

Regiospecific *O*-Methylation of Naphthoic Acids Catalyzed by NcsB1, an *O*-Methyltransferase Involved in the Biosynthesis of the Eneidyne Antitumor Antibiotic Neocarzinostatin*[§]

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Neocarzinostatin, a clinical anticancer drug, is the archetypal member of the chromoprotein family of enediynes antitumor antibiotics that are composed of a nonprotein chromophore and an apoprotein. The neocarzinostatin chromophore consists of a nine-membered enediyne core, a deoxyaminosugar, and a naphthoic acid moiety. We have previously cloned and sequenced the neocarzinostatin biosynthetic gene cluster and proposed that the biosynthesis of the naphthoic acid moiety and its incorporation into the neocarzinostatin chromophore are catalyzed by five enzymes NcsB, NcsB1, NcsB2, NcsB3, and NcsB4. Here we report the biochemical characterization of NcsB1, unveiling that: (i) NcsB1 is an *S*-adenosyl-*L*-methionine-dependent *O*-methyltransferase; (ii) NcsB1 catalyzes regiospecific methylation at the 7-hydroxy group of its native substrate, 2,7-dihydroxy-5-methyl-1-naphthoic acid; (iii) NcsB1 also recognizes other dihydroxynaphthoic acids as substrates and catalyzes regiospecific *O*-methylation; and (iv) the carboxylate and its *ortho*-hydroxy groups of the substrate appear to be crucial for NcsB1 substrate recognition and binding, and *O*-methylation takes place only at the free hydroxy group of these dihydroxynaphthoic acids. These findings establish that NcsB1 catalyzes the third step in the biosynthesis of the naphthoic acid moiety of the neocarzinostatin chromophore and further support the early proposal for the biosynthesis of the naphthoic acid and its incorporation into the neocarzinostatin chromophore with free naphthoic acids serving as intermediates. NcsB1 represents another opportunity that can now be exploited to produce novel neocarzinostatin analogs by engineering neocarzinostatin biosynthesis or applying directed biosynthesis strategies.

Neocarzinostatin (NCS),³ a clinical anticancer drug used to treat leukemia and cancers of the bladder, stomach, pancreas, liver, and brain, is an archetypal member of the chromoprotein family of enediynes antitumor antibiotics that are composed of a nonprotein chromophore and an apoprotein (1–3). NCS was originally isolated from *Streptomyces carzinostaticus* in 1965 (1–3). Its apoprotein NcsA is encoded by the *ncsA* gene and protects, carries, and delivers the reactive NCS chromophore (4). The NCS chromophore (**1**, Fig. 1*B*) is composed of a nine-membered enediyne core, a deoxyaminosugar, and a naphthoic acid moiety (2, 5). As a member of the enediyne family, the biological activity of NCS is derived from its ability to cleave DNA (6). The NCS chromophore undergoes Myers-Saito cycloaromatization to form a 2,6-indacene diradical species that subsequently abstracts hydrogen atoms from the deoxyribose of DNA, leading to single- and double-stranded DNA breaks (2, 7). For NCS, a thiol is often required to trigger the cycloaromatization, although a few enediynes have been reported to form diradicals via the cycloaromatization in a thiol-independent manner, likely the result of the intrinsic reactivity of the nine-membered ring enediyne core (1, 2, 7). The mechanism of action for NCS also involves DNA intercalation by the naphthoic acid moiety that positions the NCS chromophore into the minor groove (8–10). The naphthoate group also plays a key role in the binding of the NCS chromophore to its apoprotein NcsA (10–12). Similarly, there exist numerous examples of other cytotoxic agents whose binding to protein targets are significantly hastened by tethered naphthoate moieties (10–13). Naphthoates also serve as DNA binding affinity units for other secondary metabolites. For instance, the naphthoate groups (Fig. 1*D*) in N1999A2 (**2**), kedarcidin (**3**), azinomycins A (**4**) and B (**5**) play a significant role in DNA intercalation, as well as, non-intercalative DNA binding interactions (14–25).

We have previously cloned and sequenced the NCS biosynthetic gene cluster from *S. carzinostaticus* ATCC 15944 (26). On the basis of genetic analysis we have proposed that the biosynthesis of the naphthoic acid moiety and its incorporation

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1 and Figs. S1–S4.

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³ The abbreviations used are: NCS, neocarzinostatin; APCI-MS, atmospheric pressure chemical ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; LIC, ligation-independent cloning; DMSO, dimethyl sulfoxide; AdoMet, *S*-adenosyl-*L*-methionine.

