Molecular Biological and Biochemical Investigation of the Biosynthesis of Aminocoumarin Antibiotics

Molekularbiologische und biochemische Untersuchungen zur Biosynthese von Aminocoumarin-Antibiotika

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen
zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2004

vorgelegt von
Hui Xu

Tag der mündlichen Prüfung:	18.05.2004
Dekan:	Prof. Dr. H. Probst
1. Berichterstatter:	Prof. Dr. L. Heide
2. Berichterstatter:	PD Dr. S.-M. Li

Dedicated to

my parents

Contents

PUBLICATIONS AND PRESENTATIONS AT SCIENTIFIC MEETINGS

ABBREVIATIONS

SUMMARY/ZUSAMMENFASSUNG/综述

I INTRODUCTION

1. Antibiotics in the past and nowadays …………1
2. Aminocoumarin antibiotics—novobiocin, clorobiocin and coumermycin A_1 …………2
   2.1. Structural analysis …………3
   2.2. Mechamism of action and clinical application …………4
   2.3. Feeding experiments and identification of biosynthetic gene cluster …………5
   2.4. Generation of new aminocoumarin antibiotics …………8
3. Aims of this thesis …………9

II MATERIALS AND METHODS

1. Chemicals …………11
2. Materials for chromatography …………13
3. Enzymes and kits …………13
4. Media, buffers and solutions …………14
   4.1. Media for bacterial culture …………14
      4.1.1. Cultivation of *E. coli* …………14
      4.1.2. Cultivation of *Streptomyces* …………14
      4.1.3. Production medium for *S.rishiriensis* …………15
      4.1.4. Production media for *S. roseochromogenes* …………15
      4.1.5. Sporulation medium for *S. roseochromogenes* …………15
      4.1.6. Protein expression in *S. lividans* …………16
      4.1.7. Protoplast transformation of *Streptomyces* …………16
   4.2. Solutions of antibiotics …………17
   4.3. Buffers and solutions …………18
      4.3.1. Buffers for isolation of DNA …………18
      4.3.2. Buffers for DNA gel electrophoresis …………19
      4.3.3. Buffers and solutions for Southern blot analysis …………19
      4.3.4. Solutions for blue/white selection of *E. coli* …………20
      4.3.5. Buffers for preparation of protoplasts and transformation of *Streptomyces* …………20
      4.3.6. Buffers for protein purification by nickel affinity chromatography and for gel filtration …21
      4.3.7. Bradford reagent for protein quantification …………21
      4.3.8. Solutions for protein gel electrophoresis (SDS-PAGE) and for Coomassie staining ….22
5. Plasmids, bacterial strains and primers …………22
   5.1. Vectors, cosmids and plasmids …………23
   5.2. Bacterial strains …………25
   5.3. PCR primers …………26
   5.4. Probes used in Southern blot analysis …………26
6. Culture conditions …………26
   6.1. Cultivation of *E. coli* …………26
   6.2. Cultivation of *Streptomyces* …………26
      6.2.1. General cultivation …………26
      6.2.2. Production of secondary metabolites …………27
      6.2.3. Preparation of permanent culture and spore suspension …………27
7. Methods of molecular biology and biochemistry …………28

7.1. Methods of molecular biology ……28
7.1.1. Purification, concentration and quantification of DNA ……28
7.1.2. Agarose gel electrophoresis of DNA ……28
7.1.3. DNA manipulation with enzymes ……29
7.1.4. DNA isolation ……29
7.1.4.1. Isolation of plasmids from *E. coli* ……29
7.1.4.2. Isolation of plasmids from *Streptomyces* ……29
7.1.4.3. Isolation of genomic DNA from *Streptomyces* ……30
7.1.5. DNA denaturation for ssDNA transformation in *Streptomyces* ……30
7.1.5.1. Glycerol-heat denaturation ……30
7.1.5.2. Alkaline treatment ……30
7.1.6. PCR amplification ……30
7.1.7.Southern blot analysis ……31
7.1.7.1. Probe preparation ……31
7.1.7.2. Blot preparation ……31
7.1.7.3. Prehybridization and hybridization ……32
7.1.7.4. Detection ……32
7.1.7.5. Removal of probe ……32
7.1.8. $CaCl_2$-mediated transformation of *E. coli* ……32
7.1.8.1. Preparation of competent cells ……32
7.1.8.2. Transformation ……33
7.1.9. Preparation of protoplasts and transformation of *Streptomyces* ……33
7.1.9.1. Preparation of protoplasts ……33
7.1.9.2. PEG-mediated protoplast transformation and regeneration ……34
7.1.10. DNA sequencing and computer-assisted sequence analysis ……35
7.2. Methods of biochemistry ……35
7.2.1. Protein quantification ……35
7.2.2. SDS-PAGE ……35
7.2.3. Gel filtration and determination of molecular weight ……35
7.2.4. Overexpression and purification of recombinant protein from *E. coli* ……37
7.2.5. Overexpression and purification of NovN as Histidine fusion protein from *S. lividans* T7
7.2.5.1. Cultivation ……37
7.2.5.2. Preparation of cell-free extract ……37
7.2.5.3. Purification by nickel affinity chromatography ……37
7.2.6. Carbamoyltransferase assay ……38
7.2.7. Determination of kinetic parameters ……38

8. Construction of vectors ……38
8.1. Construction of pN3 and pN4 for inactivation of *cou*N3 and *cou*N4 in *S. rishiriensis* (WT) …38
8.2. Construction of vector pN2 for inactivation of *clo*N2 in *S. roseochromogenes* (WT) and *clo*-hal⁻ mutant ……39
8.3. Construction of pN2C for complementation of *clo*N2⁻ mutant with gene *clo*N2 ……39
8.4. Construction of pXHN for heterologous expression of NovN in *cou*N3⁻ mutant ……40
8.5. Construction of pXHNEG for expression of NovN as N-terminal 6×His-tagged protein in *S. lividans* T7 ……40

9. Analysis and isolation of secondary metabolites ……40
9.1. Extraction and HPLC analysis ……40
9.1.1. Analysis of coumermycin A_1 and derivatives ……40
9.1.2. Analysis of clorobiocin and derivatives ……41
9.1.3. Analysis of pyrrole-2-carboxylic acid ……41
9.1.4. Analysis of enzymatic reaction products ……41
9.2. Preparative isolation of new aminocoumarin derivatives ……42
9.2.1. Column chromatography using Sephadex® LH-20 ……42
9.2.2. Preparative HPLC ……42
9.3. Spectroscopic methods for structural elucidation ……42
9.3.1. LC/MS and selected reation monitoring (SRM) ……42
9.3.2. FAB (-) mass spectroscopy ……43
9.3.3. NMR ……43

10. Methods of biology ……43
10.1. Supercoiling assay ……43
10.2. Bioassay with *Bacillus subtilis* ……43

III RESULTS

1. Involvements of *cou*N3 and *cou*N4 in the biosynthesis of the pyrrole moiety of coumermycin A_1..45
   1.1. Inactivation of *cou*N3 47
   1.2. Secondary metabolites production in the wild-type and *cou*N3⁻ mutant 48
   1.3. Inactivation of *cou*N4 50
   1.4. Secondary metabolites production in the *cou*N4⁻ mutant 51
2. Identification of *clo*N2, an acyltransferase gene responsible for the attachment of the pyrrole moiety to the deoxysugar of clorobiocin 52
   2.1. Inactivation of *clo*N2 in *S. roseochromogenes* (WT) 53
   2.2. Analysis of secondary metabolites and identification of novclobiocin 104 and 105 54
   2.3. Identification of pyrrole-2-carboxylic acid in the culture extract of *clo*N2⁻ mutant by LC/MS and ^{1}H-NMR 56
   2.4. Complementation of *clo*N2⁻ mutant with pN2C 57
3. Production of carbamoylated aminocoumarin derivatives 58
   3.1. 3´´-Unsubstituted aminocoumarin derivatives 58
      3.1.1. Production of novclobiocins 107 and 108 by inactivation of *clo*N2 in *clo*-hal⁻ mutant ...59
   3.2. Production of carbamoylated aminocoumarin derivatives *in vivo* 62
      3.2.1. Overexpression of NovN in the *cou*N3⁻ mutant 62
      3.2.2. Secondary metabolite production of the *cou*N3⁻ mutant expressing NovN 63
   3.3. Production of carbamoylated aminocoumarin derivatives *in vitro* 66
      3.3.1. Overexpression of NovN as 6×His-tagged protein in *S. lividans* 66
      3.3.2. Carbamoyltransferase assay 68
      3.3.3. Characterization of NovN 69
         3.3.3.1. Molecular weight of native protein 69
         3.3.3.2. Cofactor, linear dependence over time and protein amount 69
         3.3.3.3. Determination of kinetic parameters for descarbamoyl novobiocin 69
      3.3.4. Chemoenzymatic synthesis of new aminocoumarin antibiotics 70
      3.3.5. Kinetic parameters for novclobiocin 104 and 105 72
      3.3.6. Further investigations of substrate specificity 73
4. Testing the biological activity of new aminocoumarin derivatives 74
   4.1. Inhibitory activity on *E. coli* gyrase 74
   4.2. Antibacterial activity against *Bacillus subtilis* ATCC 14893 75

IV DISCUSSION

1. Biosynthesis of pyrrole-2-carboxyl moiety in coumermycin A_1 and clorobiocin 77
2. Different biosynthetic pathway of central pyrrole moiety in coumermycins 78
3. Attachment of pyrrole-2-carboxyl moiety to the deoxysugar in clorobiocin and coumermycinA_1 ...78
4. Production of novel carbamoylated aminocoumarin derivatives 82
   4.1. Production of new carbamoylated coumermycin derivatives *in vivo* 82
   4.2. Production of new carbamoylated aminocoumarin antibiotics *in vitro* 82
5. Structure—activity relationships within the aminocoumarin antibiotics 83

REFERENCES

APPENDIX I—Summary of MS and NMR spectral data

APPENDIX II—MS and NMR spectra

ACADEMIC TEACHERS

ACKNOWLEDGMENTS

CURRICULUM VITAE

LIST OF TABLES

Table 1 Chemicals and media compounds 11

Table 2 Liquid chromatography media 13

Table 3 Enzymes and kits 13

Table 4 Solutions of antibiotics 18

Table 5 Buffers for isolation of plasmids from *E. coli* 18

Table 6 Buffers for isolation of plasmids from *Streptomyces* 18

Table 7 Buffers for isolation of genomic DNA from *Streptomyces* 19

Table 8 Buffers for DNA gel electrophoresis 19

Table 9 Buffers and solutions for Southern blot analysis 19

Table 10 Stock solutions for blue/white selection 20

Table 11 Buffers for preparation of protoplasts and transformation of *Streptomyces* 20

Table 12 Buffers for protein purification 21

Table 13 Buffers and solutions for SDS-PAGE and Coomassie staining 22

Table 14 Vectors, cosmids and plasmids 23

Table 15 Bacterial strains of *E. coli* and *Streptomyces* 25

Table 16 Primers for PCR amplification 26

Table 17 DIG-labelled probes 26

Table 18 PCR mixture using GC-Rich PCR system and amplification conditions 31

Table 19 Calibration of the Sephadex® 200 column 32

Table 20 Similarity of the gene products of *cou*N3, *cou*N4 and *cou*N5 with products of the clorobiocin, pyoluteorin and undecylprodiginine genes 46

Table 21 Chemical structures of aminocoumarin derivatives 71

Table 22 Comparison of ^{1}HNMR data of aminocoumarin derivatives (400 MHz, CD_3OD) 72

LIST OF FIGURES

Fig. 1 Structures and biosynthetic gene clusters of novobiocin, clorobiocin and coumermycin A_1 3

Fig. 2 Interaction between GyrB and novobiocin as well as between GyrB and clorobiocin 5

Fig. 3 Calibration curve of gel filtration 36

Fig. 4 Proposed biosynthetic pathway for the pyrrole-2-carboxylic acid moieties of coumermycin A_1, clorobiocin, pyoluteorin and undecylprodiginine 46

Fig. 5 Inactivation of *cou*N3 and *cou*N4 in *S. rishiriensis* DSM 40489 48

Fig. 6 HPLC analysis of the secondary metabolites in wild-type and mutants of *S. rishiriensis* 50

Fig. 7 Inactivation of *clo*N2 in *S. roseochromogenes var. oscitans* DS 12.976........ 53

Fig. 8 HPLC analysis of the secondary metabolites in *S. roseochromogenes* strains........ 55

Fig. 9 Analysis of pyrrole-2-carboxylic acids in extracts of bacterial cultures by SRM 57

Fig. 10 Structures of the 3´´-unsubstituted aminocoumarin derivatives 59

Fig. 11 Inactivation of *clo*N2 in *clo*-hal$^-$ mutant........ 60

Fig. 12 HPLC analysis of the secondary metabolites in *clo*-hal$^-$ and *clo*N2$^-$/ *clo*-hal$^-$ mutants of *S. roseochromogenes* 61

Fig. 13 Map of the vector pXHN and restriction analysis of the intact plasmid pXHN from two different transformands 63

Fig. 14 LC/MS and selected reaction monitoring (SRM) analysis of the secondary metabolites in *S. rishiriensis* wild-type and mutants........ 65

Fig. 15 Map of expression vector pXHNEG 67

Fig. 16 Analysis of proteins expressed in *S. lividans* T7 by SDS-PAGE (10%) 68

Fig. 17 HPLC analysis of the carbamoyltransferase assay with native substrate descarbamoyl novobiocin 68

Fig. 18 Lineweaver-Burk plot for calculation of kinetic parameters for descarbamoyl novobiocin 69

Fig. 19 HPLC analysis of the carbamoyltransferase assays with 3´´-unsubstituted aminocoumarin derivatives 70

Fig. 20 Lineweaver-Burk plots for calculation of kinetic parameters for novclobiocin 104 and 105.. 72

Fig. 21 HPLC analysis of the carbamoyltransferase assay with coumermycin D 73

Fig. 22 HPLC analysis of the carbamoyltransferase assay with acetyl phosphate 73

Fig. 23 *In vitro* inhibition of the DNA supercoiling reaction catalysed by *E. coli* gyrase 74

Fig. 24 Bioassay against *Bacillus subtilis* 76

Fig. 25 Hypothetical scheme of the last steps of clorobiocin biosynthesis 81

Publications and presentations at scientific meetings

Research publications:

H. Xu, Z.-X. Wang, J. Schmidt, L. Heide, S.-M. Li (2002) Genetic analysis of the biosynthesis of the pyrrole and carbamoyl moieties of coumermycin A_1 and novobiocin. Mol. Genet. Genomics. 268:387-396

H. Xu, R. Kahlich, B. Kammerer, L. Heide, S.-M. Li (2003) CloN2, a novel acyltransferase involved in the attachment of the pyrrole-2-carboxyl moiety to the deoxysugar of clorobiocin. Microbiology. 149:2183-2191

C. Meyers, M. Obertür, **H. Xu**, L. Heide, D. Kahne, C. T. Walsh (2004) Characterization of NovP and NovN: completion of novobiocin biosynthesis *via* sequential tailoring of the noviosyl ring. Angew. Chem. Eng. Int. Ver. 43:67-70

H. Xu, L. Heide, S.-M. Li. (2004) New aminocoumarin antibiotics formed by a combined mutational and chemoenzymatic approach, utilizing the carbamoyltransferase NovN. Chem. Biol. 11:655-662

Presentations at scientific meetings:

November 15-17 2001: Poster presentation at the VAAM Workshop (“Biologie bakterieller Naturstoffproduzenten”) in Berlin, Germany

September 24-26 2002: Poster presentation at the VAAM Workshop (“Biologie bakterieller Naturstoffproduzenten”) in Freiburg, Germany

September 27-29 2003: Oral presentation at the International Meeting on the Biology of Bacteria Producing Natural Compounds in Groningen, Holland

March 24-26 2004: Oral presentation at the DPhG Graduate Student Meeting 2004 (“Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft DPhG”) in Freudenstadt-Lauterbach, Germany

ABBREVIATIONS

°C	degree celsius
µ	micro
6×His	hexahistidines
aa	amino acids
Amp	ampicillin
AMP	adenosine monophosphate
APS	ammonium persulphate
ATCase	aspartate carbamoyltransferase
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CAP-Li_2	carbamoyl phosphate dilithium salt
CSPD	chemiluminescence substrate
Da	dalton
DIG	digoxingenine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5´-triphosphates
dsDNA	double-stranded deoxyribonucleic acid
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ESI	electrospray ionization
eV	electron volt
FAB	fast atom bombardment
Fig.	figure
g	gram
GyrB	gyrase B subunit
h	hour
HAc	acetic acid
HCl	hydrochloric acid
HCOOH	formic acid
HPLC	high performance liquid chromatography
Hz	hertz
IPTG	isopropyl-β-thiogalactoside
k	kilo
KAc	potassium acetate
kb	kilobase
kDa	kilodalton
K_m	Michaelis-Menten constant
l	liter
*lac*Z	β-galactosidase gene
LC-ESI-CID	liquid chromatography-electrospray ionization-collision induced dissociation
M	molar
m	milli
min	minute
MW	molecular weight
MS	mass spectroscopy

n	nano
NaAc	sodium acetate
NaOH	sodium hydroxide
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
ORF	open reading frame
p	pico
PCR	polymerase chain reaction
PCP	peptidyl carrier protein
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
Ring A	3-dimethylallyl-4-hydroxybenzoic acid
RNase	ribonuclease
RP	reverse phase
rpm	rotation per minute
RT	room temperature
s	second
s.	see
S.	*Streptomyces*
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRM	selected reaction monitoring
ssDNA	single-stranded deoxyribonucleic acid
TCA	trichloroacetic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
TES	N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-maleate	Tris-(hydroxymethyl)-aminomethane-maleate
U	unit
UV	ultraviolet
WT	wild-type
×g	ground acceleration
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Summary

The structurally related aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of bacterial gyrase and represent interesting starting compounds for the development of new antibacterial agents. X-ray crystallographic analysis has shown that the acyl moieties at the 3´´-hydroxy group of the deoxysugars of these antibiotics, namely the carbamoyl group in novobiocin and 5-methylpyrrole-2-carboxylic acid moiety in clorobiocin and coumermycin A_1, are particularly important for their binding to the biological target, i.e. the B subunit of gyrase. In this thesis, genes involved in the biosynthesis of the methylpyrrole moiety and those responsible for acyl transfer to the deoxysugar were identified by gene inactivation, heterologous expression as well as biochemical experiments. Several aminocoumarin derivatives were obtained from different defective mutants of the coumermycin and clorobiocin producers. Based on these findings, a series novel carbamoylated aminocoumarin antibiotics were generated by *in vivo* overexpression of carbamoyltransferase NovN from the novobiocin gene cluster and *in vitro* chemoenzymatic biosynthesis using NovN.

Coumermycin A_1 contains a central and two terminal pyrrole moieties. The coumermycin gene cluster from *Streptomyces rishiriensis* DSM 40489 contains three genes (*cou*N3, *cou*N4 and *cou*N5) which show sequence similarity to genes involved in the biosynthesis of the pyrrole moieties of pyoluteorin in *Pseudomonas fluorescens* and of undecylprodiginine in *Streptomyces coelicolor*. The gene *cou*N3, coding for a putative L-prolyl-*S*-PCP dehydrogenase, and the gene *cou*N4, coding for a putative L-prolyl-AMP ligase, were disrupted using in-frame deletion and insertional inactivation, respectively. HPLC analysis of culture extracts showed that the formation of coumermycin A_1 was abolished in the *cou*N3$^-$ und *cou*N4$^-$ mutants. The mutants accumulated coumermycin D, which contains only the central pyrrole moiety. This result not only confirmed the involvement of *cou*N3 and *cou*N4 in the biosynthesis of the terminal pyrrole-2-carboxylic acid moieties of coumermycin A_1, but also demonstrated, for the first time, that the central 3-methylpyrrole-2,4-dicarboxylic acid unit of the coumermycins is formed by a biosynthetic pathway different from that of the terminal pyrrole moieties.

The terminal 5-methylpyrrole-2-carboxylic acid units in clorobiocin and coumermycin A_1 are attached *via* an ester bond to the 3´´-OH group of the deoxysugar moiety. In order to investigate candidate genes responsible for the formation of this ester bond, a gene inactivation experiment was carried out in the clorobiocin producer *Streptomyces roseochromogenes* var. *oscitans* DS 12.976. An in-frame deletion was created in the coding sequence of the gene *clo*N2. The production of secondary metabolites in the wild-type and in the *clo*N2$^-$ mutant was analysed. The wild-type showed clorobiocin as the main product, whereas the *clo*N2$^-$ mutant accumulated a new aminocoumarin derivative, i.e. novclobiocin 104, lacking the pyrrole moiety at 3´´-OH of the deoxysugar. Clorobiocin production was successfully restored in the *clo*N2$^-$ mutant by introducing a replicative plasmid containing the *clo*N2 sequence. In addition, free pyrrole-2-carboxylic acid accumulated in the culture extract of the *clo*N2$^-$ mutant. The structures of the metabolites were confirmed by NMR and LC/MS analysis. These results prove an involvement of *clo*N2 in the formation of the ester bond between the pyrrole moiety and the deoxysugar in clorobiocin biosynthesis. Moreover, the results indicate that the *C*-methylation at position 5´´´ of the pyrrole moiety occurs after the attachment of pyrrole-2-carboxylic acid unit to the deoxysugar moiety.

*nov*N, a putative carbamoyltransferase gene in the biosynthetic gene cluster of novobiocin from *Streptomyces spheroides* NCIMB 11891, was expressed in the *cou*N3$^-$ mutant. This led to the formation of bis-carbamoylated coumermycin D, a novel compound of the coumermycin series. This experiment also proved that NovN is responsible for the carbamoylation of 3´´-OH of the noviose in novobiocin.

Five further carbamoylated aminocoumarin derivatives were produced by a combined mutational and chemoenzymatic approach. For this purpose, the carbamoyltransferase NovN was overexpressed in the heterologous host *S. lividans* as N-terminal 6×His-tagged protein and purified by nickel affinity chromatography. Five different 3´´-unsubstituted aminocoumarin derivatives were isolated from mutants of the clorobiocin producer *S. roseochromogenes*, carrying single or multiple gene defects. All five compounds were readily accepted as substrates by NovN, demonstrating that structural variation can be introduced by this method in the deoxysugar moiety, the aminocoumarin moiety as well as in the benzoyl moiety. The 3´´-carbamoylated products were isolated on a preparative scale and their structures

were elucidated by ^{1}H-NMR and mass spectroscopy. Testing the biological activity of the 3´´-carbamoylated products gave further insights into the structure–activity relationships of aminocoumarin antibiotics, especially about the importance of the acyl moiety and the methyl group in position 3´´- and 4´´-OH of the deoxysugar, respectively. Removing either of these structural elements resulted in complete loss of activity.

Zusammenfassung

Die Gyrase-Hemmstoffe Novobiocin, Clorobiocin und Coumermycin A_1 werden von verschiedenen *Streptomyces*-Arten produziert. Aufgrund ihrer strukturellen Ähnlichkeiten und Unterschiede stellen sie sehr interessante Ausgangssubstanzen für die Herstellung neuer pharmazeutischer Produkte durch Gentechnik dar. Um diese Untersuchungen durchführen zu können, braucht man jedoch genaue Informationen über die Biosynthese von Aminocoumarin-Antibiotika. Die biosynthetischen Gencluster für alle drei Antibiotika wurden in der Arbeitsgruppe von Prof. Dr. Heide und PD Dr. Li kloniert und sequenziert und die Funktion von vielen Genen aufgeklärt. In der vorliegenden Arbeit wurden die Gene, die an der Biosynthese der 5-Methylpyrrol-2-carbonsäure-Einheit beteiligt sind sowie die für die Übertragung der Acylkomponenten an den Desoxyzucker verantwortlich sind, durch Geninaktivierung, heterologe Expression und biochemische Untersuchungen identifiziert. Zudem wurden neue Aminocoumarin-Derivate erzeugt und deren biologische Aktivität untersucht.

Coumermycin A_1 enthält zwei endständige 5-Methylpyrrol-2-carbonsäure-Einheiten und eine zentrale 3-Methylpyrrol-2,4-dicarbonsäure-Einheit. Im biosynthetischen Gencluster für Coumermycin A_1 von *Streptomyces rishiriensis* DSM 40489 kommen drei Gene (*cou*N3, *cou*N4 und *cou*N5) vor, die Homologie zu den Genen der Biosynthese der Pyrroleinheit von Pyoluteorin in *Pseudomonas fluorescens* und von Undecylprodiginine in *Streptomyces coelicolor* aufweisen. Das Gen *cou*N3*, das* vermutlich für eine L-Prolyl-*S*-PCP-Dehydrogenase codiert, wurde durch eine „in-frame“ Deletion inaktiviert. Das Gen *cou*N4*,* ein Homolog zur L-Prolyl-AMP-Ligase, wurde durch Insertion eines Neomycinresistenzgens inactiviert. Während Coumermycin A_1 als Hauptprodukt im Wildtyp zu detektieren war, wurde die Produktion von Coumermycin A_1 in *cou*N3-Defektmutante und *cou*N4-Defektmutante vollständig blockiert und stattdessen wurde Coumermycin D akkumuliert. Im Vergleich zu Coumermycin A_1, enthält Coumermycin D nur die zentrale Pyrroleinheit und die zwei endständigen Pyrroleinheiten am Zucker sind nicht mehr vorhanden. Dieses Ergebnis bewies, dass die Gene *cou*N3 und *cou*N4 an der Biosynthese der 5-Methylpyrrol-2-carbonsäure-Einheit beteiligt sind, jedoch nicht im Zusammenhang mit der Biosynthese der zentralen 3-Methylpyrrol-2,4-dicarbonsaüre-Einheit stehen.

Die zentrale Pyrroleinheit muss also durch einen anderen Biosyntheseweg gebildet sein.

Sowohl in Clorobiocin als auch in Coumermycin A_1 sind die 5-Methylpyrrol-2-carbonsäure-Einheiten an der 3´´-OH Gruppe des Desoxyzuckers durch eine Esterbindung verknüpft. Durch Sequenzvergleich in Datenbanken konnte festgestellt werden, dass *clo*N2/*cou*N2 die möglichen Acyltransferasen codieren. Die Vermutung wurde durch die Inaktivierung des Gens *clo*N2 im Clorobiocin-Produzenten *Streptomyces roseochromogenes* var. *oscitans* DS 12.976 experimentell bestätigt. Die inaktivierung von *clo*N2 erfolgte durch eine „in-frame" Deletion. Die Sekundärstoffproduktion vom Wildtyp und von der *clo*N2-Defektmutante wurde analysiert. Während der Wildtyp Clorobiocin als das Hauptprodukt zeigte, akkumulierte die *clo*N2-Defektmutante ein neues Aminocoumarin-Derivat, nämlich Novclobiocin 104, wobei die Pyrrol-Einheit an der 3´´-OH des Desoxyzuckers fehlt. Nach Überexpression des Gens *clo*N2 in der *clo*N2-Defektmutante konnte Clorobiocin wieder hergestellt werden. Darüber hinaus war Pyrrol-2-carbonsäure im Kulturextrakt der *clo*N2-Defektmutante detektierbar. Der Nachweis der zwei Bestandteile von Clorobiocin, i.e. Novclobiocin 104 und Pyrrol-2-carbonsäure, im Kulturextrakt der *clo*N2-Defektmutante bewies, dass Gen *clo*N2 für die Übertragung der Pyrroleinheit an den Desoxyzucker verantwortlich ist. Weiterhin wiesen die Ergebnisse darauf hin, dass die *C*5-Methylierung der Pyrroleinheit erst nach der Übertragung stattfindet.

*nov*N aus dem Novobiocin-Biosynthesegencluster von *Streptomyces spheroides* NCIMB 11891 zeigt Sequenzähnlichkeiten mit Carbamoyltransferase-Genen. Heterologe Expression von NovN in der *cou*N3-Defektmutante führte zur Produktion von einem neuen Coumermycin-Derivat, und zwar Dicarbamoylcoumermycin D, bei dem die 5-Methylpyrrol-2-carbonsäurereste von Coumermycin A_1 durch Carbamoylgruppen ersetzt sind. Das Ergebnis bewies gleichzeitig, dass NovN für die Carbamoylierung der 3´´-Hydroxylgruppe des Desoxyzuckers verantwortlich ist.

Fünf weitere carbamoylierte Aminocoumarin-Derivate wurden durch Mutation und anschließende chemoenzymatische Synthese hergestellt. Dafür wurde NovN als 6×Histidin-Fusionsprotein in S. *lividans* T7 überexprimiert. Nach der Reinigung wurde das Protein charakterisiert. Fünf Aminocoumarin-Derivate mit freier 3´´-OH Gruppe

wurden aus verschiedenen Mutanten von *S. roseochromogenes* gewonnen und für die Carbamoylierung verwendet. Dadurch entstanden fünf neue carbamoylierte Aminocoumarin-Derivate, deren Strukturen mit Hilfe von MS und NMR aufgeklärt wurden. Die Untersuchung der biologischen Aktivität von natürlichen Aminocoumarinen und deren Derivaten ermöglichte es, weitere Struktur-Aktivitäts-Beziehungen aufzuklären. Es wurde gezeigt, dass die Acylkomponenten an 3´´-OH bzw. die Methylgruppe an 4´´-OH vom Desoxysugar von großer Bedeutung für die biologische Aktivität sind. Die Entfernung von einem der beiden Elemente führt zum nahezu vollständigen Verlust der Aktivität.

综述

氨基香豆素类抗生素新生霉素(novobiocin), 氯新生霉素(clorobiocin)和香豆霉素A_1(coumermycin A_1)分别由不同的链霉菌属细菌产生。它们通过与 ATP 竞争结合到细菌拓扑异构酶 II 中 B 亚单位上而产生抗菌作用。但是，由于存在副作用较大，水溶性差，对格兰氏阴性细菌不起作用等缺点，限制了氨基香豆素类抗生素在临床上的应用。因此， 对它们的结构进行改造，合成新的性能更好的氨基香豆素类抗菌素将是非常有意义的。在过去的几年里，新生霉素, 氯新生霉素和香豆霉素 A_1 的生物合成基因组已陆续被鉴定，许多基因的功能也已阐明，这就使得利用现代的基因杂交技术及酶反应等方法来合成新的氨基香豆素类抗生素成为可能。本研究工作主要包括以下三个方面。

1. 脱氢酶基因(*cou*N3)和单磷酸腺苷结合酶基因(*cou*N4)的鉴定及末端吡咯的合成

香豆霉素 A_1 含有一个中央 3-甲基-2,4-吡咯二羧酸和两个末端 5-甲基-2-吡咯羧酸。通过蛋白质序列比较，在香豆霉素生物合成基因组中可以找到三个基因(*cou*N3, *cou*N4 和 *cou*N5)，它们和 Pyoluteorin 以及 Undecylprodiginine 中吡咯生物合成基因有很高的同源性。用基因工程的方法分别阻断香豆霉素产生菌 *Streptomyces rishiriensis* DSM 40489 中的 *cou*N3 和 *cou*N4，得到了两种突变株。与野生株相比，突变株都只产生香豆霉素 D（含中央吡咯酸，不含末端吡咯酸）。这个实验一方面证明了 *cou*N3 和 *cou*N4 参与了香豆霉素 A_1 末端吡咯羧酸的生物合成，另一方面，也表明了中央吡咯羧酸是通过另外一个生物合成途径形成的。

2. 酰基转移酶基因(*clo*N2)的鉴定及末端吡咯羧酸与脱氧糖的链接

氯新生霉素和香豆霉素 A_1 末端吡咯羧酸都是通过一个酯键与脱氧糖相连的。为了寻找催化这个反应的酰基转移酶，进行了氯新生霉素产生菌 *Streptomyces roseochromogenes* 基因 *clo*N2 的失活实验。*clo*N2 突变株, 与野生株相比, 其二级代谢产物为去吡咯氯新生霉素（novclobiocin 104）, 此外还可以监测到游离 2-吡咯羧酸, 从而证明了 CloN2 是一个酰基转移酶, 催化氯新生霉素中脱氧糖和吡咯羧酸间酯键的形成. 由于在变异株中只能检测到 2-吡咯羧酸，而不是 5-甲基-2-吡咯羧酸，因此该实验也证明了吡咯羧酸是先连接到脱氧糖上后，才被甲基化的。

3. 甲酰胺基转移酶基因(*nov*N)的鉴定与甲酰胺化氨基香豆素类衍生物的合成

新生霉素生物合成基因组中有一基因 *nov*N，蛋白质序列比较表明，和甲酰胺基转移酶有很高的同源性。将此基因（来自新生霉素产生菌 *Streptomyces spheroides*）通过可自我复制质粒转化到 *cou*N3 突变株中，可得到一个新的香豆霉素的衍生物，也就是甲酰胺化的香豆霉素 D。这个实验同时也证明了 NovN 确实是一甲酰胺基转移酶。

此外，利用基因突变和酶催化反应的有机结合，合成了另外五个新的甲酰胺化氨基香豆素类抗生素的衍生物。首先，将酰胺基转移酶 NovN 在 *Streptomyces lividans* T7 中表达成有活性的组氨酸结合蛋白质，经纯化后进行生化性质的研究。用五个来自不同 *S. roseochromogenes* 突变株的含游离的 3´´-OH 脱氧糖的氨基香豆素类衍生物做底物，进行甲酰胺基转移酶催化反应。经分离纯化后，得到了五种新的甲酰胺化氨基香豆素类衍生物。这些化合物抗菌性能的预实验，阐明了结构和生物活性之间的联系，揭示了脱氧糖上 3 位的酰基取代和 4 位的甲基取代对于它们的生物活性来说是至关重要的。

Introduction

1 Antibiotics in the past and nowadays

Since the introduction in the 1940s, antibiotics have been widely used in human and veterinary medicine. 1940-1960 was the so-called ‘golden era’ of antibiotic discovery from natural products, and new classes of antimicrobial agents, such as chloramphenicol, tetracycline, erythromycin, among many others, rapidly entered the market (Walsh 2003). Many of the antibiotics in the marketplace today are semi-synthetic variants of natural antibiotic compounds. On the other hand, antibiotics with fundamentally new structures were obtained by chemical synthesis. Drugs arising from such approaches include the therapeutically successful sulphonamides and quinolones.

The chemical structures of antibiotics are extremely varied, including β-lactams, aminoglycosides, glycopeptides, macrolides, quinolones, aminocoumarins and so on. Correspondingly, their antibacterial mechanisms also vary greatly. For example, the β-lactams such as penicillin or cephalosporins inhibit the cell-wall biosynthesis. The bacterial targets of quinolones and aminocoumarin antibiotics are DNA gyrase A und B subunit, respectively. Aminoglycosides, macrolides, chloramphenicol and tetracyclines are inhibitors of protein synthesis (Coates *et al.,* 2002).

Antibiotic natural products are secondary metabolites of bacteria, fungi or plants. The bacterial genus *Streptomyces* has, over the last sixty years, been the richest source of antibiotics. More than half of the world's antibiotics are isolated from *Streptomyces*.

Over 8000 antibiotics are known to science, but there is still a constant need for new antibacterial agents owing to the global emergence of resistance to antibiotics (Walsh 2003). Classic methods as well as novel ones are employed to develop new antibiotics. Recent interest in the screening approach has been rekindled to discover naturally antimicrobial peptides such as cathelicidins (Nizet *et al.,* 2001) and piscidins (Noga & Silphaduang 2003). Potentially, new antimicrobial agents could also result from combinatorial chemistry (Bax *et al.,* 2000). Through structural changes, whole families of drugs have been made that are based on, for example, penicillin or quinolone. Another variant of the combinatorial approach is combinatorial

biosynthesis to make hybrid antimicrobial agents (Hopwood *et al.,* 1985; Omura *et al.,* 1986). This method is based on the principles of heterologous gene expression, molecular biology and the promiscuous nature of enzymes that are involved in the biosynthesis of antibiotics. It has been successfully used to generate novel polyketide and peptides by re-programming the polyketide and non-ribosomal peptide pathway through swapping protein domains, modules and subunits between sets of polyketide synthesis and non-ribosomal peptides synthetase 'assemble lines' (McDaniel *et al.,* 1999; Mootz *et al.,* 2000; Walsh 2003). Numerous novel glycosylated compounds have been produced using sugar-flexible glycosyltransferases (Hoffmeister *et al.,* 2002; Losey *et al.,* 2002). Two further methods are the development of novel antibiotics that aim at new bacterial targets. Since many bacterial genomes have been sequenced in recent years, it could be possible to seek potential bacterial targets that are not hit by existing antimicrobials. New antibiotics, which inhibit the target enzymes, may avoid encountering the previously generated resistance (Allsop 1998; Black & Hare 2000; Coates *et al.,* 2002). At present, however, there are no products yet derived from this technology. Finally, it is attempted to find new potential antibiotics that target non-multiplying bacteria and kill them with one-dose therapy. This will avoid the emergence of antibiotic resistance caused by extended treatment and prolong the effectiveness of new antimicrobial agents (Coates *et al.,* 2002).

2 Aminocoumarin antibiotics—novobiocin, clorobiocin and coumermycin A_1

The three "classical" aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 (for structures see Fig. 1A) are produced by different *Streptomyces* strains. Novobiocin was first reported in the middle of 1950s (Hoeksema *et al.,* 1955; Smith *et al.,* 1956), while coumermycin and clorobiocin were found between the 1960s and the 1970s. In the literature, two producers of novobiocin are described, i.e. *Streptomyces spheroids* NCIMB 11891 and *Streptomyces niveus* (Smith *et al.,* 1956; Wallick *et al.,* 1955). However, Southern blot analysis has shown that these two strains may actually be independent isolates of the same strain. Recently, a novobiocin-producing *Streptomyces* strain was isolated from the stem of the plant *Aucuba japonica* (Sasaki *et al.,* 2001). Clorobiocin is produced by *Streptomyces hygroscopicus*, *S. albocinerscens* and *S. roseochromogenes* var. *oscitans* (Dolak 1973; Ninet *et al.,* 1972). Coumermycin A_1 is obtained from several *Streptomyces*

strains, i.e. *S. rishiriensis*, *S. hazeliensis* var. *hazeliensis*, *S. spinichromogenes* and *S. spinicoumarensis* (Berger *et al.,* 1965; Umezawa *et al.,* 1971; Whitaker 1968). So far, two further aminocoumarin antibiotics, simocyclinones (Schimana *et al.,* 2000) and rubradirin (Sohng *et al.,* 1997), have been discovered in nature.

2.1 Structural analysis

All three antibiotics contain 3-amino-4,7-dihydroxy coumarin core. The aminocoumarin ring links to an acyl component *via* an amide bond at position 3´, to a deoxysugar *via* a glycosidic bond at position 7´. The 3´´-OH group of the deoxysugar is acylated, in novobiocin with a carbamoyl group, in the other two with a 5-mehtylpyrrole-2-carboxylic acid. Clorobiocin, as indicated by its name, carries a chlorine atom at position 8´ of aminocoumarin ring, whereas novobiocin and coumermycin A_1 show a methyl group at the corresponding positions. At the amino group of aminocoumarin ring, novobiocin und clorobiocin carry the same acyl substituent, 3-dimethylallyl-4-hydroxybenzoyl moiety (also referred as Ring A). In contrast, the acyl component in coumermycin A_1 is a 3-methylpyrrole-2,4-dicarboxylic acid. To both carboxyl groups, the same aminocoumarin-deoxysugar moiety is attached, making the molecule nearly, but not completely symmetrical in structure.

