The Nonribosomal Peptide Synthetase Enzyme DdaD Tethers N_{β} -fumaramoyl-L-2,3-diaminopropionate for Fe(II)/ α -ketoglutarate-Dependent Epoxidation by DdaC During Dapdiamide Antibiotic Biosynthesis.

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I. Supplemental Materials and Methods

A. Materials and General Methods for Preparation of N_β RREpSmDAP and N_β SSEpSmDAP.

Chemicals used for synthesis were purchased from Sigma-Aldrich unless otherwise noted. Reported yields are unoptimized. Nuclear magnetic resonance (NMR) solvents were purchased from Cambridge Isotopes Laboratories. NMR spectra were recorded on a Varian 400 MHz or 600 MHz spectrometer. Chemical shifts are reported in parts-per-million (ppm) downfield from tetramethylsilane. For ^1H -NMR experiments, residual solvent resulting from incomplete deuteration served as the internal standard (methanol- d_4 δ 3.31, D_2O δ 4.75).¹ For ^{13}C -NMR experiments in $\text{DMSO}-d_6$ and methanol- d_4 , the solvent served as the internal standard ($\text{DMSO}-d_6$ δ 39.51, methanol- d_4 δ 49.15). For ^{13}C -NMR experiments in D_2O , 2% methanol was added as an internal standard (methanol δ 49.15). NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet), coupling constants, and integration. Mass spectrometry (MS) analysis was performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF liquid chromatography (LC)-mass spectrometry (MS) instrument.

B. Preparation of N_β RREpSmDAP and N_β SSEpSmDAP.

N_β -(R,R)-epoxysuccinoyl-DAP(BOC)-OtBu (**1a**).

In a 25 mL pear flask with magnetic stir bar, N_α -Boc-DAP-OtBu (Bachem) (195 mg, 750 μmol , 1 equiv.) was dissolved in dimethylformamide (DMF) (8 mL). (R,R)-epoxysuccinate (Wako) (498 mg, 3.8 mmol, 5 equiv.) was added, followed by N,N -diisopropylethylamine (DIEA) (130 μL , 750 μmol , 1 equiv.), then 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (173 mg, 900 μmol , 1.2 equiv.). The reaction was stirred at room temperature for 12 h. The reaction was diluted with ethyl acetate (EtOAc) (40 mL), and the organic layer was washed with 100 mM HCl (2×40 mL) and then saturated aqueous NaCl (2×40 mL). The organic layer was dried (Na_2SO_4) and then concentrated *in vacuo* to afford a white solid (222 mg, 79% yield). ^1H NMR (600 MHz, METHANOL- d_4) δ ppm 1.45 (s, 9 H) 1.47 (s, 9 H) 3.49 (dd, $J=13.5$, 7.8 Hz, 1 H) 3.54 (d, $J=1.8$ Hz, 1 H) 3.58 - 3.64 (m, 2 H) 4.18 (t, $J=5.9$ Hz, 1 H). ^{13}C NMR (151 MHz, METHANOL- d_4) δ ppm 28.35, 28.85, 41.58, 53.25, 54.54, 55.50, 80.87, 83.41, 157.86, 169.12, 170.31, 171.21. HRMS (ESI⁺): $[\text{M}+\text{H}]^+$ m/z calcd. for $\text{C}_{16}\text{H}_{27}\text{N}_2\text{O}_8^+$, 375.1762; found, 375.1754.

N_β -(S,S)-epoxysuccinoyl-DAP(BOC)-OtBu (**1b**).

1b was prepared as described above for **1a** except that the reaction was stirred at room temperature for 17 h. This yielded a white solid (98.1 mg, 69% yield). ^1H NMR (600 MHz, METHANOL- d_4) δ ppm 1.45 (s, 9 H) 1.47 (s, 9 H) 3.51 - 3.61 (m, 4 H) 4.18 (dd, $J=6.9$, 5.1 Hz, 1 H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ ppm 27.56, 28.15, 48.62, 51.52, 52.86, 53.91, 78.41, 80.83, 155.31, 165.72, 168.54, 169.62. HRMS (ESI⁺): $[\text{M}+\text{H}]^+$ m/z calcd. for $\text{C}_{16}\text{H}_{27}\text{N}_2\text{O}_8^+$, 375.1762; found, 375.1764.

N_β -(R,R)-epoxysuccinamoyl-DAP(BOC)-OtBu (**2a**).

