led to the production of intact caprazamycins. In vitro studies showed that Cpz31 is responsible for the attachment of the L-rhamnose to the caprazamycin aglycones, generating a rare acylated deoxyhexose. An L-rhamnose gene cluster was identified elsewhere on the *Streptomyces* sp. MK730-62F2 genome and its involvement in caprazamycin formation was demonstrated by insertional inactivation of *cpzDIII*. The L-rhamnose subcluster was assembled to cpzLK09 using Red/ET-mediated recombination.

Heterologous expression of the resulting cosmid led to the production of caprazamycins, demonstrating that both set of genes are required for caprazamycin biosynthesis. Knock-outs of *cpzDI* and *cpzDV* in the L-rhamnose subcluster confirmed that four genes, *cpzDII*, *cpzDIII*, *cpzDIV* and *cpzDVI*, are sufficient for the biosynthesis of the deoxysugar moiety.

The liposidomycins are potent inhibitors of the bacterial translocase I and differ from the caprazamycins only in the absence of the permethylated L-rhamnose and the presence of a sulfate group at the aminoribosyl moiety. In the next part of the thesis the liposidomycin biosynthetic gene cluster was identified in *Streptomyces* sp. SN-1061M using *cpz11*, a putative *N*-methyltransferase from the caprazamycin gene cluster, as a probe. Heterologous expression of the identified cosmid in *Streptomyces coelicolor* M512 led to the production of liposidomycins. A comparison of the liposidomycin gene cluster and the caprazamycin gene cluster revealed strong similarities in both clusters though other parts reflect the structural differences of the two compounds. A set of genes were assigned to be involved in the characteristic sulfation reaction of the liposidomycins, including a putative sulfotransferase gene. Surprisingly, similar genes were found adjacent to the caprazamycin gene cluster in *Streptomyces* sp. MK630-62F2. Reinvestigation of extracts from both, *Streptomyces* sp. MK630-62F2 and the heterologous caprazamycin producer *S. coelicolor* M512/cpzLK09 led to the identification of novel sulfated caprazamycin derivatives.

The last part of the thesis (carried out in collaboration with Diploma student Kornelia Eitel) demonstrates that Cpz4 from *Streptomyces* sp. MK730-62F2 is an arylsulfate sulfotransferase (ASST) responsible for the formation of sulfated liponucleoside antibiotics. Gene deletion mutants showed that *cpz4* is required for the production of sulfated caprazamycin derivatives. Cloning, overproduction and purification of Cpz4 resulted in a 58 kDa soluble protein. The enzyme catalyzed the transfer of a sulfate group from *p*-nitrophenol sulfate (pNS; K_m 48.1 µM, k_{cat} 0.14 s^{-1}) and methylumbelliferone sulfate (MUS; K_m 34.5 µM, k_{cat} 0.15 s^{-1}) onto phenol (K_m 25.9 mM and 29.7 mM, respectively). The Cpz4 reaction proceeds by a ping pong bi-bi mechanism. Several synthetic structural analogs of intermediates of the caprazamycin biosynthetic pathway were tested as substrates of Cpz4. Des-*N*-methyl-acyl-caprazol was converted with highest efficiency 100 times faster than phenol. The fatty acyl side chain and the uridyl moiety seem to be important for substrate recognition by Cpz4. Liponucleosides, partially purified from various mutant

strains, were readily sulfated by Cpz4 using pNS. No product formation could be observed with PAPS as the donor substrate. Sequence homology of Cpz4 to the previously examined ASSTs is low. However, numerous orthologs are encoded in microbial genomes and represent interesting subjects for future investigations.

In vivo, in vitro and *in silico* analysis of the caprazamycin and the liposidomycins biosynthetic gene clusters conducted in this thesis allowed a first proposal of the biosynthetic pathway to the liponucleosides and provides insights into the formation of related uridyl-antibiotics.

ZUSAMMENFASSUNG

Caprazamycine sind Liponukleosid-Antibiotika die aus *Streptomyces* sp. MK730-62F2 isoliert wurden und den Translokase I-Inhibitoren zugerechnet werden. Sie weisen eine sehr gute Wirksamkeit gegen Mykobakterien und anderen Gram-positiven Mikroorganismen auf. Die Caprazamycine besitzen eine hochkomplexe chemische Struktur, bestehend aus einem 5'-(*β*-*O*-Aminoribosyl)-Glycyluridin und einem *N*-Methyl-Diazepanonring. Über die Biosynthese dieser ungewöhnlichen Naturstoffe ist bislang nichts bekannt und auch über die Biosynthese anderer Translokase I-Inhibitoren weiß man nur wenig.

Im ersten Teil dieser Arbeit wird die Identifizierung, Klonierung und Sequenzierung des Caprazamycin-Genclusters beschrieben; des ersten identifizierten Biosynthese-Genclusters eines Translokase I-Inhibitors. Die Sequenzanalyse ergab, dass 23 mögliche Gene auf dem Cluster am Export, der Resistenz, der Regulation und an der Biosynthese der Caprazamycine beteiligt sind. Die heterologe Expression des Clusters, auf dem Cosmid cpzLK09, in *Streptomyces coelicolor* M512 führte zur Produktion nicht-glykosylierter Caprazamycin-Derivate. Durch verschiedene Deletions-Experimenten konnten die Grenzen des Clusters bestimmt werden. Im nächsten Teil der Arbeit, durchgeführt in Zusammenarbeit mit der Diplomandin Liane Lutsch, wird gezeigt, dass durch die Inaktivierung der Gene *cpz20*, *cpz21* und *cpz25* nur noch die Hydroxyacyl-Caprazole produziert werden. Diesen neuen Substanzen fehlt die Methylglutaryl-Einheit der Caprazamycine. Dennoch weisen sie Bioaktivität gegen Mykobakterien auf und stellen damit strukturell vereinfachte Liponukleosid-Antibiotika dar. Aufgrund dieser Ergebnisse kann angenommen werden, dass die Methylglutarat-Gruppe der Caprazamycine aus Methylglutaconat entsteht, durch Hydrogenierung katalysiert von Cpz25, CoA-Aktivierung katalysiert von Cpz20 und dem abschliessenden Transfer der Einheit an die Hydroxyacyl-Caprazole katalysiert durch Cpz21.

Der nächste Teil der Arbeit beschäftigt sich mit der Herkunft der Desoxyzucker-Gruppe der Caprazamycine. Auf dem Cosmid cpzLK09, welches das Caprazamycin-Gencluster trägt, konnten keine Gene identifiziert werden die für die Biosynthese der benötigten dTDP-L-Rhamnose zuständig sein könnten. Durch die Co-Expression von cpzLK09 mit dem Plasmid pRHAM, das die ensprechenden Gene zur Herstellung von dTDP-L-Rhamnose trägt, wurden die glykosilierten Caprazamycine im

heterologen Stamm *S. coelicolor* M512 akkumuliert. In vitro-Experimente zeigten, dass Cpz31 für den Transfer der Desoxyzucker-Einheit zuständig ist. Im Caprazamycin-Produzenten *Streptomyces* sp. MK730-62F2 konnten daraufhin die fehlenden Gene *cpzDII*, *cpzDIII*, *cpzDIV* und *cpzDVI*, für die Biosynthese der dTDP-L-Rhamnose auf einem separaten Gencluster identifiziert werden. Die Vereinigung und Expression des neu gefundenen Clusters mit dem Caprazamycin-Gencluster führte zur heterologen Produktion der glykosilierten Caprazamycine.

Die Liposidomycine sind mit den Caprazamycinen strukturell verwandte Translokase I-Inhibitoren. Sie unterscheiden sich von Caprazamycinen durch die sulfatierte Aminoribose-Gruppe und durch die Abwesenheit der Desoxyzucker-Einheit. In diesem Teil der Arbeit wird die Identifizierung des Liposidomycin-Genclusters aus *Streptomyces* sp. SN-1061M beschrieben. Heterologe Expression des Genclusters in *S. coelicolor* M512 erlaubte die Produktion der Liposidomycine. Ein Vergleich mit dem Caprazamycin-Gencluster zeigte starke Ähnlichkeiten, aber auch Unterschiede, entsprechend der strukturellen Besonderheiten beider Liponukleosid-Antibiotika. Eine Reihe von Genen wurde der Sulfatierungsreaktion in der Liposidomycin-Biosynthese zugeordnet. Interessanterweise, wurden ähnliche Gene auch nahe des Caprazamycin-Genclusters gefunden. Die daraufhin durchgeführte Untersuchung der Caprazamycin-Produzenten *Streptomyces* sp. MK730-62F2 und *S. coelicolor* M512/cpzLK09 zeigte dass in den Extrakten beider Stämme sulfatierte Caprazamycin-Derivate zu finden waren.

Im letzten Teil der Arbeit, durchgeführt in Zusammenarbeit mit der Diplomandin Kornelia Eitel, wird gezeigt, dass es sich bei dem Enzym Cpz4 aus *Streptomyces* sp. MK730-62F2 um eine neuartige Arylsulfatsulfotransferase (ASST) handelt, welches die Sulfatierung von Liponukleosid-Antibiotika katalysiert. Die Untersuchung von Deletionsmutanten zeigte, dass *cpz4* für die Produktion sulfatierter Caprazamycin-Derivate essentiell ist. Cpz4 wurde kloniert, heterolog überproduziert und als 58-kDa großes lösliches Protein aufgereinigt. Das Enzym katalysiert den Transfer einer Sulfatgruppe von *p*-Nitrophenol (pNS; K_m 48.1 µM, k_{cat} 0.14 s^{-1}) bzw. Methylumbelliferonsulfat (MUS; K_m 34.5 µM, k_{cat} 0.15 s^{-1}) auf Phenol (K_m 25.9 mM bzw. 29.7 mM) mittels eines Ping-Pong Reaktionsmechanismus. Die Untersuchung verschiedener synthetisch hergestellter Strukturanaloga von Intermediaten der Caprazamycin-Biosynthese ergab, dass Des-*N*-Methyl-Acyl-Caprazol von Cpz4 100fach besser umgesetzt wird als Phenol. Der Fettsäure-Rest und die Uridyl-Einheit

der Caprazamycin-Derivate scheinen wichtig zu sein für die Substraterkennung durch Cpz4. Die von bakteriellen Mutantenstämmen akkumulierten und partiell aufgereinigten Liponukleosid-Antibiotika wurden in Anwesenheit von pNS als Substratgruppen-Donor ebenfalls von Cpz4 sulfatiert. Kein Umsatz wurde hingegen mit PAPS als Sulfatgruppen-Donor beobachtet. Während die bisher bekannten Arylsulfatsulfotransferasen nur geringe Sequenzhomologie zu Cpz4 aufweisen, scheinen etliche Cpz4-orthologe Proteine in Genomen verschiedenster Mikroorganismen kodiert zu sein.

Die in dieser Arbeit vorgestellte in vivo-, in vitro- und in silico-Analyse der Caprazamycin- und Liposidomycin-Gencluster ermöglichte es ein erstes Model zur Biosynthese der Liponukleosid-Antibiotika vorzuschlagen.

I. INTRODUCTION

1. The search for new antibiotics

Natural products have been the fundamental source of new bioactive compounds. In the last 25 years, more than 60% of the small-molecule chemicals which were introduced as drugs are derived from natural products (Newman, 2008). In the 1990s the focus of the pharmaceutical industries switched to combinatorial chemistry, high-throughput screening of synthetic chemical libraries and computer assisted drug design (Christoffersen, 2006). However, natural products still represent unprecedented structural diversity and are regarded as privileged scaffolds for drug lead development (Kumar & Waldmann, 2009). Substantial progress in the areas of DNA sequencing, genetic engineering, analytical chemistry and the accessibility of so far unexplored microbial habitats has now renewed the interest in natural product-based drug discovery (Harvey, 2007; Lam, 2007; Li & Vederas, 2009; Newman, 2008). Historically, the actinomycetes have been a prolific source of chemotherapeutic agents for the treatment of infectious diseases (Pelaez, 2006). Numerous compounds were isolated over the last decades, which showed antimicrobial properties (Saleem *et al.*, 2010). However, only a small fraction of these are in clinical use. With the emergence of multi-drug resistance pathogens in recent years there is now an urgent need for new antibiotics (Arias & Murray, 2009). In order to avoid cross-resistance with the antimicrobial agents on the market, attention is especially drawn to compounds which consist of novel structural scaffolds and act against new targets (Clardy *et al.*, 2006; Falconer & Brown, 2009; Fischbach & Walsh, 2009).

2. Translocase I inhibitors

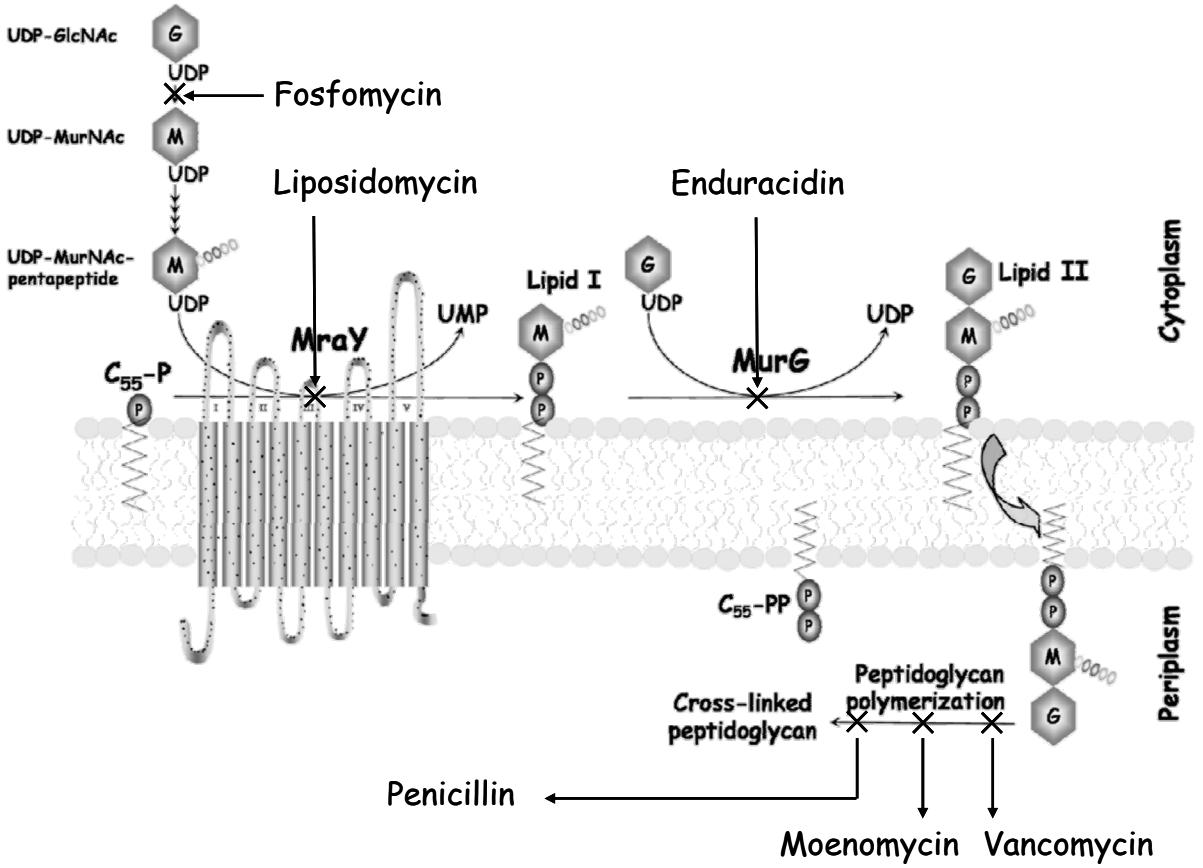


Figure I.2.1 Overview of the biosynthesis of the bacterial cell wall and antibiotics targeting the same. Figure modified from (Al-Dabbagh *et al.*, 2008).

The biosynthesis of the bacterial cell wall (Figure I.2.1) is a well precedent target for antibacterial action. Peptidoglycan, the main chemical component of the cell wall consists of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units which are *β*-1,4-glycosidically linked to form a polysaccharide. A pentapeptide side chain is attached to the lactyl group of the Mur*N*Ac unit via an amide bond. The pentapeptide generally consists of an L-alanine, a γ-D-glutamate, two D-alanines and either an L-lysine (in the case of Gram-positive bacteria) or diaminopimelic acid (DAP; in the case of Gram-negative bacteria). Cross-linking confers stability to the peptidoglycan scaffold and occurs between the lysine/DAP residue and the D-alanine residue of another polysaccharide chain by transpeptidation. Formation of the peptidoglycan takes place in different compartments of the bacterial cell. The Mur*N*Ac-pentapeptide and the Glc*N*Ac are synthesized in the cytoplasm. Two lipid-linked glycosylation steps, the first at the inner cytoplasmic membrane and the second at the outer membrane, generate the

glycan polysaccharide. The transpeptidation occurs in the periplasm. The biosynthetic steps at the outer membrane and in the periplasm are extensively exploited as targets for the treatment infectious disease e.g. by the application of the β-lactam antibiotics, the penicillin or the cephalosporines, and the glycopeptide antibiotic vancomycin. However, inhibitors of other reactions in the formation of the microbial cell wall are comparably unexplored for their use as leads in drug development.

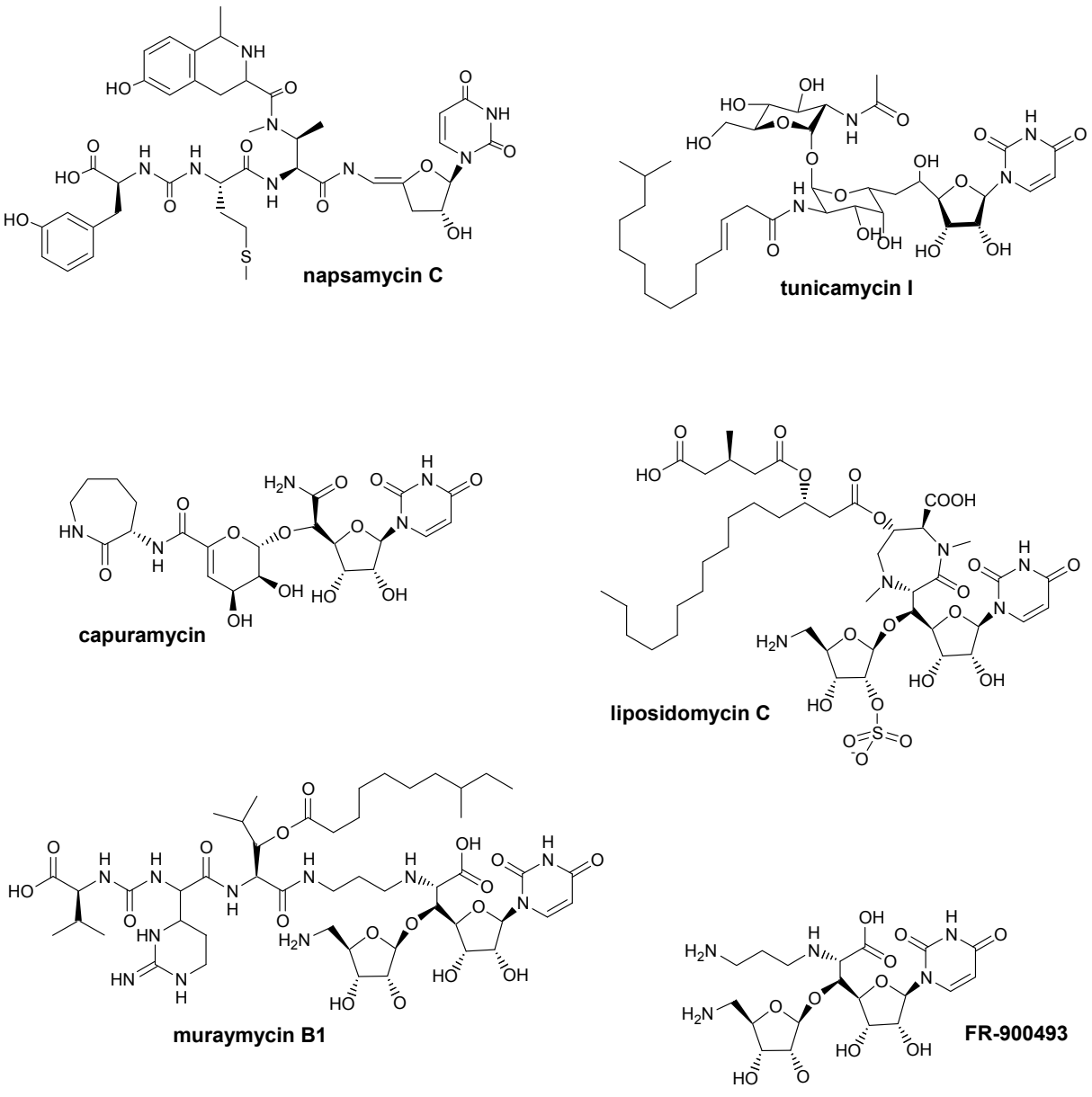


Figure I.2.2 Selected nucleoside antibiotics which target the translocase I, an essential enzyme in peptidoglycan biosynthesis.

A growing group of structurally diverse nucleoside antibiotics have been found to interfere in peptidoglycan biosynthesis by targeting the translocase I. This enzyme

(also referred to as MraY) is an integral membrane protein and responsible for the transfer of the Mur*N*Ac-pentapeptide on the lipid carried undecaprenyl phosphate to generate lipid I (Struve *et al.*, 1966) (Figure I.2.1). The translocase I inhibitors have been classified into three different groups (Winn *et al.*). The peptidyl nucleoside antibiotics include the mureidomycins (Isono *et al.*, 1989a), the pacidamycins (Chen *et al.*, 1989), the napsamycins (Chatterjee *et al.*, 1994) (Figure I.2.2) and the sansanmycins (Xie *et al.*, 2008). These compounds share a 3'-deoxyuridine and a non-ribosomal derived peptide backbone which are linked via a 4',5'-enamide bond. Peptidyl nucleoside antibiotics show specific inhibitory activity against Pseudomonades e.g. MICs 0.1-6.5 µg/mL against different *Pseudomonas aeruginosa* strains in the case of mureidomycin C (Isono *et al.*, 1989b). A second class of translocase I inhibitors comprise the liponucleoside antibiotics of the tunicamycin-type, the tunicamycins (Takatsuki *et al.*, 1971) (Figure I.2.2), the streptoviridins (Eckardt *et al.*, 1975) and the corynetoxins (Vogel *et al.*, 1981). Structurally, they contain a unique 11-carbon dialdose sugar, the tunicamine, an N-acetylglucosamine and a fatty acid side chain. Unlike other nucleoside translocase I inhibitors these compounds are not only antimicrobials but also exhibit high mammalian toxicity, as they effect *N*-linked protein glycosylation in eucaryotic cells (Heifetz *et al.*, 1979). The liponucleoside antibiotics of the liposidomycins-type constitute the third class and include the liposidomycins (Figure I.2.2) and the caprazamycins. A detailed description of these structurally complex nucleosides is given in the next sections. Recently, the isolation of two other liposidomycins analogs, the muraminocins (Muramatsu *et al.*, 2004) and the A-97065 (Fujita *et al.*, 2006) were reported.

Other nucleoside antibiotics of the translocase I inhibitor family can not be classified to these three groups, which are e.g. the antimycobacterial capuramycin (Yamaguchi *et al.*, 1986), the muraymycins (McDonald *et al.*, 2002) and FR-900493 (Ochi *et al.*, 1989), a compound isolated from *Bacillus cereus* (Figure I.2.2).

2.1. The liposidomycins

The liposidomycins (LPMs, Figure I.2.3, **1**) were identified in a culture broth of *Streptomyces griseosporeus* in 1985 through screening for inhibitors of peptidoglycan synthesis (Isono *et al.*, 1985). They show strong and selective activity against the *Escherichia coli* translocase I MraY with IC_{50} from 30 ng mL^{-1} (LPM C) but inhibit eucaryotic glycoconjugate formation, e.g. glycoprotein, glycosaminoglycan and

teichoic acid biosynthesis only at high dosage (Kimura *et al.*, 1989; Muroi *et al.*, 1997). Structurally, the liposidomycins are liponucleoside antibiotics of unusual complexity (Ubukata *et al.*, 1988). They comprise a 5'-substituted uridine, a 5-amino-5-deoxyribose-2-sulfate and an *N*-methylated perhydro-1,4-diazepine. A fatty acid side chain of variable length and conformation distinguishes the different known liposidomycins and is linked to a rare 3-methylglutaryl moiety. The 5'-(*β*-*O*-aminoribosyl)-glycyluridyl core moiety is also known from the translocase I inhibitors FR-900493 (Ochi *et al.*, 1989) and the muraymycins (McDonald *et al.*, 2002). However, the overall structure resembles only the caprazamycins (**2**, Figure I.2.3) (Igarashi *et al.*, 2005), which lack the sulfate group at the 2''-position of the aminoribose. Generally, the introduction of a sulfate group in a molecule alters its characteristics significantly e.g. size, charge and water solubility. This is used by mammals for detoxification of xenobiotics. In bacterial secondary metabolism, however, sulfation is uncommon and not many of the compounds have been investigated in detail. In one of the few examples, incorporation of the sulfate group into the glycopeptide A47934 from *Streptomyces toyocaensis* has been linked to the PAPS-dependent enzyme StaL (Lamb *et al.*, 2006). Though, the liposidomycins are potent inhibitors of the translocase I in vitro, they exhibited only weak antimicrobial activity. However, change of medium components and UV-radiation of the original producer strain led to the production of liposidomycin derivatives with strong activity against mycobacteria (Kimura *et al.*, 1998c). Interestingly, the change of capacity was mainly due to the compounds which lost the sulfate moiety. It seems that the hydrophilic sulfate attached to the 2''-position of the aminoribose confers deficient membrane permeability to the liposidomycins. On the other hand, a comparison of the sulfated liposidomycin A-(I) (Figure I.2.3, **1**) and non-sulfated liposidomycin A-(III) (Figure I.2.3, **3**) showed liposidomycin A-(I) to be more selective *in vitro* against peptidoglycan biosynthesis versus glycoprotein biosynthesis by one order of magnitude (Kimura *et al.*, 1998a). Thus, the role of the sulfate group remains an intriguing subject to investigate.

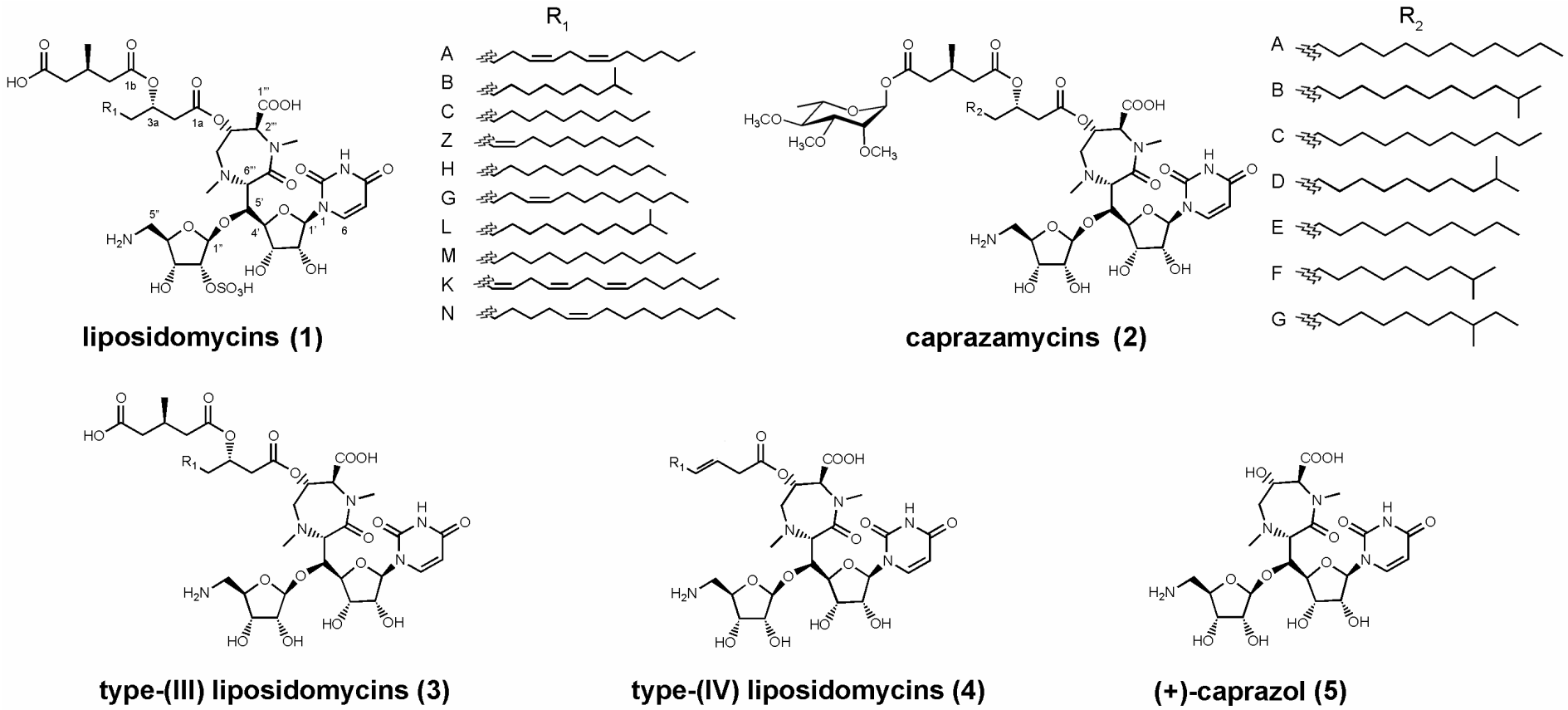


Figure I.2.3. Liponucleoside antibiotics and derivatives.

2.2. The caprazamycins

Caprazamycins (CPZs, Figure I.2.3, **2**) are liponucleoside antibiotics isolated from a fermentation broth of *Streptomyces* sp. MK730-62F2 (Igarashi *et al.*, 2003; Igarashi *et al.*, 2005). They show excellent activity *in vitro* against gram-positive bacteria, in particular against the genus *Mycobacterium* including *M. intracellulare* (MIC = 0.78 – 1.56 µg/mL), *M. avium* (MIC = 0.025 – 0.1 µg/mL) and *M. tuberculosis* (MIC = 0.78 – 1.56 µg/mL) (Takeuchi *et al.*, 2004). In a pulmonary mouse model with *M. tuberculosis* H37Rv, nasal administration of caprazamycin B exhibited a therapeutic effect at 1.5 mg kg^{-1} day^{-1} but no significant toxicity (Igarashi *et al.*, 2002). Structural elucidation (Igarashi *et al.*, 2005) revealed a complex and unique composition of elements the caprazamycins share only with the closely related liposidomycins (Isono *et al.*, 1985). They differ from the liposidomycins in the absence of a sulfate group at the 2''-position of the aminoribose and the presence of a permethylated L-rhamnose *ß*-glycosidically linked to the 3-methylglutaryl (3-MG) moiety. Similar to the liposidomycins the 3-MG motif is attached to *ß*-hydroxy-fatty acids of different chain length resulting in caprazamycins A-G (Figure I.2.3, **2**).

The liposidomycins have been shown to inhibit the biosynthesis of the bacterial cell wall by targeting the formation of lipid I (Brandish *et al.*, 1996). The caprazamycins are expected to act in the same way and are assigned to the growing number of translocase I inhibitors (Kimura & Bugg, 2003).

Recent investigations indicate that the 3''-OH group (Dini *et al.*, 2001b), the amino group of the aminoribosyl-glycyluridine and an intact uracil moiety (Dini *et al.*, 2001a) are essential for the inhibition of the *Escherichia coli* translocase I MraY. The chemical synthesis of the (+)-caprazol (Figure I.2.3, **5**) was recently accomplished (Hirano *et al.*, 2005), however, this compound only shows weak antibacterial activity. In contrast, the acylated compounds (Figure I.2.3, **3** and **4**) exhibit strong growth inhibition of mycobacteria, suggesting a potential role of the fatty acid side chain in penetration of the bacterial cell (Hirano *et al.*, 2008; Kimura *et al.*, 1998a). Apparently, the acyl-caprazols (Figure I.2.3, **4**) represent the most simplified antibiotically active liponucleosides and a good starting point for further optimization of this class of potential therapeutics.

Although chemical synthesis and biological activity of caprazamycins and liposidomycins has been studied in some detail, their biosynthesis remains

speculative and only few data existed about the formation of other translocase I inhibitors (Ohnuki *et al.*, 2003; Price & Tsvetanova, 2007) upon the start of my studies. Nevertheless, we assumed that the caprazamycin biosynthetic pathway is partially similar to that of liposidomycins, FR-90043 and muraymycins and presents a model for the comprehension and manipulation of liponucleoside formation. Considering the unique structural features of the caprazamycins we also expected some unusual biotransformations to be involved in the formation of e.g. the (+)-caprazol.

3. Aims of this study

My general objective was to investigate the biosynthesis of the liposidomycin-type of liponucleoside translocase I inhibitors using molecular methods. Therefore I had to identify the biosynthetic gene clusters of these antibiotics. However, no gene cluster of a structurally related compound or another translocase I inhibitor had been reported so far. From the different structural motifs which compose the scaffold of the liponucleosides only the permethylated L-rhamnose moiety of the caprazamycin has been investigated in the biosynthesis of other secondary metabolites. Thus, probe development for screening of a genomic library was most promising for the caprazamycin gene cluster.

Consequently, the identification of the caprazamycin gene cluster was my first objective. In order to clone and sequence the biosynthetic gene cluster of the caprazamycins, the following experiments were necessary:

- Optimization of culture conditions for caprazamycin production and development of extraction methods for the isolation of secondary metabolites. Establishment of a reliable method for the analysis of caprazamycin derivatives by liquid chromatography (LC) and mass spectrometry (MS).
- Development of a specific probe for the identification of the caprazamycin gene cluster. Generation and screening of cosmid library from genomic DNA of *Streptomyces* sp. MK730-62F2.
- Sequencing and analysis of cosmids containing the caprazamycin gene cluster.
- Functional proof of the identity of the caprazamycin gene cluster by gene deletion experiments and heterologous expression in *S. coelicolor* M512.
- Determination of the borders of the caprazamycin gene cluster by gene deletion experiments.
- Establishment of a model for caprazamycin biosynthesis.

The analysis of liponucleosides from bacterial culture extracts by LC-ESI-MS was conducted in cooperation with Stefanie Siebenberg and Bernd Kammerer (Clinical Pharmacology, University Hospital Tübingen, Germany).

My second objective was to elucidate the role of the putative methyltransferases Cpz11 and Cpz26 in caprazamycin biosynthesis. I also aimed to investigate the putative acyltransferases Cpz21 and Cpz23, the putative acyl-CoA synthase Cpz20 and the hypothetical dehydrogenase Cpz25. Thereby I hoped to provide evidence of the biosynthetic origin and attachment of the methylglutaryl moiety and to produce structurally simplified liponucleoside antibiotics. This involved the following experiments:

- Generation of mutant cosmids by single in-frame gene deletions and heterologous expression in *S. coelicolor* M512.
- Cultivation of the mutant strains and analysis of extracts for the identification of caprazamycin derivatives by LC-MS.

The generation and investigation of mutant strains was carried out in cooperation with Liane Lutsch as diploma student.

In the caprazamycin gene cluster, potential genes responsible for the biosynthesis of dTDP-L-rhamnose could not be found. And only the caprazamycin aglycones were accumulated in a heterologous host containing the caprazamycin gene cluster. Consequently, my third objective was the identification of the dTDP-L-rhamnose gene cluster and investigations in the formation and attachment of the deoxysugar moiety in caprazamycin biosynthesis. The following experiments were required for this task:

- Heterologous co-expression of the caprazamycin gene cluster and plasmid pRHAM, which contains genes directing the formation of dTDP-L-rhamnose. Analysis of mutant extracts for the accumulation of intact caprazamycins.
- Expression and purification of the putative glycosyltransferase Cpz31. In vitro studies for its role in the attachment of the rhamnosyl moiety in caprazamycin biosynthesis.
- Introduction of the caprazamycin gene cluster in different *Streptomyces* strains for the production of caprazamycin derivatives with altered deoxysugar moiety.

- Screening of the cosmid library of *Streptomyces* sp. MK730-62F2 for the dTDP-L-rhamnose biosynthetic gene cluster using primers for the amplification of a dTDP-glucose 4,6-dehydratase.
- Sequencing and analysis of a cosmid containing the dTDP-L-rhamnose gene cluster.
- Functional proof of the involvement of the dTDP-L-rhamnose gene cluster in caprazamycin biosynthesis by inactivation of the dTDP-glucose 4,6-dehydratase gene in *Streptomyces* sp. MK730-62F2 and complementation with pRHAM.
- Assembling of the caprazamycin and the dTDP-L-rhamnose gene clusters onto one cosmid. Heterologous expression of the mutant cosmid in *S. coelicolor* M512 and analysis of extracts for the accumulation of intact caprazamycins by LC-MS.
- Confirmation of *cpzDI* and *cpzDV* as non-essential in caprazamycin biosynthesis by gene deletion experiments performed on the assembled cluster.

With the caprazamycin biosynthetic gene cluster at hand I had all necessary information to proceed with my fourth objective: the identification of the liposidomycin gene cluster. The following experiments had to be conducted:

- Generation and screening of a cosmid library from genomic DNA of *Streptomyces* sp. SN-1061M for the liposidomycin biosynthetic gene cluster using *cpz11* as probe.
- Sequencing and analysis of a cosmid containing the liposidomycin gene cluster. And comparison of the sequence with the caprazamycin gene cluster.
- Functional proof of the identity of the liposidomycin gene cluster by heterologous expression in *S. coelicolor* M512.
- Establishment of a model for liposidomycin biosynthesis.
- Analysis of extracts from *Streptomyces* strains producing caprazamycin derivatives for the presence of sulfated liponucleosides.

From the comparison of the caprazamycin and the liposidomycins gene cluster we could identify a set of genes which are responsible for the sulfation of the

aminoribose moiety in liposidomycin biosynthesis. Interestingly, similar genes were found adjacent to the caprazamycin gene cluster and the expected sulfated caprazamycin derivatives were readily detected in extracts of caprazamycin producer strains. One of these genes, Cpz4, was annotated as potential arylsulfate sulfotransferase. My fifth aim was therefore to elucidate the role of Cpz4 in the sulfation reaction of liponucleoside antibiotics. In order investigate the function of Cpz4, the following experiments were necessary:

- Verification of Cpz4 to be involved in the sulfation of caprazamycin derivatives by gene deletion experiments.
- Expression and purification of the Cpz4. Biochemical characterization of Cpz4 and investigations in the reaction mechanism.
- In vitro studies of Cpz4 for the sulfation of caprazamycin derivatives and synthetic precursor analogs of the biosynthetic pathway.

The biochemical investigations on Cpz4 were carried out in cooperation with Kornelia Eitel as diploma student.

II. MATERIALS AND METHODS

1. Chemicals

Table II.1.1 Chemicals and media components

Supplier	Chemical / Media component
Amersham Biosciences, Freiburg, Germany	Agarose
Bacto-Difco, Heidelberg, Germany	Agar Casaminoacids Malt extract Peptone Tryptic soy broth Tryptone Yeast extract
Calbiochem-Novabiochem, Bad Soden, Germany	L-proline Thiostrepton
Fluka, Ulm, Germany	Apramycin Tetramethyl ethylendiamine (TEMED) Potassium sulfate (K_2SO_4)
Genaxxon Bioscience, Ulm, Germany	Hygromycin
Merck, Darmstadt, Germany	L-asparagine Chloramphenicol Coomassie brilliant blue R250 Copper chloride ($CuCl_2$) Dipotassium phosphate (K_2HPO_4) Disodium phosphate (Na_2HPO_4) Ethylene-diamine tetraacetic acid (EDTA) Ethanol Glucose D-maltose Iron(II) sulfate ($FeSO_4$) Manganese(II) chloride ($MnCl_2$) Methanol Monopotassium phosphate (KH_2PO_4) Monosodium phosphate (NaH_2PO_4) Potassium acetate (KAc) Sodium borate ($Na_2B_4O_6$)
Roth, Karlsruhe, Germany	1,4-dithiothreitol (DTT) 5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal)

	Carbenicillin Glacial acetic acid Glycerol [86%] Glycine Isopropanol Isopropyl-β-thiogalactoside (IPTG) Mannitol MES Nickel(II) chloride ($NiCl_2$) Phenol/Chloroform/Isoamylalcohol(25:24:1) Polyethylene glycol (PEG) 1000 Rotiphorese® Gel 30 Sodium dodecyl sulphate (SDS) Tris-(hydroxymethyl)-aminomethane-maleate (Tris-maleate) N-tris-(Hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES)
Serva, Heidelberg, Germany	N-lauroylsarcosine (Na-Salt, 35%)
Sigma-Aldrich, Deisenhofen, Germany	4-methylumbelliferone 4-nitrophenol (pN) 4-nitrophenolsulfate (pNS) Ammonium persulfate (APS) Bromophenol blue Calcium chloride ($CaCl_2$) *n*-butanol Diethyl pyrocarbonate (DEPC) Dimethyl formamide (DMF) Dimethyl sulfoxide (DMSO) Imidazole Iron(III) chloride ($FeCl_3$) Kanamycin Magnesium chloride ($MgCl_2$) Magnesium sulfate ($MgSO_4$) Methylumbelliferone sulfate (MUS) Nalidixic acid Phenol, crystalline Phenylmethylsulfonyl fluoride (PMSF) 3’-phosphoadenosine 5’-phosphosulfate (PAPS) Polyoxyethylenesorbitan monolaurate (Tween 20) Sodium chloride (NaCl) Sodium hydroxide (NaOH) Spectinomycin Streptomycin

	Tetracycline Tris base
SOBO Naturkost, Köln, Germany	Soy flour
Südzucker, Mannheim, Germany	Sucrose
VWR Intern. Prolabo, Leuben, Belgium	Acetonitrile β-mercaptoethanol

2. Materials for chromatography

The liquid chromatography media were obtained as dry beads, suspensions or commercial columns. The storage of the media or columns was carried out according to the manufacturers' instructions.

