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ARTICLE *in* BIOCHEMISTRY · MAY 2003

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Initial Characterization of Novobiocic Acid Noviosyl Transferase Activity of NovM in Biosynthesis of the Antibiotic Novobiocin†

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Received January 6, 2003

ABSTRACT: The aminocoumarin class of antibiotics, exemplified by novobiocin, is composed of tripartite L-noviosylaminocoumarin prenylbenzoate natural products. The decorated noviosyl sugar component interacts with the target bacterial enzyme DNA gyrase. We have subcloned the putative 40 kDa L-noviosyl transferase from *Streptomyces spheroides* into *Escherichia coli*, expressed it in soluble form, and purified it to homogeneity as a C-terminal His_8 fusion protein. The aglycone novobiocic acid, obtained from selective degradation of novobiocin, and TDP-L-noviose, obtained by an 11-step chemical synthesis from L-rhamnose, were shown to be robust substrates for NovM to produce the desmethyldescarbamoyl novobiocin intermediate with a k_{cat} of >300 min^{-1}. NovM displays activity with variant coumarin aglycones, suggesting it may be a promiscuous catalyst for noviosylation of a range of planar scaffolds. Conversely, NovM shows no activity with and is inhibited by TDP-L-rhamnose ($K_i = 83.5 \pm 5.5$ μM), the sugar donor that most closely structurally resembles the natural substrate TDP-L-noviose. The NovM reaction products generated during the course of this work will serve as substrates for subsequent analysis of the NovP and NovN tailoring enzymes that impart the noviose decorations required for DNA gyrase binding and antibiotic activity.

The glycosylation of many natural products is required for the associated antibiotic and/or antitumor activities (*1*). Such glycosylated natural products include the polyketide and macrolide class of antibiotics as well as the nonribosomal peptide family of antibiotics. Novobiocin, clorobiocin, and coumermycin A_1 (Figure 1) are members of the coumarin family of antibiotics produced from various *Streptomyces* species, and each bears a noviosyl sugar component that imparts the functionality essential for biological activity. This family of antibiotics exerts its antibacterial activity via the inhibition of type II DNA topoisomerase DNA gyrase. Specifically, the aminocoumarins inhibit ATP hydrolysis in the GyrB subunit (*2*−*6*), the same mode of action exhibited by the quinolone antibacterials such as ciprofloxacin. In general, the coumarin antibiotics have had limited use in the clinic because of problems associated with poor solubility and pharmacological properties (*7*−*9*). Novobiocin has, however, received renewed attention as a result of its potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strains (*10*).

The coumarin antibiotics contain three structural moieties: a noviose sugar, a 3-substituted coumarin ring, and a prenylated 4-hydroxybenzoic acid moiety. Clorobiocin differs structurally from novobiocin at two positions: C-8 of the coumarin ring and the 3 position of the noviose moiety. Novobiocin bears a carbamoyl group at the 3 position of the noviose moiety, while clorobiocin bears a 5-methyl-2-pyrrolecarboxy group, a structural modification that results in enhanced inhibitory activity of clorobiocin relative to novobiocin (*11*). In addition, novobiocin bears a methyl substituent at C-8 while clorobiocin bears a chloro substituent. Coumermycin A_1 is a dimer of the noviosyl coumarin components linked by a 3-methyl-2,4-dicarboxylpyrrole moiety and bears the 5-methyl-2-pyrrolecarboxy group at the 3 position of the noviose ring. Like novobiocin, coumermycin A_1 bears a methyl substituent at C-8.

Early investigation of the biosynthesis of the coumarin antibiotics revealed that the noviose component is derived from D-glucose, and the coumarin and prenylated 4-hydroxybenzoic acid components are derived from tyrosine (*12*−*14*). The recent sequence analysis of the novobiocin gene cluster from *Streptomyces spheroides* (*15*) has provided additional insight into the biosynthesis of the coumarin antibiotics and motivated recent studies carried out to characterize the enzymes (NovH and -I) involved in the early steps of coumarin ring formation (*16*).

Further scrutiny of the *nov* gene cluster reveals a putative glycosyltransferase (NovM) implicated in the transfer of the noviose component to the novobiocic acid aglycone of novobiocin (Scheme 1). We have an ongoing interest in the reactions catalyzed by antibiotic glycosyltransferases and the utilization of these enzymes in the formation of novel glycosylated antibiotics (*17*, *18*). In this study, we focus on the characterization of the 40 kDa novobiocin glycosyltransferase NovM as part of an effort to expand the coumarin

† This work was supported by National Institutes of Health Grants 49338 (to C.T.W.) and 66174 (to D.K.).

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10.1021/bi0340088 CCC: $25.00
Published on Web 03/14/2003

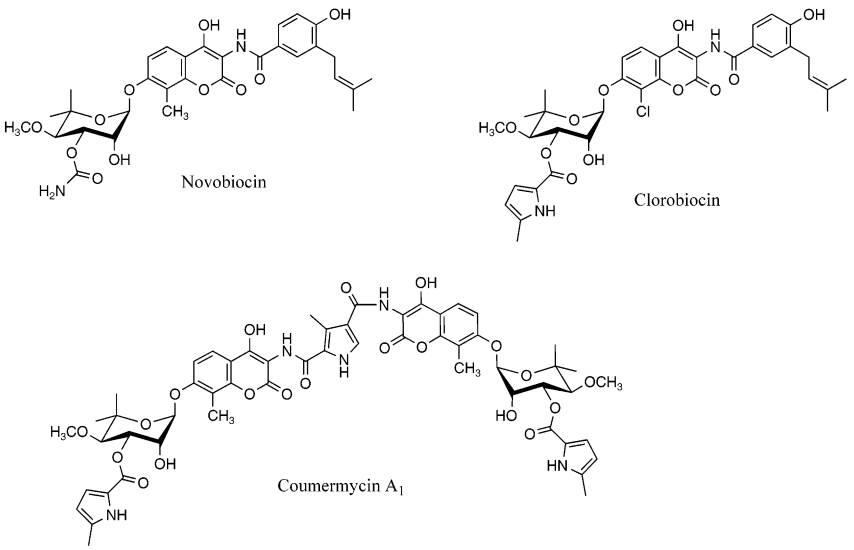
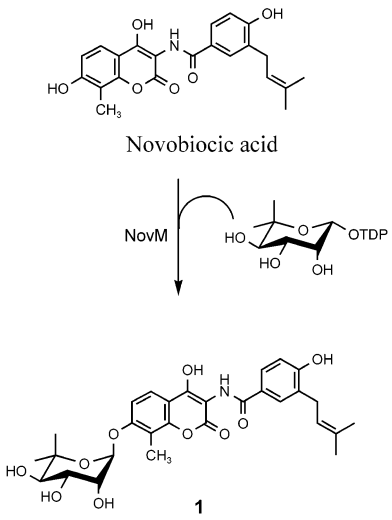


FIGURE 1: Aminocoumarin antibiotics.

Scheme 1: Glycosylation of Novobiocic Acid Catalyzed by NovM



antibiotic repertoire. We report herein the cloning, heterologous overproduction, and purification of active NovM from *Escherichia coli*. In addition, we report the first synthesis of TDP-L-noviose and demonstrate that it is a substrate for NovM in the glycosylation of novobiocic acid. Finally, we describe initial efforts to probe the substrate specificity of NovM and establish that this glycosyltransferase exhibits somewhat relaxed specificity for the aglycone substrate.

