ChemPubSoc Europe

DOI: 10.1002/cbic.201300712

New Aminocoumarins from the Rare Actinomycete *Catenulispora acidiphila* DSM 44928: Identification, Structure Elucidation, and Heterologous Production

Judith Zettler,[a, b] Haiyang Xia,[a, b, c] Nadja Burkard,[d] Andreas Kulik,[e] Stephanie Grond,[d] Lutz Heide,[a, b] and Alexander K. Apel*[a, b]

Genome mining led to the discovery of a novel aminocoumarin gene cluster in the rare actinomycete *Catenulispora acidiphila* DSM 44928. Sequence analysis revealed the presence of genes putatively involved in export/resistance, regulation, and biosynthesis of the aminocoumarin moiety and its halogenation, as well as several genes with so far unknown function. Two new aminocoumarins, cacibiocin A and B, were identified in the culture broth of *C. acidiphila*. Heterologous expression of the putative gene cluster in *Streptomyces coelicolor* M1152 confirmed that this cluster is responsible for cacibiocin biosynthesis. Furthermore, total production levels of cacibiocins could be increased by heterologous expression and screening of different culture media from an initial yield of 4.9 mg L^{-1} in *C. acidiphila* to 60 mg L^{-1} in *S. coelicolor* M1152. By HR-MS and NMR analysis, cacibiocin A was found to contain a 3-amino-4,7-dihydroxycoumarin moiety linked by an amide bond to a pyrrole-2,5-dicarboxylic acid. The latter structural motif has not been identified previously in any natural compound. Additionally, cacibiocin B contains two chlorine atoms at positions 6′ and 8′ of the aminocoumarin moiety.

Introduction

Aminocoumarin antibiotics are produced by *Streptomyces* strains and defined by their 3-amino-4,7-dihydroxycoumarin moiety. So far, five aminocoumarins: novobiocin, clorobiocin, coumermycin A_1, simocyclinone D8, and rubradirin (Scheme 1), have been discovered, and their biosynthetic gene clusters have been published and investigated over the last two decades.[1] Novobiocin (Albamycin) was licensed in the USA in 1964 as an antibiotic against methicillin-resistant *Staphylococcus aureus* strains (MRSA),[2] but was withdrawn from sale by the Food and Drug Administration in 2011 because of its side effects and the availability of better therapeutic agents. It is still widely used in veterinary medicine (Albadry, Biodry) for preventing mastitis of dairy cows.[3] Clorobiocin and coumermycin A_1 are even more potent as antibacterial agents.[4]

The three classical aminocoumarins novobiocin, clorobiocin, and coumermycin A_1 are well-known as potent inhibitors of bacterial gyrase.[5] Bacterial DNA gyrases are among the best-investigated antibacterial targets.[6] They are heterotetrameric enzymes consisting of two GyrA and two GyrB subunits that catalyze the ATP-dependent negative supercoiling of DNA. The three classical aminocoumarins target the GyrB subunit, in contrast to fluoroquinolones, which attack the GyrA subunit of bacterial gyrase. X-ray crystallographic analysis showed that both the aminocoumarin moiety and the substituted desoxysugar moiety (Scheme 1) are involved in binding to the GyrB subunit and, therefore, both moieties are essential for antibiotic activity.[7] More recently, novobiocin and its derivatives have also been identified as potent inhibitors of heat shock protein Hsp90[8] and, recently, as inhibitors of HIF1α and the p300/CBP interaction,[9] suggesting their potential as antitumor compounds and against protozoan infections such as trypanosomiasis.[10] Simocyclinone D8 has no desoxysugar moiety at the 7-OH of the aminocoumarin moiety but was unexpectedly found to be a more powerful gyrase inhibitor than novobiocin.[11] A completely new mechanism of gyrase inhibition was subsequently reported for simocyclinone D8,[12] in which it interacts with two separate pockets within the N-terminal domain of the GyrA subunit. Rubradirin is methylated at the 7-OH of the aminocoumarin ring and, in contrast to the previously mentioned aminocoumarin antibiotics, exhibits antibacterial activity by a distinct mechanism, inhibiting translation at the bacterial ribosomes.[13]

[a] J. Zettler, Dr. H. Xia, Prof. Dr. L. Heide, Dr. A. K. Apel
Eberhard-Karls-Universität Tübingen, Pharmazeutische Biologie
Auf der Morgenstelle 8, 72076 Tübingen (Germany)
E-mail: kristian.apel@uni-tuebingen.de

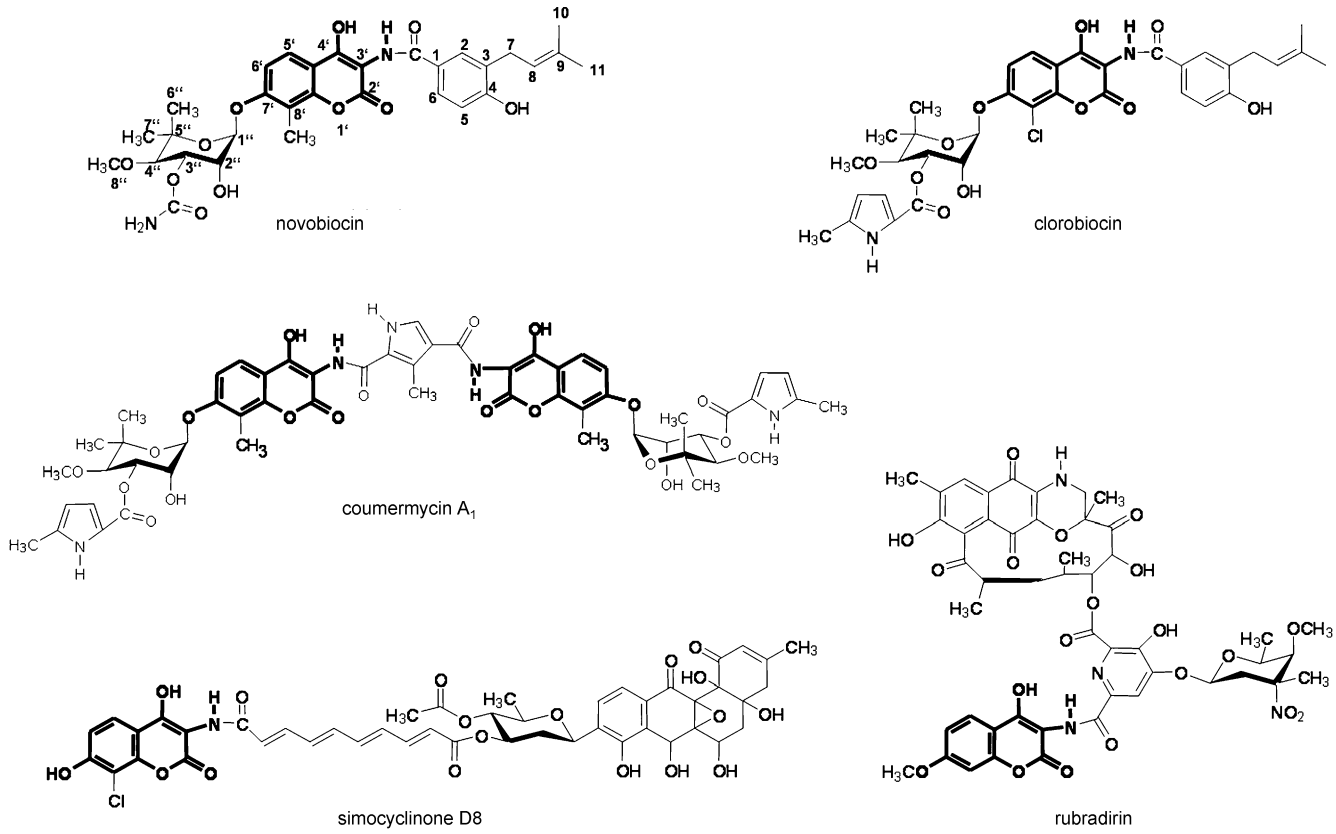
[b] J. Zettler, Dr. H. Xia, Prof. Dr. L. Heide, Dr. A. K. Apel
German Centre for Infection Research (DZIF)
Partner site Tübingen (Germany)

