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Improved Mutasynthetic Approaches for the Production of Modified Aminocoumarin Antibiotics

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SUMMARY

This study reports improved mutasynthetic approaches for the production of aminocoumarin antibiotics. Previously, the mutasynthetic production of aminocoumarins with differently substituted benzoyl moieties was limited by the substrate specificity of the amide synthetase CloL. We expressed two amide synthetases with different substrate specificity, CouL and SimL, in appropriately engineered producer strains. After feeding of precursor analogs that were not accepted by CloL, but by SimL or CouL, a range of aminocoumarins, unattainable in our previous experiments, was produced and isolated in preparative amounts. Further, we developed a two-stage mutasynthesis procedure for the production of hybrid antibiotics that showed the substitution pattern of novobiocin in the aminocoumarin moiety and that of clorobiocin in the deoxysugar moiety. The substitution pattern of the benzoyl moiety was determined by external addition of an appropriate precursor. Twenty-five aminocoumarin compounds were prepared by these methods, and their structures were elucidated with mass and ^{1}H-NMR spectroscopy.

INTRODUCTION

Mutasynthesis, i.e., the feeding of synthetic precursor analogs to mutants of microbial producer strains of natural products, is an important and powerful tool for drug discovery and lead optimization. However, this technique has three principal limitations [1]: (1) the range of precursors that can be incorporated into the final molecule is limited by the substrate specificity of the biosynthetic enzymes; (2) usually, only the structural features generated in the early steps of the biosynthesis can be modified; and (3) the range of precursors that can be taken up into the producing cells is limited. Here, we present examples of how the first two of these limitations, and thereby the structural diversity of compounds generated by mutasynthesis, can be significantly expanded by appropriate metabolic engineering of the employed mutant strains, as well as by utilization of two-stage feeding procedures.

The aminocoumarin antibiotics novobiocin, clorobiocin, coumermycin A_1, and simocyclinone D8 (Figure 1) are produced by different *Streptomyces* strains. These compounds are potent inhibitors of bacterial gyrase [2]. Novobiocin has been licensed in the United States under the name Albamycin for the treatment of infections with Gram-positive bacteria in humans.

All aminocoumarin antibiotics contain a 3-amino-4,7-dihydroxycoumarin moiety (= Ring B; Figure 1). This is substituted at position 8 by a chlorine atom in the case of clorobiocin and simocyclinone D8, or by a methyl group in the case of novobiocin and coumermycin. The aminocoumarin moiety is attached, via an amide bond, to structurally very different acyl moieties (Figure 1). In novobiocin, clorobiocin, and coumermycin A_1, the aminocoumarin moiety is furthermore glycosidically linked to a deoxysugar.

Our group has cloned and sequenced the biosynthetic gene clusters of all four aminocoumarin antibiotics, and the functions of most of the genes contained therein were elucidated [3, 4]. These findings now form the basis for the development of new aminocoumarin derivatives by metabolic engineering and chemoenzymatic synthesis [3].

A mutant strain of the clorobiocin producer, which was defective in the gene for the prenyltransferase CloQ, was successfully used for mutasynthesis experiments. The prenyltransferase CloQ is required for the biosynthesis of the prenylated 4-hydroxybenzoate moiety (= Ring A) of clorobiocin (Figure 1). Feeding of different synthetic Ring A analogs to the *cloQ*-defective mutant resulted in the formation of several new aminocoumarin derivatives with modified substitution patterns [5].

However, this method was limited by the substrate specificity of CloL. In clorobiocin biosynthesis, the amide

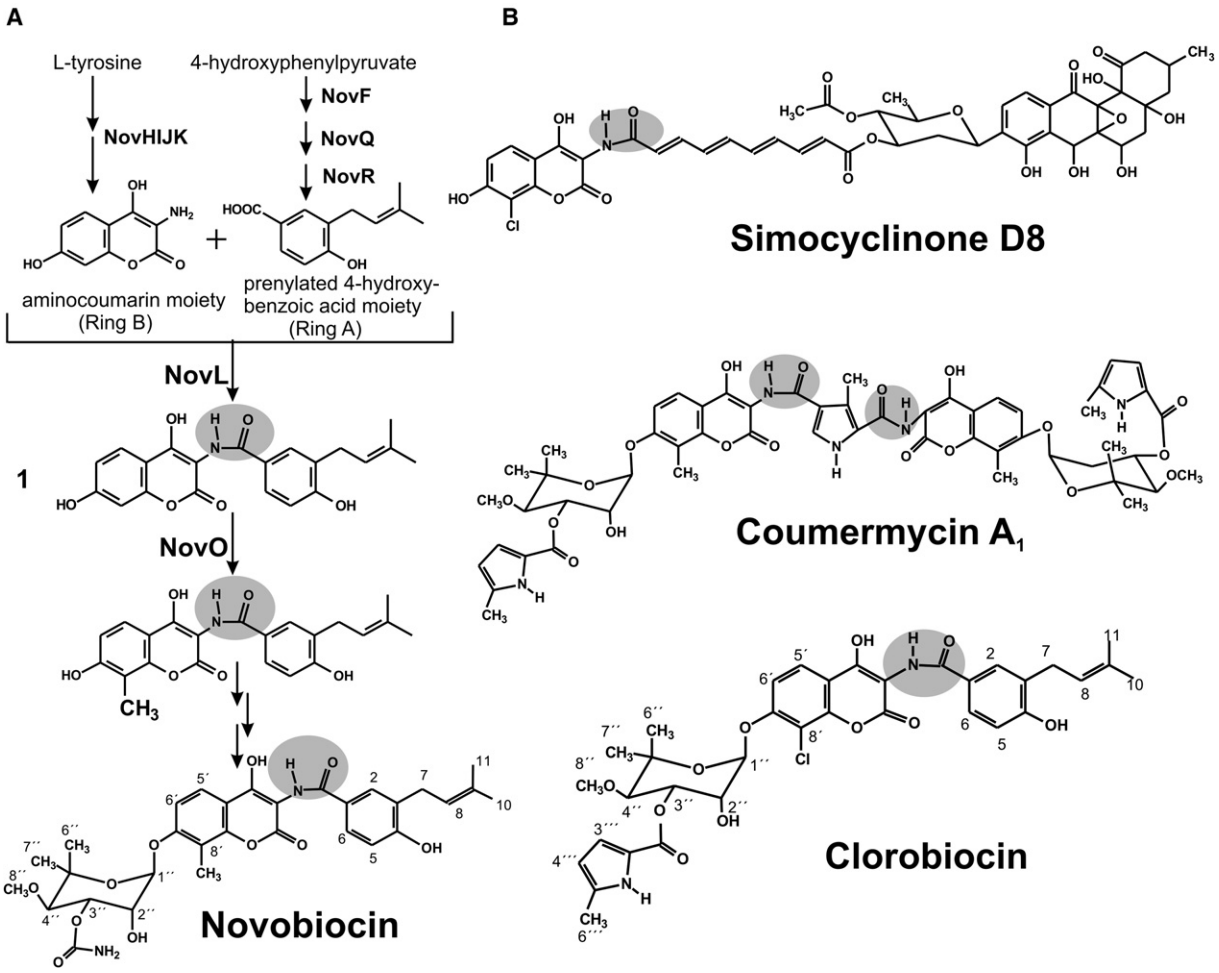


Figure 1. Aminocoumarin Antibiotics

(A) Structure and biosynthesis of novobiocin.

(B) Structures of simocyclinone D8, coumermycin A_1, and clorobiocin.

The amide bonds generated by the amide synthetases NovL, SimL, CouL, and ClоL are marked in gray.

synthetase ClоL attaches the externally added Ring A analog to the 3-amino group of the aminocoumarin ring. As shown by Galm et al. [5], ClоL accepts different Ring A analogs with very different efficiency in vitro. The product yields in the amide synthetase reaction in vitro correlated with the yields of the respective aminocoumarin antibiotics obtained in vivo in mutasynthetic feeding experiments [5]. Compounds that were poor substrates of ClоL in vitro, such as 3,5-dimethyl-4-hydroxybenzoic acid, did not lead to the formation of aminocoumarin antibiotics in mutasynthesis experiments.

The aim of the present study was to expand the range of new aminocoumarin antibiotics producible from mutasynthesis experiments, by using metabolic engineering of the producer strains. For this purpose, we expressed, in an appropriate mutant of the producer strain, the amide synthetases CouL or SimL, involved in the biosynthesis of coumermycin A_1 and simocyclinone D8. Both enzymes utilize completely different acyl substrates than ClоL (Figure 1).

In a second approach, we attempted to generate hybrid antibiotics, i.e., aminocoumarin antibiotics that contain structural features of both clorobiocin and novobiocin. For this purpose, we developed a two-step feeding strategy that successively uses mutants of the novobiocin and the clorobiocin producer strains.

RESULTS

Utilization of Different Amide Synthetases to Overcome Limitations of Substrate Specificity

As may be expected from the structures of clorobiocin, novobiocin, coumermycin A_1, and simocyclinone D8 (Figure 1), the substrate specificities of the amide synthetases ClоL and NovL are similar to each other, whereas both CouL and SimL accept many substrates that are not converted by any of the other three enzymes [5–8]. Therefore, the genes *couL* and *simL* were cloned into the expression vector pUWL201, which contains the constitutive *ermE**

promotor [9], resulting in the plasmids pMS91 and pSH2, respectively.