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 Strategene. 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 mm × 4.6 mm). Enzymatic reaction products were confirmed by LC−MS using a Shimadzu LCMS-QP8000α instrument.

For the synthesis of TDP-L-noviose, novobiocic acid, and cyclonovobiocin, all chemicals were purchased from Aldrich or Sigma, unless otherwise noted, and used without further purification. Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates precoated with silica gel (250 μm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%) and heating. 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). Compounds bearing a thymidine chromophore were monitored at an absorbance of 270 nm. Reactions were monitored by HPLC using gradient A (5 min of an H_2O/0.1% NH_4HCO_3 mixture and then a linear gradient to a 100% MeOH/0.1% NH_4HCO_3 mixture over the course of 15 min). Preparative HPLC was performed on a Varian ProStar instrument using a Phenomenex Luna 10 μm C18 column (250 mm × 50 mm). NMR spectra were recorded on Varian Mercury 300 MHz and Varian Inova 400 or 500 MHz spectrometers. Mass spectra (ESI) were obtained at the Mass Spectroscopy Facility at the Department of Chemistry, Princeton University.

Preparation of the pNovM-pET37b Overexpression Construct. The gene encoding NovM was amplified from *S. spheroides* (ATCC 23965) genomic DNA. Amplification was accomplished using the foward primer 5′-GGTCATCATATGAGAGTGCTGTTGACGAG-3′ and the reverse primer 5′-ACGGAACTGGAGCGCGGGAGTCATTGACCGTTG-3′. The forward primer introduced an *Nde*I restriction site (restriction site underlined above), and the reverse primer introduced an *Xho*I restriction site. PCRs 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 and *Xho*I. Expression of pNovM-pET37b was accomplished following transformation into *E. coli* TOP10 competent cells; the construct was subsequently sequenced and compared to the

novM gene sequence from *S. spheroides* NCIB 11891 reported by Heide et al. (GenBank entry AAF67506). Twenty base changes out of 1140 were observed, resulting in a total of three amino acid alterations. The changes in the *novM* gene sequence from the *S. spheroides* NCIB 11891 strain to the *S. spheroides* ATCC 23965 strain are as follows: ACC → GCC (Thr30 → Arg), GTG → GCC (Val42 → Ala), and GTC → GAC (Val66 → Asp). It is not known whether the observed changes in amino acid sequence between strains have any functional consequences; the NovM reported here has robust activity.

Overproduction and Purification of NovM. The purified pNovM-pET37b plasmid was transformed into BL21(DE3) competent *E. coli* cells. Transformants harboring the pNovM-pET37b construct were grown in LB medium supplemented with kanamycin (50 μg/mL). The cells were grown at 25 °C for 24 h without IPTG induction. Cells were harvested by centrifugation (20 min at 6000*g*) and frozen at −20 °C. Cells were thawed and resuspended in 90 mL of buffer A [25 mM Tris-HCl (pH 8.0), 400 mM NaCl, 2 mM imidazole, and 10% glycerol]. Resuspended cells were lysed with a French press (two passes at 15 000 psi), and the cell debris was removed by centrifugation (30 min at 9500*g*). 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, and 10% glycerol]. NovM was eluted from the column in a stepwise imidazole gradient (from 20 to 200 mM imidazole). Fractions containing pure NovM (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, and 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 DTT, and 10% glycerol]. The protein was flash-frozen in liquid nitrogen and stored at −80 °C. The concentration of purified NovM was measured spectrophotometrically at 280 nm using a calculated extinction coefficient of 33 010 M^{-1} cm^{-1}. Large-scale overproduction afforded an overall yield of >9 mg/L NovM.

Synthesis of Novobiocic Acid, Cyclonovobiocic Acid, and TMP-Morpholidate. Novobiocic acid and cyclonovobiocic acid were obtained by acidic hydrolysis of novobiocin (*19*). Thymidine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt (TMP-morpholidate) was prepared by DCC-mediated coupling of TMP with morpholine (*20*).

*Synthesis of 5′,5′-Di-C-methyl-L-lyxose (L-Noviose, **2**).* L-Noviose (**2**) was prepared starting from commercially available L-rhamnose by the method of Klemer and Waldmann (*21*).

*Synthesis of 1,2,3,4-Tetra-O-acetyl-5,5-di-C-methyl-α/β-L-lyxopyranose (**3**).* Crude L-noviose **2** (4.47 g, 25 mmol) was dissolved in a mixture of pyridine (80 mL) and Ac_2O (50 mL). After being stirred for 16 h at room temperature, the mixture was evaporated. The residue was dissolved in CH_2Cl_2 (300 mL), washed with 1 N HCl, saturated aqueous $NaHCO_3$, and brine, and evaporated. Flash chromatography (7:2 petroleum ether/EtOAc mixture) of the residue gave tetraacetate **3** (6.23 g, 72%) as a clear syrup (3:1 mixture of α- and β-anomers). α-Anomer: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.02 (d, $J_{1,2}$ = 1.2 Hz, 1 H, 1-H), 5.45 (dd, $J_{1,2}$ = 1.2 Hz, $J_{2,3}$ = 3.2 Hz, 1 H, 2-H), 5.23 (d, $J_{3,4}$ = 10.4 Hz, 1 H, 4-H), 5.16 (dd, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 10.4 Hz, 1 H, 3-H), 2.19, 2.07, 2.05, 1.98 (4 s, each 3 H, $COCH_3$), 1.37, 1.29 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 170.1, 169.9, 168.8, 87.0, 75.5, 71.3, 68.9, 68.7, 27.9, 21.0, 20.7, 18.9; MS (ESI) for $C_{15}H_{22}O_9$ (346.3) 346 ($[M]^+$).

*Phenyl 2,3,4-Tri-O-acetyl-5,5-di-C-methyl-1-thio-α/β-L-lyxopyranoside (**4**).* To a solution of tetraacetate **3** (5.3 g, 15.3 mmol) in CH_2Cl_2 (20 mL) was added thiophenol (2.36 mL, 23.0 mmol) followed by boron trifluoride diethyl etherate (0.78 mL, 6.1 mmol) under argon. After being stirred overnight at room temperature, the reaction mixture was diluted with CH_2Cl_2 (300 mL) and washed with 300 mL portions of H_2O (once), saturated aqueous $NaHCO_3$ (twice), and brine (once). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by flash chromatography (3:1 petroleum ether/EtOAc mixture) to give **4** (5.5 g, 91%, 6:1 mixture of α- and β-anomers) as a clear syrup. α-Anomer: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50 and 7.31 (2 m, 5 H, aromatic), 5.64 (dd, $J_{1,2}$ = 1.5 Hz, $J_{2,3}$ = 3.6 Hz, 1 H, 2-H), 5.28 (d, $J_{3,4}$ = 10.8 Hz, 1 H, 4-H), 5.10 (dd, $J_{2,3}$ = 3.6 Hz, $J_{3,4}$ = 10.8 Hz, 1 H, 3-H), 5.08 (d, $J_{1,2}$ = 1.2 Hz, 1 H, 1-H), 2.21, 2.06, 1.98 (3 s, each 3 H, $COCH_3$), 1.36, 1.28 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR ($CDCl_3$) δ 170.6, 170.5, 170.0, 131.8, 129.3, 128.1, 82.3, 80.8, 71.6, 71.2, 69.6, 28.3, 21.1, 20.9, 20.8, 18.4; MS (ESI) for $C_{19}H_{24}O_7S$ (396.5) 396 ($[M]^+$).

