CouO and NovO: C-Methyltransferases for Tailoring the Aminocoumarin Scaffold in Coumermycin and Novobiocin Antibiotic Biosynthesis†

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ABSTRACT: During the biosynthesis of the streptomycete aminocoumarin antibiotics novobiocin and the dimeric coumermycin A_1, the bicyclic coumarin scaffold is C-methylated adjacent to the phenolic oxygen. The SAM-dependent C-methyltransferases NovO and CouO have been heterologously expressed and purified from *Escherichia coli* and shown to act after the aminocoumarin ring has been constructed by prior action of Nov/CouHIJK. Neither C-methyltransferase works on the tyrosyl-derived *S*-pantetheinyl intermediates tethered to NovH or on the subsequently released free aminocoumarin. NovL ligates the aminocoumarin to prenylhydroxybenzoate to yield novobiocic acid, which is the substrate for NovO before it is O-glycosylated by NovM. In coumermycin assembly, the corresponding ligase CouL makes the bis-amide by tandem ligation of two aminocoumarins to a dicarboxypyrrole. CouO works on both the mono- and bis-amides for mono- and di-C-methylation adjacent to the phenolic hydroxyl before it is glycosylated by CouM. Thus, the specific timing of C-methylation in the aminocoumarin antibiotic pathways is established.

The three aminocoumarin antibiotics, novobiocin **1**, clorobiocin **2**, and the dimeric coumermycin A_1 **3** (Figure 1) all target the GyrB[1] subunit of bacterial DNA gyrase where they act as competitive inhibitors of ATP substrate binding (*1*−*3*). Novobiocin and clorobiocin are assembled from three types of building blocks: the aminocoumarin scaffold **4**, 3-prenyl-4-hydroxybenzoate **5**, and the 5,5-dimethyl-L-deoxyhexose L-noviose via dTDP-L-noviose **8** (Figure 2A). Coumermycin A_1 uses a 2,5-dicarboxy-3-methylpyrrole **9** in place of the prenylhydroxybenzoate, with both carboxylates being ligated to the aminocoumarin and then noviosylated (Figure 2B).

Much of the biosynthetic logic of assembly of this class of antibiotics is understood from a combination of biosynthetic gene cluster sequencing (*4*−*6*), in vivo gene disruptions (*7*, *8*), and in vitro biochemistry with purified enzymes (*9*−*15*). The aminocoumarin bicyclic ring system is elaborated from tyrosine on a nonribosomal peptide synthetase module, NovH (in novobiocin) (*9*) (CloH and CouH in clorobiocin and coumermycin), and then ligated enzymatically by NovL (CloL, CouL) (*14*, *15*) to produce desmethyl-novobiocic acid **6**. This bipartite intermediate uses the phenolic-hydroxyl as a nucleophile to attack the C_1 of the novoisyl moiety of **8**, forming desmethyl, descarbamoyl novobiocin by action of NovM (CloM, CouM) (*11*, *13*). The final two steps in novobiocin maturation are 4′-O-methylation (catalyzed by NovP, CouP, CloP) (*12*, *13*) and 3′-O-carbamoylation (catalyzed by NovN) (*12*). Clorobiocin and coumermycin differ from novobiocin in the acyl group added to the noviosyl-3′-OH: a 5-methylpyrrolyl group is transferred (in coumermycin and clorobiocin) instead of the carbamoyl group (in novobiocin). The heterocyclic methylpyrrole is

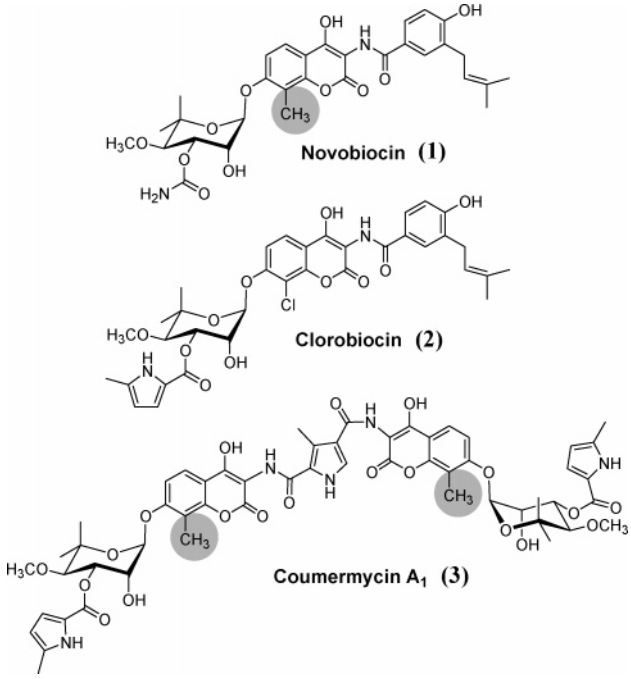

FIGURE 1: Structures of aminocoumarin antibiotics.

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[1] Abbreviations: GyrB, DNA gyrase B subunit; RP-HPLC, reverse phase high performance liquid chromatography; SAM, *S*-adenosylmethionine.

