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for

Rational Manipulation of Carrier Domain Conformation in Nonribosomal Peptide Synthetase Assembly Lines

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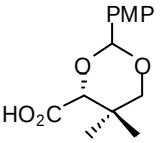
General procedures: ^{1}H NMR spectra were recorded on a Varian 500, 400 or 300 MHz spectrometer. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0 ppm) or with the solvent reference relative to TMS employed as the internal standard (D_2O, δ 4.79 ppm). Data are reported as follows: chemical shift (multiplicity, coupling constants, integration). ^{13}C NMR spectra were recorded on a Varian 500 (126 MHz) or 400 (100 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm relative to TMS with the $CDCl_3$ as the external standard (δ 77.26 ppm). ^{31}P NMR spectra were recorded on a Varian 300 (121 MHz) spectrometer with complete proton decoupling employing an external standard (85% H_3PO_4, δ 0.0 ppm). High resolution mass spectra were obtained at the Mass Spectrometry Facility of either Boston College (Chestnut Hill, MA) or the University of Illinois (Urbana-Champaign, IL). The method of ionization is given in parentheses.

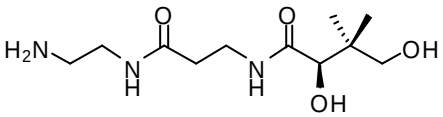
Preparative HPLC was performed on a Shimadzu (LC-6AD) instrument equipped with a multiwavelength UV-Vis detector. Preparative HPLC was carried out using a reversed-phase Vydac 218TP1022 C18 column (22 mm ID× 250 mm). Reagents and chemicals

were purchased from the Sigma-Aldrich Chemical Company unless otherwise noted. All the compounds synthesized were stored at -20ºC. For all the solid phase synthetic steps, the Kaiser test was employed to monitor the completeness of each step.

Experimental Procedures.



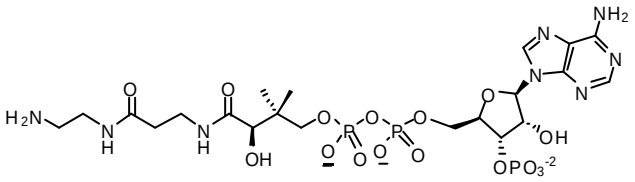
PMP-protected pantoic acid was synthesized using the published procedure[1].



Amino-pantetheine (3). Pantetheine analogue **3** was synthesized on solid support using trityl chloride polystyrene resin (Novabiochem, loading 1.6 mmol/g) in a solid phase synthesis vessel. The resin (1.00 g, 1.60 mmol) was swelled in DCM (8 mL) for 1 h before 1,2-diaminoethane (1.07 mL, 16.0 mmol) was added. The suspension was agitated on a shaker for 12 h, then drained and rinsed with NMP (*N*-methyl-2-pyrrolidinone) (5× 20mL) to give 1,2-diaminoethane-functionalized resin. A solution of FMOC-β-alanine (1.49 g, 4.80 mmol), PyBOP (2.50 g, 4.80 mmol), and DIPEA (1.67 mL, 9.60 mmol) in NMP (4 mL) was added to the resin and the suspension was agitated for 2 h, then drained and rinsed with NMP (5× 20 mL). The FMOC protecting group was removed by treating the resin with 20% (v/v) piperidine/NMP (20 mL) for 20 min, and then rinsed with NMP (5× 20 mL). A solution of PMP-protected pantoic acid (1.28 g, 4.80 mmol), PyBOP (2.50 g, 4.80 mmol) and DIPEA (1.67 mL, 9.60 mmol) in NMP (4 mL) was added to the resin and the suspension was then agitated for 4 h. After rinsing with NMP (5× 20 mL) and DCM (5× 20 mL), the resin was treated with 15 mL of 5% (v/v) trifluoroacetic acid (TFA) and 2% (v/v) triethylsilane (TES) in DCM for 20 min to cleave the product from the resin. The resin was washed by 5% TFA in DCM (10 mL). The cleavage solution and wash solution were combined and concentrated in vacuo. The residue was redissolved in 10 mL 10% (v/v) TFA/H_2O. The aqueous solution was extracted twice with diethyl ether (10 mL), and then lyophilized to give **3** as a yellow oil. The product was further purified by preparative HPLC (9 mL/min flow rate; gradient: 0-5min, 5% B; 5-25 min, 5-98% B, where

