Supporting Information

The Puromycin Route to Assess Stereo- and Regiochemical Constraints on Peptide Bond Formation in Eukaryotic Ribosomes

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General Information. ^{1}H and ^{13}C NMR spectra were recorded on a Varian, Inc. UNITY INOVA instrument operating at 500 MHz using D_2O or DMSO-*d6* as the solvent. ^{1}H NMR data are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet; dd, doublet of doublets. High-resolution mass spectra (FAB) were recorded on a JMS-600H double-focusing, high-resolution, magnetic sector mass spectrometer at the Mass Spectrometry Laboratory, Division of Chemistry and Chemical Engineering, California Institute of Technology. Column chromatography was carried out on silica gel (40-63 μm, EM Science). Analytical HPLC was performed using a Vydac C18 column (5 mm, 4.5 x 250 mm) with buffer A (5 mM NH_4OAc, pH 5.5 with 10% acetonitrile) and buffer B (5 mM NH_4OAc, pH 5.5 with 90% acetonitrile); a linear gradient of 100% buffer B in 50 min was used with a flow rate of 1 mL/min. All reagents were of highest available commercial quality and were used without further purification. Puromycin aminonucleoside (3'-amino-3'-deoxy-*N*, *N'*-dimethyl-adenosine) (PANS) was purchased from Sigma Chemical Co. FMOC-(4-methoxy-D-phenylalanine) and FMOC-(D-alanine) were purchased from Bachem. FMOC-(4-methyl-L-phenylalanine), FMOC-(L-alanine), and FMOC-(L-β-homoalanine) were purchased from Fluka. FMOC-(4-

methyl-D-phenylalanine) and FMOC-(4-methyl-L-β-phenylalanine) were from Peptech. Puromycin and puromycin analog concentrations were determined with the following extinction coefficients ($M^{-1}cm^{-1}$) at 260 nm: L- and D-puromycin (**1a** and **1b**) [ε = 11,790] in H_2O; L-(4-Me)-Phe-PANS, D-(4-Me)-Phe-PANS, and L-β-(4-Me)-Phe-PANS (**2a** – **2c**) [ε = 10,500] in H_2O; and L-Ala-PANS, D-Ala-PANS, and L-β-Ala-PANS (**3a** – **3c**) [ε = 11,000] in phosphate buffered saline (pH 7.3).

Rabbit reticulocyte lysate was purchased from Novagen. Rabbit globin mRNA was obtained from Life Technologies Gibco BRL. L-Puromycin (**1a**) was purchased from Sigma Chemical Co. Ras mRNA was prepared by using two DNA primers complementary to the 5'- and 3'-ends of the coding region for H-Ras (pProEX HTb vector, a kind gift from Dafna Bar-Sagi)[1] to amplify the gene using PCR. mRNA was produced by T7 runoff transcription[2] of the H-Ras DNA in the presence of RNAsecure (Ambion) followed by gel purification via denaturing urea-PAGE and 'crush and soak' RNA isolation. L-[^{35}S]methionine (1,175 Ci/mmol) was purchased from NEN Life Science Products. Carboxypeptidase Y was obtained from Pierce. GF/A glass microfiber filters were from Whatman. Scintillation counting was carried out using a Beckman LS-6500 liquid scintillation counter.

General Procedure for Preparation of Puromycin Analogs. *N*, *N*'-dicyclohexylcarbodiimide (DCC) (0.0539 mmol) was added to a cold (0 °C) solution of PANS (0.0520 mmol), FMOC-protected amino acid (0.0541 mmol), and *N*-hydroxysuccinimide (NHS) (0.0556 mmol) in dried *N*,*N'*-dimethylformamide (DMF) (0.900 mL). The solution was stirred for 30 min in an ice-water bath and then for 25 h at ambient temperature. *N*,*N'*-dicyclohexylurea was filtered and washed (EtOAc, 4 mL),

and the filtrate was concentrated *in vacuo*. For **1b**, the residue was resuspended in EtOAc, sonicated, and the mixture was filtered and then dried. The material was purified by gradient flash chromatography using $CHCl_3 \rightarrow$ MeOH/$CHCl_3$ (4:96) for **1b** or MeOH/$CHCl_3$ (7:93) for **2a**-**2c** and **3a**-**3c**. Homogenous product fractions were dried *in vacuo* to yield the FMOC-protected product. FMOC-deprotection was carried out in 20% (v/v) piperidine in DMF (5mL) with stirring for 30 min at ambient temperature. The solvent was removed *in vacuo* and the residue was subjected to gradient flash chromatography using $CHCl_3 \rightarrow$ MeOH/$CHCl_3$ (8:92) for **1b**, **2a**, and **2b** and TEA/MeOH/$CHCl_3$ (2:10:88) for **2c** and **3a**-**3c** to afford the titled products. Confirmation of purity was assessed using analytical HPLC.

