Supplementary Material (ESI) for Molecular BioSystems

Biosynthesis of 3-Methoxy-5-Methyl Naphthoic Acid and Its Incorporation into the Antitumor Antibiotic Azinomycin B

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Supplemental Data

Table S1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristic(s)	Reference
Escherichia coli		
DH5α	Host for general cloning	Invitrogen
ET12567 (pUZ8002)	Donor strain for conjugation between *E.coli* and *Streptomyces*	(1)
BL21 (ED3)	Host for protein expression	Novagen
AL1032	BL21 (ED3) derivative, containing pAL1045 for expression of *aziB1*	This study
AL1033	BL21 (ED3) derivative, containing pAL1047 for expression of *aziB2*	This study
AL1034	BL21 (ED3) derivative, containing pAL1049 for expression of *aziA1*	This study
Streptomyces albus		
J1074	Host for heterologous expression	(2)
TL1006	J1074 containing the vector pTGV2, negative control	(3)
AL1031	J1074 containing pAL1043, heterologous expression of *aziB* and *aziB1* together, 3-hydroxy-5-methyl-NPA (**3**) producing	This sturdy
Plasmids		
pSP72	*E. coli* subcloning vector	Promega
pGEM-7zf	*E. coli* subcloning vector	Promega
pGEM-3zf	*E. coli* subcloning vector	Promega
pET-28a	Vector for expression of the *N*-terminal 6 x His-tagged protein in *E. coli*	Novagen
pET-37b	Vector for expression of the *C*-terminal 6 x His-tagged protein in *E. coli*	Novagen
pTGV2	*E.coli-Streptomyces* shuttle vector for heterologous expression	(3)
pAL1012	pTGV2 derivative, carrying *aziB* alone under the control of the *PermE** promoter	(3)
pAL1021	pGEM-7zf derivative, containing a 3.1 kb fragment that carries *aziB1* and *aziB2* under the control of *PermE**	(3)
pAL1024	*S.sahachiroi* NRRL 2485 genomic libarary cosmid	(3)
pAL1041	pGEM-3zf derivative, containing a 2.5 kb fragment that carries *aziB1* under the control of	This study

	*PermE**	
pAL1042	pGEM-7zf derivative, containing a 2.5 kb fragment that carries *aziB1* under the control of *PermE**	This study
pAL1043	pTGV2 derivative, carrying *aziB* and *aziB1* with the *PermE*-aziB* + *PermE*-aziB1* genotype	This study
pAL1044	pSP72 derivative, containing a 1.2 kb PCR product that encodes AziB1	This study
pAL1045	pET-28a derivative, containing a 1.2 kb PCR product that encodes AziB1	This study
pAL1046	pSP72 derivative, containing a 1.0 kb PCR product that encodes AziB2	This study
pAL1047	pET-28a derivative, containing a 1.0 kb PCR product that encodes AziB2	This study
pAL1048	pSP72 derivative, containing a 1.9 kb PCR product that encodes AziA1	This study
pAL1049	pET37b derivative, containing a 1.9 kb PCR product that encodes AziA1	This study

Fig. S1. Characterization of AziB1 as a P450 enzyme by detection of absorptions varying in the range of 380-600 nm. I, recorded baseline of the 1 ml of 3.5 μM AziB1 in 100 mM Tris-HCl buffer (pH 8.0); and II, absorptions of this solution after bubbling of CO for 0.5 min followed by reduction of dithionite for 1 min.

