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In Vitro and In Vivo Production of New Aminocoumarins by a Combined Biochemical, Genetic, and Synthetic Approach

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Summary

The aminocoumarin antibiotics clorobiocin, novobiocin, and coumermycin A_1 are inhibitors of bacterial gyrase. Their chemical structures contain amide bonds, formed between an aminocoumarin ring and an aromatic acyl component, which is 3-dimethylallyl-4-hydroxybenzoate in the case of novobiocin and clorobiocin. These amide bonds are formed under catalysis of the gene products of *cloL*, *novL*, and *couL*, respectively. We first examined the substrate specificity of the purified amide synthetases CloL, NovL, and CouL for the various analogs of the prenylated benzoate moiety. We then generated new aminocoumarin antibiotics by feeding synthetic analogs of the 3-dimethylallyl-4-hydroxybenzoate moiety to a mutant strain defective in the biosynthesis of the prenylated benzoate moiety. This resulted in the formation of 32 new aminocoumarin compounds. The structures of these compounds were elucidated using FAB-MS and ^{1}H-NMR spectroscopy.

Introduction

The aminocoumarin antibiotics clorobiocin, novobiocin, and coumermycin A_1 (Figure 1) are potent inhibitors of DNA gyrase produced by different *Streptomyces* sp. strains [1–3]. Novobiocin (Albamycin, Pharmacia & Upjohn) has been licensed in the USA for the treatment of human infections with gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Its efficacy has been demonstrated in preclinical and clinical trials [4–6]. However, due to their toxicity in eukaryotes, their poor water solubility, and their low activity against gram-negative bacteria, clinical use of these antibiotics remains restricted [2]. Therefore, it is of interest to test whether new, structurally modified aminocoumarin antibiotics may overcome the limitations of the known compounds [1, 7].

The characteristic structural moiety of the aminocoumarin antibiotics is a 3-amino-4,7-dihydroxycoumarin unit (= ring B) that is attached to the deoxysugar noviose (= ring C). X-ray crystallographic examinations [2, 8–10] have demonstrated that the aminocoumarin moiety and the substituted deoxysugar moiety are essential for the binding of these compounds to the B subunit of gyrase. The prenylated 4-hydroxybenzoate moiety (= ring A) of novobiocin and clorobiocin is important for the uptake of these antibiotics by bacteria [8, 10] and contributes weakly to the binding of the compounds to bacterial gyrase [8–10]. Biosynthetically, the 3-dimethylallyl-4-hydroxybenzoic acid moiety of clorobiocin and novobiocin is derived from 4-hydroxyphenylpyruvate and an isoprenoid precursor [11–14].

Our group has cloned and sequenced the biosynthetic gene clusters of clorobiocin (*clo*) [15], novobiocin (*nov*) [16], and coumermycin A_1 (*cou*) [17]. The aminocoumarin moiety (ring B) of all three substances is linked to the acyl component (ring A) via amide bonds, and correspondingly all three gene clusters contain an amide synthetase gene, i.e., *cloL*, *novL*, and *couL*, respectively.

The aim of the present study was the generation of new aminocoumarins by feeding of synthetic analogs of the prenylated 4-hydroxybenzoate moiety (= ring A) to ring A defective mutants of the aminocoumarin antibiotic producers. To be incorporated into aminocoumarin antibiotics, these ring A analogs need first to be accepted by the amide synthetases of the producers, i.e., by CloL, NovL, and CouL, respectively. NovL was shown previously to activate the acyl component (ring A) by adenylate formation and to transfer it onto the amino group of the aminocoumarin ring without involvement of a 4-phosphopantetheinyl cofactor [18]. In order to identify the most promising compounds as well as the most suitable organism for the feeding experiments, we overexpressed and purified the three amide synthetases and tested which ring A analogs were accepted by them. CloL was found to accept the most suitable range of compounds, and therefore the clorobiocin producer was chosen for subsequent mutasynthesis studies.

Results and Discussion

Overexpression and Purification of the Amide Synthetases CloL, NovL, and CouL

For overexpression of the three amide synthetases in *Escherichia coli* (see Experimental Procedures), the genes *novL*, *cloL*, and *couL* were cloned into the expression vector pQE70, resulting in the plasmids pMS80 [18], pUG018, and pMS90, respectively. The C-terminal His_6-tagged fusion proteins CloL, NovL, and CouL were purified by nickel affinity chromatography as described in [18], yielding proteins of near homogeneity (Figure 2). Purified NovL showed a specific activity of 14.5 nkat/mg protein and CloL 4.2 nkat/mg protein, both with 3-dimethylallyl-4-hydroxybenzoate (ring A) as substrate (1 nkat catalyzes a product formation of 1 nmol per second; product formation was assayed by HPLC). CouL showed a specific activity of 7.4 nkat/mg protein using its genuine acyl substrate 3-methylpyrrole-2,4-dicar-

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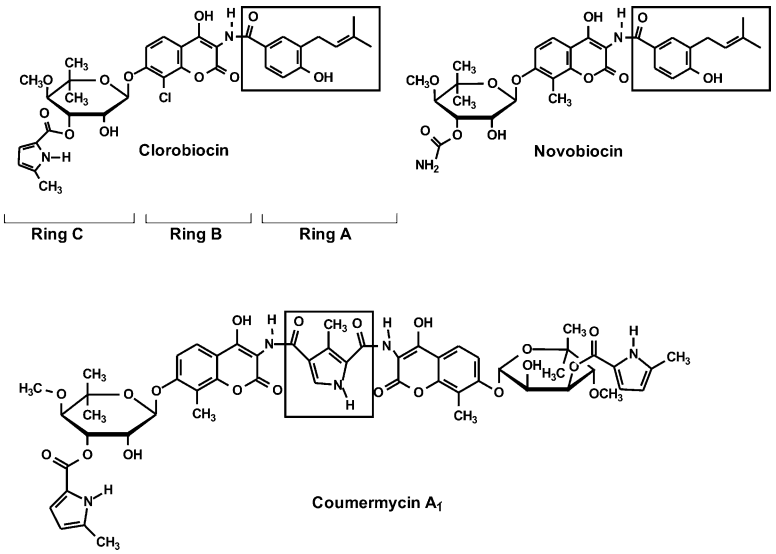


Figure 1. Structures of the Aminocoumarin Antibiotics Clorobiocin, Novobiocin, and Coumermycin A_1

The acyl components involved in amide bond formation are indicated by boxes.

boxylic acid (= 3-MePy-2,4-COOH) as substrate. All amide synthetase activities were tested using the aminocoumarin moiety (ring B) of novobiocin, ATP, and the respective acyl component as substrates.

Substrate Specificity of CloL, NovL, and CouL

To compare the substrate specificities of CloL, NovL, and CouL for different acyl components, the genuine substrates (ring A and 3-MePy-2,4-COOH) and 22 synthetic ring A analogs (see Table 1 for structures) were used in the amide synthetase assay. Formation of the resulting amides was observed by HPLC. Figure 3 shows the product formation expressed as relative activities in comparison to the product formation with the respective genuine substrate.

Ring A (= 3-dimethylallyl-4-hydroxybenzoic acid) is the genuine substrate of CloL and NovL but was also very well accepted by CouL (Figure 3). In contrast, 3-MePy-2,4-COOH, the genuine substrate of CouL, was not accepted by CloL and NovL. 4-hydroxybenzoic acid was accepted by the amide synthetases CloL and CouL and to a lesser extent by NovL (see Figure 3). The presence of a linear alkyl side chain at C-3 of 4-hydroxybenzoic acid (in RAA 200, 210, 220, and 300) increased the reaction velocity of the CloL and NovL reactions. In contrast, a cyclohexyl substituent in the same position (RAA 520) led to complete loss of activity. Compounds containing a hydroxy, keto, or ether function in the side chain (RAA 530, 510, 230) were poor substrates. On the other hand, compounds with an amide bond in the side chain were well accepted, especially by CloL, if they contained a sufficiently large *N*-alkyl side chain (RAA 240, 250, and 260). 3-halogenated 4-hydroxybenzoic acids (RAA 270, 280) were accepted by all three amide synthetases. Interestingly, 3,5-dibromo-4-hydroxybenzoic acid (RAA 290) was very well accepted by CouL but not by CloL and NovL. A similar observation was made for 3,5-dimethyl-4-hydroxybenzoic acid (RAA 200). Of the 4-aminobenzoic acid derivatives (RAA 560, 380, 570, and 390), only the 3-methyl compound RAA 380 was accepted by CloL and CouL.