In a 25 mL round bottom flask with magnetic stir bar under argon, acid **1a** (49.9 mg, 130 μ mol, 1 equiv.) was dissolved in tetrahydrofuran (THF) (5 mL). The solution was stirred at -10 °C, and isobutyl chloroformate (21 μ L, 160 μ mol, 1.2 equiv.) dissolved in 500 μ L THF was added dropwise to the reaction. The reaction was stirred at -10 °C for 4 min. A 1.48 M aqueous NH_4OH solution¹ (J.T. Baker) (270 μ L, ~400 μ mol, ~3 equiv.) was added dropwise to the stirring solution. The reaction was warmed to room temperature and stirred for 3 h. The crude product was purified by silica gel chromatography (15:1 dichloromethane-methanol) to afford a clear solid (15.3 mg, 31% yield). ¹H NMR (600 MHz, METHANOL-*d*₄) δ ppm 1.45 (s, 9 H) 1.47 (s, 9 H) 3.48 - 3.53 (m, 2 H) 3.55 (d, *J*=1.8 Hz, 1 H) 3.59 (dd, *J*=13.8, 5.1 Hz, 1 H) 4.15 (dd, *J*=7.2, 5.1 Hz, 1 H) ¹³C NMR (151 MHz, METHANOL-*d*₄) δ ppm 28.37, 28.85, 41.58, 54.67, 54.90, 55.67, 80.91, 83.44, 157.94, 169.25, 171.27, 171.30. HRMS (ESI⁺): [M+Na]⁺ *m/z* calcd. for $\text{C}_{16}\text{H}_{27}\text{N}_3\text{NaO}_7^+$, 396.1741; found, 396.1751.

*N*_β-(*S,S*)-epoxysuccinamoyl-DAP(BOC)-*Orbu* (**2b**).

2b was prepared as described above for **2a** to yield a clear solid (46 mg, 65% yield). ¹H NMR (600 MHz, METHANOL-*d*₄) δ ppm 1.45 (s) 1.47 (s) 3.49 - 3.61 (m) 4.17 (t). ¹³C NMR (100 MHz, METHANOL-*d*₄) δ ppm 28.37, 28.86, 41.53, 54.76, 54.87, 55.66, 80.89, 83.41, 157.91, 169.24, 171.25, 171.27. HRMS (ESI⁺): [M+H]⁺ *m/z* calcd. for $\text{C}_{16}\text{H}_{28}\text{N}_3\text{O}_7^+$, 374.1922; found, 374.1924.

*N*_β-(*R,R*)-epoxysuccinamoyl-DAP (**3a**).

In a 25 mL round bottom flask with magnetic stir bar, amide **2a** (8.2 mg, 22 μ mol, 1 equiv.) was dissolved in dichloromethane (4 mL). The solution was stirred at 0 °C. Trifluoroacetic acid (TFA) (1 mL) was added dropwise to the stirring solution. The solution was warmed to room temperature and stirred at room temperature for 12 h. Solvent and TFA were removed *in vacuo* to afford a tan solid (6.5 mg, 88% yield). ¹H NMR (600 MHz, DEUTERIUM OXIDE) δ ppm 3.67 (d, *J*=1.8 Hz, 1 H) 3.70 (d, *J*=1.8 Hz, 1 H) 3.73 (dd, *J*=15.0, 6.3 Hz, 1 H) 3.83 (dd, *J*=15.0, 4.0 Hz, 1 H) 4.06 (dd, *J*=6.3, 4.0 Hz, 1 H). ¹³C NMR (100 MHz, DEUTERIUM OXIDE + 2% Methanol) δ ppm 39.25, 53.68 (2C), 53.87, 170.07, 170.40, 171.17. HRMS (ESI⁺): [M+H]⁺ *m/z* calcd. for $\text{C}_7\text{H}_{12}\text{N}_3\text{O}_5^+$, 218.0771; found, 218.0771.

*N*_β-(*S,S*)-epoxysuccinamoyl-DAP (**3b**).

In a 1 dram vial with magnetic stir bar, amide **2b** (15.3 mg, 41 μ mol, 1 equiv.) was dissolved in dichloromethane (1 mL), and TFA (250 μ L) was added. The solution was stirred at room temperature for 15 h. Solvent and TFA were removed *in vacuo* to afford a white solid (8.9 mg, 66% yield). ¹H NMR (600 MHz, DEUTERIUM OXIDE) δ ppm 3.67 - 3.73 (m, 3 H) 3.82 (dd, *J*=15.0, 4.1 Hz, 1 H) 4.02 (dd, *J*=6.5, 4.1 Hz, 1 H). ¹³C NMR (100 MHz, DEUTERIUM OXIDE + 2% Methanol) δ ppm 39.94, 53.71, 53.88, 54.94, 169.91, 171.22, 171.88. HRMS (ESI⁺): [M+H]⁺ *m/z* calcd. for $\text{C}_7\text{H}_{12}\text{N}_3\text{O}_5^+$, 218.0771; found, 218.0784.