In cosmid clo-BG1, which harbors the complete biosynthetic gene cluster of clorobiocin, the gene *cloL*, coding for the genuine amide synthetase, and the gene *cloQ*, required for Ring A biosynthesis [10], were inactivated by λ RED-mediated recombination, resulting in cosmid clo-CA5. This cosmid was integrated into the genome of *Streptomyces coelicolor* M512 [11], resulting in the strain *S. coelicolor*(clo-CA5). The genotype of the resulting mutant was confirmed by Southern blot (Figure 2). As expected, cultivation and HPLC analysis of *S. coelicolor*(clo-CA5) showed that no clorobiocin was produced, while integration of an intact clorobiocin cluster (i.e., clo-BG1) into *S. coelicolor* M512 resulted in a strain producing approximately ~35 μg/ml clorobiocin [11]. Since in *S. coelicolor*-(clo-CA5) the amide synthetase gene *cloL* had been deleted, this strain could not produce clorobiocin even when the prenylated 4-hydroxybenzoate moiety (= Ring A) was added externally. However, when the *couL* expression plasmid pMS91 or the *simL* expression plasmid pSH2 were introduced into *S. coelicolor*(clo-CA5), external feeding of Ring A readily resulted in the formation of clorobiocin (data not shown). This was expected, as the amide synthetases CouL and SimL can accept Ring A as a substrate [5, 7, 8].

In order to investigate whether the introduction of *couL* and *simL* could expand the range of aminocoumarin antibiotics accessible from mutasynthesis experiments, we added synthetic Ring A analogs to four different strains. Three of these strains were obtained from *S. coelicolor*-(clo-CA5) by transformation with (1) the empty expression vector pUWL201, with (2) the *couL* expression plasmid pMS91, or with (3) the *simL* expression plasmid pSH2. The fourth strain was a previously described *cloQ*-defective mutant [5, 10] that still contained the genuine amide synthetase gene *cloL*. In a first experiment, we added to these strains 3,5-dimethyl-4-hydroxybenzoic acid, which is readily accepted by CouL, but is poorly accepted by the other amide synthetases. The extracts of the four cultures were investigated by HPLC and by LC-MS (Figure 3A). The expected new antibiotic, which carries 3,5-dimethyl-4-hydroxybenzoic acid as Ring A analog (novclobiocin 201, Table 1), was detected only in the extract of the strain harboring *couL*, not in the other three strains.

In a second experiment, we added benzoic acid, which is accepted by SimL, but is poorly accepted by the other amide synthetases. In this case, the antibiotic containing benzoic acid as a Ring A analog was detected only in the strain harboring *simL* (Figure 3B). This proved that the expression of different amide synthetases indeed allowed for the production of aminocoumarin antibiotics which were unattainable in our previous experiments. The amide synthetase SimL appears to be especially suitable for this approach, as it accepts many substrates that are poorly utilized by NovL, CloL, or CouL [7, 12], including 4-methylthiobenzoic acid, cinnamic acid, and ferulic acid. Feeding of these three compounds to *S. coelicolor*(clo-CA5) harboring *simL* readily gave the expected clorobiocin analogs (Table 1). All compounds, including the products resulting from the feeding of benzoic acid and 3,5-dimethyl-4-hydroxybenzoic acid, were isolated in preparative amounts, and their structures were confirmed by ^{1}H-NMR and mass spectrometry (MS) (see Experimental Procedures).

In four of these five feeding experiments, a minor product was formed that showed the same mass as the respective clorobiocin analog. ^{1}H-NMR spectroscopy proved that these compounds were structural isomers of the dominant clorobiocin analogs, carrying the 5-methylpyrrole-2-carboxylic acid esterified to 2″-OH instead of 3″-OH of the deoxysugar [13]. This type of isomer has also been observed in previous studies [5].

In the feeding experiments with benzoic acid and 3,5-dimethyl-4-hydroxybenzoic acid, an additional product could be isolated by preparative HPLC with suitable solvent systems. Mass spectrometric analysis showed a mass that was 34 Da lower than that of the respective clorobiocin analog, consistent with the loss of a chlorine atom. ^{1}H-NMR spectroscopy showed an additional proton in the aminocoumarin system, proving that these compounds carry a hydrogen instead of a chlorine atom at C-8′ of the aminocoumarin moiety. Therefore, halogenation of position 8′ by Clo-hal had not been carried out completely, and similar observations had been made in previous studies [5, 11].

When we fed 4-hydroxybenzoic acid (4-HBA) to *S. coelicolor*(clo-CA5) harboring *simL*, an 8′-chlorinated aminocoumarin antibiotic as well as the corresponding 8′-nonsubstituted compound could be isolated. However, MS and ^{1}H-NMR analysis proved that these two compounds carried a vanilloyl (i.e., 3-methoxy-4-hydroxybenzoyl) moiety as the Ring A analog, indicating that the externally added 4-HBA has been hydroxylated and methylated in position 3. As observed previously [6], small amounts of these two compounds were formed even without feeding of 4-HBA, i.e., from an endogenous pathway leading to vanillic acid.

Hybrid Aminocoumarin Antibiotics from Two-Stage Mutasynthesis Experiments

In a second series of experiments, we investigated a two-stage mutasynthesis procedure. In the first stage, Ring A analogs were fed to a strain with an engineered novobiocin biosynthetic gene cluster. This strain (*S. coelicolor*-(nov-CA7), see the Experimental Procedures) could form the 3-amino-4,7-dihydroxycoumarin moiety, attach it to the externally added Ring A analog (by utilizing NovL as a catalyst), and methylate the resulting product at position 8′ (Figure 4) [14]. However, the strain could not produce the complete antibiotic, due to defects in Ring A and deoxysugar biosynthesis. The feeding therefore resulted in analogs of the aglycon of novobiocin, which is termed novobiocic acid (Figure 4). The culture extract from this first mutasynthetic feeding step was subsequently added, without purification, to a second strain. That strain was *S. coelicolor*(clo-CA5), described above, which formed

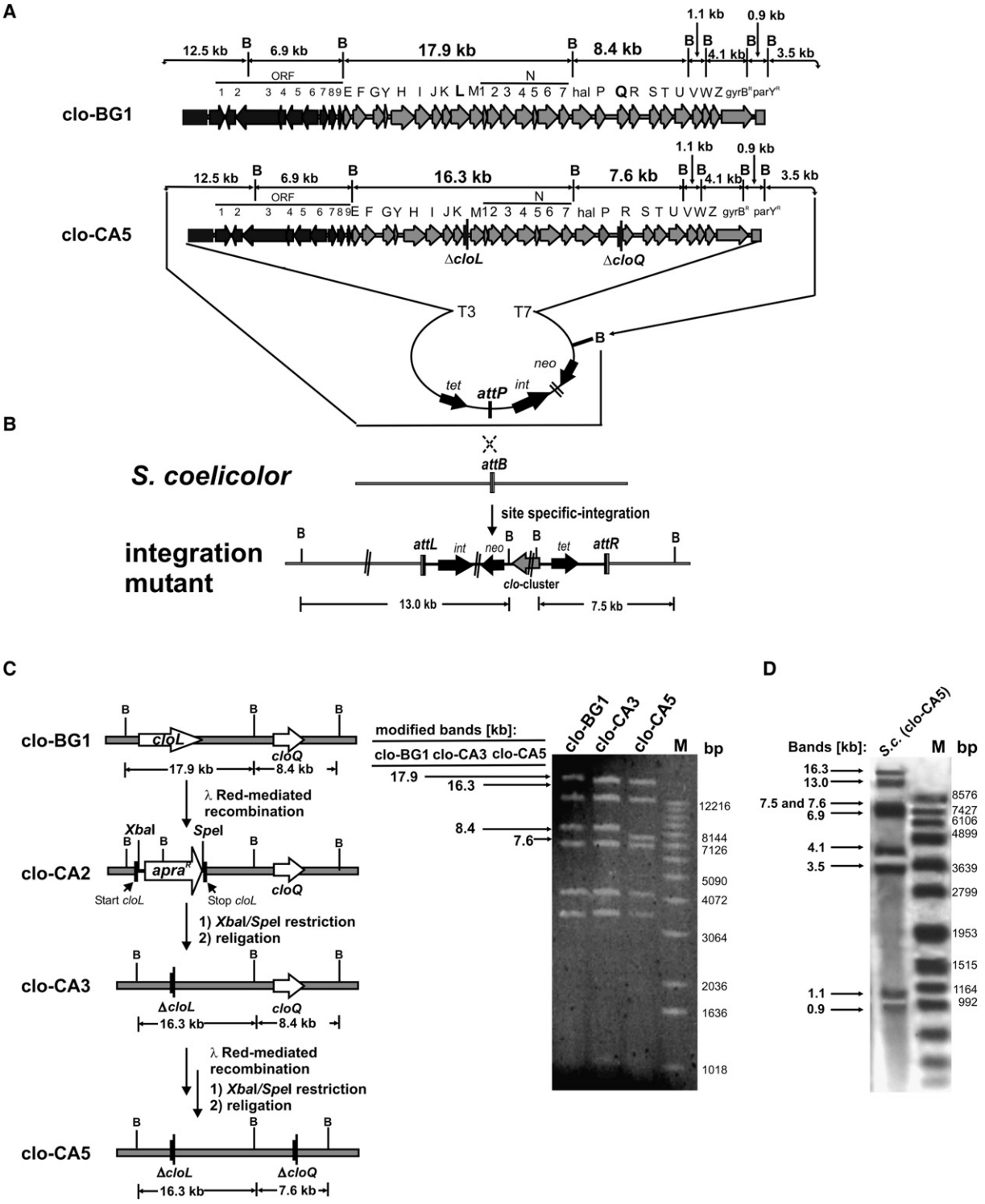


Figure 2. Construction of clo-CA5 and Southern Blot Analysis of the Mutant *S. coelicolor*(clo-CA5)

(A) Cosmid constructs clo-BG1 (intact) and clo-CA5 (Δ*cloL*Δ*cloQ*) containing the clorobiocin biosynthetic gene cluster and the ΦC31 integration functions. T3, T7 = T3, and T7 promoter of the SuperCos1 vector. The cosmid backbone is not to scale.

