Chapter 5

The Puromycin Route to Assess Amine Substitution Constraints on Peptide Bond Formation

Abstract

The use of puromycin analogs to probe the stereo- and regiospecificity of the ribosome shows that our protein synthesis machinery tolerates a wide range of unnatural residues (e.g., D-residues and β-amino acids). This approach offers a direct means to study the allowances of translation without the drawbacks of using chemically misacylated-tRNAs. Here, the limits of translation are studied with respect to substitution of the primary amine (e.g., secondary amine or N-methyl amine). Surprisingly, the amine is extremely sensitive to substitution since the activity of N-substituted analogs are dramatically lower than analogues with primary amines (7- to 500-fold loss in activity relative to the unsubstituted counterpart). The loss in activity upon primary amine substitution is thought to result in part from a change in the rate-determining step of peptide bond formation or from altered basicity of the substituted amine.

5.1 Background

N-substituted amino acids are a class of residues that show potential utility in the area of peptide- or protein-based therapeutics. Several properties render these unnatural amino acids useful for such therapies because they are 1) less polar than normal α-amino acids, which increases their bioavailability and 2) resistant to common human proteases. Nature appears to already utilize substituted amino acids such as proline and hydroxyproline in type I collagen to insure conformational rigidity in the collagen triple helix (1, 2). Further, substitution of an *N*-substituted amino acid (*N*-methyl-glycine) for proline in Src homology 3 (SH3) ligands maintains the high-specificity recognition and affinity for the SH3 scaffold (3). Recently, the utility of *N*-substituted amino acids in therapeutics led to the discovery that *N*-methyl-*N*-pyrimidin-2-yl glycine derivatives have antiphlogistic activity (4). In addition, *N*-substituted peptide antagonists of the Bradykinin receptor, a receptor directly involved with several inflammatory disease states, prove to be therapeutic leads (5). For these reasons, incorporation of *N*-substituted amino acids into peptides and proteins has been an area of intense research.

There are a diverse set of *N*-substituted amino acids that have been incorporated into peptides and proteins through aminoacyl-tRNA delivery (Figure 5.1). These experiments show that incorporation of the *N*-substituted residues is measurable, with a ~5 to ~70 suppression efficiency (Figure 5.1) (6, 7). This result is encouraging but is convoluted by the necessity for an elongation factor (EF-Tu in prokaryotes and EF1A in eukaryotes) to escort the *N*-substituted amino acid-tRNA (aa-tRNA) complex into the ribosome. Improper interactions between the aa-tRNA complex and elongation factor are expected to decrease the efficiency amino acid incorporation. Amino acids that bind to EF-Tu too tightly or too weakly may offset the optimum affinity balance (8, 9). Although, the optimal affinity may be generated by using a tRNA body that compensates for the amino acid affinity. This is a solution inferred from data showing that affinity for EF-Tu is a function of the amino acid identity

