Enzymatic De Novo Pyrimidine Nucleotide Synthesis

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Abstract: The use of stable isotope labeling has revolutionized NMR studies of nucleic acids, and there is a need for methods of incorporation of specific isotope labels to facilitate specific NMR experiments and applications. Enzymatic synthesis offers an efficient and flexible means to synthesize nucleoside triphosphates from a variety of commercially available specifically labeled precursors, permitting isotope labeling of RNAs prepared by in vitro transcription. Here, we recapitulate de novo pyrimidine biosynthesis in vitro, using recombinantly expressed enzymes to perform efficient single-pot syntheses of UTP and CTP that bear a variety of stable isotope labeling patterns. Filtered NMR experiments on 13C, 15N, 2H-labeled HIV-2 TAR RNA demonstrate the utility and value of this approach. This flexible enzymatic synthesis will make implementing detailed and informative RNA stable isotope labeling schemes substantially more cost-effective and efficient, providing advanced tools for the study of structure and dynamics of RNA molecules.

Introduction

Enzymatic synthesis has emerged as an important tool in the production of biochemicals. Enzyme catalyzed reactions are often more specific, efficient, cost-effective, and afford reduced environmental impact over traditional chemical methods.1-4 Because of these advantages, many straightforward enzymatic schemes have been adopted in industrial biochemical production.5 Recent advances in metabolic engineering have greatly broadened the targets and applications for enzymatic synthesis, which now contributes significantly to the biofuel industry.6,7 and natural products synthesis.8 In particular, the development of enzymatic tools to synthesize nucleotides has had a tremendous impact on the study of RNA, DNA, and other nucleic acids by nuclear magnetic resonance (NMR) methods.9 Multinuclear NMR experiments require the incorporation of one or more stable isotope labels, and therefore synthetic methods to incorporate isotope labels into nucleic acid building blocks have driven a significant advance in structural studies, including allowing the study of larger molecules.10 In particular, there is a need for pyrimidine nucleotides that are selectively isotope labeled to complement the specifically labeled purine nucleotides that are commercially available and/or obtained efficiently using published methods.11

The primary method to obtain stable isotope labeled nucleotides is the harvest of uniformly 13C, 15N-labeled nucleotides from bacteria grown on labeled media containing 15NH4Cl and 13C-glucose or 13C-methanol.12-14 This biomass method is suitable for economic production of uniformly labeled nucleotides in large quantity, but does not allow for efficient site-specific incorporation of stable isotopes at designated locations without specialized labeled medium, metabolically modified bacteria, or both. Selective incorporation of 15N, 13C into nucleic acids is extremely useful to highlight specific resonances, to facilitate selective magnetization transfer pathways, and to simplify spectra. Using 13C, 2H-glucose and unlabeled bases, enzymatic methods for the selective deuteration of the ribose moiety of nucleotides have allowed the study of RNA of increasing size by effectively editing spectra to allow for the assignment of intermolecular and sugar—sugar NOEs.15 Using this method, the H2 and H2 protons can be selectively observed, providing important structural information, and a variety of

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labeling patterns are possible due to the availability of labeled glucose. Incorporation of isotope labeling into the base moiety of nucleotides is also extremely important due to their structural role in hydrogen bonding and base stacking.