C. LC-MS Assay for DdaF Activity with *N*_βEpSmDAP and Val.

Reaction mixtures (50 μ L) contained 9.5 μ M DdaF, 500-600 μ M *N*_βEpSmDAP, 1 mM Val, 3 mM ATP, 4 mM MgCl_2 , and 50 mM HEPES (pH 8). Reactions were incubated for 2 h at room temperature, then

¹ This concentration was calculated by assuming that the commercial solution of “28-30% Ammonium Hydroxide” (density 0.9 g/mL) contains 28 g NH_3 per 100 g of solution. This 14.8 M solution was diluted 1:10 with water to provide the 1.48 M solution.

filtered through 5 kDa molecular weight cut-off (MWCO) filter tubes at $12,000 \times g$ for 15 min at 4 °C. Filtered reactions were diluted 1:100 in water and 10 μ L of this solution was analyzed by LC-MS. A Thermo Scientific Hypercarb (50 $\times$ 2.1 mm) column was used for LC separation, with a flow rate of 0.4 mL/min. The 15 min separation gradient used for LC-MS analysis is shown in **Table S4**, where solvent A was H₂O + 0.1% formic acid and solvent B was acetonitrile (MeCN) + 0.1% formic acid. MS analysis was performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-MS in the positive ion mode with a gas temperature of 300 °C and a fragmentor voltage of 105.

D. Cloning, Overexpression, and Purification of DdaC and DdaD.

ddaC and *ddaD* were PCR amplified from pUC19 A10A using the primers shown in **Table S5**. Unpurified PCR products were mixed with the pENTR/D-TOPO (*ddaC*) or pENTR/SD/D-TOPO (*ddaD*) vectors and the TOPO construct was then transformed into TOP10 *E. coli*. Purified vector was analyzed by PCR to confirm the presence of insert. The *ddaC* insert was recombined into pDEST17, encoding an N-terminal His₆ tag and the *ddaD* insert was recombined into pET301/CT-DEST, encoding a C-terminal His₆ tag. The product of this reaction was used to transform TOP10 *E. coli*. Purified vector was sequenced to confirm the insert. BL21(DE3) *E. coli* were transformed with pDEST17-*ddaC* or pET301/CT-DEST-*ddaD*. 75 mL cultures were grown overnight at 37 °C in Luria-Bertani (LB) medium supplemented with 50 μ g/mL carbenicillin. 2 L LB with 50 μ g/mL carbenicillin was inoculated with 10 mL of this overnight culture. The cultures were incubated at 37 °C for 3-4 h, then 15 °C for 30 min. Cultures were induced with 100 μ M IPTG. The cultures were incubated at 15 °C overnight, and then the cells were harvested by centrifugation at $2248 \times g$ (4,200 rpm in a Beckman JS-4.2 rotor) for 12 min. Cell pellets were resuspended in 10 mL lysis buffer (DdaC lysis buffer: 20 mM Tris-HCl (pH 8), 300 mM NaCl, 10% glycerol; DdaD lysis buffer: 20 mM Tris-HCl (pH 7.5), 300 mM NaCl) and lysed at 5,000-10,000 psi in an Avestin EmulsiFlex-C5 high-pressure homogenizer. The cell debris was removed by ultracentrifugation at $49167 \times g$ (35,000 rpm in a Beckman Type 45 Ti rotor) for 35 min. Imidazole was added to the supernatants to a final concentration of 2 mM, and then the supernatants were applied to a low pressure chromatography column containing Ni-NTA resin pre-equilibrated in lysis buffer. The resin was washed with lysis buffer containing 25 mM imidazole. The protein was eluted with lysis buffer containing 80 mM and then 200 mM imidazole. Protein was concentrated and exchanged into storage buffer (DdaC storage buffer: 20 mM Tris-HCl (pH 8), 50 mM NaCl, 10% glycerol; DdaD storage buffer: 20 mM Tris-HCl (pH 7.5), 50 mM NaCl) using 10 or 30 kDa MWCO Amicon Ultra filters (Millipore) and/or dialysis in Slide-A-Lyzer 10 kDa MWCO dialysis cassettes (Thermo Scientific). Aliquots of the proteins were flash-frozen in N₂(l) and stored at -80 °C, or in some cases for DdaC, subjected to further anaerobic purification.

