Metabolic Engineering of Aminocoumarins: Inactivation of the Methyltransferase Gene *cloP* and Generation of New Clorobiocin Derivatives in a Heterologous Host

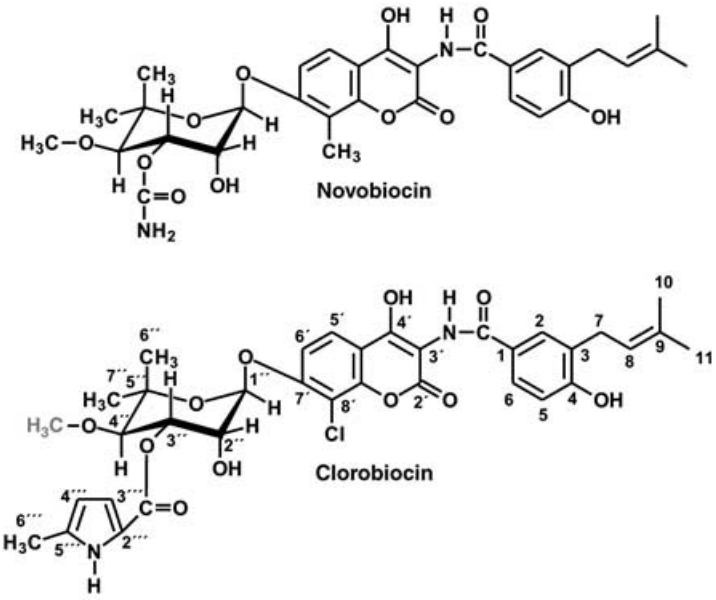
Anja Freitag, Heike Rapp, Lutz Heide, and Shu-Ming Li*[a]

Aminocoumarin antibiotics are highly potent inhibitors of bacterial gyrase and represent a class of antibiotics that are very suitable for the generation of new compounds by metabolic engineering. In this study, the putative methyltransferase gene cloP *in the biosynthetic gene cluster of clorobiocin was inactivated. Expression of the modified gene cluster in the heterologous host* Streptomyces coelicolor *M512 gave three new aminocoumarin antibiotics. The structures of the new compounds were elucidated by MS and ^{1}H NMR, and their antibacterial activities were determined. All three compounds lacked clorobiocin's methyl group at 4-OH of the deoxysugar moiety, noviose. They differed from each other in the position of the 5-methylpyrrole-2-carbonyl group, which was found to be attached to either 2-OH, 3-OH or 4-OH of noviose. Attachment at 4-OH resulted in the highest antibacterial activity. This is the first time that an aminocoumarin antibiotic acylated at 4-OH in noviose has been detected.*

Introduction

Antibiotics are a drug class that presents a unique challenge to pharmaceutical research, since resistance has developed to every antibiotic some years or a few decades after its introduction into clinical use.[1] Consequently, there is a need for the continuous development of new antibiotics.[2] Genetic engineering of antibiotic-producing bacteria has emerged as a new and powerful tool for the development of new bioactive compounds, either through rational metabolic engineering or by random combinatorial biosynthesis.[3] A class of antibiotics particularly suitable for such approaches is that of the aminocoumarin antibiotics.[4] Synthetic methods for the generation of aminocoumarin antibiotics and their analogues have been explored, but remain difficult and time-consuming.[5–7]

The aminocoumarins are very potent inhibitors of gyrase, a validated drug target.[8] Novobiocin (Albamycin®) has been introduced into the US drug market as an antibiotic for use in humans, but its clinical use has remained limited due to problems of poor solubility, low activity against Gram-negative bacteria, resistance development and several adverse effects. Nevertheless, clinical studies have confirmed the therapeutic potential of the aminocoumarin antibiotics.[9–11]



Scheme 1. The aminocoumarin antibiotics novobiocin and clorobiocin. The 4″-Me group of clorobiocin is shown in grey.

Clorobiocin (Scheme 1) is considerably more powerful than novobiocin as a gyrase inhibitor and an antibacterial agent[12] and therefore represents an interesting starting compound for the development of new, modified aminocoumarin antibiotics that could be clinically superior to novobiocin.