We have previously developed a method to selectively incorporate $^{13}$C, $^{15}$N into the purine bases via a total enzymatic synthesis of ATP and GTP starting from serine, glucose, NH$_4^+$ and CO$_2$ with up to 66% isolated yield. Historically, labeled UTP and CTP have been prepared from chemically synthesized glucose giving an isolated yield of 60%. Specifically labeled 2, $^{13}$C$_2$, $^{15}$N$_3$- uridine has been synthesized from K$^{13}$CN and $^{15}$N-urea with overall yields of 6.1% and 35.2% based on respective starting materials. A chemical synthesis of $^{13}$C$_6$-UTP from K$^{13}$CN was also achieved with an overall yield of 24%. Specifically labeled $^{13}$N-labeled cytidine has been chemically synthesized from uridine and $^{15}$NH$_4$Cl with yields of 94% for $^{15}$Namino-cytidine, 72% for $^{15}$N$_2$-cytidine, and 62% for $^{15}$Namino, 3-cytidine. Specifically deuterated CDP has been chemically synthesized from tert-butyldimethylsilyl cytidine derivatives with $^{13}$C, $^{15}$N into the purine bases via a total enzymatic synthesis of U$^{13}$C, $^{15}$N-UTP has also been demonstrated starting from U.$^{15}$N, $^{15}$C-uracil and U.$^{15}$C-glucose giving an isolated yield of 60%. Specifically labeled $^{13}$C$_2$, $^{15}$N$_3$- uridine has been synthesized from K$^{13}$CN and $^{15}$N-urea with overall yields of 6.1% and 35.2% based on respective starting materials. A chemical synthesis of $^{13}$C$_6$-UTP from K$^{13}$CN was also achieved with an overall yield of 24%. Specifically labeled $^{15}$N-labeled cytidine has been chemically synthesized from uridine and $^{15}$NH$_4$Cl with yields of 94% for $^{15}$Namino-cytidine, 72% for $^{15}$N$_2$-cytidine, and 62% for $^{15}$Namino, 3-cytidine. Specifically deuterated CDP has been chemically synthesized from tert-butyldimethylsilyl cytidine derivatives with $^{13}$C, $^{15}$N into the purine bases via a total enzymatic synthesis of U$^{13}$C, $^{15}$N-UTP has also been demonstrated starting from U.$^{15}$N, $^{15}$C-uracil and U.$^{15}$C-glucose giving an isolated yield of 60%. Specifically labeled $^{13}$C$_2$, $^{15}$N$_3$- uridine has been synthesized from K$^{13}$CN and $^{15}$N-urea with overall yields of 6.1% and 35.2% based on respective starting materials. A chemical synthesis of $^{13}$C$_6$-UTP from K$^{13}$CN was also achieved with an overall yield of 24%.

Results and Discussion

Design of the Enzymatic Synthesis. In contrast to de novo purine synthesis, where the base is constructed step by step onto the ribose moiety, in de novo pyrimidine synthesis, the preformed nucleobase is enzymatically coupled to the ribose moiety, as shown in Scheme 1A. While the reconstitution of entire E. coli pathways in vitro has proven successful in many cases, several key substitutions proved necessary for efficient in vitro orotate synthesis using the de novo pyrimidine pathway. The carbamoyl-phosphate synthase-like carbamate kinase enzyme from thermophile Pyrococcus furiosus (cpkA) was substituted for E. coli carbamoyl phosphate synthase (carAB) due to three advantages. First, cpkA utilizes NH$_4^+$ as the nitrogen source, in contrast to the strict requirement for glutamine by carAB, making the reaction more cost-effective. Second, the single subunit Pyrococcus enzyme cpkA is extremely stable and is easy to prepare. In contrast, E. coli carbamoyl phosphate carAB proved unstable and required a challenging preparation of both subunits for optimal activity. Third, Pyrococcus cpkA utilized aspartate, many specific pyrimidine base labeled patterns can be synthesized including a combination of $^{13}$C$_2$, $^{15}$N, and $^3$H. The exchange of C$_6$ proton with solvent during the decarboxylation of OMP is convenient for specific deuteration schemes. Deuteration at the C$_5$ position is often desired since the H$_5$ and H$_1$ chemical shifts are very close. With all of the proper components in place, the engineered de novo pyrimidine pathway was combined with pentose phosphate enzymes and cofactor regeneration of ATP and NADP$^+$ to synthesize UTP from HCO$_3^-$, NH$_4^+$, aspartate, and glucose in one pot. Although the E. coli enzymes for the de novo pyrimidine pathway downstream from E. coli carAB functioned well, the requirement for ubiquinone as a hydrogen acceptor by E. coli dihydro-orotase dehydrogenase (pyrD) in the fourth step of de novo pyrimidine synthesis was very inconvenient. Possibility of ubiquinone recycling was daunting because the multisubunit membrane associated enzymes such as succinate dehydrogenase or NADH dehydrogenase would be required. However, dihydro-orotase dehydrogenase A (pydA) from Lactococcus lactis (a class 1A member of the dihydro-orotase dehydrogenase family) was found to utilize glucose as its hydrogen acceptor, and this enzyme-cofactor pair proved to be a suitable replacement.

