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General Methods

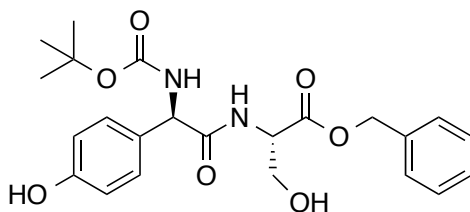
¹H-NMR spectra were recorded on a Bruker Avance (Billerica, MA) 400 or 300 MHz spectrometer. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0 ppm) or with the solvent reference relative to TMS (residual HDO, δ 4.79 ppm, CHCl₃, δ 7.26 ppm, DMSO-d₅, 2.50 ppm). Data are reported as follows: chemical shift {multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration}. ¹³C-NMR spectra were recorded on a Bruker 400 (101 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the (CD₃)₂SO (δ 39.52 ppm) or CDCl₃ (δ 77.16 ppm) as the internal standard. High-resolution mass spectrometry was performed by fast atom bombardment (FAB) or electrospray ionization (ESI) at the Mass Spectrometry Facility of The Johns Hopkins University. Column chromatography was carried out on Silica Gel 60 Merck (Whitehouse Station, NJ), 230-400 mesh ASTM. Reagents and chemicals were purchased from the Sigma-Aldrich Chemical Company (Milwaukee, WI) unless otherwise noted and used without further purification. Pearlman's catalyst (Pd-OH/C) was purchased and used without further purification from Strem Chemicals, Inc. (Newburyport, MA). All solvents used for reactions were distilled prior to use (THF over Na/benzophenone, CH₂Cl₂ and CH₃CN over CaH).

Preparative HPLC purifications were performed on an Agilent model 1100 (Santa Clara, CA) equipped with a multi-wavelength UV-Vis detector in conjunction with a reverse-phase Phenomenex Luna 10 μ C18(2) 100 Å preparatory column (250 x 21.20 mm ID). Mobile phase conditions included one of the following: **Prep Method A** (water + acetonitrile (ACN) +0.1% TFA): 0–5 min isocratic 87% water 13% ACN + 0.1% TFA, 5-25 min gradient 13% to 50% ACN + 0.1% TFA, 25-30 min 50% ACN to 13% ACN, 30-35 min isocratic 87% water 13% ACN + 0.1% TFA. Flow rate = 6.5 mL/min. **Prep Method B** (water + ACN + 0.1% TFA): 0-25 min gradient 15-80% ACN + 0.1% TFA, 25-30 min 80% to 15% ACN + 0.1% TFA, 30-35 min 15% ACN + 85% water + 0.1% TFA. Flow rate = 6.5 mL/min.

Analytical HPLC purifications of synthetic substrates were performed on an Agilent model 1200 equipped with a multi-wavelength UV-Vis detector in conjunction with a reverse phase Phenomenex Luna 5 μ phenyl/hexyl analytical column (250 x 4.60 mm ID). **Analytical Method A** (water + ACN + 0.1% TFA): 0-5 min isocratic 93% water + 7% ACN +0.1% TFA, 5-22 min gradient 7% to 50% ACN + 0.1% TFA, 22-25 min gradient 50% to 7% ACN + 0.1% TFA, 25-35 min isocratic 93% water + 7% ACN + 0.1% TFA. Flow rate = 1.0 mL/min.

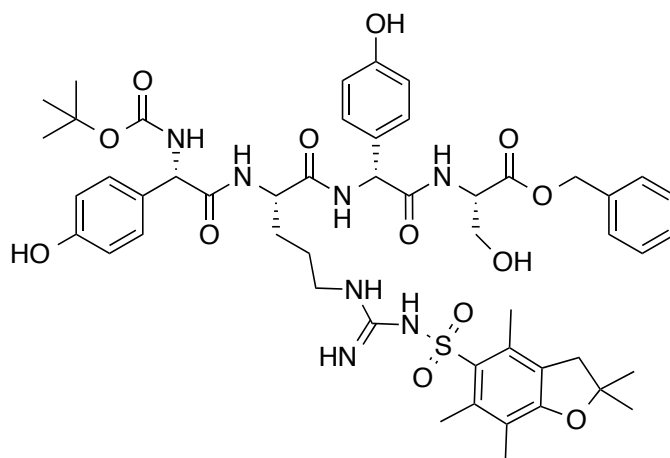
Synthesis of Substrates

I. Synthesis of L-pHPG-L-Arg-D-pHPG-L-Ser-S-Coenzyme A (1)



S1

N-tert-Butyloxycarbonyl-D-[p-(hydroxy)phenyl]glycyl-L-serine Benzyl Ester (S1). To a 250 mL round-bottomed flask equipped with a magnetic stir bar, Boc-D-pHPG¹ (4.36 g, 16.3 mmol) and DIPEA (8.50 mL, 49.0 mmol) was dissolved in 30 mL of reagent grade DMF and cooled to 0 °C in an ice-bath. When the solution had come to temperature, PyBOP (8.50 g, 16.3 mmol) was added and after 1 min benzyl L-serine hydrochloride (5.00 g, 13.61 mmol) was added. The reaction mixture was allowed to stir at 0 °C to room temperature for 3 h. The solution was diluted with 200 mL of EtOAc and washed with sat. aq. NH₄Cl (2 x 75 mL), sat. aq. NaHCO₃ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography with a gradient of 40: 60 EtOAc: Hex to 50: 50 EtOAc: Hex over 3 L to obtain the product as a white foam (4.54 g, 75%). **¹H-NMR** (400 MHz; DMSO-d₆): δ 9.37 (s, 1H), 8.42 (d, *J* = 7.6 Hz, 1H), 7.38-7.32 (m, 6H), 7.20 (d, *J* = 8.6 Hz, 2H), 7.17 (d, *J* = 8.6 Hz, 1H), 6.67 (d, *J* = 8.6 Hz, 2H), 5.20 (d, *J* = 8.6 Hz, 1H), 5.14 (s, 2H), 4.35 (q, *J* = 6.3 Hz, 1H), 3.71 (dd, *J* = 10.9, 5.2 Hz, 1H), 3.59 (dd, *J* = 10.9, 4.6 Hz, 1H), 1.38 (s, 9H). **¹³C-NMR** (101 MHz, DMSO-d₆): δ 171.4, 170.8, 157.3, 136.4, 129.7, 128.93, 128.91, 128.4, 128.1, 115.4, 78.8, 66.4, 61.7, 57.5, 55.3, 28.6. **HRMS** (FAB) calculated for C₂₃H₂₉N₂O₇ 445.1969; Found 445.1954 [M+H]⁺.

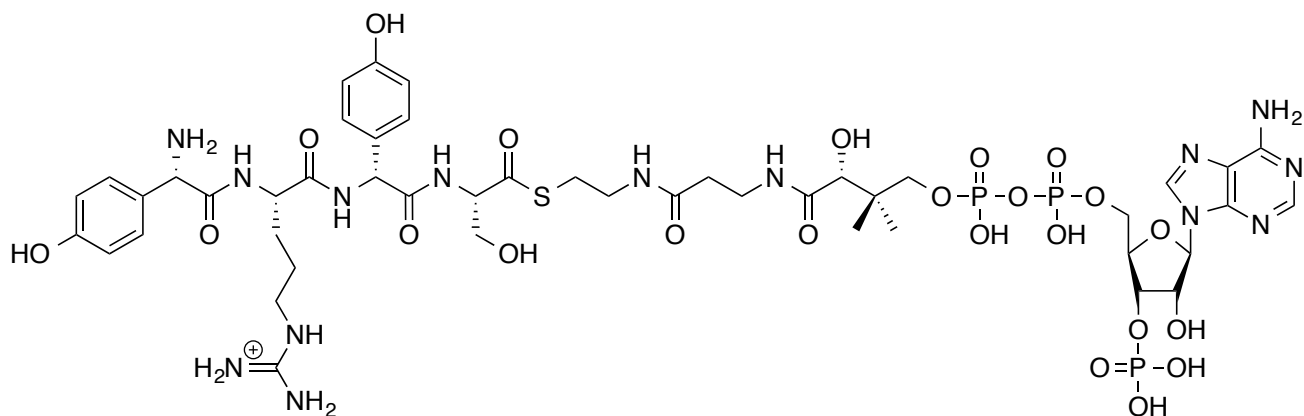


S2

***N*-tert-Butyloxycarbonyl-L-[*p*-(hydroxy)phenyl]glycyl-L-arginyl(Pbf)-D-[*p*-(hydroxy)phenyl]-glycyl-L-serine Benzyl Ester (S2)** To a 250 mL round-bottomed flask equipped with a magnetic stir bar, *N*-tert-butyloxycarbonyl-L-[*p*-(hydroxy)phenyl]glycyl-L-arginine(Pbf) benzyl ester¹ (1.22 g, 1.59 mmol) was dissolved in 20 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the dipeptide was hydrogenated under 1 atm. of H₂ for 12 h. The mixture was filtered through Celite, washed with THF (3 x 50 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification. In a separate 250 mL round-bottomed flask equipped with a magnetic stir bar, dipeptide **S1** (779 mg, 1.59 mmol) was dissolved in 50 mL of TFA and stirred at room temperature for 30 min. The solution was concentrated *in vacuo* and the solvents were removed by azeotropic distillation with toluene (3 x 50 mL).

