Uncovering the Enzymatic pK_a of the Ribosomal Peptidyl Transferase Reaction Utilizing a Fluorinated Puromycin Derivative†

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ABSTRACT: The ribosome-catalyzed peptidyl transferase reaction displays a complex pH profile resulting from two functional groups whose deprotonation is important for the reaction, one within the A-site substrate and a second unidentified group thought to reside in the rRNA peptidyl transferase center. Here we report the synthesis and activity of the *β*,*β*-difluorophenylalanyl derivative of puromycin, an A-site substrate. The fluorine atoms reduce the pK_a of the nucleophilic α-amino group (<5.0) such that it is deprotonated at all pHs amenable to ribosomal analysis (pH 5.2−9.5). In the 50S modified fragment assay, this substrate reacts substantially faster than puromycin at neutral or acidic pH. The reaction follows a simplified pH profile that is dependent only upon deprotonation of a titratable group within the ribosomal active site. This feature will simplify characterization of the peptidyl transferase reaction mechanism. On the basis of the reaction efficiency of the doubly fluorinated substrate compared to the unfluorinated derivative, the Brönsted coefficient for the nucleophile is estimated to be substantially smaller than that reported for uncatalyzed aminolysis reactions, which has important mechanistic implications for the peptidyl transferase reaction.

The ribosome is the macromolecular machine that catalyzes protein synthesis in all cells. The ribosome has two substrate binding sites and utilizes two tRNAs as the reaction substrates: an aminoacyl-tRNA bound to the A-site and a peptidyl-tRNA bound to the P-site (*1*). The peptidyl transferase (PT)[1] reaction involves aminolysis of the ester bond linking the peptide to the P-site tRNA by the α-amino group of the A-site aminoacyl-tRNA (Figure 1). The tRNA substrates are aligned in the active site by base pairing between the CCA sequence at the end of each tRNA and complementary sequences within the A- and P-sites of the rRNA (*2*, *3*). The ribosome can also catalyze amide bond formation using the minimal A-site substrate puromycin (Pmn), a nucleoside antibiotic comprised of 3′-deoxy-3′-amino-N^6,N^6-dimethyladenosine coupled to the amino acid *O*-methyltyrosine (*4*). Pmn functions as an A-site substrate because it is analogous to the amino acid and the terminal adenosine of an aminoacylated tRNA.

The catalytic mechanism of the PT reaction is an area of ongoing research interest both for its medicinal and for its evolutionary relevance. Medicinally, the PTC with its surrounding substrate and product channels is the binding site for several naturally and synthetically derived antibiotics (*5*, *6*). Evolutionarily, RNA catalysis of peptide bond formation is the reaction required to bridge between a prebiotic world dominated by RNA catalysts and modern biology dominated by protein catalysts (*7*). Catalytic strategies postulated for the PT reaction have included general acid−base catalysis (*8*, *9*), substrate-assisted catalysis (*10*−*12*), and catalysis derived solely from substrate alignment (*13*). To differentiate between these possibilities, additional tools that facilitate mechanistic analysis are needed.

A potentially important clue regarding the PT catalytic mechanism is the significant pH dependence of the reaction (*14*−*16*). Two ionizable functional groups contribute to the mechanism, and both have acidity constants (pK_as) near neutrality (*16*). The close proximity of these two values makes them difficult to differentiate. The pK_a of the A-site tRNA α-amino group, which serves as the nucleophile, is approximately 7.0 (*17*), so its deprotonation from the ammonium to the amino form was predicted to be responsible for one of the titratable groups in the PT reaction (*16*). This model was tested using the antibiotic derivative α-hydroxypuromycin (Pmn-OH), in which the α-amino was replaced with an α-hydroxyl group, which has a substantially higher pK_a (>11) than the amine (*16*, *17*). The resulting transacylation reaction remained pH dependent, though the magnitude of the dependence changed. The plot of the pH vs observed rate (k_{obs}) was consistent with a single pK_a of 7.5. Because the titratable group with a near neutral pK_a on the substrate had been removed, this suggested that one pK_a is that of the

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[1] Abbreviations: PTC, peptidyl transferase center; PT, peptidyl transferase; Pmn, puromycin; pK_a, acidity constant; Pmn-OH, α-hydroxypuromycin; CPmn, 5′-cytidylyl-3′−5′-phosphorylpuromycin; dFPhe, *β*,*β*-difluorophenylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; Alloc, allyloxycarbonyl; DMTr, *p*,*p*′-dimethoxytrityl; tBDMS, *tert*-butyldimethylsilyl; CCApcb, CC-3′-(biotinyl-*ϵ*-aminocaproyl-L-phenylalanyl)A; CPmn-pcb, biotinyl-*ϵ*-aminocaproyl-L-phenylalanyl-CPmn.

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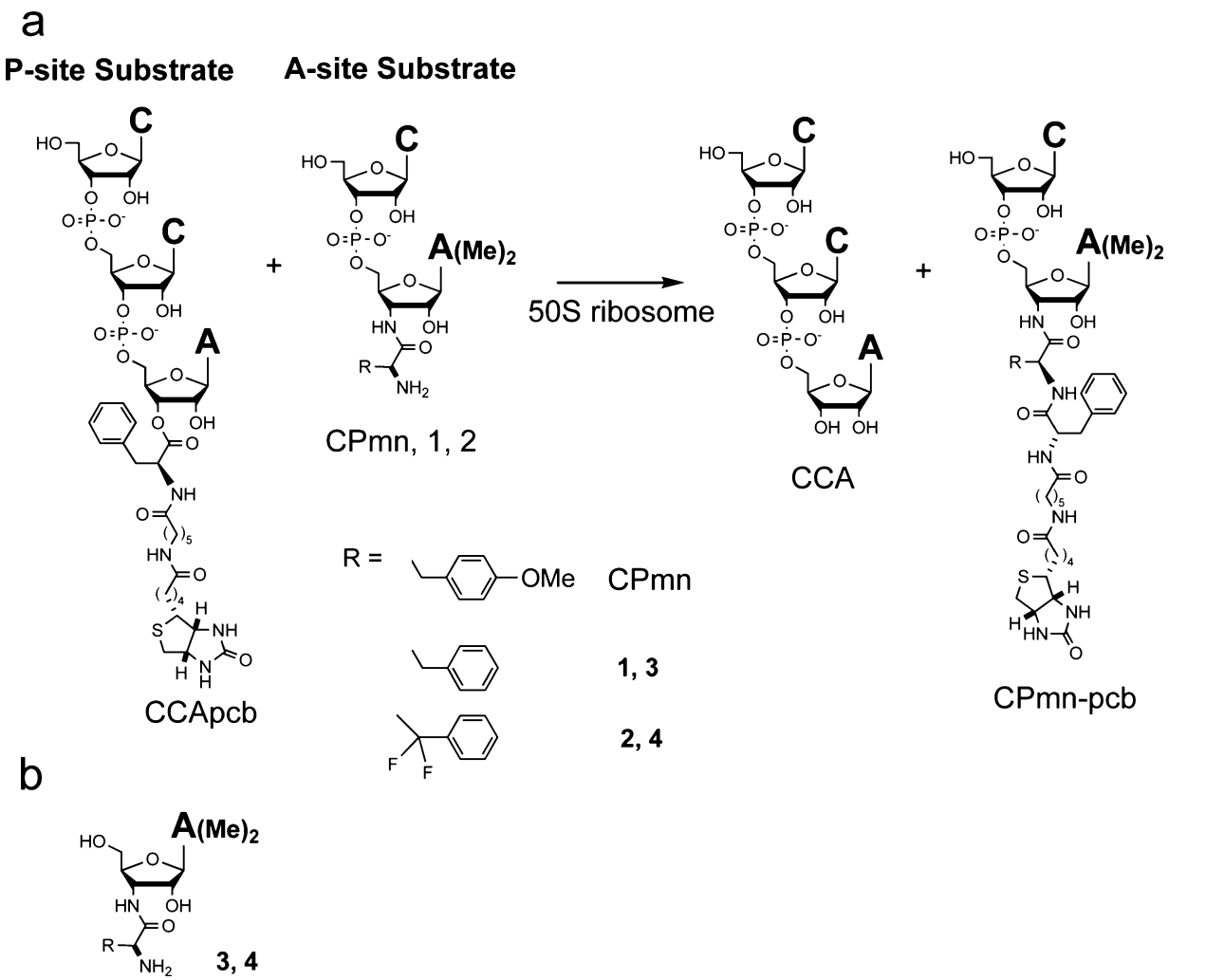

