

Assembly of Dimeric Variants of Coumermycins by Tandem Action of the Four Biosynthetic Enzymes CouL, CouM, CouP, and NovN[†]

Caren L. Freel Meyers,[‡] Markus Oberthür,[§] Lutz Heide,^{||} Daniel Kahne,[§] and Christopher T. Walsh^{*,‡}

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115,

Department of Chemistry, Princeton University, Princeton, New Jersey 08544, and Pharmaceutical Institute, Tuebingen University, 72076 Tuebingen, Germany

Received July 19, 2004; Revised Manuscript Received September 20, 2004

ABSTRACT: Coumermycin A₁ is a member of the aminocoumarin family of antibiotics. Unlike its structural relatives, novobiocin and clorobiocin, coumermycin A₁ is a dimer built on a 3-methyl-2,4-dicarboxypyrrole scaffold and bears two decorated noviose sugar components which are the putative target binding motifs for DNA gyrase. Starting with this scaffold, we have utilized the ligase CouL for mono- and bisamide formation with aminocoumarins to provide substrates for the glycosyltransferase CouM. CouM was subsequently shown to catalyze mono- and bisnoviosylation of the resulting CouL products. CouP was shown to possess 4'-O-methyltransferase activity on products from tandem CouL, CouM assays. A fourth enzyme, NovN, the 3'-O-carbamoyltransferase from the novobiocin operon, was then able to carbamoylate either or both arms of the CouP product. The tandem action of CouL, CouM, CouP, and NovN thus generates a biscarbamoyl analogue of the pseudodimer coumermycin A₁. Starting from alternative dicarboxy scaffolds, these four enzymes can be utilized in tandem to create additional variants of dimeric aminocoumarin antibiotics.

The bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, are required for DNA replication and catalyze the biochemical reactions that control the topology of DNA including steps in DNA decatenation and topoisomer relaxation number control (1). Both DNA gyrase and topoisomerase IV (topo IV)¹ are antibiotic targets in bacterial pathogens. Among the known inhibitors of gyrase and topo IV are the synthetic fluoroquinolone class of antibiotics which target the A subunits (2–4) and the natural product aminocoumarins which target the B subunits of both enzymes, respectively (5–9). The fluoroquinolones ciprofloxacin and levofloxacin are potent inhibitors of gyrase and topo IV and have experienced widespread clinical use as antibiotics. However, the aminocoumarin family of antibiotics including novobiocin, clorobiocin, and coumermycin A₁ has found limited use in the clinic because of problems

associated with low solubility, poor pharmacokinetics, and poor cell wall penetration (10–12). Novobiocin has, however, received renewed attention as a result of its potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strains (13). The increasing prevalence of antibiotic resistance to the synthetic antibacterial fluoroquinolones in addition to the recent sequencing of the biosynthetic gene clusters for novobiocin (14), clorobiocin (15), and the pseudodimer coumermycin A₁ (16) has prompted an interest in the optimization of aminocoumarin antibiotics by structural variation.

A comparison of the biosynthetic gene clusters for novobiocin, clorobiocin, and coumermycin A₁ reveals a similar logic for the biosynthetic assembly of all three family members. The components of the tripartite scaffolds of novobiocin and clorobiocin are constructed by enzymes encoded in subclusters of *nov* and *clo* genes. The prenylated 4-hydroxybenzoate component is derived from 4-hydroxyphenylpyruvate (17) while the 7-hydroxy-3-aminocoumarin and the L-hexose substrate TDP-L-noviose are derived from tyrosine and D-glucose, respectively (18–20). The planar scaffolds in novobiocin and clorobiocin are formed following enzymatic ligation of the prenylated benzoate and aminocoumarin by the ligases NovL and CloL (15, 21). Attachment of the noviose moiety to the planar scaffold is catalyzed by the dedicated glycosyltransferases NovM and CloM (15, 22). Antibiotic activity is only attained following two subsequent enzymatic tailoring reactions on the noviose ring. 4'-O-Methylation is catalyzed by methyltransferases NovP (23) and CloP (15), and 3'-O-carbamoylation (novobiocin) or 3'-O-acylation (clorobiocin) is catalyzed by the carbamoyltransferase NovN (23) and acyltransferase CloN2 (24). The

[†] We gratefully acknowledge support from National Institutes of Health Grants 49338 (to C.T.W.), 66174 (to D.K.), and A10-54007-01 (to C.L.F.M.).

* To whom correspondence should be addressed. E-mail: christopher_walsh@hms.harvard.edu. Phone: (617) 432-1715. Fax: (617) 432-0438.

[‡] Harvard Medical School.

[§] Princeton University.

^{||} Tuebingen University.

¹ Abbreviations: topo IV, topoisomerase IV; MRSA, methicillin-resistant *Staphylococcus aureus*; TDP, thymidine diphosphate; ATP, adenosine triphosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; DMF, dimethylformamide; PyBOB, benzotriazol-1-yloxypropylidino-phosphonium hexafluorophosphate; DMSO, dimethyl sulfoxide; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; TCEP, tris(2-carboxyethyl)phosphine; BSA, bovine serum albumin; MES, 2-morpholinoethanesulfonic acid; SAM, S-adenosylmethionine; MIC, minimum inhibitory concentration; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; HATU, 2-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, diisopropylethylamine.

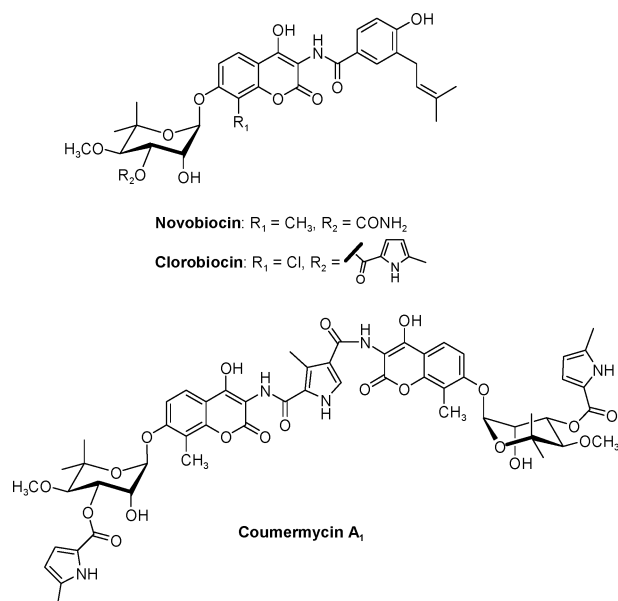


FIGURE 1: Aminocoumarin family of antibiotics.

aminocoumarins target DNA gyrase by inhibiting ATP hydrolysis in the B subunit. The 3'-substituent on the noviose ring, carbamoyl in novobiocin and methylpyrrolyl in clorobiocin, is presented by the planar aminocoumarin scaffold to the ATP binding site of the GyrB subunit (5, 6).

Coumermycin A_1 bears resemblance to the tripartite aminocoumarin antibiotic novobiocin in the aminocoumarin component and to clorobiocin in the noviose sugar component (Figure 1). The most striking feature of coumermycin A_1 , however, is that it is pseudodimeric, containing five structural elements consisting of two decorated noviosyl-aminocoumarin elements linked through a dicarboxypyrrole scaffold. Furthermore, coumermycin A_1 shows the highest antibiotic potency in the aminocoumarin family (25). The four enzymes that catalyze the five-part assembly and tailoring in coumermycin biosynthesis are the ligase CouL, the glycosyltransferase CouM, the methyltransferase CouP, and the acyltransferase CouN2. In principle, the five-part enzymatic assembly could be achieved using purified enzymes. This would establish a foundation for variation of one or more of the elements of the scaffold and pharmacophores. CouL has previously been reported by Heide and co-workers to catalyze the double ligation of 3-methyl-2,4-dicarboxylic acid with two aminocoumarin components (26).

We report herein the overproduction and purification of both CouM and CouP from *Escherichia coli* and confirm the catalytic functions of each enzyme for single and double glycosylation and 4'-O-methylation, respectively. A full reconstitution of late stage coumermycin A_1 biosynthesis has not been possible because the methylpyrrolyl transferase CouN2 is not soluble when heterologously overproduced in *E. coli*. Alternatively, we have used the novobiocin carbamoyltransferase NovN to effect biscarbamoylation on the coumermycin scaffold. Thus, the four-enzyme sequence CouL, CouM, CouP, NovN has been employed to achieve a total of eight enzymatic transformations to create variants of the dimeric aminocoumarin coumermycin A_1 . With the tandem action of the four enzymes we demonstrate that it is possible to vary all five structural elements in the coumermycin scaffold to generate novel coumermycin analogues.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Materials, and Instrumentation. Chemically competent *E. coli* TOP10 and BL21(DE3) cell strains were purchased from Invitrogen. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs. The pET37b overexpression vector was purchased from Novagen. *Pfu* DNA polymerase was purchased from Stratagene. DNA primers for PCR amplification were purchased from Integrated DNA Technologies. HPLC analysis of crude enzymatic reaction mixtures was carried out using a Beckman Gold Nouveau System Gold with a Vydac small-pore C18 column (250 × 4.6 mm). Enzymatic reaction products were confirmed by LCMS using a Shimadzu LCMS-QP8000α.

All chemicals were purchased from Aldrich or Sigma and used without further purification. Solvents were of reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates pre-coated with silica gel (250 μm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32–63 μm), purchased from Sorbent Technologies. Analytical HPLC of reaction mixtures was performed on a Hewlett-Packard 1100 series instrument using a Phenomenex Luna 5 μm C18 column (250 mm × 4.6 mm). Reactions were monitored by HPLC using a linear gradient from 20% CH_3CN in H_2O /0.1% TFA to 100% CH_3CN /0.1% TFA over 20 min. Preparative HPLC was performed on a Varian ProStar instrument (flow rate 45 mL/min) using a Phenomenex Luna 10 μm C18 column (250 × 50 mm) or on a Hitachi L6200 instrument (flow rate 7.5 mL/min) using a Phenomenex Luna 5 μm C18 column (250 × 21.2 mm). NMR spectra were recorded on Varian Mercury 300 MHz and Inova 400 or 500 MHz spectrometers. Designation of NMR peaks: H-5, H-6, H-8, or 8-Me (coumarin); H-1', H-2', H-3', ... (noviose). Mass spectra (ESI) were obtained at the Mass Spectroscopy Facility at the Department of Chemistry, Princeton University.

TDP-L-noviose (22) and 8-demethylaminocoumarin **33** (27) were prepared according to previously reported procedures. CouL was overproduced and purified as previously reported (26).

Preparation of Aglycon Substrates. (A) 3-Amino-4,7-dihydroxy-8-methylcoumarin (**1**) was prepared according to a previously reported method (28) with modifications in the isolation procedure. Novobiocin sodium salt (Sigma, 5.0 g) was heated in a mixture of pyridine and acetic anhydride (5:1, 120 mL) under reflux for 4 h. After being cooled to room temperature, the mixture was then carefully acidified with 5 N HCl while the temperature of the reaction mixture was kept below 25 °C, upon which a brown gum precipitated. After the mixture was allowed to settle, the aqueous phase was decanted. Precipitation of the crude aminocoumarin cleavage product was induced by the addition of EtOAc (250 mL). Following filtration, the gray precipitate was refluxed in EtOAc (125 mL) for 1 h, cooled to room temperature, filtered, and dried, which afforded the 3,4-oxazole derivative of 7-O-(2'-O-acetyl-3'-O-carbamoyl-4'-O-methyl-α-L-noviosyl)-3-amino-4,7-dihydroxy-8-methylcoumarin (3.3 g) as a light brown amorphous powder: $R_f = 0.13$, petroleum ether/EtOAc, 1:2. The oxazole derivative (1.4 g) was suspended

in anhydrous MeOH (20 mL) and 10% HCl/MeOH (70 mL) and was refluxed for 2 h. The clear solution was evaporated until precipitation started (ca. 15 mL final volume) and kept at 4 °C overnight. The precipitate was filtered and washed with ice-cold MeOH to give a first crop of aminocoumarin **1**. Evaporation of the mother liquor to a small volume to induce precipitation was repeated twice, which afforded a total of 510 mg of **1**.

