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New Aminocoumarin Antibiotics Formed by a Combined Mutational and Chemoenzymatic Approach Utilizing the Carbamoyltransferase NovN

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Summary

Five new aminocoumarin antibiotics were produced by a combined mutational and chemoenzymatic approach. For this purpose, the 3″-carbamoyltransferase NovN from the novobiocin producer *Streptomyces spheroides* was overexpressed in the heterologous host *S. lividans* as an N-terminal His_6 fusion protein and purified by nickel affinity chromatography. Five different 3″-unsubstituted aminocoumarin derivatives were isolated from mutants of the clorobiocin producer *S. roseochromogenes*, carrying single or multiple gene defects. All five compounds were readily accepted as substrates by NovN, and the 3″-carbamoylated products were isolated on a preparative scale. Their structures were elucidated by ^{1}H-NMR and mass spectroscopy, and their inhibitory activity on gyrase in vitro as well as their antibacterial activity was determined. The results give further insight into the structure-activity relationships of aminocoumarin antibiotics.

Introduction

The aminocoumarin antibiotics novobiocin and clorobiocin (structures see Table 1) as well as the related coumermycin A_1 are potent inhibitors of DNA gyrase. They compete with ATP for binding to the B subunit of this enzyme and inhibit the ATP-dependent DNA supercoiling catalyzed by gyrase [1]. X-ray crystallographic analysis showed that the acyl moieties attached to the 3″-OH of the deoxysugar, i.e., a carbamoyl group in case of novobiocin and a 5-methylpyrrole-2-carboxyl moiety in case of clorobiocin, are important for the hydrogen bonding network between the antibiotic and the GyrB subunit [2, 3]. In addition, the chlorine atom at position 8′ of the coumarin core of clorobiocin contributes to the higher biological activity of this compound in comparison to novobiocin, as 8′-chlorinated compounds show at least a 2-fold higher inhibitory activity than 8′-methylated compounds [4]. This makes clorobiocin an interesting starting material for the development of new antibiotics.

Our group has cloned and sequenced the biosynthetic gene clusters for novobiocin [5], coumermycin A_1 [6], and clorobiocin [7], all of which permit the development of new aminocoumarin antibiotics by combinatorial biosynthesis. A putative carbamoyltransferase gene *novN* was identified in the novobiocin gene cluster. Its function was experimentally confirmed by the heterologous expression of *novN* in a mutant of the coumermycin producer, *Streptomyces rishiriensis*, resulting in the formation of a carbamoylated coumermycin derivative [8]. However, the yield was low [8], and no carbamoylated clorobiocin derivatives could be detected after expression of *novN* in a *clo*N2-defective mutant of the clorobiocin producer, *Streptomyces roseochromogenes* (our unpublished data). It appears that insufficient availability of the substrate carbamoyl phosphate, rather than the specificity of the carbamoyltransferase, has limited the formation of carbamoylatd products in these experiments. Therefore, expression of NovN in vivo seems not to be a promising method to obtain new carbamoylated aminocoumarin antibiotics in useful quantities.

Recently, NovN was successfully overexpressed as an N-terminal His_8 fusion protein in *E. coli* and used for in vitro formation of novobiocin from 3″-descarbamoyl novobiocin [9]. Furthermore, our group has been able to create mutants of the clorobiocin producer that accumulate 3″-unsubstituted clorobiocin derivatives, i.e., compounds lacking the pyrrole-2-carboxyl moiety [10]. These compounds represent interesting new substrates for the carbamoyltransferase NovN, setting the stage for the in vitro production of new carbamoylated aminocoumarin antibiotics.

We here report the generation of further 3″-unsubstituted clorobiocin derivatives by specific mutagenesis of the clorobiocin producer, the overexpression and purification of NovN by a convenient method using *Streptomyces lividans* as host, the chemoenzymatic synthesis of new carbamoylated clorobiocin derivatives using NovN, and the testing of these compounds for gyrase inhibition and antibacterial activity.

Results

Production of 3″-Unsubstituted Clorobiocin Derivatives Using Specific Mutants of the Clorobiocin Producer

cloN2 is involved in the transfer of the pyrrole-2-carboxyl moiety to the 3″-OH group of clorobiocin [10]. In a previous study, we have inactivated the gene *cloN2* in the clorobiocin producer by in-frame deletion. The resulting mutant accumulated two 3″-unsubstituted clorobiocin derivatives, i.e., novclobiocin 104 and novclobiocin 105 (Table 1) [10].

A similar strategy was now used to inactivate the gene *cloN2* in a *clo-hal*$^-$ mutant of the clorobiocin producer, which accumulates clorobiocin derivatives lacking the chlorine atom. The plasmid pN2, containing an in-frame deletion in the coding sequence of *cloN2*, was introduced into protoplasts of the *clo-hal*$^-$ mutant. Homologous recombination led to a single crossover mutant (Figure 1A). Southern analysis proved the presence of the disrupted gene *cloN2* in the genome of strain XHC26 (Figure 1B). Chemical analysis showed that this mutant

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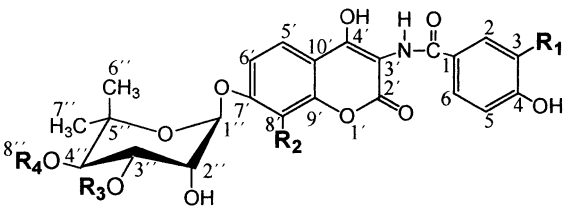
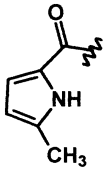
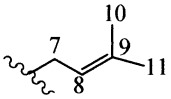
Table 1. Chemical Structures of Aminocoumarin Derivatives