To a third 250 mL round-bottomed flask equipped with a magnetic stir bar, the freshly hydrogenolyzed Boc-L-pHPG-L-Arg(Pbf)-COOH dipeptide was added in 30 mL of reagent grade DMF. To this was solution added DIPEA (415 μ L, 2.35 mmol) and the solution was cooled to 0 °C in an ice-bath. The freshly deprotected peptide **S1** was dissolved in 20 mL of reagent grade DMF, DIPEA (415 μ L, 2.35 mmol) was added and the solution was cooled to 0 °C in an ice-bath. After both solutions were sufficiently cooled, PyBOP (911 mg, 1.75 mmol) was added to the flask containing the carboxylate and after 1 min, freshly deblocked **S1** was added dropwise over 2 min. The reaction mixture was stirred from 0 °C to room temperature over 3 h. The reaction mixture was diluted with 150 mL of EtOAc and washed with sat. aq. NH₄Cl (2 x 75 mL), sat. aq. NaHCO₃ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography using a gradient of 50: 50 EtOAc: Hex to 80: 20 EtOAc: Hex over 3 L to obtain the product as a white foam (0.95 g, 65%). ¹H-NMR (400 MHz; DMSO-d₆): δ 9.38 (br.s, 2H), 8.55 (d, *J* = 7.6 Hz, 1H), 8.49 (d, *J* = 7.8 Hz, 1H), 8.23-8.20 (m, 1H), 7.42-7.32 (m, 5H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.13 (d, *J* = 8.5 Hz,

2H), 6.69 (d, $J = 8.7$ Hz, 2H), 6.67 (d, $J = 8.6$ Hz, 2H), 5.47 (d, $J = 8.3$ Hz, 1H), 5.14 (s, 2H), 4.41 (dd, $J = 13.1, 7.5$ Hz, 1H), 4.34 (q, $J = 6.2$ Hz, 1H), 3.71-3.67 (m, 1H), 3.55 (dd, $J = 10.8, 4.8$ Hz, 2H), 3.01 (br. s, 2H), 2.96 (s, 3H), 2.48 (s, 3H), 2.43 (s, 3H), 1.61 (br. s, 2H), 1.41 (s, 9H), 1.38 (s, 6H). **$^{13}\text{C-NMR}$** (101 MHz, DMSO- d_6): δ 171.05, 170.79, 157.91, 157.21, 156.49, 137.75, 136.37, 131.90, 129.80, 128.86, 128.82, 128.69, 128.44, 128.13, 124.79, 116.74, 115.45, 86.76, 66.43, 61.65, 55.34, 42.95, 28.79, 28.64, 21.24, 19.44, 18.08, 14.57, 12.78. **HRMS** (FAB) calculated for $\text{C}_{50}\text{H}_{64}\text{N}_7\text{O}_{13}\text{S}$ 1002.42828; Found 1002.42870 $[\text{M}+\text{H}]^+$.



1

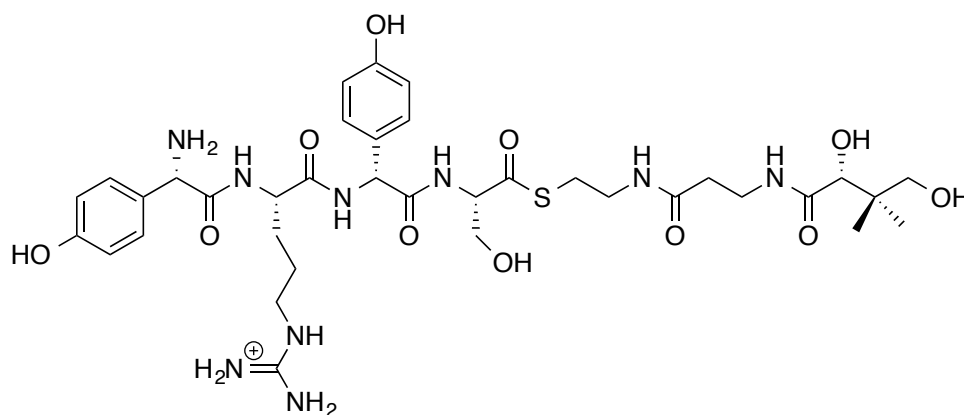
L-[p-(Hydroxy)phenyl]glycyl-L-arginyl-D-[p-(hydroxy)phenyl]glycyl-L-seryl-S-coenzyme A (1).

To a 50 mL round-bottomed flask equipped with a magnetic stir bar, **S2** (400 mg, 0.39 mmol) was dissolved in 10 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the tetrapeptide was hydrogenated under 1 atm. of H_2 for 12 h. The mixture was filtered through Celite, washed with THF (3 x 20 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification.

To a 10 mL round-bottomed flask equipped with a magnetic stir bar, freshly hydrogenolyzed tetrapeptide **S2** (40.0 mg, 0.044 mmol) was dissolved in 500 μL of THF and to this solution was added K_2CO_3 (18.0 mg, 0.132 mmol). In a separate vial, coenzyme A (42.0 mg, 0.055 mmol) was dissolved in 500 μL of HPLC grade water. PyBOP (27.0 mg, 0.055 mmol) was added to the solution containing hydrogenolyzed **S2**, followed by freshly prepared aqueous coenzyme A solution. The reaction mixture was stirred at room temperature for 30 min, filtered through a 0.2 μm filter and purified directly without any intermediate work-up. The filtered solution was purified by Prep Method B and the desired product was collected on dry ice and lyophilized to dryness. The freshly lyophilized product was dissolved in 5 mL of reagent grade TFA and stirred at room temperature for 15 min. The TFA was removed *in vacuo* and the residue was re-suspended in 2 mL of 70: 30 H_2O : ACN supplemented with 0.1% TFA. The reaction mixture was purified by Prep Method A, collected, frozen

on dry ice and lyophilized to dryness as a white TFA salt (6.9 mg, 12%). The product was further purified using Analytical Method B to obtain 4 mg of highly purified compound **1**. **¹H-NMR** (400 MHz; D₂O): δ 8.60 (s, 1H), 8.34 (s, 1H), 7.18 (d, *J* = 8.7 Hz, 2H), 7.12 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 6.14 (d, *J* = 5.5 Hz, 1H), 5.32 (s, 1H), 5.05 (s, 1H), 4.54-4.53 (m, 1H), 4.50 (d, *J* = 5.3 Hz, 1H), 4.32 (t, *J* = 7.3 Hz, 1H), 4.22-4.19 (m, 2H), 3.97 (s, 1H), 3.84-3.74 (m, 5H), 3.52 (dd, *J* = 10.2, 4.5 Hz, 1H), 3.37 (app. t, *J* = 6.9 Hz, 3H), 3.27-3.22 (m, 2H), 3.04 (app. t, *J* = 7.1 Hz, 3H), 3.00-2.92 (m, 2H), 2.34 (app. t, *J* = 6.9 Hz, 2H), 1.70 (q, *J* = 7.7 Hz, 2H), 1.54-1.40 (m, 4H), 0.87 (s, 3H), 0.74 (s, 3H). **HRMS** (ESI) calculated for C₄₆H₆₈N₁₄O₂₃P₃S 1309.3510; Found 1309.3520 [M+H]⁺.

II. Synthesis of L-pHPG-L-Arg-D-pHPG-L-Ser-S-pantetheine (**3**)



3

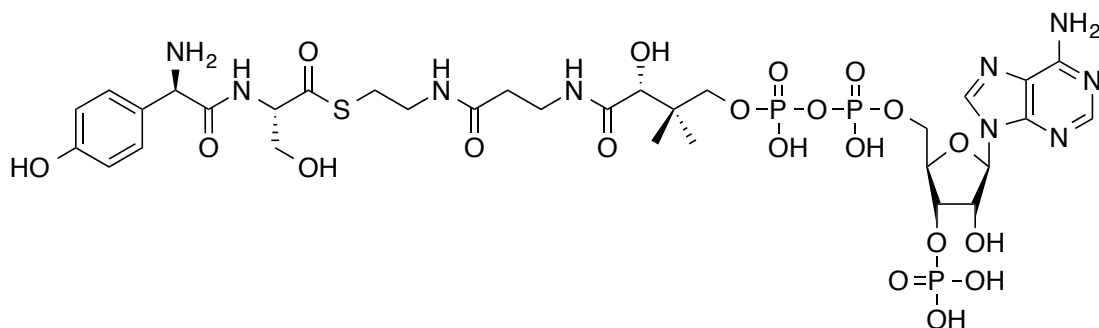
L-[*p*-(Hydroxy)phenyl]glycyl-L-arginyl-D-[*p*-(hydroxy)phenyl]glycyl-L-seryl-S-pantetheine (**3**).

To a 10 mL round-bottomed flask, protected peptide **S2** (210 mg, 0.318 mmol) was dissolved in 4 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the protected tetrapeptide was deblocked at 1 atm. of H₂ for 12 h. The mixture was filtered through Celite, washed with THF (3 x 10 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification.

