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# Production of 8′-Halogenated and 8′-Unsubstituted Novobiocin Derivatives in Genetically Engineered *Streptomyces coelicolor* Strains

**Alessandra S. Eustáquio,[1] Bertolt Gust,[1,2] Shu-Ming Li,[1] Stefan Pelzer,[3,4] Wolfgang Wohlleben,[3] Keith F. Chater,[2] and Lutz Heide[1,*]**
[1]Pharmazeutische Biologie
Pharmazeutisches Institut
Eberhard-Karls-Universität Tübingen
Auf der Morgenstelle 8
72076 Tübingen
Germany
[2]Department of Molecular Microbiology
John Innes Centre
Norwich Research Park
Colney, Norwich NR4 7UH
United Kingdom
[3]Fakultät für Biologie
Mikrobiologisches Institut
Mikrobiologie/Biotechnologie
Eberhard-Karls-Universität Tübingen
Auf der Morgenstelle 28
72076 Tübingen
Germany

## Summary

**In the present study, we produced a hybrid antibiotic, carrying a chlorine atom instead of a methyl group at position 8 of the aminocoumarin moiety of novobiocin. This compound was not accessible by conventional gene inactivation/gene expression experiments due to difficulties in the genetic manipulation of the novobiocin producer *Streptomyces spheroides*. However, the desired compound was obtained after modification of the novobiocin biosynthetic gene cluster by using λ-Red-mediated recombination in *Escherichia coli*, followed by integration of the resulting modified cosmid into the φC31 attachment site of *Streptomyces coelicolor* and coexpression of the halogenase Clo-hal of clorobiocin biosynthesis. The halogenase BhaA, responsible for chlorination of tyrosyl moieties of the glycopeptide antibiotic balhimycin, was unable to functionally replace the halogenase Clo-hal, suggesting that the two enzymes have different substrate specificities.**

## Introduction

Antibiotic resistance has developed in every class of antibiotics, both natural and synthetic, within a few years after the first clinical use [1]. Therefore, there is a constant need for the development of new antibacterial drugs in order to reduce the impact of resistance in antibacterial therapy, as emphasized by the World Health Organization [2].

Natural products play a dominant role in the discovery of drugs for infectious diseases [3]. Structural modifications of these natural products are often necessary for improvements in efficacy and pharmacokinetics. Yet, introduction of structural changes by chemical synthesis is not always easy, especially if the compound's scaffold is very complex. Combinatorial biosynthesis, namely, the shuffling of genes with related functions but different specificities via genetic engineering to create combinatorial libraries of novel natural product-like molecules [4], offers a recently developed alternative way to introduce structural modifications. In addition, rational and directed generation of structurally modified antibiotics can be achieved by metabolic engineering of biosynthetic pathways, i.e., by the modification of specific biochemical reactions or the introduction of new ones by using recombinant DNA technology [5], as demonstrated, e.g., by Yanai et al. [6].

Clorobiocin (=chlorobiocin) and novobiocin (Figure 1) belong to the class of aminocoumarin antibiotics, which are highly suitable for drug development by metabolic engineering. They are potent inhibitors of bacterial DNA gyrase and are produced by different *Streptomyces* strains [7–9]. The efficacy of novobiocin against gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* has been demonstrated in preclinical and clinical trials [10–12]. Novobiocin and its derivatives have also been investigated as potential anticancer drugs [13–15]. Our group has recently cloned and sequenced the biosynthetic gene clusters of clorobiocin (*clo*) and novobiocin (*nov*) [16, 17].

Clorobiocin is the more potent of the two aminocoumarin antibiotics [18]. It differs from the clinically approved drug novobiocin in the substitution pattern at C-8′ of the aminocoumarin ring, carrying a chlorine atom instead of a methyl group, and in the presence of a 5-methylpyrrole-2-carboxyl moiety instead of a carbamoyl group at 3″-OH of the deoxysugar unit (Figure 1). We aimed at the production of hybrid antibiotics by combining structural features of these two compounds. A clorobiocin analog with -$CH_3$ instead of -Cl at C-8′ (=novclobiocin 102; Figure 1) was readily obtained by inactivation of the halogenase gene *clo-hal* in the clorobiocin producer *Streptomyces roseochromogenes* and by subsequent expression of the methyltransferase gene *novO* from the novobiocin producer *S. spheroides* in the *clo-hal*⁻ mutant [19].

However, when we attempted an inactivation of *novO* in the novobiocin producer *S. spheroides*, followed by expression of the halogenase *clo-hal* in the *novO*⁻ mutant in order to generate a hybrid antibiotic with a chlorine atom at C-8′ and a carbamoyl group at 3″-OH (novclobiocin 114; Figure 1), this strategy remained unsuccessful (F. Pojer and L.H., unpublished data). *S. spheroides* is very difficult to manipulate genetically [20], and this may have been the cause for the failure of our experiment. Similar problems encountered in many important antibiotic-producing strains severely limit the

*Correspondence: heide@uni-tuebingen.de
[4]Present address: Combinature Biopharm AG, Robert-Rössle-Str. 10, 13125 Berlin, Germany.

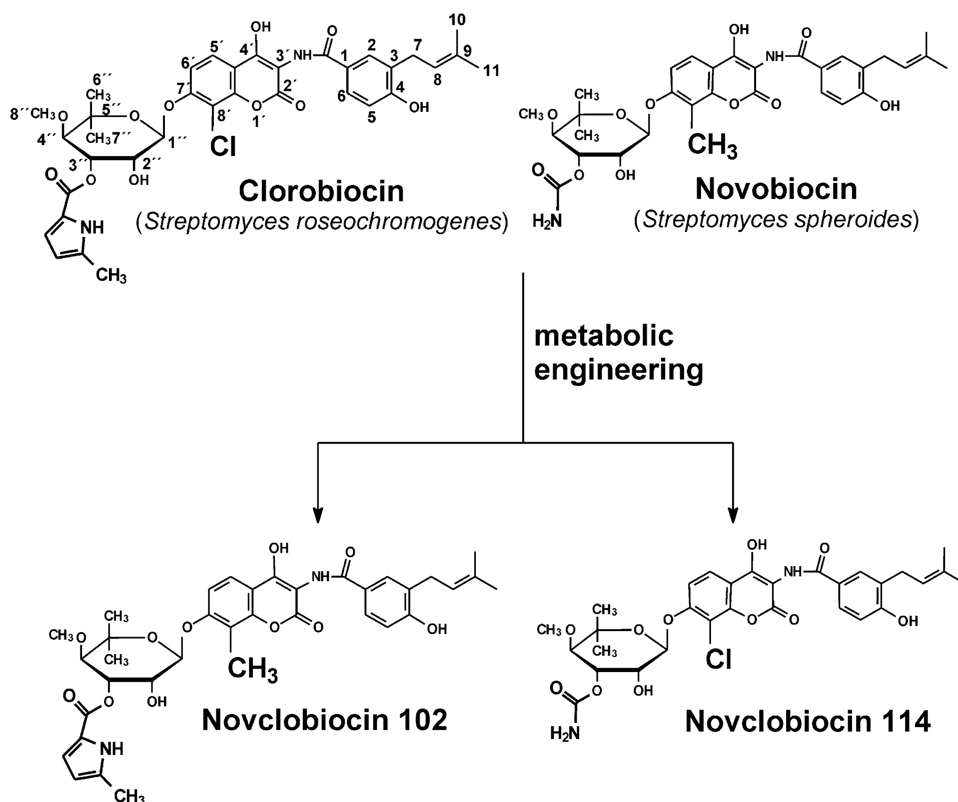



Figure 1. Structures of Aminocoumarins
Structures of the aminocoumarin antibiotics novobiocin and clorobiocin, and of the hybrid antibiotics novclobiocin 102 and 114.

possibilities for combinatorial biosynthesis and metabolic engineering.

