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Clorobiocin Biosynthesis in *Streptomyces*: Identification of the Halogenase and Generation of Structural Analogs

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Summary

Clorobiocin (*clo*) and novobiocin (*nov*) are potent inhibitors of bacterial DNA gyrase. The two substances differ in the substitution pattern at C-8′ of the aminocoumarin ring, carrying a chlorine atom or a methyl group, respectively. By gene inactivation, *clo-hal* was identified as the gene of the halogenase responsible for the introduction of the chlorine atom of clorobiocin. Inactivation of *cloZ* did not affect clorobiocin formation, showing that this ORF is not essential for clorobiocin biosynthesis. Expression of the methyltransferase gene *novO* in the *clo-hal*$^-$ mutant led to the very efficient formation of a hybrid antibiotic containing a methyl group instead of a chlorine atom at C-8′. Comparison of the antibacterial activity of clorobiocin analogs with -Cl, -H, or -CH_3 at C-8′ showed that chlorine leads to 8-fold higher activity than hydrogen and to 2-fold higher activity than a methyl group.

Introduction

The aminocoumarin antibiotics clorobiocin (also spelled chlorobiocin), novobiocin, and coumermycin A_1 (Figure 1A) are potent inhibitors of DNA gyrase produced by different *Streptomyces* strains [1–3]. Their characteristic structural moiety is a 3-amino-4,7-dihydroxycoumarin unit (Ring B), which is attached to the deoxysugar novirose (Ring C). Early investigations of structure-activity relationships [4, 5] demonstrated that both the aminocoumarin and the substituted deoxysugar moiety are essential for antibacterial activity. More recent X-ray crystallographic examinations [6–8] showed that both these moieties are involved in binding to the B subunit of DNA gyrase.

Novobiocin (Albamycin®, Pharmacia & Upjohn) is licensed in the USA for the treatment of human infections with gram-positive bacteria such as *Staphylococcus aureus* and *S. epidermidis*. Its efficacy has been demonstrated in preclinical and clinical studies [9–11]. Novobiocin and its derivatives have also been investigated as potential anticancer drugs [12–14]. However, due to their toxicity in eukaryotes, their poor solubility in water, and their low activity against gram-negative bacteria, clinical use of these antibiotics remains restricted [2]. Therefore, it is of interest to test whether new, structurally modified aminocoumarin antibiotics may be able to overcome the limitations of the known compounds [15]. Such new aminocoumarins may be developed by the methods of combinatorial biosynthesis [16]. A functional analysis of biosynthetic genes for the aminocoumarins is a crucial prerequisite for such approaches [17].

Our group has cloned and sequenced the biosynthetic gene clusters of novobiocin (*nov*) [18], coumermycin A_1 (*cou*) [19], and clorobiocin (*clo*) [20]. A uniform nomenclature for the genes in these clusters has recently been introduced [20], replacing the previous names of the coumermycin biosynthetic genes (see GenBank entry AF235050 for old and new names).

The characteristic aminocoumarin moiety is substituted at position 8 in novobiocin and coumermycin A_1 with a methyl group and in clorobiocin with a chlorine atom (Figure 1A). This structural difference is perfectly reflected in the organization of the gene clusters: the novobiocin and coumermycin A_1 clusters contain a C-methyltransferase gene, i.e., *novO* and *couO*, respectively [21], whereas the clorobiocin cluster contains the gene *clo-hal*, which shows sequence similarity to $FADH_2$-dependent halogenases [22], at the corresponding position (Figure 1B). In addition, the clorobiocin cluster also contains the ORF *cloZ*, which shows no sequence similarity to known genes. For all genes of the clorobiocin cluster, with the exception of *clo-hal* and *cloZ*, homologs exist in the novobiocin and/or coumermycin cluster. Since the halogen atom represents the only structural characteristic of clorobiocin absent from both novobiocin and coumermycin, we were prompted to question whether *cloZ*, together with *clo-hal*, may play a role in the halogenation of clorobiocin.

The aims of the present study were, first, to provide functional proof for the role of *clo-hal* and *cloZ* in clorobiocin biosynthesis by gene inactivation, and, second, to generate structural analogs of clorobiocin by these inactivation experiments and by heterologous expression of the putative C-methyltransferase *novO*.

Results

Sequence Analysis of *clo-hal* and *cloZ*

The predicted gene product of *clo-hal* comprises 525 amino acids and resembles $FADH_2$-dependent halogenases, which use phenols or pyrrole derivatives as substrates, such as BhaA of the balhimycin gene cluster [23, 24], ComH of the complestatin gene cluster [25], and PltA of the pyoluteorin gene cluster [26]. The sequence identity of Clo-hal to those three proteins is 36%, 35%, and 31%, respectively.

The predicted gene product of *cloZ* comprises 254 amino acids and shows no homology to known proteins.

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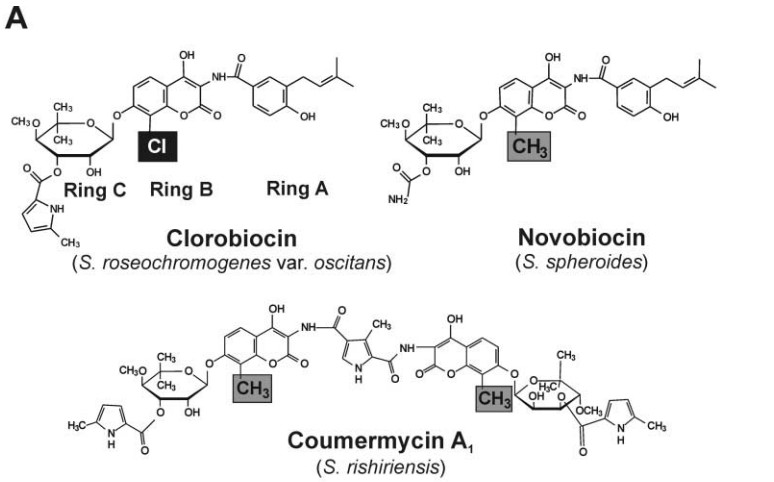


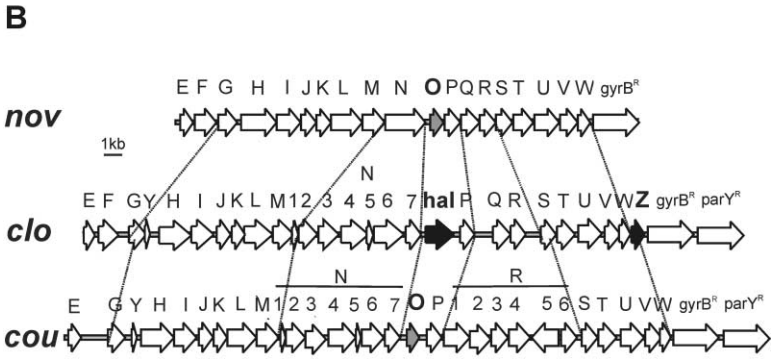


Figure 1. Aminocoumarin Antibiotics

(A) Structure of the aminocoumarin antibiotics.

