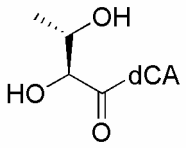
Appendix

Synthesis of Unnatural Amino Acids

A.1 Preparation of α-Hydroxythreonine (Tah)



Synthesis of α-hydroxy threonine (Tah, 2*R*, 3*S*-dihydroxy-butanoic acid)[1]

L-Threonine (2.2 g, 18.5 mmol), suspended in 5 ml of water at -5 °C, was treated simultaneously with a solution of 1.38 g $NaNO_2$ (20 mmol) in 2 ml of water and 557 µl of concentrated H_2SO_4 (10 mmol) in 1.5 ml H_2O. The two solutions were added slowly while stirring so that the temperature remained between 0 °C and 5 °C. The reaction turned yellow upon addition. The solution was then stirred overnight at room temperature. The reaction mixture was concentrated, the mixture was treated with 3 ml of EtOH, and the salts were filtered. The solution was concentrated. The material was dry loaded onto a flash silica gel column and run in 1:1 hexanes/ethyl acetate with 1% acetic acid to give 730 mg (38 %) of hydroxythreonine: ^{1}H NMR (D_2O) δ 1.17 (d, 3 H, J = 6 Hz), 4.1 (m, 2H); ^{13}C NMR 18.2, 68.4, 74.2, 176.0: Electrospray MS Calcd for $C_4H_8O_4$ minus H: 119.1. Found *m/z:* 119.0.

Synthesis of Tah cyanomethyl ester (2*R*, 3*S*-dihydroxy-butanoate cyanomethyl ester)[2]

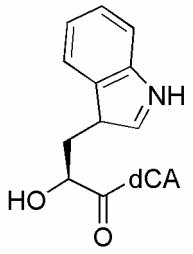
The hydroxy acid (385 mg, 3.21 mmol) was dissolved in 5.1 ml of $ClCH_2CN$ (80.1 mmol) and 1.2 ml Et_3N (8.44 mmol). Upon stirring under Ar for 30 min, the solution turned yellow. A gradient flash silica gel column from 20 % to 80 % ethyl acetate/hexanes was run, and the isolated product was dried on vacuum to yield 50.9 mg

(10 %) of hydroxythreonine cyanomethyl ester: ^{1}H NMR (D_2O) δ 1.27 (d, 3H, J= 6 Hz), 4.22 (m, 1H), 4.34 (d, 1H, J= 3 Hz), 5.01 (s, 2H), ^{13}C 18.2, 49.7, 68.4, 74.4, 115.5, 172.6; FAB MS Calcd for $C_6H_9O_4N$ plus H: 160.17. Found *m/z*: 160.03 (M+H), 75.02, 103.07.

Synthesis of dCA-Tah

Hydroxythreonine cyanomethyl cyanomethyl ester (5.7 mg, 35.8 μmol) was dissolved in 250 μl dry DMF in a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (14.4 mg, 11.9 μmol) was added, and the reaction stirred under Ar for 24 h. Upon completion of the reaction, the pure dCA-Tah compound was obtained by preparative HPLC. Electrospray MS Calcd for $C_{23}H_{31}N_8O_{16}P_2$ minus H: 736.13. Found *m/z* (M-H): 737.4.

A.2 Preparation of α-Hydroxytryptophan (Wah)



Synthesis of Wah cyanomethyl ester (3-(3-Indolyl)-2-hydroxypropanoic acid cyanomethyl ester)

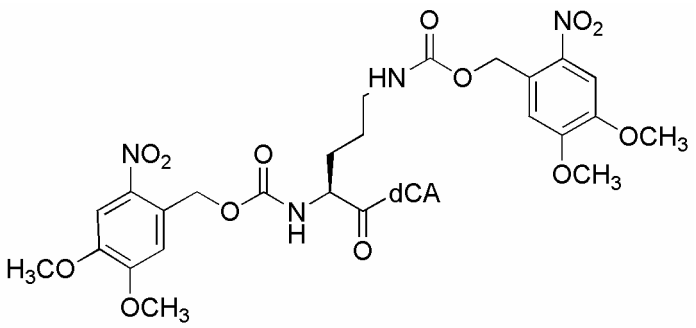
The hydroxy acid (255 mg, 1.24 mmol) was dissolved in 1.9 ml of $ClCH_2CN$ (30 mmol) and 514 μl Et_3N (3.65 mmol). Upon stirring under Ar for 45 min, the solution turned pale yellow. The reaction mixture was concentrated and dried under vacuum. The material was dry loaded onto a flash silica gel column and run in 9:1 methylene