In the present study, we therefore attempted to circumvent the problem of genetic manipulation in *S. spheroides* by heterologous expression of the entire biosynthetic gene cluster of novobiocin in *S. coelicolor*, which is well characterized and easy to manipulate [21, 22]. Indeed, the desired compound, novclobiocin 114, was readily produced by this method.

Parallel expression experiments in the heterologous producer strain, using either *clo-hal* from the clorobiocin producer or the very similar gene *bhaA* from the balhimycin producer [23], showed that these two halogenases are not interchangeable, despite their sequence similarity and their common involvement in a 3-chlorination of a β-hydroxy-tyrosine derivative.

## Results

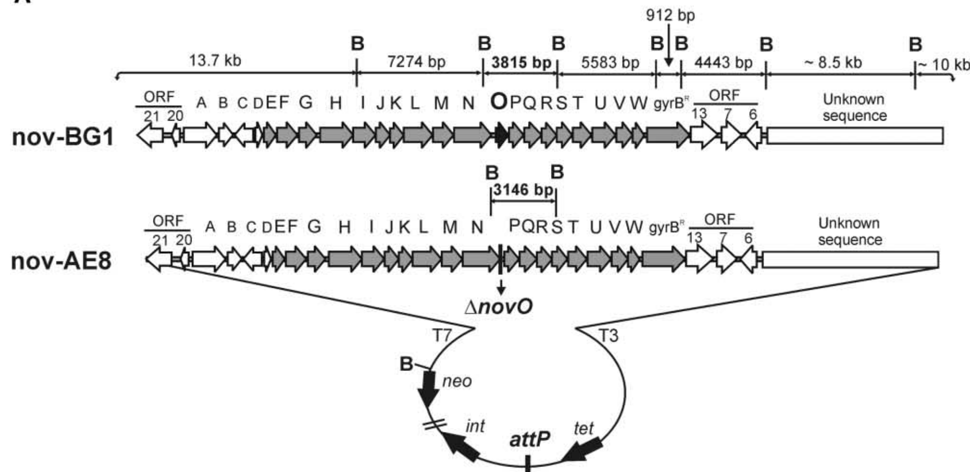
### Heterologous Expression of the Intact Novobiocin Biosynthetic Gene Cluster

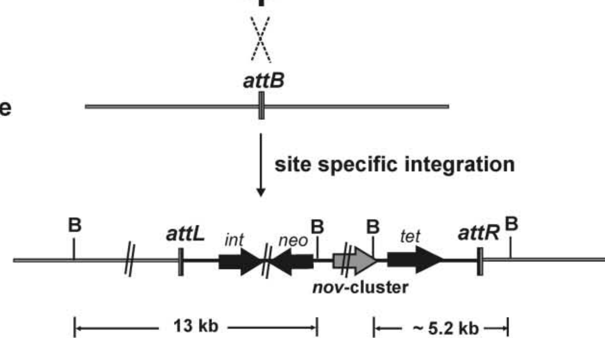
We have previously cloned the biosynthetic gene cluster of novobiocin into the SuperCos I vector, resulting in cosmid 10-9C [16]. For the heterologous expression, the ampicillin resistance gene in the SuperCos I backbone of cosmid 10-9C was replaced by a cassette containing the integrase gene (*int*) and attachment site (*attP*) of phage ϕC31 by using λ-Red-mediated recombination (see the Experimental Procedures). The resulting cosmid nov-BG1 is depicted in Figure 2A. Of the 45 kb insert, the entire novobiocin cluster and parts of the flanking regions have been sequenced, while approximately 10 kb of insert sequence are yet unknown.

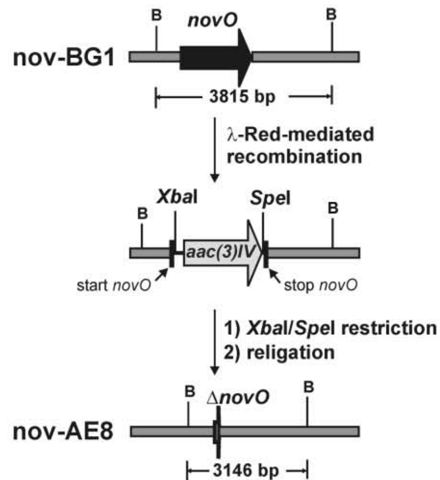
The cosmid nov-BG1 was then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation. Southern blot analysis confirmed its integration into the genome of *S. coelicolor*. After hybridization with the DIG-labeled cosmid nov-BG1, *S. coelicolor* integration mutants showed all of the bands expected from the specific integration of nov-BG1 into the ϕC31 attachment site, while the parental strain gave no hybridization signals (Figure 2D, lanes 1 and 2).

Two independent integration mutants as well as the parental strain were cultured in novobiocin production medium. The analysis of secondary metabolites by HPLC showed that the mutants (Figure 3B), in contrast to the parental strain (Figure 3A), accumulated novobiocin. This compound was isolated on a preparative scale. The identity of the produced novobiocin was confirmed by $^1$H NMR analysis (Table 1; Figure 4). The obtained signals were identical to those from authentic novobiocin. Negative-ion FAB MS analysis showed a molecular ion $[M-H]^-$ at *m/z* 611 (novobiocin is $C_{31}H_{36}N_2O_{11}$; molecular weight = 612).

The two *S. coelicolor* strains that had integrated the novobiocin biosynthetic gene cluster produced on aver-



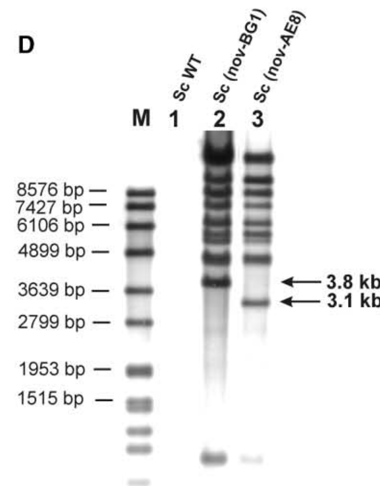




Figure 2. Heterologous Expression of the Novobiocin Biosynthetic Gene Cluster in *S. coelicolor* and Inactivation of *novO*

(A) Schematic presentation of cosmids nov-BG1, containing the intact novobiocin biosynthetic gene cluster, and nov-AE8, in which *novO* was deleted. The novobiocin cluster is presented in gray, the flanking regions are presented in white, and *novO* is presented in black. B = BglII restriction site. T3 and T7 indicate the orientation of the cluster in the SuperCos I vector. *tet* = tetracycline resistance gene; *neo* = neomycin/kanamycin resistance gene; *int*, *attP* = integrase and attachment site, respectively, of phage $\phi$C31. The cosmid backbone is not shown to scale.

(B) Schematic presentation of site-specific integration. The integrase, *int*, derived from *Streptomyces* phage $\phi$C31 catalyzes integration via recombination between *attP* (from phage or vector) and *attB* (from *Streptomyces* genome) sites, generating the hybrid sites *attL* and *attR* [47]. Junction fragments that prove specific integration into the $\phi$C31 attachment site of the *S. coelicolor* genome are indicated. This figure is not shown to scale.