(B) *3-Methylpyrrole-2,4-dicarboxylic acid diethyl ester* (**34**) was prepared in analogy to a previously published procedure (29). A mixture of ethyl isocynoacetate (4.4 mL, 40 mmol) and DBU (6.0 mL, 40 mmol) in THF (60 mL) was heated to 50 °C. A solution of acetaldehyde (2.8 mL, 25 mmol) in THF (20 mL) was added dropwise over 15 min, and stirring of the mixture was continued for 1 h at this temperature. The solution was cooled to room temperature, neutralized with AcOH, and evaporated. The residue was dissolved in EtOAc (300 mL) and washed with 1 N HCl, saturated aqueous NaHCO₃, and brine. The organic phase was dried (MgSO₄), filtered, and evaporated, and the residue was purified by flash chromatography using silica gel (petroleum ether/EtOAc, 6:1 to 4:1). Evaporation of the product-containing fractions gave the pyrrole diethyl ester (3.2 g, 70%) as a light yellow solid: *R*_f = 0.20, petroleum ether/EtOAc, 6:1. ¹H NMR (300 MHz, CDCl₃): δ 9.65 (br s, 1 H, NH), 7.48 (d, 1 H, *J*_{5,NH} = 3.5 Hz, H-5), 4.34, 4.28 (2 q, each 2 H, *J* = 7.1 Hz, 2 CH₂CH₃), 2.59 (s, 3 H, 3-Me), 1.37 and 1.34 (2 t, each 3 H, *J* = 7.1 Hz, 2 CH₂CH₃). ¹³C NMR (75 MHz, CDCl₃): δ 165.0, 162.0, 129.9, 127.3, 121.0, 117.0, 60.7, 59.9, 14.6, 11.5. MS(ESI) for C₁₁H₁₅NO₄ (225.24): 226 [M + H]⁺, 248 [M + Na]⁺.

(C) *3-Methylpyrrole-2,4-dicarboxylic Acid* (**2**). *3-Methylpyrrole-2,4-dicarboxylic acid diethyl ester* (**34**) (1.0 g, 4.44 mmol) and NaOH (1.76 g, 44 mmol) were heated to reflux in a H₂O/EtOH mixture (1:1, 60 mL) overnight. After being cooled to room temperature, H₂O (30 mL) was added, and the solution was acidified with concentrated HCl (pH = 2) to precipitate the acid. The mixture was kept at 4 °C for 4 h and then filtered, and the residue was washed with cold H₂O and cold acetone and dried under high vacuum to yield **2** (0.6 g, 80%) as a yellow powder. ¹H NMR (500 MHz, CD₃OD): δ 7.43 (s, 1 H, H-5), 2.58 (s, 3 H, 3-Me); ¹³C NMR (125 MHz, CDCl₃): δ 167.3, 163.2, 129.9, 127.8, 121.1, 116.0, 10.3. MS(ESI) for C₇H₇NO₄ (169.14): 168 [M - H]⁻.

(D) *3-Methylpyrrole-2,4-dicarboxylic Acid 4-Ethyl Ester* (**35**). Diethyl ester **34** (500 mg, 2.22 mmol) and KOH (148 mg, 2.64 mmol) were heated to reflux in a H₂O/EtOH mixture (1:4, 9 mL) for 2.5 h. After being cooled to room temperature, the yellow solution was poured into ice-water (60 mL) and acidified with 1 N HCl (pH = 3). The aqueous phase containing a white precipitate was extracted with EtOAc (3 × 150 mL), and the combined organic phases were washed with brine (2 × 100 mL), dried (MgSO₄), filtered, and evaporated. The residue, which contained small impurities of starting material (*R*_f = 0.85, CH₂Cl₂/MeOH, 9:1) and 2-ethyl ester (*R*_f = 0.28, CH₂Cl₂/MeOH 9:1), was purified by flash chromatography using silica gel (CH₂Cl₂/MeOH, 15:1) to afford the monoester **35** as a colorless powder (290 mg, 66%): *R*_f = 0.21, CH₂Cl₂/MeOH, 9:1. ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.00 (br s, 2 H, COOH, NH), 7.39 (d, 1 H, *J*_{5,NH} = 3.6 Hz, H-5), 4.16 (q, 2 H, *J* = 7.1 Hz, CH₂-

CH₃), 2.48 (s, 3 H, 3-Me), 1.24 (t, 3 H, *J* = 7.1 Hz, CH₂CH₃); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 164.0, 162.3, 128.1, 127.1, 121.1, 115.2, 59.0, 14.3, 11.1. MS(ESI) for C₉H₁₁NO₄ (197.19): 196 [M - H]⁻.

(E) *3-Methylpyrrole-2,4-dicarboxylic Acid 2-(3-Amino-4,7-dihydroxy-8-methylcoumarin Amide) 4-Ethyl Ester* (**7**). To a solution of aminocoumarin **1** (24 mg, 0.1 mmol) and monoester **35** (20 mg, 0.1 mmol) in DMF (1.5 mL) was added *N*-methylmorpholine (33 μL, 0.3 mmol) and PyBOP (52 mg, 0.1 mmol) under argon. After being stirred at room temperature for 40 h, the mixture was purified by preparative reversed-phase HPLC (20% CH₃CN in H₂O/0.1% TFA to 100% CH₃CN/0.1% TFA over 40 min) to give 4-ethyl ester **36** (30 mg, 78%). ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.00 (br s, 2 H, OH, NH), 10.50, 8.72 (2 s, each 1 H, NH, OH), 7.58 (d, 1 H, *J*_{5,NH} = 3.4 Hz, H-5_{pyr}), 7.57 (d, 1 H, *J*_{5,6} = 8.7 Hz, H-5), 6.90 (d, 1 H, *J*_{5,6} = 8.7 Hz, H-6), 4.19 (q, 2 H, *J* = 7.2 Hz, CH₂CH₃), 2.48 (s, 3 H, 3-Me_{pyr}), 2.17 (s, 3 H, 8-Me), 1.28 (t, 3 H, *J* = 7.2 Hz, CH₂CH₃).

To this compound was added concentrated H₂SO₄ (0.4 mL), and the mixture was stirred at 40 °C for 2 h. The solution was carefully diluted with DMF/H₂O (1:1, 1.5 mL) and purified by reversed-phase HPLC to give monomer **7** (8 mg, 66%) as a powder. ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.00 (br s, 2 H, OH, NH), 10.45, 8.65 (2 s, each 1 H, NH, OH), 7.59 (d, 1 H, *J*_{5,6} = 8.6 Hz, H-5), 7.55 (d, 1 H, *J*_{5,NH} = 3.2 Hz, H-5_{pyr}), 6.89 (d, 1 H, *J*_{5,6} = 8.6 Hz, H-6), 2.57 (s, 3 H, 3-Me_{pyr}), 2.18 (s, 3 H, 8-Me).

Preparation of pCouM-pET37b and pCouP-pET28a Overexpression Constructs. The gene encoding CouM was amplified from *Streptomyces rishiriensis* (DSM 40489) genomic DNA. Amplification was accomplished using the forward primer 5'-AATTCACATATGAGAGTGCTGTTTACGAGC-3' and the reverse primer 5'-TGCGGCCGCAAGCTTTTACTGTCGACCGTGCGA-3'. The forward primer introduced an *Nde*I restriction site (restriction site underlined above), and the reverse primer introduced a *Hind*III restriction site. PCR reactions were carried out using *Pfu* DNA polymerase as described by Stratagene. The amplified gene was inserted into the linearized pET37b vector following a restriction digest with *Nde*I/*Hind*III. Expression of pCouM-pET37b was accomplished following transformation into *E. coli* TOP10 competent cells. The gene sequence was confirmed by comparison to the *S. rishiriensis* sequence reported by Heide et al. (Accession Number AF235050).

Generation of the expression construct pCouP-pET28a was accomplished as described for pCouM-pET37b. The forward primer used for PCR amplification was 5'-GCGTATCATATGGAGGTGGCACCTATCGTAAGC-3' and introduced an *Nde*I restriction site. The reverse primer used was 5'-ACGGAAAAGCTTTCACTCGGTCTGCCAGTA-3' and introduced a *Hind*III restriction site.

Overproduction and Purification of CouM. Purified pCouM-pET37b plasmid was transformed into BL21(DE3) competent *E. coli* cells. Transformants harboring the pCouM-pET37b construct were grown in LB medium supplemented with kanamycin (50 μg/mL). The cells were grown at 25 °C to an OD of ~0.6 and induced with 60 μM IPTG. Shaking was continued overnight at 25 °C. Cells were harvested by centrifugation (20 min at 6000g) and frozen at -20 °C. Cells were thawed and resuspended in 75 mL of buffer A (25 mM Tris-HCl, pH 8.0, 400 mM NaCl, 2 mM imidazole, 10%

glycerol). Resuspended cells were lysed by French press (two passages at 15000 psi), and the cell debris was removed by centrifugation (30 min at 9500g). The supernatant was incubated with 2 mL of superflow Ni(II) affinity resin (Qiagen) at 4 °C for 1.5 h. The resin was loaded onto a column and washed with 10 mL of buffer B (25 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM imidazole, 10% glycerol). CouM was eluted from the column in a stepwise imidazole gradient (20–200 mM imidazole). Fractions containing pure CouM (as determined by SDS–PAGE) were combined and dialyzed against 1 L of buffer C (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol) overnight at 4 °C. A second dialysis was carried out in 1 L of buffer D (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM TCEP, 10% glycerol). The protein was flash frozen in liquid nitrogen and stored at –80 °C. The concentration of purified CouM was measured spectrophotometrically at 280 nm using the calculated extinction coefficient 38820 M^{–1} cm^{–1}. Large-scale overproduction afforded an overall yield of >2 mg/L CouM. CouM could not be concentrated above 0.9 mg/mL.

Overproduction and Purification of CouP. Purified pCouP-pET28a plasmid was transformed into BL21(DE3) competent *E. coli* cells. Transformants harboring the pCouP-pET28a construct were grown in LB medium supplemented with kanamycin (50 µg/mL). The cells were grown at 25 °C to an OD of ~0.6 and induced with 60 µM IPTG. Shaking was continued overnight at 25 °C. Cell lysis and CouP purification were carried out as described for CouM above. Large-scale overproduction afforded an overall yield of >23 mg/L CouP.

Characterization of CouM. Initial characterization of CouM was carried out in a tandem CouL, CouM reaction (360 µL) at ambient temperature and contained 75 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 5 mM ATP, 1 mg/mL BSA, 5% DMSO, 100 µM aminocoumarin **1**, 100 µM dicarboxylic acid **2**, 200 µM TDP-L-noviose, 500 nM CouL, and 500 nM CouM. Aliquots (50 µL) were quenched at specified time points (5 min, 10 min, 30 min, 60 min, and 2 h) in 100 µL of cold methanol. Quenched aliquots were incubated at 4 °C for 20 min, and the supernatant was analyzed by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min]. The bisnoviosyl CouM product **4** was confirmed by LCMS (ESI for C₄₁H₄₅N₃O₁₈: calcd, 867.3; obsd, 866.4 [M – H][–]).

The distributive action of CouM for mononoviosylation followed by bisnoviosylation was confirmed in a reaction (370 µL) carried out at ambient temperature containing 75 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 1 mg/mL BSA, 5% DMSO, 100 µM diamide **3**, 200 µM TDP-L-noviose, and 200 nM CouM. Aliquots (50 µL) were quenched at 1, 5, 10, 30, and 60 min and analyzed as described above. The formation of the monoglycosylated intermediates **5** and **6** was observed by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min] followed by formation of the bisglycosylated CouM product **4** characterized above. The mixture of monoglycosylated intermediates **5** and **6** was confirmed by LCMS (ESI for C₃₄H₃₃N₃O₁₄: calcd, 707.2; obsd, 706.0 [M – H][–]). Subsequent optimization of reaction conditions revealed an optimal buffer and pH of MES, pH 6.

The glycosylation of monoamide **7** was carried out at ambient temperature in a reaction mixture (370 µL) contain-

ing 75 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 1 mg/mL BSA, 5% DMSO, 100 µM monoamide **7**, 100 µM TDP-L-noviose, and 200 nM CouM. Aliquots (50 µL) were quenched at the time points specified above and analyzed as previously described. The formation of glycosylated monoamide **8** was monitored by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min] and confirmed by LCMS (ESI for C₂₄H₂₆N₂O₁₁: calcd, 518.1; obsd, 569.0 [M – H][–]).

Characterization of CouP. Methyltransferase activity of CouP was confirmed in a reaction (410 µL) containing 75 mM Tris-HCl (pH 7.5), 1 mg/mL BSA, 10% DMSO, 100 µM CouM product **4** (obtained from a tandem enzymatic incubation of diamide **3** with TDP-L-noviose and CouM), 500 µM *S*-adenosylmethionine (SAM), and 1 µM CouP. The distributive action of CouP to catalyze monomethylation of **4** followed by bismethylation to form CouP product **11** was monitored by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min]. Aliquots (50 µL) were quenched at 5 min, 30 min, 60 min, 90 min, 2 h, and 3 h. The formation of the monomethylated intermediates **9** and **10** was observed by reversed-phase HPLC and confirmed by LCMS (ESI for C₄₂H₄₇N₃O₁₈: calcd, 881.3; obsd, 880.3 [M – H][–]). The formation of CouP product **11** was confirmed by LCMS (ESI for C₄₃H₄₉N₃O₁₈: calcd, 895.3; obsd, 894.3 [M – H][–]).

