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Substrate Specificity of NovM: Implications for Novobiocin Biosynthesis and Glycorandomization

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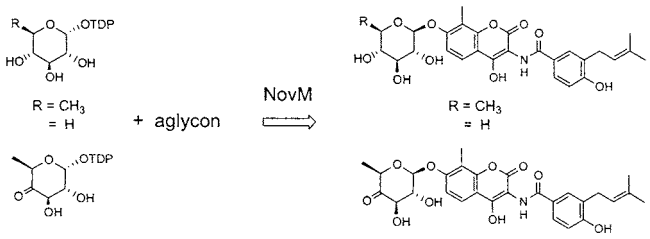
ABSTRACT



In an effort to expand the scope of natural product in vitro glycorandomization (IVG), the substrate specificity of NovM was investigated. A test of four aglycon analogues and over 40 nucleotide sugars revealed NovM has a surprisingly stringent substrate specificity and provided only three new "unnatural" natural products. On the basis of the determined substrate specificity, an alternative to the sugar nucleotide biosynthetic dogma and a cautionary note for the general applicability of IVG are introduced.

Aminocoumarin antibiotics, such as novobiocin (Albamycin, **1**), clorobiocin (**2**), and coumermycin A_1 (**3**, Figure 1), are secondary metabolites produced by various *Streptomyces* strains and are very potent against Gram-positive bacteria, including methicillin-resistant *Staphylococcus* strains. These effective agents function as potent competitive inhibitors of ATP-binding to the type II DNA topoisomerase DNA gyrase B subunit.[1] The aminocoumarins share a common core structure consisting of a 3-amino-4-hydroxy-coumarin and a substituted deoxysugar (noviose) that is essential for their biological activity. Structural studies reveal a significant overlap of the noviose and the gyrase ATP-binding site and suggest that alteration of the noviose ligand may lead to the rational design of better gyrase inhibitors and/or potentially other ATP-binding/utilizing target proteins.[2]

The early stages of the novobiocin aglycon (Scheme 1, **10**) assembly have recently been studied in vitro.[3] On the basis of precursor labeling studies, the recently elucidated novobiocin biosynthetic gene (*nov*) locus from *Streptomyces spheroides*,[4] and the current level of understanding in deoxy sugar biosynthesis,[5,6] a pathway for noviobicin biosynthesis

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(1) (a) Hooper, D. C.; Wolfson, J. S.; McHugh, G. L.; Winters, M. B.; Swartz, M. N. *Antimicrob. Agents Chemother*. **1982**, *22*, 662−671. (b) Maxwell, A. *Mol. Microbiol.* **1993**, *9*, 681−686. (c) Maxwell, A. *Trends Microbiol.* **1997**, *5*, 102−109. (d) Maxwell, A. *Biochem. Soc. Trans.* **1999**, *27*, 48−53.

(2) (a) 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.; Wigley, D. B. *Proteins* **1997**, *28*, 41−52. (b) Lewis, R. J.; Singh, O, M,; Smith, C. V.; Skarzynski, T.; Maxwell, A.; Wonacott, A. J.; Wigley, D. B. *EMBO J* **1996**, *15*, 1412−1420.

(3) (a) Steffensky, M.; Li, S.-M.; Heide, L. *J. Biol. Chem.* **2000**, *275*, 21754−21760. (b) Chen, H.; Walsh, C. T. *Chem. Biol*. **2001**, *8*, 301−312. (c) Pojer, F.; Li, S.-M.; Heide, L. *Microbiology* **2002**, *148*, 3901−3911.

