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Synthesis of a Fluorine-Substituted Puromycin Derivative for Brønsted Studies of Ribosomal-Catalyzed Peptide Bond Formation

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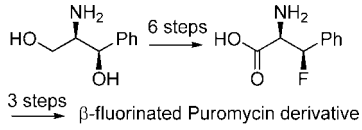

The mechanism by which the ribosome catalyzes peptide bond formation remains controversial. Here we describe the synthesis of a nucleoside 3′-amino-3′-deoxy-3′-[(3″*R*)-3-fluoro-L-phenylalanyl]-N^6,N^6-dimethyladenosine, which was prepared from (1*R*,2*R*)-2-amino-1-phenylpropane-1,3-diol. This substrate is active in peptide bond formation on the ribosome and is a useful probe for Brønsted analysis experiments on the ribosome.

The ribosome is the ribonucleoprotein complex responsible for protein synthesis in all living systems. The structure of the 50S ribosomal subunit, which was determined by X-ray crystallography,[1,2] revealed that RNA surrounds the peptidyl transferase (PT) center. The ribosome catalyzes peptide bond formation between two tRNA substrates, the aminoacyl tRNA (A-site tRNA) and the peptidyl tRNA (P-site tRNA). These two substrates bind to the ribosome in their respective active sites, and then a nucleophilic substitution reaction of the α-amino group of the A-site tRNA on to the ester−carbonyl linkage of the P-site tRNA occurs.

The ribosome enhances the rate of peptide bond formation by more than 10^6-fold compared to nonenzymatic reactions.[3] To explain this acceleration, several catalytic strategies have been proposed such as general acid−base catalysis,[1,4] substrate-assisted catalysis,[5] and catalysis derived solely from substrate alignment.[6] To distinguish these possibilities, additional chemical probes that facilitate mechanistic analysis are needed.

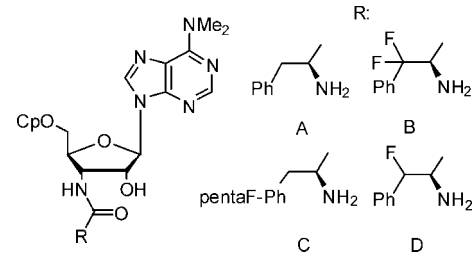

FIGURE 1. The puromycin derivatives used as A-site substrates.

To determine the chemical and catalytic mechanism of the ribosome, a detailed understanding of the transition state of peptidyl transfer is necessary. Brønsted analysis is a direct experimental technique that reports the charge state of the transition state. The Brønsted coefficient is a linear free energy relationship between the log of the reaction rate and the pK_a of a particular reactive group and reflects the change in charge on that group between the ground state and the transition state.[7] This information can then be used to determine the change in bonding that occurs in the transition state, which helps to define the reaction mechanism. We previously reported a limited Brønsted analysis of the PT reaction on 50S ribosomes using dinucleotide C-puromycin (C-Pmn) derivatives (Figure 1, parts A and B) as A-site substrates and the P-site substrate CC-3′-(biotinyl-ε-aminocaproyl-L-phenylalanyl) A.[8] Using these small molecules as substrates, this model reaction enabled us to examine the rate difference due to the perturbed nucleophilic pK_a values of the C-Pmn derivatives. The rates between both substrates did not vary significantly, leading to an initial estimate of the Brønsted coefficient for the nucleophile (β_{nuc}) value to be close to zero. These results suggest that the transition state of the ribosomal PT reaction was fairly different from that of the simple ester aminolysis reaction in solution where β_{nuc} values are 0.8−0.9. However, two substrates alone are insufficient to define the β_{nuc}.