Table II.1.2 Materials for chromatography

Supplier	Medium
Amersham Biosciences, Freiburg, Germany	Sephadex® LH-20 (dry beads) Sephadex® G-25 NAP-10 (commercial column)
C+S Chromatographie Service, Düren, Germany	Multosphere® RP 18-5 (commercial colum, 5 µm, 250×4 mm) Multosphere® 100 RP 18-5 (commercial column, 5 µm, 250×20 mm)
Qiagen, Hilden, Germany	Ni-NTA Agarose (Suspension)

3. Enzymes and kits

Table II.1.2 Enzymes and kits

Supplier	Enzymes and kits
Amersham Biosciences, Freiburg, Germany	Restriction endonucleases T4 DNA Ligase
Fermentas	Deoxyribonuclease I (1 U/µL) 10 x buffer with $MgCl_2$ (100 mM Tris-HCl (pH 7.5 at 25 °C), 25 mM $MgCl_2$, 1mM $CaCl_2$) Revert-Aid™M-MuLV Reverse Transcriptase (200 U/µL) 5 x reaction buffer (250 mM Tris-HCl (pH: 8.3 at 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) 10 mM dNTP-mix (10 mM each) 1 kb DNA Ladder
Fluka, Ulm, Germany	Lysozyme (47 000 U/mg)

	Lysozyme (85 400 U/mg)
Macherey-Nagel, Düren, Germany	Nucleobond® AX100 NucleoSpin® Extract 2 in 1 Nucleo-spin® RNA Clean-up
New England Biolabs, Schwalbach, Germany	Restriction endonucleases T4 DNA Ligase
Plant Bioscience Limited, Norwich, UK	REDIRECT© technology: PCR-targeting system in *Streptomyces coelicolor*
Promega, Madison, WI, USA	pGEM-T Easy® Vector System 1 *Taq* DNA polymerase *Pfu* DNA polymerase
Qiagen, Hilden, Germany	RNase A (100g/mL)
Roche, Mannheim, Germany	DNA Molecular Weight Marker VII, DIG-Labelled Expand High Fidelity PCR System
Strategene, Taufkirchen, Germany	Restriction endonucleases SuperCos1 cosmid vector kit Gigapack® III Gold Packaging Extract

4. Media, buffers and solutions

4.1. Media for bacterial cultivation

The media used in this study were as follows. Unless otherwise stated, the media were prepared with distilled water and autoclaved for 20 min at 121 °C (15 psi). To obtain solid media, 2% (w/v) agar was added before autoclaving. If necessary, sterile supplementary components like antibiotics and other heat-labile substances were added in the sterile media at time of use. The media were stored at room temperature or at 4 °C.

Cultivation of E. coli

LB (Luria-Bertani) Medium (Sambrook & Russell, 2001)

NaCl	10.0 g
Tryptone	10.0 g
Yeast extract	5.0 g

Dissolve the ingredients in about 900 mL water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving.

SOB Medium

Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.5 g

Dissolve the ingredients in about 900 mL water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving. Add 10 mL of a sterile solution of 1 M $MgCl_2$.

TB Medium

Tryptone	12.0 g
Yeast extract	24.0 g
Glycerol (87%)	4.6 mL

Dissolve the ingredients in 900 mL water. Sterilize by autoclaving.

KH_2PO_4	2.31 g
K_2HPO_4	12.54 g

are dissolved in 100 mL water, sterilized by autoclaving and added to the medium.

Cultivation of Streptomyces

2YT Medium (Kieser *et al.*, 2000)

Tryptone	16.0 g
Yeast extract	10.0 g
NaCl	5 g

Dissolve the ingredients in 100 mL water. Sterilize by autoclaving.

TSB (Tryptone Soya Broth) Medium (Kieser *et al.*, 2000)

Tryptone Soya Broth	30.0 g

Dissolve the ingredient in up to 1 litre water, and sterilize by autoclaving.

YEME (Yeast Extract – Malt Extract) Medium (Kieser *et al.*, 2000)

Yeast extract	3.0 g
Peptone	5.0 g
Malt extract	3.0 g
Glucose	10.0 g

Sucrose	340 g

Dissolve the ingredients in 1 litre water. Sterilize by autoclaving. Add 5 mL of a sterile solution of 1 M $MgCl_2$.

MS (Mannitol Soya flour) Agar (Kieser *et al.*, 2000)

Mannitol	20.0 g
Soya flour	20.0 g
Agar	20.0 g

Dissolve the mannitol in 1 litre tap water and pour 100 mL into 300 mL Erlenmeyer flasks each containing 2 g agar and 2 g soy flour. Sterilize by autoclaving twice (115 ºC, 15 min) with gentle shaking between the two runs.

MM Medium (Kieser *et al.*, 2000)

L-asparagine	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Mannitol	5 g
Agar	10 g
Distilled water	ad 1000ml

Dissolve the ingredients, except agar, in the distilled water, adjust to pH 7.0-7.2 and dispense 200 mL into 300 mL Erlenmeyer flasks each containing 2 g agar. Close the flasks and sterilize by autoclaving.

Caprazamycin and liposidomycin production medium (P-medium)

Soytone	10 g
Soluble starch	10 g
D-maltose	20 g
Trace elements solution	5 mL (see protoplast transformation)

Dissolve the ingredients in water, adjust to pH 6.7. Add water to 1000 mL Sterilize by autoclaving.

Protoplast transformation of Streptomyces

Trace elements solution

$ZnCl_2$	40 mg
$FeCl_3 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	10 mg
$Na_2B_4O_6 \cdot 10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10 mg

Dissolve in 1 litre distilled water and autoclave.

R5 Medium (Kieser *et al.*, 2000)

Sucrose	103.0 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	10.12 g
Glucose	10.0 g
Difco Casaminoacids	0.1 g
Trace elements solution	2.0 mL
DifcoYeast extract	5.0 g
TES buffer	5.73 g
Agar (plates)	23.0 g

Dissolve in water to a final volume of 1 litre and sterilize by autoclaving. To prepare soft agar, 6 g Agar instead of 23 g, were added.
After autoclaving, add the following sterile solutions:

KH_2PO_4 (0.5%)	10 mL
$CaCl_2 \cdot 2H_2O$ (5 M)	4 mL
L-Proline (20% (w/v))	15 mL
NaOH (1M)	7 mL

4.2. Antibiotic solutions

Antibiotics were dissolved in appropriate solvents as stock solutions and kept at -20 °C. The aqueous solutions were sterilized by passing through a 0.22 µm filter. The solutions in ethanol and DMSO were autosterile. For antibiotic selection, the

required antibiotics were added to the cooled media (room temperature to 60 °C) in appropriate concentration.

Table II.3.1 Solutions of antibiotics

Antibiotic	Concentration in		Solvent
	stock solution (mg/mL)	media (µg/mL)	
Apramycin	50	15-50[a]	H_2O
Carbenicillin	50-100	50-100	H_2O
Chloramphenicol	25-50	25-50	ethanol
Kanamycin	50	15-50[a]	H_2O
Tetracycline	12	12	ethanol
Thiostrepton	50	15-50[a]	DMSO
Nalidixic acid	25	25	0.3 M NaOH
Spectinomycin	200	200	H_2O
Streptomycin	10	10	H_2O
Hygromycin	40	40	H_2O

[a]15 µg/mL in liquid and 50 µg/mL in solid media for selection of *Streptomyces* strains; otherwise, 50 µg/mL.

4.3. Buffers and solutions

Unless otherwise stated, the buffers and solutions were prepared with distilled water, autoclaved and stored at room temperature.

Buffers and Solutions for DNA isolation

Table II.3.2 Buffers and solutions for plasmid and cosmid isolation from *E.coli*

Buffer	Components	Final concentration	Preparation
Solution MP1	Tris-HCl EDTA RNase A	50 mM 10 mM 100 µg/mL	Adjust the pH to 8.0. Add RNase A just before use.
Solution MP2	NaOH SDS	0.2 M 1% (w/v)	

Solution MP3	KAc · $3H_2O$	3 M	Adjust the pH to 4.8. Store at 4 °C.

Table II.3.3 Buffers for isolation of genomic DNA from *Streptomyces*

Buffer	Components	Final concentration	Preparation
Lysozyme solution	Sucrose Tris-HCl EDTA RNase A Lysozyme	10.3% 25 mM 25 mM 100 µg/mL 3 mg/mL	Adjust the pH to 8.0. Add RNase A and lysozyme just before use.
SDS solution	SDS 2 M Tris-HCl pH 8	2 g 12 g	Dissolve the SDS in up to 89 mL distilled water; add the Tris-HCl buffer. Do not autoclave.
KAc solution	KAc · $3H_2O$	3 M	Adjust the pH to 4.8.
Tris-Buffer	Tris-HCl	10 mM	Adjust the pH to 7.5.

Buffers for DNA gel electrophoresis

Table II.3.4 Buffers for DNA gel electrophoresis

Buffer/solution	Components	Final concentration	Preparation
50×TAE	Tris base EDTA Glacial acetic acid	2 M 0.05 M 57.1 mL/l	Adjust the pH to 8.0 with glacial acetic acid.
Loading buffer	Glycerol Bromophenol blue	30% (w/v) 0.25% (w/v)	Store at 4 °C
Ethidium bromide solution for staining the agarose gel	Ethidium bromide	1 µg/mL	

Solutions for blue/white selection of E. coli

The storage was carried out at -20°C.

Table II.3.5 Stock solutions for blue/white selection.

Solution	Composition	Per plate
IPTG	80 mg/mL in distilled water, sterilize by filtering	15 µL
X-Gal	20 mg/mL in DMF, autosterile	60 µL

Buffers for preparation of protoplasts and transformation of Streptomyces

The following sterile solutions were prepared separately. To obtain P-buffer and T-buffer, they were mixed according to the description and stored at -20°C.

Table II.3.6 Buffers for preparation of protoplasts and transformation of *Streptomyces*

Buffer	Components	Amount (mL)
P(protoplast)-buffer (100 mL) (Kieser *et al.*, 2000)	Sucrose (12% (w/v) in H_2O)	85.5
	$MgCl_2 \cdot 6H_2O$ (1M)	1.0
	$K_2SO_4 \cdot$ (140 mM)	1.0
	Trace elements solution	0.2
	KH_2PO_4 (40 mM)	1.0
	$CaCl_2 \cdot 2H_2O$ (250 mM)	1.0
	TES (0.25M, pH 7.2)	10.0
T(transformation)-buffer (10 mL) (Kieser *et al.*, 2000)	Sucrose (25% (w/v) in H_2O)	1.0
	Trace elements solution	0.03
	K_2SO_4 (140 mM)	0.1
	KH_2PO_4 (40 mM)	0.1
	$MgCl_2 \cdot 6H_2O$ (1 M)	0.1
	$CaCl_2 \cdot 2H_2O$ (5 M)	1.0
	Tris-maleate (0.5 M, pH 8.0)	1.0
	PEG 1000 (50% (w/v) in H_2O);	5.0

Buffers for protein purification by nickel affinity chromatography

The buffers for protein purification were prepared with distilled water, autoclaved and stored at 4 °C. If required, lysozyme, imidazole, DTT and PMSF were added just before use.

Table II.3.7 Buffers for protein purification

Buffer	Components	Amount
Buffer A	Tris-HCl	50 mM (pH 8.0)
	NaCl	500 mM
	Glycerol	10%
	Imidazole	20 mM
	β-mercaptoethanol	10 mM

Buffers and solutions for protein gel electrophoresis (SDS-PAGE) and for Coomassie staining

All the buffers and solutions were prepared according to the user manual for protein gel electrophoresis of Bio-Rad (Bio-Rad, München, Germany).

Table II.3.8 Buffers and solutions for SDS-PAGE and Coomassie staining

Buffer/solution	Components	Amount	Preparation
Stacking gel (4%)	Distilled water	6.1 mL	Combine all the components, except APS and TEMED; degas under vacuum for about 15 min. Add APS and TEMED just before pouring the gel.
	0.5 M Tris-HCl (pH 6.8)	2.5 mL	
	10% (w/v) SDS	0.1 mL	
	Rotiphorese® Gel 30	1.3 mL	
	10% (w/v) APS	0.05 mL	
	TEMED	0.01 mL	
Resolving gel (12%)	Distilled water	3.35 mL	(See above).
	1.5 M Tris-HCl (pH 8.8)	2.5 mL	
	10% (w/v) SDS	0.1 mL	
	Rotiphorese® Gel 30	4.0 mL	
	10% (w/v) APS	0.05 mL	

	TEMED	0.005 mL	
Sample buffer	Distilled water 0.5 M Tris-HCl (pH 6.8) Glycerol SDS (10% (w/v) in H_2O) Bromophenol blue (0.5% (w/v) in H_2O)	3.55 mL 1.25 mL 2.5 mL 2.0 mL 0.2 mL	Add 5 µL β-mercapto-ethanol to 95 µL sample buffer prior to each use. Store at 4 °C.
10×running buffer	Tris base Glycine SDS	30.0 g 144.0 g 10.0 g	Dissolve in distilled water to a final volume of 1 litre. Store at 4 °C.
Fixing buffer	Distilled water Acetic acid Methanol	70% (w/v) 10% (w/v) 20% (w/v)	Store at RT.
Coomassie Brilliant Blue G-250 solution	Coomassie Brilliant Blue G-250 Distilled water Acetic acid Methanol	0.25% (w/v) 45% (w/v) 10% (w/v) 45% (w/v)	Store at RT.
Bleaching solution for SDS-PAGE	Distilled water Acetic acid Methanol	45% (w/v) 10% (w/v) 45% (w/v)	Store at RT.

5. Plasmids, bacterial strains, primers and probes

5.1. Vectors, cosmids and plasmids

Table II.4.1 Vectors, cosmids and plasmids

Name	Description	Source or reference
Vector		
pGEM-T	Linearized vector with T-overhang for direct cloning of PCR fragments with A-overhang, *lacZ*α, ori, f1-origin, Amp^R	Promega

pUWL201	*E. coli* - *Streptomyces* shuttle vector, *ermE** promoter, colE1, *rep*; Amp^R, $Thio^R$	(Doumith *et al.*, 2000)
pHis8	General expression vector for protein overproduction; pET-28a(+) (Merck, Darmstadt, Germany) derivative providing an His_8-tag; Kan^R	(Jez *et al.*, 2000)
SuperCos1	Cloning vector for the construction of genomic libraries; cos p_{SV40} ori_{pUC}; Amp^R, Kan^R	Stratagene
Plasmid		
pIJ787	SuperCos1-derivative, *bla* gene replaced by a cassette containing *oriT*, *tet*, *attP*, *int* ΦC31, Kan^R	(Eustáquio *et al.*, 2004)
pIJ773	*aac(3)IV* ($Apra^R$), *oriT,* FRT	(Gust *et al.*, 2003)
pIJ774	*aac(3)IV* ($Apra^R$), *oriT*, loxP	(Gust *et al.*, 2003)
pIJ778	*aadA* (Spc^R, Str^R), *oriT,* FRT	(Gust *et al.*, 2003)
pIJ10700	*hyg* (Hyg^R), *oriT,* FRT	John Innes Centre, Norwich, UK
pIJ790	λ RED (*gam bet exo*), *ara*C, *rep*101^{ts}; Cml^R	(Gust *et al.*, 2003)
pUZ8002	*tra,* RP4 ; Kan^R	(Paget *et al.*, 1999)
pUB307	„Driver"-Plasmid for triparental conjugation; *tra oriT* ; Kan^R, Cml^R	(Flett *et al.*, 1997)
pRHAM	Derivative of pUK21 (Vieira & Messing, 1991) containing the genes *oleL*, *oleS*, *oleE* and *oleU* from *S. antibioticus* ATCC 1891 for dTDP-L-rhamnose biosynthesis, p_{ermE}, $Thio^R$	(Rodriguez *et al.*, 2000)

Table II.4.2 Plasmids and cosmids produced in this study

Name	Description
Plasmid	
pLK01	1.2 kb PCR product comprising *cpz31* flanked by *Eco*RI/*Hind*III restriction sites in pGEM®-T; $Carb^R$
pLK02	*Eco*RI/*Hind*III restriction fragment from pLK01 comprising *cpz31* into the same sites of pHis8; Kan^R
pLK03	1.6 kb PCR product comprising *cpz4* flanked by *Sac*I/*Xho*I restriction sites in pGEM®-T; $Carb^R$
pLK04	*Sac*I/*Xho*I restriction fragment from pLK03 comprising *cpz4* cloned into the same sites of pHis8; Kan^R
pLL05	*Eco*RI/*Hind*III restriction fragment from a 0.6 kb PCR product comprising *cpz11* cloned into the same sites of pUWL201; $Thio^R$

pLL06	*Eco*RI/*Hind*III restriction fragment from a 1.5 kb PCR product comprising *cpz21* cloned into the same sites of pUWL201; Thio^R
pLL07	*Eco*RI/*Hind*III restriction fragment from a 1.0 kb PCR product comprising *cpz23* cloned into the same sites of pUWL201; Thio^R
Cosmid	
4H11	SuperCos1-based cosmid containing the dTDP-L-rhamnose biosynthetic genes from *Streptomyces* sp. MK730-62F2, Amp^R, Kan^R
31C2	SuperCos1-based cosmid containing the caprazamycin biosynthetic gene cluster from *Streptomyces* sp. MK730-62F2, Amp^R, Kan^R
3G5	SuperCos1-based cosmid containing the liposidomycin biosynthetic gene cluster from *Streptomyces* sp. SN-1061M, Amp^R, Kan^R
cpzLK01	from 4H11; *cpzDIII* is replaced by an apramycin resistance cassette from pIJ773; Apra^R, Kan^R
cpzLK02	from 31C2; *cpz28-30* are replaced by an apramycin resistance cassette from pIJ773; Amp^R, Apra^R, Kan^R
cpzLK04	from cpzLK02; Δ*cpz28-30* (apramycin resistance cassette removed by *Spe*I restriction); Amp^R, Apra^R, Kan^R
cpzLK09	from 31C2; *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ϕC31); Tet^R, Kan^R.
cpzEW01	from 4H11; *orf1-9* are replaced by an apramycin resistance cassette from pIJ774; Apra^R, Amp^R, Kan^R
cpzEW02	from cpzEW01; *orf10-25* are replaced by an hygromycin resistance cassette from pIJ10700; ; Hyg^R, Apra^R, Amp^R, Kan^R
cpzEW03	from cpzLK09; *orf32-33* are replaced by an apramycin resistance cassette from pIJ774; Apra^R, Tet^R, Kan^R
cpzEW05	from cpzEW03; *orf34* is replaced by an spectinomycin/streptomycin resistance cassette from pIJ778; Spc^R, Str^R, Apra^R, Tet^R, Kan^R
cpzEW07	from cpzEW05; a 4116 bp region upstream of *cpz32* is replaced by a 9950 bp *Nhe*I/*Pme*I restriction fragment from cpzEW02 containing the genes *cpzDI-cpzDVII*; Tet^R, Hyg^R, Apra^R, Kan^R
cpzEW08	from cpzEW07; apramycin resistance cassette removed by *Spe*I restriction; Tet^R, Hyg^R, Kan^R
cpzEW09	from cpzEW08; *cpzDI* is replaced by an apramycin resistance cassette from pIJ774; Tet^R, Hyg^R, Apra^R, Kan^R
cpzEW10	from cpzEW09; ∆*cpzDI* (apramycin resistance cassette removed by Cre-recombinase mediated recombination); Tet^R, Hyg^R, Kan^R
cpzEW11	from cpzEW08; *cpzDV* is replaced by an apramycin resistance cassette from pIJ774; Tet^R, Hyg^R, Apra^R, Kan^R
cpzEW12	from cpzEW11; ∆*cpzDV* (apramycin resistance cassette removed by Cre-recombinase mediated recombination); Tet^R, Hyg^R, Kan^R
cpzLL05	from cpzLK09; *cpz11* is deleted (81 bp scar); Tet^R, Kan^R

cpzLL06	from cpzLK09; *cpz21* is deleted (81 bp scar); Tet^R, Kan^R
cpzLL07	from cpzLK09; *cpz23* is deleted (81 bp scar); Tet^R, Kan^R
cpzLL08	from cpzLK09; *cpz26* is deleted (81 bp scar); Tet^R, Kan^R
cpzWP01	from cpzLK09; *cpz33-34* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP02	from cpzLK09; *cpz32-34* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP04	from cpzLK09; *cpz1-3* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP05	from cpzLK09; *cpz1-4* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP06	from cpzLK09; *cpz1-5* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP07	from cpzLK09; *cpz1-6* are deleted (81 bp scar); Tet^R, Kan^R
cpzWP22	from cpzLK09; *cpz20* is deleted (81 bp scar); Tet^R, Kan^R
cpzWP25	from cpzLK09; *cpz25* is deleted (81 bp scar); Tet^R, Kan^R
lipLK01	from 3G5; *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ϕC31), Tet^R, Kan^R.

5.2. Bacterial strains

Table II.4.3 Bacterial strains

Strain	Relevant characteristics	Source or reference
E. coli XL1Blue MRF'	General cloning host (*rec*A1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB *lacI*^{q}ZDM15 Tn10 (Tet^R)]), Tet^R	Stratagene
E. coli ET12567	Strain triply defective in DNA methylation (*dam*$^-$ *dcm*$^-$ *hsdM*$^-$), Tet^R, Cm^R	(MacNeil *et al.*, 1992)
E. coli BW25113	*Δ(araD-araB)567 ΔlacZ4787::rrnB-4;lacIP-4000(*lac^Q*) λ*$^-$ *rpoS369(*Am*) rph-1 Δ(rhaD-rhaB)568 hsdR514*	(Datsenko & Wanner, 2000)
E. coli BT340	DH5α/pCP20	(Cherepanov & Wackernagel, 1995)
E. coli SURE®	Host strain for cosmid cloning (*end*A1 *gln*V44 thi-1 *gyr*A96 *rel*A1 *lac rec*B *rec*J *sbc*C *umu*C::Tn5 *uvr*C e14- *Δ(mcrCB-hsdSMR-mrr)171* F'[*pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15 Tn10]); Tet^R, Kan^R	Stratagene
E. coli Rosetta(DE3) pLysS	Host for the heterologous expression of His_8 tagged proteins (F^- *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]) pLysSRARE); Cm^R	Invitrogen
S. coelicolor M512	Δ*redD* Δ*actII-ORF4* SCP1$^-$ SCP2$^-$ (no production of actinorhodin,	(Floriano and Bibb, 1996)

	undecylprodigiosin, and methylenomycin)	
Streptomyces sp. MK730-62F2	caprazamycin producer; wildtype strain.	acc. no. FERM-BP7218 (National Institute of Bioscience and Human Technology, Tsukuba, Japan)
Streptomyces sp. SN-1061M	liposidomycin producer; obtained from UV-mutagenesis of the wildtype strain.	acc. no. FERM-BP5800 (National Institute of Bioscience and Human Technology, Tsukuba, Japan)
Streptomyces sp. MK730-62F2 *Δcpz28-30*-(1),-(2) and -(3)	Three independent *cpz28-30*$^{-}$ mutants of *Streptomyces* sp. MK730-62F2	this study
Streptomyces sp. MK730-62F2 *ΔcpzDIII*-(1),-(2) and -(3)	Three independent *cpzDIII*$^{-}$ mutants of *Streptomyces* sp. MK730-62F2; Apra^{R}	this study
Streptomyces sp. MK730-62F2 *ΔcpzDIII*-(1)/pRHAM	*cpzDIII*$^{-}$ mutant of *Streptomyces* sp. MK730-62F2 complemented with pRHAM; Thio^{R}, Apra^{R}	this study
S. coelicolor M512/ cpzLK09-(1),-(2) and -(3)	*S. coelicolor* M512 containing the caprazamycin gene cluster, three independent mutants; Kan^{R}	this study
S. coelicolor M512/ cpzLK09-(1)/pRHAM	*S. coelicolor* M512 mutant co-expressing the caprazamycin gene cluster and pRHAM; Thio^{R}, Kan^{R}	this study
S. coelicolor M512/ lipLK01-(1),-(2) and -(3)	*S. coelicolor* M512 containing the liposidomycin gene cluster, three independent mutants; Kan^{R}	this study
S. coelicolor M512/ cpzEW07-(1),-(2) and -(3)	*S. coelicolor* M512 containing the assembled caprazamycin gene cluster and the genes *cpzDI-cpzDVII* for dTDP-L-rhamnose biosynthesis, three independent mutants; Hyg^{R}, Apra^{R}, Kan^{R}	this study
S. coelicolor M512/ cpzEW10-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpzDI* deficient assembled caprazamycin gene cluster, three independent mutants; Hyg^{R}, Kan^{R}	this study
S. coelicolor M512/ cpzEW12-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpzDV* deficient assembled caprazamycin gene cluster, three independent mutants; Hyg^{R}, Kan^{R}	this study

S. coelicolor M512/ cpzLL05-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz11* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzLL05-(1)/pLL05	A heterologous *cpz11*$^{-}$ mutant complemented with pLL05; $Thio^R$, Kan^R	this study
S. coelicolor M512/ cpzLL06-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz21* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzLL06-(1)/pLL06	A heterologous *cpz21*$^{-}$ mutant complemented with pLL06; $Thio^R$, Kan^R	this study
S. coelicolor M512/ cpzLL07-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz23* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzLL07-(1)/pLL07	A heterologous *cpz23*$^{-}$ mutant complemented with pLL07; $Thio^R$, Kan^R	this study
S. coelicolor M512/ cpzLL08-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz26* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP01-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz33-34* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP02-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz32-34* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP04-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz1-3* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP05-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz1-4* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP06-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz1-5* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP07-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz1-6* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP22-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz20* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study
S. coelicolor M512/ cpzWP25-(1),-(2) and -(3)	*S. coelicolor* M512 containing a *cpz25* deficient caprazamycin gene cluster, three independent mutants; Kan^R	this study

S. fradiae A0	*urdGT1a* $^{-}$, *urdGT1b* $^{-}$, *urdGT1c* $^{-}$ mutant of the urdamycin producer *S. fradiae* TÜ2171	(Trefzer *et al.*, 2000)
S. fradiae A0/cpzLK09-(1), -(2) and -(3)	*S. fradiae* A0 containing the caprazamycin gene cluster, three independent mutants; Kan^{R}	this study
Streptomyces sp. TÜ6071*ΔplaA6*	*plaA6* $^{-}$ mutant of the phenalinolactone producer *Streptomyces* sp. TÜ607	A. Luzhetskyy, Pharmaceutical Biotechnology, University of Freiburg
Streptomyces sp. TÜ6071*ΔplaA6*/cpzLK09-(1), -(2) and -(3)	*Streptomyces* sp. TÜ6071*ΔplaA6* containing the caprazamycin gene cluster, three independent mutants; Kan^{R}	this study
Mycobacterium phlei	Indicator strain for caprazamycins and derivatives	acc. no. DSM750 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)

6. Culture conditions

6.1. Cultivation of *E. coli*

For cloning experiments, *E. coli* strains were grown overnight (16-18 h) in liquid or solid LB medium with appropriate antibiotic at 37 °C (Sambrook and Russell, 2001).
For protein overproduction, the incubation temperature was 20 ºC.
Permanent cultures of *E. coli* were prepared by mixing 600 µL of overnight culture with 400 µL of glycerol solution (50% (w/v) in distilled water) and stored at -70°C.

6.2. Cultivation of *Streptomyces*

General cultivation

Streptomyces strains were routinely cultured in liquid or solid TSB medium. Liquid cultures were carried out in baffled Erlenmeyer flasks containing a stainless steel spring at 180-200 rpm and 28-30 °C for 2 to 3 days. For preparation of protoplasts, *Streptomyces* were cultured in YEME medium containing 0.5% glycine; For isolation

of genomic DNA, *Streptomyces* were cultured in YEME medium. An appropriate concentration of antibiotic was added, if required.

Production of secondary metabolites

For the production of caprazamycins, liposidomycins and derivatives thereof, 1 mL of a two-day-old TSB culture of the respective *Streptomyces* strain was inoculated into 50 mL P-medium and grown at 30 °C and 210 rpm for 7 days.

Preparation of spore suspensions of Streptomyces

To prepare spore suspensions, *Streptomyces* strains were spread on MS agar and incubated at 30 °C for about a week.

The plates were grown till they were well sporulated. 4 mL of sterile ddH_2O were added to each plate and the spores scraped off of the top of the plates and into suspension. The resulting spore suspension was poured into a falcon tube and vortexed vigorously (about 1 min). The spores were separated from the mycelium by passing the suspension through sterile cotton plugged in a disposable syringe. Spores were collected by centrifugation (2,100×g, 10 min, 4 °C), and resuspended in 1-3 mL of 20% glycerol. The spore suspensions were stored at -70°C.

7. Methods of molecular biology

7.1. Purification, concentration and quantification of DNA

Standard methods for DNA isolation and manipulation were performed as described elsewhere (Kieser *et al.*, 2000; Sambrook & Russell, 2001).

Phenol/chloroform extraction and ion exchange column chromatography were used for purification of DNA. Ethanol or isopropanol precipitation was used for concentration.

Quantification of DNA was carried out by using a GeneQuant photometer (Pharmacia, Freiburg, Germany) at 260 nm as well as by comparing the fluorescent intensity with DNA markers on agarose gels.

7.2. Agarose gel electrophoresis of DNA

Gel electrophoresis with 0.8-1.5% (w/v) agarose was used to separate DNA fragments between 0.5 and 50 kb. The buffer system employed was 1×TAE buffer. After running the gels, they were stained with the fluorescent dye ethidium bromide,

detected under the UV light at 312 nm and photographed by using Eagle Eye II System (Strategene, Heidelberg, Germany) (Sambrook & Russell, 2001).
DNA fragments were isolated from agarose gels using a NucleoSpin® 2 in 1 extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

7.3. DNA manipulation with enzymes

Restriction of DNA with endonucleases was carried out according to the manufacturer's instructions.
DNA ligation was achieved by using T4-DNA ligase. The ligation preparation, containing 1U T4 DNA ligase, 1×ligation buffer and a 1:1 (mole ratio) mixture of insert and linearized vector (about 100 ng) in a final volume of 10 µL, was incubated at room temperature for 2 h or at 16 °C or 4 °C overnight.

7.4. DNA isolation

Isolation of plasmids from E. coli

Mini-preps employing alkaline lysis were used to isolate recombinant plasmids from *E. coli* for routine screening. 5 mL LB-medium was inoculated with a single colony and grown overnight at 37 °C, 170 rpm. 4 mL of this culture were harvested by centrifugation (10000×g, 4 °C, 1 min) and resuspended in 200 µL solution MP1 by vortexing. The suspension was mixed with 400 µL solution MP2 by inversion and incubated at room temperature for 1-5 min. 300 µL solution MP3 was added and the mixture was incubated on ice for 5 min. After centrifugation (20,000×g, 4 °C, 15 min), the supernatant was poured into a fresh microfuge tube. The DNA was precipitated by addition of 0.7-fold isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µL 70% ethanol, air dried and resuspended in 50 µL distilled water or Tris-HCl pH 8 buffer. The mentioned solutions are listed in Table II.5.
Preparative isolation of plasmids from *E. coli* was carried out from 50 mL overnight culture. 2 mL MP1, 4 mL MP2 and 3 mL MP1 were applied as described above. The DNA was precipitated with 2.5 vol. ethanol instead of Isopropanol.
For the isolation of cosmids a similar protocol was used. In this case, after addition of solution MP2, the suspension was inverted and immediately treated with solution MP3.

Isolation of genomic DNA from Streptomyces coelicolor

Genomic DNA was isolated by the following procedure (Kieser *et al.*, 2000). 2 mL of a 2-day-old culture in TSB medium were harvested by centrifugation (17,000×g, 4 °C, 1 min). The cells were washed with 0.5 mL 0.3 M sucrose and resuspended in 500 µL Lysozyme solution by vortexing. The suspension was incubated for 30 min at 37 °C. 250 µL of SDS solution were added and the mixture was vortexed vigorously for 1 min. 250 µL phenol/chloroform/isoamyl alcohol (25:24:1) were added and the mixture was vortexed vigorously for 15 s and centrifuged at 17,000×g and 4 °C for 10 min. The supernatant was poured into a fresh microfuge tube and extracted a second time with 250 µL phenol/chloroform/isoamyl alcohol (25:24:1) (1 min vigorous vortexing). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.1 volume of KAc solution and 0.8 volume of isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µL 70% ethanol, air dried and resuspended in 50 to 100 µL Tris-HCl pH 8 buffer.

A similar procedure was used for the isolation of high-molecular genomic DNA. In order to avoid mechanical forces on the DNA, mixing was done by gentle inverting instead of vortexing in all steps. Cells were harvested from a 50 mL culture and treated with 10 mL Lysozyme solution and 8ml SDS solution. Extraction was done at least 5 times with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) until the aqueous phase appeared clear.

7.5. PCR amplification

General conditions

PCR amplifications were carried out with the GeneAmp® PCR System 2400 or GeneAmp® PCR-System 9700 (Perkin-Eimer, Weiterstadt, Germany).

Table II.6.1 PCR reaction and amplification conditions using the Expand High Fidelity PCR system

Substance	**Final concentration**
Reaction buffer (10×)	1×
DMSO	5% (v/v)
Template DNA	about 100 ng

Cyclus	**Temperatur e**	**Time**	**Cycles**
Hot start	94 °C	2 min	1
Denaturing	94 °C	45 s	30
Annealing	**50-60 °C**	45 s	

dNTPs	0.2 mM each
Primer	50 pmol each
DNA-Polymerase	2.5 U
Add distilled water to make up to 50 µl	

Elongation	72 °C	90 s	
Final elongation	72 °C	5 min	1
End	4 °C	∞	1

For PCR amplification with *Taq* or *Pfu* polymerase, the PCR mixture (50-100 µL) contained 50 pmol each primer, 100-300 ng template DNA, 0.2 mM each dNTP, 1× reaction buffer, 5% (v/v) DMSO and 2-3 U polymerase. Amplification conditions were according to the supplier's instructions.
In general, 5 to 10 µL of the PCR reaction were analysed by gel electrophoresis.

Conditions for the amplification of resistance cassettes from pIJ773, pIJ774, pIJ778 and pIJ10700

The conditions for amplification of a resistance cassette from pIJ773, pIJ774, pIJ778 and pIJ10700 (REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003)) using the Expand High Fidelity PCR system (Roche) are given in Table II.21. Template DNA was prepared by digesting about 10 µg of the respective plasmid with *Kpn*I and *Sac*II, and by isolating the respective cassette fragment from an agarose gel (Gust *et al.*, 2003).

Table II.6.2 Conditions for amplification of resistance cassettes

Substance	**Final concentration**
Reaction buffer (10×)	1×
DMSO	5% (v/v)
Template DNA	about 100 ng
dNTPs	0.2 mM each
Primer	50 pmol each
DNA-Polymerase	2.5 U
Add distilled water to make up to 50 µl	

Cyclus	**Temperatur e**	**Time**	**Cycles**
Hot start	94 °C	2 min	1
Denaturing	94 °C	45 s	10
Annealing	**50 °C**	45 s	
Elongation	72 °C	90 s	
Denaturing	94 °C	45 s	15
Annealing	**55 °C**	45 s	
Elongation	72 °C	90 s	
Final elongation	72 °C	5 min	1
End	4 °C	∞	1

7.6. Introduction of DNA in *E. coli*

The methods described in this section were modified from (Sambrook & Russell, 2001).

Preparation of electro-competent cells

50 mL LB-medium was inoculated with 1 mL of an overnight culture of *E. coli* and cultivated at 30 to 37 °C (see note below), 170 rpm till the OD_{600} reached 0.6 (2.5-4 h). The cells were harvested by centrifugation (3,000×g, 4°C, 5 min), and washed twice with 50 and 25 mL ice-cold 10% (w/v) glycerol solution, respectively. The cell pellet was suspended in the remaining drops after discarding the supernatant. Competent cells could be used immediately or dispensed in 50-µL aliquots in 1.5-mL microfuge tubes, and stored at -70 °C.

Electroporation

DNA (about 100 ng in 1-2 µL distilled water) was added to 50 µL competent cells in 1.5-mL microfuge tube and incubated on ice for about 1 min. The mixture was then carefully transferred to an ice-cold electroporation cuvette (0.2 cm), avoiding formation of air bubbles, and electroporation was carried out using a BioRad electroporator set to 2.5 kV. The optimal time constant is 4.5 – 5.0 ms. 1 mL LB medium was immediately pipetted into the cuvette, and the suspension was transferred to a microfuge tube and incubated on a water bath or on a shaker (170 rpm) for 1 h at 30 to 37 °C. The mixture was spread on LB agar plates containing the appropriate antibiotic(s) (no more than 200 µL per plate) and the plates were incubated at 30 to 37 °C.

Note: To maintain plasmid pIJ790 in *E. coli* BW25113 (REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003)), cells must be cultured at 30 °C, since pIJ790 contains a temperature sensitive origin of replication. Otherwise, the cultivation temperature was 37 °C.

Blue/white selection

If a *lacZ*α-containing cloning vector was used to prepare the recombinant plasmid, blue/white selection can facilitate the identification of the expected clones. For this purpose, first 15 µL of IPTG solution (80 mg/mL) in up to 100 µL H_2O (sterile) were pipetted on the top of the plates and spread evenly, and then 60 µL of X-Gal solution (20 mg/mL in DMF) was plated in the same way. The plates were air dried under the

laminar flow for 30-45 min in order to evaporate the toxic DMF. Colonies containing the recombinant plasmid lack β-galactosidase activity and remain white.

7.7. Site-directed mutagenesis

Cpz4 variants were generated by site-directed mutagenesis using plasmid pLK03 as template and primer His180Leu_fw GCGGGAGCCGATCTCCTTGACA TGCAGATCACT and His180Leu_rv AGTGATCTGCATGTCAAGGAGATCGGCT CCCGC, His253Leu_fw CCGTATGACTACGTCCTCATCAACTCCATGTCC and His253Leu_rv GGACATGGAGTTGATGAGGACGTAGTCATACGG, His308Leu_fw TCCTTCGCCTGGCAGCTCGACGTGAGCCGGGAA and His308Leu_rv TTCCCGG CTCACGTCGAGCTGCCAGGCGAAGGA. PCR and subsequent *Dpn*I restriction was performed according to the QuickChange™ manual (Stratagene, Heidelberg, Germany). *cpz4* variants were verified by sequencing and cloned into vector pHis8 (Jez *et al.*, 2000).