Carbamoylation of the Coumermycin Scaffold by NovN. The ability of NovN to catalyze carbamoylation on a coumermycin scaffold was confirmed in a reaction (210 µL) containing 75 mM Tris-HCl (pH 7.5), 1 mg/mL BSA, 10% DMSO, 100 µM CouP product **11** (obtained from a tandem enzymatic incubation of diamide **3** with TDP-L-noviose/CouM and SAM/CouP), 500 µM carbamoyl phosphate, and 1 µM NovN. The distributive action of CouN to catalyze monocarbamoylation of **11** followed by biscarbamoylation to form NovN product **14** was monitored by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min]. Aliquots (50 µL) were quenched at 1, 5, 30, and 60 min. The formation of the monocarbamoylated intermediates **12** and **13** was observed by reversed-phase HPLC and confirmed by LCMS (ESI for C₄₄H₅₀N₄O₁₉: calcd, 938.3; obsd, 937.2 [M – H][–]). The formation of NovN product **14** was confirmed by LCMS (ESI for C₄₅H₅₁N₅O₂₀: calcd, 981.3; obsd, 980.2 [M – H][–]).

Small-Scale Generation of Coumermycin Variants via Four Enzyme Tandem Incubations. A general procedure is described for the generation of coumermycin variants **16**–**22** on a small scale via four enzyme tandem incubations. For the generation of compounds **16** and **19**, the reaction mixture (100 µL) contained 75 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 4 mM ATP, 1 mg/mL BSA, 10% DMSO, 150 µM diacid substrate, and 200 µM aminocoumarin **1**. For the generation of compounds **17**, **18**, and **20**–**22**, a 1:1 mixture of aminocoumarin **1** (200 µM) and 8-demethylaminocoumarin **33** (200 µM) was added to the reaction mixture. CouL was added to a final concentration of 1 µM, and the reaction mixture was incubated at room temperature for 4 h. TDP-L-noviose (final concentration = 150 µM) and CouM (final concentration = 1 µM) were added, and the reaction mixture was incubated for an additional 4 h at room temperature. *S*-Adenosylmethionine (SAM) (final concentration = 1 mM) and CouP (final concentration = 1 µM) were added, and

the reaction mixture was incubated overnight at room temperature. Finally, carbamoyl phosphate (final concentration = 500 μ M) and NovN (1 μ M) were added, and the reaction mixture was incubated for 4 h at room temperature. The reaction was then quenched with methanol (200 μ L) and incubated at 4 °C for 20 min. The supernatant was analyzed by reversed-phase HPLC and LCMS.

For the generation of mixed diamide coumermycin analogue **15**, the reaction mixture (200 μ L) contained 75 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 4 mM ATP, 1 mg/mL BSA, 10% DMSO, 50 μ M monoamide substrate **7**, and 100 μ M 8-demethylaminocoumarin **33**. CouL (final concentration = 1 μ M) was added, and the four-enzyme tandem incubation sequence was carried out as described for compounds **16**–**22** above. The formation of compound **15** was monitored by reversed-phase HPLC and confirmed by LCMS.

LCMS Characterization of Analogues 15–22. The formation of the coumermycin analogues described above was confirmed by LCMS. It is assumed that a mixture of 2-aminoterephthalic acid mixed diamide analogues **20** and **21** is formed as a result of adding a 1:1 mixture of aminocoumarins **1** and **33** to the reaction mixture. Further analysis of the fragmentation patterns of these analogues is necessary in order to confirm this assumption: mixed diamide analogue **15** (ESI for C₄₄H₄₉N₅O₂₀: calcd, 967.3; obsd, 966.1 [M – H][–]); terephthalic acid analogue **16** (ESI for C₄₆H₅₀N₄O₂₀: calcd, 978.3; obsd, 977.1 [M – H][–]); terephthalic acid analogue **17** (ESI for C₄₅H₄₈N₄O₂₀: calcd, 964.3; obsd, 963.4 [M – H][–]); terephthalic acid analogue **18** (ESI for C₄₄H₄₆N₄O₂₀: calcd, 950.3; obsd, 950.2 [M – H][–]); 2-aminoterephthalic acid analogue **19** (ESI for C₄₆H₅₁N₅O₂₀: calcd, 993.3; obsd, 992.3 [M – H][–]); 2-aminoterephthalic acid analogues **20** and **21** (ESI for C₄₅H₄₉N₅O₂₀: calcd, 979.3; obsd, 978.1 [M – H][–]); 2-aminoterephthalic acid analogue **22** (ESI for C₄₄H₄₇N₅O₂₀: calcd, 965.3; obsd, 964.2 [M – H][–]).

LCMS Characterization of Intermediates 23–30. Intermediates arising from incomplete ligation, methylation, and carbamoylation in the tandem incubation to produce analogue **16** (Figure 9) were characterized by LCMS: intermediate **23** (ESI for C₄₃H₄₆N₂O₁₈: calcd, 878.3; obsd, 877.4 [M – H][–]); intermediate **24** (ESI for C₄₂H₄₄N₂O₁₈: calcd, 864.3; obsd, 863.4 [M – H][–]); intermediate **25** (ESI for C₂₇H₂₈N₂O₁₂: calcd, 572.2; obsd, 571.2 [M – H][–]); intermediate **26** (ESI for C₂₅H₂₅N₂O₁₁: calcd, 515.1; obsd, 514.2 [M – H][–]); intermediate **27** (ESI for C₂₆H₂₆N₂O₁₂: calcd, 558.2; obsd, 557.2 [M – H][–]); intermediate **28** (ESI for C₄₃H₄₅N₃O₁₉: calcd, 907.3; obsd, 906.3 [M – H][–]); intermediate **29** (ESI for C₄₄H₄₆N₄O₂₀: calcd, 950.3; obsd, 949.2 [M – H][–]); intermediate **30** (ESI for C₄₄H₄₈N₂O₁₈: calcd, 892.3; obsd, 891.4 [M – H][–]).

Large-Scale Preparation of Coumermycin Analogues. (A) **Coumermycin Analogue 14.** For the preparation of the biscarbamoyl coumermycin analogue **14**, aminocoumarin **1** (9.3 mg, 38.3 μ mol) and dicarboxylic acid **2** (2.6 mg, 15.3 μ mol) were each dissolved in DMSO (0.50 mL) and added to a MES-buffered solution (131 mL, pH 7) containing DMSO (14.3 mL, 10% v/v). ATP and MgCl₂ were added to a final concentration of 4 and 10 mM, respectively, followed by the addition of BSA (153 mg, final concentration = 1 mg/mL). CouL was added to a final concentration of 500 nM (1.6 mL of 48.6 μ M stock), and the reaction was

incubated at ambient temperature for 24 h. The reaction was followed by reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min]. Additional CouL (1.6 mL of 48.6 μ M stock) was added two times over the next 48 h to ensure complete conversion of the diacid and aminocoumarin substrates to the CouL diamide product **3**. When ligation was >90% complete (as determined by HPLC), TDP-L-noviose (21.4 mg, 38.3 μ mol) was added followed by the addition of CouM (0.74 mL of 20.8 μ M stock) to a final concentration of 100 nM. The reaction mixture was incubated overnight at room temperature. Reversed-phase HPLC analysis indicated that glycosylation was complete after 10 h.

The reaction mixture was diluted by the addition of 50 mL of 10% DMSO in 75 mM MES, pH 7, since CouP operates more efficiently at low substrate concentration. CouP was added to a final concentration of 1 μ M (3.3 mL of 60.2 μ M stock) followed by the addition of SAM (33.3 mg, 76.6 μ mol). The reaction was monitored by reversed-phase HPLC. Additional CouP (3.3 mL of 60.2 μ M stock) was added after 24 h, and monitoring was continued by HPLC. Slow but complete conversion of CouM product **4** to the bismethyl CouP product was observed over 4 days, indicating that CouP retains activity for an extended period of time at ambient temperature.

Carbamoyl phosphate (11.6 mg, 76.6 μ mol) was added followed by the addition of NovN (2.2 mL of 45.5 μ M stock) to a final concentration of 500 nM. The reaction was incubated at room temperature and monitored by HPLC and LCMS. Very slow conversion of the CouP product **11** was observed under these conditions. It was necessary to add NovN and carbamoyl phosphate three additional times over the next 72 h in order to ensure complete conversion to the biscarbamoyl NovN product **14**.

The crude reaction mixture was adjusted to pH 6 by the addition of concentrated HCl and desalted and concentrated by passage over a C18 SepPak (900 mg bed) conditioned with 75 mM MES, pH 6. Following loading of the crude reaction product, the SepPak was washed with 75 mM MES, pH 6 (10 mL), followed by water (10 mL). The crude reaction product was eluted from the SepPak using DMSO (5 mL). Fractions containing product **14** (as determined by HPLC) were combined, and the product was purified by preparative reversed-phase HPLC [gradient 15:85 CH₃CN/H₂O (0.1% TFA) to 100% CH₃CN over 20 min]. Lyophilization of the product-containing fractions afforded coumermycin analogue **14** (9.6 mg, 65% yield) as a white powder. ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.45–12.25 (br s, 2 H, OH, NH), 11.93, 9.00, 8.69 (3 s, 3 H, NH, OH), 7.82 (br s, 1 H, H-5_{pyrr}), 7.76 (2 d, 2 H, *J*_{5,6} = 8.7 Hz, H-5), 7.17 (2 d, 2 H, *J*_{5,6} = 8.7 Hz, H-6), 6.73, 6.52 (2 br s, 4 H, CONH₂), 5.62 (d, 2 H, *J*_{1,2} = 5.1 Hz, H-1'), 5.54 (s, 2 H, 2'-OH), 5.15 (dd, 2 H, *J*_{2,3} = 3.1 Hz, *J*_{3,4} = 9.9 Hz, H-3'), 4.08–4.01 (m, 2 H, H-2'), 3.48 (d, 2 H, *J*_{3,4} = 9.9 Hz, H-4'), 3.47 (s, 6 H, 4'-OMe), 2.62 (s, 3 H, 3-Me_{pyrr}), 2.23 (s, 6 H, 8-Me), 1.26, 1.05 (2 s, 12 H, 6'-CH₃).

(B) **Coumermycin Analogue 19.** Analogue **19** was prepared as described above starting with 2-aminoterephthalic acid (2.8 mg, 15.3 μ mol) and aminocoumarin **1** (9.3 mg, 38.3 μ mol). Following lyophilization, **19** (10.4 mg, 68% yield) was obtained as a bright yellow solid. ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.90 (d, 1 H, H-6_{tereph}), 7.76 (d, 2 H, *J*_{5,6} =

8.8 Hz, H-5), 7.35 (s, 1 H, H-3_{tereph}), 7.22–7.14 (m, 3 H, H-6, H-5_{tereph}), 6.78–6.60 (br s, 4 H, CONH₂), 5.62 (d, 2 H, $J_{1,2} = 5.2$ Hz, H-1'), 5.54 (s, 2 H, 2'-OH), 5.18 (dd, 2 H, $J_{2,3} = 3.2$ Hz, $J_{3,4} = 9.8$ Hz, H-3'), 4.10–4.00 (m, 2 H, H-2'), 3.48 (d, 2 H, $J_{3,4} = 9.9$ Hz, H-4'), 3.48 (s, 6 H, 4'-OMe), 2.21 (s, 6 H, 8-Me), 1.26, 1.06 (2 s, 12 H, 6'-CH₃).

(C) *Mixed Diamide Coumermycin Analogue 17*. For the preparation of mixed diamide analogue **17**, 8-demethylaminocoumarin **33** (3 mg, 13.1 μ mol) and terephthalic acid (21.8 mg, 131 μ mol) were each dissolved in DMSO (0.50 mL) and added to a MES-buffered solution (56 mL, pH 7) containing DMSO (9 mL). ATP and MgCl₂ were added to a final concentration of 4 and 10 mM, respectively, followed by the addition of BSA (100 mg, final concentration = 1 mg/mL). CouL was added to a final concentration of 1 μ M (3.2 mL of 30.9 μ M stock), and the reaction was incubated at ambient temperature for 1 h. The reaction was analyzed by HPLC as described above; the mixture contained >95% monoamide **31** and <5% diamide.

The reaction mixture was adjusted to pH 3 and extracted three times with ethyl acetate (50 mL). The organic extractions were combined and concentrated under reduced pressure. The crude reaction product was purified by preparative reversed-phase HPLC and lyophilized to afford monoamide **31** as a bright yellow solid (3.2 mg, 72% yield).