Compound Name	R_1	R_2	R_3	R_4
Clorobiocin		Cl		CH_3
Novobiocin		CH_3	$CONH_2$	CH_3
Novclobiocin 104[a]		Cl	H	CH_3
Novclobiocin 114[b]		Cl	$CONH_2$	CH_3
Novclobiocin 105[a]		Cl	H	H
Novclobiocin 115[b]		Cl	$CONH_2$	H
Novclobiocin 107[a]		H	H	CH_3
Novclobiocin 117[b]		H	$CONH_2$	CH_3
Novclobiocin 108[a]		H	H	H
Novclobiocin 118[b]		H	$CONH_2$	H
Novclobiocin 283[a]	Br	Cl	H	CH_3
Novclobiocin 284[b]		Cl	$CONH_2$	CH_3

[a] Substrates for carbamoyltransferase assays
[b] Products of carbamoyltransferase assays

accumulated novclobiocin 107 (Table 1), which lacked both the pyrrole moiety at 3″-OH and chlorine atom at position 8′. In addition, another derivative, novclobiocin 108 (Table 1) in which the methyl group at 4″-OH was also absent was accumulated.

The single crossover mutant created in this study contained both an inactivated copy and an intact copy of *cloN2* (Figure 1). Integration of the vector sequence apparently disrupted the transcription unit containing *cloN2* and additional genes involved in the biosynthesis of the pyrrole moiety [8], as indicated by the lack of this structural moiety in the metabolites accumulated by the mutant. Since a single crossover was sufficient to generate the desired compounds, no further attempts were made to obtain double crossover mutants.

The new compounds, novclobiocin 107 and 108, were produced in amounts of 25.7 mg/l and 6.3 mg/l, respectively. They were extracted from the culture with ethyl acetate, purified by preparative HPLC, and their structures were elucidated by ^{1}H-NMR and mass spectroscopy (see Experimental Procedures).

A further 3″-unsubstituted compound, novclobiocin 283, was derived from a mutasynthetic experiment [11]. Novclobiocin 283 contained a bromine atom instead of the dimethylallyl moiety at position 3 of the 4-hydroxybenzoyl moiety but otherwise had the same structure as novclobiocin 104 (see Table 1).

Overexpression of NovN as an His_6 Fusion Protein in *Streptomyces lividans*

NovN has been successfully overexpressed as an N-terminal His_8 tag protein in *E. coli* [9]. However, in our hands expression in *E. coli* gave only low yields of active enzyme. It has been observed repeatedly that proteins from *Streptomyces* strains that could not be well expressed in *E. coli* could be effectively expressed in *Streptomyces lividans* [12–14]. Therefore, we expressed NovN as an N-terminal His_6 fusion protein in *S. lividans* T7 (see Experimental Procedures). The protein yield was quite high (6.7 mg/g cells, fresh weight), but much of the protein was insoluble. The amount of soluble protein did not increase significantly when the growth temperature was reduced to 20°C. Nevertheless, sufficient amounts of the His_6-NovN fusion protein could be readily purified from the soluble fraction by metal affinity chromatography. SDS-PAGE analysis showed the expected band of 78 kDa (calculated mass 78.5 kDa) in the eluate (Figure 2). An additional band appeared at 76 kDa that had also been observed upon expression in *E. coli* [9] and may represent a degradation product. The protein yield of purified NovN was about 16 μg/g cells (fresh weight), determined by SDS-PAGE.

Characterization of NovN

By using gel filtration, the molecular mass of native NovN was determined as 79 kDa (calculated mass 78.5 kDa), indicating that the protein was active as a monomer. The NovN reaction was strictly dependent on the presence of ATP and divalent cations such as Mg^{2+} or Mn^{2+}. The optimal concentrations of both Mg^{2+} and ATP were 2 mM. The product formation in the carbamoyltransferase assay showed a linear dependence on the protein amount up to 0.5 μM of purified protein and on incubation time up to 90 min. The K_m value for descarbamoyl novobiocin was determined as 2.4 μM and turnover rate K_{cat} as 2.4 min^{-1}, similar to the data obtained for NovN expressed in *E. coli* [9].

Chemoenzymatic Synthesis of New Aminocoumarin Antibiotics

The five 3″-unsubstituted clorobiocin derivatives (i.e., novclobiocins 104, 105, 107, 108 as well as novclobiocin 283) were used as substrates for carbamoylation. HPLC analysis revealed that all of them were readily accepted by NovN. As shown in Figure 3, 87% of novclobiocin 104 were converted to the carbamoylated derivative after overnight incubation under the assay conditions described in Experimental Procedures. The average conversion rate of the other four substrates was about 54%. The structures of the enzymatic substrates and products are given in Table 1. For structural elucidation, each product was isolated in preparative amounts and subjected to ^{1}H-NMR and MS analysis. Compared to the parent compounds, the NMR spectra of the enzymatic products in CD_3OD showed no additional proton signals but marked downfield shifts of the signals of the protons H-2″, H-4″, and especially H-3″ of the deoxysugar (Table 2), indicating a substitution at position 3″of the deoxysugar. FAB-MS analysis showed that the molecular mass of the enzymatic products was 43 Da larger than that of the respective substrates, consistent with the attachment of a carbamoyl group.

