Methyltransferase genes in *Streptomyces rishiriensis*: new coumermycin derivatives from gene-inactivation experiments

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The coumarin antibiotic coumermycin A₁ contains at least eight methyl groups, presumably derived from S-adenosylmethionine. Two putative methyltransferase genes, *couO* and *couP*, of the coumermycin A₁ biosynthetic gene cluster were inactivated by in-frame deletion. In the resulting mutants, coumermycin A₁ production was abolished. New coumermycin derivatives were accumulated instead, and were identified by HPLC-MS using selected reaction monitoring via electrospray ionization.

*couO* mutants accumulated a coumermycin derivative lacking the methyl groups at C-8 of the characteristic aminocoumarin rings, whereas in the *couP* mutant a coumermycin derivative lacking the methyl groups at the 4-hydroxyl groups of the two deoxysugar moieties was identified. These results provided evidence that *couO* encodes a C-methyltransferase responsible for the transfer of a methyl group to C-8 of the aminocoumarin ring, and *couP* an O-methyltransferase for methylation of 4-OH of the sugar in the biosynthesis of coumermycin A₄, respectively. C-methylation of the aminocoumarin ring is considered as an early step of coumermycin biosynthesis. Nevertheless, the intermediates with the non-methylated aminocoumarin ring were accepted by the enzymes catalysing the subsequent steps of the pathway. The new, demethylated secondary metabolites were produced in an amount at least as high as that of coumermycin A₁ in the wild-type.

**Keywords:** aminocoumarin antibiotics, biosynthesis, methyltransferase

INTRODUCTION

Aminocoumarin antibiotics, such as novobiocin, clorobiocin and coumermycin A₁ (Berger & Batcho, 1978), are produced by various *Streptomyces* strains and are very potent against Gram-positive bacteria, including methicillin-resistant *Staphylococcus* strains. Novobiocin is licensed in the US as an antibiotic for use in humans (Albamycin; Pharmacia & Upjohn).

The mechanism of action of the aminocoumarin antibiotics is well studied. Bacterial DNA gyrase is their target (Hooper *et al*., 1982; Maxwell, 1999); X-ray crystallographic examinations demonstrated that the aminocoumarin moiety and the substituted deoxysugar moieties are essential for the binding of these compounds to the B subunit of gyrase (Ali *et al*., 1993; Lewis *et al*., 1996; Maxwell, 1993; Tsai *et al*., 1997). The affinity of coumermycin A₁ for intact gyrase is extremely high: 50% inhibition of gyrase is reportedly achieved by coumermycin A₁ in a concentration of only 0·004 µM, compared to 0·1 µM for novobiocin, 1·8 µM for norfloxacin and 110 µM for nalidixic acid (Peng & Marians, 1993). Correspondingly, coumermycin A₁ has been found to exhibit a much higher antibacterial activity than novobiocin (Ryan, 1979). These features make coumermycin A₁ a most interesting starting compound for the development of new aminocoumarin antibiotics, which may serve as anti-infective agents against multi-resistant Gram-positive bacteria.

Coumermycin A₁ (1, Fig. 1), produced by *Streptomyces*
S.-M. Li and others

Fig. 1. (a) Proposed biosynthetic pathway of coumermycin A₁ in *S. rishiriensis*. (b) Coumermycin A₁ analogues produced by *couO* and *couP* mutants.

**nishiriensis** DSM 40489, contains two 3-amino-4,7-dihydroxy-8-methylcoumarin moieties, which are attached via amide bonds to a central pyrrole unit, i.e. 3-methylpyrrole 2,4-dicarboxylic acid. Two unusual 5-gem-dimethyl sugar units are linked to the 7-hydroxyl groups of the two aminocoumarin rings via glycosidic bonds. Both deoxysugars are acylated with 5-methylpyrrole-2-carboxylic acid at position 3.
In the structurally similar novobiocin, it has been shown that the aminocoumarin moiety is derived from l-tyrosine, and the deoxysugar (called noviose) is formed from glucose (Bunton et al., 1963; Li et al., 1998). The pyrrole moieties of coumermycin A₁ have been reported to be derived from l-proline (Scannell & Kong, 1969).

