Molecular and biochemical investigation of the biosynthesis of clorobiocin in *Streptomyces roseochromogenes* DS 12.976

Molekularbiologische und biochemische
Untersuchungen zur Biosynthese
von Clorobiocin
in *Streptomyces roseochromogenes* DS 12.976

DISSERTATION

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Florence Pojer

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Dekan: Prof. Dr. H. Probst

1. Berichterstatter: Prof. Dr. L. Heide

2. Berichterstatter: Prof. Dr. A. Bechthold

A ma famille française et allemande.

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ABBREVIATIONS

3DMA-4HB	3-Dimethylallyl-4-hydroxybenzoic acid (Ring A)
3DMA-4HMA	3-Dimethylallyl-4-hydroxymandelic acid
3DMA-4HPP	3-Dimethylallyl-4-hydroxyphenylpyruvate
4HPP	4-Hydroxyphenylpyruvate
aa	Amino acids
ACP	Acyl-carrier protein
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ß-OH-Tyr	ß-hydroxytyrosine
bp	Base pair
Bq	Bequerel
CoA	Coenzyme A
cpm	Counts per minute
Da	Dalton
DIG	Digoxigenine
DMAPP	Dimethylallylpyrophosphate
DMAT	Dimethylallyltryptophane synthase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
DTT	1,4-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiamintetraacetic acid
ESI	Electrospray ionization
g	Gram
gyrB	Gyrase B subunit
h	Hour
HCOOH	Formic acid
HPLC	High performance liquid chromatography
IPP	Isopentenyldiphosphate
IPTG	Isopropyl-ß-thiogalactoside
k	Kilo
kb	Kilobase
kD	Kilodalton
K_m	Michaelis-Menten constant
L	Liter
LB	Luria and Broth
LC-ESI-CID	Liquid chromatography- Electrospray ionization- Collision induced dissociation
M	Molar
min	Minute
M_r	Molecular weight
MS	Mass spectrum
NCIBM	National Collection of Industrial, Food and Marine Bacteria
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction

PEG	Polyethylenglycol
ppm	parts per million
Ring A	3-Dimethylallyl-4-hydroxybenzoic acid
Ring B	3-Amino-4,7-dihydroxy-8-methylcoumarin
Ring C	Noviose
s	Second
SAM	S-Adenosylmethionin
SDS	Sodiumdodecylsulfate
Tyr	Tyrosine
UV	Ultraviolet
WT	Wild type

Publications and presentations at scientific meetings

Research publications:

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

F. Pojer, E. Wemakor, B. Kammerer, H. Chen, C.T. Walsh, S.-M. Li and L. Heide (2003a). CloQ, a prenyltransferase involved in clorobiocin biosynthesis. *PNAS* 100, 2316-2321.

F. Pojer, R. Kahlich, B. Kammerer, S.-M. Li and L. Heide (2003b). CloR, a bifunctional non-heme iron oxygenase involved in clorobiocin biosynthesis. *JBC*, in press.

Presentations at scientific meetings:

September 3-7 2000: Poster presentation at the GA congress ("Natural products research in the new millennium") in Zürich, Switzerland.

September 24-26 2000: Poster presentation at the VAAM Workshop ("Biologie bakterieller Naturstoffproduzenten") in Bonn, Germany.

August 5-9 2001: Poster presentation at the ISBA conference in Vancouver, Canada.

November 15-17 2001: Oral 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.

SUMMARY/ ZUSAMMENFASSUNG

Summary

Aminocoumarin antibiotics, such as novobiocin, clorobiocin and coumermycin A_1, are produced by various *Streptomyces* strains and are very potent against gram-positive pathogenic bacteria including methicillin-resistant *Staphylococcus* strains. Bacterial DNA gyrase is the target of the aminocoumarin antibiotics. Until recently, novobiocin (Albamycin®, Pharmacia-Upjohn) was licensed in the United States for the treatment of infections with gram-positive bacteria and has been shown to enhance the cytotoxic activities of the anti-tumor drugs etoposide and teniposide. So far, the therapeutic use of aminocoumarin antibiotics is limited due to their low solubility in water, toxicity in eukaryotes and poor penetration in gram-negative bacteria. Combinatorial biosynthesis may offer a chance to develop novel aminocoumarins with improved properties. The biosynthetic gene clusters of novobiocin and coumermycin A_1 were already sequenced in our laboratory.

The first task of my thesis was to identify the biosynthetic gene cluster of the third "classical" aminocoumarin antibiotic, clorobiocin. The cluster was cloned by screening a cosmid library of *Streptomyces roseochromogenes* DS 12.976 with two heterologous probes from the novobiocin biosynthetic gene cluster. Sequence analysis revealed 29 open reading frames with striking similarity to the biosynthetic gene clusters of novobiocin and coumermycin A_1. A comparison of the gene clusters of clorobiocin, novobiocin and coumermycin A_1 showed that the structural differences between the three antibiotics were remarkably well reflected by differences in the organization of the biosynthetic gene clusters.

The second part of my thesis was to elucidate the biosynthesis of 3-dimethylallyl-4-hydroxybenzoate moiety (Ring A) of clorobiocin and novobiocin by biochemical and molecular biological studies. Comparison of the three aminocoumarin clusters allowed us to identify three genes in the novobiocin and clorobiocin clusters for which no homologues existed in the coumermycin cluster. We speculated that these genes might be involved in the biosynthesis of Ring A (which is absent in coumermycin A_1). These genes were: a) *cloR* and *novR*, which showed sequence similarity to putative

aldolases; b) cloF and novF, which showed sequence similarities to dehydrogenases; c) cloQ and novQ, which did not show sequence similarities to known genes in the database. A biosynthetic pathway for clorobiocin was proposed in which activated ß-hydroxytyrosine was a common intermediate in the formation of the aminocoumarin ring (Ring B) and Ring A (Fig. 7, p. 40).

However, analysis of *S. roseochromogenes* mutants (*cloI⁻* mutant, *cloQ⁻* mutant and *cloR⁻* mutant) revealed that Ring A and Ring B are formed by two distinct and independent pathways and that *cloQ* and *cloR* are essential for the formation of Ring A.

CloQ was expressed in *E. coli*, purified and identified as an aromatic prenyltransferase. It is a soluble, monomeric, 35 kDa protein. 4-Hydroxyphenylpyruvate (4HPP) and dimethylallyl diphosphate (DMAPP) were identified as the substrates of this enzyme, with K_m values determined as 25 and 35 µM, respectively. CloQ was found to be dissimilar from most prenyltransferases described so far and may indicate the existence of a new class of prenyltransferases. CloR was expressed in *E.* coli, purified and identified as a bifunctional non-heme iron oxygenase. It is a soluble, tetrameric protein. CloR converts 3-dimethylallyl-4-hydroxyphenylpyruvate *via* 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA) to Ring A. Therefore it catalyzes two consecutive oxidative decarboxylation steps. $^{18}O_2$ labelling experiments showed that two oxygen atoms are incorporated into the intermediate 3DMA-4HMA in the first reaction step, but only one further oxygen is incorporated into the final product Ring A during the second reaction step. CloR does not show sequence similarity to known oxygenases. It apparently presents a novel member of the diverse family of the non-heme iron (II) and a-keto dependent oxygenases, with 3DMA-4HPP functioning both as a-ketoacid and as hydroxylation substrate. The reaction catalyzed by CloR represents a new pathway to benzoic acids in nature.

In the third part of my thesis, a *novO⁻* mutant was created from the novobiocin producer *S. spheroides,* using a new inactivation method: the PCR targeting system. This mutant produced a derivative of novobiocin lacking the methyl group on the aminocoumarin ring.This provided functional proof for the role of *novO* in novobiocin biosynthesis. The mutant will be used for further experiments in combinatorial biosynthesis.

Zusammenfassung

Aminocoumarin-Antibiotika wie Novobiocin, Clorobiocin und Coumermycin A_1 werden von verschiedenen Streptomycetenstämmen produziert und besitzen große Wirksamkeit gegenüber grampositiven pathogenen Bakterien, einschließlich Methicillin-resistenter Staphylococcusstämme. Das Zielmolekül der Aminocoumarin-Antibiotika ist die bakterielle DNA-Gyrase. Bis vor kurzen wurde Novobiocin (Albamycin®, Pharmacia-Upjohn) in den Vereinigten Staaten zur Behandlung von Infektionen mit grampositiven Bakterien eingesetzt. Es konnte gezeigt werden, dass es die cytotoxische Aktivität von Cytostatika wie Etoposid und Teniposid verstärkt. Aufgrund ihrer schlechten Wasserlöslichkeit, ihrer Toxizität für Eukaryoten und ihrer schlechten Aufnahme in gramnegative Bakterien ist der therapeutische Einsatz der Aminocoumarin-Antibiotika bislang stark eingeschränkt. Kombinatorische Biosynthese könnte eine Möglichkeit zur Entwicklung neuer Aminocoumarine mit verbesserten Eigenschaften darstellen. Voraussetzung dafür ist die genaue Kenntnis der Biosynthese der betreffenden Antibiotika. Die Biosynthesegencluster von Novobiocin und Coumermycin A_1 waren bereits in unserem Labor sequenziert worden.

Die erste Aufgabe meiner Doktorarbeit war die Identifizierung des Biosynthesegenclusters des dritten „klassischen" Aminocoumarin-Antibiotikums Clorobiocin. Das Cluster wurde durch Screening einer Cosmidbank von *Streptomyces roseochromogenes* DS 12.976 mit zwei heterologen Sonden des Novobiocin-Biosynthesegenclusters kloniert. Vergleichende Sequenzanalyse ergab 29 offene Leserahmen mit deutlicher Übereinstimmung zu den Biosynthesegenclustern von Novobiocin und Coumermycin A_1. Ein Vergleich der Gencluster von Clorobiocin, Novobiocin und Coumermycin A_1 zeigte, dass sich die strukturellen Unterschiede zwischen den drei Antibiotika erstaunlich genau in der Organisation der Cluster widerspiegeln.

Der zweite Teil meiner Arbeit bestand in der Aufklärung der Biosynthese der 3-Dimethylallyl-4-hydroxybenzoat Gruppe (Ring A) von Clorobiocin und Novobiocin, sowohl durch biochemische als auch durch molekularbiologische Untersuchungen. Der Vergleich der drei Aminocoumarin-Cluster ermöglichte uns die Identifizierung von

drei Gene, die sowohl im Novobiocin- als auch im Clorobiocin-Cluster vorkommen, für die aber keine Homologe im Coumermycin A$_1$-Cluster existieren. Wir vermuteten, dass diese Gene an der Biosynthese von Ring A beteiligt sein könnten, da dieser in Coumermycin A$_1$ nicht vorhanden ist.

Diese Gene sind: a) cloR und novR, die Sequenzähnlichkeit zu putativen Aldolasen zeigten; b) cloF und novF, die Sequenzähnlichkeit zu Dehydrogenasen zeigten; und c) cloQ und novQ, welche keinerlei Sequenzähnlichkeit zu bekannten Genen in den Datenbanken aufwiesen. Es wurde ein Weg für die Biosynthese von Clorobiocin vorgeschlagen demzufolge aktiviertes ß-Hydroxytyrosin als gemeinsames Zwischenprodukt bei der Bildung des Aminocoumarinrings (Ring B) und des Ring A fungiert (Fig. 7, p. 40).

Es konnte jedoch durch die Herstellung und Analyse von *S. roseochromogenes* Mutanten (*cloI⁻*, *cloQ⁻*, und *cloR⁻*) gezeigt werden, dass Ring A und Ring B auf zwei verschiedenen, unabhängigen Wegen gebildet werden.

CloQ wurde in *E. coli* exprimiert und gereinigt und als eine aromatische Prenyltransferase identifiziert. CloQ ist ein lösliches, monomeres, 35 kD Protein. 4-Hydroxyphenylpyruvat (4HPP) und Dimethylallyldiphosphat (DMAPP) wurden als Substrate dieses Enzyms identifiziert und die K_m-Werte mit 25 µM bzw. 35 µM bestimmt. Wir konnten zeigen, dass CloQ sich von den meisten bisher bekannten Prenyltransferasen unterscheidet und möglicherweise einer neuen Klasse von Prenyltransferasen angehört.

CloR wurde in *E. coli* exprimiert und gereinigt und als eine bifunktionale non-Häm Eisen-Oxygenase identifiziert. Es ist ein lösliches, tetrameres Protein. CloR setzt 3-Dimethylallyl-4-hydroxyphenylpyruvat über 3-Dimethylallyl-4-hydroxymandelsäure zu Ring A um. Dies erfolgt in zwei aufeinanderfolgenden Decarboxylierungsschritten. $^{18}O_2$-Markierungsexperimente zeigten, dass im ersten Reaktionsschritt zwei Sauerstoffatome in das 3DMA-4HMA Zwischenprodukt eingebaut werden, wohingegen aber im zweiten Schritt nur ein weiteres Sauerstoffatom in das Ring A Endprodukt inkorporiert wird. CloR zeigt keine Sequenzähnlichkeit zu bekannten Oxygenasen. Anscheinend stellt es ein neues Mitglied der divergenten Familie der non-Häm Eisen (II) und a-Ketosäure-abhängigen Oxygenasen dar, bei dem 3DMA-4HPP sowohl als a-Ketosäure als auch als Hydroxylierungssubstrat fungiert. Die von

CloR katalysierte Reaktion stellt einen neuen Weg zur Biosynthese von Benzoesäuren dar.

Weiterhin wurde aus dem Novobiocin-Produzenten *S. spheroides* eine *novO*⁻ Mutante mit Hilfe einer neuen Inaktivierungsmethode erzeugt, dem „PCR targeting"-System. Diese Mutante produzierte ein Derivat von Novobiocin, das keine Methylgruppe mehr am Aminocoumarin-Ring trug. Dies bewies die Funktion von *novO* in der Biosynthese von Novobiocin. Die Mutante soll in zukünftigen Experimenten zu kombinatorischen Biosynthese eingesetzt werden.

I.   INTRODUCTION

1.   Aminocoumarin antibiotics

Novobiocin, clorobiocin and coumermycin A_1 are the "classical" members of the class of aminocoumarin antibiotics. They all contain three structural moieties: a 3-amino-4,7-dihydroxy coumarin moiety (called Ring B), a deoxysugar moiety (called Ring C) and an acyl component (Fig. 1, p. 22). Ring B is linked to the acyl component *via* an amide bond, and to Ring C *via* a glycoside bond. Clorobiocin and novobiocin share the same 3-dimethylallyl 4-hydroxybenzoate moiety (called Ring A) as acyl component. Clorobiocin differs from novobiocin at two positions: novobiocin bears a carbamoyl group at position 3 of the noviose moiety, while clorobiocin possesses a 5-methylpyrrole 2-carboxylic acid, and clorobiocin, as indicated by its name, carries a chlorine atom at position 8 of Ring B, while novobiocin carries a methyl group. Coumermycin A_1 contains two noviosyl aminocoumarin moieties, and carries a different acyl component as novobiocin and clorobiocin, i.e. a 3-methyl-pyrrole 2,4-dicarboxylic acid. Coumermycin A_1 shares with novobiocin the same methyl group at position 8 of Ring B, and with clorobiocin the same 5-methylpyrrole 2-carboxylic acid at position 3 of the noviose moiety (Fig. 1, p. 22).

The simocyclinones of the D class and rubradirin are two further aminocoumarins found in nature (Fig. 1, p. 22). They also show antibacterial activity, but their mechanism of action is unknown (Bhuyan *et al.*, 1965; Holzenkämpfer *et al.*, 2002; Yoo *et al.*, 2000). They possess the same aminocoumarin ring as the "classical" aminocoumarins described above. Simocyclinone D8 carries a chlorine atom at position 8 of its aminocoumarin ring just as clorobiocin. In contrast to the "classical" aminocoumarins, simocyclinones and rubradirins do not carry a noviose moiety, and their acyl components are large and complicated structures (Galm *et al.*, 2002; Schimana *et al.*, 2000; Schimana *et al.*, 2001; Trefzer *et al.*, 2002; Bannister and Zapotocky, 1992; Bhuyan *et al.*, 1965; Yoo *et al.*, 2000).
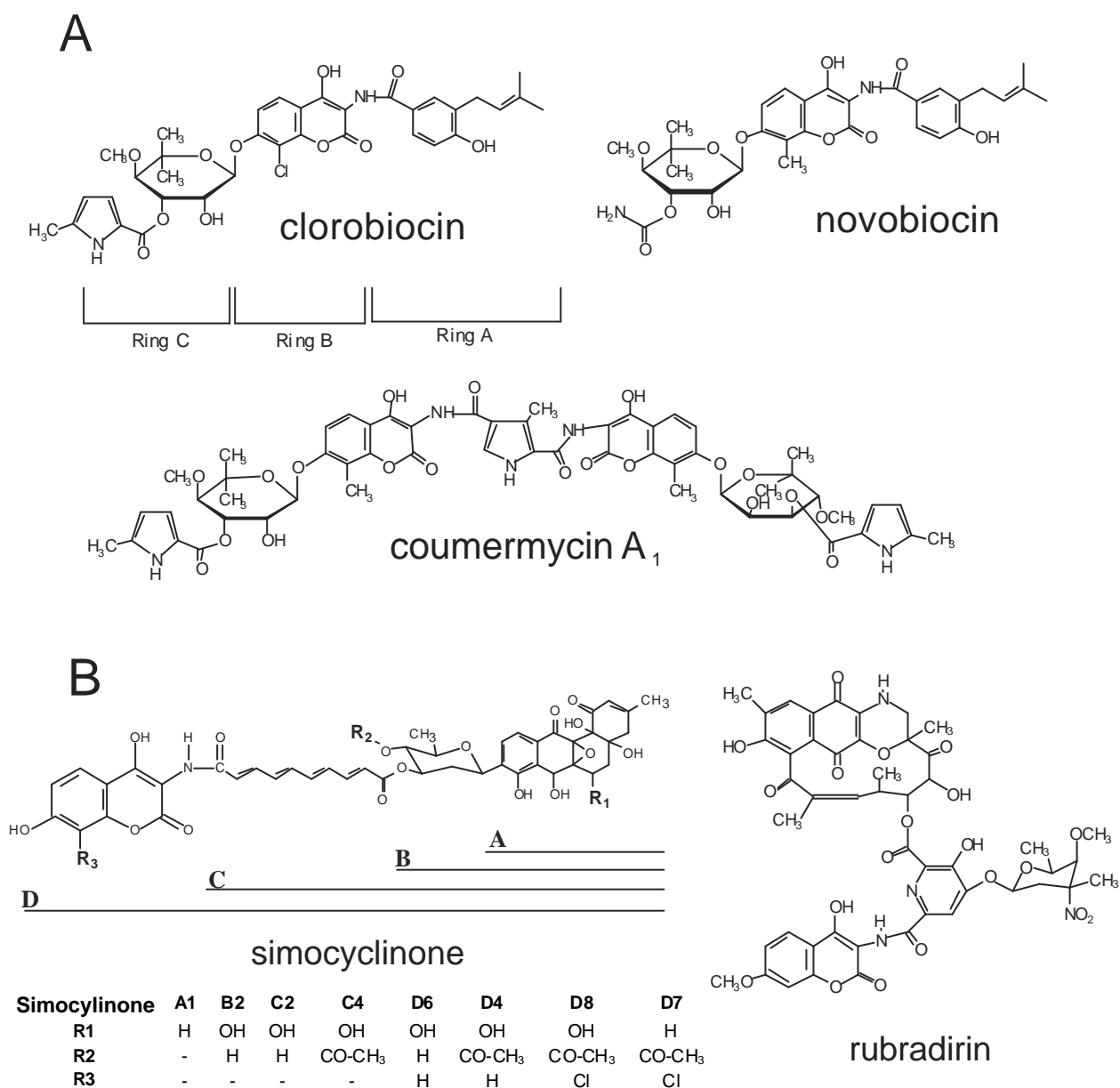
Fig. 1: Structure of aminocoumarin antibiotics.

(A) The three "classical" aminocoumarins: clorobiocin, novobiocin and coumermycin A_1.

(B) The "unusual" aminocoumarins: simocyclinones and rubradirin.

Early investigation of the biosynthesis of the aminocoumarin antibiotics revealed that the deoxysugar moiety is derived from intact glucose, while the aminocoumarin ring and the prenylated 4-hydroxybenzoate moiety are derived from tyrosine (Bunton *et al.*, 1963; Calvert *et al.*, 1972; Kominek and Sebek, 1974). Recently, $^{18}O_2$ incorporationexperiments on simocyclinone D have shown that the ring oxygen of the aminocoumarin ring is derived from molecular oxygen, not from the carboxyl group of tyrosine (Holzenkämpfer and Zeeck, 2002). The dimethylallyl side chain of Ring A is derived from the methylerythritol phosphate pathway (mevalonate-independent pathway) (Li *et al.*, 1998; Orihara *et al.*, 1998).

All these aminocoumarins are secondary metabolites from various *Streptomyces* (*S.*) species. Novobiocin is produced by *S. spheroides* NCIMB 11891 and *S. niveus*. However, Southern blot experiments have shown that these two strains may actually be independent isolates of the same strain. Recently, Sasaki *et al.* (2001) have isolated a Streptomyces strain from the stem of the plant *Aucuba japonica* that also produces novobiocin. Clorobiocin is produced by *Streptomyces hygroscopicus* DS 9.751, *S. albocinerescens* DS 21.647 and *S. roseochromogenes* var. *oscitans* DS 12.976 (Ninet *et al.*, 1972). Coumermycin is produced by *S. rishiriensis* DSM 40489, *S. hazeliensis* var. *hazeliensis, S. spinichromogenes* and *S. spinicoumarensis* (Claridge, 1968; Kawaguchi *et al.*, 1965). Besides the main product coumermycin A_1, several closely related metabolites have been identified in these strains, e.g. coumermycin D, which lacks the two acyl groups at the 3-OH of the deoxysugars (Fig. 1, p. 22) (Claridge *et al.*, 1984). Berger and Batcho (1978) have reviewed the production and isolation of novobiocin, clorobiocin and coumermycin A_1.
The simocyclinones are produced by *S. antibioticus* TÜ 6040 (Schimana *et al.*, 2000; Theobald *et al.*, 2000) and Rubradirin by *S. achromogenes* var. *rubradiris* NRRL 3061 (Bannister and Zapotocky, 1992; Bhuyan *et al.*, 1965; Meyer, 1965).

The mechanism of action of the three "classical" aminocoumarin antibiotics is well known. They exert their antibacterial activity *via* the inhibition of the DNA gyrase (Lewis *et al.*, 1996; Maxwell, 1999). Recently, novobiocin was also shown to target the DNA topoisomerase IV (Hardy *et al.,* 2003). Gyrase and topoisomerase IV have vital roles in DNA replication, chromosome segregations, and DNA compaction. Gyrase is responsible for maintaining negative supercoiling of the bacterial

chromosome, whereas topoisomerase IV's primary role is in the decatenation of daughter chromosomes following DNA replication. Both enzymes carry out their functions by coupling the energy of ATP hydrolysis to the directional passage of one double-strand of DNA through another. The enzymatic activity is divided between the two subunits of each enzyme: GyrA and GyrB for gyrase and ParC and ParE for topoisomerase IV. The GyrB and ParE subunits contain nucleotide binding sites, whereas the GyrA and ParC subunits are responsible for DNA breakage and reunion. Gyrase and topoisomerase IV are A_2B_2 and C_2E_2 heterotetramers (Fig. 2, p. 25).

The aminocoumarin antibiotics target the GyrB subunit of gyrase, and/or the ParE subunit of topoisomerase IV (Fig. 2, p. 25). In contrast, the fluoroquinolone drugs, such as ciprofloxacin, target the GyrA subunit of gyrase, and also the ParC subunit of topoisomerase IV (Fig. 2, p. 25). X-ray crystallographic examinations demonstrated that the aminocoumarin moiety, the substituted deoxysugar moiety and the prenylated 4-hydroxybenzoate moiety of novobiocin and clorobiocin are important for binding to the B subunit of bacterial gyrase (Lafitte *et al.*, 2002; Maxwell and Lawson, 2003). Coumermycin A_1 contains two aminocoumarin-deoxysugar moieties and has been shown to stabilize a dimeric form of the 43 kDa fragment of GyrB (Farrar *et al.*, 2000). The affinity of the aminocoumarin antibiotics for bacterial gyrase is very high. The inhibition constants of these antibiotics are in the 10 nM range, i.e. two orders of magnitude lower than those of modern fluoroquinolones.

An additional mechanism of action of these antibiotics appears to be the formation of ion channels (Feigin *et al.*, 1995). Recently, novobiocin was shown to interact with the eukaryotic chaperone heat shock protein 90 (Hsp90) (Marcu *et al.*, 2000). Novobiocin also exhibits immunomodulating properties, downregulating the secretion of tumour necrosis factor α in human peripheral blood mononuclear cells (Luhrmann *et al.*, 1998).

Aminocoumarin antibiotics are very potent against gram-positive pathogenic bacteria including methicillin-resistant *Staphylococcus* strains. In addition, the aminocoumarins act synergistically with anticancer compounds such as etoposide and can be used to overcome drug resistance in tumour cell lines (Rappa *et al.*, 2000a; Rappa *et al.*, 2000b).
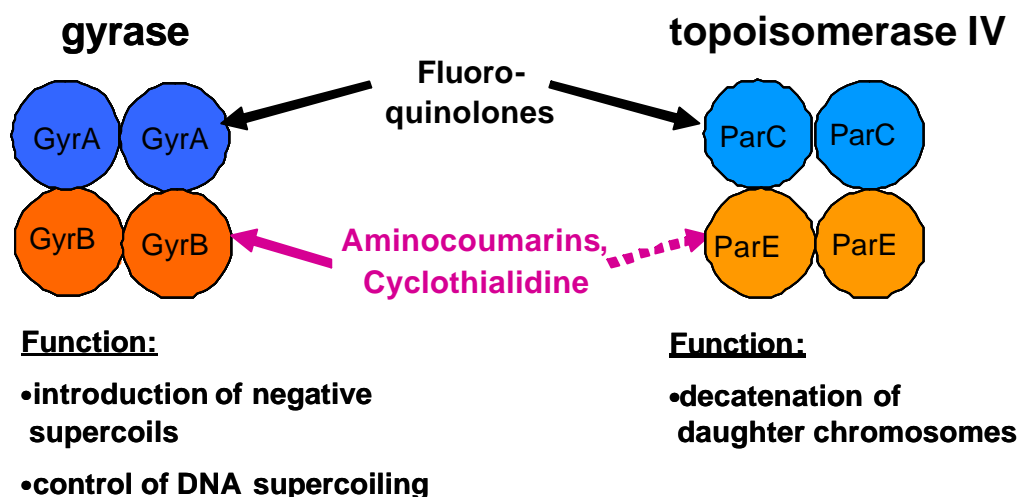
Fig. 2: Schematic model of gyrase and topoisomerase IV.

Dramatic increases in the number of antibiotic-resistant pathogenic bacteria in the past decade have focused attention on the need for new antibiotics (Walsh, 2002). The antibacterial activity of novobiocin has been demonstrated in preclinical and clinical studies (Arathoon *et al.*, 1990; Eder *et al.*, 1991;. Raad *et al.*, 1995; Raad *et al.*, 1998). However, due to their toxicity in eukaryotes, their poor solubility in water, and their low activity against gram-negative bacteria, clinical use of these antibiotics remains restricted (Maxwell, 1993). Therefore, it is of interest to test whether new, structurally modified aminocoumarin antibiotics may be able to overcome the limitations of existing compounds. Such new aminocoumarins could be created by combinatorial biosynthesis (Hutchinson, 1998). Up-to-now, the derivatives of novobiocin were only obtained by chemical synthesis (Ferroud *et al.*, 1999; Laurin *et al.*, 1999b; Laurin *et al.*, 1999a; Musicki *et al.*, 2000; Periers *et al.*, 2000; Schio *et al.*, 2001).