FIGURE 1: (a) Modified fragment reaction. A peptidyl-tRNA in the P-site reacts with an aminoacyl-tRNA in the A-site to create a peptide bond via aminolysis of the P-site ester. The substrates are analogues of the full-sized tRNAs and are utilized in this modified fragment assay. The A-site substrates are CPmn derivatives that include the amino acids *O*-methyltyrosine (Pmn version), Phe (**1**), or dFPhe (**2**). (b) Puromycin derivatives used for pK_a measurements.

substrate and that the remaining titratable group resides within the PTC (*16*, *18*).

Although Pmn-OH has played a valuable role for investigating the mechanism of peptide bond formation (*16*, *18*, *19*), there are several reasons why it is not an ideal substrate for such studies. Reaction with Pmn-OH changes not only the pK_a of the substrate but also the identity of the nucleophile, which may alter the mechanism of the reaction. Reaction with Pmn-OH results in ester rather than amide bond formation, and the reaction is 500-fold slower than the equivalent reaction with Pmn, consistent with the possibility that the active site may be less well suited to this substrate (*16*). Additional concerns are not specific to Pmn-OH but reflect problems resulting from the use of an A-site substrate that includes only a single nucleotide. Recent studies have found that most ribosomal active site mutations, which show strong effects when tested with Pmn, show little or no effect when tested with full-sized tRNAs or with the slightly larger dinucleotide substrate, CPmn (Figure 1) (*20*) (J. Brunelle, E. Youngman, and R. Green, Johns Hopkins Medical Institute, unpublished results).

On the basis of these considerations, we set out to synthesize an alternative puromycin derivative that retains the α-amino group but also has an acidic pK_a and includes a 5′-cytidine (CPmn derivatives) (Figure 1). The ideal substrate would exist primarily in the deprotonated amino form at neutral pH (*21*), thus simplifying analysis of the ribosomal pH rate profile. Toward this goal we targeted CPmn derivatives with phenylalanine (Phe) or *β*,*β*-difluorophenylalanine (dFPhe) in place of the *O*-methyltyrosine present on Pmn (Figure 1). The strong electronegative character of the difluoro substitution is expected to reduce the pK_a of the α-amino group (*21*), though the magnitude of the effect has not been reported for this fluorinated amino acid.

Here we report the chemical synthesis of the unfluorinated and the fluorinated phenylalanine derivatives of CPmn (**1** and **2**) and the pH dependence of their reactivity in a 50S ribosomal peptidyl transferase assay. The chemical properties of these substrates make it possible to separate the pK_a of the substrate from that of the ribosome without reducing the reaction rate. We anticipate that this will facilitate exploration of the ribosomal catalytic mechanism.

MATERIALS AND METHODS

General Synthetic Procedures. All reactions were stirred magnetically and monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60 F254 precoated plates

(0.25 mm). Chromatography was performed with the indicated solvent system using Silicycle 0.040−0.060 mm silica gel. ^{1}H and ^{13}C NMR chemical shifts were recorded using tetramethylsilane as an internal standard, ^{19}F NMR chemical shifts were recorded using trichlorofluoromethane as an internal standard, and ^{31}P NMR chemical shifts were recorded using 85% phosphoric acid as an external standard. Pyridine was dried using Molecular Sieves. All other chemicals were used as received from commercial suppliers. 3′-Amino-3′-deoxy-3′-(*N*-Fmoc-L-phenylalanyl)-N^6,N^6-dimethyladenosine (**13**), 3′-amino-3′-deoxy-3′-(*N*-Fmoc-L-phenylalanyl)-5′-*O*-DMTr-N^6,N^6-dimethyladenosine (**18**) (*47*), (*S*)-2-carbomethoxy-3-phenyl-2*H*-azirine (**5**) (*24*, *48*, *49*), and diethylammonium hydrogen carbonate (*50*) were prepared as previously described. Puromycin aminonucleoside was purchased from Sigma.

*L-β,β-Difluorophenylalanine Methyl Ester Hydrochloride (**6**).* (*S*)-2-Carbomethoxy-3-phenyl-2*H*-azirine (*24*) (**5**, 2.28 g, 13.0 mmol) was dissolved in anhydrous THF (12.0 mL). Hydrogen fluoride in pyridine (70%, 18.0 mL) was added dropwise to the solution at 0 °C over 8 min. The reaction mixture was stirred at 0 °C for 10 min and then at room temperature for 1 h. The mixture was poured into ice and extracted with CH_2Cl_2. The aqueous phase was alkalized with concentrated ammonia at 0 °C and extracted with CH_2Cl_2. The organic phase was combined, dried over $MgSO_4$, evaporated in vacuo, and coevaporated with toluene to remove the residual pyridine. The remaining oil was dissolved in hydrochloric acid in dioxane (4.0 M, 7.5 mL) and evaporated in vacuo. The remaining foam was washed with diethyl ether successively to yield **6** (1.59 g, 49%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): 10−9 (br, 3H), 7.59 (d, 2H, J = 7.2 Hz), 7.52−7.44 (m, 3H), 4.93 (t, 1H, J = 18.2, 4.6 Hz), 3.65 (s, 3H). ^{19}F NMR (376 MHz, $CDCl_3$): −93.2 (d, 1F, J = 249.7 Hz), −107.4 (dd, 1F, J = 249.7, 18.8 Hz). ESI-MS (ES^+): m/z calcd for $C_{10}H_{11}F_2NO_2$ 215.1, found 216.0 (MH^+). $[\alpha]^{21.5}_D$: +32.3 (*c* 0.4, methanol). The enantiomeric excess of **6** was determined by dissolving it in $CDCl_3$ (1.0 mL). *N*,*N*-Diisopropylethylamine (0.2 mL) and (−)-menthyl chloroformate (0.1 mL) were added. The reaction was incubated at room temperature for 2 h. The ^{19}F NMR of the crude reaction mixture showed that the enantiomeric excess was >99%. ^{19}F NMR (376 MHz, $CDCl_3$): −103.0 (d, 1F, J = 247.6 Hz), −105.2 (d, 1F, J = 248.0 Hz).

