J|A|C|S

COMMUNICATIONS

Enediyne Antitumor Antibiotic Maduropeptin Biosynthesis Featuring a *C*-Methyltransferase That Acts on a CoA-Tethered Aromatic Substrate

Jianya Ling,[†,∥] Geoffrey P. Horsman,[†] Sheng-Xiong Huang,[†] Yinggang Luo,[†] Shuangjun Lin,[†] and Ben Shen*,[†,‡,§]

Division of Pharmaceutical Sciences, University of Wisconsin National Cooperative Drug Discovery Group, Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53705, and State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China

Received June 10, 2010; E-mail: bshen@pharmacy.wisc.edu

Abstract: The enediyne antitumor antibiotic maduropeptin (MDP) is produced by *Actinomadura madurae* ATCC 39144. The biosynthetic pathway for the 3,6-dimethylsalicylic acid moiety of the MDP chromophore is proposed to be comprised of four enzymes: MdpB, MdpB1, MdpB2, and MdpB3. Based on the previously characterized biosynthesis of the naphthoic acid moiety of neocarzinostatin (NCS), we expected a biosynthetic pathway featuring carboxylic acid activation by the MdpB2 CoA ligase immediately before its coupling to an enediyne core intermediate. Surprisingly, the MDP aromatic acid biosynthetic pathway employs an unusual logic in which MdpB2-catalyzed CoA activation occurs before MdpB1-catalyzed C-methylation, demonstrating that MdpB1 is apparently unique in its ability to C-methylate a CoA-tethered aromatic acid. MdpB2 is a promiscuous CoA ligase capable of activating a variety of salicylic acid analogues, a property that could be potentially exploited to engineer MDP analogues.

The enediyne natural products are potent antitumor antibiotics.[1] They share a common unprecedented structural feature, an enediyne core containing two acetylenic groups conjugated by a double bond or incipient double bond (Figure 1A), that imparts cytotoxicity via unique mechanisms of DNA damage.[2] Recent progress in elucidating enediyne biosynthesis[3] has enabled rational engineering of new analogues with altered activities.[4] For example, manipulations of the C-1027 (**1**, Figure 1A) biosynthetic pathway produced analogues with DNA interstrand cross-linking activity, a property that could be exploited to target hypoxic tumors.[4]

The successful design of C-1027 analogues inspires parallel efforts to manipulate the neocarzinostatin (NCS, **2**, Figure 1A) biosynthetic pathway for novel analogues. The naphthoic acid moiety of **2** is proposed to arise by the activities of the NcsB iterative type I polyketide synthase (PKS),[5] the NcsB3 hydroxylase, and the NcsB1 *O*-methyltransferase.[6] The NcsB2 CoA ligase finally activates the resultant naphthoic acid as a CoA thioester,[7] setting the stage for its coupling to the enediyne core, catalyzed by the NcsB4 acyltransferase[5,7] (Figure 1B). Significantly, both NcsB1 and NcsB2 exhibit broad substrate promiscuity, a property that can be exploited to engineer NCS analogues.[6,7]

Maduropeptin (MDP, **3**, Figure 1A)[8a] also contains an aromatic acid moiety, a 3,6-dimethylsalicylic acid (**4**) whose construction might be targeted to engineer analogues. We recently cloned and
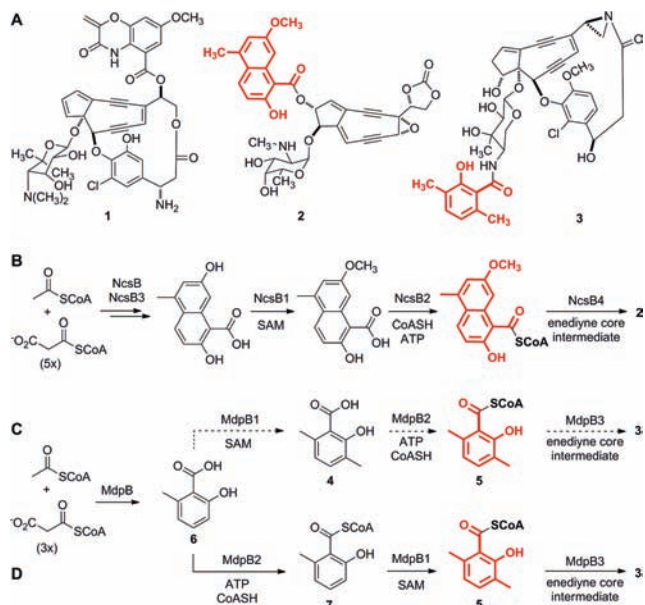