Kinetic parameters were determined for novclobiocin 104 and 105. K_{cat} values were determined as 1.32 min^{-1}

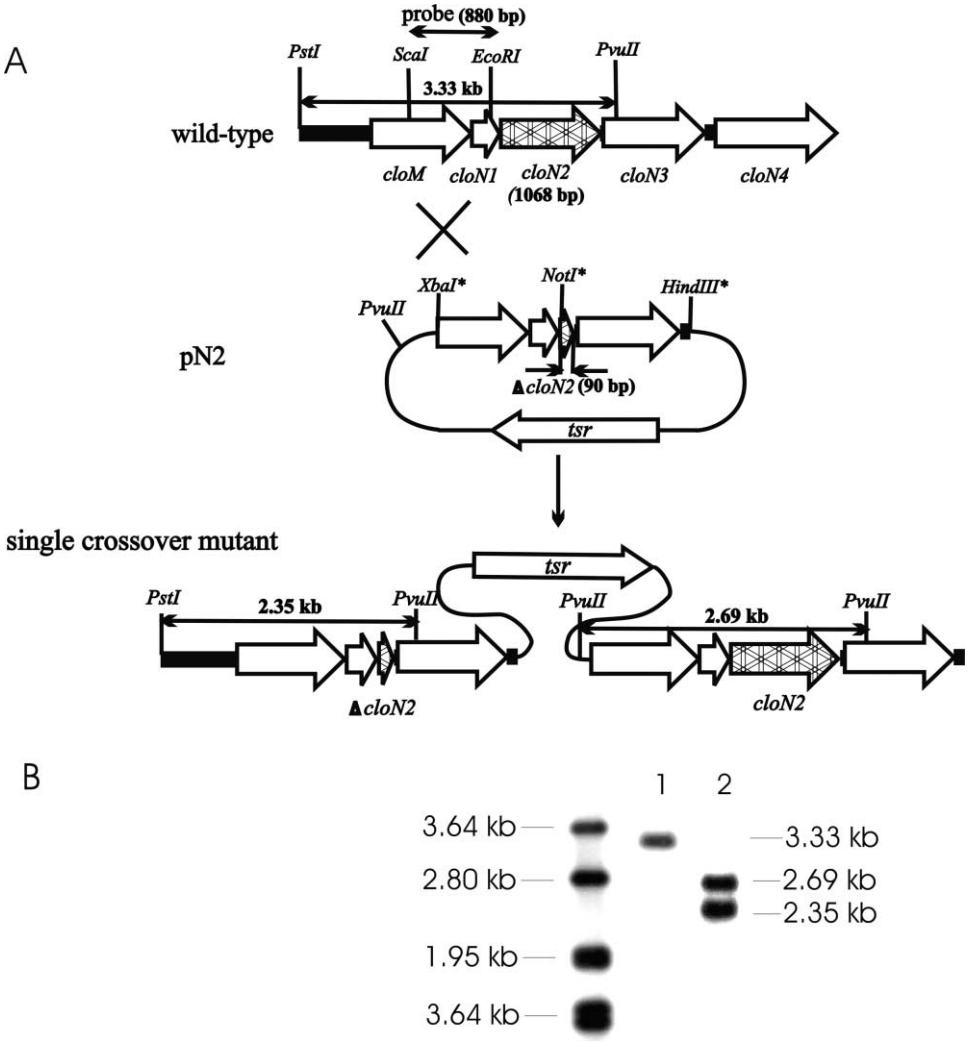


Figure 1. Inactivation of *cloN2* in the *clo-hal*$^-$ Mutant

(A) Schematic representation of the gene inactivation.

(B) Southern blot analysis. Genomic DNA was restricted with PstI/PvuII. *clo-hal*$^-$ mutant (lane 1), *cloN2*$^-$*clo-hal*$^-$ double mutant XHC26 (lane 2). The indicated ScaI-EcoRI frament of 880 bp was used as a probe.

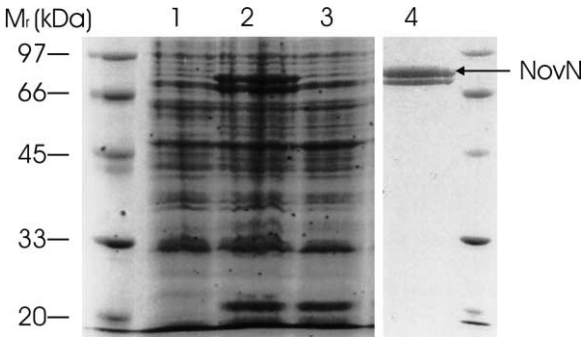


Figure 2. Analysis of Proteins Expressed in *S. lividans* T7 by 10% SDS-PAGE

Lane 1, total protein before induction; lane 2, total protein after induction with 25 μg/ml thiostrepton for 24 hr; lane 3, soluble protein after induction; lane 4, eluate from nickel affinity chromatography.

and 1.53 min^{-1}, respectively, similar to the value obtained for descarbamoyl novobiocin. The K_m value for novclobiocin 104 (1.9 μM) was similar to that of descarbamoyl novobiocin. In contrast, the K_m for novclobiocin 105 was 14.3 μM, i.e., five times higher than that of genuine substrate, indicating that the 4″-methoxy group may play a role in substrate binding. NovN was specific for carbamoyl phosphate as substrate. With acetyl phosphate, no product formation was observed.

Testing for Biological Activity

The new aminocoumarin derivatives were tested in vitro for their inhibitory effect on *E. coli* gyrase in comparison with the natural compounds novobiocin and clorobiocin. The estimated IC_{50} of novobiocin resulted as 0.9 μM, and the activity of the other substances was expressed relative to that of novobiocin (Figure 4). As observed previously [15, 16], clorobiocin was the most active substance in vitro, three times as active as novobiocin.