E. Anaerobic Purification of DdaC.

Ni-NTA purified N-His₆-DdaC was further purified by gel filtration on a Hi Load 26/60 Superdex 75 prep grade column (Amersham Biosciences) using Ar-sparged DdaC storage buffer as the eluent. Fractions were collected in a glove box under a nitrogen atmosphere. Fractions containing apo-DdaC were concentrated using an Amicon Stirred Ultrafiltration Cell (Millipore). The protein was incubated with 2 mM dithiothreitol (DTT), 2 mM α -ketoglutarate (α -KG), and 1 mM Fe(NH₄)₂(SO₄)₂ for 3-4 min, and then desalted using Bio-Gel P6-DG desalting gel (Biorad), pre-equilibrated with DdaC storage buffer. Protein-containing fractions were concentrated as described previously. Aliquots were prepared, removed from the glove box, and immediately flash-frozen in N₂(l). The tubes were stored at -80 °C.

F. Ferene Assay for DdaC Iron Determination.

A ferene² solution containing 0.75 mM ferene (3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt), 10 mM ascorbic acid, and 45% (weight/volume) sodium acetate trihydrate was prepared fresh the day of the assay, 0.45 μ m syringe filtered, and kept in the dark on ice. A standard curve was generated by preparing 800 μ L solutions containing 0-32 nmol FeCl₃ in 13 $\times$ 100 mm borosilicate glass tubes. To each of these solutions was added 100 μ L 12 N HCl, 100 μ L 80% trichloroacetic acid (TCA), 200 μ L 45% sodium acetate trihydrate, and 1.8 mL ferene solution. In a microcentrifuge tube, water was added to the protein sample to bring the volume to 800 μ L, then 100 μ L 12 N HCl was added, and the sample was mixed every 2 min for 10 min. 100 μ L 80% TCA was added, and the sample was centrifuged at 16,000 $\times$ g for 10 min at room temperature. The supernatant was transferred to a 13 $\times$ 100 mm borosilicate glass tube, and 200 μ L 45% sodium acetate trihydrate was added. The solution was mixed, then 1.8 mL ferene solution was added. The optical density (OD) at 593 nm of each sample was measured using a Cary 50 Bio UV-Visible Spectrophotometer (Varian).

G. DdaC/D Enzymatic Assays with Anaerobically Purified DdaC.

Phosphopantetheinylation reaction conditions were the same as those for the reactions with aerobically purified DdaC (See DdaC/D Enzymatic Assays with Aerobically Purified DdaC in the Materials and Methods Section). The *N* _{β} FmmDAP-*S*-DdaD preparations were transferred to a glovebox under a nitrogen atmosphere and allowed to equilibrate during the incubation with *N* _{β} FmmDAP and ATP. In the glovebox, a stock solution of 100 mM α -KG in 100 mM HEPES (pH 7.5) was added to the reactions to a final concentration of 2 mM. Then, an aliquot of anaerobically purified DdaC was thawed and immediately added to the reaction to a final concentration of 6.8 μ M (final volume 100 μ L). Reactions were removed from the glove box, exposed to ambient atmosphere, and incubated for 1 h at room temperature. Reactions were quenched and analyzed as described for the reactions with aerobically purified DdaC in the main text.

H. DdaD ATP-[³²P]PP_i Exchange Kinetics.

Assays were performed as described in the main text with the following exceptions: reaction mixtures (60 μ L) contained 100 nM DdaD for *N* _{β} FmmDAP reactions and 5 μ M DdaD for *N* _{β} SSEpSmDAP reactions. Reactions additionally contained 13.3 mM borate·NaOH (pH 9.5).

I. BODIPY-Coenzyme A Fluorescent Phosphopantetheinylation Assay.

Reaction mixtures (50 μ L) contained 5 μ M DdaD, 1 μ M Sfp, 5 μ M BODIPY-CoA³, 10 mM MgCl₂, 1 mM dithiothriitol (DTT), and 50 mM HEPES (pH 7.5). Reactions were incubated in the dark at room temperature for 1 h. Reaction mixtures were diluted 1:1 in 2 $\times$ Laemmli sample buffer (Biorad), boiled for 10 min, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% Tris-glycine gel. The gel was first imaged at λ =365 nm, then stained with Coomassie and imaged again.