Regeneration of ATP and NADP$^+$, as shown in Scheme 1B, is often desired since the H$_5$ and H$_1$ chemical shifts are very close. With all of the proper components in place, the engineered de novo pyrimidine pathway was combined with pentose phosphate enzymes and cofactor regeneration of ATP and NADP$^+$ to synthesize UTP from HCO$_3^-$, NH$_4^+$, aspartate, and glucose in one pot. Although de novo pyrimidine enzymes cpkA and pydG accept dATP with slightly reduced activity, the deoxynucleotide byproducts can be easily separated from the product NTPs during the affinity purification step. With all of the proper components in place, the engineered de novo pyrimidine pathway was combined with pentose phosphate enzymes and cofactor regeneration of ATP and NADP$^+$ to synthesize UTP from HCO$_3^-$, NH$_4^+$, aspartate, and glucose in one pot. Although de novo pyrimidine enzymes cpkA and pydG accept dATP with slightly reduced activity, the deoxynucleotide byproducts can be easily separated from the product NTPs during the affinity purification step.

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successful in a single pot reaction. There is one limitation placed on possible labeling patterns using this scheme due to the production of CO₂ by decarboxylation of OMP to UMP and 6-phosphogluconate to ribulose-5-phosphate during PRPP generation. These steps couple the carbon source for the C2 of the pyrimidine ring to C1 of aspartate and C1 of glucose. Degassing and inert atmosphere are sufficient to prevent isotope dilution from air and solvent at the C2 position (see Supplementary Figure 8). If C2 labeling is desired without ribose labeling, PRPP can be generated enzymatically from unlabeled ribose. In addition, ¹³C₁-aspartate is readily available and can be included to label C2 without C₄, C₅, C₆ labeling.

Implementation of the Enzymatic Synthesis. There are 18 enzymes required to implement the de novo enzymatic synthesis of UTP and CTP. The majority of the pentose phosphate pathway and cofactor recycling enzymes are commercially available or have been cloned previously. The pyrimidine specific enzymes were cloned from E. coli given our previous success with other E. coli pathways. In addition, carbamoylphosphate synthase like carbamate kinase from Pyrococcus furiosus was cloned and substituted in the first step of the de novo pyrimidine pathway. Similarly, dihydro-orbate dehydrogenase was cloned from Lactococcus lactis and used in place of the E. coli enzyme in the fourth step of de novo pyrimidine synthesis. Due to the lack of suitable activity assays, it was not possible to determine the specific activities for steps 2–4 of de novo pyrimidine synthesis. Instead, an assumed specific activity of 0.5 U/mg was used for aspartate carbamoyl transferase, dihydro-ornotase, and dihydro-ornotate dehydrogenase. The amounts for the unassayed enzymes were empirically optimized during pilot scale reactions. For best results, it is advisable to use fresh preparations all of the enzymes to prepare nucleotides on a scale sufficient for transcription reactions. To illustrate the flexibility and utility of the enzymatic synthesis, five different labeled nucleotides were synthesized, as shown in Figure 2.

