Abstract

Plants and microorganisms are the most important sources of secondary metabolites in nature. For research in the functional genomics of secondary metabolism, and for the biotechnological application of such research by genetic engineering and combinatorial biosynthesis, most microorganisms offer a unique advantage to the researcher: the biosynthetic genes for a specific secondary metabolite are not scattered over the genome, but rather are clustered in a well-defined, contiguous region – the biosynthetic gene cluster of that metabolite. This is exemplified in this review for the biosynthetic gene clusters of the aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁, which are potent inhibitors of DNA gyrase. Cloning, sequencing and analysis of the biosynthetic gene clusters of these three anti-

biotics revealed that the structural differences and similarities of the compounds are perfectly reflected by the genetic organisation of the biosynthetic gene clusters. The function of most biosynthetic genes could be identified by gene inactivation experiments as well as by heterologous expression and biochemical investigation. The prenylated benzoic acid moiety of novobiocin and clorobiocin, involved in the interaction with gyrase, is structurally similar to metabolites found in plants. However, detailed investigations of the biosynthesis revealed that the biosynthetic pathway and the enzymes involved are totally different from those identified in plants.

Key words

Antibiotics · aminocoumarins · biosynthesis · gene clusters · gyrase inhibitors · *Streptomyces*

Chemical Structures of the Aminocoumarin Antibiotics

The aminocoumarin antibiotics comprise three classical compounds, i.e., novobiocin, clorobiocin and coumermycin A₁ (Fig. 1). They are potent inhibitors of gyrase, competing with ATP for binding to the B subunit of DNA gyrase and thereby inhibiting the ATP-dependent DNA supercoiling catalysed by gyrase [1]. All three are produced by different *Streptomyces* strains [2], [3], [4].

The novobiocin molecule comprises three structural moieties: the deoxy sugar noviose, a substituted 3-aminocoumarin moiety and a 3-prenylated 4-hydroxybenzoate moiety. Noviose is related to L-rhamnose, but shows an unusual 5,5-dimethyl structure and carries a carbamyl group at 3-OH and a methyl group at 4-OH. The aminocoumarin ring is substituted with hydroxy groups in positions 3 and 7. Novobiocin and coumermycin A₁ carry a methyl group at position 8 of the aminocoumarin unit, while clorobiocin (also called chlorobiocin) is chlorinated in this position. In clorobiocin and coumermycin A₁, the 3-OH of the deoxy sugar is

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Dedication

In memory of Prof. Dr. Ernst Reinhard.

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Fig. 1 **A** Structures of aminocoumarin antibiotics. **B** Organisation of the biosynthetic gene clusters of novobiocin (*nov*), clorobiocin (*clo*) and coumermycin A₁ (*cou*).

connected with a 5-methylpyrrole-2-carboxyl moiety rather than with a carbamyl group. While clorobiocin contains the same prenylated 4-hydroxybenzoate moiety as novobiocin, the coumermycin A₁ molecule contains a central 3-methylpyrrole-2,4-dicarboxylic acid moiety, which links two aminocoumarin-deoxy sugar assemblies in a nearly but not completely symmetrical fashion.

The affinity of the aminocoumarin antibiotics to bacterial gyrase is extremely high, with equilibrium dissociation constants in the 10 nM range [1], i.e., much lower than those of modern fluoroquinolones.

The Biosynthetic Gene Clusters

In the last few years, we have been able to clone and sequence the entire biosynthetic gene clusters of novobiocin [2], coumermycin A₁ [3] and clorobiocin [4].

As shown in Fig. 1, the structural differences and similarities between the three antibiotics were reflected by differences and similarities in the organisation of the biosynthetic gene clusters, respectively. The functions of the genes identified in the clusters are summarised in Table 1. The biosynthetic pathway of clorobiocin is illustrated in Fig. 2.