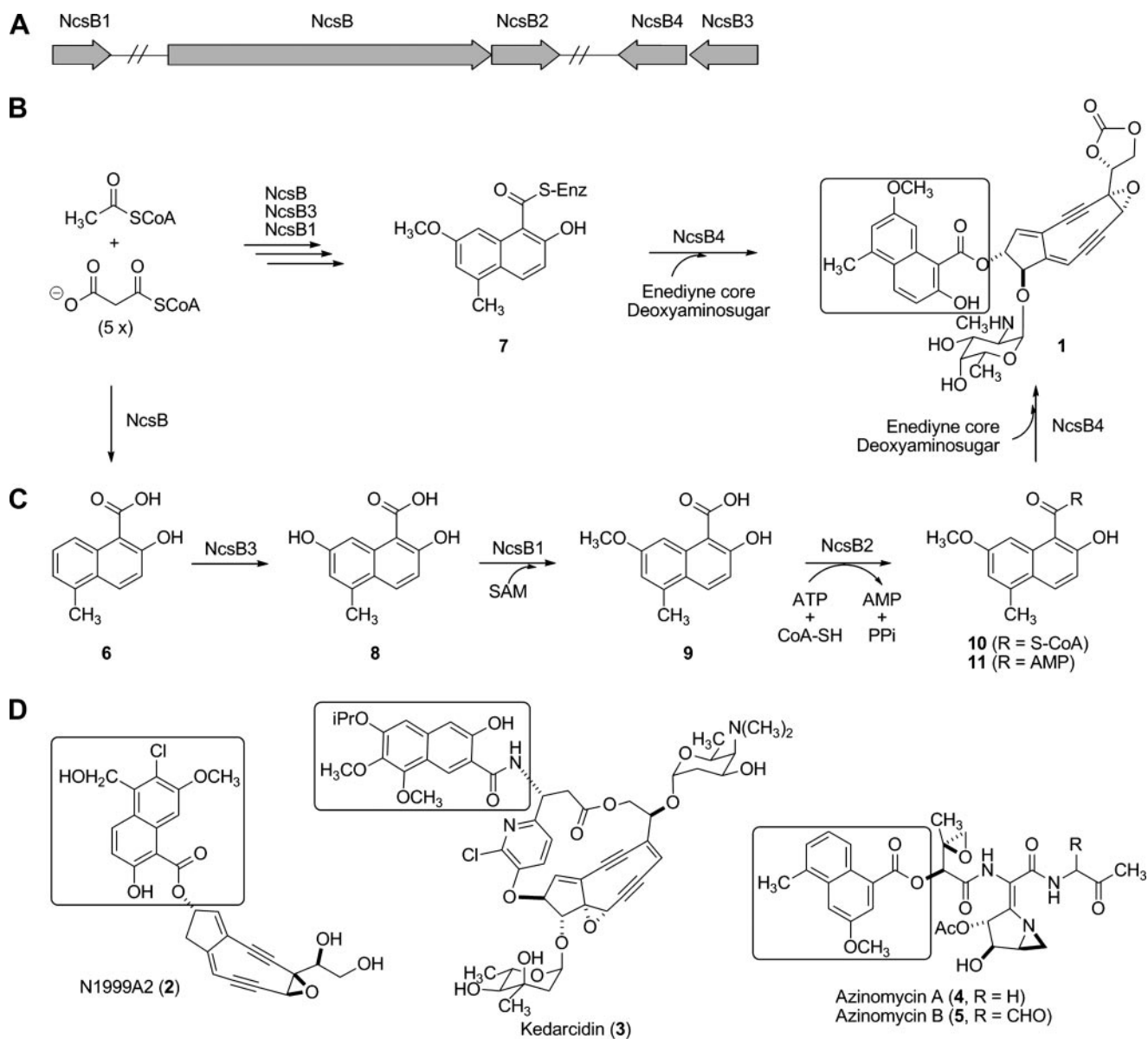


FIGURE 1. **Biosynthetic pathway for the 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid moiety (boxed) of the NCS chromophore (1).** A, subcluster of genes within the NCS biosynthetic cluster encoding enzymes for the biosynthesis of the naphthoic acid moiety, B) early and C) revised proposal for the biosynthesis of **9** and its incorporation into **1**, and D) other antitumor antibiotics containing structurally related naphthoic acid moieties (boxed).

into **1** are catalyzed by five enzymes NcsB, NcsB1, NcsB2, NcsB3, and NcsB4 (Fig. 1A). We predicted that NcsB, a unique iterative type I polyketide synthase, first catalyzes the condensation of acetyl-CoA with five malonyl-CoA units to form the 2-hydroxy-5-methyl-1-naphthoic acid (**6**) (Fig. 1, B and C) (26). Our original proposal featured the direct coupling between a protein-tethered intermediate naphthoyl-S-NcsB (**7**) and an enediyne core intermediate (Fig. 1B) (26) but was later revised to involve free naphthoic acids as intermediates (Fig. 1C) (27). Thus, hydroxylation at the C-7 position of **6** by the NcsB3 cytochrome P-450 hydroxylase as the second step should generate 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**). Subsequent methylation of the C-7 hydroxy group of **8** by NcsB1, a *S*-adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferase, as the third step would afford 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid (**9**). Activation of **9** into the corresponding

acyl-CoA (**10**) by the NcsB2 CoA ligase as the fourth step would set the stage for the incorporation of **10** into **1**. The NcsB4 acyltransferase has been proposed to catalyze this final coupling reaction (Fig. 1C) (27).

The revised pathway (Fig. 1C) is consistent with the finding that expression of *ncsB* in heterologous hosts resulted in the accumulation of **6** (28). We have also demonstrated previously that NcsB2 is an ATP-dependent CoA ligase that catalyzes the activation of **9** into **10** via the acyl-AMP (**11**) as an intermediate *in vitro* (26, 27). Here we now report the *in vitro* biochemical characterization of NcsB1 as a AdoMet-dependent *O*-methyltransferase that catalyzes the regiospecific methylation of the 7-hydroxy moiety of **8** to yield **9** (Fig. 1C). These findings establish that NcsB1 catalyzes the third step in the biosynthesis of the naphthoic acid moiety of the NCS chromophore, further supporting the revised proposal that biosynthesis of the naphthoic

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acid moiety and its incorporation into the NCS chromophore proceeds with free naphthoic acids as intermediates (27). *NcsB1* absolutely requires the *ortho*-hydroxy naphthoic acid scaffold for substrate recognition but displays significant substrate promiscuity by catalyzing regiospecific O-methylation of other available hydroxyl groups. Together with the demonstrated substrate promiscuity of the ensuing *NcsB2* CoA ligase (26, 27), these findings could now be exploited to produce novel NCS analogs by engineering NCS biosynthesis or applying directed biosynthesis strategies.

EXPERIMENTAL PROCEDURES

Materials and Methods—Dithiothreitol and isopropyl 1-thio- β -D-galactopyranoside were purchased from Research Products International Corp. (Mt. Prospect, IL), and AdoMet was purchased from Sigma. Complete protease inhibitor tablet, EDTA-free, was from Roche Applied Science (Indianapolis, IN). Medium components and chemicals were from Fisher Scientific (Fairlawn, NJ).

2-Hydroxy-5-methyl-1-naphthoic acid (**6**), 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**), and 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid (**9**) were synthesized following a convergent procedure by Myers (29) and characterized by spectroscopic methods (27). 2-Hydroxy-1-naphthoic acid (**12**), 3,5-dihydroxy-2-naphthoic acid (**13**), 3,7-dihydroxy-2-naphthoic acid (**14**), and 1,4-dihydroxy-2-naphthoic acid (**15**) were purchased from Sigma. Chemicals from standard commercial sources were used directly without further purification.

E. coli DH5 α was used as the host for general subcloning, and *E. coli* BL21(DE3) was used as the host for protein overproduction (Novagen, Madison, WI). They were grown at 37 °C in Luria-Bertani medium and prepared using standard procedures (30). Synthetic DNA oligonucleotides were purchased from the University of Wisconsin-Madison Biotechnology Center (Madison, WI). PCR was performed with a PerkinElmer GeneAmp 2400 (PerkinElmer Life Sciences, Inc.).

High performance liquid chromatography (HPLC) analyses were carried out on a Varian HPLC system equipped with Prostar 210 pumps, a photodiode array detector, and a Waters SunFire™ C18 reverse-phase column (5 μ m, 4.6 \times 250 mm), using a mobile phase system that consisted of 0.1% trifluoroacetic acid in Milli-Q water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The following gradient was applied: 0–12 min a linear gradient from 30% B to 80% B, 12–15 min a linear gradient from 80% B to 100% B, 15–18 min isocratic at 100% B at a flow rate of 1 ml/min with UV detection at 340 nm.

The electrospray ionization-mass spectroscopy (ESI-MS) or high resolution electrospray ionization-mass spectroscopy (high resolution ESI-MS) was performed with an Agilent 1100 HPLC-MSD SL ion trap mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA). Atmospheric pressure chemical ionization-mass spectroscopy (APCI-MS) was measured with an Agilent 1100 VL APCI Mass Spectrometer. One- and two-dimensional NMR spectral data were recorded on Varian UI-400 or 500 spectrometers (Varian, Inc., Palo Alto, CA). ¹H NMR spectral data were calibrated to the residual solvent at 2.50 ppm for DMSO-*d*₆, 3.33 ppm for CD₃OD, or 7.26 ppm for CDCl₃.