(B) Map of the clorobiocin biosynthetic gene cluster of *Streptomyces roseochromogenes* var. *oscitans* DS 12.976 (middle), compared to the biosynthetic gene clusters of novobiocin (top) and coumermycin A_1 (bottom).

Inactivation of *clo-hal* and *cloZ* in *Streptomyces roseochromogenes* var. *oscitans* DS 12.976

Datsenko and Wanner [27] have recently developed a rapid method to disrupt chromosomal genes in *Escherichia coli* by replacement with a selectable marker. The marker is generated by PCR, using primers with 36–50 nt extensions that are homologous to the targeted gene. Recombination of these short homologous sequences with chromosomal DNA is promoted by the λ RED functions (*gam*, *bet*, *exo*). This strategy was adapted for use in *Streptomyces coelicolor* by Gust et al. [28]. We have used this PCR targeting system to inactivate *clo-hal* and *cloZ* in *S. roseochromogenes* var. *oscitans* DS 12.976.

Within cosmid D1A8, which contained the biosynthetic gene cluster of clorobiocin in the SuperCos1 vector (carrying a kanamycin resistance gene), *clo-hal* was replaced by an apramycin resistance/*oriT* cassette (see Experimental Procedures). The modified cosmid (named D1A8-h-773) was introduced into *S. roseochromogenes* var. *oscitans* by conjugation. Apramycin-resitant, kanamycin-sensitive colonies, resulting from double crossover events, were selected. Southern blot analysis confirmed that in these mutants *clo-hal* was replaced by the apramycin resistance/*oriT* cassette (Figures 2A and 2B).

cloZ was inactivated in the same way, and the correct genotype of the resulting double crossover mutants was confirmed by Southern blot analysis (Figures 2C and 2D).

With some modifications of the conjugation procedure (see Experimental Procedures), the PCR targeting system could therefore be used successfully in *S. roseochromogenes* var. *oscitans*, allowing gene inactivation experiments to be carried out much more simply and quickly than by previous methods [29].

Characterization of Secondary Metabolites in the *clo-hal*$^-$ and *cloZ*$^-$ Mutants

Three independent *clo-hal*$^-$ mutants (AE-h2, AE-h10, and AE-h11) and three independent *cloZ*$^-$ mutants (AE-Z4, AE-Z25, and AE-Z40) as well as the wild-type were cultured in clorobiocin production medium (see Experimental Procedures). After extraction of the cultures with ethyl acetate, secondary metabolites were analyzed by HPLC in comparison with clorobiocin standard.

The production of clorobiocin was abolished in all *clo-hal*$^-$ mutants (Figure 3B). These mutants produced, instead, a new substance with a shorter retention time than clorobiocin. This compound was isolated on a preparative scale. Negative-ion FAB MS analysis showed a molecular ion $[M-H]^-$ at *m/z* 661, consistent with the loss of a chlorine atom in comparison to clorobiocin ($[M-H]^-$ at *m/z* 695). Clorobiocin shows the typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl (mass [intensity]: 695 [100.0%], 696 [32.6%], 697 [31.8%], 698 [11.1%]), whereas the negative-ion FAB MS of the new substance did not show this pattern, indicating the absence of chlorine (mass [intensity]: 661 [100.0%], 662 [25.9%], 663 [9.5%]). ^1H NMR (Table 1) and ^{13}C NMR (Table 2) unequivocally confirmed that the