To a 10 mL round-bottomed flask equipped with a magnetic stir bar, freshly hydrogenolyzed tetrapeptide was dissolved in 2 mL of reagent grade DMF and to this solution was added DIPEA (166 μL, 0.95 mmol) and cooled to 0 °C with an ice-bath. To this solution was added PyBOP (182 mg, 0.350 mmol) and pantetheine dimethyl ketal¹ (112 mg, 0.350 mmol) and the reaction mixture was stirred for 30 min at room temperature. The solution was diluted with 50 mL of EtOAc and washed with sat. aq. NH₄Cl (2 x 20 mL), sat. aq. NaHCO₃ (1 x 20 mL) and brine (1 x 20 mL). The EtOAc

extract was concentrated *in vacuo* to a viscous oil, which was re-dissolved in 2 mL 70: 30 ACN: H₂O. The product was purified by Prep Method B, collected on dry ice and lyophilized to dryness. The freshly lyophilized product was dissolved in 5 mL of reagent grade TFA and stirred at room temperature for 15 min. The TFA was removed *in vacuo* and the residue was re-suspended in 2 mL of 70: 30 H₂O: ACN supplemented with 0.1% TFA. The reaction mixture was purified by Prep Method A, and the product was collected, frozen on dry ice and lyophilized to dryness as a white TFA salt (167 mg, 54%). The product was further purified using Analytical Method B to obtain 7 mg of highly pure compound **3**. **¹H-NMR** (400 MHz; D₂O): δ 7.17 (d, *J* = 8.5 Hz, 2H), 7.12 (d, *J* = 8.5 Hz, 2H), 6.81 (d, *J* = 8.2 Hz, 2H), 6.73 (d, *J* = 8.2 Hz, 1H), 5.30 (s, 1H), 4.99 (s, 1H), 4.50 (t, *J* = 4.8 Hz, 1H), 4.29 (t, *J* = 7.4 Hz, 1H), 3.87 (s, 1H), 3.79 (d, *J* = 5.0 Hz, 1H), 3.69 (dd, *J* = 12.1, 4.2 Hz, 1H), 3.39 (d, *J* = 11.0 Hz, 1H), 3.37-3.32 (m, 2H), 3.27 (d, *J* = 11.0 Hz, 1H), 3.25-3.23 (m, 1H), 3.15 (t, *J* = 6.2 Hz, 1H), 3.04-3.00 (m, 2H), 2.96 (q, *J* = 6.0 Hz, 1H), 2.82 (app. t, *J* = 7.6 Hz, 1H), 2.31 (q, *J* = 6.1 Hz, 2H), 1.68-1.63 (m, 2H), 1.52-1.34 (m, 2H), 0.80 (s, 3H), 0.77 (s, 3H). **¹³C-NMR** (101 MHz; D₂O): δ 200.9, 175.0, 174.0, 172.6, 163.1, 157.2, 156.6, 156.1, 129.6, 129.3, 126.9, 123.2, 116.3, 115.9, 75.7, 68.3, 61.7, 60.9, 57.1, 55.8, 53.9, 40.4, 38.6, 35.37, 35.18, 27.93, 27.76, 24.3, 20.5, 19.1 **HRMS** (FAB) calculated for C₃₉H₅₄N₉O₁₁ S 820.36635; Found 820.36592 [M+H]⁺.

III. Synthesis of D-pHPG-L-Ser-S-Coenzyme A (**4**)



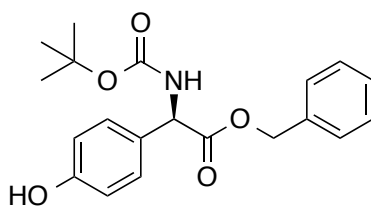
4

D-[p-(hydroxy)phenyl]glycyl-L-seryl-S-coenzyme A (4**)**. To a 10 mL round-bottomed flask equipped with a magnetic stir bar, freshly hydrogenolyzed dipeptide **S1** (25.0 mg, 0.057 mmol) was dissolved in 500 μL of THF and to this solution was added K₂CO₃ (21.0 mg, 0.169 mmol). In a separate vial coenzyme A (66.0 mg, 0.086 mmol) was dissolved in 500 μL of HPLC grade water. PyBOP (44.0 mg, 0.086 mmol) was added to the solution containing hydrogenolyzed **S1** followed by the addition of the aqueous solution of coenzyme A. The reaction was stirred at room temperature for

7

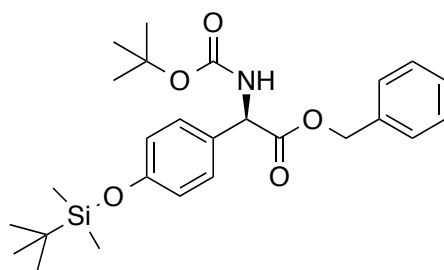
30 min, filtered through a 0.2 μm filter and purified directly by HPLC without any intermediary work-up. The reaction solution were purified by Prep Method A, collected on dry ice and lyophilized to dryness. The desired freshly lyophilized product was dissolved in 5 mL of reagent grade TFA and stirred at room temperature for 15 min. The TFA was removed *in vacuo* and the residue was re-suspended in 2 mL of 70: 30 H_2O : ACN supplemented with 0.1% TFA. The reaction mixture was purified by Prep Method A again, collected, frozen on dry ice and lyophilized to a white TFA salt (20.0 mg, 35%). **$^1\text{H-NMR}$** (400 MHz; D_2O): δ 8.57 (s, 1H), 8.33 (s, 1H), 7.26 (d, $J = 8.6$ Hz, 2H), 6.81 (d, $J = 8.6$ Hz, 2H), 6.12 (d, $J = 5.3$ Hz, 1H), 5.11 (s, 1H), 4.78 (d, $J = 6.8$ Hz, 2H), 4.56 (t, $J = 4.9$ Hz, 1H), 4.51 (br. s, 1H), 4.18 (br. s, 2H), 3.96 (s, 1H), 3.80-3.76 (m, 2H), 3.68 (dd, $J = 11.8, 4.3$ Hz, 1H), 3.55 (dd, $J = 9.9, 4.6$ Hz, 1H), 3.38 (app. t, $J = 6.1$ Hz, 2H), 3.28 (app. t, $J = 5.1$ Hz, 2H), 2.97 (app. t, $J = 6.1$ Hz, 2H), 2.36 (app. t, $J = 6.4$ Hz, 2H), 0.86 (s, 3H), 0.76 (s, 3H). **HRMS** (ESI) calculated for $\text{C}_{32}\text{H}_{49}\text{N}_9\text{O}_{20}\text{P}_3\text{S}$ 1004.2022; Found 1004.2031 $[\text{M}+\text{H}]^+$.

IV. Synthesis of L-pHPG-L-Arg-D-pHPG-dehydroalanyl-S-3'-dephospho Coenzyme A (6)



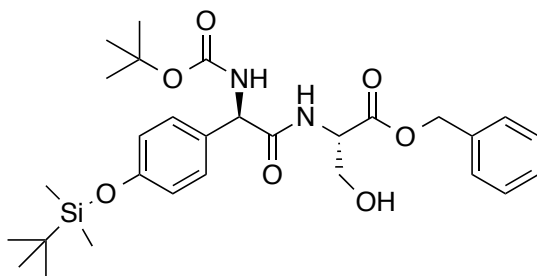
S3

N-tert-Butyloxycarbonyl-D-[p-(hydroxy)phenyl]glycine Benzyl Ester (S3). To a 500 mL round-bottomed flask, equipped with a magnetic stir bar, Boc-D-pHPG (7.00g, 26.19 mmol) was dissolved in 50 mL of freshly distilled THF. To this solution was added TEA (4.38 mL, 31.43 mmol) and benzyl bromide (3.43 mL, 28.81 mmol). The solution was left to stir at room temperature overnight. The reaction solvent was removed *in vacuo* and 300 mL of EtOAc was added to the concentrate and the organic solution was washed with 2 x 100 mL of sat. NH_4Cl (aq) and 1 x 100 mL sat. NaCl (aq). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography with an isocratic mixture of 80: 20 Hex: EtOAc to afford the product as a white foam (4.99 g, 53%). **$^1\text{H-NMR}$** (400 MHz; DMSO-d_6): δ 9.50 (s, 1H), 7.67 (d, $J = 7.8$ Hz, 1H), 7.32-7.24 (m, 5H), 7.20 (d, $J = 8.5$ Hz, 2H), 6.72 (d, $J = 8.5$ Hz, 2H), 5.16-5.08 (m, 3H), 1.39 (s, 9H). **$^{13}\text{C-NMR}$** (101 MHz; DMSO-d_6): δ 171.7, 157.8, 155.8, 136.4, 129.6, 128.8, 128.4, 127.9, 126.9, 115.6, 78.9, 66.4, 57.9, 28.7. **HRMS** (FAB) calculated for $\text{C}_{20}\text{H}_{24}\text{N}_1\text{O}_4$ 358.16490; Found 358.16509 $[\text{M}+\text{H}]^+$.



