Investigations on the Biosynthesis of the Central Pyrrole Moiety of Coumermycin A_1 and Identification of new Caprazamycins by LC/ESI-MS/MS

Untersuchungen zur Biosynthese der zentralen Pyrroleinheit von Coumermycin A_1 und Identifizierung neuer Caprazamycine mittels LC/ESI-MS/MS

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„Was wir wissen, ist ein Tropfen;
was wir nicht wissen, ein Ozean“

Sir Isaac Newton

CONTENTS

PUBLICATIONS AND PRESENTATIONS

SCIENTIFIC PUBLICATIONS

Stefanie Siebenberg, Nadja Burkard, Anna Knuplesch, Bertolt Gust, Stephanie Grond, and Lutz Heide. **„Two pathways for pyrrole formation in coumermycin A_1 biosynthesis: the central pyrrole moiety is formed from L-threonine".** Chembiochem 2011 Sep 27; doi: 10.1002/cbic.201100494. [Epub ahead of print]

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Stefanie Siebenberg, Leonard Kaysser, Lutz Heide, Bertolt Gust, and Bernd Kammerer. **"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".** New Directions in Molecular Genetics and Genomics – Applications in natural product producing organisms, Freiburg, 2008.

Stefanie Siebenberg, Leonard Kaysser, Lutz Heide, Bertolt Gust, and Bernd Kammerer. **"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".** Analytica Conference 2008, 21. Internationale Leitmesse für instrumentelle Analytik, Labortechnik und Biotechnologie, München, 2008.

Oral Presentations:

Stefanie Siebenberg, Nadja Burkard, Anna Knuplesch, Bertolt Gust, Stephanie Grond, and Lutz Heide. **„Biosynthesis of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety of coumermycin A_1 from L-threonine".** International VAAM-Workshop 2011: Biology of Bacterial Producers of Natural Compounds. September 2011, Bonn.

CONTRIBUTION OF OTHER PEOPLE TO THIS WORK

Biosynthesis of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety of coumermycin A_1

Inactivation of the *couR* genes on cosmid couMW16 by RED/ET mediated recombination and construction of plasmids pAK06 and pAK10 for complementation was carried out by Anna Knuplesch under supervision of Dr. Manuel Wolpert during her diploma work in the group of Prof. Dr. L. Heide.

Initial work on the inactivation of the genes *couM-couN7*, resulting in cosmid couMW22, was done by Dr. Manuel Wolpert

Feeding experiments with ^{13}C-labeled precursors were carried out in cooperation with the group of Prof. Dr. S. Grond (Organic Chemistry Department, University of Tübingen). The NMR analysis of the labeled and unlabeled CPM monoamide, including the data evaluation, was done by Nadja Burkard (Grond group).

Identification of new caprazamycins from *Streptomyces sp*. MK730-62F2

Cultivation of the caprazamycin and liposidomycin producer strains was carried out by Emmanuel Wemakor.

ABBREVIATIONS

°C	degree celsius
µ	micro
aa	amino acids
aac(3)IV	apramycin resistance gene from pIJ773
Amp	ampicillin
Apra	apramycin
Arb	Arbitrary units
ATP	adenosine triphosphate
bp	base pair
CA	collisional activation
CID	collision-induced dissociation
Cml	chloramphenicol
COSY	correlation spectroscopy; 2D-NMR experiment
CPM	central pyrrole moiety of coumermycin A_1 (3-methylpyrrole-2,4-dicarboxylic acid)
CPZ	caprazamycin
Da	Dalton
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5´-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
EIC	extracted ion chromatogram
ESI	electrospray ionization
FAB	fast atom bombardment
FMN	flavin mononucleotide
g	gram
GlcNAc	*N*-acetylglucosamine
GyrA	gyrase A subunit
GyrB	gyrase B subunit
h	hour
HCl	hydrochloric acid
HCOOH	formic acid
HMBC	heteronuclear multiple bond coherence; 2D-NMR experiment
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence; 2D-NMR experiment
k	kilo
Kan	kanamycin
kb	kilo base pairs
KOAc	potassium acetate
L	litre
LC	liquid chromatography
LPM	liposidomycin
M	molar
M.	*Mycobacterium*

m	milli
MIC	minimal inhibitory concentration
min	minute
MS	mass spectrometry; mass spectrometer
MS/MS	tandem mass spectrometry
MurNAc	*N*-acetylmuramic acid
MW	molecular weight
m/z	mass-to-charge ratio
n	nano
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
neo	neomycin/kanamycin resistance gene
NH_4OAc	ammonium acetate
NMR	nuclear magnetic resonance
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriT	origin of transfer from RK2
p	pico
ParC	topoisomerase IV subunit C
ParE	topoisomerase IV subunit E
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLP	pyridoxal phosphate
Q1	first quadrupole
Q3	third quadrupole
R (superscript)	resistant
RP	reverse phase
rpm	rotation per minute
RT	room temperature
s	second
S (superscript)	sensitive
S.	*Streptomyces*
SAM	S-adenosylmethionine
SD	standard deviation
sp.	*species*
t	time
TES	N-Tris-(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid
THF	tetrahydrofolate
Thio	thiostrepton
TIC	total ion chromatogram
Topo	topoisomerase
TPP	thiamine pyrophosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	unit
UDP	uridine diphosphate
UV	ultraviolet
WT	wild-type
xg	ground acceleration

SUMMARY

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of DNA gyrase and are produced by different *Streptomyces* strains. Cloning and sequencing of the corresponding biosynthetic gene clusters allowed detailed investigations of their biosynthetic pathways in recent years and now the functions of nearly all genes in biosynthesis, regulation and resistance are known.

In the first part of this thesis the production of novobiocin was investigated in 24-square deepwell plates, an alternative cultivation system to Erlenmeyer flasks. Investigations on antibiotic production are often hampered by the notorious variability of production rates observed in Erlenmeyer flasks, even between parallel cultivation batches of the same strain. The high standard deviation in the production rates makes it necessary to increase the number of parallel cultures if significant differences need to be distinguished from chance variations. Therefore a cultivation method which shows less variability in secondary metabolite production could significantly reduce the workload and cost of research in antibiotic biosynthesis. Comparison of novobiocin production in Erlenmeyer flasks and 24-square deepwell plates showed that the production was originally higher in the Erlenmeyer flasks (24 mg/L vs. 11 mg/L). However, the variability was much lower in the deepwell plate (SD 6% vs. 39%). Optimization of the inoculation ratio and preparation of frozen inoculum from a defined section of the growth phase further reduced the variability, especially between independent cultivation batches (SD 6% vs. 52%). Addition of the water soluble siloxylated ethylene oxide/propylene oxide copolymer Q2-5247, as artificial oxygen carrier, as well as the overexpression of the pathway-specific positive regulator NovG increased novobiocin production to levels comparable to those in the Erlenmeyer flasks (54 mg/L and 48 mg/L, respectively). Combination of the positive effects of NovG and of copolymer Q2-5247 led to a novobiocin production of 163 mg/L, exceeding all previous production levels observed in Erlenmeyer flasks in our laboratory.

For the production of coumermycin A_1 in the 24-square deepwell plates, two alternative production media were tested because the originally used SK medium was not suitable, due to the contained lard oil. In the first medium, a coumermycin production of 17 mg/L was observed. However, the main product was not coumermycin A_1 but coumermycin C, which lacks one of the terminal pyrrole moieties. In the second medium coumermycin production was not only higher (53 mg/L) than in the first medium, but coumermycin A_1 was now the main product. Therefore, the second medium was chosen for all further experiments.

Coumermycin A_1 contains two types of pyrrole moieties, two terminal 5-methylpyrrole-2-carboxylic acid moieties and the central 3-methylpyrrole-2,4-dicarboxylic acid moiety. The terminal pyrrole moiety is also present in clorobiocin and its biosynthesis from L-proline by action of CouN1-7 is well investigated. However, the biosynthetic precursors of the central pyrrole moiety have remained unknown, and none of the genes or enzymes involved in its formation have been identified so far. In the second part of this thesis evidence is provided that five genes, termed *couR1-couR4*, contained in a contiguous 4.7 kb region within the

coumermycin biosynthetic gene cluster, are required for the biosynthesis of the central pyrrole moiety. Each of these genes was deleted individually, resulting in a strong reduction (by at least 92%) or an abolishment of coumermycin production. External feeding of the central pyrrole moiety, i.e. 3-methylpyrrole-2,4-dicarboxylic acid, restored coumermycin production. One of the enzymes involved, i.e. CouR3, shows similarity to the L-threonine kinase PduX from *Salmonella enterica*, and feeding of *O*-phospho-L-threonine to a *couR3* defective mutant resulted in a moderate increase of coumermycin production, while feeding of L-threonine (0.197 mg/L vs. 0.143 mg/L) did not, indicating that the first step in the biosynthesis might be the phosphorylation of L-threonine. Feeding of [U-^{13}C,^{15}N]L-threonine and ^{13}C NMR analysis of the resulting labeled CPM monoamide unequivocally proved that threonine is incorporated intact into the central pyrrole moiety (19 % enrichment), providing the heterocyclic nitrogen as well as four of the seven carbons of this moiety. Therefore, the central pyrrole moiety is formed via a new, hitherto unknown biosynthetic pathway. A hypothesis for the reaction sequence leading to this moiety, starting from L-threonine and oxaloacetate, is presented. A second feeding experiment with [U-^{13}C]fumaric acid did not show significant incorporation.

Caprazamycins are potent anti-mycobacterial liponucleoside antibiotics that belong to the translocase I inhibitors and are produced by *Streptomyces* sp. MK730-62F2. Their complex structure is derived from 5'-(*β*-*O*-aminoribosyl)-glycyluridine and comprises a unique *N*-methyl-diazepanone ring. Recently, the caprazamycin biosynthetic gene cluster was identified by our group.

In the third part of the presented thesis, a liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method is reported for the structural elucidation of caprazamycins and liposidomycins from culture extracts of the respective producer strains. Both caprazamycins and liposidomycins were readily detected in positive as well as in negative ionization mode and the comparison of the fragmentation pattern revealed several characteristic product ions. These product ions can be divided into two groups, according to whether they still contain the fatty acid side chain or not. Those that lack the fatty acid side chain are characteristic for all caprazamycins and/or liposidomycins, respectively, and were used for the identification of six new caprazamycins that probably contain unsaturated fatty acid side chains. The proposed chemical formula of these new compounds fit that of the fatty acid moieties of the liposidomycins Y, Z, A, G, K and N, respectively. On the whole, the positive ionization mode provided more structural information, while the negative ionization mode seems to be of advantage if informations regarding the sulfate group or the fatty acid side chains are required.

A chromatographic method using a boronic acid stationary phase, which was originally used for the purification of modified nucleosides from cell cultures, was adapted for the partial purification of the culture extracts. The fractions containing the caprazamycins were identified by a bioactivity assay with *Mycobacterium phlei* and by LC-MS/MS analysis, respectively. To prevent an early elution of the compounds, the methanol content in the second washing step needed to be reduced from 50% to 20%.

ZUSAMMENFASSUNG

Die Aminocoumarin-Antibiotika Novobiocin, Clorobiocin und Coumermycin A_1 sind potente Inhibitoren der DNA-Gyrase und werden von verschiedenen Streptomyceten gebildet. Die Klonierung und Sequenzierung der entsprechenden Biosynthese-gencluster ermöglichte in den letzten Jahren eine detaillierte Untersuchung ihrer Biosynthesewege und die Funktion fast aller Gene in Biosynthese, Regulation und Resistenz ist mittlerweile bekannt.

Im ersten Teil dieser Arbeit wurde die Produktion von Novobiocin in 24-Kammer-Platten untersucht, welche ein alternatives Kultivierungssystem zu Erlenmeyerkolben darstellen. Untersuchungen zur Antibiotikaproduktion in Streptomyceten werden oft durch die stark schwankenden Produktionsraten in Erlenmeyerkolben behindert, welche man selbst zwischen parallel angezogenen Kulturen ein und desselben Stammes beobachten kann. Die großen Standardabweichungen machen es daher oft nötig viele parallele Kultivierungen durchzuführen wenn man echte Unterschiede in den Produktionsraten von zufälligen Schwankungen unterscheiden will. Aus diesem Grund könnte eine Kultivierungsmethode, welche geringere Schwankungen in den Produktionsraten aufweist, den Arbeitsaufwand und die Kosten in der Antibiotikaforschung deutlich reduzieren. Ein Vergleich der Novobiocinproduktion in Erlenmeyerkolben und einer 24-Kammer-Platte ergab zunächst eine deutlich höhere Produktion im Erlenmeyerkolben (24 mg/L vs. 11 mg/L). Die Kulturen aus der 24-Kammer-Platte wiesen im Vergleich zu denen aus dem Erlenmeyerkolben allerdings deutlich geringere Produktionsschwankungen auf (Standardabweichung: 6% vs. 39%). Durch die Optimierung des Animpfverhältnisses sowie die Herstellung von eingefrorenem Inoculum aus Vorkulturen in der Wachstumsphase, konnte eine weiteren Abnahme der Variabilität, besonders zwischen unabhängige Kultivierungen (Standardabweichung: 6% vs. 52%), erreicht werden. Der Zusatz eines wasserlöslichen Copolymers aus Ethylenoxid und Propylenoxid sowie die Überexpression des spezifischen positiven Regulators NovG führte jeweils zu vergleichbaren Produktionen wie im Erlenmeyerkolben (54 mg/L bzw. 48 mg/L). Diese positiven Effekte von Copolymer-Zusatz und NovG-Überexpression lassen sich auch kombinieren, sodass schließlich eine Novobiocinproduktion von 163 mg/L erreicht werden konnte, was alle bisher von uns beobachteten Produktionsraten im Erlenmeyerkolben deutlich übersteigt.

Für die Produktion von Coumermycin A_1 in den 24-Kammer-Platten wurden zwei alternative Produktionsmedien getestet, weil das ursprüngliche verwendete SK-Medium aufgrund des enthaltenen Fettes leider ungeeignet war. In dem ersten getesteten Medium ergab sich eine Gesamtproduktion von 17 mg/L. Das Hauptprodukt war in diesem Fall allerdings nicht Coumermycin A_1 sondern Coumermycin C, welchem eine der beiden endständigen Pyrroleinheiten fehlt. Im zweiten Medium fiel die Produktion dann nicht nur deutlich höher aus (53 mg/L), sondern Coumermycin A_1 war diesmal auch das Hauptprodukt. Aus diesem Grund wurde entschieden das zweite Medium für alle weiteren Versuche zu verwenden.

Coumermycin A_1 enthält zwei verschiedene Pyrroleinheiten, die beiden endständigen 5-Methylpyrrol-2-carbonsäure-Einheiten und die zentrale 3-Methylpyrrol-2,4-dicarbonsäure. Die endständige Pyrroleinheit findet sich auch im Clorobiocin wieder und ihre Biosynthese, ausgehend von L-Prolin und katalysiert durch CouN1-7, ist gut untersucht. Die Biosynthesevorstufen der zentralen Pyrroleinheit, andererseits, sind noch vollkommen unbekannt und keines der beteiligten Gene oder Enzyme wurden bisher identifiziert. Der zweite Teil dieser Arbeit erbringt nun den Nachweis, dass fünf Gene, *couR1-couR4*, die sich in einem 4,7 kb großen Abschnitt innerhalb des Coumermycin-Genclusters befinden, für die Biosynthese der zentralen Pyrroleinheit verantwortlich sind. Jedes dieser Gene wurde einzeln inaktiviert, was zu einer starken Reduzierung (wenigstens 92%) oder sogar zum Ausfall der Coumermycin-produktion führte. Durch Fütterung der zentralen Pyrroleinheit, 3-Methylpyrrol-2,4-dicarbonsäure, konnte die Produktion dann teilweise wiederhergestellt werden. Eines der beteiligten Enzyme, und zwar CouR3, zeigt Ähnlichkeit zu der Threoninkinase PduX aus *Salmonella enterica* und die Fütterung von *O*-Phospho-L-Threonin zu einer *couR3*-defekten Mutante führte zu einer leichten Produktionssteigerung im Vergleich zur Fütterung von L-Threonin (0,197 mg/L vs. 0,143 mg/L). Dies deutet darauf hin, dass der erste Schritt in der Biosynthese der zentralen Pyrroleinheit womöglich in der Phosphorylierung von L-Threonin besteht. Die Fütterung von [U-^{13}C,^{15}N]L-Threonin und die anschließende ^{13}C NMR-Analyse des resultierenden gelabelten Monoamids der zentralen Pyrroleinheit zeigen eindeutig, dass das intakte Threonin in diese Einheit eingebaut wird (Anreicherung 19%) und dass es sowohl den heterozyklischen Stickstoff als auch vier der sieben Kohlenstoffatome liefert. Das bedeutet, dass die zentrale Pyrroleinheit von Coumermycin A_1 über einen neuen, bisher vollkommen unbekannten, Biosyntheseweg gebildet wird. Eine Hypothese für den Ablauf dieser Biosynthese, ausgehend von Threonin und Oxalacetat, wurde aufgestellt. Leider war ein zweiter Fütterungsversuch, diesmal mit [U-^{13}C]Fumarsäure, nicht erfolgreich.

Caprazamycine sind Liponukleosid-Antibiotika und werden zu den Translokase I-Inhibitoren gezählt. Sie werden von *Streptomyces sp.* MK730-62F2 produziert und weisen eine sehr gute Wirksamkeit gegen Mykobakterien und anderen grampositive Mikroorganismen auf. Die Caprazamycine besitzen eine hochkomplexe chemische Struktur, bestehend aus einem 5'-(*β*-*O*-Aminoribosyl)-Glycyluridin und einem *N*-Methyl-Diazepanonring. Das entsprechende Biosynthesegencluster wurde erst kürzlich in unserem Arbeitskreis identifiziert.

Im dritten Teil dieser Arbeit wird eine HPLC/ESI-MS/MS-Methode für die Identifizierung von Caprazamycinen und Liposidomycinen, in Kulturextrakten der jeweiligen Produktionsstämme, vorgestellt. Beide Verbindungsklassen konnten sowohl bei positiver als auch bei negativer Ionisierung eindeutig identifiziert werden und der Vergleich der Fragmentierungsmuster zeigte mehrer charakteristische Produktionen. Diese lassen sich in zwei Klassen einteilen, je nachdem ob sie noch die Fettsäure-Seitenkette enthalten oder nicht. Produktionen denen die Fettsäure fehlt, sind charakteristisch für alle Caprazamycine bzw. Liposidomycine, und wurden daher für die Identifizierung von insgesamt sechs neuen Caprazamycinen herangezogen, welche wahrscheinlich ein- und zweifach ungesättigte Fettsäuren enthalten. Die vorgeschlagenen Summenformeln der neuen Verbindungen passen jeweils zu den Fettsäuren der Liposidomycine Y, Z, A, G, K und N. Insgesamt liefern die Spektren bei positiver Ionisierung mehr Strukturinformation, aber die negative

Ionisierung könnte immer dann von Vorteil sein, wenn Informationen bezüglich der Sulfatgruppe oder der Fettsäure benötigt werden.

Eine chromatographische Methode unter Verwendung eines Boronsäure-Gels, welche ursprünglich für die Aufreinigung von modifizierten Nukleosiden aus Zellkulturen verwendet wurde, wurde für die teilweise Aufreinigung der Kulturextrakte angepasst. Die Fraktion, welche die Caprazamycine enthält, wurde anschließend mittels eines Bioaktivitäts-Assays mit *Mycobacterium phlei* sowie mittels HPLC-MS/MS identifiziert. Um ein vorzeitiges Eluieren der Substanzen zu verhindern, war es nötig den Methanolgehalt im zweiten Waschschritt von 50% auf 20% zu reduzieren.

I. INTRODUCTION

1. Antibiotic resistance and the need for new antibiotics

Infectious diseases continue to be a major health problem (Watve *et al.*, 2001), mainly due to the increasing prevalence of antibiotic resistance in pathogenic bacteria in hospitals and in the community (Fischbach & Walsh, 2009).

Penicillin-resistant *Staphylococcus aureus* emerged soon after the introduction of penicillin in the 1940s and by 1986 methicillin-resistant *S. aureus* (MRSA) became so prevalent that vancomycin became a front-line antibiotic in the treatment of MRSA infections. Since then, MRSA have evolved to vancomycin-resistant *S. aureus* (VRSA) (Walsh, 2003). In the developing world, multidrug-resistant (MDR) and extensively-drug-resistant (XDR) strains of *Mycobacterium tuberculosis* are an increasing problem (Fischbach & Walsh, 2009), especially in co-infections with HIV, since the risk of developing active tuberculosis is increased (Ma *et al.*, 2010). Furthermore, rifampicin, a first-line drug in tuberculosis treatment, renders protease inhibitors ineffective due to the induction of CYP3A4 and p-glycoprotein expression (Ma *et al.*, 2010). The emergence of multidrug-resistant Gram-negative pathogens, especially in intensive care units, is another serious problem. Some Entero-bacteriaceae, like *Enterobacter* and *Klebsiella*, carry extended-spectrum β-lactamases, which are able to destroy even the latest generations of penicillins and cephalosporins and the metallo-β-lactamases can even inactivate carbapenems, often the “last resort” in infections caused by Gram-negative bacteria (Levy & Marshall, 2004). Furthermore, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are sometimes resistant to all antibiotics (Levy & Marshall, 2004).

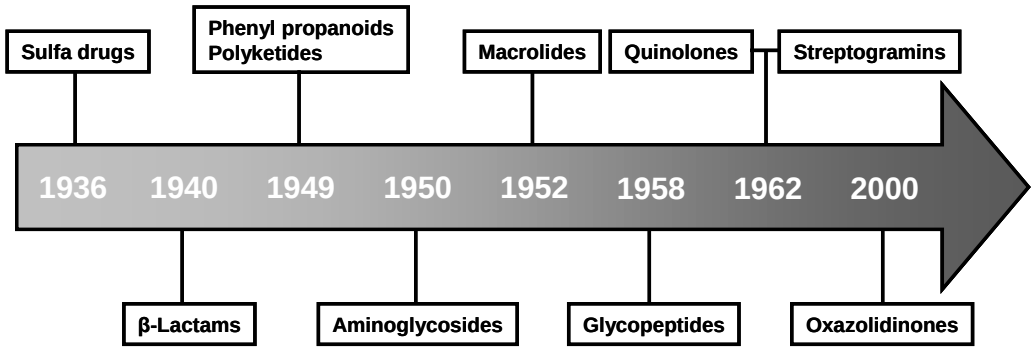


Figure I.1
Introduction of new classes of antibiotics into clinical practice between 1936 and 2000. Modified from Walsh (2003).

The use of antibiotics inevitably selects for resistant microbes, and therefore there is a continuing and cyclical need for new and more effective antibiotics (Clardy *et al.*, 2006). However, progress in developing them has been slow and only three new classes of antibiotics have been introduced in the past 50 years: the quinolones, the streptogramins and the oxazolidinones (**Figure I.1**) (Fischbach & Walsh, 2009; Walsh, 2003). Among the 15 antibiotics currently in the pipeline are three cephalo-

sporins, three glycopeptides and four quinolones, but not one representative of a new antibiotic class and especially the lack of new antibiotics against Gram-negative bacteria is concerning (Freire-Moran *et al.*, 2011; Schmitz & Holzgrabe, 2011).

Natural products are among the most important sources for new drug candidates (Kumar & Waldmann, 2009), though structural modifications of these compounds are often necessary to optimize the pharmacological properties and to minimize toxicity (Baltz, 2008). More than 60% of the antibacterial and anticancer drugs introduced between 1981 and 2002 originated from natural sources (Lam, 2007), mostly from actinomycetes or fungi (Baltz, 2007). Despite the success of natural products as sources of new drug leads, many pharmaceutical companies terminated their natural product research programs, focusing now on combinatorial chemistry and high-throughput, target-based screening of synthetic compound libraries (Baltz, 2007; Lam, 2007; Li & Vederas, 2009). However, the structural diversity of natural products is still unmatched (Lam, 2007) and significant progress in the areas of genome sequencing, bioinformatics, genetic engineering and analytical chemistry, the development of new screening methods and the access to so far unexplored microbial niches, will hopefully renew the interest in natural product research (Baltz, 2008; Clardy *et al.*, 2006; Fischbach & Walsh, 2009; Lam, 2007; Li & Vederas, 2009).

2. Aminocoumarin antibiotics

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are produced by different *Streptomyces* strains, e.g. novobiocin by *S. spheroides* (syn. *S. caeruleus*) NCIMB 11891 (Lanoot *et al.*, 2002; Steffensky *et al.*, 2000), clorobiocin by *S. roseochromogenes var. oscitans* DS12.976 (Mancy *et al.*, 1974) and coumermycin A_1 by *S. rishiriensis* (Kawaguchi *et al.*, 1965; Weinstein *et al.*, 1978). The more recently discovered simocylinone D8 (Schimana *et al.*, 2000) and rubradirin (Sohng *et al.*, 1997), which are structurally more complex than the three “classical” aminocoumarin antibiotics, are also produced by different *Streptomyces* strains.

2.1. Chemical structure

The aminocoumarin antibiotics are characterized by their 3-amino-4,7-dihydroxy-coumarin moiety, which is attached to an acyl component via an amide bond and to an deoxysugar moiety, i.e. 4-*O*-methyl-5-*C*-methyl-L-rhamnose, via a glycosidic bond. Novobiocin and clorobiocin share the same acyl component, i.e. 3-dimethylallyl-4-hydroxybenzoate, while coumermycin A_1 contains a 3-methylpyrrole-2,4-dicarboxylic acid moiety (central pyrrole moiety). The same aminocoumarin-deoxysugar moiety is attached in coumermycin A_1 to both carboxyl groups of the central pyrrole moiety, leading to a nearly symmetric molecule. The 3-OH group of the deoxysugar moiety is acylated in all three aminocoumarin antibiotics, either with a carbamyl group (novobiocin) or with a 5-methylpyrrole-2-carboxyl group (clorobiocin and coumermycin A_1). As suggested by the name, clorobiocin contains a chlorine atom at position 8 of the aminocoumarin moiety, whereas novobiocin and coumermycin A_1 carry a methyl group at the same position (**Figure I.2**).

Simocylinone D8 and rubradirin share the 3-amino-4,7-dihydroxycoumarin moiety with the “classical” aminocoumarins, but their acyl components are structurally more complex and they contain different deoxysugar moieties, which are attached to the acyl moiety rather than to the aminocoumarin ring (**Figure I.2**).

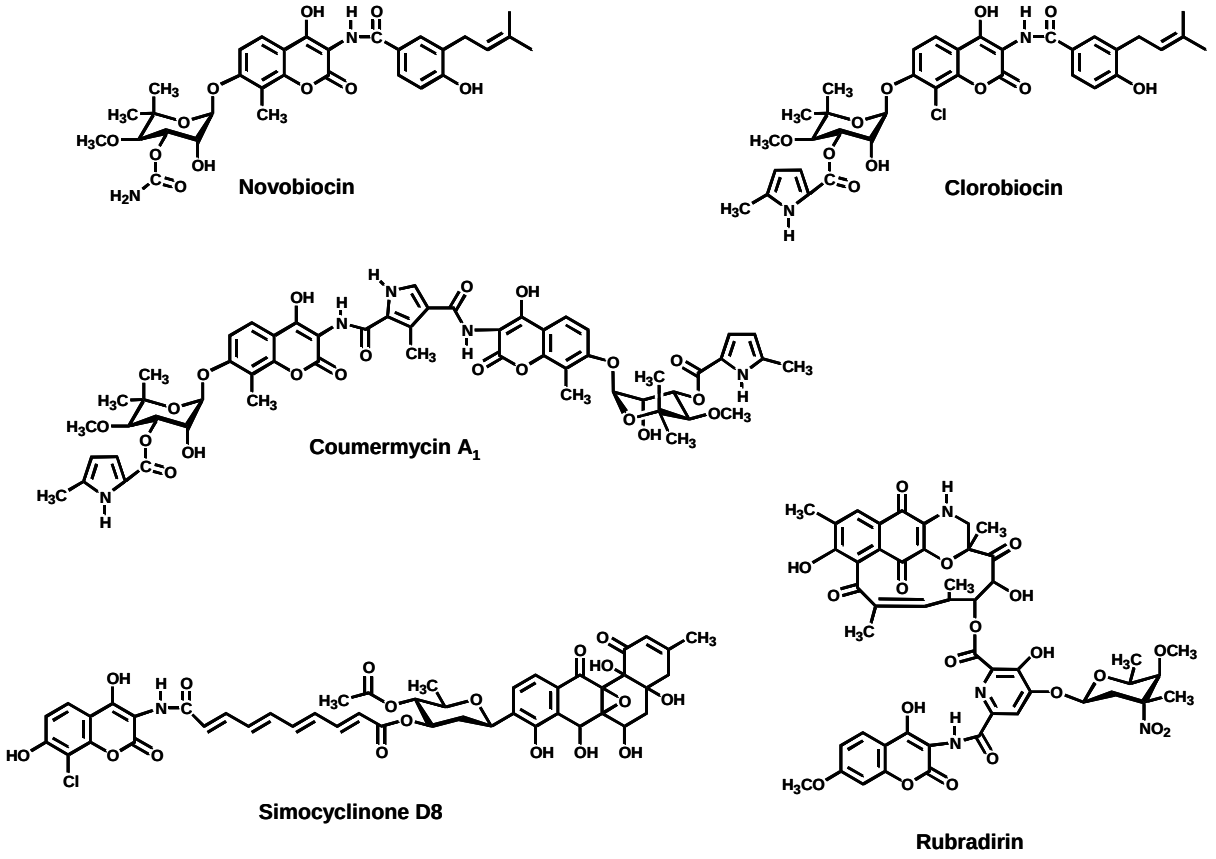


Figure I.2
Chemical structures of the aminocoumarin antibiotics

2.2. Biosynthetic gene clusters

The novobiocin biosynthetic gene cluster was the first gene cluster of an aminocoumarin antibiotic that was cloned and sequenced (Steffensky *et al.*, 2000). Subsequently, the gene clusters of coumermycin A_1 (Wang *et al.*, 2000) and clorobiocin (Pojer *et al.*, 2002) followed. The clusters span between 23 and 38 kb and comprise between 20 and 32 coding sequences (Heide, 2009). Nearly all the genes have been investigated by genetic and biochemical methods and their functions in biosynthesis, regulation and resistance are known (Heide, 2009). Although the structural differences between the three antibiotics are well reflected by the differences in the gene clusters (Li & Heide, 2006), the genes responsible for the biosynthesis of a certain moiety, e.g. for the biosynthesis of the aminocoumarin moiety, have the same order in all three clusters and show high sequence similarity (**Figure I.3**).

All three gene clusters contain two positive regulators e.g. *novE* and *novG* in the novobiocin gene cluster (Dangel *et al.*, 2008; Eustáquio *et al.*, 2005; Eustáquio *et al.*, 2003), as well as a gene coding for an aminocoumarin-resistant gyrase B subunit, termed *gyrB*^{R} (Schmutz *et al.*, 2003a; Steffensky *et al.*, 2000). The gene clusters of clorobiocin and coumermycin A_1 additionally contain a gene immediately downstream of *gyrB*^{R} which showed, on average, 44% identity and 57% similarity to *gyrB*^{R} on the amino acid level. This gene, named *parY*^{R}, codes for an aminocoumarin-resistant topoisomerase IV subunit and is not present in the novobiocin gene cluster (Schmutz *et al.*, 2003a).

The gene clusters of simocyclinone and rubradirin are larger and span approximately 65 and 105 kb, respectively. Their precise borders are not defined yet (Heide, 2009).

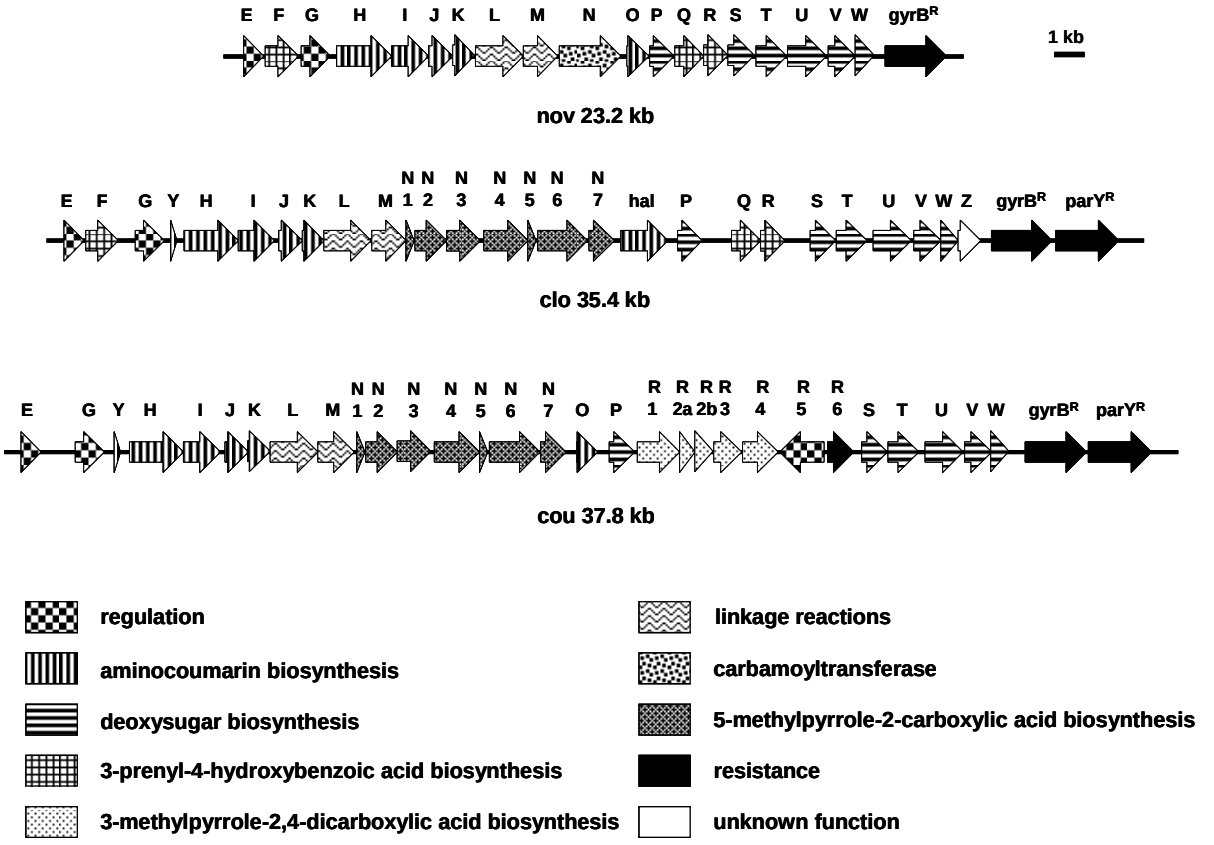


Figure I.3
The biosynthetic gene clusters of novobiocin (nov), clorobiocin (clo) and coumermycin A_1 (cou)

2.3. Biosynthesis of coumermycin A_1

Coumermycin A_1 consists of three structural moieties, i.e. the 3-amino-4,7-dihydroxy-coumarin moiety, the central 3-methylpyrrole-2,4-dicarboxylic acid moiety and a substituted deoxysugar moiety (**Figure I.2**).

The genes *couHIJK* are responsible for the biosynthesis of the aminocoumarin moiety (Chen & Walsh, 2001; Pacholec *et al.*, 2005a), which is derived from L-tyrosine, as was demonstrated by feeding experiments with isotope-labeled precursors (Bunton *et al.*, 1963). The small gene *couY*, which is located directly upstream of *couH*, shows sequence similarity to *mbtH* from the mycobactin gene cluster of *Mycobacterium tuberculosis* (Baltz, 2011; Quadri *et al.*, 1998) and is probably involved in the adenylation of L-tyrosine catalyzed by CouH (Baltz, 2011; Wolpert *et al.*, 2007; Zhang *et al.*, 2010). The origin of the heterocyclic oxygen atom is yet unknown, although Bunton *et al.* (1963) provided evidence that it is derived from the carboxyl group of L-tyrosine. However, Holzenkämpfer and Zeeck (2002) later showed for simocyclinone that it originated from molecular oxygen, which contradicts the earlier finding. The position 8 of the aminocoumarin ring is methylated by the methyltransferase CouO (**Figure I.4**) (Pacholec *et al.*, 2005b).

