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SUPPLEMENTARY INFORMATION

ENGINEERING THE SYNTHETIC POTENTIAL OF β-LACTAM SYNTHETASE AND THE IMPORTANCE OF CATALYTIC LOOP DYNAMICS

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Materials and Methods

Materials. Unless otherwise specified, buffers, coupled enzymes, and assay components were purchased from Sigma-Aldrich (St. Louis, MO) NADH was purchased from Roche Applied Sciences (Mannheim, Germany); nickel-nitrilotriacetic acid–agarose resin from Qiagen (Düsseldorf, Germany). *E. coli* Rosetta2(DE3) cells were obtained from EMD Biosciences(Darmstadt, Germany). Primers were ordered from Sigma/Genosys. Synthetic reagents were purchased from either Sigma-Aldrich or Acros (Geel, Belgium). CEA (**5**) was synthesized as previously described.[1]

Characterization of Compounds. $[\alpha]_D$ values are given in degrees•cm^2/(10 g). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300- or 400-MHz NMR spectrometer. Chemical shifts for ^{1}H NMR spectra are reported in ppm relative to chloroform (7.26 ppm), water (4.79 ppm), or methanol (3.31 ppm). Chemical shifts for ^{13}C NMR spectra are reported in ppm relative to chloroform (77.0 ppm) or dioxane (66.5 ppm) in water. Accurate masses were determined with either a VG Analytical VG-70SE magnetic sector or Shimadzu LCMS-IT-TOF hybrid mass spectrometer.

Preparation of (*S*)- & (*R*)-3-Hydroxy-2-methylpropionic Acid (11), Potassium Salt. In a 500-mL round-bottomed flask with magnetic stirbar, 4.7 mL methyl (*S*)- or (*R*)- 3-hydroxy-2-methylpropionate (5.0 g, 42 mmol, 1.0 eq.) and 1.0 M KOH (42 mL, 42 mmol, 1.0 eq.) were stirred for 5 h in 80 mL water and 100 mL tetrahydrofuran. The THF and methanol were removed on a rotary evaporator to leave a white solid after several hours, which was triturated in

acetone and then suction filtered to leave 5.7 g (95% yield). ^{1}H-NMR (300 MHz, D_2O): δ 1.05 (d, *J* = 7.0 Hz, 3H), 2.46 (m, 1H), 3.51 (dd, *J* = 6.3, 10.7 Hz, 1H), 3.68 (dd, *J* = 7.3, 10.7 Hz, 1H) ppm.

Preparation of *N*^{5}-CBz-L-Ornithine Benzyl Ester (12), *p*-Toluenesulphonate Salt. In a 500-mL round-bottomed flask with magnetic stirbar, *N*^{5}-CBz-L-ornithine[2] (10.00 g, 39.95 mmol, 1.0 eq.) and *p*-toluenesulfonic acid monohydrate (8.360 g, 43.95 mmol, 1.1 eq.) were dissolved in 250 mL benzene. Benzyl alcohol (41 mL, 43 g, 0.40 mol, ~10 eq.) was added, and a Dean-Stark trap filled with benzene and a condenser were attached. The apparatus was wrapped in aluminum foil and heated to reflux overnight. The clear solution turned yellow as the reaction progressed, and the Dean-Stark trap collected ~2 mL water. The solvent was evaporated to leave an oil. Addition of diethyl ether with stirring caused white solid to precipitate. The solid was collected by suction filtration and washed with copious amounts of diethyl ether to leave 27.76 g (98% yield) of the tosyl salt, which was air dried. mp 84–86 °C (lit.[2] 84–86 °C); $[\alpha]_D^{28}$ = -1.4 *(c* 1.0 in $CDCl_3$) [lit.,[2] $[\alpha]_D^{24}$ = -2.4 (*c* 1.0 in $CHCl_3$)]; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.4–1.6 (2 m, 2H), 1.89 (m, 2H), 2.29 (s, 3H), 3.02 (m, 2H), 4.04 (br, 1H), 5.0–5.2 (m, 4H), 5.48 (br, 1H), 7.05 (d, *J* = 8.1 Hz, 2H), 7.32 (br s, 10H), 7.76 (d, *J* = 8.1 Hz, 2H) ppm.