There are some basic requirements that have to be met for the production of hybrid antibiotics by combinatorial biosynthesis. First, this method requires the existence of microorganisms that produce antibiotics whose biosynthetic pathways show common features. Second, it has to be possible to manipulate and analyze pathways by creating mutants that accumulate intermediate compounds. The third requirement is the identification of the genes encoding the biosynthetic enzymes. In addition, the

creation of hybrid antibiotics is dependent on the ability to introduce one or more genes of interest into these microorganisms (Hutchinson, 1999).

Aminocoumarin antibiotics fullfill these criteria and are therefore ideal candidates for the production of new hybrid antibiotics by combinatorial biosynthesis. Our group worked towards this goal by sequencing the biosynthetic gene cluster of novobiocin from *S. spheroides* NCIB 11891 (Steffensky *et al.*, 2000b) and of coumermycin A_1 from *S. rishiriensis* (Wang *et al.*, 2000) (Fig. 3, p. 31).

The sequence of the novobiocin cluster revealed the presence of 20 putative open reading frames (ORFs), including a gene for novobiocin resistance, *gyrB*[R], and at least 11 additional ORFs to which a possible role in novobiocin biosynthesis could be assigned. The sequence of the coumermycin cluster revealed the presence of 29 putative ORFs upstream of two aminocoumarin resistance genes *gyrB*[R] *and parY*[R]. The coumermycin cluster shows striking similarity to the novobiocin biosynthetic gene cluster: 15 of the identified ORFs were found to display, on average, 84% amino acid identity to corresponding ORFs of the novobiocin cluster, and all of these ORFs were arranged in identical orientation in both clusters. Comparison of the clusters of novobiocin with the one of coumermycin A_1 allowed us to predict the function of a number of these genes in the biosynthesis. *NovL*, for example, was predicted to encode an amide synthetase, which catalyzed the formation of the amide bond between Ring A and Ring B of novobiocin. *In vitro* experiments demonstrated that, indeed, NovL is a novobiocic acid synthetase (Steffensky *et al.*, 2000a). Recently, the biosynthetic genes for the aminocoumarin moiety of simocyclinone (Galm *et al.*, 2002; Trefzer *et al.*, 2002) and for rubradirin (Sohng *et al.,* 1997) have been cloned and sequenced.

2. Objectives of this study

My first objective was to identify the biosynthetic gene cluster of the third "classical" aminocoumarin antibiotics, clorobiocin. Comparison of the biosynthetic gene clusters of novobiocin, coumermycin A_1 and clorobiocin was expected to allow the generation of hypotheses for the function of the genes contained in these clusters in the biosynthesis of the aminocoumarin antibiotics, especially in the formation of Ring A.

In order to clone and sequence the biosynthetic gene cluster of clorobiocin, the following experiments were necessary:

- Optimisation of culture conditions for clorobiocin production and establishment of extraction methods for analysis of secondary metabolites by mass spectrometry (MS) and nuclear magnetic resonance (NMR).
- Construction of a cosmid library from *S. roseochromogenes* and screening with probes from novobiocin biosynthetic genes (*novT* and *novL*).
- Sequencing and analysis of cosmids containing the clorobiocin biosynthetic gene cluster, and comparison of the sequence with the novobiocin and coumermycin A_1 clusters.
- Establishment of a model for clorobiocin biosynthesis.

Comparison of the three biosynthetic gene clusters indeed allowed us to determine three genes which were present in clorobiocin and novobiocin clusters but not in coumermycin A_1 cluster and which might therefore be involved in the formation of Ring A.

The second objective of my thesis was to elucidate in detail the biosynthesis of Ring A, i.e. the prenylated 4-hydroxybenzoate moiety of clorobiocin and novobiocin. This involved following experiments:

- sequence analysis of the three genes (*cloR*, *cloQ* and *cloF*) presumably involved in Ring A formation and subsequently establishment of a model for the formation of Ring A.
- Creation of three *S. roseochromogenes* mutants (*cloQ*⁻, *cloR*⁻ and *cloI*⁻) by in-frame deletion, analysis of their secondary metabolites by HPLC, mass

spectrometry or LC-ESI-CID, and by feeding experiments in order to determine the roles of these genes in clorobiocin biosynthesis.

- Expression and purification of CloQ. Biochemical investigation of this enzyme and confirmation of its role in Ring A biosynthesis.

- Expression and purification of CloR. Biochemical investigation of this enzyme and confirmation of its role in Ring A biosynthesis.

My third objective was the inactivation of *novO* in the novobiocin producer *S. spheroides*, using a new method of gene inactivation (PCR targeting system). This should provide functional evidence for *novO*, and form a basis for future experiments in combinatorial biosynthesis.

II.   RESULTS

1. Identification of the clorobiocin biosynthetic gene cluster.

1.1 Introduction

The biosynthetic gene clusters of novobiocin from *Streptomyces* (*S.*) *spheroides* NCIMB 11891 and of coumermycin A_1 from *S. rishiriensis* DSM 40489 were recently identified (Steffensky *et al.*, 2000b; Wang *et al.*, 2000). Comparison of these two biosynthetic gene clusters provided first indications about the function of some gene in the biosynthesis of Ring B and the sugar moiety. Unfortunately, this comparison did not provide any information about the biosynthesis of Ring A of novobiocin and clorobiocin or of the centrale pyrrol ring of coumermycin A_1 (Fig. 1, p. 22). The first task of my thesis was to identify the biosynthetic gene cluster of the third "classical" aminocoumarin antibiotic, clorobiocin. Comparison of the three biosynthetic gene clusters was expected to allow a better understanding of the genes involved in the formation of the aminocoumarin antibiotics, and would also offer prospects for the production of new aminocoumarins by combinatorial biosynthesis.

Clorobiocin is produced by three different *Streptomyces* strains (see Introduction, p. 21). *S. roseochromogenes* var. *oscitans* DS 12.976 was shown to be the best producer of clorobiocin (see Materials and methods, p. 79) and was therefore used in all experiments.

1.2 Cloning and sequencing of the clorobiocin biosynthetic gene cluster.

Novobiocin (Steffensky *et al.*, 2000b) and coumermycin A_1 (Wang *et al.*, 2000) biosynthetic gene clusters were previously cloned and sequenced (Fig. 3, p. 31). In the novobiocin cluster, the gene *nov*T codes for a dNDP-glucose 4,6-dehydratase involved in the biosynthesis of the deoxysugar moiety of novobiocin. The gene *nov*L encodes the novobiocic acid synthetase which catalyzes the formation of the amide bond between Ring A and Ring B of novobiocin (Fig. 3, p. 31) (Steffensky *et al.*, 2000a). Similar reactions were expected to be involved in clorobiocin biosynthesis.

Therefore, Southern hybridizations of genomic DNA of the clorobiocin producer *S. roseochromogenes* were carried out with probes for *nov*T and *nov*L, each resulting in a single band.

A cosmid library from *S. roseochromogenes* was constructed into Supercos-1, and screened with the *novT* and *novL* probes (see Materials and methods, p. 81). Four cosmids (VIIIA7-c, K1F2, D1A8 and F1A4) hybridize with both probes. These cosmids are different but overlapp each other (Fig. 34, p. 82). Cosmid K1F2 was sequenced on both strands. 36 open reading frames (ORFs) were identified. 29 of these ORFs showed striking similarity to genes of the novobiocin and/or coumermycin A_1 biosynthetic gene cluster (Table 1, p. 32). In addition a partial sequence of the aminocoumarin resistance gene $gyrB^R$ was found at the 3' end of the cluster. Recently, our group has sequenced the genes downstream of $gyrB^R$, and also found a gene with homology to topoisomerase IV ($parY^R$), as in the cluster of coumermycin A_1 (Fig. 3, p. 31) (Eustáquio *et al.*, 2003b; Schmutz *et al.,* 2003). Strikingly, in all three clusters, the corresponding ORFs were arranged in exactly the same order and oriented in the same direction (Fig. 3, p. 31).

The sequence of cosmid K1F2 was deposited in the GenBank database under accession no. AF 329398.
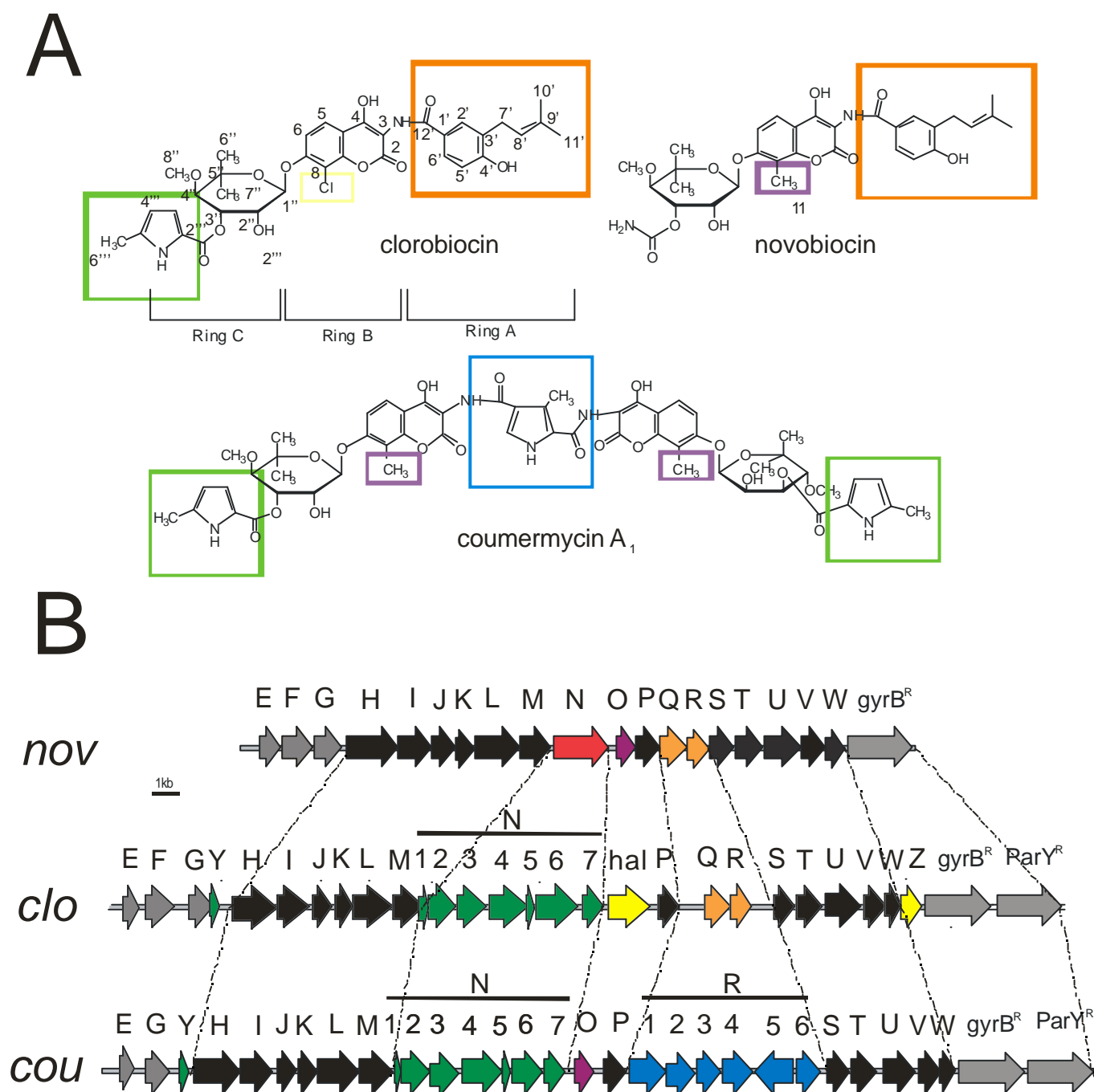
Fig. 3: Comparison of the three "classical" aminocoumarin biosynthetic gene clusters.

(A) Structures of the aminocoumarin antibiotics.

(B) Map of the clorobiocin biosynthetic gene cluster of *S. roseochromogenes* DS 12 976 (*clo*), compared to the biosynthetic gene clusters of novobiocin (*nov*) and coumermycin A₁ (*cou*).

Table 1: Identified ORFs in the biosynthetic gene cluster of clorobiocin (cosmid K1F2).

ORF	size of the product (amino acids [aa])	Similar entity or entities[a]	% identity of products	accession number
ORF1	306	FkbI (lysine cyclodeaminase) from *S. hygroscopicus*	37	AAF86391
ORF2	197	sarcosine oxidase gamma subunit from *Corynebacterium sp.*	44	Q46338
ORF3	962	sarcosine oxidase alpha subunit from *Corynebacterium sp.*	59	Q46337
ORF4	93	sarcosine oxidase delta subunit from *Corynebacterium sp.*	66	Q46336
ORF5	406	sarcosine oxidase beta subunit from *Corynebacterium sp.*	80	P40875
ORF6	406	serine hydroxymethyltransferase from *S. coelicolor*	75	O86565
ORF7	218	putative transcriptional regulator *from S. coelicolor*	45	AL596248.1
ORF8	149	unknown protein		
ORF9	78	transposase from *S. coelicolor*	68	AL109949.1
cloE	217	**novE** (217 aa)/**couE** (217 aa)	**82**	
		lmbU protein from *S. lincolnensis*	43	S44974
cloF	362	**novF** (362 aa)	**73**	
		prephenate dehydrogenase from *S. lavendulae*	30	AAK81837
cloG	319	**novG** (318 aa)/ **couG** (319 aa)	**79/80**	
		regulatory protein (StrR) from *S. glaucescens*	40	S44506
cloY	71	**couY** (71 aa)	**81**	
		hypothetical protein from *S. coelicolor*	68	T36310
cloH	600	**novH** (600 aa)/ **couH** (599 aa)	**75/80**	
		peptide synthetase-like protein *from S. antibioticus*	50	AAG34184.1
cloI	407	**novI** (407 aa)/ **couI** (407 aa)	**90/95**	
		cytochrome P-450 enzyme from *S. antibioticus*	66	AAK06805.1
cloJ	258	**novJ** (262 aa)/ **couJ** (258 aa)	**72/77**	
		3-oxoacyl reductase from *bacillus halodurans*	48	BAB06210
cloK	245	**novK** (244 aa)/ **couK** (245 aa)	**77/81**	
		reductase from *S. antibioticus*	35	AAG34182
cloL	527	**novL** (527 aa)/ **couL** (529 aa)	**86/86**	
		amide synthetase from *S. antibioticus*	37	AAG34183
cloM	390	**novM** (379 aa)/ **couM** (402 aa)	**78/78**	
		glycosyltransferase from *S. argillaceus*	38	AAC64927
cloN1	95	**couN1** (95 aa)/ hypothetical protein	**86**	
cloN2	355	**couN2** (355 aa)	**86**	
		dpsC from *S. peucetius*	32	1093565
cloN3	376	**couN3** (373 aa)	**81**	
		acyl-CoA dehydrogenase (*pltE*) from *Pseudomonas fluorescens*	45	AAD24879
cloN4	501	**couN4** (501 aa)	**87**	
		acyl-CoA synthetase (*pltF*) from *Pseudomonas fluorescens*	45	AAD24881
cloN5	89	**couN5** (89 aa)	**91**	
		hypothetical protein (*pltL*) from *Pseudomonas fluorescens*	34	T17418
cloN6	561	**couN6** (560 aa)	**94**	
		hypothetical protein		
cloN7	278	**couN7** (281 aa)	**82**	
		putative hydrolase from *S. coelicolor*	34	CAB95984
clo-hal	524	non-heme halogenase from *S. lavendulae*	35	AAK81830
cloP	277	**novP** (262 aa)/ **couP** (276 aa)	**91/89**	
		O-methyltransferase III from *S. olivaceus*	58	CAC32469
cloQ	324	**novQ** (271 aa)/ **4HPP-prenyltransferase (this work)**	**80**	
cloR	277	**novR** (270 aa)/ **non-heme iron oxygenase (this work)**	**95**	
		putative aldolase *from S.coelicolor*	47	CAB82026
cloS	288	**novS** (288 aa)/ **couS** (288 aa)	**84/87**	
		4-ketoreductase from *S. antibioticus*	50	AAD55455
cloT	336	**novT** (336 aa)/ **couT** (336 aa)	**82/87**	
		dNDP-glucose-4,6-dehydratase from *S. globisporus*	66	AAF13998
cloU	420	**novU** (420 aa)/ **couU** (420 aa)	**88/90**	
		D-mycarose 3-C-methyltransferase from *S.argillaceus*	48	CAB96549
cloV	296	**novV** (297 aa)/ **couV** (296 aa)	**89/92**	
		dTDP-glucose synthase from *S.spectabilis*	74	AAD31796
cloW	198	**novW** (207 aa)/ **couW** (198 aa)	**86/91**	
		dTDP-4-keto-6-deoxyglucose 3,5-epimerase from *S. glaucescens*	54	S44236
cloZ	253	hypothetical protein		
gyrB[R]	partial sequence	**gyrB**[R]-**nov** (novobiocin cluster) /**gyrB**[R]-**cou** (coumermycin cluster)		

[a] *nov* genes are from the novobiocin biosynthetic gene cluster of *S. spheroides* (accession number AF170880), *cou* genes are from the coumermycin A₁ biosynthetic gene cluster of *S. rishiriensis* (accession number AF235050)

1.3 Genes involved in the biosynthesis of deoxysugar moiety (Ring C).

At the 3' end of the cluster, five ORFs with high homology to genes involved in the deoxysugar biosynthesis were discovered (*cloSTUVW*) (Table 1, p. 32). Homologous genes are found in the same position in the clusters of novobiocin (*novSTUVW*) and coumermycin A$_1$ (*couSTUVW*) (Fig. 3, p. 31). Based upon their homology to known genes of deoxysugar biosynthesis, we previously assigned these genes to the five steps required for the biosynthesis of the deoxysugar moiety (Fig. 4), and evidence for this hypothesis was provided by an inactivation experiment with *novT* in the novobiocin producer (Steffensky *et al.*, 2000b). The presence of these genes in the clorobiocin cluster provides additional support to our previous functional assignment of these genes.

O-methylation at position 4 of the deoxysugar moiety is regarded as the last step in aminocoumarin biosynthesis (Fig. 30, p. 69) (Queener S.W. *et al.*, 1978). The genes *cloP*, *novP* and *couP*, situated in the same relative position of the clusters, show homology to known deoxysugar O-methyltransferases. *couP* was shown, by gene inactivation, to methylate the hydroxyl group on position 4 of the deoxysugar (Li *et al.*, 2002). *cloP* shows 89 % homology to c*ouP* and is likely to catalyze the same reaction in clorobiocin biosynthesis.
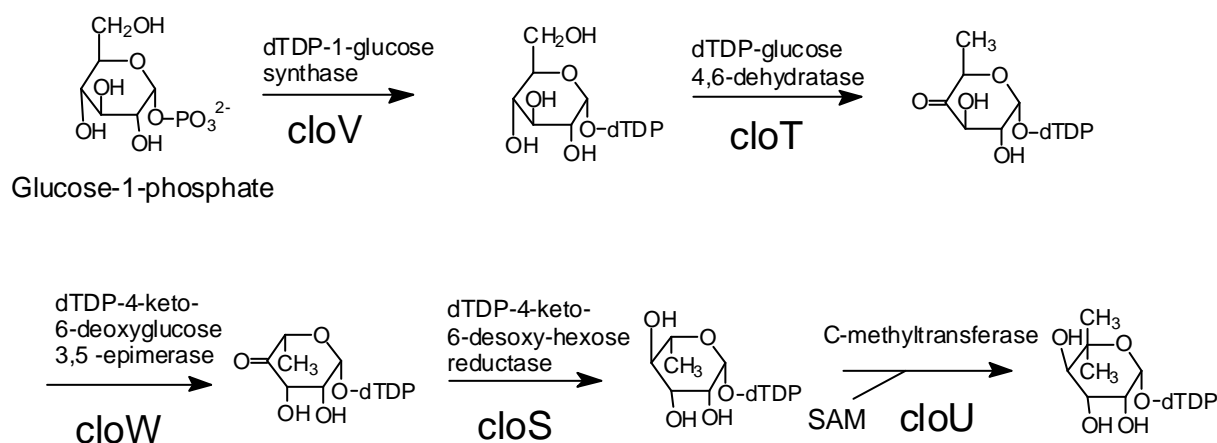
Fig. 4: Proposed biosynthetic pathway of the deoxysugar moiety.

1.4 Genes presumably involved in the formation of pyrrole ring.

Clorobiocin and coumermycin A_1 contain pyrrole carboxylic acid rings attached to position 3 of their deoxysugar moieties (Fig. 3, p. 31). Novobiocin contains a carbamoyl group at the corresponding position.

These structural similarities and differences between the three antibiotics are reflected in the organization of the gene clusters (Fig. 3, p. 31): downstream of the glycosyltransferase gene *novM*, the novobiocin cluster contains a gene (*novN*) with homology to carbamoyl transferases, whereas in the same relative position of the clorobiocin and coumermycin clusters, a group of seven genes is found (*cloN1-N7* or *couN1-N7*, respectively) which show very high homology between the two clusters (87% aa identity on average). These genes can be assigned to pyrrole biosynthesis, which has very recently been elucidated genetically as well as biochemically. *cloN3*, *cloN4* and *cloN5* show sequence similarity to *pltE*, *pltF* and *pltL*, respectively, involved in the biosynthesis of the pyrrole moiety of pyoluteorin in *Pseudomonas fluorescens* Pf-5 (Nowak-Thompson *et al.*, 1999) and to *redW, redM* and *redO*, respectively, involved in the biosynthesis of the pyrrole moiety of undecylprodiginin in *S. coelicolor* (Cerdeno *et al.*, 2001). PltF and RedM convert L-proline into its acyl adenylate (Fig. 5, p. 35) and the small proteins PltL and RedO act as peptidyl carrier proteins (PCP) (Thomas *et al.*, 2002). The same functions may therefore be assigned to the homologous CloN4 and CloN5, respectively. CloN3, like CouN3, PltE and RedW, shows homologies to flavine-dependent acyl-coenzyme A dehydrogenases. PltE and RedW catalyze the dehydrogenation of the PCP-bound proline (Thomas *et al.*, 2002). The resulting pyrroline derivative (presumably ?[2]) undergoes spontaneous oxidation to the aromatic pyrrole derivative (Fig. 5, p. 35).

Recently, members of our group showed by gene inactivation in the coumermycin producer *S. rishiriensis*, that CouN3 and CouN4 are indeed involved in the biosynthesis of the pyrrole ring (Xu *et al.*, 2002). Expression of the putative carbamoyltransferase gene, *novN*, in these mutants led to the formation of bis-carbamoylated coumermycin D, also providing evidence for the function of the *novN* gene in novobiocin biosynthesis (Xu *et al.*, 2002).

CloN6 (CouN6) belongs to the BchE-like/methyltransferase subgroup of radical SAM proteins, which has recently been identified by bioinformatic techniques (Sofia *et al.*,

2001), and catalyzes the transfer of a methyl group to position 5 of the pyrrole-2-carboxylic acid (Westrich *et al.*, 2003).

cloN2 (*couN2*) shares homology with *dpsC*, which encodes an enzyme with acyltransferase activity. *cloN2* is indeed involved in the transfer of the activated pyrrole-2-carboxylic acid to the 3-OH of the deoxysugar moiety (Xu *et al.*, 2003).

The small ORF *cloN1* (95 amino acids) does not show homology to other database entries, and its function remains unknown at present.
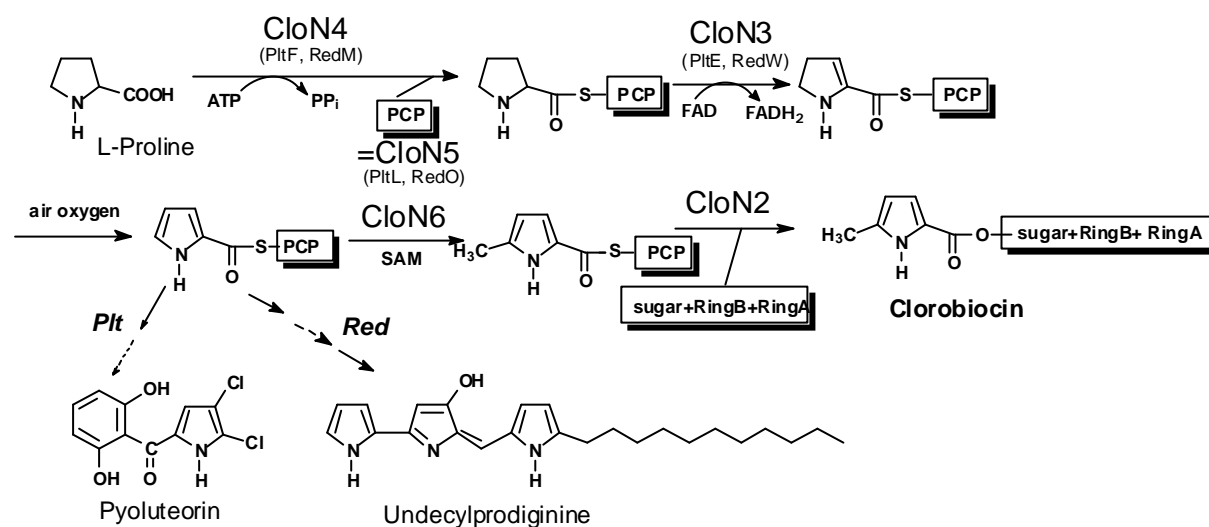
Fig. 5: Proposed biosynthetic pathway of the pyrrole ring.

1.5 Genes presumably involved in the biosynthesis of the aminocoumarin ring (Ring B).

The genes for the biosynthesis of the characteristic aminocoumarin ring must be present in all three clusters, and a comparison of the three clusters therefore presents an obvious method to identify possible candidate genes for the biosynthesis of this ring. In the clorobiocin cluster, *cloHIJK* showed, on average, 85% homology to the corresponding genes in the novobiocin (*novHIJK*) and the coumermycin A_1 (*couHIJK*) clusters (Table 1, p. 32). It appears likely that the gene products of *these genes* are involved in the formation of the aminocoumarin ring from tyrosine (Fig. 6, p. 36). A detailed discussion of the role of these genes is presented page 67.

Clorobiocin contains a chlorine atom at position 8 of the aminocoumarin ring, whereas novobiocin and coumermycin A_1 contain a methyl group at the same position (Fig. 3, p. 31). This structural difference of the antibiotics is perfectly reflected in the organization of the gene clusters: the novobiocin and coumermycin clusters contain a C-methyltransferase gene, *novO* or *couO*, respectively. In the clorobiocin cluster, in contrast, *clo-hal,* a homologue of non-heme halogenase genes, is found at the same relative position. Another gene, *cloZ*, is also exclusively found in clorobiocin and could, therefore, play a role in the halogenation of clorobiocin together with *clo-hal*. Recently, gene inactivations of *cloZ* and *clo-hal* have shown that Clo-hal is responsible for the halogenation of clorobiocin. However, CloZ was demonstrated not to be involved in the halogenation, nor to be essential for clorobiocin biosynthesis (Eustáquio *et al.*, 2003a).
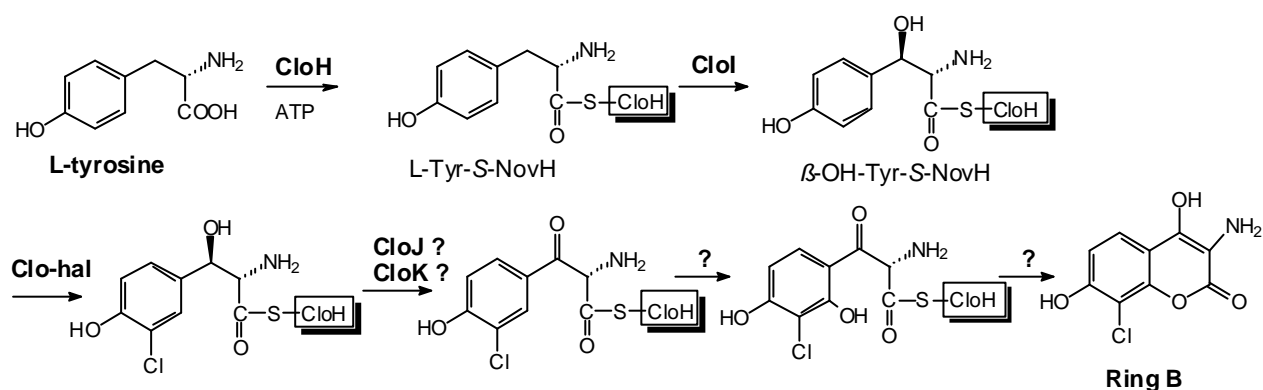
Fig. 6: Proposed biosynthetic pathway of Ring B.