9-{3'-Deoxy-3'-[(4-methoxy-D-phenylalanyl)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine (D-puromycin) (1b).[3] White solid (31.5 mg, 87.3%): ^{1}H (DMSO-*d6*) δ 1.85 (br s, 2H), 2.58-2.63 (m, 1H), 2.93 (dd, *J* = 4.5, 14 Hz, 1H), 3.42 (dd, *J* = 4.5, 8.5 Hz, 2H), 3.51-3.56 (m, 2H), 3.72 (s, 6H), 3.93-3.96 (m, 1H), 4.47-4.51 (m, 4H), 5.17 (t, *J* = 5.5 Hz, 1H), 5.97 (d, *J* = 2.0 Hz, 1H), 6.17 (d, *J* = 5.0, 1H), 6.85 (d, *J* = 9.0 Hz, 2H), 7.15 (d, *J* = 8.5 Hz, 2H), 8.08 (br s, 1H), 8.24 (s, 1H), 8.45 (s, 1H); ^{13}C (DMSO-*d6*) δ 25.4, 50.6, 56.0, 56.8, 61.7, 73.8, 84.4, 90.2, 114.3, 131.0, 138.7, 150.4, 152.6, 158.0, 175.5; HRMS (FAB), *m/z* calculated for $C_{22}H_{30}N_7O_5$ $(M+H)^+$ 472.2311, found 472.2307.

9-{3'-Deoxy-3'-[(4-methyl-L-phenylalanyl)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine [L-(4-Me)-Phe-PANS] (2a). Pale white solid (18.8 mg, 80.8%): ^{1}H NMR (DMSO-*d6*) δ 1.84 (br s, 2H), 2.26 (s, 6H), 2.52-2.57 (m, 1H), 2.94 (dd, *J* = 4.5, 14 Hz, 1H), 3.44-3.52 (m, 2H), 3.67-3.70 (m, 2H), 3.92-3.95 (m, 1H), 4.44-

4.50 (m, 4H), 5.14 (t, *J* = 5.5 Hz, 1H), 5.98 (d, *J* = 3.0 Hz, 1H), 6.14 (d, *J* = 4.0 Hz, 1H), 7.10 (dd, *J* = 8.0, 18 Hz, 4H), 8.07 (d, *J* = 5.5 Hz, 1H), 8.24 (s, 1H), 8.45 (s, 1H); ^{13}C (DMSO-*d6*) δ 21.3, 41.2, 50.7, 56.9, 61.7, 73.9, 84.3, 90.2, 120.3, 129.4, 129.8, 135.7, 136.3, 138.7, 150.4, 152.6, 155.0, 175.5; HRMS (FAB), *m/z* calculated for $C_{22}H_{30}N_7O_4$ $(M+H)^+$ 456.2362, found 456.2367.

9-{3'-Deoxy-3'-[(4-methyl-D-phenylalanyl)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine [D-(4-Me)-Phe-PANS] (2b). Pale white solid (20.7 mg, 88.8%): ^{1}H NMR (DMSO-*d6*) δ 1.85 (br s, 2H), 2.26 (s, 6H), 2.61 (dd, *J* = 8.0, 13 Hz, 1H) 2.96 (dd, *J* = 4.5, 14 Hz, 1H), 3.42-3.45 (m, 1H), 3.51-3.56 (m, 1H), 3.71-3.73 (m, 2H), 3.94-3.96 (m, 1H), 4.40-4.49 (m, 4H), 4.48 (d, *J* = 12 Hz, 1H), 5.17 (t, *J* = 5.5 Hz, 1H), 5.97 (d, *J* = 2.5 Hz, 1H), 6.19 (br s, 1H), 7.11 (dd, *J* = 8.0, 16 Hz, 4H), 8.10 (br s, 1H), 8.23 (s, 1H), 8.45 (s, 1H); ^{13}C (DMSO-*d6*) δ 21.4, 41.0, 50.6, 56.8, 61.7, 73.8, 84.4, 90.2, 120.3, 129.4, 129.9, 135.8, 136.1, 138.7, 150.4, 152.6, 155.0, 175.5; HRMS (FAB), *m/z* calculated for $C_{22}H_{30}N_7O_4$ $(M+H)^+$ 456.2362, found 456.2360.