The deoxysugar moiety, a 5-*C*-methyl-L-rhamnose, is derived from glucose-1-phosphate and the additional methyl group at C-5 from S-adenosylmethionine (Birch *et al.*, 1960). The genes responsible for the biosynthesis of this moiety are *couSTUVW* (Heide, 2009), which are located at the right border of the cluster (**Figure I.3**). The activated deoxysugar moiety is transferred to the 7-hydroxy group of the aminocoumarin ring by the glycosyl transferase CouM. Methylation of the 4-hydroxy group, which is catalyzed by CouP (Li *et al.*, 2002), and acylation of the 3-hydroxy group with a 5-methylpyrrole-2-carboxylic acid moiety occur only after glycoside formation (**Figure I.4**) (Heide, 2009).

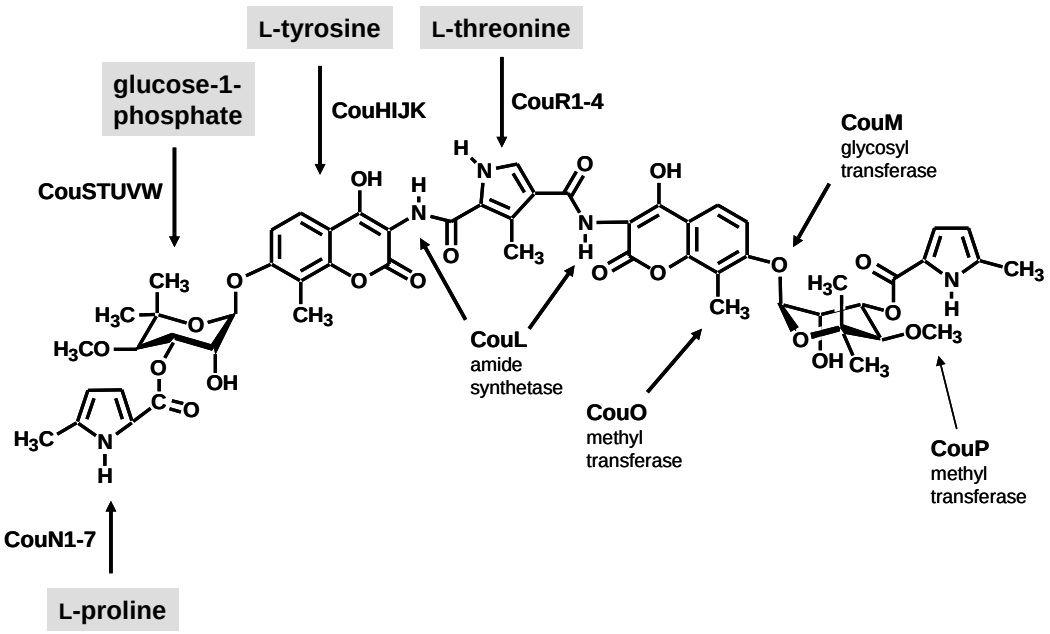


Figure I.4
Structure of coumermycin A_1 and function of the gene products of *couHIJKLMNOPRSTUVW* in coumermycin A_1 biosynthesis

Coumermycin A_1 contains three pyrrole moieties, i.e the central 3-methylpyrrole-2,4-dicarboxylic acid moiety and two terminal 5-methylpyrrole-2-carboxylic acid moieties. The terminal pyrrole moieties, which are also present in clorobiocin, are derived from L-proline (Scannell & Kong, 1969). In the first reaction step proline is activated by thioester formation with an acyl carrier protein and then subsequently oxidized to the pyrrole, methylated by a cobalamine-dependent methyltransferase and transferred to the deoxysugar moiety in a two-step acyl transfer process. This reaction sequence is catalyzed by the gene products of the seven genes *couN1-7* (Balibar *et al.*, 2007; Fridman *et al.*, 2007; Garneau-Tsodikova *et al.*, 2006; Garneau *et al.*, 2005). However, these genes are not responsible for the biosynthesis of the central pyrrole moiety, which is derived from L-threonine rather than from L-proline, as demonstrated in this thesis. The genes responsible for the biosynthesis are *couR1-4* (**Figure I.4**). The formation of the amide bonds between the central pyrrole moiety and the aminocoumarin moieties is catalyzed by the amide synthetase CouL (Schmutz *et al.*, 2003b).

2.4. Mechanism of action and resistance

Novobiocin, clorobiocin and coumermycin A_1 are all potent inhibitors of the bacterial DNA gyrase (Maxwell, 1999) and show a considerably higher affinity for this enzyme than the fluoroquinolones (Maxwell & Lawson, 2003). However, the affinity of coumermycin A_1 towards intact gyrase is higher than that of any other aminocoumarin antibiotic. A 50 % inhibition of gyrase was reported to occur at a concentration of 4 nM, in comparison to 100 nM for novobiocin (Peng & Marians, 1993b).

Bacterial DNA gyrase is a type II topoisomerase, which uniquely catalyses the negative supercoiling of closed-circular DNA using the free energy of ATP hydrolysis. Its unique presence in prokaryotes makes it an attractive drug target (Gormley *et al.*, 1996; Tsai *et al.*, 1997). DNA gyrase is composed of two subunits, GyrA and GyrB, forming an A_2B_2 heterotetramer (Maxwell, 1999; Schmutz *et al.*, 2004). GyrA is involved in the DNA breakage-resealing reactions while GyrB catalyzes ATP hydrolysis (Maxwell, 1999). Topoisomerase IV is another type II topoisomerase which is target by the aminocoumarin antibiotics (Peng & Marians, 1993b). Its primary role is the decatenation of daughter chromosomes following DNA replication. Like gyrase it consists of two subunits, ParC and ParE, forming a C_2E_2 heterotetramer, with ParE containing the nucleotide binding site and ParC being responsible for DNA breakage and reunion (Hardy & Cozzarelli, 2003; Peng & Marians, 1993a).

Aminocoumarin antibiotics target the B subunit of gyrase and were shown to be competitive inhibitors of the ATP hydrolysis reaction, although they show little structural resemblance with ATP (Maxwell, 1993; Tsai *et al.*, 1997). X-ray christallography studies with an 24 kDa N-terminal fragment of GyrB from *Escherichia coli* in complex with the non-hydrolysable ATP analogue ADPNP (5'-adenylyl-β,γ-imidodiphosphate), novobiocin (Lewis *et al.*, 1996) and clorobiocin (Tsai *et al.*, 1997), respectively, revealed that the deoxysugar moiety of the aminocoumarins overlaps with the binding site for the adenine ring of ATP. While the substituted deoxysugar and the aminocoumarin ring are essential for the inhibition of gyrase, the 3-dimethylallyl-4-hydroxybenzoate moiety is not required, though it may influence the uptake of the compounds into the cells (Maxwell & Lawson, 2003). The

novobiocin and clorobiocin binding site shows a network of hydrogen bonds and a number of hydrophobic interactions. Key hydrogen bonds are those between Arg136 and the aminocoumarin ring, Asn46 and the 2-hydroxyl group of the deoxysugar moiety, and between Asp73 and the 3-acyl group of the deoxysugar moiety (Lewis *et al.*, 1996; Maxwell & Lawson, 2003; Tsai *et al.*, 1997). In contrast to novobiocin and clorobiocin, coumermycin A_1 was shown to stabilize a dimer of a 43 kDa N-terminal fragment of GyrB, which is consistent with its pseudo-dimeric structure (**Figure I.2**) (Gormley *et al.*, 1996; Maxwell & Lawson, 2003).

Simocyclinone also is a potent inhibitor of DNA gyrase, although with a different mode of action. Instead of inhibiting the ATPase reaction of GyrB it prevents the binding of the enzyme to DNA (Flatman *et al.*, 2005). Rubradirin is structurally related to the ansamycin family of antibiotics. Most members of this group act as potent inhibitors of the bacterial RNA polymerase (RNAP), e.g. the rifamycins. However, intact rubradirin was shown to inhibit the bacterial protein synthesis rather than RNAP, although its aglycone is a potent inhibitor of this enzyme (Reusser, 1979).

Analysis of aminocoumarin-resistant bacterial strains from several species led to the identification of point mutations in the N-terminal domain of GyrB that cause aminocoumarin-resistance. The most frequently mutated residue was an arginine (Arg136 in *E. coli* GyrB), which was found to form a hydrogen bond with the aminocoumarin moiety (Maxwell, 1997; Maxwell & Lawson, 2003). The accumulation of mutations in both *gyrB* and *parE* was shown to be associated with high-level resistance to novobiocin in *S. aureus*. However, mutations in *parE* only occured in mutants that had previously acquired mutations in *gyrB*, indicating that gyrase is the primary target of novobiocin (Fujimoto-Nakamura *et al.*, 2005). Another mechanism that contributes to bacterial antibiotic resistance are multidrug efflux pumps, which are able to recognize a large number of compounds other than their natural substrates. Aminocoumarin antibiotics are recognized by several of them (Nikaido, 1998), e.g. by the multidrug transporter ABC (Nagakubo *et al.*, 2002).

2.5. Clinical applications

The aminocoumarin antibiotics are active against Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA). However, due to their toxicity in eukaryotes, their poor solubility in water, preventing the development of parenteral dosage forms, and their low activity against Gram-negative bacteria, their clinical use remains limited (Maxwell, 1993). In contrast to clorobiocin and coumermycin A_1, novobiocin (Albamycin®, Pharmacia & Upjohn) was licensed for clinical use in the USA. It was initially approved by FDA in 1964 for the treatment of “serious infections due to susceptible strains of *S. aureus* when other less toxic antibiotics e.g. penicillins, vancomycin and tetracyclines, cannot be used” (FDA, 2011) and its efficacy was shown in several preclinical and clinical trials (Arathoon *et al.*, 1990; Raad *et al.*, 1998; Walsh *et al.*, 1993). However, in 2009 FDA withdraw its approval for reasons of safety or effectiveness after Pharmacia & Upjohn (now Pfizer, Inc.) already stopped production in 1999. Among the safety concerns reported were adverse reactions (e.g. skin reactions, hepatic failure and blood dyscrasias), the development of novobiocin-resistance during treatment, and the potential for drug interactions (FDA, 2011).

Further studies showed that the aminocoumarin antibiotics act synergistically with certain antitumor drugs, e.g. with the topoisomerase II inhibitors etoposide and teniposide. It was demonstrated that novobiocin enhances the cytotoxicity of etoposide and increases the formation of drug-stabilized topoisomerase II-DNA complexes in several drug-sensitive and -resistant tumor cell lines. Although novobiocin was shown to inhibit topoisomerase II at higher concentrations, the mechanism involved is more likely the inhibition of etoposide efflux resulting in a higher intracellular concentration of this drug (Rappa *et al.*, 1993; Rappa *et al.*, 2000a; Rappa *et al.*, 2000b).

The eukaryotic heat shock protein 90 (Hsp90) plays a key role in the stability and function of multiple cell-signalling molecules including several oncogenic tyrosine and serine-threonine kinases. It is therefore considered as a novel target for anticancer drugs (Marcu *et al.*, 2000). The aminocoumarins, novobicin, clorobiocin and coumermycin A_1 respectively, were shown to bind to the C-terminal part of Hsp90 and thereby interfere with its chaperon function. Subsequently, tumor cells were depleted of a series of Hsp90-dependent signalling proteins, e.g. Raf-1 (Allan *et al.*, 2006; Marcu *et al.*, 2000). However, the affinity of novobiocin for Hsp90 is poor (IC_{50} 700 µM) and therefore, several analogs of novobiocin and coumermycin A_1 with higher affinity and selectivity for Hsp90 were generated in the last years (Burlison & Blagg, 2006; Burlison *et al.*, 2006).

Recently, coumermycin A_1 was also shown to possess potent antiretroviral activity. It impairs viral gene expression in acutely infected cells probably by preventing Hsp90 dimerization and is additionally able to inhibit HIV-1 integration by targeting the capsid protein. Novobiocin also shows some antiretroviral activity but lower than that of coumermycin A_1 (Vozzolo *et al.*, 2010).

3. Liponucleoside antibiotics

The caprazamycins (CPZ) and liposidomycins (LPM) are liponucleoside antibiotics of unusual complexity that inhibit the bacterial peptidoglycan synthesis. The liposidomycins were isolated in 1985 from a culture broth of *Streptomyces griseosporeus* and, based on the HPLC profile, consisted of at least 12 compounds (Isono *et al.*, 1985; Ubukata *et al.*, 1988). The caprazamycins are produced by *Streptomyces sp*. MK730-62F2 and were identified in 2003 in a screening for new anti-mycobacterial antibiotics (Igarashi *et al.*, 2003). Recently, the gene clusters of caprazamycins (Kaysser *et al.*, 2009) and liposidomycins (Kaysser *et al.*, 2010a), respectively, were identified by our group.

3.1. Chemical structure

The core structure of both liposidomycins and caprazamycins is the (+)-caprazol (**Figure I.5**), consisting of a 5'-substituted uridine, a 5-amino-5-deoxyribose and a *N*-methylated perhydro-1,4-diazepine moiety. Fatty acids of different chain lengths and conformations are attached to the 3'''-OH group of the diazepanone ring and distinguish the different liposidomycins and caprazamycins, respectively. Attached to the β-hydroxy group of the fatty acid side chain is a rare 3-methylglutaryl (3-MG)

moiety which, in case of the caprazamycins, is linked to a permethylated L-rhamnose. A sulfate group at the 2''-position of the aminoribose is the only other difference between the liposidomycins and the caprazamycins (**Figure I.5**). The muraymycins (McDonald *et al.*, 2002) and FR-900493 (Ochi *et al.*, 1989) also exhibit the 5'-β-*O*-aminoribosyl-5'-*C*-glycyluridine core moiety contained in the caprazamycins and liposidomycins.

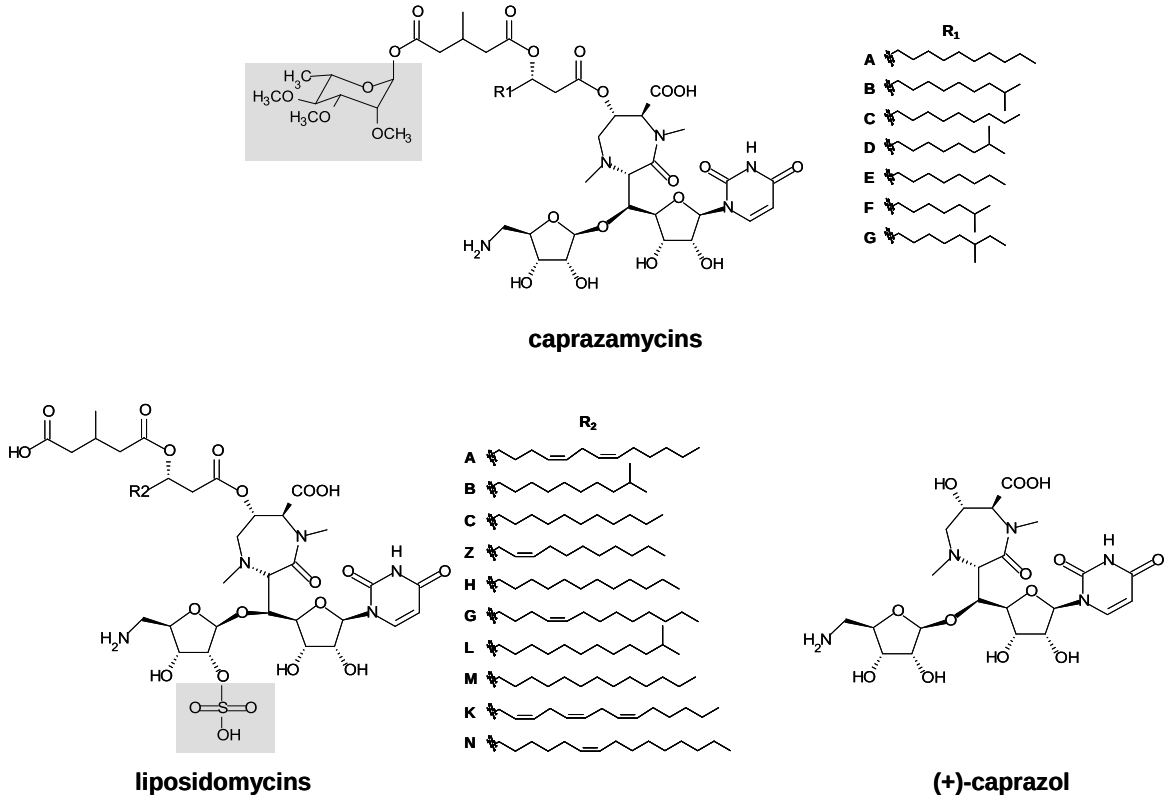


Figure I.5
Chemical structures of caprazamycins, liposidomycins and (+)-caprazol. The structural differences between caprazamycins and liposidomycins are shaded in grey.

3.2. Mechanism of action and bioactivity

The biosynthesis of peptidoglycan, a major component of the bacterial cell wall, is essential for all bacteria and therefore represents one of the most important targets for antibiotics. It consists of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units, that are *β*-1,4-glycosidically linked. Attached to the D-lactoyl group of MurNAc is a pentapeptide side chain, which consists of L-alanine, γ-D-glutamate, L-lysine or *meso*-diaminopimelic acid (DAP), and two D-alanines. Cross-linking of the peptidoglycan generally occurs between the carboxyl group of the D-alanine at position 4 and the amino group of lysine/DAP at position 3 (Barreteau *et al.*, 2008; Kimura & Bugg, 2003). The assembly of peptidoglycan involves several steps and takes place in different compartments of the cell, i.e. in the cytoplasm and at the inner and outer side of the cytoplasmatic membrane. The first step is the synthesis of the nucleotide precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, which takes place in the cytoplasm. Subsequently, the phospho-

MurNAc-pentapeptide moiety is transferred from UDP to the membrane acceptor undecaprenyl phosphate (C_{55}-P), generating lipid I. This reaction is catalysed by the translocase I MraY. A second transferase, MurG, then catalyzes the transfer of GlcNAc to lipid I yielding lipid II. After its passage through the membrane, by a yet unknown mechanism, lipid II is used as the substrate for the polymerization reaction (**Figure I.6**) (Al-Dabbagh *et al.*, 2008; Bouhss *et al.*, 2008).

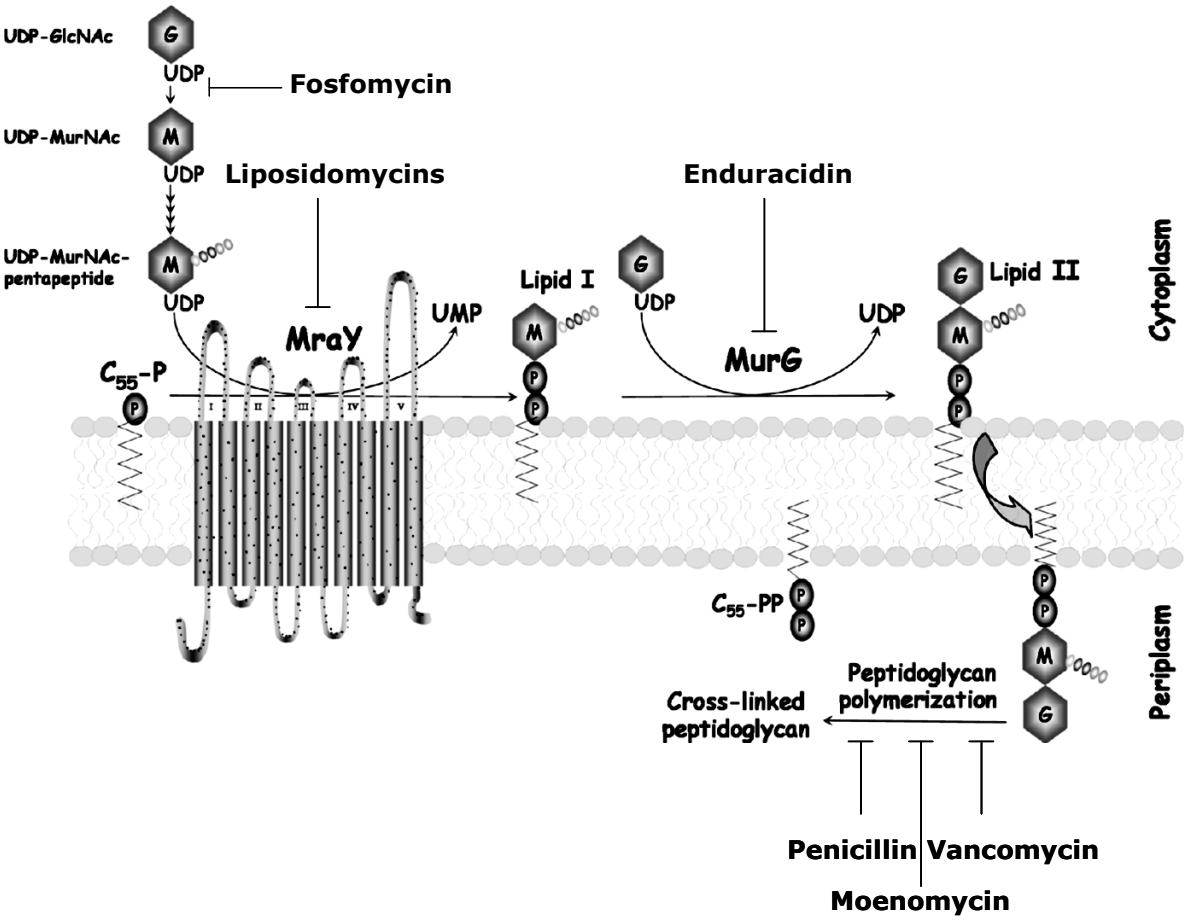


Figure I.6
Biosynthesis of the bacterial cell wall and some antibiotics inhibiting it. Modified from Al-Dabbagh *et al.* (2008).

The liposidomycins are potent inhibitors of the peptidoglycan biosynthesis (ID_{50} 0.03 µg/mL) and were shown to target translocase I with high specificity (Isono *et al.*, 1985; Kimura *et al.*, 1989). Eukaryotic glucoconjugate formation, in comparison, was only inhibited at high dosages (Muroi *et al.*, 1997) and no toxic effects of the liposidomycins were observed in mice after intravenous administration of 180 mg/kg (Isono *et al.*, 1985). Although their in vitro activity is high, the antibacterial activity of the liposidomycins is limited, with *Mycobacterium phlei* (MIC 0.16 µg/mL) being the most sensitive bacterium (Isono *et al.*, 1985; Kimura *et al.*, 1989). Kimura and co-workers (Kimura *et al.*, 1998a; 1998b; 1998c) suggested that, due to the presence of the hydrophilic sulfate group, the permeability of the liposidomycins is limited. Accordingly, its removal resulted in an improved antimicrobial activity, while the in vitro activity remained nearly the same (Kimura *et al.*, 1998a; 1998b; 1998c). Further studies indicate that the 3''-OH group (Dini *et al.*, 2001b), the amino group of the

aminoribose and an intact uracil moiety (Dini *et al.*, 2001a) are essential for the inhibition of *Escherichia coli* translocase I. The fatty acid side chain was suggested to play a role in the penetration of the bacterial cell (Hirano *et al.*, 2008).

The caprazamycins resemble the liposidomycins and were therefore also assigned to the translocase I inhibitor family (Kimura & Bugg, 2003). They show excellent activity in vitro against Gram-positive bacteria, especially against Mycobacteria, e.g. *M. tuberculosis* (MIC 0.78 – 1.56 µg/mL), *M. avium* (MIC <0.025 – 0.1 µg/mL) and *M. intracellulare* (MIC 0.78 – 1.56 µg/mL) (Takeuchi *et al.*, 2001). In a pulmonary mouse model with *M. tuberculosis* H37Rv, caprazamycin B showed a therapeutic effect at 1.5 mg/kg/day but no significant toxicity was observed (Igarashi *et al.*, 2002).

The peptidyl nucleoside antibiotics, e.g. the mureidomycins, pacidamycins and napsamycins, and the tunicamycin-type liponucleoside antibiotics, e.g. the tunicamycins, streptoviridins and corynetoxins, also belong to the translocase I inhibitor family (Kimura & Bugg, 2003).

4. Aims of this study

The main objectives of this study were:

1) To establish 24-square deepwell plates with sandwich covers in our laboratory as a new cultivation system for *Streptomyces*, which shows a reduced variability of the production rates in comparison to Erlenmeyer flasks.

Investigations on antibiotic formation in streptomycetes are often hampered by the notorious variability of production rates, observed between different cultivation batches, in Erlenmeyer flasks, and therefore a cultivation method that shows less variability in secondary metabolite production could probably reduce the workload and cost of research in antibiotic biosynthesis significantly. In order to test whether the variability of antibiotic production in *Streptomyces* can be reduced by cultivation in 24-square deepwell plates (Duetz, 2007), novobiocin production in the heterologous producer strain *S. coelicolor* M512(novBG01) was investigated in this new cultivation system. To obtain similar production rates as in the Erlenmeyer flasks as well as a low variability, it was necessary to optimize the inoculation method, to prepare frozen inoculum either from the early stationary phase or from a defined section of the growth phase and to test the influence of aeration and of a siloxylated ethylene oxide/propylene oxide copolymer as artificial oxygen carrier as well as the overexpression of the pathway-specific positive regulator NovG.

For the production of coumermycin A_1 and its derivatives in the 24-square deepwell plates, it was necessary to optimize the inoculation ration and to test different production media, since the originally used SK medium was not suitable, due to the lard oil contained therein.

2) To show that the genes *couR1-4*, but not *couN1-7*, are involved in the biosynthesis of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety of coumermycin A_1.

Coumermycin A_1 contains two types of pyrrole moieties, i.e. two terminal 5-methylpyrrole-2-carboxyl moieties and the central 3-methylpyrrole-2,4-dicarboxylic acid moiety. While the biosynthesis of the terminal pyrrole moieties from L-proline by action of CouN1-7 is well investigated, the biosynthetic pathway to the central pyrrole moiety is still unknown. Deletion of the genes *couN1-7* together with *couM*, coding for the glycosyl transferase responsible for the attachment of the deoxysugar moiety to the aminocoumarin ring, was attempted to show whether these genes are also involved in the biosynthesis of the central pyrrole moiety.

The genes *couR1-4* within the coumermycin gene cluster have no orthologs in the gene clusters of novobiocin and clorobiocin and are not yet

functionally assigned. In order to prove that these genes are involved in the biosynthesis of coumermycin A_1, *couR1-couR4* were deleted separately on cosmid couMW16 by RED/ET mediated recombination. This work was carried out by Anna Knuplesch and is described in her diploma thesis. We now attempted heterologous expression of the resulting cosmids couAK06-couAK10 in *S. coelicolor* M1146 followed by complementation and feeding of 3-methylpyrrole-2,4-dicarboxylic acid, to prove that all five genes are not only involved in the biosynthesis of coumermycin A_1 but specifically in the biosynthesis of the central pyrrole moiety.

3) To prove that L-threonine is one of the precursors of the central pyrrole moiety of coumermycin A_1 and that the intact L-threonine molecule is incorporated.

CouR3 shows similarity to the L-threonine kinase PduX from *Salmonella enterica* and therefore the first step in the biosynthesis of the central pyrrole moiety may be the phosphorylation of L-threonine catalysed by CouR3. Consequently, we attempted to show that CouR3 acts as a L-threonine kinase by feeding of *O*-phospho-L-threonine to a *couR3*-defective mutant.

In order to prove that L-threonine is one of precursor of the central pyrrole moiety a feeding experiment with [U-^{13}C,^{15}N]L-threonine followed by LC-MS and ^{13}C-NMR analysis was attempted. The identification of the second precursor necessitates an additional feeding experiment and therefore feeding of [U-^{13}C]fumaric acid was tried. The NMR analysis was conducted in cooperation with Nadja Burkard and Prof. Stephanie Grond (Organic chemistry department, University of Tübingen, Germany).

4) To establish a LC/ESI-MS/MS method for the identification of caprazamycins and liposidomycins in culture extracts of the respective producer strains and to adapt a chromatographic method using a boronic acid stationary phase for the partial purification of caprazamycins and liposidomycins

The identification of caprazamycins, liposidomycins, and derivatives thereof, e.g. resulting from gene inactivations, requires a rapid and precise analytical method that also supplies sufficient structural information. High-performance liquid chromatography (HPLC) coupled to a triple-stage quadrupole mass spectrometer (MS) is capable of both, separating complex mixtures of secondary metabolites and providing reliable information on the analyte's mass and structure. In order to get as much structural information as possible LC/ESI-MS/MS analysis was attempted in positive as well as in negative ionization mode followed by the assignment of characteristic fragment ions.

Affi-Gel® boronate is a boronic acid-containing gel that specifically binds cis-diol structures, like the ribose of nucleosides, at alkaline pH values by formation of a cyclic boronate ester. At acidic pH values the ester bond is

cleaved resulting in the elution of the cis-diol-containing compounds. Since the liponucleoside antibiotics also contain a nucleosidic structure, purification was attempted using a method originally developed for the purification of modified nucleosides from cell cultures.

5) To identify new caprazamycins in the culture extracts of *Streptomyces sp.* MK730-62F2.

Previously, 27 liposidomycin derivatives containing 13 different fatty acid side chains have been isolated from *Streptomyces sp.* SN-1061. In contrast, only seven caprazamycins were reported from *Streptomyces sp*. MK730–62F2. Since both gene clusters are highly conserved and do not contain possible genes for fatty acid biosynthesis, the fatty acid moiety of the liponucleoside antibiotics is most likely provided by the primary metabolism of the host strain. We therefore attempted to identify new caprazamycins in the culture extracts of *Streptomyces sp*. MK730-62F2 by precursor ion scans in positive and negative mode, respectively. This method allows the identification of new derivatives from a known structural class of compounds by scanning for product ions that are characteristic for the whole class.

II. MATERIALS AND METHODS

1. Chemicals, materials for chromatography and enzymes

1.1. Chemicals

Chemicals used in this thesis were obtained mainly from Fluka (Ulm), Merck (Darmstadt), Roth (Karlsruhe) and Sigma-Aldrich (Taufkirchen), media components mainly from Becton-Dickinson (Heidelberg). Special chemicals and media components are listed in Table II.1.

Table II.1 Special chemicals and media components

Supplier	Chemical / Media component
Becton-Dickinson, Heidelberg, Germany	Malt extract Proteose Pepton No. 3 Soytone Soluble starch Tryptone Tryptone Soya Broth (TSB) Yeast extract
Biozyme Scientific GmbH, Oldendorf, Germany	Agarose
Calbiochem-Novabiochem, Bad Soden, Germany	Thiostreptone
CGeneTech, Indianapolis, USA	3-methylpyrrole-2,4-dicarboxylic acid diethyl ester
Dow Corning, Auburn, USA	siloxylated ethylene oxide/propylene oxide copolymer Q2-5247
Eurisotop, Saarbrücken, Germany	[U-^{13}C] fumaric acid
Fluka, Ulm, Germany	Novobiocin
Fisher Scientific, Schwerte, Germany	Acetonitrile LCMS grade Methanol LCMS grade
Genaxxon Biosciences, Biberach, Germany	Apramycin Chloramphenicol Kanamycin
Merck, Darmstadt, Germany	Ethanol D-Maltose Methanol
Roth, Karlsruhe, Germany	Agar Bromphenol blue Carbenicillin EDTA Formic acid Glacial acetic acid Glucose Glycerol Mannitol Phenol/Chloroform/Isoamyl alcohol (25:24:1) Phosphorus pentoxide L-Proline SDS

Supplier	Chemical / Media component
Sigma-Aldrich, Taufkirchen, Germany	Acetonitrile HPLC grade Brewers yeast Coumermycin A_1 Dimethyl sulfoxide (DMSO) Lard oil Methanol HPLC grade Nalidixic acid [U-^{13}C,^{15}N]L-threonine Tris base
SOBO Naturkost, Köln, Germany	Soy flour
Südzucker, Mannheim, Germany	Sucrose
VWR, Darmstadt, Germany	Acetonitrile HPLC grade Methanol HPLC grade

1.2. Materials for chromatography

The liquid chromatography media were obtained as commercial columns or dry material. Columns and media were stored according to the manufacturers' instructions.

Table II.2 Materials for chromatography

Supplier	Medium
Bio-Rad, Munich, Germany	Affi-Gel® boronate (dry material)
C+S Chromatographie Service, Düren, Germany	Multoprep® RP18 – 25/40 µm (dry material)
Dr. Maisch GmbH, Ammerbuch, Germany	Reprosphere® C18-DE (commercial column, 5 µm, 150 x 3 mm) Reprosphere® C18-DE (commercial column, 5 µm, 150 x 4.6 mm) Reprosil-Pur® Basic C18 (commercial column, 5 µm, 250 x 2 mm)
Ziemer Chromatographie, Langerwehe, Germany	Multosphere® 120 RP18 (commercial column, 5 µm, 250 x 8 mm)

1.3. Enzymes and kits

Enzymes and kits used in this study are listed in Table II.3 and were stored according to the manufacturers' instructions.

Table II.3 Enzymes and kits

Supplier	Enzymes and kits
Fermentas	GeneRuler® 1kb DNA ladder
Fluka, Ulm, Germany	Lysozyme 85400 U/mg
Genaxxon Biosciences, Biberach, Germany	10 mM dNTP-mix (2.5 mM each)
New England Biolabs, Frankfurt, Germany	Restriction endonucleases T4 DNA Ligase
Quiagen, Hilden, Germany	RNase A 100 mg/mL QIAquick PCR Purification kit

2. Media, buffers and solutions

2.1. Media for bacterial cultivation

Unless stated otherwise, the media were prepared with distilled water and autoclaved for 20 min at 121°C. To obtain solid media, 2% (w/v) agar was added before autoclaving. All recipes are given for 1 L of final volume. If necessary, supplementary components like antibiotics or other heat labile substances were added to the media as sterile solutions (filtered through 0.22 µm pore sized filters) at the time of use. The media were stored at 4°C or at room temperature.

2.1.1. Cultivation of *E. coli*

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

NaCl	10.0 g
Tryptone	10.0 g
Yeast extract	5.0 g

The components were dissolved in 1 L water, adjusted to pH 7.0 and sterilized by autoclaving.

2.1.2. Cultivation of *Streptomyces*

YMG (Yeast-Malt-Glucose) medium

Yeast extract	4.0 g
Malt extract	10.0 g
Glucose	4.0 g

The components were dissolved in 800 mL water, filled up to 1 L after the pH was adjusted to 7.3 and sterilized by autoclaving.

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

Tryptone Soya Broth	30.0 g

The component was dissolved in 1 L water and sterilized by autoclaving.

2 x YT medium (Kieser *et al.*, 2000)

Tryptone	16.0 g
Yeast extract	10.0 g
NaCl	5.0 g

The components were dissolved in 1 L water and sterilized by autoclaving

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

Mannitol	20.0 g
Soya flour	20.0 g
Agar	20.0 g

The mannitol was dissolved in 1 L tap water and 100 mL were poured into each flask containing 2 g agar and 2 g soya flour. The medium was sterilized twice by autoclaving (115°C, 15 min) with gentle shaking of the medium between the two runs.

2.1.3. Media for secondary metabolite production

2.1.3.1. Media for novobiocin production

CDM (Chemical Defined Medium) (Kominek, 1972)

tri-sodium citrate · $2H_2O$	6.0 g
L-proline	6.0 g
K_2HPO_4 · $3H_2O$	2.0 g
$(NH_4)_2SO_4$	1.5 g
NaCl	5.0 g

The components were dissolved in 800 mL water and adjusted to pH 7.2

$MgSO_4$ · $7H_2O$	2.05 g
$CaCl_2$ · $2H_2O$	0.4 g
$FeSO_4$ · $7H_2O$	0.2 g
$ZnSO_4$ · $7H_2O$	0.1 g
Glucose 30% (w/v)	100 mL

The components, except the glucose solution, were dissolved in the first solution and water was added to 900 mL. The pH was again adjusted to 7.2 and medium and glucose solution were sterilized by autoclaving and mixed prior to use.