S4

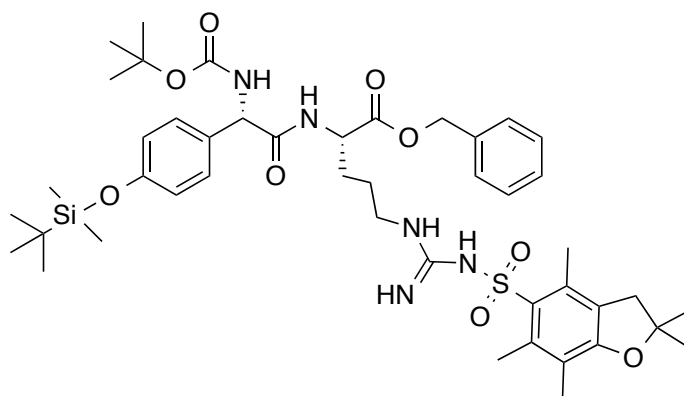
***N*-tert-Butyloxycarbonyl-D-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycine Benzyl Ester (S4).** To a 250 mL round-bottomed flask, equipped with a magnetic stir bar **S3** (4.99 g, 13.96 mmol) was dissolved in 50 mL of anhydrous DMF. To this was added imidazole (2.85 g, 41.88 mmol) followed by the addition of *t*-BDMS-Cl (2.52 g, 16.75 mmol). The reaction was stirred at room temperature overnight under Ar. The reaction mixture was poured into a separatory funnel containing 300 mL of EtOAc and the organic mixture was washed 2 x 100 mL sat. NH₄Cl (aq) and 1 x 100 mL sat. NaCl (aq). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography with an isocratic mixture of 90: 10 Hex: EtOAc to afford the product as a white foam (6.32 g, 96%). **¹H-NMR** (400 MHz; DMSO-d₆): δ 7.75 (d, *J* = 8.0 Hz, 1H), 7.31-7.20 (m, 7H), 6.81 (d, *J* = 8.5 Hz, 2H), 5.20 (d, *J* = 8.0 Hz, 1H), 5.11 (t, *J* = 16.2 Hz, 2H), 1.39 (s, 9H), 0.95 (s, 9H), 0.19 (s, 6H). **¹³C-NMR** (101 MHz; DMSO-d₆): δ 171.5, 155.7, 155.4, 136.4, 129.8, 128.7, 128.3, 127.8, 120.2, 78.9, 66.4, 57.7, 28.6, 26.0, 18.4, -4.1. **HRMS** (FAB) calculated for C₂₆H₃₈N₁O₅Si 472.25193; Found 472.24715 [M+H]⁺.



S5

***N*-tert-Butyloxycarbonyl-D-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-serine Benzyl Ester (S5).** To a 25 mL round-bottomed flask, protected peptide **S4** (3.21 g, 6.81 mmol) was dissolved in 10 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the protected amino acid was deblocked at 1 atm. of H₂ for 12 h. The mixture was filtered through Celite, washed with THF (3 x 20 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification.

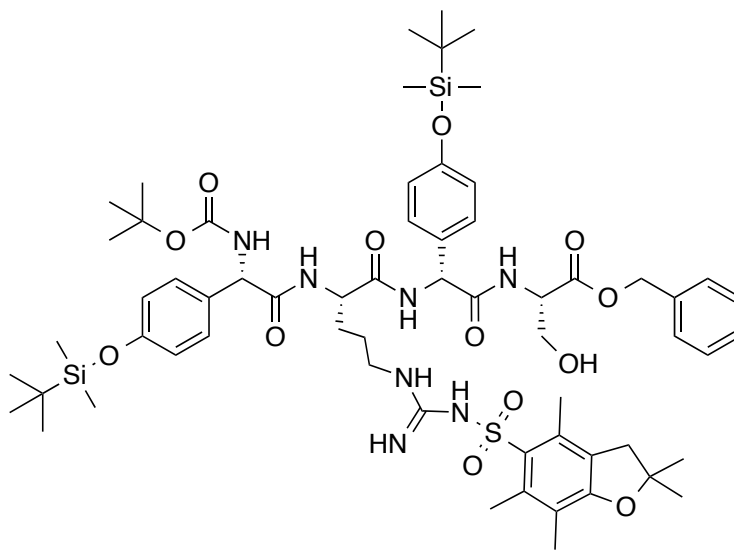
To a 250 mL round-bottomed flask equipped with a magnetic stir bar, freshly deblocked **S4** (2.60 g, 6.81 mmol) was dissolved in 30 mL of reagent grade DMF, cooled to 0 °C in an ice-bath and to this was added DIPEA (3.56 mL, 20.43 mmol). When the solution had come to temperature, PyBOP (3.90 g, 7.50 mmol) was added and after 1 min benzyl L-serine hydrochloride (2.75 g, 7.50 mmol) was added. The reaction mixture was allowed to stir at 0 °C to room temperature for 3 h. The solution was diluted with 200 mL of EtOAc and washed with sat. aq. NH₄Cl (2 x 75 mL), sat. aq. NaHCO₃ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography with a gradient of 80: 20 Hex: EtOAc to 60: 40 Hex: EtOAc over 3 L to obtain the product as a white foam (3.08 g, 81%). **¹H-NMR** (400 MHz; DMSO-d₆): δ 8.50 (d, *J* = 7.4 Hz, 1H), 7.37-7.33 (m, 5H), 7.29 (d, *J* = 8.4 Hz, 2H), 6.75 (d, *J* = 8.4 Hz, 2H), 5.27 (d, *J* = 8.1 Hz, 1H), 5.14-5.07 (m, 3H), 4.37 (q, *J* = 5.8 Hz, 1H), 3.76-3.70 (m, 1H), 3.61-3.57 (m, 1H), 1.39 (s, 9H), 0.95 (s, 9H), 0.18 (s, 6H). **¹³C-NMR** (101 MHz; DMSO-d₆): δ 171.1, 170.8, 155.2, 154.9, 136.4, 132.2, 129.03, 128.83, 128.38, 128.36, 119.9, 78.9, 66.4, 61.6, 55.3, 28.6, 26.0, 18.4, -4.0. **HRMS** (FAB) calculated for C₂₉H₄₃N₂O₇Si 559.28340; Found 559.28333 [M+H]⁺.



S6

***N*-tert-Butyloxycarbonyl-L-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-arginine(Pbf) Benzyl Ester (**S6**).** To a 250 mL round-bottomed flask, equipped with a magnetic stir bar *N*-tert-butyloxycarbonyl-L-[*p*-(hydroxy)phenyl]glycyl-L-arginine(Pbf) benzyl ester¹ (2.00 g, 2.62 mmol) was dissolved in 50 mL of anhydrous DMF. To this was added imidazole (0.53 g, 7.86 mmol) followed by the addition of *t*-BDMS-Cl (0.47 g, 3.14 mmol). The reaction was stirred at room temperature overnight under Ar. The reaction mixture was poured into a separatory funnel containing 300 mL of EtOAc and the organic mixture was washed 2 x 100 mL sat. NH₄Cl (aq) and 1 x 100 mL sat. NaCl (aq). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography with an isocratic mixture of 90: 10 Hex: EtOAc to afford the product as a white foam (2.17 g, 94%). **¹H-NMR**

(400 MHz; CDCl₃): δ 7.32-7.19 (m, 9H), 6.72 (d, *J* = 8.5 Hz, 2H), 6.29 (br. s, 2H), 6.14 (br. s, 1H), 5.68 (br. s, 1H), 5.24 (br. s, 1H), 5.02 (s, 2H), 4.54 (q, *J* = 6.3 Hz, 1H), 3.17 (br. s, 2H), 2.95 (s, 2H), 2.56 (s, 3H), 2.50 (s, 3H), 2.10 (s, 3H), 1.93-1.83 (m, 1H), 1.72 (dq, *J* = 14.6, 7.3 Hz, 1H), 1.54 (quintet, *J* = 7.3 Hz, 2H), 1.47 (s, 6H), 1.39 (s, 9H), 0.99 (s, 9H), 0.18 (s, 6H). ¹³C-NMR (101 MHz; CDCl₃): δ 181.5, 171.34, 171.18, 158.7, 156.2, 154.1, 138.4, 135.1, 132.3, 128.67, 128.62, 128.45, 128.27, 128.24, 124.6, 120.7, 120.5, 117.4, 86.3, 80.4, 67.2, 46.37, 46.33, 43.3, 28.6, 28.3, 26.47, 26.39, 25.7, 25.1, 19.3, 17.9, 12.5, -4.4. **HRMS** (FAB) calculated for C₄₅H₆₆N₅O₉SSi 880.43450; Found 880.43250 [M+H]⁺.