*N-(9-Fluorenylmethoxycarbonyl)-L-β,β-difluorophenylalanine Methyl Ester (**7**).* To the solution of **6** (1.32 g, 5.25 mmol) in DMF (10 mL) and *N*,*N*-diisopropylethylamine (1.92 mL, 11.0 mmol) was added FmocCl (1.43 g, 5.53 mmol) at 0 °C. The resulting solution was stirred at room temperature for 1 h, mixed with water, and extracted with CH_2Cl_2. The combined organic phase was extracted with brine and dried over $MgSO_4$. After evaporation, the oily residue was subjected to column chromatography using a gradient of 15−50% ethyl acetate in hexanes to give the product **7** (1.81 g, 79%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): 7.77 (d, 2H, J = 7.6 Hz), 7.53 (t, 2H, J = 6.8 Hz), 7.49−7.29 (m, 9H), 5.62 (d, 1H, J = 10.0 Hz), 5.07 (q, 1H, J = 11.5 Hz), 4.41−4.25 (m, 2H), 4.15 (t, 1H, J = 7.2 Hz), 3.72 (s, 3H). ^{19}F NMR (376 MHz, DMSO-d_6): −103.2 (dd, 1F, J = 247.6, 10.7 Hz), −104.0 (dd, 1F, J = 248.3, 13.0 Hz). ESI-MS (ES^+): m/z calcd for $C_{25}H_{21}F_2NO_4$ 437.1, found 460.4 ($M + Na^+$).

*N-(9-Fluorenylmethoxycarbonyl)-L-β,β-difluorophenylalanine (**8**).* Compound **7** (1.64 g, 3.75 mmol) was dissolved in dioxane (70 mL), concentrated HCl (70 mL) was added, and the mixture was refluxed for 1 h. The resulting semisolid was dried in vacuo, and water was slowly added. The mixture was extracted with diethyl ether, washed with brine, dried over $MgSO_4$, and then evaporated to give a slight reddish solid (1.53 g). The product was recrystallized from $CHCl_3$ at −20 °C to yield **8** (0.99 g, 62%) as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$): 7.77 (d, 2H, J = 7.6 Hz), 7.54−7.29 (m, 11H), 5.57 (d, 1H, J = 10.0 Hz), 5.19−5.10 (m, 1H), 4.41−4.37 (m, 1H), 4.31−4.27 (m, 1H), 4.15 (t, 1H, J = 7.0 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): −102.3 (dd, 1F, J = 248.9, 10.3 Hz), −103.8 (dd, 1F, J = 250.8, 13.5 Hz). ESI-MS (ES^+): m/z calcd for $C_{24}H_{19}F_2NO_4$ 423.1, found 446.4 ($M + Na^+$).

*N-(9-Fluorenylmethoxycarbonyl)-L-β,β-difluorophenylalanyl Fluoride (**9**).* Cyanuric fluoride was added dropwise (0.30 mL, 3.55 mmol) to a solution of **8** (501.8 mg, 1.19 mmol) dissolved in CH_2Cl_2 (15 mL) and pyridine (0.144 mL, 1.78 mmol). After addition of CH_2Cl_2 the mixture was washed with ice water, dried over $MgSO_4$, and evaporated in vacuo. The resulting residue was washed with hexanes to give the product **9** as a colorless powder (430.4 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.77 (d, 2H, J = 7.6 Hz), 7.52−7.30 (m, 11H), 5.49 (d, 1H, J = 9.2 Hz), 5.31−5.23 (m, 1H), 4.44−4.32 (m, 2H), 4.15 (t, 1H, J = 6.8 Hz). ^{19}F NMR (376 MHz, DMSO-d_6): 41.8 (dd, 1F, J = 15.2, 8.6 Hz), −101.1 (ddd, 1F, J = 251.2, 15.0, 10.5 Hz), −103.7 (ddd, 1F, J = 250.8, 13.2, 6.8 Hz).

*3′-Amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-L-β,β-difluorophenylalanyl]-N^6,N^6-dimethyladenosine (**10**).* Puromycin aminonucleoside (247.2 mg, 0.840 mmol) was dried by repeated coevaporation with pyridine and then dissolved in DMF (23.75 mL) and pyridine (1.25 mL). To this was added **9** (357.3 mg, 0.840 mmol), and the solution was stirred at room temperature for 1.5 h. After evaporation in vacuo, the oily residue was subjected to a column chromatography gradient of 2−4% methanol in CH_2Cl_2 to give the product **10** as a slight yellowish white powder (525.5 mg, 89%). ^{1}H NMR (400 MHz, DMSO-d_6): 8.62 (d, 1H, J = 7.6 Hz), 8.46 (s, 1H), 8.24 (s, 1H), 8.20 (d, 1H, J = 10.0 Hz), 7.87 (d, 2H, J = 7.6 Hz), 7.72 (dd, 2H, J = 13.8, 7.4 Hz), 7.60−7.58 (m, 2H), 7.47−7.38 (m, 5H), 7.33−7.28 (m, 2H), 6.18 (s, 1H), 5.99 (d, 1H, J = 2.4 Hz), 5.19 (m, 2H), 4.52 (m, 2H), 4.18−4.01 (m, 3H), 3.87 (s, 1H), 3.65 (d, 1H, J = 10.4 Hz), 3.42 (d, 1H, J = 12.2, 3.8 Hz), 3.45 (br, 6H). ^{19}F NMR (376 MHz, DMSO-d_6): −95.3 (dd, J = 246.3, 5.3 Hz), −97.3 (dd, 1F, J = 244.8, 6.4 Hz), −103.9 (dd, 1F, J = 245.2, 19.4 Hz), −109.8 (s). ESI-MS (ES^+): m/z calcd for $C_{36}H_{35}F_2N_7O_6$ 699.3, found 722.6 ($M + Na^+$).

*3′-Amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-L-β,β-difluorophenylalanyl]-5′-O-(p,p′-dimethoxytrityl)-N^6,N^6-dimethyladenosine (**11**).* Compound **10** (329.7 mg, 0.471 mmol) was dried by repeated coevaporation with pyridine and dissolved in pyridine (12.0 mL). Triethylamine (0.18 mL, 1.3 mmol) and DMTrCl (506.0 mg, 1.183 mmol) were added, and the solution was stirred at room temperature for 2.5 h. The reaction was quenched by the addition of methanol (5 mL), and the mixture was evaporated in vacuo. Coevaporation twice with toluene was followed by column chromatography (gradient from 1% to 3% methanol in CH_2Cl_2) to

give the pure product **11** as a white foam (384.3 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.26 (s, 1H), 8.03 (s, 1H), 7.75 (d, 2H, J = 7.6 Hz), 7.53 (t, 2H, J = 8.6 Hz), 7.43−7.15 (m, 18H), 6.76 (d, 4H, J = 8.4 Hz), 6.63 (br, 1H), 5.82 (d, 1H, J = 8.4 Hz), 5.70 (br, 1H), 4.95 (m, 1H), 4.77 (br, 1H), 4.44 (br, 1H), 4.35 (t, 1H, J = 8.6 Hz), 4.28−4.21 (m, 2H), 4.13 (t, 1H, J = 7.0 Hz), 3.76 (s, 6H), 3.55 (br, 6H), 3.46 (d, 1H, J = 10.8 Hz), 3.31 (d, 1H, J = 10.8, 2.4 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): −102.4 (dd, 1F, J = 248.0, 13.3 Hz), −104.3 (d, 1F, J = 250.4 Hz). ESI-MS (ES^+): m/z calcd for $C_{57}H_{54}F_2N_7O_8$ 1002.4, found 1025.1 ($M + Na^+$).