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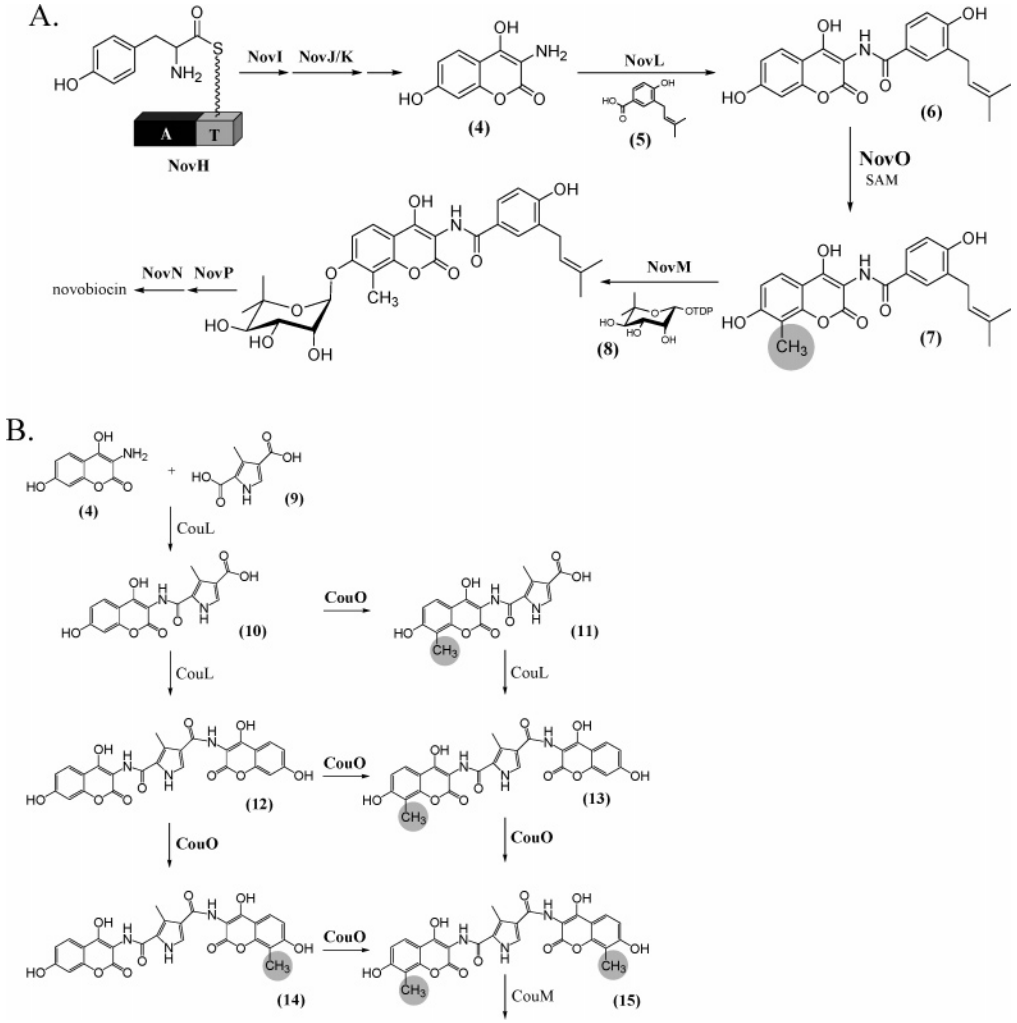


FIGURE 2: Aminocoumarin C-methylation (A) in novobiocin biosynthesis and (B) in coumermycin A_1 biosynthesis.

derived from proline and is generated and transferred by a series of six proteins, $CloN_{1-6}$/$CouN_{1-6}$ (*16*, *17*).

The aminocoumarin trio of antibiotics, **1**−**3**, also differ in the substituent at C_8 of the aminocoumarin scaffold, the carbon adjacent to the phenolic-OH. In novobiocin and coumermycin, there is a C-methyl group, while clorobiocin contains a chlorine atom at that position (Figure 1). Where CouO and NovO are conserved as predicted SAM-dependent methyltransferases in the two gene clusters, there is no CloO equivalent, but instead a predicted $FADH_2$-dependent halogenase CloHal (*18*). Disruption of CouO or CloHal results in the production of C_8−H in place of the C_8−CH_3 or C_8−Cl versions of antibiotics **3** and **2** (*7*, *18*), suggesting that the absence of C-methylation or C-chlorination does not inhibit recognition by downstream biosynthetic enzymes.

While the identity of the coumermycin aminocoumarin C_8-methyltransferase is thus established genetically, nothing is known about the timing of C-methylation of the aminocoumarin scaffold. In novobiocin, this is one of three methyl groups installed from SAM during biosynthesis (the others being one of the two 5′-CH_3 and the 4′-O−CH_3 in the noviose sugar), while it is one of six methyl groups in coumermycin. Purification of NovO and CouO in active forms by heterologous expression in *Escherichia coli* has allowed us to assign the substrates for C-methylation during novobiocin and coumermycin assembly.

MATERIALS AND METHODS

General. All analytical RP-HPLC experiments were performed using a Phenomenex Luna 5u C18(2) 100 Å column (250 mm × 4.6 mm) (at a flow rate of 1 $mLmin^{-1}$). All preparative RP-HPLC experiments were performed using a Vydac Selectapore 10u smallpore C18 90 Å column (250 mm × 22 mm) (at a flow rate of 10 mL min^{-1}). All experiments were performed at room temperature.