1.6 Genes presumably involved in the biosynthesis of the 3-dimethylallyl-4-hydroxybenzoic acid (Ring A).

Clorobiocin and novobiocin contain a prenylated 4-hydroxybenzoate moiety (Ring A). Coumermycin A_1 contains a pyrrole dicarboxylic acid moiety instead, linking the two aminocoumarin rings of this molecule (Fig. 3, p. 31). The aromatic nucleus of Ring A of clorobiocin and novobiocin is derived from tyrosine (Bunton *et al.*, 1963; Kominek and Sebek, 1974), but the exact reaction sequence is unknown.

Sequencing of the clorobiocin gene cluster revealed three genes, which were also present in the novobiocin cluster but not in the coumermycin cluster, i.e. *cloF, cloQ* and *cloR*. This fact led us to hypothesize that these genes may be involved in Ring A biosynthesis.

cloF shows homology to putative oxido-reductases (49% from *S. coelicolor*) and to putative prephenate dehydrogenases (36% from *Amycolatopsis orientalis*).

CloR has 47% identity to a putative Class II aldolase from *S. coelicolor*. *cloQ* did not show homologies to other genes in the database, except *novQ*. *cloQ* and *cloR*, like *novQ* and *novR*, show transcriptional coupling (i.e. the stop codon of *cloQ* is fused with the start codon of *cloR*) and are likely to be transcribed as a single operon. Unusually large intergenic regions are found upstream and downstream of *cloQR* (1001 bp and 830 bp, respectively).

The role of these proteins in the formation of Ring A was elucidated in the second part of my thesis (see Results, p. 39).

1.7 Genes involved in the linkage of Ring A, B and C of clorobiocin.

Attachment of the deoxysugar to the 7-OH group of the aminocoumarin ring should require very similar glycosyl transferases in clorobiocin, novobiocin and coumermycin A_1 biosynthesis, and indeed three very similar putative glycosyltransferase genes, *cloM*, *novM* and *couM*, are found at the same relative position in all three clusters (Fig. 3, p. 31). Recently, NovM was expressed in *E. coli* and purified as a C-terminal His_8 fusion protein. The aglycone novobiocic acid and TDP-L-noviose were shown to be the preferred substrates for NovM (Freel Meyers *et al.*, 2003).

In clorobiocin and novobiocin, the aminocoumarin moiety (Ring B) and the prenylated 4-hydroxybenzoate moiety (Ring A) are linked by an amide bond (Fig. 3, p. 31). It has been demonstrated that the enzyme NovL catalyzes this reaction, i.e. adenylation of the substituted benzoyl moiety and its transfer to the amino group (Steffensky *et al.*, 2000a). The gene *cloL* shows high homology to *novL* and is most probably involved in the formation of the amide bond of clorobiocin.

1.8 Resistance and regulatory genes.

A gene encoding for a aminocoumarin-resistant gyrase B subunit (*gyr*B^R) is located downstream of the deoxysugar biosynthesis genes *cloSTUVW* in the clorobiocin cluster, and similarly at the corresponding position of the novobiocin and coumermycin clusters. This gene has previously been identified as the principal

novobiocin resistance gene in the novobiocin producer *S. spheroides* (Thiara and Cundliffe, 1988).

Unexpectedly, the clorobiocin and coumermycin A_1 clusters were found to contain an additional, similar gene, named *parY^R*. Its predicted gene product showed sequence similarity with the B unit of type II topoisomerases. Recently, expression of *gyrB^R*, and likewise of *parY^R*, in *Streptomyces lividans* TK24 resulted in resistance against novobiocin and coumermycin A_1, suggesting that both genes function as resistance genes against the aminocoumarins (Schmutz *et al.*, 2003).

cloG, *novG* and *couG* are homologous to *strR*, a regulatory gene from the streptomycin cluster. Streptomycin biosynthesis is known to be regulated by (-butyrolactones (Horinouchi and Beppu, 1995). It may therefore be speculated that (-butyrolactones are involved in the regulation of the biosynthesis of clorobiocin and other aminocoumarin antibiotics. A catalytic function of the gene product of these genes appears very unlikely.

cloE has homology to the *lmbU* gene of the lincomycin biosynthetic gene cluster of *S. lincolnensis* 78-11. It was suggested that LmbU may have a regulatory function, but no experimental evidence is available so far (Peschke *et al.*, 1995). Recently, inactivation of *novE* by gene replacement demonstrated that *novE* does not have an essential catalytic role in novobiocin biosynthesis, but is likely to have a regulatory function (Eustáquio *et al.*, 2003b).

1.9 Genes with unknown function.

At present, no function can be suggested for the small ORFs *cloY*, *cloN1* and *cloN7*, which have homologues in the coumermycin A_1 cluster.

2. Identification of CloQ as an aromatic prenyltransferase.

2.1 Introduction.

The 3-prenylated 4-hydroxybenzoic acid moiety of novobiocin (called Ring A) has been shown to be derived from tyrosine and an isoprenoid precursor (Li *et al.*, 1998), but conflicting suggestions have been made for the prenylation substrate (Calvert *et al.*, 1972; Chen and Walsh, 2001; Kominek, 1972; Steffensky *et al.*, 1998). 3-Prenylated 4-hydroxybenzoic acid moieties are known as intermediates in the biosynthesis of ubiquinones (Melzer and Heide, 1994) and shikonin (Yazaki *et al.*, 2002), where they are formed from 4-hydroxybenzoic acid (4HB) under catalysis of membrane-bound prenyltransferases. These enzymes contain the characteristic prenyl diphosphate binding site ((N/D)DxxD) known from *trans*-prenyltransferases (Koyama *et al.*, 1996; Liang *et al.*, 2002). Surprisingly, cloning of the novobiocin and the clorobiocin biosynthetic gene clusters (Pojer *et al.*, 2002; Steffensky *et al.*, 2000b) revealed neither a gene with sequence similarity to known prenyltransferases, nor genes which could be assigned to 4HB biosynthesis, e. g. similar to the benzoate biosynthesis genes recently described in *S. maritimus* (Fig. 3, p. 31 and Table 1, p. 32) (Hertweck and Moore, 2000).

A new hypothesis for the formation of Ring A was derived from studies on the biosynthesis of the aminocoumarin moiety (Ring B) of novobiocin. Chen and Walsh (2001) showed that the first two steps in Ring B formation are the activation of L-tyrosine by NovH, and the subsequent hydroxylation to ß-hydroxytyrosyl-NovH (ß-OH-Tyr-*S*-NovH) under catalysis of the cytochrome P_{450} enzyme NovI (Fig. 7, p. 40). Alkali treatment of this product led to the formation of 4-hydroxybenzaldehyde (4HBAL), resulting from a retro-aldol cleavage of ß-OH-Tyr-*S*-NovH. It therefore appeared possible that Ring A of novobiocin may also be derived from ß-OH-Tyr-*S*-NovH by a retro-aldol reaction, occurring either before or after prenylation of the aromatic nucleus (Fig. 7, p. 40). Further support for this hypothesis came from the detection of *novR* and *cloR* in the biosynthetic gene clusters of novobiocin and clorobiocin since their gene products show similarity to Class II aldolases, and the

involvement of *cloR* in Ring A biosynthesis was demonstrated by a gene inactivation experiment (see Results ,p. 54).

CloQ shows no homology to any database entries. To test if *cloQ* is involved in the formation of Ring A, a gene inactivation was done.
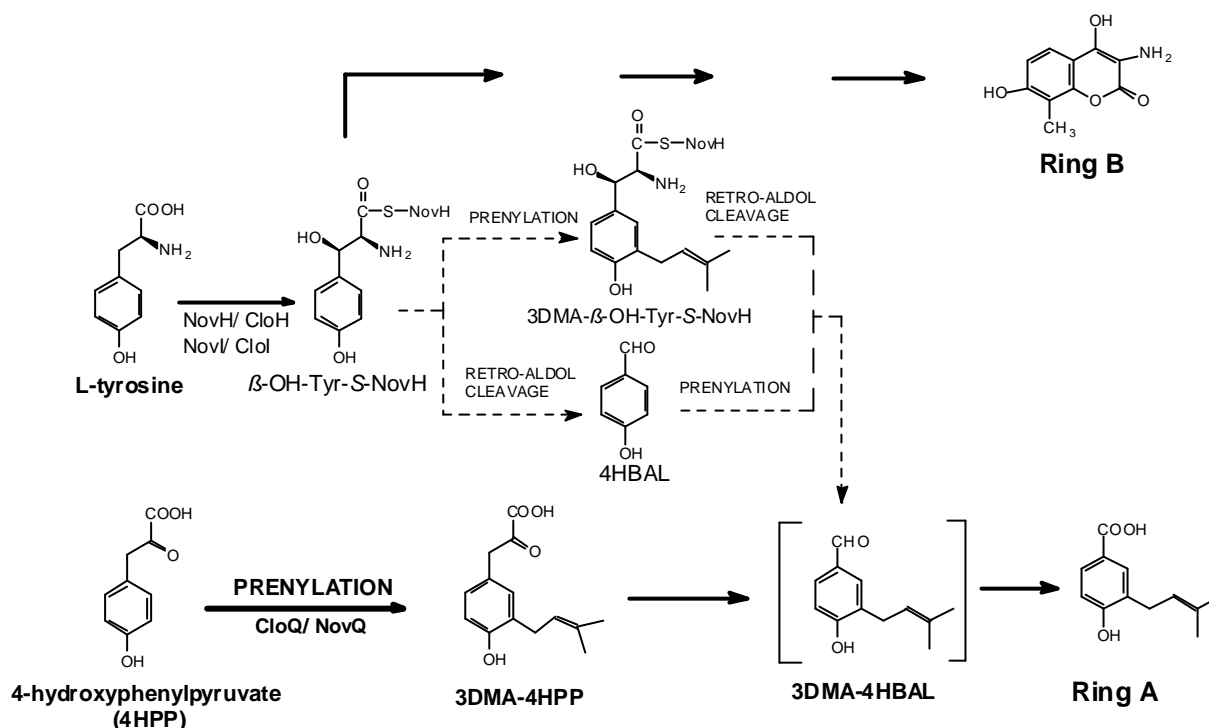
Fig. 7: Possible biosynthetic pathways to the prenylated 4-hydroxybenzoate moiety (Ring A).

4HBAL, 4-hydroxybenzaldehyde; 3DMA, 3-dimethylallyl.

2.2 Inactivation of cloQ and feeding of Ring A.

Sequence analysis of the novobiocin and clorobiocin gene clusters (see Results, p. 29) (Pojer *et al.*, 2002; Steffensky *et al.*, 2000b) did not reveal any genes with similarity to known prenyltransferases, nor genes containing the typical prenyl diphosphate binding site (N/D)DxxD (Koyama *et al.*, 1996) (Table 1, p. 32). The identification of candidates for prenyltransferase genes was facilitated, however, by a comparison of the biosynthetic gene clusters of novobiocin (Steffensky *et al.*, 2000b), clorobiocin (Pojer *et al.*, 2002) and coumermycin A₁ (Wang *et al.*, 2000) (Fig. 3, p. 31). The prenylated 4-hydroxybenzoate moiety (Ring A) is present in novobiocin and

clorobiocin, but not in coumermycin A_1 (Fig. 3, p. 31). Correspondingly, three genes were found to be present in the novobiocin and the clorobiocin clusters, but absent in the coumermycin A_1 cluster: i) the putative aldolase genes *novR* and *cloR*; ii) *novF* and *cloF*, with sequence similarity to dehydrogenases; iii) *novQ* and *cloQ*, which did not show any similarity to known genes in the database. If the prenyltransferases were contained within the clusters and were dissimilar to prenyltransferases described previously, *novQ* and *cloQ* would be possible candidate genes for these enzymes.

A gene inactivation experiment was therefore carried out with *cloQ*. To avoid polar effects on the genes downstream of *cloQ*, especially on *cloR*, an in-frame deletion of 810 base pairs within the coding sequence of *cloQ* was created (see Materials and methods, p. 85), and the correct genotype of the mutant was confirmed by Southern blotting (Fig. 8 and Fig. 9, p. 42). Chromosomal DNA from *S. roseochromogenes* wild-type as well as from mutants QSCO8 and *cloQ⁻* mutant (QDCO30) was digested by *Bam*HI and *Pvu*II and hybridized with a probe containing a part of the *cloR* gene immediately downstream of *cloQ*. A band at 1.5 kb was detected in the *S. roseochromogenes* wild-type, while chromosomal DNA from *cloQ⁻* mutant showed the expected band of 2.9 kb corresponding to the in-frame deletion of *cloQ* (Fig. 9, p. 42).

HPLC analysis of the culture extracts showed that clorobiocin production was completely abolished in the *cloQ⁻* mutant (Fig. 10, p. 43). However, upon addition of Ring A to the culture, the production of clorobiocin was restored to one third of the wild type level (Fig. 10, p. 43). This showed that *cloQ* is involved in Ring A biosynthesis. 3-dimethylallyl 4-hydroxybenzaldehyde (3-DMA-4HBAL) is a possible intermediate of Ring A biosynthesis (Fig. 7, p. 40). Adding this compound to *cloQ⁻* mutant culture proved to be equally effective in restoring clorobiocin production as the addition of Ring A.
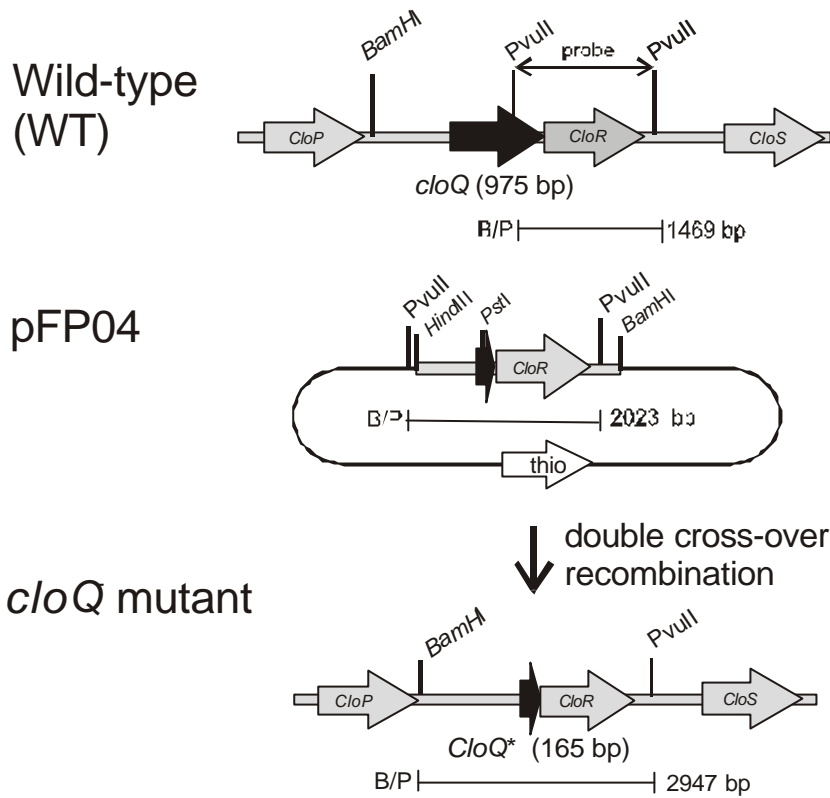
Fig. 8: Schematic representation of the inactivation of *cloQ* by in-frame deletion.

The indicated 1469 bp *Pvu*II fragment was used as a probe.
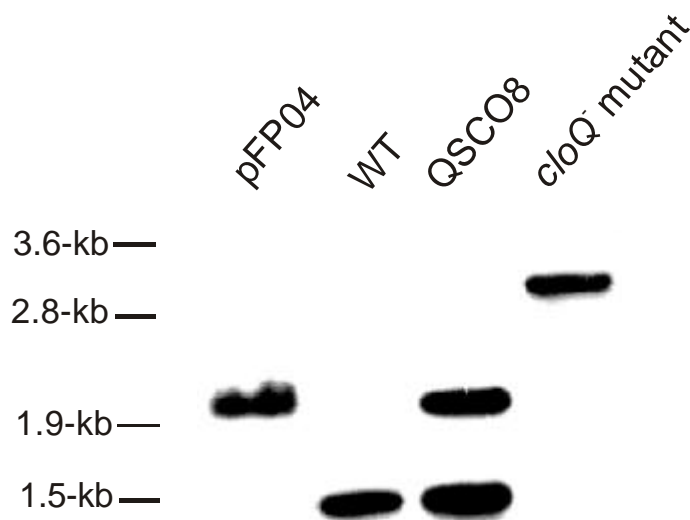
Fig. 9: Southern blot analysis of *cloQ* defective mutants.

Wild-type *S. roseochromogenes* (WT), single cross-over mutant (QSCO8) and double cross-over mutant (*cloQ*⁻ mutant) are depicted. Genomic DNA was restricted by *Pvu*II *and Bam*HI.

Fig. 10: HPLC analysis of culture extracts of the *cloQ* defective mutant.

The identity of clorobiocin ([M-H]⁻ = 695) was confirmed by LC-ESI-CID and the mass spectroscopic fragments are indicated.

2.3 Expression and purification of CloQ.

CloQ was expressed in *E. coli* as a soluble GST fusion protein of 61.6 kDa. After purification, GST was cleaved from CloQ by thrombin treatment and removed (see Materials and methods, p. 90). This resulted in apparently homogenous CloQ protein as judged by SDS-PAGE (Fig. 11, p. 44). The observed molecular weight corresponded to the calculated mass (35.6 kDa). A protein yield of 6 mg pure CloQ per liter of culture was obtained.
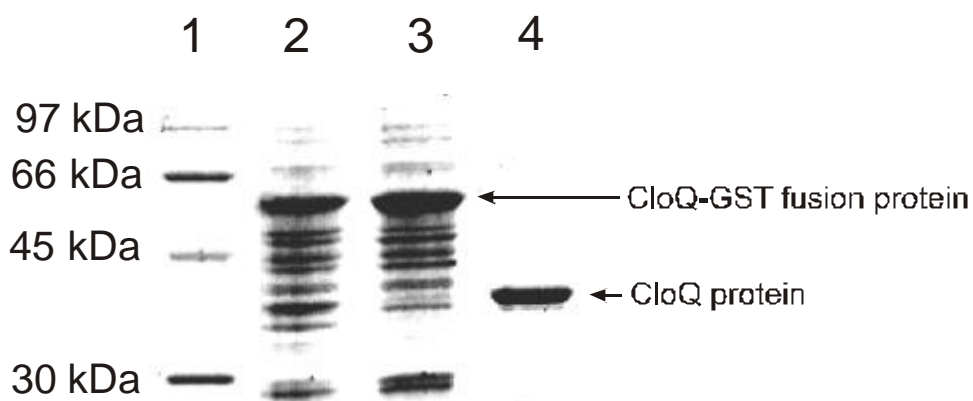
Fig. 11: Purification of CloQ after overexpression as a fusion protein with glutathion-*S*-transferase.

The 12% SDS-PAGE gel was strained with Coomassie Brilliant Blue. Lane 1, molecular weight standard; lane 2, total protein after IPTG induction; lane 3, soluble protein after induction, lane 4, eluate from glutathion sepharose chromatography after thrombin treatment.

2.4 Investigation of 4HB and ß-hydroxytyrosyl-S-NovH as substrate of CloQ

4-Hydroxybenzaldehyde (4HBAL) can be formed from *ß*-hydroxytyrosyl-*S*-NovH (*ß*-OH-Tyr-*S*-NovH) by retro-aldol cleavage (Chen and Walsh, 2001). Subsequent prenylation and oxidation may lead to the formation of Ring A of novobiocin or clorobiocin (Fig. 7, p. 40). However, when the purified CloQ protein was incubated with 4HBAL, or with the corresponding acid 4HB, in the presence of DMAPP and Mg^{2+}, no prenylated products were observed (see Materials and methods, p. 92). Alternatively, prenylation of *ß*-OH-Tyr-*S*-NovH may preceed the retro-aldol cleavage reaction in novobiocin and clorobiocin formation, rendering 3-dimethylallyl-*ß*-hydroxytyrosine, in its enzyme-bound form, as an intermediate of Ring A biosynthesis (Fig. 7, p. 40). A natural product containing a 3-dimethylallyl *ß*-hydroxytyrosyl residue has been identified previously in a fungus (Barrow *et al.*, 1994). We therefore prepared *ß*-OH-Tyr-*S*-NovH by incubation of L-[U-^{14}C]tyrosine with NovH and NovI, as described by Chen and Walsh (2001). When the resulting products were precipitated with trichloroacetic acid and cleaved with alkali, [^{14}C]4HBAL was

detected by HPLC, representing the retro-aldol reaction product from ß-OH-Tyr-*S*-NovH. However, when CloQ, DMAPP and Mg^{2+} were included in the enzyme incubation, no prenylated products were observed (Fig. 12, p. 45) (see Materials and methods, p. 90).

Therefore, neither ß-OH-Tyr-*S*-NovH nor 4HB or 4HBAL were readily identified as substrates for CloQ.

A
NovHI+ CloQ with [UL¹⁴C] tyrosine

B
NovHI+ CloQ denatured with [UL¹⁴C] tyrosine
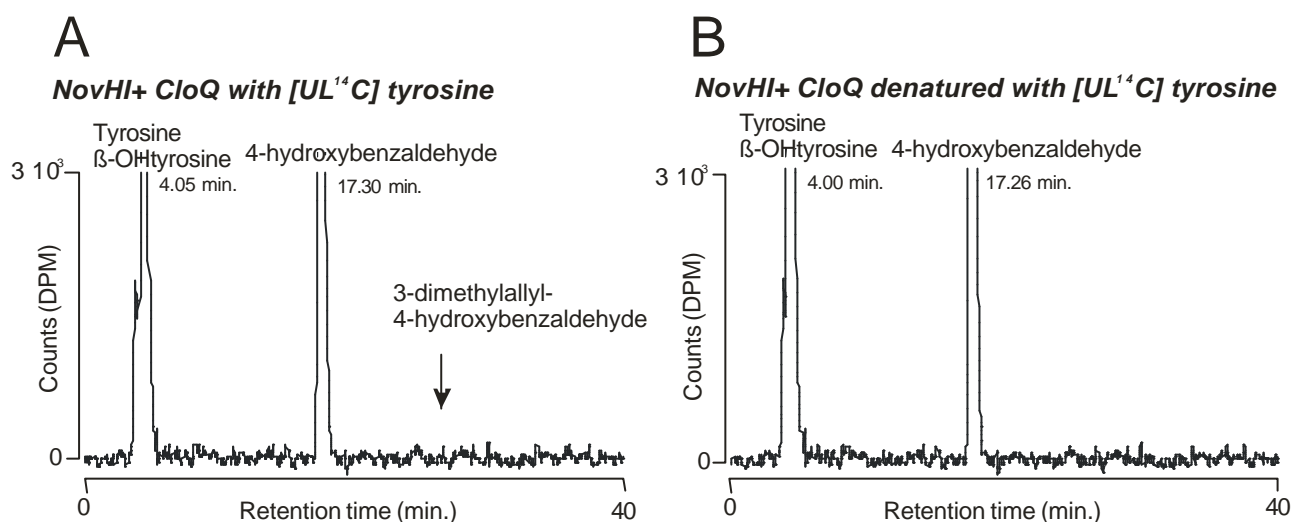
Fig. 12: HPLC analysis of [UL¹⁴C]-tyrosyl-S-NovH/NovI/CloQ incubation products released by KOH treatment.

Plot of radioactivity versus retention time (RT) from the complete reaction (A) and from the control reaction (B). The realized radioactive compounds were proven to be tyrosine + ß-OHtyrosine (RT= 4 min) and 4-hydroxybenzaldehyde (RT= 17.3 min) by co-chromatography. No peak was found corresponding to 3-dimethylallyl-4-hydroxybenzaldehyde (RT= 26.3 min).

2.5 Inactivation of *cloI*: Proof for an independent pathway for Ring A biosynthesis.

The cytochrome P_{450} enzyme CloI is responsible for the hydroxylation of Tyr-*S*-CloH to ß-OH-Tyr-*S*-CloH (Fig. 6, p. 36) (Chen and Walsh, 2001). If the latter compound is indeed an intermediate in Ring A biosynthesis, inactivation of *cloI* should lead to the abolishment of Ring A formation. *cloI* was inactivated by in-frame deletion (Fig. 13, p. 47) (see Materials and methods, p. 85), and the genotype of the resulting mutant was confirmed by Southern blotting (Fig. 14, p. 47). Chromosomal DNA from *S.*

roseochromogenes wild-type as well as from mutants ISCO4 and *cloI* mutant (IDCO169) was digested by *Nco*I and hybridized with a 2 kb probe containing *cloI* gene. A band at 2 kb was detected in the *S. roseochromogenes* wild-type, while chromosomal DNA from *cloI* mutant showed the expected band of 0.9 kb corresponding to the in-frame deletion of *cloI*.

HPLC analysis of culture extracts of the *cloI* mutant showed complete abolishment of the clorobiocin production (Fig. 15A, p. 48). This was expected since CloI had been shown to catalyze an essential step in Ring B biosynthesis (Chen and Walsh, 2001). The same cultures were subsequently analyzed for the accumulation of Ring A, using a different UV wavelength for detection (Fig. 15B, p. 48). The wild-type did not accumulate detectable amounts of this compound. However, Ring A was clearly detected in the *cloI* mutant (approximately 3.5 mg/l culture). This compound was identified mass spectroscopically, using LC-ESI-CID, by comparison to an authentic reference sample. Moreover, addition of Ring B of novobiocin to the *cloI* mutant led to the formation of a new clorobiocin analogue, termed novclobiocin C102, in which the chlorine atom of clorobiocin is replaced by a methyl group. This compound was identified mass spectroscopically, using negative LC-ESI-CID (m/z= 675 [M-H]⁻, 488, 206).

