Chemistry & Biology
Article

An Artificial Pathway to 3,4-Dihydroxybenzoic Acid Allows Generation of New Aminocoumarin Antibiotic Recognized by Catechol Transporters of *E. coli*

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DOI 10.1016/j.chembiol.2010.12.016

SUMMARY

An artificial operon was synthesized, consisting of the genes for chorismate pyruvate-lyase of *E. coli* and for 4-hydroxybenzoate 3-hydroxylase of *Corynebacterium cyclohexanicum*. This operon, directing the biosynthesis of 3,4-dihdroxybenzoate, was expressed in the heterologous expression host *Streptomyces coelicolor* M512, together with a modified biosynthetic gene cluster for the aminocoumarin antibiotic clorobiocin. The resulting strain produced a clorobiocin derivative containing a 3,4-dihdroxybenzoyl moiety. Its structure was confirmed by MS and NMR analysis, and it was found to be a potent inhibitor of the gyrases from *Escherichia coli* and *Staphylococcus aureus*. Bioassays against different *E. coli* mutants suggested that this compound is actively imported by catechol siderophore transporters in the cell envelope. This study provides an example of the structure of a natural product that can be rationally modified by synthetic biology.

INTRODUCTION

The first experiment showing that a new antibiotic can be produced by genetic engineering, i.e., by the combination of biosynthetic genes from different organisms, was published in 1985 (Hopwood et al., 1985). Since then, the number of biosynthetic genes and gene clusters available for such experiments, and the genetic techniques available for recombination and expression have expanded greatly, and many new bioactive compounds have been generated by genetic engineering of microorganisms (Hopwood, 2009a, 2009b). Advances in the methods of chemical DNA synthesis now allow to readily adapt the codon usage in a given gene to the codon usage of different expression hosts. This greatly expands the possibilities for the generation of new bioactive compounds by the combination of genes from very different organisms in a suitable host strain using a synthetic biology approach. The present paper reports the rational design of a structurally modified antibiotic by such a strategy.

The aminocoumarin antibiotics are characterized by their 3-amino-4,7-dihydroxycoumarin moiety (Heide, 2009a). This family of antibiotics comprises potent DNA gyrase inhibitors, including clorobiocin and the structurally related novobiocin (Figure 1). These compounds interact with the subunit B of bacterial DNA gyrase and inhibit ATP-dependent supercoiling of DNA, which is essential for transcription and DNA replication in bacteria. The therapeutic potential of the aminocoumarins lies especially in their very high affinity to gyrase. Their equilibrium dissociation constants (K_D) for gyrase is in the 10 nM range, i.e., much lower than that of modern fluoroquinolones (Maxwell and Lawson, 2003). Even though clorobiocin is a more potent inhibitor of DNA gyrase, only novobiocin (Albamycin) has been licensed for clinical use in human infections with Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* strains (Maxwell and Lawson, 2003). However, due to its poor solubility in water and its low activity against Gram-negative pathogens, clinical application of novobiocin remains restricted.

Over the last years, the biosynthetic gene clusters of five different aminocoumarin antibiotics have been cloned and sequenced (Heide, 2009a), and this group of antibiotics has become a successful example for the generation of new antibiotics by genetic techniques, e.g., metabolic engineering, mutasynthesis, and chemoenzymatic synthesis (Heide, 2009b). Structurally, clorobiocin is composed of three moieties: the acylated noviose deoxysugar (Ring C), the 3-amino-4,7-dihydroxycoumarin which is chlorinated at 8′-position (Ring B), and the 3-dimethylallyl-4-hydroxybenzoyl moiety (Ring A). These three moieties are linked by a glycosidic and an amide bond (Figure 1). X-ray crystallographic studies have shown that Ring B and Ring C are essential for the interactions of clorobiocin (as well as novobiocin) with gyrase, while Ring A is much less involved in the binding of the antibiotic to the target (Maxwell and Lawson, 2003). Therefore, it appears possible to vary the structure of Ring A without severely affecting the DNA gyrase inhibitory activity.

A principal shortcoming of the aminocoumarin antibiotics is their poor activity against Gram-negative organisms which is partly due to their poor permeation across the outer membrane of these organisms (Tropp et al., 1995). Gram-negative bacteria have efficient iron acquisition systems which consist of outer membrane proteins that bind iron complexes of specific siderophores and facilitate their active transport into the cell (Neilands, 1995). Many siderophores, like enterobactin of *Escherichia coli*,

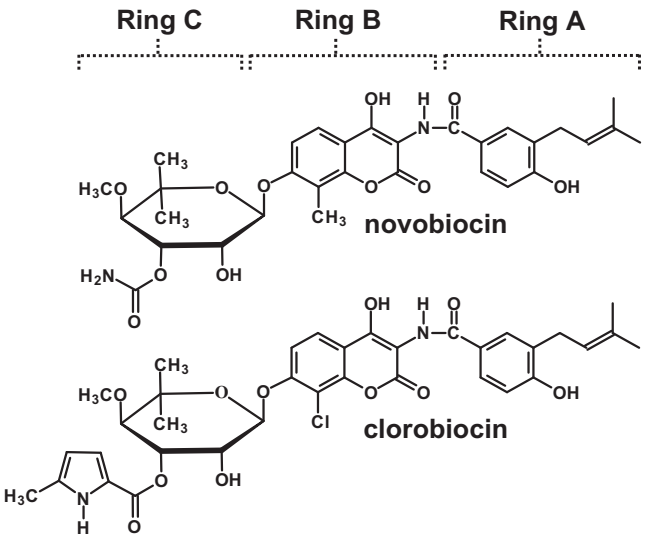

Figure 1. The Aminocoumarin Antibiotics Novobiocin and Clorobiocin

contain catechol (= o-diphenol) motifs. Their hydroxyl groups are responsible for chelating the Fe^{3+} ion (Neilands, 1995). The bacterial iron uptake mechanisms offers a possibility to overcome membrane-associated drug resistance by a Trojan horse approach (Miethke and Marahiel, 2007; Möllmann et al., 2009). It has been shown that beta-lactam antibiotics to which a catechol moiety had been chemically attached were transported by siderophore transporters into the Gram-negative cell, resulting in an enhanced antibacterial activity (Möllmann et al., 2009). In the present study, we attempted to develop a clorobiocin derivative with a catechol moiety instead of the genuine Ring A, using a synthetic biology approach, and to test whether it would be transported into the cell under involvement of catechol siderophore transporters. We (1) isolated the gene cluster for clorobiocin from a *Streptomyces* strain; (2) deleted the pathway to the genuine Ring A moiety of this antibiotic from the cluster by gene inactivation; (3) constructed a new, artificial biosynthetic pathway to a catechol moiety by combining one gene from the Gram-negative *Escherichia coli* and one gene from the Gram-positive *Corynebacterium cyclohexanicum* into a synthetic operon; (4) expressed these genes from three different organisms in a fourth organism, i.e., *Streptomyces coelicolor* M512, as heterologous host. This genetic strategy resulted in the production of the desired compound, i.e., a clorobiocin derivative with a catechol (= o-diphenol) moiety. We could provide evidence that this compound was transported across the cell envelope of *E. coli* under involvement of TonB-dependent transporters, i.e., bacterial siderophore transporters which recognize catechol moieties.