(B) Schematic representation of site-specific integration of clo-CA5 into the *S. coelicolor* chromosome. B = BglII restriction site. Fragment sizes resulting from digestion with BglII are indicated.

(C) Schematic presentation of the *cloL* and *cloQ* inactivation and restriction fragment analysis of the modified cosmids. *cloL* was replaced by an apramycin cassette that was subsequently excised by restriction enzyme digestion and religated, leaving an in-frame "scar" of 18 nucleotides, resulting in

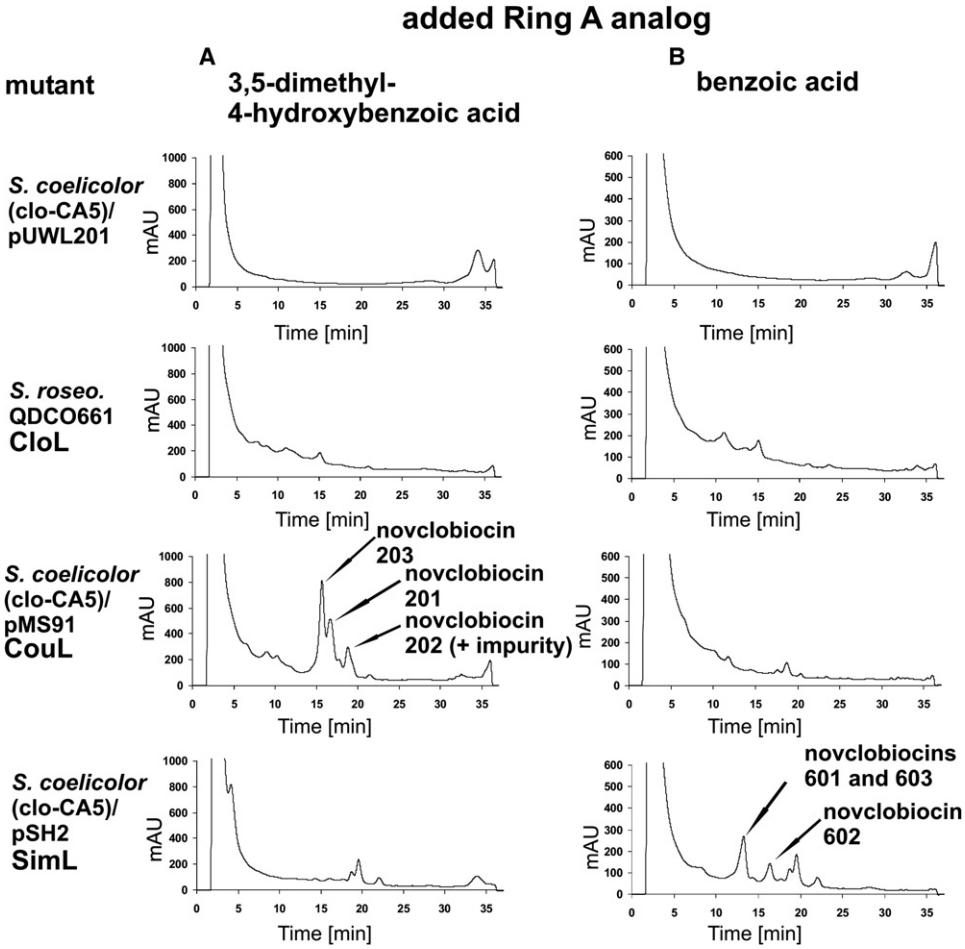


Figure 3. HPLC Analysis of Culture Extracts

(A and B) HPLC analysis of culture extracts of different *S. coelicolor*(clo-CA5) mutants after feeding of (A) 3,5-dimethyl-4-hydroxybenzoic acid or (B) benzoic acid. The identity of the marked peaks was confirmed by LC-MS as well as by ^{1}H-NMR. Unmarked peaks are metabolites unrelated to aminocoumarins, as confirmed by LC-MS.

the deoxysugar, the 4″-methyl group, and the 5-methylpyrrole-2-carbonyl moiety, which is typical of clorobiocin, and attached them to the novobiocic acid analog (Figure 4). Therefore, this two-stage procedure led to the formation of hybrid antibiotics that carry structural features of both novobiocin and clorobiocin.

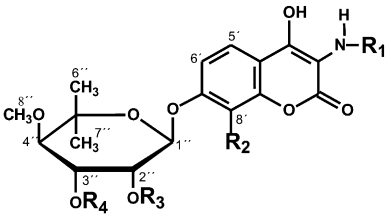
Five Ring A analogs (Table 2, A compounds), known to be accepted by the amide synthetase NovL, were subjected to this two-stage mutasynthesis procedure. The resulting aminocoumarin antibiotics were isolated by preparative HPLC, and their structures were elucidated by ^{1}H-NMR and MS.

In all five experiments, the expected aminocoumarin antibiotics containing the externally added Ring A analog were obtained. However, when Ring A analogs that carried either a bromine atom or a polar side chain with an amide bond at position 3 were used, the resulting aminocoumarin antibiotics were unexpectedly found to contain a chlorine atom rather than a methyl group at position 8′ of the aminocoumarin ring (Table 2, A compounds). Their structures were unequivocally confirmed by both ^{1}H-NMR and MS (see the Experimental Procedures). This finding suggests that the novobiocic acid analogs containing these polar Ring A analogs are poor substrates of the 8′-methyl transferase NovO (Figure 4), but a good substrate for the halogenase Clo-hal [15], present in *S. coelicolor*(clo-CA5). Control experiments proved that no 8′-halogenated compounds were produced by *S. coelicolor*(nov-CA7) alone, which does not contain the halogenase Clo-hal. *S. coelicolor*(clo-CA5), which

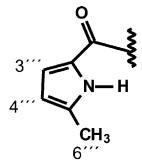
cosmid clo-CA3. In the same way, *cloQ* was inactivated, resulting in cosmid clo-CA5. This figure is not to scale. M = 1 kb DNA ladder (Invitrogen). DNA of clo-BG1, clo-CA3, and clo-CA5 were digested with BglII.

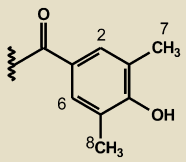
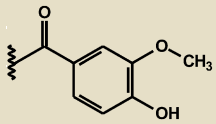
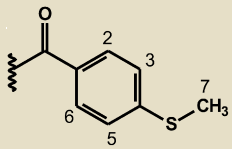
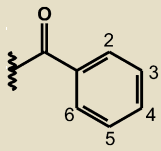
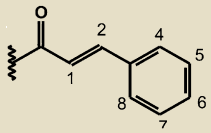
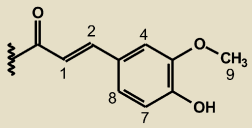
(D) Southern blot analysis of the *S. coelicolor* M512 (*S. c.*) integration mutant harboring clo-CA5. M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic DNA was digested with BglII. The DIG-labeled cosmid clo-BG1 was used as a probe.

Table 1. Chemical Structures of Aminocoumarin Antibiotics Produced by One-Stage Mutasynthesis Experiments, Utilizing the Amide Synthetase Genes *couL* or *simL*



Pyr =



Compound Name	R_1	R_2	R_3	R_4
Novclobiocin 201		Cl	OH	Pyr
Novclobiocin 202		Cl	Pyr	OH
Novclobiocin 203		H	OH	Pyr
Novclobiocin 311[a]		Cl	OH	Pyr
Novclobiocin 312[a]		Cl	Pyr	OH
Novclobiocin 313[a]		H	OH	Pyr
Novclobiocin 371		Cl	OH	Pyr
Novclobiocin 372		Cl	Pyr	OH
Novclobiocin 601		Cl	OH	Pyr
Novclobiocin 602		Cl	Pyr	OH
Novclobiocin 603		H	OH	Pyr
Novclobiocin 701		Cl	OH	Pyr
Novclobiocin 702		Cl	Pyr	OH
Novclobiocin 731		Cl	OH	Pyr

couL was used for production of novclobiocin 201, 202, and 203. *simL* was used for all other experiments.
[a] These products were already isolated and identified by Freitag et al. [6].

does not contain CloQ and CloL, produced these 8′-chlorinated aminocoumarin antibiotics only upon feeding with 8′-unsubstituted novobiocic acid analogs, not upon feeding with Ring A analogs. This supports the hypothesis formulated by Pacholec et al. [14] that 8′-desmethyl novobiocic acid may be the genuine substrate of Clo-hal.