*3′-Amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-*L*-β,β-difluorophenylalanyl]-2′-O-(tert-butyldimethylsilyl)-N^6,N^6-dimethyladenosine (**12**).* Compound **11** (63.3 mg, 0.063 mmol) was dried by repeated coevaporation with pyridine and then dissolved in pyridine (1.5 mL). *tert*-Butyldimethylsilyl trifluoromethanesulfonate (tBDMSOTf) (0.12 mL, 0.50 mmol) was added dropwise to the solution at 0 °C and stirred at room temperature for 1 h. The reaction was quenched by the addition of methanol (0.5 mL) and neutralized with $NaHCO_3$(aq). The mixture was extracted with CH_2Cl_2, and the organic phase was dried over $MgSO_4$, evaporated in vacuo, and coevaporated twice with toluene. The remaining oil was dissolved in CH_2Cl_2 (10 mL). Chloroacetic acid (0.25 g) was added to the solution and stirred at room temperature for 1 h. $NaHCO_3$(aq) was added carefully, and the mixture was extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and evaporated in vacuo. The resulting oil was subjected to a column chromatography gradient of 1−2% methanol in CH_2Cl_2 to give the product **12** (37.7 mg, 73%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): 8.28 (s, 1H), 7.77 (d, 2H, J = 7.6 Hz), 7.71 (s, 1H), 7.76−7.48 (m, 7H), 7.41 (dd, 2H, J = 7.2, 7.2 Hz), 7.32 (dd, 2H, J = 7.2, 7.2 Hz), 6.48 (br s, 1H), 6.38 (d, 1H, J = 10.4 Hz), 5.87 (d, 1H, J = 8.4 Hz), 5.36 (d, 1H, J = 6.4 Hz), 5.17 (dd, 1H, J = 6.8, 6.8 Hz), 4.92 (dd, 1H, J = 20.4, 11.6 Hz), 4.43−4.39 (m, 2H), 4.31−4.24 (m, 2H), 4.17 (t, 1H, J = 7.2 Hz), 3.93 (d, 1H, J = 12.8 Hz), 3.82 (t, 1H, J = 11.8 Hz), 3.53 (br, 6H), 0.80 (s, 9H), −0.14 (s, 3H), −0.34 (s, 3H). ^{19}F NMR (376 MHz, $CDCl_3$): −101.3 (dd, 1F, J = 249.1, 11.3 Hz), −103.8 (dd, 1F, J = 249.1, 11.7 Hz). ESI-MS (ES^+): m/z calcd for $C_{42}H_{49}F_2N_7O_6Si$ 813.4, found 814.4 (MH^+), 836.5 ($M + Na^+$).

*N^4-Allyloxycarbonyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(p,p′-dimethoxytrityl)-cytidine-3′-O-H-phosphonate Triethylammonium Salt (**17**).* To a stirred solution of imidazole (3.00 g, 43.6 mmol) and triethylamine (9.0 mL, 64.6 mmol) in CH_2Cl_2 (240 mL) was added phosphorus trichloride (0.75 mL, 8.42 mmol) dropwise at −78 °C, and then the resultant solution was warmed to room temperature. *N*^{4}-Alloc-2′-*O*-tBDMS-5′-*O*-DMTr-cytidine (*27*) (1.79 g, 2.41 mmol), dried by successive coevaporation with pyridine and dissolved in CH_2Cl_2 (60 mL), was added dropwise to the $POCl_3$/imidazole solution over 10 min at 0 °C. The solution was stirred at room temperature for 15 min, and the reaction mixture was poured into 1 M triethylammonium bicarbonate buffer (240 mL, pH 7.6). The aqueous phase was washed with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and evaporated in vacuo. The remaining oil was subjected to column chromatography using a gradient from 5% to 10% methanol in CH_2Cl_2 to give the product **17** (2.12 g, 97%) as a white foam. ^{31}P NMR (161.9 MHz, $CDCl_3$): 2.70 (d, J = 606.9 Hz). ESI-MS (ES^+): m/z calcd for $C_{40}H_{50}N_3O_{11}PSi$ 807.3, found 808.0 (MH^+), 830.0 ($M + Na^+$).

*3′-Amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-3′-*L*-phenylalanyl]-2′-O-(tert-butyldimethylsilyl)-N^6,N^6-dimethyladenosine (**14**).* 3′-Amino-3′-deoxy-3′-(*N*-Fmoc-L-phenylalanyl)-5′-*O*-DMTr-*N*^{6},*N*^{6}-dimethyladenosine (*47*) (**13**, 412.7 mg, 0.427 mmol) was dried by repeated coevaporation with pyridine and then dissolved in pyridine (8.0 mL). tBDMSOTf (0.80 mL, 3.41 mmol) was added dropwise to the solution at 0 °C and stirred at room temperature for 1 h. The reaction was quenched by the addition of methanol (2 mL) and neutralized with $NaHCO_3$(aq). The mixture was extracted with CH_2Cl_2, and the organic phase was dried over $MgSO_4$, evaporated in vacuo, and coevaporated twice with toluene. The remaining oil was dissolved in CH_2Cl_2 (10 mL). Chloroacetic acid (1.7 g) was added to the solution and stirred at room temperature for 1 h. $NaHCO_3$(aq) was added carefully, and the mixture was extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and evaporated in vacuo. The resulting oil was subjected to a column chromatography gradient of 1−2% methanol in CH_2Cl_2 to give the product **14** (305.9 mg, 92%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): 8.26 (s, 1H), 7.78 (d, 2H, J = 7.5 Hz), 7.62 (s, 1H), 7.58 (dd, 2H, J = 6.0, 6.0 Hz), 7.43−7.28 (m, 9H), 6.40 (d, 1H, J = 11.5 Hz), 6.11 (br, 1H), 5.55 (br, 1H), 5.05 (br, 2H), 4.47−4.21 (m, 6H), 3.91 (d, 1H, J = 13.0 Hz), 3.83 (t, 1H, J = 12.0 Hz), 3.45 (br, 6H), 3.37−3.33 (m, 1H), 2.90−2.80 (m, 1H), 0.70 (s, 9H), −0.24 (s, 3H), −0.44 (s, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$): 170.7, 155.7, 155.1, 152.0, 148.9, 143.74, 143.66, 141.3, 137.9, 136.9, 129.3, 129.1, 127.8, 127.09, 127.08, 125.1, 125.0, 121.5, 120.1, 120.0, 91.2, 86.1, 71.7, 67.2, 63.4, 56.4, 53.2, 47.2, 39.5, 38.4 (br), 25.4, 17.7, −5.5, −5.7. ESI-MS (ES^+): m/z calcd for $C_{42}H_{51}N_7O_6Si$ 777.4, found 778.2 (MH^+), 800.1 ($M + Na^+$).

*N^4-Allyloxycarbonyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(p,p′-dimethoxytrityl)-cytidylyl-(3′−5′-phosphityl)-3′-amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-*L*-phenylalanyl]-2′-O-(tert-butyldimethylsilyl)-N^6,N^6-dimethyladenosine (**16**).* Compounds **17** (0.33 g, 0.363 mmol) and **14** (186.0 mg, 0.239 mmol) were dried by repeated coevaporation with pyridine and then dissolved in pyridine (6.0 mL). Pivaloyl chloride (0.125 mL, 1.02 mmol) was added to the solution and stirred at room temperature for 1 h. The reaction mixture was poured into 1 M triethylammonium bicarbonate buffer (50 mL, pH 7.6), and the mixture was extracted with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$, evaporated in vacuo, and coevaporated twice with toluene. The remaining oil was subjected to column chromatography (2% methanol in CH_2Cl_2) to give the product **16** (374.0 mg, 100%) as a white foam. ^{31}P NMR (161.9 MHz, $CDCl_3$): 9.79, 9.09.