J. Hydrolysis of the *N* _{β} FmmDAP-*S*-DdaD Thioester and Detection of Free *N* _{β} FmmDAP by LC-MS.

Phosphopantetheinylation reactions (150 μ L) contained 50 μ M DdaD, 12 μ M Sfp, 400 μ M CoA, 10 mM MgCl₂, 1.5 mM DTT, and 50 mM HEPES (pH 7.5). Reactions were incubated at room temperature for 1.5 h. 123 μ M *N* _{β} FmmDAP and 500 μ M ATP were added (final volume 155 μ L) and the reactions incubated an additional 1 h at room temperature. To wash away free substrate, the reactions were applied

to 10 kDa MWCO filter tubes and centrifuged at $16,000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 15 min, then 450 μL 50 mM HEPES (pH 7.5) was added to the retentate and the samples were mixed. The tubes were centrifuged and this process was repeated five times. The volume of the retentate was adjusted to 140 μL with 50 mM HEPES (pH 7.5), and the reactions incubated at room temperature for 70 min. The reactions were centrifuged at $16,000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 15 min, and the flow through was lyophilized overnight. The residue was dissolved in 20 μL H_2O , and 10 μL of this solution was analyzed by LC-MS. A Thermo Scientific Hypercarb ($50 \times 2.1\text{ mm}$) column was used for LC separation, with a flow rate of 0.4 mL/min. The 15 min separation gradient used for LC-MS analysis is shown in **Table S1**, where solvent A was $\text{H}_2\text{O} + 0.1\%$ formic acid and solvent B was MeCN + 0.1% formic acid. MS analysis was performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-MS in the positive ion mode with a gas temperature of $300\text{ }^{\circ}\text{C}$ and a fragmentor voltage of 105.

K. MSⁿ Analysis of DdaD Phosphopantetheinyl (Ppant) Ejection Ions.

The following methods were used to characterize the tandem mass spectral fragmentation patterns of Ppant ejection ions generated from 1) HS-DdaD loaded with synthetic $N_{\beta}\text{RREpSmDAP}$, 2) HS-DdaD loaded with synthetic $N_{\beta}\text{SSEpSmDAP}$, and 3) $N_{\beta}\text{EpSmDAP-S-DdaD}$ generated by incubation of $N_{\beta}\text{FmmDAP-S-DdaD}$ with DdaC. The fragment ions used for MSⁿ analyses were selected based on the observed shift of +16 Da when compared to the fragment ions generated from MSⁿ analysis of the Ppant ejection ion from $N_{\beta}\text{FmmDAP-S-DdaD}$.

Each reaction was completed as previously described, quenched with an equal reaction volume of 25% formic acid, and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. For these analyses, the same reversed phase (RP)LC columns, RPLC methods, and Fourier Transform mass spectrometry (FTMS) instrumentation used for analysis of intact DdaD (as described in the accompanying manuscript) were used. A reaction aliquot containing 20 μg of DdaD was injected onto the column, separated according to the gradient shown in **Table S5**, and all proteins eluting into the mass spectrometer were subjected to the following MS analyses. The MS analyses of each species included the following: (1) full scan measurement of all intact proteins (all ions detected in the ion trap MS in profile mode; m/z range detected 400-2000), (2) the Ppant ejection assay using nozzle-skimmer dissociation (NSD) (all ions detected in the FTMS in profile mode, resolution 50 000, m/z range 250-500, SID = 75 V), (3) MS³ on the Ppant ejection ion generated at m/z 460.2, (4) MS⁴ on the fragment ion at m/z 311 generated from MS³ on the Ppant ejection ion at m/z 460.2, (5) MS⁵ on the fragment ion at m/z 200 generated from MS⁴ on the fragment ion at m/z 311 generated from MS³ on the Ppant ejection ion at m/z 460.2, (6) MS⁵ on the fragment ion at m/z 216 generated from MS⁴ on the fragment ion at m/z 311 generated from MS³ on the Ppant ejection ion at m/z 460.2. All MSⁿ analyses used collision induced dissociation (CID) with the following parameters: detection of all ions in the ion trap MS in profile mode, isolation width 5 m/z , activation q value 0.25, activation time 30 ms, NCE 35. All data were analyzed using Qualbrowser (Xcalibur) as described previously.

II. Supplemental Tables

Table S1. Gradient used for RPLC separation of tryptic digests of DdaD-containing reactions.

Time (min)	% Solvent B
0	10
5	10
35	55
42	75
47	95
50	10
70	10

Table S2. Gradient used for RPLC separation of reactions containing intact DdaD.

Time (min)	% Solvent B
0	10
5	10
20	55
25	75
30	95
31	10
45	10

Table S3. Summary of all DdaD-related peptides and Ppant ejection ions observed in this study.