9-{3'-Deoxy-3'-[(4-methyl-L-β-phenylalanyl)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine [L-β-(4-Me)-Phe-PANS] (2c). Pale white solid (17.8 mg, 73.0%): ^{1}H NMR (D_20) δ 2.06 (s, 6H), 2.45-2.49 (m, 1H), 2.67 (d, *J* = 6.5 Hz, 1H), 3.18 (t, *J* = 6.0 Hz, 1H), 3.28 (br s, 3H), 3.37-3.39 (m, 1H), 3.49-3.50 (m, 1H), 3.59-3.62 (m, 1H), 3.80 (dd, *J* = 2.0, 13 Hz, 1H), 4.08-4.10 (m, 1H), 4.35 (dd, *J* = 6.0, 8.5 Hz, 1H), 4.46 (dd, *J* = 3.0, 5.5 Hz, 1H), 5.94 (d, *J* = 2.5 Hz, 1H), 7.03 (s, 4H), 8.03 (s, 1H), 8.15 (s, 1H); ^{13}C NMR (D_20) δ 19.1, 26.0, 40.5, 49.8, 50.5, 54.5, 60.8, 73.6, 82.7, 89.7, 111.0, 120.0, 129.4, 129.6, 134.0, 137.2, 138.0, 148.8, 152.3, 173.6; HRMS (FAB), *m/z* calculated for $C_{23}H_{32}N_7O_4$ $(M+H)^+$ 470.2519, found 470.2508.

9-{3'-Deoxy-3'-[(L-alanine)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine (L-Ala-PANS) (3a). Pale yellow solid (5.7 mg, 30.2%): ^{1}H NMR (D_2O) δ 1.41 (d, *J* = 7.0 Hz, 3H), 3.28 (br s, 6H), 3.63 (dd, *J* = 3.5, 13 Hz, 1H), 3.82 (dd, *J* = 2.5, 13 Hz, 1H), 3.97 (q, *J* = 7.0 Hz, 1H), 4.17-4.18 (m, 1H), 4.55-4.58 (m, 2H), 4.62-4.64 (m, 1H), 5.98 (d, *J* = 3.0 Hz, 1H), 8.03 (s, 1H), 8.17 (s, 1H); ^{13}C NMR (D_2O) δ 17.3, 39.0, 49.4, 51.0, 60.7, 73.5, 82.7, 89.6, 119.5, 138.0, 148.8, 152.2, 154.6, 172.4; HRMS (FAB), *m/z* calculated for $C_{15}H_{24}N_7O_4$ $(M+H)^+$ 366.1892, found 366.1889.

9-{3'-Deoxy-3'-[(D-alanine)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine (D-Ala-PANS) (3b). Pale yellow solid (8.6 mg, 45.4%): ^{1}H NMR (D_20) δ 1.43 (d, *J* = 7.5 Hz, 3H), 3.28 (br s, 6H), 3.65 (dd, *J* = 4.0, 13 Hz, 1H), 3.83 (dd, *J* = 2.5, 13 Hz, 1H), 4.02 (q, *J* = 7.5 Hz, 1H), 4.14-4.17 (m, 1H), 4.55-4.58 (m, 2H), 4.64-4.66 (m, 1H), 5.98 (d, *J* = 3.0 Hz, 1H), 8.04 (s, 1H), 8.17 (s, 1H); ^{13}C NMR (D_20) δ 17.0, 39.0, 49.3, 50.9, 60.8, 73.3, 82.8, 89.6, 106.0, 119.6, 138.1, 152.3, 154.8, 172.0; HRMS (FAB), *m/z* calculated for $C_{15}H_{24}N_7O_4$ $(M+H)^+$ 366.1892, found 366.1898.

