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Synthesis of isotopically labeled puromycin derivatives for kinetic isotope effect analysis of ribosome catalyzed peptide bond formation

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Abstract—The mechanism by which the ribosome catalyze peptide bond formation remains controversial. Here we describe the synthesis of dinucleotides that can be used in kinetic isotope effect experiments to assess the transition state of ribosome catalyzed peptide bond formation. These substrates are the isotopically labeled dinucleotide cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-L-phenylalanyl-N^6,N^6-dimethyladenosine (Cm^6A_NPhe-NH_2) and cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-(L-2-hydroxy-3-phenylpropionyl)-N^6,N^6-dimethyl-adenosine (Cm^6A_NPhe-OH). These substrates are active in peptide bond formation and can be used to measure kinetic isotope effects in ribosome catalyzed protein synthesis.

1. Introduction

The ribosome is the ribonucleoprotein-complex responsible for protein synthesis in all cells. The structure of the 50 S ribosomal subunit was determined recently by X-ray crystallography.[1,2] The structure revealed that the peptidyl transferase center of the ribosome resides solely within the 23 S ribosomal RNA (rRNA), that is, the ribosome is an RNA enzyme or ribozyme.[1] The ribosome catalyses peptide bond formation between two substrates, the aminoacyl tRNA bound in the ribosomal A site, and the peptidyl tRNA bound in the ribosomal P site. The reaction involves nucleophilic attack of the A-site tRNA α-amino group on the carbonyl-ester linking the nascent peptide to the P-site tRNA. The products of this reaction are a deacylated P-site tRNA and an A-site tRNA linked to the nascent peptide, which has been extended by one amino acid.

The ribosome enhances the rate of peptide bond formation by more than 100,000 fold.[3] The pH dependence of the reaction suggests that an ionizable functional group in the ribosome is catalytically important.[3–5] This group could act as a general base to deprotonate the nucleophile, or deprotonation may cause a conformation change in the ribosome that enhances the reaction rate. Several chemical pathways have been proposed for the reaction, all of which invoke a tetrahedral transition state.[6–8] They differ with regard to the point along the reaction coordinate at which the nucleophile is deprotonated and the leaving group is protonated.

Transition state stabilization is a fundamental strategy employed by enzymes to promote chemical reactions. Characterization of the transition state would provide information essential to understanding how the ribosome enhances the peptidyl transferase reaction; but the transient nature of the free energy maxima between products and reactants makes such investigations extremely challenging. One approach to transition state analysis is the measurement of kinetic isotope effects.[9–12] Reactive functional groups in the substrates are isotopically labeled and the relative reaction rates of the heavy and light substrates determined by enzyme kinetics. Kinetic isotope effects arise from changes in vibrational states between the ground state and the transition state in a chemical reaction.[13] The magnitude and direction of these effects provides the information needed to successfully predict the reaction transition state, including reactions catalyzed by enzymes such as the ribosome.[13–18]

Keywords: Ribosome; Puromycin; Kinetic isotope effect; Solid-phase synthesis.
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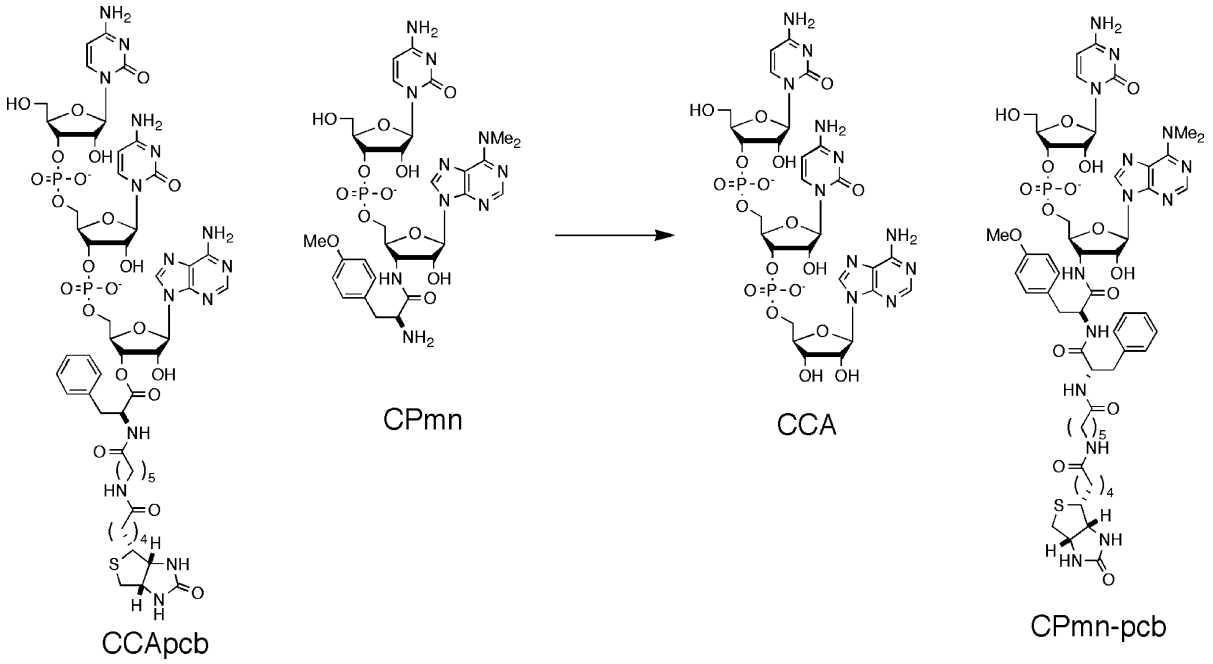


Scheme 1. Schematic of the modified fragment assay.

In order to measure a kinetic isotope effect, the isotope sensitive step must be rate-limiting. Ribosomal protein synthesis involves a multi-step process aided by GTP dependent protein factors. It is known from kinetic studies that accommodation of the amino-acyl tRNA is rate-limiting during the elongation step of protein synthesis.[19] Therefore, an assay that does not include accommodation is necessary for kinetic isotope effect measurements. The 50 S ribosomal subunit alone can catalyze peptide bond formation using two small synthetic substrates that mimic the A-site and P-site tRNAs (Scheme 1).[20] In this modified fragment assay, cytidylyl-(3′-5′)-puromycin (CPmn) functions in place of the A-site tRNA, while cytidylyl-(3′-5′)-cytidylyl-(3′-5′)-3′-(biotinyl-ε-aminocaproyl-L-phenylalanyl)adenosine (CCApcb) serves in place of the P-site tRNA. Like the standard peptidyl transferase reaction, the α-amino group of the A-site substrate attacks the ester bond in the P-site substrate, to produce a new peptide bond.[21] This simplified reaction is ideal for measuring kinetic isotope effects for two reasons: (i) the small substrates can be chemically synthesized to include specific isotopic substitutions, and (ii) the reaction is mechanistically simplified to the reversible binding of two small substrates, a chemical reaction, and product release.

In order to measure kinetic isotope effects on the ribosome, it was necessary to synthesize substrates with heavy atom substitutions on the reactive functional groups. Here we describe the synthesis of CPmn derivatives with ^{15}N substitution at the α-amino group and remote positions (**1**, **1*** and **1*****), and derivatives in which the α-amino is substituted with an ^{18}O α-hydroxyl group (**2** and **2***) (Fig. 1). We also demonstrate that these molecules serve as ribosome substrates.

2. Results and discussion

2.1. Synthesis of $Cm^6A_NPhe\text{-}NH_2$

The CPmn analogs $Cm^6A_NPhe\text{-}NH_2$ (**1** and **1***),[22] where **N** is either ^{14}N or ^{15}N, were prepared by solid phase synthesis.

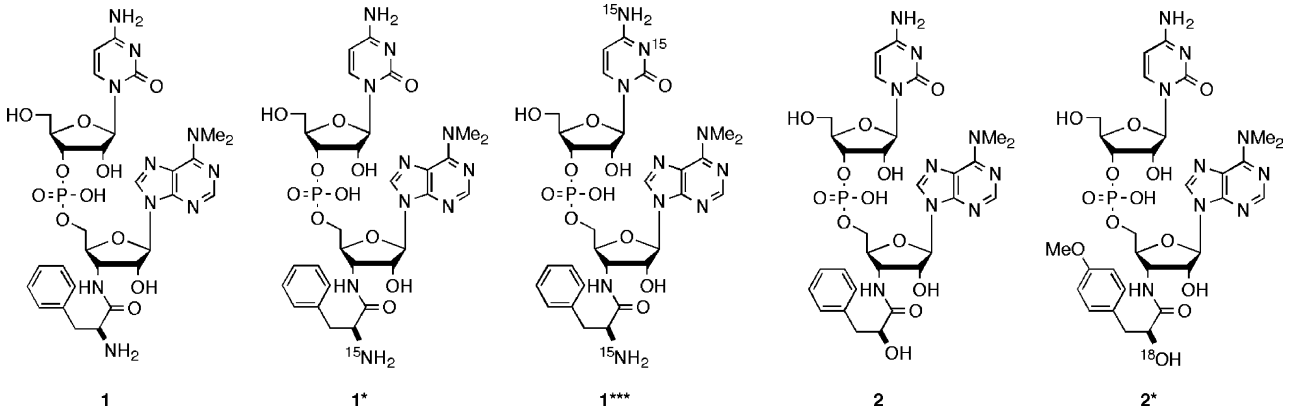


Figure 1. Synthetic targets $Cm^6A_NPhe\text{-}NH_2$ and $Cm^6A_NPhe\text{-}OH$ and their isotopic derivatives.

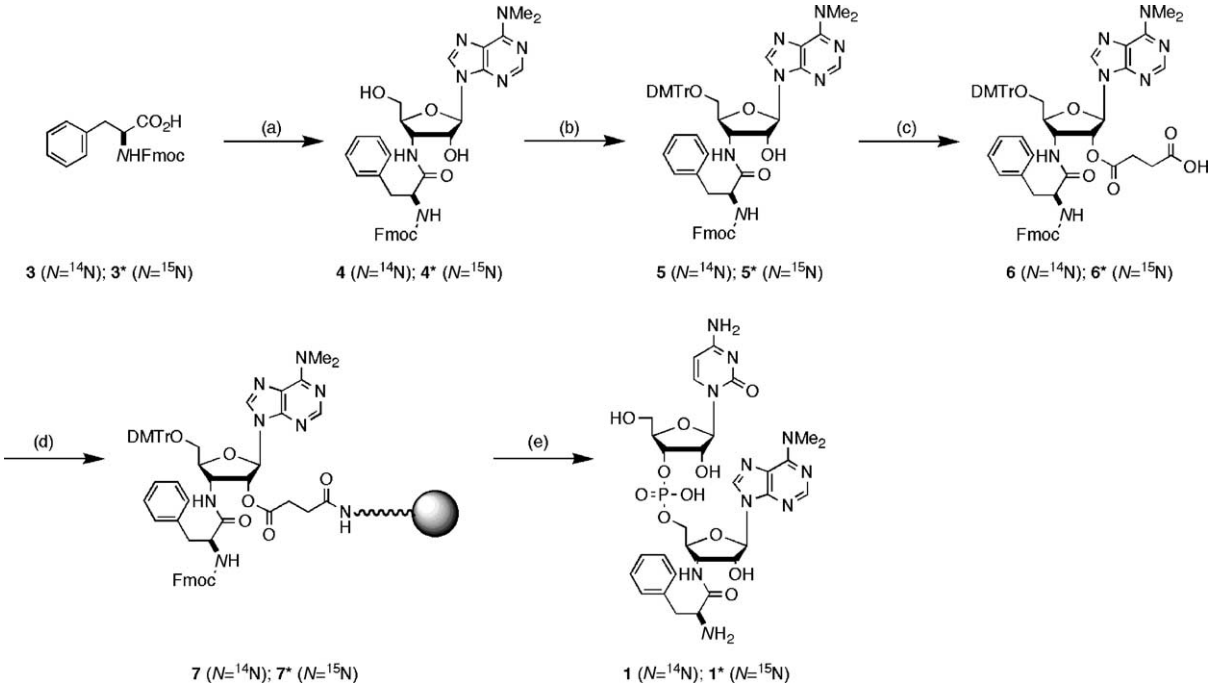
For this purpose, the appropriately protected puromycin analogs were attached to solid support and coupled to cytidine as shown in Scheme 2.