We have recently cloned the biosynthetic gene cluster of clorobiocin from its natural producer, *Streptomyces roseochromogenes* var. *oscitans* DS 12.976, and have elucidated the function of several genes contained therein.[4,13] Modifications of this gene cluster by genetic engineering led to the formation of several new clorobiocin derivatives.[4] In this study, we

[a] *A. Freitag,[+] H. Rapp,[+] Prof. Dr. L. Heide, Priv.-Doz. Dr. S.-M. Li*
Pharmazeutische Biologie, Pharmazeutisches Institut
Eberhard-Karls-Universität Tübingen
Auf der Morgenstelle 8, 72076 Tübingen (Germany)
Fax: (+49) 7071-295-250
E-mail: shuming.li@uni-tuebingen.de
[+] *These authors contributed equally to this work*

 DOI: 10.1002/cbic.200500019

aimed to produce a clorobiocin derivative lacking the Me group at the 4″-position (Scheme 1); that is, with a free OH group in this position. We were interested in this experiment for three reasons:

i) The biosynthetic gene cluster of clorobiocin contains three putative methyltransferase genes: *cloN6*, *cloP* and *cloU*;[13] however, only the function of *cloN6* has been confirmed experimentally.[14] This study should provide experimental evidence for the role of *cloP*, expected to be responsible for the *O*-methylation reaction at the 4″-position.
ii) Removal of the 4″-Me group should allow the importance of this group for antibacterial activity to be determined, and at the same time could provide a starting material for the generation of further aminocoumarin derivatives through chemical or chemoenzymatic acylation or alkylation of this group, potentially affording antibiotics with improved properties.
iii) We have very recently succeeded in producing aminocoumarin antibiotics in a heterologous host, using the integration functions of the bacteriophage ΦC31 for the integration of the biosynthetic gene clusters into the heterologous host genome.[15] Modification of the biosynthetic gene clusters prior to their integration into the heterologous host may provide an efficient method for rational generation of antibiotics with modified structures,[16] and this study should also provide a further example of the applicability of this method.

Results

1. Construction of the cloP inactivation construct clo-HR1

The *cloP* gene of the clorobiocin biosynthetic gene cluster codes for a 31 kDa protein with sequence similarity to SAM-dependent methyltransferases such as TylF.[17] A BLAST search shows closest similarity to *couP*, which is responsible for the 4″-methylation reaction in coumermycin A_1 biosynthesis.[18] For *cloP* inactivation, the *cloP* gene was replaced by an apramycin resistance gene, *aac(3)IV*, in a cosmid containing the entire biosynthetic gene cluster of clorobiocin. The modified gene cluster was subsequently expressed in a heterologous producer strain.

Cosmid clo-BG1, containing the clorobiocin gene cluster, the *attP* and the *int* genes of bacteriophage ΦC31 and a tetracycline, as well as a neomycin resistance gene, had been constructed in a previous study.[15] Site-specific integration of this cosmid into the *attB* site of the genome of *Streptomyces coelicolor* M512 had resulted in the formation of clorobiocin by the heterologous host.[15]

For *cloP* inactivation, the apramycin resistance gene *aac(3)IV* was amplified from plasmid pUG019[15] by PCR, by use of primers with 39 bp extensions homologous to the regions upstream and downstream of *cloP*. The PCR product was used to replace the entire open reading frame of *cloP* on cosmid clo-BG1, only the start and the stop codon being left intact (Figure 1A and C). The resulting cosmid was termed clo-HR1. In this construct, the *aac(3)IV* gene is under control of its own promoter. As shown in Figure 1C, the resistance gene was placed between two restriction sites rare in *Streptomyces* DNA: *Xba*I and *Spe*I. If removal of the *aac(3)IV* cassette were to become necessary due to, for example, unwanted polar effects of the cassette on downstream genes, digestion of the cosmid with *Xba*I and *Spe*I and subsequent religation of the compatible overhangs would allow complete removal of the cassette.[15]

2. Integration of cosmid clo-HR1 into the genome of *Streptomyces coelicolor* M512.

Cosmid clo-HR1 was introduced into protoplasts of *Streptomyces coelicolor* M512 as described in the Experimental Section, and mutants resulting from integration of this cosmid into the host genome were selected by their apramycin and kanamycin resistance. Eight independent clones were obtained, and their genotype was confirmed by Southern blotting, with use of the entire cosmid clo-BG1 as probe. All eight integration mutants showed all expected bands of the integrated cosmid clo-HR1 (Figure 1A and D). Therefore, no deletions or rearrangements had taken place during gene inactivation and during integration of the cosmid. The *cloP*$^-$ genotype was corroborated by the absence of the 8.4 kb band and the presence of the expected 2.5 and 5.9 kb bands in the mutants (Figure 1C and D). Site-specific integration was verified by the expected 13.0 and 7.5 kb bands, resulting from integration of the cosmid at the *attP* site of the fully sequenced *S. coelicolor* chromosome (Figure 1B and D).

