Surprising Biosynthetic Logic for the Enediyne Antitumor Antibiotic Maduropeptin Featuring a *C*-Methyltransferase That Acts on a CoA-Tethered Aromatic Substrate

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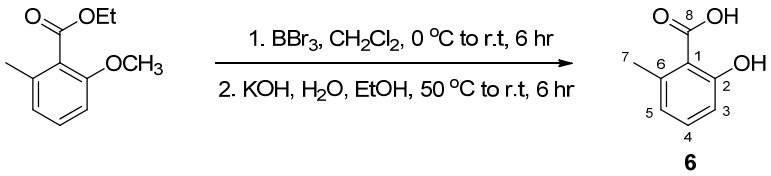
Supporting Information

General experimental procedures

DNA oligonucleotide primers were obtained from Integrated DNA Technology (Coralville, IA). Restriction enzymes and bovine serum albumin (BSA) were purchased from New England Biolabs (Ipswich, MA). DNA purification kits and Ni-NTA resin were from Qiagen (Valencia, CA). Competent *E. coli* cells were purchased from Invitrogen (Carlsbad, CA) and Fisher Scientific (Pittsburgh, PA). Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or VWR (West Chester, PA). Chemical reactions, unless otherwise noted, were run in solvents that were dried using stills by Glass Contour (Santa Monica, CA). Deuterated NMR solvents were purchased from Cambridge Isotopes (Andover, MA). The high resolution electrospray ionization-mass spectroscopy (HR ESI-MS) was performed with an Agilent 1100 HPLC-MSD SL ion trap mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA). Atmospheric pressure chemical ionization-mass spectroscopy (APCI-MS) was measured with an Agilent 1100 VL APCI mass spectrometer. Direct matrix-assisted laser desorption/ionization experiments were performed on a ProMALDI-Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Varian IonSpec, Lake Forest, CA). ^{1}H NMR spectra were recorded on either a Varian 500 or 400 MHz spectrometer. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration, assignment). ^{13}C NMR spectra were recorded on a Varian 500 (125 MHz) or 400 (100 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the DMSO as the external standard (δ 39.51 ppm).

Synthesis of 6-methylsalicylic acid (6)



6

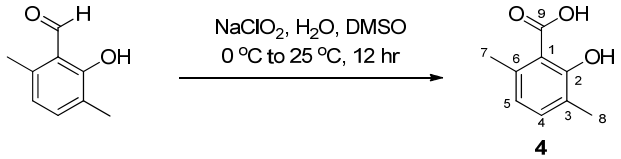
To a solution of commercial 2-methoxy-6-methylbenzoic acid ethyl ester (194 mg, 1 mmol) in 2 mL of absolute CH_2Cl_2 was added 1.1 mL of a solution of BBr_3 (1 M in anhydrous CH_2Cl_2) at 0 °C under an argon atmosphere. The whole reaction mixture was stirred for 30 min at 0 °C, then 6 h at room temperature. The reaction solution was diluted with methanol, and the solvents were removed on a rotary evaporator under reduced pressure. This process was repeated three times to give a residue (6-methylbenzoic acid ethyl ester) which was dissolved in 5 mL of a H_2O: EtOH (2:1) solution of KOH (3.5 M). The resulting solution was heated at 50 °C for 6 h, cooled to room temperature and then acidified with concentrated HCl. EtOAc was introduced to extract the product 6-methylsalicylic acid from the acidic solution. The organic layers were combined, dried over anhydrous Na_2SO_4, and evaporated to about 0.5 mL. 6-Methylsalicylic acid was crystallized from the EtOAc solution to yield 135 mg (89% yield) of white crystalline material.

^{1}H NMR ($CDCl_3$/CD_3OD, 500 MHz): δ 7.24 (t, J = 8.0 Hz, 1H, H-4), 6.75 (d, J = 8.5 Hz, 1H, H-5), 6.71 (d, J = 7.5 Hz, 1H, H-3), 2.55 (s, 3H, H-7).

^{13}C NMR ($CDCl_3/CD_3OD$, 125 MHz): δ 173.7 (C-8), 162.4 (C-2), 141.6 (C-6), 133.6 (C-4), 122.6 (C-5), 114.9 (C-3), 113.2 (C-1), 22.9(C-7).

APCI-MS at *m/z* 151 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_8H_8O_3$, 151.05), 107 ($[M-CO_2-H]^-$).