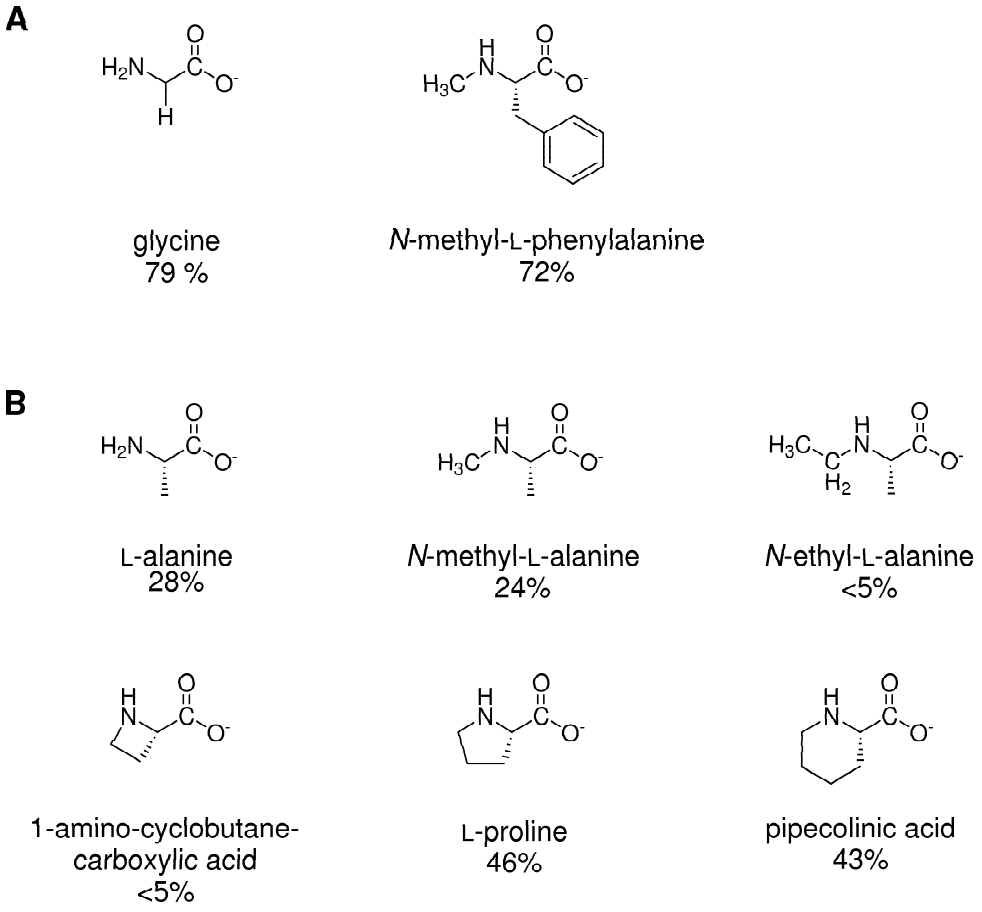

Figure 5.1: *N*-substituted amino acids inserted into (A) peptides (6) and (B) protein (7). Percent values indicate the extent of nonsense codon suppression using chemically misacylated-tRNAs

and the tRNA body sequence (8, 9).

Identification of *N*-substituted residues that are recognized by the ribosome and participate in peptidyl transferase is important for preparation of peptide-based libraries, such as those used in mRNA display (10, 11). Unnatural amino acid suppression techniques offer an excellent means to decipher those variants incorporated into protein. However, this strategy is labor intensive, burdened by low yields of unnatural aa-tRNA product, and costly. Further, extrapolation of ribosome toleration for *N*-substituted amino acids can not be directly assessed using this technique. In contrast, a direct means to examine the peptidyl transferase activity of *N*-substituted amino acids is to use puromycin analogs bearing *N*-methyl or other primary amine substitutions (12). Once the *N*-substituted amino acid is shown to participate in peptide-bond formation, use of an appropriate tRNA body to thermodynamically compensate for the unnatural amino acid, may yield an aa-tRNA complex readily recognized by EF-Tu or EF1A (8, 9). In this chapter, the peptidyl transferase activity of various *N*-substituted puromycin analogs is investigated.

5.2 Results and Discussion

Proline is the only naturally occurring substituted amino acid, bearing a secondary amine. Proline should be called an imino acid since the side chain is continuous within the backbone yielding a five-member ring. Proline is present 5.1% in proteins (13) and collagen is comprised from 25% of proline and hydroxyproline (14), its incorporation is thought to be readily accommodation by the ribosome. Therefore, it was of interest whether a puromycin analog of proline, called L-Pro-PANS (**1**) (Figure 5.2), would be recognized by the ribosome. The literature already had demonstrated that small, non-aromatic amino acids such as proline or alanine function poorly in translation (15). But this is thought to result from poor interactions within the A-site, which could be favorable between aromatic rings such as phenylalanine and tyrosine with the RNA