2.1.3.2. Media for coumermycin A_1 production

SK medium (Scannell & Kong, 1969)

Soluble starch	20.0 g
Pharma medium	20.0 g
Lard oil	20.0 g
Yeast extract	4.0 g
K_2HPO_4 · $3\ H_2O$	2.5 g
$CaCl_2$ · $2\ H_2O$	0.5 g
$CoCl_2$ · $6\ H_2O$	200 µg

The components were dissolved in 1 L water and sterilized by autoclaving.

Coumermycin production medium 1 (Weinstein *et al.*, 1978)

Soluble starch	60.0 g
Brewers yeast	30.0 g
$CaCO_3$	10.0 g

The components were dissolved in 1 L water and sterilized by autoclaving.

Coumermycin production medium 2 (Kawaguchi *et al.*, 1965)

Glucose	30.0 g
Pharma medium	35.0 g
Glycerol 86%	2.3 g
Yeast extract	2.0 g
$K_2HPO_4 \cdot 3\ H_2O$	5.0 g
KCl	2.0 g
$CaCO_3$	8.0 g
$CoCl_2 \cdot 6\ H_2O$	200 µg

The components were dissolved in 1 L water and sterilized by autoclaving.

2.1.3.3. Media for caprazamycin and liposidomycin production

P medium (Kaysser *et al.*, 2009)

Soytone	10.0 g
Soluble starch	10.0 g
D-maltose	20.0 g
Trace elements solution	5.0 mL

The components were dissolved in water, filled up to 1 L after adjustment to pH 6.7 and sterilized by autoclaving.

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
$Na_2B_4O_7 \cdot 10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10 mg

The components were dissolved in 1 L water and sterilized by autoclaving

2.2. Antibiotic solutions

The antibiotics were dissolved in an appropriate solvent and the stock solutions were kept at -20°C. Aqueous solutions were sterilized by filtration through 0.22 µm pore size filters, solutions in ethanol and DMSO are autosterile. The required antibiotics were added to the media (RT to 60°C) in the appropriate concentration.

Table II.4 Antibiotic solutions

Antibiotic	Concentration in		Solvent
	Stock solution (mg/mL)	Medium (µg/mL)	
Apramycin	50	15-50[a]	H_2O
Chloramphenicol	15-50	25-50	ethanol
Nalidixic acid	25	25	0.3 M NaOH
Kanamycin	50	15-50[a]	H_2O
Thiostrepton	50	15-50[a]	DMSO

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

2.3. Buffers and solutions

Unless stated otherwise, buffers and solutions were prepared with distilled water, autoclaved (121°C, 20 min) and stored at room temperature.

2.3.1. Buffers and solutions for DNA isolation

Buffers and solutions for the isolation of plasmids and cosmids from *E. coli* are listed in Table II.5, the buffers and solutions required for the isolation of genomic DNA from *Streptomyces* in Table II.6.

Table II.5 Buffers and solutions for plasmid and cosmid isolation from *E.coli*

Buffer	Components	Final concentration	Preparation
MP 1	Glucose	50 mM	Adjust to pH 8.0. Add RNase A just before use
	Tris-HCl	25 mM	
	EDTA	10 mM	
	RNase A	100 µg/mL	
MP 2	NaOH	0.2 N	
	SDS	1% (w/v)	
MP 3	$K(CH_3COO)$	3 M	Adjust to pH 4.8. Store at 4°C

Table II.6 Buffers for isolation of genomic DNA from *Streptomyces*

Buffer	Components	Final concentration	Preparation
TSE buffer	Sucrose Tris-HCl EDTA	10.3% 25 mM 25 mM	Adjust to pH 8.0
TE buffer	Tris-HCl EDTA	10 mM 1 mM	Adjust to pH 7.5
Lysozyme solution	TSE buffer Lysozyme RNase A	- 3 mg/mL 100 µg/mL	Add RNase A and lysozyme just before use
SDS solution	SDS	2% (w/v)	
NaOAc solution	$Na(CH_3COO)$	3 M	Adjust to pH 4.8
Tris buffer	Tris-HCl	10 mM	Adjust to pH 8.0

2.3.2. Buffers for DNA gel electrophoresis

Table II.7 Buffers for DNA gel electrophoresis

Buffer	Components	Final Concentration	Preparation
50x TAE	Tris base EDTA Glacial acetic acid	2 M 0.05 M 57.1 mL/L	Adjust to pH 8.0 with glacial acetic acid
Loading buffer	Glycerol Bromphenol blue	30% (w/v) 0.25% (w/v)	Store at 4°C
Ethidium bromide solution	Ethidium bromide	1 mg/L	

2.3.3. Buffers and solutions for affinity chromatography with Affi-Gel® boronate

Table II.8 Buffers and solutions for affinity chromatography with Affi-Gel® boronate

Buffer	Components	Final Concentration	Preparation
NH_4OAc buffer	$NH_4(CH_3COO)$	0.25 M	Adjust to pH 8.8 with NH_3 25%
Formic acid solution	$MeOH/H_2O$ 1:1 (v/v) Formic acid	- 0.2 M	
NaCl solution	NaCl	0.1 M	

3. Plasmids, cosmids, bacterial strains and primers

3.1. Vectors, plasmids and cosmids

All vectors, plasmids and cosmids used in this study are listed with their respective sources in Table II.9.

Table II.9 Vectors, plasmids and cosmids

Name	Description	Source or reference
Vectors		
pUWL-Apra-oriT	*E. coli* - *Strepromyces* shuttle vector, *oriT*, *ermE** promoter; $Apra^R$, $Carb^R$	Andrease Günther, Albert Ludwigs Universität Freiburg
pWHM3	*E. coli* - *Strepromyces* shuttle vector, *lacZα*; Amp^R, $Thio^R$	(Vara *et al.*, 1989)
Plasmids		
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)
pIJ773	*aac(3)IV* ($Apra^R$), *oriT*, FRT	(Gust *et al.*, 2003)
pAE12	1.35 kb *Pst*I-*Sph*I fragment of pAE11 cloned into the same sites of pWHM3, containing *novG*; Amp^R $Thio^R$	(Eustáquio, 2004)
pAK06	1.4 kb *Hind*III/*Spe*I fragment of pAK01 cloned into the same sites of pUWL-Apra-oriT, containing *couR1*; $Apra^R$	(Knuplesch, 2008)
pAK10	1.2 kb *Hind*III/*Spe*I fragment of pAK05 cloned into the same sites of pUWL-Apra-oriT, containing *couR4*; $Apra^R$	(Knuplesch, 2008)
Cosmids		
novBG01	From cosmid 10-9C, *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ΦC31); Kan^R.	Bertolt Gust (Eustáquio *et al.*, 2004)
couMW16	Integrative cosmid with complete coumermycin A_1 gene cluster; *attP*, *int*, *tet*, *oriT*; Kan^R, $Carb^R$	(Wolpert *et al.*, 2008)
couMW22	From couMW16, *couM-couN7* (position 10985-19284 in AF235050) replaced by the apramycin resistance cassette from pIJ773; $Apra^R$, Kan^R	Final report of Manuel Wolpert
couSS01	From couMW22, apramycin resistance cassette removed by *Xba*I digestion and religation; Kan^R	This thesis
couAK06	From couMW16, *couR1* (position 21980-22753 in AF235050) replaced by the apramycin resistance cassette from pIJ773; resistance cassette removed by *Xba*I/*Spe*I digestion and religation; Kan^R	(Knuplesch, 2008)
couAK07	From couMW16, *couR2a* (position 23206-23472 in AF235050) replaced by the apramycin resistance cassette from pIJ773; resistance cassette removed by *Xba*I/*Spe*I digestion and religation; Kan^R	(Knuplesch, 2008)

Name	Description	Source or reference
couAK08	From couMW16, *couR2b* (position 23752-24056 in AF235050) replaced by the apramycin resistance cassette from PIJ773; resistance cassette removed by *Xba*I/*Spe*I digestion and religation	(Knuplesch, 2008)
couAK09	From couMW16, *couR3* (position 24248-25150 in AF235050) replaced by the apramycin resistance cassette from pIJ773; resistance cassette removed by *Xba*I/*Spe*I digestion and religation	(Knuplesch, 2008)
couAK10	From couMW16, *couR4* (position 25211-26374 in AF235050) replaced by the apramycin resistance cassette from pIJ773 ; resistance cassette removed by *Xba*I/*Spe*I digestion and religation	(Knuplesch, 2008)

3.2. Bacterial strains

The bacterial strains used in this study are listed with their respective sources in Table II.10.

Table II.10 *E. coli* and *Streptomyces* strains used in this study

Strain	Description	Source or reference
E.coli		
E. coli XL1 Blue MRF$^-$	General cloning host; *rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17, *sup*E44, *rel*A1, *lac* [F´, *pro*AB, *lacI*^{q}, ZΔM15, Tn10]; Tet^R	Stratagene
E. coli ET12567	DNA methylase negative strain (*dam*$^-$ *dcm*$^-$ *hsdM*); Tet^R, Cml^R	(MacNeil *et al.*, 1992)
Streptomyces		
S. coelicolor M512	*S. coelicolor* M145 derivative; Δ*redD,* Δ*actII-ORF4,* SCP1$^-$, SCP2$^-$	(Floriano & Bibb, 1996)
S. coelicolor M1146	*S. coelicolor* M145 derivative; SCP1$^-$, SCP2$^-$, Δ*act*, Δ*red*, Δ*cda*, Δ*cpk*	(Gomez-Escribano & Bibb, 2011)
S. coelicolor M512 (novBG01)	*S. coelicolor* M512 containing cosmid novBG01	(Eustáquio *et al.*, 2004)
S. coelicolor M512 (novBG01)/pWHM3	*S. coelicolor* M512(novBG01) containing the empty vector pWHM3; Kan^R, Thio^R	(Siebenberg *et al.*, 2010)
S. coelicolor M512 (novBG01)/pAE12	*S. coelicolor* M512(novBG01) containing the plasmid pAE12 (*novG*); Kan^R, Thio^R	(Siebenberg *et al.*, 2010)
S. coelicolor M1146 (couMW16)	*S. coelicolor* M1146 containing cosmid couMW16; Kan^R	(Flinspach *et al.*, 2010)
S. coelicolor M1146 (couSS01)	*S. coelicolor* M1146 containing cosmid couSS01 (Δ*couM-couN7*); Kan^R	This thesis
S. coelicolor M1146 (couAK06)	*S. coelicolor* M1146 containing cosmid couAK06 (Δ*couR1*); Kan^R	This thesis
S. coelicolor M1146 (couAK06)/pAK06	*S. coelicolor* M1146(couAK06) containing plasmid pAK06 (*couR1*); Kan^R, Apra^R	This thesis
S. coelicolor M1146 (couAK07)	*S. coelicolor* M1146 containing cosmid couAK07 (Δ*couR2a*); Kan^R	This thesis

Strain	Description	Source or reference
S. coelicolor M1146 (couAK09)	*S. coelicolor* M1146 containing cosmid couAK09 (Δ*couR3*); Kan^R	This thesis
S. coelicolor M1146 (couAK10)	*S. coelicolor* M1146 containing cosmid couAK10 (Δ*couR4*); Kan^R	This thesis
S. coelicolor M1146 (couAK10)/pAK10	*S. coelicolor* M1146(couAK10) containing plasmid pAK10 (*couR4*); Kan^R, $Apra^R$	This thesis
Streptomyces sp. MK730-62F2	caprazamycin producer; wildtype strain	acc. no. FERMBP7218 (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. FERMBP5800 (National Institute of Bioscience and Human Technology, Tsukuba, Japan)

3.3. PCR primers

All primers listed in Table II.11 were purchased from Eurofins MWG (Ebersberg, Germany).

Table II.11 Primers

Name	Sequence (5' → 3')[a]
Primers for the deletion of couM-couN7	
couM_fwd	CCG GAC GTG CTG GTG GAC AGG TGA GGT GAG TCA TTC GAT GTC TAG A**TG TAG GCT GGA GCT GCT TC**
couN7_rev	CGG ATT CCG TGG GCG TGA TGC CGG TCG GTT CCG GAT CTT AAC TAG T**AT TCC GGG GAT CCG TCG ACC**
Primers for testing of couSS01 (ΔcouM-couN7)	
couSS01_f	GTG GAT GCG TGC GTG TAC G
couSS01_r	CGA TGC GAT GGT GTG GAA GG
Primers for testing of couAK06 – couAK10 (ΔcouR1-ΔcouR4)	
kont_couR1_f	CGG GTA TGT GGA CCA CCC GG
kont_couR1_r	CGA CCG GTC GTC AGG ATC CG
kont_couR2a_f	CTG TTG CGC GGC GCC GAC TT
kont_couR2a_r	ACT CGG CGG CCA GCA ACA GC
kont_couR2b_f	ACG GTC ATC AGC GTC CGG GG
kont_couR2b_r	CAT CGC ACA TCC GGC GGA CC
kont_couR3_f	GCC GCC GAG TTC ACC GGG TT

Name	Sequence (5' → 3')[a]
Primers for testing of couAK06 – couAK10 (ΔcouR1-ΔcouR4) *(continuation)*	
kont_couR3_r	CGA TCC TGC TAC CGG CTG CG
kont_couR4_f	TGG ACC GGG GAT TCC GGC TG
kont_couR4_r	AGC GGG CTG GTG ATC GGA CC
Primers for amplification of couE, couO and gyrB (presence of coumermycin gene cluster)	
couE_fwd	ACT TCC GTC GGA AGC CGT CG
couE_rev	ACC GTG AGG ACC TTC CGC C
couO_fwd	GAG ACC TCC CTA CCG CGA AC
couO_rev	CCG ATG ACT CCT TCG ACG TC
gyrB_fwd	GAC TGG TTC GAC CGC AAC CC
gyrB_rev	CCG TCG AGC ACC ATC AGG TG

[a] the priming sequence for pIJ773 is shown in bold, *Spe*I (ACTAGT) and *Xba*I (TCTAGA) restriction sites are underlined

4. Culture Conditions

4.1. Cultivation of *E. coli*

E. coli strains were cultivated in liquid or on solid LB medium, supplemented with the appropriate antibiotic(s), at 37°C overnight (16-18 h). Standard methods for cultivation were performed as described by Sambrook & Russell (2001).

4.2. Cultivation of *Streptomyces*

4.2.1. General cultivation conditions

Streptomyces strains were cultivated either on MS agar or in liquid YMG or TSB medium. Cultivations in liquid media were carried out in 300 mL baffled Erlenmeyer flasks, containing a stainless steel spring and 50 mL of the respective medium, at 180-220 rpm and 30°C for 2 days. If required, an appropriate amount of antibiotic(s) was added to the medium.

4.2.2. Production of secondary metabolites

Cultivations were carried out either in 300 mL baffled Erlenmeyer flasks containing a stainless steel spring or in 24-square deepwell plates (Duetz *et al.*, 2000), without the addition of antibiotic(s).

4.2.2.1. Production of novobiocin

Cultivation in Erlenmeyer flasks

For novobiocin production, 1 mL of a YMG preculture of *S. coelicolor* M512(novBG01) was used to inoculate a 300 mL baffled Erlenmeyer flask containing 50 mL of CDM medium. The cultivation was carried out at 30°C and 200 rpm for 7 days.

Cultivation in 24-square deepwell plates

For cultivation in 24-square deepwell plates, 40 mL of CDM medium were mixed either with 0.5 mL of YMG preculture or with 0.1 mL of frozen inoculum (see 4.2.3). 3 mL of this mixture were placed into each well of the deepwell plate. The cultivation was carried out at 30°C and 300 rpm for 7 days.

4.2.2.2. Production of coumermycin A_1 and its derivatives

Cultivation in Erlenmeyer flasks

For coumermycin A_1 production, 5-10 mL of a TSB preculture of *S. coelicolor* M1146 containing the entire or modified coumermycin gene cluster were used to inoculate a 300 mL baffled Erlenmeyer flask containing 50 mL of SK medium, coumermycin production medium 1 (Weinstein *et al.*, 1978) or coumermycin production medium 2 (Kawaguchi *et al.*, 1965). The cultivation was carried out at 30°C and 200 rpm for 7 days.

Cultivation in 24-square deepwell plates

For cultivation in 24-square deepwell plates, 3 mL aliquots of the coumermycin production medium 1 (Weinstein *et al.*, 1978) or 2 (Kawaguchi *et al.*, 1965) were distributed to the wells of the deepwell plate. Each well was inoculated either with 75 µL of fresh preculture or 15 µL of frozen inoculum of *S. coelicolor* M1146 containing the entire or modified coumermycin gene cluster. The cultivation was carried out at 30°C and 300 rpm for 7 days.

For the production of the CPM mono- and diamide, 40 mL of CDM medium were mixed with 0.2 mL of frozen inoculum of *S. coelicolor* M1146(couSS01) and 3 mL of this mixture were placed into each well of the 24-square deepwell plate. The cultivation was carried out at 30°C and 300 rpm for 6-7 days.

4.2.2.3. Production of caprazamycins and liposidomycins

For the production of caprazamycins and liposidomycins, 1 mL of a TSB preculture of the respective *Streptomyces* strain was used to inoculate a 300 mL baffled Erlenmeyer flask containing 50 mL of P medium. The cultivation was carried out at 30°C and 210 rpm for 7 days.

4.2.3. Growth curves

Growth curves were recorded either by measuring the optical density of the culture at 600 nm or by determining the dry cell weight at different time points during the cultivation.

4.2.3.1. Determination of optical density at 600 nm (OD_{600})

1 mL of a YMG or TSB preculture was transferred to a 1 mL polystyrene cuvette and the optical density was measured at 600 nm using a spectrophotometer (Pharmacia LKB Novaspec II, Pharmacia, Freiburg/Germany). Samples were taken at different time points over 48 h of cultivation. During the growth phase (approx. after 12-24 h of cultivation) samples were taken every 2-3 hours.

4.2.3.2. Determination of dry cell weight

3 mL of CDM culture from a 24-square deepwell plate were filtered through a preweighted membrane filter with 0.45 µm pore size (Millipore Corporation, Billerica, MA, USA). The cells were washed with 10 mL of water and dried with the filters at 80°C to constant weight. Samples were taken every 24 h for 7-10 days.

4.2.4. Preparation of frozen inoculum

For the preparation of frozen inoculum, the cells of a YMG or TSB preculture were harvested by centrifugation (10 min, 4°C, 2800 ×*g*) at an OD_{600} of 0.4-0.7. The cells were resuspended in 1/5 of the original culture volume of an aqueous solution of peptone 20% (w/v) and gently homogenized using a potter homogenizer operated manually (B. Braun Biotech, Sartorius AG, Göttingen, Germany). The resulting mixture was divided in aliquots of 1-2 mL and stored at -70°C.

4.2.5. Preparation of spore suspensions

For the preparation of spore suspensions *Streptomyces* strains were spread on MS agar and incubated at 30°C till dense sporulation was observed (about 1-2 weeks). 4 mL of sterile ddH_2O were added to each plate and the spores were scraped off of the top of the plates using a sterile cotton bud. The resulting spore suspension was poured into a falcon tube, vortexed and separated from the mycelium by passing through sterile cotton wool. After centrifugation (2100 x*g*, 4°C, 10 min) the spores were resuspended in 0.5-2.0 mL of glycerol solution 20% (v/v) and stored at -70°C.

5. Molecular biology methods

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

5.1. DNA isolation

5.1.1. Isolation of plasmid and cosmid DNA from *E. coli*

Alkaline lysis was used to isolate recombinant plasmids and cosmids from *E. coli* (Sambrook & Russell, 2001). 3 mL LB medium were inoculated with a single colony and grown overnight at 37°C and 200 rpm. 1.5 mL of this culture was harvested by centrifugation (20000 x*g*, RT, 1 min) and the cells were resuspended in 100 µL solution MP1. The suspension was mixed with 200 µL solution MP2 by inversion and incubated on ice for 5 min (only for plasmid isolation). 150 µL solution MP3 was added and the mixture was incubated on ice for 5 min (only for plasmid isolation). After centrifugation (20000 x*g*, 4°C, 5 min) the supernatant was transferred to a fresh tube and the DNA was precipitated by addition of 500 µL isopropanol and centrifugation (20000 x*g*, 4°C, 30 min). The DNA pellet was washed with 500 µL ethanol 70% (v/v), air dried and resuspended in 50 µL Tris-HCl buffer (10 mM, pH 8.0). The required solutions are listed in Table II.5.

5.1.2. Isolation of genomic DNA from *Streptomyces*

Genomic DNA was isolated by the following procedure. 2 mL of a 2-day-old culture in TSB medium were harvested by centrifugation (1500 x*g*, 4°C, 10 min). The cells were washed with 1 mL TSE buffer, resuspended in 500 µL TSE buffer with lysozyme (3 mg/mL) and RNase A (100 µg/mL) and incubated for 30 min at 37°C. 250 µL of SDS solution were added and, after incubation for 10 min at 60°C, 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 1-2 min. After centrifugation (20000 x*g*, 4°C, 10 min) the supernatant was transferred to a fresh tube and, after addition of 150 µL TE buffer, was extracted for a second time with 250 µL phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was separated and the genomic DNA was precipitated by addition of 70 µL NaOAc solution and 700 µL isopropanol and centrifugation (20000 ×*g*, 4°C, 30 min). The DNA pellet was washed with 500 µL ethanol 70% (v/v), air dried and resuspended in 50 -100 µL Tris-HCl buffer (10 mM, pH 8.0). The required solutions are listed in Table II.6.

5.2. Purification, concentration and quantification of DNA

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

For quantification of DNA, a Nano Drop 1000 spectrophotometer (PeqLab, Erlangen, Germany) at 260 nm was used

5.3. DNA manipulation with enzymes

Restriction of DNA with endonucleases was performed according to the manufacturers' instructions (New England Biolabs).

DNA ligation was performed by using 1U T4-DNA ligase (New England Biolabs), 1x ligation buffer and the mixture of insert and linearized vector at a 1:1 ratio in a total volume of 10 µL. The mixture was incubated at 16°C overnight.

5.4. PCR amplification of DNA

PCR amplifications were carried out with a GenAmp® PCR system 2400 (Perkin-Elmer, Weiterstadt, Germany). The primers listed in Table II.11 were purchased from Eurofins MWG (Ebersberg, Germany). Taq-DNA polymerase was used for all amplifications.

For the purification of PCR products the QIAquick PCR Purification kit (Qiagen) was used and 5-10 µL of the purified PCR product were analysed by gel electrophoresis.

Table II.12 General PCR reaction and amplification conditions

Substance	Final concentration
10x amplification buffer	1x
Primer	50 pmol each
Template	about 100 ng
dNTPs	0.2 mM each
DNA polymerase	2.5 U
DMSO	5 % (v/v)
Final volume	50 µL

Cyclus	Temperature	Time	Cycles
Hot start	94 °C	2 min	1
Denaturing	94 °C	45 sec	
Annealing	50-60 °C	45 sec	25-30
Elongation	72 °C	1 min	
Final elongation	72 °C	5 min	1
End	4 °C	∞	-

5.5. Agarose gel electrophoresis of DNA

Gel electrophoresis with 1% (w/v) agarose was used to separate DNA fragments between 0.25 and 10 kb. The buffer system employed was 1xTAE buffer (Table II.7). The gels were stained with ethidium bromide for 15 min and analysed under UV light (312 nm) using a gel documentation system from Biostep equipped with Argus X1 software.

5.6. Introduction of DNA in *E. coli* and *Streptomyces*

The methods described in this section were modified from Kieser *et al.* (2000) and Sambrook & Russell (2001).

5.6.1. Introduction of DNA in *E. coli*

5.6.1.1. Electroporation

Preparation of electrocompetent cells

50 mL LB medium were inoculated with 1 mL of an overnight culture of *E. coli* and cultivated at 37°C and 200 rpm till the OD_{600} reached 0.6 (2-4 h). The cells were harvested by centrifugation (3000 x*g*, 4°C, 5 min) and washed twice with 50 mL and 25 mL ice-cold glycerol solution 10% (v/v), respectively. The supernatant was discarded and the cell pellet was suspended in the remaining drops. The competent cells could be used immediately or stored as 50 µL aliquots at -70°C.

Electroporation

1-3 µL DNA (about 100 ng) were mixed with 50 µL competent cells. The mixture was transferred into an ice-cold electroporation cuvette (0.2 cm) 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 ice-cold LB medium was immediately added to the cells and the suspension was transferred to a fresh tube. After incubation for 1 h at 37°C and 200 rpm, 200 µL of the suspension were spread on LB agar containing the appropriate antibiotic(s). The agar plates were incubated overnight at 37°C.

5.6.2. Introduction of DNA in *Streptomyces*

5.6.2.1. Intergeneric conjugation

Intergeneric conjugation was used to transfer modified plasmid DNA into *Streptomyces.* Therefore, the plasmid (containing an *ori*T) was introduced into the non-methylating *E. coli* strain ET12567/pUZ8002 harbouring the *tra*-genes required for mobilization and transfer of circular DNA. *E. coli* ET12567/pUZ8002 containing the plasmid was grown in LB medium supplemented with the appropriate antibiotic(s) at 37°C till the OD_{600} reached 0.6. The cells were harvested by centrifugation (3000 x*g*, 4°C, 5 min) and washed twice with 20 mL ice-cold LB medium without antibiotics. The supernatant was discarded and the cell pellet was suspended in the remaining drops.

In parallel 50 µL of spores of the target *Streptomyces* strain were incubated in 500 µL 2xYT medium at 50°C for 10 min. The spore suspension was mixed with 500 µL of the *E. coli* ET12567/pUZ8002 suspension followed by centrifugation (3000 x*g*, 4°C, 2 min). About 500 µL of the supernatant were discarded and the pellet was suspended in the remaining volume. Different volumes (20-100 µL) of the suspension were spread on MS agar supplemented with 10 mM $MgCl_2$. After incubation at 30°C for 16-

24 h, the plates were flooded with 1 mL sterile ddH_2O containing the required antibiotics for selection of the mutants. Incubation was continued for further 3-7 days.

Triparental conjugation was used for the transfer of modified cosmid DNA. Therefore, the cosmid DNA was introduced into *E. coli* ET12567 and cultivated as described above. In parallel *E. coli* ET12567/pUB307 was cultivated. After washing and resuspension of the cells, 500 µL of the *Streptomyces* spore suspension were mixed with 250 µL of each *E. coli* culture.

6. Biochemistry methods

6.1. Bioassay with *Mycobacterium phlei*

Antibacterial activity of extracts, containing caprazamycins or liposidomycins, 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 the growth inhibition zones were measured (Kaysser, 2010).

7. Construction of *Streptomyces* mutant strains

7.1. Deletion of *couM-couN7* on cosmid couMW16 and heterologous expression

A 7.1 kb fragment comprising the genes *couM-couN7* was replaced on cosmid couMW16 by an apramycin resistance cassette using RED/ET mediated recombination (Gust *et al.*, 2004), resulting in cosmid couMW22. The cassette for the replacement of *couM-couN7* was generated by PCR using the primer pair couM_fwd and couN7_rev (Table II.11, Final report of Dr. Manuel Wolpert). The resistance cassette was removed by *Xba*I digestion and religation and the resulting cosmid couSS01 was introduced into *S. coelicolor* M1146 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 (Flett *et al.*, 1997) as described in section 5.6.2.1. Kanamycin resistant clones were selected, checked by PCR and designated *S. coelicolor* M1146(couSS01) 1-5.

7.2. Deletion of *couR1*, *couR2a*, *couR2b*, *couR3* and *couR4* on cosmid couMW16 and heterologous expression

The genes *couR1, couR2a, couR2b, couR3* and *couR4* were separately replaced on cosmid couMW16 by an apramycin resistance cassette using RED/ET mediated recombination. The resistance cassette was removed by *Xba*I/*Spe*I digestion and religation, resulting in the cosmids couAK06, couAK07, couAK08, couAK09 and couAK10, respectively (Knuplesch, 2008). The cosmids were introduced into *S. coelicolor* M1146 by triparental intergeneric conjugation with the help of *E. coli*

ET12567/pUB307 as described in section 5.6.2.1. Kanamycin resistant clones were selected, checked by PCR and designated as *S. coelicolor* M1146(couAK06) 1-3, *S. coelicolor* M1146(couAK07) 1-2, *S. coelicolor* M1146(couAK08) 1-3, *S. coelicolor* M1146(couAK09) 1-3 and *S. coelicolor* M1146(couAK10) 1-3, respectively.

7.3. Complementation of the Δ*couR1* and the Δ*couR4* mutant

For the complementation of the Δ*couR1* and the Δ*couR4* mutant, the genes *couR1* and *couR4* were amplified by PCR and the PCR products were cloned into pGEM-T (Promega), resulting in plasmids pAK01 (*couR1*) and pAK05 (*couR4*), respectively (Knuplesch, 2008). After digestion with *Hind*III and *Spe*I, *couR1* and *couR4* were cloned into the same sites of pUWL-Apra-oriT resulting in plasmids pAK06 and pAK10 (Knuplesch, 2008). pAK06 and pAK10 were introduced into *S. coelicolor* M1146(couAK06) and *S. coelicolor* M1146(couAK10), respectively, by intergeneric conjugation as described in section 5.6.2.1. Kanamycin resistant clones were selected, designated *S. coelicolor* M1146 (couAK06)/pAK06 1.1-1.5 and *S. coelicolor* M1146 (couAK10)/pAK10 1.1-1.5 and checked for coumermycin A_1 production.

8. Chemical synthesis

8.1. Synthesis of 3-methylpyrrole-2,4-dicarboxylic acid

3-methylpyrrole-2,4-dicarboxylic acid, the central pyrrole moiety (CPM) of coumermycin A_1, was synthesized from its diethyl ester using a method described by Freel-Meyers *et al.* (2004).

3-methylpyrrole-2,4-dicarboxylic acid diethyl ester (1.0 g, 4.44 mmol) and NaOH (1.76 g, 44.4 mmol) were solved in 60 mL of EtOH/H_2O 1:1 (v/v) and heated to reflux overnight (16-18 h). After cooling to room temperature, 30 mL H_2O were added, and the solution was acidified (pH 2) with HCl 37 % to precipitate the 3-methylpyrrole-2,4-dicarboxylic acid. After 4 h at 4°C the precipitate was harvested by filtration, washed with ice-cold H_2O and ice-cold acetone and dried under vacuum. The substance was subjected to ^{1}H NMR analysis to confirm complete ester hydrolysis: ^{1}H NMR (250 MHz, DMSO-d_6) δ 12.21 (s, 2 H, OH), 11.87 (m, 1 H, NH), 7.34 (d, 1 H, H-5), 2.46 (s, 3 H, 3-Me) (see Appendix).

9. Feeding experiments

9.1. Feeding of 3-Methylpyrrole-2,4-dicarboxylic acid and *O*-Phospho-L-threonine

S. coelicolor M1146 containing the entire or modified coumermycin gene cluster was cultivated in coumermycin production medium 2 as described in section 4.2.2.2. 3-methylpyrrole-2,4-dicarboxylic acid, *O*-phospho-L-threonine and L-threonine (negative control) were added to the cultures in two equal aliquots after 24h and 48h of cultivation, to give a final concentration of 0.1 mmol/L. 3-methylpyrrole-2,4-

dicarboxylic acid was added as an ethanolic solution (15 mmol/L), *O*-phospho-L-threonine and L-threonine as sterile aqueous solutions (15 mmol/L). The cultures were harvested after 7 days and the production of coumermycin A_1 was determined by HPLC analysis.

9.2. Feeding of ^{13}C-labeled precursors

S. coelicolor M1146(couSS01) was cultivated in CDM medium as described in section 4.2.2.3 (total culture volume: 360 mL). [U-^{13}C,^{15}N]L-threonine and [U-^{13}C]fumaric acid were added to the cultures as sterile aqueous solutions (64.5 mmol/L and 34 mmol/L, respectively) in 5 equal aliquots after 72, 84, 96, 108 and 120 h of cultivation, to give final concentrations of 2.15 mmol/L and 2.3 mmol/L, respectively. The cultures were harvested after 144 h of cultivation and the CPM monoamide was isolated from the culture as described in section 10.1.2.2.

10. Analytical methods

10.1. Sample preparation

10.1.1. Novobiocin

S. coelicolor M512(novBG01) was cultivated in CDM medium for 7 days as described in section 4.2.2.1. For analytical purposes 1 mL CDM culture was centrifuged twice (20000 x*g*, 4°C, 20 min) and the supernatant was directly used for HPLC analysis.

10.1.2. Coumermycin A_1 and its derivatives

10.1.2.1. Coumermycin A_1

S. coelicolor M1146 containing the entire or modified coumermycin gene cluster was cultivated in SK medium, coumermycin production medium 1 or coumermycin production medium 2 for 7 days as described in section 4.2.2.2. For analytical puposes 1 mL culture was adjusted to pH 4 with 1N HCl and extracted three times with 600 µL ethyl acetate. The organic phase was evaporated and the residue resolved in 200 µL ethanol. If SK medium was used the culture was extracted with petroleum ether prior to extraction with ethyl acetate to remove the lard oil.

10.1.2.2. CPM mono- and diamide

S. coelicolor M1146(couSS01) was cultivated in CDM medium for 6-7 days as described in section 4.2.2.3. For analytical purposes 1 mL CDM culture was centrifuged twice (20000 x*g*, 4°C, 20 min) and the supernatant was directly used for HPLC and LCMS analysis.

For preparative isolation of the CPM monoamide, the cultures were centrifuged (6000 x*g*, RT, 30 min) and the cell pellet was discarded. The supernatant was adjusted to

pH 2 by addition of 1N HCl and left on ice for 30 min to precipitate the CPM monoamide, which was collected by centrifugation (4000 x*g*, 4°C, 15 min). After a washing step with ice cold 0.1N HCl, the precipitate was extracted three times with 6 mL methanol and the insoluble material was discarded. The solvent was evaporated and the residue was resolved in 5 mL methanol at 70°C. Further purification of the CPM monoamide was achieved by semipreparative HPLC (see 10.2.2).

10.1.3. Caprazamycins and liposidomycins

10.1.3.1. Extraction of caprazamycins and liposidomycins

Streptomyces sp. MK730-62F2 and *Streptomyces sp*. SN1061-M were cultivated in P medium as described in section 4.2.2.4. The culture supernatant was adjusted to pH 4 by addition of 1N HCl and extracted twice with an equal volume of *n*-butanol. The organic phase was evaporated and the residue was resolved in 1 mL methanol.

10.1.3.2. Affinity chromatography with Affi-Gel® boronate

A *cis*-diol specific affinity chromatography was used for further purification of the extracts. Affi-Gel® boronate is a boronic acid stationary phase which specifically binds *cis*-diol structures, like the ribose of nucleosides, by formation of a cyclic boronate ester at alkaline pH values. At acidic pH values the ester bond is cleaved, resulting in the elution of the *cis*-diol-containing compounds (Bullinger *et al.*, 2007).

Preparation of the column

500 mg Affi-Gel® boronate were suspended in ddH_2O and the material was transferred to the column (150 x 15 mm) after 15 min of soaking. For conditioning of the column the scheme depicted in Table II.13 was used and the required buffers and solutions are listed in Table II.8. Compressed air was applied to maintain a moderate flow rate.

Purification of caprazamycins and liposidomycins

Caprazamycins and liposidomycins were extracted as described in section 10.1.3.1. 300 µL of the culture extract were dissolved in 10 mL NH_4OAc buffer (0.25 M, pH 8.8) and then loaded on the column, preconditioned with NH_4OAc buffer (0.25 M, pH 8.8). After washing with NH_4OAc buffer (0.25 M, pH 8.8) and MeOH/H_2O 2:8 (v/v) elution was carried out with formic acid solution (0.2 M, MeOH/H_2O 1:1 (v/v)), as shown in Table II.14. The solvent of the elution step was evaporated and the residue was dissolved in 1 mL methanol. The column can be stored on NaCl solution (0.1 M) at 4-8°C for several weeks.