Coumermycin A₁ contains at least eight methyl groups, presumably derived from S-adenosylmethionine. Feeding experiments on novobiocin biosynthesis, using [14C]glucose (Birch et al., 1960) and [13C]glucose (Li et al., 1998), have shown that the methyl groups at C-8 of the aminocoumarin ring, the methyl groups at the 4-OH of the deoxysugar and one of the two methyl groups at C-5 of the deoxysugar probably originated from S-adenosylmethionine. The other methyl group at C-5 of the deoxysugar is derived from C-6 of glucose after reduction. Coumermycin A₁ contains two further methyl groups at C-5 of the terminal pyrrole moieties. Coumermycin derivatives lacking one or both of these methyl groups were identified in the coumermycin-producing strains (Claridge et al., 1984). It might therefore be expected that these methyl groups at the terminal pyrrole units are also derived from S-adenosylmethionine. The origin of the methyl group at C-3 of the central pyrrole unit has not been established, but this group also may be derived from S-adenosylmethionine. Therefore, at least four different methyltransferases are expected to be involved in the biosynthesis of coumermycin A₁.

None of the methyltransferases involved in novobiocin or coumermycin biosynthesis has been functionally identified. Our group has recently cloned and sequenced the biosynthetic gene clusters of novobiocin from Streptomyces spheroides NCIMB 11891 (Steffensky et al., 2000b), of coumermycin A₁ from S. rishiriensis DSM 40489 (Wang et al., 2000) and of clorobiocin from Streptomyces roseochromogenes DS 12.976 (unpublished results). The three clusters share extensive similarities. To create a consistent nomenclature for the corresponding biosynthetic genes of these clusters, we have recently revised the nomenclature of the coumermycin biosynthetic genes (see GenBank entry AF235050).

Sequence analysis of the coumermycin biosynthetic gene cluster (Wang et al., 2000) led to the identification of three putative methyltransferase genes, i.e. couU, couO and couP (formerly designated as cumW, cumM and cumN, respectively). The predicted gene product of couU, as well as that of the very similar novU of the novobiocin cluster, showed high sequence similarity to enzymes that have been functionally identified as C-methyltransferases carrying out the C-methylation of deoxysugar during antibiotic biosynthesis. CouU, for example, shows 48% identity to MtmC, which is responsible for the 3-C-methylation reaction in the biosynthesis of d-mycarose, a deoxysugar moiety of mithramycin (Gonzalez et al., 2001). Likewise, CouO shows 37% identity to TyfCIII and 35% to EryBIII, found in the biosynthetic gene clusters of tylosin and erythromycin A, respectively. Like MtmC, TyfCIII and EryBIII catalyse the methylation of dTDP-l-mycarose at C-3 during the biosynthesis of these two antibiotics (Bate et al., 2000; Gaisser et al., 1998). CouU shows also 35% identity to AviG1, a 3-C-methyltransferase involved in the biosynthesis of the deoxysugar 2-deoxy-t-d-xylo-2'-furanose present in the antibiotic avilamycin (Weitnauer et al., 2002). Therefore couU was assigned to the C-methyltransferase reaction involved in the biosynthesis of the deoxysugar noviose (Wang et al., 2000).

Recently, a new subgroup of radical S-adenosylmethionine proteins has been identified by bioinformatic techniques (Sofia et al., 2001). The gene couN6 of the coumermycin cluster (formerly designated as cumK) shows sequence similarity to these enzymes and is therefore likely to represent a methyltransferase. Since couN6 is contained in a putative transcription unit with genes of pyrrole biosynthesis, and since no homologue for couN6 is found in the novobiocin cluster, it appears likely that couN6 may be responsible for the introduction of the methyl groups into the pyrrole moieties of coumermycin A₁.

In this study, we report the functional identification of two further methyltransferase genes, couO and couP, from the coumermycin A₁ cluster by means of gene inactivation and the identification of the new secondary metabolites accumulated in the deficient mutants.

**METHODS**

**Bacterial strains and plasmids.** S. rishiriensis DSM 40489 (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was routinely cultivated at 28°C for 2 days in HA medium (Steelsky et al., 2000b) containing 10% malt extract, 0.4% yeast extract, 0.4% glucose and 1 M CaCl₂ (pH 7.3).

