CloN6, a Novel Methyltransferase Catalysing the Methylation of the Pyrrole-2-carboxyl Moiety of Clorobiocin

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The aminocoumarin antibiotic clorobiocin contains a 5-methylpyrrole-2-carboxylic acid unit. This pyrrole unit is derived from L*-proline, and it would be expected that its 5-methyl group should be introduced by a methylation reaction. However, sequence analysis of the clorobiocin biosynthetic gene cluster did not reveal a gene with sequence similarity to the SAM-dependent methyltransferases that could be assigned to this reaction. This study, however, has provided evidence that the gene* cloN6 *is involved in this methylation reaction. Its gene product CloN6 shares conserved sequence motifs with the recently identified radical SAM protein superfamily, and it has been suggested that members of this family can catalyse methylcobalamin-dependent methylation reactions.* cloN6 *was inactivated in the clorobiocin producer* Streptomyces roseochromogenes var. oscitans *DS 12.976 by use of the PCR-targeting method. The* cloN6$^-$ *mutants accumulated, instead of clorobiocin, a derivative lacking the 5′′′-methyl group of the pyrrole moiety (termed novclobiocin 109). A structural isomer carrying the pyrrole-2-carboxyl moiety at 2′′-OH rather than at the 3′′-OH of the deoxysugar (novclobiocin 110), and a derivative completely lacking the pyrrole unit (novclobiocin 104) were also identified. The structures of the metabolites were confirmed by NMR and MS analysis. Antibacterial activity tests against* Bacillus subtilis *showed that novclobiocin 109 and novclobiocin 110 have antibacterial activities about eight times less than that of clorobiocin, whereas novclobiocin 104 showed no activity under the test conditions.*

KEYWORDS:

aminocoumarin antibiotics · antibiotics · biosynthesis · pyrrole-2-carboxylic acid · methyltransferases

Introduction

The structurally related aminocoumarin antibiotics clorobiocin, novobiocin and coumermycin A_1 are potent inhibitors of DNA gyrase and are produced by different *Streptomyces* strains.[1–3] Both clorobiocin and coumermycin A_1 contain 5-methylpyrrole-2-carboxyl moieties attached to the 3′′-OH group of the deoxysugar by ester bonds (Scheme 1), whereas novobiocin carries a carbamyl group at this position.

It has been reported that clorobiocin is at least ten times more active than novobiocin, as determined by its affinity for gyrase and by its antibacterial activity.[1, 4] A comparison of the crystal structures of the complexes of clorobiocin and novobiocin with a 24 kDa fragment of the gyrase B subunit from *E. coli* showed the overall structures of the two complexes to be very similar. The 5-methylpyrrole-2-carboxyl moiety of clorobiocin sits in a hydrophobic pocket and displaces two ordered water molecules which are present in the complex of novobiocin with gyrase. This displacement is entropically favourable and is likely to contribute to the increased affinity of gyrase for clorobiocin.[3]

The 5-methylpyrrole-2-carboxyl moieties of clorobiocin and coumermycin A_1 are derived from L-proline,[5–7] so proline thereby contributes the pyrrole ring and the carboxyl group of that moiety. It is likely that the 5-methyl group is introduced by a methylation reaction.

We have recently cloned the clorobiocin biosynthetic gene cluster.[8] Sequence analysis revealed two genes with sequence similarity to S-adenosylmethionine(SAM)-dependent methyltransferases: *cloP* and *cloU*. However, previous evidence strongly suggests that the products of these two genes are responsible for the 4-*O*-methylation and the 5-*C*-methylation reaction of the deoxysugar moiety of clorobiocin.[8–10] No additional gene for a putative SAM-dependent methyltransferase that might have been assigned to the postulated 5-methylation of the pyrrole-2-carboxyl moiety was identified. However, the biosynthetic gene clusters of clorobiocin and coumermycin A_1 each contain a contiguous group of seven genes—*cloN1*–*cloN7* and *couN1*–*couN7*, respectively—for which no homologues exist in the novobiocin cluster.[8] This group contains several genes involved in the biosynthesis of the pyrrole-2-carboxyl moieties. CloN3, CloN4 and CloN5, and their homologues encoded by the coumermycin A_1 cluster, catalyse the conversion of L-proline