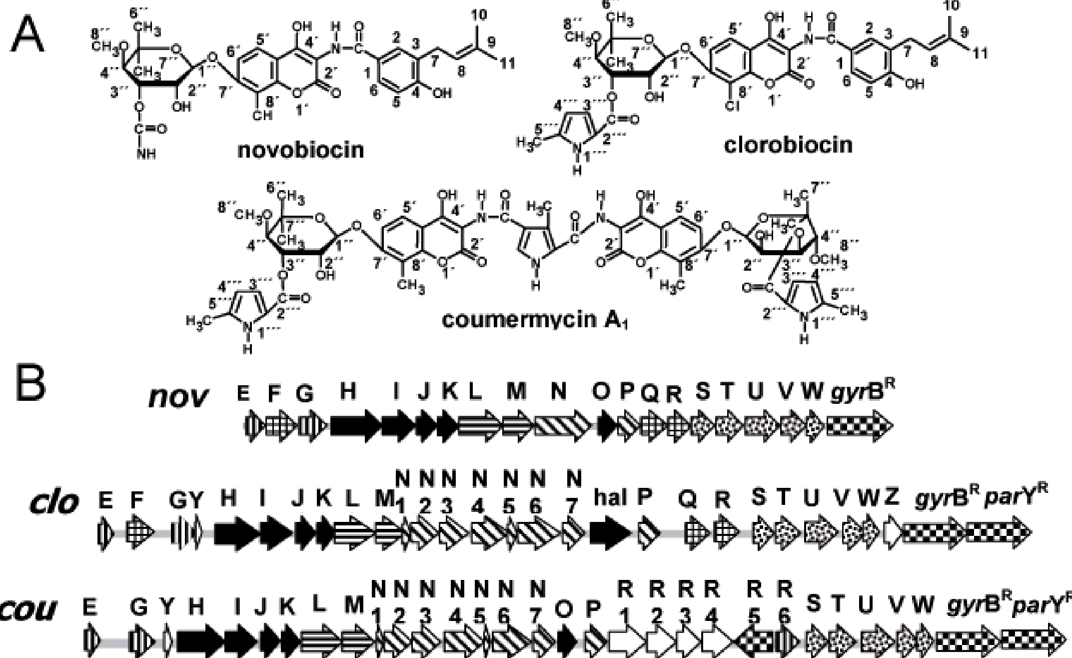


Fig. 1 Structures (A) and biosynthetic gene clusters (B) of novobiocin, clorobiocin and coumermycin A_1, respectively

2.2 Mechanism of action and clinical application

Novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of bacterial gyrase. DNA gyrase is a type II topoisomerase that is found in prokaryotes, but not in eukaryotes. The active gyrase molecule is an A_2B_2 tetramer. It is involved in maintaining the negative superhelicity of the chromosome in replication and transcription (Gross *et al.,* 2003). All three classical aminocoumarins compete with ATP for binding to the B subunit of bacterial gyrase and inhibit the ATP-dependent DNA supercoiling catalysed by this enzyme (Lewis *et al.,* 1996; Tsai *et al.,* 1997). X-ray crystallographic analysis showed that the aminocoumarin ring and the substituted deoxysugar moiety of these compounds are essential for their binding to GyrB (Celia *et al.,* 1994; Lewis *et al.,* 1996; Tsai *et al.,* 1997; Wigley *et al.,* 1991). Coumermycin A_1 molecule contains two of these active aminocoumarin-deoxysugar moieties and therefore may crosslink the two gyrase B subunits of the gyrase heterotetramer (Ali *et al.,* 1993; Gormley *et al.,* 1996; Maxwell 1997). In addition, the acyl components at the 3´´ position of the deoxysugar of these antibiotics are important for the hydrogen bonding network between the antibiotic and the GyrB subunit (Lewis *et al.,* 1996; Tsai *et al.,* 1997). In the interaction of gyrase and novobiocin, an aspartate residue forms a hydrogen bond directly with the carbamoyl moiety, and two further hydrogen bonds are formed *via* ordered water molecules (Fig.2) (Lewis *et al.,* 1996). When clorobiocin binds to gyrase, these two water molecules are displaced by the more voluminous pyrrole moieties present in this compound (Fig. 2) (Tsai *et al.,* 1997), and the same is expected for the binding of coumermycin A_1. Also the chlorine atom at position 8´ of the coumarin core of clorobiocin contributes to the higher biological activity of this compound in comparison to novobiocin, as clorobiocin with 8´-chlorine at the aminocoumarin ring show at least a 2-fold higher inhibitory activity than 8´-methylated derivative (Eustáquio *et al.,* 2003a). Ring A of novobiocin and clorobiocin was supposed to facilitate the absorption of these antibiotics but have little effect on the interaction between gyrase and the compounds (Lewis *et al.,* 1996), which, however, has been disproved with recent studies (Galm *et al.,* 2004b; Lafitte *et al.,* 2002).

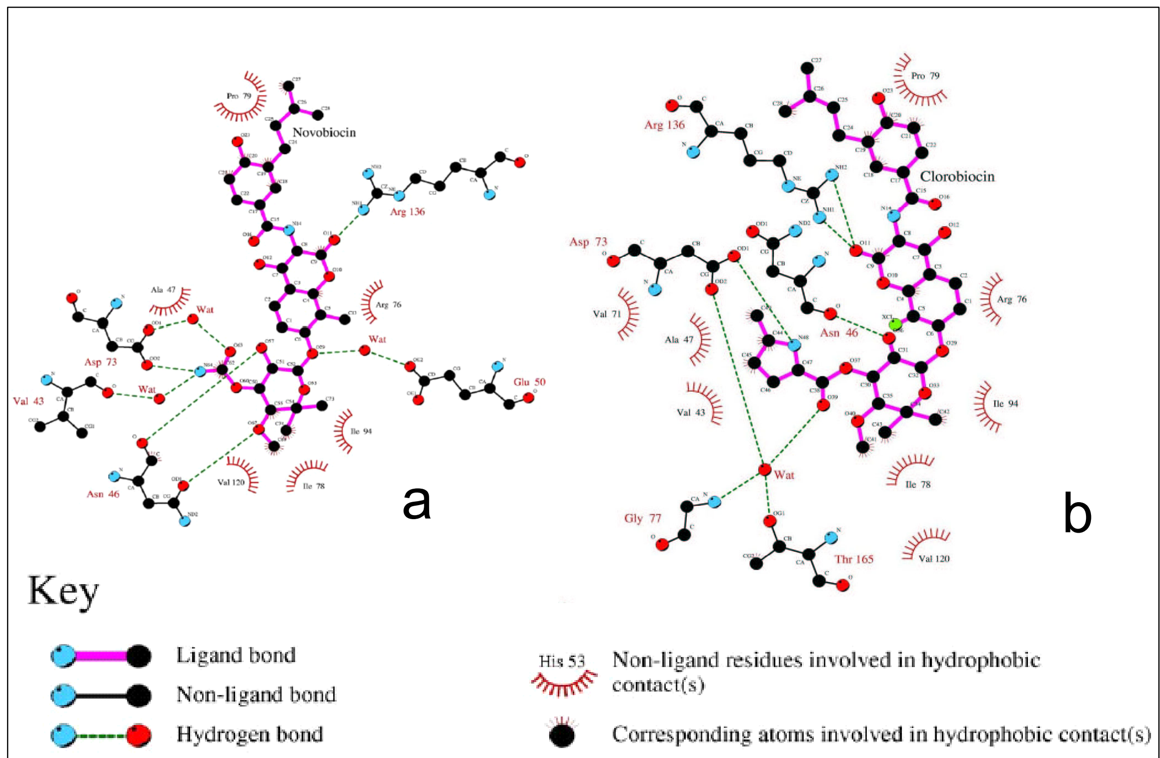


Fig. 2 Interaction between GyrB and novobiocin (left) as well as between GyrB and clorobiocin (right), adapted from Maxwell & Lawson, 2003

Aminocoumarin antibiotics are very potent against Gram-positive bacteria, including methicillin- and vancomycin-resistant *Staphylococcus* strains. Additionally, the aminocoumarins show synergistic effect with anticancer compounds such as etoposide and teniposid. Novobiocin (Albamycin®, Pharmacia & Upjohn) was licensed in the USA as an antibiotic for the treatment of infections with multiresistant Gram-positive bacteria. Its antibacterial activity has been demonstrated in preclinical and clinical studies (Raad *et al.,* 1995; Raad *et al.,* 1998). Unfortunately, it was called back from the market because of eukaryotic toxicity. Clorobiocin and coumermycin have not been used clinically.

2.3 Feeding experiments and identification of biosynthetic gene cluster

Heide and Li have reviewed the biosynthesis of aminocoumarin antibiotics (Heide & Li 2002). The biosynthese of novobiocin was firstly studied by feeding experiments in the 1960s and 1970s. The deoxysugar is derived from intact glucose (Birch *et al.,* 1962; Li *et al.,* 1998). Tyrosine is the precursor of both the aminocoumarin ring and Ring A (Bunton *et al.,* 1963). The amino group in position 3´ of the coumarin ring is derived from the nitrogen of tyrosine (Calvert *et al.,* 1972). Feeding of [carboxyl-^{18}O]

labelled tyrosine resulted in the incorporation of ^{18}O into the ring oxygen of the aminocoumarin ring. However, it has been proven recently by Holzenkämpfer and Zeeck (2002) that the ring oxygen of aminocoumarin of simocyclinone is derived from molecular oxygen, not from the carboxyl group of tyrosine. Feeding of [^{14}C] labelled L-proline to a coumermycin producer led to the incorporation of radioactivity into the pyrrole groups of coumermycin A_1 (Scannell & Kong 1969). But the degradation method used in this experiment did not allow to distinguishing between the central and the two terminal pyrrole moieties of this compound.

The earlier molecular biological studies were restricted to the investigation of the novobiocin resistance gene, especially $GyrB^r$ (Thiara & Cundliffe 1988; Thiara & Cundliffe 1993), and the production of novobiocin-deficient mutants (Hoggarth *et al.,* 1995). Only in 2000, the complete biosynthetic gene cluster for novobiocin from *Streptomyces spheroides* NCIMB 11981 was cloned and sequenced using dNTP-glucose-4,6-dehydratase gene fragments as probes by our group (Steffensky *et al.,* 2000b). Subsequently, the biosynthetic gene cluster for coumermycin A_1 from *Streptomyces rishiriensis* DSM 40489 (Wang *et al.,* 2000) and for clorobiocin from *Streptomyces roseochromogenes* var. *oscitans* DS 12.976 (Pojer *et al.,* 2002) were also identified. A comparison of these gene clusters showed that the structural differences and similarities between the three antibiotics are reflected perfectly by the differences and similarities in the organization of the corresponding biosynthetic gene clusters (Fig. 1B) (Pojer *et al.,* 2002).

At the 3´ end of the cluster, five ORFs with high homology to known genes of deoxysugar biosynthesis are found in all three gene clusters, i.e. *nov*STUVW, *clo*STUVW, *cou*STUVW, respectively.

Resistance gene (*gyr*B^r) is located downstream of the deoxysugar biosynthesis genes. An additional gene *par*Y^r is discovered immediately downstream of *gyr*B^r in clorobiocin and coumermycin gene clusters, which is identified as another resistance gene by heterologous expression in *Streptomyces lividans* TK24 (Schmutz *et al.,* 2003a). *cou*R5 in the gene cluster for coumermycin A_1, encodes a transporter and its expression in *S. lividans* TK24 resulted in resistance against novobiocin and coumermycin A_1 (Schmutz *et al.,* 2003a).

The genes for the biosynthesis of the characteristic aminocoumarin ring must be present in all three clusters, and a comparison reveals that *nov*HIJK in the novobiocin cluster, *clo*HIJK in the clorobiocin cluster and *cou*HIJK in the coumermycin cluster show very high homology with each other. It appears that the products of these genes are involved in the formation of the aminocoumarin ring from tyrosine. NovH activates tyrosine and P450 enzyme NovI catalyses the β-hydroxylation of the activated tyrosine (Chen & Walsh 2001). It was proposed that the activated β-hydroxytyrosine may be further oxidised by NovJ together with NovK (Chen & Walsh 2001), however, no evidences were provided.

The biosynthesis of Ring A was elucidated by biochemical studies very recently. The precursor of Ring A is 4-hydroxyphenylpyruvate, but not β-tyrosine, as reported by Bunton *et al.* (1963). CloQ is a prenyltransferase, which prenylates 4-hydroxyphenylpyruvate (Pojer *et al.,* 2003b). CloR, a bifunctional non-heme iron oxygenase, catalyses the conversion of 3-dimethylallyl-4-hydroxyphenylpyruvate to Ring A in two oxidative decarboxylation steps, *via* 3-dimethylallyl-4-hydroxymandelic acid as intermediate (Pojer *et al.,* 2003a). The same functions may be postulated for the corresponding genes of the novobiocin gene cluster, i.e. *nov*QR.

The novobiocin gene cluster contains a gene (*nov*N) with homology to carbamoyltransferase, whereas at the same relative position of the clorobiocin and coumermycin cluster, a contiguous group of seven genes (*clo*N1-N7, *cou*N1-N7, respectively) are found. *clo*N3/*cou*N3, *clo*N4/*cou*N4 and *clo*N5/*cou*N5 show sequence similarity to *plt*E, *plt*F and *plt*L of the pyoluteorin gene cluster, and to *redW*, redM and redO of the undecylprodiginine gene cluster, respectively. The *plt* and *red* genes have been shown to be involved in the biosynthesis of pyrrole moiety in the respective antibiotics (Nowak-Thompson *et al.,* 1999; Thomas *et al.,* 2002). Analogously, *clo*N1-N7, *cou*N1-N7 might be responsible for the synthesis and attachment of pyrrole moiety in clorobiocin and coumermycin A_1, respectively.

*nov*OP and *cou*OP were identified as methyltransferases by gene inactivation (Li *et al.,* 2002). *nov*O and *cou*O code for a C-methyltransferase, catalysing the methylation of position 8´ of the aminocoumarin ring. In contrast, *nov*P and *cou*P are O-methyltransferase genes, catalysing the methylation of the 4´´-OH group of the deoxysugar. Gene *clo*P, homologous to *nov*P and *cou*P, is found in the clorobiocin

gene cluster, however, no gene homologous to *nov*O and *cou*O exists in the clorobiocin cluster. Instead, a halogenase gene *clo*-hal is located in the same relative position as *nov*O and *cou*O.

*nov*M and its homologs *clo*M, *cou*M catalyse the formation of the glycosidic bond between the deoxysugar and the aminocoumarin ring. Recently, NovM was expressed in *E. coli* as 6×His-tagged protein and the aglycone novobiocic acid and TDP-L-noviose were shown to be the preferred substrates (Freel Meyers *et al.,* 2003).

NovL and CloL are responsible for the amide bond formation between the aminocoumarin ring and Ring A (Galm *et al.,* 2004a; Steffensky *et al.,* 2000a). *cou*L encodes an unusual amide synthestase, which catalyses the formation of both amide bonds of coumermycin A_1, i.e. between the central pyrrole moiety and the two aminocoumarins (Schmutz *et al.,* 2003b).

*cou*R1-R4 are only found in coumermycin gene cluster, but no homologues have been found in the novobiocin gene cluster and clorobiocin gene cluster. It is speculated they are involved in the biosynthesis of the central pyrrole unit of coumermycin A_1 (Wang *et al.,* 2000).

Besides the genes above, the gene clusters still contain regulatory genes, *nov*EG/*clo*EG/*cou*EG (Eustáquio *et al.,* 2003b) and some other genes like *clo*Z, *clo*Y, *cou*Y and *cou*R6, of which the functions are still unknown.

2.4 Generation of new aminocoumarin antibiotics

Aminocoumarin antibiotics have not been highly successful as pharmaceuticals for various reasons, e.g. poor solubility in water, poor oral absorption, low activity against Gram-negative bacteria and eukaryotic toxicity as mentioned above. Therefore, it is of great interest to test whether new, structurally modified aminocoumarin antibiotics may be overcome the limitations of existing compounds. Furthermore, the dramatic increase in the number of antibiotic-resistant pathogenic bacteria in the past decade makes the aminocoumarin antibiotics with their high antibacterial activity attractive compounds for drug discovery. Knowledge of the sequences and the genes involved in the biosynthesis of aminocoumarin antibiotics permits the development of modified

or even novel, potent aminocoumarin antibiotics by gene engineering and combinatorial biosynthesis.

The final steps of the biosynthesis of novobiocin, clorobiocin and coumermycin A_1 include a number of tailoring reactions, such as methylation, chlorization, carbamoylation, acylation and so forth. The structural modifications are likely to be significiant for antimicrobial activity. For examples, removing the chlorine atom from C-8´ of the aminocoumarin ring in aminocoumarin derivatives reduced the activity by the factor ~2-4, whereas removing the methoxyl group at 4´´-OH of deoxysugar resulted in the complete loss of activity (Galm *et al.,* 2004b). Therefore, it is of interest to investigate the tailoring enzymes involved in the biosynthesis of aminocoumarin antibiotics, which may provide a convenient and effective way to generate new potential, structurally modified aminocoumarin antibiotics.

3 Aims of this thesis

This thesis focused on the acyl moieties attached to the 3´´-OH of the deoxysugar of aminocoumarin antibiotics, i.e. a carbamoyl group in case of novobiocin and a 5-methylpyrrole-2-carboxyl moiety in case of clorobiocin and coumermycin A_1, which are shown to be important for the binding of the antibiotics to the GyrB subunit. The first aim of this thesis is to elucidate on the genetic level the biosynthesis and the attachment of acyl components of the deoxysugar in aminocoumarins, i.e.:

- Biosynthesis of 5-methylpyrrole-2-carboxylic acid moiety of clorobiocin and coumermycin A_1
- Attachment of 5-methylpyrrole-2-carboxylic acid moiety to the deoxysugar in clorobiocin and coumermycin A_1
- Attachment of the carbamoyl group to the deoxysugar in novobiocin

Secondly, the aim of this thesis is to generate new, carbamoylated aminocoumarin antibiotics using genetical and biochemical methods.

For these purposes, the following experiments have to be carried out,

(1) Creation of two *S. rishiriensis* mutants (*cou*N3$^-$, *cou*N4$^-$), analysis of the secondary metabolites by HPLC and LC/MS;

(2) Creation of two *S. roseochromogenes* mutants (*clo*N2$^-$, *clo*N2$^-$/*clo*-hal$^-$), analysis of the secondary metabolites by HPLC, preparative isolation of new products, structural elucidation by MS and NMR;

(3) Overexpression of NovN in the *cou*N3$^-$ mutant and in the *clo*N2$^-$ mutant, analysis of the secondary metabolites by HPLC and LC/MS;

(4) Expression and purification of NovN, characterization, enzyme assay, analysis of the enzymatic reaction products by HPLC, isolation of the new products and structural elucidation by MS and NMR;

(5) Testing of biological activity of new compounds by supercoiling assay and bioassay.

Materials and methods

1. Chemicals

Chemicals and components of the media used in this thesis are listed in Table 1. Descarbamoyl novobiocin was kindly provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI, USA). Novclobiocin 283 was obtained by feeding experiment with 3-Br-4-OH-benzoic acid as described elsewhere (Galm *et al.,* 2004a).

Table 1 Chemicals and media components

Chemical/Media component	Supplier
Clorobiocin	Aventis, Bad Soden, Germany
Agar Casaminoacids Corn starch (soluble) Malt extract Nutrient agar Peptone Tryptic soy broth Tryptone Yeast extract	Becton-Difco, Heidelberg, Germany
Thiostrepton	Calbiochem-Novabiochem, Bad Soden, Germany
Apramycin Novobiocin	Fluka, Ulm, Germany
L-Proline Pyrrole-2-carboxylic acid	Lancaster, Mühlheim a. M., Germany
Agarose	USB, Cleveland, OH, USA
Bovine serum albumine (BSA) Chloramphenicol EDTA Ethanol Glucose Meat extract β-Mercaptoethanol Methanol Phosphorus pentoxide Uvasol® DMSO-d6 Uvasol® methanol-d4	Merck, Darmstadt, Germany
Acetonitrile	Roth, Karlsruhe, Germany

Ammonium persulphate 5-Bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal) Carbenicillin 1,4-Dithiothreitol (DTT) Glacial acetic acid Glycine Isopropanol Isopropyl-β-thiogalactoside (IPTG) Maleic acid Phenol/Chloroform/Isoamylalkohol(25:24:1) Polyethyleneglycol (PEG) 1000 (50%) Rotiphorese®Gel 30 (30% acrylamid, 0.8% bisacrylamide) Sodium dodecyl sulphate (SDS) N,N,N´,N´-Tetramethylethylenediamine (TEMED) Tris-(hydroxymethyl)-aminomethane-maleate (Tris-maleate) N-Tris-(Hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES)	
Coomassie Brilliant Blue R250 N-Lauroylsarcosine (Na-Salt, 35%)	Serva, Heidelberg, Germany
Adenosine 5´-triphosphate dipotassium salt (ATP) Bromophenol blue Carbamoyl phosphate dilithium salt (CAP-Li_2) Coumermycin A_1 Dimethyl formamide (DMF) Dimethyl sulfoxide (DMSO) Distillers grains and solubles Ethyleneglycol Imidazol Kanamycin Lard oil Lithium potassium acetyl phosphate Novobiocin t-Octylphenoxypolyethoxyethanol (Triton X-100) Phenylmethylsulfonyl fluoride (PMSF) Polyoxyethlenesorbitan monolaurate (Tween 20) Tetracycline Tris base	Sigma-Aldrich, Deisenhofen, Germany
Sucrose	Südzucker, Mannheim, Germany

2. Materials for Chromatography

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

Table 2 Liquid chromatography media

Medium	Supplier
Sephadex® LH-20 (dry beads) Sephadex® G-25 NAP-10 (commercial column) HiLoad 26/60 Superdex® 200 (commercial column, 13 µm, 26×600 mm)	Amersham Biosciences, Freiburg, Germany
Multosphere® RP 18-5 (commercial colum, 5 µm, 250×4 mm) Multosphere® 100 RP 18-5 (commercial colum, 5 µm, 250×20 mm)	C+S Chromatographie Service, Düren, Germany
Nucleosil® RP 120-5 (commercial column, 5 µm, 250×2 mm)	Macherey-Nagel, Düren, Germany
Ni-NTA Agarose (Suspension)	Qiagen, Hilden, Germany

3. Enzymes and kits

Table 3 Enzymes and kits

Enzymes and kits	Supplier
Low Molecular Weight Calibration Kit for SDS-Electrophoresis Low Molecular Weight Gel Filtration Calibration Kit Restriction endonucleases T4 DNA Ligase	Amersham Biosciences, Freiburg, Germany
Lysozyme (76000 U/mg)	Fluka, Ulm, Germany
1kb DNA Marker	GibcoBRL Life Technologies, Karlsruhe, Germany
DNA Gyrase Assay Kit	John Innes Enterprises Ltd., Norwich, UK
Nucleobond® Kit A×100 NucleoSpin® Extract 2 in 1	Macherey-Nagel, Düren, Germany

Restriction endonucleases	New England Biolabs, Schwalbach, Germany
Taq DNA polymerase *Pfu* DNA polymerase	Promega, Madison, WI, USA
RNase A	Qiagen, Hilden, Germany
DIG-High Prime DNA Labelling and Detection Starter Kit II DNA Molecular Weight Marker VII, DIG-Labelled Expand High Fidelity PCR System GC-Rich PCR System Kit	Roche, Mannheim, Germany
Restriction endonucleases	Strategene, Taufkirchen, Germany

4. Media, buffers and solutions

4.1. Media for bacterial culture

The media used in this thesis were as follows. Unless otherwise stated, the media were prepared with distilled water and autoclaved for 20 min at 121°C (15 psi). To obtain agar media, 1.5-2.5% (w/v) agar was added before autoclave. If necessary, sterile supplementary components like antibiotics and other heat-labile substances were added in the sterile media at time of use. The media were stored at RT or at 4°C for long periods.

4.1.1. Cultivation of *E. coli*

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

Per liter	NaCl	10.0 g
	Tryptone	10.0 g
	Yeast extract	5.0 g

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

4.1.2. Cultivation of *Streptomyces*

Yeast-Malt-Glucose Medium (YMG medium)

Per liter	Yeast extract	4.0 g
	Malt extract	10.0 g
	Glucose	4.0 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.3, and add water to make up to 1 liter. Sterilize by autoclaving.

HA Medium

To 1 liter sterile YMG-Medium, add 1ml sterile $CaCl_2 \cdot 2H_2O$ (1M).

4.1.3. Production medium for *S. rishiriensis*

SK Medium (Scannell & Kong 1969)

Per liter	Corn starch	20.0 g
	Lard oil	20.0 g
	Yeast extract	4.0 g
	$K_2HPO_4 \cdot 3H_2O$	2.5 g
	$CoCl_2 \cdot 6H_2O$	202 µg

Dissolve the ingredients in about 900 ml water, add 20 g Cotton seed flour and mix up, adjust the volume to 1 liter with water. Autoclave.

4.1.4. Production media for *S. roseochromogenes*

Corn starch medium (pre-culture medium) (Mancy *et al.,* 1974)

Per liter	Corn starch	10.0 g
	Peptone	10.0 g
	Meat extract	5.0 g

Dissolve in about 900 ml water, adjust the pH to 7.0, and add water to make up to 1 liter. Sterilize by autoclaving.

Distillers solubles medium (production medium) (Mancy *et al.,* 1974)

Solution A	Distillers solubles	48.0 g
	Glucose	12.0 g
	Cobalt chloride	24 mg

Dissolve in water, adjust the pH to 7.8, add 6.0 g calcium corbonate and then add water to make up to 887 ml.

Solution B Ammonium sulphate 32.0 g

Dissolve in 200 ml water and autoclave.

Solution C Glucose 75.0 g

Dissolve in 300 ml water and autoclave.

After autoclave, add 13 ml solution B and 100 ml solution C to solution A.

4.1.5. Sporulation medium for *S. roseochromogenes*

Grundy starch medium

Per liter Soluble starch 5.0 g

$(NH_4)_2HPO_4$	1.0 g
KCl	0.2 g
$Mg_2Cl{\cdot}6H_2O$	0.2 g

Dissolve in about 900 ml water, adjust the pH to 7.3 and add

Agar	15.0 g

Adjust the volume with water to 1 liter and sterilize for 25 min at 121°C. For sporulation of *S. roseochromogenes*, mix 2-3 ml 2-day-old culture with 1 g sterile powdered soil and plate the mixture on two grundy starch medium plates.

4.1.6. Protein expression in *S. lividans*

Yeast extract-malt extract medium (YEME medium)

Per liter	Yeast extract	3.0 g
	Peptone	5.0 g
	Malt extract	3.0 g
	Glucose	10.0 g
	Sucrose	340.0 g

Dissolve in water to a final volume of 1 liter and autoclave. After that, add 2 ml $Mg_2Cl{\cdot}6H_2O$ (2.5 M).

4.1.7. Protoplast transformation of *Streptomyces*

CRM medium

Per liter	Sucrose	103.0 g
	Tryptic soy broth	20.0 g
	$MgCl_2{\cdot}6H_2O$	10.12 g
	Yeast extract	10.0 g

Dissolve in about 900 ml water, adjust the pH to 7.0 and adjust the volume to 1 liter. Sterilize by autoclaving. To 100 ml sterile medium, add 1 ml $CaCl_2$ (1M). If the culture was used to prepare protoplasts, 0.4-0.75% glycine should be added to the medium before autoclave (0.4% for *S. rishiriensis* and 0.75% for *S. roseochromogenes*, respectively).

Trace elements solution

Per liter	$ZnCl_2$	40 mg
	$FeCl_3{\cdot}6H_2O$	200 mg
	$CuCl_2{\cdot}2H_2O$	10 mg
	$MnCl_2{\cdot}4H_2O$	10 mg
	$Na_2B_4O_6{\cdot}10H_2O$	10 mg

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

Dissolve in 1 liter distilled water and autoclave.

R2YE Medium (Kieser *et al.,* 2000)

Per liter	Sucrose	103.0 g
	K_2SO_4	0.25 g
	$MgCl_2 \cdot 6H_2O$	10.12 g
	Glucose	10.0 g
	Casaminoacids	0.1 g
	Trace elements-solution	2.0 ml
	Yeast extract	5.0 g
	TES	5.73 g
	Agar (plates)	23.0 g

Dissolve in water to a final volume of 1 liter and sterilize by autoclaving. To prepare the soft agar, 6 g Agar, instead of 23 g, were added.

After autoclave, add:

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

R3 soft agar

Per liter	Sucrose	171.0 g
	Glucose	10.0 g
	Peptone	4.0 g
	KCl	0.5 g
	$CaCl_2 \cdot 2H_2O$	2.2 g
	$MgCl_2 \cdot 6H_2O$	8.1 g
	Agar	8.0 g

Dissolve in water to a final volume of 1 liter and autoclave. After that, add:

KH_2PO_4 (0.5%)	40 ml
TES (0.25 M, pH 7.2)	100 ml

Soft nutrient agar (Kieser *et al.,* 2000)

Per liter	nutrient Broth	8 g
	Agar	5 g

Dissolve in water to a final volume of 1 liter and autoclave.

4.2. Solutions of antibiotics

Antibiotics were dissolved in appropriate solvents as stock solutions and kept at -20°C. The aqueous solutions were sterilized by passing through a 0.22 µm filter. The

solutions in ethanol and DMSO were autosterile. For antibiotic selection, the required antibiotics were added to the cooled media (45-50°C) in appropriate concentration.

Table 4 Solutions of antibiotics

Antibiotic	Concentration in		Solvent
	stock solution (mg/ml)	media (µg/ml)	
Apramycin	50	50	H_2O
Carbenicillin	50	50	H_2O
Chloramphenicol	25	12.5	ethanol
Kanamycin	50	10-25	H_2O
Tetracycline	25	12.5	ethanol
Thiostrepton	50	20-50	DMSO

4.3. Buffers and solutions

Unless otherwise stated, the buffers were prepared with distilled water and stored at room temperature (RT).

4.3.1. Buffers for isolation of DNA

Table 5 Buffers for isolation of plasmids from *E.coli*

Buffer	Components	Final concentration	
Solution A1	Tris-HCl EDTA RNase A	50 mM 10 mM 100 µg/ml	Adjust the pH to 8.0 and autoclave. Store at 4°C. Add RNase A just before use.
Solution A2	NaOH SDS	0.2 N 1% (w/v)	
Solution A3	KAc·3H_2O	3 M	Adjust the pH to 4.8 and autoclave. Store at 4°C.

Table 6 Buffers for isolation of plasmids from *Streptomyces*

Buffer	Components		
Solution B1	Glucose Tris-HCl EDTA RNase A Lysozyme	50 mM 25 mM 10 mM 100 µg/ml 4-8 mg/ml	Adjust the pH to 8.0 and autoclave. Store at 4°C. Add RNase A and lysozyme prior to use.
Solution B2	a. NaOH b. SDS	0.4 N 2% (w/v)	Mix a and b at the rate of 1:1 just before use.
Solution B3	KAc·3H_2O	5 M	Adjust the pH to 4.8 and autoclave. Store at 4°C.

Table 7 Buffers for isolation of genomic DNA from *Streptomyces*

Buffer	Components	Final concntration	
TSE buffer	Sucrose Tris-HCl EDTA RNase A Lysozyme	10.3% 25 mM 25 mM 100 µg/ml 3 mg/ml	Adjust the pH to 8.0 and autoclave. Add RNase A and lysozyme just before use. Store at 4°C.
TE buffer	Tris-HCl EDTA	10 mM 1 mM	Adjust the pH to 7.5 and autoclave.

4.3.2. Buffers for DNA gel electrophoresis

Table 8 Buffers for DNA gel electrophoresis

Buffer/solution	Components	Final concentration	
50×TAE	Tris base EDTA (0.5 M, pH 8.0) Glacial acetic acid	2 M 0.05 M 57.1 ml/l	Adjust the pH to 8.0 with glacial acetic acid.
Load buffer	Glycerol Bromophenol blue	30% (w/v) 0.25% (w/v)	Store by 4°C
Ethidium bromide solution for staining the agarose gel	Ethidium bromide	1 µg/ml	

4.3.3. Buffers and solutions for Southern blot analysis

Table 9 Buffers and solutions for Southern blot analysis

Buffer/solution	Components		
Denaturing solution	NaOH NaCl	0.5 M 1.5 M	
Neutralizing solution	Tris-HCl NaCl	0.5 M 3 M	Adjust the pH to 7.5 with HCl.
20×SSC buffer	Trisodium citrate NaCl	0.3 M 3 M	Adjust the pH to 7.0
Pre-hybridizing solution	Skimmed milk powder SDS (10% in H_2O) N-Lauroylsarkosin (35% (w/v) in H_2O)	3% 0.02% 0.1%	Add to 5×SSC buffer before use.
Hybridizing solution	Skimmed milk powder SDS (10% (w/v) in H_2O) N-Lauroylsarkosine (35% (w/v) in H_2O)	1.5% 0.02% 0.1%	Add to 5×SSC buffer before use.
2×Wash buffer	SDS (10% (w/v) in H_2O)	0.1%	Add to 2×SSC buffer before use.
0.5×Wash buffer	SDS (10% (w/v) in H_2O)	0.1%	Add to 0.5×SSC buffer before use.

Maleic acid buffer	Maleic acid NaCl	0.1 M 0.15 M	Adjust the pH to 7.5
Tween wash buffer	Tween 20	0.3%	Add to the maleic acid buffer before use.
Blocking solution	Skimmed milk powder	3%	Add to the maleic acid buffer just before use
Detection buffer	Tris-HCl NaCl	0.1 M 0.1 M	Adjust the pH to 9.5
Antibody solution	Blocking solution Maleic acid buffer Anti-DIG-AP-conjugate	20 ml 20 ml 4 µl	Mix just before use.
Stripping solution for blot	NaOH SDS (2% (w/v) in H_2O)	0.2 M 0.1%	

4.3.4. Solutions for blue/white selection of *E. coli*

The storage was carried out at -20°C.

Table 10 Stock solutions for blue/white selection.

Solution	**Composition**	**Per plate**
IPTG	80 mg/ml in distilled water, sterilize by filtering	12 µl
X-Gal	20 mg/ml in DMF, autosterile	50 µl

4.3.5. Buffers for preparation of protoplasts and transformation of *Streptomyces*

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

Table 11 Buffers for preparation of protoplasts and transformation of *Streptomyces*

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

	For *S. rishiriensis*, add PEG 1000 (50% (w/v) in H_2O); or for *S. roseochromogenes*, add PEG 1000 (67.25 % (w/v) in H_2O)	5.0 ml 7.5 ml adjust the volume with distilled water to 10 ml.
Denaturing reagent	25×TE buffer (s. Table 7) EDTA (0.1 M) Glycerol (86-87.7%) Ethyleneglycol	400 µl 100 µl 5 ml 5 ml

4.3.6. Buffers for protein purification by nickel affinity chromatography and for gel filtration

The buffers for protein purification and gel filtration were stored at 4°C.

Table 12 Buffers for protein purification

Buffer	Components	
Lysis buffer	NaH_2PO_4/Na_2HPO_4 NaCl Lysozyme Imidazol If necessary, add Triton X-100 (1% (w/v) in H_2O)	50 mM (pH 8.0) 300 mM 4-8 mg/ml 10-15 mM 0.2%
Wash buffer	NaH_2PO_4/Na_2HPO_4 NaCl Imidazol	50 mM (pH 8.0) 300 mM 20-30 mM
Elution buffer	NaH_2PO_4/Na_2HPO_4 NaCl Imidazol	50 mM (pH 8.0) 300 mM 250 mM
Storage buffer	Tris-HCl Glycerol (87%)	0.2 M (pH 7.5) 15%
PMSF stock solution	PMSF	50 mM in isopropanol, stored at -20°C
Buffer G1	Tris-HCl DTT PMSF	50 mM (pH 8.0) 5 mM 50 µM
Buffer G2	Buffer G1 + NaCl	 150 mM

4.3.7. Bradford reagent for protein quantification (Bradford 1976)

70 mg Brilliant Serva Blau G was dissolved in 50 ml ethanol. After addition of 100 ml H_3PO_4 (85% (w/v)), the volume was adjusted with distilled water to 200 ml. This stock solution was kept at 4°C.

After diluting the stock solution with 5 volumes of distilled water, the solution was filtered and ready for use.

4.3.8. Solutions for protein gel electrophoresis (SDS-PAGE) and for Coomassie staining

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

Table 13 Buffers and solutions for SDS-PAGE and Coomassie staining

Buffer/solution	Components		
Stacking gel (4%)	Distilled water 0.5 M Tris-HCl (pH 6.8) 10% (w/v) SDS Rotiphorese®Gel 30 10% (w/v) APS TEMED	6.1 ml 2.5 ml 0.1 ml 1.3 ml 0.05 ml 0.01 ml	Combine all the components, except APS and TEMED, degas under vacuum for at least 15 min. Add APS and TEMED just before pouring the gel.
Resolving gel (10%)	Distilled water 1.5 M Tris-HCl (pH 8.8) 10% (w/v) SDS Rotiphorese®Gel 30 10% (w/v) APS TEMED	4.1 ml 2.5 ml 0.1 ml 3.3 ml 0.05 ml 0.005 ml	(See above)
Sample puffer	Distilled water 0.5 M Tris-HCl (pH 6.8) Glycerol SDS (10% (w/v) in H_2O) Bromophenol blue (0.5% (w/v) in H_2O)	3.55 ml 1.25 ml 2.5 ml 2.0 ml 0.2 ml	Add 50µl β-mercaptoethanol to 950 µl sample buffer prior to use.
10×running buffer	Tris base Glycine SDS	30.0 g 144.0 g 10.0 g	Dissolve in distilled water to a final volume of 1 liter. Store at 4°C
Fixing buffer	Distilled water Acetic acid Methanol	70% (w/v) 10% (w/v) 20% (w/v)	
Coomassie Brilliant Blue G-250 solution	Coomassie Brilliant Blue G-250 Distilled water Acetic acid Methanol	0.25% (w/v) 45% (w/v) 10% (w/v) 45% (w/v)	
Stripping solution for SDS-PAGE	Distilled water Acetic acid Methanol	45% (w/v) 10% (w/v) 45% (w/v)	

5. Plasmids, bacterial strains and primers

5.1. Vectors, cosmids and plasmids

Table 14 Vectors, cosmids and plasmids

Name	Markers and comments	Source or reference
pBluescript SK(-)	Cloning vector, Amp^r, lacZ´(α-Komplementation), f1(-)-origin, ColE1-origin	Stratagene
pBSKT	pBluescript SK(+) derivative, *Streptomyces*-*E.coli* shuttle vector, non-replicative in *Streptomyces*, Amp^r, Tsr^r, lacZ´(α-Komplementation), ColE1-origin	Lombo *et al.*, 1997
pcDNA 2.1	Cloning vector, Amp^r, lacZ´(α-Komplementation)	Invitrogen
pEM4	*Streptomyces* expression vector, LacZα,Amp^r,Tsr^r, *ermE** up promotor	Quiros *et al.*, 1998
pGEM-T	Linearized vector with T-overhang, for direct cloning of PCR fragments with A-overhang	Promega
pGM9	*Streptomyces* replicative vector, *ble*,*aphII*	Muth *et al.*, 1989
pKC1132	non-replicative vector in *Streptomyces*, $Apra^r$	Bierman *et al.*, 1992
pRSET B	Expression vector for *E. coli*, T7 promotor, N-terminal 6×His-tagged, *Amp^r*, ColE1-origin, pUC-origin	Invitrogen
pUWL 201	*Streptomyces* expression vector, LacZα,Amp^r,Tsr^r, *ermE** up promoter, pIJ101-origin, ColE1-origin	Doumith *et al.*, 2000
pNeo4	*aphII* neomycin resistance gene	Wang *et al.* 2000
p9-6GE9	9.7 kb *EcoRI* fragment from cosmid 9-6G of *S. spheroides* cosmid library in pBluescript SK(-)	Steffensky *et al.* 2000b
Cosmid 10-9C	Cosmid containing the novobiocin biosynthetic gene cluster (sequence AF170880)	Steffensky *et al.* 2000b
Cosmid K1F2	Cosmid containing the cloroibiocin biosynthetic gene cluster (sequence AF 329398)	Pojer *et al.*, 2002
pZW11	14736 bp *Bgl*II fragment (positions 5388 to 20123 in sequence AF235050), cloned into the same site of pBluescript SK(-)	Wang *et al.* 2000
pZW33	3103 bp *EcoR*I fragment (positions 13696 to 16798 in sequence AF235050), cloned into the same site of pBluescript SK(-); the insert	Wang *et al.* 2000

	contains the complete sequence of *cou*N3	
pZW334	3419 bp *EcoR*I-*Pst*I fragment (positions 13696 to 17594 in sequence AF235050) in pBluescript SK(-) with the deletion of a 480bp *Pst*I Fragment (positions 15449 to 15928) in coding region of *cou*N3	Constructed by Z.-X. Wang
pN3	3.4 kb *EcoR*V-*Xba*I fragment from pZW334, containing the defective *cou*N3 gene, cloned into the same sites of pKC1132	Constructed by Z.-X. Wang
pN4	2.65 kb *Pst*I-fragment containing the *cou*N4 gene inactivated by insertion of gene *aphII* into the *EcoR*I site, cloned in pBluescript SK(-)	Constructed by Z.-X. Wang
pN21	1.3 kb PCR fragment, containing the left franking region of *clo*N2 from the clorobiocin biosynthetic gene cluster (sequence AF 329398), cloned into the vector pGEM-T	This thesis
pN22	1.3 kb PCR fragment, containing the right franking region of *clo*N2 from the clorobiocin biosynthetic gene cluster (sequence AF 329398), cloned into the vector pGEM-T	This thesis
pN23	1276 bp *Not*I-*Hind*III fragment (position 22151 to 23426 in sequence AF 329398) from pN22, cloned into the same sites of pcDNA 2.1	This thesis
pN24	1308 bp *Xba*I-*Not*I fragment (position 19866-21173 in sequence AF 329398) from pN22, cloned into the same sites of pN23	This thesis
pN2	2584 bp *Xba*I-*Hind*III fragment from pN24, containing the defective gene *clo*N2, cloned into the same sites of pBSKT	This thesis
pXHN	2499 bp *Bac*I-*EcoR*I fragment from plasmid p9-6GE9 (positions 15066 to 17564 in sequence AF170880), containing the complete sequence of *nov*N, cloned into the *BamH*I-*EcoR*I sites of pEM4,	This thesis
pXHNET	2.1 kb PCR-fragment, containing the complete sequence of *nov*N from the novobiocin gene cluster (AF 170880), cloned into the vector pGEM-T	This thesis
pXHNER	2.1 kb *Bcl*I-*Hind*III fragment from pXHNET, containing *nov*N (position 15222 to 17255 in sequence AF 170880), cloned into the sites of	This thesis

	BamHI/HindIII of vector pRSET B	
pXHNEG	The fusion product of pXHNER and vector pGM9 over their common *Hind*III restriction site	This thesis

5.2. Bacterial strains

Table 15 Bacterial strains of *E. coli* and *Streptomyces*

Strain	Relevant characteristics	Source or reference
E. coli XL1Blue MRF'	*rec*A1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB *lacI*^{q}ZDM15 Tn10 (Tet^r)]	Stratagene
E. coli ET 12567	DNA methylase-negative strain	MaNeil *et al*., 1992
E. coli BL21(DE3)pLysS	F$^-$ *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)pLysS(Cam^R)	invitrogen
S. lividans T7	*S. lividans* TK23-derivative, *tsr ble* T7-RNA polymerase gene	Provided by W. Wohlleben, originally obtained from J. Altenbuchner (Heinzelmann *et al.,* 2001)
S. rishiriensis DSM 40489	Wild-type, coumermycin A_1 producer	DSMZ[1]
S. rishiriensis XH12	*cou*N3$^-$ mutant of *S. rishiriensis* DSM 40489	This thesis
S. rishiriensis XH13	*cou*N3$^-$ mutant of *S. rishiriensis* DSM 40489	This thesis
S. rishiriensis XH14	*cou*N3$^-$ mutant of *S. rishiriensis* DSM 40489	This thesis
S. rishiriensis ZW20	*cou*N4$^-$ mutant of *S. rishiriensis* DSM 40489	This thesis
S. rishiriensis ZW21	*cou*N4$^-$ mutant of *S. rishiriensis* DSM 40489	This thesis
S. roseochromogenes var. *oscitans* DS 12.976	Wild-tpye, clorobiocin producer	Aventis
S. roseochromogenes XHB12	*clo*N2$^-$ mutant of *S. roseochromogenes* var. *oscitans* DS 12.976	This thesis
S. roseochromogenes AE-h10	*clo*-hal$^-$ mutant of *S. roseochromogenes* var. *oscitans* DS 12.976	Eustáquio *et al*., 2003a
S. roseochromogenes XHC26	*clo*N2$^-$ *clo*-hal$^-$ mutant of *S. roseochromogenes* var. *oscitans* DS 12.976	This thesis
[1] Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany		

5.3. PCR primers

Table 16 Primers for PCR amplification

Primer	Sequence	Restriction site	Accession number and positions	Amplified gene(s)
cloN2-1	5´-TTG ACC CGG TC**T** AG**A** TGT TCA GAC-3´	*Xba*I	19856-19879 in AF 329398	The left franking region of the gene *clo*N2
cloN2-2	5´-CTC CGA TC**G** CGG CC**G** CGT ATA TAC-3´	*Not*I	21162-21185 in AF 329398	
cloN2-3	5´-CAT ACA AGG CGG CCG **C**CA TCC GAA-3´	*Not*I	22141-22164 in AF 329398	The right franking region of the gene *clo*N2
cloN2-4	5´-GCA GTA GGA AA**G** CTT GGT TGG TCA-3´	*Hind*III	23419-23442 in AF 329398	
cloN2C-1	5´-CTA TCG CGG ATC **C**TG TTC TGC GA-3´	*BamH*I	21082-21104 in AF 329398	*clo*N2 (1068 bp)
cloN2C-2	5´-TAG ACC **T**GC **A**GT TGC TGC TGT G-3´	*Pst*I	22230-22251 in AF 329398	
novN-*Bcl*I	5´- GTG CTC GC**T** GAT **CA**G AAC GAC ATG -3´	*Bcl*I	15198-15221 in AF 170880	*nov*N (2034 bp)
novN-*Hind*III	5´- AAG GG**A** AG**C** **TT**T ACG GCC GCG AC -3´	*Hind*III	17285-17307 in AF 170880	

* The letters shown in bold represent the mutations inserted in the original sequence to give desired restriction sites (underlined).

