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Assembly and Heterologous Expression of the Coumermycin A_1 Gene Cluster and Production of New Derivatives by Genetic Engineering

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Many secondary metabolites of clinical importance have been isolated from different Streptomyces *species. As most of the natural producers remain difficult to handle genetically, heterologous expression of an entire biosynthetic gene cluster in a well characterised host allows improved possibilities for modifications of the desired compound by manipulation of the biosynthetic genes. However, the large size of a functional gene cluster often prevents its direct cloning into a single cosmid clone. Here we describe a successful strategy to assemble the entire coumermycin A_1 biosynthetic gene cluster (38.6 kb) into a single cosmid clone by λ RED recombination technology. Heterologous expression of the reconstituted gene cluster in* Streptomyces coelicolor *M512 resulted in the heterologous production of coumermycin A_1. Inactivation of the methyltransferase gene* couO*—responsible for the C-methylation at the 8-positions of the aminocoumarin moieties in coumermycin A_1—and heterologous expression of the modified cluster resulted in an accumulation of a C-8-unsubstituted coumermycin A_1 derivative. Subsequent expression of the halogenase gene* clo-hal *from the clorobiocin gene cluster in the heterologous producer strain led to the formation of two new hybrid antibiotics, containing either one or two chlorine atoms. The identities of the new compounds were verified by LC-MS, and their antibacterial activities were tested against* Bacillus subtilis *in an agar diffusion assay.*

Introduction

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of bacterial DNA gyrase and are of therapeutic interest both as antibiotics and as anticancer agents.[1] Their common characteristic structural moiety is a 3-amino-4,7-dihydroxycoumarin ring, substituted at position C-8 either with a methyl group or with a chlorine atom. In all three compounds, the 7-hydroxy group of the aminocoumarin moiety is glycosidically linked to an unusual deoxy sugar, 4-*O*-methyl-5-*C*-methyl-L-rhamnose, which is acylated at its 3-hydroxy group with a 5-methylpyrrole-2-carboxyl group or a carbamyl group. Coumermycin A_1 is unique among the aminocoumarins in incorporating two 3-amino-4,7-dihydroxycoumarin moieties, which are connected through amide bonds to a central pyrrole unit—3-methylpyrrole-2,4-dicarboxylic acid—resulting in a nearly but not completely symmetric molecule (Figure 1).

Aminocoumarin antibiotics bind to the 43 kDa subunit of gyrase, which contains the ATP binding site. It has been shown that the action of coumermycin A_1 is unique, in stabilizing a dimer form of the 43 kDa fragment of GyrB.[2,3] The affinity of coumermycin A_1 towards intact gyrase is extremely high. Peng and Marians (1993) reported that 50% inhibition of gyrase was achieved by coumermycin A_1 at a concentration of 4 nM, comparison to 100 nM for novobiocin, 1.8 μM for norfloxacin and 110 μM for nalidixic acid.[4] These features make coumermycin A_1 a very interesting starting compound for the production of new structurally modified antibiotics that may show improved properties for clinical application. Structural analogues of novobiocin and clorobiocin have been generated successfully by genetic engineering, especially through the heterologous expression of modified, recombinant gene clusters of these antibiotics in *Streptomyces coelicolor* M512.[5–7] However, no similar success has so far been achieved with coumermycin A_1 analogues. The natural producer of coumermycin A_1, *Streptomyces rishiriensis* DSM40489,[8] shows low and unreliable productivity and is not easy to manipulate genetically. In addition, the coumermycin A_1 gene cluster is the largest of the three aminocoumarin clusters, spanning approximately 38.6 kb from the putative regulatory gene *couE*[9] to the resistance genes *gyrB*^{R} and *parY*^{R} (Figure 1).[10] Therefore, the cluster has been sequenced and analysed from different cosmids with overlapping inserts.[11] No single cosmid containing the entire gene cluster was available for heterologous expression experiments.

Here, we report the assembly of two overlapping cosmid inserts into a single cosmid spanning the entire coumermycin A_1 gene cluster by an efficient strategy based on λ RED recombination.[12,13] Heterologous expression of the obtained cosmid in *S. coelicolor* M512 resulted in the ready formation of coumermycin A_1. Targeted manipulation of the biosynthetic gene cluster in combination with the heterologous expression of an additional gene allowed the formation of three structural analogues of coumermycin A_1. Preparative isolation of the result-

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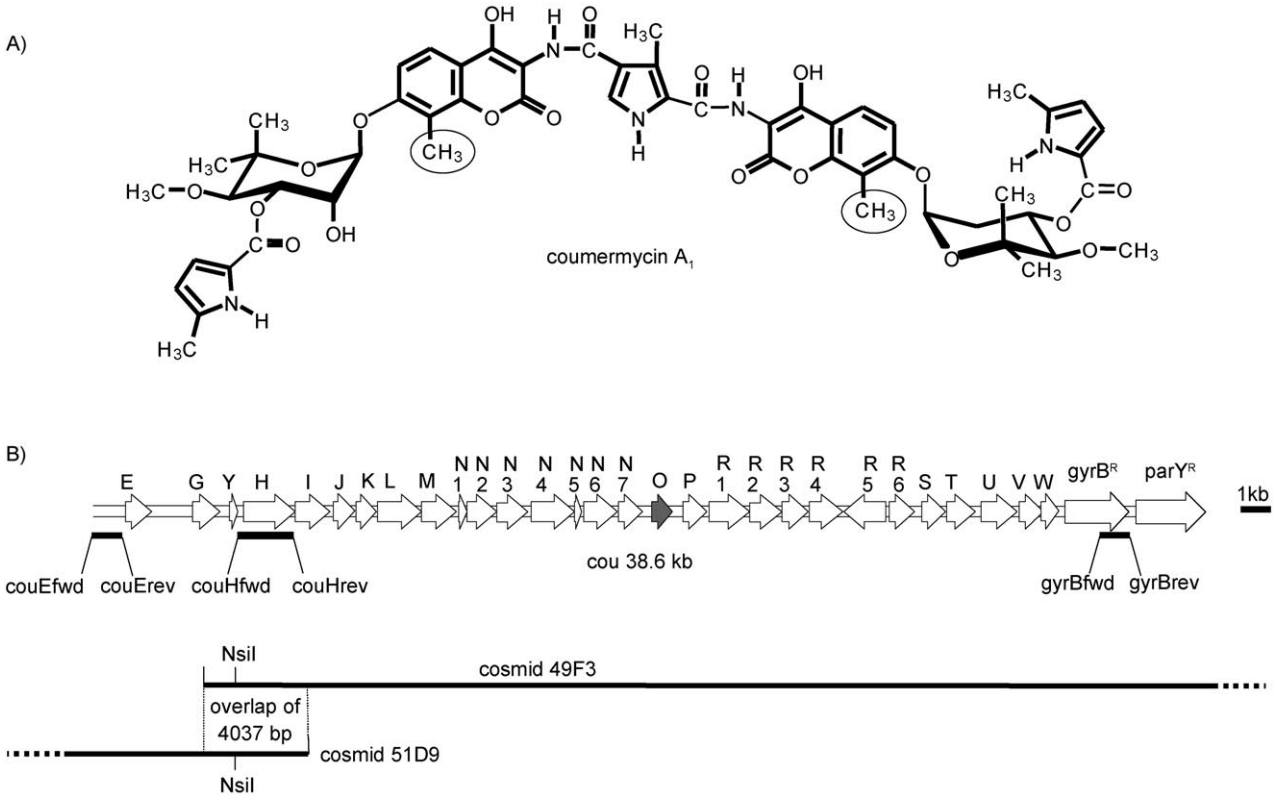