Again, structural isomers containing the 5-methyl-pyrrole-2-carbonyl moieties at 2″-OH rather than 3″-OH were formed in several of these feeding experiments (Table 2, A compounds).

Utilization of a Heterologous Amide Synthetase in Two-Stage Mutasynthesis Experiments

We had shown that both the expression of heterologous amide synthetases and the utilization of a two-stage procedure can expand the range of aminocoumarin antibiotics accessible from mutasynthesis experiments.

We now attempted to combine these two strategies. For this purpose, the *simL* expression plasmid pSH2 was transformed into strain *S. coelicolor*(nov-CA7). Subsequently, three Ring A analogs, known to be accepted by the amide synthetase SimL, but to be poorly accepted

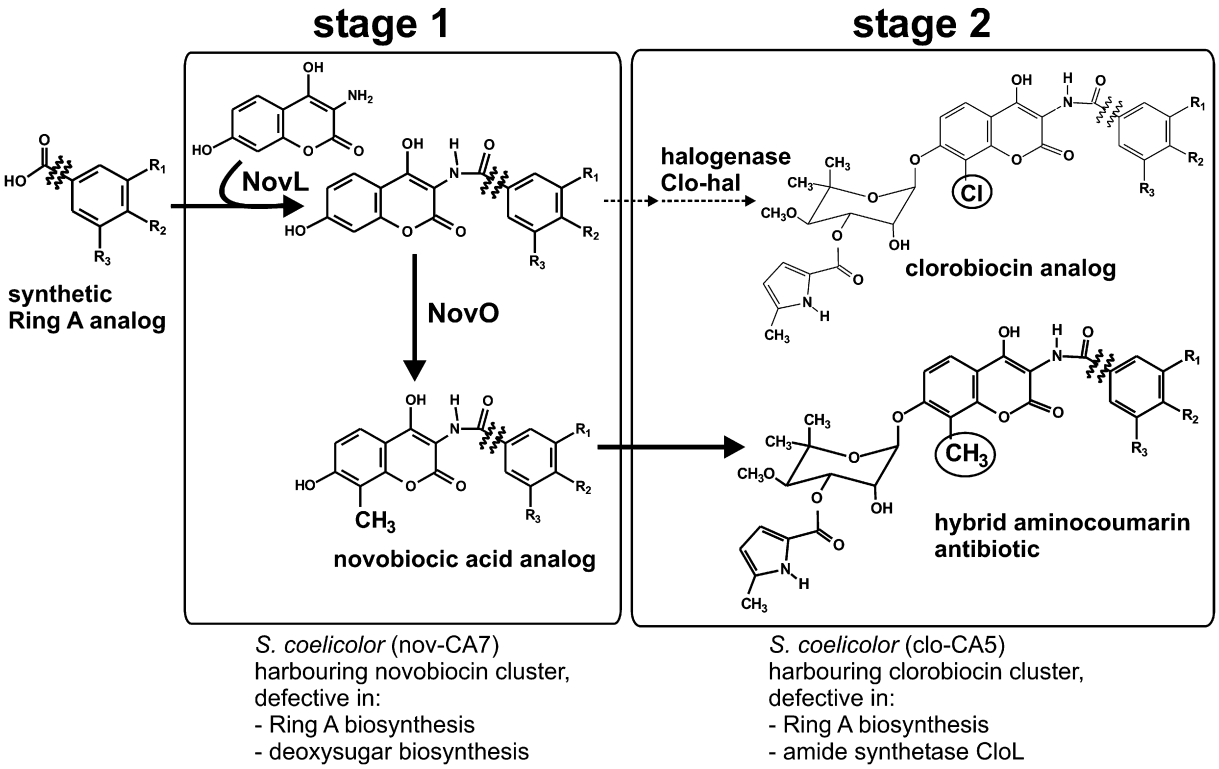


Figure 4. Schematic of the Two-Stage Feeding Procedure
See text for explanations.

by NovL [7], were fed to this strain (Table 2, B compounds). As described above, the resulting culture extracts containing novobiocic acid analogs were fed, without purification, to *S. coelicolor*(clo-CA5).

In all three experiments, the expected aminocoumarin antibiotics could be isolated in preparative amounts and analyzed by ^{1}H-NMR and MS (see the Experimental Procedures). In the feeding experiment with cinnamic acid, additionally a compound was isolated that carried a chlorine rather than a methyl group in position 8′ (Table 2, B compounds, novclobiocin 701).

Antibacterial Activity

The antibacterial activity of selected compounds, representing novclobiocin with different Ring A analogs and carrying the pyrrole-2-carboxl group attached to the 3-OH group of the deoxysugar, were investigated against *Staphylococcus aureus* ATCC 43300, a Gram-positive, methicillin-resistant pathogen strain, and *Pseudomonas aeruginosa* K799/wt, a Gram-negative bacterium (Table 3). Aminocoumarin antibiotics have been reported to show poor activity against Gram-negative pathogens, but high activity against Gram-positive strains, especially against staphylococci [16]. Correspondingly, *Pseudomonas aeruginosa* K799/wt was not sensitive to the tested aminocoumarin derivatives. In contrast to this, *Staphylococcus aureus* ATCC 43300 was sensitive to all of the tested derivatives. Novclobiocins 217 and 225, which include Ring A analogs similar to the genuine Ring A, were most active. Novclobiocins 201, 314, 371, and 701 also showed activities comparable to that of novobiocin. However, low activities resulted from a polar side chain in the Ring A analog (novclobiocin 241) and from the lack of a substituent at position 8 of the coumarin moiety (novclobiocin 203).

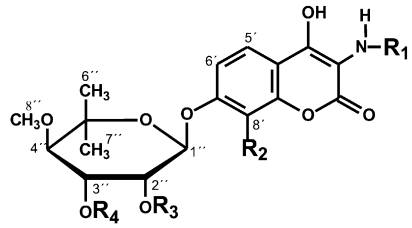
DISCUSSION

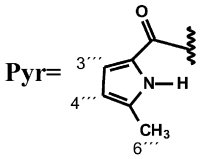
In the present study, we demonstrated that limitations of mutasynthesis experiments can be overcome by exploiting the natural diversity of biosynthetic genes and enzymes. Utilizing the amide synthetases SimL and CouL, which use different substrates than CloL, we could generate a range of modified aminocoumarins which were not accessible from our previous mutasynthetic experiments.

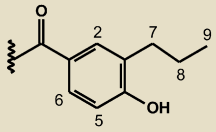
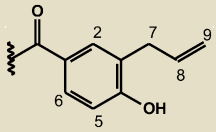
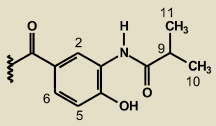
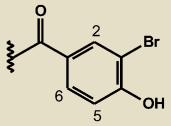
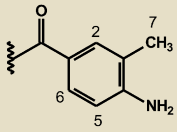
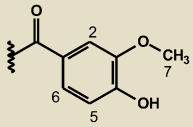
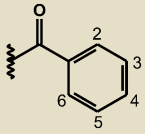
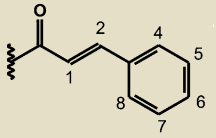
For the present experiments, we generated heterologous producer strains by stable integration of biosynthetic gene clusters into the genome of *Streptomyces coelicolor* M512. The genetic modification of gene clusters by λ RED-mediated recombination [17] and the heterologous expression of these clusters in the fully sequenced host *S. coelicolor* [11] greatly expand the possibilities for mutasynthetic experiments.

In our mutasynthesis approach, the structure of the substituted benzoyl moiety of the resulting antibiotic could be chosen by external addition of the respective Ring A analog, and the structures of the other parts of the

Table 2. Chemical Structures of Aminocoumarin Antibiotics Produced by Two-Stage Mutasynthesis Experiments





	Compound Name	R_1	R_2	R_3	R_4
A	Novclobiocin 217		CH_3	OH	Pyr
	Novclobiocin 218		CH_3	Pyr	OH
	Novclobiocin 225		CH_3	OH	Pyr
	Novclobiocin 226		CH_3	Pyr	OH
	Novclobiocin 241[a]		Cl	OH	Pyr
	Novclobiocin 281[a]		Cl	OH	Pyr
	Novclobiocin 386		CH_3	OH	Pyr
	Novclobiocin 387		CH_3	Pyr	OH
B	Novclobiocin 314		CH_3	OH	Pyr
	Novclobiocin 604		CH_3	OH	Pyr
	Novclobiocin 703		CH_3	OH	Pyr
	Novclobiocin 701		Cl	OH	Pyr

(A) Compounds obtained without additional amide synthetase.
(B) Compounds obtained with expression of the amide synthetase SimL.
[a] These compounds were already isolated by Galm et al. [5].

molecule could be chosen by use of an appropriately engineered strain. Strains that produce many differently substituted aminocoumarin and deoxysugar moieties are available from previous work [18–21]. Therefore, the present procedure opens a route to a large number of structurally different aminocoumarin antibiotics.

