Chapter 3

Probing Flexibility of Protein Synthesis *In Vitro* by Puromycin Analogs

Part of this work has previously appeared as: Starck, S. R., Qi, X., Olsen, B. N. & Roberts, R. W. The puromycin route to assess stereo- and regiochemical constraints on Peptide bond formation in eukaryotic ribosomes. *J Am Chem Soc* **125**, 8090-1 (2003).

Abstract

Puromycin is a small-molecule mimic of aminoacyl-tRNA (aa-tRNA), and can be mistakenly inserted in place of aminoacyl-tRNA by ribosome and acts as a universal protein translation inhibitor. We have constructed a series of puromycin analogs with natural and unnatural amino acids side chains in order to systematically test the effects of side chain characteristics of amino acid moiety on the activity of puromycin. We then measured the activity of each compound in a high dynamic-range IC_{50} potency assay using the rabbit reticulocyte protein synthesis system. Both IC_{50} values and carboxypeptidase Y (CPY)-resistant results lead us to conclude that all of the compounds we synthesized both inhibit translation and participate in ribosome-mediated peptide bond formation.

Introduction

Puromycin has played an important role in our understanding of the ribosome and protein synthesis. Puromycin is a small-molecule mimic of aminoacyl-tRNA (aa-tRNA), and can be mistakenly inserted in place of aminoacyl-tRNA by ribosome and acts as a universal protein translation inhibitor (Figure 1). It enters the ribosomal A site and participates in peptide bond formation with the nascent peptidyl chain,[1] resulting in truncated proteins containing the drug at their C-terminus. Puromycin and puromycin analogs have been extremely useful in exploring the activity of various nucleophiles (-OH vs. -NH_2 vs. -SH) in the ribosome active site.[2-5]

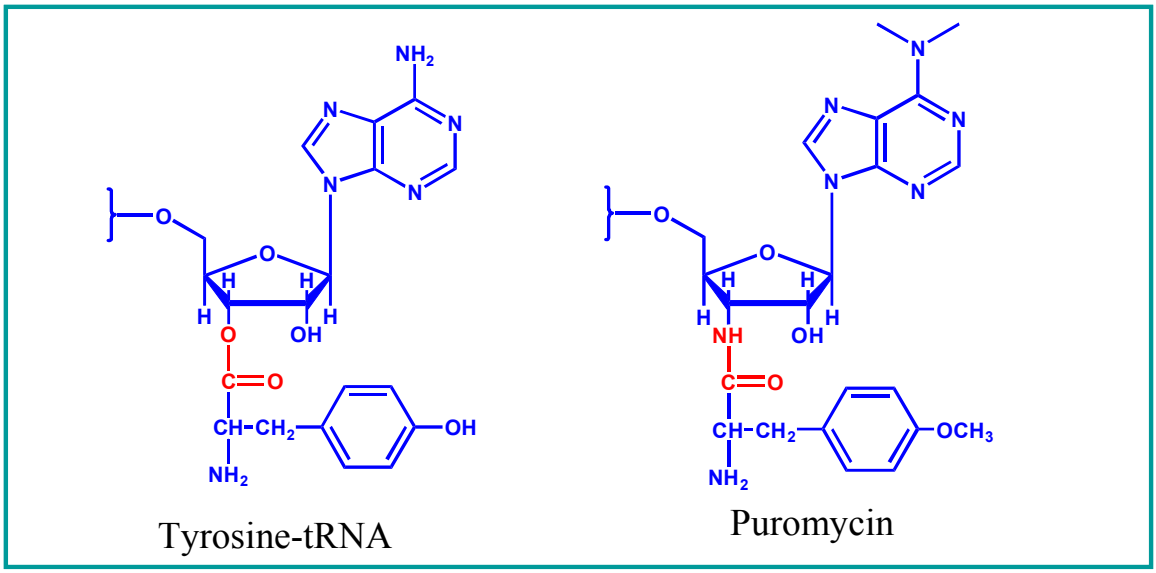


Figure 1. Structural comparison between Tyrosine-tRNA and puromycin.

Unlike aa-tRNA, puromycin is able to enter the ribosome independently (Figure 2), does not induce EF-Tu•GTPase activiy,[6] and does not appear to require soluble

translation factors for function.[7] Puromycin and related analogs thus provide a direct means to address ribosome-mediated peptide bond formation even in an intact translation extract.

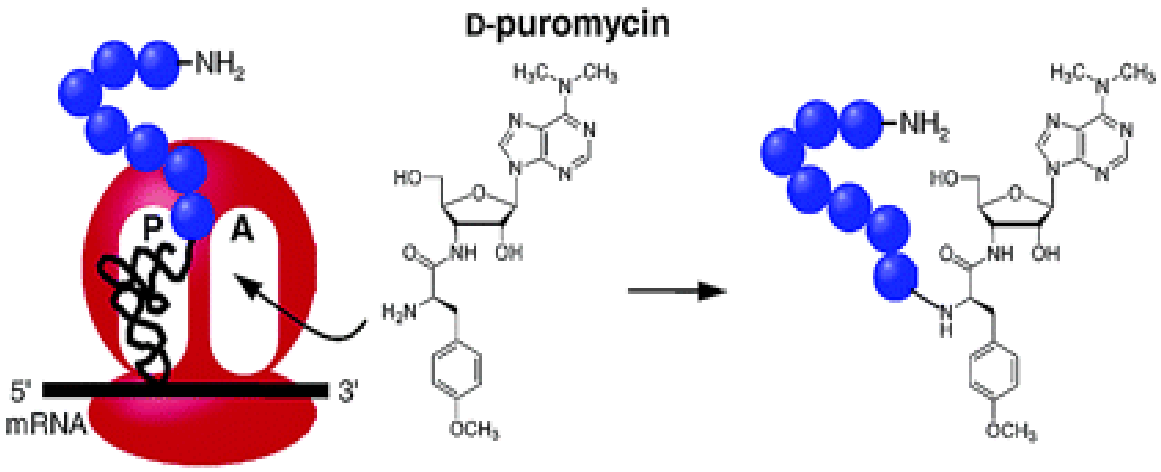


Figure 2. D-puromycin incorporated in the peptidyltransferase center of the ribosome.