Preparation of (2'*S*)- & (2'*R*)-*N*^{5}-CBz-*N*^{2}-(3'-Hydroxy-2'-methylpropanoyl)-L-ornithine Benzyl Ester (13). A flame-dried 100-mL round-bottomed flask with magnetic stirbar was charged with (*S*)- or (*R*)-3-hydroxy-2-methylpropionic acid (**11**), potassium salt (1.00 g, 7.03 mmol, 1.0 eq.), and protected L-ornithine, tosyl salt, **12** (3.72 g, 7.03 mmol, 1.0 eq.) and placed

under N_2. Distilled dichloromethane (45 mL) and anhydrous *N*,*N*-dimethylformamide (4.5 mL) were added by syringe, followed in order by *N*,*N*-diisopropylethylamine (1.32 mL, 1.00 g, 7.74 mmol, 1.1 eq.), 1-hydroxybenzotriazole (950 mg, 7.03 mmol, 1.0 eq.), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.48 g, 7.74 mmol, 1.1 eq.). The reaction was stirred under N_2 overnight, after which the solvent was evaporated and the residue was taken up in ethyl acetate and brine, and the organic layer was separated and washed consecutively with water (1×), saturated sodium bicarbonate solution (2×), 2 M hydrochloric acid (2×), water (1×), and brine (1×). The organic layer was then dried over Na_2SO_4 and evaporated to give 2.60 g white solid (84% yield). R_f: ~0.4 (1:1 hexanes/ethyl acetate); (2′*S*) diastereomer (**13a**): ^{1}H-NMR (400 MHz, $CDCl_3$, broadened by amide isomers): δ 1.12 (d, J = 6.8 Hz, 3H), 1.4–1.6 (m, 2H), 1.6–1.8 (m, 1H), 1.8–2.0 (m, 1H), 2.53 (m, 1H), 3.17 (m, 2H), 3.66 (m, 2H), 4.62 (m, 1H), 4.89 (m, 1H), 5.0–5.3 (m, 4H), 6.63 (m, 1H), 7.34 (br s, 10H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.35, 25.98, 28.76, 40.28, 42.48, 52.05, 65.03, 66.60, 67.29, 128–130 (6C), 135.05, 136.39, 156.52, 172.55, 175.80 ppm; HRMS (MH^+) calcd. for $C_{24}H_{31}N_2O_6$ 443.2182 g/mol, found 443.2187 g/mol. (2′*R*) diastereomer (**13b**): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.11 (d, J = 5.3 Hz, 3H), 1.4–1.7 (m, 3H), 1.8–2.0 (m, 1H), 2.51 (m, 1H), 3.14 (m, 2H), 3.66 (m, 2H), 4.65 (m, 1H), 5.0–5.3 (m, 4H), 6.64 (m, 1H), 7.33 (br s, 10H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.51, 25.68, 29.13, 40.31, 42.53, 51.72, 64.85, 66.65, 67.17, 128–130 (6C), 135.13, 136.39, 156.61, 172.09, 175.58 ppm; HRMS (MH^+) calcd. for $C_{24}H_{31}N_2O_6$ 443.2182 g/mol, found 443.2190 g/mol.

Preparation of Benzyl (3'*S*)- & (3'*R*)-*N*-CBz-3'-Methyldeoxyproclavaminate. A 25-mL round-bottomed flask with a magnetic stirbar was charged with **13** (1.00 g, 2.26 mmol, 1.0 eq.) and placed under N_2. Distilled tetrahydrofuran (40 mL) was added by syringe. Triethylphosphite (0.39 mL, 375 mg, 2.26 mmol, 1.0 eq.) and 40% diethyl azodicarboxylate in toluene (1.03 mL, 394 mg, 2.26 mmol, 1.0 eq.) were added successively, and the yellow-orange solution was stirred overnight. After completion, the solvent was removed in vacuo and to the resulting oil was added diethyl ether to precipitate diethyl hydrazodicarboxylate, which was filtered off. After partial purification by flash column (1:1 hexanes/ethyl acetate, R_f: ~0.2), the compound was used directly for the next step.