Figure 1. A) Chemical structure of the aminocoumarin antibiotic coumermycin A_1. The substituents at the 8-positions in the aminocoumarin moieties are circled. B) Organisation of the coumermycin A_1 gene cluster.[5] Primer pairs used for the screening of the cosmid library and the borders of the inserts of cosmids 49F3 (the parent cosmid of couMW12) and 51D9 (the parent cosmid of couMW13) are indicated. The gene *couO* involved in the inactivation experiment for the generation of new coumermycin analogues is depicted in grey.

ing compounds and investigation of their antibacterial activities gave further insights into the structure–activity relationships of the aminocoumarin antibiotics.

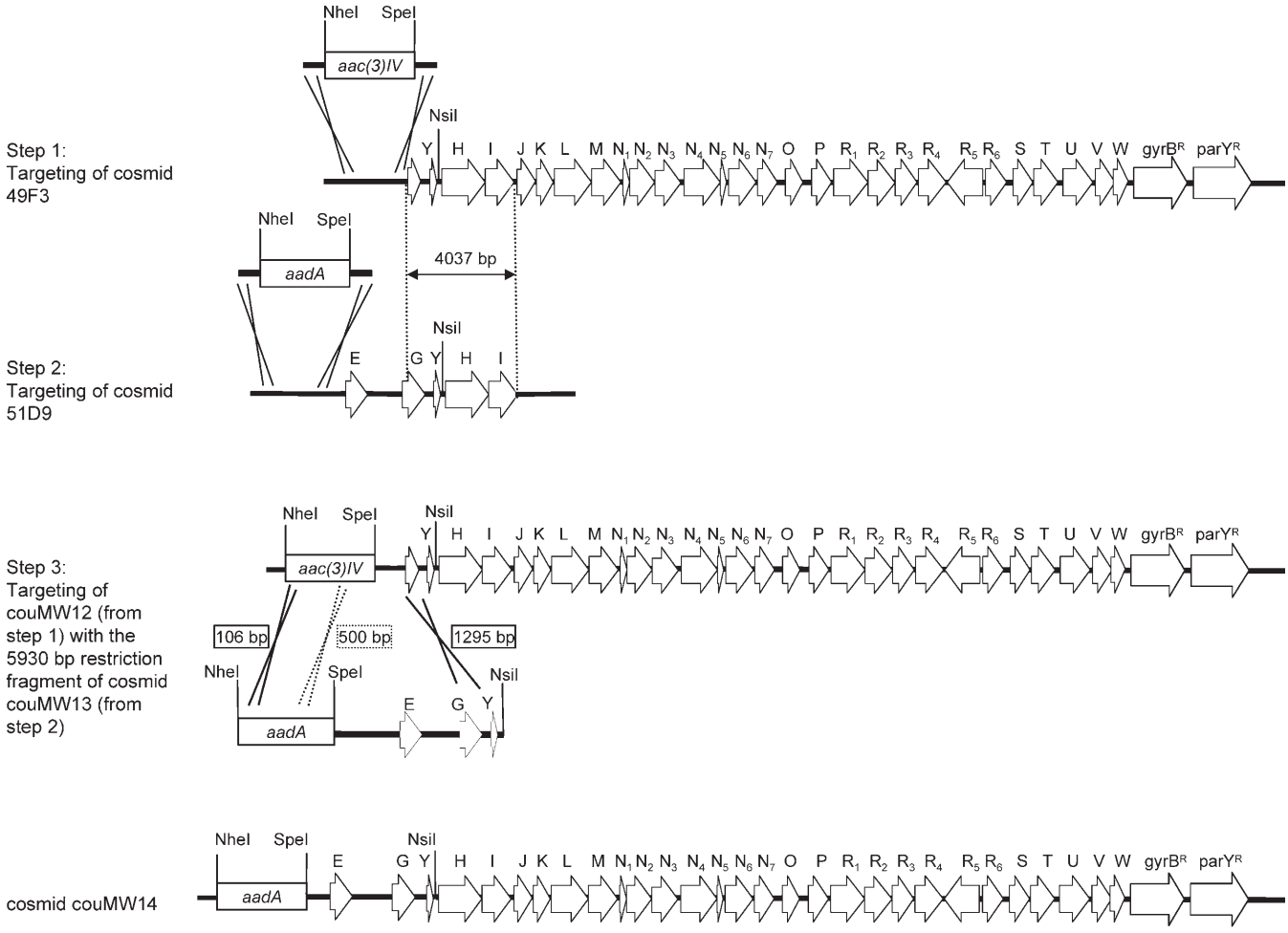
Results and Discussion

Generation of a *S. rishiriensis* cosmid library and construction of cosmid couMW16 harbouring the entire coumermycin A_1 gene cluster

The gene clusters for novobiocin and clorobiocin have already been expressed successfully in *S. coelicolor* M512, which resulted in a heterologous production of the corresponding compound in amounts comparable to those found in the natural producer.[7] However, because of the size of the coumermycin A_1 gene cluster (38.6 kb), heterologous expression of this antibiotic had not yet been achieved. In a first attempt to obtain a cosmid clone containing the entire coumermycin A_1 gene cluster, a cosmid library from *Streptomyces rishiriensis* DSM40489 was constructed in the SuperCos1 vector. Approximately 10000 clones were screened by PCR with two different primer pairs amplifying either a fragment upstream of *couE* localized at the left end of the gene cluster or a fragment localized at the right end of the gene cluster within the resistance gene *gyrB*^{R} (Figure 1). The cluster contains a second resistance gene, *parY*^{R}, located downstream of *gyrB*^{R}. The inclusion of *parY*^{R}, however, seemed not to be essential for a successful heterologous expression experiment, as it has been shown that *gyrB*^{R} alone is sufficient to convey resistance against coumermycin A_1 in *Streptomyces*.[10] Omission of *parY*^{R} by screening for the presence only of *gyrB*^{R} reduced the required insert size from 38.6 to 36.6 kb and thereby increased the probability of obtaining a single cosmid clone harbouring a functional coumermycin A_1 gene cluster. Unfortunately, though, no cosmid containing the complete sequence spanning the region from *couE* to *gyrB*^{R}, and therefore giving the expected PCR products with both primer pairs, was obtained.