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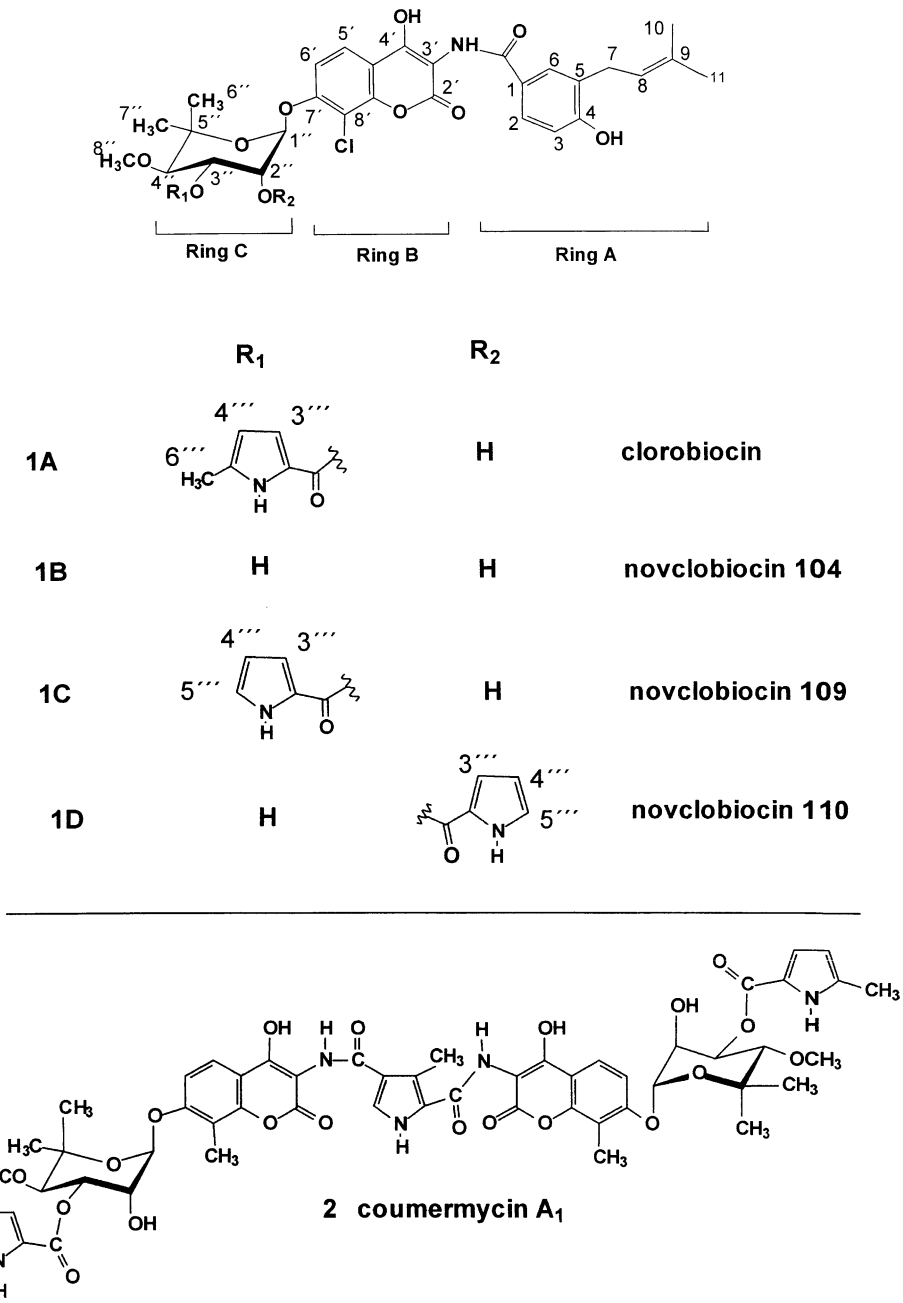

Scheme 1. Structures of aminocoumarin antibiotics.

into pyrrole-2-carboxyl-PCP (PCP = peptidyl carrier protein).[6, 7] CloN2 shows similarity to acyltransferases and it has been suggested that it transfers the pyrrole-2-carboxyl moiety to the 3″-OH group of the deoxysugar.[8, 10] We speculated that the methyltransferase responsible for the 5-methylation of the pyrrole-2-carboxyl moiety may also be encoded within the *cloN1 – cloN7* fragment. *cloN7* shows sequence similarity to hydrolases, and *cloN1* encodes a small hypothetical protein of 95 amino acids. These genes therefore appeared unlikely to encode methyltransferases. Therefore, *cloN6* was left as a candidate for the structural gene of the pyrrole-5-methyltransferase.

