Biosynthesis of the unusual 5,5-*gem*-dimethyl-deoxysugar noviose: investigation of the C-methyltransferase gene *cloU*

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The aminocoumarin antibiotic clorobiocin contains an unusual branched deoxysugar with a 5,5-*gem*-dimethyl structure. Inactivation of the putative C-methyltransferase gene *cloU* was carried out, which led to the loss of the axial methyl group at C-5 of this deoxysugar moiety. This result establishes the function of *cloU*, and at the same time it proves that the biosynthesis of the deoxysugar moiety of clorobiocin proceeds via a 3,5-epimerization of the dTDP-4-keto-6-deoxyglucose intermediate. The inactivation was carried out on a cosmid which contained the entire clorobiocin biosynthetic gene cluster. Expression of the modified cluster in a heterologous host led to the formation of desmethyl-clorobiocin and a structural isomer thereof. Both compounds were isolated on a preparative scale, their structures were elucidated by ¹H-NMR and mass spectroscopy and their antibacterial activity was assayed.

INTRODUCTION

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ are potent inhibitors of bacterial gyrase (Maxwell, 1999). All three compounds contain an unusual branched deoxysugar with a 5,5-*gem*-dimethyl structure (Piepersberg & Distler, 1997). In novobiocin, the 3-OH group of this sugar is acylated with a carbamoyl group, resulting in noviose. In clorobiocin and coumermycin A₁, the 3-OH group is acylated with a 5-methyl pyrrole-2-carboxyl moiety (Fig. 1). Feeding experiments with isotope-labelled precursors showed that one of the methyl groups at C-5 of noviose is derived from glucose (Birch *et al.*, 1962), the other from S-adenosylmethionine (SAM) (Birch *et al.*, 1960). Cloning of the biosynthetic gene clusters of novobiocin, clorobiocin and coumermycin A₁ (Pojer *et al.*, 2002; Steffensky *et al.*, 2000; Wang *et al.*, 2000) revealed a contiguous group of five genes, *novSTUVW* and the homologous *cloSTUVW* and *couSTUVW*, which are contained in all three clusters in identical order. Sequence analysis suggested that these genes are responsible for the biosynthesis of the deoxysugar moieties of the antibiotics: NovV shows sequence similarity to dTDP-glucose synthases, NovT to dTPD-glucose-4,6-dehydratases, NovW to dTPD-4-keto-6-deoxyglucose epimerases and NovS to dTPD-4-keto-hexose reductases. Such enzymes are commonly found in deoxysugar pathways (He & Liu, 2002).

NovU, CloU and CouU show sequence similarity to SAM-dependent methyltransferases, e.g. on average 30 % identity

to TylC3 which catalyses C-3 methylation in the biosynthesis of the branched deoxysugar mycarose during tylosin formation (Chen *et al.*, 2001). It has therefore been speculated that NovU catalyses C-5 methylation in the biosynthesis of noviose, and CloU and CouU the corresponding reactions in clorobiocin and coumermycin A₁ biosynthesis (Li & Heide, 2004).

NovW, and its homologues CloW and CouW, show sequence similarity to (d)NDP-4-keto-6-deoxyglucose epimerases. These include, for example, the di-epimerase RmlC which carries out an epimerization of both C-3 and C-5 in the biosynthesis of L-rhamnose in *E. coli* (Giraud *et al.*, 2000) and the mono-epimerase WbcA which carries out an epimerization of only C-3 in the biosynthesis of 6-deoxy-D-glucose during the formation of the O-antigen of *Yersinia enterocolitica* (Trefzer *et al.*, 1999). L-Rhamnose and 6-deoxy-L-gulose differ only in the stereochemistry at C-5.

From chemical reasoning, three pathways appear equally plausible for the formation of noviose from dTPD-4-keto-6-deoxyglucose: (a) NovW may catalyse a 3,5-epimerization, followed by a C-5 methylation by NovU and a 4-keto reduction by NovS (this pathway is depicted in Fig. 1); (b) NovW may catalyse only a 3-epimerization, followed by a C-5 methylation by NovU (in opposite stereochemistry to above) and a 4-keto reduction by NovS; (c) NovU may methylate C-5 of dTPD-4-keto-6-deoxyglucose, followed by a 3-epimerization by NovW and a 4-keto reduction by NovS. All three reaction sequences would lead to the same activated deoxysugar, which is known to be transferred to the aglycon under catalysis of the glycosyltransferase NovM

Abbreviation: SAM, S-adenosylmethionine.