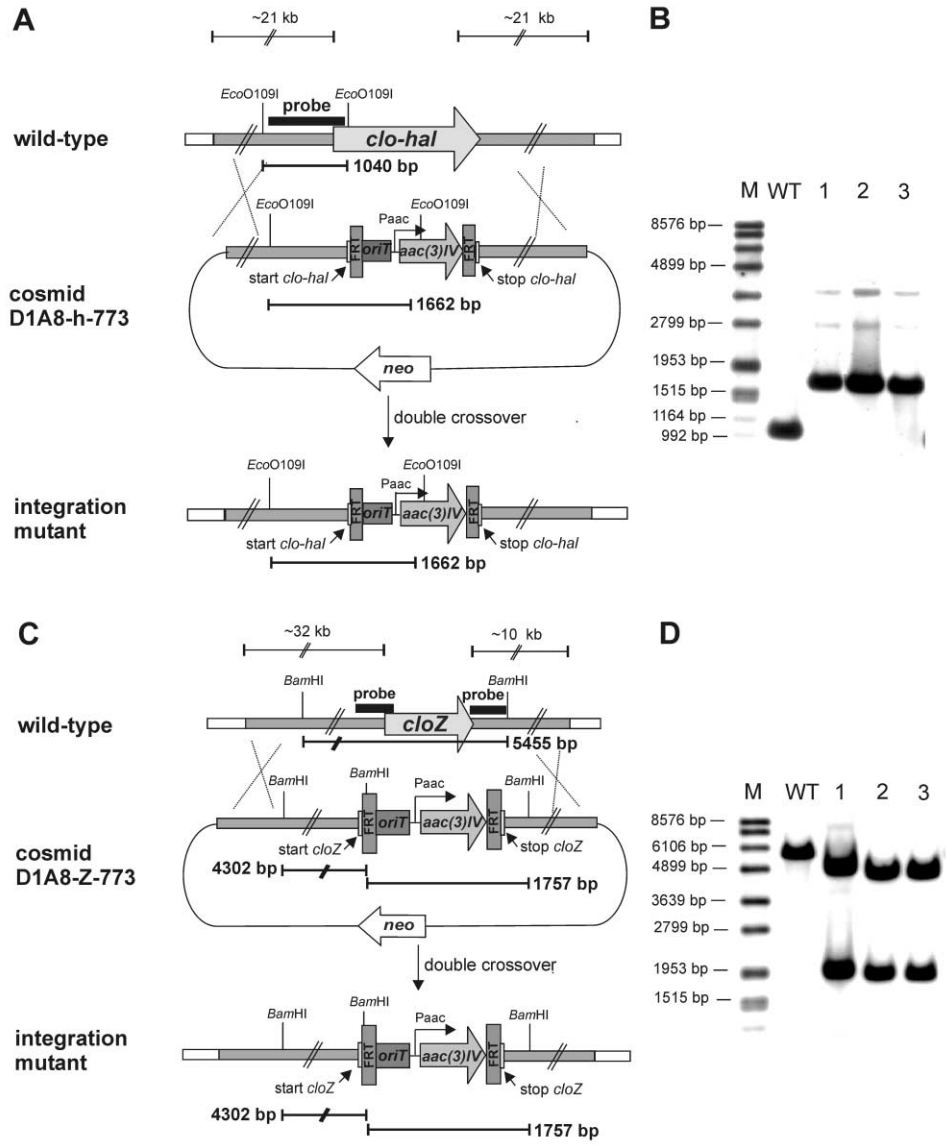


Figure 2. Inactivation of *clo-hal* and *cloZ* in *S. roseochromogenes*

(A) Schematic presentation of the *clo-hal* inactivation experiment. The DNA fragment used as probe is indicated as a black bar. *aac(3)IV*, apramycin resistance gene; Paac, promoter of the apramycin resistance gene; FRT, FLP recognition target; *oriT*, origin of transfer from RK2; *neo*, kanamycin resistance gene. Out of scale.

(B) Southern blot analysis of wild-type and *clo-hal*$^-$ mutants. Genomic DNA was restricted by *Eco*0109I. Abbreviations: M, DIG-labeled DNA Molecular Weight Marker VII (Roche); WT, *S. roseochromogenes* var. *oscitans* wild-type; 1–3: *clo-hal*$^-$ mutants (strains AE h2, AE h10, and AE h11).

(C) Schematic presentation of the *cloZ* inactivation experiment. The DNA fragment used as probe is indicated as a black bar. Out of scale.

(D) Southern blot analysis of wild-type and *cloZ*$^-$ mutants. Genomic DNA was restricted by *Bam*H1. Abbreviations: M, DIG-labeled DNA Molecular Weight Marker VII (Roche); WT, *S. roseochromogenes* var. *oscitans* wild-type; 1–3, *cloZ*$^-$ mutants (strains AE-Z4, AE-Z25, and AE-Z40).

new substance was a clorobiocin derivative containing a hydrogen instead of a chlorine atom at C-8′ of Ring B. In the ^{1}H NMR spectrum of the new compound, the signal at 7.33 ppm corresponding to H-6′ of clorobiocin had disappeared. Instead, a signal at 7.06 ppm for two protons was observed as a broad singlet, which could be assigned to H-6′ and H-8′ by correlated spectroscopy (COSY). The coincidence of the signals of H-6′ and H-8′ as a broad singlet is in accordance with the spectrum reported from a naturally occurring novobiocin derivative lacking the 8′-methyl group [30]. In the ^{13}C NMR spectrum, the signal corresponding to C-8′ was found at 104.0 ppm (instead of 110.7 ppm as for clorobiocin), and the signal corresponding to C-6′ was at 115.0 ppm (clorobiocin, 112.5 ppm). This is also in accordance with literature data [30].

The new compound was named novclobiocin 101. The *clo-hal*$^-$ mutants produced 35–45 μg novclobiocin

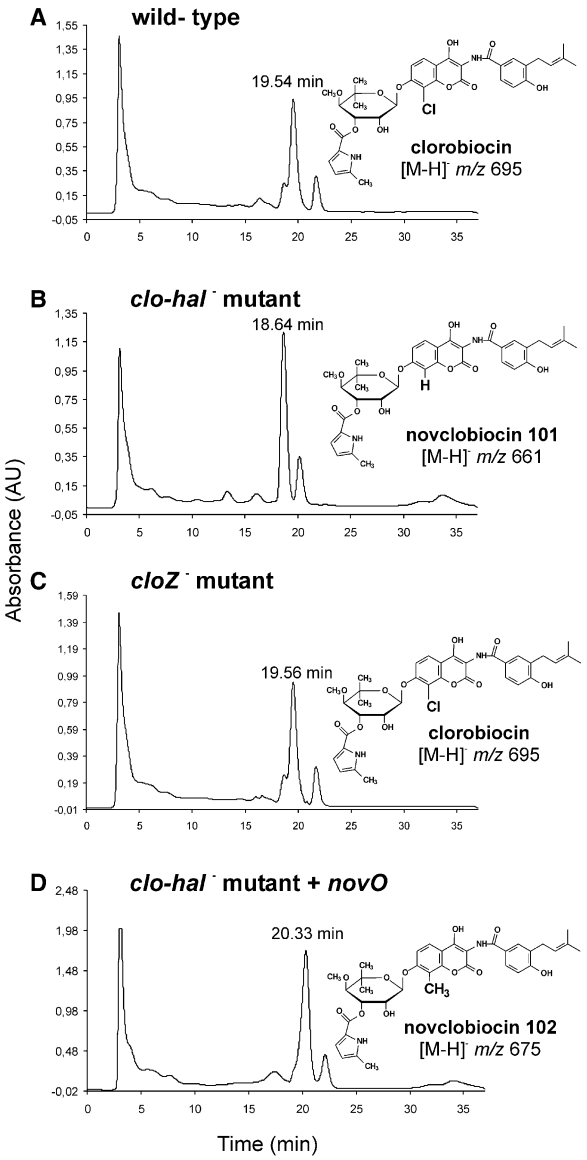


Figure 3. HPLC Analysis of Secondary Metabolites

(A) *S. roseochromogenes* var. *oscitans* wild-type.