Genes Responsible for the Biosynthesis of the Aminocoumarin Moiety

Feeding experiment in the 1970s showed that the aminocoumarin ring is derived from tyrosine [5]. The sequencing of the biosynthetic gene clusters revealed a contiguous group of four genes, i.e., *novHIJK*, *cloHIJK* and *couHIJK*, found in the clusters of novobiocin, clorobiocin and coumermycin A₁, respectively. We speculated [3] that tyrosine is activated by Nov/Clo/CouH, which show similarity to acyl-CoA ligases, and subsequently hydroxylated in the β -position by Nov/Clo/CouI, which show similarity to

Table 1 Genes identified in the biosynthetic gene clusters of novobiocin, clorobiocin and coumermycin A₁ (numbers in parentheses show the number of amino acids of the encoded protein)

<i>Novobiocin cluster</i>	<i>Clorobiocin cluster</i>	<i>Coumermycin A₁ cluster</i>	<i>Function of the encoded protein</i>	<i>Ref.</i>
<i>novE</i> (217)	<i>cloE</i> (217)	<i>couE</i> (217)	regulatory protein (similar to LmbU)	[39]
<i>novF</i> (362)	<i>cloF</i> (362)		prephenate dehydrogenase (?)	[17]
<i>novG</i> (318)	<i>cloG</i> (319)	<i>couG</i> (319)	regulatory protein (similar to StrR)	[37]
	<i>cloY</i> (71)	<i>couY</i> (71)	unknown (similar to MbtH)	–
<i>novH</i> (600)	<i>cloH</i> (600)	<i>couH</i> (599)	formation of L-tyrosyl-S-[enzyme] thioester	[6], [14]
<i>novI</i> (407)	<i>cloI</i> (407)	<i>coul</i> (407)	β -hydroxylation of L-tyrosyl-S-[enzyme] thioester	[6], [14]
<i>novJ</i> (262)	<i>cloJ</i> (258)	<i>couJ</i> (258)	oxidation of β -hydroxy-L-tyrosyl-S-[enzyme] thioester	[7]
<i>novK</i> (244)	<i>cloK</i> (245)	<i>couK</i> (245)	oxidation of β -hydroxy-L-tyrosyl-S-[enzyme] thioester	[7]
<i>novL</i> (527)	<i>cloL</i> (527)	<i>coul</i> (529)	amide synthetase	[24], [25], [26]
<i>novM</i> (379)	<i>cloM</i> (390)	<i>couM</i> (402)	glycosyltransferase	[27, 28]
<i>novN</i> (677)			carbamyltransferase	[29], [30]
	<i>cloN1</i> (95)	<i>couN1</i> (95)	acyl carrier protein	[31]
	<i>cloN2</i> (355)	<i>couN2</i> (355)	pyrrole-2-carboxyl transferase	[31]
	<i>cloN3</i> (376)	<i>couN3</i> (373)	oxidation of L-prolyl-S-[acyl carrier protein] thioester	[19], [20]
	<i>cloN4</i> (501)	<i>couN4</i> (501)	formation of L-prolyl-S-[acyl carrier protein] thioester	[20], [19]
	<i>cloN5</i> (89)	<i>couN5</i> (89)	acyl carrier protein	[31]
	<i>cloN6</i> (561)	<i>couN6</i> (560)	C-methyltransferase (methylating C-5'')	[33]
	<i>cloN7</i> (278)	<i>couN7</i> (281)	pyrrole-2-carboxyl transferase	[31]
	<i>clo-hal</i> (524)		halogenase (halogenating C-8')	[9]
<i>novO</i> (230)		<i>couO</i> (230)	C-methyltransferase (methylating C-8')	[9], [10]
<i>novP</i> (262)	<i>cloP</i> (277)	<i>couP</i> (276)	O-methyltransferase (methylating O-4')	[29], [32]
<i>novQ</i> (323)	<i>cloQ</i> (324)		Prenyltransferase	[14]
<i>novR</i> (270)	<i>cloR</i> (277)		bifunctional non-haeme iron(II)-dependent oxygenase	[17]
		<i>couR1</i> (474)	? biosynthesis of central pyrrole unit of coumermycin	–
		<i>couR2</i> (377)	? biosynthesis of central pyrrole unit of coumermycin	–
		<i>couR3</i> (302)	? biosynthesis of central pyrrole unit of coumermycin	–
		<i>couR4</i> (389)	? biosynthesis of central pyrrole unit of coumermycin	–
		<i>couR5</i> (491)	? transport protein	–
		<i>couR6</i> (290)	? regulator of <i>couR5</i> expression	–
<i>novS</i> (288)	<i>cloS</i> (288)	<i>couS</i> (288)	dTDP-4-keto-5-methyl-L-rhamnose 4-ketoreductase	[21]
<i>novT</i> (336)	<i>cloT</i> (336)	<i>couT</i> (336)	dTDP-glucose 4,6-dehydratase	[2]
<i>novU</i> (420)	<i>cloU</i> (420)	<i>couU</i> (420)	dTDP-4-keto-L-rhamnose 5-C-methyltransferase	[21], [23]
<i>novV</i> (297)	<i>cloV</i> (296)	<i>couV</i> (296)	dTDP-glucose synthase	–
<i>novW</i> (207)	<i>cloW</i> (198)	<i>couW</i> (198)	dTDP-4-keto-6-deoxyglucose 3,5-epimerase	[21], [22]
	<i>cloZ</i> (253)		not essential for clorobiocin biosynthesis	[9]
<i>gyrB^R</i> (677)	<i>gyrB^R</i> (677)	<i>gyrB^R</i> (677)	aminocoumarin-resistant B subunit of gyrase	[35], [36]
	<i>parY^R</i> (702)	<i>parY^R</i> (702)	aminocoumarin-resistant B subunit of topoisomerase IV	[35], [36]