The 3′-amino group of puromycin aminonucleoside was selectively derivatized with commercially available *N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanine (**3** and **3***) using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI). Further derivatization of the 5′ and 2′ hydroxyl groups was performed using *p*,*p*′-dimethoxytrityl chloride (DMTrCl) and succinic anhydride, respectively. This transformation was accomplished using methods analogous to those reported in the literature[23,24] to yield suitably protected compounds **6** and **6***. These were attached to LCAA-polystyrene support and the loading quantified spectroscopically by the DMTr cation method.[25] The solid support (**7** and **7***) was coupled to the protected cytidine phosphoramidite, 4-acetyl-5′-*O*-[benzhydroxybis-(trimethylsiloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]-cytidine-3′-(methyl-*N*,*N*-diisopropyl)phosphoramidite (**8**). The dinucleotide was cleaved from the solid support and deprotected as described previously.[26] Subsequent purification by reversed-phase HPLC yielded **1** and **1***, respectively.

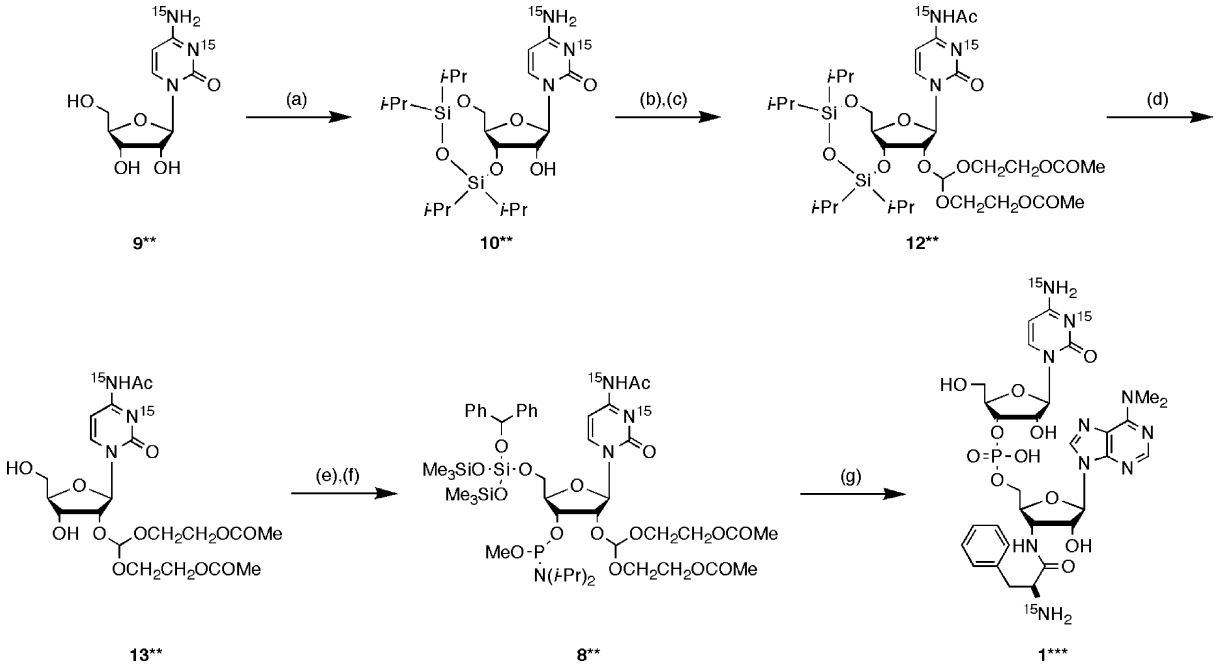
2.2. Synthesis of [3-^{15}N,4-$^{15}NH_2$]Cm^6A_NPhe-$^{15}NH_2$

The mass difference between Cm^6A_NPhe-$^{14}NH_2$ (**1**) and Cm^6A_NPhe-$^{15}NH_2$ (**1***) is only 1 Da. Whole molecule mass spectroscopic analysis of the two isotopes would be complicated by the significant size of the M+1 peaks that arises from natural ^{13}C abundance. To increase the mass difference between the two isotopes, we incorporated two additional remote substitutions in the ^{15}N labeled substrate. This is reminiscent of the remote labeling method that used radioactive isotopes as markers for heavy atom substitutions in kinetic isotope effect studies.[27] Because the cytidine moiety does not participate directly in the chemical reaction, heavy isotope substitution should not affect the reaction, nor should it show an isotope effect. Any affects can be controlled for by characterization of a dinucleotide containing only the remote labels. We selected [3-^{15}N,4-$^{15}NH_2$]cytidine[28] (**9****) for this purpose. The preparation of the [3-^{15}N,4-$^{15}NH_2$]cytidine phosphoramidite (**8****) was accomplished as follows (Scheme 3).[29]

Compound **9**** was prepared from uridine in five steps.[28] The ^{15}N at position N3 was introduced by the rearrangement reaction caused by the attack of $^{15}NH_3$ at the C4 of 2′,3′,5′-tri-*O*-acetyl-3-nitrouridine. The $^{15}NH_2$ substitution at the N4 position was introduced from the 4-(tetrazol-1-yl) intermediate by the $^{15}NH_3$ replacement reaction. Compound **9**** was treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDSCl$_2$) in pyridine to simultaneously protect the 3′ and 5′ hydroxyl groups to produce **10****. The N4 amino group was acetyl protected (**11****), and the 2′ hydroxyl group protected with tris(2-acetoxyethoxy)orthoformate in the presence of 4-*tert*-butyldimethylsiloxy-3-penten-2-one in CH_2Cl_2 under reflux to give **12****. The 5′-3′ silyl protecting group was removed by fluoride (**13****), and the 5′ hydroxyl group was again protected by benzhydroxybis(trimethylsiloxy)silyl chloride (BzhCl) and diisopropylamine (**14****). Finally the 3′ hydroxyl group was derivatized with methyl tetraisopropyl phosphorodiamidite and 1*H*-tetrazole to yield target phosphoramidite (**8****). Coupling of **8**** to **7*** and deprotection was performed by standard



Scheme 2. Synthesis of Cm^6A_NPhe-NH_2 (**1**, **1***). Reagents and conditions: (a) puromycin aminonucleoside, EDCI, *N*-hydroxysuccinimide, DMF, 0 °C to room temperature, 74% (**4**), 77% (**4***); (b) DMTrCl, triethylamine, pyridine, room temperature, 86% (**5**), 89% (**5***); (c) succinic anhydride, DMAP, pyridine, room temperature, 65% (**6**), 60% (**6***); (d) LCAA-polystyrene, EDCI, DMAP, triethylamine, pyridine, room temperature, 109 μmol/g (**7**), 110 μmol/g (**7***); (e) as described in Ref. 26.



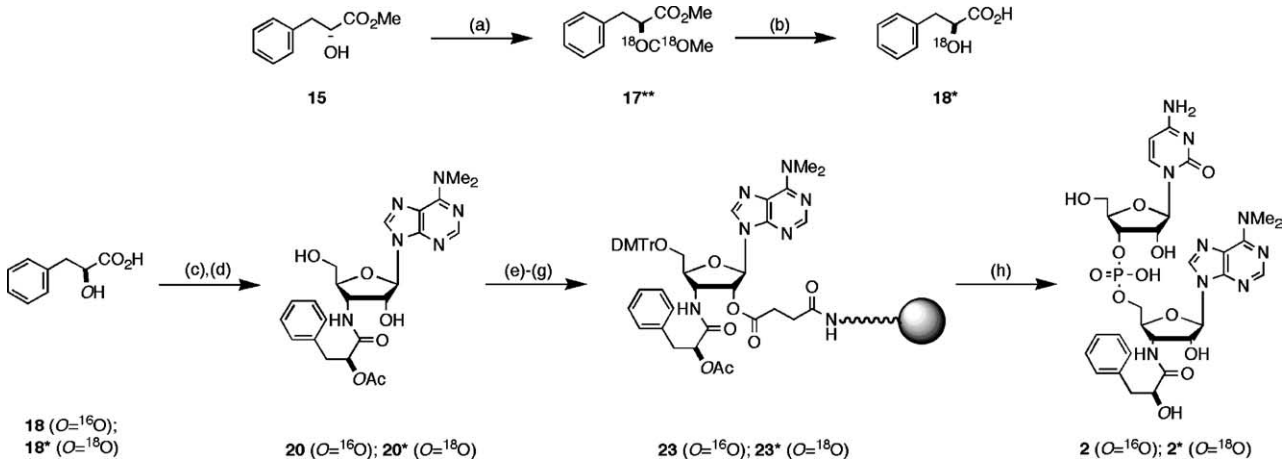
Scheme 3. Synthesis of [3-^{15}N,4-^{15}NH$_2$]Cm^{6}A$_N$Phe-^{15}NH$_2$ (**1*****). Reagents and conditions: (a) TIPDSCl$_2$, pyridine, 0 °C to room temperature, 78%; (b) acetic anhydride, DMF, room temperature, 100%; (c) tris(2-acetoxyethoxy)orthoformate, pyridnium *p*-toluenesulfonate, 4-*tert*-butyldimethylsiloxy-3-penten-2-one, CH_2Cl_2, reflux, 85%; (d) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 48% HF aq, acetonitrile, room temperature, 99%; (e) BzhCl, diisopropylamine, CH_2Cl_2, 0 °C, 96%; (f) methyl tetraisopropyl phosphorodiamidite, 1*H*-tetrazole, CH_2Cl_2, 0 °C, 89%; (g) as described in Ref. 26.

methods of solid phase oligoribonucleotide synthesis.[26] Purification by reverse-phase HPLC yielded [3-^{15}N,4-^{15}NH$_2$]-Cm^{6}A$_N$Phe-^{15}NH$_2$ (**1*****), which has three ^{15}N labels.