Cloning of NovO and CouO. The *novO* gene was amplified by PCR from *Streptomyces spheroides* (ATCC26935) genomic DNA. PCR amplification of a C-terminally Histidine-tagged NovO construct, pNovO−C, was accomplished using the forward primer novO-1 (5′-ACGAGGGGCATC-CATATGAAGATTGAAGCG −3′) and the reverse primer novO-2 (5′-TCGGGTCCAGAAGCTTGTTCGGGACAATT−3′). These primers introduced the respective *Nde*I and *Hin*dIII restriction sites (underlined above). PCR amplification was performed with *Pfu* Turbo polymerase (Stratagene). Similarly, the PCR amplification of an N-terminally histidine-tagged NovO construct, pNovO−N, was accomplished using the same forward primer novO-1 and the reverse primer novO-3 (5′-TCGGGTCCAGAAGCTTGTTTCAGACAATT−3′), introducing the same restriction sites. The PCR products were gel-purified, digested with *Nde*I and *Hin*dIII, and ligated into linearized pET37b and pET16b vectors (Novagen) to

give the C-terminal His8-tagged pNovO−C-pET37b and the N-terminal His10-tagged construct pNovO−N-pET16b.

The *couO* gene was amplified by PCR from *Streptomyces rishiriensis* (DSM 40489) genomic DNA. PCR amplification of a C-terminally Histidine-tagged CouO construct, pCouO−C, was accomplished using the forward primer couO-1 (5′-GGCGGGATCCATATGAAGATTGAACCGATTAC-GGGA−3′) and the reverse primer couO-2 (5′-CCGAACG-GCAAGCTTGGCAGCCGCCCGACG−3′). Similarly, the PCR amplification of an N-terminally Histidine-tagged CouO construct, pCouO−N, was accomplished using the same forward primer couO-1 and the reverse primer couO-3 (5′-GCGGGACCGAAGCTTCGGTCAGGCAGCGGC−3′). Ultimately, the C-terminal His8-tagged pCouO−C-pET37b and the N-terminal His10-tagged pCouO−N-pET16b constructs were obtained by similar procedures to those described above.

Expression and Purification of NovO and CouO. The pNovO−C-pET37b, pNovO−N-pET16b, pCouO−C-pET37b, and pCouO−N-pET16b expression constructs were transformed into *E. coli* BL21(DE3) competent cells (Invitrogen) for protein overproduction. Transformants harboring the desired constructs were grown at 25 °C in LB supplemented with 50 μg/mL kanamycin (for pNovO−C-pET37b and pCouO−C-pET37b) and 100 μg/mL ampicillin (for pNovO−N-pET16b and pCouO−N-pET16b) to an OD_{600} of 0.6, then induced with isopropyl-D-thiogalactopyranoside (IPTG) to a final concentration of 60 μM, and grown for an additional 14 h at 25 °C. The cells were harvested by centrifugation (15 min at 6000*g*) and frozen at −80 °C. Thawed cells were resuspended in buffer A [25mM Tris-HCl (pH 8.0), 400 mM NaCl, 2 mM imidazole, and 10% glycerol], lysed by French press (three passes at 15000 psi), or Avestin EmulsiFlex-C5 high-pressure homogenizer (10000−15000 psi), and the resultant cell debris removed by centrifugation (30 min at 10000*g*). The supernatant was incubated with 3 mL of Ni−NTA resin (Qiagen) for 2 h at 4 °C. The recovered resin was washed with 50 mL buffer A, packed into a column, and the protein was eluted using a stepwise gradient of 5−500 mM imidazole. Fractions containing the target protein (judged by SDS−PAGE) were pooled and dialyzed against buffer B [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 10% glycerol] overnight. The protein was dialyzed a second time in buffer C [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM TCEP, and 10% glycerol], concentrated, flash frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined spectrophotometrically at 280 nm using the calculated molar extinction coefficients (29640 $M^{-1}cm^{-1}$ for NovO and 30800 $M^{-1}cm^{-1}$ for CouO).

Preparation of NovO and CouO Substrates and Products. The coumermycin and novobiocin ligases, CouL and NovL, were isolated by previously described methods (*14*, *15*) and used to enzymatically prepare the desired substrates for NovO (desmethyl-novobiocic acid **6**) and CouO (desmethyl-monoamide **10**). The CouL ligase was also used to enzymatically prepare **10** and **11** as intermediates that were used to make CouO substrates **12**, **13**, and **14** by subsequent chemical coupling. This combined approach was used in order to bypass the difficulties in isolating significant quantities of the diamide substrates using CouL. Direct chemical coupling was similarly avoided in order to bypass the difficulties in selectively protecting the pyrrole-dicarboxylic acid **9**.

*Enzymatic Preparation of Desmethyl-monoamide **10**, Methyl-monoamide **11**, and Desmethyl-novobiocic Acid **6**.* Three separate 10 mL reactions containing 75 mM Tris-HCl (pH 8.0), 1 mg/mL BSA, 10 mM $MgCl_2$, 5 mM ATP, 10% DMSO and the following pairs of substrates were prepared: 2 mM desmethyl-coumarin **4** prepared in a previous effort (*19*) and 2 mM 3-methyl-pyrrole dicarboxylic acid **9** (*13*), 6 mM 2-amino-3,7-dihydroxy-8-methyl-coumarin and 2 mM 3-methyl-pyrrole dicarboxylic acid **9**, and 1 mM desmethyl-coumarin **4** and 1 mM 3-prenyl-4-hydroxy-benzoic acid **5**. The reactions were initiated with either NovL or CouL to a final concentration of 5−10 μM and allowed to proceed at ambient temperature for 48−72 h. Reaction progress was monitored by reverse-phase HPLC (RP-HPLC). The products were purified by preparative RP-HPLC [linear gradient of 100% (H_2O, 0.1%TFA) to 100% CH_3CN over 40 min]. Fractions containing the desired product were pooled, rotoevaporated, and concentrated under vacuum. Products were confirmed by LC-MS: des-methyl monoamide **10** (λ_{max} = 330, 228 nm) [ESI for $C_{16}H_{12}N_2O_7$; calcd 344.1, obsd 343.0 $(M - H)^-$], methyl-monoamide **11** (λ_{max} = 332, 225 nm) [ESI for $C_{17}H_{14}N_2O_7$; calcd 358.1, obsd 357.1 $(M - H)^-$], desmethyl novobiocic acid **6** (λ_{max} = 336, 209 nm) [ESI for $C_{21}H_{19}NO_6$; calcd 381.1, obsd 380.1 $(M - H)^-$]. Enzymatic reactions were performed multiple times as needed, with ∼3−6 mg of material isolated from each enzymatic reaction (yields ranged from of 62%−90%). The methyltransferase substrates/intermediates were presumed to be >95% pure as only a single peak was observed by RP-HPLC.