Subsequently, five randomly selected cosmids containing the gene *gyrB*^{R} were tested by PCR for the presence of the gene *couH*, which maps 3.6 kb downstream of *couE* (Figure 1). Two cosmids yielded the expected PCR product, and one of them—49F3—was used for further analyses. Sequencing of the ends of cosmid 49F3 by use of T7 and T3 primers from the SuperCos1 vector determined the left end of the cloned insert within the gene *couG*, 1.5 kb downstream of the stop codon of *couE* (Figure 1). The right end was identified 7.2 kb downstream of the stop codon of the resistance gene *gyrB*^{R}, so the cosmid therefore also included the second resistance gene *parY*^{R}. Although cosmid 49F3 contains most of the biosynthetic gene cluster, both putative regulatory genes, *couE* and *couG*, were absent. Subsequently, five randomly selected cosmids containing the upstream region of *couE* were also tested for

the presence of *couH* by PCR. One cosmid, 51D9, which gave the expected PCR products with the *couE* and *couH* primer pairs, was isolated and used for further analyses. Sequencing of the ends of cosmid 51D9 determined an overlap of 4037 bp with cosmid 49F3. We subsequently attempted to reconstitute the complete gene cluster into one cosmid by homologous recombination mediated by λ RED. For this purpose, the inserts of the two conjoining cosmids have to contain two stretches of identical DNA sequences. Since one stretch of identical sequence resulted from the overlap of the cosmids, the second stretch was obtained by introducing different resistance cassettes into both cosmids, whereas both cassettes contain identical sequences of 106 and 500 bp. A spectinomycin/streptomycin resistance cassette (*aadA*) was amplified by PCR and inserted upstream of *couE* in cosmid 51D9 by λ RED-mediated recombination (Figure 2).[12,13] Additionally, a NheI site was included in the forward primer sequence. The resulting cosmid couMW13 was digested with NsiI (with cutting at the native site between *couY* and *couH*) and NheI, resulting in a 5930 bp fragment that contained the spectinomycin/streptomycin resistance cassette (*aadA*) as well as the genes *couE*, *couG* and *couY* (Figure 2). In a parallel experiment, an apramycin resistance cassette (*aac(3)IV*) was amplified by PCR and inserted within the truncated sequence of *couG* in cosmid 49F3, resulting in cosmid couMW12 (Figure 2). Both resistance cassettes were flanked by unique NheI and SpeI restriction sites for later elimination of the cassettes by NheI/SpeI digestion and religation of the compatible overhangs. As shown in Figure 2,



Step 4: Elimination of the resistance cassette by NheI/SpeI restriction and religation resulting in cosmid couMW15

Step 5: Insertion of the integration cassette resulting in cosmid couMW16

Figure 2. Strategy for reassembling the entire coumermycin A_1 gene cluster and construction of the integrative cosmid couMW16. Cosmid couMW12 was obtained by insertion of an apramycin resistance cassette (*aac(3)IV*) into cosmid 49F3 upstream of the truncated *couG* sequence. Cosmid couMW13 was obtained by insertion of a spectinomycin/streptomycin resistance cassette (*aadA*) upstream of *couE* in cosmid 51D9. The 5930 bp restriction fragment containing the spectinomycin/streptomycin resistance cassette, the genes *couE*, *couG*, *couY* as well as 1020 bp upstream of *couE* was derived by NheI/NsiI digestion of cosmid couMW13. The three stretches of identical DNA sequences of 106 bp, 500 bp and 1295 bp that allow λ RED-mediated recombination are indicated. The desired recombination event resulting in cosmid couMW14 is depicted in bold lines, whereas dotted lines show an additional undesired recombination event. NheI, SpeI and NsiI restriction sites are indicated.

homologous recombination of the 5930 bp NheI/NsiI fragment with cosmid couMW12 was possible in three regions: 1) the 1295 bp region representing a part of the original overlap of the cosmids, 2) the 500 bp region, and 3) the 106 bp region of the resistance cassettes. λ RED-mediated recombination would lead to spectinomycin/streptomycin resistant transformants only if homologous recombination were to occur within the 106 bp region and one of the two other regions of homology: that is, either the 1295 bp or the 500 bp region (Figure 2). To obtain the desired cosmid harbouring the entire coumermycin A_1 gene cluster, recombination of the 106 bp and the 1295 bp regions is required. Since λ RED efficiency depends on the sizes of identical sequences for recombination,[14] most spectinomycin/streptomycin resistant clones were expected to be the result of this recombination event. Nine of ten isolated transformants showed the expected restriction pattern, indicating the successful integration of the desired genes *couE*, *couG* and *couY* into couMW12. The resulting cosmid was termed couMW14 (Figure 2). To eliminate the spectinomycin/streptomycin resistance cassette (*aadA)* from cosmid couMW14, a NheI/SpeI digestion and a religation of the compatible overhangs was performed, resulting in cosmid couMW15. A further recombination step was performed to provide the integrative cosmid couMW16, as described previously for the novobiocin and clorobiocin gene cluster.[7]

In this work we have used λ RED recombination to assemble the entire coumermycin A_1 biosynthetic gene cluster from two cosmids with overlapping inserts. This procedure is generally applicable to many biosynthetic gene clusters in cases in which their sizes prevent their direct cloning into a single clone. The usefulness of λ RED recombination in reassembling gene clusters has also been demonstrated in the work of Perlova et al., who reconstituted the myxothiazol biosynthetic gene cluster in a single construct in 2006.[15] These authors used a combination of standard cloning techniques and RED/ET recombination because of the presence of large repeats within the gene cluster. Another variant of this method, assembling the inserts of a cosmid clone and a recovery plasmid, was published by Wenzel et al. in 2005.[16]