7.8. PEG-mediated protoplast transformation for introduction of DNA in *Streptomyces*

Preparation of protoplasts from Streptomyces

Slightly modified from (Kieser *et al.*, 2000). Mycelium from a 40 h old culture (50 mL TSB medium containing 0.4 % glycine) was washed twice with 15 mL of a 10.3% sucrose solution, resuspended in 10 mL of lysozyme solution (2 mg/mL in P buffer) and incubated at 30 ºC for 15-60 min with gentle agitation. Protoplast formation was monitored using the microscope. After most cells became protoplasts, the reaction was stopped by incubation on ice. The following steps were carried out on ice. 10 mL of ice-cold P buffer were added and the suspension was drawn in and out of a 10 mL pipette three times and filtered through glass wool. Protoplasts were sedimented gently by centrifugation (e.g. 1,000xg, 7 min). The supernatant was discarded, the pellet was first carefully resuspended in the remaining drop of liquid by tapping the tube, and then in 1 mL P buffer. The protoplast suspension can be immediately used for transformation or 100 µL aliquots can be stored at -70 ºC. To freeze protoplasts for storage, tubes were placed in ice contained in a plastic beaker, and the beaker was placed at -70 ºC overnight. To assess the protoplast regeneration, dilution series of the protoplast suspension in P buffer were prepared and plated on R5 agar plates. The plates were incubated at 30 °C for 3-7 days. The regenerable protoplasts per mL

suspension were calculated. To assess the proportion of non-protoplasted units in the suspension, samples were also diluted in distilled water and plated on regeneration plates (R5 agar).

Transformation

The transformation of *Streptomyces* strains was carried out by a modification of the method described by Kieser *et al.* (2000).

Before transformation of *Streptomyces* strains the plasmids were propagated in *E. coli* ET 12567 to bypass methyl-sensing restriction.

1-20 µg DNA (in maximal 10-20 µL Tris buffer) were added to 100-200 µL of a protoplast suspension, containing at least 10^8 protoplasts per mL; 400-500 µL T-buffer containing PEG 1000 (25% (w/v) were immediately added, mixed by pipetting carefully three times and incubated at room temperature for 1 min. Increasing volumes of the resulting suspension (e.g. 10 µL, 100 µL, 200 µL, rest) were mixed with warm R5 soft agar (about 50 °C, 4×3 mL) and plated on four R5 plates. After 16-24 h incubation at 30 °C, the plates were overlaid with 1 mL of dH_2O including the required antibiotics for selection of mutants, and incubation was continued for further 3-7 days.

7.9. Intergeneric conjugation for introduction of DNA in *Streptomyces*

Intergeneric conjugation was performed for the transfer of modified cosmid DNA into *Streptomyces*. Therefore, the cosmid (containing an *ori*T) was introduced into the non-methylating strain *E. coli* ET12567/pUZ8002 harbouring the *tra*-genes required for mobilization and transfer of circular DNA. A culture of *E. coli* ET12567/pUZ8002 containing the cosmid was grown in LB supplemented with the appropriate antibiotics on 37°C until OD_{600} 0.6. The cells were harvested by centrifugation (3,000×g, 4°C, 5 min), and washed twice with 25 mL ice-cold LB medium without antibiotics. The cell pellet was suspended in LB to give a final OD_{600} of 0.5. In parallel 50 µL of spores of the target Streptomyces strain were incubated in 500 µL 2YT medium for 10 min at 50°C and incubated on ice for 5 min. 500 µL of the *E. coli* ET12567/pUZ8002 suspension was added and centrifuged (3,000×g, 4°C, 2 min). The supernatant was discarded and the pellet resuspended in 200 µL LB. The suspension was spread on four MS agar plates supplemented with 10 mM $MgCl_2$. After 16-24 h incubation at 30 °C, the plates were overlaid with 1 mL of dH_2O including the required antibiotics for selection of mutants, and incubation was continued for further 3-7 days.

For triparental conjugation cosmid DNA was introduced into *E. coli* ET12567. Two parallel cultures, one with *E. coli* ET12567 containing the cosmid and one with *E. coli* ET12567/pUB307 were set up as described above. After washing and resuspension of the cells, 250 µL of each *E. coli* culture was added to 500 µL 2YT medium containing the *Streptomyces* spores.

7.10. Generation and screening of a cosmid library

Genomic cosmid libraries were constructed from the caprazamycin producer strain *Streptomyces* sp. MK730-62F2 and from the liposidomycins producer strain *Streptomyces* sp. SN-1061M. In both cases chromosomal DNA was prepared and ligated into the SuperCos1 vector according to the manufacturer's instructions (SuperCos1 cosmid vector kit, Stratagene, Heidelberg).

Preparation of SuperCos1 vector DNA

20 µg of the cosmid vector DNA was linearized by *Xba*I digestion to dissect the cos-sites. After phenol/chloroform extraction and precipitation the vector DNA solution (1 µg/µL) was treated with 2 µL Calf Intestinal Alkaline Phosphatase (CIAP). Again the DNA was extracted with phenol/chloroform and precipitated. The vector DNA was than resuspended to a 1 µg/µL solution and digested with BamHI to dissect the cloning sites. After another round of phenol/chloroform extraction and precipitation the vector was ready to use.

Preparation of chromosomal DNA

Preparation of the chromosomal DNA started with partial digestion by *Bsp*1431. The digestion was checked by agarose gel electrophoresis to result in DNA fragments of ~40 kb. The partially digested chromosomal DNA was extracted with phenol/chloroform, precipitated and treated with CIAP. Again the DNA was extracted with phenol/chloroform, precipitated and resuspended to a 1 µg/µL solution.
Subsequently, the partially digested chromosomal DNA was ligated with the *Bam*HI sites of the digested SuperCos1 DNA. The ligation was checked by agarose gel electrophoresis.

Packaging

The packaging was performed using Gigapack® III XL Packaging Extract (Stratagene, Heidelberg, Germany). This kit packages preferentially large inserts (i.e., 40- to 51-Kb recombinants). *E. coli* SURE® was used as host strain. After transfection, the cells were plated on LB with carbenicillin (50 µg/mL) plates and incubated overnight at 37°C. 3000 carbenicillin resistant *E. coli* clones were obtained from genomic DNA of *Streptomyces* sp. MK730-62F2 and 1100 clones from *Streptomyces* sp. SN-1061M.

Screening for the caprazamycin gene cluster

A 0.45 kb fragment of *cpz28* was amplified from genomic DNA of *Streptomyces* sp. MK730-62F2 using primer omt1CH_fw CCGTCCGCTACGGCTCNSMNAARTGG and omt1CH_rv GCGGTCCACAGGTCCTCNAYNACRTA. Perfect matching primers were deduced from the sequence of the obtained fragment. Oligonucleotides omt7218_fw GGCTGCACTGGTTCACGGG and omt7218_rv CCAGAGGTCCTCGATCACG amplifying a 0.39 kb fragment of *cpz28* were applied in a PCR screening of the cosmid library. Clones were pooled in order to facilitate the screening procedure. In the first round, pools of 48 clones were screened, followed by a screening of subpools of 8 clones and eventually the testing of single colonies.

Screening for the genes for dTDP-L-rhamnose biosynthesis

A 0.5 kb fragment of *cpzDIII* was amplified from genomic DNA using degenerated primer as described by Decker et al. (Decker *et al.*, 1996). Perfect matching primers GDHspf_fw CGTAGTTGTTGGAGCAGCGCG and GDHspf_rv GGTTCATCGGCTCCCGCTACG amplifying a 0.5 kb fragment of *cpzDIII* were applied in a PCR screening of the cosmid library as described above.

Screening for the liposidomycin gene cluster

LipI was amplified from genomic DNA of *Streptomyces* sp. SN-1061M using primer MT2_fw GTGACGAACTTGGTGGCGC and MT2_rv TCACCGGCTCACCCGGAAC. Perfect matching primers MTLip_fw ACCTACTCGCTGCTGTTC and MTLip_rv TTGGAGATGCTCGACCAC amplifying a 0.42 kb fragment of *lipI* were applied in a PCR screening of the cosmid library as described above.

7.11. DNA sequencing and computer-assisted sequence analysis

Double-stranded sequencing of recombinant plasmids was done by the dideoxynucleotide chain termination method on a LI-COR automatic sequencer (MWG-Biotech AG, Ebersberg, Germany).

Double-stranded sequencing of entire cosmid clones (~40 000 bp insert) was performed by GenoTech (Baejeon, Korea) by using a shotgun library with DNA fragments of approximately 0.5–1.0 kb in length.

The DNASIS software package (Hitachi Software Engineering, Tokyo, Japan) and Artemis (Wellcome Trust Genome Campus, Cambridge, UK) were used for sequence analysis and annotation. Database comparisons were carried out in the GenBank database by using the BLAST program (Altschul *et al.*, 1997). Alignment and comparison of sequences were performed using ClustalX algorithm (Jeanmougin *et al.*, 1998) and GeneDoc alignment editor (http://www.psc.edu/biomed/genedoc).

8. Methods of biochemistry and biology

8.1. Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous SDS-PAGE was carried out according to the method of Laemmli (1970). Probes were mixed with sample buffer in ration 1:1 to 1:4 and incubated for 1 min in boiling water. 4% and 12% Polyacrylamide gel were used as stocking and resolving gel, respectively. Gel electrophoresis was carried out with working voltage of 200 V using the Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad, München, Germany). Protein bands were stained with Coomassie Brilliant Blue G-250.:1-5 min in Fixing buffer, 15 min in Coomassie Brilliant Blue G-250 solution, and 90 min in Bleaching solution. To determine protein sizes, the Low Molecular Weight Calibration Kit for SDS gel electrophoresis (Amersham Biosciences, Freiburg, Germany) was used.

8.2. Heterologous overproduction and purification of recombinant protein from *E. coli*

Heterologous overproduction and purification of 8×His-tagged proteins from *E. coli* were carried out using expression vector pHis8 and *E. coli* Rosetta2pLys (Novagen, Darmstadt, Germany) as the recombinant host strain. Different conditions for cultivation and purification were tested (e.g. different cultivation temperatures,

amount of IPTG used for induction). The preparative method described bellow yielded the best results.

Cloning and purification of Cpz31

cpz31 was amplified from cosmid DNA using the primers cpzGTEco_fw GAATTCATGCGCGTGCTCTTCACG and cpzGTHind_rv AAGCTTCTAGGCCCTCC TCGCCAG (restriction sites for *Eco*RI and *Hind*III are underlined). A 1.2 kb PCR product was cloned into the pGEM®-T cloning vector (Promega, Mannheim, Germany). The resulting plasmid pLK01 was verified by sequencing. *cpz31* was subsequently cloned into the expression vector pHis8 (Jez *et al.*, 2000) taking advantage of the *Eco*RI and *Hind*III restriction sites and confirmed by sequencing. The resulting plasmid was named pLK02.

E. coli Rosetta2™ (DE3)pLys containing plasmid pLK02 were cultivated in 1 l TB broth supplemented with 50 µg mL^{-1} kanamycin and 50 µg mL^{-1} chloramphenicol at 37°C. At OD_{600} 0.7 the temperature was adjusted to 20°C and isopropyl thiogalactoside (IPTG) was added to 0.5 mM final concentration. After additional 10 h cultivation at 20°C the culture was harvested and 10 mL Buffer A supplemented with 0.5 mg mL^{-1} lysozyme and 0.5 mM PMSF was added to the pellet (12 g). Cells were disrupted by sonication (Branson, Danbury, CT) at 4°C. The lysate was centrifuged (55000×g, 45 min) and the supernatant was applied to affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 4 mL of the resin was loaded onto a column and equilibrated with 20 mL Buffer A. Subsequently the column was loaded with the lysate and washed using 25 mL of Buffer A. His_8-Cpz31 was eluted from the column with 6 mL 250 mM imidazole in Buffer A. Buffer exchange was carried out with PD10 columns (Amersham Bioscience, Freiburg, Germany) and Buffer A without *β*-mercaptoethanol and imidazole. The protein was concentrated by centrifugation using the Centriprep YM-10 columns (Millipore, Billerica, US). Low solubility of His_8-Cpz31 resulted in a yield of 0.46 mg of partially purified protein per litre culture. The protein was stored at -80°C in aliquots of 50 µL.

Heterologous overproduction and purification of the Cpz4 derivatives obtained from site-directed mutagenesis were performed similarly.

Cloning and purification of Cpz4

cpz4 was amplified from cosmid 31C2 containing the caprazamycin gene cluster (Kaysser *et al.*, 2009). Primers cpz4Sac_fw 5'-GAGCTCATGACTGTGTTGGTCCGCAG-3' and cpz4Xho_rv 5'-CTCGAGTCAGCGCACCCGGGAGGCCT-3' (restriction sites for *Sac*I and *Xho*I are underlined) were used. The PCR product with 1.6 kb was cloned into the pGEM®-T cloning vector (Promega, Mannheim, Germany). The resulting plasmid pLK03 was verified by sequencing. The gene was subsequently cloned into the expression vector pHis8 (Jez *et al.*, 2000), taking advantage of the *Sac*I and *Xho*I restriction sites, and confirmed by sequencing. The resulting plasmid was named pLK04. *E. coli* Rosetta2™ (DE3)pLys containing pLK04 was cultivated in 2 L TB broth as described for Cpz31. Induction of the Cpz4-expression with IPTG, and harvesting and disruption of the cells were performed analogous to Cpz31. The supernatants of the lysates were applied to affinity chromatography using an Äktapurifier™ platform (GE Healthcare, Freiburg, Germany) equipped with a HisTrap™ (34 µm, 1.6 x 2.5 cm) HP column (GE Healthcare, Freiburg, Germany). The His-tagged proteins were eluted from the column using a linear gradient from 0-100% imidazole (250 mM) in Buffer A over 60 min and collected by a Frac-920™ system (GE Healthcare, Freiburg, Germany). Fractions were checked for the presence of the desired proteins by SDS-PAGE and further purified by gel filtration, using a Superdex 200 (*CV* 120 mL) column (GE Healthcare, Freiburg, Germany), on the platform described above. A yield of 12.5 mg of purified His_8-Cpz4 was obtained per litre culture. Cpz4 was stored at -80°C in aliquots.

8.3. In vitro studies on Cpz31

Caprazamycin aglycones were obtained from cultures of *S. coelicolor* M512/cpzLK09 by butanolic extraction, evaporation of the organic solvent and resuspension in methanol (as described in sections II.5.2 and II.9.1). 5 µL of this methanolic solution containing partially purified caprazamycin aglycones were applied to a reaction tube, air dried and dissolved in 50 µL reaction mixture containing 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM dTDP-L-rhamnose and 9 µM protein. dTDP-L-rhamnose was kindly provided to us by Prof. Jae Kyung Sohng (Asansi, Korea) The assay was performed at 30°C for 2 h and stopped by the addition of 150 µL methanol. The activity of His_8-Cpz31 was measured by LC-ESI-MS.

8.4. In vitro studies on Cpz4 using aromatic substrates

Assays for the sulfotransferase activity of Cpz4 and mutants were performed at 30°C using phenol as sulfate acceptor substrate. Conversion of pNS as sulfate donor substrate was measured on a UV/VIS Spectrometer Lambda™ 25 (Perkin Elmer, Waltham, United States) at 405 nm. For this purpose, assays were adjusted to pH 8 after incubation by the addition of 500 µL 1 M Tris-HCl pH 8. Conversion of MUS as sulfate donor was monitored on a Synergy™ HT Multi-Detection Microplate Reader (BioTek, Bad Friedrichshall, Germany) at 460 nm (excitation 360 nm). Both systems were calibrated with at least 5 different concentrations of *p*-nitrophenol or methylumbelliferone for each single experiment. Sulfatase activity of Cpz4 was checked in parallel control reactions without phenol for each single assay. All experiments were run as duplicates and included a duplicate control assay lacking the protein. The reactions were started by the addition of Cpz4.

Analysis of Cpz4 as arylsulfate sulfotransferase

For the analysis of the Cpz4 products by LC-ESI-MS, 500 µL containing 50 mM Tris-HCl pH 7.4, 50 mM MES pH 7.4, 140 mM NaCl, 0,1 mM EDTA, 10 mM Phenol, 750 nM Cpz4 and 100 µM pNS or MUS were incubated overnight. The reactions were stopped by the addition of 1 volume of methanol and analysed by LC-ESI-MS.

Dependency of the reaction on Mg2+, Ni2+ and EDTA

800 µL of 20 mM Tris-HCl pH 8, 140 mM NaCl, 10 mM Phenol and 25 µM pNS with 750 nM Cpz4 contained either 1-50 mM $MgCl_2$ or 2 mM $NiCl_2$ or 0.1 mM, 1 mM or 10 mM EDTA. Incubation time was 60 min.

Dependency of the reaction on pH

Assays of 500 µL contained 1 M NaCl, 0.1 mM EDTA, 10 mM Phenol, 25 µM pNS and 250 nM Cpz4. The reactions were buffered either with 100 mM MES for pH 4, 5, 6, 7 or 80 mM Tris-HCl and 20 mM glycine for pH 8 or 60 mM Tris-HCl and 40 mM glycine for pH 9 or 100 mM glycine for pH 10, 11. The reaction solutions were adjusted to the respective pH values (pH 4-11) prior to the addition of protein. Incubation time was 60 min.

Dependency of the reaction on protein concentration and time

For the reaction with pNS as sulfate donor substrate an assay of 500 µL contained 100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA, 75 mM Phenol, 300 µM pNS and 50-3000 nM Cpz4. Incubation time was 60 min. For the reaction with MUS as sulfate donor substrate an assay of 200 µL contained 100 mM MES pH 6.7, 1 M NaCl, 0.1 mM EDTA, 60 mM Phenol, 450 µM MUS and 25-450 nM Cpz4. Incubation time was 15 min.

Dependency of the reaction on time

For the reaction with pNS as sulfate donor substrate an assay of 500 µL contained 100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA, 75 mM Phenol, 300 µM pNS and 125 nM Cpz4. Conversion of pNS was measured every 15 min over 7 hours of incubation. For the reaction with MUS as sulfate donor substrate an assay of 200 µL contained 100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA, 60 mM Phenol, 450 µM MUS and 100 nM Cpz4. Conversion of MUS was measured every 15 min over 5 hours of incubation.

Influence of inhibitors on Cpz4 activity

An assay containing 100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA, 75 mM Phenol, 300 µM pNS and 125 nM Cpz4 was supplemented either with 0%, 0.05%, 0.1% or 0.3% DEPC or 1 mM PMSF. Reaction volume was 500 µL and incubation time 60 min.

Analysis of Cpz4 mutants

Cpz4 mutants Cpz4_His180Leu, Cpz4_His253Leu and Cpz4_His308Leu, derived from site-directed mutagenesis, were analysed for the conversion of pNS in 500 µL reaction buffer containing 100 mM MES pH 6.7, 1 M NaCl, 0.1 mM EDTA, 75 mM Phenol, 300 µM pNS. ~90 nM of protein was added and incubated for 60 min.

Kinetic analysis of Cpz4

Reaction velocities of Cpz4 with pNS as donor substrate (27 – 270 µM) and phenol as acceptor substrate (6.5 – 160 mM) were measured in 500 µL reaction buffer (100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA) containing 125 nM protein. Incubation time was 25 min. Reaction velocities of Cpz4 with MUS as donor substrate (25 – 550 µM) and phenol as acceptor substrate (12.5 – 160 mM) were monitored over 15 min

in 200 µL reaction buffer (100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA) containing 100 nM protein. Initial rates were globally fitted with GraFit 6 (Erithacus Software, Surrey, United Kingdom) to the equation

$$v = V[A][B]/(K_a[B](1+[B]/K_{ib})+K_b[A]+[A][B]$$

describing ping pong kinetics with substrate inhibition (Malojcic *et al.*, 2008). [A] and [B] are the initial concentrations of pNS or MUS and phenol, respectively. K_a and K_b are the respective Michealis constants for pNS or MUS and phenol. The inhibition constant of phenol is K_{ib}. V is the maximum and v the measured initial velocity.

8.5. In vitro studies on Cpz4 using nucleosidic substrates

Sulfation of caprazamycin derivatives

Partially purified caprazamycin derivatives were obtained from culture extracts of *S. coelicolor* M512/cpzWP05 (CPZ aglycones), *S. coelicolor* M512/cpzEW07 (CPZs) and *S. coelicolor* M512/cpzLL06 (hydroxyacyl-caprazols) as described in section II.5.2 and II.9.1. Caprazamycins, caprazamycins aglycones or hydroxyacyl-caprazols were dissolved in 500 µL reaction buffer (100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA) containing 500 µM pNS and 1.5 - 2 µM Cpz4. Conversion of pNS was measured at 405 nm. The reactions were stopped by the addition of 1 volume of methanol. Product formation was analyzed by LC-ESI-MS.

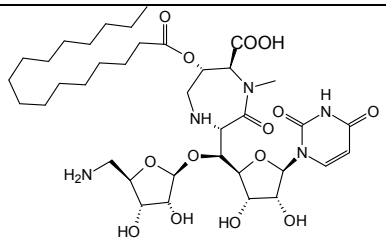
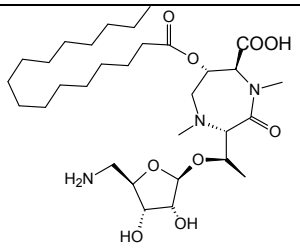
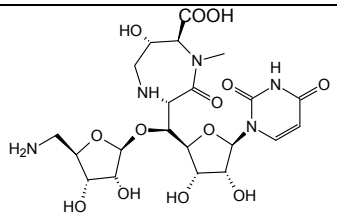
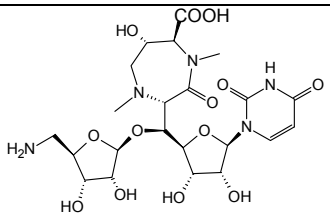
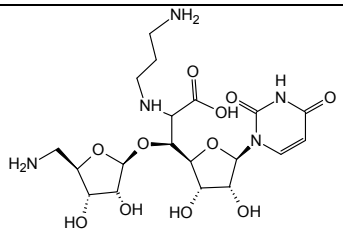
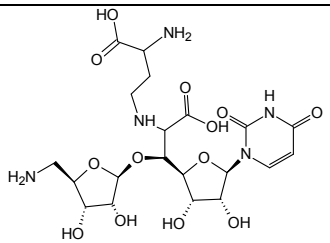
Investigations on PAPS as sulfate donor

Assays with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (500 µM) as sulfate donor substrate (instead of pNS) were performed under the same conditions as described above but included 0.1 mM sodium ascorbate to avoid oxidation. A control experiment showed that 0.1 mM sodium ascorbate in the assay had no effect on the activity of Cpz4.

Sulfation of synthetic nucleoside analogs

Different analogs of potential precursors for liponucleoside biosynthesis were synthesized by Satoshi Ichikawa and colleagues from the Faculty of Pharmaceutical Science, Hokkaido University, Japan as described in (Kaysser *et al.*, 2010). The compounds were kindly provided to us for the testing as substrates of Cpz4.

Table II.7.1 Synthetic nucleoside analogs kindly provided by Satoshi Ichikawa from the Hokkaido University, Japan

	
Compound 1: des-N-Me-palmitoylcaprazol **Exact Mass:** 799,4215	**Compound 2:** des-uridyl-palmitoylcaprazol **Exact Mass:** 615,4095
	
Compound 3: des-N-Me-caprazol **Exact Mass:** 561,1918	**Compound 4:** (+)-caprazol **Exact Mass:** 575,2075
	
Compound 5: N-aminopropyl-O-aminoribosyl-glycyluridine **Exact Mass:** 505,2020	**Compound 6:** N-carboxy-aminopropyl-O-aminoribosyl-glycyluridine **Exact Mass:** 549,1918

In order to assay the sulfation of the synthetic caprazamycin precursor, compounds **1** - **6** were added to a final concentration of 200 µM to the reaction buffer (100 mM MES pH 6.7, 1 M NaCl, 0,1 mM EDTA) containing 300 µM pNS and 125 nM protein. The reaction was measured at 405 nm after 60 min. The assays were stopped by the addition of 1 volume of methanol prior to LC-ESI-MS analysis of product formation.

8.6. Bioassay with Mycobacterium phlei

Antibacterial activity was determined by a disc diffusion assay using *Mycobacterium phlei* as the indicator strain. 10 µL methanolic culture extracts were applied to filter paper disks (Ø 6 mm; MN 440 B blotting paper, Macherey-Nagel), air dried under the clean bench and placed on the top of nutrient agar plates, which were spread with

Mycobacterium phlei for confluent growth. Plates were cultured overnight at 37 °C, and the diameters of growth inhibition zones measured.

9. Construction of *Streptomyces* mutant strains

Generally, manipulation of *Streptomyces* DNA was performed in *E. coli* using the PCR targeting system according to the instructions of the REDIRECT© technology kit (Gust *et al.*, 2003; Gust *et al.*, 2004). This strategy is to replace a chromosomal sequence within a genomic library clone (e.g. cosmid) by a selectable marker that has been generated by PCR using primers with homologous extensions. Amplification was performed using the Expand High Fidelity PCR system. The PCR product was introduced into the cells by electroporation. The recombination event in *E. coli* between the cosmid and the PCR-product is mediated by the λ-Red system (Red/ET-recombineering). Cosmids which had integrated the cassette were isolated and analyzed by restriction enzyme digestion. The inclusion of *oriT* in the disruption cassette allows the conjugation of the modified cosmid DNA from *E. coli* into the actinomycete. The PCR-targeting cassettes are flanked by FLP recognition target (FRT) sites which can be used to remove the resistance marker by the co-expression of the mutant cosmid and the FLP-recombinase in *E. coli*. The residual scar sequence (81 bp) represents a nonpolar in-frame gene deletion and allows the repeated use of the same cassette for making multiple knock-outs. Alternatively, restriction sites can be introduced into the PCR primers and later used to excise the resistance marker by restriction and relegation.

9.1. Deletion of cpz28-30 in *Streptomyces* sp. MK730-62F2

An apramycin resistance *(aac(3)IV)* cassette was amplified from plasmid pIJ773 using primers KOomtbox_fw TTCTCGAAGACGAACCTCACCAGGGAGTTGCT GAAGATGACTAGT**ATTCCGGGGATCCGTCGACC** and KOomtbox_rv CAGCCCG GGCGACGTCGTGAAGAGCACGCGCATGGCTCAACTAGT**TGTAGGCTGGAGCTG CTTC** with flanking regions homologous to the target sequence. Priming sequence for pIJ773 is shown in bold, SpeI restriction sites are underlined. The genes were replaced in *E. coli* BW25113/pIJ790/31C2 by using the PCR-targeting system (Gust *et al.*, 2003). Resulting cosmid cpzLK02 was confirmed by restriction analysis. *Spe*I digestion and religation led to the excision of the cassette resulting in cosmid cpzLK04 which was further verified by restriction analysis and PCR using

omtboxtest_fw CCTCACCAGGGAGTTGCTG and omtboxtest_rv CGACGTCGTGAAGAGCACG as primers. Cosmid cpzLK04 was introduced into *S.* sp. MK730-62F2 by PEG-mediated protoplast transformation with non-methylated DNA isolated from *E. coli* ET12567. Double-crossover mutants were confirmed by their kanamycin sensitivity, checked by PCR and designated as *Streptomyces* sp. MK730-62F2 *Δcpz28-30* (1-3).

9.2. Inactivation of *cpzDIII* in *Streptomyces* sp. MK730-62F2

An apramycin resistance cassette *(aac(3)IV)* was amplified from plasmid pIJ773 (Gust *et al.*, 2003) using primer pair gdhKO_fw GTGCTGGACAAGCTCACCTACGC CGGCACCCTCGACGAC**ATTCCGGGGATCCGTCGACC** and gdhKO_rv GAGCTG GCACGGGCCGTAGTTGTTGGAGCAGCGCGTGAC**TGTAGGCTGGAGCTGCTTC**. Priming sequence for pIJ773 is shown in bold. The gene was replaced in *E. coli* BW25113/pIJ790/4H11 by using Red/ET-mediated recombination (Gust *et al.*, 2003) and the resulting cosmid cpzLK01 was confirmed by restriction analysis. cpzLK01 was transferred into the non-methylating strain *E. coli* ET12567/pUZ8002 (Paget *et al.*, 1999) and introduced into *Streptomyces* sp. MK730-62F2 by conjugation. Exconjugants, resistant to apramycin, were isolated and tested for the loss of their kanamycin resistance indicating a successful double-crossover. The mutants were further analysed by PCR with chromosomal DNA as template. Mutant strains were designated as *Streptomyces* sp. MK730-62F2*ΔcpzDIII*-(1-3).

9.3. Heterologous expression of the caprazamycin and the liposidomycin gene cluster

Plasmid is a SuperCos1 derivative, in which the ampicillin resistance gene (*bla*) was replaced by a cassette containing the integrase gene (*int*) and attachment site (*attP*) of phage ϕC31 as well as a resistance marker (*tet*, tetracycline resistance gene) (Eustaquio *et al.*, 2005).

The *Dra*I-*Bsa*I-fragment of pIJ787, containing the integrase cassette and flanked by about 100 bp *bla* sequence on one site and about 300 bp *bla* sequence on the other side was isolated. This fragment was used to replace the respective *bla* gene in the SuperCos1 backbone of cosmids 31C2 carrying the caprazamycin gene cluster and 3G5 carrying the liposidomycin gene cluster in *E. coli* BW25113/pIJ790, via λ-Red-mediated recombination (Datsenko and Wanner, 2000; Gust *et al.*, 2003). The generated cosmids were termed cpzLK09 and lipLK01 respectively.

The modified cosmid cpzLK09, still carrying the kanamycin resistance gene *neo*, was then introduced into *S. coelicolor* M512 via PEG-mediated protoplast transformation. Kanamycin resistant clones were selected, checked by PCR and designated as *S. coelicolor* M512/cpzLK09 (1-3). Cosmid lipLK01 was introduced into *S. coelicolor* M512 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 (Flett *et al.*, 1997). Kanamycin resistance clones were selected, confirmed by PCR and designated as *S. coelicolor* M512/lipLK01 (1-3).

9.4. Introduction of pRHAM into *Streptomyces* mutant strains

Plasmid pRHAM (Rodriguez *et al.*, 2000), containing all required genes for the biosynthesis of dTDP-L-rhamnose *oleL*, *oleS*, *oleE* and *oleU* from *S. antibioticus* ATCC 1891, was kindly provided to us by Prof. Jose A. Salas (Oviedo, Spain). pRHAM was introduced into the non-methylating strain *E. coli* ET12567 (MacNeil, 1988) and re-isolated. The isolated plasmid DNA was used for PEG-mediated protoplast transformation with either *Streptomyces* sp. MK730-62F2*ΔcpzDIII* for complementation of the *cpzDIII* knock-out or with *S. coelicolor/cpzLK09* for the production of intact caprazamycins. Thiostrepton resistance mutants were selected and termed as either *Streptomyces* sp. MK730-62F2/pRHAM-(1-3) or *S. coelicolor*/cpzLK09/pRHAM-(1-3).

9.5. Deletion of genes for the determination of the boundaries of the caprazamycin gene cluster

An apramycin resistance *(aac(3)IV)* cassette was amplified from plasmid pIJ773 (Gust *et al.*, 2003) using primer pairs B4_rv (CACCCTCCACACCCGCACCACCCG ACTGGCCCACGCCCTACTAGT**TGTAGGCTGGAGCTGCTTC**)/B5_fw (CGGCAAC CGCCGGTTCCTGACCGTCCACGTCCGAGGGAGACTAGT**ATTCCGGGGATCCGT CGACC**) (*Δcpz1-4*), B4_rv/B6_fw (AACCGGGACAACGAGACAGCAAAGGTCG GTGTTTGGGACACTAGT**ATTCCGGGGATCCGTCGACC**) (*Δcpz1-5*), B4_rv/B7_fw (TGCCGGCCATCACAGTGACCGGCTTCGCAACGTGTACGGACTAGT**ATTCCGGG GATCCGTCGACC**) (*Δcpz1-6*), B1_rv (TCGCCGCGGTGGCGCACCACGACGAA CTGGTGCCGCGGGACTAGT**TGTAGGCTGGAGCTGCTTC**)/B1_fw (AACCCGGGC CATCCGGCCGACGCTCCGTCGAGGCCGCACACTAGT**ATTCCGGGGATCCGTC GACC**) (*Δcpz33-34*) and B1_rv/B2_fw (TGCGCGCCGGGTGGCTGCCCTGCCCC CCAGGGCCATCGGACTAGT**ATTCCGGGGATCCGTCGACC**) (*Δcpz32-34*). Priming sequence for pIJ773 is shown in bold, *Spe*I restriction sites are underlined.

The genes were replaced in *E. coli* BW25113/pIJ790/cpzLK09. Resulting cosmids were confirmed by restriction analysis. Excision of the cassette was performed in *E. coli* BT340 taking advantage of the FLP/FRT recognition sites adjacent to the resistance cassette (Cherepanov & Wackernagel, 1995). Positive cosmids were screened for their apramycin sensitivity and verified by restriction analysis and PCR using primers B1_test_rv (CGGGACTAGTTGTAGGCTG), B1_test_fw (GCACTTCGACGGTGCG GTG), B2_test_fw (GTCCTGGCCGACTGGTTGC), B4_test_fw (CACCGAATACGGC ACCCTC), B5_test_rv (CTTCGCCGGGACTCTCGTC), B6_test_rv (CGGCAGCGTC CAGGAGGTG), B7_test_rv (CGAGGACGGCGGGCAGTAC). Cosmids cpzWP01 (*Δcpz33-34*), cpzWP02 (*Δcpz32-34*), cpzWP05 (*Δcpz1-4*), czWP06 (*Δcpz1-5*) and cpzWP07 (*Δcpz1-6*) were transferred into *E. coli* ET12567 (MacNeil, 1988) and introduced into *S. coelicolor* M512 by triparental intergeneric conjugation. Kanamycin resistance clones were selected, confirmed by PCR and designated as *S. coelicolor* M512/cpzWP01 (1-3), *S. coelicolor* M512/cpzWP02 (1-3), *S. coelicolor* M512/cpzWP05 (1-3), *S. coelicolor* M512/cpzWP06 (1-3) and *S. coelicolor* M512/cpzWP07 (1-3).

9.6. Generation of single gene deletions on cpzLK09

Deletion mutants for the genes *cpz11*, *cpz20*, *cpz21*, *cpz23*, *cpz25* and *cpz26* were generated in accordance to the generation of the mutants for the detection of the cluster boundaries. Primer pairs cpz11_rv (TCGGCAGCGCCCAGGGGCCCGTGAC GACGATGATCACCGACTAGT**TGTAGGCTGGAGCTGCTTC**)/cpz11_fw (GCGAGT CGAAGTAGCGGCCGGAGGAGTCGGGGCGTGACGACTAGT**ATTCCGGGGATCC GTCGACC**), cpz20_F (TCCGCACCGACGAAACGGGGACCGTTCATGTCTGACG GGACTAGT**ATTCCGGGGATCCGTCGACC**)/cpz20_R (GGACCTTCTGCCCGTCTC GGCCGTTCGCCATCAGTAGATACTAGT**TGTAGGCTGGAGCTGCTTC**), cpz21_fw (GTTCGAAGGCAGTCGGGTAGTCGAGGGGATCTACTGATGACTAGT**ATTCCGGG GATCCGTCGACC**)/cpz21_rv (CCATCCATGACGGCGACACCCGCACTTCCGTG CTCACCAACTAGT**TGTAGGCTGGAGCTGCTTC**), cpz23_fw (AGCAACGAGCAAC CCAGGACAAGGAGACGAAGCGTGAAGACTAGT**ATTCCGGGGATCCGTCGACC**)/ cpz23_rv (CATGTGTGGCTGTTCGTCACGGGGCCGAGCGGTTCATCCACTAGT**T GTAGGCTGGAGCTGCTTC**), cpz25_F (TGGGAACGCCATCCGGCAGCCCGAGG ACGACGACTCATGACTAGT**ATTCCGGGGATCCGTCGACC**)/cpz25_R (TGGTCT GCATGTGCGCTCCGCGCGGGTCCTGGGCCGTCAACTAGT**TGTAGGCTGGAGC**

TGCTTC) and cpz26_rv (TGATCCACACCAGCAAGGGCTTCTCCGTCCTGGT CACGTACTAGT**TGTAGGCTGGAGCTGCTTC**)/cpz26_fw (CGGAGCGCACATGCA GACCATGTTCCAGAAAGGGTGATGACTAGT**ATTCCGGGGATCCGTCGACC**) were used to amplify the apramycin resistance cassette from pIJ773. The resulting mutants were designated *S. coelicolor* M512/cpzLL05 (1-3) (*Δcpz11*), *S. coelicolor* M512/cpzLL06 (1-3) (*Δcpz21), S. coelicolor* M512/cpzLL07 (1-3) (*Δcpz23*) and *S. coelicolor* M512/cpzLL08 (1-3) (*Δcpz26)*.

9.7. Complementation of single gene deletions

To generate the expression plasmids for mutant complementation *cpz11*, *cpz21* and *cpz23* were amplified from cosmid cpzLK09 using primer pairs (restriction sites are underlined) cpz11Hind_fw (AAGCTTGTGACGAACTTGGTGGCGC)/cpz11Eco_rv (GAATTCTCACCGGCTCACCCGGAAC), cpz21Eco_fw (GAATTCATGGCGAACGG CCGAGACG)/cpz21Hind_rv (AAGCTTTCACCACCGGTGGTCCTCG) and cpz23Eco_fw (GAATTCGTGAAGTCGTTGACGCACG)/cpz23Hind_rv (AAGCTTTCA TCCGAGGCACTTCCGG) and cloned into the vector pGEM®-T (Promega, Mannheim, Germany). The genes were subcloned into the *Eco*RI/*Spe*I sites of the expression vector pUWL201 (Doumith *et al.*, 2000) under the control of the *ermE** promoter. This resulted in plasmids pLL05 (*cpz11*), pLL06 (*cpz21*) and pLL07 (*cpz23*) respectively. DNA sequencing of these plasmids confirmed the correct sequence of all constructs. Transformation of the *S. coelicolor* mutant strains by polyethylene glycol-mediated protoplast transformation finally generated the strains *S. coelicolor* M512/cpzLL05/pLL05 *S. coelicolor* M512/cpzLL06/pLL06 and *S. coelicolor* M512/cpzLL07/pLL07.

9.8. Assembly of the caprazamycin gene cluster and the genes cpzDI-cpzDVII onto one cosmid

An apramycin resistance cassette (*aac(3)IV*) was amplified by PCR from plasmid pIJ774 (Gust *et al.*, 2004) with primers 4H11_774_fw *GCCCGAGCACCCGCAGCC GGTTCGCCCGCGAGCCCAGCA*GCTAGCTTATGAGCAGCCAATCGAC, including a *Nhe*I restriction site (underlined) and 4H11_774_rv *CAGAGGGTGCGCTCGGAAG ACGGGCGCGGAAGCCGCCCG*ACTAGTATTCCGGGGATCCGTCGACC, including a *Spe*I restriction site (underlined). Italic letters represent 39 nt homologous extensions to an internal region of *orf1* and to a region downstream of *orf9* respectively. The resulting 1310 bp PCR product contained the *aac(3)IV* gene and a

single loxP site. The PCR-product was inserted into cosmid 4H11 by Red/ET-mediated recombination (Gust *et al.*, 2003) thereby replacing a 13531 bp region (*orf1* - *orf9*) upstream of *cpzDI* resulting in the apramycin and kanamycin resistant cosmid cpzEW01. A hygromycin resistance cassette (*hyg*) was amplified from pIJ10700 by PCR with primers 4H11_10700_fw *TGACGGGCGGGCGGGGCTCGC AGCCCCTAGGCTGAGGAG*ATTTAAATTGTAGGCTGGAGCTGCTTC including a *Swa*I restriction site (underlined) and 4H11_10700_rv *GGGGGCCGTCGCCGA AGTGCGGGCCGCCGGGCGGCACGG*GTTTAAACATTCCGGGGATCCGTCGACC, including a *Pme*I restriction site (underlined). Italic letters represent 39 nt homologous extensions to sequences upstream of *orf10* and to an internal region of *orf25* respectively. The resulting 1678 bp PCR product contained the hygromycin resistance gene *hyg*, an origin of transfer *oriT* and was flanked by two FLP-recombinase recognition sites (FRT). The PCR-product was inserted into cosmid cpzEW01 by Red/ET-mediated recombination thereby replacing a 16144 bp region (*orf10* – *orf25*) upstream of *cpzDVII* resulting in the hygromycin, apramycin and kanamycin resistant cosmid cpzEW02. Restriction of cpzEW02 with *Nhe*I and *Pme*I generated the 9950 bp L-rhamnose subcluster fragment (*cpzD1* – *cpzDVII*) flanked by the apramycin and the hygromycin resistance cassettes.

