Preparation of Specifically Deuterated RNA for NMR Studies Using a Combination of Chemical and Enzymatic Synthesis†

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Abstract: The syntheses of ATP, GTP, UTP, and CTP with deuterium labels on the 3′, 4′, and 5′ carbons (**2**−**5**) is described. A combination of chemical and enzymatic synthesis is used where D,L-ribose-3,4,5,5′-d_4 (±**1**) is first produced from glycerol-d_8 by chemical methods, and then the four 3′,4′,5′,5′-labeled NTPs (**2**−**5**) are prepared from (−**1**) using enzymes from the purine salvage and pyrimidine biosynthetic metabolic pathways. New procedures were developed for the large scale preparation of GTP and CTP, and existing procedures were modified for the preparation of ATP and UTP. A 30-nucleotide RNA derived from the HIV-2 TAR RNA was prepared with unlabeled NTPs and deuterated NTPs (**2**−**5**) to illustrate the dramatic effects of deuteration on the NMR spectra of RNA. The NOESY spectra of the deuterated RNA exhibits greatly reduced spectral crowding compared to that of the unlabeled RNA, and assignment of NOEs to the H2′ protons is simplified due to the specific deuteration pattern. Also, the nonselective T_1 and T_2 relaxation rates were measured for the deuterated RNA and found to be approximately twice as long as the T_1 and T_2 relaxation rates of the unlabeled RNA. The spectral simplification and improved relaxation properties of the deuterated RNA should prove useful in the study of large RNAs by NMR.

Introduction

NMR spectroscopy is a powerful tool for studying the structure of RNA oligonucleotides.[1] Short sequences forming simple structures can be studied using ^{1}H NMR methods, and recently, heteronuclear methods have been developed that permit the study of structures up to ≈35 nucleotides.[2−16] Two significant problems encountered in application of NMR to the study of larger RNA molecules are spectral crowding in the ribose region and the large line widths. While the base, imino, and H1′ protons in RNA have good chemical shift dispersion, the remaining protons of the ribose ring, comprising over half of the nonexchangeable protons in RNA, resonate between 4 and 5 ppm. Since there are many biologically interesting RNAs that are significantly larger than 35 nucleotides, it is important to develop techniques that would allow the study of these larger RNAs using NMR. We have developed a combined chemical and enzymatic synthesis of ribonucleotides deuterated at the 3′, 4′, and 5′ carbons of ribose. These nucleotides will help reduce the spectral crowding in the ribose region and also reduce the proton line widths by reducing dipolar relaxation. Specifically deuterated nucleotides should prove to be a valuable tool in extending the size of RNAs that can be studied by NMR.

Deuteration has been used advantageously in the study of proteins and nucleic acids with NMR spectroscopy by simplification of coupling patterns, reduction of line widths, removal of unessential resonances, increasing NOE intensities, and as a probe of dynamics.[17−31] Although uniform ^{13}C and ^{15}N labeling of proteins and RNA have been applied to macromolecular

† Abbreviations: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; NTP, nucleoside triphosphate; RNA, ribonucleic acid; nt, nucleotides; NMR, nuclear magnetic resonance; ppm, parts per million; NOE, nuclear Overhauser effect; DIBAL-H, diisobutylaluminum hydride; PRPP, 5-phospho-D-ribosyl α-1-pyrophosphate; PEP, phosphoenolpyruvate; OMP, orotidine 5′-monophosphate; IR, infrared; UV, ultraviolet; EtOAc, ethyl acetate; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; calcd, calculated; HRMS, high-resolution mass spectrometry; HRFABMS, high-resolution fast atom bombardment mass spectrometry; DMSO, dimethyl sulfoxide; Et_2O, diethyl ether; Et, ethyl; Bn, benzyl; THF, tetrahydrofuran; AcOH, acetic acid; tBuOH, *tert*-butyl alcohol; NADH, β-nicotinamide adenine dinucleotide, reduced form; NAD^+, β-nicotinamide adenine dinucleotide; Tris, tris-(hydroxymethyl)aminomethane; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; LB medium, Luria-Bertani medium; OD, optical density; OPRT, orotate phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; XGPRT, xanthine−guanine phosphoribosyltransferase; TEABC, triethylammonium bicarbonate; 3PGA, 3-phosphoglycerate; R5P, ribose 5-phosphate; PP_i, inorganic pyrophosphate; DTT, dithiothreitol.

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(1) Varani, G.; Tinoco, I. J. *Q. Rev. Biophys.* **1991**, *24*, 479−532.
(2) Puglisi, J. D.; Tan, R.; Calnan, B. J.; Frankel, A. D.; Williamson, J. R. *Science* **1992**, *257*, 76−80.
(3) Peterson, R. D.; Bartel, D. P.; Szostak, J. W.; Horvath, S. J.; Feigon, J. *Biochemistry* **1994**, *33*, 5357−5366.
(4) Battiste, J. L.; Tan, R.; Frankel, A. D.; Williamson, J. R. *Biochemistry* **1994**, *33*, 2741−2747.
(5) Battiste, J. L.; Tan, R.; Frankel, A. D.; Williamson, J. R. *J. Biomol. NMR* **1995**, *6*, 375−389.
(6) Marino, J. P.; Gregorian, R. S.; Csankovszki, G.; Crothers, D. M. *Science* **1995**, *268*, 1448−1454.
(7) Puglisi, J. D.; Chen, L.; Blanchard, S.; Frankel, A. D. *Science* **1995**, *270*, 1200−1203.
(8) Allain, F.; Varani, G. *J. Mol. Biol.* **1995**, *250*, 333−353.
(9) Aboul-ela, F.; Karn, J.; Varani, G. *J. Mol. Biol.* **1995**, *253*, 313−332.
(10) Greenbaum, N. L.; Radhakrishnan, I.; Hirsh, D.; Patel, D. J. *J. Mol. Biol.* **1995**, *252*, 314−327.
(11) Nowakowski, J.; Tinoco, I. J. *Biochemistry* **1996**, *35*, 2577−2585.
(12) Jucker, F. M.; Pardi, A. *Biochemistry* **1995**, *34*, 14416−14427.
(13) Hall, K. B. *Methods Enzymol.* **1995**, *261*, 542−559.
(14) Pardi, A. *Methods Enzymol.* **1995**, *261*, 350−380.
(15) Ye, X.; Kumar, R. A.; Patel, D. J. *Chem. Biol.* **1995**, *2*, 827−840.
(16) Allain, F. H.; Gubser, C. C.; Howe, P. W.; Nagai, K.; Neuhaus, D.; Varani, G. *Nature* **1996**, *380*, 646−650.
(17) LeMaster, D. M. *Prog. NMR Spectrosc.* **1994**, *26*, 371−419.
(18) Arrowsmith, C. H.; Pachter, R.; Altman, R. B.; Iyer, S. B.; Jardetzky, O. *Biochemistry* **1990**, *29*, 6332−6341.
(19) Hsu, V. L.; Armitage, I. M. *Biochemistry* **1992**, *31*, 12778−12784.
(20) Wagner, G. *J. Biomol. NMR* **1993**, *3*, 375−385.
(21) Farmer, B. T.; Venters, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 4187−4188.
(22) Yamazaki, T.; Lee, W.; Arrowsmith, C. H.; Muhandiram, D. R.; Kay, L. E. *J. Am. Chem. Soc.* **1994**, *116*, 11655−11666.
(23) Nietlispach, D.; Clowes, R. T.; Broadhurst, R. W.; Ito, Y.; Keeler, J.; Kelly, M.; Ashurst, J.; Oschkinat, H.; Domaille, P. J.; Laue, E. D. *J. Am. Chem. Soc.* **1996**, *118*, 407−415.
(24) Grzesiek, S.; Anglister, J.; Ren, H.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 4369−4370.

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structure determination with great advantage, introduction of the isotopic label results in broader line widths for protons due to the strong dipolar interaction with directly bonded ^{13}C and ^{15}N nuclei.[17,21−24] This effect is particularly problematic for larger molecules. Deuterium labeling offers the alternative advantage of spectral simplification without causing broader line widths. One deuteration pattern that would benefit NMR of RNA is the selective deuteration of the ribose 3′, 4′, and 5′ carbons. Much of the structural information available from NMR spectra of RNA can be derived from the base protons, the ribose H1′ and H2′ protons, and the exchangeable protons. The glycosidic torsion can be estimated from the magnitude of base to H1′ NOEs, the sugar pucker can be estimated from the scalar coupling between the H1′ and H2′, and base pairing information is obtained from the exchangeable protons. Deuteration of the 3′, 4′, and 5′ carbons would still permit this information to be obtained; however, the spectral assignment would be greatly simplified.

Since large RNAs are most readily prepared by transcription with T7 RNA polymerase, our goal was to prepare deuterated nucleoside triphosphates (NTPs).[32] One method for producing isotopically labeled NTPs is to harvest total cellular RNA from bacteria that have been grown on isotopically labeled media, enzymatically digest the RNA to nucleoside monophosphates (NMPs), and finally enzymatically convert the NMPs to NTPs. Uniform ^{13}C, ^{15}N, and $^{13}C/^{15}N$ labeling of NTPs has been accomplished by this method by growing *Escherichia coli* or *Methylophilus methylotrophus* on ^{13}C and/or ^{15}N labeled media.[33−36] Unfortunately it is difficult to incorporate specific isotopic labels with this method due to isotopic scrambling during biosynthesis. A second method that can be used for producing deuterated NTPs involves selective oxidation of one of the hydroxyl groups of ribose, followed by reduction with deuteride. This method has been used to deuterate both H5′ protons of thymidine, as well as the H3′ of adenosine.[37,38] Schemes using selective oxidation/reduction of nucleosides to incorporate deuterium labels work well for incorporation of deuterium on a single carbon, but become cumbersome when applied to deuteration of multiple carbons, and also do not readily offer the possibility for ^{13}C and ^{15}N incorporation. A third method for incorporating isotopic labels into nucleotides is to isotopically label the ribose or base separately, followed by chemical coupling to form a nucleoside.[39] This method allows a large flexibility in isotopic labeling since the ribose or the bases can be prepared and labeled separately and then combined. A variety of isotopic labels have been incorporated into nucleosides using this general scheme including ribose deuterated by Raney nickel−^2H_2O exchange,[40] H1-deuterated ribose,[41] H2-deuterated ribose,[42] and ribose ^{13}C labeled at the C1 or C2.[43,44] This method also presents problems, however, since to chemically couple ribose to a base, D-ribose must first be protected as acetyl 2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranoside or some equivalent, which is a multistep synthetic process. Furthermore, once nucleosides have been formed chemically, they must still be converted into NTPs, which requires additional chemical and enzymatic steps.[45] Another method that has been used, and the one that we have adopted, is isotopic labeling of the ribose or base separately, followed by enzymatic coupling to form a nucleotide. Enzymatic coupling allows the direct conversion of ribose into nucleotides or nucleosides without the use of protecting groups, which greatly simplifies the synthetic effort required to produce isotopically labeled NTPs from ribose. This strategy has been used to produce [1′-^{14}C]AMP and [1′-^{3}H]AMP starting with isotopically labeled ribose and adenine using adenine phosphoribosyltransferase.[46] Purine nucleoside phosphorylases have also been used to couple [C8-^{13}C]purines to ribose-1-phosphate to form [C8-^{13}C]purine nucleosides.[45]

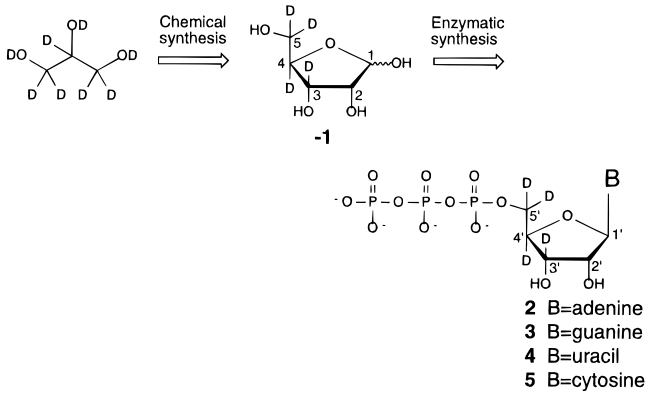

Figure 1. Strategy for synthesis of deuterated nucleotides.