Heterologous expression of cosmid couMW16 and coumermycin A_1 production in *S. coelicolor* M512

To confirm the functionality of the assembled gene cluster (couMW16) and to test whether *S. coelicolor* M512 (Δ*redD,* Δ*actII-ORF4,* SCP1$^-$, SCP2$^-$) is a suitable host strain for heterologous production of coumermycin A_1, cosmid couMW16 harbouring the entire coumermycin A_1 gene cluster was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation. Selection for kanamycin resistance identified the desired integration mutant, termed *S. coelicolor*(couMW16). Seven independent mutant strains were selected for further examinations. These strains were cultured in production medium, and secondary metabolite formation was analysed by HPLC and LC-MS (Figure 3). The integration mutants accumulated a substance with a retention time identical to that of the coumermycin A_1 standard. The UV absorption spectrum of this compound was also identical to that of coumermycin A_1, with two maxima at 275 and 345 nm (data not shown). Negative-ion FAB mass spectrometry further confirmed that the molecular mass of this substance was identical to that of coumermycin A_1, while the negative-ion ESI-CID fragmentation pattern of the isolated compound was also identical to that of coumermycin A_1 (Figure 3). The level of coumermycin A_1 production of the *S. coelicolor*(couMW16) integration mutants was on average 7 μg mL^{-1} and thereby superior to that found in the natural producer *Streptomyces rishiriensis* DSM40489 (2–5 μg mL^{-1}).[17]

Inactivation of the *C*-methyltransferase gene *couO*

After heterologous expression of a functional coumermycin A_1 gene cluster had been achieved successfully, we attempted to use genetic engineering to generate a structurally modified antibiotic containing chlorine atoms in place of the methyl groups at C-8 in the two aminocoumarin moieties in coumermycin A_1. It has been shown previously that the methyltransferase CouO is responsible for *C*-methylation at position 8 in the aminocoumarin moiety in coumermycin A_1.[17,18] Inactivation of *couO* was performed by the PCR targeting method.[12,13] The complete open reading frame of *couO* within cosmid couMW16 was replaced by an apramycin resistance cassette flanked by unique XbaI restriction sites, resulting in cosmid couMW17. To avoid possible polar effects of the *couO* inactivation on downstream genes, the resistance cassette was removed by XbaI digestion and religation to generate cosmid couMW18, leaving a 6 bp "scar" sequence in place. Cosmid couMW18 was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation. Genomic integration mutants—termed *S. coelicolor*(couMW18)—were isolated on the basis of their kanamycin resistance, and the correct genotype was verified by PCR with use of oligonucleotides priming 125 bp upstream and 158 bp downstream of the remaining "scar" sequence. The expected 289 bp PCR product was obtained for all couMW18 clones, while neither the wild-type gene *couO* (969 bp) nor the apra-marked *couO* deletion in couMW17 (1663 bp) were detected (data not shown).

Identification of secondary metabolites in the *couO* defective mutant

Three independent *S. coelicolor*(couMW18) transformants and the reference strain *S. coelicolor*(couMW16) were cultivated in production medium, and the extracts were analysed by HPLC and LC-MS (Figure 3). The production of coumermycin A_1 (retention time 9.8 min) was clearly detectable in the reference strain *S. coelicolor*(couMW16), but not in the *ΔcouO* mutant *S. coelicolor*(couMW18). Instead, cultures of *S. coelicolor* (couMW18) showed a dominant peak at a retention time of 8.1 min, displaying a UV spectrum indistinguishable from that of coumermycin A_1 (data not shown). This compound was isolated by HPLC, and negative-ion ESI-CID analysis showed a molecular ion $[M-H]^-$ at *m/z* 1080, corresponding to the loss of two methyl groups relative to coumermycin A_1 (M_r 1109;