RESULTS

Investigation of the Substrate Tolerance of Different Aminocoumarin Acyl Ligases for Acyl Substrates with Catechol Moieties

The aim of our study was the replacement of the genuine 3-dimethylallyl-4-hydroxybenzoyl moiety (= Ring A) in clorobiocin

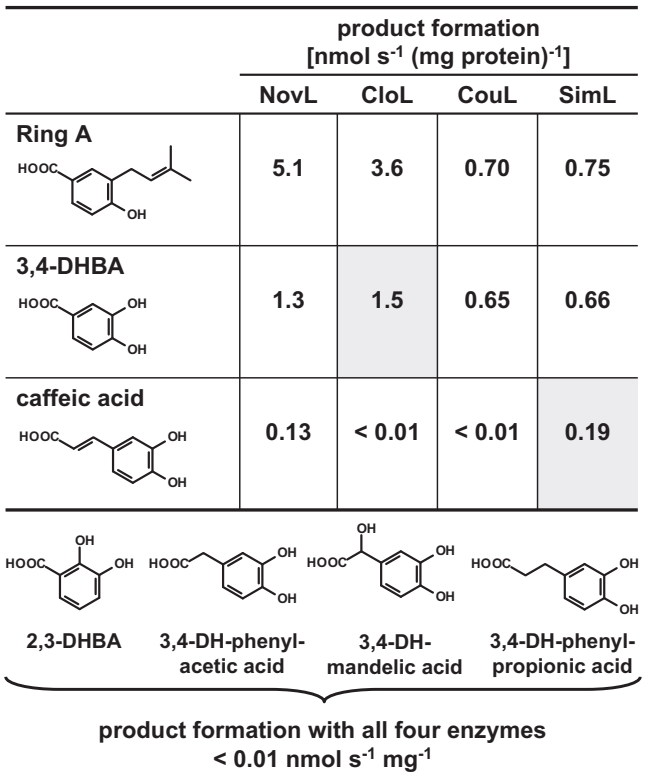

Figure 2. Substrate Tolerance of Different Aminocoumarin Acyl Ligases

Activity of different aminocoumarin acyl ligases with acyl substrates containing catechol motifs. Amide bond formation was determined with 3-amino-4,7-dihydroxy-8-methyl-coumarin as amino substrate in the presence of ATP and Mg^{2+}, as described in the Experimental Procedures.

(Figure 1) with an acyl moiety containing a catechol structure. We considered six different acids which contain catechol motifs (Figure 2). Previous mutasynthetic experiments, aimed at the replacement of Ring A by other acyl groups, had shown that the success of those experiments is dependent primarily on the acceptance of the acyl substrate by the aminocoumarin acyl ligase (= amide synthetase) which attaches the acyl moiety to the 3-amino group of the aminocoumarin moiety (Anderle et al., 2007; Galm et al., 2004a). The later biosynthetic steps, i.e., glycosylation and final tailoring steps, appeared not to be affected by modifications of the structure of the acyl moiety. We therefore tested the acceptance of the six acyl substrates with catechol motifs (Figure 2) by four different aminocoumarin acyl ligases, i.e., NovL, CloL, CouL, and SimL, obtained from the biosynthetic gene cluster of novobiocin, clorobiocin, coumermycin A_1, and simocyclinone D8, respectively. These four enzymes were expressed and purified as described previously (Galm et al., 2004a; Luft et al., 2005; Schmutz et al., 2003; Steffensky et al., 2000a). Aminocoumarin acyl ligase activity was tested using 3-amino-4,7-dihydroxy-8-methyl-coumarin as amino substrate and the six catechols as acyl substrates in an in vitro assay for amide formation (see Experimental Procedures). The identity of the resulting compounds was confirmed by LC-MS. As shown in Figure 2, the best accepted catechol substrate was 3,4-dihydroxybenzoic acid (3,4-DHBA). In case

of CloL, amide bond formation with this substrate reached 42% of the reaction velocity observed with the genuine substrate Ring A. Caffeic acid was not accepted by CloL but by SimL, albeit only to a low extent. The other four catechols were not accepted by any of the investigated enzymes. We therefore concentrated further experiments on 3,4-DHBA and the aminocoumarin acyl ligase of clorobiocin biosynthesis, i.e., CloL.

Inactivation of *cloQ* in the Biosynthetic Gene Cluster of Clorobiocin, and Heterologous Expression of the Modified Cluster

In order to replace Ring A in clorobiocin with 3,4-DHBA, the biosynthesis of Ring A had to be abolished to avoid competition between the genuine and the artificial acyl moiety as precursor in the antibiotic biosynthesis. The first committed step in Ring A biosynthesis is the prenylation of 4-hydroxyphenylpyruvate under catalysis of CloQ (Pojer et al., 2003). Therefore, we carried out an inactivation of the gene *cloQ* within the biosynthetic gene cluster of clorobiocin.

The heterologous expression cosmid clo-BG1, containing the clorobiocin biosynthetic gene cluster, had been prepared previously (Eustáquio et al., 2005). It contains the ΦC31 attachment site for stable integration into the genome of different *Streptomyces* host strains. Using Red/ET-mediated recombination, we replaced the coding sequence of *cloQ* with an apramycin resistance gene (see Experimental Procedures). The resistance cassette was subsequently removed by restriction digestion and religation, using XbaI and SpeI sites introduced into the PCR primer sequence as described previously (Gust, 2009). This *cloQ*-deficient gene cluster was integrated into the genome of *S. coelicolor* M512, resulting in *S. coelicolor* M512(clo-SA2).

Cultivation of this strain in clorobiocin production medium did not result in production of clorobiocin, while corresponding heterologous expression strains containing the intact cluster produce this antibiotic (Eustáquio et al., 2005; Flinspach et al., 2010). However, feeding of Ring A (1 mg per 80 ml culture medium) restored production of clorobiocin, as confirmed by HPLC and HPLC-MS analysis in comparison to an authentic reference compound. Therefore, the inactivation of *cloQ* had led to the abolishment of the production of Ring A, but had not affected the subsequent steps of clorobiocin biosynthesis.

Mutasynthetic Experiments with 3,4-DHBA and Caffeic Acid

Feeding of different amounts of 3,4-DHBA to cultures of *S. coelicolor* M512(clo-SA2) did not lead to the formation of a clorobiocin derivative, as shown in HPLC and LC-MS analysis. In a parallel experiment, we also fed caffeic acid. In these experiments, we additionally introduced a SimL expression plasmid, pSH2 (Anderle et al., 2007), since SimL but not CloL was able to accept caffeic acid (Figure 2). Also these experiments remained unsuccessful. However, we noticed that caffeic acid was clearly detected in the cultures after feeding, while no detectable amounts of 3,4-DHBA were present in the cultures already one day after feeding. We speculated that 3,4-DHBA may rapidly be oxidized in the medium, or may quickly be metabolized by catabolic pathways similar to those described in *Corynebacterium* (Merkens et al., 2005). We therefore considered whether a continuous in vivo biosynthesis of 3,4-DHBA might be superior to an external feeding of this compound in order to supply 3,4-DHBA for aminocoumarin antibiotic formation.

Creating an Artificial Pathway to 3,4-Dihydroxybenzoic Acid

3,4-DHBA can be formed by the well-characterized 4-hydroxybenzoate-3-hydroxylase PobA of *Corynebacterium cyclohexanicum* (Figure 3) (Fujii and Kaneda, 1985; Huang et al., 2008). This 44 kDa flavoprotein monooxygenase is involved in catabolic processes, e.g., lignin degradation. In all Gram-positive bacteria it uses NADH in order to provide the required reduction equivalents. However, the substrate of PobA, 4-hydroxybenzoic acid (4HBA), is not expected to be present in *Streptomyces* in high concentrations. 4HBA is an intermediate of ubiquinone biosynthesis. In most organisms, it is formed by degradation of tyrosine (Meganathan, 2001). However, Gram-negative bacteria such as *E. coli* derive 4HBA directly from chorismate by elimination of the enol-pyruvyl side chain under catalysis of chorismate pyruvate lyase, a 19 kDa protein encoded by the gene *ubiC* (Figure 3) (Siebert et al., 1992). The reaction does not require cofactors. By heterologous expression of *ubiC* in the chloroplasts of plants, which do not contain an ortholog of this gene, very large amounts of 4HBA could be generated without any detrimental effect on growth (Viitanen et al., 2004). Also *Streptomyces* genomes do not contain an ortholog of *ubiC*. An expression of both *ubiC* and *pobA* therefore presented an attractive possibility to generate 3,4-DHBA in our *Streptomyces* strains in vivo.