To synthesize 3′,4′,5′,5′-labeled NTPs (d_4-NTPs) we have developed a synthetic strategy that uses chemical and enzymatic methods, outlined in Figure 1. Chemical synthesis is used to convert glycerol-d_8 into D-ribose-*3,4,5,5*-d_4 (D-d_4-ribose, **−1**), and then four enzymatic reactions are used to convert that ribose into the four d_4-NTPs (**2**−**5**) with a minimum amount of effort. The strategy utilizes chemical synthesis to place deuterium at specific positions, and enzymatic synthesis to perform a series of complex transformations in a single coupled reaction. These deuterated ribonucleotides should prove useful for the study of large RNA molecules, and the preparative methods we have developed will prove useful for a wide variety of labeling applications.

(25) Brush, C. K.; Stone, M. P.; Harris, T. M. *Biochemistry* **1988**, *27*, 115−122.
(26) Puglisi, J. D.; Wyatt, J. R.; Tinoco, I. *J. Mol. Biol.* **1990**, *214*, 437−453.
(27) Yamakage, S.-I.; Maltseva, T. V.; Nilson, F. P.; Földesi, A.; Chattopadhyaya, J. *Nucleic Acids Res.* **1993**, *21*, 5005−5011.
(28) Agback, P.; Maltseva, T. V.; Yamakage, S.-I.; Nilson, F. P. R.; Földesi, A.; Chattopadhyaya, J. *Nucleic Acids Res.* **1994**, *22*, 1404−1412.
(29) Muhandiram, D. R.; Yamazaki, T.; Sykes, B. D.; Kay, L. E. *J. Am. Chem. Soc.* **1995**, *117*, 11536−11544.
(30) Wang, A. C.; Kennedy, M. A.; Reid, B. R.; Drobny, G. P. *J. Mag. Reson. Ser. B* **1994**, *105*, 1−10.
(31) Földesi, A.; Yamakage, S. I.; Nilsson, F. P.; Maltseva, T. V.; Chattopadhyaya, J. *Nucleic Acids Res.* **1996**, *24*, 1187−1194.
(32) Wyatt, J. R.; Chastain, M.; Puglisi, J. D. *BioTechniques* **1991**, *11*, 764−769.
(33) Batey, R. T.; Inada, M.; Kujawinski, E.; Puglisi, J. D.; Williamson, J. R. *Nucleic Acids Res.* **1992**, *20*, 4515−4523.
(34) Nikonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, *20*, 4507−4513.
(35) Hoffman, D. W.; Holland, J. A. *Nucleic Acids Res.* **1995**, *23*, 3361−3362.
(36) Batey, R. T.; Battiste, J. L.; Williamson, J. R. *Methods Enzymol.* **1995**, *261*, 300−322.
(37) Frank, B. L.; Worth, L. J.; Christner, D. F.; Kozarich, J. W.; Stubbe, J.; Kappen, L. S.; Goldberg, I. H. *J. Am. Chem. Soc.* **1991**, *113*, 2271−2275.
(38) Robins, M. J.; Samano, V.; Johnson, M. D. *J. Org. Chem.* **1990**, *55*, 410−412.
(39) Vorbrüggen, H.; Krolikiewicz, K.; Bennua, B. *Chem. Ber.* **1981**, *114*, 1234−1255.
(40) Földesi, A.; Nilson, F. P. R.; Glemarec, C.; Gioeli, C.; Chattopadhyaya, J. *Tetrahedron* **1992**, *48*, 9033−9072.
(41) Toyama, A.; Takino, Y.; Takeuchi, H.; Harada, I. *J. Am. Chem. Soc.* **1993**, *115*, 11092−11098.
(42) Cook, G. P.; Greenberg, M. M. *J. Org. Chem.* **1994**, *59*, 4704−4706.
(43) Lancelot, G.; Chanteloup, L.; Beau, J.; Thuong, N. T. *J. Am. Chem. Soc.* **1993**, *115*, 1599−1600.
(44) Kline, P. C.; Serianni, A. S. *J. Am. Chem. Soc.* **1990**, *112*, 7373−7381.
(45) SantaLucia, J.; Shen, L. X.; Cai, Z.; Lewis, H.; Tinoco, I. *Nucleic Acids Res.* **1995**, *23*, 4913−4921.
(46) Parkin, D. W.; Leung, H. B.; Schramm, V. L. *J. Biol. Chem.* **1984**, *259*, 9411−9417.

Results and Discussion

Chemical Synthesis. For the chemical synthesis, our strategy was to build upon well-established carbohydrate syntheses to produce D-d_4-ribose (**-1**) from commercially available, isotopically labeled starting materials. The ribose synthesis was based on acyclic carbohydrate syntheses, where 2,3-*O*-isopropylidene-D-glyceraldehyde is extended by a Wittig reaction, and the resulting double bond is then dihydroxylated with osmium tetroxide, or by Sharpless epoxidation with subsequent ring opening.[47−49] The desired labeling pattern can be obtained if the protected D-glyceraldehyde is prepared perdeuterated, since the 3, 4, and 5 carbons of ribose will arise from D-glyceraldehyde. Unfortunately, common methods of synthesizing 2,3-*O*-isopropylidene-D-glyceraldehyde, such as the periodate oxidation of 1,2:5,6-di-*O*-isopropylidene-D-mannitol,[50] do not conveniently allow for deuteration, so a method for obtaining perdeuterated 2,3-*O*-isopropylidene-D-glyceraldehyde was developed. Initial attempts involved preparation of perdeuterated D-glyceraldehyde from perdeuterated allyl alcohols using asymmetric dihydroxylation.[51] This method had the advantage of producing a single enantiomer, but the multistep synthesis of the perdeuterated allyl alcohol had a low overall yield. A higher yield of the cyclohexyl ketal of perdeuterated D-glyceraldehyde (**7**) was obtained from the racemic mixture of perdeuterated glyceraldehydes produced from a simple two-step synthesis starting with commercially available glycerol-d_8. Since the ultimate product of the chemical synthesis, D,L-ribose, can be subsequently resolved by the enzymes used to convert D-ribose into NTPs, the disadvantage of a racemic synthesis was offset by the higher total yield of D-glyceraldehyde and ease of preparation.

The synthesis of D,L-d_4-ribose (±**1**) from glycerol-d_8 is shown in Figure 2. Protection of glycerol-d_8 is followed by Swern oxidation of ketal (**6**), resulting in D,L-glyceraldehyde-1,2,3,3-d_4 ketal (**7**). The triethylamine from the Swern oxidation was carefully neutralized to prevent deuterium exchange by enolization that can occur under basic protic conditions.[48] The crude aldehyde was unstable to silica gel purification, and was directly submitted to a Wittig reaction with $Ph_3P{=}CHCO_2Et$ in methanol at 0 °C, which preferentially forms the *Z* olefin,[49] to produce (**8**) in a 76% yield. Reduction of the ester with DIBAL-H and protection of the resulting alcohol (**9**) afforded the benzyl ether (**10**). Facial diastereoselectivity in the osmium tetroxide cis dihydroxylation of the *Z* olefin[47] resulted in the desired protected ribitol (±**11**) and protected lyxitol diastereomers in a 7:3 ratio. After chromatographic separation of the diastereomers, protection of the *cis*-diol, and removal of the benzyl ether, Swern oxidation of ±**13** gave the protected ribose (±**14**). Again, the triethylamine was carefully neutralized by an aqueous workup, to prevent epimerization under basic conditions.[48] The aldehyde (±**14**) was unstable to silica gel purification, and hydrolysis of the crude aldehyde (±**14**) afforded crude D,L-d_4-ribose (±**1**). The yield of ribose was quantitated by enzymatic assay of the crude racemic ribose mixture. The overall yield of the 10 step synthesis was 12% D-d_4-ribose (−**1**), (24% ±**1**), as calculated from the starting material glycerol-d_8. The crude D,L-d_4-ribose (±**1**) prepared in this manner was suitable for direct use in the subsequent enzymatic reactions.

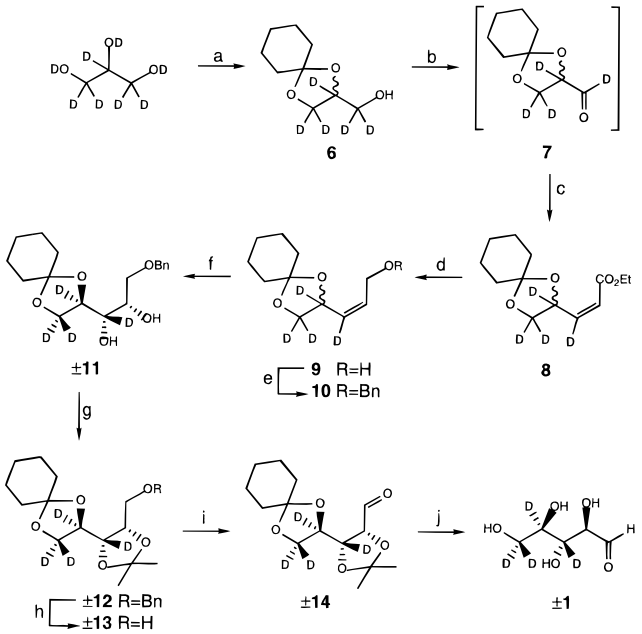

Figure 2. Synthesis of d_4-ribose. Reagents: (a) cyclohexanone, $(MeO)_3$-CH, H^+ (b) oxalyl chloride, DMSO, Et_3N (c) $Ph_3P{=}CHCO_2Et$, MeOH (d) DIBAL-H, CH_2Cl_2 (e) BnBr, NaH, $(nBu)_4NI$, THF (f) OsO_4, *N*-methylmorpholine *N*-oxide, acetone:H_2O (8:1) (g) 2-methoxypropene, H^+ (h) Pd on C, H_2 (i) oxalyl chloride, DMSO, Et_3N (j) H^+, THF, H_2O.

Enzymatic Synthesis. Our strategy for the enzymatic synthesis was to utilize phosphoribosyltransferases for coupling of 5-phospho-D-ribosyl α-1-pyrophosphate (PRPP) with nitrogenous bases to form nucleoside monophosphates. Reactions catalyzed by phosphoribosyltransferases can be easily linked to enzymatic PRPP synthesis and NTP formation, allowing conversion of ribose into a nucleoside triphosphate in a single coupled enzymatic reaction, as has been shown previously by Schramm.[46] Preparative scale enzymatic reactions for PRPP synthesis from ribose[52] and for NTP formation from NMPs[53] have been described previously. Enzymatic procedures for converting ribose into AMP and UMP have been reported, offering enzymatic routes to ATP[46] and UTP.[52] We have developed enzymatic reactions that produce GTP from ribose and CTP from UTP, to complete the set of four NTPs required for RNA synthesis. By combining these methods we were able to form d_4-NTPs (**2**−**5**) from D-d_4-ribose (−**1**) in a minimum number of steps in high yield.