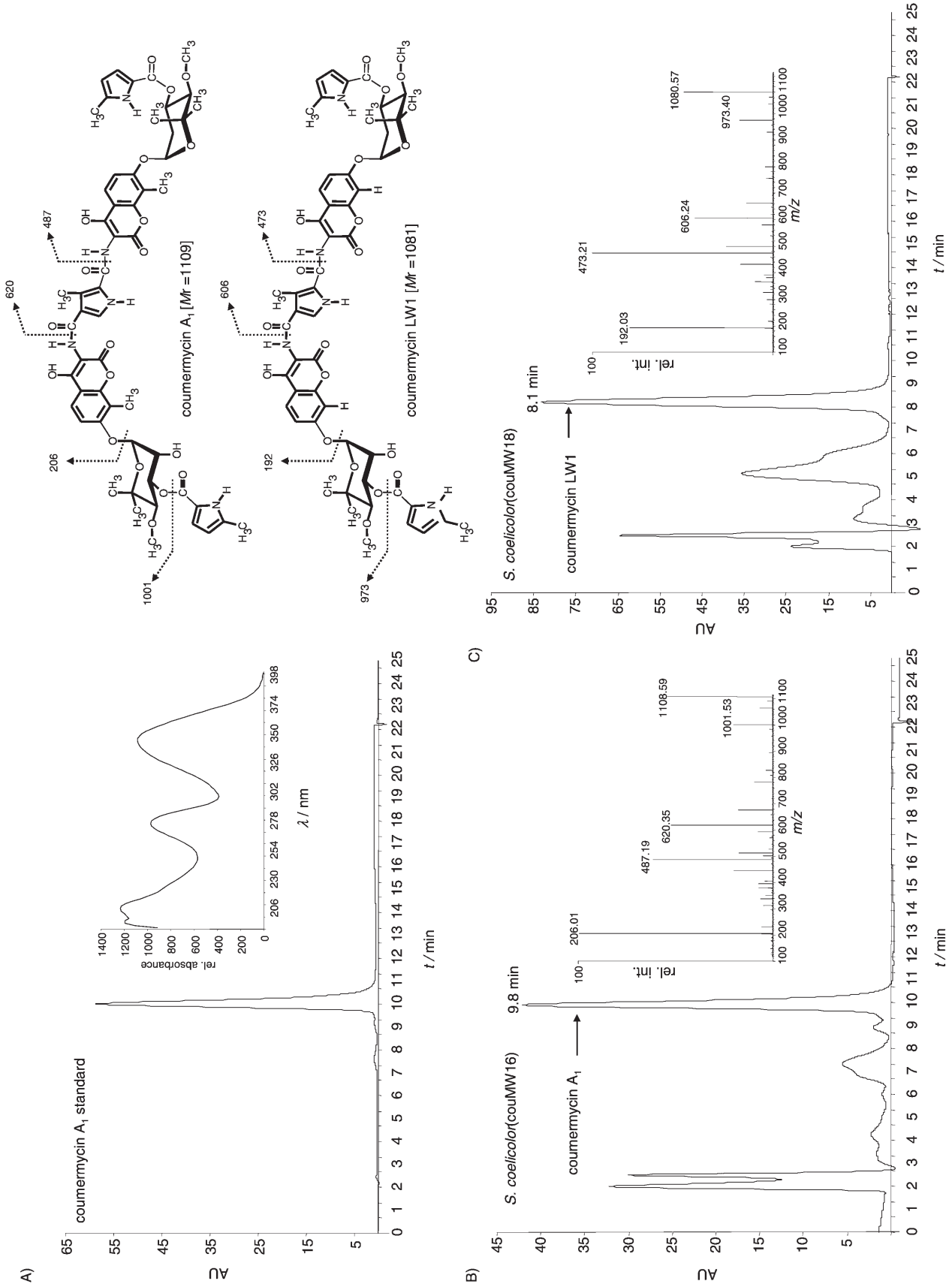


Figure 3. HPLC and LC-MS analyses of culture extracts from heterologous producer strains. HPLC diagrams of A) coumermycin A_1 standard, B) extract of *S. coelicolor*(couMW16) containing the intact coumermycin A_1 gene cluster, and C) *S. coelicolor*(couMW18) containing the *couO*-defective gene cluster. The observed MS fragmentation patterns of isolated coumermycin A_1 and coumermycin LW1 are shown next to the chromatograms. The suggested fragmentation schemes for coumermycin A_1 and coumermycin LW1 are shown separately.

$[M-H]^-$ at *m/z* 1108; Figure 3). Fragmentation of coumermycin A_1 occurred during negative electrospray ionisation ((−)-ESI) mass spectrometry at the glycosidic, the amide and the ether bonds, producing ions at *m/z* 1108, 1001, 620, 487 and 206 (Figure 3). The ion at *m/z* 206, representing the methylated aminocoumarin ring, was observed as the main fragment (rel. int. 100). In contrast, an ion of *m/z* 192 (rel. int. 80) but no fragment at *m/z* 206 was detected in the *S. coelicolor*(couMW18) mutant extract (Figure 3), indicating that both aminocoumarin moieties now lacked the C-8 methyl group. This bis-desmethyl-coumermycin A_1 has been designated previously as coumermycin LW1.[17] The production level of coumermycin LW1 was found to be similar to that of coumermycin A_1 in *S. coelicolor* M512 expressing the intact coumermycin A_1 gene cluster.

Heterologous expression of the halogenase gene *clo-hal* in *S. coelicolor*(couMW18)

To obtain a new chlorinated coumermycin A_1 derivative, the gene *clo-hal* from the clorobiocin gene cluster was introduced into *S. coelicolor*(couMW18). The halogenase Clo-hal has been shown to be responsible for the chlorination of the aminocoumarin moiety at position 8 in clorobiocin biosynthesis.[19] *Clo-hal* had previously been placed under control of the constitutive promoter *ermEp** in the replicative plasmid pUWL201[20] harbouring a thiostrepton resistance marker, resulting in pAE_ha7.[21] This plasmid was introduced into *S. coelicolor* (couMW18), generating *S. coelicolor*(couMW18)/pAE_ha7. Eight independent thiostrepton-resistant transformants were isolated, and the presence of pAE_ha7 in all mutants was verified by retransformation of total DNA into *E. coli* and restriction analyses of the isolated pAE_ha7 plasmid. As shown by HPLC, the *clo-hal* transformants produced two new peaks with retention times at 8.8 min and 9.6 min (Figure 4). A third, minor peak with a retention time at 8.2 min could be assigned by LC-MS to the unsubstituted coumermycin A_1 derivative coumermycin LW1 (Figure 4).

Negative-ion ESI-CID fragmentation of the first new peak, which appeared at an HPLC retention time of 8.8 min, displayed an ion at *m/z* 1114 ((−)-ESI) corresponding to the molecular mass of a coumermycin A_1 derivative lacking two methyl groups but containing instead one chlorine atom and one hydrogen. Two ions with *m/z* 192 and *m/z* 226 were detected, representing an 8-unsubstituted and an 8-chlorinated aminocoumarin moiety, respectively (Figure 4). The presence of two further ions with *m/z* 507 and *m/z* 473, representing the 8-unsubstituted and the 8-chlorinated aminocoumarin moiety tethered to the substituted deoxy sugar, confirmed our assumption that in this fraction one of the two aminocoumarin moieties is substituted with a chlorine atom. It appears likely that this peak represents a mixture of two compounds, carrying the chlorine atom at either of the two aminocoumarin moieties of the antibiotic molecule. This fraction was termed coumermycin MW1.

For the second peak, termed coumermycin MW2 and showing a retention time of 9.6 min, LC-MS analysis revealed a molecular ion at 1148 ($[M-H]^-$); this suggests the substitution of both aminocoumarin moieties with chlorine atoms (Figure 4). This was further confirmed by negative-ion ESI-CID fragmentation, in which the ion at *m/z* 226 (rel. int. 100; (−)-ESI) was again observed, indicating the presence of chlorine in the aminocoumarin moiety. In contrast, the ion of the 8-unsubstituted aminocoumarin moiety at *m/z* 192 was now missing. The presence of chlorine in both aminocoumarin moieties was further confirmed by an ion at *m/z* 586, representing two chlorinated aminocoumarin moieties tethered to the central 3-methylpyrrole-2,4-dicarboxylic acid.