3. Analysis of secondary metabolite production

The eight *cloP*$^-$ integration mutants of *S. coelicolor* M512 were cultured in clorobiocin production medium, and secondary metabolite formation was analysed by HPLC. A *cloP*$^+$ integration mutant of *S. coelicolor* M512, carrying the unmodified cosmid clo-BG1, was used as comparison. All eight *cloP*$^-$ mutants showed identical patterns of secondary metabolites, clearly different from that of the *cloP*$^+$ strain (Figure 2). In the *cloP*$^+$ strain, clorobiocin was the dominant product, accompanied by its nonchlorinated derivative novclobiocin 101 and its structural isomer isoclorobiocin (Figure 2A). These compounds have similarly been observed in previous studies.[16] In contrast, the *cloP*$^-$ strains showed two dominant peaks (Figure 2B), both of which showed the same molecular ion ($m/z = 681$, $[M-H]^-$) in FAB MS analysis, thus indicating the loss of a methyl group relative to clorobiocin ($[M-H]^- = 695$). Extracts prepared from the *cloP*$^-$ mutants did not show any mass peak at $m/z = 695$, showing the complete absence of clorobiocin or its structural isomers.

For the elucidation of the structures of the new compounds, the *cloP*$^-$ mutants were cultured in 1000 mL medium. The culture was extracted with ethyl acetate and subjected to column chromatography on Sephadex LH-20, followed by preparative reversed-phase HPLC (see Experimental Section). This resulted