Preparation of (3'*S*)- & (3'*R*)-3'-Methyldeoxyproclavaminic Acid (3'-Me-DPC, 14). The crude protected 3'-methyl-DPC above was dissolved with 3:1 THF-phosphate buffer (20 mM, pH = 7.6) in a Parr bottle. The solution was sparged, and a spatula-full of 30% Pd/C was added. The flask was placed on a standard Parr hydrogenation apparatus, and the system was evacuated and flashed with hydrogen three times. The reaction mixture was left overnight under a hydrogen atmosphere (35 psi). The mixture was filtered to remove catalyst and the THF was evaporated. The compounds were purified by HPLC (250×21.20-mm, 10-micron Phenomenex® Luna C18 column; flow rate of 5.0 mL/min with an eluent of 5:95 acetonitrile/water). The elution was monitored at 210 nm, and each diastereomer had a retention time of ~13 min. Lyophilization gave 59.3 mg (13% over two steps). (3′*S*) diastereomer (**14a**): ^{1}H-NMR (400 MHz, D_2O): δ 1.25 (d, J = 7.3 Hz, 3H), 1.6–1.7 (m, 2H), 1.7–1.9 (m, 2H), 3.01 (t, J = 8.1 Hz, 2H), 3.12 (dd, J = 2.0, 5.8 Hz, 1H), 3.21 (sym m, 1H), 3.55 (t, J = 5.6 Hz, 1H), 4.06 (dd, J = 5.3, 9.3 Hz, 1H) ppm;

(3′*R*) diastereomer (**14b**): ^{1}H-NMR (400 MHz, D_2O): δ 1.26 (d, *J* = 7.6 Hz, 3H), 1.6–1.7 (m, 2H), 1.7–1.9 (m, 2H), 3.04 (t, *J* = 7.3 Hz, 2H), 3.07 (dd, *J* = 1.8, 5.8 Hz, 1H), 3.26 (sym m, 1H), 3.62 (t, *J* = 5.3 Hz, 1H), 4.11 (dd, *J* = 5.1, 9.6 Hz, 1H) ppm.

Preparation of (3'*S*)- & (3'*R*)-3'-Methyldeoxyguanidinoproclavaminic Acid (3'-Me-DGPC, 15). 9.7 mg 3'-Me-DPC (**14**) was dissolved in 0.5 mL H_2O, and 6.7 mg aminoiminomethanesulfonic acid[3] (0.054 mmol, 1.2 eq.) and 16 mg K_2CO_3 (0.11 mmol, 2.5 eq.) were added. The solution was stirred uncapped (to allow for escape of sulfur dioxide) at room temperature for 12 h. The products were purified by HPLC (250×9.40-mm, 10-micron Phenomenex® Partisil 10 ODS-3 column; flow rate of 2.0 mL/min with an eluent of 100% water). The elution was monitored at 210 nm, and each diastereomer had a retention time of ~35 min. (3′*S*) diastereomer (**15a**): ^{1}H-NMR (400 MHz, D_2O): δ 1.29 (d, *J* = 7.3 Hz, 3H), 1.6–1.7 (m, 2H), 1.8–2.0 (m, 2H), 3.16 (dd, *J* = 2.3, 5.8 Hz, 1H), 3.2–3.3 (t & m, 3H), 3.58 (t, *J* = 5.6 Hz, 1H), 4.08 (dd, *J* = 5.3, 9.6 Hz, 1H) ppm; (*R*) diastereomer (**15b**): ^{1}H-NMR (400 MHz, D_2O): δ 1.27 (d, *J* = 6.1 Hz, 3H), 1.6–1.7 (m, 2H), 1.7–1.8 (m, 1H), 1.8–1.9 (m, 1H), 3.06 (app d, *J* = 5.8 Hz, 1H), 3.2–3.3 (m, 3H), 3.63 (app t, *J* = 4.8 Hz, 1H), 4.11 (dd, *J* = 4.8, 10.1 Hz, 1H) ppm.

Preparation of (2'*S*)- & (2'*R*)-N^2-(2'-Carboxypropyl)arginine (2'-Me-CEA, 10) Hydrochloride. 3'-methyl-DGPC (**15**) was dissolved in 2 M HCl and stirred for several hours at room temperature. After lyophilization 2'-Me-CEAs were obtained quantitatively. (2′*S*) diastereomer (**10a**): ^{1}H-NMR (400 MHz, D_2O): δ 1.30 (d, *J* = 7.1 Hz, 3H), 1.6–1.9 (2 x m, 2H), 1.9–2.1 (m, 2H), 2.9–3.1 (sym m, 1H), 3.15 (dd, *J* = 4.0, 12.9 Hz, 1H), 3.26 (app t, *J* = 6.3 Hz,