Some of the enzymes necessary for these conversions were commercially unavailable or only available in costly or low specific activity forms. These enzymes were purified from overexpressing strains using a general purification scheme which readily afforded partially purified enzymes. The enzyme purification scheme used requires at most one chromatographic step and results in preparations that are free from undesirable contaminating activities, such as nonspecific ATPases. Each enzyme preparation from 1 L of bacterial culture yielded enough enzyme to catalyze several, from 7 to 180 depending on the enzyme, reactions of the type described here.

PRPP Synthesis. The PRPP used in NTP formation was generated in situ from D-ribose in a manner similar to that used by Gross *et al.*[52] D-d_4-ribose (−**1**) was first phosphorylated by ribokinase to produce ribose-3′,4′,5′,5′-d_4 5-phosphate (d_4-R5P), which was pyrophosphorylated by PRPP synthetase to form PRPP-3′,4′,5′,5′-d_4 (d_4-PRPP, **15**), as shown in Figure 3a. In

(47) Cha, J. K.; Christ, W. J.; Kishi, Y. *Tetrahedron* **1984**, *40*, 2247−2255.
(48) Ko, Y. S.; Lee, A. W. M.; Masamune, S.; Reed, L. A. I.; Sharpless, K. B.; Walker, F. J. *Tetrahedron* **1990**, *46*, 245−264.
(49) Katsuki, T.; Lee, A. W. M.; Ma, P.; Martin, V. S.; Masamune, S.; Sharpless, K. B.; Tuddenham, D.; Walker, F. J. *J. Org. Chem.* **1982**, *47*, 1373−1378.
(50) Jackson, D. Y. *Synth. Commun.* **1988**, *18*, 337−341.
(51) Sharpless, K. B.; Amberg, W.; Bennani, Y. L.; Crispino, G. A.; Hartung, J.; Jeong, K.; Kwong, H.; Morikawa, K.; Wang, Z.; Xu, D.; Zhang, X. *J. Org. Chem.* **1992**, *57*, 2768−2771.
(52) Gross, A.; Abril, O.; Lewis, J. M.; Geresh, S.; Whitesides, G. M. *J. Am. Chem. Soc.* **1983**, *105*, 7428−7435.
(53) Simon, E. S.; Grabowski, S.; Whitesides, G. M. *J. Org. Chem.* **1989**, *55*, 1834−1841.

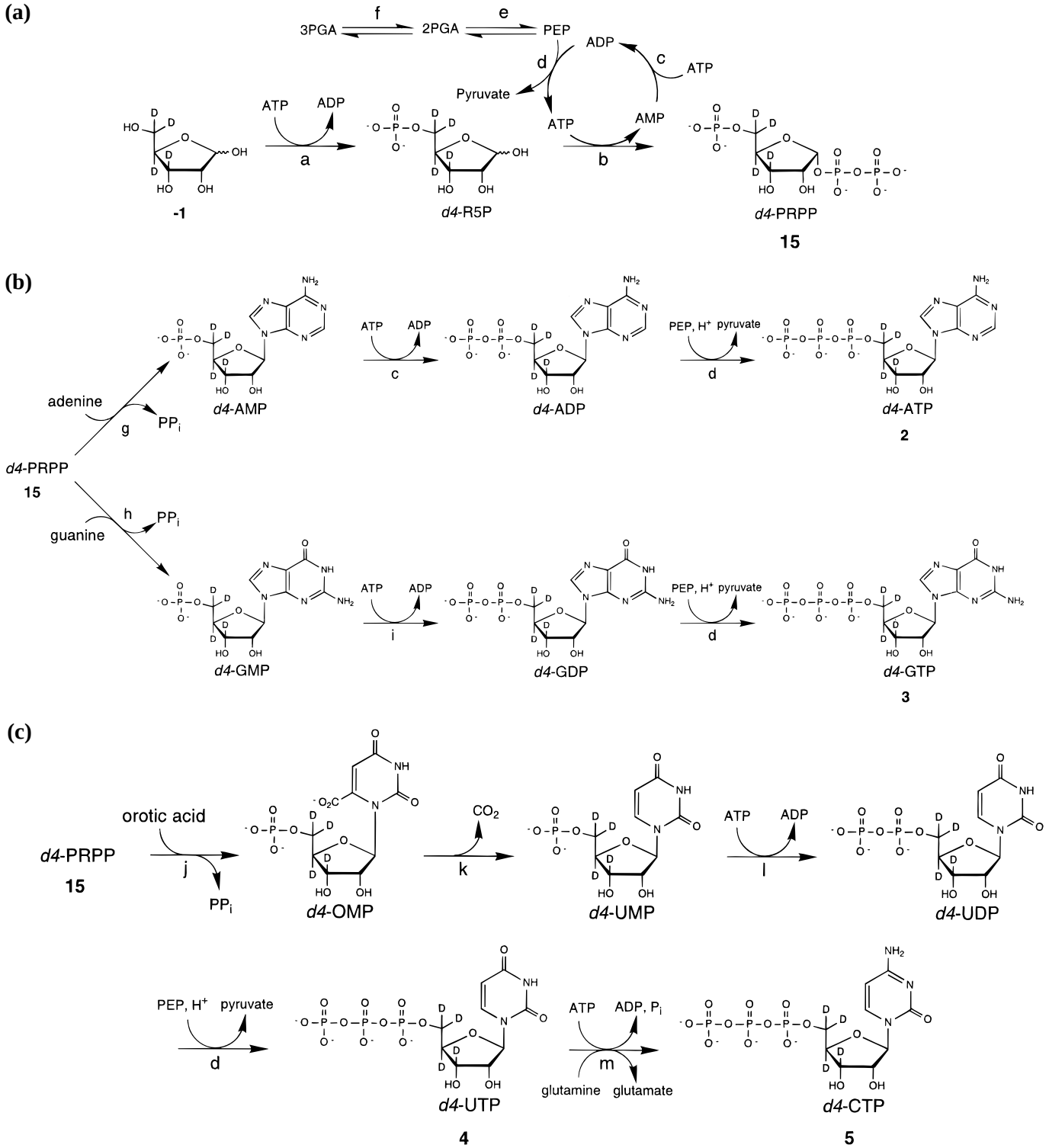

Figure 3. (**a**) Enzymatic synthesis of labeled PRPP from labeled D-ribose. Also shown is ATP regeneration by PEP/PK/MK system driven by 3PGA. Enzymes are (a) ribokinase, (b) PRPP synthetase, (c) myokinase, (d) pyruvate kinase, (e) enolase, and (f) 3-phosphoglycerate mutase. PRPP synthesis was coupled directly to ATP, GTP and UTP formation shown in parts b and c. (**b**) Enzymatic synthesis of purines ATP and GTP using purine salvage. Enzymes are (c) myokinase, (d) pyruvate, kinase, (g) adenine phosphoribosyltransferase, (h) xanthine−guanine phosphoribosyltransferase, and (i) guanylate kinase. (**c**) Enzymatic synthesis of UTP and CTP using pyrimidine biosynthesis. Enzymes are (d) pyruvate kinase, (j) orotate phosphoribosyltransferase, (k) OMP decarboxylase, (l) nucleoside monophosphate kinase, and (m) CTP synthetase. In the preparative reactions, CTP synthesis is performed in a reaction separate from UTP formation.

all of the enzymatic reactions, the ATP consumed was regenerated by a PEP/pyruvate kinase/myokinase system, where the phosphoenolpyruvate (PEP) used to drive the reaction was generated in situ from an excess of 3-phosphoglycerate,[53,54] also shown in Figure 3a. Reactions containing PRPP synthetase were carried out in potassium phosphate buffer to prevent the inactivation of PRPP synthetase that occurs at low phosphate concentration.[55] One difficulty encountered in PRPP synthesis was the inhibition of PRPP synthetase by ADP in the presence of ribose 5-phosphate, resulting in extremely slow nucleotide formation in our reactions due to a buildup of ADP and ribose 5-phosphate.[56] The buildup of ADP was prevented by reducing the amount of myokinase and increasing the amount of pyruvate

(54) Hirschbein, B. L.; Mazenod, F. P.; Whiteside, G. M. *J. Org. Chem.* **1982**, *47*, 3765−3766.

(55) Switzer, R. L.; Gibson, K. J. *Methods in Enzymology* **1978**, *LI*, 3−11.

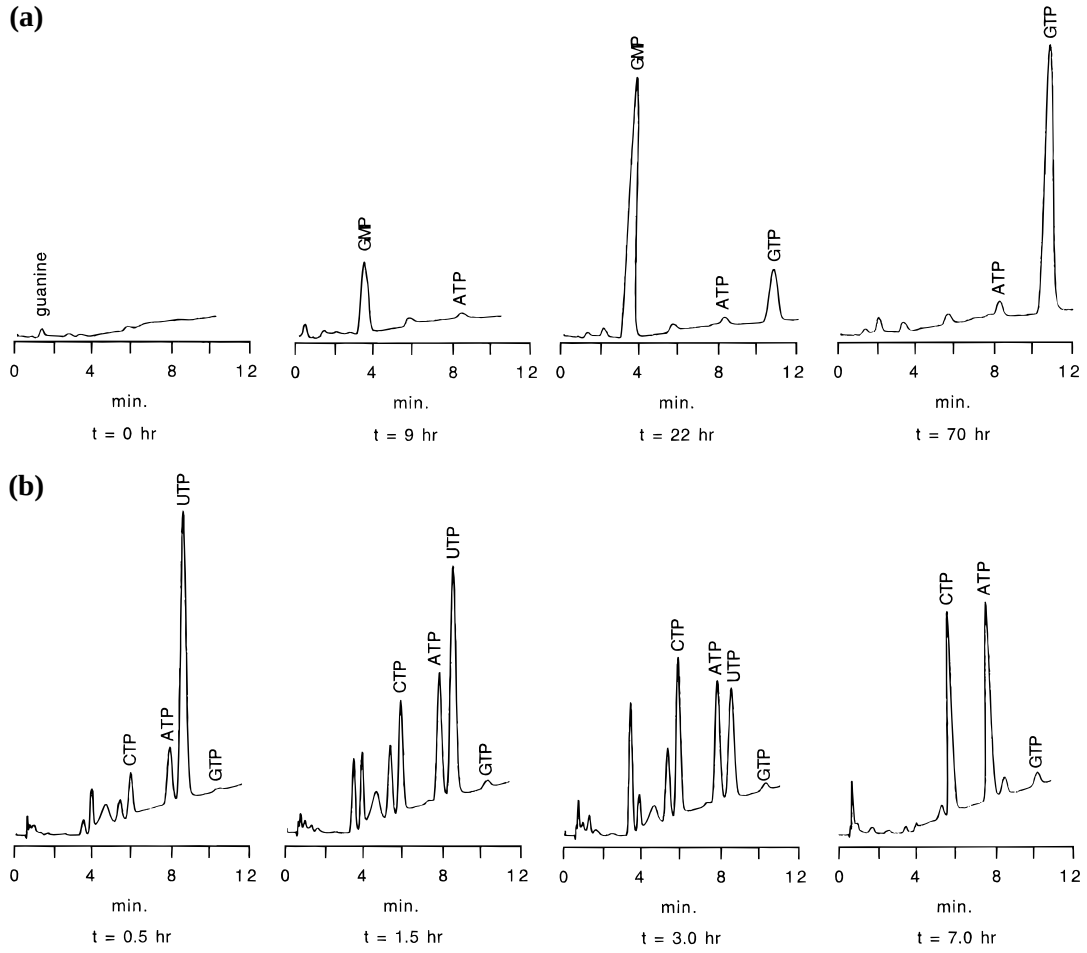

Figure 4. HPLC chromatograms of (a) GTP- and (b) CTP-forming reactions.

kinase added to the reactions, to insure that the rate of ADP formation from AMP was much less than the rate of ATP formation from ADP and PEP.

