VOL. 57 NO. 3, MAR. 2004 THE JOURNAL OF ANTIBIOTICS pp. 205 — 209

New Aminocoumarin Antibiotics from a *cloQ*-Defective Mutant of the Clorobiocin Producer *Streptomyces roseochromogenes* DS12.976

ANJA FREITAG, UTE GALM, SHU-MING LI and LUTZ HEIDE*

Pharmazeutische Biologie, Pharmazeutisches Institut, Eberhard-Karls-Universität Tübingen
Auf der Morgenstelle 8, 72076 Tübingen, Germany

(Received for publication November 13, 2003)

Three new antibiotics, vanillobiocin, isovanillobiocin and declovanillobiocin, were isolated from the culture broth of a *cloQ*-defective mutant of the clorobiocin producer *Streptomyces roseochromogenes*, which is blocked in the biosynthesis of the prenylated 4-hydroxybenzoic acid moiety of clorobiocin. Spectroscopic analysis showed that the isolated compounds were similar to clorobiocin, but contained vanillic acid as the acyl component instead of the prenylated 4-hydroxybenzoic acid present in clorobiocin. Isovanillobiocin differs from vanillobiocin by the position of the pyrrole unit attached to the sugar moiety of the antibiotic. Declovanillobiocin lacks the chlorine atom at the aminocoumarin ring. All three compounds had lower antibiotic activity against *Bacillus subtilis* than clorobiocin.

The aminocoumarin antibiotic clorobiocin is produced by *Streptomyces roseochromogenes*. Its biological target is the bacterial DNA gyrase[1,2]. Clorobiocin (Fig. 1) consists of a 3-dimethylallyl-4-hydroxybenzoate moiety (=ring A), a 3-amino-4,7-dihydroxycoumarin moiety (=ring B) and the deoxysugar noviose (=ring C). The gene *cloQ* codes for a prenyltransferase and is essential for the biosynthesis of the 3-prenylated 4-hydroxybenzoate moiety (Fig. 1). We recently created a *cloQ*-defective mutant by an in-frame deletion within the coding sequence of *cloQ*, and showed that the resulting mutant was unable to produce clorobiocin unless the culture medium was supplemented with the prenylated 4-hydroxybenzoate moiety[3]. However, even without supplementation, the culture extract of the *cloQ*$^-$ mutant had some antibacterial activity. In this paper, we report the isolation, structural elucidation and the biological activity testing of three new aminocoumarin antibiotics, vanillobiocin, isovanillobiocin and declovanillobiocin, that are produced by the *cloQ*$^-$ mutant.