Synthesis of U-¹⁵N, ²H₁, ₄F₃, ₅F₅-UTP. A 0.75 mmol scale UTP synthesis was performed with U-¹⁵N, ²H₁, ₄F₃, ₅F₅-aspartate, and ¹⁵NH₄Cl. The labeled glucose, aspartate and ammonium chloride were combined with stoichiometric substrates (KHCO₃ and sodium fumarate), fuel reagents (α-ketoglutarate, creatine phosphate), catalytic cofactors (dATP, NADP⁺), and the 16 enzymes listed in column 1 of Table 1 in
Affinity purification afforded a 30% isolated yield of U-15N, reaction is shown in Supporting Information Figure S6A. When the reaction ceased to progress. The time course for this formation was monitored by HPLC over the course of 3 days, were more efficient.

Using different preparations of the unassayed de novo enzymes otides. (1) U-15N,2H5,3

Supporting Information Figure S6B. Affinity purification afforded a 48% isolated yield of U-15N, 2H3,5,4,5,5′-CTP based on input UTP.

Synthesis of 13C6-UTP. A 0.3 mmol scale synthesis was performed using 13C2-glucose and 13C2-aspartate. The labeled glucose and aspartate were combined with stoichiometric substrates (KHCO3, sodium fumarate, NH4Cl), fuel reagents (α-ketoglutarate, creatine phosphate), catalytic cofactors (dATP, NADP+), and the 15 enzymes listed in column 3 of Table 1 in a 30 mL buffered reaction. UTP formation was monitored by HPLC over the course of 3 days when the reaction ceased to progress. The time course of this synthesis is shown in Supporting Information Figure S6C. Affinity purification afforded a 25% yield based on input glucose.

Synthesis of U-13C, 15N-UTP. A 0.75 mmol scale synthesis was performed using U-13C-glucose, U-13C, 15N-aspartate, 15NH4Cl, and NaH13CO3. The labeled substrates were combined with stoichiometric substrate (sodium fumarate), fuel reagents (α-ketoglutarate, creatine phosphate), catalytic cofactors (dATP, NADP+), and the 16 enzymes listed in column 4 of Table 1 in a 75 mL buffered reaction. UTP formation was monitored by HPLC over the course of 1 day when the reaction ceased to progress. The time course of this synthesis is shown in Supporting Information Figure S6D. Affinity purification afforded a 45% yield based on input glucose. Since equimolar amounts of aspartate and glucose are included in the reaction, the yield is also 45% based on input aspartate.

Synthesis of U-13C, 15N-UTP. A 0.75 mmol scale synthesis was performed using U-C-glucose, U-13C-aspartate, and NaH13CO3. The labeled substrates were combined with stoichiometric substrates (sodium fumarate, ammonium chloride), fuel reagents (α-ketoglutarate, creatine phosphate), catalytic cofactors (dATP, NADP+), and the 15 enzymes listed in column 5 of Table 1 in a 75 mL buffered reaction. UTP formation was monitored by HPLC over the course of 2 days when the reaction ceased to progress. The time course of this synthesis is shown in Figure 3. Affinity purification afforded a 40% yield based on input glucose.

Incorporation of Labeled Nucleotides into RNA. To demonstrate the utility of the labeled nucleotides, HIV-2 TAR RNA was transcribed in vitro using U-13C, 15N-ATP, U-13C, 15N-GTP, and the U-13N, 2H3,5,4,5,5′-CTP and U-13N, 2H3,5,4,5,5′-CTP prepared as described above. Relative to the 13C-edited NOESY spectrum, these two spectra are of particular interest since vital secondary and tertiary structural information can be gained through understanding the network of hydrogen bonding, base stacking and conformational features of base and ribose moieties. However, the informative areas of RNA proton spectra are naturally crowded, and it is extremely difficult to discern specific correlations.