Cosmid cpzLK09 which already contained the ΦC31 integration cassette from pIJ787 (Eustaquio *et al.*, 2005) was prepared as the L-rhamnose subcluster acceptor cosmid. An apramycin resistance cassette (*aac(3)IV*) was amplified from pIJ774 (Gust *et al.*, 2003) by PCR with primers 31C2_774_fw2 *TGCCGTACCCGCGGTCA CCGGTTCCGCCTCGGCGGGTGC*ACTAGTTGTAGGCTGGAGCTGCTTC including a *Spe*I restriction site (underlined) and 31C2_774_rv *CGTGCTGGTGCT CGCCAACCACCCCATGCGGCTGGGCAT*GCTAGCAAATGCCGGCCTTTGAATGG including a *Nhe*I restriction site (underlined). Italic letters represent 39 nt homologous extensions to sequences downstream of *cpz31* and to an internal region of *cpz33* respectively. The resulting 1079 bp PCR product contained the *aac(3)IV* gene and a single loxP site. The PCR-product was inserted into cosmid cpzLK09 by Red/ET-mediated recombination thereby replacing a 1000 bp region downstream of *cpz31* and resulted in the apramycin and kanamycin resistant cosmid cpzEW03. A spectinomycin/streptomycin resistance cassette (*aadA*) was amplified from pIJ778 (Gust *et al.*, 2003) by PCR with primers 31C2_778_fw *TCGTCAGATGCAGATGGACGTCGTGCCCGGCCGCGTTCT*ATTTAAATTTATTTG

CCGACTACCTTGG including a *Swa*I restriction site (underlined) and 31C2_778_rv *CCGCGGTGGCGCACCACGACGAACTGGTGCCGCGGGCCG*GTTTAAACATTCC GGGGATCCGTCGACC including a *Pme*I restriction site (underlined). Italic letters represent 39 nt homologous extensions to an internal sequence of *cpz34* and to a region 351 bp upstream of *cpz34* respectively. The resulting 1406 bp PCR product contained the *aadA* gene, an *oriT* and a single FRT site. The PCR-product was inserted into cosmid cpzEW03 thereby replacing the 613 bp region between *aac(3)IV* and the beginning of the SuperCos1 backbone resulting in the spectinomycin/streptomycin, apramycin and kanamycin resistant cosmid cpzEW05.

For the generation of cpzEW07, the cosmid containing the caprazamycin gene cluster and the L-rhamnose subcluster, Red/ET-mediated recombination was used. A Red/ET-proficient *E. coli* BW25113/pIJ790 harbouring cpzEW05 was transformed of with the 9950 bp *Nhe*I/*Pme*I fragment from cpzEW02. Homologous recombination took place between a 924 bp region, represented by the apramycin resistance gene *aac(3)IV,* and a 523 bp region of identical sequences between the hygromycin- and the spectinomycin/streptomycin-resistance cassettes. A positive selection with hygromycin was possible for this recombination event due to the exchange of the resistance gene *aadA* by *hyg*. Note that after Red/ET-mediated recombination, the apramycin resistance cassette is now flanked by loxP sites and the hygromycin resistance cassette by FRT sites, allowing removal of the resistance cassettes by the use of Cre-recombinase or FLP-recombinase respectively. The isolated cosmid DNA of cpzEW07 was verified by restriction analysis. cpzEW07 was introduced into *S. coelicolor* M512 by triparental intergeneric conjugation. Apramycin, hygromycin and kanamycin resistant clones were selected and termed as *S. coelicolor* M512/cpzEW07-(1-3).

9.9. Generation of single gene deletions on the assembled caprazamycin gene cluster

The apramycin resistance cassette in cosmid cpzEW07 was eliminated by *Spe*I restriction and religation. The resulting cosmid cpzEW08 was verified by restriction analysis. An apramycin resistance cassette *(aac(3)IV)* was amplified from plasmid pIJ774 (Gust *et al.*, 2003) by PCR with primers containing an *Afl*II site (underlined) KOcpzDI_fw *GTGGAAGCAGAAGGAAGATGATGAGTCGTGGAGTCCATG*CTTAAG ATTCCGGGGATCCGTCGACC and KOcpzDI_rv *GCTCGGATCGTCACCGCGCT GTCCACGAGGGAGGCGTCA*CTTAAGTGTAGGCTGGAGCTGCTTC for the

inactivation of *cpzDI* or KOcpzDV_fw *GCATCCGCAAGGAAGGTCTTCCT CGTGAAACGCCATGAG*CTTAAGATTCCGGGGATCCGTCGACC and KOcpzDV_rv *CGCCGGCCGCCCCCGGGCGGTGGCCGGCGCCGTCGTTCA*CTTAAGTGTAGGC TGGAGCTGCTTC for the inactivation of *cpzDV*. Italic letters represent 39 nt homologous extensions to sequences up- and downstream of the target genes for Red/ET-mediated recombination. Resulting cosmids cpzEW09 (Δ*cpzDI*::*aac(3)IV*) and cpzEW11 (Δ*cpzDV*::*aac(3)IV*) were confirmed by restriction analysis. Excision of the cassette was performed by *in vitro* application of the Cre-recombinase (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's instructions, taking advantage of the loxP recognition sites flanking the apramycin resistance cassette. The obtained cosmids cpzEW10 (Δ*cpzDI*) and cpzEW12 (Δ*cpzDV*) were screened for their apramycin sensitivity and verified by restriction analysis. Cosmids cpzEW10 and cpzEW12 were introduced into *S. coelicolor* M512 by triparental intergeneric conjugation. Kanamycin resistance exconjugants were selected and designated as *S. coelicolor* M512/cpzEW10-(1-3) (*ΔcpzDI*) and *S. coelicolor* M512/cpzEW12-(1-3) (*ΔcpzDV*).

10. Analysis and isolation of secondary metabolites

10.1. LC-MS analysis

Methods for LC-MS analysis were developed in cooperation with Stefanie Siebenberg and Bernd Kammerer. The analysis was routinely carried out by Stefanie Siebenberg.

Analysis of caprazamycin and liposidomycin derivatives

Streptomyces sp. MK730-62F2, Streptomyces SN1061-M, *S. coelicolor* M512 or derivatives thereof were cultured in production medium as described in section II.6.2.2. Partial purification of caprazamycins was achieved by extraction of the culture supernatant (adjusted to pH 4) with an equal volume of *n*-butanol. The organic phase was evaporated and extracts were resolved in 500 µL methanol. LC-ESI-MS analysis was performed on a Surveyor HPLC system equipped with a Reprosil-Pur Basic C18 (5 µm, 250x2 mm) column (Dr. Maisch, Ammerbuch, Germany) coupled to a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (heated capillary temperature 320°C; sheath gas nitrogen, collision gas argon). Analysis of extracts from the co-expression of pRHAM experiments was

performed with a linear gradient from 2% to 40% acetonitrile in aqueous formic acid (0.1%) over 5 min followed by a linear gradient from 40% to 100% acetonitrile in aqueous formic acid (0.1%) over 20 min. Otherwise, a linear gradient from 2% to 40% acetonitrile in aqueous formic acid (0.1%) over 4 min was used followed by a linear gradient from 40% to 100% acetonitrile in aqueous formic acid (0.1%) over 31 min. The flow rate was 0.2 l min^{-1} and detection at 262 nm. Positive electrospray ionisation ((+)-ESI) was performed with electrospray voltage of 3.8 kV and collision induced dissociation (CID) spectra were recorded with collision energy of 35 eV. The respective parameters in negative mode ((-)-ESI) were 4.0 kV and 25 eV.

Analysis of phenolic compounds

In order to analyze product formation in the assays with Cpz4, pNS, MUS and phenol a similar set up was used as described above. Conditions for sample separation were changed to a linear gradient from 2 to 100% acetonitrile in aqueous formic acid (0.1%) over 20 min.

III. RESULTS

1. Identification of the caprazamycin biosynthetic gene cluster

1.1. Generation and screening of a cosmid library

The unusual structure of caprazamycins and the lack of information about the biosynthetic origin made it difficult to select genetic probes for the identification of the gene cluster. However, the formation of the permethylated L-rhamnose moiety is known from other antibiotics like elloramycin (Patallo *et al.*, 2001) and spinosyn (Waldron *et al.*, 2001). Oligonucleotides deduced from a multiple sequence alignment based on the elloramycin methyltransferase *elmM1* led to the amplification of a partial sequence of *cpz28* with high similarity to sugar *O*-methyltransferases. Primer walking revealed two adjacent genes *cpz29* and *cpz30* to be homologous to other *O*-methyltransferases suggesting the presence of the caprazamycin gene cluster. To our knowledge, this demonstrates for the first time the successful application of degenerated primers for *O*-methyltransferases for probe development. Commonly, methyltransferases are considered to be too diverse on nucleotide sequence level and too widely distributed in bacterial metabolism to be useful in the identification of a specific gene cluster.

Perfect matching primers were applied to a genomic library of *Streptomyces* sp. MK730-62F2 constructed in a SuperCos1 vector. Eight positive cosmids out of 3000 could be identified and proven to contain overlapping DNA by restriction mapping. Cosmid 31C2 was finally selected for further investigations.

1.2. Deletion of the genes *cpz28-30*

In order to confirm that the identified putative *O*-methyltransferase genes *cpz28*, *cpz29* and *cpz30* on cosmid 31C2 indeed direct the biosynthesis of caprazamycins, a gene disruption experiment was performed. To avoid possible cross-complementation all three genes were deleted at once. Therefore, a chromosomal 6.9 kb fragment was replaced with an apramycin-resistance cassette from pIJ773 by the use of Red/ET-mediated recombination (Gust *et al.*, 2003). The cassette was flanked by compatible restrictions sites which allowed subsequent removal of the cassette generating cosmid cpzLK04. After transformation of the cosmid into

Streptomyces sp. MK730-62F2 double-crossover mutants were selected and positive candidates were verified by PCR. Three independent mutants were obtained and named *Streptomyces* sp. MK730-62F2 *Δcpz28-30* (1), (2) and (3).

We applied positive ESI-CID mass spectrometry coupled to reverse phase HPLC for the analysis of caprazamycin formation in the cultivation broth of the mutants and the wildtype strain.

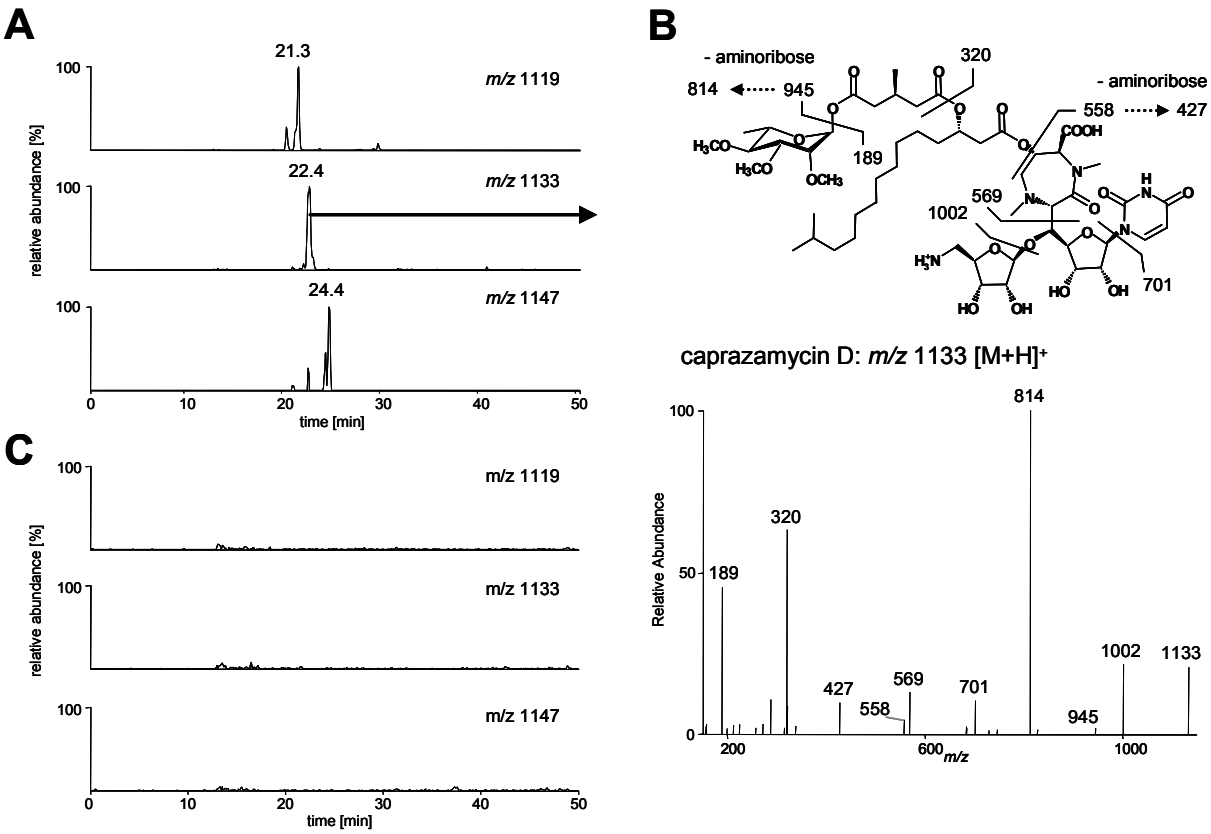


Figure III.1.1 Analysis *n*-butanolic culture extracts from (**A**) wildtype *Streptomyces* sp. MK730-62F2 and (**C**) *Streptomyces* sp. MK730-62F2*Δcpz28-30* by LC-ESI-MS. Production ion chromatograms are obtained from mass scans in positive mode with *m/z* 1119 $[M+H]^+$ (CPZ E/F), *m/z* 1133 $[M+H]^+$ (CPZ C/D/G) and *m/z* 1147 $[M+H]^+$ (CPZ A/B). Mass spectrometric fragmentation pattern in the CID experiment is shown for CPZ C/D/G (**B**) from (**A**).The suggested fragmentation schemes is depicted for CPZ D.

In the wildtype strain the known caprazamycins A-G (Figure I.2.3), whose different fatty acid side chains result in three different masses, were detected readily as depicted in the selected ion monitoring (SIM) chromatograms of Figure III.1.1A (*m/z* 1119 $[M+H]^+$ for CPZ E and F at Rt = 21.3 min, *m/z* 1133 $[M+H]^+$ for CPZ C, D and G at Rt = 22.4 min and *m/z* 1147 $[M+H]^+$ for CPZ A and B at Rt = 24.4 min). Characteristic MS/MS fragmentation patterns were observed by collision-induced

dissociation of the methylated rhamnosyl-group, aminoribose, uracil and fatty acid group. Similar fragmentations have been reported for liposidomycins in FAB-MS (Ubukata *et al.*, 1992). For all caprazamycins, product ions of *m/z* 189, *m/z* 320, *m/z* 427 and *m/z* 558 could be found correlating with the deacyl-components (Figure III.1.1B). For the parent ion *m/z* 1133 $[M+H]^+$ fragments of *m/z* 1002, *m/z* 945, *m/z* 814, *m/z* 701 and *m/z* 569 were detected. Corresponding fragments with a mass shift of −14 Da and +14 Da were observed for the parent ions *m/z* 1119 $[M+H]^+$ and *m/z* 1147 $[M+H]^+$ respectively. Additional evidence was obtained by product ion scans in negative mode using the same extracts. While the presence of the caprazamycins in the culture broth of the wildtype strain could be confirmed unequivocally, they were absent in cultures of all three independent isolates of the mutant *S.* sp. MK730-62F2 *Δcpz28-30* (Figure III.1.1C). However, product ion scans for non-glycosylated caprazamycin derivatives (*m/z* 931 $[M+H]^+$, *m/z* 945 $[M+H]^+$, *m/z* 959 $[M+H]^+$) resulted in prominent mass peaks in extracts from *Streptomyces* sp. MK730-62F2 *Δcpz28-30*. This shows that the generated mutant strains are only affected in the biosynthesis of the deoxysugar moiety, as desired. Overall, our data proves the involvement of the *O*-methyltransferase genes in caprazamycin biosynthesis and strongly indicates that the genes identified on cosmid 31C2 indeed represent the caprazamycin biosynthetic gene cluster. In addition, a reliable method for the detection of caprazamycins in bacterial culture extracts could be established. The LC-ESI-MS/MS mass spectra provided substantial information on the structural characteristics of these unusual compounds. Subsequently, cosmid 31C2 was send for complete shot-gun sequencing (nucleotide sequence of the gene cluster has been deposited at GenBank, accession number FJ490409).

1.3. Sequence analysis of the caprazamycin gene cluster

A contiguous 42.3 kb region could be assembled with an average GC content of 70.2%, a typical value for *Streptomyces* DNA. *In silico* sequence analysis guided by BLAST homology searches (Altschul *et al.*, 1997), conserved protein domain searches (Marchler-Bauer *et al.*, 2007) and the GC frame plot method (Bibb *et al.*, 1984) revealed 34 candidate genes. A total of 23 open reading frames, designated *cpz9-31*, were assigned to the caprazamycin gene cluster putatively encoding for biosynthesis, resistance, transport and regulatory functions (Figure III.1.2). Table II.1.1 summarizes the orthologs and proposed functions of the annotated genes. Notably, most of the putative gene products did not show homology to proteins found

previously in other secondary metabolite gene clusters, which reflects the unusual structure of the caprazamycins.

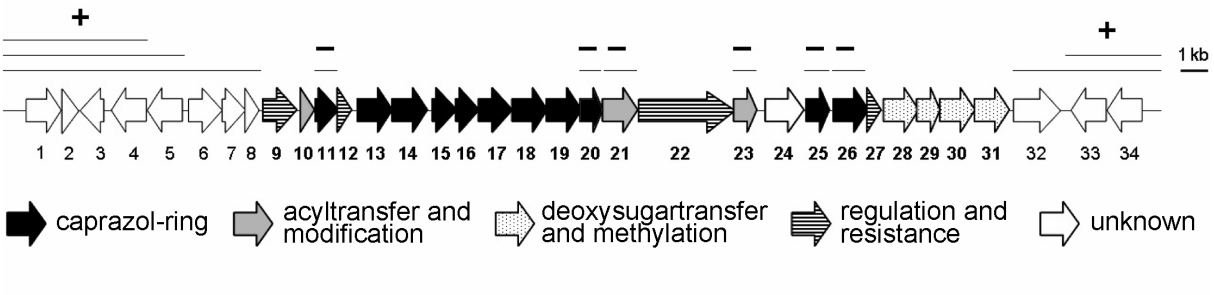


Figure III.1.2 Organisation of the caprazamycin gene cluster**.** The putative assignment of the genes to different steps in the biosynthesis is indicated. Bars above the cluster mark the gene deletions performed in this study. (-) indicates that the deletion of the respective region led to an abolishment of caprazamycin production. (+) indicates that caprazamycin production was not influenced.

As proposed, the caprazamycin gene cluster would start with *cpz9* which encodes for a putative regulator of the AraC family. Most members of this family are positive transcriptional activators containing a helix-turn-helix motif. They are known from sugar degradation and other pathways but are rarely found in gene clusters of secondary metabolism (Gallegos *et al.*, 1997). As an example, the AraC-like regulator TxtR from *S. scabies* activates thaxtomin biosynthesis and recognizes cellobiose as a ligand (Joshi *et al.*, 2007). The predicted gene product of *cpz22* shows homology to ABC-transporters. Similar proteins can be found in many antibiotic gene clusters and are usually involved in self-resistance and export (Rodriguez *et al.*, 1993). *Cpz12* and *cpz27* are two putative sugar kinase genes similar to tunicamycin resistance proteins e. g. TmrD from *Deinococcus radiodurans* which structure has been reported recently (Kapp *et al.*, 2008). The 2'-, 3'- and 5'-hydroxy groups of the uridine have been suggested as potential targets for phosphorylation by TmrD, resulting in the inactivation of the nucleoside antibiotic tunicamycin.

Cpz10 shows only low similarity to proteins from the database but exhibits a conserved protein domain for aspartyl/asparaginyl *β*-hydroxylases from the Fe(II)/2-oxoglutarate dependent oxygenase family (Stenflo *et al.*, 1989). Cpz11 and Cpz26 have weak homology to putative methyltransferases including possible phosphatidylethanolamine *N*-methyltransferases.

Table II.1.1 Deduced functions of genes in the caprazamycin gene cluster

Gene	AA	Protein homolog	Identity/ similarity*	Proposed function
cpz1	477**	SchA30, *Streptomyces* sp. SCC 2136	84/90	feruloyl-CoA synthase
cpz2	151	SSEG_02365, *S. sviceus* ATCC 29083	90/95	acyl dehydratase
cpz3	278	SchA31, *Streptomyces* sp. SCC 2136	76/84	transcriptional regulator
cpz4	513	SACE_7046, *Saccharopolyspora erythraea*	44/58	hypothetical protein
cpz5	419	hmgs, *Streptomyces* sp. CL190	70/79	HMG-CoA synthase
cpz6	349	SaRppA, *S. antibioticus*	30/46	type III pks
cpz7	260	SACE_3529, *Saccharopolyspora erythraea*	57/65	PAPS 3'phosphatase
cpz8	212	SACE_5947, *Saccharopolyspora erythraea*	43/60	hypothetical protein
cpz9	348	SSEG_03332, *S. sviceus* ATCC 29083	27/41	transcriptional regulator
cpz10	182	RSK20926_04892, *Roseobacter* sp.	25/40	*β*-hydroxylase
cpz11	210	SCO_1731, *S. coelicolor* A3(2)	29/40	methyltransferase
cpz12	189	SSEG_10045, *S. sviceus* ATCC 29083	23/34	kinase
cpz13	441	SACE_4299, *Saccharopolyspora erythraea*	16/30	aminotransferase
cpz14	424	Orf-4, *S. atroolivaceus*	36/53	hydroxymethyltransferase
cpz15	274	Xccb100_2413, *Xanthomonas campestris*	26/40	dioxygenase
cpz16	233	Smed_4814, *Sinorhizobium medicea*	20/30	nucleotidyltransferase
cpz17	377	AprG2, *S. tenebrarius*	19/30	glycosyltransferase
cpz18	424	CetH, *Actinomyces* sp. LU 9419	34/49	aminotransferase
cpz19	460	GK_2312, *Geobacillus kaustophilus*	39/54	pyrimidine phosphorylase
cpz20	354	TMCL4, *Streptomyces* sp. CK 4412	44/58	acyl-CoA synthase
cpz21	500	TMCL1, *Streptomyces* sp. CK 4412	35/46	carboxyesterase
cpz22	1238	SAV_5299, *S. avermitilis* MA 4680	36/53	ABC transporter
cpz23	344	AviX9, *S. viridochromogenes*	31/46	lipase
cpz24	598	Cja_1569, *Cellvibrio japonicus*	26/40	hypothetical protein
cpz25	341	SAV_2980, *S. avermitilis* MA 4680	45/63	dehydrogenase
cpz26	417	SSDG_05270, *S. pristinaespiralis* ATCC 25486	15/24	methyltransferase
cpz27	192	SSEG_10045, *S. sviceus* ATCC 29083	39/54	kinase
cpz28	396	spnK, *Saccharopolyspora spinosa*	50/66	*O*-methyltransferase
cpz29	265	elmM3, *S. olivaceus*	46/60	*O*-methyltransferase
cpz30	419	elmM1, *S. olivaceus*	43/53	*O*-methyltransferase
cpz31	396	elmGT, *S. olivaceus*	40/53	deoxysugar transferase
cpz32	543	Tfu_2432, *Thermobifida fusca*	22/28	nucleotidyltransferase
cpz33	556	SchA32, *Streptomyces* sp. SCC 2136	81/88	hypothetical protein
cpz34	544	SchA33, *Streptomyces* sp. SCC 2136	85/93	metallophosphoesterase

* Overall homology [%]

** Gene lacks an appropriate stop codon and is considered to be incomplete on cosmid 31C2

cpz Bold genes proposed to be essential for caprazamycin production

The predicted proteins contain conserved SAM-binding domains (cd02440). Interestingly, both genes are translationally coupled to possible resistance genes *cpz12* and *cpz27* by overlap of start and stop codon. *Cpz14* is predicted to encode a serine hydroxymethyltransferase known to generate glycine from serine. Like other PLP-dependent enzymes, they have been shown to catalyze a great diversity of different reactions (Ogawa *et al.*, 2000). Upstream and translationally coupled to *cpz14* is *cpz13* with weak homology to aminocyclopropan-carboxylate synthases and aminotransferases.

Several genes seem to be co-transcribed in a *cpz15-23* sub-cluster. The first, *cpz15*, displays domain homology on a protein level with clavaminate synthases and other α-ketoglutarate dependent dioxygenases. They are known to catalyze various oxidation reactions including *β*-hydroxylation, desaturation and oxidative ring closure (Townsend, 2002). A putative nucleotidyltransferase is encoded by *cpz16* while Cpz17 shows similarity to the glycosyltransferase AprG2 from *S. tenebrarius*. The deduced product of c*pz18* seems to belong to the class-III aminotransferases and bears 38% homology to CetH from the cetoniacytone gene cluster (Wu *et al.*, 2009). Cpz19 resembles pyrimidine-nucleoside phosphorylases which catalyze the formation of *β*-ribose-1-phosphate and a pyrimidine from either uridine or thymidine. The genes *cpz20* and *cpz21* encode proteins similar to a putative acyl-CoA synthase (TMCL4) and a carboxyesterase (TMCL1) from the tautomycetin gene cluster (Choi *et al.*, 2007). Another possible esterase is Cpz23. It displays similarity to AviX9, a protein with unknown function from the avilamycin gene cluster from *S. viridochromogenes* (Weitnauer *et al.*, 2001). The deduced gene product of *cpz25* is a putative zinc-dependent alcohol dehydrogenase.

Cpz28, *cpz29*, *cpz30* and *cpz31* apparently constitute an operon for the attachment and the methylation of a deoxysugar. They show strong similarity to *O*-methyltransferases and glycosyltransferases from known antibiotic gene clusters in particular to proteins participating in the formation of elloramycin (Blanco *et al.*, 2001; Patallo *et al.*, 2001) and spinosyn (Waldron *et al.*, 2000). Both these compounds contain the same permethylated L-rhamnose moiety as found in the caprazamycins. In elloramycin biosynthesis, the L-rhamnose is transferred to the aglycone utilizing the dTDP-activated deoxysugar substrate and is subsequently methylated in three successive steps. Interestingly, we could not identify genes for the dTDP-L-rhamnose biosynthesis on the cosmid. This was initially surprising, because all genes for the

production of a bacterial secondary metabolite are usually clustered. However, neither the gene cluster of elloramycin (Ramos *et al.*, 2008) nor of spinosyn (Madduri *et al.*, 2001), steffimycin (Gullon *et al.*, 2006) or arranciamycin (Luzhetskyy *et al.*, 2007) contain genes for dTDP-L-rhamnose formation. We therefore suggested the genes for the caprazamycins deoxysugar biosynthesis to be located elsewhere on the genome of the natural producer.

1.4. Heterologous expression of the caprazamycin gene cluster

In order to investigate whether the genes found on cosmid 31C2 were sufficient for the biosynthesis of caprazamycins we intended to express the cosmid heterologously. For this purpose, the beta-lactamase (*bla*) gene on the backbone of 31C2 was replaced with an integration cassette of pIJ787 (Eustaquio *et al.*, 2005; Gust *et al.*, 2004) containing the *attP* attachment site and the integrase gene (*int*) of phage ΦC31, a tetracycline resistance gene (*tet*) and an origin of transfer (*oriT*) using Red/ET-mediated recombination. The generated cosmid cpzLK09 was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser *et al.*, 2000) and three kanamycin resistance clones were selected, referred to as *S. coelicolor* M512/cpzLK09 (1), (2) and (3). Extracts of cultures of the wildtype and the mutant strains were applied to LC-ESI-MS.

In HPLC-UV-chromatograms peaks appeared with *S. coelicolor* M512/cpzLK09 at Rt. 18.1 min, Rt. 19.3 min and Rt. 20.8 min which were not found in extracts of *S. coelicolor* M512 without the cluster (Figure III.1.3A and B). While the masses for the caprazamycins could not be detected, prominent mass peaks for the caprazamycin aglycones were observed in *S. coelicolor* M512/cpzLK09 (SIM chromatograms in Figure III.1.3B; for the CPZ E and F aglycones *m/z* 931 $[M+H]^+$ at Rt = 18.3 min, for CPZ C, D and G aglycones *m/z* 945 $[M+H]^+$ at Rt = 19.5 min and for CPZ A and B aglycones *m/z* 959 $[M+H]^+$ at Rt = 21.0 min). Displaying the more hydrophilic character of the free carboxylic acid group, the aglycones elute three minutes earlier from the reversed phase HPLC column than the corresponding intact caprazamycins (compare Figure III.1.1). Positive ESI-CID fragmentation of the new compounds was identical to caprazamycins except for the absence of the L-rhamnose moiety. Fragments of *m/z* 814, *m/z* 701 and *m/z* 569 were obtained from the parent ion *m/z* 945 $[M+H]^+$ (Figure III.1.3C). Analogous fragments with a mass shift of −14 Da and +14 Da resulted from *m/z* 931 $[M+H]^+$ and *m/z* 959 $[M+H]^+$ representing the caprazamycin derivatives with fatty acids of different chain length.

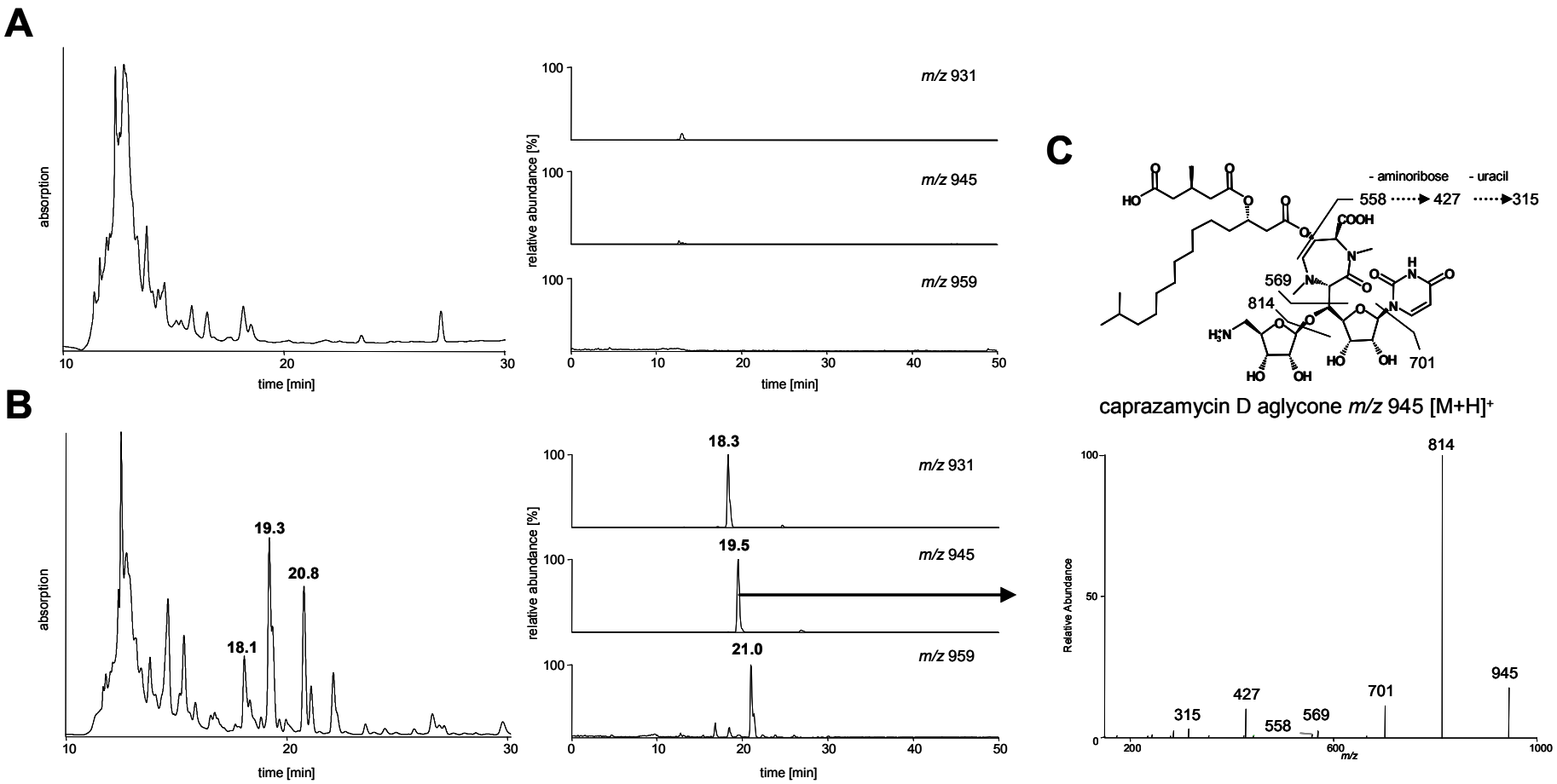


Figure III.1.3 Analysis of *n*-butanolic culture extracts from (**A**) *S. coelicolor* M512 and (**B**) *S. coelicolor* M512/cpzLK09 containing the caprazamycin gene cluster. UV-chromatograms are depicted. Production ion chromatograms are obtained from mass scans in positive mode with *m/z* 931 $[M+H]^+$ (CPZ E/F aglycone), *m/z* 945 $[M+H]^+$ (CPZ C/D/G aglycone) and *m/z* 959 $[M+H]^+$ (CPZ A/B aglycone). Mass spectrometric fragmentation pattern in the CID experiment is shown for CPZ C/D/G aglycone (**C**) from (**B**).The suggested fragmentation scheme is depicted for CPZ D aglycone.

Molecular ions of *m/z* 558, *m/z* 427 and *m/z* 315, assigned to components of the caprazol structure, were found in all three spectra. *S. coelicolor* M512 without the gene cluster did not produce any of these new substances (Figure III.1.3A). The analytical data strongly implicates the production of non-glycosylated caprazamycins which are similar to the type-(III) liposidomycins (Figure I.2.3) isolated previously (Kimura *et al.*, 1998b). These compounds have been reported to show excellent activity against *Mycobacteria*. Thus, culture extracts from the heterologous producer were applied to an agar diffusion assay against *M. phlei*. An inhibition zone of similar size could be observed with extracts from the wildtype and the mutant strains, whereas extracts from *S. coelicolor* M512 without the gene cluster showed no bioactivity in this assay (Figure III.2.1D).

The production of the non-glycosylated caprazamycin derivatives correlates with the absence of genes for the dTDP-L-rhamnose biosynthesis on the cosmid. Apparently, *S. coelicolor* M512 is unable to provide the dTDP-L-rhamnose in compensation as the corresponding enzymes are not encoded on the genome.

Both, analytical and biological data verify that the genes identified on cosmid 31C2 indeed represent the caprazamycin biosynthetic gene cluster

1.5. Determination of the boundaries of the gene cluster

A set of gene disruption experiments was carried out to determine the cluster boundaries. Sequence analysis of overlapping cosmids suggested the caprazamycin gene cluster to be inserted into a highly conserved genomic region. *cpz1* and *cpz3* at the one end of the cluster and *cpz33* and *cpz34* at the other are almost identical with a continuous part of sequence from *Streptomyces.* sp. SCC 2136 (Basnet *et al.*, 2006). Primer walking and terminal sequencing of overlapping cosmids showed that this similarity extends further upstream of *cpz1*.

While *cpz4* encodes for a hypothetical protein with unknown function, *cpz5* showed homology to 3-hydroxy-methylglutaryl (HMG)-CoA synthases. HMG-CoA synthases catalyze the aldol-addition of acetyl-CoA onto acetoacetyl-CoA and usually participate in the mevalonate pathway (Dairi, 2005). A biosynthetic route to the uncommon 3-MG moiety was proposed involving a HMG-CoA synthase, a dehydratase and a hydrogenase. No function in caprazamycin formation could be assigned to a putative type III PKS encoded by *cpz6* and the possibly co-transcribed genes *cpz7* and *cpz8*.

In order to validate the left border of the cluster *cpz1*, *cpz2*, *cpz3* and *cpz4* were deleted in cpzLK09 to generate cosmid cpzWP05 and *cpz5* was additionally deleted to generate cpzWP06. By inactivation of the suggested biosynthetic pathway to 3-MG in cpzWP06 we hoped to produce compounds similar to the highly bioactive type-(IV) liposidomycins (Figure I.2.3). *Cpz6* was inactivated in addition to *cpz1-cpz5* in cosmid cpzWP07. At the right end of the cluster *cpz33* and *cpz34* encoding for a hypothetical protein and a metallophosphoesterase were deleted in cosmid cpzWP01. A possible nucleotidyltransferases encoded by *cpz32* was additionally deleted in cosmid cpzWP02.

After introducing the modified cosmids into *S. coelicolor* M512, positive candidates were selected by their kanamycin resistance and verified by PCR. Cultivation and analysis by LC-ESI-MS revealed production of caprazamycin aglycones in all mutants. In addition, bioassays of the culture extracts against *M. phlei* did not show any difference in inhibitory activity compared to *S. coelicolor* M512/cpzLK09 containing the intact gene cluster. In case of *Δcpz4*, *Δcpz5* and *Δcpz32* complementation by host genes seems unlikely as the *S. coelicolor* genome contains no homologs. Therefore we concluded *cpz1-6* and *cpz32-34* to be non-essential in caprazamycin biosynthesis. Given that *cpz6* is most likely co-transcribed with its downstream positioned genes, a functional knock-out of *cpz7* and *cpz8* can be assumed in cosmid cpzWP07. Consequently, the biosynthetic gene cluster for caprazamycins is predicted to span from *cpz9* to *cpz31* (Figure III.1.2).

2. Gene deletion experiments on the caprazamycin gene cluster

Since the putative HMG-CoA synthase Cpz5 seems not to be required for caprazamycin formation and the corresponding mutant *S. coelicolor* M512/cpzWP06 did not accumulate the desired *β*-hydroxyacyl-caprazols, we searched for possible acyltransferases within the gene cluster. Two acyl moieties, the 3-methylglutarate (3-MG) and the *β*-hydroxy fatty acids have to be attached during caprazamycin biosynthesis and the two putative hydrolases Cpz21 and Cpz23 could be considered for these transfer reactions. Cpz21 is predicted to contain a typical α/β-hydrolase fold, the catalytical triade Ser208-Glu326-His409 and a GxSxG-motif (Holmquist, 2000). The overall homology of Cpz21 is strongest with TMCL1 from *Streptomyces* sp. CK4412 (Choi *et al.*, 2007). TMCL1, also named TmcC, is assigned to the esterification of a dialkylmaleic anhydride moiety to the linear polyketide during tautomycetin formation.

The amino acid sequence deduced from *cpz23* shows highest overall homology to several hypothetical proteins from Streptomycetes including AviX9 of the avilamycin gene cluster from *S. viridochromogenes* (Weitnauer *et al.*, 2001). According to the conserved protein domain search the C-terminus of Cpz23 is similar to SGNH-hydrolases, a diverse family of lipases and carboxyesterases (Akoh *et al.*, 2004).

2.1. Deletion of the putative acyltransferase genes *cpz21* and *cpz23*

This part of the thesis was carried out in collaboration with Diploma student Liane Lutsch. Both genes were individually deleted from cosmid cpzLK09 using Red/ET-mediated recombination. To create in-frame deletions, the disruption cassette from pIJ773 was subsequently removed by the use of FLP-recombinase (Cherepanov & Wackernagel, 1995) generating cosmid cpzLL06 (*Δcpz21*) and cpzLL07 (*Δcpz23*). After introduction of the cosmids in *S. coelicolor* M512 kanamycin resistant mutants were cultivated and extracts were analyzed by LC-ESI-MS. Production of the caprazamycin aglycones was abolished in *S. coelicolor* M512/cpzLL06 and *S. coelicolor* M512/cpzLL07. This proves that both, Cpz21 and Cpz23 play an essential role in caprazamycin biosynthesis. Metabolites were only identified in extracts of *S. coelicolor* M512/cpzLL06 (*Δcpz21*) with *m/z* 803 $[M+H]^+$ at Rt = 16.1 min, *m/z* 817

$[M+H]^+$ at Rt = 17.2 min and *m/z* 831 $[M+H]^+$ at Rt = 18.6 min by LC-ESI-MS mass scan in positive mode (UV- and product ion chromatograms in Figure III.2.1A and B).

