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Synthesis of Isotopically Labeled P-Site Substrates for the Ribosomal Peptidyl Transferase Reaction

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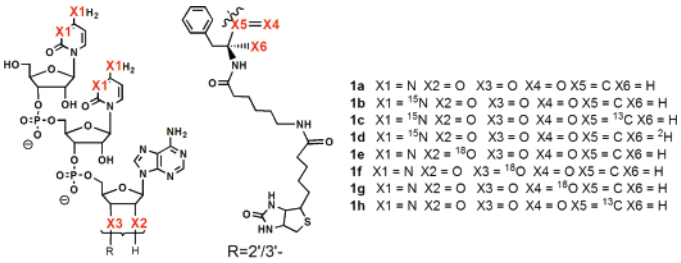

Isotopomers of the ribosomal P-site substrate, the trinucleotide peptide conjugate CCA-pcb (Zhong, M.; Strobel, S. A. *Org. Lett.* **2006**, *8*, 55−58), have been designed and synthesized in 26−35 steps. These include individual isotopic substitution at the α-hydrogen, carbonyl carbon, and carbonyl oxygen of the amino acid, the O2′ and O3′ of the adenosine, and a remote label in the N3 and N4 of both cytidines. These isotopomers were synthesized by coupling cytidylyl-(3′,5′)-cytidine phosphoramidite isotopomers as the common synthetic intermediates, with isotopically substituted A-Phe-cap-biotin (A-pcb). The isotopic enrichment is higher than 99% for 1-^{13}C (Phe), 2-^{2}H (Phe), and 3,4-$^{15}N_2$ (cytidine), 93% for 2′/3′-^{18}O (adenosine), and 64% for 1-^{18}O (Phe). A new synthesis of highly enriched [1-$^{18}O_2$]phenylalanine has been developed. The synthesis of [3′-^{18}O]adenosine was improved by Lewis acid aided regioselective ring opening of the epoxide and by an economical S_N2-S_N2 method with high isotopic enrichment (93%). Such substrates are valuable for studies of the ribosomal peptidyl transferase reaction by complete kinetic isotope effect analysis and of other biological processes catalyzed by nucleic acid related enzymes, including polymerases, reverse transcriptases, ligases, nucleases, and ribozymes.

Introduction

The catalytic mechanism of peptide bond formation by the ribosome is an area of ongoing research. Several aspects of the reaction mechanism are well-established.[2] The reaction uses two substrate tRNAs, an aminoacyl-tRNA that binds to the A site and a peptidyl tRNA that binds to the P site. The reaction involves aminolysis of the P-site ester by the A-site α-amino group. Relative to the rate in solution, the peptidyl transferase center of the ribosome provides a rate enhancement of approximately 10^7-fold, a contribution that has been largely attributed to entropy.[3] The nascent peptide in the P site and the amino acid in the A site are both linked via an ester to the 3′-O of the terminal nucleoside of the tRNA, A76. The 2′-OH vicinal to the P-site ester is essential for the reaction. Deletion or alteration of this functional group results in a complete loss of peptidyl transferase activity (>10^6-fold loss of activity).[4] This contribution is significantly greater than that made by any of the rRNA nucleotides within the active site, though the 2′-OH of A2451 also makes an important contribution.[5] The ribosome aligns the two substrates such that reaction proceeds through a chiral transition state with a *S* stereochemistry.[6] This configuration places the critical A76 2′-OH between the α-amino

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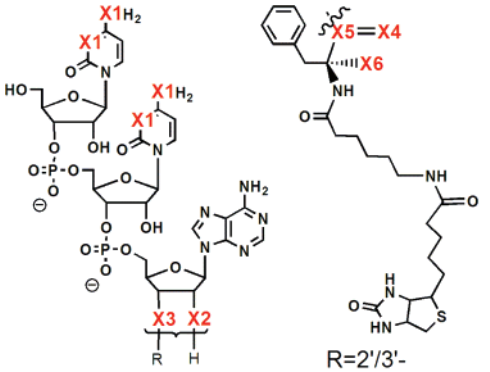
nucleophile and the 3′-O leaving group. Possible roles for this hydroxyl in proton transfer from the α-amine to the 3′-O have been proposed.[7c,i,k]

Although the essential contributors to the reaction have been largely defined, both biochemically and structurally, several substantially different mechanisms have been drawn to explain these observations.[7,8] One model assumes a mechanism equivalent to that observed in solution in which the reaction proceeds through a zwitterionic $T^{\pm}$ intermediate followed by deprotonation to a T^{-} intermediate that is then resolved into products.[7a,b] It is suggested that the A76 2′-OH could accept the proton from the α-amino group. A second alternative involves resolution of the $T^{\pm}$ intermediate directly to products by the simultaneous transfer of a proton from the amine, through the A76 2′-OH to the 3′-O leaving group as the C−O bond is broken.[7c,i] A third suggestion, which has appeared in several reviews, involves the simultaneous N−C bond formation with C−O bond cleavage coupled to proton transfer through the 2′-OH.[7d−g] This mechanism does not involve a tetrahedral intermediate. Other mechanistic proposals have also been made that invoke additional intermediates and transition states.[7h,j]

In order to understand the role the ribosome plays in promoting the peptidyl transferase reaction, it is essential to characterize the reaction mechanism. The most powerful approach for transition-state characterization is kinetic isotope effect (KIE) analysis in which heavy isotopes are introduced at atoms expected to have altered bonding in the transition state. By measuring and analyzing the small differences in reaction rate that result from isotopic substitution, it is possible to characterize the nature of the chemical transition state.

Toward this goal, we prepared an A-site substrate in which the α-amino group was ^{15}N substituted and used this substrate to measure a KIE of 1.009 for this substitution.[9] This analysis provided evidence that chemistry is at least partially rate limiting in the modified fragment reaction between CCA-pcb and CCPmn catalyzed by 50S ribosomes. The magnitude of the ^{15}N KIE suggests either that the transition state is early, with little N−C bond order, or that there is significant deprotonation of the amine in the transition state. It is impossible to distinguish these possibilities or to speculate on the nature of other changes in the reaction coordinate based upon this single measurement. Our goal is to expand the KIE measurements to include substitutions at other atoms involved in the aminolysis reaction, all of which are located within the P-site substrate. This necessitates that various isotopic substitutions be introduced into the substrate CCA-pcb.



1a X1 = N X2 = O X3 = O X4 = O X5 = C X6 = H
1b X1 = ^{15}N X2 = O X3 = O X4 = O X5 = C X6 = H
1c X1 = ^{15}N X2 = O X3 = O X4 = O X5 = ^{13}C X6 = H
1d X1 = ^{15}N X2 = O X3 = O X4 = O X5 = C X6 = ^{2}H
1e X1 = N X2 = ^{18}O X3 = O X4 = O X5 = C X6 = H
1f X1 = N X2 = O X3 = ^{18}O X4 = O X5 = C X6 = H
1g X1 = N X2 = O X3 = O X4 = ^{18}O X5 = C X6 = H
1h X1 = N X2 = O X3 = O X4 = O X5 = ^{13}C X6 = H

FIGURE 1. Isotopomers of the P-site substrate for kinetic isotope effect analysis of ribosomal peptide bond formation.