Table 3. Antibacterial Activity of Selected Compounds

Compound	*Staphylococcus aureus* ATCC 43300	*Pseudomonas aeruginosa* K799/wt
Clorobiocin	≤0.06	16
Novobiocin	0.25	>32
Novclobiocin 201	0.25	>32
Novclobiocin 203	4	>32
Novclobiocin 217	≤0.06	>32
Novclobiocin 225	≤0.06	>32
Novclobiocin 241	4	>32
Novclobiocin 281[a]	1	>32
Novclobiocin 311	1	>32
Novclobiocin 314	0.25	>32
Novclobiocin 371	0.25	>32
Novclobiocin 386	1	>32
Novclobiocin 701	1	>32
Novclobiocin 731	0.5	>32

Antibacterial activity is given as MIC (μg/ml).
[a] Tested against *Staphylococcus aureus* ATCC 29213 [16].

The structural diversity of compounds may be further expanded by two-stage mutasynthesis experiments. In the first stage, the externally added Ring A analog is attached to a chosen substituted aminocoumarin, and then, in the second stage, the resulting culture extract is fed to another strain that attaches differently substituted deoxysugar moieties to the aminocoumarin (see Figure 4). We have shown that such two-stage mutasynthesis experiments can be performed successfully. The two-stage mutasynthesis strategy was also combined with the expression of different amide synthetases in the first stage, further expanding the range of accessible antibiotic structures (Table 2, B compounds). No purification was required between the first and second stage. As expected, the overall yields of two-stage experiments were lower than those of one-stage approaches: by feeding of 10 mg of a given Ring A analog, up to 4 mg of the respective aminocoumarin antibiotic could be obtained in one-stage experiments, but only up to 1 mg could be obtained in two-stage experiments.

SIGNIFICANCE

Our study exemplifies that the efficiency and versatility of mutasynthesis experiments can be significantly improved by utilization of the natural diversity of biosynthetic genes and enzymes for the generation of metabolically engineered microbial producer strains and by utilization of two-stage feeding procedures. These methods offer the potential to significantly increase the structural diversity of natural products obtainable by mutasynthesis experiments in drug discovery programs.

EXPERIMENTAL PROCEDURES

Chemicals

Novobiocic acid was isolated from *Streptomyces spheroides* AM1T2 [22]; Ring A (3-dimethylallyl-4-hydroxybenzoic acid) was obtained by hydrolysis of novobiocin [23]; commercially available Ring A analogs were purchased from Aldrich, Fluka, Lancaster, Merck, and Sigma; and further substituted benzoic acids were synthesized as described by Dessoy [24].

Genetic Procedures

Escherichia coli XL1 Blue MRF′ (Stratagene, Heidelberg, Germany), BW 25113 [25], and ET 12567 [26] were used for cloning. The REDIRECT technology kit for PCR targeting was obtained from Plant Bioscience Limited (Norwich, UK). Standard methods for DNA isolation and manipulation were performed as described by Sambrook et al. [27] and by Kieser et al. [28]. Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kit; Macherey-Nagel). Genomic DNA was isolated from *Streptomyces* strains by lysozyme treatment and phenol/chloroform extraction described by Kieser et al. [28].

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

Construction of Plasmids pMS91 and pSH2

The *E. coli-Streptomyces* shuttle vector pUWL201, containing the *ermE** promoter [9], was used for the construction of the expression plasmids pMS91 and pSH2.

For the construction of pMS91, *couL* was amplified by PCR. A HindIII site was introduced before the start codon by using primer cumG1 (5′-CCGCTGCCC**AAGCTT**GAGTGCCTC-3′). At the C terminus, an XbaI restriction site was introduced after the stop codon by using primer cumG2 (5′-CGGGCAGGC**TCTAGA**ACAGCACTC-3′). Bold letters represent the restriction sites. The resulting PCR fragment was digested with HindIII and XbaI and was subsequently cloned into the same sites of pUWL201 to give pMS91.

For the construction of pSH2, the gene *simL* was isolated by restriction digest with NotI from cosmid VII-8g, containing a part of the simocyclinone biosynthetic gene cluster of *S. antibioticus* Tü 6040 [4], and was cloned into pcDNA2.1 (Invitrogen, La Jolla, California, USA), resulting in pTLsimLfori. The correct orientation of *simL* was confirmed. pTLsimLfori was digested with XbaI and HindIII, and the fragment containing *simL* was cloned into pUWL201 to give pSH2.

Inactivation of *cloL* and *cloQ* in Cosmid clo-BG1 and Heterologous Expression

In cosmid clo-BG1 [11], first *cloL* and then *cloQ* were replaced, through λ RED-mediated recombination, with an apramycin-resistance (*aac(3)IV*) cassette that was flanked by XbaI and SpeI recognition sites. For replacement of *cloL*, the cassette was generated by PCR by using pUG019 [11] as template and the primers cloL_for (5′-**AGCGGAAG TACCTCTACTTCGCGAAAGGTAGTCACTGGTG**ATTCCGGGGATC TCTAGATC-3′) and cloL_rev (5′-**CATCGAATGACTCACCTCACC TGTCCACCAGCACGTCCGG**ACTAGTCTGGAGCTGCTTC-3′). Bold letters represent the homologous extensions to the DNA regions immediately upstream and downstream of *cloL*. Underlined letters indicate the XbaI and SpeI restriction sites. PCR amplification was performed in 50 μl volume with 50 ng template, 0.2 mM dNTPs, 50 pmol of each primer, and 5% (v/v) DMSO with the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94°C for 2 min, then 10 cycles with denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 90 s, followed by 15 cycles with annealing at 55°C for 45 s, and the last elongation step at 72°C for 5 min. The PCR product was introduced by electroporation into *E. coli* BW25113/pIJ790 harboring cosmid clo-BG1 [17]. The resulting modified cosmid clo-CA2 was isolated, transformed into the nonmethylating strain *E. coli* ET12567, reisolated, and digested with XbaI

and SpeI to remove the apramycin-resistance cassette. Religation overnight at 4°C gave the cosmid clo-CA3.

The apramycin-resistance cassette for replacement of *cloQ* was generated by PCR amplification with the primers cloQ_for (5′-**GGCGCGCCCATTGCTCACCGTCTTACCGACACCGTCCTT**ATTCCGGGGATCTCTAGATC-3′) and cloQ_rev (5′-**CCCATGGTCGATTCCGTGTGTTGGTGAAGTGCGCGCAG**ACTAGTCTGGAGCTGCTTC-3′) and the same template and conditions as described above. The PCR product was introduced by electroporation into *E. coli* BW25113/pIJ790 harboring cosmid clo-CA3. Again, the modified cosmid was isolated, and the resistance cassette was removed with XbaI and SpeI. Religation gave the cosmid clo-CA5.

The cosmid clo-CA5 isolated from *E. coli* ET12567 was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation [28]. Clones resistant to kanamycin were selected, and integration of clo-CA5 into the genome was confirmed by Southern blot analysis (Figure 2).

Transformation with the Plasmids pMS91 or pSH2

Transformation of the different integration mutants was carried out by PEG-mediated protoplast transformation [28].

Inactivation of *novQ* in the Cosmid nov-BG1 and Heterologous Expression

In cosmid nov-BG1 [11], *novQ* was inactivated by a similar strategy to that described above. The apramycin-resistance cassette was generated by PCR with the primers novQ_P01f (5′-**ACCGGTAATTCACTGTGAGTTGATCACGGAGGAATTC*ATG***ATTCCGGGGATCTCTAGATC-3′) and novQ_P02rev (5′-**AGTCGTCTCCGGGCATGTTCGCCAGAGCCTCGC*TCA*TCG**ACTAGTCTGGAGCTGCTTC-3′). The PCR product was introduced by electroporation into *E. coli* BW25113/pIJ790 harboring cosmid nov-BG1 [17]. The modified cosmid was isolated and the apramycin-resistance cassette was excised to give the cosmid nov-CA7.

Cosmid nov-CA7 isolated from *E. coli* ET12567 was introduced into *S. coelicolor* M512 [28]. Clones resistant to kanamycin were selected, and integration of nov-CA7 into the genome was confirmed by Southern blot analysis (data not shown). Feeding of Ring A to this strain resulted in the accumulation of novobiocic acid, but not of novobiocin, showing that the deoxysugar biosynthesis, encoded by *novSTUVW* situated downstream of *novQ* (Figure 2), had been abolished, presumably due to downstream effects. Since the inactivation of *novQ* had been carried out by in-frame deletion, the precise nature of the downstream effects was not clear. To our surprise, *novQ* inactivations utilizing four different strategies resulted in this same phenotype: (1) replacement of the *novQ* gene with an apramycin-resistance cassette in the wild-type producer *Streptomyces spheroides*; (2) in-frame deletion of the entire coding sequence of *novQ* between the start codon and the stop codon, and expression of this modified novobiocin cluster in the heterologous host *Streptomyces coelicolor* M512; (3) repetition of experiment (2), but leaving the last codon of *novQ* (CGA) before the stop codon intact in order not to affect the presumed translational coupling between *novQ* and *novR* (**CGATGA**GC; bold letters indicate the last two codons of *novQ*; underlined letters indicate the first two codons of *novR*); (4) introduction of a frameshift into *novQ* by deletion of a single base (position 19059 in accession number AF170880). All four mutant strains produced, upon feeding of Ring A, the aglycon novobiocic acid, but not the glycoside novobiocin; therefore, all four inactivation methods affected the downstream deoxysugar biosynthesis genes *novSTUVW*. Our experiments did not allow us to determine whether this effect was due to termination of transcription, altered mRNA stability, termination of translation, disrupted protein-protein interactions, or other factors. However, this result made the originally planned, separate inactivation of the deoxysugar biosynthesis genes unnecessary. HPLC-MS analysis of the products obtained by feeding of Ring A analogs to strain *S. coelicolor* M512(nov-CA7), generated by inactivation strategy (3), proved that these products represented the desired aglyca. HPLC-MS and NMR analysis of the products of the two-stage feeding procedure proved that these represented the desired glycosides, carrying the pyrrole-2-carboxyl moiety typical of clorobiocin.