(C) Schematic presentation of the *novO* inactivation. *novO* (693 bp) was first replaced by an apramycin resistance (*aac(3)IV*) cassette. Afterwards, the cassette was excised by digestion with XbaI and SpeI and religated, leaving an in-frame "scar" of 18 nucleotides between the start and stop codons of *novO*. This figure is not shown to scale.

(D) Southern blot analysis of *S. coelicolor* M512 (lane 1) and M512-derived integration mutants bearing cosmid nov-BG1 (lane 2) or the $novO^-$ cosmid nov-AE8 (lane 3). M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic DNA was digested with BglII. The DIG-labeled cosmid nov-BG1 was used as a probe. The *novO* inactivation is confirmed by the 3.1 kb band (lane 3) instead of a 3.8 kb band (lane 2); otherwise, the band pattern should be identical.

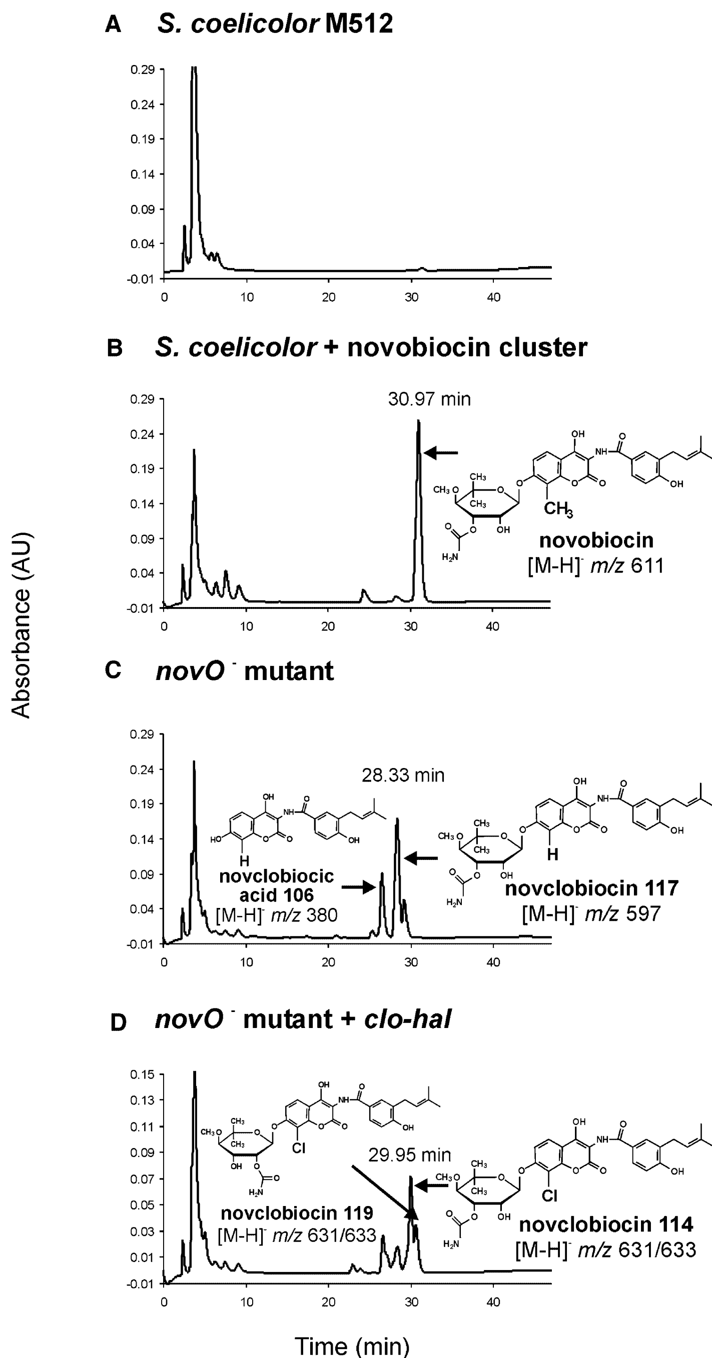



Figure 3. HPLC Analysis of Secondary Metabolites

(A) *S. coelicolor* M512 parental strain.
(B) *S. coelicolor* (nov-BG1), carrying the novobiocin biosynthetic gene cluster.
(C) *S. coelicolor* (nov-AE8), *novO*⁻ mutant.
(D) *S. coelicolor* (nov-AE8)/pAE-ha7, *novO*⁻ mutant transformed with the *clo-hal* expression construct (pAE-ha7). Detection at 305 nm. The major products are emphasized by a larger font size.

age 31 mg novobiocin per liter, comparable to the productivity of the natural producer *S. spheroides* (35 mg/l).

### Heterologous Expression of a Modified Novobiocin Cluster (*novO*⁻)

Since heterologous expression of the intact novobiocin biosynthetic gene cluster was successful, we attempted to generate a modified (8′-unsubstituted) antibiotic by inactivation of the methyltransferase gene *novO* in cosmid nov-BG1 and introduction of this modified cosmid into the genome of *S. coelicolor*.

Therefore, *novO* was replaced by an apramycin resistance cassette flanked by XbaI and SpeI recognition sites via λ-Red-mediated recombination [24]. The cassette was then removed by digestion with XbaI and SpeI, enzymes that create compatible ends, allowing religation of the outer ends and consequent excision of the cassette (Figure 2C). This procedure leaves a minimal in-frame "scar" of 18 nucleotides inside the targeted sequence. The modified cosmid (named nov-AE8) was then introduced into *S. coelicolor* by transformation. Southern blot analysis confirmed the integration into the

Table 1. $^1$H NMR Data of Novobiocin, Novclobiocic Acid 106, Novclobiocin 117, Novclobiocin 114, and Novclobiocin 119 in *d4*-Methanol

| | Compound | | | | |
|---|---|---|---|---|---|
| | Novobiocin | Novclobiocic Acid 106 | Novclobiocin 117 | Novclobiocin 114 | Novclobiocin 119 |
| Position | δ, Multiplicity (J/Hz) | δ, Multiplicity (J/Hz) | δ, Multiplicity (J/Hz) | δ, Multiplicity (J/Hz) | δ, Multiplicity (J/Hz) |
| 2-H | 7.75 br[a] s | 7.76 br s | 7.76 br s | 7.75 br s | 7.76 br s |
| 5-H | 6.84 d (8.3) | 6.83 d (8.4) | 6.82 d (8.2) | 6.84 d (8.3) | 6.82 d (8.3) |
| 6-H | 7.72 br d (8.3) | 7.72 br d (8.4) | 7.72 br d (8.2) | 7.72 br d (8.3) | 7.72 br d (8.3) |
| 7-$H_2$ | 3.34 d (7.3) | 3.34 d (7.5) | 3.33[b] | 3.33 d (7.1) | 3.33 d (6.9) |
| 8-H | 5.34 mt (7)[c] | 5.35 br t (7.5) | 5.35 mt (7)[d] | 5.35 mt (7)[c] | 5.35 br t (7)[d] |
| 10-$H_3$ | 1.74 s | 1.74 br s | 1.74 br s | 1.74 s | 1.74 br s |
| 11-$H_3$ | 1.75 s | 1.74 br s | 1.74 br s | 1.75 s | 1.74 br s |
| 5′-H | 7.81 d (8.9) | 7.81 br d (8.0) | 7.91 br d (7.5) | 7.88 d (8.9) | 7.88 d (8.8) |
| 6′-H | 7.23 br d (8.9) | 6.78 br d (8.3) | 7.00 br d (7.6)[c] | 7.29 d (8.9) | 7.26 d (9.1) |
| 8′-H | – | 6.68 s | 7.00 s | – | – |
| 11′-$H_3$ | 2.31 s | – | – | – | – |
| 1″-H | 5.57 br s | – | 5.55 d (2.3) | 5.67 br s | 5.74 br s |
| 2″-H | 4.23 t[b] | – | 4.18 t (2.8) | 4.28 t[b] | 5.10 t[b] |
| 3″-H | 5.31 dd (3; 10)[c] | – | 5.25 dd (3.1; 9.6) | 5.36 dd (2.8; 10.0)[c] | 4.35 dd (3.4; 9.9) |
| 4″-H | 3.57 d (9.9) | – | 3.54 d (10.2) | 3.58 d (10.0) | 3.40 d (9.9) |
| 6″-$H_3$ | 1.14 s | – | 1.17 s | 1.15 s | 1.14 s |
| 7″-$H_3$ | 1.34 s | – | 1.35 s | 1.32 s | 1.32 s |
| 8″-$H_3$ | 3.55 s | – | 3.53 s | 3.55 s | 3.60 s |