*Dibenzyl (2,3,4-Tri-O-acetyl-5,5-di-C-methyl-L-lyxopyranosyl Phosphate) (**6**).* *N*-Iodosuccinimide (1.57 g, 7.0 mmol) was added to a solution of **4** (1.86 g, 4.7 mmol) in CH_3CN and H_2O (21:1, 66 mL) and the mixture stirred at room temperature for 1 h. The reaction mixture was diluted with EtOAc (200 mL) and the reaction quenched with aqueous $Na_2S_2O_3$ (10%, 200 mL). After extraction of the aqueous phase with EtOAc, the combined organic phases were washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash chromatography (7:3 petroleum ether/EtOAc mixture) to give lactol **5** as a waxy solid (1.1 g, 76%): R_f = 0.35 (7:3 petroleum ether/EtOAc mixture).

Dibenzyl diisopropyl phosphoramidite (2.4 mL, 7.1 mmol) was added to a stirred suspension of lactol **5** (1.1 g, 3.6 mmol) and tetrazole (982 mg, 14.2 mmol) in CH_2Cl_2 (70 mL) at −40 °C under argon. The reaction mixture was warmed to 0 °C over 1 h and then cooled again to −40 °C. *m*CPBA (3.1 g, 17.8 mmol) was dissolved in CH_2Cl_2 and added to the mixture. The reaction mixture was then warmed to 0 °C over 1 h and the reaction quenched with aqueous Na_2SO_3 (10%, 50 mL). The organic layer was washed with equal volumes of H_2O, saturated aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), filtered, and evaporated to dryness. The ^{1}H NMR of the crude reaction mixture indicated a 4:1 mixture (β:α) of dibenzyl phosphates. The residue was purified by flash chromatography (4:1 petroleum ether/EtOAc mixture); the first to elute was the α-dibenzyl phosphate, followed by the desired β-dibenzyl phosphate **6**, which was obtained as a white solid (1.3 g, 61%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 10 H, aromatic), 5.58 (dd, $J_{1,2}$ = 1.6 Hz, $J_{1,P}$ = 7.2 Hz, 1 H, 1-H), 5.46 (dd, $J_{1,2}$ = 1.6 Hz, $J_{2,3}$ = 3.3 Hz, 1 H, 2-H), 5.22 (d, $J_{3,4}$ = 10.3 Hz, 1 H, 4-H), 5.11 (dd, $J_{2,3}$ = 3.3 Hz, $J_{3,4}$ = 10.3 Hz, 1 H, 3-H),

5.00−5.10 (m, 4 H, 2 OCH_2), 2.15, 2.07, 2.00 (3 s, each 3 H, $COCH_3$), 1.30, 1.29 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 170.4, 170.0, 128.9, 128.8, 128.7, 128.2, 128.1, 91.2 (d, $J_{C-1,P}$ = 4.1 Hz, C-1), 75.6, 71.2, 69.8, 69.7, 69.1, 68.5, 27.9, 21.0, 20.9, 20.8, 19.3; ^{31}P NMR (162 MHz, $CDCl_3$) δ −1.64; MS (ESI) for $C_{27}H_{33}O_{11}P$ (564.5) 587 ($[M + Na]^+$).

2,3,4-Tri-O-acetyl-5,5-di-C-methyl-L-lyxopyranosyl Phosphate (7). A suspension of dibenzyl phosphate **6** (1.3 g, 2.2 mmol) and Pd-C (600 mg) in MeOH (20 mL) was stirred under hydrogen at room temperature for 1 h. The reaction mixture was filtered through a Celite pad and evaporated to dryness. The free acid **7** was obtained as a white solid (924 mg, quantitative) and used without further purification: ^1H NMR (500 MHz, CD_3OD) δ 5.55 (dd, $J_{1,2}$ = 0.8 Hz, $J_{1,P}$ = 8.0 Hz, 1 H, 1-H), 5.45 (dd, $J_{1,2}$ = 0.8 Hz, $J_{2,3}$ = 3.0 Hz, 1 H, 2-H), 5.20 (dd, $J_{2,3}$ = 3.0 Hz, $J_{3,4}$ = 10.0 Hz, 1 H, 3-H), 5.12 (d, $J_{3,4}$ = 10.0 Hz, 1 H, 4-H), 2.20, 2.02, 1.92 (3 s, each 3 H, $COCH_3$), 1.40, 1.24 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR (125 MHz, CD_3OD) δ 172.4, 171.9, 171.6, 91.0, 75.8, 72.9, 72.0, 70.7, 28.4, 20.9, 20.9, 20.7, 19.2; ^{31}P NMR (162 MHz, CD_3OD) δ −1.56; MS (ESI) for $C_{13}H_{21}O_{11}P$ (384.3) 383 ($[M - H]^-$).

Thymidine 5′-(2,3,4-Tri-O-acetyl-5,5-di-C-methyl-L-lyxopyranosyl Diphosphate) (8). A mixture of the free acid **7** (780 mg, 1.8 mmol) and trioctylamine (0.8 mL, 1.8 mmol) was coevaporated with pyridine (3 × 10 mL). To the residue was added TMP-morpholidate (2.5 g, 3.7 mmol), and the mixture was coevaporated with pyridine (3 × 20 mL). After the mixture was redissolved in pyridine (25 mL), tetrazole (500 mg, 7.2 mmol) was added, and the reaction mixture was stirred under argon at room temperature for 4 days. The progress of the reaction was monitored by analytical HPLC (gradient A, retention times of 13.2 min for TMP-morpholidate and 14.4 min for **8**). The reaction mixture was then evaporated to dryness under reduced pressure. The residue was suspended in 0.1 M aqueous NH_4HCO_3 (40 mL) and extracted three times with equal volumes of diethyl ether. The product **8** was purified by preparative HPLC. The fractions containing **8** were concentrated under vacuum and lyophilized to give the diammonium salt of **8** as a white powder (670 mg, 52%): ^1H NMR (400 MHz, CD_3OD) δ 7.80 (s, H, thymidine H), 6.30 (t, $J_{1',2'}$ = 6.8 Hz, 1 H, 1′-H), 5.68 (dd, $J_{1,2}$ = 0.8 Hz, $J_{1,P}$ = 9.6 Hz, 1 H, 1-H), 5.54 (dd, $J_{1,2}$ = 0.8 Hz, $J_{2,3}$ = 3.2 Hz, 1 H, 2-H), 5.22 (dd, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 10.5 Hz, 1 H, 3-H), 5.11 (d, $J_{3,4}$ = 10.5 Hz, 1 H, 4-H), 4.52 (m, 1 H, 3′-H), 4.16 (m, 2 H, 5′-H_2), 4.00 (m, 1 H, 4′-H), 2.15−2.30 (m, 2 H, 2′-H_2) 1.91 (s, 3 H, thymidine CH_3), 2.12, 2.03, 1.90 (3 s, each 3 H, $COCH_3$), 1.38, 1.22 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR (100 MHz, CD_3OD) δ 172.2, 171.9, 171.5, 166.6, 152.6, 138.2, 112.1, 91.5, 87.3 (d, $J_{C-1,P}$ = 6.9 Hz, C-1), 86.2, 75.9, 73.0, 72.9, 72.5, 71.8 (d, $J_{C-2,P}$ = 5.0 Hz, C-2), 70.6, 40.7, 28.4, 21.0, 20.9, 20.7, 19.2, 12.8; ^{31}P NMR (162 MHz, CD_3OD) δ −9.9, −11.9; MS (ESI) for $C_{23}H_{34}N_2O_{18}P_2$ (688.5) 687 ($[M - H]^-$).