chloride/ethyl acetate to give 242 mg (80 % yield) of hydroxyl-tryptophan cyanomethyl ester: ^{1}H NMR (DMSO) δ 3.04 (m, 2H), 3.32 (broad s, 1H), 4.38 (broad s, 1H), 4.94 (s, 2H), 6.96 (t, 1H, J = 7.2 Hz), 7.05 (t, 1H, J= 6.9 Hz), 7.12 (d, 1H, J = 2 Hz), 7.32 (d, 1H, J = 7.8 Hz), 7.51 (d, 1H, J = 7.5 Hz) , 10.85 (s, 1H); ^{13}C NMR 30.0, 48.9, 70.7, 109.7, 111.3, 115.8, 118.3, 118.3, 120.8, 123.8, 127.3, 136.0, 172.7: Electrospray MS Calcd for $C_{13}H_{12}N_2O_3$ plus H: 245.08. Found *m/z* (M+H)*:* 245.0.

Synthesis of dCA-Wah

Hydroxy-tryptophan cyanomethyl ester (11 mg, 45μmol) was dissolved in 315 μl dry DMF n a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (20 mg, 16.7 μmol) was added, and the reaction was stirred under Ar for 9 h. Upon completion of the reaction, the pure compound was obtained by preparative HPLC. Electrospray MS Calcd for $C_{30}H_{35}N_9O_{15}P_2$ minus H: 823.17. Found *m/z* (M-H): 822.0.

A.3 Preparation of $(NVOC)_2$Ornithine (Orn)



Synthesis of $(NVOC)_2$Ornithine ((S)-2,5-bis((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)pentanoic acid)

L-Ornithine-hydrochloride (Advanced Chem Tech Y02595) (90 mg, 0.53 mmol) was added to 1.2 ml of 10 % Na_2CO_3 (0.53 mmol) and 1.8 ml dioxane. The reaction was stirred over an ice bath, and 4,5 dimethoxy-2-nitrobenzyl chloroformate (NVOC-Cl, Aldrich) (453 mg, 1.6 mmol) was slowly added to the mixture. The reaction was allowed to warm to ambient temperature. After 4 h, the reaction was poured into 30 ml of water and extracted 3 times with 20 ml of diethyl ether. The precipitate was filtered to give 475.4 mg of crude $(NVOC)_2$–Ornithine (45.8 % crude yield): Electrospray MS Calcd for $C_{25}H_{30}N_4O_{14}$ minus H: 609.18. Found *m/z* (M-H)*:* 609.2.

Synthesis of $(NVOC)_2$Ornithine cyanomethyl ester ((S)-cyanomethyl 2,5-bis((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)pentanoate

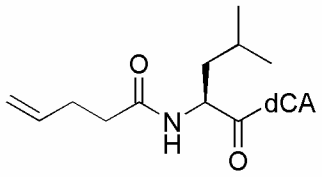
The crude (NVOC)2-Ornithine (250 mg, 0.41 mmol) was dissolved in 1 ml of $ClCH_2CN$ (15.8 mmol) and 200 µl Et_3N (1.4 mmol). The reaction was stirred under Ar for 50 min, and was concentrated and dried under vacuum. The material was purified on

a flash silica gel column and run in 3:1 methylene chloride/ethyl acetate to give 130 mg (49 % yield) of $(NVOC)_2$Ornithine cyanomethyl ester: ^{1}H NMR ($CDCl_3$) δ 1.64 (m, 2 H), 1.76 (m, 2H), 3.25 (m, 2H), 3.96 (t, 12H, J = 7 Hz), 4.44 (m, 1H), 4.77 (m, 2H) or q, 2H, 4.99 (broad s, 1H, αNH), 5.52 (m, 4H), 6.98 (d, 2H, J = 5 Hz), 7.67 (d, 2H, J = 5Hz); ^{13}C NMR 26.27, 40.32, 49.12, 53.60, 56.44, 56.51, 63.86, 64.26, 108.25, 110.16, 110.71, 113.84, 127.52, 139.75, 140.05, 148.29, 153.47, 153.70, 155.56, 156.15, 170.96: Electrospray MS Calcd for $C_{27}H_{31}N_5O_{14}$ plus Na: 672.18. Found *m/z* (M+Na+)*:* 672.2.