*Cytidylyl-(3′−5′-phosphoryl)-3′-amino-3′-deoxy-3′-*L*-phenylalanyl-N^6,N^6-dimethyladenosine (**1**).* Compound **16** (360.3 mg, 0.230 mmol) was dissolved in 2% iodine solution (10 mL, 98% pyridine:2% water) and stirred at room temperature for 10 min. $Na_2S_2O_4$(aq) (5%, 40 mL) was added, and the mixture was extracted with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$, evaporated in vacuo, and coevaporated twice with toluene. The resulting oily residue was dissolved in a solution of chloroacetic acid (1.50 g) in CH_2Cl_2 (15 mL) and stirred at room temperature for 1.5 h.

$NaHCO_3$(aq) was added, and the mixture was extracted with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and evaporated in vacuo. The remaining residue was subjected to column chromatography using a gradient from 4% to 10% methanol in CH_2Cl_2 to give the oxidized intermediate (225.3 mg) as a light yellowish white powder. This oxidized intermediate (185.8 mg, 0.143 mmol) was dissolved in CH_2Cl_2 (13 mL), mixed with piperidine (1.3 mL), and stirred at room temperature for 1.5 h. The reaction mixture was evaporated in vacuo and coevaporated twice with toluene. The oily residue was partially purified by column chromatography using a gradient from 5% to 10% methanol in CH_2Cl_2 to give the Fmoc-deprotected intermediate (145.3 mg) as a white powder. ^{31}P NMR (161.9 MHz, $CDCl_3$): 0.65. To the solution of this Fmoc-deprotected intermediate (125.3 mg) in CH_2Cl_2 (5 mL) was added diethylammonium hydrogen carbonate (313.3 mg, 2.32 mmol), tetrakis(triphenylphosphine)palladium(0) (27.0 mg, 0.0231 mmol), and triphenylphosphine (10.0 mg, 0.0377 mmol) (*50*). The resulting mixture was stirred at room temperature for 2 h. After addition of water, the mixture was extracted with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and evaporated in vacuo. The foamy residue was partially purified by column chromatography using a gradient of 10−30% methanol in CH_2Cl_2 to give the Fmoc- and Alloc-deprotected intermediate (67.2 mg) as a light yellowish white powder. ^{31}P NMR (161.9 MHz, $CDCl_3$): −2.62 (with peak at 28.90 resulting from triphenylphosphine). This crude intermediate (19.3 mg) was dissolved in 1-methyl-2-pyrrolidinone (1 mL) and mixed with triethylamine (0.5 mL, 3.59 mmol) and triethylamine trihydrofluoride (0.67 mL, 4.11 mmol). The reaction was stirred at 65 °C for 1.5 h. After concentration in vacuo, the residue was dissolved in water and extracted with CH_2Cl_2. The aqueous phase was lyophilized, and the fully deprotected product was purified over a C-18 column using a 0−40% acetonitrile gradient in 0.1 M triethylammonium acetate. The product fractions were lyophilized to yield the purified **1** in 9% yield (4.3 μmol) based upon UV absorbance. ESI-MS (ES^+): *m*/*z* calcd for $C_{30}H_{39}N_{10}O_{11}P$ 746.3, found 747.4 (MH^+). HRMS *m*/*z* calcd for $C_{30}H_{39}N_{10}O_{11}P$ 746.2435 ($M + Na^+$), found 769.2454.

*N^4-Allyloxycarbonyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(p,p′-dimethoxytrityl)-cytidylyl-(3′−5′-phosphityl)-3′-amino-3′-deoxy-3′-[N-(9-fluorenylmethoxycarbonyl)-*L*-β,β-difluorophenylalanyl]-2′-O-(tert-butyldimethylsilyl)-N^6,N^6-dimethyladenosine (**15**).* The product **15** (46.5 mg, 67%) was obtained as a colorless powder from **12** (35.4 mg, 0.0435 mmol) and **17** (58.4 mg, 0.0642 mmol) using the method described above for the synthesis of **16**.

*Cytidylyl-(3′−5′-phosphoryl)-3′-amino-3′-deoxy-3′-*L*-β,β-difluorophenylalanyl-N^6,N^6-dimethyladenosine (**2**).* The product **2** was obtained from **15** using the method described above for the synthesis of **1**. The second column chromatography step was omitted (17% yield in four steps based upon UV analysis). ESI-MS (ES^+): *m*/*z* calcd for $C_{30}H_{37}F_2N_{10}O_{11}P$ 782.2, found 783.2 (MH^+). HRMS *m*/*z* calcd for $C_{30}H_{37}F_2N_{10}O_{11}P$ 783.2427 (MH^+), found 783.2436.

*3′-Amino-3′-deoxy-3′-*L*-phenylalanyl-N^6,N^6-dimethyladenosine (**3**).* 3′-Amino-3′-deoxy-3′-(*N*-Fmoc-*L*-phenylalanyl)-N^6,N^6-dimethyladenosine (*47*) (**18**, 145.0 mg, 0.250 mmol) was dissolved in pyridine (10 mL), tris(2-aminoethyl)amine (0.34 mL, 2.27 mmol) was added, and the solution was stirred at room temperature for 2 h. The resulting cloudy reaction mixture was evaporated in vacuo and coevaporated with toluene to remove the residual pyridine. The oily residue was purified by the column chromatography using a gradient of 4−10% methanol in CH_2Cl_2 to give **3** (89.7 mg, 93%) as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$): 8.16 (s, 1H), 8.06 (s, 1H), 8.01 (br, 1H), 7.34−7.21 (m, 5H), 5.86 (d, 1H, J = 4.0 Hz), 4.73 (dd, 1H, J = 6.2, 3.8 Hz), 4.49 (dd, 1H, J = 12.2, 6.0 Hz), 4.20 (br, 1H), 3.99 (dd, 1H, J = 13.0, 2.2 Hz), 3.81 (dd, 1H, J = 13.0, 2.2 Hz), 3.68 (dd, 1H, J = 9.0, 4.6 Hz), 3.54 (br, 6H), 3.23 (dd, 1H, J = 13.6, 4.4 Hz), 2.74 (dd, 1H, J = 13.4, 9.0 Hz). ESI-MS (ES^+): *m*/*z* calcd for $C_{21}H_{27}N_7O_4$ 441.2, found 442.4 (MH^+), 464.4 ($M + Na^+$).

*3′-Amino-3′-deoxy-3′-*L*-β,β-difluorophenylalanyl-N^6,N^6-dimethyladenosine (**4**).* Using the method described above, **4** was obtained as a white power (73.2 mg, 61%) from **10** (175 mg, 0.250 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): 8.06 (s, 1H), 7.93 (s, 1H), 7.51−7.43 (m, 5H), 7.27 (br, 1H), 5.66 (d, 1H, J = 5.2 Hz), 4.76 (t, 1H, J = 5.8 Hz), 4.41 (dd, 1H, J = 11.2, 6.0 Hz), 4.09−4.05 (m, 2H), 3.89 (dd, 1H, J = 12.8, 1.6 Hz), 3.70 (dd, 1H, J = 12.8, 2.0 Hz), 3.45 (br, 6H). ^{19}F NMR (376 MHz, $CDCl_3$): −102.4 (d, 1F, J = 247.8 Hz), −106.1 (d, 1F, J = 248.2 Hz). ESI-MS (ES^+): *m*/*z* calcd for $C_{21}H_{25}F_2N_7O_4$ 477.2, found 478.5 (MH^+).