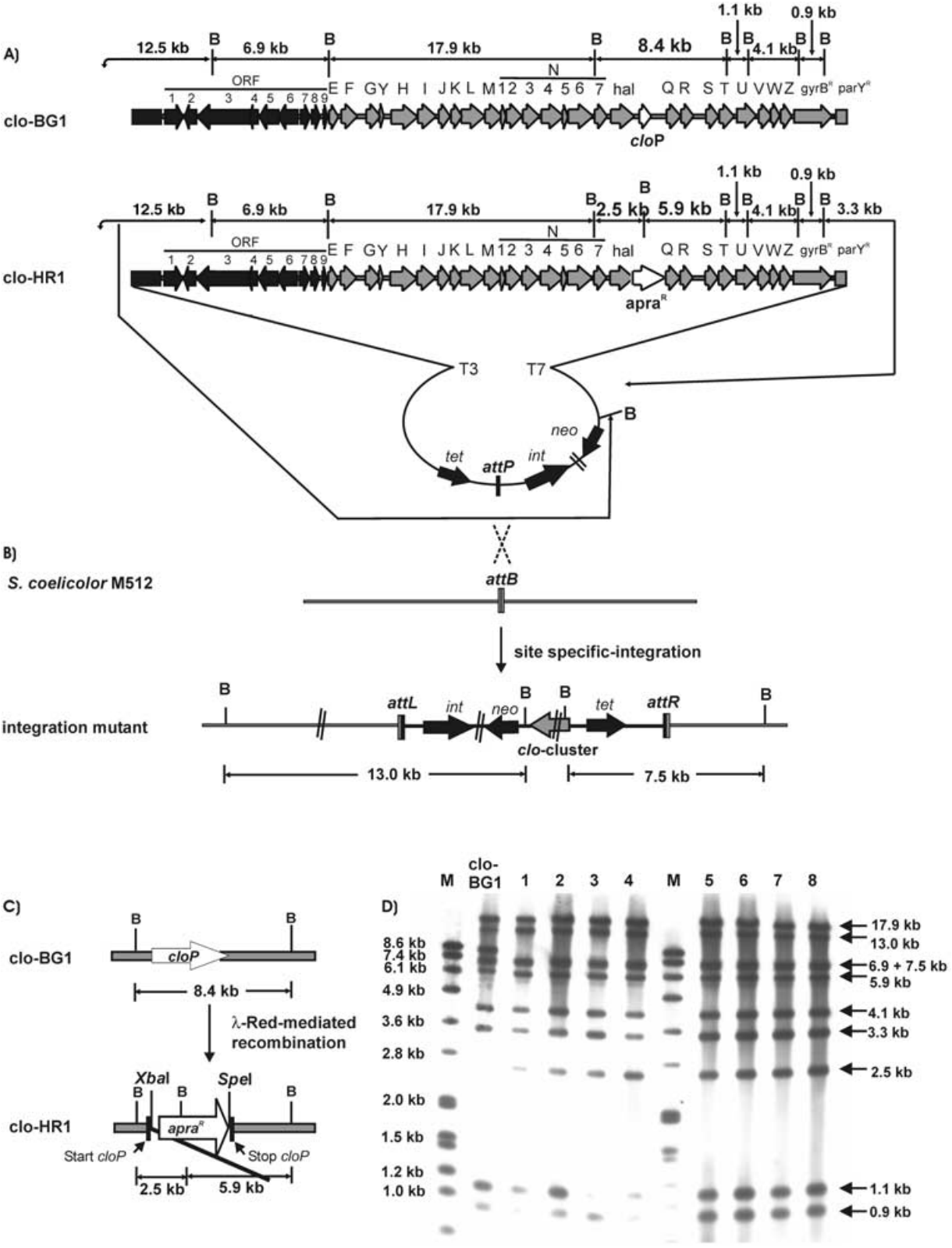


Figure 1. A) Cosmid constructs clo-BG1 (intact) and clo-HR1 (*cloP*$^-$) containing the ΦC31 integration functions and the clorobiocin biosynthetic gene cluster. B) Schematic representation of site-specific integration of clo-HR1 into the *S. coelicolor* chromosome. B = *BglII* restriction site. T3, T7 = T3 and T7 promoter of the SuperCos1 vector. Fragment sizes resulting from digestion with *BglII* are indicated. Cosmid backbone out of scale. See ref. [32] for details of the integration mechanism. C) Detail of the replacement of *cloP* by an apramycin resistance gene, flanked by *XbaI* and *SpeI* sites. D) Southern blot analysis of eight *S. coelicolor M512* integration mutants harbouring clo-HR1. M = DIG-labelled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with *BglII*. The DIG-labelled cosmid clo-BG1 was used as probe.

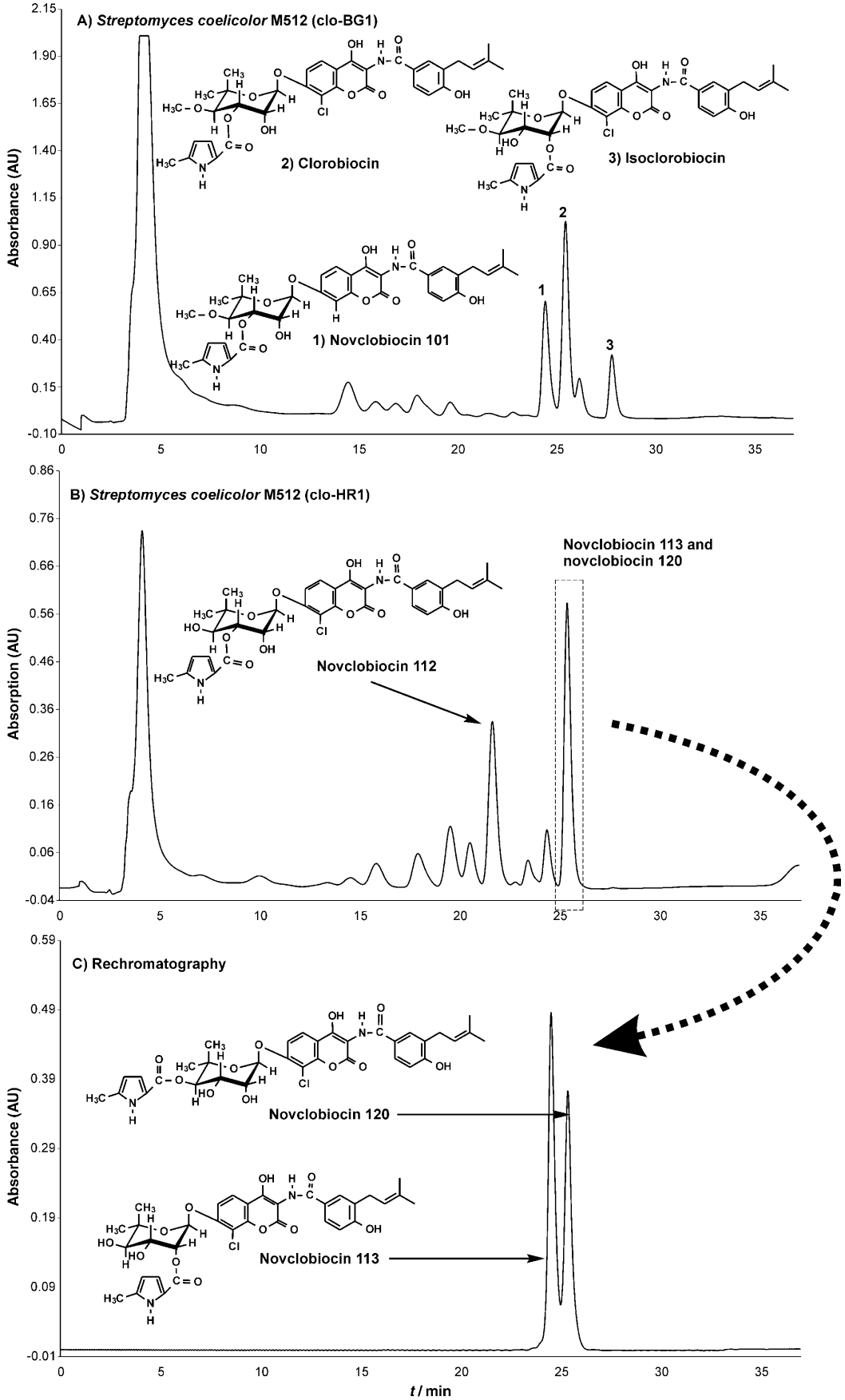


Figure 2. HPLC analysis of secondary metabolite production from: A) *Streptomyces coelicolor* M512 harbouring cosmid clo-BG1 with the intact clorobiocin biosynthetic gene cluster. B) *Streptomyces coelicolor* M512 harbouring cosmid clo-HR1 with the *cloP*$^{-}$ clorobiocin biosynthetic gene cluster; MeOH/water gradient. C) Rechromatography of the major peak of (B); acetonitrile/water gradient.

in one compound, termed novclobiocin 112, in pure form. However, two other compounds co-eluted in this purification procedure, and were eventually separated by an additional preparative HPLC purification step with a different solvent system (Figure 2C). The resulting pure compounds were termed novclobiocins 113 and 120.