Employing our specific labeling synthesis scheme, we set out to observe a particular interaction between the H7 and H3 protons of pyrimidine nucleotides with protons bonded to 13C atoms on purines in HIV-2 TAR. The experiment focuses on the internucleotide distance constraints between the H7 and H3 protons of pyrimidine nucleotides and the 13C bound protons of purine nucleotides. The 1H, 13C filtered-edited NOESY experiment, shown in Figure 4B, revealed the two expected NOE correlations in HIV-2 TAR where the protons are ~2 Å apart: H7 of C4 to 13C-H3 of A5 and H2 of U26 to 13C-H6 of G27. Relative to the 13C-edited NOESY spectrum, these two correlations represent a small fraction of the total number of

Figure 1. Substrates and products for biosynthesis of (A) UTP and (B) CTP. Metabolic origin of pyrimidine atoms for (C) uracil and (D) cytidine. The substrates and metabolic source for each pyrimidine atom are color-coded: bicarbonate (green), ammonium (blue), and aspartate (red). The H6 hydrogen is derived from water.

Figure 2. Molecular structures of specifically labeled pyrimidine nucleotides. (1) U-15N,2H5,3-UTP, (2) U-15N,2H5,3-CTP, (3) 13C6,15N-UTP, (4) 13C-UTP, (5) 13C-UTP. Hydrogens are indicated by black circles and 15N labels are indicated by open circles.

A 0.125 mmol scale synthesis was performed in two steps. UTP formation was monitored by HPLC over the course of 3 days, when the reaction ceased to progress. The time course for this reaction is shown in Supporting Information Figure S6A. Affinity purification afforded a 30% isolated yield of U-15N, 2H3,5,4,5,5′-UTP based on input glucose. Subsequent reactions using different preparations of the unassayed de novo enzymes were more efficient.

Synthesis of U-15N,2H3,5,4,5,5′-CTP. A 0.125 mmol scale CTP synthesis was performed with U-15N, 2H3,5,4,5,5′-UTP and 15NH4Cl. The labeled UTP and ammonium chloride were combined with fuel reagent (creatine phosphate), catalytic cofactor (dATP) and the 4 enzymes listed in column 2 of Table 1 in a 125 mL buffered reaction. CTP formation was monitored by HPLC over the course of 20 h when the reaction ceased to progress. The time course for this synthesis is shown in

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NOE correlations typically observed. The application of filtered-edited and edited—edited experiments using this labeling scheme would reveal proton—proton distance constraints within the populations of pyrimidines and purines, respectively. Accessible site-specific labeling of pyrimidine bases will also advance the collection and quantitative analysis of dynamics measurements on the ps—ns and μs—ms time scales. To date, 13C relaxation studies have been conducted on uniformly labeled samples and the associated data are complicated by magnetic interactions between adjacent 13C atoms. Through the use of the one-pot synthesis scheme we present here, a variety of pyrimidine nucleotides that have 13C at isolated positions in the base can be rapidly generated. In addition to extending the size of RNA molecules that can be studied, this labeling strategy will also enable the use of sensitive relaxation dispersion experiments to measure dynamics on the μs—ms time scale, instead of depending on the power dependence methodologies.9 The relative ease and efficiency with which these nucleotides are synthesized should facilitate informative structural and dynamic studies of RNA molecules and complexes of increasing size.

Conclusions

Utilizing enzymes from several different species, we have designed an efficient and flexible method for site-specific labeling of pyrimidine nucleotides in the base and ribose moiety from readily available precursors. This method has allowed the synthesis of new labels with up to 45% yield based on input glucose and aspartate. We have shown that when used in well-designed combinations, these labeled nucleotides enable extremely specific secondary structural features in RNA to be detected by NMR. In the future, we hope to expand our enzymatic synthesis to deoxyribonucleotides to create an equally valuable set of isotope editing tools for probing the secondary structures of DNA molecules.