The GC content of *ubiC* of *E. coli* is 53%, much lower than the average content in genes of the GC-rich *Streptomyces*. Therefore, the sequence of *ubiC* was modified in order to adapt it to the codon preference of *Streptomyces*. The Gene Designer program (DNA 2.0, Menlo Park, CA) was employed for this purpose, using the codon usage table of *Streptomyces coelicolor*. The same was done for the gene *pobA* of *Corynebacterium cyclohexanicum*, although fewer modifications were required for this gene from a GC-rich species. The two genes were translationally coupled in order to increase translation efficiency and to facilitate co-regulation. The nucleotide sequence of the synthetic *ubiC/pobA* construct is given in Figure S1 (available online). After commercial synthesis, this DNA fragment was cloned into the *Streptomyces* expression vector pUWL201 (Doumith et al., 2000), placing it under control of the strong constitutive *ermE** promoter. The resulting plasmid, pSA11, was introduced into *S. coelicolor*(clo-SA2) by protoplast transformation. Thereby, all genes required for the biosynthesis of the desired compound were assembled in the heterologous expression strain (Figure 3).

Production of Novclobiocin 401 by *Streptomyces coelicolor*(clo-SA2) Harboring Plasmid pSA11

Strain *S. coelicolor*(clo-SA2) harbouring pSA11 was cultivated in three different media: (1) the complex clorobiocin production medium described for the wild-type producer strain *Streptomyces roseochromogenes* var. *oscitans* DS 12.976 (Mancy et al., 1974); (2) the complex GYM medium described for *S. coelicolor* A3(2) fermentation (Shima et al., 1996); (3) the chemically defined medium (CDM) developed for novobiocin production in the wild-type producer strain *Streptomyces niveus* (Kominek, 1972). Ethyl acetate extracts from cultures in the three media

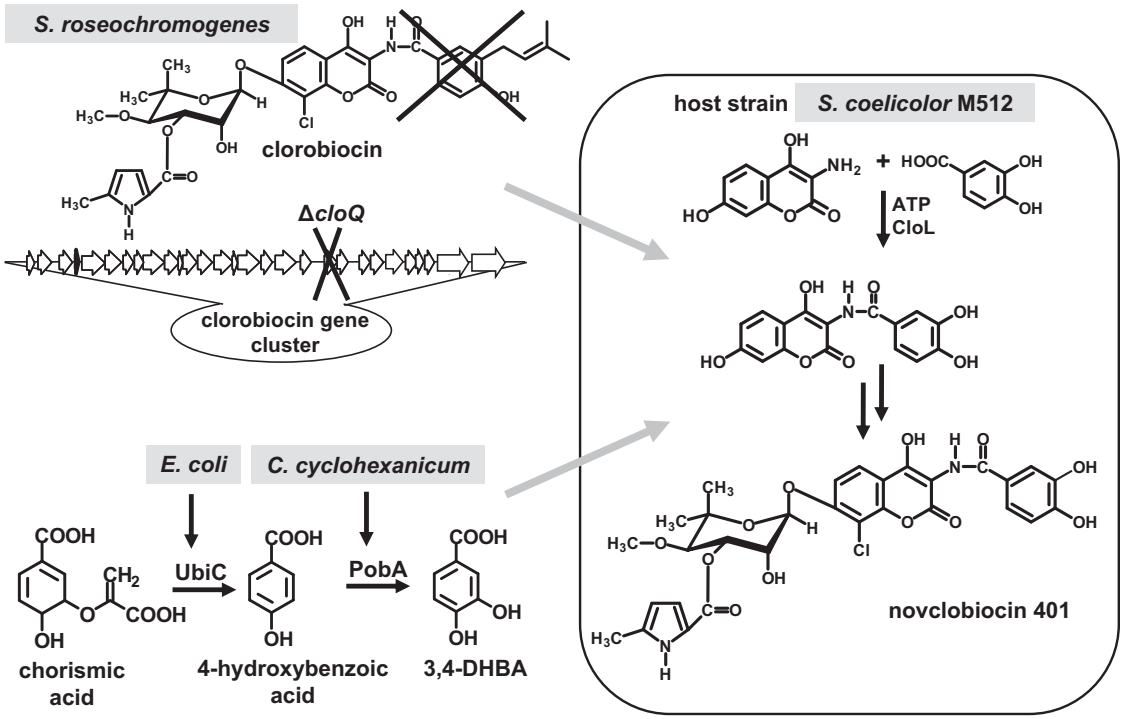

Figure 3. Strategy for the Generation of a Clorobiocin Derivative Containing a 3,4-Dihydroxybenzoyl Moiety
The sequence of the synthetic *ubiC/pobA* operon is shown in Figure S1.

were analyzed by HPLC and LC-MS. The formation of a new compound with the expected molecular ion of *m/z* 643 $[M-H]^-$ was detected in all media. The amount of this compound was moderate using the two complex media (4 and 7 μg/ml, respectively), but was much higher using the chemically defined medium (64 μg/ml). In this case, the new metabolite clearly represented a major compound in the extract (Figure 4A). Preparative isolation of this metabolite from 2 l of culture resulted in 18 mg of pure compound (Figure 4B). This new clorobiocin derivative was termed novclobiocin 401.

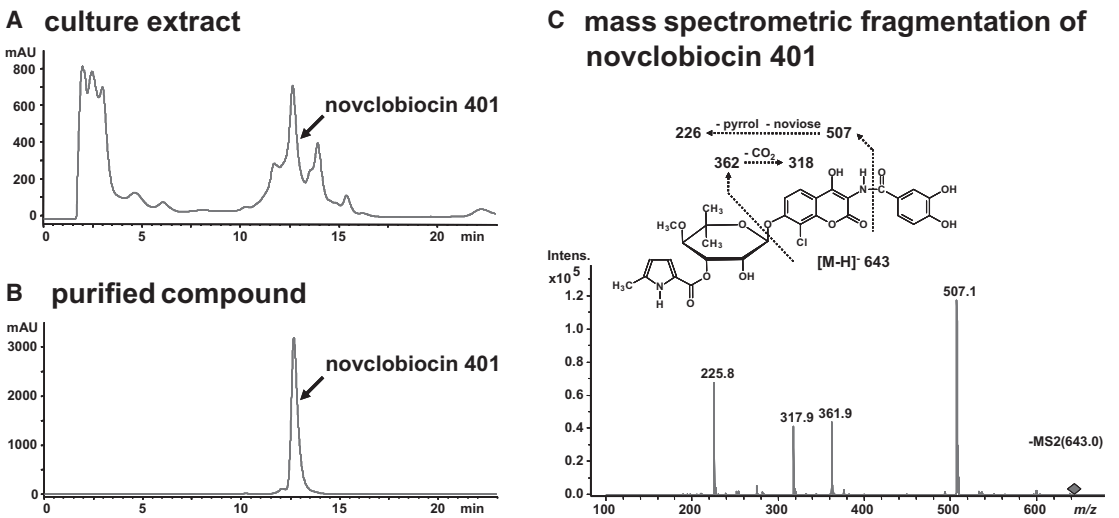

Figure 4. HPLC and LC-MS Analysis
(A) HPLC chromatogram of a culture extract from the heterologous producer strain *Streptomyces coelicolor* M512(clo-SA2)/pSA11 cultivated in CDM medium.
(B) Purified compound isolated from cultures in CDM medium.
(C) Mass spectrometric fragmentation of novclobiocin 401 obtained by selected ion monitoring chromatograms. LC-ESI-MS mass scans were performed in negative mode for *m/z* 643. The suggested fragmentation scheme for the compound is shown.

Structure Elucidation of Novclobiocin 401

LC-MS analysis in negative mode showed the presence of the molecular ion of *m/z* 643 $[M-H]^-$, corresponding to the expected molecular mass of 644 for novclobiocin 401. MS/MS analysis (Figure 4C) showed a fragmentation pattern corresponding to that identified in previous mass spectrometric studies of aminocoumarin antibiotics (Kammerer et al., 2004). High resolution ESI-MS in positive mode showed a molecular ion of *m/z* = 645.14858, which is in agreement with the calculated value of *m/z* = 645.14818 ($C_{30}H_{30}N_2O_{12}Cl$ $[M+H]^+$; Δ 0.62 ppm).