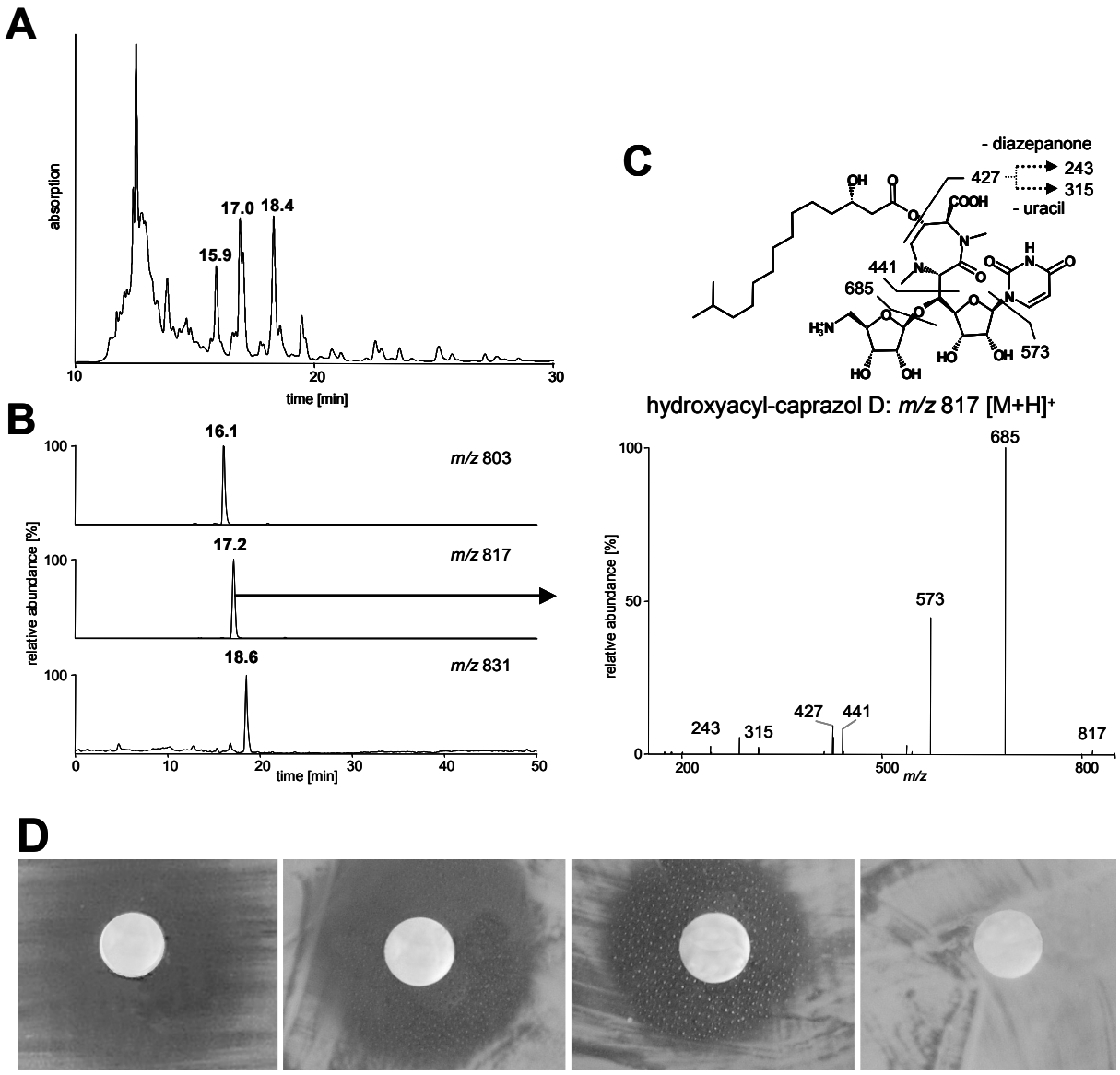


Figure III.2.1 Analysis of *n*-butanolic culture extracts from *S. coelicolor* M512/cpzLL06 containing the caprazamycin gene cluster by LC-ESI-MS (see also diploma thesis of Liane Lutsch). UV-chromatogram is depicted (**A**). (**B**) Production ion chromatograms are obtained from mass scans in positive mode with *m/z* 803 $[M+H]^+$ (hydroxyacyl-caprazols E/F), *m/z* 817 $[M+H]^+$ (hydroxyacyl-caprazols C/D/G) and *m/z* 831 $[M+H]^+$ (hydroxyacyl-caprazols A/B). Mass spectrometric fragmentation pattern in the CID experiment is shown for hydroxyacyl-caprazols C/D/G (**C**) from (**B**).The suggested fragmentation schemes is depicted for hydroxyacyl-caprazol D. (**D**) Bioactivity of culture extracts. Butanolic culture extracts from *Streptomyces* sp. MK730-62F2, *S. coelicolor* M512/cpzLK09, *S. coelicolor* M512/cpzLL06 and *S. coelicolor* M512 (in this order from left to right) were applied to an agar diffusion assay against *M. phlei.*

Collision-induced fragmentation corresponds to the caprazamycin aglycones by sequential loss of the aminoribose (-131 Da), the uracil (-111 Da) and the ribose (-132 Da) but *m/z*-values of product ions indicate the absence of the 3-methylglutarate (129 Da) (Figure III.2.1C). Overall, fragments matched exactly the predicted characteristics of *β*-hydroxyacyl-caprazols, structurally minimized liponucleosides antibiotics. In a bioassay against *M. phlei* (Figure III.2.1D) extracts of a *S. coelicolor* M512/cpzLK09 and *S. coelicolor* M512/cpzLL06 (*Δcpz21*) cultivation broths showed both similar inhibiting activities, whereas no growth inhibition could be observed with *S. coelicolor* M512 extracts. Co-expression of intact copies of the deleted genes under the constitutive *ermE** promoter in the respective mutants restored the production of caprazamycin aglycones.

Although similar to the type-(IV) liposidomycins (Figure I.2.3) the *β*-hydroxyacyl-caprazols are expected to be slightly more hydrophilic due to the additional OH-group at the 3a-C-position. Thus, they represent interesting novel compounds for further investigations e.g. in structure/activity relationships. Moreover, the data indicated that Cpz21 is most likely involved in the attachment of the 3-MG moiety. Beside Cpz21 two other enzymes with homology to para-nitrobenzyl esterases are known from bacterial secondary metabolism. Both these enzymes, TmcC and TtmK from the tautomycin gene cluster (Li *et al.*, 2008) were proposed to catalyze the attachment of an acyl group. However, inactivation of the corresponding genes in the gene clusters did not lead to the identification of an accumulated intermediate. Therefore the data presented here provides first functional evidence that these family of enzymes indeed act as acyltransferases.

2.2. Deletion of the genes *cpz20* and *cpz25*

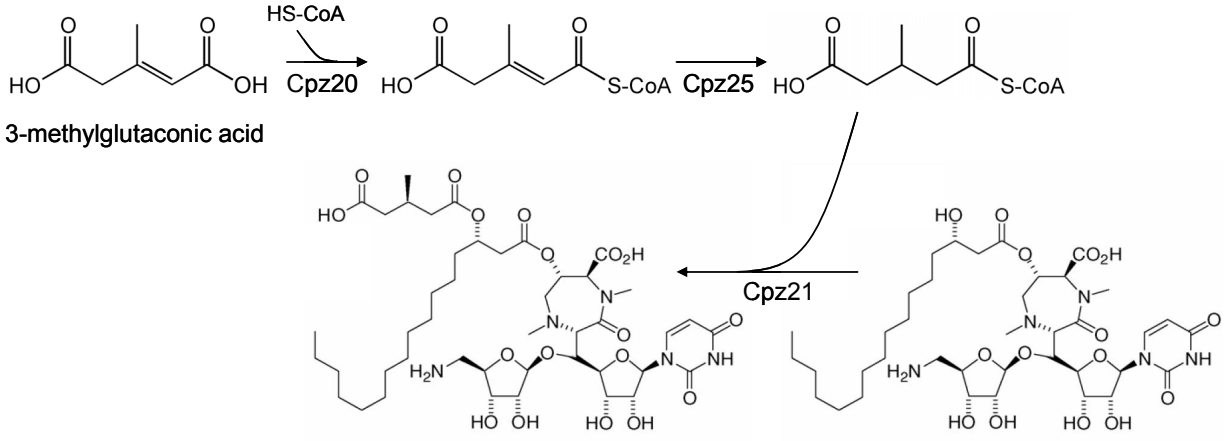


Figure III.2.2 Proposed reactions for activation and attachment of the methylglutaryl moiety

The assignment of Cpz21 as the methylglutaryl transferase in caprazamycin biosynthesis leads to the question of the biosynthetic origin of the 3-MG motif. From the previous results an involvement of the HMG-CoA synthase Cpz5 could be excluded. As an alternative we postulated a biosynthetic pathway via 3-methylglutaconate an intermediate in the leucine/isovaleriate catabolism. The leucine/isovaleriate utilization pathway was extensively investigated in *Pseudomonas* and has been shown to comprise the proteins LiuA, LiuB, LiuC, LiuD and LiuE (Forster-Fromme *et al.*, 2006; Forster-Fromme & Jendrossek, 2008; Hoschle *et al.*, 2005). By BLAST searches we found homologs of the *liu* genes in the genome of *S. coelicolor* A3(2) to be clustered together (*sco2776-sco2779*). Therefore we speculated that a similar pathway as in *Pseudomonas* exists in *S. coelicolor* and may provide methylglutaconate as a precursor for caprazamycin biosynthesis. The following reaction sequence for the generation of the 3-MG moiety would require a hydrogenase and possibly an acyl-CoA transferase (Figure III.2.2). Two such enzymes are encoded close to *cpz21*, which are Cpz20 and Cpz25. Cpz20 contains an AMP-binding domain and shows highest homology to TmcF (44%/58% identity/similarity) from the tautomycetin gene cluster (Choi *et al.*, 2007). The putative dehydrogenase Cpz25 is similar to MupE, a postulated crotonyl reductase involved in mupirocin biosynthesis in *Pseudomonas fluorescens* (El-Sayed *et al.*, 2003; Hothersall *et al.*, 2007). Cpz25 was initially assigned to the to first distinctive reaction step in a hypothetical biosynthetic pathway to the caprazamycins, the oxidation of uridine to uridine 5'-aldehyde. Though, Cpz25 seemed the only reasonable candidate for the postulated hydrogenation of 3-methylglutaconate.

Both genes were individually deleted from cosmid cpzLK09 using Red/ET-mediated recombination. To create in-frame deletions, the disruption cassette from pIJ773 was subsequently removed by the use of FLP-recombinase (Cherepanov & Wackernagel, 1995) generating cosmid cpzWP22 (*Δcpz20*) and cpzWP25 (*Δcpz25*). After introduction of the cosmids in *S. coelicolor* M512 kanamycin resistant mutants were cultivated and extracts were analyzed by LC-ESI-MS. Production of the caprazamycin aglycones was abolished in *S. coelicolor* M512/cpzWP22 and *S. coelicolor* M512/cpzWP25. However, the hydroxyacyl-caprazols could still be detected by LC-ESI-MS mass scans (Figure III.2.3). Similar to the *Δcpz21* mutant *S. coelicolor* M512/cpzLL06, extracts of *S. coelicolor* M512/cpzWP22 and *S. coelicolor*

M512/cpzWP25 still showed bioactivity in an agar diffusion assay against *Mycobacterium phlei* (Figure III.2.3). These results strongly indicate, that both Cpz20 and Cpz25, participate in the formation and/or attachment of the methylglutaryl moiety of caprazamycins. Moreover, our data supports the postulated biosynthetic pathway to the 3-MG motif, though biochemical investigations e.g. in vitro studies have to verify our conclusions.

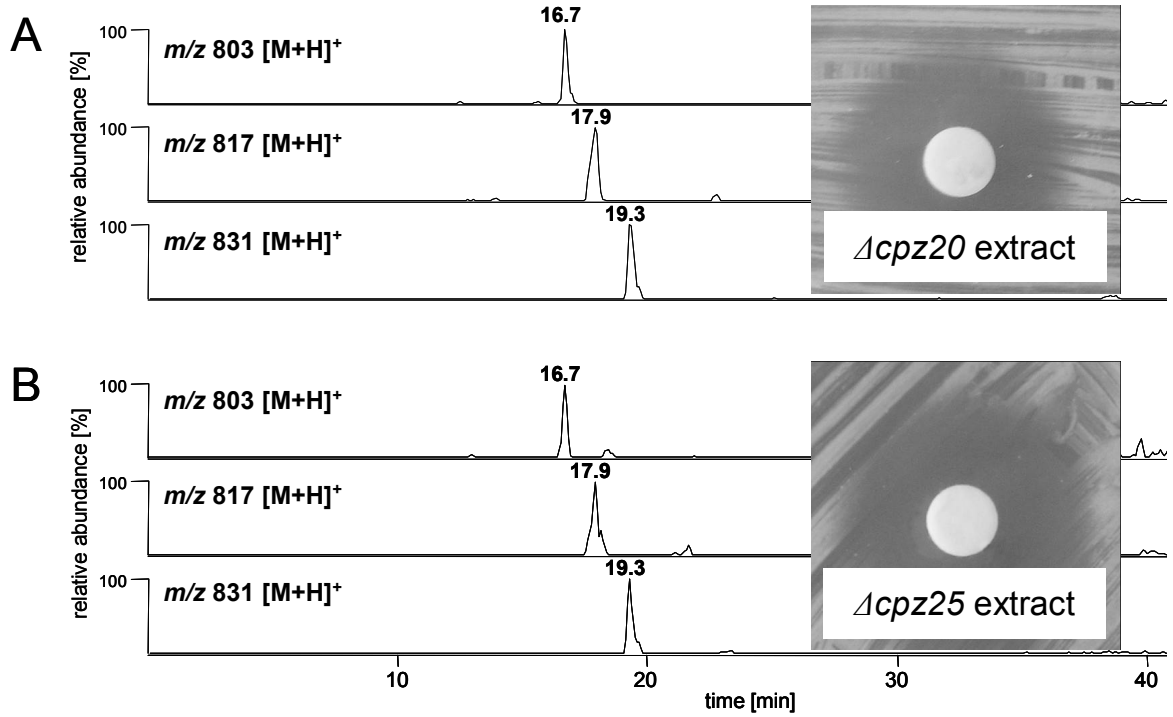


Figure III.2.3 Analysis of *n*-butanolic culture extracts from (**A**) *S. coelicolor* M512/cpzWP22 (*Δcpz20*) and (**B**) *S. coelicolor* M512/cpzWP25 (*Δcpz25*) by LC-ESI-MS. Production ion chromatograms are obtained from mass scans in positive mode with *m/z* 803 $[M+H]^+$ (hydroxyacyl-caprazols E/F), *m/z* 817 $[M+H]^+$ (hydroxyacyl-caprazols C/D/G) and *m/z* 831 $[M+H]^+$ (hydroxyacyl-caprazols A/B). Butanolic culture extracts were applied to agar diffusion assays against *M. phlei.*

2.3. Deletion of the putative methyltransferase genes *cpz11* and *cpz26*

This part of the thesis was carried out in collaboration with Diploma student Liane Lutsch. In the diazepanone ring, both nitrogens are methylated. Five putative methyltransferases are encoded by the caprazamycin gene cluster, which are Cpz11, Cpz26, Cpz28, Cpz29 and Cpz30. Cpz28, Cpz29 and Cpz30 exhibit high sequence homology to rhamnosyl-*O*-methyltransferases from other antibiotic gene clusters. Therefore, the *N*-methylation reactions are likely to be catalyzed by Cpz11 and/or Cpz26. Notably, Cpz11 shows sequence similarity (55%) to one of the few

characterized *N*-methyltransferases AtM1 from the gene cluster of AT2433 (Gao *et al.*, 2006).

Both genes were individually deleted from cosmid cpzLK09 using Red/ET-mediated recombination. To create in-frame deletions, the disruption cassette from pIJ773 was subsequently removed by the use of FLP-recombinase (Cherepanov & Wackernagel, 1995) generating cosmid cpzLL05 (*Δcpz11*) and cpzLL08 (*Δcpz26*). After introduction of the cosmids in *S. coelicolor* M512 kanamycin resistant mutants were cultivated and extracts were analyzed by LC-MS/MS. Production of the caprazamycin aglycones was abolished in *S. coelicolor* M512/cpzLL05 and *S. coelicolor* M512/cpzLL08. This proves that both, Cpz11 and Cpz26 play an essential role in caprazamycin biosynthesis. However, accumulated metabolites could not be identified in the mutant strains. LC-ESI-MS mass scans for various possible intermediates did not result in the detection of a certain compound, neither in the *Δcpz11* nor in the *Δcpz26* mutant. Co-expression of an intact copy of *cpz11* under the constitutive *ermE** promoter in the *Δcpz11* mutant restored the production of caprazamycin aglycones. A corresponding complementation plasmid with *cpz26* could not be generated.

3. Investigation of the sugartransfer reaction in caprazamycin biosynthesis

Above I described the cloning and heterologous expression of cosmid cpzLK09 containing the first identified gene cluster of a translocase I inhibitor, the caprazamycins. The caprazamycins are glycosylated with a 2,3,4-*O*-methyl-L-rhamnose and therefore belong to the large number of bioactive compounds containing 6-deoxyhexoses. Usually, these moieties contribute significantly to the compounds properties influencing e.g. molecule/target interactions, cell import and export, pharmacokinetics and solubility (Williams *et al.*, 2008). The biosynthesis of deoxysugars has been studied in detail and generally starts from NDP-activated hexoses via 4-keto-6-deoxy intermediates (Liu & Thorson, 1994). However, genes for the formation of the dTDP-L-rhamnose could not be identified on cosmid 31C2 and therefore only caprazamycin aglycones were produced by the heterologous host *S. coelicolor* M512/cpzLK09. Since potential genes for *O*-methylation (*cpz28* – *cpz30*) and a glycosyltransferase (*cpz31*) are encoded in the caprazamycin gene cluster, we speculated that only four genes are missing for successful heterologous production of intact caprazamycins.

3.1. Co-expression of plasmid pRHAM and the caprazamycin gene cluster

The production of the non-glycosylated caprazamycin derivatives correlates with the absence of putative genes for the deoxysugar formation within the gene cluster. In order to investigate if all other genes, required for the biosynthesis of the intact caprazamycins, are functionally expressed on cpzLK09, we introduced plasmid pRHAM (Rodriguez *et al.*, 2000) into the heterologous host *S. coelicolor* M512/cpzLK09. pRHAM contains all genes required for L-rhamnose biosynthesis from *S. antibioticus; oleS,* encoding a dTDP-glucose synthase, *oleE,* a dTDP-glucose 4,6-dehydratase, *oleL,* a dTDP-deoxyglucose 3,5-epimerases and *oleU,* a 4-ketoreductase. Three individual clones termed as *S. coelicolor* M512/cpzLK09/pRHAM were selected by their thiostrepton resistance and cultivated in production media. Analysis of the culture extracts by LC-ESI-MS revealed that in comparison to *S. coelicolor* M512/cpzLK09 which only produced the caprazamycin

aglycones, all mutant strains now accumulated additional metabolites (Figure III.3.1). These new compounds correspond to the caprazamycins E/F (at Rt. 20.0 min), caprazamycins C/D/G (at Rt. 20.9 min) and caprazamycins A/B (at Rt. 22.3 min) as found by ESI-MS product ion scans. MS^2 spectra from collision-induced dissociation (CID) experiments matched exactly the characteristic fragmentation profiles of the intact caprazamycins. This verified that the enzymes for methylation and attachment of the L-rhamnose moiety have to be encoded on the gene cluster. A set of genes *cpz28-31* encoding for three hypothetical sugar *O*-methyltransferases and a putative rhamnosyltransferase are the most likely candidates for these reactions.

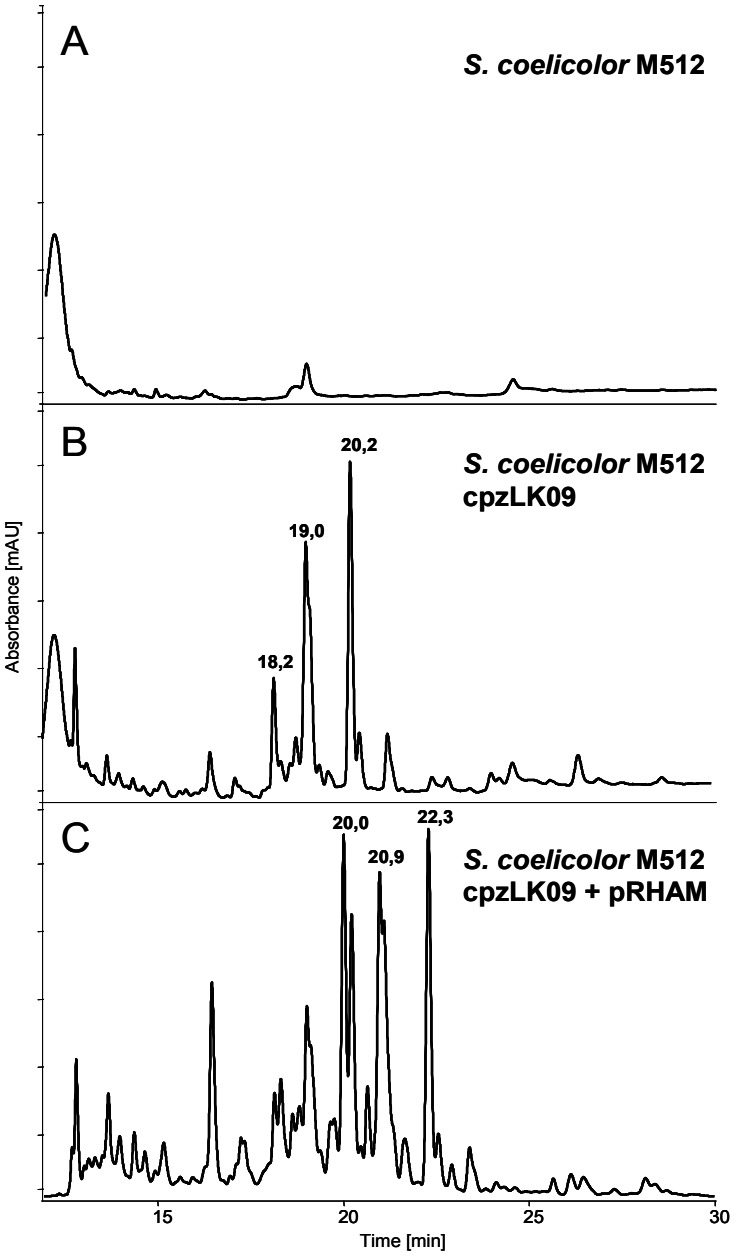


Figure III.3.1 HPLC chromatograms of *n*-butanolic extracts from **A** *S. coelicolor* M512 **B** *S. coelicolor* M512 containing the caprazamycin gene cluster cpzLK09 and **C** *S. coelicolor* M512/cpzLK09 containing pRHAM co-expressing the biosynthetic genes for dTDP-L-rhamnose.

3.2. Expression and purification of Cpz31

Cpz31 shows high sequence homology to known rhamnosyltransferases from other antibiotic gene clusters, e.g. ElmGT (38% identity/50% similarity) from *S. olivaceus* (Blanco *et al.*, 2001). In order to investigate the role of Cpz31 in caprazamycin biosynthesis, plasmid pLK02 containing *cpz31* was overexpressed in *E. coli* and the resulting protein purified for in vitro studies. His_8-Cpz31 consists of 420 amino acids with a calculated molecular mass of 44.7 kDa. Expression of a corresponding protein could be observed after induction with IPTG. Solubility of the enzyme was low and the yield could not be improved using different culture temperatures or IPTG concentrations for expression. However, His_8-Cpz31 was partially purified by Ni-NTA chromatography resulting in 0.85 mg of protein from 1 litre culture.

3.3. Reaction of Cpz31 with dTDP-L-rhamnose and caprazamycin aglycones

A butanolic extract from a *S. coelicolor* M512/cpzLK09 culture containing caprazamycin aglycones was incubated with His_8-Cpz31 in the presence of dTDP-L-rhamnose. After 2 h, caprazamycin aglycones could not be observed in the assay anymore but instead three new peaks appeared in the LC-chromatograms (Figure III.3.2B; at Rt. 17.2 min, Rt. 18.2 min and Rt. 19.7 min) indicating product formation. Precursor ions for the rhamnosylated caprazamycins E/F with *m/z* 1076.6 $[M+H]^+$, C/D/G with *m/z* 1090.6 $[M+H]^+$ and A/B with *m/z* 1104.6 $[M+H]^+$ were readily detected by ESI-MS product ion scans at Rt. 17.4 min, Rt. 18.5 min and Rt. 19.9 min, respectively (Figure III.3.2C). The MS^2 spectrum obtained from the precursor ion with *m/z* 1090.6 $[M+H]^+$ (Figure III.3.2D) was identical to the predicted fragmentation of the rhamnosylated caprazamycins C/D/G (Figure III.3.2A). Characteristic ions with *m/z* 1091, *m/z* 960 and *m/z* 848 display the presence of the rhamnosyl moiety. Similar spectra were produced by the precursor ions with *m/z* 1076.6 $[M+H]^+$ and *m/z* 1104.6 $[M+H]^+$ representing the rhamnosylated caprazamycins with different fatty acid chain length. These compounds were not detectable in assays without Cpz31 or dTDP-L-rhamnose. Therefore, we conclude that Cpz31 glycosylates the caprazamycin aglycones using dTDP-L-rhamnose as a substrate (Figure III.3.2A)

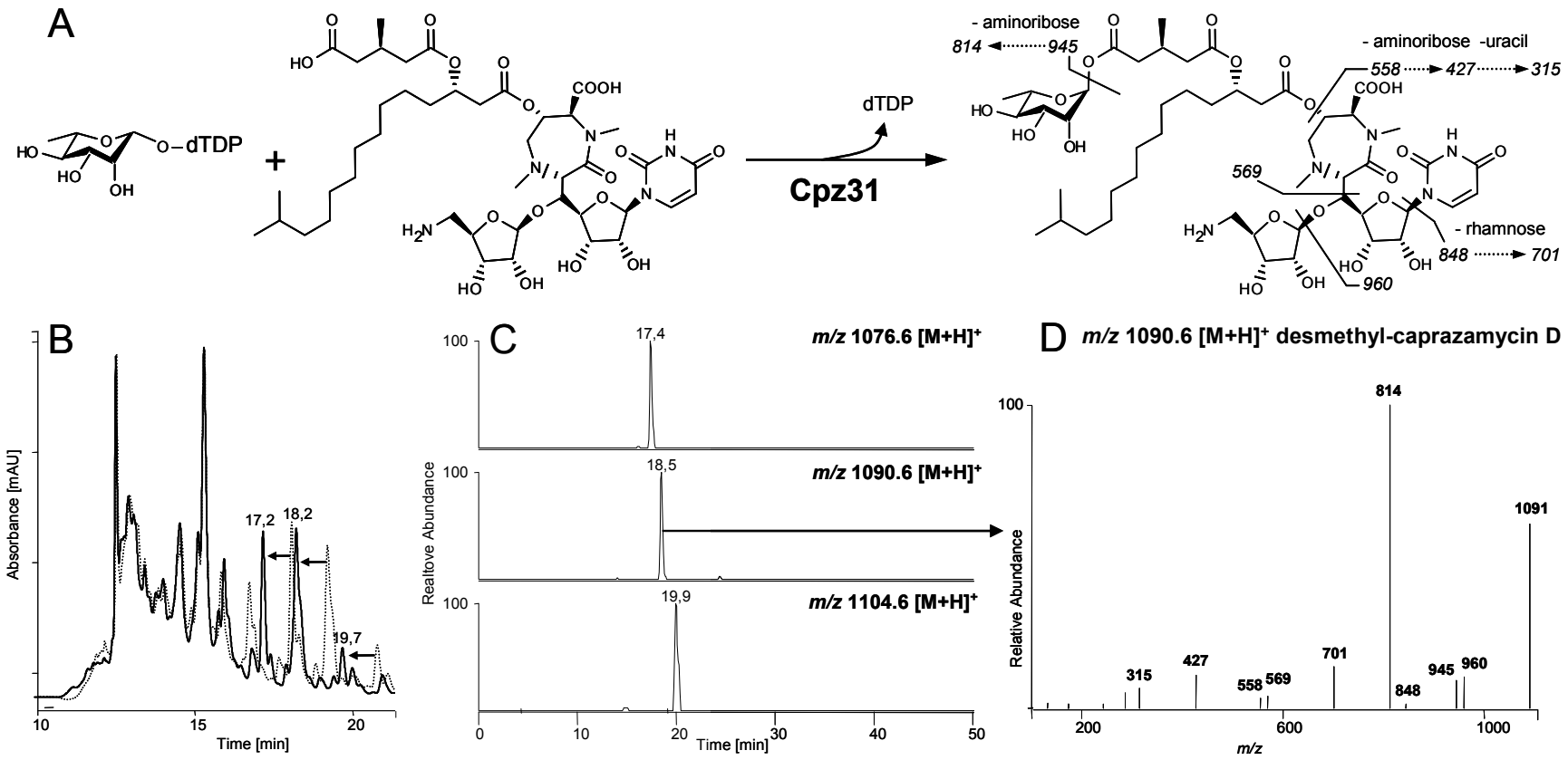


Figure III.3.2 Glycosylation of caprazamycins by Cpz31. **A** Reaction scheme of Cpz31 with dTDP-L-rhamnose and caprazamycin D aglycone as substrates. The suggested fragmentation of the reaction product 2c,3c,4c-desmethyl-caprazamycin D is depicted. **B** Overlay of HPLC chromatograms (262 nm) from activity assays with Cpz31 (black line) and without protein (dashed line). The observed shift in retention time by the complete conversion of caprazamycin aglycones to the rhamnosylated product is indicated by bold arrows. **C** LC-ESI-MS product ion scans in positive mode for desmethyl-caprazamycins E/F with *m/z* 1076.6 $[M+H]^+$, for desmethyl-caprazamycins C/D/G with *m/z* 1090.6 $[M+H]^+$ and for desmethyl-caprazamycins A/B with *m/z* 1104.6 $[M+H]^+$ in the activity assay with Cpz31. **D** Mass spectrometric fragmentation pattern in the CID experiment is shown for 2c,3c,4c-desmethyl-caprazamycin C/D/G.

3.4. Introduction of the caprazamycin gene cluster into different *Streptomyces* host strains

Rhamnosyltransferases have been frequently exploited for the structural diversification of glycosylated antibiotics (Blanco *et al.*, 2001; Gaisser *et al.*, 2009; Luzhetskyy *et al.*, 2007; Olano *et al.*, 2008) as they often exhibit high flexibility towards the donor substrate. In order to generate caprazamycin derivatives with altered sugar moieties the cosmid cpzLK09 was introduced into *S. fradiae* A0 which produce the deoxysugars D-olivose and L-rhodinose and into *Streptomyces* sp. TÜ6071*ΔplaA6* producing L-amicetose. Transfer of the cosmid DNA into the host strains was conducted by intergeneric triparental conjugation. Three individual clones each were selected by their kanamycin resistance and termed as *S. fradiae* A0/cpzLK09-(1-3) and *Streptomyces* sp. TÜ6071*ΔplaA6*/cpzLK09-(1-3), respectively. However, cultivation and analysis of culture extracts from the mutant strains did not lead to the identification of new caprazamycin derivatives. Since the caprazamycin aglycones were readily detected in the culture extracts, it can be speculated that Cpz31 is more specific for dTDP-L-rhamnose than related glycosyltransferases e.g. ElmGT from *S. olivaceus* (Blanco *et al.*, 2001) or StfG from *S. steffisburgensis* (Olano *et al.*, 2008).

4. Identification of the gene cluster for dTDP-L-rhamnose biosynthesis

In bacteria, genes directing the biosynthesis of a certain secondary metabolite are generally clustered together. However, cosmid 31C2 containing the caprazamycin gene cluster missed the genes for the biosynthesis of the deoxysugar moiety. The absence of gene for the biosynthesis of dTDP-L-rhamnose within the corresponding gene cluster has previously been reported for aranciamycin (Luzhetskyy *et al.*, 2007), steffimycin (Gullon *et al.*, 2006), spinosyn (Waldron *et al.*, 2001) and elloramycin (Decker *et al.*, 1995). The formation of L-rhamnose involves four enzymes a dTDP-D-glucose synthase, a dTDP-D-glucose 4,6-dehydratase, a dTDP-3,5-deoxyglucose epimerase and a 4-ketoreductase. Since these genes are required for caprazamycin biosynthesis, they have to be located elsewhere on the chromosome of the original producer strain *Streptomyces* sp. MK730-62F2.

4.1. Cloning and sequencing of a dTDP-L-rhamnose biosynthetic gene cluster

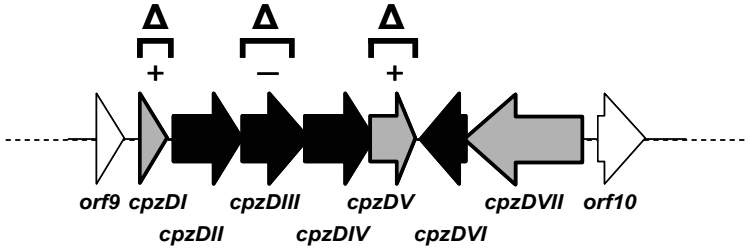


Figure III.4.1 Organization of the L-rhamnose gene cluster *cpzDI* to *cpzDVII* on cosmid 4H11. Black arrows indicate genes proposed to be essential in dTDP-L-rhamnose biosynthesis. Grey arrows indicate genes with unknown function. Squared brackets above the cluster mark the gene deletions performed in this study. (-) indicates that the deletion of the respective region led to an abolishment of caprazamycin production. (+) indicates that caprazamycin production was not influenced.

Using degenerated primers for the amplification of NDP-glucose 4,6-dehydratases (Decker *et al.*, 1996), we obtained a 500 bp partial sequence of *cpzDIII* from genomic DNA of the caprazamycin producer. This fragment was cloned and sequenced. Annotation revealed high homology to dTDP-glucose 4,6-dehydratases on protein level.

Table III.4.1 Deduced functions of genes within the L-rhamnose subcluster

Gene	AA	Protein homolog (Accession number)	Homology*	Proposed function
orf9	140	SvirD4_010100037399, *S. viridochromogenes* DSM 40736	87/93	oxidoreductase
cpzDI	58	SAMR0798, *S. ambofaciens* ATCC 23877)	64/75	unknown
cpzDII	356	MtmD, *S. argillaceus*	76/86	dTDP-glucose synthase
cpzDIII	327	NanG2, *S. nanchangensis*	77/83	dTDP-glucose dehydratase
cpzDIV	296	OleU, *S. antibioticus*	63/71	4-ketoreductase
cpzDV	268	SvirD4_010100037424, *S. viridochromogenes* DSM 40736	96/99	unknown
cpzDVI	202	StrM, *S. glaucescens*	65/77	sugar 3,5-epimerase
cpzDVII	647	SghaA1_010100002568, *S. ghanaensis* ATCC 14672	76/82	cell wall biosynthesis glycosyltransferase
orf10	170	SAMR0794, *S. ambofaciens* ATCC 23877	83/89	phosphoesterase

*Overall homology [%]

Bold genes represent the proposed L-rhamnose subcluster

Perfect matching primers were constructed to screen the cosmid library of *Streptomyces* sp. MK730-62F2. Sequencing and annotation of a positive cosmid, 4H11, revealed a stretch of 6.8 kb DNA putatively involved in the biosynthesis of dTDP-L-rhamnose (Figure III.4.1; GenBank accession number HM051054). According to the gene function predictions (Table III.4.1) *cpzDII* encodes for a putative dTDP-glucose synthase, *cpzDIII* for a dTDP-glucose 4,6-dehydratase, *cpzDIV* for a 4-ketoreductase and *cpzDVI* for a dTDP-deoxyglucose 3,5-epimerases. The identified gene cluster consists of two putative operons (Figure III.4.1). *CpzDII*, *cpzDIII* and *cpzDIV* are translationally coupled to *cpzDV* as indicated by overlap of stop- and start-codons. The deduced gene product of *cpzDV* shows homology to hypothetical proteins from various *Streptomyces* strains which are annotated as methyltransferases. In the opposite direction, *cpzDVI,* is translationally coupled with *cpzDVII,* encoding for a putative glycosyltransferase. Homologs of CpzDVII have been assigned to bacterial cell wall biosynthesis. The putative gene *cpzDI,* 105 bp upstream of *cpzDII,* encodes for a small protein of 57 amino acids with several homologs from *Streptomyces* genomes.

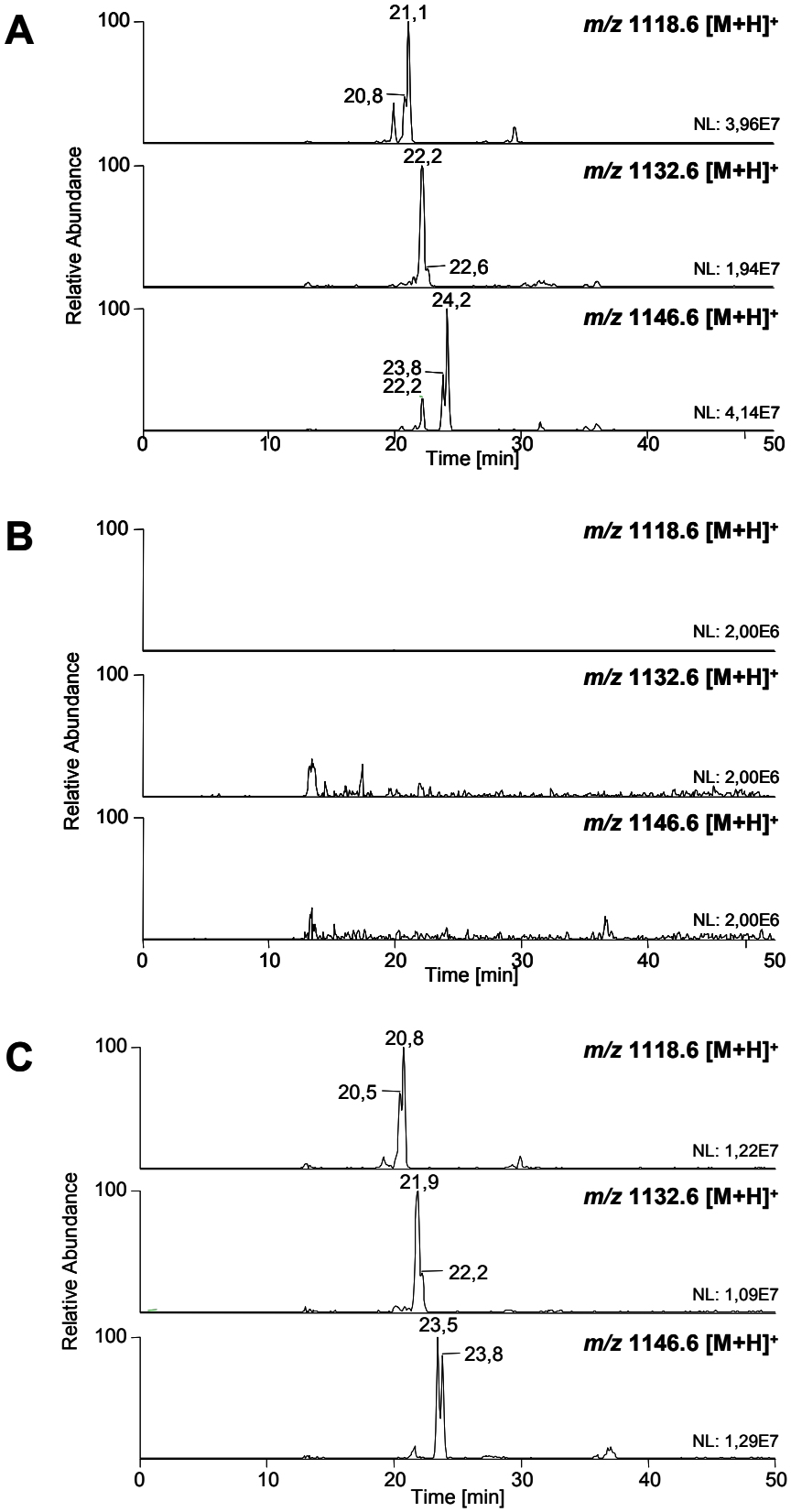


Figure III.4.2 LC-ESI-MS product ion scans for caprazamycins E/F with *m/z* 1118.6 $[M+H]^+$, for caprazamycins C/D/G with *m/z* 1132.6 $[M+H]^+$ and for caprazamycins A/B with *m/z* 1146.6 $[M+H]^+$ in butanolic extracts of **A** *Streptomyces sp.* MK730-62F2 **B** *Streptomyces sp.* MK730-62F2 *ΔcpzDIII* and **C** *Streptomyces sp.* MK730-62F2 *ΔcpzDIII*/pRHAM.

Many of them, e.g. SvirD4_37404 from *S. viridochromogenes* DSM40736 and SAMR0798 from *S. ambofaciens* ATCC 23877, are located next to hypothetical NDP-glucose dehydratase genes. Since *orf9,* a putative oxidoreductase gene and *orf10,* a possible phosphodiesterase gene, have no apparent role in deoxysugar metabolism, we proposed the L-rhamnose biosynthetic gene cluster to span from *cpzDI* to *cpzDVII*.