Synthesis of 3,6-dimethylsalicylic acid (4)



4

$NaH_2PO_4 \cdot H_2O$ (197.2 mg, 1.43 mmol, dissolved in 0.5 mL H_2O) was added to a solution of 2-hydroxy-3,6-dimethylbenzaldehyde (100 mg, 0.67 mmol) in DMSO (2 mL) at 0 °C. $NaClO_2$ (142.6 mg, 1.58 mmol, dissolved in 0.5 mL H_2O) was then added and the resulting mixture was warmed slowly to 25 °C and stirred for 12 h. The reaction mixture was diluted with saturated aqueous Na_2CO_3 (1 mL) and washed with EtOAc (0.5 mL). The aqueous layer was then acidified to pH 4 with aqueous HCl and extracted with EtOAc. The organic layer was dried overnight with anhydrous Na_2SO_4 and concentrated. The residue was purified by HPLC to afford 3,6-dimethylsalicylic acid (51.2 mg, 46.2 % yield).

^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.18 (d, J = 8.0 Hz, 1H, H-4), 6.65 (d, J = 8.0Hz, 1H, H-5), 2.44 (s, 3H, H-8), 2.13 (s, 3H, H-7).

^{13}C NMR (DMSO-d_6, 100 MHz,): δ 174.1 (C-9), 160.3 (C-2), 138.3 (C-1), 134.8 (C-4), 123.7 (C-3), 114.1 (C-5), 122.2 (C-6), 23.5 (C-8), 16.3 (C-7).

HR ESI-MS at *m/z* 165.0567 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_9H_{10}O_3$,165.0557).

Cloning of *mdpB1*

The *mdpB1* gene was PCR-amplified from cosmid pBS10002[1] as a template and Platinum Pfx DNA polymerase from Invitrogen (Carlsbad, CA) using the following primers: forward 5′-GGA ATT CCA TAT GGT CGA CAG TCG CAC CG-3′ and reverse 5′-CCC AAG CTT TCA CTT GCG GCC GAC GAC G-3’ (the *Nde*I and *Hind*III sites are underlined). The purified PCR product was sequenced to confirm PCR fidelity and cloned into the pET-28a(+) vector (Novagen, Madison, WI) as an *Nde*I-*Hind*III fragment to afford the pBS10017 plasmid, in which MdpB1 will be overproduced as an N-terminal His_6-tagged fusion protein.

Overproduction and purification of MdpB1

Plasmid pBS10017 was transformed into *E.coli* BL21(DE3) chemically competent cells and grown in LB media supplemented with 50 μg mL^{-1} kanamycin at 18 °C and 250 rpm. The culture was induced with 0.1 mM IPTG when OD_{600} reached 0.6-0.8, and overexpression was

continued for an additional 16-20 h. Cells were harvested by centrifugation at 4 °C at 4100 rpm for 25 min and resuspended in buffer A (100 mM sodium phosphate, pH 7.5, containing 300 mM NaCl) supplemented with a complete protease inhibitor tablet, EDTA-free (Roche, Mannheim, Germany). The cells were lysed by sonication (4 × 30 s pulsed cycle), and then centrifuged (15,000 rpm for 50 min at 4 °C) to remove cell debris. The clarified supernatant was loaded onto a pre-equilibrated Ni-NTA agarose (Qiagen, Valencia, CA) column with chilled buffer B (buffer A plus 10% glycerol and 1% Triton X-100). After washing with 5 column volumes of cold Buffer C (Buffer B with 20 mM imidazole), the expected His_6-tagged MdpB1 protein was eluted with six column volumes of buffer D (Buffer B with 250 mM imidazole).

After dialyzing in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol, the purified MdpB1 protein solution was concentrated by ultrafiltration using an Amicon Ultra-4 (10K, GE Healthcare) and stored at -78 °C in 20% glycerol for use in assays. The purity of the purified His_6-tagged MdpB1 was examined by 12% SDS-PAGE analysis, migrating as a single band with a molecular mass that is consistent with the predicted size of 40.5 kDa (Figure S1). Protein concentration was determined from the absorbance at 280 nm ($\varepsilon = 2.05 \times 10^4\ M^{-1}\ cm^{-1}$).