(B) *clo-hal*$^-$ mutant.

(C) *cloZ*$^-$ mutant.

(D) *clo-hal*$^-$ mutant transformed with the *novO* expression construct (pTLO5).

101 per milliliter medium, which exceeded the clorobiocin production of the wild-type (25 μg/ml).

In contrast to the *clo-hal*$^-$ mutants, all *cloZ*$^-$ mutants produced clorobiocin (Figure 3C). The production level was similar to that of the wild-type strain (25 μg/ml). The identity of the clorobiocin produced by the *cloZ*$^-$ mutants was confirmed by negative-ion FAB MS (molecular ion $[M-H]^-$ at *m/z* 695) and ^{1}H NMR analysis (the spectrum obtained was identical to that of authentic clorobiocin; Table 1).

Although the presence of *cloZ* within the clorobiocin cluster is suggestive of a function related to the formation of this antibiotic, the above experiments show that *cloZ* is clearly not essential for clorobiocin biosynthesis under the present culture conditions, and apparently is not involved in the halogenation reaction.

Complementation of the *clo-hal*$^-$ Mutation

To prove that only the inactivation of *clo-hal* was responsible for the loss of the chlorine atom, we complemented the *clo-hal*$^-$ mutant by expression of an intact copy of *clo-hal* under the control of the constitutive *ermE** promoter, using the expression vector pUWL201 (plasmid pAE-ha7). HPLC analysis showed a chromatogram identical to that of the wild-type, i.e., clorobiocin production could be fully restored by expression of *clo-hal*. The authenticity of the obtained peak was confirmed by negative-ion FAB MS analysis (mass [intensity]: 695 [100.0%], 696 [36.5%], 697 [30.2%], 698 [10.5%]).

Combinatorial Biosynthesis: Expression of *novO* in the *clo-hal*$^-$ Mutant

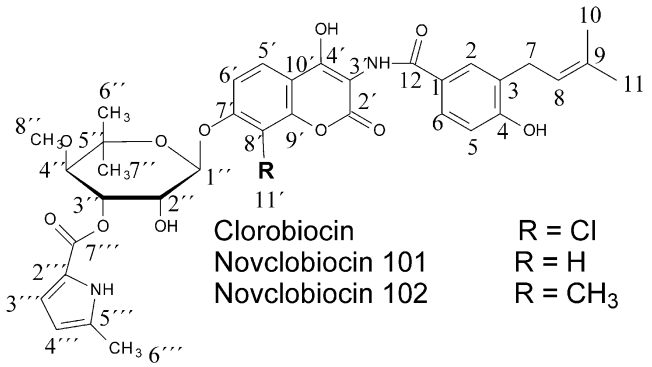
novO encodes a putative methyltransferase [18]. A very similar gene, *couO*, in the coumermycin A_1 biosynthetic cluster [19] (Figure 1B) has been experimentally confirmed to determine the methylation of C-8′ of the aminocoumarin ring [21]. NovO shows 84% identity to CouO, and both proteins are of equal size (230 aa). The conserved motif III [LL(R/K)PGG(R/I/L)(L/I)(L/F/I/V)(I/L)] for S-adenosyl-methionine-dependent methyltransferases [31] is found in both gene products from amino acid 135 to 144 (CouO, LVKPGGAILN; NovO, LAKPGGAVLN).

In order to produce a hybrid antibiotic, the putative methyltransferase gene *novO* was expressed in the *clo-hal*$^-$ mutant. For this purpose, *novO* was cloned into the replicative expression vector pUWL201 (see Experimental Procedures), placing it under the control of the constitutive *ermE** promoter. The resulting construct, pTLO5, was introduced into the *clo-hal*$^-$ mutant by protoplast transformation. As control, the *clo-hal*$^-$ mutant was transformed with the empty vector pUWL201.

Culture extracts of the two strains were analyzed by HPLC. In each case, three independent transformants were examined. While transformants containing the empty vector still produced novclobiocin 101 (data not shown), transformants containing the *novO* construct showed instead a new compound (Figure 3D) with a longer retention time than novclobiocin 101. Negative-ion FAB MS analysis of the isolated substance showed a molecular ion $[M-H]^-$ at *m/z* 675, corresponding to the addition of a methyl group to the molecule of novclobiocin 101. ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) analysis unequivocally confirmed that this substance was a clorobiocin derivative in which the chlorine atom at C-8′ of the aminocoumarin ring is replaced with a methyl group. In comparison to the ^{1}H NMR spectrum of clorobiocin (Table 1), an additional singlet at 2.34 ppm corresponding to three protons was detected, which could be assigned to 8′-CH_3, based on literature data [30]. In comparison to novclobiocin 101, the signal of H-8′ had disappeared, and the signal of H-6′ was found as doublet at 7.24 ppm, as reported in the literature [30]. In the ^{13}C NMR spectrum (Table 2), a new signal at 8.7 ppm was observed, which could be assigned to C-11′, using ^{1}H, ^{13}C correlated spectroscopy (COSY). This was also in accordance with the literature [30].

The new compound was named novclobiocin 102. It

Table 1. ^{1}H NMR Data of Clorobiocin, Novclobiocin 101, and Novclobiocin 102 in d_4-Methanol