2.3. Synthesis of Cm^{6}A$_N$Phe-OH

The A-site substrate hydroxy-puromycin (Pmn-OH) has proven useful for investigating the peptidyl transferase reaction, because it is a substrate that does not have a neutral pK_a.[5] Pmn-OH participates in a transesterification reaction in which the peptide in the P-site is transferred to the A-site substrate via a new ester linkage. Pmn-OH has been used to deconvolute the contribution of the subtrate pK_a from that of the ribosome.[3]

In order to explore kinetic isotope effects on this transesterification reaction we set out to prepare the CPmn-OH derivatives (**2** and **2***) with ^{18}O substitution at the nucleophilic hydroxyl. Preparation of Cm^{6}A$_N$Phe-OH followed a synthetic scheme analogous to that described above (Scheme 4). The 2 hydroxyl group of D-methyl 2-hydroxy-3-phenylpropionate[30] (**15**) was converted to the triflate by trifluoromethanesulfonic anhydride in the



Scheme 4. Synthesis of Cm^{6}A$_N$Phe-OH (**2** and **2***); Reagents and conditions: (a) trifluoromethanesulfonic anhydride, pyridine, CH_2Cl_2, 0 °C to room temperature, then acetic $^{18}O_2$-acid, K_2CO_3, acetonitrile, room temperature, 83%; (b) 5 N KOH aq, MeOH, room temperature, 54%; (c) acetic anhydride, pyridine, room temperature, 100% (**19**), 95% (**19***); (d) puromycin aminonucleoside, EDCI, *N*-hydroxysuccinimide, DMF, 0 °C to room temperature, 73% (**20**), 71% (**20***); (e) DMTrCl, triethylamine, pyridine, room temperature, 89% (**21**), 89% (**21***); (f) succinic anhydride, DMAP, pyridine, room temperature, 57% (**22**), 62% (**22***); (g) LCAA-polystyrene, EDCI, DMAP, triethylamine, pyridine, room temperature, 70 μmol/g (**23**), 103 μmol/g (**23***); (h) as described in Ref. 26.

presence of pyridine and CH_2Cl_2.[31] The crude intermediate (**16**) was directly transformed to the ^{18}O-labeled acetoxy compound (**17****) with inverted stereochemistry using acetic $^{18}O_2$-acid and K_2CO_3 in acetonitrile. The L-[2-^{18}OH]-3-phenyllactic acid (**18***) was obtained by alkaline hydrolysis. L-3-Phenyllactic acid (**18** and **18***) was converted to acetate by acetic anhydride in pyridine to give **19** and **19***, respectively. As above, the 3′-amino group of puromycin aminonucleoside was selectively derivatized with **19** or **19*** using EDCI, the 5′ and 2′ hydroxyl groups protected with DMTrCl and succinic anhydride, respectively, and the resulting compounds attached to solid support. Solid-phase coupling of the cytidine phosphoramidite (**8**), followed by deprotection and HPLC purification yielded substrates, **2** and **2***. Unlike the ^{15}N containing compound **1***, the difference of two mass units between ^{16}O and ^{18}O is sufficient for kinetic isotope effect measurements by whole molecule mass spectrometry.

2.4. Ribosome 50 S subunit reaction assay

We tested if **1** and **2** serve as acceptors in the ribosomal peptidyl transferase reaction. Each substrate was incubated with 50 S ribosomal subunits in the presence of the P-site substrate CCApcb (Dharmacon Inc.)[21] that had been 5′-^{32}P radiolabeled with the enzyme polynucleotide kinase. Production of the P-site product CCA was monitored by gel electrophoresis (Fig. 2a). A new band of increased mobility increased as a function of time. The aminolysis and alcoholysis reactions proceed at a rate >100-fold and >10-fold above the background rate of hydrolysis, respectively. The A-site product was visualized by a peak of increased retention on the HPLC and the assignment confirmed by mass spectroscopic analysis (Fig. 2b, data shown for **1**). Similar results were obtained for compounds **1***, **1*****. These data indicate that **1**, **1***, **1*****, **2** and **2*** serve as acceptors in the peptidyl transferase reaction. These substrates will make it possible to perform kinetic isotope experiments on the ribosome.

3. Experimental

3.1. General

All reactions were 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. NMR spectra were measured on Brucker Avance

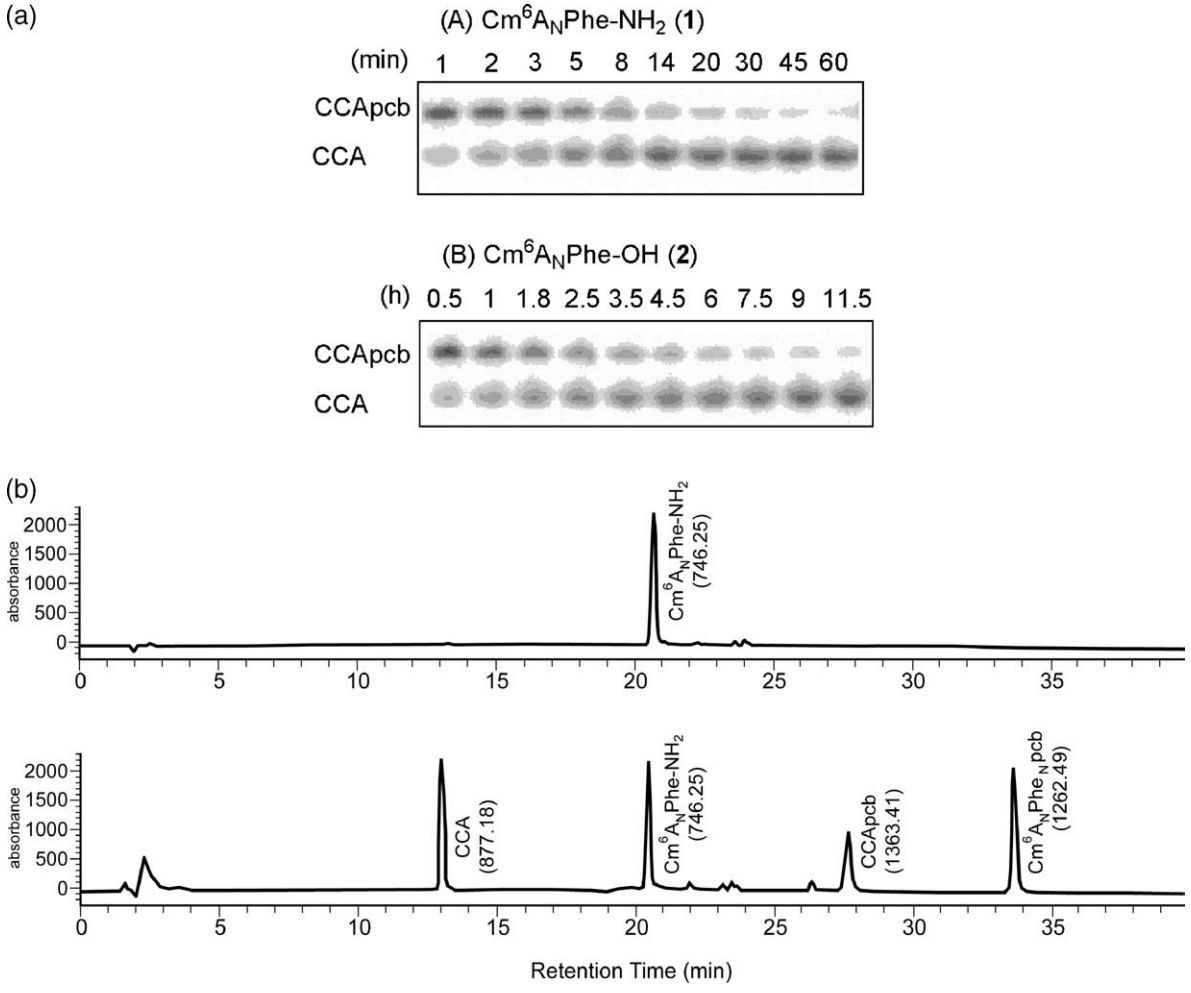


Figure 2. a. Demonstration of the peptidyl transferase reaction of **1** (A) and **2** (B) with ^{32}pCCApcb catalyzed by the 50 S ribosome subunit. The top bands are ^{32}pCCApcb and the bottom bands are the deacylated product ^{32}pCCA. b. HPLC trace of the reaction of **1** with CCApcb at an intermediate time point. Peaks for each of the reactants and nucleotide containing products are visible. Their observed molecule weights are indicated.

DPX-400 and Brucker Avance DPX-500 spectrometers. ^{1}H and ^{13}C NMR chemical shifts were recorded relative to the standard of tetramethylsilane, ^{15}N NMR chemical shifts were recorded relative to nitromethane as an external standard, and ^{31}P NMR chemical shifts were recorded relative to 85% phosphoric acid as an external standard. Mass spectra were collected on Waters Micromass LCT and Waters Micromass ZQ mass spectrometers. Optical rotation was performed on Perkin–Elmer Polarimeter 341.

Amino-derivatized polystyrene beads (Primer Support 30 HL, Amino-derivatized with a loading level of 161 μmol/g) was purchased from Amersham Biosciences. Pyridine was dried using Molecular Sieves. All other chemicals were used as received from commercial suppliers. [3-^{15}N,4-$^{15}NH_2$]cytidine[28] (**9****) and D-methyl 3-phenyllactate[30] (**15**) were synthesized according to the literature procedure.

3.1.1. 3′-Amino-3′-deoxy-3′-[*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-*N*^{6},*N*^{6}-dimethyladenosine (4). To the solution of puromycin aminonucleoside (Sigma) (200.3 mg, 0.681 mmol), *N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanine (**3**, 290.4 mg, 0.750 mmol), and *N*-hydroxysuccinimide (290.4 mg, 0.748 mmol) in DMF (9.0 ml), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (145.0 mg, 0.756 mmol) was added at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, then stirred at room temperature for 24 h. After evaporation, the oily residue was crystallized with ethyl acetate (15 ml). This crude product was washed with ethyl acetate (30 ml), water (15 ml), ethyl acetate (10 ml), successively to give the pure product **4** as a colorless powder (333.9 mg, 74%). ^{1}H NMR (400 MHz, DMSO-d_6): 8.44 (s, 1H, H8), 8.23 (s, 1H, H2), 8.17 (d, 1H, J=7.2 Hz, 3′-NH), 7.87 (d, 2H, J=8.0 Hz, Fmoc-aromatic), 7.64 (m, 3H, Fmoc-aromatic, Phe-NH), 7.42–7.18 (m, 9H, Fmoc-aromatic, Phe-aromatic), 6.10 (d, 1H, J=4.4 Hz, 2′-OH), 6.00 (s, 1H, H1′), 5.19 (t, 1H, J=4.0 Hz, 5′-OH), 4.52–4.47 (m, 2H, H2′, H3′), 4.42–4.37 (m, 1H, Phe-CH), 4.17–4.08 (m, 3H, Fmoc-CH, Fmoc-CH_2), 3.94 (m, 1H, H4′), 3.72–3.65 (m, 1H, H5′), 3.49 (br, 7H, H5′, NCH_3), 3.04–2.97 (m, 1H, Phe-CH_2), 2.85–2.72 (m, 1H, Phe-CH_2); ^{13}C NMR (125.8 MHz, DMSO-d_6/$CDCl_3$=3:1): 171.8, 155.7, 154.3, 151.6, 149.6, 143.7, 140.7, 140.6, 137.9, 137.7, 129.3, 127.9, 127.5, 126.9, 126.9, 126.1, 125.2, 125.1, 119.8, 119.8, 89.4, 83.5, 73.2, 65.7, 60.9, 56.1, 50.4, 46.6, 37.9 (*N*^{6},*N*^{6}-dimethyl carbon was overlapped by DMSO-d_6, confirmed by DEPT.); ESI-MS (ES^+): *m*/*z* calcd for $C_{36}H_{37}N_7O_6$ 663.3, found 664.5 (MH^+); HRMS *m*/*z* calcd for $C_{36}H_{37}N_7O_6$ 664.2883 (MH^+), found 664.2871.

