The aminocoumarins: biosynthesis and biology

Lutz Heide*

Received 29th June 2009
First published as an Advance Article on the web 10th August 2009
DOI: 10.1039/b808333a

Covering: up to 2009

The aminocoumarin antibiotics are characterized by their 3-amino-4,7-dihydroxycoumarin moiety. This family of antibiotics comprises highly potent gyrase inhibitors, including novobiocin and the structurally related compounds clorobiocin and coumermycin A_1. These compounds interact with the B subunit of bacterial gyrase and inhibit ATP-dependent supercoiling of DNA. The structurally more complex simocyclinone D8, which contains two polyketide moieties, inhibits gyrase by a completely different mechanism, *i.e. via* interaction with the A subunit. Rubradirin and its aglycone, which contain an ansamacrolide moiety, interfere with protein or RNA synthesis, respectively. The biosynthetic gene clusters of all five aminocoumarin antibiotics have been identified, and the gene functions have been studied by genetic and biochemical methods. The biosynthesis of novobiocin and clorobiocin is now one of the best-understood pathways of secondary metabolism in streptomycetes.

1 Chemical structures and biosynthetic gene clusters of aminocoumarin antibiotics

The aminocoumarin antibiotics (Scheme 1) comprise three "classical" aminocoumarins, *i.e.* novobiocin, clorobiocin and coumermycin A_1, as well as the structurally more complex simocyclinones and rubradirins. They are produced by different *Streptomyces* strains. The aminocoumarin antibiotics are characterized by their 3-amino-4,7-dihydroxycoumarin moiety. The occurrence of this moiety has not been reported in any other natural product, neither within the genus *Streptomyces* nor in other organisms.

Pharmaceutical Institute, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany. E-mail: heide@uni-tuebingen.de; Fax: +49 7071 295250; Tel: +49 7071 2972460

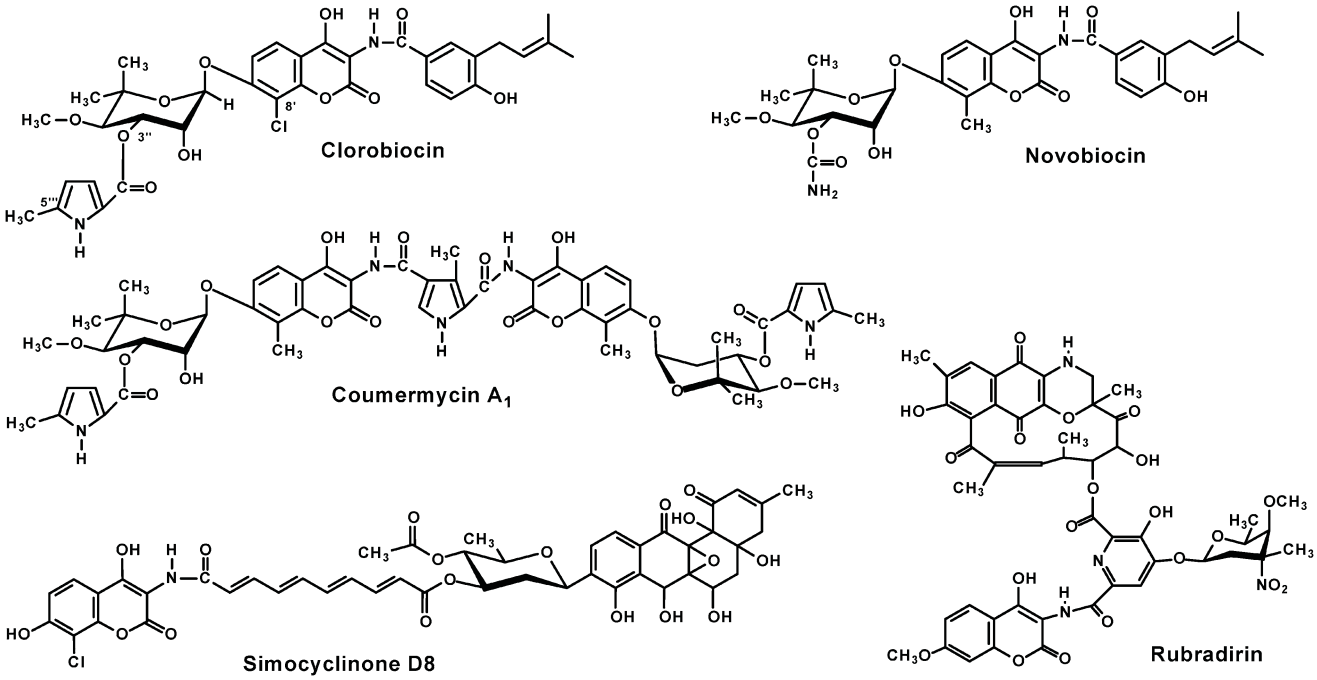
The biosynthetic gene clusters of all five aminocoumarins have been cloned and sequenced.[1–6] The clusters of novobiocin, clorobiocin and coumermycin span between 23 and 38 kb and comprise between 20 and 32 coding sequences. The clusters of simocyclinone and rubradirin are larger, spanning approximately 65 and 105 kb, respectively, and their precise borders have not yet been defined.

Nearly all genes contained in the novobiocin, clorobiocin and coumermycin biosynthetic gene clusters have been investigated by genetic and biochemical experiments, and their precise function in the catalysis of biosynthetic reactions, in regulation and in resistance is known. On the other hand, few experimental data are available for simocyclinone and rubradirin biosyntheses.

2 Biosynthesis of the aminocoumarin moiety

Incorporation studies with isotope-labelled precursors had shown that the aminocoumarin moiety is derived from L-tyrosine.[7] Cloning and sequence analysis of the biosynthetic gene cluster of novobiocin[1] suggested that the gene products of *novHIJK* might be responsible for the biosynthesis of the aminocoumarin moiety. Biochemical investigations[8] indeed revealed that L-tyrosine is first activated by the adenylation domain of the 65 kDa protein NovH and attached covalently *via* a thioester bond to the phosphopantetheinyl cofactor of the peptidyl carrier protein (PCP) domain of NovH (Scheme 2). Subsequently, the 45 kDa heme protein NovI hydroxylates this enzyme-bound tyrosine in a monooxygenase reaction, generating β-hydroxytyrosyl-*S*-NovH.[8] The stereochemistry of this compound has been elucidated as (2*S*,3*R*) (Scheme 2).This intermediate is oxidized by the heterotetrameric enzyme NovJ/NovK to β-ketotyrosyl-*S*-NovH, using NADP as electron acceptor.[9] NovJ and NovK both show similarity to 3-oxoacyl-[acylcarrierprotein]-reductases from polyketide and fatty acid biosynthesis.

The attachment of the phosphopantetheinyl cofactor to the PCP domain of NovH and its orthologs requires



Scheme 1 Structures of the aminocoumarin antibiotics clorobiocin, novobiocin, coumermycin A_1, simocyclinone D8 and rubradirin.

a phosphopantetheinyl transferase. Such an enzyme is not encoded within the biosynthetic gene clusters of novobiocin, clorobiocin, coumermycin and rubradirin. The transfer therefore appears to be catalyzed be unspecific phosphopantetheinyl transferases encoded elsewhere in the genome. The simocyclinone cluster[5] contains two putative phosphopantetheinyl transferase genes, but sequence comparison with database entries suggests that these are involved in the biosynthesis of the two polyketide moieties of simocyclinone.