Monoamide **31** (2.5 mg, 7.33 μ mol) and aminocoumarin **1** (8.9 mg, 36.6 μ mol) were dissolved in DMSO (0.50 mL) and added to a MES-buffered solution (60 mL, pH 7) containing DMSO (6.9 mL). ATP and MgCl₂ were added to a final concentration of 4 and 10 mM, respectively, followed by the addition of BSA (100 mg, final concentration = 1 mg/mL). CouL was added to a final concentration of 1 μ M (3.2 mL of 30.9 μ M stock), and the four-enzyme tandem incubation and purification were carried out as described for compounds **14** and **19** above. Lyophilization of the purified product afforded mixed diamide coumermycin analogue **17** (4.7 mg, 67% yield) as a yellow solid. ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.10–11.90 (br s, 2 H, OH), 9.65 (br s, 2 H, NH), 8.12 (s, 4 H, H-2,3,5,6_{tereph}), 7.85, 7.76 (2 d, 2 H, $J_{5,6} = 8.6$ Hz, H-5), 7.17 (d, 1 H, $J_{5,6} = 8.6$ Hz, H-6), 7.02–7.00 (m, 2 H, H-6, H-8), 6.80–6.50 (br s, 4 H, CONH₂), 5.64–5.62 (m, 2 H, H-1'), 5.59, 5.55 (2 d, 2 H, $J_{2-OH} = 1.7$ Hz, 2'-OH), 5.16–5.07 (2 dd, 2 H, $J_{2,3} = 3.1$ Hz, $J_{3,4} = 9.9$ Hz, H-3'), 4.10–4.05 (m, 2 H, H-2'), 3.48–3.44 (m, 8 H, H-4', 4'-OMe), 2.33 (s, 3 H, 8-Me), 1.27, 1.23, 1.08, 1.06 (4 s, 12 H, 6'-CH₃).

Determination of Minimum Inhibitory Concentrations for Analogues 14, 17, and 19. Twenty-two hour minimum inhibitory concentrations (MICs) were determined against strains grown in brain–heart infusion broth in a microdilution format according to NCCLS guidelines (33). The following strains were used for the MIC determination: *Enterococcus faecium*, 49624; *E. faecium*, resistant (VanA), CL4931; *Enterococcus faecalis*, 29212; *E. faecalis*, resistant (VanB), CL4877; *S. aureus*, 29213.

RESULTS

Overproduction and Purification of S. rishiriensis CouM and CouP in E. coli. CouM is the putative glycosyltransferase in coumermycin A₁ biosynthesis as it bears significant sequence homology to the family of glycosyltransferases

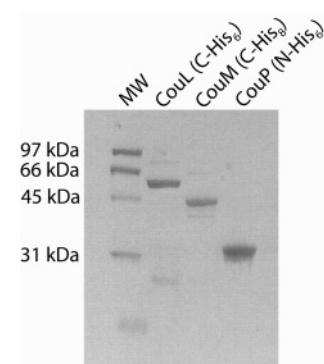
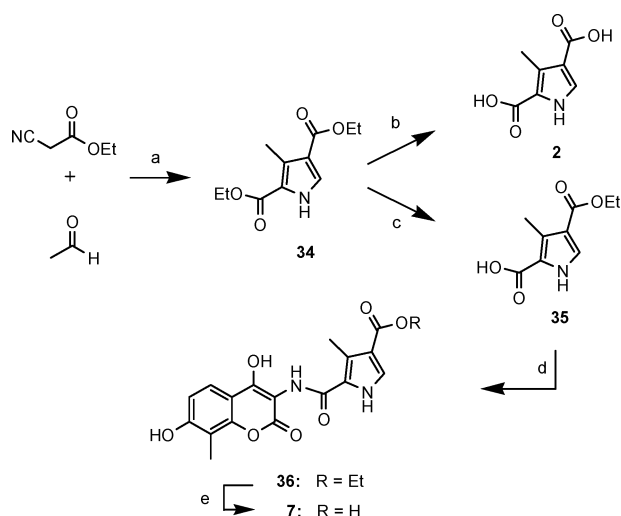
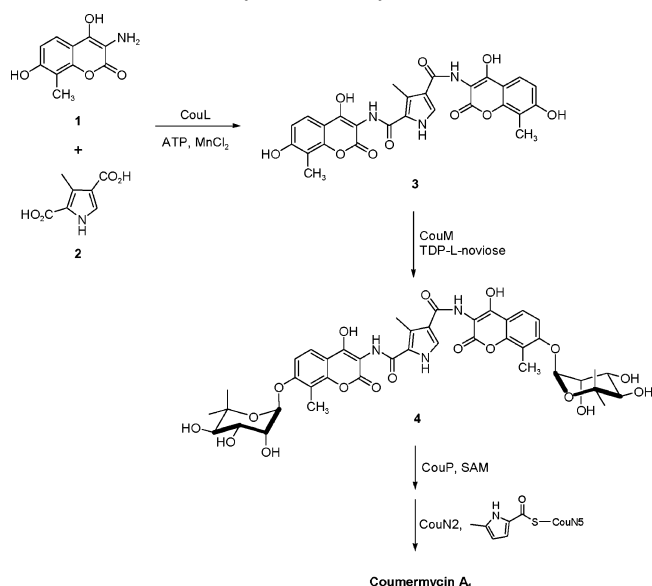


FIGURE 2: Heterologous overproduction of CouL, CouM, and CouP from *E. coli*.

including the novobiocin glycosyltransferase NovM (16, 22). CouM presumably catalyzes the transfer of two noviosyl moieties from TDP-L-noviose to the diamide aglycon **3** (Scheme 2) to form the bisnoviosyl aminocoumarin diamide **4**. The 42 kDa enzyme was overproduced heterologously and purified from *E. coli* BL21(DE3) cells harboring the CouM expression construct. Cells were grown at 25 °C to an OD of ~0.6 followed by induction with IPTG (60 μ M). The C-terminal His₆ fusion protein CouM was then purified to homogeneity by Ni(II) affinity chromatography (Figure 2).

CouP bears homology to the family of *O*-methyltransferases including the novobiocin *O*-methyltransferase NovP (16, 23). CouP presumably catalyzes the first step in sugar decoration by *O*-methylation at the 4'-position of each noviose component (Figure 6A) to form the precursor to coumermycin A₁. The 31 kDa enzyme was overproduced heterologously and purified from *E. coli* BL21(DE3) cells harboring the CouP expression construct as described above for the overproduction and purification of CouM. The N-terminal His₆ fusion protein CouP was purified to homogeneity by Ni(II) affinity chromatography (Figure 2).

Preparation of Aglycon Substrates. Aminocoumarin **1** was obtained from novobiocin through a two-step degradation procedure (step 1, Ac₂O/pyridine; step 2, HCl/MeOH) (28). 3-Methylpyrrole-2,4-dicarboxylic acid (**2**) was obtained by DBU-catalyzed condensation of acetaldehyde with ethyl isocyanoacetate (Scheme 1) (29), which afforded diethyl ester **34** (70%). Both ester groups could be cleaved by heating **34** and sodium hydroxide (10 equiv) in a water/ethanol mixture under reflux for 16 h to give diacid **2** (80%). Monoethyl ester **35** was required for the synthesis of monomer **7** and could be prepared from diester **34** based on the higher reactivity of the 2-carboxyethyl group toward base (30). Thus, saponification using 1.2 equiv of potassium hydroxide in an ethanol/water mixture under reflux for 2.5 h afforded the 2-carboxylic acid **35** in 66% yield. Coupling of aminocoumarin **1** with acid **35** using HATU as the activating agent proceeded in unsatisfying yields (only 10% of protected monomer **36** could be isolated). When the corresponding acid chloride (obtained from **35** using oxalyl chloride/DMF) was coupled with **1** in DMF in the presence of DIPEA, the yield of **36** was slightly improved (40%). Finally, it was found that activation of acid **35** with PyBOP proceeded smoothly and afforded **36** in 76% yield. Deprotection of the remaining carboxylic ester under acidic conditions then gave monomer **7** in 66% yield.

Scheme 1^aScheme 2: Coumermycin A₁ Biosynthesis

Characterization of CouM Glycosyltransferase Activity. Heide and co-workers recently demonstrated that the ligase CouL catalyzes two amide bond forming reactions (26), the first between the aminocoumarin **1** and 3-methylpyrrole-2,4-dicarboxylic acid (**2**) to form a monoamide which then undergoes subsequent ligation with a second molecule of aminocoumarin **1** to form diamide **3**. Glycosylation by CouM in coumermycin A₁ biosynthesis presumably follows amide bond formation (Scheme 2), and CouM is expected to process the distinct scaffolds required for the first and second glycosylation reactions in the same way that CouL processes two distinct carboxylic acid substrates.

Initial confirmation of glycosyltransferase activity was accomplished in a tandem CouL, CouM enzyme incubation (Figure 3). The appearance of the bisnoviosyl product **4** was monitored by reversed-phase HPLC, and the CouM product was confirmed by LCMS (m/z 866.40 [M - H]⁻). Further HPLC analysis of CouM activity was carried out using purified diamide scaffold **3** as substrate for CouM (Figure 4). Disappearance of substrate **3** in the presence of CouM (100

nM) and TDP-L-noviose was accompanied by the accumulation of an intermediate product peak, presumably corresponding to a mixture of mononoviosyl intermediates **5** and **6** (m/z 706.00 [M - H]⁻) and the bisnoviosyl CouM product **4** (m/z 866.45 [M - H]⁻). Under these conditions, greater than 70% of diamide **3** underwent glycosylation over 60 min (Figure 4C). In contrast, less than 25% of monoamide **7** underwent glycosylation to afford the corresponding glycosylated monoamide **8** (m/z 517.00, [M - H]⁻) under the same conditions (Figure 5), suggesting that while CouM will accept variant aminocoumarin scaffolds as substrates, diamide **3** is most likely the preferred initial aglycon substrate for CouM.

The general pattern of partial accumulation of a monoglycosylated intermediate that then undergoes bisglycosylation by tandem action of CouM is evident from Figure 4. However, a detailed kinetic analysis of both initial monoglycosylation and tandem bisglycosylation has not yet been carried out to obtain kinetic parameters k_{cat} and K_M . It is assumed that a mixture of regioisomers resulting from glycosylation at either 7-hydroxy group of the aglycon scaffold is formed during the initial sugar transfer from TDP-L-noviose (**5** and **6**, Figure 4A). Until each regioisomer has been prepared and characterized separately, and conditions have been determined for the chromatographic separation of **5** and **6**, the efficiency of CouM in the formation of either isomer **5** or **6** and the extent to which CouM prefers each intermediate for bisglycosylation cannot be accurately determined. However, it is apparent from the studies carried out during the course of this work that CouM will effectively process a diamide scaffold to the corresponding bisglycosylated product, and efforts to utilize CouM as a glycosyltransferase for the large-scale preparation of variant coumermycin analogues are described below.

Characterization of CouP Methyltransferase Activity. Decarboxylation of the noviose sugar components in coumermycin A₁ biosynthesis is expected to follow the same biosynthetic logic as that confirmed for sugar tailoring in novobiocin biosynthesis (22). Thus, it is anticipated that the putative methyltransferase CouP catalyzes bis-O-methylation at the 4'-position of each noviose ring following bisglycosylation by CouM.

The methyltransferase activity of CouP was confirmed using as substrate the CouM product **4** obtained via enzymatic glycosylation of synthetic scaffold **3**. Not surprisingly, CouP exhibits a reaction profile similar to that observed during glycosylation by CouM (Figure 6C). The disappearance of **4** in the presence of CouP (1 μ M) and *S*-adenosylmethionine (SAM) was monitored by reversed-phase HPLC (Figure 6B), and the formation of the presumed mixture of monomethyl intermediates **9** and **10** (m/z 880.30, [M - H]⁻) followed by formation of CouP product **11** (m/z 894.30, [M - H]⁻) was confirmed by LCMS. As noted above, a detailed kinetic analysis of a reaction profile involving the probable formation of a mixture of regioisomers is not feasible unless the reaction intermediates are prepared and studied separately. Therefore, a comprehensive kinetic analysis has not yet been carried out for the reactions catalyzed by CouP.