Measurement of Puromycin Analogue pK_a Values. The pK_a of the dihydrochloride salt of puromycin as well as the free bases of derivatives **3** and **4** was determined by monitoring the pH while titrating a 10 mM nucleoside solution in water with 0.1 N KOH(aq) (Pmn), in 50% (v/v) aqueous methanol with 0.1 N HCl(aq) (**3**), or in 50% (v/v) aqueous acetonitrile with 0.1 N HCl(aq) (**3** and **4**) at 25 °C. It was necessary to include methanol or acetonitrile to improve the solubility of the derivatives. The pK_a of Pmn in water was found to be 7.2. The observed pK_a of **3** in 50% aqueous methanol was 7.1 and in 50% acetonitrile was 6.9. On the basis of the data in aqueous methanol, the pK_a of **3** in water is predicted to be 7.0 (*51*). The observed pK_a of **4** was less than 5.0 in 50% aqueous acetonitrile.

Modified Fragment Assay. Large ribosomal subunits were isolated from *Escherichia coli* MRE600 cells by a procedure modified from the literature (*52*). CCApcb was 5′-^{32}P end labeled by phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP. Reaction of 1.2 mM **1** or **2** with trace [^{32}P]-CCApcb and a saturating concentration of 50S ribosomes (9 μM) was performed in 7 mM Mg^{2+}, 7 mM K^+, 150 mM NH_4^+, 0.1 mM EDTA, 0.2 mM DTT, 25 mM MES, 25 mM MOPS, and 50 mM Tris-HCl buffer at 25 °C. Neither CPmn nor CCApcb shows a pH dependence to their binding (*31*). The ribosomes were incubated for 2 min at 37 °C before the reaction was begun. The samples were analyzed by polyacrylamide gel electrophoresis [7 M urea/50 mM Tris−sodium phosphate (pH 6.5)/12% polyacrylamide gel with 50 mM Tris−sodium phosphate buffer (pH 6.5) at 30 W]. The products were quantitated by phosphoimager analysis on a Storm Imaging System 820 (Molecular Dynamics). For derivative **1**, plots of log(k_{obs}) vs pH were fit to

$$\log(k_{obs}) = \log[(K_1K_2V_{max})/(H + K_1)(H + K_2)]$$

where H is the proton concentration, K_1 is the first dissocia-

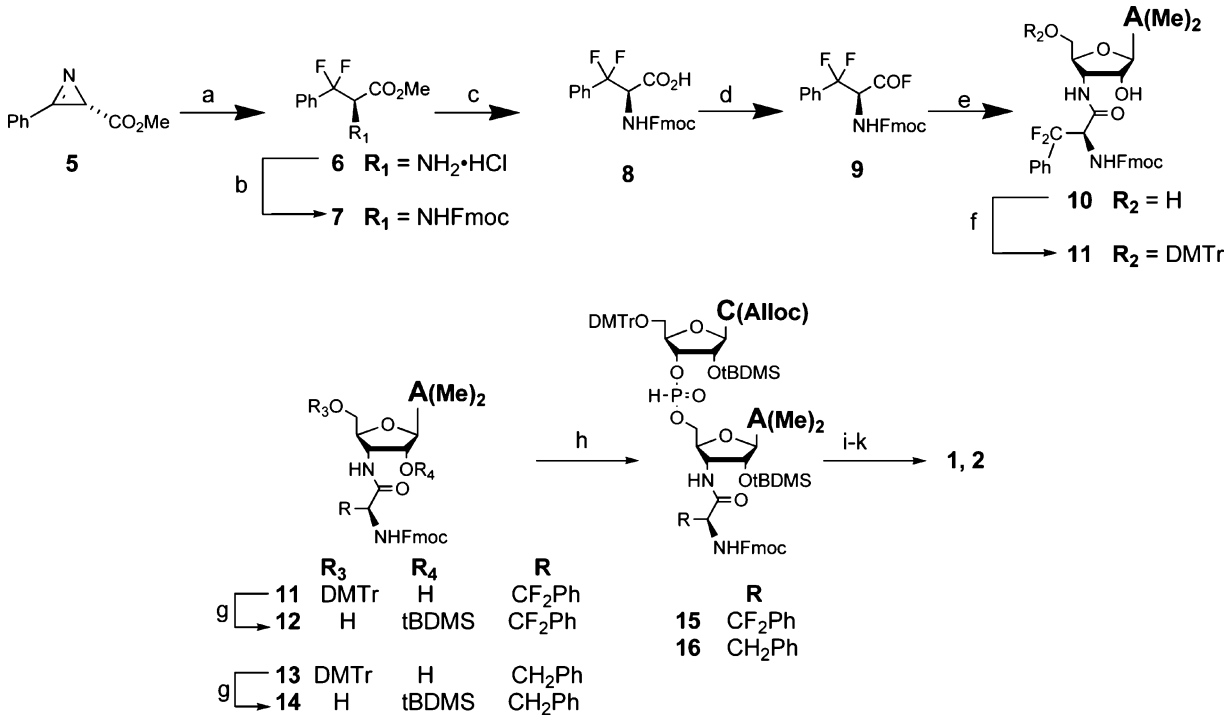

FIGURE 2: Solution-phase synthesis of CPmn derivatives containing β-fluoro substitution. Reagents and conditions: (a) HF−pyridine, THF, 0 °C to room temperature, then HCl, dioxane, 49%; (b) FmocCl, *N*,*N*-diisopropylethylamine, DMF, room temperature, 79%; (c) concentrated HCl, dioxane, reflux, 62%; (d) cyanuric fluoride, pyridine, CH_2Cl_2, room temperature, 85%; (e) puromycin aminonucleoside, pyridine, DMF, room temperature, 89%; (f) DMTrCl, triethylamine, pyridine, room temperature, 60%; (g) tBDMSOTf, pyridine, 0 °C to room temperature, then CH_2ClCO_2H, CH_2Cl_2, room temperature, 92% **14** and 73% **12**; (h) **17**, pivaloyl chloride, pyridine, room temperature, 100% **16** and 67% **15**; (i) I_2, wet pyridine, room temperature, then CH_2ClCO_2H, CH_2Cl_2, room temperature; (j) piperidine, CH_2Cl_2, room temperature; $Et_2NH_2^+ \cdot HCO_3^-$, Ph_3P, $Pd(PPh_3)_4$, CH_2Cl_2, room temperature; (k) $Et_3N \cdot 3HF$, Et_3N, 1-methyl-2-pyrrolidinone, 65 °C, 9% **1** from **16** and 17% **2** from **15**.

tion constant, and K_2 is the second dissociation constant. For derivative **2**, plots of $\log(k_{obs})$ vs pH were fit to

$$\log(k_{obs}) = \log[V_{max}/(1 + H/K_1)]$$

Data were fit by the nonlinear least squares approach using KaleidaGraph (Synergy Software). CCApcb hydrolysis was monitored in the absence of **1** or **2** across the pH range and found to be significantly slower than the peptidyl transferase reaction. Peptide bond formation was confirmed under multiple turnover conditions with saturating concentrations of A-site and P-site substrates by resolving the products by HPLC and observing the formation of a new peak of a mass consistent with the CPmn-pcb derivatives of **1** and **2** (Figure 1). Peptide bond formation at high pH was also confirmed using radiolabeled **1** or **2**, saturating 50S ribosome concentration (11.5 μM), and saturating amounts of CCApcb (400 μM).