	Compound		
	Clorobiocin	Novclobiocin 101	Novclobiocin 102
Position	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)
2-H	7.76 d (2.5)	7.77 d (2.1)	7.76 br s
5-H	6.84 d (8.4)	6.84 d (8.4)	6.84 d (8.3)
6-H	7.72 dd (8.4; 2.5)	7.72 br d (8.0)	7.72 br d (8.5)
7-H_2	3.34 d (7.1)	3.34 d (7.0)	3.34 d (7.2)
8-H	5.35 br[a] t (7.1)	5.35 br t (7.3)	5.35 br t (7.2)
10-H_3	1.74 s	1.74 s	1.74 s
11-H_3	1.75 s	1.75 s	1.75 s
5′-H	7.90 d (9.2)	7.92 d (9.4)	7.82 d (8.8)
6′-H	7.33 d (9.2)	7.06[b]	7.24 d (8.7)
8′-H	-	7.06[b]	-
11′-H_3	-	-	2.34 s
1″-H	5.73 d (1.8)	5.63 d (2.0)	5.63 d (1.6)
2″-H	4.34 t (2.7)	4.24 t (2.6)	4.29 br s
3″-H	5.71 dd (10.3; 2.9)	5.59 dd (9.8; 3.2)	5.67 dd (9.9; 3.1)
4″-H	3.72 d (10.3)	3.70 d (9.8)	3.71 d (9.9)
6″-H_3	1.18 s	1.20 s	1.18 s
7″-H_3	1.35 s	1.37 s	1.36 s
8″-OCH_3	3.52 s	3.51 s	3.52 s
3‴-H	6.90 d (3.6)	6.90 d (3.6)	6.90 d (3.6)
4‴-H	5.94 br d (3.6)	5.94 d (3.6)	5.94 br d (3.4)
6‴-H_3	2.29 s	2.29 s	2.29 s

δ is given in ppm. The solvent signal (3.30 ppm) was used as reference. Spectra were obtained at 400 MHz (clorobiocin and novclobiocin 102) or at 600 MHz (novclobiocin 101).

[a] br indicates broad signal.

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

was produced in an amount of 28–58 μg per ml medium (data of three independent transformants), exceeding the amount of clorobiocin produced in the wild-type (25 μg/ml).

In the wild-type and in all mutant strains, an additional peak with a slightly longer retention time than the respective main product was observed (Figures 3A–3D). Negative-ion FAB MS analysis of these minor peaks showed the same molecular ion as the corresponding main product. These compounds are likely to represent isomers of the main products, possibly carrying the pyrrole carboxylic acid moiety in position 2 instead of position 3 of the deoxysugar [20].

Antibacterial Activity of Novclobiocin 101 and Novclobiocin 102 in Comparison to Clorobiocin

Authentic clorobiocin (Aventis), novclobiocin 101 (from the *clo-hal*$^-$ mutant), and novclobiocin 102 (from the *clo-hal*$^-$ mutant transformed with *novO*) were assayed for antibiotic activity against *Bacillus subtilis* (Figure 4). Clorobiocin showed the highest antibacterial activity, followed by novclobiocin 102 (approximately half as active as clorobiocin) and novclobiocin 101 (approximately eight times less active than clorobiocin). The results show that a substitution at C-8′ of Ring B strongly enhances the activity of the aminocoumarin antibiotics against this test organism. A chlorine atom at this position appears to be superior to a methyl group, although the difference is moderate.

Discussion

The present work provides experimental proof for the function of *clo-hal* of the clorobiocin gene cluster and of *novO* of the novobiocin gene cluster, and shows that *cloZ* is inessential for clorobiocin biosynthesis. Moreover, two clorobiocin analogs with different substitutions at C-8′ of the aminocoumarin ring were produced by genetic manipulation.

The *clo-hal* gene product is responsible for the intro-

Table 2. ^{13}C NMR Data of Clorobiocin, Novclobiocin 101, and Novclobiocin 102 in d_4-Methanol at 100 MHz

	Compound		
	Clorobiocin	Novclobiocin 101	Novclobiocin 102
Position	δ (ppm)	δ (ppm)	δ (ppm)
1	124.2	124.3	124.3
2	130.9	130.8	130.8
3	129.9	129.8	129.8
4	161.0	160.9	160.9
5	115.6	115.6	115.6
6	128.5	128.5	128.4
7	29.2	29.2	29.2
8	123.2	123.2	123.2
9	133.8	133.8	133.8
10	17.9	17.9	17.9
11	26.0	26.0	26.0
12	170.0	169.8	169.7
2′	162.4[a]	163.3[a]	163.5[a]
3′	103.7	103.4	103.3
4′	157.8[a]	158.7[a]	158.8[a]
5′	123.9	126.4	123.2
6′	112.5	115.0	111.7
7′	161.8[a]	161.3[a]	158.8[a]
8′	110.7	104.0	114.9
9′	156.5	154.1	151.7
10′	113.3	112.4	112.4
11′	-	-	8.7
1″	100.4	100.1	100.0
2″	71.0	70.9	71.0
3″	71.6	71.6	71.8
4″	82.7	82.7	82.7
5″	80.5	80.15	80.1
6″	22.9	23.4	23.2
7″	29.3	29.0	29.2
8″	62.1	62.0	62.0
2‴	121.8	121.8	121.8
3‴	118.33	118.4	118.4
4‴	109.8	109.8	109.8
5‴	136.3	136.4	136.3
6‴	12.9	12.9	12.9
7‴	161.8[a]	161.9[a]	161.9[a]

The signal of the solvent (49.0 ppm) was used as reference. Assignments were made with the help of ^1H, ^{13}C COSY, and the literature [30, 40].
[a] Signal of carbon 2′, 4′, 7′, and 7‴ showed similar chemical shift (157.8–163.5 ppm), and their assignment may be interchanged.

duction of the halogen atom of clorobiocin. The mechanism of halogenation reactions, which are involved in the biosynthesis of at least 3000 natural halometabolites [32], is not yet completely understood. Clo-hal shows sequence similarity to the recently discovered class of $FADH_2$-dependent halogenases [22], such as are involved in the biosynthesis of chlortetracycline [33], the glycopeptide antibiotic balhimycin [24], and pyoluteorin [26]. It has been speculated that the reactions catalyzed by these enzymes may proceed via an epoxide intermediate [22]. In vitro, these enzymes require the presence of an unspecific flavin reductase generating $FADH_2$. This reductase appears not to be encoded within the gene clusters of the above mentioned secondary metabolites, similar to our finding for the clorobiocin cluster. Our functional identification of *clo-hal* reinforces the importance of this new class of halogenases for natural product biosynthesis.