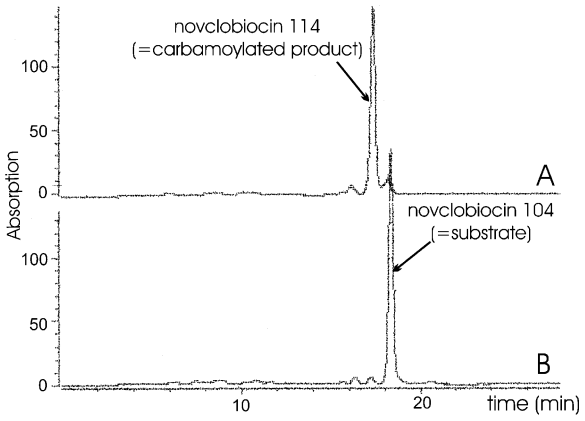


Figure 3. HPLC Analysis of the Carbamoyltransferase Assays with Novclobiocin 104 as Substrate

The assays were incubated overnight. (A) Assay with purified NovN. (B) Negative control with heat-denatured enzyme.

Removal of the pyrrole carboxylic acid moiety from clorobiocin resulted in a 100-fold drop of the activity (novclobiocin 104). The subsequent removal of the methyl group at 4″-OH (novclobiocin 105), of the chlorine atom at position 8′ (novclobiocin 107), or of both these substituents (novclobiocin 108) further reduced the activity. When the pyrrole carboxylic acid moiety was removed from clorobiocin and subsequently replaced by a carbamoyl group, activity was restored: the resulting compound (novclobiocin 114) was nearly as active as novobiocin but still four times less active than clorobiocin. In contrast, carbamoylation of novclobiocins 105, 107, and 108 led only to compounds of low activity (novclobiocins 115, 117, and 118, respectively), proving the importance of the 4″-methoxyl group and the 8′-chlorine atom for gyrase inhibition. Replacement of the 3-dimethylallyl moiety in novclobiocin 114 by a bromine atom (novclobiocin 284) resulted in a 7-fold drop in activity.

These results on the inhibition of gyrase in vitro were subsequently confirmed by determination of the antibacterial activity in a disc-diffusion assay by using *Bacillus subtilis* as test organism (Figure 5). Activity was expressed relative to novobiocin. As observed previously [15], novobiocin showed higher activity than clorobiocin in this assay. Otherwise, however, the antibacterial assays largely confirmed the results of the in vitro gyrase inhibition assays. Removal of the pyrrole carboxylic acid moiety from clorobiocin resulted in a sharp drop of activity, and the additional removal of the methyl group at 4″-OH and/or the chlorine atom at position 8′ further reduced growth inhibition. Carbamoylation of novclobiocin 104 led to an active antibacterial compound (novclobiocin 114), which, however, was less active than novobiocin or clorobiocin. Removal of the methyl group at 4″-OH or of the chlorine atom at the 8′ position or replacement of the 3-dimethylallyl group by a bromine atom strongly reduced the activity.

Discussion

The present study demonstrates the potential of a combined mutational and chemoenzymatic approach for the generation of new amincouamrin antibiotics. In previous studies [4, 17, 18], our group has generated a variety of new aminocoumarins by methods of combinatorial biosynthesis, i.e., by combination of gene inactivation and heterologous gene expression experiments. However, expression of the carbamoyltransferase NovN in mutants of aminocoumarin antibiotic producers resulted only in low yields of carbamoylated products [8]. In the present study, we have overcome the problem by producing 3″-unsubstituted aminocoumarin derivatives by using specific mutants with single or multiple gene defects followed by efficient carbamoylation of these compounds in vitro, which resulted in five new carbamoylated aminocoumarin antibiotics in preparative amounts.

The carbamoyltransferase NovN could readily be obtained in nearly pure form after expression of its structural gene in *Streptomyces lividans*. Although the natural substrate of NovN is 3″-descarbamoyl novobiocin, the enzyme readily accepted all five other aminocoumarin substrates tested in our study, showing that structural variations can be introduced by this method in the deoxysugar moiety, in the aminocoumarin moiety, and in the benzoyl moiety of the antibiotics. On the other hand, acetyl phosphate was not accepted as substrate by NovN, limiting the range of products to carbamoylated compounds.

Structural variation may be further increased by the

Table 2. Comparison of ^{1}HNMR Data of Aminocoumarin Derivatives

	H-2″ δ (ppm), Multi (*J*/Hz)	H-3″ δ (ppm), Multi (*J*/Hz)	H-4″ δ (ppm), Multi (*J*/Hz)
Novclobiocin 104	4.12, t (3.1)	4.18, dd (3.1, 9.9)	3.40, d (9.9)
Novclobiocin 114	4.29, t (3.0)	5.37, dd (3.1, 10.1)	3.65, br s
Novclobiocin 105	4.13, br s	4.12, dd (3.1, 10.0)	3.76, d (10.0)
Novclobiocin 115	4.33, br s	5.27, dd (3.1, 10.3)	3.93, d (10.4)
Novclobiocin 107	4.02, t (3.1)	4.09, dd (3.1, 9.4)	3.37, d (9.4)
Novclobiocin 117	4.17, t (2.8)	5.25, dd (2.8, 8.8)	3.54[a]
Novclobiocin 108	4.04, br s	4.03, dd (3.4, 9.5)	3.73, d (9.5)
Novclobiocin 118	4.24, t (3.3)	5.15, dd (3.3, 10.0)	3.90, d (10.0)
Novclobiocin 283	4.11, s	4.19, dd (3.3, 9.9)	3.40, d (9.9)
Novclobiocin 284	4.27, t (3.1)	5.37, dd (3.1, 10.0)	3.55[a]

The NMR spectra were recorded at 400 MHz, in CD_3OD. Abbreviations: t, triplet; d, doublet; dd, double doublet; s, singlet; and br, broad signal; see table 1 for numbering of the structures.