The protein synthesis machinery can be used to incorporate unnatural amino acids into peptides,[8-10] proteins,[11,12] and molecular libraries.[13-18] These studies indicate that the ribosome displays a broad ability to utilize residues beyond the 20 naturally occurring amino acids. Chemically misacylated tRNA fragments and tRNAs provide one route to probe the stereo- and regiospecificity of isolated ribosomes[19-22] and intact translation systems.[23] This approach has expanded our understanding of the range of residues that can be incorporated by the ribosome.[24-27] However, entry of both β- and D-amino acids has proved challenging.[9,11] Analysis of incorporation of these residues would be valuable and deepen our understanding of the stereo- and regiochemical constraints of ribosome-mediated peptide bond formation.

Materials and Methods

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 $CHCl_3$ → 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 $CHCl_3$ → 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).

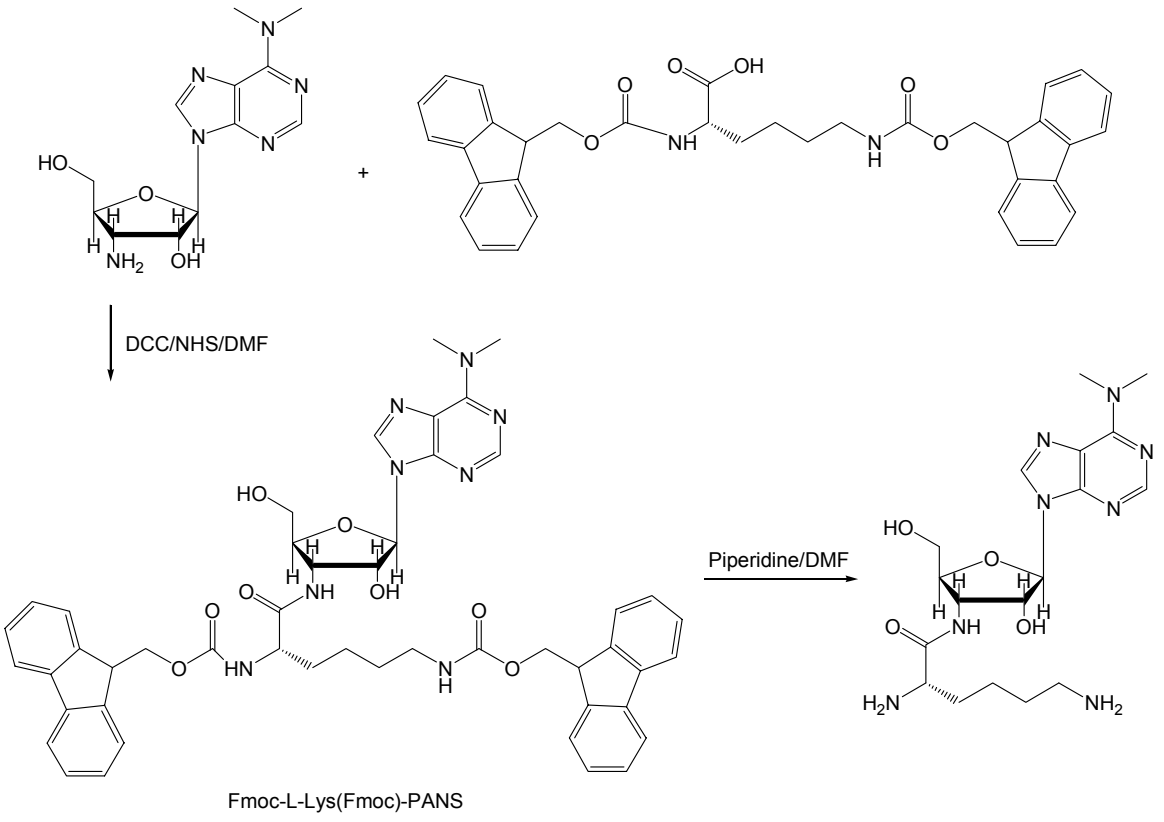
Carboxypeptidase Assay.

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.

Results

Here, we have used a series of synthetic puromycin analogs with a variety of α-amino acid moieties in both L- and D-configurations, and β-amino acids, to measure their activity in an intact eukaryotic translation system. We have synthesized a series of puromycin derivatives (Figure 3) that differ in the 1) amino acid moiety, 2) amino acid stereochemistry, and 3) number of carbon units in the amino acid backbone. The synthesis route of these compounds is outlined in Scheme 1.



Scheme 1. Synthesis of puromycin analogs (Lys- derivative is used here as an example)