Expression of *novO* from the novobiocin cluster in the *clo-hal*$^-$ mutant led to the very efficient formation of an

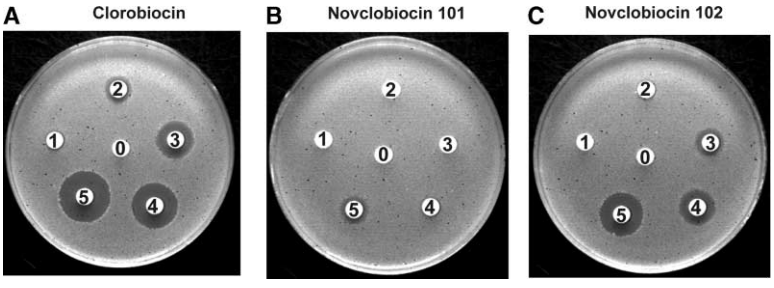


Figure 4. Antibacterial Activity of Clorobiocin and Derivatives

(A) clorobiocin, (B) novclobiocin 101, and (C) novclobiocin 102.

Bioassay with *Bacillus subtilis*. 0, methanol; 1–5, 0.5 μg, 1 μg, 2 μg, 4 μg, and 8 μg of the respective substance. For structures see Table 1.

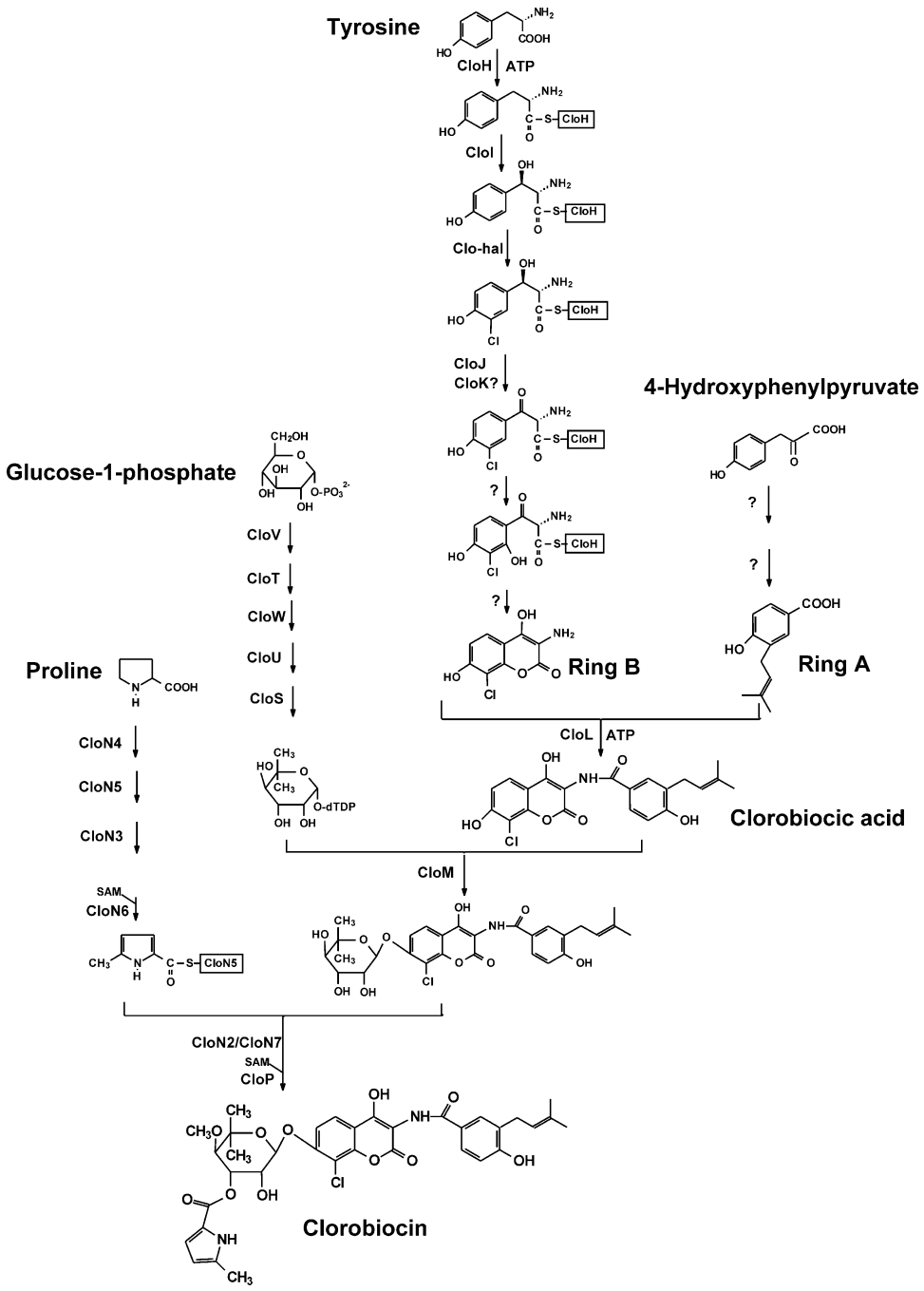


Figure 5. Hypothetical Biosynthetic Pathway of Clorobiocin

It is not clear at which exact step during the biosynthesis halogenation takes place (see Discussion).

8′-methylated compound, i.e., novclobiocin 102. Together with the previously published inactivation of the very similar gene *couO* from the coumermycin cluster [21], this result provides conclusive evidence that NovO and CouO catalyze the C-methylation reaction in the biosynthesis of the aminocoumarin rings of novobiocin and coumermycin.

It is not clear at present at which step of aminocoumarin biosynthesis the methylation or halogenation reactions takes place. For novobiocin biosynthesis, Chen and Walsh [34] provided evidence that the methylation occurs after activation of tyrosine, which is the first step in aminocoumarin formation (Figure 5), and an inactivation experiment by Steffensky et al. [18] demonstrated that methylation takes place before glycosylation of novobiocic acid. In Figure 5, we tentatively suggest the activated form of β-hydroxy-tyrosine as the substrate for the halogenation. In any case, for the assembly of the entire novobiocin or clorobiocin molecule, several enzymatic steps are required to take place after the

methylation or halogenation, respectively (Figure 5). It is remarkable that the yields of novclobiocin 101 (8′-H), produced by the *clo-hal*$^-$ mutant, and 102 (8′-CH_3), produced by the *clo-hal*$^-$ mutant expressing *novO*, were at least as high as that of clorobiocin (8′-Cl) in the wild-type. This indicates a low specificity of the subsequent biosynthetic enzymes for the substituent at the 8′ position, a very useful feature for the development of new aminocoumarin antibiotics by combinatorial approaches. In the present study, this allowed the production of the two clorobiocin analogs mentioned above, and a first comparison of these compounds with clorobiocin regarding their antibacterial activity.