It is interesting to note that the glycosyltransferase NovM and the methyltransferase NovP from the novobiocin biosynthetic cluster (22, 23) catalyze comparable transformations on the coumermycin scaffold with similar relative efficiencies to CouM and CouP (data not shown). This suggests there is

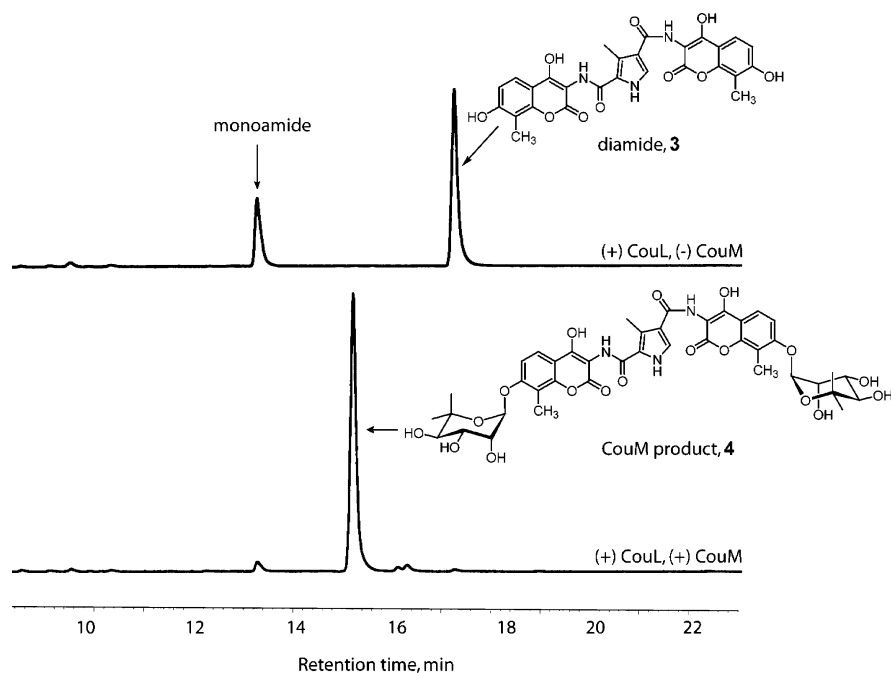


FIGURE 3: Confirmation of CouM activity in a tandem CouL, CouM incubation of substrates **1** and **2**.

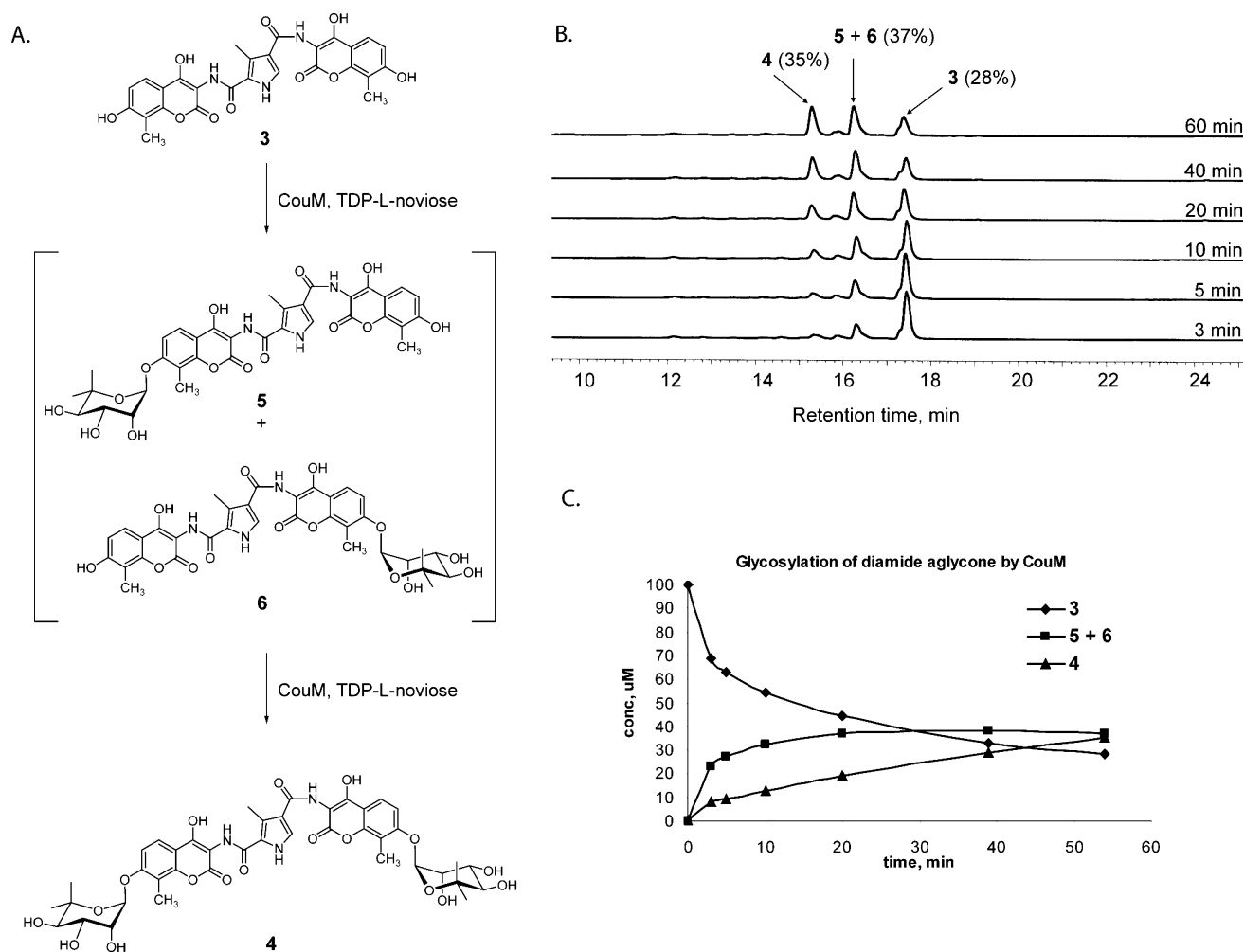


FIGURE 4: (A) CouM-catalyzed bisglycosylation of diamide substrate **3** to form CouM product **4**. (B) Reversed-phase HPLC stack plot showing conversion of **3** to **4** (75 mM MES, pH 6, 10% DMSO, 1 mg/mL BSA). (C) Reaction profile of bisglycosylation by CouM.

little deviation of the fundamental enzyme mechanisms for glycosylation and methylation by CouM and CouP compared to NovM and NovP.

Carbamoyltransferase Activity on a Coumermycin Scaffold. The characterization of the ligase CouL (26) and preliminary confirmation of CouM and CouP functions

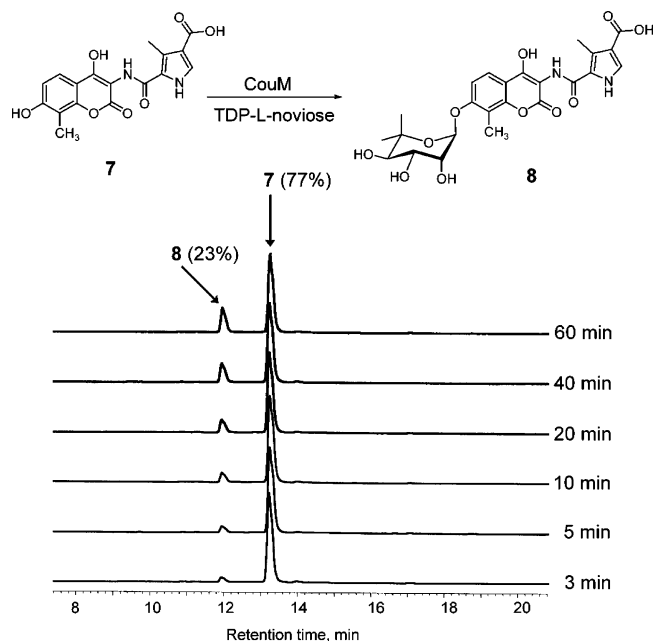


FIGURE 5: CouM-catalyzed glycosylation of monoamide **7** (75 mM MES, pH 6, 10% DMSO, 1 mg/mL BSA).

described here present an opportunity for diversification of coumermycin structural components. However, functionalization at the 3'-position of the noviose ring with either a carbamoyl substituent (novobiocin) or a 2-methylpyrrole-5-carboxy ester substituent (clorobiocin and coumermycin A₁) is essential for antibiotic activity in the aminocoumarin family of natural products. As we are not yet able to obtain soluble, active acyltransferase CouN2 (Scheme 2) for further diversification of the noviose ring, a study was carried out in order to determine whether the novobiocin carbamoyltransferase NovN would catalyze biscarbamoylation on a coumermycin scaffold. Carbamoylation by NovN was monitored by reversed-phase HPLC (Figure 7). The disappearance of substrate **11** was accompanied by the accumulation of the presumed mixture of monocarbamoylated intermediates **12** and **13** followed by subsequent disappearance of this mixture to form the biscarbamoylated coumermycin analogue **14**. The formation of monocarbamoylated and biscarbamoylated coumermycin analogues was confirmed by LCMS (**12** + **13**, m/z 937.22, $[M - H]^-$; **14**, m/z 980.20, $[M - H]^-$).

Combinatorial Biosynthetic Generation of Novel Coumermycin Analogues. The activity of NovN on CouP product **11** to form the novel coumermycin analogue **14** (Figure 7) shows that completion of sugar decoration in dimeric aminocoumarin biosynthesis is possible via carbamoylation, and the generation of novel coumermycin analogues with potential antibiotic activity is conceivable. Furthermore, an initial substrate specificity study of CouL carried out by Heide and co-workers (26) suggests that alteration of the coumermycin scaffold is also feasible. Our investigation confirms that the combinatorial biosynthetic generation of coumermycin analogues via four-enzyme tandem incubations (CouL, CouM, CouP, NovN) is achievable, and the nine coumermycin variants generated as a result of these studies are shown in Figure 8.

The introduction of structural diversity in the scaffold of coumermycin to generate compounds **16**–**22** (Figure 8) required some additional analysis of substrate usage by CouL

for potential large-scale tandem incubations. Table 1 summarizes ligase substrate usage as a result of a brief study aimed at identifying aromatic diacid substrates for CouL that are distinct from the natural pyrrole dicarboxylic acid substrate. Interestingly, none of the isophthalic acids or pyridine-2,5-dicarboxylic acid were accepted as substrates for CouL whereas both terephthalic acid and 2-aminoterephthalic acid were. Clearly, a more rigorous study of substrate specificity is required in order to better understand the structural requirements of CouL for its diacid substrate.

The generation of compounds **16** and **19** via four-enzyme tandem incubation (CouL, CouM, CouP, and NovN) was studied by reversed-phase HPLC using both terephthalic acid and 2-aminoterephthalic acid as alternate CouL diacid substrates. Enzymes were added sequentially, and reactions were allowed to incubate for varying periods of time in order to permit the accumulation of intermediates to be used subsequently as substrates for the succeeding enzymes. As expected, the production of diacid-modified biscarbamoylated analogues **16** (m/z 977.05, $[M - H]^-$; Figure 9A) and **19** (m/z 992.27, $[M - H]^-$; data not shown) was observed and confirmed by LCMS. Not surprisingly, the presence of intermediates arising from incomplete ligation, methylation, and carbamoylation were also detected in the final enzyme reaction mixture as well as in control reactions, each lacking one of the enzymes (Figure 9).

Compound **15** was generated chemoenzymatically starting from the synthetically prepared monoamide **7** as substrate for CouL. The second ligation by CouL was carried out using 8-demethylaminocoumarin **33**, available from a prior synthetic effort in our laboratory (27), as the second substrate followed by tandem action of CouM, CouP, and NovN as described above. The production of the mixed diamide coumermycin analogue **15** was monitored by HPLC as described above (data not shown) and confirmed by LCMS (m/z 966.10, $[M - H]^-$). As noted above, the presence of intermediates arising from incomplete ligation, methylation, and carbamoylation was also detected in the final enzyme reaction mixture.

Compounds **17**–**18** and **20**–**22** were generated enzymatically using either terephthalic acid or 2-aminoterephthalic acid as the diacid substrate for CouL and a 1:1 mixture of aminocoumarins **1** and **33**. All possible diamide and mixed diamide coumermycin analogues **16**–**18** and **19**–**22** were detected by LCMS (data not shown).

Large-Scale Enzymatic Preparation of Coumermycin Analogues for Biological Evaluation. As further demonstration of the utility of the four-enzyme tandem incubation using coumermycin and novobiocin enzymes to produce novel coumermycin variants, three analogues (**14**, **17**, and **19**, Figure 8) were selected for enzymatic synthesis on sufficient scale for biological evaluation. In each case, the individual enzymatic reactions were monitored by HPLC and LCMS to ensure complete conversion of substrates to products before proceeding to the subsequent step. In some cases, complete conversion of substrate to product was only achievable by the repeated addition of enzyme or extended incubation times. As shown in Figure 10 for compound **19**, satisfactory conversion to the final product was accomplished using this strategy, and little or no formation of intermediates arising from incomplete ligation, methylation, or carbamoylation was detected.