These experiments demonstrated that Ring A can still be formed in the absence of CloI. Therefore, *ß*-OH-Tyr-*S*-CloH cannot be an intermediate in the biosynthesis of Ring A of clorobiocin (Fig. 7, p. 40). This prompted us to test additional compounds as substrates for CloQ.
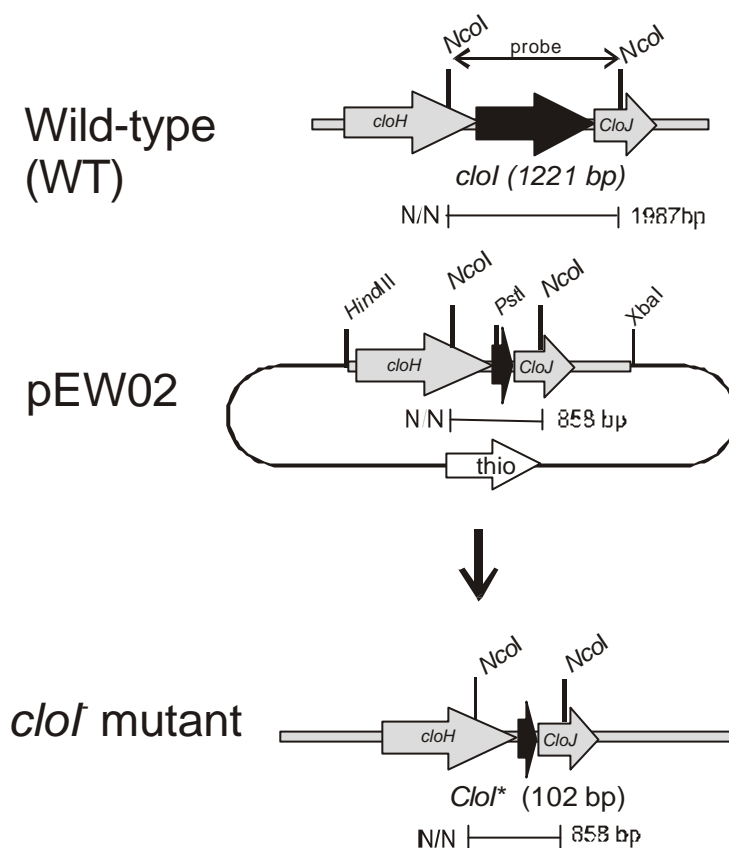
Fig. 13: Schematic representation of the inactivation of *cloI* by in-frame deletion.

thio, thiostrepton resistance gene.
The indicated 1987 bp *Nco*I fragment was used as probe.
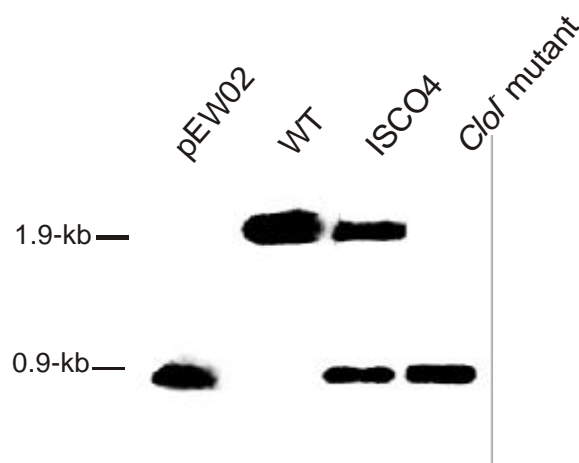
Fig. 14: Southern blot analysis of *cloI* defective mutant.

Wild-type S. roseochromogenes (WT), single cross-over event (ISCO4) and double cross-over event (*cloI* mutant) are depicted. Genomic DNA was restricted by *Nco*I.
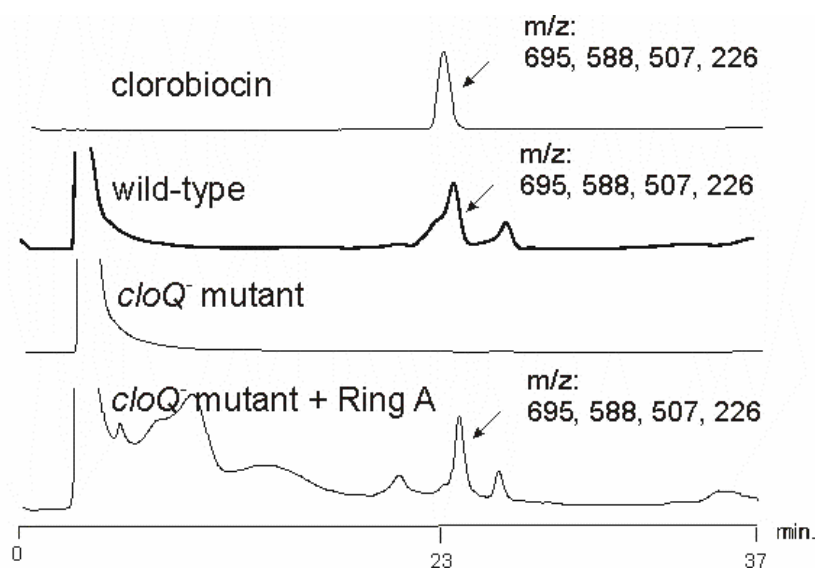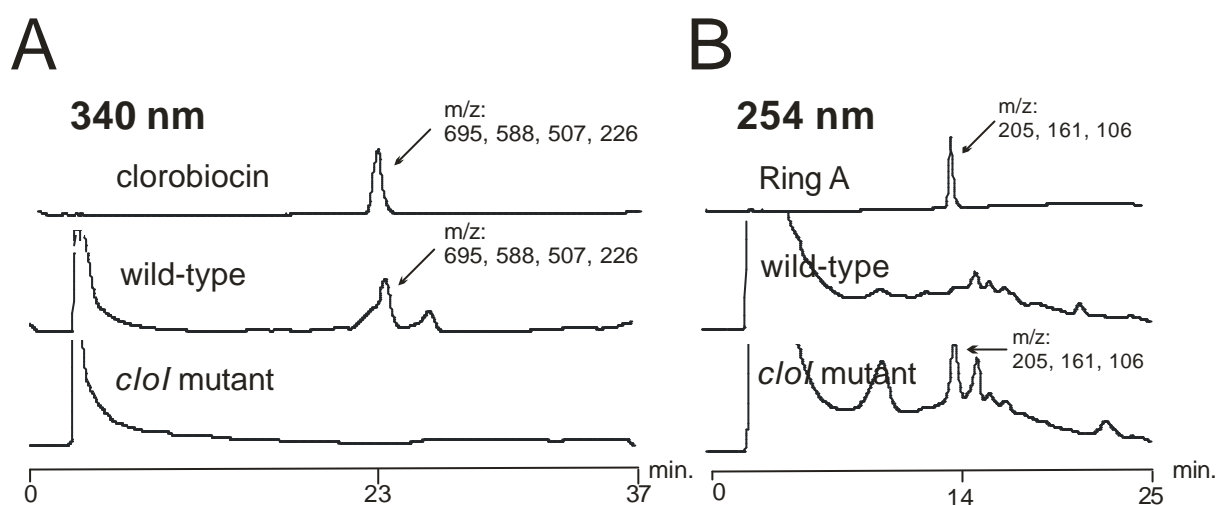
Fig. 15: HPLC analysis of culture extracts of the *cloI* defective mutant.

(A) Clorobiocin standard; wild-type; *cloI* mutant. Detection at 340 nm. (B) Ring A standard; wild-type; *cloI* mutant. Detection at 254 nm. The identity of clorobiocin ([M-H]⁻ = 695) and Ring A ([M-H]⁻ = 205) were confirmed by LC-ESI-CID. Mass spectroscopic fragments are indicated.

2.6 Identification of 4-hydroxyphenylpyruvate as substrate of CloQ.

When CloQ was incubated with 4-hydroxyphenylpyruvate (4HPP) and [1-^{14}C]DMAPP, the formation of a prenylation product was readily detected (Fig. 16A, p. 49) (see Materials and methods, p. 92). This product was absent in control incubations with heat-denatured CloQ. The incubation was repeated with non-radioactive substrates, and the product was analyzed mass spectroscopically using LC-ESI-CID, resulting in the following ions: m/z= 247 ([M-1]⁻), 203 ([M-44]⁻), 175 ([M-72]⁻), 119 ([M-128]⁻) (Fig. 17, p. 50). This is identical to the mass spectrum of a previously isolated sample of this compound, which had been identified by both mass spectroscopy and ^{1}H-NMR (Steffensky *et al.*, 1998).

4HPP is an unstable compound and decomposes to 4-hydroxybenzaldehyde (4HBAL), especially in the presence of alkali (DOY, 1960). When the enzymatic prenylation products were treated with 0.5 M NaOH, a nearly complete conversion of 3DMA-4HPP to 3DMA-4HBAL was observed (Fig. 16B, p. 49). The latter compound was identified in comparison to an authentic reference sample, synthesized according to Gluesenkamp and Buechi (1986).
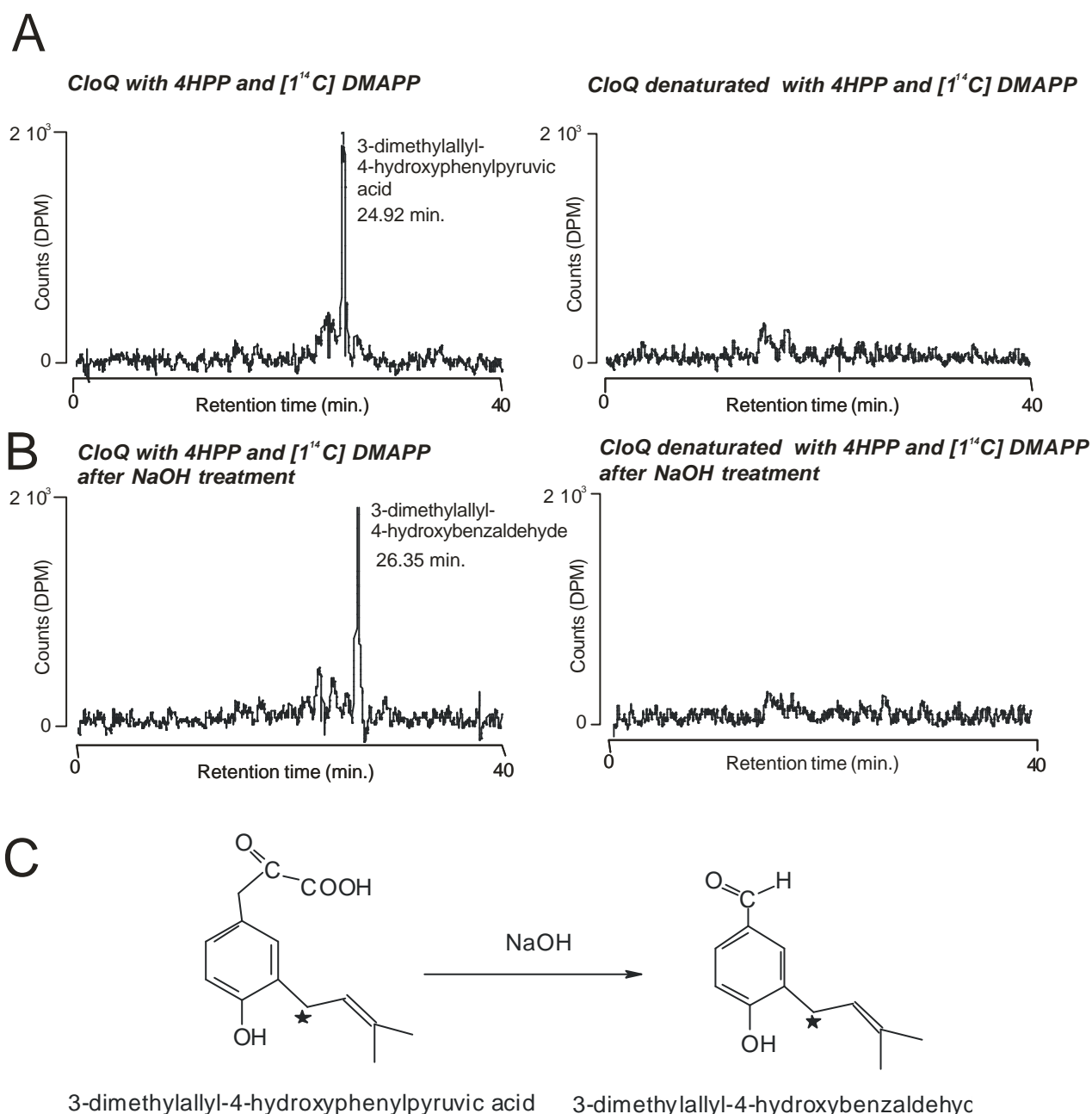
Fig. 16: HPLC analysis of the prenyltransferase assay.

(A)   HPLC analysis of the incubation product of CloQ with [1 ^{14}C] DMAPP and 4-hydroxyphenylpyruvic acid before NaOH treatment: plot of radioactivity versus retention time (RT) from the complete reaction and from the control reaction in which CloQ was denaturated. The radioactive peak (RT= 24.9 min) was proven to be 3-dimethylallyl-4-hydroxyphenylpyruvate by LC-ESI-CID.

(B)   HPLC analysis of the incubation product of CloQ with [1 ^{14}C] DMAPP and 4-hydroxyphenylpyruvic acid after NaOH treatment: plot of radioactivity versus retention time from the complete reaction and from the control reaction in which CloQ was denaturated. The new radioactive peak (RT= 26.35) has the same RT as 3-dimethylallyl-4hydroxybenzaldehyde.

(C)   Retro-aldol conversion of radioactive 3DMA-4HPP to radioactive 3DMA-4HBAL by NaOH treatment.
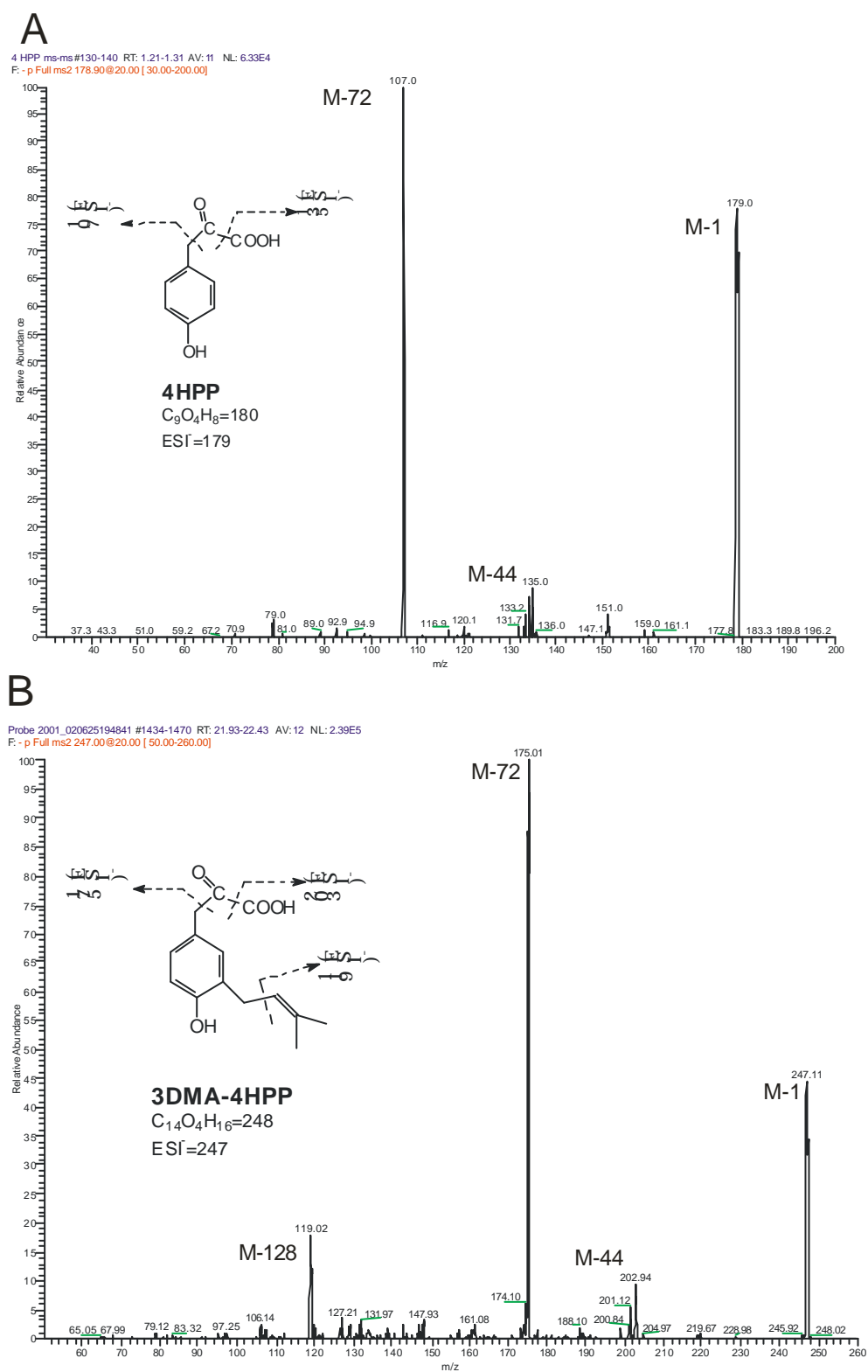
Fig. 17: Mass spectrometry data obtained of 4HPP and 3DMA-4HPP.

(A). Negative-ion-ESI-CID data of authentic 4-hydroxyphenylpyruvic acid (4HPP).

(B) Negative-ion-ESI-CID data of prenylated 4-hydroxyphenylpyruvic acid (3DMA-4HPP)

2.7 Biochemical properties and kinetic parameters of 4HPP dimethylallyltransferase.

The native molecular mass of CloQ was determined as 34.6 kDa using gel chromatography, showing that the protein was monomeric in solution. Moreover, the enzyme was soluble in the absence of detergents.

Product formation showed a linear dependence on the amount of protein (up to 3 µg per assay), and on the reaction time (up to 15 min).

The reaction was strictly dependent on the presence of active CloQ, 4HPP and DMAPP. The presence of Mg^{2+} enhanced prenyltransferase activity, with 2.5 mM being the most effective concentration (Fig. 18, p. 52). However, in the absence of divalent cations and in the presence of 5 mM of EDTA, the enzyme retained 25% of its original activity. This is in contrast to the absolute requirement for divalent cations reported for most prenyltransferases (Liang *et al.*, 2002). The addition of Ca^{2+} (2.5 mM) instead of Mg^{2+} resulted in 70% of the activity obtained with Mg^{2+}. No product formation could be detected by addition of $ZnCl_2$, $CuCl_2$, $CoCl_2$ or $MnCl_2$ due to degradation of the substrate 4HPP by these metal ions.

In contrast to many protein prenyltransferases that contain a tightly bound zinc atom (Harris *et al.*, 2002), purified CloQ protein was found to contain neither zinc nor magnesium (see Materials and methods, p. 95).

CloQ was found to be specific for the substrate 4HPP. No product formation was observed using L-tyrosine, 4HB, 4HBAL or prephenic acid. Only with *ß*-hydroxy-L-tyrosine, formation of dimethylallyl-*ß*-hydroxytyrosine was observed. The identity of the product was confirmed by LC-ESI-CID (m/z: 264 $[M-H]^-$, 189, 134). The reaction velocity, however, was only 2% of that obtained with 4HPP. When DMAPP was replaced with isopentenyl diphosphate (IPP) or geranyl diphosphate (GPP), no product formation was observed.

The CloQ reaction apparently followed Michaelis-Menten kinetics, and the K_m values were determined by the Lineweaver-Burk method as approximately 25 µM for 4HPP and 35 µM for DMAPP (Fig. 19 and Fig. 20, p. 52, respectively). The maximum reaction velocity observed was 3690 pKat mg^{-1}, corresponding to a turnover number of 7.9 min^{-1}.
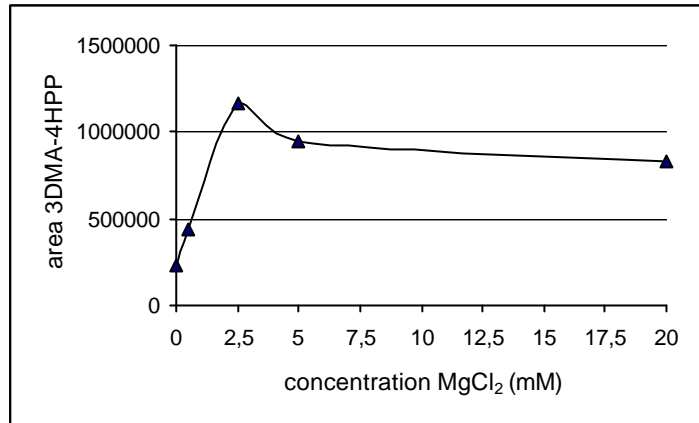
Fig. 18: Influence of MgCl$_2$ in the 4HPP prenyltransferase assay.
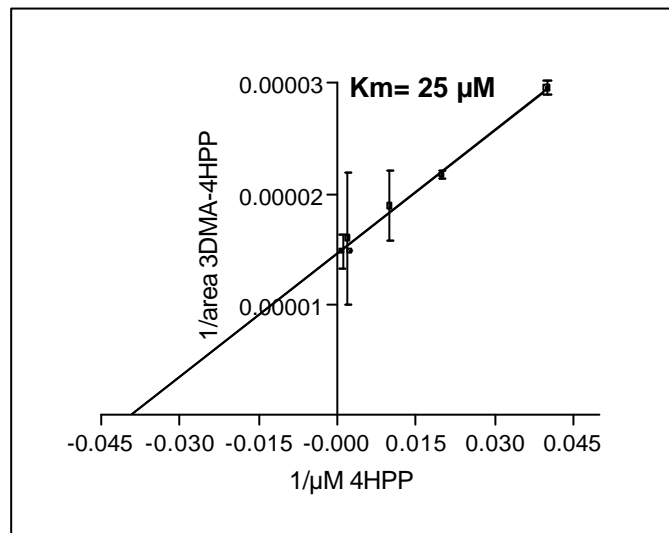
Fig. 19: Lineweaver-Burk Plot for calculation of the K$_m$ of 4HPP.
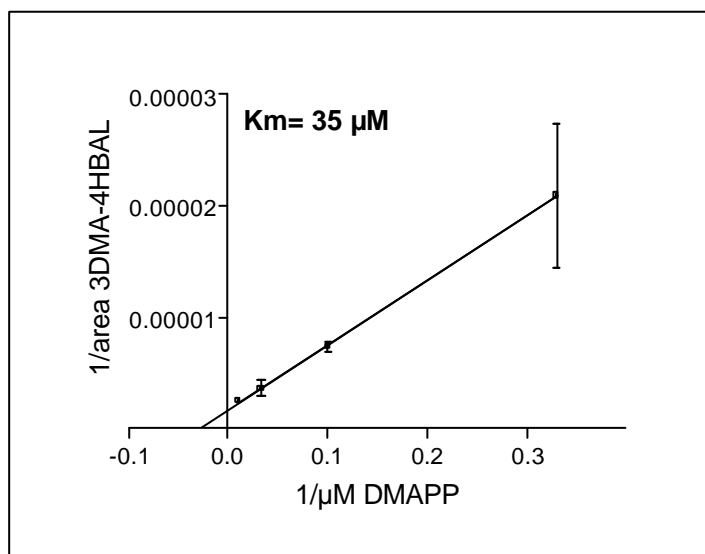
Fig. 20: Lineweaver-Burk Plot for calculation of the K$_m$ of DMAPP.

3. Identification of CloR as a non-heme iron dependent oxygenase.

3.1 Introduction.

Three genes were identified in the novobiocin and clorobiocin clusters for which no homologues existed in the coumermycin cluster (Fig. 3, p. 31). We speculated that these genes might be involved in the biosynthesis of Ring A (which is absent in coumermycin A$_1$). These genes were: a) *cloR* and *novR*, which showed sequence similarity to putative class II aldolases; b) cloF and novF, which show sequence similarities to prephenate dehydrogenases; c) cloQ and novQ; the protein CloQ was identified to code for a prenyltransferase (see Results, p. 39-52). The substrate of this enzyme is 4-hydroxyphenylpyruvate (Fig. 7, p. 40). The product of the reaction, i.e. 3-dimethylallyl-4HPP, was easily degraded to 3-dimethylallyl-4-hydroxybenzaldehyde (3DMA-4HBAL), suggesting a biosynthesis of Ring A by the mechanism depicted in Fig. 7, p. 40, i.e. *via* oxidation of 3DMA-4HBAL to the corresponding acid.

My work was to investigate the conversion of 3DMA-4HPP to Ring A and the role of CloR in this reaction sequence.

3.2 Sequence analysis of CloR and NovR

The gene *cloR* from the clorobiocin cluster and the corresponding gene *novR* from the novobiocin cluster (Fig. 3, p. 31 and Table 1, p. 32) encode proteins of 277 and 270 amino acids, respectively, and show 95% identity with each other. A database search revealed sequence similarity to class II aldolases which are represented e.g. by L-fuculose-1-phosphate aldolase (Dreyer and Schulz, 1996) and L-rhamnose-1-phosphate aldolase (Kroemer and Schulz, 2002), and to the structurally related L-ribulose-5-phosphate-4-epimerase (Luo *et al.*, 2001). These enzymes contain a catalytic zinc residue in their active center and are involved in the catabolism of sugars in *E. coli*.

cloR is transcriptionally coupled to the gene *cloQ*, which encodes the aromatic prenyltransferase involved in Ring A formation (see Results, p. 39) (Pojer *et al.*, 2003). The same situation is found in the novobiocin cluster for the corresponding genes *novQ* and *novR*.

3.3 Inactivation of cloR and feeding of Ring A.

In order to test whether *cloR* was involved in Ring A biosynthesis, a gene inactivation experiment was carried out. An inactivation vector carrying a thiostrepton resistance gene (pFP02) was constructed in which *cloR* was disrupted by in-frame deletion (see Materials and methods, p. 84) (Fig. 21, p. 55). Chromosomal DNA from *S. roseochromogenes* wild-type as well as from mutants RSCO2, RDCO30 and RDCO32 was digested by *Sac*II and hybridized with a probe containing a part of the *cloR* gene (Fig. 21, p. 55). A band of approximately 1.1 kb in size was detected upon hybridization of the wild-type *S. roseochromogenes*, whereas hybridization of the chromosomal DNA from mutant strain RDCO30 with the probe produced the expected 2.2 kb band, which corresponded to the in-frame deletion of *cloR* (Fig. 22, p. 55).

The deletion mutant, RDCO30, was subsequently cultured and examined by HPLC for secondary metabolites. As shown in Fig. 23, p. 56, the production of clorobiocin was abolished in this mutant. Another thiostrepton sensitive strain obtained in the screening for double cross-over mutants, RDCO32, represented a reversion to the wild-type (Fig. 22, p. 55) and showed clorobiocin production identical to that in the wild-type strain.

In order to restore clorobiocin biosynthesis in the *cloR* defective mutant RDCO30, Ring A (3-dimethylallyl-4-hydroxybenzoic acid) was added to the culture of this strain. This led to the reconstitution of clorobiocin production (Fig. 23, p. 56), to a third of the original wild-type level. The identity of this product was confirmed by MS and ^{1}H-NMR, in comparison to authentic reference substance (see Materials and methods, p. 86).