The novobiocin biosynthetic cluster of *Streptomyces spheroides* is available in GenBank under accession numbers AF170880 and AF205854, the clorobiocin biosynthetic cluster of *S. roseochromogenes* under accession numbers AF329398 and AY136281, and the coumermycin A₁ biosynthetic cluster of *S. rishiriensis* under accession numbers AF235050 and AF205853.

cytochrome P450 enzymes. Indeed, Chen and Walsh [6] proved this reaction sequence by biochemical experiments using purified NovH and NovI *in vitro*. Apparently, the products of the genes *nov/clo/couK* and *nov/clo/couJ* are involved in the oxidation of covalently enzyme-bound β -hydroxytyrosine to β -ketotyrosine [7]. The ring oxygen atom of the aminocoumarin unit of the antibiotic simocyclinone is derived from oxygen of the air [8], suggesting that a 2-hydroxylation takes place before lactonisation. However, the enzyme which introduces this 2-hydroxy group is still unknown, and this reaction presents one of the few remaining mysteries in aminocoumarin biosynthesis (Fig. 2).

Clorobiocin contains a chlorine atom at position 8 of the aminocoumarin ring, whereas novobiocin and coumermycin A₁ contain a methyl group at the same position (Fig. 1). This structural dif-

ference of the antibiotics is perfectly reflected in the gene clusters: the novobiocin and the coumermycin A₁ clusters contain a C-methyltransferase gene, *novO* or *couO*, respectively, whereas in the clorobiocin cluster, *clo-hal*, a gene with sequence similarity to FADH₂-dependent halogenases is found at the same relative position.

When *clo-hal* was inactivated in the clorobiocin producer *Streptomyces roseochromogenes*, an aminocoumarin derivative containing no chlorine atom was produced [9]. Expression of the methyltransferase *novO* in this mutant resulted in the accumulation of a 8'-methylated derivative [9]. Recently, biochemical experiments showed that methylation by NovO takes place after acylation of the amino group of the aminocoumarin moiety [10].

Fig. 2 Biosynthetic pathway of clorobiocin.

Genes Responsible for the Biosynthesis of the Prenylated 4-Hydroxybenzoate Moiety

Novobiocin and clorobiocin contain a prenylated 4-hydroxybenzoate moiety. A similar 3-prenylated 4-hydroxybenzoate moiety is found as intermediate in the biosynthesis of the plant metabolite shikonin [11]. In shikonin biosynthesis, the 4-hydroxybenzoate moiety is derived from *p*-coumaroyl-CoA in a reaction sequence similar to the β -oxidation of fatty acids [12]. 4-Hydroxybenzoate is then prenylated in the 3-position by a membrane-bound prenyltransferase similar to that involved in the biosynthesis of ubiquinones [13].