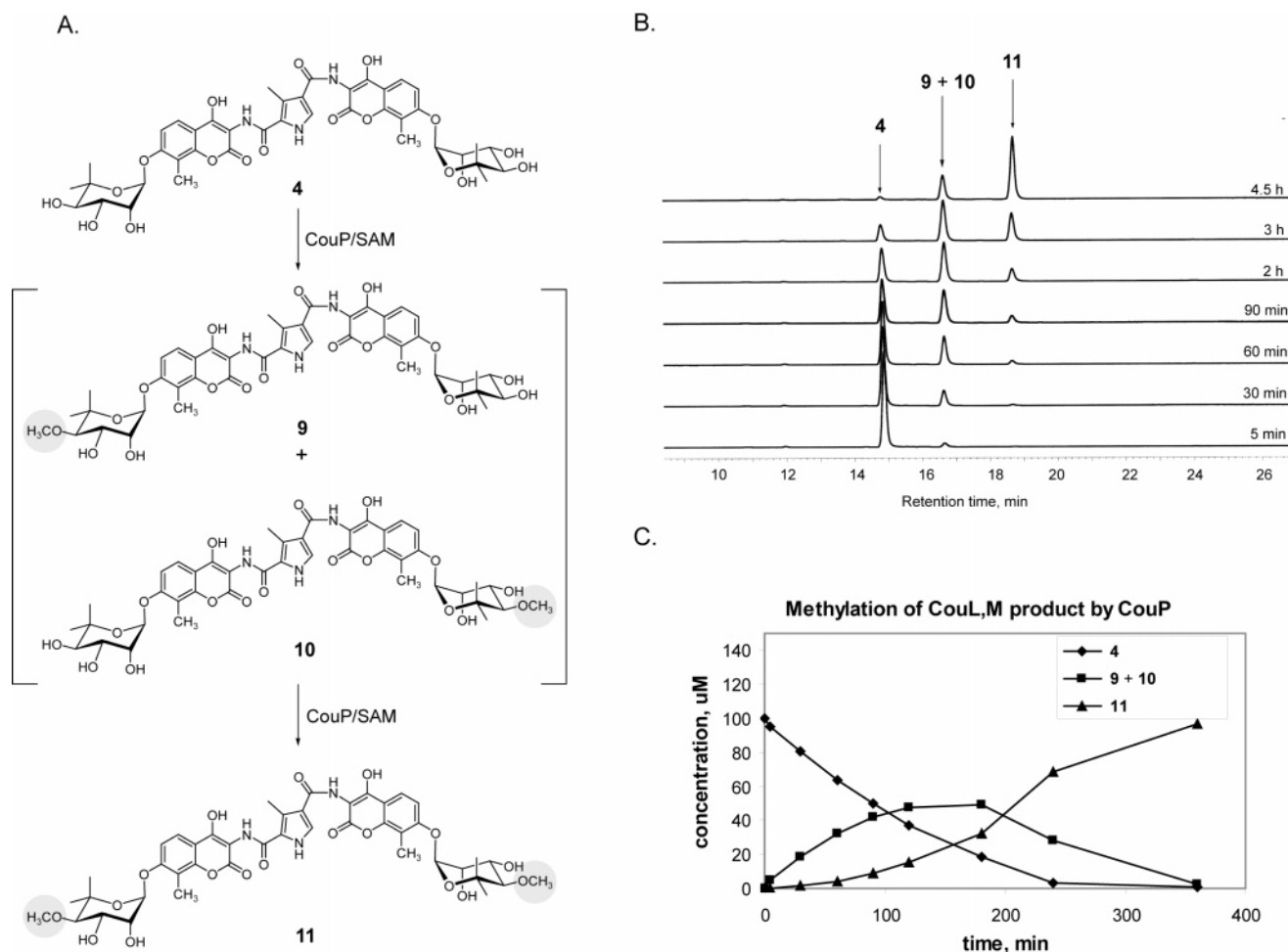


FIGURE 6: (A) CouP-catalyzed bismethylation of CouM product **4** to form bismethylation product **11**. (B) Reversed-phase HPLC stack plot showing conversion of **4** to **11** (75 mM Tris-HCl, pH 7.5, 10% DMSO, 1 mg/mL BSA). (C) Reaction profile of bismethylation by CouP.

For the preparation of mixed diamide analogue **17**, monoamide **31** was prepared (>95% by HPLC) by ligation of 8-demethylaminocoumarin **33** to terephthalic acid (in excess) in the presence of CouL (Scheme 3). Monoamide **31** was purified by preparative HPLC following an acidic workup, and the purified product was reacted with aminocoumarin **1** (in excess) in the presence of CouL. Subsequent tandem reaction with CouM, CouP, and NovN was carried out as described above for the preparation of analogues **14** and **19**. In all cases, the final products were confirmed by LCMS prior to concentration by passage through a C18 SepPak and elution in DMSO. The crude products were then purified by preparative reversed-phase HPLC to yield **14** (65% yield), **17** (67% yield), and **19** (68% yield). Compounds **14**, **17**, and **19** were tested for antibiotic activity, and the minimum inhibitory concentrations (MICs) are summarized in Table 2.

The biological activity data obtained here (Table 2) for novobiocin and coumermycin A₁ are in agreement with the literature (31), suggesting that novobiocin is ~1 log less potent than coumermycin A₁ against *S. aureus* and 30–60-fold less potent than coumermycin A₁ against the four enterococcal strains. Compound **14**, which resembles coumermycin A₁ but instead bears a carbamoyl group at the 3'-O-noviosyl position as in novobiocin, displays activity similar to novobiocin. This observation is consistent with the 3'-O-carbamoyl moiety as the prime pharmacophore on the

aminocoumarin scaffolds for presentation to the GyrB subunit. Compounds **17** and **19**, bearing the terephthalic and 2-aminoterephthalic spacer elements in the dimer, display antibiotic potencies 1–2 logs lower than compound **14** and are 3 logs less active than coumermycin A₁ itself.

DISCUSSION

The aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A₁ (Figure 1), produced by various strains of *Streptomyces*, use the 4'-O-methyl-3'-O-acyl- or 4'-O-methyl-3'-O-carbamoyl-L-noviosyl moiety as the pharmacophore to inhibit ATP hydrolysis in the B subunits of DNA gyrase and topoisomerase IV. The decorated noviosyl component is presented to the ATP binding site by a two-part scaffold comprised of an aminocoumarin linked through an amide bond to a prenylated hydroxybenzoic acid moiety in novobiocin and clorobiocin. X-ray crystallographic analyses of novobiocin and clorobiocin each bound to the ATPase domain of GyrB illustrate that both the 3'-O-carbamoyl substituent in novobiocin and the 3'-O-methylpyrrole carboxy ester substituent of clorobiocin are positioned in the active site (5, 6) and support the observed requirement of these substituents for gyrase inhibitory activity and antibiotic potency. Consequently, it is presumed that the 3'-O-methylpyrrole carboxy ester substituent on noviose in the pseudodimeric coumermycin A₁ is also the competing ligand which blocks ATP binding. It is unknown whether the

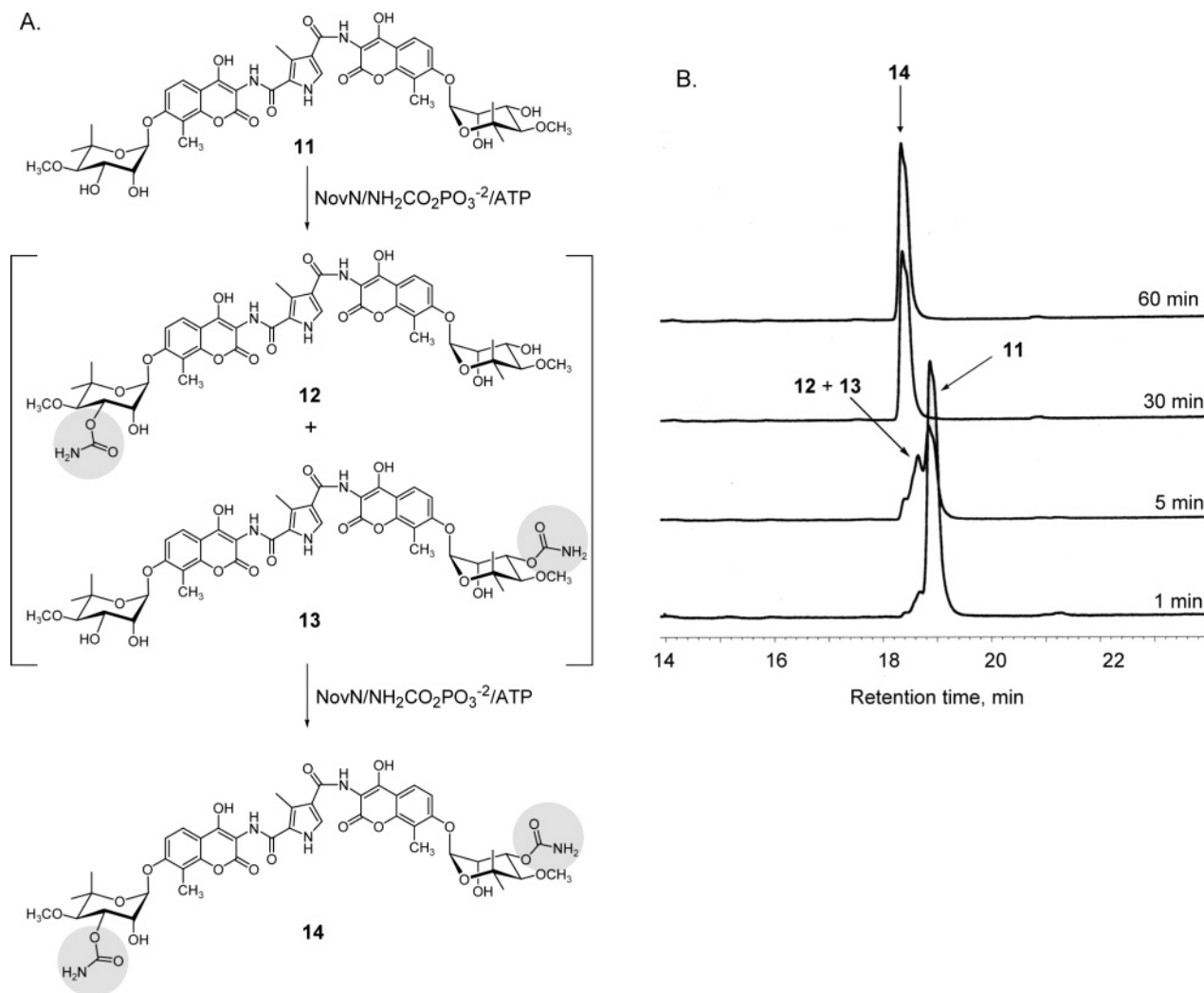


FIGURE 7: (A) NovN-catalyzed biscarbamylation of CouP product **11** to form biscarbamoylated coumermycin analogue **14**. (B) Reversed-phase HPLC stack plot showing conversion of **11** to **14** (75 mM Tris-HCl, pH 7.5, 10% DMSO, 1 mg/mL BSA).

enhanced potency and gyrase inhibitory activity of coumermycin A₁ are merely the concentration effect of two pharmacophores in one molecule or if there is some likelihood of simultaneous occupancy of two gyrase B subunits in an A₂B₂ tetramer.

The implication that the late stage biosynthetic enzymes act twice in coumermycin biosynthesis makes coumermycin A₁ an intriguing molecule to study. The prenyl hydroxybenzoate element in novobiocin and clorobiocin is replaced by a 3-methyl-2,4-dicarboxypyrrole linker in coumermycin, and the biosynthetic origin of this linking element is yet unknown. The ligase CouL catalyzes the double ligation of 3-amino-7-hydroxycoumarin with the 3-methyl-2,4-dicarboxylic acid linker to create the corresponding diamide, the first step in elongation of the two halves of coumermycin A₁ (26). We have demonstrated here that CouM and CouP, as well as the novobiocin enzymes NovM and NovP, will indeed catalyze double glycosylation and 4'-O-methylation in the subsequent elongation steps. The accumulation of mononoviosyl and monomethyl intermediates during glycosylation and methylation reactions catalyzed by CouM and CouP, respectively, suggests that these enzymes act distributively, not processively. It is not clear if such partially elongated intermediates accumulate in the producing microorganisms,

but completion of double glycosylation and methylation can be accomplished in vitro by treatment of substrates for extended periods of incubation with the purified enzymes.

