Supporting Information

Molecular basis of substrate promiscuity for the SAM-dependent *O*-methyltransferase, NcsB1, involved in the biosynthesis of the enediyne antitumor antibiotic neocarzinostatin

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Table S1: Crystallization Conditions for NcsB1				
	SAH	**SAM and 2**	**SAH and 4**	**SAH and 6**
NcsB1 (mg/mL)	2	2	2	2
cofactor (mM)	2	2	2	2
NA (mM)	0	2	2	1
βME (mM)	1	1	1	10
Sodium Formate (M)	3.7	3.6	3.66	3.9
Space Group	$C222_1$	$P6_5$	$P6_5$	$P6_5$
Resolution (Å)	2.1	2.6	2.7	3.0

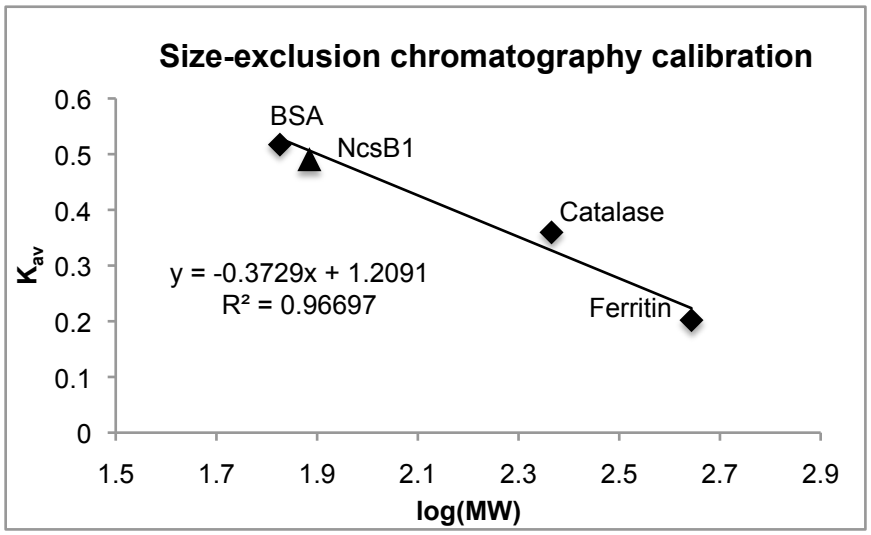


Figure S1: Size-exclusion chromatography calibration for estimating biologically active unit of NcsB1 (▲). K_{av} for NcsB1 = 0.491; Calculated size = 84.3 kDa, ~2 monomers. Actual size = 69.0 kDA.

Table S2: Dali Structural Homolog Search (*1*)							
Position[a]	**PDB**[b]	**Z**[c]	**RMSD$_{Ca}$ (Å)**	**# of eq. res.**	**# of res.**	**%ID of eq. res.**	**Description**
1 (*2*)	1TW3	39.2	1.8	324	340	44	DnrK, *Streptomyces peucetius*
5 (*3*)	1XDS	37.5	1.7	316	336	45	RdmB, *Streptomyces purpurascens*
10 (*4*)	1FPX	29.6	3.8	309	345	23	IOMT, alfalfa
12	2R3S	29.1	3.6	314	330	18	NPOMT, *Nostoc punctiforme*
13 (*5*)	2IP2	28.7	4.8	317	330	26	PhzM, *Pseudomonas aeruginosa*
16 (*6*)	1KYW	27.0	4.3	313	361	25	COMT, alfalfa

[a] Position in listing of structural homologs. Gaps indicate additional structures(s) of identical proteins. Reference in parentheses.