5.4. Probes used in Southern blot analysis

Table 17 DIG-labelled probes

DNA sequence, Accession number	Positions	objects
880 bp *Sca*I-*EcoR*I fragment vor *clo*N2 in AF 329398	20145-21025	*clo*N2
826 bp *Sph*I-*Pst*I fragment in AF 235050	10512-11338	*cou*N3
870 bp *Pst*I-*EcoR*I fragment in AF 235050	11338-12688	*cou*N4

6. Culture conditions

6.1. Cultivation of *E. coli*

For cloning experiments, *E. coli* strains were grown overnight (16-18 h) in liquid or solid LB medium with appropriate antibiotic(s) at 37°C, 170 rpm.

The permanent culture of *E. coli* was prepared by mixing 500 µl of overnight culture with 500 µl of glycerol solution (40% (w/v) in distilled water) and stored at -70°C.

6.2. Cultivation of *Streptomyces*

6.2.1. General cultivation

Streptomyces strains were routinely cultured in liquid or solid YMG or HA medium in baffled Erlenmeyer flasks containing a stainless steel spring at 180 rpm for 2 to 3 days. The suitable culture temperature was between 28-30°C. For preparation of

protoplasts or isolation of genomic DNA, *Streptomyces* were cultured in CRM medium with glycine was used. For expression experiment, *S. lividans* T7 was grown in YEME medium at 28°C, 170 rpm for 2 day. An appropriate concentration of antibiotic(s) was added, if required.

6.2.2. Production of secondary metabolites

For the production of coumermycin A_1 and other secondary metabolites, *S. rishiriensis* (wild-type) and mutants were precultured in 50 ml HA-medium at 28°C and 180 rpm for two days. 2 ml of this preculture were inoculated into 500-ml baffled flask containing 100 ml production medium (SK medium) and grown at 28°C and 180 rpm for 7-10 days.

For the production of clorobiocin and other secondary metabolites, *S. roseochromogenes* (wild-type) and mutants were grown in 50 ml corn starch medium at 33°C and 210 rpm for 2 days. 5 ml of this preculture were inoculated into 500 ml baffled flasks containing 50 ml of production medium, Distillers solubles-medium. Cultivation was carried out at 33°C and 210 rpm for 5 to 8 days.

6.2.3. Preparation of permanent culture and spore suspension

For preparation of permanent culture, 1 ml 2-day-old culture in YMG or HA medium was harvested by centrifugation (4,300 ×g, 10 min, 4°C). After wash with 1 ml 20% glycerol, the cells were resuspended in 0.5 ml 20% glycerol. The storage was carried out at -70°C.

To prepare the spore suspension, *S. rishiriensis* were sporulated on HA agar medium at 30°C for 3 to 6 days and *S. roseochromogenes* on Grundy starch medium at 28°C for 1 to 1.5 month. 1-3 ml 2-day-old culture was transferred to each plate, spread evenly and dried under clean bench. The plates (two plates for good sporulaters, four for more sparely sporulating strains) were grown till they were well sporulated. 8 ml of Tween 20 (0.1% (w/v)) were added to the top of each plate and the spores scraped off of the top of the plates and into suspension. The resulting spore suspension was poured into a falcon tube and vortexed vigorously (about 1 min). The spores were separated from the mycelium by passing the suspension through sterile cotton pluged in a disposable syringe. Spores was collected by centrifugation (2,100×g, 10

min, 4°C), washed with 20% glycerol and resuspended in 1-3 ml of 20% glycerol. Spore suspension was kept at -70°C.

7. Methods of molecular biology and biochemistry

7.1. Methods of molecular biology

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

7.1.1. Purification, concentration and quantification of DNA

Phenol/chloroform extraction and ethanol precipitation were used for purification and concentration of DNA.

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

7.1.2. Agarose gel electophoresis of DNA (Sambrook & Russell 2001)

Gel electrophoresis with 0.4-1.5% (w/v) agarose (USB, Cleveland, USA) was used to separate DNA fragments between 0.5 and 50 kb. The buffer system employed was 1×TAE buffer (Table 8). After running the gels, they were stained with the fluorescent dye ethidium bromide, detected under the UV light at 312 nm and photographed by using Eagle Eye II System (Strategene, Heidelberg, Germany).

DNA markers used in this thesis were

- 1 kb DNA ladder (Gibco BRL, Karlsruhe, Germany): 0.5-12 kb
- DNA Molecular Weight Marker VII, DIG-labelled (Boehringer Mannheim, Mannheim, Germany), 0.359-8.576 kb for Southern blot analysis

DNA fragments were isolated from agarose gels using a NucleoSpin 2 in 1 extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol.

7.1.3. DNA manipulation with enzymes

Restriction of DNA with endonucleases was carried out according to the manufacturer´ instructions.

DNA ligation was achieved by using T4-DNA ligase. The ligation preparation, containing 1U T4 DNA ligase (Amersham Bioscience, Freiburg, Germany), 1×ligation buffer and a 1:1 (mole ratio) mixture of insert (about 50-200 ng) and linearized vector in a final volume of 10 µl, was incubated at RT for 2h or at 16°C overnight.

7.1.4. DNA isolation

7.1.4.1. Isolation of plasmids from *E. coli*

Mini-preps employing alkaline lysis were used to isolate recombinant plasmids from *E. coli* for routine screening. 3 ml LB-medium was inoculated with a single colony and grown overnight at 37°C, 170 rpm. 1.5 ml of this culture were harvested by centrifugation (2,000 ×g, 4°C, 10 min) and resuspended in 250 µl solution A1 by vortexing. The suspension was mixed with 250 µl solution A2 by inversion and incubated at RT for 5 min. And then 250 µl solution A3 was added and incubated on ice for 10 min. After centrifugation for 20-30 min (20,000×g, 4°C), the supernatant was poured into a fresh microfuge tube. DNA was precipitated by addition of 0.8-1.0 fold Isopropanol and centrifugation (20,000×g, 4°C, 30 min). DNA pellet was washed once with 500 µl 70% ethanol, air dried and resuspended in 50-100 µl distilled water or TE buffer. The relative solutions are listed in Table 5.

Preparative isolation of plasmids from *E. coli* was carried out with ion-exchange columns (Nucleobond® A×100, Macherey-Nagel, Düren, Germany) according to the manufacter´s protocol.

7.1.4.2. Isolation of plasmids from *Streptomyces*

Plasmids were isolated from *Streptomyces* as described by Kieser *et al.* (2000). 2 ml of 2-day-old culture in CRM medium were harvested by centrifugation (2,100 ×g, 4°C, 10 min). After wash with 1ml of solution B1, the cells were resuspended in 500 µl of solution B1 with Lysozyme (4-8 mg/ml) and RNase (100 µl/ml) by vortexing. The suspension was incubated at 37°C for 30-60 min, then mixed with 650 µl of solution A2 by inversion and again incubated at RT for 10 min. 650 µl solution A3 was added and the mixture incubated on ice for 10 min. After 20-30 min centrifugation (20,000×g,

4°C), the supernatant was poured into two fresh microfuge tubes and extracted with 300 µl Rotiphenol® once or twice. DNA was precipitated by addition of 0.8-1.0 volume of Isopropanol and centrifugation (20,000×g, 4°C, 30 min). DNA pellet was washed once with 500 µl 70% ethanol, air dried and resuspended in 20-50 µl distilled water or TE buffer. The relative solutions are listed in Table 6.

7.1.4.3. Isolation of genomic DNA from *Streptomyces*

Genomic DNA was isolated from *Streptomyces* by lysozyme treatment and phenol-chloroform extraction (Kieser *et al.,* 2000). 2 ml of 2-day-old culture in CRM or HA medium were harvested by centrifugation (2,100 ×g, 4°C, 10 min). The cells were washed with 1 ml TSE buffer and resuspended in 500 µl TSE buffer with lysozym (3 mg/ml) and RNase (100 µl) by vortexing. The suspension was incubated for 30 min at 37°C and in between mixed well every ten minutes, then added with 250 µl of 2% SDS solution and incubated for 10 min at 60°C, for 10 min at RT, again at 60°C for 10 min. The lysate was extracted with 250 µl Rotiphenol® for three times. The third extraction was carried out with addition of 70 µl NaAc (pH 4.8). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.8-1.0 volume of isopropanol and centrifugation (20,000×g, 4°C, 30 min). DNA pellet was washed once with 500 µl 70% ethanol, air dried and resuspended in 20-30 µl TE buffer.

7.1.5. DNA denaturation for ssDNA transformation in *Streptomyces*

7.1.5.1. Glycerol-heat denaturation

8 µl dsDNA was mixed with 5 µl denaturing reagent (s. Table 11) and incubated for 5 min at 90-100°C. To stop the reaction, the mixture was replaced on ice.

7.1.5.2. Alkaline treatment (Oh & Chater 1997)

9 µl dsDNA in H_2O was mixed with 2 µl 1 M NaOH and incubated for 10 min at 37°C. The mixture was replaced on ice and the reaction was terminated with addition of 2 µl 1 M HCl.

7.1.6. PCR amplification

PCR amplification was carried out with GeneAmp PCR System 2400 (Perkin-Eimer, Weiterstadt, Germany). The PCR mixture using GC-Rich PCR system (Roche,

Mannheim, Germany) and amplification conditions are given in Table 18. For PCR amplification with Taq or Pfu polymerase, the PCR mixture (100µl) contained 20 pmol each primer, 250-500 ng template DNA, 0.2 mM dNTP each and 1× reaction buffer, 5% DMSO and 1U polymerase.

Tab. 18 PCR mixture using GC-Rich PCR system and amplification conditions

Substance	Final concentration
GC-Rich PCR reaction buffer with DMSO(5×)	1×
GC-Rich resolution solution (5 M)	0.5 mM
dNTP-Mix	0.2 mM each
Primer 1	20 pmol
Primer 2	20 pmol
template DNA	250 ng
GC-Rich PCR System enzyme mix	1U/100µl
Add distilled water to make up to 100µl	

Cyclus	Temperature	time	cycles
Hot start	95 °C	5 min	1
Denaturing	95°C	90 s	30-35
Annealing	56-60°C	90 s	
Elongation	72°C	45s/ 1 kb	
	4°C	∞	1

7.1.7. Southern blot analysis

Southern blot analysis was performed on Hybond-N membranes (Amersham, Braunschweig, Germany) by using DIG high prime DNA labelling and detection starter kit II (Roche, Mannheim, Germany). All the buffers and solutions are given in Table 9.

7.1.7.1. Probe preparation

Probe for Southern hybridization was prepared by random priming method using DIG high prime DNA labelling and detection starter Kit II according to the user manual. All the probes used in this thesis are listed in Table 17.

7.1.7.2. Blot preparation

An agarose gel with DNA digested with appropriated enzymes, 1 kb ladder and DIG Marker No. VII was prepared before bloting. DNA in gel was denaturized by soaking the gel in denaturing buffer for 2×15 min, and then neutralized by soaking in neutralizing buffer for 2×15 min, with gentle agitation. After that, the gel was placed on prewetted filter paper and overlaid with prewetted Hybond-N nylon membrane and prewetted filter paper. 10 cm paper towels with 1-2 kg weight were stacked on top. Blot overnight. Capillar action with 20 ×SSC solution transferred DNA from the gel to

the membrane. Air dry membrane and crosslink with UV light (312 nm, 60 s on the front side and 30 s on the backside).

7.1.7.3. Prehybridization and hybridization

The membrane was put in prehybridization solution (20 ml/100 cm^2) and incubated for 4 h at 68°C with gentle rotation. Die probe was added to hybridization solution (5-25 ng/ml) and denaturized by heat (10 min at 100°C), chilled with liquid nitrogen. The prehybridization solution was changed with hybridization solution (3.5-5 ml/100 cm^2) with probe and incubated overnight at 68°C.

7.1.7.4. Detection

The membrane was washed twice with 2×SSC wash buffer for 10-15 min at RT, followed with 0.5×SSC wash buffer at 68°C for 20 min, with gentle agitation. After equilibration in maleic acid buffer for 5 min, the membrane was incubated with blocking solution for 30-60 min and then with antibody solution for 30 min. To remove the rest of antibody, the membrane was washed twice in tween wash buffer, 15 min each at RT. Finally, the membrane was equilibrated in detection buffer for 5 min and placed in a plastic sheet. A 1:1000 dilution of CSPD stock solution in detection buffer was dropped onto the membrane (0.5 ml/100cm^2) and spread over the entire membrane. Incubate at RT for 5 min and expose a Hyperfilm ECL-X-ray film (Amersham Biosciences, Freiburg, Germany) at 37°C for 1 h. The film was developed using standard method.

7.1.7.5. Removal of probe

Probe on the membrane was removed by washing the membrane twice with stripping solution for 15 min at 37°C, followed with 2×SSC solution for 30 min at RT. After that, the membrane could be used for further hybridization or stored in 2×SSC solution at 4°C.

7.1.8. $CaCl_2$-mediated transformation of *E. coli* (a method modified from Sambrook and Russel (2001)

7.1.8.1. Preparation of competent cells

100 ml LB-medium was inoculated with 3 ml of an overnight culture of *E. coli* and cultivated at 37°C, 170 rpm till the OD_{600} reached 0.6 (2-4 h). The cells was harvested by centrifugation (2,000×g, 4°C, 10 min), resuspended in 30 ml ice-cold 0.1 M $MgCl_2$ (no vortex) and again centrifuged (2,000×g, 4°C, 10 min). The cell pellet was suspended in 20 ml ice-cold $CaCl_2$ (0.1 M) and incubated on ice for 20 min. After centrifugation (2,000×g, 4°C, 10 min), the pellet was suspended in 5 ml of $CaCl_2$ (0.1 M) solution containing 15 % glycerol (no vortex). Competent cells could be used immediately or dispensed in 200-µl aliquots in 1.5-ml microfuge tubes, shock frozen and stored at -70°C.

7.1.8.2. Transformation

DNA (0.2-5 µg in 1-5 µl) was added to 100-200 µl competent cells in 1.5-ml microfuge tube and incubated on ice for 30 min. The tube was replaced in heat block or water bath at 42°C for 1-2 min and cooled down on ice (about 5 min). 1 ml LB medium (without antibiotics) was pipetted into the tube and incubated with gentle rotation for 1 h at 37°C. 200 µl of the mixture was plated on a LB agar plate and the rest was centrifuged (2,000×g, 4°C, 10 min), resuspended in the runback (about 300 µl) and plated on another LB agar plate. For transforming a circular plasmid, the incubation with LB medium at 37°C might be omitted.

Blue/white selection

If *lac*Z cloning vector was used to prepare the recombinant plasmid, blue/white selection could facilitate the identification of the expected clones. Before the transformation cells were plated on agar plates, 50 µl of X-Gal solution (20 mg/ml), 12 µl of IPTG solution (80 mg/ml) and 100 µl H_2O (sterile) were pipetted on the top of the plates and spread evenly, air dried under the clean bench (about 30 min). The colonies containing the recombinant plasmid lack β-galactosidase activity and remain white.

7.1.9. Preparation of protoplasts and transformation of *Streptomyces*

7.1.9.1. Preparation of protoplasts

50 ml of CRM medium were inoculated with 100-200 µl glycerol stock culture of *Streptomyces* and grown at required temperature with rotation of 180 rpm for 42-48 h.

The cells was harvested by centrifugation (2,100×g, 4°C, 10 min), washed with 10 ml TE buffer containing 10% sucrose (pH 8) and subsequently with 10 ml P-buffer. The cell pellet was resuspended in P-buffer with lysozyme (3-4 ml P-buffer/g cells, 0.3 mg lysozyme/ml P- buffer for *S. rishiriensis*; 5 ml P-buffer/g cells, 1 mg lysozyme/ml P-buffer for *S. roseochromogenes*; s. Table 11). The suspension was incubated at 30°C for 15-60 min with gentle agitation and in between the protoplast formation was controlled under microscopy at intervals of 15 min. After most cells became protoplasts, the reaction was stopped by incubation on ice. The following steps were carried out on ice. The suspension was filtered through sterile glass wool and centrifuged for 10 min at 2,100×g, 4°C. The supernatant was poured off and the pellet was suspended in 0.5-2 ml P-buffer. The protoplast suspension could be used immediately or dispensed in 200-µl aliquots in 1.5 ml microfuge tube, shock frozen and stored at -70°C.

To observe the protoplast regeneration, dilution series of the protoplast suspension were prepared and plated on R2YE agar plate. The plates were incubated at 30°C for 3-7 days. The regenerable protoplasts per ml suspension were calculated.

7.1.9.2. PEG-mediated protoplast transformation and regeneration

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

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

100-200 µl of Protoplasts, containing at least 10^8 protoplasts per ml, was added with 5-20 µg (in maximal 20 µl) unmethylated DNA and 500 µl T-buffer containing PEG 1000 (25% (w/v) for *S. rishiriensis*, 40% (w/v) for *S. roseochromogenes*; s. Table 11), mixed by inversion and incubated at RT for 1 min. The resulting suspension was mixed with warm R3 or R2YE soft agar (40-50°C, 4×3 ml) and plated on four R2YE plates. After 16-24 h incubation at 28°C-30°C, the plates were overlaid with 3 ml of soft nutrient agar including the required antibiotics for selection of mutants. The plates were left to grow for further 5-14 days. In order to allow homologous recombination, denatured DNA was used to transform *Streptomyces*.

7.1.10. DNA sequencing and computer-assisted sequence analysis

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

The DNASIS software package (Version 2.1, Hitachi Software Engineering, San Bruno, CA, USA) was used for sequence analysis. Amino acid sequence homology searches were performed by using BLAST program (Version 2.0) in internet at www. ncbi.nim.nih.gov/BLAST/.

7.2. Methods of biochemistry

7.2.1. Protein quantification

Protein concentrations were determined by Bradford method (1976) using bovine serum albumin as a standard. 100 µl of protein solution was mixed with 1 ml Bradford reagent (s. 4.3.7). After incubation at RT for 5 min, the absorption was determined with a photometer at 595 nm. As reference, 100 µl H_2O was mixed with 1 ml Bradford reagent.

7.2.2. SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970). 4% and 10% Polyacrylamide gel were used as stocking gel and resolving gel, respectively. Gel electrophoresis was carried out with working voltage of 150-200 V using the Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad, München, Germany). Protein bands were stained with Coomassie Brilliant Blue R250 (s. Table 13). To determine protein size, Low Molecular Weight Calibration Kit for SDS gel electrophoresis (Amersham Biosciences, Freiburg, Germany) was used.

7.2.3. Gel filtration and determination of molecular weight.

Gel filtration was carried out on a Hiload 26/60 Superdex® 200 column with a flow rate of 1 ml/min at 4°C. The column was calibrated with blue dextran 2000 (MW 2,000,000), aldolase (MW 158,000), albumin (MW 66,000), ovalbumin (MW 45,000) and ribonuclease A (MW 13,700) (Amersham Biosciences, Freiburg, Germany).

Buffer G1 and G2 (Table 12) were used to elute the standard proteins and probe, respectively.

The molecular mass of native proteins was determined from the calibration curve of the log of molecular weight of protein versus partition coefficient K_{av}, given by the following equation,

$$K_{av}=\frac{V_e-V_0}{V_t-V_0}$$

V_t: the total bed volume
V_0: the void/exclusion volume
V_e: the elution volume

In the mixture of proteins listed above, blue dextran 2000 was totally excluded (V_0) and acetone totally included (V_t). K would be between 0 and 1 for the other proteins, which are within the fractionation range of the column.

Table 19 Calibration of the Sephadex® 200 column

Protein/substance	MW (Da)	V_e (ml)	K_{av}
Blue dextran	2,000,000	107.9	
Aldolase	158,000	158.6	0.2724
Albumin	66,000	178.4	0.3788
Ovalbumin	45,000	212.4	0.5615
Ribonuclease A	13,700	252.4	0.7765
Acetone	58	294.0	

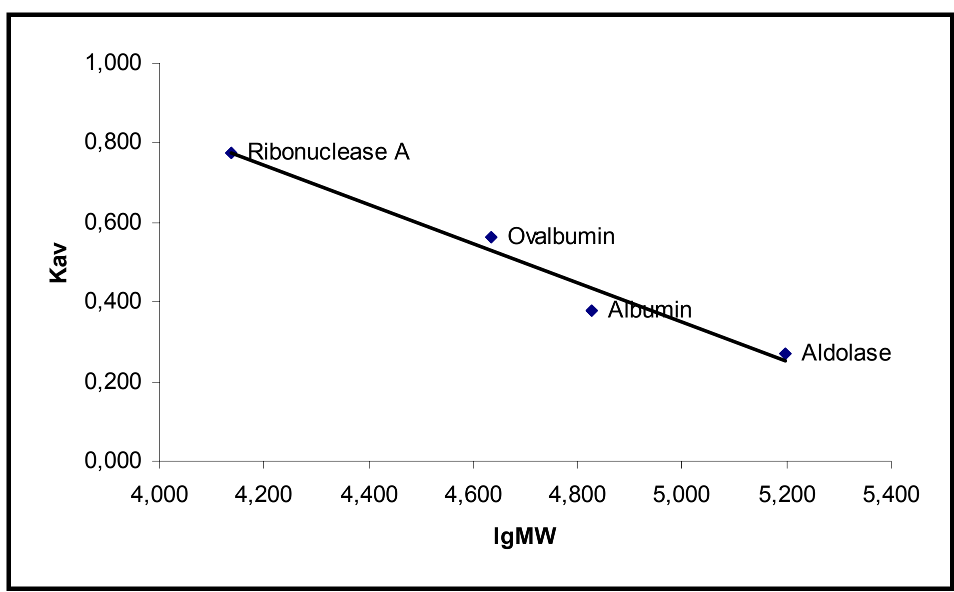


Fig. 3 Calibration curve of gel filtration

7.2.4. Overexpression and purification of recombinant protein from *E. coli*

Overexpression and purification of 6×His-tagged protein from *E. coli* were carried out as decribed by the user manual of Qiagen (Qiagen, Hilden, Germany), “a handbook for high level expression and purification of 6×His-tagged proteins”.

7.2.5. Overexpression and purification of NovN as Histidine fusion protein from *S. lividans* T7

Expression and purification of NovN was carried out as described elsewhere (Heinzelmann *et al.,* 2001; Schmutz *et al.,* 2004). For buffers see Table 12.

7.2.5.1. Cultivation

S. lividans T7 harbouring the expression vector were pre-cultured in YEME medium with kanamycin (10 µg/ml) at 28°C, 180 rpm for 48 h. 1 ml of the preculture was transferred to 100 ml new YEME medium with kanamycin (10 µg/ml) and thiostrepton (inductor, 25 µg/ml) and grown for further 24 h. In order to get a fine culture, an insulin syringe might be employed to inoculate the media.

7.2.5.2. Preparation of cell-free extract

All the following procedures were carried out at 4°C or on ice. The cells after expression were harvested by centrifugation (10 min at 2,500 × g) and incubated in ice-cold lysis buffer (1-2 ml/ g cells) for 30 min. The cell suspension was sonicated (Branson Sonifier 250) for 10 min at intervals of 2 min ultrasonication followed with 0.5 min break. Subsequently, cell debris was removed by centrifugation (30 min at 15,000 × g) and protein content in the cell-free extract was determined by Bradford method.

7.2.5.3. Purification by nickel affinity chromatography

Histidine fusion protein was purified from the soluble cell extract by metal affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. 6-8 ml cell free extract was added with 1 ml Ni-NTA Agarose (Qiagen, Hilden, Germany) and mixed gently for 1 h at 4°C. The mixture was loaded into a plastic column, washed with wash buffer (s.

4.3.6) for three times. 6×His-tagged protein was eluted with elution buffer (2-3 ml). To remove salt and imidazol, the Ni-NTA resin eluate was applied onto a NAP 10 column (Amersham Biosciences, Freiburg, Germany) and eluted with 0.2 M Tris-HCl (pH 8.0).

7.2.6. Carbamoyltransferase assay

The carbamoyltransferase assay mixture contained 0.2 M Tris-HCl (pH 8.0), 1 mM descarbamoyl novobiocin or analogues, 5 mM CAP-Li_2, 2 mM ATP, 2 mM Mg^{2+} and purified NovN (0.4 µM) in a final volume of 100 µl. The reactions were carried out at 30°C and terminated by the addition of 5 µl 1.5 M trichloroacetic acid. As negative control, assay was carried out with heat-denatured protein. For preparative experiments, the assay mixtures were incubated overnight, the enzymatic reaction products were isolated by HPLC and subjected to MS and ^{1}H-NMR analysis.

7.2.7. Determination of kinetic parameters

The determination of K_m for descarbamoyl novobiocin and novclobiocin 104, 105 was accomplished at a constant concentration of carbamoyl phosphate (5 mM) and NovN (37.5 nM) and over a range of descarbamoyl novobiocin or novclobiocins concentration (2.5-40 µM). The reaction was quenched at 5 min for each concentration. Each experiment was carried out in duplicate. The kinetic parameters were calculated using Lineweaver-Burk plot.

8. Construction of vectors

8.1. Construction of pN3 and pN4 for inactivation of *cou*N3 and *cou*N4 in *S. rishiriensis* (WT)

Plasmid pN3, for *cou*N3 inactivation, carried an in-frame deletion of 480 bp at the 3´ end of *cou*N3. The 3’ region of the insert was obtained from the plasmid pZW11 (Table 14) as a *Pst*I-fragment of 1666 bp, including the C-terminal region of *cou*N3 and most of the *cou*N4 gene. This fragment was ligated to a 4.7 kb fragment obtained by *Pst*I digestion of the plasmid pZW33, containing the pBluescript SK(-) vector as well as a 1.8 kb fragment with the 5’ end of *cou*N3 and the adjacent *cou*N2 and *cou*N1 genes. The resulting plasmid pZW334 was restricted with *EcoR*V and

*Xba*I and religated into the same sites of pKC1132 (Bierman *et al.,* 1992), an integrative vector containing the apramycin resistance gene, to give pN3.

The vector pN4 for *cou*N4 inactivation carried a neomycin resistance gene inserted into the sequence of *cou*N4. It was constructed from pZW331 and pZW32 (Wang *et al.,* 2000). pZW331, which contained the 5' region of *cou*N4, was restricted with *EcoR*I and *Hind*III and ligated to a 0.99 kb fragment (containing the *aph*II gene), obtained by restriction of pNeo (Wang *et al.,* 2000) with the same enzymes. The resulting plasmid pZW2 was restricted with *Hind*III and *Xho*I and ligated to a 0.96 kb *Hind*III-*Xho*I fragment from pZW32 (containing the 3' region of *cou*N3) to give pZW3. pZW3 was restricted with *PstI*, and the resulting 2.65 kb fragment containing the disrupted *cou*N3 gene was cloned into the *Pst*I site of pBluescript SK (-) to give the inactivation vector pN4. In pN4, the *aph*II gene fragment had the same orientation as the *cou*N4 gene.

8.2. Construction of vector pN2 for inactivation of *clo*N2 in *S. roseochromogenes* (WT) and *clo*-hal$^-$ mutant

Vector pN2 was constructed for in-frame deletion of the *clo*N2 as follows: Two fragments cloN2-1 and cloN2-2, containing the flanking regions of *clo*N2, were generated by PCR-amplification using cosmid K1F2 as template. Primer pairs used were cloN2-1/*Xba*I and cloN2-1/*Not*I (Table 16); cloN2-2/*Not*I and cloN2-2/*Hind*III (Table 16). The PCR fragments of cloN2-1 and cloN2-2 were purified and ligated into the linearized vector pGEM-T (Promega, Madison, WI, USA), to give plasmids pN21 and pN22, respectively. The 1276 bp *Not*I-*Hind*III fragment (bp 22151 to 23426 in sequence AF 329398) was excised from pN22 and cloned into the same sites of pcDNA 2.1 (Invitrogen, Carlsbad, CA, USA) to give plasmid pN23. The *Xba*I-*Not*I fragment of 1308 bp (bp 19866 to 21173 in sequence AF 329398) was isolated from plasmid pN21 and ligated into the same sites of pN23, resulting in plasmid pN24, which contained an in-frame deletion of 978 bp in the coding region of *clo*N2. The inactivation vector pN2 was obtained by releasing the *Xba*I-*Hind*III fragment of 2584 bp from pN24 and cloned into the same sites of pBSKT (Lombo *et al.,* 1997), a non-replicative vector containing a thiostrepton resistance gene.

8.3. Construction of pN2C for complementation of *clo*N2$^-$ mutant with gene *clo*N2

For the complementation with *clo*N2, vector pN2C was constructed. A fragment of 1.15 kb containing the whole sequence of *clo*N2 (bp 21090 to 22242 in sequence AF329398) was obtained by PCR amplification with the primer pair cloN2C/*BamH*I and cloN2C/*Pst*I (Table 16.) using cosmid K1F2 as template. The purified PCR product was cloned into the vector pGEM-T, to give pN2C-T. The *BamH*I-*Spe*I fragment of 1.2 kb from the vector pN2C-T was religated into the restriction sites of *BamH*I and *Xba*I of pUWL201 (Steffensky *et al.,* 2000b). The resulting vector pN2C, containing a thiostrepton resistance gene for selection in *Streptomyces*, was used for complementation of the *clo*N2$^-$ mutant.

8.4. Construction of pXHN for heterologous expression of NovN in *cou*N3$^-$ mutant

A 2.5 kb *Bcl*I-*EcoR*I fragment from plasmid p9-6GE9 (Table 14), comprising the complete *nov*N gene, was cloned into the *BamH*I-*EcoR*I sites of expression vector pEM4 (Quiros *et al.,* 1998), resulting in plasmid pXHN.

8.5. Construction of pXHNEG for expression of NovN as N-terminal 6×His-tagged protein in *S. lividans* T7

*nov*N was amplified from cosmid 10-9C (Table 14) using the GC-RICH PCR system (Roche, Mannheim, Germany), using the primer pair novN-*Bcl*I and novN-*Hind*III (Table 16). The DNA fragment was directly ligated into the linearized vector pGEM-T, resulting in plasmid pXHNET. *nov*N was released from pXHNET by digestion with *Bcl*I and *Hind*III and ligated into pRSET-B, which had been restricted with *BamH*I and *Hind*III, to give plasmid pXHNER. To obtain the plasmid pXHNEG, pXHNER was fused with the vector pGM9 *via* the restriction site *Hind*III. pXHNEG contained a kanamycin resistance gene.

9. Analysis and isolation of secondary metabolites

9.1. Extraction and HPLC analysis

9.1.1. Analysis of coumermycin A_1 and derivatives

6 ml of the whole culture of wild-type and mutant strains of *S. rishiriensis* in SK-medium (s. 4.1.3) were acidified with HCl to pH 6, washed twice with 6 ml petroleum ether and subsequently extracted twice with 6 ml ethyl acetate. The solvent was

removed and the residue was dissolved in 0.6 ml ethanol. This solution is used for HPLC and LC/MS analysis.

Metabolites were analyzed by HPLC with a Nucleosil RP 120-5 column (5 µm, 250×2 mm, Macherey-Nagel GmbH & Co., Düren, Germany) with a linear gradient from 30 to 100% acetonitrile in 0.1% aqueous phosphoric acid. Flow rate was 0.2 ml/min and UV absorption was recorded at 345 nm. Authentic coumermycin A_1 (Sigma-Aldrich, Taufkirchen, Germany) was used as standard.

9.1.2. Analysis of clorobiocin and derivatives

20 ml of the whole culture of wild-type and mutant strains of *S. roseochromogenes* in distiller solubles medium (s. 4.1.4) were acidified with 1N HCl to pH 2-4, and extracted twice with an equal volume of ethyl acetate. The solvent of the organic phase was removed and the residue was dissolved in 1 ml ethanol. This solution is used for HPLC and LC/MS analysis.

HPLC analysis was carried out on a Multosphere RP18-5 column (5 µm, 250×4 mm; C+S Chromatographie Service, Düren, Germany) at a flow rate of 1 ml/min, using a linear gradient from 70% to 100% methanol in 1% aqueous formic acid over 25 min; UV detection was carried out at 340 nm. Authentic clorobiocin (Aventis, Bad Soden, Germany) was used as standard.

9.1.3. Analysis of pyrrole-2-carboxylic acid

The analysis of pyrrole-2-carboxylic acid by HPLC was also carried out on a Multosphere RP18-5 column. An isocratic elution with 20% methanol in 1% aqueous formic acid was used; UV detection was at 262 nm. Pyrrole-2-carboxylic acid (Lancaster, Mühlheim a. M. Germany) was used as standard.

9.1.4. Analysis of enzymatic reaction products

The assay products were extracted twice with 500 µl ethyl acetate. After evaporation of the organic solvent, the residue was dissolved in methanol and analyzed by HPLC with a Nucleosil RP 120-5 column (5 µm, 250×2 mm, Macherey-Nagel, Düren, Germany) using a linear gradient from 70 to 100% methanol in 1% aqueous formic acid over 25 min and UV absorption was recorded at 325 nm. Authentic clorobiocin

(Aventis, Bad Soden, Germany) and descarbamoyl novobiocin (Pharmacia & Upjohn, Kalamazoo, MI, USA) was used as standard.

9.2. Preparative isolation of new aminocoumarin derivatives

9.2.1. Column chromatography using Sephadex® LH-20

Column chromatography was used to pre-purify the culture extract. Crude culture extract (max. 3 ml) was loaded onto a glass column (100×2.6 cm) with Sephadex LH-20 (Amersham Biosciences, Freiburg, Germany). The column was eluted with degased methanol with a flow rate of 1 ml/min. Each 15 ml eluate was collected by fraction collector and controlled by HPLC. The expected fractions were pooled and the solvent removed in vacuum. The residue was dissolved in methanol and ready for further purification by HPLC.

9.2.2. Preparative HPLC

Preparative HPLC was carried out on a Multosphere 120 RP 18-5 column (5 µm, 250×20 mm; C+S Chromatographie Service, Düren Germany) with a flow rate of 3 ml. Methanol and 1% aqueous formic acid were used as the solvents. Concentrated culture extract, fractions after LH-20 or enzymatic reaction products were injected and analysed. The expected eluates after HPLC analysis were collected and the solvent was evaporated in vacuum. The residues were dried over phosphorus pentoxide and subjected to MS and NMR (^{1}H-NMR and ^{13}C-NMR) analysis.

9.3. Spectroscopic methods for structural elucidation

9.3.1. LC/MS and selected reaction monitoring (SRM)

LC/MS was carried out in the lab of J. Schmidt (Universität Halle, Halle, Germany) as well as of B. Kammerer (Universität Tübingen, Tübingen, Germany). The positive and negative electrospray ionization (ESI) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 3-4.5 kV; heated capillary temperature 220-300 °C; sheath and auxillary gas: nitrogen) equipped with RP18-columns (5 µm, 1x100 mm, SepServ, Berlin, Germany, for analysis of coumermycin derivatives / 5 µm, 2x250 mm, Macherey-Nagel, Düren, Germany, for analysis of pyrrole-2-carboxylic acid). For separation an acidic gradient system of H_2O:CH_3CN was used; flow rate 70 µl min^{-1}/ 0.2 ml min^{-1} for the two different columns,

respectively. The collision-induced dissociation (CID) spectra and the selected reaction monitoring (SRM) during a HPLC run were recorded with a collision energy of -25 eV for positive ions as well as +40 eV for negative ions, respectively; collision gas: argon, collision pressure: 1-1.8 x 10^{-3} Torr.

9.3.2. FAB (-) mass spectroscopy

Negative fast atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using diethylethanol amine as matrix.

9.3.3. Nuclear magnetic resonance (NMR)

^{1}H-NMR spectra were measured on an AMX 400 spectrometer (400 MHz; Bruker, Karlsruhe, Germany), using CD_3OD or DMSO-d6 as solvent.

10. Methods of biology

10.1. Supercoiling assay (Galm *et al.,* 2004b)

Aminocoumarin derivatives in this thesis were tested for inhibitory effect on supercoiling activity of *E. coli* gyrase, using the DNA gyrase kit from John Innes Enterprises Limited (John Innes Centre and Norwich Research Park, Colney, Norwich) according to the manufacturer's instruction. Reaction mixtures (20 µl) contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (w/v) glycerol, 2 µg bovine serum albumin (Merck, Darmstadt, Germany), 1 U DNA gyrase, 100 ng relaxed pBR322 DNA, and various concentrations of aminocoumarins dissolved in methanol/water (0-20% methanol) After incubation of the mixtures at 37°C for 1 h, the reactions were terminated by cooling to 0°C, then 5 µl of 30% glycerol containing 0.25% bromophenol blue were added and the DNA was analysed by electrophoresis in 0.8% agarose. The inhibitory effect of different aminocoumarins was expressed as IC_{50}, i.e. the aminocoumarin concentration at which the DNA gyrase supercoiling activity was inhibited by 50%.

10.2. Bioassay with *Bacillus subtilis* (Eustáquio *et al.,* 2003a; Westrich *et al.,* 2003)

Antibacterial activity against *Bacillus subtilis* ATCC 14893 was determined by a disc diffusion assay using *Bacillus subtilis* ATCC 14893 as the indicator strain. Different

amounts of aminocoumarin dissolved in 2-10 µl of methanol were applied to filter paper disks (Ø 3 mm; MN 440 B blotting paper, Macherey-Nagel, Düren, Germany), air dried under the clean bench and placed on the top of nutrient agar plates, which was seeded with 2 x 10^5 *B. subtilis* spores per ml of solid medium. Plates were cultured overnight at 37°C, and the diameters of growth inhibition zones measured. Novobiocin (Sigma, Deisenhof, Germany) and clorobiocin (Aventis Biosciences, Freiburg, Germany) were used as reference substances.