Figure 1. Selected enediynes and biosynthesis of the aromatic acid moieties: (A) the enediyne chromophores of C-1027 (**1**), NCS (**2**), and MDP (**3**) with the aromatic acid moieties highlighted in red; (B) biosynthesis of the naphthoic acid moiety of **2**; and (C) early and (D) revised biosynthesis of the salicylyl moiety of **3**.

characterized the MDP biosynthetic gene cluster.[8b] Parallel to that of the NCS naphthoic acid,[6,7] the biosynthesis of **4** was proposed to be catalyzed by the MdpB iterative type I PKS and the MdpB1 *C*-methyl transferase; the MdpB2 CoA ligase then activated **4** as a CoA thioester (**5**) prior to its coupling to the enediyne core intermediate, catalyzed by the MdpB3 acyltransferase (Figure 1C).[8] We were therefore inspired to study MdpB1 and MdpB2 with the hope that, like NcsB1 and NcsB2, similar substrate promiscuity may be uncovered and exploited to engineer MDP analogues. Here we report the surprising finding that MdpB2 acts *before* MdpB1, leading to a revised pathway for the biosynthesis of the salicylyl moiety of **3** (Figure 1D). Moreover, we demonstrate that MdpB2 is a promiscuous CoA ligase capable of activating a variety of salicylic acids.

We first attempted to characterize MdpB1 as a *C*-methyltransferase with 6-methylsalicylic acid (**6**) as a substrate in vitro.[9] MdpB1 was overproduced in *E. coli* and purified to homogeneity (Figure S1), and **6** was synthesized.[9] Unexpectedly, MdpB1, under all conditions examined, was unable to methylate **6** in the presence of *S*-adenosyl-L-methionine (SAM) to yield the predicted product **4** (Figure 1C), suggesting that the proposed pathway should be revised.

† Division of Pharmaceutical Sciences.
∥ Shandong University.
‡ University of Wisconsin National Cooperative Drug Discovery Group.
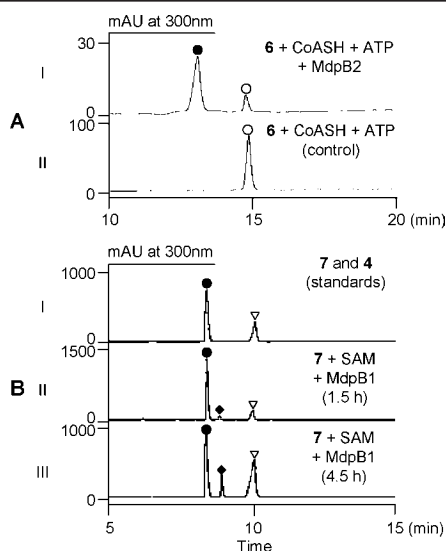§ Department of Chemistry, University of Wisconsin—Madison.

Figure 2. HPLC analysis of MdpB2- and MdpB1-catalyzed sequential conversion of **6** (○) to **5** (◆): (A) MdpB2-catalyzed conversion of **6** (○) to **7** (●) (I) and control without enzyme (II) and (B) authentic standards of **7** (●) and **4** (▽) (I) and time course of MdpB1-catalyzed conversion of **7** (●) to **5** (◆) and its degradation product **4** (▽) upon 1.5 h (II) and 4.5 h (III) incubation.

We next overproduced MdpB2 in *E. coli* and purified it to homogeneity (Figure S1) to examine the predicted CoA ligase activity in vitro. Upon incubation of **6** with MdpB2 in the presence of ATP and CoA, a new product was detected by HPLC analysis (Figure 2A) and identified as the corresponding CoA thioester **7**.[9] This suggested that MdpB2 may act, *before* MdpB1, on **6** as depicted in Figure 1D.