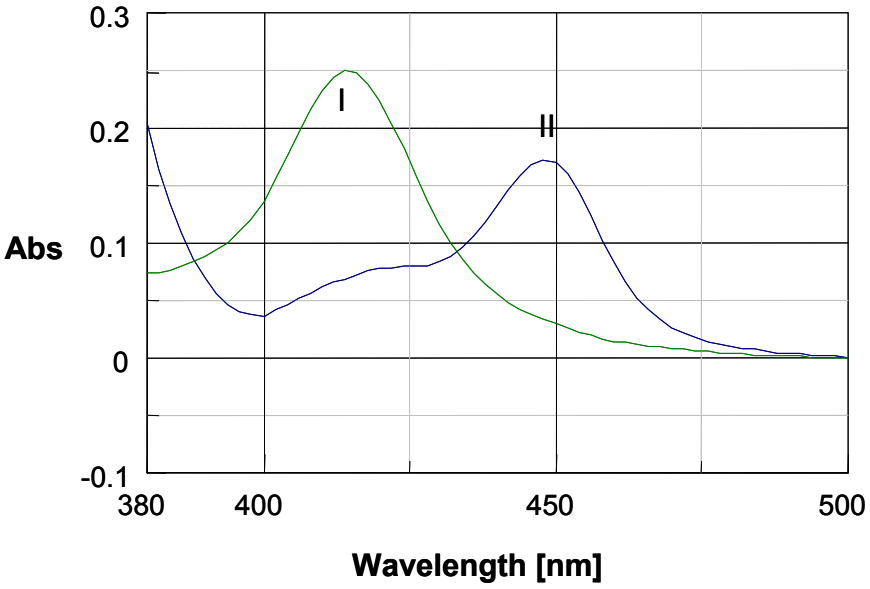


Fig. S2. Effect of pH on the relative activity of AziB1 as determined with 5-methyl-NPA (**2**) as the substrate in 50 mM reaction buffer varying in pH from 4.5 to 10.0 at 25^{o}C.

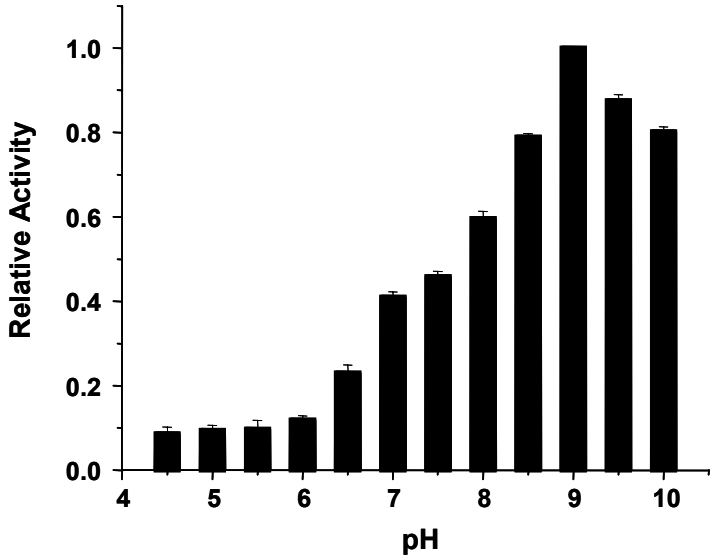


Fig. S3. Time course of AziB1-catalyzed C-3 hydroxylation as determined by product formation. The reaction mixtures contained 1 mM NADPH, 0.1 U/ml Ferredoxin-$NADP^+$ Reductase, 50 μg/ml Ferredoxin, 10 mM glucose-6-P, 1 U/ml glucose-6-P dehydrogenase, and 500 μM 5-methyl-NPA (**2**) in 50 mM Tris-HCl (pH 9.0). The assays were performed at 25°C in the presence of 1 μM Azi B1 (●) and terminated at 1, 2, 3, 4, 5, 8, 15, 30, 60, 120, 200, 300 min, or in the presence of 5 μM Azi B1 (■) and terminated at 2, 4, 6, 8, 10, 12, 15, 20, 30, 60, 120, 150, and 240 min. The increase of the hydroxylated product 3-hydroxyl-5-methyl-NPA (**3**) corresponded with the concomitant consumption of 5-methyl-NPA (**2**), and **3** production was linear with respect to time until approximately 15 min under these conditions.

