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Use of a Halogenase of Hormaomycin Biosynthesis for Formation of New Clorobiocin Analogues with 5-Chloropyrrole Moieties

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The depsipeptide antibiotic hormaomycin, which is produced by Streptomyces griseoflavus *W-384, contains a 5-chloropyrrole moiety. In the producer strain we identified the gene* hrmQ *that shows sequence similarity to $FADH_2$-dependent halogenases. This gene was cloned and heterologously expressed in* Streptomyces roseochromogenes *var.* oscitans *DS12.976, which is the producer of the aminocoumarin antibiotic clorobiocin, which contains a 5-methylpyrrole moiety. For the present experiment, we used a mutant of this strain in which the respective pyrrole-5-methyltransferase had been inactivated. Expression of the halogenase* hrmQ *in this mutant strain led to the formation of two new clorobiocin derivatives that carried a 5-chloropyrrole moiety. These compounds were isolated on a preparative scale, their structures were elucidated by ^1H NMR spectroscopy and mass spectrometry, and their antibacterial activity was determined. The substrate of HrmQ is likely to be a pyrrole-2-carboxyl-S-[acyl carrier protein] thioester. If this assumption is true, this study presents the first experiment in combinatorial biosynthesis that uses a halogenase that acts on an acyl carrier protein-bound substrate.*

Introduction

Nature's inventory of secondary metabolites comprises thousands of halogenated compounds.[1] Incorporation of halogen atoms into the respective molecules alters the chemical and physical properties and often has a profound effect on the biological activity of the compound. The biochemical mechanisms of the halogenation of natural products by different enzyme classes have only recently been elucidated.[2,3] Especially the $FADH_2$-dependent halogenases, which were first discovered by genetic experiments of Dairi et al.[4] and investigated biochemically by van Pée and co-workers,[5,6] are of pivotal importance for the generation of many bioactive natural products, predominantly in microorganisms. Genes for $FADH_2$-dependent halogenases are contained in many biosynthetic gene clusters, for example, those of chlortetracycline, vancomycin and chloramphenicol.[5] It is tempting to speculate that halogenases could be a powerful tool for combinatorial biosynthesis, that is, for the generation of new compounds by recombination of biosynthetic genes from different organisms. Many examples have been published for successful combinatorial biosynthesis by using the genes for modules of polyketide synthases of nonribosomal peptide synthases, for oxygenases, methyltransferases, deoxysugar biosynthesis enzymes and glycosyltransferases. In contrast, only four examples are found in the literature for the use of halogenases in such experiments.[7–10] Here, we describe a further example for the successful use of a halogenase in combinatorial biosynthesis; this is likely to be the first such experiment that uses a halogenase that acts on a acyl carrier protein bound substrate.

The aminocoumarin antibiotics clorobiocin and coumermycin A_1 (Scheme 1) are powerful inhibitors of bacterial gyrase and topoisomerase IV and have strong antibacterial activity.[11] The binding mode of these compounds to gyrase has been investigated by X-ray crystallography.[12] The 5-methylpyrrole-2-carboxylic acid moiety, which is attached by an ester bond to the deoxysugar, is of central importance for the interaction of these antibiotics with their targets. This moiety occupies a hydrophobic pocket of gyrase and is crucial for the exceptionally high affinity of clorobiocin and coumermycin A_1 to gyrase.[11] The pyrrole-2-carboxylic acid moiety of clorobiocin and coumermycin A_1 is biosynthetically derived from proline, and the 5-methyl group is subsequently attached under catalysis of the C-methyltransferases CloN6 or CouN6, respectively.[13]

Hormaomycin is a depsipeptide antibiotic formed by *Streptomyces griseoflavus* W-384.[14–16] It also contains a pyrrole-2-carboxylic acid moiety, which is in this case attached by an amide bond to the peptide backbone of the molecule. However, in contrast to clorobiocin and coumermycin A_1, the pyrrole

