Supporting Information

The ATP-dependent amide ligases DdaG and DdaF assemble the fumaramoyl-dipeptide scaffold of the dapdiamide antibiotics.

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Supplemental Figures and Tables

Figure S1. The dapdiamide gene cluster and protein homologies.

DdaA: Cysteine synthase

DdaB: Ornithine cyclodeaminase

DdaC: Fe(II)/ α -ketoglutarate dependent dioxygenase

DdaD: Adenylation-thiolation enzyme

DdaE: Thioesterase

DdaF: Biotin carboxylase

DdaG: Phenylacetate-CoA ligase

DdaH: Asparagine synthetase

Figure S2. SDS-PAGE of purified DdaF and DdaG.

Figure S3. HPLC analysis showing DdaG-catalyzed ligation of succinate and DAP.

Table S1. MS analysis of FMOC-derivatized DdaG products.

	Calculated [M-H] ⁻	Measured [M-H] ⁻
FMOC-Fumaroyl-DAP	423.11977	423.11816
FMOC-Succinoyl-DAP	425.13542	425.13477

Figure S4. HPLC analysis showing that DdaG does not ligate fumaramate, succinamate, or *trans*-epoxysuccinate and DAP. Each reaction contained the indicated carboxylate substrate, DAP, ATP, and DdaG.

Table S2. DdaG kinetics with respect to fumarate and succinate determined at a fixed DAP concentration of 10 mM. Values are best fit $\pm$ standard error from two or three independent experiments.

	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
Fumarate	11.6 $\pm$ 0.4	0.55 $\pm$ 0.07	21
Succinate	3.8 $\pm$ 0.1	20 $\pm$ 2	0.19

Figure S5. HPLC analysis showing that DdaF does not accept either isomer of fumaroyl-DAP.

Figure S6. HPLC analysis showing ATP-dependent ligation of N_β -fumaramoyl-DAP and amino acids by DdaF. A) Ligation of N_β -fumaramoyl-DAP and Ile to form daptiamide B. B) Ligation of N_β -fumaramoyl-DAP and Leu to form daptiamide C.

Table S3. MS analysis of Fmoc-derivatized DdaF products.

	Calculated [M-H] ⁺	Measured [M-H] ⁺
Fmoc-Dapdiamide A	521.20417	521.20648
Fmoc-Dapdiamide B	535.21982	535.22297
Fmoc-Dapdiamide C	535.2198	535.2189

Figure S7. HPLC analysis showing that DdaF does not accept *N*_α-fumaramoyl-DAP.

Figure S8. DdaF reaction velocity with varying concentrations of *N*_β-fumaramoyl-DAP determined at a fixed Val concentration of 5 mM.

Table S4. Apparent kinetic parameters for DdaF with respect to Val, Ile, and Leu determined at a fixed N_β -fumaramoyl-DAP concentration of 125 μ M. Values are best fit $\pm$ standard error from two independent experiments.

	Apparent k_{cat} (min^{-1})	Apparent K_m (mM)	Apparent k_{cat}/K_m ($\text{mM}^{-1}\text{min}^{-1}$)
Val	8.1 ± 0.5	0.26 ± 0.07	31
Ile	9.5 ± 0.7	0.83 ± 0.21	12
Leu	11.2 ± 0.9	7.82 ± 1.94	1

Figure S9. ^1H -NMR spectra of dapdiamide A in D_2O before and after treatment with 500 mM NH_4OH .

Figure S10. Stability of N_β -fumaramoyl-DAP at pH 8 for three days. Comparison of FMO-derivatized sample of N_β -fumaramoyl-DAP incubated at pH 8 with authentic FMO- N_β -fumaramoyl-DAP standards.

Supplemental Methods

Synthesis of N_α -fumaroyl-DAP.

Scheme S1. Synthesis of N_α -fumaroyl-DAP.

***t*-Bu ethyl fumarate (2).** *t*-butanol (40 mL) was transferred to a 250 mL round bottom flask with magnetic stir bar, and then fumarate monoethyl ester (**1**) (1.00 g, 6.94 mmol, 1.0 equiv.) followed by di-*t*-Bu¹ dicarbonate (2.20 g, 10.08 mmol, 1.5 equiv.) were added and the reaction was stirred until both compounds dissolved completely. DMAP (366 mg, 3.00 mmol, 30 mol%) was added and the dark red solution stirred for 4 hours. The crude mixture was purified by silica gel column chromatography (dichloromethane) to afford a clear, colorless liquid (1.24 g, 89%). ¹H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 1.31 (t, $J=7.2$ Hz, 3 H) 1.50 (s, 9 H) 4.25 (q, $J=7.0$ Hz, 2 H) 6.74 (d, $J=15.8$ Hz, 1 H) 6.78 (d, $J=15.8$ Hz, 1 H).