[c] Dr. H. Xia
Key laboratory of Synthetic Biology
Shanghai Institute of Plant Physiology and Ecology
Shanghai Institutes for Biological Sciences
The Chinese Academy of Sciences
Fenglin Road 300, 200032 Shanghai (P. R. China)

[d] N. Burkard, Prof. Dr. S. Grond
Eberhard-Karls-Universität Tübingen, Organische Chemie
Auf der Morgenstelle 18, 72076 Tübingen (Germany)

[e] A. Kulik
Eberhard-Karls-Universität Tübingen
Interfakultäres Institut für Mikrobiologie und Infektionsmedizin
Auf der Morgenstelle 28, 72076 Tübingen (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201300712.



Scheme 1. Structures of aminocoumarin antibiotics. The aminocoumarin moiety is shown in bold.

The aminocoumarin moiety is rare in nature and has only been reported in the five aminocoumarin antibiotics described above. All five biosynthetic gene clusters contain orthologues of the genes involved in the formation of the 3-amino-4,7-dihydroxycoumarin moiety, first described for novobiocin as *novH*, *novI*, *novJ*, and *novK*.[14] NovH activates tyrosine by adenylate formation and subsequent binding to a phosphopantetheinyl cofactor. The activated tyrosine is then hydroxylated at the β-position by NovI, a cytochrome P450 enzyme.[14b] The MbtH-like proteins CloY and SimY are strictly required for the activity of the NovH orthologues CloH and SimH, and they also stimulate NovH activity.[15] NovJ and NovK are involved in the oxidation of covalently enzyme-bound β-hydroxytyrosine to β-ketotyrosine.[16] The ring oxygen atom of the aminocoumarin moiety is derived from atmospheric oxygen,[17] suggesting that 2-hydroxylation takes place before lactonization. However, the final steps leading to the formation of the aminocoumarin moiety are still unknown. In the biosynthesis of all five aminocoumarins, an amide synthetase such as NovL links the 3-amino-4,7-dihydroxycoumarin moiety to an acyl moiety.[18] The aminocoumarin moiety can be further modified at position 8. In case of novobiocin or coumermycin A_1, it is methylated by NovO or CouO, and in the case of clorobiocin and simocyclinone D8, it is chlorinated by ClohaI or SimC6.[14a]

Recently, the identification of coumermycins from an actinomycete strain of the genus *Actinoallomurus* was reported.[19] This was the first time aminocoumarins were described outside of the genus *Streptomyces*. The fast-growing number of bacterial genome sequences in the databases, including an increasing number of rare actinomycetes, offers a new chance to discover aminocoumarins by genome mining. In this study, we describe the structure of two new aminocoumarins, cacibiocin A and B, which were identified by genome mining and heterologous expression of a newly discovered aminocoumarin gene cluster from the rare actinomycete *Catenulispora acidiphila* DSM 44928.

Results

Identification of a new aminocoumarin gene cluster by genome mining and bioinformatic sequence analysis

Screening of actinobacteria sequences deposited in the NCBI GenBank database, by using the tblastx algorithm, for genes with high similarity to the four aminocoumarin biosynthesis genes *novH*, *novI*, *novJ*, and *novK* led to the identification of a putative aminocoumarin gene cluster in the genome of *Catenulispora acidiphila* DSM 44928.[20] *C. acidiphila* has been studied previously for the production of bioactive compounds,[21] but no aminocoumarins have been described. The identified gene cluster contains genes homologous to *novH*, *novI*, *novJ*, and *novK*, that is, *caci_2723* (hereafter termed *cabH*), *caci_2722* (*cabI*), *caci_2720* (*cabJ*), and *caci_2718* (*cabK*). In addition, *caci_2721* (*cabY*) encodes an MbtH-like protein with high similarity to SimY and CloY from the simocyclinone and clorobiocin biosynthetic gene clusters. However, the C terminus of CabY con-

Table 1. Identified coding sequences (cds) in the biosynthetic gene cluster of cacibiocin.

ORF	Size of the product (amino acids)	Locus tag[a]	Similar entity	Identity with similar entity [%]	Accession No.
cabR7	264	*caci_2727*	*schA4*, transcriptional regulator angocyclinon Sch 47554 cluster, TetR familiy	40	CAH10095
cabR5	500	*caci_2726*	*couR5*, drug resistance transporter	50	AAG29799.1
cabR8	431	*caci_2725*	*rubRg2*, transcriptional regulator rubradirin cluster, LuxR family	43	CAI94711
cabhal	408	*caci_2724*	*simC6*, putative halogenase simocyclinone cluster	72	AEU17894
cabH	591	*caci_2723*	*cloH*, peptide synthetase-like protein	59	AAN65224.1
cabI	407	*caci_2722*	*novI*, cytochrome P450	75	AAF67502.1
cabY	104	*caci_2721*	*simY*, MbtH domain protein	49	AAG34186
cabJ	255	*caci_2720*	*novJ*, NADP dependent oxidase	67	AAF67503
cabR9	164	*caci_2719*	hypothetical protein	40	XM_002365945
cabK	260	*caci_2718*	*novK*, NADP dependent oxidase	45	AAF67504
cabL	544	*caci_2717*	*couL*, amide synthetase	58	AAG29784.2
cabR10	427	*caci_2716*	Pyroxidal-5′-phosphate-dependent protein beta subunit	45	WP_014985127
cabR11	424	*caci_2715*	FAD dependent oxidoreductase	39	ACM37038
cabR12	439	*caci_2714*	*prlI*, putative Na^+/K^+ antiporter	46	AGC24263

[a] from the genome of *Catenulispora acidiphila* DSM 44928 (accession no. NC_013131).