The successful heterologous expression of coumermycin A_1 in *S. coelicolor* M512 opened the route to a rapid modification of the coumermycin A_1 structure by genetic engineering of the biosynthetic gene cluster. This possibility was demonstrated by the generation of the coumermycin A_1 derivatives coumermycin LW1, MW1 and MW2. All of them were produced in levels similar to the production level of coumermycin A_1 in the heterologous producer strain *S. coelicolor* M512. It is not clear at which step of coumermycin MW1 and MW2 biosynthesis these chlorine atoms are introduced. Walsh and colleagues provided biochemical evidence that in novobiocin biosynthesis the SAM-dependent *C*-methyltransferase NovO uses desmethyl-novobiocic acid—that is, the 3-amino-4,7-dihydroxycoumarin moiety linked through an amide bond to 3-dimethylallyl-4-hydroxybenzoic acid—as substrate.[22] In coumermycin A_1 biosynthesis, the methyltransferase CouO works on the corresponding mono- and bis-amides, which contain one or two aminocoumarin moieties tethered to a central 3-methylpyrrole-2,4-dicarboxylic acid moiety. In each case, methylation occurs at the 8-positions in the aminocoumarin moieties.[22] It appears plausible that in clorobiocin biosynthesis the halogenase Clo-hal may utilise desmethyl-novobiocic acid as substrate for halogenation, but this reaction has not been demonstrated in vitro. Our finding of the production of the mono- and dichlorinated coumermycins MW1 and MW2 indicates that Clo-hal can also accept the mono- and bisamides of the 3-methyl-2,4-dicarboxylic acid moiety, showing an extraordinarily broad substrate tolerance. Of course, it cannot at present be excluded that halogenation may occur at an earlier step of the biosynthetic pathway.

Antibacterial activity of the novel coumermycin A_1 derivatives

All three coumermycin A_1 derivatives were isolated by HPLC and tested for their antibacterial activities in an agar diffusion assay against *Bacillus subtilis*. Coumermycin LW1, which lacks the methyl groups at the 8-positions of both aminocoumarin moieties, showed less activity than the reference coumermycin A_1 (Figure 5). Substitution of one aminocoumarin moiety by a chlorine atom at position 8 (coumermycin MW1) restored antibacterial activity to the same level as observed for coumermycin A_1. Somewhat unexpectedly, substitution of both aminocoumarin moieties with chlorine atoms (coumermycin MW2) again resulted in a decrease of activity to a level comparable to that of the unsubstituted derivative coumermycin LW1.

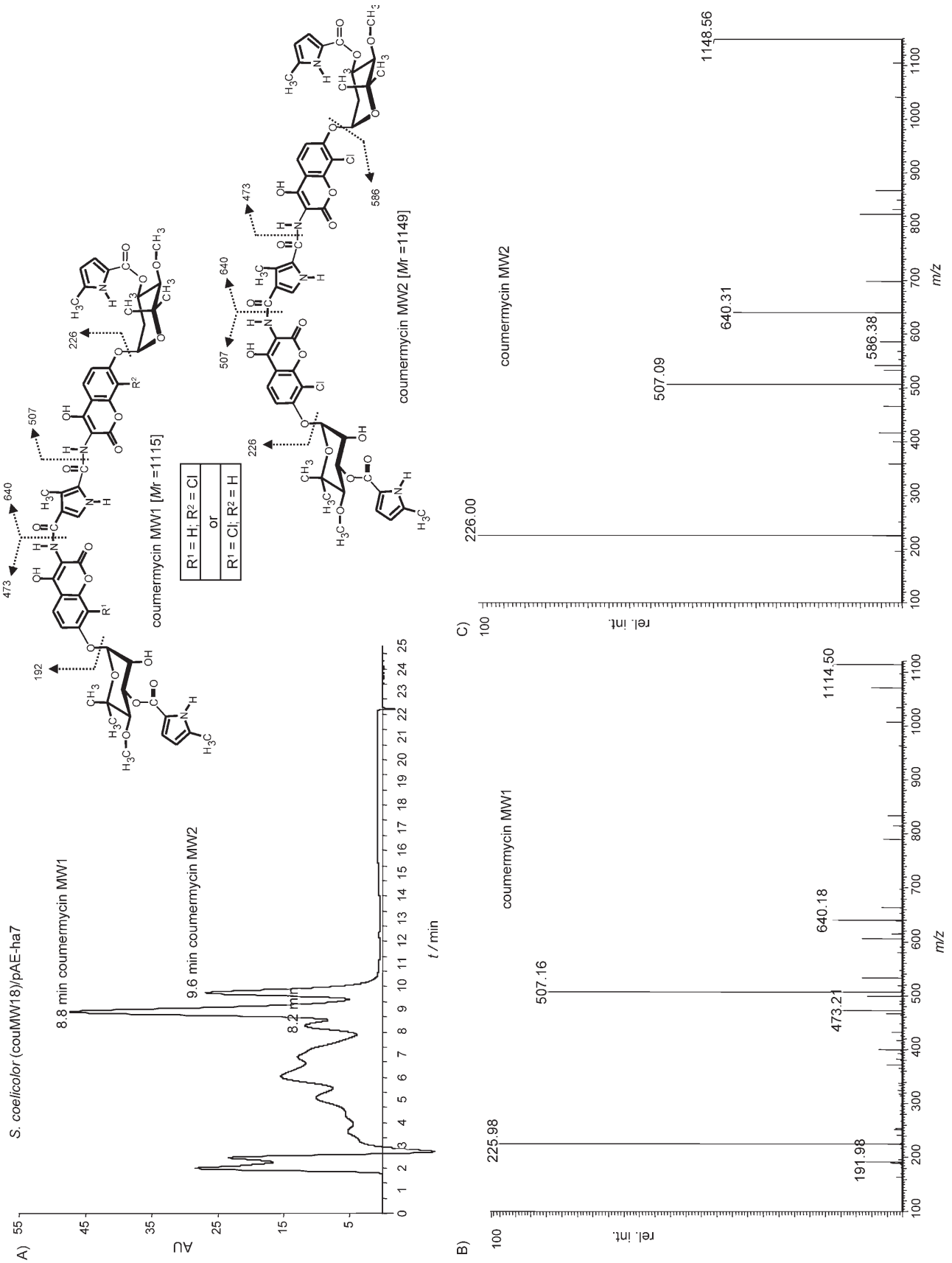


Figure 4. HPLC and LC-MS analyses of culture extracts from *S. coelicolor*(couMW18) harbouring the *couO*-defective coumermycin A_1 cluster and the halogenase gene *clo-hal*. A) HPLC diagram of *S. coelicolor*(couMW18)/pAE_ha7. The retention times of the new coumermycin derivatives coumermycin MW1 and coumermycin MW2 are shown at the corresponding peaks. Observed MS fragmentation patterns of: B) coumermycin MW1 (retention time in HPLC at 8.8 min), and C) coumermycin MW2 (retention time in HPLC at 9.6 min). The suggested fragmentation schemes for coumermycin MW1 ($R^1 = H$, $R^2 = Cl$) and coumermycin MW2 are shown separately.