The numbering of the structures is shown in Figure 4.
δ is given in ppm. The solvent signal (3.30 ppm) was used as reference. Spectra were obtained at 400 MHz.
[a] br = broad signal.
[b] *J* is not determinable.
[c] Complex, overlapping signals; *J* is not exactly determinable.
[d] Complex signal; *J* is not exactly determinable.

genome, and the deletion of *novO* was clearly shown by the shortening of the relevant BglII restriction fragment (Figure 2D, lane 3).

The analysis of secondary metabolites by HPLC (Figure 3C) showed the complete absence of novobiocin.

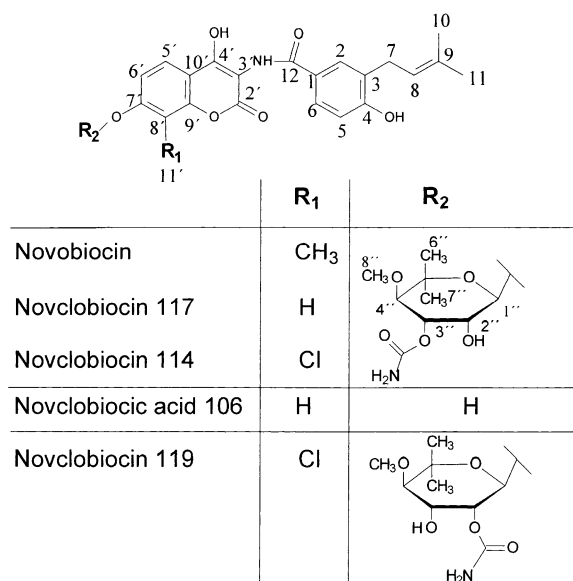



Figure 4. Chemical Structure of Aminocoumarin Antibiotics Generated in This Study

Instead, the novobiocin derivative novclobiocin 117, carrying a hydrogen instead of a methyl group at C-8′, accumulated. The structure was confirmed by negative-ion FAB MS (molecular ion $[M-H]^-$ at *m/z* 597 corresponding to the loss of a methyl group in comparison to novobiocin) and by $^1$H NMR analysis (Table 1) of the isolated compound, which clearly showed the absence of the signal for 8′-$CH_3$. These spectroscopic data are in accordance with the literature [25, 26]. The *novO* mutant also produced a minor product (=novclobiocic acid 106) with shorter retention time than novclobiocin 117. This compound lacked the entire deoxysugar moiety, as shown by a molecular ion $[M-H]^-$ at *m/z* 380 in MS analysis and by the absence of sugar protons in $^1$H NMR analysis (Table 1). In addition, a third compound was detected with a slightly higher retention time than novclobiocin 117. This substance showed a molecular ion $[M-H]^-$ at *m/z* 597, i.e., identical to novclobiocin 117. As explained below, this compound is likely to represent an isomer of novclobiocin 117, carrying the carbamoyl moiety in position 2 instead of 3 of the deoxysugar. The production of such 2″-acylated isomers has previously been observed in strains of *S. roseochromogenes*, the clorobiocin producer [27, 28].

The *S. coelicolor* strain harboring the *novO*-defective cluster produced 30 mg/L novclobiocin 117 and 10 mg/L novclobiocic acid 106. Its productivity was therefore similar to that of the *S. coelicolor* strains carrying the intact novobiocin biosynthetic gene cluster (see above).

### Complementation of the *novO* Mutation

To prove that only the inactivation of *novO* was responsible for the loss of the methyl group, we complemented

the mutant by expressing an intact copy of *novO*. For this purpose, we used a derivative of the replicative vector pUWL201, in which *novO* was placed under the control of the constitutive *ermE** promoter (plasmid pTLO5). After pTLO5 was used to transform the *novO* mutant, HPLC analysis showed that the novobiocin production was restored to about 80% of the productivity observed before *novO* inactivation (data not shown). The identity of novobiocin was confirmed by negative-ion FAB MS analysis (molecular ion $[M-H]^-$ at *m/z* 611).

### Expression of the Halogenase *clo-hal* in the *novO*$^-$ Mutant

In order to obtain the desired novobiocin analog carrying a chlorine atom instead of a methyl group at C-8′, the halogenase gene *clo-hal* from the clorobiocin biosynthetic gene cluster was expressed in the *S. coelicolor* strain harboring the *novO*-defective cluster by using the replicative plasmid pAE-ha7 in which *clo-hal* was under the control of the constitutive *ermE** promoter [19]. As shown by HPLC (Figure 3D), the resulting *clo-hal* transformants produced a substance that was absent from transformants carrying the empty replicative vector pUWL201 (data not shown). Negative-ion FAB MS of the isolated compound showed a molecular ion $[M-H]^-$ at *m/z* 631, corresponding to the substitution of a hydrogen by a chlorine atom in the molecule of novclobiocin 117. The typical isotopic pattern caused by the chlorine isotopes $^{35}Cl$ and $^{37}Cl$ was clearly visible (mass [intensity]: 631 [100.0%], 632 [30.6%], 633 [31.9%], 634 [11.2%]). $^1H$ NMR analysis (Table 1) unequivocally confirmed that the chlorine atom was attached at C-8′ of the aminocoumarin core: the signal at 7.00 ppm corresponding to the two protons, H-6′ and H-8′, of novclobiocin 117 had disappeared, and instead a signal at 7.27 ppm (doublet, 1H) was observed, which could be assigned to H-6′. In comparison, the H-6′ signal of novobiocin was seen as a doublet (1H) at 7.23 ppm (Table 1). As observed previously [19, 26], the chlorine atom at C-8′ in clorobiocin causes a slightly different magnetic shift of the H-6′ signal than a methyl group at the same position. The accumulated substance was therefore unequivocally identified as novclobiocin 114 (Figure 1). The three independent *clo-hal* transformants tested produced on average 14 mg novclobiocin 114 per liter of medium. The minor peak with slightly longer retention time represents the isomer of novclobiocin 114, i.e., novclobiocin 119, which carries the carbamoyl moiety in position 2 instead of 3 of the deoxysugar. In the negative-ion FAB MS, it showed the same molecular ion $[M-H]^-$ at *m/z* 631 and a similar isotopic pattern (mass [intensity]: 631 [100.0%], 632 [34.7%], 633 [32.1%], 634 [12.5%]) as novclobiocin 114. Furthermore, in the $^1H$ NMR analysis (Table 1), the signal of H-2″ was seen at 5.10 ppm instead of 4.28 ppm, and the signal of H-3″ was seen at 4.35 instead of 5.36 ppm. Therefore, the chemical shift of the sugar protons demonstrates the presence of the carbamoyl moiety in position 2 instead of 3, in accordance with published data on novobiocin and isonovobiocin [29].