Table II.13 Conditioning of the column

Step	Buffer / Solution	Volume
1	H_2O	10 mL
2	MeOH/H_2O 2:8 (v/v)	10 mL
3	H_2O	10 mL
4	MeOH/H_2O 2:8 (v/v)	10 mL
5	H_2O	10 mL
6	NaCl solution	20 mL
7	MeOH/H_2O 2:8 (v/v)	30 mL
8	Formic acid solution	30 mL
9	NH_4OAc buffer	30 mL
10	MeOH/H_2O 2:8 (v/v)	30 mL
11	NH_4OAc buffer	30 mL
12	MeOH/H_2O 2:8 (v/v)	30 mL
13	Formic acid solution	30 mL
14	MeOH/H_2O 2:8 (v/v)	30 mL
15	NaCl solution	50 mL

Table II.14 Affinity chromatography for the purification of caprazamycins and liposidomycins (optimized method)

Step		Buffer / Solution	Volume
1	Preconditioning	NH_4OAc buffer (0.25 M, pH 8.8)	45 mL
2	Sample	Culture extract	0.3 mL
		NH_4OAc buffer (0.25 M, pH 8.8)	10 mL
3	Washing step 1	NH_4OAc buffer (0.25 M, pH 8.8)	20 mL
4	Washing step 2	MeOH/H_2O 2:8 (v/v)	6 mL
5	Elution	Formic acid solution (0.2 M)	50 mL
6	Regeneration	MeOH/H_2O 2:8 (v/v)	25 mL

10.2. HPLC analysis

For HPLC analysis a Agilent 1200 system with PDA detector or a Agilent 1100 system with VWD detector, both equipped with ChemStation software for data recording and evaluation, were used. A Reprosphere® C18-DE column (5 µm, 3 x 150 mm) with a flow rate of 0.6 mL/min or a Reprosphere® C18-DE colum (5 µm, 4.6 x 150 mm) with a flow rate of 1.0 mL/min were used for analytical purposes and a Multosphere® 120 RP18 column (5 µm, 250 x 8 mm) with a flow rate of 1 mL/min was used for preparative HPLC.

10.2.1. Novobiocin

For analysis of novobiocin, the conditions depicted in Table II.15 were used. Authentic novobiocin was used as standard.

Table II.15 HPLC conditions for novobiocin analysis

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate	Wavelength	Temperature
0.0	40	60	1 mL/min	305 nm	not controlled
2.0	40	60			
13.0	0	100			
19.0	0	100			
19.1	40	60			
25.0	40	60			
Solvent A:	H_2O : HCOOH (99:1)				
Solvent B:	CH_3OH : HCOOH (99:1)				

10.2.2. Coumermycin A_1 and its derivatives

For analysis of coumermycin A_1 and its derivatives, the conditions depicted in Table II.16 were used. For all coumermycin derivatives authentic coumermycin A_1 was used as standard, only for the CPM monoamide authentic novobiocin was used. For the preparative isolation of the CPM monoamide, the conditions depicted in Table II.17 were used

Table II.16 HPLC conditions for coumermycin A_1 analysis

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate	Wavelength	Temperature
0.0	30	70	0.6 mL/min	345 nm	40°C
2.0	30	70			
22.0	0	100			
26.0	0	100			
26.1	30	70			
30.0	30	70			
Solvent A:	H_2O : HCOOH (99:1)				
Solvent B:	CH_3CN : HCOOH (99:1)				

Table II.17 HPLC conditions for the purification of CPM monoamide

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate	Wavelength	Temperature
0.0	60	40	1 mL/min	345 nm	not controlled
5.0	60	40			
25.0	0	100			
30.0	0	100			
31.0	60	40			
35.0	60	40			
Solvent A:	H_2O : HCOOH (99.9:0.1)				
Solvent B:	CH_3CN : HCOOH (99.9:0.1)				

10.3. LCMS analysis

For LC/ESI-MS analysis, a Thermo Finnigan TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Finnigan, San Jose, USA) coupled to a Surveyor HPLC system was used. Reversed-phase chromatography was carried out on a Reprosil-Pur® Basic C18 column (5 µm, 250 x 2 mm) at a flow rate of 0.2 mL/min. The mobile phase consisted of (A) water/formic acid (99.9:0.1) and (B) acetonitrile/formic acid (99.9:0.1). Mass spectrometry settings for electrospray ionization (ESI) in positive and negative mode are shown in Table II.18.

Table II.18 Mass spectrometry settings

Parameter	Positive mode	Negative mode	
	caprazamycins	caprazamycins	coumermycins
Electrospray voltage	3.8 kV	- 4.0 kV	- 3.8 kV
Heated capillary temperature	320°C	320°C	300°C
Collision gas	argon	argon	argon
Collision gas pressure	1.0 mTorr	1.0 mTorr	1.0 mTorr
Sheath/auxillary gas	nitrogen	nitrogen	nitrogen
Sheath gas pressure	30 Arb	35 Arb	35 Arb
Auxillary gas pressure	12 Arb	10 Arb	10 Arb

10.3.1. Coumermycin A_1 and its derivatives

10.3.1.1. HPLC conditions

The HPLC conditions for the LCMS analysis of coumermycin A_1 and its derivatives are listed in Table II.19.

Table II.19 HPLC conditions for coumermycin A_1 and its derivatives.

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate	Wavelength
0.0	98	2	0.2 mL/min	345 nm
5.0	98	2		
30.0	0	100		
39.0	0	100		
40.0	98	2		
45.0	98	2		
Solvent A:	H_2O : HCOOH (99.9:0.1)			
Solvent B:	CH_3CN : HCOOH (99.9:0.1)			

10.3.1.2. MS/MS product ion scans

Product ion scans were performed in negative ionization mode on the respective $[M-H]^-$ ions of coumermycin A_1 and its derivatives, e.g. CPM mono- and diamide. The collision energy was set to 40V for coumermycin A_1 and 25V for CPM mono- and diamide, respectively. Argon was used as collision gas. Product ion spectra were recorded with a scan time of 0.7 s for each mass and a peak width of 0.7 for the first

quadrupole (Q1) and the third quadrupole (Q3), respectively. The mass range was set according to the *m/z* values of the molecular ion and the expected fragment ions.

10.3.2. Caprazamycins and liposidomycins

10.3.2.1. HPLC conditions

The HPLC conditions for the LCMS analysis of caprazamycins and liposidomycins are listed in Table II.20.

Table II.20 HPLC conditions for caprazamycins and liposidomycins

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate	Wavelength
0.0	98	2		
5.0	98	2		
9.0	70	30		
38.0	0	100	0.2 mL/min	261 nm
45.0	0	100		
46.0	98	2		
50.0	98	2		
Solvent A:	H_2O : HCOOH (99.9:0.1)			
Solvent B:	CH_3CN : HCOOH (99.9:0.1)			

10.3.2.2. MS/MS product ion scans

In positive ionization mode product ion scans were performed on the respective $[M+H]^+$ ions of the caprazamycins and liposidomycins and in negative ionization mode on the respective $[M–H]^-$ ions. The collision energy was set to 35V in positive mode and 30V in negative mode. Product ion spectra were recorded with a scan time of 0.7 s for each mass and a peak width of 0.7 for the first quadrupole (Q1) and the third quadrupole (Q3), respectively. The mass range was set according to the *m/z* values of the molecular ion and the expected fragment ions.

10.3.2.3. MS/MS precursor ion scan

Precursor ion spectra of the permethylated L-rhamnose at *m/z* 189 in positive mode were obtained by scanning the mass range *m/z* 150–1500. Spectra were recorded with a scan time of 0.7 s and a collision energy of 35V.

10.4. NMR analysis

For NMR analysis the compounds were dissolved in d_6-dmso and NMR spectra were recorded on spectrometer Varian Inova 500 equipped with ^{13}C-sensitive cryoprobe (^1H: 500 MHz, ^{13}C: 125.7 MHz) or on Bruker AMX 600 (^1H: 600 MHz, ^{13}C: 156.7 MHz). Chemical shifts in d_6-dmso are reported as δ values relative to the respective solvent as an internal reference.

III. RESULTS

1. Antibiotic production in 24-square deepwell plates

Investigations on antibiotic formation in streptomycetes are often hampered by the notorious variability of production rates observed between successive and even between parallel cultivation batches of the same strain in Erlenmeyer flasks. A cultivation method which shows less variability in secondary metabolite production therefore would significantly reduce the workload and cost of research in antibiotic biosynthesis.

1.1. Comparison of novobiocin production in Erlenmeyer flasks and 24-square deepwell plates

In a first experiment, the novobiocin production of *S. coelicolor* M512(novBG01) in a 24-square deepwell plate with sandwich-cover, as described by Duetz and co-workers (Duetz, 2007; Duetz & Witholt, 2004), was compared to that in baffled Erlenmeyer flasks equipped with a stainless steel spring. The polypropylene plate contained 24 square wells (18x18 mm in the horizontal plane, 40 mm deep) and each well was filled with 3 mL of liquid medium while the Erlenmeyer flasks each contained 50 mL (**Figure III.1A**). Both, deepwell plate and Erlenmeyer flasks, were inoculated with the same batch of fresh preculture, cultivated in YMG medium, and the cultures were agitated on orbital shakers (25 mm shaking diameter) at 300 and 200 rpm, respectively. As shown in **Figure III.1B**, novobiocin production after 7 days was higher in the Erlenmeyer flasks (24 mg/L vs. 11 mg/L) but the variability was much lower in the deepwell plate (relative standard deviation 39% and 6%, respectively).

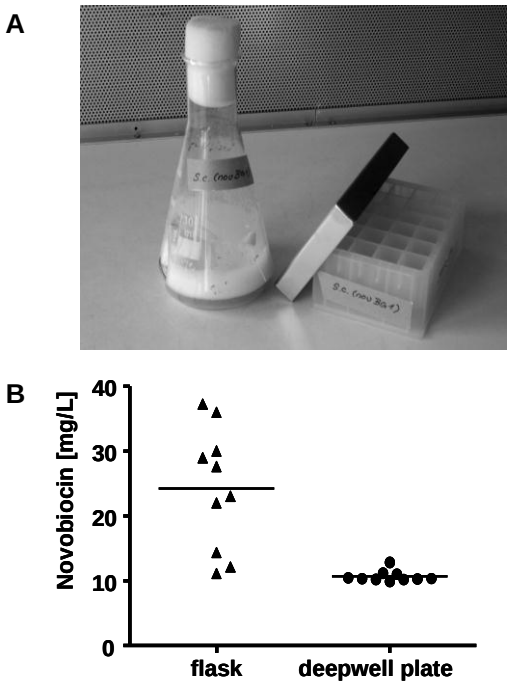


Figure III.1
Comparison of novobiocin production in Erlenmeyer flasks and a 24-square deepwell plate. A) Cultures of *S. coelicolor* M512 (novBG01) in Erlenmeyer flask and deepwell plate. B) Novobiocin production and variability in 10 Erlenmeyer flasks and 10 wells of a deepwell plate in a parallel cultivation.

1.2. Optimization of the inoculation method

In order to further reduce the variability of novobiocin production in the 24-square deepwell plates, especially between independent cultivation batches, the inoculation method was optimized. This involved the optimization of the inoculation ratio with and without homogenization of the inoculum and preparation of frozen inoculum either from the early stationary phase or from a defined section of the growth phase.

1.2.1. Homogenization of the preculture and optimization of the inoculation ratio

As *Streptomyces* cultures with their mycelial growth form cell aggregates of variable size in liquid culture, it was tested whether homogenization of the preculture with a potter homogenizer prior to mixing with the fresh medium would further reduce variability. Therefore the appropriate amount of homogenized or non-homogenized preculture of *S. coelicolor* M512(novBG01) was mixed with the fresh medium and then 3 mL aliquots of this mixture were dispensed to the wells of the deepwell plates. Since pre-mixing of inoculum and fresh medium may bear a certain risk of unequal inoculation due to sedimentation of cells in the mixture, separate dispensing of medium and homogenized inoculum to the deepwell plates was also tested. However, all of these method variations resulted in similar antibiotic production and similar variability (rel. SD 2.0 – 4.7%). Subsequently, the inoculation ratio, i.e. the ratio of preculture volume to production medium volume, was varied from 1:20 to 1:160, using both homogenized and non-homogenized inoculum. Using non-homogenized inoculum the highest novobiocin production was obtained with an inoculation ratio of 1:40 (**Figure III.2**). As may be expected, homogenized inoculum with its higher number of colony-forming units could be used in a slightly smaller amount (1:80), but the difference between the two ratios was small. An inoculation ratio of 1:80 was therefore used in all further experiments.

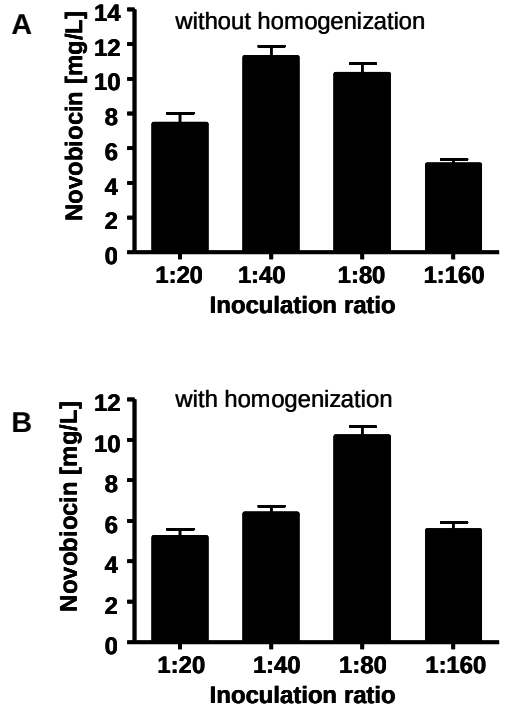


Figure III.2
Influence of the inoculation ratio on novobiocin production in *S. coelicolor* M512(novBG01) in 24-square deepwell plates. A) Direct inoculation with the preculture and B) Inoculation with homogenized inoculum using a potter homogenizer. During homogenization, cells from 5 Vol preculture were resuspended in 1 Vol production medium or peptone solution 20% (w/v). Inoculation volumes were adjusted accordingly.

1.2.2. Use of frozen inoculum and preparation of inoculum from a defined section of the growth phase

In order to provide a uniform inoculum for a series of experiments over a range of time, a large batch of frozen inoculum which could be stored at -70°C was prepared, following the procedure described in the materials and methods section (4.2.4). This procedure involves centrifugation of the preculture, resuspension of the cells in 20% (w/v) peptone as cryoprotectant, gentle homogenization with a potter homogenizer and freezing at -70°C. Freezing and re-thawing of an inoculum of *S. coelicolor* M512(novBG01) did not affect novobiocin production (**Figure III.3**) and independent cultivation experiments from the same batch of frozen inoculum over a period of nine months showed standard deviations of 8 % between different time points, and 4% to 9% within parallel experiments at the same time point. However, when different batches of precultures from the same strain were used, the variability between these experiments was found to be high (53 % relative standard deviation). Harvesting of the precultures in a defined section of the growth phase might reduce this batch-to-batch variability.

The growth curve of *S. coelicolor* M512(novBG01) in 50 mL YMG medium in a 300 mL baffled Erlenmeyer flask with stainless steel spring was recorded by monitoring the optical density at 600 nm over 48 hours (**Figure III.4**). Rapid growth occurred between 12 and 20 hours after inoculation when OD_{600} values rose quickly from 0.3 to 0.7. The final OD_{600} after 48 hours was 0.8. Five independent batches of frozen inoculum were prepared from precultures harvested at OD_{600} values between 0.5 and 0.7 and, notably, novobiocin production between cultures, prepared from these five different batches, varied only by 6% (relative standard deviation), i.e. much less than using inocula from the early stationary phase. Also the absolute novobiocin production (30 mg/L) was higher than using inoculum from the early stationary phase. Harvesting of the precultures in the second half of the growth phase therefore is clearly of advantage. The precise time when the OD value is reached varied slightly from flask to flask, but the range from OD_{600} 0.5 - 0.7 proved large enough to conveniently obtain cultures at the desired growth stage. It was not necessary to make adjustments from the slight differences in cell density of the homogenized inoculum batches as such adjustments did not reduce variability of the novobiocin production any further.

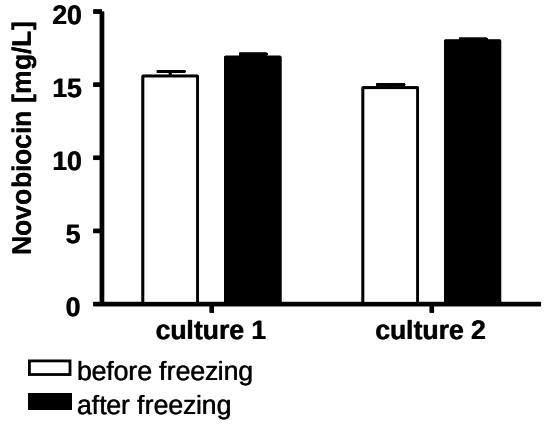


Figure III.3
Effect of freezing of the mycelial inoculum of *S. coelicolor* M512(novBG01) (in the presence of 20% (w/v) peptone) on novobiocin production

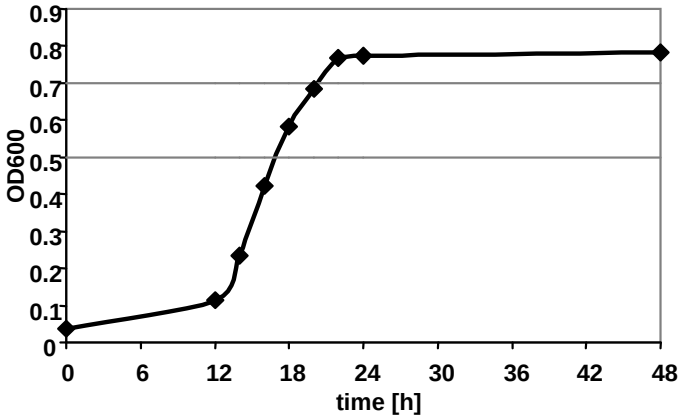


Figure III.4
Growth curve of *S. coelicolor* M512(novBG01) in YMG medium

1.3. Influence of aeration on novobiocin production

One reason for the originally lower novobiocin production in the deepwell plates, in comparison to Erlenmeyer flasks (**Figure III.1B**), may be insufficient aeration. To increase aeration, sandwich covers with 4 mm holes instead of 2 mm ones were tested. However, this did not cause a difference in the novobiocin production, in accordance with previous observations that not aeration of the headspace but gas–liquid transfer is limiting for oxygen supply of the culture (Duetz *et al.*, 2000; Duetz & Witholt, 2001; Duetz & Witholt, 2004). All further experiments therefore were carried out with the standard sandwich covers with 2 mm holes. However, a smaller culture volume should lead to better aeration of the culture due to the increased surface/volume ratio. Indeed novobiocin production in 1.5 mL culture volume was slightly higher than in 3 mL (5.5 versus 4.5 mg/L), but so was the standard deviation (14.5 versus 6.2%). A culture volume of 3 mL was therefore used in all further experiments, and alternative methods were explored to increase oxygen supply, e.g. the addition of an artificial oxygen carrier.

1.4. Investigation of a siloxylated ethylene oxide/propylene oxide copolymer as artificial oxygen carrier

Oxygen carriers, such as the natural compound haemoglobin or the biotechnologically used perfluorodecalin, can be used to increase the concentration of oxygen in aqueous solutions and improve the efficiency of oxygen-consuming biochemical processes. Siloxylated ethylene oxide/propylene oxide copolymers such as Q2-5247, which are water-soluble compounds in contrast to perfluorodecalin, have recently been shown to increase growth of *Bacillus thuringensis* and production of the polyketide antibiotic actinorhodin in *S. coelicolor* A3(2) (Dey *et al.*, 2004). Therefore the effect of the addition of the copolymer Q2-5247 on novobiocin production in *S. coelicolor* M512(novBG01) was tested. When this compound was added to the medium in concentrations between 0.3% and 2.4%, a strong increase of novobiocin production was observed. The most effective concentration was found to be 0.6%, which led to a 5-fold overproduction of novobiocin in comparison to the control (**Figure III.5**). Production rates in the deepwell plates supplemented with Q2-5247 were comparable to those observed in Erlenmeyer flasks. Cell growth and novobiocin production were compared with and without the addition of polymer over a culture period of 10 days (**Figure III.6**). This showed that the copolymer did not

influence cell growth. Notably, the cell aggregates were smaller in the presence of Q2-5247, and novobiocin production started 1 day earlier (**Figure III.6**).

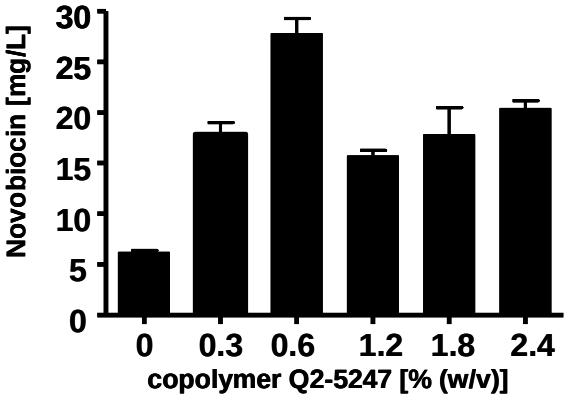


Figure III.5
Optimization of the final concentration of the siloxylated ethylene oxide/propylene oxide copolymer Q2-5247 for *S. coelicolor* M512(novBG01) in the novobiocin production medium

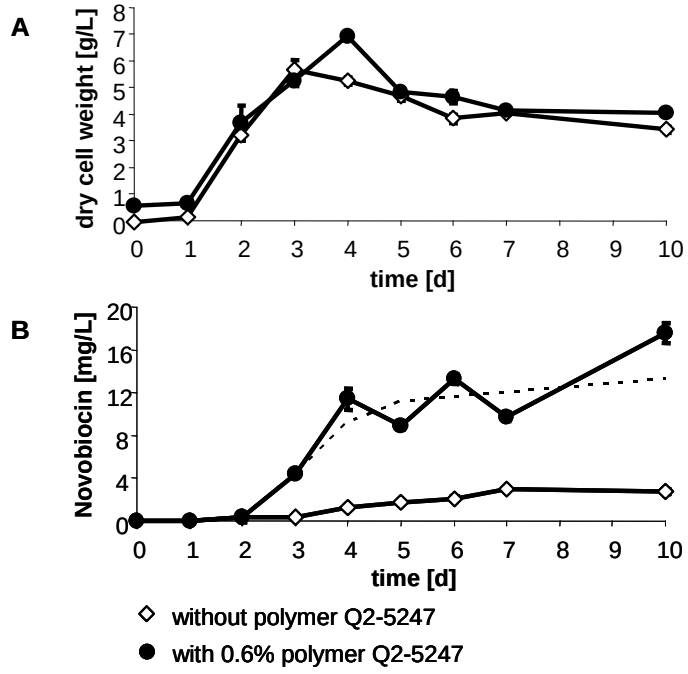


Figure III.6
A) Growth curves and B) production curves of an frozen inoculum of *S. coelicolor* M512 (novBG01) without copolymer and with addition of 0.6% siloxylated ethylene oxide/ propylene oxide copolymer Q2-5247

1.5. Overexpression of the pathway-specific positive regulator *novG*

Another possibility to increase novobiocin production is the overexpression of *novG*, a pathway-specific positive regulator of novobiocin biosynthesis. In Erlenmeyer flasks, overexpression of this gene was already shown to increase novobiocin production (Eustáquio *et al.*, 2005) and when cultures of *S. coelicolor* M512 (novBG01) harbouring the *novG* expression plasmid pAE12 were compared with those containing the empty vector pWHM3 in the 24-square deepwell plates, a three-fold increase of novobiocin production was observed. Furthermore, the positive effects of *novG* and of copolymer Q2-5247 on novobiocin production could be combined. By simultaneous copolymer addition and *novG* expression an 11-fold increase of novobiocin formation was achieved, reaching 163 mg/L and thereby

exceeding all previous production levels observed in Erlenmeyer flasks in our laboratory (**Figure III.7**).

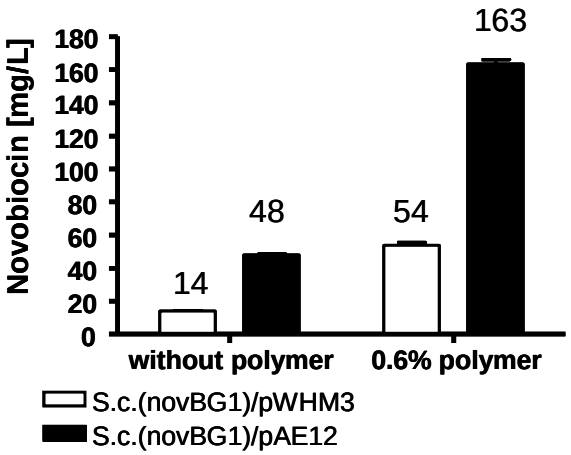


Figure III.7
Effect of overexpression of the positive regulator *novG* and the addition of 0.6% of the siloxylated ethylene oxide/ propylene oxide copolymer Q2-5247 on novobiocin production in *S. coelicolor* M512(novBG01); pAE12: *novG* expression plasmid; pWHM3: empty vector

1.6. Production of coumermycin A_1 and derivatives thereof in the 24-square deepwell plates

Since the suitability of 24-square deepwell plates for investigations on antibiotic production in *Streptomyces* was now demonstrated at the example of novobiocin, it was decided, that this method should also be used for the investigation of the biosynthesis of the central pyrrole moiety (CPM) of coumermycin A_1, described in the following chapter. However, SK medium, which was originally used for coumermycin production, was not suitable for cultivations in the deepwell plates, due to the lard oil contained therein. Therefore, two previously described production media without lard oil, in this thesis termed coumermycin production medium 1 (Weinstein *et al.*, 1978) and 2 (Kawaguchi *et al.*, 1965), were tested.

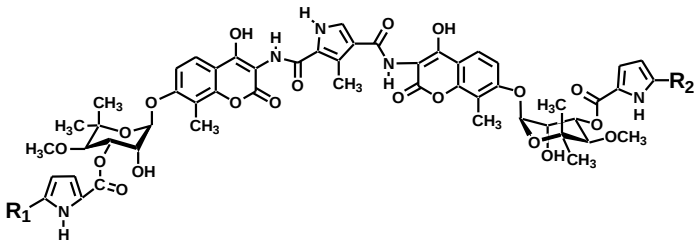
In a previous study the coumermycin gene cluster was assembled into a single construct, termed couMW16, and then this construct was integrated into the ΦC31 attachment site of the genome of *S. coelicolor* M512. The resulting mutants produced on average 7 mg/L coumermycin A_1, exceeding the productivity of the wild-type producer strain *Streptomyces rishiriensis* DSM 40489 under the same cultivation conditions (5 mg/L) (Flinspach *et al.*, 2010; Wolpert *et al.*, 2008). For unknown reasons, coumermycin production suddenly dropped dramatically (< 1 mg/L) at the beginning of this study and therefore it was decided to use the recently developed host strain *S. coelicolor* M1146 for the heterologous expression of cosmid couMW16 (Gomez-Escribano & Bibb, 2011). The average coumermycin A_1 production of the resulting strain in SK medium, using baffled Erlenmeyer flasks as the cultivation system, was 52 mg/L (SD = 16.9 mg/L), 7.5 times higher than the average productivity originally found in *S. coelicolor* M512 containing the same cosmid (Flinspach *et al.*, 2010).

Cultivation of *S. coelicolor* M1146(couMW16) in medium 1 (Weinstein *et al.*, 1978) led to a total coumermycin production of 19 mg/L. However, the main product was not coumermycin A_1 but coumermycin C, containing only one terminal pyrrole moiety which additionally lacks the methyl group at position 5 (**Figure III.8** and **III.9**). Besides

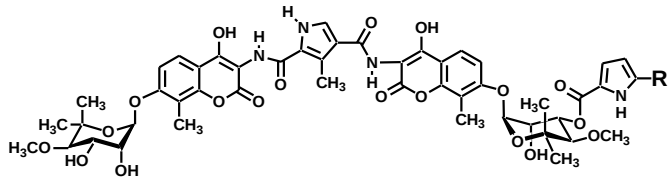
these two compounds, significant amounts of coumermycin A_2, B and D-1b/1c were detected, also lacking one of the terminal pyrrole moieties and/or one or both methyl groups (**Figure III.8** and **III.9**). Since the methylation reaction catalysed by CouN6 is methylcobalamine-dependent (Westrich *et al.*, 2003), supplementation of the medium with 0.2 mg/L cobalt chloride, as in SK medium, should be able to shift the production to coumermycin A_1 (Claridge *et al.*, 1966). As shown in **Figure III.9**, production of coumermycin A_1 was indeed significantly increased after the addition of cobalt chloride (4 mg/L vs. 0.6 mg/L) although an influence on the total coumermycin production was not observed (17 mg/L vs. 19 mg/L). However, coumermycin A_1 was still not the main product but coumermycin B (11 mg/L). Therefore it was decided to test a second production medium, which is more similar to the originally used SK medium.

In production medium 2 (Kawaguchi *et al.*, 1965), also supplemented with 0.2 mg/L cobalt chloride, the total coumermycin production was significantly higher than in medium 1 (53 mg/L vs. 17 mg/L). Additionally, with an average production of 36 mg/L, coumermycin A_1 was now the main product (**Figure III.9**). Therefore, production medium 2 was used in all further experiments, except for the production of the CPM mono- and diamide (**Figure III.10**), where CDM medium was used.

The inoculation ratio was varied from 1:5 to 1:160, using a homogenized inoculum of *S. coelicolor* M1146(couSS01). The differences in the production between the tested ratios were small (4.5 - 6.5 mg/L of CPM diamide) and a ratio of 1:40 was chosen for all further experiments. Since the growth curve of *S. coelicolor* M1146(couSS01) in TSB medium looked similar to that of *S. coelicolor* M512(novBG01) shown in **Figure III.4**, frozen inocula were also prepared from precultures harvested at OD_{600} values between 0.5 and 0.7.



	R_1	R_2
Coumermycin A_1	CH_3	CH_3
Coumermycin D-1b/1c	CH_3	H
	H	CH_3
Coumermycin A_2	H	H



	R
Coumermycin B *	CH_3
Coumermycin C *	H

*** isomeric structure possible**

Figure III.8
Chemical structures of coumermycins A-D. In case of coumermycin B and C isomeric structures, i.e. the terminal pyrrole moiety at the other deoxysugar moiety, are possible

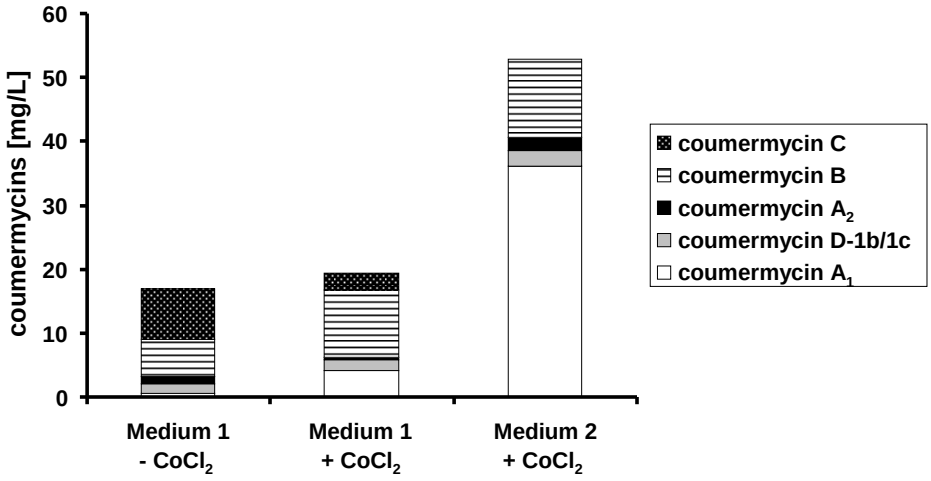


Figure III.9
Test of two different media for coumermycin production with and without addition of cobalt chloride (0.2 mg/L). Medium 1: coumermycin production medium 1 (Weinstein *et al.*, 1978); Medium 2: coumermycin production medium 2 (Kawaguchi *et al.*, 1965).

2. Biosynthesis of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety of coumermycin A_1

Coumermycin A_1 contains two types of pyrrole moieties, i.e. a 3-methylpyrrole-2,4-dicarboxylic acid moiety (hereafter called the central pyrrole moiety, **Figure III.10A**) and two 5-methylpyrrole-2-carboxylic acid groups (hereafter called the terminal pyrrole moieties), which are also found in clorobiocin (**Figure III.10A**). While the terminal pyrrole moieties are formed from L-proline by action of CouN1-7 and most of the reaction steps involved have been investigated in vitro (Balibar *et al.*, 2007; Fridman *et al.*, 2007; Garneau-Tsodikova *et al.*, 2006; Garneau *et al.*, 2005; Walsh *et al.*, 2006), the biosynthetic pathway to the central pyrrole moiety is still unknown.

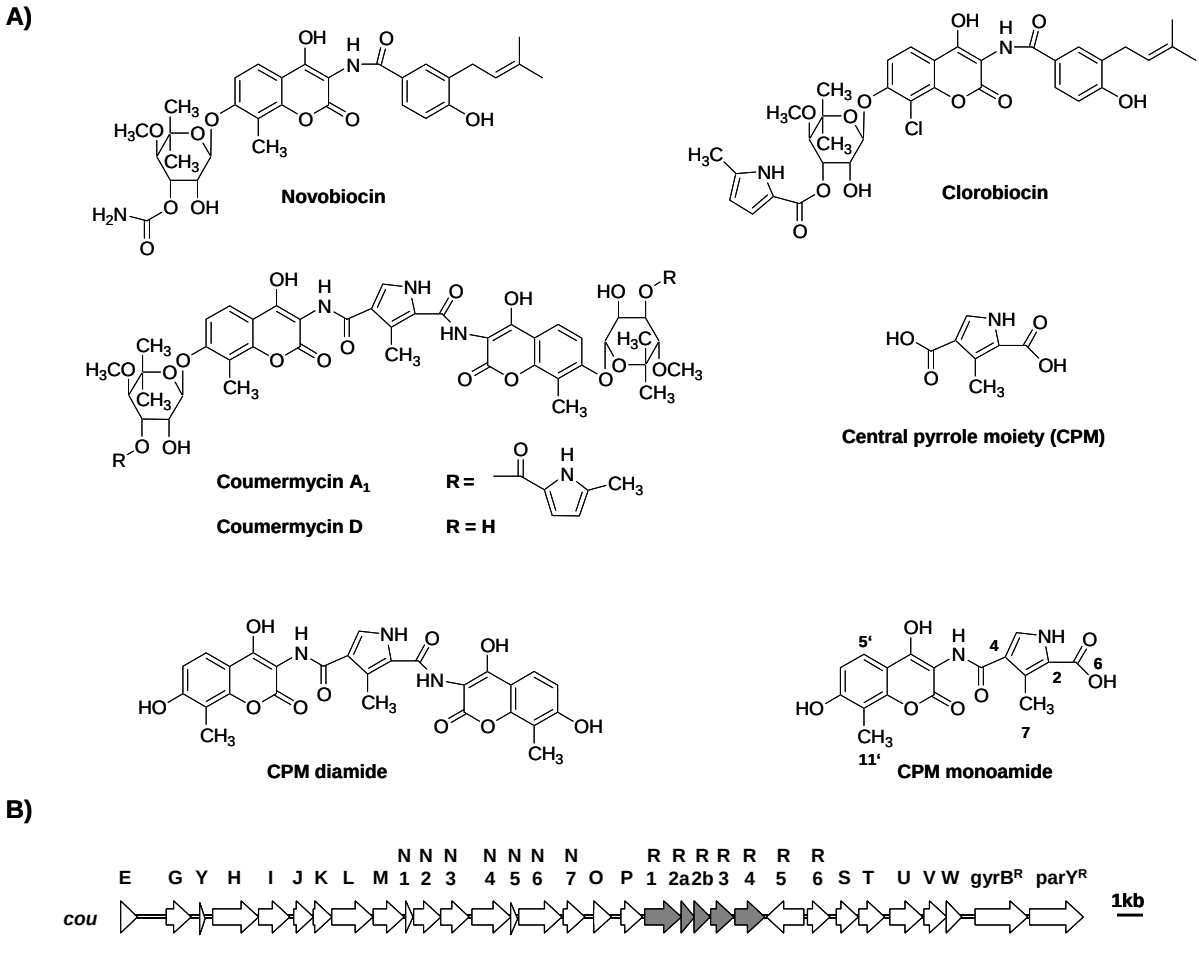


Figure III.10
A) Chemical structures of the aminocoumarin antibiotics novobiocin, clorobiocin, coumermycin A_1, coumermycin D, of the central pyrrole moiety (CPM), of CPM diamide and CPM monoamide. B) The biosynthetic gene cluster of coumermycin A_1. The genes investigated in this study for their participation in the biosynthesis of the central pyrrole moiety are shaded in grey.