Fig. 1. Proposed pathway for the biosynthesis of the deoxysugar moiety of clorobiocin.

(Albermann *et al.*, 2003; Freel Meyers *et al.*, 2003). 4-*O*-Methylation and 3-*O*-acylation have been shown to occur after the glycosyl transfer (Li & Heide, 2004).

In a first biochemical analysis of NovWUS, Thuy *et al.* (2005) incubated dTDP-4-keto-6-deoxyglucose with NovU in the presence or absence of NovW. Only in the presence of NovW did LC-MS analysis show a product with the expected mass of a methylated dTDP-4-keto-6-deoxyhexose, suggesting that epimerization by NovW must precede methylation by NovU. However, no structural elucidation of the products could be provided, and the analytical method used for investigation of the NovW product (i.e. HPLC of the dTDP-deoxysugar, either directly or after decomposition to maltol) does not allow conclusions about the stereochemistry at C-5 (Sohng *et al.*, 2004).

Recently, the crystal structure of NovW was reported (Jakimowicz *et al.*, 2006). It was observed that the structure of the active centre of NovW was different from that of the di-epimerase RmlC, but resembled that of the mono-epimerase EvaD. Therefore, Jakimowicz *et al.* (2006) raised the question whether NovW might function as a 3-mono-epimerase rather than as a 3,5-di-epimerase.

We decided to carry out an in-frame inactivation of the *cloU* gene in the clorobiocin biosynthetic gene cluster. NMR investigation of a (potentially) resulting clorobiocin derivative lacking one of the methyl groups at C-5 of the

deoxysugar would allow unequivocal determination of whether the sugar moiety is an L-rhamnose derivative or a 6-deoxy-D-glucose derivative, and would therefore clarify whether deoxysugar biosynthesis proceeds via 3,5-epimerization or via 3-epimerization. At the same time, such an experiment would present the first example of metabolic engineering of the deoxysugar biosynthetic pathway of the aminocoumarin antibiotics. This antibiotic class has been very successfully used in the past for the generation of new compounds, using metabolic engineering of the biosynthetic pathways of the aglycon of the antibiotic as well as engineering of the tailoring reactions (Li & Heide, 2005). Finally, identification of the function and the substrate of *cloU* would make this unusual 5-*C*-methyltransferase gene available for use in the metabolic engineering of deoxysugar pathways, as described by Salas and coworkers (Lombo *et al.*, 2004; Perez *et al.*, 2005; Rodriguez *et al.*, 2002).

METHODS

Bacterial strains and culture conditions. *Streptomyces coelicolor* M512 ($\Delta redD \Delta actII-ORF4$ SCP1[−] SCP2[−]) (Floriano & Bibb, 1996) was cultured as described previously (Eustáquio *et al.*, 2004; Kieser *et al.*, 2000). *Escherichia coli* strains ET12567 (MacNeil *et al.*, 1992) and XL-1 Blue MRF' (Stratagene) were used for cloning experiments and grown as described by Sambrook & Russell (2001). The REDIRECT technology kit for PCR targeting (Gust *et al.*, 2003) was obtained from Plant Bioscience Limited. Kanamycin (50 µg ml^{−1}), chloramphenicol (25–50 µg ml^{−1}), apramycin (50 µg ml^{−1}) and

thiostrepton (25 µg ml⁻¹) were used for selection of recombinant strains. Cosmid clo-BG1 and plasmid pUG019 have been described previously (Eustáquio *et al.*, 2005a).

Genetic procedures. Standard procedures for DNA isolation and manipulation were performed as described by Kieser *et al.* (2000) and Sambrook & Russell (2001). Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kits; Macherey–Nagel), according to the manufacturer's protocol. Genomic DNA was isolated from *Streptomyces* strains by lysozyme treatment and phenol/chloroform extraction (Kieser *et al.*, 2000). Southern blot analysis was performed on Hybond-N nylon membrane (Amersham Biosciences) with digoxigenin-labelled probe using the DIG high prime DNA labelling and detection starter kit II (Roche Applied Science).