Unidimensional (^{1}H NMR, ^{13}C NMR) and multidimensional (^{1}H-^{1}H COSY, ^{1}H HSQC, and ^{1}H HMBC) NMR spectroscopy confirmed the expected structure. Chemical shifts for the substituted pyrrole, the deoxysugar and the aminocoumarin moiety were in accordance to those of clorobiocin (Eustáquio et al., 2003). The chemical shifts and coupling patterns of the protons in ^{1}H NMR spectra of novclobiocin 401 showed the catechol structure of the new acyl moiety. A strong high-field ^{13}C NMR shift for carbon C-2 (δ_C = 116.1) and C-4 (δ_C = 150.8) in novclobiocin 401 compared to clorobiocin ($\delta_{C\text{-}2}$ = 130.9, $\delta_{C\text{-}4}$ = 161.0) indicated a new hydroxyl substitution in ortho-position (C-3). Additionally, the NMR spectra of novclobiocin 401 lack all proton and carbon NMR signals of the dimethylallyl group of the native clorobiocin. Full comparative ^{1}H and ^{13}C NMR spectroscopic data of novclobiocin 401 and clorobiocin as well as ^{1}H-^{1}H COSY, ^{1}H HSQC and ^{1}H HMBC correlations from 2D NMR experiments for novclobiocin 401 are given in Table S1, Figures S2 and S3, and Supplemental Experimental Procedures.

Inhibitory activities against *E. coli* and *S. aureus* DNA Gyrase and Topoisomerase IV

The new compound was investigated in vitro for its inhibitory effects on *E. coli* and *S. aureus* DNA gyrase and topoisomerase IV in comparison with the natural antibiotics clorobiocin and novobiocin. Two different assays were used: a DNA gyrase supercoiling assay and a topoisomerase IV decatenation assay. The 50% inhibitory concentrations (IC_{50}s) of novclobiocin 401 were determined as 0.006 μM for *S. aureus* DNA gyrase and 0.03 μM for *E. coli* DNA gyrase (Figure 5). These inhibitory concentrations are identical to those observed with clorobiocin and lower than those observed with the clinically used novobiocin (inhibitory concentration: 0.01 μM and 0.08 against the two gyrases, respectively). This clearly proves that the replacement of the genuine Ring A moiety with a 3,4-DHBA moiety had not affected the potency of the compound as gyrase inhibitor.

DNA gyrase inhibitors of the fluoroquinolone class also inhibit topoisomerase IV which is very similar to gyrase (Maxwell and Lawson, 2003). Thus, we examined whether novclobiocin 401 also inhibits the decatenation activity of *E. coli* and *S. aureus* topoisomerase IV. However, just as novobiocin and clorobiocin the new compound inhibited topoisomerase IV from *S. aureus* and *E. coli* only at much higher concentrations than required for gyrase inhibition (IC_{50} values: 35 μM *for S. aureus* topoisomerase IV and >50 μM for *E. coli* topoisomerase IV) (Figure 5). Therefore, novclobiocin 401 is expected to act primarily as gyrase inhibitor in both *S. aureus* and *E. coli*.

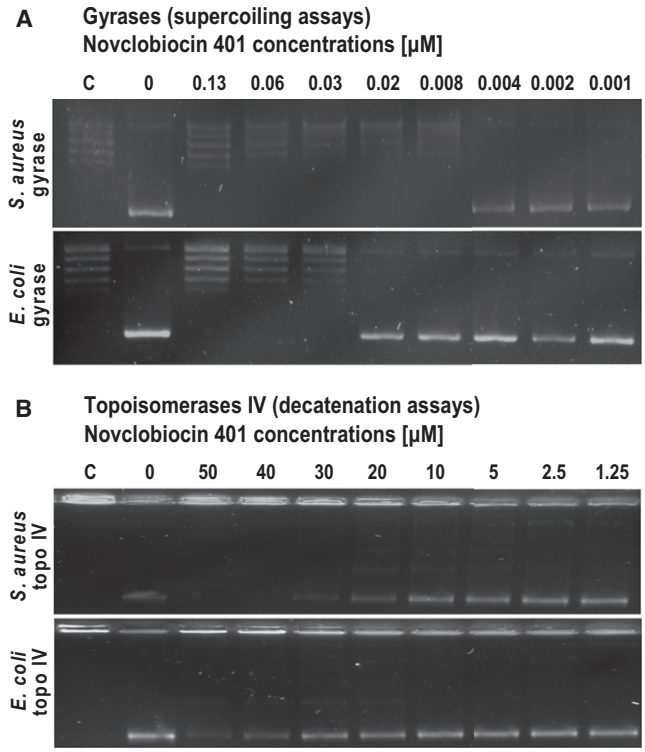

Figure 5. Determination of the Inhibitory Activity by Supercoiling and Decatenation Assays

Supercoiling (A) and decatenation (B) assays with *E. coli* and *S. aureus* DNA gyrase and topoisomerase IV. The first lane labeled with C contains control assays without enzyme. The lane labeled 0 contains assays with addition of 3 μl solvent (5% aqueous DMSO). The following lanes contain assays to which the indicated amount of antibiotic, dissolved in 5% DMSO, has been added. In the supercoiling assays, the lower band shows supercoiled pBR322 DNA, formed under catalysis of DNA gyrase. In the decatenation assays, the lower band shows decatenated kinetoplast DNA, formed under catalysis of topoisomerase IV.

Construction of *E. coli* Mutants for Investigation of Antibiotic Import by Catechol Siderophore Transporters

As reported above, *E. coli* gyrase is equally sensitive to clorobiocin and novclobiocin 401. A difference in the antibacterial activity of these two compounds may therefore be primarily caused by either increased influx or decreased efflux. In order to observe differences in influx without interferences by differences in efflux, we inactivated the gene *tolC* which codes for an essential part of the AcrAB/TolC and AcrAD/TolC drug efflux pumps of *E. coli* (Blair and Piddock, 2009).

E. coli possesses three outer membrane transporters for active import of catechol siderophores, i.e., Fiu, Cir, and FepA (Braun and Hantke, 2001). All three transporters receive the energy for active transport from the periplasmic binding protein TonB. Expression of these proteins is upregulated under conditions of iron starvation. Bacterial growth during an infection in the human body represents a condition of extreme iron starvation (Chu et al., 2010). For functional studies of catechol siderophore transport in *E. coli*, conditions of iron starvation are usually created by (1) mutation of *entC* which abolishes the biosynthesis of the important siderophore enterobactin (Crosa and Walsh, 2002); (2)

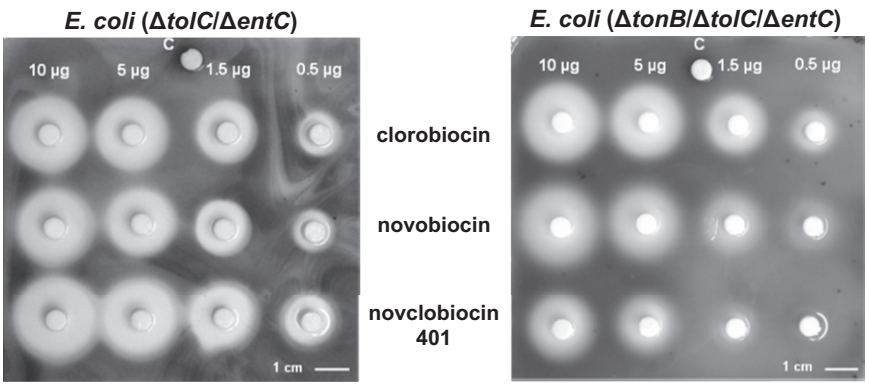

Figure 6. Determination of the Antibacterial Activity by Disk Diffusion Assays
The antibacterial activity of clorobiocin, novobiocin, and novclobiocin 401 was determined against *E. coli* mutants with and without active TonB. To improve visualization of the inhibition zones, living cells were stained with 2,3,5-triphenyltetrazolium chloride.

addition of the iron chelator 2,2′-bipyridyl to the culture medium (Alves et al., 2010; Möllmann et al., 2009). We therefore decided to use Δ*entC* mutants and 2,2′-bipyridyl containing media.