ATP Synthesis. ATP-*3′,4′,5′,5′-d*$_4$ (*d*$_4$-ATP, **2**) was formed in a coupled enzymatic reaction from D-*d*$_4$-ribose (−**1**) in a manner similar to that described by Schramm.[46] *d*$_4$-PRPP (**15**) was first generated in situ as described above (Figure 3a), and the purine salvage enzyme adenine phosphoribosyltransferase (APRT) was used to catalyze the condensation of PRPP and adenine to form *d*$_4$-AMP, shown in Figure 3b. *d*$_4$-ATP (**2**) was then formed in the same reaction by the sequential action of myokinase and pyruvate kinase on *d*$_4$-AMP (Figure 3b). A catalytic amount of unlabeled ATP was added to the *d*$_4$-ATP-forming reaction to initiate the reaction. In all of the other reactions, *d*$_4$-ATP was used as a cofactor to prevent dilution of the isotopic labels. The initial rate of ATP formation was very slow, but the reaction rate increases quickly as the concentration of ATP increases. *d*$_4$-ATP (**2**) was produced from (**-1**) in 78% isolated yield by this method.

GTP Synthesis. The synthesis of GTP-*3′,4′,5′,5′-d*$_4$ (*d*$_4$-GTP, **3**) was accomplished by utilizing the *E. coli* purine salvage enzyme xanthine−guanine phosphoribosyltransferase (XGPRT). First, *d*$_4$-PRPP (**15**) was generated in situ (Figure 3a) and then xanthine−guanine phosphoribosyltransferase was used to couple *d*$_4$-PRPP (**15**) and guanine to form *d*$_4$-GMP, as shown in Figure 3b. Once *d*$_4$-GMP had been formed, the sequential action of guanylate kinase and pyruvate kinase in the same reaction produced *d*$_4$-GTP (**3**) (Figure 3b). Although guanine has a very low solubility in water, GMP formation occurs at an appreciable rate when solid guanine is added to the reaction in a slurry. Figure 4a shows HPLC traces during the course of a preparative GTP synthesis. Only a small guanine peak can be detected in the HPLC traces, but 2 equiv (per ribose) of solid guanine were present in the reaction. During the course of the reaction, GMP formation can be observed at 9 and 22 h, and the conversion of GMP to GTP is seen in the final HPLC trace. *d*$_4$-GTP (**3**) was produced in 75% yield from D-*d*$_4$-ribose (−**1**) by this method.

UTP Synthesis. UTP-*3′,4′,5′,5′-d*$_4$ (*d*$_4$-UTP, **4**) was synthesized from D-*d*$_4$-ribose (−**1**) using two pyrimidine biosynthetic enzymes, orotate phosphoribosyltransferase and orotidine 5′-monophosphate decarboxylase (OMP decarboxylase), which catalyze the formation of UMP from PRPP and orotate. Gross *et al*.[52] have used these two enzymes to form UMP from PRPP and orotate, and we have extended this method to produce UTP from D-ribose and orotate. As in the *d*$_4$-ATP (**2**) and *d*$_4$-GTP (**3**) forming reactions, *d*$_4$-PRPP (**15**) was first generated in situ (Figure 3a), and then orotate phosphoribosyltransferase was used to catalyze the condensation of orotate and PRPP to form *d*$_4$-OMP, which was then decarboxylated by OMP decarboxylase forming *d*$_4$-UMP, shown in Figure 3c. The sequential action of nucleoside monophosphate kinase and pyruvate kinase then produced *d*$_4$-UTP (**4**) in 90% isolated yield from (−**1**) (Figure 3c).

CTP Synthesis. CTP-*3′,4′,5′,5′-d*$_4$ (*d*$_4$-CTP, **5**) was synthesized from *d*$_4$-UTP (**4**) by ATP dependent amination of UTP with glutamine or ammonia using the *E. coli* pyrimidine biosynthetic enzyme CTP synthetase, as shown in Figure 3c. CTP synthetase proved to be a difficult enzyme to work with since the activity of the enzyme is regulated by the concentration of substrates and products in solution.[57,58] The substrates ATP and UTP are activators for CTP synthetase, and the product

(56) Switzer, R. L.; Sogin, D. C. *J. Biol. Chem.* **1973**, *248*, 1063−1073.
(57) Long, C. W.; Pardee, A. B. *J. Biol. Chem.* **1967**, *242*, 4715−4721.
(58) Anderson, P. M. *Biochemistry* **1983**, *22*, 3285−3292.

CTP can act as an inhibitor. Using conditions similar to those used for synthesis of the other nucleotides, no CTP production was observed with CTP synthetase. After trying several different reaction conditions, it was found that by diluting UTP concentration from approximately 5.5 to 1 mM and increasing the ATP concentration from 0.17 to 0.22 mM, it was possible to convert UTP into CTP. Under these conditions CTP concentration is low enough to prevent feedback inhibition, and the ATP concentration is high enough to keep the enzyme active at low UTP concentrations. Because of the problems encountered with CTP synthetase, CTP was produced from UTP rather than the large coupled enzymatic reactions where ATP, GTP, and UTP were produced directly from ribose. In addition, a small amount of GTP was added as a positive affector of glutamine dependent amine transfer.[57] The conversion of UTP into CTP catalyzed by CTP synthetase was monitored by HPLC as shown in Figure 4b. The amount of ATP relative to the other nucleotides is much greater in the CTP-forming reaction than in the GTP-forming reaction (Figure 4a), and additional ATP was added after 3.5 h in order to increase the rate of the reaction. During the course of the conversion of UTP into CTP, a number of NDP species become visible, but these are eventually converted to NTPs in the final HPLC trace. After careful optimizing, this deceptively simple single enzyme reaction coupled with ATP regeneration produced d_4-CTP (**5**) in 87% yield from d_4-UTP (**4**).

NMR of d_4-RNA. To illustrate the effects of the 3′,4′,5′,5′-deuteration pattern, we prepared a 30-nucleotide RNA derived from the HIV-2 TAR bulged loop by in vitro transcription with both unlabeled and deuterated NTPs. The ^{1}H NMR spectra of both in D_2O are shown in Figure 5. The spectral crowding in the ribose region of the deuterated RNA is greatly reduced, and individual H2′ resonances are clearly observed between 4 and 5 ppm. The advantages of this deuteration pattern are dramatically illustrated in a comparison of the base to ribose region of a NOESY spectrum of TAR RNA acquired on unlabeled RNA and d_4-RNA, shown in Figure 6, parts a and b, respectively. The base to H2′ NOEs are unambiguously identified in Figure 6b, and the spectral overlap in this region is greatly reduced. A similarly dramatic effect of the ribonucleotides-d_4 is observed in the ribose region of the NOESY spectrum, shown in Figure 7. The H1′ to H2′ cross peaks can be unambiguously identified in Figure 7b. The diagonal for the region between 4 and 5 ppm is completely devoid of cross peaks, except for one weak peak that results from an unusual H2′−H2′ NOE from the loop region of TAR. This peak was not previously identified in 3D- or 4D-^{13}C resolved NOESY spectra using uniformly ^{13}C-labeled TAR RNA, even though all of the ribose protons were assigned.[59]

Although the cross peaks in the NOESY spectra of d_4-RNA in Figures 6b and 7b appear marginally narrower than the unlabeled RNA in Figures 6a and 7a, relaxation rates were determined to provide a more quantitative measure of the effects of deuteration on dipolar relaxation. Nonselective T_1 and T_2 measurements were performed on unlabeled TAR and d_4-TAR RNAs, and the relaxation rates were determined for a number of resolved peaks, summarized in Table 1. The effective T_1 and T_2 relaxation times for the d_4-RNA were increased by approximately a factor of 2 compared to unlabeled RNA. These results are comparable to the effects of deuteration observed in proteins[60] and DNA.[28] Although for the 30-nucleotide TAR RNA studied here the deuteration does not qualitatively improve the NOESY spectrum by a relaxation effect, a 50% reduction of relaxation efficiency could significantly improve the spectra of RNAs that are much larger.

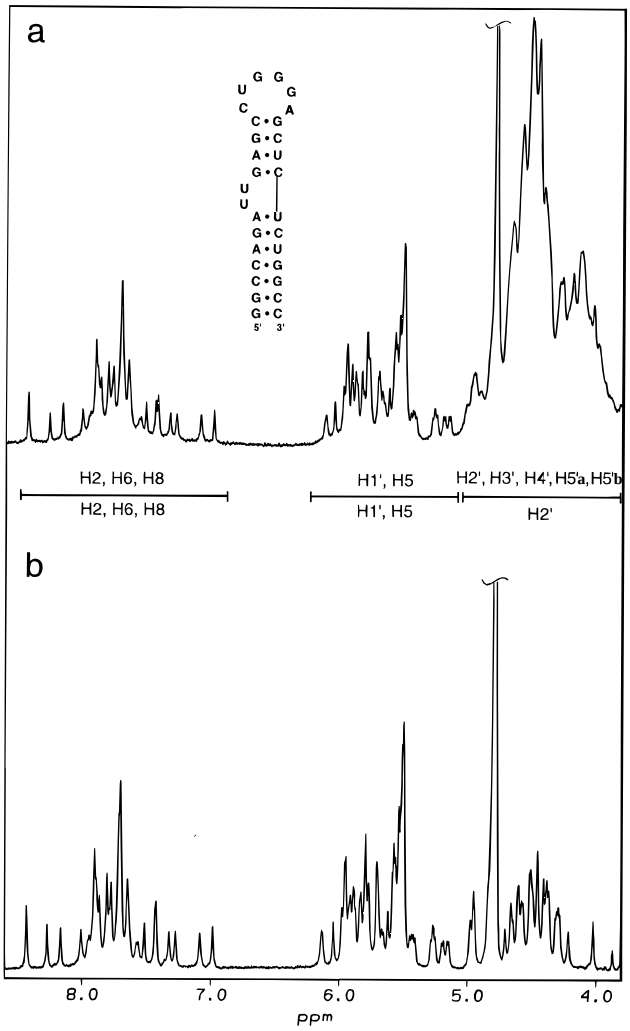

Figure 5. 500 MHz ^{1}H NMR spectra of HIV-2 TAR RNA (a) unlabeled (inset is the secondary structure of HIV-2 TAR RNA) (b) 3′,4′,5′,5′-$^2H_4$-labeled.