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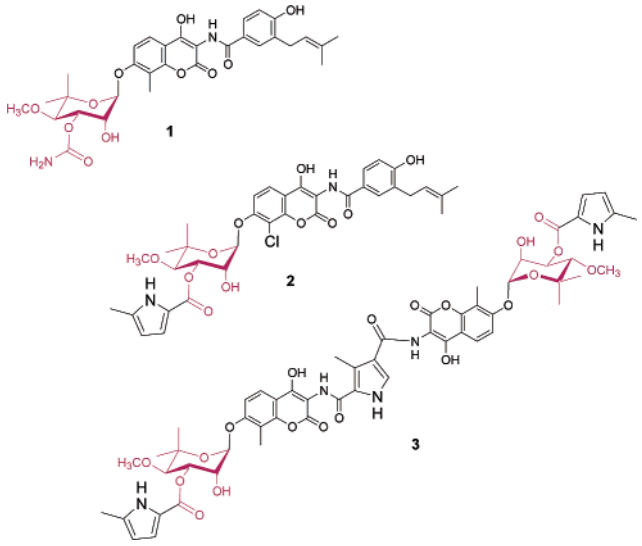
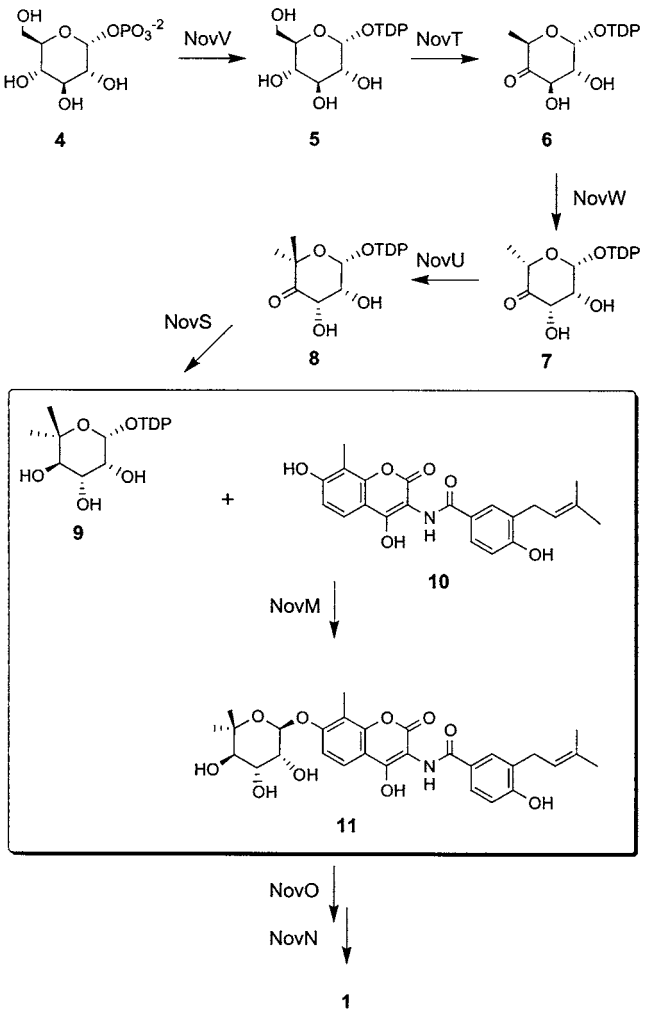


Figure 1. Naturally occurring coumarin antibiotics. The glycosyl moiety is highlighted in color.

can be postulated (Scheme 1).[4] This pathway is expected to proceed via the epimerization (NovW), methylation (NovU), and reduction (NovS) of a common 6-deoxy-4-keto-nucleotide sugar (Scheme 1, **6**), the order of which (e.g. NovW and U) could be altered. Also within the *nov* locus is a single gene (*nov*M) encoding for the putative noviosyl transferase, NovM, expected to catalyze the culminating formation of the α-glycoside **11**.[3a]

Since the noviose ligand is key to the biological activity of **1**, NovM presents a potential opportunity to test the in vitro "glycorandomization" (IVG)[7] of **1** in an effort to generate novel aminocoumarin analogues. IVG, which relies upon an engineered nucleotidylyltransferase to generate a vast "unnatural" nucleotide sugar library in conjunction with the typical promiscuity of glycosyltransferases in secondary metabolism, has recently been applied to the production of >50 vancomycin analogues.[8] In an effort to expand the IVG methodology, we now report the overexpression of *novM* in *E. coli* and the purification and characterization of NovM.