Structurally, clorobiocin differs from novobiocin in the substitution at C-8′ of the aminocoumarin ring and at the C-3″ of the deoxysugar moiety (Figure 1). Clorobiocin has been reported to show a six times higher antibacterial activity than novobiocin in vitro [4] and to bind more efficiently to isolated gyrase [6–8]. Most authors have attributed the higher activity of clorobiocin primarily to the pyrrole moiety at C-3″ [7, 35]. Interestingly, however, clorobiocic acid (Figure 5), but not novobiocic acid, was found to inhibit both DNA synthesis in vivo and gyrase activity in vitro [5, 36], suggesting that the chlorine atom makes an important contribution to the biological activity of this molecule. The clorobiocin analogs produced in the present study allowed a comparison of aminocoumarin compounds, which differ only in the substitution at C-8′, i.e., carrying a chlorine, a methyl group or a hydrogen atom at that position. In a bioassay with *Bacillus subtilis* (Figure 4), clorobiocin (8′-Cl) was twice as active as novclobiocin 102 (8′-CH_3) and eight times more active than novclobiocin 101 (8′-H).

Significance

Clorobiocin (*clo*) and novobiocin (*nov*) are potent inhibitors of bacterial DNA gyrase. Their characteristic aminocoumarin moiety is substituted at C-8′ with a chlorine atom in clorobiocin and with a methyl group in novobiocin.

The results presented here demonstrate that the gene *clo-hal* is responsible for halogenation of position 8 of the aminocoumarin ring of clorobiocin, while *novO* is responsible for methylation of the corresponding position of novobiocin. *cloZ* is not involved in the halogenation, nor is it essential for clorobiocin biosynthesis under the present culture conditions. The functional identification of antibiotic biosynthetic genes provides important information for the generation of structurally modified compounds by combinatorial biosynthesis. An example of this approach is given by heterologous gene expression of the methyltransferase *novO* in a *clo-hal*$^-$ mutant, which led to production of a hybrid antibiotic in excellent yield. Similar inactivation/expression experiments, possibly combined with feeding of synthetic precursors, are expected to produce a range of new aminocoumarin antibiotics, possibly with improved properties. The PCR targeting system proved to be a fast and effective method for gene inactivation in *Streptomyces roseochromogenes* var. *oscitans* and opens improved possibilities for genetic engineering.

Bioassays showed that the chlorine atom of clorobiocin is important for biological activity, resulting in higher antibacterial activity than a methyl group in the same position.

Experimental Procedures

Bacterial Strains, Plasmids, and Culture Conditions

E. coli-Streptomyces shuttle vector pUWL201 was kindly provided by A. Bechthold (Freiburg, Germany) and was originally obtained from W. Piepersberg (Wuppertal, Germany). Cosmid D1A8 contained the clorobiocin biosynthetic gene cluster [20] in the SuperCos1 vector. Cosmid 9-6G, used for construction of pTLO5, was described previously [18].

Streptomyces roseochromogenes var. *oscitans* DS 12.976 was kindly provided by Aventis and 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 30°C and 180 rpm for 2 to 3 days. For preparation of genomic DNA, wild-type and mutant strains of *S. roseochromogenes* were grown in YEME medium without glycine [29]. For preparation of protoplasts, CRM medium [20] was used. For conjugation, cells were cultured in a 1:1 mixture of TSB and YEME medium [29] without glycine for 2 to 3 days at 30°C and 180 rpm. 2×YT broth, containing 1.6% tryptone, 1% yeast extract, and 0.5% NaCl (pH 7.0), was used during conjugation (see below). For analysis of secondary metabolites, cells were precultured in 50 ml cornstarch medium (1% cornstarch, 1% peptone, 0.5% meat extract [pH 7.0]) for 2 days at 33°C and 210 rpm. Five milliliters of this preculture was inoculated into 50 ml of production medium adapted from [37], prepared from 4.8% distillers' solubles, 3.7% glucose, 0.0024% cobalt chloride (at this point, the pH of the mixture was adjusted to 7.8), 0.6% calcium carbonate, and 0.2% ammonium sulfate. Cultivation was carried out in 500 ml baffled flasks for 5 to 8 days at 33°C and 210 rpm.

Escherichia coli XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) was used for cloning experiments and grown in liquid or on solid Luria-Bertani medium (1.5% agar) at 37°C [38]. Before transformation of *S. roseochromogenes*, the recombinant plasmids were amplified in *E. coli* ET12567 to bypass methyl-sensing restriction [39].

The REDIRECT© technology kit containing *E. coli* ET12567, *E. coli* ET12567/pUZ8002, *E. coli* BW25113/pIJ790, and *E. coli* DH5α/pIJ773 [28] was obtained from Plant Bioscience Limited (Norwich Research Park, Colney, Norwich).

Thiostrepton (50 μg/ml for solid media and 40 μg/ml for liquid media), apramycin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25–50 μg/ml), nalidixic acid (25 μg/ml), and carbenicillin (50–100 μg/ml) 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 et al. [38] and Kieser et al. [29]. 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 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 *S. roseochromogenes* strains using the Kirby mix procedure [29].

Southern blot analysis was performed on Hybond-N nylon membrane (Amersham, Braunschweig, Germany) with digoxigenin-labeled probe by using the DIG high prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals).

Inactivation of *clo-hal* in *S. roseochromogenes*

clo-hal was inactivated using the PCR targeting system [28], which takes advantage of the λ RED recombination functions (*gam*, *bet*, *exo*) to promote gene replacement. An *aac(3)IV* (apramycin resistance gene)/*oriT* cassette for replacement of *clo-hal* was generated by PCR using the primer pair P1-clo-hal (5′-ATTGGCGATTTATCGTCAGCGGTTGGAGGAAGTAGCGTGATTCCGGGGATCCGTCGACC-3′)

and P2-clo-hal (5′-AGCCTTCGGGCGAGAAGTCCTCGTCACGTACCGCGCTTATGTAGGCTGGAGCTGCTTC-3′). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *clo-hal*, respectively, including the putative start and stop codons of *clo-hal*. 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 and PCR using test primers TP1-clo-hal (5′-GGAACGGAAGCTTGGCTATC-3′) and TP2-clo-hal (5′-GAAGTGCGACAGGATCTGGA-3′).