Replacement of *cloU* with *aac(3)IV* and construction of cosmids clo-AF1/clo-AF2. In cosmid clo-BG1, *cloU* was replaced, via λ -Red-mediated recombination (Eustáquio *et al.*, 2005a), by an apramycin resistance cassette, *aac(3)IV*. The resistance cassette was excised from pUG019 (Eustáquio *et al.*, 2005a) by digestion with *EcoRI* and *HindIII*, and was generated by PCR with the primer pair cloU-f (5'-GACCGGCGGTTTGCATCCCGTGAGGGGGAGGAAG-AGTTGATTCCGGGGATCTCTAGATC-3') and cloU-r (5'-TGGAACGGGATCACCGCAGCCGACACCGTGGCCACGTCAACTAGTCTGGAGCTGCTTC-3'). Bold letters represent 39-nt homologous extensions to the DNA regions immediately upstream and downstream of *cloU*, including the putative start and stop codons of *cloU*. Underlined letters indicate *XbaI* and *SpeI* restriction sites.

PCR was carried out in a 50 µl volume with 50 ng template, 0.2 mM dNTPs, 50 pmol each primer and 5% (v/v) DMSO, using the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles of denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s and extension at 72 °C for 90 s, followed by 20 cycles of annealing at 48 °C, and the last elongation step at 72 °C for 5 min. The PCR product was introduced by electroporation into *E. coli* BW25113(pIJ790) harbouring cosmid clo-BG1 (Gust *et al.*, 2003). The modified cosmid was isolated and analysed by restriction enzyme digestion. This cosmid was termed clo-AF1. For the excision of the apramycin resistance cassette from clo-AF1, cosmid DNA was isolated from *E. coli* ET12567, digested with *XbaI* and *SpeI* and religated overnight at 16 °C to give cosmid clo-AF2.

Heterologous expression of clo-AF2 in *S. coelicolor* M512. Because of the potent methylation restriction system of *S. coelicolor*, cosmid DNA had to be passed through a non-methylating host. We used *E. coli* ET12567 for this purpose (Eustáquio *et al.*, 2005a). The modified cosmid clo-AF2, still carrying the kanamycin resistance gene *aphII*, was then introduced into *S. coelicolor* M512 via PEG-mediated protoplast transformation (Kieser *et al.*, 2000). Clones resistant to kanamycin were selected and checked for site-specific integration into the genome by Southern blot analysis.

Complementation of the *cloU* mutant with the *cloU* gene. For the complementation assays with *cloU*, vector pAF16 was constructed. A fragment of 1353 bp containing the whole sequence of *cloU* [position 36566–37919 in sequence AF329398 (GenBank accession no.)] was obtained by PCR amplification with primer pair k-*cloU*/*Bam*HI (5'-GACCGGCGGTTTGGATCCCGTGA-3') and k-*cloU*/*XbaI* (5'-TTCCGACCTTCTAGACACGTGG-3') using cosmid clo-BG1 as template. The underlined letters represent mutations inserted into the original sequence to give the desired *Bam*HI and *XbaI* restriction sites, respectively. The purified PCR product was restricted with *Bam*HI and *XbaI* and ligated into the same sites of pBluescript SK(–) (Stratagene) to give pAF15. The *Bam*HI–*XbaI* fragment of 1.3 kb from pAF15 was religated into the *Bam*HI and

XbaI restriction sites of the replicative vector pUWL201 (Doumith *et al.*, 2000), downstream of the constitutive *ermE** promoter, to give pAF16.

After passing through the non-methylating strain *E. coli* ET12567, pAF16 was introduced into the *cloU*-defective mutant using PEG-mediated protoplast transformation (Kieser *et al.*, 2000). After transformation, thiostrepton-resistant clones were selected. To confirm the presence of intact pAF16, the plasmid was reisolated by alkaline lysis and potassium acetate precipitation, procedure D (Kieser *et al.*, 2000). Subsequently, plasmid DNA isolated from *Streptomyces* was amplified in *E. coli* XL-1 Blue MRF' before restriction analysis.