Cloning of *mdpB2*

The *mdpB2* gene was PCR-amplified from cosmid pBS10002[1] as a template and Platinum Pfx DNA polymerase from Invitrogen (Carlsbad, CA) using the following primers: forward 5′- GGA ATT CCA TAT GAC CAG CAT TCC GCG CA -3′ and reverse 5′- CCC AAG CTT TCA GCG GGT CGG GGC G-3’ (the *Nde*I and *Hind*III sites are underlined). The purified PCR product was sequenced to confirm PCR fidelity and cloned as an *Nde*I-*Hind*III fragment into pET-28a(+) to afford pBS10018, in which MdpB2 will be overproduced as an N-terminal His_6-tagged fusion protein.

Overproduction and purification of MdpB2

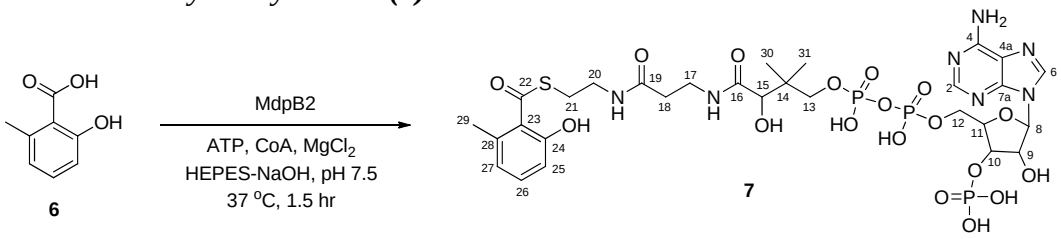
Plasmid pBS10018 was transformed into *E.coli* BL21(DE3) chemically competent cells and grown in LB media supplemented with 50 μg mL^{-1} kanamycin at 18 °C and 250 rpm. The culture was induced with 0.1 mM IPTG when OD_{600} reached 0.6-0.8, and overexpression was continued for an additional 16-20 h. Cells were harvested by centrifugation at 4 °C at 4100 rpm for 25 min and resuspended in buffer A (100 mM sodium phosphate, pH 7.5, containing 300 mM NaCl) supplemented with a complete protease inhibitor tablet, EDTA-free. The cells were lysed by sonication (4 × 30 s pulsed cycle), and then centrifuged (15,000 rpm for 50 min at 4 °C) to remove cell debris. The clarified supernatant was loaded onto a pre-equilibrated Ni-NTA agarose (Qiagen, Valencia, CA) column with chilled buffer B (buffer A plus 10% glycerol and 1% Triton X-100). After washing with 5 column volumes of cold Buffer C (Buffer B with 20 mM imidazole), the expected His_6-tagged MdpB2 protein was eluted with six column volumes of buffer D (Buffer B with 250 mM imidazole).

After dialyzing in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol, the purified MdpB2 protein solution was concentrated using an Amicon Ultra-4 (10K, GE Healthcare) and stored at -78 °C in 20% glycerol for use in assays. The purity of the purified His_6-tagged MdpB2 was examined by 12% SDS-PAGE analysis, migrating as a single band with a molecular

mass that is consistent with the predicted size of 60.5 kDa (Figure S1). Protein concentration was determined from the absorbance at 280 nm ($\varepsilon = 7.04 \times 10^4\ M^{-1}\ cm^{-1}$).

Coenzyme A arylation assay of MdpB2

*Assay towards 6-methylsalicylic acid **(6)***



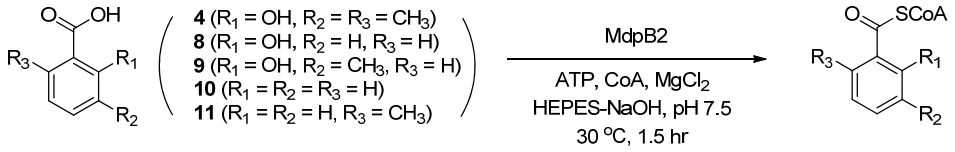
The ability of MdpB2 to couple the activated substrate **6** to CoA was assayed with similar conditions used by Linne *et al*.[2] An assay mixture containing 20 μM MdpB2, 4 mM ATP, 1 mM CoA, 0.2 mg/mL BSA, 0.5 mM **6** in assay buffer (50 mM HEPES-NaOH, 100 mM NaCl, 1 mM ethylendiaminetetraacetic acid, 10 mM $MgCl_2$, pH 7.5) was incubated at 37 °C for 1.5 hours. The reaction was terminated by ultrafiltration with a Microcon YM-10 (Millipore), and the clarified supernatant was transferred to HPLC vials. A control without CoA was also performed. The assay products were separated on an APOLLO C_{18} reverse phase column (250 × 4.6 mm, 5 μm; Grace Davison, Deerfield, IL) equilibrated with 100% solvent A (2 mM triethylamine/water) and 0% solvent B (2 mM triethylamine/80% acetonitrile/20% water), and developed with linear gradients in the following manner (beginning time and ending time with linear increase to % B): 0-20 min, 20% B; 20-28 min, 100% B; 28-32 min, 100% B; and 32-35 min, 0% B. The flow rate was kept constant at 1.0 ml/min, and elution was monitored at 300 nm. The MdpB2-catalyzed enzymatic reaction of **6** was scaled up, and the CoA-ester product **7** was purified by HPLC. After removal of the solvent under reduced pressure, the residue was lyophilized overnight and subsequently subjected to high-resolution MALDI-MS in negative mode. Analysis of the ^{1}H NMR spectrum and complete 2D spectroscopic experiments were consistent with the structural assignment of **7** as the product of MdpB2.