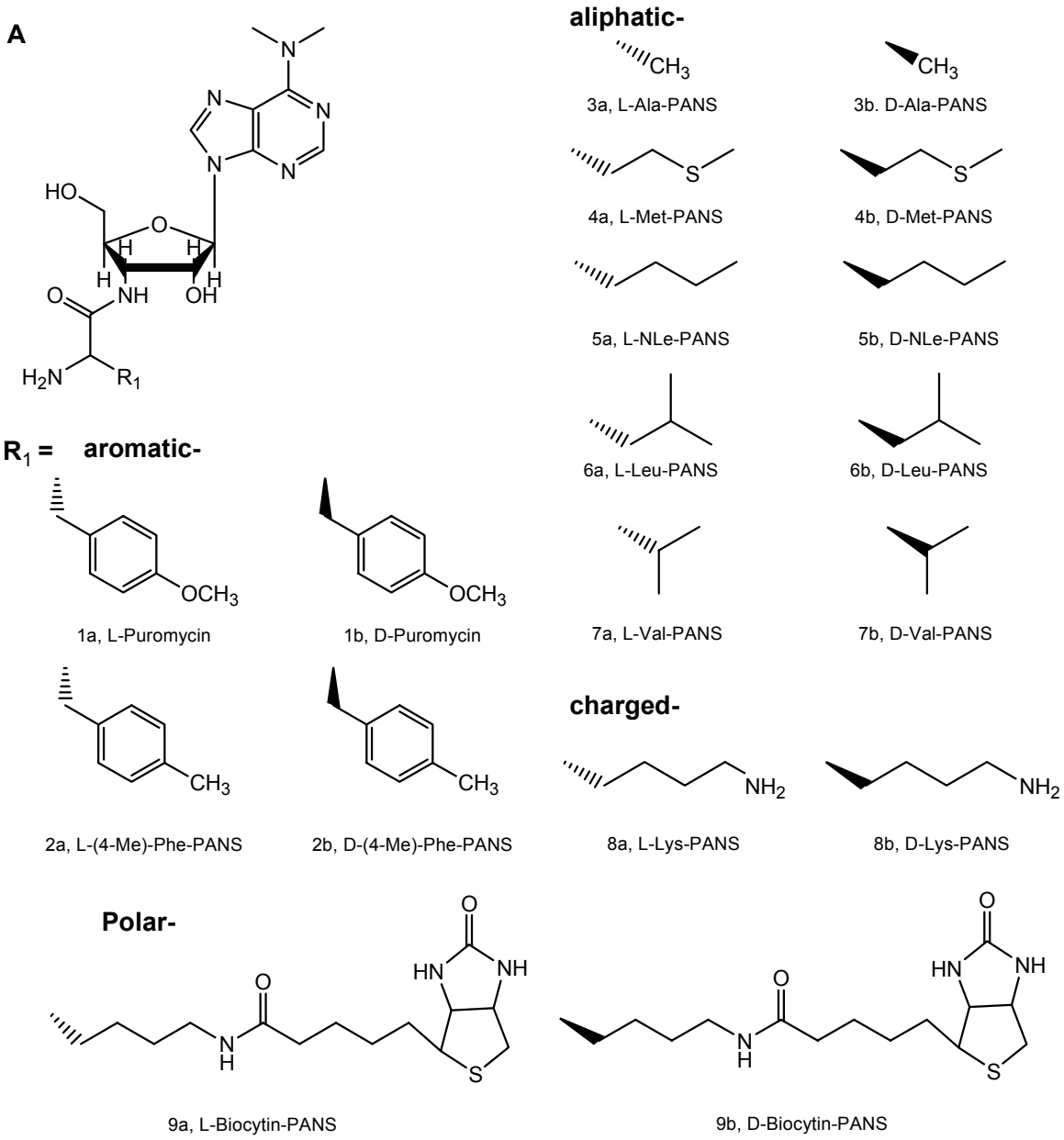


Figure 3A. Puromycin analogs with L- and D- α-amino acid side chains.

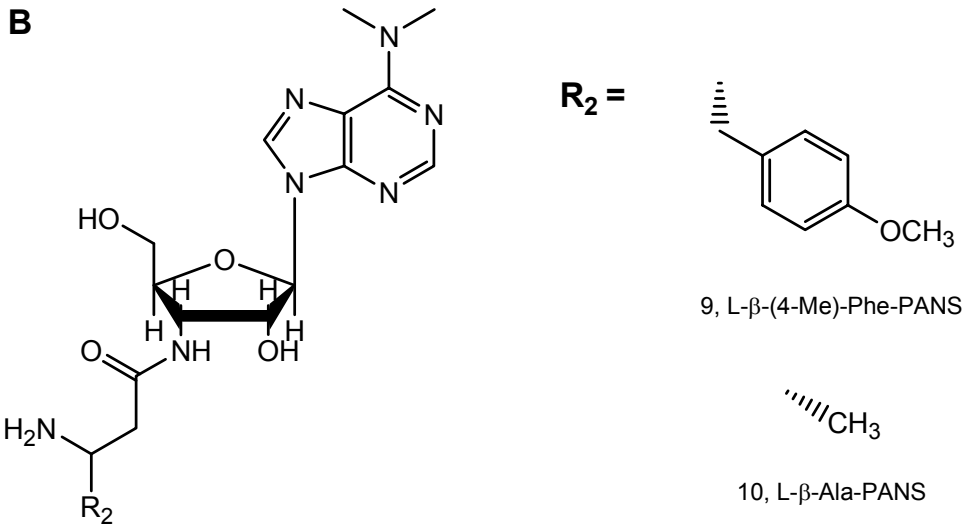


Figure 3B. Puromycin analogs with L-β- amino acid sidechains.

We then measured the activity of each compound in a high dynamic-range IC_{50} potency assay using the rabbit reticulocyte protein synthesis system.[7] The naturally occurring compound, L-puromycin (**1a**), inhibits globin mRNA translation with an IC_{50} of 1.8 μM (Table 1). Surprisingly, D-puromycin (**1b**) also inhibited translation giving an IC_{50} of 280 μM, a difference of 150-fold (Figure 4).