RESULTS

1 Involvement of *cou*N3 and *cou*N4 in the biosynthesis of the pyrrole moiety of coumermycin A_1

Structurally, clorobiocin and coumermycin A_1 share the same terminal 5-methylpyrrole-2-carboxylic acid moiety attached to the 3´´-OH group of the deoxysugar via a glycosidic bond. Besides the terminal pyrrole moieties, coumermycin A_1 still contains a central 3-methylpyrrole-2,4-dicarboxyl moiety, which combines the two aminocoumarin rings via two amide bonds. The only experimental study published on the biosynthesis of the pyrrole moieties of coumermycin A_1 was carried out by Scannell and Kong (1969), who showed that radioactivity from [U-^{14}C]L-proline and from [methyl-^{14}C]L-methionine, but not from [4-^{14}C]δ-aminolevulinic acid was incorporated into the pyrroles. They concluded that "proline serves as a direct precursor of the pyrrole groups of coumermycin A_1", and that S-adenosyl-methionine contributes the methyl groups attached to these moieties. No explanation was offered for the different structure of the central pyrrole moiety as compared to the terminal pyrroles.

Pyrrole-2-carboxylic acid moieties are found also in the tripyrrole prodiginines and the chlorinated pyoluteorin (Fig. 4) as well as in other natural products. The biosynthetic gene clusters of pyoluteorin (*plt* genes) (Nowak-Thompson *et al.,* 1999) and undecylprodiginine (*red* genes) (Cerdeno *et al.,* 2001; Malpartida *et al.,* 1990) have been identified. Recently, three key enzymes involved in the conversion of L-proline to pyrrole in the biosynthesis of these compounds have been expressed, purified and investigated biochemically (Thomas *et al.,* 2002). PltF (or RedM, respectively) activates proline in form of an prolyl adenylate and transfers the prolyl moiety onto the 4-phosphopantetheinyl cofactor of the small peptidyl carrier protein (PCP) PltL (or RedO, respectively) to form a thioester. The FAD-dependent dehydrogenase PltE (or RedW, respectively) oxidises this compound to Δ^2-pyrrolinyl-2-carboxyl-*S*-PCP, similar to the acyl-CoA dehydrogenase reaction in the β-oxidation of fatty acids. The product appears to undergo spontaneous, non-enzymatic air oxidation, yielding pyrrolyl-2-carboxyl-*S*-PCP (Fig. 4).

Fig. 4 Proposed biosynthetic pathway for the pyrrole-2-carboxylic acid moieties of coumermycin A_1, clorobiocin, pyoluteorin and undecylprodiginine

In the biosynthetic gene clusters of coumermycin A_1 and clorobiocin, three pairs of genes (*cou*N3/*clo*N3, *cou*N4/*clo*N4 and *cou*N5/*clo*N5) were identified which showed high sequence similarity to genes involved in pyrrole formation in pyoluteorin and undecylprodiginine biosynthesis (Table 20). These genes were therefore likely to be involved in the formation of the pyrrole moieties of coumermycin A_1 and clorobiocin. In order to provide experimental proof for this hypothesis, an inactivation of the putative L-prolyl-*S*-PCP dehydrogenase gene *cou*N3 was carried out in the coumermycin producer *S. rishiriensis* DSM 40489.

Table 20 Similarity of the gene products of *cou*N3, *cou*N4 and *cou*N5 with products of the clorobiocin, pyoluteorin and undecylprodiginine genes

Coumermycin A_1 cluster (AF 235050)	Clorobiocin cluster (AF 329398)		Pyoluteorin cluster (AF 081920)		Undecylprodiginine cluster (AL021530, AL021409)		Proposed function
	Gene product	Identity[§] (%)	Gene product	Identity[§] (%)	Gene product	Identity[§] (%)	
CouN3 373 aa	**CloN3** 376 aa	81	**PltE** 380 aa	47	**RedW** 391 aa	43	L-prolyl-*S*-PCP dehydrogenase
CouN4 501 aa	**CloN4** 501 aa	87	**PltF** 499 aa	51	**RedM** 532 aa	43	L-prolyl-AMP ligase
CouN5 89 aa	**CloN5** 89 aa	91	**PltL** 88 aa	38	**RedO** 87 aa	38	peptidyl carrier protein (PCP)

* The Genbank accession numbers for each of the clusters are shown in parentheses.

[§] The identity is referred to CouN3, CouN4 and CouN5, respectively.

1.1 Inactivation of *cou*N3

For this purpose, plasmid pN3 was constructed, which contained a 480 bp in-frame deletion in the C-terminal region of the gene *cou*N3, thereby shortening the predicted gene product from 373 to 213 amino acids (Fig. 5A). The deletion site was flanked on either side by approximately 1.7 kb of the adjacent regions of the coumermycin A_1 cluster in order to allow homologous recombination, and this fragment was cloned into the non-replicative vector pKC1132 carrying an apramycin resistance marker.

The construct was introduced into the coumermycin A_1 producer *S. rishiriensis* (wild-type) by protoplast transformation. Mutants resulting from integration of the vector were selected with apramycin, sporulated and plated on agar medium with and without antibiotic. Four antibiotic-sensitive strains resulting from double cross-over events were obtained, and Southern blot examination (Fig. 5B) revealed that three of them (strains XH12, XH13 and XH14) represented the desired genotype with the inactivated *cou*N3 gene, while the fourth one had reverted to wild-type.

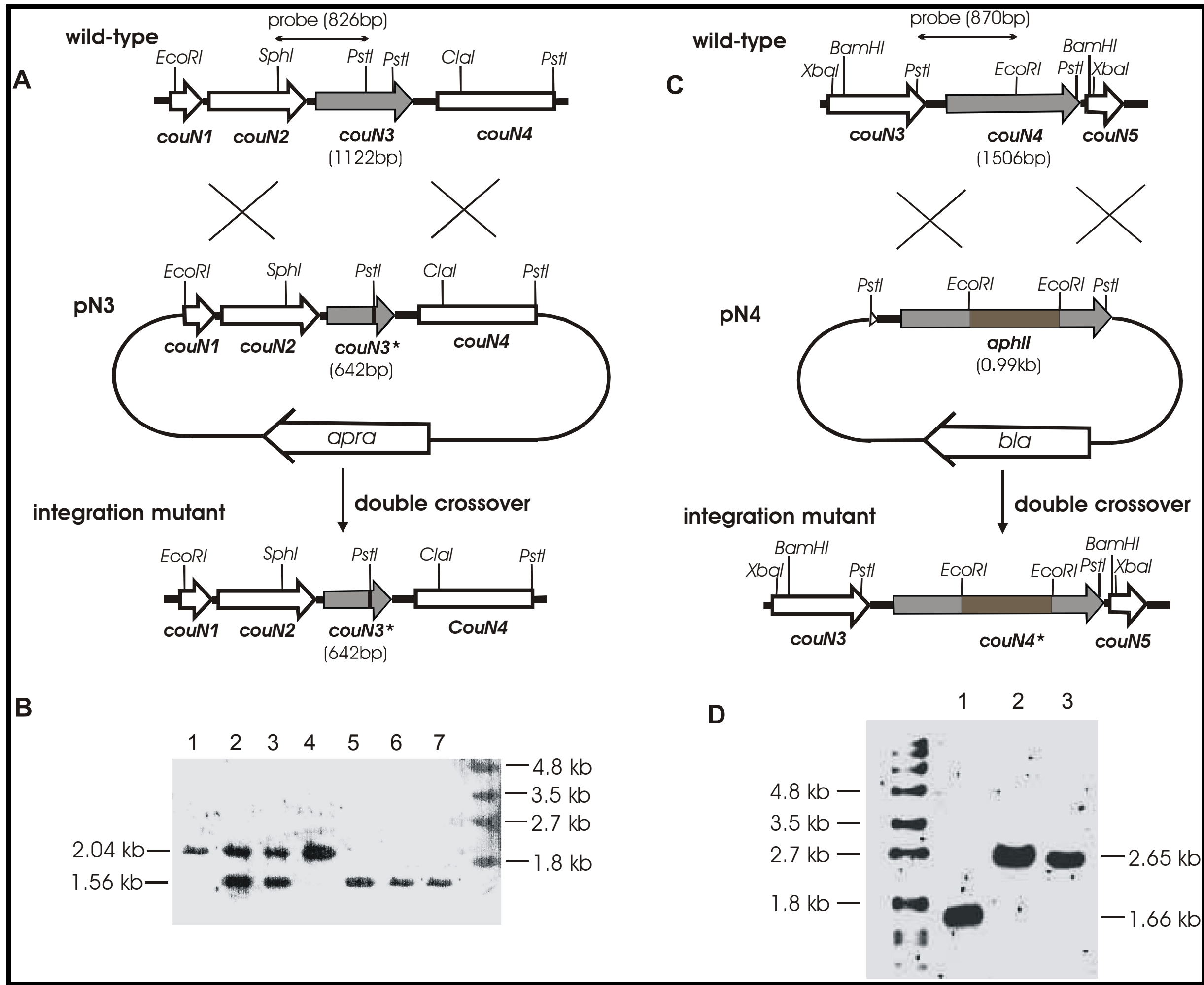


Fig. 5 Inactivation of *cou*N3 and *cou*N4 in *S. rishiriensis* DSM 40489. (A) Schematic presentation of the *cou*N3 gene inactivation. (B) Southern blot analysis of strains obtained in the *cou*N3 gene inactivation. Genomic DNA was restricted with *Sph*I/*Cla*I from wild-type (lane 1), single crossover mutants (lanes 2 und 3), reversion to wild-type (lane 4), and *cou*N3⁻ mutants XH12, XH13 and XH14 (lanes 5, 6 and 7, respectively). (C) Schematic presentation of the *cou*N4 gene inactivation. (D) Southern blot analysis of strains obtained in the *cou*N4 gene inactivation. Genomic DNA was restricted with *Pst*I from wild-type (lane 1), and *cou*N4⁻ mutants ZW20 and ZW21 (lanes 2 and 3, respectively)

1.2 Secondary metabolite production in the wild-type and *cou*N3⁻ mutant

To investigate the secondary metabolites, *S. rishiriensis* (wild-type) and *cou*N3⁻ mutants were cultured in the production medium for coumermycin A_1 and culture extracts were analyzed by HPLC (Fig. 6) The wild-type strain showed coumermycin A_1 as the dominant product (Fig. 6B). This compound was identified by HPLC in comparison to an authentic reference substance, as well as by LC/MS analysis which confirmed the expected molecular mass ($[M-H]^-$=1108).

In the *cou*N3$^-$ mutant, coumermycin A_1 production was completely abolished (Fig. 6C). This mutant accumulated instead another product, which was identified as coumermycin D by LC/MS analysis ($[M\text{-}H]^-$=894) (Fig. 6C). As described by Berger and Batcho (1978), the UV spectrum of this compound did not show the maximum at 275 nm, which is observed in coumermycin A_1 and all other coumermycins except coumermycin D.

The finding that the *cou*N3$^-$ mutant accumulated coumermycin D instead of coumermycin A_1 (Fig. 6) had two implications: first, it confirmed experimentally that *cou*N3 is involved in the biosynthesis of the two terminal pyrrole moieties of coumermycin A_1 as had been speculated (Fig. 4). But secondly, it proved that the central pyrrole moiety was still formed in the absence of an intact *cou*N3 protein, indicating a different biosynthesis of this moiety.

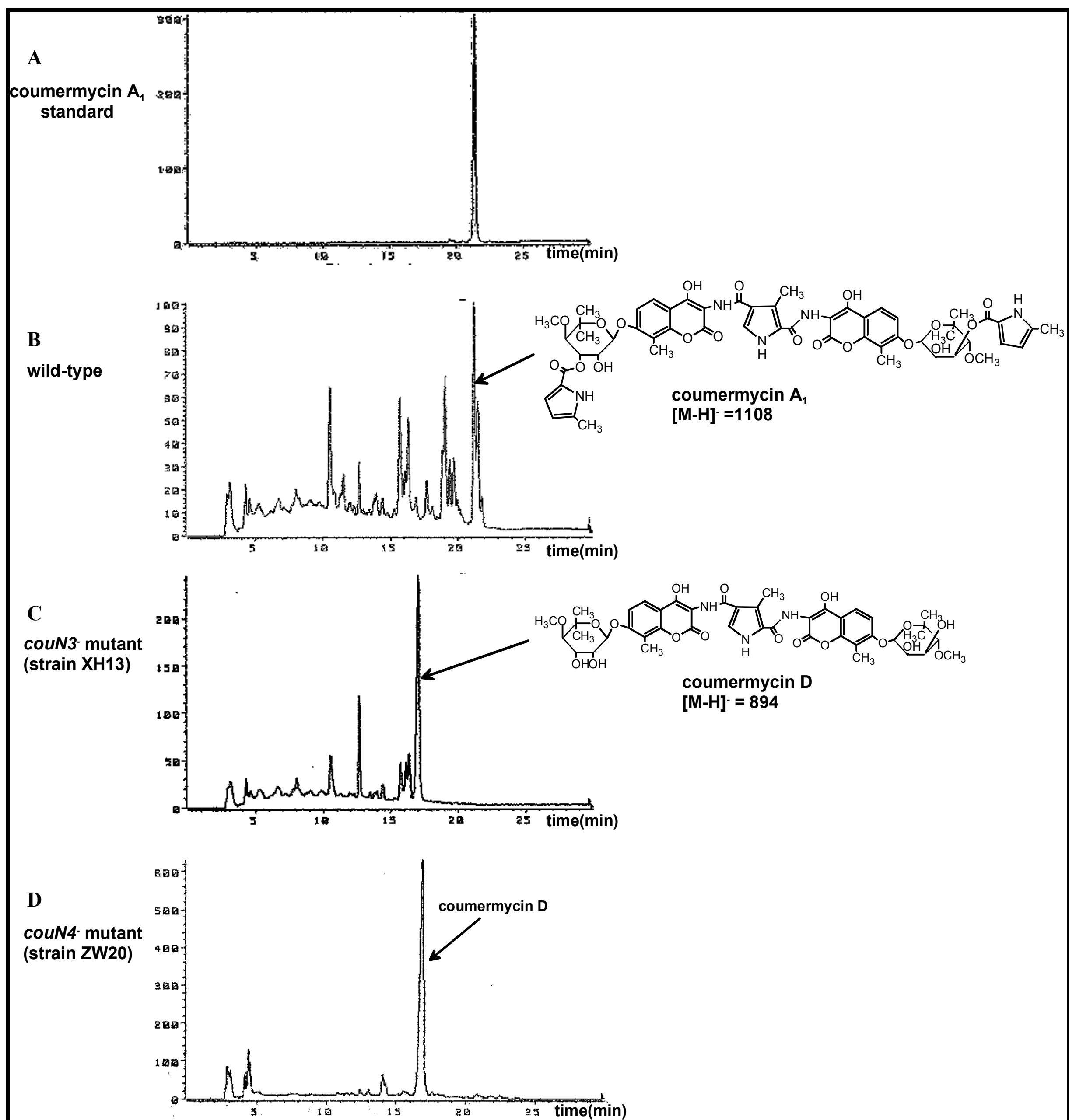


Fig. 6 HPLC analysis of the secondary metabolites in wild-type and mutants of *S. rishiriensis*

1.3 Inactivation of *cou*N4 (This experiment was carried out by Z.-X. Wang.)

The hypothesis that the biosynthetic pathways of the central and the terminal pyrrole moieties of coumermycin A_1 are different was confirmed by a second gene inactivation experiment. *cou*N4, encoding a putative L-prolyl-AMP ligase, was targeted in this experiment, since its gene product might catalyse the first reaction step in the biosynthesis of pyrroles from L-proline (Fig. 4).

The suicide vector pN4, was constructed for this purpose (Fig. 5C). In this vector, the structural gene of *couN4* was disrupted by insertion of a neomycin resistance gene, i.e. *aphII*. pN4 was introduced into protoplasts of *S. rishiriensis* (wild-type). Mutants resulting from integration of the construct were selected with neomycin and investigated further. Two mutants (strains ZW20 und ZW21) were obtained, which showed the desired genotype resulting from a double cross-over event, i.e. a replacement of the intact *couN4* gene by the disrupted gene copy (Fig. 5D).

The absence of the intact *couN4* gene and the presence of the disrupted gene were also confirmed by PCR, using two different pairs of primers.

1.4 Secondary metabolite production in the *cou*N4$^-$ mutant

In HPLC analysis, culture extracts of the *cou*N4$^-$ mutants showed complete abolishment of coumermycin A_1 production (Fig. 6D). Similar to the *cou*N3$^-$ mutants, *cou*N4$^-$ mutants accumulated coumermycin D as the dominant product (Fig. 6D), which was identified by HPLC and by UV spectroscopy. Therefore, as speculated, also *cou*N4 was involved in the formation of the two terminal pyrrole rings of coumermycin A_1 (Fig. 4). *cou*N4 was, however, not required for the biosynthesis of the central pyrrole moiety of coumermycin A_1 and coumermycin D.

Surprisingly, the amount of coumermycin D produced by both *cou*N3$^-$ and *cou*N4$^-$ mutants (varied from 12-17 µmol/l) was about five time higher than that of coumermycin A_1 produced by the wild-type (ca 2.7 µmol/l).

2 Identification of *clo*N2, an acyltransferase gene responsible for the attachment of the pyrrole moiety to the deoxysugar of clorobiocin

Upstream of the genes *clo*N3*, clo*N4 and *clo*N5, which are expected to encode enzymes for the conversion of L-proline to pyrrole-2-carboxyl-*S*-PCP, the gene *clo*N2 is located in the clorobiocin biosynthetic cluster. A homologous gene, i.e. *cou*N2, is located at the same relative position in the coumermycin A_1 biosynthetic cluster (Pojer *et al.,* 2002).

Database searches revealed that *clo*N2 show sequence similarity to genes in the biosynthetic gene clusters of certain antibiotics and antitumor agents, e.g. *cal*O4 of the calicheamicin biosynthetic gene cluster in *Micromonospora echinospora* ssp. *calichensis* (47% identity on the amino acid level) (Ahlert *et al.,* 2002), *avi*N of the avilamycin A cluster in *Streptomyces viridochoromogenes* (45% identity) (Weitnauer *et al.,* 2001) and *evr*I of the evernimicin cluster in *Micromonospora carbonacea* var*. africana* ATCC 39149 (44% identity) (Hosted *et al.,* 2001). *clo*N2 also shows 38% identity (aa level) to *dpsC* of the daunorubicin/doxorubicin cluster in *Streptomyces peuceticus* (Grimm *et al.,* 1994). DpsC was shown to catalyse the transfer of propionate to an acyl carrier protein in daunorubicin biosynthesis (Bao *et al.,* 1999a; Bao *et al.,* 1999b). CalO4, AviN and EvrI were proposed to be involved in orsellenic acid biosynthesis and to control the nature of the starter unit of the biosynthesis (Ahlert *et al.,* 2002; Weitnauer *et al.,* 2001), but no experimental evidence has been published for the function of these enzymes yet.

By comparison of the structures of calicheamicin, avilamycin A and evernimicin with those of clorobiocin and coumermycin A_1, it is notable that all these compounds contains one or two aromatic acyl moieties attached to a deoxysugar via an ester bond. In the cases of calicheamicin, avilamycin A and evernimicin, the acyl components are orsellenic acid derivatives and in cases of clorobiocin and coumermycin A_1, it is 5-methylpyrrole-2-carboxylic acid. We speculated that CloN2/CouN2, and possibly CalO4, AviN and EvrI, may be involved in the transfer of the acyl component to the sugar moiety. To provide evidence for this hypothesis, the inactivation of *clo*N2 was carried out in the clorobiocin producer, *S. roseochromogenes* var. *oscitans* DS 12.976.

2.1 Inactivation of *clo*N2 in *S. roseochromogenes* (WT)

For inactivation of *clo*N2 by in-frame deletion, plasmid pN2 was constructed by cloning two PCR fragments into the vector pBSKT, a pBluescript derivative containing a thiostrepton resistance marker. Thereby, 978 bp were deleted from the coding sequence of *clo*N2, shortening the predicted gene product from 355 to 29 amino acids (Fig. 7A).

pN2 was introduced into the clorobiocin producer *S. roseochromogenes* (wild-type) by protoplast transformation. After appropriate selection procedures, three antibiotic-sensitive strains resulting from double cross-over events were obtained. Southern blot examination (Fig. 7B) revealed that one of them (strain XHB12), showing the expected band of 2.35 kb, represented the desired genotype with the inactivated *clo*N2 gene, while the other two (strains XHB11 und XHB13) gave the same band of 3.33 kb as wild-type, indicating the reversion to the wild-type.

The absence of the intact *clo*N2 gene and the presence of the disrupted gene were also confirmed by PCR, using primers cloN2-1/*Xba*I and cloN2-2/*Hind*III (Table 16).

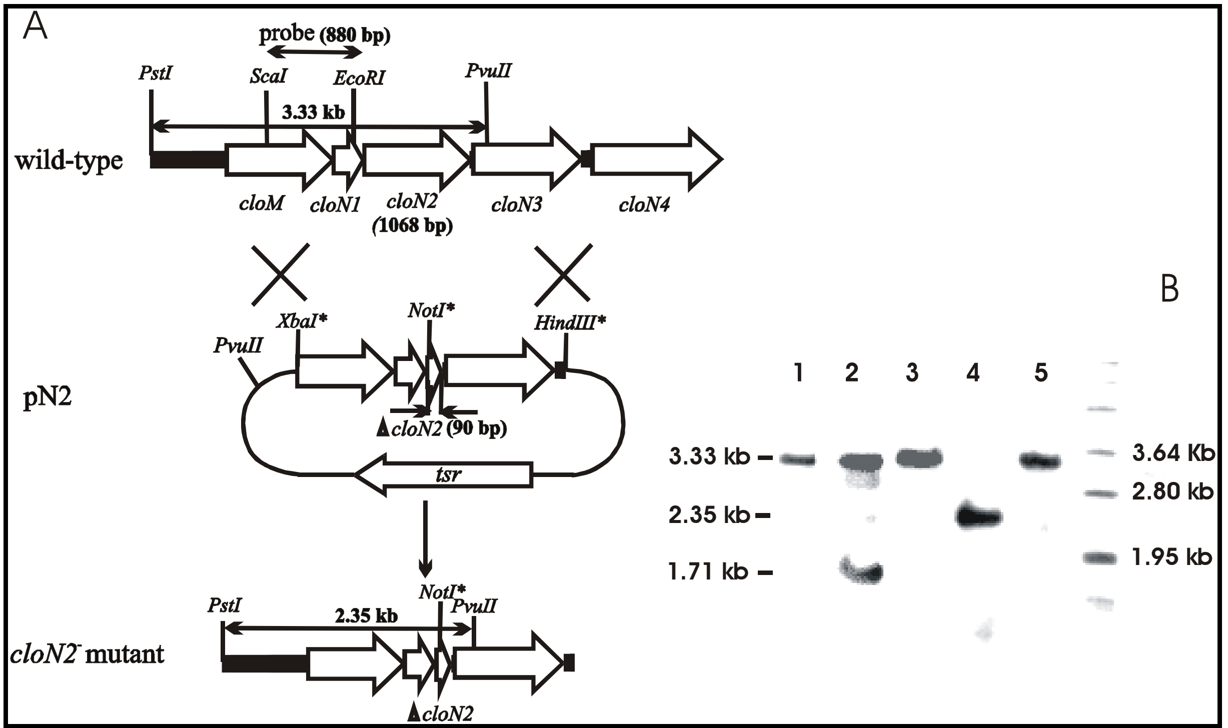


Fig. 7 Inactivation of *clo*N2 in *S. roseochromogenes* var. *oscitans* DS 12.976. (A) Schematic presentation of the *clo*N2 gene inactivation. (B) Southern blot analysis of strains obtained in

the *clo*N2 gene inactivation experiment. Genomic DNA was restricted with *Pst*I/*Pvu*II from wild-type (lane 1), single crossover mutant XHB1 (lane 2), reversion to wild-type XHB11 and XHB13 (lane 3 and 5), and *clo*N2$^-$ mutant XHB12 (lane 4). The indicated *Sca*I-*EcoR*I fragment of 880 bp was used as a probe.

2.2 Analysis of secondary metabolites and identification of novclobiocin 104 and 105

For analysis of secondary metabolites, both wild-type and the *clo*N2$^-$ mutant XHB12 were cultured in production medium (s. 6.2.2) (Mancy *et al.,* 1974; Pojer *et al.,* 2002). Ethyl acetate extracts of the cultures were analysed by HPLC with UV detection at 340 nm. The wild-type strain showed clorobiocin with a retention time of 20.9 min as the dominant product (Fig. 8A), which was identified by co-chromatography with an authentic reference substance as well as by LC/MS (MW = 696).

By contrast, the *clo*N2$^-$ mutant accumulated, instead of clorobiocin, two new products with retention times of 15.5 min and 18.2 min, respectively (Fig. 8B), which were designated novclobiocin 105 and 104, respectively. To elucidate their structures, these two products were isolated on a preparative scale and analysed by MS, ^{1}H-NMR and ^{13}C-NMR. The original spectra are shown in Appendix II and the MS and NMR data are summarized in Appendix I.

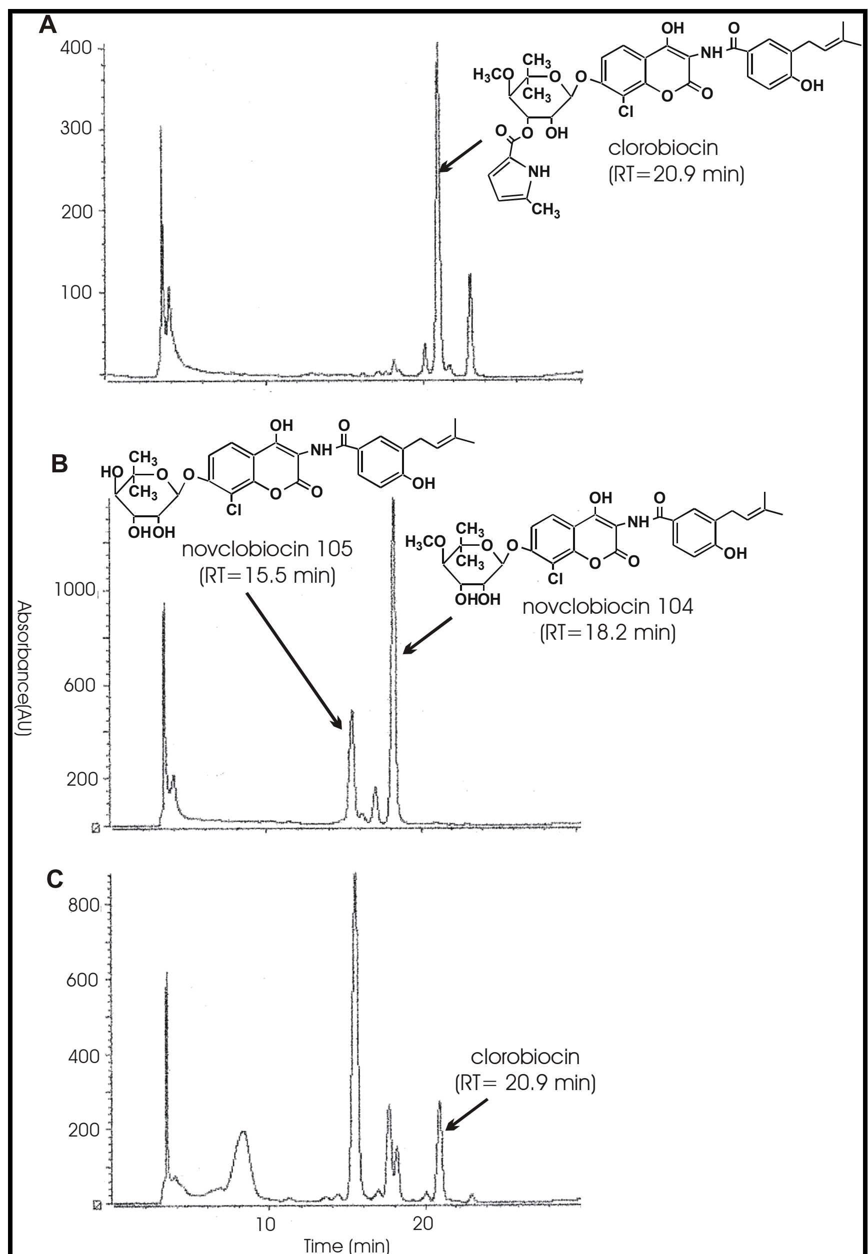


Fig. 8 HPLC analysis of the secondary metabolites in S. roseochromogenes strains. (A) DS 12.976 (wild-type); (B) XHB12 (*clo*N2$^-$ mutant); (C) XHBC12 (*clo*N2$^-$ mutant harbouring pN2C)

The mass spectrum of novclobiocin 104 showed a negative ion at *m/z* 588 $[M-H]^-$, 107 mass units less than that of clorobiocin, corresponding to the lack of the methylpyrrole-carboxyl moiety. The ^{1}H-NMR spectrum of novclobiocin 104 is very similar to that of clorobiocin, with the exception that the signals for protons at H-3´´´ (6.90 ppm), H-4´´´ (5.94 ppm) and H-6´´´ (2.29 ppm) of the pyrrole unit in clorobiocin were absent (s. Appendixs). Furthermore, the absence of the signals for carbons C-7´´´, C-2´´´, C-3´´´, C-4´´´, C-5´´´, C-6´´´ in ^{13}C-NMR spectrum of novclobiocin 104 also confirmed the lack of the pyrrole unit in its structure (s. Appendixes). This proved

novclobiocin 104 is a clorobiocin derivative lacking the 5-methylpyrrole-2-carboxyl moiety at 3´´ of the deoxysugar (Fig. 8B).

The mass spectrum of novclobiocin 105 showed a negative ion at *m/z* 574 $[M-H]^-$, 14 mass units less than that of novclobiocin 104, indicating the lack of a methyl group. The signals at 3.59 ppm in the ^{1}H-NMR spectrum and at 62.3 ppm in the ^{13}C-NMR spectrum of novclobiocin 104, corresponding to the methoxy group at 4´´ of the deoxysugar, were absent in the corresponding spectra of novclobiocin 105. The other signals in the spectra of both compounds corresponded to each other very well (s. Appendixes). Obviously, both the 3´´ and 4´´of the deoxysugar in novclobiocin 105 were not substituted (Fig. 8B).

The amount of novclobiocin 104 and 105 produced by the *clo*N2$^-$ mutant was, in total, about 100-180 mg/l (corresponding to 172-309 µmol/l), i.e. ten times higher than the clorobiocin content in the wild-type, which varied from 15 – 20 mg/l (22-29 µmol/l) under the culture condition in this thesis (s. 6.2.2). The similar phenomenon was also observed with the production of coumermycin D by the *cou*N3$^-$ and *cou*N4$^-$ mutants (s. "Results 1.4"). Till now, no obvious explanation could be given for this unexpected result.

2.3 Identification of pyrrole-2-carboxylic acid in the culture extract of *clo*N2$^-$ mutant by LC/MS and ^{1}H-NMR

Culture extracts of both the wild-type and the *clo*N2$^-$ mutant XHB12 of *S. roseochromogenes* were further analysed for free pyrrole-2-carboxylic acid by LC/MS using selected reaction monitoring (SRM). In SRM, authentic pyrrole-2-carboxylic acid gave the reaction of *m/z* 110 →*m/z* 66, corresponding to the decarboxylation of $[M-H]^-$. This reaction was observed in the wild-type as well as in the *clo*N2$^-$ mutant XHB12 (Fig. 9), demonstrating the presence of pyrrole-2-carboxylic acid in both cultures. The biosynthesis of the pyrrole moiety in the *clo*N2$^-$ mutant was therefore not blocked.

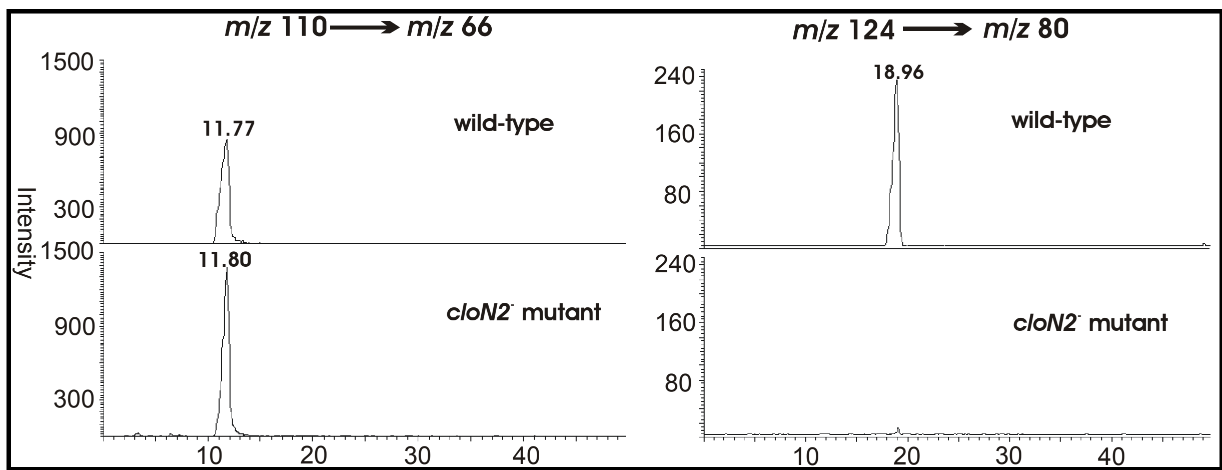


Fig. 9 Analysis of pyrrole-2-carboxylic acids in extracts of bacterial cultures by SRM. The reactions *m/z* 110→ *m/z* 66 and *m/z* 124→ *m/z* 80 were used to detect pyrrole-2-carboxylic acid and 5-methylpyrrole-2-carboxylic acid, respectively.

The presence of pyrrole-2-carboxylic acid in the *clo*N2$^-$ mutant was further confirmed by ^{1}H-NMR analysis. The pyrrole-2-carboxylic acid (retention time 7.7 min) was isolated from the culture extract of the *clo*N2$^-$ mutant on a preparative scale and analysed by ^{1}H-NMR. The spectrum showed three doublet-doublet signals at 6.93, 6.84 and 6.17 ppm, respectively, which were identical to those of an authentic substance of pyrrole-2-carboxylic acid and consistent with the literature data (Shimokawa *et al.,* 1970). The amount of pyrrole-2-carboxylic acid accumulated in the cloN2$^-$ mutant was about 1.2 mg/l (corresponding to 11 µmol/l). Comparable amounts of this substance were found in the wild-type.

The ethyl acetate extracts of the cultures were also used to investigate the presence of 5-methylpyrrole-2-carboxylic acid (Mr = 125) by LC/MS with SRM. Using SRM, the reaction of *m/z* 124 →*m/z* 80, representing the decarboxylation of [M-H]$^-$, was observed in wild-type, but not in the *clo*N2$^-$ mutant (Fig. 9), which indicated the complete absence of 5-methylpyrrole-2-carboxylic acid in the *clo*N2$^-$ mutant.

2.4 Complementation of *clo*N2$^-$ mutant with pN2C

To complement the *clo*N2$^-$ mutant XHB12, a vector pN2C was constructed, containing the entire sequence of *clo*N2 in pUWL201 under the control of the constitutive *erm*E*p promoter. After transformation of vector pN2C into the *clo*N2$^-$ mutant, thiopstrepton-resistant clones were selected. To confirm the presence of the intact vector pN2C, plasmid was reisolated and controlled by restriction digest and

agarose gel electrophoresis. HPLC analysis showed that the *clo*N2⁻ mutant harboring plasmid pN2C (strain XHBC12) produced clorobiocin in an amount similar to the wild-type (Fig. 8C). At the same time, this strain still produced considerable amounts of novclobiocin 104 and 105. No clorobiocin production was detected in transformants with empty vector pUWL201.

3 Production of carbamoylated aminocoumarin derivatives

Carbamoylation of the 3´´-hydroxy group of the deoxysugar is considered to be one of the last steps of the biosynthesis of novobiocin (Fig. 1). In the novobiocin biosynthetic gene cluster, a putative carbamoyl transferase gene *nov*N was identified (Steffensky *et al.,* 2000b). Since the aminocoumarin core and the deoxysugar moieties of coumermycin, clorobiocin and novobiocin are identical, it was attempted to produce new carbamoylated aminocoumarin derivatives using NovN.

3.1 3´´-Unsubstituted aminocoumarin derivatives

The inactivation of gene *cou*N3, *cou*N4 and *clo*N2 not only provided experimental evidence for the functions of these genes in the biosynthesis of coumermycin A_1 and clorobiocin, but also led to the production of some 3´´-unsubstituted aminocoumarins, namely coumermycin D (Fig. 10), novclobiocin 104 (Fig. 10) and novclobiocin 105 (Fig. 10). These compounds represent interesting substrates for the carbamoyltransferase NovN, setting the stage for the production of new carbamoylated aminocoumarin antibiotics.

Two further 3´´-unsubstituted aminocoumarin derivatives, novclobiocin 107 and novclobiocin 108 (Fig. 10), were obtained by the inactivation of gene *cloN2* in *clo*-hal⁻ mutant (Eustáquio *et al.,* 2003a), as described as follows. Another 3´´-unsubstituted compound, novclobiocin 283, was derived from a mutasynthetic experiment (Galm *et al.,* 2004a). Novclobiocin 283 contained a bromine atom instead of the dimethylallyl moiety at position 3 of the 4-hydroxybenzoyl moiety, but otherwise had the same structure as novclobiocin 104 (Fig. 10).

Fig. 10 Structures of the 3´´-unsubstituted aminocoumarin derivatives

3.1.1 Production of novclobiocins 107 and 108 by inactivation of *clo*N2 in *clo*-hal$^-$ mutant

The halogenase gene *clo*-hal in the clorobiocin producer *S. roseochromogenes*, was inactivated by PCR-targeting system. The resulting *clo*-hal$^-$ mutant produced novclobiocin 101, lacking the chlorine atom at position 8´ of aminocoumarin (Fig. 12A) (Eustáquio *et al.,* 2003a).

The *clo*-hal$^-$ mutant was transformed with plasmid pN2 (Fig. 11A), which contained an in-frame deletion in the coding region of *clo*N2 (Xu *et al.,* 2003). Thiostrepton-resistant colonies were selected and analysed by Southern blotting. While the *clo*-hal$^-$ mutant gave a band of 3.33 kb, strain XHC26 showed two bands of 2.35 kb and 2.69 kb, representing the desired genotype of single crossover events (Fig. 11B).

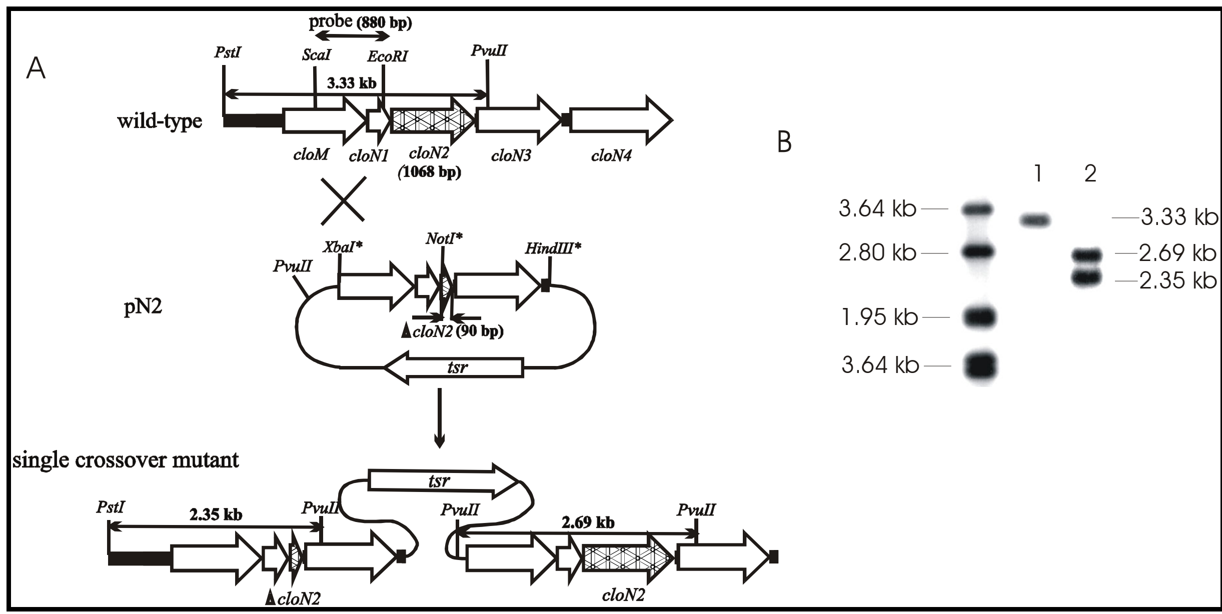


Fig. 11 Inactivation of *cloN2* in the *clo*-hal⁻ mutant. (A) Schematic representation of the gene inactivation (B) Southern blot analysis. Genomic DNA was restricted with *Pst*I/*Pvu*II. *clo*-hal⁻ mutant (lane 1), *cloN2*⁻/*clo*-hal⁻ double mutant XHC26 (lane 2). The indicated *Sca*I-*EcoR*I frament of 880 bp was used as a probe.

The secondary metabolites of *clo*N2⁻/*clo*-hal⁻ mutant were investigated. Compared to the wild-type and the *clo*-hal⁻ mutant, two new peaks were found in the chromatogram of the *clo*N2⁻/*clo*-hal⁻ double mutant (Fig. 12B).