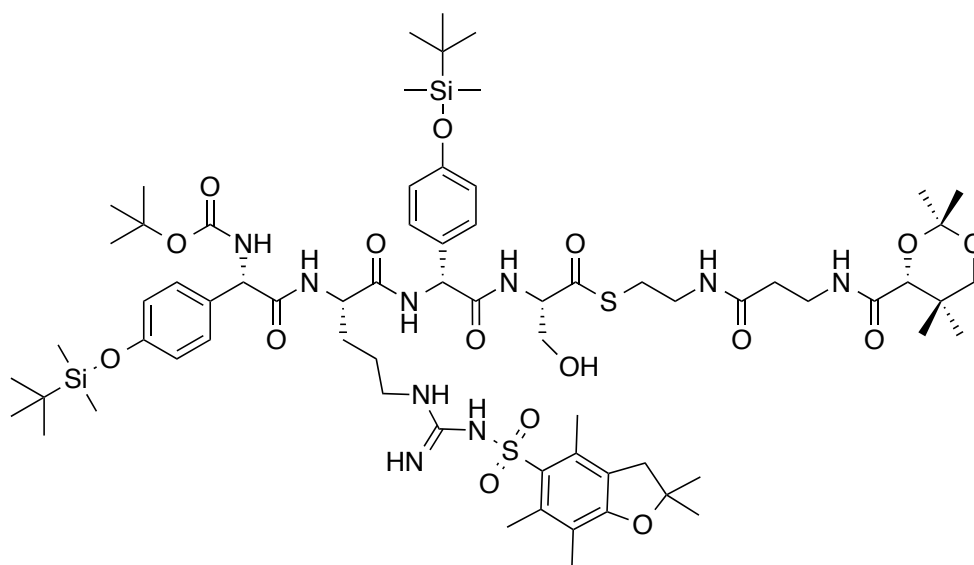


S7

***N*-tert-butyloxycarbonyl-L-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-arginyl(Pbf)-D-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-serine Benzyl Ester (S7).** To a 250 mL round-bottomed flask equipped with a magnetic stir bar, the *N*-terminal dipeptide **S6** (1.70 g, 1.93 mmol) was dissolved in 20 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the protected dipeptide was hydrogenated under 1 atm. of H₂ for 12 h. The mixture was filtered through Celite, washed with THF (3 x 50 mL) and the organic filtrate was concentrated *in vacuo* and used without further purification. In a separate 250 mL round-bottomed flask equipped with a magnetic stir bar, the *C*-terminal dipeptide **S5** (1.29 g, 2.32 mmol) was dissolved in 50 mL of TFA and stirred at room temperature for 30 min. The solution was concentrated *in vacuo* and residual solvent was removed by azeotropic distillation with toluene (3 x 50 mL).

To a 250 mL round-bottomed flask equipped with a magnetic stir bar, freshly hydrogenolyzed **S6** was added in 30 mL of freshly distilled DCM. To this was solution added DIPEA (0.50 mL, 2.90 mmol) and the solution was cooled to 0 °C in an ice-bath. Freshly deblocked *C*-terminal dipeptide **S5**

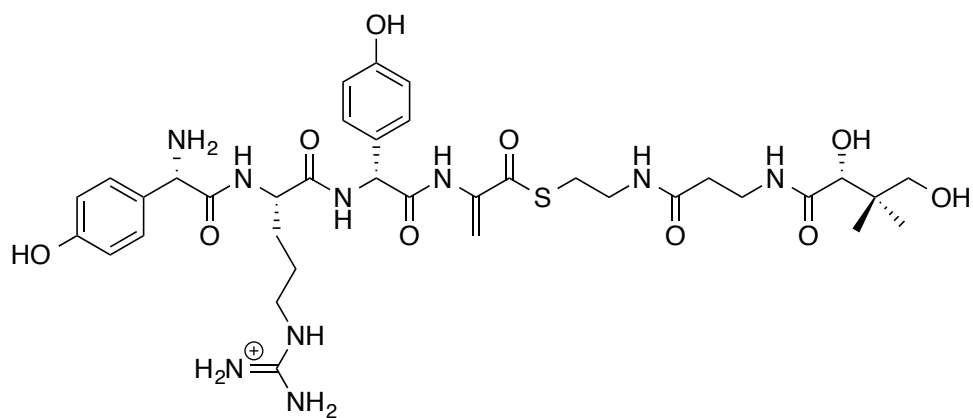
was dissolved in 20 mL of freshly distilled DCM, DIPEA (0.50 mL, 2.90 mmol) was added and the solution was cooled to 0 °C in an ice-bath. After both dipeptide containing solutions were sufficiently cooled, PyBOP (1.21 g, 2.32 mmol) was added to the flask containing the carboxylate and after 1 min, freshly deblocked **S5** was added dropwise over 2 min. The reaction mixture was stirred from 0 °C to room temperature over 3 h. The solvent was removed *in vacuo* and the concentrate was dissolved in 150 mL of EtOAc, washed with sat. aq. NH₄Cl (2 x 75 mL), sat. aq. NaHCO₃ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and purified by silica gel chromatography using a gradient of 60: 40 EtOAc: Hex to 80: 20 EtOAc: Hex over 3 L to obtain the product as a white foam (2.38 g, 62%). **¹H-NMR** (400 MHz; DMSO-d₆): δ 8.64 (d, *J* = 7.3 Hz, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 8.33 (d, *J* = 7.7 Hz, 1H), 7.38-7.31 (m, 6H), 7.27 (d, *J* = 8.7 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 2H), 6.77 (d, *J* = 8.5 Hz, 2H), 6.74 (d, *J* = 8.6 Hz, 2H), 6.39 (br. s, 1H), 5.54 (d, *J* = 8.2 Hz, 1H), 5.19 (d, *J* = 8.3 Hz, 1H), 5.14 (s, 2H), 5.07 (t, *J* = 5.6 Hz, 1H), 4.46-4.38 (m, 1H), 4.34 (dt, *J* = 7.6, 4.8 Hz, 1H), 3.69 (dt, *J* = 10.9, 5.4 Hz, 1H), 3.54 (dt, *J* = 10.9, 5.2 Hz, 1H), 3.01 (q, *J* = 6.3 Hz, 2H), 2.95 (s, 2H), 2.48 (s, 3H), 2.43 (s, 3H), 2.01 (s, 3H), 1.65-1.61 (m, 1H), 1.52-1.47 (m, 1H), 1.41 (s, 9H), 1.38 (s, 9H), 0.94 (s, 9H), 0.17 (s, 6H), 0.16 (s, 3H), 0.15 (s, 3H). **¹³C-NMR** (101 MHz; DMSO-d₆): δ 171.1, 170.80, 170.73, 170.5, 157.9, 156.5, 155.3, 154.9, 153.3, 140.5, 137.7, 136.3, 131.99, 131.89, 128.94, 128.89, 128.84, 128.77, 128.4, 128.1, 124.7, 119.98, 119.89, 116.7, 86.7, 78.9, 66.4, 61.6, 55.3, 43.0, 28.77, 28.63, 25.9, 19.4, 18.4, 18.1, 14.6, 12.8, -4.1. **HRMS** (FAB) calculated for C₆₂H₉₂N₇O₁₈SSi₂ 1230.60069; Found 1230.60234 [M+H]⁺.



S8

***N*-tert-Butyloxycarbonyl-L-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-arginyl(Pbf)-D-[*p*-(*tert*-butyldimethylsilyl)phenyl]glycyl-L-seryl-S-pantetheine dimethyl ketal (**S8**).** To a 100 mL round-bottomed flask, protected peptide **S7** (1.00 g, 0.81 mmol) was dissolved in 20 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the protected tetrapeptide was deblocked at 1 atm. of H₂ for 12 h. The mixture was filtered through Celite, washed with THF (3 x 10 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification.

The freshly hydrogenolyzed tetrapeptide was dissolved in 10 mL of freshly distilled DCM and to this solution was added DIPEA (0.31 mL, 2.43 mmol) and cooled to 0 °C with an ice-bath. To this solution was added PyBOP (0.51 g, 0.97 mmol) and pantetheine dimethyl ketal¹ (0.31 g, 0.97 mmol) and the reaction mixture was stirred for 30 min at room temperature. The reaction mixture was diluted with 150 mL of EtOAc and washed with sat. aq. NH₄Cl (2 x 20 mL), sat. aq. NaHCO₃ (1 x 20 mL) and brine (1 x 20 mL). The organic layer was concentrated *in vacuo* and the viscous oil was partially purified through a plug of silica, eluting with 2% MeOH in EtOAc. Product was verified via mass-spectrometry analysis. **HRMS** (FAB) calculated for C₆₉H₁₁₀N₉O₁₆S₂Si₂ 1440.70450; Found 1440.70727 [M+H]⁺.



S9

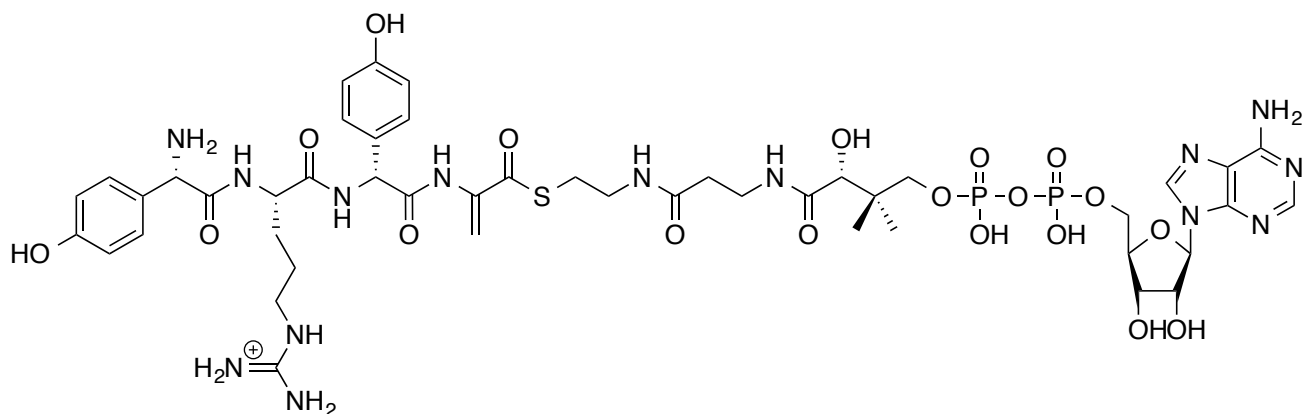
L-[p-(Hydroxy)phenyl]glycyl-L-arginyl-D-[p-(hydroxy)phenyl]glycyl-dehydroalanyl-S-

pantetheine (S9). To a 100 mL round-bottomed flask, protected peptide **S8** (0.28 g, 0.81 mmol) was dissolved in 20 mL of reagent grade THF. To this solution was added a catalytic amount of Pd-OH/C and the protected tetrapeptide was deblocked at 1 atm. of H₂ for 1 h. The mixture was filtered through Celite, washed with THF (3 x 10 mL) and the organic filtrate was concentrated *in vacuo* and used in the next reaction without further purification.