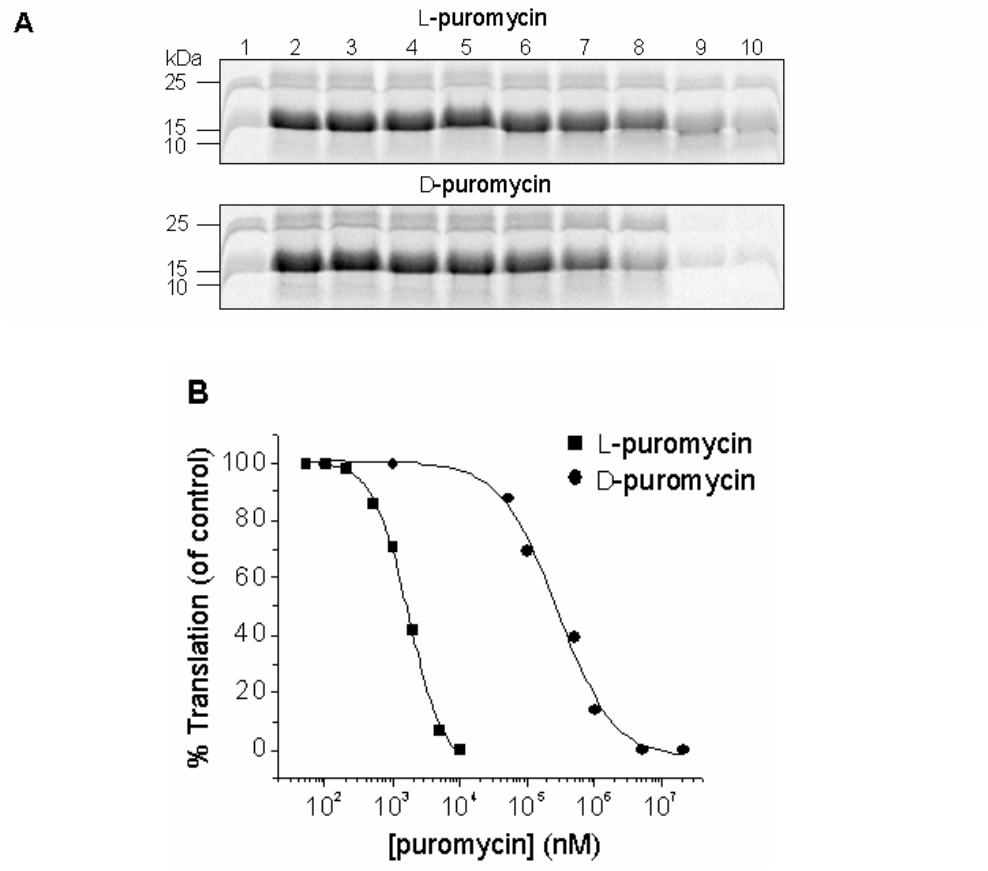


Figure 4. IC_{50} determination for puromycin. (A) Tricine-SDS-PAGE analysis of [^{35}S]Met-globin translation reactions in the presence of L-puromycin (**1a**) and D-puromycin (**1b**): Lane 1, no template; lane 2, globin alone; lanes 3-10, concentrations from 50 nM to 10 mM for **1a** and from 100 nM to 20 mM for **1b**. (B) Percent globin translation relative to the no drug control for L-puromycin (**1a**) and D-puromycin (**1b**).

We reasoned that stereoselectivity should be a function of the side chain size and geometry. To test this, we constructed compounds where the puromycin side chain was altered to bear either a bulky aromatic side chain (L- and D-4-methyl-phenylalanine; **2a** and **2b**), aliphatic side chains of different length (L- and D-alanine, **3a** and **3b**; L- and D-methionine, **4a** and **4b**; L- and D-norleucine, **5a** and **5b**; L- and D-leucine, **6a** and **6b**; L- and D-valine, **7a** and **7b**), a charged side chains (L- and D-lysine, **8a** and **8b**), or a polar side chains (L- and D-Biocytin, **9a** and **9b**).

Table 1. IC_{50} values for puromycin analogs.

	Puromycin Analogs	**IC50 (μM)**		**D/L**
		L- (a)	**D- (b)**	
1	Puromycin	1.8	280	156
2	(4-Me)-Phe-PANS	1.0	2400	2400
3	Ala-PANS	730	1900	2.6
4	Met-PANS	15	765	51
5	Nle-PANS	61	937	15
6	Leu-PANS	105	776	7.4
7	Val-PANS	3500	5300	1.5
8	Lys-PANS	289	347	1.2
9	Biocytin-PANS	2900	2900	1.0
10	β-(4-Me)-Phe-PANS	600	-	-
11	β-Ala-PANS	1700	-	-

Compound **2a** inhibits translation better than puromycin itself (IC_{50} = 1.0 μM) and is the most potent compound we constructed. The D-amino acid variant (**2b**) shows significantly reduced activity (IC_{50} = 2400 μM) with ~9-fold reduced activity compared to D-puromycin (**1b**), and is 2400-fold less potent than the L-isomer.

Compared to the natural occurring L-puromycin (1.8 μM), the other analogs we constructed, including Methionine (Met), Leucine (Leu), NorLeucine (Nle), Lysine (Lys), Biocytin, and Valine (Val), Alanine (Ala), are all less potent as inhibitor of protein synthesis (Table 1), but they show some interesting trend. For example, the IC_{50} values for L-Lys and D-Lys analogs are very similar, 289 μM and 347 μM, respectively. This shows that the ribosome perhaps does not discriminate L- and D-configurations for charged amino acid analogs. To be consistent with this observation, the puromycin analogs with biocytin side chain (biotinylated lysine) also have the same IC_{50} (2900 μM) between the L- and D-configuration, though much less potent.

The IC_{50} values for the puromycin analog with unnatural amino acid L-Nle and D-Nle are 61 μM and 937 μM, respectively. The Nle has the same aliphatic side chain as Lys but lacks the terminal amino group therefore lacks the positive charge. In contrast to Lys derivatives, L-Nle is 15-fold more potent than its D- counterpart. This allows us to conclude that the more hydrophobic the amino acid moiety, the more different is the potency of the D- and L- puromycin analog, consistent with our previous observation that larger hydrophobic aromatic side chain is a key element of ribosome discrimination.