Cloning, Gene Overexpression, and Protein Purification—The *ncsB1* gene was amplified with cosmid pBS5002 (26) as a template and Platinum *pf*x DNA polymerase from Invitrogen, using the forward primer 5'-GGTATTGAGGGTTCGCATGGGAAAAAGGGCTGCACAC-3' and the reverse primer 5'-AGAGGAGAGTTAGAGCCTCAGAGGGCGGTCATTTC-3'. Pure PCR products were cloned into pET-30Xa/LIC vector following the ligation-independent cloning (LIC) strategy and its procedure as described by Novagen (Madison, WI) to give plasmid pBS5039. The fidelity of *ncsB1* in the plasmid pBS5039 was confirmed by DNA sequencing.

Plasmid pBS5039 was transformed into *E. coli* BL21(DE3). The cells were cultured in Luria-Bertani medium supplemented with 50 μ g/ml of kanamycin at 18 °C, 250 rpm (Series 25 Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ). The culture was induced with 0.1 mM isopropyl 1-thio- β -D-galactopyranoside once the A₆₀₀ reached 0.5–0.8, and overexpression was continued at 18 °C, at 250 rpm for an additional 15–18 h. The resulting cells were harvested by centrifugation at 4 °C at 4100 \times g (Sorvall Legent RT 75006441K rotor, Thermo Fisher Scientific Inc., Waltham, MA) for 10 min and the cell pellet stored at –78 °C until protein purification.

Cells were suspended in Buffer A (100 mM sodium phosphate, pH 7.5, containing 300 mM NaCl) supplemented with Roche complete protease inhibitor, EDTA-free. Lysozyme was added to the suspended solution to a final concentration of 1 mg/ml. The cells were lysed by sonication (4 \times 30-s pulsed cycle), and the resulting solution was centrifuged at 4 °C, 15,000 \times g (Beckman JA-25.50 rotor, Beckman Coulter, Inc., Fullerton, CA) for 50 min to remove the debris. The supernatant was filtered with a 0.8- μ m MF-Millipore MCE membrane (Corrigtwohill, Co., Cork, Ireland), and the filtrate was loaded onto a pre-equilibrated nickel-nitrilotriacetic acid-agarose (Qiagen) column with chilled Buffer B (Buffer A plus 10% glycerol and 1% Triton X-100). After washing with 5 column volumes of cold Buffer C (Buffer B with 20 mM imidazole additive), followed by 5 column volumes of Buffer D (Buffer C without Triton X-100), the expected His₆-tagged *NcsB1* protein was eluted with 6 column volumes of Buffer E (Buffer A containing 250 mM imidazole and 10% glycerol). The homogeneous protein solutions were dialyzed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM dithiothreitol. The resulting His₆-tagged *NcsB1* protein solution was concentrated by using an Amicon Ultra-4 concentrator (10 K, GE Healthcare) and stored at –25 °C as 40% glycerol stocks. The purity of the purified *NcsB1* was examined by 12% SDS-PAGE analysis. Protein concentration was determined from the absorbance at 280 nm (ϵ = 3.30 \times 10⁴ M⁻¹ cm⁻¹).

Characterization of *NcsB1*-catalyzed O-Methylation Activity *in Vitro*—For *NcsB1* activity assay, each 100 μ l of reaction mixture contained 100 μ M *NcsB1*, 5.0 mM **8**, 2.5 mM AdoMet in 50 mM sodium phosphate (pH 6.0). As a negative control, *NcsB1* was boiled at 100 °C for 10 min. Reactions were initiated by the addition of *NcsB1* and then incubated at 25 °C for 1 h. To terminate the reaction, trifluoroacetic acid was added to a final concentration of 16% (v/v) to precipitate the enzyme. All precipitates were removed by centrifugation at 14,000 \times g (Eppendorf Centrifuge 5415c, Brinkmann Instruments, Inc., West-

bury, NY) for 10 min. The resulting supernatant was subjected to HPLC analysis as described above.

The NcsB1-catalyzed enzymatic reaction of **8** was scaled up, and the product **9** was purified by HPLC. After removal of the solvent under reduced pressure, the residue was lyophilized overnight and subjected to ^1H NMR and MS analyses. ^1H NMR (CDCl_3 , 500 MHz) assignments for **9** are δ 12.21 (1H, brs, C2-OH), 8.18 (1H, brs, C8-H), 8.08 (1H, d, $J = 9.0$ Hz, C4-H), 7.04 (1H, d, $J = 9.0$ Hz, C3-H), 6.91 (1H, brs, C6-H), 3.92 (3H, s, C7-OCH₃), and 2.64 (3H, s, C5-CH₃), and APCI-MS (negative mode) for **9** yielded m/z (relative intensity) at 231 ($[\text{M}-\text{H}]^-$, 100), 187 ($[\text{M}-\text{H}-\text{CO}_2]^-$, 70).

Optimization of NcsB1-catalyzed O-Methylation in Vitro—To optimize the O-methylation catalyzed by NcsB1, we first determined the optimal pH for NcsB1 activity. The 100 μl of reaction mixture contained 20 μM NcsB1, 100 μM **8**, 300 μM AdoMet in 50 mM sodium acetate (pH 4.5–5.5), or 50 mM sodium phosphate (pH 5.5–8.5), with varying pH values. All reactions were carried out in duplicate, and each assay was initiated by the addition of NcsB1 and then incubated at 25 °C for 30 min. The workups and the HPLC analyses of the reaction mixture were identical to those noted above.

A time course for the NcsB1-catalyzed O-methylation of **8** was carried out to determine the initial rate conditions. The 50 μl of reaction mixture contained 5.0 or 50 μM NcsB1, 1 mM **8**, 1.5 mM AdoMet in 50 mM sodium phosphate (pH 6.0), and all assays were carried out in duplicate. The reactions were initiated by the addition of NcsB1, incubated at 25 °C, and then terminated at 2, 4, 8, 15, 30, 60, 120, and 300 min (for assays using 5 μM NcsB1) or 5, 15, 35, 60, 120, 180, 240, and 300 min (for assays using 50 μM NcsB1), respectively. The assays were subjected to the same workups and HPLC analyses as described above. Formation of **9** was fitted into a linear equation to obtain the initial velocity, maintaining initial rate conditions by calculating the rate at less than 10% substrate conversion. To investigate the effect of divalent cations on NcsB1 activity, 5 mM of different metal salts with Cl^- as the anion were added, and the assays were carried out under identical conditions as described above to generate initial velocity values.

Quantification of AdoMet Bound to NcsB1—The 50 μl of reaction mixture contained 5 mM **8** and 50 or 100 μM NcsB1 in 50 mM sodium phosphate (pH 6.0), respectively. Reactions were initiated by the addition of NcsB1, incubated at 25 °C, and terminated by the addition of trifluoroacetic acid to a final concentration of 16% (v/v) at 2, 5, or 8 h. The reactions were subjected to the same workups and HPLC analysis as described above to determine the amount of **9** formed.