Freshly deprotected **S8** was dissolved in 4 mL of freshly distilled DCM and to this solution was added TEA (30 μL, 0.23 mmol) followed by methanesulfonyl chloride (17 μL, 0.23 mmol). After 45 min, DBU (57 μL, 0.38 mmol) was added and the reaction was stirred at room temperature for 1 h. The solvent was removed *in vacuo* and the product was partially purified through a thick plug of silica gel, eluting with 1% MeOH in EtOAc. The obtained yellow foam was verified to contain the desired dehydroalanyl intermediate product by HRMS. (**HRMS** (FAB) calculated for C₆₉H₁₀₇N₉NaO₁₅S₂Si₂ 1444.67643; Found 1444.67643 [M+Na].)

To a 25 mL round-bottomed flask, equipped with a magnetic stir-bar, dehydroalanyl intermediate product (140 mg, 98 μmol) was dissolved in 5 mL of reagent grade THF. To this solution was added 20 μL of AcOH and TBAF (30 μL of 1.0 M solution in THF, 30 μmol) and the silyl-deprotection was monitored by TLC. After the O-silyl-groups were deblocked, the THF was removed *in vacuo* and the resulting yellow oil was left to stand in 10 mL of neat TFA for 25 min. The TFA was removed *in vacuo* and the residue was re-suspended in 2 mL of 70: 30 H₂O: ACN supplemented with 0.1% TFA. The reaction mixture was purified by Prep Method A, the product was collected over multiple injections, frozen on dry ice and lyophilized to afford a yellow TFA salt (30 mg, 38%). **¹H-NMR** (400 MHz; D₂O): δ 7.17 (d, *J* = 8.5 Hz, 2H), 7.12 (d, *J* = 8.5 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 6.72 (d, *J* = 8.7 Hz, 2H), 6.03 (s, 1H), 5.80 (s, 1H), 5.28 (s, 1H), 4.99 (s, 1H), 4.31 (t, *J* = 7.3 Hz, 1H),

3.86 (s, 1H), 3.38 (d, $J = 11.3$ Hz, 2H), 3.26 (d, $J = 11.3$ Hz, 2H), 3.04 (app. t, $J = 6.9$ Hz, 2H), 2.99 (app. t, $J = 6.4$ Hz, 2H), 2.32 (app. t, $J = 6.3$ Hz, 2H), 1.71-1.63 (m, 2H), 1.53-1.37 (m, 2H), 0.79 (s, 3H), 0.75 (s, 3H). ^{13}C -NMR (101 MHz; D_2O): δ 175.0, 174.0, 171.2, 168.8, 164.0, 163.2, 157.2, 156.2, 153.5, 149.1, 129.6, 129.3, 117.8, 116.26, 116.24, 115.9, 114.9, 76.9, 68.4, 55.8, 53.8, 40.4, 38.6, 38.3, 35.38, 35.19, 24.3, 20.5, 19.0, 14.1. HRMS (ESI) calculated for $\text{C}_{36}\text{H}_{52}\text{N}_9\text{O}_{10}\text{S}$ 802.3552; Found 802.3552 $[\text{M}+\text{H}]^+$.