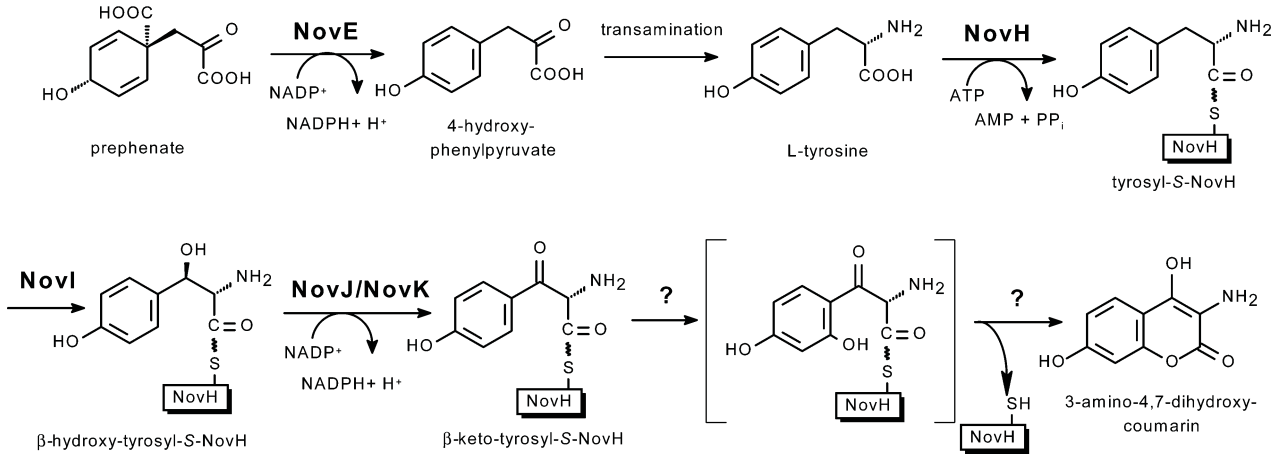
The final step of the biosynthesis of the aminocoumarin moiety, *i.e.* from β-keto-tyrosyl-*S*-NovH to 3-amino-4,7-dihydroxycoumarin, has not been elucidated yet. It may be speculated that a hydroxyl group is introduced into position 2 of the aromatic ring of β-keto-tyrosyl-*S*-NovH, followed by a nucleophilic attack of this hydroxyl group on the thioester moiety (Scheme 2). Cleavage of the carbon–sulfur bond and enolization of the β-keto group would readily yield the expected 3-amino-4,7-dihydroxycoumarin. However, an enzyme which catalyzes the hydroxylation of the aromatic ring of β-keto-tyrosyl-*S*-NovH has yet not been identified. Chen and Walsh[8] originally suggested that the enzyme encoded by the putative oxidoreductase gene *novC* of the novobiocin cluster may be responsible for this hydroxylation. Meanwhile, however, heterologous expression of a minimal novobiocin cluster proved that *novC* is not required for the biosynthesis of the aminocoumarin moiety of novobiocin,[10] and cloning of the biosynthetic gene clusters of the four other aminocoumarin antibiotics has shown that these clusters do not contain an ortholog of *novC*.

Holzenkämpfer and Zeeck[11] have demonstrated the incorporation of an isotope label from $^{18}O_2$ into the ring oxygen of the aminocoumarin moiety of simocyclinone, consistent with the hypothetical cyclization mechanism described above. In contrast, however, earlier isotope-labelling studies by Bunton *et al.*[7] have concluded that the ring oxygen of the aminocoumarin moiety of novobiocin is not derived from molecular oxygen but from one of the oxygens of the carboxyl group of tyrosine, and the authors suggested a completely different cyclization mechanism. Therefore, the cyclization reaction in aminocoumarin biosynthesis remains to be elucidated.

In the novobiocin biosynthetic gene cluster, the four genes *novHIJK* form part of a single operon. Very similar operon structures, *i.e. cloHIJK* and *couHIJK*, are found in the biosynthetic gene clusters of clorobiocin and coumermycin. The simocyclinone cluster also contains orthologs of these genes, *i.e. simH* and *simI*, situated adjacent to each other, and the genes *simK* and *simJ1*, situated in different locations upstream and downstream of *simHI*. A second gene with similarity to *novJ*, termed *simJ2*, is found nearby, but an inactivation experiment proved that *simJ1* is required for the biosynthesis of the aminocoumarin moiety of simocyclinone.[4]

Lutz Heide

Lutz Heide obtained his PhD at the University of Münster, Germany. After postdoctoral time at Kyoto University, Japan, he worked at the University of Bonn and later at the University of Freiburg. Since 1994 he has been Full Professor of Pharmaceutical Biology at the University of Tübingen, Germany. His main research interests are the biosynthesis of secondary metabolites and the genetic engineering of antibiotic biosynthesis in microorganisms.



Scheme 2 Biosynthesis of the 3-amino-4,7-dihydroxycoumarin moiety of novobiocin.

Also the recently reported biosynthetic gene cluster for rubradirin[6] contains genes with sequence similarity to *novHIJ*, *i.e.* the putative operon formed by *rubC1*, *rubC2* and *rubC3*. No ortholog for *novK* is found in the vicinity of these genes, suggesting that in this case RubC3 alone may catalyse the oxidation of β-hydroxy-tyrosyl-*S*-NovH to β-keto-tyrosyl-*S*-NovH.

The biosynthetic gene clusters of clorobiocin, coumermycin A_1 and simocyclinone, but not the clusters of novobiocin and rubradirin, contain a small open reading frame, termed *cloY*, *couY* and *simY*, respectively. These genes code for 70-71 aa proteins which show sequence similarity to *mbtH* from the mycobactin biosynthetic gene cluster of *Mycobacterium tuberculosis*. *mbtH*-like genes are frequently found in the biosynthetic gene clusters of peptide antibiotics and siderophores, but their function remains enigmatic. Inactivation of *cloY* and feeding of the resulting mutant with 3-amino-4,7-dihydroxycoumarin showed that *cloY* is specifically required for the formation of the aminocoumarin moiety of the clorobiocin molecule.[12] However, its role in either catalysis or regulation or protein–protein interactions is as yet unknown.

In novobiocin and coumermycin, the aminocoumarin moiety is substituted in position 8 with a methyl group. It was originally believed that this methylation takes place either during or immediately after the biosynthesis of the aminocoumarin moiety.[8] However, biochemical investigations proved that the SAM-dependent methyltransferase NovO, which catalyzes this methylation reaction in novobiocin biosynthesis, does not utilize the free aminocoumarin moiety or precursors thereof, but rather the amide formed from the aminocoumarin and the prenylated 4-hydroxybenzoyl moieties.[13] In coumermycin biosynthesis, the orthologous methyl transferase CouO accepts in *vitro* both the mono- and the bis-amide formed from the central dicarboxylpyrrole moiety with either one or two aminocoumarin moieties.[13]

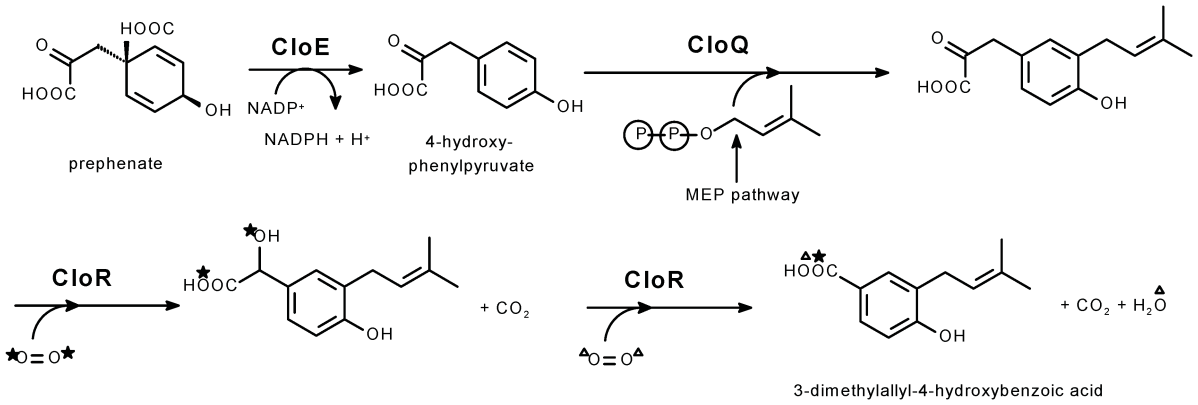
Clorobiocin and simocyclinone D8 contain a chlorine atom rather than a methyl group in position 8 of their aminocoumarin moieties. Genetic studies on clorobiocin biosynthesis proved that the FAD-dependent halogenase Clo-hal is responsible for the introduction of this halogen.[14,15] However, the reaction has not been demonstrated *in vitro* yet. Indirect evidence from feeding of intermediates to mutant strains suggests that also in this case amide formation precedes halogenation.[16] Also the simocyclinone cluster contains a similar halogenase, termed *simD4*,[5] but no experimental data are available on this halogenation reaction.

3 Biosynthesis of the prenylated 4-hydroxybenzoyl moiety of novobiocin and clorobiocin

Both in novobiocin and clorobiocin, a 3-prenylated 4-hydroxybenzoyl moiety is attached *via* an amide bond to the amino group of the aminocoumarin moiety (Scheme 1). Feeding studies had shown the incorporation of labelled L-tyrosine into this substituted benzoyl moiety.[7] Chen and Walsh[8] had originally speculated that β-hydroxy-tyrosyl-*S*-NovH, a proven intermediate in aminocoumarin biosynthesis, may undergo a retro-aldol cleavage, resulting in 4-hydroxybenzaldehyde, which could be prenylated and oxidized to give the 3-prenylated 4-hydroxybenzoyl moiety.