tains 30 additional amino acids, which is unusual in the superfamily of MbtH-like proteins (Table 1). In between *cabJ* and *cabK* (see Figure 1), we found an additional coding sequence (CDS), *caci_2719* (*cabR9*), coding for a 164 amino acid (aa) protein with unknown function, for which no close homologues can be found in the NCBI databases. Gene *caci_2717* (*cabL*), located downstream of *cabK*, codes for an amide synthetase with 58% amino acid identity to CouL, which catalyzes the formation of the amide bond between the central pyrrole moiety and the aminocoumarin moieties in coumermycins.[22]

All seven genes named above are transcribed in the same direction. It is possible that they form an operon together with the three adjacent genes: *caci_2716* (*cabR10*), *caci_2715* (*cabR11*), and *caci_2714* (*cabR12*), as the predicted coding sequences of *cabL*, *cabR10*, and *cabR11* overlap, and the intergenic region in front of *cabR12* comprises only 6 bp. The genes *cabR10* and *cabR11* encode a putative pyroxidal-5′-phosphate-dependent protein and an FAD-dependent oxidoreductase, whereas *cabR12* encodes a putative Na^+/H^+ antiporter possibly involved in the export of cacibiocins.

Six further CDSs situated downstream of *cabR12* are transcribed in the same direction: hypothetical protein *caci_2713* (*cds1*), calcium binding protein *caci_2712* (*cds2*), DUF556 domain-containing protein *caci_2711* (*cds3*), putative aldehyde dehydrogenase *caci_2710* (*cds4*), putative alcohol dehydrogenase *caci_2709* (*cds5*), and methyltransferase *caci_2708* (*cds6*). It remains unclear whether these six genes are involved in secondary metabolism. Two putative promoters could be situated in the intergenic regions upstream of *cds1* (78 bp) and *cds2* (160 bp).

On the other side of the cluster, upstream of *cabH* (see Figure 1), gene *caci_2724* (*cabhal*) is transcribed in the opposite direction and encodes a halogenase with 72% amino acid identity to SimC6 from the simocyclinone biosynthetic gene cluster, suggesting that the identified cluster might be responsible for production of a chlorinated aminocoumarin. Downstream of the halogenase *cabhal*, we identified two regulatory genes: *caci_2725* (*cabR8*), belonging to the luxR-family, and *caci_2727* (*cabR7*), belonging to the tetR-family, as well as gene *caci_2726* (*cabR5*), which encodes a transporter with 50% amino acid identity to CouR5 from the coumermycin A_1 cluster.[23]

We speculated that the borders of the identified aminocoumarin cluster might be represented by genes *cabR7* and *cabR12*. However, it cannot be overlooked that the cluster also comprises *cds1–6*. Downstream of *cabR7*, the genes *caci_2728* and *caci_2729* encode two putative α-L-arabinofuranosidases, whereas on the other site of the cluster, downstream of *cds6*, the genes *caci_2707* and *caci_2706* encode two low-conserved hypothetical proteins, followed by the genes *caci_2705* and *caci_2704*, encoding a putative heat shock protein Hsp18 transcriptional regulator and a putative heat shock protein Hsp20. These gene annotations are not suggestive of an involvement in secondary metabolism.

Two new aminocoumarins produced by *Catenulispora acidiphila*

To discover new possible aminocoumarins produced by *C. acidiphila*, the bacteria were grown in a chemically defined medium (CDM) originally developed for novobiocin production,[24] extracted, and examined by HPLC and LC-MS. HPLC analysis yielded two product peaks with UV absorption spectra similar to that of the aminocoumarin antibiotic clorobiocin, characterized by absorption maxima at 280 nm and 320 nm (Figure S1 in the Supporting Information). Using both parent ion scan MS and product ion scan MS, we could confirm that one of the two compounds, termed cacibiocin A, gave the expected fragment for an unsubstituted dihydroxyaminocoumarin moiety (*m/z* 192). The other compound, termed cacibiocin B, gave a fragment of *m/z* 260, consistent with a dichlorinated dihydroxyaminocoumarin moiety. The molecular masses of cacibiocin A and cacibiocin B were determined to be 330 g mol^{-1} and 397.9 g mol^{-1}. As expected, a clear pattern of a dichlorinated compound was visible for cacibiocin B with three masses of