To investigate the possible involvement of *cloN6* in the methyltransfer reaction, we have now inactivated this gene in the clorobiocin producer *Streptomyces roseochromogenes var. oscitans* DS 12.976. This experiment also offered the prospect of producing new clorobiocin derivatives lacking the methyl group at the pyrrole unit and of investigating the contribution of this methyl group to the biological activity of clorobiocin.

Results

Sequence analysis of *cloN6*

The predicted gene products of *cloN6* of the clorobiocin biosynthetic cluster, and its homologue *couN6* of the coumermycin A_1 biosynthetic cluster, comprise 561 and 560 amino acids, respectively, and have 94% similarity with each other.

When we first published the sequence of *couN6* (formerly called *cumK*,[10] BLAST searches did not reveal meaningful homologies of this open reading frame (ORF) to proteins with known functions in the database. In the meantime, Sofia et al.[11] have identified, by bioinformatic techniques, the radical SAM protein superfamily, and CloN6 and CouN6 both show the cysteine motif CxxxCxxC (from amino acids 219 to 226) conserved in all members of this family. These cysteines are believed to form part of an iron – sulfur cluster, situated close to the *N*-terminus of the "classical" radical SAM enzymes such as lysine 2,3-aminomutase, pyruvate formate-lyase activating enzyme and biotin synthase.[12] In contrast to these enzymes, CloN6 and CouN6 each display a long *N*-terminal extension comprising a putative cobalamin-binding site. This places CloN6 and CouN6 in a certain subgroup within the radical SAM superfamily identified by Sofia et al.[11] This so-called "BchE-like/methyltransferase subgroup" incorporates several methyltransferases which have been suggested to catalyse methylcobalamin-dependent methylation

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reactions in the biosynthesis of different antibiotics, such as fortimicin KL1, bialaphos and fosfomycin.[13]

Inactivation of *cloN6* in *Streptomyces roseochromogenes*

An inactivation of *cloN6* in the clorobiocin producer *Streptomyces roseochromogenes* was performed by use of the PCR-targeting method described by Gust et al.[14] In cosmid D1A8, the entire coding sequence of *cloN6* was replaced by an apramycin resistance/*oriT* cassette (see Experimental Section). The modified cosmid LW17 was introduced into *S. roseochromogenes* by conjugation. Apramycin-resistant, kanamycin-sensitive colonies, resulting from double crossover events, were selected. Southern blot analysis confirmed that *cloN6* had been replaced by the apramycin resistance/*oriT* cassette in these mutants (Figure 1).