Escherichia coli XL-1 Blue MRF’ (Stratagene) was grown in liquid or solid Luria–Bertani medium at 37°C (Sambrook & Russell, 2001). pGem-3Zf(−) and pUC18 were purchased from Promega and Amersham Biosciences, respectively, and pKC1132, a non-replicative vector carrying an apramycin resistance determinant, was described by Bierman et al. (1992). Carbenicillin (50 µg ml⁻¹) and apramycin (50 µg ml⁻¹) were used for selection of recombinant plasmids and strains.

**Genetic procedures.** Standard methods for DNA isolation and manipulation were performed as described by Sambrook & Russell (2001) and Kieser et al. (2000). DNA fragments were isolated from agarose gels using a NucleoSpin 2 in 1 extraction kit (Macherey-Nagel). Genomic DNA was isolated from Streptomyces strains by lysozyme treatment and phenol/chloroform extraction as described elsewhere (Kieser et al., 2000).

Southern blot analysis was performed on Hybond-N membranes (Amersham Biosciences) with digoxigenin-labelled probes by using DIG high prime DNA labelling and detection kit II (Roche Molecular Biochemicals).

**Construction of the vector pLW3 for in-frame gene inactivation of couO.** A SpfI–PstI fragment of 3.89 kb containing genes couN6, couN7, couO and couP was isolated from cosmid 4-2H (Wang et al., 2000) and cloned into the same sites of pGem-3Zf(−), resulting in vector pLW1. Vector pLW2, containing a deletion of 366 bp within couO, was constructed by ligation of two restriction fragments of pLW1, i.e. a 5·09 kb EcoRI–SfoI fragment and a 0·98 kb SmaI–EcoRI

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3319
Fig. 2. Inactivation of the gene couO of the coumermycin biosynthetic gene cluster. (a) Schematic presentation of the gene-inactivation experiment. apra, apramycin-resistance gene. (b) Southern blot analysis of wild-type and mutants. Genomic DNA was restricted by BamHI. The indicated 1.43 kb BamHI fragment containing couO was used as probe. SCO, single-crossover mutant; DCO, double-crossover mutant; RV, reversion to wild-type.

Fig. 3. Inactivation of the gene couP of the coumermycin biosynthetic gene cluster. (a) Schematic presentation of the gene-inactivation experiment. (b) Southern blot analysis of wild-type and mutants. Genomic DNA was restricted by HincIII. The indicated 1.1 kb PstI–HincIII fragment containing couR1 was used as probe. Abbreviations are as given in Fig. 2.

fragment. pLW3 was obtained by cloning of a 3.52 kb HindIII–PstI fragment from pLW2 into the same sites of vector pKC1132 (Fig. 2a).

Construction of the vector pLW9 for in-frame gene inactivation of couP. A NarI fragment of 3.08 kb containing couO, couP and couR1 was isolated from cosmid 4-2H and cloned into the AccI sites of pUC18 to give vector pLW7. pLW7 was restricted with BclI, and the 5.09 kb fragment was religated, resulting in a deletion of 678 bp within the couP gene. The vector obtained was termed pLW8. The 2.56 kb XbaI–HindIII fragment was released from pLW8 and ligated into the same sites of pKC1132, resulting in the inactivation vector for couP, pLW9 (Fig. 3a).

Transformation of S. rishiriensis and selection for recombinant mutants. Transformation of the S. rishiriensis wild-type with pLW3 and pLW9 was carried out by polyethylene glycol-mediated protoplast transformation (Kieser et al., 2000). For preparation of protoplasts, mycelia of S.
risibiensis were grown in CRM medium, containing 10.3% sucrose, 24% tryptic soy broth, 1.0% MgCl₂·6H₂O, 1.0% yeast extract and 0.4% glycine (pH 7.0), for 42–48 h, harvested, and incubated in 4 ml P-buffer per g mycelia, containing 0.3 mg lysozyme (Sigma) ml⁻¹, for 30–60 min at 30 °C.

