

Characterization of NcsB2 as a Promiscuous Naphthoic Acid/Coenzyme A Ligase Integral to the Biosynthesis of the Enediyne Antitumor Antibiotic Neocarzinostatin

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Neocarzinostatin (NCS) is the archetypal member of the chromoprotein family of antitumor antibiotics that consists of an apoprotein and an enediyne chromophore.¹ The NCS chromophore (**1**) is composed of a nine-membered enediyne core, a deoxy-aminosugar, and a naphthoic acid moiety (Figure 1). We have previously cloned and sequenced the NCS biosynthetic gene cluster from *Streptomyces carzinostaticus* ATCC15944 and proposed a convergent pathway featuring a NcsB2-catalyzed direct coupling between a naphthoyl-S-NcsB and an enediyne core intermediate (Figure 1B).² While this hypothesis was consistent with the sequence-based prediction of NcsB2 acting as a CoA ligase, expression of *ncsB* in heterologous hosts resulted in the accumulation of 2-hydroxy-5-methyl-1-naphthoic acid (**2**).^{3a} Similar results were also obtained upon the expression of *aviM*^{3b} and *chlB1*^{3c}, two homologues of *ncsB*, in several heterologous hosts. These findings would argue against the involvement of an naphthoyl-S-NcsB tethered intermediate in NCS biosynthesis as originally proposed. Here we report the in vivo and in vitro characterization of NcsB2 as a CoA ligase that catalyzes the activation of 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid (**3**) into its CoA-ester (**4**). Additionally, we have revised the pathway for biosynthesis of **4** featuring its incorporation into **1** with free naphthoic acids as intermediates (Figure 1A and 1C). The finding that NcsB2 exhibits promiscuous substrate specificity presents a promising opportunity to produce novel analogues of **1** by engineering NCS biosynthesis.

We first confirmed that *ncsB2* is essential for **1** biosynthesis by inactivating *ncsB2* and complementing the resultant Δ *ncsB2* mutants in vivo.⁴ Replacing *ncsB2* with a mutant copy yielded an *S. carzinostaticus* SB5006 mutant strain that completely lost its ability to produce **1**. Production of **1** was partially restored to SB5006 by overexpressing a functional copy of *ncsB2* in trans, alleviating any concern over potential polar effects from *ncsB2* inactivation on the expression of downstream genes in SB5006 (Figure 2A).

We next characterized NcsB2 as a naphthoyl CoA ligase in vitro.⁴ The *ncsB2* gene was expressed in *E. coli*, and the overproduced NcsB2 enzyme was purified to homogeneity. Substrate **3** was synthesized by following a literature procedure.⁵ CoA ligases have two activities: (i) ATP-dependent activation of carboxylic acids as acyl AMP esters and (ii) formation of acyl CoAs by coupling the activated acyl group with the thiol nucleophile of CoA. We examined the first half of the CoA ligase activity of NcsB2 by the ATP-[³²P]pyrophosphate exchange assay, which has been widely used in the characterization of adenylating enzymes.⁶ Efficient

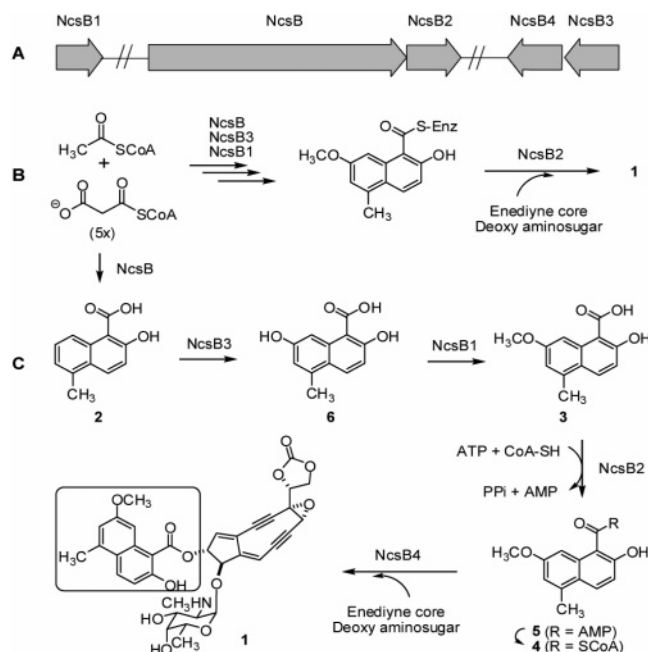


Figure 1. Biosynthetic pathway for the 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid moiety of NCS (**1**, boxed): (A) subcluster of genes within the NCS cluster encoding enzymes and (B) early and (C) revised proposal for the biosynthesis of **4** and its incorporation into **1**.

exchange was readily observed, indicative of the formation of naphthoyl-AMP ester **5**. The reaction occurs with a k_{cat} of 38 min⁻¹ and K_M of 0.59 μ M, comparable to those for other characterized adenylating enzymes such as the 2,3-dihydroxybenzoate activating enzyme, DhBE.⁷ We verified the second half of the CoA ligase reaction of NcsB2 by HPLC to directly monitor the formation of **4** from **3** in the presence of ATP and CoA. As shown in Figure 2B, **3** was specifically converted to **4**, and CoA was the only nucleophile among a range of potential coupling partners tested that efficiently supported this conversion.⁴