Determination of the Kinetic Parameters for the NcsB1-catalyzed O-Methylation of 8 and AdoMet—For the determination of the kinetic parameters for methylation of **8**, 50- μl reaction mixtures contained 10 μM NcsB1, 2.5 mM AdoMet, and **8** varying from 25 μM to 3.2 mM in 50 mM sodium phosphate (pH 6.0). For the determination of the kinetic parameters of AdoMet, 50- μl reaction mixtures contained 10 μM NcsB1, 2.5 mM **8**, and AdoMet varying from 25 μM to 3.2 mM in 50 mM sodium phosphate (pH 6.0). All assays were carried out in duplicate. The reactions were initiated by the addition of NcsB1, incubated at 25 °C for 15 min, and terminated by the addition of trifluoro-

acetic acid to a final concentration of 16% (v/v). HPLC analyses of **9** formation were carried out as noted above. The resulting initial velocities were then fitted to the Michaelis-Menten equation by nonlinear regression analysis using Origin software (OriginLab, Northampton, MA) to extract K_m and k_{cat} parameters.

Substrate Specificity of NcsB1—Under the identical reaction conditions determined for NcsB1-catalyzed O-methylation of **8**, a series of hydroxylated naphthoic acids were evaluated as **8** analogs to serve as potential substrates for NcsB1. These included 1-naphthoic acid analogs **6**, **9**, and **12**, and 2-naphthoic acid analogs **13**, **14**, and **15**. The assays were subjected to the same workups and HPLC analyses as those described for **8**.

Enzymatic Preparation and Characterization of 3-Hydroxy-5-methoxy-2-naphthoic Acid (16), 3-Hydroxy-7-methoxy-2-naphthoic Acid (17), and 1-Hydroxy-4-methoxy-2-naphthoic Acid (18)—For enzymatic synthesis of **16**, **17**, or **18**, 30 ml of the reaction mixture contained 300 μM NcsB1, 2 mM AdoMet, and 10 mM **13**, **14**, or **15**, respectively, in 100 mM sodium phosphate (pH 6.0). For **18**, 10 mM dithiothreitol was also included in the reaction solution to avoid adventitious oxidation of **15** by air. The reactions were incubated at 25 °C for 22 h with occasional shaking and similarly terminated by the addition of trifluoroacetic acid. The resulting mixture was centrifuged at $4,100 \times g$ at 4 °C for 10 min. The precipitate was extracted with 3 ml of acetonitrile twice. The supernatant was loaded onto a C₁₈ Sep-Pak cartridge (Waters, Milford, MA), washed with 5-column volumes of water, and then eluted with 3-column volumes of acetonitrile. The acetonitrile eluent from the C₁₈ Sep-Pak cartridge and the acetonitrile extract from the precipitate were combined and concentrated *in vacuo*. The crude products were then subjected to HPLC purification to afford pure products of **16**, **17**, and **18**. The HPLC-purified products were concentrated under reduced pressure to give a residue, lyophilized overnight to remove the remaining solvents, and subjected to MS and ^1H NMR and NOESY analyses.

3-Hydroxy-5-methoxy-2-naphthoic Acid (16)—APCI-MS (negative mode) yielded m/z (relative intensity) at 217 ($[\text{M}-\text{H}]^-$, 100). ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) assignments are δ 8.48 (1H, s, C1-H), 7.53 (1H, d, $J = 8.4$ Hz, C8-H), 7.46 (1H, s, C4-H), 7.27 (1H, t, $J = 8.4$ Hz, C7-H), 6.99 (1H, d, $J = 8.4$ Hz, C6-H), and 3.95 (3H, s, C5-OCH₃). NOESY ($\text{DMSO}-d_6$, 400 MHz) correlations are between Hs at δ 8.48/7.53, 7.53/7.27, 7.27/6.99, 6.99/3.95, and 3.95/7.46 (see Fig. S1A in supplemental data).

3-Hydroxy-7-methoxy-2-naphthoic Acid (17)—APCI-MS (negative mode) yielded m/z (relative intensity) at 217 ($[\text{M}-\text{H}]^-$, 100). ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) assignments are δ 8.43 (1H, s, C1-H), 7.69 (1H, d, $J = 9.0$ Hz, C5-H), 7.40 (1H, d, $J = 2.5$ Hz, C8-H), 7.28 (1H, s, C4-H), 7.22 (1H, dd, $J = 9.0, 2.5$ Hz, C6-H), and 3.84 (3H, s, C7-OCH₃). NOESY ($\text{DMSO}-d_6$, 500 MHz) correlations are between Hs at δ 8.43/7.40, 7.40/3.84, 3.84/7.22, 7.22/7.69, and 7.69/7.28 (see Fig. S1B in supplemental data).

1-Hydroxy-4-methoxy-2-naphthoic Acid (18)—APCI-MS (negative mode) yielded m/z (relative intensity) at 217 ($[\text{M}-\text{H}]^-$, 100). ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) assignments are δ 8.28 (1H, d, $J = 8.0$ Hz, C8-H), 8.14 (1H, d, $J = 8.0$ Hz, C5-H), 7.71 (1H, t, $J = 8.0$ Hz, C6-H), 7.64 (1H, t, $J = 8.0$ Hz,

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C7-H), 7.08 (1H, s, C3-H), and 3.93 (3H, s, C4-OCH₃). NOESY (DMSO-*d*₆, 400 MHz) correlations are between Hs at δ 8.28/7.64, 8.14/7.71, 7.71/7.64, 7.08/3.93, and 3.93/8.14 (see Fig. S1C in supplemental data). The NMR spectral data for **18** are consistent with previous reports (31).

Determination of the Kinetic Parameters for NcsB1-catalyzed O-Methylation of 13, 14, and 15—To determine the kinetic parameters of **13**, **14**, or **15**, respectively, the initial velocities were determined according to the procedures described above for **8**. Product (*i.e.* **16**, **17**, or **18**) formation was fitted into a linear equation to obtain the initial velocity. The 50- μ l reaction mixtures contained varying concentrations of **13** (from 50 μ M to 1.6 mM), **14** (from 50 μ M to 0.8 mM), or **15** (from 50 μ M to 1.6 mM), 2.5 mM AdoMet, and 50 (for **13**), 50 (for **14**), or 100 μ M (for **15**) NcsB1, respectively, in 50 mM sodium phosphate (pH 6.0). All assays were carried out in duplicate. The reactions were initiated by the addition of NcsB1, incubated at 25 °C for 30 (for **13**), 50 (for **14**), and 50 (for **15**) min, respectively, and terminated by the addition of trifluoroacetic acid to a final concentration of 16% (v/v). HPLC analyses for the formation of **16**, **17**, or **18** were carried out under the same conditions as described for **9** except that the UV detection wavelengths of 376 (for **16**), 388 (for **17**), and 366 nm (for **18**) were used, respectively. The resulting initial velocities for the formation of **16**, **17**, and **18** were fitted to the Michaelis-Menten equation by nonlinear regression analysis using Origin software (OriginLab, Northampton, MA) to extract the K_m and k_{cat} parameters.