3-Prenylated 4-hydroxybenzoate is an intermediate in the biosynthesis of ubiquinones in all living organisms, and the enzyme catalyzing the 3-prenylation of 4-hydroxybenzoate in ubiquinone biosynthesis has been investigated in bacteria and in eukaryotes.[17,18] A similar reaction is involved in the biosynthesis of the plant secondary metabolite shikonin.[19] However, the biosynthetic gene clusters of novobiocin and clorobiocin did not contain any gene with similarity to known prenyltransferases. Eventually, genetic and biochemical experiments proved that the prenyltransferase of clorobiocin biosynthesis was encoded by *cloQ*.[20] The 35 kDa soluble, monomeric protein CloQ was found to be the first member of a new class of prenyltransferases which catalyze the *C*-prenylation of different aromatic substrates.[21,22] These enzymes show a new type of protein fold.[23,24] Due to their αββα architecture, these enzymes have been termed ABBA prenyltransferases.[21]

The aromatic substrate of the prenyltransferase CloQ is 4-hydroxyphenylpyruvate,[20,25] and the isoprenoid substrate is dimethylallyl diphosphate (DMAPP) (Scheme 3). Feeding experiments with isotope-labelled precursors have shown that this DMAPP is derived from the methylerythritol phosphate (MEP) pathway rather than from the mevalonate pathway.[26,27] In streptomycetes, the MEP pathway is responsible for the supply of isoprenoids of primary metabolism; therefore, the



Scheme 3 Biosynthesis of the 3-prenylated 4-hydroxybenzoyl moiety of clorobiocin.

genes for DMAPP biosynthesis are not encoded in the biosynthetic gene clusters of novobiocin and clorobiocin, but elsewhere in the genome.

The product of prenylation by CloQ is 3-dimethylallyl-4-hydroxyphenylpyruvate (Scheme 3). Adjacent to the gene *cloQ* in the clorobiocin cluster is a gene *cloR*, the function of which could not be predicted by database comparisons. Overexpression, purification and biochemical investigation[28] proved that *cloR* codes for a bifunctional non-heme iron(II)-dependent oxygenase which converts 3-dimethylallyl-4-hydroxyphenylpyruvate, *via* two consecutive oxidative decarboxylation steps, first to 3-dimethylallyl-4-hydroxymandelate and further to 3-dimethylallyl-4-hydroxybenzoate. Labeling experiments with $^{18}O_2$ proved that two oxygen atoms are incorporated in the first reaction step, but only one further oxygen is incorporated during the second reaction step.[28] CloR was thereby identified as a novel non-heme iron(II)- and α-ketoacid-dependent oxygenase, which utilizes 3-dimethylallyl-4-hydroxyphenylpyruvate both as the substrate for hydroxylation and as the α-ketoacid cosubstrate. The first reaction step catalyzed by CloR is similar to the reaction catalyzed by the well-examined 4-hydroxyphenylpyruvate dioxygenase which generates homogentisate.[29] However, CloR hydroxylates the benzylic position of the side chain of 4-hydroxyphenylpyruvate rather than the aromatic ring.

The different groups of iron(II)- and α-ketoacid-dependent oxygenases possess little overall sequence similarity to each other, and it is therefore not surprising that CloR does not show sequence similarity to known members of this family. However, CloR does show significant similarity to several proteins of so far unknown function, deduced from genome sequences of different microorganisms. These may possibly represent enzymes of similar function to CloR.

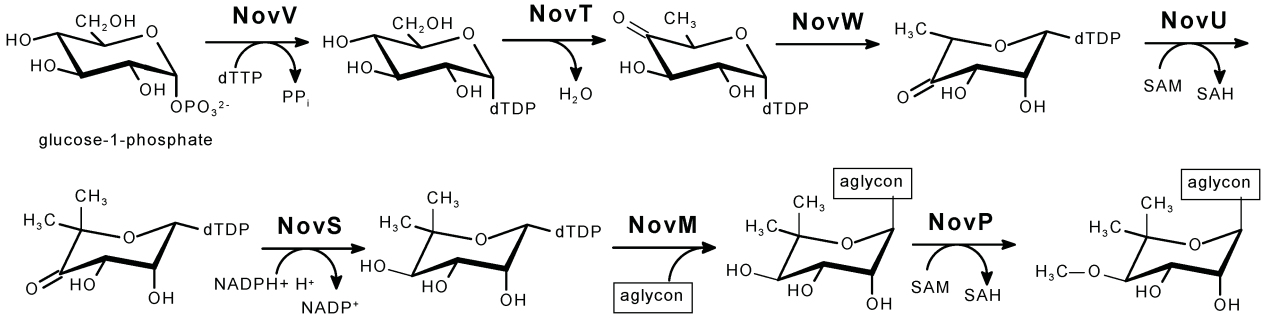
The novobiocin biosynthetic gene cluster contains two genes with high similarity to *cloQ* and *cloR*, *i.e. novQ* and *novR*, which carry out the corresponding reactions in novobiocin biosynthesis.[30] NovR has been crystallized, and a preliminary structural elucidation has been reported.[31] Additionally, both gene clusters contain a gene termed *cloF* or *novF*, respectively, which shows sequence similarity to prephenate dehydrogenase genes. The product of the prephenate dehydrogenase reaction is 4-hydroxyphenylpyruvate, the precursor of the prenylated 4-hydroxybenzoyl moieties of novobiocin and clorobiocin. 4-Hydroxyphenylpyruvate can also be converted by transamination to L-tyrosine, the precursor of the aminocoumarin moiety. Therefore it appears likely that the function of CloF and NovF is to increase the supply of precursors for the biosynthesis of the aromatic moieties of novobiocin and clorobiocin. However, even after the inactivation of NovF considerable amounts of novobiocin were still accumulated, showing that NovF is not essential for novobiocin biosynthesis (Dangel, Heide and Gust, unpublished results). Notably, the coumermycin gene cluster does not contain a functional ortholog of *novF*, but the corresponding position of the cluster (*i.e.* the large intergenic region between *couE* and *couG*) still shows nucleotide sequence similarity to the coding sequence of *novF*, indicating a close evolutionary relationship between the clusters. No *novF* ortholog is found in the gene clusters of simocyclinone and rubradirin. However, the biosynthetic gene clusters of glycopeptide antibiotics like balhimycin, which contain β-hydroxylated tyrosyl residues, do comprise an *novF* ortholog.[32]

Also in coumermycin, rubradirin and simocyclinone, acyl moieties are attached to the amino group of the 3-amino-4,7-dihydroxycoumarin moiety. However, these acyl groups are structurally completely different from the 3-dimethylallyl-4-hydroxybenzoyl moiety of novobiocin and clorobiocin (Scheme 1), and their biosynthesis has not yet been experimentally examined.

4 Biosynthesis of the deoxysugar moieties

Novobiocin, clorobiocin and coumermycin A_1 all contain the same deoxysugar, noviose (*i.e.* 4-*O*-methyl-5-*C*-methyl-L-rhamnose). This sugar moiety is acylated at its 3-OH group either with a carbamoyl or with a 5-methyl-pyrrole-2-carboxyl moiety (Scheme 1).

L-Rhamnose is a frequently encountered 6-deoxysugar in glycosidic natural products, and its biosynthetic pathway is known from different organisms.[33] The unusual feature of the deoxysugar moieties of novobiocin, clorobiocin and coumermycin is the additional methyl group at C-5, resulting in a 5,5-*gem*-dimethyl structure. Early feeding experiments with [methyl-^{14}C]methionine showed that one of these methyl groups is derived from *S*-adenosylmethionine.[34] With this fact established, a biosynthetic pathway from glucose-1-phosphate to dTDP-5-*C*-methyl-L-rhamnose can readily be suggested (Scheme 4), involving the activation of glucose by its attachment to deoxythymidyl diphosphate (dTDP), followed by the 4,6-dehydratase



Scheme 4 Biosynthesis of noviose, the deoxysugar moiety of novobiocin.

reaction which is common in deoxysugar biosynthesis and results in a dTDP-activated 4-keto-6-deoxysugar. Epimerization at positions 3 and 5 (or at position 3 alone) and 5-*C*-methylation, followed by 4-ketoreduction, would result in dTDP-5-*C*-methyl-L-rhamnose. Indeed, cloning of the biosynthetic gene cluster of novobiocin showed a group of five genes termed *novSTUVW* which, by comparison to known genes of deoxysugar biosynthesis from other organisms, could be assigned to the five enzymatic reactions in the pathway postulated above. Close orthologs of these five genes are found in the gene clusters of clorobiocin and coumermycin, arranged in exactly the same order.