The structure of all enzymatically formed amides shown in Figure 3 was confirmed by LC-MS- and LC-MS/MS experiments (see Experimental Procedures). Although the three amide synthetases CloL, NovL, and CouL show 80%–86% identity on amino acid level and differ only by two amino acids in size, they showed marked differences in their substrate specificity. CloL readily accepted many of the synthetic substrates. Therefore, the clorobiocin producer (*Streptomyces roseochromogenes*) was chosen for the subsequent feeding experiments. This was also advantageous since clorobiocin has been shown to be an especially potent inhibitor of DNA gyrase, six times more active than novobiocin [19].

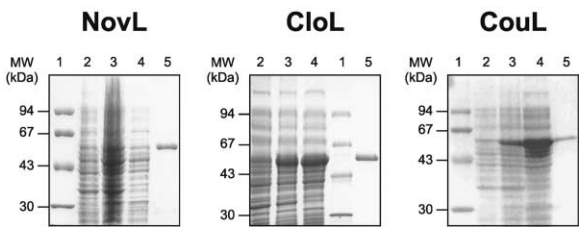


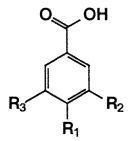
Figure 2. Purification of the Amide Synthetases NovL, CloL, and CouL after Overexpression as a Fusion Protein with a C-terminal His_6 Tag in *E. coli* XL1 Blue MRF′

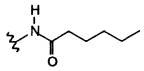
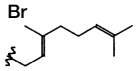
SDS-PAGE was carried out as described in Experimental Procedures. Lanes 1, molecular weight (MW) marker; lanes 2, total protein before induction; lanes 3, total protein after IPTG induction; lanes 4, soluble protein after IPTG induction; lanes 5, eluate from Ni^{2+} affinity chromatography.

Mutasynthesis Experiments: Feeding of Ring A Analogs to a *cloQ*$^-$ Mutant

For production of novel aminocoumarin antibiotics by feeding of ring A analogs, we decided to use a mutant strain of the clorobiocin producer, defective in biosynthesis of ring A. The gene *cloQ*, encoding a prenyltransferase essential for the biosynthesis of 3-dimethylallyl-4-hydroxybenzoic acid (ring A) in *S. roseochromogenes*,

Table 1. RAAs Used in This Study

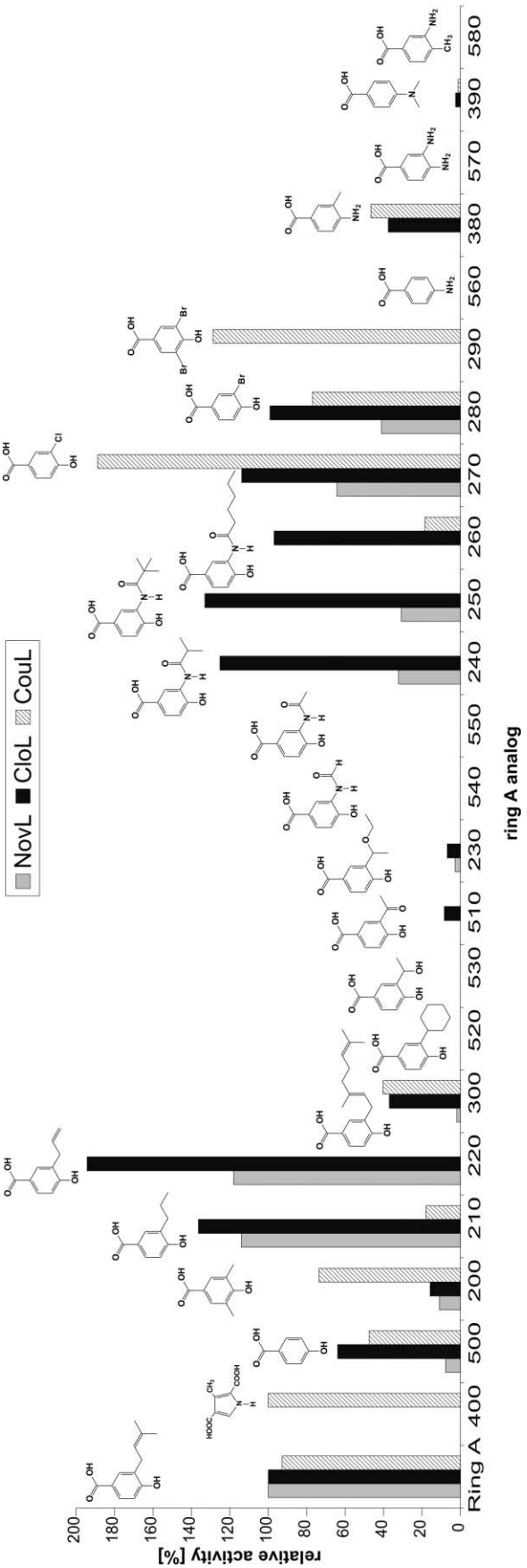


Compound Name	R1	R2	R3	Reference or Source
ring A	OH		H	[27]
RAA 200	OH	CH_3	CH_3	Lancaster
RAA 210	OH		H	[28]
RAA 220	OH		H	[28]
RAA 230	OH		H	[28]
RAA 240	OH		H	[28]
RAA 250	OH		H	[28]
RAA 260	OH		H	[28]
RAA 270	OH	Cl	H	[28]
RAA 280	OH	Br	H	[28]
RAA 290	OH	Br	Br	Aldrich
RAA 300	OH		H	[28]
RAA 380	NH_2	CH_3	H	Sigma
RAA 390		H	H	Fluka
RAA 500	OH	H	H	Fluka
RAA 510	OH		H	[28]
RAA 520	OH		H	[28]
RAA 530	OH		H	[28]
RAA 540	OH		H	[28]
RAA 550	OH		H	[28]
RAA 560	NH_2	H	H	Merck
RAA 570	NH_2	NH_2	H	Fluka
RAA 580	CH_3	NH_2	H	Fluka

was inactivated by in-frame deletion [14], and the resulting *cloQ*$^-$ mutant was used for mutasynthesis experiments.

Feeding experiments were performed with those ring A analogs that had been accepted by CloL in vitro (with the exception of RAA 300, which was not available in sufficient amount). In parallel control cultures, *S. roseochromogenes* wild-type produced 15 μg/ml clorobiocin, while the *cloQ*$^-$ mutant produced no clorobiocin. In the feeding experiments, usually 10 mg of the respective ring A analog were added to 500 ml of the culture medium of the *cloQ*$^-$ mutant, 2 days after inoculation. 5–8 days later, the cultures were extracted and the culture extracts were analyzed by HPLC. In total, 13 different ring A analogs were fed. Nine of these experiments led to the formation of new aminocoumarins, which were isolated on a preparative scale using a Sephadex LH 20 column and HPLC. Their structures (Table 2) were identified by negative-ion FAB-MS analysis and by ^{1}H-NMR (see Experimental Procedures).

In each of the nine successful feeding experiments, the expected direct clorobiocin analog could be isolated, i.e., a compound that contained the externally added ring A analog but was otherwise identical to clorobiocin in structure. All newly isolated compounds were called novclobiocins, as in previous studies by our group [20–22]. The isolated compounds were designated as novclobiocins 211, 221, etc. (Table 2), corresponding to the numbering of the ring A analogs (Table 1). In addition to these direct clorobiocin analogs, another series of compounds was isolated (designated novclobiocin 212, 222, etc.), which showed slightly longer retention times



in HPLC. The ^{1}H-NMR data showed that in these "isoclorobiocins" the 5-methylpyrrole-2-carboxylic acid was attached to the 2-OH instead of the 3-OH of the deoxysugar. This was demonstrated by a shift of the signal of the proton at C-2″ from 4.34 ppm in the clorobiocin derivative to 5.39 ppm in the isoclorobiocin derivative and of the signal of the proton at C-3″ from 5.71 ppm to 4.44 ppm. The molecular weight of the isoclorobiocins was confirmed by FAB-MS. The natural occurrence of a similar isomer of novobiocin has been reported previously [23], showing similar ^{1}H-NMR data.