3.1.2. 3′-Amino-3′-deoxy-3′-[[2-^{15}NH]-*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-*N*^{6},*N*^{6}-dimethyladenosine (4*). The product (**4***) was obtained from the coupling of puromycin aminonucleoside (250.7 mg, 0.852 mmol) and [2-^{15}NH]-*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanine (**3***, 398.9 mg, 0.938 mmol) as described in Section 3.1.1. **4*** was obtained as a colorless powder (435.0 mg, 77%). ^{1}H NMR (400 MHz, DMSO-d_6): 8.44 (s, 1H, H8), 8.23 (s, 1H, H2), 8.20 (d, 1H, J=7.6 Hz, 3′-NH), 7.87 (d, 2H, J=7.6 Hz, Fmoc-aromatic), 7.64 (t, 2H, J=7.8 Hz, Fmoc-aromatic), 7.63 (dd, 1H, J_{HN}=92.4 Hz, J_{HH}=8.8 Hz, Phe-NH), 7.42–7.16 (m, 9H, Fmoc-aromatic, Phe-aromatic), 6.10 (d, 1H, J=4.0 Hz, 2′-OH), 5.98 (d, 1H, J=0.8 Hz, H1′), 5.18 (t, 1H, J=4.2 Hz, 5′-OH), 4.50–4.46 (m, 2H, H2′, H3′), 4.42–4.37 (m, 1H, Phe-CH), 4.18–4.08 (m, 3H, Fmoc-CH, Fmoc-CH_2), 3.96 (m, 1H, H4′), 3.67 (m, 1H, H5′), 3.44 (br, 7H, H5′, NCH_3), 3.04–2.97 (m, 1H, Phe-CH_2), 2.84–2.75 (m, 1H, Phe-CH_2); ^{13}C NMR (125.8 MHz, DMSO-d_6/$CDCl_3$=3:1): 171.8, 155.6 (d, J_{CN}=27.5 Hz), 154.3, 151.6, 149.6, 143.7, 140.6, 140.6, 137.9, 137.7, 129.3, 127.9, 127.5, 126.9, 126.9, 126.1, 125.3, 125.1, 119.9, 119.8, 89.4, 83.4, 73.2, 65.7, 60.9, 56.0 (d, J_{CN}=11.3 Hz), 50.3, 46.6, 37.9 (*N*^{6},*N*^{6}-dimethyl carbon was overlapped by DMSO-d_6, confirmed by DEPT.); ^{15}N NMR (50.7 MHz, DMSO-d_6): −292.4 (d, J_{HN}=94.7 Hz); ESI-MS ($ES+$): *m*/*z* calcd for $C_{36}H_{37}N_6{}^{15}NO_6$ 664.3, found 665.3 (MH^+), 687.3 ($M+Na^+$); HRMS *m*/*z* calcd for $C_{36}H_{37}N_6{}^{15}NO_6$ 665.2854 (MH^+), found 665.2865.

3.1.3. 3′-Amino-3′-deoxy-3′-[*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-*O*-(*p*,*p*′-dimethoxytrityl)-*N*^{6},*N*^{6}-dimethyladenosine (5). 4 (175.0 mg, 0.264 mmol) was dried by repeated co-evaporation with pyridine, then dissolved in pyridine (8.0 ml). Triethylamine (0.12 ml, 0.861 mmol) and *p*,*p*′-dimethoxytrityl chloride (DMTrCl) (283.6 mg, 0.795 mmol) were added to the solution, then stirred at room temperature for 3 h. After addition of MeOH (2 ml) to quench the reaction, the mixture was evaporated. Further co-evaporation with toluene twice was followed by column chromatography (gradient from 1% MeOH in CH_2Cl_2 to 2% MeOH in CH_2Cl_2) to give the pure product (**5**) as a white foam (219.7 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.22 (s, 1H, H8), 7.99 (s, 1H, H2), 7.75 (d, 2H, J=7.2 Hz, Fmoc-aromatic), 7.54 (t, 2H, J=7.0 Hz, Fmoc-aromatic), 7.40–7.11 (m, 18H, Fmoc-aromatic, DMTr-aromatic, Phe-aromatic), 6.76 (d, 4H, J=8.4 Hz, DMTr-aromatic), 6.40 (br, 1H, 3′-NH), 5.58 (br, 1H, H1′), 5.45 (br, 1H, J=6.4 Hz, Phe-NH), 4.67 (br, 1H, J=4.4 Hz, H2′), 4.41(br, 1H, H3′), 4.36 (br.d, 3H, J=6.4 Hz, Phe-CH, Fmoc-CH_2), 4.19 (m, 2H, H4′, Fmoc-CH), 3.76 (s, 6H, DMTr-OCH_3), 3.54 (br, 6H, NCH_3), 3.44 (d, 1H, J=8.8 Hz, H5′), 3.32 (dd, 1H, J=10.6, 3.4 Hz, H5′), 3.10 (br, 1H, Phe-CH_2), 2.91 (br, 1H, Phe-CH_2); ^{13}C NMR (125.8 MHz, $CDCl_3$): 171.2, 158.5, 155.9, 154.9, 151.7, 149.1, 144.4, 143.8, 143.6, 141.3, 141.3, 136.4, 136.0, 135.7, 135.6, 130.1, 130.0, 129.2, 128.8, 128.2, 127.8, 127.8, 127.7, 127.1, 127.0, 126.8, 125.1, 120.6, 120.0, 119.9, 113.1, 91.3, 86.5, 84.1, 74.5, 67.1, 63.6, 56.4, 55.2, 52.6, 47.1, 39.2, 38.7 (br); ESI-MS (ES^+): *m*/*z* calcd for $C_{57}H_{55}N_7O_8$ 965.4, found 988.6 ($M+Na^+$); HRMS *m*/*z* calcd for $C_{57}H_{55}N_7O_8$ 966.4190 (MH^+), found 966.4185.

3.1.4. 3′-Amino-3′-deoxy-3′-[[2-^{15}NH]-*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-*O*-(*p*,*p*′-dimethoxytrityl)-*N*^{6},*N*^{6}-dimethyladenosine (5*). The product (**5***) was obtained as a white powder from **4*** (199.4 mg, 0.300 mmol) using the method described in Section 3.1.3 (257.1 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.22 (s, 1H, H8), 7.98 (s, 1H, H2), 7.74 (d, 2H, J=7.6 Hz, Fmoc-aromatic), 7.53 (t, 2H, J=7.6 Hz, Fmoc-aromatic), 7.40–7.10 (m, 18H, Fmoc-aromatic, DMTr-aromatic, Phe-aromatic), 6.76 (d, 4H, J=8.4 Hz, DMTr-aromatic), 6.42 (br, 1H, 3′-NH), 5.59–5.35 (m, 2H, H1′, Phe-NH), 4.66 (t, 1H, J=5.0 Hz, H2′), 4.41 (m., 1H, H3′), 4.35 (br.d, 3H, J=6.0 Hz, Phe-CH, Fmoc-CH_2), 4.18 (m, 2H, H4′, Fmoc-CH),

3.76 (s, 6H, DMTr-OCH_3), 3.54 (br, 6H, NCH_3), 3.43 (dd, 1H, J=10.8, 2.0 Hz, H5′), 3.29 (dd, 1H, J=11.0, 3.4 Hz, H5′), 3.08 (br, 1H, Phe-CH_2), 2.89 (br, 1H, Phe-CH_2); ^{13}C NMR (125.8 MHz, $CDCl_3$): 171.2, 158.5, 154.9, 151.7, 149.2, 144.4, 143.7 (d, J_{CN}=13.1 Hz), 141.3, 136.4, 136.0, 135.7, 135.6, 130.1, 129.2, 128.8, 128.2, 127.8, 127.7, 127.1, 126.8, 125.1, 120.6, 120.0, 120.0, 113.1, 91.3, 86.5, 84.2, 74.5, 67.2, 63.7, 56.4 (d, J_{CN}=12.7 Hz), 55.2, 52.7, 47.1, 39.2, 38.7 (br); ^{15}N NMR (50.7 MHz, $CDCl_3$): −294.1 (d, J_{HN}=91.6 Hz); ESI-MS (ES^+): m/z calcd for $C_{57}H_{55}N_6{}^{15}NO_8$ 966.4, found 989.5 ($M+Na^+$); HRMS m/z calcd for $C_{57}H_{55}N_6{}^{15}NO_8$ 967.4160 (MH^+), found 967.4136.

3.1.5. 3′-Amino-3′-deoxy-3′-[*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-succinate (6). **5** (167.7 mg, 0.173 mmol) was dried by repeated co-evaporation with pyridine and dissolved in pyridine (1.4 ml). Succinic anhydride (52.1 mg, 0.521 mmol) and 4-(dimethylamino)pyridine (DMAP) (11.0 mg, 0.090 mmol) was added to the solution and stirred at room temperature for 24 h. The mixture was evaporated, followed by addition of 0.1 M $NaHCO_3$ aq (25 ml) and extraction with CH_2Cl_2 (25 ml×7). The combined organic phase was evaporated. Further co-evaporation with toluene twice was followed by column chromatography (gradient from 2% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) to give the pure product (**6**) as a white foam (120.7 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.30 (s, 1H, H8), 7.94 (s, 1H, H2), 7.75 (d, 2H, J=7.6 Hz, Fmoc-aromatic), 7.50 (t, 2H, J=7.8 Hz, Fmoc-aromatic), 7.45–7.00 (m, 18H, Fmoc-aromatic, DMTr-aromatic, Phe-aromatic), 6.80 (d, 4H, J=7.6 Hz, DMTr-aromatic), 6.46 (d, 1H, J=9.2 Hz, 3′-NH), 6.10 (d, 1H, J=1.2 Hz, H1′), 5.79 (dd, 1H, J=5.4, 1.8 Hz, H2′), 5.72 (d, 1H, J=9.6 Hz, Phe-NH), 5.19 (m, 1H, H3′), 4.90 (m, 1H, Phe-CH), 4.33–4.20 (m, 2H, Fmoc-CH_2), 4.11 (t, 1H, J=7.0 Hz, Fmoc-CH), 3.83 (br, 1H, H4′), 3.74 (s, 3H, DMTr-OCH_3), 3.73 (s, 3H, DMTr-OCH_3), 3.51 (br, 6H, NCH_3), 3.40–3.28 (m, 2H, H5′), 2.85–2.58 (m, 6H, Phe-CH_2, succinic ester-CH_2); ^{13}C NMR (125.8 MHz, $CDCl_3$): 175.4 (br), 171.2, 171.0, 158.5, 156.5, 154.9, 152.5, 149.8, 144.4, 143.5, 141.2, 141.2, 136.2, 136.1, 135.6, 130.2, 129.3, 128.5, 128.4, 127.8, 127.8, 127.1, 126.9, 126.9, 125.1, 125.0, 120.4, 120.0, 113.1, 87.6, 86.4, 82.3, 75.7, 67.6, 62.6, 55.2, 49.5, 46.9, 40.2, 38.6 (br), 29.8; ESI-MS (ES^+): m/z calcd for $C_{61}H_{59}N_7O_{11}$ 1,065.4, found 1,066.6 (MH^+); HRMS m/z calcd for $C_{61}H_{59}N_7O_{11}$ 1066.4350 (MH^+), found 1066.4330.