In addition, small quantities of diamides **12** and **15** were isolated from these reactions by described methods and characterized: desmethyl-diamide **12** [ESI for $C_{25}H_{17}N_3O_{10}$; calcd 519.1, obsd 518.1 $(M - H)^-$], bismethyl-diamide **15** [ESI for $C_{27}H_{21}N_3O_{10}$; calcd 547.1, obsd 546.1 $(M - H)^-$]. These compounds were used as standards to compare with synthetically prepared diamides.

*Preparation of desmethyl-diamide **(12)**, monomethyl-diamide **(13)**, and monomethyl-diamide **(14)**.*

To prepare monomethyl-diamide **13**, 10 mg of methyl-monoamide **11** (1 equiv) in ∼250 uL of DMF, 6.4 mg of desmethyl-coumarin (1 equiv), 5 uL of diisopropyl ethylamine (2 equiv), and 34 uL of 0.5 M solution of HBTU in DMF (1.2 equiv) were added and stirred at room temperature for ∼8 h. Reaction progress was monitored by reverse-phase HPLC (RP-HPLC). The same ratios of substrates were used to make the other diamides, except to prepare monomethyl-diamide **14**, desmethyl-monoamide **10** was coupled to 8-methyl-aminocoumarin and to prepare desmethyl-diamide **12**, desmethyl-monoamide **10** was coupled to des-methyl-aminocoumarin **4**. The products were purified by preparative RP-HPLC [linear gradient of 100% (H_2O, 0.1%TFA) to 100% CH_3CN over 40 min]. Fractions containing the desired product were pooled, rotoevaporated and concentrated under vaccum. Products were confirmed by coelution with enzymatically made standards (see above) and by LC-MS: monomethyl-diamide **13** (λ_{max} = 343, 224 nm) [ESI for $C_{26}H_{19}N_3O_{10}$; calcd 533.1, obsd 532.1 $(M - H)^-$], monomethyl-diamide **14** (λ_{max} = 343, 224 nm) [ESI for $C_{26}H_{19}N_3O_{10}$; calcd 533.1, obsd 532.1 $(M - H)^-$], and desmethyl-

diamide **12** (λ_{max} = 342, 231 nm) [ESI for $C_{25}H_{17}N_3O_{10}$; calcd 519.1, obsd 518.1 $(M - H)^-$]. For each reaction, ~3−4 mg of material was obtained (with yields ranging from 21%-35%), and the products were presumed to be >95% pure as only a single peak was observed by RP-HPLC.

Characterization of NovO and CouO Activity. Preliminary attempts to reconstitute NovO in vitro activity were made on the expected substrate, modified tyrosine-acyl thioesters linked to the non-ribosomal peptide synthetase (NRPS) NovH (*9*) (see Supporting Information). In addition, NovO activity on the soluble desmethyl-aminocoumarin **4** substrate, the glycosylated desmethyl-novobiocic acid, desmethyl-descarbamoyl novobiocin, and desmethyl-novobiocin was tested (see Supporting Information).

Reconstitution of NovO was performed in reactions containing: 250 μM desmethyl-novobiocic acid **6**, 75 mM Tris-HCl (pH 8.5), 2 mM *S*-adenosylmethionine (SAM), 1 mg/mL bovine serum albumin (BSA), and 10% DMSO. Reactions were initiated with 1 μM NovO and quenched at specific time points with an equal volume of methanol at 4 °C. The quenched reactions were incubated at −20 °C for 20 min and centrifuged to remove precipitated protein (5min, 13000 rpm). The supernatant of each reaction was analyzed by analytical RP-HPLC [linear gradient of 100% (H_2O, 0.1%TFA) to 100% CH_3CN over 25 min]. Product formation was confirmed by LC-MS.

Preliminary attempts to reconstitute CouO activity were performed on the modified tyrosyl-NovH thioesters and soluble desmethyl-coumarin (see Supporting Information). Initial reconstitution of CouO was performed in reactions containing: 250 μM desmethyl-monoamide **10**, 75 mM Tris-HCl (pH 8.5), 2 mM *S*-adenosylmethionine (SAM), 1 mg/mL bovine serum albumin (BSA), and 10% DMSO. CouL-CouO coincubations were performed with 500 μM desmethyl-monoamide **10**, 75 mM Tris-HCl (pH 8.5), 2 mM *S*-adenosylmethionine (SAM), 1 mg/mL bovine serum albumin (BSA), 10% DMSO, and 5 μM CouL. Following an 18 h incubation at ambient temperature, CouO was added to a final concentration of 500 nM, and the reaction was monitored over time. All reactions were analyzed as described above.