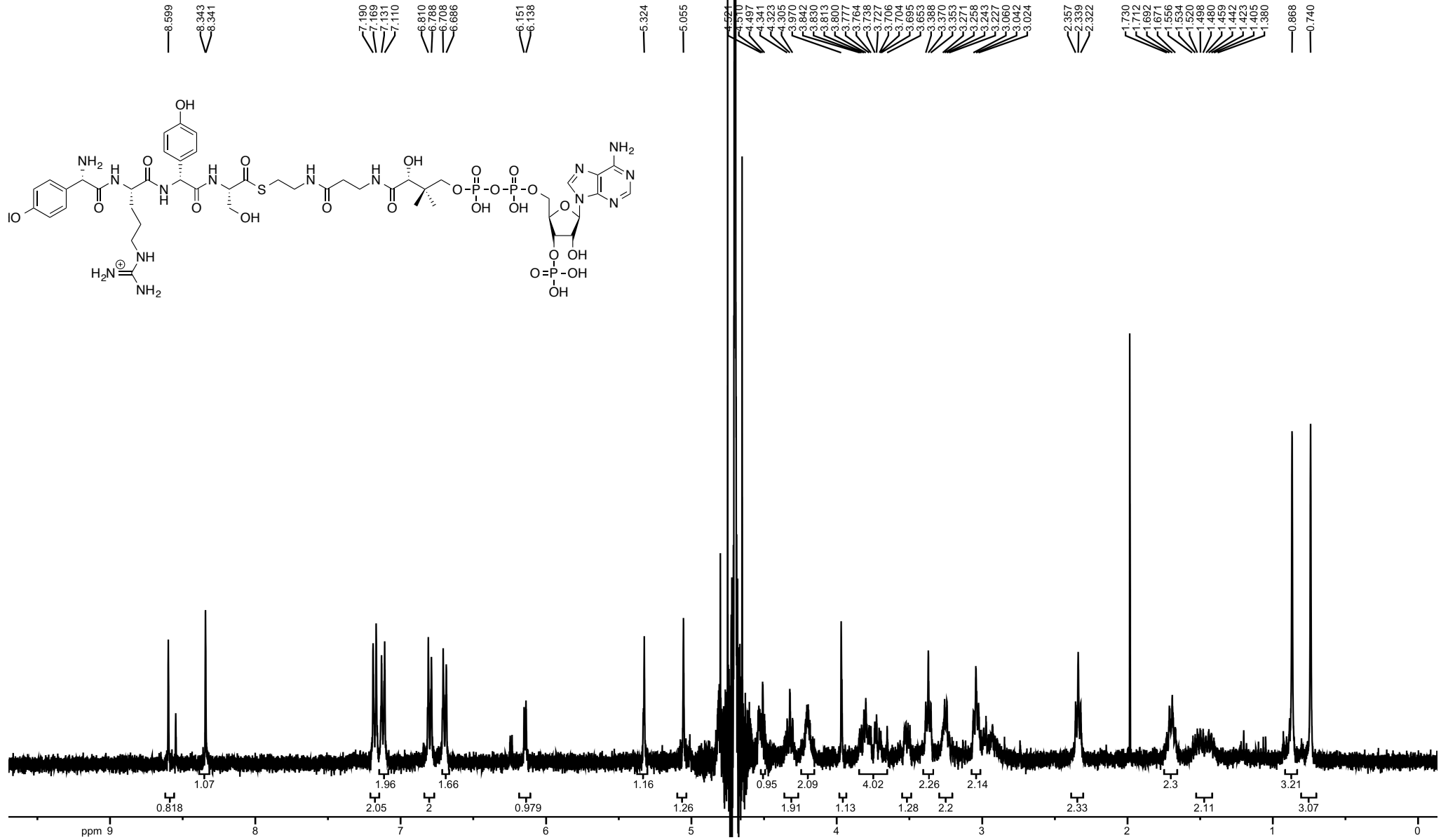


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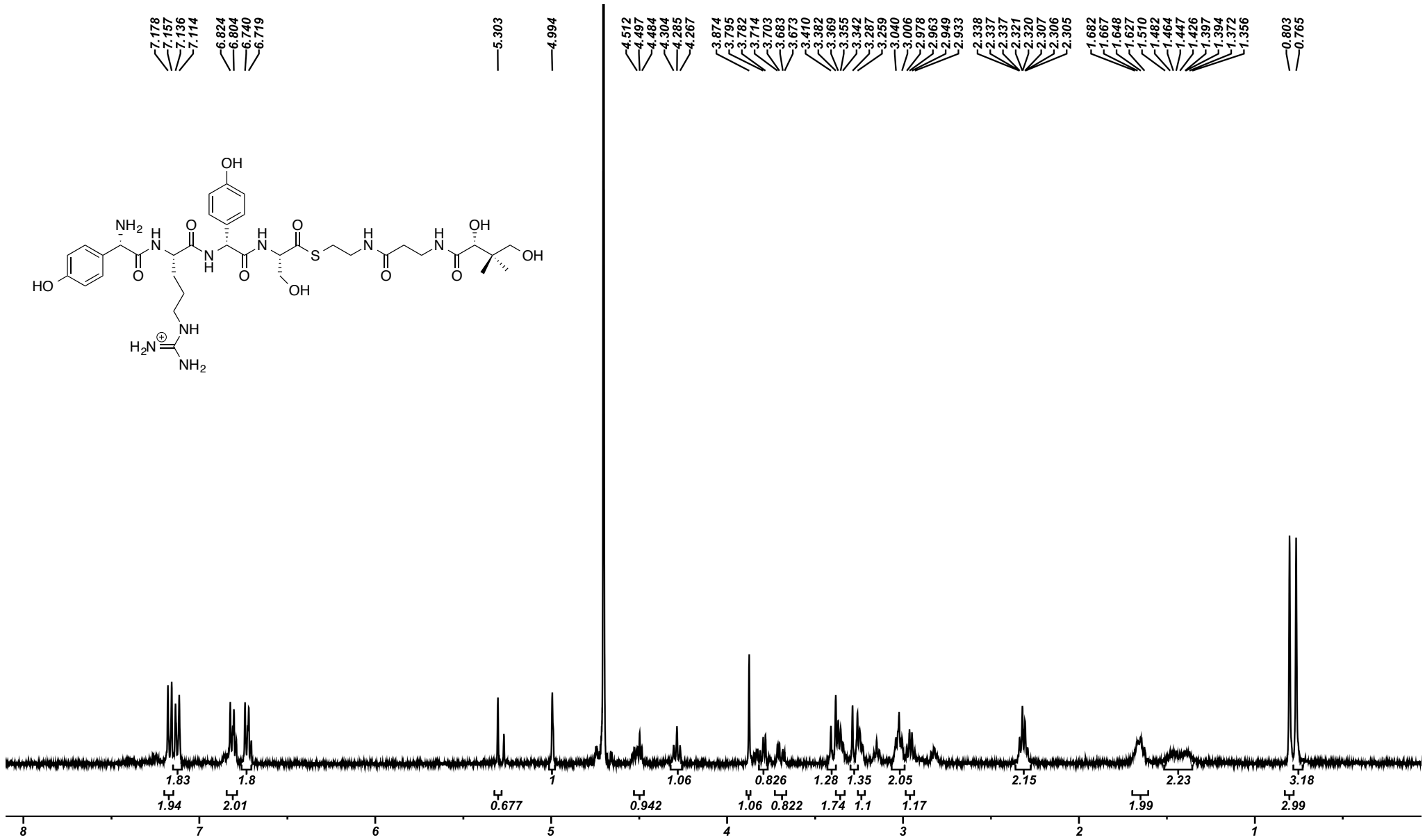
L-[p-(hydroxy)phenyl]glycyl-L-arginyl-D-[p-(hydroxy)phenyl]glycyl-dehydroalanyl-S-3'-diphospho-coenzyme A (6).

The title compound was obtained through established procedures utilizing the previously reported *E. coli* coenzyme A biosynthetic enzymes Pank and Ppat.^{2,3} To a 2.0 mL centrifugal tube was added freshly purified compound **S9** (6 mg, 7.31 μmol) in 500 μL of buffer consisting of 50 mM Tris, 25 mM NaCl, 5 mM ATP and 1 mM MgCl_2 , pH 7.5. To this mixture was added 1.5 eq of Pank enzyme. After 45 min, 1.5 eq of Ppat was added and the reaction was allowed to proceed for 1 h. Enzymes were removed with a 10 kMWCO Amicon Ultra centrifugal filter. The flow-through was purified by HPLC using Prep Method A, the product was collected over multiple injections, frozen on dry ice and lyophilized to dryness to afford a white TFA salt (1 mg, 11%). ^1H -NMR (400 MHz; D_2O): δ 8.43 (s, 1H), 8.16 (s, 1H), 7.13 (d, $J = 8.6$ Hz, 2H), 7.08 (d, $J = 8.5$ Hz, 2H), 6.77 (d, $J = 8.6$ Hz, 2H), 6.64 (d, $J = 8.6$ Hz, 2H), 6.02 (d, $J = 5.5$ Hz, 1H), 5.97 (s, 1H), 5.77 (s, 1H), 5.26 (s, 1H), 5.00 (s, 1H), 4.43 (t, $J = 4.6$ Hz, 1H), 4.32-4.28 (m, 1H), 4.14 (br. s, $J = 2.4$ Hz, 1H), 3.91 (s, 1H), 3.78-3.72 (m, 1H), 3.66-3.63 (m, 2H), 3.56-3.53 (m, 1H), 3.46 (m, $J = 2.3$ Hz, 1H), 3.36-3.20 (m, 4H), 3.00 (app. t, $J = 6.9$ Hz, 2H), 2.94-2.90 (m, 2H), 2.29 (app. t, $J = 6.3$ Hz, 2H), 1.70-1.61 (m, 2H), 1.49-1.35 (m, 2H), 0.80 (s, 3H), 0.66 (s, 3H). HRMS (ESI) calculated for $\text{C}_{46}\text{H}_{65}\text{N}_{14}\text{O}_{19}\text{P}_2\text{S}$ 1211.3741; Found 1211.3724 $[\text{M}+\text{H}]^+$.

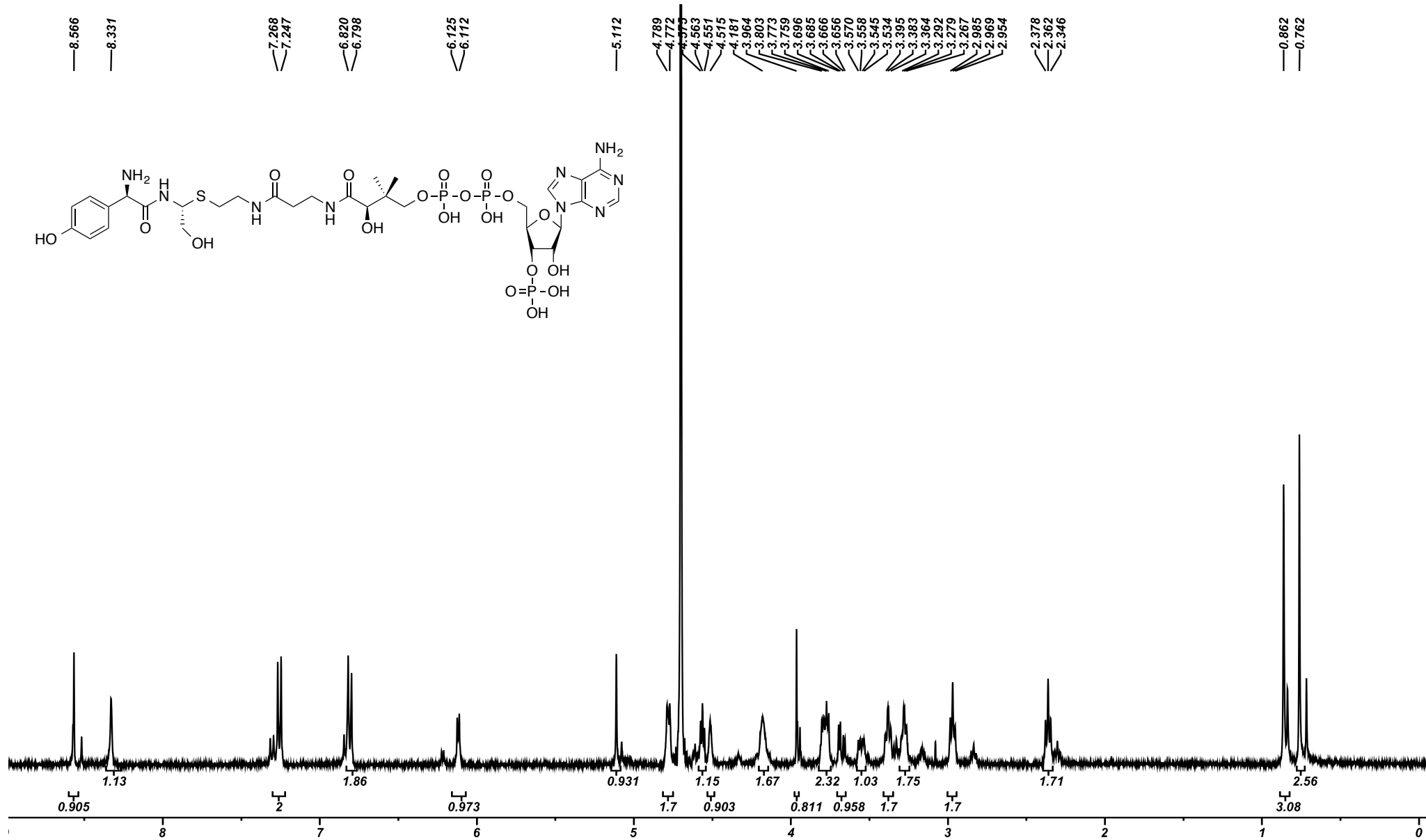
L-pHPG-L-Arg-D-pHPG-L-Ser-S-coenzyme A (1)