For transformation, plW3 and plW9 were isolated from E. coli ET 12567 (MacNeil et al., 1992) using ion-exchange columns (Nucleobond AX kits; Macherey-Nagel), according to the manufacturer’s protocol, and denaturated by alkaline treatment (Oh & Chater, 1997). The resulting single-stranded DNA (10 μg in 10 μl TE-buffer) was mixed with 200 μl P-buffer containing 10⁻⁸ S. risibiensis protoplasts and 500 μl T-buffer containing 25% (w/v) polyethylene glycol 1000 (Roth) (Kieser et al., 2000). The resulting suspension was plated on three R2YE agar plates (Kieser et al., 2000). After incubation for 20 h at 30 °C, each plate was overlaid with 3 ml soft R2YE agar containing a total of 500 μg apramycin, for selection of recombinant mutants.

Apramycin-resistant, single-crossover mutants were cultured in liquid HA medium without antibiotics and sporulated after four subcultures on solid HA medium without antibiotics. The spores were tested for loss of apramycin resistance by culturing the colonies on HA plates with and without apramycin. Sensitive colonies were subjected to Southern blotting.

Production and analysis of secondary metabolites. For the production of coumermycin derivatives, wild-type and mutant strains of S. risibiensis were pre-cultured in 300 ml baffled flasks containing 50 ml HA medium. After growth for 48 h at 28 °C and 180 r.p.m., 3 ml of this pre-culture was inoculated into 500 ml baffled flasks containing 100 ml production medium (Scannell & Kong 1969). In this medium, cells were cultured at 28 °C and 180 r.p.m. for 7 days.

Aliquots (6 ml) of the cultures were then extracted with 2 x 6 ml ethyl acetate after treatment with 2 x 6 ml petroleum ether to remove lipophilic substances. After evaporation of the solvent, the residue was dissolved in 0.6 ml ethanol and analysed on a Hewlett Packard HPLC system with a photo diode-array detector. The analysis was performed with a Nucleosil 120-5 C₁₈ column (2 x 250 mm; Macherey-Nagel) and a linear gradient from 30 to 100% acetonitrile in 0.1% aqueous phosphoric acid. Detection was at 345 nm.

HPLC-MS and selected reaction monitoring. The positive and negative electrospray ionization (ESI) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage, 4.5 kV; heated capillary temperature, 220 °C; sheath and auxiliary gas, nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with an RP18 column (5 μm, 1 x 100 mm; SepServ). For all samples, a gradient system ranging from 80:20 H₂O/CH₃CN (each of them containing 0.2% HOAc) to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 25 min, was used; the flow rate was 70 μl min⁻¹. The collision-induced dissociation (CID) spectra of coumermycin A₁ and the selected reaction monitoring during an HPLC run were recorded with a collision energy of −25 eV for positive ions and +40 eV for negative ions, respectively; collision gas, argon; collision pressure, 1.8 x 10⁻³ torr (240 x 10⁻³ Pa). The positive-ion ESI-CID mass spectrum of coumermycin A₁ (m/z, rel. int.) was as follows: 1110 ([M + H]⁺, 3), 960 (8), 622 (10), 282 (100), 108 (9). The negative-ion ESI-CID mass spectrum of coumermycin A₁ (m/z, rel. int.) was as follows: 1108 ([M – H]⁻, 22), 620 (24), 594 (36), 513 (42), 487 (100), 206 (54). The reactions monitored by selected reaction monitoring were as shown below.

Coumermycin A₁ (I, Mᵋ=1109), positive-ion reactions: m/z 1110 ([M + H]⁺)→m/z 108, m/z 1110→m/z 282 and m/z 1110→m/z 622; negative-ion reactions: m/z 1108 ([M – H]⁻)→m/z 206 and m/z 1108→m/z 487.

Coumermycin LW1 (2, Mᵋ=1081): positive-ion reactions: m/z 1082 ([M + H]⁺)→m/z 108 and m/z 1082→m/z 282; negative-ion reactions: m/z 1080 ([M – H]⁻)→m/z 192 and m/z 1080→m/z 473.

Coumermycin LW2 (3, Mᵋ=1081): positive-ion reactions: m/z 1082 ([M + H]⁺)→m/z 108 and m/z 1082→m/z 268; negative-ion reactions: m/z 1080 ([M – H]⁻)→m/z 206 and m/z 1080→m/z 473.