Thymidine 5′-(5,5-Di-C-methyl-L-lyxopyranosyl Diphosphate) (9). The protected diammonium salt **8** (670 mg, 0.93 mmol) was dissolved in a mixture of H_2O, MeOH, and Et_3N (5:5:2, 36 mL) and stirred at room temperature. The reaction was monitored by analytical HPLC (gradient A, t_R of 14.4 min for **8** and 10.9 min for **9**). After 3 h, the reaction mixture was reduced in volume under vacuum and the remaining solution was lyophilized. After purification of the residue by preparative HPLC, fractions containing **9** were concentrated under vacuum and lyophilized to give the diammonium salt **9** as a white powder (482 mg, 87%): ^1H NMR (500 MHz, D_2O) δ 7.74 (s, 1 H, thymidine H), 6.29 (t, $J_{1',2'}$ = 6.8 Hz, 1 H, 1′-H) 5.32 (d, $J_{1,P}$ = 8.0 Hz, 1 H, 1-H), 4.56 (m, 1 H, 3′-H), 4.11 (m, 3 H, 4′-H, 5′-H_2), 4.04 (d, $J_{2,3}$ = 3.2 Hz, 1 H, 2-H), 3.75 (dd, $J_{2,3}$ = 3.3 Hz, $J_{3,4}$ = 10.3 Hz, 1 H, 3-H), 3.50 (d, $J_{3,4}$ = 10.3 Hz, 1 H, 4-H), 2.31 (m, 2 H, 2′-H_2) 1.90 (s, 3 H, thymidine CH_3), 1.26, 1.19 [2 s, each 3 H, 5-$(CH_3)_2$]; ^{13}C NMR (125 MHz, D_2O) δ 166.8, 152.0, 137.5, 111.9, 92.3, 85.5 (d, $J_{C-1,P}$ = 8.6 Hz, C-1), 85.2, 76.6, 73.0, 71.2, 71.1, 69.4, 65.6, 38.7, 27.3, 17.4, 11.9; ^{31}P NMR (162 MHz, D_2O) δ −10.5, −12.7; MS (ESI) for $C_{17}H_{28}N_2O_{15}P_2$ (562.4) 560 ($[M - 2H]^-$).

Characterization of NovM. Reactions (total volume of 200 μL) were carried out at ambient temperature, and the mixtures contained novobiocic acid and TDP-L-noviose substrates in 75 mM MES (pH 6.0), 10 mM $MnCl_2$, 1 mg/mL bovine serum albumin (BSA), and 10% DMSO. Reactions were initiated by the addition of NovM and terminated at specified time points by quenching 50 μL aliquots with 100 μL of methanol at 4 °C. For the determination of kinetic parameters, NovM was added to a final concentration of 10 nM, and K_m and k_{cat} were determined with both novobiocic acid and TDP-L-noviose as variable substrates. The determination of K_m for novobiocic acid was accomplished at a constant concentration of TDP-L-noviose (150 μM) and over a range of novobiocic acid concentrations (5−60 μM). Four samples were taken between 1 and 5 min for each concentration. The determination of K_m for TDP-L-noviose was accomplished at a constant concentration of novobiocic acid (45 μM) and over a range of TDP-L-noviose concentrations (1−60 μM). For each concentration, four aliquots were removed and the reactions quenched between 0.5 and 3 min. Each experiment was carried out in triplicate.

Quenched aliquots were incubated at −20 °C for 30 min and then centrifuged to remove precipitated protein (5 min at 13 000 rpm). The supernatant in each case was analyzed by analytical reverse-phase HPLC [60:40 CH_3CN/H_2O mixture (0.1% TFA), 1 mL/min] and monitored at 340 nm. Product formation was confirmed by LC−MS {$C_{29}H_{33}NO_{10}$ calcd, 556.21 [$(M + H)^+$]; observed, 555.95}. The novobiocic acid (t_R = 10.6 min) and NovM product (t_R = 7.0 min) HPLC peak areas were measured, and the product concentration was calculated as a percent of the total peak area.

Substrate Specificity Study of NovM. Reaction mixtures were incubated at ambient temperature and contained 150 μM TDP-L-noviose, 75 mM MES (pH 6.0), 10 mM $MnCl_2$, 1 mg/mL BSA, 10% DMSO, and 5 μM NovM. Reactions were initiated by the addition of NovM and terminated by the addition of methanol at 4 °C. Quenched aliquots were analyzed by reverse-phase HPLC [60:40 CH_3CN/H_2O mixture (0.1% TFA), 1 mL/min], and the peak areas were measured as described above. The noviosyl cyclonovobiocic acid product was confirmed by LC−MS {$C_{29}H_{33}NO_{10}$ calcd, 556.21 [$(M + H)^+$]; observed, 555.95}. For the determination of kinetic parameters for cyclonovobiocic acid, a concentration range of 20−200 μM for novobiocic acid was used, and 50 μL aliquots were quenched with 100 μL of methanol at 4 °C. K_m and k_{cat} determinations were carried out in triplicate.

Reaction mixtures containing 7-hydroxycoumarin or 7-hydroxy-4-methylcoumarin were carried out as described above. The reaction product obtained following incubation of TDP-L-noviose with 7-hydroxycoumarin was confirmed by LC−MS {$C_{16}H_{18}O_7$ calcd, 323.11 [$(M + H)^+$]; observed, 323.10} as was the reaction product obtained following incubation of TDP-L-noviose with 7-hydroxy-4-methylcoumarin {$C_{17}H_{20}O_7$ calcd, 337.12 [$(M + H)^+$]; observed, 337.00}. For the determination of kinetic parameters for 7-hydroxycoumarin and 7-hydroxy-4-methylcoumarin under these conditions, a concentration range of 50 μM to 8 mM for the aglycone substrate was used. At >8 mM, 7-hydroxycoumarin was insoluble, and at >6 mM, 7-hydroxy-4-methylcoumarin was insoluble. Aliquots were quenched and analyzed as described above [elution in a 25:75 CH_3CN/H_2O mixture (0.1% TFA), 1 mL/min]. Under these conditions, saturation was not observed in either case; hence, k_{cat}/K_m values were calculated by linear regression analysis of the line generated in a plot of velocity versus substrate concentration. Experiments were carried out in triplicate in both cases.

For the determination of k_{cat}/K_m for 4-phenylphenol and 4-nitrophenol, reactions were carried out at pH 6.0 and analyzed as described above. It should be noted that a reaction pH of 6.0 is not optimal for the glycosylation of these substrates but was used for the purpose of comparison to the glycosylation of the natural substrates.

Inhibition of NovM by TDP-L-Rhamnose. Competitive inhibition studies were carried out by measuring enzyme activity in the presence of varying amounts of the inhibitor, TDP-L-rhamnose (0, 200, 400, and 600 μM) at each of six TDP-L-noviose substrate concentrations between 1 and 30 μM. The reactions were carried out at ambient temperature under the same conditions described above for the determination of kinetic parameters for TDP-L-noviose. The data were fit to the following equation:

$$v = (V_{max}[S])/[K_m(1 + 1/K_i) + [S]]$$

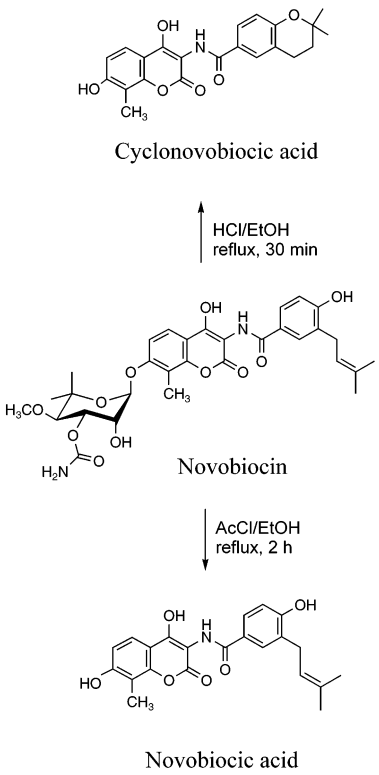
K_i was determined from these data fit and displayed as an Eadie−Hofstee plot. K_i determination was carried out in triplicate.