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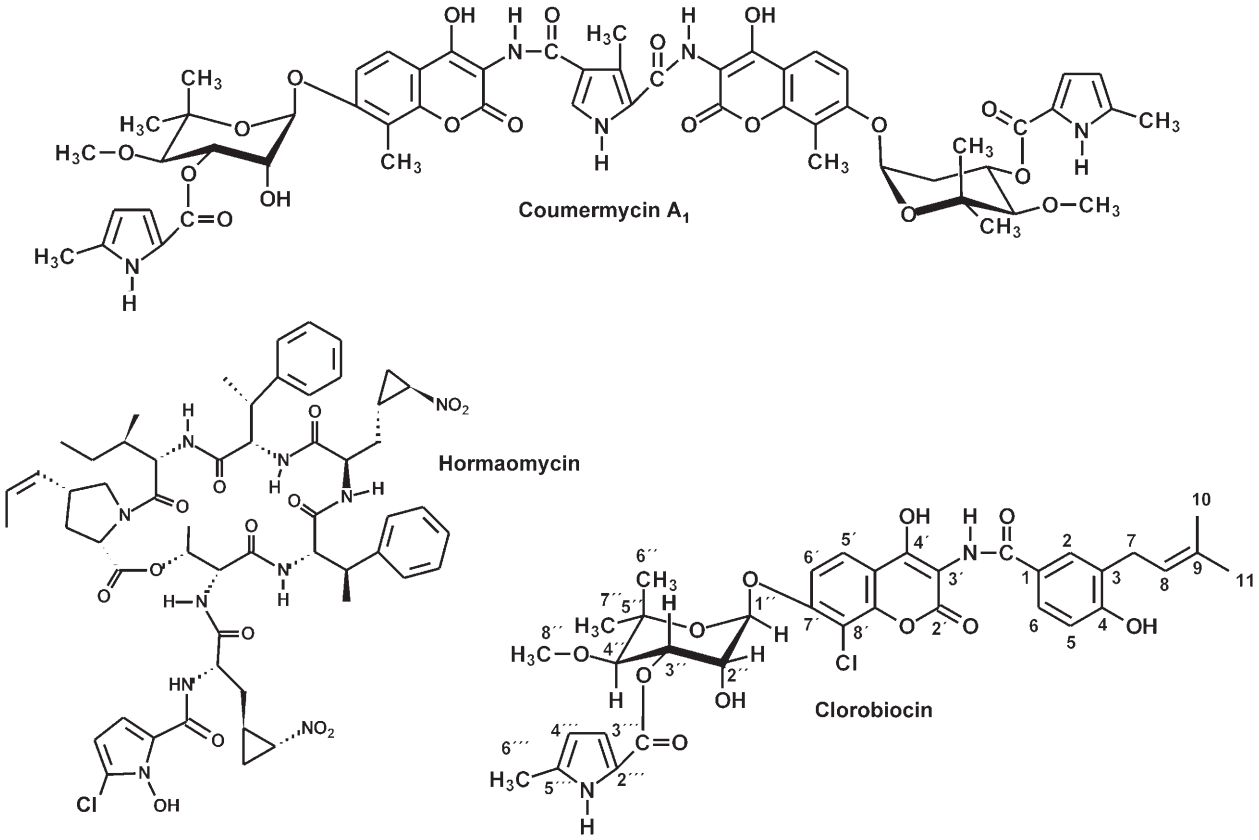


Scheme 1. Structures of coumermycin A1, clorobiocin and hormaomycin.

moiety of hormaomycin carries a chlorine rather than a methyl group at C5, and in addition it is N-hydroxylated. The biosynthetic origin of the N-hydroxyl group is unknown. Recently, the gene cluster for hormaomycin biosynthesis has been identified (J. Piel et al., unpublished results). It was found to contain a gene, termed *hrmQ*, with sequence similarity to $FADH_2$-dependent halogenases. We speculated that it might be responsible for the chlorination of C5 of the pyrrole moiety of hormaomycin.

We examined whether the expression of *hrmQ* in a producer strain of aminocoumarin antibiotics can lead to formation of a hybrid compound that contains the skeleton of the aminocoumarin antibiotic, but carries a chlorine rather than a methyl group at position 5 of the pyrrole moiety.

$FADH_2$-dependent halogenation reactions require two enzymes: the halogenase itself and a flavin reductase for the regeneration of the oxidised flavin cofactor. Only the putative halogenase *hrmQ* had been found in the hormaomycin gene cluster, not the reductase. It has been shown that flavin-dependent halogenases can functionally interact with flavin reductases from other pathways.[5] However, to minimise any problems that result from the possible lack of a suitable reductase, we carried out the experiment using the clorobiocin producer strain rather than the coumermycin producer strain. Clorobiocin already contains a chlorine atom in position 8 of the aminocoumarin moiety, which is attached under catalysis of the $FADH_2$-dependent halogenase Clo-hal.[17] Therefore, a suitable reductase for the regeneration of the flavin cofactor was expected to be present in this organism.

Results

Sequence analysis of the putative halogenase gene *hrmQ*

The gene *hrmQ* codes for a protein of 448 amino acids. A BLAST search revealed sequence similarity to $FADH_2$-dependent halogenases, and the predicted amino acid sequence of HrmQ showed the two motifs that are conserved in flavin-dependent halogenases.[5] These are the GxGxxG motif at the N-terminal end, which is involved in the binding of the flavin cosubstrate, and the WxWxIP motif, which is located near the middle of the protein and is suggested to prevent the enzyme from catalyzing a monoxygenase rather than a halogenase reaction.[6]

Three proteins in the database showed especially high sequence similarity to HrmQ (Table 1 and Figure S1 in the Supporting Information): PltA, which catalyses the two chlorination reactions at positions 5 and 4 of the pyrrole moiety of pyoluteorin;[18] Pyr29, which is suggested to catalyze the same reaction in the biosynthesis of pyrrolomycin;[19] and, less expectedly, ChlB4, which is suggested to catalyse 5-chlorination of the 6-methyl salicylic acid moiety of chlorothricin.[20]

In contrast, HalB of pentachloropseudilin biosynthesis[21] and PrnC of pyrrolnitrin biosynthesis,[22] both of which have been

Table 1. Sequence comparison of HrmQ with other $FADH_2$-dependent halogenases.