Assay for antibacterial activity. The antibacterial activities of coumermycin derivatives were tested using Bacillus subtilis ATCC 14893. Cultures of S. risibiensis (wild-type and mutant strains) were cultured and extracted as described for HPLC analysis. For the bioassays, 30 μl of an ethanolic solution of the ethyl acetate extracts was applied to filter-paper discs (5 mm diameter) and air-dried for 30 min. The discs were then placed on Difco nutrient agar plates (Kieser et al., 2000) containing approximately 2 x 10⁸ spores of B. subtilis per ml agar medium. After culture overnight at 37 °C, the diameter of the growth-inhibition zone was determined.

RESULTS

Sequence analysis of couO and couP

Two adjacent ORFs, couO and couP, encoding putative methyltransferases were found in the coumermycin A₁ biosynthetic gene cluster (Wang et al., 2000). Two very similar genes, novO and novP, are found in the novobiotic biosynthetic cluster. The functions of these genes have not be established.

The predicted gene product of couO comprises 230 amino acids and shows only low sequence similarity to putative methyltransferases in the database, e.g. 29% identity (at amino acid level) to the C-methyltransferase UbiE (GenBank accession no. AE010901) of the ubiquinone/menaquinone biosynthesis in Methanosarcina acetivorans (Galagan et al., 2002). Conserved motif III [LL/FR/K]PGG(R/I/L)[L/D](L/F/V)/Y(1/L)] for S-adenosylmethionine-dependent methyltransferases (Kagan & Clarke 1994) is found from amino acid 135 to 144 of CouO (LVKPGGAILN).

In the biosynthesis of coumermycin A₁, C-methyltransferases may be involved in the methylation of C-8 of the aminocoumarin ring, of C-5 of the terminal pyrrole, and possibly of C-3 of the central pyrrole unit, besides the C-methylation reaction at C-5 of the deoxysugar.

The predicted gene product of couP consists of 276 amino acids. In contrast to CouO, CouP shows high similarities to known deoxysugar O-methyltransferases in the database, e.g. 57% identity to 3-O-methyltransferase MycF catalysing the methylation at the 3-position of the deoxysugar mycinosine of mycaminicin III (Inouye et al., 1994), 59% to ElnmIII responsible for 4-O-methylation of the permethylated l-rhamnose in the biosynthesis of elloramycin A (Patallo et al., 2001), and 53% to 3-O-methyltransferase TyIF converting macrocin to tylosin by O-methylation at C-3 of the deoxysugar.
mycinose (Fouces et al., 1999). Conserved motif I [(V/V/L)(L/V)/(D/E)/(V/I)G(G/C)(G/T/P)G] for S-adenosylmethionine-dependent methyltransferases (Kagan & Clarke, 1994) was found in the predicted CouP from amino acid 105 to 113 (LVETGVRG).

Construction of the inactivation vectors
To provide experimental evidence for the functions of couO and couP, inactivation vectors were prepared for both genes. A vector containing an in-frame deletion within the coding sequence of couO was obtained by the cloning of a SphI–SfoI fragment of 1926 bp and a SmaI–EcoRI fragment of 983 bp from a cosmid containing the coumermycin cluster into the non-replicative vector pKC1132 (containing an apramycin resistance determinant), via the cloning vector pGem-3Zf(−). The resulting inactivation vector was termed pLW3 (Fig. 2a). The predicted product of couO is thereby expected to be shortened from 230 (wild-type) to 108 (mutants) amino acids.

The inactivation vector for couP (pLW9) (Fig. 3a) contained an in-frame deletion within the structural gene of couP. This could be achieved by excision of a 678 bp BclI fragment from a subclone of the coumermycin gene cluster, and ligation of the resulting fragment into pKC1132. The predicted product of the shortened couP consists of only 50 amino acids, in comparison to 276 amino acids in the wild-type.