Materials and Methods

Fermentation

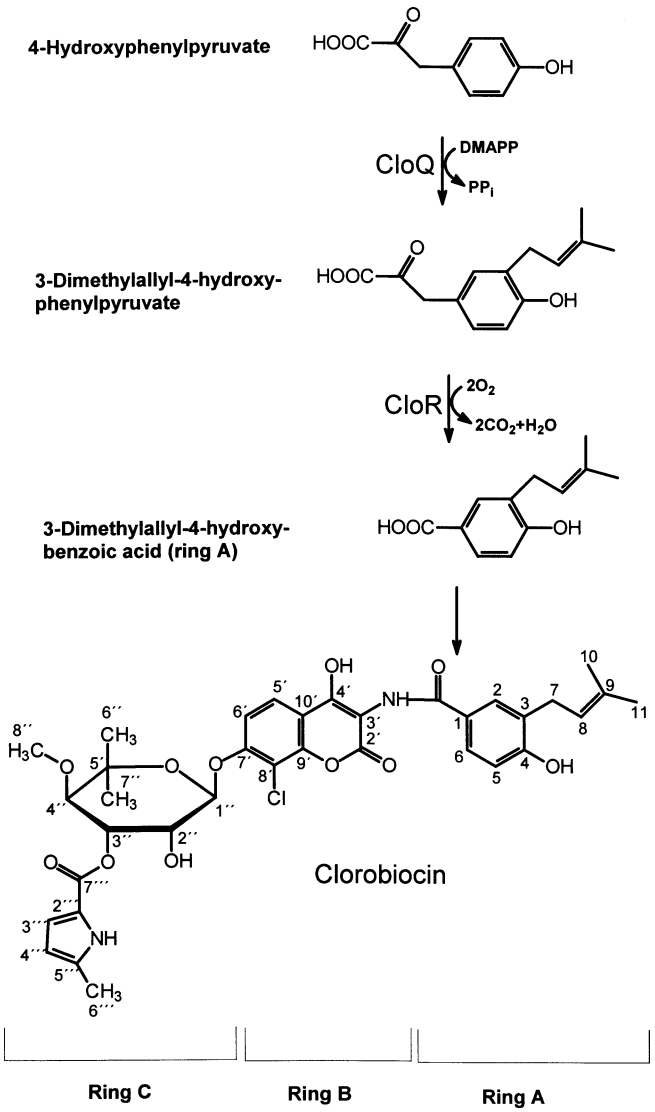
For the production of clorobiocin and vanillobiocins, wild-type and the *cloQ*-defective mutant of *Streptomyces roseochromogenes* were first inoculated in 300 ml flasks each containing 50 ml of YMG liquid medium consisting of yeast extract (Difco Laboratories) 0.4%, malt extract (Difco Laboratories) 1.0% and glucose 0.4% (pH 7.3). The flasks were incubated for 2 days at 30°C and 210 rpm on a rotary shaker. Cells (1 ml culture) were then inoculated into three 300 ml flasks each containing 50 ml of corn starch medium consisting of soluble starch 1%, pepton (Difco Laboratories) 1% and meat extract 0.5% (pH 7.0). The flasks were incubated for another 2 days at 33°C and 210 rpm on a rotary shaker. Five ml of these seed cultures were transferred into twenty 500 ml Erlenmeyer flasks each containing 50 ml of the production medium adapted from[4], prepared from distillers' solubles 4.8%, glucose 3.7%, cobalt chloride 0.0024% (at this point, the pH of the mixture was adjusted to 7.8), calcium carbonate 0.6% and ammonium sulphate 3.2%. The flasks were kept on a rotary shaker (210 rpm) at 33°C for 9 days before harvest.

Isolation Procedure

For analytical purpose, 1 ml of bacterial culture was acidified with 1 M HCl to pH 4 and extracted twice with an equal volume of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 0.2 ml methanol.

* Corresponding author: heide@uni-tuebingen.de

Fig. 1. Structure of clorobiocin and biosynthesis of the prenylated benzoate moiety (ring A).



After centrifugation, 50 μl of the clear supernatant was analysed by HPLC with a Multosphere RP18-5 column (5 μm; 250×4 mm; C+S Chromatographie Service, Düren, Germany) at a flow rate of 1 ml/minute, using a linear gradient from 60 to 100% solvent B (1% HCOOH in CH_3OH) in solvent A (1% HCOOH in 79% aqueous CH_3OH) with a detection at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation, the fermented whole broth (800 ml) was acidified with 1 M HCl to pH 4 and washed once with 600 ml of petroleum ether. The resulting aqueous solution was extracted twice with 600 ml ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to dryness. The residue was dissolved in 3 ml of methanol and passed through a Sephadex LH-20 column (100×5 cm; Amersham Biosciences, Freiburg, Germany) and eluted with methanol. The fractions from the Sephadex LH-20 column were analysed with HPLC using the method mentioned above. Fractions containing vanillobiocins 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) using the same gradient as for the analytical column, but with a flow of 2.5 ml/minute 1.44 mg of vanillobiocin, 0.84 mg of isovanillobiocin and 0.4 mg of declovanillobiocin were obtained, respectively.

Structural Elucidation

The isolated compounds were analysed by ^{1}H-NMR spectroscopy and by negative-ion FAB mass spectrometry. ^{1}H-NMR spectra were measured on an AMX 400 spectrometer (Bruker, Karlsruhe, Germany), using CD_3OD as solvent. The NMR data of clorobiocin, vanillobiocin (**1**), isovanillobiocin (**2**) and declovanillobiocin (**3**) are shown in Table 1.