RESULTS

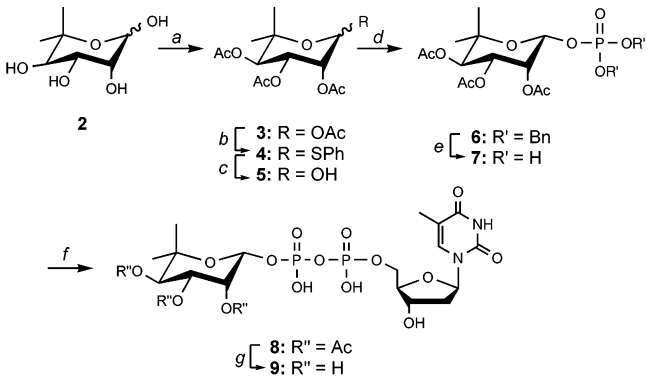
Preparation of NovM Substrates TDP-L-Noviose, Novobiocic Acid, TDP-L-Rhamnose, and Cyclonovobiocic Acid. The main obstacle in evaluation of the catalytic activity and substrate specificity in the family of antibiotic glycosyltransferases has been the availability of substrates. For the purposes of NovM characterization in this study, the natural substrates were prepared via chemical synthesis. The aglycone substrate novobiocic acid was prepared in a single step by acid-catalyzed degradation of novobiocin as shown in Scheme 2. Slightly harsher acidic conditions resulted in cleavage of the noviose component from the parent antibiotic and concomitant cyclization of the prenyl moiety to form cyclonovobiocic acid (Scheme 2) (*19*). TDP-L-rhamnose was synthesized as part of an earlier effort to develop a novel method for the preparation of NDP-bound sugars (*22*) and was used here to test the substrate specificity of NovM.

The noviose donor TDP-L-noviose was prepared in 11 steps starting from commercially available L-rhamnose. The

Scheme 2: Preparation of Novobiocic Acid and Cyclonovobiocic Acid Aglycones by Degradation of Novobiocin



Scheme 3: Preparation of TDP-l-Noviose[a]



[a] (a) Ac_2O, pyridine, 72%; (b) PhSH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 91%; (c) NIS, CH_3CN/H_2O, 76%; (d) (1) $iPr_2NP(OBn)_2$, tetrazole, CH_2Cl_2, (2) *m*CPBA, CH_2Cl_2, 61%; (e) H_2/Pd-C, MeOH, quantitative; (f) TMP-morpholidate, tetrazole, pyridine, 52%; (g) $Et_3N/MeOH/H_2O$ (5:5:2), 87%.

synthesis accomplishes the introduction of the key 5-methyl substituent present in TDP-L-noviose (prepared according to the procedure outlined in ref *21*) and further elaborates the sugar moiety to a nucleoside diphosphate. The synthesis of TDP-L-noviose starting from 5,5-dimethylhexose **2** (*21*) is shown in Scheme 3. Briefly, hydroxyl protection of **2** with acetic anhydride in pyridine afforded tetraacetate **3**. Conversion of the anomeric acetate to the corresponding thiophenyl glycoside **4** followed by treatment with wet *N*-iodosuccinimide resulted in the formation of lactol **5**. Standard phosphitylation at the 1 position followed by oxidation with *m*CPBA afforded a mixture of anomeric benzyl phosphate esters (1: 4 α:β), from which pure β-phosphate **6** could be isolated in 61% yield. Removal of the benzyl protecting

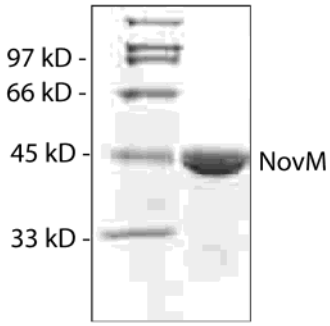


FIGURE 2: Heterologous overproduction and purification of N-terminal His_8 glycosyltransferase NovM (40 kDa) from *E. coli*.

groups via hydrogenolysis to generate **7** followed by standard phosphate coupling with TMP-morpholidate afforded acetoxy-protected TDP-L-noviose **8**. Subsequent treatment of **8** with triethylamine in a methanol/water mixture resulted in the generation of TDP-L-noviose (**9**).

Overproduction and Purification of S. spheroides NovM in Escherichia coli. NovM is the presumed glycosyltransferase in novobiocin biosynthesis as its sequence is significantly homologous to that of the family of glycosyltransferases (*15*). Specifically, NovM was evaluated for catalysis of the transfer of the noviose component from TDP-L-noviose to the aglycone novobiocic acid. The 40 kDa enzyme was heterologously overproduced in good yield (>9 mg/L) and purified from *E. coli* BL21(DE3) cells harboring the NovM expression construct and grown at 25 °C for 24 h without IPTG induction. A C-terminal His_8-tagged NovM was subsequently purified to homogeneity by Ni(II) affinity chromatography (Figure 2).

Characterization of Glycosyltransferase Activity. The function of NovM was established using synthetically prepared novobiocic acid and TDP-L-noviose (Scheme 1) as substrates. The appearance of glycosylation product **1** in the presence of 100 nM NovM was monitored by reverse-phase HPLC (RP-HPLC, Figure 3A), and the NovM reaction product was confirmed by LC−MS {*m*/*z* 555.95 [(M + 1)$^+$]}. The reaction conditions were subsequently optimized, revealing maximum enzyme activity at pH 6 and a requirement for Mn^{2+}.

The kinetic parameters for NovM were defined with either novobiocic acid or TDP-L-noviose as the variable substrate (Figure 3B,C). The catalyst, NovM, was added to a final concentration of 10 nM. Typical hyperbolic saturation is observed when TDP-L-noviose is the variable substrate (novobiocic acid concentration held constant at 45 μM), resulting in a K_m of 8.6 ± 1.1 μM for the nucleotide sugar (Figure 3C). Under these conditions, a maximal rate constant of 226 ± 27 min^{-1} was observed and is in good agreement with the rate constant measured in parallel experiments carried out using 45 μM novobiocic acid under saturating TDP-L-noviose conditions (227 ± 46 min^{-1}). Enzyme inhibition is observed when novobiocic acid is the variable substrate and occurs at >60 μM novobiocic acid. A Michaelis−Menten curve fit of these data up to 60 μM novobiocic acid provides an apparent K_m of 32 ± 9.9 μM and a k_{cat} of 313 ± 16 min^{-1} for novobiocic acid (Figure 3B).