Transformation of S. rishiriensis with pLW3, and selection of couO-defective mutants
Vector pLW3 was transformed into S. rishiriensis protoplasts (Kieser et al., 2000; Wang et al., 2000), and apramycin-resistant colonies were selected. Southern blotting confirmed that pLW3 had been integrated into these mutants via a single-crossover recombination event (Fig. 2b). One of these single-crossover mutants (LW-O11) was grown in the absence of apramycin, sporulated, and examined for loss of resistance as a consequence of double-crossover events. Four sensitive colonies, LW-O41, LW-O61, LW-O316 and LW-O318, were obtained and examined further. Chromosomal DNA from these four strains and from the DSM 40489 wild-type, the couO-defective mutant, LW-O41 (b), and the couP-defective mutant, LW-P634 (c). The samples were analysed with an RP C-18 column. Detection was at 345 nm.

Identification of secondary metabolites in the defective mutants
S. rishiriensis wild-type, the couO mutants (LW-O41 and LW-O316) and the couP mutant (LW-P634) were cultured under the conditions described by Scannell & Kong (1969). After extraction of the cultures with ethyl acetate, secondary metabolites were analysed by HPLC. As shown in Fig. 4, the production of coumermycin A₁ was abolished in all mutants. The HPLC chromatogram of strain LW-O316 was identical to that of LW-O41 (data not shown). Instead, the couO mutant LW-O41 showed a prominent peak at 19.7 min (2) and the couP mutant showed a dominant peak at 17.5 min (3). The
two peaks 2 and 3 showed UV spectra similar to that for coumermycin A₁ with two maxima at 275 and 345 nm (data not shown). HPLC-MS showed both substances to have identical molecular ions: \( m/z \) at 1082 \([M+H]^+\) in the positive mode and \( m/z \) at 1080 \([M−H]^−\) in the negative mode. Therefore substances 2 and 3 have a molecular mass of 1081, corresponding to a loss of two methyl groups relative to coumermycin A₁ (\( M^+\), 1109).

To determine the positions of the remaining methyl groups in 2 and 3, we employed MS using selected reaction monitoring via ESI during positive and negative ionization. Fragmentation of coumermycin A₁ occurred at the amide, the glycosidic and the ester bonds, producing ions at \( m/z \) 487 (ESI−), \( m/z \) 282 (ESI+) and \( m/z \) 108 (ESI+), respectively (Fig. 5a). In addition, the ion at \( m/z \) 206 (ESI−), representing the methylated aminocoumarin ring (4), was observed. Fig. 5 shows, as examples, the selected reaction monitoring of the reactions of \( m/z \) 1110 \([M+H]^+\)→\( m/z \) 282 as well as \( m/z \) 1108 \([M−H]^−\)→\( m/z \) 206.

In compound 2 produced by the couO mutant, reaction of \( m/z \) 1082 \([M+H]^+\)→\( m/z \) 282 was observed, confirming the presence of the same sugar and the same terminal pyrrole units as from coumermycin A₁. 1. In addition, a reaction of \( m/z \) 1080 \([M−H]^−\)→\( m/z \) 192 (rather than \( m/z \) 1080→\( m/z \) 206) was observed, indicating that the aminocoumarin moiety now lacked a methyl group, as shown in structure 5 (Fig. 5). This proved that the couO mutant produced a new coumermycin, lacking the 8-methyl groups at both aminocoumarin rings (Fig. 5, 2). This new coumermycin was designated coumermycin LW1.

In compound 3 produced by the couP mutant, selected reaction monitoring showed the reaction confirming the presence of the methylated aminocoumarin moiety 4. However, instead of the \( m/z \) 1082→\( m/z \) 282 reaction, a reaction of \( m/z \) 1082 \([M+H]^+\)→\( m/z \) 268 was now observed, indicating the loss of a methyl group from either the deoxysugar or the terminal pyrrole unit. However, since the fragment at \( m/z \) 108 (ESI+) was still observed, it is obvious that the pyrrole moiety is unchanged in comparison to coumermycin A₁, and that therefore a methyl group is lacking from the deoxysugar in comparison to coumermycin A₁.

The S. risbiriensis wild-type produced about 5 mg coumermycin A₁ per litre medium under the culture conditions described. Judging from the peak areas in the HPLC chromatograms, the couO and couP mutants produced at least as much of the new metabolites, i.e. coumermycin LW1 and LW2, respectively (Fig. 4).