RESULTS

Cloning, Gene Overexpression, and Purification of NcsB1—The *ncsB1* gene was PCR amplified from pBS5002 (26) and directly cloned into the pET-30 Xa/LIC vector to afford pBS5039 that was transformed into *E. coli* BL21(DE3) according to standard protocols for overexpression (30). After standard inoculation, growth, and induction by isopropyl 1-thio- β -D-galactopyranoside, NcsB1 was overproduced as an N-terminal His₆-tagged fusion protein and purified to homogeneity by nickel-nitrilotriacetic acid-agarose affinity chromatography. The purified NcsB1 protein was examined on 12% SDS-PAGE, migrating as a single band with a molecular mass that is consistent with the predicted size of 39.5 kDa (Fig. 2A).

Substrate and Product Preparation and O-Methyltransferase Assay of NcsB1 in Vitro—The proposed substrate **8** and product **9** for NcsB1 were synthesized according to literature methods (27). Incubation of **8** and AdoMet, or **8**, boiled NcsB1, and AdoMet in 50 mM sodium phosphate (pH 6.0), followed by standard workup procedures and HPLC analysis showed only unmodified **8** (Fig. 3B, panel I). Alternatively, incubation of **8**, AdoMet, and NcsB1 led to production of a new product displaying the same retention time and UV-visible spectrum as that of synthetic authentic **9** (Fig. 3B, panels II and III). This enzymatic reaction was scaled up, and the resultant product was purified by HPLC. MS and ¹H NMR data of this product are consistent with those reported for **9** (27, 29, 32), establishing that NcsB1 catalyzed regioselective methylation of the 7-OH group of **8** to afford **9** (Fig. 3A).

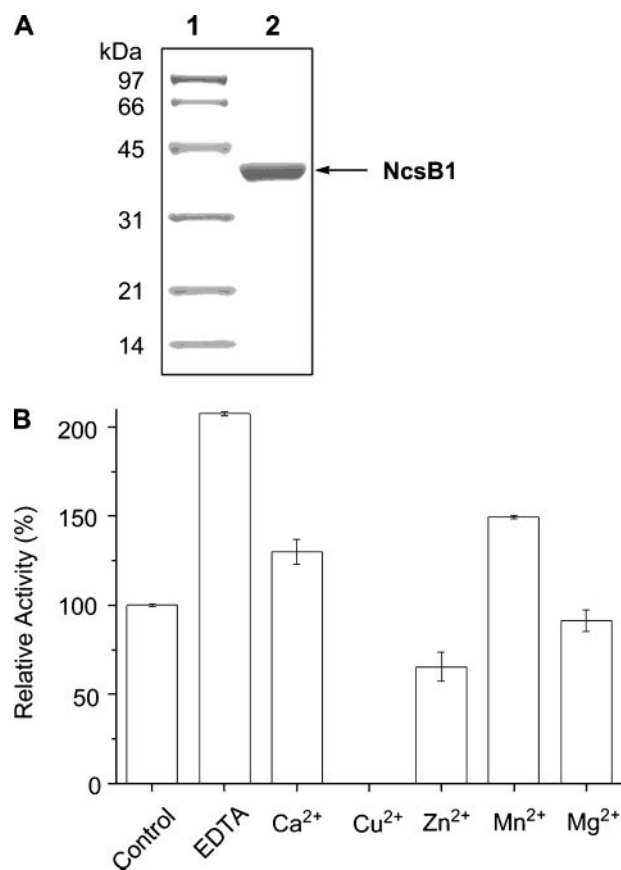


FIGURE 2. NcsB1 and optimization of its O-methyltransferase activity in vitro. A, NcsB1 purified from *E. coli* BL21(DE3)/pBS5039 used in this study as judged by analysis of a 12% SDS-PAGE (lane 1, low range molecular weight protein standards; lane 2, purified His₆-tagged NcsB1 with the predicted molecular mass of 39.5 kDa); and B, effect of 5 mM divalent metals on NcsB1-catalyzed O-methylation of 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) *in vitro*.

Optimization of NcsB1-catalyzed O-Methylation in Vitro—To determine the conditions for optimal activity, the pH dependence of NcsB1 was first investigated with **8** as a substrate in 50 mM sodium acetate (pH 4.5–5.5) or sodium phosphate (pH 5.5–8.5). No formation of **9** was detected in assays at pH \leq 5.5, and NcsB1 was found to precipitate completely at low pH. In contrast, formation of **9** was detected in all assays with pH \geq 6.0, displaying an optimal activity at pH 6.0 (Fig. S2 supplemental data).

Methyltransferases requiring divalent cations for optimal activity are known (33–38), and we next evaluated the activity of NcsB1 in the presence of different metal ions. Notably, the presence of EDTA resulted in a 2-fold higher activity; inclusion of Mn²⁺ and Ca²⁺ in the reactions resulted in a slightly higher activity, whereas inclusion of Mg²⁺ afforded a slight decrease in enzyme activity. Inclusion of Zn²⁺ in the reactions resulted in a 2-fold decrease of enzyme activity, and the presence of Cu²⁺ completely abolished the activity (Fig. 2B). Similar patterns of metal-dependent enzyme activities have been reported for the MmcR mitomycin 7-O-methyltransferase (39) and the Rif-Orf14 rifamycin 27-O-methyltransferase (40). Thus, all subsequent enzyme assays were performed in 50 (for analytical assays) or 100 mM (for preparative reactions) sodium phosphate (pH 6.0) without exogenous metal ions.