Gene	Organism	Substrate (position of chlorination)	Number of amino acids	Identity to HrmQ	Similarity to HrmQ
hrmQ	*S. griseoflavus* W-384	Pyrrole-2-carboxyl-*S*-ACP[a] (C-5 of pyrrole)	448	(100)	(100)
chlB4	*S. antibioticus* DSM 40725	6-Methylsalicoyl-*S*-ACP[a] (C-5 of salicyloyl moiety)	449	64	78
pyr29	*Actinosporangium vitaminophilum* ATCC 31673	Pyrrole-2-carboxyl-*S*-ACP[a] (C-4 and C-5 of pyrrole)	447	65	78
pltA	*P. fluorescens* PF-5	Pyrrole-2-carboxyl-*S*-ACP[b] (C-4 and C-5 of pyrrole)	449	59	74
halB	*Actinoplanes* sp. ATCC 33002	2-(3,5-dibromophenyl)-pyrrole[c]	562	27	44
prnC	*P. fluorescens* BL915	Monodechloroamino-pyrrolnitrin[b] (C-3 of pyrrole)	567	25	40
clo-hal	*S. roseochromogenes* var. *oscitans* DS12.976	8-desmethyl-novobiocic acid[a] (C-8′ of aminocoumarin)	524	32	51

[a] Putative substrate; [b] experimentally confirmed substrate; [c] artificial substrate used in vitro; position of halogenation of the pyrrole moiety unknown; ACP: acyl carrier protein.

biochemically proven to chlorinate pyrrole moieties, showed only low sequence similarity to HrmQ (Table 1).

Cloning of *hrmQ* and expression in a genetically modified producer strain of clorobiocin

The gene *hrmQ* was amplified by PCR from a cosmid that contained part of the hormaomycin cluster of *S. griseoflavus* W-384, and was cloned into the expression vector pUWL201 (*thio*^{R});[23] this placed it under control of the constitutive *ermE** promoter. The resulting plasmid was termed pLW42.

Clorobiocin is produced by *Streptomyces roseochromogenes* var. *oscitans* DS12.976. This strain contains the methyltransferase gene *cloN6* that is responsible for the methylation of position 5 of the pyrrole-2-carboxylic acid moiety.[13] To avoid that the desired 5-chlorination by HrmQ had to compete with 5-methylation by the genuine CloN6, we used a mutant strain in which the *cloN6* gene had been inactivated. This strain accumulates a clorobiocin derivative, novclobiocin 109, which is not substituted at position 5 of the pyrrole-2-carboxylic acid moiety.[13]

The *hrmQ* expression plasmid pLW42 was introduced into this mutant by protoplast transformation. After selection of transformants by thiostrepton resistance, cultures were established from single spores of the transformants. The presence of the intact plasmid was confirmed by DNA isolation and restriction analysis.

Analysis of secondary metabolites

Reversed-phase HPLC of extracts from the strain transformed with pLW42 in comparison to the parent strain showed two new peaks (compounds **1** and **2**), which had higher retention times than novclobiocin 109 (Figure 1). LC–MS analysis with ionisation in the positive mode showed a pseudomolecular ion $[M+H]^+$ at *m/z* 717 for both compounds, in comparison to *m/z* 697 for clorobiocin. This corresponds to the loss of a

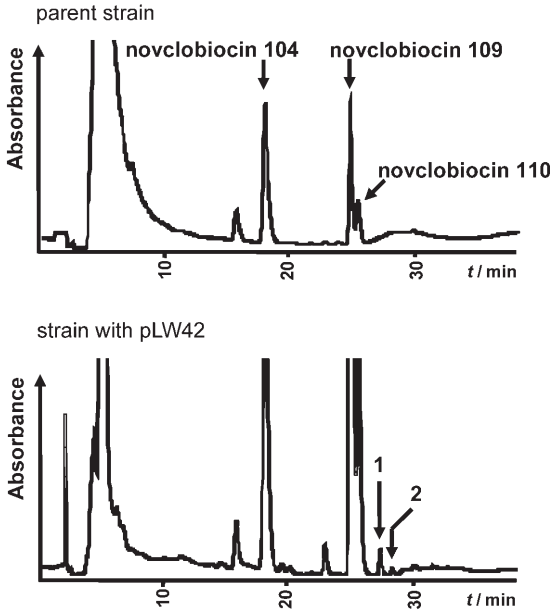


Figure 1. HPLC analysis of a strain transformed with the halogenase expression plasmid pLW42 in comparison to the parent strain. The parent strain was a derivative of *Streptomyces coelicolor* M512, which expresses a clorobiocin cluster that is defective in the gene *cloN6*.

methyl group and the gain of a chlorine atom. Both new compounds showed the characteristic isotopic signature of a dichlorinated compound that results from the high abundance of the heavy isotope ^{37}Cl (24.2%) besides the dominant ^{35}Cl. The predicted isotopic pattern, which was calculated by using the Xcalibur 1.3 software suite (ThermoFinnigan, San Jose, CA), was identical with the measured one. Tandem-MS analysis of the pseudomolecular ion showed the fragments of the prenylated 4-hydroxybenzoyl moiety (*m/z* 189) for both compounds (Figure 2) and of the aglycon clorobiocic acid (*m/z* 416), which is identical to the mass spectrum of clorobiocin. However, in comparison to clorobiocin, the mass of the substituted deoxy-