In five feeding experiments, compounds with slightly shorter HPLC retention times than the direct clorobiocin analogs were observed and identified as "desclorobiocins." FAB-MS analysis showed that their molecular mass was 34 Da smaller than that of the parent compound, consistent with the loss of a chlorine atom. While the mass spectra of the clorobiocin and isoclorobiocin compounds showed the typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl, the latter substances did not show this pattern, confirming the absence of chlorine. ^{1}H-NMR data unequivocally proved that the new substances carried a hydrogen instead of a chlorine atom at C-8′ of ring B. The signal at 7.20–7.29 ppm corresponding to H-6′ of the clorobiocin derivative was missing. Instead, a signal at 7.01–7.04 ppm for two protons was observed as a broad singlet, which could be assigned to H-6′ and H-8′. The coincidence of the signals of H-6′ and H-8′ as a broad singlet has been observed previously from a naturally occurring novobiocin derivative lacking the 8′-methyl group [24]. These new desclorobiocin derivatives were named novclobiocin 214, 233, 243, 253, and 383.

From two feeding experiments, compounds completely lacking the 5-methylpyrrole-2-carboxylic acid moiety were isolated. This was obvious from their molecular mass, reduced by 107 compared to the respective clorobiocin derivative, and from the signals in the ^{1}H-NMR spectra. These two substances were designated novclobiocin 213 and 283.

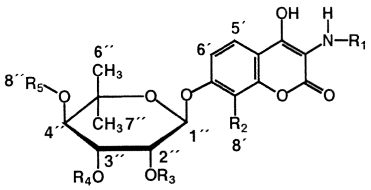
Feeding of 3-methyl-4-aminobenzoic acid (RAA 380) resulted in similar clorobiocin, isoclorobiocin, and desclorobiocin derivatives, but also in two further compounds. One of these substances lacked the chlorine atom at position 8 of the aminocoumarin moiety and at the same time carried the 5-methylpyrrole-2-carboxylic acid at 2″-OH instead of 3″-OH, as could be deduced from the ^{1}H-NMR data. This compound was named novclobiocin 384. The second compound contained both the chlorine and the 3″-acyl group, but its ^{1}H-NMR spectrum lacked the signal at 3.51 ppm. This showed the absence of a *O*-methyl group at position 4 of the deoxysugar. The molecular mass was confirmed by FAB-MS. This substance was designated novclobiocin 385.

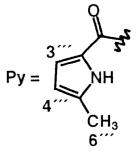
In several feeding experiments, aminocoumarin aglycons, i.e., compounds lacking the deoxysugar moiety, could be found; their structures were confirmed by mass

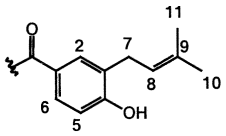
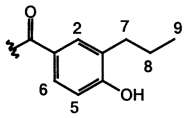
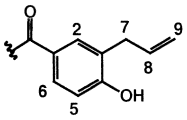
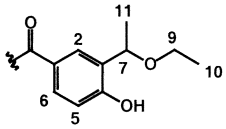
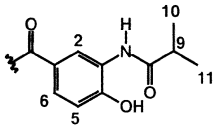
Figure 3. Relative Activities of the Amide Synthetases NovL, CloL, and CouL with Different Carboxylic Acids

Specific activities with the genuine substrates of the enzymes were set as 100%.

Table 2. Chemical Structures of the Novel Aminocoumarins Derived from Mutasynthesis Experiments





Compound Name	R1	R2	R3	R4	R5
novobiocin		CH_3	H	$CONH_2$	CH_3
clorobiocin		Cl	H	Py	CH_3
novclobiocin 211		Cl	H	Py	CH_3
novclobiocin 212		Cl	Py	H	CH_3
novclobiocin 213		Cl	H	H	CH_3
novclobiocin 214		H	H	Py	CH_3
novclobiocic acid 215[a]		Cl	-	-	-
novclobiocic acid 216[a]		H	-	-	-
novclobiocin 221		Cl	H	Py	CH_3
novclobiocin 222		Cl	Py	H	CH_3
novclobiocic acid 223[a]		Cl	-	-	-
novclobiocic acid 224[a]		H	-	-	-
novclobiocin 231		Cl	H	Py	CH_3
novclobiocin 232		Cl	Py	H	CH_3
novclobiocin 233		H	H	Py	CH_3
novclobiocin 241		Cl	H	Py	CH_3
novclobiocin 242		Cl	Py	H	CH_3
novclobiocin 243		H	H	Py	CH_3
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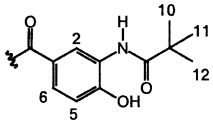
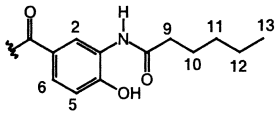
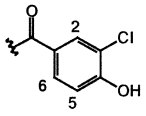
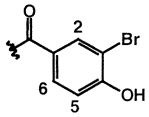
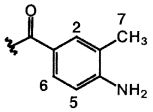
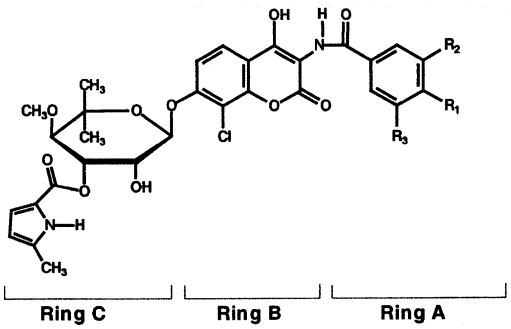
novclobiocin 252		Cl	Py	H	CH_3
novclobiocin 253		H	H	Py	CH_3
novclobiocin 261		Cl	H	Py	CH_3
novclobiocin 262		Cl	Py	H	CH_3
novclobiocic acid 265[a]		H	-	-	-
novclobiocin 271		Cl	H	Py	CH_3
novclobiocin 272		Cl	Py	H	CH_3
novclobiocin 281		Cl	H	Py	CH_3
novclobiocin 282		Cl	Py	H	CH_3
novclobiocin 283		Cl	H	H	CH_3
novclobiocin 381		Cl	H	Py	CH_3
novclobiocin 382		Cl	Py	H	CH_3
novclobiocin 383		H	H	Py	CH_3
novclobiocin 384		H	Py	H	CH_3
novclobiocin 385		Cl	H	Py	H

[a] The novclobiocic acids lack the entire deoxysugar moiety.

Table 3. Negative Ion FAB Mass Spectral Data of the Isolated Aminocoumarins



Compound	MW	Negative Ion FAB-MS (*m/z*)			
		[M-H]$^-$	[M-Ring C]$^-$	Ring A Analog	Ring B
novclobiocin 211	670	669/671	388	162	225
novclobiocin 212	670	669/671	n.d.	162	n.d.
novclobiocin 213	563	562	n.d.	162	n.d.
novclobiocin 214	636	635	354	162	191
novclobiocic acid 215	389	388/390	-	162	n.d.
novclobiocic acid 216	355	354	-	162	191
novclobiocin 221	668	667/669	386/388	n.d.	225
novclobiocin 222	668	667/669	386	n.d.	225
novclobiocic acid 223	387	386/388	-	n.d.	n.d.
novclobiocic acid 224	353	352	-	n.d.	n.d.
novclobiocin 231	700	699/701	418	n.d.	225
novclobiocin 232	700	699/701	418	n.d.	225
novclobiocin 233	666	665	384	192	191
novclobiocin 241	713	712/714	431/433	205	225
novclobiocin 242	713	712/714	431	205	225
novclobiocin 243	679	678	397	205	191
novclobiocin 251	727	726/728	445	n.d.	225
novclobiocin 252	727	726/728	445	219	225
novclobiocin 253	693	692	411	219	191
novclobiocin 261	741	740/742	459/461	233	225
novclobiocin 262	741	740/742	459	233	225
novclobiocic acid 265	426	425	-	233	191
novclobiocin 271	662	661/663/665	380	n.d.	225
novclobiocin 272	662	661/663/665	n.d.	n.d.	225
novclobiocin 281	706	705/707/709	424	n.d.	225
novclobiocin 282	706	705/707/709	424	198	225
novclobiocin 283	599	598/600/602	n.d.	198	225
novclobiocin 381	641	640/642	359/361	n.d.	225
novclobiocin 382	641	640/642	359/361	n.d.	225
novclobiocin 383	607	606	325	n.d.	191
novclobiocin 384	607	606	325	n.d.	191
novclobiocin 385	627	n.d.	n.d.	n.d.	n.d.

