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New Aminocoumarin Antibiotics Derived from 4-Hydroxycinnamic Acid are Formed after Heterologous Expression of a Modified Clorobiocin Biosynthetic Gene Cluster

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Abstract Three new aminocoumarin antibiotics, termed ferulobiocin, 3-chlorocoumarobiocin and 8′-dechloro-3-chlorocoumarobiocin, were isolated from the culture broth of a *Streptomyces coelicolor* M512 strain expressing a modified clorobiocin biosynthetic gene cluster. Structural analysis showed that these new aminocoumarins were very similar to clorobiocin, with a substituted 4-hydroxycinnamoyl moieties instead of the prenylated 4-hydroxybenzoyl moiety of clorobiocin. The possible biosynthetic origin of these moieties is discussed.

Keywords antibiotics, biosynthesis, clorobiocin, ferulic acid, 3-chloro-4-hydroxycinnamic acid, *Streptomyces*

Introduction

Clorobiocin and coumermycin A_1 (Fig. 1) are members of the family of aminocoumarin antibiotics. They are potent inhibitors of bacterial gyrase [1] and represent interesting starting compounds for the development of new antibacterial agents by genetic and mutasynthetic methods [2].

By expression of the clorobiocin biosynthetic gene cluster in *Streptomyces coelicolor* M512, the antibiotic can be conveniently produced [3]. Clorobiocin contains a 3-dimethylallyl-4-hydroxybenzoyl moiety (Ring A) which is formed under involvement of the aromatic prenyltransferase CloQ. Inactivation of the structural gene *cloQ* leads to abolishment of clorobiocin production [4]. In order to generate a strain suitable for mutasynthesis experiments, we expressed a *cloQ*-defective gene cluster in the heterologous host *S. coelicolor* M512, and replaced the genuine amide synthetase gene of the clorobiocin biosynthesis, *cloL* (Fig. 1), with the similar *couL* from coumermycin biosynthesis. *couL* encodes for an enzyme with different substrate specificity to CloL [5, 6]. As expected, the *cloQcloL*-defective heterologous expression strain did not produce clorobiocin. Unexpectedly, however, introduction of *couL* into this strain resulted in the formation of three new aminocoumarin antibiotics.

In this paper, we report the isolation, structure elucidation and antibacterial activities of these three new antibiotics, termed ferulobiocin, 3-chlorocoumarobiocin and 8′-dechloro-3-chlorocoumarobiocin. The occurrence of these compounds has interesting implications for the current hypotheses on phenylpropanoid and aminocoumarin biosynthesis in *Streptomycetes*.

Materials and Methods

Strains and Plasmids

S. coelicolor M512 was kindly provided by E. Takano (Groningen, Netherlands) and Janet White (Norwich, United Kingdom). The integrative cosmid clo-BG1 was described by Eustáquio *et al.* [3].

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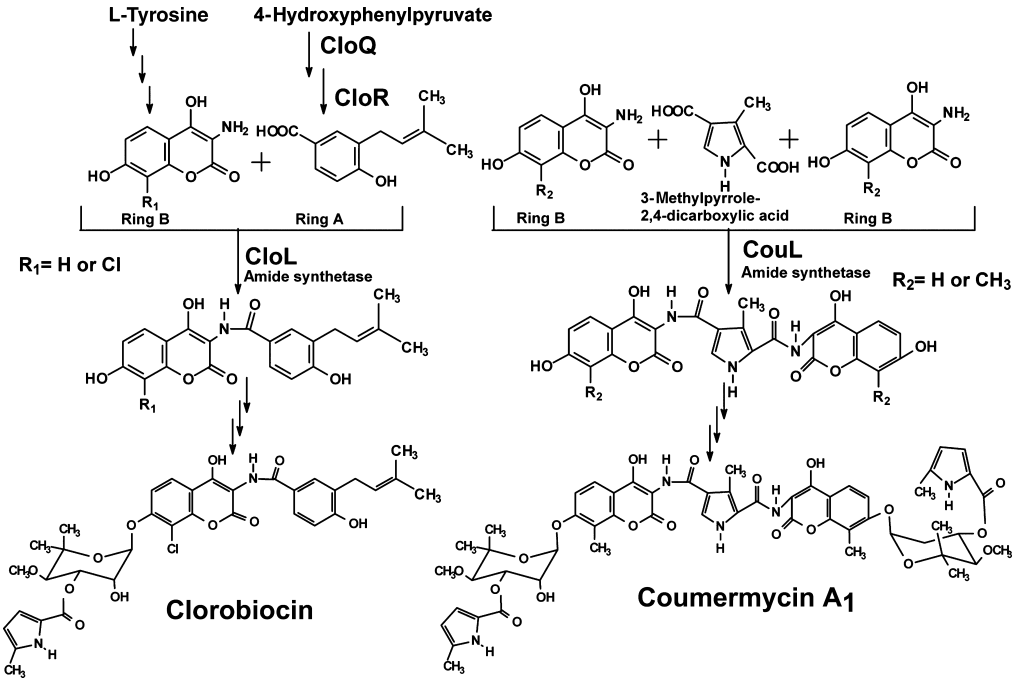