Scheme 1. Postulated Novobiocin Biosynthetic Pathway Highlighting the NovM Reaction



While this work confirms the functional assignment of *nov*M and presents the chemoenzymatic synthesis of three new glycosylated variants of **1**, NovM displayed a suprisingly stringent sugar donor specificity toward D-sugar nucleotides related to **6**, contrasting previous biosynthetic postulates.

Soluble overexpression of NovM was accomplished in *E. coli* in both a construct designed to express wild-type protein (pET22*nov*M) and a *C*-terminal His_6-fusion (pET20*nov*M).[9] Purified NovM-His_6 was used for the described studies.[10] For the NovM in vitro assays,[11] the native acceptor aglycon, novobiocic acid (**10**), was generated as previously described from commercially available **1**.[12] Other acceptor aglycons tested include the commercially available coumarin analogues 7-hydroxycoumarin (Figure 2, **11**), 7-hydroxycoumarin-4-acetic acid (**12**), 7-hydroxy-4-methylcoumarin (**13**), and 7-hydroxy-3,4,8-trimethylcoumarin (**14**). The natural and "unnatural" NDP-sugar donors tested were generated in situ via the nucleotidylyltransferase (E_p)-catalyzed conversion of

(4) (a) Steffensky, M.; Muhlenweg, A.; Wang, Z. X.; Li, S.-M.; Heide, L. *Antimicrob. Agents Chemother*. **2000**, *44*, 1214−1222. (b) Wang, Z. X.; Li, S.-M.; Heide, L. *Antimicrob. Agents Chemother*. **2000**, *44*, 3040−3048.
(5) He, X. M.; Liu, H.-w. *Annu. Rev. Biochem*. **2002**, *71*, 701−754.
(6) Birch, A. J.; Holloway, P. W.; Richards, R. W. *Biochim. Biophys. Acta* **1962**, *57*, 143−145.
(7) (a) Jiang, J.; Biggins, J. B.; Thorson, J. S. *J. Am. Chem. Soc*. **2000**, *122*, 6803−6804. (b) Thorson, J. S.; Hosted, T. J., Jr.; Jiang, J.; Biggins, J. B.; Ahlert, J.; Ruppen, M. *Curr. Org. Chem*. **2001**, *5*, 139−167. (c) Jiang, J.; Biggins, J. B.; Thorson, J. S. *Angew. Chem., Int. Ed*. **2001**, *40*, 1502−1505. (d) Barton, W. A.; Biggins, J. B.; Lesniak, J.; Jeffrey, P. D.; Jiang, J.; Rajashankar, K. R.; Thorson, J. S.; Nikolov, D. B. *Nature Struct. Biol*. **2001**, *8*, 545−551. (e) Barton, W. A.; Biggins, J. B.; Jiang, J.; Thorson, J. S.; Nikolov, D. B. *Proc. Natl. Acad. Sci. U.S.A*. **2002**, *99*, 13397−13402. (f) Thorson, J. S.; Barton, W. A.; Albermann, C.; Hoffmeister, D.; Nikolov, D. B. *ChemBioChem*. In press. (g) Jiang, J.; Albermann, C.; Thorson, J. S. *Angew Chem., Int. Ed*. Submitted for publication.
(8) (a) Solenberg, P. J.; Matsushima, P.; Stack, D. R.; Wilkie, S. C.; Thompson, R. C.; Baltz, R. H. *Chem. Biol*. **1997**, *4*, 195. (b) Losey, H.; Jiang, J.; Biggins, J. B.; Oberthur, M.; Ye, X.-Y.; Dong, S. D.; Kahne, D.; Thorson, J. S.; Walsh, C. T. *Chem. Biol*. **2002**, *9*, 1305−1314. (c) Albermann, C.; Xun-Fu, A.; Jiang, J.; Thorson, J. S. Unpublished.

(9) PCR-amplified *nov*M with engineered flanking *Nde*I/*Xho*I restriction sites was blunt-end ligated into pBS and the insert sequence confirmed to give plasmid pKMACTW. The corresponding *Nde*I/*Xho*I insert bearing *nov*M was cloned into the *Nde*1/*Xho*1 site of pET20/22.