4.2. Inactivation of *cpzDIII* and complementation with pRHAM

In order to confirm the involvement of the postulated L-rhamnose biosynthetic gene cluster in caprazamycin biosynthesis, the hypothetical dTDP-glucose 4,6-dehydratase gene *cpzDIII* was inactivated. The resulting cosmid cpzLK01 was introduced into *Streptomyces sp.* MK730-62F2 by intergeneric conjugation. Four double-crossover mutants of *Streptomyces sp.* MK730-62F2 *ΔcpzDIII* were finally selected and confirmed by PCR. After cultivation, extracts of the mutant strains were applied to LC-ESI-MS analysis. In contrast to the wildtype strain, production of intact caprazamycins was completely abolished in the mutants (Figure III.4.2). However, caprazamycin aglycones could be clearly detected and extracts of the mutants still showed bioactivity in an agar diffusion assay against *Mycobacterium phlei*. In order to validate that the disruption of *cpzDIII* is responsible for the abolishment of the caprazamycin production, we complemented the Δ*cpzDIII* mutant by introducing pRHAM. After cultivation, LC-ESI-MS analysis showed that caprazamycin production was restored (Figure III.4.2). Analysis of the Δ*cpzDIII* mutant and the complementation confirms that the L-rhamnose biosynthetic gene cluster on cosmid 4H11 is required for the formation of the L-rhamnose moiety in caprazamycin biosynthesis.

4.3. Assembly of caprazamycin gene cluster

In order to proof that the identified genes *cpzDI-DVII* are sufficient for L-rhamnose biosynthesis, we assembled the subcluster to cosmid cpzLK09 containing the caprazamycin gene cluster (Figure III.4.3). It has been shown that the genes *cpz32* – *cpz34* are not involved in caprazamycin biosynthesis by inactivation experiments. Therefore, an apramycin resistance cassette and a spectinomycin/streptomycin resistance cassette were used to replace *cpz32* – *cpz34* by Red/ET-mediated recombination generating cosmid cpzEW05.

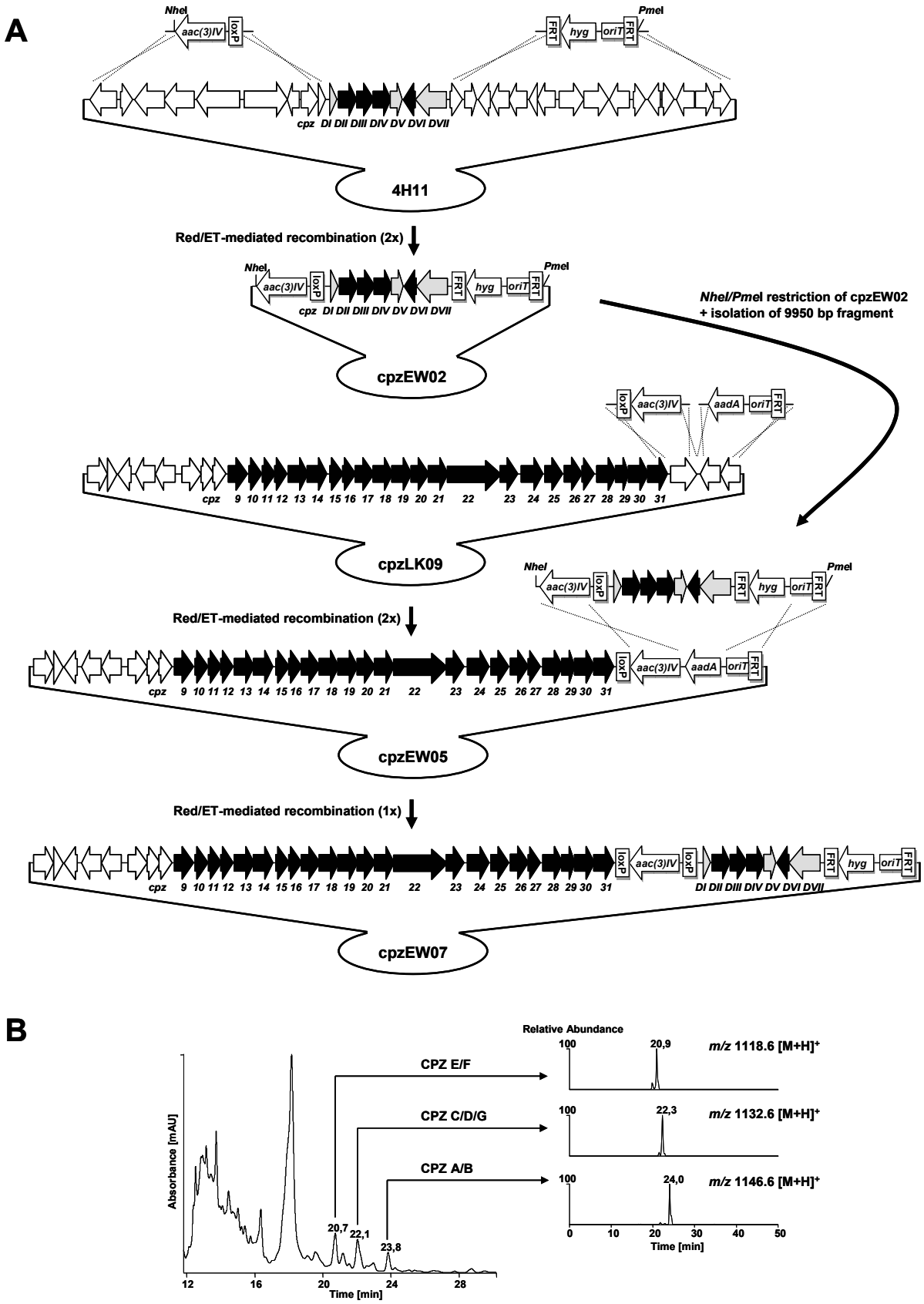


Figure III.4.3 A Strategy for the assembly of the caprazamycin gene cluster and the L-rhamnose subcluster. Black arrows indicate genes proposed to be essential in CPZ biosynthesis. *aac(3)IV*, apramycin resistance gene; *aad4*, spectinomycin/streptomycin resistance gene; *hyg*, hygromycin resistance gene; FRT, FLP recognition site; loxP, Cre recognition site; *oriT*, origin of transfer. The size

of the resistance cassettes and their components are out of scale. **B** LC-ESI-MS UV-chromatogram and product ion scans for caprazamycins E/F with *m/z* 1118.6 $[M+H]^+$, for caprazamycins C/D/G with *m/z* 1132.6 $[M+H]^+$ and for caprazamycins A/B with *m/z* 1146.6 $[M+H]^+$ in butanolic extracts of *S. coelicolor* M512/cpzEW07.

Within cosmid 4H11, containing the L-rhamnose gene cluster, a 14 kb stretch of DNA upstream of *cpzDI* was replaced with an apramycin resistance cassette introducing a unique *Nhe*I restriction site. A 17 kb fragment upstream of *cpzDVII* was replaced by a hygromycin resistance cassette introducing a unique *Pme*I restriction site. From the generated cosmid cpzEW02, a 10 kb *Pme*I/*Nhe*I restriction fragment, containing the *cpzDI-DVII* subcluster, was transferred into Red/ET-proficient *E. coli* cells harbouring cpzEW05. Homologous recombination of the restriction fragment with cosmid cpzEW05 was possible between the apramycin resistance gene *aac(3)IV* (924 bp) and 523 bp identical sequences of the hygromycin and the spectinomycin/streptomycin resistance cassettes. These sequences include an *oriT* and a FRT site. Red/ET-mediated recombination would lead to an apramycin, hygromycin and kanamycin resistant transformant only if homologous recombination occurred within these regions.

The resulting cosmid cpzEW07 was introduced into *S. coelicolor* M512 by intergeneric triparental conjugation. Positive clones were selected by their apramycin, hygromycin and kanamycin resistance and termed as *S. coelicolor* M512/cpzEW07. The mutant strain was cultivated and extracts were analysed by LC-ESI-MS. Three new peaks appeared in the UV-chromatograms at retention times 20.7 min, 22.1 min and 23.8 min (Figure III.4.3B). These peaks were missing in extracts of *S. coelicolor* M512 and *S. coelicolor* M512/cpzLK09. Corresponding mass peaks were detected by product ion scans for the caprazamycins E/F with *m/z* 1118.8 $[M+H]^+$ (Rt. 20.9 min), caprazamycins C/D/G with *m/z* 1132.8 $[M+H]^+$ (Rt. 22.3 min) and the caprazamycins A/B with *m/z* 1146.8 $[M+H]^+$ (Rt. 24.0 min) (Figure III.4.3B). MS^2 fragmentation patterns matched exactly the intact caprazamycins. The heterologous production of caprazamycins in *S. coelicolor* M512 containing the assembled gene cluster proofs that the *cpzDI-DVII* subcluster is sufficient for the biosynthesis of dTDP-L-rhamnose.

4.4. Deletion of *cpzDI* and *cpzDV*

The production of intact caprazamycins by expression of the assembled gene cluster now enabled us to investigate on other genes in caprazamycin biosynthesis in a

heterologous background. However, to allow further gene deletion experiments, the existing apramycin resistance cassette needed to be eliminated from cosmid cpzEW07. This was achieved by a *Spe*I digestion and religation procedure generating cosmid cpzEW08. Corresponding *Spe*I sites were added to the primer sequence during the generation of cosmid cpzEW02 and cpzEW05 (see section II.9.8).

Three of the hypothetical genes in the L-rhamnose gene cluster have no obvious role in dTDP-L-rhamnose biosynthesis. The potential glycosyltransferase CpzDVII contains several conserved domains typical for glycan synthases and is most likely involved in cell wall biogenesis. *cpzDI* is similar to small hypothetical genes often located next to genes from deoxysugar metabolism and *cpzDV* encodes for a putative protein with unknown function. The genes *cpzDI* and *cpzDV* were individually deleted by replacement with an apramycin resistance cassette flanked by loxP sites. Subsequently, the resistance cassettes were eliminated *in vitro* using Cre-recombinase. The resulting cosmids were termed cpzEW10 (*ΔcpzDI*) and cpzEW12 (*ΔcpzDV*). After introduction of the cosmids into *S. coelicolor* M512, hygromycin and kanamycin resistant exconjugants were selected and designated as *S. coelicolor* M512/cpzEW10 and *S. coelicolor* M512/cpzEW12. After cultivation, culture extracts of the mutants showed no differences in LC-ESI-MS analysis in comparison to *S. coelicolor* M512/cpzEW07 containing the complete L-rhamnose cluster. Caprazamycins were accumulated by all of the strains. Therefore we conclude that *cpzDI* and *cpzDV* have no role in caprazamycin biosynthesis and are probably involved in primary metabolism.

5. Identification of the liposidomycin biosynthetic gene cluster

5.1. Cloning and sequencing of the liposidomycins gene cluster

Due to the structural resemblance between the liposidomycins and caprazamycins we assumed similar enzymes to be involved in the formation of both compounds. Oligonucleotides deduced from the sequence of the hypothetical *N*-methyltransferase Cpz11 generated a PCR product with sequence homology to *cpz11* (81%) and other putative SAM-dependent methyltransferase genes. Perfect matching primers were applied to a genomic library of *Streptomyces* sp. SN-1061M constructed in a SuperCos1 vector. Two positive cosmids out of 1100 could be identified and proven to contain overlapping DNA by restriction mapping. Cosmid 3G5 was finally selected for complete shot-gun sequencing. The nucleotide sequence of the gene cluster has been deposited in GenBank under accession number GU219978. Sequencing of the cosmid clone revealed a 45.8 kb insert with an average GC content of 70.2%, similar to other sequences from *Streptomyces* DNA. *In silico* analysis using BLAST homology searches, conserved protein domain searches and the GC frame plot method revealed 39 putative genes. A total of 25 open reading frames, designated *lipA-lipY*, were assigned to the liposidomycin gene cluster presumably encoding for biosynthesis, resistance, transport and regulation (Figure III.5.1). Proposed functions of the annotated genes and best matches in homology search are shown in Table III.5.1.

5.2. Sequence analysis of the liposidomycins gene cluster

A comparison of the annotated genes on this cosmid and the caprazamycin biosynthetic cluster reveals striking similarities (Figure III.5.1). The genes *cpz9* to *cpz27* have been assigned previously to the formation of the caprazamycin aglycones. In the liposidomycin cluster, the genes *lipG* – *lipY* show 78% - 86% sequence identity to *cpz9* – *cpz27* on DNA level. All of these genes are transcribed in the same direction and several subcluster seem to be translational coupled, indicated by overlap of stop and start codons. The only significant difference concerns the organization of the genes *cpz10-12* and *cpz13/14* in two putative operons while their homologs *lipH-L* form a single 4.4 kb subcluster. This indicates that the biosynthesis of the non-sulfated, non-glycosylated liponucleoside core structure, the regulation

and the resistance mechanism are analog for both compounds (see Discussion section).

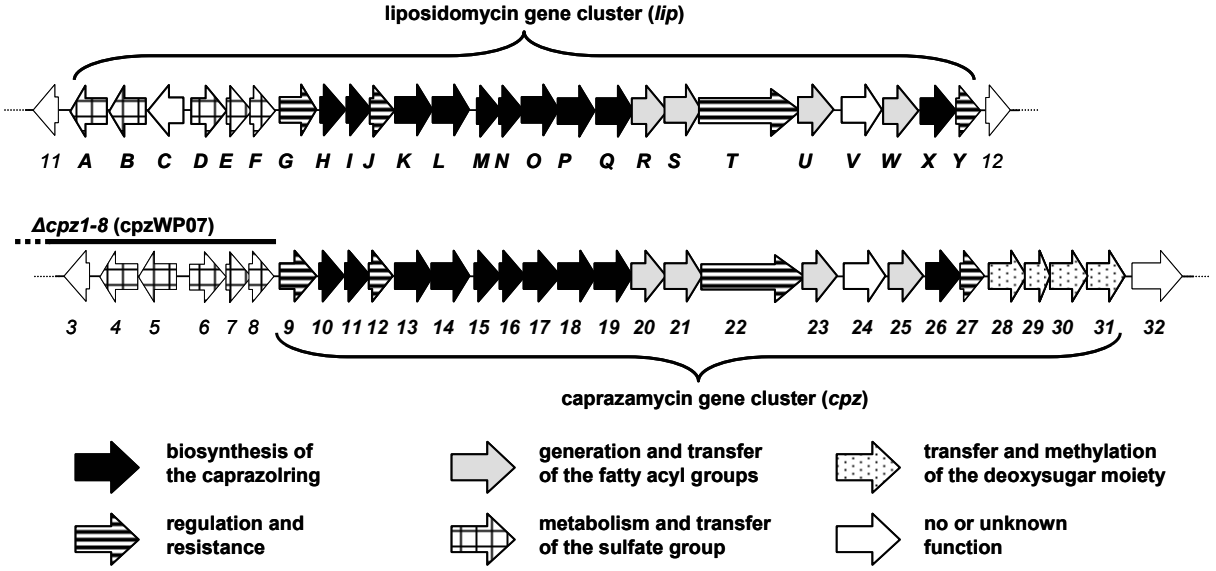


Figure III.5.1 Organization of the liposidomycin and the caprazamycin gene cluster. Assignment of genes to different steps of their biosynthesis is indicated by different colours. A black bar above genes indicate the gene deletion in the mutant strain *S. coelicolor* M512/cpzWP07. Note that *lipA* is a homolog of *cpz5* and *lipB* a homolog of *cpz4*.

Genes directing the attachment and methylation of a deoxysugar, similar to the *cpz28-31* genes at the right end of the caprazamycin cluster, were not identified on cosmid 3G5 reflecting the absence of the rhamnosyl moiety in the liposidomycins (Figure III.5.1). Instead, downstream of *lipY* we found an open reading frame *orf12* encoding for a hypothetical protein with no apparent function in liposidomycin biosynthesis. The adjacent genes *orf13* and *orf14* have sequence similarity to protein kinases (61%) and DNA helicases (59%) respectively and are most likely involved in primary metabolism. Consequently, *lipY* was defined to be the right border of the liposidomycin cluster. On the left end, upstream of the *lipG*, a set of genes *lipA* – *lipF* are proposed to play a role in sulfation of the liposidomycins. Similar genes were identified adjacent to the caprazamycin gene cluster and have been shown not to be involved in caprazamycin formation by inactivation experiments. *lipA* is flanked by the genes *orf1-10* which can not be assigned to the liposidomycin biosynthesis but are highly homologous to a contiguous part of sequence from *Streptomyces coelicolor* (SCO2293 – SCO2301). Therefore the biosynthetic gene cluster for liposidomycins is predicted to span 29.7 kb from *lipA* – *lipY*.

Table III.5.1 Deduced functions of genes in the liposidomycin gene cluster

Gene	AA	Protein homolog (BLAST)	Identity/ Similarity[a]	Proposed function
orf1[b]	198	SCO2301, *Streptomyces coelicolor* A3(2)	87/91	NGG1-interacting factor 3
orf2	197	SCO2300, *S. coelicolor* A3(2)	85/91	hypothetical protein
orf3	452	SCO2299, *S. coelicolor* A3(2)	70/76	RNase H/acid phosphatase
orf4	223	SCO2298, *S. coelicolor* A3(2)	80/88	KHG/KDPG aldolase
orf5	261	SCO2297, *S. coelicolor* A3(2)	87/92	hypothetical protein
orf6	478	SAV5880, *S. avermitilis* MA4680	79/85	ribonuclease R
orf7	208	SCO2296, *S. coelicolor* A3(2)	62/70	integral membrane protein
orf8	250	SCO2295, *S. coelicolor* A3(2)	66/75	transcriptional regulator
orf9	275	SCO2294, *S. coelicolor* A3(2)	81/87	transcriptional regulator
orf10	310	SCO2293, *Streptomyces coelicolor* A3(2)	80/87	integral membrane protein
orf11	173	RHA1_ro01178, *Rhodococcus jostii* RHA1	31/36	hypothetical protein
lipA[c]	391	hmgs , *S. anulatus*	70/79	HMG-CoA synthase
lipB	413	MkanA1_04597, *Mycobacterium kansasii*	36/51	sulfotransferase
lipC	246	Mpop_3269, *Methylobacterium populi*	23/38	hypothetical protein
lipD	350	SaRppA, *S. antibioticus*	30/45	type III pks
lipE	260	Francci3_1340, *Frankia* sp. CcI3	56/65	PAPS 3'phosphatase
lipF	212	gll2605, *Gleobacter violaceus* PCC7421	41/56	hypothetical protein
lipG	336	Cpz9, *Streptomyces* sp. MK730-62F2	65/75	transcriptional regulator
lipH	173	Cpz10, *Streptomyces* sp. MK730-62F2	82/88	*β*-hydroxylase
lipI	213	Cpz11, *Streptomyces* sp. MK730-62F2	76/84	methyltransferase
lipJ	185	Cpz12, *Streptomyces* sp. MK730-62F2	71/80	kinase
lipK	443	Cpz13, *Streptomyces* sp. MK730-62F2	80/86	aminotransferase
lipL	424	Cpz14, *Streptomyces* sp. MK730-62F2	87/91	hydroxymethyltransferase
lipM	276	Cpz15, *Streptomyces* sp. MK730-62F2	82/90	dioxygenase
lipN	233	Cpz16, *Streptomyces* sp. MK730-62F2	81/91	nucleotidyltransferase
lipO	377	Cpz17, *Streptomyces* sp. MK730-62F2	81/87	glycosyltransferase
lipP	424	Cpz18, *Streptomyces* sp. MK730-62F2	82/88	aminotransferase
lipQ	457	Cpz19, *Streptomyces* sp. MK730-62F2	82/88	pyrimidine phosphorylase
lipR	354	Cpz20, *Streptomyces* sp. MK730-62F2	88/91	acyl-CoA synthase
lipS	495	Cpz21, *Streptomyces* sp. MK730-62F2	83/88	carboxyesterase
lipT	1237	Cpz22, *Streptomyces* sp. MK730-62F2	78/85	ABC transporter
lipU	344	Cpz23, *Streptomyces* sp. MK730-62F2	83/93	lipase
lipV	599	Cpz24, *Streptomyces* sp. MK730-62F2	70/80	hypothetical protein
lipW	342	Cpz25, *Streptomyces* sp. MK730-62F2	90/95	dehydrogenase
lipX	417	Cpz26, *Streptomyces* sp. MK730-62F2	75/84	methyltransferase
lipY	185	Cpz27, *Streptomyces* sp. MK730-62F2	70/79	kinase
orf12	207	StAA4_29435, *Streptomyces* sp. AA4	61/71	hypothetical protein
orf13	399	SSDG_03666, *S. pristinaespiralis* 25486	45/61	Ser/Thr protein kinase
orf14	997	FRAAL6332, *Frankia alni* ACN14a	46/59	DNA/RNA helicase

[a] Overall homology [%]
[b] Gene lacks an appropriate stop codon and is considered to be uncomplete on cosmid 3G5
[c] Bold genes proposed to be essential for liposidomycin production

The characteristic feature of the liposidomycins is the sulfate group at the 2''-OH of the aminoribose. The deduced gene product LipE shows high homology to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) 3'-phosphatases in the database (56%/64%; identity/similarity). This family of enzymes is predicted to take part in the regulation of the intracellular level of PAPS, which is the most important sulfate donor in sulfation reactions. Proteins involved in the metabolism of PAPS are encoded in other gene clusters of sulfated natural products, e.g. cylindrospermopsin (Mihali *et al.*, 2008) and saxitoxin (Kellmann *et al.*, 2008). Translationally coupled to the *lipE* gene is *lipD*, a putative type III pks gene, and *lipF* encoding for a hypothetical protein with unknown function. The gene product of *lipB* is similar to several conserved hypothetical proteins from bacteria and fungi with unknown function.

Notably, a LipB-homolog from *Thermobaculum terrenum* is annotated as an arylsulfotransferase due to the presence of a corresponding conserved domain (pfam05935). Therefore, we propose that the region *lipA-F*, including the genes for a PAPS-phosphatase and a potential sulfotransferase is responsible for the supply and incorporation of the sulfate group in liposidomycin biosynthesis. Surprisingly, similar genes can also be found upstream of *cpz9*, adjacent to the caprazamycin gene cluster (Figure III.5.1). The genes *lipD-F* show 82%, 80% and 83% sequence identity to *cpz6-8*, respectively. In addition, homology is observable for the putative HMG-CoA synthases LipA and Cpz5 (81%/89% identity/similarity) and to a lesser extend for the hypothetical proteins LipB and Cpz4 (32%/50% identity/similarity).

5.3. Heterologous expression of the liposidomycin gene cluster

In order to verify that the identified genes were sufficient for the biosynthesis of liposidomycins, we prepared the cosmid 3G5 for heterologous expression. For this purpose, the beta-lactamase (*bla*) gene on the backbone of 3G5 was replaced with an integration cassette of pIJ787 containing the *attP* attachment site and the integrase gene (*int*) of phage ΦC31, a tetracycline resistance gene (*tet*) and an origin of transfer (*oriT*) using Red/ET-mediated recombination (Gust *et al.*, 2003). The generated cosmid lipLK01 was introduced into *S. coelicolor* M512 by triparental intergeneric conjugation and three kanamycin resistance clones were selected. Extracts of cultures from *S. coelicolor* M512 and the mutant strains were applied to HPLC and ESI-MS/MS analysis.

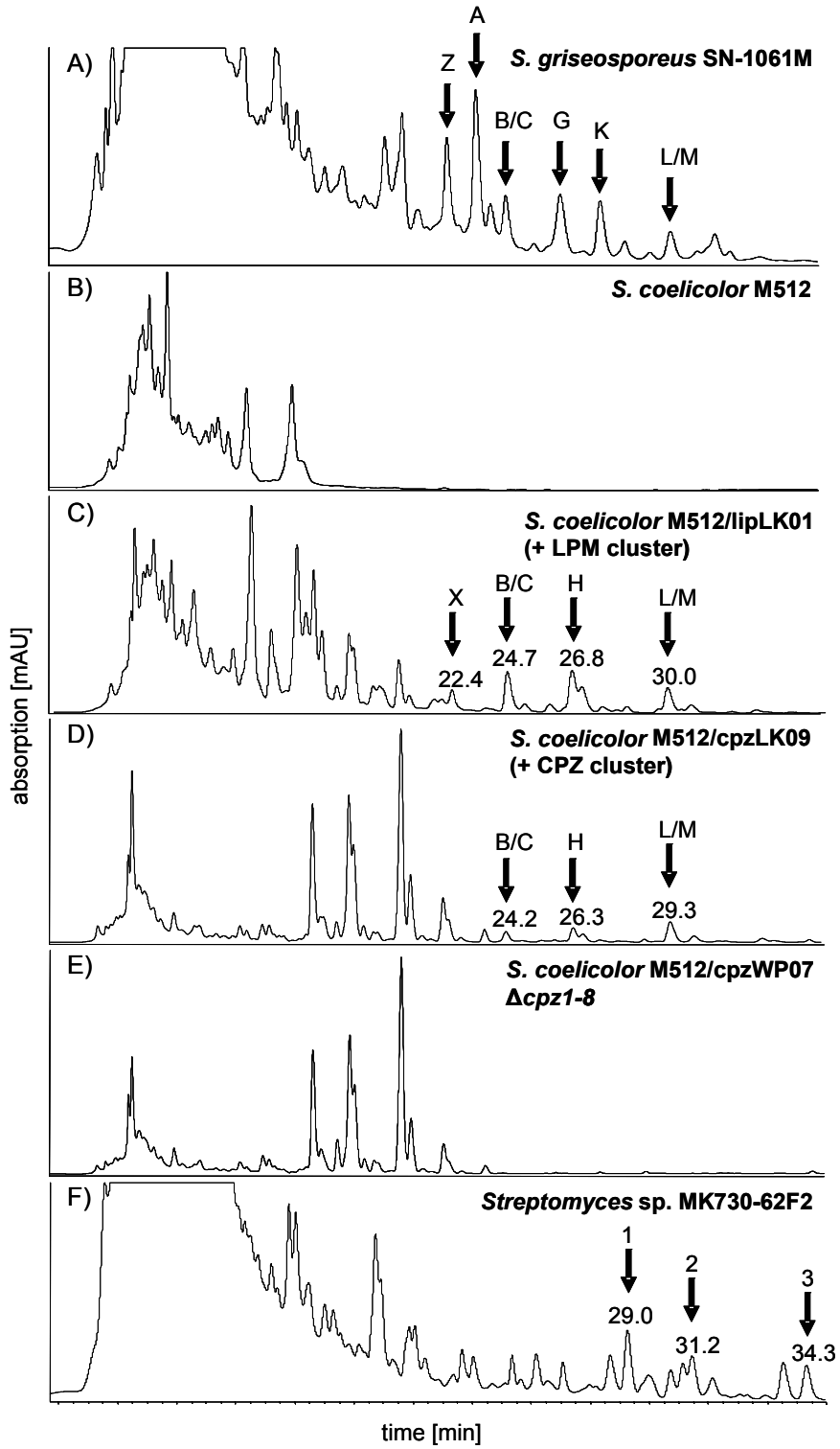


Figure III.5.2 HPLC chromatograms of *n*-butanolic extracts from **A** the liposidomycin (LPM) producer strain *Streptomyces* sp. SN-1061M **B** *S. coelicolor* M512 **C** *S. coelicolor* M512 containing the LPM gene cluster lipLK01 **D** *S. coelicolor* M512 containing the caprazamycin gene cluster cpzLK09 **E** *S. coelicolor* M512 containing the caprazamycin gene cluster cpzWP07, lacking the flanking genes *cpz1-8* and **F** the caprazamycin (CPZ) producer *Streptomyces* sp. MK730-62F2. The different LPMs in **A**, **C** and **D** have been assigned by LC-ESI-MS/MS and are indicated by black arrows and bold letters. The black arrows in **F** indicate the identified sulfo-CPZs E/F (**1**), C/D/G (**2**) and A/B (**3**).

In UV-chromatograms of *S. coelicolor* M512/lipLK01 extracts (Figure III.5.2C) four prominent peaks at Rt = 22.4 min, Rt = 24.7 min, Rt = 26.8 min and Rt = 30.0 min appeared which could not be found in extracts of *S. coelicolor* M512 without the liposidomycin cluster (Figure III.5.2B). Mass peaks for known liposidomycins with corresponding retention times were observed in product ion chromatograms; for liposidomycin X with *m/z* 996.5 $[M+H]^+$ at Rt = 22.7 min, for liposidomycin B and C with *m/z* 1010.5 $[M+H]^+$ at Rt = 25.0 min, for liposidomycin H with *m/z* 1024.5 $[M+H]^+$ at Rt = 27.1 min and for liposidomycin L and M with *m/z* 1038.5 $[M+H]^+$ at Rt = 30.3 min. Characteristic fragmentation patterns were observed by collision-induced dissociation of the sulfate-group, aminoribose, uracil and fatty acid group as published for FAB-MS (Ubukata *et al.*, 1992). Similar fragmentations have been shown for the caprazamycins in section III.1. For further evidence, extracts of the genuine liposidomycin producer *Streptomyces* sp. SN-1061M were analysed by LC-ESI-MS/MS. In UV-chromatograms, the wildtype strain produced several peaks which represent the liposidomycins Z, A, B, C, G, K, L and M (Figure III.5.2A). Product ion scans clearly detected liposidomycins X, B/C, H and L/M and the fragmentation patterns matched exactly the liposidomycins found in the heterologous producer. In summary, the analytical data proves that *S. coelicolor* M512 containing lipLK01 produces liposidomycins which confirms the identification of the liposidomycin gene cluster.

5.4. Identification of new sulfated caprazamycin derivatives

Postulating that the genes *lipA-F* confer to the capability of a sulfotransfer reaction, the same should apply to the *cpz4-8* genes flanking the caprazamycin cluster. In order to verify this assumption, we carefully reinvestigated *S. coelicolor* M512 containing the caprazamycin gene cluster and the genuine producer *Streptomyces* sp. MK730-62F2 for the accumulation of sulfated caprazamycin derivatives. And indeed, HPLC-chromatograms of extracts from *S. coelicolor* M512/cpzLK09, showed peaks at similar retention times as the liposidomycins at 24.2 min, 26.3 min and 29.3 min (Figure III.5.2D). The extracts were further analyzed by LC-ESI-MS/MS to confirm the identity of the new compounds. Ions with *m/z* 1010.5 $[M+H]^+$ at Rt = 24.4 min, *m/z* 1024.5 $[M+H]^+$ at Rt = 26.5 min and *m/z* 1038.5 $[M+H]^+$ at Rt = 29.6 min in product ion chromatograms matched the liposidomycins B/C, H and L/M. CID fragmentation in positive mode was characteristic for the liposidomycins, with the initial loss of the sulfate group (80 Da). For precursor ion with *m/z* 1010.5 $[M+H]^+$

fragments with *m/z* 931, *m/z* 800 and *m/z* 687 were detected which presumably derive from acylated fragments (Figure III.5.3A).

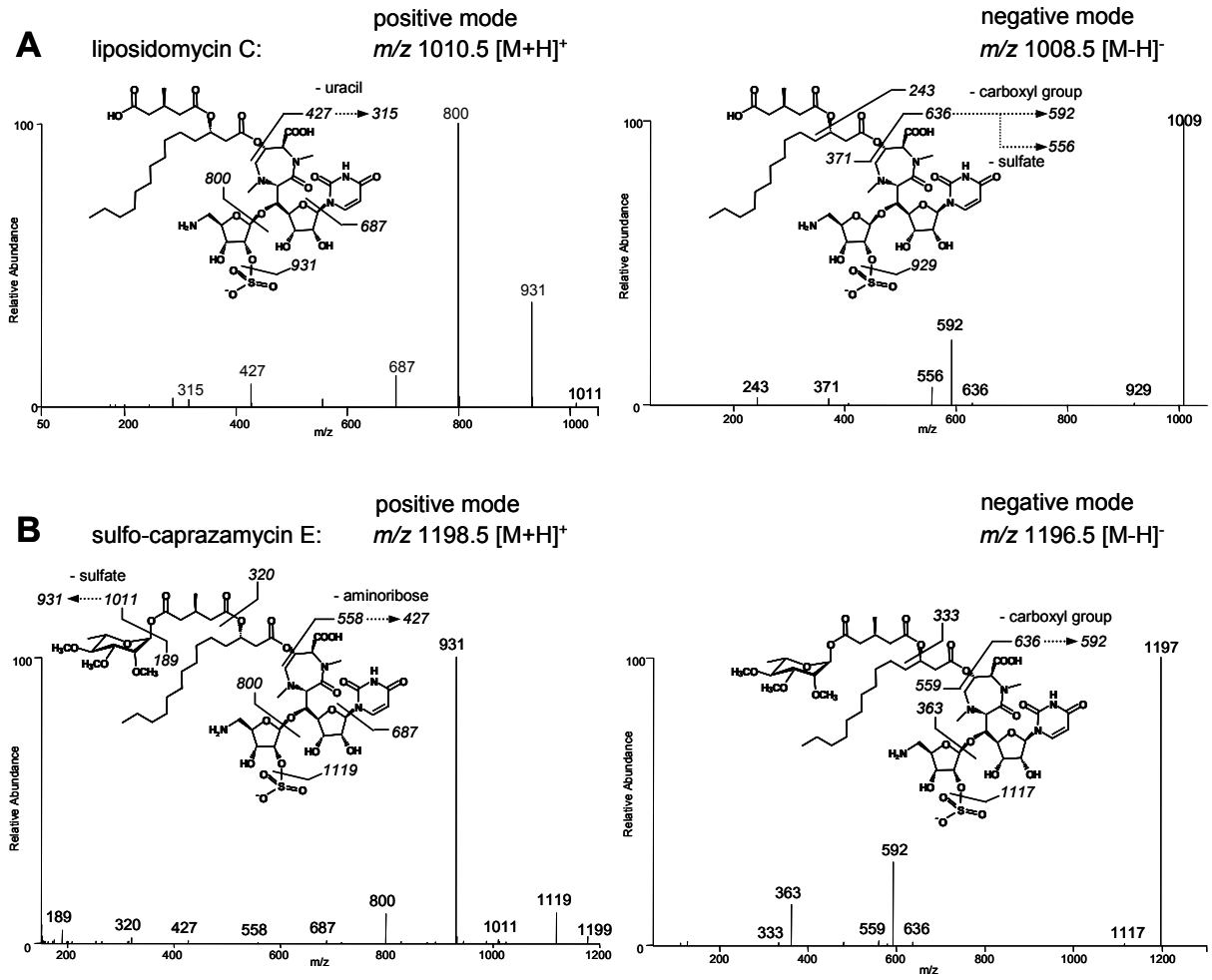


Figure III.5.3 Mass spectrometric fragmentation pattern for collision-induced dissociation experiments in positive and negative mode. **A** LPM B/C from *S. coelicolor* M512/cpzLK09 and **B** sulfo-CPZ E/F from *Streptomyces* sp. MK730-62F2. Suggested fragmentation schemes are depicted for LPM C and its glycosylated derivative sulfo-CPZ E.

Molecular ions with *m/z* 427 and *m/z* 315 were assigned to components of the caprazol structure. The observed CID fragmentation pattern of these new compounds matched perfectly to the liposidomycins produced by the heterologous host *S. coelicolor* M512/lipLK01. Using the same extracts, product ion scans in negative mode provided additional evidence (Figure III.5.3A). Dissociation of precursor ion with *m/z* 1008.5 $[M-H]^-$ (LPM B/C) gave ions with *m/z* 243 and *m/z* 371 which probably represent the acyl part, ions with *m/z* 556, *m/z* 592 and *m/z* 636 for the caprazol core structure and with *m/z* 929, the desulfated (-80 Da) compound. The identification of liposidomycins in the culture broth of a strain expressing the

caprazamycin gene cluster shows, that enzymes for a sulfation reaction have to be encoded on both cosmids, cpzLK09 and lipLK01. We analyzed another mutant strain, *S. coelicolor* M512/cpzWP07, which contains an intact gene cluster for the production of the caprazamycin aglycones but additionally possesses a functional knock-out of the genes *cpz1-8* . Peaks for liposidomycins could be found neither in HPLC (Figure III.5.2E) nor in LC-ESI-MS analysis. This proves that the homologous genetic regions *lipA-F* and *cpz4-8* are involved in an enzymatic mechanism for the sulfation of liponucleoside antibiotics.

Our findings prompted us to search for sulfated caprazamycin derivatives in the wildtype strain *Streptomyces* sp. MK730-62F2. Product ion scans revealed prominent mass peaks with *m/z* 1198.5 $[M+H]^+$ (Rt. 29.4 min; CPZ E/F + 80 Da), *m/z* 1212.5 $[M+H]^+$ (Rt. 31.6 min; CPZ C/D/G + 80 Da) and *m/z* 1226.5 $[M+H]^+$ (34.7 min; CPZ A/B + 80 Da) in extracts from the caprazamycin producer strain. Analogous peaks were found with *m/z* 1196.5 $[M-H]^-$, *m/z* 1210.5 $[M-H]^-$ and *m/z* 1224.5 $[M-H]^-$ in negative mode. Positive CID of the new substances was similar to the caprazamycin as shown in section III.1. Precursor ion *m/z* 1198.5 $[M+H]^+$ produced fragments with *m/z* 189, *m/z* 320, *m/z* 427, *m/z* 558, *m/z* 687, *m/z* 800, *m/z* 931, *m/z* 1011 and *m/z* 1119 (Figure III.5.3B) displaying the loss of the rhamnosyl-moiety, the aminoribose, the uracil, the lipid components and a sulfate group. In negative mode (Figure III.5.3B), fragmentation of precursor ion with *m/z* 1196.5 $[M-H]^-$ resulted in ions with *m/z* 333 and *m/z* 559 which possibly represent the glycosylated acyl part of the substance, in fragment ions with *m/z* 592 and *m/z* 636, the sulfated caprazol core structure and with *m/z* 1117, the desulfated (-80 Da) compound. Though the position of the sulfate group is still unclear, a comparison of the ions *m/z* 363, *m/z* 592 and *m/z* 636 indicate that the sulfate is bound to the aminoribosyl moiety similar to the liposidomycins. The prominent fragment *m/z* 363 matches well with a caprazol substructure (592 Da) lacking the sulfated aminoribose. Therefore we propose that the substances identified in the extract of the caprazamycin producer *Streptomyces* sp. MK730-62F2 (Figure III.5.F) are indeed sulfated caprazamycins, which so far have not been described.

6. Characterization of Cpz4 as a novel arylsulfate sulfotransferase

This part of the thesis was carried out in collaboration with Diploma student Kornelia Eitel. Sulfation describes the transfer of a sulfate group onto an acceptor molecule. The responsible enzymes, the sulfotransferases, participate in different processes such as intracellular signaling, extracellular interaction and detoxification of xenobiotics (Chapman *et al.*, 2004). Sulfotransferases have been extensively studied in humans and other mammals and are usually classified in the membrane associated and the cytosolic type. In contrast, little is known about bacterial sulfotransferases. Recent reports suggested their function as important modulators of e.g. plant host interaction in *Sinorhizobium meliloti* (Cronan & Keating, 2004) and virulence in *Mycobacterium tuberculosis* (Goren *et al.*, 1974). Several sulfated bioactive compounds were isolated from microorganisms over the last decades, including the micafungin precursor FK463, (Iwamoto *et al.*, 1994), the cyanobacterial toxin cylindrospermopsin (Harada *et al.*, 1994) and the liponucleoside antibiotic liposidomycins (Isono *et al.*, 1985). Though, in bacterial secondary metabolism only few sulfotransferases have been studied in vitro. StaL, involved in the biosynthesis of the glycopeptide A47934, catalyzes the transfer of a sulfate group from 3'-phosphoadenosin 5'-phosphosulfate (PAPS) onto the acceptor substrate (Lamb *et al.*, 2006; Shi *et al.*, 2007). Another PAPS-dependent sulfotransfer reaction has been investigated in curacin biosynthesis (Gu *et al.*, 2009).

The vast majority of known sulfotransferases from eukaryotes and prokaryotes uses PAPS as the sulfate donor substrate. However, a small class of microbial sulfotransferases is PAPS-independent and transfers a sulfate group from an aromatic donor to an aromatic acceptor molecule. These enzymes were termed arylsulfate sulfotransferases (ASSTs). The reaction of ASSTs proceeds by a ping pong bi-bi mechanism with a covalent modification of the enzyme by the sulfation of an active site histidine (Malojcic *et al.*, 2008). In vitro studies of eight arylsulfate sulfotransferases (ASSTs) have been published from bacteria (Baek *et al.*, 1996; Kang *et al.*, 2001a; Kang *et al.*, 2001b; Kim *et al.*, 2007; Konishi-Imamura *et al.*, 1994; Kwon *et al.*, 1999; Kwon *et al.*, 2001; Malojcic *et al.*, 2008), but for all of them their precise physiological role is yet unknown.