Experimental Methods

Materials. Chemicals were purchased from Sigma. D-glucose (13C1, 99%), D-glucose (13C2, 98%), d-glucose (13C1, 2, 3, 4, 5, 6, 98%), D-ascorbic acid (13C1, 99%), d-ascorbic acid (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%), l-lysine (13C1, 2, 3, 4, 5, 6, 98%)...
Supporting Information Table S1 lists typical yields and specific preparations of each enzyme were generally consistent. Purified protein per liter of culture depending on the enzyme, but where 1 U is the amount of enzyme required to convert 1 µmol substrate to product per minute. Special activity is reported as U per mg of enzyme. Enzymatic activities were determined for all enzymes except pyrB, pyrC, and pydA by coupling the reaction to the consumption of ATP monitored by the change in A$_{340}$ due to the action of pyruvate kinase and lactate dehydrogenase ADP and NADH.

**Enzymatic Assays.** Enzymatic activity is reported in units (U), where 1 U is the amount of enzyme required to convert 1 µmol of substrate to product per minute. Specific activity is reported as U per mg of enzyme. Enzymatic activities were determined for all enzymes except pyrB, pyrC, and pydA by coupling the reaction to the consumption of ATP monitored by the change in A$_{340}$ due to the action of pyruvate kinase and lactate dehydrogenase ADP and NADH. For the other enzymes, the estimated specific activity was assumed to be 0.5 U/mg. Table 1 shows the amount of enzyme added in units or milligrams to each synthesis.

**Nucleotide Synthesis.** Synthesis of $^{13}$C$_{1-2,3,4,5}$-UTP. Stoichiometric substrates: 18 mg (100 µmol) U-$^{13}$C-glucose, 20 mg (200 µmol) NaH$_{13}$CO$_3$, 54 mg (1 mmol) NH$_4$Cl, 34.2 mg (200 µmol) U-$^{13}$C-aspartate, 32 mg (200 µmol) sodium fumarate. Fuel reagents: 90.4 mg (400 µmol) α-ketoglutarate, 0.5 g (1.55 mmol) creatine phosphate. Catalytic cofactors: 0.7 mg (1 µmol) NADP$^+$, 5.5 mg (10 µmol) dATP. Substrates and cofactors were combined in 20 mM MgCl$_2$, 20 mM DTT, 50 mM KCl, 100 µg/mL ampicillin, 50 µg/mL kanamycin. The pH was adjusted to 9 with 1 M KOH and the solution was thoroughly flushed with Argon. Enzymes were added 0.17 U cpkA, 1.3 mg pyrIB, 1.5 mg pyrC, 1.6 mg pydA, 0.78 U pyrE, 0.86 U pyrF, 0.94 U cmk, 3.5 U ckmT, 0.48 U hxA, 0.53 U zwf, 0.58 g udpA, 0.64 U rpiA, 0.70 U prsA, 2.4 U gdhA, 2.6 U pIsA to give a final volume of 10 mL. Argon was gently bubbled again, and the reaction was sealed in a glass flask. This label was synthesized for use in the C$_2$-labeling efficiency experiment, shown in Supplementary Figure 8.

**Synthesis of U-$^{15}$N, $^{12}$H$_5$, $^{13}$C$_2$-UTP.** Stoichiometric substrates: 140 mg (0.75 mmol) $^{12}$H$_{1-2,3,4,5,6}$-glucose, 413 mg (7.5 mmol) $^{15}$NH$_4$Cl. Fuel reagents: 0.5 g (1.5 mmol) creatine phosphate. Catalytic cofactor: 41 mg (0.75 mmol) dATP. Substrates and cofactor were combined in 20 mM MgCl$_2$. The pH was adjusted to 8.5 with 1 M KOH. Enzymes were added: 3.6 U hxA, 15 U pgI, 4 U pIsA, 6 U ckmT to a final volume of 50 mL. The reaction was incubated for 90 h to allow sufficient exchange with solvent to protonate the C2 position of glucose. After $\sim$90 h the remaining reaction components for UTP synthesis were added. Stoichiometric substrates: 150 mg (1.5 mmol) KHCO$_3$, 263 mg (1.5 mmol) $^{13}$C$_2$-aspartic acid, 240 mg (1.5 mmol) sodium fumarate. Fuel reagents: 4 g (12 mmol) creatine phosphate, 0.68 g (3 mmol) α-ketoglutarate. Catalytic cofactor: 5.5 mg (7.5 µmol) NADP$^+$. Substrates and cofactors were added and pH was readjusted to 8.5 with 1 M KOH. The remaining enzymes were added as listed in Table 1 (1) to give a final volume of 75 mL.