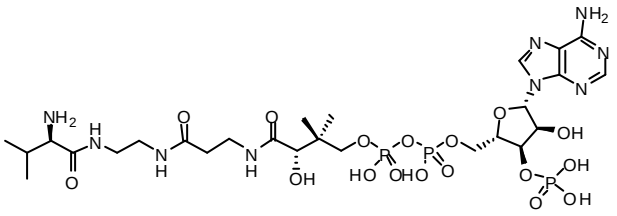
A=0.05% TFA/H_2O and B=0.05% TFA/CH_3CN). Chromatographs were monitored at 220nm and **3** was eluted at 11.2 min. Removal of solvents gave **3** as a colorless oil (combined purified yield 70%, 290 mg). ^{1}H NMR (D_2O, 300 MHz) δ 3.94 (s, 1H), 3.49-3.43 (m, 5H), 3.35 (d, *J* = 11.1 Hz, 1H), 3.10 (t, *J* = 5.9 Hz, 2H), 2.48 (t, *J* = 6.5 Hz, 2H), 0.88 (s, 3H), 0.85 (s, 3H). ^{13}C NMR (D_2O, 100 MHz) δ 175.1, 174.9, 75.9, 68.5, 39.4, 38.8, 37.0, 35.6, 20.7, 19.4. HRMS (ESI-) *m/z* Calcd for $C_{11}H_{24}N_3O_4$ 262.1767, found 262.1768.

Kaiser test procedure. Reagent #1 was made by dissolving 250 mg of ninhydrin in 5 mL ethanol. Reagent #2 was made by dissolving 4.0 g of phenol in 1.0 mL ethanol. Reagent #3 was made by dissolving 5 mg of KCN in one drop of water and added 5 mL of pyridine (cloudy suspension). A few sampled resin beads were transferred to a small glass test tube and 5 drops of reagent #1, 4 drops of reagent #2 and 1 drop of reagent #3 were added. The suspension was swirled and heated at 100 ºC for 1 min. The presence of visibly blue beads indicated a positive test for free amines.



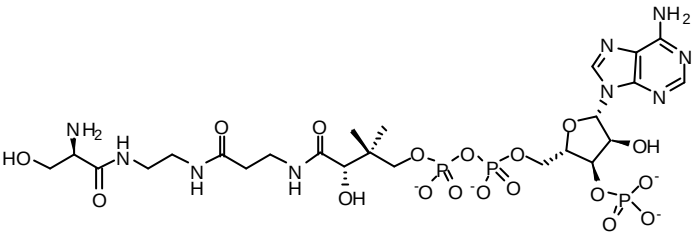
Enzymatic preparation of amino-coenzyme A (1). The genes encoding the PanK, PPAT, and DPCK enzymes were each amplified from *E. coli* genomic DNA, digested and ligated into the pET28a vector following a published protocol.[2] The vectors were individually transformed and overexpressed in *E. coli* BL21(DE3) cells (Novagen). For each enzyme: cells were harvested by centrifugation at 3500 rpm for 20 min. The cell pellets were suspended in 40 mL lysis buffer (20 mM Tris·HCl pH 7.5, 500 mM NaCl, 20 mM imidazole) and lysed by passing through a French Press cell at 1000 psi. The lysate was clarified by centrifugation at 10 000 rpm for 20 min. The supernatant was added 2 mL of Ni-NTA resin (Qiagen) suspension and incubated for 1 h. The resin was washed with lysis buffer (4× 15 mL), and then the protein was eluted by 3× 15 mL elution buffer (20 mM Tris·HCl pH 7.5, 500 mM NaCl, 250 mM imidazole). The first elution fraction for each enzyme was dialyzed against 1 L of 50 mM Hepes pH 8.0, 250 mM NaCl, 2 mM $MgCl_2$ buffer overnight at 4 ºC.