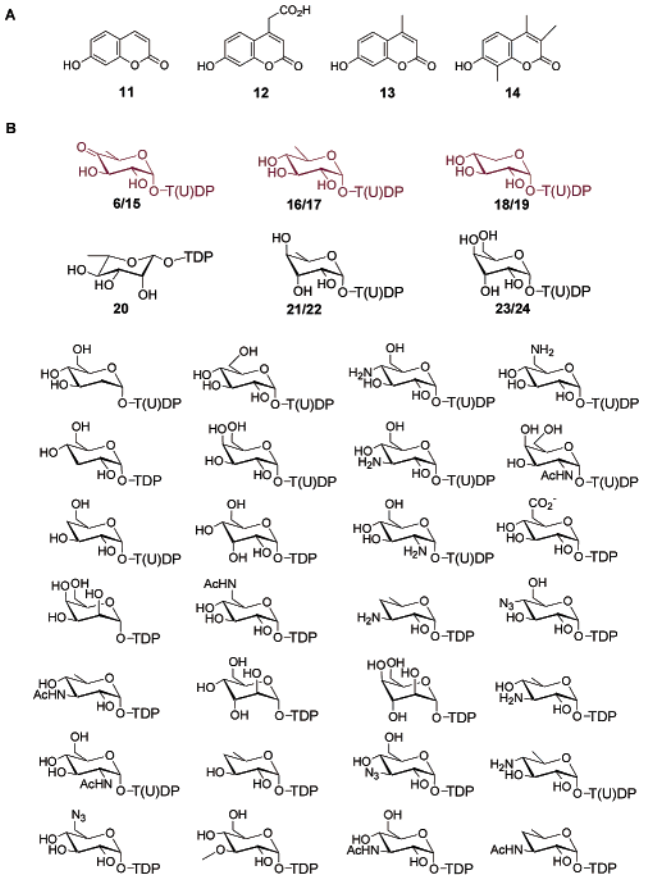
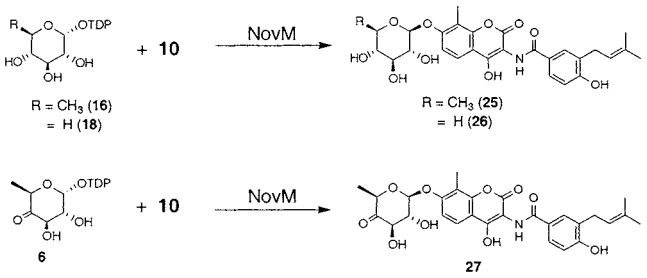


Figure 2. Putative NovM substrates tested: (a) aglycon analogues and (b) nucleotide sugar analogues.

hexopyranosyl- and pentopyranosyl-α-D-phosphates as previously reported.[7] In addition, commercially available NDP-sugars (UDP-Xyl, TDP-Glc, UDP-Glc, UDP-Gal, UDP−GalNAc, UDP-GlcNAc, UDP-GlcA) were utilized and TDP-β-L-rhamnose (**20**) was synthesized as previously described.[13]

Scheme 2. NovM-Catalyzed Production of New Coumarin Antibiotics



As a control, assays lacking NovM or NDP-sugar gave no product formation.

Of the entire substrate pool tested with NovM (Figure 2), only four "unnatural" NDP-sugars (Figure 2, highlighted in color), TDP-6-deoxy-Glc (**16**, 90% conversion), UDP-6-deoxy-Glc (**17**, 20%), TDP-4-keto-6-deoxy-Glc (**6**, 18%), and TDP-Xyl (**18**, 8%), led to detectable NovM-catalyzed transfer to **10** and none of the "unnatural" aglycons were accepted by NovM. The kinetic parameters for **16** ($K_m = 156 \pm 17$ μM, $V_{max} = 3.0 \pm 0.1$ μM min^{-1})[14] were determined to be consistent with values found with other characterized glycosyltransferases of secondary metabolism.[8] NMR analysis of the **16** product was consistent with the 6-deoxy-β-D-glucopyranoside **25** (Scheme 2; d, $J_{1'2'} = 7.2$ Hz).[15] Of the three new **1** analogues produced (Scheme 2), **25** showed the best antibiotic activity (MIC 5 μg mL^{-1}), yet all analogues produced were significantly less effective than **1** (MIC 0.06 μg mL^{-1}).[16] This is consistent with the critical role of the **1** sugar carbamoyl moiety for interaction with DNA gyrase.[2]