Fig. 1 Biosynthesis of clorobiocin and coumermycin A.

The *E. coli - Streptomyces* shuttle vector pUWL201, containing the *ermE** promoter [7] was used for the construction of the *couL* expression plasmid pMS91. Therefore, *couL* was amplified by PCR. A *Hin*dIII site was introduced before the start codon using primer cumG1 (5′-CCGCTGCCC**AAGCTT**GAGTGCCTC-3′). At the C-terimnus, an *Xba*I restriction site was introduced after the stop codon using primer cumG2 (5′-CGGGCAGGC**TCTAGA**ACAGCACTC-3′). Bold letters represent the corresponding restriction sites. The resulted PCR fragment was digested with *Hin*dIII and *Xba*I and subsequently cloned into pUWL201 to give pMS91.

Modification of the cosmid clo-BG1, and stable integration into the genome of *S. coelicolor* M512, were carried out as described by Eustáquio *et al.* [3]. Transformation with the *couL* expression plasmid pMS91 was carried out as described by Kieser *et al.* [8].

Fermentation and Preparative Isolation of New Antibiotics

The genetically engineered *S. coelicolor* M512 strain was pre-cultured for 2 days in YMG medium, for 2 further days in corn starch medium and subsequently for 8～10 days in production medium as described previously [9]. Cultures were treated with petrol ether to remove lipids, and subsequently the antibiotics were extracted with ethyl acetate [9].

Analytical HPLC was carried out on an Agilent Eclipse XDB-C18 column (5 μm, 150×4.6 mm) at a flow rate of 1.0 ml/minute. A linear gradient from 40 to 100% solvent B in solvent A (solvent A=MeOH/H_2O/HCOOH 50 : 49 : 1; solvent B=MeOH/HCOOH 99 : 1) over 28 minutes was used, followed by elution with 100% solvent B for 3 minutes and equilibration with 40% solvent B for 6 minutes; UV detection was carried out at 340 nm. Authentic clorobiocin was used as standard.

Purification by preparative HPLC was carried out on a Multosphere 120 RP18-5 column (5 μm, 250×10 mm, C&S Chromatographie Service, Düren, Germany) at a flow rate of 2.5 ml/minute and the same gradient as for the analytical column.

Structure Elucidation

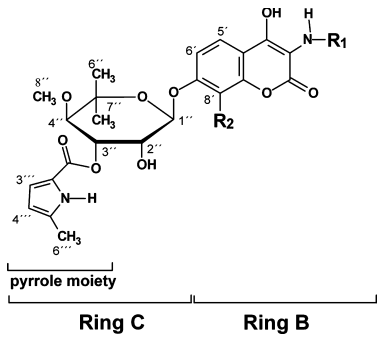
The isolated products were analyzed by ^{1}H-NMR spectroscopy and by ESI-MS.

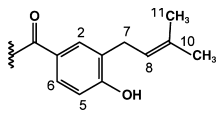
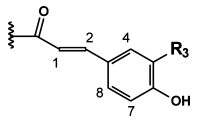
^{1}H-NMR spectra were measured on an Avance 400 MHz spectrometer (Bruker, Karlsruhe, Germany), using CD_3OD as solvent. The NMR data are shown in Table 1.