Negative fast-atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using diethanolamine as matrix. The negative ion mass spectrum of vanillobiocin (**1**) is characterized by signals at *m/z* 657 ([M−H]$^-$), 623, 376 and 281. Isovanillobiocin (**2**) gave the following negative ions: *m/z* 657 ([M−H]$^-$), 623, 376 and 281. Declovanillobiocin (**3**) yielded the following negative ions: *m/z* 623 ([M−H]$^-$), 342, 281 and 191.

Bioassay

Antibacterial activities of the isolated compounds were tested against *Bacillus subtilis* ATCC 14893. Authentic clorobiocin and novobiocin were used as comparison. For the bioassays, different amounts of the respective substances dissolved in methanol were applied onto filter paper disks (i.d. 3 mm; MN 440 B blotting paper; Macherey-Nagel, Düren, Germany). The filter discs were placed on the surface of nutrient agar (Difco Laboratories, Detroit, MI 48232-7058, USA) containing approximately 2×10^5 spores of *Bacillus subtilis* per ml agar medium, and then dried in the air for 30 minutes. After culturing overnight at 37°C, the diameter of the growth-inhibition zone was determined.

Table 1. ^{1}H-NMR data of clorobiocin, vanillobiocin, isovanillobiocin and declovanillobiocin.

Position	Clorobiocin δ, Multiplicity (*J*/Hz)	Vanillobiocin (1) δ, Multiplicity (*J*/Hz)	Isovanillobiocin (2) δ, Multiplicity (*J*/Hz)	Declovanillobiocin (3) δ, Multiplicity (*J*/Hz)
2-H	7.76, d (2.5)	7.63, br s	7.63, br s	7.62, br s
5-H	6.84, d (8.4)	6.85, d (7.9)	6.85, d (7.3)	6.87, d (7.9)
6-H	7.72, dd (8.4; 2.5)	7.54, d (7.9)	7.55, d (7.3)	7.55, d (7.9)
7-H_2	3.34, d (7.1)	-	-	-
ArOCH_3	-	3.92, s	3.92, s	3.93, s
8-H	5.35, br[a] t (7.1)	-	-	-
9-H_2	-	-	-	-
10-H_3	1.74, s	-	-	-
11-H_3	1.75, s	-	-	-
5′-H	7.90, d (9.2)	7.90, d (8.0)	7.90, d (9.1)	7.93, d (9.1)
6′-H	7.33, d (9.2)	7.22, d (8.0)	7.21, d (9.1)	7.04[b]
8′-H	-	-	-	7.04[b]
1′′-H	5.73, d (1.8)	5.69, br s	5.78, s	5.62, s
2′′-H	4.34, t (2.7)	4.33, br s	5.39, br s	4.23, br s
3′′-H	5.71, dd (10.3; 2.9)	5.71, dd (12.9, 3.1)	4.44, dd (7.9, 3.2)	5.59, dd (9.8, 3.1)
4′′-H	3.72, d (10.3)	3.71, d (10.1)	3.55, d (9.9)	3.70, d (7.4)
6′′-H_3	1.18, s	1.20, s	1.18, s	1.20, s
7′′-H_3	1.35, s	1.35, s	1.37, s	1.37, s
8′′-OCH_3	3.52, s	3.51, s	3.64, s	3.51, s
3′′′-H	6.90, d (3.6)	6.90, d (3.5)	6.89, d (3.7)	6.90, d (3.6)
4′′′-H	5.94, br d (3.6)	5.94, d (3.5)	5.95, d (3.4)	5.94, d (3.5)
6′′′-H_3	2.29, s	2.29, s	2.30, s	2.29, s

δ is given in ppm. Spectra were obtained at 400 MHz. The spectra were taken in CD_3OD.
[a] br indicates broad signal.
[b] Complex, overlapping signals; *J* not determinable.
See Figs. 1 and 2 for numbering of the structures.