Inactivation of the putative 4,6-dehydratase gene *novT* in the novobiocin producer led, as expected, to the abolishment of deoxysugar biosynthesis and to the accumulation of the aglycone of novobiocin, *i.e.* of novobiocic acid.[1] However, in this experiment the expression of deoxysugar biosynthesis genes located downstream of *novT*, *i.e. novUVW*, might also have been affected, and this may have contributed to the observed phenotype.

Thuy *et al.*[35] expressed *novW*, *novU* and *novS* in *Escherichia coli* and purified the enzymes by Ni^{2+} affinity chromatography. *In vitro* investigations proved that the epimerization catalyzed by NovW precedes the 5-*C*-methylation, catalyzed by NovU. Additional experiments proved that, as expected, NovU does not accept dTDP-L-rhamnose as a substrate, but only its precursor that still contains the 4-keto group. Therefore, the methylation catalyzed by NovU precedes the 4-keto-reduction catalyzed by NovS (Scheme 4).

The well-examined biosynthesis of L-rhamnose from D-glucose requires epimerization at both C-3 and C-5, which is catalyzed by a 3,5-epimerase like OleL of oleandomycin biosynthesis.[36] Correspondingly, Thuy *et al.*[35] postulated that NovW catalyzes a 3,5-epimerization. However, the X-ray crystallographic elucidation of the structure of NovW[37] showed that a tyrosine side chain in the active centre is oriented differently from in the 3,5-epimerase RmlC, but similarly to in the 5-epimerase EvaD. Therefore, Jakimowicz *et al.*[37] raised the question as to whether NovW may function as a 3-mono-epimerase rather than as a 3,5-epimerase. In subsequent *in vitro* experiments, measuring the incorporation of deuterium into the substrate dTDP-4-keto-6-deoxyglucose during its incubation with NovW in D_2O, Tello *et al.*[38] observed a fast incorporation of deuterium into position 3, but only a very slow incorporation into position 5 of dTDP-4-keto-6-deoxyglucose. Based on this result, they suggested that NovW should be functionally assigned as a 3-epimerase.

This assignment is in conflict, however, with results from *in vivo* experiments in a clorobiocin producer strain.[39] After inactivation of the methyltransferase *cloU*, a close ortholog of *novU*, the resulting strain was found to accumulate clorobiocin glycosides that contained derivatives of L-rhamnose. Therefore, both position 3 and 5 had been epimerized during the biosynthesis of the deoxysugar. The structure of the deoxysugar was proven by ^{1}H NMR investigations, showing the strong coupling of the axial protons in positions 3 and 5 with the axial proton in position 4,[39] identical to NMR signals of other L-rhamnosides. Furthermore, the natural occurrence of an L-rhamnoside analogue of novobiocin[40] proved that 3,5-epimerization also occurs in a genuine producer strain. In contrast, aminocoumarins carrying a 6-deoxy-D-gulose moiety (which would result from 3-epimerization alone) have not been reported.

In a further *in vivo* experiment, the L-rhamnose biosynthesis genes *oleS*, *oleE*, *oleL* and *oleU* from the oleandomycin biosynthetic gene cluster were introduced into a clorobiocin producer strain which was impaired in deoxysugar biosynthesis.[41] The clorobiocin glycosides produced by the resulting strain contained the same derivatives of L-rhamnose as previously produced by the action of the four genes *cloV*, *cloT*, *cloW* and *cloS* in the *cloU*-defective mutant. Therefore, these four genes of clorobiocin biosynthesis must be assumed to direct the same reaction sequence as catalyzed by OleS/OleE/OleL/OleU. OleL is a 3,5-epimerase.[36,42]

Possibly, the very slow deuterium incorporation into position 5 of dTDP-4-keto-6-deoxyglucose observed by Tello *et al.*[38] in an equilibrium reaction with NovW can be explained by a sequential mechanism for the enzymatic reaction, in which 3-epimerization precedes 5-epimerization. An example for such a sequential mechanism of a 3,5-epimerase has been reported.[43]

The 3-*O*-acylation and the 4-*O*-methylation of the deoxysugar moieties of novobiocin, clorobiocin and coumermycin occur only after attachment of the sugar to the aglycone and are discussed in Section 6.

Simocyclinone and rubradirin contain different deoxysugars than novobiocin, clorobiocin and coumermycin. A hypothetical pathway for the formation of the 2,6-dideoxsugar D-olivose in simocyclinone biosynthesis has been discussed by Trefzer *et al.*[5] Rubradirin contains D-rubranitrose, an unusual 2,6-dideoxy-3-methyl-3-nitro sugar, and a biosynthetic pathway to this compound has been proposed by Kim *et al.*[6]

5 Linkage of the aminocoumarin, the acyl and the deoxysugar moieties

In all aminocoumarin antibiotics, the amino group of the 3-amino-4,7-dihydroxyaminocoumarin moiety is attached *via* an amide bond to an acyl moiety (Scheme 1). The enzymes catalyzing the formation of this amide bond have been biochemically investigated in novobiocin, clorobiocin, coumermycin and simocyclinone biosynthesis and are termed NovL,[44] CloL,[45] CouL[46,47] and SimL,[48,49] respectively. In the biosynthesis of non-ribosomally formed peptide antibiotics, the amide bond formation is catalyzed by modular enzymes which comprise an adenylation domain for the activation of the acyl moiety, a peptidyl carrier protein (PCP) domain with a phosphopantetheinyl cofactor for attachment of the activated acyl moiety *via* a thioester bond, and a condensation domain for transfer of the acyl moiety to the amino group of the acceptor substrate.[50,51] In contrast, the enzymes involved in amide bond formation in aminocoumarin antibiotic biosynthesis do not form covalent thioester intermediates with their acyl substrates and do not show domains with similarity to PCP or condensation domains. They are monomeric proteins of approximately 60 kDa and catalyze both the activation of the acyl substrate *via* formation of an acyl adenylate and the transfer of the acyl group to the amino group. All four enzymes mentioned above contain only a single adenylation domain. Remarkably, CouL catalyzes two consecutive reactions, forming first the monoamide and then the diamide of 3-methylpyrrole-2,4-dicarboxylic acid with 3-amino-4,7-dihydroxycoumarin. As may be expected, the substrate specificities of the amide synthetases involved in the biosynthesis of different aminocoumarin antibiotics are different, and these differences have been exploited for the mutasynthetic generation of new aminocoumarin antibiotics in genetically engineered producer strains.[16] Also the rubradirin cluster contains candidate genes for the amide synthetase reactions, but these have not yet been biochemically investigated.

In novobiocin, coumermycin and clorobiocin biosynthesis, formation of the amide bonds between the acyl and the aminocoumarin moieties is followed by methylation or chlorination of position 8 of the aminocoumarin moieties under catalysis of NovO, CouO or Clo-hal, as discussed above. This concludes the assembly of the aglycones of these antibiotics and is followed by transfer of the 5-*C*-methyl-L-rhamnosyl moieties onto the 7-hydroxyl group of the aminocoumarin moiety under catalysis by the glycosyltransferase NovM[52–54] or CouM.[47] The same reaction is expected to occur in clorobiocin biosynthesis, catalyzed by the gene product of *cloM*, but this enzyme has not yet been investigated *in vitro*.