Mass spectra of the purified compounds were obtained from a Finnigan TSQ Quantum instrument using positive electrospray ionisation (ESI) (electrospray voltage 3.8 kV; heated capillary temperature 320°C; sheath and auxiliary gas: nitrogen; sheath gas flow rate: 30.1 AU; aux gas flow rate: 12.0 AU). The mass spectra are shown in Table 2.

For LC-MS analysis, a Nucleosil 120 RP ODS-column (8 μm, 2×250 mm, Macherey-Nagel) and a gradient of

Table 1 ^{1}H-NMR data of three new aminocoumarin antibiotics isolated from a genetically engineered *Streptomyces* strain, in comparison with clorobiocin



R_1	R_2	R_3	Compound name
	Cl	—	Clorobiocin
	Cl	OCH_3	Ferulobiocin
	Cl	Cl	3-Chlorocoumarobiocin
	H	Cl	8′-Dechloro-3-chlorocoumarobiocin

Position	Clorobiocin	Ferulobiocin	3-Chlorocoumarobiocin	8′-Dechloro-3-chloro-coumarobiocin
	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)
1-H	—	6.75 d (15.8)	6.71 d (15.6)	6.80 d (14.4)
2-H	7.76 d (2.5)	7.57 d (15.8)	7.49 d (15.6)	7.54 d (14.4)
4-H	—	7.19 br[a] s	7.56 br[a] s	7.61 br[a] s
5-H	6.84 d (8.4)	—	—	—
6-H	7.72 dd (8.4; 2.5)	—	—	—
7-H	—	6.80 d (8.2)	6.92 d (8.8)	6.91 d (8.2)
7-H_2	3.34 d (7.1)			
8-H	5.35 br[a] t (7.1)	7.07 d (8.2)	7.36 d (8.8)	7.41 d (8.2)
9-OCH_3	—	3.90 s	—	—
10-H_3	1.74 s	—	—	—
11-H_3	1.75 s	—	—	—
5′-H	7.90 d (9.2)	7.90 d (8.8)	7.89 d (8.6)	7.92 d (8.0)
6′-H	7.33 d (9.2)	7.23 d (8.8)	7.19 d (8.6)	7.03[b]
8′-H	—	—	—	7.03[b]
1″-H	5.73 d (1.8)	5.69 br[a] s	5.68 br[a] s	5.61 d (1.6)
2″-H	4.34 t (2.8)	4.33 br[a] s	4.33 br[a] s	4.23 br[a] s
3″-H	5.71 dd (10.3; 2.8)	5.71 dd (11.6; 2.0)	5.71 dd (11.0; 4.8)	5.59 dd (10.0; 2.8)
4″-H	3.72 d (10.3)	3.71 d (11.6)	3.71 d (11.0)	3.68 d (10.0)
6″-H_3	1.18 s	1.19 s	1.19 s	1.20 s
7″-H_3	1.35 s	1.35 s	1.34 s	1.37 s
8″-OCH_3	3.52 s	3.51 s	3.51 s	3.51 s
3‴-H	6.90 d (3.6)	6.90 d (3.6)	6.90 d (3.4)	6.90 d (3.4)
4‴-H	5.94 d (3.6)	5.94 d (3.6)	5.94 d (3.4)	5.94 d (3.4)
6‴-H_3	2.29 s	2.29 s	2.29 s	2.29 s

δ is given in ppm. Spectra were obtained at 400 MHz. The spectra were taken in CD_3OD.

[a] br=broad signal.

[b] Complex, overlapping signals; *J* not determinable.

acetonitrile in water (each containing 0.1% HCOOH) from 0 to 50% acetonitrile over 20 minutes were used (flow rate 0.2 ml/minute). The collision-induced dissociation (CID) spectra during the HPLC run were recorded with collision energy +25 eV, collision gas argon, and collision pressure 1.0×10^{-3} torr (133 mPa).