The observed NovM substrate specificity was unexpected for a number of reasons. First, the postulated natural NovM

(10) NovM-His$_6$ was grown in BL21 (DE3) cells at 37 °C to an OD$_{600}$ = 0.6, induced with IPTG (1.0 mM) and the temperature decreased to 30 °C for overnight growth (~12−14 h). Harvested cultures (12 L) were lysed in 250 mL of 50 mM sodium phosphate buffer, 300 mM NaCl, 10% glycerol (pH 8.0) using a cell disruptor. Insoluble debris was removed by microcentrifugation (14 000 rpm, 1 h) and NovM-His$_6$ purified by cobalt affinity chromatography according to manufacturer (Qiagen). Fractions containing NovM-His$_6$ (~80% pure) were pooled and dialyzed extensively against 20 mM Tris, 50 mM NaCl (pH 8.0) and then further purified by Mono-Q column chromatography. NovM-His$_6$ eluted at ~250−300 mM NaCl (50 to 1000 mM NaCl linear gradient). Protein was pooled, concentrated to 10 mg mL^{-1}, and chromatographed on a 16/60 Superdex 75 column (Pharmacia) preequilibrated in 20 mM Tris, 200 mM NaCl, 10% glycerol (pH 7.5). Fractions containing NovM-His$_6$ were pooled and concentrated to 10 mg mL^{-1} and stored at −80 °C.

(11) In a typical assay, aglycon (1 mM), NovM-His$_6$ (0.1 U), and NDP-sugar (2 mM) in a total volume of 50 μL of reaction buffer (50 mM Tris/HCl pH 8, 10 mM $MgCl_2$, 1 mg/mL BSA) were incubated at 37 °C for 1 h. The reaction was quenched by addition of an equal volume of chilled MeOH and proteins removed via microcentrifugation (10 min, 14 000 rpm). Product formation was monitored via Varian HPLC by analyzing 20-mL aliquots on a Phenomenex Luna C18 reverse phase column (10 min 70% MeOH/1% formic acid isocratic run followed by 80% MeOH/1% formic acid for 20 min in 1% formic acid, 0.75 mL min^{-1}, 305 nm). Retention times: **10** (21.3 min), **25** (17.9 min), **26** (15.5 min), and **27** (18.7 min).

(12) Kominek, L. A.; Meyer, H. F. *Methods Enzymol.* **1975**, *43*, 502−508.

(13) Zhao, Y.; Thorson, J. S. *J. Org. Chem.* **1998**, *63*, 7568−7572.

(14) A series of 25-μL assays containing 1.8 mM novobiocic acid, 26.3 μM NovM, 1 mg mL^{-1} of BSA, 1.0 mM $MgCl_2$, and varying concentrations of **16** (0.031−4 mM) in 49 mM potassium phosphate buffer, pH 8, were analyzed by HPLC (ref 11). Initial velocities were calculated from the product peak area per unit time (incubation time range up to 1 h). Product concentration was calculated from the integration (μM) using novobiocic acid as standard. The initial velocities (μM min^{-1}) were plotted against corresponding **16** concentrations (μM). At saturating concentrations of novobiocic acid, the rate equation simplifies to $v_o = V_{max}[S]/K_m + [S]$, where [S] is the concentration of TDP-6-deoxy glucose. K_m for **16** was estimated by fitting the plot of initial velocity versus substrate concentration to the above equation using the Kaleidograph.