For the determination of kinetic parameters for the various substrates accepted by NovO and CouO, the concentration of the SAM substrate was kept constant at 2 mM while the concentration of the other substrates was varied in the above-described reaction buffer. RP-HPLC analysis of each reaction was performed as described above. For desmethyl-novobiocic acid **6**, the substrate concentration was varied from 1 to 100 μM (for NovO) and 10−500 μM (for CouO), initiated with 2 μM NovO or CouO, quenched at 3 min, and monitored at the λ_{max} of 333 nm. For desmethyl-monoamide **10**, the substrate concentration was varied from 1 to 400 μM (for CouO) and 0.05−10 mM (for NovO), initiated with 500 nM CouO or 2 μM NovO, quenched at 15 min (for CouO) or 1 h (for NovO), and monitored at the λ_{max} of 330 nm. For monomethyl-diamides **13** and **14**, the substrate concentration was varied from 2 to 350 μM, initiated with 500 nM CouO, quenched at 15 min, and monitored at the λ_{max} of 343 nm. For desmethyl-diamide **12**, the substrate concentration was varied from 2 to 500 μM, initiated with 250 nM CouO, quenched at 10 min, and monitored at the λ_{max} of 343 nm. Under these conditions, less than 1% of bismethyl-diamide product **15** accumulated. All described determinations of k_{cat} and K_m were performed in triplicate using two separate substrate stock solutions. To ensure accuracy, the product concentration was calculated in two ways: by comparison with a product standard curve (described below) as well as by direct calculation of relative percentage of product. Standard curves were generated for each product using analytical reverse phase HPLC over the following specific concentration ranges: methyl-monoamide **11** (5−250 pmol), monomethyl-diamide **13** (10−200 pmol), monomethyl-diamide **14** (2−100 pmol), bismethyl-diamide **15** (10−200 pmol), and novobiocic acid **7** (2−300 pmol). The calculated product concentration was compared between both methods and agreed well (data not shown).

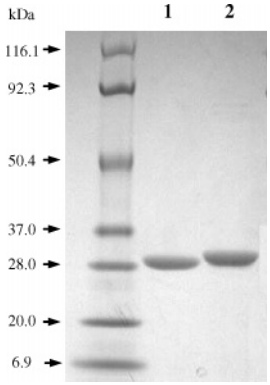


FIGURE 3: SDS polyacrylamide gel of NovO and CouO: lane 1, NovO (C−His); lane 2, CouO (C−His).

RESULTS

Expression and Purification of NovO and CouO. The 26.5 kDa NovO and 27.4 kDa CouO methyltransferases from *Streptomyces spheroides* and *Streptomyces rishiriensis* were heterologously expressed in *E. coli* and purified to homogeneity by Ni(II)-affinity chromatography as both C-terminally His8-tagged and N-terminally His10-tagged proteins. Yields ranged from 10 to 30 mg/L for NovO and 5−10 mg/L for CouO. For both proteins, the C-terminally tagged methyltransferases were purified in higher yields (Figure 3) and also exhibited higher activity in vitro and were therefore used in all the experiments described herein.

Characterization of NovO and CouO Activity. Previous investigations into aminocoumarin ring biosynthesis demonstrated that the initial steps involve activation of L-tyrosine by the NovH adenylation domain to form L-tyrosyl-*S*-PCP (*9*). The tethered tyrosine is then subject to modification: first, β-hydroxylation mediated by NovI, and then β-oxidation to the β-keto-Tyr-*S*-enzyme mediated by NovJ/NovK (*10*). With these precedents, we sought to determine if NovO acted to C-methylate either of the three aminoacyl-*S*-NovH covalent intermediates. However, attempts to reconstitute in vitro activity of NovO and CouO on the aminoacyl-*S*-NovH species were unsuccessful. No activity was observed by NovO or CouO (in the presence of [^{3}H]-SAM) with the following substrates: Tyr-*S*-NovH, β-OH-Tyr-*S*-NovH, or β-keto-Tyr-*S*-NovH (each generated by coincubations of NovH, I, J/K with tyrosine, ATP, NADPH, O_2 and NADP).

The next predicted intermediate would arise from cyclization of the 2-amino-3-keto-tyrosyl moiety to release the free aminocoumarin as the desmethyl molecule **4**. This was available by our prior synthetic efforts (*19*). Again, no

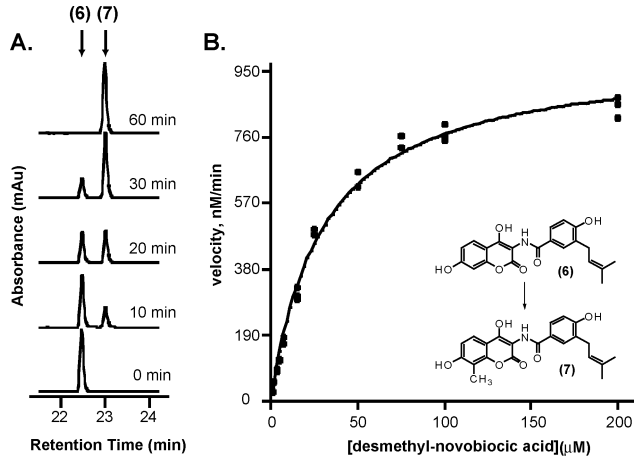


FIGURE 4: Characterization of NovO activity: (A) HPLC time course of NovO-mediated methylation of desmethyl-novobiocic acid **6**: (B) MM-kinetics for desmethyl-novobiocic acid **6**.

activity with either NovO or CouO was detected with this potential substrate. The last three steps in the novobiocin pathway are catalyzed by NovM (the noviosyl transferase that yields glycosylated desmethyl-novobiocic acid), NovP (which mediates 4′-O-methylation of the noviosyl ring to produce descarbamoyl novobiocin), and last NovN (which catalyzes 3′-O-carbamoylation to complete novobiocin assembly) (*11*, *12*). None of these glycosylated compounds obtained by action of NovM or NovM, P or NovM, P, N, respectively, were accepted as a substrate by NovO.