Active Site Peptide*	Mass (Da, Theor)	Mass (Da, Exp)	Error (ppm)	Shift from <i>apo</i> -DdaD (Da, Theor)	Shift from <i>apo</i> -DdaD (Da, Exp)	Ppant Ejection Ion (1+, Da, Theor)	Ppant Ejection Ion (1+, Da, Exp)	Error (Ppant Ejection Ion, ppm)
<i>apo</i> -DdaD	2919.415	2919.420	1.7	0.00	0.01	-	N/A	-
HS-DdaD	3259.505	3259.506	0.4	340.09	340.09	261.1267	261.1273	2.7
<i>N</i> _β FmmDAP- <i>S</i> -DdaD	3442.569	3442.572	0.9	523.15	523.16	444.1911	444.1920	1.8
<i>N</i> _β EpSmDAP- <i>S</i> -DdaD	3458.564	3458.568	1.1	539.15	539.15	460.1860	460.1870	2.2
<i>N</i> _β EpSmDAP- <i>S</i> -DdaD (labeled with ¹⁸ O from H ₂ ¹⁸ O)	3460.569	N/D	-	541.15	-	462.1908	N/D	-
<i>N</i> _β EpSmDAP- <i>S</i> -DdaD (labeled with ¹⁸ O from ¹⁸ O ₂)	3460.569	-	-	541.15	-	462.1908	462.1898	-1.1
Dapdiamide A- <i>S</i> -DdaD	3541.638	N/D	-	622.22	-	543.2601	N/D	-

Theor: theoretical

Exp: experimental

ppm: parts-per-million

*N*_βFmmDAP: *N*_β-fumaramoyl-DAP*N*_βEpSmDAP: *N*_β-epoxysuccinamoyl-DAP

N/D: not detected

-: calculation not possible

*: all active site peptide masses are reported as the neutral monoisotopic mass

Table S4. Gradient used for RPLC separation of DdaF reactions and *N*_βFmmDAP-*S*-DdaD thioester hydrolysis reactions.

Time (min)	% Solvent B
0	2
2	2
12	100
14	100
15	2

Table S5. Sequences of primers used for amplification of *ddaC* and *ddaD*.

Primer	Sequence (5' to 3')
<i>ddaC</i>	
Forward	CACCAGCCATTCTTATAATGTTTCGTCCCTTG
Reverse	TCAACGCTGTGCATGAGGATTCTCCTC
<i>ddaD</i>	
Forward	CACCATGCACAGCGTTGAGACTTTTAACTTG
Reverse	ACTCTCCGCCTGCACAGAAGATGCATC

III. Supplemental Figures

Figure S1. LC-MS observation of N_β EpSmDAP-Val following incubation of DdaF, N_β EpSmDAP, Val, and ATP. DdaF ligates both diastereomers ((2*R*, 3*R*) and (2*S*,3*S*)) of N_β -*trans*-EpSmDAP to Val in an ATP-dependent manner.

Reaction	N_β EpSmDAP observed <i>m/z</i>	N_β EpSmDAP-Val observed <i>m/z</i>
N_β RREpSmDAP + Val + ATP	Not observed	317.1457
N_β RREpSmDAP + Val - ATP	218.0771	Not observed

Reaction	N_β EpSmDAP observed <i>m/z</i>	N_β EpSmDAP-Val observed <i>m/z</i>
N_β SSEpSmDAP + Val + ATP	Not observed	317.1455
N_β SSEpSmDAP + Val - ATP	218.0769	Not observed

Figure S2. Reaction velocity vs. substrate concentration curves determined for DdaF by coupled spectrophotometric ADP production assay. The concentration of DdaF in these assays was 250 nM. Curve fits determined using GraphPad Prism are shown. A nonlinear, least squares fitting method was used for N_{β} FmmDAP and N_{β} RREpSmDAP, and a linear regression was used for N_{β} SSEpSmDAP.

Figure S3. SDS-PAGE of purified His₆-tagged DdaC, DdaD, and DdaF. 4-15% Tris-HCl gel (Biorad). (MW = PageRuler™ Prestained Protein Ladder (Fermentas)).

Figure S4. DdaD ATP- ^{32}P PP_i exchange velocity vs. substrate concentration plot for N_{β} FmmDAP and N_{β} SSEpSmDAP.² The concentration of DdaD was 100 nM for N_{β} FmmDAP reactions and 5 μM for N_{β} SSEpSmDAP reactions. Curve fits determined using GraphPad Prism are shown. A nonlinear, least squares fitting method was used for N_{β} FmmDAP and a linear regression was used for N_{β} SSEpSmDAP.

Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\text{min}^{-1}\cdot\text{mM}^{-1}$)
N_{β} FmmDAP	64 ± 5	420 ± 80	150
N_{β} SSEpSmDAP*	ND	ND	2.3 ± 0.1

*Saturation not achieved at concentrations up to 1.6 mM.

Figure S5. Loading of DdaD with BODIPY-CoA by Sfp.

² Data are presented as value $\pm$ standard deviation. ND = not determined.

Figure S6. Predicted structures of all theoretical Ppant ejection ions.