4. Structural elucidation of new clorobiocin derivatives

The negative FAB mass spectra of the isolated compounds—novclobiocins 112, 113 and 120—were nearly identical, showing peaks at $m/z = 681$ $[M-H]^-$, 517, 501, 357, 281 and 255 (see Experimental Section), and hence suggesting that these compounds may be closely related structural isomers. All three compounds showed the typical pattern of compounds containing one chlorine atom, due to the isotopes ^{35}Cl and ^{37}Cl.

Their structures were elucidated by ^1H NMR spectroscopy (see Table 1, below), through comparison with clorobiocin and novclobiocin 104, a clorobiocin analogue lacking the 5-methylpyrrole-2-carbonyl moiety.[19] The three new compounds obtained in this study—novclobiocins 112, 113 and 120—showed the same signals as clorobiocin for the protons of the aminocoumarin moiety, of the prenylated 4-hydroxybenzoyl moiety and of the 5-methylpyrrole-2-carbonyl moiety. However, they differed from clorobiocin and from one another in the signals obtained from the deoxysugar moiety. The three principal aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 all contain the same deoxysugar moiety—noviose—present in the $^1C_4$ conformation (Scheme 1). The protons at 4″ and 3″ are thus in axial positions, whereas those at 2″ and 1″ are in equatorial positions. Correspondingly, 3″-H shows strong coupling with 4″-H ($J = 10$ Hz), but weaker coupling with 2″-H ($J = 3$ Hz), resulting in a characteristic doublet of doublets signal for 3″-H ($J = 10$, 3 Hz) and a doublet for 4″-H ($J = 10$ Hz). The equatorial 2″-H shows additional coupling with the equatorial 1″-H ($J = 1.7$ Hz), resulting in a broad singlet or triplet signal for 2″-H.

In the previously obtained compound novclobiocin 104, none of the hydroxy groups in these three positions is acylated, so the signals of 2″-H, 3″-H and 4″-H appear at 4.12, 4.18 and 3.40 ppm, respectively (Table 1). In clorobiocin, the 5-methylpyrrole-2-carbonyl moiety is attached to 3″-OH, causing a strong downfield shift of the 3″-H signal to 5.71. The same was now observed for the new compound novclobiocin 112; this indicated that in this compound the 5-methylpyrrole-2-carbonyl moiety is attached to the same position as in clorobiocin. The lack of the 4-Me group resulted in a shift of the signal of 4″-H to 4.08 ppm, compared to 3.72 ppm in clorobiocin. Novclobiocin 112 was therefore 4″-*O*-demethylclorobiocin, the principal target compound of this study.

In novclobiocin 113, the proton at 3″ appears at 4.40 ppm, similarly to the corresponding signal in the nonacylated compound novclobiocin 104. In contrast, the signal for 2″-H is shifted strongly downfield to 5.42 ppm; this shows that the acyl moiety is attached to 2″-OH rather than to 3″-OH.

In the ^1H NMR spectrum of the third compound, novclobiocin 120, the signals for both 2″-H and 3″-H were similar to those in the nonacylated compound novclobiocin 104. Now, however, the doublet signal of 4″-H showed a strong downfield shift (to 5.42 ppm), showing that the acyl group in this compound was attached to the 4″-OH. This also influenced the chemical shifts of the methyl groups attached to the neighbouring 5″-C, resulting in near coincidence of the proton signals of 6″-CH_3 and 7″-CH_3.

It was therefore possible to assign the structures depicted in Figure 2 to novclobiocins 112, 113 and 120.

5. Testing of antibacterial activity

Aminocoumarin antibiotics are primarily active against Gram-positive bacteria. The activities of the three new compounds against *Bacillus subtilis* ATTC 14893 were tested in a disc diffusion assay. Novclobiocin 112 showed approximately 5% of the activity of clorobiocin, showing that the 4″-OMe group was very important for antibacterial activity. Transfer of the acyl moiety from 3″-OH to 2″-OH resulted in some further reduction of activity, as novclobiocin 113 was half as active as novclobiocin 112. In contrast, novclobiocin 120, with the acyl moiety at 4″-OH, was twice as active as novclobiocin 112, reaching 10% of the activity of clorobiocin.