Enzymatic synthesis reactions were carried out following a similar procedure as reported[2] with several modifications: the enzymatic reactions were performed in Tris·HCl buffer at pH 9.0 as no product was observed at pH 7.5; the reactions were run at 37 ºC with twice the amount of enzymes and ATP as reported and the reactions were terminated by trichloroacetic acid (TCA) precipitation (added 50% (w/v) TCA solution to give a 10% (w/v) final concentration, incubated for 20 min on ice, then centrifuged at 5000 rpm for 10 min). The supernatant was lyophilized and the compound was purified on preparative reverse-phase C18 HPLC (9 mL/min; 0-5 min, 0% B; 5-25 min, 0-5% B, where A=0.05% TFA/H_2O and B=0.05% TFA/CH_3CN; **1** eluted at 25.5 min). Removal of solvent gave **1** as a white powder. The combined purified yield was 50% for **1**. ^{1}H NMR (D_2O, 500 MHz) δ 8.69 (s, 1H), 8.45 (s, 1H), 6.24 (d, *J* = 5.5 Hz, 1H), 4.89-4.87 (m, 2H), 4.62 (br s, 1H), 4.29-4.27 (m, 2H), 4.04 (s, 1H), 3.84 (q, *J* = 5.0 Hz, 1H), 3.66 (q, *J* = 5.0 Hz, 1H), 3.53-3.48(m, 4H), 3.14 (t, *J* = 6.0 Hz, 2H), 2.51 (t, *J* = 6.5 Hz, 2H), 0.95 (s, 3H), 0.89 (s, 3H). ^{13}C NMR (D_2O, 126 MHz) δ 175.4, 175.0, 150.1, 148.7, 144.9, 142.7, 118.8, 87.7, 83.8, 74.7, 74.3, 74.2, 71.9, 65.3, 39.4, 38.6, 37.0, 35.8, 35.6, 20.8, 19.1. ^{31}P NMR (D_2O, 121 MHz) δ 0.81, -9.87, -10.30. HRMS (ESI+) *m/z* Calcd for $C_{21}H_{38}N_8O_{16}P_3$ 751.1619, found 751.1653.

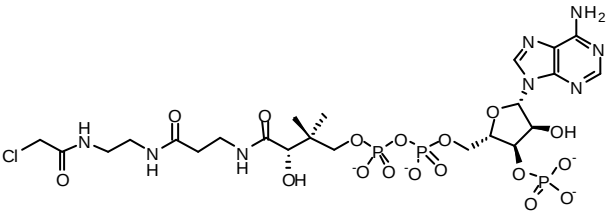


Valyl-amide CoA (4). Amino-CoA **1** (5.0 mg, 6.7 µmol) was reacted with *N*-FMOC-Valine (5.7 mg, 17 µmol), PyBOP (8.7 mg, 17 µmol), and DIPEA (5.8 µL, 33 µmol) in DMF/H_2O (350 µL, 2.5:1) for 3 h. The protecting group was removed by treating the solution with 40% (v/v) piperidine/DMF (350 µL) for 30 min. When lyophilized, the crude product was treated with 8 mL of DCM/H_2O/TFA (50:50:0.1). The aqueous layer was lyophilized again and compound **4** was purified by preparative reverse phase HPLC (8 mL/min; 0-3 min, 0% B; 3-40 min 0-30% B, where A=0.1% TFA/H_2O; B= CH_3CN; **4** eluted at 20.4 min). Removal of solvent gave **4** (3.2 mg, 65%) as white powder. ^{1}H NMR (D_2O, 400 MHz) δ 8.66 (s, 1H), 8.42 (s, 1H), 6.21 (d, *J* = 5.2 Hz, 1H), 4.86-4.84 (m, 2H), 4.59 (br s, 1H), 4.25-4.21 (m, 2H), 4.01 (s, 1H), 3.84-3.80 (m, 1H), 3.72 (d, *J* = 6.4 Hz, 1H), 3.63-3.59 (m, 1H), 3.50-3.41 (m, 2H), 3.38-3.24 (overlapping m, 4H), 2.44 (t, *J* = 6.4 Hz, 2H), 2.19-2.10 (m, 1H), 0.98 (d, *J* =

6.8 Hz, 6H), 0.92 (s, 3H), 0.83 (s, 3H). HRMS (ESI-) *m/z* Calcd for $C_{26}H_{45}N_9O_{17}P_3$ 848.2146, found 848.2125.