The mutated cosmid (termed D1A8-h-773) was introduced into *Streptomyces roseochromogenes* by conjugation from *E. coli* ET12567 carrying the nontransmissible pUZ8002. The conjugation procedure was adapted from Gust et al. [28] and Kieser et al. [29]. Fresh mycelium from 50 ml TSB/YEME 1:1 liquid culture was used instead of spores. After centrifugation, the mycelium was washed with 10.3% sucrose solution and resuspended in 5 ml 2×YT broth. Five hundred microliters of the *Streptomyces* suspension was gently mixed with 500 μl of the *E. coli* suspension (treated as described in [29]). The mixture was spread on two MS plates [29] and incubated at 30°C for about 18 hr, then overlaid with 1 ml water containing 1.25 mg apramycin and 0.5 mg nalidixic acid. Incubation at 30°C was continued for about a week to allow outgrowth of the exconjugants. Apramycin-resistant, kanamycin-sensitive colonies were identified by replica plating and characterized by PCR (using the test primers mentioned above) and Southern blot analysis.

Inactivation of *cloZ* in *S. roseochromogenes*

cloZ was inactivated as described for *clo-hal*. The primer pair used for amplification of the *aac(3)IV/oriT* cassette was P1-cloZ (5′-GGCCTGTCAGGCGTTAACGGCGTTGACTCGTCGATGATGATTCCGGGGATCCGTCGACC-3′) and P2-cloZ (5′-CCGGCGTAACCCTCCG CGGTCGCCAGTCGCGCTGCGTCATGTAGGCTGGAGCTGCTTC-3′).

Test primers were TP1-cloZ (5′-GATCCTGAATTGGCCATCAAC-3′) and TP2-cloZ (5′-ATGCGTCAGACGAGAACTCAC-3′).

Construction of Plasmids pTLO5 and pAE-ha7

The *E. coli-Streptomyces* shuttle vector pUWL201, containing the *ermE** promotor, was used for the construction of the expression plasmids pTLO5 and pAE-ha7.

pTLO5

novO was amplified by PCR using cosmid 9-6G as template and the primer pair novO-E1 (5′-CTACGCCAAGCTTGTTCAGATCAG-3′) and novO-E2 (5′-CTCACTCGAGTCCAGGCG CTCTG-3′); underlined letters represent mutations inserted in comparison to the original sequence to give the desired restriction sites *Hind*III and *Xho*I, respectively. The *Hind*III/*Xho*I fragment, containing the *novO* gene and its natural ribosome binding site (CGAGG), was ligated into the same sites of pGEM7Zf(-) to give pTLO1. After isolation, pTLO1 was digested using *Hind*III and *Xba*I and ligated into the same sites of pUWL201 to give pTLO5.

pAE-ha7

clo-hal was amplified by PCR using cosmid D1A8 as template and the primer pair Pclo-hal/BamHI (5′-GGTCGCGGATCCCAGAAAC-3′) and Pclo-hal/XbaI (5′-GCCTTCGGTCTAGAAGTCC-3′); underlined letters represent mutations inserted in comparison to the original sequence to give the desired restriction sites *Bam*HI and *Xba*I, respectively. After restriction, the fragment containing the *clo-hal* gene and its natural ribosome binding site (GGAGG) was ligated into the same sites of pUWL201 to give pAE-ha7.

Transformation of the *clo-hal*⁻ Mutant with Plasmid pTLO5 or pAE-ha7

Transformation of the *clo-hal*⁻ mutant of *S. roseochromogenes* with pTLO5 or pAE-ha7 was carried out by polyethylene glycol-mediated protoplast transformation [20, 29].

Production and Analysis of Secondary Metabolites

Mutants, transformants, and the wild-type strain of *S. roseochromogenes* were cultured in cornstarch medium and in production medium as described above. Five milliliters of bacterial culture were acidified with HCl to pH 3 and extracted twice with an equal volume of ethylacetate. After evaporation of the solvent, the residue was redissolved in 0.5 ml ethanol. After centrifugation, 10 to 100 μl of the clear supernatant was analyzed by HPLC with a Multosphere RP18-5 column (250 × 4 mm; 5 μm; C+S Chromatographie Service, Düren, Germany) at flow rate of 1 ml/min, using a linear gradient from 40% to 100% of solvent B in 25 min (solvent A, MeOH:H_2O:HCOOH, 50:49:1; solvent B, MeOH:HCOOH 99:1), with detection at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation, an extract of 500 ml bacterial culture was prepared and purified by HPLC as described above. The product was analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy and by negative-ion FAB mass spectrometry. Negative-ion FAB mass spectra were recorded on a TSQ70 spectometer (Finnigan, Bremen, Germany) using diethanolamine as matrix.

^{1}H NMR and ^{13}C NMR spectra were measured either on an AMX 400 or on an AMX600 spectrometer (Bruker, Karlsruhe, Germany), using CD_3OD as solvent.

Bioassay

Antibacterial activity of authentic clorobiocin (Aventis), novclobiocin 101, and novclobiocin 102 was tested using *Bacillus subtilis* ATCC 14893. For the bioassays, 0.5, 1, 2, 4, and 8 μg of the respective substance in 10 to 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 [29] containing approximately 2 × 10^5 spores of *Bacillus subtilis* per milliliter agar medium. After culturing overnight at 37°C, the diameter of the growth inhibition zone was determined.

Computer-Assisted Sequence Analysis

The DNASIS software package (version 2.1; Hitachi Software Engineering, San Bruno, CA) and the BLAST program (release 2.2.3) were used for sequence analysis and for homology searches in the GenBank database, respectively.

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Accession Numbers

The nucleotide sequences reported in this study are available in the GenBank database under accession number AF 329398 (clorobiocin cluster), AF170880 (novobiocin cluster), and AF235050 (coumermycin A_1 cluster).