**Synthesis of U-$^{15}$N, $^{12}$H$_5$, $^{13}$C$_2$-CTP.** 125 µmol purified U-$^{15}$N, $^{12}$H$_5$, $^{13}$C$_2$-UTP, 7 mg (12.5 µmoles) dATP, 380 mg (69 µmol) $^{13}$C$_2$-aspartic acid, and 327 mg (1 mmol) creatine phosphate were combined in 8 mM MgCl$_2$, 4 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.6) with 40 µg/mL ampicillin and 20 µg/mL kanamycin. Enzymes were added and pH was readjusted to 8.5 with 1 M KOH. The remaining enzymes were added according to Table 1 (3) giving a final volume of 30 mL.

**Synthesis of U-$^{15}$C-$^{18}$N, UTP.** Stoichiometric substrates: 139.5 mg (0.75 mmol) U-$^{15}$C-glucose, 151.5 mg (1.5 mmol) NaH$_{15}$CO$_3$, 262.5 mg (1.5 mmol) U-$^{15}$C-1-aspartic acid, 412 mg (7.5

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mmol) $^{15}$NH$_4$Cl, 240 mg (1.5 mmol) sodium fumarate. Fuel reagents: 678 mg (3 mmol) R-ketoglutarate, 3.4 g (10.5 mmol) creatine phosphate. Catalytic cofactors: 5.5 mg (7.5 µmol) NADP$^+$, 40.1 mg (75 µmol) dATP. Substrates and cofactors were combined in 20 mM MgCl$_2$, 20 mM DTT, 100 µg/mL ampicillin, 50 µg/mL kanamycin and the pH was adjusted to 8.5 with 1 M KOH. Enzymes were added according Table 1(4) giving a final volume of 75 mL.

**Synthesis of U-$^{13}$C-UTP.** Stoichiometric substrates: 139.5 mg (0.75 mmol) U-$^{13}$C-glucose, 151.5 mg (1.5 mmol) NaH$^{13}$CO$_3$, 253 mg (1.4 mmol) U-$^{13}$C- l-aspartic acid, 405 mg (7.5 mmol) NH$_4$Cl, 240 mg (1.5 mmol) sodium fumarate. Fuel reagents: 689 mg (3 mmol) α-ketoglutarate, 3.5 g (10.7 mmol) creatine phosphate. Catalytic cofactors: 5.5 mg (7.5 µmol) NADP$^+$, 40.1 mg (75 µmol) dATP. Substrates and cofactors were combined in 20 mM MgCl$_2$, 22 mM DTT, 100 µg/mL ampicillin, 50 µg/mL kanamycin and the pH was adjusted to 8.5 with 1 M KOH. Enzymes were added according Table 1(5) giving a final volume of 75 mL.

UTP and CTP formation was monitored by HPLC using a Vydac nucleotide ion exchange column (250 mM × 4.6 mM), using a linear gradient of Buffer A (25 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ (1:1) adjusted to pH 2.8 with acetic acid) and Buffer B (125 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ (1:1) adjusted to pH 2.9 with acetic acid) over 30 min at a flow rate of 2 mL/min. NTPs were monitored at 260 nm. HPLC chromatograms depicting the time course for each synthesis are included in Supporting Information.