Conclusion

This paper describes a synthesis of d_4-NTPs (**2**−**5**) that utilizes both chemical and enzymatic techniques. A chemical synthesis of D,L-d_4-ribose (±**1**) that uses perdeuterated glycerol as a starting material and produces ±**1** in 24% overall yield is reported. Three multienzyme reactions are described that mimic purine salvage and pyrimidine biosynthetic pathways to convert D-d_4-ribose (−**1**) into d_4-ATP (**2**), d_4-GTP (**3**), and d_4-UTP (**4**) in 78%, 75%, and 90% yields, respectively. Another enzymatic reaction is reported where d_4-UTP (**4**) is converted into d_4-CTP (**5**) in 87% yield. These enzymatic reactions represent an efficient method for incorporating isotopically labeled ribose or bases into the four NTPs required for RNA transcription. The d_4-ribonucleotides described here should prove extremely valuable in the structural characterization of large RNAs using NMR spectroscopy. The method preparation of these nucleotides is sufficiently flexible that a number of different labeling patterns with ^{15}N/^{13}C/^{2}H can be readily prepared. In this way, ribonucleotides can be tailor-made to extract particular information of interest from simplified and heteronuclear spectra. Specific deuteration, in particular, offers the advantages of reduced line width and spectral simplification, without the sacrifice of sensitivity inherent in random fractional deuteration.

(59) Brodsky, A. S.; Williamson, J. R., personal communication 1996.
(60) Markus, M. A.; Dayie, K. T.; Matsudaira, P.; Wagner, G. *J. Magn. Reson. Ser. B* **1994**, *105*, 192−195.

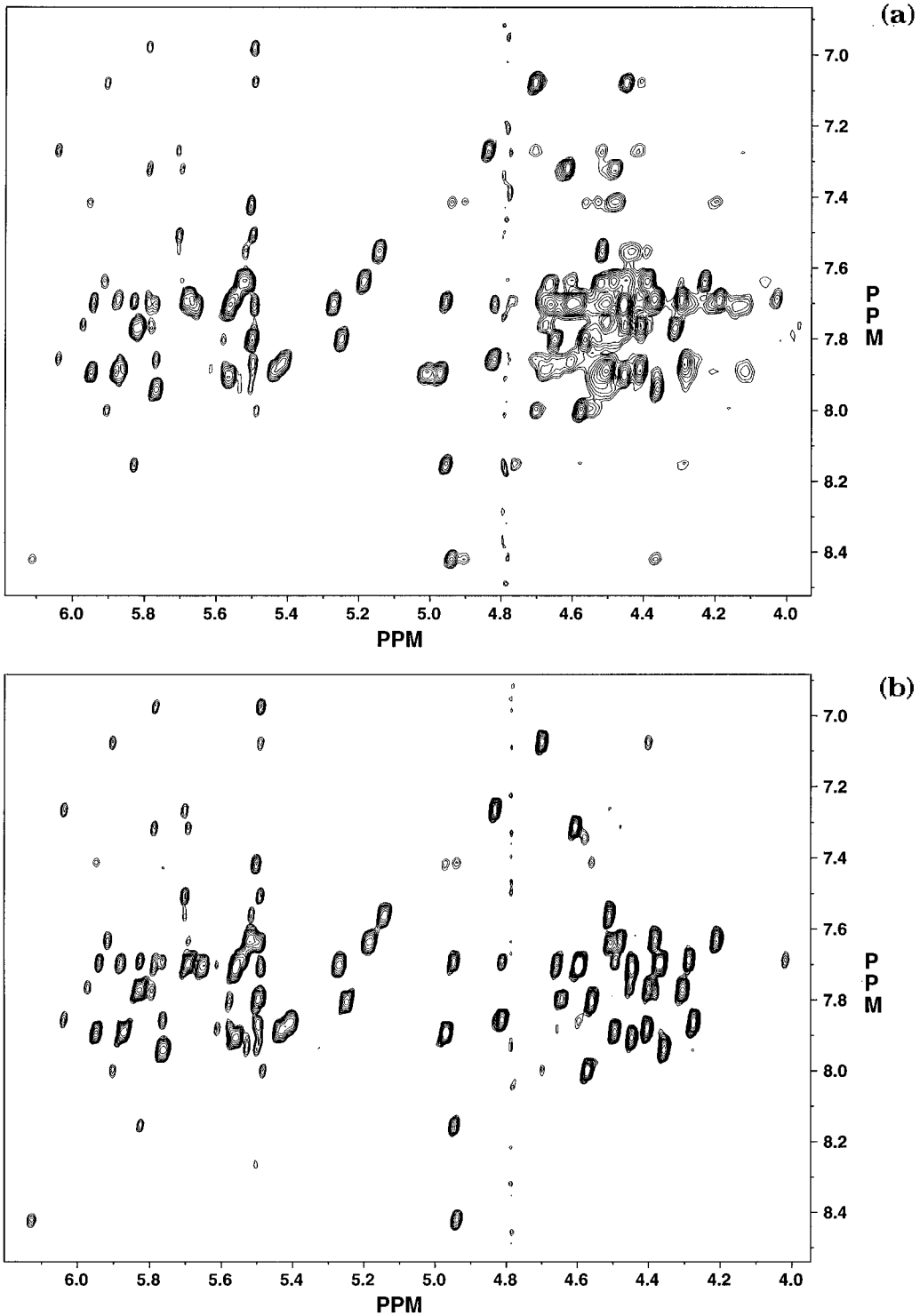

Figure 6. Expansion of the base to ribose region of a NOESY spectrum of (a) TAR RNA and (b) d_4-TAR RNA.

Experimental Section

Materials and Methods/General Procedures. Reactions were conducted under an argon atmosphere unless otherwise noted, and solvents were dried and distilled.[61] Chemicals were purchased from Aldrich and Sigma. Glycerol-d_8, D_2O, and $CDCl_3$ were purchased from Cambridge Isotopes Laboratories. Phosphoglycerate mutase from rabbit muscle and nucleoside monophosphate kinase from beef liver were purchased from Boehringer Mannheim. Enolase from baker's yeast, myokinase from chicken muscle, pyruvate kinase from rabbit muscle, and guanylate kinase from porcine brain were purchased from Sigma. One unit of enzyme corresponds to 1 μmol/min of activity under assay conditions. The pH of aqueous solutions was determined by ColorpHast Indicator Strips pH 0−14 made by EM Science. NMR spectra were recorded on a Varian XL-300 or a Varian VXR-500 spectrometer, infrared spectra were recorded on a Perkin Elmer 1600 series FT-IR, and ultraviolet spectra were recorded on a Hitachi U-2000 UV/Vis spectrophotometer. Mass spectra were obtained with a Hewlett Packard 5890/5971 GC/mass spectrometer for low resolution and a Finnigan MAT 8200 for high resolution.

D,L-2,3-*O*-Cyclohexylideneglycerol-*1,1′,2,3,3′*-d_5 (6). Cyclohexanone (3.49 g, 35.6 mmol) and trimethylorthoformate (3.77 g, 35.6 mmol) were dissolved in CH_2Cl_2 (150 mL). Glycerol-d_8 (98%) (3.56 g, 35.6 mmol) and a catalytic amount of *p*-toluenesulfonic acid (10 mg, 50 μmol) were added, and the heterogeneous mixture was stirred for 9 h at room temperature, after which the solution was homogeneous and the reaction complete. The reaction mixture was concentrated and the product purified by flash chromatography (5−40% EtOAc in

(61) Perrin, D. D.; Armarego, W. L. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon Press Inc.: New York, 1988.

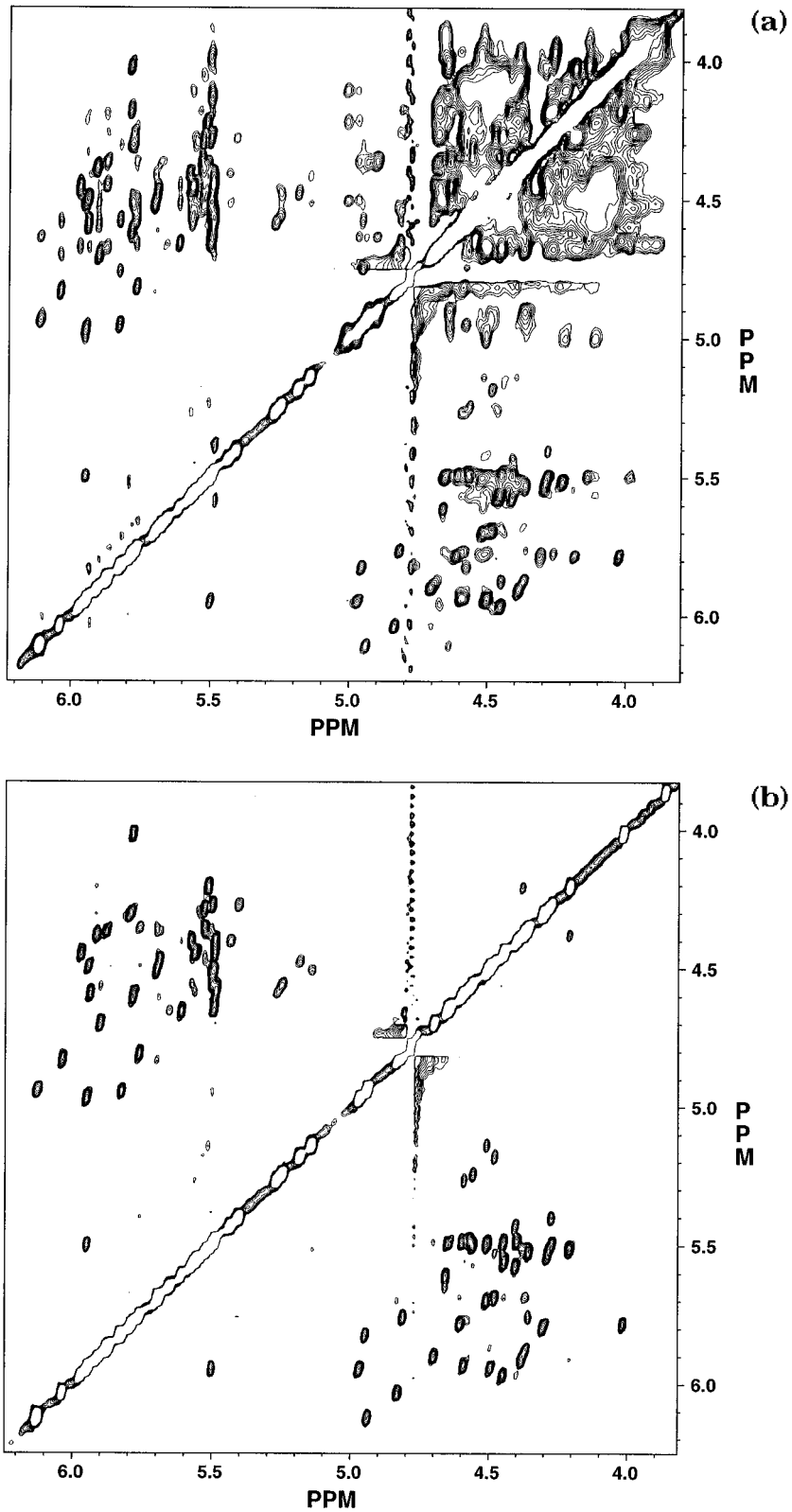

Figure 7. Expansion of the ribose to ribose region of a NOESY spectrum of (a) TAR RNA and (b) d_4-TAR RNA.

hexane) to yield 6.07 g (34.1 mmol, 95%) of **6** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.86 (s br, 1H), 1.85−1.28 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 109.7, 75.0 (t, J = 23 Hz), 64.6 (quintet, J = 23 Hz), 62.2 (quintet, J = 21 Hz), 36.2, 34.6, 24.9, 23.8, 23.6; IR (neat) 3442, 2944, 2860, 2215, 2106, 1449, 1367, 1333, 1288, 1257, 1231, 1169, 1105, 1063, 1011, 974, 941, 907; HRMS *m*/*z* 177.1409 (177.1408 calcd for $C_9H_{11}O_3{}^2H_5$, M^+).