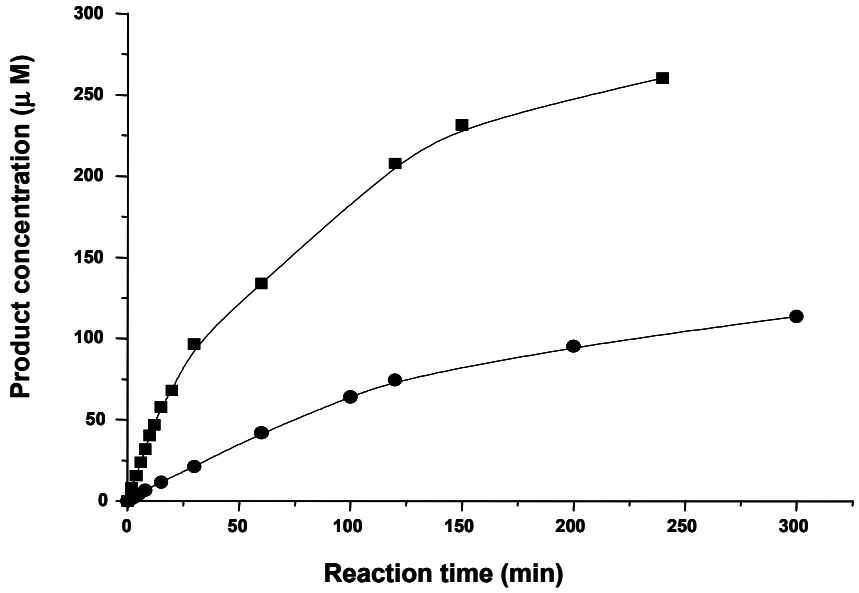


Fig. S4. HPLC-MS analysis of the conversion of SAM to SAH (yielding a $[M + H]^+$ ion at *m/z* = 385.05 and consistent with the molecular formula $C_{15}H_{24}N_6O_4S$. 385.16 calculated) in AziB2-catalyzed reaction, by using inactivated AziB2 (I) or AziB2 (II) as the catalyst.

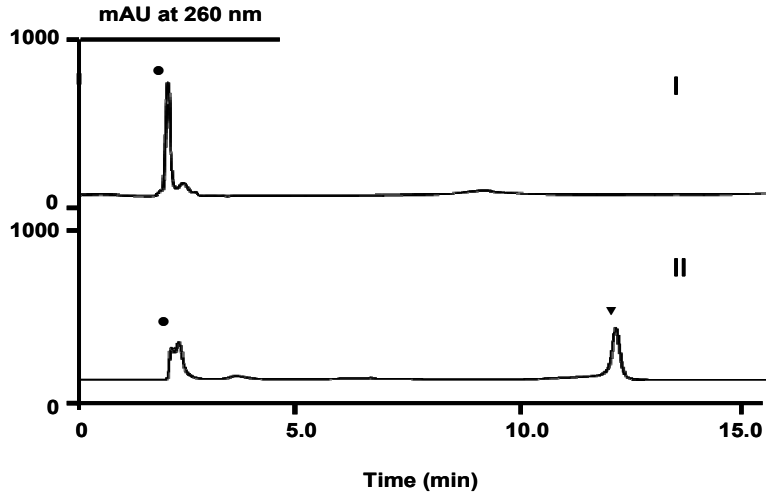


Fig. S5. Effect of pH on the relative activity of AziB2 as determined with 3-hydroxyl-5-methyl-NPA (**3**) as the substrate in 50 mM reaction buffer varying in pH from 4.5 to 10.0 at 25°C.