The P-site substrate contains the last three nucleotides at the 3′-end of the tRNA (CCA) linked to a peptidyl mimic (pcb) by an ester linkage. Kinetic isotope effects of P-site atoms that are directly or closely involved in the reaction are expected to provide specific information about the reaction mechanism (Figure 1, X1 = N).[10] For example, the carbonyl carbon is directly involved in peptide bond formation, and the ^{13}C isotope effect at this position is always normal (>1.00) in solution aminolysis reactions with a magnitude as large as 1.04 (a 4% KIE). This substitution provides information about the extent of N−C bond formation in the transition state. Deuterium substitution at the adjacent α-H is expected to result in an inverse KIE ($k^D > k^H$), and its magnitude (reported to be between 0 and 11%) will define the orbital hybridization at the reaction center carbon, with large KIEs correlating to sp^3 hybridization of the tetrahedral intermediate and small KIEs to sp^2 hybridization of a concerted mechanism. The primary isotope effect of the carbonyl oxygen should be normal, and its magnitude should also correlate with its bonding characteristics and environment, such as its bond order with the reaction center carbon in the transition state (TS). The kinetic isotope effect of the leaving group ($^{18}k_{lg}$) for the ester linkage can further define the chemical mechanism of this catalyzed reaction. The $^{18}k_{lg}$ for the alkaline hydrolysis of methyl formate, where formation of tetrahedral intermediate is rate limiting, is 0.9%, while the hydrazinolysis reaction has a KIE of 6.2%. This isotope effect provides valuable information about the degree of C−O3′ bond cleavage in the transition state. The value of the isotope effect at the O2′ should correlate with its catalytic role and define the bonding details of this atom as a possible proton shuttle.

In the KIE study of the A-site α-amine, we used a competition method in which the heavy and the light isotopomer were mixed together and the change in substrate ratio was monitored using mass spectroscopy with a remote label. This approach necessitates that isotopic substitutions are also made at a site distant from the reactive groups. For this purpose, we have selected the N3 and N4 nitrogens of the two cytidines for ^{15}N substitution

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(Figure 1). This provides a convenient mass change of 4 Da that is expected to fully separate the light and heavy substrate peaks on the mass spectrometer.

Isotopically labeled nucleosides, nucleotides, and their polymers (RNA and DNA) have potential applications in exploring the mechanistic details of fundamental biological processes such as protein synthesis, DNA and RNA synthesis, mRNA post-transcriptional modifications and processing, and formation of small RNAs, as shown in recent reviews on biological kinetic isotope effects.[11] However, there are few reports of this kind of synthetic efforts, and the probes that are used are usually prepared by enzymatic methods on small scales. We have reported the solid-phase synthesis of CCPmn with ^{15}N substitutions at the α-amine of puromycin and the N3 and N4 of cytidine.[12] However, this synthetic strategy, as well as other routine synthetic methods for oligonucleotides, cannot be applied to the synthesis of CCA-pcb isotopomers for the following reasons. First, the 3′-ester linkage is highly labile and will not survive the coupling reactions and final basic deprotection steps; second, the chirality of the amino acids is sensitive to the basic conditions used in oligonucleotide synthesis. Therefore, we have developed a compatible solution-phase method to synthesize these isotopically labeled P-site substrates.[1]

We have reported the synthesis of CCA-pcb in two different schemes by phosphoramidite chemistry, using acid-labile 2′-*O*-bis(acetoxyethoxy)methyl (AcE),[13] 2′-*O*-bis(methoxyethoxy)-methyl (MeE), and 4/6-*N*-(4,4′-dimethoxytrityl) (DMTr) as protecting groups and imidazolium salt as the promoter.[1] Here, we report the synthesis of a complete collection of isotopically labeled and remotely tagged P-site substrates. Improved methods for synthesis of ^{18}O-adenosine with the highest reported isotope enrichment and a general method for incorporation of ^{18}O into amino acids are also described.

Results and Discussion

The synthesis of CCA-pcb was first reported by coupling the trinucleotide CCA with cyanomethyl pcb.[14] Although this synthesis provided synthetic access to the molecule, there were multiple byproducts that were difficult to separate from the authentic compound resulting in low overall yields. Furthermore, this approach could not be readily adapted to the synthesis of the target molecule with [2′-^{18}O]- or [3′-^{18}O]adenosine or with the remote isotope tags. We recently reported an alternative synthetic scheme for this compound that produced high yields and few side products.[1] We used bulky bis(trimethylsiloxy)-cyclododecyloxylsilyl (DOD) at the O5′ of cytidine 3′-*O*-phosphoramidite (C74 in tRNA) and AcE at the O2′ of the second cytidine (C75 in tRNA). This ensured the coupling between the 3′-*O*-phosphoramidite of the first nucleoside and the 5′-OH in the second nucleoside with its free 3′-OH site reserved for the coupling to aminoacyladenosine, which is the last coupling step. We used the methyl phosphoramidite, which can be cleaved under mild conditions, and DMTr and ortho ester (AcE, MeE) to protect unwanted nucleophilic sites. MeE was selected to protect 2′-OH to exclude possible hydrolysis of AcE during the cleavage of Fmoc. Both DMTr and ortho ester were removed under weak acidic conditions to ensure the full retention of the 3′-ester linkage and chirality within the peptide chain mimic. After exploring several nucleotide coupling conditions, we found that the phenylimidazolium salt of the phosphoramidite provided high coupling yields. Using this synthetic approach, the isotopomers of the P-site substrate were synthesized by coupling two fragments of similar sizes: cytidinyl-(3′5′)-cytidine phosphoramidite (or its N3 and N4 substituted isotopomer) and isotopomers of A-pcb with substitutions at each of the relevant positions.

Synthesis of C(3′5′)C Phosphoramidite and Its Isotopomer. There are three general methods to prepare [3-^{15}N,4-$^{15}NH_2$]cytidine. The compound was synthesized by S_NAr displacement of the 4-oxo of uridine followed by a Dimroth rearrangement of cytidine.[15] An alternative method by a Dimroth-like rearrangement of N^3-activated uridine followed by S_NAr reaction was also reported.[16] The compound can also be prepared by construction of the pyrimidine ring followed by glycosylation. We used the well-studied second method, and [3-^{15}N,4-$^{15}NH_2$]cytidine (**1b**) was prepared according to a reported procedure by a Dimroth-like rearrangement of 2′,3′,5′-*O*-acetyl-*N*^{3}-nitrouridine and S_NAr reaction of the intermediate 2′,3′,5′-*O*-acetyl[$^{15}N^3$]uridine with in situ produced (^{15}N)H_3 as a nucleophile, respectively, followed by deacetylation.[16] Cytidine (**1a)** and its isotopomer (**1b**) were then treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$) in pyridine, respectively, to afford 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (92%) and its isotopomer (90%).[12] Compounds **2a**,**b** were selectively tritylated at the N4 to produce **3a**,**b** (91%, 88%), which was further protected at the 2′-position by reflux with a mixture of trisacetoxyethoxy orthoformate, 4-*tert*-butyldimethylsiloxy-3-penten-2-one, and PPTS in DCM. The syrup that resulted after chromatography was treated with HF-TMEDA in CH_3CN to provide compounds **5a**,**b** (71%, 86%), which were further protected at the 5′-position by DOD (89%, 89%). The cytidylyl phosphoramidites **7a**,**b** were obtained by treatment of compound **6a**,**b** with methyl tetraisopropylphosphorodiamidite (POMe) in the presence of tetrazole in DCM overnight (81%, quant).