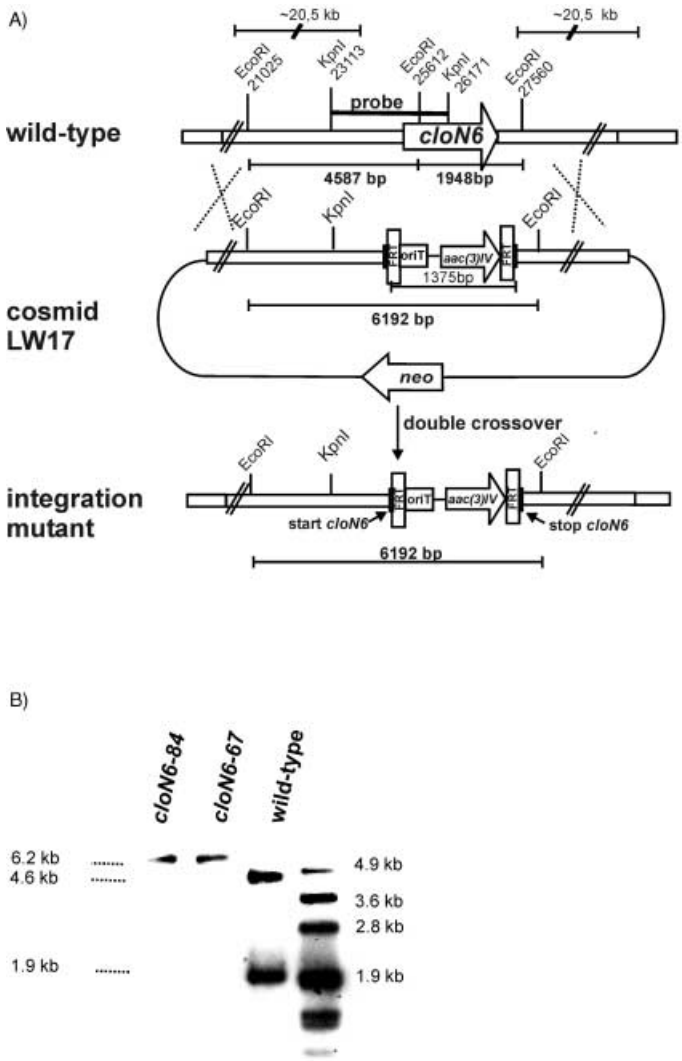

Figure 1. Inactivation of cloN6 *in* S. roseochromogenes. *A) Schematic presentation of the* cloN6 *inactivation experiment. The DNA fragment used as probe is indicated as a black bar. B) Southern blot analysis of wild-type and* cloN6⁻ *mutants. Genomic DNA was restricted by* EcoRI. *Strains* cloN6 – 67 *and* cloN6 – 84 *are two independent double crossover mutants.* aac(3)IV: *apramycin resistance gene, FRT: FLP recognition target,* oriT: *origin of transfer from RK2,* neo: *kanamycin resistance gene.*

Analysis of secondary metabolite production in *cloN6*⁻ mutants

For analysis of secondary metabolite formation, both wild type and *cloN6*⁻ mutants were cultured in production medium as described previously,[8, 15] Ethyl acetate extracts of the cultures were analysed by HPLC. The wild-type strain extract contained clorobiocin, with a retention time of 19.4 min, as the dominant product (Figure 2 A), identified by co-chromatography with an authentic reference substance.

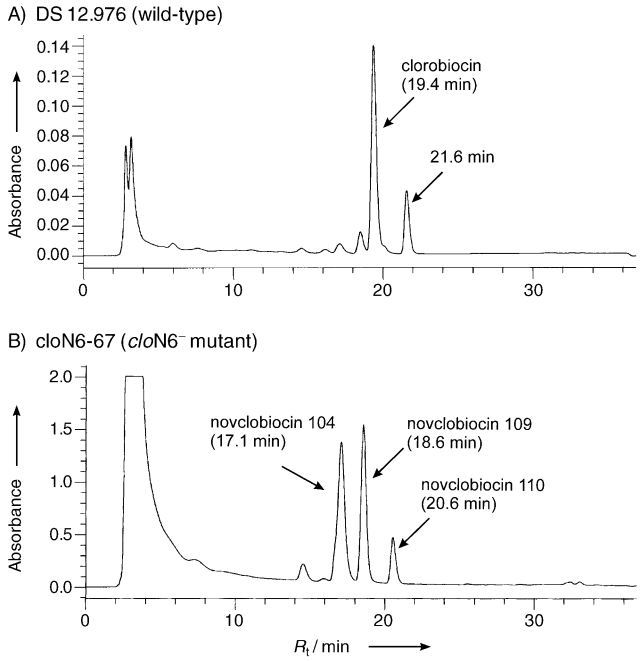

Figure 2. HPLC analysis of secondary metabolite production. (A) S. roseochromogenes *var.* oscitans *wild type. (B)* cloN6⁻ *mutant. More culture extract was injected for the HPLC chromatogram in B than for the chromatogram in A. For chromatograms A and B, extracts from 0.2 and 6.7 mL bacterial culture were injected, respectively.*

In contrast, the *cloN6*⁻ mutants accumulated not clorobiocin but three new products with retention times of 17.1, 18.6 and 20.6 min, respectively (Figure 2 B). These were designated novclobiocin 104, novclobiocin 109 and novclobiocin 110, respectively. To elucidate their structures, these products were isolated on a preparative scale by column chromatography on Sephadex LH 20 and preparative reversed-phase HPLC. The structures of the purified compounds were elucidated by MS and ^{1}H NMR. The NMR data are summarized in Table 1.