Analysis of secondary metabolites. Transformants of *S. coelicolor*, harbouring clo-BG1, clo-AF2 and clo-AF2 with pAF16, respectively, were cultured and assayed for the production of clorobiocin derivatives by HPLC as described previously (Eustáquio *et al.*, 2004) using a Multisphere RP18-5 column (250 × 4 mm; 5 µm; CS Chromatographie Service, Düren, Germany) at a flow rate of 1 ml min⁻¹, using a linear gradient from 60 to 100% solvent B over 30 min (solvent A, MeOH/H₂O/HCOOH 20:79:1; solvent B, MeOH/HCOOH 99:1) with 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 production medium as described above. A total of 5 l bacterial cultures of the *cloU*-defective strain was pooled, acidified with HCl to pH 4 and extracted with ethyl acetate after prior removal of the lipophilic components by extraction with petroleum ether. The residue of the ethyl acetate extract after evaporation of the solvent was dissolved in 30 ml methanol and further purified on a semipreparative HPLC column (Multisphere 120 RP18-5, 5 µm, 250 × 10 mm; CS Chromatographie Service, Düren, Germany) using the same solvents and gradient as for the analytical column, but with a flow rate of 2.5 ml min⁻¹. The purified compounds were subjected to ¹H-NMR and MS analysis.

Negative-ion FAB mass spectra were recorded on a TSQ70 spectrometer (Finnigan) using diethanolamine as matrix.

The negative ion fast atom bombardment mass spectrum of novclobiocic acid 106 showed molar ion at *m/z* = 380 ([M-H]⁻). Clorobiocic acid gave the following negative ions: *m/z* = 414 ([M-H]⁻) and 225. Novclobiocin 122 and novclobiocin 123 showed the molar ion at *m/z* = 681 ([M-H]⁻).

¹H-NMR spectra were acquired on an AC 250 spectrometer (Bruker) or an AMX 400 spectrometer (Bruker). The NMR data for clorobiocin, novclobiocic acid 106, clorobiocic acid, novclobiocin 122 and 123 are shown in Table 1.

Bioassay. The antibacterial activities of authentic clorobiocin (Aventis) and novclobiocin 122 and 123 against *Bacillus subtilis* ATCC 14893 were determined by a disc-diffusion assay, as described previously (Eustáquio *et al.*, 2003).

RESULTS

Sequence analysis of *cloU*

CloU encodes a protein which consists of 420 aa and shows 90% identity at the amino acid level to CouU from *Streptomyces rishiriensis* (Wang *et al.*, 2000) and 88% to NovU from *Streptomyces spheroides* (Steffensky *et al.*, 2000). CouU and NovU were putatively assigned to the C-methyltransferase reactions involved in the deoxysugar biosynthesis of

Table 1. ^1H -NMR data of clorobiocin and clorobiocin analogues

The numbering of the structures is shown in Fig. 3. δ is given in p.p.m.; J in Hz. Spectra were obtained at 250 or 400 MHz. The NMR spectra of clorobiocin, clorobiocic acid, novclobiocic acid 106 and novclobiocin 123 were measured in CD_3OD . The NMR spectrum of novclobiocin 122 was measured in CD_3SOCD_3 . br, Broad signal; s, singlet; d, doublet; dd, double doublet; dq, double quartet; m, multiplet; t, triplet.

Position	δ , multiplicity (J)				
	Clorobiocin	Novclobiocic acid 106	Clorobiocic acid	Novclobiocin 122	Novclobiocin 123
2-H	7.76, d (2.5)	7.67, d (2.5)	7.74, d*	7.68, br s	7.75, br s
5-H	6.84, d (8.4)	6.81, d (8.3)	6.85, d (8.4)	6.78, d (8.4)	6.79, d (7.8)
6-H	7.72, dd (8.4, 2.5)	7.71, dd (2.5, 8.3)	7.69, d*	7.63, br d (8.4)	7.70, br d (7.8)
7-H ₂	3.34, d (7.1)	3.33, d (7.4)	3.30, d*	3.24, d (7.2)	3.30*
8-H	5.35, br t (7.1)	5.35, t (7.4)	5.34, t (6.7)	5.32, t (7.2)	5.33, t*
10-H ₃	1.74, s	1.73, s	1.72, s	1.69, s	1.72, s
11-H ₃	1.75, s	1.73, s	1.72, s	1.69, s	1.72, s
5'-H	7.90, d (9.2)	7.80, d (8.6)	8.10, d (8.8)	7.71, d (9.0)	7.87, d (8.6)
6'-H	7.33, d (9.2)	6.70, dd (2.2, 8.6)	6.80, d (8.8)	7.09, d (9.0)	7.19, d (8.6)
8'-H	—	6.66, d (2.2)	—	—	—
1''-H	5.73, d (1.8)	—	—	5.60, br s	5.77, br s
2''-H	4.34, t (2.8)	—	—	4.20, br s	5.36, t*
3''-H	5.71, dd (10.3; 2.8)	—	—	5.29, dd (2.8, 9.6)	4.29, dd (2.8, 9.0)
4''-H	3.72, d (10.3)	—	—	3.44, t (9.6)	3.38, t (9.0)
5''-H	—	—	—	3.71, dq (6.4, 9.2)	3.74, m†
6''-H ₃	1.18, s	—	—	—	—
7''-H ₃	1.35, s	—	—	1.18, d (6.4)	1.19, d (6.4)
8''-OCH ₃	3.52, s	—	—	3.42, s	3.33, s
3'''-H	6.90, d (3.6)	—	—	6.76, t (2.8)	6.89, d (3.4)
4'''-H	5.64, d (3.6)	—	—	5.91, br s	5.94, d (3.4)
6'''-H ₃	2.29, s	—	—	2.23, s	2.30, s