Seryl-amide CoA (5). Amino-CoA **1** (3.3 mg, 4.4 µmol) was reacted with *N*-FMOC-(*O*-trityl)-serine (5.0 mg, 8.8 µmol), PyBOP (4.6 mg, 8.8 µmol), and DIPEA (3.1 µL, 18 µmol) in DMF/H_2O (300 µL, 4:1) for 2 h. Protecting groups were removed by treating the solution with 40% (v/v) piperidine/DMF (300 µL) for 30 min and then slowly adding 50% (v/v) TFA/H_2O until the solution turned orange. When lyophilized, the crude product was treated with 6 mL of DCM/H_2O/TFA (50:50:0.1). The aqueous layer was lyophilized again and compound **5** was purified by preparative reversed-phase HPLC (8 mL/min; 0-3 min, 0% B; 3-40 min 0-50% B, where A=0.1% TFA/H_2O; B= CH_3CN; 7 eluted at 16.9 min). Removal of solvent gave **5** (1.7 mg, 50%) as white powder. ^{1}H NMR (D_2O, 300 MHz) δ 8.66 (s, 1H), 8.41 (s, 1H), 6.22 (d, *J* = 5.7 Hz, 1H), 4.85-4.83 (m, 2H), 4.66 (br s, 1H), 4.24-4.22 (m, 2H), 4.08-4.05 (m, 1H), 4.00 (s, 1H), 3.92-3.89 (m, 1H), 3.82-3.78 (m, 1H), 3.71-3.66 (m, 1H), 3.61-3.56 (m, 1H), 3.49-3.41(m, 2H), 3.38-3.24 (overlapping m, 4H), 2.44 (t, *J* = 7.0 Hz, 2H), 0.91 (s, 3H), 0.83 (s, 3H). MS (ESI+) *m/z* Calcd for $C_{24}H_{43}N_9O_{18}P_3$ 838.19, found 838.3.



α**-Chloroacetyl-amide CoA (6).** Amino-CoA **1** (2.9 mg, 3.8 µmol) was treated with α-chloroacetic acid (1.1 mg, 12 µmol), PyBOP (6.0 mg, 12 µmol), and DIPEA (4.0 µL, 23 µmol) in 200 µL DMF/H_2O (4:1) for 2 h. The crude product was lyophilized and compound **6** was purified by preparative reverse phase HPLC (8 mL/min; 0-3 min, 0% B; 3-40 min 0-10% B, where A=0.1% TFA/H_2O; B= CH_3CN; **6** eluted at 29.7 min). Removal of solvent gave **6** (2.3 mg, 80%) as white powder ^{1}H NMR (D_2O, 300 MHz) δ 8.70 (s, 1H), 8.46 (s, 1H), 6.25 (d, *J* = 5.1 Hz, 1H), 4.90-4.88 (m, 2H), 4.62 (br s, 1H), 4.26-4.22 (m, 2H), 4.11 (s, 2H), 4.04 (s, 1H), 3.88-3.85 (m, 1H), 3.64-3.61 (m, 1H), 3.48

(t, J = 6.3 Hz, 2H), 3.33-3.28 (overlapping m, 4H), 2.47 (t, J = 6.3 Hz, 2H), 0.95 (s, 3H), 0.84 (s, 3H). HRMS (ESI+) *m/z* Calcd for $C_{23}H_{38}N_8O_{17}P_3Cl$ 827.1329, found 827.1331.

Quantitation of covalent incorporation of amino-CoA (1) and serine into apo-EntF (Figure 1). Overproduction and purification of EntF was done as described by Gehring et al[3]. Detection of the covalent incorporation of amino-CoA **1** and serine into EntF was quantified using a trichloroacetic acid (TCA) precipitation radioassay[4]. The reaction mixture (200 µL final volume) included 75 mM Tris·HCl (pH 9.0 for amino-CoA **1,** or pH 7.5 for blank control and CoA), 1 mM tris-(2-carboxyethyl)phosphine (TCEP), 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM amino-CoA **1** (or CoA, or blank)**,** 5 µM SVP PPTase (from *Streptomyces verticillus*), 800 µM L-[^{14}C]-serine (150 mCi/mmol) (PerkinElmer)/30 µM EntF. Apo protein was preincubated for 45 min at 37°C with SVP before addition of L-[^{14}C]-serine and ATP. Reactions were initiated by addition of ATP, and incubated at 37°C for 30 min. 100 µL reaction mixture was quenched with 0.8 mL 10% TCA with BSA (375 µg) added as a carrier. Precipitated proteins were centrifuged and washed three times with 10% TCA. The protein pellet was dissolved in 150 µL 1 M Tris base and the amount of incorporated [^{14}C]seryl-*N*-ppant quantified by liquid scintillation counting.