2.1. The genes *couN1-couN7* are not involved in the biosynthesis of the central pyrrole moiety of coumermycin A_1

Previous inactivation experiments in the genuine coumermycin producer strain had shown that *couN3* and *couN4* are not required for the biosynthesis of the central pyrrole moiety (CPM) of coumermycin A_1 (Xu *et al.*, 2002). We now carried out a deletion of all seven genes involved in the biosynthesis and transfer of the terminal pyrrole moieties of coumermycin, i.e. of the entire region from *couN1* to *couN7*. In order to ease analysis of the products, we decided to delete at the same time the gene *couM*, adjacent to *couN1* (**Figure III.11**). *couM* codes for the glycosyl transferase which is responsible for the transfer of the deoxysugar moiety to the aminocoumarin ring (Freel Meyers *et al.*, 2004). Deletion of *couM* is therefore expected to result in accumulation of the coumermycin aglycone (CPM diamide, **Figure III.10A**) and possibly its immediate precursor, CPM monoamide (**Figure III.10A**). Both compounds have been identified previously as enzymatic reaction products of the amide synthetase CouL (Schmutz *et al.*, 2003b).

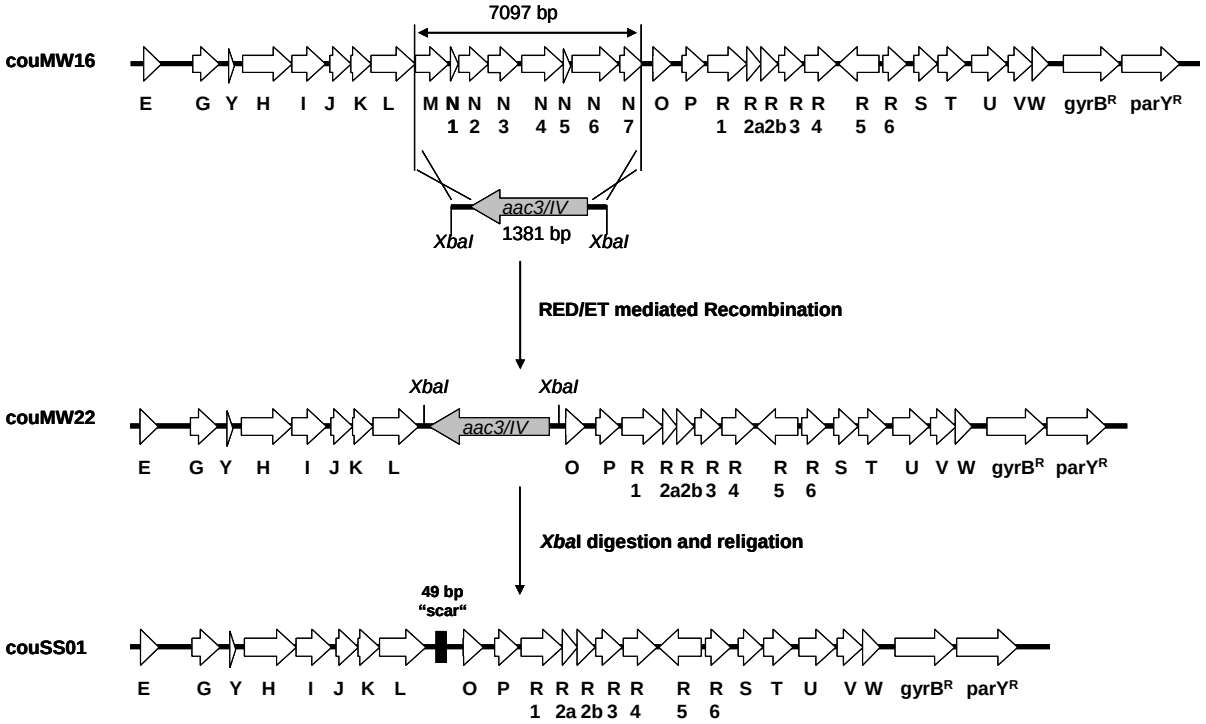


Figure III.11
Deletion of the genes *couN1-couN7* responsible for the formation of the terminal pyrrole moieties of coumermycin A_1. *couM* codes for the glycosyltransferase attaching the deoxysugars of coumermycin. Genes *couM-couN7* were replaced on cosmid couMW16 by an apramycin resistance cassette, resulting in cosmid couMW22. Removal of the resistance cassette resulted in cosmid couSS01.

Integration of the coumermycin biosynthetic gene cluster (contained in cosmid couMW16) into the ΦC31 attachment site of the genome of *Streptomyces coelicolor* M1146 results in heterologous production of coumermycin A_1 (Flinspach *et al.*, 2010; Gomez-Escribano & Bibb, 2011; Wolpert *et al.*, 2008). We now replaced the 7.1 kb fragment comprising the genes *couM-couN7* on cosmid couMW16 by an apramycin

resistance cassette, using RED/ET mediated recombination (Gust *et al.*, 2003). This initial work was conducted by Dr. Manuel Wolpert and resulted in cosmid couMW22 (**Figure III.11**). To avoid a possible polar effect of the inactivation on downstream genes, the resistance cassette was removed by digestion with *Xba*I and religation. Originally, digestion with *Xba*I and *Spe*I was planned to remove the resistance cassette, but the *Spe*I site got lost during recombination. Therefore, the resulting cosmid couSS01 contained a "scar" sequence of 49 bp instead of 6 bp, which resulted in a frame-shift and the loss of the *couN7* stop codon. However, the frame-shift also resulted in another stop codon, located in the intergenic region upstream of *couO.* Hence cosmid couSS01 was introduced into *S. coelicolor* M1146 by conjugation and the genotype of the integration mutant was verified by PCR.

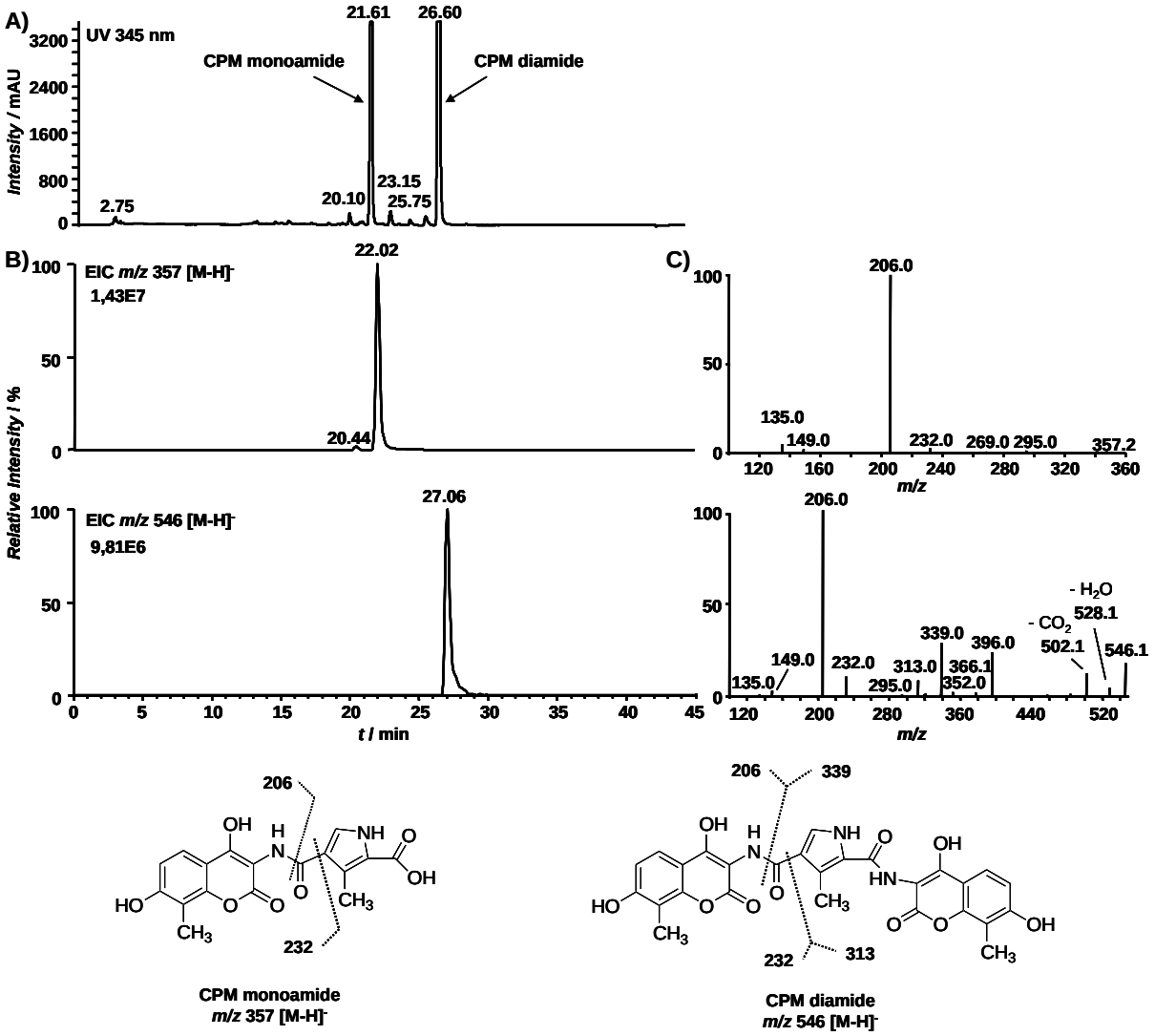


Figure III.12
Production of CPM monoamide and CPM diamide by *S. coelicolor* M1146(couSS01). A) HPLC-UV analysis of the culture supernatant. B) HPLC-MS extracted ion chromatograms (EIC) showing CPM monoamide (*m/z* 357) and CPM diamide (*m/z* 546). C) Collision-induced dissociation (CID)-MS/MS spectra. See Figure VI.2 in the Appendix for suggested structural formulas of the mass spectrometric fragments**.**

When *S. coelicolor* M1146(couSS01) was cultured in the production medium CDM, CPM diamide and CPM monoamide were readily detected after seven days by HPLC-UV and HPLC-MS analysis in 4 mg/L and 12 mg/L yield, respectively (**Figure III.12**). Since both compounds contain the complete central pyrrole moiety of coumermycin A_1, this experiment provided conclusive evidence that none of the genes from *couN1* to *couN7* is required for the formation of CPM (and neither is *couM*). Therefore, the pathways to the central and the terminal pyrrole moieties of coumermycin do not share the same biosynthetic enzymes.

2.2. The genes *couR1-couR4* are involved in the biosynthesis of the central pyrrole moiety of coumermycin

The genes *couR1-couR4* within the coumermycin biosynthetic gene cluster do not have orthologs in the gene clusters of novobiocin or clorobiocin, and their function is yet unknown. By comparison to database entries, these genes were originally annotated as hypothetical protein (*couR1*), decarboxylase (*couR2*), threonine kinase PduX (*couR3*) and dehydrogenase (*couR4*) (Wang *et al.*, 2000). The predicted product of *couR2* was a 377 aa protein. However, renewed sequencing of cosmid couMW16 proved that the original sequence AF235050 was incorrect: one nucleotide (i.e. C24843) needed to be deleted. This results in a TGA stop codon 5 bp downstream of the deleted cytidine. A putative GTG start codon overlaps with this stop codon (GTGA, stop codon underlined). Therefore, this DNA region codes for two proteins, i.e. CouR2a (172 aa) and CouR2b (205 aa). Database comparison shows several decarboxylases (e.g. sulfopyruvate decarboxylase) consisting of an α- and a β-subunit which closely correspond in sequence and size to CouR2a and CouR2b. Therefore, we corrected the sequence AF235050 in GenBank.

The five genes *couR1*, *couR2a*, *couR2b*, *couR3* and *couR4* were separately replaced on cosmid couMW16 by an apramycin resistance cassette, using RED/ET mediated recombination (Gust *et al.*, 2003). This work was done by Anna Knuplesch and is described in detail in her diploma thesis (Knuplesch, 2008). In case of *couR3* and *couR4* the complete open reading frame was replaced, leaving only the start and stop codon intact. In case of *couR1*, *couR2a* and *couR2b* an internal sequence, comprising approximately half of each gene, was replaced, because the start and/or stop codons of these genes overlap and a complete replacement may have affected the presumed translational coupling. The resistance cassette was removed in all cases by digestion with *Xba*I and *Spe*I and religation, leaving only a 6 bp “scar” sequence. The resulting integrative cosmids, termed couAK06 – couAK10, were introduced into *S. coelicolor* M1146 by conjugation and the genotype of the mutants was verified by PCR.

Three independent clones of each mutant (two independent clones in case of Δ*couR2a*) were cultivated and coumermycin A_1 production was determined by HPLC-UV and HPLC-MS analysis. Strain *S. coelicolor* M1146(couMW16), harbouring the intact gene cluster, was used as positive control. The results are shown in **Table III.1**. Deletion of *couR1* and *couR4* led to the total abolishment of coumermycin A_1 production. Deletion of *couR2a*, *couR2b* and *couR3* reduced coumermycin A_1 production by 99.8 %, 92% and 99% in comparison to the reference strain, but HPLC-UV and HPLC-MS analysis still showed a residual production of the antibiotic.

We speculated that genes of the expression host compensated for the gene deletions to some extent, allowing the residual coumermycin A_1 production. Indeed, a BLAST search revealed that the genome of the expression host *Streptomyces coelicolor* contains orthologs of *couR2a/2b* and *couR3*, i.e. *sco6824* (NP_733715) similar to *couR2a/2b* and *sco0991* (NP_625287) similar to *couR3*.

The Δ*couR1* and Δ*couR4* mutants were complemented with intact copies of the deleted genes, cloned into replicative expression vectors (see Materials and Methods). The respective plasmids pAK06 and pAK10 were constructed by Anna Knuplesch and are also described in her diploma thesis (Knuplesch, 2008). Coumermycin A_1 production was restored by the complementation, but only to 2-3 % of the production level found in the reference strain, *S. coelicolor* M1146(couMW16). Low production rates in complemented mutants are encountered frequently and may be due to e.g. incorrect regulation of the gene expression, or to problems of protein folding or protein interactions.

2.3. Feeding of the central pyrrole moiety to the deletion mutants

The above described inactivation experiments showed that the genes *couR1-couR4* are involved in the biosynthesis of coumermycin A_1, but did not prove that they are involved specifically in the biosynthesis of the central pyrrole moiety. In order to show that these genes are responsible for the biosynthesis of the central pyrrole moiety, 3-methylpyrrole-2,4-dicarboxylic acid was added to the cultures of the mutants after 24h and 48h of cultivation, to give a final concentration of 0.1 mmol/L (i.e. 16.9 mg/L). Coumermycin production was restored by this external feeding in the Δ*couR1*, Δ*couR2b*, Δ*couR3* and Δ*couR4* mutants (**Table III.1**). In the Δ*couR2a* mutant, production was increased under external feeding by a factor of ten, but still remained lower than in the other strains. The reason for this is unknown, but we noticed that only few integration mutants could be obtained using the Δ*couR2a* cosmid, and the obtained mutants showed relatively slow growth.

These results provided evidence that the four genes *couR1*, *couR2b*, *couR3* and *couR4*, and probably also *couR2a*, are involved in the biosynthesis of the central pyrrole moiety of coumermycin A_1.

Table III.1
Restoration of coumermycin A_1 production in gene deletion mutants by feeding of the central pyrrole moiety (CPM)

	coumermycin A_1 [mg/L]	
	no CPM added	+ CPM (16.9 mg/L)
intact gene cluster	10.2	n.d.[a]
Δ*couR1*	< 0.01	3.8
Δ*couR2a*	0.02	0.2
Δ*couR2b*	0.8	5.1
Δ*couR3*	0.1	6.6
Δ*couR4*	< 0.01	5.0

[a] n.d. = not determined

2.4. Feeding of *O*-Phospho-L-threonine to the Δ*couR3* mutant

CouR3 (302 aa) shows similarity to kinases of the GHMP family, e.g. 34% identity to the threonine kinase PduX involved in adenosyl-cobalamine biosynthesis in *Salmonella enterica* (Fan & Bobik, 2008; Fan *et al.*, 2009). We therefore speculated that the biosynthesis of the central pyrrole moiety of coumermycin may possibly involve an *O*-phosporylation of threonine. Fan and Bobik (2008) were able to show that feeding of *O*-phospho-L-threonine to a *pduX* defective mutant of *Salmonella enterica* was able to compensate for the growth defect caused by the deletion of *pduX*. Therefore, we tested whether feeding of *O*-phospho-L-threonine to the Δ*couR3* mutant could restore the production of coumermycin A_1.

O-Phospho-L-threonine was added to the cultures of three independent clones of the *couR3* defective mutant after 24h and 48h of cultivation, to give a final concentration of 0.1 mmol/L. In a parallel experiment, L-threonine was fed as control. Four independent replicates were carried out for each experiment. However, the results were not fully conclusive (**Table III.2**): feeding of *O*-phospho-L-threonine reproducibly increased coumermycin production, but only from 0.137 to 0.197 mg/L (i.e. by 44 %). In contrast, feeding of L-threonine had no significant effect on coumermycin production. Though this experiment showed a positive effect of *O*-phospho-L-threonine on coumermycin production in the Δ*couR3* mutant, the effect is too small in order to provide strong support for a role of *O*-phospho-L-threonine in the biosynthesis of CPM.

Table III.2
Coumermycin A_1 production of the Δ*couR3* mutant after feeding of L-threonine (Thr) and *O*-phospho-L-threonine (Thr-P)

clone[a]	Δ*couR3* mean (mg L^{-1})	Δ*couR3* SD (mg L^{-1})	Δ*couR3* + Thr mean (mg L^{-1})	Δ*couR3* + Thr SD (mg L^{-1})	Δ*couR3* + Thr-P mean (mg L^{-1})	Δ*couR3* + Thr-P SD (mg L^{-1})
1	0.14 (100%)	0.05	0.16 (114%)	0.03	0.24 (171%)	0.08
2	0.15 (100%)	0.07	0.14 (93%)	0.03	0.17 (113%)	0.05
3	0.12 (100%)	0.01	0.13 (108%)	0.01	0.18 (150%)	0.02
average	0.137 (100%)		0.143 (104%)		0.197 (144%)	

[a] Three independent clones were generated of the Δ*couR3* strain *Streptomyces coelicolor* M1146(couAK08). Four independent feeding experiments were carried out for each clone, and mean and standard deviation (SD) of these experiments are reported.

2.5. Intact incorporation of [U-^{13}C,^{15}N]L-threonine into the central pyrrole moiety of coumermycin

The sequence similarity of CouR3 to a threonine kinase and the above feeding experiment with *O*-phospho-L-threonine hinted at the possibility that L-threonine may be a precursor of the central pyrrole moiety of coumermycin A_1. To provide unambiguous proof, we carried out a feeding experiment with [U-^{13}C,^{15}N]L-threonine, followed by MS and NMR analysis. To ease the investigation of the coupling patterns in NMR analysis, we used the above described heterologous expression strain containing the construct couSS01 (**Figure III.11**) and isolated the produced CPM

monoamide (M_R = 358.3, **Figure III.10A**), instead of using a strain producing coumermycin A_1 (M_R = 1110.1, **Figure III.10A**). NMR analysis and data evaluation were in all cases conducted by Nadja Burkard (AK Grond, Organic Chemistry Department, University of Tübingen).

Initially, unlabeled monoamide was isolated from cultures of *S. coelicolor* M1146(couSS01) in an amount of 8.3 mg from two successive cultivations, and the ^{13}C-NMR signals of this compound were assigned by 2D-NMR methods (see Appendix). The CPM monoamide was isolated as a pure regioisomer, with the aminocoumarin moiety linked to the carboxyl group at position 4 of the pyrrole ring (**Figure III.14**).

In a preliminary, small-scale feeding experiment, [U-^{13}C,^{15}N]L-threonine was added to 6 mL culture volume, using a 24-square deepwell plate for cultivation (Siebenberg *et al.*, 2010). The culture supernatant was analysed by HPLC-MS in comparison to cultures without feeding of labeled threonine (**Figure III.13**). The CPM monoamide formed in the feeding experiment showed a strong MS signal with *m/*z 362 [M-H]$^-$, additionally to the regular molecular ion (M-H$^-$ = 357, **Figure III.13**). This fragment (M-H+5) indicated that the four ^{13}C carbons and the ^{15}N nitrogen of the labeled L-threonine had been incorporated into the CPM monoamide. The fragmentation pattern of the *m/z* 362 ion showed *m/z* 206, i.e. the unlabeled aminocoumarin ring, as the most abundant fragment, demonstrating that the label had been incorporated into the pyrrole rather than into the aminocoumarin moiety.

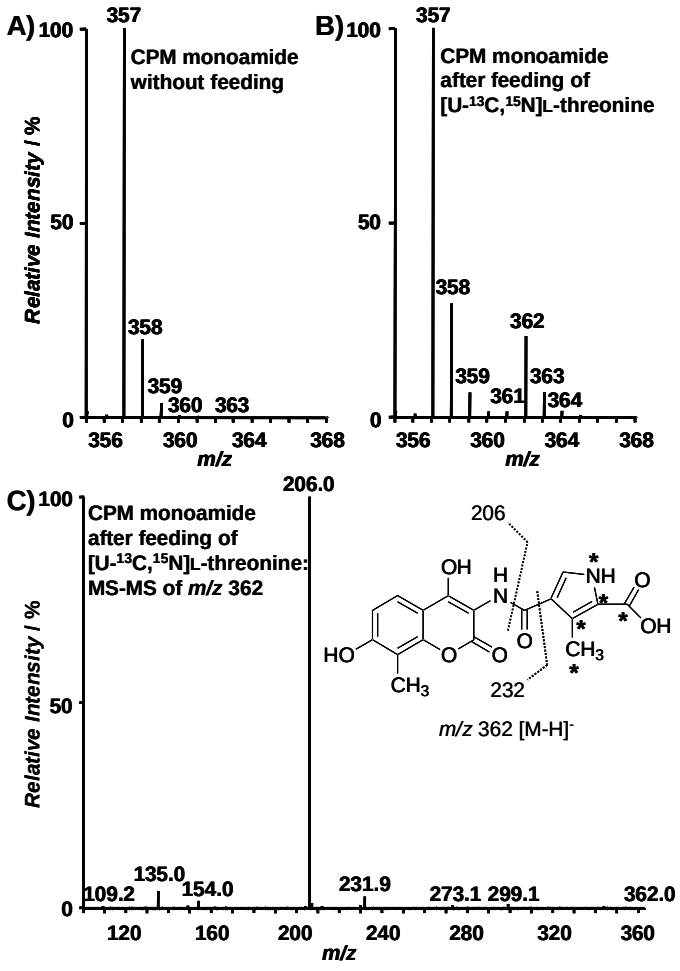


Figure III.13
Incorporation of [U-^{13}C,^{15}N]L-threonine into CPM monoamide, shown by mass spectrometric analysis. Mass spectra A) of the unlabeled CPM monoamid and B) of the labeled CPM monoamide. C) CID-MS/MS spectrum of the labeled CPM monoamide. The expected positions of the label are indicated by asterisks. For the CID-MS/MS spectrum of the unlabeled CPM monoamide see Figure III.12.

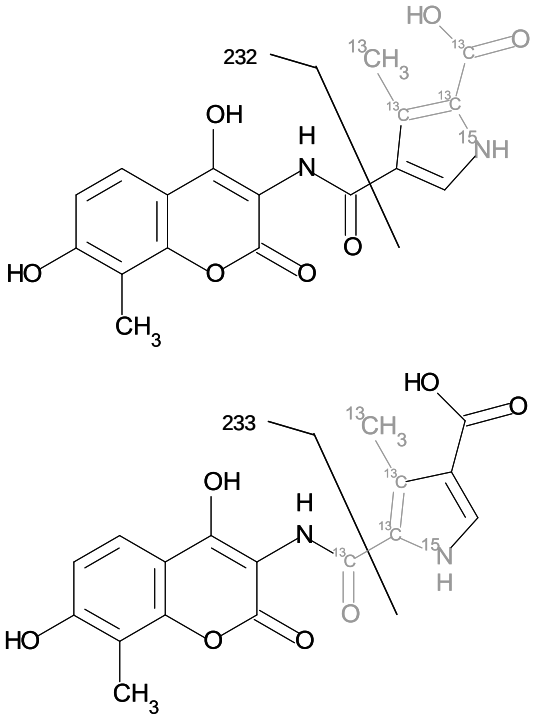


Figure III.14
Expected fragmentation of the two possible regioisomers of CPM monoamide labeled by feeding of [U-^{13}C,^{15}N]L-threonine.
Experimentally, the $[M-H]^-$ = 232 fragment was observed (see Figure III.13), indicating that the CPM monoamide has the upper structure.

The exact regiochemistry of the incorporation of the labeled L-threonine was determined by ^{13}C-NMR spectroscopy of CPM monoamide isolated from a subsequent large-scale experiment. Therefore, [U-^{13}C,^{15}N]L-threonine (96 mg; final concentration 2.15 mmol/L) was added to 360 mL of growing cultures of *S. coelicolor* M1146(couSS01) in five 24-square deepwell plates (see Materials and Methods). The cultures were harvested six days after inoculation, and cells were removed by centrifugation. The culture medium was acidified to pH 2.0, resulting in the precipitation of the monoamide. This was collected by centrifugation, dissolved in methanol and purified by preparative HPLC (yield 2.1 mg). NMR analysis and data evaluation were then conducted by Nadja Burkard (AK Grond, Organic Chemistry Department, University of Tübingen). The ^{13}C-NMR spectrum (**Figure III.15**) showed an average isotope enrichment of 19.5 % for pyrrole carbons C-2, C-3, C-6 and C-7. The clear coupling pattern with $^{1}J_{C\text{-}C}$, $^{2}J_{C\text{-}C}$ and $J_{C\text{-}N}$ couplings (**Table III.3**) proved unequivocally that the intact carbon skeleton and the nitrogen of [U-^{13}C,^{15}N]L-threonine were incorporated into the CPM monoamide (**Figure III.16**). Therefore, the above mentioned four carbon atoms and the ring nitrogen of the central pyrrole moiety of coumermycin A_1 are derived from threonine. A conversion of threonine to proline does not occur in general amino acid metabolism. Accordingly, proline is not the precursor of the central pyrrole moiety of coumermycin A_1, but the producing organism uses two different biosynthetic pathways towards the central and the terminal pyrrole moieties of the antibiotic.

A second feeding experiment with [U-^{13}C]fumaric acid was conducted to identify the second precursor of CPM. Unfortunately, no ^{13}C-enrichment was observed in the NMR analysis of the isolated CPM monoamide. Therefore, fumaric acid is probably not a precursor of CPM. However, fumaric acid was only used because no ^{13}C-labeled oxaloacetate, the postulated second precursor (**Figure IV.2**), was available. We expected fumaric acid to be converted to oxaloacetate in the citric acid cycle via maleic acid, which also is a probable precursor of CPM (Berg *et al.*, 2011).

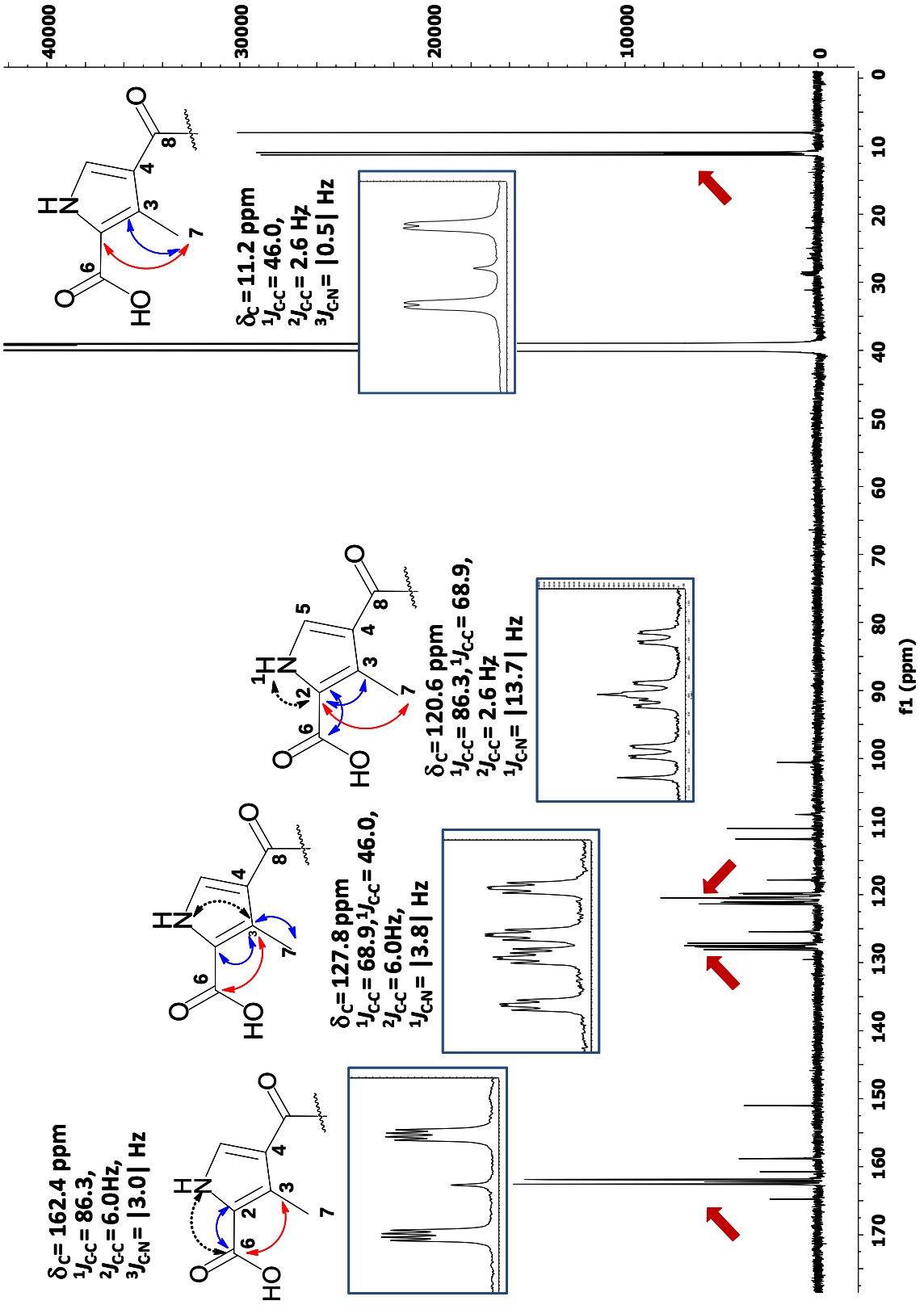


Figure III.15
^{13}C-NMR analysis of the incorporation of [U-^{13}C,^{15}N]L-threonine into CPM monoamide (125.7 MHz, d_6-DMSO, cold probe). Expansions of ^{13}C-NMR coupling patterns of all enriched pyrrole carbon atoms with respective coupling constants (in Hz). Structural fragments with respective couplings.

Table III.3
^{13}C NMR chemical shifts and enrichment ratios of CPM monoamide, isolated after feeding of [U-^{13}C,^{15}N]L-threonine in d_6-DMSO

Carbon	δ_C (ppm)[b]	J_{CC} (Hz)[c]	J_{CN} (Hz)	Enrichment (%)[d]
C-2	120.6	86.3 (C-6) 68.9 (C-3) 02.6 (C-7)	|13.7|	20
C-3	127.8	68.9 (C-2) 46.0 (C-7) 06.0 (C-6)	|3.8|	19
C-4	118.0	[a]	[a]	[a]
C-5	125.5	[a]	[a]	[a]
C-6	162.4	86.3 (C-2) 06.0 (C-3)	|3.0|	19
C-7	011.2	46.0 (C-3) 02.6 (C-2)	|0.5|	20
C-8	160.9	[a]	[a]	[a]
C-2’	164.8	[a]	[a]	[a]
C-3’	100.5	[a]	[a]	[a]
C-4’	159.3	[a]	[a]	[a]
C-5’	121.5	[a]	[a]	[a]
C-6’	111.9	[a]	[a]	[a]
C-7’	158.9	[a]	[a]	[a]
C-8’	110.3	[a]	[a]	[a]
C-9’	151.2	[a]	[a]	[a]
C-10’	108.3	[a]	[a]	[a]
C-11’	008.1	[a]	[a]	1.8

[a] not detected. [b] Chemical shifts were referenced to residual DMSO. [c] coupling partners are shown in parentheses. [d] Method for the calculation of enrichment is shown in the Appendix

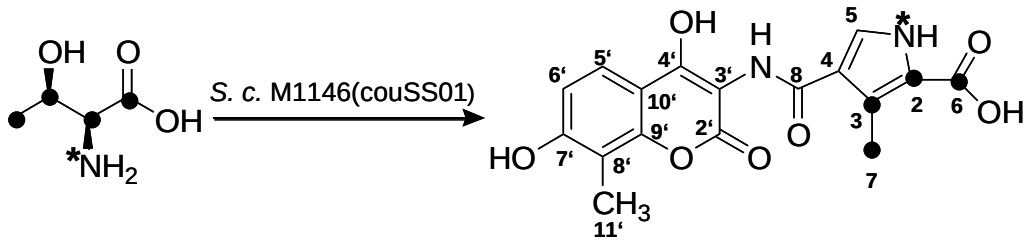


Figure III.16
^{13}C enrichment in CPM monoamide after feeding of [U-^{13}C,^{15}N]L-threonine.

3. Identification and structural elucidation of new caprazamycins from *Streptomyces sp.* MK730-62F2 by LC/ESI-MS/MS

For the identification of new caprazamycin and liposidomycin derivatives, e.g. resulting from inactivation experiments, a rapid and precise analytical method is required that also supplies sufficient structural information. High-performance liquid chromatography coupled to a triple-stage quadrupole mass spectrometer is capable of both, separating complex mixtures of secondary metabolites and providing reliable information on the analyte's mass and structure.

3.1. Identification of caprazamycins A-G and of liposidomycin A in culture extracts of the respective producer strains

Caprazamycins and liposidomycins are structurally complex compounds showing acidic as well as basic functional groups (**Figure III.17**). Therefore, the liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) analysis was conducted both in positive and in negative ionization mode, using culture extracts of the caprazamycin producer strain *Streptomyces sp.* MK730–62F2 and the liposidomycin producer strain *Streptomyces sp.* SN-1061M. The $[M+H]^+$ and $[M-H]^-$ ions of the caprazamycins A–G and liposidomycin A (**Figure III.17**) were readily detected in the extracts, showing that both positive and negative ionization mode are suitable for the analysis of those compounds.