^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.54 (s, 1H, H-6), 8.22 (s, 1H, H-2), 7.19 (t, J = 8.1Hz, 1H, H-26), 6.77 (d, J =8.1 Hz, 2H, H-25/27), 6.16 (d, J = 7.0 Hz, 1H, H-8), 4.81 (m, 1H, H-9), 4.61 (brs, 1H, H-11), 4.26 (brs, 2H, H-12), 4.02 (s, 1H,), 3.82 (dd, J = 9.9, 5.0 Hz, 1H, H-13), 3.75 (m, 0.5H, H-10), 3.67 (m, 0.5H, H-10), 3.57 (dd, J = 9.9, 5.1 Hz, 1H, H-13), 3.47-3.51 (m, 4H, H-17/20), 3.21 (m, 2H, H-21), 2.49 (t, J = 6.5 Hz, 2H, H-18), 2.21 (s, 3H, H-29), 0.89 (s, 3H, H-30), 0.76 (s, 3H, H-31).

^{13}C NMR (DMSO-d_6, 125 MHz): δ 200.1 (C-22), 175.0 (C-19), 174.4 (C-16), 155.8 (C-4), 153.2 (C-2), 152.5 (C-24), 149.6 (C-7a), 140.1 (C-6), 135.8 (C-28), 131.6 (C-26), 127.5 (C-23), 122.2 (C-27), 118.9 (C-4a), 114.0 (C-25), 86.8 (C-8), 84.3 (C-11), 74.8 (C-9), 74.6 (C-10), 72.3 (C-13), 66.1 (C-12), 38.8 (C-20), 35.6 (C-17), 29.4 (C-21), 21.0 (C-30), 18.3 (C-31), 18.2 (C-29).

HR MALDI MS at *m/z* 900.1421 for the $[M-H]^-$ ion (calcd $[M-H]^-$ ion for $C_{29}\ H_{43}N_7O_{18}\ P_3S$, 900.1447).

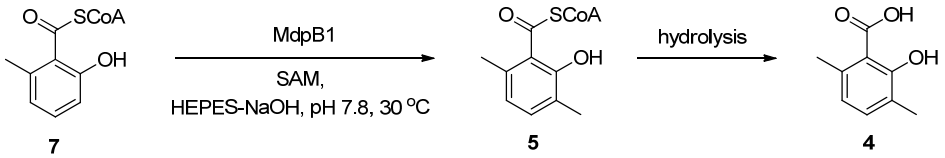
*Assays towards **4**, **8**, **9**, **10**, and **11***



The ability of MdpB2 to couple the activated substrates **4**, **8**, **9**, **10**, and **11** to CoA was assayed as above with minor modifications. An assay mixture containing 20 μM MdpB2, 5 mM ATP, 1.5 mM CoA, 0.1 mg/mL BSA, 1 mM substrate in assay buffer (50 mM HEPES-NaOH, 100 mM NaCl, 1 mM ethylendiaminetetraacetic acid, 10 mM $MgCl_2$, pH 7.5) was incubated at 30 °C. At various time points, aliquots of the reaction were removed and acidified with 15% TFA and analyzed by HPLC. A control reaction without CoA was also analyzed. The assay products were separated on an Alltech Alltima C_{18} reverse phase column (250 × 4.6 mm, 5 μm; Grace Davison, Deerfield, IL) equilibrated with 70% solvent A (0.1% trifluoroacetic acid in water) and 30% solvent B (0.1% trifluoroacetic acid/90% acetonitrile/10% water), and developed with linear gradients in the following manner (beginning time and ending time with linear increase to % B): 0-20 min, 100% B, 20-25 min, 100% B; 25-27 min, 30% B; 27-32 min, 30% B. The flow rate was kept constant at 1.0 mL/min, and elution was monitored at 300 nm.