Compounds **7a**,**b** were directly coupled to compound **5a**,**b** in the presence of PhImOTf[17] and molecular sieves in CH_3CN/DCM (1:1), followed by oxidation with *t*-BuOOH in toluene. The reaction selectively produced **8a**,**b** in good yield (81% and quant) after chromatography. Due to the large size of DOD at the O5′ of compound **7a**,**b**, the relatively large 2′-*O*-AcE of compound **5a**,**b**, and large leaving group of phenylimidazole, only the 3′−5′ coupling products were detected, which were fully deprotected to give cytidylyl(3′,5′)cytidine. The diribonucleotide was transformed into its phosphoramidite **9a**,**b** as before (73% and 51%) (Scheme 1). Synthesis of the dinucleotide with tetrazole and 5-(ethylthio)-1*H*-tetrazole gave only moderate yields, which might be caused by the bulky protecting groups as reasoned above.

Synthesis of [3′/2′-^{18}O]Adenosine Derivative. Methods for synthesis of ^{18}O nucleosides are limited, and this is especially

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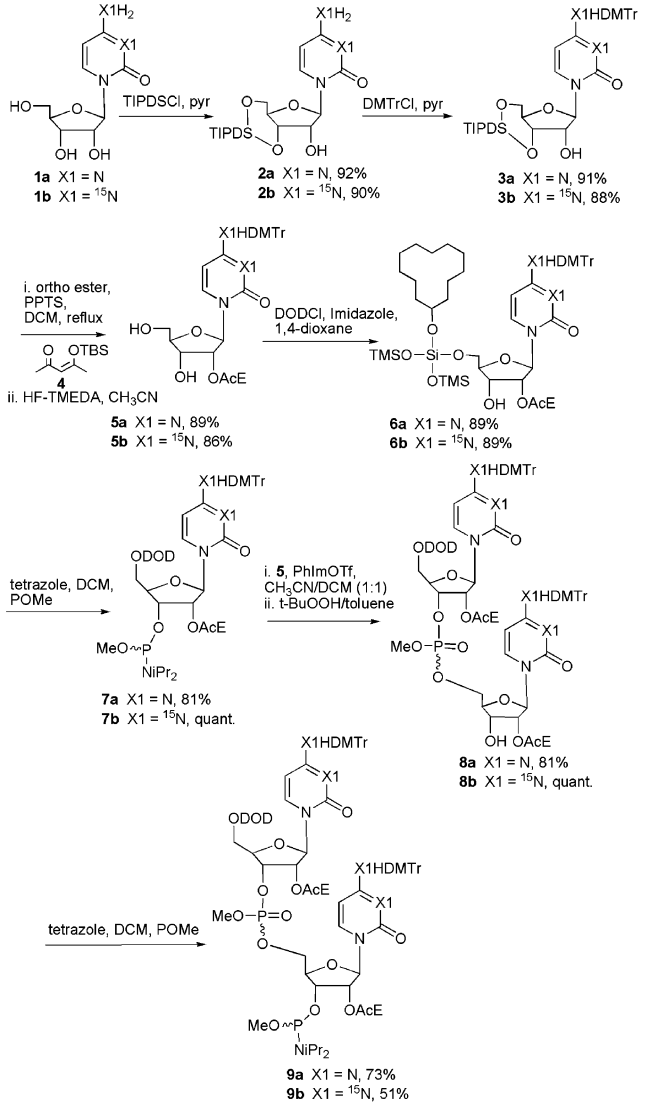
SCHEME 1. Preparation of Cytidinyl-(3′5′)-cytidine Phosphoramidite and Its Isotopomer



true for substitutions within the ribosyl sugar.[18−21] Hogencamp et al. synthesized [3′/2′-^{18}O]adenosine by condensation of adenine with ribosyl chlorides to produce an isotope enrichment of 27−33%.[18] Reversible hydration ($HCl/H_2{}^{18}O$) of a 3′-ketouridine derivative followed by reduction ($NaBH_4$) and separation from the xylo epimer (major) gave [3′-^{18}O]uridine in low yield.[21] Alternatively, the configurations of the 2′-C or 3′-C were inverted, the resulting 2′-OH or 3′-OH were transformed into good leaving groups, and these were replaced to incorporate ^{18}O. Using this strategy, Jiang et al. reported the synthesis of [2′-^{18}O]adenosine; however, they had difficulty during triflation for the synthesis of [3′-^{18}O]adenosine, which was finally prepared via an epoxide intermediate.[20] For pyrimidine nucleosides, the inversion can be realized by formation of 2,2′- or 2,3′-anhydronucleosides.[19,21]

We initially attempted to prepare the [3′-^{18}O]adenosine according to a reported procedure via an epoxide intermediate.[20] The epoxide **17** was prepared as reported. Cesium [$^{18}O_2$]-propionate was prepared by acidic hydrolysis of propionitrile in $HCl-H_2{}^{18}O$ in a pressure tube, which gave 94% of ^{18}O enrichment. This was in contrast to less than 50% enrichment reported for hydrolysis of propionyl chloride. Ring opening of the epoxide by cesium [$^{18}O_2$]propionate (94% ^{18}O) gave multiple spots on TLC, in contrast to the reported regioselective ring opening. After deprotection, ion-exchange chromatography revealed a 1.6:1 mixture of two major products, due to the two possible attacking sites. We reasoned that the formed alkoxide in the ring-opening reaction may cause the disruption of the glycosidic bond and hydrolysis of the 5′ ester. To avoid these side reactions, we employed a Lewis acid to neutralize the resulting alkoxide. In the presence of phenylimidazolium triflate, TLC showed a clean reaction with two products. A moderate Lewis acid induced regioselectivity (3.6:1) for the production of compound **18**. This may result from the increased steric hindrance in the possible hydrogen-bonding complex between imidazolium and N3. Deprotection of 5′-O and 3′-O gave compound **18**, which was selectively crystallized in the reaction mixture (63%). The final incorporation of ^{18}O at the 3′ position was 93%. Silylation of compound **18** by $TIDPSCl_2$ at the 3′ and 5′ positions (73%), followed by similar inversion of configuration of the 2′-OH, gave compound **21f** (42% for three steps) (Scheme 2).

Although we improved the epoxide method, we still expected that the steroselective inversion (S_N2-S_N2) strategy should provide an economical synthesis of the target compound **16f**. We reasoned that the inaccessible triflation might be due to the bulky spherical TBS, which could be substituted with more suitable protecting groups. DMTr is propeller shaped just like diphenylimidazole, which in a reported alkylation study inefficiently excluded N7 alkylation of purine.[22] Selective protection of 5′-O by DMTr is straightforward and may leave the 3′-OH accessible to triflation. Based upon this observation, compound **10** was prepared according to a reported procedure.[23] The 3′-OH was triflate substituted in good yield (84%). Nucleophilic displacement by cesium propionate (70%) and hydrolysis (77%) gave the xylofuranosyl nucleoside. Reactivation of 3′-OH by triflation and nucleophilic substitution incorporated ^{18}O at 3′ position to give compound **15** in good yield (82%). Full deprotection by sequential treatments with NaOH in dioxane−H_2O and HCOOH in MeOH at 50 °C gave compound **16f** (61% with 93% ^{18}O incorporated at 3′). Because ^{18}O was introduced at a later stage in a stereoselective way, this more economical S_N2-S_N2 method was used in subsequent syntheses (Scheme 2).