**Antibiotic activity of culture extracts from the couO and the couP mutants**

Cultures of S. risbiriensis wild-type as well as of the couO and the couP mutants were extracted with ethyl acetate and assayed (against B. subtilis) for antibiotic activity (Fig. 6). All three strains showed similar antibacterial activity, indicating that neither the methyl group at C-8 of the aminocoumarin ring nor the methyl group at 4-OH of the deoxysugar was essential for the antibiotic activity of the coumermycins.

**DISCUSSION**

In the present study, we have inactivated the putative methyltransferase genes couO and couP by in-frame deletion, and have identified the products formed in the resulting mutants by HPLC-MS analysis, using selected reaction monitoring for structure elucidation.

Inactivation of couO led to a new product (coumermycin LW1) which lacked the methyl groups of the aminocoumarin moieties. This provides strong evidence that couO encodes the C-methyltransferase responsible for the methylation of C-8 of these aminocoumarin rings. However, it is still unclear at which stage of coumermycin biosynthesis (or correspondingly of novobiocin biosynthesis) this reaction occurs. The aminocoumarin ring is derived from tyrosine, and the methylation reaction may take place prior to, during, or after the conversion of 1-tyrosine to 3-amino-4,7-dihydroxycoumarin (Chen & Walsh, 2001). We have previously inactivated an early step of deoxysugar biosynthesis in novobiocin formation, and the mutant accumulated large amounts of novobiocinic acid, the aglycone of novobiocin (Steffensky et al., 2000b). This indicates that methylation of C-8 takes place prior to glycosylation, and probably before acylation of the amino group of the aminocoumarin (Steffensky et al., 2000a). Nevertheless, the unmethylated aminocoumarin produced by the couO mutant was accepted as substrate by all subsequent enzymes of coumermycin biosynthesis (Fig. 1), and the resulting product, coumermycin LW1, was accumulated in an amount at least as high as that of coumermycin A₁ in the wild-type. This proves that there is no strict substrate specificity for the substituent in this position of the aminocoumarin. This is also indicated by the natural occurrence of novobiocin analogues lacking the 8-methyl group, as recently reported by Sasaki et al. (2001), and by the simultaneous occurrence of simocyclinones with and without a substituent at the corresponding position of the aminocoumarin ring (Theobald et al., 2000).

Inactivation of another methyltransferase gene, couP, in the present study also led to the formation of a new coumermycin (termed coumermycin LW2) lacking two methyl groups. Mass spectroscopic analysis with selected reaction monitoring proved that these methyl groups were lacking from the deoxysugar moiety. Since C-methylation at C-5 had been assigned to couU, this result suggests that couP encodes a methyltransferase responsible for the methylation of the 4-hydroxyl groups of the deoxysugar moieties of coumermycin. This is also in accordance with the sequence similarity of couP to known deoxysugar O-methyltransferases (see Results). In novobiocin biosynthesis, acylation of 3-OH and methylation of 4-OH of the deoxysugar moiety have been suggested as the two final steps of the biosynthetic
Fig. 5. Fragmentation scheme for coumermycin derivatives, and the corresponding LC-MS chromatograms with detection by selected reaction monitoring.
pathway, since novobiocin derivatives lacking either or both substituent(s) have been identified in mutants of the novobiocin producer *Streptomyces niveus* (Kominek & Sebek, 1974). At first sight, the very high accumulation of coumarmycin LW2 in the couP mutant may appear to indicate that 4-O-methylation is the last step of coumar- mycin A₁ biosynthesis. In the study by Kominek & Sebek (1974) mentioned above, however, it was shown by mutation and biotransformation experiments that 4- O-methylation of the deoxysugar could be achieved before or after acylation of the 3-hydroxy group. It has been shown repeatedly that several enzymes of coumarin antibiotic biosynthesis are not strictly substrate specific. The flexibility for the substituent at the position 8 of the antibiotic biosynthesis are not strictly substrate specific. The flexibility of the enzymes of coumarin antibiotic biosynthesis depicted in Fig. 1 remains tentative. The flexibility of the enzymes of coumarin antibiotic biosynthesis for different substrates presents a very useful feature in experiments for the production of new antibiotics by combinatorial biosynthesis (Tang & McDaniel, 2001; Yoon et al., 2002), and this study has provided two examples of the production of new antibiotics by genetic methods.

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