The remaining intermediate to test was the putative desmethyl-novobiocic acid **6** that would arise from action of the NovL ligase that joins a desmethyl-aminocoumarin **4** to prenylbenzoate **5**. We generated an authentic sample of **6** by the preparative use of purified NovL (*14*) with the synthetic desmethyl-aminocoumarin **4**. NovO finally showed C-methyltransferase activity on **6** (Figure 4), as did CouO. NovO and CouO methyltransferase activity was found to be dependent on the presence of SAM, the anticipated methyl donor, and the pH optimum was determined to be 8.5 in Tris-HCl buffer. In RP-HPLC analysis of coincubations of NovO, desmethyl-novobiocic acid **6**, and SAM, the appearance of the novobiocic acid product **7** is observed over time, accompanied by a corresponding decrease in the desmethyl-novobiocic acid substrate (Figure 4A). The identity of the product was confirmed by coelution with authentic novobiocic acid and by LC-MS [ESI for $C_{22}H_{21}NO_6$; calcd 395.1, obsd 394.1 $(M - H)^-$].

By analogy, we then anticipated that CouO would function at the equivalent step in the coumermycin biosynthetic pathway (Figure 2B). The corresponding substrate would be the monoamide **10** arising from CouL-mediated ligation (*15*) of the desmethyl-aminocoumarin **4** with 3-methyl-2,4-dicarboxypyrrole **9**. We were able to enzymatically prepare this monoamide via CouL action (see Materials and Methods section). Indeed, CouO was found to be active on desmethyl-monoamide **10** in the presence of SAM. RP-HPLC analysis of this reaction reveals the appearance of the methyl-monoamide product over time, accompanied by a corresponding decrease in the desmethyl-monoamide substrate (Figure 5A). The identity of the product was confirmed by LC-MS [ESI for $C_{17}H_{14}N_2O_7$; calcd 358.1, obsd 357.1 $(M - H)^-$].

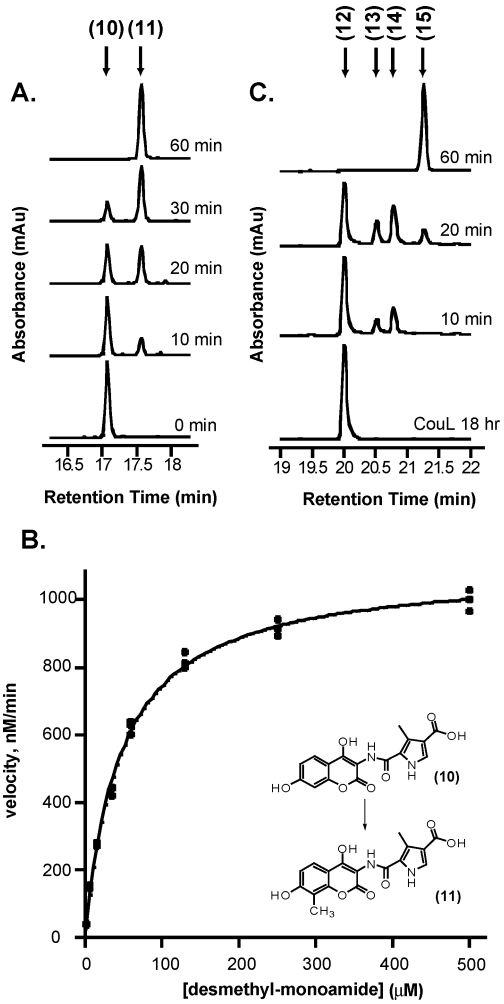
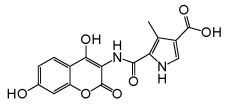
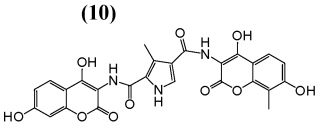
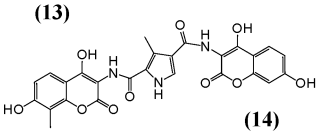
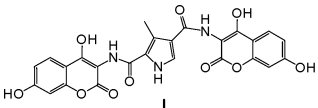
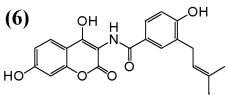


FIGURE 5: Characterization of CouO activity: (A) HPLC time course of CouO-mediated methylation of desmethyl-monoamide **10**; (B) MM-kinetics for desmethyl-monoamide **10**; (C) HPLC time course of a CouL−CouO tandem incubation depicting resolution of **12**, **13**, **14**, and **15**—bottom trace, CouL 18 h incubation; top three traces, 10, 20, and 60 min incubations of CouL product **12** with CouO.

Since CouO must ultimately act twice to yield the bismethyl-diamide **15**, which presumably is then subsequently glycosylated by CouM, preliminary experiments were performed to deduce the order of CouL-CouO action. In principle, as noted in Figure 2, CouO could work on the monoamide **11** (just noted in the preceding paragraph) and the diamide **12** which results from tandem action of CouL. In turn, monomethylation by CouO of diamide **12** could yield either monomethylated regioisomers **13** and **14**, either of which could be substrates for the second C-methylation by CouO. In the absence of any regiospecificity, CouO could work on **11**, **12**, **13**, and **14** as substrates.