Besides the major peak of clorobiocin (peak A), a minor peak of identical mass (peak B) was detected both in the wild-type and in the complemented mutant. This substance is likely to present an isomer of clorobiocin, possibly carrying the pyrrole carboxylic acid moiety in position 2 instead of position 3 of the deoxysugar. Recently, ^{1}H-NMR confirmed that peak B is, indeed, an isomer of clorobiocin (S.-M. Li, personal communication). Such isomers have been reported previously for novobiocin (Hinman *et al.*, 1957).
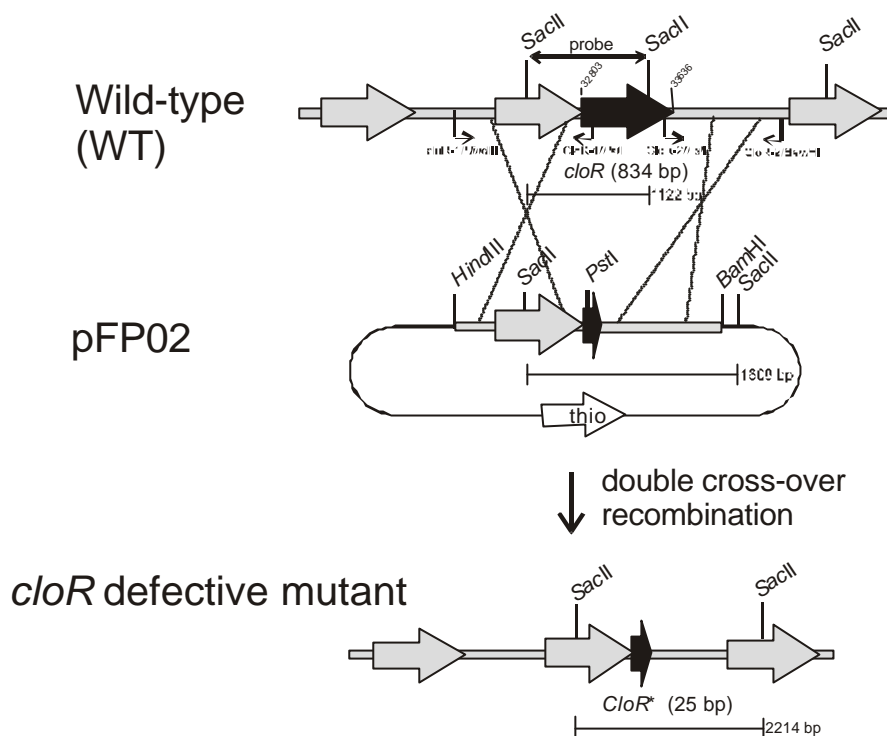
Fig. 21: Schematic representation of the inactivation of cloR by in-frame deletion.
thio, thiostrepton resistance gene. A 1122 bp SacII fragment was used as probe.
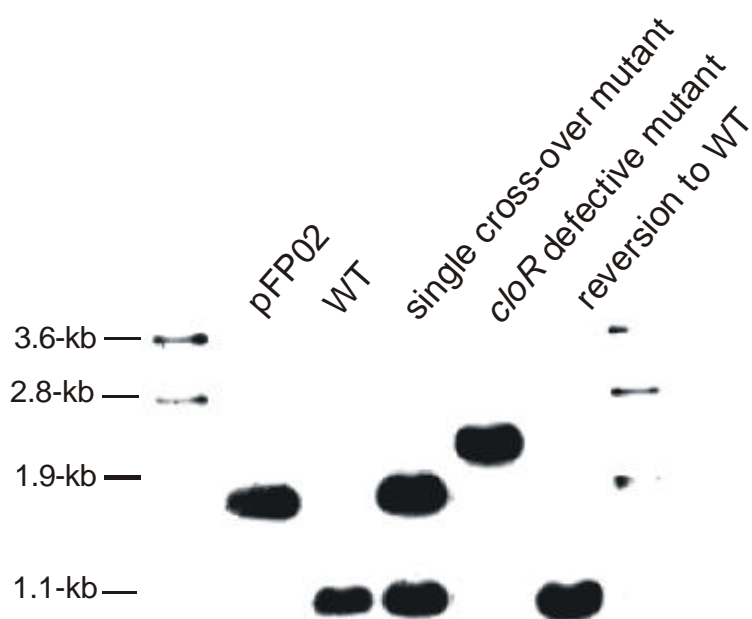
Fig. 22: Southern blot analysis of cloR defective mutant.
Genomic DNA was restricted by SacII (S/S).
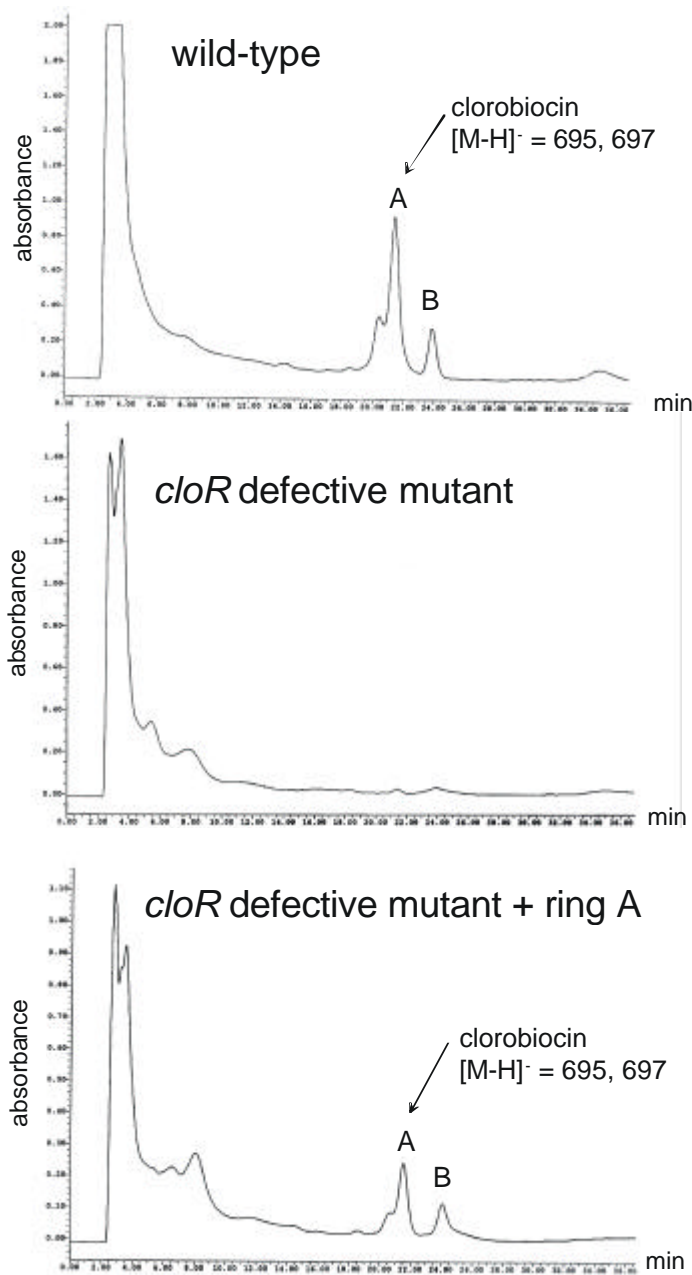
Fig. 23: HPLC analysis of the *cloR* defective mutant.

Analysis of secondary metabolites produced by *S. roseochromogenes* wild-type, *cloR* defective mutant , and complementation of *cloR* defective mutant with 3-dimethylallyl-4-hydroxybenzoic acid (Ring A). Peak A and B correspond to clorobiocin ([M-H]⁻= 695, 697).

3.4 Expression and purification of CloR

We decided to express CloR as a glutathion-*S*-transferase (GST) fusion protein rather than as a His-tagged protein, since CloR showed sequence similarity to class II aldolases, and these enzymes require zinc for their activity (Dreyer and Schulz, 1993). A purification as His-tagged protein can lead to a complete loss of aldolase activity due to interactions between the hexahistidyl tag and the metal ion (Zn^{2+}).

E. coli cells harboring CloR expression constructs yielded only insoluble protein when grown at temperatures of 20°C or higher. To obtain soluble CloR-GST fusion protein, cells were cultured at 15°C and induced with 250 µM IPTG. After purification, GST was cleaved from CloR by thrombin treatment and removed. This procedure resulted in apparently homogenous CloR protein as judged by SDS-PAGE (Fig. 24). The molecular mass observed in SDS corresponded to the calculated mass of the protein (30.5 kDa). A protein yield of 1 mg of pure CloR per liter of culture was obtained. By using gel chromatography, the molecular mass of native CloR was determined as 124.5 kDa showing that the protein was tetrameric in solution (see Materials and methods, p. 88).
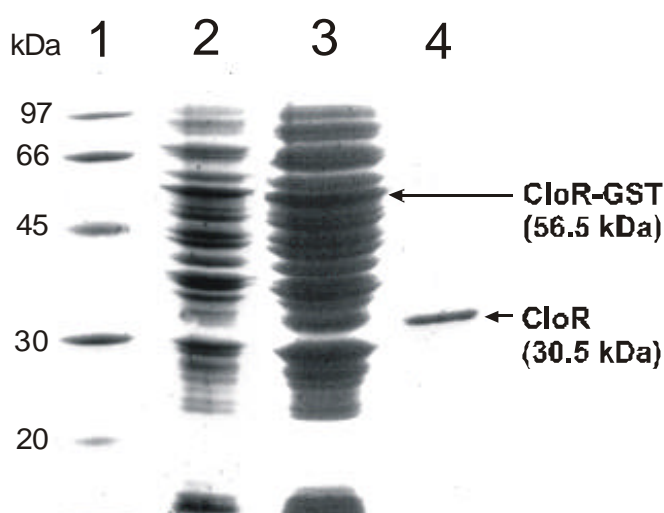
Fig. 24: Purification of CloR after overexpression as a fusion protein with glutathion-*S*-transferase.

The 12% SDS-PAGE gel was stained with Coomassie Brilliant Blue. Lane 1, molecular weight standard; lane 2, total protein after IPTG induction; lane 3, soluble protein after induction, lane 4, eluate after thrombin treatment. The calculated molecular weights were 56.5 kDa (CloR-GST) and 30.5 kDa (CloR).

3.5 Characterization of the reaction products of the CloR reaction

In order to investigate the catalytic activity of CloR, we first produced 3DMA-4HPP (Fig. 7, p. 40), the putative substrate of CloR, by incubation of 4-hydroxyphenylpyruvate (4HPP) with DMAPP and the prenyltransferase CloQ (see Materials and methods, p. 92). Subsequently, CloR and different cofactors, e.g. Zn^{2+} and NADH (Wehmeier, 2001), were added to the reaction mixture. However, no formation of 4-hydroxybenzaldehyde (3DMA-4HBAL) or Ring A could be detected by HPLC.

A more sensitive analysis using a radioactive assay with 4HPP and $[1-^{14}C]$ DMAPP as substrates, however, revealed the presence of a small amount of a new radioactive compound (termed product X), with a retention time of 11.1 min in HPLC (Ring A: 16.7 min; 3DMA-4HBAL: 20.7 min; 3DMA-4HPP: 18.4 min). This product was absent if heat-denaturated CloR was used, or if 4HPP or DMAPP were omitted from the prenylation assay. This indicated that the new metabolite X was derived enzymatically from 3DMA-4HPP.

In the biosynthesis of chloroeremomycin in *Amycolatopsis orientalis*, 4HPP is converted to 4-hydroxymandelic acid under catalysis of the non-heme iron dioxygenase HmaS (=ORF21) (Choroba *et al.*, 2000; Hubbard *et al.*, 2000). As described by Hubbard *et al.* (2000), HmaS and similar enzymes need to be activated by preincubation with an excess of Fe^{2+} immediately before incubation and Fe^{3+}, generated by oxidation, has to be reduced to Fe^{2+} by ascorbic acid in order to maintain an active enzyme. Although CloR did not show sequence similarity to HmaS or other non-heme iron dependent enzymes, we decided to test CloR under similar conditions. After preincubation of CloR for 20 min with 1 mM $FeSO_4$, ascorbic acid and enzymatically generated 3DMA-4HPP were added (see Materials and methods, p. 93). After incubation for 1 hour, the products of the reaction were analyzed by HPLC (Fig. 25, p. 60). Under these conditions, formation of the new product X was approximately 15-fold higher than in the absence of Fe^{2+} and ascorbate. Furthermore, an additional product was detected which showed the same retention time as an authentic Ring A standard. LC-ESI-CID analysis in comparison with an authentic reference compound confirmed that this latter product was indeed Ring A (m/z: 205, 161, 150, 106).

When product X was isolated by HPLC and incubated with holo-CloR in presence of ascorbic acid, again the formation of Ring A was clearly demonstrated by LC-ESI-CID analysis, proving that product X was an intermediate in the formation of Ring A.

LC-ESI-CID analysis of product X revealed that this compound showed the calculated mass ($[M-H]^- = 235$) of 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA). Furthermore, it showed the characteristic fragmentation pattern of a 4-hydroxymandelic acid derivative (m/z: 235 $[M-1]^-$, 191 $[M-44]^-$, 189 $[M-46]^-$); the same pattern was observed from an authentic 4-hydroxymandelic acid (m/z: 167 $[M-1]^-$, 123 $[M-44]^-$, 121 $[M-46]^-$).

Therefore, CloR catalyzes two consecutive reactions (Fig. 25, p. 60): first, the conversion of 3DMA-4HPP to 3DMA-4-hydroxymandelic acid (3DMA-4HMA), and second, the conversion of 3DMA-4HMA to 3DMA-4-hydroxybenzoic acid, i.e. Ring A.

CloR was found to be specific for 3DMA-4HPP and 3DMA-4HMA as substrates. No product formation was observed with the non-prenylated substrates 4-hydroxyphenylpyruvate or DL-4-hydroxymandelic acid, nor with D-mandelic acid, L-mandelic acid, ß-hydroxytyrosine or 4-hydroxyphenyllactic acid. Additional experiments confirmed that the prenyltransferase CloQ specifically prenylated 4HPP and was not able to react with DL-4-hydroxymandelic acid or 4-hydroxybenzoic acid.

Replacement of Fe^{2+} with other monovalent or divalent metal ions (1 mM Cu^+, Zn^{2+}, Mg^{2+} or Mn^{2+}) resulted in almost complete (95- 98%) loss of enzymatic activity of CloR, as described previously for non-heme iron oxygenases (Que and Ho, 1996). Many non-heme iron dependent oxygenases require, besides Fe^{2+}, an a-ketoacid as cofactor, e.g. a-ketoglutaric acid (Choroba et al., 2000). However, the activity of CloR was not stimulated by addition of a-ketoglutarate.

Purified CloR was a colorless protein. UV-VIS spectrometry showed an absorption maximum at 283 nm, but no absorption in the visual range. Therefore, CloR is not a heme protein, as are e.g. the cytochrome P_{450} monooxygenases
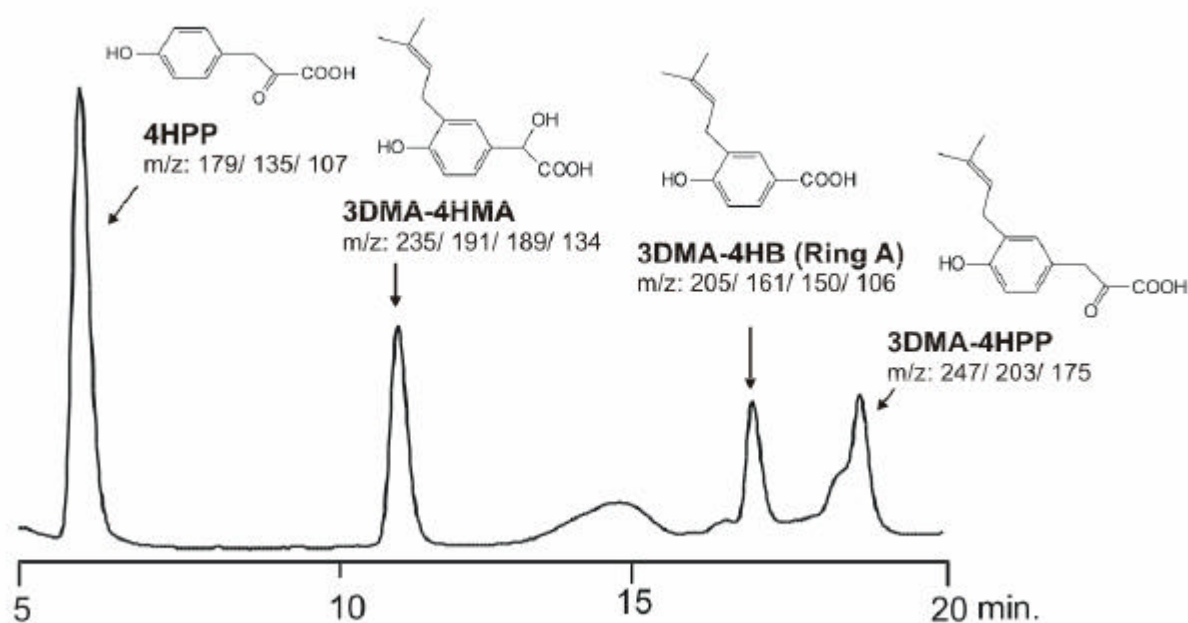
Fig. 25: HPLC analysis of the CloR reaction products.

Detection was carried out at 277 nm. The identity of each product was confirmed by LC-ESI-CID analysis, and the fragmentation pattern is indicated.

3.6 Investigation of the reaction mechanism of CloR

The requirement of CloR for Fe^{2+} and ascorbate suggested that it belongs to the non-heme iron oxygenases (Prescott and Lloyd, 2000; Que and Ho, 1996; Serre *et al.*, 1999). To confirm whether indeed molecular oxygen was the substrate of the CloR reaction, and whether one or both oxygen atoms of O_2 were incorporated into the product, we carried out isotope-labelling experiments with $^{18}O_2$ (see Materials and methods, p. 93). Incorporation of the label was analyzed by LC-ESI-CID analysis.

In the first experiment, CloR (after pre-incubation with Fe^{2+}) was incubated with 3DMA-4HPP and ascorbate in an $^{18}O_2$ atmosphere. A control incubation was carried out in the usual $^{16}O_2$ atmosphere. The reaction products 3DMA-4HMA and 3DMA-4HB were separated by HPLC and analyzed by MS-MS. Fig. 26A (p. 62) shows the molecular ions obtained in usual and in $^{18}O_2$ atmosphere. Unlabeled 3DMA-4HMA showed the molecular ion at [M-H]⁻=235. In contrast, most of the 3DMA-4HMA produced under $^{18}O_2$ atmosphere showed the molecular ion at [M-H]⁻=239, demonstrating incorporation of two ^{18}O atoms. The position of the label was revealed by MS-MS analysis: the decarboxylation product (m/z= 193) was 46 Da smaller than

the parent compound, indicating that one of the ^{18}O atoms had been incorporated into the carboxyl group of 3DMA-4HMA. Both the direct decarboxylation product (m/z= 193) and the corresponding keto compound arising from oxidation (m/z= 191) were 2 Da larger than the corresponding ions of the unlabeled 3DMA-4HMA, indicating that the other $^{18}O_2$ had been incorporated into the a-hydroxyl group. For the molecular ion of 3DMA-4HMA, an additional minor peak at [M-H]$^-$=237 was detected (Fig. 26A, p. 62), resulting from the incorporation of a single ^{18}O atom into the product. This shows a certain dilution of the label and has been reported previously for HmaS as well as for other non-heme iron oxygenases (Choroba *et al.*, 2000). This dilution has been suggested to result from an exchange of a presumed Fe^{IV}=O intermediate with water (Choroba *et al.*, 2000; Rohde *et al.*, 2003). The product mixture also contained some unlabeled 3DMA-4HMA ([M-H]$^-$=235), most likely due to the presence of residual $^{16}O_2$ in the incubation vial. In addition to 3DMA-4HMA, the CloR reaction also produced 3DMA-4HB. Incorporation of two ^{18}O atoms into this product was demonstrated by the molecular ion at [M-H]$^-$=209, in comparison to [M-H]$^-$=205 for the unlabeled compound (Fig. 26A, p. 62). As expected both these labeled oxygens were located in the carboxyl group (Fig. 26A, p. 62).

For a second labelling experiment, the intermediate 3DMA-4HMA was first produced in unlabeled form and isolated by HPLC. This compound was then incubated in an $^{18}O_2$ atmosphere with CloR (preincubated with Fe^{2+}) and ascorbate. The resulting 3DMA-4HB was analyzed by LC-ESI-CID (Fig. 26B, p. 62), and this clearly showed the incorporation of one ^{18}O atom into the carboxyl group of the product, as demonstrated by the molecular ion at [M-H]$^-$=207, and the decarboxylation product at m/z= 161 (Fig. 26B, p. 62).

Fig. 26C (p. 62) summarizes the Results of the $^{18}O_2$ incorporation experiments. Two ^{18}O atoms are incorporated in the first reaction step, and one ^{18}O atom in the second reaction step. The other ^{18}O atom involved in this second step is most likely converted to water (see Discussion, p. 73).
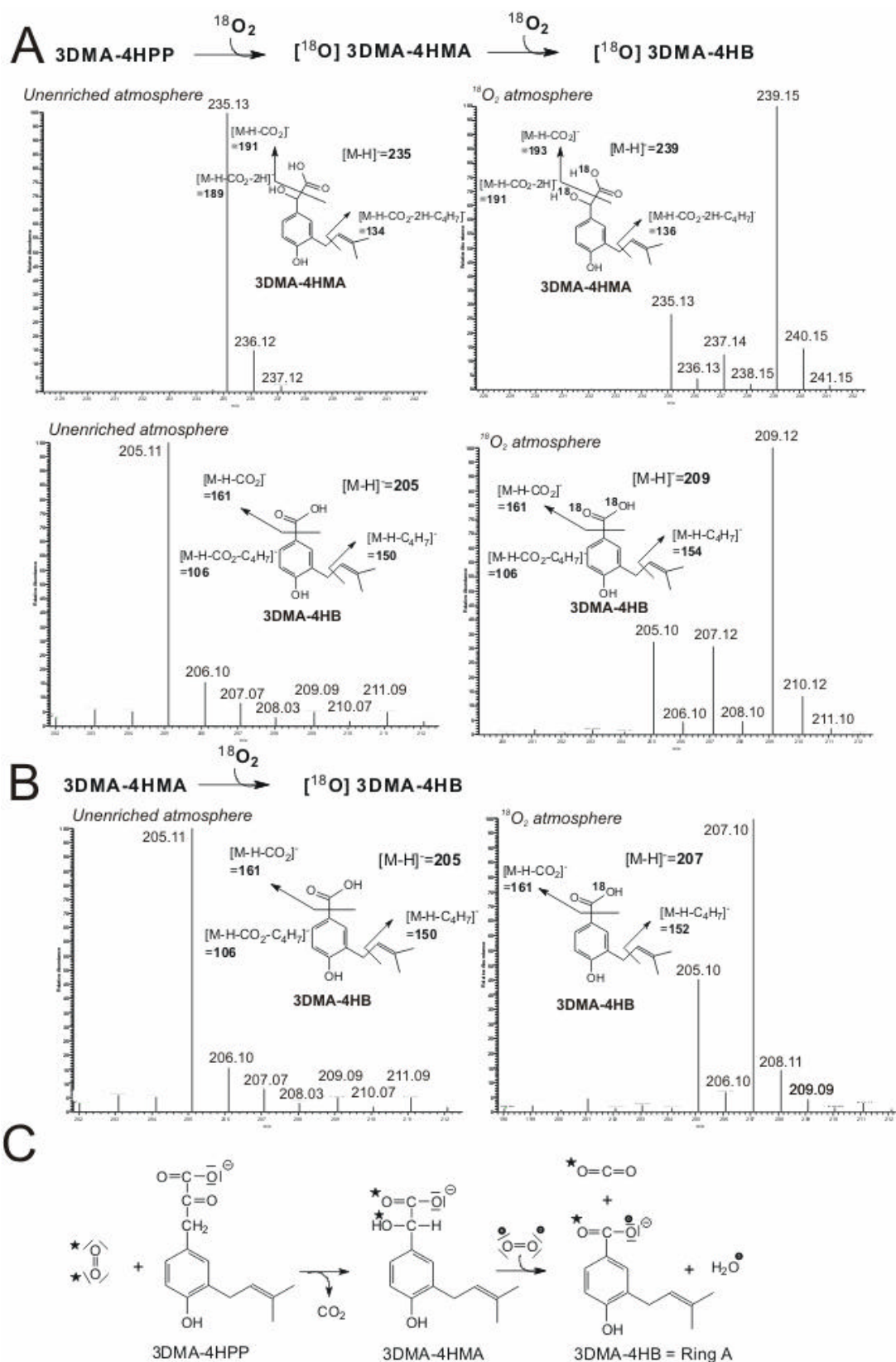
Fig. 26: $^{18}O_2$ labelling experiment with the bifunctional oxygenase CloR.
(A) Molecular ions of 3DMA-4HMA and 3DMA-4HB resulting from 3DMA-4HPP under unenriched (left) and $^{18}O_2$-enriched (right) atmospheres. Fragmentation patterns are indicated. (B) Molecular ions of 3DMA-4HB resulting from 3DMA-4HMA under unenriched (left) and $^{18}O_2$-enriched (right) atmospheres. Fragmentation patterns are indicated. (C) Schematic summary of the incorporation of $^{18}O_2$ into 3DMA-4HB (Ring A) during the CloR reaction.

4. Inactivation of the methyltransferase gene *novO* in *S. spheroides* by PCR targeting.

Introduction

The characteristic aminocoumarin moiety of the aminocoumarin antibiotics is substituted with a methyl group at position 8 in novobiocin and coumermycin A_1 and with a chlorine atom in clorobiocin (Fig. 3, p. 31). This structural difference is perfectly reflected in the organization of the gene clusters: the novobiocin and coumermycin A_1 clusters contain a putative C-methyltransferase gene, i.e. *novO* and *couO*, respectively (Li *et al.*, 2002), whereas the clorobiocin cluster contains the gene *clo-hal*, encoding for a halogenase (Eustáquio *et al.*, 2003a; van Pée, 2001), at the corresponding position (Fig. 3, p. 31).

The aims of the present experiment were, first, to provide functional proof for the role of *novO* in novobiocin biosynthesis by gene inactivation, second, to generate a new derivative of novobiocin lacking the methyl group on the aminocoumarin ring, and third, to further establish the new inactivation method called PCR-targeting in *S. spheroides*.

Inactivation of novO by PCR targeting

Recently, a rapid method to disrupt chromosomal genes in *Escherichia coli* was adapted for use in *Streptomyces coelicolor* by Gust *et al.* (Gust *et al.*, 2002; Gust *et al.*, 2003) and for use in *S. spheroides* NCIMB 11891 by A. Eustáquio in our laboratory (Eustáquio *et al.*, 2003b). This method was developed by Datsenko and Wanner (2000). A given chromosomal locus is disrupted by replacement of the endogenous gene with a selectable marker. The marker is generated by PCR, using primers with 36-50 nt extensions which are homologous to the targeted gene. Recombination of these short homologous sequences with chromosomal DNA is promoted by the λ RED functions (*gam*, *bet*, *exo*). I used this PCR targeting system to inactivate *novO* in *S. spheroides*

Within cosmid 10-9C, which contained the biosynthetic gene cluster of novobiocin in the Supercos-1 vector (carrying a kanamycin resistance gene), *novO* was replaced by an apramycin resistance/*oriT* cassette (Fig. 27, p. 64) (see Materials and methods, p. 85). The modified cosmid (named 10-9C-novO) was introduced into *S.*

spheroides by conjugation. Apramycin-resistant, kanamycin-sensitive colonies, resulting from double crossover events, were selected. One colony was found named *novO*⁻-1. The presence of a ~ 1.7 kb PCR fragment instead of a ~ 1 kb PCR fragment confirmed that *novO* was replaced by the apramycin resistance*/oriT* cassette (Fig. 28, p. 65).