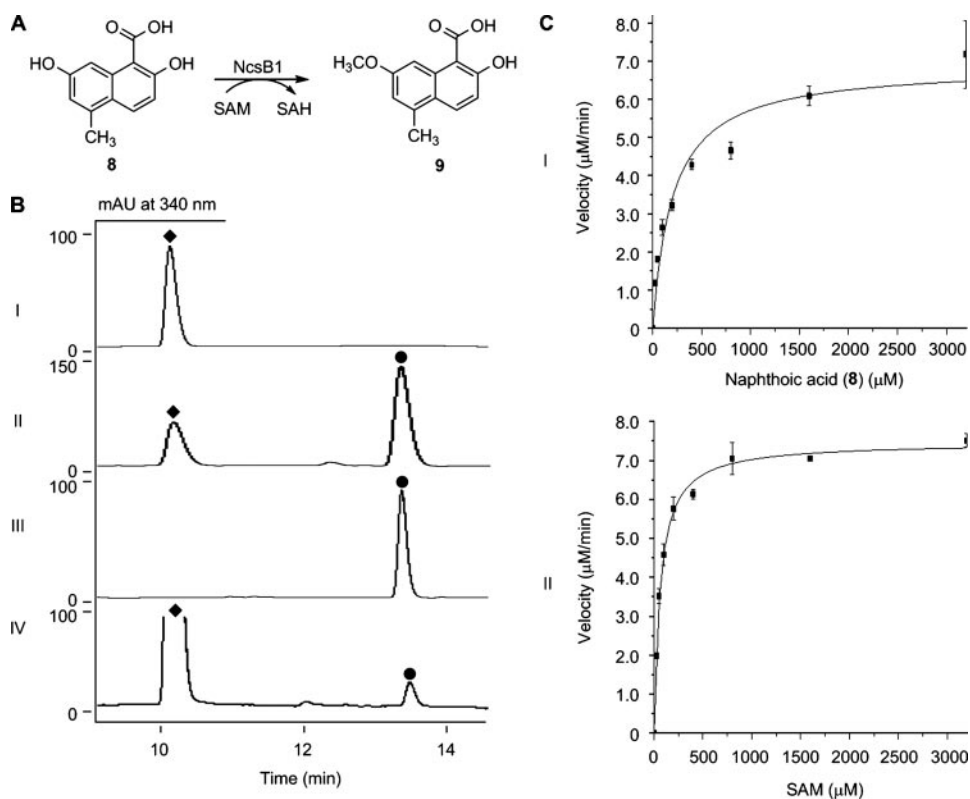


FIGURE 3. **Characterization of NcsB1 as an O-methyltransferase *in vitro*.** A, NcsB1-catalyzed regiospecific methylation of 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) to afford 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid (**9**); B, HPLC analyses of (I) the incubation of **8** with AdoMet alone or **8** and AdoMet with boiled NcsB1 at 25°C for 1 h; (II) incubation of **8** with NcsB1 and AdoMet at 25°C for 1 h; (III) authentic standard of product **9**; and (IV) incubation of **8** and NcsB1 without AdoMet at 25°C for 2 h (◆ **8** and ● **9**); C, kinetic characterization of NcsB1 by plotting the initial velocities of **9** formation as a function of **8** (I) at a saturating concentration of AdoMet or AdoMet (II) at a saturating concentration of **8**.

TABLE 1
Michaelis-Menten constants for NcsB1 as an AdoMet-dependent O-methyltransferase towards selected hydroxynaphthoic acids

Hydroxynaphthoic acids	Methylated products	K_m μM	k_{cat} min^{-1}	Relative k_{cat}/K_m
8	9	206 ± 49	0.69 ± 0.05	1
13	16	352 ± 71	0.27 ± 0.02	0.23
14	17	66 ± 12	0.030 ± 0.002	0.14
15	18	24 ± 11	0.0080 ± 0.0005	0.10

NcsB1 is predicted to be a AdoMet-dependent O-methyltransferase on the basis of bioinformatics analysis (26). This was confirmed by *in vitro* assay of NcsB1 in the absence of exogenous AdoMet (panel IV, Fig. 3B). The amount of AdoMet endogenously bound to the purified NcsB1 was then determined on the basis of **9** formed from **8** without exogenous AdoMet. It was found that the NcsB1-bound AdoMet was consumed exhaustively within 2 h and that 1 mol of NcsB1 bound ~ 0.8 mol of AdoMet (Fig. S3 and Table S1 in supplemental data).

Kinetic Analysis for NcsB1-catalyzed O-Methylation of Native Substrates 2,7-Dihydroxy-5-methyl-1-naphthoic Acid (8**) and AdoMet**—A time course of NcsB1-catalyzed O-methylation of 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) displayed an increase of the methylated product **9** with the concomitant decrease of **8**, and, under these conditions, product formation was linear with respect to time until ~ 15 min (Fig. S4

in supplemental data). The kinetic parameters for the NcsB1-catalyzed O-methylation of **8** were first determined with saturating AdoMet (2.5 mM). The conversion of **8** to **9** followed Michaelis-Menten kinetics with a K_m value of $206 \pm 49 \mu\text{M}$ for **8** and a k_{cat} value of $0.69 \pm 0.05 \text{ min}^{-1}$ (Fig. 3C, panel I). These experiments were repeated with saturating **8** (2.5 mM), and the NcsB1-catalyzed O-methylation of **8** displayed similar Michaelis-Menten kinetics with a K_m value of $62 \pm 4.5 \mu\text{M}$ for AdoMet and a k_{cat} value of $0.75 \pm 0.01 \text{ min}^{-1}$ (Fig. 3C, panel II). The k_{cat} value determined with saturating AdoMet is in good agreement with that obtained with saturating **8** (Table 1).

Substrate Specificity of NcsB1—To test substrate specificity of NcsB1, a series of hydroxylated naphthoic acid analogs, **6**, **9**, **12**, **13**, **14**, and **15**, were tested as potential substrates with **8** as a positive control (Fig. 4, A and B). Together with the native substrate **8**, naphthoic acids **13**, **14**, and **15** can also serve as substrates for NcsB1 (Fig. 4B). Enzymatic reactions involving **13**, **14**, and **15** were scaled up, and the

resulting products were isolated and characterized on the basis of APCI-MS, ^1H NMR, and NOESY analyses. The products obtained (**16**, **17**, and **18**) display masses that are 14 Da greater than their corresponding substrates, consistent with O-methylation of the corresponding naphthoic acids **13**, **14**, and **15**. ^1H NMR spectra of **16**, **17**, and **18** displayed substitution patterns identical to their parent materials but bearing additional methoxy groups with δ 3.95 (for **16**), 3.84 (for **17**), and 3.93 (for **18**) ppm, respectively. All ^1H NMR data are consistent with the assigned substitution patterns that are further supported by NOESY spectra. NOESY analysis of **16** revealed key correlations between δ 6.99 (C6-H) and 3.95, 3.95 and 7.46 (C4-H) consistent with NcsB1-catalyzed methylation of the 5-OH of **13**. Regiospecific methylations at the 7-hydroxy group of **14** and the 4-hydroxy group of **15** were similarly established on the basis of key correlations between δ 7.40 (C8-H) and 3.84, 3.84 and 7.22 (C6-H) in the NOESY spectra of **17** and that between δ 7.08 (C3-H) and 3.93, 3.93, and 8.14 (C5-H) in the NOESY spectra of **18**, respectively. The kinetic parameters of **13**, **14**, and **15** were next determined to compare with that of the native substrate **8** (Fig. 4D). Although NcsB1 exhibited a slightly higher K_m value for **13**, significantly smaller K_m values are observed for **14** and **15**. Conversely, NcsB1 turned over these analogs with slightly (for **13**) or significantly slower (for **14** and **15**) k_{cat} values than that of **8** (Table 1).