¹ Abbreviations: *t*Bu, *tert*-butyl; DMAP, 4-dimethylaminopyridine; DIEA, *N,N*-diisopropylethylamine; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; Boc, *tert*-butyloxycarbonyl; DMSO, dimethyl sulfoxide

Fumarate mono-*t*-Bu ester (3). In a 25 mL pear flask with magnetic stir bar, diester **2** (156 mg, 777 μmol , 1 equiv.) was dissolved in 3:1 methanol-water (5 mL). The solution was cooled to 0 °C and then lithium hydroxide monohydrate (161 mg, 3.84 mmol, 5 equiv.) was added. The reaction was stirred at 0 °C for 40 minutes, then the reaction mixture was partitioned between ethyl acetate (50 mL) and water (25 mL). The aqueous layer was acidified to approximately pH 6 with HCl. The acidified water layer was removed and the organic layer washed again with water. The aqueous layers were combined and acidified to approximately pH 4, then extracted with ethyl acetate (3 $\times$ 50 mL). The combined organic layers were dried (MgSO_4) and solvent removed *in vacuo* to afford a solid (56.9 mg, 43%). ^1H NMR (600 MHz, CHLOROFORM-d) δ ppm 1.52 (s, 9 H) 6.76 (d, $J=15.8$ Hz, 1 H) 6.86 (d, $J=15.8$ Hz, 1 H).

***N* $_{\alpha}$ -*t*-Bu-fumaroyl-DAP(Boc)-OMe (5).** In a 25 mL pear flask with magnetic stir bar, carboxylic acid **3** (98.7 mg, 573 μmol , 1 equiv.) was suspended in dichloromethane (5 mL). DIEA (300 μL , 1720 μmol , 3 equiv.) was added and **3** dissolved. PyBOP (328 mg, 631 μmol , 1.1 equiv.) was added followed by *N* $_{\beta}$ -Boc-DAP-OMe (**4**) (148 mg, 573 μmol , 1 equiv.). The reaction was stirred for 1 hour at room temperature. Dichloromethane (30 mL) was added to the reaction, and the organic layer was washed with 5% citric acid in water (30 mL) followed by saturated aqueous NaHCO_3 (30 mL). The organic layer was dried (Na_2SO_4) and solvent removed *in vacuo*. The crude product was purified by silica gel column chromatography (4% methanol in dichloromethane) to yield a clear, colorless oil (185 mg, 87%) which could be solidified by dissolving in dichloromethane followed by solvent removal *in vacuo*. ^1H NMR (600 MHz, CHLOROFORM-d) δ ppm 1.43 (s, 9 H) 1.50 (s, 9 H) 3.52 - 3.67 (m, 2 H) 3.78 (s, 3 H) 4.62 - 4.72 (m, 1 H) 4.86 (br. s, 1 H) 6.72 (d, $J=15.6$ Hz, 1 H) 6.83 (d, $J=15.8$ Hz, 1 H) 7.12 (br. s, 1 H).

***N*_α-fumaroyl-DAP (6).** In a screw-cap 1 dram vial with magnetic stir bar, **5** (51.5 mg, 138 μmol, 1 equiv.) was dissolved in 3:1 methanol-water (800 μL). The reaction solution was cooled to 0 °C and lithium hydroxide monohydrate (31.0 mg, 734 μmol, 5 equiv.) added. The reaction was stirred 0 °C for 1 hour. The reaction mixture was partitioned between ethyl acetate (8 mL) and water (8 mL). The aqueous layer was cooled to 0 °C, acidified to approximately pH 3. with HCl, then washed with ethyl acetate (3 × 8 mL). The combined organic layers were dried (Na₂SO₄) and solvent removed *in vacuo*. To this crude product, dichloromethane (1 mL) was added followed by TFA (250 μL). The solution was stirred at room temperature for 7.5 hours, then water (10 mL) was added. The aqueous layer was washed with dichloromethane (3 × 10 mL) and dried *in vacuo*. ¹H-NMR in D₆-DMSO indicated that the reaction was complete. Residual solvent was removed by azeotropic distillation with benzene to afford a white solid (15.8 mg, 36%). MS (ESI) *m/z* = 203.0684 [M+H]⁺; ¹H NMR (600 MHz, DMSO-d₆) δ ppm 2.99 - 3.12 (m, 1 H) 3.20 - 3.31 (m, 1 H) 4.47 - 4.65 (m, 1 H) 6.55 (d, *J*=15.6 Hz, 1 H) 6.92 (d, *J*=15.6 Hz, 1 H) 7.98 (br. s., 3 H) 9.00 (br. s., 1 H). **6** was dissolved in 100 mM HEPES, pH 8 and stored at -20 °C.

Synthesis of N_β-fumaroyl-DAP.