Minimal antibiotic activity is observed in novobiocin and clorobiocin analogues lacking the 3'-O-substituents on the noviose ring (25). Carbamylation (novobiocin) and acylation (clorobiocin) are catalyzed by NovN (23) and CloN2 (24), respectively. Double acylation of the coumermycin scaffold is presumably catalyzed by CouN2 (16), and a similar antibiotic potency profile is anticipated. The 5-methylpyrrolyl moiety at the 3'-position on the noviose ring in clorobiocin and coumermycin A₁ is derived from proline by nonribosomal peptide synthetase module logic (15, 16). The pathway is assumed to generate a pyrrolyl-S-peptidyl carrier protein species that is most likely transferred to the CloP or CouP product by the action of acyltransferases CloN2 and CouN2, respectively. C-Methylation at the 5-position of the pyrrolyl moiety is thought to be the final step in both clorobiocin and coumermycin biosynthesis (24). Although the heterologous overproduction of both CloN2 and CouN2 is possible in *E. coli*, to date only insoluble protein is obtainable; thus, acyl transfer to the noviose ring in coumermycin A₁ biosynthesis cannot be assayed with purified enzymes. Alternatively, we have evaluated the novobiocin carbam-

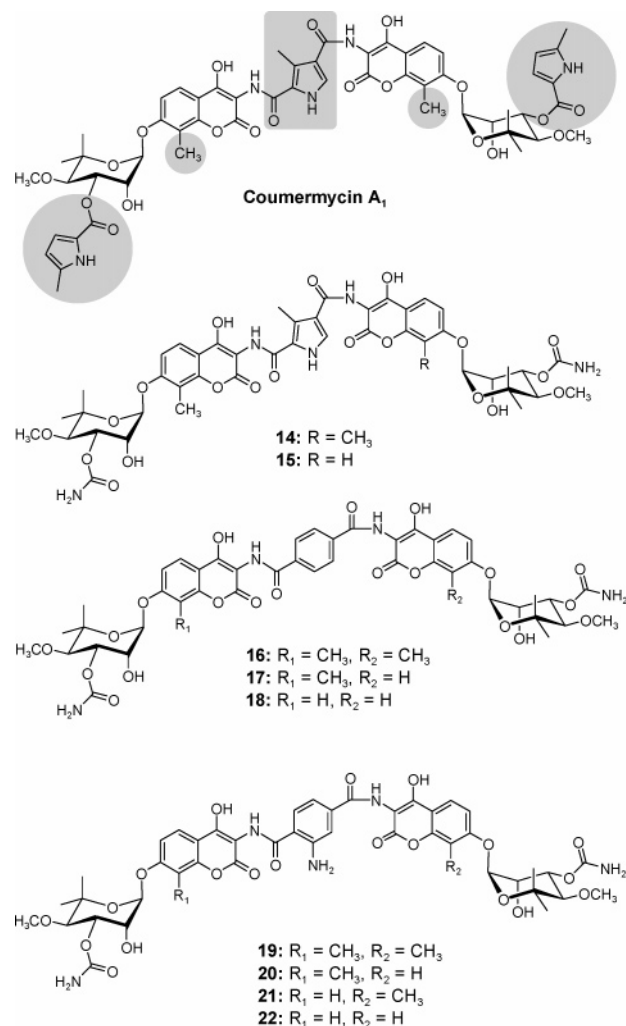
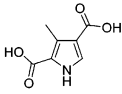
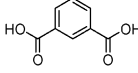
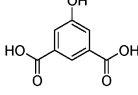
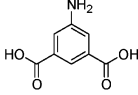
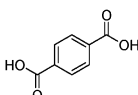
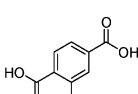
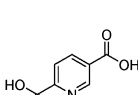


FIGURE 8: Coumermycin analogues generated via four-enzyme CouL, CouM, CouP, and NovN tandem incubation.

oyltransferase NovN as a catalyst for biscarbamoylation of the CouL, CouM, CouP enzymatic product and have observed mono- and biscarbamoylation to create the biscarbamoyl analogue **14**, an analogue previously obtained in low yield *in vivo* (32). Furthermore, we were able to carry out the tandem four-enzyme incubation and facilitate the complete conversion of intermediates in all eight enzymatic steps to produce multimilligram quantities of **14** for biological evaluation against the authentic natural product coumermycin A₁ (Table 2).

The *in vitro* assembly of the coumermycin A₁ scaffold and alternate carbamoyl substitution at the 3'-position of noviose by NovN led to additional studies aimed at introducing diversity into the planar scaffold of coumermycin A₁. Heide and co-workers recently demonstrated that the ligase CouL will accept alternative dicarboxylic acids as substrates (26). We have extended this study to include a few additional commercially available diacids, and the usage of these diacids as substrates for CouL is summarized in Table 1. Of the six aromatic diacids tested, terephthalic acid and 2-aminoterephthalic acid underwent ligation to aminocoumarin **1** in the presence of CouL to form the corresponding mono- and diamides. The resulting diamides were then shown to undergo glycosylation, methylation, and carbamoylation in the six subsequent steps to generate coumermycin analogues

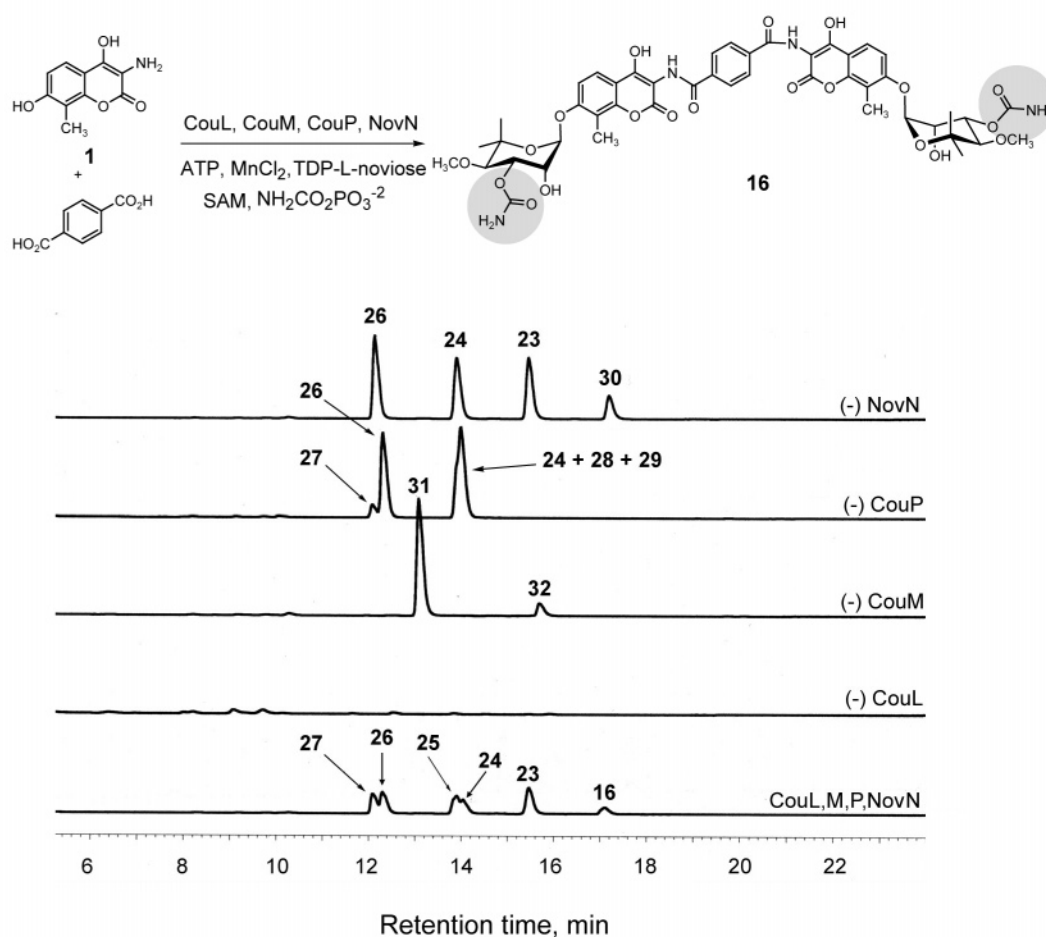
Table 1: Diacid Substrate Usage by CouL

Diacid Substrate	Usage by CouL
 3-Methyl-pyrrole-2,4-dicarboxylic acid	yes
 Isophthalic acid	no
 5-hydroxyisophthalic acid	no
 5-aminoisophthalic acid	no
 Terephthalic acid	yes
 2-Aminoterephthalic acid	yes
 Pyridine-2,5-dicarboxylic acid	no

16 and **19** (Figure 8) exemplifying modification of three of the five components in the coumermycin scaffold. Introduction of the 2-aminoterephthalic acid moiety in analogue **19** significantly improves the aqueous solubility properties relative to coumermycin A₁ and coumermycin analogue **16** and could, in principle, be further modified at the 2-amino position. As for compound **14**, we are able to facilitate the complete conversion of intermediates in all eight enzymatic steps to produce multimilligram quantities of **19** for biological evaluation against the authentic natural product coumermycin A₁ (Table 2).

Further study of the ligation by CouL revealed that a mixture of aminocoumarin **1** and 8-demethylaminocoumarin **33** easily undergoes ligation in the presence of terephthalic acid or 2-aminoterephthalic acid to give the corresponding mixtures of diamides. The subsequent tandem action of CouM, CouP, and NovN resulted in the formation of a mixture of compounds **16–18** and **19–22**, which include examples of mixed diamide coumermycin analogues. Likewise, treatment of synthetic monoamide **7** with 8-demethylaminocoumarin **33** in the presence of CouL afforded the corresponding mixed diamide. Tandem action of CouM, CouP, and NovN provided coumermycin analogue **15**, illustrating the modification of three components in the coumermycin scaffold. Compounds **17**, **20**, and **21** demonstrate structural modification to four of the five components

A.



B.

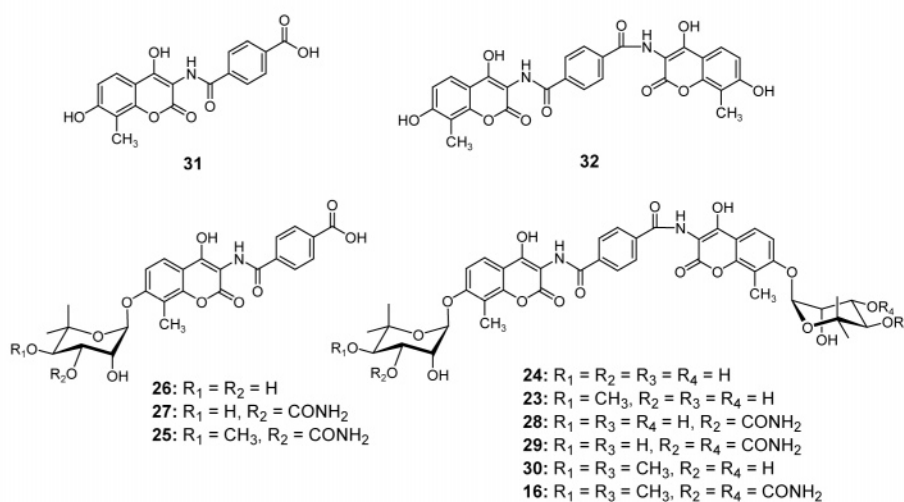


FIGURE 9: (A) Nonoptimized small-scale generation of coumermycin analogue **16** via four-enzyme CouL, CouM, CouP, and NovN tandem incubation. The HPLC traces show the product distribution for the four-enzyme reaction and for incubations each lacking one of the enzymes. (B) Reaction products and intermediates detected by LCMS.

of the coumermycin scaffold while compounds **18** and **22** incorporate changes to all five components.

The rapid accumulation of monoamide **31** relative to diamide **32** in ligation reactions carried out using terephthalic acid and aminocoumarin **1** (Figure 9) permitted the preparative isolation of monoamide **31**. 8-Demethylaminocoumarin **33** was utilized in a second preparative ligation step with CouL and monoamide **31**. The resulting mixed diamide CouL product could be carried through the subsequent six enzy-

matic steps of CouM, CouP, NovN to generate the coumermycin A_1 variant **17** in multimilligram quantities for biological evaluation (Table 2).