[a] Overlapping with the signal of 3H-8″, *J* not determinable.

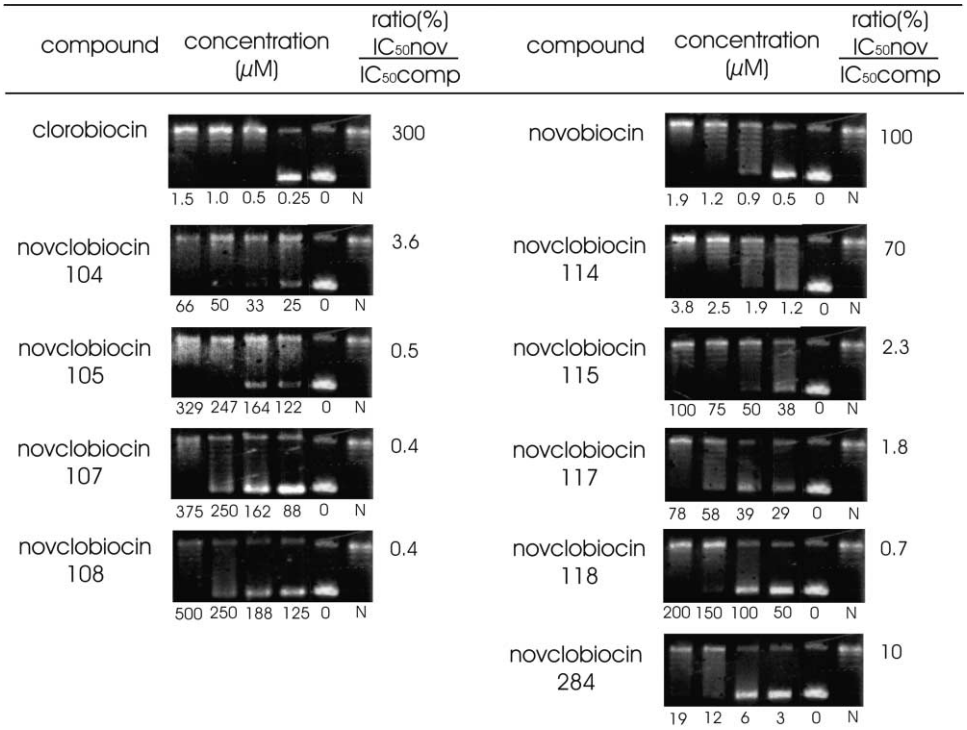


Figure 4. In Vitro Inhibition of the DNA Supercoiling Reaction Catalyzed by *E. coli* Gyrase

IC_{50} values were estimated as described in the Experimental Procedures.

utilization of mutasynthetic strategies, as demonstrated by the generation of novclobiocin 284. First, the biosynthesis of the natural benzoyl moiety was blocked by an inactivation experiment [19]; then, a synthetic analog of the benzoyl moiety was fed to this mutant [15], resulting in the formation of novclobiocin 283; finally, this compound was carbamoylated by chemoenzymatic methods by using NovN.

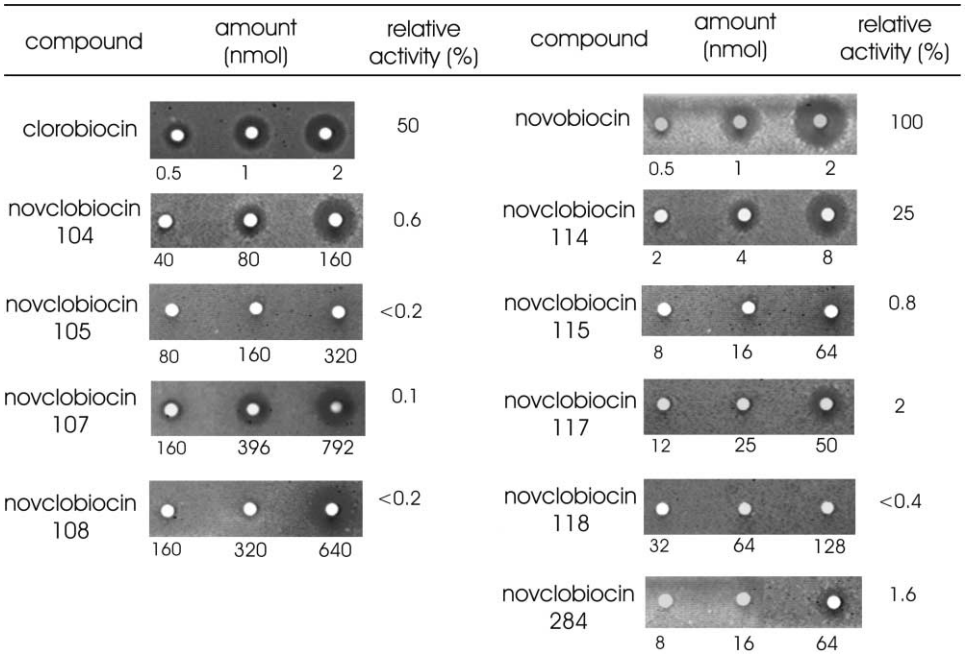


Figure 5. Bioassay against *Bacillus subtilis*

Activities are expressed relative to novobiocin (100%).