Measuring β_{nuc} for ribosomal peptide bond formation requires more A-site substrates with perturbed pK_a values in the middle of the pK_a range of the available Pmn derivatives (<5.0 (B) to 7.0 (A)). The first candidate was a Pmn derivative with a pentafluorophenyl moiety substituted for the phenyl moiety (Figure 1C). This pentafluorophenyl derivative was prepared

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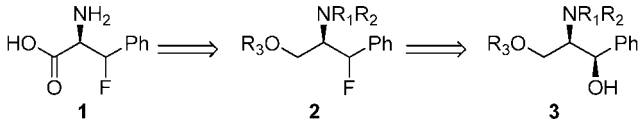
SCHEME 1. Retrosynthesis for the Production of Nonracemic 3F-L-Phe (1)



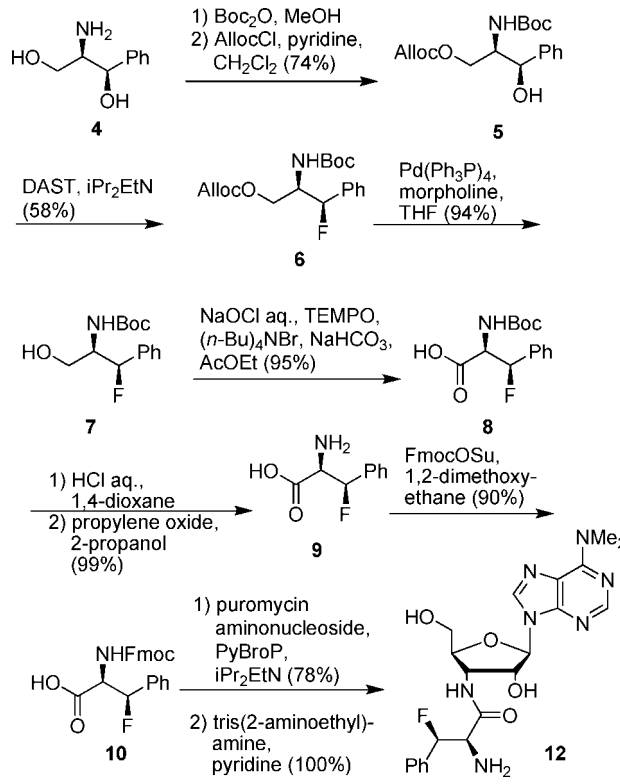
from the commercially available (*S*)-2-Fmoc-amino-3-(perfluorophenyl)propanoic acid by an analogous method described previously.[8] The α-amino group pK_a of this substrate is perturbed to 5.9 and it bound to the ribosomal PT center, but it was found to be inactive as a substrate (data not shown). The reason for the inactivity of the pentafluorophenyl-Pmn derivative is unknown. An alternative target is a monofluorine-substituted L-phenylalanine analogue at the 3-position (Figure 1D). This is expected to reduce the pK_a of the amine, but by an amount less than that of the disubstituted analogue. Here we describe the synthesis of the mononucleoside and demonstrate it is an active ribosomal substrate for peptide bond formation by the 70S initiation complex assay, a standard ribosomal peptide bond assay.[3]

The first synthetic goal was the synthesis of chiral 3-substituted L-phenylalanine (3F-L-Phe). An efficient asymmetric synthesis of chiral 3F-D-Phe was already reported by Davis.[9] Their stereodirecting group at the 2-position (α-position) was (*R*)-(−)-*p*-toluenesulfinamide. Therefore, using (*S*)-(+)-*p*-toluenesulfinamide should lead to a chiral 3F-L-Phe, but the reagents are rather expensive. Furthermore, we felt that the easily racemizable α-fluoro aldehyde preparation by Dess−Martin oxidation was technically too demanding. There are two other reported synthetic strategies for producing chiral 3F-L-Phe. One used the enzymatic hydrolysis of the racemic precursor ester, but was inefficient for asymmetric synthesis.[10,11] The other strategy used the Schöllkopf's bis lactim ether strategy and resulted in low overall yields and mixtures of products.[12] The infeasibility of these precedents required that we explore another synthetic route to produce chiral 3F-L-Phe.