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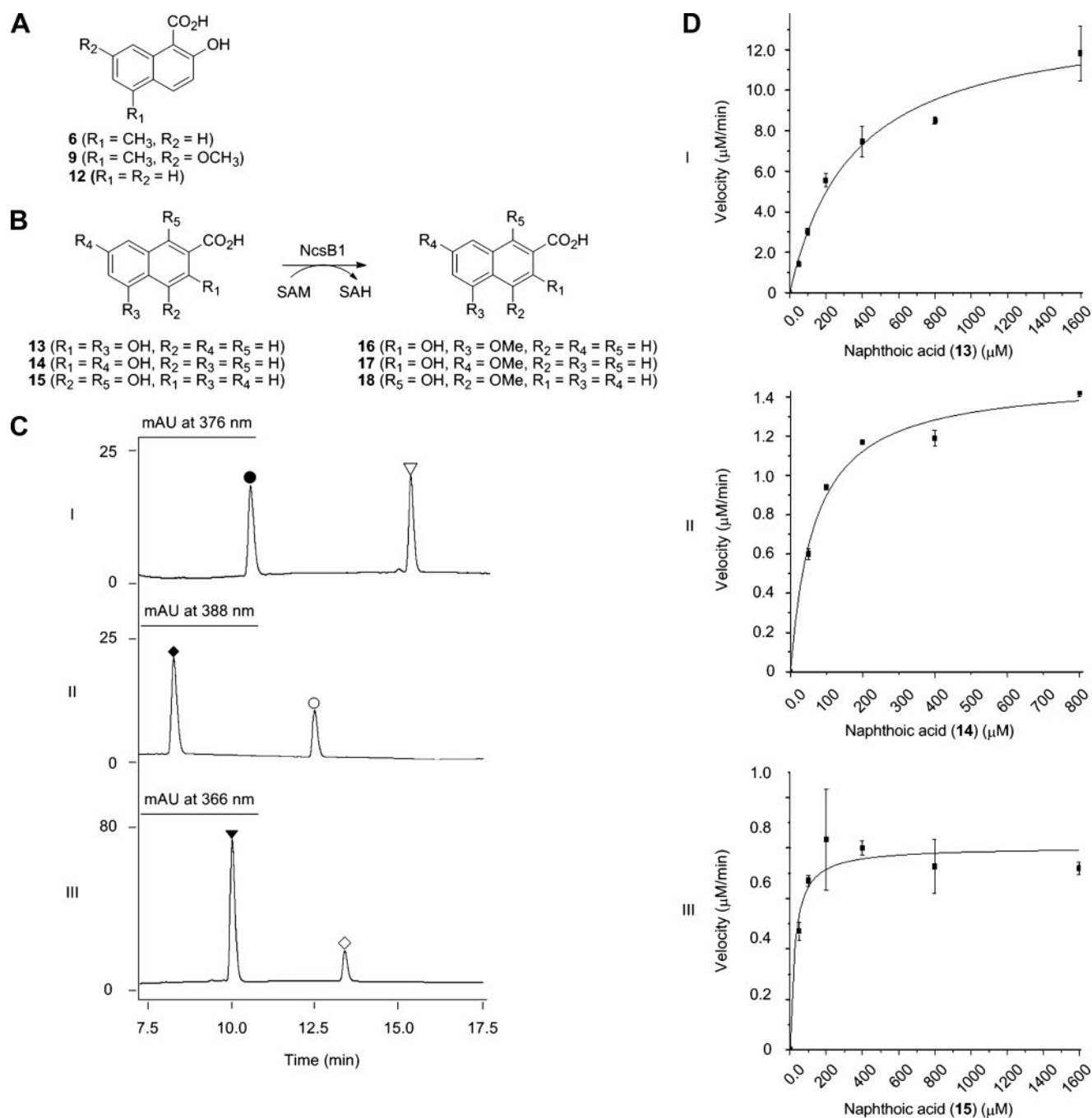


FIGURE 4. Substrate specificity of NcsB1-catalyzed regiospecific O-methylation of other hydroxylated naphthoic acids. *A*, hydroxynaphthoic acids (**6**, **9**, and **12**) that failed to serve as a substrate for NcsB1; *B*, hydroxynaphthoic acids (**13**, **14**, and **15**) that can serve as substrates for NcsB1 and their corresponding methyl ether products (**16**, **17**, and **18**); *C*, HPLC analyses of the incubation of NcsB1 and AdoMet at 25 °C for 1 h with (I) **13** (●) to afford **16** (▽), (II) **14** (◆) to afford **17** (○), and **15** (▼) to afford **18** (◇); and *D*, kinetic characterization of NcsB1 by plotting the initial velocities of formation of (I) **16** as a function of **13**, (II) **17** as a function of **14**, and (III) **18** as a function of **15** at saturating concentration of AdoMet.

DISCUSSION

The enediyne antitumor antibiotics are among the most cytotoxic natural products ever described. Each reactive enediyne core responsible for the bioactivity is decorated with a variety of chemical moieties that alter the properties, including cytotoxicity, of a given enediyne antibiotic (2). NCS is currently used clinically to treat leukemia and various other cancers by inhibiting tumor cell growth at nanomolar concentrations (1–3, 41). However, the clinical use of NCS is restricted by its

instability and substantial toxicity. As such, there is a clear need for more stable NCS congeners with improved chemotherapeutic properties that retain similar biological activity; this is a significant challenge that could be potentially addressed through engineering NCS biosynthesis.

Several strategies could be envisaged to enhance the clinical viability of NCS, such as to increase its lipophilicity or stability or to introduce tumor cell targeting motifs (42). Indeed, conjugation of NCS with poly(styrene-co-maleic acid), which has

afforded SMANCS (styrene maleic acid neocarzinostatin) that is both more lipophilic and stable than the natural product alone, has been very effective to improve the clinical efficacy of NCS. However, functional group tuning on the NCS chromophore has yet to be exploited to any large extent (43).

The NCS chromophore is composed of a nine-membered enediyne core, a deoxyaminosugar, and a naphthoic acid moiety. All three components of NCS play a role in its biological activity (2, 5). The naphthoic acid component of the NCS chromophore plays two important roles in the action of NCS bioactivity: (i) it aids in binding the NCS chromophore to its apoprotein NcsA, which protects, carries, and delivers the drug to its DNA target; (ii) it intercalates into DNA, hence positioning the NCS chromophore into the minor groove. It is reasonable to speculate that alteration of the naphthoic acid moiety of NCS may allow attenuation of DNA binding effects in a way that reduces dose limiting toxicity or that such modifications may enhance NCS lipophilicity and stability.

We have previously cloned the NCS biosynthetic gene cluster and identified a subcluster of five genes encoding enzymes responsible for the biosynthesis and incorporation of the naphthoic acid moiety into the NCS chromophore (Fig. 1A) (26). We subsequently showed that NcsB2 serves as a gatekeeper responsible for selection and activation of the naphthoic acid **9** prior to its incorporation into NCS. NcsB2 is a CoA ligase with promiscuous substrate specificity, which is particularly exciting because it presents an opportunity to potentially produce novel analogs of **1** by engineering NCS biosynthesis (27). Inspired by the substrate promiscuity of NcsB2, we set out to investigate if other enzymes involved in naphthoic acid biosynthesis, such as the NcsB1 methyltransferase, might also display relaxed substrate specificity (Fig. 1C).