The minor peaks with shorter retention times than novclobiocin 114 (Figure 3D) represent the remaining nonhalogenated compounds, i.e., novclobiocin 117 and novclobiocic acid 106.

### Antibacterial Activity of Novclobiocins in Comparison to Novobiocin

The obtained novclobiocins (i.e., novclobiocin 114, 119, 117, and novclobiocic acid 106; see Figure 4 for structures) were assayed for antibiotic activity against *Bacillus subtilis* in comparison to authentic novobiocin (Figure 5). In accordance with results obtained previously [19, 26, 30], substitution at C-8′ with either $-CH_3$ or -Cl was important for biological activity: novclobiocin 117 (8′-H) showed only 4% of the activity of novobiocin, while novclobiocin 114 (8′-Cl) was half as active as novobiocin (8′-$CH_3$). Further investigations of the structure-activity relationships of these compounds are in progress.

As reported for clorobiocin derivatives [30], the transfer of the acyl group from 3″-OH to 2″-OH resulted in a reduction but not in a complete loss of activity: novclobiocin 119 still showed approximately 20% of the activity of novclobiocin 114. In contrast, the removal of the deoxysugar moiety and the 8′-substituent (=novclobiocic acid 106) resulted in a 99% loss of activity.

### Expression of the Halogenase *bhaA* in the *novO*$^-$ Mutant

The glycopeptide antibiotic balhimycin (Figure 6) and the aminocoumarin antibiotic clorobiocin both contain 3-chloro-β-hydroxy-tyrosyl-derived moieties. In both compounds, the biosynthesis of these moieties starts from L-tyrosine, which forms a thioester bond with an activating enzyme, i.e., BpsD or CloH, respectively. Subsequently, the cytochrome $P_{450}$ enzymes OxyD or CloI, respectively, introduce the β-hydroxy group (Figure 6) [31, 32]. The predicted protein sequences of CloH and CloI show clear sequence similarity to BpsD and OxyD. The introduction of the chlorine atom into position 3 of the aromatic nucleus of the β-hydroxy-tyrosyl moieties is catalyzed by enzymes with sequence similarity to $FADH_2$-dependent halogenases [33]. The predicted amino acid sequence of BhaA of the balhimycin cluster [34] shows 36% identity to Clo-Hal of the clorobiocin cluster [19]. In the biosynthesis of both antibiotics, it is unknown which exact intermediate serves as a substrate for the halogenation.

In order to verify whether *bhaA* could functionally replace *clo-hal*, we placed *bhaA* under the control of the constitutive *ermE** promoter by using the same expression vector, pUWL201, as employed for the experiments with *clo-hal* described above. The resulting plasmid was termed pAE-B3. pAE-B3 was constructed by cloning a BamHI-XbaI fragment of plasmid pSETbhaA, containing the *bhaA* gene and its natural ribosome binding site, [34] into the same sites of pUWL201. pSETbhaA has been successfully used for complementation of a *bhaA*$^-$ mutant of *Amycolatopsis balhimycina* [34].

pAE-B3 was introduced by protoplast transformation into the *S. coelicolor* strain containing the *novO*-defective novobiocin cluster. In three independent transformants, the presence of the intact plasmid was confirmed by plasmid isolation and restriction analysis. However, HPLC analysis of the three transformants showed no changes in comparison to the untransformed control, or to controls transformed with the empty vector

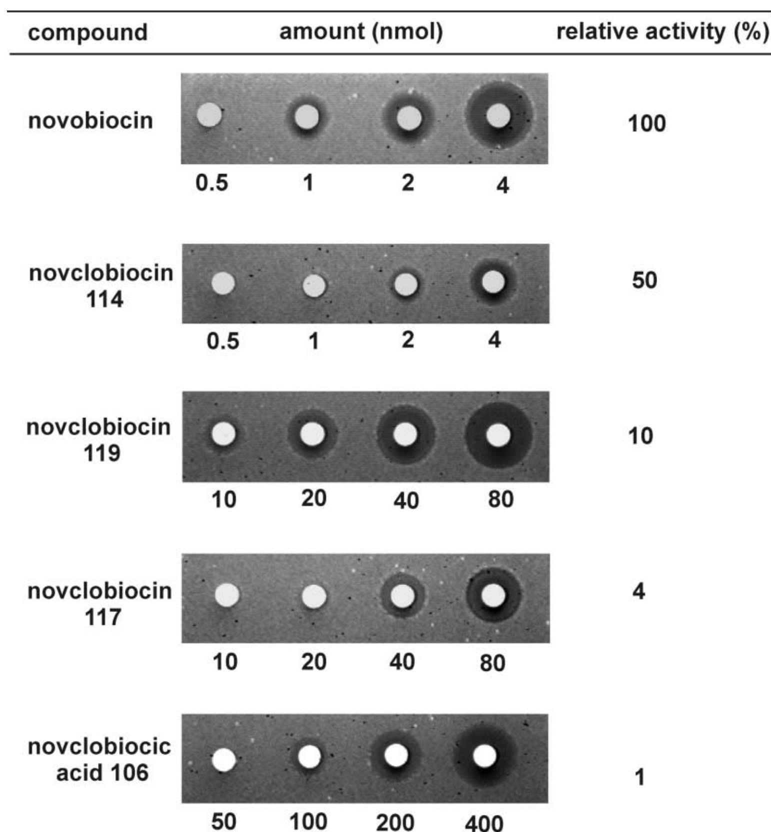



Figure 5. Antibacterial Activity of Novobiocin and Derivatives

Bioassay against *Bacillus subtilis*. Activities are expressed relative to novobiocin (100%). For structures, see Figure 4.

pUWL201. The detected compounds still showed the same molecular ions in the negative-ion FAB MS analysis as those seen in the *S. coelicolor* strain with the *novO*$^-$ cluster ([M-H]$^-$ at *m/z* 597, corresponding to novclobiocin 117; and [M-H]$^-$ at *m/z* 380, corresponding to novclobiocic acid 106). Therefore, no chlorinated products could be observed after expression of *bhaA* in the *novO*$^-$ mutant.

In order to confirm this result, we also introduced the *bhaA* expression plasmid pAE-B3 into the *clo-hal*$^-$ mutant of the clorobiocin producer *S. roseochromogenes*, constructed in a previous study [19]. Again, *bhaA* was unable to complement the mutant. HPLC analysis of three independent transformants, as well as FAB MS analysis of the isolated compounds, showed only the same metabolites as detected in the *clo-hal*$^-$ mutants prior to transformation. The dominant product was a clorobiocin derivative with a hydrogen instead of a chlorine atom at C-8′ ([M-H]$^-$ at *m/z* 661). If, however, the *clo-hal*$^-$ mutant was transformed with plasmid pAE-ha7, which contains *clo-hal* rather than *bhaA* and is otherwise identical to pAE-B3, clorobiocin production was readily restored. The resulting chromatogram corresponded to that of the wild-type *S. roseochromogenes*, and the identity of clorobiocin was confirmed by negative-ion FAB MS analysis (mass [intensity]: 695 [100.0%, [M-H]$^-$], 696 [36.5%], 697 [30.2%], 698 [10.5%]). These experiments provide clear evidence that *clo-hal* cannot be functionally replaced by *bhaA*.