Our retrosynthetic strategy for the production of nonracemic 3F-L-Phe (**1**) is outlined in Scheme 1. Deprotection of the hydroxyl group, oxidation of the alcohol to the carboxylic acid, and deprotection of the amino group of the *N*,*O*-protected 3F-L-phenylalaninol **2** will afford the amino acid **1**. Compound **2** is obtained by diethylaminosulfur trifluoride (DAST) deoxofluorination of **3**, usually with stereoinversion.[13] **3** is derived by *N*,*O*[3]-protection from the commercially available, nonracemic (1*R*,2*R*)-2-amino-1-phenylpropane-1,3-diol (**4**).

We screened the DAST deoxofluorination reaction of **3**. A solution of **3** in CH_2Cl_2 was added dropwise to the DAST (3 equiv) solution in CH_2Cl_2 at −78 °C and then the solution was warmed to rt for 18−19 h under N_2 atmosphere. TBS, DPS, and Tr groups were used to protect the hydroxyl group and the amino group was protected with Boc, trifluoroacetyl, formyl, and dibenzyl groups. All of these combinations resulted in diastereomeric mixtures[14−16] determined by ^{1}H and ^{19}F NMR. Although some of the diastereomers were separable by careful silica gel column chromatography purification, these procedures were inefficient. Perhaps S_N1 mechanism competed with the S_N2 mechanism[16] due to benzyl position.

SCHEME 2. Preparation of Pmn(3-F-Phe) (12)



Finally, we found that *tert*-butyl (1*R*,2*R*)-3-(allyloxycarbonyloxy)-1-hydroxy-1-phenylpropan-2-ylcarbamate (Scheme 2, **5**) could be converted to a single diastereomer (37%).[17] To improve the relatively low yield, we added *i*-Pr$_2$EtN (3 equiv) to boost reactivity of DAST to give the product with slightly better yield (54%) and some recovered starting material (18%). Increasing the equivalents of DAST and *i*-Pr$_2$EtN (each 3.5 equiv) gave the best result in 58% yield with some recovered starting material (6%).

At first, it was assumed that this reaction proceeded via S_N2 reaction, so the product should be *erythro*-(1*S*,2*R*). However, the product was later confirmed to be the *threo* isomer judged by the analytical data of 3F-L-Phe (**9**),[9,10] so it must be *threo*-(1*R*,2*R*) that has retention of its stereocenter (described later). We speculate the mechanism of this stereochemistry is either (1) participation of the carbonate protecting group via a 6-*exo*-tet intermediate or (2) a S_N1-like pathway. The first scenario is plausible because only the carbonate protective group (Alloc)

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(17) TLC analysis of the crude reaction mixture did not show any considerable spot indicating possible diastereomer. R_f of spots on silica gel TLC (ethyl acetate/*n*-hexane, 25:75): 0.38 (**6**), 0.15 (**5**), 0.02 (a byproduct), 0 (byproducts).

gave the *threo* diastereomer. Incorporating other protective groups (TBS, DPS, and Tr) did not lead to similar results. If the reaction does proceed by the first scenario a 6-membered cyclic carbonate byproduct should be observed upon loss of the allyl group. However, this byproduct was not detected during the reaction. Alternatively, the second scenario is supported by some of the α-fluoro phosphonate literature. While simple secondary α-hydroxyphosphonates usually show stereoinversion by DAST deoxofluorination reaction,[18] this reaction proceeds via S_N1 pathways in α-hydroxy-α-phenylphosphonates.[19] Like the Shibuya study, shielding of one face of the carbocation intermediate gives some diastereomeric excess. If our reaction went via a S_N1-like pathway, one might expect the *erythro* diastereomer as a byproduct in the reaction mixture. We did not detect such a spot during TLC analysis of the crude reaction mixtures.[17]