The involvement of the catechol siderophore transporters Fiu, Cir, and FepA in the uptake of novclobiocin 401 can be investigated by comparing mutants with and without active TonB for their sensitivity to clorobiocin and novclobiocin 401. As described above, TonB energizes all three catechol transporters.

Due to these considerations, we decided to generate an *E. coli* Δ*tolC*/Δ*entC* mutant as well as an *E. coli* Δ*tonB*/Δ*tolC*/Δ*entC* mutant and to compare their sensitivity to clorobiocin and novclobiocin 401. Single gene in-frame deletion mutants of *entC, tolC,* and *tonB* in *E. coli* K-12 were available from the Keio collection (Baba et al., 2006). Using these strains, we generated *E. coli* Δ*tolC*/Δ*entC* and *E. coli* Δ*tonB*/Δ*entC* double mutants as well as an *E. coli* Δ*tonB*/Δ*tolC*/Δ*entC* triple mutant by Red/ET-mediated recombination (see Experimental Procedures). The correct genotype of the mutants was verified by PCR analysis of the genomic DNA of the mutant.

Antibacterial Activity of Novclobiocin 401

The antibacterial activity of novclobiocin 401, in comparison to clorobiocin and novobiocin, was first tested in disk diffusion assays with the *E. coli* Δ*tolC*/Δ*entC* and the *E. coli* Δ*tonB*/Δ*tolC*/Δ*entC* mutants. To improve visualization of the inhibition zones, living cells on the agar plates were stained with 2,3,5-triphenyltetrazolium chloride. The results are shown in Figure 6. Against the mutant with intact TonB, novclobiocin 401 shows approximately 2-fold higher activity than clorobiocin, and 4-fold higher activity than novobiocin. In contrast, against the *E. coli* mutant with deleted *tonB* novclobiocin 401 is approximately 10-fold less active than clorobiocin. This shows that TonB-dependent transport plays an important role in the antibacterial activity of novclobiocin 401 but not in the activity of clorobiocin and novobiocin.

We also determined the minimal inhibitory concentrations in a liquid culture, following the procedure described by Wiegand et al. (2008), but including 50 μM 2,2′-bipyridyl into the culture media. Under these conditions, the minimal inhibitory concentrations of novobiocin and clorobiocin against *E. coli* Δ*tolC*/Δ*entC* double mutants were 23 and 12 μg/ml, respectively. The MIC of novclobiocin 401 was 6 μg/ml, showing again that novclobiocin 401 had a higher antibacterial activity than its parent compound clorobiocin against this mutant. Against the Δ*tonB*/Δ*tolC*/Δ*entC* triple mutant, novobiocin and clorobiocin gave the same MIC values as observed against the Δ*tolC*/Δ*entC* double mutant (23 and 12 μg/ml, respectively). However, novclobiocin 401 showed much less activity against the *tonB*-deficient triple mutant (MIC: 47 μg/ml) than against the double mutant with active *tonB* (MIC: 6 μg/ml). This shows again that TonB-dependent transport is important for the antibacterial activity of novclobiocin 401, but not of novclobiocin and clorobiocin.

Against the wild-type strain *E. coli* K-12, the MICs of novobiocin, clorobiocin and novclobiocin 401 resulted as 375, 47, and 95 μg/ml. Therefore, against the wild-type novclobiocin 401 was less active than its parent compound clorobiocin (see Discussion). The poor activity of aminocoumarins against wild-type strains of *E. coli* is in accordance with previous observations (Anderle et al., 2008; Galm et al., 2004b).

DISCUSSION

The present study provides an example for the rational design of a structurally modified antibiotic by utilizing synthetic biology principles. Our engineering strategy involved genes from four different organisms: (1) the Gram-negative organism *E. coli* as the source of *ubiC*, which codes for an enzyme of an anabolic pathway; (2) the Gram-positive organism *Corynebactericum cyclohexanicum* as the source of *pobA*, coding for an enzyme of a catabolic pathway; (3) *Streptomyces roseochromogenes* as the source of the biosynthetic gene cluster of clorobiocin, which was modified by deletion of *cloQ*; (4) *Streptomyces coelicolor* M512 as heterologous expression host. *S. coelicolor* M512 (Floriano and Bibb, 1996) is a derivative of the strain *S. coelicolor* A3(2). Strain M512 does not produce three of the genuine antibiotics of strain A3(2), i.e., methylenomycin, actinorhodin, and undecylprodigiosine, which facilitates the detection and isolation of heterologously produced compounds. The modified clorobiocin gene cluster was stably integrated into the chromosome of this organism, and the synthetic operon containing *ubiC* and *pobA* under control of the strong constitutive *ermE** promoter was expressed from a replicative plasmid. This readily resulted in the production of the desired compound novclobiocin 401. The yield of novclobiocin 401 exceeded that of clorobiocin reported for both the wild-type and for a heterologous producer strain (Eustáquio et al., 2005). Even without extensive optimization experiments, novclobiocin 401 represented a major compound in the culture extract (Figure 4A) which facilitated the preparative isolation of the compound. Recently, improved heterologous expression strains have been derived from *S. coelicolor* and may offer a benefit in future experiments (Gomez-Escribano and Bibb, 2010).

The production of novclobiocin 401 did not result in any impairment of growth of the heterologous producer strain. Apparently, both the diversion of chorismate for the production of 3,4-DHBA and the accumulation of the potent gyrase inhibitor novclobiocin 401 were tolerated well. Self-resistance of the heterologous producer strain was expected, as the clorobiocin gene cluster, expressed in *S. coelicolor* M512, contains an aminocoumarin resistance gene (Eustáquio et al., 2005).

The genetic engineering experiment resulted in a clorobiocin derivative in which the genuine Ring A (Figure 1) is replaced with a 3,4-DHBA moiety. In vitro investigation of the inhibitory effect of this compound on the gyrases of *E. coli* and *S. aureus* showed no change of activity in comparison to the parent compound clorobiocin. This confirms that the structure of Ring A is of little importance for the interaction of aminocoumarin antibiotics with their principal target, and can be modified to introduce desirable motifs into the molecule. It should be noted that the IC_{50} of novclobiocin 401 against the gyrases of *E. coli* and *Staphylococcus aureus* is two to three orders of magnitude lower than that of modern fluoroquinolones (Takei et al., 2001), confirming the strong potency of aminocoumarins.

Novclobiocin 401, containing a catechol motif, showed higher antibacterial activity than its parent compound clorobiocin against *E. coli* mutants which were defective in their TolC-dependent efflux pumps. When additionally *tonB* was deleted, the activity of novclobiocin 401 was reduced eightfold (as calculated from the MIC values), while the activity of clorobiocin remained unchanged. TonB supplies the energy for the catechol siderophore transporters Cir, Fiu, and FepA, and therefore this result strongly suggests that these transporters are involved in the active import of novclobiocin 401. Previous experiments showed that especially Cir and Fiu have a broad substrate specificity and can import catechol compounds with quite different structures (Heinisch et al., 2003).

Uptake of drug-siderophore conjugates by Cir and Fiu has been demonstrated previously, especially for cephalosporin-catechol conjugates (Heinisch et al., 2003; Möllmann et al., 2009). In these experiments, the catechol moiety conjugated to the antibiotic added considerably to the molecular weight of the drug; often the size of the catechol moiety exceeded the size of the antibiotic moiety. In the present study, a preexisting moiety (i.e., Ring A) of the antibiotic was replaced by a catechol moiety, and the molecular weight of the new catechol compound (645 Da) was lower than that of the parent compound clorobiocin (697 Da).