The reactions were scaled up, the CoA-thioester products were HPLC-purified and analyzed by high resolution MALDI-MS in negative ion mode. For CoA-thioesters of: **4**, *m/z* at 914.1604 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_{30}H_{44}N_7O_{18}P_3S$, 914.1604); **8**, *m/z* at 886.1281 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_{28}H_{40}N_7O_{18}P_3S$, 886.1291); **9**, *m/z* at 900.1457 for the $[M-H]^-$ ion (calcd the $[M-H]^-$ ion of $C_{29}H_{42}N_7O_{18}P_3S$, 900.1447); **10**, at *m/z* 870.1332 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_{28}H_{40}N_7O_{17}P_3S$, 870.1342); **11**, at *m/z* 884.1524 (calcd for the $[M-H]^-$ ion of $C_{29}H_{42}N_7O_{17}P_3S$, 884.1498).

In vitro characterization of MdpB1-catalyzed *C*-methylation of-methylsalicylyl CoA (7)



For the MdpB1 activity assay, each 100 μL of reaction mixture contained 2.0 mM MdpB1, 5.0 mM substrate, 10 mM *S*-adenosyl-L-methionine in assay buffer (50 mM HEPES-NaOH, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM $MgCl_2$, pH 7.8). As a negative control, MdpB1 was boiled at 100 °C for 10 min. Reactions were initiated by the addition of MdpB1 and then incubated at 30 °C. The reaction was terminated by ultrafiltration with a Microcon YM-10 (Millipore), and the clarified supernatant was subjected to HPLC analysis using an Apollo C18 column equilibrated with 100% solvent A (2 mM triethylamine/water) and 0% solvent B (2 mM triethylamine/80% acetonitrile/20% water), and developed with linear gradients in the following manner (beginning time and ending time with linear increase to % B): 0-8 min, 30% B, 8-15min, 30% B; 15-18min, 100% B; 18-22 min, 100% B; and 22-25 min, 0% B. The flow rate was kept constant at 1.0 ml/min and elution was monitored at 300 nm.

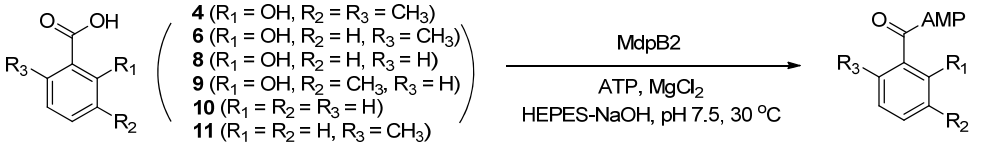
The MdpB1-catalyzed reaction of **7** was scaled up, and the two new products were purified by HPLC. After removal of the solvent under reduced pressure, the residue was lyophilized overnight and subjected to MS and NMR detection. Upon MALDI-MS analysis, One product yielded *m/z* at 914.1584 for the $[M-H]^-$ ion (calcd $[M-H]^-$ ion for $C_{30}H_{44}N_7O_{18}P_3S$, 914.1604), as expected for **5**. Another product was consistent with the structural assignment of **4** on the basis of the following MS and NMR data.

^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.18 (d, J = 8.0 Hz, 1H, H-4), 6.65 (d, J = 8.0 Hz, 1H, H-5), 2.44 (s, 3H, H-8), 2.13 (s, 3H, H-7).

^{13}C NMR (DMSO-d_6, 100 MHz,): δ 174.1 (C-9), 160.3 (C-2), 138.3 (C-1), 134.8 (C-4), 123.7 (C-3), 114.1 (C-5), 122.2 (C-6), 23.5 (C-8), 16.3 (C-7).

HR ESI MS: *m/z* at 165.0566 for the $[M-H]^-$ ion (calcd for the $[M-H]^-$ ion of $C_9H_{10}O_3$,165.0557).