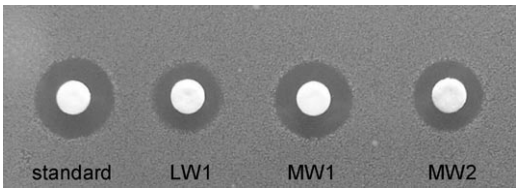


Figure 5. Antibacterial activities against *Bacillus subtilis* of coumermycin A_1 (standard), coumermycin LW1, coumermycin MW1 and coumermycin MW2. Equal quantities (0.75 nmol) of each compound were used for the agar diffusion assay.

As might be expected from previous studies,[6,17,19] removal of the 8-methyl group from the aminocoumarin moiety reduced the antibacterial activity, while subsequent introduction of a chlorine atom at this position restored the activity. At first glance, it may appear surprising that halogenation of one of the two aminocoumarin moieties of the molecule was sufficient for optimal activity and that halogenation of the second aminocoumarin moiety did not further increase, but rather decreased, the activity. It must be kept in mind, however, that X-ray crystallographic studies have shown that only one aminocoumarin moiety and the attached deoxysugar with its substituents occupy the aminocoumarin antibiotic binding pocket of the gyrase B subunit, which overlaps with the functionally important ATP binding site. The substituent attached to the 3-amino group of the aminocoumarin moiety, in contrast, enters only into weak interactions with the target enzyme.[23]

Experimental Section

Bacterial strains, plasmids and culture conditions: *Streptomyces rishiriensis* DSM40489 was provided by the "Deutsche Sammlung von Mikroorganismen" (Braunschweig, Germany). *Streptomyces coelicolor* M512 (Δ*redD,* Δ*actII-ORF4,* SCP1$^-$, SCP2$^-$) was kindly provided by E. Takano (Groeningen, Netherlands) and Janet White (Norwich, UK). *Escherichia coli* XL1 Blue MRF$^-$ (Stratagene, Heidelberg, Germany) was used for cloning experiments and grown in liquid or on solid Luria–Bertani medium at 37 °C. For analyses of secondary metabolite production of *S. coelicolor* mutants, cells were precultured in Trypticase Soy Broth (30 g L^{-1}) for 3 days at 30 °C and 210 rpm. A preculture (5 mL) was inoculated into production medium (SK medium, 50 mL) containing Pharma media (20 g L^{-1}), corn starch (20 g L^{-1}), lard oil (20 g L^{-1}), yeast extract (4 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (2.5 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.5 g L^{-1}) and $CoCl_2 \cdot 6H_20$ (202 μg L^{-1}) and routinely cultivated in baffled flasks at 30 °C and 210 rpm for 7 days. *E. coli* ET12567 was used for isolation of non-methylated plasmid and cosmid DNA prior to the transformation into *S. coelicolor* M512 to bypass methyl-sensing restriction in *S. coelicolor*.[24] The REDIRECT technology kit containing *E. coli* ET12567, *E. coli* ET12567/pUZ8002, *E. coli* BW25113/pIJ790, *E. coli* DH5α/pIJ773 and *E. coli* DH5α/pIJ778 was obtained from Plant Bioscience Limited (Norwich Research Park, Colney, Norwich, UK). Plasmid pAE_ha7 has been described previously.[21] Kanamycin (50 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}), apramycin (50 μg mL^{-1}), carbenicillin (100 μg mL^{-1}) and thiostrepton (10 μg mL^{-1}) were used for selection of recombinant strains.

Genetic procedures: Standard procedures for DNA isolation and manipulations were performed as described previously.[25,26] Isolation of DNA fragments from agarose gel and purification of PCR products were carried out with the NucleoSpin 2 in 1 Extract Kit (Macherey–Nagel, Düren, Germany). Isolation of cosmids and plasmids was carried out with ion exchange columns (Nucleobond AX kit, Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA was isolated from *S. coelicolor* strains by the Kirby mix procedure.[26]

Construction and screening of a SuperCos1-based *S. rishiriensis* cosmid library: Chromosomal DNA of *S. rishiriensis* DSM40489 was partially digested with Sau3A, dephosphorylated and ligated with BamHI/XbaI-digested SuperCos1 vector. The ligation products were subsequently packaged with Gigapack III XL (Stratagene, Heidelberg, Germany) and transduced into *E. coli* Sure®. PCR screening of the cosmid library was performed with primers couE_fwd (5′-ACTTCCGTCGGAAGCCGTCG-3′) and couE_rev (5′-ACCGTGAGGATC-CTTCCGCC-3′), gyrB_fwd (5′-GACTGGTTCGACCGCAACCC-3′) and gyrB_rev (5′-CCGTCGAGCACCATCAGGTG-3′), couH_fwd (5′-AGGGC-GTGATTCCATCACGC-3′) and couH_rev (5′-GGACGGGTGCTCATCTC-TCA-3′) to amplify a 1020, a 1009 and a 2014 bp fragment, respectively.

Assembling of the entire coumermycin A_1 gene cluster by λ RED recombination: The λ RED recombination was described previously.[12,13] An apramycin resistance cassette (*aac(3)IV*) was amplified by PCR from plasmid pIJ773 with primers fwd49F3 (5′-CGTCTTCA-AGAATTCGCGGCCGCAATTAACCCTCACTAAA**GCTAGC**TGTAGGCTGG-AGCTGCTTC-3′) and rev49F3 (5′-GGCCGCCAGTCCAGTGGTACGT-GCGATCTTGCGGTCGGAC**ACTAGT**ATTCCGGGGATCCGTCGACC-3′). Restriction sites for enzymes NheI and SpeI are shown in bold letters, and underlined letters represent 40 nucleotide extensions to the sequence 979 and 868 bp upstream of *couY* within *couG*. The resulting 1381 bp PCR product was inserted into cosmid 49F3, resulting in the apramycin/kanamycin resistant cosmid couMW12.