2H), 3.42 (app t, J = 11.0 Hz, 1H), 3.92 (t, J = 4.7 Hz, 1H) ppm; (2′*R*) diastereomer (**10b**): ^{1}H-NMR (400 MHz, D_2O): δ 1.25 (d, J = 7.1 Hz, 3H), 1.6–1.8 (sym m, 2H), 1.9–2.0 (sym m, 2H), 2.9–3.0 (m, 1H), 3.13 (dd, J = 4.8, 12.9 Hz, 1H), 3.25 (app t, J = 6.6, 2H), 3.32 (app t, J = 10.1 Hz, 1H), 3.80 (t, J = 5.6 Hz, 1H) ppm.

Preparation of (*R*)- & (*S*)-4-Benzyl-3-butyryl-2-oxazolidinone (19). A 250-mL round-bottomed flask with magnetic stirbar was charged with (*R*)- or (*S*)-4-benzyl-2-oxazolidinone (**18**) (4.2 g, 24 mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (290 mg, 2.4 mmol, 0.1 eq.), which were dissolved in freshly distilled dichloromethane (60 mL) to give a yellow solution. Butyric acid (2.6 mL, 2.5 g, 28 mmol, 1.2 eq.) was added by syringe while stirring, followed by *N*,*N*′-dicyclohexylcarbodiimide (DCC) (4.9 g, 24 mmol, 1.0 eq.) in one portion. A white precipitate began to form immediately, but the reaction was stirred overnight. The reaction mixture was filtered through fluted paper and the powder was washed with dichloromethane and diethyl ether. The combined filtrate and washes were concentrated on a rotary evaporator to leave a yellow liquid, which was triturated in diethyl ether to precipitate more dicyclohexylurea (DCU). The DCU was filtered off on Celite and washed with diethyl ether. The filtrate was washed with saturated sodium bicarbonate, dried, evaporated, and purified by silica gel chromatography (1:1 hexanes/ethyl acetate, 30-mm, 6 in.) to collect a yellow oil (~65% typical yield). R_f: ~0.65 (1:1 hexanes/ethyl acetate); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.07 (t, J = 7.3 Hz, 3H), 1.7–1.9 (sym m, 2H), 2.82 (dd, J = 9.6, 13.1 Hz, 1H), 2.98 (sym m, 2H), 3.36 (dd, J = 3.3, 13.4 Hz, 1H), 4.1–4.3 (sym m, 2H), 4.74 (m, 1H), 7.1–7.4 (m, 5H) ppm.

Preparation of (4*S*,2'*R*)-4-Benzyl-3-(2'-benzyloxymethylbutyryl)-2-oxazolidinone (20b). A flame-dried 250-mL round-bottomed flask with magnetic stirbar was charged with **19b** (5.06 g, 20.5 mmol, 1.0 eq.) and placed under N_2. Distilled dichloromethane (80 mL) was transferred by syringe and the solution was cooled in an ice bath. 1.0 M titanium(IV) chloride in dichloromethane (21.3 mL, 21.3 mmol, 1.04 eq.) was added dropwise with stirring to give a yellow reaction mixture. After 5 min, *N*,*N*-diisopropylethylamine (3.74 mL, 2.78 g, 21.5 mmol, 1.05 eq.) was added slowly by syringe, which caused fuming and turned the mixture dark red. By syringe, freshly distilled benzyloxymethyl chloride (5.7 mL, 41 mmol, 2.0 eq.) was added, and the reaction was stirred for 1 h in an ice bath before quenching with 20 mL saturated NH_4Cl solution, which turned the mixture dark yellow and caused precipitation. Water was added to dissolve the precipitate, and the layers were separated, followed by extraction into dichloromethane (2×). The orange combined organic layers were washed consecutively with water, saturated sodium bicarbonate solution, water, and brine. The organic layer was then dried over anhy. Na_2SO_4 and evaporated to leave an amber liquid, which was purified by silica gel column (9:1 hexanes/ethyl acetate, 70-mm, 6 in. silica) to collect 5.30 g white solid (70% yield). R_f: ~0.4 (9:1 hexanes/ethyl acetate); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.93 (t, J = 7.6 Hz, 3H), 1.5–1.7 (sym m, 1H), 1.7–1.8 (sym m, 1H), 2.70 (dd, J = 9.3, 13.4 Hz, 1H), 3.23 (dd, J = 5.1, 13.6 Hz, 1H), 3.66 (dd, J = 5.1, 9.1 Hz, 1H), 3.81 (t, J = 8.3 Hz, 1H), 4.0–4.2 (m, 3H), 4.54 (s, 2H), 4.7–4.8 (m, 1H), 7.1–7.4 (m, 10H) ppm.