Protein (or Active Site Peptide)	Theoretical Ppant Ejection Ion Structure	Theoretical Exact Mass (1+)
H S-DdaD		261.1267 Da
N_{β} FmmDAP-S-DdaD		444.1911 Da
N_{β} EpSmDAP-S-DdaD		460.1860 Da
N_{β} EpSmDAP-S-DdaD (labeled with ^{18}O)		462.1908 Da
Dapdiamide A-S-DdaD		543.2601 Da

Figure S7. Localization of the Ppant arm to the active site serine (Ser) of the DdaD thiolation (T) domain. MS/MS of the DdaD active site peptide using CID resulted in localization of the Ppant arm to a region of the peptide containing the predicted active site Ser residue. Fragment ions containing the +340.09 Da mass shift from phosphopantetheinylation are shown in red, while fragment ions not showing the mass shift are shown in black. Note that the peptide shown (corresponding to residues I(514)-K(545)) contains one N-terminal and one C-terminal missed trypsin cleavage, while the peptides shown in all other figures are the fully trypsin-digested active site peptides.

Figure S8. Hydrolysis of the N_β FmmDAP-*S*-DdaD thioester and detection of free N_β FmmDAP by LC-MS. HS-DdaD was incubated with ATP and N_β FmmDAP. Substrate-loaded DdaD was washed and then incubated at room temperature for 70 min. The filtered hydrolysis reaction was analyzed by LC-MS.

Total ion current chromatogram

UV 220nm

Extracted ion chromatogram

MS

Figure S9. N_β RREpSmDAP and N_β SSEpSmDAP are loaded onto HS-DdaD, as determined by Top Down FTMS analysis and the Ppant ejection assay. (A) In the absence of the substrate N_β FmmDAP and presence of ATP, only the Ppant ejection ion from HS-DdaD (red) is observed. (B) In the presence of N_β FmmDAP and ATP, the Ppant ejection ion from N_β FmmDAP-S-DdaD (blue) is observed, confirming loading of this substrate onto HS-DdaD. (C) In the presence of ATP, N_β FmmDAP and DdaC, HS-DdaD is loaded with the substrate and DdaC catalyzes formation of the epoxide to generate N_β EpSmDAP-S-DdaD (the Ppant ejection ion from this species is shown in orange). (D) In the presence of N_β RREpSmDAP and ATP, the Ppant ejection ion from N_β RREpSmDAP-S-DdaD (pink) is observed, confirming loading of this substrate onto HS-DdaD. (E) In the presence of N_β SSEpSmDAP and ATP, the Ppant ejection ion from N_β SSEpSmDAP-S-DdaD (purple) is observed, confirming loading of this substrate onto HS-DdaD. The species in purple and pink are the exact same mass as the species in orange. For the predicted structures of the Ppant ejection ions, see **Figure S6**.

Figure S10. HS-DdaD is not loaded with dapdiamide A in the presence of substrate and ATP. (A) In the presence of ATP and absence of dapdiamide A, only the active site peptide from HS-DdaD (red) is observed. (B) In the presence of ATP and dapdiamide A, the only active site peptide observed is from HS-DdaD (red). No active site peptide corresponding to the loading of dapdiamide A onto HS-DdaD is observed. The theoretical m/z value of the 3+ charge state of the dapdiamide A-S-DdaD active site peptide is denoted by the green box. (C) Subjecting the peptides to MS/MS using NSD after incubation with both ATP and dapdiamide A results only in a Ppant ejection ion from HS-DdaD and not dapdiamide A-S-DdaD (the theoretical m/z value of the 1+ Ppant ejection ion is denoted by the green box). For the predicted structures of the Ppant ejection ions, see **Figure S6**.

Figure S11. Aerobically purified DdaC converts N_{β} FmmDAP-S-DdaD to N_{β} EpSmDAP-S-DdaD. (A) In the presence of α -KG but the absence of DdaC, only the active site peptide from N_{β} FmmDAP-S-DdaD (blue) is observed. (B) In the presence of both α -KG and DdaC (purified aerobically), N_{β} FmmDAP-S-DdaD is converted to N_{β} EpSmDAP-S-DdaD and the active site peptide from N_{β} EpSmDAP-S-DdaD (orange) is observed. (C) In the presence of α -KG and the absence of DdaC, only the small molecule Ppant ejection ion from N_{β} FmmDAP-S-DdaD (blue) is observed when the peptides are subjected to MS/MS using NSD. (D) In the presence of both α -KG and DdaC (purified aerobically), the Ppant ejection ions from both N_{β} FmmDAP-S-DdaD and N_{β} EpSmDAP-S-DdaD (orange) are observed when the peptides are subjected to MS/MS using NSD. For the predicted structures of the Ppant ejection ions, see **Figure S6**.