[b] Protein Data Bank code

[c] Strength of structural similarity

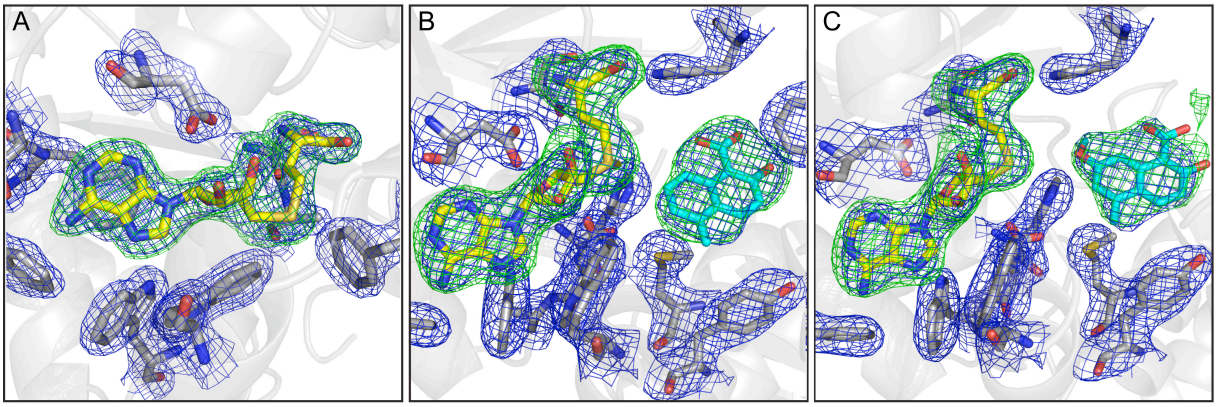
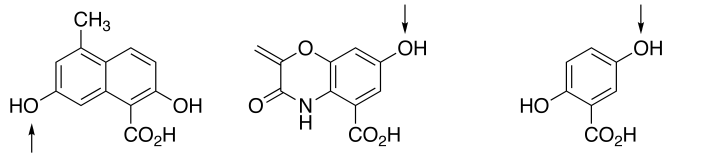


Figure S2: $2F_o - F_c$ maps contoured to 1.5 σ and $F_o - F_c$ map contoured to 3.0. Maps generated without ligands modeled into active site (A) NcsB1/SAH. (B) NcsB1/SAM/**2**. (C) NcsB1/SAH/**4**.

A



NcsB1 substrate naphthoic acid (**3**) | SgcD4 substrate benzoxazolinate (**S1**) | 2,5-dihydroxybenzoic acid (**S2**)

B

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NcsB1   -MG-KRAAHIGLRALADLATPMAVRVAATLRVADHIAAGHRTAAEIASAAGAHADSLDRL 58
SgcD4   MLGDEDGKAAELWSMANLGTPMAVRVAATLRIADHITAGAHTAGEIAEAAAVHEESLDRL 60
         :* : .    * ::*:*.************:****:** :**.***.**..* :*****

NcsB1   LRHLVAVGLFTRDGQGVYGLTEFGEQLRDDHAAGKRKWLDMNSAVGRGDLGFVELAHSIR 118
SgcD4   LRYLTVRGLLDRDGLGRYTLTPLGRPLCEDHPAGVRAWFDMEG-AGRGELSFVDLLHSVR 119
        **:*.. **: *** * * ** :*. * :**.** * *:**:. .***:*.**:* **:*

NcsB1   TGQPAYPVRYGTSFWEDLGSDPVLSASFDTLMSHHLELDYTGIAAKYDWAALGHVVDVGG 178
SgcD4   TGKAAFPLRYGREFWEDLAEDPRRAESFNRLLGQDVATRAPAVVAGFDWASTGHVIDLGG 179
        **:.*:*:*** .*****..**  : **: *:.:.:    ..:.* :***: ***:*:**

NcsB1   GSGGLLSALLTAHEDLSGTVLDLQGPASAAHRRFLDTGLSGRAQVVVGSFFDPLPAGAGG 238
SgcD4   GDGSLLTALLTACPSLRGTVLDLPEAVQRAKESFAVSGLDDRANAVAGSFFDALPAGAGA 239
        *.*.**:*****  .* ******  ... *:. *  :**..**:.*.*****.******.

NcsB1   YVLSAVLHDWDDLSAVAILRRCAEAAGSGGVVLVIEAVAGDEHAG-TGMDLRMLTYFGGK 297
SgcD4   YVLSLVLHDWDDEASVAILRRCAEAAGQTGSVFVIESTGSAGDAPHTGMDLRMLCIYGAK 299
        **** ******* ::************. * *:***:...  .*  ********  :*.*

NcsB1   ERSLAELGELAAQAGLAVRAAHPISYVSIVEMTAL 332
SgcD4   ERRVEEFEELAGRAGLRVVAVHPAGPSAIIQMSAV 334
        ** : *: ***.:*** * *.** .  :*::*:*:
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Figure S3: Comparison of NcsB1 and SgcD4 substrates and sequence homology (56% homologous). (A) NcsB1 substrate (**3**), SgcD4 substrate (**S1**), and substrate analog 2,5-dihydroxybenzoic acid (**S2**). The site of methylation is indicated with an arrow. (B) NcsB1/SgcD4 sequence alignment with key residues boxed. Naphthoic acid binding pocket, solid line; SAM binding pocket, dashed line; Catalytic residues, dotted line.

Determination of Substrate Specificity of NcsB1 for Benzoic Acid

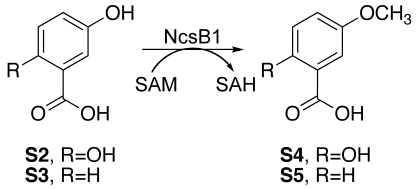

Figure S4: Benzoic acids tested for activity with NcsB1 and observed products.