Prior to our work, it was reported that L-tyrosine was incorporated into the 4-hydroxybenzoic moiety of clorobiocin and novobiocin [5], but the exact reaction sequence was unknown. To our surprise, sequencing of the novobiocin and clorobiocin biosynthetic gene clusters revealed that neither genes with similarity to those involved in the β -oxidation of fatty acids nor genes with similarity to prenyltransferases were present, forcing us to draw a new hypothesis for the origin of the prenylated benzoic acid moiety.

This prenylated benzoic acid moiety is present in the structures of novobiocin and clorobiocin, but absent in coumermycin A₁. A comparison of the biosynthetic gene clusters of novobiocin, clorobiocin and coumermycin revealed that, correspondingly, three genes (*clo/novF*, *clo/novQ* and *clo/novR*) are present in the novobiocin and clorobiocin cluster but have no homologue in the coumermycin A₁ cluster. This fact led us to hypothesise that these genes may be involved in the biosynthesis of the prenylated benzoic acid moiety.

We subsequently provided experimental evidence for the involvement of *cloQ* and *cloR* in the biosynthesis of this moiety by inactivation of *cloQ* and *cloR* in the clorobiocin producer *Streptomyces roseochromogenes* [4], [14]. The production of clorobiocin was abolished in the *cloQ*- and *cloR*-defective mutants. However, feeding of 3-prenyl-4-hydroxybenzoic acid restored clorobiocin production in both mutants, demonstrating that CloQ and CloR are involved in the biosynthesis of that structural element.

CloQ was subsequently expressed in *E. coli*, purified and identified as a prenyltransferase which catalyses the transfer of a dimethylallyl moiety to position 3 of 4-hydroxyphenylpyruvate (Fig. 2) [14]. CloQ showed no homology to known proteins in the database, except to NovQ and a hypothetical protein of *Streptomyces coelicolor*. In contrast to the prenyltransferases involved in the biosynthesis of ubiquinones [13], menaquinones, tocopherols and plastoquinones, and to the prenyltransferase involved in formation of the plant secondary metabolite shikonin [11], CloQ is a soluble protein and does not contain the typical prenyl diphosphate binding motif (N/D)DxxD. The reaction catalysed by CloQ did not require magnesium or other divalent cations [14]. We therefore speculated that CloQ may represent the first member of an entirely new class of aromatic prenyltransferases. This has recently been confirmed by the identification and crystallisation of a similar prenyltransferase, involved in naphtherpine biosynthesis in another *Streptomyces* strain [15]. This enzyme showed a completely novel protein architecture,

characterised by a barrel with antiparallel β -strands. Modelling suggested the same protein architecture for CloQ, NovQ and the recently identified Fur7 of furaquinocin biosynthesis [16]. The term ABBA prenyltransferases is suggested for this enzyme class, due to the alpha-beta-beta-alpha architecture of the proteins.

CloR was also overexpressed in *E. coli*, purified and identified as a bifunctional non-haeme iron oxygenase, which converts 3-dimethylallyl-4-hydroxyphenylpyruvate via 3-dimethylallyl-4-hydroxymandelate to 3-dimethylallyl-4-hydroxybenzoate by two consecutive oxidative decarboxylation steps (Fig. 2) [17]. ¹⁸O₂ labelling experiments showed that both oxygen atoms of molecular oxygen are incorporated into the intermediate 3-dimethylallyl-4-hydroxymandelate in the first reaction step, but only one further oxygen is incorporated into the final product 3-dimethylallyl-4-hydroxybenzoate during the second reaction step [17]. CloR presents a novel member of the diverse family of the non-haeme iron(II)- and α -keto acid-dependent oxygenases, with 3-dimethylallyl-4-hydroxyphenylpyruvate functioning both as α -keto acid and as hydroxylation substrate. The reaction catalysed by CloR represents a new pathway to benzoic acids in nature, and is principally different from the biosynthesis of the 4-hydroxybenzoic acid moiety in the course of shikonin formation in the plant *Lithospermum erythrorhizon* [11].