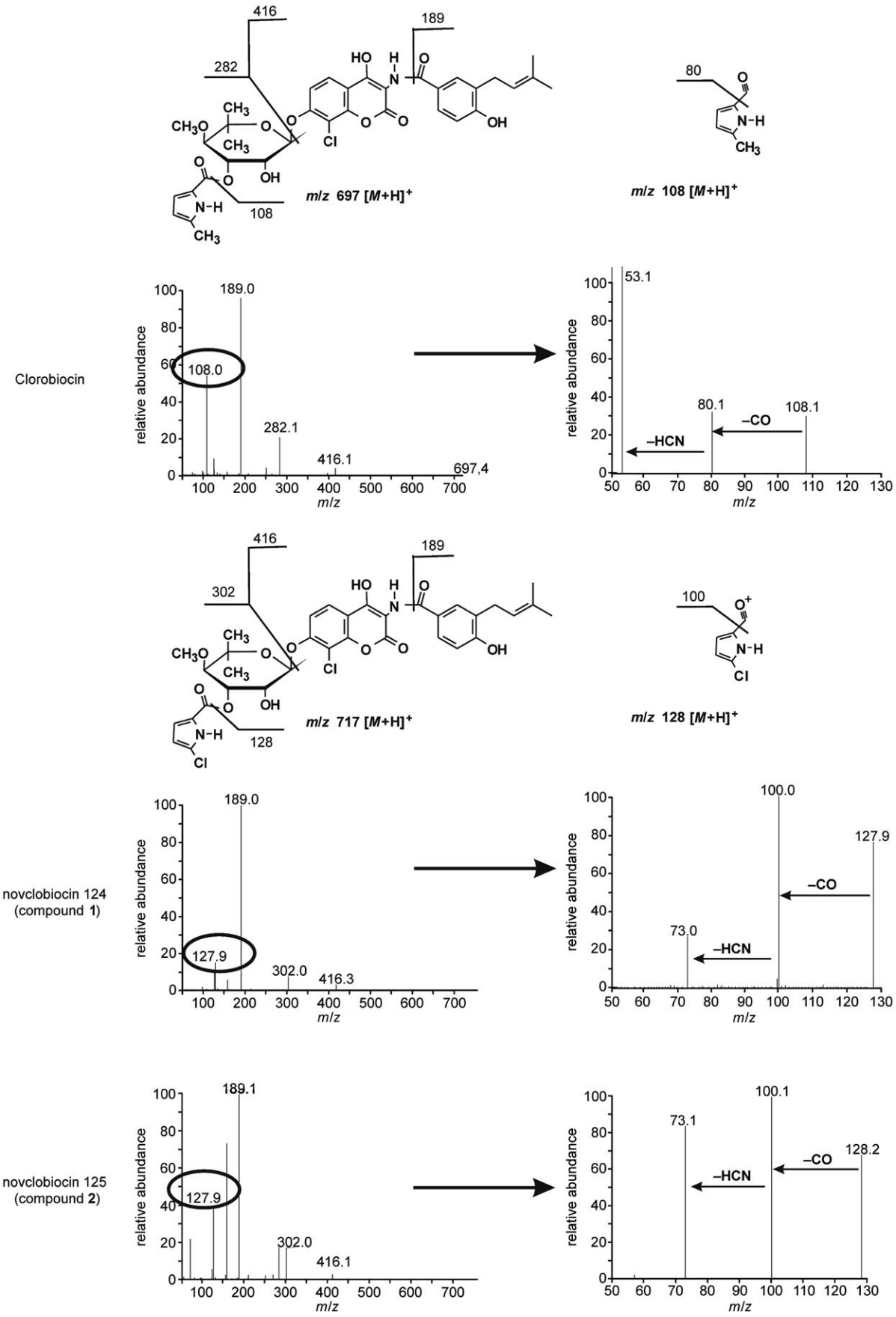


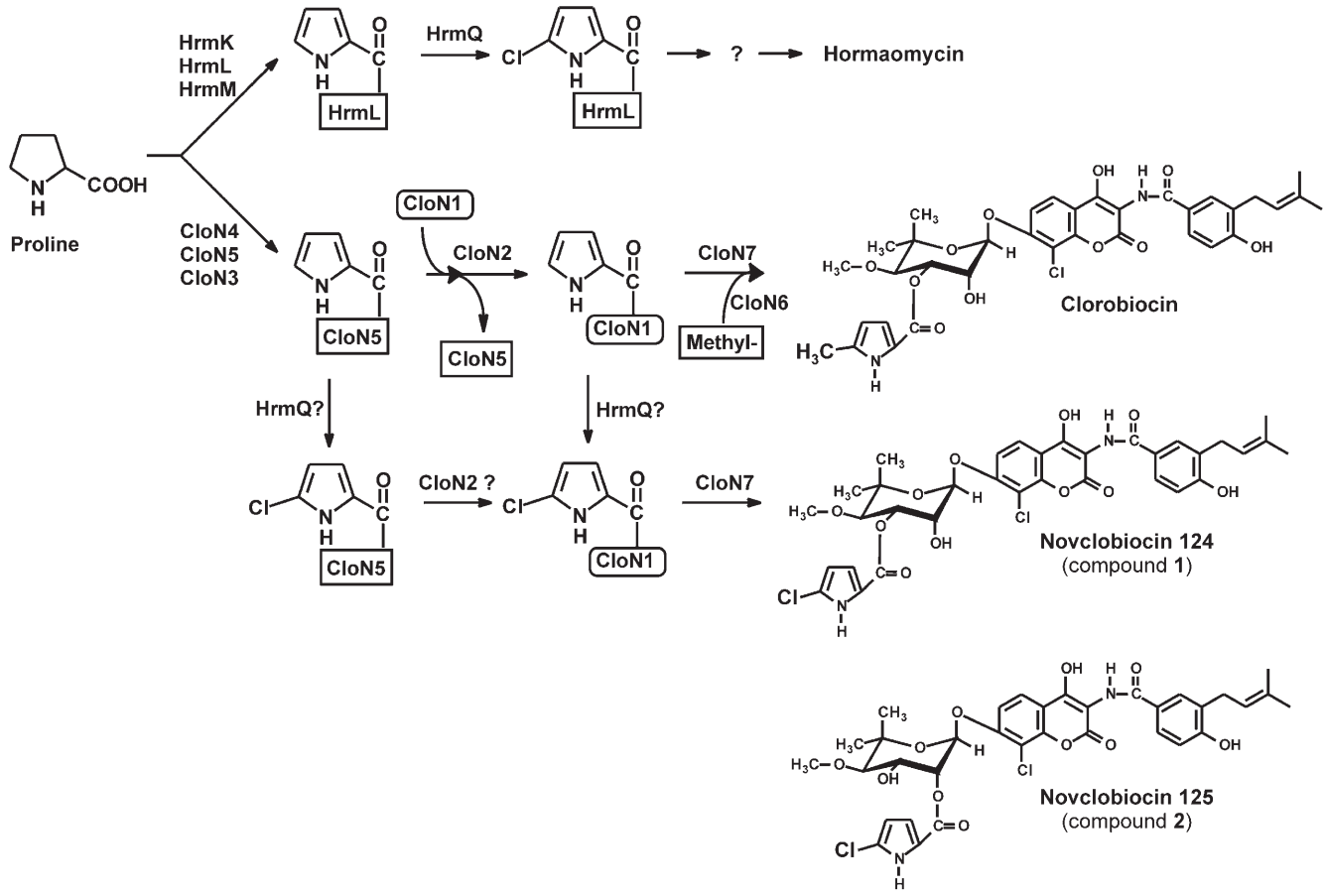
Figure 2. Tandem MS analysis of novclobiocin 124 and 125 in comparison to clorobiocin. Left-hand panels show the fragmentation pattern of the pseudomolecular ion (MS^2). Right-hand panels show fragmentation of the substituted pyrrole-2-carbonyl moiety (pseudo-MS^3 measurements).

sugar moiety showed an increase of 20 Da (i.e., *m/z* 302 rather than 282). Likewise, the fragment that corresponded to the substituted pyrrole-2-carboxylic acid moiety showed a mass increase from *m/z* 108 to 128. Therefore, it was the pyrrole moiety that had lost a methyl group and gained a chlorine atom. Both for clorobiocin and for the two new compounds, pseudo-MS^3 analysis (in-source CID fragmentation followed by MS/MS measurements) showed the characteristic fragmentation pattern of the differently substituted pyrrole-2-carboxyl moieties (Figure 2).[24]

Therefore, LC–MS analysis showed that the expression of *hrmQ* had indeed resulted in the formation of two new clorobiocin derivatives, chlorinated rather than methylated in the pyrrole moiety. However, mass spectroscopic analysis was insufficient to prove which position of the pyrrole moiety was substituted with the chlorine, and likewise it was insufficient to show the structural difference between compounds **1** and **2**. We therefore isolated both compounds on a preparative scale and subjected them to ^{1}H NMR analysis (see the Experimental Section).

Compound **1** showed the same ^{1}H NMR signals as clorobiocin for the prenylated 4-hydroxybenzoyl moiety, the aminocoumarin moiety and the deoxysugar moiety. However, the signal of the methyl group at 5‴ (Scheme 1), which is present in the spectrum of clorobiocin was missing in compound **1**. As in clorobiocin, the pyrrole protons at positions 3‴ and 4‴ coupled with each other; this resulted in doublet signals for both protons ($J = 3.6$ Hz). The absence of the proton signal of H5‴, which is observed in novclobiocin 109 at 7.02 ppm,[13] as well as the multiplicity of the signals of H4‴ and H3‴ proved that no proton was present at 5‴, that is, the chlorine had been introduced at this position. Correspondingly, the signal of H4‴ in compound **1** (6.12 ppm) showed a slight downfield shift in comparison to H4‴ of clorobiocin (5.94 ppm). Therefore, the structure shown in Scheme 2 was assigned to compound **1**, which was then designated as novclobiocin 124.