6.1. Analysis of the *Δcpz1-3* and the *Δcpz1-4* deletion mutants

In the last section I showed that the heterologous host strain *S. coelicolor* M512 harbouring the entire caprazamycin gene cluster not only produces the caprazamycin aglycones but also sulfated derivatives thereof i.e. the liposidomycins. In our search for the gene, which might be responsible for the sulfation reaction, we focused on *cpz4* (GenBank accession number GU323955) located 4.2 kb upstream of the gene cluster. Cpz4 exhibits significant sequence homology to a large number of hypothetical proteins from bacteria and fungi. A conserved protein domain (Pfam05935) was found, characteristic for the arylsulfate sulfotransferases (ASSTs) (Baek *et al.*, 1996). Overall, the known ASSTs show only low sequence similarity to Cpz4 e.g. 26% in the case of the ASST from *E.coli* CFT073 (Malojcic *et al.*, 2008). In order to investigate the role of *cpz4* we compared the previously generated deletion mutant *S. coelicolor* M512/cpzWP05 (*Δcpz1-4*) with mutant *S. coelicolor* M512/cpzWP04 (*Δcpz1-3*). Culture extracts of both mutants were applied to LC-ESI-MS/MS and analyzed for the presence of liposidomycins. As expected, the caprazamycin aglycones were accumulated by both mutants (Figure III.6.1). However, the sulfated liposidomycins (LPM E/F with *m/z* 1010.5 $[M+H]^+$ at Rt. 24.4 min, LPM C/D/G with *m/z* 1024.5 $[M+H]^+$ at Rt. 26.5 min, LPM A/B with *m/z* 1038.5 $[M+H]^+$ at Rt. 29.6 min) could only be identified in the mutant containing an intact copy of the *cpz4* gene as demonstrated by HPLC-UV and HPLC-MS analysis (Figure III.6.1). This result proves that Cpz4 is essential for the sulfation of caprazamycin aglycones.

A homolog of Cpz4 was also found in the liposidomycin gene cluster of *Streptomyces* sp. SN-1061M. *lpmB* is located at one end of the cluster and its gene product shows 32% identity and 50% similarity to the central 300 amino acids of Cpz4. Notably, LpmB (412 aa) lacks 101 amino acids which form the C-terminus of Cpz4 (513 aa). It appears likely that *lpmB* has a similar function as Cpz4 in liposidomycin biosynthesis though a conserved protein domain such as Pfam05935 could not been identified.

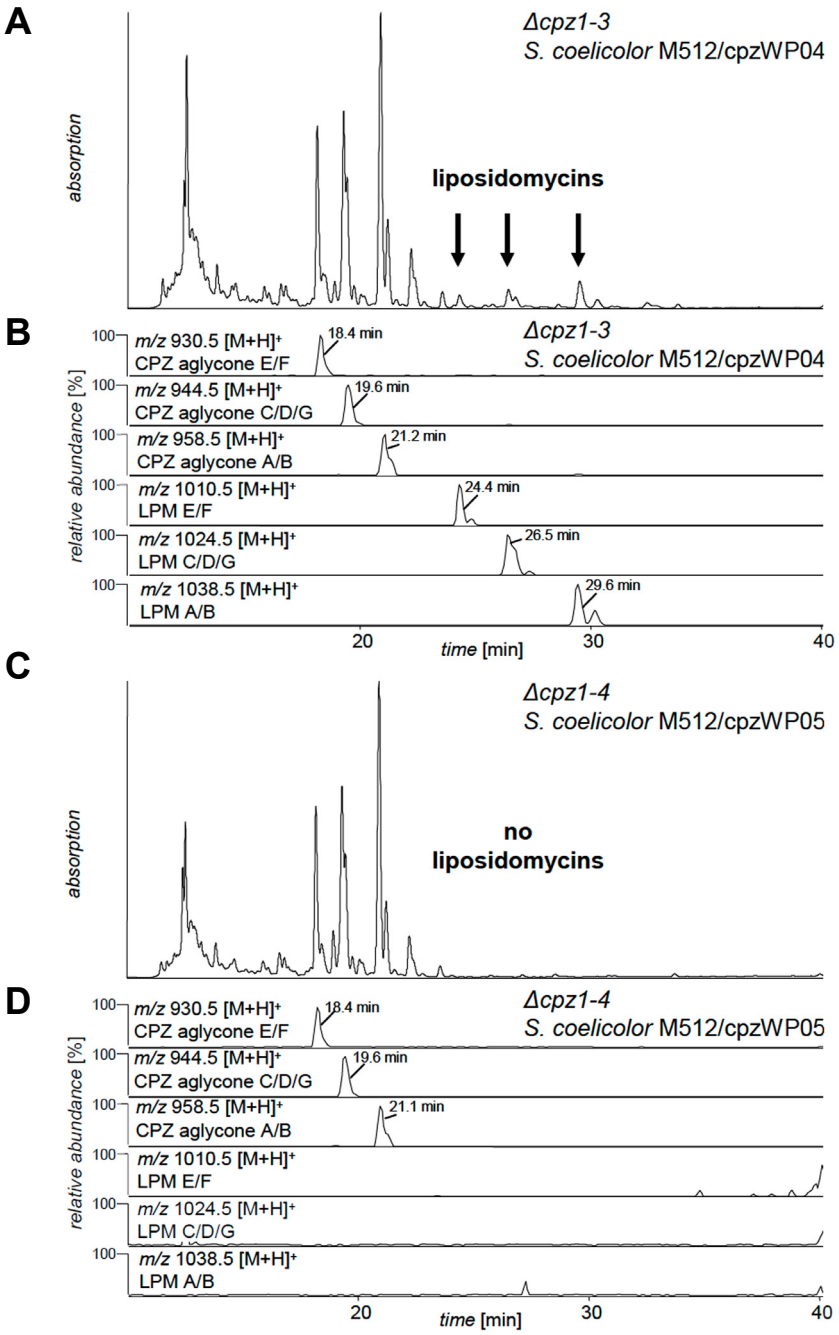


Figure III.6.1 HPLC-UV and HPLC-MS analysis of *n*-butanolic extracts from a *cpz4* mutant strain. **A** UV-chromatogram of an extract from *S. coelicolor* M512/cpzWP04 containing a derivative of cosmid cpzLK09 in which the genes *cpz1-cpz3* are deleted and **B** the respective product ion chromatograms. **C** UV-chromatogram of an extract from *S. coelicolor* M512/cpzWP05 containing a derivative of cosmid cpzLK09 in which the genes *cpz1-cpz4* are deleted and **D** the respective product ion chromatograms. UV-absorption was monitored at 262 nm. Product ion chromatograms were obtained from (+)-ESI-MS mass scans for CPZ E/F aglycones with *m/z* 930.5 $[M+H]^+$, CPZ C/D/G aglycones with *m/z* 944.5 $[M+H]^+$, CPZ A/B aglycones with *m/z* 958.5 $[M+H]^+$ and for their corresponding sulfated derivatives LPM E/F with *m/z* 1010.5 $[M+H]^+$, LPM C/D/G with *m/z* 1024.5 $[M+H]^+$, LPM A/B with *m/z* 1038.5 $[M+H]^+$. LPM (liposidomycins); CPZ (caprazamycin).

6.2. Expression and purification of Cpz4

For the biochemical investigation of the potential sulfotransferases, *cpz4* and *lpmB* were cloned into a vector for the expression as *N*-terminal His-tagged proteins and introduced into *E. coli* Rosetta2™ (DE3)pLys. Induction with IPTG led to the production of a protein with ~57.7 kDa matching the calculated molecular mass of His_8-Cpz4 (57.626 kDa). However, LpmB could not be obtained as a soluble protein. Hence, we proceeded with Cpz4 alone. Ni^{2+} affinity chromatography and subsequent gel chromatography resulted in 25 mg of protein from 2 L of culture, showing approximately 95% purity in SDS-PAGE. This protein was used for further biochemical studies.

6.3. Analysis of Cpz4 as an arylsulfate sulfotransferase

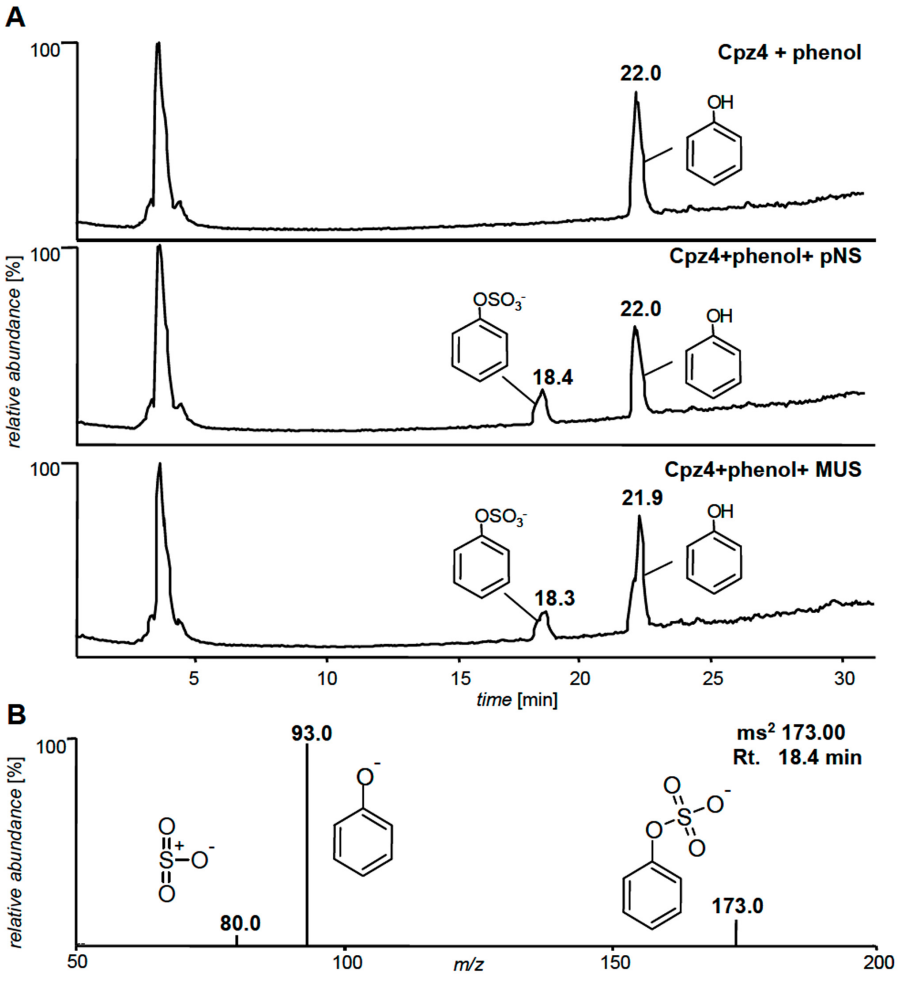


Figure III.6.2 LC-ESI-MS/MS analysis of sulfotransferase assays with Cpz4 using phenol as sulfate acceptor and either pNS (*p*-nitrophenol sulfate) or MUS (methylumbelliferone sulfate) as sulfate donor substrates (see also diploma thesis of Kornelia Eitel). **A** TIC (total ion current)-chromatograms. **B** MS^2 spectrum of the enzymatic reaction product phenolsulfate with *m/z* 173 $[M-H]^-$ at Rt. 18.4 min from the assay with Cpz4, phenol and pNS.

Identification of a conserved protein domain typical for ASSTs suggested that Cpz4 may catalyze a PAPS-independent sulfation reaction. Accordingly, Cpz4 was incubated with either *p*-nitrophenol sulfate (pNS) or methylumbelliferone sulfate (MUS) as sulfate donor substrates and phenol as an acceptor substrate. Product formation was monitored by LC-ESI-MS/MS analysis. In the total ion current (TIC) chromatograms a peak appeared at ~18.3 min both in assays with pNS and in assays with MUS as donor substrate (Figure III.6.2A). The formation of this product was dependent on the presence of active Cpz4. The mass spectrometric data of the new compound (Figure III.6.2B) corresponded to the expected product phenolsulfate with *m/z* 173 $[M-H]^-$ and MS^2 fragments with *m/z* 93 (phenol) and *m/z* 80 (sulfate). These experiments showed that Cpz4 can act as an arylsulfate sulfotransferase.

6.4. Biochemical properties of Cpz4

The biochemical properties of Cpz4 were determined with pNS as the sulfate donor and phenol as the acceptor substrate. Reaction velocities were photometrically measured at 405 nm, the absorption maximum for the product *p*-nitrophenol (pN). The aryl sulfotransfer reaction was strictly dependent on the presence of active Cpz4 and pNS. However, a low formation of pN was detected in assays without phenol, indicating a certain sulfatase activity of Cpz4. Therefore, hydrolysis of pNS was monitored in parallel assays without phenol and the amount subtracted from the initial results for all subsequent experiments. The reaction (as described in section II.7.4) showed a linear dependency on the amount of Cpz4 (up to 250 nM) and the incubation time (up to 90 min). In the absence of NaCl pH values ≤7.0 resulted in precipitation of the protein in the assay. Inclusion of 1 M NaCl prevented precipitation and allowed to assay the protein in a range from pH 4.0 to pH 11.0. Maximal activity was observed at pH 6.7 with half-maximal values at pH 5.0 and pH 7.6. Low concentrations of Mg^{2+} (1 mM, 5 mM, 10 mM) had no significant effect on the reaction velocity. The presence of 20 mM Mg^{2+} or 2 mM Ni^{2+} in the assay led to precipitation of the protein. Notably, addition of 0.1 mM EDTA increased product formation of Cpz4 by 2.5-fold and was therefore routinely included in the assay.

6.5. Influence of inhibitors on Cpz4 activity

While PAPS-dependent sulfotransferases follow a sequential mechanism, the ASSTs e.g. from *Eubacterium* A-44 (Kim & Kobashi, 1991) and from *E. coli* CFT073

(Malojcic *et al.*, 2008) have been shown to react by a ping-pong bi-bi mechanism with sulfation of an active site residue of the enzyme. Until recently it had been assumed that the sulfate is transferred first to a histidine residue and subsequently to a tyrosine residue (Chapman *et al.*, 2004). However, (Malojcic *et al.*, 2008) could only show the formation of a sulfohistidine in the *E. coli* ASST. Specific inhibitors were added to assays containing Cpz4, pNS and phenol. In the presence of 1 mM PMSF, which targets nucleophilic hydroxy groups, the turnover of pNS by Cpz4 was not affected. In contrast, addition of 3 mM of diethylpyrocarbonate (DEPC), which specifically modifies histidine side chains (Sambrook & Russell, 2001), led to a ~90% decrease of the activity. These findings indicate that one or more histidine residues are essentially involved in the catalysis of the reaction.

6.6. Site-directed mutagenesis

A multiple sequence alignment of characterized and putative ASSTs including Cpz4 revealed three highly conserved histidine residues i.e. His180, His253 and His308. The corresponding histidine residues in the ASST from *E. coli* CFT073 were reported to be essential for the catalysis of the sulfotransfer reaction (Malojcic *et al.*, 2008). In this enzyme, the sulfate group is covalently bound to His436 and further stabilized by His252 and His356. Consequently, each of these histidines was replaced with leucine in Cpz4 by site-directed mutagenesis. Heterologous production of the mutant enzymes as His-tagged proteins and subsequent purification led to 17.4 mg (His180Leu), 1 mg (His253Leu) and 6.2 mg (His308Leu) of soluble protein from 1 L cultures. Activities of all three mutant enzymes against pNS and phenol were reduced to ≤5% in comparison to the wildtype enzyme. On the basis of these findings and the results obtained from the addition of specific inhibitors, we would assume a catalytic role of the histidines in Cpz4 similar to the ASST from *E. coli* CFT073.

6.7. Kinetic analysis of Cpz4

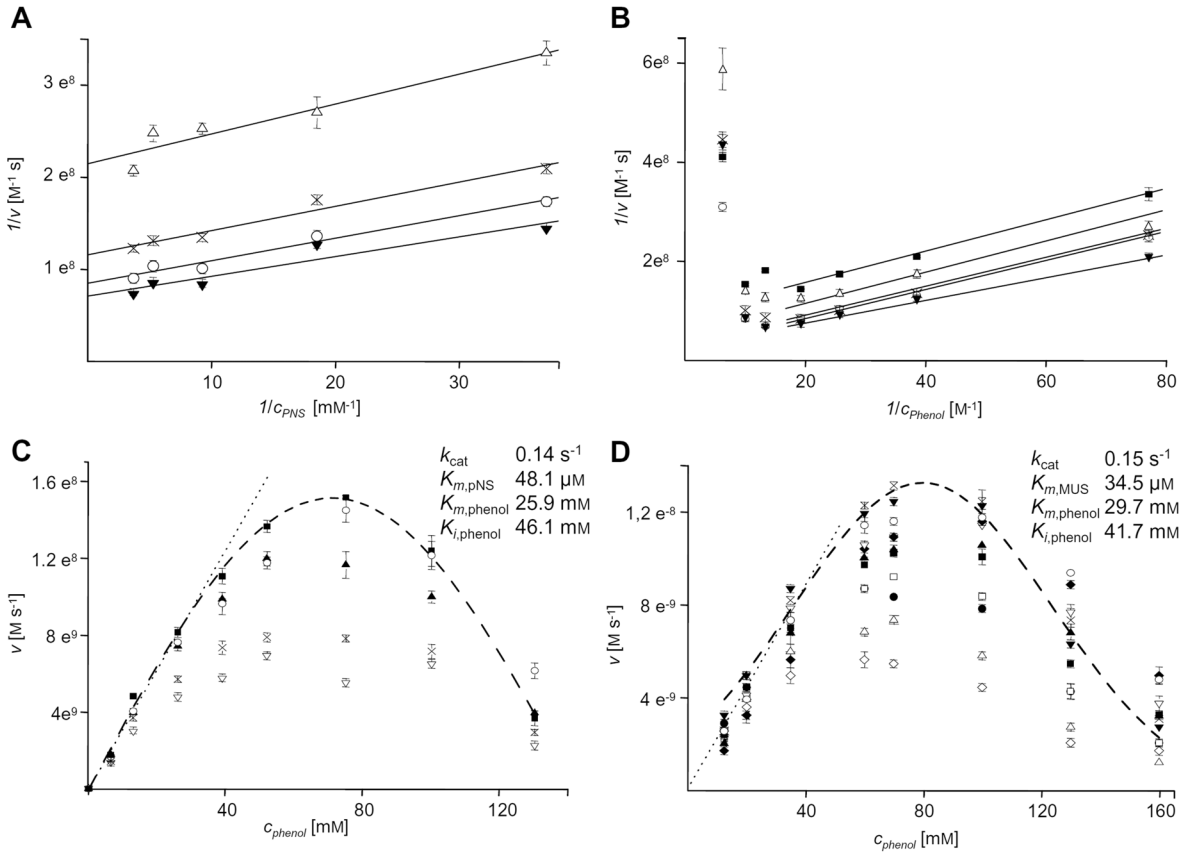


Figure III.6.3 Kinetic analysis of the Cpz4-catalyzed sulfate transfer reaction from the aromatic donor substrates pNS and MUS to phenol (see also diploma thesis of Kornelia Eitel). **A** Double reciprocal plot of reaction velocities, obtained with pNS and phenol, over pNS concentration (27 µM, 54 µM, 108 µM, 189 µM, 270 µM pNS). Partial regression of velocities from data sets with fixed initial concentrations of phenol (13 mM (Δ), 26 mM (×), 39 mM (O) and 52 mM (▼)) shows parallel lines indicating a ping pong kinetic. **B** Double reciprocal plot of reaction velocities, obtained with pNS and phenol, over phenol concentration (13 mM, 26 mM, 39 mM, 52 mM, 75 mM, 100 mM and 160 mM phenol). Partial regression of velocities from data sets with fixed initial concentrations of pNS (27 µM (■), 54 µM (Δ), 108 µM (×), 189 µM (O), 270 µM (▼)) shows parallel lines up to a phenol concentration of ~50 mM. **C** Initial velocities with pNS at initial fixed concentrations of 27 µM (Δ), 54 µM (×), 108 µM (▲), 189 µM (O), 270 µM (■) over phenol (0 mM, 6.5 mM, 13 mM, 26 mM, 39 mM, 52 mM, 75 mM, 100 mM and 130 mM). This data set was globally fitted (see section II.7.4) to obtain the depicted kinetic parameters for Cpz4 with pNS and phenol as substrates. **D** Initial velocities with MUS at initial fixed concentrations of 25 µM (♢), 50 µM (Δ),75 µM (●), 100 µM (□), 150 µM (■), 200 µM (▲), 250 µM (Δ), 300 µM (O), 350 µM (◆), 450 µM (▼) and 550 µM (×) over phenol (12.5 mM, 20 mM, 36 mM, 60 mM, 70 mM, 100 mM, 130 mM and 160 mM). This data set was globally fitted (see section II.7.4) to obtain the depicted kinetic parameters for Cpz4 with MUS and phenol as substrates. Spotted lines in **C** and **D** indicate linear dependency of the reaction velocities and phenol concentration on low phenol concentrations.

Applying a constant concentration of phenol (75 mM) and varying concentrations of pNS, a plot of product formation over substrate concentration gave a hyperbolic curve, as expected for Michaelis-Menten kinetics. Using different concentrations of phenol with 25µM of PNS, the reaction velocities increased up to a phenol concentration of 75 mM and thereafter declined, indicating substrate inhibition. In order to investigate the reaction mechanism, Cpz4 was incubated with different fixed concentrations of phenol (13 mM, 26 mM, 39 mM and 52 mM) and various concentrations of pNS. Double reciprocal plots of initial velocities over pNS concentrations resulted in a series of parallel lines (Figure III.6.3A), characteristic for ping-pong kinetics (Cleland, 1963). Increasing concentrations of phenol lead to a proportional increase of V_{max} and K_m. When the intercepts of the lines ($1/V_{max}$) were plotted against the corresponding phenol concentrations ($1/c_{phenol}$), a linear relation was observed (Figure III.6.4A).

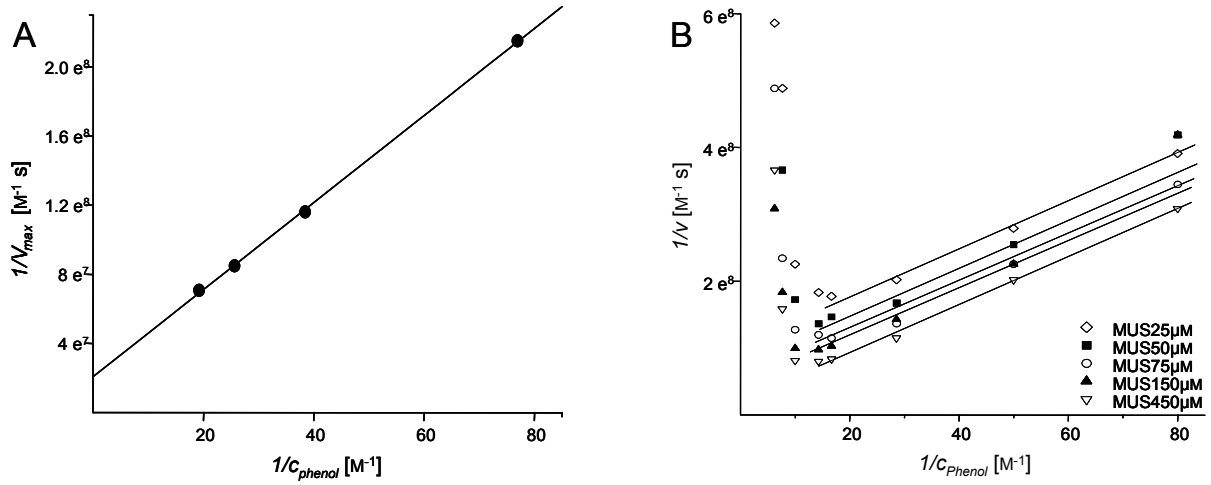


Figure III.6.4 Kinetic analysis of the Cpz4-catalyzed sulfate transfer reaction from aromatic donor substrates pNS and MUS to phenol (see also diploma thesis of Kornelia Eitel). **A** Double reciprocal plot of maximal reaction velocities with pNS and phenol (obtained from Figure 4) over initial fixed phenol concentration (13 mM, 26 mM, 39 mM, 52 mM). **B** Double reciprocal plot of reaction velocities with MUS and phenol over phenol concentration (12.5 mM, 20 mM, 36 mM, 60 mM, 70 mM, 100 mM, 130 mM and 160 mM). Partial regression of velocities from data sets with fixed initial concentrations of MUS (25 – 450 µM) shows parallel lines up to a phenol concentration of ~40 mM.

A similar set of assays using different fixed pNS concentration (27 µM, 54 µM, 108 µM, 189 µM, 270 µM) and various phenol concentrations was performed. Again, the double reciprocal plot shows a series of parallel lines but only up to a phenol concentration of ~50 mM (Figure III.6.3B). At higher phenol concentrations the curves rise asymptotic towards the y axis, a manifestation of substrate inhibition by phenol

(Goldstein, 1944). Another series of assays with various concentrations of both substrates phenol and pNS was performed to determine the kinetic constants of the reaction. A global fit of the data plotted in Figure III.6.3C (as described in section II.7.4) resulted in the following parameters: V_{max} 19.8 nM s^{-1}, $K_{m,pNS}$ 48.1 µM, $K_{m,phenol}$ 25.9 mM and $K_{i,phenol}$ 46.1 mM. k_{cat} was determined as 0.14 s^{-1}.

A similar approach was initiated using MUS as the sulfate donor substrate. Conversion of MUS was determined by measuring fluorescence at 460 nm and an excitation wavelength of 360 nm. The reaction showed linear dependence on the amount of active Cpz4 (up to 200 nM) and the incubation time (up to 25 min). The reaction was dependent on the presence of MUS and phenol. No sulfatase activity could be observed in a control without phenol. A dataset was generated applying various phenol concentrations (12.5 mM - 160 mM) against constant initial MUS concentrations in a range from 25 µM to 550 µM (Figure III.6.3D). Double reciprocal plotting of the reaction velocities over substrate concentration confirmed the ping pong bi-bi kinetics with substrate inhibition of phenol as observed with pNS as the sulfate donor (Figure III.6.4B).

Kinetic parameters were calculated as V_{max} 15.3 nM s^{-1}, $K_{m,MUS}$ 34.5 µM, $K_{m,phenol}$ 29.7 mM and $K_{i,phenol}$ 41.7 mM. k_{cat} was determined as 0.15 s^{-1}.

The K_m values for pNS (48.1 µM) and MUS (34.5 µM) are similar to the K_m for MUS published for the ASST from *E. coli* CFT073 i.e. 44.5 µM (Malojcic *et al.*, 2008). However, catalytic efficiency for phenol (k_{cat}/K_m) is low at 5.5 M s^{-1} and 5.1 M s^{-1} as may be expected for an artificial substrate.

6.8. Sulfation of caprazamycin derivatives extracted from bacterial cultures

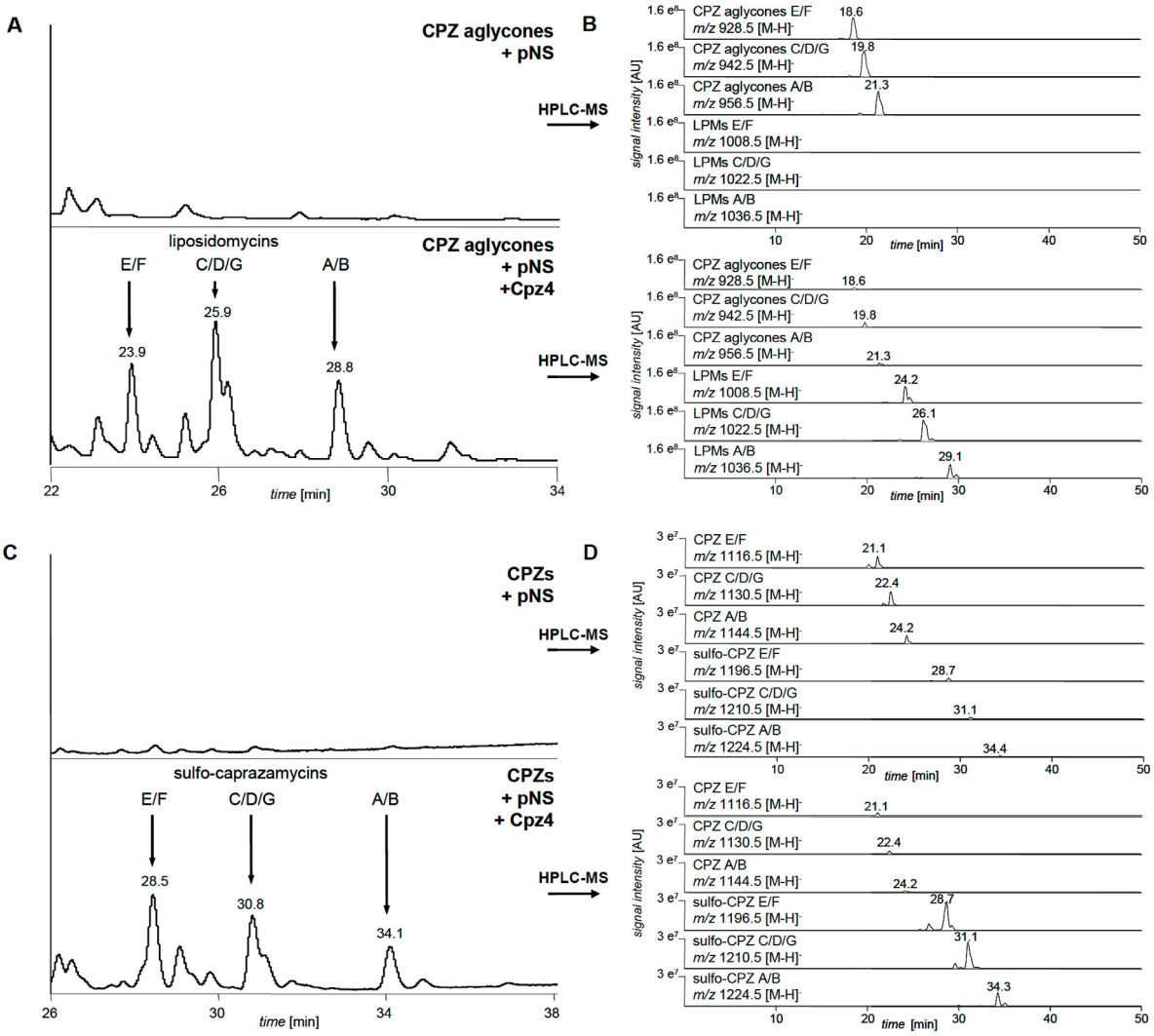


Figure III.6.5 HPLC-UV and HPLC-MS analysis of Cpz4-catalyzed sulfation using different liponucleoside derivatives, isolated from cultures of mutant strains, as acceptor substrates (see also diploma thesis of Kornelia Eitel). **A** Formation of liposidomycins from partially purified caprazamycin aglycones as substrates and pNS as sulfate donor in HPLC-UV chromatograms and **B** product ion chromatograms. (-)-ESI-MS mass scans for the substrates caprazamycin E/F aglycones with *m/z* 928.5 $[M-H]^-$, caprazamycin (CPZ) C/D/G aglycones with *m/z* 942.5 $[M-H]^-$, caprazamycin A/B aglycones with *m/z* 956.5 $[M-H]^-$ and for the products liposidomycins (LPMs) E/F with *m/z* 1008.5 $[M-H]^-$, liposidomycins C/D/G with *m/z* 1022.5 $[M-H]^-$, liposidomycins A/B with *m/z* 1036.5 $[M-H]^-$ are depicted. Signal intensity was adjusted to 1.6 10^8 for all experiments. **C** Formation of sulfo-caprazamycins from partially purified caprazamycins as substrates and pNS as sulfate donor in HPLC-UV chromatograms and **D** product ion chromatograms. (-)-ESI-MS mass scans for the substrates caprazamycins (CPZs) E/F with *m/z* 1116.5 $[M-H]^-$, caprazamycins C/D/G with *m/z* 1130.5 $[M-H]^-$, caprazamycins A/B with *m/z* 1144.5 $[M-H]^-$ and for the products sulfo-caprazamycins E/F with *m/z* 1196.5 $[M-H]^-$, sulfo-caprazamycins C/D/G with *m/z* 1210.5 $[M-H]^-$and sulfo-caprazamycins A/B with *m/z* 1224.5 $[M-H]^-$ are depicted. Signal intensity was adjusted to 3 10^7 for all experiments.

In order to investigate the role of Cpz4 in the sulfation of liponucleosides, caprazamycin aglycones were partially purified and tested as possible substrates. Extracts from *S. coelicolor* M512/cpzWP05 which does not produce the sulfated liposidomycins were incubated with Cpz4 and pNS. In a second experiment, pNS was replaced by PAPS to test the sulfate donor specificity of Cpz4. The conversion of the caprazamycin aglcones was observed with pNS in HPLC-UV chromatograms resulting in product peaks at Rt. 23.9 min, 25.9 min and 28.8 min (Figure III.6.5A). The new compounds were determined as liposidomycins (LPM E/F with *m/z* 1008.5 $[M-H]^-$ at Rt. 24.2 min, LPM C/D/G with *m/z* 1022.5 $[M-H]^-$ at Rt. 26.1 min, LPM A/B with *m/z* 1036.5 $[M-H]^-$ at Rt. 29.1 min) by LC-ESI-MS analysis (Figure III.6.5B). MS^2 fragmentation patterns matched exactly the liposidomycins from the original producer *Streptomyces* sp. SN-1061M. In the structure of liposidomycins the sulfate group is attached to the aminoribosyl moiety. Our results indicate a similar position in the Cpz4 product molecule. No liposidomycins could be identified in HPLC-UV chromatograms of assays containing PAPS as the donor substrate. However, traces of liposidomycins could be detected by ESI-MS product ion scans in control assays with Cpz4 lacking pNS or PAPS but not in assays without Cpz4. A similar phenomenon has been observed by Malojcic et al. (Malojcic *et al.*, 2008) for the ASST from *E. coli* CFT073 and may indicate a covalently bound sulfate in the Cpz4 active site.

Thus, we conclude that Cpz4 is responsible for a PAPS-independent sulfate transfer reaction in liponucleoside biosynthesis. In contrast to the previously reported ASST reactions Cpz4 uses a glycosidic instead of an aromatic acceptor substrate.

Additionally, we tested whether Cpz4 sulfates intact caprazamycins and hydroxyacyl-caprazols. These compounds were extracted from *S. coelicolor* M512/cpzEW07 and *S. coelicolor* M512/cpzLL06, partially purified and assayed with pNS and Cpz4. Note that in both extracts some sulfated derivatives are already present. However, after incubation with Cpz4 a substantial increase of the sulfo-caprazamycins at Rt. 28.5 min, 30.8 min and 34.1 min was observed in HPLC-UV chromatograms (Figure III.6.5C). The identity of the products was confirmed by ESI-MS mass scans for sulfo-caprazamycins E/F with *m/z* 1196.5 $[M-H]^-$ (Rt. 28.7 min), C/D/G with *m/z* 1210.5 $[M-H]^-$ (Rt. 31.1 min) and A/B with *m/z* 1224.5 $[M-H]^-$ (Rt. 34.3 min).

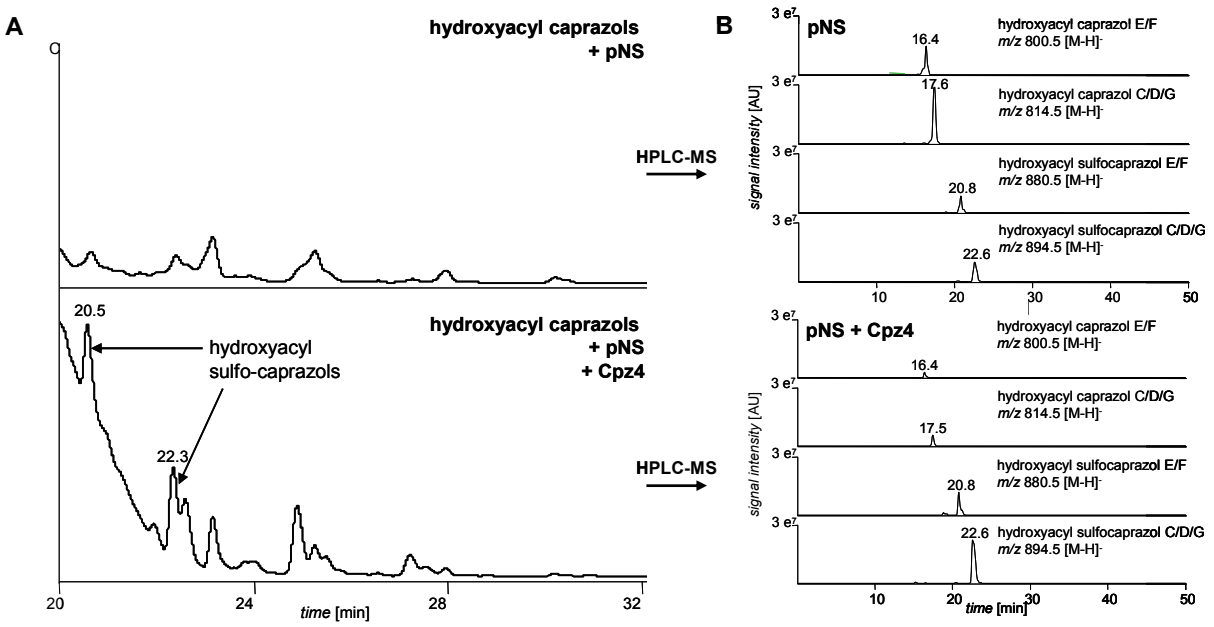


Figure III.6.6 Cpz4-catalyzed sulfation of hydroxyacyl caprazols from culture extracts with pNS as sulfate donor analysed by LC-ESI-MS (see also diploma thesis of Kornelia Eitel). **A** HPLC-UV chromatograms before and after the formation of hydroxyacyl sulfo-caprazols from partially purified hydroxyacyl caprazols (Fig. 1, **4**) as substrates and pNS as sulfate donor. **B** Respective product ion chromatograms obtained from (-)-ESI-MS mass scans for the substrates hydroxyacyl caprazols E/F with *m/z* 800.5 $[M-H]^-$and hydroxyacyl caprazols C/D/G with *m/z* 814.5 $[M-H]^-$. Product formation was determined by mass scans for hydroxyacyl sulfo-caprazols E/F with *m/z* 880.5 $[M-H]^-$ and hydroxyacyl sulfo-caprazols C/D/G with *m/z* 894.5 $[M-H]^-$. Signal intensity was adjusted to 3 10^7 for all experiments.

Similarly, product formation was found for Cpz4 and the hydroxyacyl-caprazols as substrates (Figure III.6.6). These findings suggest that Cpz4 is promiscuous in regard to the liponucleoside acceptor substrate.