A panel of selected analogs of 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**), the native substrate of NcsB1, were prepared to assess the potential for substrate flexibility of NcsB1. NcsB1 efficiently catalyzed the regiospecific *O*-methylation of **8** to afford the methyl ether **9** (Fig. 3A); three of the six naphthoic acid analogs (**13**, **14**, and **15**) were also turned over by NcsB1 to the corresponding methyl ether products (**16**, **17**, and **18**) (Fig. 4, A and B). These findings suggest an absolute requirement of the naphthoic acid scaffold as a substrate by NcsB1. Complete structural characterization of the methylated products (**16**, **17**, and **18**) from **13**, **14**, and **15**, respectively, further revealed that the *ortho*-hydroxy moiety of the naphthoic acids might be necessary for NcsB1 substrate recognition but are, themselves, not enzymatically modified (Fig. 4B). This is supported by the fact that hydroxy groups *ortho* to the carboxylates of the substrate analogs tested are recalcitrant to NcsB1 modification (Fig. 4A). On the other hand, the hydroxy groups not *ortho* to the corresponding carboxylate group do not appear necessary for NcsB1 binding but rather, can serve as sites for regiospecific *O*-methylation as exemplified by the hydroxy groups at C-5 (for **13**), C-7 (for **14**), C-4 (for **15**), and C-7 (for **8**) positions, respectively (Fig. 4B). The *ortho*-hydroxynaphthoic acid scaffold therefore provides the key substrate recognition needed for NcsB1, as evidenced from the comparable or even favored K_m values for the analogs (**13**, **14**, and **15**), but these analogs suffered a significant decrease in k_{cat} ranging from 2.5- to 86-fold reduction

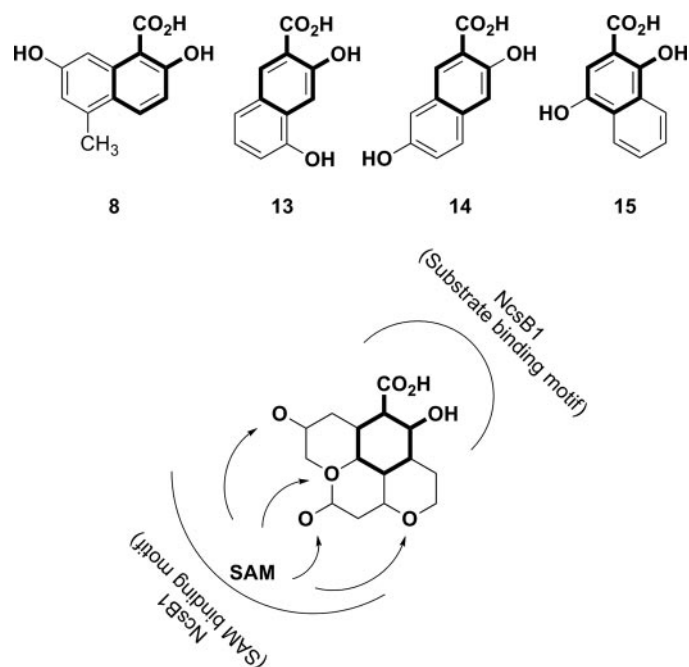


FIGURE 5. Superimposition of hydroxynaphthoic acids, **8**, **13**, **14**, and **15**, depicting the *ortho*-hydroxynaphthoic acid moiety, the hydroxyl groups competent for methylation, and their relative positions to the substrate and AdoMet binding motifs of NcsB1 (shown in bold).

(Table 1). Overall, NcsB1 catalysis was 4–10-fold more efficient with **8** than any substrate analogs tested, further supporting **8** as the native substrate for NcsB1, hence the timing of the NcsB1-catalyzed *O*-methylation of **8** in the biosynthesis of the naphthoic acid and its incorporation into **1** (Fig. 1C). Given the role of naphthoates as DNA intercalating components, our findings that bicyclic aromatic moieties bearing the *ortho*-hydroxynaphthoic acid scaffold are effective substrates for NcsB1 could potentially be exploited to produce novel NCS analogs with improved therapeutic properties by engineering NCS biosynthesis or by applying a directed biosynthesis strategy.

The fact that NcsB1 absolutely requires *ortho*-hydroxynaphthoic acids as substrates but exhibits great tolerance in the relative position of the methylated hydroxy groups is intriguing. One could speculate that specific interaction between NcsB1 and the substrates, as anchored by the *ortho*-hydroxynaphthoic acid motif, would project the hydroxy groups into a wide range of space depending on their relative positions at the naphthoic acid scaffold. NcsB1 therefore must be very flexible to accommodate the interactions between the tightly bound AdoMet and substrates within the active site pocket, hence methylating hydroxy groups at all positions (Fig. 5).

Although AdoMet is the natural substrate for many enzymatic methylations, a number of methyltransferases have been shown to be capable of transferring non-natural alkyl, alkenyl, or alkynyl group through the substitution of AdoMet with the corresponding *S*-alkyl, *S*-akenyl, and *S*-alkynyl analogs (44, 45). Preliminary investigation with *S*-ethyl and *S*-*n*-propyl analogs of AdoMet indeed demonstrated that NcsB1 can turnover 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) into the corresponding 7-ethyl ether and 7-*n*-propyl ether, respectively, the identities of which have been confirmed by high resolution ESI-MS.

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Although these products were produced with significantly reduced efficiency relative to **9**, the fact that NcsB1 is promiscuous toward AdoMet analogs presents yet another opportunity that potentially could be exploited to generate new NCS analogs with improved therapeutic properties (46, 47).

In conclusion, NcsB1 is a flexible AdoMet-dependent O-methyltransferase involved in the biosynthesis of the NCS chromophore. NcsB1 regioselectively methylates 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) to produce 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid (**9**) but is also capable of regioselectively alkylating the hydroxy moiety of a variety of *ortho*-hydroxynaphthoic acids. These alternative substrates retain a hydroxy moiety *ortho* to a carboxylate, as is seen for the native substrate **8**, but possess another free hydroxy group that serves then as the site of modification by NcsB1. NcsB1 is also capable of using *S*-alkyl analogs of AdoMet to produce the corresponding alkyl ethers. These findings unambiguously establish NcsB1 as the enzyme that catalyzes the third step in the biosynthesis of the naphthoic acid moiety of the NCS chromophore and further support the revised proposal for the biosynthesis of the naphthoic acid and its incorporation into the NCS chromophore to proceed with free naphthoic acids as intermediates (27). NcsB1 represents another opportunity that could be exploited to produce novel NCS analogs by engineering NCS biosynthesis or by applying directed biosynthesis strategies.

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