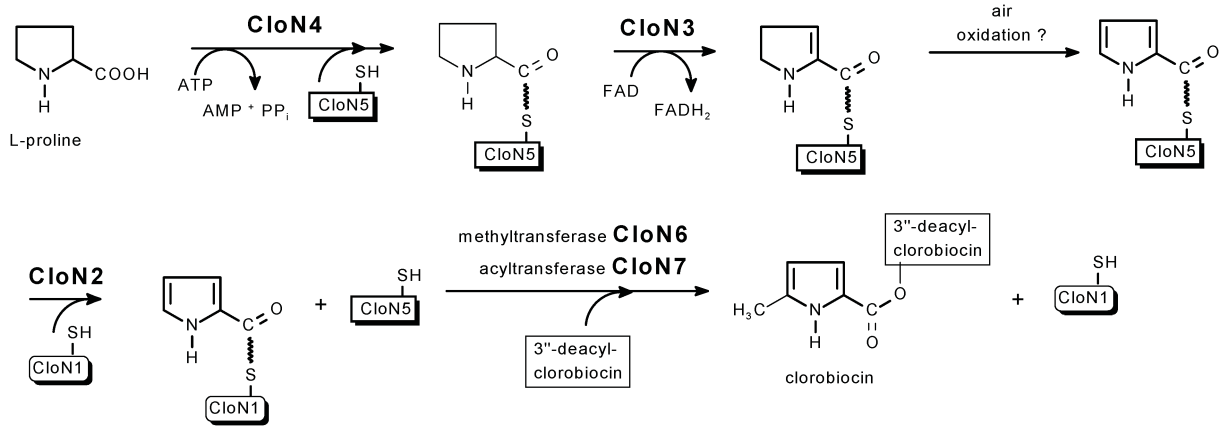
No glycosylation of the 7-hydroxyl group of the aminocoumarin moiety occurs in simocyclinone and rubradirin biosynthesis (Scheme 1). In rubradirin, this hydroxyl group is methylated, but the responsible methyltransferase is unknown.

After the 5-*C*-methyl-L-rhamnosyl moiety has been transferred to the aglycone of novobiocin, clorobiocin and coumermycin, its 4-OH group is methylated by the SAM-dependent methyltransferases NovP, CloP and CouP, respectively. Inactivations of the genes *cloP* and *couP* resulted in the accumulation of clorobiocin and coumermycin derivatives which lacked the respective methyl groups.[55,56] NovP and CouP have been expressed and purified in *E. coli*, and their reactions have been characterized *in vitro*.[47,54,57] NovP has been crystallized, and a preliminary structural elucidation by X-ray crystallography has been reported.[58]

6 Acylation of the deoxysugar moieties of novobiocin, clorobiocin and coumermycin

The final reaction of novobiocin biosynthesis is the carbamoylation of the 3-OH group of the deoxysugar moiety. This reaction is catalyzed by NovN, which has been heterologously expressed and purified in *Streptomyces lividans*[59] and in *E. coli*.[57] The enzyme uses carbamoyl phosphate as the carbamoyl donor, and in contrast to most other carbamoyl transferases requires ATP for its reaction. NovN has been crystallized,[60] but its structure has not been reported yet. It has been used for the generation of new aminocoumarin antibiotics *in vitro* and *in vivo*.[47,59,61]

Also, in clorobiocin and coumermycin A_1, the 3-OH group of the deoxysugar moiety is acylated, but not with a carbamoyl but with a 5-methyl-pyrrole-carboxyl moiety (Scheme 1). This moiety is derived from L-proline, which is activated by acyl adenylate formation under catalysis of CloN4 or CouN4 in clorobiocin or coumermycin biosynthesis, respectively (Scheme 5). It is subsequently transferred by CloN4/CouN4 onto the phosphopantetheinyl cofactor of the small acyl carrier protein CloN5/CouN5 and oxidized to a pyrrole derivative by



Scheme 5 Biosynthesis and transfer of the 5-methyl-pyrrole-2-carboxyl moiety of clorobiocin.

the flavoprotein CloN3/CouN3. These reactions have been demonstrated *in vitro*.[62,63] Inactivation of the gene *cloN2*, which showed sequence similarity to acyl transferases, in a clorobiocin producer strain led to the accumulation of free pyrrole-2-carboxylic acid and of a clorobiocin derivative lacking the 5-methyl-pyrrole-2-carboxyl moiety.[64] Furthermore, inactivation of the putative methyltransferase gene *cloN6* led to accumulation of a clorobiocin derivative lacking the 5-methyl group at the pyrrole-2-carboxyl moiety.[65] This suggested that CloN2 might catalyze the transfer of the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5 to the 3-OH group of the deoxysugar, and that subsequent methylation of C-5 of the pyrrole moiety might be the last step of clorobiocin biosynthesis.

However, detailed genetic and biochemical investigations revealed that the true reaction sequence is more complicated. Unexpectedly, inactivations of the gene *cloN1*, coding for a small 95 aa protein with no sequence similarity to known genes from the database, as well as inactivation of *cloN7*, coding for a protein with sequence similarity to the α/β hydrolase family, both resulted in the accumulation of free pyrrole-2-carboxylic acid and of a clorobiocin derivative lacking the 5-methyl-pyrrole-2-carboxyl moiety, *i.e.* to the same phenotype as produced by inactivation of *cloN2*.[66] The logical explanation was that CloN1, CloN2 and CloN7 were all required for the transfer of the pyrrole-2-carboxyl group from CloN5 to the deoxysugar. Closer inspection of the sequence of CloN1 revealed that it showed an Asp-Ser-Leu motif at the second of four predicted α-helices, reminiscent of a phosphopantetheinyl attachment site of an acyl carrier protein. This, and a study of the reported functions of thioesterases with sequence similarity to CloN7 in the biosynthesis of other antibiotics, led to the suggestion that the transfer of the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5 to the 3-OH group of the deoxysugar is a two-step process:[66]

1. Transfer of the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5 to a second acyl carrier protein, CloN1, catalyzed by the acyl transferase CloN2.

2. Transfer of the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN1 to the 3-OH group of the deoxysugar moiety of clorobiocin, catalyzed by the acyl transferase CloN7.

Biochemical evidence for this hypothesis was provided by Walsh and co-workers.[67] They proved that CouN1, orthologous to CloN1, is indeed an acyl carrier protein that can covalently bind the pyrrole-2-carboxyl moiety *via* a phosphopantetheinyl cofactor, and that CouN7, orthologous to CloN7, can catalyze the transfer of the pyrrole-2-carboxyl moiety to descarbamoylnovobiocin, which was used as an analog of the genuine coumermycin precursor. These authors also postulated that CouN2 catalyzes the transfer of the pyrrole-2-carboxyl moiety from CouN5 to CouN1, but this reaction has not yet been shown *in vitro*. In further enzymatic studies, they proved that during the transfer from CouN1 to the 3-OH group of the deoxysugar moiety of clorobiocin, the pyrrole-2-carboxyl group binds covalently to the hydroxyl group of Ser101 of the CouN7 protein.[68] The reversibility of this reaction allowed the transfer of the pyrrole-2-carboxyl group between different aminocoumarin scaffolds using CouN7 as catalyst.[68]

The 5-methylation of the pyrrole-2-carboxyl moiety has not yet been demonstrated *in vitro*, and its precise substrate is unknown. Biochemical investigations did not allow the determination of whether pyrrole-2-carboxyl-CouN1 or rather its 5-methylated form is the preferred substrate of CouN7.[67] As mentioned, inactivation of *cloN2* led to the accumulation of free pyrrole-2-carboxylic acid and of a clorobiocin derivative lacking the 5-methyl-pyrrole-2-carboxyl moiety.[64] Feeding of a synthetic analog of pyrrole-2-carboxyl-CloN1, *i.e.* pyrrole-2-carboxyl-*S*-(*N*-acetyl-)cysteamine, led to the formation not of clorobiocin but of a clorobiocin derivative lacking the methyl group at C-5 of the pyrrole moiety.[69] The fact that this compound was not methylated by the strain, despite the presence of an intact *cloN6* gene, disproved the earlier hypothesis[65] that the methyltransferase CloN6 utilizes 5‴-desmethyl-clorobiocin as a substrate. The obvious alternative would be that CloN6 utilizes the intermediate pyrrole-2-carboxyl-CloN1, *i.e.* that the pyrrole-2-carboxyl moiety is first methylated and then transferred to the deoxysugar. However, in a mutant strain defective in the gene *cloN7* (which codes for the enzyme which catalyzes the acyl transfer to the deoxysugar), only the non-methylated pyrrole-2-carboxylic acid but no trace of 5-methyl-pyrrole-2-carboxylic acid was found, even using sensitive LC-MS techniques (selective reaction monitoring). Therefore, the 5-methylation and the transfer of the pyrrole-2-carboxyl moiety may occur simultaneously, requiring the interaction of CloN1, CloN6 and CloN7.[69]

7 Regulation of aminocoumarin biosynthesis

The gene clusters of novobiocin, clorobiocin and coumermycin A_1 each contain a regulatory gene termed *novG*, *cloG* or *couG*, respectively, which shows sequence similarity to *strR*, a positive regulator of streptomycin biosynthesis.[70] Electrophoretic mobility shift assays have shown that NovG binds to a well-conserved inverted repeat located in the intergenic region between *novG* and *novH*.[71] This sequence is similar to the StrR binding sites in the streptomycin cluster, and to the binding sites of the closely related Bbr in the balhimycin cluster.[72] These data suggested that NovG and its orthologs CloG and CouG act as positive regulators of the transcription of the genes located downstream of their respective binding sites.