## Discussion

In this study, we have developed a strategy for the production of a hybrid antibiotic, novclobiocin 114, which carries a chlorine atom instead of a methyl group at position 8′ of the aminocoumarin unit of novobiocin. This compound could not be produced by gene inactivation and heterologous gene expression experiments in the novobiocin producer *Streptomyces spheroides* because of difficulties in the genetic manipulation of this strain. Yet, through heterologous expression of the entire novobiocin biosynthetic gene cluster in *S. coelicolor*, novobiocin and, after appropriate modification of the cluster, both the 8′-H compound (novclobiocin 117) and the 8′-Cl compound (novclobiocin 114) were readily obtained from the resulting strains.

Obviously, further novobiocin derivatives may be obtained by modifying other genes of the novobiocin cluster, offering a strategy for the production of a number of 3″-carbomylated aminocoumarin antibiotics, in addition to the previously described chemoenzymatic methods [26, 35].

Clorobiocin (Figure 1) has been reported to show a higher inhibition of *Escherichia coli* gyrase and bacterial growth than novobiocin [18] and to bind more tightly to isolated gyrase [36–38]. The relative contribution of the 5-methylpyrrole-2-carboxyl moiety at 3″-OH of clorobiocin and of the chlorine atom at C-8′ to this effect is not known [19, 38, 39].

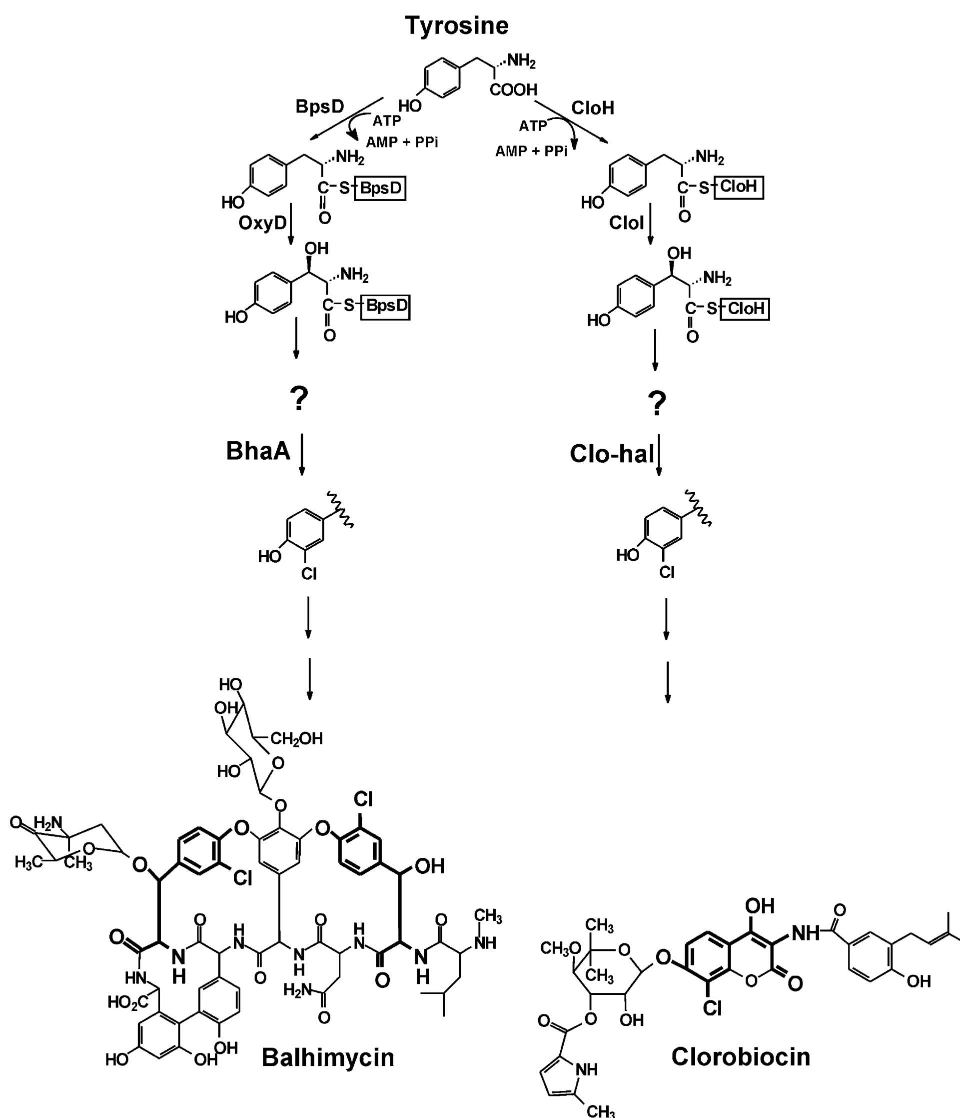



Figure 6. Biosynthesis of the 3-Chloro-β-Hydroxy-Tyrosyl-Derived Moieties of Clorobiocin and Balhimycin

In both pathways, it is unknown at which exact step during biosynthesis the halogenation takes place (see text). The 3-chloro-β-hydroxy-tyrosyl-derived moieties of clorobiocin and balhimycin are emphasized by a larger font size.

The substances produced in this study (novobiocin analogs with -$CH_3$, -H, or -Cl at C-8′) and in additional experiments will allow an investigation of the structure-activity relationships of such aminocoumarin compounds through the use of both antibacterial assays and in vitro investigations with purified gyrase.

An accumulation of truncated intermediates, lacking the deoxysugar moiety, was observed in mutants carrying modified biosynthetic pathways, i.e., in the *novO*$^-$ mutant (Figure 3C) and in the *novO*$^-$ mutant complemented with *clo-hal* (Figure 3D). This may indicate that the remaining biosynthetic machinery does not accept the modified substrates as efficiently as the genuine intermediates.

The aminocoumarin antibiotic clorobiocin and the glycopeptide antibiotic balhimycin both contain 3-chloro-β-hydroxy-tyrosyl-derived moieties, and the biosynthesis of these moieties shows striking similarities (see Results section and Figure 6). It is not clear at present at which step of clorobiocin biosynthesis the halogenation reaction takes place. In novobiocin biosynthesis, the methylation at C-8′ (corresponding to the chlorination at C-8′ in clorobiocin biosynthesis) occurs only after activation of tyrosine (Figure 6) [31], and before glycosylation of novobiocic acid [16]. Possible substrates of Clo-hal therefore include β-hydroxy-tyrosyl-CloH, 3-amino-4, 7-dihydroxycoumarin, and the amide of this aminocoumarin unit with 3-dimethylallyl-4-hydroxybenzoic acid.

Likewise, the substrate of the halogenase BhaA of balhimycin biosynthesis is still elusive. Possible candidates are tyrosine, β-hydroxytyrosine in either free or enzyme bound form, or substrates derived from the later stages of peptide assembly [32].

Our present study clearly showed that BhaA was un-

able to functionally replace Clo-hal. We have cloned the structural genes for both halogenases into the same expression vector, which contains the constitutive promoter *ermE**. Furthermore, the identical *bhaA* gene under control of this promoter has been used successfully for complementation of a *bhaA*-defective mutant [34]. However, only expression of *clo-hal* resulted in the formation of halogenated aminocoumarins in the present study.