The initial antibiotic potencies obtained for compounds **14**, **17**, and **19** on five Gram-positive pathogenic strains raise two issues. First, these data confirm the observation that the 5-methylpyrrolacetyl moiety at the 3'-position of the noviose ring is a key pharmacophore, whether displayed on cloribocin or coumermycin, and imparts ~ 1 log greater potency

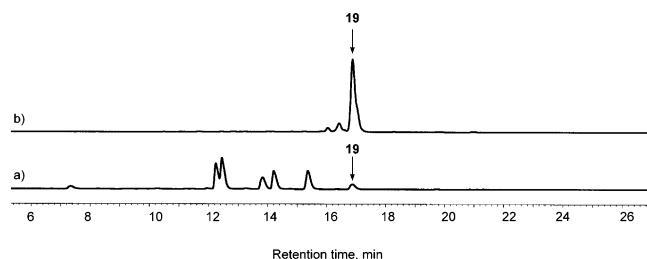


FIGURE 10: (a) HPLC detection of coumermycin analogue **19** formation on a small scale. (b) HPLC analysis of analogue **19** formation on a large scale following optimization of each step in the four-enzyme tandem incubation.

Scheme 3: Biosynthetic Preparation of Mixed Diamide Coumermycin Analogue **17**

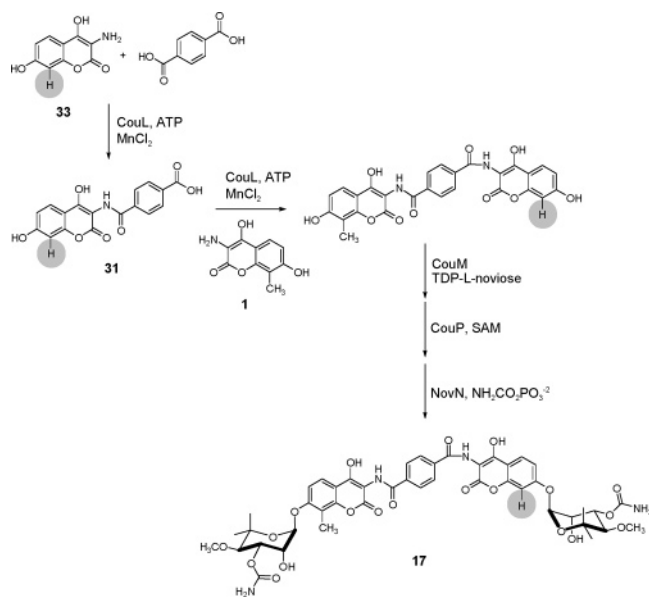


Table 2: Minimum Inhibitory Concentrations (MICs) against Various Gram-Positive Strains^a

	<i>S. aureus</i>	<i>E. faecium</i>	<i>E. faecium</i> (VanA)	<i>E. faecalis</i>	<i>E. faecalis</i> (VanB)
novobiocin	0.4	1.6	0.4	6.25	12.5
coumermycin	<0.05	0.05	0.01	0.1	0.2
14	0.8	12.5	12.5	12.5	25
17	25	>100	>100	>100	>100
19	>100	>100	>100	>100	>100

^a MIC values are in $\mu\text{g/mL}$.

than the 3'-O-carbamoyl group found in novobiocin. Subsequent efforts in diversification of the dimeric aminocoumarins at this position should focus on obtaining soluble, active pyrrolyl transferase CouN2 to use instead of NovN. Second, the antibiotic activity data obtained during the course of this study suggest that the dicarboxy-2-methylpyrrole spacer between the two aminocoumarins also appears to play an important role in coumermycin A₁ antibiotic activity. While the hydrophobic terephthalic moiety might not be expected to mimic the nitrogen-containing pyrrole in coumermycin A₁, it is somewhat more surprising that the 2-amino-terephthalic linker is unsuitable as a pyrrole mimic. Subsequent assay of compounds **17** and **19** against purified DNA gyrase may be worthwhile to determine whether the lack of whole cell activity is indeed failure of compounds

17 and **19** to be recognized by the GyrB target or simply a bacterial cell uptake problem. Delineation of the role played by the dicarboxy-2-methylpyrrole spacer element may also require cocrystallization of the dimeric coumermycin with the same GyrB fragment that has previously been crystallized with clorobiocin and novobiocin.

The consecutive action of the four enzymes CouL, CouM, CouP, and NovN reconstitutes and redirects late stage coumermycin assembly and demonstrates the versatility of this enzymatic route for the generation of novel coumermycin analogues. Further combinatorial variation of the coumermycin scaffold is contingent only upon the permissivity of the four enzymes. The results obtained during the course of this work establish how such combinatorial manipulation can be practiced in vitro in this biosynthetic pathway to effect up to five structural modifications in a single coumermycin scaffold. The preparation and preliminary biological evaluation of selected analogues demonstrate the utility of this system for the practical study of biological and physical properties of novel coumermycin analogues which will foster future studies of this dimeric natural product family.

ACKNOWLEDGMENT

The synthesis of aminocoumarin **33** by Dr. Junhua Tao, construction of the CouL expression vector pMS90 by Marion Steffensky, and contributions by Shu-Ming Li are gratefully acknowledged.

REFERENCES

- Drlica, K., and Zhao, X. (1997) DNA Gyrase, topoisomerase IV, and the 4-quinolones, *Microbiol. Mol. Biol. Rev.* **61**, 377–392.
- Pan, X. S., and Fisher, L. M. (1996) Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: Role in fluoroquinolone resistance, *J. Bacteriol.* **178**, 4060–4069.
- Munoz, R., and De La Campa, A. G. (1996) ParC subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase a subunit in forming resistance phenotype, *Antimicrob. Agents Chemother.* **40**, 2252–2257.
- Kumagai, Y., Kato, J. I., Hoshino, K., Akasaka, T., Sato, K., and Ikeda, H. (1996) Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene, *Antimicrob. Agents Chemother.* **40**, 710–714.
- Lewis, R. J., Singh, O. M., Smith, C. V., Skarzynski, T., Maxwell, A., Wonacott, A. J., and Wigley, D. B. (1996) The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography, *EMBO J.* **15**, 1412–1420.
- Tsai, F. T., Singh, O. M., Skarzynski, T., Wonacott, A. J., Weston, S., Tucker, A., Pauptit, R. A., Breeze, A. L., Poyser, J. P., O'Brien, R., Ladbury, J. E., and Wigley, D. B. (1997) The high-resolution crystal structure of a 24-kDa gyrase B fragment from *E. coli* complexed with one of the most potent coumarin inhibitors, clorobiocin, *Proteins* **28**, 41–52.
- Kampranis, S. C., Gormley, N. A., Tranter, R., Orphanides, G., and Maxwell, A. (1999) Probing the binding of coumarins and cyclothialidines to DNA gyrase, *Biochemistry* **38**, 1967–1976.
- Gormley, N. A., Orphanides, G., Meyer, A., Cullis, P. M., and Maxwell, A. (1996) The interaction of coumarin antibiotics with fragments of DNA gyrase B protein, *Biochemistry* **35**, 5083–5092.
- Chatterji, M., Unniraman, S., Maxwell, A., and Nagaraja, V. (2000) The additional 165 amino acids in the B protein of *Escherichia coli* DNA gyrase have an important role in DNA binding, *J. Biol. Chem.* **275**, 22888–22894.
- Downes, C. S., Ord, M. J., Mullinger, A. M., Collins, A. R., and Johnson, R. T. (1985) Novobiocin inhibition of DNA excision repair may occur through effects on mitochondrial structure and

- ATP metabolism, not on repair topoisomerases, *Carcinogenesis* 6, 1343–1352.
11. Castora, F. J., Vissering, F. F., and Simpson, M. V. (1983) The effect of bacterial DNA gyrase inhibitors on DNA synthesis in mammalian mitochondria, *Biochim. Biophys. Acta* 740, 417–427.
 12. Sung, S. C. (1974) Effect of novobiocin on DNA-dependent DNA polymerases from developing rat brain, *Biochim. Biophys. Acta* 361, 115–117.
 13. Martin, M. A. (1994) Methicillin-resistant *Staphylococcus aureus*: The persistent resistant nosocomial pathogen, *Curr. Clin. Top. Infect. Dis.* 14, 170–191.
 14. Steffensky, M., Muhlenweg, A., Wang, Z. X., Li, S. M., and Heide, L. (2000) Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891, *Antimicrob. Agents Chemother.* 44, 1214–1222.
 15. Pojer, F., Li, S. M., and Heide, L. (2002) Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: New insights into the biosynthesis of aminocoumarin antibiotics, *Microbiology* 148, 3901–3911.
 16. Wang, Z. X., Li, S. M., and Heide, L. (2000) Identification of the coumermycin A(1) biosynthetic gene cluster of *Streptomyces rishiriensis* DSM 40489, *Antimicrob. Agents Chemother.* 44, 3040–3048.
 17. Pojer, F., Kahlich, R., Kammerer, B., Li, S. M., and Heide, L. (2003) CloR, a bifunctional non-heme iron oxygenase involved in clorobiocin biosynthesis, *J. Biol. Chem.* 278, 30661–30668.
 18. Birch, A. J., Holloway, P. W., and Rickards, R. W. (1962) The biosynthesis of noviose, a branched-chain monosaccharide, *Biochim. Biophys. Acta* 57, 143–145.
 19. Bunton, C. A., Kenner, G. W., Robinson, M. J. T., and Webster, B. R. (1963) Experiments related to the biosynthesis of novobiocin and other related coumarins, *Tetrahedron* 19, 1001–1010.
 20. Kominek, L. A., and Sebek, O. K. (1974) Biosynthesis of novobiocin and related coumarin antibiotics, *Dev. Ind. Microbiol.* 15, 60–69.
 21. Steffensky, M., Li, S. M., and Heide, L. (2000) Cloning, overexpression, and purification of novobiocin acid synthetase from *Streptomyces spheroides* NCIMB 11891, *J. Biol. Chem.* 275, 21754–21760.
 22. Freel Meyers, C. L., Oberthür, M., Anderson, J. W., Kahne, D., and Walsh, C. T. (2003) Initial characterization of novobiocin acid noviosyl transferase activity of NovM in biosynthesis of the antibiotic novobiocin, *Biochemistry* 42, 4179–4189.
 23. Freel Meyers, C. L., Oberthür, M., Xu, H., Heide, L., Kahne, D., and Walsh, C. T. (2004) Characterization of NovP and NovN: Completion of novobiocin biosynthesis by sequential tailoring of the noviosyl ring, *Angew. Chem., Int. Ed. Engl.* 43, 67–70.
 24. Xu, H., Kahlich, R., Kammerer, B., Heide, L., and Li, S. M. (2003) CloN2, a novel acyltransferase involved in the attachment of the pyrrole-2-carboxyl moiety to the deoxysugar of clorobiocin, *Microbiology* 149, 2183–2191.
 25. Hooper, D. C., Wolfson, J. S., McHugh, G. L., Winters, M. B., and Swartz, M. N. (1982) Effects of novobiocin, coumermycin A1, clorobiocin, and their analogues on *Escherichia coli* DNA gyrase and bacterial growth, *Antimicrob. Agents Chemother.* 22, 662–671.
 26. Schmutz, E., Steffensky, M., Schmidt, J., Porzel, A., Li, S. M., and Heide, L. (2003) An unusual amide synthetase (CouL) from the coumermycin A1 biosynthetic gene cluster from *Streptomyces rishiriensis* DSM 40489, *Eur. J. Biochem.* 270, 4413–4419.
 27. Tao, J., Hu, S., Pacholec, M., and Walsh, C. T. (2003) Synthesis of proposed oxidation-cyclization-methylation intermediates of the coumarin antibiotic biosynthetic pathway, *Org. Lett.* 5, 3233–3236.
 28. Hinman, J. W., Caron, E. L., and Hoeksema, H. (1957) The structure of novobiocin, *J. Am. Chem. Soc.* 79, 3789–3800.
 29. Suzuki, M., Miyoshi, M., and Matsumoto, K. (1974) Synthesis of amino acids and related compounds. 7. Convenient synthesis of 3-substituted pyrrole-2,4-dicarboxylic acid esters, *J. Org. Chem.* 39, 1980.
 30. Corwin, A. H., and Viohl, P. (1944) The condensation of pyrroles with bromine. A self-oxidation and a new type of displacement reaction, *J. Am. Chem. Soc.* 66, 1137–1146.
 31. Gellert, M., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1976) Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase, *Proc. Natl. Acad. Sci. U.S.A.* 73, 4474–4478.
 32. Xu, H., Wang, Z. X., Schmidt, J., Heide, L., and Li, S. M. (2002) Genetic analysis of the biosynthesis of the pyrrole and carbamoyl moieties of coumermycin A1 and novobiocin, *Mol. Genet. Genomics* 268, 387–396.
 33. NCCLS (1997) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* (approved standard, NCCLS Document M7-A4) 4th ed., National Committee for Clinical Laboratory Standards, Wayne, PA.

BI048457Z