After deprotection of the allyloxycarbonyl group by Pd catalysis (94%), the oxidation reaction of Boc-amino alcohol (**7**) to Boc-amino acid (**8**) was screened. Jones oxidation (3 equiv)[20] gave a poor result (27%). Under Parikh−Doering oxidation (5 equiv) condition, only starting material was recovered.[21] The $RuCl_3$ (5.5 mol %)−$NaIO_4$ (4 equiv) oxidation system gave moderate results (79%).[22] The TEMPO (1 mol %)−*n*-Bu_4NBr (0.5 mol %)–NaOCl (3.5 equiv) oxidation system gave the best result (95%).[23] Furthermore, the TEMPO−*n*-Bu_4NBr–NaOCl oxidation system only needed simple recrystallization to be purified, in contrast to $RuCl_3$−$NaIO_4$ oxidation, which required purification by silica gel column chromatography.

The Boc group of **8** was removed by HCl (99%) to give the previously characterized compound, 3F-L-Phe (**9**). The most distinguishable feature was the 14.0 Hz J_{H2-F} for 3F-L-Phe (**9**), which was in good accordance with the 14.3 Hz reported for *threo*-(2*S*,3*S*)-3F-D-Phe, as opposed to the 27.4 Hz reported for *erythro*-(2*S*,3*R*)-3F-D-Phe.[9]

To confirm its enantio purity, we converted **8** to (1*R*)-α-methylbenzylamide.[24] Standard DCC coupling failed, but the acyl fluoride method produced (2*R*,3*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-fluoro-3-phenyl-*N*-[(1*R*)-1-phenylethyl]propanamide **8′** in good yield (70%).[25] Analogously, we prepared (2*S*,3*S*)-**8** (*ent*-**8**) starting from (1*S*,2*S*)-**4** (*ent*-**4**) to give diasetero-**8′**. We confirmed that the newly introduced chiral centers provided different sets of signals for each diastereomer in ^1H NMR which showed their ee were >99% (see the Supporting Information for details).

Next this amino acid was coupled to puromycin aminonucleoside. **8** was coupled by the acyl fluoride method, but the Boc group deprotection gave disappointing results likely due to the acid cleavage conditions. This is similar to the problems reported by Robins when they prepared a related compound.[26] Boc protection (**8**) was substituted with Fmoc protection (**10**) (89% for 2 steps). Bromotris(pyrrolidino)phosphonium hexafluorophosphate was used to couple **10** to puromycin aminonucleoside to give **11** (78%). Finally, deprotection of the Fmoc group by tris(2-aminoethyl)amine gave the target compound (**12**) in a quantitative yield.

The pK_a value of the α-amino group of **12** was measured by the titration method. As anticipated, the pK_a of **12** is 5.6, which fills in the gap between the 7.0 pK_a of the Phe derivative and <5.0 pK_a of the 3″,3″-difluoroPhe derivative.

Finally, this pK_a perturbed probe (**12**) was an active substrate for the ribosomal peptidyl transferase reaction as measured by the 70S initiation complex assay (see the Supporting Information for details).[3] K_M of **12** was similar to K_M of puromycin (2.9 mM vs. 2.5 mM), and reaction rates between **12** and puromycin were no more than 3-fold different (37.5 s^{-1} vs. 12 s^{-1}). These kinetic data were included in the determination of the Brønsted coefficient for the nucleophile of ribosomal peptidyl transferase.[27]

In conclusion, an efficient synthesis of a monofluorinated puromycin analogue was accomplished and the product was active as a ribosomal substrate in peptide bond formation. This analogue provided essential data in the middle of the pK_a range used for the Brønsted analysis that was unattainable by any other puromycin derivative. In the process of producing the monofluorinated puromycin analogue, a diastereoselective synthesis of 3-fluoroPhe was accomplished in 6 steps.

A protected *threo*-1-substituted 2-amino-1,3-propanediol as well as Ph group should be available via addition of the appropriate organometallics to a Garner aldehyde with chelation control.[28] Therefore, this deoxofluorination methodology of protected *threo* 3-hydroxy-L-phenylalaninol with stereoretention would pave a general procedure for syntheses of *threo* selective, chiral 3-fluorinated amino acids.