In line with this observation, the Leu analogs have IC_{50} values of 105 µM and 776 µM for L- and D-configuration, a 7-fold difference (Figure 5). These are very similar to the Nle analogs because Leu and Nle have similar aliphatic side chains. For Met, L-configuration (15 µM) is about 51-fold different than the D-configuration (765 µM). On the contrary, Val analogs are much less potent, having IC_{50} values of 3500 µM and 5300 µM, with little discrimination, similar to the case of Ala. The alanine analogs show only a 3-fold selectivity for the L- versus D- isomers (**3a** vs. **3b**).

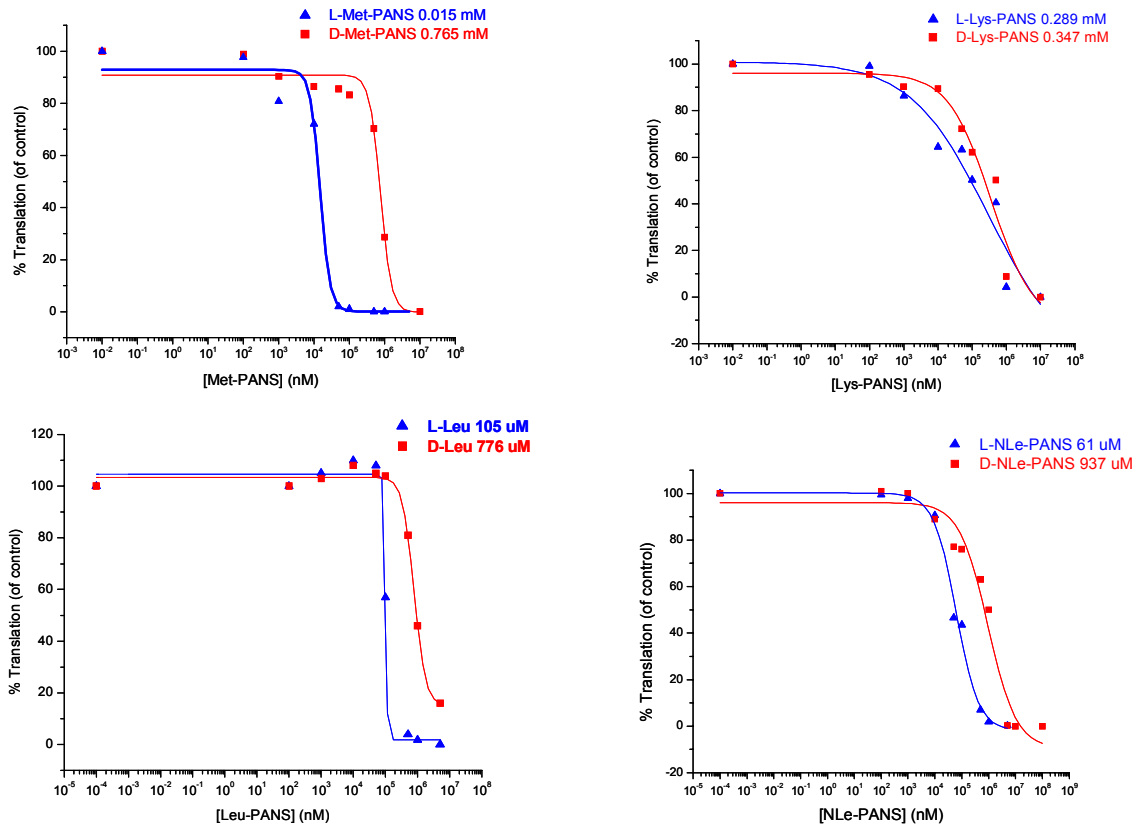


Figure 5. IC_{50} comparison for L- and D- puromycin.

We then next examined puromycin derivatives bearing β-amino acids. β-amino acids have been previously incorporated at low levels using nonsense suppression techniques.[10,11] In our experiments, both L-β-(4-Me)-Phe-PANS (**10**) and L-β-Ala-PANS (**11**) gave measurable inhibition constants (IC_{50} = 600 and 1700 μM, respectively) (Table 1).

Finally, we wished to confirm that our puromycin analogs participated in peptide bond formation within the ribosome. Incorporation of puromycin blocks the C-terminus, rendering the protein carboxypeptidase resistant.[1] Previous work in our laboratory indicated that puromycin incorporation is measureable when the analog concentration is near the IC_{50}.[7] Protein synthesis performed in the presence of our puromycin derivatives resulted in a 12- to 16-fold increase in carboxypeptidase Y (CPY)-resistant protein compared with the no puromycin, Ras only control (Figures 6A & B). The production of truncated protein fragments in the presence of each puromycin derivative, previously shown to result from puromycin attachment to globin,[7] along with the CPY-resistance results lead us to conclude that all of the compounds we synthesized both inhibit translation and participate in ribosome-mediated peptide bond formation.