Symbols and abbreviations: x/y, two or more signals resulting from chlorine and/or bromine isotopes contained in the molecule; n.d., not detectable.

spectrometric and ^{1}H-NMR data. In analogy to novobiocic acid, a precursor of novobiocin, these compounds were called novclobiocic acids, and their structures are described in Table 3.

In the nine successful mutasynthetic feeding experiments, we could isolate on average 1.7 mg of pure clorobiocin derivative (range: 0.7–2.9 mg), 0.7 mg of pure isoclorobiocin derivative, and 0.1 mg of pure desclorobiocin derivative after feeding of 10 mg of the respective ring A analog. There was a statistically significant correlation between the in vitro conversion of the ring A analogs by CloL (Figure 3) and the total yield of new aminocoumarins obtained in all 13 feeding experiments (correlation coefficient $r = 0.649$; $p = 0.0164$). For example, feeding of RAA 200, 390, and 510, which had been poor substrates of CloL in vitro, did not result in the formation of detectable amounts of new aminocoumarin antibiotics. An exception was 4-hydroxybenzoate (RAA 500), which was well accepted in vitro but did not lead to the formation of new aminocoumarins in vivo.

In contrast, feeding of RAA 210, 220, 230, 240, 250, 260, 270, 280, and 380 resulted in the isolation of a total of 32 novel aminocoumarins. Compared to former mutasynthetic experiments, e.g., by Ankenbauer et al., who fed 13 different precursors and succeeded in the isolation of 3 new compounds [25], and by Dutton et al., who fed >800 precursor substrates and succeeded in the isolation of 36 new compounds [26], this is a very

successful outcome and demonstrates the usefulness of mutasynthetic approaches for the generation of new aminocoumarin derivatives.

Significance

This study describes the mutasynthetic generation, isolation, and structure elucidation of novel aminocoumarin antibiotics. In the past, mutasynthesis experiments often yielded only small amounts of compounds, being of limited use for further evaluation. Therefore, the present study started with the overexpression of key biosynthetic enzymes, i.e., the amide synthetases CloL, NovL, and CouL, from different strains and the investigation of their substrate specificity in vitro. These experiments revealed CloL as a very promiscuous enzyme, which is why the clorobiocin producer was selected for mutasynthesis.

The incorporation of various precursors by the recombinant amide synthetase CloL was compared in vitro and could then be recapitulated by in vivo feeding experiments. There was a statistically significant correlation between the enzymatic in vitro conversion of precursor analogs by CloL and the subsequent yields of new aminocoumarins obtained from feeding experiments in vivo. Therefore, in vitro investigations of the substrate specificity of the respective enzymes may provide useful data to predict the success of subsequent mutasynthetic experiments.

Following this strategy, a whole set of pure, novel aminocoumarins in often substantial quantities could be obtained. The number of substances generated was unusually high: feeding of the 13 substances that had been accepted by CloL in vitro to a mutant of the clorobiocin producer *S. roseochromogenes* resulted in the formation of 32 new aminocoumarin derivatives. This sets the stage for the generation of further derivatives with improved pharmacological properties and is an example of the powerful potential of mutasynthesis to generate novel chemotypes of natural-product-derived antimicrobials. The present success in generating new compounds may reinvigorate similar efforts with other antimicrobial natural products.

Experimental Procedures

Chemicals

Ring B and novobiocic acid were kindly provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI). Ring A was obtained by hydrolysis of novobiocin as described previously [27]. Commercially available ring A analogs (RAAs) were purchased from Aldrich, Fluka, Lancaster, Merck, and Sigma. Additional substituted benzoic acids were synthesized as described by M.A. Dessoy [28]. The carboxylic acids used in the amide synthetase assay are listed in Table 1.

Construction of Plasmids pMS80, pUG018, and pMS90 for Expression of NovL, CloL, and CouL as a C-Terminal His_6-Tag Fusion Protein

pMS80 for overexpression of NovL has been described previously [18].

cloL was amplified by PCR using pCloLM (F. Pojer, personal communication) as template. An SphI site was introduced at the place of the natural start codon using primer cloL_P03f (5′-TCAC**GCATG**CCGAACAAGGACCAC-3′, bold letters represent the SphI site). At the C terminus, a BglII site was introduced before the stop codon using primer cloL_P02r (5′-CTC**AGATCT**CCTGTCCACCAGCAC-3′, bold letters represent the BglII site). The PCR mixture (100 μl) contained 100 ng of pCloLM template, 20 pmol of each primer, 0.2 mM dNTPs (Stratagene), *Pfu* DNA polymerase reaction buffer, 5% (v/v) DMSO, and 3 units of cloned *Pfu* DNA polymerase (Stratagene). The PCR product was digested with SphI and BglII before ligation into the same sites of the expression vector pQE70 (Qiagen), resulting in a C-terminal in-frame fusion with the His_6 tag of pQE70. The resulting plasmid was designated as pUG018.

pMS90 for overexpression of CouL was constructed in the same way using primers couL-1 (5′-TAGTCACT**GCATGC**CGAACAGG GAC-3′, bold letters represent the SphI site) and couL-2 (5′-TGACT C**AGATCT**CCTGTCCACCAG-3′, bold letters represent the BglII site) and was kindly provided by E. Schmutz from our group [29].

Expression and Purification of the Amide Synthetases NovL, CloL, and CouL

Escherichia coli XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) was used as host for the expression of the amide synthetases NovL, CloL, and CouL from the plasmids pMS80 [18], pMS90, and pUG018, respectively. Expression and purification procedures using Ni-NTA-agarose were carried out as described previously [18]. Protein concentrations were determined by the Bradford method [30] using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli [31], and protein bands were stained with Coomassie Brilliant Blue R 250.

Amide Synthetase Assay

The amide synthetase assay contained 1 mM ring A or ring A analog, 1 mM ring B of novobiocin (bearing a methyl group at C-8), 5 mM ATP, 5 mM $MnCl_2$, 50 mM Tris-HCl (pH 8.0), and 0.5–2 μg of the respective protein in a final volume of 100 μl. The reaction was carried out for 30 min at 30°C and stopped by addition of 5 μl 1.5 M trichloroacetic acid. The reaction mixture was extracted with ethyl acetate and analyzed using HPLC as described elsewhere [18].

Structure Elucidation of Enzymatic Products

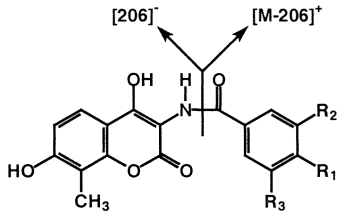
The positive and negative ion electrospray (ES) mass spectra were obtained using a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV, heated capillary temperature 220°C, sheath and auxillary gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1 × 100 mm, SepServ, Berlin). UV detection was at 305 nm. A gradient 10% to 90% MeOH in 0.2% aqueous acetic acid was used; flow rate 70 μl min^{-1}. Mass spectra were averaged and background was subtracted. The collision-induced dissociation (CID) spectra of the $[M-H]^-$ and $[M+H]^+$ ions, respectively, during a HPLC run were recorded with a collision energy of −20 eV for positive ions as well as +25 eV for negative ions, respectively; collision gas: argon, collision pressure: 1.8×10^{-3} Torr.