The compounds obtained in this study allowed us to obtain further insights into the structure-activity relationships within the aminocoumarin class of gyrase inhibitors by comparing their biological activities. Firstly, our results confirmed the importance of the acyl moiety attached to the 3″-OH of the deoxysugar. Lack of this acyl group led to a sharp reduction of activity. Novclobiocin 114, which contained the carbamoyl instead of the pyrrole carboxylic acid group at 3″-OH but otherwise had an identical structure as clorobiocin, showed a 4-fold lower inhibitory activity on gyrase than clorobiocin.

Furthermore, our study demonstrates a crucial importance of the methyl group attached to the 4″-OH group of the deoxysugar; lack of this group led to a strong decrease of activity. In accordance with earlier results [4], lack of the chlorine (or methyl) substituent at C-8′ of the aminocoumarin ring also leads to a considerable decrease of activity.

Interestingly, novclobiocin 284, which contains a bromine instead of the dimethylallyl moiety at C-3 of the benzoyl moiety, not only showed lower antibacterial activity than novclobiocin 114, but also less gyrase inhibition in vitro. This finding gives further experimental support to the hypothesis of Lafitte et al. [20] that the dimethylallyl moiety directly contributes to the binding of the antibiotic to gyrase and not just facilitates the uptake through the bacterial membrane as speculated earlier [2].

Significance

Novobiocin and clorobiocin are potent inhibitors of bacterial DNA gyrase. We reported here a method to generate new carbamoylated aminocoumarin derivatives by a combined mutational and chemoenzymatic approach. The 3″-carbamoyltransferase NovN from the novobiocin producer *Streptomyces spheroides* was overexpressed in *S. lividans* and purified to near homogeneity. Different 3″-unsubstituted aminocoumarin derivatives were isolated from mutants of the clorobiocin producer *S. roseochromogenes*, carrying single or multiple gene defects. All these compounds were readily accepted as substrates by NovN, demonstrating that structural variation can be introduced by our method in the deoxysugar moiety, the aminocoumarin moiety, as well as in the benzoyl moiety. Testing of biological activity of the 3″-carbamoylated products gave further insights into the structure-activity relationships of aminocoumarin antibiotics, especially about the importance of the acyl moiety and the methoxy group at the deoxysugar. This work is another example for generating novel, structurally modified compounds for drug discovery.

Experimental Procedures

General Chemicals

Descarbamoyl novobiocin was kindly provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI). Carbamoyl phosphate dilithium salt (CAP-Li_2), lithium potassium acetyl phosphate, and adenosine 5′-triphosphate dipotassium salt (ATP) were purchased from Sigma (Taufkirchen, Germany). Novclobiocin 283 was obtained as described elsewhere [11]. All the other reagents and chemicals were of analytical grade.

Bacterial Strains, Vectors, and DNA Manipulation

The gene *novN* was cloned from cosmid 10-9C of the cosmid library of the novobiocin producer *Streptomyces spheroides* NCIB 11891 [5]. Cloning experiments were performed in *Escherichia coli* XL1 Blue MRF'. The *clo-hal*$^-$ mutant of *S. roseochromogenes* is described in Eustáquio et al. [4]. The replicative vector pGM9 and the heterologous expression host *S. lividans* T7, possessing a thiostrepton-inducible T7 RNA polymerase gene, were kindly provided by W. Wohlleben (Tübingen, Germany); *S. lividans* T7 was originally obtained from J. Altenbuchner (Stuttgart, Germany). The cloning vector pGEM-T and the expression vector pRSET B were purchased from Promega (Mannheim, Germany) and Invitrogen (Karlsruhe, Germany), respectively. DNA manipulation and standard genetic techniques in *E. coli* and *Streptomyces* species were carried out as described previously [21, 22].

Inactivation of *cloN2* in the *clo-hal*$^-$ Mutant and Production of the 3″-Unsubstituted Clorobiocin Derivatives Novclobiocin 107 and 108

The *clo-hal*$^-$ mutant was transformed with plasmid pN2 (Figure 1A), which contained an in-frame deletion in the coding region of *cloN2* [10]. Thiostrepton-resistant colonies were selected and analyzed by Southern blotting. While the *clo-hal*$^-$ mutant gave a band of 3.33 kb, strain XHC26 showed two bands of 2.35 kb and 2.69 kb, representing the desired genotype of single crossover events (Figure 1B).

The wild-type, the *clo-hal*$^-$ mutant, and the *cloN2*$^-$*clo-hal*$^-$ double mutant were cultured for the production of secondary metabolites, as described previously [7, 10, 23]. The culture was extracted twice with ethyl acetate and analyzed by HPLC with a Multosphere RP18-5 column (5 μm, 250 × 4 mm; C + S Chromatographie Service, Düren, Germany) at flow rate of 1 ml/min by using a linear gradient from 70%–100% methanol in 1% aqueous formic acid over 25 min and UV detection at 340 nm. Compared to the wild-type and the *clo-hal*$^-$ mutant, two new peaks were found in the chromatogram of the *cloN2*$^-$*clo-hal*$^-$ double mutant. These two substances, designated as novclobiocin 107 and novclobiocin 108, were isolated on a preparative scale by HPLC as described above. Their structures were elucidated by MS and ^{1}H-NMR. Negative fast atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) by using diethylethanolamine as matrix. ^{1}H-NMR spectra were measured on an AMX 400 spectrometer (400 MHz; Bruker, Karlsruhe, Germany) by using CD_3OD as solvent.

MS and ^{1}H-NMR spectral data were as follows (see Table 1 for numbering of the structures; br, broad signal).