Results

Isolation Procedure

When the *cloQ*-defective mutant[3] of the clorobiocin producer *Streptomyces roseochromogenes* DS 12.976 was cultured in production medium (see methods), the ethyl acetate extract of the culture broth clearly showed antibacterial activity. RP-HPLC analysis (detection: 340 nm) showed three new compounds, all with shorter retention times than that of clorobiocin. These three compounds were not observed in the extract of the wild-type strain (data not shown).

To investigate the structure and the biological activities of these compounds, 800 ml of bacterial culture of *cloQ*-defective mutant were extracted with ethyl acetate, and the extract was chromatographed on a Sephadex LH-20 column. Fractions were analysed for the desired compounds by analytical HPLC. The respective fractions were pooled and further purified by preparative HPLC. Three compounds, *i.e.* **1**～**3** could be isolated by this procedure.

The structures of the compounds were elucidated by spectroscopic methods, including MS and ^{1}H-NMR.

Vanillobiocin (**1**)

The FAB mass spectrum of compound **1** showed a $[M-H]^-$ ion at *m/z* 657. The ^{1}H-NMR spectrum of **1** is closely related to that of clorobiocin. They differ in the signals of the acyl components: clorobiocin shows signals for a 3-dimethylallyl-4-hydroxybenzoyl moiety at δ=1.74 (s), 1.75 (s), 3.34 (d), 5.35 (t), 6.84 (d), 7.72 (dd) and 7.76 (d) ppm, whereas the signals of a vanilloyl moiety (3-methoxy-4-hydroxybenzoyl moiety) were observed in spectrum of **1** at δ=3.92 (s), 6.85 (d), 7.54 (br d) and 7.63 (br s) ppm. Compound **1** was identified as a clorobiocin analogue, containing a vanilloyl moiety attached to the amino group of the 3-aminocoumarin ring. This compound was termed vanillobiocin (Fig. 2).

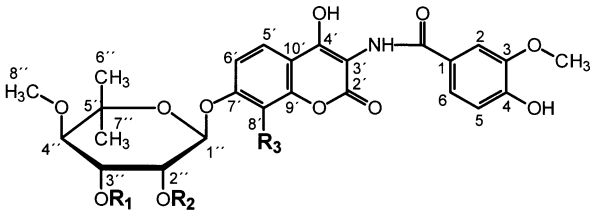
Isovanillobiocin (**2**)

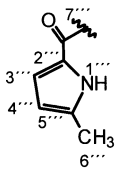
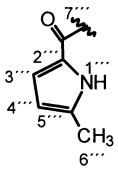
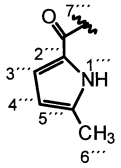
The negative FAB mass spectrum of compound **2** showed a $[M-H]^-$ peak identical to that of **1** (*m/z* 657) and

identical main fragments at *m/z* 623, 376 and 281.

Comparison of the ^{1}H-NMR spectra of **2** and **1** indicated that the signals for all the protons of the vanilloyl moiety, for ring B and for ring C of compound **1** were also present in the spectrum of **2**. However, significant differences were observed for protons H-2″ and H-3″ of the deoxysugar, with a downfield shift of 1.06 ppm for H-2″ (from 4.33 ppm in **1** to 5.39 ppm in **2**) and an upfield shift of 1.27 ppm for H-3″ (from 5.71 ppm in **1** to 4.44 ppm in **2**) (Table 1). These changes can be explained by the attachment of the pyrrole unit at 2″-OH in **2**, instead of at 3″-OH in **3**. Similar aminocoumarin antibiotics, with an acyl moiety at 2″-OH rather than 3″-OH, have been described previously[5,6]. Compound **2** was named isovanillobiocin (Fig. 2).

Fig. 2. Structures of the isolated vanillobiocins.