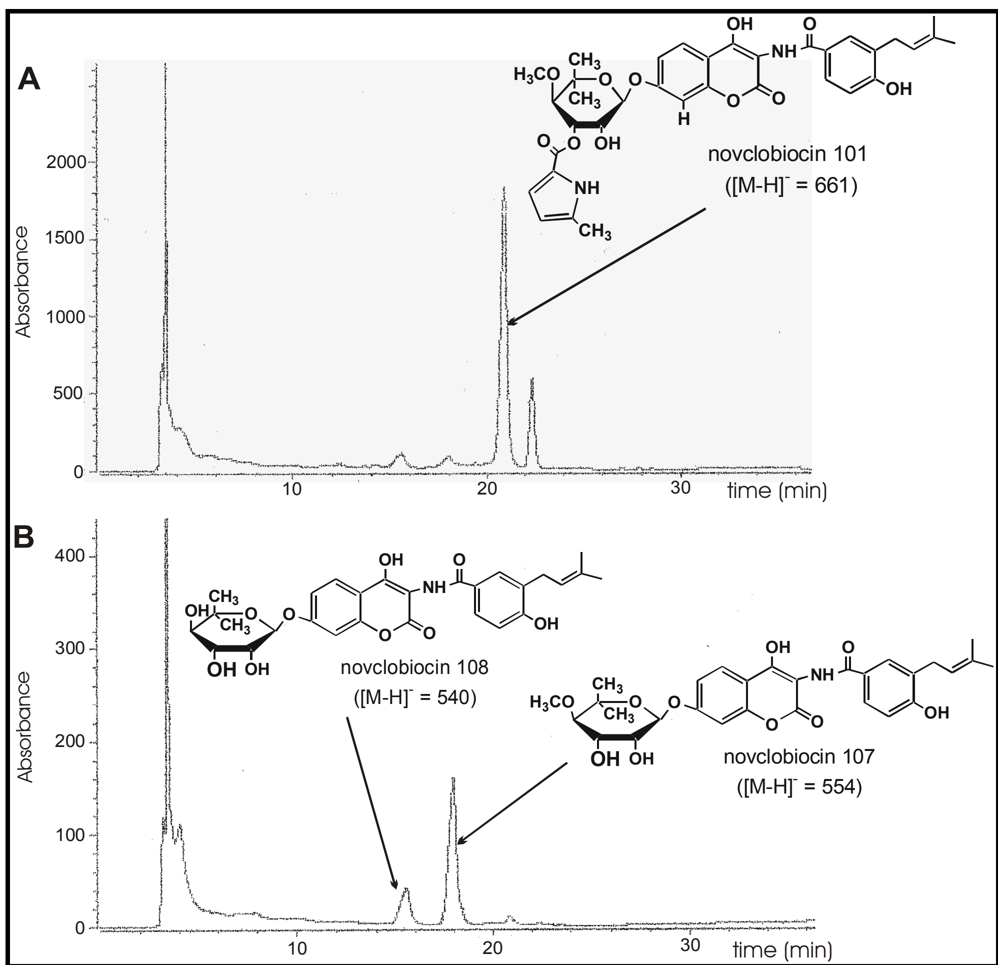


Fig. 12 HPLC analysis of the secondary metabolites of *clo*-hal⁻ and *clo*N2⁻/*clo*-hal⁻ mutants of *S. roseochromogenes*. (A) Strain AE-h10 (*clo*-hal⁻ mutant); (B) Strain XHC26 (*clo*N2⁻/*clo*-hal⁻ mutant)

The two new substances, designated as novclobiocin 107 and novclobiocin 108, were isolated on a preparative scale by column chromatography with Sephadex® LH20, followed by preparative HPLC. Their structures were elucidated by MS and ^{1}H-NMR (s. Appendixes for data and spectra). Negative-ion FAB mass spectrum of novclobiocin 107 showed a molecular ion $[M-H]^-$ at *m*/z 554, consistent with the loss of a chlorine atom in comparison to novclobiocin 104 ($[M-H]^-$ at *m*/z 588). Novclobiocin 104 showed the typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl (mass [intensity in %]: 588 [100], 589 [28], 590 [24], 591 [10]), whereas the mass spectrum of novclobiocin 107 did not show this pattern, indicating the absence of chlorine (mass [intensity in %]: 554 [100], 555 [27], 556 [9]). In the ^{1}H-NMR spectrum of this new compound, the signal at 7.29 ppm corresponding to the H-6´ of novclobiocin 104 has disappeared. Instead, a signal at 7.04 ppm for two protons was observed as a broad singlet, which could be assigned to H-6´ and H-8´, as

reported previously (Eustáquio *et al.,* 2003a; Sasaki *et al.,* 2001). Therefore, novclobiocin 107 lacks the chlorine atom at position 8´ of aminocoumarin ring as well as the pyrrole moiety at 3´´-OH of deoxysugar (Fig. 12). The mass spectrum of novclobiocin 108 showed a negative ion at *m*/z 540 $[M-H]^-$, 14 mass unit less than that of novclobiocin 107, indicating the lack of a methyl group. Similar to novclobiocin 107, no chlorine isotopic pattern was observed in the mass spectrum. The signal at 3.58 ppm in the ^{1}H-NMR spectrum of novclobiocin 107, corresponding to the methoxy group at 4´´ of deoxysugar, were absent in the corresponding spectrum of novclobiocin 108. The other signals in the spectra of both compounds corresponded to each other very well. In comparison to novclobiocin 107, the methyl group at 4´´-OH of the deoxysugar was also absent in novclobiocin 108 (Fig. 12).

The single crossover mutant created in this thesis contained both an inactivated copy and an intact copy of *clo*N2 (Fig. 11). Integration of the vector sequence apparently disrupted the transcription unit containing *clo*N2 and further genes involved in the biosynthesis of the pyrrole moiety (Xu *et al.,* 2002), as indicated by the lack of this structural moiety in the metabolites accumulated by the mutant. Since a single crossover was sufficient to generate the desired compounds, no further attempts were made to obtain double crossover mutants.

The new compounds, novclobiocin 107 and 108, were produced in amounts of 26 mg/l and 6 mg/l (corresponding to 47 µmol/l and 11µmol/l), respectively.

3.2 Production of carbamoylated aminocoumarin derivatives *in vivo*

Firstly, it was attempted to try whether expression of NovN in the *cou*N3$^-$ mutant would result in the formation of carbamoylated coumermycin D derivatives.

3.2.1 Overexpression of NovN in the *cou*N3$^-$ mutant

For this purpose, the *nov*N gene was cloned into the expression vector pEM4, which contains a pIJ101 origin of replication, a thiostrepton resistance marker and a *ermE** promotor for foreign gene expression (Quiros *et al.,* 1998). The resulting construct, pXHN (Fig. 13), was introduced into the *cou*N3$^-$ mutant of *S. rishiriensis* by protoplast transformation. Thiostrepton-resistant colonies were selected and the presence of the intact plasmid was confirmed by isolation and restriction analysis (Fig. 13).

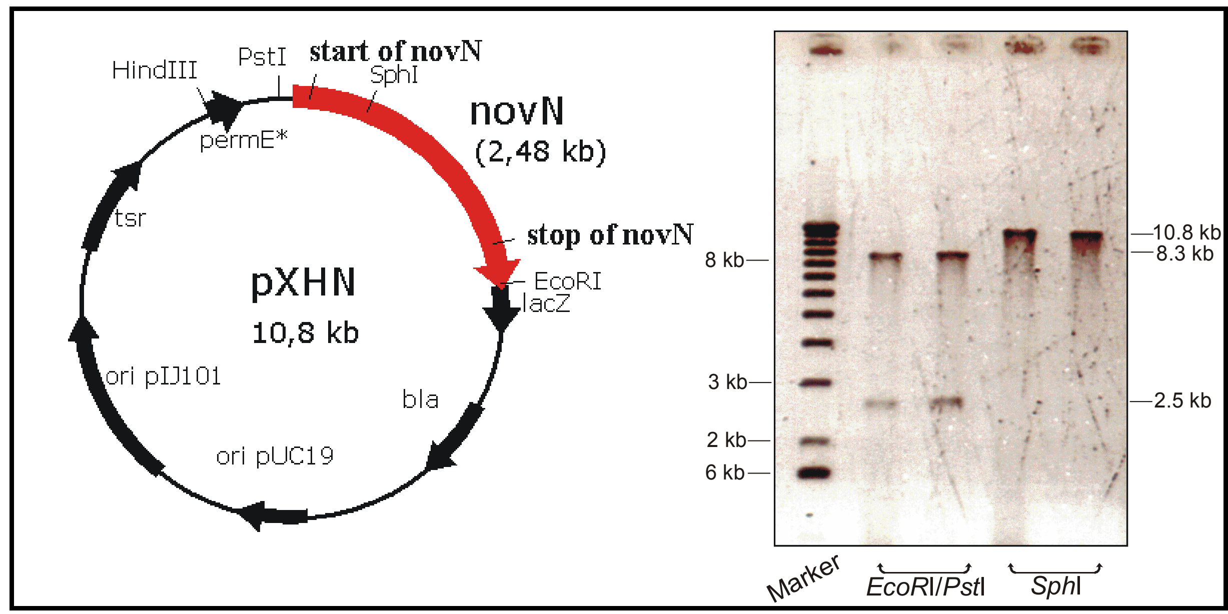


Fig. 13 Map of the vector pXHN (left) and restriction analysis of the intact plasmid pXHN from two different transformands (right). The expected bands after restriction with *EcoR*I/*Pst*I and *Sph*I are at 8.3 kb/2.5 kb and 10.8 kb, respectively.

3.2.2 Secondary metabolite production of the *cou*N3$^-$ mutant expressing NovN

Culture extracts of the *cou*N3$^-$ mutant with and without the *nov*N expression vector pXHN were analysed by LC/MS, in comparison with the wild-type. This allowed selective detection of coumermycin A_1 (MW 1109), coumermycin D (MW 895) and bis-carbamoylated coumermycin D (MW 981). The collision–induced dissociation (CID) mass spectra and the selected reaction monitoring (SRM) during a HPLC run were recorded after both positive and negative electrospray ionization (ESI). MS data are listed in Appendix I. As shown in Fig. 14, this analysis confirmed the identity of coumermycin A_1 and coumermycin D as the main products of the wild-type and the *cou*N3$^-$ mutants, respectively. Bis-carbamoylated coumermycin D was detected in the *cou*N3$^-$ mutant expressing NovN, but not in the corresponding strain without *nov*N, and not in the wild-type. The ESI-CID spectra of the $[M+H]^+$ and $[M-H]^-$ ions, respectively, of coumermycin A_1 display key fragments characterizing the several subunits (Fig. 14A). Thus, the complementary ions at *m/z* 487 (negative ion ESI) and 622 (positive ion ESI) are formed by scission of the amide bond. While the positive ion at *m/z* 108 represents the methylpyrrole moiety, the base peak ion at *m/z* 282 comprises the sugar unit with the attached methylpyrrole subunit. The negative ion at *m/z* 206 represents a key ion of the methylated aminocoumarin moiety. For detecting

coumermycin A_1 the fragment ions at *m/z* 282 and 622 were used for SRM measurements in the positive ion mode.

In coumermycin D ($[M+H]^+$ at *m/z* 896) corresponding reactions from the $[M+H]^+$ ion leading to ions at *m/z* 175 and 515 could be detected in the SRM mode (Fig. 14B).

The bis-carbamoylated coumermycin D ($[M+H]^+$ at *m/z* 982) displays in the SRM mode the correponding mass shifts to *m/z* 218 and 558 (Fig. 14C), indicating the same fragmentation pattern as coumermycin A_1 and coumermycin D, respectively.

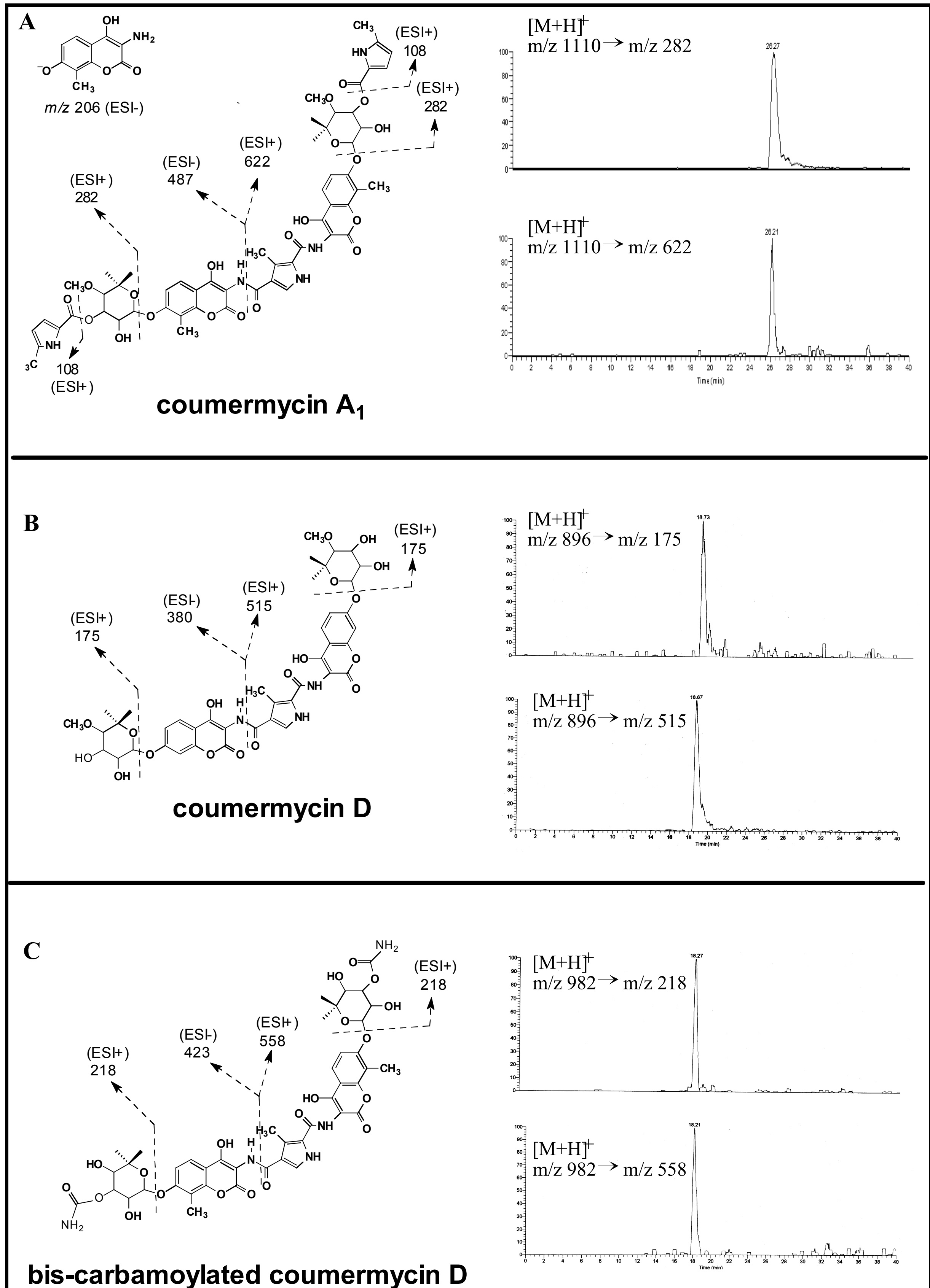


Fig. 14 LC/MS and selected reaction monitoring (SRM) analysis of the secondary metabolites in *S. rishiriensis* wild-type and mutants

The retention times of coumermycin D and bis-carbamoylated coumermycin D were very similar, so that a chromatographic separation was not achieved. Coumermycin D was the dominant product in the *cou*N3⁻ mutant expressing NovN, and the carbamoylated compound was present in smaller quantities. Addition of NH_4NO_3 (12 mM) or carbamoyl phosphate (4 mM) to the culture medium did not increase the proportion of carbamoylated coumermycin D.

3.3 Production of carbamoylated aminocoumarin derivatives *in vitro*

As described above, the heterologous expression of NovN in *cou*N3⁻ mutant of the coumermycin producer, *S. rishiriensis*, resulted in the formation of a carbamoylated coumermycin derivative (Xu *et al.,* 2002). However, the yield was low (Xu *et al.,* 2002), and no carbamoylated clorobiocin derivatives could be detected after expression of NovN in a *clo*N2⁻ mutant of the clorobiocin producer, *S. roseochromogenes* (data not shown). It appears like that insufficient availability of the substrate carbamoyl phosphate, rather than the specificity of the carbamoyltransferase, has limited the formation of carbamoylatd products in these experiments. Therefore, expression of NovN *in vivo* appears not to be a promising method to obtain new carbamoylated aminocoumarin antibiotics in useful quantities.

Subsequently, it was attempted to overexpress the carbamoyltransferase NovN as histidine fusion protein by a convenient method using *S. lividans* as host and synthesize new carbamoylated clorobiocin derivatives using carbamoyltransferase assay.

3.3.1 Overexpression of NovN as histidine fusion protein in *S. lividans*

NovN has been successfully overexpressed as N-terminal 8×His-tagged protein in *E. coli* (Freel Meyers *et al.,* 2004). However, in our hands expression in *E. coli* gave only low yields of active enzyme. It has been observed repeatedly that proteins from *Streptomyces* strains which could not be well expressed in *E. coli* could be effectively expressed in *Streptomyces lividans* (Heinzelmann *et al.,* 2001; Pelletier *et al.,* 1994; Pfeifer *et al.,* 2001). Therefore, NovN was expressed as an N-terminal 6×His-tagged protein in *S. lividans* T7, a strain possessing a thiostrepton-inducible T7 RNA polymerase gene.

For this purpose, the vector pXHNEG (Fig. 15) containing the whole sequence of *nov*N from the novobiocin gene cluster behind the sequence coding for six histidine residues and an origin for replication in *Streptomyces* from the vector pGM9 (s. Table 14) were constructed. pXHNEG was transformed into the protoplasts of *S. lividans* T7 and the kanamycin-resistent mutants were selected. NovN was achieved by overexpression of mutants in YEME medium using thiostrepton as inductor (s. 7.2.5).

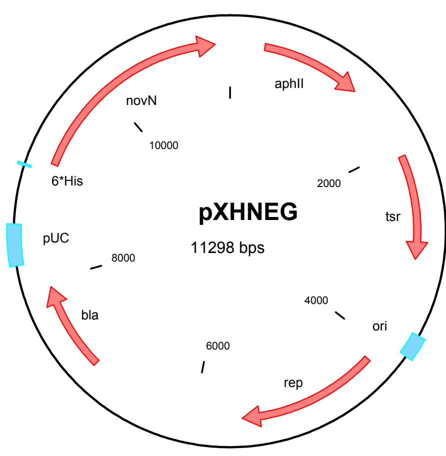


Fig. 15 Map of expression vector pXHNEG

The yield of NovN was quite high (6.7 mg/g cells, fresh weight; s. Fig. 16, lane 2), but much of it was insoluble. The amount of soluble protein did not increase significantly when the growth temperature was reduced to 20°C. Nevertheless, sufficient amounts of the 6×His-tagged NovN could be readily purified from the soluble fraction by metal affinity chromatography. SDS-PAGE analysis showed the expected band of 78 kDa (calculated mass 78.5 kDa) in the eluate (Fig. 16). An additional band appeared at 76 kDa, which had also been observed upon expression in *E. coli* (Freel Meyers *et al.,* 2004) and may represent a degradation product. The protein yield of purified NovN was about 16 µg/g cells (fresh weight), determined by SDS-PAGE.

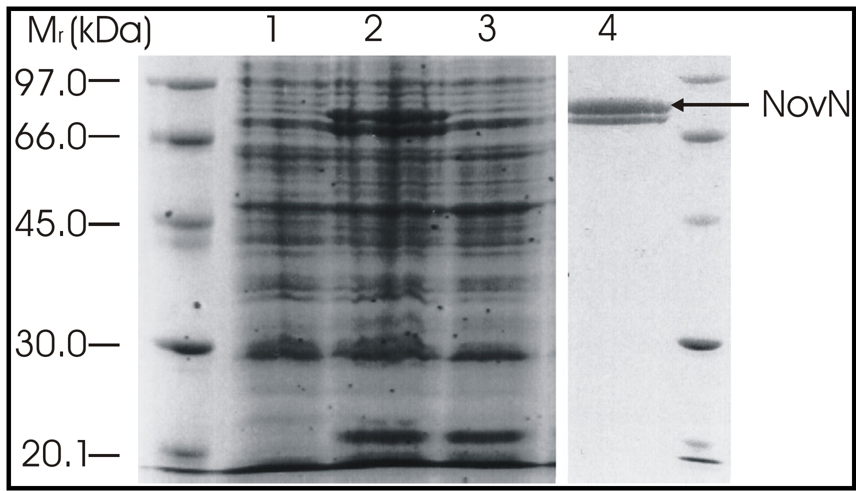


Fig. 16 Analysis of proteins expressed in *S. lividans* T7 by SDS-PAGE (10%). Lane 1, total protein before induction; lane 2, total protein after induction with 25 µg/ml thiostrepton for 24h; lane 3, soluble protein after induction; lane 4, eluate from nickel affinity chromatography.

3.3.2 Carbamoyltransferase assay

The carbamoyltransferase assay was carried out as described in “Materials and methods 7.2.6.”. The catalytic activity of NovN as a carbamoyltransferase was confirmed by HPLC following conversion of descarbamoyl novobiocin to novobiocin (Fig. 17).

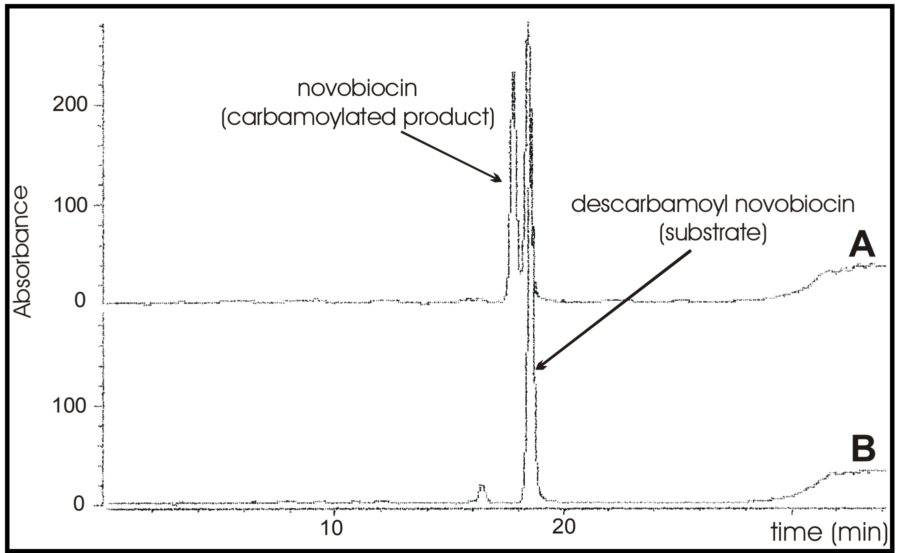


Fig. 17 HPLC analysis of the carbamoyltransferase assay with native substrate descarbamoyl novobiocin. The assays were incubated overnight. (A) Assay with purified NovN; (B) Negative control with heat-denatured enzyme.

3.3.3 Characterization of NovN

3.3.3.1 Molecular weight of native protein

The native molecular weight was determined by gel filtration. The elution volume V_e of NovN was 182 ml, corresponding to 79 kDa. The calculated molecular mass of NovN was 78.5 kDa. Therefore, the protein is active as a monomer.

3.3.3.2 Cofactor, linear dependence over time and protein amount

The NovN reaction was strictly dependent upon the presence of ATP and divalent cations such as Mg^{2+} or Mn^{2+}. The absence of ATP in assay mixture resulted in the complete verlust of the carbamoyltransferase activity. Only 1% activity could be detected, when ATP but no Mg^{2+}/Mn^{2+} cations were included. The optimal concentrations of both Mg^{2+} and ATP were 2 mM. The product formation in the carbamoyltransferase assay showed a linear dependence over the protein amount up to 0.5 µM of purified protein and over incubation time up to 90 min.

3.3.3.3 Determination of kinetic parameters for descarbamoyl novobiocin

The K_m value for the native substrate, descarbamoyl novobiocin, was determined as 2.4 µM and turnover rate K_{cat} as 2.4 min^{-1} (Fig. 18), similar to the data obtained for NovN expressed in *E. coli* (Freel Meyers *et al.,* 2004).

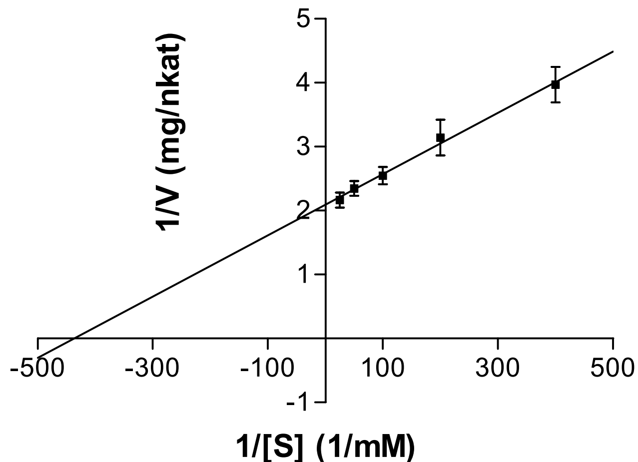


Fig. 18 Lineweaver-Burk plot for calculation of kinetic parameters for descarbamoyl novobiocin (native substrate)

3.3.4 Chemoenzymatic synthesis of new aminocoumarin antibiotics

The five 3´´-unsubstituted clorobiocin derivatives, i.e. novclobiocins 104, 105, 107, 108 as well as novclobiocin 283, were used as substrates for carbamoylation. HPLC analysis revealed that all of them were readily accepted by NovN (Fig. 19). 87% of novclobiocin 104 were converted to the carbamoylated derivative after overnight incubation under the assay conditions described in "Materials and Methods 7.2.6". The average conversion rate of the other four substrates was about 54%. The structures of the enzymatic substrates and products are given in Table 21.

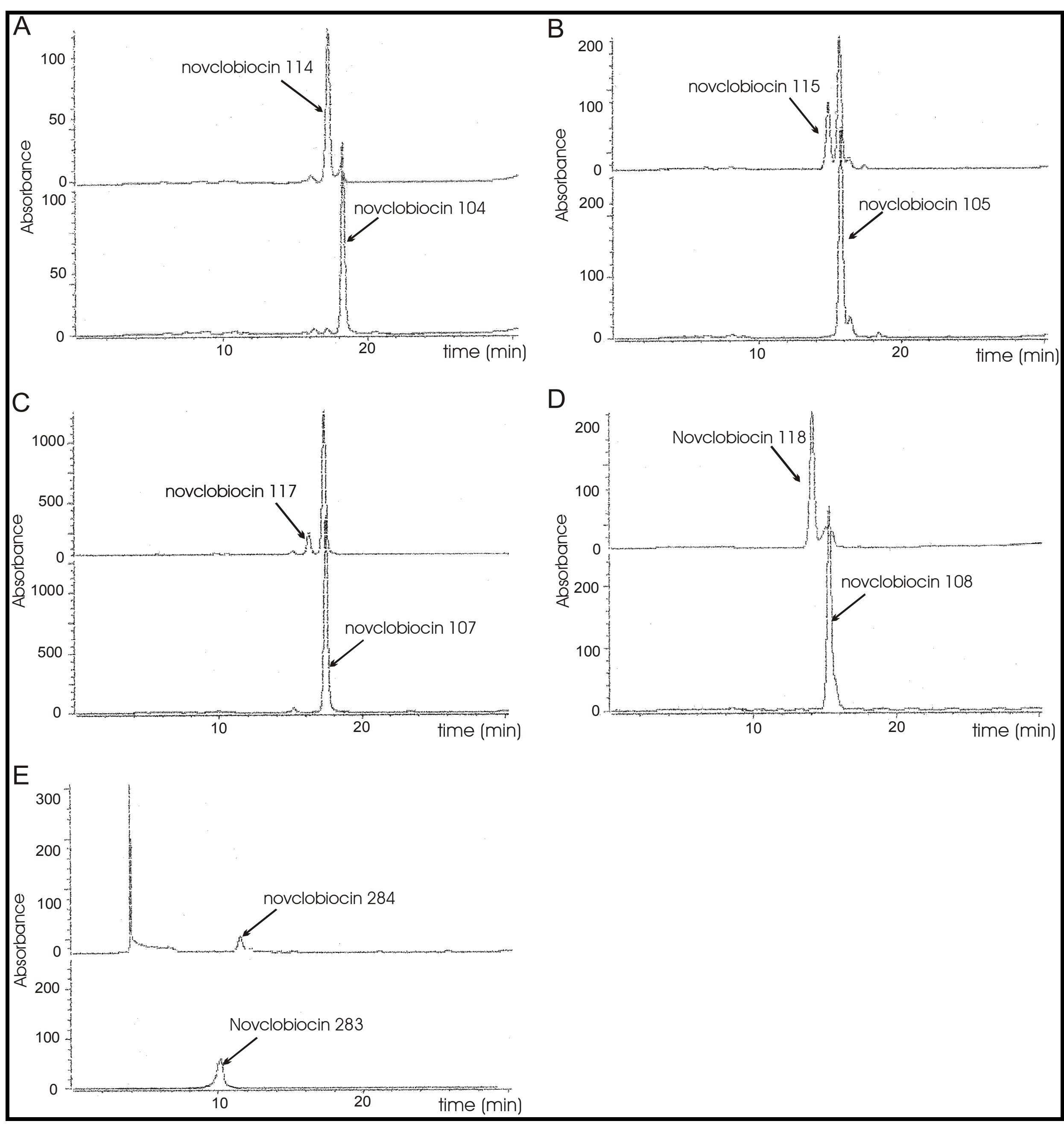


Fig. 19 HPLC analysis of the carbamoyltransferase assays with 3´´-unsubstituted aminocoumarin derivatives. (A) novclobiocin 104; (B) novclobiocin 105; (C) novclobiocin 107; (D) novclobiocin; (E) novclobiocin 283. Assay with purified NovN (top) and negative control with heat-denatured enzyme (bottom). The assays were incubated overnight.

For structural elucidation, each product was isolated in preparative amounts and subjected to ^{1}H-NMR and MS analysis (s. Appendixes). Compared to the parent compounds, the NMR spectra of the enzymatic products in CD_3OD showed no additional proton signals but marked downfield shifts of the signals of the protons H-2´´, H-4´´ and especially H-3´´ of the deoxysugar (Table 22), indicating a substitution at position 3´´ of the deoxysugar. FAB-MS analysis showed that the molecular mass of the enzymatic products was 43 Da larger than that of the respective substrates, consistent with the attachment of a carbamoyl group.

Table 21 Chemical structures of aminocoumarins derivatives

Compound name	R_1	R_2	R_3	R_4
clorobiocin		Cl		CH_3
novobiocin		CH_3	$CONH_2$	CH_3
novclobiocin 104[a]		Cl	H	CH_3
novclobiocin 114[b]		Cl	$CONH_2$	CH_3
novclobiocin 105[a]		Cl	H	H
novclobiocin 115[b]		Cl	$CONH_2$	H
novclobiocin 107[a]		H	H	CH_3
novclobiocin 117[b]		H	$CONH_2$	CH_3
novclobiocin 108[a]		H	H	H
novclobiocin 118[b]		H	$CONH_2$	H
novclobiocin 283[a]	Br	Cl	H	CH_3
novclobiocin 284[b]		Cl	$CONH_2$	CH_3

[a] substrates for carbamoyltransferase assays
[b] products of carbamoyltransferase assays

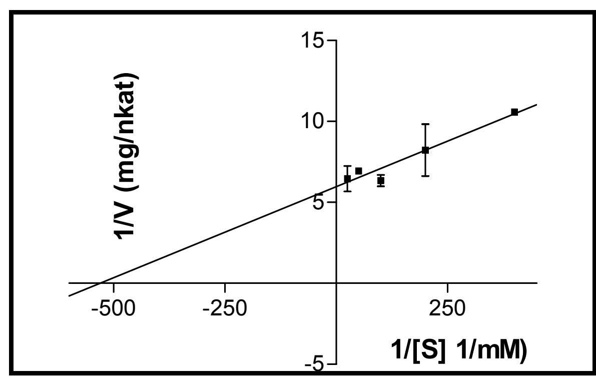
Table 22 Comparison of ^{1}HNMR data of aminocoumarin derivatives (400 MHz, CD_3OD)

	H-2´´ δ (ppm),multi (*J*/Hz)	H-3´´ δ (ppm),multi (*J*/Hz)	H-4´´ δ (ppm),multi (*J*/Hz)
novclobiocin 104	4.12,t (3.1)	4.18,dd (3.1, 9.9)	3.40,d (9.9)
novclobiocin 114	4.29,t (3.0)	5.37,dd (3.1, 10.1)	3.65,br s
novclobiocin 105	4.13,br s	4.12,dd (3.1, 10.0)	3.76,d (10.0)
novclobiocin 115	4.33,br s	5.27,dd (3.1, 10.3)	3.93,d (10.3)
novclobiocin 107	4.02,t (3.1)	4.09,dd (3.1, 9.4)	3.37,d (9.4)
novclobiocin 117	4.17,t (2.8)	5.25,dd (2.8, 8.8)	3.54§
novclobiocin 108	4.04,br s	4.03,dd (3.4, 9.5)	3.73,d (9.5)
novclobiocin 118	4.24,t (3.3)	5.15,dd (3.3, 10.0)	3.90,d (10.0)
novclobiocin 283	4.11,s	4.19,dd (3.3, 9.9)	3.40,d (9.9)
novclobiocin 284	4.27,t (3.1)	5.37,dd (3.1, 10.0)	3.56§

Abbreviation: t, triplet; d, doublet; dd, double doublet; s, singlet; br, broad signal
See Table 21 for numbering of the structures
§ overlapping with the Signal of 3H-8´´, *J* not determinable

3.3.5 Kinetic parameters for novclobicn 104 and 105

Kinetic parameters were determined for novclobiocin 104 and 105 (Fig. 20). K_{cat} values were determined as 1.32 min^{-1} and 1.53 min^{-1}, respectively, similar to the value obtained for descarbamoyl novobiocin. The K_m value for novclobiocin 104 (1.9 µM) was similar to that of descarbamoyl novobiocin. In contrast, the K_m for novclobiocin 105 was 14.3 µM, i.e. five times higher than that of genuine substrate, indicating that the 4´´-methoxy group may play a role in substrate binding.



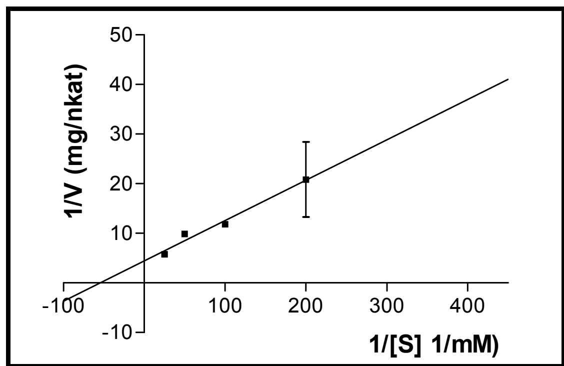


Fig. 20 Lineweaver-Burk plots for calculation of kinetic parameters for novclobiocin 104 and 105

3.3.6 Further investigations of substrate specificity

In contrast to the novclobiocins, which were well accepted by NovN, the assay with coumermycin D in the crude extract of *cou*N3⁻ mutant (not in pure form) did not lead to the production of expected carbamoylated coumermycin (Fig. 21).

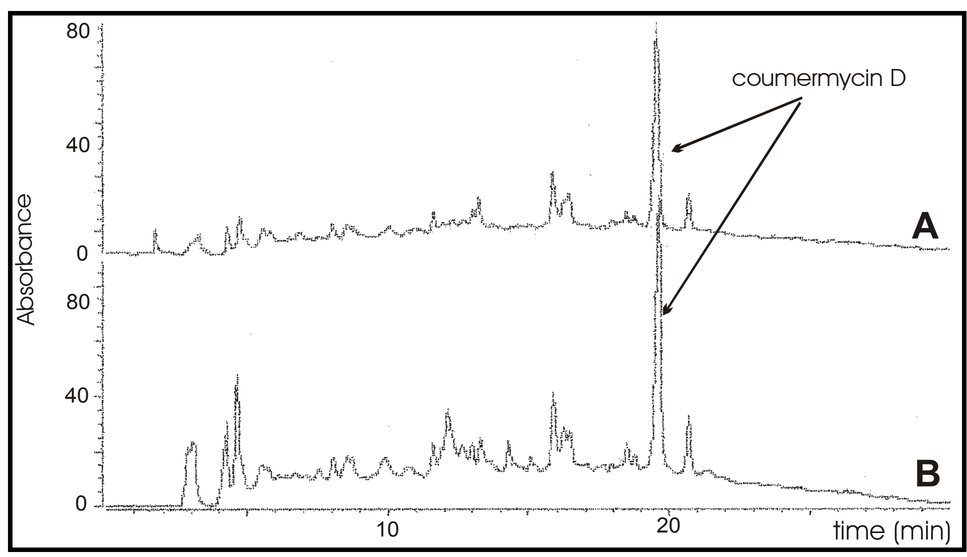


Fig. 21 HPLC analysis of the carbamoyltransferase assay with coumermycin D. (A) negative control, the crude extract of couN3⁻ mutant; (B) assay with coumermycin D in the crude extract of couN3⁻ mutant. The assay mixture containing 0.1 mM coumermycin D (calculated as coumermycin A_1), was incubated overnight.

On the other hand, NovN was specific for carbamoyl phosphate as substrate. No product formation was observed, after incubating acetyl phosphate with descarbamoyl novobiocin and NovN overnight (Fig. 22).

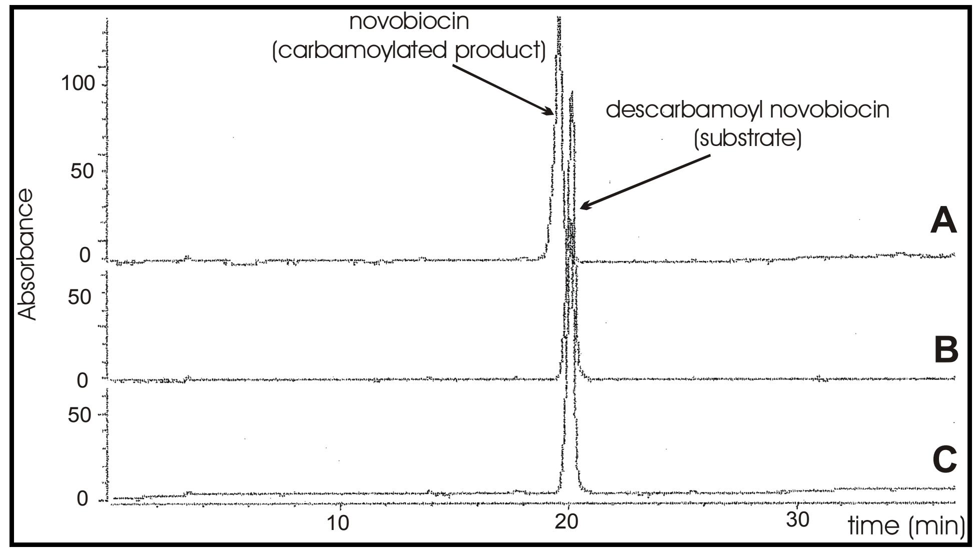


Fig. 22 HPLC analysis of the carbamoyltransferase assays with acetyl phosphate. (A) positive control, assay with carbamoylphosphate; (B) assay with acetyl phosphate (5 mM); (C) negative control, descarbamoyl novobiocin. The assays were incubated overnight.

4 Testing the biological activity of new aminocoumarin derivatives

4.1 Inhibitory activity on *E. coli* gyrase

The new aminocoumarin derivatives were tested *in vitro* for their inhibitory effect on *E. coli* gyrase, in comparison with the natural compounds novobiocin and clorobiocin. The IC_{50} of novobiocin resulted as 0.9 µM, and the activity of the other substances was expressed relative to that of novobiocin (Fig. 23). As observed previously (Galm *et al.,* 2004b; Maxwell & Lawson 2003), clorobiocin was the most active substance *in vitro*, three times as active as novobiocin.