*Complex, overlapping signals; J could not be determined.

† J could not be determined.

coumermycin A₁ and novobiocin (Li & Heide, 2004). CloU contains a possible SAM-binding site (Kagan & Clarke, 1994), located at position 108–116 in CloU (VVEFGSNTG). NovU and CouU contain the same motif at the same position.

Inactivation of *cloU*

For inactivation of *cloU*, the gene was replaced by an apramycin resistance gene, *aac(3)IV*, in cosmid clo-BG1 containing the complete biosynthetic gene cluster of clorobiocin. The resistance cassette was then removed and the modified cosmid was expressed in a heterologous producer strain as described below.

Cosmid clo-BG1, containing the clorobiocin gene cluster, the attachment site *attP*, the integrase gene of bacteriophage ΦC31 , and tetracycline and neomycin resistance genes, had been constructed in a previous study (Eustáquio *et al.*, 2005a). Site-specific integration of this cosmid into the *attB* site of genome of *S. coelicolor* M512 had resulted in the formation of clorobiocin by the heterologous host, in amounts comparable to the wild-type producer strain (Eustáquio *et al.*, 2005a).

The apramycin resistance gene, *aac(3)IV*, was amplified from plasmid pUG019 (Eustáquio *et al.*, 2005a) by PCR using primers with 39 bp extensions homologous to the regions upstream and downstream of *cloU*. The PCR product was used to replace the entire ORF of *cloU* in cosmid clo-BG1, leaving only the start and the stop codons intact. This gave the modified cosmid clo-AF1. The resistance cassette was then removed by digestion with *Xba*I and *Spe*I, enzymes which create compatible ends, allowing religation of the outer ends and subsequent excision of the cassette. The removal of the *aac(3)IV* cassette was carried out to avoid possible polar effects of this cassette on downstream genes. The resulting cosmid was termed clo-AF2 (Fig. 2a, c).

The modified cosmid clo-AF2 was introduced into *S. coelicolor* M512 by protoplast transformation (Kieser *et al.*, 2000). Mutants resulting from integration of this cosmid into the host genome were selected by their kanamycin resistance. Five independent mutants were examined by Southern blot analysis, showing identical genotypes. Results of one such mutant are presented in Fig. 3. The desired deletion of *cloU* in the mutant was clearly shown by the

Fig. 2. (a) Cosmid constructs clo-BG1 (intact) and clo-AF2 (*cloU*⁻) containing the Φ C31 integration functions and the chlorobiocin biosynthetic gene cluster. (b) Schematic presentation of site-specific integration of clo-AF2 into the *S. coelicolor* chromosome. B, *Bgl*II restriction site; T3, T7, T3 and T7 promoters of the SuperCos1 vector. Fragment sizes resulting from digestion with *Bgl*II are indicated. The cosmid backbone is not drawn to scale. See Thorpe & Smith (1998) for details of the integration mechanism. (c) Detail of the replacement of *cloU* by an apramycin resistance gene flanked by *Xba*I and *Spe*I sites. (d) Southern blot analysis of two *S. coelicolor* M512 integration mutants harbouring clo-AF2. M, DIG-labelled DNA molecular mass marker VII (Roche). Genomic and cosmid DNA were digested with *Bgl*II. The DIG-labelled cosmid clo-BG1 was used as probe.