Compound [2′-^{18}O]adenosine (**16e)** was synthesized according to the reported procedure from arabinofuranosyladenine,[20] with high enrichment of ^{18}O (>90%), using the highly ^{18}O-enriched cesium propionate. Compounds **21a**,**e**,**f** were selectively tritylated at the N6 to produce **22a**,**e**,**f** (91%, 84%, and 93%), which were further protected at the 2′-position by reflux with a mixture

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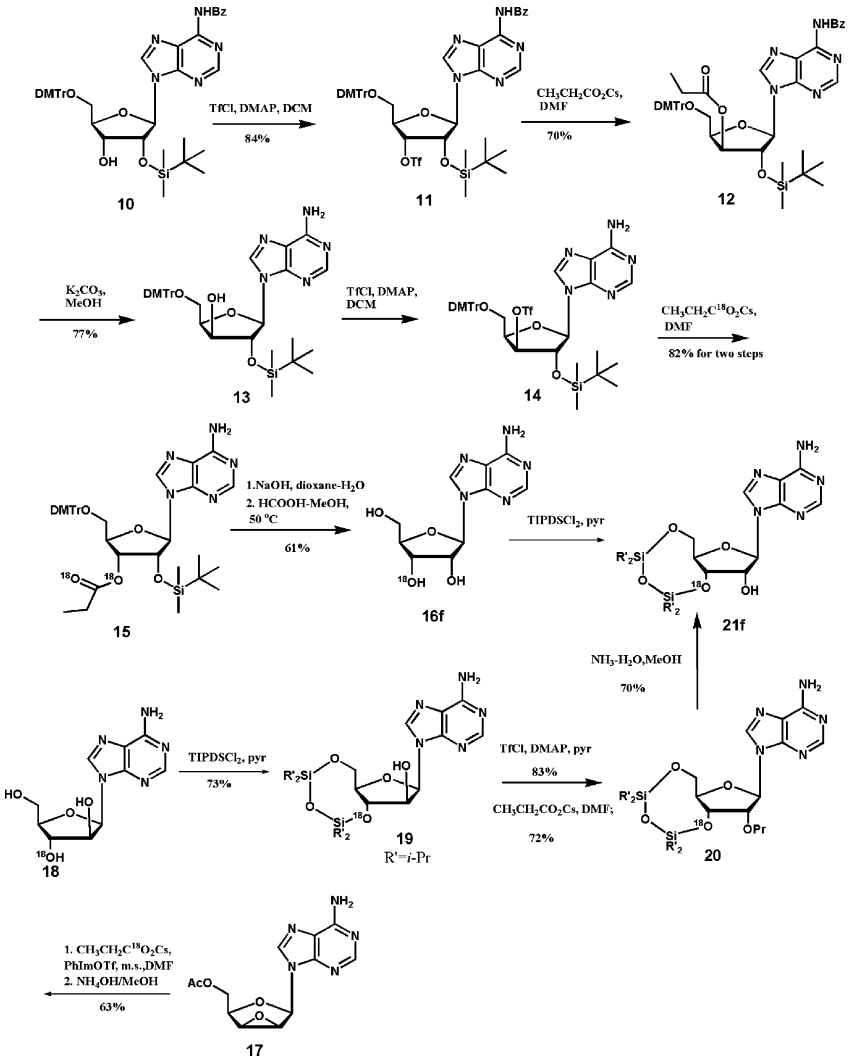
SCHEME 2. Preparation of [3′-^{18}O]Adenosine

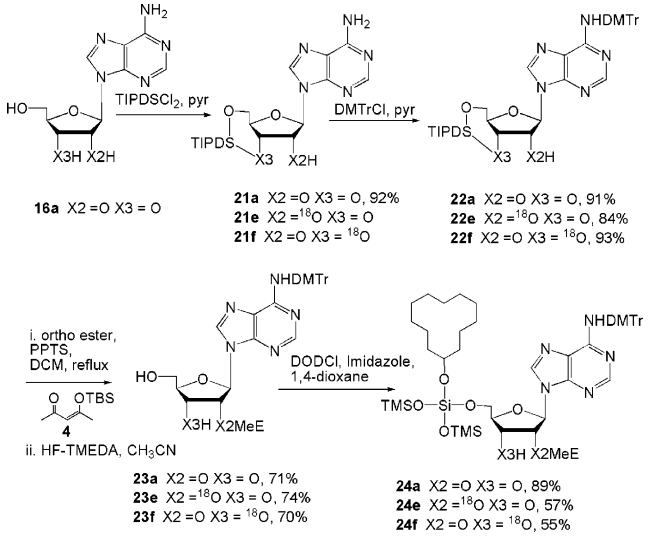


of trismethoxyethoxy orthoformate, 4-*tert*-butyldimethylsiloxy-3-penten-2-one, and PPTS in DCM. The syrups that resulted after chromatography were treated with HF−TMEDA in CH_3-CN to provide compounds **23a**,**e**,**f** (71%, 74%, and 70%), which were further protected by DOD to give **24a**,**e**,**f** (89%, 57%, and 55%) (Scheme 3).

Synthesis of Isotopically Substituted *N*-Fmoc-L-phenylaniline. To synthesize isotopically substituted CCA-pcb, isotopically substituted *N*-Fmoc-L-phenylaniline was first prepared. Stereoselective preparation of L-[α-D]phenylaniline is challenging because of the sensitivity of its chirality to even weak bases. The reported syntheses include the introduction of deuterium by an enzymatic reductive amination[24] and full isotope exchange or chemical preparation of a mixture followed by enzymatic resolution of two enantiomers.[25,26]

Because of the readily available deuterium source and acylase, we synthesized L-[α-D]phenylaniline by a reported method using enzymatic resolution.[26] *N*-Acetyl-DL-phenylalanine was treated with NaOH−D_2O at room temperature, and the resulting highly

SCHEME 3. Synthesis of Adenosine and [2′-^{18}O/3′-^{18}O]Adenosine Intermediates



deuterium enriched (>98% by NMR) *N*-acetyl-[α-D]-DL-phenylalanine was resolved by porcine kidney acylase at pH 7 to give L-[α-D]phenylaniline in high purity, which was protected

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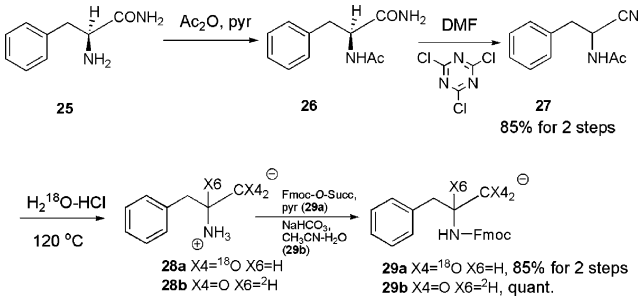
SCHEME 4. Preparation of l-[$^{18}O_2$]Phenylaniline