We then chemically synthesized **4**[9] and compared the steady-state kinetic parameters of MdpB2 toward **4** (Figure 1C) and **6** (Figure 1D) to differentiate the two pathways. CoA ligase catalysis occurs in two steps: (i) ATP-dependent activation of carboxylic acids as acyl-AMPs and (ii) addition of CoA to the acyl-AMP to generate the acyl-CoA product. Although the rate-limiting step of the MdpB2 reaction is unknown, we probed adenylation specificity using the ATP-[^{32}P]pyrophosphate exchange assay, which has been widely used for characterizing the substrate specificities of adenylating enzymes.[10] Although **4** was also converted to its CoA thioester **5**, whose identity was verified by HPLC analysis (Figure S3A) and high resolution mass spectrometry (HRMS),[9] MdpB2 apparently prefers **6** (k_{cat} of 2.3 min^{-1} and K_M of 0.041 μM) over **4** (k_{cat} of 0.59 min^{-1} and K_M of 2.1 μM) (Table 1 and Figure S2). The 200-fold higher specificity (k_{cat}/K_M) of MdpB2 for **6** over **4** supports the proposed pathway in Figure 1D, implying that MdpB1 *C*-methylates the CoA activated **7** rather than the free acid **6** as a substrate.

The kinetic insight from MdpB2 prompted re-evaluation of MdpB1 in light of the pathway proposed in Figure 1D. Incubation of MdpB1 with **7** and SAM yielded two products in a time-dependent fashion upon HPLC analysis (Figure 2B). One was the expected product **5**,[9] and the other product was identified as **4**, indicating that **5** was not stable under the assay conditions. These results corroborate the revised pathway of Figure 1D and highlight its unusual biosynthetic logic of CoA-activation one step earlier than chemically necessary and demonstrate that MdpB1 is a *C*-methyltransferase specific for a CoA-tethered aromatic substrate.

Finally we characterized MdpB2 substrate promiscuity and observed a requirement for the *ortho*-hydroxy and carboxylate groups of the aromatic acid substrate. As summarized in Table 1,

Table 1. Kinetic Parameters of MdpB2 towards Aromatic Substrates Featuring 1,2-Hydroxy Acid Functionality (highlighted in red)

Substrate	K_M (μM)	k_{cat} (min^{-1})	rel k_{cat}/K_M
6	0.041 ± 0.005	2.32 ± 0.06	1
4	2.1 ± 0.3	0.59 ± 0.02	0.0049
8	0.23 ± 0.05	0.90 ± 0.03	0.069
9	1.2 ± 0.2	0.86 ± 0.04	0.013
10		≥0.0042[a]	
11		≥0.0013[a]	

[a] Values represent specific activities at 100 μM substrate concentration.

MdpB2 can also utilize salicylic acid (**8**) and its 3-methyl derivative (**9**), in addition to **4**, as a substrate, albeit with 15-, 80-, and 200-fold lower specificities than **6**, respectively (Figure S2). In contrast, MdpB2 barely recognized benzoic acid (**10**) and its methyl derivative (**11**) as substrates, exhibiting specific activities of only 0.25 and 0.08 h^{-1}, respectively, at substrate concentrations of 100 μM. Although the kinetic analyses were based on the ATP-[^{32}P]pyrophosphate exchange assays,[9] formation of the corresponding CoA thioester product was confirmed in all cases by HPLC (Figure S3) and HRMS.[9] In contrast to other aryl-CoA ligases such as NcsB2[7] or SsfL1,[13] MdpB2 is apparently more specific for the 1,2-hydroxy acid functionality, reminiscent of that reported for the NcsB1 *O*-methyltransferase.[6]

In summary, this study characterizes a novel *C*-methyltransferase that acts on a CoA-tethered aromatic substrate in natural product biosynthesis. Relatively few *C*-methyltransferases act on aromatic substrates in natural product biosynthesis.[11] Notably, polyketomycin (POK) also possesses the **4** moiety (Figure S4A) and the POK biosynthetic gene cluster predicts proteins with high sequence identities (65−73%) to MdpB (PokM1), MdpB1 (PokMT1), and MdpB2 (PokM3) (Figure S4B).[11a] Indeed, a Δ*pokMT1* strain produced a **6**-containing POK analogue, confirming PokMT1 as a *C*-methyltransferase (Figure S4A). Our present in vitro work using purified MdpB1 sets the stage to further characterize this unusual enzyme capable of *C*-methylating a CoA-tethered aromatic substrate. To our knowledge, only an *O*-methyltransferase acting on caffeoyl-CoA has been described to date.[12] Although the significance of the unusual biosynthetic logic is unclear, MdpB1 may represent an emerging class of *C*-methyltransferases exploitable for tailoring CoA thioester-tethered intermediates in natural product biosynthetic pathways.