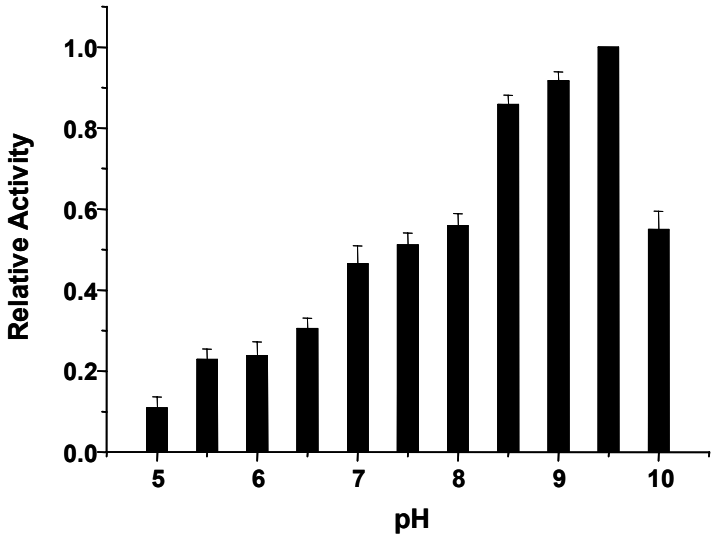


Fig. S6. Time course of AziB2-catalyzed *O*-methylation as determined by product formation. The reaction mixture contained 1 mM 3-hydroxyl-5-methyl-NPA (**3**), 2 mM SAM in 50 mM Gly-sodium hydroxide buffer (pH 9.5). The assays were performed at 25°C in the presence of 5 μM AziB2 (■) and terminated at 1, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 120, 150, and 240 min, or in the presence of 20 μM Azi B2 (▲) and terminated at 2, 4, 8, 15, 20, 30, 60, 120, 150, and 240 min. The increase of the *O*-methylated product 3-methoxy-5-methyl-NPA (**1**) corresponded with the concomitant consumption of 3-hydroxyl-5-methyl-NPA (**3**), and **1** production was linear with respect to time until approximately 20 min.

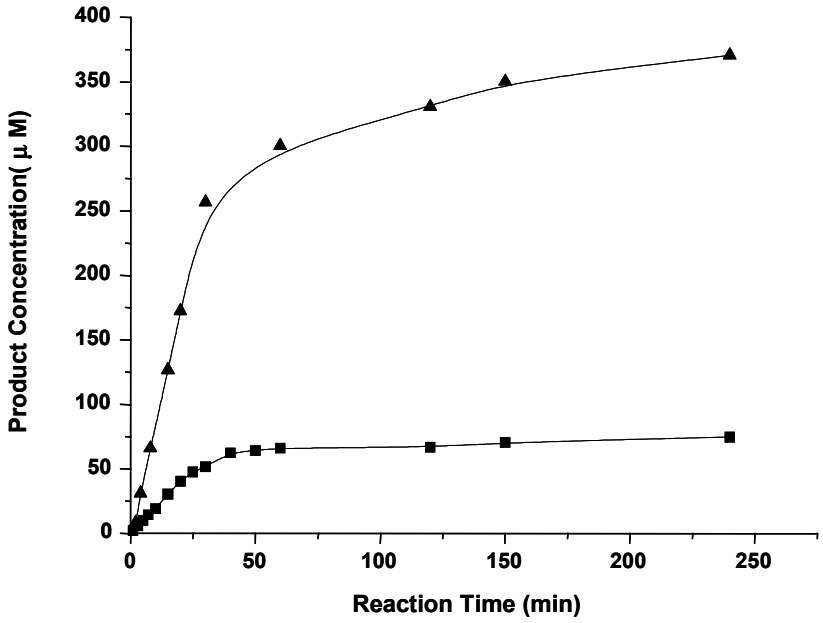


Fig. S7. Effect of divalent metal ions on the relative activity of AziB2 as an *O*-methyltransferase by using 3-hydroxyl-5-methyl-NPA (**3**) as the substrate.