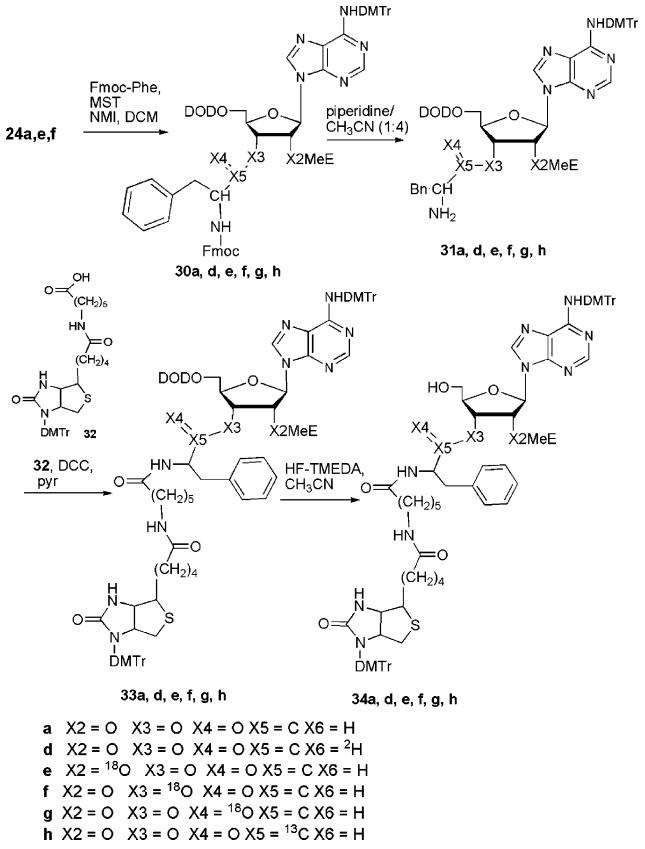


with Fmoc by treatment of Fmoc *O*-succinimide in CH_3CN-H_2O (1:1) in the presence of $NaHCO_3$ (quant).

We were unable to identify a synthesis of L-[$^{18}O_2$]phenylaniline despite an extensive literature search. Sharpless and co-workers reported a stereoselective synthesis of an L-phenylglycinonitrile derivative.[27] We thought acidic hydrolysis of a nitrile may not epimerize the chiral center. According to this procedure, we synthesized compound **27** by acetylating L-phenylglycinamide and treating the resulting *N*-acetyl-L-phenylglycinamide with cyanuric chloride.[28] The overall yield was 85% for two steps. *N*-Fmoc-L-[$^{18}O_2$]phenylaniline was then synthesized by hydrolysis of compound **27** to give [$^{18}O_2$]phenylaniline with high ^{18}O enrichment (quant, >92% ^{18}O). Because of $^{18}O/^{16}O$ exchange between amino acids and H_2O in aqueous solution, compound **28a** was treated with Fmoc *N*-hydroxysuccinimide ester (Fmoc-OSu) (85%) in pyridine (Scheme 4). Significant epimerization was observed in spite of the much lower pK_a of pyridine than that of α-H in phenylalanine.

Synthesis of A-pcb and Its Isotopomers. Aminoacylation of adenosine derivatives (**24a**,**e**,**f**) was challenging because of the steric hindrance caused by the bulky 5′- and 2′-protecting groups (DOD and MeE) and the relatively low nucleophilicity of the 3′-OH. Several coupling strategies such as acylation by acyl halides and DCC-promoted esterification were attempted, but we achieved our best coupling using the imidazolium salt of the amino acid. Compounds **24a**,**e**,**f** were treated with *N*-Fmoc-L-phenylalanines in the presence of mesitylenesulfonyl tetrazole (MST)[29,30] and excess *N*-methylimidazole in DCM at ambient temperature to produce the fully protected derivatives **30a**,**d**−**h** in reasonable to excellent yields (59% to quant). The reaction involves the imidazolium cation as shown by ^1H NMR. The imidazole was nucleophilically displaced as a neutral leaving group[31] by the 3′-OH of **24a**,**e**,**f**. All isotopomers are single distereomers as shown by ^1H NMR, except for **30g**. Compound **30g** is a mixture of two distereomers as a result of the corresponding racemic *N*-Fmoc-[$^{18}O_2$]phenylalanine. Removal of Fmoc by treatment of **30a**,**d**−**h** with 20% piperidine in CH_3CN resulted in **31a**,**d**−**h**. The two diostereomers of **31g** were separated by careful column chromatography, and the correct component was used for later steps. DCC-mediated amide formation with compound **32** followed by desilylation

SCHEME 5. Preparation of Protected A-pcb and Its Isotopomers



by treatment with HF−TMEDA in CH_3CN gave **34a**,**d**−**h** in fair to good yields (Scheme 5). Compound **32** was prepared as reported[1] with an improved high yield tritylation of a methyl ester of biotin in the presence of both Et_3N and DMAP in pyridine at 70 °C (88%).

Synthesis of Protected CCA-pcb Isotopomers and Their Deprotection. With compounds **9a**,**b** and **34a**,**d**−**h** in hand, we performed the final steps of CCA-pcb isotopomer syntheses using the previously reported coupling method.[1] Diribonucleotide phosphoramidites **9a**,**b** were coupled to 1 molar equiv of compound **34a**,**d**−**h** using phenylimidazolium triflate (PhIm-OTf) and molecular sieves as the promotor in CH_3CN/DCM (1:1) to form the fully protected CCA-pcb **35a**−**h** in reasonable yields (33−72%). The efficient 1:1 coupling reaction involves imidazolium cation, which was displaced by the 5′-OH to release a neutral leaving group. Desilylation of compound **35a**−**h** by treatment with HF−TMEDA in CH_3CN and cleavage of methyl phosphate ester with S_2Na_2,[13] followed by complete removal of DMTr and orthoformate ester in 0.5 M HCOOH in MeOH−DCM (1:1) at 55 °C for 5 h, gave compounds **1a** (160 mg, 59%), **1b** (40 mg, 26%), **1c** (53 mg, 28%), **1d** (21 mg, 13%), and **1h** (54 mg, 62%) as essentially single isotopomers and isotopically highly enriched **1e** (86 mg, 30% with 93% of ^{18}O in A76 (2′-O)), **1f** (16 mg, 8% with 93% of ^{18}O in A76 (3′-O)), and **1g** (12 mg, 13% with 64% of ^{18}O (C═O)) as a mixture of two regioisomers (2′/3′-ester as shown by ^1H NMR), respectively (Scheme 6). The final relatively low enrichment (64%) of ^{18}O in the carbonyl of compound **1g** may be caused by $^{18}O/^{16}O$ exchange with adventitious H_2O and with triflate in MST-promoted aminoacylation.

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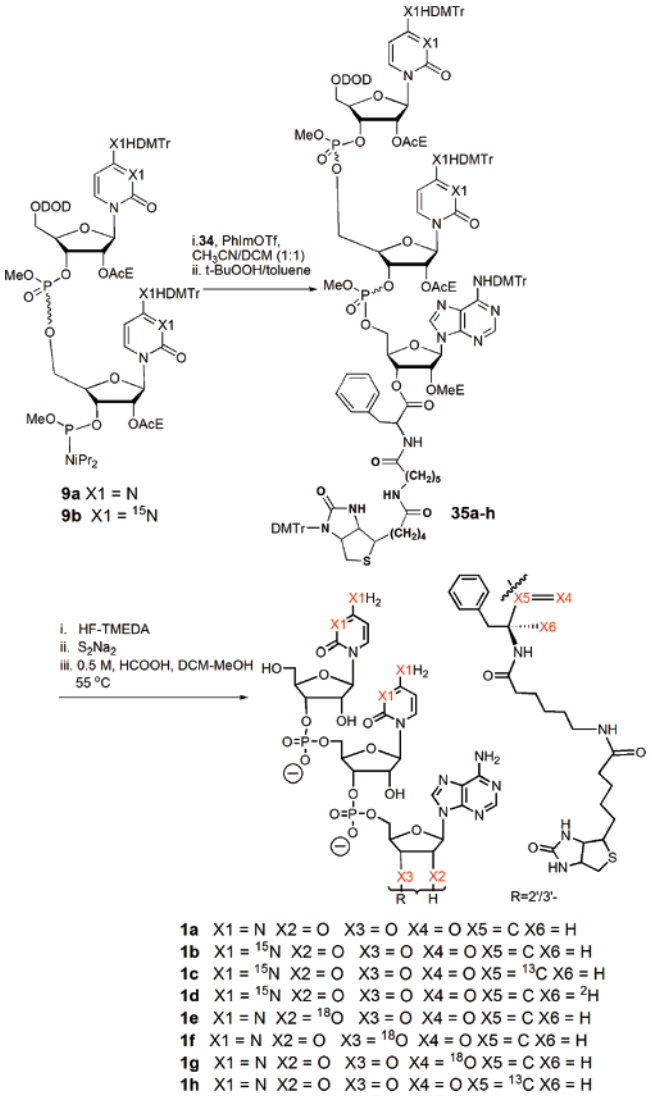
SCHEME 6. Coupling and Deprotection for CCA-pcb and Its Isotopomers