Substrate Specificity of NovM. Numerous efforts have been made to introduce diversity into the glycosyl substituents of many natural products to enhance the biological activities and produce new therapeutic agents (*23*−*25*). Few studies have been carried out in which the antibiotic aglycone specificity has been examined for the transfer of glycosyl substituents by dedicated glycosyltransferases. Recent studies in our laboratory have revealed a notable relaxed specificity of vancomycin glycosyltransferases for nonribosomal peptide aglycones as well as NDP-sugar substrates (*17*, *18*). Both the noviose and coumarin components of novobiocin are key structural elements for the recognition of the bacterial topoisomerase gyrase B (*2*, *3*, *26*). It is therefore of interest to study the substrate specificity of NovM in an effort to expand the coumarin antibiotic repertoire. Some commercially available 7-hydroxycoumarins (Table 1) were tested as substrates for NovM as well as cyclonovobiocic acid and some simple phenols. The appearance of novel noviosylated coumarin analogues was monitored by RP-HPLC as described above. For experiments outlined in entries 1−4 (Table 1), the resulting glycosylated reaction product was confirmed by LC−MS.

Cyclonovobiocic acid is readily accepted by NovM, maintaining a K_m (39 μM) similar to that of the natural substrate (32 μM) and an only 2.5-fold lower observed k_{cat} (118 min^{-1}). Removal of the 3-amido and 4-hydroxyl substituents from novobiocic acid is also tolerated by the enzyme, although these transfers on the simple 7-hydroxycoumarin scaffold result in a reduction in the NovM catalytic efficiency by 5−6 orders of magnitude under these conditions (Table 1, entries 3 and 4).

Simple phenols are also noviosylated by the enzyme (Table 1, entries 5 and 6), exhibiting catalytic efficiencies 6−7 orders of magnitude lower than that observed with the natural substrates. Nonetheless, these data suggest that NovM tolerates removal of the α,β-unsaturated cyclic ester comprising the coumarin system. Transfer of the noviose component to phenolic aglycones is pH-dependent; 4-phenylphenol and 4-nitrophenol undergo noviosylation more readily at a pH (pH 7.5 and 6.5, respectively) that is higher than the optimal pH for enzyme activity with the natural substrates (data not shown). The manner in which substitution of the coumarin nucleus affects the catalytic efficiency of noviosylation remains under investigation.

Inhibition of NovM by TDP-L-Rhamnose. Some antibiotic glycosyltransferases are known to have relaxed substrate specificity for NDP-sugars (*1*, *27*), and have been utilized in the combinatorial generation of novel polyketide and macrolide antibiotics (*23*−*25*). As an initial effort to study the specificity of NovM toward NDP-sugars, TDP-L-rhamnose was assayed as a potential NDP-sugar substrate, and the reactions were monitored by RP-HPLC. L-Rhamnose serves as the starting material for TDP-L-noviose in the synthesis reported here. It is a close structural relative of TDP-L-noviose differing only at the 5 position of the pyranose ring (Figure 4A). When no glycosyltransferase activity was detected in initial incubations of NovM with TDP-L-rhamnose and novobiocic acid, additional assays were performed over a 500-fold range of enzyme concentrations (10 nM to 5 μM NovM). Even under these conditions, formation of the rhamnosyl novobiocic acid product was not observed, indicating a >10^5-fold reduction in the catalytic efficiency for NovM in the transfer of TDP-L-rhamnose to novobiocic acid. TDP-L-rhamnose was found to inhibit the enzyme with an apparent K_i of 83.5 ± 5.5 μM (Figure 4B),

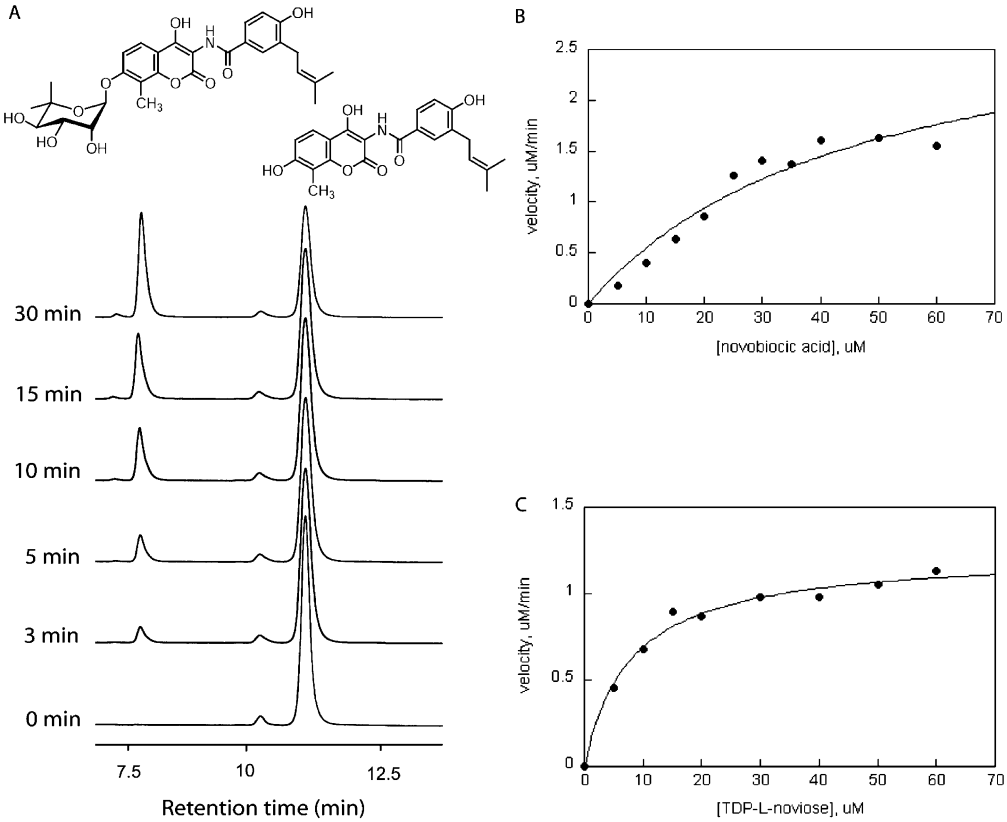


FIGURE 3: Characterization of NovM activity with novobiocic acid and TDP-L-noviose. (A) Conversion of novobiocic acid to desmethyldescarbamoyl novobiocin monitored by RP-HPLC. Product confirmed by LC−MS {*m*/*z* 555.95 [(M + 1)$^+$]}. Conditions: 1 mM novobiocic acid, 1 mM TDP-L-noviose, 10 mM $MnCl_2$, 75 mM MES (pH 6), and 100 nM NovM at 25 °C. (B) Michaelis−Menten plot for measurement of kinetic parameters for the novobiocic acid substrate. The TDP-L-noviose concentration was fixed at 150 μM, and NovM was added to a final concentration of 10 nM. (C) Michaelis−Menten plot for measurement of kinetic parameters for the TDP-L-noviose substrate. The novobiocic acid concentration was fixed at 45 μM, and NovM was added to a final concentration of 10 nM.

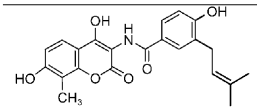
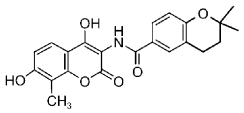
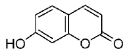
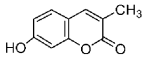
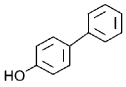
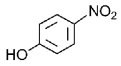
a 10-fold increase over the observed K_m for TDP-L-noviose. These data suggest that the unique 5,5-dimethyl substituent of the noviosyl component of TDP-L-noviose is essential for glycosyltransferase activity during transfer of the pyranose ring to novobiocic acid.