6.9. Activity of Cpz4 with synthetic liponucleoside precursor

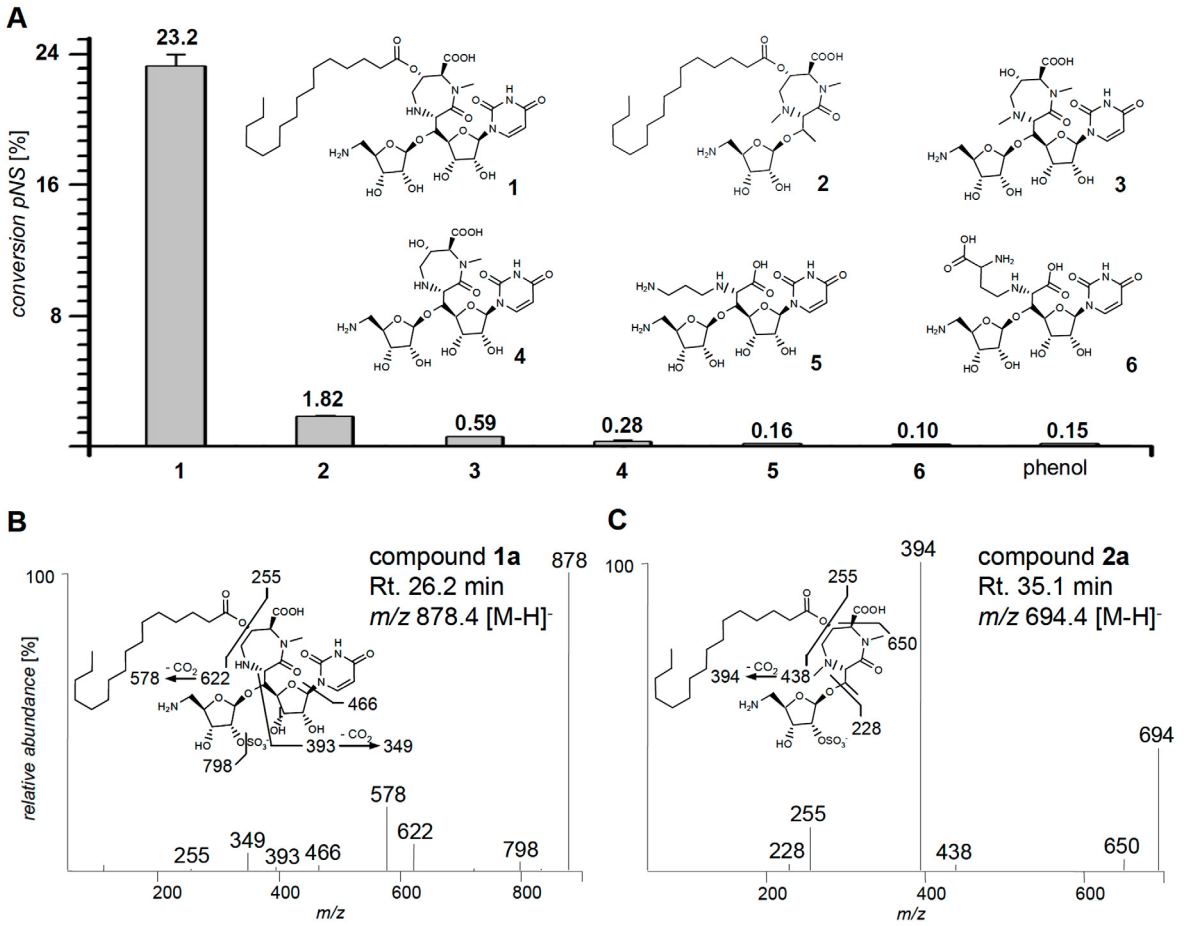


Figure III.6.7 Activity of Cpz4 with synthetic liponucleoside precursor and phenol as sulfate acceptor substrates (see also diploma thesis of Kornelia Eitel). **A** Conversion of pNS [%] with the different substrates (**1** - **6** and phenol) is noted above the respective bars. Concentrations of pNS and acceptor substrates were 500 µM and 200µM respectively. **B** MS^2 spectrum of the product **1a** (*m/z* 878.4 $[M-H]^-$, Rt. 26.2 min) formed by Cpz4 from compound **1** and pNS. The proposed fragmentation scheme for **1a** is depicted. **C** MS^2 spectrum of the product **2a** (*m/z* 694.4 $[M-H]^-$, Rt. 35.1 min) formed by Cpz4 from compound **2** and pNS. The proposed fragmentation scheme for **2a** is depicted.

Several structural precursor of liponucleosides (Figure III.6.7A) were synthesized as described in (Kaysser *et al.*, 2010). They were investigated as substrates for Cpz4 and compared to phenol in a photometric assay. All acceptor substrates were used in a concentration of 0.2 mM. Conversion (%) of pNS (300 µM) was very low for compounds **4** (0.28%), **5** (0.16%), **6** (0.10%) and phenol (0.15%) (Figure III.6.7A). A higher turnover of 0.59% was observed with caprazol (compound **3**) a putative intermediate in liponucleoside biosynthesis. Notably the acylated compounds **2** and **1,** were sulfated by Cpz4 with 10-fold (1.82%) and 100-fold (23.2%) higher conversion rates than phenol. This clearly suggests that Cpz4 is a specific

sulfotransferase for the biosynthesis of sulfated liponucleosides. It requires both, the fatty acyl and the uridyl moiety of the liponucleoside for an efficient structural recognition of the substrate.

Product formation was verified by LC-ESI-MS/MS analysis in negative mode. Mass peaks for the sulfated products **1a** and **2a** were found with *m/z* 878.4 $[M-H]^-$ at Rt. 26.2 min and *m/z* 694.4 $[M-H]^-$ at Rt. 35.2 min, respectively. Ions with *m/z* 848, *m/z* 622, *m/z* 578 and *m/z* 466 in the **1a** MS^2-spectrum (Figure III.6.7B) match the expected fragments containing a sulfated aminoribose. The ion with *m/z* 349 may derive from an uridyl-diazepanone substructure. The lack of an ion with *m/z* 429, corresponding to a sulfated analog of the *m/z* 349 fragment, suggests that the sulfation of compound **1** takes place at the aminoribosyl group similar to the sulfation of liposidomycins. MS^2 fragmentation pattern of product **2a** (Figure III.6.7C) displays an analogous situation.

IV. DISCUSSION

The caprazamycins and liposidomycins are potent anti-mycobacterial liponucleoside antibiotics and belong to the translocase I inhibitor family. Their complex structure is derived from 5'-(*β*-*O*-aminoribosyl)-glycyluridine and comprises a unique *N*-methyl-diazepanone ring. The biosynthesis of these unusual compounds was unknown at the beginning of this study.

My thesis describes the identification and analysis of the caprazamycin biosynthetic gene cluster from *Streptomyces* sp. MK730-62F2. Heterologous expression led to the production of non-glycosylated bioactive caprazamycin derivatives and a set of gene deletions established the borders of the cluster. Inactivation of *cpz20, cpz21* and *cpz25* resulted in the accumulation of novel simplified liponucleoside antibiotics which lack the 3-methylglutaryl moiety. Heterologous co-expression of the gene cluster with pRHAM, led to the production of intact caprazamycins. In vitro studies showed that Cpz31 is responsible for the attachment of the L-rhamnose to the caprazamycin aglycones. An L-rhamnose gene cluster was identified elsewhere on the *Streptomyces* sp. MK730-62F2 genome. This subcluster was assembled to the caprazamycin gene cluster using Red/ET-mediated recombination. Heterologous expression of the resulting cosmid led to the production of caprazamycins. From the genetic information obtained from the caprazamycin gene cluster, the liposidomycin biosynthetic gene cluster could be identified in *Streptomyces* sp. SN-1061M. A heterologous host accumulated the liposidomycins. Comparison of the liposidomycin gene cluster and the caprazamycin gene cluster finally led to the identification of novel sulfated caprazamycin derivatives. Cpz4 from *Streptomyces* sp. MK730-62F2 was shown to be an arylsulfate sulfotransferase responsible for the formation of the sulfated liponucleoside antibiotics by in vivo and in vitro experiments. The enzyme was biochemically characterized using *p*-nitrophenol sulfate, methylumbelliferone sulfate and phenol as substrates. Biogenic and synthetic nucleosidic compounds were readily sulfated by Cpz4. Overall, the *in vivo, in vitro* and *in silico* analysis of the caprazamycin and the liposidomycins biosynthetic gene clusters provides first insights into the formation of the liposidomycin-type liponucleoside antibiotics and sets basis for detailed investigations of the biosynthetic pathway.

1. Identification of the gene cluster for dTDP-L-rhamnose biosynthesis

Based on the identification of the L-rhamnose biosynthetic genes in this study, we propose two gene clusters, the caprazamycin and the L-rhamnose subcluster to participate in caprazamycin biosynthesis. Co-expression of pRHAM, containing all genes required for the biosynthesis of dTDP-L-rhamnose, led to the accumulation of intact caprazamycins in a heterologous producer strain harbouring cosmid cpzLK09. This clearly demonstrates that only the genes for the formation of dTDP-L-rhamnose are missing in the heterologous caprazamycin producer. Thus, the genes directing the attachment and methylation of the L-rhamnosyl moiety have to be encoded on cosmid cpzLK09. Three putative methyltransferases, Cpz28, Cpz29 and Cpz30 show highest sequence homology to known rhamnose *O*-methyltransferases from elloramycin (Patallo *et al.*, 2001) and spinosyn biosynthesis (Waldron *et al.*, 2000). Most likely, these proteins have a similar function in caprazamycin formation.

Screening of a cosmid library of the caprazamycin producer *Streptomyces* sp. MK730F-62F2 led to the identification of *cpzDII*, *cpzDIII*, *cpzDIV* and *cpzDVI* elsewhere on the genome. The deduced gene products match the required enzymes for the biosynthesis of dTDP-L-rhamnose. Involvement in caprazamycin biosynthesis was demonstrated by inactivation of *cpzDIII* resulting in the accumulation of caprazamycin aglycones. In contrast, inactivation of the collocated genes *cpzDI* and *cpzDV* had no influence on caprazamycin formation. Based on the presented results, we propose the biosynthetic pathway for the latter steps in caprazamycin biosynthesis as follows: generation of the deoxysugar would start with the activation of glucose-1-phosphate by the addition of deoxythymidyldiphosphate (dTDP) catalyzed by the putative NDP-glucose synthase CpzDII. Subsequently, the glucose dehydratase CpzDIII would dehydrate dTDP-glucose to dTDP-4-keto-6-deoxyglucose. This product would serve as a substrate for the sugar 3,5-epimerase CpzDVI resulting in dTDP-4-keto-L-rhamnose which is further reduced to dTDP-L-rhamnose by the 4-ketoreductase CpzDIV. Cpz31 would than attach the L-rhamnose to the caprazamycin aglycones generating 2c,3c,4c,-demethyl caprazamycins. Sequential methylation of the deoxysugar moiety by the putative *O*-methyltransferases Cpz28, Cpz29 and Cpz30 would finally lead to the caprazamycins.

Usually, genes involved in the formation of a bacterial secondary metabolite are clustered together. However, biosynthetic gene clusters of rhamnosylated compounds often lack the genes for L-rhamnose formation (Decker *et al.*, 1995; Gullon *et al.*, 2006; Luzhetskyy *et al.*, 2007; Waldron *et al.*, 2000). In the case of elloramycin (Ramos *et al.*, 2008) and spinosyn (Madduri *et al.*, 2001) these genes were identified elsewhere in the genome. Inactivation experiments in *Saccharopolyspora spinosa* suggested that the *gtt*, *gdh*, *epi* and *kre* genes provide the rhamnosyl moiety for both, spinosyn and cell wall biosynthesis. The presence of *cpzDVII,* a putative cell-wall biosynthesis glycosyltransferase gene may indicate a similar role for the L-rhamnose cluster in *Streptomyces* sp. MK730F-62F2. L-rhamnose seems not to be essential for the strain as inactivation of the glucose dehydratase gene *cpzDIII* did result in a viable corresponding mutant.

In order to allow investigation of the entire biosynthetic pathway of caprazamycins, we assembled the L-rhamnose and caprazamycin gene cluster on cosmid cpzLK09, using a new strategy based on Red/ET-mediated recombination. This technology, designated as recombineering, was initially established for DNA manipulations in *E. coli* (Datsenko & Wanner, 2000; Zhang *et al.*, 1998; Zhang *et al.*, 2000) and has been adapted to *Streptomyces* (Gust *et al.*, 2003). It was successfully applied for functional studies and engineering of antibiotic biosynthetic machineries (Gust *et al.*, 2003) e.g. heterologous expression (Eustaquio *et al.*, 2005) and combinatorial biosynthesis (Eustaquio *et al.*, 2003). More recently, recombineering was used for the reconstitution of large biosynthetic gene clusters from overlapping inserts (Binz *et al.*, 2008; Hu *et al.*, 2008; Wolpert *et al.*, 2008). This “stitching” overcomes the problem, that genomic library clones rarely contain an entire gene cluster due to limitations of the average insert sizes. While “stitching” relies on the presence of overlapping, identical DNA sequences in both clones, we now show for the first time that assembling is possible without the need of overlapping DNA regions. For the presented strategy, Red/ET-mediated recombination is restricted to sequences in form of resistance cassettes. These cassettes were introduced into the L-rhamnose donor cosmid and the recipient cosmid harbouring the caprazamycin gene cluster. Final assembly was achieved by Red/ET-mediated recombination between a restriction fragment from the donor cosmid with the recipient cosmid. FRT and loxP recognition sites and *Spe*I restriction sites were introduced for the latter excision of the resistance cassettes. The *in vivo* usage of Cre- (Fedoryshyn *et al.*, 2008b) and

the Flp-recombinase (Fedoryshyn *et al.*, 2008a) has now been successfully established in *Streptomyces* and allows the rapid generation of unmarked deletions. However, if Cre- or Flp-recombinase is used, a “scar” sequence comprising a loxP or an FRT site is maintained which can cause undesirable recombination events in further recombineering experiments. Therefore, elimination of the apramycin resistance cassettes was performed with *Spe*I digestion and religation of the cosmid-DNA. In this study, *cpzDI* and *cpzDV* were successfully deleted on the assembled cosmid and the corresponding mutants analysed.

Since manipulation of natural producer strains can be difficult and time-consuming, heterologous expression of entire biosynthetic gene clusters in more amenable and fully sequenced host strains is desirable. In addition, formation of a natural compound often relies on the supply of specific precursors provided by genes encoded outside the gene cluster, e.g. deoxysugars (Ramos *et al.*, 2008), isoprenoid derived moieties (Haagen *et al.*, 2006) or other small subunits (Ostash *et al.*, 2007; Yu *et al.*, 2002). Therefore, the new assembling approach described in this study may help to establish heterologous expression and genetic engineering of scattered gene clusters.

2. Investigations on the role of the glycosyltransferase Cpz31

Translationally coupled to the genes *cpz28-cpz30* is the putative glycosyltransferase gene *cpz31*. An in vitro assay with His_8-Cpz31 using dTDP-L-rhamnose and caprazamycin aglycones as substrates showed a complete conversion of the aglycones after two hours incubation time. The products were unambiguously identified as 2c,3c,4c-desmethyl caprazamycins by LC-ESI-MS/MS analysis. This result implies that attachment of the deoxysugar occurs prior to its sequential methylation. A similar order of sugartransfer and subsequent tailoring reactions was found in the biosynthesis of other glycosylated compounds such as tylosin (Bate & Cundliffe, 1999), oleandomycin (Rodriguez *et al.*, 2001), mycinamycin (Inouye *et al.*, 1994) and chromomycin (Menendez *et al.*, 2004). Cpz31, together with most glycosyltransferases involved in natural product biosynthesis, belongs to the GT-1 family of glycosyltransferases as classified by the CAZy system (Coutinho *et al.*, 2003). Cpz31 exhibit significantly higher homology to e.g. the 4,6-dideoxy-4-hydroxylamino-a-D-glucosyltransferase CalG3 (36%/50%) from *Micromonospora echinospora* (Zhang *et al.*, 2008) than to the rhamnosyltransferases AraGT (29%/38%) from *S. echinatus* (Luzhetskyy *et al.*, 2007) and SpnG (29%/39%) from *Saccharopolyspora spinosa* (Chen *et al.*, 2009). Luzhetskyy et al. (Luzhetskyy *et al.*, 2005) proposed that similarity based relationships of glycosyltransferases is better understood in consideration of the aglycone substrate then of the sugar donor. In this aspect, Cpz31 is distinctive from other known glycosyltransferases involved in natural product biosynthesis. The rhamnosyl moiety of caprazamycins is linked to the methylglutaryl moiety by an ester bond and not by a glycosidic bond. While acylated deoxyhexoses are common in plants, they are rare in bacterial secondary metabolism. So far, only the phenazine derivatives aestivophoenins (Kunigami *et al.*, 1998) and phenazoviridins (Kato *et al.*, 1993) and the rhamnopyranosides (Hu *et al.*, 2000) have been reported. Thus, Cpz31 may serve as a new enzymatic tool for the modification of bioactive compounds.

Rhamnosyltransferases have been frequently exploited for the structural diversification of glycosylated antibiotics (Blanco *et al.*, 2001; Gaisser *et al.*, 2009; Luzhetskyy *et al.*, 2007; Olano *et al.*, 2008) as they often exhibit high flexibility towards the donor substrate. Though, heterologous expression of cosmid cpzLK09 in

S. fradiae A0 which produce the deoxysugars D-olivose and L-rhodinose and *Streptomyces* sp. TÜ6071*ΔplaA6* producing L-amicetose did not lead to the identification of new caprazamycin derivatives. Since the caprazamycin aglycones were readily detected in the culture extracts, it can be speculated that Cpz31 is more specific for dTDP-L-rhamnose than related glycosyltransferases e.g. ElmGT from *S. olivaceus* (Blanco *et al.*, 2001) or StfG from *S. steffisburgensis* (Olano *et al.*, 2008). Further investigations will have to proof this assumption.

3. Characterization of Cpz4 as an arylsulfate sulfotransferase in liponucleoside biosynthesis

In this study we show, for the first time, the physiological function of an arylsulfate sulfotransferase type enzyme. Gene deletion experiments proved the requirement of *cpz4* for the biosynthesis of sulfated liponucleoside antibiotics, since in the absence of *cpz4* the liposidomycins were not produced. Purified Cpz4 protein accepted different liponucleoside derivatives and synthetic caprazamycin precursor as substrates for a sulfotransfer reaction. Therefore, we conclude that Cpz4 is the sulfotransferase responsible for the biosynthesis of sulfated caprazamycins in *Streptomyces* sp. MK730-62F2. We isolated the hydroxyacyl-caprazols, the caprazamycin aglycones and the intact caprazamycins from suitable engineered producer strains. All of them were sulfated by Cpz4. In this case, conversion rates could not be quantified as the substrates represent mixtures rather than pure compounds. Structural analogs of putative intermediates of the caprazamycin biosynthetic pathway were synthesized and investigated as substrates of the enzyme. Clearly the fatty acyl-containing caprazol derivative (**1**, Figure III.6.7) was sulfated with much higher reaction velocity than non-acylated analogs or phenol. Compound **2** (Figure III.6.7) lacking the uridyl moiety was converted 13 times slower than **1** (Figure III.6.7). These findings allow us to hypothesize at which stage of the caprazamycin biosynthetic pathway the sulfation may occur. We would propose that after cyclization of the diazepanone ring and attachment of a hydroxy-fatty acid side chain all subsequent intermediates represent genuine substrates for Cpz4. This could imply that the sulfated liponucleosides are either dead-end products which are not further processed. Alternatively, the enzymes catalyzing the subsequent reactions in caprazamycin biosynthesis would be flexible towards a sulfated substrate. Recently, we observed that the rhamnosyltransferase Cpz31 from the caprazamycin gene cluster is able to glycosylate not only the caprazamycin aglycones but also the liposidomycins (data not shown). Therefore, we would favour the latter hypothesis. However, the physiological or ecological function of the sulfated liponucleosides is still unknown (Malojcic *et al.*, 2008).

Cpz4 represents one of the few biochemically characterized sulfotransferases from bacterial secondary metabolism. However, in sharp contrast to StaL from *S.*

toyocaensis (Lamb *et al.*, 2006), CurM-ST from the curacin gene cluster (Gu *et al.*, 2009) and to most other known sulfotransferases (Chapman *et al.*, 2004), Cpz4 does not utilize PAPS as preferred sulfate donor. Instead, it efficiently uses aromatic sulfate donor substrates such as pNS or MUS resembling the ASSTs isolated from other bacteria and fungi. A genuine sulfate donor of the ASST-type sulfotransferases remains to be identified. Kinetic investigations showed that the reaction catalyzed by Cpz4 follows a ping pong bi-bi mechanism as has been reported for previously investigated ASSTs (Burns *et al.*, 1977; Kim & Kobashi, 1991; Kwon *et al.*, 2001; Malojcic *et al.*, 2008). X-ray structural analysis and biochemical characterization of the ASST from *E. coli* CFT073 indicated the transient sulfation of an active site histidine residue (Malojcic *et al.*, 2008). The results obtained for Cpz4 by site-directed mutagenesis and by use of inhibitors may suggest a similar role for one of the conserved histidine residues in Cpz4.

For none of the ASSTs reported so far, the genuine substrates are known. They have been biochemically investigated using phenol or other synthetic aromatic compounds as acceptor molecules. Cpz4 now represents the first ASST-type enzyme shown to preferentially sulfate the hydroxy group of the sugar moiety of a glycosidic substrate rather than a phenolic hydroxy group. Recently another ASST-type sulfotransferase involved in A-90289 biosynthesis has been identified (Funabashi *et al.*, 2010).

Notably, a BLAST search identifies numerous genes with significant sequence homology to Cpz4 in bacterial and fungal genomes. Almost all of them are annotated as hypothetical proteins and it remains to be shown whether they are structural genes for sulfotransferases. A sequence homology based clustering analysis (Frickey & Lupas, 2004) of Cpz4, functionally characterized ASSTs and homologs thereof separates two major clusters (Figure IV.3). One of them comprises the *E. coli* ASST (Malojcic *et al.*, 2008) and all of the other five previously characterized sulfotransferases (Baek *et al.*, 1996; Kang *et al.*, 2001a; Kang *et al.*, 2001b; Kim *et al.*, 2007; Kwon *et al.*, 1999). Within this group, the ASSTs from enterobacteria form a well-defined subcluster, while the enzyme from *Eubacterium* A-44 (Kim *et al.*, 2007) and putative ASSTs from other firmicutes form another subcluster. A second, clearly separated cluster comprises Cpz4, LpmB as well as further hypothetical proteins mainly from actinobacteria and fungi. The biochemical and physiological investigation of these potential ASSTs represents an exiting challenge for future research.

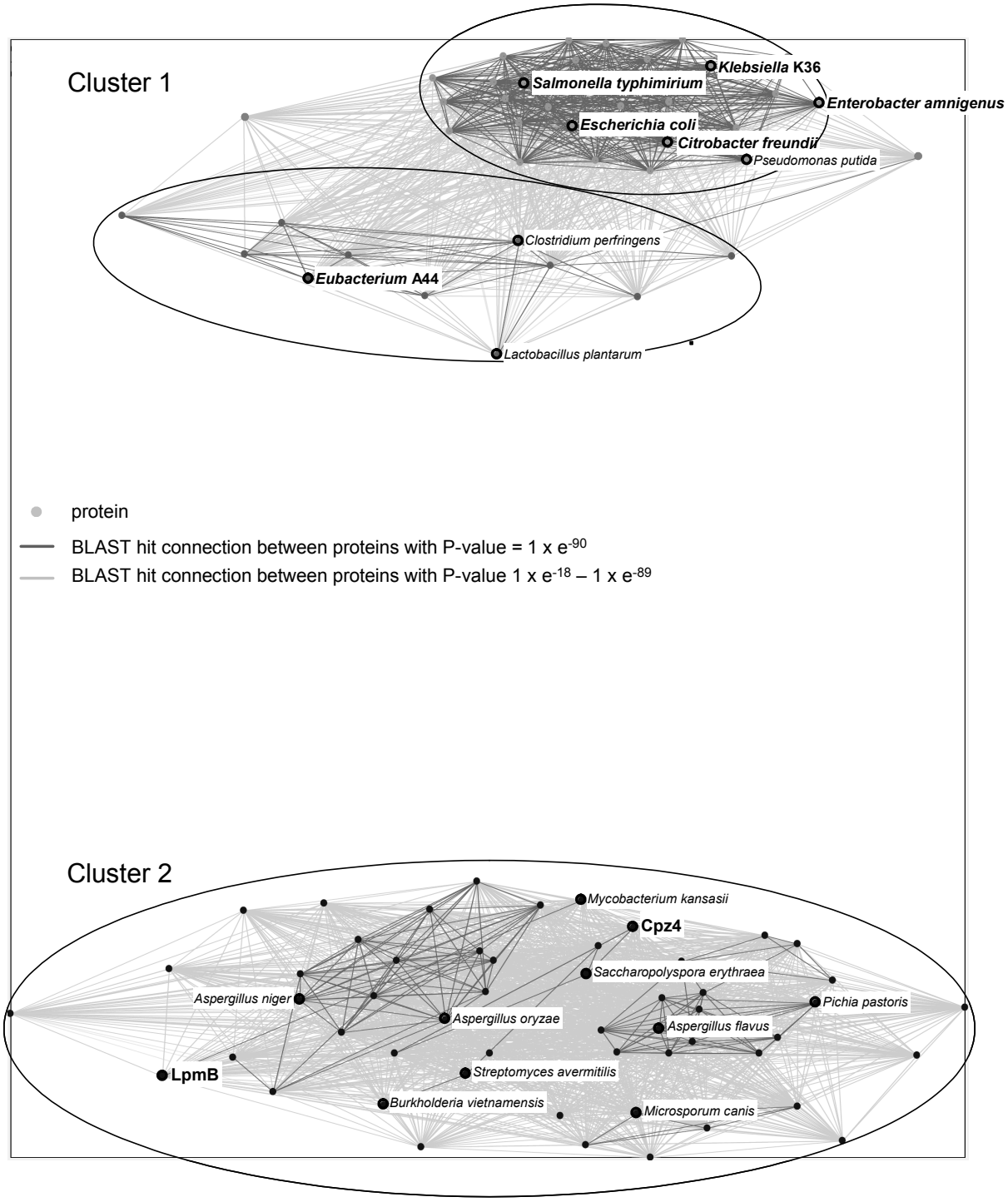


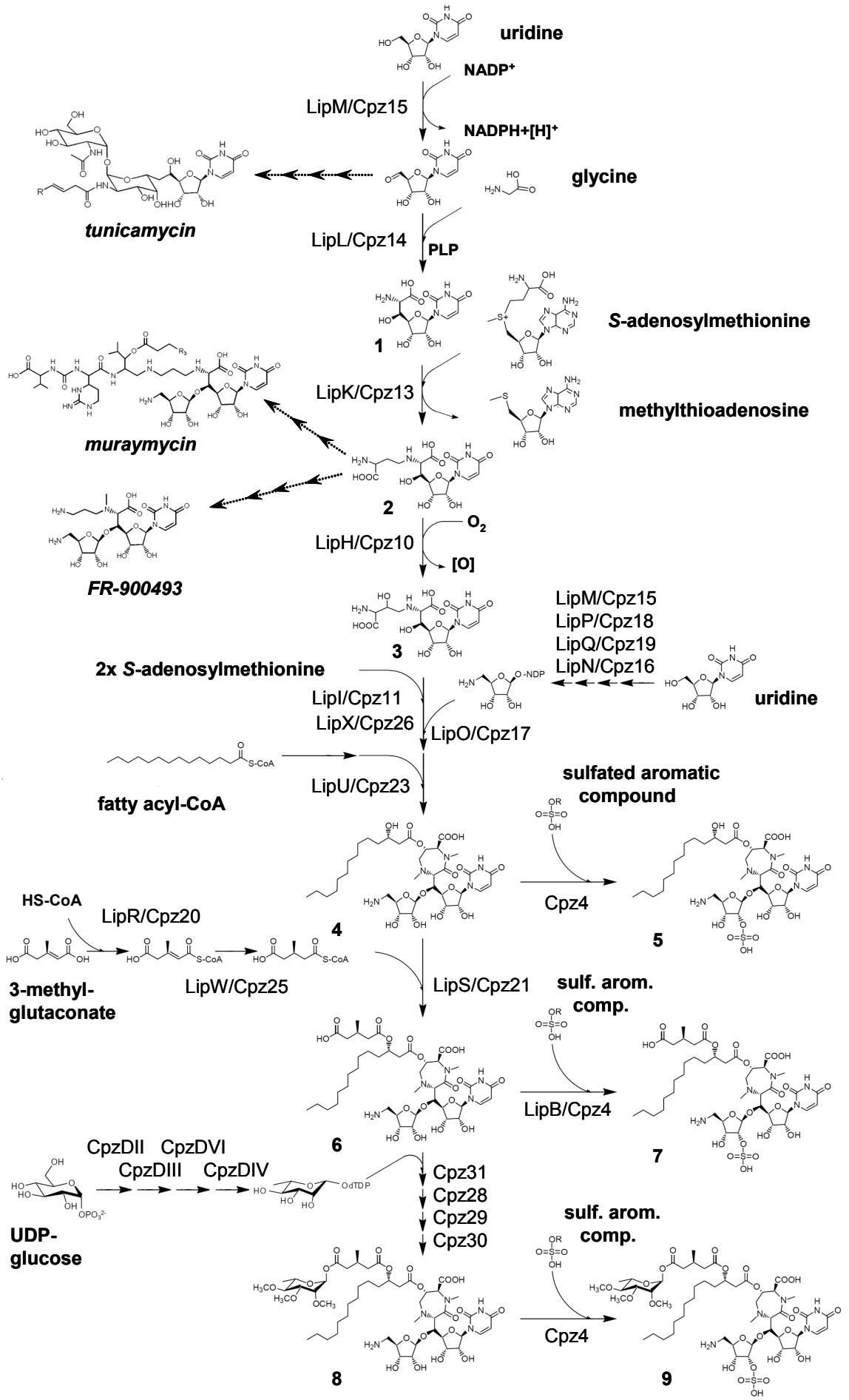
Figure IV.3 Cluster analysis of ASST-type sulfotransferases and homologous hypothetical proteins. Protein sequences were obtained from PSI-BLAST searches using Cpz4 (accession GU323955), LpmB (accession GU219978), the ASST from *Eubacterium* A44 (accession ABG76784) and the ASST from *Escherichia coli* CFT073 (accession NP_755656). All data bank entries with at least 25% identity to the respective query protein sequence were included. If the database contained entries from different strains of the same bacterial species, only the entry with the highest sequence identity was included. Alignment and clustering of the resulting 93 sequences was performed with the help of the CLANS (Frickey & Lupas, 2004) online tool (http://toolkit.tuebingen.mpg.de/clans#). Pairwise sequence similarities were calculated using BLASTP and the BLOSUM80 matrix. The proteins were

clustered by their pairwise P-values (up to 1 x e^{-3}). Attraction and repulsion was set at 10 and the attraction exponent at 1. In the figure, selected proteins are labelled by their origin, including the functional investigated ASST-type sulfotransferases (bold).

4. A model for the biosynthesis of liponucleoside antibiotics

Sequence analysis of the caprazamycin and liposidomycin gene clusters combined with the analytical data from heterologous expression, gene inactivation experiments and in vitro studies may allow a first proposal of the biosynthetic pathway (Figure IV.4.1) to the liponucleoside antibiotics although many of the suggested reactions remain speculative at present. Below, the postulated biosynthesis is described for the caprazamycins, though the liposidomycins would be generated analogously, except for the formation of the permethylated L-rhamnose moiety.

A key question in the biosynthesis of caprazamycins and translocase I inhibitors of the same class is the origin of the glycyluridine (Figure IV.4.1, **1**). Metabolic labelling studies have shown that uridine is incorporated directly into related uridyl antibiotics such as tunicamycins (Tsvetanova *et al.*, 2002). A pathway to the tunicamycins has been proposed to start with the oxidation of uridine to form uridine-5'-aldehyde (Price & Tsvetanova, 2007). We suggest a similar reaction for caprazamycin biosynthesis, which may be catalyzed by the putative oxygenase Cpz15. The resulting product could undergo a subsequent aldol addition with a PLP-glycine adduct to generate (**1**). This mechanism would be very similar to that of the well studied serine hydroxymethyltransferases, which are known to produce *β*-hydroxy-*α*-amino acids from glycine and various aldehydes (Makart *et al.*, 2007). Cpz14, with significant sequence similarity to serine hydroxymethyltransferases, is an obvious candidate for the catalysis of this reaction. The next step would be the transfer of a 3-amino-3-carboxypropyl group to the 5'''-amino group of (**1**) to form (**2**). A corresponding reaction occurs in the nocardicin biosynthesis (Gunsior *et al.*, 2004). In this pathway, the gene product Nat utilizes *S*-adenosyl methionine (SAM) to transfer the 3-amino-3-carboxypropyl moiety to a nucleophilic acceptor (Reeve *et al.*, 1998). Nat shows conserved domains of SAM-dependent methyltransferases, which are also found in Cpz11 and Cpz26. Hence, both genes may be candidates for a 3-amino-3-carboxypropyl transfer in caprazamycin biosynthesis, although we consider them rather to be involved in the two *N*-methylation steps discussed below. Notably, Cpz13, a putative PLP-dependent enzyme of the aspartate aminotransferase family (Pfam00155), exhibits homology to known aminocyclopropane carboxylic acid (ACC) synthases from plants.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muraymycin
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LipM/Cpz15
LipP/Cpz18
LipQ/Cpz19
LipN/Cpz16
2x S-adenosylmethionine
uridine
LipI/Cpz11
LipX/Cpz26
LipO/Cpz17
fatty acyl-CoA
LipU/Cpz23
sulfated aromatic compound
4
Cpz4
5
HS-CoA
LipR/Cpz20
3-methyl-glutaconate
LipW/Cpz25
LipS/Cpz21
sulf. arom. comp.
6
LipB/Cpz4
7
CpzDII CpzDVI
CpzDIII CpzDIV
UDP-glucose
Cpz31
Cpz28
Cpz29
Cpz30
sulf. arom. comp.
8
Cpz4
9

Figure IV.4.1 A model for the biosynthetic pathway of the liposidomycins (**7**) and caprazamycins (**8**). Isolated metabolites, caprazamycin aglycones (**6**), hydroxyacyl-caprazols (**4**) sulfo-hydroxyacyl-caprazols (**5**) and sulfo-caprazamycins (**9**) are indicated as well as the putative intermediates glycyluridine (**1**), 5'''-*N*-(3-amino-3-carboxypropyl)-uridine (**2**) and 6-*N*-(β-hydroxy-3-amino-3-carboxypropyl)-uridine (**3**). Structurally related nucleoside antibiotics the tunicamycins, the muraymycins and FR900493 are included. PLP is pyridoxalphosphate.

As the formation of ACC from SAM resembles the postulated reaction for the attachment of the 3-amino-3-carboxypropyl moiety in caprazamycin biosynthesis we would favour Cpz13 as a candidate for the catalysis of this step to generate (**2**). We further speculate that (**2**) could be a common intermediate in the caprazamycin (**8**), liposidomycin (**7**), FR900493 and the muraymycin biosynthesis. *β*-hydroxylation of the 3-amino-3-carboxypropyl group of (**2**) would lead to (**3**) and could be catalyzed by either Cpz10 or Cpz15. Both proteins show homology to oxygenases.

Subsequent biosynthetic steps, including formation and transfer of the aminoribose, cyclization and *N*-methylation of the diazepanone ring and attachment of the fatty acid would finally lead to the hydroxyacyl-caprazols (Figure IV.4.1, **4**). Compounds of this structure were accumulated in the *Δcpz21* mutant strain and are probable intermediates of the caprazamycin pathway. Reasonable candidate genes for these biosynthetic steps can be found in the cluster. However, the sequence of these reactions, described in the following paragraphs, is speculative at present.

Cyclization of (**3**) by amide bond formation between the carboxyl group and the secondary amino group would immediately result in the characteristic diazepanone ring. For this reaction, a previous activation of the carboxyl group e.g. in form of an acyl adenylate, a coenzyme A ester or an acyl phosphate would be required. The putative kinases Cpz12 and Cpz27 may be involved in this reaction.

Interestingly, a contiguous set of genes, *cpz16-19,* was found in the caprazamycin cluster which can be assigned to all steps required for the generation and attachment of the aminoribosyl moiety. This reaction sequence may start from a second molecule of uridine-5'-aldehyde, derived from uridine by a Cpz15 mediated oxidation as described above. Subsequently, the 5-aldehyde group could undergo an aminotransfer reaction, yielding a 5-aminated nucleoside possibly catalyzed by the hypothetical aminotransferase Cpz18. CetH, an ortholog of Cpz18, has recently been assigned to the aminotransfer reaction in biosynthesis of the aminocyclitol cetoniacytone (Wu *et al.*, 2009). 5-Amino-ribose-1-phosphate and uracil would be

generated from the aminated nucleoside by Cpz19, a putative pyrimidine-nucleoside phosphorylase. A similar reaction has been shown in flourothreonine biosynthesis where a 1-phospho-ribosyl derivative is formed under catalysis of the pyrimidine phosphorylase FlB (Huang *et al.*, 2006). Subsequently, the potential nucleotidyltransferase Cpz16 may convert the 5-amino-ribose-1-phosphate to dNDP-5-aminoribose. Then, the putative glycosyltransferase Cpz17 could transfer the aminoribose moiety forming a glycosidic bond.

Generally, ribosyl moieties are attached by phosphoribosyltransferases (Sinha & Smith, 2001) using 5-phosphribosyl-1-diphosphate as a donor to generate a 5'-phosphoribosylated product. Then, the 5'-phospho group is removed by a phosphatase. Similar reactions have recently been shown to lead to the ribosyl moiety in butirosin biosynthesis involving BtrL and BtrP (Kudo *et al.*, 2007). However, no orthologoues to BtrL and BtrP were found in the caprazamycin gene cluster, making the pathway described above a more likely alternative.

The fatty acid moieties of liposidomycins and caprazamycins are probably derived from primary metabolism, as feeding studies with labelled palmitic acid in *S. griseosporeus* showed the direct incorporation into liposidomycins (Kagami *et al.*, 2003). Hydroxylation of the fatty acids could either occur within primary metabolism or by the oxygenases Cpz10 or Cpz15. Cpz23 may be involved in the attachment of the hydroxy-fatty acids, due to its homology to lipases.

In the diazepanone ring, both nitrogens are methylated. The *N*-methylation reactions are likely to be catalyzed by Cpz11 and/or Cpz26. Notably, Cpz11 exhibits sequence similarity (55%) to one of the few characterized *N*-methyltransferases AtM1 from the gene cluster of AT2433 (Gao *et al.*, 2006). Gene deletion experiments showed *cpz11* and *cpz26* to be essential in caprazamycin biosynthesis. However, a possible intermediate of the biosynthetic pathway could not be identified.

First insights could be obtained concerning the biosynthetic origin of the 3-MG moiety. By our inactivation experiments we could exclude an involvement of the putative HMG-CoA synthase Cpz5. As indicated by functional investigations in this study we would assign the catalysis of the transfer of the methylglutarate to Cpz21. The substrate of Cpz21 is probably provided in form of a coenzyme A ester. The deletion of *cpz20* and *cpz25* resulted in a similar phenotype, which is the accumulation of the hydroxyacyl-caprazols. Therefore, we would speculate that 3-MG-CoA is generated from 3-methylglutaconate by hydrogenation and CoA-

activation catalyzed by the putative alcohol dehydrogenase Cpz25 and the hypothetical acyl-CoA synthase Cpz20, respectively.

Cpz21 generates the caprazamycin aglycones (**6**) which are similar to the type-(III) liposidomycins (Figure I.2.3). Based on the sequence homology to Cpz4, LpmB is assigned to catalyze the subsequent and final step in liposidomycin biosynthesis, the transfer of the sulfate group from a sulfated aromatic donor. In contrast the formation of the caprazamycin would proceed with the reactions which finally lead to the permethylated L-rhamnose moiety.

Analogous to the biosynthesis of elloramycin (Ramos *et al.*, 2008), the L-rhamnosyl group of the caprazamycins is synthesized from enzymes encoded on another cluster on the genome of *Streptomyces* sp. MK730-62F2. *cpzDII*, *cpzDIII*, *cpzDIV* and *cpzDVI* have been shown to be sufficient and essential for the provision of the deoxysugar in caprazamycin formation. These genes encode for the required dTDP-glucose synthase, dehydratase, epimerase and 4-ketoreductase to generate dTDP-L-rhamnose as discussed in section IV.1. Subsequently, the rhamnosyltransferase Cpz31 catalyzes the attachment of the deoxysugar to the caprazamycin aglycone as demonstrated by in vitro studies. Sequential methylation of the deoxysugar moiety is likely catalyzed by the hypothetical sugar *O*-methyltransferases Cpz28, Cpz29 and Cpz30.

As shown in this study and discussed in section IV.3, Cpz4 produces the sulfated liponucleoside derivatives (**5**), (**7**) and (**9**) from (**4**), (**6**) and (**8**) and a so far unidentified aromatic sulfate donor.

The identification and analysis of the caprazamycin and liposidomycin gene clusters provide the first molecular basis for the proposal of a translocase I inhibitor biosynthetic pathway. Since the formation of intermediate (**2**) can be speculated to be similar for other structurally related compounds, this work may help in the development of probes for the discovery of gene clusters of other uridyl-antibiotics. As proposed, several biosynthetic steps to the caprazamycins and liposidomycins seem to be distinctive and unique in bacterial secondary metabolism. Apparently they represent intriguing subjects for further functional investigations.

A detailed understanding of the caprazamycin and liposidomycin biosynthetic pathways combined with the successful establishment of a heterologous expression system sets the basis for genetic and metabolic engineering towards the production of new liponucleoside antibiotics with improved properties.

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