Summary and Conclusions

In conclusion, we have accomplished the syntheses of a complete set of CCA-pcb isotopomers that will be used for a kinetic isotope effect analysis of the ribosomal peptidyl transfer reaction. These syntheses were made possible by improved methods for high-yield synthesis of CCA-pcb.[1] With its compatible coupling methods for nucleotide synthesis, amino acylation, and mild deprotection conditions, 3′,5′-linkages were constructed exclusively, and the chirality of the amino acid and the labile 2′/3′-ester linkage was conserved. The improved syntheses of 3′-[^{18}O]adenosine with high heavy isotope enrichment have been developed, which provide an important small molecule probe for studies of nucleic acid related enzymes.

Experimental Section

2′-*O*-((Bisacetoxyethoxy)methyl)-5′-*O*-bis(trimethylsiloxy)cyclododecyloxysilyl-4-*N*-(4,4′-dimethoxytrityl)[3-^{15}N,4-^{15}NH$_2$]-cytidylyl-(3′,5′)-2′-*O*-((bisacetoxyethoxy)methyl)-4-*N*-(4,4′-dimethoxytrityl)[3-^{15}N,4-^{15}NH$_2$]cytidine 3′-(*N*,*N*-diisopropylmethoxy)phosphoramidite (9b). To compound **8b** (3.2 g, 1.6 mmol) in a dry flask was added tetrazole in CH_3CN (3 wt %, 5.5 mL, 1.9 mmol) and volatiles were evaporated under vacuum to give a solid residue. DCM (10 mL) and methyl tetraisopropylphosphorodiamidite (1.3 mL, 1.2 g, 1.8 mmol) were added, and the resulting solution was stirred under Ar at room temperature for 11 h. TLC (EtOAc/hexanes, 85:15) showed complete reaction. The reaction mixture was then diluted by addition of DCM (50 mL), and the resulting solution was washed with NaCl/H_2O (30 × 2 mL). The organic layer was separated and dried ($MgSO_4$), and volatiles were evaporated under vacuum. The derived residue was chromatographed (DCM/hexanes, 3:7 → 2:3 with 10% Et_3N) to give compound **9b** (1.3 g, 51%) (four distereomers) with ^{1}H and ^{31}P NMR spectra identical to those of compound **9a**; ESI-MS (ES^+) *m*/*z* 2156.88 ($[M + H]^+$ $[C_{104}H_{147}N_3{}^{15}N_4O_{32}P_2Si_3]$ = 2156.88).

6-*N*-Benzoyl-2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-trifluoromethanesulfonyladenosine (11). 2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-benzoyladenosine (0.98 g, 1.2 mmol) and DMAP (230 mg, 1.9 mmol) were dried by coevaporation with DCM (10 mL). The mixture was then dissolved in DCM (15 mL) and cooled in ice−water bath under Ar. To this solution was added trifluoromethanesulfonyl chloride (0.32 g, 1.9 mmol), and the reaction mixture was stirred at 0 °C under Ar for about 1 h (TLC showed complete reaction). The reaction mixture was poured into ice-cold saturated $NaHCO_3$−H_2O (50 mL) and extracted with DCM (50 × 2 mL). The organic layer was separated, washed twice with saturated $NaHCO_3$−H_2O (50 × 2 mL), and dried ($MgSO_4$). Volatiles were evaporated under vacuum, and the resulting mixture was chromatographed (EtOAc/hexanes, 3:7) to give compound **11** (0.96 g, 84%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.36 (s, 1H), 8.81 (s, 1H), 8.67 (s, 1H), 8.12 (d, J = 7.3 Hz, 2H), 7.74−6.94 (m, 16H), 6.22 (d, J = 7.5 Hz, 1H), 5.64−5.66 (m, 1H), 5.57−5.58 (m, 1H), 4.61 (t, J = 5.4 Hz, 1H), 3.82 (s, 6H), 3.65 (dd, J = 5.5, 8.3 Hz, 1H), 3.50 (dd, J = 5.5, 8.3 Hz, 1H), 0.77 (s, 9H), 0.00 (s, 3H), −0.41 (s, 3H).

6-*N*-Benzoyl-[2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-propionyl-9-xylofuranosyl]adenine (12). A mixture of compound **11** (0.96 g, 1 mmol) and cesium propionate (0.46 g, 2.1 mmol) was dissolved in anhydrous DMF (10 mL), and the solution was stirred under Ar at room temperature overnight. Volatiles were evaporated under vacuum, and the residue was chromatographed (EtOAc/hexanes, 3:7) to give compound **12** (0.61 g, 70%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.17 (s, 1H), 8.67 (s, 1H), 8.34 (s, 1H), 8.00 (d, J = 7.4 Hz, 2H), 7.06−6.81 (m, 16H), 6.07 (d, J = 3.4 Hz, 1H), 5.21−5.19 (m, 1H), 4.97−4.96 (m, 1H), 4.57−4.54 (m, 1H), 3.69 (s, 6H), 3.36 (dd, J = 6.5, 8.4 Hz, 1H), 3.15 (dd, J = 4.7, 7.5 Hz, 1H), 2.03−1.92 (m, 2H), 0.78 (s, 9H), 0.75 (t, J = 7.5 Hz, 3H), 0.00 (s, 3H), −0.10 (s, 3H).

(2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-9-xylofuranosyl)adenine (13). To a solution of compound **12** (0.61 g, 0.72 mmol) in MeOH (20 mL) was added K_2CO_3 (0.5 g, 3.6 mmol), and the mixture was stirred at room temperature overnight (TLC showed complete reaction). Volatiles were evaporated under vacuum, and the residue was chromatographed (EtOAc/hexanes, 1:1 → 7:3 → EtOAc) to give compound **13** (0.38 g, 77%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.07 (s, 1H), 8.01 (s, 1H), 7.37−6.85 (m, 15H), 5.86 (br s, J = 1.5 Hz, 1H), 5.68 (d, J = 4.8 Hz, 1H), 4.38 (br s, 1H), 4.28−4.25 (m, 1H), 3.92−3.90 (m, 1H), 3.68 (s, 6H), 3.45 (dd, J = 9.9, 10.2 Hz, 1H), 3.50 (dd, J = 1.4, 5.2 Hz, 1H), 0.80 (s, 9H), 0.00 (s, 6H).