Though novclobiocin 401 was transported into *E. coli* cells under involvement of TonB-dependent transporters, it did not show higher activity than clorobiocin against wild-type and *ΔentC* mutants of *E. coli*. The MIC values of clorobiocin and novclobiocin 401 against *ΔentC* mutants were 23 and 95 μg/ml, respectively. Under identical conditions, the MICs against *ΔentC*/*ΔtolC* double mutants were 12 and 6 μg/ml, respectively. These results suggest that novclobiocin 401 is more rapidly exported than clorobiocin by TolC-dependent drug efflux pumps. The introduction of a catechol moiety into the clorobiocin molecule therefore had the desired effect to facilitate active import by catechol siderophore transporters, but also the undesired effect to accelerate TolC-dependent efflux. Future attempts to improve the activity of aminocoumarins against Gram-negative organism may therefore aim at a reduced efflux, either by modification of the drug or by combination with an efflux pump inhibitor, and at a further improvement of active import, e.g., by inclusion of a 2,3-DHBA moiety which is the catechol moiety present in enterobactin. The chemical synthesis of a clorobiocin derivative with a 2,3-DHBA moiety is not straightforward, since a clorobiocin derivative lacking Ring A (e.g., containing only Rings B and C, Figure 1) can not be obtained from any producer strain, due to the fact that Ring A is the starter moiety for clorobiocin biosynthesis. The de novo chemical synthesis of an entire aminocoumarin antibiotic is a complicated multistep procedure (Laurin et al., 1999). Generation of a clorobiocin derivative with a 2,3-DHBA moiety by genetic engineering depends on the identification of an aminocoumarin acyl ligase which, in contrast to the presently known enzymes, can accept 2,3-DHBA as substrate (Figure 2). A search for such an aminocoumarin acyl ligase is currently under way in our laboratory.

SIGNIFICANCE

The present study provides an example that the rational modification of the structure of an antibiotic can be achieved by a synthetic biology approach. Specifically, we planned to replace a structural moiety which was not required for antibiotic activity by a different moiety which would serve as an "import handle," facilitating the import of the antibiotic into the bacterial cell by catechol siderophore transporters. Our study provides a proof-of-principle for this strategy. It confirms earlier results that catechol siderophore transporters can be used in a "Trojan horse" approach for the active import of an antibiotic. We achieved the incorporation of a catechol motif into the structure of the antibiotic utilizing a synthetic biology strategy. Such strategies are promising for the generation of new bioactive molecules in future. The growing number of sequenced biosynthetic gene clusters and microbial genomes provides a rich pool for enzymes which can be reassembled to generate artificial biosynthetic pathways. In the present study, genes from three different organisms were combined in a fourth organism, resulting in the biosynthesis of the desired antibiotic. DNA synthesis was used to adapt the codon usage of two of the introduced genes to the requirement of the expression host. We used a genetically engineered and fully sequenced expression host which is amenable to rapid genetic manipulation and devoid of several interfering biosynthetic pathways. Considering the current advances in the technologies for DNA sequencing, DNA synthesis, DNA manipulation, and engineering of expression hosts, many new bioactive compounds may be accessible in future by the generation of artificial biosynthetic pathways. The present study provides an example for such an approach.

EXPERIMENTAL PROCEDURES

Chemicals

Novobiocic acid was isolated from *Streptomyces spheroides* AM1T2 (Steffensky et al., 2000b); 3-amino-4,7-dihydroxy-8-methyl-coumarin (Ring B) was kindly provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI). 3-Dimethylallyl-4-hydroxybenzoic acid (Ring A) was obtained by hydrolysis of novobiocin (Kominek and Meyer, 1975). Novobiocin and catechol compounds were

purchased from Sigma-Aldrich. Clorobiocin was kindly provided by A. Maxwell (John Innes Centre, Norwich, UK).

Bacterial Strains, Plasmids, Cosmids, and Culture Conditions

The *E. coli* and *Streptomyces* strains, plasmids, and cosmids used in this study are listed in Table S2. *E. coli* strains were cultivated in liquid or on solid LB medium at 37°C. *Streptomyces* strains were routinely precultivated in liquid TSB medium (BD Bioscience) at 30°C for 2 days. The cultivation was continued in distillers' solubles medium (Mancy et al., 1974), GYM medium (Shima et al., 1996), and CDM medium (Kominek, 1972) for antibiotic production at 30°C for 5–8 days. Standard methods for cultivation, DNA isolation and manipulation were performed as described by Sambrook and Russell, (2001) and Kieser et al. (2000).

Assay for Aminocoumarin Acyl Ligase

The assay contained 2 mM of the respective acyl substrate (3,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxypropionic acid or 3,4-dihydroxymandelic acid), 2 mM 3-amino-4,7-dihydroxy-8-methyl-coumarin (Ring B), 5 mM ATP, 5 mM $MgCl_2$, 10 mM ascorbic acid, 100 mm Tris-HCl (pH 8.0) and 10 μg of the respective enzyme in a final volume of 100 μl. The reaction was carried out for 45 min at 30°C and stopped by addition of 5 μl 1.5 M trichloroacetic acid. The reaction mixture was extracted with 100 μl ethyl acetate. The organic layer was used for analysis. After evaporation of the solvent and dissolution in methanol, the sample was analyzed by HPLC with a Multosphere RP18-5 column (250 × 4 mm, 5 μm; Agilent) at a flow rate of 1 ml·min^{-1}. A linear gradient from 60% to 100% solvent B (solvent A H_2O/HCOOH 99:1; solvent B MeOH/HCOOH 99:1) over 30 min was used. UV detection was carried out at 330 nm. Novobiocic acid was used as standard.

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

In cosmid clo-BG1 (Eustáquio et al., 2005) *cloQ* was replaced by Red/ET-mediated recombination with an apramycin-resistance (aac(3)IV) cassette that was flanked by XbaI and SpeI recognition sites. For replacement of *cloQ*, the cassette was generated by PCR using pUG019 (Eustáquio et al., 2005) as template and the primers *cloQ*_f (5′-GGC GCG CCC ATT GCT CAC CGT CTT ACC GAC ACC GTC CTT ATT CCG GGG ATC TCT AGA TC-3′) and *cloQ*_r (5′-TCC CAT GGT CGA TTC CGT GTG TTG GTG AAG TGC GCG CAG ACT AGT CTG GAG CTG CTT C-3′). Underlined letters indicate the XbaI and SpeI restriction sites. PCR amplification was performed in 50 μl volume with 100 ng template, 0.25 mM dNTPs, 50 pmol of each primer, and 5% (v/v) DMSO with the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94°C for 2 min, then 10 cycles with denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 90 s, followed by 15 cycles with annealing at 55°C for 45 s, and the last elongation step at 72°C for 5 min. The PCR product was introduced by electroporation into *E. coli* BW25113/pIJ790 harboring cosmid clo-BG1 (Eustáquio et al., 2005). The resulting modified cosmid was isolated, transformed into the nonmethylating strain *E. coli* ET12567, reisolated, and digested with XbaI and SpeI to remove the apramycin-resistance cassette. Religation overnight at 4°C gave the cosmid clo-SA2.

The cosmid clo-SA2 isolated from *E. coli* ET12567 was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser et al., 2000). Clones resistant to kanamycin were selected. Feeding experiments were carried out by addition of 1 mg Ring A (3-dimethylallyl-4-hydroxybenzoic acid) dissolved in 100 μl ethanol to 80 ml *S. coelicolor* M512(clo-SA2) culture in distillers' solubles medium (Eustáquio et al., 2003).

For mutasynthesis experiments, 3 mg of the respective catechol compounds were dissolved in 100 μl ethanol and added to 80 ml of the culture of the *cloQ*-defective strain *S. coelicolor* M512(clo-SA2) in distillers' solubles medium one day after inoculation. After 5–8 days cultivation at 30°C and 210 rpm, the cultures were extracted and analyzed by HPLC described below.

Design of the Synthetic Gene Operon

The nucleotide sequences of the genes *pobA* coding for a 4-hydroxybenzoate hydroxylase from *Corynebacterium cyclohexanicum* (GenBank AB210281) and *ubiC* coding for a chorismate pyruvate-lyase from *Escherichia coli* 536 (GenBank NC_008253) were redesigned using the codon preference of *Streptomyces coelicolor* (DNA2.0 Gene Designer Software). The two genes were linked by translational coupling and flanked by SpeI and HindIII restriction sites. The DNA fragment was commercially synthesized by DNA2.0 (Menlo Park, CA) and provided in the vector pJ201. The nucleotide sequence of the synthetic *ubiC*/*pobA* DNA fragment is shown in Figure S1.