D,L-2,3-*O*-Cyclohexylideneglyceraldehyde-*1,2,3,3′-d*$_4$ (7). Alcohol **6** was dissolved in CH_3OD and evaporated two times to exchange the hydroxyl proton, remove protons, and prevent ^{1}H exchange that can occur with the aldehyde under basic conditions. Oxalyl chloride (2.18 g, 17.2 mmol) was added to CH_2Cl_2 (200 mL) in a flask cooled to −78 °C, and DMSO (2.68 g, 34.4 mmol) was added slowly via syringe. The resulting mixture was stirred for 5 min before **6** (2.78 g, 15.6 mmol) was added. The mixture was stirred for 30 min at −78 °C, and then triethylamine (6.32 g, 62.5 mmol) was added slowly. After stirring for 15 min at −78 °C, the dry ice/acetone bath was removed. After 30 min, the reaction was quenched with 75 mL of 0.55M HCl. The organic layer was separated and washed with saturated $NaHCO_3$ (75 mL) and saturated NaCl (75 mL). The organic layer was dried over $MgSO_4$ and then concentrated. The crude aldehyde **3** was carried on directly to the next reaction without purification.

Table 1

	$\langle T_1 \rangle^a$			$\langle T_2 \rangle^c$		
	TAR	d_4-TAR	ratio[b]	TAR	d_4-TAR	ratio[d]
H2′/(H3′,H4′,H5′a,H5′b)[e]	4.3 ± 0.7	9.3 ± 1.0	2.2 ± 0.2	0.04 ± 0.01	0.12 ± 0.02	2.6 ± 0.3
H1′/H5	5.6 ± 0.7	10.3 ± 1.3	1.8 ± 0.1	0.10 ± 0.02	0.17 ± 0.03	1.8 ± 0.3
H8/H2/H6	5.4 ± 1.2	10.2 ± 1.6	1.9 ± 0.2	0.09 ± 0.03	0.17 ± 0.06	2.0 ± 0.3

[a] Average T_1 relaxation time [b] Ratio of d_4-TAR and TAR T_1 relaxation times. [c] Average T_2 relaxation time. [d] Ratio of *d4*-TAR and TAR T_2 relaxation times. [e] The global average of relaxation times were calculated for these protons in the unlabeled sample due to spectral overlap in this region.

Ethyl (*Z*)-D,L-4,5-*O*-Cyclohexylidene-2-pentenoate-*3,4,5,5-d*$_4$ (8). The crude aldehyde **7** was dissolved in 100 mL of methanol and cooled to 4 °C. $Ph_3P{=}CHCO_2Et$, (carbethoxymethylene)triphenylphosphorane, was added, and the reaction was stirred for 24 h at 4 °C. After concentration in vacuo the residue was purified by flash chromatography (5% Et_2O in petroleum ether) to yield 2.91 g (11.9 mmol, 76%) of a light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.83 (s, 1 H), 4.17 (q, 2 H, J = 7.1 Hz), 1.75−1.33 (m, 10 H), 1.29 (t, 3 H, J = 7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.5, 149.0 (t, J = 23 Hz), 120.4, 110.1, 72.5 (t, J = 24 Hz), 68.2 (quintet, J = 23 Hz), 60.2, 36.1, 34.8, 25.0, 23.8, 23.7, 14.0; IR (neat) 2981, 2935, 2862, 2229, 2174, 2111, 1716, 1634, 1448, 1372, 1349, 1272, 1198, 1164, 1117, 1036; HRMS *m*/*z* 244.1610 (244.1608 calcd for $C_{13}H_{16}O_4{}^2H_4$, M^+).

(*Z*)-D,L-4,5-*O*-Cyclohexylidene-2-penten-*3,4,5,5-d*$_4$-1-ol (9). *Z* ester **8** (4.21 g, 17.2 mmol) was added to 200 mL of CH_2Cl_2 and was cooled to 0 °C. DIBAL-H (1 M in hexanes, 43.1 mmol) was slowly added with a syringe over a period of 10 min. The mixture was stirred at 0−4 °C for 30 min, then the reaction was quenched with 100 mL of 1 M NaOH. The sodium hydroxide solution was added dropwise to prevent explosive evolution of hydrogen. The quenched reaction was stirred for 8 h at room temperature, then the aqueous and organic layers were separated. The aqueous layer was extracted with 3 × 100 mL of EtOAc. The organic layers were combined and dried over $MgSO_4$, concentrated, and purified by flash chromatography (15−30% EtOAc in hexane), affording 3.24 g (16.0 mmol, 93%) of a light yellow oil (**9**). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.81 (t, 1 H, J = 6.5 Hz), 4.28 (dd, 1 H, J = 13.2, 6.9 Hz), 4.15 (dd, 1 H, J = 13.2, 6.1 Hz), 2.95 (s br, 1 H), 1.75−1.30 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 132.9, 129.0 (t, J = 24 Hz), 109.9, 70.8 (t, J = 22 Hz), 68.2 (quintet, J = 24 Hz), 58.2, 36.2, 35.3, 24.9, 23.8, 23.7; IR (neat) 3416, 2934, 2861, 2226, 2175, 2108, 1448, 1366, 1338, 1285, 1234, 1166, 1116, 1081, 1035, 978, 928; HRMS *m*/*z* 202.1503 (202.1503 calcd for $C_{11}H_{14}O_3{}^2H_4$, M^+).

(*Z*)-D,L-1-(Benzyloxy)-4,5-*O*-cyclohexylidene-2-pentene-*3,4,5,5-d*$_4$ (10). Sodium hydride, 60% dispersion in mineral oil (2.56 g, 64.1 mmol), was washed with hexane to remove the mineral oil. THF (100 mL) was added to the sodium hydride. Allylic deuterated alcohol **9** (3.24 g, 16.0 mmol), benzyl bromide (3.01 g, 17.6 mmol), and tetrabutylammonium iodide (100 mg, 0.3 mmol) were added to this mixture. The reaction was stirred for 24 h at room temperature and quenched by addition of saturated NaCl (75 mL). Water was added to dissolve precipitated salts, and the aqueous layer was separated. The aqueous layer was extracted with three 50 mL portions of EtOAc, then the combined organic layers were dried over $MgSO_4$ and concentrated. Purification by flash chromatography (5−15% Et_2O in petroleum ether) yielded 4.49 g of the yellow oil (**6**) (15.4 mmol, 96%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.20 (m, 5 H), 5.80 (t, 1 H, J = 6.4 Hz), 4.53 (d, 1 H, J = 11.8 Hz), 4.48 (d, 1 H, J = 11.8 Hz), 4.11 (d, 2 H, J = 6.4 Hz), 1.75−1.33 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.9, 130.5 (t, J = 24 Hz), 129.8, 128.1 (2 C), 127.5 (2 C), 127.4, 109.6, 71.9, 70.9 (t, J = 23 Hz), 68.1 (quintet, J = 22 Hz), 65.4, 36.1, 35.2, 24.9, 23.7, 23.6; IR (neat) 2934, 2860, 2226, 2174, 2107, 1451, 1365, 1166, 1086, 1028, 1011, 738, 698; HRMS *m*/*z* 292.1970 (292.1972 calcd for $C_{18}H_{20}O_3{}^2H_4$, M^+).

D,L-1-(Benzyloxy)-4,5-*O*-cyclohexylideneribitol-*3,4,5,5-d*$_4$ (±11). Benzyl deuterated allyl alcohol **10** (1.71 g, 5.85 mmol), *N*-methylmorpholine *N*-oxide (1.37 g, 11.7 mmol), and osmium tetroxide 2.5% in tBuOH (3.05 g, 0.3 mmol OsO_4) were combined in a solution of 1:8 D_2O/acetone (72 mL) and stirred for 12 h at room temperature. The reaction was quenched by addition of 40 mL of 30% Na_2SO_3. After stirring for 1 h, the solution was extracted with four 75 mL portions of EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated. Separation of diastereomers was accomplished by careful flash chromatography (15% EtOAc in hexane). The slower running diastereomer corresponds to the ribitol configuration (±**11**), 1.21 g (3.7 mmol, 63%) of the yellow oil ±**11** was separated from the faster eluting diastereomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.22 (m, 5 H), 4.56 (d, 1 H, J = 11.9 Hz), 4.51 (d, 1 H, J = 11.9 Hz), 3.85 (t, 1 H, J = 4.9 Hz), 3.68 (d, 2 H, J = 4.9 Hz), 3.31 (s br, 1 H), 3.10 (s br, 1 H), 1.70−1.30 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.5, 128.3 (2 C), 127.8, 127.7 (2 C), 109.7, 75.2 (t, J = 23 Hz), 73.5, 72.3 (t, J = 22 Hz), 71.5, 70.9, 65.2 (quintet, J = 23 Hz), 36.1, 34.6, 25.0, 23.9, 23.6; IR (neat) 3442, 2934, 2861, 2153, 1452, 1367, 1286, 1106, 1008, 909, 738, 699; HRMS *m*/*z* 326.2028 (326.2027 calcd for $C_{18}H_{22}O_5{}^2H_4$, M^+).

D,L-1-(Benzyloxy)-4,5-*O*-cyclohexylidene-2,3-*O*-isopropylideneribitol-*3,4,5,5-d*$_4$ (±12). Diol ±**11** (2.03 g, 6.2 mmol), 2-methoxypropene (0.54 g, 7.5 mmol), and CH_2Cl_2 (100 mL) were combined, and a single crystal of *p*-toluenesulfonic acid was added. The reaction was stirred for 1 h and then concentrated. Purification by flash chromatography (10−20% Et_2O in petroleum ether) yielded 2.11 g of the yellow oil ±**12** (5.8 mmol, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46−7.22 (m, 5 H), 4.67 (d, 1 H, J = 12.1 Hz), 4.55 (d, 1 H, J = 12.1 Hz), 4.41 (dd, 1 H, J = 7.9, 3.1 Hz), 3.83 (dd, 1 H, J = 10.6, 3.1 Hz), 3.63 (dd, 1 H, J = 10.6, 7.9 Hz), 1.42 (s, 3 H), 1.34 (s, 3 H), 1.67−1.24 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.9, 128.1 (2 C), 127.7 (2 C), 127.4, 109.9, 108.7, 77.2 (t, J = 22 Hz), 76.7, 73.3, 72.2 (t, J = 23 Hz), 68.5, 66.7 (quintet, J = 23 Hz), 36.3, 34.7, 27.7, 25.2, 24.9, 23.8, 23.7; IR (neat) 2934, 2861, 2117, 1452, 1380, 1370, 1271, 1243, 1219, 1182, 1109, 1070, 1052, 736, 698; HRMS *m*/*z* 366.2339 (366.2340 calcd for $C_{21}H_{26}O_5{}^2H_4$, M^+).