Preparation of (4*R*,2'*S*)-4-Benzyl-3-(2'-benzyloxymethylbutyryl)-2-oxazolidinone (20a). Same as above, except beginning with **19a**.

Preparation of (*S*)- & (*R*)-2-Benzyloxymethylbutyric Acid. A 25-mL round-bottomed flask with magnetic stirbar was charged with **20** (460 mg, 1.3 mmol, 1.0 eq.) via pipette. 5 mL tetrahydrofuran and 0.75 mL water were added, and the solution was stirred and chilled in an ice bath. 30% hydrogen peroxide (0.57 mL, 5.0 mmol, 4.0 eq.) was added, followed by a solution of lithium hydroxide monohydrate (84 mg, 1.6 mmol, 1.6 eq.) in 0.5 mL water. The cloudy solution was stirred overnight and then quenched with saturated sodium sulfite. The THF was removed on a rotary evaporator. The pH was increased to ~12 with 2 M NaOH, and the solution was washed with dichloromethane three times to remove the chiral auxiliary **18** (which was recycled for future use). The pH was adjusted to ~1 with 2 M HCl to give a cloudy white mixture, and the product was extracted into ethyl acetate three times. The extracted layers were dried, filtered, and evaporated to leave a colorless oil, which was used without further purification for the next step. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.94 (t, J = 7.3 Hz, 3H), 1.6–1.8 (2 x sym m, 2H), 2.66 (sym m, 1H), 3.59 (dd, J = 5.3, 9.3 Hz, 1H), 3.67 (dd, J = 7.8, 9.1 Hz, 1H), 4.55 (s, 2H), 7.2–7.4 (m, 5H) ppm.

Preparation of (*S*)- & (*R*)-2-Hydroxymethylbutyric Acid (17*)*. The 2-benzyloxymethylbutyric acid synthesized above was dissolved in 5 mL tetrahydrofuran and 5 mL water and transferred to a Parr pressure bottle and sparged with nitrogen. A pinch of palladium on carbon (10%) was added, and the mixture was placed on the Parr apparatus and shaken under H_2 at 40 psi overnight. The solvents were removed to leave 132 mg product (90% over two steps). ^{1}H-NMR (400 MHz, CD_3OD): δ 0.95 (t, J = 7.3 Hz, 3H), 1.5–1.6 (m, 2H), 2.43 (quin, J = 7.6 Hz, 1H), 3.62 (dd, J = 5.6, 10.6 Hz, 1H), 3.72 (dd, J = 7.8, 10.9 Hz, 1H) ppm.

Preparation of (2'*S*)- & (2'*R*)-N^5-CBz-N^2-(2'-hydroxymethylbutyryl)-L-ornithine Benzyl Ester (21). Same as for **13**, except beginning with **17**. (2′*S*) diastereomer (**21a**): R_f: ~0.45 (1:9 hexanes/ethyl acetate); ^{1}H-NMR (400 MHz, $CDCl_3$, broadened owing to amide isomers): δ 0.93 (t, J = 7.3 Hz, 3H), 1.4–1.6 (m, 3H), 1.6–1.8 (m, 2H), 1.8–2.0 (m, 1H), 2.2–2.4 (m, 1H), 3.16 (m, 2H), 3.70 (m, 2H), 4.63 (m, 1H), 4.87 (br t, 1H), 5.0–5.2 (m, 4H), 6.57 (br d, 1H), 7.2–7.4 (m, 10H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.90, 21.47, 26.12, 28.79, 40.32, 50.54, 52.14, 63.54, 66.74, 67.43, 128–130 (6C), 135.07, 136.40, 156.53, 172.49, 175.38 ppm; HRMS (MH^+) calcd. for $C_{25}H_{33}N_2O_6$ 457.2339 g/mol, found 457.2341 g/mol. (2′*R*) diastereomer (**21b**) : R_f: ~0.4 (1:9 hexanes/ethyl acetate); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.94 (t, J = 7.3 Hz, 3H), 1.4–1.8 (m, 5H), 1.8–2.0 (m, 1H), 2.2–2.4 (m, 1H), 3.15 (m, 2H), 3.73 (m, 2H), 4.69 (m, 1H), 4.87 (br t, 1H), 5.0–5.2 (m, 4H), 6.43 (br d, 1H), 7.2–7.4 (m, 10H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.78, 21.79, 25.75, 29.07, 40.28, 50.50, 51.71, 63.28, 66.67, 67.19, 128–130 (6C), 135.13, 136.41, 156.62, 172.08, 175.17 ppm; HRMS (MH^+) calcd. for $C_{25}H_{33}N_2O_6$ 457.2339 g/mol, found 457.2339 g/mol.