Supporting Information Available: Full experimental details, SDS-PAGE gels, kinetic data, HPLC chromatograms, and comparison between MDP and POK biosynthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Maeda, H. *Adv. Drug Delivery Rev.* **2001**, *46*, 169–185. (b) Sievers, E. L.; Linenberger, M. *Curr. Opin. Oncol.* **2001**, *13*, 522–527.

(2) (a) Nicolaou, K. C.; Dai, W.-M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1387–1416. (b) Galm, U.; Hager, M. H.; Van Lanen, S. G.; Ju, J.; Thorson, J. S.; Shen, B. *Chem. Rev.* **2005**, *105*, 739–758.

(3) Van Lanen, S. G.; Shen, B. *Curr. Top. Med. Chem.* **2008**, *8*, 448–459.

(4) (a) Kennedy, D. R.; Gawron, L. S.; Ju, J. H.; Liu, W.; Shen, B.; Beerman, T. A. *Cancer Res.* **2007**, *67*, 773–781. (b) Kennedy, D. R.; Ju, J.; Shen, B.; Beerman, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17632–17637.

(5) Liu, W.; Nonaka, K.; Nie, L. P.; Zhang, J.; Christenson, S. D.; Bae, J.; Van Lanen, S. G.; Zazopoulos, E.; Farnet, C. M.; Yang, C. F.; Shen, B. *Chem. Biol.* **2005**, *12*, 293–302.

(6) (a) Luo, Y.; Lin, S.; Zhang, J.; Cooke, H. A.; Bruner, S. D.; Shen, B. *J. Biol. Chem.* **2008**, *283*, 14694–14702. (b) Cooke, H. A.; Guenther, E. L.; Luo, Y.; Shen, B.; Bruner, S. D. *Biochemistry* **2009**, *48*, 9590–9598.

(7) Cooke, H. A.; Zhang, J.; Griffin, M. A.; Nonaka, K.; Van Lanen, S. G.; Shen, B.; Bruner, S. D. *J. Am. Chem. Soc.* **2007**, *129*, 7728–7729.

(8) (a) Komano, K.; Shimamura, S.; Norizuki, Y.; Zhao, D.; Kabuto, C.; Sato, I.; Hirama, M. *J. Am. Chem. Soc.* **2009**, *131*, 12072–12073. (b) Van Lanen, S. G.; Oh, T. J.; Liu, W.; Wendt-Pienkowski, E.; Shen, B. *J. Am. Chem. Soc.* **2007**, *129*, 13082–13094.

(9) See Supporting Information for full experimental details.

(10) Linne, U.; Marahiel, M. A. *Methods Enzymol.* **2004**, *388*, 293–315.

(11) (a) Daum, M.; Peintner, I.; Linnenbrink, A.; Frerich, A.; Weber, M.; Paululat, T.; Bechthold, A. *ChemBioChem* **2009**, *10*, 1073–1083. (b) Lozano, M. J.; Remsing, L. L.; Quiros, L. M.; Brana, A. F.; Fernandez, E.; Sanchez, C.; Mendez, C.; Rohr, J.; Salas, J. A. *J. Biol. Chem.* **2000**, *275*, 3065–3074. (c) Zhang, W.; Watanabe, K.; Wang, C. C. C.; Tang, Y. *J. Biol. Chem.* **2007**, *282*, 25717–25725. (d) Ishida, K.; Fritzsche, K.; Hertweck, C. *J. Am. Chem. Soc.* **2007**, *129*, 12648–12649. (e) Pacholec, M.; Tao, J.; Walsh, C. T. *Biochemistry* **2009**, *44*, 14969–14976.

(12) Ibdah, M.; Zhang, X.-H.; Schmidt, J.; Vogt, T. *J. Biol. Chem.* **2003**, *278*, 43961–43972.

(13) Pickens, L. B.; Kim, W.; Wang, P.; Zhou, H.; Watanabe, K.; Gomi, S.; Tang, Y. *J. Am. Chem. Soc.* **2009**, *131*, 17677–17689.

JA1050814