Discussion

The production of new antibiotics by genetic engineering of the producer strain has emerged as an important new tool in drug development. Recently developed methods for the expression of entire biosynthetic gene clusters in heterologous hosts[15] may allow a significant acceleration of such approaches. In this study we have used these methods for the inactivation of the putative methyltransferase gene *cloP* and for the generation of new clorobiocin derivatives.

Sequence analysis had suggested that *cloP* may code for an SAM-dependent methyltransferase that methylates the 4″-OH group in clorobiocin biosynthesis. Our study now provides experimental confirmation of this hypothesis. Inactivation of *cloP* resulted in the loss of the 4″-Me group—that is, in the accumulation of novclobiocin 112—while other parts of the molecule, including the methyl groups attached to C-5″ and to C-5‴, were not affected.

The inactivation of *cloP* in this study was not carried out in the genome of the natural producer, *Streptomyces roseochromogenes* var. *oscitans* DS 12.976, but rather in a cosmid containing the biosynthetic gene cluster of clorobiocin, which was subsequently expressed in the heterologous host *S. coelicolor* M512. As observed previously,[16] production levels of clorobiocin and its derivatives were quite high in the heterologous host, amounting to 12.8, 5.8 and 4.1 mg L^{-1} for novclobiocins 112, 113 and 120, respectively. This provides a further illustration that modified aminocoumarin antibiotics can be successfully produced by heterologous expression of genetically modified clusters, as shown recently by our group.[16]

In this study we have inactivated *cloP* by gene replacement with a resistance gene (*aac(3)IV*) under control of its own promoter. Desmethylclorobiocin production was readily observed

in the *cloP*$^-$ integration mutants, which still contained the *aac(3)IV* cassette, showing that transcription and translation of the genes *cloQR*, responsible for the biosynthesis of the prenylated 4-hydroxybenzoate moiety[20,21] and of *cloSTUVW*, responsible for the biosynthesis of the deoxysugar moiety,[13] had not been affected. Downstream of *cloP*, a relatively large (1000 bp) intergenic region is found that might contain a promoter region driving the transcription of the downstream genes *cloQRSTUVW*. Alternatively, transcription of these genes in our mutants might have been driven by the promoter of the inserted resistance gene *aac(3)IV*.

Inactivation of *cloP* had resulted in the production of 4″-desmethylclorobiocin (= novclobiocin 112), together with its isomer novclobiocin 113, with the 5-methylpyrrole-2-carbonyl moiety attached to 2″-OH rather than to 3″-OH. Since the corresponding isomers of clorobiocin and novobiocin are always found as minor products in the natural producers of these antibiotics,[22,23] this result was expected. However, for the first time we have now detected a compound in which the acyl group is attached to the 4″-OH group of the deoxy sugar. No clorobiocin or novobiocin derivatives of such a structure have been reported before.

As we recently discussed,[4] 4″-O-methylation and 3″-O-acylation belong to the last steps of the biosynthesis of the antibiotic clorobiocin, but the sequence of these two steps has not been unequivocally determined yet. This study shows that, if the 4″-OH group is not blocked by methylation, the producing organism accumulates a mixture of compounds acylated at 3″, 2″ and 4″. Since 4″-acylated clorobiocin isomers have never been observed in *cloP*$^+$ strains, this might indicate that 4″-methylation precedes acylation in the natural biosynthetic reaction sequence.

X-ray crystallographic studies of the complexes formed between novobiocin and clorobiocin with the gyrase B subunit did not suggest that hydrophobic interaction of the 4″-O-CH_3 group with *E. coli* gyrase contributes significantly to the binding of the antibiotic to its target.[24] However, our study now shows that removal of this 4″-Me group strongly reduced antibacterial activity, by a factor of approximately 20. Still, removal of the 5-methylpyrrole-2-carbonyl moiety has an even more pronounced effect, resulting in a hundredfold reduction in activity[19,25] and showing that the hydrogen bonds between the latter group and gyrase are even more important for the biological activity than the hydrophobic interactions between the 4″-Me group and the enzyme. In accordance with previous studies,[15,25] shifting of the 5-methylpyrrole-2-carbonyl moiety from 3″-OH to 2″-OH moderately reduced the activity. In contrast, shifting of this group to 4″-OH increased the activity. Apparently, there is sufficient flexibility in the clorobiocin–gyrase B interaction to allow attachment of the bulky 5-methylpyrrole-2-carbonyl group at 3″-, 4″- or 2″-OH while retaining its ability to occupy the binding pocket in the gyrase protein. The binding of the new clorobiocin derivatives to gyrase in vitro, in comparison with other novobiocin and clorobiocin analogues, is currently being investigated.