Preparation of (3'*S*)- & (3'*R*)-3'-Ethyldeoxyproclavaminic Acid (3'-Et-DPC, 22). Same as for **14**, except beginning with **21**. The (3′*S*) diastereomer (**22a**) had a retention time of ~18 min; the (3′*R*) diastereomer (**22b**) ~20 min. (3′*S*) diastereomer (**22a**) : ^{1}H-NMR (400 MHz, D_2O): δ 0.94 (t, J = 7.6 Hz, 3H), 1.6–1.9 (2 x m, 6H), 3.01 (app t, J = 7.6 Hz, 2H), 3.1–3.2 (m, *2*H), 3.51 (app t, J = 5.6 Hz, 1H), 4.06 (dd, J = 5.6, 9.3 Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.68, 20.64, 23.94, 26.37, 38.83, 43.86, 48.86, 57.03, 174.23, 176.46 ppm; HRMS (MH^+) calcd. for $C_{10}H_{19}N_2O_3$ 215.1396 g/mol, found 215.1398 g/mol. (3′*R*) diastereomer (**22b**) : ^{1}H-NMR

(400 MHz, D_2O): δ 0.95 (t, *J* = 7.3 Hz, 3H), 1.6–1.8 (m, 5H), 1.8–2.0 (m, 1H), 3.03 (app t, *J* = 7.33 Hz, 2H), 3.17 (sym m, 1H), 3.25 (sym m, 1H), 3.56 (app t, *J* = 5.8 Hz, 1H), 4.12 (dd, *J* = 4.8, 9.3 Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.67, 21.52, 24.76, 27.04, 39.51, 44.22, 49.68, 57.09, 175.11, 177.25 ppm; HRMS (MH^+) calcd. for $C_{10}H_{19}N_2O_3$ 215.1396 g/mol, found 215.1395 g/mol.

Preparation of (3'*S*)- & (3'*R*)-3'-Ethyldeoxyguanidinoproclavaminic Acid (3'-Et-DGPC, 23). Same as for **15**, except beginning with **22**. Each diastereomer had retention times of both ~20 min and ~35 min due to different protonation states. (3′*S*) diastereomer (**23a**): ^{1}H-NMR (400 MHz, D_2O): δ 0.95 (t, *J* = 7.3 Hz, 3H), 1.5–1.9 (m, 6H), 3.1–3.3 (m, 4H), 3.50 (app t, *J* = 4.3 Hz, 1H), 4.06 (dd, *J* = 5.8, 7.8 Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.66, 21.36, 25.76, 27.24, 41.12, 44.58, 49.50, 57.98, 157.36, 174.91, 177.55 ppm; HRMS (MH^+) calcd. for $C_{11}H_{21}N_4O_3$ 257.1608 g/mol, found 257.1610 g/mol; (3′*R*) diastereomer (**23b**): ^{1}H-NMR (400 MHz, D_2O): δ 0.96 (t, *J* = 7.5 Hz, 3H), 1.6–1.8 (2 x m, 5H), 1.8–2.0 (m, 1H), 3.15 (m, 1H), 3.2–3.3 (m, 3H), 3.57 (app t, *J* = 5.6 Hz, 1H), 4.12 (dd, *J* = 5.1, 10.1 Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.66, 21.54, 26.00, 27.24, 41.10, 44.11, 49.63, 57.16, 157.39, 175.12, 177.57 ppm; HRMS (MH^+) calcd. for $C_{11}H_{21}N_4O_3$ 257.1608 g/mol, found 257.1609 g/mol.