Structural elucidation of the novclobiocins

Negative FAB-MS of novclobiocin 109 showed $[M-H]^-$ peaks with an isotopic pattern typical of monochlorinated compounds at $m/z = 681$ and 683, 14 mass units less than those of clorobiocin, corresponding to the loss of a methyl group. Comparison of the ^{1}H NMR spectra of novclobiocin 109 and clorobiocin indicated that the signals for all the protons of rings A, B and C of clorobiocin were also present in the spectrum of novclobiocin 109 (Table 1). Significant differences, however,

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Table 1. ^{1}H NMR data for clorobiocin and novclobiocins (CD_3OD, 250 MHz)

position	clorobiocin	novclobiocin 104	novclobiocin 109	novclobiocin 110
H-2	7.76, d, (2.5)	7.76, brs	7.77, brs	7.76, brs
H-5	6.84, d, (8.4)	6.84, d, (8.4)	6.82, d, (8.3)	6.82, d, (8.4)
H-6	7.72, dd, (8.4,2.5)	7.72, dd, (8.5, 2.2)	7.72, dd, (8.3, 2.2)	7.70,d, (8.4)
2H-7	3.34, d, (7.1)	3.34, d, (7.3)	3.34, d, (7.2)	3.34,d, (7.2)
H-8	5.35, brt (7.1)	5.35, brt, (7.2)	5.36, brt, (7.2)	5.36, brt, (7.2)
3H-10	1.74, s	1.75, s	1.74, s	1.73, s
3H-11	1.75, s	1.74, s	1.74, s	1.73, s
H-5′	7.90, d, (9.2)	7.88, d, (9.0)	7.91, d, (9.0)	7.89, d, (8.9)
H-6′	7.33, d, (9.2)	7.29, d, (9.0)	7.16, d, (9.0)	7.24, d, (8.9)
H-1″	5.73, d, (1.8)	5.65, d, (1.0)	5.72 m[b]	5.80, d, (1.4)
H-2″	4.34, t (2.7)	4.12, t (2.9)	4.35, t (2.7)	5.44, t, (2.5)
H-3″	5.71, dd (10.0, 3.0)	4.18, dd, (10.0, 3.3)	5.72, m[b]	4.45, dd, (9.9, 3.5)
H-4″	3.72, dd (10.3)	3.40, d, (9.8)	3.74, d, (10.2)	3.55, d, (9.9)
3H-6″	1.18, s	1.10, s	1.19, s	1.18, s
3H-7″	1.35, s	1.31, s	1.35, s	1.38, s
3H-8″	3.52, s	3.59, s	3.53, s	3.64, s
H-3‴	6.90, d, (3.6)	-	7.02, m[c]	7.03, dd, (2.8,1.8)
H-4‴	5.94, brd (3.6)	-	6.24, dd (3.7, 2.5)	5.80, dd, (3.8, 2.8)
H-5‴	-	-	7.02, m[c]	6.99, dd, (3.8,1.8)
3H-6‴	2.29, s	-	-	-

For numbering of the protons see Scheme 1. [a] δ are given in ppm and coupling constants (J) in Hz. [b] Signals of H-1″ and H-3″ of novclobiocin 109 overlapped with each other. [c] Signals of H-3‴ and H-5‴ of novclobiocin 109 overlapped with each other

were observed for signals of the pyrrole moiety. The two doublets at 6.90 and 5.94 ppm for H-3‴ and H-4‴ of the pyrrole unit of clorobiocin had disappeared, as had the singlet at 2.29 ppm for the methyl group at C-5‴. Instead, signals for three protons at 7.02 (2 H) and 6.24 (1 H) were observed, representing the signals for H-3‴, H-4‴ and H-5‴ of the pyrrole unit of novclobiocin 109. This confirmed that novclobiocin 109 is a clorobiocin derivative lacking the methyl group at the pyrrole moiety (Scheme 1 C).

The negative FAB mass spectrum of novclobiocin 110 showed $[M-H]^-$ peaks identical to those of novclobiocin 109 ($m/z = 681$ and 683), an identical isotopic pattern indicating a monochlorinated compound and identical main fragments at $m/z = 647$ and 493. It can be deduced that these two substances are isomers. Inspection of the ^{1}H NMR spectra of novclobiocin 109 and novclobiocin 110 revealed that the signals for the protons of ring A of both substances were unchanged. The chemical shift of H-6′ of ring B was slightly shifted from 7.16 ppm in novclobiocin 109 to 7.24 ppm in novclobiocin 110. However, significant changes were observed for protons of the deoxysugar, especially for the protons H-2″ and H-3″, with a downfield shift of 1.09 ppm for H-2″ (from 4.35 ppm in novclobiocin 109 to 5.44 ppm in novclobiocin 110) and an upfield shift of 1.27 ppm for H-3″ (from 5.72 ppm in novclobiocin 109 to 4.45 ppm in novclobiocin 110) (Table 1). These shifts can be explained by the migration of the pyrrole unit from 3″-OH in novclobiocin 109 to 2″-OH in novclobiocin 110 (Scheme 1 D). Similar observations were reported for novobiocin and isonovobiocin.[16] The three doublet of doublets for H-3‴, H-4‴ and H-5‴ in the pyrrole unit in novclobiocin 110 could be clearly identified at 7.03, 5.80 and 6.99 ppm, respectively. The coupling pattern and coupling constants of these three protons are consistent with those of free pyrrole-2-carboxylic acid.[17]