Experimental Section

***tert*-Butyl (1*R*,2*R*)-3-(Allyloxycarbonyloxy)-1-hydroxy-1-phenylpropan-2-ylcarbamate (5).** To a cooled solution (0 °C) of (1*R*,2*R*)-2-amino-1-phenylpropane-1,3-diol (**4**, 2.51 g, 15.0 mmol) in methanol (15 mL) was added Boc_2O (3.77 mL, 15.9 mmol) dropwise over 1 min, then the mixture was stirred at rt for 13 h. The resulting yellow solution was evaporated in vacuo to give an orange viscous oil. Silica gel chromatography (ethyl acetate/*n*-hexane, 1:1) gave *N*-Boc-**4** as a colorless oil. To the cooled solution (0 °C) of *N*-Boc-**4** and pyridine (4.3 mL, 53.3 mmol) in CH_2Cl_2 (200 mL) was added allyl chloroformate (3.97 mL, 37.5 mmol) dropwise over 1 min, then the mixture was stirred at rt for 20 min. Water was added and then extracted. The aqueous phase was further extracted with CH_2Cl_2. The combined organic phase was washed with saturated brine, re-extracted with CH_2Cl_2, dried over Na_2SO_4, and then evaporated in vacuo. The resulting yellow oily residue was repeatedly coevaporated with toluene, followed by silica gel column chromatography (ethyl acetate/*n*-hexane, 12:88 to 100:0) to give **5** as a white waxy solid (3.74 g, 71%) with recovered *N*-Boc-**4** (19%).

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***tert*-Butyl (1*R*,2*R*)-3-(Allyloxycarbonyloxy)-1-fluoro-1-phenylpropan-2-ylcarbamate (6).** A solution of **5** (1.23 g, 3.50 mmol) in anhydrous CH_2Cl_2 (35 mL) and *i*-Pr_2EtN (2.13 mL, 12.2 mmol) was added dropwise to a cooled solution (−78 °C) of DAST (1.62 mL, 12.3 mmol) in anhydrous CH_2Cl_2 (35 mL) under N_2 atmosphere over 1 h. The reaction mixture was then warmed to rt over 18 h. Water was added and then extracted. The aqueous phase was further extracted with CH_2Cl_2. The combined organic phase was washed with saturated brine, dried over Na_2SO_4, and then evaporated in vacuo to give a dark reddish brown oil. This oily residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane, 1:19 to 1:4) to give **6** as a white solid (711.3 mg, 58%) with **5** also recovered (5%).

***tert*-Butyl (1*R*,2*R*)-1-Fluoro-3-hydroxy-1-phenylpropan-2-ylcarbamate (7).** To a solution of **6** (713.7 mg, 2.02 mmol) in THF (30 mL) was added $Pd(Ph_3P)_4$ (94.5 mg, 0.081 mmol) and morpholine (0.27 mL, 3.09 mmol) successively, then the mixture was stirred at rt for 1 h under N_2 atmosphere. Saturated brine was added, and then extracted with ethyl acetate. The combined organic phase was dried over Na_2SO_4, and then evaporated in vacuo to give a light orange semisolid. This residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane, 1:4) to give **7** as a white solid (512.6 mg, 94%).

(2*R*,3*R*)-2-(*tert*-Butoxycarbonylamino)-3-fluoro-3-phenylpropanoic Acid ((3*R*)-*N*-Boc-3-fluoro-L-Phe) (8). 7 (512.6 mg, 1.90 mmol) was dissolved in ethyl acetate (4 mL), then water (2 mL), TEMPO (2.9 mg), $(n\text{-Bu})_4NBr$ (2.9 mg), and $NaHCO_3$ (0.60 g) were added successively. NaOCl aq (10%, 5 mL) was added dropwise to this mixture (ice bath) with vigorous stirring, and then the solution was stirred for 30 min at 0 °C. Ethyl acetate was added, and then extracted with saturated $NaHCO_3$ aq. To the combined aqueous phase was added $NaHSO_3$ (0.80 g) slowly, followed by dropwise addition of 10% HCl aq to pH ca. 3. This cloudy mixture was extracted by ethyl acetate, washed with saturated brine, dried over Na_2SO_4, then evaporated in vacuo. The white solid residue was recrystallized from cyclohexane to give **8** as a colorless feather crystal (513.9 mg, 95%).