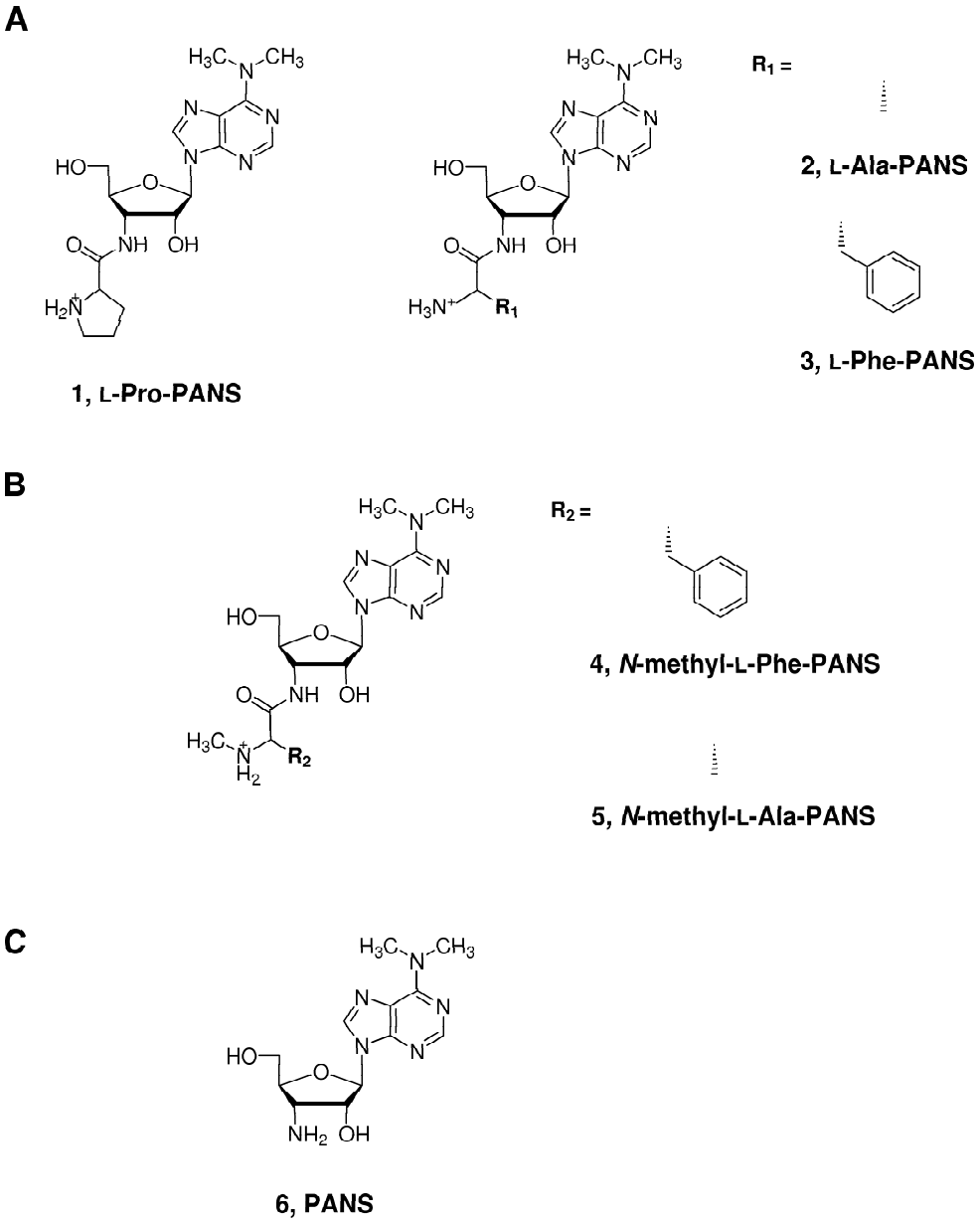

Figure 5.2: Puromycin analogs. (A) Analogs with naturally occurring amino acids, (B) *N*-methyl-substituted amino acid analogs, and (C) puromycin aminonucleoside (PANS)