Scheme S2. Synthesis of *N*_β-fumaroyl-DAP.

***N* β -*t*-Bu-fumaroyl-DAP(Boc)-OtBu (8).** In a 50 mL pear flask with magnetic stir bar carboxylic acid **3** (56.9 mg, 330 μ mol, 1 equiv.) was dissolved in dichloromethane (3 mL), and DIEA (173 μ L, 991 μ mol, 3 equiv.) was added followed by PyBOP (189.0 mg, 364 μ mol, 1.1 equiv.) and then *N* α -Boc-DAP-OtBu (**7**) (87.9 mg, 338 μ mol, 1 equiv.). The red solution was stirred at room temperature for 17 hours and then diluted with dichloromethane (30 mL). The organic layer was washed with 5% citric acid in water (30 mL) and then saturated aqueous NaHCO₃ (30 mL). The organic layer was dried (MgSO₄) and then concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography (20:1 dichloromethane-methanol) to afford an orange oil (110 mg, 80%). ¹H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 1.45 (s, 9 H) 1.47 (s, 9 H) 1.50 (s, 9 H) 3.64 - 3.75 (m, 2 H) 4.31 (br. s., 1 H) 5.37 - 5.47 (m, 1 H) 6.57 (br. s, 1 H) 6.69 (d, *J*=15.0 Hz, 1 H) 6.76 (d, *J*=15.0 Hz, 1 H).

***N* β -fumaroyl-DAP (9).** In a screw-cap 1 dram vial with magnetic stir bar **8** (55.0 mg, 133 μ mol) was dissolved in dichloromethane (1 mL) and TFA (250 μ L) was added. The reaction was stirred at room temperature for 24 hours, then water (10 mL) was added. The aqueous layer was washed with dichloromethane (3 $\times$ 10 mL). The combined aqueous layers were dried *in vacuo* to afford a white solid (35.2 mg, 84% yield). (ESI) *m/z* = 203.0681 [M+H]⁺; ¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 3.52 (dt, *J*=14.4, 6.5 Hz, 1 H) 3.70 (dt, *J*=14.7, 5.6 Hz, 1 H) 4.03 (t, *J*=5.6 Hz, 1 H) 6.53 (d, *J*=15.6 Hz, 1 H) 6.90 (d, *J*=15.6 Hz, 1 H) 8.25 (br. s, 2 H) 8.77 (t, *J*=6.2 Hz, 1 H). **9** was dissolved in 100 mM HEPES, pH 8 and stored at -20 °C.

Synthesis of N α -fumaramoyl-DAP.

Scheme S3. Synthesis of *N* α -fumaramoyl-DAP.

***N* α -fumaramoyl-DAP(Boc)-OMe (**11**).** In a screw-cap 1 dram vial with magnetic stir bar, fumaramate (**10**) (4.1 mg, 36 μ mol, 1 equiv.) was dissolved in DMSO (200 μ L). DIEA (18.6 μ L, 107 μ mol, 3 equiv.) was added followed by PyBOP (20.6 mg, 40 μ mol, 1 equiv.), then *N* β -Boc-DAP-OMe (**4**) (10.4 mg, 41 μ mol, 1 equiv.). The reaction was stirred at room temperature for 3 hours. Solvent was removed by lyophilization and then the mixture was purified by silica gel column chromatography (10:1 dichloromethane-methanol) followed by preparative HPLC using a gradient of 0-100% solvent B (0.1% TFA in methanol) in solvent A (0.1% TFA in water) over 40 minutes. Solvent was removed by evaporation followed by lyophilization to yield a white solid (3.9 mg, 35%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.33 (s, 9 H) 3.58 (s, 3 H) 4.32 - 4.40 (m, 1 H) 6.82 (d, J =15.6 Hz, 1H) 6.71 (d, J =15.2 Hz, 1H) 6.87 (t, J =5.9 Hz, 1 H) 7.28 (s, 1 H) 7.77 (s, 1 H) 8.61 - 8.72 (m, 1 H) One DAP β -H is obscured by the water peak, the other is partially obscured but can be assigned as a multiplet centered at 3.28 ppm.

***N* α -fumaramoyl-DAP(Boc)-OH (**12**).** In a screw-cap 1 dram vial with magnetic stir bar, methyl ester **11** (2.0 mg, 6 μ mol, 1 equiv) was dissolved in methanol (150 μ L). Water (31 μ L) was added followed by 0.5 M LiOH in water (19 μ L). The reaction was stirred at room temperature for 1.5 hours. The crude mixture was purified by preparative HPLC using a gradient of 0-100% solvent B (0.1% TFA in methanol) in solvent A (0.1% TFA in water) over 40 minutes

to yield a white solid (0.9 mg, 47%). ^1H NMR (600 MHz, METHANOL- d_4) δ ppm 1.40 (s, 9 H) 3.38 - 3.48 (m, 1 H) 3.50 - 3.61 (m, 1 H) 4.50 - 4.70 (m, 1 H) 6.91 (d, $J=15.0$ Hz, 1H) 6.95 (d, $J=15.6$ Hz, 1H).