Tandem incubations of CouL, desmethyl-aminocoumarin **4**, pyrrole-diacid **9**, and then CouO and SAM, revealed that CouO is, in fact, active on all the desmethyl intermediates (Figure 2B): desmethyl-monoamide **10**, desmethyl-diamide **12**, monomethyl-diamide **13**, monomethyl-diamide **14**. When CouL is preincubated with **4** and **9**, only the CouL products monoamide **10** and diamide **12** are present (Figure 5C). Upon the addition of CouO (Figure 5C, 10 min), desmethyl-monoamide **10** is converted to methyl-monoamide **11** (see above), and desmethyl-diamide **12** is converted to mono-

Table 1: Summary of Kinetic Parameters for CouO

Substrate	k_{cat} (min^{-1})	K_m (μM)
 **(10)**	2.2 ± 0.25	52.0 ± 4.5
 **(13)**	1.23 ± 0.2	63.4 ± 11.6
 **(14)**	1.38 ± 0.2	63.8 ± 6.0
 **(12)**	1.71 ± 0.22 (total)	
→ **(13)**	1.1 ± 0.12	50.4 ± 4.9
→ **(14)**	0.61 ± 0.1	51.0 ± 5.5
 **(6)**	0.23 ± 0.05	108.5 ± 8.7

methyl-diamides **13** and **14**. The assignment of these products was confirmed by coelution with the synthetic standards (data not shown, see Materials and Methods section) and LC-MS: **13** [ESI for $C_{26}H_{19}N_3O_{10}$; calcd 533.1, obsd 532.1 $(M - H)^-$]; **14** [ESI for $C_{26}H_{19}N_3O_{10}$; calcd 533.1, obsd 532.1 $(M - H)^-$]. As the CouO incubation progresses over time, monomethyl-diamides **13** and **14** are methylated a second time by CouO to yield the final product bis-methyl-diamide **15** [ESI for $C_{27}H_{21}N_3O_{10}$; calcd 547.1, obsd 546.1 $(M - H)^-$] (Figure 5C, 1 h).

Somewhat surprisingly, despite its specificity for the NovL product, NovO was able to methylate both the coumermycin desmethyl-monoamide [ESI for $C_{17}H_{14}N_2O_7$; calcd 358.1, obsd 357.1 $(M - H)^-$] and the desmethyl-diamide [ESI for $C_{27}H_{21}N_3O_{10}$; calcd 547.1, obsd 546.1 $(M - H)^-$]. Similarly, CouO was able to methylate desmethyl-novobiocic acid [ESI for $C_{22}H_{21}NO_6$; calcd 395.1, obsd 394.1 $(M - H)^-$]. This promiscuity suggests that the amide linkage on the aminocoumarin is an enabling recognition element for both methyltransferase enzymes.

Kinetic Characterization of CouO and NovO. The kinetic parameters for CouO and NovO were defined by holding the concentration of SAM constant at 2 mM. The K_m for SAM for NovO and CouO was determined to be <100 μM, and no substrate inhibition was observed at 2 mM SAM (data not shown).

Addition of CouO yields a typical hyperbolic saturation curve over varying desmethyl-monoamide **10** concentrations, resulting in a K_m of 52.0 ± 4.5 μM for **10** (Figure 5B, Table 1). The maximal rate for this first C-methylation was observed to be 2.2 ± 0.25 min^{-1}. Kinetic parameters for CouO were also determined for the four other accepted amide substrates. With the exception of the noncognate substrate, desmethyl-novobiocic acid, all four coumermycin amides tested exhibited very similar k_{cat} values (no greater than a 2-fold difference) and had no significant differences in their K_m values. In the case of the regioisomeric monomethyl-diamide **13** (k_{cat} = 1.23 ± 0.2 min^{-1}, K_m = 62.4 ± 11.6 μM) and methyl diamide **14** (k_{cat} = 1.38 ± 0.2 min^{-1}, K_m = 63.8 ± 6.0 μM), there was no significant difference in the observed maximal rates and K_ms. In the case of desmethyl-diamide **12**, the total rate of conversion to **13** and **14** was comparable (k_{cat} = 1.71 ± 0.22 min^{-1}) to that of the desmethyl-monoamide **10**. Since **13** and **14** could be resolved by RP-HPLC, their maximal rates of formation from **12** were individually measured. Although the K_m values are the same (as expected), the k_{cat} for monomethyl-diamide **13** formation is nearly double that of monomethyl-diamide **14**, 1.1 ± 0.12 min^{-1} versus 0.61 ± 0.1 min^{-1} (Table 1).

The kinetic parameters were also determined for the novobiocin precursor, desmethyl-novobiocic acid for both CouO (Table 1) and NovO. NovO exhibits twice the apparent k_{cat} than does CouO, 0.50 ± 0.02 min^{-1} versus 0.23 ± 0.05 min^{-1}. As might be expected, however, CouO had a K_m value ~5-fold greater than NovO does for its natural substrate, desmethyl-novobiocic acid, 108.5 ± 8.7 μM for CouO versus 26.5 ± 5.5 μM for NovO. The kinetic parameters for NovO mediated methylation of the CouO substrate desmethyl-monoamide **10** were also determined (k_{cat} = 0.48 ± 0.02 min^{-1}, K_m = 1.7 ± 0.1 mM. While there is no significant reduction in the k_{cat} compared to the natural desmethyl-novobiocic acid substrate, the K_m is ~65-fold greater for the desmethyl-monoamide substrate **10**. Although there is little information about the kinetic competence of C-methyltransferases in the literature, all the observed k_{cat} values do fall within the range reported for O-methyltransferases.