The key ions obtained by the CID spectra are listed in Table 4. The negative ion CID spectra show the aminocoumarin moiety (*m/z* 206), and the positive ion ones show the complementary part ($[M-206]^+$).

Production and Analysis of New Aminocoumarins from Mutasynthesis Experiments

Streptomyces roseochromogenes var. *oscitans* DS 12.976 was kindly provided by Aventis and was routinely cultured in baffled Erlenmeyer flasks containing a stainless steel spring. For the production of clorobiocin and novclobiocins, wild-type *S. roseochromogenes* and the *cloQ*$^-$ mutant defective in ring A biosynthesis [14] were first precultured in YMG liquid medium containing 1% malt extract, 0.4% yeast extract, 0.4% glucose (pH 7.3) at 30°C and 180 rpm for 2 to 3 days. Cells (1 ml culture) were then precultured in 50 ml cornstarch medium (1% cornstarch (Becton-Dickinson, Heidelberg, Germany), 1% peptone (Roth, Karlsruhe, Germany), 0.5% meat extract (Merck, Darmstadt, Germany) (pH 7.0) for 2 days at 33°C and 210 rpm. Five milliliters of this preculture was inoculated into 50 ml of production medium adapted from [32], prepared from 4.8% distillers' solubles (Sigma-Aldrich, Deisenhofen, Germany), 3.7% glucose, 0.0024% $CoCl_2 \times 6\ H_2O$ (at this point, the pH of the mixture was adjusted to 7.8), 0.6% $CaCO_3$, and 0.2% $(NH_4)_2SO_4$. Two days after inoculation of the production medium, 1 mg of ring A or of the

Table 4. Enzymatic Products Monitored by Full Scan CID Spectra



Compound	MW	Key Ions in the Full Scan CID Spectra of the $[M-H]^-$ and $[M+H]^+$ Ions (*m/z*), Respectively		
		$[M-H]^-$	$[206]^-$	$[M-206]^+$
novclobiocic acid 200	355	354	+	149
novclobiocic acid 210	369	368	+	163
novclobiocic acid 220	367	366	+	161
novclobiocic acid 230	399	398	+	193
novclobiocic acid 240	412	411	+	206
novclobiocic acid 250	426	425	+	220
novclobiocic acid 260	440	439	+	234
novclobiocic acid 270	361	**360**/362	+	155 (^{35}Cl)
novclobiocic acid 280	406	**404**/406	+	199 (^{79}Br)
novclobiocic acid 290	485	482/**484**/486	+	279 (^{79}Br^{81}Br)
novclobiocic acid 300	464	structure elucidation was performed previously [18]		
novclobiocic acid 380	340	339	(+)	134
novclobiocic acid 390	354	353	n.d.	148
novclobiocic acid 500	327	326	+	121
novclobiocic acid 510	369	368	+	163

Symbols and abbreviations: +, detectable; n.d., not detectable; x/y, two or more signals resulting from chlorine or bromine isotopes contained in the molecule. For structures of R_1, R_2, and R_3, see Table 1.

respective ring A analog dissolved in 200 μl ethanol was added to 50 ml of culture medium of the *clo*Q^- mutant. Cultivation was carried out in 500 ml baffled flasks for 5 to 8 days at 33°C and 210 rpm.

For analytical purposes, 10 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 0.5 ml methanol. After centrifugation, 100 μl of the clear supernatant was analyzed by HPLC with a Multosphere RP18-5 column (250 × 4 mm; 5 μm; C&S Chromatographie Service, Düren, Germany) at a flow rate of 1 ml/min, using a linear gradient from 68% to 100% MeOH in 0.2% aqueous acetic acid in 37 min with detection at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation, cultivation was carried out in 500 ml baffled flasks in 50 ml of medium as described above. Usually, a total of 500 ml bacterial culture was pooled, acidified with HCl to pH 4, and extracted with ethyl acetate after removing the lipophilic components by treatment with petrol ether. The residue of the ethyl acetate extract after evaporation of the solvent was dissolved in 3 ml methanol and passed through a glass column (100 × 2.6 cm) filled with Sephadex LH 20 (Amersham Biosciences, Freiburg, Germany) and eluted with methanol. The fractions after separation on Sephadex LH 20 were analyzed with HPLC using the conditions mentioned above. Fractions containing novclobiocins were pooled and further purified on a preparative HPLC column (Multosphere 120 RP18-5; 5 μm; 250 × 20 mm, C&S Chromatographie Service, Düren, Germany) using the same solvents and gradient as for the analytical column but with a flow of 2.5 ml/min. The purified compounds were subjected to ^{1}H-NMR and MS analysis.

Structure Elucidation and Characterization

The products were analyzed by ^{1}H-NMR spectroscopy and by negative ion FAB mass spectrometry. Negative fast atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using diethanolamine as matrix. The mass spectral data of the resulting novclobiocins are shown in Table 3. ^{1}H-NMR spectra were measured on an AMX 400 spectrometer (Bruker, Karlsruhe, Germany) using CD_3OD as solvent.

^{1}H-NMR spectral data (400 MHz, CD_3OD; br, broad) follow.

Novclobiocin 211

δ 0.97 (t, *J* = 7.6 Hz, 3H-9), 1.18 (s, 3H-6″), 1.35 (s, 3H-7″), 1.65 (sext., *J* = 7.6 Hz, 2H-8), 2.29 (s, 3H-6‴), 2.62 (t, *J* = 7.6 Hz, 2H-7), 3.51 (s, 3H-8″), 3.72 (d, *J* = 10.3 Hz, H-4″), 4.34 (t, *J* = 2.7 Hz, H-2″), 5.71 (dd, J_1 = 10.3 Hz, J_2 = 2.7 Hz, H-3″), 5.72 (d, *J* = 2.7 Hz, H-1″), 5.94 (d, *J* = 3.5 Hz, H-4‴), 6.82 (d, *J* = 8.4 Hz, H-5), 6.90 (d, *J* = 3.5 Hz, H-3‴), 7.29 (d, *J* = 9.1 Hz, H-6′), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, H-6), 7.78 (d, *J* = 2.2 Hz, H-2), 7.89 (d, *J* = 9.1 Hz, H-5′).

Novclobiocin 212

δ 0.96 (t, *J* = 7.4 Hz, 3H-9), 1.17 (s, 3H-6″), 1.37 (s, 3H-7″), 1.65 (sext., *J* = 7.4 Hz, 2H-8), 2.30 (s, 3H-6‴), 2.61 (t, *J* = 7.4 Hz, 2H-7), 3.55 (d, *J* = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.7 Hz, J_2 = 3.1 Hz, H-3″), 5.39 (t, *J* = 3.1 Hz, H-2″), 5.79 (d, *J* = 3.1 Hz, H-1″), 5.95 (d, *J* = 3.6 Hz, H-4‴),6.81 (d, *J* = 8.4 Hz, H-5), 6.89 (d, *J* = 3.6 Hz, H-3‴), 7.23 (d, *J* = 8.9 Hz, H-6′), 7.72 (dd, J_1 = 8.4 Hz, J_2 = 2.1 Hz, H-6), 7.78 (d, *J* = 2.1 Hz, H-2), 7.88 (d, *J* = 8.9 Hz, H-5′).

Novclobiocin 213

δ 0.96 (t, *J* = 7.5 Hz, 3H-9), 1.10 (s, 3H-6″), 1.30 (s, 3H-7″), 1.65 (sext., *J* = 7.5 Hz, 2H-8), 2.62 (t, *J* = 7.5 Hz, 2H-7), 3.40 (d, *J* = 9.9 Hz, H-4″), 3.59 (s, 3H-8″), 4.11 (t, *J* = 2.1 Hz, H-2″), 4.19 (dd, J_1 = 9.9 Hz, J_2 = 2.1 Hz, H-3″), 5.63 (d, *J* = 2.1 Hz, H-1″), 6.80 (d, *J* = 8.1 Hz, H-5), 7.22 (d, *J* = 9.0 Hz, H-6′), 7.72 (dd, J_1 = 8.1 Hz, J_2 = 2.0 Hz, H-6), 7.79 (d, *J* = 2.0 Hz, H-2), 7.89 (d, *J* = 9.0 Hz, H-5′).

