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

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Running title: The NcsB1 *O*-Methyltransferase in Neocarzinostatin Biosynthesis

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SUPPLEMENTAL DATA

Fig. S1. Graphic explanation of the nOe from the NOESY experiments for (A) 3-hydroxy-5-methoxy-2-naphthoic acid (**16**), (B) 3-hydroxy-7-methoxy-2-naphthoic acid (**17**), and (C) 1-hydroxy-3-methoxy-2-naphthoic acid (**18**), respectively. The curves between two protons depict nOe observed in the NOESY experiments. Key nOes correlations that unambiguously establish the regiospecific *O*-methylation are highlighted in red.

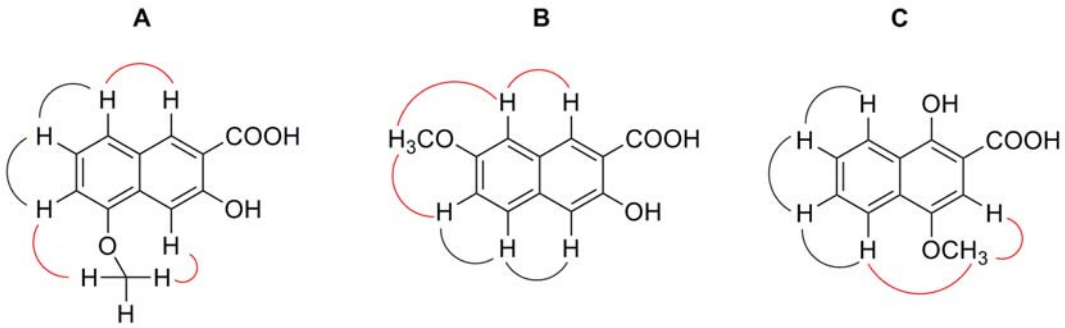

Fig. S2. Effect of pH on the relative activity of NcsB1 as an *O*-methyltransferase as determined with 2,7-dihydroxy-5-methyl-1-naphthoic acid (**8**) as a substrate in 50 mM sodium acetate (pH 4.5-5.5) or 5.5-8.5 in 50 mM sodium phosphate (pH5.5-8.5), respectively, at 25°C.

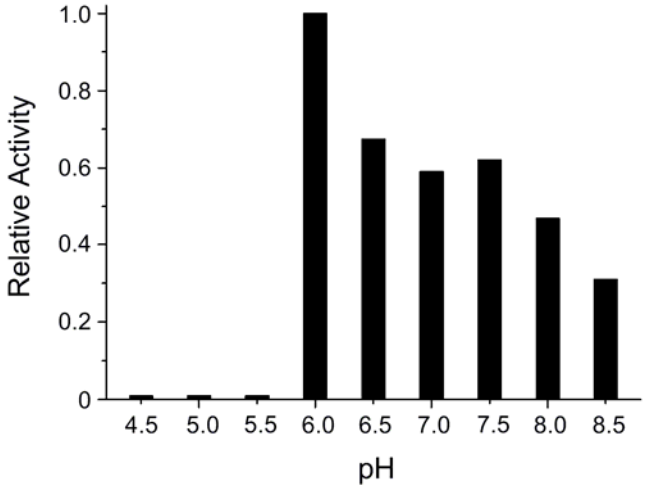

Fig. S3. HPLC analyses of incubation of 2,7-dihydroxy-5-methyl-1-naphathoic acid **(8**) and NcsB1 without exogenously supplemented SAM in 50 mM sodium phosphate (pH 6.0) at 25°C and quantification of 2-hydroxy-7-methoxy-5-methyl-1-naphathoic acid formed to determine SAM endogenously bound to NcsB1: enzyme reactions contained 50 μM NcsB1 and 5.0 mM **8** and terminated at (I) 2, (II) 5, and (III) 8 h, and enzyme reaction contained 100 μM NcsB1 and 5.0 mM **8** and terminated at (I) 2, (II) 5, and (III) 8 h, respectively. (♦), **8** and (•), **9**.