(3*R*)-3-Fluoro-L-Phe (9). To a solution of **8** (425.1 mg. 1.50 mmol) in 1,4-dioxane (36 mL) was added 12% HCl aq (12 mL) dropwise, then the mixture was stirred at rt for 3.5 h. After evaporation in vacuo at 30 °C, excess water was removed through repeated coevaporation with 1,4-dioxane. The resulting white solid was added to 2-propanol (24 mL) and then propylene oxide (0.6 mL) was added dropwise with stirring at rt for 3.5 h to give a white precipitate. After evaporation in vacuo, the resulting white powder was filtrated and washed with 2-propanol repeatedly to give **9** as a white powder (271.7 mg, 99%).

(3*R*)-*N*-Fmoc-3-fluoro-L-Phe (10). To a solution of Fmoc-OSu (608.1 mg, 1.77 mmol) in 1,2-dimethoxyethane (6 mL) was added **9** (220.1 mg, 1.20 mmol), then the mixture was stirred at rt for 12 h. The resulting white precipitate was filtrated and washed successively with 1,4-dioxane. The filtrate was slightly acidified by dropwise addition of 2 N HCl aq to pH 2−3, followed by ethyl acetate extraction. The collected organic layer was washed with saturated brine, re-extracted by ethyl acetate, dried over Na_2SO_4, and then evaporated in vauo. The resulting oily residue was purified by silica gel column chromatography (methanol/CH_2Cl_2, 0:100 to 1:99) to give a white solid residue, which was washed successively by cyclohexane to give pure **10** as a white powder (437.2 mg, 90%).

3′-Amino-3′-deoxy-3′-[(3″*R*)-*N*-Fmoc-3″-fluoro-L-phenylalanyl]-N^6,N^6-dimethyladenosine (11). Puromycin aminonucleoside (30.6 mg, 0.104 mmol) was dried by repeated coevaporation with pyridine, then solvated in anhydrous CH_2Cl_2 (1.0 mL). To this mixture was added **10** (44.5 mg, 0.110 mmol), PyBroP (51.2 mg, 0.107 mmol), *i*-Pr_2EtN (0.06 mL, 0.344 mmol), and DMF (1.0 mL) successively at 0 °C. The solution was stirred at rt for 1.5 h. After evaporation in vacuo, the oily residue was purified by silica gel column chromatography (methanol/CH_2Cl_2, 0:100 to 2:98) to give a white solid powder, which was washed successively by water followed by methanol to give pure **11** as a white powder (55.2 mg, 78%).

3′-Amino-3′-deoxy-3′-[(3″*R*)-3″-fluoro-L-phenylalanyl]-N^6,N^6-dimethyladenosine (12). 11 (195.0 mg, 0.286 mmol) was dissolved in pyridine (15 mL) and tris(2-aminoethyl)amine (0.46 mL, 3.07 mmol) was added, then the solution was stirred at rt for 4 h. The resulting cloudy reaction mixture was evaporated in vacuo and coevaporated with toluene. The oily residue was purified by silica gel column chromatography (methanol/CH_2Cl_2, 4:96 to 6:94) to give a white solid, which was recrystallized from $CHCl_3$−*n*-hexane to give pure **12** as a white powder (131.9 mg, 100%).

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Supporting Information Available: Experimental procedures for **8′**, characterization, ^1H and ^{13}C NMR spectra of all compounds, and details for the 70S ribosome initiation complex assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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