Antibacterial Activities

The antibacterial activities of the new compounds were tested against *Bacillus subtilis* ATCC 14893 in a disc

Table 2 Mass spectrometric analysis of three new aminocoumarin antibiotics, isolated from a genetically engineered *Streptomyces* strain, in comparison to clorobiocin

Compound	MW	$[M+H]^+$	$[M-\text{Ring C}]^+$	$[\text{Ring A}]^+$	$[\text{Ring C}]^+$	$[\text{Pyrrole moiety}]^+$
Clorobiocin	696.2	697.3/699.3	416.1	189.1	282.2	108.0
Ferulobiocin	684.2	685.3/687.3	404.1	177.0	282.1	108.0
3-Chlorocoumarobiocin	688.2	689.3/691.2	n.d.	181.0	282.0	108.0
8′-Dechloro-3-chlorocoumarobiocin	654.2	655.2/657.4	n.d.	181.0	282.2	108.0

Measurements were carried out using electrospray ionization in the positive mode.
x/y signal resulting from chlorine isotopes contained in the molecule; n.d. not detectable.

diffusion assay as described previously [9]. Authentic clorobiocin was used as comparison. The activities were quantified from inhibition zone diameters and expressed relatively to clorobiocin.

Results

Detection and Isolation of the New Aminocoumarin Antibiotics

In cosmid clo-BG1, which harbours the complete biosynthetic gene cluster of clorobiocin, the genes *cloQ* and *cloL* were inactivated by λ RED-mediated recombination [10] using previously described methods for the successive deletion of both genes [11]. Subsequently, this modified cosmid was heterologously expressed in *S. coelicolor* M512 [3]. The amide synthetase gene *couL* of the coumermycin biosynthetic gene cluster was cloned into the *Streptomyces* expression vector pUWL201 [7] and introduced into the mutant by protoplast transformation as described previously [12].

When the resulting strain was cultivated as described in the Experimental, the formation of three aminocoumarin antibiotics was observed. The same compounds were observed as side products in later mutasynthesis feeding experiments using the same strain (data not shown). The new compounds were extracted from the culture broth, isolated by reversed-phase HPLC and investigated by spectroscopic methods.

Structure Elucidation

Ferulobiocin

The ESI-MS spectrum of this compound showed an $[M+H]^+$ ion at $m/z=685$. Furthermore, the spectrum showed the same characteristic fragments of the deoxysugar moiety ($m/z=282$) and the 5-methylpyrrole-2-carbonyl moiety ($m/z=108$) as observed for clorobiocin. However, while the mass spectrum of clorobiocin shows a fragment at $m/z=189$, representing the prenylated 4-hydroxybenzoyl moiety (=Ring A), and a fragment at $m/z=416$, representing the moiety comprising both the aminocoumarin and the prenylated 4-hydroxybenzoyl moieties (see Fig. 1), the new compound showed fragments at $m/z=177$ and $m/z=404$ instead. These fragments indicate that the new compound represent a clorobiocin derivative containing a different Ring A analog.

The ^{1}H-NMR spectrum of this compound confirmed this hypothesis. It showed the same signals as clorobiocin for the aminocoumarin, the substituted deoxysugar and the 5-methylpyrrol-2-carbonyl moieties (Table 1). However, instead of the signals for the genuine Ring A, signals of a different structural moiety were detected. Two doublets at $\delta=7.57$ ppm (1H; 15.8 Hz) and $\delta=6.75$ ppm (1H; 15.8 Hz) indicate the presence of two vinylic protons in *trans*-position attached to adjacent carbon atoms of a double bond, and the singlet at $\delta=3.90$ ppm (3H) indicate a methoxygroup attached to an aromatic nucleus. Finally, the signals at $\delta=7.19$ (br s, 1H), 6.80 (d, 1H, 8.2 Hz) and 7.07 (d, 1H, 8.2 Hz) showed the presence of a 1,3,4-trisubstituted aromatic ring. Comparison with literature data [13～15] and data obtained from commercially available ferulic acid showed that these NMR signals are in perfect agreement with those of ferulic acid (=3-methoxy-4-hydroxycinnamic acid) and its corresponding esters, and the observed mass spectrometric data were also consistent with the presence of a feruloyl moiety.