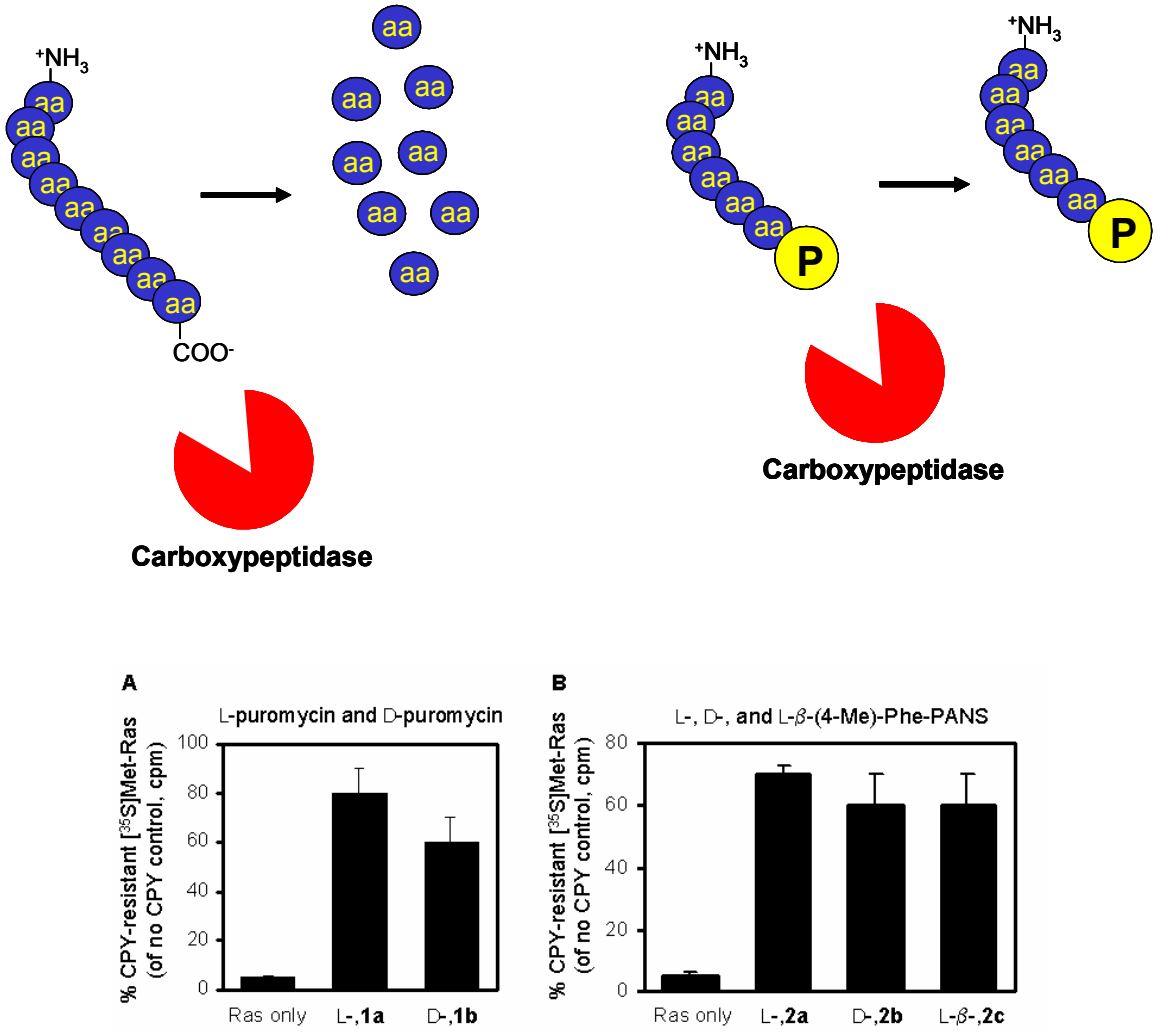


Figure 6. Carboxypeptidase Y (CPY) analysis of protein-puromycin products.[11] TCA precipitation of [^{35}S]Met-protein (Ras) from translation reactions after CPY treatment containing (A) Ras only, L-puromycin (**1a**) at 2 μM and D-puromycin (**1b**) at 500 μM and (B) Ras only, L-(4-Me)-Phe-PANS (**2a**) at 1 μM, D-(4-Me)-Phe-PANS (**2b**) at 1500 μM, and L-β-(4-Me)-Phe-PANS (**2c**) at 1000 μM. Data represent the mean ± standard error for at least three independent experiments.

Discussions

We vision that stereoselectivity should be a function of the side chain size and geometry. In order to systematically test the effects of size chain characteristics of amino acid moiety on the activity of puromycin, we have constructed a series of puromycin analogs with natural and unnatural amino acids side chains. These compounds include 4-methyl-Phenylalanine, Alanine(Ala), Methionine (Met), Leucine (Leu), NorLeucine (Nle), Lysine (Lys), Biocytin, and Valine (Val), in addition to naturally occurring puromycin, in both L- and D-configurations. They have either aromatic, aliphatic hydrophobic side chains of different length, or charged/polar side chains. This allows us to specifically probe different type of interactions between these analogs with the ribosome. We have used the same high dynamics IC_{50} assay to measure the activities of these puromycin analogs in inhibition of globin translation.

These observations argue that ribosomal stereoselectivity falls over a broad range and is primarily dictated by the size and geometry of the pendant side chain. Within the L-amino acid series (**1a**, **2a**, and **3a**), marked variation is also seen based solely on side chain identity. Larger, hydrophobic side chains provide improved function. In the L-amino acid series, 4-methylphenylalanine and naturally occurring puromycin are the best, followed by long aliphatic amino acids (Met, Nle, Leu). This is consistent with previous observations.[4,5] In the D-amino acid series, the 4-O-methyltyrosine derivative (**1b**) functions the best overall, and shows even better activity than the natural L-Ala and L-Val variants.

Overall, an aromatic side chain provides the highest stereospecificity by ribosome between L- and D-configuration, followed by long hydrophobic aliphatic side chains. Charged and polar side chains provide little discrimination.

We proposed that steric hindrance is the main reason that L-puromycin analogs are in general more potent than D-analogs (Figure 7), and our observation that aromatic and hydrophobic amino acid side chains exhibit higher discrimination than either charged or smaller side chains is consistent with this hypothesis.