3.1.6. 3′-Amino-3′-deoxy-3′-[[2-^{15}NH]-*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-succinate (6*). The product (**6***) was obtained as a white foam from **5*** (242.2 mg, 0.250 mmol) using the method described in Section 3.1.5 (161.2 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.30 (s, 1H, H8), 7.94 (s, 1H, H2), 7.75 (d, 2H, J=7.6 Hz, Fmoc-aromatic), 7.50 (t, 2H, J=7.6 Hz, Fmoc-aromatic), 7.44–7.01 (m, 18H, Fmoc-aromatic, DMTr-aromatic, Phe-aromatic), 6.80 (d, 4H, J=8.4 Hz, DMTr-aromatic), 6.47 (d, 1H, J=7.2 Hz, 3′-NH), 6.09 (s, 1H, H1′), 5.78 (d, 1H, J=5.6 Hz, H2′), 5.73 (dd, 1H, J_{HN}=91.6 Hz, J_{HH}=8.4 Hz, Phe-NH), 5.18 (m, 1H, H3′), 4.90 (m, 1H, Phe-CH), 4.34–4.20 (m, 2H, Fmoc-CH_2), 4.11 (t, 1H, J=7.2 Hz, Fmoc-CH), 3.83 (br, 1H, H4′), 3.74 (s, 3H, DMTr-OCH_3), 3.73 (s, 3H, DMTr-OCH_3), 3.51 (br, 6H, NCH_3), 3.40–3.29 (m, 2H, H5′), 2.85–2.58 (m, 6H, Phe-CH_2, succinic ester-CH_2); ^{13}C NMR (125.8 MHz, $CDCl_3$): 175.2, 171.3, 170.9, 158.5, 156.5 (d, J_{CN}=27.8 Hz), 154.9, 152.6, 149.8, 144.4, 143.5, 141.2, 141.2, 136.3, 136.1, 135.6, 135.6, 130.2, 129.3, 128.5, 128.4, 127.8, 127.8, 127.2, 126.9, 126.9, 125.1, 125.0, 120.4, 120.0, 113.1, 87.5, 86.5, 82.3, 75.8, 67.5, 62.6, 55.1 (2C), 49.4, 46.9, 40.2, 38.6 (br), 29.8, 29.7; ^{15}N NMR (50.7 MHz, $CDCl_3$): −291.5 (d, J_{HN}=91.4 Hz); ESI-MS (ES^+): m/z calcd for $C_{61}H_{59}N_6{}^{15}NO_{11}$ 1,066.4, found 1,067.6 (MH^+); HRMS m/z calcd for $C_{61}H_{59}N_6{}^{15}NO_{11}$ 1067.4320 (MH^+), found 1067.4310.

3.1.7. 3′-Amino-3′-deoxy-3′-[*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-(LCAA-polystyrene)succinate (7). Amino-derivatized polystyrene support (850.0 mg) was suspended in pyridine (8.5 ml) with **6** (180.0 mg, 0.169 mmol), DMAP (10.7 mg, 0.0876 mmol), EDCI (327.2 mg, 1.71 mmol), and triethylamine (70 μl, 0.498 mmol). The mixture was rocked gently for 26 h. The support was filtered and washed successively with pyridine (8.5 ml), MeOH (17 ml), and CH_2Cl_2 (25.5 ml). The support was capped to acetylate unreacted amino residues by suspension in 0.5 M acetic anhydride, 0.5 M pyridine, 1 M *N*-methyl imidazole solution in THF (8.5 ml) and rocked for 2.5 h. The support was filtered and successively washed with MeOH (17 ml) and CH_2Cl_2 (25.5 ml) to yield the product (**7**). The nucleoside loading was 109 μmol/g.

3.1.8. 3′-Amino-3′-deoxy-3′-[[2-^{15}NH]-*N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl]-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-(LCAA-polystyrene)succinate (7*). The product (**7***) was obtained from **6*** (180.0 mg, 0.169 mmol) using the method described in Section 3.1.7. The nucleoside loading was 110 μmol/g.

3.1.9. Cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-L-phenylalanyl-N^6,N^6-dimethyladenosine (1). The coupling of 4-acetyl-5′-*O*-[benzhydroxybis(trimethylsiloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]cytidine-3′-(methyl-*N*,*N*-diisopropyl)phosphoramidite (**8**) to 5′ hydroxyl group of **7** and successive deprotection was performed as described previously.[26] After lyophilization, the deprotected product was purified over a C-18 column with 0.1 M triethylammonium acetate buffer (pH 6.5) and acetonitrile (from 10:0 to 6:4). Lyophilization resulted in the purified product **1**. ^{1}H NMR (500 MHz, D_2O): 8.19 (s, 1H, H8-puromycin), 8.04 (s, 1H, H2-puromycin), 7.54 (d, 1H, J=7.5 Hz, H6-cytosine), 7.35–7.15 (m, 5H, Phe-aromatic), 5.93 (br, 1H, H1′-puromycin), 5.60 (d, 1H, J=7.5 Hz, H5-cytosine), 5.42 (br, 1H, H1′-cytosine), 4.32 (m, 1H, Phe-CH), 4.22 (dd, 1H, J=13.0, 8.1 Hz, H5′-cytosine), 4.09 (m, 1H), 4.03 (m, 2H), 3.85 (m, 1H), 3.71 (dd, 1H, J=12.8, 2.1 Hz, H5′-puromycin), 3.63 (dd, 1H, J=13.2, 4.2 Hz, H5′-puromycin), 3.61 (m, 1H), 3.25 (br, 6H, NCH_3), 3.12–2.93 (m, 4H); ESI-MS (ES^+): m/z calcd for $C_{30}H_{39}N_{10}O_{11}P$ 746.3, found 747.0 (MH^+); HRMS m/z calcd for $C_{30}H_{39}N_{10}O_{11}P$ 769.2437 ($M+Na^+$), found 769.2433.

3.1.10. Cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-([2-$^{15}NH_2$]-L-phenylalanyl)-N^6,N^6-dimethyladenosine (1*). The product (**1***) was obtained from **7*** using the method described in Section 3.1.9; ESI-MS (ES^+): *m/z* calcd for $C_{30}H_{39}N_9{}^{15}NO_{11}P$ 747.3, found 748.0 (MH^+); HRMS *m/z* calcd for $C_{30}H_{39}N_9{}^{15}NO_{11}P$ 748.2586 (MH^+), found 748.2585.

3.1.11. [3-^{15}N,4-$^{15}NH_2$]-3′,5′-*O*-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)cytidine (10).** [3-^{15}N,4-$^{15}NH_2$]-cytidine[28] (**9****, 1.63 g, 6.65 mmol) was dried by co-evaporation twice with pyridine (25 ml) and then dissolved in pyridine (55 ml). 1,3-Dichloro-1,1,3,3-tetraisopropyl-disiloxane (2.34 ml, 7.31 mmol) was added to the mixture dropwise at 0 °C over 1 min. The reaction mixture was stirred for 1 h at 0 °C, then stirred at room temperature for 13 h. After evaporation, addition of water (100 ml) was followed by extraction using CH_2Cl_2 (100 ml×3). The organic phases were combined, dried over $MgSO_4$, then evaporated followed by co-evaporation twice with toluene (40 ml). The pure product **10**** was obtained by column chromatography (gradient from 4% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) as a white solid (2.53 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$=3:1): 7.96 (d, 1H, *J*=7.2 Hz, H6), 5.81 (d, 1H, *J*=7.2 Hz, H5), 5.68 (s, 1H, H1′), 4.29–4.17 (m, 3H, H3′, H4′, H5′), 4.09 (d, 1H, *J*=3.6 Hz, H2′), 4.02 (dd, 1H, *J*=13.2, 2.0 Hz, H5′), 1.13–0.96 (m, 28H, 4×$CH(CH_3)_2$); ^{13}C NMR (125.8 MHz, $CDCl_3/CD_3OD$=3:1): 166.3 (dd, J_{CN}=21.4, 4.2 Hz), 156.5 (d, J_{CN}=5.0 Hz), 141.0, 94.7, 91.8, 81.8, 75.3, 68.4, 60.2, 17.6, 17.6, 17.4, 17.4, 17.1, 17.1, 17.0, 16.9, 13.7, 13.3, 13.2, 12.7; ^{15}N NMR (50.7 MHz, $CDCl_3/CD_3OD$=3:1): −176.9 (d, J_{HN}=4.5 Hz), −292.5 (br); ESI-MS (ES^+): *m/z* calcd for $C_{21}H_{39}N^{15}N_2O_6Si_2$ 487.2, found 488.5 (MH^+); HRMS *m/z* calcd for $C_{21}H_{39}N^{15}N_2O_6Si_2$ 488.2396 (MH^+), found 488.2414.

3.1.12. [3-^{15}N,4-$^{15}NH_2$]-4-Acetyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)cytidine (11).** To a mixture of **10**** (2.47 g, 5.06 mmol) and DMF (50 ml) was added acetic anhydride (2.39 ml, 25.3 mmol). The reaction mixture became clear soon after acetic anhydride addition. The solution was stirred for additional 6 h, evaporated, and co-evaporated twice with MeOH (30 ml). The pure product **11**** was obtained by column chromatography (3% MeOH in CH_2Cl_2) as a white foamy powder (2.72 g, 100%). ^{1}H NMR (400 MHz, $CDCl_3$): 10.11 (d, 1H, J_{HN}=90.4 Hz, NH), 8.21 (d, 1H, *J*=7.2 Hz, H6), 7.44 (d, 1H, *J*=7.2 Hz, H5), 5.82 (s, 1H, H1′), 4.29–4.20 (m, 4H, H2′, H3′, H4′, H5′), 4.01 (dd, 1H, *J*=13.6, 2.8 Hz, H5′), 2.30 (d, 3H, J_{HN}=1.6 Hz, N4-$COCH_3$), 1.11–0.90 (m, 28H, 4×$CH(CH_3)_2$); ^{13}C NMR (125.8 MHz, $CDCl_3$): 171.2 (d, J_{CN}=11.2 Hz), 163.2 (dd, J_{CN}=18.1, 6.9 Hz), 155.0 (dd, J_{CN}=6.9, 4.8 Hz), 144.4, 96.6, 91.5, 82.0, 75.2, 68.6, 60.0, 24.9 (d, J_{CN}=9.1 Hz), 17.5, 17.4, 17.3, 17.3, 17.0, 17.0, 16.9, 16.8, 13.4, 13.0, 12.9, 12.5; ^{15}N NMR (50.7 MHz, $CDCl_3$): −152.0, −233.1 (d, J_{HN}=86.0 Hz); ESI-MS (ES^+): *m/z* calcd for $C_{23}H_{41}N^{15}N_2O_7Si_2$ 529.2, found 530.5 (MH^+); HRMS *m/z* calcd for $C_{23}H_{41}N^{15}N_2O_7Si_2$ 530.2502 (MH^+), found 530.2511.