The novobiocin gene cluster contains, besides *novG*, only one additional regulatory gene, *i.e. novE*. Orthologs of *novE* are found in the biosynthetic gene clusters of clorobiocin, coumermycin A_1, rubradirin and lincomycin (*cloE*, *couE*, *rubC4* and *lmbU*, respectively), and further orthologs have been identified in other bacterial genomes. Inactivation of *novE* in a heterologously expressed novobiocin cluster led to a strong reduction in novobiocin formation, and overexpression of *novE* led to a two-fold increase in production. Notably, inactivation of *novE* could be complemented by overexpression of *novG*.[73] This suggested a role of *novE* as a positive regulator of novobiocin biosynthesis. However, no DNA-binding activity of the NovE protein to the DNA region upstream of *novG* was found in electrophoretic mobility shift assays.[73]

No ortholog of *novG* is found in the biosynthetic gene clusters or simocyclinone and rubradirin. The rubradirin cluster, but not the simocyclinone cluster, contains an ortholog of *novE*, termed *rubC4*, and both clusters contain additional regulators. However, no experimental data are available on the regulation of gene expression in these two gene clusters.

8 Resistance genes in the aminocoumarin biosynthetic gene clusters

Novobiocin is a well-established inhibitor of bacterial gyrase and interacts with the B subunit of the heterotetrameric $(GyrA)_2(GyrB)_2$ gyrase holoenzyme. The novobiocin producer must protect its own gyrase from the inhibitory effect of the antibiotic. Already before the novobiocin biosynthetic gene cluster was cloned, Thiara and Cundliffe[74–76] reported that the principal resistance mechanism of the novobiocin producer strain is the *de novo* synthesis of an aminocoumarin-resistant gyrase B subunit, which replaces the sensitive GyrB subunit in the active $(GyrA)_2(GyrB)_2$ heterotetramer. They cloned the promoter region of $gyrB^R$ into a promoter probe vector and showed, by expression in *Streptomyces lividans* TK24, that the promoter was induced by cultivation in the presence of novobiocin. The authors suggested that the induction of $gyrB^R$ is mediated by the change of superhelical density of chromosomal DNA (*i.e.* loss of negative supercoils), caused by the gyrase inhibitor novobiocin.[75] Besides the resistance gene $gyrB^R$, the novobiocin producer strain was shown to contain a constitutively expressed gene $gyrB^S$, encoding the aminocoumarin-sensitive gyrase B subunit which is expressed under normal growth conditions, *i.e.* when no novobiocin is formed.

Cloning and sequencing of the novobiocin biosynthetic gene cluster[1] showed that the $gyrB^R$ resistance gene is located at the border of this cluster, while the $gyrB^S$ gene is encoded elsewhere in the genome. Orthologs of the $gyrB^R$ resistance gene are present in the clusters of clorobiocin and coumermycin.[77] Expression, purification and biochemical investigation of $gyrB^R$ from the coumermycin cluster proved that it indeed encodes an aminocoumarin-resistant GyrB subunit.[78]

The biosynthetic gene clusters of clorobiocin and coumermycin, but not of novobiocin, were found to contain an additional gene, $parY^R$, which was located immediately downstream of $gyrB^R$ and showed sequence similarity to genes coding for gyrase B subunits.[77] Upon expression in *Streptomyces lividans*, both $gyrB^R$ and $parY^R$ conferred resistance to aminocoumarin antibiotics. Expression, purification and biochemical investigation of $parY^R$ from the coumermycin cluster proved that it encodes an aminocoumarin-resistant topoisomerase IV subunit.[78] Gyrase as well as topoisomerase IV are type II topoisomerases, involved in controlling the topological state of bacterial DNA by an ATP-dependent reaction mechanism.[79] Topoisomerase IV is composed of the subunits ParC and ParE, which form a $(ParC)_2(ParE)_2$ heterotetrameric holoenzyme. The function of topoisomerase IV is the decatenation of daughter chromosomes following DNA replication, and the relaxation of superhelical DNA. Most, but not all bacteria possess both these type II topoisomerases. The investigation of the resistance genes of the clorobiocin and the coumermycin cluster provided the first evidence for the existence of a separate topoisomerase IV in the class of actinobacteria.

The simocyclinone cluster contains no resistance genes related to gyrase or topoisomerase IV, but two putative transporter genes which may be involved in antibiotic export.[5] One of them, termed *simX*, and the neighbouring, divergently transcribed gene *simR* resemble the TetR/TetA repressor–efflux pump pair that causes resistance to tetracyclines in many clinical isolates of pathogenic strains. Recently, *simX* was expressed from a strong, heterologous promoter in *Streptomyces lividans*, and this conferred high level simocyclinone D8 resistance.[80] Therefore, *simX* indeed codes for a simocyclinone efflux pump. The repressor protein SimR binds to two operator sites in the *simX–simR* intergenic region. Binding of simocyclinone D8 leads to release of SimR from its binding site, providing a mechanism that couples the biosynthesis of simocyclinone to its export.

The rubradirin cluster contains a putative ABC transporter gene, *rubT1*, and several other genes for which a function as resistance genes appears possible,[6] but no experimental data have been provided yet.

9 Targets of the aminocoumarin antibiotics

Novobiocin, clorobiocin and coumermycin A_1 are inhibitors of bacterial gyrase, a well-validated drug target.[79] X-ray crystallographic analysis showed that both the aminocoumarin and the substituted deoxysugar moieties are involved in binding to the B subunit of DNA gyrase.[81,82] The carbamoyl group of novobiocin and the 5-methylpyrrole-2-carboxyl moiety of clorobiocin are important for the hydrogen bonding network between the antibiotic and the GyrB subunit. The binding site of the aminocoumarin antibiotics overlaps with the binding site of ATP, and the aminocoumarins competitively inhibit the ATP-dependent supercoiling of DNA, which is a key function of bacterial gyrase. The prenylated 4-hydroxybenzoyl moiety may facilitate the uptake of these antibiotics across the bacterial membrane,[83] and possibly contribute to the binding of the antibiotic to gyrase.[84] The coumermycin A_1 molecule has been shown to stabilize a dimer form of the 43 kDa fragment of GyrB.[85,86]

The affinity of the aminocoumarin antibiotics to bacterial gyrase is extremely high. The equilibrium dissociation constants (KDs) are in the 10 nM range.[79] Novobiocin (Albamycin®) has been licensed as a drug for the treatment of bacterial infections in humans. However, due to their toxicity in eukaryotes, their poor solubility in water, and their low activity against Gram-negative bacteria, clinical use of the aminocoumarin antibiotics remains restricted.

Topoisomerase IV is an additional target of some aminocoumarin antibiotics.[79] A comparison of the inhibition of gyrase and topoisomerase IV by different aminocoumarin antibiotics showed that clorobiocin, but not novobiocin, is a potent inhibitor of topoisomerase IV.[87] Clorobiocin shows not only 10-fold higher gyrase-inhibitory activity than novobiocin but also 70-fold-higher topoisomerase IV inhibition. The biological significance of the topoisomerase IV inhibition is underlined by the above-mentioned fact that the clorobiocin producer strain, but not the novobiocin producer strain, contains a gene for an aminocoumarin-resistant topoisomerase IV subunit. Since clorobiocin attacks two distinct targets, gyrase and topoisomerase IV, the development of resistance against clorobiocin is expected to proceed less readily than development of resistance against novobiocin, which has been a limiting factor in the clinical use of the latter antibiotic.