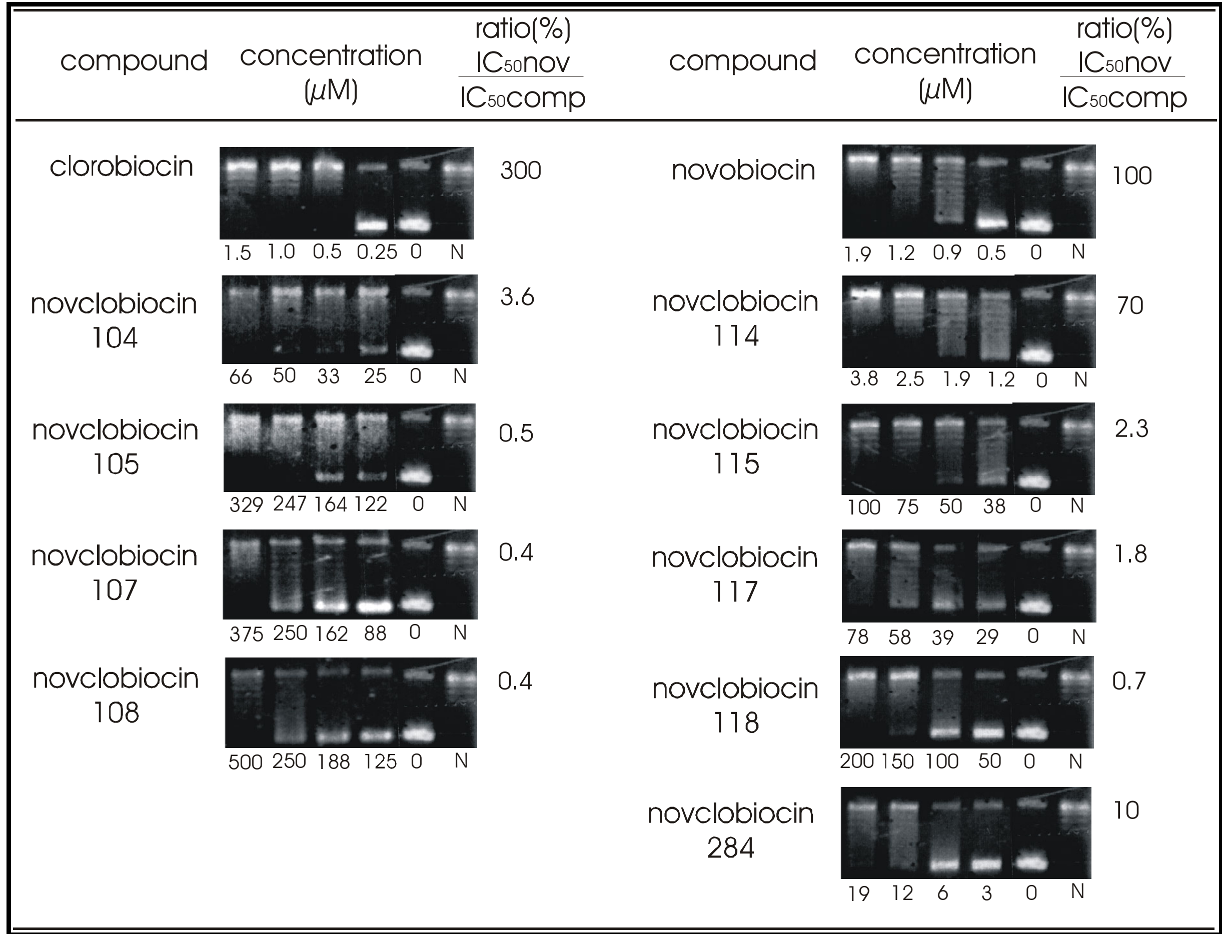


Fig. 23 *In vitro* inhibition of the DNA supercoiling reaction catalysed by *E. coli* gyrase. (upper band: relaxed pBR322; lower band: supercoiled pBR322; N: negative control without gyrase)

Removal of the pyrrole carboxylic acid moiety from clorobiocin resulted in a 100-fold drop of the activity (novclobiocin 104). The subsequent removal of the methyl group at 4´´-OH (novclobiocin 105), of the chlorine atom at position 8´ (novclobiocin 107), or of both these substituents (novclobiocin 108) further reduced the activity. When the pyrrole carboxylic acid moiety was removed from clorobiocin and subsequently replaced by a carbamoyl group, activity was restored: the resulting compound (novclobiocin 114) was nearly as active as novobiocin, but still four times less active than clorobiocin. In contrast, carbamoylation of novclobiocins 105, 107 and 108 led only to compounds of low activity (novclobiocins 115, 117 and 118, respectively), proving the importance of the 4´´-methoxyl group and the 8´-chlorine atom for gyrase inhibition.

Replacement of the 3-dimethylallyl moiety in novclobiocin 114 by a bromine atom (novclobiocin 284) resulted in a sevenfold drop in activity, indicating that also the substituted Ring A contributes to the biologische activity.

4.2 Antibacterial activity against *Bacillus subtillis* ATCC 14893

The results on the inhibition of gyrase *in vitro* were subsequently confirmed by determination of the antibacterial activity in a disc diffusion assay using *Bacillus subtilis* ATCC 14893 as test organism (Fig. 24). Activity was expressed relative to novobiocin. As observed previously (Galm *et al.,* 2004b), novobiocin showed higher activity than clorobiocin in this assay. Otherwise, however, the antibacterial assays largely confirmed the results of the *in vitro* gyrase inhibition assays. Removal of the pyrrole carboxylic acid moiety from clorobiocin resulted in a sharp drop of activity, and the additional removal of the methyl group at 4´´-OH and/or the chlorine atom at position 8´ further reduced growth inhibition. Carbamoylation of novclobiocin 104 led to an active antibacterial compound (novclobiocin 114), which, however, was less active than novobiocin or clorobiocin. Removal of the methyl group at 4´´-OH or of the chlorine atom at the 8´ position, or replacement of the 3-dimethylallyl group by a bromine atom, strongly reduced the activity.

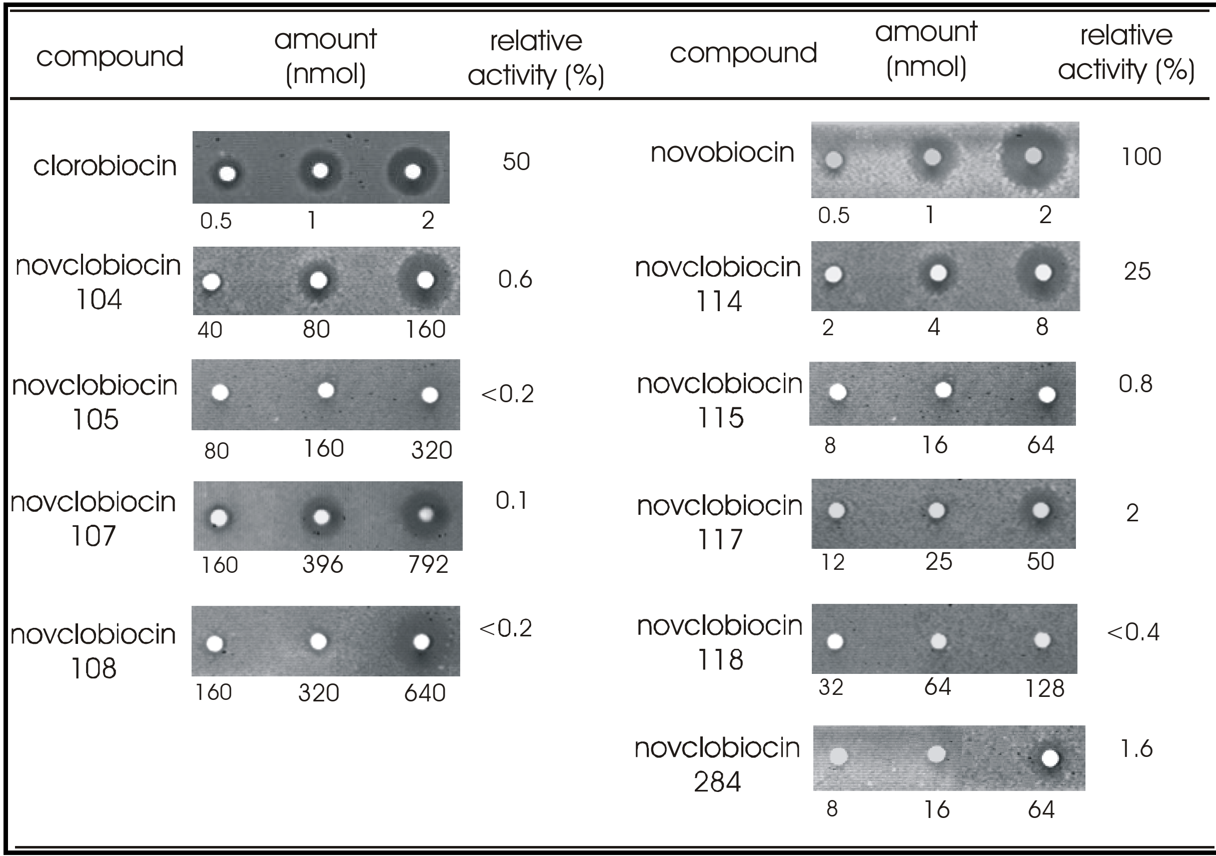


Fig. 24 Bioassay against *Bacillus subtilis*. Activities are expressed relative to novobiocin (100%).

DISCUSSION

1 Biosynthesis of pyrrole-2-carboxyl moiety of coumermycin A_1 and clorobiocin

In pyoluteorin and undecylprodiginine biosynthesis, the three essential steps in the conversion of L-proline to pyrrole-2-carboxylic acid (Fig. 4) have been elucidated on the biochemical and the genetic level (Cerdeno *et al.,* 2001; Nowak-Thompson *et al.,* 1999; Thomas *et al.,* 2002). As shown in Table 20, genes *cou*N3/*cou*N4/*cou*N5 and *clo*N3/*clo*N4/*clo*N5 from the coumermycin and clorobiocin clusters, respectively, show sequence similarity with these established pyrrole biosynthesis genes and were likely to carry out the reaction steps depicted in Fig. 4. Experimental proof for their involvement in the formation of the terminal pyrrole moieties of coumermycin A_1 has now been provided by the inactivation of *cou*N3 and *cou*N4, resulting in a complete abolishment of coumermycin A_1 formation (Fig. 6).

The same functions may be postulated for the corresponding genes of the clorobiocin biosynthetic gene cluster, i.e. *clo*N3, *clo*N4.

As reported previously (Berger & Batcho 1978; Claridge *et al.,* 1984), *S. rishiriensis* produces a multiplicity of related antibiotics, which differ by the substitutents at the 3´´-hydroxy groups of the two deoxysugars. At either sugar moiety, a 5-methylpyrrole-2-carboxyl group, a non-methylated pyrrole-2-carboxyl group, or no substituent at all can be found, resulting in 9 different possible coumermycins. In culture extracts of our wild-type strain (Fig. 6B), therefore a multiplicity of compounds could be detected by HPLC, with coumermycin A_1 as the dominant product. The presence of coumermycin D was confirmed by LC/MS analysis ($[M-H]^-$ = *m/z* 894), but this compound was present only in minor quantities in the wild-type. In contrast, coumermycin D was the dominant product in the *cou*N3$^-$ and *cou*N4$^-$ mutants, accompanied by relatively few other metabolites (Fig. 6C and 6D).

2 Different biosynthetic pathway of central pyrrole moiety in coumermycins

Coumerycin A_1 contains a central 3-methylpyrrole-2,4-dicarboxylic acid moiety and two terminal 5-methyl-pyrrole-2-carboxylic acid moieties. The fact that neither the inactivation of the dehydrogenase gene *cou*N3 nor the inactivation of the L-prolyl-AMP ligase gene *cou*N4 led to an abolishment of the biosynthesis of the central pyrrole moiety indicates that the central pyrrole may be formed by a different pathway from that responsible for synthesis of the two terminal pyrrole moieties.

Based on the results of a previous feeding experiment with [U-^{14}C]L-proline, Scannell and Kong (Scannell & Kong 1969) had assumed that all three pyrrole rings of coumermycin are formed from L-proline. A closer examination of their method, however, reveals that their chemical degradation procedure (pyrolysis and short-column gas chromatography) did not differentiate between the central and the two terminal pyrrole moieties. Therefore, the incorporation of L-proline reported in their study would also be consistent with a biosynthesis of the terminal pyrroles from proline and a different biosynthetic pathway of the central pyrrole.

The mechanism of the biosynthesis of the central pyrrole moiety and the genes involved in this process are currently under investigation in our group.

3 Attachment of pyrrole-2-carboxyl moiety to the deoxysugar in clorobiocin and coumermycin A_1

In the clorobiocin producer *Streptomyces roseochromogenes*, inactivation of the gene *clo*N2 by in-frame deletion resulted in the accumulation of two new aminocoumarin derivatives, novclobiocin 104 and 105, both lacking the pyrrole moiety at C-3´´ of the deoxysugar. However, free pyrrole-2-carboxylic acid was still produced by the *clo*N2$^-$ mutant, proving that the mutation did not affect the biosynthesis of this moiety, but rather its transfer to the deoxysugar unit of clorobiocin. Clorobiocin production could be restored by expression of *clo*N2 in the *clo*N2$^-$ mutant, which demonstrates that only gene *clo*N2 had been inactivated. Our results prove that CloN2 is involved in the formation of the ester bond between the pyrrole-2-carboxylic acid moiety and the deoxysugar. Most likely, CloN2 transfers the acyl

moiety from a pyrrole-2-carboxyl-*S*-CloN5 intermediate to the 3´´-OH of the deoxysugar moiety of clorobiocin (Fig. 25).

The same function may be postulated for the corresponding gene of the coumermycin biosynthetic gene cluster, i.e. *cou*N2. CouN2 shows 86% identity to CloN2, and both proteins comprise 355 amino acids.

Genes with sequence similarity to *clo*N2, i.e. *cal*O4*, avi*N and *evr*I, are found in the biosynthetic clusters of calicheamicin (Ahlert *et al.,* 2002), avilamycin A (Weitnauer *et al.,* 2001) and evernimicin (Hosted *et al.,* 2001), respectively. It has been proposed that they may control the starter unit for orsellenic acid biosynthesis, based on their sequence similarity to *dps*C (Hosted *et al.,* 2001; Weitnauer *et al.,* 2001). However, their function has not been proven experimentally. All three compounds (calicheamicin, avilamycin and evernimicin) contain an orsellinic acid unit attached *via* an ester bond to a deoxysugar moiety. Since we have now provided evidence that *clo*N2 is involved in the acyl transfer of an aromatic carboxylic acid to the deoxysugar moiety of clorobiocin, it should be considered whether also CalO4, AviN and EvrI may be responsible for the acylation of the deoxysugar moieties of the respective antibiotics.

The biosynthesis of the terminal pyrrole moieties of clorobiocin and oumermycin A_1 requires two additional steps, i.e. methylation at position 5 of the pyrrole ring, and transfer of the entire 5-methypyrrole-2-carboxyl moiety onto the deoxysugar of coumermycin D. In our experiments, only pyrrole-2-carboxylic acid, but not its 5-methyl derivative was found in *clo*N2$^-$ mutant. This indicates that the methylation of the pyrrole unit occurs only after its transfer to the deoxysugar moiety (Fig. 25). The wild-type contained a small quantity of free 5-methylpyrrole-2-carboxylic acid, possibly produced by hydrolysis of clorobiocin. This compound was not found in the *clo*N2$^-$ mutant, which is consistent with the above hypothesis.

A clorobiocin derivative lacking the methyl group at the pyrrole moiety (designated as antibiotic 2562 B) has been isolated previously from *Streptomyces griseovariabilis* (Lysenkova *et al.,* 1980). Similarly, coumermycin derivates lacking the methyl group at *C*-5 of one or both the pyrrole-2-carboxylic acid moieties have been identified in the coumermycin producer (Claridge *et al.,* 1984).

The gene *clo*N6, immediately downstream of *clo*N5 (Fig. 1B), shows sequence similarity to a new class of radical SAM proteins identified recently (Sofia *et al.,* 2001). Inactivation of this gene has proved its involvement in the *C*-methylation of pyrrole-2-carboxylic acid moiety (Westrich *et al.,* 2003).

Fig. 25 is a schematic representation of the biosynthesis of the terminal pyrrole moiety and its attachment to the 3´´-OH group of deoxysugar of clorobiocin. CloN3, CloN4 and CloN5 are expected to catalyse the conversion of L-proline to pyrrole-2-carboxyl-*S*-PCP (Thomas *et al.,* 2002; Xu *et al.,* 2002). And then the acyl component is transferred to the deoxysugar moiety by CloN2. Finally, CloN6 catalyses the *C*-methylation at position 5 of the pyrrole-2-carboxyl moiety (Westrich *et al.,* 2003). The same biosynthetic pathway may be postulated for the terminal pyrrole moieties of coumermycin A_1.

Fig. 25 Hypothetical scheme of the late steps of clorobiocin biosynthesis. The extract sequence of the CloP and CloN2 reactions has not yet been established.

Among the contiguous group of seven genes in the clorobiocin and in the coumermycin A_1 gene cluster (*clo*N1-*clo*N7 and *cou*N1-*cou*N7, respectively), only the roles of the small ORF *clo*N1 and the putative hydrolase gene *clo*N7 are yet unclear. An inactivation of these genes may provide evidence of their importance for the biosynthesis of aminocoumarin antibiotics.

4 Production of novel carbamoylated aminocoumarin derivatives

4.1 Production of new carbamolated coumermycin derivatives *in vivo*

Carbamoylated derivatives of coumermycin D have never been identified before. Expression of the putative carbamoyltransferase gene *nov*N from the novobiocin biosynthetic gene cluster in the *cou*N3^{-} mutant led to the formation of bis-carbamoylated coumermycin D (Fig. 14), which was identified by its molecular mass and fragmentation in LC/MS analysis. This result provides experimental evidence for the presumed function of NovN as a carbamoyltransferase and also demonstrates that new aminocoumarin antibiotics may be obtained by genetic modification of the producing strains.

4.2 Production of new carbamoylated aminocoumarin antibiotics *in vitro*

Generation of the novel carbamoylated novclobiocins using NovN demonstrates the potential of a combined mutational and chemoenzymatic approach for the generation of new amincouamrin antibiotics.

In previous studies (Eustáquio *et al.,* 2003a; Li *et al.,* 2002; Westrich *et al.,* 2003), our group has generated a variety of new aminocoumarins by methods of combinatorial biosynthesis, i.e. by combination of gene inactivation and heterologous gene expression experiments. However, expression of the carbamoyltransferase NovN in mutants of aminocoumarin antibiotic producers resulted only in low yields of carbamoylated products (Xu *et al.,* 2002). This problem was overcome by producing 3´´-unsubstituted aminocoumarin derivatives using specific mutants with single or multiple gene defects, followed by efficient carbamoylation of these compounds *in vitro*, which resulted in five new carbamoylated aminocoumarin antibiotics in preparative amounts.

The carbamoyltransferase NovN could readily be obtained in nearly pure form after expression of its structural gene in *Streptomyces lividans*. Surprisingly, the carbamoyltransferase activity of NovN is strictly dependent upon divalent cations Mg^{2+}/Mn^{2+} and ATP. Activation of O-carbamoyltransferase by ATP in secondary metabolites biosynthesis was investigated so far for 3´-hydroxymethylcephem-*O*-carbamoyltransferase from *Streptomyces clavuligerus* (Brewer *et al.,* 1980). However, the role of ATP activation in *O*-carbamoylation remains unclear. Mg-ATP

complex has been shown to be an allosteric effector (activator) for class B aspartate N-carbamoyltransferases (ATCases) from *E. coli* (Sakash *et al.,* 2000) and a number of enterobacteria (Chen *et al.,* 1998; Fetler & Vachette 2001). Is it possible that Mg-ATP complex has the similar function in the NovN carbamoylation reaction?

Although the natural substrate of NovN is 3´´-descarbamoyl novobiocin, the enzyme readily accepted all five other aminocoumarin substrates tested in this thesis, showing that structural variations can be introduced by this method in the deoxysugar moiety, in the aminocoumarin moiety and in the benzoyl moiety of the antibiotics. On the other hand, acetyl phosphate was not accepted as substrate by NovN, limiting the range of products to carbamoylated compounds.

Structural variation may be further increased by the utilization of mutasynthetic strategies, as demonstrated by the generation of novclobiocin 284: first, the biosynthesis of the natural benzoyl moiety was blocked by an inactivation experiment (Pojer *et al.,* 2003b); then, a synthetic analogue of the benzoyl moiety was fed to this mutant , resulting in the formation of novclobiocin 283 (Galm *et al.,* 2004a); finally, this compound was carbamoylated by chemoenzymatic methods using NovN.

Bis-carbamoyl coumermycin D could be generated by expressing NovN *in vivo*, but not *in vitro*. Probably, the carbamoyltransferase assay conditions used in this thesis was not optimal for the carbamoylation of coumermycin D. And the much larger molecule of coumermycin D may also influence its effective binding to the enzyme.

5 Structure–activity relationships within the aminocouamrin antibiotics

The compounds obtained in this thesis allowed to obtaining further insights into the structure-activity relationships within the aminocoumarin class of gyrase inhibitors. Firstly, our results confirmed the importance of the acyl moiety attached to the 3´´-OH of the deoxysugar. Lack of this acyl group led to a sharp reduction of activity. Novclobiocin 114, which contained the carbamoyl instead of the pyrrole carboxylic acid group at 3´´-OH, but otherwise had an identical structure as clorobiocin, showed a fourfold lower inhibitory activity on gyrase than clorobiocin.

Furthermore, our study demonstrates a crucial importance of the methyl group attached to the 4´´-OH group of the deoxysugar; lack of this group led to a strong

decrease of activity. In accordance with earlier results (Eustáquio *et al.,* 2003a), lack of the chlorine (or methyl) substituent at C-8´ of the aminocoumarin ring also leads to a considerable decrease of activity.

Interestingly, novclobiocin 284 which contains a bromine atom instead of the dimethylallyl moiety at C-3 of the benzoyl moiety not only showed lower antibacterial activity than novclobiocin 114, but also less gyrase inhibition *in vitro*. This finding gives further experimental support to the hypothesis of Lafitte *et al*. (Lafitte *et al.,* 2002) that the dimethylallyl moiety directly contributes to the binding of the antibiotic to gyrase, and not only facilitates the uptake through the bacterial membrane as speculated earlier (Lewis *et al.,* 1996).

REFERENCE

Ahlert, J., Shepard, E., Lomovskaya, N., Zazopoulos, E. et al. (2002). The calicheamicin gene cluster and its iterative type I enediyne PKS. *Science* **297**, 1173-1176.

Ali, J. A., Jackson, A. P., Howells, A. J. & Maxwell, A. (1993). The 43-kilodalton N-terminal fragment of the DNA gyrase B protein hydrolyzes ATP and binds coumarin drugs. *Biochemistry* **32**, 2717-2724.

Allsop, A. E. (1998). New antibiotic discovery, novel screens, novel targets and impact of microbial genomics. *Curr. Opin. Microbiol.* **1**, 530-534.

Bao, W., Sheldon, P. J. & Hutchinson, C. R. (1999a). Purification and properties of the *Streptomyces peucetius* DpsC beta-ketoacyl:acyl carrier protein synthase III that specifies the propionate-starter unit for type II polyketide biosynthesis. *Biochemistry* **38**, 9752-9757.

Bao, W., Sheldon, P. J., Wendt-Pienkowski, E. & Hutchinson, C. R. (1999b). The *Streptomyces peucetius* dpsC gene determines the choice of starter unit in biosynthesis of the daunorubicin polyketide. *J. Bacteriol.* **181**, 4690-4695.

Bax, R., Mullan, N. & Verhoef, J. (2000). The millennium bugs - the need for and development of new antibacterials. *International Journal of Antimicrobial Agents* **16**, 51-59.

Berger, J. & Batcho, A. D. (1978). Coumarin-glycoside antibiotics. *J. Chromatogr. Libr.* **15**, 101-158.

Berger, J., Schocher, A. J., Batcho, A. D., Pecherer, B. et al. (1965). Production, isolation, and synthesis of the coumermycins (sugordomycins), a new streptomycete antibiotic complex. *Antimicrobial. Agents Chemother.* **5**, 778-785.

Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N. & Schoner, B. E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**, 43-49.

Birch, A. J., Holloway, R. W. & Rickards, R. W. (1962). Biosynthesis of noviose, a branched-chain monosaccharide. *Biochim. Biophys. Acta* **57**, 148-5.

Black, T. & Hare, R. (2000). Will genomics revolutionize antimicrobial drug discovery? *Curr. Opin. Microbiol.* **3**, 522-527.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brewer, S. J., Taylor, P. M. & Turner, M. K. (1980). An adenosine triphosphate-dependent carbamoylphosphate--3-hydroxymethylcephem O-carbamoyltransferase from *Streptomyces clavuligerus*. *Biochem. J.* **185**, 555-564.

Bunton, C. A., Kenner, G. W., Robinson, M. J. T. & Webster, B. R. (1963). Experiments related to the biosynthesis of novobiocin and other coumarins. *Tetrahedron* **19**, 1001-1010.

Calvert, R. T., Spring, M. S. & Stoker, J. R. (1972). Investigations of the biosynthesis of novobiocin. *J. Pharm. Pharmacol.* **24**, 972-978.

Celia, H., Hoermann, L., Schultz, P., Lebeau, L., Mallouh, V., Wigley, D. B., Wang, J. C., Mioskowski, C. & Oudet, P. (1994). Three-dimensional model of *Escherichia coli* gyrase B subunit crystallized in two-dimensions on novobiocin-linked phospholipid films. *J. Mol. Biol.* **236**, 618-628.

Cerdeno, A. M., Bibb, M. J. & Challis, G. L. (2001). Analysis of the prodiginine biosynthesis gene cluster of *Streptomyces coelicolor* A3(2): new mechanisms for chain initiation and termination in modular multienzymes. *Chem. Biol.* **8**, 817-829.

Chen, H. & Walsh, C. T. (2001). Coumarin formation in novobiocin biosynthesis: ß-hydroxylation of the aminoacyl enzyme tyrosyl-S-NovH by a cytochrome P450 NovI. *Chem. Biol.* **8**, 301-312.

Chen, P., Van Vliet, F., Van de, C. M., Legrain, C., Cunin, R. & Glansdorff, N. (1998). Aspartate transcarbamylase from the hyperthermophilic eubacterium Thermotoga maritima: fused catalytic and regulatory polypeptides form an allosteric enzyme. *J. Bacteriol.* **180**, 6389-6391.

Claridge, C. A., Elander, R. P. & Price, K. E. (1984). The coumermycins: properties, biosynthesis, and fermentation. *Drugs Pharm. Sci.* **22**, 413-425.

Coates, A., Hu, Y., Bax, R. & Page, C. (2002). The future challenges facing the development of new antimicrobial drugs. *Nat. Rev. Drug Discov.* **1**, 895-910.

Dolak, L. (1973). The structure of RP 18,631. *Journal of Antibiotics* **26**, 121-125.

Doumith, M., Weingarten, P., Wehmeier, U. F., Salah-Bey, K., Benhamou, B., Capdevila, C., Michel, J.-M., Piepersberg, W. & Raynal, M.-C. (2000). Analysis of genes involved in 6-deoxyhexose biosynthesis and transfer in *Saccharopolyspora erythraea*. *Mol. Gen. Genet.* **264**, 477-485.

Eustáquio, A. S., Gust, B., Luft, T., Li, S.-M., Chater, K. F. & Heide, L. (2003a). Clorobiocin biosynthesis in *Streptomyces*. Identification of the halogenase and generation of structural analogs. *Chem Biol* **10**, 279-288.

Eustáquio, A. S., Luft, T., Wang, Z.-X., Gust, B., Chater, K. F., Li, S.-M. & Heide, L. (2003b). Novobiocin biosynthesis: inactivation of the putative regulatory gene *nov*E and heterologous expression of genes involved in aminocoumarin ring formation. *Arch. Microbiol.* **180**, 25-32.

Fetler, L. & Vachette, P. (2001). The allosteric activator Mg-ATP modifies the quaternary structure of the R-state of Escherichia coli aspartate transcarbamylase without altering the T<-->R equilibrium. *J. Mol. Biol* **309**, 817-832.

Freel Meyers, C. L., Oberthuer, M., Xu, H., Heide, L., Kahne, D. & Walsh, C. T. (2004). Characterization of NovP and NovN: Completion of novobiocin biosynthesis by sequential tailoring of the noviosyl ring. *Angew. Chem. , Int. Ed.* **43**, 67-70.

Freel Meyers, C. L., Oberthur, M., Anderson, J. W., Kahne, D. & Walsh, C. T. (2003). Initial characterization of novobiocic acid noviosyl transferase activity of NovM in biosynthesis of the antibiotic novobiocin. *Biochemistry* **42**, 4179-4189.

Galm, U., Dessoy, M. A., Schmidt, J., Wessjohann, L. A. & Heide, L. (2004a). *In vitro* and *in vivo* production of new aminocoumarins by a combined biochemical, genetic and synthetic approach. *Chem Biol*.

Galm, U., Heller, S., Shapiro, S., Page, M., Li, S.-M. & Heide, L. (2004b). Antimicrobial and DNA gyrase-Inhibitory activities of novel clorobiocin derivatives prepared by mutasynthesis. *Antimicrob. Agents Chemother.* **in press**.

Gormley, N. A., Orphanides, G., Meyer, A., Cullis, P. M. & Maxwell, A. (1996). The interaction of coumarin antibiotics with fragments of DNA gyrase B protein. *Biochemistry* **35**, 5083-5092.

Grimm, A., Madduri, K., Ali, A. & Hutchinson, C. R. (1994). Characterization of the *Streptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. *Gene* **151**, 1-10.

Gross, C. H., Parsons, J. D., Grossman, T. H., Charifson, P. S. et al. (2003). Active-site residues of *Escherichia coli* DNA gyrase required in coupling ATP hydrolysis to DNA supercoiling and amino acid substitutions leading to novobiocin resistance. *Antimicrob. Agents Chemother.* **47**, 1037-1046.

Heide, L. & Li, S.-M. (2002). Aminocoumarin antibiotics. In *Microbial secondary metabolites: biosynthesis, genetics and regulation*, pp. 63-83. Edited by. F.Fierro & J.F.Martín. Kerala, India: Research Signpost.

Heinzelmann, E., Kienzlen, G., Kaspar, S., Recktenwald, J., Wohlleben, W. & Schwartz, D. (2001). The phosphinomethylmalate isomerase gene pmi, encoding an aconitase-like enzyme, is involved in the synthesis of phosphinothricin tripeptide in *Streptomyces viridochromogenes*. *Appl. Environ. Microbiol.* **67**, 3603-3609.

Hoeksema, H., Johnson, J. L. & Hinman, J. W. (1955). Structural studies on streptonivicin, a new antibiotic. *J. Am. Chem. Soc.* **77**, 6710-6711.

Hoffmeister, D., Wilkinson, B., Foster, G., Sidebottom, P. J., Ichinose, K. & Bechthold, A. (2002). Engineered urdamycin glycosyltransferases are broadened and altered in substrate specificity. *Chem Biol.* **9**, 287-295.

Hoggarth, J. H., Cushing, K. E. & Ritchie, D. A. (1995). Genetic and functional analysis of novobiocin non-producing mutants of *Streptomyces niveus*. *J. Appl. Bacteriol.* **79**, 625-630.

Holzenkämpfer, M. & Zeeck, A. (2002). Biosynthesis of simocyclinone D8 in an 18O2-rich atmosphere. *Journal of Antibiotics* **55**, 341-342.

Hopwood, D. A., Malpartida, F., Kieser, H. M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B. A., Floss, H. G. & Omura, S. (1985). Production of 'hybrid' antibiotics by genetic engineering. *Nature* **314**, 642-644.

Hosted, T. J., Wang, T. X., Alexander, D. C. & Horan, A. C. (2001). Characterization of the biosynthetic gene cluster for the oligosaccharide antibiotic, Evernimicin, in *Micromonospora carbonacea* var. *africana* ATCC39149. *J. Ind. Microbiol. Biotechnol.* **27**, 386-392.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000) *Practical Streptomyces Genetics*. Norwich, UK: John Innes Foundation.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lafitte, D., Lamour, V., Tsvetkov, P. O., Makarov, A. A., Klich, M., Deprez, P., Moras, D., Briand, C. & Gilli, R. (2002). DNA gyrase interaction with coumarin-based inhibitors: the role of the hydroxybenzoate isopentenyl moiety and the 5'-methyl group of the noviose. *Biochemistry* **41**, 7217-7223.

Lewis, R. J., Singh, O. M. P., Smith, C. V., Skarzynski, T., Maxwell, A., Wonacott, A. J. & Wigley, D. B. (1996). The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. *EMBO J.* **15**, 1412-1420.

Li, S.-M., Hennig, S. & Heide, L. (1998). Biosynthesis of the dimethylallyl moiety of novobiocin via a non-mevalonate pathway. *Tetrahedron Letters* **39**, 2717-2720.

Li, S.-M., Westrich, L., Schmidt, J., Kuhnt, C. & Heide, L. (2002). Methyltransferase genes in *Streptomyces rishiriensis*: new coumermycin derivatives from gene-inactivation experiments. *Microbiology* **148**, 3317-3326.

Lombo, F., Siems, K., Braña, A. F., Méndez, C., Bindseil, K. & Salas, J. A. (1997). Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in the earliest steps of biosynthesis of the sugar moieties of the antitumor polyketide mithramycin. *J. Bacteriol.* **179**, 3354-3357.

Losey, H. C., Jiang, J., Biggins, J. B., Oberthur, M., Ye, X. Y., Dong, S. D., Kahne, D., Thorson, J. S. & Walsh, C. T. (2002). Incorporation of glucose analogs by GtfE and GtfD from the vancomycin biosynthetic pathway to generate variant glycopeptides. *Chem Biol* **9**, 1305-1314.

Lysenkova, L. N., Brazhnikova, M. G., Borisova, V. N., Fedorova, G. B., Rubasheva, L. M., Potapova, N. P. & Rozynov, B. V. (1980). Production and physicochemical study of components of the new antibacterial antibiotic 2562. *Antibiotiki (Moscow)* **25**, 483-488.

MacNeil, D. J., Gewain, K. M., Ruby, C. L., Dezeny, G., Gibbons, P. H. & MacNeil, T. (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61-68.

Malpartida, F., Niemi, J., Navarrete, R. & Hopwood, D. A. (1990). Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. *Gene* **93**, 91-99.

Mancy, D., Ninet, L., & Preud´Homme, J. (1974). Antibiotic 18631 RP. 3,793,147. U.S.

Maxwell, A. (1997). DNA gyrase as a drug target. *Trends Microbiol.* **5**, 102-109.

Maxwell, A. & Lawson, D. M. (2003). The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. *Curr. Top. Med. Chem.* **3**, 283-303.

McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M. & Ashley, G. (1999). Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc. Natl. Acad. Sci. U. S. A* **96**, 1846-1851.

Mootz, H. D., Schwarzer, D. & Marahiel, M. A. (2000). Construction of hybrid peptide synthetases by module and domain fusions. *Proc. Natl. Acad. Sci. U. S. A* **97**, 5848-5853.

Muth, G., Nussbaumer, B., Wohlleben, W. & Puehler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. *MGG, Mol. Gen. Genet.* **219**, 341-348.

Ninet, L., Benazet, F., Charpentie, Y., Dubost, M., Florent, J., Mancy, D., Preud'Homme, J., Threlfall, T. L. & Vuillemin, B. (1972). Clorobiocin (18.631 R.P.), a new chlorinated antibiotic produced by several *Streptomyces species*. *C. R. Acad. Sci. , Ser. C* **275**, 455-458.

Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J. et al. (2001). Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **414**, 454-457.

Noga, E. J. & Silphaduang, U. (2003). Piscidins: A novel family of peptide antibiotics from fish. *Drug News & Perspectives* **16**, 87-92.

Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J. & Loper, J. E. (1999). Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* **181**, 2166-2174.

Oh, S. H. & Chater, K. F. (1997). Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. *J. Bacteriol.* **179**, 122-127.

Omura, S., Ikeda, H., Malpartida, F., Kieser, H. M. & Hopwood, D. A. (1986). Production of new hybrid antibiotics, mederrhodins A and B, by a genetically engineered strain. *Antimicrob. Agents Chemother.* **29**, 13-19.

Pelletier, I., Pfeifer, O., Altenbuchner, J. & van Pee, K. H. (1994). Cloning of a second non-haem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762: sequence analysis, expression in *Streptomyces lividans* and enzyme purification. *Microbiology* **140 (Pt 3)**, 509-516.

Pfeifer, V., Nicholson, G. J., Ries, J., Recktenwald, J., Schefer, A. B., Shawky, R. M., Schröder, J., Wohlleben, W. & Pelzer, S. (2001). A polyketide synthase in glycopeptide biosynthesis: the biosynthesis of the non-proteinogenic amino acid (S)-3,5-dihydroxyphenylglycine. *J. Biol. Chem.* **276**, 38370-38377.

Pojer, F., Kahlich, R., Kammerer, B., Li, S.-M. & Heide, L. (2003a). CloR, a bifunctional non-heme iron oxygenase involved in clorobiocin biosynthesis. *J. Biol Chem* **278**, 30661-30668.

Pojer, F., Li, S.-M. & Heide, L. (2002). Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: new insights into the biosynthesis of aminocoumarin antibiotics. *Microbiology* **148**, 3901-3911.

Pojer, F., Wemakor, E., Kammerer, B., Chen, H., Walsh, C. T., Li, S.-M. & Heide, L. (2003b). CloQ, a prenyltransferase involved in clorobiocin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A* **100**, 2316-2321.

Quiros, L. M., Aguirrezabalaga, I., Olano, C., Mendez, C. & Salas, J. A. (1998). Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. *Mol. Microbiol.* **28**, 1177-1185.

Raad, I., Darouiche, R., Hachem, R., Sacilowski, M. & Bodey, G. P. (1995). Antibiotics and prevention of microbial colonization of catheters. *Antimicrob. Agents Chemother.* **39**, 2397-2400.

Raad, I. I., Hachem, R. Y., Abi-Said, D., Rolston, K. V. T., Whimbey, E., Buzaid, A. C. & Legha, S. (1998). A prospective crossover randomized trial of novobiocin and rifampin prophylaxis for the prevention of intravascular catheter infections in cancer patients treated with interleukin-2. *Cancer* **82**, 403-411.

Sakash, J. B., Tsen, A. & Kantrowitz, E. R. (2000). The use of nucleotide analogs to evaluate the mechanism of the heterotropic response of Escherichia coli aspartate transcarbamoylase. *Protein Sci.* **9**, 53-63.

Sambrook, J. & Russell, D. W. (2001) *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Sasaki, T., Igarashi, Y., Saito, N. & Furumai, T. (2001). TPU-0031-A and B, new antibiotics of the novobiocin group produced by *Streptomyces sp*. TP-A0556. *J. Antibiot.* **54**, 441-447.

Scannell, J. & Kong, Y. L. (1969). Biosynthesis of coumermycin A1: incorporation of L-proline into the pyrrole groups. *Antimicrobial. Agents Chemother.* **9**, 139-143.

Schimana, J., Fiedler, H. P., Groth, I., Süssmuth, R., Beil, W., Walker, M. & Zeeck, A. (2000). Simocyclinones, novel cytostatic angucyclinone antibiotics produced by *Streptomyces antibioticus* Tü 6040. I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.* **53**, 779-787.

Schmutz, E., Hennig, S., Li, S.-M. & Heide, L. (2004). Identification of a topoisomerase IV in actinobacteria: Purification and characterization of ParYR and

GyrBR from the coumermycin A1 producer *Streptomyces rishiriensis* DSM 40489. *Microbiology* **150**, 641-647.

Schmutz, E., Mühlenweg, A., Li, S.-M. & Heide, L. (2003a). Resistance genes of aminocoumarin producers: Two type II topoisomerase genes confer resistance against coumermycin A1 and clorobiocin. *Antimicrob. Agents Chemother.* **47**, 869-877.

Schmutz, E., Steffensky, M., Schmidt, J., Porzel, A., Li, S.-M. & Heide, L. (2003b). An unusual amide synthetase (CouL) from the coumermycin A1 biosynthetic gene cluster from *Streptomyces rishiriensis* DSM 40489. *European Journal of Biochemistry* **270**, 4413-4419.

Shimokawa, S., Fukui, H. & Sohma, J. (1970). Analyses of the N.M.R. spectra of pyrrole derivatives: Pyrrole-2-carboxylic acid and pyrrole-2-aldehyde. *Mol. Phys.* **19**, 695-702.

Smith, C. G., Dietz, A., Sokolski, W. T. & Savage, G. M. (1956). Streptonivicin, a new antibiotic. I. Discovery and biologic studies. *Anitbiotics & Chemotherapy* **6**, 135-142.

Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F. & Miller, N. E. (2001). Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.* **29**, 1097-1106.

Sohng, J. K., Oh, T. J., Lee, J. J. & Kim, C. G. (1997). Identification of a gene cluster of biosynthetic genes of rubradirin substructures in *S. achromogenes* var. rubradiris NRRL3061. *Mol. Cells* **7**, 674-681.

Steffensky, M., Li, S.-M. & Heide, L. (2000a). Cloning, overexpression, and purification of novobiocic acid synthetase from *Streptomyces spheroides* NCIMB 11891. *J. Biol. Chem.* **275**, 21754-21760.

Steffensky, M., Mühlenweg, A., Wang, Z.-X., Li, S.-M. & Heide, L. (2000b). Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. *Antimicrob. Agents Chemother.* **44**, 1214-1222.

Thiara, A. S. & Cundliffe, E. (1988). Cloning and characterization of a DNA gyrase B gene from *Streptomyces sphaeroides* that confers resistance to novobiocin. *EMBO J.* **7**, 2255-2259.

Thiara, A. S. & Cundliffe, E. (1993). Expression and analysis of two gyrB genes from the novobiocin producer, *Streptomyces sphaeroides*. *Mol. Microbiol.* **8**, 495-506.