These experiments proved again that the PCR targeting system could be used successfully in *S. spheroides* allowing gene inactivation experiments to be carried out much more simply and quickly than by previous methods (Steffensky *et al.*, 2000b).
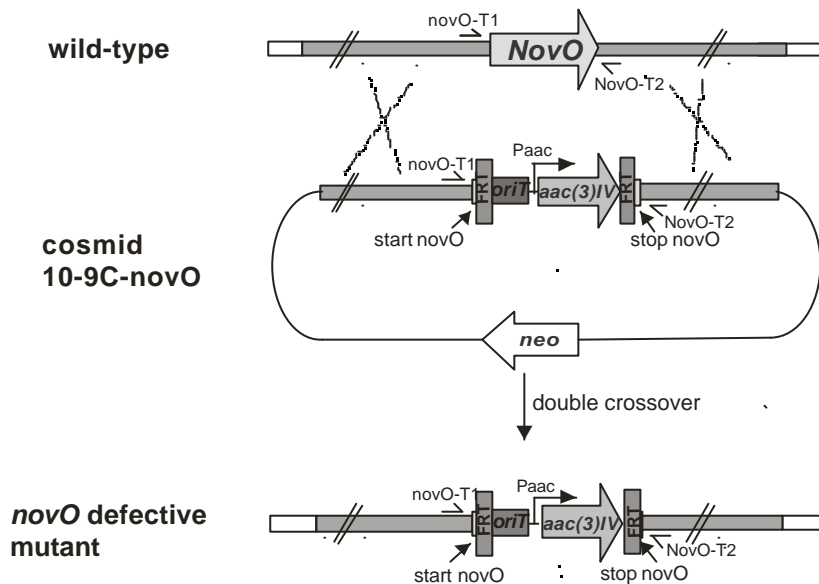
Fig. 27: Schematic representation of the inacti vation of *novO* by PCR targeting.

aac(3)IV: apramycin resistance gene, Paac: promoter of the apramycin resistance gene, FRT: FLP recognition target, *oriT*: origin of transfer from RK2, *neo*: kanamycin resistance gene.
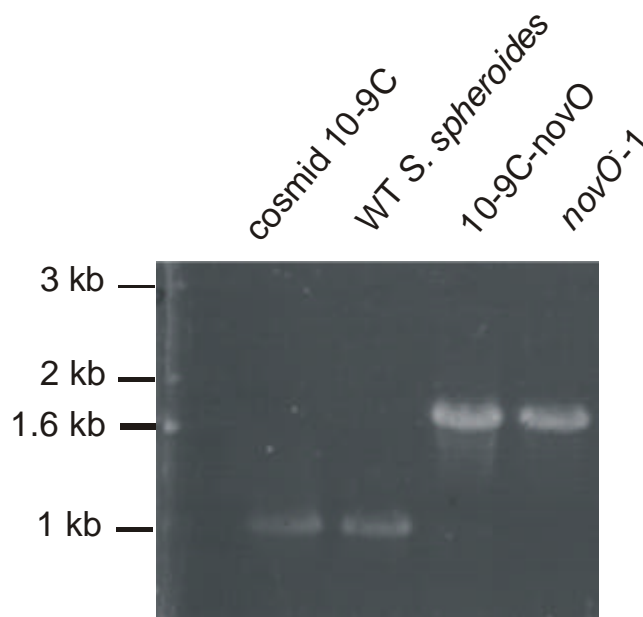
Fig. 28: PCR analysis of *novO* defective mutant.

Characterization of the secondary metabolites of novO defective mutant

novO defective mutant (*novO⁻* -1) as well as the wild-type were cultured in novobiocin production medium (see Materials and methods, p. 79). The secondary metabolites were analyzed by HPLC in comparison with novobiocin standard (Fig. 29, p. 66) (see Materials and methods, p. 87).

The production of novobiocin was abolished in *novO* defective mutant. This mutant produced, instead, a new substance with a shorter retention time than novobiocin. LC-ESI-CID analysis showed a fragmentation pattern with molecular ion [M-H]⁻ at *m/z* 597/ 554/ 409/ 366/ 192, consistent with the loss of a methyl group on the aminocoumarin ring in comparison to novobiocin ([M-H]⁻ at *m/z* 611/ 568/ 423/ 380/ 206). In the ^{1}H-NMR spectrum of the new compound, the signal at 2.31 ppm corresponding to H-11 of the methyl group of novobiocin had 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 (Eustáquio *et al.*, 2003a). The coincidence of the signals of H-6 and H-8 as a broad singlet is in accordance with the spectrum reported from a naturally occurring novobiocin derivative lacking the 8-methyl group (Sasaki *et al.*, 2001). The new compound was named novclobiocin B106.
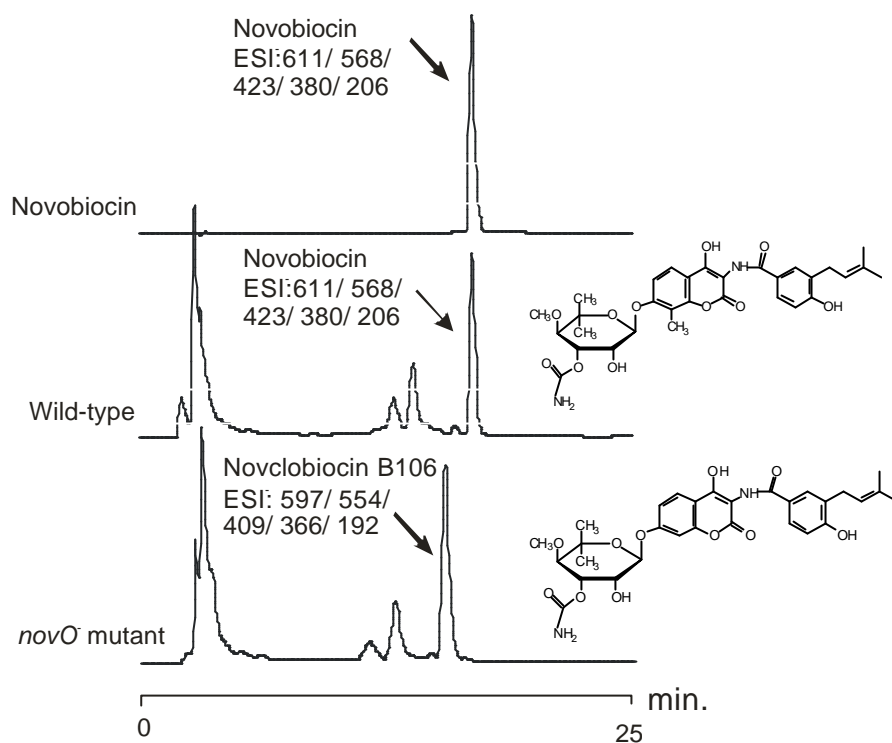
Fig. 29: HPLC analysis of culture extracts of the *novO* defective mutant.

The identity of novobiocin ([M-H]⁻ = 611) and novclobiocin B106 were confirmed by LC-ESI-CID and the mass spectroscopic fragments are indicated.

Conclusion

This experiment provides experimental evidence for the function of *novO* of the novobiocin gene cluster. Moreover, a new novobiocin analogue lacking the methyl group at C-8 of the aminocoumarin ring was produced by genetic manipulation.

The PCR targeting system was confirmed to be a fast and effective method for gene inactivation in *S. spheroides* and improves our ability for genetic engineering of *Streptomyces*.

It is not clear at present at which step of aminocoumarin biosynthesis the methylation takes place. Chen and Walsh (2001) provided some evidence that the methylation occurs after activation of tyrosine (Fig. 30, p. 69), and an inactivation experiment by Steffensky et al. (2000b) indicated that methylation takes place before glycosylation of novobiocic acid. In Fig. 30 (p. 69), we suggest that the aminocoumarin ring is the substrate of the methylation.

III. DISCUSSION

1. Comparison of the three classical aminocoumarin biosynthetic gene clusters.

The first objective of my thesis was to clone and sequence the clorobiocin biosynthetic gene cluster. A comparison of the three "classical" aminocoumarin biosynthetic gene clusters was expected to allow a better understanding of the role of the different genes in the biosynthesis of these antibiotics, which is one of the prerequisite for creating new hybrid antibiotics by combinatorial biosynthesis.

The clorobiocin cluster spans approximately 35.5 kb and comprises 29 ORFs. The suggested functions of these ORFs in clorobiocin biosynthesis are depicted in Fig. 30, p. 69.

Upstream of the *cloE* gene, primary metabolic genes were found (Table 1, p. 32) suggesting that *cloE* represents the 5´ border of the cluster. The gene adjacent to *cloE*, i.e. ORF9, encodes a putative transposase, and it may be speculated that this gene is related to the introduction of the cluster into the *S. roseochromogenes* genome. Also at the 3´ end, downstream of the *gyr*B^R and *parY*^{R} resistance genes, primary metabolic genes were found, suggesting that *parY*^{R} is the right border (Fig. 3, p. 31). It cannot be excluded, however, that additional biosynthetic enzymes for clorobiocin formation are encoded at different loci of the genome.

The putative role of the genes found in the clorobiocin cluster were already discussed in the results part (p. 29). In this discussion part, I would like to discuss in detail the genes involved in the important common ring of the aminocoumarin antibiotics, Ring B.

The clorobiocin cluster contains the genes *cloHIJK*, for which homologues exist in the novobiocin cluster (*novHIJK*) and the coumermycin cluster (*couHIJK*) and which therefore could be involved in Ring B biosynthesis. NovH has recently been shown to activate tyrosine by covalent binding to the 4-phosphopantetheinyl cofactor, and the P450 enzyme NovI catalyzes the ß-hydroxylation of the activated tyrosine (Chen and Walsh, 2001). A central, unresolved question in aminocoumarin biosynthesis is how activated ß-hydroxytyrosine is then converted to the coumarin ring, especially how the ring oxygen is introduced. *cloJ* and *cloK*, the genes immediately downstream of *cloH* and *cloI*, are homologous to *novJK* and *couJK* of the novobiocin and the

coumermycin clusters. The detection of *cloJ* and *cloK* in the clorobiocin cluster and the homologous *simJ1* and *simK* in the biosynthetic gene cluster of the aminocoumarin antibiotic simocyclinone (Galm *et al.*, 2002; Trefzer *et al.*, 2002) now strongly supports the hypothesis that these genes are indeed involved in the aminocoumarin biosynthesis. *cloJ* shows homology to 3-oxo-acyl-[ACP] reductases and may likely be involved in the oxidation of a ß-hydroxytyrosyl to a ß-ketotyrosyl intermediate (Fig. 30, p. 69). Also, *cloK* shows homology to oxidoreductases, but this homology is not very high (average 35% on the amino acid level). Chen and Walsh (2001) speculated that NovJ and NovK might act together to oxidize the ß-hydroxyl function to a keto group. The unresolved step in the postulated Ring B biosynthesis, however, is the hydroxylation of the activated tyrosyl derivative in position 2 of the aromatic nucleus (Fig. 30, p. 69). Bunton *et al.* (1963) had reported that the ring oxygen of the aminocoumarin might be derived from the carboxyl group of tyrosine rather than from molecular oxygen. This was recently disproven by Holzenkämpfer and Zeeck (2002). It was shown that the ring oxygen of the aminocoumarin moiety of simocyclinone is in fact derived from molecular oxygen. Therefore, coumarin ring formation most likely proceeds *via* the 2-hydroxylation of a tyrosine derivative. It has been speculated that the predicted flavine dioxygenase NovC, encoded by a gene near the novobiocin cluster, may catalyze this reaction (Chen and Walsh, 2001). An important finding of our study is that no *novC* homologue was detected in or near the clorobiocin gene cluster. Likewise, no *novC* homologue was detected in the simocyclinone cluster (Galm *et al.*, 2002; Trefzer *et al.*, 2002). We therefore suggest that *novC* is not related to aminocoumarin biosynthesis. The enzyme responsible for the 2-hydroxylation of the ß-ketotyrosyl intermediate remains unknown at present. Whether *cloK* is involved in this or in another reaction of aminocoumarin biosynthesis has yet to be demonstrated.

Halogenation of the aminocoumarin ring by Clo-hal may occur after ring formation, as depicted in Fig. 30 (p. 69) or at an earlier stage.

The prenylated 4-hydroxybenzoate moiety (Ring A) of clorobiocin and novobiocin is formed from tyrosine (Kominek and Sebek, 1974) and an isoprenoid precursor. The reaction sequence and the genes involved are unknown. The second objective of my thesis was to elucidate the formation of Ring A by *in vivo* and *in vitro* experiments.
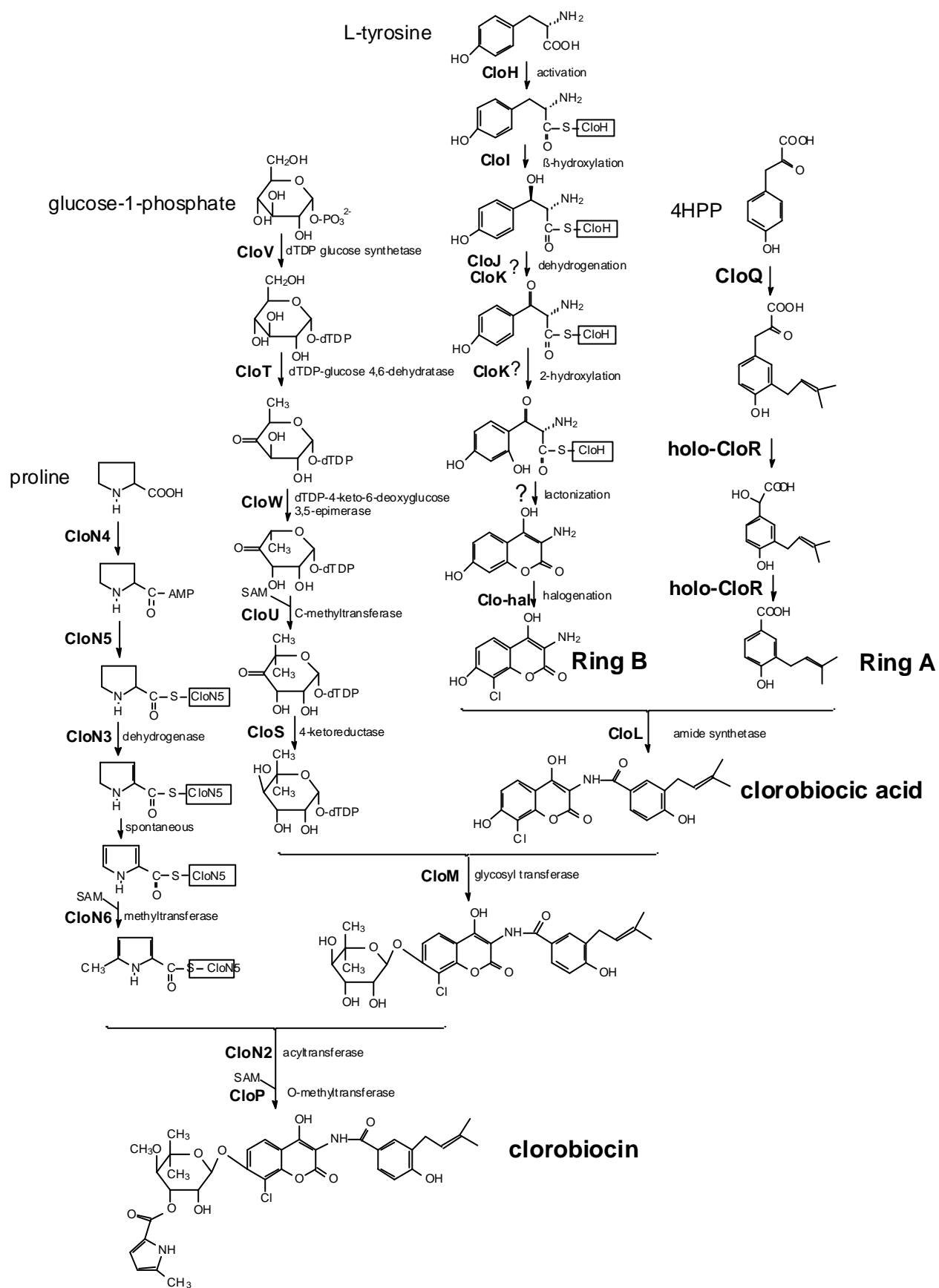
Fig. 30: A model for the biosynthesis of clorobiocin.

The role of CloQ and CloR has been elucidated in the present thesis.

The cloning and sequencing of the clorobiocin gene cluster has completed the genetic information on the biosynthesis of three "classical" aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1. Comparison of the three gene clusters revealed a striking correspondence between the structures of the antibiotics and the organization of the biosynthetic genes (Fig. 3, p. 31), unprecedented so far in any class of natural products outside the polyketide and the peptide antibiotics. For each structural moiety of the aminocoumarin antibiotics, the biosynthetic genes are grouped together, resulting in a "modular" structure of the clusters. The order of the modules, and the order of the genes within each module are perfectly identical for the three "classical" aminocoumarins, and nearly all the genes within the clusters are oriented in the same direction. The comparison of the three clusters greatly facilitates the prediction of functions for the different genes. The similarity between the three clusters also provides excellent opportunities for the production of hybrid aminocoumarins by genetic methods.

2. Complete identification of Ring A biosynthesis of clorobiocin.

The second objective of my thesis was to elucidate the formation of Ring A of clorobiocin and novobiocin.

The biosynthesis of Ring A requires: a) the assembly of the isoprenoid precursor (probably dimethylallyl diphosphate) *via* the methylerythritol phosphate pathway (Li *et al.*, 1998); b) the conversion of the phenylpropanoid compound tyrosine to a benzoic acid derivative; c) the prenylation of the aromatic nucleus in a prenyltransferase reaction. The conversion of the phenylpropanoid intermediate to a benzoic acid derivative may proceed by a mechanism analogous to the oxidation of fatty acids, as demonstated in *S. maritimus* (Hertweck and Moore, 2000). Alternatively, this conversion may occur by retro-aldol cleavage of a 3-hydroxylated phenylpropanoid compound as found in *Pseudomonas fluorescens* (Gasson *et al.*, 1998) and *Amycolatopsis sp.* (Achterholt *et al.*, 2000). Retro-aldol cleavage would result in a benzaldehyde derivate, which would subsequently be oxidized to the benzoic acid derivative.

An additional model for the formation of Ring A was derived from studies on the biosynthesis of the aminocoumarin moiety (Ring B) of novobiocin. It was proposed

that ß-hydroxytyrosyl-NovH might be a common intermediate in the biosynthesis of Ring B and Ring A (Fig. 7, p. 40) (Chen and Walsh, 2001). However, creation of a *S. roseochromogenes* mutant blocked in the biosynthesis of Ring B (*cloI* mutant) and accumulation of Ring A by this mutant demonstrated that Ring A and Ring B are formed by two distinct and independent pathways.

Comparison of the clorobiocin biosynthetic gene cluster with the one of novobiocin and coumermycin revealed three genes which could be involved in the formation of Ring A. CloQ was identified as a soluble aromatic prenyltransferase, which prenylates 4-hydroxyphenylpyruvate in clorobiocin biosynthesis. CloQ was found to be dissimilar from most prenyltransferases described so far and may indicate the existence of a new class of prenyltransferases. CloR was identified as a bifunctional non-heme iron oxygenase, which catalyzes the conversion of 3-dimethylallyl-4-hydroxyphenylpyruvate (3DMA-4HPP) to Ring A in two oxidative decarboxylation steps, *via* 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA) as intermediate. The formation of Ring A of clorobiocin or novobiocin represents a new pathway to benzoic acid.

2.1 First step: prenylation of 4-hydroxyphenylpyruvate by CloQ.

cloQ was identified as the structural gene for the prenyltransferase involved in the biosynthesis of the 3-dimethylallyl-4-hydroxybenzoate moiety of clorobiocin (Ring A) (Fig. 30, p. 69). 4-Hydroxyphenylpyruvate (4HPP) and dimethylallyl diphosphate (DMAPP) were identified as the substrates of CloQ.

Inactivation of *cloQ* led to abolishment of clorobiocin formation, which could be restored by addition of Ring A. This showed that the reaction catalyzed by CloQ is an essential step of the biosynthesis of Ring A of clorobiocin.

Novobiocin, just as clorobiocin, contains a 3-dimethylallyl-4-hydroxybenzoate moiety, and the novobiocin biosynthetic gene cluster contains the gene *novQ*. The predicted gene product NovQ (323 amino acids) shows 84 % identity on the amino acid level to CloQ (324 amino acids) (Pojer *et al.*, 2002; Steffensky *et al.*, 2000b). Therefore, NovQ is likely to catalyze the prenyltransferase reaction in novobiocin biosynthesis.

Surprisingly, database searches did not reveal any similarities between NovQ or CloQ and known prenyltransferases. The only match found for CloQ in a BLAST

search was a hypothetical protein of *S. coelicolor* (e= 4×10^{-08}). At present, very few sequences are available for "aromatic" prenyltransferases, i. e. enzymes catalyzing the formation of a carbon-carbon bond between a prenyl group and an aromatic nucleus. Among the few aromatic prenyltransferases, which have been cloned are those involved in the biosynthesis of ubiquinones (Meganathan, 2001), menaquinones (Suvarna *et al.*, 1998), tocopherols (Schledz *et al.*, 2001), plastoquinones (Collakova and DellaPenna, 2001), and the prenyltransferase involved in formation of the plant secondary metabolite shikonin (Yazaki *et al.*, 2002). All these enzymes are integral membrane proteins, and their active centers include the prenyl diphosphate binding site (N/D)DxxD similar to that of the *trans*-prenyltransferases (Koyama *et al.*, 1996; Liang *et al.*, 2002). In contrast, CloQ and NovQ do not show this motif and represent soluble enzymes, without membrane-spanning domains.

The only other soluble aromatic prenyltransferase cloned so far is dimethylallyltryptophan synthase (DMAT), which is involved in ergot alkaloid biosynthesis in the fungus *Claviceps* (Cress *et al.*, 1981; Gebler and Poulter, 1992; Lee *et al.*, 1976; Tsai *et al.*, 1995; Tudzynski *et al.*, 1999). DMAT was found to be active in a metal-free buffer containing EDTA, in contrast to all previously known prenyltransferases (Cress *et al.*, 1981; Gebler and Poulter, 1992). It has been suggested that the reaction catalyzed by DMAT may not proceed *via* a carbonium ion (Cress *et al.*, 1981), unlike the reaction of farnesyl diphosphate synthase (Poulter and Rilling, 1976). In common with DMAT, CloQ was also active in the presence of EDTA, i. e. the reaction did not show an absolute requirement for divalent cations. Moreover, the K_m values, the specific activity and the turnover number determined for CloQ are similar to those reported for DMAT (Cress *et al.*, 1981; Gebler and Poulter, 1992; Lee *et al.*, 1976). However, the bacterial enzyme CloQ (35 kDa) shows only low sequence similarity (19% identity, 33% similarity) to the 50 KDa protein *cpd1*, suggested as the structural gene for the fungal enzyme DMAT (Fig. 31, p. 73) (Tudzynski *et al.*, 1999). Nevertheless, CloQ, NovQ and DMAT may belong to a novel class of prenyltransferases that exist as soluble enzymes, do not contain the prenyl diphosphate binding motif (N/D)DxxD, and are able to form carbon-carbon bonds between isoprenoid and aromatic substrates.

```
              10         20         30         40         50
CloQ    1 MPAL--PIDQ EFDCERFRAD IRATAAAI-- --GAPIAHRL TDTVLEAFRD
Cpd1    1 MSTAKDPGNG VYEILSLIFD FPSNEQRLWW HSTAPMFAAM LDNAGYNIHD
          *       *          *          **    *      *
              60         70         80         90        100
CloQ   51 NFAQGATLWK TT-------S QPGDQLSYRF FSR------L KMDTVSRAID
Cpd1   51 QYRHLGIFKK HIIPFLGVYP TKDKERWLSI LTRCGLPLEL SLNCTDSVVR
                   *               *          *
             110        120        130        140        150
CloQ  101 AGLLDAAHPT LAVVDAWSSL YGGAPVQS-G DFDAGRGMAK TWL-YFGG--
Cpd1  101 YTYEPINEVT GTEKDPFNTL AIMASVQKLA QIQAGIDLE- -WFSYFKDEL
                   *        *     * **      **      *  **
             160        170        180        190        200
CloQ  151 -LRPAEDI-L TVPALPASVQ ARLKDFLALG LAHVRFAAVD WRHHSANVYF
Cpd1  151 TLDESESATL QSNELVKEQ- IKTQNKLALD LKESQFALKV YFYPHLKSIA
             *     *    *           ***   *    **
             210        220        230        240        250
CloQ  201 RGKGPLDTVQ FARIHALSGS TPPAAHVVEE VLAYMP---- --------ED
Cpd1  201 TGKSTHDLI- FDSVFKLSQK HDSIQPAXQV LCDYVSRRN- ---HSAESDQ
            **    *    *     **          *
             260        270        280        290        300
CloQ  251 YCVAITL--- ----DLHSGD IERVCFYALK ---------- ----------
Cpd1  251 HI---ALHAR LLSCDLIDPA KSRVXIYLLE KTVSLSVMED LWTLGGQRVD
            *          **       ** * *
             310        320        330        340        350
CloQ  301 ---------- ---------- ---------- -----VPKNA LPRIP-----
Cpd1  301 ASTMDGLDML RELWSLLKVP TGHLEYPKGY LELGEIPNEQ LPSMANYTLH
                                               *       **
             360        370        380        390        400
CloQ  351 -----TRIAR FLEVAPSHDV EECNVIGWSF GRSGDYVKAE R-------SY
Cpd1  351 HNNPMPEPQV YFTVFGMNDA EISNALTIFF QRHGFDDMAK KYRVFLQDSY
                    *    * * *    * *     *  * *    *       **
             410        420        430        440        450
CloQ  401 TGNMAEILAG WNCF----FH GEEGR----D HDLRALH--- -QHTESTMGG
Cpd1  401 PYHDFESLNY LHAYISFSYW PVGESTELYQ HGLRKLNNFL VADSPISFDA
               * *                      * ** *
CloQ  451 AR
Cpd1  451 YR
           *
```

Fig. 31: Multiple alignment of the amino acid sequences of CloQ with Cpd1.

Cpd1 is a dimethylallyl-tryptophan-synthase of *Claviceps purpurea* (accession number: CAC37397). Identical amino acids are marked by asterisk and bold print.

2.2 Second and last step: formation of Ring A from prenylated 4-hydroxyphenylpyruvate by CloR.

CloR was identified as a bifunctional oxygenase which converts 3-dimethylallyl-4-hydroxyphenylpyruvate (3DMA-4HPP) in two consecutive reaction steps to 3-dimethylallyl-4-hydroxybenzoate (3DMA-4HB), i.e. to the Ring A moiety of clorobiocin. An $^{18}O_2$ labelling experiment unequivocally confirmed that molecular oxygen is used as substrate by CloR. The purified CloR protein did not contain a heme prosthetic group, and its activation by Fe^{2+} and ascorbate indicated that it belongs to the non-heme iron oxygenase.

The first reaction catalyzed by CloR, the conversion of 3DMA-4HPP to 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA), has a well-established precedent in the HmaS reaction in chloroeremomycin biosynthesis. HmaS belongs to the iron(II) and α-ketoacid dependent dioxygenases (reviewed in Prescott and Lloyd, 2000; Que, 2000; Que and Ho, 1996). These enzymes utilize O_2 and an α-ketoacid as cosubstrates. During the reaction, the α-ketoacid looses CO_2 and the keto function is oxidized to a carboxyl group by introduction of one of the oxygen atoms of O_2. The other oxygen may be used for a hydroxylation reaction, exemplified by the prolyl 3-hydroxylase reaction. However, iron(II) and α-ketoacid dependent oxygenases have been shown to catalyze not only hydroxylations but a wide range of diverse oxidative transformations, including epoxidations, desaturations, ring formation and ring expansion reactions. Some of these enzymes are bifunctional (e.g. deacetoxy-/deacetylcephalosporin synthase) or even trifunctional (e. g. clavaminic acid synthase or thymine hydroxylase), catalyzing several consecutive oxidative transformations within a single biosynthetic pathway.

In the conversion of 4-hydroxyphenylpyruvate (4HPP) to homogentisate by 4HPP dioxygenase, 4HPP serves both as α-ketoacid and as hydroxylation substrate. CloR (in its first reaction step) and HmaS carry out a very similar reaction as 4HPP dioxygenase, but hydroxylate the benzylic position of the substrate instead of the phenyl ring. However, while HmaS shows obvious sequence similarity to 4HPP dioxygenase, CloR does not.

The second reaction step catalyzed by CloR is the conversion of 3DMA-4HMA to 3DMA-4HB. It is tempting to speculate that this reaction may involve a hydroxylation of the α-position of 3DMA-4HMA, resulting in a α,α-*gem*-diol which eliminates water to give the corresponding α-ketoacid, i.e. 4-hydroxybenzoylformate. Oxydative decarboxylation of this compound would result in the final product 3DMA-4HB. Thereby the overall reaction mechanism would be similar to that of the first reaction and resemble that of other iron(II) and α-ketoacid dependent dioxygenases.