Fig. 3. Chemical structure of aminocoumarin antibiotics generated in this study.

disappearance of the 1 kb *Bgl*II restriction fragment arising from the *Bgl*II site within *cloU*, in comparison to the clo-BG1 strain (Fig. 2b, d).

Analysis of secondary metabolite production

For analysis of secondary metabolites, one integration mutant of *S. coelicolor* M512 carrying the unmodified cosmid clo-BG1 and five integration mutants of *S. coelicolor* M512 with the *cloU*-defective cluster were cultured in production medium as described previously (Mancy *et al.*, 1974; Pojer *et al.*, 2002). Ethyl acetate extracts of the cultures were analysed by HPLC.

The integration mutant of *S. coelicolor* M512 harbouring the intact clorobiocin cluster (clo-BG1) showed clorobiocin as the dominant product, accompanied by its non-chlorinated derivative novclobiocin 101 and its structural isomer isoclorobiocin (Figs 3 and 4a). In contrast, production of clorobiocin was not detected in the *cloU*-defective strains. Instead, these mutants produced the aglycon of clorobiocin, clorobiocic acid (20 mg l⁻¹) as well as the corresponding non-chlorinated compound, novclobiocic acid 106 (39 mg l⁻¹). Both compounds were isolated on a preparative scale from 1 l culture and identified by NMR and MS analysis (see below). Besides these major products, we found two minor peaks with retention times of 20 and 23 min which were designated novclobiocin 122 and novclobiocin 123, respectively (Figs 3 and 4b).

To elucidate the structures of these latter compounds, the *cloU*-defective strain was cultured in 5 l production medium. The culture was extracted with ethyl acetate and the compounds were isolated by preparative reversed-phase HPLC and analysed by MS and ¹H-NMR.

Structural elucidation

The mass spectrum of clorobiocic acid showed a molecular ion at *m/z* 414 ([M-H]⁻), 281 mass units less than that of clorobiocin, corresponding to the lack of the entire deoxy-sugar moiety. This result was confirmed by the absence of the signals of sugar protons and the signals for the protons of the 5-methylpyrrole-2-carbonyl moiety in ¹H-NMR analysis, in comparison to those of clorobiocin. In contrast, the signals for the protons of the aminocoumarin and 3-dimethylallyl-4-hydroxybenzoyl unit were clearly observed (Table 1).

Novclobiocic acid 106 showed a molecular ion at *m/z* 380 ([M-H]⁻) in MS analysis, 35 mass units smaller than that of clorobiocic acid, corresponding to loss of the chlorine atom. This was confirmed by the signal of an additional proton at 6.66 p.p.m. for 8'-H. The ¹H-NMR data of this compound (Table 1) corresponded well to the data obtained in a previous study (Eustáquio *et al.*, 2005b).

The ¹H-NMR analysis of novclobiocin 122 and novclobiocin 123 showed similar signals to clorobiocin for the protons of the aminocoumarin moiety, the prenylated

Fig. 4. HPLC analysis of the secondary metabolite production from: (a) *S. coelicolor* M512 carrying the intact clorobiocin biosynthetic gene cluster; (b) *S. coelicolor* M512 carrying the *cloU* clorobiocin biosynthetic gene cluster. (c) Complementation of *S. coelicolor* M512 carrying the *cloU* clorobiocin biosynthetic gene cluster by plasmid pAF16.

4-hydroxybenzoyl moiety and the 5-methylpyrrole-2-carbonyl moiety. However, the signals of the deoxysugar moiety were clearly different in comparison to those of clorobiocin. The deoxysugar moiety in all three compounds is present in the $^1C_4$ conformation. The protons at 1'' and 2'' are thus in equatorial positions, whereas those at 3'' and 4'' are in axial positions. Accordingly, in clorobiocin, 3''-H shows strong coupling with 4''-H ($J=10.3$ Hz), but weaker coupling with 2''-H ($J=2.8$ Hz), resulting in a characteristic