Simocyclinone and rubradirin do not carry deoxysugar moieties at the 7-OH group of the aminocoumarin moiety and therefore cannot interact with the well-characterized binding site for novobiocin, clorobiocin and coumermycin located on the B

subunit of gyrase. Surprisingly, however, it was found that simocyclinone D8 is nevertheless a potent inhibitor of the gyrase-catalyzed supercoiling reaction, with a 50% inhibitory concentration lower than that of novobiocin.[88,89] However, it does not competitively inhibit the DNA-independent ATPase reaction of GyrB, which is characteristic of other aminocoumarins. Binding studies suggest that simocyclinone D8 interacts with the N-terminal domain of GyrA. This finding represented a novel mechanism for a gyrase inhibitor and presents new possibilities for antibacterial drug development.[88]

No inhibitory effect on gyrase or topoisomerase IV has been reported for rubradirin. The polyketide moiety of rubradirin is structurally related to the ansamycin family of antibiotics. The most prominent member of this family is rifamycin, precursor of the semisynthetic antibacterial drug rifampicin, a specific inhibitor of bacterial RNA polymerase. Likewise, the aglycone of rubradirin is a potent inhibitor of RNA polymerase.[90] However, the glycosylated form of rubradirin does not inhibit RNA polymerase, but inhibits the peptide chain initiation process in protein biosynthesis at the ribosomes.[90]

10 Conclusions

Few antibiotics have been as thoroughly examined for their biosynthesis and biology as the "classical" aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1. In the biosynthetic gene clusters of novobiocin and clorobiocin, the precise function of nearly every gene has been established by genetic and biochemical investigations. The molecular basis of the interaction of these compounds with their principal target, DNA gyrase, has been clarified by X-ray crystallography. This provides an attractive basis for the generation of new and possibly improved aminocoumarin antibiotics by metabolic engineering, mutasynthesis and chemoenzymatic synthesis, and considerable progress has already been achieved along this road.[91]

The unexpected finding that the aminocoumarin antibiotic simocyclinone D8 is a potent gyrase inhibitor with a different mode of action opens further possibilities for antibacterial drug development. It remains to be shown whether the common biological function of simocyclinone and the classical aminocoumarins, *i.e.* inhibition of gyrase, is a coincidence or the result of the evolutionary relationship of these antibiotics.

11 Acknowledgements

The author gratefully acknowledges the contributions of many colleagues, co-workers, and collaboration partners, especially of S.-M. Li, B. Gust and B. Kammerer. Parts of the work conducted in the author's laboratory were supported by grants from the Deutsche Forschungsgemeinschaft (SFB 766) and from the European Community (IP 005244 ActinoGEN).

12 References

1 M. Steffensky, A. Mühlenweg, Z. X. Wang, S. M. Li and L. Heide, *Antimicrob. Agents Chemother.*, 2000, **44**, 1214–1222.
2 Z. X. Wang, S. M. Li and L. Heide, *Antimicrob. Agents Chemother.*, 2000, **44**, 3040–3048.
3 F. Pojer, S. M. Li and L. Heide, *Microbiology*, 2002, **148**, 3901–3911.
4 U. Galm, J. Schimana, H. P. Fiedler, J. Schmidt, S. M. Li and L. Heide, *Arch. Microbiol.*, 2002, **178**, 102–114.
5 A. Trefzer, S. Pelzer, J. Schimana, S. Stockert, C. Bihlmaier, H. P. Fiedler, K. Welzel, A. Vente and A. Bechthold, *Antimicrob. Agents Chemother.*, 2002, **46**, 1174–1182.
6 C. G. Kim, J. Lamichhane, K. I. Song, V. D. Nguyen, D. H. Kim, T. S. Jeong, S. H. Kang, K. W. Kim, J. Maharjan, Y. S. Hong, J. S. Kang, J. C. Yoo, J. J. Lee, T. J. Oh, K. Liou and J. K. Sohng, *Arch. Microbiol.*, 2008, **189**, 463–473.
7 C. A. Bunton, G. W. Kenner, M. J. T. Robinson and B. R. Webster, *Tetrahedron*, 1963, **19**, 1001–1010.
8 H. Chen and C. T. Walsh, *Chem. Biol.*, 2001, **8**, 301–312.
9 M. Pacholec, N. J. Hillson and C. T. Walsh, *Biochemistry*, 2005, **44**, 12819–12826.
10 A. S. Eustáquio, B. Gust, U. Galm, S. M. Li, K. F. Chater and L. Heide, *Appl. Environ. Microbiol.*, 2005, **71**, 2452–2459.
11 M. Holzenkämpfer and A. Zeeck, *J. Antibiot. (Tokyo)*, 2002, **55**, 341–342.
12 M. Wolpert, B. Gust, B. Kammerer and L. Heide, *Microbiology*, 2007, **153**, 1413–1423.
13 M. Pacholec, J. Tao and C. T. Walsh, *Biochemistry*, 2005, **44**, 14969–14976.
14 A. S. Eustáquio, B. Gust, T. Luft, S. M. Li, K. F. Chater and L. Heide, *Chem. Biol.*, 2003, **10**, 279–288.
15 A. S. Eustáquio, B. Gust, S. M. Li, S. Pelzer, W. Wohlleben, K. F. Chater and L. Heide, *Chem. Biol.*, 2004, **11**, 1561–1572.
16 C. Anderle, S. Hennig, B. Kammerer, S. M. Li, L. Wessjohann, B. Gust and L. Heide, *Chem. Biol.*, 2007, **14**, 955–967.
17 M. Melzer and L. Heide, *Biochim. Biophys. Acta*, 1994, **1212**, 93–102.
18 K. Ohara, K. Yamamoto, M. Hamamoto, K. Sasaki and K. Yazaki, *Plant Cell Physiol.*, 2006, **47**, 581–590.
19 K. Ohara, A. Muroya, N. Fukushima and K. Yazaki, *Biochem. J.*, 2009, **421**, 231–241.
20 F. Pojer, E. Wemakor, B. Kammerer, H. Chen, C. T. Walsh, S. M. Li and L. Heide, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 2316–2321.
21 M. Tello, T. Kuzuyama, L. Heide, J. P. Noel and S. B. Richard, *Cell. Mol. Life Sci.*, 2008, **65**, 1459–1463.
22 L. Heide, *Curr. Opin. Chem. Biol.*, 2009, **13**, 171–179.
23 T. Kuzuyama, J. P. Noel and S. B. Richard, *Nature*, 2005, **435**, 983–987.
24 S. Keller, F. Pojer, L. Heide and D. M. Lawson, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2006, **62**, 1153–1155.
25 M. Steffensky, S. M. Li, B. Vogler and L. Heide, *FEMS Microbiol. Lett.*, 1998, **161**, 69–74.
26 S. M. Li, S. Hennig and L. Heide, *Tetrahedron Lett.*, 1998, **39**, 2717–2720.
27 N. Orihara, T. Kuzuyama, S. Takahashi, K. Furihata and H. Seto, *J. Antibiot. (Tokyo)*, 1998, **51**, 676–678.
28 F. Pojer, R. Kahlich, B. Kammerer, S. M. Li and L. Heide, *J. Biol. Chem.*, 2003, **278**, 30661–30668.
29 G. R. Moran, *Arch. Biochem. Biophys.*, 2005, **433**, 117–128.
30 T. Ozaki, S. Mishima, M. Nishiyama and T. Kuzuyama, *J. Antibiot. (Tokyo)*, 2009, **62**, 385–392.
31 S. Keller, F. Pojer, L. Heide and D. M. Lawson, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2006, **62**, 1564–1570.
32 S. Pelzer, R. Süßmuth, D. Heckmann, J. Recktenwald, P. Huber, G. Jung and W. Wohlleben, *Antimicrob. Agents Chemother.*, 1999, **43**, 1565–1573.
33 A. Trefzer, J. A. Salas and A. Bechthold, *Nat. Prod. Rep.*, 1999, **16**, 283–299.
34 A. J. Birch, D. W. Cameron, P. W. Holloway and R. W. Rickards, *Tetrahedron Lett.*, 1960, 26–31.
35 T. T. T. Thuy, H. C. Lee, C. G. Kim, L. Heide and J. K. Sohng, *Arch. Biochem. Biophys.*, 2005, **436**, 161–167.
36 I. Aguirrezabalaga, C. Olano, N. Allende, L. Rodriguez, A. F. Brana, C. Mendez and J. A. Salas, *Antimicrob. Agents Chemother.*, 2000, **44**, 1266–1275.
37 P. Jakimowicz, M. Tello, C. L. Meyers, C. T. Walsh, M. J. Buttner, R. A. Field and D. M. Lawson, *Proteins: Struct., Funct., Bioinf.*, 2006, **63**, 261–265.
38 M. Tello, P. Jakimowicz, J. C. Errey, C. L. Freel Meyers, C. T. Walsh, M. J. Buttner, D. M. Lawson and R. A. Field, *Chem. Commun.*, 2006, 1079–1081.
39 A. Freitag, S. M. Li and L. Heide, *Microbiology*, 2006, **152**, 2433–2442.