3.1.13. [3-^{15}N,4-$^{15}NH_2$]-4-Acetyl-2′-*O*-[bis(2-acetoxyethoxy)methyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)cytidine (12).** To a stirred solution of **11**** (2.65 g, 5.00 mmol) in CH_2Cl_2 were added tris(2-acetoxyethoxy)orthoformate tris (ACE)orthoformate (4.51 g, 14.0 mmol), pyridinium *p*-toluenesulfonate (251 mg, 1.00 mmol) and 4-*tert*-butyldimethylsiloxy)-3-penten-2-one (2.13 ml, 9.01 mmol). The reaction mixture was refluxed under Ar atmosphere for 10 h, cooled to the room temperature, and quenched by the addition of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.38 ml, 2.50 mmol). The mixture was subjected directly to a chromatography column (gradient from 50% ethyl acetate in *n*-hexane to 100% ethyl acetate) to afford the pure product **12**** as a white foamy powder (3.19 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): 9.94 (d, 1H, J_{HN}=89.6 Hz, NH), 8.30 (d, 1H, *J*=7.2 Hz, H6), 7.44 (d, 1H, *J*=7.2 Hz, H5), 5.85 (s, 1H, H1′), 5.84 (s, 1H, ACE-CH), 4.32–4.17 (m, 8H, 2×ACE-CH_2, H2′, H3′, H4′, H5′), 4.02–3.84 (m, 5H, 2×ACE-CH_2, H5′), 2.28 (d, 3H, J_{HN}=1.2 Hz, N4-$COCH_3$), 2.07 (s, 3H, ACE-$COCH_3$), 2.06 (s, 3H, ACE-$COCH_3$), 1.11–0.91 (m, 28H, 4×$CH(CH_3)_2$); ^{13}C NMR (125.8 MHz, $CDCl_3$): 170.9, 170.9, 170.8 (d, J_{CN}=12.3 Hz), 163.1 (dd, J_{CN}=18.3, 7.1 Hz), 154.7 (dd, J_{CN}=6.5, 5.2 Hz), 144.2, 111.9, 96.4, 89.9, 82.0, 77.3, 67.5, 63.5, 63.3, 61.7, 61.2, 59.3, 24.9 (d, J_{CN}=9.1 Hz), 20.9, 20.8, 17.5, 17.4, 17.3, 17.3, 17.1, 16.9, 16.9, 16.8, 13.4, 13.1, 12.9, 12.6; ^{15}N NMR (50.7 MHz, $CDCl_3$): −151.0 (d, J_{HN}=5.9 Hz), −233.3 (dd, J_{HN}=89.7, 6.1 Hz); ESI-MS (ES^+): *m/z* calcd for $C_{32}H_{55}N^{15}N_2O_{13}Si_2$ 747.3, found 770.5 ($M+Na^+$); HRMS *m/z* calcd for $C_{32}H_{55}N^{15}N_2O_{13}Si_2$ 770.3112 ($M+Na^+$), found 770.3100.

3.1.14. [3-^{15}N,4-$^{15}NH_2$]-4-Acetyl-2′-*O*-[bis(2-acetoxyethoxy)methyl]cytidine (13).** To the mixture of acetonitrile (42 ml) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (3.13 ml, 4.14 mmol) 48% HF aq (0.53 ml, 14.6 mmol) was added dropwise over 1 min at 0 °C. **12**** (3.10 g, 4.14 mmol) was added to the solution, then stirred at room temperature for 4 h. The reaction mixture was evaporated. Pure product **13**** was obtained by column chromatography (gradient from 5% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) as a white foamy powder (2.08 g, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): 9.67 (d, 1H, J_{HN}=89.6 Hz, NH), 8.30 (d, 1H, *J*=7.6 Hz, H6), 7.43 (d, 1H, *J*=7.2 Hz, H5), 5.80 (d, 1H, *J*=2.4 Hz, H1′), 5.62 (s, 1H, ACE-CH), 4.63 (dd, 1H, *J*=5.4, 3.0 Hz, H2′), 4.39 (q, 1H, *J*=5.7 Hz, H3′), 4.26–4.02 (m, 7H, 2×ACE-CH_2, H4′, H5′, 5′-OH), 3.89–3.78 (m, 5H, 2×ACE-CH_2, H5′), 3.54 (d, 1H, *J*=6.0 Hz, 3′-OH), 2.26 (d, 3H, J_{HN}=0.8 Hz, N4-$COCH_3$), 2.07 (s, 3H, ACE-$COCH_3$), 2.06 (s, 3H, ACE-$COCH_3$); ^{13}C NMR (100.6 MHz, $CDCl_3$): 171.1 (2C), 171.0 (d, J_{CN}=13.3 Hz), 162.9 (dd, J_{CN}=18.4, 6.9 Hz), 155.5 (dd, J_{CN}=5.1, 4.5 Hz), 146.6, 112.7, 96.9, 92.1, 85.1, 76.8, 68.4, 63.1, 63.0, 62.9, 62.8, 60.6, 24.9 (d, J_{CN}=9.2 Hz), 20.9, 20.9; ^{15}N NMR (50.7 MHz, $CDCl_3$): −149.5 (d, J_{HN}=4.7 Hz), −233.2 (dd, J_{HN}=90.2, 6.6 Hz); ESI-MS (ES^+): *m/z* calcd for $C_{20}H_{29}N^{15}N_2O_{12}$ 505.2, found 506.4 (MH^+); HRMS *m/z* calcd for $C_{20}H_{29}N^{15}N_2O_{12}$ 506.1770 (MH^+), found 506.1793.

3.1.15. [3-^{15}N,4-$^{15}NH_2$]-4-Acetyl-5′-*O*-[benzhydroxybis(trimethylsiloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]cytidine (14).** A solution of **13**** (2.02 g, 4.00 mmol) and diisopropylamine (0.56 ml, 4.00 mmol)

in CH_2Cl_2 (22 ml) was cooled to 0 °C. Benzhydroxybis(trimethylsiloxy)silyl chloride (BzhCl) (3.47 g, 8.17 mmol) was dissolved in CH_2Cl_2 (13.5 ml), then diisopropylamine (0.56 ml, 4.00 mmol) was added dropwise to this mixture at 0 °C. This mixture was added dropwise to the previous solution over 2 h at 0 °C. Upon completion, the addition of 5% $NaHCO_3$ aq (50 ml) quenched the reaction. The mixture was separated, then the aqueous phase was extracted with CH_2Cl_2 (30 ml×3). The organic layers were combined, washed with brine, dried over $MgSO_4$, and evaporated. The pure product **14**** was obtained by column chromatography (gradient from 50% ethyl acetate in *n*-hexane to 10% MeOH in CH_2Cl_2) as a colorless oil (3.43 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.98 (d, 1H, J_{HN}=89.6 Hz, NH), 8.33 (d, 1H, J=7.6 Hz, H6), 7.36–7.25 (m, 9H, H5, phenyl), 7.22–7.17 (m, 2H, phenyl), 5.94 (s, 1H, Bzh-CH), 5.93 (s, 1H, H1′), 5.71 (s, 1H, ACE-CH), 4.28–4.21 (m, 5H, 2×ACE-CH_2, H2′), 4.11–3.98 (m, 3H, H3′, H4′, H5′), 3.93–3.83(m, 5H, 2×ACE-CH_2, H5′), 2.85 (d, 1H, J=8.0 Hz, 3′-OH), 2.25 (d, 3H, J_{HN}=1.6 Hz, N4-$COCH_3$), 2.08 (s, 3H, ACE-$COCH_3$), 2.05 (s, 3H, ACE-$COCH_3$), 0.09 (s, 9H, $Si(CH_3)_3$), 0.08 (s, 9H, $Si(CH_3)_3$); ^{13}C NMR (125.8 MHz, $CDCl_3$): 170.9, 170.9, 169.9 (d, J_{CN}=11.2 Hz), 162.5 (dd, J_{CN}=19.1, 7.5 Hz), 155.0 (dd, J_{CN}=6.9, 5.3 Hz), 144.7, 143.9 (d, J_{CN}=5.4 Hz), 128.3, 127.3, 126.4, 126.3, 113.0, 96.2, 89.6, 83.9, 78.6, 67.1, 63.1, 63.1, 63.0, 60.5, 25.0 (d, J_{CN}=9.4 Hz), 20.9, 20.8, 1.5 (6C); ^{15}N NMR (50.7 MHz, $CDCl_3$): −149.8 (d, J_{HN}=6.9 Hz), −233.8 (dd, J_{HN}=88.6, 7.2 Hz); ESI-MS (ES^+): *m/z* calcd for $C_{39}H_{57}N^{15}N_2O_{15}Si_3$ 893.3, found 894.6 (MH^+); HRMS *m/z* calcd for $C_{39}H_{57}N^{15}N_2O_{15}Si_3$ 916.2936 ($M+Na^+$), found 916.2926.

3.1.16. [3-^{15}N,4-$^{15}NH_2$]-4-Acetyl-5′-*O*-[benzhydroxybis(trimethylsiloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]cytidine-3′-(methyl-*N*,*N*-diisopropyl)phosphoramidite (8).** To a stirred solution of **14**** (3.38 g, 3.78 mmol) in CH_2Cl_2 (25 ml) were added methyl tetraisopropyl phosphorodiamidite (3.05 ml, 10.6 mmol) and 1*H*-tetrazole (291 mg, 4.16 mmol). The resulting solution was stirred for 19 h. The reaction was quenched by the addition of 5% $NaHCO_3$ aq (50 ml). The organic phase was separated, and the aqueous phase was extracted by CH_2Cl_2 (30 ml×4). The organic fractions were combined, dried over $MgSO_4$, and evaporated. The pure product **8**** was obtained by column chromatography (gradient from 30% CH_2Cl_2: 60% *n*-hexane: 10% triethylamine to 50% CH_2Cl_2: 40% *n*-hexane: 10% triethylamine) as a colorless oil (3.55 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): (mixture of diastereomers) 10.05 (d, 1H, J_{HN}=88.8 Hz, NH), 8.37 (d, 0.4H, J=7.6 Hz, H6), 8.35 (d, 0.6H, J=7.6 Hz, H6), 7.38–7.18 (m, 11H, H5, phenyl), 6.02 (s, 0.4H, H1′), 6.00 (s, 0.6H, H1′), 5.97 (s, 0.4H, Bzh-CH), 5.96 (s, 0.6H, Bzh-CH), 5.81 (s, 0.6H, ACE-CH), 5.75 (s, 0.4H, ACE-CH), 4.31–4.16 (m, 8H), 4.11–4.03 (m, 1H), 3.95–3.80 (m, 4H), 3.61–3.53 (m, 2H, $CH(CH_3)_2$), 3.35 (d, 1.6H, J=13.6 Hz, OCH_3), 3.32 (d, 1.4H, J=13.6 Hz, OCH_3), 2.28 (s, 3H, N4-$COCH_3$), 2.05 (s, 1.5H, ACE-$COCH_3$), 2.05 (s, 1.5H, ACE-$COCH_3$), 2.04 (s, 1.5H, ACE-$COCH_3$), 2.04 (s, 1.5H, ACE-$COCH_3$), 1.17–1.14 (m, 12H, $CH(CH_3)_2$), 0.09 (s, 4.5H, $Si(CH_3)_3$), 0.09 (s, 4.5H, $Si(CH_3)_3$), 0.07 (s, 4.5H, $Si(CH_3)_3$), 0.06 (s, 4.5H, $Si(CH_3)_3$); ^{15}N NMR (50.7 MHz, $CDCl_3$): −151.8, −233.2 (d, J_{HN}=88.4 Hz); ^{31}P NMR (161.9 MHz, $CDCl_3$): 151.8, 151.0; ESI-MS (ES^+): *m/z* calcd for $C_{46}H_{73}N_2{}^{15}N_2O_{16}PSi_3$ 1054.4, found 1055.6 (MH^+).