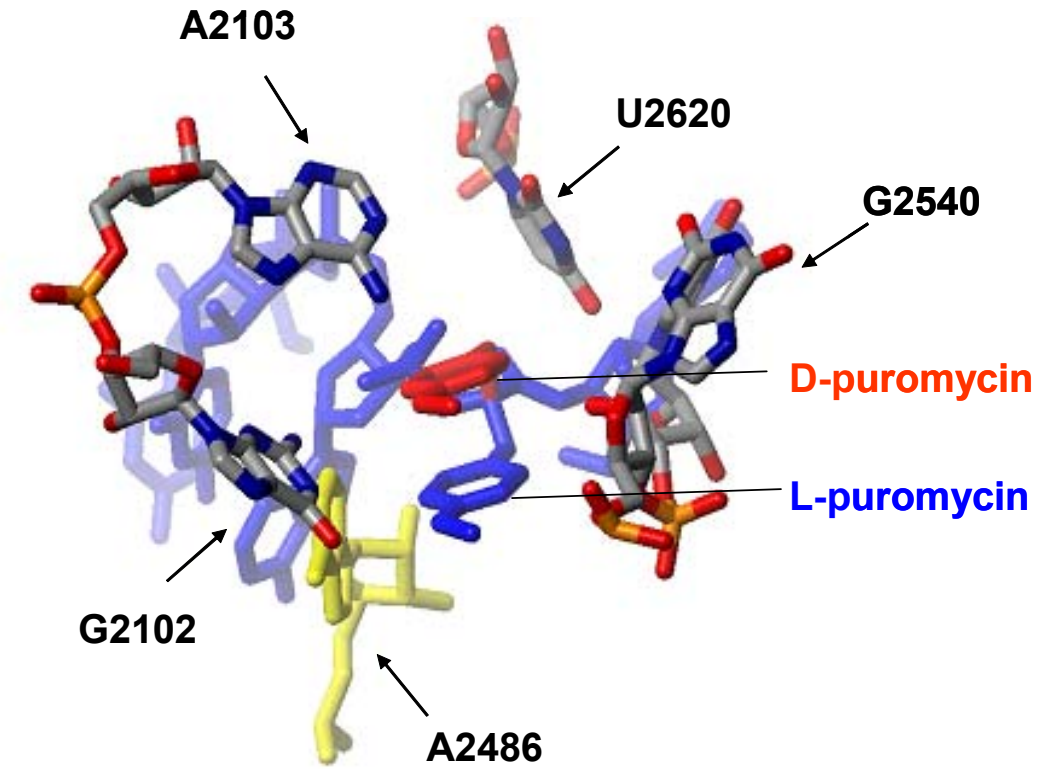


Figure 7. Model for D-puromycin (red) placement in the large 50S ribosome CCdA-p-L-puromycin (blue) complex. There is some potential hydrogen bond interactions of Met side chain with A2486.

The structural basis for stereoselectivity in rabbit ribosomes cannot be addressed presently, as there are no high-resolution structures available. However, modeling D-puromycin (**1b**) into the active site of the *Haloarcula marismortui* 50S subunit[28] is consistent with the idea that steric effects play a role in chiral discrimination. In the atomic resolution structure, U2620 (U2585 *E. coli*) is the closest nucleotide to the D-side chain. Also, while many of the ribosome active site nucleotides are highly conserved, the fact that critical residues can be mutated,[29] implies that construction of ribosomes with altered stereo- and regiospecificity may be possible. In the case of Met, there could potentially be hydrogen bonding interactions with the sulphur atom from the ribosome nucleotide for the L-derivative and this is consistent with the fact that there is about 51 fold difference between L- and D- Met-PANS.

Conclusions

Our work demonstrates that L-, D-, and β-amino acids can participate in ribosome-mediated peptide bond formation when constructed as analogs of puromycin. Our approach allows us to examine the activity of the ribosome directly in a physiologically complete protein-synthesizing system. Measurements using intact systems are critical as these can produce very different results from reconstituted or purified systems that are incapable of synthesizing proteins.[30,31] Our results provide one metric of the chiral and regiospecificity of mammalian ribosomes. We are hopeful that this data along with other information, such as the ability to optimize tRNA affinity for elongation factor Tu (EF-Tu)[32,33](EF1A in eukaryotes), will facilitate the incorporation of desirable but recalcitrant residues into peptides and proteins.

References

1. Nathans, D. Puromycin Inhibition of Protein Synthesis - Incorporation of Puromycin into Peptide Chains. *Proc Natl Acad Sci U S A* **51**, 585-& (1964).
2. Fahnesto.S, Neumann, H., Shashoua, V. & Rich, A. Ribosome-Catalyzed Ester Formation. *Biochemistry* **9**, 2477-& (1970).
3. Gooch, J. & Hawtrey, A. O. Synthesis of Thiol-Containing Analogues of Puromycin and a Study of Their Interaction with N-Acetylphenylalanyl-Transfer Ribonucleic-Acid on Ribosomes to Form Thioesters. *Biochemical Journal* **149**, 209-220 (1975).
4. Nathans, D. & Neidle, A. Structural Requirements for Puromycin Inhibition of Protein Synthesis. *Nature* **197**, 1076-& (1963).
5. Harris, R. J., Hanlon, J. E. & Symons, R. H. Peptide Bond Formation on Ribosome - Structural Requirements for Inhibition of Protein Synthesis and of Release of Peptides from Peptidyltransferrna on Bacterial and Mammalian Ribosomes by Aminoacyl and Nucleotidyl Analogues of Puromycin. *Biochimica Et Biophysica Acta* **240**, 244-& (1971).
6. Campuzano, S. & Modolell, J. Hydrolysis of Gtp on Elongation-Factor Tu.Ribosome Complexes Promoted by 2'(3')-O-L-Phenylalanyladenosine. *Proc Natl Acad Sci U S A* **77**, 905-909 (1980).