D,L-4,5-*O*-Cyclohexylidene-2,3-*O*-isopropylideneribitol-*3,4,5,5-d*$_4$ (±13). The benzyl ribitol ±**12** (2.34 g, 6.4 mmol) was dissolved in 75 mL of THF and hydrogenated over 5% palladium on carbon at 1 atm of hydrogen for 22 h. The reaction was filtered through Celite and the filtrate was concentrated. Purification by flash chromatography (20−40% Et_2O in petroleum ether) afforded 1.62 g (5.9 mmol, 92%) of alcohol ±**13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.36 (dd, 1 H, J = 7.3, 5.7 Hz), 3.90 (dd, 1 H, J = 11.9, 7.3 Hz), 3.83 (dd, 1 H, J = 11.9, 5.7 Hz), 2.99 (s, 1 H), 1.72−1.25 (m, 10 H), 1.40 (s, 3 H), 1.35 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 110.4, 108.5, 77.6 (t, J = 24 Hz), 77.4, 72.2 (t, J = 23 Hz), 66.9 (quintet, J = 22 Hz), 60.6, 36.2, 34.6, 27.6, 25.0, 24.8, 23.9, 23.7; IR (neat) 3512, 2986, 2935, 2862, 2175, 2118, 1449, 1371, 1219, 1180, 1112, 1062, 861; HRMS *m*/*z* 276.1870 (276.1871 calcd for $C_{14}H_{20}O_5{}^2H_4$, M^+).

D,L-4,5-*O*-Cyclohexylidene-2,3-*O*-isopropylideneribose-*3,4,5,5-d*$_4$ (±14). Oxalyl chloride (53 mg, 0.42 mmol) was added to CH_2Cl_2 (200 mL) and cooled to −78 °C. DMSO (66 mg, 0.84 mmol) was added slowly via syringe, and the mixture was stirred for 5 min before the deuterated alcohol ±**13** (104 mg, 0.38 mmol) was added to the flask as a solution in CH_2Cl_2 (30 mL). After the reaction was stirred for 30 min at −78 °C, triethylamine (154 mg, 1.52 mmol) was added slowly to the mixture. After stirring for 15 min at −78 °C, the dry ice/acetone bath was removed. Thirty minutes later, the reaction was quenched with an aqueous HCl solution (15 mL, 0.12 M). The organic and aqueous layers were immediately separated, and the organic layer was washed with saturated aqueous $NaHCO_3$ solution (20 mL) and saturated aqueous NaCl solution (20 mL). The organic layer was dried over $MgSO_4$ and then concentrated. Purification by flash chromatography (20−40% Et_2O in petroleum ether) yielded 94 mg (0.34 mmol, 90%) of protected ribose (±**14**). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (d, 1 H, J = 1.8 Hz), 4.63 (d, 1 H, J = 1.8 Hz), 1.73−1.28 (m, 10 H), 1.54 (s, 3 H), 1.38 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.2, 111.2, 110.7, 81.8, 78.4 (t, J = 23 Hz), 72.6 (t, J = 23 Hz), 66.5 (quintet, J

= 23 Hz), 36.5, 34.5, 27.3, 25.4, 25.0, 23.9, 23.7; HRMS *m*/*z* 274.1715 (274.1714 calcd for $C_{14}H_{18}O_5{}^2H_4$, M^+).

D,L-Ribose-*3,4,5,5-d*$_4$ (±1). The protected deuterated ribose **±14** (22 mg, 78 μmol) was dissolved in 7 mL of H_2O:THF:AcOH (3:3:1). The flask was attached to a reflux condenser and heated to 60 °C for 24 h. The mixture was evaporated under vacuum to afford crude D,L-d_4-ribose **(±1)**. The amount of D-d_4-ribose **(−1)** produced was determined by enzymatic assay to be 31 μmol, corresponding to 78% yield from starting protected D-d_4-ribose **−14** and 39% yield from **(±14)**. NMR revealed the presence of the expected complex mixture of α- and β-anomers of the furanose and pyranose forms of ribose. HRFABMS (glycerol) *m*/*z* 137.0747 (137.0748 calcd for $C_5H_5O_4{}^2H_4$, $[M - OH]^+$).

Enzymatic D-Ribose Assay. The amount of D-d_4-ribose present in the crude racemic mixture produced by the chemical synthesis was quantitated by coupling D-ribose phosphorylation by ribokinase to oxidation of NADH. This assay is similar to the ribokinase assay of Gross *et al*.,[52] except that the amount of D-ribose is limiting and the absolute amount of NADH consumed is measured. Since there is a 1:1 relationship between NADH oxidation and D-ribose phosphorylation, the concentration of D-ribose can then be calculated by determining the amount of NADH consumed. The assay solution (1 mL) contained 0.2 mM NADH, 1 mM PEP, 10 mM $MgCl_2$, 3 mM ATP, 50 mM Tris-HCl buffer pH 7.8, 2 units of lactate dehydrogenase, 2 units of pyruvate kinase, and 1 unit of ribokinase. The assay solution was equilibrated after the addition of the enzymes, and then the assay was started by addition of a small aliquot of the unknown D-ribose solution. The absorbance of NADH at 340 nm was monitored until no further change was observed, and the total change in absorbance was used to calculate the amount of NADH consumed, and therefore the amount of D-ribose present in the aliquot, using an extinction coefficient of 6220 cm^{-1} mol^{-1}.

Cloning the Gene for Adenine Phosphoribosyltransferase. The gene encoding *E. coli* adenine phosphoribosyltransferase was cloned from the *E. coli* strain JM109 genome based on the reported gene sequence[62] using PCR with the oligonucleotides dCCG CGC GAA TTC ATG ACC GCG ACT GCA CAG CAG CTT and dCCG GCG CTG CAG TTA ATG GCC CGG GAA CGG GAC AAG as primers.[62] The PCR product was digested with *Eco*RI and *Pst*I and ligated into expression plasmid pKK223-3, that had been prepared by digestion with *Eco*RI and *Pst*I and dephosphorylation with calf intestinal alkaline phosphatase. Transformation of this construct into *E. coli* strain JM109 produced an IPTG inducible adenine phosphoribosyltransferase overproducing strain JM109/pTTA6.

Cloning the Gene for Xanthine−Guanine Phosphoribosyltransferase. The gene encoding *E. coli* xanthine−guanine phosphoribosyltransferase was cloned from the *E. coli* strain JM109 genome based on the reported gene sequence[63] using PCR with the oligonucleotides dCCG CGC GAA TTC ATG AGC GAA AAA TAC ATC GTC ACC and dCCG GCG CTG CAG TTA GCG ACC GGA GAT TGG CGG GAC as primers.[63] The PCR product was digested with *Eco*RI and *Pst*I and ligated into expression plasmid pKK223-3, that had been prepared by digestion with *Eco*RI and *Pst*I and dephosphorylation with calf intestinal alkaline phosphatase. Transformation of this construct into *E. coli* strain JM109 produced an IPTG inducible xanthine−guanine phosphoribosyltransferase overproducing strain JM109/pTTG2.

General Enzyme Purification Scheme. All seven of the enzymes not obtained commercially were overexpressed and purified using a similar purification procedure. Cells were disrupted by sonication, followed by streptomycin sulfate precipitation, ammonium sulfate precipitation, and DEAE anion exchange chromatography. The general outline of this purification is given below, with specific details and exceptions for each enzyme following.

Cells were grown at 37 °C in either LB media or A+B minimal media of Clark and Maaløe[64] containing 50 μg/mL of ampicillin and supplemented as indicated. All steps after cell growth were carried out in a 4 °C cold room or on ice, unless otherwise specified. Cells were harvested by centrifugation at 6000*g* for 15 min. The cell pellets were resuspended in the appropriate buffer and disrupted with 30 30-s sonication bursts using a Fisher Scientific 550 Sonic Dismembrator on a setting of 7, with a 2.5 min interval between bursts. Cellular debris was removed by centrifugation at 31000*g* for 30 min. A 0.1 volume of a 20% streptomycin sulfate solution was then added to the protein supernatant. After stirring for 15 min, the resulting precipitate was removed by centrifugation at 31000*g* for 30 min. The supernatant was then fractionated by ammonium sulfate precipitation. The ammonium sulfate pellets were dissolved in the appropriate buffer and dialyzed against 2 L of buffer for 8 h. The resulting solution was then loaded onto a 2.5 × 14 cm DEAE column (DEAE-650M Toyopearl resin from Supelco) that had been washed thoroughly with 3 M KCl and then equilibrated with H_2O. After loading the protein solution, the column was washed with 100 mL of H_2O, and the enzymes were eluted with a salt gradient, the fractions being collected every 8 mL. The column fractions were assayed for protein and then pooled and concentrated by ammonium sulfate precipitation. Enzymes were stored at −20 °C in 40−50% glycerol buffer solutions.

Ribokinase. The partial purification of ribokinase was accomplished by a simplified form of the method of Hope *et al.*[65] Inducible strain MRi240/pJGK10 was grown on A+B minimal media[64] supplemented with glucose (4 g/L), ribose (4 g/L), and thiamine (7 mg/L). One liter of media was inoculated with a 5 mL overnight culture of MRi240/pJGK10. The cells were grown to an OD of 0.6, then 1 mL of a solution of 3-β-indoleacrylic acid dissolved in ethanol (25 mg/mL) was added to the culture to induce ribokinase production. Cells were harvested 14 h after the addition of 3-β-indoleacrylic acid, and the cell pellet was resuspended in 40 mL of 50 mM potassium phosphate buffer, pH 7.5, and 2 mM 2-mercaptoethanol (buffer A). Cell disruption, removal of cell debris, and streptomycin sulfate precipitation were carried out as described in the general procedure. The 0−80% saturation ammonium sulfate pellet was collected and dissolved in buffer A for dialysis. DEAE chromatography was performed with a 500 mL linear gradient of 0 to 300 mM KCl in buffer A. Column fractions were assayed for ribokinase activity using the assay of Gross *et al.*[52] The final ammonium sulfate pellet was dissolved in a minimum amount of buffer A containing 40% glycerol. A total of 700 units of ribokinase were obtained from 1 L of bacterial culture.

5-Phosphoribosyl-α-1-pyrophosphate Synthetase. The partial purification of 5-Phosphoribosyl-α-1-pyrophosphate synthetase (PRPP synthetase) was accomplished by a modified form of the procedure of Switzer *et al.*[55] A 5 mL overnight culture of strain HO561/pHO11,[66] which constitutively expresses PRPP synthetase, was used to inoculate a 1 L culture in LB media which was then grown for 12 h. After cell harvest the cell pellet was resuspended in 40 mL of buffer A. After sonication and removal of cellular debris, a 0.1 volume of 20% streptomycin sulfate solution was added to the supernatant and the mixture was stirred at 0 °C for 15 min. Before centrifuging, the streptomycin sulfate mixture was heat treated in a water bath at 55 °C for 5 min and then immediately cooled to 0 °C in an ice bath for at least 4 min. The resulting precipitate was removed by centrifugation at 31000 g for 30 min. The supernatant was brought to 35% saturation of ammonium sulfate and stirred for 15 min. The ammonium sulfate pellet was collected and dissolved in 25 mL of buffer A. The pH of this solution was adjusted to 4.6 by addition of 1 M acetic acid at 0 °C, followed by immediate centrifugation for 10 min at 31000*g*. The acid precipitate was collected and dissolved in buffer A. No DEAE chromatography was used in the purification of PRPP synthetase. The resulting solution was concentrated by ammonium sulfate precipitation and stored in buffer A containing 50% glycerol. PRPP synthetase activity was assayed using the method of Gross *et al.*[52] From 1 L of bacterial culture, 47 units of PRPP synthetase were obtained.