40 T. Sasaki, Y. Igarashi, N. Saito and T. Furumai, *J. Antibiot. (Tokyo)*, 2001, **54**, 441–447.
41 A. Freitag, C. Méndez, J. A. Salas, B. Kammerer, S. M. Li and L. Heide, *Metab. Eng.*, 2006, **8**, 653–661.
42 L. Rodríguez, I. Aguirrezabalaga, N. Allende, A. F. Brana, C. Méndez and J. A. Salas, *Chem. Biol.*, 2002, **9**, 721–729.
43 B. A. Wolucka and M. Van Montagu, *J. Biol. Chem.*, 2003, **278**, 47483–47490.
44 M. Steffensky, S. M. Li and L. Heide, *J. Biol. Chem.*, 2000, **275**, 21754–21760.
45 U. Galm, M. A. Dessoy, J. Schmidt, L. A. Wessjohann and L. Heide, *Chem. Biol.*, 2004, **11**, 173–183.
46 E. Schmutz, M. Steffensky, J. Schmidt, A. Porzel, S. M. Li and L. Heide, *Eur. J. Biochem.*, 2003, **270**, 4413–4419.
47 C. L. Freel Meyers, M. Oberthür, L. Heide, D. Kahne and C. T. Walsh, *Biochemistry*, 2004, **43**, 15022–15036.
48 T. Luft, S. M. Li, H. Scheible, B. Kammerer and L. Heide, *Arch. Microbiol.*, 2005, **183**, 277–285.
49 M. Pacholec, C. L. Freel Meyers, M. Oberthur, D. Kahne and C. T. Walsh, *Biochemistry*, 2005, **44**, 4949–4956.
50 E. S. Sattely, M. A. Fischbach and C. T. Walsh, *Nat. Prod. Rep.*, 2008, **25**, 757–793.
51 R. Finking and M. A. Marahiel, *Annu. Rev. Microbiol.*, 2004, **58**, 453–488.
52 C. L. Freel Meyers, M. Oberthür, J. W. Anderson, D. Kahne and C. T. Walsh, *Biochemistry*, 2003, **42**, 4179–4189.
53 C. Albermann, A. Soriano, J. Jiang, H. Vollmer, J. B. Biggins, W. A. Barton, J. Lesniak, D. B. Nikolov and J. S. Thorson, *Org. Lett.*, 2003, **5**, 933–936.
54 N. Pi, C. L. Meyers, M. Pacholec, C. T. Walsh and J. A. Leary, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 10036–10041.
55 A. Freitag, H. Rapp, L. Heide and S. M. Li, *ChemBioChem*, 2005, **6**, 1411–1418.
56 S. M. Li, L. Westrich, J. Schmidt, C. Kuhnt and L. Heide, *Microbiology*, 2002, **148**, 3317–3326.
57 C. L. Freel Meyers, M. Oberthür, H. Xu, L. Heide, D. Kahne and C. T. Walsh, *Angew. Chem., Int. Ed.*, 2004, **43**, 67–70.
58 I. Usón, C. E. Stevenson, D. M. Lawson and G. M. Sheldrick, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2007, **63**, 1069–1074.
59 H. Xu, L. Heide and S. M. Li, *Chem. Biol.*, 2004, **11**, 655–662.
60 I. Gómez Garcia, C. L. Freel Meyers, C. T. Walsh and D. M. Lawson, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2008, **64**, 1000–1002.
61 H. Xu, Z. X. Wang, J. Schmidt, L. Heide and S. M. Li, *Mol. Genet. Genomics*, 2002, **268**, 387–396.
62 C. T. Walsh, S. Garneau-Tsodikova and A. R. Howard-Jones, *Nat. Prod. Rep.*, 2006, **23**, 517–531.
63 S. Garneau, P. C. Dorrestein, N. L. Kelleher and C. T. Walsh, *Biochemistry*, 2005, **44**, 2770–2780.
64 H. Xu, R. Kahlich, B. Kammerer, L. Heide and S. M. Li, *Microbiology*, 2003, **149**, 2183–2191.
65 L. Westrich, L. Heide and S. M. Li, *ChemBioChem*, 2003, **4**, 768–773.
66 A. Freitag, E. Wemakor, S. M. Li and L. Heide, *ChemBioChem*, 2005, **6**, 2316–2325.
67 S. Garneau-Tsodikova, A. Stapon, D. Kahne and C. T. Walsh, *Biochemistry*, 2006, **45**, 8568–8578.
68 C. J. Balibar, S. Garneau-Tsodikova and C. T. Walsh, *Chem. Biol.*, 2007, **14**, 679–690.
69 C. Anderle, S. Alt, T. Gulder, G. Bringmann, B. Kammerer, B. Gust and L. Heide, *Arch. Microbiol.*, 2007, **187**, 227–237.
70 A. Tomono, Y. Tsai, H. Yamazaki, Y. Ohnishi and S. Horinouchi, *J. Bacteriol.*, 2005, **187**, 5595–5604.
71 A. S. Eustáquio, S. M. Li and L. Heide, *Microbiology*, 2005, **151**, 1949–1961.
72 R. M. Shawky, O. Puk, A. Wietzorrek, S. Pelzer, E. Takano, W. Wohlleben and E. Stegmann, *J. Mol. Microbiol. Biotechnol.*, 2007, **13**, 76–88.
73 V. Dangel, A. S. Eustáquio, B. Gust and L. Heide, *Arch. Microbiol.*, 2008, **190**, 509–519.
74 A. S. Thiara and E. Cundliffe, *EMBO J.*, 1988, **7**, 2255–2259.
75 A. S. Thiara and E. Cundliffe, *Gene*, 1989, **81**, 65–72.
76 A. S. Thiara and E. Cundliffe, *Mol. Microbiol.*, 1993, **8**, 495–506.
77 E. Schmutz, A. Mühlenweg, S. M. Li and L. Heide, *Antimicrob. Agents Chemother.*, 2003, **47**, 869–877.
78 E. Schmutz, S. Hennig, S. M. Li and L. Heide, *Microbiology*, 2004, **150**, 641–647.
79 A. Maxwell and D. M. Lawson, *Curr. Top. Med. Chem.*, 2003, **3**, 283–303.
80 T. B. Le, H. P. Fiedler, C. D. den Hengst, S. K. Ahn, A. Maxwell and M. J. Buttner, *Mol. Microbiol.*, 2009, **72**, 1462–1474.
81 R. J. Lewis, O. M. P. Singh, C. V. Smith, T. Skarzynski, A. Maxwell, A. J. Wonacott and D. B. Wigley, *EMBO J.*, 1996, **15**, 1412–1420.
82 F. T. F. Tsai, O. M. Singh, T. Skarzynski, A. J. Wonacott, S. Weston, A. Tucker, R. A. Pauptit, A. L. Breeze, J. P. Poyser, R. O'Brien, J. E. Ladbury and D. B. Wigley, *Proteins*, 1997, **28**, 41–52.
83 A. Maxwell, *Biochem. Soc. Trans.*, 1999, **27**, 48–53.
84 D. Lafitte, V. Lamour, P. O. Tsvetkov, A. A. Makarov, M. Klich, P. Deprez, D. Moras, C. Briand and R. Gilli, *Biochemistry*, 2002, **41**, 7217–7223.
85 J. A. Ali, A. P. Jackson, A. J. Howells and A. Maxwell, *Biochemistry*, 1993, **32**, 2717–2724.
86 N. A. Gormley, G. Orphanides, A. Meyer, P. M. Cullis and A. Maxwell, *Biochemistry*, 1996, **35**, 5083–5092.
87 R. H. Flatman, A. Eustáquio, S. M. Li, L. Heide and A. Maxwell, *Antimicrob. Agents Chemother.*, 2006, **50**, 1136–1142.
88 R. H. Flatman, A. J. Howells, L. Heide, H. P. Fiedler and A. Maxwell, *Antimicrob. Agents Chemother.*, 2005, **49**, 1093–1100.
89 L. M. Oppegard, B. L. Hamann, K. R. Streck, K. C. Ellis, H. P. Fiedler, A. B. Khodursky and H. Hiasa, *Antimicrob. Agents Chemother.*, 2009, **53**, 2110–2119.
90 F. Reusser, *J. Antibiot. (Tokyo)*, 1979, **32**, 1186–1192.
91 L. Heide, B. Gust, C. Anderle and S. M. Li, *Curr. Top. Med. Chem.*, 2008, **8**, 667–679.