Thomas, M. G., Burkart, M. D. & Walsh, C. T. (2002). Conversion of L-proline to pyrrolyl-2-carboxyl-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis. *Chem. Biol.* **9**, 171-184.

Tsai, F. T. F., Singh, O. M., Skarzynski, T., Wonacott, A. J. et al. (1997). The high-resolution crystal structure of a 24-kDa gyrase B fragment from *E. coli* complexed with one of the most potent coumarin inhibitors, clorobiocin. *Proteins* **28**, 41-52.

Umezawa, H., Hamada, M., Takita, T., & Naganawa, H. (1971). Coumermycin A1. 46015675. JP.

Wallick, H., Harris, D. A., Reagan, M. A., Ruger, M. & Woodruff, H. B. (1955). *Antibiotic Annual* 909.

Walsh, T. (2003). Where will new antibiotics come from? *Nat. Rev. Microb.* **1**, 65-70.

Wang, Z.-X., Li, S.-M. & Heide, L. (2000). Identification of the coumermycin A1 biosynthetic gene cluster of *Streptomyces rishiriensis* DSM 40489. *Antimicrob. Agents Chemother.* **44**, 3040-3048.

Weitnauer, G., Mühlenweg, A., Trefzer, A., Hoffmeister, D. et al. (2001). Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the avi biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics. *Chem. Biol.* **8**, 569-581.

Westrich, L., Heide, L. & Li, S.-M. (2003). CloN6, a novel methyltransferase catalysing the methylation of the pyrrole-2-carboxyl moiety of clorobiocin. *Chembiochem* **4**, 768-773.

Whitaker, W. D. (1968). No. 1,111,511. British.

Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A. & Dodson, G. (1991). Crystal structure of an N-terminal fragment of the DNA gyrase B protein. *Nature* **351**, 624-629.

Xu, H., Kahlich, R., Kammerer, B., Heide, L. & Li, S.-M. (2003). CloN2, a novel acyltransferase involved in the attachment of the pyrrole-2-carboxyl moiety to the deoxysugar of clorobiocin. *Microbiology* **149**, 2183-2191.

Xu, H., Wang, Z.-X., Schmidt, J., Heide, L. & Li, S.-M. (2002). Genetic analysis of the biosynthesis of the pyrrole and carbamoyl moieties of coumermycin A(1) and novobiocin. *Mol. Genet. Genomics* **268**, 387-396.

Appendix I—MS and NMR spectral data

Coumermycin A_1: Positive ion ESI-CID mass spectrum *m/z* (relative intensity in %): 1110 ($[M+H]^+$, 3), 960 (8), 622 (10), 282 (100), 108 (9); Negative ion ESI-CID mass spectrum *m/z* (relative intensity): 1108 ($[M-H]^-$, 22), 620 (24), 594 (36), 513 (42), 487 (100), 206 (54).

Pyrrole-2-carboxylic acid: Negative ion ESI-CID mass spectrum *m/z* (relative intensity in %): 110 ($[M-H]^-$), 66 ($M-CO_2$); ^{1}H-NMR (400MHz, DMSO-d6) : δppm 6.93 (dd, J_1 = 2.5 Hz, J_2 = 1.4, H-5), 6.84 (dd, J_1 = 3.8 Hz, J_2 = 1.4 Hz, H-3), 6.17 (dd, J_1 = 3.8 Hz, J_2 = 2.5 Hz, H-4).

5-methylpyrrole-2-carboxylic acid: Negative ion ESI-CID mass spectrum *m/z* (relative intensity in %): 124 ($[M-H]^-$), 80 ($M-CO_2$).

See Table 21 for numbering of the structures; ^{1}H-NMR at 400 MHz, ^{13}C-NMR at 100 MHz, using CD_3OD as the solvent; br, broad signal.

Novclobiocin 104: Negative FAB-MS *m/z* (relative intensity in %): 588 ($[M-H]^-$), 554, 400, 225, and 209); ^{1}H-NMR (400 MHz, CD_3OD): δppm 1.10 (s, 3H-6´´), 1.31 (s, 3H-7´´), 1.74 (s, 3H-10, 3H-11), 3.34 (d, J = 7.2 Hz, 2H-7), 3.40 (d, J = 9.9 Hz, H-4´´), 3.59 (s, 3H-8´´), 4.12 (t, J = 3.1 Hz, H-2´´), 4.18 (dd, J_1 = 9.9 Hz, J_2 = 3.1 Hz, H-3´´), 5.35 (br t, J = 7.2 Hz, H-8), 5.65 (d, J = 1.6 Hz, H-1´´), 6.84 (d, J = 8.5 Hz, H-5), 7.29 (d, J = 9.0 Hz, H-6´), 7.72 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, H-6), 7.76 (br s, H-2), 7.88 (d, J = 9.0 Hz, H-5´); ^{13}C-NMR (100 MHz, CD_3OD) : δppm 17.9 (C-10), 22.9 (C-6´´), 26.0 (C-11), 29.3 (C-7), 29.6 (C-7´´), 62.3 (C-8´´), 69.4 (C-2´´), 72.7 (C-3´´), 80.1 (C-5´´), 85.2 (C-4´´), 100.5 (C-1´´), 103.6(C-3´), 110.3 (C-6´), 111.5 (C-10´), 115.4 (C-5), 123.5 (C-8), 124.5 (C-1), 125.5 (C-5´), 128.4 (C-6), 129.6 (C-3), 130.9 (C-2), 133.4 (C-9), 150.3 (C-9´), 156.2 (C-4´), 160.4 (C-4, C-7´), 170.0 (C-12), the assignment of C-2´, 4´ and 7´ may be interchangeable. The signals of C-8´ were not detectable.

Novclobiocin 105: Negative FAB-MS *m/z* (relative intensity in %): 574 ($[M-H]^-$), 540, 339, 209; ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.13 (s, 3H-6´´), 1.29 (s, 3H-7´´), 1.73 (s, 3H-10, 3H-11), 3.33 (d, J = 7.3 Hz, 2H-7), 3.76 (d, J = 10.0 Hz, H-4´´), 4.12 (dd, J_1 = 10.0 Hz, J_2 = 3.3 Hz, H-3´´), 4.13 (br s, overlapping with the signals of H-3´´, H-2´´), 5.35 (br t, H-8), 5.66 (br s, H-1´´), 6.82 (d, J = 8.5 Hz, H-5), 7.24(d, J = 8.8 Hz, H-6´), 7.72 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, H-6), 7.76 (s, H-2), 7.88 (d, J = 8.8 Hz, H-5´); ^{13}C-NMR (100 MHz, CD_3OD) : δppm 17.9 (C-10), 22.4 (C-6´´), 26.0 (C-11), 29.2 (C-7), 29.3 (C-7´´), 62.3 (C-8´´), 68.9 (C-2´´), 72.4 (C-3´´), 74.4 (C-4´´), 80.6 (C-5´´), 100.6 (C-1´´), 102.6(C-3´), 110.5 (C-6´), 111.5 (C-10´), 112.0 (C-5), 123.4 (C-8), 124.5 (C-1), 124.9 (C-5´), 128.4 (C-6), 129.6 (C-3), 130.8 (C-2), 133.6 (C-9), 150.1 (C-9´), 156.4 (C-4´), 160.5 (C-4, C-7´), 169.9 (C-12), the assignment of C-2´, 4´ and 7´ may be interchangeable. The signals of C-8´, C-8´´ were not detectable.

Novclobiocin 107: Negative FAB-MS *m/z* (relative intensity in %): 554 (48, $[M-H]^-$), 392(9), 379(20), 366(20), 336(7), 314(11), 283(11), 253(15), 237(11), 209(100), 207(39), 190(27); ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.11 (s, 3H-6´´), 1.33 (s, 3H-7´´), 1.74 (s, 3H-11), 1.75 (s, 3H-10), 3.34 (d, J = 6.8 Hz, 2H-7), 3.37 (d, J = 9.4 Hz,

H-4´´), 3.58 (s, 3H-8´´), 4.02 (t, J = 3.1 Hz, H-2´´), 4.09 (dd, J_1 = 9.4 Hz, J_2 = 3.1 Hz, H-3´´), 5.35 (tt, J_1 = 7.4 Hz, J_2 = 1.2 Hz, H-8), 5.56 (d, J = 2.3 Hz, H-1´´), 6.85 (d, J = 8.4 Hz, H-5), 7.04 (complex overlapping signals, J not determinable, H-6´, H-8´), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.75 (d, J = 2.2 Hz, H-2), 7.89 (d, J = 9.5 Hz, H-5´)

Novclobiocin 108: Negative FAB-MS *m/z* (relative intensity in %): 540(27, $[M-H]^-$), 415(7), 380(16), 352(11), 338(7), 314(15), 312(7), 283(24), 255(16), 209(100), 207(35), 191(8); ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.14 (s, 3H-6´´), 1.32 (s, 3H-7´´), 1.74 (s, 3H-11), 1.75 (s, 3H-10), 3.34 (d, J = 7.3 Hz, 2H-7), 3.73 (d, J = 9.5 Hz, H-4´´), 4.03 (dd, J_1 = 3.4 Hz, J_2 = 9.5 Hz, H-3´´), 4.04 (br s, H-2´´), 5.35 (br t, J = 7.3 Hz, H-8), 5.58 (br s, H-1´´), 6.84 (d, J = 8.4 Hz, H-5), 7.04 (complex overlapping signals, J not determinable, H-6´, H-8´), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.75 (br s, H-2), 7.90 (d, J = 9.4 Hz, H-5´)

Novclobiocin 114: Negative FAB-MS *m/z* (relative intensity in %): 631(17, $[M-H]^-$), 597(6), 524(6), 443(6), 358(6), 326(7), 283(18), 255(22), 209(100); ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.17 (s, 3H-6´´), 1.33 (s, 3H-7´´), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.55 (s, 3H-8´´), 3.65 (br s, H-4´´), 4.29 (t, J = 3.0 Hz, H-2´´), 5.35 (overlapping with the signals of H-3´´, H-8), 5.37 (dd, J_1 = 10.1 Hz, J_2 = 3.1 Hz, H-3´´), 5.63 (d, J = 1.6 Hz, H-1´´), 6.80 (d, J = 8.2 Hz, H-5), 7.19 (d, J = 9.0 Hz, H-6´), 7.71 (dd, J_1 = 8.2 Hz, J_2 = 2 Hz, H-6), 7.76 (s, H-2), 7.88 (d, J = 9.0 Hz, H-5´)

Novclobiocin 115: Negative FAB-MS *m/z* (relative intensity in %): 617(3, $[M-H]^-$), 524(2), 485(2), 462(6), 419(6), 388(6), 356(10), 314(16), 283(84), 255(61), 209(100); ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.20 (s, 3H-6´´), 1.32 (s, 3H-7´´), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.93 (d, J = 10.3 Hz, H-4´´), 4.33 (br s, H-2´´), 5.27 (dd, J_1 = 10.3 Hz, J_2 = 3.1 Hz, H-3´´), 5.35 (br s, H-8), 5.65 (br s, H-1´´), 6.81 (br s, H-5), 7.21(br s, H-6´), 7.73 (br s, H-6), 7.77 (s, H-2), 7.90 (br s, H-5´)

Novclobiocin 117: Negative FAB-MS *m/z* (relative intensity in %): 597(6, $[M-H]^-$), 554(2), 491(2), 427(2), 411(7), 370(7), 307(9), 306(29), 258(37), 257(69), 209(100), 207(25); ^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.17 (s, 3H-6´´), 1.35 (s, 3H-7´´), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.54 (complex overlapping signals, 3H-8´´, H-4´´), 4.17 (t, J = 2.8 Hz, H-2´´), 5.25 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, H-3´´), 5.35 (br t, J = 7.2 Hz, H-8), 5.55 (d, J = 2.5 Hz, H-1´´), 6.81 (d, J = 8.4 Hz, H-5), 6.97 (complex overlapping signals, J not determinable, H-6´, H-8´), 7.71 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.76 (br s, H-2), 7.91 (d, J = 9.0 Hz, H-5´)

Novclobiocin 118: Negative FAB-MS *m/z* (relative intensity in %): 583(10, $[M-H]^-$), 540(6), 522(2), 462(5), 402(5), 380(6), 325(6), 314(13), 283(97), 255(70), 209(100); ^{1}H-NMR (400 MHz, CD_3OD): δppm 1.20 (s, 3H-6´´), 1.34 (s, 3H-7´´), 1.74 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.90 (d, J = 10.0 Hz, H-4´´), 4.24 (t, J = 3.3 Hz, H-2´´), 5,15 (dd, J_1 = 10.0 Hz, J_2 = 3.3 Hz, H-3´´), 5.35 (br t, J = 7.2 Hz, H-8), 5.57 (d, J = 2.2 Hz, H-1´´), 6.82 (d, J = 8.4 Hz, H-5), 7.00 (complex overlapping signals, J not determinable, H-6´, H-8´), 7.71 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.76 (s, H-2), 7.91 (d, J = 9.3 Hz, H-5´)

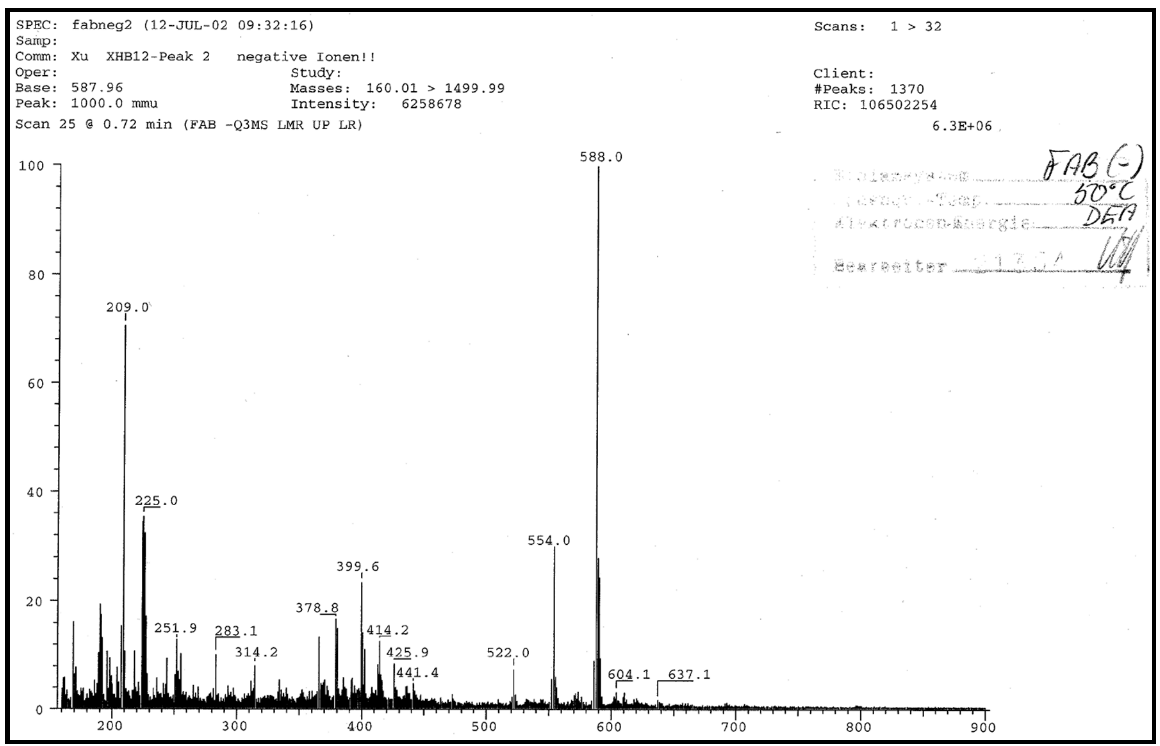
Novclobiocin 284: Negative FAB-MS *m/z* (relative intensity in %): 643(2, $[M-H]^-$), 569(2), 524(6), 491(3), 463(6), 419(6), 356(10), 314(25), 283(37), 253(41), 209(100);

^{1}H-NMR (400 MHz, CD_3OD) : δppm 1.17 (s, 3H-6´´), 1.32 (s, 3H-7´´), 3.55 (complex overlapping signals, *J* not determinable, H-4´´ and 3H-8´´), 4.27 (t, *J* = 3.1 Hz, H-2´´), 5.37 (dd, J_1 = 10.0 Hz, J_2 = 3.1 Hz, H-3´´), 5.63 (d, *J* = 1.8 Hz, H-1´´), 6.94 (d, *J* = 8.4 Hz, H-5), 7.19 (d, *J* = 8.1 Hz, H-6´), 7.87 (overlapping signals of H-6 and H-5´), 8.19 (br s, H-2).

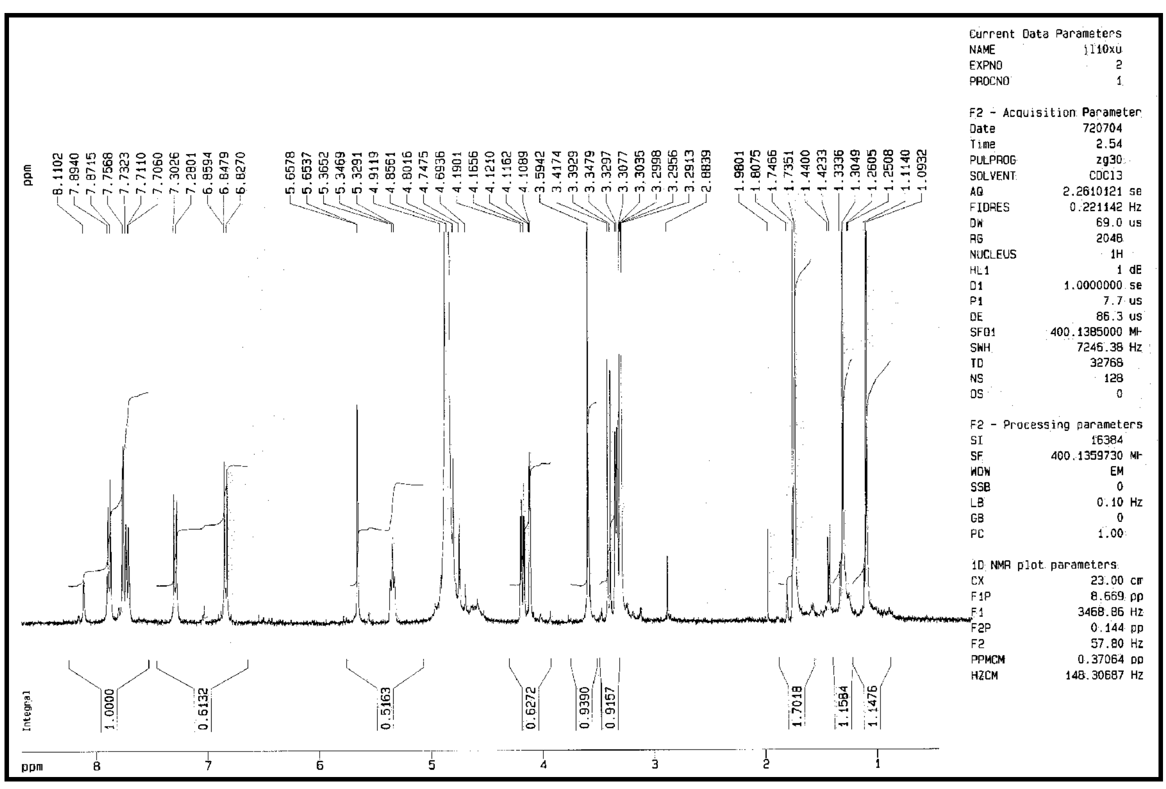
Appendix II—MS and NMR spectra

Novclobiocin 104

MS spectrum

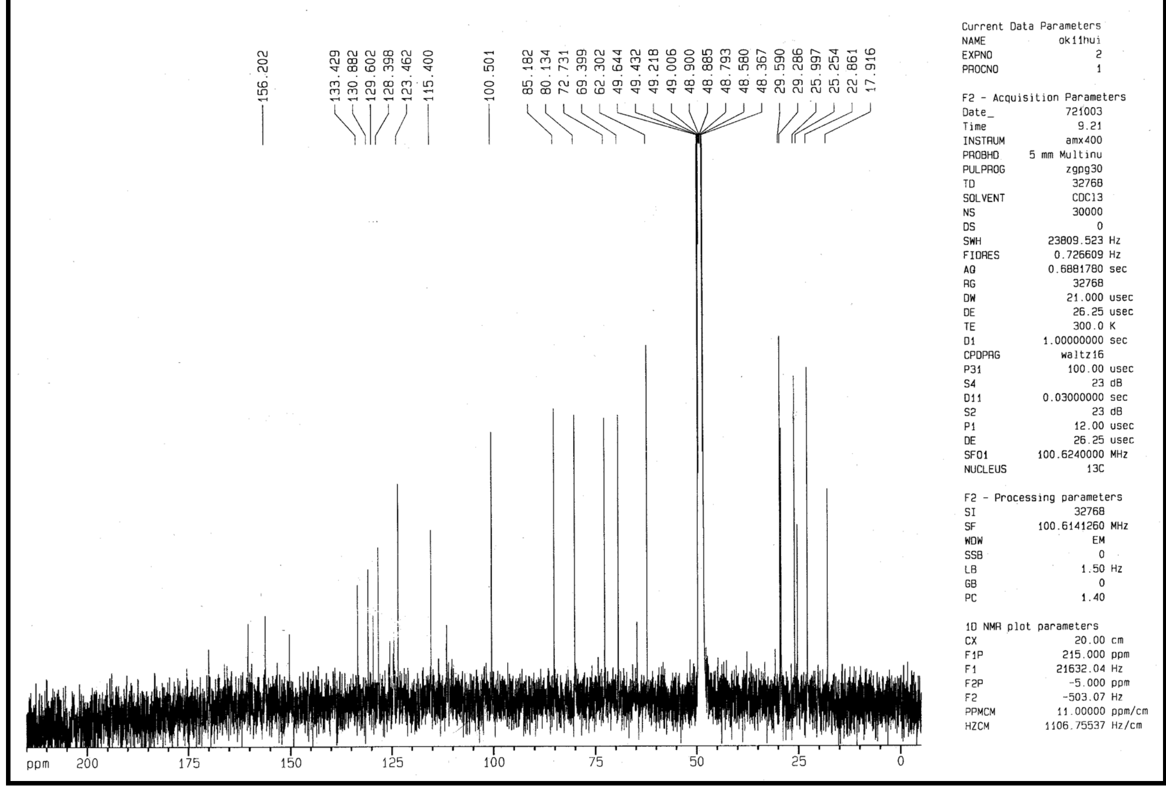


^{1}H-NMR spectrum (400 MHz, CD_3OD)



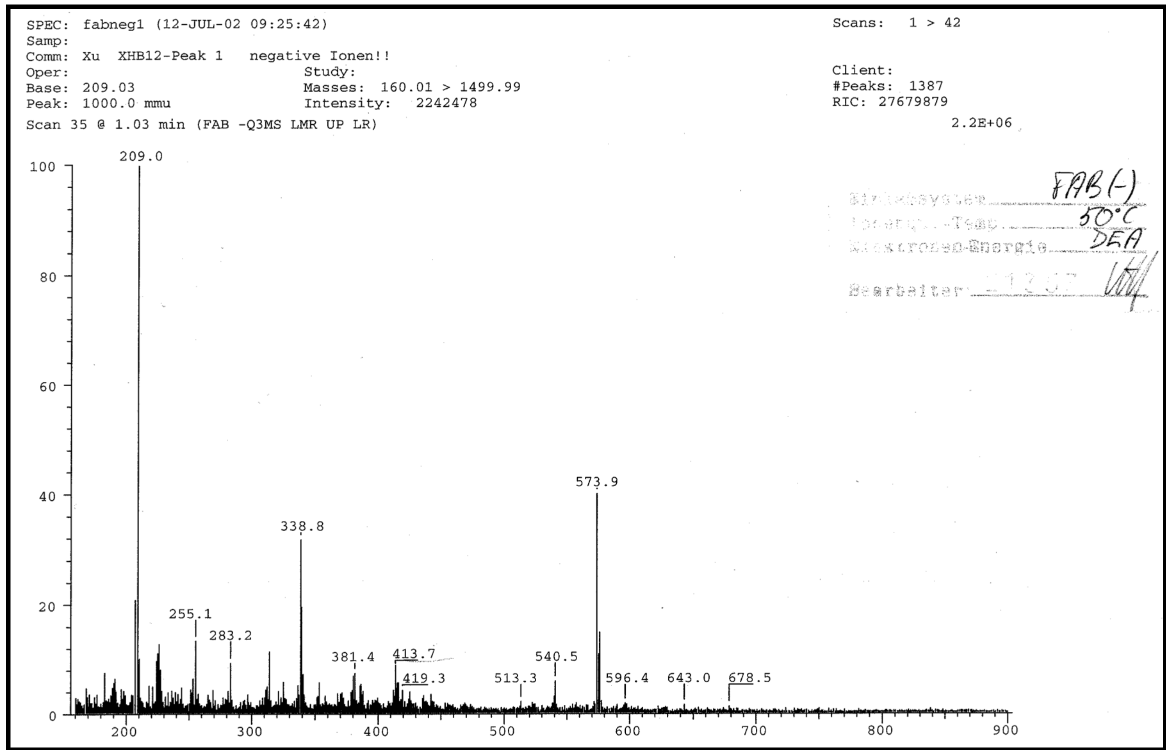
Novclobiocin 104

^{13}C-NMR spectrum (100 MHz, CD_3OD)

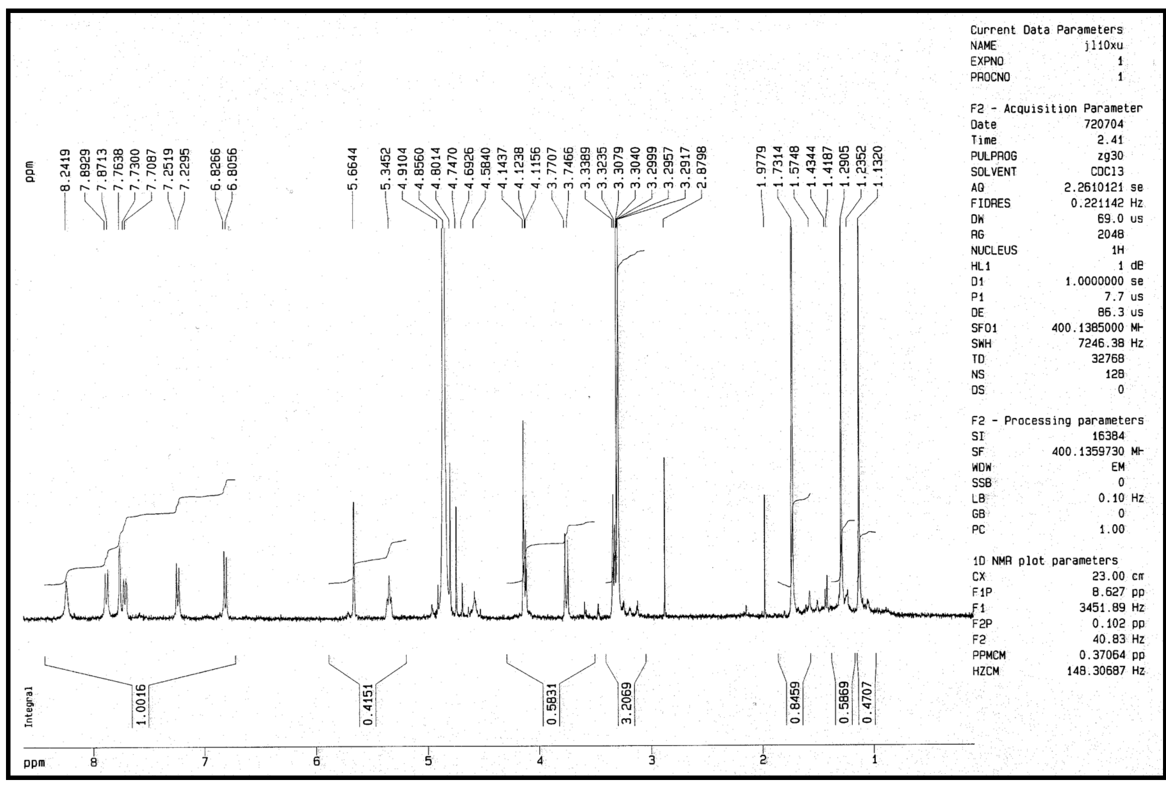


Novclobiocin 105

MS spectrum

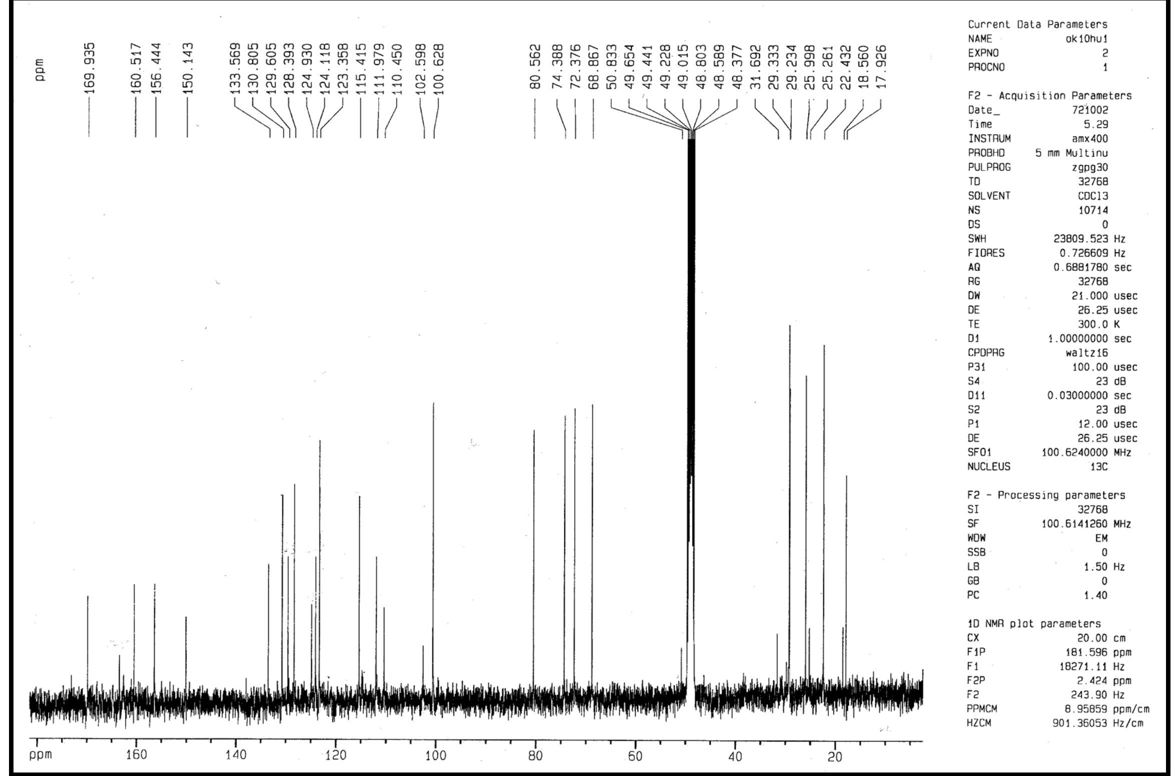


^{1}H-NMR spectrum (400 MHz, CD_3OD)



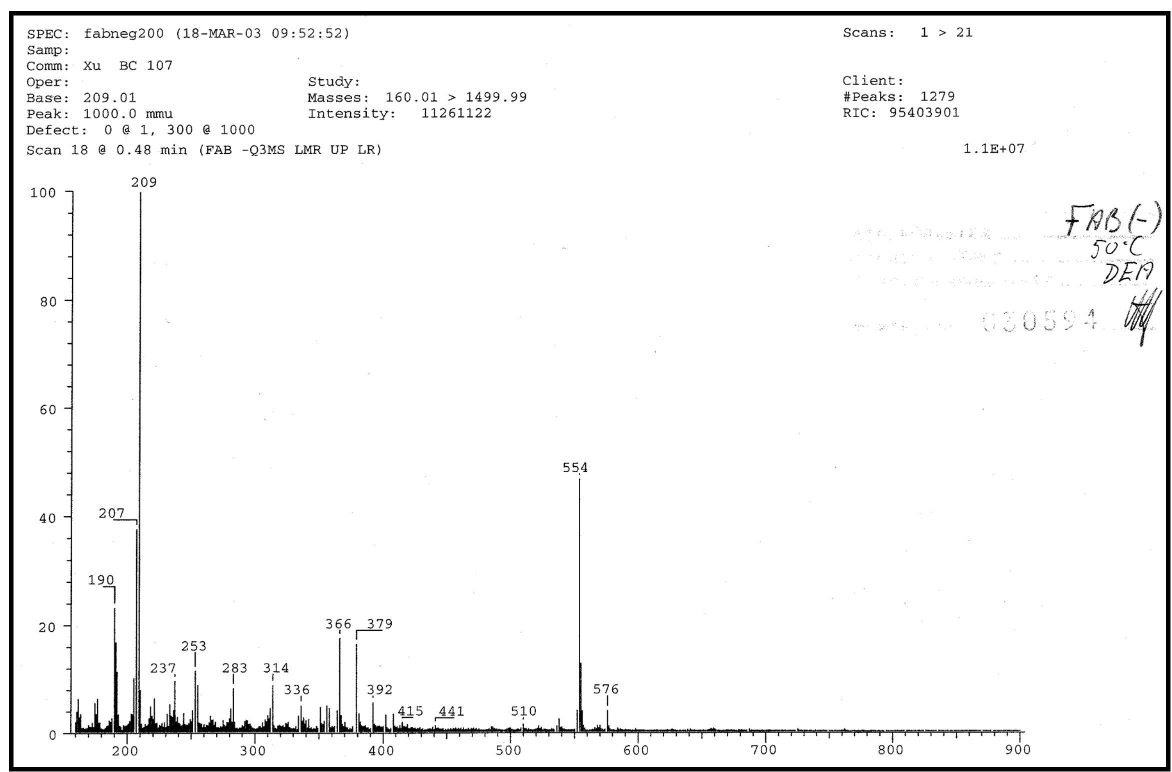
Novclobiocin 105

^{13}C-NMR spectrum (100 MHz, CD_3OD)

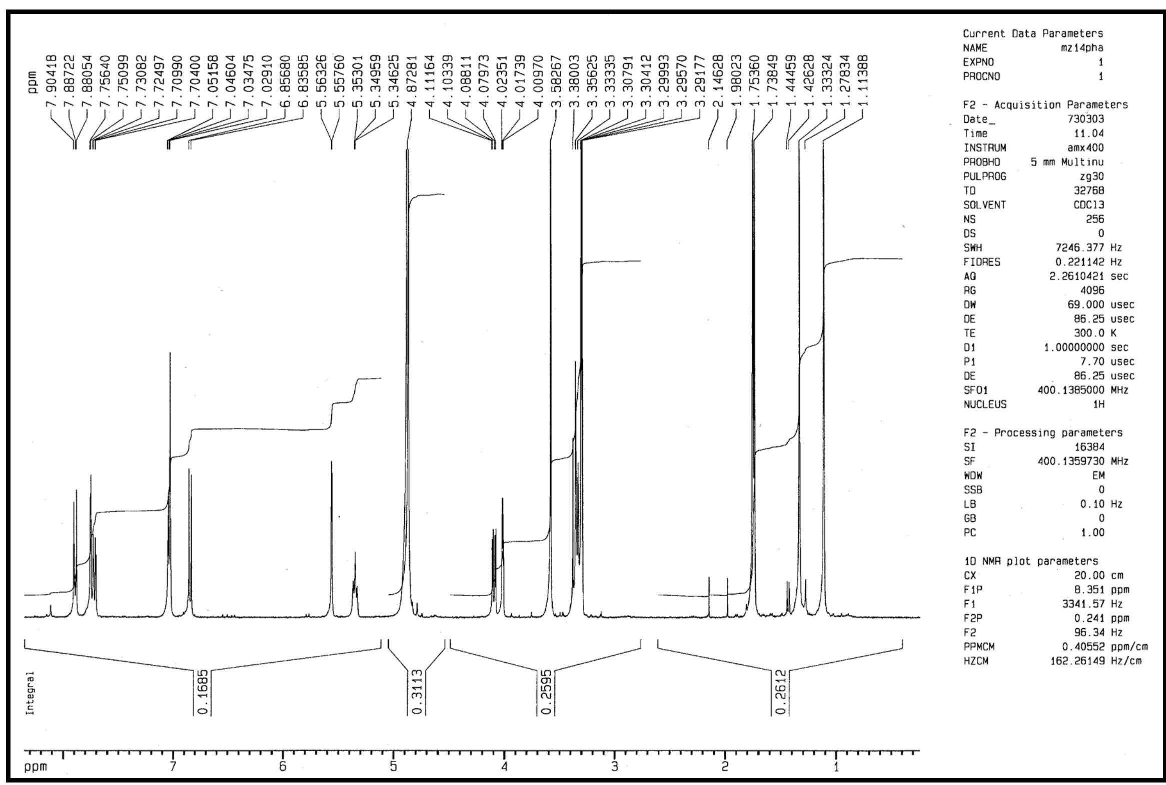


Novclobiocin 107

MS spectrum

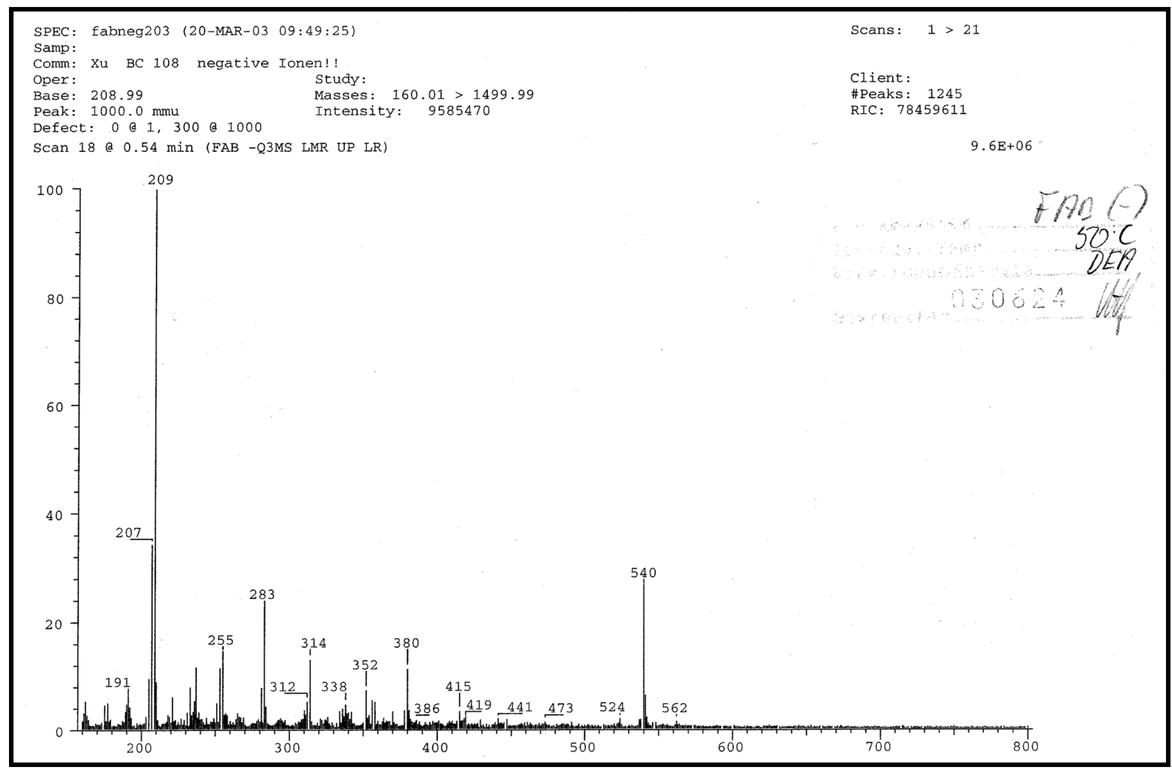


^{1}H-NMR spectrum (400 MHz, CD_3OD)