ATP-[^{32}P]pyrophosphate exchange assay



ATP-[^{32}P]pyrophosphate exchange catalyzed by MdpB2 was monitored for substrates **4**, **6**, **8**, **9**, **10**, and **11**, by following a literature procedure.[2] Initial rates were determined at several substrate concentrations using a fixed concentration of ATP. The assay mixture (0.5 mL) consisted of buffer (50 mM Na-HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.5), 1 μM [^{32}P]PPi, 0.1 mg/mL BSA, 5 mM ATP, and substrate. The reactions were initiated by the addition of MdpB2 enzyme and the reaction was incubated at 30 °C. Enzyme concentrations were 26 nM for **4**, **6**, **8**, and **9**, 1.2 μM for **10** and **11**. Aliquots (95 μL) were removed for at least 4 reaction time points (typically 0.5, 1, 1.5, and 2 min) and immediately quenched into 500 μL of 4.5% $Na_2P_2O_7$, 3.5% $HClO_4$, 1% activated charcoal. Each solution was centrifuged and the charcaol residue was subjected to successive 1 mL washes with (i) 40 mM $Na_2P_2O_7$, 1.4% $HClO_4$, (ii) water, and (iii) ethanol. The charcoal pellet was resuspended in 600 μL of water, to which was added 10 mL of scintillation cocktail (ScintSafe Econo 2; Fisher Scientific, Fair Lawn, NJ), and the sample was subjected to liquid scintillation counting. Initial rates were obtained from linear progress curves in which <10% of isotope was incorporated into ATP. Steady-state kinetic constants were extracted by fitting the Michaelis-Menten equation to the data by non-linear regression (Figure S2) using a free web-based curve fitting interface.[3]

Figure S1. SDS-PAGE analysis on a 12% acrylamide gel showing purified MdpB1 (lane 1), MdpB2 (lane 2), and molecular weight markers (lane 3). The expected molecular masses are 40.5 kDa for MdpB1 and 60.5 kDa for MdpB2 (an extra 2 kDa is attributed to the engineered His_6-tag).

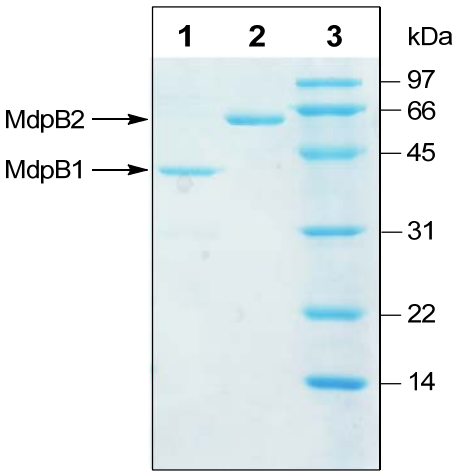


Figure S2. Determination of steady-state kinetic parameters of MdpB2 towards (A) **6**, (B) **4**, (C) **8**, and (D) **9** by fitting the Michaelis-Menten equation (solid line) to the data (black squares) of initial velocities of ATP-[^{32}P]pyrophosphate exchange versus substrate concentration.