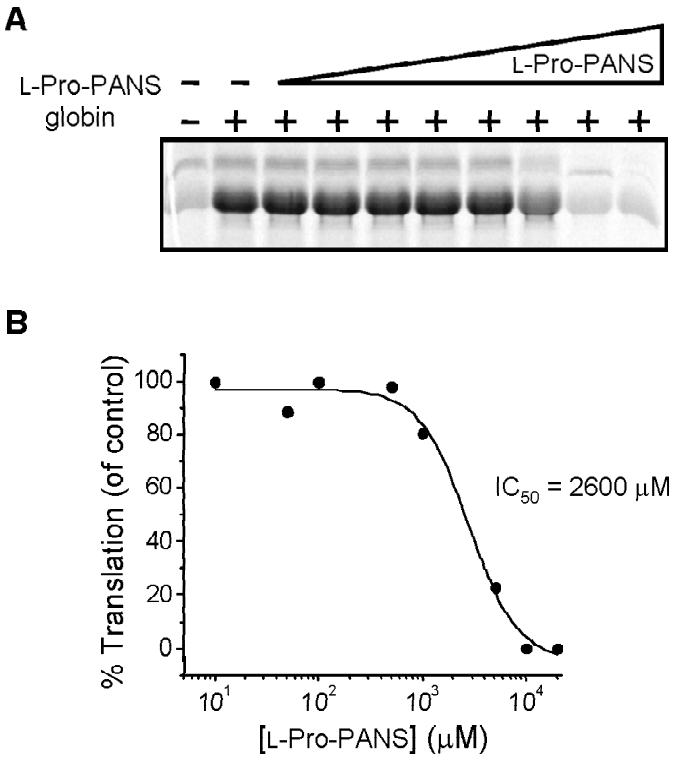

Figure 5.3: IC_{50} determination for L-Pro-PANS (**1**). Globin mRNA was translated with increasing amounts of **1**, resolved using tricine-SDS-PAGE, and quantitated by detection of [^{35}S]Met-globin. (A) Translation of globin with **1**: lane 1, no template; lane 2, globin alone; lanes 3 – 17, concentrations from 0.01 to 20 mM. (B) Percent of globin translation relative to a no-drug control.

bases. But how does L-Pro-PANS compare with previously tested small puromycin analogs (e.g., L-Ala-PANS and L-β-Ala-PANS)? Surprisingly, L-Pro-PANS inhibited translation of globin with an IC_{50} of 2.6 mM (Figure 5.3 and Table 5.1). This is nearly 4-fold less active than L-Ala-PANS (**2**) and 2-fold less potent than the unnatural amino acid analog L-β-Ala-PANS (IC_{50} = 0.73 and 1.7 mM, respectively) (Table 5.1 and reference (12)). This significant difference in activity between L-Pro-PANS (**1**) and L-Ala-PANS (**2**) is not expected to result from side chain size since L-Pro-PANS is actually larger than L-Ala-PANS (MW = 391 and 365 g/mol, respectively). Since

Puromycin analog	IC_{50} (μM)
1, L-Pro-PANS	2600
2, L-Phe-PANS	5.9
3, L-Ala-PANS	730*
4, *N*-methyl-L-Phe-PANS	2900
5, *N*-methyl-L-Ala-PANS	4800
6, PANS	3800

Table 5.1: IC_{50} values for *N*-substituted puromycin analogs (*Data from (12))

they are both non-polar, hydrophobic amino acid analogs, other conclusions based on hydrophobicity or charge-charge interactions do not apply. However, this dramatic lack of activity puts into question whether other *N*-substituted amino acid analogs would also show a significant loss in activity compared to the primary amino acid counterpart.