Production of Aminocoumarin Antibiotics by One- and Two-Stage Mutasynthesis Experiments

The strains were cultured as described previously [5]. For one-stage mutasynthesis experiments, 1 mg of the Ring A analog was dissolved in 200 μl ethanol and added to 80 ml of the culture at the time of inoculation of the production medium, followed by cultivation for 7–10 days at 33°C and 210 rpm.

For analytic purposes, 1 ml bacterial culture was acidified with HCl to pH 4 and extracted twice with an equal volume of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 100 μl methanol. After centrifugation, 80 μl supernatant was analyzed by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 150 × 4.6 mm) at a flow rate of 1 ml min^{-1}. A linear gradient from 40% to 100% solvent B (solvent A = 50:49:1 MeOH:H_2O:HCOOH; solvent B = 99:1 MeOH:HCOOH) over 28 min was used. UV detection was carried out at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation of the products, cultivation was carried out as described above, by using a total culture volume of 800 ml. The antibiotics were isolated as described previously [5].

For two-stage mutasynthesis experiments, *S. coelicolor*(nov-CA7) was precultivated in CDM medium [29] for 3–4 days and then used to inoculate ten flasks, each containing 50 ml CDM production medium. At the time of inoculation, 1 mg of the Ring A analog, dissolved in 200 μl ethanol, was added to each flask, followed by cultivation for 7–10 days at 30°C and 210 rpm. The culture was adjusted to pH 4 with hydrochloric acid and extracted twice with an equal volume of ethyl acetate. The organic layer was evaporated to dryness. The amount of the aglycon (= novobiocic acid analog) contained in this extract was determined by HPLC, and the extract was dissolved in an appropriate volume of ethanol to obtain a final concentration of 1 mg aglycon in 200 μl ethanol. A total of 200 μl of this solution was added, at the time of inoculation, to 80 ml of a culture of *S. coelicolor*(clo-CA5) in distillers solubles production medium as described previously [5]. The total culture volume varied between 160 ml and 560 ml. After cultivation for 7–10 days at 33°C and 210 rpm, the preparative isolation and purification of the products were carried out as described above.

Structure Elucidation

Positive electrospray ionisation (ESI) mass spectra were obtained from a Finnigan TSQ Quantum instrument (electrospray voltage, 3.8 kV; heated capillary temperature, 320°C; sheath and auxiliary gas, nitrogen; sheath gas flow rate, 30.1 AU; aux gas flow rate, 12.0 AU) equipped with a Nucleosil 120 RP ODS column (8 μm, 2 × 250 mm, Macherey-Nagel). For separation, a gradient of acetonitrile in water (each containing 0.1% HCOOH) ranging from 0% acetonitrile to 50% acetonitrile over 20 min was used, followed by elution with 100% acetonitrile for 5 min and equilibration with 100% water (containing 0.1% HCOOH) for 4 min; the flow rate was 0.2 ml min^{-1}. The collision-induced dissociation (CID) spectra during the HPLC run were recorded with collision energy of +25 eV, collision gas argon, and collision pressure of 1.0×10^{-3} torr (133 mPa).

^{1}H-NMR spectra were measured on an Avance 400 spectrometer (Bruker, Karlsruhe, Germany) with CD_3OD as solvent. ^{1}H-NMR spectral data (400 MHz; CD_3OD; br, broad).

Novclobiocin 201

δ 1.18 (s, 3H-6″), 1.35 (s, 3H-7″), 2.26 (s, 3H-7 and 3H-8), 2.29 (s, 3H-6‴), 3.52 (s, 3H-8″), 3.72 (d, J = 10.3 Hz, H-4″), 4.34 (br s, H-2″), 5.70 (dd, J_1 = 10.3 Hz, J_2 = 3.3 Hz, H-3″), 5.72 (br s, H-1″), 5.93 (d, J = 3.7 Hz, H-4‴), 6.90 (d, J = 3.7 Hz, H-3‴), 7.31 (d, J = 8.8 Hz, H-6′), 7.63 (s, H-2 and H-6), 7.88 (d, J = 8.8 Hz, H-5′).

MS: 657.3 ([M+H]$^+$, Cl^{35}), 659.3 ([M+H]$^+$, Cl^{37}), 282.1 [sugar+pyrrole unit]$^+$, 149.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 3.8 mg.

Novclobiocin 202

δ 1.17 (s, 3H-6″), 1.38 (s, 3H-7″), 2.26 (s, 3H-7 and 3H-8), 2.30 (s, 3H-6‴), 3.56 (d, J = 9.6 Hz, H-4″), 3.65 (s, 3H-8″), 4.44 (dd, J_1 = 9.6 Hz, J_2 = 2.8 Hz, H-3″), 5.41 (br s, H-2″), 5.82 (br s, H-1″), 5.95 (d, J = 3.4 Hz, H-4‴), 6.90 (d, J = 3.4 Hz, H-3‴), 7.31 (d, J = 6.1 Hz, H-6′), 7.62 (s, H-2 and H-6), 7.85 (d, J = 6.1 Hz, H-5′).

MS: 657.3 ([M+H]$^+$, Cl^{35}), 659.1 ([M+H]$^+$, Cl^{37}), 282.0 [sugar+pyrrole unit]$^+$, 149.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 3.1 mg.

Novclobiocin 203

δ 1.20 (s, 3H-6″), 1.37 (s, 3H-7″), 2.27 (s, 3H-7 and 3H-8), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.69 (d, J = 9.9 Hz, H-4″), 4.24 (br s, H-2″), 5.59 (dd, J_1 = 9.9 Hz, J_2 = 2.2 Hz, H-3″), 5.63 (br s, H-1″), 5.94 (d, J = 3.5 Hz, H-4‴), 6.90 (d, J = 3.5 Hz, H-3‴), 7.07 (overlapping signals, J is not determinable, H-8′ and H-6′), 7.63 (s, H-2 and H-6), 7.90 (d, J = 6.8 Hz, H-5′).

MS: 623.3 [M+H]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 149.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 3.0 mg.

Novclobiocin 217

δ 0.97 (t, J = 7.4 Hz, 3H-9), 1.19 (s, 3H-6″), 1.37 (s, 3H-7″), 1.66 (sext., J = 7.4 Hz, 2H-8), 2.29 (s, 3H-6‴), 2.34 (s, 3H-8′), 2.63 (t, J = 7.4 Hz, 2H-7), 3.53 (s, 3H-8″), 3.71 (d, J = 10.0 Hz, H-4″), 4.28 (br s, H-2″), 5.62 (d, J = 2.0 Hz, H-1″), 5.67 (dd, J_1 = 10.0 Hz, J_2 = 3.2 Hz, H-3″), 5.94 (d, J = 3.8 Hz, H-4‴), 6.83 (d, J = 8.6 Hz, H-5), 6.90 (d, J = 3.8 Hz, H-3‴), 7.21 (d, J = 8.8 Hz, H-6′), 7.73 (d, J = 8.6 Hz, H-6), 7.79 (br s, H-2), 7.83 (d, J = 8.8 Hz, H-5′).

MS: 651.4 [M+H]$^+$, 370.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 163.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 1.0 mg after the second feeding step.

Novclobiocin 218

δ 0.96 (t, J = 7.4 Hz, 3H-9), 1.19 (s, 3H-6″), 1.42 (s, 3H-7″), 1.65 (complex and broad signal, J is not determinable, 2H-8), 2.28 (s, 3H-8′), 2.29 (s, 3H-6‴), 2.62 (t, J = 7.4 Hz, 2H-7), 3.51 (d, J = 9.2 Hz, H-4″), 3.63 (s, 3H-8″), 4.41 (dd, J_1 = 9.2 Hz, J_2 = 3.2 Hz, H-3″), 5.37 (t, J = 2.6 Hz, H-2″), 5.69 (d, J = 2.6 Hz, H-1″), 5.94 (d, J = 3.8 Hz, H-4‴), 6.81 (d, J = 8.4 Hz, H-5), 6.88 (d, J = 3.8 Hz, H-3‴), 7.15 (d, J = 8.8 Hz, H-6′), 7.72 (d, J = 8.4 Hz, H-6), 7.78 (br s, H-2), 7.81 (d, J = 8.8 Hz, H-5′).