3.1.17. [3-^{15}N, 4-$^{15}NH_2$]Cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-([2-^{15}NH]-phenylalanyl)-N^6,N^6-dimethyladenosine (1*).** The product (**1*****) was obtained from **9*** and **8**** using the method described in Section 3.1.9; ESI-MS (ES^+): *m/z* calcd for $C_{30}H_{39}N_7{}^{15}N_3O_{11}P$ 749.2, found 750.3 (MH^+); HRMS *m/z* calcd for $C_{30}H_{39}N_7{}^{15}N_3O_{11}P$ 750.2526 (MH^+), found 750.2537.

3.1.18. D-Methyl 2-trifluoromethanesulfonyl-3-phenyllactate (16). Trifluoromethanesulfonic anhydride (2.4 ml, 14.3 mmol) was added dropwise over 2 min to a solution of D-methyl 3-phenyllactate[30] (**15**, 500.0 mg, 2.77 mmol) in CH_2Cl_2 (25 ml) and pyridine (1.2 ml) at 0 °C. After addition of CH_2Cl_2 (20 ml), the mixture was stirred at room temperature for 2 h. The mixture was further diluted by addition of CH_2Cl_2 (60 ml), and successively washed with 0.8 N $NaHCO_3$ aq (120 ml), 1 N HCl aq (120 ml), and brine (120 ml). The organic layer was dried over $MgSO_4$, and evaporated to give crude product (**16**) as an oil (0.70 g). ^{1}H NMR (400 MHz, $CDCl_3$): 7.37–7.20 (m, 5H, phenyl), 5.25 (dd, 1H, J=8.8, 4.4 Hz, CH), 3.84 (s, 3H, CH_3), 3.35 (dd, 1H, J=14.8, 4.4 Hz, CH_2), 3.21 (dd, 1H, J=14.8, 8.4 Hz, CH_2).

3.1.19. L-Methyl [2-^{18}O,1′-^{18}O]-2-acetoxy-3-phenyllactate (17).** Crude **16** (0.70 g) was dissolved in an acetic $^{18}O_2$-acid (200 mg, 3.12 mmol) solution in acetonitrile (15 ml). K_2CO_3 (402.6 mg, 2.914 mmol) was added and the mixture was stirred at room temperature for 29 h. CH_2Cl_2 (100 ml) was added and the organic phase was washed successively with 0.8 N $NaHCO_3$ aq (100 ml), 1 N HCl aq (100 ml), and brine (120 ml). The organic layer was dried over $MgSO_4$, and evaporated to give product (**17****) as an oil (521.6 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.33–7.21 (m, 5H, phenyl), 5.22 (dd, 1H, J=8.8, 4.4 Hz, CH), 3.73 (s, 3H, CO_2CH_3), 3.17 (dd, 1H, J=14.2, 4.6 Hz, CH_2), 3.09 (dd, 1H, J=14.0, 8.8 Hz, CH_2), 2.08 (s, 3H, acetoxy-CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{12}H_{14}O_2{}^{18}O_2$ 226.1, found 249.2 ($M+Na^+$).

3.1.20. L-[2-^{18}OH]-3-Phenyllactic acid (18*). 17** (496.1 mg, 2.19 mmol) was dissolved in MeOH (10 ml) and 5 N KOH aq (10 ml), and stirred at room temperature for 5 h. After addition of conc. HCl (10 ml), the mixture was evaporated at 60 °C to give a white semi-solid. The crude product was washed with ethyl acetate (40 ml×4). The combined organic phase was filtered and evaporated. Recrystallization from $CHCl_3$/*n*-hexane gave the product (**18***) as needles (200.6 mg, 54%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.35–7.26 (m, 5H, phenyl), 4.53 (br.s, 1H, CH), 3.23 (d, 1H, J=11.6 Hz, CH_2), 3.01 (dd, 1H, J=13.4, 7.0 Hz, CH_2); ESI-MS (ES^+): *m/z* calcd for $C_9H_{10}O_2{}^{18}O$ 168.1, found 190.9 ($M+Na^+$); $[\alpha]_D^{22}$: −27.8 (*c*0.18, MeOH).

3.1.21. L-2-Acetoxy-3-phenyllactic acid.[32] (19) Acetic anhydride (0.3 ml, 3.15 mmol) was added to a L-3-phenyllactic acid (**18**) (250.4 mg, 1.48 mmol) solution in pyridine (3.0 ml), and the mixture was stirred at room

temperature for 1 day. The reaction was quenched by addition of MeOH (5 ml), and the mixture was evaporated. The resulting oil was dissolved in 0.1 N HCl aq (20 ml), and extracted with CH_2Cl_2 (20 ml×3). The combined organic phase was washed with brine (30 ml), dried over $MgSO_4$, and evaporated to give product (**19**) as a colorless oil (313.2 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.34–7.24 (m, 5H, phenyl), 5.25 (dd, 1H, J=9.0, 4.2 Hz, CH), 3.23 (dd, 1H, J=14.2, 3.8 Hz, CH_2), 3.12 (dd, 1H, J=14.4, 8.8 Hz, CH_2), 2.08 (s, 3H, CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{11}H_{12}O_4$ 208.1, found 231.1 ($M+Na^+$).

3.1.22. L-[2-^{18}O]-Acetoxy-3-phenyllactic acid (19*). The product (**19***) was obtained as a colorless oil from **18*** (154.6 mg, 0.919 mmol) using method described in Section 3.1.21 (184.0 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.34–7.24 (m, 5H, phenyl), 4.53 (dd, 1H, J=9.0, 4.2 Hz, CH), 3.24 (dd, 1H, J=14.4, 4.0 Hz, CH_2), 3.12 (dd, 1H, J=14.2, 8.6 Hz, CH_2), 2.08 (s, 3H, CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{11}H_{12}O_3{}^{18}O$ 210.1, found 232.9 ($M+Na^+$).

3.1.23. 3′-Amino-3′-deoxy-3′-(L-2-acetoxy-3-phenylpropionyl)-*N*^{6},*N*^{6}-dimethyladenosine (20). EDCI (176.0 mg, 0.900 mmol) was added at 0 °C to a solution of puromycin aminonucleoside (240.4 mg, 0.817 mmol), **19** (172.5 mg, 0.828 mmol), and *N*-hydroxysuccinimide (107.1 mg, 0.903 mmol) in DMF (10 ml). The reaction mixture was stirred at 0 °C for 1 h, then stirred at room temperature for 24 h. After evaporation, the oily residue was subjected to column chromatography (gradient from 2% MeOH in CH_2Cl_2 to 4% MeOH in CH_2Cl_2) to give the crude product as a white powder. This material was washed with ethyl acetate (10 ml) to give the pure product **20** (288.9 mg, 73%) as a colorless fine powder. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD=1:1): 8.29 (s, 1H, H8), 8.24 (s, 1H, H2), 7.32–7.21 (m, 5H, phenyl), 5.85 (d, 1H, J=2.8 Hz, H1′), 5.30 (dd, 1H, J=7.4, 5.8 Hz, Phe-CH), 4.49–4.41 (m, 2H, H2′, H3′), 4.05 (dt, 1H, J=7.2, 2.0 Hz, H4′), 3.95 (dd, 1H, J=12.8, 2.0 Hz, H5′), 3.69 (dd, 1H, J=12.8, 2.4 Hz, H5′), 3.53 (br, 6H, NCH_3), 3.36 (m, 1H, 3′-NH), 3.18 (dd, 1H, J=14.2, 5.4 Hz, Phe-CH_2), 3.11 (dd, 1H, J=13.8, 7.4 Hz, Phe-CH_2), 2.12 (s, 3H, acetyl-CH_3); ^{13}C NMR (125.8 MHz, $CDCl_3$/CD_3OD=1:1): 171.4, 171.1, 155.5, 152.2, 149.4, 138.2, 136.2, 129.9, 129.0, 127.5, 121.3, 91.5, 84.6, 75.1, 74.3, 61.8, 50.8, 39.0 (br), 38.3, 20.7; ESI-MS (ES^+): *m/z* calcd for $C_{23}H_{28}N_6O_6$ 484.2, found 485.2 (MH^+), 507.2 ($M+Na^+$); HRMS *m/z* calcd for $C_{23}H_{28}N_6O_6$ 485.2148 (MH^+), found 485.2154.

3.1.24. 3′-Amino-3′-deoxy-3′-(L-[2-^{18}O]-2-acetoxy-3-phenylpropionyl)-*N*^{6},*N*^{6}-dimethyladenosine (20*). The product (**20***) was obtained as a colorless powder from **19*** (173.1 mg, 0.823 mmol) using the method described in Section 3.1.23 (306.1 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD=3:1): 8.28 (s, 1H, H8), 8.24 (s, 1H, H2), 7.32–7.21 (m, 5H, phenyl), 5.85 (d, 1H, J=2.8 Hz, H1′), 5.30 (dd, 1H, J=7.2, 5.6 Hz, Phe-CH), 4.49–4.42 (m, 2H, H2′, H3′), 4.06 (dt, 1H, J=6.9, 2.1 Hz, H4′), 3.95 (dd, 1H, J=13.0, 2.2 Hz, H5′), 3.70 (dd, 1H, J=12.8, 2.4 Hz, H5′), 3.54 (br, 6H, NCH_3), 3.36 (m, 1H, 3′-NH), 3.18 (dd, 1H, J=13.8, 5.8 Hz, Phe-CH_2), 3.11 (dd, 1H, J=14.2, 7.4 Hz, Phe-CH_2), 2.12 (s, 3H, acetyl-CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{23}H_{28}N_6O_5{}^{18}O$ 486.2, found 487.2 (MH^+); HRMS *m/z* calcd for $C_{23}H_{28}N_6O_5{}^{18}O$ 487.2191 (MH^+), found 487.2209.

3.1.25. 3′-Amino-3′-deoxy-3′-(L-2-acetoxy-3-phenylpropionyl)-5′-(*p*,*p*′-dimethoxytrityl)-*N*^{6},*N*^{6}-dimethyladenosine (21). The product (**21**) was obtained as a white foam from **20** (242.0 mg, 0.499 mmol) using the method described in Section 3.1.3 (350.9 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.26 (s, 1H, H8), 7.97 (s, 1H, H2), 7.35–7.14 (m, 14H, DMTr-aromatic, Phe-aromatic), 6.77 (d, 4H, J=9.2 Hz, DMTr-aromatic), 6.65 (d, 1H, J=5.2 Hz, 3′-NH), 6.01 (s, 1H, 2′-OH), 5.77 (d, 1H, J=4.0 Hz, H1′), 5.32 (dd, 1H, J=7.2, 5.6 Hz, Phe-CH), 4.65 (t, 1H, J=5.4 Hz, H2′), 4.44 (dd, 1H, J=11.6, 5.6 Hz, H3′), 4.33 (m, 1H, H4′), 3.77 (s, 6H, DMTr-OCH_3), 3.54 (br, 6H, NCH_3), 3.47 (dd, 1H, J=10.4, 2.4 Hz, H5′), 3.37 (dd, 1H, J=10.8, 3.6 Hz, H5′), 3.17 (dd, 1H, J=14.2, 5.4 Hz, Phe-CH_2), 3.09 (dd, 1H, J=14.2, 7.4 Hz, Phe-CH_2), 2.07 (s, 3H, acetyl-CH_3); ^{13}C NMR (125.8 MHz, $CDCl_3$): 169.7, 169.6, 158.5, 155.0, 151.7, 149.2, 144.4, 136.1, 135.9, 135.7, 135.6, 130.1, 129.5, 128.5, 128.2, 127.8, 127.0, 126.8, 120.7, 113.1, 91.5, 86.5, 84.4, 74.6, 74.4, 63.7, 55.2, 52.6, 38.6 (br), 37.7, 20.8; ESI-MS (ES^+): *m/z* calcd for $C_{44}H_{46}N_6O_8$ 786.4, found 787.5 (MH^+), 809.5 ($M+Na^+$); HRMS *m/z* calcd for $C_{44}H_{46}N_6O_8$ 787.3435 (MH^+), found 787.3447.