Compound **2** showed the same signals for the prenylated 4-hydroxybenzoyl moiety, the aminocoumarin moiety and the 5-chloropyrrole-2-carboxylic acid moiety as compound **1**, including the doublet signals of the pyrrole protons H3‴ and H4‴ at 6.95 and 6.12 ppm, respectively. However, the chemical shift of the signals of the deoxysugar protons at 2″ and 3″ had changed markedly. The signal of the axial proton H3″ (Scheme 1), which showed strong coupling with the axial H4″ ($J = 10.5$ Hz) had shifted upfield from 5.72 ppm (compound **1** and clorobiocin) to 4.45 ppm. In contrast, the signal of the



Scheme 2. Hypothetical scheme of the pyrrole-5-halogenation reactions in the biosynthesis of hormaomycin and in the formation of the new clorobiocin derivatives novclobiocin 124 and 125. HrmK, CloN4: prolyl-AMP ligases; HrmL, CloN5, CloN1: acyl carrier proteins; HrmM, CloN3: flavin-dependent dehydrogenases; CloN2, CloN7: acyltransferases; HrmQ: halogenase.

equatorial proton H2″, which coupled more weakly with the axial H3″ and the equatorial H1″ had shifted downfield from 4.35 ppm (compound **1** and clorobiocin) to 5.40 ppm. This showed that in compound **2** the 5-chloropyrrole-2-carboxylic acid moiety was attached to the 2″-OH group rather than to the 3″-OH group (Schemes 1 and 2). Similar 2″-acylated compounds have been found in earlier studies[13,25,26] and showed very similar NMR spectroscopic signals of H2″ and H3″ as compound **2**. Therefore, the structure shown in Scheme 2 was assigned to compound **2**, and this second new clorobiocin derivatives was designated as novclobiocin 125.

Antibacterial activity of the new clorobiocin derivatives

The antibacterial activities of novclobiocin 124 and 125 were determined in a disc-diffusion assay against *Bacillus subtilis* and compared to authentic clorobiocin (Figure 3). Novclobiocin 124 showed similar activity as clorobiocin; this suggests that the 5-chloropyrrole-2-carboxylic acid moiety is nearly equivalent to the genuine 5-methylpyrrole-2-carboxylic acid moiety in ensuring a tight binding of the inhibitor to gyrase. In contrast, novclobiocin 109, which does not carry a substituent at position 5 of the pyrrole ring, has been found to be eight-times less active than clorobiocin.[13] Novclobiocin 125, with the 5-chloropyrrole-2-carboxylic acid moiety attached to the 2-OH group of the deoxysugar, showed only 25% of the activity of clorobiocin.

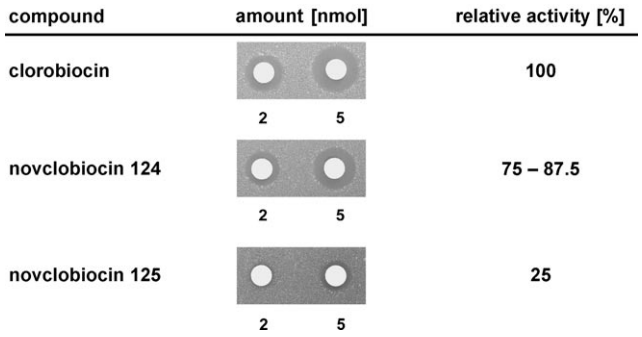


Figure 3. Antibacterial activity of novclobiocin 124 and 125 in comparison to clorobiocin.

Discussion

In the present study, expression of the halogenase gene *hrmQ* in a clorobiocin producer strain, which is defective in the 5‴-methyltransferase gene *cloN6*, was shown to result in the formation of 5‴-chlorinated clorobiocin derivatives. This finding supports the functional assignment of *hrmQ* to the halogenation reaction at position 5 of the pyrrole moiety of hormaomycin. Many $FADH_2$-dependent halogenases have been putatively identified from DNA sequence data, but functional proof from biochemical experiments is only available for eight of these enzymes, and proof from genetic experiments for a further three.[5,27,28] Our study now provides functional proof for a further halogenase.

For hormaomycin biosynthesis, no experimental data are available to indicate at which stage of the biosynthetic reaction sequence the halogenation of the pyrrole moiety takes place. However, investigations on the biosynthesis of pyoluteorin in *Pseudomonas fluorescens* Pf-5 have revealed the mechanism of the formation of the 4,5-dichloropyrrole-2-carbonyl moiety of that molecule (Scheme 2).[18] Proline is first adenylated under catalysis of the prolyl-AMP-ligase PltF, then transferred to the 4′-phosphopantheteinyl cofactor of the small acyl carrier protein PltL and finally oxidised by the flavoprotein PltE to the pyrrole derivative. Subsequently, the $FADH_2$-dependent halogenase PltA catalyses two successive halogenations at position 5 and 4 of the pyrrole-2-carboxyl-*S*-[acyl carrier protein] intermediate. HrmQ shows high sequence similarity with PltA (Table 1). Notably, both the hormaomycin and the clorobiocin biosynthetic gene cluster also contain genes with sequence similarity to the prolyl-AMP-ligase PltF, the acyl carrier protein PltL and the flavoprotein PltE. It appears therefore likely that in hormaomycin biosynthesis, HrmQ acts on a pyrrole-2-carboxyl-*S*-[HrmL] thioester (Scheme 2), although no biochemical evidence has yet been provided for the HrmQ reaction.

Detailed evidence is available on the function of the corresponding genes of clorobiocin biosynthesis.[29–33] In contrast to pyoluteorin biosynthesis, the formation of clorobiocin involves the successive attachment of the pyrrole-2-carboxylic acid moiety to two different acyl carrier proteins, that is, CloN5 and CloN1, before 5-methylation and transfer to the deoxysugar (Scheme 2).