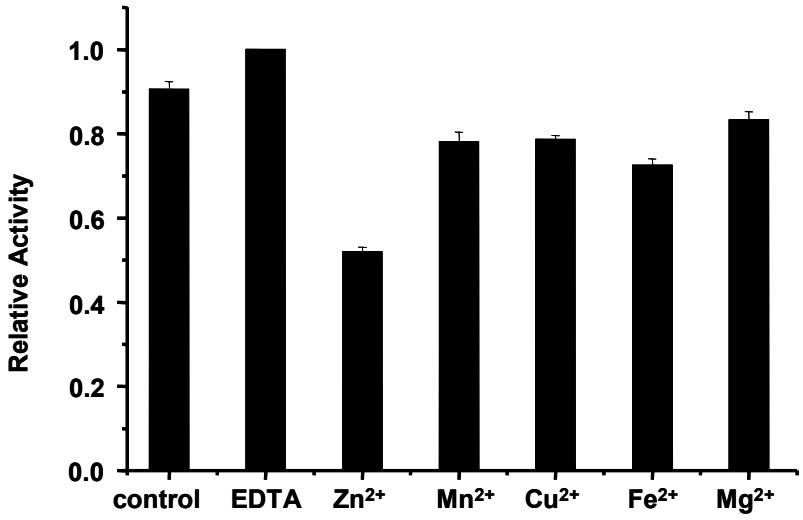
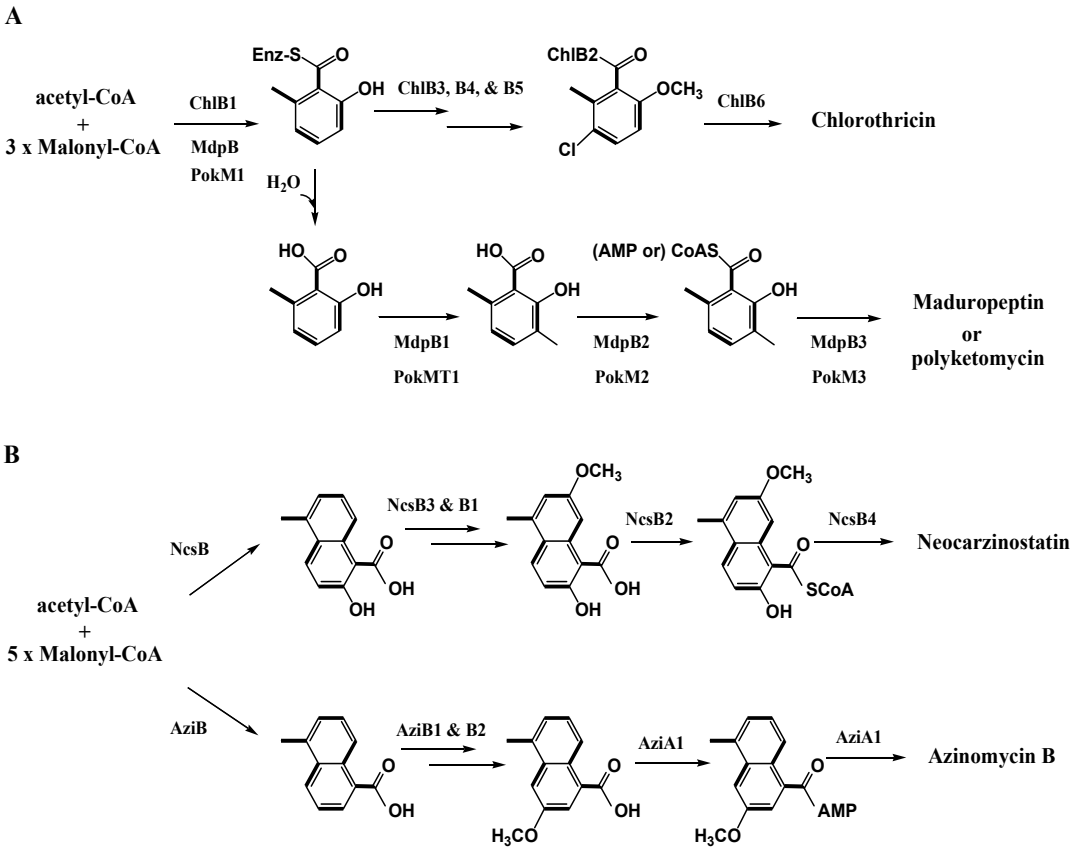


Figure S8. Selected modes of bacterial iterative type I PKSs-post modifications and incorporation for mono-cyclic (A) and (B) aromatic compounds, respectively.



References

1. MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H., and MacNeil, T. (1992). *Gene* **111**, 61-68.
2. Chater K.F., and Wilde L.C. (1980). *J. Gen. Microbiol.* **116**, 323-334.
3. Zhao, Q., He, Q., Ding, W., Tang, M., Kang, Q., Yu, Y., Deng, W., Zhang, Q., Fang, J., Tang, G., and Liu, W. (2008). *Chem. Biol.* **15**, 693-705.