To address this question, a series of analogs with and without *N*-methylation were prepared and tested for inhibition of translation (Figure 5.2B). Once again, the presence of a *N*-methyl group appears to abolish the potency of the analog, even a highly active analog, such as L-phenylalanine-PANS (L-Phe-PANS, **3**). For example, L-Phe-PANS inhibits translation with an IC_{50} of 5.9 μM, while *N*-methyl-L-Phe-PANS (**4**) shows ~500-fold loss in activity (Table 5.1). This trend is seen further with *N*-methyl-L-Ala-PANS (**5**) which is ~7-fold less potent than L-Ala-PANS (**2**) (Table 5.1). The IC_{50} for puromycin aminonucleoside (PANS, **6**) (Figure 5.2C) was determined to be 3.8 mM (Table 5.1). Not only did addition of a methyl group on L-Ala-PANS diminish its potency, but to the extent that the analog was less active than PANS itself, which does not even bear a reactive amine. Although, it is unknown exactly why PANS inhibits translation, biochemical and structural data show that the equivalent base in aa-tRNA does make interactions with the ribosomal RNA within

the A-site (16, 17).

These results directly implicate the presence of a methyl group as responsible for activity loss. Apparently, substitution on the reactive nucleophile of puromycin analogs severely inhibits activity in the peptidyl transferase reaction. However, why does the addition of a methyl group or a natural amino acid analog such as L-Pro-PANS lack the expected level of potency?

Peptide bond formation is simply attack of an amine on an activated carbon through a tetrahedral intermediate (Figure 5.4). Depending on the natures of the ester and the amine, the breakdown of the intermediate or the attack of the amine on the carbonyl carbon of the ester can be the rate-limiting step in solution (19). Further insights may be inferred from experiments where the aminolysis of methyl formate is measured using various *N*-substituted amines (20). In this study, the rate of amine attack and the rate of the tetrahedral intermediate breakdown are determined using primary, secondary, and tertiary amines (20). The rate of amine attack (K_2) appeared to correlate positively with the basicity or pKa of the molecule (Figure 5.5A). More basic amines tended to attack methyl formate faster than less basic amines, which is expected. However, the rate of tetrahedral breakdown (K_4) showed that there is negative correlation between pKa and rate (Figure 5.5A). The increased basicity of the amine actually stabilizes the tetrahedral intermediate considerably.

The loss in activity with *N*-substitution, either with addition of a methyl group or with L-Pro-PANS, could reflect the stability of the tetrahedral intermediate with the P-site held aa-tRNA. While the IC_{50} determination assays are under multiple turnover conditions where the rate of the tetrahedral intermediate breakdown can not be derived, the effect of a more stable intermediate may decrease the inhibitory potential of the analog (Figure 5.5B). The stability of the intermediate may allow incoming aa-tRNAs to compete more effectively and exclude the *N*-substituted analog from the peptidyl transferase reaction.

Ab initio calculations of amino acids with and without *N*-substitution reveal that