	R_1	R_2	R_3
Vanillobiocin		H	Cl
Isovanillobiocin	H		Cl
Declovanillobiocin		H	H

Declovanillobiocin (**3**)

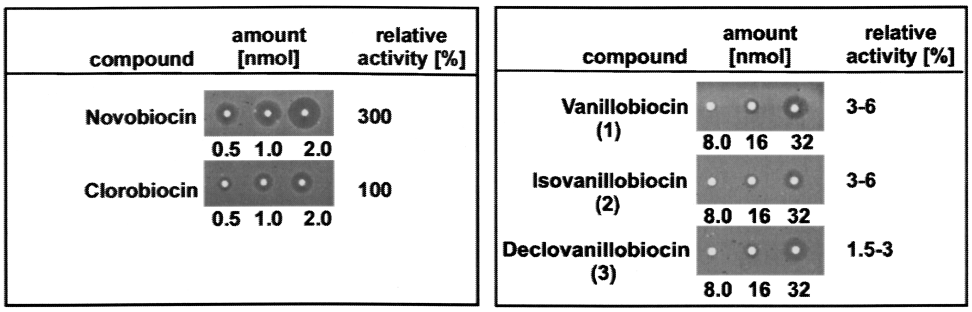
The negative FAB-MS analysis of **3** showed a molecular ion $[M-H]^-$ at *m/z* 623, *i.e.* 34 dalton less than that of **1** and **2**, corresponding to the loss of a chlorine atom. The typical isotopic pattern for compounds with chlorine atoms, observed for **1** and **2**, was absent in **3**.

The ^{1}H-NMR spectra of **3** showed significant changes in comparison to **1** and **2** for the protons of the aminocoumarin ring. Signals for three protons were observed at δ=7.04 (2H) and 7.93 (1H) ppm. Similar signals have been observed previously for aminocoumarin derivatives lacking the substitution at C-8 of this ring[7,8]. Therefore, compound **3**, named declovanillobiocin, differs from vanillobiocin (**1**) by lacking the chlorine atom at C-8 of the aminocoumarin ring (Fig. 2).

Antibacterial Activities of Vanillobiocin (**1**), Isovanillobiocin (**2**) and Declovanillobiocin (**3**) against *B. subtilis*

Vanillobiocin and its analogues were assayed for growth-inhibitory activity against *B. subtilis* ATCC 14893 in a disc diffusion assay using different quantities of the respective compounds. Fig. 3 shows the results and the antibacterial activities, derived from inhibition zone diameters and

Fig. 3. Antibacterial activities of the aminocoumarins.



Relative bioactivities were estimated by comparing inhibition zones with those obtained for clorobiocin (assigned a relative bioactivity of 100%).

expressed relative to clorobiocin.

Declovanillobiocin was less active against *B. subtilis* than vanillobiocin and isovanillobiocin, confirming the contribution of the chlorine atoms to the antibacterial activities, as observed previously[7]. In contrast, the activities of vanillobiocin and isovanillobiocin were similar to each other, indicating that the position of the pyrrole carboxylic acid moiety at 2″-OH or 3″-OH is not of major importance for the antibiotic action. This is at variance with the results for the position of the carbamyl moiety in novobiocin[9]. Vanillobiocin was clearly less active than clorobiocin, confirming the importance of the dimethylallyl moiety[10].

Absence of Vanillobiocin in a *cloR*-Defective Mutant

The prenylated 4-hydroxybenzoate moiety of clorobiocin is formed from 4-hydroxyphenyl-pyruvate (Fig.1) by (i) prenylation under catalysis of CloQ, and (ii) two successive oxidative decarboxylations under catalysis of CloR[11]. We have created a *cloR*-defective mutant[12]. Like the *cloQ*-defective mutant, the *cloR*$^-$ strain was unable to produce clorobiocin, unless supplemented with the prenylated 4-hydroxybenzoate moiety. However, in the culture extract of the *cloR*$^-$ mutant, we did not observe an accumulation of vanillobiocin and its derivatives (data not shown).

Discussion

Three new aminocoumarin derivatives containing a vallinoyl moiety attached to the amino group of the 3-aminocoumarin ring could be isolated from the culture broth of a *cloQ*$^-$ mutant of the clorobiocin producer, *S. roseochromogenes*, blocked in the biosynthesis of the prenylated 4-hydroxybenzoic acid (Ring A). The yield of total vanillobiocins was about 3 μg/ml culture, *i.e.* seven times lower than that of clorobiocin in the wild-type strain.