Construction of the Plasmid pSA11

The synthetic *ubiC*/*pobA* fragment was isolated from the pJ201 vector (DNA2.0) by restriction digest with SpeI and HindIII and cloned into pUWL201 (Doumith et al., 2000) which contains the constitutive *ermE** promoter for foreign gene expression. Transformation of pSA11 into the integration mutant *S. coelicolor* M512(clo-SA2) was carried out by PEG-mediated protoplast transformation (Kieser et al., 2000). Transformed colonies appeared after 5 days at 30°C.

Production and Purification of Novclobiocin 401

S. coelicolor M512(clo-SA2)/pSA11 was precultivated in TSB medium (BD Bioscience) with 50 μg/ml thiostrepton for 3 days and then used to inoculate 40 flasks, each containing 50 ml CDM production medium (Kominek, 1972) with 50 μg/ml thiostrepton. The cultivation was carried out for 5 days at 30°C and 210 rpm. The culture was adjusted to pH 4 with hydrochloric acid and extracted twice with an equal volume of ethyl acetate. The organic layer was evaporated to dryness. The residue was dissolved in methanol and purified by HPLC with a Multosphere column 120 RP18-5 (250 × 20 mm, 5 μm; C&S Chromatographie Service Düren, Germany) at a flow rate of 2 ml/min. A linear gradient from 70% to 100% solvent B in solvent A over 36 min was used (solvent A H_2O/HCOOH 99:1; solvent B MeOH: HCOOH 99:1).

For analytical purposes, 1 ml bacterial culture was acidified with HCl to pH 4 and extracted with equal volume of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 100 μl methanol. Eighty microliters was analyzed by HPLC with a Multosphere RP18-5 column (250 × 4 mm, 5 μm; Agilent) at a flow rate of 1 ml/min. A linear gradient from 60% to 100% solvent B over 23 min was used. UV detection was carried out at 280 nm.

DNA Gyrase and Topoisomerase IV Activity Assays

Relaxed pBR322 DNA and kDNA (from *Crithidia fasciculata*), DNA gyrase and topoisomerase IV enzymes from *E. coli* and *S. aureus* were obtained from Inspiralis (Norwich, UK). DNA gyrase and topoisomerase IV holoenzymes were reconstituted by mixing approximately equimolar amounts of recombinant *E. coli* and *S. aureus* GyrA and GyrB and ParC and ParE subunits, respectively. DNA gyrase activity was measured by a supercoiling assay that monitored the ATP-dependent conversion of relaxed pBR322 DNA to the supercoiled form. Topoisomerase IV activity was measured by a decatenation assay that monitored the ATP-dependent unlinking of DNA minicircles from kDNA. Details of the assay procedures are described in the Supplemental Experimental Procedures.

Agar Diffusion Tests

Agar plates (40 ml medium) were prepared by suspending 1 ml of a culture of the respective *E. coli* mutant (overnight culture in LB medium, OD_{600} 1.2) and 50 μM 2,2′-bipyridyl in melted Mueller-Hinton agar (Roth). To paper disks of 7 mm diameter, different amounts of the respective antibiotic, dissolved in 15 μl methanol, were applied. These disks were placed on the agar plates which were incubated at 37°C for 16 hr. For visualization of living cells, 5 ml of 0.5% aqueous 2,3,5-triphenyltetrazolium chloride (Roth) were added to the plates, and after further incubation for 10 min, the inhibition zones were determined.

Generation of *E. coli* Mutants

E. coli double and triple mutants were generated from *E. coli* K-12 MG1655 mutants obtained from the Keio collection (Baba et al., 2006). The gene *entC* was replaced in *E. coli* JW5195-1/pIJ790 and JW5503-1/pIJ790 using Red/ET-mediated recombination (Gust et al., 2003). An apramycin resistance cassette [acc(3)IV] was amplified from plasmid pIJ773 (Gust et al., 2003). The primers used for PCR were as follows: *entC*_f: (5′-*TCA TTA TTA AAG CCT TTA TCA TTT TGT GGA GGA TGA TAT* GAT TCC GGG GAT CCG TCG ACC-3′) and *entC*_r (5′-*CCG GCC AGC GGG TGA ATG GAA TGC TCA TCC TCG CTC CTT A*TG TAG GCT GGA GCT GCT TC-3′). Italic letters represent

39 nucleotide homologous extensions for Red/ET-mediated recombination. The gene *tolC* was replaced in *E. coli* JW5195-1/pIJ790 using Red/ET-mediated recombination (Gust et al., 2003). For this purpose, a streptomycin resistance cassette [aadA] was amplified from plasmid pIJ778 (Gust et al., 2003) using the primer pair *tolC*_f (5′-*GAT CGC GCT AAA TAC TGC TTC ACC ACA AGG AAT GCA AAT* GAT TCC GGG GAT CCG TCG ACC-3′) and *tolC*_r (5′-*ACG TTC AGA CGG GGC CGA AGC CCC GTC GTC GTC ATC* ATG TAG GCT GGA GCT GCT TC-3′). The genotype of the resulting mutants was confirmed by PCR with chromosomal DNA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.12.016.

ACKNOWLEDGMENTS

We are grateful to K. Hantke (Tübingen University) and U. Möllmann (Hans Knöll Institut, Jena) for their valuable advice and support. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 766).

Received: November 2, 2010
Revised: December 1, 2010
Accepted: December 2, 2010
Published: March 24, 2011

REFERENCES

Alves, J.R., Pereira, A.C., Souza, M.C., Costa, S.B., Pinto, A.S., Mattos-Guaraldi, A.L., Hirata-Junior, R., Rosa, A.C., and Asad, L.M. (2010). Iron-limited condition modulates biofilm formation and interaction with human epithelial cells of enteroaggregative *Escherichia coli* (EAEC). J. Appl. Microbiol. *108*, 246–255.

Anderle, C., Hennig, S., Kammerer, B., Li, S.M., Wessjohann, L., Gust, B., and Heide, L. (2007). Improved mutasynthetic approaches for the production of modified aminocoumarin antibiotics. Chem. Biol. *14*, 955–967.

Anderle, C., Stieger, M., Burrell, M., Reinelt, S., Maxwell, A., Page, M., and Heide, L. (2008). Biological activities of novel gyrase inhibitors of the aminocoumarin class. Antimicrob. Agents Chemother. *52*, 1982–1990.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006–2008.

Blair, J.M., and Piddock, L.J. (2009). Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Curr. Opin. Microbiol. *12*, 512–519.

Braun, V., and Hantke, K. (2001). Mechanisms of bacterial iron transport. In Microbial Transport Systems, G. Winkelmann, ed. (Weinheim, Germany: Wiley-VCH Verlag GmbH), pp. 289–311.

Chu, B.C., Garcia-Herrero, A., Johanson, T.H., Krewulak, K.D., Lau, C.K., Peacock, R.S., Slavinskaya, Z., and Vogel, H.J. (2010). Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. Biometals *23*, 601–611.

Crosa, J.H., and Walsh, C.T. (2002). Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol. Mol. Biol. Rev. *66*, 223–249.

Doumith, M., Weingarten, P., Wehmeier, U.F., Salah-Bey, K., Benhamou, B., Capdevila, C., Michel, J.M., Piepersberg, W., and Raynal, M.C. (2000). Analysis of genes involved in 6-deoxyhexose biosynthesis and transfer in *Saccharopolyspora erythraea*. Mol. Gen. Genet. *264*, 477–485.

Eustáquio, A.S., Gust, B., Luft, T., Li, S.M., Chater, K.F., and Heide, L. (2003). Clorobiocin biosynthesis in *Streptomyces*. Identification of the halogenase and generation of structural analogs. Chem. Biol. *10*, 279–288.

Eustáquio, A.S., Gust, B., Galm, U., Li, S.M., Chater, K.F., and Heide, L. (2005). Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters. Appl. Environ. Microbiol. *71*, 2452–2459.