Figure S12. The conversion of N_{β} FmmDAP-*S*-DdaD to N_{β} EpSmDAP-*S*-DdaD is dependent on the presence of α -KG. (A) In the presence of DdaC and the absence of α -KG, N_{β} FmmDAP-*S*-DdaD (blue) is not oxidized. (B) In the presence of both DdaC and α -KG, N_{β} FmmDAP-*S*-DdaD is oxidized to N_{β} EpSmDAP-*S*-DdaD (orange). (C) In the presence of DdaC and the absence of α -KG, when the DdaD active site peptide is subjected to MS/MS using NSD, only the Ppant ejection ion corresponding to N_{β} FmmDAP-*S*-DdaD (blue) is observed. (D) In the presence of DdaC and α -KG, when the DdaD active site peptides are subjected to MS/MS using NSD, the Ppant ejection ions corresponding to both N_{β} FmmDAP-*S*-DdaD and N_{β} EpSmDAP-*S*-DdaD (orange) are observed. For the predicted structures of the Ppant ejection ions, see **Figure S6**.

Figure S13. Comparison of Ppant ejection ion tandem mass spectral fragmentation patterns between HS-DdaD loaded with synthetic N_{β} RREpSmDAP, HS-DdaD loaded with synthetic N_{β} SSEpSmDAP, and N_{β} EpSmDAP-S-DdaD generated by incubation of N_{β} FmmDAP-S-DdaD with DdaC. The peaks selected for analysis all showed a +16 Da shift when compared to fragmentation of the Ppant ejection ion from N_{β} FmmDAP-S-DdaD. (A) Comparison of fragmentation patterns from MS³ on the target ion at m/z 460.2 (the N_{β} EpSmDAP-S-DdaD Ppant ejection ion) generated from MS² on loaded DdaD. (B) Comparison of fragmentation patterns from MS⁵ on the target ion at m/z 311, generated from MS⁴ on the target ion at m/z 444.2, generated by water loss from the Ppant ejection ion at m/z 460.2 during MS³. (C) Comparison of fragment ions generated from MS⁶ on the target ion at m/z 200, generated from MS⁵ on the fragment ion at m/z 311, generated from MS⁴ on the fragment ion at m/z 442.2, the peak that is generated by a water loss from the Ppant ejection ion at m/z 460.2. (D) Comparison of fragment ions generated from MS⁶ on the target ion at m/z 216, generated from MS⁵ on the fragment ion at m/z 311, generated from MS⁴ on the fragment ion at m/z 442.2, the peak that is generated by a water loss from the Ppant ejection ion at m/z 460.2.

Figure S14. An oxygen atom from the reaction solvent is not incorporated into N_{β} EpSmDAP-S-DdaD, as determined from analysis of the epoxidation reaction in $H_2^{18}O$. (A) In the presence of α -KG and the absence of DdaC, only the active site peptide from N_{β} FmmDAP-S-DdaD (blue) is observed. (B) In the presence of α -KG and DdaC, and under reaction conditions in $H_2^{18}O$, a +16 Da mass increase is observed in the tryptic active site peptide from N_{β} EpSmDAP-S-DdaD (orange). A +18 Da mass shift would be observed if the ^{18}O atom from the solvent was incorporated during oxidation; this indicates that the oxygen atom is not incorporated from the solvent. (C) In the presence of α -KG and the absence of DdaC, only the Ppant ejection ion from N_{β} FmmDAP-S-DdaD (blue) is observed when the peptides are subjected to MS/MS using NSD. (D) In the presence of α -KG and DdaC, and under reaction conditions in $H_2^{18}O$, a +16 Da mass increase is observed in the Ppant ejection ion generated from DdaD during MS/MS using NSD. Again, because a +18 Da shift was not observed, it is concluded that the oxygen atom in the epoxide is not solvent-derived. If the oxygen atom from the solvent was incorporated, a Ppant ejection at m/z 462.1903 would be observed. For the predicted structures of the Ppant ejection ions, see **Figure S6**.

IV. Supplemental Schemes

Scheme S1. Ppant ejection assay. The Ppant ejection assay relies on the elimination of the Ppant functionality from intact or trypsin-digested T domains during tandem mass spectrometry to generate two different Ppant ejection peptide marker ions and two different small molecule ejection ions.^{4,5} R is H or an acyl group. Dha is dehydroalanine.

Scheme S2. Proposed mechanism for DdaC epoxidation. An Fe(IV)-oxo intermediate could be formed via oxidative decarboxylation of α -KG.⁶ The conjugated enamide substrate could undergo one-electron β -addition by the Fe(IV)-oxo intermediate to generate a resonance-stabilized radical that could collapse to generate the epoxide.

V. References

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