This study demonstrates a route for the production of clorobiocin derivatives lacking the 4″-Me group, while our previous studies showed methods to produce clorobiocin derivatives lacking the 3″-acyl group.[19,26] Clorobiocin derivatives lacking substituents at 4″-OH and/or 3″-OH can thus be produced and used for the generation of new aminocoumarin antibiotics by chemical methods.

Experimental Section

Bacterial strains, cosmids and culture conditions: *Streptomyces coelicolor* M512 (Δ*redD* Δ*actII-ORF4* SCP1$^-$ SCP2$^-$)[27] was kindly provided by E. Takano (Tübingen, Germany) and Janet White (Norwich, UK). The strains were cultured as described previously.[16,28]

Escherichia coli strains ET12567 and XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) were used for cloning experiments and grown as described.[29]

The REDIRECT technology kit for PCR targeting,[30] was obtained from Plant Bioscience Limited (Norwich, UK).

Kanamycin (50 μg mL^{-1} for *Streptomyces* and for *E. coli*), chloramphenicol (25–50 μg mL^{-1}) and apramycin (50 μg mL^{-1}) were used for selection of recombinant strains.

Cosmid clo-BG1 and plasmid pUG019 have been described previously.[16]

DNA isolation, manipulation and cloning: Standard procedures for DNA isolation and manipulation were performed as described by Sambrook et al.[29] and Kieser et al.[28] Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kits, Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA was isolated from *Streptomyces* strains by lysozyme treatment and phenol/chloroform extraction.[28]

Southern blot analysis was performed on Hybond-N nylon membrane (Amersham Biosciences, Freiburg, Germany) with digoxigenin-labelled probe with the DIG high prime DNA labelling and detection starter kit II (Roche Applied Science, Mannheim, Germany).

PCR reactions were carried out with the Vent Polymerase (New England Biolabs) according to the manufacturer's instructions.

Replacement of cloP with aac(3)IV and generation of clo-HR1: The apramycin resistance cassette (approximately 1 kb) was excised from pUG019 by digestion with *Eco*RI and *Hind*III and amplified by PCR by using the forward primer 5′-**CCTCGTCAACCTTCTGAGTCGAGGCACTGGACCCGAATG**ATTCCGGGGATCTCTAGATC-3′ and the reverse primer 5′-**GAAACCAGCATCCCGCATTGATCAACTCGCTGTGGACTA**ACTAGTCTGGAGCTGCTTC-3′. The *Xba*I and *Spe*I restriction sites are underlined, whilst bold print shows the homologous extensions to the DNA regions immediately upstream and downstream of *cloP*, respectively. PCR was performed with an annealing temperature of 55 °C. The PCR product was used for gene replacement in cosmid clo-BG1 by λ-Red-mediated recombination as described in ref. [30] resulting in cosmid clo-HR1.

Heterologous expression of clo-HR1 in S. coelicolor M512: Because of the potent methylation restriction system of *S. coelicolor*, cosmid DNA had to be passed through a nonmethylating host. We used *E. coli* ET12567 for this purpose.[15] The modified cosmid clo-HR1, still carrying the kanamycin resistance gene *neo*, was then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation.[28] Clones resistant to kanamycin and apramycin were selected and checked for site-specific integration into the genome by Southern blot analysis.

Table 1. ^{1}H NMR data for clorobiocin and clorobiocin analogues.[a]

Position	Novclobiocin 104	Clorobiocin	δ, multiplicity (J [Hz]) Novoclobiocin 112	Novoclobiocin 113	Novoclobiocin 120
2-H	7.76, brs	7.76, d (2.5)	7.74, brs	7.76, brs	7.77, brs
5-H	6.84, d (8.5)	6.84, d (8.4)	6.85, d (8.2)	6.80, d (8.2)	6.80, d (8.7)
6-H	7.72, dd (8.5, 2.2)	7.72, dd (8.4, 2.5)	7.69, dd (8.2, 2.2)	7.71, brd (8.2)	7.72, brd (8.7)
7-H2	3.34, d (7.2)	3.34, d (7.1)	3.33[b]	3.32[b]	3.32[b]
8-H	5.35 brt (7.2)	5.35, brt (7.1)	5.35, brt (7.3)	5.34, brt (7.0)	5.36, brt (6.8)
10-H3	1.74, s	1.74, s	1.74, s	1.73, s	1.73, s
11-H3	1.75, s	1.75, s	1.76, s	1.73, s	1.73, s
5′-H	7.88, d (9.0)	7.90, d (9.2)	7.89, d (9.0)	7.86, d (9.0)	7.90, d (8.7)
6′-H	7.29, d (9.0)	7.33, d (9.2)	7.35, d (9.2)	7.22, d (9.0)	7.24, d (8.9)
1″-H	5.65, d (1.6)	5.73, d (1.8)	5.75, brs	5.82, brs	5.74, brs
2″-H	4.12, t (3.1)	4.34, t (2.7)	4.43, brs	5.42, brs	4.21, brs
3″-H	4.18, dd (9.9, 3.1)	5.71, dd (10.3; 2.9)	5.58, dd (10.4, 3.1)	4.40, dd (10.1, 3.4)	4.37, dd (9.8, 3.3)
4″-H	3.40, d (9.9)	3.72, d (10.3)	4.08, d (10.4)	3.92, d (10.1)	5.42, d (9.8)
6″-H3	1.10, s	1.18, s	1.21, s	1.21, s	1.25, s
7″-H3	1.31, s	1.35, s	1.35, s	1.36, s	1.28, s
8″-OCH3	3.59, s	3.52, s	–	–	–
3‴-H	–	6.90, d (3.6)	6.92, d (3.7)	6.87, d (3.6)	6.82, d (3.7)
4‴-H	–	5.94, d (3.6)	5.93, d (3.6)	5.94, d (3.5)	5.91, d (3.2)
6‴-H3	–	2.29, s	2.29, s	2.28, s	2.27, s