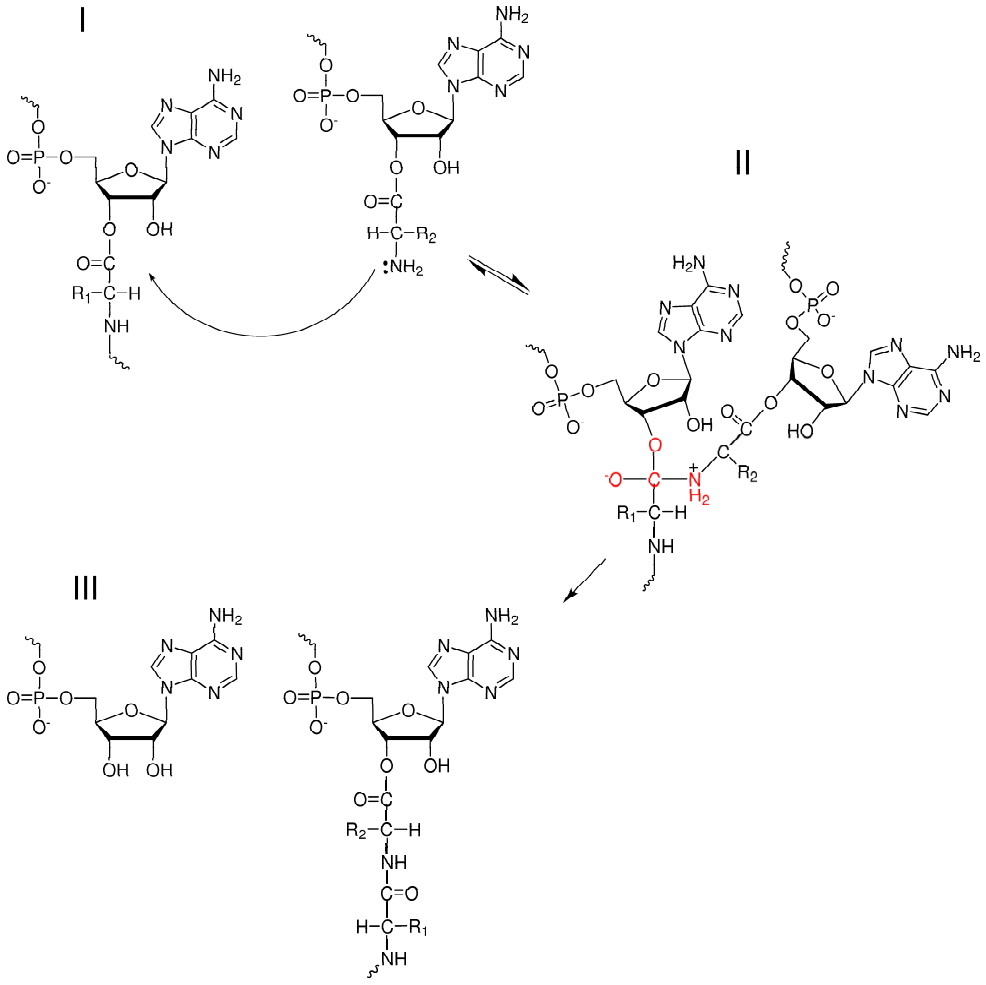

Figure 5.4: Mechanism of peptide bond formation in the ribosome. I, a free amine nucleophilically attacks an activated carbonyl carbon of a P-site held aa-tRNA; II, a zwitterionic tetrahedral intermediate (atoms in red) is formed; and III, the intermediate loses a proton to yield a new peptide bond and an empty P-site tRNA. Figure adapted from (18).