Novclobiocin 107

Negative FAB-MS *m/z* (relative intensity in %): 554 (48, $[M-H]^-$), 392(9), 379(20), 366(20), 336(7), 314(11), 283(11), 253(15), 237(11), 209(100), 207(39), 190(27); ^{1}H-NMR: δppm 1.11 (s, 3H-6″), 1.33 (s, 3H-7″), 1.74 (s, 3H-11), 1.75 (s, 3H-10), 3.34 (d, J = 6.8 Hz, 2H-7), 3.37 (d, J = 9.4 Hz, H-4″), 3.58 (s, 3H-8″), 4.02 (t, J = 3.1 Hz, H-2″), 4.09 (dd, J_1 = 9.4 Hz, J_2 = 3.0 Hz, H-3″), 5.35 (tt, J_1 = 7.4 Hz, J_2 = 1.2 Hz, H-8), 5.56 (d, J = 2.3 Hz, H-1″), 6.85 (d, J = 8.4 Hz, H-5), 7.04 (complex overlapping signals, J not determinable, H-6′, H-8′), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.75 (d, J = 2.2 Hz, H-2), 7.89 (d, J = 9.5 Hz, H-5′).

Novclobiocin 108

Negative FAB-MS *m/z* (relative intensity in %): 540(27, $[M-H]^-$), 415(7), 380(16), 352(11), 338(7), 314(15), 312(7), 283(24), 255(16), 209(100), 207(35), 191(8); ^{1}H-NMR: δppm 1.14 (s, 3H-6″), 1.32 (s, 3H-7″), 1.74 (s, 3H-11), 1.75 (s, 3H-10), 3.34 (d, J = 7.3 Hz, 2H-7), 3.73 (d, J = 9.5 Hz, H-4″), 4.03 (dd, J_1, = 9.5 Hz, J_2 = 3.4 Hz, H-3″), 4.04 (br s, H-2″), 5.35 (br t, J = 7.3 Hz, H-8), 5.58 (br s, H-1″), 6.84 (d, J = 8.4 Hz, H-5), 7.04 (complex overlapping signals, J not determinable, H-6′, H-8′), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.75 (br s, H-2), 7.90 (d, J = 9.4 Hz, H-5′).

Construction of pXHNEG for Expression of NovN as an N-Terminal His_6 Fusion Protein in *S. lividans* T7

novN was amplified from cosmid 10-9C by using the GC-RICH PCR system (Roche, Mannheim, Germany) with the primer pair novN-BclI

(5′-GTGCTCGCTGATCAGAACGACATG-3′) and novN-HindIII (5′-AAGGGAAGCTTTACGGCCGCGAC-3′). Introduced restriction sites are underlined. The DNA fragment was directly ligated into the linearized vector pGEM-T, resulting in plasmid pXHNET. *novN* was released from pXHNET by digestion with BclI and HindIII and ligated into pRSET-B, which had been restricted with BamHI and HindIII, to give plasmid pXHNER. To obtain the plasmid pXHNEG, pXHNER was fused with the vector pGM9 via the restriction site *Hind*III. pXHNEG contained a kanamycin resistance gene.

Expression of pXHNEG and Purification of Carbamoyltransferase

After transforming *S. lividans* T7 protoplasts with plasmid pXHNEG, cells were regenerated on R_2YE plates and kanamycin- and thiostrepton-resistant colonies were selected. They were grown in YEME medium with kanamycin (10 μg/ml) at 28°C for 48 hr, and then 1 ml culture was transferred to 100 ml new YEME medium with kanamycin (10 μg/ml) and thiostrepton (25 μg/ml) and cultured for further 24 hr. The cells were harvested by centrifugation (10 min at 5000 × g) and incubated in ice-cold lysis buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 15 mM imidazole, 0.2% triton 100, and 6 mg/ml lyzozyme) for 30 min. The cell suspension was sonicated for 8 min (Branson Sonifier 250) and the cell debris was removed by centrifugation (30 min at 15,000 × g). The protein was purified from the soluble cell extract by metal affinity chromatography by using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, the Ni-NTA resin eluate was applied onto a NAP 10 column (Amersham Biosciences, Freiburg, Germany) and eluted with 0.2 M Tris-HCl (pH 8.0).

Protein Analysis

Protein concentrations were determined by the Bradford method [24] by using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli [25], and protein bands were stained with Coomassie brilliant blue R250. The molecular mass of native NovN was determined by gel filtration on a HiLoad 26/60 Superdex 200 column by using 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 5 mM dithiothreitol, and 50 μM phenylmethylsulfonyl fluoride. The column was calibrated with blue dextran 2000, aldolase (M_r 158,000), albumin (M_r 66,000), ovalbumin (M_r 45,000), and ribonuclease A (M_r 13,700) (Amersham Biosciences, Freiburg, Germany).

Enzyme Assay and Identification of the Enzymatic Products

The carbamoyltransferase assay mixture contained 0.2 M Tris-HCl (pH 8.0), 1 mM descarbamoyl novobiocin or analogs, 5 mM CAP-Li_2, 2 mM ATP, 2 mM Mg^{2+}, and purified NovN (0.4 μM) in a final volume of 100 μl. The reactions were carried out at 30°C and terminated by the addition of 5 μl 1.5 M trichloroacetic acid. Kinetic parameters were determined with 37.5 nM NovN over 5 min. The assay products were extracted with ethyl acetate. After evaporation of the organic solvent, the residue was dissolved in methanol and analyzed by HPLC with the same gradient as described for analysis of novclobiocin 107 and 108. UV absorption was recorded at 325 nm. For preparative experiments, the assay mixtures were incubated overnight, and the enzymatic products were isolated by HPLC and subjected to MS and ^{1}H-NMR analysis.