[a] The numbering of the structures is shown in Scheme 1. Novclobiocins 112, 113 and 120 (see Figure 2 for formulas) were generated in this study. Novclobiocin 104 corresponds to clorobiocin in structure, but lacks the 5-methylpyrrole-2-carbonyl moiety.[19] [b] Overlap with solvent signal.

Production and analysis of secondary metabolites: Transformants of *S. coelicolor*, harbouring clo-BG1 or clo-HR1, respectively, were cultured and assayed for the production of clorobiocin derivatives by HPLC as described previously,[16] by using a Multosphere RP18–5 column (250×4 mm; 5 μm; Chromatographie Service, Düren, Germany) at a flow rate of 1 mL min^{-1}, with a linear gradient from 60 to 100% of solvent B in 30 min (solvent A: MeOH/H_2O/HCOOH 20:79:1; solvent B: MeOH/HCOOH 99:1) and detection at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation, cultivation was carried out in 500 mL baffled flasks containing 50 mL medium as described above. A total of 1000 mL bacterial culture was pooled, acidified with HCl to pH 4 and extracted with ethyl acetate after removal of the lipophilic components by treatment with petroleum ether. The residue from the ethyl acetate extract after evaporation of the solvent was dissolved in methanol (4 mL) and passed through a glass column (2.6 cm×65 cm) filled with Sephadex LH 20 (Amersham Biosciences, Freiburg, Germany) and eluted with methanol. The fractions obtained after separation on Sephadex LH 20 were analysed by HPLC under the conditions described above. Fractions containing aminocoumarin derivatives were pooled and further purified on a preparative HPLC column (Multosphere 120 RP18–5, 5 μm, 250×10 mm, Chromatographie Service, Düren, Germany) with the same solvents and gradient as for the analytical column, but with a flow rate of 2.5 mL min^{-1}. Novclobiocins 113 and 120 coeluted in this procedure and had to be separated in a second preparative HPLC step, with a linear gradient from 30 to 100% of solvent D in 30 min (solvent C: acetonitrile/H_2O/HCOOH 79:20:1; solvent D: acetonitrile/HCOOH 99:1). The purified compounds were subjected to ^{1}H NMR and MS analysis.

Negative-ion FAB mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) with use of diethanolamine as matrix. MS data [*m/z* (relative intensity in %)] were as follows:

Novclobiocin 112: 681 (18, [*M*−H]$^-$), 517 (6), 501 (12), 414 (14), 357 (7), 283 (77) 281 (62), 255 (100).

Novclobiocin 113: 681 (8, [*M*−H]$^-$), 517 (12), 501 (16), 415 (12), 357 (29), 281 (74), 255 (100).

Novclobiocin 120: 681 (5, [*M*−H]$^-$), 517 (5), 515 (8), 501 (25), 415 (11), 357 (38), 281 (100), 255 (79).

^{1}H NMR spectra were measured on an AMX 400 spectrometer (Bruker, Karlsruhe, Germany), with CD_3OD as solvent. The ^{1}H NMR data for the isolated compounds are given in Table 1.

Bioassay: The antibacterial activities of authentic clorobiocin (Aventis) and the isolated compounds against *Bacillus subtilis* ATCC 14893 were determined by a disc-diffusion assay, as described elsewhere.[31]

Nucleotide sequence accession number: The nucleotide sequence employed in this study is available in the GenBank database under the accession numbers AF329398 (clorobiocin cluster) and AY136281 (*gyrB*^{R} and *parY*^{R} of the clorobiocin cluster).

Acknowledgements

This work was supported by a grant from the European Commission (503466 CombiGyrase) to L.H. and S.-M.L.

Keywords: antibiotics · biosynthesis · heterologous expression · methyltransferase · *Streptomyces roseochromogenes*

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Received: January 18, 2005
Published online on June 24, 2005