The characterization of NcsB2 as a **3** CoA ligase prompted us to revise the biosynthetic pathway of **4** from **2** and its subsequent incorporation into **1**. The previous functional assignments of NcsB as the naphthoic acid synthase, NcsB3 as the 7-hydroxylase and NcsB1 as the *O*-methyltransferase all acting on naphthoyl-S-NcsB tethered intermediates are consistent with the current finding of NcsB2 as a **3** CoA ligase.² However, we now prefer a scheme where NcsB3 and NcsB1 act on the free acid intermediates **2** and 2,7-dihydroxy-5-methyl-1-naphthoic acid (**6**), respectively (Figure 1C). The new proposal would require an additional enzyme to catalyze the coupling of **4** to the enediyne core of **1**. Reexamination of the

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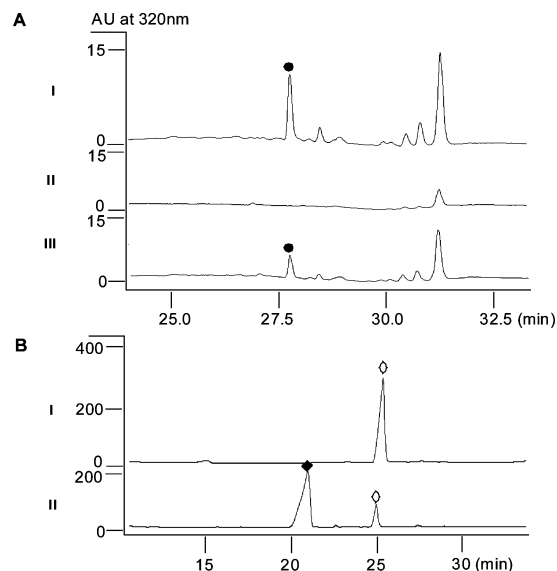


Figure 2. HPLC analysis (A) NCS chromophore (●) isolated from *S. carzinostaticus* ATCC15944 (I), SB5006 (II), and SB5006 complemented by expressing a functional copy of *ncsB2* in trans (III) and (B) NcsB2-catalyzed conversion of naphthoic acid 3 (◆) into its CoA-ester 4 (◇).

Table 1. Naphthoic Acid Substrate Scope of the NcsB2 CoA Ligase

naphthoic acid	K_m (μ M)	k_{cat} (min^{-1})	rel k_{cat}/K_m
3	0.59 ± 0.2	38 ± 2.0	1
2	0.25 ± 0.1	8.1 ± 0.6	0.50
6	3.1 ± 0.1	93 ± 0.6	0.47
7	2.3 ± 0.7	7.1 ± 0.8	4.8×10^{-2}
8	>500		
9	1.1 ± 0.4	25 ± 2.4	0.35
10	3.4 ± 0.9	2.8 ± 0.2	1.3×10^{-2}
11	18 ± 9	6.7 ± 1.4	5.8×10^{-3}

NCS biosynthetic gene cluster indeed led to the identification of an additional open reading frame, *orf27*, immediately downstream of *ncsB3*, whose function was not assigned previously.² We now renamed *orf27* as *ncsB4*. NcsB4 shows high sequence similarity to known esterases and acyltransferases,⁴ serving as a candidate to catalyze the coupling between 4 and the enediyne core to afford 1 (Figure 1A and 1C).

Finally, we directly compared the kinetic parameters of 3 as a substrate for NcsB2 with those of 2 and 6, two alternative substrates for NcsB2 depending on the timing of the CoA-ester formation step in 4 biosynthesis. Both 2 and 6 can be activated by NcsB2 in the presence of ATP but with the k_{cat}/K_m values ~ 2 fold lower than 3 (Table 1).⁶ Taken together, these results are consistent with the assignment of 3 as the preferred substrate of NcsB2 and support the timing of the individual steps as proposed for 4 biosynthesis (Figure 1C).

The fact that NcsB2 activates all three naphthoic acid biosynthetic intermediates 2, 3, and 6 inspired us to further probe the substrate specificity of NcsB2. A selected set of 1-naphthoic acid analogues with different substitutions at the 2-, 5-, or 7-position (7–11) were prepared by incorporating substituted benzaldehydes into the flexible synthetic scheme.⁵ Each of the analogues was subjected to the ATP-

[³²P]pyrophosphate exchange assay to examine if they can serve as substrates for NcsB2 with 3 as the positive control. Remarkably, substitutions at all three positions were well tolerated with their observed k_{cat} and K_m values ranging within 30-fold of the natural substrate 3 (Table 1). The one exception was 8, which showed essentially no detectable activity presumably because of the strong electron-withdrawing effect of the nitro group. We also tested 2-hydroxybenzoic acid and benzoic acid as potential substrates for NcsB2 but no measurable activity was detected (data not shown), suggesting the naphthoic acid moiety as the minimal substrate recognition element for NcsB2.

The characterization of NcsB2 as a CoA ligase with promiscuous substrate specificity is exciting because it presents an opportunity to produce novel analogues of 1 by engineering NCS biosynthesis. Analogous to adenylation domains found in nonribosomal peptide biosynthesis, NcsB2 could be viewed as the “gate-keeper” that selects and activates naphthoic acids to be incorporated into 1, assuming that the ensuing NcsB4 acyltransferase also possesses relaxed substrate specificity. Generation of novel enediynes has been successful as exemplified by the production of C-1027 and calicheamicin analogues,⁸ the availability of which has already unveiled new insight into the mode of action and drug discovery for the enediynes.⁹ This work now sets the stage to explore these possibilities within the NCS scaffold, one of two enediyne natural products currently used in clinic as anticancer drugs.¹

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Supporting Information Available: Full experimental details for *ncsB2* inactivation, complementation, and expression; NcsB2 overproduction, purification, and biochemical assays; and synthesis of the NcsB2 substrate and its analogues. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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