NovF and CloF show sequence similarity to prephenate dehydrogenases and are likely to supply 4-hydroxyphenylpyruvate for the biosynthesis of the substituted benzoate moiety of novobiocin and clorobiocin (Fig. 2). The dimethylallyl moiety of novobiocin and clorobiocin is formed via the methylerythritol 4-phosphate pathway [18].

Genes Involved in the Biosynthesis of the Pyrrole Moieties

Clorobiocin and coumermycin A₁ contain a terminal 5-methylpyrrole-2-carboxylic acid moiety, attached to the deoxy sugar (Fig. 1). Gene inactivation experiments have demonstrated that *couN3* and *couN4* are involved in the biosynthesis of this moiety in coumermycin A₁ [19]. CouN3, CouN4 and CouN5, encoded by genes of the coumermycin A₁ gene cluster, apparently convert L-proline to a pyrrole-2-carboxyl-S-[acyl carrier protein] intermediate [20]. Similar functions are expected for their homologues encoded by the clorobiocin biosynthetic gene cluster, i.e., CloN3, CloN4 and CloN5 (Fig. 2).

CouN3 and CouN4 are not involved in the formation of the central 3-methylpyrrole-2,4-dicarboxylic acid moiety, which is present only in coumermycin A₁ (Fig. 1) [19]. Four genes, *couR1*, *couR2*, *couR3* and *couR4* are found in the coumermycin A₁ gene cluster, but no similar genes exist in the novobiocin or clorobiocin cluster. We speculate that these four genes are involved in the biosynthesis of the central pyrrole unit of coumermycin.

Genes Responsible for the Biosynthesis of the Deoxy Sugar

Similar genes for the biosynthesis of the deoxy sugar moiety are found in all three clusters, i.e., *nov/clo/couSTUVW* (Fig. 1 and Table 1). These are responsible for the conversion of glucose 1-phosphate to dTDP-5-methyl-L-rhamnose (Fig. 2). First biochemical experiments with these enzymes have been reported [21],

and recently the crystal structure of the sugar epimerase NovW was solved [22]. Nov/Clo/CouU represent unusual C-methyltransferases of deoxy sugar biosynthesis [23].

Genes Responsible for the Formation of the Glycosidic, Amide and Ester Bonds

In clorobiocin and novobiocin, the aminocoumarin moiety and the prenylated 4-hydroxybenzoyl moiety are linked by an amide bond. We could show that the enzyme NovL catalyses the amide bond formation in novobiocin biosynthesis, a reaction comprising the adenylation of the substituted benzoate moiety and its subsequent transfer to the amino group of the aminocoumarin moiety (Fig. 2) [24]. Two amide synthetase genes similar to *novL*, i.e., *cloL* and *couL*, were found in the clorobiocin and coumermycin clusters, directly downstream of the genes for the biosynthesis of the aminocoumarin moiety (Fig. 1).

We have overexpressed and purified all three amide synthetases and investigated their substrate specificities, as a preparation for subsequent mutasynthesis experiments [25], [26]. Although the three amide synthetases CloL, NovL and CouL show 80–86% identity on the amino acid level and are nearly identical in size, they showed marked differences in their substrate specificity [25].

In the biosynthesis of novobiocin, clorobiocin and coumermycin A₁, the deoxy sugar is attached to the 7'-OH group of the aminocoumarin ring. Three very similar putative glycosyl transferase genes, *cloM*, *novM* and *couM*, are found at the same relative position in all three clusters (Fig. 1). NovM was overexpressed and purified and shown to catalyse the glycosylation *in vitro* [27], [28].

In novobiocin, the 3-OH group of the deoxy sugar is acylated by a carbamyl group (Fig. 1). The novobiocin gene cluster contains a carbamyl transferase gene, i.e., *novN*. The function of *novN* was confirmed by the heterologous expression in a *couN3*-defective mutant of the coumermycin producer, *Streptomyces rishiriensis*, resulting in the formation of a carbamylated coumermycin derivative [19]. NovN was also expressed in *E. coli* [29] and *Streptomyces lividans* [30] and investigated biochemically. Surprisingly, ATP is absolutely necessary for the NovN reaction, which is unusual for carbamyl transfer reactions.