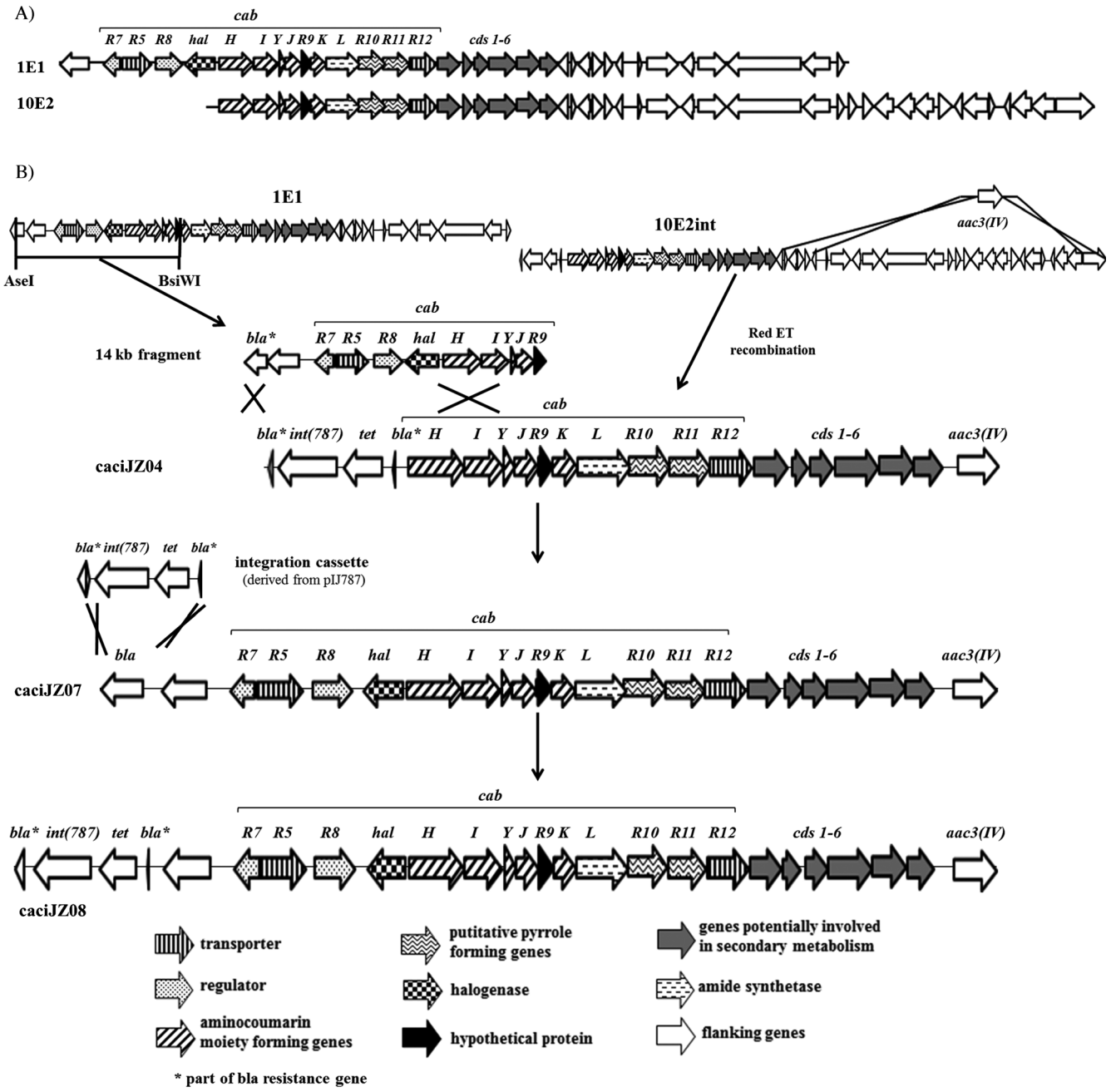


Figure 1. Construction of the heterologous expression cosmid caciJZ08 containing the whole cacibiocin biosynthetic gene cluster (see text for details).

397.9, 399.9, and 401.9 g mol^{-1} in a ratio of 57.4:36.7:5.9%, due to the natural abundance of ^{35}Cl (75.78%) and ^{37}Cl (24.22%).

Studies of cultures over 12 days of cultivation showed that maximum amounts of cacibiocins were reached after 10 days in CDM medium, with no further changes observed after that time (data not shown). Therefore, 11-day-old cultures were chosen for all further cacibiocin extractions. Total production was determined in CDM in Erlenmeyer flasks to be 2.4 mg L^{-1} for cacibiocin A and 2.5 mg L^{-1} for cacibiocin B.

Separate extraction of pellet and supernatant showed that the major fraction of cacibiocin A and B was detected in the supernatant. Extraction of the aqueous culture supernatant, adjusted to pH 4, with ethyl acetate reproducibly yielded 90% of the total cacibiocins. However, cacibiocin A and B production levels in CDM and eight other media (CDM, ISP medium 2, TSB medium, distillers solubles medium, simocyclinone fermentation medium, SSM medium, medium M, and SK medium; Table S1) were quite low. We therefore decided to heterologously express the cacibiocin gene cluster in *Streptomyces coelicolor* M1152, a strain optimized for the heterologous expression of secondary metabolites.[25] This experiment would also facilitate the preparative isolation of sufficient amounts of cacibiocins for structure elucidation and, at the same time, could provide proof that the identified aminocoumarin biosynthetic gene cluster was indeed responsible and sufficient for biosynthesis of cacibiocins A and B.

Cloning and heterologous expression of the cacibiocin biosynthetic gene cluster

A SuperCos 1-based cosmid library containing 3000 clones was constructed from *C. acidiphila* DNA and screened with PCR primers for genes situated within or adjacent to the identified gene cluster (see the Experimental Section). Two cosmids were identified (Figure 1): cosmid 1E1 contained the whole putative cacibiocin biosynthetic gene cluster, together with upstream and downstream flanking regions. Cosmid 10E2 contained only a part of the putative gene cluster, that is, the biosynthetic genes from *cabH* to *cabR12*, as well as genes downstream thereof. Cosmid 10E2 therefore lacked the C-terminal region of the halogenase *cabhal*, the two regulatory genes *cabR7* and *cabR8*, and the putative transporter gene *cabR5*. Unexpectedly, attempts to heterologously express cosmid 1E1 failed, due to an instability of this cosmid that was observed whenever the integration cassette from pIJ787[26] (containing the genes and the *attP* site required for integration into a *Streptomyces* genome) was introduced into the *bla* sequence of the cosmid backbone.[27] It remains unclear whether the size of the cosmid insert or a defect in the SuperCos 1 backbone caused this instability problem. Introduction of the integration cassette from pIJ787 into cosmid 10E2, in contrast, resulted in the stable construct 10E2int. As expected, however, heterologous expression of this construct in *S. coelicolor* M1152 did not result in cacibiocin formation, as parts of the biosynthetic gene cluster were missing. In order to generate a stable cosmid containing the complete cluster for heterologous expression, and in order to eliminate unnecessary flanking sequences adjacent to the cluster, we designed the two-step strategy depicted in Figure 1. In the first step, the genes from *caci_2664* to *caci_2706* were eliminated from 10E2int. This was achieved by replacing these genes using Red ET recombination with the apramycin resistance cassette from pIJ773,[28] resulting in cosmid caciJZ04. In the second step, a 14 kb restriction fragment was generated from cosmid 1E1 containing the genes from *cabR7* to *cabR9* and the *bla* resistance gene. This fragment was introduced into caciJZ04 by Red ET recombination, resulting in cosmid caciJZ07, which contained the complete cluster and a functional *bla* resistance gene. Reintroduction of the integration cassette from pIJ787 into the *bla* resistance gene by Red ET recombination finally resulted in the stable integrative cosmid caciJZ08. This was subsequently introduced by conjugation into the genome of *S. coelicolor* M1152.[25]

Four independent integration mutants were selected for analysis. Strain *S. coelicolor* M1152 (caciJZ08)-4 showed the highest production level and was chosen for all subsequent studies. Cultivation in CDM medium resulted in the formation of cacibiocin A (10 mg L^{-1}) and cacibiocin B (0.7 mg L^{-1}). The identity of these compounds to those formed by the wild-type strain *C. acidiphila* was confirmed by HPLC and ESI-MS/MS.

Improvement of production rates by a media screen

To further improve the yield of cacibiocin A and B in the heterologous producer strain, as well as in the wild-type strain, 31 media (Table S1) were prepared and used for cultivation in 24-well deep-well plates.[29] Cacibiocin production was determined and compared to production in CDM medium.[24] Results are given in Figure 2 and Table S2.