To date, three different halogenating enzymes are known: haloperoxidases, perhydrolases, and $FADH_2$-dependent halogenases. Clo-hal and BhaA shows sequence similarity to the recently discovered class of $FADH_2$-dependent halogenases, which in contrast to haloperoxidases and perhydrolases show substrate specificity and regioselectivity. The mechanism of halogenation catalyzed by $FADH_2$-dependent halogenases is not yet completely understood. It has been speculated that the reaction may proceed via an epoxide intermediate. In vitro, these enzymes require the presence of an unspecific flavin reductase generating $FADH_2$ [33]. Recently, the cocrystallization of tryptophan 7-halogenase with tryptophan and FAD was described, representing an important step toward understanding the reaction mechanism of such enzymes [40].

Therefore, the inability of BhaA to functionally replace Clo-hal is unlikely to result from the specificity of the flavin reductase. Rather, our results suggest that the genuine substrates for the halogenation reactions catalyzed by BhaA and Clo-hal are different. This is in accordance with recent evidence showing that in balhimycin biosynthesis halogenation apparently takes place during the biosynthesis of the oligopeptide backbone [41].

## Significance

**Metabolic engineering of biosynthetic pathways for antibiotics, namely, the modification of specific biochemical reactions or the introduction of new ones by using recombinant DNA technology, represents a useful way of creating novel drugs, especially if the introduction of structural modifications by chemical synthesis is difficult due to the complex structure of the respective compound. In this study, we generated a novobiocin analog bearing a chlorine atom instead of a methyl group at C-8′ of the aminocoumarin core. Attempts to produce this substance in the natural novobiocin producer *Streptomyces spheroides* were unsuccessful, due to the difficulties in the genetic manipulation of this strain. Therefore, we modified the cosmid containing the novobiocin biosynthetic gene cluster by introducing the integrase gene, *int*, and the attachment site, *attP*, of phage $\phi$C31, via $\lambda$-Red-mediated recombination. Heterologous expression of this cosmid in *S. coelicolor* M512 resulted in the production of novobiocin in good yield, providing a novobiocin producer strain that is highly amenable for genetic manipulation. Inactivation of the methyltransferase *novO* could be rapidly achieved in the cosmid by $\lambda$-Red-mediated recombination in *Escherichia coli*. Subsequent integration of the *novO*$^-$ cosmid into the *S. coelicolor* genome, followed by expression of *clo-hal* by using a replicative expression plasmid, readily allowed the production of the desired hybrid antibiotic. This approach opens improved possibilities for genetic engineering of biosynthetic pathways of natural products in actinomycetes. At the same time, it allows a rapid functional investigation of genes, as demonstrated by our finding that the halogenase BhaA, responsible for 3-chlorination of the $\beta$-hydroxy-tyrosyl moieties of the glycopeptide antibiotic balhimycin, is unable to replace the halogenase Clo-hal of aminocoumarin biosynthesis. This finding also suggests that the two enzymes have different substrate specificities.**

## Experimental Procedures

### Bacterial Strains, Plasmids, and Culture Conditions

*Streptomyces coelicolor* M512 ($\Delta$*redD* $\Delta$*actII-ORF4* SCP1$^-$ SCP2$^-$) [42] was kindly provided by E. Takano (Tübingen, Germany) and was originally obtained from Janet White (Norwich, UK). *S. spheroides* NCIMB 11891 [16] was obtained from E. Cundliffe (Leicester, UK). The *clo-hal*$^-$ mutant of *S. roseochromogenes* was described previously [19]. The strains were cultured as described in the cited references. *Escherichia coli* XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) was used for cloning experiments and was grown as described [43]. The REDIRECT technology kit for PCR targeting [24] was obtained from Plant Bioscience Limited (Norwich, UK). Kanamycin (15 $\mu$g/ml in liquid medium and 50 $\mu$g/ml in solid medium for *Streptomyces*; 50 $\mu$g/ml for *E. coli*), chloramphenicol (25–50 $\mu$g/ml), apramycin (50 $\mu$g/ml), carbenicillin (50–100 $\mu$g/ml), and thiostrepton (15 $\mu$g/ml in liquid medium and 50 $\mu$g/ml in solid medium) were used for selection of recombinant strains. Before transformation of *S. coelicolor* and *S. roseochromogenes* strains, the recombinant plasmids and cosmids were amplified in *E. coli* ET12567 to avoid methyl-sensing restriction [44]. *E. coli-Streptomyces* shuttle vector pUWL201 was kindly provided by A. Bechthold (Freiburg, Germany) and was originally obtained from W. Piepersberg (Wuppertal, Germany). Cosmid 10-9C contains the novobiocin biosynthetic gene cluster in the SuperCos I vector [16]. Plasmid pTLO5, derived from pUWL201 and containing the *novO* gene, was described previously [19].

### Construction of Plasmids pAE-ha7, pAE-B3, and pUG019

#### *pAE-ha7*

*clo-hal* was amplified by PCR by 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 BamHI and XbaI, respectively. After restriction, the fragment containing the *clo-hal* gene and its natural ribosome binding site (GGAGG) was ligated into the same sites of the replicative vector pUWL201, downstream of the constitutive *ermE** promoter, to give pAE-ha7 [19].

#### *pAE-B3*

A 1.6 kb BamHI-XbaI fragment of pSETbhaA [34], containing the *bhaA* gene and its natural ribosome binding site (AGAGG), was ligated into the same sites of the replicative vector pUWL201, downstream of the constitutive *ermE** promoter, to give pAE-B3.

#### *pUG019*

pUG019, containing an apramycin resistance cassette and flanked by XbaI and SpeI recognition sites, was generated by PCR amplification of two fragments from pIJ773 (REDIRECT technology kit [24]). The first fragment of about 100 bp was amplified with primers FRT_P01f (5′-CTGCAGGAATTCGATATTCCGGGGATCTCTAGATCT-3′; the EcoRI and XbaI restriction sites are underlined) and FRT_P01r (5′-TGGCGGGGATATCGAAGTTCC-3′; the EcoRV restriction site is underlined). After digestion with EcoRI and EcoRV, this fragment was ligated into the same sites of pBlueskript SK(−) (Stratagene, Heidelberg, Germany) to give pUG017. The second fragment of about 1 kb containing the apramycin resistance gene *aac(3)IV* was amplified with primers apra_P03f (5′-GGGGATGATATCTTTATCACCACCGACTATTTG-3′; the EcoRV restriction site is underlined) and

apra_P02r (5′-TCGATAAGCTTGATGACTAGTCTGGAGCTGGAGCTG CTTCGA-3′; the HindIII and the SpeI restriction sites are underlined). After digestion with EcoRV and HindIII, this fragment was ligated into the same sites of pUG017 to give pUG019.

### DNA Isolation, Manipulation, and Cloning

Standard procedures for DNA isolation and manipulation were performed as described by Sambrook et al. [43] and Kieser et al. [22]. 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 from *E. coli* was carried out with ion-exchange columns (Nucleobond AX kits, Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Isolation of plasmid DNA from *Streptomyces* strains was carried out by alkaline lysis and potassium acetate precipitation, procedure D [22]. If required, the plasmid DNA isolated from *Streptomyces* was amplified in *E. coli* XL1 Blue MRF′ before restriction analysis.

Genomic DNA was isolated from *S. coelicolor* strains by using the Kirby mix procedure [22]. Southern blot analysis was performed on Hybond-N nylon membrane (Amersham Biosciences, Freiburg, Germany) with a digoxigenin-labeled probe by using the DIG high prime DNA labeling and detection starter kit II (Roche Applied Sciences, Mannheim, Germany).