doublet of doublets signal for 3''-H ($J=2.8$ and 10.3 Hz) and a doublet for 4''-H ($J=10.3$ Hz). The equatorial 2''-H shows additional coupling with the equatorial 1''-H with a coupling constant of 1.8 Hz, resulting in a triplet signal for 2''-H due to low resolution of the measurement. The two methyl groups at position 5 of the deoxysugar appear as singlets at 1.18 and 1.35 p.p.m. in the NMR spectrum of clorobiocin. In contrast, in novclorobiocin 122, only one methyl group was observed as a doublet at 1.18 p.p.m. with

a coupling constant of 6.4 Hz. The proton at 4'' appeared as a triplet with a coupling constant of 9.6 Hz. This means that the proton at 4'' has a strong coupling both with 5''-H and 3''-H. This proves that all of these protons are located in axial positions and therefore the deoxysugar moiety is an L-rhamnose derivative. The negative mass spectra of this compound showed a molar ion peak at m/z 681 ($[M-H]^-$), 14 mass units smaller than that of clorobiocin, corresponding to a loss of a methyl group in comparison to clorobiocin.

In novclobiocin 123, the proton at 3'' appears at 4.29 p.p.m. In contrast, the signal for 2''-H is shifted strongly downfield to 5.36 p.p.m. This shows that the acyl moiety is attached to 2''-OH rather than to 3''-OH, similar to observations in previous studies (Freitag *et al.*, 2005; Galm *et al.*, 2004). The negative mass spectra showed the same molar ion as observed for novclobiocin 122.

The amount of novclobiocin 122 produced by the *cloU*-defective strain was 1.5 mg l^{-1} ; the amount of novclobiocin 123 was 0.4 mg l^{-1} .

Complementation of the *cloU* mutation

To prove that only the inactivation of *cloU* was responsible for the changes in secondary metabolite production, the *cloU*-defective strain was complemented by expression of an intact copy of *cloU* under the control of the constitutive *ermE** promoter, using the expression vector pUWL201. HPLC analysis showed that the *cloU*-defective strain harbouring the *cloU* expression plasmid pAF16 produced clorobiocin at a similar level to the *S. coelicolor* M512 strain carrying the complete biosynthetic gene cluster of clorobiocin (Fig. 4c).

Antibacterial activity of the new novclobiocins in comparison to clorobiocin

The novclobiocins obtained (novclobiocin 122 and novclobiocin 123) were assayed for antibiotic activity against

Bacillus subtilis ATCC 14893 in comparison to authentic clorobiocin. Novclobiocin 122 exhibited only 20 % of the activity of clorobiocin, showing that the methylation at position 5 of the deoxysugar is important for the biological activity. Transfer of the acyl moiety from 3''-OH to 2''-OH resulted in a further reduction of activity, as novclobiocin 123 was half as active as novclobiocin 122 (Fig. 5). Results from an earlier study showed that the removal of the deoxysugar moiety resulted in a 99 % activity loss (Eustáquio *et al.*, 2004). Therefore the two acids were not analysed for their biological activity.

DISCUSSION

In the present study, we have demonstrated that inactivation of the *cloU* gene leads to a loss of the axial methyl group at C-5 of the deoxysugar moiety of clorobiocin. This proves that the function of the *cloU* gene product is as a C-methyltransferase in the biosynthesis of this antibiotic. The same function may be assumed for the close homologues of *cloU* in gene clusters for novobiocin and coumermycin A₁, i.e. *novU* and *couU*, respectively. The current study completes the functional identification of the three methyltransferases contained in the clorobiocin cluster, i.e. CloP (Freitag *et al.*, 2005), CloN6 (Westrich *et al.*, 2003) and CloU (present study).

CloU and its two homologues are, to our knowledge, the first 5-C-methyltransferases identified in the biosynthesis of branched deoxysugars. Several enzymes are known to catalyse a 3-C-methylation of deoxysugars, such as TylC3 (He & Liu, 2002), EryBIII (Lombo *et al.*, 2004) and AviG1 (Weitnauer *et al.*, 2002). But the 5-C-methyltransferase CloU and its homologues add a unique, new function to the toolbox of deoxysugar biosynthesis genes, which can be assembled in a combinatorial fashion for the generation of a large diversity of activated deoxysugars (Lombo *et al.*, 2004; Perez *et al.*, 2005; Rodriguez *et al.*, 2002; Salas & Mendez, 2005).