DISCUSSION

Many classes of antibiotics are glycosylated with specific deoxyhexose residues, and the aglycones are of diverse structural types. Polyketide-based antibiotics are comprised of aglycones exhibiting various polycyclic scaffolds, such as the aklavinone class of antitumor agents that are elaborated to daunomycin and congeners. Additional structurally diverse scaffolds are found in the macrolide class, including the 14-membered erythromycins and the 16-membered relatives tylosin and carbomycin (*1*, *28*). The nonribosomal peptide family of antibiotics are varied in the decoration of the peptide aglycone. The cross-linked heptapeptide scaffold of the vancomycin and teicoplanin aglycones is further tailored by enzymatic glycosylation (*1*), while ramoplanin bears a dimannosyl chain which is added late in biosynthesis (*29*). The nonribosomal peptide−polyketide hybrid natural product bleomycin bears L-gulose and D-mannose sugar components which are added sequentially to a β-hydroxyhistidine residue (*1*). In each of these examples, the glycosylations are carried out by dedicated glycosyltransferases encoded in the biosynthetic cluster and acting late in the antibiotic maturation process. The sugar components serve a variety of purposes, from enhancing solubility to imparting specific interactions with the biological target. One example is the interaction of the C5−O sugars on the macrolides with the peptidyl transferase region of the 23S rRNA in the 50S ribosomal subunit (*30*, *31*).

The biosynthesis of the aminocoumarin-containing antibiotics, such as novobiocin and clorobiocin, occurs in three distinct stages: assembly of the bicyclic aminocoumarin nucleus on a nonribosomal peptide synthetase module (NovH), ligation of the aminocoumarin to the prenylated hydroxybenzoate to form novobiocic acid (NovL), and glycosylation with the L-deoxyhexose, L-noviose (NovM). At this point in the biosynthesis of novobiocin, the backbone connectivities have been established; however, the NovM product is two steps from the generation of a molecule that can bind to the target DNA gyrase and facilitate bacterial cell death. The two key tailoring steps are thought to occur immediately following introduction of the noviosyl ring by NovM, and include 4-O-methylation (NovP) and 3-O-carbamoylation (NovN). X-ray structure analysis of novobiocin bound to an N-terminal fragment of the GyrB subunit (*2*) reveals key interactions between the carbamoylated noviosyl moiety of the antibiotic and the ATP binding site of the GyrB subunit. The NovM-mediated glycosylation of novobiocic acid produces the essential platform for these final tailoring steps in novobiocin biosynthesis and provides the necessary scaffold for presentation of the tailored L-noviose as the specific pharmacophore in DNA gyrase inhibition.

Table 1: Kinetic Parameters for Natural and Alternative Aglycone Substrates

Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)	Relative k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
 Novobiocic acid	313 ± 16	32 ± 9.9	9.8	1
 Cyclonovobiocic acid	118 ± 4	39 ± 6.5	3.0	0.3
 7-Hydroxycoumarin	n/a[a]	n/a[a]	5.7×10^{-5}	5.8×10^{-6}
 7-Hydroxy-4-methylcoumarin	n/a[a]	n/a[a]	6.9×10^{-5}	7.0×10^{-5}
 4-Phenylphenol	n/a[a]	n/a[a]	5.2×10^{-6}	5.3×10^{-7}
 4-Nitrophenol	n/a[a]	n/a[a]	4.7×10^{-5}	4.8×10^{-6}

[a] Kinetic parameters could not be determined for this substrate using the Michaelis−Menten equation.

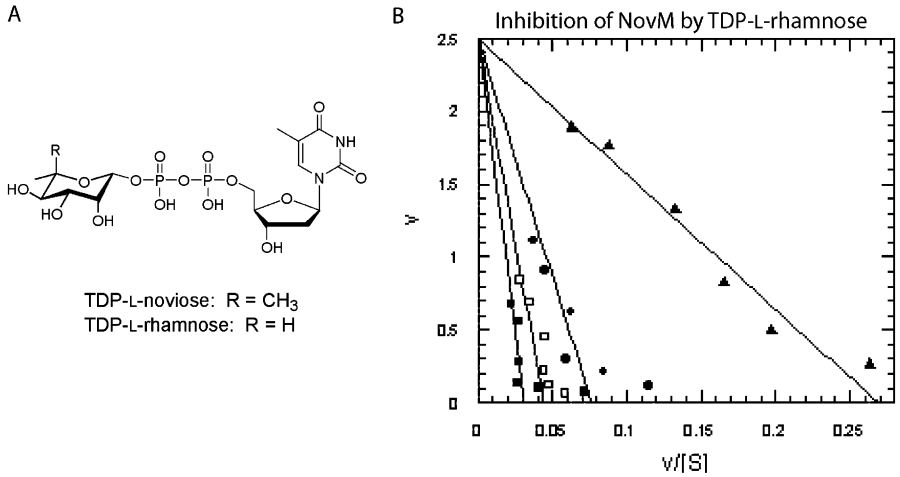


FIGURE 4: Competitive inhibition of NovM by TDP-L-rhamnose. (A) TDP-L-noviose and TDP-L-rhamnose structures. (B) Eadie−Hofstee plot demonstrating competitive inhibition of NovM by TDP-L-rhamnose [0 (▲), 200 (●), 400 (□), and 600 μM (■)]. $K_i = 83.5 \pm 5.5$ μM. Other experimental conditions are outlined in Materials and Methods.

The aim of this study has been to validate the role of NovM and address its specificity and mechanism as a starting point for the subsequent evaluation of its potential to utilize alternate aglycone or NDP-L-deoxyhexose substrates. This work will ultimately provide a foundation for combinatorial biosynthetic approaches to novel coumarin-based antibiotics. Our investigation has required the purification of NovM and the development of an assay for measuring enzyme activity. Few reports of heterologous expression and purification of antibiotic glycosyltransferases in a soluble, folded form or relevant catalytic assays have appeared in the literature. Notable successes include the isolation of the vancomycin and chloroeremomycin glycosyltransferases (*18*, *32*) and the autoprotection glucosyl transferase from the oleandomycin-

producing *Streptomyces antibioticus* (*33*). In the work reported here, overproduction of C-terminally His_8-tagged NovM from *S. spheroides* in *E. coli* produced a soluble protein in good yields that could be purified readily.

The major hurdle in evaluation of the catalytic activity and permissivity of a broad range of antibiotic glycosyltransferases has been the availability of substrates. Many studies have thus been limited to *in vivo* fermentations where the metabolic pathways of the producer organisms supply substrates (*27*). Promiscuity for NDP-sugar substrates has been demonstrated *in vivo* by several of the polyketide antibiotic glycosyltransferases, including OleG2 (*23*, *34*), TylM2 (*23*), Des VII (*35*), and UrdGT2 (*1*) in oleandomycin, tylosin, daunomycin, and urdamycin biosynthesis, respectively. These investigations were executed via gene transfer and/or exogenous feeding of aglycones and resulted in the formation of altered glycosylated products. Access to various aglycone substrates for these studies is often achieved by fermentation of blocked mutants (*36*) and by partial degradation of the mature antibiotic. The selective degradation route was employed in our previous studies on the vancomycin and chloroeremomycin glycosyltransferases (*18*) and in this study on NovM. The acid-catalyzed degradation of novobiocin, in fact, gave both novobiocic acid and cyclonovobiocic acid (*19*), each bearing the coumarin 7-hydroxyl group as the nucleophile, and both were evaluated as substrates.