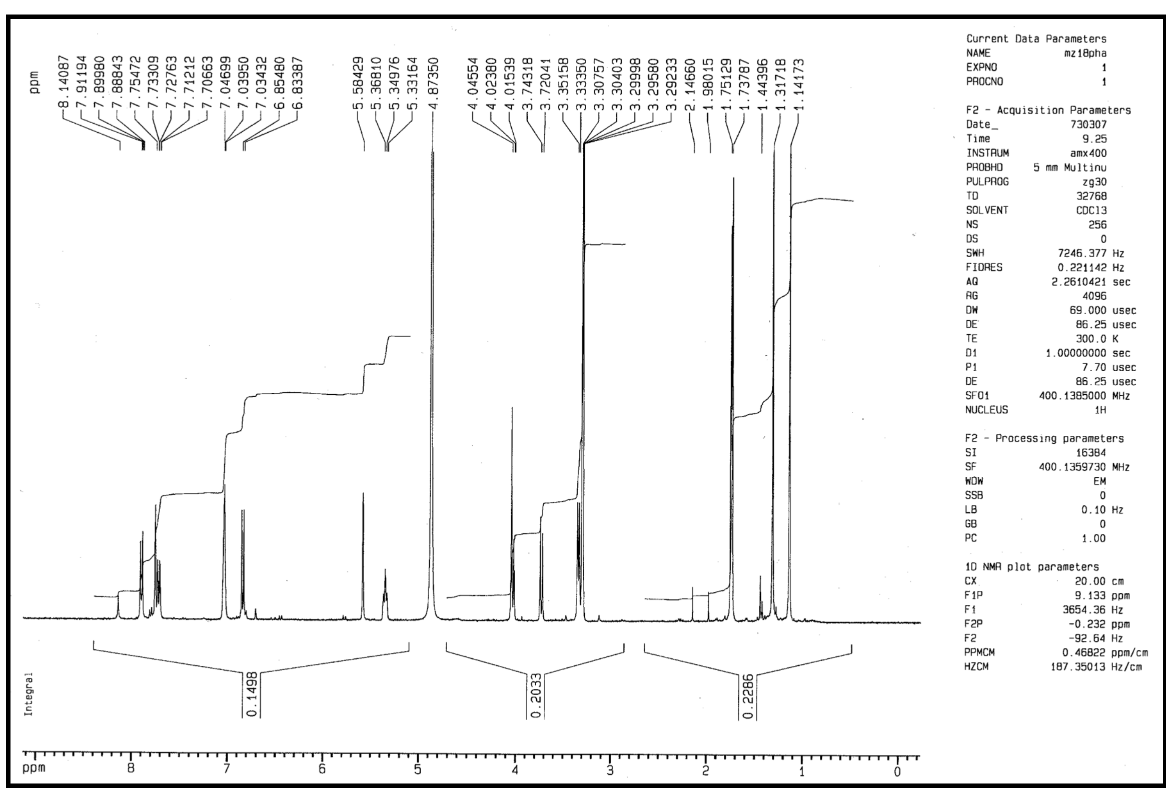


Novclobiocin 108

MS spectrum

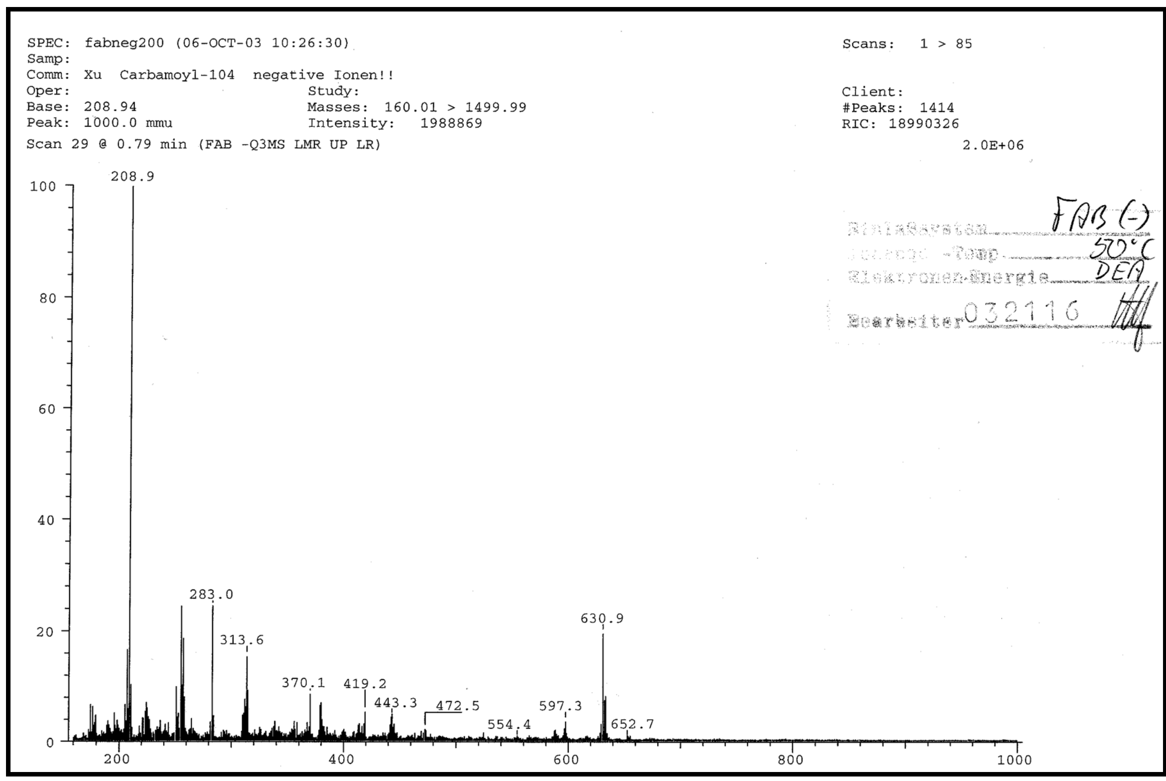


^{1}H-NMR spectrum (400 MHz, CD_3OD)

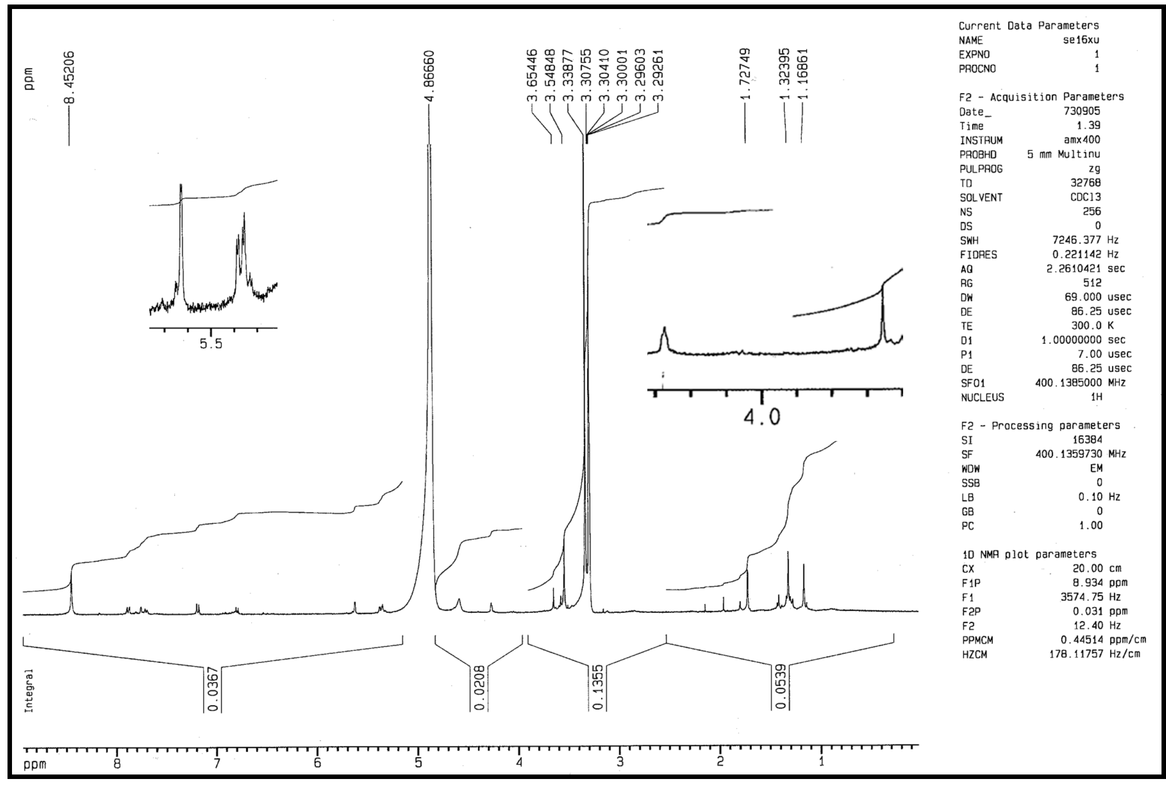


Novclobiocin 114

MS spectrum

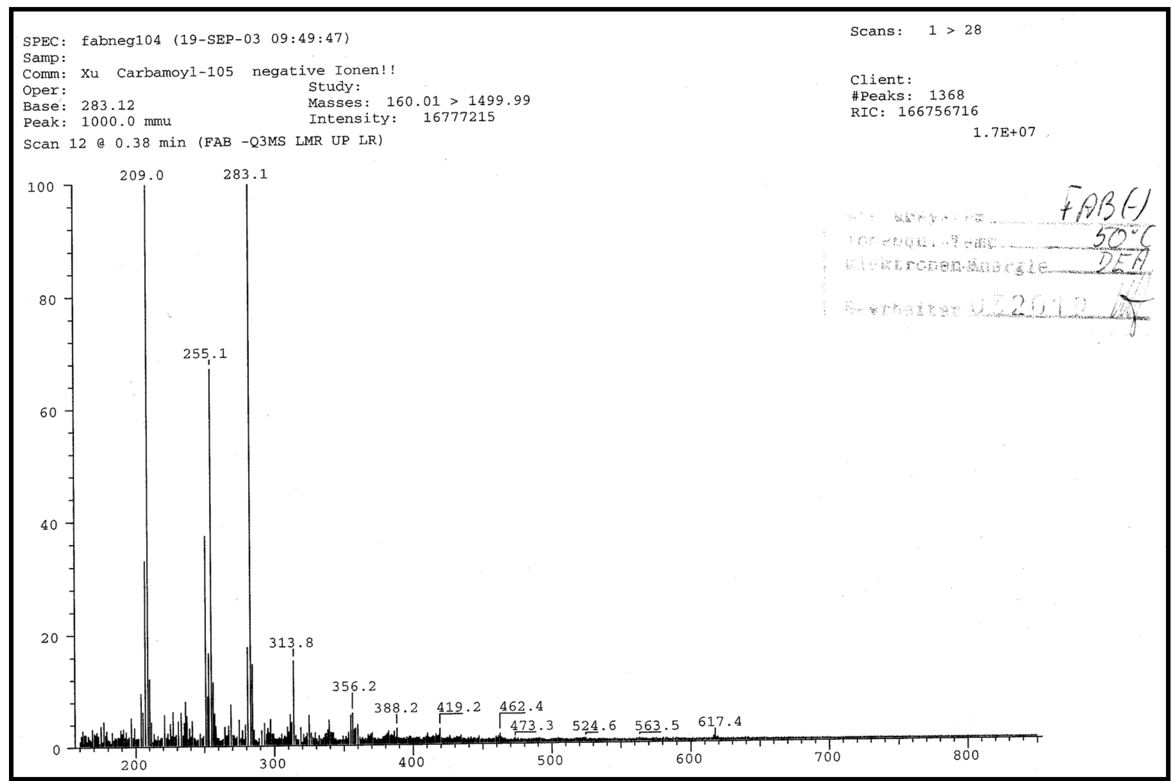


^{1}H-NMR spectrum (400 MHz, CD_3OD)

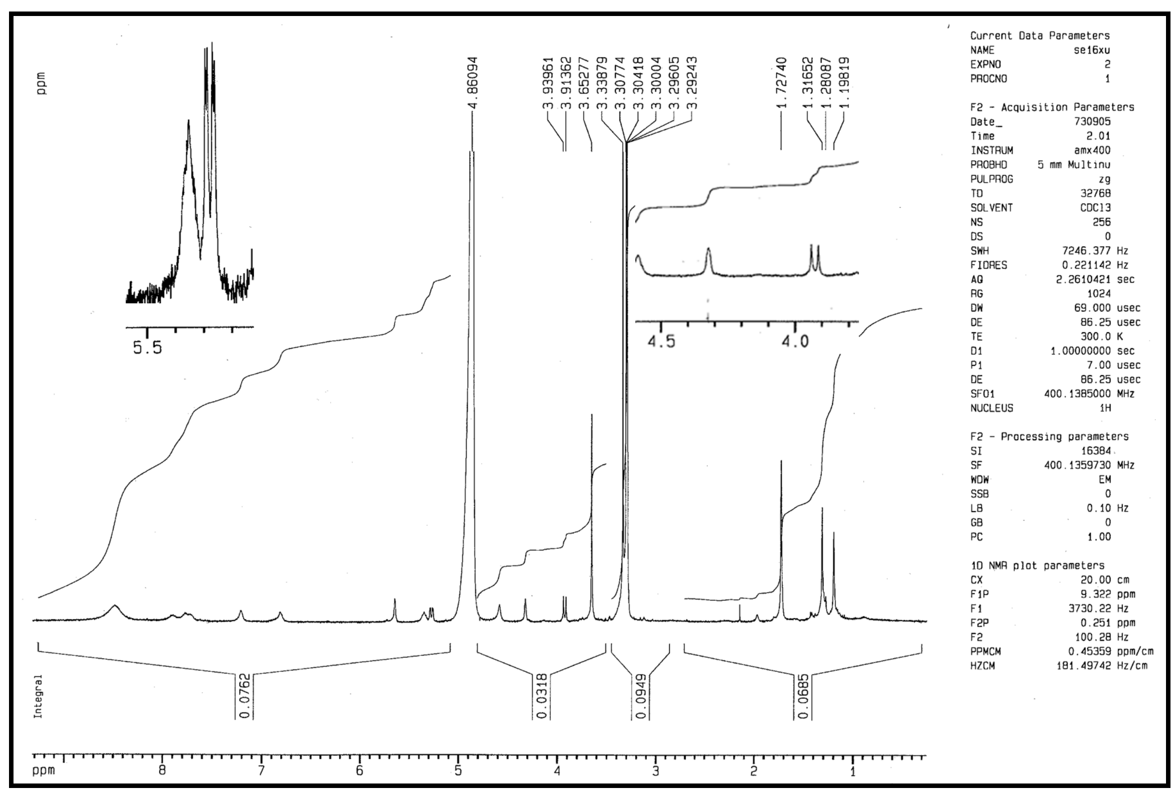


Novclobiocin 115

MS spectrum

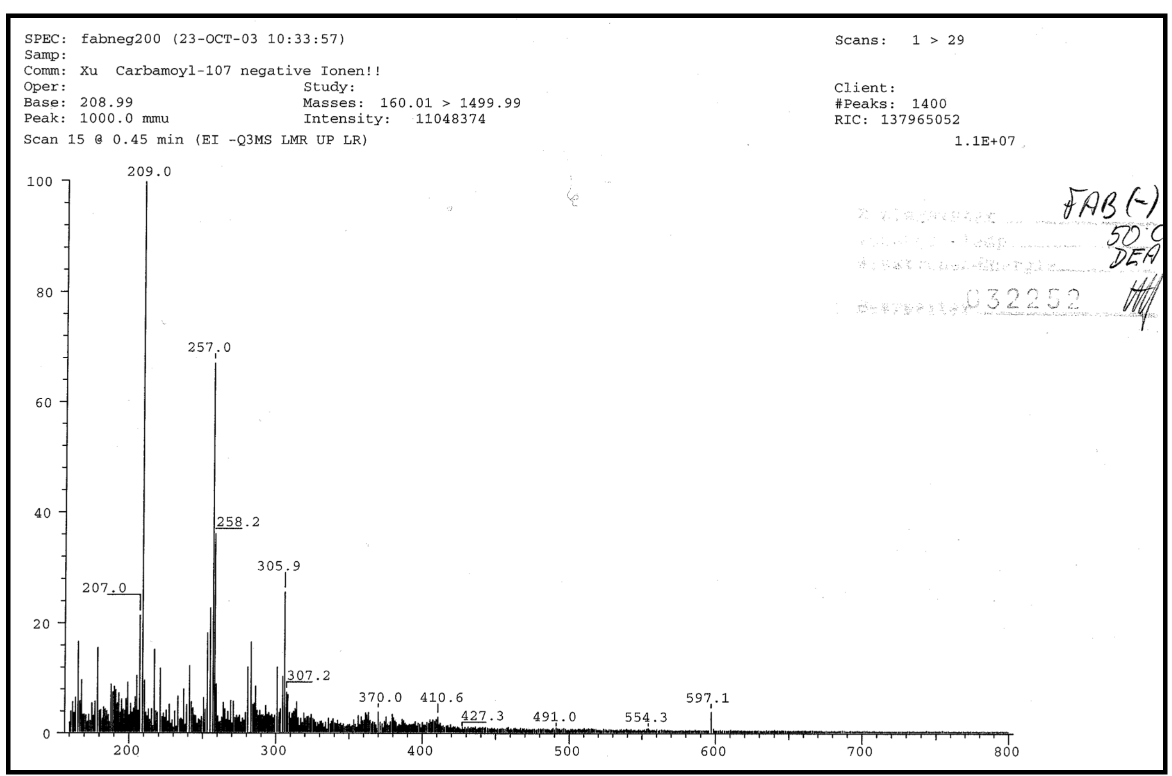


^{1}H-NMR spectrum (400 MHz, CD_3OD)

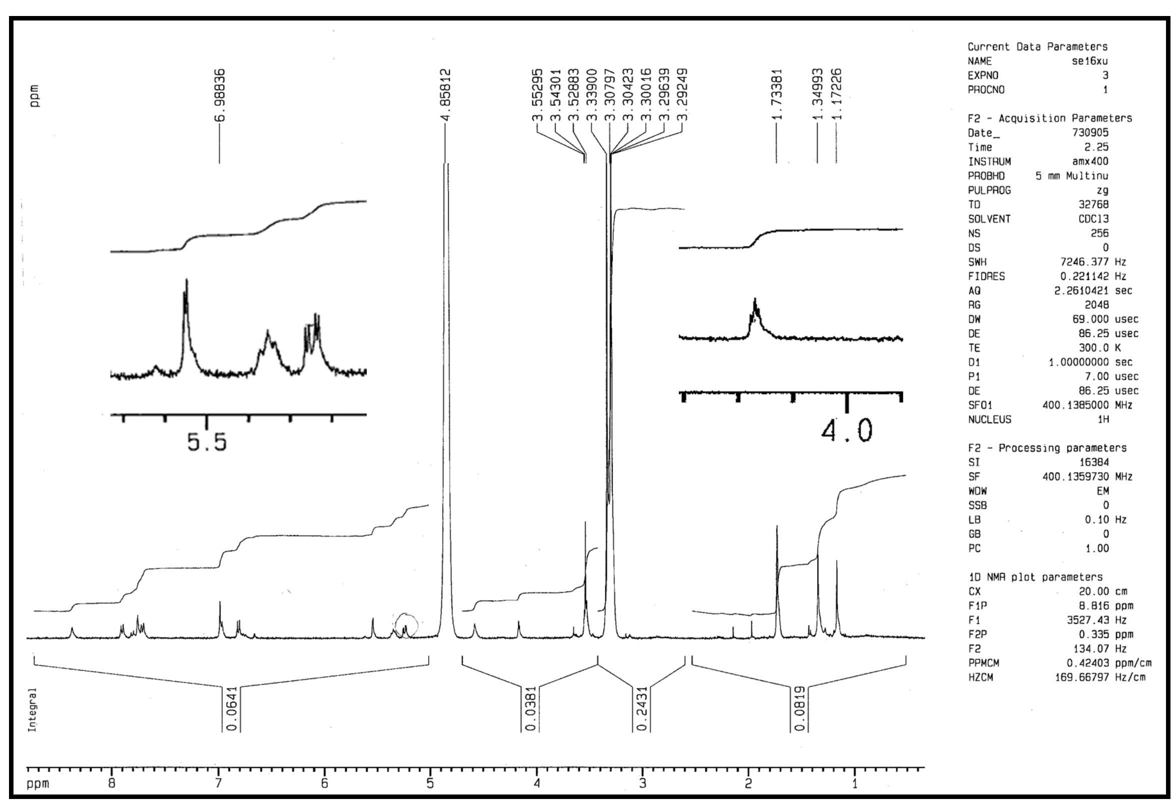


Novclobiocin 117

MS spectrum

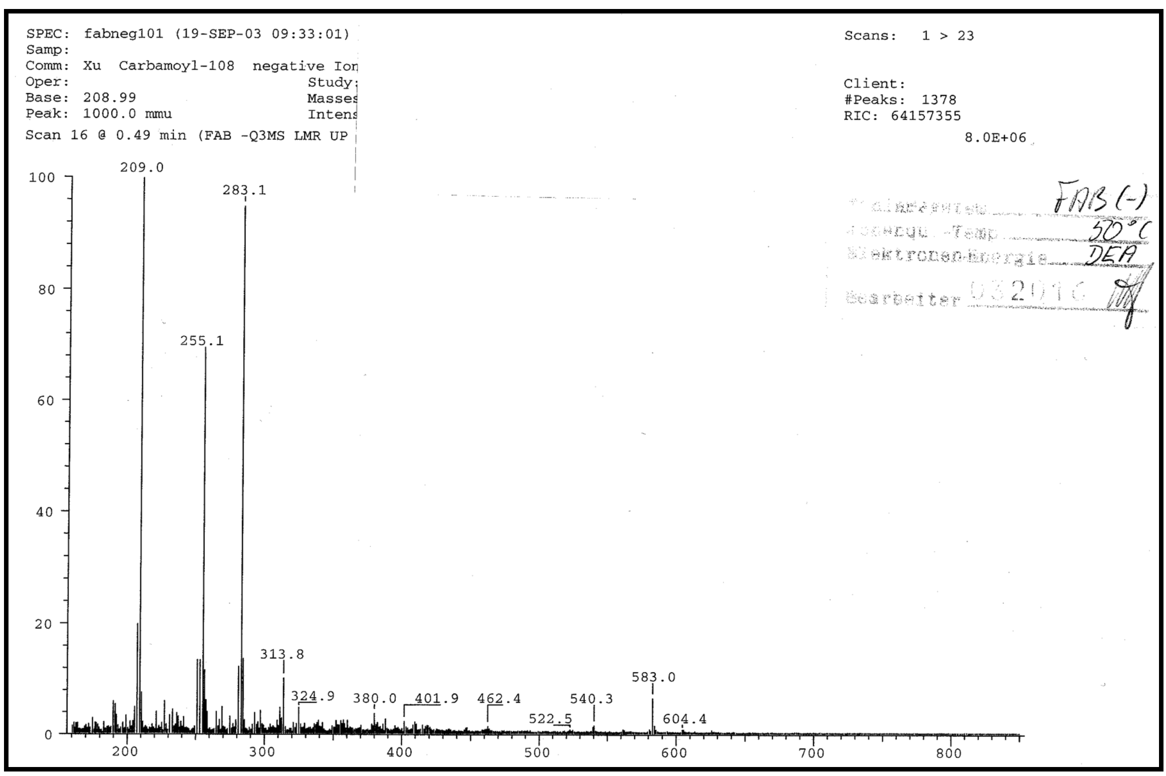


^{1}H-NMR spectrum (400 MHz, CD_3OD)

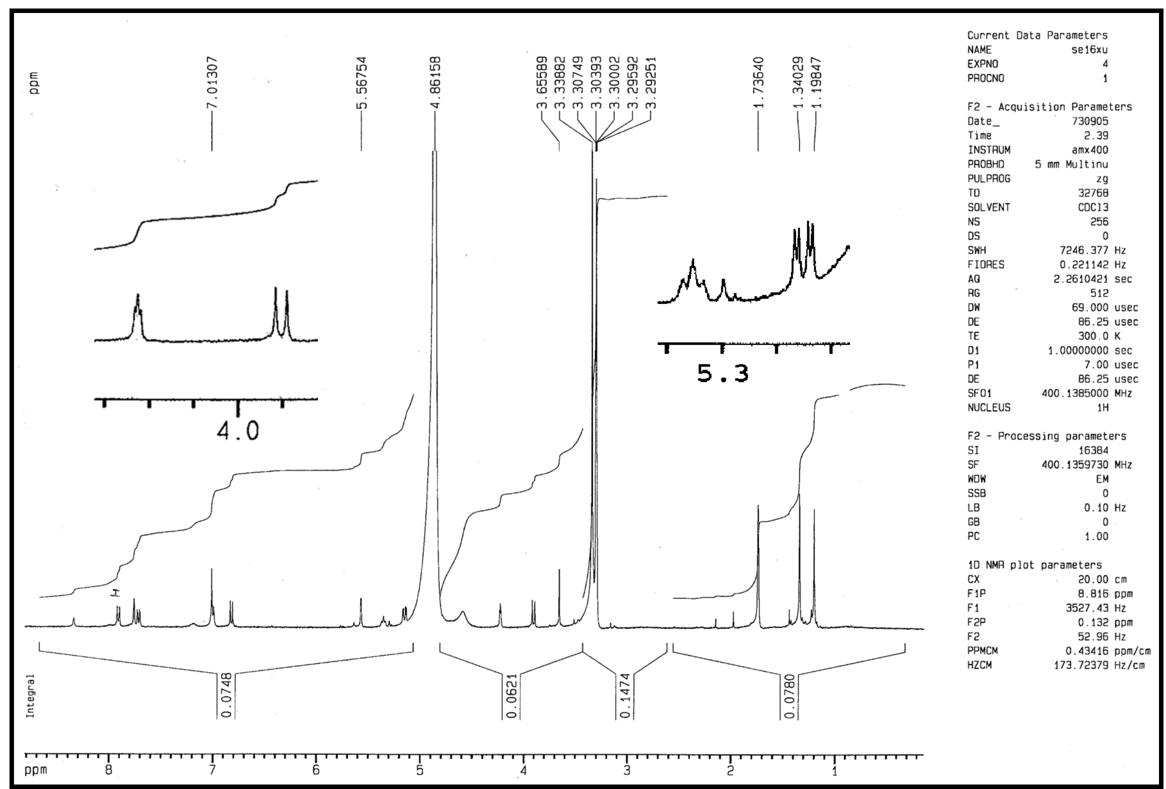


Novclobiocin 118

MS spectrum

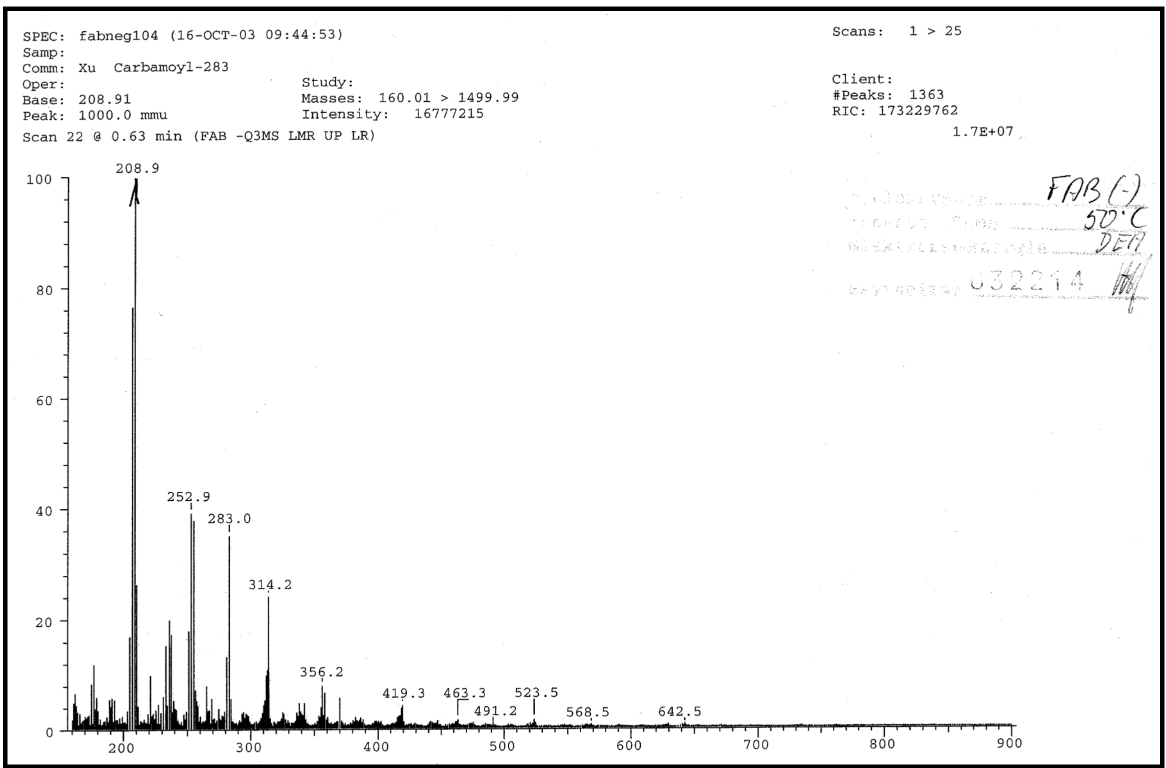


^{1}H-NMR spectrum (400 MHz, CD_3OD)

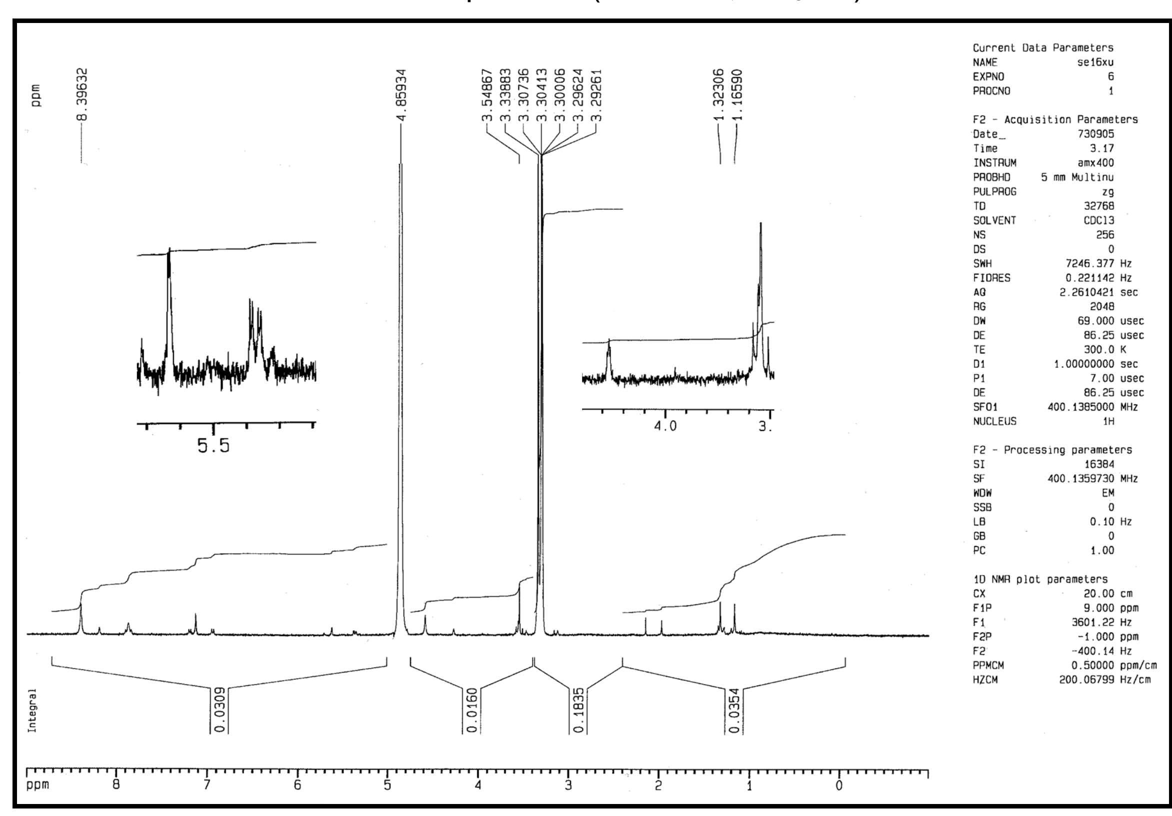


Novclobiocin 284

MS spectrum

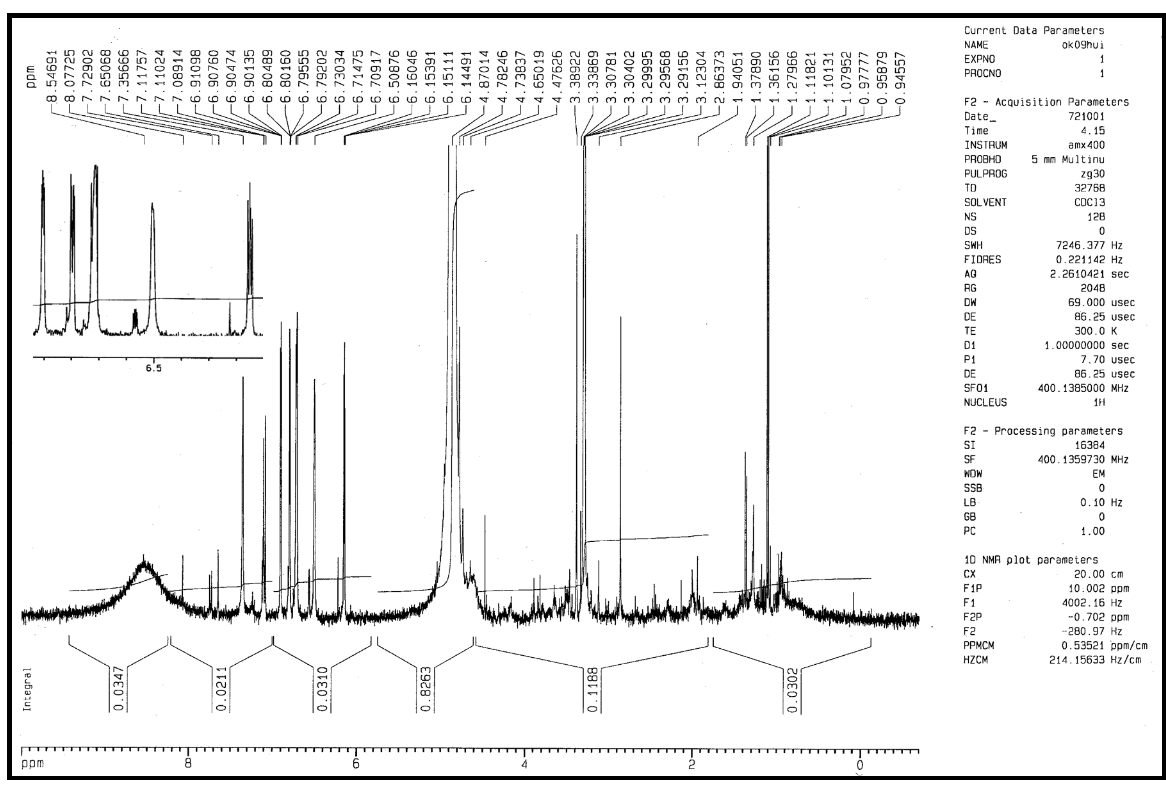


^{1}H-NMR spectrum (400 MHz, CD_3OD)



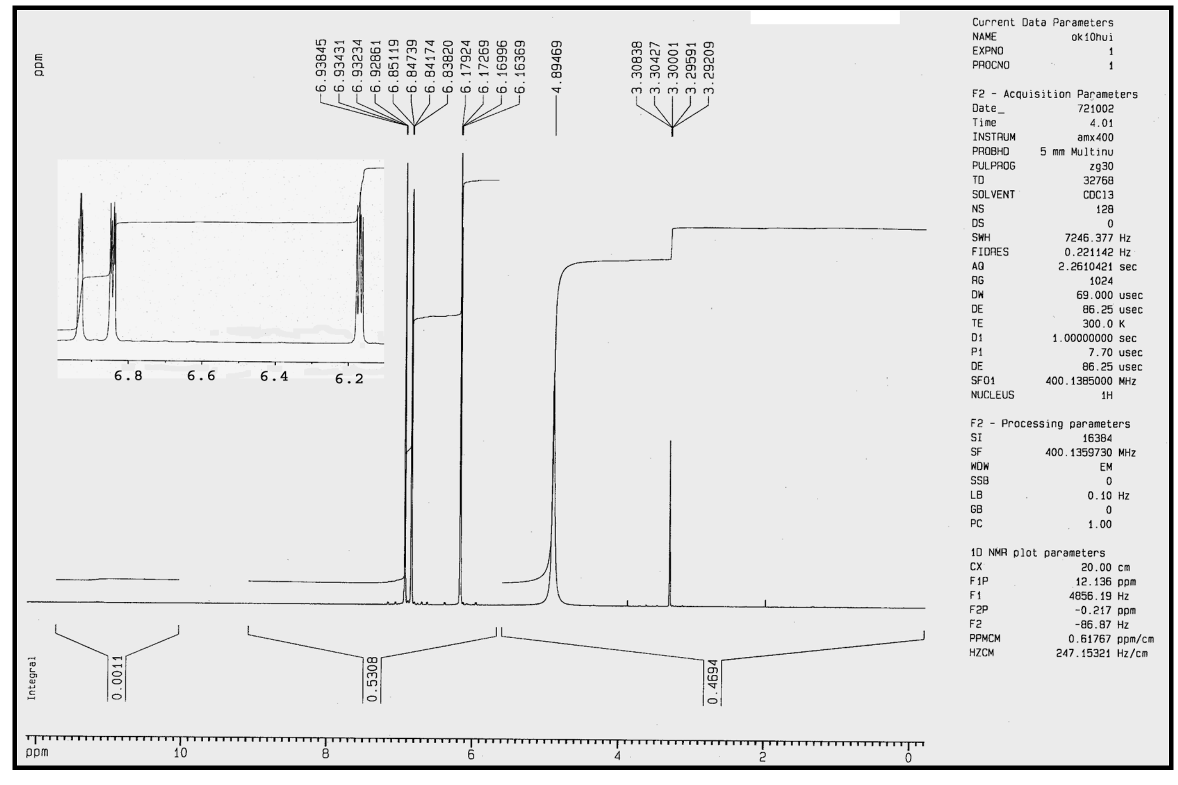
Pyrrole-2-carboxylic acid isolated from *clo*N2$^-$ mutant

^{1}H-NMR spectrum (400 MHz, DMSO-d6)



Authentic pyrrole-2-carboxylic acid

^{1}H-NMR spectrum (400 MHz, DMSO-d6)



ACADEMIC TEACHERS

I express my gratitude to all my academic teachers:

Zhongshan University, Department of Chemistry

Prof. Qipeng Huang

Prof. Jinyuan Mo

Prof. Xiuhuan Yang

Prof. Peixiang Cai

Prof. Pinyan Kong

Prof. Guangtao Zhong

Prof. Zhanxia Zhang

Prof. Kailiang Shi

University of Tuebingen, Pharmaceutical Institute

Prof. Dr. Lutz Heide

PD Dr. Shu-Ming Li

Acknowledgments

This study is performed in Pharmaceutical Institute, University of Tuebingen.

I am indebted to Prof. Dr. Lutz Heide for the opportunity to study in his group and important advice during this study. His support and encouragement have contributed decisively to the success of this thesis.

I express my greatest gratitude to Dr. Shu-Ming Li for his longstanding support, stimulating discussions and valuable counsels through out the study period. Many thank to his wife, Mrs Zhao-Xin Wang, for her help and the excellent preliminary study of my thesis. Their friendship is invaluable for me.

I am grateful to Dr. Jürgen Schmidt, Dr. Bernd Kammerer and Rainer Kahlich for LC/MS analysis.

I am especially grateful to Mrs. Lörcher and Mrs. Bauer for their effort in organizing the labs and for the loveliest spring, Eastern and Christmas atmosphere they brought in the lab.

I would like to thank Elisabeth, Ute and Alessandra for lots of helpful advice and for the good time we had together. Thomas is thanked for his prompt help in computer disasters.

Special thanks to Dr. Katja Taxis for the time she took for checking the languages of this thesis.

I also wish to thank Lucy, an expert in experimental works, for her help through out this study. And many thanks to Susanne for her help with “Sally”.

I offer my warmest gratitude to all the other colleagues and ex-colleagues of “Pharmazeutische Biologie”, Alex, Anja, Anna, Christine, Claudia, Emmanuel, Florence, Inge, Irmela, Marion, Munuel, Yvonne, for their support at anytime and the pleasant atmosphere in the lab.

At last, I want to think my family for their patience in waiting for me and a special thank to “Zhutou Bing”, who always had to carry my super heavy suitcases during my journeys between Guangzhou and Hongkong airport.

Curriculum vitae

Name:	Hui XU
Date of Birth:	10.01.70
Place of Birth:	Fujian, PR. China

1977-1982	Elementary school in Fuan, Fujian Province, PR China
1982-1983	Secondary school in Fuan, Fujian Province, PR China
1983-1988	Secondary school in Ningde, Fujian Province, PR China
09/1988-07/1992	Undergraduate student at Zhongshan University, Department of Chemistry in Guangzhou, PR China
07/1992	Bachelor of Science in Chemistry
09/1992-07/1995	Graduate student at Zhongshan University, Department of Chemistry in Guangzhou, PR China; Master thesis supervised by Prof. Guangtao Zhong, Title: “Multivariate analysis of trace elements in hair and blood samples of diabetes patients as well as in alloxan-induced diabetic rats”
07/1995	Master of Science in Analytical Chemistry
08/1995-08/1999	Academic staff at Guangzhou University of Traditional Chinese Medicine, College of Traditional Chinese Drugs, PR China
11/1997	Position of lecturer
9/1999-07/2000	German course at Tongji University, College of German, Shanghai, PR China, finished with certificate of level 2
10/2000-05/2004	PhD student at the University of Tuebingen, Department of Chemistry and Pharmacy, Tuebingen, Germany. Doctoral thesis supervised by Prof. Dr. Lutz Heide and Dr. Shu-Ming Li, Title: “Molecular biological and biochemical investigation of the biosynthesis of aminocoumarin antibiotics”
05/2004	Doctor of Science