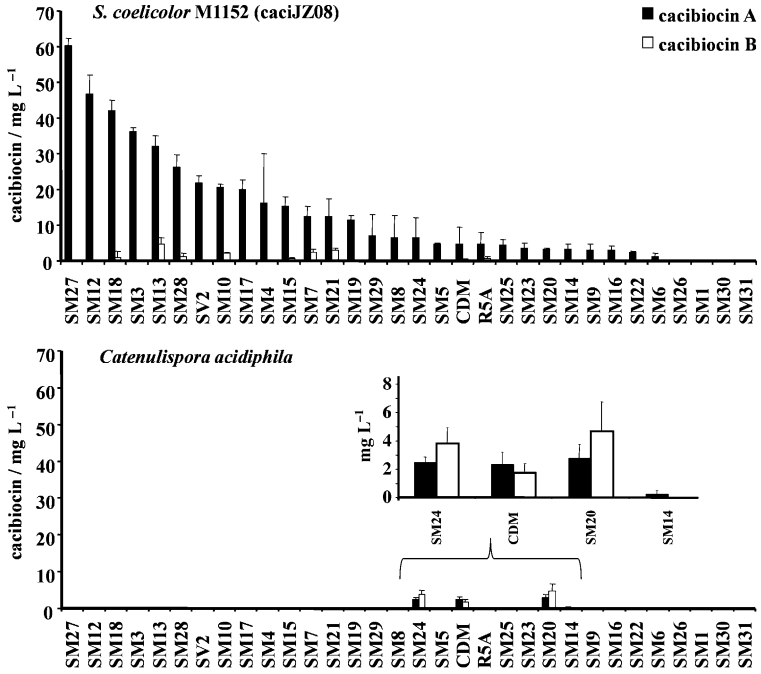


Figure 2. Cacibiocin production in 31 different media compared with the originally employed CDM medium in 24-well deep-well plates. Data are mean values of triplicate measurements. Extracts were prepared after 11 days of cultivation.

Cacibiocin production by the native producer *C. acidiphila* was observed only in three other media (SM14, SM20, and SM24). In two of these, production was significantly higher than in CDM, reaching a total yield of 7.5 mg L^{-1}. Notably, cacibiocin production by the heterologous producer was observed in 27 of the investigated media, with a maximum production of 60 mg L^{-1} in SM27. In only four media, no production was observed. Most of the cacibiocin was produced in form of the non-halogenated cacibiocin A, whereas the di-halogenated cacibiocin B was produced in smaller amounts. As shown in Figure 2, the best production media for *C. acidiphila* (SM20 and SM24) differed strikingly from those for our heterologous producer strain *S. coelicolor* M1152 (caciJZ08) (SM27, SM12, SM18, SM3, and SM13).

The time course of cacibiocin production was re-examined in Erlenmeyer flasks (50 mL culture volume), which yielded similar amounts of cacibiocins, as observed in deep-well plates (3 mL culture volume; Figure S2). Notably, the heterologous producer strain not only yielded much higher amounts of cacibiocins, but production also started much earlier. In the initial days of culture, the heterologous producer strain predominant-

ly formed cacibiocin A. After day 10, however, the amount of cacibiocin A decreased, with a concomitant increase in cacibiocin B (Figure S2). This might indicate that cacibiocin A was taken up from the medium, halogenated to form cacibiocin B, and excreted again. In contrast, in *C. acidiphila*, cacibiocin B was the dominant product at all time points, indicating a very efficient halogenation system.

Structure elucidation of cacibiocin A and B

Preparative amounts of cacibiocin A were isolated from the heterologous producer strain *S. coelicolor* M1152 (caciJZ08)-4, and cacibiocin B was isolated from the native producer strain *C. acidiphila*. HPLC-MS analysis in negative mode showed the presence of molecular ions with *m/z* 329.0 $[M-H]^-$ for cacibiocin A and *m/z* 396.9 $[M-H]^-$ for cacibiocin B. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) showed molecular ions with *m/z* 329.041527 $[M-H]^-$ (calcd 329.041524 for $C_{15}H_9N_2O_7$ $[M-H]^-$; Δ 0.009 ppm) for cacibiocin A and *m/z* 396.963791 $[M-H]^-$ (calcd 396.963580 for $C_{15}H_7Cl_2N_2O_7$ $[M-H]^-$; Δ 0.53 ppm) for cacibiocin B. As mentioned before, the isotope pattern for cacibiocin B is characteristic of a dichlorinated substance. ESI-HR-MS/MS analysis resulted in characteristic fragmentations of pyrrole and coumarin moieties for cacibiocin A (*m/z* 149.0354, 192.0313) and cacibiocin B (*m/z* 149.0359, 259.9535), respectively,[30] and clear assignment of the dichlorinated coumarin moiety of cacibiocin B due to the isotopic pattern (Figures 3 and S5). Unidimensional (^1H, ^{13}C) and multidimensional ($^1H,^1H$ COSY, $^1H,^1H$ TOCSY[1], HSQC[1], HMBC[1], NOESY) NMR studies resulted in 5-[(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)carbamoyl]-1*H*-pyrrole-2-carboxylic acid for cacibiocin A ($C_{15}H_{10}N_2O_7$, $M_R = 330.25\ g\,mol^{-1}$). The 2,5-substitution pattern of the pyrrole moiety was determined via ^1H NMR with 3-H (6.80 ppm, $^3J = 2.9\ Hz, ^4J = 2.1\ Hz$) and 4-H (6.91 ppm, $^3J = {}^4J = 2.9\ Hz$) as a double doublet pattern in [D_6]DMSO and with a strong vicinal coupling constant for 3-H and 4-H ($^3J = 4.0\ Hz$) in [D_4]MeOH. NOESY experiments resulted in a strong signal between 4-H of the pyrrole and the proton of the 4′-OH at the aminocoumarin moiety; this confirmed the 2,5-substitution of the pyrrole. Minimum energy calculations (DFT), including solvent effects in Gaussian 09, resulted in a distance of 3.393 Å for 4-H and 4′-OH and are in accordance with the NOESY range. Other possible substitution patterns are not in accordance with the observed consistent ^1H and ^{13}C NMR signals and NOESY correlations. The ^1H and ^{13}C NMR spectroscopic data of cacibiocin A and $^1H,^1H$ COSY, ^1H HSQC, ^1H HMBC, and NOESY correlations are given in the Supporting Information (Figure S3 and S4 and Table S3). NMR data for cacibiocin A and B show distinct differences between the coumarin signals, as expected (Figure S4). The chlorine substitution of cacibiocin B was assigned to a 6′,8′-dichloro coumarin moiety via comparative ^1H NMR and increment calculations. Cacibiocin B lacks the ^1H NMR signals for protons 6′ and 8′ of cacibiocin A. However, it shows the same doublets for the protons of the pyrrole moiety (cf Figure S4), with the singlet for proton H-5′ shifted +0.1 ppm. Hence, cacibiocin B was assigned as 5-[(6,8-dichloro-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)carbamoyl]-1*H*-pyrrole-2-carboxylic acid ($C_{15}H_8Cl_2N_2O_7$, $M_R = 399.14\ g\,mol^{-1}$).