Preparation of (2'*S*)- & (2'*R*)-N^2-(2'-Carboxybutyl)arginine (2'-Et-CEA, 16) Hydrochloride. Same as for **10**, except beginning with **23**. (2′*S*) diastereomer (**16α**): ^{1}H-NMR (300 MHz, H_2O): δ 0.93 (t, *J* = 7.1 Hz, 3H), 1.6–1.8 (m, 4H), 1.9–2.1 (m, 2H), 2.86 (br dd, 1H), 3.18 (br dd, *J* = 2.7, 12.6 Hz, 1H), 3.24 (app t, *J* = 6.1 Hz, 2H) , 3.43 (app t, *J* = 11.4 Hz, 1H),

3.96 (app t, *J* = 5.8, Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.55, 23.69, 24.33, 26.56, 40.94, 43.77, 47.58, 61.72, 157.40, 172.18, 177.41 ppm; HRMS (MH^+) calcd. for $C_{11}H_{23}N_4O_4$ 275.1714 g/mol, found 275.1722 g/mol; (2′*R*) diastereomer (**16b**) : ^{1}H-NMR (400 MHz, D_2O): δ 0.93 (t, *J* = 6.8 Hz, 3H), 1.6–1.8 (m, 4H), 1.9–2.1 (sym m, 2H), 2.86 (br dd, 1H), 3.1–3.3 (br dd & t, *3*H), 3.41 (app t, *J* = 11.4 Hz, 1H), 3.40 (app t, *J* = 11.1, Hz, 1H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 10.67, 23.64, 24.51, 26.66, 40.92, 43.74, 47.50, 61.30, 157.38, 171.80, 177.27 ppm; HRMS (MH^+) calcd. for $C_{11}H_{23}N_4O_4$ 275.1714 g/mol, found 275.1722 g/mol.

Structural Visualization and Modeling. All computer modeling, surface area generations, minimizations, and visualizations were carried out within the Accelrys Discovery Studio 2.1 Suite. Superimpositions of βLS subunits were performed by sequence alignment. The tetrahedral intermediate model was generated by manual docking of the intermediate such that the Mg^{2+}, PP_i, and AMP atoms superimposed with those of PDB ID#1MBZ (subunit A) and the carboxylate and guanidino atoms with those of PDB ID#1MC1 (subunit A). All waters, except for those found in the majority of βLS structures, were removed. This was followed by CHARMm[4] minimization with default settings unless specified. An adopted basis NR (ABNR) algorithm with a generalized Born implicit solvent model with molecular volume (GBMV) was selected. Minimization constraints were established to limit the distances between atoms seen to make hydrogen bonds in all of the relevant crystal structures (to ±0.25 Á). The algorithm was allowed to exit after an RMS gradient of less than 0.1 kcal/(mol Á) was achieved between runs.

Protein Preparation. Mutations were introduced into *bls* as described previously[1] using the overlap extension method. Genes were cloned into the pET29b vector and amplified in DH5α. The primers for the V446A mutation were 5'-CC AAG CTG GGC GCT CAC GAG GGC TC-3' (forward) and 3'-GG TTC GAC CCG CGA GTG CTC CCG AG-5' (reverse), with the mutated bases underlined. The primers for the V446G mutation were 5'-CC AAG CTG GGC GGC CAC GAG GGC TC-3' (forward) and 3'-GG TTC GAC CCG CCG GTG CTC CCG AG-5' (reverse). The mutations were confirmed by complete gene sequencing at the DNA Sequence Facility at Johns Hopkins University, Baltimore, MD. Overproduction of WT βLS and variants followed established procedures,[1] with induction in *E. coli* Rosetta2(DE3) cells grown in 2×YT or Terrific Broth media. Purification of enzymes was as described previously.[1]

Steady-State Kinetic Assays. AMP release catalyzed by βLS was monitored at 340 nm by coupled enzyme assay through the consumption of NADH as described previously.[1,5] Reactions were initiated by addition of enzyme at 25 °C in a final volume of 100 μL. A three-buffer system of 40 mM MES, 40 mM HEPES, 35 mM TAPS, and 25 mM TAPS (pH 8.8, I=100 mM) was used for all assays. ATP and MgCl were held at saturating levels (2 and 12.5 mM, respectively) for all reactions. Kinetic parameters were determined from initial velocities with the Kaleidograph 4.1 software by nonlinear regression using

$$k_{obs} = \frac{k_{cat}[S]}{K_M + [S]}$$

where k_{obs} is equal to $v/[E]_0$.

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