9-{3'-Deoxy-3'-[(L-β-homoalanine)amino]-β-D-ribofuranosyl}-6-(*N*,*N*'-dimethylamino)purine (L-β-Ala-PANS) (3c). Pale yellow solid (4.6 mg, 25.6%): ^{1}H NMR (D_20) δ 1.16 (d, *J* = 6.5 Hz, 3H), 1.74 (s, 1H), 2.52 (d, *J* = 3.5 Hz, 2H), 3.24 (br s, 6H), 3.52-3.61 (m, 2H), 3.78 (d, *J* = 13 Hz, 1H), 4.11 (d, *J* = 5.5 Hz, 1H), 4.62-4.64 (m, 2H), 5.93 (s, 1H), 7.99 (s, 1H), 8.13 (s, 1H); ^{13}C NMR (D_20) δ 18.2, 39.0, 39.5, 45.0, 50.7, 60.7, 73.5, 82.7, 89.6, 119.5, 138.0, 148.8, 152.2, 172.7; HRMS (FAB), *m/z* calculated for $C_{16}H_{26}N_7O_4$ $(M+H)^+$ 380.2049, found 380.2054.

IC_{50} Determination. Translation reactions containing [^{35}S]Met were made up in batch on ice and added in aliquots to microcentrifuge tubes containing an appropriate

amount puromycin or puromycin analog dried *in vacuo*. Typically, a 20 µl translation mixture consisted of 0.8 µL of 2.5 M KCl, 0.4 µL of 25 mM MgOAc, 1.6 µL of 12.5X Translation Mixture without methionine (25 mM dithiothreitol (DTT), 250 mM HEPES (pH 7.6), 100 mM creatine phosphate, and 312.5 µM of 19 amino acids, except methionine), 3.6 µL of nuclease-free water, 0.6 µL (6.1 µCi) of [^{35}S]Met (1175 Ci/mmol), 8 µL of Red Nova® nuclease-treated lysate, and 5 µL of 0.05 µg/µL globin mRNA. Inhibitor, lysate preparation (include all components except template), and globin mRNA were mixed simultaneously and incubated at 30 ºC for 60 min. Then 2 µL of each reaction was combined with 8 µL of tricine loading buffer (80 mM Tris-Cl (pH 6.8), 200 mM DTT, 24% (v/v) glycerol, 8% sodium dodecyl sulfate (SDS), and 0.02 % (w/v) Coomassie blue G-250), heated to 90 ºC for 5 min, and applied entirely to a 4% stacking portion of a 16% tricine SDS-polyacrylamide gel containing 20% (v/v) glycerol[4] (30 mA for 1.5h). Gels were fixed in 10% acetic acid (v/v) and 50% (v/v) methanol, dried, exposed overnight on a PhosphorImager screen, and analyzed using a Storm PhosphorImager (Molecular Dynamics). Analysis in Figure S1 was carried out as described above except 6 µL of each reaction and 24 µL of tricine loading buffer were loaded (1.5-fold increase in stacking and resolving portion of gel; 30mA for 7 h).

Carboxypeptidase Assay. Translation reactions were prepared as described for IC_{50} determination except reactions (50 µL) contained 2 µL of 2.5 M KOAc, 1 µL of 25 mM MgOAc, 4 µL of 12.5X Translation Mixture without methionine (25 mM dithiothreitol (DTT), 250 mM HEPES (pH 7.6), 100 mM creatine phosphate, and 312.5 µM of 19 amino acids, except methionine), 16 µL (163 µCi) of [^{35}S]Met (1175 Ci/mmol), 20 µL of Red Nova® nuclease-treated lysate, and 6.96 µL of 230 µg/mL Ras mRNA.[3]

Inhibitor, lysate components, and Ras mRNA were mixed simultaneously and incubated at 30 ºC for 60 min. Then 2 µL of reaction was combined with 150 µL of 0.1 M sodium acetate (pH 5.0) and 17 µL carboxypeptidase Y (CPY) (1 mg/mL in 0.05 M sodium citrate (pH 5.3) Pierce), and incubated at 37 ºC for 18 h. After incubation, reactions were mixed with 100 µL of 1 N NaOH/2% H_2O_2 (hydrolyzes charged tRNAs and removes the red color that may quench scintillation counting) and incubated at 37 ºC for 10 min to hydrolyze the charged tRNAs. Then 0.9 mL of 25% trichloroacetic acid (TCA)/2% casamino acids was added to the samples, vortexed, and put on ice for 10 min. The samples were filtered on GF/A filters (pre-soaked in 5% TCA), washed 3 times with 3-mL portions of cold 5% TCA, and scintillation counted to determine the amount of [^{35}S]Met-Ras. For the no CPY-treated samples, [^{35}S]Met-Ras (2 µL of reaction) was TCA precipitated without CPY treatment as described above.