RESULTS AND DISCUSSION

Synthesis of CPmn and Pmn Derivatives. The construction of CPmn analogues is depicted in Figure 2. This strategy involves the acylation of the appropriate L-amino acid to puromycin aminonucleoside. A solution phase methodology utilizing *H*-phosphonate chemistry is then employed to afford the desired puromycin derivatives. A detailed synthesis is described in the Materials and Methods section.

Fluorinated analogue **2** requires the acid fluoride intermediate **9** (*22*). We achieved an enantioselective synthesis of this compound utilizing an azirine ring-opening approach (*23*). (*R*)-2-Carbomethoxy-3-phenyl-2*H*-azirine (**5**) (*24*) was subjected to a cold THF solution containing HF−pyridine to afford L-β,β-difluorophenyalanine hydrochloride salt **6**. Protection of the α-amino group followed by acid hydrolysis gave **8** in good yield. Carbodiimide coupling of **8** and puromycin aminonucleoside gave poor yields of **10**. However, acylation with acid fluoride **9** delivered **10** in good yield (*25*), which was tritylated to give **11**.

Generally, solid-phase organic synthesis is employed for the construction of oligonucleotides. This strategy involves the immobilization of a protected nucleotide followed by the addition of phosphoramidites building blocks. When the desired sequence is completed, the oligonucleotide product is cleaved from the polymer by the action of 40% methylamine at 55 °C (*26*). This condition also removes the exocyclic protecting groups of the bases. For synthetic ease and the high efficiency of deprotection, we attempted this approach for the synthesis of **2** by first grafting the 2′-succinyl derivative of **11** onto polystyrene support, followed by coupling of cytidine phosphoramidite. Alkaline treatment of the dFPhe-derivatized polymer support led to the decomposition of the desired dinucleotide, most likely by β-elimination of HF as suggested by its ESI-MS spectra. Efforts to identify milder deprotection conditions including lower temperatures and shorter reaction times were unsuccessful.

The susceptibility of fluorinated amino acid to alkaline decomposition on the solid support led us to explore an alternative route, namely, a solution phase methodology. First, we changed the identity of the exocyclic protecting group of the base. Commercially available cytidine phosphoramidites contain benzoyl-protecting groups, which are removed by the action of methylamine. To avoid using base treatment, we selected the allyloxycarbonyl protecting group,

which is removed when activated by a palladium catalyst (*27*). Second, we changed the nature of the building block. Phosphoramidite chemistry is commonly performed using acetonitrile as solvent. However, puromycin analogue precursors **12** and **14** were insoluble in this solvent. Consequently, coupling of **12** with N^4-allyloxycarbonylphosphoramidite (*28*) failed using acetonitrile, acetonitrile/DMF, or DMF as solvent. Alternatively, *H*-phosphonate chemistry uses pyridine as solvent. When **12** was added to a solution of *H*-phosphonate **17** in pyridine, dinucleotide **15** was formed. Oxidation of the phosphite with iodine followed by the removal of the allyloxycarbonyl group afforded the desired dFPhe derivative of CPmn **2**. This methodology was also successful for the syntheses of the Phe derivative **1**.

The integrity of the α-carbon stereo center was demonstrated by fluorine NMR of the (−)-menthylcarbonylated derivative of intermediate **6**. The retention of chirality through subsequent steps of synthesis is supported by the efficient use of substrate **2** by the ribosome (see below). The peptidyl transferase center utilizes only the L and not the D isomer (*29*). The clean reaction kinetics (>94% reacted with single exponential decay) suggests that the fraction of D isomer is no greater than 6%.

The pK_a values of the α-amino groups of Pmn and its derivatives **3** and **4** were measured by titration with KOH or HCl. Under these conditions, the pK_a of Pmn was 7.2, a value consistent with the previous report (*17*). The pK_a of the Phe derivative **3** was 7.0. The dFPhe derivative **4** did not show a distinct inflection point above pH 5. Below pH 5, the pK_a approaches that of the adenosine N1, which significantly complicates the analysis. Thus, while it was not possible to determine the absolute pK_a of the fluorinated Pmn derivative, it is less than 5.0. A pK_a shift of greater than 2 units is consistent with that anticipated for difluoro substitution β to an amino group (*21*). For example, difluoro substitution of alanine reduces the pK_a of the α-amino group by 1.5 units, while trifluoro substitution results in a 4.5 unit reduction. A pK_a change of this magnitude should be sufficient to fully separate the substrate pK_a from the ribosomal pK_a for the PT reaction.

Activity of the Fluorinated Derivative in the Peptidyl Transferase Reaction. The ability of the dFPhe CPmn derivative **2** to serve as a substrate in the PT reaction was tested using the modified fragment assay with 50S ribosomal subunits (Figure 1) (*30*). In this simplified system, the P-site substrate is the peptidyl−nucleic acid conjugate CC-3′-(biotinyl-ϵ-aminocaproyl-L-phenylalanyl)A (CCApcb), and the A-site substrate is either of the two CPmn derivatives described above (**1** or **2**). The substrates are loaded into the ribosome without the assistance of additional protein factors, and chemistry is at least partially rate limiting based upon kinetic isotope effect analysis (*31*). This is not the case for full-length tRNA A-site substrates where accommodation into the active site is limiting (*32*). Crystallographic studies show that CPmn is bound in the 50S ribosomal A-site and is competent to react (*30*).

The unfluorinated Phe derivative **1** reacted with CCApcb to produce the aminolysis product at a rate of 1.2 min^{-1} at alkaline pHs (pH >8) (Figure 3) under single-turnover conditions with saturating A-site substrate and a concentration of 50S subunits saturating for the trace P-site substrates. The reaction was highly pH dependent with a slope of 2.0

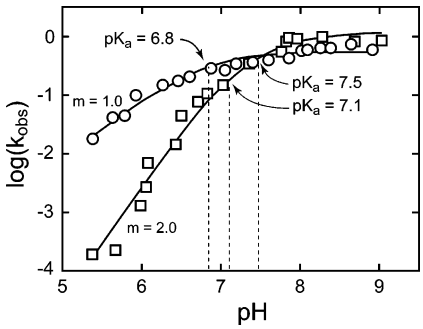

FIGURE 3: pH dependence of the 50S modified fragment reaction tested under conditions of saturating A-site and P-site substrates: Phe derivative **1** (squares); dFPhe derivative **2** (circles). The slope of each line in the log-linear range is indicated, as is the pK_a values determined for each curve.

below pH 6.5. This pH-dependent rate curve can be deconvoluted into two ionizable groups, one with a pK_a of 7.1 ± 0.7 and the second with a pK_a of 7.5 ± 0.7. The estimated error is high due to the proximity of the two titratable groups. Given the pK_a of 7.0 for the unfluorinated derivative **1**, one of these pK_as is likely due to the α-amino group. If so, the other pK_a would be the ionizable group within the peptidyl transferase center.