Flinspach, K., Westrich, L., Kaysser, L., Siebenberg, S., Gomez-Escribano, J.P., Bibb, M., Gust, B., and Heide, L. (2010). Heterologous expression of the biosynthetic gene clusters of coumermycin A_1, clorobiocin and caprazamycins in genetically modified *Streptomyces coelicolor* strains. Biopolymers *93*, 823–832.

Floriano, B., and Bibb, M. (1996). *AfsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). Mol. Microbiol. *21*, 385–396.

Fujii, T., and Kaneda, T. (1985). Purification and properties of NADH/NADPH-dependent *p*-hydroxybenzoate hydroxylase from *Corynebacterium cyclohexanicum*. Eur. J. Biochem. *147*, 97–104.

Galm, U., Dessoy, M.A., Schmidt, J., Wessjohann, L.A., and Heide, L. (2004a). *In vitro* and *in vivo* production of new aminocoumarins by a combined biochemical, genetic, and synthetic approach. Chem. Biol. *11*, 173–183.

Galm, U., Heller, S., Shapiro, S., Page, M., Li, S.M., and Heide, L. (2004b). Antimicrobial and DNA gyrase-inhibitory activities of novel clorobiocin derivatives produced by mutasynthesis. Antimicrob. Agents Chemother. *48*, 1307–1312.

Gomez-Escribano, J.P., and Bibb, M. (2010). Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. Microb. Biotechnol., in press. Published online October 26, 2010. doi:10.1111/j.1751–7915.2010.00219.x.

Gust, B. (2009). Cloning and analysis of natural product pathways. Methods Enzymol. *458*, 159–180.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA *100*, 1541–1546.

Heide, L. (2009a). The aminocoumarins: biosynthesis and biology. Nat. Prod. Rep. *26*, 1241–1250.

Heide, L. (2009b). Genetic engineering of antibiotic biosynthesis for the generation of new aminocoumarins. Biotechnol. Adv. *27*, 1006–1014.

Heinisch, L., Wittmann, S., Stoiber, T., Scherlitz-Hofmann, I., Ankel-Fuchs, D., and Mollmann, U. (2003). Synthesis and biological activity of tris- and tetrakiscatecholate siderophores based on poly-aza alkanoic acids or alkylbenzoic acids and their conjugates with beta-lactam antibiotics. Arzneimittelforschung *53*, 188–195.

Hopwood, D.A. (2009a). Complex Enzymes in Microbial Natural Product Biosynthesis; Part A: Overview Articles and Peptides. Methods in Enzymology (San Diego, CA: Elsevier/ Academic Press).

Hopwood, D.A. (2009b). Complex Enzymes in Microbial Natural Product Biosynthsis. Part B: Polyketides, Aminocoumarins and Carbohydrates. Methods in Enzymology (San Diego, CA: Elsevier/ Academic Press).

Hopwood, D.A., Malpartida, F., Kieser, H.M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B.A., Floss, H.G., and Omura, S. (1985). Production of "hybrid" antibiotics by genetic engineering. Nature *314*, 642–644.

Huang, Y., Zhao, K.X., Shen, X.H., Jiang, C.Y., and Liu, S.J. (2008). Genetic and biochemical characterization of a 4-hydroxybenzoate hydroxylase from *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. *78*, 75–83.

Kammerer, B., Kahlich, R., Laufer, S., Li, S.M., Heide, L., and Gleiter, C.H. (2004). Mass spectrometric pathway monitoring of secondary metabolites: systematic analysis of culture extracts of *Streptomyces* species. Anal. Biochem. *335*, 17–29.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics (Norwich, UK: John Innes Foundation).

Kominek, L.A. (1972). Biosynthesis of novobiocin by *Streptomyces niveus*. Antimicrob. Agents Chemother. *1*, 123–134.

Kominek, L.A., and Meyer, H.F. (1975). Novobiocic acid synthetase. Methods Enzymol. *43*, 502–508.

Laurin, P., Ferroud, D., Klich, M., Dupuis-Hamelin, C., Mauvais, P., Lassaigne, P., Bonnefoy, A., and Musicki, B. (1999). Synthesis and *in vitro* evaluation of novel highly potent coumarin inhibitors of gyrase B. Bioorg. Med. Chem. Lett. *9*, 2079–2084.

Luft, T., Li, S.M., Scheible, H., Kammerer, B., and Heide, L. (2005). Overexpression, purification and characterization of SimL, an amide synthetase involved in simocyclinone biosynthesis. Arch. Microbiol. *183*, 277–285.

Mancy, D., Ninet, L., and Preudi Homme, J. (1974). U.S. Patent 3793147.

Maxwell, A., and Lawson, D.M. (2003). The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. Curr. Top. Med. Chem. *3*, 283–303.

Meganathan, R. (2001). Ubiquinone biosynthesis in microorganisms. FEMS Microbiol. Lett. *203*, 131–139.

Merkens, H., Beckers, G., Wirtz, A., and Burkovski, A. (2005). Vanillate metabolism in *Corynebacterium glutamicum*. Curr. Microbiol. *51*, 59–65.

Miethke, M., and Marahiel, M.A. (2007). Siderophore-based iron acquisition and pathogen control. Microbiol. Mol. Biol. Rev. *71*, 413–451.

Möllmann, U., Heinisch, L., Bauernfeind, A., Kohler, T., and Ankel-Fuchs, D. (2009). Siderophores as drug delivery agents: application of the "Trojan Horse" strategy. Biometals *22*, 615–624.

Neilands, J.B. (1995). Siderophores: structure and function of microbial iron transport compounds. J. Biol. Chem. *270*, 26723–26726.

Pojer, F., Wemakor, E., Kammerer, B., Chen, H., Walsh, C.T., Li, S.M., and Heide, L. (2003). CloQ, a prenyltransferase involved in clorobiocin biosynthesis. Proc. Natl. Acad. Sci. USA *100*, 2316–2321.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning. A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schmutz, E., Steffensky, M., Schmidt, J., Porzel, A., Li, S.M., and Heide, L. (2003). An unusual amide synthetase (CouL) from the coumermycin A_1 biosynthetic gene cluster from *Streptomyces rishiriensis* DSM 40489. Eur. J. Biochem. *270*, 4413–4419.

Shima, J., Penyige, A., and Ochi, K. (1996). Changes in patterns of ADP-ribosylated proteins during differentiation of *Streptomyces coelicolor* A3(2) and its development mutants. J. Bacteriol. *178*, 3785–3790.

Siebert, M., Bechthold, A., Melzer, M., May, U., Berger, U., Schröder, G., Schröder, J., Severin, K., and Heide, L. (1992). Ubiquinone biosynthesis. Cloning of the genes coding for chorismate pyruvate-lyase and 4-hydroxybenzoate octaprenyl transferase from *Escherichia coli*. FEBS Lett. *307*, 347–350.

Steffensky, M., Li, S.M., and Heide, L. (2000a). Cloning, overexpression, and purification of novobiocic acid synthetase from *Streptomyces spheroides* NCIMB 11891. J. Biol. Chem. *275*, 21754–21760.

Steffensky, M., Mühlenweg, A., Wang, Z.X., Li, S.M., and Heide, L. (2000b). Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. Antimicrob. Agents Chemother. *44*, 1214–1222.

Takei, M., Fukuda, H., Kishii, R., and Hosaka, M. (2001). Target preference of 15 quinolones against *Staphylococcus aureus*, based on antibacterial activities and target inhibition. Antimicrob. Agents Chemother. *45*, 3544–3547.

Tropp, B.E., Ragolia, L., Xia, W., Dowhan, W., Milkman, R., Rudd, K.E., Ivanišević, R., and Savić, D.J. (1995). Identity of the *Escherichia coli cls* and *nov* genes. J. Bacteriol. *177*, 5155–5157.

Viitanen, P.V., Devine, A.L., Khan, M.S., Deuel, D.L., Van Dyk, D.E., and Daniell, H. (2004). Metabolic engineering of the chloroplast genome using the *Echerichia coli ubiC* gene reveals that chorismate is a readily abundant plant precursor for *p*-hydroxybenzoic acid biosynthesis. Plant Physiol. *136*, 4048–4060.

Wiegand, I., Hilpert, K., and Hancock, R.E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. *3*, 163–175.