In clorobiocin and coumermycin A₁, the 3-OH group of the deoxy sugar is acylated with a pyrrole-2-carboxyl group. The transfer of the activated pyrrole-2-carboxyl moiety (mentioned above) to the deoxy sugar moiety was unexpectedly found to involve two acyltransferases, CloN2 and CloN7, and two acyl carrier proteins, CloN1 and CloN5 (Fig. 2) [31].

Late Methylation Reactions

Three very similar O-methyltransferase genes, *novP*, *cloP* and *couP*, were found in the gene clusters of novobiocin, clorobiocin and coumermycin A₁, respectively (Fig. 1 and Table 1). Inactivation of *cloP* in the clorobiocin producer resulted in the accumulation of a new clorobiocin derivative, lacking the methyl group at 4''-OH of the deoxy sugar [32]. Freil Meyers et al. [29] have overexpressed and purified NovP and identified the 4''-O-methyl transfer reaction *in vitro*. It appears likely that the 4''-O-methyla-

tion takes place before the acylation of 3''-OH with the carbamyl or pyrrole-2-carboxyl moieties, respectively [29], [32].

The 5-C-methylation of the terminal pyrrole-2-carboxyl moieties occurs only after their transfer to the deoxy sugar moiety (Fig. 2) and is catalysed by the gene product of *cloN6* [33], which shares conserved sequence motifs with the recently identified radical SAM protein superfamily [34].

Resistance Genes

Antibiotic-producing organisms protect themselves from the toxic effects of their antibiotics by genes coding for self-resistance. The resistance genes are usually located in the biosynthetic gene cluster of the respective antibiotic.

All three clusters for aminocoumarin antibiotics contain a gene coding for an aminocoumarin-resistant gyrase B subunit, *gyrB^R*. The clorobiocin and the coumermycin A₁ clusters contain an additional, similar gene, *parY^R* (Fig. 1), coding for an aminocoumarin-resistant subunit of topoisomerase IV [35], [36]. This represented the first topoisomerase IV identified in the class of actinobacteria. At the same time, it was the first demonstration of the formation of a topoisomerase IV as a resistance mechanism of an antibiotic producer.

Regulatory Genes

The predicted gene products of *novG*, *cloG* and *couG* share sequence similarity with StrR, a well-studied pathway-specific transcriptional activator of streptomycin biosynthesis. We showed that NovG acts as a positive regulator of novobiocin biosynthesis [37]. Strains defective in *novG* produced only 2% of the novobiocin amount formed by the strains carrying the intact novobiocin cluster. The production could be restored by introducing an intact copy of *novG*. Furthermore, purified NovG protein showed specific DNA-binding activity upstream of the genes coding for the biosynthesis of the aminocoumarin moiety. The DNA sequence of the NovG binding site was very similar to the binding site for StrR.

The *novE*, *cloE* and *couE* genes have sequence similarity to the *lmbU* gene of the lincomycin biosynthetic gene cluster, suggested to have a regulatory function [38]. We generated *novE*-defective mutants [39] and found that they still produced novobiocin, but to a significantly lower level than the wild-type (only 5% in comparison to wild-type). This finding rules out the possibility that NovE has an essential catalytic function in the novobiocin biosynthesis. Rather, NovE may act as a positive regulator in novobiocin biosynthesis.

Conclusions

Comparison of the gene clusters of the three “classical” aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ revealed a strikingly stringent correspondence between the structures of the antibiotics and the organisation of the biosynthetic genes. For each structural moiety of the aminocoumarin antibiotics, the biosynthetic genes are grouped together, resulting in a “modular” structure of the clusters. The orders of the modules, and the order of the genes within each module, are perfectly identical for these three aminocoumarins, and nearly all

the genes within the clusters are oriented in the same direction. The comparison of the three clusters greatly facilitated the prediction of functions for the different genes. Transfer of the entire biosynthetic gene clusters of novobiocin and clorobiocin to other *Streptomyces* strains resulted in the formation of these antibiotics by the engineered host organisms [40], and provided excellent opportunities for the production of new aminocoumarins by genetic methods [41].

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