[2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-trifluoromethanesulfonyl-9-xylofuranosyl]adenine (14). Compound **13** (0.38 g, 0.6 mmol) and DMAP (115 mg, 0.9 mmol) were dried by coevaporation with DCM (10 mL). The mixture was then dissolved in DCM (15 mL) and cooled in an ice−water bath under Ar. To this solution was added trifluoromethanesulfonyl chloride (0.16 g, 0.98 mmol), and the reaction mixture was stirred at 0 °C under Ar for about 1 h (TLC showed complete reaction). The reaction mixture was poured into ice-cold saturated $NaHCO_3$−H_2O (50 mL) and extracted with DCM (50 × 2 mL). The organic layer was separated, washed twice with saturated $NaHCO_3$−H_2O (50 × 2 mL), and dried ($MgSO_4$). Volatiles were evaporated under

vacuum, and the resulting mixture was chromatographed (MeOH/DCM, 1:30) to give the compound (0.46 g, quant): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.19 (s, 1H), 8.12 (s, 1H), 7.42−6.83 (m, 13H), 6.03 (d, J = 4.8 Hz, 1H), 5.71−5.69 (m, 1H), 5.42−5.40 (m, 1H), 4.67−4.64 (m, 1H), 3.72 (s, 6H), 3.49−3.48 (m, 1H), 3.25−3.20 (m, 1H), 0.77 (s, 9H), 0.00 (s, 3H), −0.29 (s, 3H).

2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-[3′-^{18}O]-3′-*O*-propionyladenosine (15). [$^{18}O_2$]Propionate cesium was prepared by acidic hydrolysis of propionitrile in HCl−$H_2{}^{18}O$. $H_2{}^{18}O$ (10 g) was saturated with HCl at 0 °C, and to this cold solution was added propionitrile (3 mL, 2.32 g, 42 mmol). The solution was stirred in a pressure tube at 120 °C for 48 h. The yellow solution was cooled in an ice−water bath and the solid precipitated out. The acidic $H_2{}^{18}O$ was collected for recycling, and the solid was dissolved in DCM (50 mL). The solution was extracted with ice-cold $NaHCO_3$−H_2O, and the aqueous solution was washed with DCM. The aqueous solution was acidified by addition of HCl−H_2O to about pH 3 and extracted with DCM. The organic layer was separated and dried ($MgSO_4$). Vacuum fractional distillation gave [$^{18}O_2$]propionoic acid as a clear colorless liquid.

The liquid was added to MeOH (40 mL) in a flask, and to this solution was added Cs_2CO_3 (1.0 g, 3.1 mmol). The mixture was stirred at room temperature for 3 h, and volatiles were evaporated under vacuum to give a solid residue, which was thoroughly washed with Et_2O to give the cesium salt (1. 4 g, 95%): ESI-MS (ES$^-$) m/z 75 ([M − H]$^-$ [$C_3H_5{}^{18}OO$] = 75, 12%), 77 ([M − H]$^-$ [$C_3H_5{}^{18}O_2$] = 77, 88%).

Compound **14** (0.46 g, 0.56 mmol) and cesium [$^{18}O_2$]propionate (0.23 g, 1.1 mmol) were dissolved in anhydrous DMF (10 mL), and the resulting solution was stirred at room temperature under Ar overnight. Volatiles were removed under vacuum at 37 °C, and the solid residue was chromatographed (EtOAc/hexanes, 1:1 → 7:3) to give compound **15** (0.34 g, 81%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (s, 1H), 8.16 (s, 1H), 7.55−6.97 (m, 13H), 6.03 (d, J = 7.1 Hz, 1H), 5.51−5.51 (m, 1H), 5.40−5.39 (m, 1H), 4.36 (br s, 1H), 3.86 (s, 6H), 3.48−3.44 (m, 2H), 2.58−2.49 (m, 2H), 1.19 (t, J = 7.6 Hz, 3H), 0.78 (s, 9H), 0.77 (t, J = 7.5 Hz, 3H), 0.00 (s, 3H), −0.23 (s, 3H).

[3′-^{18}O]Adenosine (16f). To a solution of compound **15** (0.34 g, 0.46 mmol) in dioxane (5 mL) was added 1 M NaOH−H_2O (5 mL), and the turbid solution was stirred at room temperature overnight. The solution was neutralized by bubbling through CO_2. Volatiles were evaporated under vacuum, and the residue was chromatographed (MeOH/DCM. 1:15) to give a white solid.

This white solid was then dissolved in MeOH/DCM (1:1, 10 mL), and to the clear solution was added 88% formic acid (0.22 mL, 0.26 g, 5 mmol). The solution was stirred at 50 °C for 11 h (TLC showed complete reaction). The volatiles were evaporated under vacuum, and the residue was thoroughly washed (DCM) to give a white solid (77 mg, 63%) with ^{1}H and ^{13}C NMR spectra identical to the authentic sample: ESI-MS (ES$^+$) m/z 268 ([M + H]$^+$ [$C_{10}H_{13}N_5O_4$] = 268, 8.4%), 270 ([M + H]$^+$ [$C_{10}H_{13}N_5O_3{}^{18}O$] = 270, 91.6%).

(9-β-D-[3′-^{18}O]-Arabinofuranosyl)adenine (18). A mixture of compound **17** (0.86 g, 3.0 mmol), phenylimidazolium triflate (0.97 g, 3.3 mmol), and [$^{18}O_2$]propionate cesium (0.93 g, 4.4 mmol) in DMF was stirred at 120 °C in the presence of dried molecular sieves for 6 h. Volatiles were evaporated under vacuum. The derived residue was dissolved in MeOH (40 mL) in a sealed flask, and to this solution was added NH_3·H_2O (4 mL). The mixture was stirred at ambient temperature overnight. The precipitated solid was collected by filtration to give compound **18** (0.5 g, 63%) with with ^{1}H and ^{13}C NMR spectra identical to the authentic unlabeled sample: ESI-MS (ES$^+$) m/z 268 ([M + H]$^+$ [$C_{10}H_{13}N_5O_4$] = 268, 7.4%), 270 ([M + H]$^+$ [$C_{10}H_{13}N_5O_3{}^{18}O$] = 270, 92.6%). The mother liquor was dried by evaporation in vacuo, and the solid residue was washed thoroughly with MeOH to give a crude mixture of the needed product and (9-β-D-2′-^{18}O-xylofuranosyl)adenine, which was treated with $TIPDSCl_2$ to give silylated byproduct (0.20 g).

L-[$^{18}O_2$]Phenylaniline (28a). $H_2{}^{18}O$ (10 g) was saturated with HCl at 0 °C, and to this cold solution added compound **27** (1.5 g, 7.8 mmol). The suspension was stirred at 120 °C to give a clear brown solution after 10 min, and the solution was further stirred for 48 h. The yellow solution was cooled in ice−water bath and solid precipitated out. The acidic $H_2{}^{18}O$ was collected for future use, and the solid was dissolved in MeOH (50 mL). Volatiles were evaporated in vacuo, and the residue was thoroughly washed with DCM to give crude compound **28a** (including NH_4Cl): ESI-MS (ES$^+$) m/z 168 (M + H$^+$ [$C_9H_{12}{}^{18}OO$] = 168, 9.5%), 170 (M + H$^+$ [$C_9H_{12}{}^{18}O_2$] = 170, 90.5%).