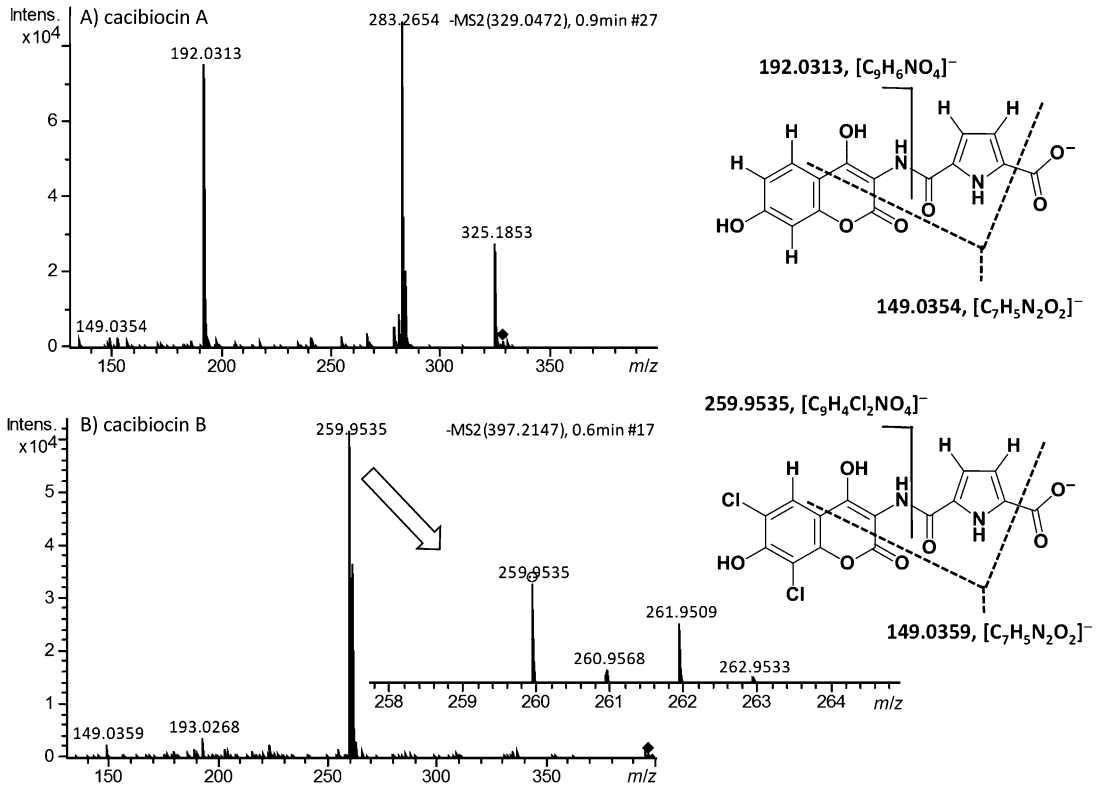


Figure 3. ESI–high-resolution MS-MS fragmentation spectra. A) Cacibiocin A. B) Cacibiocin B, with expansion of the mass range from 258 to 264.

Lack of gyrase-inhibitory and antibacterial activity of cacibiocin A and B

Classical aminocoumarin antibiotics such as novobiocin (Scheme 1) are potent inhibitors of gyrase. The substituted deoxysugar moiety of these compounds is essential for this activity.[5] As mentioned in the introduction, simocyclinone D8 (Scheme 1) was unexpectedly also found to be a potent gyrase inhibitor, despite the lack of a deoxysugar moiety, and it acts via a different binding mechanism.[12] We tested cacibiocin A and B for gyrase inhibitory activity in DNA supercoiling assays with clorobiocin as a positive control.[4] Clorobiocin inhibited *Staphylococcus aureus* gyrase at concentrations of 0.5 μM, but no gyrase inhibition was exhibited by cacibiocin A or B, even at concentrations up to 50 μM (Figure S6). Antibacterial activity was tested in disc diffusion assays against the Gram-negative strains *E. coli* DH5α and *E. coli* Δ*tolC* and the Gram-positive *Bacillus subtilis* ATCC 6633. Clorobiocin showed clear growth inhibition of *E. coli* Δ*tolC* and *B. subtilis* ATCC 6633 when 1 nmol was applied to the filter disc. In contrast, no antimicrobial activity could be observed for cacibiocin A or B at amounts of 20 nmol and 50 nmol per disc, respectively (data not shown). Thus, cacibiocins did not act as gyrase inhibitors and did not show antimicrobial activity against the tested bacterial strains. Their role in the producing organism is still unclear.

Discussion

Aminocoumarins are a rare class of compounds in nature. Only five biosynthetic gene clusters for aminocoumarins have been identified thus far (for the five compounds depicted in Scheme 1), all within the genus *Streptomyces*. The cacibiocin cluster is the first one found outside of this genus.

The cacibiocins comprise the same 3-amino-4,7-dihydroxycoumarin moiety that is characteristic of other aminocoumarins, but they contain two interesting new structural features. First, the aminocoumarin moiety of cacibiocin B is chlorinated in two positions, that is, in positions 6′ and 8′ (Figure 3). Previous structure–activity relationship investigations have proven that the chlorine atom of clorobiocin (Scheme 1) contributes significantly to the very high activity of this antibiotic.[4,31] Combinatorial biosynthesis experiments with the halogenase gene *clohal* have enabled generation of chlorinated derivatives of novobiocin and coumermycin.[31,32] The discovery of the halogenase *cabhal* of cacibiocin B biosynthesis might open a route to the combinatorial biosynthesis of dichlorinated novobiocin, clorobiocin, coumermycin, and simocyclinone derivatives, increasing the chemical diversity of this class of gyrase inhibitors for drug development programs and potentially aiding the generation of compounds with increased antibiotic activity.

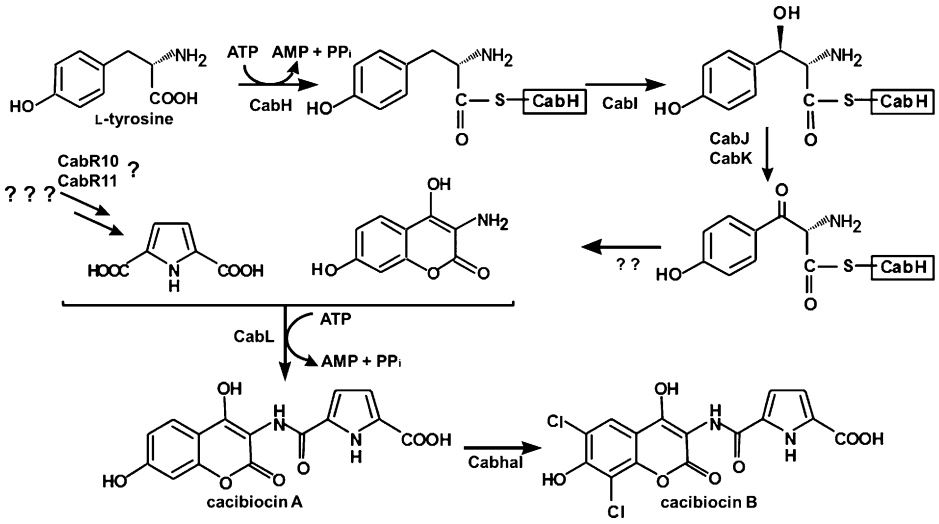
The second new structural feature of the cacibiocins is the pyrrole-2,5-dicarboxylic acid moiety. It is different from the central pyrrole moiety of coumermycin A_1 (Scheme 1). This latter moiety was recently found to be derived from threonine under catalysis of the gene product of *couR1*, *couR2a/b*, *couR3*, and *couR4*.[33] This represented a new biosynthetic pathway to the pyrrole moiety, which has important functions in biomolecules and pharmaceuticals.[34] The structure of the pyrrole-2,5-dicarboxylic acid moiety of the cacibiocins is not consistent with biosynthesis from threonine, and the cacibiocin cluster does not contain orthologues of the genes *couR1–couR4*. Therefore, the cacibiocin cluster apparently contains the genes for a further, yet unknown biosynthetic pathway to the pyrrole moiety. Identification of these genes might open a route to the combinatorial biosynthesis of new coumermycin derivatives, which is of interest as coumermycin is the most potent known aminocoumarin antibiotic.[4]