N α -fumaramoyl-DAP (13). In a screw-cap 1 dram vial with magnetic stir bar, **12** (0.9 mg, 3 μmol) was dissolved in dichloromethane (500 μL) and TFA (125 μL) was added. The reaction was stirred at room temperature for 5 hours. The reaction was diluted with water (10 mL), then washed dichloromethane (3×10 mL). The aqueous layer was lyophilized to yield a white solid (0.9 mg, 96%). MS (ESI) $m/z = 202.0834$ $[\text{M}+\text{H}]^+$; ^1H NMR (600 MHz, METHANOL- d_4) δ ppm 3.24 (dd, $J=13.4, 8.4$ Hz, 1 H) 3.49 (dd, $J=13.2, 5.3$ Hz, 1 H) 4.77 (dd, $J=8.5, 6.2$ Hz, 1 H) 6.95 (d, $J=15.6$ Hz, 1 H) 6.99 (d, $J=15.0$ Hz, 1 H). **13** was dissolved in 50 mM sodium borate, pH 9.5 and stored at -20°C .

Synthesis of N_β -fumaramoyl-DAP.

Scheme S4. Synthesis of N_β -fumaramoyl-DAP.

N β -fumaramoyl-DAP (14). In a screw-cap 1 dram vial with magnetic stir bar, **10** (22.0 mg, 191 μmol , 1 equiv.) was dissolved in DMSO (700 μL). DIEA (100 μL , 573 μmol , 3 equiv.) was added followed by PyBOP (114 mg, 220 μmol , 1.2 equiv.), then N_α -Boc-DAP-OtBu (**7**) (49.5 mg, 190 μmol , 1.0 equiv.). The reaction was stirred at room temperature for 15.5 hours. Solvent was removed by lyophilization, yielding a brown/orange oil. This crude mixture was purified by preparative HPLC using a gradient of 0-80% solvent B (0.1 % TFA in methanol) in solvent A (0.1 % TFA in water) over 10 minutes and 80-100% solvent B over 15 minutes. Solvent was

removed *in vacuo*, then dichloromethane (1 mL) and TFA (250 μ L) were added. The opaque mixture was stirred at room temperature for 3 hours, then dichloromethane (500 μ L) and TFA (125 μ L) were added and the opaque mixture was stirred for another 2 hours. Solvent was removed by rotary evaporation followed by lyophilization. The material was dissolved in a solution of 5% TFA in water and filtered through a 0.2 μ m syringe filter. Solvent was removed by lyophilization to provide the product as a tan solid (26.9 mg, 45%). MS (ESI) m/z = 202.0835 $[M+H]^+$; 1H NMR (600 MHz, 5% TRIFLUOROACETIC ACID in DEUTERIUM OXIDE) δ ppm 3.51 (dd, $J=15.0, 6.0$ Hz, 1H), 3.64 (dd, $J= 15.0$ Hz, 4.2 Hz, 1H), 3.99 (dd, $J=6.0, 4.2$ Hz, 1 H) 6.52 (d, $J=15.6$ Hz, 1 H) 6.57 (d, $J=15.6$ Hz, 1 H). **14** was dissolved in 50 mM sodium borate, pH 9.5 and stored at -20 $^{\circ}$ C.

Fumaroyl-DAP isomerization assays. 50 μ L reactions contained 5 mM N_{β} -fumaroyl-DAP, 6 mM ATP, 10 mM $MgCl_2$, and 100 mM HEPES, pH 8. Reactions were incubated at room temperature. After 3 days, 20 μ L was removed and combined with 64 μ L CH_3CN , 80 μ L water, 32 μ L 10 mM FMOC-Cl (freshly prepared solution in CH_3CN) and incubated at room temperature for 20 minutes. 64 μ L of 100 mM aminoadamantane was added and reactions incubated at room temperature for 10 minutes before chiral HPLC analysis using a gradient of 20-70% solvent B (0.1 % TFA in CH_3CN) in solvent A (0.1 % TFA in water) over 35 minutes. The absorbance was monitored at 263 nm.

Dapdiamide A isomerization assay. 2 mg synthetic dapdiamide A was dissolved in 1 mL 500 mM NH_4OH and incubated at room temperature for 16 hours. Solvent was removed *in vacuo*, then the compound was dissolved in water acidified by addition of a few drops of 2% TFA in

water. The solvent was removed *in vacuo* and the ^1H -NMR spectrum of this compound was compared to that of the untreated compound.