Cytidylyl-(3′,5′)-cytidylyl-(3′,5′)-3′(2′)-*O*-(*N*-(6-D-(+)-biotinoyl-aminohexanoyl)-L-phenylalanyl)adenosine Isotopomers (CCA-pcb, 1a−h). A freshly prepared HF-TMEDA solution in DMF (0.4 M, 12.5 equiv) was added to a flask containing compound **35a/b/c/d/e/f/g/h** (0.95 g, 0.27 mmol/0.21 g, 0.06 mmol/0.50 g, 0.14 mmol/0.41 g, 0.12 mmol/0.77 g, 0.21 mmol/0.53 g, 0.15 mmol/0.23 g, 0.06 mmol/0.28 g, 0.08 mmol), respectively, and the resulting solution was stirred for 0.5 h. TLC showed complete reaction. Volatiles were evaporated under vacuum and coevaporated with toluene, and the residue was separated by preparative TLC (MeOH/DCM, 1:15 → 1:10) to give a solid.

S_2Na_2·$3H_2O$ was dissolved in H_2O/DMF (0.1 M, 2% H_2O). To the clear lightly yellow solution was added the solid, and the resulting clear solution was stirred at room temperature for 1 h. TLC showed complete reaction. S_2Na_2 was then partially precipitated by addition of DCM (25 mL) and removed by centrifuge. The solid was extracted with DCM (5 mL), and the clear solutions were combined. Volatiles were evaporated under vacuum, and the residue was suspended in limited amount of MeOH/DCM (1:3). The resulting solution was separated by preparative TLC (MeOH/DCM, 1:6 → 1:3.6) to give a solid.

To the obtained solid in a dry flask was added freshly prepared 0.5 M HCOOH in DCM/CH_3OH (1:1). The clear solution was stirred at 55 °C for 5 h with precipitated product observed. Volatiles were evaporated and coevaporated with toluene twice. The residue was dried under vacuum overnight and then washed with MeOH/DCM (1:2) at 37 °C to give compound **1a**−**h** (two regioisomers).

Compound **1a** (160 mg, 59%): ESI-MS (ES$^-$) m/z 1362.51 ([M − H]$^-$ [$C_{53}H_{70}N_{15}O_{22}P_2S$] =1362.40), 1384.54 ([M − 2H + Na]$^-$ [$C_{53}H_{69}N_{15}O_{22}P_2S$ Na] = 1384.40).

Compound **1b** (40 mg, 26%): ESI-MS (ES$^-$) m/z 682.90 ([M − 2H]$^{2-}$ [$C_{53}H_{69}{}^{15}N_4N_{11}O_{22}P_2S$] = 682.70), 1366.81 ([M − H]$^-$ [$C_{53}H_{70}{}^{15}N_4N_{11}O_{22}P_2S$] =1366.40), 1388.77 ([M − 2H + Na]$^-$ [$C_{53}H_{69}{}^{15}N_4N_{11}O_{22}P_2S$ Na] = 1386.40).

Compound **1c** (53 mg, 28%): ESI-MS (ES$^-$) m/z 683.49 ([M − 2H]$^{2-}$ [$C_{52}{}^{13}CH_{69}{}^{15}N_4N_{11}O_{22}P_2S$] = 683.20), 1367.95 ([M − H]$^-$ [$C_{52}{}^{13}CH_{70}{}^{15}N_4N_{11}O_{22}P_2S$] = 1367.40), 1389.91 ([M − 2H + Na]$^-$ [$C_{52}{}^{13}CH_{69}{}^{15}N_4N_{11}O_{22}P_2S$ Na] = 1389.40) with an extremely abundant peak at 170.7 ppm in ^{13}C NMR.

Compound **1d** (21 mg, 13%): ESI-MS (ES$^-$) m/z 683.41 ([M − 2H]$^{2-}$ [$C_{53}{}^2HH_{68}{}^{15}N_4N_{11}O_{22}P_2S$] = 683.20), 1367.82 ([M − H]$^-$ [$C_{53}{}^2HH_{69}{}^{15}N_4N_{11}O_{22}P_2S$] = 1367.40).

Compound **1e** (86 mg, 30%): ESI-MS (ES$^-$) m/z 681.65 ([M − 2H]$^{2-}$ [$C_{53}H_{69}N_{15}O_{21}{}^{18}OP_2S$] = 681.70), 1364.40 ([M − H]$^-$ [$C_{53}H_{70}N_{15}O_{21}{}^{18}O\ P_2S$] = 1364.40), 1375.90 ([2M + Na − 2H]$^{2-}$ [$C_{106}H_{140}N_{30}O_{42}{}^{18}O_2P_4S_2Na$] = 1375.90), 1386.37 ([M − 2H + Na]$^-$ [$C_{53}H_{69}N_{15}O_{21}{}^{18}O\ P_2SNa$] = 1386.40).

Compound **1f** (16 mg, 8%): ESI-MS (ES$^-$) m/z 681.69 ([M − 2H]$^{2-}$ [$C_{53}H_{69}N_{15}O_{21}{}^{18}OP_2S$] = 681.70), 1364.42 ([M − H]$^-$ [$C_{53}H_{70}N_{15}O_{21}{}^{18}O\ P_2S$] =1364.40), 1386.39 ([M − 2H + Na]$^-$ [$C_{53}H_{69}N_{15}O_{21}{}^{18}O\ P_2SNa$] = 1386.40).

Compound **1g** (12 mg, 13%): ESI-MS (ES$^-$) m/z 680.72 ([M − 2H]$^{2-}$ [$C_{53}H_{69}N_{15}O_{22}\ P_2S$] = 680.70, 35.7%), 681.72 ([M − 2H]$^{2-}$ [$C_{53}H_{69}N_{15}O_{21}{}^{18}OP_2S$] = 681.70, 64.3%), 1362.38 ([M − H]$^-$ [$C_{53}H_{70}N_{15}O_{22}\ P_2S$] = 1362.40, 33.5%), 1364.38 ([M − H]$^-$ [$C_{53}H_{70}N_{15}O_{21}{}^{18}O\ P_2S$] = 1364.40, 66.5%).

Compound **1h** (54 mg, 62%): ESI-MS (ES^-) *m*/*z* 681.45 ([M − 2H]$^{2-}$ [$C_{52}{}^{13}C\ H_{69}N_{15}O_{22}P_2S$] = 681.20), 1363.74 ([M − H]$^-$ [$C_{52}{}^{13}CH_{70}N_{15}O_{22}P_2S$] = 1363.40), 1375.24 ([2M + Na − 2H]$^{2-}$ [$C_{104}{}^{13}C_2H_{140}N_{30}O_{44}P_4S_2Na$] = 1375.20), 1385.73 ([M − 2H + Na]$^-$ [$C_{52}{}^{13}C\ H_{69}N_{15}O_{22}\ P_2SNa$] = 1386.40) with an extremely abundant peak at 170.7 ppm in ^{13}C NMR.

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Supporting Information Available: General experimental procedures, experimental details, and synthetic procedures for compounds **2b**, **3b**, **5b**−**8b**, **22**−**24e**,**f**, **27**, **29a**,**b**, **30**−**31a**, **d**−**h**, **34a**,**d**−**h**, **35a**−**h**; ^1H NMR for new compounds (**11**−**15**, **27**, **29b**, and **31g**) and $^1H-^{15}N$ HMBC for compounds **6b** and **9b**; and mass spectra of **1a**−**h**, **9b**, **16f**, **28a**, **34e**, and **34f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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