Novclobiocin 214

δ 0.97 (t, *J* = 7.6 Hz, 3H-9), 1.20 (s, 3H-6″), 1.37 (s, 3H-7″), 1.66 (sext., *J* = 7.6 Hz, 2H-8), 2.29 (s, 3H-6‴), 2.63 (t, *J* = 7.6 Hz, 2H-7), 3.51 (s, 3H-8″), 3.68 (d, *J* = 9.8 Hz, H-4″), 4.23 (br s, H-2″), 5.60 (dd, J_1 = 9.8 Hz, J_2 = 3.5 Hz, H-3″), 5.70 (br s, H-1″), 5.94 (d, *J* = 3.7 Hz, H-4‴), 6.82 (d, *J* = 8.5 Hz, H-5), 6.90 (d, *J* = 3.7 Hz, H-3‴), 7.04 (complex overlapping signal; *J* not determinable, H-6′ and H-8′), 7.73 (d, *J* = 8.5 Hz, H-6), 7.79 (br s, H-2), 7.92 (d, *J* = 8.7 Hz, H-5′).

Novclobiocic Acid 215

δ 0.96 (t, *J* = 7.3 Hz, 3H-9), 1.65 (q, *J* = 7.3 Hz, 2H-8), 2.62 (t, *J* = 7.3 Hz, 2H-7), 6.80 (d, *J* = 8.6 Hz, H-5), 6.85 (d, *J* = 8.7 Hz, H-6′),

7.71 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, H-6), 7.77 (d, J = 8.7 Hz, H-5′), 7.78 (br s, H-2).

Novclobiocic Acid 216

δ 0.96 (t, J = 7.4 Hz, 3H-9), 1.65 (sext., J = 7.4 Hz, 2H-8), 2.62 (t, J = 7.4 Hz, 2H-7), 6.65 (d, J = 1.3 Hz, H-8′), 6.74 (dd, J_1 = 8.7 Hz, J_2 = 1.3 Hz, H-6′), 6.81 (d, J = 8.3 Hz, H-5), 7.72 (dd, J_1 = 8.3 Hz, J_2 = 2.6 Hz, H-6), 7.78 (br s, H-2), 7.82 (d, J = 8.7 Hz, H-5′).

Novclobiocin 221

δ 1.34 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.39 (d, J = 6.6 Hz, 2H-7), 3.51 (s, 3H-8″), 3.71 (d, J = 10.2 Hz, H-4″), 4.34 (br s, H-2″), 5.02 (dd, J_1 = 10.2 Hz, J_2 = 1.8 Hz, H-9a trans), 5.06 (dd, J_1 = 17.2 Hz, J_2 = 1.8 Hz, H-9b *cis*), 5.70 (complex overlapping signal, *J* not determinable, H-3″), 5.72 (d, J = 3.6 Hz, H-1″), 5.94 (d, J = 3.4 Hz, H-4‴), 6.02 (m, H-8), 6.83 (d, J = 8.4 Hz, H-5), 6.90 (d, J = 3.4 Hz, H-3‴), 7.25 (d, J = 9.3 Hz, H-6′), 7.75 (d, J = 8.4 Hz, H-6), 7.78 (s, H-2), 1.18 (s, 3H-6″), 7.88 (d, J = 9.3 Hz, H-5′).

Novclobiocin 222

δ 1.18 (s, 3H-6″), 1.37 (s, 3H-7″), 2.30 (s, 3H-6‴), 3.39 (d, J = 6.6 Hz, 2H-7), 3.55 (d, J = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.7 Hz, J_2 = 2.8 Hz, H-3″), 5.01 (dd, J_1 = 11.0 Hz, J_2 = 1.3 Hz, H-9a trans), 5.05 (dd, J_1 = 16.8 Hz, J_2 = 1.3 Hz, H-9b *cis*), 5.38 (t, J = 2.8 Hz, H-2″), 5.78 (d, J = 2.8 Hz, H-1″), 5.95 (d, J = 3.3 Hz, H-4‴), 6.02 (m, H-8), 6.83 (d, J = 8.0 Hz, H-5), 6.89 (d, J = 3.3 Hz, H-3‴), 7.22 (d, J = 8.6 Hz, H-6′), 7.75 (d, J = 8.0 Hz, H-6), 7.79 (s, H-2), 7.89 (d, J = 8.6 Hz, H-5′).

Novclobiocic Acid 223

δ 3.39 (d, J = 6.5 Hz, 2H-7), 5.02 (dd, J_1 = 10.2 Hz, J_2 = 1.7 Hz, H-9a trans), 5.06 (dd, J_1 = 17.1 Hz, J_2 = 1.7 Hz, H-9b *cis*), 6.02 (m, H-8), 6.88 (d, J = 8.7 Hz, H-6′), 7.75 (complex overlapping signal, *J* not determinable, H-6), 7.75 (complex overlapping signal, *J* not determinable, H-5′), 7.78 (s, H-2), 6.83 (d, J = 8.3 Hz, H-5).

Novclobiocic Acid 224

δ 3.40 (d, J = 6.5 Hz, 2H-7), 5.02 (dd, J_1 = 10.4 Hz, J_2 = 1.9 Hz, H-9a trans), 5.06 (dd, J_1 = 17.5 Hz, J_2 = 1.9 Hz, H-9b *cis*), 6.02 (m, H-8), 6.67 (d, J = 1.9 Hz, H-8′), 6.76 (dd, J_1 = 9.1 Hz, J_2 = 1.9 Hz, H-6′), 6.84 (d, J = 8.4 Hz, H-5), 7.75 (d, J = 8.4 Hz, H-6), 7.78 (s, H-2), 7.81 (d, J = 9.1 Hz, H-5′).

Novclobiocin 231

δ 1.19 (s, 3H-6″), 1.20 (t, J = 7.0 Hz, 3H-10), 1.35 (s, 3H-7″), 1.41 (d, J = 6.4 Hz, 3H-11), 2.29 (s, 3H-6‴), 3.45 (q, J = 7.0 Hz, 2H-9), 3.51 (s, 3H-8″), 3.72 (d, J = 9.3 Hz, H-4″), 4.34 (s, H-2″), 4.87 (obscured by water, H-7), 5.71 (dd, J_1 = 9.3 Hz, J_2 = 2.9 Hz, H-3″), 5.72 (s, H-1″), 5.94 (d, J = 3.5 Hz, H-4‴), 6.86 (d, J = 8.5 Hz, H-5), 6.90 (d, J = 3.5 Hz, H-3‴), 7.28 (d, J = 8.8 Hz, H-6′), 7.81 (d, J = 8.5 Hz, H-6), 7.90 (d, J = 8.8 Hz, H-5′), 7.98 (s, H-2).

Novclobiocin 232

δ 1.18 (s, 3H-6″), 1.20 (t, J = 6.9 Hz, 3H-10), 1.38 (s, 3H-7″), 1.41 (d, J = 6.4 Hz, 3H-11), 2.30 (s, 3H-6‴), 3.45 (q, J = 6.9 Hz, 2H-9), 3.55 (d, J = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.45 (dd, J_1 = 9.7 Hz, J_2 = 3.0 Hz, H-3″), 4.87 (obscured by water, H-7), 5.39 (t, J = 3.0 Hz, H-2″), 5.79 (d, J = 3.0 Hz, H-1″), 5.95 (d, J = 3.6 Hz, H-4‴), 6.84 (d, J = 8.3 Hz, H-5), 6.89 (d, J = 3.6 Hz, H-3‴), 7.23 (d, J = 9.0 Hz, H-6′), 7.80 (dd, J_1 = 8.3 Hz, J_2 = 3.5 Hz, H-6), 7.89 (d, J = 9.0 Hz, H-5′), 7.96 (d, J = 3.5 Hz, H-2).