DISCUSSION

Novobiocin and clorobiocin, antibiotics that target DNA gyrase by binding to the GyrB ATPase domain, have three distinct subparts: the aminocoumarin nucleus, the prenylated hydroxybenzoate, and the decorated L-noviosyl sugar. The bicyclic coumarin ring acts as a scaffold to present the L-deoxysugar moiety and the 3′-O-acyl groups (carbamoyl for novobiocin and methyl-pyrrolecarboxy for clorobiocin) as the key pharmacophores in the active site (*20*, *21*). The bis-aminocoumarin antibiotic, coumermycin A_1, is a pseudo-symmetric dimer with two noviosylated aminocoumarins in amide linkage to a dicarboxypyrrole element. Like clorobiocin, coumermycin has a methylpyrrole carboxylate as the 3′-O-acyl moiety on the noviose, but like novobiocin, each coumarin ring has an 8-methyl rather than an 8-chloro species.

The logic of molecular assembly of the three parts of novobiocin and clorobiocin and the corresponding five parts of coumermycin have been addressed by a series of genetic and biochemical studies over the past five years (*7*−*15*). This work has been enabled by sequencing of the three biosynthetic clusters in the producing streptomycetes by Heide and his colleagues (*4*−*6*).

There are three methylation steps in novobiocin assembly, two in clorobiocin, and six to nine in coumermycin (the origins of the 3-methyl in the central pyrrole linker and the two 5-methyls on the 3′-pyrroleccarboxy groups are unclear).

In this work we have focused on the C_8-methylation of the coumarin ring system. Of the three SAM-dependent methylations in novobiocin construction, one is an O-methylation where the nucleophile is the 4′-OH of the noviosyl moiety. The other two are C-methylations that require a carbanion equivalent in the cosubstrate. One installs the C_5′ methyl, found on the noviosyl ring, during sugar biosynthesis at the stage of dTDP-4-keto-6-deoxy-D-glucose (*22*). The other is the C-methylation under consideration here. As a class, C-methylases are relatively understudied, and the carbanion nucleophiles not well characterized.

The coumarin scaffold is a highly functionalized 2-amino-3,7-dihydroxy-8-methyl bicyclic lactone in novobiocin and coumermycin. Of these four functional groups, two (the 2-amino and 7-hydroxy) are provided by the original proteinogenic amino acid substrate tyrosine. The third functional group, the 3-hydroxy, is provided by a remarkable series of oxidative transformations at the C_3 benzylic CH_2 of the tyrosine moiety while it is covalently tethered as a pantotheinyl thioester on the carrier protein domain of NovH. NovI and NovJ/NovK carry out a net four electron oxidation of the CH_2 to C═O (on Tyr-*S*-NovH), via the CH−OH intermediate (*9*, *10*).

The fourth functional group installed on the coumarin scaffold is the C_8 methyl group. Given the precedence of tailoring proteinogenic to nonproteinogenic aminoacyl moieties while they are sequestered as pantetheinyl thioesters on A−T didomain proteins (*23*, *24*) (e.g., NovH (*9*)), we anticipated that the C_8 methylation by CH_3 transfer from SAM would happen at the β-keto-Tyr-*S*-NovH stage. This work vitiates that expectation. Instead, NovO and CouO show exquisite specificity for an N-acylated aminocoumarin as the C-methylation substrate. The N-acylated derivative is novobiocic acid in the novobiocin pathway and the corresponding mono- and di-amides of the aminocoumaryl-dicarboxypyrrole in coumermycin biogenesis.

The biosynthetic pathway appears to clearly favor C-methylation at the level of the N-acylated coumarins before glycosylation occurs. This accords with the chemical expectation that a free C_7-phenolic-OH is required to undergo deprotonation and generate the stabilized C_8 carbanion as the requisite nucleophile to attack the CH_3^+ equivalent being transferred from SAM. This explains the observed regioselectivity of C-methylation in the aminocoumarin antibiotics. Once glycosylation of the C_7−OH has been achieved by NovM/CouM the C_8 would be deactivated for carbanion formation, leaving no obvious mechanism for C−CH_3 bond formation.

In the dimeric coumermycin pathway, the ligase CouL, the methyltransferase CouO, and the glycosyltransferase CouM, all must act twice, on both arms of the growing antibiotic. It is reasonable, therefore, to expect promiscuity toward each arm as the antibiotic is built up. That is clearly observed in this initial study of CouO toward its suite of potential methylation substrates and suggests a stochastic set of C-methylations once the free amino group of the aminocoumarin moiety has been acylated to eliminate the positive charge.

Clorobiocin differs from novobiocin and coumermycin in that the biosynthetic gene cluster lacks a NovO/CouO homologue and instead has a putative $FADH_2$-dependent halogenase in that locus (*18*). It is not yet known when C_8-chlorination occurs. However, we suppose a comparable C_8 carbanion is attacking an oxidized "Cl^+" equivalent (*25*) and that suggests a free C_7−OH but does not necessarily predict that chlorination also occurs following amide ligation by CloL. Subsequent mechanistic and structural studies on NovO/CouO will reveal how useful these C-methylation catalysts may be in the combinatorial biosynthesis of other scaffolds.

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SUPPORTING INFORMATION AVAILABLE

NovO and CouO methods for activity assays on aminoacyl-*S*-NovH intermediates, desmethyl-coumarin and glycosylated novobiocic acids and NMR spectra for compounds **4** and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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