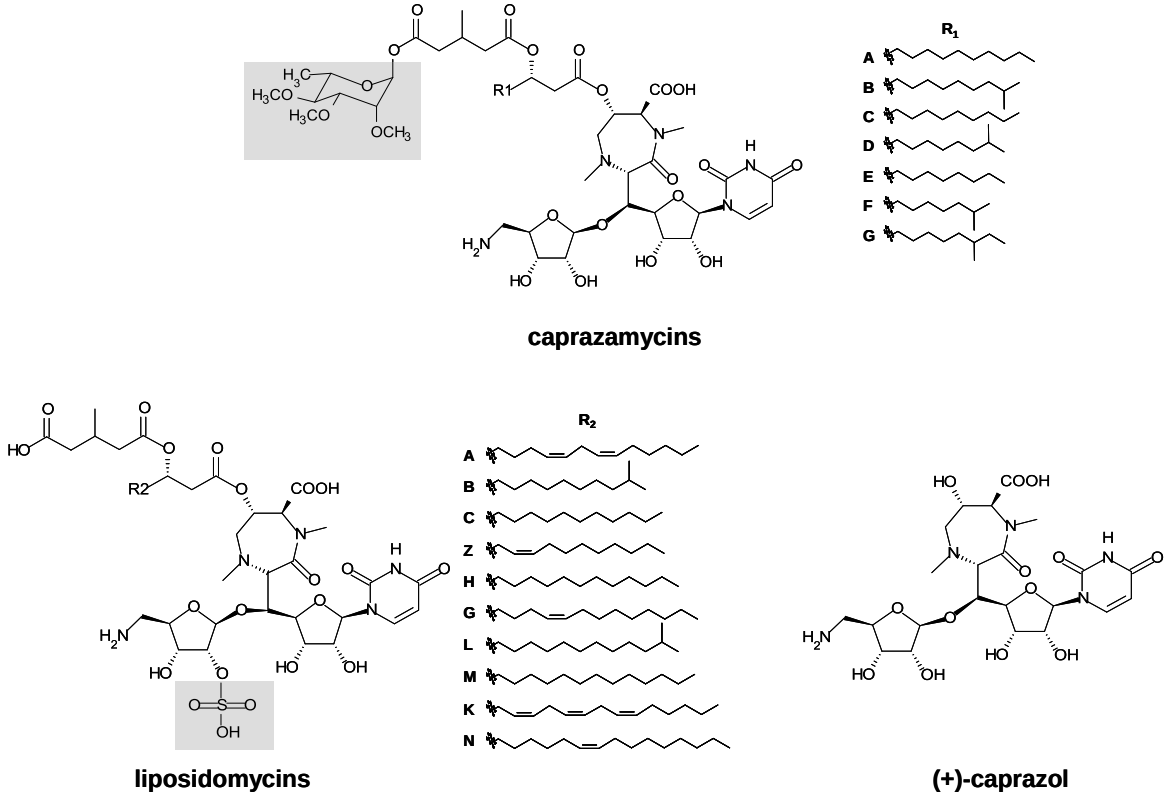


Figure III.17
Chemical structures of caprazamycins, liposidomycins and (+)-caprazol. The structural differences between caprazamycins and liposidomycins are shaded in grey.

The collision-induced dissociation (CID) MS/MS spectra of the caprazamycins A–G and liposidomycin A were recorded in positive and negative ionization mode as well. Caprazamycins and liposidomycins show a characteristic fragmentation pattern, which reflects the structural similarities as well as the structural differences of the analysed compounds (**Figure III.18** and **Table VI.1**, see Appendix). The product ions formed by CID of the $[M+H]^+$ and $[M–H]^-$ precursor ions can be divided into two groups. Product ions from the first group show distinctive *m/z* values for the different caprazamycins or liposidomycins, respectively, according to the differences in the mass of the fatty acid side chain. Product ions from the second group lack the fatty acid side chain and are therefore characteristic for all caprazamycins and/or liposidomycins. Characteristic product ions in positive ionization mode for both caprazamycins and liposidomycins, with *m/z* 558 and *m/z* 427, result from the successive loss of the fatty acid side chain and of the aminoribose. Product ions with *m/z* 320 and *m/z* 189 are only present in the mass spectra of caprazamycins, since liposidomycins lack the permethylated L-rhamnose moiety (**Figure III.17**). Further product ions, *m/z* 1002, *m/z* 813 and *m/z* 701 in the case of caprazamycin C, derive from the loss of the aminoribose and of the uracil prior to the loss of the fatty acid side chain. For the liposidomycins the first step in the fragmentation pathway seems to be the loss of the sulfate group, which is consistent with the published fast atom bombardment mass spectrometry (FAB-MS) data (Kimura *et al.*, 1998b; Ubukata *et al.*, 1988). In negative ionization mode characteristic fragments resulted from the successive loss of the fatty acid, of the carboxyl group at the caprazol and of the aminoribose with *m/z* values of 556, 512 and 363, respectively. Since the loss of the sulfate group occurs less frequently in negative mode, characteristic product ions with *m/z* 636 and 592 can be found in the mass spectra of the liposidomycins. The sulfated product ions show higher relative intensities than the corresponding non-sulfated product ions (2% for *m/z* 636 vs. <0.1% for *m/z* 556 and 100% for *m/z* 592 vs. <0.1% for *m/z* 512), which can be explained by the good ionization of the sulfate group in negative mode. Product ions containing the fatty acid and the 3-methylglutaryl moiety can also be detected as well as the fatty acid side chain alone.

3.2. Purification of culture extracts by affinity chromatography

The samples used in this study were derived from the extraction of the acidified culture supernatant with n-butanol. However, not only the caprazamycins or liposidomycins were extracted by that but a wide range of culture components which could interfere with the caprazamycins during HPLC and LC/MS analysis. Affi-Gel® boronate is a boronic acid-containing gel which specifically binds cis-diol structures, like the ribose of nucleosides, by formation of a cyclic boronate ester at alkaline pH values. At acidic pH values the ester bond is cleaved, resulting in elution of the cis-diol-containing compounds. Since caprazamycins and liposidomycins contain a nucleosidic structure, purification by the described method should be possible. The protocol used was modified from Bullinger *et al.* (2007) and is shown in **Table III.5**.

A bioactivity assay with *Mycobacterium phlei* showed the highest bioactivity for washing step 2 (6 mL methanol/water 1:1 (v/v)), comparable to the bioactivity of the unpurified culture extract (**Figure III.19A**). It was confirmed later by LC/ESI-MS/MS analysis that the caprazamycins already eluted in the second washing step and not in the elution step.

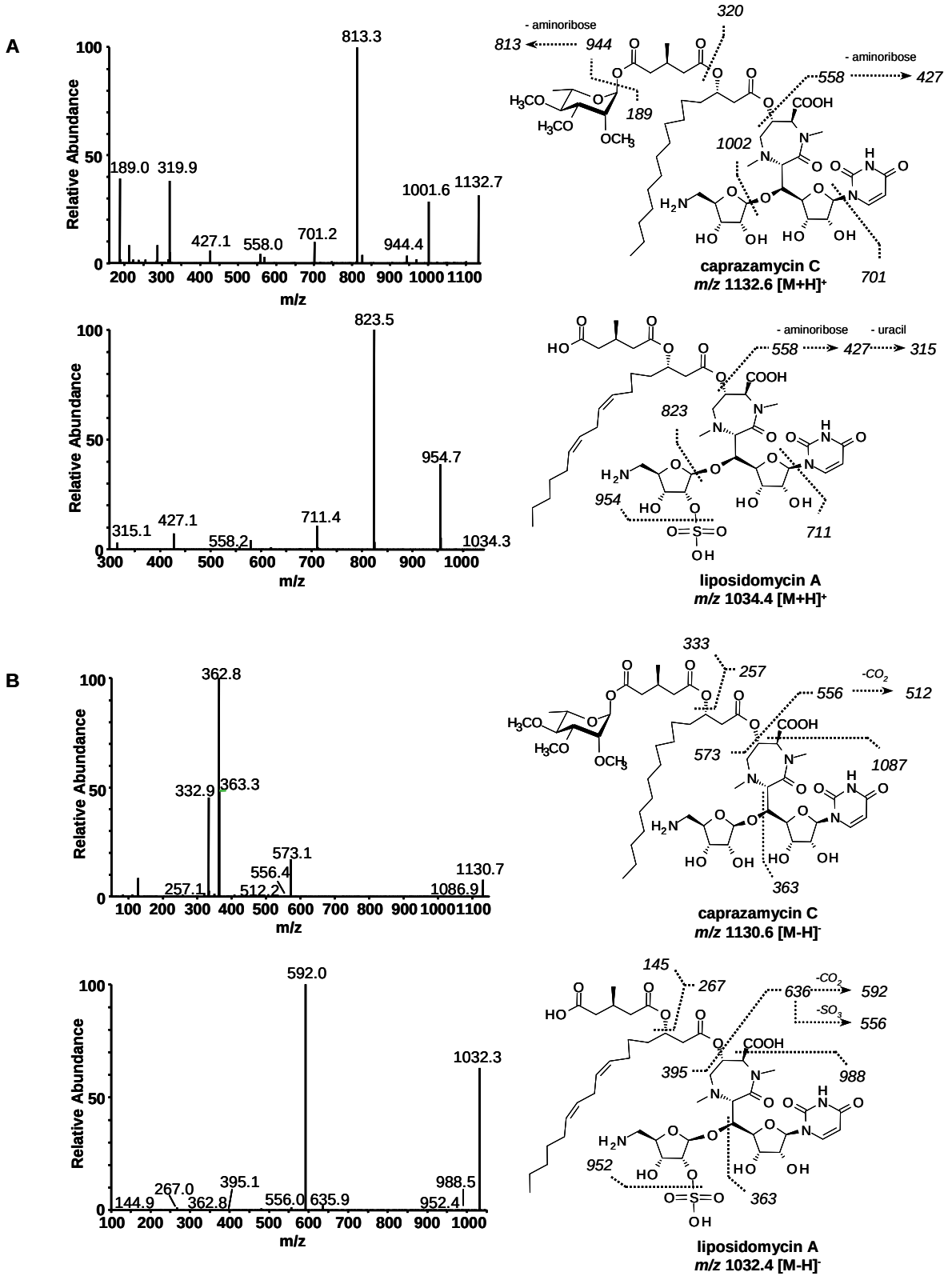


Figure III.18
Characteristic CID-MS/MS spectra of caprazamycin C from *Streptomyces sp*. MK730–62F2 and liposidomycin A from *Streptomyces sp.* SN-1061M in A) positive and B) negative ionization mode with the suggested fragmentations. The collision energy applied was 35V for positive and 30V for negative ionization mode, respectively.

However, after the washing step was changed to 6 mL of methanol/water 2:8 (v/v) the caprazamycins eluted, as desired, in the following elution step with 0.2 M formic acid in methanol/water 1:1 (v/v) (**Figure III.19B**). The colour of the extract changed visibly after affinity chromatography from dark red to light yellow and the peaks of the caprazamycins in the Q3 full scan, between 16 and 25 minutes, became more distinct. Additionally, the inconsistent broad peak at about 13 minutes significantly decreased (**Figure III.20**). Interestingly, the Q3 full scan showed more peaks between 16 and 25 min than there are known caprazamycins and therefore, a scan for new caprazamycins was conducted.

Table III.5
Affinity chromatography with Affi-Gel® boronate. Comparison of the original method modified by Bullinger *et al*. (2007) and the optimized method for the purification of caprazamycins and liposidomycins.

Step		Buffer / Solution		Volume
		Original method (Bullinger *et al.*, 2007)	Optimized method	
1	Preconditioning	NH_4OAc buffer (0.25 M, pH 8.8)	NH_4OAc buffer (0.25 M, pH 8.8)	45 mL
2	Sample	Culture extract (0.3 mL) in NH_4OAc buffer (0.25 M, pH 8.8)	Culture extract (0.3 mL) in NH_4OAc buffer (0.25 M, pH 8.8)	10 mL
3	Washing step 1	NH_4OAc buffer (0.25 M, pH 8.8)	NH_4OAc buffer (0.25 M, pH 8.8)	20 mL
4	Washing step 2	***MeOH/H_2O 1:1 (v/v)***	***MeOH/H_2O 2:8 (v/v)***	6 mL
5	Elution	Formic acid solution (0.2 M)	Formic acid solution (0.2 M)	50 mL
6	Regeneration	***MeOH/H_2O 1:1 (v/v)***	***MeOH/H_2O 2:8 (v/v)***	25 mL

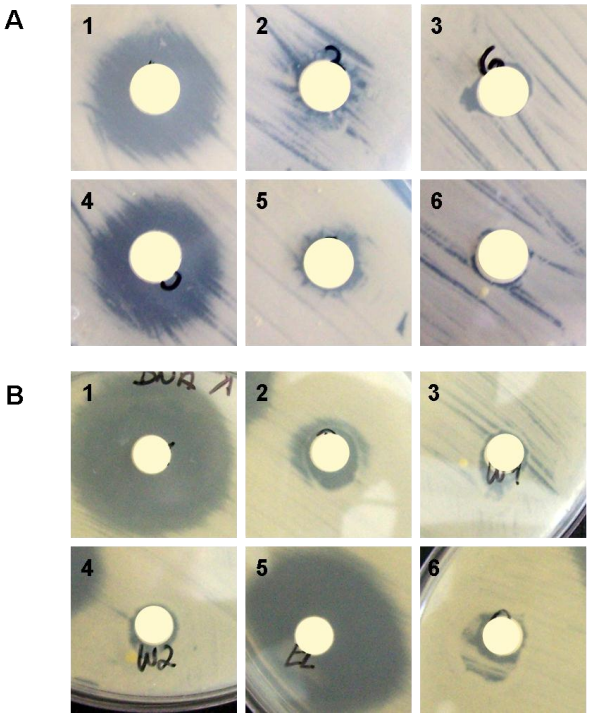


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Figure III.19
Bioactivity assay with *Mycobacterium phlei* after purification of the caprazamycins by affinity chromatography with Affi-Gel® boronate. A) Original method modified by Bullinger *et al.* (2007) and B) optimized method. 1: culture extract (unpurified); 2: sample flow through; 3: washing step 1; 4: washing step 2; 5: eluate; 6: regeneration of the column (see Table III.5 for details)

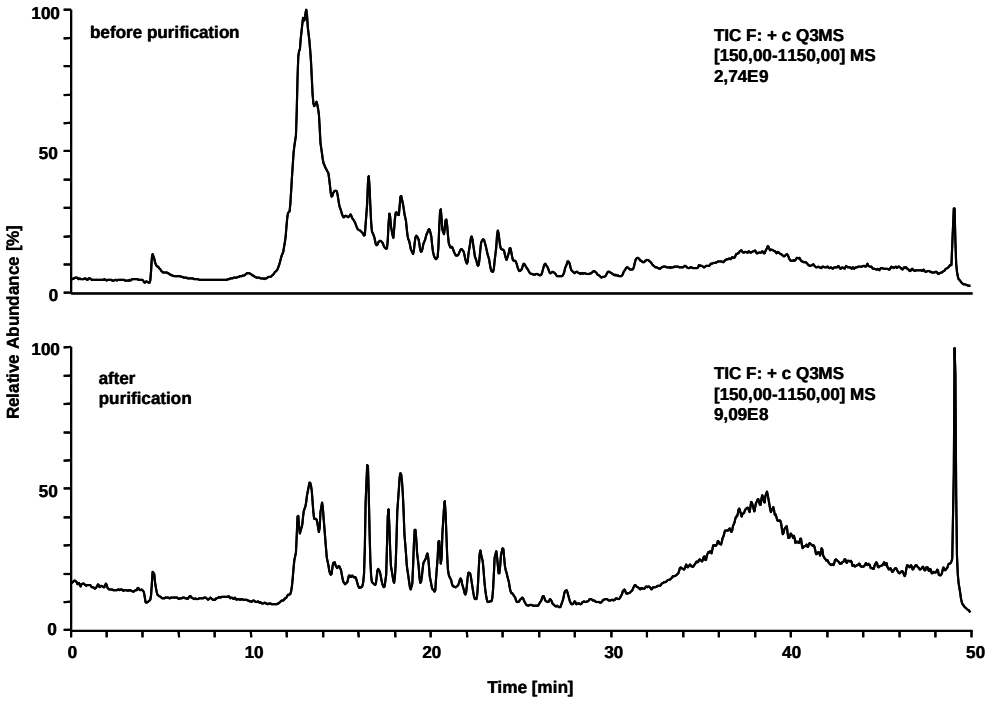

Figure III.20
Mass chromatograms of Q3 full scans before and after purification of the culture extract by affinity chromatography on a boronic acid gel (optimized method). The peaks of the caprazamycins can be found at retention times between 16 and 25 min.

3.3. Identification of new caprazamycins

The search for new caprazamycins was performed by precursor ion scans in positive ion mode with *m/z* 558, 427, 320 and 189 and in negative ion mode with *m/z* 333 and 363. Precursor ion scans allow the identification of new derivatives from a known structural class of compounds by scanning for characteristic product ions. Besides the known caprazamycins A–G with *m/z* 1146.6, 1132.6 and 1118.5 six new compounds with *m/z* 1114.5, 1116.5, 1142.5, 1144.6, 1170.6 and 1172.6 were detected in the precursor ion scans with *m/z* 189 (permethylated L-rhamnose) (**Figure III.21**) and 320 (3-methylglutaryl moiety + permethylated L-rhamnose; data not shown) but not with the two other product ions. The same result was achieved in negative ion mode with *m/z* 333 and 363 (data not shown). The CID-MS/MS spectra of the six new compounds in positive ionization mode show the typical fragmentation pattern of the caprazamycins (**Figure III.22** and **III.23**) and the mass differences to the known caprazamycins suggest the incorporation of mono and di-unsaturated fatty acids. In rising order of retention time these new caprazamycins were termed caprazamycin H, I, J, K, L and M. Complete structural elucidation of their fatty acid moieties was not possible with the available mass spectrometric instrumentation, but the proposed chemical formula fit that of the fatty acid moieties from the liposidomycins Y, Z, A, G, K and N, respectively (**Figure III.21** and **Table VI.2**, see Appendix). Since there could be several fatty acids with the same chemical formula the caprazamycins H–M could each consist of not only one but several compounds. A full structural elucidation of the fatty acid side chains will therefore be mandatory.

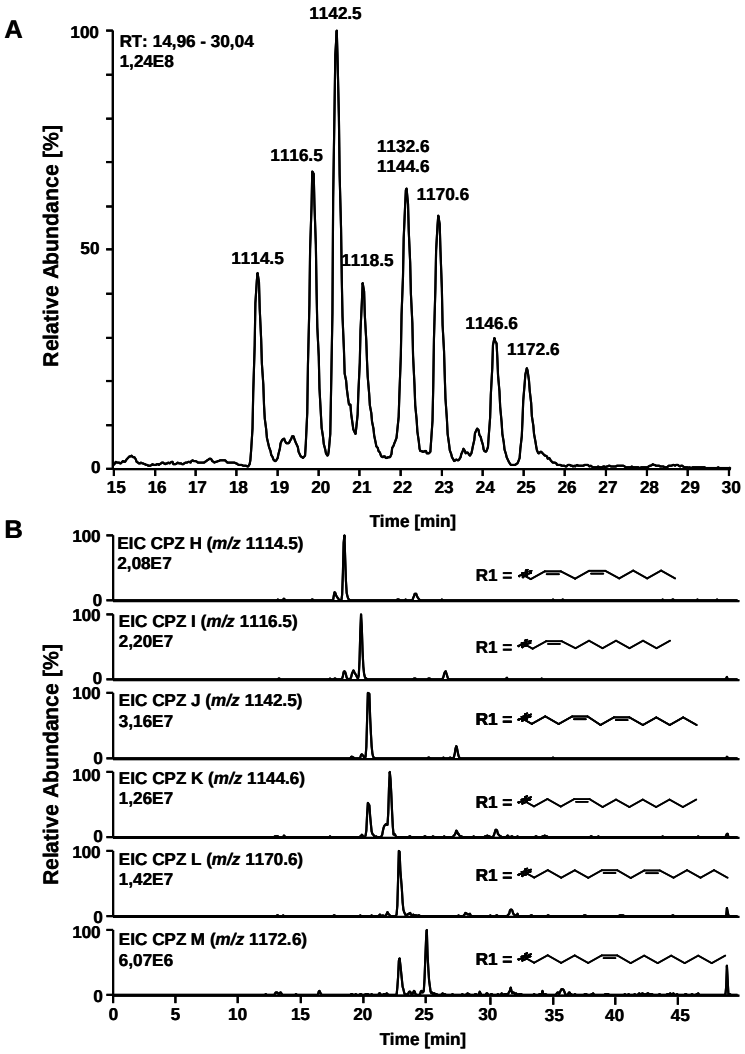

Figure III.21
A) Precursor ion scan with *m/z* 189 (L-rhamnose moiety) and B) extracted ion chromatograms (EIC) of the new caprazamycins (CPZ) H–M with the suggested fatty acid residues (R1) found in the liposidomycins.

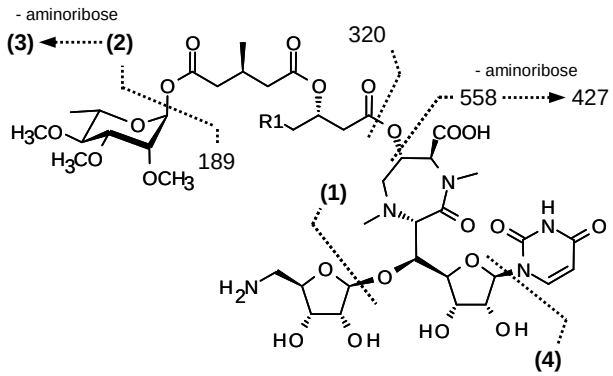

Figure III.22
Fragmentation scheme of the caprazamycins H–M in positive mode. The CID-MS/MS spectra of the new caprazamycins are shown in Figure III.23.

	CPZ	R1	(1)	(2)	(3)	(4)
A)	H	$C_{11}H_{19}$	984	926	795	683
B)	I	$C_{11}H_{21}$	986	928	797	685
C)	J	$C_{13}H_{23}$	1012	954	823	711
D)	K	$C_{13}H_{25}$	1014	956	825	713
E)	L	$C_{15}H_{27}$	1040	982	851	739
F)	M	$C_{15}H_{29}$	1042	984	853	741

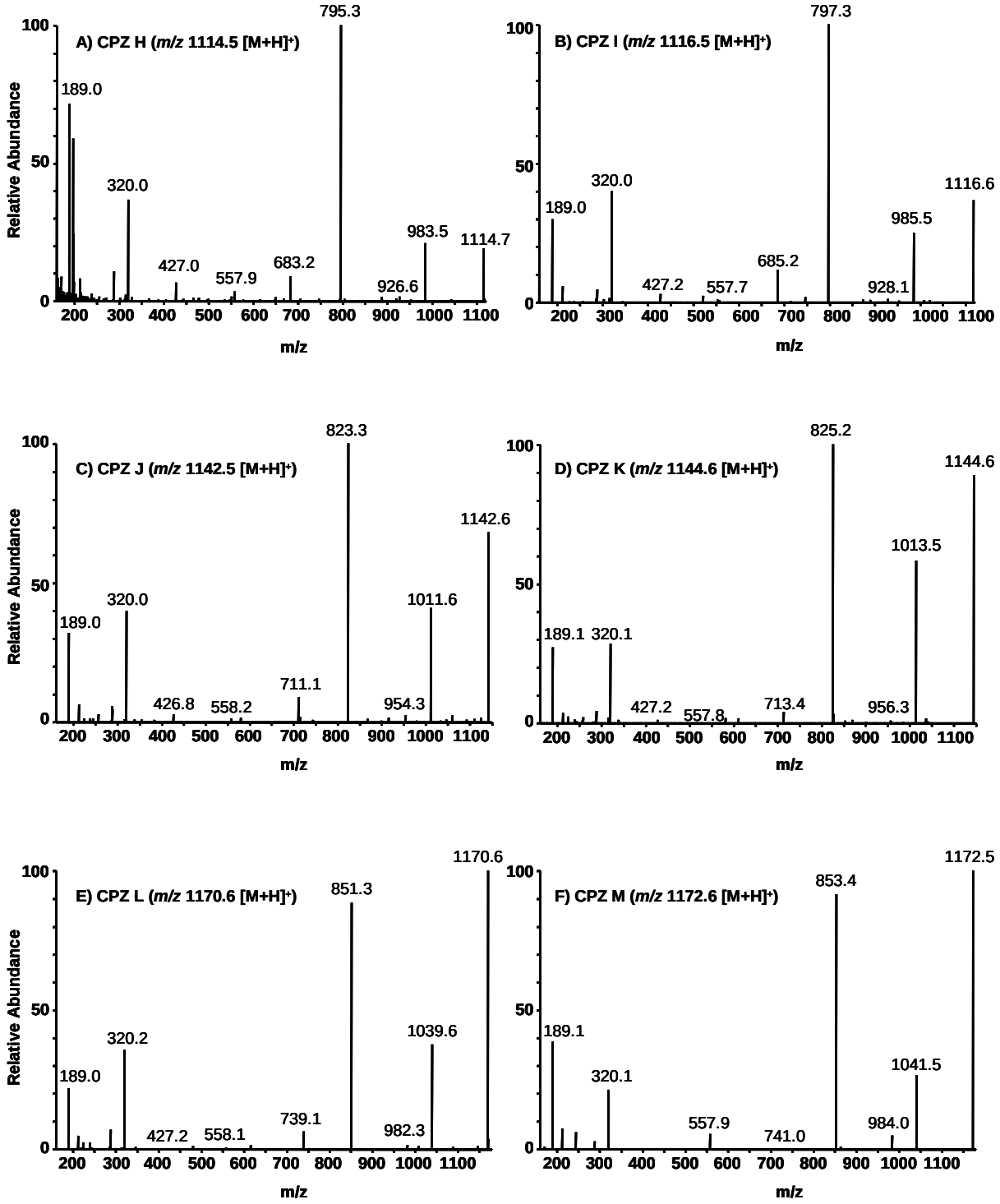

Figure III.23
CID-MS/MS spectra of the six new caprazamycins in positive ionization mode: A) caprazamycin H (*m/z* 1114.5), B) caprazamycin I (*m/z* 1116.5), C) caprazamycin J (*m/z* 1142.5), D) caprazamycin K (*m/z* 1144.6), E) caprazamycin L (*m/z* 1170.6) and F) caprazamycin M (*m/z* 1172.6). The fragmentation scheme is presented in **Figure III.22**.

IV. DISCUSSION

1. Antibiotic production in 24-square deepwell plates

Investigations on antibiotic formation in *Streptomyces* are often hampered by the notorious variability of production rates observed between successive and even between parallel cultivation batches of the same strain in Erlenmeyer flasks (Minas *et al.*, 2000). Often these flasks are equipped with a baffle and/or a stainless steel spring in order to increase aeration and to reduce the size of cell aggregates which are formed by the hyphal growth of the streptomycetes. However, this also leads to splashing of the liquid medium and to wall growth of the cells, probably contributing to the problem of variability (Betts & Baganz, 2006; Büchs, 2001). The high standard deviation in the production rates makes it necessary to increase the number of parallel cultures if significant differences need to be distinguished from chance variations. This increases both, the workload and the demand for shakers, flasks, media and analytical equipment. A cultivation method which shows less variability in secondary metabolite production therefore would significantly reduce the workload and costs of research in antibiotic biosynthesis.

Micro-culture systems based on various designs of microtiter plates have been investigated in the past few years as an alternative system to Erlenmeyer flasks (Duetz, 2007; Duetz *et al.*, 2000; Minas *et al.*, 2000). However, previous investigations mostly focused on bacteria with unicellular dispersion and on the determination of growth and primary metabolism. Only a single study has been published previously on the cultivation of streptomycetes in such a culture system, investigating growth and production of the polyketide antibiotic actinorhodin (Minas *et al.*, 2000). Recently, the utilization of 24-square deepwell plates has been adopted for systems biology research in *Streptomyces coelicolor* (Prashant M. Bapat, personal communication). Based on these methods, the production of the aminocoumarin antibiotic novobiocin was investigated in the heterologous producer strain *S. coelicolor* M512(novBG01) which has been generated by site-specific integration of the entire novobiocin biosynthetic gene cluster into the genome of the host strain (Eustáquio *et al.*, 2005).

In the present study it was shown, that 24-square deepwell plates provide a very suitable system for investigations on the production of novobiocin in the heterologous producer strain. Compared to Erlenmeyer flasks, the main advantage of this system is the low variability (4–9%) of novobiocin production rates between parallel cultures, as well as the low requirement for shaker space and culture medium. Furthermore, handling of the deepwell plates is faster than that of Erlenmeyer flasks, and can be automated. Therefore, the use of 24-square deepwell plates may help to reduce the costs and the workload of investigations on antibiotic production in microorganisms.

Under the conditions used in this study, novobiocin production rates in the deepwell plates were originally lower than in the Erlenmeyer flasks (**Figure III.1B**), probably due to insufficient aeration. It was shown by Duetz and co-workers (Duetz *et al.*, 2000; Duetz & Witholt, 2001; Duetz & Witholt, 2004) that gas–liquid transfer rather than aeration of the headspace is the limiting factor for oxygen supply in the cultures and therefore the use of sandwich covers with bigger holes (4 mm instead of 2 mm)

had no effect on the production of novobiocin. However, artificial oxygen carriers such as the water soluble compound Q2-5247, a siloxylated ethylene oxide/propylene oxide copolymer, are able to increase the concentration of oxygen in aqueous solutions and improve the efficiency of oxygen-consuming biochemical processes. Accordingly, the addition of Q2-5247 resulted in an increase of growth of *Bacillus thuringensis* and of the production of the polyketide antibiotic actinorhodin in *S. coelicolor* A3(2) (Dey *et al.*, 2004). In the present study, inclusion of 0.6% of copolymer Q2-5247 was able to significantly enhance the production of novobiocin, presumably by increasing the oxygen supply to the cells. An alternative way to increase the oxygen supply may have been an increase of shaker speed (currently 300 rpm) or shaker diameter (25 mm). However, the commonly available commercial shakers operate with 25 mm shaking diameter and do not allow speeds significantly above 300 rpm.

The deepwell plates offer a convenient system e.g. to investigate the influence of external factors like media components on antibiotic production or for feeding experiments with biosynthetic precursors. In such studies, it is preferable to use the same batch of inoculum for all experiments, resulting in the best reproducibility of production rates. As long as the precultures used as inoculum were harvested in the early stationary phase we were not successful in obtaining reproducible novobiocin production between independently prepared batches of (genetically identical) inoculum, even when the homogenized inoculum mixture was standardized for the optical density or for the number of colony-forming units. However, reproducible novobiocin production values between independently prepared batches of inoculum could be readily obtained when the cells of the preculture were harvested in a defined section of the growth phase. These inocula also resulted in higher novobiocin production than inocula from the stationary phase, which is in accordance with previous findings on clavulanic acid production (Neves *et al.*, 2001). It is well known that in the transition from growth phase to stationary phase, bacterial cultures undergo fundamental changes in the gene expression pattern which have a profound effect on secondary metabolite production (Bibb, 2005). This may contribute to the variability between independent batches of inoculum harvested in the early stationary phase. It is therefore preferable to harvest precultures in a defined section of the growth phase and this procedure appears mandatory when antibiotic production is to be compared between genetically different mutant or transformant strains.

The production of coumermycin A_1 in the 24-square deepwell plates required a new production medium, since the originally used SK medium was not suitable due to the lard oil contained therein. However, cultivation of the heterologous producer stain *S. coelicolor* M1146(couMW16) in the tested media resulted in the production of not only coumermycin A_1 but of several coumermycin derivatives, e.g. coumermycin A_2, lacking the methyl groups at position 5 of the terminal pyrrole moieties (Claridge *et al.*, 1984; Claridge *et al.*, 1966). The methyltransferase CouN6, which is responsible for the methylation of the terminal pyrrole moieties, is methylcobalamine-dependent (Westrich *et al.*, 2003). Therefore, the addition of cobalt chloride to the tested production media was able to shift coumermycin production in favour of coumermycin A_1 (Claridge *et al.*, 1966).

Although this study was originally prompted by the need for a production system with low variability for investigations on the regulatory genes of aminocoumarin antibiotic formation in the heterologous producer strains (Dangel *et al.*, 2008; Eustáquio *et al.*,

2005), 24-square deepwell plates are now widely used in our laboratory, e.g. for the investigation of the biosynthesis of the central pyrrole moiety of coumermycin A_1 described in this thesis or for the comparison of genetically modified *Streptomyces coelicolor* strains as hosts for the heterologous production of aminocoumarin and liponucleoside antibiotics (Flinspach *et al.*, 2010).

2. Biosynthesis of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety of coumermycin A_1

Coumermycin A_1 contains two types of pyrrole moieties, i.e. two terminal 5-methyl-pyrrole-2-carboxylic acid moieties and the central 3-methylpyrrole-2,4-dicarboxylic acid moiety. While the pyrrole-2-carboxylate scaffold of the terminal pyrrole moieties can be found in several other natural products (Walsh *et al.*, 2006), the 3-methylpyrrole-2,4-dicarboxylic acid moiety is to our knowledge unique.

The electron-rich, planar pyrrole ring system is an important structural element in many biomolecules of primary and secondary metabolism. It can coordinate metals, form hydrogen bonds and provide stacking interactions (Walsh *et al.*, 2006). The best-known pathway for pyrrole biosynthesis proceeds via δ-aminolevulinic acid to the porphyrins. Another important route is the dehydrogenation of L-proline to pyrrole-2-carboxyl units. That route leads to the terminal pyrrole moieties of coumermycin A_1 and clorobiocin (**Figure III.10A**), but also to one of the three pyrrole rings found in the prodiginines of *Streptomyces* and *Serratia* species. The biosynthesis of the terminal pyrrole moieties of coumermycin A_1 from L-proline is catalysed by CouN1-7 and involves the activation of L-proline by thioester formation with an acyl carrier protein, the oxidation to the pyrrole, the methylation at position 5 and the transfer to the deoxysugar moiety in a two-step acyl transfer process (**Figure IV.1**). These and other enzymatic machineries for the biological formation of pyrroles have been reviewed by Walsh *et al.* (2006). The present study now reveals a further, hitherto unknown pathway to a pyrrole ring system. While the terminal pyrrole moieties of coumermycin A_1 are derived from L-proline, the central pyrrole moiety (CPM) is not. Inactivation of the genes *couN1-couN7* proved that none of the enzymes involved in the formation and transfer of the terminal pyrrole moieties is required for the biosynthesis of the central pyrrole moiety. Rather, inactivation experiments followed by feeding of CPM established that the genes *couR1*, *couR2a/b*, *couR3* and *couR4* are involved in the formation of CPM (**Table III.1**).

Heterologous expression of the cosmid couSS01 (**Figure III.11**) resulted in the formation of CPM monoamide and CPM diamide, indicating that all enzymes required for the biosynthesis of CPM are encoded in couSS01. In detailed previous studies, the function of all genes contained in couSS01 in biosynthesis, regulation and self-resistance has been elucidated (Heide, 2009), except for the genes *couR1-couR6*. The gene product of *couR5* shows similarity to transporters of the major facilitator superfamily (Schmutz *et al.*, 2003a), and the gene product of *couR6* is similar to transcriptional regulators of the LysR family. Therefore, these two genes are not likely to code for biosynthetic enzymes, but the genes *couR1-couR4* may encode all enzymes required for the assembly of the central pyrrole moiety.