We expressed only the halogenase HrmQ, and not the acyl carrier protein HrmL in the mutated clorobiocin producer strain. Therefore, our transgenic strain HrmQ is expected to act on either pyrrole-2-carboxyl-*S*-[CloN5] or pyrrole-2-carboxyl-*S*-[CloN1] (Scheme 2). HrmL, CloN5 and CloN1 are of similar size (91, 89 and 95 amino acids, respectively), and all contain the characteristic Asp(Asn)-Ser-Leu site for attachment of the 4′-phosphopantheteinyl cofactor. However, HrmL shares only 29% sequence identity (and 57% sequence similarity) with CloN5, and no significant sequence similarity with CloN1. This difference between HrmL and CloN5/CloN1 might be responsible for the relatively low yield of 5‴-chlorinated compounds obtained in the present study. In the strain that expressed the halogenase *hrmQ*, only about 5% of the aminocoumarin antibiotics were accumulated in the form of 5‴-chlorinated compounds, while the major part remained in 5‴-unsubstituted form. In contrast, when we inactivated the methyltransferase genes *novO* and *couO*, which are responsible for 8′-methylation of the aminocoumarin moieties of novobiocin and clorobiocin, and expressed the corresponding 8′-halogenase from the clorobiocin cluster in the resulting mutants, more than 75% of the aminocoumarins were accumulated as 8′-chlorinated compounds.[7,9] In these two cases, the substrates of the halogenation reactions are likely to be identical to those of the methylation reactions,[34,35] although the Clo-hal reaction has not yet been demonstrated in vitro.

It has been speculated that the use of halogenases for combinatorial biosynthesis in vivo could be very problematic because many of the substrates are halogenated only when tethered to an acyl carrier protein (ACP), and the substrate specificity of the halogenases might prevent their action on substrates bound to a heterologous ACP.[5] Our study suggests that in vivo halogenation of an ACP-bound substrate by a heterologous halogenase is possible, and similar evidence has been provided from an in vitro study with PltA.[18]

The amino acid sequences of several halogenases that act on different ACP-bound substrates are extremely similar (Table 1 and Figure S1). This offers prospects for both combinatorial biosynthesis and for directed evolution of such enzymes. In contrast, the $FADH_2$-dependent halogenases acting on free aromatic substrates show little sequence similarity to HrmQ, even if they also chlorinate pyrrole moieties (Table 1). These enzymes are unlikely to accept ACP-bound pyrroles. In our experiment, the mutant strain used for *hrmQ* expression contained an active halogenase, Clo-hal,[17] even before the introduction of the *hrmQ* expression plasmid pLW42. However, by using LC–MS analysis novclobiocin 124 and 125 could not be detected in the strain that lacked pLW42; this proves that the substrate specificity of Clo-hal allowed, as expected, only the halogenation of the aminocoumarin moiety, and not of the pyrrole moiety.

Hormaomycin biosynthesis involves not only the 5-chlorination but also the *N*-hydroxylation of the pyrrole moiety (Scheme 1). However, LC–MS analysis of the *ΔcloN6* mutant expressing *hrmQ* did not show any *N*-hydroxylated analogues of novclobiocin 124 and 125. This suggests that a further enzyme is required for the N-hydroxylation of the pyrrole moieties in hormaomycin biosynthesis.

Halogenation is a powerful tool, both in synthetic medicinal chemistry and in secondary metabolism in nature, to modify the physical and chemical properties and thereby the bioactivities of small molecules. With our growing knowledge of the mechanism of biological halogenations, and the rapidly growing number of known genes for halogenases with different substrate specificities, halogenases may become a useful tool for combinatorial biosynthesis.

Experimental Section

Bacterial strains and culture conditions: *E. coli* XL1 Blue MRF′ (Stratagene) was used for cloning experiments and grown in liquid or on solid Luria–Bertani medium (1.5% agar) at 37 °C.[36] The mutant strain *S. roseochromogenes* var. *oscitans* (*ΔcloN6*) has been described previously.[13]

Thiostrepton (12 $\mu g\,mL^{-1}$ for solid media and 40 $\mu g\,mL^{-1}$ for liquid media) and carbenicillin (50–100 $\mu g\,mL^{-1}$) were used for selection of recombinant strains.

DNA isolation, manipulation and cloning: Standard methods for DNA isolation and manipulation were performed as described by Sambrook and Russell[36] and by Kieser et al.[37] Isolation of plasmids was carried out with ion exchange columns (Nucleobond AX kits, Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol.

Construction of plasmid pLW42: The *E. coli–Streptomyces* shuttle vector pUWL201, which contained the *ermE** promoter,[23] was used for the construction of the expression plasmid pLW42. The gene *hrmQ* was amplified by PCR. A HindIII site was introduced before the start codon by using primer hrmQ_F (5′-**AAGCTT**ATGAGCGACTTCGACTACGAC-3′). At the C terminus, an XbaI restriction site was introduced after the stop codon by using primer hrmQ_R (5′-**TCTAGA**TGGGCGAGCTCAGAAGAGCGG-3′). Bold letters represent the introduced restriction sites. PCR amplification was performed in a 50 μL volume with template (600 ng), dNTPs (0.2 mM), each primer (10 pmol) and DMSO (5%, *v*/*v*) with the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, 24 cycles with denaturation at 94 °C for 1 min, annealing at 68 °C for 45 s and elongation at 72 °C for 90 s, followed by the last elongation step at 72 °C for 5 min. The resulting PCR fragment was cloned into pGEM-T (Promega); this resulted in pLW41. The plasmid pLW41 was digested with HindIII and XbaI and subsequently *hrmQ* was cloned into the same sites of pUWL201 to give pLW42.