3.1.26. 3′-Amino-3′-deoxy-3′-(L-[2-^{18}O]-2-acetoxy-3-phenylpropionyl)-5′-(*p*,*p*′-dimethoxytrityl)-*N*^{6},*N*^{6}-dimethyladenosine (21*). The product (**21***) was obtained as a white foam from **20*** (249.4 mg, 0.513 mmol) using the method described in Section 3.1.3 (359.9 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.27 (s, 1H, H8), 7.96 (s, 1H, H2), 7.32–7.16 (m, 14H, DMTr-aromatic, Phe-aromatic), 6.75 (d, 4H, J=9.2 Hz, DMTr-aromatic), 6.69 (d, 1H, J=4.8 Hz, 3′-NH), 6.02 (s, 1H, 2′-OH), 5.73 (d, 1H, J=4.4 Hz, H1′), 5.34 (dd, 1H, J=7.0, 5.4 Hz, Phe-CH), 4.71 (t, 1H, J=5.6 Hz, H2′), 4.41 (dd, 1H, J=11.4, 5.0 Hz, H3′), 4.37 (m, 1H, H4′), 3.78 (s, 3H, DMTr-OCH_3), 3.77 (s, 3H, DMTr-OCH_3), 3.54 (br, 6H, NCH_3), 3.46 (dd, 1H, J=10.8, 2.8 Hz, H5′), 3.37 (dd, 1H, J=10.6, 3.4 Hz, H5′), 3.18 (dd, 1H, J=14.0, 5.2 Hz, Phe-CH_2), 3.10 (dd, 1H, J=14.2, 7.4 Hz, Phe-CH_2), 2.08 (s, 3H, acetyl-CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{44}H_{46}N_6O_7{}^{18}O$ 788.3, found 811.4 ($M+Na^+$); HRMS *m/z* calcd for $C_{44}H_{46}N_6O_7{}^{18}O$ 789.3487 (MH^+), found 789.3477.

3.1.27. 3′-Amino-3′-deoxy-3′-(L-2-acetoxy-3-phenylpropionyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)-*N*^{6},*N*^{6}-dimethyladenosine 2′-*O*-succinate (22). The product (**22**) was obtained as a white foam from **21** (299.7 mg, 0.380 mmol) using the method described in Section 3.1.5 (192.7 mg, 57%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.29 (s, 1H, H8), 7.94 (s, 1H, H2), 7.32–7.16 (m, 14H, DMTr-aromatic, Phe-aromatic), 6.77 (d, 4H, J=8.8 Hz, DMTr-aromatic), 6.43 (br, 1H, 3′-NH), 6.10 (s, 1H, H1′), 5.61 (d, 1H, J=3.2 Hz, H2′), 5.41 (t, 1H, J=5.2 Hz, Phe-CH), 5.18 (dd, 1H, J=15.0, 8.2 Hz, H3′), 3.91 (br, 1H, H4′), 3.73 (s, 3H, DMTr-OCH_3), 3.72 (s, 3H, DMTr-OCH_3), 3.50 (br, 6H, NCH_3), 3.38–3.31 (m, 2H, H5′), 3.03 (dd, 1H, J=14.0, 7.6 Hz, Phe-CH_2), 2.92 (dd, 1H, J=14.2, 5.4 Hz, Phe-CH_2), 2.65 (br, 2H, succinic ester-CH_2), 2.60 (br, 2H, succinic ester-CH_2), 2.08 (s, 3H, acetyl-CH_3); ^{13}C NMR (100.6 MHz, $CDCl_3$): 170.7, 170.2, 169.1, 158.4, 154.9, 152.3, 149.4, 144.3,

139.4, 136.3, 135.7, 135.6, 135.5, 130.1, 129.7, 129.5, 129.1, 128.5, 128.2, 127.9, 127.8, 127.1, 127.0, 126.8, 120.3, 113.1, 113.0, 87.2, 86.6, 77.2, 75.9, 74.1, 62.5, 55.3, 55.2, 49.6, 38.6 (br), 37.8, 29.9, 29.7 (br), 20.9; ESI-MS (ES^+): *m/z* calcd for $C_{48}H_{50}N_6O_{11}$ 886.4, found 887.6 (MH^+); HRMS *m/z* calcd for $C_{48}H_{50}N_6O_{11}$ 887.3538 (MH^+), found 887.3550.

3.1.28. 3′-Amino-3′-deoxy-3′-(L-[2-^{18}O]-2-acetoxy-3-phenylpropionyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-succinate (22*). The product (**22***) was obtained as a white foam from **21*** (312.5 mg, 0.396 mmol) using the method described in Section 3.1.5 (217.0 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.29 (s, 1H, H8), 7.94 (s, 1H, H2), 7.32–7.16 (m, 14H, DMTr-aromatic, Phe-aromatic), 6.77 (d, 4H, J=8.8 Hz, DMTr-aromatic), 6.43 (br, 1H, 3′-NH), 6.10 (d, 1H, J=2.0 Hz, H1′), 5.62 (d, 1H, J=4.4 Hz, H2′), 5.41 (t, 1H, J=6.4 Hz, Phe-CH), 5.18 (dd, 1H, J=14.2, 8.6 Hz, H3′), 3.91 (br, 1H, H4′), 3.74 (s, 3H, DMTr-OCH_3), 3.73 (s, 3H, DMTr-OCH_3), 3.50 (br, 6H, NCH_3), 3.39–3.32 (m, 2H, H5′), 3.03 (dd, 1H, J=14.4, 7.2 Hz, Phe-CH_2), 2.93 (dd, 1H, J=13.6, 5.2 Hz, Phe-CH_2), 2.65 (br, 2H, succinic ester-CH_2), 2.61 (br, 2H, succinic ester-CH_2), 2.08 (s, 3H, acetyl-CH_3); ESI-MS (ES^+): *m/z* calcd for $C_{48}H_{50}N_6O_{10}{}^{18}O$ 888.4, found 889. 6 (MH+), 911.7 ($M+Na^+$); HRMS *m/z* calcd for $C_{48}H_{50}N_6O_{10}{}^{18}O$ 889.3658 (MH^+), found 889.3656.

3.1.29. 3′-Amino-3′-deoxy-3′-(L-2-acetoxy-3-phenylpropionyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-(LCAA-polystyrene)succinate (23). The product (**23**) was obtained from **22** (180.0 mg, 0.169 mmol) using the solid support derivatization method described in Section 3.1.7. The nucleoside loading was 70 μmol/g.

3.1.30. 3′-Amino-3′-deoxy-3′-(L-[2-^{18}O]-2-acetoxy-3-phenylpropionyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)-N^6,N^6-dimethyladenosine 2′-*O*-(LCAA-polystyrene)succinate (23*). The product (**23***) was obtained from **22*** (180.0 mg, 0.169 mmol) using the solid support derivatization method described in Section 3.1.7. The nucleoside loading was 103 μmol/g.

3.1.31. Cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-(L-2-hydroxy-3-phenylpropionyl)-N^6,N^6-dimethyladenosine (2). The product (**2**) was obtained by coupling the cytidine phosphoramidite (**8**) to **23** using the method described in Section 3.1.9. ESI-MS (ES^+): *m/z* calcd for $C_{30}H_{38}N_9O_{12}P$ 747.2, found 748.3 (MH^+); HRMS *m/z* calcd for $C_{30}H_{38}N_9O_{12}P$ 770.2275 ($M+Na^+$), found 770.2284.

3.1.32. Cytidylyl-(3′-5′)-3′-amino-3′-deoxy-3′-(L-[2-^{18}OH]-2-hydroxy-3-phenylpropionyl)-N^6,N^6-dimethyladenosine (2*). The product (**2***) was obtained by coupling the cytidine phosphoramidite (**8**) to **23*** using the method described in Section 3.1.9. ESI-MS (ES^+): *m/z* calcd for $C_{30}H_{38}N_9O_{11}{}^{18}OP$ 749.2, found 750.4 (MH^+); HRMS *m/z* calcd for $C_{30}H_{38}N_9O_{11}{}^{18}OP$ 750.2498 (MH^+), found 750.2509.

3.2. 50 S subunit reaction assay

Large ribosomal subunits were isolated from *E. coli*MRE600 cells by a procedure modified from the literature.[33] CCApcb was 5′-^{32}P end labeled by phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction of 1.0 mM **1** or **2** with ^{32}pCCApcb and 9 μM 50 S ribosome was performed in 7 mM Mg^{2+}, 7 mM K^+, 166 mM $NH_4{}^+$, 0.1 mM EDTA, 0.2 mM DTT, 25 mM MES, 25 mM MOPS, 50 mM Tris-HCl buffer (pH 7.0) at 25 °C. The ribosomes were incubated for 2 min at 37 °C before beginning the reaction. 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).

For HPLC analysis, 20 nmol of A-site substrate was added to 30 nmol of CCApcb (Fig. 2b). The buffer conditions were the same as above. 4.5 μM 50 S ribosomal subunits were added to begin the reaction. Once the reaction had proceeded to greater than 50% reacted, it was quenched by addition of ~50 mM EDTA. The reaction was purified on an Agilent Technologies XBD-C_{18} reverse phase HPLC column using 10 mM triethylamine acetate (TEAA), pH 6.5 as the mobile phase. Substrates and products were separated by HPLC using a gradient of 0–30% acetonitrile over 30 min followed by an isocratic run for 10 min at 30% acetonitrile. Mass spectrometry was used to determine the identity of each HPLC peak. All HPLC fractions were frozen, lyophilized to dryness, and desalted by multiple rounds of lyophilization. The samples were analyzed on an Applied Biosystems PE SciEX API 3000 triple quadrupole mass spectrometer with an electrospray ion source (ESI-MS). For ESI-MS analysis, samples were resuspended in 1:1 10 mM TEAA:acetonitrile and injected by direct infusion at a rate of 10 μl/min. The same procedure was used for reactions with **1**, **1***, and **1***** each serving as the A-site substrate. As expected, the ^{15}N substitutions did not effect the HPLC retention time. The exact mass for each of the A-site substrates **1**, **1***, and **1***** and their products $Cm^6A_NPhe_N$-pcb, $Cm^6A_NPhe_{15N}$pcb, and [3-^{15}N,4-$^{15}NH_2$]-$Cm^6A_NPhe_{15N}$pcb are 746.25, 747.25, 749.25, 1262.49, 1263.49, and 1265.49, respectively.

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