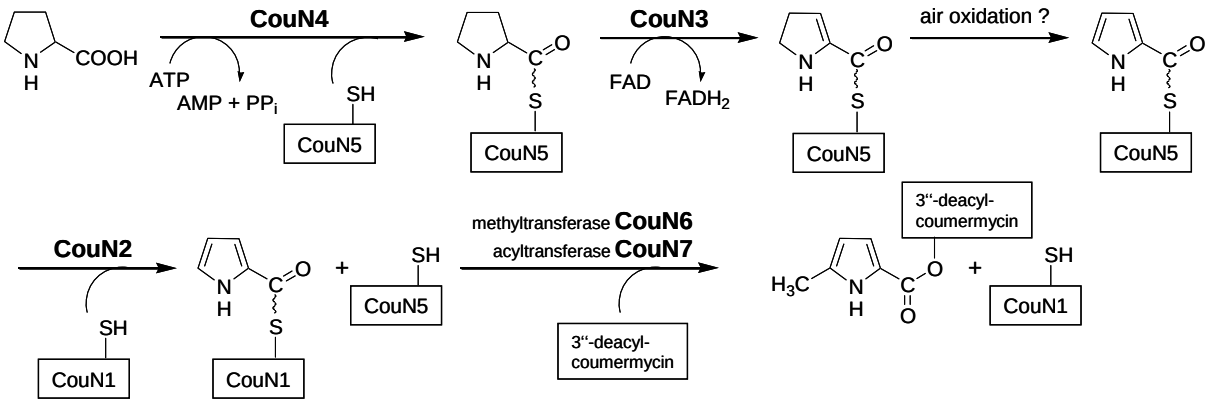

Figure IV.1
Biosynthesis and transfer of the terminal pyrrole moiety of coumermycin A_1 from L-proline by action of CouN1-CouN7. Modified from Heide (2009).

CouR3 (302 aa) shows sequence similarity to the threonine kinase PduX involved in adenosyl-cobalamine biosynthesis in *Salmonella enterica* (Fan *et al.*, 2009). The biosynthesis of the central pyrrole moiety of coumermycin A_1 may therefore involve the *O*-phosporylation of threonine. In case of CouR1 (474 aa), BLAST searches revealed only five proteins in the database with significant similarity and all of these are annotated as hypothetical proteins. However, the PHYRE server (Kelley & Sternberg, 2009) indicated a similarity of CouR1 to "tryptophan synthase beta-subunit like PLP-dependent enzymes" (SCOP 53686). Tryptophan synthase catalyzes the replacement of the OH-group of serine with an indole moiety, resulting in L-tryptophan (Dunn *et al.*, 2008). CouR2a (172 aa) and CouR2b (205 aa) show similarity to many thiamine pyrophosphate-dependent decarboxylases. Some of these consist of two subunits which correspond in sequence and size to the separate proteins CouR2a and CouR2b, e.g. the sulfopyruvate decarboxylase ComDE from *Methanococcus jannaschii* (Graupner *et al.*, 2000), some others include both subunits in a single polypeptide chain (Zhang *et al.*, 2003). ComDE is a dodecamer consisting of six alpha (D) and six beta (E) subunits and catalyses the decarboxylation of sulfopyruvic acid to sulfoacetaldehyde in the coenzyme M pathway (Graupner *et al.*, 2000). CouR4 (389 aa) shows similarity to many FMN-dependent dehydrogenases.

This study unequivocally proved that L-threonine is incorporated intact into CPM and provides the heterocyclic nitrogen as well as four of the seven carbons of this moiety. It is tempting to speculate that the biosynthesis of CPM may start from L-threonine (**Figure IV.2**), and that the first step in this pathway may be the formation of *O*-phospho-L-threonine catalysed by CouR3, identical to the reaction catalysed by PduX in adenosyl-cobalamine biosynthesis in *Salmonella enterica* (Fan *et al.*, 2009). However, feeding of *O*-phospho-L-threonine to a *couR3* defective mutant increased coumermycin production only by 44 %, i.e. to a level of 0.2 mg/L (**Table III.2**), while feeding of CPM to the same mutant increased coumermycin production >50-fold, i.e. to a level of 6.6 mg/L (**Table III.1**). This may indicate that L-threonine first forms another intermediate, e.g. a Schiff's base with a compound containing a carbonyl group, and that the hydroxyl group of threonine is phosphorylated in a later step. On the other hand, *O*-phospho-L-threonine is a very polar compound which may not easily be taken up into the cell, and is also easily hydrolysable by phosphatases. This

may provide an alternative explanation for the relatively small increase of coumermycin A_1 production after feeding of *O*-phospho-L-threonine. Notably, in a total of 12 feeding experiments with this compound coumermycin production was consistently higher than observed after feeding of free threonine (**Table III.2**). Therefore, our results do not exclude that *O*-phospho-L-threonine may be an intermediate in the biosynthesis of the central pyrrole moiety of coumermycin.

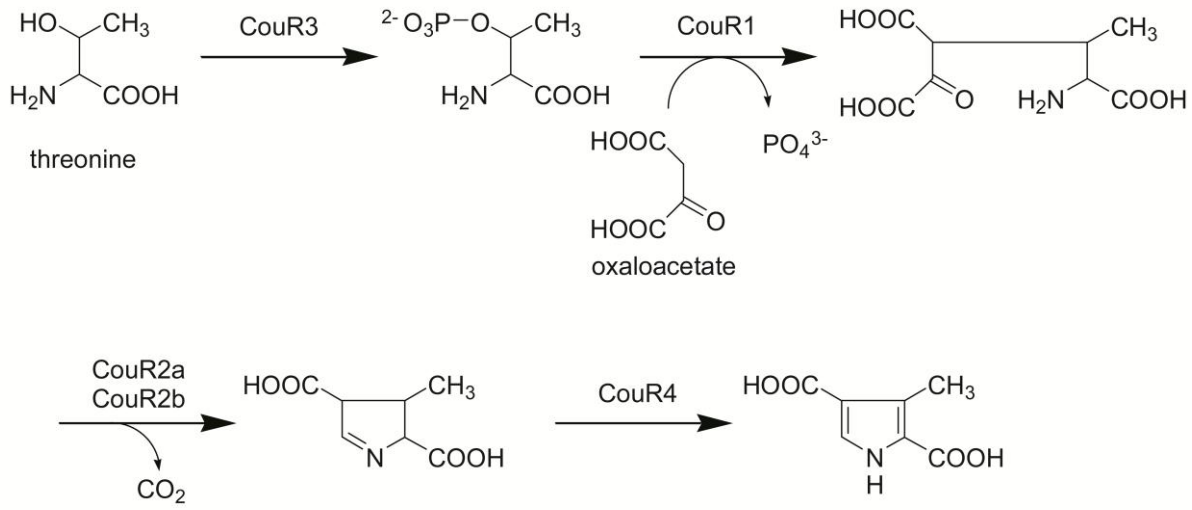

Figure IV.2
Hypothetical biosynthetic pathway to the central pyrrole moiety of coumermycin A_1 (i.e. 3-methylpyrrole-2,4-dicarboxylic acid).

The biosynthetic origin of the other three carbons of CPM is unknown. **Figure IV.2** shows oxaloacetate as a hypothetical precursor of this part of the molecule, with CouR1 catalysing a ß-replacement of the hydoxyphosphate group of *O*-phospho-L-threonine, followed by a (possibly spontaneous) Schiff's base formation. CouR2a/b may catalyse a decarboxylation of the resulting intermediate, and the putative FMN-dependent dehydrogenase CouR4 may oxidize the pyrroline to the pyrrole (**Figure IV.2**). However, other molecules than oxaloacetate are equally conceivable as precursors of this part of CPM, e.g. maleic acid, and both, feeding studies and biochemical experiments, are required to elucidate this biosynthetic reaction sequence.

Feeding experiments with isotope-labeled precursors are widely used for the identification of biosynthetic building blocks, especially in PKS- and NRPS-derived compounds (Erol *et al.*, 2010; Ohlendorf *et al.*, 2008; Schuhmann & Grond, 2004; Surup *et al.*, 2007; Weber *et al.*, 2008). The origin of the isoprenoid moiety of furanonaphthoquinone I and endophenazine A from the mevalonate pathway, for example, was shown by feeding experiments with [$^{13}C_2$]acetate and [2-^{13}C]glycerol (Bringmann *et al.*, 2007) and Bunton *et al.* (1963) showed that the aminocoumarin moiety of novobiocin is derived from L-tyrosine by feeding of [^{14}C]L-tyrosine.

In the conducted feeding experiment with [U-^{13}C,^{15}N]L-threonine, a very strong (19.5 %) ^{13}C enrichment was observed for the pyrrole carbons C-2, C-3, C-6 and C-7. For the other carbons of CPM monoamide no significant incorporation was observed, except for a weak (1.8 %) enrichment for the methyl group attached to position 8 of the aminocoumarin ring. This methyl group is derived from *S*-adenosylmethionine (SAM) (Pacholec *et al.*, 2005b) and its labeling can be readily explained by the degradation of L-threonine to glycine via an aldol cleavage (Balk *et al.*, 2007; Liu *et al.*, 1998). Glycine, like serine and formaldehyde, is a carbon-donor for the generation of N^5,N^{10}-methylene-THF from tetrahydrofolate (THF), which in turn provides the methyl group of SAM (Berg *et al.*, 2011).

3. Identification and structural elucidation of new caprazamycins from *Streptomyces sp.* MK730-62F2 by LC/ESI-MS/MS

The biosynthetic gene clusters of caprazamycins and liposidomycins, respectively, were recently identified by our group (Kaysser *et al.*, 2009; Kaysser *et al.*, 2010a). The heterologous expression of the caprazamycin gene cluster originally resulted in the production of the caprazamycin aglyca, since the genes for the biosynthesis of the L-rhamnose moiety are located in a second cluster elsewhere on the genome (Kaysser *et al.*, 2010b). Inactivation of *cpz20, cpz21* and *cpz25* led to the production of new simplified liponucleoside antibiotics that lack the 3-methylglutaryl moiety (Kaysser *et al.*, 2009) and an in vitro assay with the putative glycosyltransferase Cpz31, using dTDP-L-rhamnose and the caprazamycin aglyca as substrates, resulted in the production of 2,3,4-desmethyl caprazamycins (Kaysser, 2010). The identification of all these compounds required a rapid and precise analytical method that also supplies sufficient structural information. High-performance liquid chromatography (HPLC) coupled to a triple-stage quadrupole or ion trap mass spectrometer (MS) is capable of both, separating complex mixtures of secondary metabolites and providing reliable information on the analyte's mass and structure. Therefore a LC-ESI-MS/MS method for the identification of caprazamycins and liposidomycins in the culture extracts of the respective producer strains using a triple-stage quadrupole mass spectrometer was established in this study.

The liponucleoside antibiotics are complex compounds that contain acidic as well as basic functional groups. Therefore the caprazamycins and liposidomycins were readily detected in positive as well as in negative ionization mode. However, the positive mode provides more structural information and so is used for the routine analysis of caprazamycins and liposidomycins, respectively. However, if informations regarding the sulfate group or the fatty acid moiety are required the negative ionization mode is of advantage. The overall fragmentation pattern in positive as well as in negative ionization mode is consistent with the published FAB-MS data for the liposidomycins (Kimura *et al.*, 1998b; Ubukata *et al.*, 1988).

Affi-Gel® boronate is a boronate-derivatized polyacrylamide gel with an affinity for adjacent *cis* hydroxyl (*cis*-diol) groups and is able to efficiently separate low molecular weight molecules such as nucleotides, nucleosides, catecholamines, coenzymes, and sugars (Boeseken, 1949; Higa *et al.*, 1977; Liebich *et al.*, 1997). At alkaline pH values Affi-Gel® boronate forms a cyclic boronate ester with the *cis*-diol groups, which can be cleaved by shifting the pH to an acidic value. This results in the

elution of the *cis*-diol containing compound and the regeneration of the material. The general principle of this method is depicted in **Figure IV.3**. The present study now showed that the caprazamycins with their modified uridine structure are also able to bind to the boronic acid stationary phase and that a partial purification is possible by the described method. Originally the caprazamycins eluted in the second washing, consisting of 50% (v/v) methanol. This was also observed by Dino Bullinger for some nucleosides, e.g. for pseudouridine, and could be prevented by reducing the methanol content in washing step 2 from 50 to 20% (v/v) (Bullinger, 2008). A possible reason for the early elution of the caprazamycins could be the occurrence of secondary interactions between the boronic acid gel and some other structural moiety, e.g. with the amino group of the aminoribose (Tuytten *et al.*, 2007). As for the nucleosides a reduction of the methanol content to 20% (v/v) in washing step 2 was able to solve the problem.

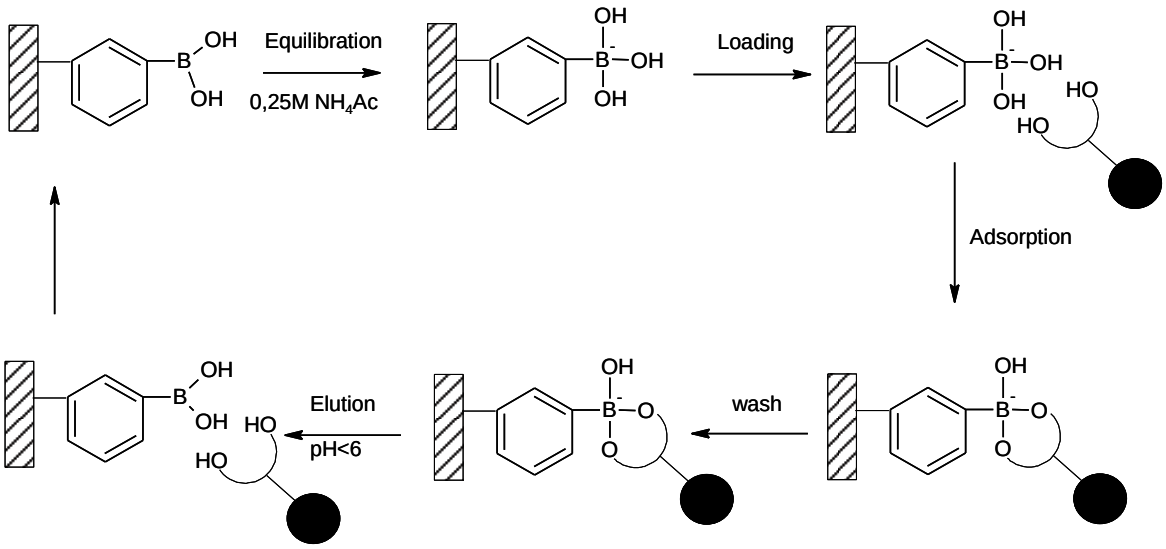

Figure IV.3
Principle of the affinity chromatography on a boronic acid gel (Affi-Gel® boronate). Modified from Bullinger (2008).

Previously, 27 liposidomycin derivatives containing 13 different fatty acid side chains have been isolated from *Streptomyces sp.* SN-1061 (Esumi *et al.*, 1999; Kimura *et al.*, 1998b). In contrast, only seven caprazamycins were reported from *Streptomyces sp*. MK730–62F2 (Igarashi *et al.*, 2005). Both gene clusters are highly conserved and do not contain possible genes for fatty acid biosynthesis. Consequently, the fatty acid moiety of the liponucleoside antibiotics is most likely provided by the primary metabolism of the host strain. We therefore wanted to investigate if the variety in the length and conformation of the fatty acid side chain known from the liposidomycins can also be found for the caprazamycins. In the present study we report the identification of at least six new caprazamycins, termed CPZ G, H, I, J, K and L. The mass differences of the new compounds to the known caprazamycins suggest the incorporation of unsaturated fatty acids and the proposed chemical formula fit that of the fatty acid side chains of the liposidomycins Y, Z, A, G, K and N, respectively. However, the exact regiochemistry of the new caprazamycins could not be determined with the means available.

The position of double bonds in unsaturated fatty acids can normally be determined by charge-remote fragmentation, a class of gas-phase decompositions that occur physically remote from the charge site (Adams, 1990; Adams & Songer, 1993; Gross, 1992). Under high-energy collisional activation (CA), e.g. in sector instruments with fast-atom bombardment (FAB) as ionization technique, fatty acids undergo charge-remote fragmentation to give a series of product ions, representing the cleavage of consecutive C-C single bonds, from which the structure of the fatty acid can be determined (Cheng & Gross, 2000; Hsu & Turk, 2008). At the position of a double bond the ion series is interrupted by a gap which is framed by two abundant ions on either side (Hsu & Turk, 1999, 2008). More recently, the classic fragmentation processes were also observed under low-energy CA, e.g. in tandem quadrupole instruments with electrospray ionization in negative mode, as used in the present study. However, these studies were mainly conducted on free fatty acid and not on such complex compounds as the liponucleoside antibiotics (Hsu & Turk, 1999, 2008). Accordingly, the structure elucidation of the fatty acid moieties of the liposidomycins was conducted under high-energy conditions (Esumi *et al.*, 1999) and also no charge-remote fragmentation was observed for the caprazamycins under low-energy conditions in the present study.

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VI. APPENDIX

1. MS data

Figure VI.1: CID-MS/MS spectrum of coumermycin A_1

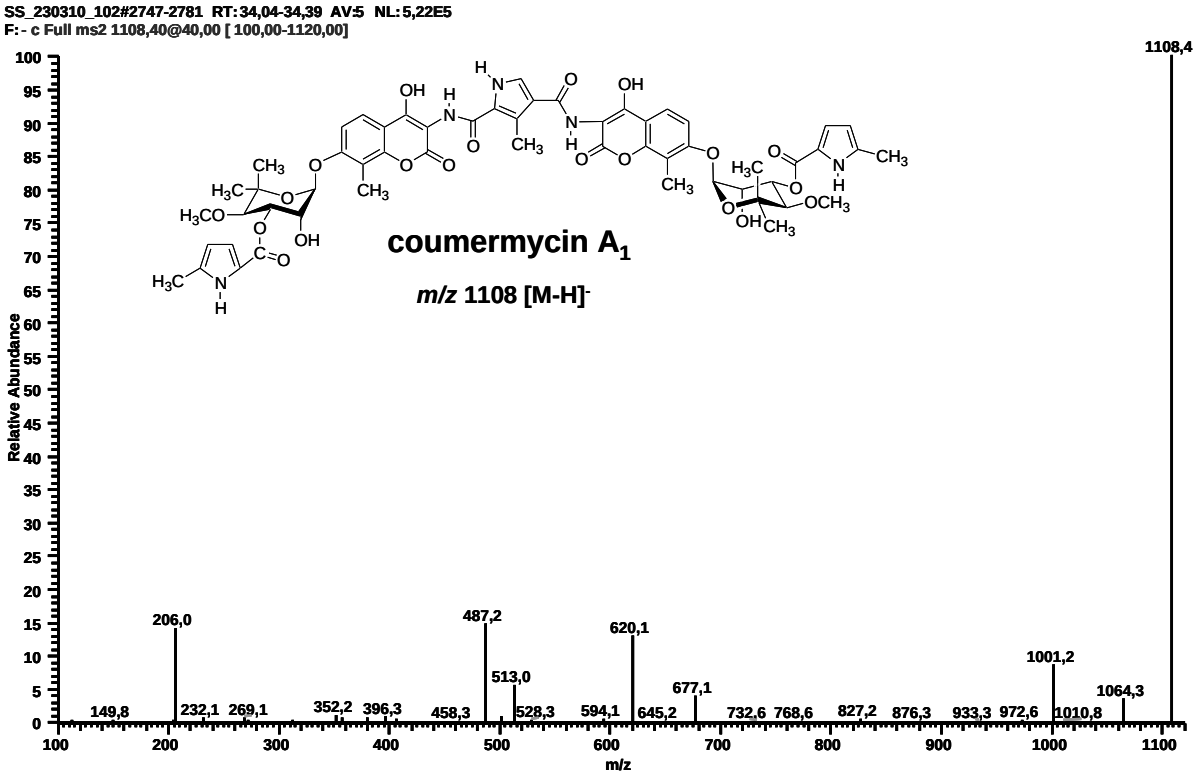


Figure VI.2: Suggested structural formulas of the mass spectrometric fragments *m/z* 206 and 232 $[M-H]^-$

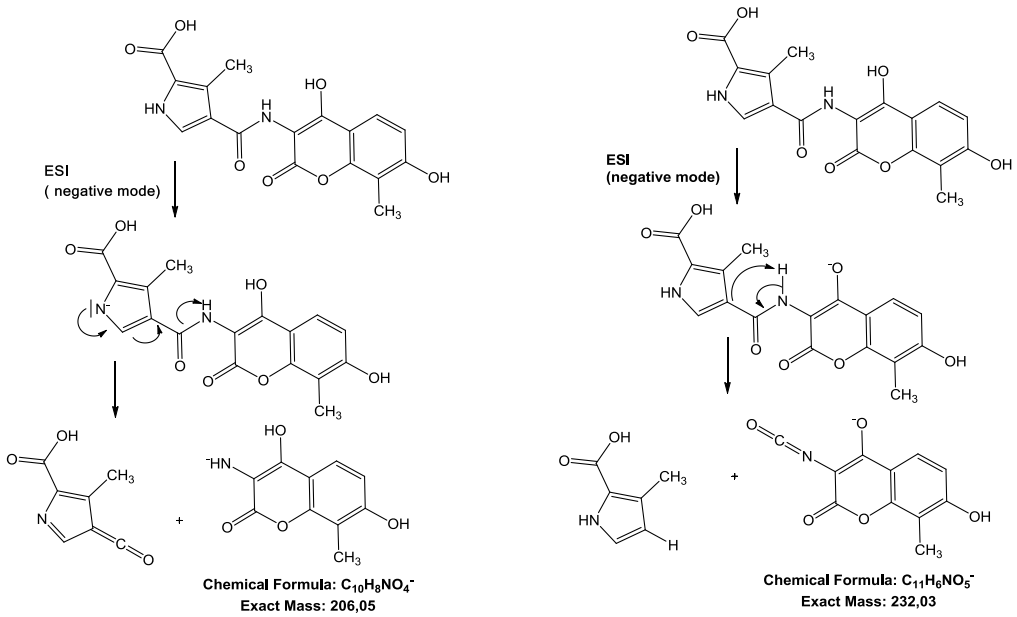


Figure VI.3: CID-MS/MS spectrum of coumermycin A_2

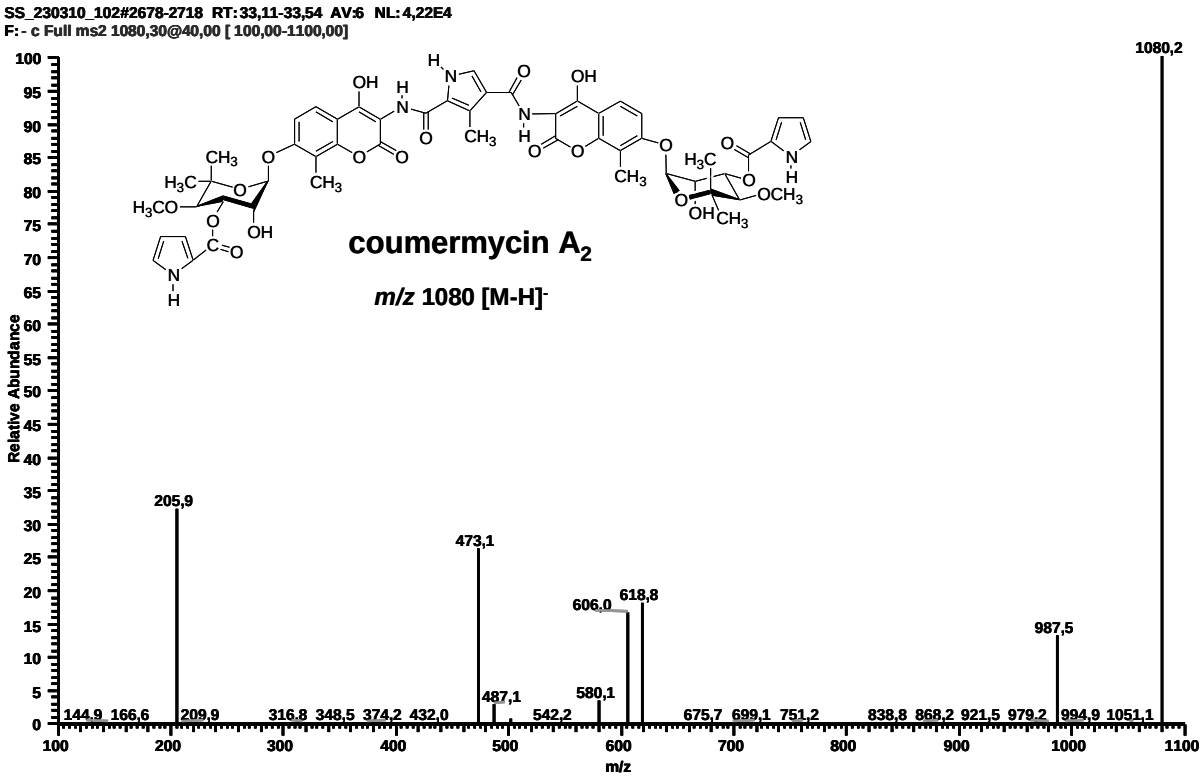


Figure VI.4: CID-MS/MS spectrum of coumermycin B

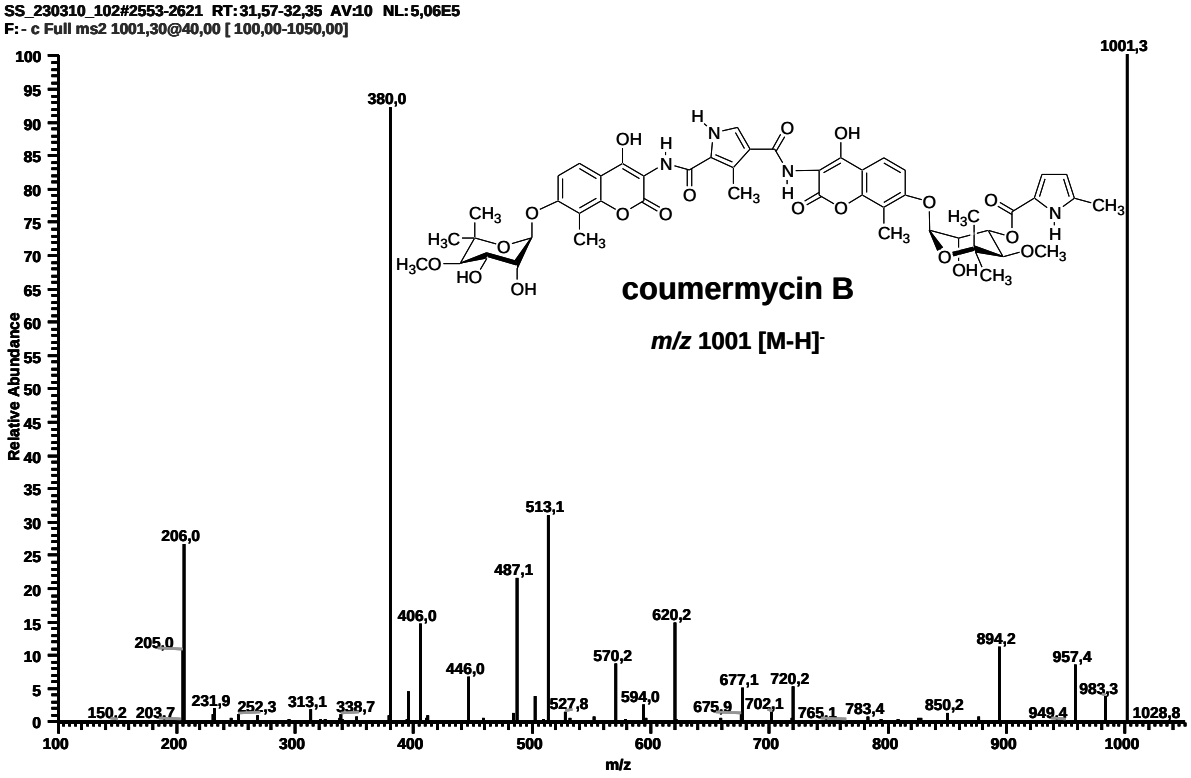


Figure VI.5: CID-MS/MS spectrum of coumermycin C

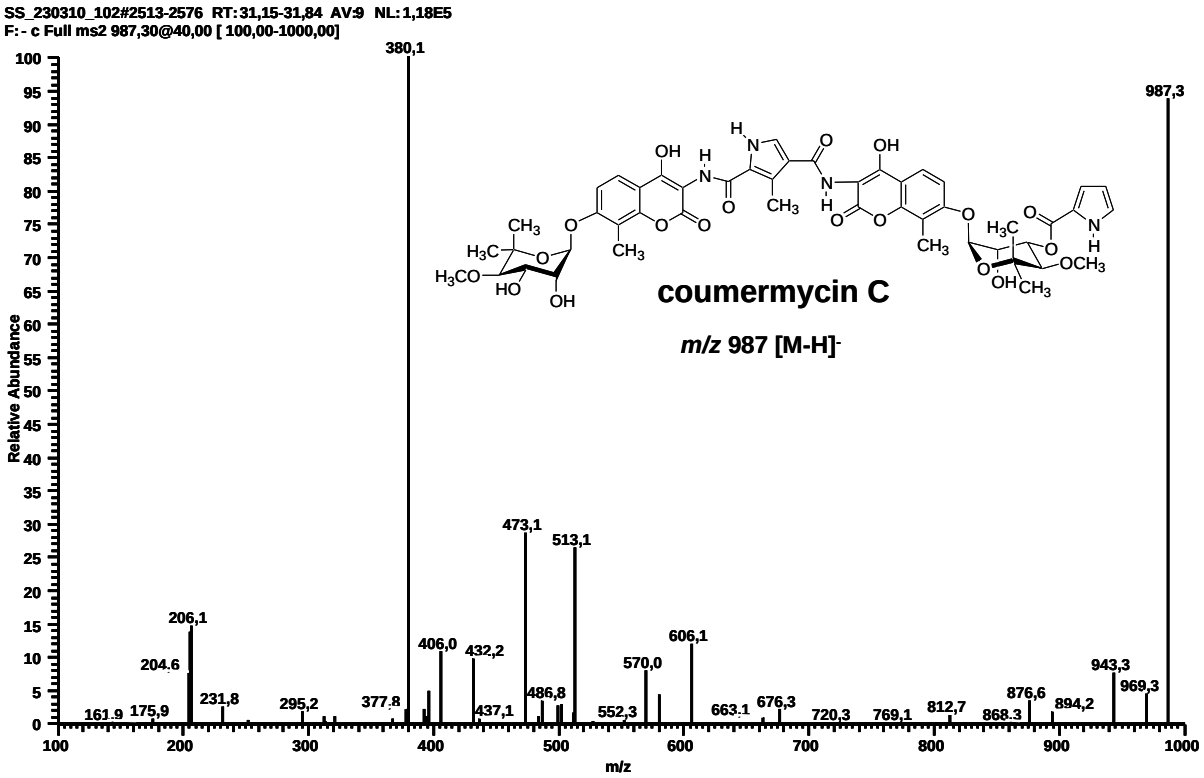


Figure VI.6: CID-MS/MS spectrum of coumermycin D-1b/1c

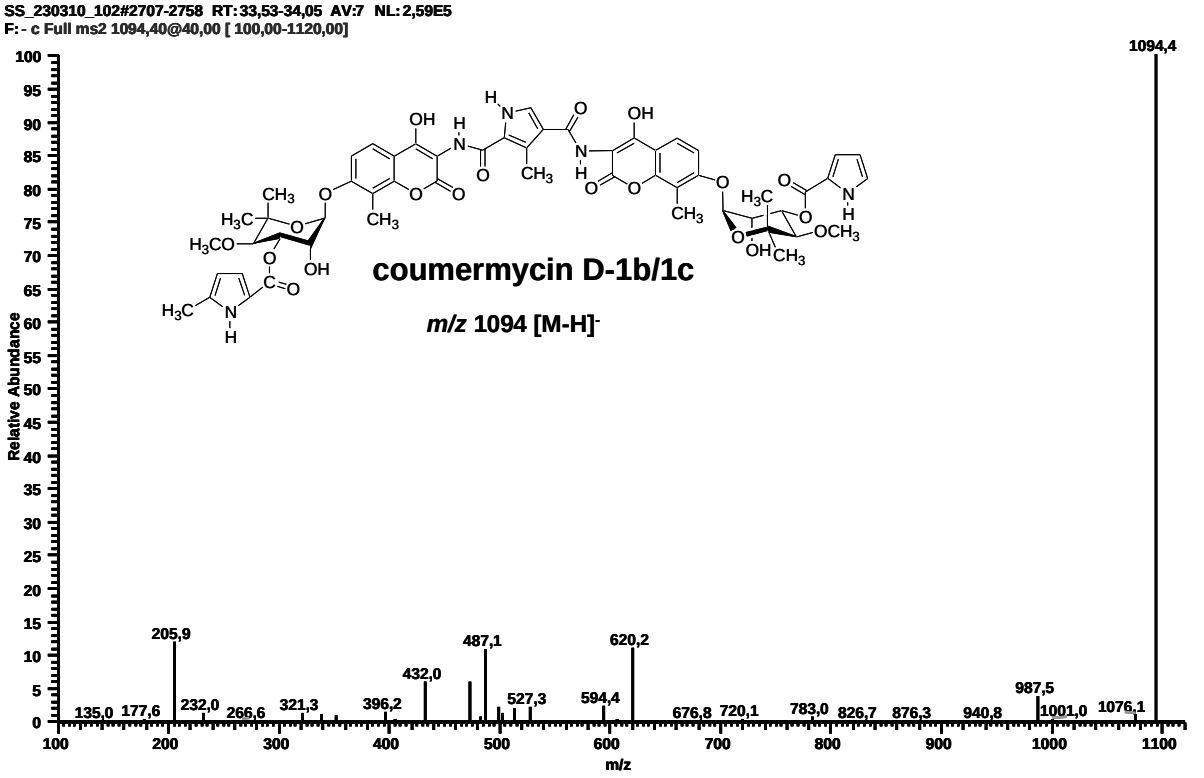


Table VI.1
LC-MS/MS analysis of caprazamycins (CPZ) and liposidomycin (LPM) A in positive and negative ionization mode

Compound	*m/z* $[M+H]^+$	MS/MS *m/z* (%)	*m/z* $[M-H]^-$	MS/MS *m/z* (%)
CPZ A/B	1146,6	1146,8 (30), 1015,6 (28), 958,5 (2), **827,3 (100)**, 715,2 (10), 558,0 (4), 427,1 (6), 320,0 (47), 189,0 (40)	1144,6	1144,7 (8), 1100,8 (1), 587,0 (12), 556,3 (<0,1), 512,2 (<0,1), **362,7 (100)**, 332,8 (42), 271,1 (1)
CPZ C/D/G	1132,6	1132,7 (32), 1001,6 (28), 944,4 (4), **813,3 (100)**, 701,2 (10), 558,0 (4), 427,1 (6), 319,9 (38), 189,0 (39)	1130,6	1130,7 (8), 1086,9 (<1), 573,1 (17), 556,4 (<1), 512,2 (<0,1), **362,8 (100)**, 332,9 (46), 257,1 (<1)
CPZ E/F	1118,6	1118,7 (26), 987,6 (28), 930,4 (2), **799,3 (100)**, 687,2 (9), 557,9 (4), 427,0 (6), 320,0 (46), 189,0 (46)	1116,6	1116,7 (4), 1072,9 (<1), 559,0 (10), 556,0 (<0,1), 512,4 (<0,1), **362,9 (100)**, 333,0 (27), 243,0 (<1)
LPM A	1034,4	1034,3 (<0,1), 954,7 (38), **823,5 (100)**, 711,4 (11), 558,2 (1), 427,1 (8), 315,1 (3)	1032,4	1032,3 (63), 988,5 (<1), 952,4 (<0,1), 635,9 (2), **592,0 (100)**, 556,0 (1), 395,1 (<1), 362,8 (<0,1), 267,0 (1), 144,9 (<1)

Table VI.2
Molecular formulae of caprazamycins A-M and calculated molecular weights

Caprazamycin	Molecular formula	R (unsaturation)	Molecular weight
A/B	$C_{53}H_{87}N_5O_{22}$	$C_{13}H_{27}$ (0)	1146
C/D/G	$C_{52}H_{85}N_5O_{22}$	$C_{12}H_{25}$ (0)	1132
E/F	$C_{51}H_{83}N_5O_{22}$	$C_{11}H_{23}$ (0)	1118
H	$C_{51}H_{79}N_5O_{22}$	$C_{11}H_{19}$ (2)	1114
I	$C_{51}H_{81}N_5O_{22}$	$C_{11}H_{21}$ (1)	1116
J	$C_{53}H_{83}N_5O_{22}$	$C_{13}H_{23}$ (2)	1142
K	$C_{53}H_{85}N_5O_{22}$	$C_{13}H_{25}$ (1)	1144
L	$C_{55}H_{87}N_5O_{22}$	$C_{15}H_{27}$ (2)	1170
M	$C_{55}H_{89}N_5O_{22}$	$C_{15}H_{29}$ (1)	1172

2. NMR data

Calculation of ^{13}C enrichment after feeding of [U-^{13}C,^{15}N]L-threonine

[U-^{13}C,^{15}N]L-threonine was purchased with 99% enrichment.