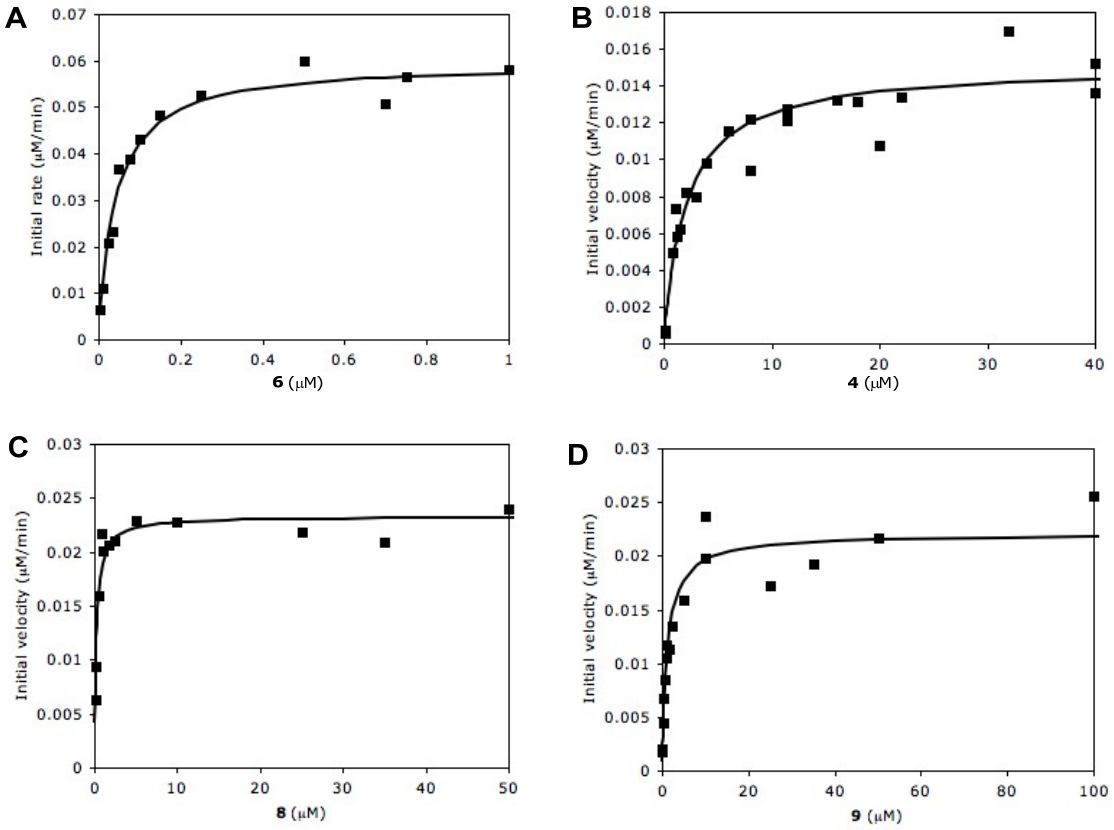


Figure S3. HPLC analysis at 300 nm of MdpB2-catalyzed formation of CoA-thioesters (●) from various aromatic acids (∇): (A) **4**, negative control (I), 5 min (II), 120 min (III); (B) **8**, negative control (I), 1 min (II), 180 min (III); (C) **9**, negative control (I), 15 min (II), 150 min (III); (D) **10**, negative control (I), 1 min (II), 180 min (III); and (E) **11**, negative control (I), 1 min (II), 180 min (III). Negative controls consisted of the reaction in the absence of either CoA or enzyme. Substrates **10** and **11** did not adsorb at 300nm.