	Blank control	CoA	Amino-CoA **1**
^{14}C DPM	386	7535	7337

SDS-PAGE autoradiography analysis. 20 µL of the reaction mixture from above was quenched by adding 10 µL (3X) SDS sample buffer prior to loading and electrophoresis on a 4-20% Tris-glycine gel (ISC Bioexpress). The gel was stained with Coomassie blue, destained, and fixed in gel fixing buffer (10% methanol, 5% acetic acid) prior to drying. The dried gel was exposed to a phosphorimaging K-screen (Bio-Rad) for 7 days prior to imaging by Molecular Imager FX (Bio-Rad).

Preparation of holo-EntF and seryl amide EntF construct for kinetic assays. The reaction mixture (2 mL final volume) included 75 mM Tris·HCl (pH 9.0 for seryl amide EntF or pH 8.0 for holo EntF), 1 mM TCEP, 10 mM $MgCl_2$, 0.5 mM amino-CoA **1** (or CoA),

5 μM SVP PPTase, 10 μM apo-EntF. The reactions were incubated for 45 min at 37°C to yield amino-ppant EntF and holo-EntF.

0.8 mM L-[^{14}C]-serine and 5 mM ATP were added to amino-ppant EntF solution, and incubated at 37°C for 30 min to give the seryl amide EntF Construct.

Preparation of a-chloroacetyl amide EntF construct. Reaction mixture (2 mL final volume) included 75 mM Tris·HCl (pH 8.0), 1 mM TCEP, 10 mM $MgCl_2$, 0.5 mM a-chloroacetyl amide CoA **6,** 5 μM SVP PPTase, 10 μM apo-EntF. The reaction was incubated for 45 min at 37°C to give the a-chloroacetyl amide EntF construct.

Assay for adenylation reaction catalyzed by EntF (or seryl amide EntF construct) A domain: ATP-[^{32}P]PP_i exchange assay. ATP-pyrophosphate exchange was assayed following the procedure described by Rusnak[5] with several modifications: Reaction mixtures included (final volume 100 μL): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 5 mM ATP, 1 mM sodium [^{32}P]pyrophosphate (~5 Ci/mol) and 20 nM holo-EntF or seryl amide EntF construct. The assay was initiated by the addition of increasing concentrations of serine and incubated at 24 °C for 5 min, respectively (each concentration was assayed in duplicate). Reactions were quenched by the addition of 0.5 mL of a charcoal suspension (1.6% (w/v) activated charcoal, 0.1 M tetrasodium pyrophosphate, 0.35 M perchloric acid), and then centrifuged. The liquid was removed, and the charcoal pellet was washed three times with 0.8 mL of washing solution (0.1 M tetrasodium pyrophosphate, 0.35 M perchloric acid). The pellet was then suspended in 0.6 mL of water and added to 3 mL of scintillation fluid; ^{32}P incorporation into ATP was quantified by liquid scintillation counting.

DTNB assay for AcSNAC substrate hydrolysis catalyzed by EntF TE domain. The assay was done by following the protocol described by Yeh *et al*[6]. Freshly prepared solutions (200 μL each) of DTNB (2 mM), potassium phosphate (20mM, pH 7.5), and increasing concentrations of *N*,*S*-diacetylcysteamine (AcSNAC). AcSNAC was dissolved in DMSO (6 μL DMSO in each assay). 50-400 μM AcSNAC, as substrate inhibition was observed at higher concentrations, were mixed with enzyme EntF or α-chloroacetyl amide EntF construct (2 μM) at 24ºC. Generation of free thol after thioester hydrolysis was assayed by DTNB (TNB^{2-}:e_{412nm}=13,600 $M^{-1}\cdot cm^{-1}$). The formation of 3-thio-5-nitrobenzoate was continuously monitored in a UV-Vis spectrophotometer (412 nm) for 5 min.

Background hydrolysis of AcSNAC was obtained by monitoring the same solution without enzyme and deducted from the initial velocities.

References.

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