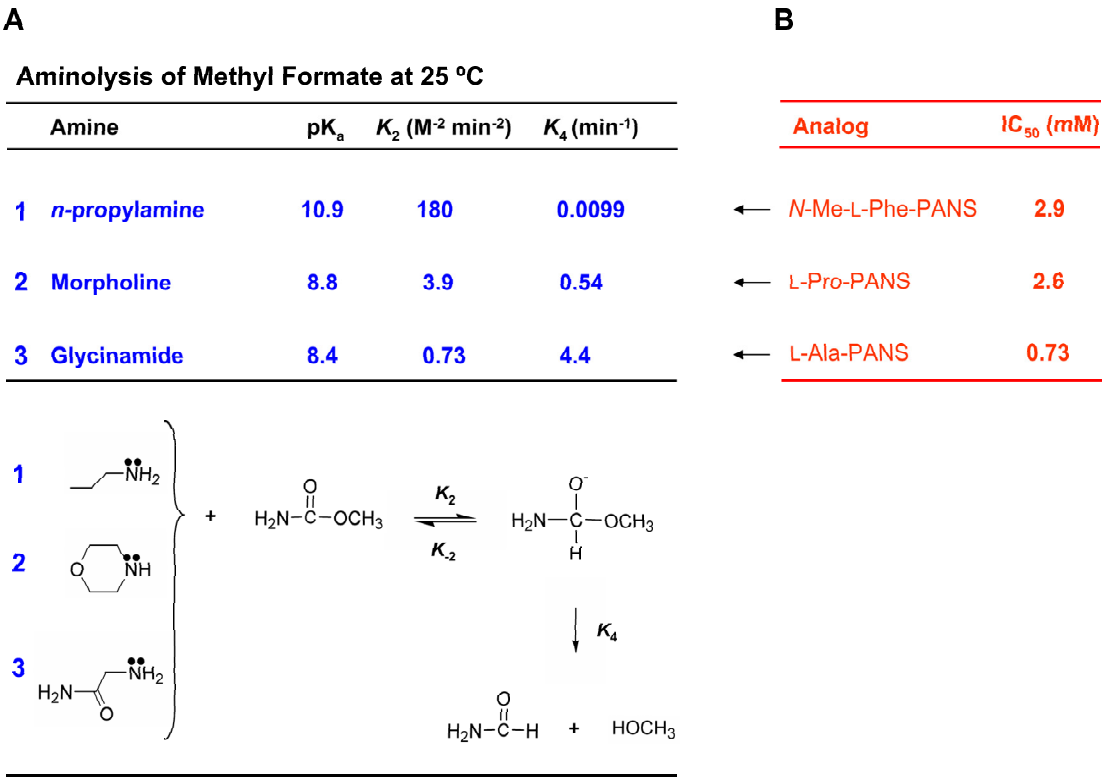

Figure 5.5: Comparison of amine attack rate on methyl formate [data from 19] to IC_{50} values for the *N*-substituted amino acid analogs *N*-Me-L-Phe-PANS (**4**), L-proline-PANS (**1**), and L-Ala-PANS (**2**) (Figure 5.2).

Amino acid	δ_N
L-Phe	-1.023
N-methyl-L-Phe	-0.761
N-ethyl-L-Phe	-0.941
N-isopropyl-L-Phe	-0.950
N-phenyl-L-Phe	-0.819

Table 5.2: Partial negative charge of *N*-substituted phenylalanine amino acids (calculations made using SPARTAN ESSENTIAL Copyright 1991-2001 by Wavefunction Inc. Hartree-Frock minimizations with a 6-31G* basis minimization set employed.)

the partial negative charge of the amine decreases with increased substitution (Table 5.2). This is a counterintuitive result based on the gas-phase basicity of amines where $NH_3 < CH_3CH_2NH_2 < (CH_3CH_2)_2NH < (CH_3CH_2)_3N$. However, the basicity of amines in solution follows a different pattern where $NH_3 < RNH_2 \sim R_3N < R_2NH$. This discontinuity results from the balance between electron release from alkyl groups (and dispersion of positive charge) and solvation effects from hydrogen bonding (Figure 5.6).

The SPARTAN calculation identifies the partial coulombic charge on the nitrogen. This calculation takes into account the electronic environment surrounding the nitrogen atom and depicts the change in bond polarity with different *N*-substituents. The decreased activity of *N*-substituted analogs may indicate that the optimum balance between positive charge dispersion and solvation through hydrogen bonding is seen with L-Phe-PANS (similar to A in Figure 5.6) instead of *N*-methyl-L-Phe-PANS (similar to B in Figure 5.6). While this calculation was carried out only with the amino acid group, not the entire amino acid-PANS derivative, there may be some validity with this analysis. As such, the presence of a larger *N*-substituent such as an ethyl or isopropyl group may preserve the nucleophilicity of the amine (greater