References

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(4) Schagger, H.; Jagow, G. V. *Anal. Biochem*. **1987**, *166*, 368-379.

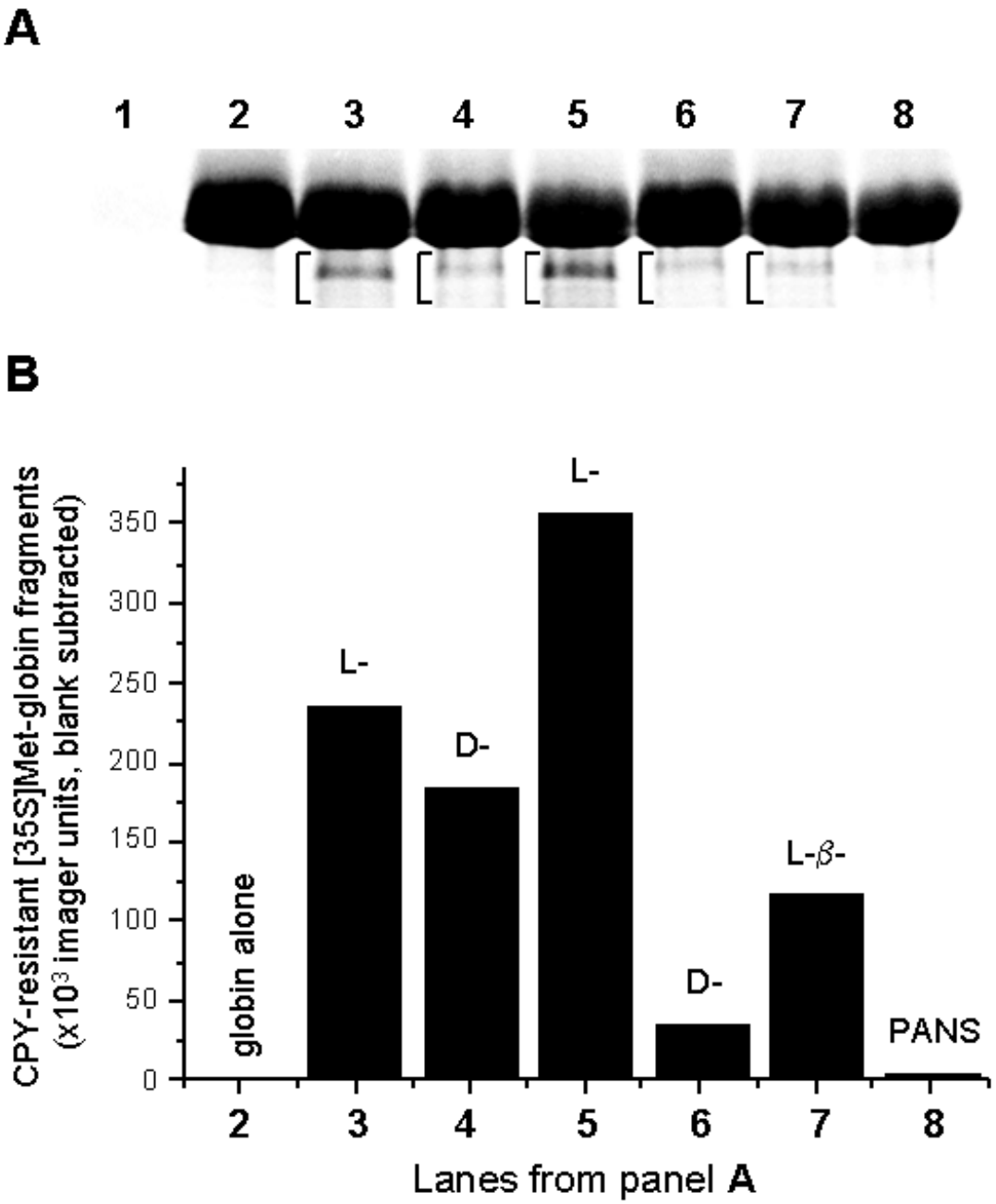

Figure S1. (A) Tricine-SDS-PAGE analysis of globin fragments resulting from puromycin and puromycin analog attachment. Lane 1, no template; lane 2, globin alone, no puromycin; lane 3, L-puromycin (2 μM); lane 4, D-puromycin (500 μM); lane 5, L-4-Me)-Phe-PANS (2 μM); lane 6, D-(4-Me)-Phe-PANS (1500 μM); lane 7, L-β-(4-Me)-Phe-PANS (1200 μM); lane 8, puromycin aminonucleoside (PANS) (5 mM). PANS is a negative control molecule (no amino acid moiety) to evaluate the production of protein fragments in the presence of high exogenous molecule concentrations. The globin fragment-puromycin complexes are indicated by brackets. (B) Quantification of the globin fragment-puromycin complexes from A.