The wild-type strain did not accumulate detectable amounts of vanillobiocins. Apparently, the genuine 3-prenylated 4-hydroxybenzoate moiety of clorobiocin is formed and incorporated preferentially. If, however, its formation is blocked by inactivation of *cloQ*, vanillic acid is incorporated into the antibiotic, instead of the genuine acyl component. Interestingly, a *cloR*-defective mutant did not accumulate vanillobiocins, suggesting that *cloR* may be involved in the biosynthesis of vanillic acid in this strain.

Acknowledgement

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to L. HEIDE and S.-M. LI).

References

1) HOOPER, D. C.; J. S. WOLFSON, G. L. MCHUGH, M. B. WINTERS & M. N. SWARTZ: Effects of novobiocin, coumermycin A1, clorobiocin, and their analogs on *Escherichia coli* DNA gyrase and bacterial growth. Antimicrob. Agents Chemother. 22: 662～671, 1982
2) MAXWELL, A.: The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. Curr. Top. Med. Chem. 3: 283～303, 2003
3) POJER, F.; E. WEMAKOR, B. KAMMERER, H. CHEN, C. T. WALSH, S.-M. LI & L. HEIDE: CloQ, a prenyltransferase involved in clorobiocin biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 100: 2316～2321, 2003
4) MANCY, D.; L. NINET & J. PREUD'HOMME: Antibiotic 18631 RP, U.S. Patent 3,793,1471974
5) WESTRICH, L.; L. HEIDE & S.-M. LI: CloN6, a novel methyltransferase catalysing the methylation of the pyrrole-2-carboxyl moiety of clorobiocin. Chembiochem 4: 768～773, 2003
6) CROW, F. W.; W. K. DUHOLKE, K. A. FARLEY, C. E. HADDEN, D. A. HAHN, B. D. KALUZNY, C. S. MALLORY, G. E. MARTIN, R. F. SMITH & T. J. THAMANN: Complete spectroscopic structural characterization of novobiocin, isonovobiocin, decarbamylnovobiocin, 2″-(O-Carbamyl) novobiocin, and novobiocin-2″,3″-carbonate. J. Heterocycl. Chem. 36: 365～370, 1999
7) EUSTÁQUIO, A. S.; B. GUST, T. LUFT, S.-M. LI, K. F. CHATER & L. HEIDE: Clorobiocin biosynthesis in *Streptomyces*: Identification of the halogenase and generation of structural analogs. Chem. Biol. 10: 279～288, 2003
8) SASAKI, T.; Y. IGARASHI, N. SAITO & T. FURUMAI: TPU-0031-A and B, new antibiotics of the novobiocin group produced by *Streptomyces* sp. TP-A0556. J. Antibiotics 54: 441～447, 2001
9) HINMAN, J. W.; E. L. CARON & H. HOEKSEMA: Novobiocin. V. Carbamoyl migration and isonovobiocin. J. Am. Chem. Soc. 79: 5321～5322, 1957
10) LAFITTE, D.; V. LAMOUR, P. O. TSVETKOV, A. A. MAKAROV, M. KLICH, P. DEPREZ, D. MORAS, C. BRIAND & R. GILLI: DNA gyrase interaction with coumarin-based inhibitors: the role of the hydroxybenzoate isopentenyl moiety and the 5′-methyl group of the noviose. Biochemistry 41: 7217～7223, 2002
11) POJER, F.; R. KAHLICH, B. KAMMERER, S.-M. LI & L. HEIDE: CloR, a bifunctional non-heme iron oxygenase involved in clorobiocin biosynthesis. J. Biol. Chem. 278: 30661～30668, 2003
12) POJER, F.; S.-M. LI & L. HEIDE: Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: new insights into the biosynthesis of aminocoumarin antibiotics. Microbiology 148: 3901～3911, 2003