The NDP-sugar donor cosubstrates, activated for capture at C_1 by the nucleophilic atom of the aglycone, are variable in their degree of accessibility. For antibiotic glycosyltransferases that utilize UDP- or TDP-glucose, such as GtfB in the chloroeremomycin pathway (*18*) and OleD in the oleandomycin self-modification pathway (*37*), substrate accessibility is not an issue. However, most antibiotic glycosyltransferases transfer deoxyhexose residues (*38*), such as TDP-D-desosamine in erythromycin biogenesis or TDP-D-olivose in mithramycin and urdamycin biogenesis (*39*, *40*). The difficulty in obtaining these specialized nucleoside diphospho-D-deoxyhexoses has been a major barrier to glycosyltransferase evaluation, and has limited investigators to conducting *in vivo* studies.

A subset of the antibiotic glycosyltransferases, including NovM, transfers L-deoxyhexoses; TDP-L-deoxyhexose substrates are even less accessible than the D-isomer counterparts. The multistep enzymology of TDP-D-deoxyhexose and TDP-L-deoxyhexose formation has been carefully deciphered over the past two decades (*38*), and the 5-epimerases that interconvert 4-keto-D- and 4-keto-L-sugars are being characterized (*41*, *42*). The biosynthetic pathway converting TDP-D-glucose to TDP-L-rhamnose is four steps (*43*); five enzymes are required for conversion of TDP-L-rhamnose to TDP-L-β-epivancosamine (*44*). In fact, the enzymatic reconstitution of the EvaA-E pathway was achieved and provided sufficient amounts of TDP-L-epivancosamine for analysis of the chloroeremomycin epivancosaminyl transferase GtfC (*18*). However, it is not a trivial matter to use five enzymes sequentially to produce substrate for the sixth enzyme, the glycosyltransferase under investigation.

The novobiocin gene cluster encodes proteins homologous to the enzymes involved in TDP-L-rhamnose biosynthesis (*15*), suggesting a five-enzyme pathway for the production of TDP-L-noviose in the *S. spheroides* antibiotic producer starting with TDP-D-glucose. In this pathway, TDP-D-glucose most likely undergoes deoxygenation at C_6, epimerization at C_3, C_4, and C_5, and C-methylation at C_5. However, enzymatic synthesis is not practical for the preparation of the quantities of TDP-L-noviose required for the screening of coumarin cosubstrates and the large-scale preparations of analogues for subsequent incubations with the tailoring enzymes, NovP and NovN. A complementary strategy for the production of the sugar donor is the chemical synthesis of UDP- or TDP-L-deoxysugars. This approach was used for evaluation of GtfC, GtfD, and GtfE in the tailoring of the vancomycin and chloroeremomycin peptide scaffolds, and was the method of choice for providing enough TDP-L-deoxyhexose for large-scale reactions enabling the production of novel glycosyl variants of those antibiotics (*17*, *18*). Thus, the chemical synthesis of TDP-L-noviose was undertaken during the course of this work and has removed the highest barrier to evaluation of the activity of NovM.

L-Noviose differs from the more common L-6-deoxyhexose, L-rhamnose, at the 5 position. L-Rhamnose was employed as the starting material for the synthesis reported here which affords 100 mg quantities of the NDP-L-sugar product. Although incomplete control of the stereochemistry during formation of the anomeric phosphate ester (4:1 β:α) remains an issue for further optimization of TDP-L-β-noviose preparations, the synthesis reported here nevertheless provides pure β-phosphate **6** in good yield (61%) and establishes an efficient preparation of TDP-L-β-noviose amenable to scaling up.

The availability of the two substrates, novobiocic acid and TDP-L-noviose, in addition to the efficient overproduction and purification of NovM from *E. coli* made characterization of glycosyltransferase activity facile. The enzyme was found to be robustly active, with turnover numbers of >300 min^{-1}. This level of activity permits several directions of investigation. The first is to evaluate the tolerance of NovM for variation in the coumarin scaffold of novobiocic acid. The data of Table 1 show that cyclonovobiocic acid is an acceptable aglycone, suggesting variants in the prenylbenzoate moiety will be tolerated. Removal of the 3-hydroxyl group and 2-amido moiety from the coumarin ring is tolerated, albeit with a significant reduction in the catalytic efficiency in the simple coumarin scaffolds. Even simple phenols can be noviosylated, again with low catalytic efficiency. These data suggest a range of planar bicyclic aromatic compounds that replace the coumarin ring system may serve as aglycone platforms for NovM action.

The modification of the TDP-L-deoxyhexose substrate is more difficult to achieve. In a preliminary study of NovM substrate specificity for the NDP-sugar donor, TDP-L-rhamnose was examined as a potential substrate. In this study, a more than 10^5-fold reduction in catalytic efficiency of NovM for transfer of TDP-L-rhamnose was observed, although TDP-L-rhamnose retains recognition as an inhibitor of the enzyme. These data suggest the 5,5-dimethyl substituent on the pyranose ring is essential for transfer of the sugar component to the aglycone scaffold and support the notion that the unusual 5,5-dimethyl substituent on the pyranose ring of L-noviose may be crucial to either antibiotic biosynthesis or biological activity.

The NovP reaction product, descarbamoyl novobiocin, was isolated from fermentations and shown in 1982 (*11*) to exhibit a >200-fold reduction in antibiotic activity. Likewise, it is

anticipated that the NovM reaction product, desmethyldescarbamoyl novobiocin, will also exhibit a reduction in antibiotic activity. Thus, the subsequent predicted decorations of the noviosyl ring 3-hydroxyl and 4-hydroxyl groups catalyzed by NovP and NovN, respectively, are the critical tailoring steps to be addressed next. The cocrystal structures of novobiocin and clorobiocin with the ATPase fragment of GyrB (*2*) confirm the requirement of the noviosyl ring tailoring enzymes for high-affinity targeting. A large-scale NovM reaction to yield tens of milligrams of noviosyl novobiocic acid is clearly feasible and will provide the substrates needed for NovP and NovN assays. Decoration of the noviose ring will be studied on both the natural novobiocic acid scaffold and the variant coumarin and noncoumarin scaffolds carried forward by NovM. The NovP product, descarbamoyl novobiocin, will also be a useful substrate for the assay of CouN2 in coumermycin A1 biosynthesis and the analogous acyltransferase in clorobiocin biosynthesis (*45*, *46*). In both coumermycin A1 and clorobiocin, a 5-methylpyrrole-2-carboxyester replaces the carbamoyl moiety at the 3-hydroxyl of the noviosyl coumarin and is thought to account for the 10-fold greater potency of inhibition of the DNA gyrase target by clorobiocin (*47*). The NovM reaction reported here, if coupled with NovP assays, would enable a CouN2 and/or CloN2 assay for antibiotic diversification efforts.

CONCLUSIONS

In conclusion, the ability to purify NovM from heterologous expression in *E. coli*, coupled with the chemical synthesis of TDP-β-L-noviose, has enabled the characterization of NovM as the L-noviosyl transferase in glycosylation of the aminocoumarin scaffold in novobiocin biosynthesis. These studies provide a foundation for evaluation of the enzymatic tailoring of the noviosyl ring and are the groundwork for future studies aimed at determination of the factors that control DNA gyrase interaction and antibacterial activity.

ACKNOWLEDGMENT

We gratefully acknowledge Raymond Chen for his preparation of the *S. spheroides* genomic DNA used for subcloning the *novM* gene and Richard Borch for providing TDP-L-rhamnose.

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BI0340088