Transformation: Transformation of *S. roseochromogenes* (*ΔcloN6*)[13] was carried out by PEG-mediated protoplast transformation.[37] For preparation of protoplasts, CRM medium[38] was used. Before transformation of the *S. roseochromogenes* mutant, the recombinant plasmids were amplified in *E. coli* ET12567 to bypass methyl-sensing restriction.[39]

Production of new aminocoumarin antibiotics: The *Streptomyces* strains were cultured as described previously[25] for seven days at 33 °C and 210 rpm. For analytical purposes, bacterial culture (1 mL) was acidified with HCl to pH 4 and extracted twice with an equal volume of EtOAc. After evaporation of the solvent, the residue was redissolved in MeOH (100 μL). After centrifugation, the supernatant (80 μL) was analyzed by HPLC with a Multosphere 120 RP18–5 column (5 μm, 250×8 mm, C&S Chromatographie Service, Düren, Germany) at a flow rate of 2 $mL\,min^{-1}$. A linear gradient from 60% to 100% solvent B (solvent A = H_2O/HCOOH 99:1; solvent B = acetonitrile/HCOOH 99:1) over 30 min was used. UV detection was carried out at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

For preparative isolation of the products, cells were cultured as described above by using a total culture volume of 3.75 L. The antibiotics were isolated as described previously.[25] Novclobiocin 124 (1.5 mg) and novclobiocin 125 (0.5 mg) were obtained.

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: N_2; sheath gas flow rate: 30.1 AU; aux gas flow rate: 12.0 AU) that was equipped with a Supersphere 100 RP 18 endcapped column (4 μm, 2×150 mm, Merck). For HPLC separation a H_2O/acetonitrile gradient (each contained 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% H_2O (that contained 0.1% HCOOH) for 4 min; the flow rate was set to 0.2 $mL\,min^{-1}$. The collision-induced dissociation (CID) spectra during the HPLC run were recorded with collision energy + 20 eV, collision gas argon, and collision pressure of 1.0×10^{-3} Torr (133 MPa).

The ^1H NMR spectra were measured by using an Avance 400 spectrometer (Bruker, Karlsruhe, Germany, 400 MHz) with CD_3OD as solvent.

Clorobiocin: $\delta = 7.76$ (d, $J = 2.5$ Hz; H2), 6.84 (d, $J = 8.4$ Hz; H5), 7.72 (dd, $J = 8.4$ Hz, 2.5 Hz; H6), 3.34 (d, $J = 7.1$ Hz, 2H; H7), 5.35 (brt, $J = 7.1$ Hz; H8), 1.74 Hz (s, 3H; H10), 1.75 (s, 3H; H11), 7.90 (d, $J = 9.2$ Hz; H5′), 7.33 (d, $J = 9.2$ Hz; H6′), 5.73 (d, $J = 1.8$ Hz; H1″), 4.34 (t, $J = 2.7$ Hz; H2″), 5.71 (dd, $J = 10.0$ Hz, 3.0 Hz; H3″), 3.72 (d, $J = 10.3$ Hz; H4″), 1.18 (s, 3H; H6″), 1.35 (s, 3H; H7″), 3.52 (s, 3H; H8″), 6.90 (d, $J = 3.6$ Hz; H3‴), 5.94 (brd, $J = 3.6$ Hz; H4‴), 2.29 ppm (s, 3H; H6‴).

Novclobiocin 124: $\delta = 7.71$ (H2), 6.81 (H5), 7.71 (H6), 3.33 (2H; H7), 5.34 (H8), 1.72 (6H; H10, H11), 7.86 (H5′), 7.20 (H6′), 5.70 (brs; H1″), 4.34 (brt; H2″), 5.72 (brd, 10.5 Hz; H3″), 3.71 (d, 10.5 Hz; H4″), 1.18 (s, 3H; H6″), 1.34 (s, 3H; H7″), 3.50 (s, 3H; H8″), 6.96 (d, 3.8 Hz; H3‴), 6.12 ppm (brd, 3.8 Hz; H4‴).

Novclobiocin 125: $\delta = 7.71$ (H2), 6.80 (H5), 7.71 (H6), 3.33 (2H; H7), 5.34 (H8), 1.72 (6H; H10, H11), 7.88 (H5′), 7.19 (H6′), 5.78 (brs; H1″), 5.40 (brt; H2″), 4.45 (brd, 10.6 Hz; H3″), 3.54 (d, $J = 10.6$ Hz; H4″), 1.18 (s, 3H; H6″), 1.34 (s, 3H; H7″), 3.64 (s, 3H; H8″), 6.95 (d, $J = 3.7$ Hz; H3‴), 6.13 ppm (brd, $J = 3.7$ Hz; H4‴).

Antibacterial activities: The antibacterial activities of the new compounds were tested against *Bacillus subtilis* ATCC 14893 in a disc diffusion assay as described previously.[40] Authentic clorobiocin was used as comparison. The activities were quantified from inhibition zone diameters and expressed relatively to clorobiocin.

Sequence information: The nucleotide sequence of the gene *hrmQ* was deposited in the GenBank database under accession no. EU583477.

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