^{13}C enrichment in the isolated CPM monoamide was calculated as described by Scott *et al.* (1974)

$$enrichment[\%] = 1.1 \bullet \frac{I[labeled]}{I[unlabeled]} - 1.1\ \%$$

Integrals of coupling signals of carbon atoms were calculated as the sum of the integral of the full multiplets. Carbon C-9' of the coumarin moiety was used as the non-^{13}C-enriched internal reference signal.

Integrals for singlet signals of respective carbon atoms were calculated using the integral of the central line (I_{CL}) of the multiplets and the usual singlet signals. Only carbon C-11' turned out to be enriched (see Main Text, Table 2).

$$enrichment[\%] = 1.1 \bullet \frac{I_{CL}[labeled]}{I[unlabeled]} - 1.1$$

Figure VI.7: ^{1}H-NMR spectrum of unlabeled CPM monoamide (400 MHz, d_6-DMSO); Signal assignments are shown in Table VI.3

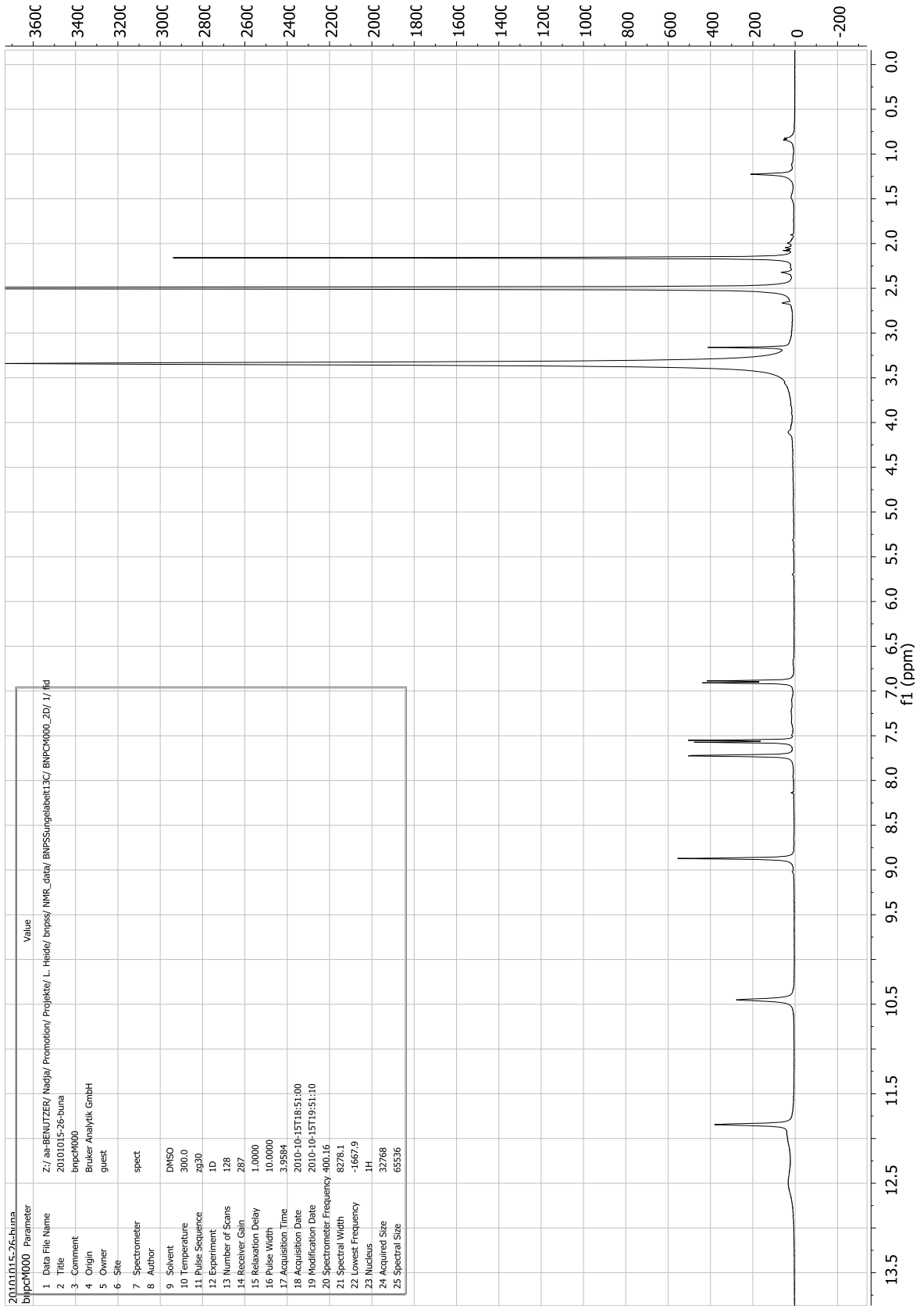


Figure VI.8: ^{13}C-NMR spectrum of unlabeled CPM monoamide (100.6 MHz, d_6-DMSO); Signal assignments are shown in Table VI.3

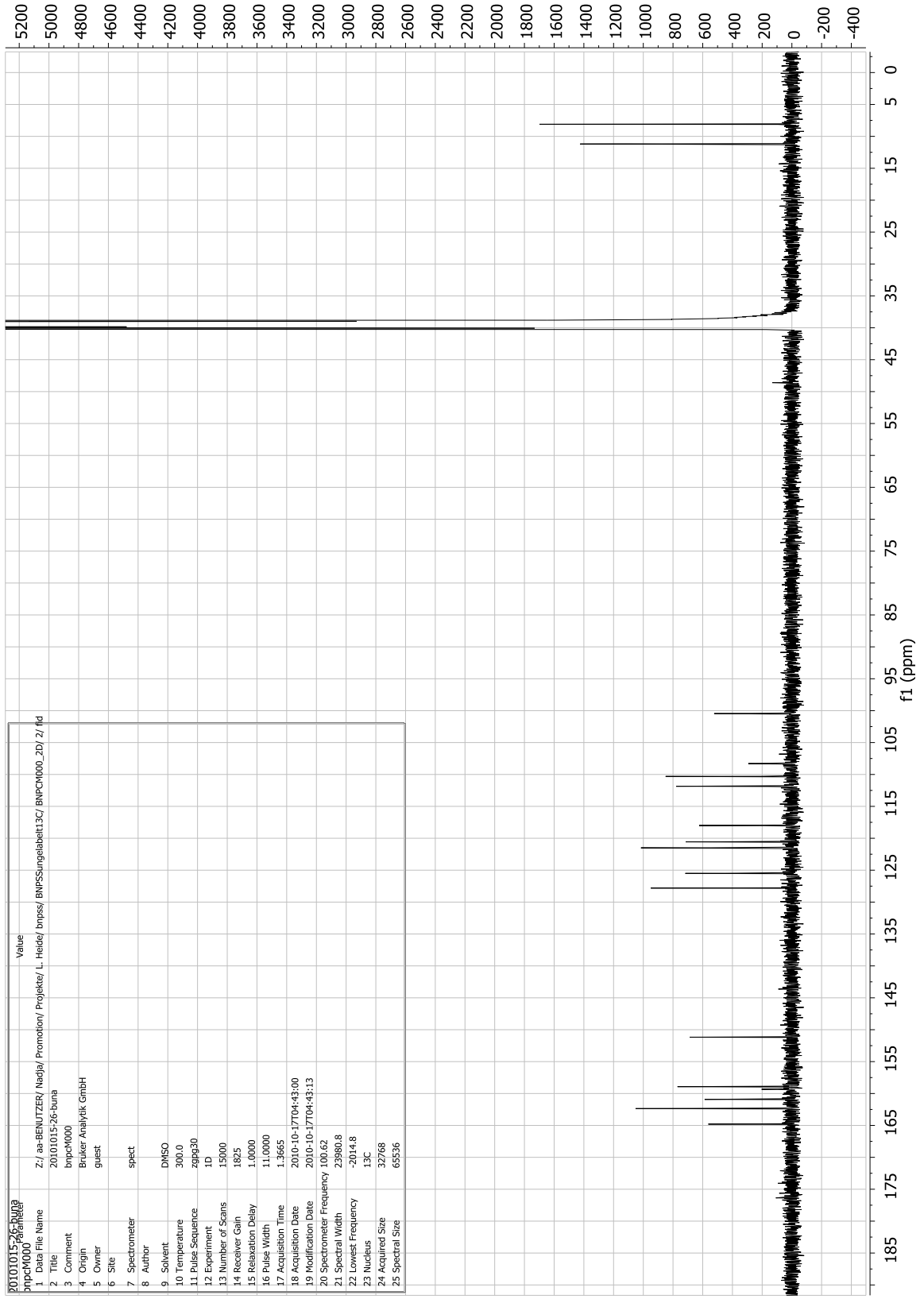


Figure VI.9: H,H-COSY of unlabeled CPM monoamide (600 MHz, d_6-DMSO); selected correlations are shown in Figure VI.12.

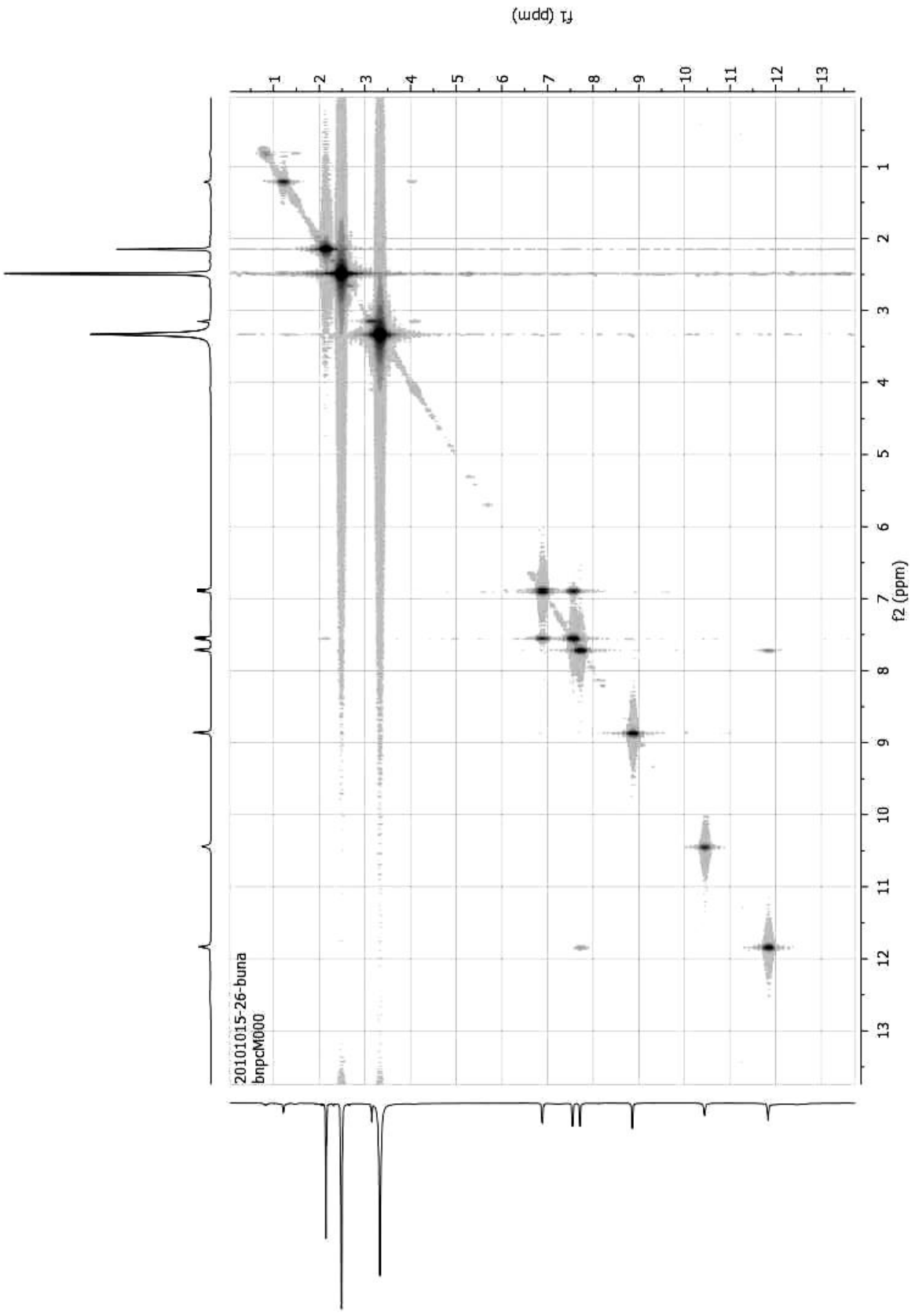


Figure VI.10: HSQC of unlabeled CPM monoamide (600 MHz, d_6-DMSO); selected correlations are shown in Figure VI.12.

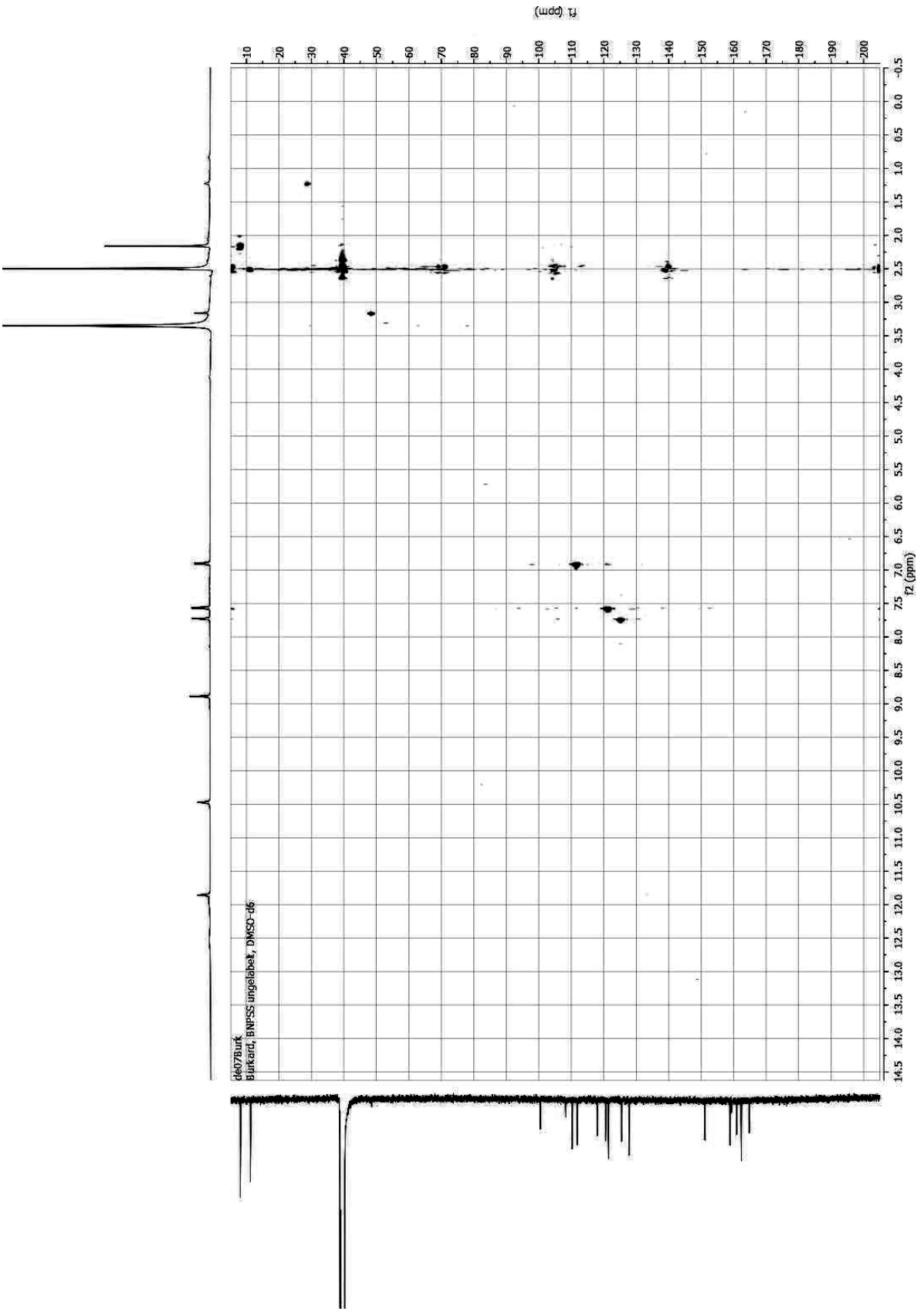


Figure VI.11: HMBC of unlabeled CPM monoamide (600 MHz, d_6-DMSO); selected correlations are shown in Figure VI.12.

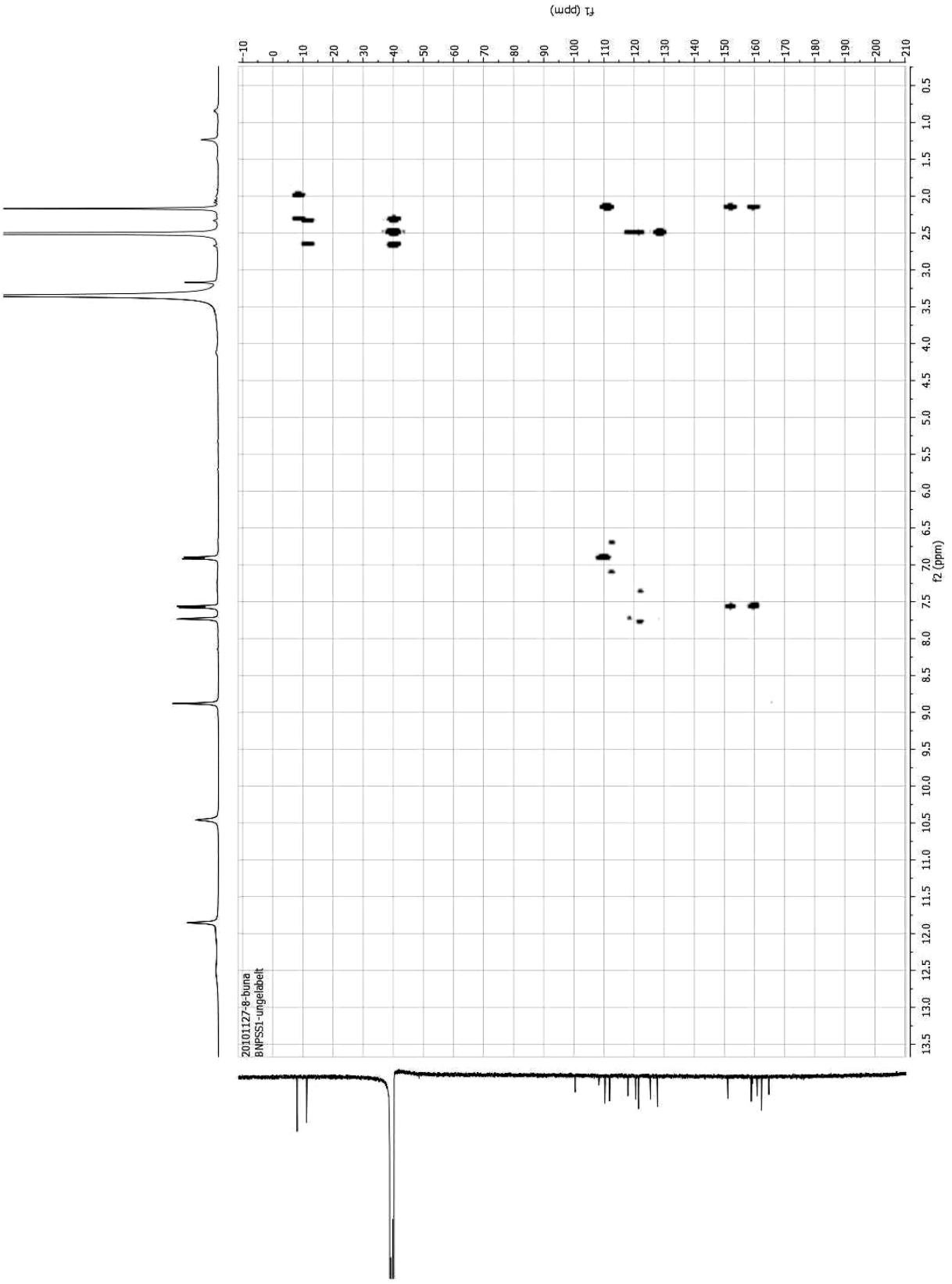


Figure VI.12: Selected ^{1}H-^{1}H-COSY (↔), HSQC (→) and HMBC (-----) correlations from 2D NMR experiments for unlabeled CPM monoamide (600 MHz, d_6-DMSO)

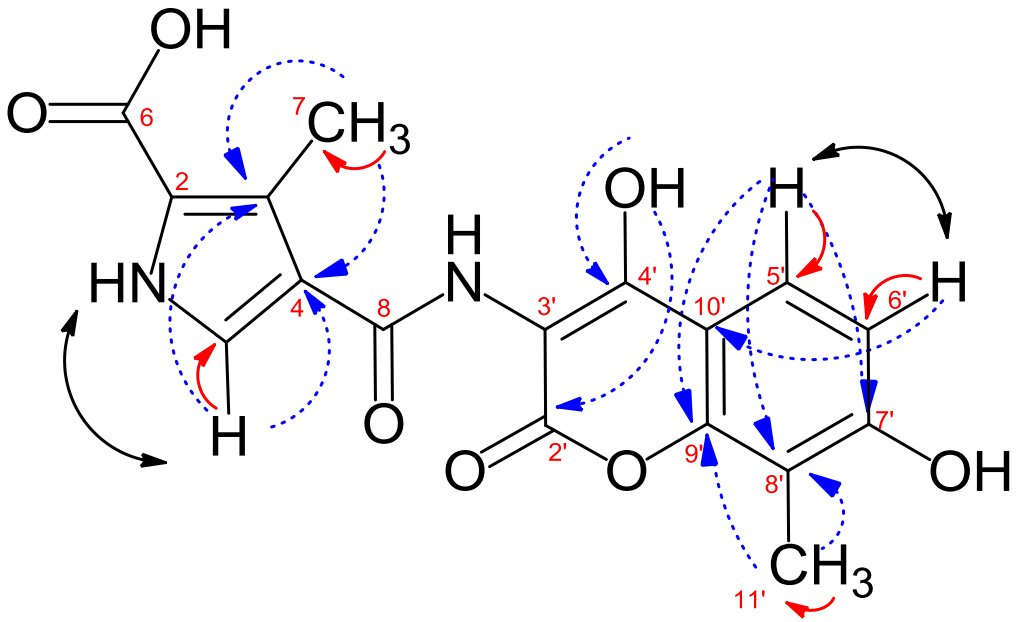


Table VI.3
Assignment of ^{1}H and ^{13}C NMR signals of unlabeled CPM monoamide

Position	^{1}H-NMR data (600 MHz, d_6-DMSO) δ_H [ppm]	^{13}C-NMR data (125.7 MHz, d_6-DMSO) δ_C [ppm]
1	11.85, 1H, bs, NH	
2	-	120.6
3	-	127.8
4	-	118.0
5	7.73, 1H, d, *J* = 2.8	125.5
6	12.50, 1H, bs, COOH	162.4
7	2.50, 3H, s, CH_3	11.2
8	-	160.9
9	11.95, 1H, bs, NH	
1‘	-	
2‘	-	164.8
3‘	-	100.5
4‘	10.47, 1H, s, OH	159.3
5‘	7.57, 1H, d, *J* = 8.7	121.5
6‘	7.90, 1H, d, *J* = 8.7	111.9
7‘	8.89, 1H, s, OH	158.9
8‘	-	110.3
9‘	-	151.2
10‘	-	108.3
11’	2.16, 3H, s, CH_3	8.1

bs = broad signal

Figure VI.13: ^{1}H-NMR spectrum of coumermycin A_1 (400 MHz, d_6-DMSO)

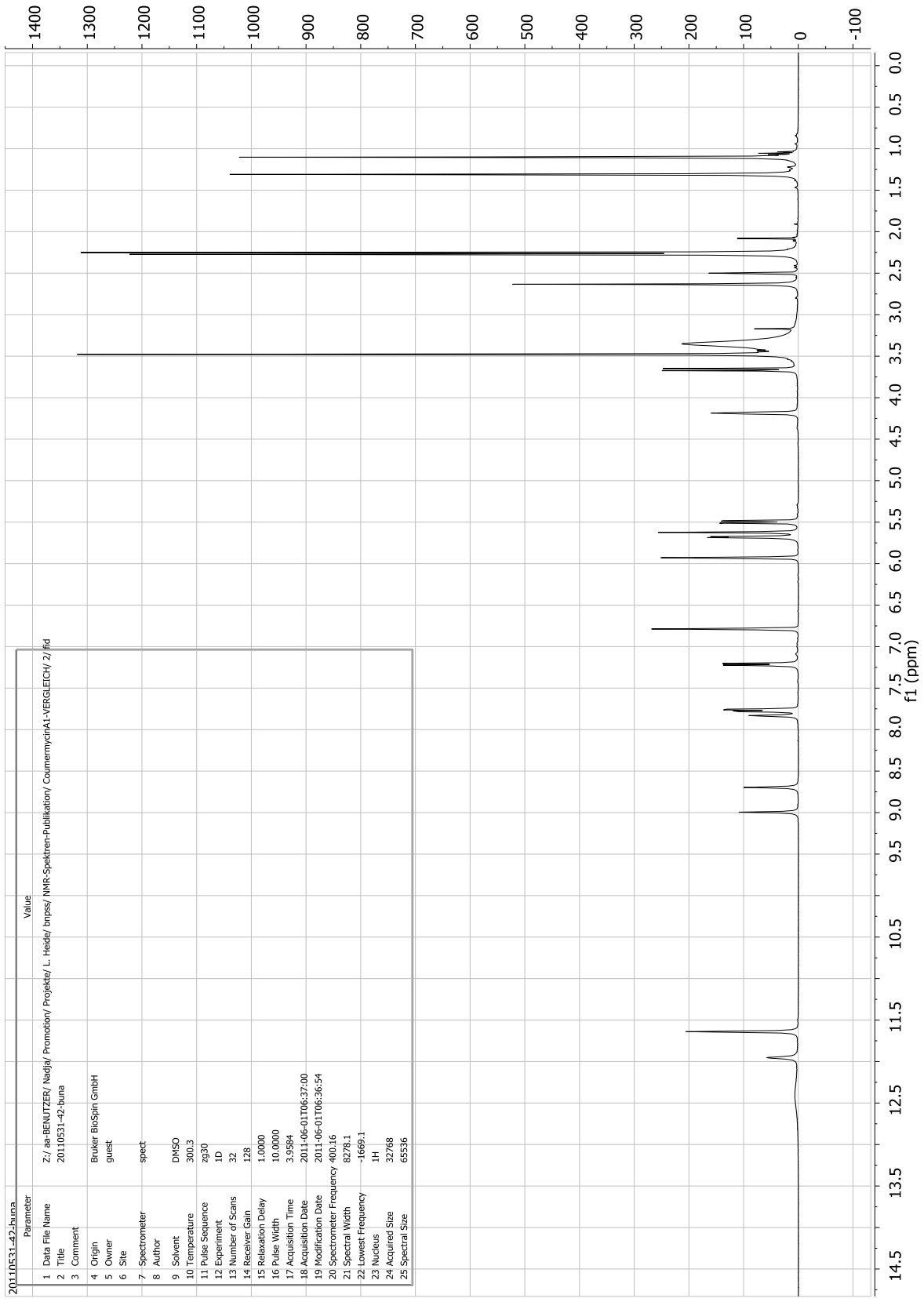


Figure VI.14: ^{13}C-NMR spectrum of coumermycin A_1 (100.6 MHz, d_6-DMSO)

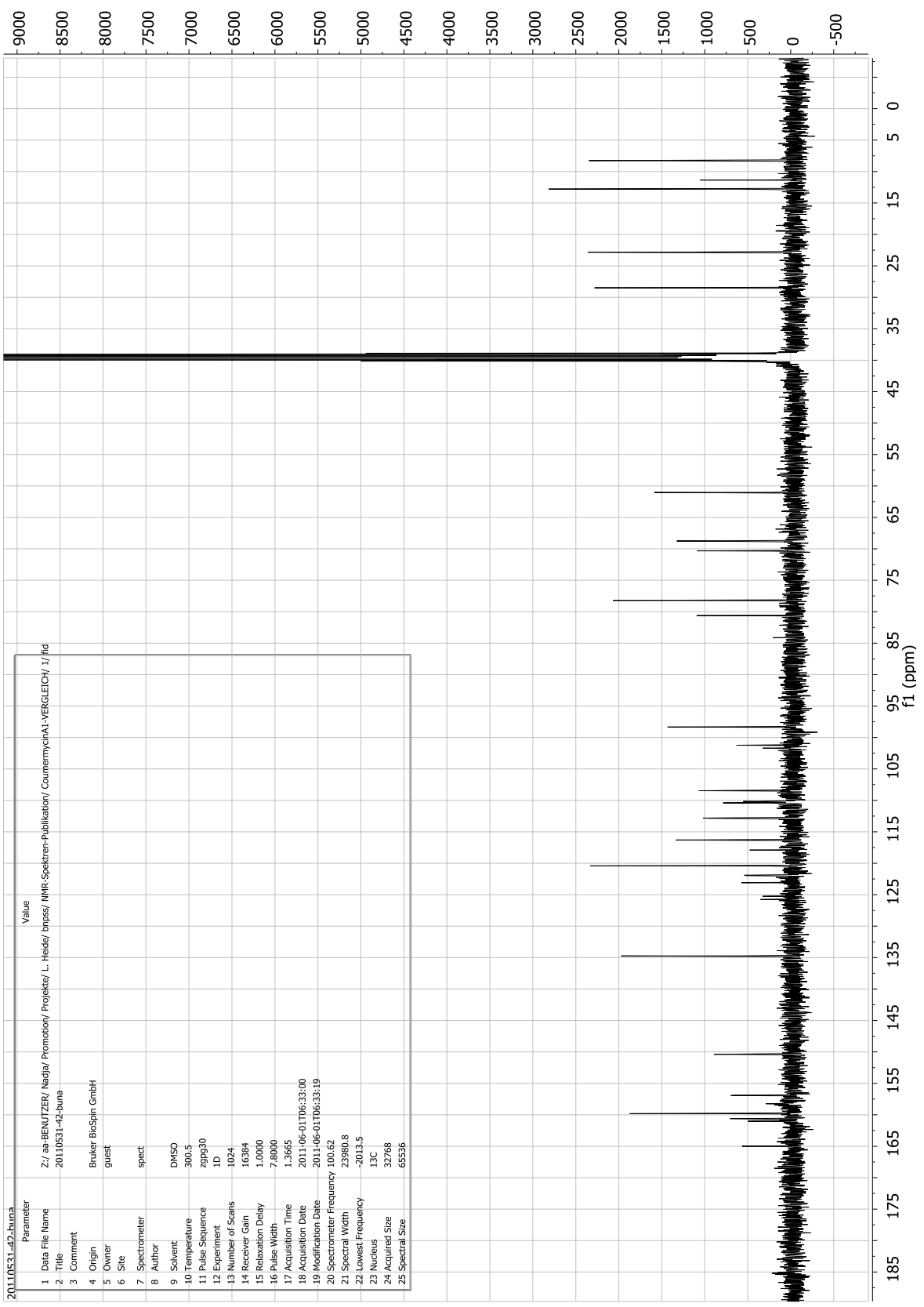
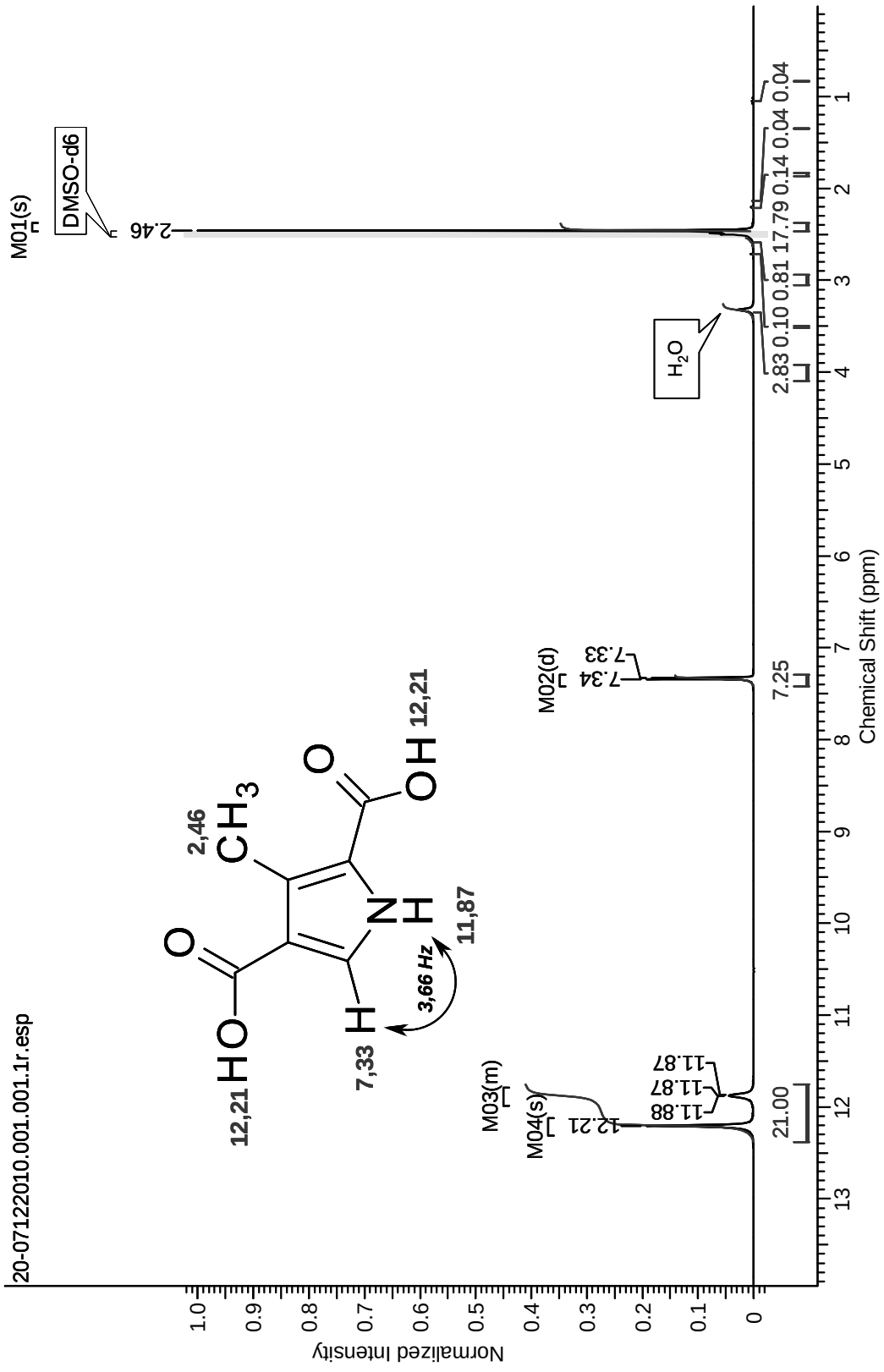


Figure VI.15: ^{1}H-NMR spectrum of 3-methylpyrrole-2,4-dicarboxylic acid (250 MHz, d_6-DMSO).



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