Therefore, this new antibiotic represented a clorobiocin derivative containing a feruloyl moiety instead of the prenylated 4-hydroxybenzoyl moiety. This compound was termed ferulobiocin.

3-Chlorocoumarobiocin

The ESI-MS spectrum of the second compound showed an $[M+H]^+$ ion at $m/z=689$, in contrast to ferulobiocin ($m/z=685$). Consistently, the ion assigned to the acyl moiety appeared at $m/z=181$ (ferulobiocin: $m/z=177$).

Otherwise, the spectrum showed the same characteristic peaks of the deoxysugar moiety and the 5-methylpyrrole-2-carbonyl moiety (m/z=282 and 108, respectively) as found in the spectra of ferulobiocin and clorobiocin.

The ^{1}H-NMR spectrum of this compound showed the signals derived from the substituted deoxysugar and the aminocoumarin moieties, identical to these of clorobiocin and ferulobiocin (Table 1). Very similar to the ^{1}H-NMR spectrum of ferulobiocin, two doublets at δ=7.49 ppm (1H, 15.6 Hz) and δ=6.71 ppm (1H; 15.6 Hz) indicated the presence of two vinylic protons and the signals at δ=7.56 (br s, 1H), 7.36 (d, 1H) and 6.92 ppm (d, 1H) again showed the presence of a 1,3,4-trisubstituted aromatic ring. However, in contrast to ferulobiocin, the singulett of the methoxygroup at δ=3.90 ppm (3H) was missing, and no other proton signal was found instead. This suggested that at position 3, the methoxygroup (31 Da) had been replaced by a chlorine atom (35 Da). Therefore, the new compound could be identified as a clorobiocin derivative containing 3-chloro-4-hydroxycinnamic acid instead of the genuine prenylated 4-hydroxybenzoyl moiety. This compound was termed 3-chlorocoumarobiocin.

8′-Dechloro-3-chlorocoumarobiocin

The ESI-MS spectrum of the third compound showed an $[M+H]^+$ ion at m/z=655, *i.e.* 34 dalton less than that of 3-chlorocoumarobiocin, corresponding to the loss of a chlorine atom. However, both the ^{1}H-NMR spectrum and the mass spectrum still showed the same signals derived from the 3-chloro-4-hydroxycinnamoyl moiety (Tables 1 and 2). In contrast, the ^{1}H-NMR spectrum now showed significant changes for the protons of the aminocoumarin ring (Table 1), proving the presence of three rather than two protons (δ=7.03 ppm, 2H; δ=7.92 ppm, 1H). Exactly the same signals had been observed previously for aminocoumarin derivatives lacking the chlorine atom at position 8′ [16]. Therefore, this compound differed from 3-chlorocoumarobiocin by lacking the chlorine atom at position 8′ of the aminocoumarin ring.

Dependence of the Formation of Ferulobiocin, 3-Chlorocoumarobiocin and 8′-Dechloro-3-chlorocoumarobiocin on the Presence of CouL

As mentioned above, the three new aminocoumarin antibiotics were found in a *S. coelicolor* M512 strain expressing a modified (*i.e. cloQcloL*-defective) clorobiocin cluster and additionally the amide synthetase gene *couL*. However, these products were not observed in strains not expressing CouL.

The amide synthetase CouL could not be replaced by CloL, which catalyzes a very similar reaction but has a different substrate specificity [5, 6], since in a *cloQ*-defective strain with intact *cloL* [9], we could not detect the three new antibiotics described here, even upon specific examination with sensitive LC-MS techniques. However, CouL could be replaced by SimL of simocyclinone biosynthesis [17], cloned into expression vector pUWL201, as is consistent with the reported substrate specificity of SimL.

Antibacterial Activities

The antibacterial activities of the three new compounds were determined by an agar diffusions test against *B. subtilis* (Table 3). All three compounds showed antibacterial activity, but clearly less than the very potent clorobiocin. 8′-Dechloro-3-chlorocoumarobiocin was less active than the two other compounds. This is consistent with earlier observations, which showed 80~90% loss of antibacterial activity when the chlorine was removed from position 8′ of the aminocoumarin antibiotics [16, 18].