However, LC-ESI-CID analysis did not confirm the presence of 4-hydroxybenzoylformate in the incubation mixture, and the exact mechanism of the CloR reaction remains speculative at present. It is believed that the reactions catalyzed by iron(II) and α-ketoacid dependent oxygenases involve a reactive $Fe^{IV}=O$

species (Prescott and Lloyd, 2000; Que and Ho, 1996; Rohde *et al.*, 2003). Whether this turns out to be true for CloR remains to be shown.

The different groups of iron(II) and α-ketoacid dependent oxygenases possess little overall sequence similarity to each other (Prescott and Lloyd, 2000; Que, Jr., 2000), and it is therefore not surprising that CloR does not show sequence similarity to known members of this family. However, a database search reveals that CloR does show significant similarity to several proteins of unknown function, deduced from genome sequences of different microorganisms. These may represent a family of enzymes with similar function as CloR.

The common structural motif of iron(II) and α-ketoacid dependent enzymes is the so-called 2-His-1-carboxylate facial triad (Hegg and Que, 1997; Que, 2000). It consists of two histidyl groups and one glutaryl or asparagyl residue, which together anchor the iron atom in the active site of the enzyme. Comparison of the primary sequence of CloR with that of NovR as well as with the sequences of six of the database entries of unknown function with high sequence similarity to CloR identifies His161, His176, His178, His241 and Asp170 of CloR as strictly conserved residues. These amino acids may be candidates for a potential 2-His-1-carboxylate facial triad of this oxygenase.

The involvement of CloR in the biosynthesis of the prenylated 4-hydroxybenzoate moiety of clorobiocin was proven *in vivo* by a gene inactivation experiment. *In vitro* experiments allow to formulate a detailed hypothesis for the formation of this moiety of clorobiocin, as shown in Fig. 32 (p. 76). CloQ is a prenyltransferase which converts 4HPP to 3DMA-4HPP. CloR converts the CloQ reaction product to Ring A. CloF shows sequences similarity to prephenate dehydrogenases and is therefore likely to produce 4HPP as the substrate for CloQ, similar to ORF1 of the chloroeremomycin biosynthetic gene cluster (van Wageningen *et al.*, 1998). Notably, the coumermycin cluster does not contain a CloF homologue (Fig. 3, p. 31).

Radioactive feeding experiments with the novobiocin producer (Calvert *et al.*, 1972) showed that [U-^{14}C]L-tyrosine was incorporated preferentially into Ring B whereas [U-^{14}C]4HPP was incorporated preferentially in Ring A. This may suggest that a primary metabolic prephenate dehydrogenase provides tyrosine for the formation of Ring B, whereas CloF supplies 4HPP for Ring A biosynthesis, and that crosstalk exists between both pathways probably *via* a transaminase reaction (Fig. 32, p. 76).

Discussion

Chen and Walsh (2001) have previously speculated that Ring A and Ring B of novobiocin may both be produced via ß-hydroxytyrosyl-*S*-NovH as a common precursor (Fig. 7, p. 40). These studies on CloQ and CloR, however, establish that these two aromatic moieties of clorobiocin (and very likely those of novobiocin as well) are produced by two independent pathways (Fig. 32). The discovery of CloR adds a new interesting member to the diverse family of the non-heme iron oxygenases, and demonstrates the existence of a new pathway to benzoic acids.
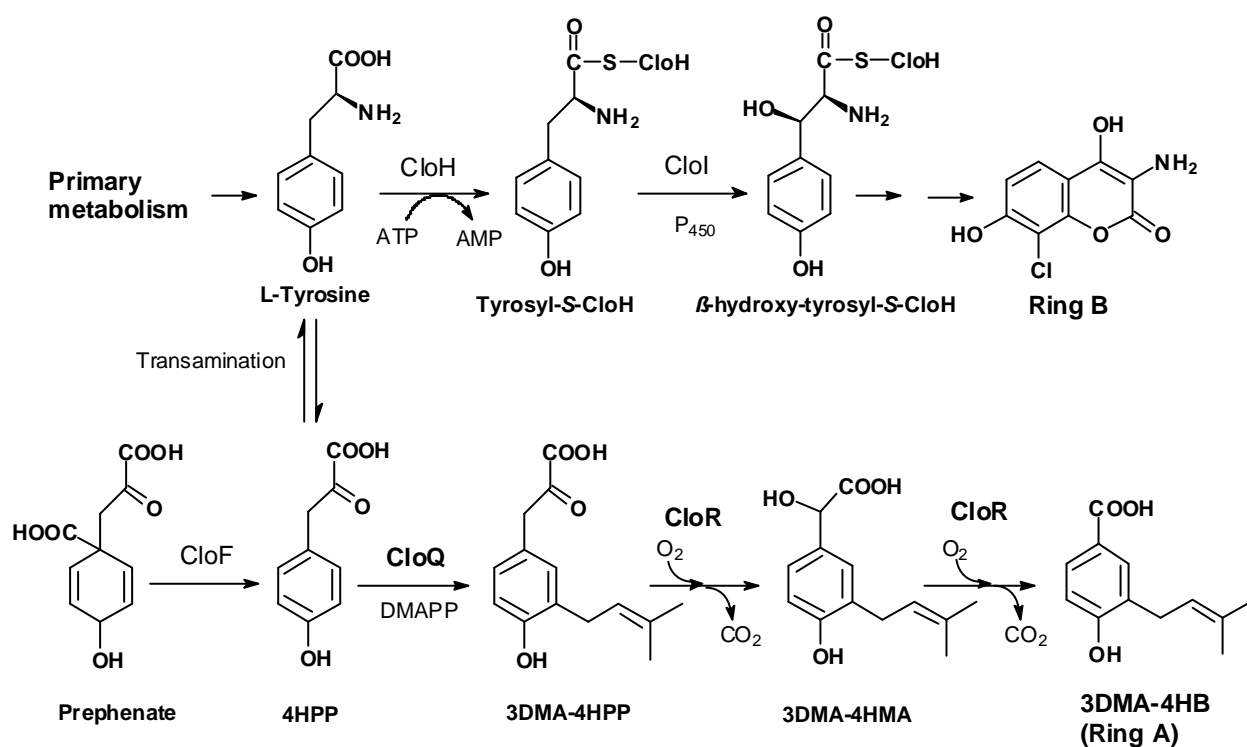
Fig. 32: Biosynthetic pathways for the aminocoumarin moiety (Ring B) and the prenylated 4-hydroxybenzoate moiety (Ring A) of clorobiocin.

IV. MATERIALS AND METHODS

1. Chemicals and Radiochemicals.

$[1-^{14}C]$Dimethylallyl diphosphate (DMAPP) and L-$[U-^{14}C]$tyrosine were obtained from Moravek Biochemicals (Brea, CA, USA). 4-Hydroxyphenylpyruvate (4HPP), D- and L-mandelic acid, DL p-hydroxymandelic acid, p-hydroxyphenyllactic acid was purchased from Sigma. ß-Hydroxy-L-tyrosine and unlabeled DMAPP were kindly provided by Dr. K.-H. van Pée (Dresden, Germany) and Dr. K. Yazaki (Kyoto, Japan), respectively. 3-Dimethylallyl-4-hydroxybenzaldehyde (3DMA-4HBAL) was synthesized as described by Gluesenkamp and Buechi (1986). Ring A of clorobiocin and novobiocin (3-dimethylallyl-4-hydroxybenzoic acid) was prepared as described by Kominek and Meyer (1975). Ring B of novobiocin (3-amino-4,7-dihydroxy-8-methyl coumarin) was kindly provided by Pharmacia & Upjohn Inc.

Oxygen-$^{18}O_2$ (99%) in a pyrex breaseal flask loaded to atmospheric pressure was purchased by Campro scientific (Berlin, Germany).

2. Plasmids and bacterial strains.

Plasmids and bacterial strains are listed in Appendix A and B, respectively, p. 95 to 98.

3. Culture conditions.

3.1 Culture of E. coli.

For cloning experiments, *Escherichia coli* (*E. coli*) strains were grown in liquid or solid Luria-Bertani medium at 37°C (Sambrook and Russell, 2001).

3.2 Culture of S. roseochromogenes.

S. roseochromogenes var. oscitans DS 12.976 was routinely cultivated at 28°C for 2 days in HA medium containing 1.0% malt extract, 0.4% yeast extract, 0.4% glucose, and 1.0 mM $CaCl_2$ (pH 7.3).

For production of clorobiocin and other secondary metabolites, wild-type and mutant strains of *S. roseochromogenes* were pre-cultured in 50 ml corn-starch medium (1% corn starch, 1% peptone, 0.5% meat extract, pH 7.0) for 2 days at 33°C and 210 rpm. 5 ml of this pre-culture were inoculated into 50 ml of production medium adapted from Mancy *et al.* (1974), prepared from 4.8% distillers solubles, 3.7% glucose, 0.0024% cobalt chloride (at this point, the pH of the mixture was adjusted to 7.8), 0.6% calcium carbonate and 0.2% ammonium sulphate. Cultivation was carried out in 500 ml baffled flasks for 4 to 6 days at 33°C and 210 rpm (Fig. 33). Clorobiocin and secondary metabolites were extracted and analyzed as described in Materials and methods, p. 86.
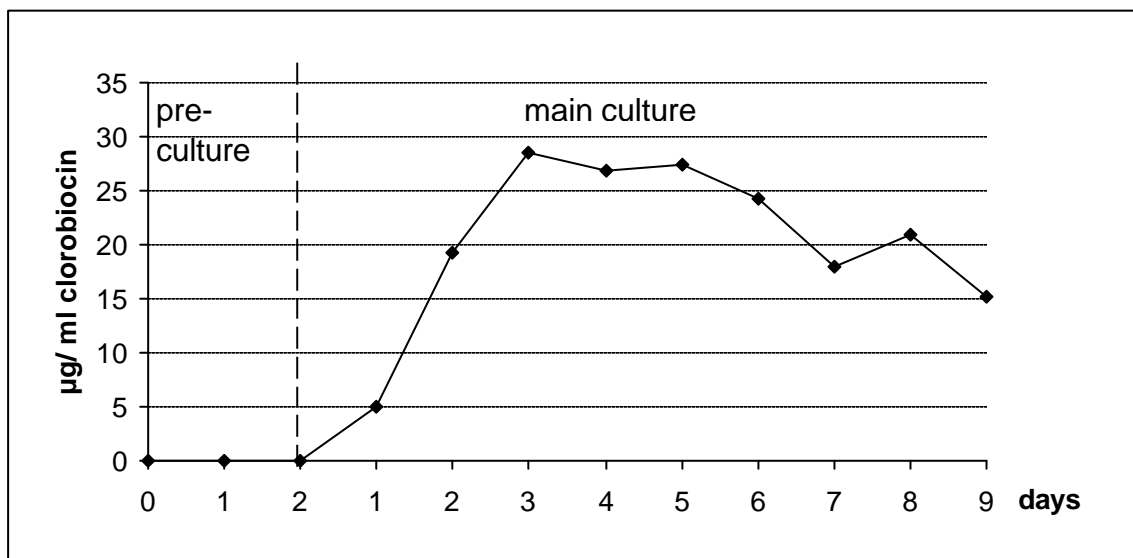
Fig. 33: Kinetic of clorobiocin production of *S. roseochromogenes*.

Preculture was carried out in corn starch medium for 2 days; main culture in distillers solubles medium for 9 days. Culture extracts were extracted and analyzed as described in p. 87.

Clorobiocin is also produced by *S. hygroscopicus* DS 9.751 and *S. albocinerescens* DS 21.647 (Mancy *et al.*, 1974). The concentration of clorobiocin obtained was too low to continue to work with these two strains.

3.3 Culture of *S. spheroides*

S. spheroides NCIMB 11891 was routinely cultivated at 28°C for 2 days in HA medium containing 1.0% malt extract, 0.4% yeast extract, 0.4% glucose, and 1.0 mM $CaCl_2$ (pH 7.3).

For production of novobiocin and other secondary metabolites, wild-type and mutant strains of *S. spheroides* were pre-cultured in 50 ml CDM medium (Kominek, 1972) at 28°C and 180 rpm for 4 days. One ml of this pre-culture was inoculated into 50 ml of CDM medium and grown for 7 days (Steffensky *et al.*, 2000).

4. Endogenous antibiotic resistances of *S. roseochromogenes*.

Endogenous resistance of *S. roseochromogenes* to different antibiotics was tested. Apramycin and thiostrepton were used. 80 µl of glycerol culture of *S. roseochromogenes* were grown in 50 ml YMG medium for 2 days. 100 µl were plated for the tests (Table 2).

Apramycin → *S. roseochromogenes* is sensitive to apramycin.

Conc. (µg/ml)	50	70	***100***	150
Nbr. of colonies	10	0	0	0

Thiostrepton→ *S. roseochromogenes* is sensitive to thiostrepton.

Conc. (µg/ml)	20	30	*50**	100
Nbr. of colonies	0	0	0	0

* concentration normally used for selection.

Table 2: Endogenous antibiotic resistances of *S. roseochromogenes*.

For inactivation and transformation of *S. roseochromogenes*, the selection could be made with thiostrepton or Apramycin.

5. Genetic procedures.

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

Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kit; Macherey-Nagel, Düren; Germany). DNA fragments were isolated from agarose gels using a NucleoSpin 2 in 1 extraction kit (Macherey-Nagel, Düren; Germany).

Genomic DNA was isolated from *S. roseochromogenes* strains using the Kirby mix procedure (Kieser *et al.*, 2000). Genomic DNA was isolated from the others *Streptomyces* strains by lysozyme treatment and phenol-chloroform extraction (Kieser *et al.*, 2000).

Southern blot analysis was performed on Hybond-N membranes (Amersham) with digoxigenin-labeled probes by using the DIG high prime DNA labeling and detection kit II (Roche Applied Science).

6. Construction and screening of the cosmid library in *S. roseochomogenes.*

The *S. roseochromogenes* cosmid library was constructed by *Sau*3A digestion of chromosomal *S. roseochromogenes* DNA and ligation into the *Bam*HI sites of Supercos-1.

Packaging:

The packaging was performed using Gigapack® III XL Packaging Extract (Stratagene). This kit packages preferentially large inserts (i.e., 47- to 51-Kb recombinants). *E. coli* XL1-Blue MRF' was used as host strain. Some modifications were made to the protocol. Different OD_{600} of the cells were tried (OD=0,5; OD=1 and OD=2) with different concentrations of cosmid packaging reaction (concentration:2 X; 1X and 1/10). After transfection, the cells were plated on LB with carbenicillin (50 µg/ml) plates and incubated overnight at 37°C.

Screening of the cosmid Library:

After packaging recombinant cosmids and transfection into *E. coli*.. 850 carbenicillin resistant clones were obtained. Clones were pooled in order to facilitate the screening procedure. In the first round, pools of 48 clones were screened, followed by a screening of subpools of 8 clones and eventually the testing of single colonies.

Southern blot analysis was performed on Hybond-N membranes (Amersham, Braunschweig; Germany) with digoxigenin-labeled probes using the DIG high prime DNA labeling and detection kit II (Roche Molecular Biochemicals, Mannheim; Germany). Two probes, one containing a part of the dTDP-glucose 4,6-dehydratase gene *novT* and the other containing a part of *gyrB^R* of novobiocin biosynthetic gene cluster were used for hybridization (Fig. 34, p. 82) (see Appendix A, p. 95).

After this first screening, three cosmids were obtained. Cosmids VIA4-e and VIIIA7-c hybridized as well with *novT* probe as with *gyrB^R* probe. Cosmid VA6-d hybridized only with *gyrB^R* probe. Cosmid VIIIA7-c has been subcloned by digestion with *Bam*HI and ligation of the fragments into pBluescript SK(-). The obtained plasmids pofl1A, pofl1B, pofl2, pofl3, polfl4, pofl5, pofl6, pofl6A, pofl6B, pofl6C, pofl7 and pofl8 are listed in Appendix A, p. 95 and depicted in Fig. 34, p. 82. Upon subcloning and partial sequencing, it was found that cosmid VIIIA7-c does not span the complete clorobiocin biosynthetic gene cluster (Fig. 34, p. 82).

Therefore, a second screening was made in order to find another cosmid that spans the complete cluster. 1300 clones were screened with an heterologous probe containing a part of the amide synthetase *novL* gene of novobiocin biosynthetic gene cluster (*novL* probe) and a homologous probe containing a part of the glycosyl transferase of clorobiocin biosynthetic gene cluster (*cloM* probe) (see Appendix A, p. 96). Three independent cosmids (K1F2, D1A8 and F1A4) were found to hybridize with *novL*, *cloM* and *gyrB^R* probes (Fig. 34, p. 82). After restriction analysis, cosmid K1F2 was chosen to be entirely sequenced (Fig. 34, p. 82).
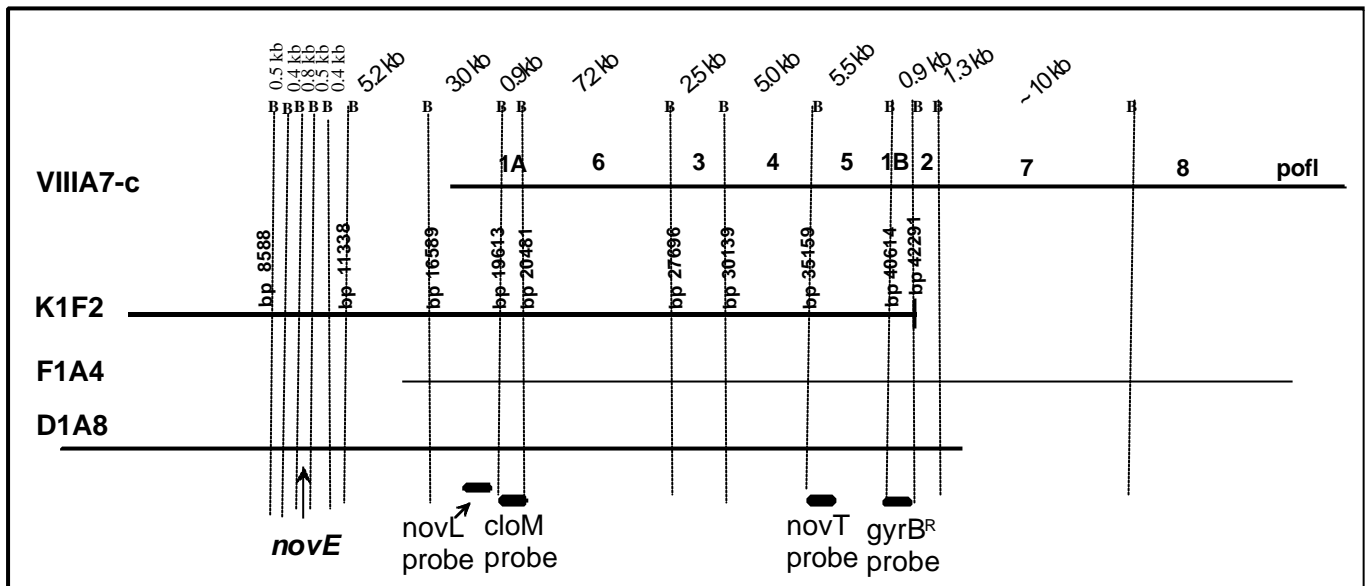
Fig. 34: Approximate alignment of cosmids VIIIA7-c, K1F2, F1A4 and D1A8.

Cosmids were digested with *Bam*HI (B). Cosmid K1F2 was completely sequenced and the *Bam*HI (B) restriction sites are indicated. Cosmid VIIIA7-c was subcloned and the plasmids obtained are depicted. Both ends of cosmid D1A8 were sequenced by A. Eustáquio from our group and showed that this cosmid ends at the beginning of the *parY^R* gene, which is situated immediately downstream of the *gyrB^R* gene. Cosmids VIA4-e and VA6-d were not mapped. Note that this is an approximate map based on restriction analysis. No end sequencing was done for VA6-d, VIA4-e, VIIIA7-c and F1A4.

7. DNA sequencing and computer-assisted sequence analysis.

Double-stranded sequencing of the entire cosmid K1F2 (carrying an insert of 42, 291 bp) was performed by the dideoxynucleotide chain termination method on a LI-COR automatic sequencer (MWG-Biotech AG, Ebersberg; Germany) using a shotgun library with DNA fragments of approximately 1.5 to 2.0-kb in length.

The DNASIS software package (version 2.1; Hitachi Software EngineeRing, San Bruno; Calif.) was used for sequence analysis. Amino acid sequence homology searches were carried out in the GenBank database by using the BLAST program (release 2.0).

8. Construction of deletion mutants by In-frame deletion in *S. roseochromogenes*.

8.1 Inactivation of cloR.

For inactivation of *cloR* in *S. roseochromogenes*, the fragment cloR-1 (1282 bp) and the fragment cloR-2 (1301 bp) were amplified by PCR. The primer pairs were: cloR-1/*Hind*III, 5´-GTCACCGG<u>AAGCTT</u>TGCCTG-3´; cloR-1/*Pst*I, 5´-GCATGTT<u>CTGCAG</u>AGCCTTG-3´; cloR-2/*Pst*I, 5´-GCCTGCA<u>CTGCAG</u>GCCCCAA-3´; cloR-2/*Bam*HI, 5´-TCGTA<u>GGATCC</u>TCCCGTCGTC-3´. Restriction sites introduced into the sequence are underlined in the primer sequences. The amplified DNA fragment cloR-1 was digested with *Hind*III and *Pst*I and cloned into the corresponding sites of vector pBSKT, a pBluescript SK(+) derivative containing carbenicillin and thiostrepton resistances, resulting in pFP01. The PCR fragment cloR-2 was digested with *Pst*I and *Bam*HI and ligated into the same sites of pFP01 to give pFP02.

Transformation of *S. roseochromogenes* with pFP02 was carried out by polyethylene glycol-mediated protoplast transformation (Kieser *et al.*, 2000). For preparation of protoplasts, mycelia of *S. roseochromogenes* were grown in CRM medium, containing 10.3% sucrose, 2.0% tryptic soy broth, 1.0% $MgCl_2.6H_2O$, 1.0% yeast extract, and 0.75% glycine (pH 7.0), for 48 h, harvested, and incubated in 5 ml P-buffer per gram mycelia, containing 1 mg of lysozyme per ml, for 30 to 60 min at 30°C. For transformation, pFP02 was mixed with 200 µl P-buffer containing 10^9 *S. roseochromogenes* protoplasts and 500 µl T-buffer containing 50 % (wt/vol) polyethylene glycol 1000 (Roth, Karlsruhe; Germany). The resulting suspension was plated on R2YE agar medium (Kieser *et al.*, 2000). After incubation for 20 h at 30°C, the plates were overlaid with 3 ml of soft R2YE agar containing a total of 500 µg thiostrepton for selection of recombinant mutants.

After transforming *S. roseochromogenes* protoplasts with plasmid pFP02, thiostrepton-resistant colonies were obtained. The single cross-over mutant RSCO2 was grown in the absence of thiostrepton, sporulated, and examined for loss of resistance as consequence of double cross-over events. Two mutants, named RDCO30 and RDCO32, were further examined by Southern blot analysis (see Results, p. 54).

8.2 Inactivation of cloQ.

For in-frame deletion of *cloQ* in *S. roseochromogenes*, two fragments cloQ-1 (727 bp) and cloQ-2 (1259 bp) were amplified. Primer pairs used were: cloQ-1/*Hind*III, 5´-GTGCGCG<u>AAGCTT</u>GGCCCCG-3´, and cloQ-1/*Pst*I, 5´-GAAGGC<u>CTGCAG</u>GACGGT-3´; cloQ-2/*Pst*I, 5´-CACGAC<u>CTGCAG</u>GCACTTCA-3´, and cloQ-2/*Bam*HI, 5´-ACCGGG<u>GGATCC</u>CCTGCAA-3´. Introduced restriction sites are underlined. The fragments were cloned into the corresponding sites of pBSKT (Lombo *et al.*, 1997), containing a thiostrepton resistance gene, resulting in pFP04. Transformation of *S. roseochromogenes* with pFP04 and selection for mutants resulting from single (QSCO8) and double cross-over recombination events were carried out as described above. One mutant, named *cloQ⁻* mutant (QDCO661), was further examined by Southern blot analysis (see Results, p. 40).

8.3 Inactivation of cloI.

For the in-frame deletion of *clo*I in *S. roseochromogenes*, two fragments, denoted cloI-1(1249 bp) and cloI-2 (1013 bp), were amplified. Primer pairs used were: cloI-1/HindIII, 5'-CGGCC<u>AAGCTT</u>GCCACGATG-3', and cloI-1/PstI, 5'-GTACTCGGC<u>CTGCAG</u>AATCGG-3'; cloI-2/PstI, 5'-GCATC<u>CTGCAG</u>GGGATGAGC-3', and cloI-2/XbaI, 5'-GCCGGACT<u>TCTAGA</u>TCCGTC-3'. As for pFP04, the two fragments cloI-1 and cloI-2 were cloned into pBSKT, resulting in pEW02. After transformation, a single cross-over mutant (ISCO4), and a double cross-over mutant (*cloI⁻* mutant: IDCO169) were obtained as described above and analyzed by Southern blot (see Results, p. 45).

9. Feeding of deletion mutants with Ring A, Ring B and 3DMA-4HBAL.

The defective mutants were grown in 50 ml "corn starch" pre-culture medium supplemented with 1 mg of Ring A. After 48 h, 5 ml of this pre-culture were inoculated into 50 ml " distillers solubles" production medium, again supplemented with 1 mg of Ring A. Another 1 mg of Ring A was added after two days. After 7 days of cultivation, secondary metabolites were analyzed (see Results, p. 54).

The feeding of *cloI* and *cloQ* defective mutants with Ring B of novobiocin was done in the same way.

The feeding of the *cloQ* defective mutant with 3DMA-4HBAL was done in the same way.

10. Construction of *novO* mutant by PCR-targeting in *S. spheroides*.

novO was inactivated using the PCR targeting system (Gust *et al*, 2002, Gust *et al.*, 2003), which takes advantage of the λ RED recombination functions (*gam*, *bet*, *exo*) to promote gene replacement. An *aac(3)IV* (=apramycin resistance gene)*/oriT* cassette for replacement of *novO* was generated by PCR using the primer pair NovO/P1 (5´-AGATCAGCTCACTGACCCAACACGAGGGGCATCGAGATGATTCCGGGGATCCGTCGACC-3´) and NovO/P2 (5´-CGGGTCCAGGCGCTCTGTTCGGGACAATTCCGCCGCTCATGTAGGCTGGAGCTGCTTC-3´). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *novO,* respectively, including the putative start and stop codons of *novO*. This cassette was introduced into *E. coli* BW25113/pIJ790, containing cosmid 10-9C (Supercos-1-based, kanamycin-resistant) which included the entire biosynthetic gene cluster of novobiocin. The gene replacement was confirmed by restriction analysis and PCR using test primers NovO/T1 (5´- CTGTGTCCGTGTAGGCTCAATTCA-3´) and NovO/T2 (5´-AGCACTTTCGATCATGAGGTCCAGA-3´).

The mutated cosmid (termed 10-9C-novO) was introduced into *Streptomyces spheroides* by conjugation from *E. coli* ET12567 carrying the non-transmissible pUZ8002. The conjugation procedure was made as described in "REDIRECT© technology" handbook (Gust *et al.*, 2002). 500 µl of the heat-shocked *S. Spheroides* spores was gently mixed with 500 µl of the *E. coli* suspension. The mixture was spread on two MS plates, and incubated at 30 ºC for about 18 h, then overlaid with 1 ml water containing 1.25 mg apramycin and 0.5 mg nalidixic acid. Incubation at 30 °C was continued for about a week to allow outgrowth of the exconjugants. Apramycin-resistant, kanamycin-sensitive colonies were identified by replica plating and characterized by PCR using the test primers mentioned above (see Results, p. 63).