MS and ^{1}H-NMR spectral data were as follows (see Table 1 for numbering of the structures; ^{1}H-NMR at 400 MHz, with CD_3OD as the solvent; br, broad signal).

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Negative FAB-MS *m/z* (relative intensity in %): 631(17, $[M-H]^-$), 597(6), 524(6), 443(6), 358(6), 326(7), 283(18), 255(22), 209(100); ^{1}H-NMR: δppm 1.17 (s, 3H-6″), 1.33 (s, 3H-7″), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.55 (s, 3H-8″), 3.65 (br s, H-4″), 4.29 (t, J = 3.0 Hz, H-2″), 5.35 (overlapping with the signals of H-3″, H-8), 5.37 (dd, J_1 = 10.1 Hz, J_2 = 3.1 Hz, H-3″), 5.63 (d, J = 1.6 Hz, H-1″), 6.80 (d, J = 8.2 Hz, H-5), 7.19 (d, J = 9.0 Hz, H-6′), 7.71 (dd, J_1 = 8.2 Hz, J_2 = 2 Hz, H-6), 7.76 (s, H-2), 7.88 (d, J = 9.0 Hz, H-5′).

Novclobiocin 115

Negative FAB-MS *m/z* (relative intensity in %): 617(3, $[M-H]^-$), 524(2), 485(2), 462(6), 419(6), 388(6), 356(10), 314(16), 283(84), 255(61), 209(100); ^{1}H-NMR: δppm 1.20 (s, 3H-6″), 1.32 (s, 3H-7″), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.93 (d, J = 10.4 Hz, H-4″), 4.33 (br s, H-2″), 5.27 (dd, J_1 = 10.3 Hz, J_2 = 3.1 Hz, H-3″), 5.35 (br s, H-8), 5.65 (br s, H-1″), 6.81 (br s, H-5), 7.21(br s, H-6′), 7.73 (br s, H-6), 7.77 (s, H-2), 7.90 (br s, H-5′).

Novclobiocin 117

Negative FAB-MS *m/z* (relative intensity in %): 597(6, $[M-H]^-$), 554(2), 491(2), 427(2), 411(7), 370(7), 307(9), 306(29), 258(37), 257(69), 209(100), 207(25); ^{1}H-NMR: δppm 1.17 (s, 3H-6″), 1.35 (s, 3H-7″), 1.73 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.54 (complex overlapping signals, 3H-8″, H-4″), 4.17 (t, J = 2.8 Hz, H-2″), 5.25 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, H-3″), 5.35 (br t, J = 7.2 Hz, H-8), 5.55 (d, J = 2.5 Hz, H-1″), 6.81 (d, J = 8.4 Hz, H-5), 6.97 (complex overlapping signals, J not determinable, H-6′, H-8′), 7.71 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.76 (br s, H-2), 7.91 (d, J = 9.0 Hz, H-5′).

Novclobiocin 118

Negative FAB-MS *m/z* (relative intensity in %): 583(10, $[M-H]^-$), 540(6), 522(2), 462(5), 402(5), 380(6), 325(6), 314(13), 283(97), 255(70), 209(100); ^{1}H-NMR: δppm 1.20 (s, 3H-6″), 1.34 (s, 3H-7″), 1.74 (s, 3H-10, 3H-11), 3.34 (overlapping with the signal of CH_3OH, 2H-7), 3.90 (d, J = 10.0 Hz, H-4″), 4.24 (t, J = 3.3 Hz, H-2″), 5,15 (dd, J_1 = 10.0 Hz, J_2 = 3.3 Hz, H-3″), 5.35 (br t, J = 7.2 Hz, H-8), 5.57 (d, J = 2.2 Hz, H-1″), 6.82 (d, J = 8.4 Hz, H-5), 7.00 (complex overlapping signals, J not determinable, H-6′, H-8′), 7.71 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.76 (s, H-2), 7.91 (d, J = 9.3 Hz, H-5′).

Novclobiocin 284

Negative FAB-MS *m/z* (relative intensity in %): 643(2, $[M-H]^-$), 569(2), 524(6), 491(3), 463(6), 419(6), 356(10), 314(25), 283(37), 253(41), 209(100); ^{1}H-NMR: δppm 1.17 (s, 3H-6″), 1.32 (s, 3H-7″), 3.55 (complex overlapping signals, J not determinable, H-4″ and H-8″), 4.27 (t, J = 3.1 Hz, H-2″), 5.37 (dd, J_1 = 10.0 Hz, J_2 = 3.1 Hz, H-3″), 5.63 (d, J = 1.8 Hz, H-1″), 6.94 (d, J = 8.4 Hz, H-5), 7.19 (d, J = 8.1 Hz, H-6′), 7.87 (overlapping signals of H-6 and H-5′), 8.19 (br s, H-2).

Determination of Biological Activity

The carbamoylated aminocoumarin derivatives were tested for inhibitory effect on supercoiling activity of *E. coli* gyrase by using the DNA gyrase kit from John Innes Enterprises Limited (John Innes Centre and Norwich Research Park, Colney, Norwich) according to the manufacturer's instruction. IC_{50} was defined as the concentration at which the DNA gyrase supercoiling activity was inhibited by 50%.

Antibacterial activity against *Bacillus subtilis* ATCC 14893 was determined by a disc-diffusion assay, as described elsewhere [4, 17], by using filter paper disks of 3 mm diameter.

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