(15) Novobiocic acid (15 mg, 39 μmol), **16** (16 mg, 27 μmol), and 10 U His-tag NovM in 30 mL of reaction buffer (50 mM Tris/HCl pH 8, 10 mM $MgCl_2$, 1 mg/mL BSA) were incubated for 48 h at 37 °C. The proteins were removed by adding 30 mL of cold MeOH and subsequent centrifugation at 10 000 × *g* for 30 min. The supernatant was concentrated, lyophilized, and resuspended in 1 mL of MeOH and the sample was purified by HPLC, using a semiprep C18 HPLC column (10 × 250 mm, 4 mL min^{-1}, linear gradient of 10% CH_3CN/H_2O to 80% CH_3CN/H_2O over 15 min). The product fractions were pooled and lyophilized (yield 9 mg, 60%). ^{1}H NMR (CD_3OD) 1.29 (d, 3 H, $J = 6.1$ Hz), 1.70 (s, 6 H), 2.33 (s, 3 H), 3.08 (t, 1 H, $J = 9.2$ Hz), 3.31 (d, 2 H, $J = 7.2$ Hz), 3.38 (t, 1 H, $J = 9.2$ Hz), 3.48 (dd, 1 H, $J = 7.6, 9.2$ Hz), 4.95 (d, 1 H, $J = 7.2$ Hz), 5.33 (t, 1 H, $J = 7.2$ Hz), 6.78 (d, 1 H, $J = 8.4$ Hz), 7.00 (d, 1 H, $J = 8.8$ Hz), 7.69 (d, 1 H, $J = 8.4$ Hz), 7.74 (s, 1 H), 7.78 (d, 1 H, $J = 8.8$ Hz); ^{13}C NMR (CD_3OD) 7.6, 16.6, 16.9, 24.7, 28.1, 72.3, 74.0, 75.6, 76.6, 101.1, 110.2, 110.5, 114.0, 114.5, 117.5, 122.4, 123.1, 125.8, 126.8, 127.9, 129.4, 131.8, 152.1, 157.7, 158.4, 165.5, 168.7, 173.0; MS calcd for $C_{28}H_{31}NO_{10}$ 541.19, found *m*/*z* 542.3 (M + H).

substrate in Scheme 1 is 5-*C*-methyl-substituted TDP-6-deoxy-β-L-Man (TDP-β-L-Rha, 20) or TDP-6-deoxy-α-D-Gul (**21**), yet no product was observed with **20** and **23**. Instead, product formation was only observed in the presence of sugar nucleotides resembling **6**. The dogma in deoxysugar biosynthesis supports the notion that most sugar modifications occur at the NDP-sugar level prior to attachment to the aglycon.[5] Interestingly, much of the experimental support for this dogma is in the form of in vivo genetics. For example, disruption of genes postulated to participate in nucleotide sugar modification leads to novel products and has been construed as suggesting that glycosyltransferases in these pathways are capable of accepting the many shunt nucleotide sugar derivatives generated in the disrupted mutant strains.[7b] Our results could also be consistent with a scenario in which the glycosyltransfer event occurs early (e.g. Scheme 1, **6**) after which the sugar modification enzymes complete the synthesis of **1**. Disruption of sugar modifying genes in this scenario would also lead to novel products but is *clearly independent* of the inherent flexibility of NovM, since these modifications would now occur downstream of the NovM reaction. Thus, these results present an intriguing, albeit speculative in the absence of the postulated substrate **9**, alternative to the present dogma in sugar nucleotide biosynthesis as it applies to secondary metabolism. This work also provides a cautionary note for the general applicability of IVG.

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(16) Products were tested for antibacterial activity against *Streptomyces griseoruber* (ATCC). A 10-mL sample of overnight cultures (in YEME broth) was applied to ISP agar plates containing varying amounts of the **1** analogues (0.06 mg/mL to 20 mg/mL). Similar amounts were applied to ISP agar plates containing varying concentrations of **1** or **10**. Control plates consisted of ISP agar only. The plates were incubated at 28 °C and monitored for growth overnight and after 2 days.