Discussion

In this study, we have generated a *S. coelicolor* M512 strain expressing a modified clorobiocin biosynthetic gene cluster, defective in an essential gene for the biosynthesis of Ring A of clorobiocin. We have additionally expressed a heterologous amide synthetase, CouL. We previously identified that this amide synthetase has a remarkably broad substrate specificity, accepting not only different pyrrole dicarboxylic acids as substrates, but also Ring A of clorobiocin, and analogs thereof [5, 6]. As discussed

Table 3 Antibacterial activities of three new aminocoumarin antibiotics isolated from a genetically engineered *Streptomyces* strain.

Compound	Amount (nmol)	Relative activity (%)
Clorobiocin	0.5 1 2	100
Ferulobiocin	16 32 64	3.3
3-Chlorocoumarobiocin	16 32 64	5.0
8′-Dechloro-3-chlorocoumarobiocin	32 64 128	1.4

Relative bioactivities were estimated by comparing inhibition zones with those obtained for clorobiocin.

previously [6, 19], this is in agreement with the hypothesis that *couL* may have evolved from an acestral gene very similar to *cloL*. We intended to use this strain for mutasynthesis experiments, *i.e.* for the external addition of various Ring A analogs, similar to a previous series of experiments by our group [5].

To our surprise we found that our strain formed modified aminocoumarin antibiotics even without the addition of any Ring A analogs. Isolation and structural elucidation of these compounds showed that they were clorobiocin analogs containing substituted coumaroyl moieties instead of the prenylated 4-hydroxybenzoyl moiety. The latter moiety can only be formed in the presence of active CloQ. The three new aminocoumarin antibiotics described here have not been reported in the literature before.

The new compounds were observed in *cloQ*-defective strains expressing the heterologous amide synthetase CouL, but not in a previously generated *cloQ*-defective strain expressing CloL [4, 9]. This is consistent with the substrate specificities of CloL and CouL, since CouL but not CloL is capable of accepting substituted cinnamic acid substrates.

The fact that coumaric acid derivatives were formed in our strain was a surprise. The biosynthetic pathway to clorobiocin is known in detail [20] and does not involve coumaric acid derivatives. Neither have such compounds been described from *S. coelicolor*.

Coumaric acid derivatives belong to the most abundant biomolecules on earth, since they form part of lignin in plants. In Actinomycetes, however, these compounds are relatively rare, and only recently it was demonstrated that several Actinomycetes possess phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) genes for the biosynthesis of cinnamic and coumaric acid [21, 22]. However, neither in the biosynthetic gene cluster of clorobiocin, nor in the completely sequenced genome of *S. coelicolor*, PAL or TAL genes have been described so far.

The host strain utilized in this study, *S. coelicolor* M512, is a derivative of *S. coelicolor* A3(2) [23]. Our present findings stimulated us to re-examine the genome sequence of *S. coelicolor* [24]. Blast search revealed one gene with 32% identity to the tyrosine ammonia-lyase Sam8 of *Saccharothrix espanaensis* [21]. This gene, SCO4932, is currently annotated as histidine ammonia-lyase. It remains to be shown whether it is involved in the formation of coumaric acid derivatives observed in this study.

Interestingly, we also detected two chlorinated compounds apparently derived from a 3-halogenation of 4-coumarate or tyrosine. The halogenase of clorobiocin biosynthesis, Clo-hal [12], may be involved in the formation of these two compounds. So far, the genuine substrate of Clo-hal is unknown. However, also the genome of *S. coelicolor* contains a gene (SCO1275) with very high similarity to FAD-dependent halogenases [25].

When various Ring A analogs were externally added to our strain, the three new compounds described here were commonly detected as side products, besides the clorobiocin analogs derived from incorporation of the added precursors (data not shown). Often the yields of the three compound described here were even higher than without external feeding, possibly due to the induction of enzymes of clorobiocin biosynthesis. Our study exemplifies that mutasynthesis experiments may result in the formation of unexpected compounds and therefore should not be evaluated purely on the basis of bioassays, but by proper chemical analysis of the products.

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