To measure the ability of NcsB1 to methylate hydroxybenzoic acids **S2** and **S3** (Figure S4), the previously reported biochemical assay was used with minor modifications (*7*). Benzoic acid substrates were purchased from Aldrich and used without purification. A 100 μL reaction containing 100 mM sodium phosphate pH 6.0, 2.5 mM SAM, and 2.5 mM benzoic acid was initiated with 100 μM NcsB1 at 25 ºC. A 50 μL aliquot was quenched with trifluoroacetic acid to a final concentration of 16% (Aldrich) at 1.5 and 24 hr. Samples were centrifuged at 14,000 *g* for 2 min and the supernatant was analyzed by HPLC (eluent A: water with 0.1% trifluoroacetic acid; eluent B: acetonitrile; gradient: 0-12 min at 10-60% acetonitrile, 12-17 min at 0% at a flow rate of 1 mL/min on a VYDAC C18 Protein and Peptide column, 4.6 x 250 mm). UV detection was varied based on the UV_{max} of the substrate: **S2** was observed at 320 nm, **S3** was observed at 287 nm. The product(s) of the enzymatic reactions of NcsB1 with the various benzoic acid substrates were analyzed on an Agilent 6210 time-of-flight LC/MS in negative mode. Enzymatic reactions were set up as above. The reactions were quenched after 18 hr at 25 ºC with trifluoroacetic acid and were submitted for LC-MS analysis (eluent A: water with 0.1% formic acid; eluent B: acetonitrile with 0.1% formic acid; gradient: 0-12 min at 10-60% B, 12-15 min at 60% B, 15-17 min at 60-10% B, 17-20 min at 10% B at a flow rate of 0.2 mL/min). The product of the enzymatic reaction with **S2** was isolated from a large-scale reaction, purified by HPLC and subjected to ^{1}H NMR and NOE analysis.

2,5-dihydroxybenzoic acid (**S2**) to 2-hydroxy-5-methoxybenzoic acid (**S4**) LC-MS (negative mode) yielded *m/z* 153.1 and 167.1, respectively ($[M-H]^-$ for both). **S4**: ^{1}H NMR (CD_3OD, 500MHz) assignments are δ 7.36 (1H, d, *J* = 5Hz, C6-H), 7.10 (1H, dd, *J* = 10, 5Hz, C4-H), 6.87 (1H, d, *J* = 10Hz, C3-H), 3.78 (3H, s, C5-OCH_3). NOE (CD_3OD, 500MHz) correlations are between Hs at δ 7.36/3.78 and 7.10/3.78.

3-hydroxybenzoic acid (**S3**) to 3-methoxybenzoic acid (**S5**) LC-MS (negative mode) yielded *m/z* 137.1 and 151.1, respectively ($[M-H]^-$ for both)

To determine the kinetic parameters of NcsB1 with **S2** and **S3** and mutant constructs with **S2**, 50 μL reaction mixtures containing 2.5 mM SAM, 100 mM phosphate buffer at pH 6.5 and varying concentrations of benzoic acid ranging from 50 μM to 2.0 mM were set up. Reactions were initiated by the addition of 100 mM enzyme, and run at 25 ºC. Reactions were quenched with trifluoroacetic to a final concentration of 16% at a specified time to determine the initial rate. Reaction times were as follows: **S2** with WT, Arg11Ala, and Arg11Lys for 90 min, **S2** with Tyr293Ile and **S3** with WT for 120 min, **S2** with Ala11Trp for 60 min. Reactions were analyzed by UV detection as described above. To obtain kinetic parameters, initial rates were plotted against substrate concentration and were fitted to the Michaelis-Menten equation using KaleidaGraph (Synergy Software, Reading, PA).

Table S3: Kinetics of NcsB1 and mutant constructs

Protein/substrate	K_m **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m **(rel to WT/NA)**	k_{cat}/K_m **(rel to WT/BA)**
WT/**S2**	280 ± 30	0.0043 ± 0.0001	0.005	1
WT/**S3**	137 ± 22	0.00064 ± 0.00002	0.001	0.3
Tyr293Ile/**S2**	558 ± 113	0.0033 ± 0.0003	0.002	0.4
Arg11Trp/**S2**	183 ± 27	0.0071 ± 0.0003	0.012	2.6
Arg11Ala/**S2**	216 ± 17	0.0022 ± 0.0005	0.003	0.7
Arg11Lys/**S2**	348 ± 41	0.0040 ± 0.0002	0.003	0.7

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