**Nucleotide Purification.** The reaction was sterilized by filtration through a 0.2 µm filter, ammonium bicarbonate was added to a final concentration of 0.5 M, and the pH was adjusted to 10 with ammonium hydroxide. The solution was filtered again and loaded to a 20 g column of Affi-gel 601 (Biorad) boronate affinity resin equilibrated with 0.5 M ammonium bicarbonate pH 10 at 4°C. The column was washed with the same buffer and the nucleotides were eluted with water acidified with CO$_2$. The products were verified by HPLC, NMR, and mass spectrometry. Final HPLC chromatograms and mass spectra are given in Supporting Information.

**RNA Synthesis.** HIV-2 TAR RNA (5′GGCCAGAUUGAGCCUGGGAGCUCUCUGGCC3′) was synthesized by in vitro transcription with T7 RNA polymerase using a mixture of U-$^{13}$C, $^{15}$N-ATP purchased from Cambridge Isotope Laboratories, U-$^{13}$C, $^{15}$N-GTP synthesized as described previously,$^{16}$ and U-U-$^{13}$N, $^3$H$_{5,3',5',3''}$-UTP and U-$^{13}$N, $^3$H$_{5,3',5',3''}$-CTP. RNA was synthesized in a 20 mL reaction under optimized conditions: 8 mM total NTPs (2 mM

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**Figure 4.** Filtered-edited NOESY experiments on the HIV-2 RNA synthesized from U-$^{13}$N, $^2$H$_{5,3',5',5''}$ pyrimidine nucleotides and U-$^{13}$C-purine nucleotides reveal the two pyrimidine-purine dinucleotide helical segments in HIV-2 TAR. (A) The homonuclear NOESY for HIV-2 TAR is shown in black with $^1$H-edited spectra overlaid in red. (B) The $^1$H, $^{13}$C-$^1$H HSQC of the aromatic protons from the $^{13}$C-labeled purines. Resonance assignments were transferred from published values,$^{16}$ which were determined under similar conditions.
each), 40 mM Tris HCl (pH 8.1), 0.1 mM spermidine, 10 mM DTT, 24 mM MgCl₂, 0.001% Triton X-100, 80 mg/mL poly(ethylene glycol) (8000 MW), 300 nm each DNA strand (Invitrogen), and 1 mg/mL T7 RNA polymerase, incubated at 37 °C for 5 h. The RNA was purified on denaturing 20% polyacrylamide gels, electro-eluted and desalted, lyophilized and diluted in 10 mM Na₂HPO₄/NaH₂PO₄ (pH 6.5), 150 mM NaCl, 10% D₂O for recording NMR spectra.

**NMR Experiments.** The spectral benefits of site-specifically labeling base residues with ^13^C and ^15^N isotopes were assessed with two-dimensional correlation spectroscopy. All spectra were collected at 25 °C on a 900 MHz Bruker Avance spectrometer equipped with a triple resonance 5 mm TXI-HCN probe with triple-axis gradients. A ^13^C, ^15^N-filtered-edited NOESY spectrum of type “F1fF2e” was optimized for aromatic C–H correlations, acquired using a 250 ms mixing time according to published pulse programs,[^33] and compared to the analogous ^13^C–F2-edited NOESY spectrum. ^1H, ^15^N-, and ^1H, ^13^C-HSQC experiments were also collected. Water suppression for most of the edited experiments was achieved through the use of flip-back pulses and the Watergate scheme.[^35,36]

[^1] H, ^13^C-HSQC spectra employed echo/antiecho gradient selection to remove the water signal. All spectra were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate, processed with NMRPipe and viewed in NMRDraw.[^37]

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**Supporting Information Available:** HPLC chromatograms and mass spectra for each nucleotide synthesis, ^13^C NMR spectra for C₂–^13^C labeling efficiency, ^1H, ^15^N- and ^1H, ^13^C-HSQC spectra of specifically labeled HIV-2 TAR, and a table of enzyme yields and specific activities. This material is available free of charge via the Internet at http://pubs.acs.org.