Fig. 5. Antibacterial activity of clorobiocin and derivatives: (a) clorobiocin, (b) novclobiocin 122 and (c) novclobiocin 123. The bioassay was carried out with *Bacillus subtilis* ATCC 14893. For details of structures, see Fig. 3.

Inactivation of *cloU* led to the formation of novclobiocin 122 and 123. The deoxysugar moieties of these two clorobiocin analogues show substituted L-rhamnose structures, as clearly shown by the characteristic coupling of the deoxysugar protons. The NMR data of these compounds are in accordance with those reported for other L-rhamnosides (Igarashi *et al.*, 2005; Ströch, 2003), and in contrast to those of 6-deoxy-D-gulosides (Patroni & Stick, 1987). This proves that in the biosynthesis of the deoxysugar moieties of the aminocoumarin antibiotics, the intermediate dTDP-4-keto-6-deoxyglucose undergoes epimerization at both C-3 and C-5. It appears most probable that the epimerase CloW catalyses both of these epimerizations, similar to the established 3,5-epimerase RmlC (Giraud *et al.*, 2000), and in contrast to the 3-epimerase WbcA (Trefzer *et al.*, 1999).

The recent elucidation of the structure of NovW, a close homologue of CloW, showed that a tyrosine side chain in the active centre is oriented differently than in RmlC (Jakimowicz *et al.*, 2006), but similarly to 5-epimerase EvaD involved in chloroeremomycin biosynthesis. The genuine substrate of EvaD is a sugar which carries an amino and a methyl group at position 3; therefore, only position 5 can be epimerized easily. However, when EvaD was incubated with dTDP-4-keto-6-deoxyglucose (i.e. the genuine substrate of NovW and CloW), a rapid epimerization at C-5, but also a slow epimerization at C-3 was observed (Merkel *et al.*, 2004). Therefore, EvaD acts as 5-monoepimerase *in vivo*, but can also catalyse a 3,5-double epimerization of a suitable substrate *in vitro*. From the structure of NovW, Jakimowicz *et al.* (2006) raised the question whether NovW may function as a 3-monoepimerase. Our present results, however, suggest that at least the similar CloW acts as a 3,5-double epimerase of dTDP-4-keto-6-deoxyglucose *in vivo*.

When the intact clorobiocin cluster was expressed in *S. coelicolor* M512, the major part of the aminocoumarin antibiotics was produced in form of glycosylated compounds (Fig. 4a). In contrast, after *cloU* inactivation, approximately 97 % of the aminocoumarins were produced in the form of aglycons and only 2–3 % in glycosylated form. This indicates that either deoxysugar biosynthesis or glycosyl transfer was impaired as a result of *cloU* inactivation. The possibility that *cloU* inactivation reduced transcription or translation of downstream genes, namely the deoxysugar biosynthesis genes *cloV* and *cloW*, could be ruled out by a complementation experiment of the *cloU* mutant with a *cloU* expression plasmid, which restored production of the glycosylated compounds to the original level. Since dTDP-L-rhamnose has been reported to be a poor substrate of the glycosyltransferase NovM (Albermann *et al.*, 2003; Freil Meyers *et al.*, 2003), a possible explanation is that the glycosyltransferase CloM may transfer the modified deoxysugar, which results from *cloU* inactivation, with less catalytic efficiency than the natural substrate.

After transfer of the deoxysugar moieties, final tailoring reactions consist of 4-O-methylation and 3-O-acylation of the deoxysugar (Li & Heide, 2004). Apparently, the substrate

specificity of the enzymes involved is broad enough to allow the utilization of clorobiocin analogues lacking the methyl group at C-5 of the deoxysugar, as no metabolites were detected which lacked the substituents at 3''-OH or 4''-OH.

Novclobiocin 122 and 123 represent new clorobiocin analogues. Investigation of their antibacterial activity showed that novclobiocin 122 exhibits only 20 % of the activity of clorobiocin, indicating that the hydrophobic contacts between the 5,5-*gem*-dimethyl group of the deoxysugar and gyrase contribute to the binding of the antibiotic to its target (Maxwell, 1999). As observed previously (Li & Heide, 2005), shifting the 5-methylpyrrole-2-carboxyl moiety from position 3 to position 2 further reduced activity.

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