In the ^{1}H NMR spectrum of novclobiocin 104, all proton signals of the pyrrole unit were missing and no additional signals were observed. The signals of the deoxysugar, but not of ring A and B, have changed in comparison to those in clorobiocin, novclobiocin 109 and novclobiocin 110 (Table 1). All of the signals for H-1″, H-2″, H-3″ and H-4″ in novclobiocin 104 have been shifted upfield relative to the corresponding protons in the other three substances. These changes indicate the lack of the pyrrole unit in novclobiocin 104, and this was also confirmed by MS, showing two isotopic peaks of $[M-H]^-$ at $m/z = 590/588$ and by a fragmentation pattern similar to that of clorobiocin and its derivatives.

The *cloN6*$^-$ mutants produced novclobiocins 104, 109 and 110 in amounts of approximately 26.3, 5.3 and 1.6 nmol per mL bacterial culture, respectively. The total production of clorobiocin derivatives (33.2 nmol per mL) in the mutants was even higher than the clorobiocin production of the wild type (16 nmol per mL).

Antibacterial activity of novclobiocin 104, novclobiocin 109 and novclobiocin 110 in comparison to clorobiocin

Authentic clorobiocin and novclobiocins 104, 109 and 110 were assayed for antibiotic activity against *Bacillus subtilis*; an example of an assay plate is shown in Figure 3. Clorobiocin showed the highest antibacterial activity, followed by novclobiocin 109 and novclobiocin 110 (both approximately eight times less active than clorobiocin). Novclobiocin 104 showed no activity. The results show that the pyrrole-2-carboxyl substituent at the sugar moiety is essential for antibacterial activity. The presence of the

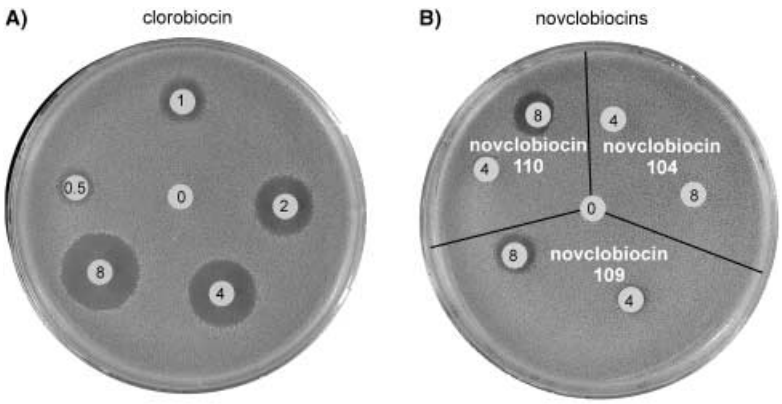

Figure 3. Antibacterial activity of clorobiocin and derivatives: A) clorobiocin, B) novclobiocins. The bioassay was carried out with Bacillus subtilis *and the numbers on the filter paper indicate the amount of the applied substance in nmol. For structures see Scheme 1.*

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methyl group at C-5‴ of the pyrrole ring clearly enhances the activity of the aminocoumarin antibiotics against this test organism. However, the shifting of the pyrrole substituent from 3″-OH to 2″-OH of the deoxysugar does not significantly influence antibacterial activity.

Discussion

In this study we have provided experimental evidence that CloN6 is involved in the methylation of the pyrrole unit of clorobiocin at C-5‴. Since CloN6, and its homologue CouN6, share characteristic sequence motifs with methyltransferases involved in fortimicin KL1, bialaphos and fosfomycin biosynthesis,[13] it may be speculated that all these enzymes catalyse similar reactions. Preliminary evidence suggests that methylcobalamin, rather than SAM, is the immediate methyl donor in the described methylation reactions in bialaphos and fosfomycin biosynthesis.[18, 19] A methylcobalamin-dependent methyl transfer reaction is well characterized in the case of methionine synthase,[20] but few data exist for other enzymes (see, for example, ref. [21, 22]). So far, no methylcobalamin-dependent methyltransferase involved in antibiotic biosynthesis has been purified and shown to be enzymatically active in vitro.