The spectinomycin/streptomycin resistance cassette (*aadA*) was amplified from plasmid pIJ778 with primers fwdSpecStrep (5′-CCGCCGCAACCGGCGCCGACCTTTTGTGGCGGGTCAAGAA**GCTAGC**T-GTAGGCTGGAGCTGCTTC-3′) and revSpecStrep (5′-GTTCAAGTTC-GCCGTGGGCAGTGGCAAGGTCATCAAGGGC**ACTAGT**ATTCCGGGGAT-CCGTCGACC-3′) and inserted into cosmid 51D9 1020 bp upstream of *couE*. The underlined letters represent 40 nucleotide extensions with sequence homology for insertion of the resistance cassette. Restriction sites for enzymes NheI and SpeI are shown in bold. The resulting spectinomycin/streptomycin resistant cosmid couMW13 was subsequently digested with the enzymes NheI and NsiI to give a 5930 bp fragment, which was isolated from an agarose gel and further used for transformation experiments. For recombination of cosmid couMW12 and the 5930 bp restriction fragment, couMW12 was introduced into *E. coli* BW25113/pIJ790 by electroporation, and resulting transformants were isolated by use of chloramphenicol (25 μg mL^{-1}), apramycin (50 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) for selection. Electroporation of BW25113/pIJ790/couMW12 with the purified restriction fragment resulted in transformants that were selected on LB solid media containing spectinomycin/streptomycin (each 50 μg mL^{-1}). Isolated cosmid DNAs from the originated clones were tested by restriction analyses. The resulting cosmid harbouring the entire coumermycin A_1 gene cluster was termed couMW14. After digestion of couMW14 with NheI and SpeI and religation of the compatible overhangs, the resulting cosmid lacking the spectinomycin/streptomycin resistance cassette was termed couMW15. Subsequent insertion of an integration cassette from pIJ787 into the *bla* sequence of the SuperCos1 vector as described previously[7] resulted in cosmid couMW16. CouMW16 was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transfor-

mation according to Kieser et al.,[26], and transformants were selected on R2YE plates containing kanamycin (50 μg mL^{-1}).

Inactivation of *couO*: For the replacement of the methyltransferase gene *couO* on cosmid couMW16, an apramycin resistance cassette (*aac(3)IV*) was generated by PCR with use of primers couO_Xba_f (5′-GATCAGTTCACTGACGCAGCACCAGGGGGGATCGAGATG-**TCTAGA**ATTCCGGGGATCCGTCGACC-3′) and couO_Xba_r (5′-TCCGCCAGATCCTGAGCAGCGGGACCGAACGGCCGGTCA**TCTAGA**TGTAGGCTGGAGCTGCTTC-3′). XbaI restriction sites introduced into the primers are shown in bold letters and are used for later elimination of the inserted cassette. Underlined letters represent 40 nucleotide extensions with sequence identity to regions upstream and downstream of *couO*, including the putative start and stop codon. The gene *couO* was replaced in *E. coli* BW25113/pIJ790/couMW16 by use of the PCR targeting system,[12] and the correct genotype of the resulting *ΔcouO*/apramycin resistant cosmid (couMW17) was confirmed by restriction analyses, as well as by PCR with test primers couO_test_f (5′-GAGAACTCCCTACCGCGAAC-3′) and couO_test_r (5′-CCGATGACTCCTTCGACGTC-3′) amplifying a fragment of 1663 bp. To avoid possible polar effects of the apramycin resistance cassette (*aac(3)IV*) cassette, XbaI digestion and religation led to the *ΔcouO*/apramycin sensitive cosmid couMW18 containing only a 6 bp "scar" within the coding sequence of *couO*. To verify the correct genotype of the *S. coelicolor* mutants carrying couMW18, PCR with genomic mutant DNA was performed with the primers couO_test_f and couO_test_r. The expected PCR product of 289 bp was obtained for all mutants, indicating the absence of the apramycin resistance cassette (*aac(3)IV*).

Analysis of secondary metabolite formation: *S. coelicolor* wild-type and mutants were routinely cultured in Trypricase Soy Broth (50 mL, 30 g L^{-1}) at 30 °C and 210 rpm for 3 days. The preculture (5 mL) was inoculated into SK production medium (50 mL) and grown for 7 days at 30 °C and 210 rpm in baffled flasks. Aliquots (1 mL) of the cultures were adjusted to pH 5 by the addition of hydrochloric acid and extracted three times with an equal volume of ethyl acetate, after prior treatment with an equal volume of petroleum ether to remove lipophilic substances. After evaporation of the solvent, the residue was dissolved in ethanol and analysed on an Agilent HPLC system with a photodiode array detector. The analysis was performed with a Nucleosil 120–5 C18 column (2× 250 mm; Macherey–Nagel) with a linear gradient from 60% to 100% acetonitrile in aqueous phosphoric acid (0.1%) and detection at 345 nm. Coumermycin A_1 (Sigma–Aldrich) was used as standard.

HPLC-MS coupling in the selected reaction monitoring mode: The negative electrospray ionization ((−)-ESI) mass spectra were obtained with a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (electrospray voltage, 3.7 kV; heated capillary temperature 320 °C; sheath and auxiliary gas, nitrogen) coupled with a Surveyor HPLC system equipped with a Nucleosil 120–5 C18 (5 μm, 250×2 mm) column (Macherey–Nagel, Düren, Germany). For all samples, a gradient system ranging from 98:2 H_2O/CH_3CN (each of them containing 0.05% formic acid) to 70:30 over 35 min, followed by isocratic elution with CH_3CN (100%, +0.1% formic acid) for 15 min, was used; the flow rate was 200 μL min^{-1}. The collision-induced dissociation (CID) spectra of coumermycin A_1 during a HPLC run were recorded with a collision energy of 45 eV; collision gas, argon; collision pressure, 1.0×10^{-3} torr.[27] Fragments obtained in negative ESI-CID mass spectrometry were as follows: coumermycin A_1 (*m/z*, rel. int.): 1108 (52), 1001 (19), 620 (53), 487 (60), 206 (100); coumermycin LW1 (*m/z*, rel. int.): 1080 (51), 973 (19) 606 (44), 473 (100), 192 (80); coumermycin MW1 (*m/z*, rel. int.): 1114 (16), 640 (19), 507 (88), 473 (16), 226 (100), 192 (10); coumermycin MW2 (*m/z*, rel. int.): 1148 (47), 640 (40), 586 (4), 507 (59), 226 (100).

DNA sequencing and computer analysis: End-sequencing of cosmids 49F3 and 51D9 was performed by the dideoxynucleotide chain termination method with primers T3 and T7. Amino acid sequence homology searches were carried out in the GenBank database with the aid of the BLAST program.

Assay for antibacterial activity: The antibacterial activities of coumermycin A_1 and its derivatives coumermycin LW1, MW1 and MW2 was tested against *Bacillus subtilis* ATCC 14893 in an agar diffusion test. The compounds were isolated from production cultures as described above and were further purified by HPLC. After evaporation of the solvent, the residues were dissolved in DMSO (100%) and equal amounts of each compound (0.75 nmol) were further applied to filter-paper discs (5 mm diameter) and subsequently placed on Difco nutrient agar plates (Kieser et al.[26]) containing approximately 2×10^5 spores of *B. subtilis* per mL agar medium. After cultivation overnight at 37 °C, the diameter of the growth-inhibition zone was determined.

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