MS: 651.4 [M+H]$^+$, 370.2 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 163.0 [Ring A analog]$^+$.

The total yield of the compound was 0.28 mg after the second feeding step.

Novclobiocin 225

δ 1.19 (s, 3H-6″), 1.36 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.34 (s, 3H-8′), 3.40 (d, J = 6.4 Hz, 2H-7), 3.52 (s, 3H-8″), 3.71 (d, J = 10.0 Hz, H-4″), 4.28 (br s, H-2″), 5.03 (d, J = 10.0 Hz, H-9a *trans*), 5.07 (dd, J_1 = 18.8 Hz, J_2 = 1.2 Hz, H-9a *cis*), 5.62 (d, J = 1.6 Hz, H-1″), 5.67 (dd, J_1 = 10.0 Hz, J_2 = 3.2 Hz, H-3″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.05 (m, H-8), 6.85 (d, J = 8.4 Hz, H-5), 6.90 (d, J = 3.6 Hz, H-3‴), 7.20 (d, J = 8.8 Hz, H-6′), 7.76 (d, J = 8.4 Hz, H-6), 7.79 (s, H-2), 7.82 (d, J = 8.8 Hz, H-5′).

MS: 649.3 [M+H]$^+$, 368.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 161.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.80 mg after the second feeding step.

Novclobiocin 226

δ 1.19 (s, 3H-6″), 1.42 (s, 3H-7″), 2.28 (s, 3H-8′), 2.29 (s, 3H-6‴), 3.40 (d, J = 6.8 Hz, 2H-7), 3.51 (d, J = 9.0 Hz, H-4″), 3.63 (s, 3H-8″), 4.41 (dd, J_1 = 9.0 Hz, J_2 = 3.2 Hz, H-3″), 5.02 (d, J = 10.4 Hz, H-9a *trans*), 5.06 (dd, J_1 = 17.2 Hz, J_2 = 2.0 Hz, H-9a *cis*), 5.37 (t, J = 2.6 Hz, H-2″), 5.69 (d, J = 2.6 Hz, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.03 (m, H-8), 6.84 (d, J = 8.4 Hz, H-5), 6.88 (d, J = 3.6 Hz, H-3‴), 7.16 (d, J = 8.8 Hz, H-6′), 7.75 (d, J = 8.4 Hz, H-6), 7.79 (s, overlapping signal, H-2), 7.81 (d, overlapping signal, J is not determinable, H-5′).

MS: 649.3 [M+H]$^+$, 368.2 [M-(sugar+pyrrole unit)]$^+$, 282.4 [sugar+pyrrole unit]$^+$, 161.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.30 mg after the second feeding step.

Novclobiocin 241

δ 1.19 (s, 3H-6″), 1.23 (d, J = 6.8 Hz, 3H-10 and 3H-11), 1.34 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.75 (sept., J = 6.8 Hz, H-9), 3.51 (s, 3H-8″), 3.71 (d, J = 10.3 Hz, H-4″), 4.35 (br s, H-2″), 5.70 (br s, H-1″), 5.71 (dd, J_1 = 10.3 Hz, J_2 = 3.0 Hz, H-3″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.90 (d, J = 3.6 Hz, H-3‴), 6.94 (d, J = 7.8 Hz, H-5), 7.21 (d, J = 7.8 Hz, H-6′), 7.71 (d, J = 8.0 Hz, H-6), 7.88 (d, J = 7.8 Hz, H-5′), 8.28 (s, H-2). These data are consistent with those reported previously [5].

MS: 714.3 ([M+H]$^+$, Cl^{35}), 716.5 ([M+H]$^+$, Cl^{37}), 282.0 [sugar+pyrrole unit]$^+$, 107.9 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.77 mg after the second feeding step.

Novclobiocin 281

δ 1.20 (s, 3H-6″), 1.35 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.71 (d, J = 10.4 Hz, H-4″), 4.32 (br s, H-2″), 5.65 (br s, H-1″), 5.71 (dd, J_1 = 10.4 Hz, J_2 = 3.2 Hz, H-3″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.90 (d, J = 3.4 Hz, H-3‴), 6.94 (d, J = 8.2 Hz, H-5), 7.21 (d, J = 9.0 Hz, H-6′), 7.85 (d, J = 8.2 Hz, H-6), 7.90 (d, J = 9.0 Hz, H-5′), 8.19 (br s, H-2). These data are consistent with those reported previously [5].

MS: 707.1 ([M+H]$^+$, Cl^{35}), 709.3 ([M+H]$^+$, Cl^{37}), 282.2 [sugar+pyrrole unit]$^+$, 108.2 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.01 mg after the second feeding step.

Novclobiocin 314

δ 1.20 (s, 3H-6″), 1.36 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.33 (s, 3H-8′), 3.52 (s, 3H-8″), 3.70 (d, J = 9.9 Hz, H-4″), 3.92 (s, 3H-7), 4.28 (t, J = 2.8 Hz, H-2″), 5.68 (dd, J_1 = 9.9 Hz, J_2 = 3.0 Hz, H-3″), 5.60 (d, J = 2.8 Hz, H-1″), 5.94 (d, J = 4.1 Hz, H-4‴), 6.84 (d, J = 8.1 Hz, H-5), 6.90 (d, J = 4.1 Hz, H-3‴), 7.13 (d, J = 9.1 Hz, H-6′), 7.54 (dd, J_1 = 8.1 Hz, J_2 = 2.0 Hz, H-6), 7.64 (d, J = 2.0 Hz, H-2), 7.83 (d, J = 9.1 Hz, H-5′).

MS: 639.3 [M+H]$^+$, 358.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 151.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.52 mg after the second feeding step.

Novclobiocin 371

δ 1.20 (s, 3H-6″), 1.35 (s, 3H-7″), 2.30 (s, 3H-6‴), 2.51 (s, 3H-7), 3.51 (s, 3H-8″), 3.71 (d, J = 10.0 Hz, H-4″), 4.34 (br s, H-2″), 5.69 (br s, H-1″), 5.72 (dd, J_1 = 10.0 Hz, J_2 = 3.0 Hz, H-3″), 5.94 (d, J = 3.8 Hz, H-4‴), 6.91 (d, J = 3.8 Hz, H-3‴), 7.19 (d, J = 8.4 Hz, H-6′), 7.28 (d, J = 8.0 Hz, H-2 and H-6), 7.89 (d, J = 8.4 Hz, H-5′), 7.92 (d, J = 8.1 Hz, H-3 and H-5).

MS: 659.3 ([M+H]$^+$, Cl^{35}), 661.3 ([M+H]$^+$, Cl^{37}), 282.1 [sugar+pyrrole unit]$^+$, 151.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.81 mg.

Novclobiocin 372

δ 1.18 (s, 3H-6″), 1.38 (s, 3H-7″), 2.30 (s, 3H-6‴), 2.52 (s, 3H-7), 3.55 (d, J = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.45 (dd, J_1 = 9.7 Hz, J_2 = 3.3 Hz, H-3″), 5.39 (br s, H-2″), 5.78 (br s, H-1″), 5.95 (d, J = 3.4 Hz, H-4‴), 6.89 (d, J = 3.4 Hz, H-3‴), 7.19 (d, J = 8.8 Hz, H-6″), 7.31 (d, J = 8.3 Hz, H-2 and H-6), 7.88 (d, J = 8.8 Hz, H-5′), 7.93 (d, J = 8.3 Hz, H-3 and H-5).

MS: 659.5 ([M+H]$^+$, Cl^{35}), 281.8 [sugar+pyrrole unit]$^+$.

The total yield of the compound was 0.05 mg.

Novclobiocin 386

δ 1.18 (s, 3H-6″), 1.36 (s, 3H-7″), 2.19 (s, 3H-7), 2.29 (s, 3H-6‴), 2.34 (s, 3H-8′), 3.52 (s, 3H-8″), 3.71 (d, J = 9.8 Hz, H-4″), 4.29 (t, J = 1.4 Hz,

H-2″), 5.62 (d, J = 1.4 Hz, H-1″), 5.67 (dd, J_1 = 9.8 Hz, J_2 = 2.8 Hz, H-3″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.72 (d, J = 9.2 Hz, H-5), 6.90 (d, J = 3.4 Hz, H-3‴), 7.22 (d, J = 9.4 Hz, H-6′), 7.67 (d, J = 9.2 Hz, H-6), 7.70 (br s, H-2), 7.83 (d, J = 9.4 Hz, H-5′).

MS: 622.3 $[M+H]^+$, 341.2 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 134.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.48 mg after the second feeding step.

Novclobiocin 387

δ 1.19 (s, 3H-6″), 1.41 (s, 3H-7″), 2.19 (s, 3H-7), 2.28 (s, 3H-8′), 2.29 (s, 3H-6‴), 3.51 (d, J = 8.8 Hz, H-4″), 3.63 (s, 3H-8″), 4.41 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, H-3″), 5.37 (t, J = 3.2 Hz, H-2″), 5.69 (d, J = 2.4 Hz, H-1″), 5.94 (d, J = 3.8 Hz, H-4‴), 6.71 (d, J = 8.4 Hz, H-5), 6.88 (d, J = 3.8 Hz, H-3‴), 7.16 (d, J = 8.8 Hz, H-6′), 7.66 (d, J = 8.4 Hz, H-6), 7.70 (br s, H-2), 7.81 (d, J = 8.8 Hz, H-5′).