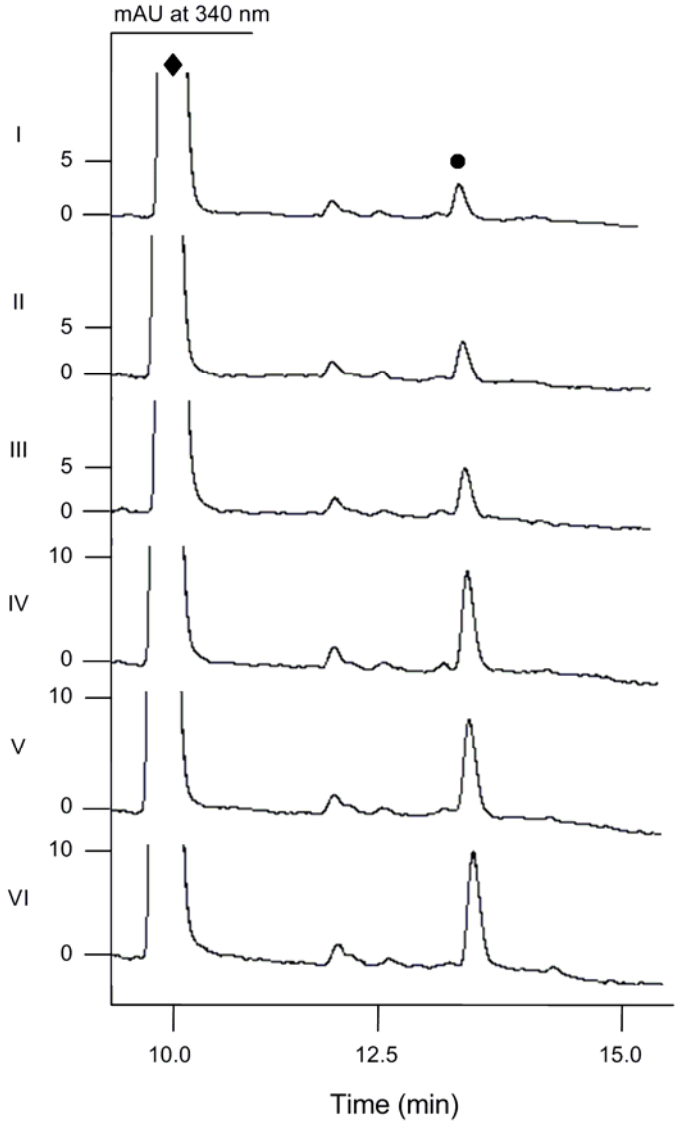

Table S1. Determination of SAM endogenously bound to NcsB1 purified and used in this study

NcsB1 (μM)	Product **9** formed (μM)		
	Reaction time (2 h)	Reaction time (5 h)	Reaction time (8 h)
50	38.0	41.4	40.1
100	81.8	80.4	82.6

[**9**] formed was estimated to be 40.1 μM with 50 μM NcsB1 or 81.6 μM with 100 μM NcsB1, which corresponds to 0.8 mole of SAM bound to 1 mole of the purified NcsB1 protein.

Fig. S4. Time course of NcsB1-catalyzed *O*-methylation as determined by production formation. Assays contained 1 mM 2,7-dihydroxy-5-methyl-1-naphathoic acid (**8**), 1.5 mM SAM in 50 mM sodium phosphate (pH 6.0) at 25°C in the presence of 50 μM NcsB1 (□) and terminated at 5, 15, 35, 60, 120, 180, 240, or 300 min, or 5 μM (△) NcsB1 and terminated at 2, 4, 8, 15, 30, 60, 120, or 300 min. Increases in observed methylation product 2-hydroxy-7-methoxy-5-methyl-1-naphathoic acid (**9**) corresponded with the concomitant consumption of **8**, and under these conditions **9** was linear with respect to time until approximately 15 min.