Novclobiocin 233

δ 1.20 (t, J = 6.7 Hz, 3H-10), 1.20 (s, 3H-6″), 1.37 (s, 3H-7″), 1.41 (d, J = 6.4 Hz, 3H-11), 2.29 (s, 3H-6‴), 3.45 (q, J = 6.7 Hz, 2H-9), 3.51 (s, 3H-8″), 3.68 (d, J = 10.8 Hz, H-4″), 4.23 (br s, H-2″), 4.87 (obscured by water, H-7), 5.59 (dd, J_1 = 10.8 Hz, J_2 = 3.1 Hz, H-3″), 5.61 (s, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.85 (d, J = 8.1 Hz, H-5), 6.90 (d, J = 3.6 Hz, H-3‴), 7.01 (d, J = 8.1 Hz, H-6′ and 1H-8′), 7.82 (d, J = 8.1 Hz, H-6), 7.93 (d, J = 8.1 Hz, H-5′), 7.99 (br s, H-2).

Novclobiocin 241

δ 1.19 (s, 3H-6″), 1.23 (d, J = 6.8 Hz, 3H-10 and 3H-11), 1.35 (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 = 10.2 Hz, H-4″), 4.34 (s, H-2″), 5.71 (complex overlapping signal, *J* not determinable, H-1″), 5.71 (complex overlapping signal, *J* not determinable, H-3″), 5.94 (d, J = 3.5 Hz, H-4‴), 6.90 (d, J = 3.5 Hz, H-3‴), 6.93 (d, J = 8.1 Hz, H-5), 7.24 (d, J = 8.7 Hz, H-6′), 7.71 (d, J = 8.1 Hz, H-6), 7.89 (d, J = 8.7 Hz, H-5′), 8.22 (s, H-2).

Novclobiocin 242

δ 1.18 (s, 3H-6″), 1.23 (d, J = 6.8 Hz, 3H-10 and 3H-11), 1.38 (s, 3H-7″), 2.30 (s, 3H-6‴), 2.75 (sept., J = 6.8 Hz, H-9), 3.55 (d, J = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.7 Hz, J_2 = 3.1 Hz, H-3″), 5.39 (br s, H-2″), 5.79 (s, H-1″), 5.95 (d, J = 3.5 Hz, H-4‴), 6.89 (d, J = 3.5 Hz, H-3‴), 6.93 (d, J = 7.6 Hz, H-5), 7.23 (d, J = 8.7 Hz, H-6′), 7.71 (d, J = 7.6 Hz, H-6), 7.88 (d, J = 8.7 Hz, H-5′), 8.25 (br s, H-2).

Novclobiocin 243

δ 1.20 (s, 3H-6″), 1.23 (d, J = 6.9 Hz, 3H-10 and 3H-11), 1.37 (s, 3H-7″), 2.29 (s, 3H-6‴), 2.75 (sept., J = 6.9 Hz, H-9), 3.51 (s, 3H-8″), 3.70 (d, J = 9.4 Hz, H-4″), 4.23 (br s, H-2″), 5.60 (dd, J_1 = 9.4 Hz, J_2 = 3.0 Hz, H-3″), 5.62 (s, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.90 (d, J = 3.6 Hz, H-3‴), 6.95 (d, J = 8.3 Hz, H-5), 7.03 (complex overlapping signal, *J* not determinable, H-6′ and H-8′), 7.72 (d, J = 8.3 Hz, H-6), 7.92 (d, J = 5.8 Hz, H-5′), 8.32 (br s, H-2).

Novclobiocin 251

δ 1.19 (s, 3H-6″), 1.33 (s, 3H-10, 3H-11 and 3H-12), 1.33 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.71 (d, J = 9.7 Hz, H-4″), 4.33 (s, 1H-2″), 5.70 (br s, H-1″), 5.70 (complex overlapping signal, *J* not determinable, H-3″), 5.94 (s, H-4‴), 6.90 (s, H-3‴), 6.94 (d, J = 7.4 Hz, H-5), 7.23 (d, J = 8.5 Hz, H-6′), 7.71 (d, J = 7.4 Hz, H-6), 7.89 (d, J = 8.5 Hz, H-5′), 8.26 (s, H-2).

Novclobiocin 252

δ 1.18 (s, 3H-6″), 1.32 (s, 3H-10, 3H-11 and 3H-12), 1.37 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.56 (d, J = 7.9 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (br s, H-3″), 5.39 (s, H-2″), 5.78 (s, H-1″), 5.94 (br s, H-4‴), 6.88 (br s, H-3‴), 6.94 (br s, H-5), 7.20 (br s, H-6′), 7.70 (br s, H-6), 7.88 (br s, H-5′), 8.38 (br s, H-2).

Novclobiocin 253

δ 1.21 (s, 3H-6″), 1.33 (s, 3H-10, 3H-11 and 3H-12), 1.37 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.70 (d, J = 8.3 Hz, H-4″), 4.23 (s, H-2″), 5.60 (complex overlapping signal, *J* not determinable, H-3″), 5.61 (s, H-1″), 5.94 (d, J = 5.0 Hz, H-4‴), 6.90 (d, J = 5.0 Hz, H-3‴), 6.96 (d, J = 5.1 Hz, H-5), 7.01 (complex overlapping signal, *J* not determinable, H-6′ and H-8′), 7.72 (d, J = 5.1 Hz, H-6), 7.92 (br s, H-5′), 8.38 (br s, H-2).

Novclobiocin 261

δ 0.93 (t, J = 6.6 Hz, 3H-13), 1.19 (s, 3H-6″), 1.35 (s, 3H-7″), 1.39 (complex overlapping signal, *J* not determinable, 2H-11 and 2H-12), 1.72 (m, 2H-10), 2.29 (s, 3H-6‴), 2.45 (t, J = 6.6 Hz, 2H-9), 3.51 (s, 3H-8″), 3.71 (d, J = 5.5 Hz, H-4″), 4.34 (s, H-2″), 5.70 (complex overlapping signal, *J* not determinable, H-1″), 5.70 (complex overlapping signal, *J* not determinable, H-3″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.90 (d, J = 3.6 Hz, H-3‴), 6.93 (d, J = 8.4 Hz, H-5), 7.24 (d, J = 7.9 Hz, H-6′), 7.71 (d, J = 8.4 Hz, H-6), 7.89 (d, J = 7.9 Hz, H-5′), 8.21 (s, H-2).

Novclobiocin 262

δ 0.93 (t, J = 6.2 Hz, 3H-13), 1.18 (s, 3H-6″), 1.37 (complex overlapping signal, *J* not determinable, 2H-11 and 2H-12), 1.37 (s, 3H-7″), 1.72 (m, 2H-10), 2.30 (s, 3H-6‴), 2.45 (t, J = 7.2 Hz, 2H-9), 3.55 (d, J = 8.8 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 8.8 Hz, J_2 = 3.3 Hz, H-3″), 5.39 (s, H-2″), 5.79 (s, H-1″), 5.95 (d, J = 3.5 Hz, H-4‴), 6.89 (d, J = 3.5 Hz, H-3‴), 6.92 (d, J = 7.9 Hz, H-5), 7.21 (d, J = 9.1 Hz, H-6′), 7.72 (d, J = 7.9 Hz, H-6), 7.88 (d, J = 9.1 Hz, H-5′), 8.24 (br s, H-2).

Novclobiocic Acid 265

δ 0.94 (t, J = 6.8 Hz, 3H-13), 1.39 (complex overlapping signal, *J* not determinable, 2H-11 and 2H-12), 1.72 (t, J = 7.4 Hz, 2H-10), 2.46 (t, J = 7.4 Hz, 2H-9), 6.67 (d, J = 1.8 Hz, H-1″), 6.77 (br d, J = 8.6 Hz, H-6′), 6.95 (d, J = 8.3 Hz, H-5), 7.71 (d, J = 8.3 Hz, H-6), 7.81 (d, J = 8.6 Hz, H-5′), 8.29 (s, H-2).