MS: 622.3 $[M+H]^+$, 341.1 [M-(sugar+pyrrole unit)]$^+$, 282.2 [sugar+pyrrole unit]$^+$, 134.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.24 mg after the second feeding step.

Novclobiocin 601

δ 1.20 (s, 3H-6″), 1.35 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.52 (s, 3H-8″), 3.72 (d, J = 10.0 Hz, H-4″), 4.33 (br s, H-2″), 5.71 (overlapping signals, J is not determinable, H-1″ and H-3″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.90 (d, J = 3.4 Hz, H-3‴), 7.28 (d, J = 8.8 Hz, H-6′), 7.48 (t, J = 7.2 Hz, H-3 and H-5), 7.57 (t, J = 6.8 Hz, H-4), 7.91 (d, J = 8.8 Hz, H-5′), 8.02 (d, J = 7.2 Hz, H-2 and H-6).

MS: 613.2 ([M+H]$^+$, Cl^{35}), 615.3 ([M+H]$^+$, Cl^{37}), 332.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 105.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.36 mg.

Novclobiocin 602

δ 1.18 (s, 3H-6″), 1.38 (s, 3H-7″), 2.30 (s, 3H-6‴), 3.56 (d, J = 9.4 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.4 Hz, J_2 = 3.2 Hz, H-3″), 5.40 (t, J = 2.4 Hz, H-2″), 5.82 (d, J = 1.6, H-1″), 5.95 (d, J = 3.4 Hz, H-4‴), 6.89 (d, J = 3.4 Hz, H-3‴), 7.30 (d, J = 9.0 Hz, H-6′), 7.50 (t, J = 7.4 Hz, H-3 and H-5), 7.58 (t, J = 7.4 Hz, H-4), 7.89 (d, J = 9.0 Hz, H-5′), 8.02 (d, J = 7.4 Hz, H-2 and H-6).

MS: 613.2 ([M+H]$^+$, Cl^{35}), 615.4 ([M+H]$^+$, Cl^{37}), 282.2 [sugar+pyrrole unit]$^+$, 104.9 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 1.07 mg.

Novclobiocin 603

δ 1.20 (s, 3H-6″), 1.37 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.69 (d, J = 9.6 Hz, H-4″), 4.24 (br s, H-2″), 5.59 (dd, J_1 = 9.6 Hz, J_2 = 2.8 Hz, H-3″), 5.64 (br s, H-1″), 5.94 (d, J = 3.0 Hz, H-4‴), 6.90 (d, J = 3.0 Hz, H-3‴), 7.07 (br s, H-6′ and H-8′), 7.51 (t, J = 7.0 Hz, H-3 and H-5), 7.59 (t, J = 7.0 Hz, H-4), 7.93 (d, J = 9.2 Hz, H-5′), 8.02 (d, J = 7.0 Hz, H-2 and H-6).

MS: 579.2 $[M+H]^+$, 282.1 [sugar+pyrrole unit]$^+$, 105.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.74 mg.

Novclobiocin 604

δ 1.20 (s, 3H-6″), 1.36 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.34 (s, 3H-8′), 3.52 (s, 3H-8″), 3.70 (d, J = 9.8 Hz, H-4″), 4.27 (t, J = 3.2 Hz, H-2″), 5.60 (d, J = 1.6 Hz, H-1″), 5.68 (dd, J_1 = 9.8 Hz, J_2 = 2.8 Hz, H-3″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.90 (d, J = 3.4 Hz, H-3‴), 7.14 (d, J = 9.0 Hz, H-6′), 7.46 (t, J = 7.4 Hz, H-3 and H-5), 7.52 (t, J = 7.4 Hz, H-4), 7.84 (d, J = 9.0 Hz, H-5′), 8.02 (d, J = 7.4 Hz, H-2 and H-6).

MS: 593.2 $[M+H]^+$, 312.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 105.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.07 mg after the second feeding step.

Novclobiocin 701

δ 1.17 (s, 3H-6″), 1.36 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.72 (d, J = 9.8 Hz, H-4″), 4.34 (br s, H-2″), 5.68 (dd, J_1 = 9.8 Hz, J_2 = 2.4 Hz, H-3″), 5.71 (br s, H-1″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.90 (d, J = 3.4 Hz, H-3‴), 7.01 (d, J = 15.3 Hz, H-1), 7.29 (d, J = 8.2 Hz, H-6′), 7.39 (m, overlapping signals, H-5, H-6, and H-7), 7.60 (d, J = 6.8 Hz, H-4 and H-8), 7.71 (d, J = 15.3 Hz, H-2), 7.89 (d, J = 8.2 Hz, H-5′).

MS: 639.5 ([M+H]$^+$, Cl^{35}), 641.6 ([M+H]$^+$, Cl^{37}), 357.9 [M-(sugar+pyrrole unit)]$^+$, 282.0 [sugar+pyrrole unit]$^+$, 131.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 4.0 mg. The yield obtained by the two-stage feeding procedure was 0.18 mg after the second feeding step.

Novclobiocin 702

δ 1.17 (s, 3H-6″), 1.38 (s, 3H-7″), 2.30 (s, 3H-6‴), 3.64 (s, 3H-8″), 3.56 (d, J = 9.6 Hz, H-4″), 5.39 (br s, H-2″), 4.44 (dd, J_1 = 9.6 Hz, J_2 = 3.3 Hz, H-3″), 5.79 (br s, H-1″), 5.95 (d, J = 3.5 Hz, H-4‴), 6.89 (d, J = 3.5 Hz, H-3‴), 6.98 (d, J = 16.4 Hz, H-1), 7.27 (d, J = 8.5 Hz, H-6′), 7.39 (t, overlapping signals, J = 7.1 Hz, H-5, H-6, and H-7), 7.62 (d J = 7.1 Hz, H-4 and H-8), 7.69 (dd, J_1 = 16.4 Hz, J_2 = 1.3 Hz,, H-2), 7.89 (d, J = 8.5 Hz, H-5′).

MS: 639.2 ([M+H]$^+$, Cl^{35}), 642.2 ([M+H]$^+$, Cl^{37}), 282.2 [sugar+pyrrole unit]$^+$, 131.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.67 mg.

Novclobiocin 703

δ 1.19 (s, 3H-6″), 1.36 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.33 (s, 3H-8′), 3.52 (s, 3H-8″), 3.70 (d, J = 9.8 Hz, H-4″), 4.27 (t, J = 2.2 Hz, H-2″), 5.59 (d, J = 2.2 Hz, H-1″), 5.68 (dd, J_1 = 9.8 Hz, J_2 = 2.8 Hz, H-3″), 5.94 (d, J = 3.8 Hz, H-4‴), 6.89 (d, J = 14.0 Hz, H-1), 6.90 (d, J = 3.8 Hz, H-3‴), 7.14 (d, J = 8.0 Hz, H-6′), 7.38 (t, overlapping signals, J = 7.4 Hz, H-5, H-6, and H-7), 7.60 (d, J = 7.4 Hz, H-4 and H-8), 7.62 (d, J = 14.0 Hz, H-2), 7.83 (d, J = 8.0 Hz, H-5′).

MS: 619.3 $[M+H]^+$, 338.2 [M-(sugar+pyrrole unit)]$^+$, 282.2 [sugar+pyrrole unit]$^+$, 131.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 0.15 mg after the second feeding step.

Novclobiocin 731

δ 1.19 (s, 3H-6″), 1.35 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.71 (d, J = 11.6 Hz, H-4″), 3.90 (s, 3H-9), 4.33 (br s, H-2″), 5.69 (br s, H-1″), 5.71 (dd, J_1 = 11.6 Hz, J_2 = 2.0 Hz, H-3″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.75 (d, J = 15.8 Hz, H-1), 6.80 (d, J = 8.2 Hz, H-7), 6.90 (d, J = 3.6 Hz, H-3‴), 7.07 (d, J = 8.2 Hz, H-8), 7.19 (br s, H-4), 7.23 (d, J = 8.8 Hz, H-6′), 7.57 (d, J = 15.8 Hz, H-2), 7.90 (d, J = 8.8 Hz, H-5′).

MS: 685.3 ([M+H]$^+$, Cl^{35}), 687.1 ([M+H]$^+$, Cl^{37}), 404.1 [M-(sugar+pyrrole unit)]$^+$, 282.1 [sugar+pyrrole unit]$^+$, 177.0 [Ring A analog]$^+$, 108.0 [C_6H_6NO = pyrrole unit]$^+$.

The total yield of the compound was 1.1 mg.

Determination of Minimum Inhibitory Concentrations

The broth microdilution procedure recommended by the National Committee for Clinical Laboratory Standards [30] was used for the determination of the minimum inhibitory concentrations (MICs). All test strains were obtained from the bacterial collection of Basilea Pharmaceutica AG (Basel, Switzerland). The MICs were determined as described previously [16]. For testing, the aminocoumarins were dissolved in DMSO. The maximum final concentration of DMSO in the assays was 2% (v/v).

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