Inactivation of *cloN6* in *Streptomyces roseochromogenes* resulted in abolition of clorobiocin production and in the formation of new metabolites, which were evaluated for their biological activity. Novclobiocin 109 was the expected clorobiocin derivative lacking the methyl group at C-5‴ of the pyrrole unit.

As an additional metabolite, novclobiocin 110, an isomer of novclobiocin 109 carrying the pyrrole unit at 2″-OH of the deoxysugar, was also isolated from the *cloN6*$^-$ mutant. We have observed previously[8] that the wild type also produces two isomers of clorobiocin (see also Figure 2 A). It appears likely that the peak detected in the HPLC analysis of the wild type at 21.6 min (Figure 2 A) represents the clorobiocin isomer carrying the 5-methylpyrrole-2-carboxyl unit at position 2″ of the deoxysugar rather than 3″. It is unknown whether the occurrence of these isomers indicates that the reaction catalysed by the putative acyltransferase CloN2 is not completely regiospecific or that a chemical rearrangement occurs in the clorobiocin molecule.

Antibacterial test showed that novclobiocin 109, which lacks the methyl group at C-5‴ of the pyrrole unit, is clearly less active than clorobiocin, establishing an important contribution of this methyl group to the antibiotic activity. In contrast, novclobiocin 110, carrying the pyrrole-2-carboxyl unit at 2″-OH, has antibacterial activity similar to that of novclobiocin 109, indicating that the position of the pyrrole-2-carboxyl unit on the deoxysugar has no significant influence on the activity. This is at variance with results obtained with novobiocin derivatives. Isonovobiocin, the result of a migration of the carbamyl group from 3″- to 2″-OH,[23] and 2″-O-carbamylnovobiocin, the result of carbamylation both of 2″-OH and of 3″-OH,[24] both show much less antibacterial activity than novobiocin.

Novclobiocin 104, which completely lacks the pyrrole-2-carboxyl moiety, showed no antibacterial activity in the tested concentration range. This is consistent with the reported results for decarbamylnovobiocin.[4]

Experimental Section

Bacterial strains, cosmids and culture conditions

Cosmid D1A8 in the SuperCos1 vector contained the clorobiocin biosynthetic gene cluster.[8]

Streptomyces roseochromogenes was routinely cultured in baffled Erlenmeyer flasks containing a stainless steel spring. YMG medium liquid or solid (2% agar) containing 1% malt extract, 0.4% yeast extract, 0.4% glucose (pH 7.3) was used routinely for cultivation, carried out at 28–30 °C and 180 rpm for 2 to 3 days. For preparation of genomic DNA, the strains of *Streptomyces roseochromogenes* were cultured in YEME medium without glycine (0.3% yeast extract, 0.5% proteose peptone No 3, 0.3% malt extract, 1% glucose, 34% sucrose, 0.1% $MgCl_2 \times 6\ H_2O$).[25] For conjugation, *Streptomyces roseochromogenes* was cultured in a 1:1 mixture of TSB (3% trypticase soy broth) and YEME medium[25] without glycine for 2 to 3 days at 30 °C and 180 rpm. 2xYT broth, containing 1.6% tryptone, 1% yeast extract and 0.5% NaCl (pH 7.0), was used during conjugation.

Escherichia coli XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) was used for propagation of cosmids and grown in liquid or on solid Luria–Bertani medium (containing 1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0; 1.5% agar) at 37 °C.[26]

The REDIRECT© technology kit containing *E. coli* ET12567, *E. coli* ET12567/pUZ8002, *E. coli* BW25113/pIJ790 and *E. coli* DH5α/pIJ773[14] was obtained from Plant Bioscience Limited (Norwich Research Park, Colney, Norwich, UK).

Apramycin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), chloramphenicol (25–50 μg mL^{-1}), nalidixic acid (25 μg mL^{-1}) and carbenicillin (50–100 μg mL^{-1}) were used for selection of recombinant strains.