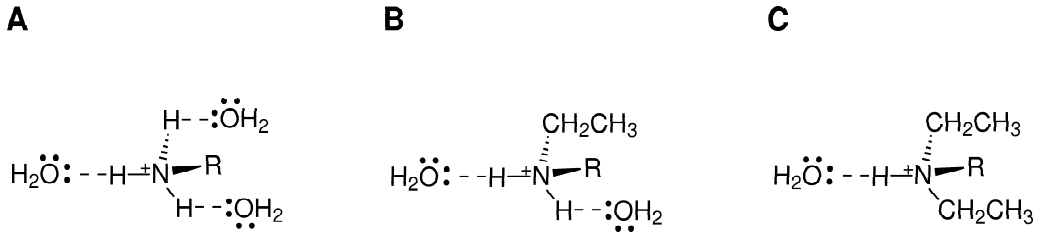

Figure 5.6: Schematic of hydrogen bonding potential for a A) primary amine with three protons on nitrogen available for H-bonding, B) secondary amine with two protons on nitrogen available for H-bonding, and C) tertiary amine with one proton on nitrogen available for H-bonding.

positive charge dispersion than by a methyl group) as evidenced by the higher δ_N values (Table 5.2). More *N*-substituted puromycin derivatives will need to prepared and tested for activity to assess the validity of these theories.

5.3 Conclusions

The peptidyl transferase reaction appears to be sensitive to substitution on the reactive amine in puromycin analogs. Even small substituents, such as a methyl group, diminish the potency of the amino acid analog. This is supported by data using puromycin analogs with *N*-substitution which shows that even a naturally occurring amino acid such as proline becomes a poor substrate when presented to the ribosome in the context of L-Pro-PANS (**1**), which is an effect that may also be seen with L-proline-tRNA at proline codons. Perhaps the kinetics of peptide bond formation vary with different amino acids even in tRNA-mediated peptidyl transferase. These studies may allow the unnatural amino acid incorporation data to be evaluated further since an amino acid bearing a substitution may still be active in peptide bond formation if the substituent does not increase the basicity (and the stability of the tetrahedral intermediate) of the amine dramatically. For example, a -CF_3 group on the α-nitrogen

would still inhibit protease activity, but not enhance the stability of the tetrahedral intermediate. However, the electronegativity of fluorine may reduce the reactivity of nitrogen or compete with nitrogen as a peptide bond acceptor. Nevertheless, these data provide a framework for the design of an *N*-substituted-unnatural amino acid that maintains high therapeutic value as well as reactivity in the peptidyl transferase reaction.

5.4 Experimental Procedures

5.4.1 General Information

Low-resolution mass spectra were recorded on a PE SCIEX API 365 triple quadruple electrospray mass spectrometer at the Beckman Institute Mass Spectrometry Laboratory, California Institute of Technology. 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-(L-phenylalanine) and FMOC-(L-alanine) were obtained from Novabiochem and Fluka, respectively. FMOC-(L-proline), FMOC-*N*-methyl-L-(phenylalanine), FMOC-*N*-methyl-L-(alanine) were purchased from Novabiochem. Puromycin analog concentrations were determined with the following extinction coefficients ($M^{-1}cm^{-1}$) at 260 nm: Non-aromatic puromycin analogs such as L-Pro-PANS (**1**), L-Ala-PANS (**2**), *N*-methyl-

L-Ala-PANS (**5**) and PANS (**6**) [$\epsilon = 11,000$] in phosphate buffered saline (pH 7.3); aromatic puromycin analogs such as L-Phe-PANS (**3**) and *N*-methyl-L-Phe-PANS (**4**) [$\epsilon = 10,500$] in H_2O. Rabbit reticulocyte lysate was purchased from Novagen. Rabbit globin mRNA was obtained from Life Technologies Gibco BRL.

5.4.2 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*. The material was purified by gradient flash chromatography using MeOH/$CHCl_3$ (7:93). 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 $CHCl_3 \rightarrow$ TEA/MeOH/$CHCl_3$ (7:10:83) for **1**; $CHCl_3 \rightarrow$ TEA/MeOH/$CHCl_3$ (2:10:88) for **2**

5.4.3 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 20μl 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, pexcept 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 (all components except template), and globin mRNA were mixed simultaneously and incubated at 30 °C 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 °C for 5 min, and applied entirely to a 4% stacking portion of a 16% tricine SDS-polyacrylamide gel containing 20% (v/v) glycerol (21)(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).

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