The successful heterologous expression of the cacibiocin cluster proves that this gene cluster is indeed responsible for the formation of the cacibiocins, and that it contains all genes required for their biosynthesis. Based on the bioinformatic analysis of the genes contained in this cluster, we propose the hypothetical biosynthetic pathway for cacibiocin A and B depicted in Scheme 2. The 3-amino-4,7-dihydroxycoumarin moiety is most likely formed by the gene products of *cabHIYJK* in the same way as in the novobiocin and clorobiocin biosynthetic pathways.[14a,15] This moiety is then linked to the pyrrole-2,5-dicarboxylic acid under catalysis of the amide synthetase CabL, forming the intermediate cacibiocin A, which is subsequently chlorinated at positions 6′and 8′of the aminocoumarin moiety by the halogenase CabHal. This is analogous to the halogenation step in clorobiocin biosynthesis.[14a] Formation of the pyrrole-2,5-dicarboxylic acid moiety occurs by a yet unknown biosynthetic pathway. As this moiety lacks the 3-methyl group found in the central pyrrole moiety of coumermycin A_1 (Scheme 1), its biosynthesis might start from serine or homoserine rather than threonine and could involve the gene products of *cabR10*, a putative pyroxidal-5′-phosphate dependent protein, and *cabR11*, an FAD-dependent oxidoreductase.

Notably, CabR5 shows high similarity to CouR5 (50% amino acid identity), a putative transporter encoded by the coumermycin A1 biosynthetic gene cluster. CouR5 has been suggested to be involved in self-resistance.[23] The expression of *couR5* is likely to be controlled by regulator gene *couR6*, situated adjacent to *couR5* (in the opposite direction) and separated by a 137 bp intergenic region that probably contains a bidirectional promotor. The same arrangement is found in the cacibiocin cluster for the putative transporter gene *cabR5* and the regulator gene *cabR7* (Figure 1), which are separated by a 56 bp region.

The genes *cabR10*, *cabR11*, and *cabR12* appear to form an operon with the biosynthetic genes from *cabH* to *cabL,* whereas the genes *cds1–6* are separated from this operon by two intergenic regions in front of *cds1* and *cds2*. As mentioned in the results section, the cacibiocin gene cluster might comprise the 16.6 kb DNA region from *cabR7* to *cabR12*. Whether or not *cds1–6* are involved in the secondary metabolism remains unclear.

Cacibiocin A and B are relatively small molecules compared to other aminocoumarins (Scheme 1) and, in contrast to all other known aminocoumarins, they lack antibiotic activities. This raises the question as to whether they merely represent intermediates of a pathway that genuinely forms a more complex, biologically active compound. As depicted in Figure 2, we



Scheme 2. Hypothetical biosynthetic pathway of cacibiocins A and B.

therefore screened secondary metabolite formation of both the native and the heterologous producer strain in a multitude of different culture media, but neither HPLC-UV (with examination of the UV spectra) nor parent ion scans in LC-MS suggested the formation of further aminocoumarin compounds.

We also searched the genome of *C. acidiphila* for homologues of genes that form additional structural motifs found in the other aminocoumarins depicted in Scheme 1. No gene cluster for the biosynthesis of a deoxysugar similar to that of other aminocoumarins, and no glycosyltransferase with close homology to the glycosyltransferases NovM, CloM, or CouM[14a] could be identified. We also were unable to find homologues of NovN, which forms the carbamoyl moiety attached to the deoxysugar of novobiocin, or homologues of CouN1–7, which are responsible for the formation and attachment of the terminal pyrrole moieties of coumermycin A1 (a similar pathway produces the pyrrole moiety of clorobiocin). As there is some structural similarity between the pyrrole-2,5-dicarboxylic acid moiety of the cacibiocins and the 3,4-dihydroxydipicolinate (DHDP) moiety of rubradirin, we also searched for homologues of 3-amino-5-hydroxybenzoic acid (AHBA) synthase, involved in the formation of the ansamacrolide moiety of rubradirin, but could find none in *C. acidiphila*. We were also unable to identify genes for the formation of an angucyclinone moiety similar to that of simocyclinone. Therefore, neither experimental nor bioinformatics studies gave any indication that *C. acidiphila* forms aminocoumarins other than cacibiocin A and B.

In the native producer strain, cacibiocin production did not exceed 7.5 mg L^{-1}. Production levels could be successfully raised to more than 60 mg L^{-1} by heterologous expression of the biosynthetic gene cluster in *S. coelicolor* M1152. This demonstrates the value of heterologous expression in specifically engineered host strains as a key method in genome mining for the discovery of new secondary metabolites. An interesting finding is the striking difference in the optimal production media of the native producer *C. acidiphila* and the heterologous producer *S. coelicolor* M1152 (caciJZ08) (Figure 2). When gene clusters are transferred to a heterologous producer strain, an extensive re-screening of optimal production media is apparently advisable in order to ensure optimal production yields. The dichlorination of the aminocoumarin moiety and presence of the pyrrole-2,5-dicarboxylic acid moiety of cacibiocin are two striking new features not previously known in aminocoumarins. Both features open new possibilities for combinatorial biosynthesis of aminocoumarins. Furthermore, investigations of the formation of the novel pyrrole-2,5-dicarboxylic acid moiety might reveal a new pathway to pyrroles in nature.

Experimental Section

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S4. *Escherichia coli* and *Bacillus subtilis* strains were cultured in lysogeny broth at 37 °C and 200 rpm.[35] *Catenulispora acidiphila* DSM 44928 was cultured at 30 °C and 200 rpm, and all media were adjusted to pH 5.6[21] before inoculation. Precultures were grown for 2 days in YMG (50 mL, yeast extract 4 g L^{-1}, malt extract 10 g L^{-1}, glucose 4 g L^{-1}) in 300 mL baffled Erlenmeyer flasks containing a stainless steel spring. Production media were inoculated with preculture (5%) and cultivated for 11 days. *Streptomyces coelicolor* M1152 strains were cultivated as described for *C. acidiphila* by using the proposed pH of the medium and supplementing with the appropriate antibiotics. Initial production studies were carried out in chemically defined medium (CDM) as described by Kominek et al.[24]

A further 31 types of medium (SM1, SM3–10, SM12–31, R5A, and SV2) were prepared according to media recipes kindly provided by EntreChem SL/Oviedo (Table S1). Screening was performed in 24-well deep-well plates[29] under the same conditions as for cultivation in flasks but with shaking at 300 rpm. Each well was cultivated with medium (3 mL) and supplemented with siloxylated ethylene oxide/propylene oxide copolymer[29] (0.6%). Triplicate measurements were performed for each medium and strain.