Novclobiocin 271

δ 1.18 (s, 3H-6″), 1.34 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.72 (d, J = 10.0 Hz, H-4″), 4.35 (t, J = 2.5 Hz, H-2″), 5.70 (dd, J_1 = 10.0 Hz, J_2 = 2.5 Hz, H-3″), 5.72 (d, J = 2.5 Hz, H-1″), 5.94 (d, J = 3.5 Hz, H-4‴), 6.90 (d, J = 3.5 Hz, H-3‴), 6.96 (d, J = 8.6 Hz, H-5), 7.25 (d, J = 9.0 Hz, H-6′), 7.81 (dd, J_1 = 8.6 Hz, J_2 = 1.8 Hz, H-6), 7.86 (d, J = 9.0Hz, H-5′), 8.02 (d, J = 1.8 Hz, H-2).

Novclobiocin 272

δ 1.17 (s, 3H-6″), 1.38 (s, 3H-7″), 2.30 (s, 3H-6‴), 3.55 (d, J = 9.7 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.7 Hz, J_2 = 2.6 Hz, H-3″), 5.39 (t, J = 2.6 Hz, H-2″), 5.79 (d, J = 2.6 Hz, H-1″), 5.95 (d, J = 3.4 Hz, H-4‴), 6.89 (d, J = 3.4 Hz, H-3‴), 6.97 (d, J = 8.5 Hz, H-5), 7.24 (d, J = 8.9 Hz, H-6′), 7.81 (dd, J_1 = 8.5 Hz, J_2 = 2.1 Hz, H-6), 7.88 (d, J = 8.9Hz, H-5′), 8.02 (d, J = 2.1 Hz, H-2).

Novclobiocin 281

δ 1.18 (s, 3H-6″), 1.35 (s, 3H-7″), 2.29 (s, 3H-6‴), 3.52 (s, 3H-8″), 3.72 (d, J = 10.2 Hz, H-4″), 4.35 (t, J = 2.4 Hz, H-2″), 5.70 (dd, J_1 = 10.2

Hz, J_2 = 2.4 Hz, H-3″), 5.73 (d, J = 2.4 Hz, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.90 (d, J = 3.6 Hz, H-3‴), 6.95 (d, J = 8.0 Hz, H-5), 7.29 (d, J = 9.0 Hz, H-6′), 7.85 (dd, J_1 = 8.0 Hz, J_2 = 1.9 Hz, H-6), 7.87 (d, J = 9.0Hz, H-5′), 8.18 (d, J = 1.9 Hz, H-2).

Novclobiocin 282

δ 1.17 (s, 3H-6″), 1.37 (s, 3H-7″), 2.30 (s, 3H-6‴), 3.55 (d, J = 9.8 Hz, H-4″), 3.64 (s, 3H-8″), 4.40 (s, H-2″), 4.44 (dd, J_1 = 9.8 Hz, J_2 = 3.3 Hz, H-3″), 5.79 (s, H-1″), 5.95 (d, J = 3.5 Hz, H-4‴), 6.89 (d, J = 3.5 Hz, H-3‴), 6.94 (d, J = 8.6 Hz, H-5), 7.21 (d, J = 9.0 Hz, H-6′), 7.85 (d, J = 8.6 Hz, H-6), 7.87 (d, J = 9.0Hz, H-5′), 8.19 (br s, H-2).

Novclobiocin 283

δ 1.10 (s, 3H-6″), 1.30 (s, 3H-7″), 3.40 (d, J = 9.9 Hz, H-4″), 3.59 (s, 3H-8″), 4.11 (s, H-2″), 4.19 (dd, J_1 = 9.9 Hz, J_2 = 3.3 Hz, H-3″), 5.63 (s, H-1″), 6.95 (d, J = 8.9 Hz, H-5), 7.22 (d, J = 9.1 Hz, H-6′), 7.85 (d, J = 8.9 Hz, H-6), 7.88 (d, J = 9.1Hz, H-5′), 8.19 (br s, H-2).

Novclobiocin 381

δ 1.18 (s, 3H-6″), 1.35 (s, 3H-7″), 2.18 (s, 3H-7), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.72 (d, J = 10.9 Hz, H-4″), 4.34 (s, H-2″), 5.70 (dd, J_1 = 10.9 Hz, J_2 = 3.0 Hz, H-3″), 5.71 (s, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.70 (d, J = 8.3 Hz, H-5), 6.90 (d, J = 3.6 Hz, H-3‴), 7.27 (d, J = 8.9 Hz, H-6′), 7.65 (d, J = 8.3 Hz, H-6), 7.68 (s, H-2), 7.87 (d, J = 8.9Hz, H-5′).

Novclobiocin 382

δ 1.17 (s, 3H-6″), 1.37 (s, 3H-7″), 2.17 (s, 3H-7), 2.30 (s, 3H-6‴), 3.55 (d, J = 9.8 Hz, H-4″), 3.64 (s, 3H-8″), 4.44 (dd, J_1 = 9.8 Hz, J_2 = 3.3 Hz, H-3″), 5.40 (s, H-2″), 5.79 (s, H-1″), 5.95 (d, J = 3.6 Hz, H-4‴), 6.69 (d, J = 8.2 Hz, H-5), 6.89 (d, J = 3.6 Hz, H-3‴), 7.22 (d, J = 8.7 Hz, H-6′), 7.65 (d, J = 8.2 Hz, H-6), 7.68 (s, H-2), 7.86 (d, J = 8.7Hz, H-5′).

Novclobiocin 383

δ 1.20 (s, 3H-6″), 1.37 (s, 3H-7″), 2.19 (s, 3H-7), 2.29 (s, 3H-6‴), 3.51 (s, 3H-8″), 3.68 (d, J = 9.8 Hz, H-4″), 4.23 (s, H-2″), 5.59 (dd, J_1 = 9.8 Hz, J_2 = 3.2 Hz, H-3″), 5.62 (s, H-1″), 5.94 (d, J = 3.6 Hz, H-4‴), 6.71 (d, J = 8.3 Hz, H-5), 6.90 (d, J = 3.6 Hz, H-3‴), 7.04 (d, J = 7.6 Hz, H-6′ and H-8′), 7.66 (d, J = 8.3 Hz, H-6), 7.69 (s, H-2), 7.92 (d, J = 7.6Hz, H-5′).

Novclobiocin 384

δ 1.19 (s, 3H-6″), 1.39 (s, 3H-7″), 2.18 (s, 3H-7), 2.29 (s, 3H-6‴), 3.52 (d, J = 9.4 Hz, H-4″), 3.62 (s, 3H-8″), 4.35 (dd, J_1 = 9.4 Hz, J_2 = 3.4 Hz, H-3″), 5.31 (br s, H-2″), 5.69 (d, J = 1.9 Hz, H-1″), 5.94 (d, J = 3.5 Hz, H-4‴), 6.70 (d, J = 8.0 Hz, H-5), 6.89 (d, J = 3.5 Hz, H-3‴), 6.97 (complex overlapping signal, J not determinable, H-6′ and H-8′), 7.66 (d, J = 8.0 Hz, H-6), 7.70 (br s, H-2), 7.91 (d, J = 8.7 Hz, H-5′).

Novclobiocin 385

δ 1.23 (s, 3H-6″), 1.34 (s, 3H-7″), 2.19 (s, 3H-7), 2.29 (s, 3H-6‴), 4.07 (d, J = 10.4 Hz, H-4″), 4.41 (br s, H-2″), 5.59 (dd, J_1 = 10.4 Hz, J_2 = 2.9 Hz, H-3″), 5.71 (br s, H-1″), 5.93 (d, J = 3.6 Hz, H-4‴), 6.71 (d, J = 8.1 Hz, H-5), 6.91 (d, J = 3.6 Hz, H-3‴), 7.25 (d, J = 8.2 Hz, H-6′), 7.67 (d, J = 8.1 Hz, H-6), 7.71 (br s, H-2), 7.91 (d, J = 8.2Hz, H-5′).

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