Synthesis of dCA-$(NVOC)_2$Ornithine

($(NVOC)_2$Ornithine cyanomethyl ester (25 mg, 45μmol) was dissolved in 315 μl dry DMF in a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (20 mg, 16.7 μmol) was added, and the reaction was stirred under Ar for 2 h. Upon completion of the reaction, the pure compound was obtained by preparative HPLC. Maldi TOF MS Calcd for $C_{44}H_{53}N_{12}O_{26}P_2$ plus H: 1228.26. Found *m/z* (M+H): 1229.4.

A.4 Preparation of 4PO-Leucine (Leu)



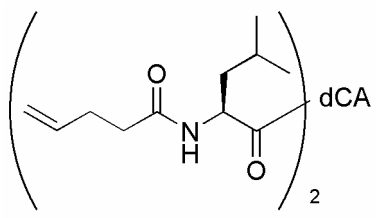
Synthesis of 4PO-Leucine. L-leucine (528 mg, 4 mmol) was added to 60 ml of 10 % Na_2CO_3 (5.6 mmol), and 30 ml dioxane. The reaction was stirred and 4-pentenoic anhydride (1 ml, 5.6 mmol) in 30 ml dioxane was added to the mixture. After 24 h, the

reaction was quenched with 100 ml of methylene chloride and 100 ml of 1N $NaHSO_4$ and extracted three times with 100 ml of methylene chloride. The organic layer was concentrated and dried to yield 4PO-Leucine, 805 mg (94 % yield) of white powder: Electrospray MS Calcd for $C_{11}H_{19}NO_3$ plus H: 214.14. Found *m/z* (M+H)*:* 214.4.

Synthesis of 4PO-Leucine cyanomethyl ester. The crude 4PO-Leucine (805 mg, 3.77 mmol) was dissolved in 7.33 ml DMF, 7.33 ml of $ClCH_2CN$ (113.1 mmol) and 1.39 ml Et_3N (9.9 mmol). The reaction was stirred under Ar for 24 h, quenched with 100 ml diethyl ether, extracted three times with water, concentrated, and dried under vacuum to give 659 mg (69 % yield) of 4PO-Leucine cyanomethyl ester: ^1H NMR ($CDCl_3$) δ 0.96 (m, 6 H), 1.65 (m, 3H), 2.35 (m, 4H), 2.51 (m, 1H), 4.77 (m, 3H), 5.06 (m, 3H), 5.83 (m, 2H): Electrospray MS Calcd for $C_{13}H_{20}N_2O_3$ plus H: 253.15. Found *m/z* (M+H): 253.2.

Synthesis of dCA-4PO-Leucine. 4PO-Leucine cyanomethyl ester (25 mg, 45μmol) was dissolved in 315 μl dry DMF in a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (20 mg, 16.7 μmol) was added, and the reaction was stirred under Ar for 2 h. Upon completion of the reaction, the pure compound was obtained by preparative HPLC. Fractions eluting at 18.5 to 27.4 minutes contained the singly-aminoacylated dCA-Leucine to yield 16.7 μmoles (46.6% recovery). Electrospray MS Calcd for $C_{30}H_{43}N_9O_{15}P_2$ minus H: 830.24. Found *m/z* (M-H): 830.4.

A.5 Preparation of (4PO-Leucine)$_2$ (DiLeu)



Synthesis of dCA-(4PO-Leucine)$_2$. 4PO-Leucine cyanomethyl ester (25 mg, 45μmol) was treated with dCA and purified as described above. Fractions eluting at 31.5 to 32.5 min contained the bis-aminoacylated dCA-Leucine to yield 0.84 μmoles (2.4% recovery). Electrospray MS Calcd for $C_{41}H_{60}N_{10}O_{17}P_2$ minus H: 1025.36. Found *m/z* (M-H): 1025.1.

A.6 References

1. Servi, S. 2,2,5-Trimethyl-1,3-Dioxolane-4-Carboxaldehyde as a Chiral Synthon-Synthesis of the 2 Enantiomers of Methyl 2,3,6-Trideoxy-Alpha-L-Threo-Hex-2-Enopyranoside, Key Intermediate in the Synthesis of Daunosamine, and of (+)- and (-)-Rhodinose. *Journal of Organic Chemistry* **50**, 5865-5867 (1985).

2. England, P. M., Lester, H. A. & Dougherty, D. A. Incorporation of esters into proteins: Improved synthesis of hydroxyacyl tRNAs. *Tetrahedron Lett.* **40**, 6189-6192 (1999).