The reaction was repeated using the dFPhe CPmn derivative **2** (Figure 3). Derivative **2** was fully active as an A-site substrate and has several properties that can be exploited to investigate the peptidyl transferase reaction mechanism. At slightly alkaline pH values, **2** reacted at a maximum rate of 0.56 min^{-1}, only 2-fold slower than that of **1**; however, the pH dependence of the reaction was substantially different. At all pHs below 7.0, **2** reacted faster than **1**, such that at pH 5.4 the fluorinated substrate reacted 90-fold faster than the nonfluorinated version. The slope of the pH dependence below pH 6.5 is 1.0, consistent with a single pK_a of 6.8. Given that the fluorinated derivative **2** has a pK_a below 5, the incoming nucleophile is expected to be in the free amino form at all pH values tested. Thus, unlike **1**, where the unreactive ammonium form is a significant fraction of the substrate population at pH values less than 8.0, derivative **2** readily reveals the underlying pK_a attributable to an ionizable group within the peptidyl transferase center. The pK_a of 6.8 ± 0.1 is within experimental error of the lower pK_a observed for derivative **1**. This suggests that the higher pK_a of substrate **1** is due to deprotonation of the substrate ammonium ion, while the slightly lower pK_a corresponds to a titratable group in the ribosome.

These values are similar to those observed in the 70S PT assay involving a full-length P-site tRNA substrate, though in that case the lower pK_a (6.9) was attributed to the substrate pK_a, while the higher pK_a (7.5) was attributed to a titratable group in the active site (*16*). These differences are within the experimental error of our measurements, so we cannot conclude that the active site pK_a is lower for the 50S than for the 70S ribosome. For the 70S assay, the dual pK_as were deconvoluted with Pmn-OH, which reacted 500-fold slower than Pmn. The difluorinated derivative **2** reacted no less than 2-fold slower at high pH and was substantially faster at low pH. Because the A-site substrate exists in the amino form at all pHs amenable to ribosomal analysis (pH 5.4−9.0), the β,β-difluoro substitution substantially simplifies characterization of the PT reaction's pH dependence.

The functional group in the ribosome responsible for the near neutral pK_a, whether that be 7.5 or 6.8, has not yet been identified. It was originally attributed to the universally conserved active site residue A2451 based upon the 50S ribosomal crystal structure and chemical modification experiments (*8*, *9*); however, A2451 and other nucleotides implicated as important for its perturbation can be mutated with little or no consequence to reactivity when substrates larger than Pmn are used in the A-site (*20*, *33*, *34*). Furthermore, studies of A2451 chemical reactivity showed that the effect resulted from a pH-dependent conformational change in the active site rather than direct titration of the adenosine (*35*). Although the possibility of A2451 as the ionizable group cannot be completely excluded, it appears increasingly unlikely. A second possibility is the noncanonical A2450 + C2063 wobble pair located immediately adjacent to the active site (*36*). The configuration of this pair within the 50S crystal structure suggests that the N1 of A2450 is protonated (*37*), which allows the N1 to hydrogen bond to the O2 of C2063. Disruption of this base pair at elevated pH may induce an altered, but more reactive PTC conformation. This possibility was explored by introducing the isosteric A2450G·C2063U mutation and exploring the pH dependence of the resulting affinity-purified ribosomes (*36*). Although the pH dependence of the PT reaction and the susceptibility of the active site to chemical modification were substantially different, the data did not unequivocally support the assignment of A2450 as the ionizable group. The ambiguity in the interpretation of the data resulted from the fact that while a G·U mutation is isosteric in the context of an isolated wobble pair, within the PTC it disrupts tertiary interactions with at least four other active site residues. The analysis was further complicated by titration of the nucleophile in any pH-dependent experiments. Another possibility is the bifurcated A2453·C2499 pair on the other side of the active site. While footprinting has been performed on a G·U mutation at this position, the pH dependence of the reaction rate has not been explored (*38*). We expect that derivative **2** will substantially simplify such analysis in the future.

Estimating the Brönsted Coefficient for the Ribosomal PT Reaction. These data can also be used to provide an initial estimate of the Brönsted coefficient (β_{nuc}) for the ribosome-catalyzed PT reaction. The Brönsted coefficient reflects the change in charge on the nucleophile between the reaction's ground state and its transition state (*39*). In its simplest interpretation, a β_{nuc} value close to 1 suggests substantial development of positive charge on the nucleophile, while a value close to 0 indicates little if any change in charge in the transition state. For model nonenzymatic aminolysis reactions, such as that between *p*-nitrophenyl acetate and primary amines, β_{nuc} is typically 0.8 (*40*, *41*). This suggests substantial charge development on the amine in the transition state. Such a value supports a model in which the nonenzymatic reaction proceeds through a T± transition state (*42*). The N−C bond forms, but the proton remains on the nitrogen, resulting in a positively charged nitrogen in the transition state.

The reaction rates for the Phe **1** and dFPhe **2** derivatives at slightly alkaline pH can be used to provide an initial estimate of β_{nuc}. β_{nuc} is measured experimentally by comparing the reaction rates (k_{obs}) for a series of chemically related substrates with different pK_as. The plot of log(k_{obs}) versus pK_a yields a line whose slope corresponds to β_{nuc}. While nonlinearity of this relationship upon large changes in pK_a can complicate the analysis, particularly for amines with very high pK_as (*43*), the relationship was observed to be linear for amine nucleophiles with pK_a values less than 10 (*43*). The amines of substrates **1** and **2** are in the free amino form at pH 8.5. As a result, k_{obs} is not complicated by differences in the relative protonation state of the two reactions. Despite the two unit difference in pK_a between these substrates, **2** reacted only 2-fold slower than **1**. Assuming a β_{nuc} comparable to the nonenzymatic reaction, the rate would be expected to be at least 60-fold different (*40*, *41*). One trivial explanation for the unexpectedly small difference would occur if chemistry was not the rate-limiting step of the reaction. However, the reaction is not limited by substrate binding or product release (*16*, *31*). Furthermore, ^{15}N substitution of the α-amino group resulted in a 0.9% kinetic isotope effect under these pH and salt conditions (*31*). This indicates that the chemical step is at least partially limiting in this reaction system.

A 2-fold difference in reaction rate for substrates differing by at least two pK_a units suggests that β_{nuc} for the 50S-catalyzed PT reaction is less than 0.2. This argues either that there is little N−C bond order or significant deprotonation of the α-amino group in the transition state (*11*, *44*). Additional data from A-site substrates with intermediate pK_as are needed to accurately measure β_{nuc}, and the synthetic efforts necessary for such studies are currently under way (*11*, *44*).

A controversial issue regarding the peptidyl transferase mechanism concerns whether the ribosome provides chemical catalysis to the reaction (*45*, *46*). There is consensus that alignment of the two tRNA substrates contributes significantly. What remains unresolved is the nature of any additional catalytic contribution. Assuming a purely entropic mechanism, where raising the effective molar concentration of the substrates is the ribosome's only catalytic role, the aligned substrates are expected to proceed through the same mechanism as observed for the uncatalyzed reaction. Consequently, β_{nuc} would be the same on and off the ribosome, i.e., equal to ∼0.8. Although β_{nuc} for the catalyzed reaction is still not known with high precision, these data estimate that it is substantially lower. Lack of equality between β_{nuc} for these two reactions suggests that the ribosome alters the transition state, which implicates the ribosome in chemical catalysis.

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SUPPORTING INFORMATION AVAILABLE

^{1}H NMR spectra of intermediate compounds **9**, **12**, **14**, **15**, and **16** and ^{19}F spectra of **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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