Extraction, analysis, and purification of cacibiocin A and B: Extractions of cultures were performed as described for clorobiocin.[1c] The cultures were acidified to pH 4 with HCl and extracted twice with equal volumes of EtOAc. Afterwards, the solvent was evaporated, and the dried extract was resuspended in MeOH for further analysis.[1c] Metabolites were analyzed by HPLC using a ReproSil-Pur C18-AQ column (5 μm, 250×4.6 mm) and a linear gradient from 30 to 100% MeOH in 0.1% aqueous formic acid over 31 min; the flow rate was 1 mL min^{-1}, with detection carried out at 320 nm.

For purification of cacibiocin A and B, 1 L of either *S. coelicolor* M1152 (caciJZ08) in SM12 medium or *C. acidiphila* DSM 44928 in CDM medium was extracted as described above. Dried extract was suspended in MeOH/CH_3CN (1:1) containing formic acid (0.1%) and eluted with the corresponding solvent in fractions over a LiChroPrep DIOL (Merck) column (flow rate: 0.6 mL min^{-1}). Fractions (6 mL each) were collected and analyzed for cacibiocin content. Fractions containing cacibiocin A or B were pooled, and the solvent was evaporated. During evaporation, cacibiocins precipitated and could be removed by centrifugation. After washing once with ice-cold MeOH and once with ice-cold CH_3CN, the cacibiocins were dried and dissolved in MeOH for LC-MS and in CD_3OD or [D_6]DMSO for NMR analysis.

Structure elucidation of cacibiocin A and B: High resolution tandem mass spectrometry was performed by using an ESI-MS Bruker MaXis 4G TOF. Measurements for aminocoumarins were performed in negative ionization mode.[30] For data analysis, the software Bruker ESI Compass 1.3, DataAnalysis 4.0 SP 5 was used. FTICR-MS (ESI-HR-MS) were recorded on a Bruker Daltonik Apex IV.

^{1}H and ^{13}C NMR data were recorded on a Bruker Avance (at 400, 100 MHz) or Bruker AMX (at 600, 150.7 MHz). Chemical shifts are expressed in δ values with the solvent as internal standard. Spectra analysis was performed with Mestrelab Research MestReNova 6.2.1–7569. For all analyses, cacibiocins were dissolved either in [D_6]DMSO or MeOD.

Agar diffusion tests and gyrase assay: Agar plates were prepared by inoculating LB agar with 3% of fully grown culture (OD_{600} = 1.2) of either *E. coli* DH5α, *E. coli* Δ*tolC*, or *B. subtilis* ATCC 6633. Sterile paper discs (Ø = 7 mm) were placed on the plates and supplied with MeOH (25 μL) containing different amounts of the respective antibiotic or secondary metabolite. A DNA gyrase supercoiling assay was performed as described by Alt et al.[4]

DNA isolation and manipulation: Standard methods for isolation and manipulation of DNA were performed as described by Kieser et al.[36]

Construction and screening of a cosmid library: Genomic DNA was partially digested with Bsp1431 at 0.1 U μg^{-1} DNA for 3–5 min, dephosphorylated, and then ligated into SuperCos 1 (Stratagene, Heidelberg, Germany). The ligation products were packaged with Gigapack III XL Packaging Extract (Stratagene) and transduced in *E. coli* SURE. A cosmid library of 3000 clones was screened with three primer pairs situated within or adjacent to the identified gene cluster: caci2698-F (5′-AGAAC ACCGC TCCGA TCAGC-3′) and caci2698-R (5′-AGGCC TGCCT GAGCA ATTCC-3′); caci2723-F (5′-TAGGC CAGGT CGGTC ACAGA-3′) and caci2723-R (5′-AGCAC CTGGT CGAAG TCCTC-3′); caci2727-F (5′-ATCTT GGGCA CCAGC AGCAC-3′) and caci2727-R (5′-TAGCG GTAGC AGGTC ATCGA CG-3′). Two cosmids were identified: 1E1, containing the whole cluster (CP001700, bases 3087901–3127663) and 10E2, containing a part of the cluster (CP001700, bases 3074836–3120624).

Construction of cosmid caciJZ08: The construction of caciJZ08 is depicted in Figure 1. First, the genes downstream of *caci_2707* in 10E2int (derived from 10E2 by introduction of the integration cassette from pIJ787[26]) were replaced by the apramycin resistance cassette from pIJ773[28b] using Red ET recombination[27,28] and the primer pair 10E2-773-F2 (5′-GTCCA TGCAG ACCAC TCTAA TCGCG GGGCG GATCG GCATT CTAGA ATTCC GGGGA TCCGT CGACC-3′) and 10E2-773-R2 (5′-TCAGA GTCCT TCCGA ATGGG TGGGT GTTGG TGGGC GATCA CTAGT TGTAG GCTGG AGCTGC TTC-3′), resulting in cosmid caciJZ04. 1E1 was then digested with AseI and BsiWI to give a 14 kb fragment containing additional genes from the cacibiocin cluster.

This fragment was purified by agarose gel extraction and subsequently introduced into caciJZ04 by Red ET recombination, resulting in cosmid caciJZ07. The integration cassette from pIJ787 containing an *attP* site[28a] was then introduced into caciJZ07 to yield caciJZ08. This cosmid was introduced into *S. coelicolor* M1152[25] by triparental conjugative transfer.

The abbreviations used are:

ESI-MS, electrospray ionization mass spectrometry; FTICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; HMBC, heteronuclear multiple bond correlation; HR-MS, high resolution mass spectrometry; HSQC, heteronuclear single quantum coherence; NCBI, National Center for Biotechnology Information; TOCSY, total correlated spectroscopy

Acknowledgements

This work was supported by the German Ministry of Education and Research (ERA IB GenoDrug, FKZ20315930, GenBioCom FKZ0315585A), and by the German Centre for Infection Research (DZIF). We also thank Alexander Müller for his work on the media screen, Lucia Westrich for help with the gyrase assays, and our collaborators from the GenoDrug project, especially Dr. Marcin Wolański and Prof. Dr. Jolanta Zakrzewska-Czerwinska from Wroclaw University (Poland), Dr. Jesus Cortes from EntreChem SL (Spain), and the company Galilaeus (Finland).

Keywords: actinobacteria · gene expression · heterologous expression · natural products · structure elucidation

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Received: November 13, 2013
Published online on February 19, 2014