11. Extraction and HPLC analysis of secondary metabolites.

11.1   Analysis of S. roseochromogenes culture.

Extraction:

Bacterial culture (20 ml) was acidified to pH 4 with HCl and extracted twice with an equal volume of ethyl acetate. After centrifugation, the solvent was evaporated and the dried extract was resuspended in 1ml methanol.

HPLC analysis:

Metabolites were analyzed by HPLC with a Multosphere RP18-5 column (250x4 mm, 5 µm) at a flow rate of 1 ml/min.

For the analysis of the clorobiocin and novclobiocin C102 production, the solvents used were solvent A (50% methanol, 49% H_2O, 1% formic acid) and solvent B (99% methanol, 1% HCOOH). The profile for separation was a linear gradient from 60%A/ 40%B to 100%B in 27 min, 100%B for 5 min, and then a equilibration time with 60%A/ 40%B for 6 min. Detection was at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For the analysis of the accumulation of Ring A by *cloI* mutant, the solvents used were solvent A (99% H_2O, 1% HCOOH) and solvent B (79% methanol, 20% H_2O, 1% HCOOH). The profile for separation was a linear gradient 40%A/ 60%B to 100%B in 13 min, 100%B for 6 min, and then equilibration with 40%A/ 60%B for 6 min. Detection was at 254 nm. Ring A was used as standard.

For preparative isolation, the fractions from HPLC analysis were collected and the solvent was evaporated.

MS and ^{1}H-NMR analysis:

Clorobiocin was analyzed by mass spectrometry (MS) and [1]H-nuclear magnetic resonance (^{1}H-NMR). Negative fast atom bombardement (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using methanol as solvent.

Peak A and B (Fig. 23, p. 56) gave identical isotopic peaks, characteristic for substances with one chlorine atom, at m/z 697 and 695 [M-H]$^-$ corresponding to clorobiocin standard.

The ^{1}H-NMR spectrum was measured on an AMX 400 spectrometer (Bruker, Karlsruhe; Germany), and peak A (Fig. 23, p. 56) gave signals corresponding to those of clorobiocin standard: $*_{ppm}$ (CD$_3$OD, 400 MHz): 7.90 (d, 9.2 Hz, H-5), 7.76 (d, 2.5 Hz, H-2´), 7.72 (dd, 8.4 Hz, 2.5 Hz, H-6´), 7.33 (d, 9.2 Hz, H-6), 6.90 (d, 3.6 Hz, H-3´´´), 6.84 (d, 8.4 Hz, H-5´), 5.94 (d, 3.6 Hz, H-4´´´), 5.73 (d, 1.8 Hz, H-1´´), 5.71 (dd, 10.3 Hz, 2.9 Hz, H-3´´), 5.35 (br.t, 7.1 Hz, H-8´), 4.34 (t, 2.7 Hz, H-2´´), 3.72 (d, 10.3 Hz, H-4´´), 3.52 (s, 3H-8´´), 3.34 (d, 7.1 Hz, 2H-7´), 2.29 (s, 3H-6´´´), 1.75 (s, 3H-11´), 1.74 (s, 3H-10´), 1.35 (s, 3H-7´´), 1.18 (s, 3H-6´´).

LC-ESI-CID analysis:

The extracts were also analyzed by liquid chromatography (LC) coupled to an electrospray ionization (ESI) mass spectrometer TSQ Quantum (ThermoFinnigan, San Jose, California) (250x4 mm, 5 µm) (electrospray voltage, 3 kV; heated capillary temperature, 300°C; sheath and auxiliary gas, nitrogen) in negative mode under addition of 10% ammonia (15 µl/min) with a Multosphere RP18-5 column. The solvents used were solvent A (99.9% H$_2$O, 0.1% HCOOH) and solvent B (99.9% acetonitrile, 0.1% HCOOH). The profile for separation was a linear gradient 70%A/ 30%B to 100%B in 14 min, 100%B for 15 min, and then equilibration time with 70%A/ 30%B for 11 min. The flow rate was 500 µl/min. The collision-induced dissociation (CID) spectra of clorobiocin standard, Ring A and culture extracts during HPLC run were recorded with a collision energy of + 20 eV; the collision gas was argon, and the collision pressure of 1.0 x 10^{-3} torr (133 x 10^{-3} Pa).

The negative-ion-ESI-CID mass spectrum of clorobiocin was as follows: (m/z)= 695 ([M-H]$^-$), 588, 507, 226.

The negative-ion-ESI-CID mass spectrum of Ring A was as follows: (m/z)= 205 ([M-H]$^-$), 161, 106 (see Results, p. 45).

11.2   Analysis of S. spheroides culture.

Extraction:

Cells were centrifugated and the clear supernatant was analyzed by HPLC.

For preparative isolation, 150 ml bacterial culture was prepared as described above. After centrifugation, the clear medium was acidified with HCl to pH 2 and extracted twice with 150 ml ethyl acetate. The organic phase was dried with sodium sulfate, and after the solvent was removed, the residue was dissolved in 1 ml ethanol and purified by HPLC.

HPLC analysis:

Metabolites were analyzed by HPLC with a Multosphere RP18-5 column (250 x 4 mm; 5 µm) at a flow rate of 1 ml/min.

For the analysis of novobiocin, a linear gradient from 60 to 100% methanol in 1% aqueous formic acid and detection at 305 nm were used. Authentic novobiocin (Fluka, Buchs, Germany) was used as standard.

For the analysis of ß-hydroxytyrosine, a linear gradient from 20 to 100% methanol in 1% aqueous formic acid and detection at 270 nm were used. ß-Hydroxy-L-tyrosine Standard was kindly provided by Dr. K.-H. van Pée (Dresden, Germany).

LC-ESI-CID analysis:

The extracts were also analyzed by liquid chromatography (LC) using a Multosphere RP18-5 column (250x4 mm, 5 µm) at a flow rate of 500 µl/min, coupled to an electrospray ionization (ESI) mass spectrometer TSQ Quantum (ThermoFinnigan, San Jose, California) in negative ion mode under the addition of 10% ammonia (15 µl/min). A linear gradient of acetonitrile (30-100%) in 0.1% aqueous formic acid was used (details see above, p. 87).

The negative-ion-ESI-CID (collision-induced dissociation) mass spectrum of novobiocin was as follows: (m/z)= 611/ 568/ 423/ 380/ 206 and for novclobiocin B106 as follows 597/ 554/ 409/ 366/ 192.

12. Protein analysis

Protein concentrations were determined by the Bradford method (1976) using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970), and protein bands were stained with Coomassie Brilliant Blue R-250.

The molecular weight of native CloQ and CloR was determined by gel filtration on a HiLoad 26/60 Superdex 200 column that has been equilibrated with 50 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2,000 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) (Amersham Pharmacia Biotech).

13. Overexpression and purification.

13.1   Holo-NovH and NovI.

The NovH construct pHC10 and the NovI construct pHC21 were described in Chen and Walsh (2001) and the phosphopantetheinyl transferase gene (*sfp*) cloned into pSU20 was described in Bartolome *et al.* (1991).

Holo-NovH was obtained by co-transformation of pHC10 and sfp/pSU20 into *E. coli* BL21(DE3). Cells were grown in LB medium supplemented with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 25°C to an OD_{600} of 0.4-0.6. After cooling to 15°C they were further cultured at this temperature to an OD_{600} of 0.6-0.7 before induction with 50 µM isopropyl ß-D-thiogalactoside (IPTG). The cell were then allowed to grow for an additional 15 hours at 15°C. Holo-NovH was subsequently purified as described in Chen and Walsh (2001). Expression of pHC21 in *E. coli* BL21(DE3) and purification of NovI was carried out as described in Chen and Walsh (2001).

13.2   CloQ

For construction of the plasmid *cloQ*-pGEX4T1, *cloQ* was amplified using the primers cloQ-Nterm-*Bam*HI (5'-GGAGGAAGTC<u>GGATCC</u>GCTCTCCCGATAGATC-3') and cloQ-Cterm-*Xho*I (5'-AAGCCT<u>CTCGAG</u>TCGGGCACCTCCCATGGTC-3'). Introduced restriction sites are underlined. The product was digested with *Bam*HI and *Xho*I and ligated into pGEX-4T-1 (Amersham) to give cloQ-pGEX4T1. *E. coli* BL21 (DE3) cells were grown in 100 ml LB medium supplemented with 50 µg/ml carbenicillin at 28°C until an OD_{600} of 0.6 was reached. IPTG was added to a final concentration of 1 mM. After 5 hours, the cells were harvested by centrifugation and broken using a French Press (SLM Instruments, Urbana, USA). Cell debris was

removed by centrifugation (30 min; 20,000 g). Purification of CloQ as a glutathione-*S*-transferase (GST) fusion protein and subsequent cleavage of the GST tag by thrombin treatment were carried out according to the manufacturer's instructions (Amersham) using Glutathione Sepharose 4B and the batch method.

13.3   CloR

For construction of the plasmid *cloR*-pGEX4T1, *cloR* was amplified using the primers cloR-Nterm-*Bam*HI (5'-AGGTGCCCG<u>GGATCC</u>AAGGCTTTGGCGAAC-3') and cloR-Cterm-*Xho*I (5'-TCGGCA<u>CTCGAG</u>CCTTGCCAGGGCGGGCGTTTG-3'). Introduced restriction sites are underlined. The product was digested with *Bam*HI and *Xho*I, gel purified and ligated into the same site of pGEX-4T-1 (Amersham) to give cloR-pGEX4T1. For over-expression of CloR, the GST (glutathione-*S*-transferase) tagged plasmid was transformed into *E. coli* strain BL21 (DE3).

E. coli BL21 (DE3)/ cloR-pGEX4T1 cells were grown in 100 ml LB medium containing 50 µg/ml carbenicillin at 15°C until an OD_{600} of 0.6 was reached (~ 20 hours). IPTG was added to a final concentration of 250 µM. After 24 hours at 15°C, the cells were harvested by centrifugation (10 min; 5,000 g) and resuspended in binding buffer PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 1 mg/ml lysozyme (76,344 U/mg, Fluka). Resuspended cells were broken by sonification (Branson sonifier, 10 min sonification with 40% on). Cell debris was removed by centrifugation (30 min; 20,000 g). Purification of CloR as a GST fusion protein and subsequent cleavage of the GST tag by thrombin treatment were carried out according to the manufacturer's instructions (Amersham) using Glutathione Sepharose 4B and the batch method.

14. Assays incubation and HPLC analysis.

14.1   Incubation of Holo-NovH, NovI, and CloQ with L-[U-^{14}C]Tyrosine.

Holo-NovH was loaded with L-[U-^{14}C]Tyrosine at 24°C for 1.5 h in a reaction mixture (110 µl) that contained 75 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 µM L-[U-^{14}C]tyrosine (500 Ci/mol), 3 mM ATP, 2 mM TCEP (Tris(2-carboxyethyl)phosphine)

and 30 µg NovH. Subsequently, NADPH and spinach ferredoxin (Sigma) were added to a final concentration of 1.5 mM and 5 µM, and 0.1 U ferredoxin reductase (Sigma) and 25 µg NovI were added. Incubation was continued for 2 h at 24°C. Then 5 µg CloQ and 1mM DMAPP were added and incubation was continued for 1 h at 30°C prior to quenching with 500 µl of 10% trichloroacetic acid. The precipitated proteins were pelleted by centrifugation, washed twice with 500 µl distilled water, and redissolved in 100 µl of 0.1 N KOH. The tethered product was released from the carrier protein by incubating for 5 min at 60°C. The proteins were removed by acidifying the mixture with 5 µl of 50% trifluoroacetic acid followed by centrifugation. Product formation was analyzed by HPLC with a Multosphere RP18-5 column (250x4 mm, 5 µm) coupled to a radiodetector (Berthold, Bad Wildbad, Germany), at a flow rate of 1ml/min. The elution profile started with 100% solvent A (99% H_2O, 1% HCOOH) for 3 min, followed by a linear gradient from 0 to 100% solvent B (99% acetonitrile, 1% HCOOH) of 27 min. A control incubation was carried out in parallel under the same conditions except that CloQ was denatured (30 min at 100°C).

14.2   Assays for 4HPP dimethylallyltransferase activity.

Non-radioactive 4HPP dimethylallyltransferase activity assay:

The reaction mixture (100 µl) contained 75 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.25 mM 4HPP, 0.5 mM DMAPP and purified CloQ. After incubation for 60 min at 30°C, the reaction was stopped with 2 µl formic acid. Products were analyzed by HPLC at 285 nm as described above.

The same conditions were used for incubation with L-tyrosine, ß-hydroxytyrosine, prephenic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde and p-hydroxymandelic acid.

For quantitative determinations, a maximum of 3 µg of purified enzyme and an incubation time of 15 min were used to ensure linearity of the product formation.

Radioactive 4HPP dimethylallyltransferase activity assay:

For radioactive assays, 19 µM [1-^{14}C] DMAPP (3.4 GBq/ µmol) was used instead of unlabeled DMAPP. Otherwise, the incubation was carried out and terminated as described above, with an incubation time of 60 min. The reaction mixture was extracted twice with 500 µl of ethyl acetate. After evaporation, the residue was

dissolved in 100 µl ethanol. For the conversion of 3DMA-4HPP to 3DMA-4HBAL, 50 µl of the ethanolic solution was mixed with 50 µl 1 M NaOH and incubated at room temperature for 15 min. The reaction was stopped with 10 µl formic acid. The products were analyzed by HPLC as described above, using a radioactivity detector.

Large scale incubation for LC-ESI-CID analysis:

For LC-ESI-CID analysis, the incubation mixture (500 µl) contained 75 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM 4HPP, 0.1 mM DMAPP, and 12.5 µg CloQ and was incubated at 30°C for 1h. The reaction was stopped with 5 µl formic acid, and the mixture was extracted twice with 500 µl of ethyl acetate. After evaporation, the residue was dissolved in 60 µl ethanol. A control incubation was carried out with denatured CloQ.

Product formation was analyzed by liquid chromatography (LC) coupled to an electrospray ionization (ESI) mass spectrometer TSQ Quantum (ThermoFinnigan, San Jose, California) (electrospray voltage, 3 kV; heated capillary temperature, 300°C; sheath and auxiliary gas, nitrogen) in negative mode with a Multosphere RP18-5 column (250x4 mm, 5 µm). The elution profile started with 100% solvent A (99.5% H_2O, 0.5% HCOOH) for 3 min, followed by a linear gradient from 0 to 100% solvent B (99.5% acetonitrile, 0.5% HCOOH) of 27 min. The flow rate was 500 µl/min. The collision-induced dissociation (CID) spectra of 4-hydroxyphenylpyruvate standard and enzymatic product during HPLC run were recorded with a collision energy of + 20 eV; the collision gas was argon, and the collision pressure of $1x\ 10^{-3}$ torr ($133\ x\ 10^{-3}$ Pa). The negative-ion-ESI-CID mass spectrum of 4-hydroxyphenylpyruvate was as follows: m/z= 179 ([M-H]⁻), 135, 107. The negative-ion-ESI-CID mass spectrum of 3-dimethylallyl-4-hydroxyphenylpyruvate was as follows: m/z= 247 ([M-H]⁻), 203, 175 .

14.3   *Incubation of holo-CloR with CloQ reaction products*

3DMA-4HPP, the substrate for CloR reaction, was obtained by incubating CloQ with 4HPP and DMAPP as described above. A reaction mixture (50 µl) containing 75 mM Tris-HCl (pH 7.5), 0.25 mM 4HPP, 0.5 mM DMAPP, 2.5 mM $MgCl_2$, and purified CloQ, was incubated at 30°C for 1 hour. This reaction produces about 7.5 nmol of 3DMA-4HPP.

Holo-CloR was reconstituted by preincubation of apo-CloR (~ 4 µg) with 1 mM FeSO$_4$ for 20 min at room temperature. Reaction conditions for the incubation of holo-CloR with 3DMA-4HPP were as described for HmaS (Choroba *et al.*, 2000; Hubbard *et al.*, 2000) with minor modifications. The reaction (150 µl) contained 50 µl of the CloQ reaction mixture (final concentration of 3DMA-4HPP approximately 50 µM), 0.2 M Tris-HCl (pH 7.5), 80 mM potassium phosphate (pH 7.5), 25 mM ascorbic acid and 4 µg holo-CloR. After incubation for 1 hour at 30°C, the reaction was stopped with 5 µl of formic acid. Product formation was analyzed by high performance liquid chromatography (HPLC) at 277 nm (absorption maximum for 4-hydroxymandelic acid) with a Multosphere RP18-5 column (250x4 mm, 5 µm) at a flow rate of 1 ml/min. A linear gradient of acetonitrile (25-75%) in 1% aqueous formic acid was used. A control incubation was carried out with denatured holo-CloR.

For incubation of holo-CloR with other substrates, 0.5 mM of the respective substrate (4-hydroxyphenylpyruvate, ß-hydroxytyrosine, 4-hydroxymandelic acid, D-mandelate, L-mandelate or 4-hydroxyphenyllactic acid) were used instead of 3DMA-4HPP, and analysis was carried out by HPLC at 254 nm with a linear gradient of acetonitrile (0-100%) in 1% aqueous formic acid at a flow rate of 1 ml/min. Control incubations were carried out with denatured holo-CloR.

CloQ was also incubated with 4-hydroxymandelic acid or 4-hydroxybenzoic acid and DMAPP as described above.

14.4 Incubation of 3-dimethylallyl-4-hydroxymandelic acid with CloR

The incubation mixture (1 ml) contained 500 µl of CloQ reaction product (~ 75 nmol of 3DMA-4HPP), 0.2 M Tris-HCl (pH 7.5), 80 mM potassium phosphate (pH 7.5), 25 mM ascorbic acid and ~ 40 µg holo-CloR and was incubated for 1 hour at 30°C. The reaction was stopped with 50 µl of formic acid and extracted twice with 1 ml ethyl acetate. After evaporation the residue was dissolved in 150 µl of ethanol. 3DMA-4HMA was isolated by HPLC as described above and its identity was confirmed by mass spectrometry.

14.5 CloR assays in the presence of $^{18}O_2$

Two flasks, one containing enzymatically produced 3DMA-4HPP (see above) and one containing holo-CloR reaction mixture (see above) were degassed by application

of a vacuum and flushed with argon for three times. The anaerobic holo-CloR solution was transferred to the substrate vial containing 3DMA-4HPP. The argon was removed by application of a vacuum and finally $^{18}O_2$ was allowed to enter into the flask. After incubation for 1 hour at 30°C, the reaction was stopped with formic acid and analyzed by liquid chromatography (LC) coupled to an electrospray ionization (ESI) mass spectrometer TSQ Quantum (ThermoFinnigan, San Jose, California) in negative ion mode. A linear gradient of acetonitrile (25-75%) in 0.1% aqueous formic acid was used. In a parallel $^{18}O_2$ labelling experiment, 3DMA-4HMA (isolated as described above) was used as substrate instead of 3DMA-4HPP.

15. Analysis of metal content of CloQ

Zinc was determined using a UNICAM atom absorption spectrometer. Magnesium was determined spectrophotometrically using the Roche/ Hitachi 902 clinical chemistry analyser. Both the zinc and the magnesium content was less than 0.1 mol per mol CloQ

APPENDIX A – Plasmids and probes.

Name	Description/ Publication
Plasmids:	
Supercos-1	Cosmid vector/ Pojer *et* al. (2002)
pBluescript SK(-)	Cloning vector/ Stratagene
pBSKT	pBluescript SK(+) derivative (Lombo *et al.*, 1997)
pFP01	1282 bp *Hind*III-*Pst*I PCR fragment, cloned into pBSKT/ Pojer *et* al. (2002)
pFP02	1301 bp *Pst*I-*Bam*HI PCR fragment, cloned into pFP01/ Pojer *et* al. (2002)
pFP04	727 bp *Hind*III-*Pst*I and 1259 bp *Pst*I-*Bam*HI PCR fragments,cloned into pBSKT/ Pojer *et* al. (2003a)
pEW02	1249 bp *Hind*III-*Pst*I and 1013 bp *Pst*I-*Xba*I PCR fragments,cloned into pBSKT/ Pojer *et* al. (2003a)
pHC10	*novH* gene from novobiocin cluster, cloned into pET24b (C-terminal Hisx6 tag)/ Chen and Walsh (2001) and Pojer *et* al. (2003a)
pHC21	*novI* from novobiocin cluster, cloned into pET16b (N-terminal Hisx6 tag)/ /Chen and Walsh (2001) and Pojer *et* al. (2003a)
sfp/pSU20	PPTase gene cloned into pSU20 (no tag)/ Bartolome *et* al. (1991) and Pojer *et* al. (2003a)
pGEX-4T-1	Glutathione-*S*-transferase fusion vector/ Pojer *et* al. (2003a and b)
cloQ-pGEX4T1	974 bp *Bam*HI-*Xho*I PCR fragment, cloned into pGEX-4T-1/ Pojer *et* al. (2003a)
cloR-pGEX4T1	833 bp *Bam*HI-*Xho*I PCR fragment, cloned into pGEX-4T-1/ Pojer *et* al. (2003b)
pofI1A	0.9 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-).
pofI1B	0.9 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
pofI2	1.3 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)

Pofl3	2.5 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
Pofl4	5.1 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
Pofl5	5.5 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
Pofl6	7 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
Pofl6A	1.5 kb *Bam*H1-*Sal*I fragment from pofl6, cloned into the same sites of pBluescript SK (-)
Pofl6B	2.5 kb *Sal*I fragment from pofl6, cloned into pBluescript SK (-)
Pofl6C	2.8 kb *Bam*H1-*Sal*I fragment from pofl6, cloned into the same sites of pBluescript SK (-)
Pofl7	10 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
Pofl8	14 Kb *Bam*HI fragment from cosmid VIIIA7-c, cloned into pBluescript SK (-)
cosmid 10-9C	cosmid vector containing the novobiocin biosynthetic gene cluster/ Steffensky *et al.* (2000)
10-9C-novO	*novO* replaced by an apramycin resistance/*oriT* cassette in cosmid 10-9C
pIJ773	disruption cassette : *aac(3)IV* (Apra^R) + *OriT*
Probes.	
novL	1.58 kb *sph*I-*Bam*HI fragment from PMS81(from Marion Steffensky)
cloM	900 bp *Bam*HI fragment from poflA1
gyrB^R	900 bp *Bgl*II from pGES11 (from Elisabeth Schmutz)
novT	Steffensky *et al.* (2000) and Wang *et al.* (2000)
cloR	1122 bp*Sac*II fragment from pofl4
cloQ	1469 bp *pvu*II fragment from pofl4
cloI	1987 bp *Nco*I fragment from K1F2

APPENDIX B – Bacterial strains.

strains	genotype	plasmid	Description/ Origin
S. roseochromogenes var. *oscitans* DS 12.976	Wild-type		Aventis
S. albocinerescens DS 21.647	Wild-type		Aventis
S. hygroscopicus DS 9.751	Wild-type		Aventis
S. spheroides NCIMB 11891	Wild-type		E. Cundliffe (Leicester, U.K.)
E.coli XL1 blue MRF´			Stratagene
E.coli XL1 blue MRF´ + supercos-1		supercos-1	cosmid vector in *E.coli* XL1 blue MRF´
E.coli ET 12567			DNA methylase-negative strain/ (MacNeil *et al.*, 1992)
S. roseochromogenes RSCO2	mutant		Single cross over event of *cloR*⁻ inactivation/ Pojer *et* al. (2002)
S. roseochromogenes RDCO30	mutant		*cloR*⁻ in-frame deletion in *S. roseochromogenes*/ Pojer *et* al. (2002)
S. roseochromogenes RDCO32	Reversion to wild-type		Reversion to *S. roseochromogenes* wild type/ Pojer *et* al. (2002)
S. roseochromogenes QSCO8	mutant		Single cross over event of *cloQ*⁻ inactivation/ Pojer *et* al. (2003a)
S. roseochromogenes QDCO661	mutant		*cloQ*⁻ in-frame deletion in *S. roseochromogenes*/ Pojer *et* al. (2003a)
S. roseochromogenes ISCO4	mutant		Single cross over event of *cloI*⁻ inactivation/ Pojer *et* al. (2003a)
S. roseochromogenes IDCO169	mutant		*cloI*⁻ in-frame deletion in *S. roseochromogenes*/ Pojer *et* al. (2003a)
E.coli BL21 (DE3)			Pojer *et* al. (2003a)
E.coli BL21 (DE3) + pHC10		pHC10	Pojer *et* al. (2003a)
E.coli BL21 (DE3) + pHC10 + sfp/pSU20		pHC10 + sfp/pSU20	Pojer *et* al. (2003a)
E.coli BL21 (DE3) + pHC21		pHC21	Pojer *et* al. (2003a)
E.coli BL21 (DE3) + sfp/pSU20		sfp/pSU20	Pojer *et* al. (2003a)
E.coli XL1 blue MRF´ + pGEX-4T-1		pGEX-4T-1	Pojer *et* al. (2003a)
E.coli BL21 (DE3) + *cloQ*-pGEX4T1		*cloQ*-pGEX4T1	Pojer *et* al. (2003a)
E.coli BL21 (DE3) + *cloR*-pGEX4T1		*cloR*-pGEX4T1	Pojer *et* al. (2003b)
S. spheroides novO⁻ -1	mutant		*novO*⁻ number 1 in *S. spheroides* created by PCR targeting
E. coli BW25113 + pIJ790		pIJ790	Gust *et al.* (2002) and Gust *et al.* (2003)
E. coli ET12567 + pUZ8002		pUZ8002	Gust *et al.* (2002) and Gust *et al.* (2003)

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ACADEMIC TEACHERS

Faculté de Pharmacie de Besançon:

Prof. J.P. Chaumont

Prof. F. Bevalot

Prof. J. Vaquette

Prof. A. Xicluna

Prof. M. Baud

Prof. C. Guinchard

Prof. A. Jacqueson

Prof. M. Mercier

Prof. J. Millet

Prof. J.F. Robert

DEA d'Oceanologie Biologique de l'Université Pierre et Marie Curie :

Dr. N. Bourgougnon

Prof. P. Duval

Pharmazeutisches Institut in Tübingen :

Prof. L. Heide

Dr. S.-M. Li

Curriculum Vitae

Name:	Florence Pojer
Geburgstag:	11 Mai 1973
Geburtsort:	Dole, Frankreich
Nationalität:	Französich

Besuchte Schulen:	**1979-1985**	Grundschule in Dole (Frankreich)
	1985-1992	Gymnasium in Dole
	1988	Halbjähriger Aufenthalt an der "Syracuse high school" in Syracuse (USA)
	1992	Abitur in Dole (Frankreich)
Universitäre Ausbildung:	**1992-1997**	Studium der Pharmazie an der Franche-Comté Universität (Frankreich)
	1997-1998	Diplomarbeit an der Universität Paris VI in Paris (Frankreich)
	1999-present	Vom 15.09.1999 bis 25.07.2003. Doktorarbeit am Lehrstuhl von Prof. Lutz Heide am Pharmazeutischen Institut in Tübingen (Deutschland). Titel der Doktorarbeit: "Molekularbiologische und biochemische Untersuchungen zur Biosynthese von Clorobiocin aus Streptomyces roseochromogenes DS 12.976".
Lehrtätigkeit:	**1999-present**	Vorbereitung und Betreuung Pharmazeutischer Praktika: Pharmazeutische Biologie I and III. Gesamtdauer der Praktika: 450 Stunden.

Publikationsliste:

- J.-P. Bergé, N. Bourgougnon, S. Alban, **F. Pojer**, S. Billaudel, J.-C. Chermann, J.M. Robert, and G. Franz (1999). Antiviral and Anticoagulant activities of a water-soluble fraction of the marine diatom *Haslea ostrearia*. *Planta Medica* 65, 604-609.

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- **F. Pojer**, E. Wemakor, B. Kammerer, H. Chen, C.T. Walsh, S.-M. Li and L. Heide (2003a). CloQ, a prenyltransferase involved in clorobiocin biosynthesis. *PNAS* 100, 2316-2321.

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