7. Starck, S. R. & Roberts, R. W. Puromycin oligonucleotides reveal steric restrictions for ribosome entry and multiple modes of translation inhibition. *RNA* **8**, 890-903 (2002).

8. Heckler, T. G., Zama, Y., Naka, T. & Hecht, S. M. Dipeptide Formation with Misacylated Transfer Rna-Phes. *Journal of Biological Chemistry* **258**, 4492-4495 (1983).

9. Bain, J. D., Glabe, C. G., Dix, T. A., Chamberlin, A. R. & Diala, E. S. Biosynthetic Site-Specific Incorporation of a Non-Natural Amino-Acid into a Polypeptide. *J Am Chem Soc* **111**, 8013-8014 (1989).

10. Bain, J. D., Wacker, D. A., Kuo, E. E. & Chamberlin, A. R. Site-Specific Incorporation of Nonnatural Residues into Peptides - Effect of Residue Structure on Suppression and Translation Efficiencies. *Tetrahedron* **47**, 2389-2400 (1991).

11. Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C. & Schultz, P. G. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182-8 (1989).

12. Nowak, M. W. et al. Nicotinic Receptor-Binding Site Probed with Unnatural Amino-Acid-Incorporation in Intact-Cells. *Science* **268**, 439-442 (1995).

13. Li, S., Millward, S. & Roberts, R. In vitro selection of mRNA display libraries containing an unnatural amino Acid. *J Am Chem Soc* **124**, 9972-3 (2002).

14. Takahashi, T. T., Austin, R. J. & Roberts, R. W. mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem Sci* **28**, 159-65 (2003).

15. Ellman, J. A., Mendel, D. & Schultz, P. G. Site-Specific Incorporation of Novel Backbone Structures into Proteins. *Science* **255**, 197-200 (1992).

16. Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C. J. & Schultz, P. G. Biosynthetic method for introducing unnatural amino acids site-specifically into proteins. *Methods Enzymol* **202**, 301-36 (1991).

17. van Hest, J. C. M. & Tirrell, D. A. Protein-based materials, toward a new level of structural control. *Chem Commun*, 1897-1904 (2001).

18. Cornish, V. W., Mendel, D. & Schultz, P. G. Probing Protein-Structure and Function with an Expanded Genetic-Code. *Ange Chem-Int Ed* **34**, 621-633 (1995).

19. Yamane, T., Miller, D. L. & Hopfield, J. J. Discrimination between D-Tyrosyl and L-Tyrosyl Transfer Ribonucleic-Acids in Peptide-Chain Elongation. *Biochemistry* **20**, 7059-7064 (1981).

20. Chladek, S. & Sprinzl, M. The 3'-End of Transfer-Rna and Its Role in Protein-Biosynthesis. *Ange Chem-Int Ed* **24**, 371-391 (1985).

21. Heckler, T. G., Roesser, J. R., Xu, C., Chang, P. I. & Hecht, S. M. Ribosomal-Binding and Dipeptide Formation by Misacylated Transfer Rnaphes. *Biochemistry* **27**, 7254-7262 (1988).

22. Roesser, J. R., Xu, C., Payne, R. C., Surratt, C. K. & Hecht, S. M. Preparation of Misacylated Aminoacyl-Transfer Rna Phes Useful as Probes of the Ribosomal Acceptor Site. *Biochemistry* **28**, 5185-5195 (1989).

23. Mendel, D., Ellman, J. & Schultz, P. G. Protein-Biosynthesis with Conformationally Restricted Amino-Acids. *J Am Chem Soc* **115**, 4359-4360 (1993).

24. Killian, J. A., Van Cleve, M. D., Shayo, Y. F. & Hecht, S. M. Ribosome-mediated incorporation of hydrazinophenylalanine into modified peptide and protein analogues. *J Am Chem Soc* **120**, 3032-3042 (1998).

25. Koh, J. T., Cornish, V. W. & Schultz, P. G. An experimental approach to evaluating the role of backbone interactions in proteins using unnatural amino acid mutagenesis. *Biochemistry* **36**, 11314-11322 (1997).

26. Eisenhauer, B. M. & Hecht, S. M. Site-specific incorporation of (aminooxy)acetic acid into proteins. *Biochemistry* **41**, 11472-11478 (2002).

27. England, P. M., Zhang, Y. N., Dougherty, D. A. & Lester, H. A. Backbone mutations in transmembrane domains of a ligand-gated ion channel: Implications for the mechanism of gating. *Cell* **96**, 89-98 (1999).

28. Nissen, P., Hansen, J., Ban, N., Moore, P. B. & Steitz, T. A. The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920-930 (2000).

29. Polacek, N., Gaynor, M., Yassin, A. & Mankin, A. S. Ribosomal peptidyl transferase can withstand mutations at the putative catalytic nucleotide. *Nature* **411**, 498-501 (2001).

30. Bhuta, P., Kumar, G. & Chladek, S. Aminoacyl Derivatives of Nucleosides, Nucleotides and Polynucleotides .36. The Peptidyltransferase Center of Escherichia-Coli Ribosomes - Binding-Sites for the Cytidine 3'-Phosphate Residues of the Aminoacyl-Trna 3'-Terminus and the Interrelationships between the Acceptor and Donor Sites. *Biochimica Et Biophysica Acta* **696**, 208-211 (1982).

31. Krayevsky, A. A. & Kukhanova, M. K. The peptidyltransferase center of ribosomes. *Prog Nucleic Acid Res Mol Biol* **23**, 1-51 (1979).

32. LaRiviere, F. J., Wolfson, A. D. & Uhlenbeck, O. C. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science* **294**, 165-168 (2001).

33. Asahara, H. & Uhlenbeck, O. C. The tRNA specificity of Thermus thermophilus EF-Tu. *Proc Natl Acad Sci U S A* **99**, 3499-504 (2002).