DNA isolation, manipulation and cloning

Standard procedures for DNA isolation and manipulation were performed as described by Sambrook and Russell[26] and Kieser et al.[25] Isolation of DNA fragments from agarose gel and purification of PCR products were carried out with the NucleoSpin® 2 in 1 Extract Kit (Macherey–Nagel, Düren, Germany). Isolation of cosmids 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 the Kirby mix procedure.[25]

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

Inactivation of *cloN6* in *S. roseochromogenes*

cloN6 was inactivated by use of the PCR targeting system.[14] An apramycin resistance gene/*oriT* cassette for replacement of *cloN6* was generated by PCR from pIJ773 by use of the primer pair *cloN6f* (5′-aatagtttggatgtcccagtgaaattgaacgcggacatgattccggggatccgtcgacc) and *cloN6r* (5′-ctctctgctgttggggagtctgccgtctccgtgggttcatgtaggctggagctgcttc). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *cloN6*, respectively, including the putative start and stop codons of *cloN6*. This cassette was introduced into *E. coli* BW25113/pIJ790, containing cosmid D1A8 (SuperCos1-based, kanamycin-resistant) which included the entire biosynthetic gene cluster of clorobiocin. The gene replacement was confirmed by restriction analysis of the cosmids.

The mutated cosmid LW17 was introduced into *Streptomyces roseochromogenes* by conjugation from *E. coli* ET12567 carrying the non-transmissible pUZ8002 as described by Eustáquio et al.[27]

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Apramycin-resistant, kanamycin-sensitive colonies were identified by replica plating and characterised by Southern blot analysis. For Southern blot analysis, genomic DNA from wild type and mutants were restricted with *Eco*RI and hybridised with a *Kpn*I fragment of 3058 bp containing the *N*-terminus of *cloN6* and sequence upstream thereof. The wild-type genomic DNA showed two bands at 4.6 and 1.9 kb, whereas that of the mutants gave a single band at 6.2 kb (Figure 1).

Analysis of secondary metabolites in *S. roseochromogenes* strains

Wild-type and mutant strains of *S. roseochromogenes* were cultured in production medium containing 4.8% distiller solubles, 3.7% glucose, 0.0024% $CoCl_2 \times 6\,H_2O$, 0.6% $CaCO_3$, and 0.2% $(NH_4)_2SO_4$[8, 15] for 7 – 8 days at 33 °C and at 210 rpm.

For analytical purposes, 10 mL of the whole culture was acidified to pH 2–4 with 1 N HCl, and extracted twice with an equal volume of ethyl acetate. The organic phase solvent was removed and the residue was dissolved in 1 mL methanol. This solution was analysed by HPLC on a Multosphere 120 RP18 – 5 column (5 μm, 250 × 4 mm, C&S Chromatographie Service, Düren, Germany) by use of a linear gradient from 60 to 100 % methanol in 1 % aqueous formic acid over 25 min with a flow rate of 1 mL min^{-1}; UV detection was carried out at 340 nm. Authentic clorobiocin was used as standard.

For preparative isolation of the secondary metabolites, 400 mL of the whole culture was acidified with 1 N HCl to pH 2 – 4 and extracted with ethyl acetate after removal of the lipophilic components by treatment with petrol ether (60 – 90 °C). The residue of the ethyl acetate extract (after evaporation of the solvent) was dissolved in 3 mL methanol and passed through a glass column (100 × 2.6 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 novclobiocins were pooled and further purified on a preparative HPLC column (Multosphere 120 RP18 – 5, 5 μm, 250 × 20 mm, C&S Chromatographie Service, Düren, Germany) with the same solvents and gradient as for the analytical column, but with a flow rate of 3 mL min^{-1}. The purified compounds were subjected to NMR and MS analysis.

Negative fast-atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan) with ethanolamine as the solvent. Novclobiocin 109 gave ions at *m/z*: 683/681 $[M-H]^-$, 647, 493 and 414. Novclobiocin 110 showed similar ions at *m/z*: 683/681 $[M-H]^-$, 647 and 493. Novclobiocin 104 gave the following negative ions: *m/z* 590/588 ($[M-H]^-$), 554, 400, 225 and 209.

The NMR spectra were measured on an AC 250 spectrometer (Bruker) in CD_3OD. The NMR data of clorobiocin, novclobiocin 104, novclobiocin 109 and novclobiocin 110 are shown in Table 1.

Bioassay

Antibacterial activities of authentic clorobiocin, novclobiocin 104, novclobiocin 109 and novclobiocin 110 were tested by use of *Bacillus subtilis* ATCC 14893. For the bioassays, different amounts of clorobioicin and of the other three substances in 20 μL methanol were applied to filter paper disks (6 mm diameter) and dried in the air for 30 min. The filter disks were then placed on Difco nutrient agar plates[25] containing approximately 2×10^5 spores of *Bacillus subtilis* per mL agar medium. After culturing overnight at 37 °C, the size of the inhibition zone was determined.

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