### Heterologous Expression of the Novobiocin Biosynthetic Gene Cluster

In the SuperCos I backbone of cosmid 10-9C, containing the complete novobiocin biosynthetic gene cluster, the ampicillin resistance gene *bla* was replaced by λ-Red-mediated recombination with a cassette containing the integrase gene, *int*, and attachment site, *attP*, of phage ϕC31, as well as a tetracycline resistance gene, *tet*.

#### *Construction of pIJ787, Containing the Integrase Cassette*

The tetracycline resistance gene (*tet*) from pBR328 was amplified with primers pIJ782forw (5′-CTATGATCGACTGATGTCATCAGCG GTGGAGTGCAATGTCATGAAATCTAACAATGCGC-3′) and pIJ782-rev (5′-GAACTTCATGAGCTCAGCCAATCGACTGGCGAGCGGCAT CTCAGGTCGAGGTGGCCCGG-3′). Underlined are the beginning and the end of the coding region of the *tet* sequence. This fragment was used to replace the apramycin resistance gene in pIJ773 [24] by using λ-Red-mediated recombination [24, 45], generating pIJ782. The structural gene *tet* was thereby placed under control of the apramycin resistance promoter, resulting in slightly lower resistance levels (5 μg/ml instead of 10 μg/ml tetracycline). *tet* was then amplified from pIJ782 with primers TetAatIIforw (5′-AAAAAAAGACGTCT TGAATGGGTTCATGTG-3′) and TetAatII rev (5′-AAAAAAAGACGTC TCAGGTCGAGGTGGCCC-3′). Underlined is the AatII restriction site. After digestion of the PCR product with AatII, it was cloned into the same site of pSET152. Clones with *tet* in the same orientation as the integrase gene *int* (named pSETtet1) were selected. The 4,590 bp MscI-PvuI fragment of pSETtet1 was ligated into the ScaI-PvuI sites (i.e., into the ampicillin resistance gene, *bla*) of SuperCos I to give pIJ787.

#### *Replacement of the Ampicillin Resistance Gene,* bla*, in the SuperCos I Backbone by the Integrase Cassette*

The DraI-BsaI-fragment of pIJ787 containing the integrase cassette and flanked by about 100 bp *bla* sequence on one side and about 300 bp *bla* sequence on the other side was used to replace the *bla* gene in the SuperCos I backbone of cosmid 10-9C, which contains the entire novobiocin biosynthetic gene cluster. The resulting cosmid was termed nov-BG1.

#### *Heterologous Expression of the Intact Novobiocin Cluster*

Cosmid nov-BG1, still carrying the kanamycin resistance gene *neo*, was then introduced into *S. coelicolor* M512 via PEG-mediated protoplast transformation [22]. Kanamycin-resistant clones were checked for specific genomic integration of the cosmid into the ϕC31 attachment site by Southern blot analysis.

### Inactivation of *novO* in Cosmid nov-BG1, and Heterologous Expression of the *novO*⁻ Cosmid

In cosmid nov-BG1, *novO* was replaced, via λ-Red-mediated recombination [24], by the apramycin resistance (*aac(3)IV*) cassette from pUG019 (see above), which is flanked by XbaI and SpeI recognition sites. The cassette for replacement of *novO* was generated by PCR with the primer pair P1-NovO (5′-AGATCAGCTCACTGACCCAAC ACGAGGGGCATCGAGATGATTCCGGGGATC**TCTAGA**TC-3′) and P2-NovO (5′CGGGTCCAGGCGCTCTGTTCGGGACAATTCCGCCGC TCA**ACTAGT**CTGGAGCTGCTTC-3′). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *novO*, respectively, including the putative start and stop codons of *novO*; the XbaI and SpeI restriction sites are presented in bold letters. The PCR reaction was carried out in 50 μl volume with 100 ng template (pUG019 digested with EcoRI, HindIII, and DraI), 0.2 mM dNTPs, 50 pmol of each primer, and 5% v/v DMSO, according to the manufacturer's instructions (Expand High Fidelity Polymerase, Roche Molecular Biochemicals). PCR conditions were as follows: denaturation at 94°C for 2 min, then 10 cycles with denaturation at 94°C for 45 s; annealing at 45°C for 45 s and extension at 72°C for 90 s, then 15 cycles with annealing at 48°C; and a last elongation step at 72°C for 5 min.

After passing through the nonmethylating *E. coli* ET12567, cosmid DNA was digested with XbaI and SpeI and ligated overnight at 4°C. $CaCl_2$-competent *E. coli* XL1 Blue MRF′ cells were transformed with 100 ng DNA. Apramycin-sensitive, kanamycin-resistant clones were analyzed by using restriction enzymes and gel electrophoresis. The generated *novO*⁻ cosmid nov-AE8, carrying the kanamycin resistance gene *neo*, was then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation [22]. Kanamycin-resistant clones were checked for specific genomic integration of cosmid nov-AE8 into the ϕC31 attachment site by Southern blot analysis.

### Transformation with Plasmid pTLO5, pAE-ha7, pAE-B3, or pUWL201

Introduction of plasmids pTLO5, pAE-ha7, pAE-B3, or pUWL201 into the *novO*⁻ mutant (strain *S. coelicolor* [nov-AE8]), or into the *clo-hal*⁻ mutant of *S. roseochromogenes*, was carried out by polyethylene glycol-mediated protoplast transformation [19, 22].

### Production and Analysis of Secondary Metabolites

Integration mutants, transformants, and the wild-type strain of *S. coelicolor* as well as *S. spheroides* were cultured at 30°C and assayed for novobiocin production by HPLC as described previously [46]. Cultivation and analysis of secondary metabolites from *S. roseochromogenes* strains were carried out as specified in [19].

Negative-ion FAB mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany), using diethanolamine as matrix. $^{1}H$ NMR spectra were measured on an AMX 400 spectrometer (Bruker, Karlsruhe, Germany), using $CD_3OD$ as solvent. For $^{1}H$ NMR data, see Table 1.

Negative-ion FAB MS data (*m/z* [relative intensity in %]) were as follows:

Novobiocin: 611 (8, $[M-H]^-$), 568 (7, $[M-CONH]^-$), 395 (7), 378 (6), 281 (10), 255 (24), 209 (100);
Novclobiocin 117: 597 (7, $[M-H]^-$), 380 (4), 314 (6), 283 (10), 255 (13), 209 (100);
Novclobiocin 114: 633 (5, $[M-H]^-$, $^{37}Cl$), 632 (5, $[M-H]^-$, $^{13}C$), 631 (15, $[M-H]^-$, $^{35}Cl$), 588 (5, $[M-CONH]^-$), 554 (4, $[M-CH_2ONCl]^-$), 380 (7), 255 (46), 209 (100);
Novclobiocin 119: 633 (10, $[M-H]^-$, $^{37}Cl$), 632 (11, $[M-H]^-$, $^{13}C$), 631 (32, $[M-H]^-$, $^{35}Cl$), 588 (12, $[M-CONH]^-$), 554 (6, $[M-CH_2ONCl]^-$), 400 (25), 255 (68), 209 (100).

### Bioassay

Antibacterial activity of authentic novobiocin (Fluka) and novclobiocins against *Bacillus subtilis* ATCC 14893 was determined by a disc-diffusion assay, as described elsewhere [19].

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### Accession Numbers

The nucleotide sequences of the genes used in this study are available in the GenBank database under accession numbers AF329398 (clorobiocin cluster), AF170880 (novobiocin cluster), and Y16952 (balhimycin cluster).