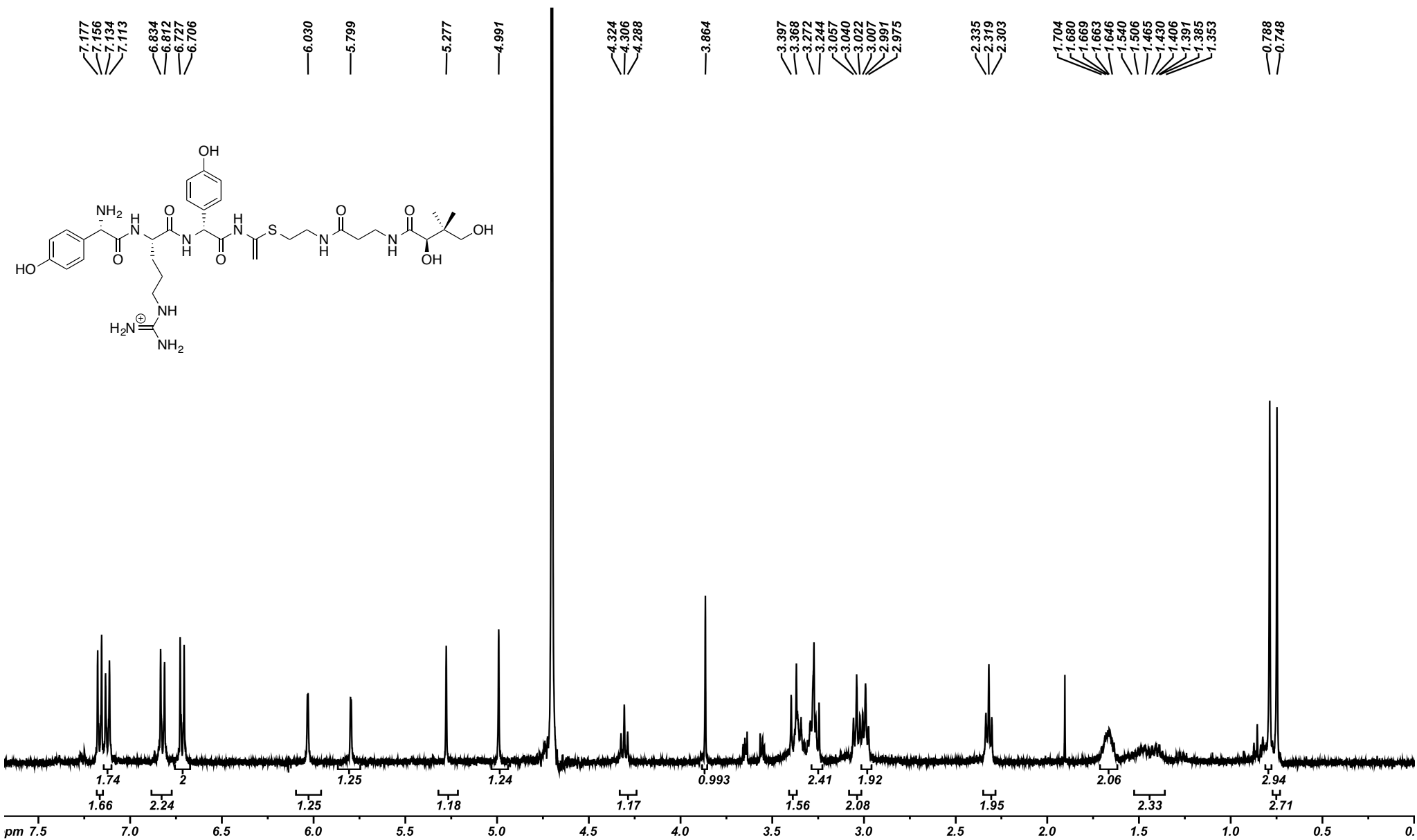
L-pHPG-L-Arg-D-pHPG-L-Ser-S-pantetheine (3)



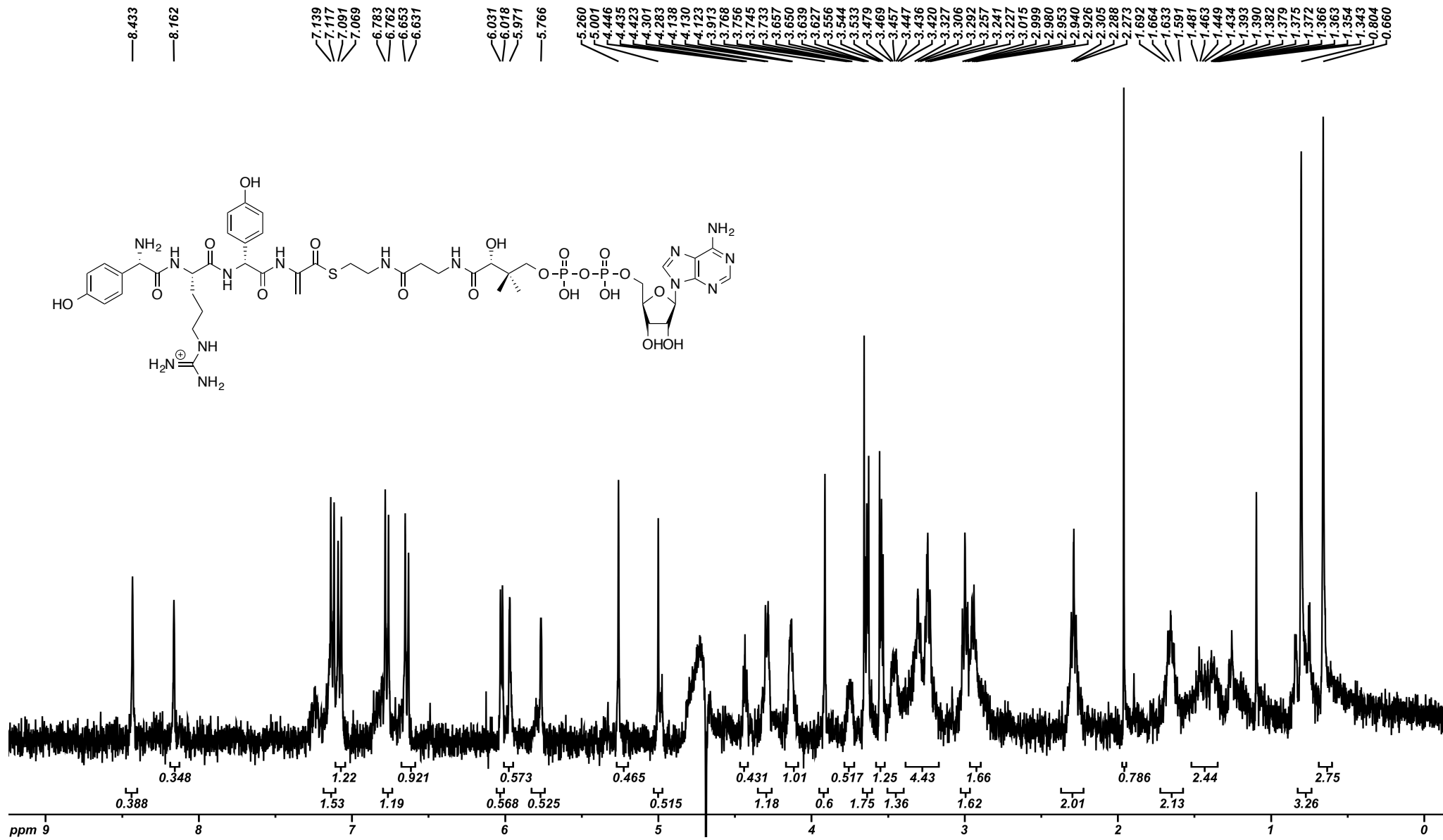
D-pHPG-L-Ser-S-coenzyme A (4)



L-pHPG-L-Arg-D-pHPG-dehydroalanyl-S-pantetheine (S9)



L-pHPG-L-Arg-D-pHPG-dehydroalanyl-S-3'-dephosho coenzyme A (6)



Supplementary Table 1: PCR primers used in this study

Primer	Nucleotide Sequence*
M5-F	5'- GGGATACATAT <u>GCGCGGCGACGACGAG</u> -3'
M5-R	5'- GGATAAAGCTTTCACCGCTCCTCCAG -3'
PCP ₄ -F	5'- GCGTAACATATGACCGCGCGGCGCT -3'
PCP ₄ -R	5'- GTAAGCGGCCGC <u>TCACTCGTCGGCGGCGG</u> -3'
M5-PstI	5'- CGCGAGCTTCTGCAGCGGCAG -3'
H790A-F	5'- TGGAGC GCC ACCACATCCTG -3'
H790A-R	5'- CAGGATGTGGT GGG CGTCCA -3'
H792A-F	5'- GAGCCACCAC GCC ATCCTGCTCG -3'
H792A-R	5'- CGAGCAGGAT GCG GTGGTGCTC -3'

Supplementary Table 2: PCR primer pairs

Construct	5' Primer	3' Primer	template
pET28b-M5	M5-F	M5-R	pMG0531 ⁴
pET28b-PCP ₄	PCP ₄ -F	PCP ₄ -R	pMG0531 ⁴
pET28b-M5*H790A	M5-F	H790A-R	pET28b-M5
	H790A-F	M5-PstI	
pET28b-M5*H792A	M5-F	H792A-R	pET28b-M5
	H792A-F	M5-PstI	

* Restriction sites are underlined, alanine codon is in bold and inserted stop codon is italicized

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1. Gaudelli, N.M. & Townsend, C.A. Stereocontrolled Syntheses of Peptide Thioesters Containing Modified Seryl Residues as Probes of Antibiotic Biosynthesis. *J. Org. Chem.* **78**, 6412-6426 (2013).
2. Worthington, A.S. & Burkart, M.D. One-pot chemo-enzymatic synthesis of reporter-modified proteins. *Org. Biomol. Chem.* **4**, 44 (2006).
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4. Gunsior, M. *et al.* The biosynthetic gene cluster for a monocyclic β -Lactam antibiotic, nocardicin A. *Chem. Biol.* **11**, 927-938 (2004).