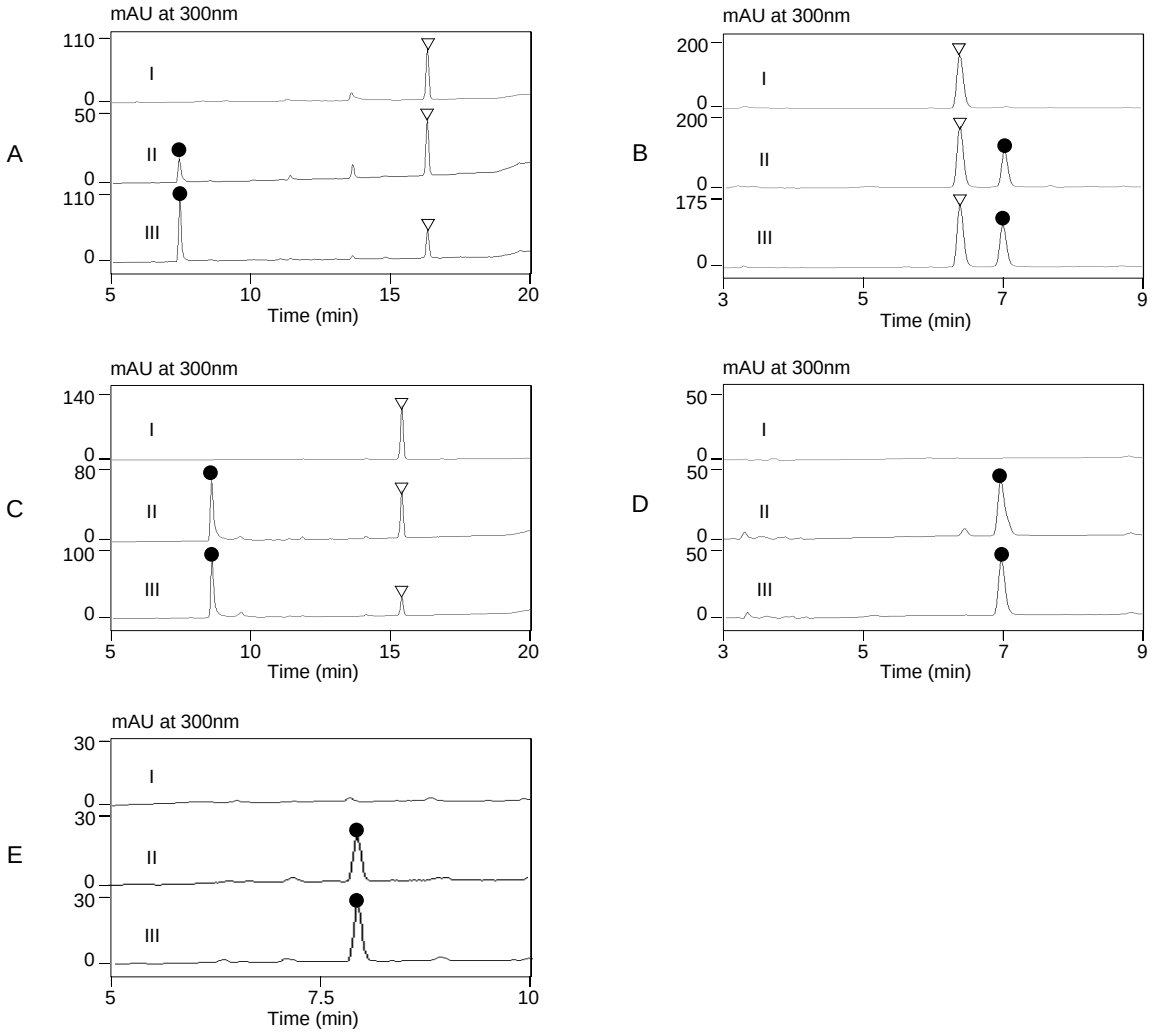
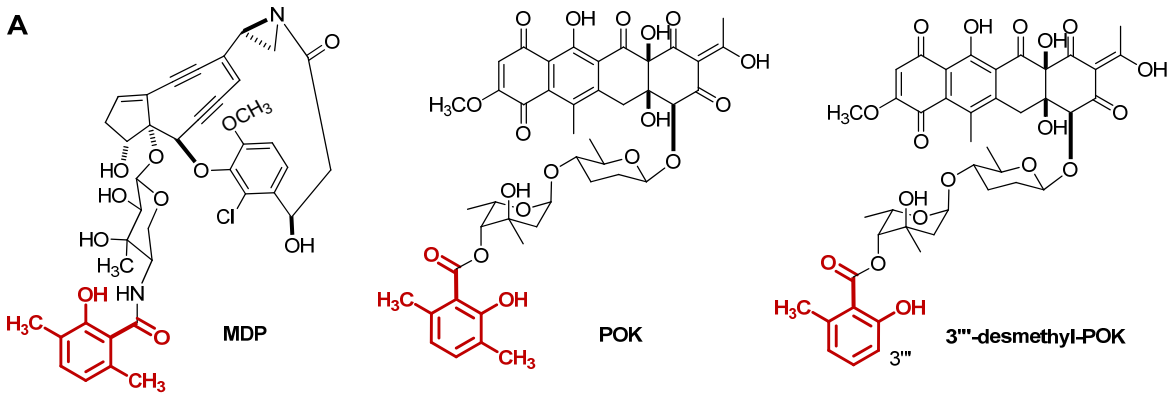


Figure S4. Comparison of biosynthesis of the salicylyl moiety in MDP and POK: (A) Structures of MDP, POK, and 3‴-desmethyl-POK with the salicylyl moiety highlighted in red and (B) sequence homology between enzymes annotated for the biosynthesis of the salicylyl moiety from the MDP and POK biosynthetic machinery.[1,4]

A



B

	% identity/% similarity		
	PokM1	PokM3	PokMT1
MdpB	65/74	-	-
MdpB2	-	73/80	-
MdpB1	-	-	72/84

References

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(2) Linne, U.; Maraheil, M. A. *Methods Enzymol.* **2004**, *388*, 293-315.

(3) http://zunzun.com/Equation/2/BioScience/Michaelis-Menten/. Accessed 4-6-10.

(4) Daum, M.; Peintner, I.; Linnenbrink, A.; Frerich, A.; Weber, M.; Paululat, T.; Bechthold, A. *ChemBioChem* **2009**, *10*, 1073-1083.