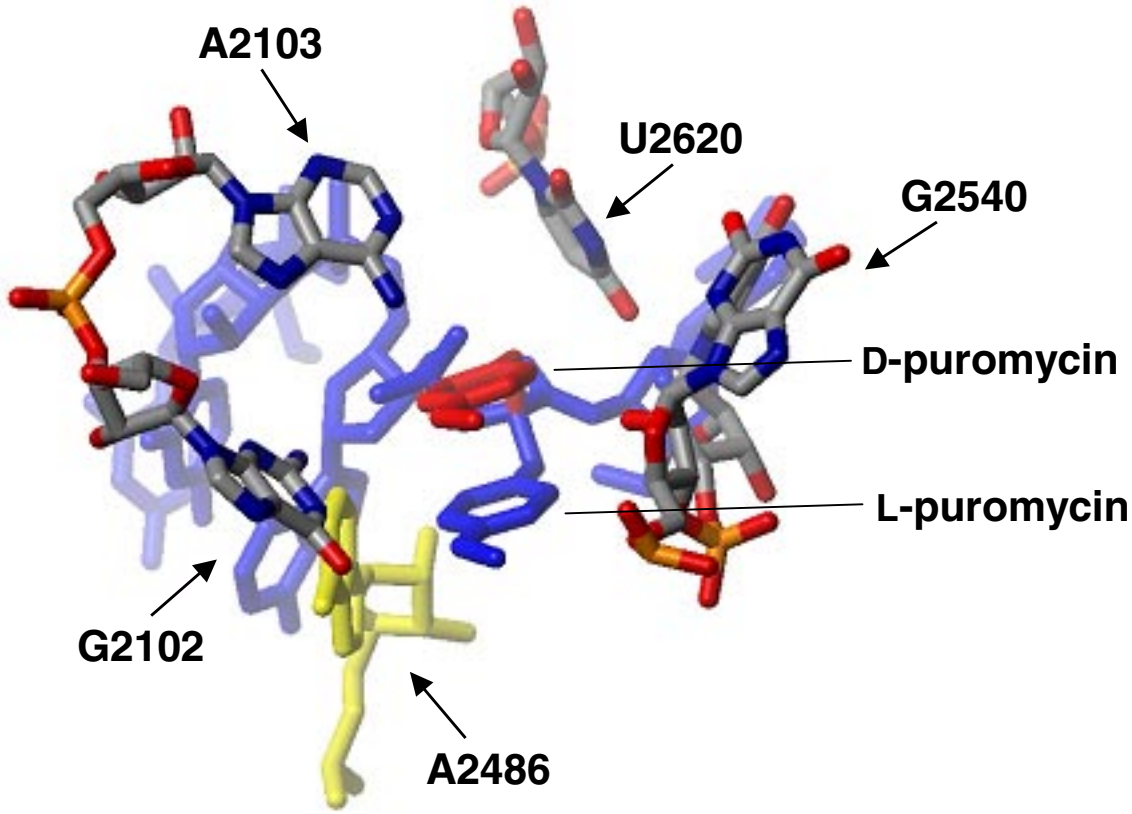

Figure S2. Model for D-puromycin (red) placement in the 50S ribosomal-CCdA-p-L-puromycin (blue) complex from *Haloarcula marismortui* (Nissen, P.; Hansen, J.; Ban, N., Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 920-930; PDB identifier 1FFZ). U2620 (U2585 in *Escherichia coli*) is the closest nucleotide to the D-puromycin side chain that may cause steric clash. A2486 (A2451 in *E. coli*) (yellow), the base possibly involved in peptidyl transferase catalysis, is shown for reference. G2102 (G2481), A2103 (A2482), and G2540 (G2505) are the next closest nucleotides (~5Å) to the D-puromycin side chain.