Orotate Phosphoribosyltransferase. The partial purification of orotate phosphoribosyltransferase (OPRT) was accomplished by a modified form of the procedure of Poulsen *et al.*[67] Strain JM109/pJTA43,[68] which constitutively expresses OPRT, was grown on LB media. One liter of medium was inoculated with a 25 mL overnight culture and shaken for 14 h at 37 °C. The cells were harvested and

(62) Hershey, H. V.; Taylor, M. W. *Gene* **1986**, *43*, 287−293.
(63) Pratt, D.; Subramani, S. *Nucleic Acids Res.* **1983**, *11*, 8817−8823.
(64) Clark, D. J.; Maaløe, O. *J. Mol. Biol.* **1967**, *23*, 99−112.
(65) Hope, J. N.; Bell, A. W.; Hermodson, M. A.; Groarke, J. M. *J. Biol. Chem.* **1986**, *261*, 7663−7668.
(66) Bjarne, H.-J. *Mol. Gen. Genet.* **1985**, *201*, 269−276.
(67) Poulsen, P.; Jensen, K. F.; Valentin-Hansen, P.; Carlsson, P.; Lundberg, L. G. *Eur. J. Biochem.* **1983**, *135*, 223−229.
(68) Jensen, K. F.; Andersen, J. T.; Poulsen, P. *J. Biol. Chem.* **1992**, *267*, 17147−17152.

resuspended in 40 mL of 100 mM Tris buffer, pH 7.6, 2 mM EDTA, and 2 mM 2-mercaptoethanol (buffer B). Sonication, removal of cellular debris, and streptomycin sulfate precipitation were carried out as described in the general procedure. The 0−75% saturation ammonium sulfate pellet was collected from the supernatant of the streptomycin sulfate precipitation and then dialyzed against buffer B. The dialysate was subjected to DEAE chromatography, by eluting with a 500 mL linear gradient from 50−300 mM KCl in buffer B. Column fractions were assayed for OPRT activity according to the method of Schawartz and Neuhard.[69] Fractions containing OPRT were pooled and ammonium sulfate precipitated. The ammonium sulfate pellet was dissolved in a minimum amount of buffer B containing 40% glycerol. From 1 L of culture, 31 units of orotate phosphoribosyltransferase were obtained.

Orotidine-5′-monophosphate Decarboxylase. The partial purification of OMP decarboxylase was accomplished by a modified version of the procedure of Turnbough *et al.*[70] Strain pDK26/N100,[70] which constitutively overproduces orotidine-5′-monophosphate decarboxylase (OMP decarboxylase), was grown on A+B minimal media[64] supplemented with 8 g of glucose, 0.5 g of casamino acids, 1 mL of 240 mM UMP, and thiamine (7 mg/L). One liter of medium was inoculated with a 5 mL overnight culture and then shaken at 37 °C for 12 h. After cell harvest, the cells were resuspended in 40 mL of 64 mM Tris buffer (pH 7.8) with 5 mM 2-mercaptoethanol (buffer C). Sonication, removal of cellular debris, and streptomycin sulfate precipitation were carried out as described in the general procedure. The 45−75% ammonium sulfate pellet was collected from the supernatant of the streptomycin sulfate precipitation and dissolved in buffer C for dialysis. The resulting dialysate was subjected to DEAE chromatography, eluting with a 500 mL linear gradient of 0−300 mM NaCl in buffer C. Column fractions containing OMP decarboxylase were detected by the method of Turnbough *et al*.[70] Active fractions were pooled, ammonium sulfate precipitated, and stored in buffer C containing 40% glycerol. From 1 L of culture, 400 units of OMP decarboxylase were obtained.

Adenine Phosphoribosyltransferase. IPTG inducible, adenine phosphoribosyltransferase (APRT) overproducing strain JM109/pTTA6 was grown on A+B minimal medium[64] supplemented with 22 mL glycerol, 2 g of glucose, and thiamine (7 mg/L). The medium was inoculated with a 5 mL overnight culture, grown for 12 h, and then induced with IPTG (0.234 g/L) for another 6 h. Cell harvest and streptomycin sulfate precipitation were carried out as described in the general procedure using buffer C. The 0−80% ammonium sulfate precipitate was collected from the supernatant of the streptomycin sulfate precipitation. The ammonium sulfate pellet was dialyzed and then subjected to DEAE chromatography with a 500 mL linear gradient of 0−300 mM KCl in buffer C. Column fractions containing APRT were detected by the nonradioactive APRT assay described below. The fractions containing APRT were pooled, ammonium sulfate precipitated, and finally dissolved in a minimum amount of buffer C containing 50% glycerol and stored at −20 °C. From 1 L of culture, 360 units of APRT were obtained.

Adenine Phosphoribosyltransferase Assay. Adenine phosphoribosyltransferase activity was measured by coupling the APRT reaction to oxidation of NADH in a manner similar to the PRPP synthetase assay.[52] Conversion of NADH to NAD^+ was monitored at 340 nm. The assay solution (1 mL) contained 0.2 mM NADH, 1 mM PEP, 10 mM $MgCl_2$, 1.5 mM PRPP, 1.5 mM adenine hydrochloride, 3 mM ATP, 50 mM Tris-HCl buffer, pH 7.8, 2 units of lactate dehydrogenase, 2 units of pyruvate kinase, and 2 units of myokinase. The assay solution was equilibrated, a 20 μL aliquot of APRT solution was added to start the assay, and the absorbance change at 340 nm was monitored as a function of time. The activity was obtained using an extinction coefficient of 6220 cm^{-1} mol^{-1} for NADH.

Xanthine−Guanine Phosphoribosyltransferase. IPTG inducible, xanthine−guanine phosphoribosyltransferase (XGPRT) overproducing strain JM109/pTTG2 was grown on 1 L of LB media inoculated with a 5 mL overnight culture for 12 h and then induced with IPTG (0.234 g/L) for another 6 h. Cells were harvested and streptomycin sulfate precipitated as in the general procedure using buffer C. The 0−75% ammonium sulfate fraction was collected from the supernatant of the streptomycin precipitation and then dialyzed against buffer C. The dialysate was subjected to DEAE chromatography with a 500 mL gradient of 50−300 mM KCl in buffer C. Column fractions containing XGPRT were identified by SDS PAGE gel electrophoresis, pooled, concentrated by ammonium sulfate precipitation, and stored in buffer C containing 50% glycerol. Xanthine−guanine phosphoribosyltransferase activity was estimated by the method described below, and from 1 L of culture, 28 units of XGPRT were obtained.

Xanthine−Guanine Phosphoribosyltransferase Assay. Xanthine−guanine phosphoribosyltransferase activity was estimated by observing GMP formation in a small GMP-forming reaction by injecting aliquots onto an HPLC column as described in the preparative nucleotide synthesis section below. The assay conditions were as follows for a 2 mL reaction: 1 mM PRPP, 10 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.5, 1 mg of solid guanine, and 1 unit of inorganic pyrophosphatase. The reaction was stirred at room temperature and initiated by adding an aliquot of XGPRT solution. The time elapsed before the reaction was complete, determined by GMP formation observed by HPLC, was used to calculate the activity of the XGPRT solution. This method provides a rough estimate of the activity of the XGPRT solution. However, the assay is very similar to our GTP-forming reactions and thus suitable for empirical determination of the amount of XGPRT required for preparative reactions.

CTP Synthetase. CTP synthetase was purified by a modified form of the procedure of Anderson.[58] One liter of LB medium was inoculated with a 5 mL overnight culture of strain JM109/pMW5,[71] which constitutively overproduces CTP synthetase. The cells were grown for 12 h and then harvested. Sonication was performed as described in the general procedure in buffer C containing 20 mM glutamine. Removal of cellular debris and streptomycin sulfate precipitation were conducted as in the general procedure. From the resulting supernatant, the 0−65% ammonium sulfate pellet was extracted. This was dissolved in buffer C containing 4 mM glutamine and was dialyzed against the same buffer. The dialysate was subjected to DEAE chromatography with a 500 mL gradient of 50−300 mM potassium phosphate buffer, pH 7.5, containing 70 mM 2-mercaptoethanol and 4 mM glutamine. Column fractions were assayed by the method of Anderson,[58] and fractions containing CTP synthetase were pooled, ammonium sulfate precipitated, and dissolved in a minimum amount of buffer B containing 50% glycerol. From 1 L of culture, 38 units of CTP synthetase activity were obtained.

Preparative Nucleotide Synthesis. All enzymatic reactions were carried out under argon. Reactions were monitored by HPLC on a 25 × 4.6 mm Vydac 303NT405 nucleotide column, using a linear gradient from 100% buffer A (0.045 M NH_4CO_2 brought to pH 4.6 with phosphoric acid) to 100% buffer B (0.5 M NaH_2PO_4 brought to pH 2.7 with formic acid) in 10 min at a flow rate of 1 mL/min, with detection at 254 nm. Potassium phosphate buffer (50mM) was used in reactions containing PRPP synthetase because the enzyme is inactivated in solutions with low phosphate concentration.[55] The pH of the reactions was monitored using pH paper and adjusted periodically with 1 M NaOH or HCl to maintain the pH between 7.0 and 7.9. NTPs were purified by using boronate affinity chromatography on Affigel 601 to remove the majority of salts and proteins in order to prepare the nucleotides for transcription. Reaction mixtures were concentrated under vacuum and then dissolved in a minimum amount of 1 M triethylammonium bicarbonate (TEABC) solution, pH 9.5, prepared by bubbling CO_2 into an aqueous solution of triethylamine. In cases where a residue could not be dissolved, the solutions were vacuum filtered, and the precipitate was washed thoroughly with TEABC. These solutions were loaded onto boronate affinity columns and purified as described elsewhere.[33]

Adenosine-*3′,4′,5′,5′-d*$_4$ 5′-Triphosphate (2). Crude D,L-*d*$_4$-ribose (±**1**) containing 42.3 mg (275 μmol) of D-*d*$_4$-ribose as determined by enzymatic ribose assay, was placed in a three-necked flask containing adenine hydrochloride (87.5 mg, 510 μmol) and sodium 3-phosphoglycerate (2.55 mmol). This was dissolved in 50 mL of a solution containing 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium phosphate buffer, pH 7.5. Argon was bubbled through this solution for 5 min to remove oxygen. To this solution was added 125 units of phosphoglycerate mutase, 50 units of enolase, 200 units of pyruvate

(69) Schwartz, M.; Neuhard, J. *J. Bacteriol.* **1975**, *121*, 814−822.

(70) Turnbough, C. L.; Kerr, K. H.; Funderburg, W. R.; Donahue, J. P.; Powell, F. E. *J. Biol. Chem.* **1987**, *262*, 10239−10245.

(71) Weng, M.; Makaroff, C. A.; Zalkin, H. *J. Biol. Chem.* **1986**, *261*, 5568−5574.

kinase, 25 units of myokinase, 5 units of ribokinase, 2 units of PRPP synthetase, and 2 units of adenine phosphoribosyltransferase. The reaction was initiated by adding a catalytic amount of ATP (0.5 mg, 0.9 μmol) to this solution. The reaction was stirred at 25 °C and monitored by HPLC. The pH was maintained between 7.0 and 7.9 with 1 M HCl. An additional 0.5 mmol of 3-phosphoglycerate was added every 5 h. Formation of ATP was initially slow but increased as the reaction proceeded due to the increase in ATP concentration. The reaction was stopped after 29 h by concentration under vacuum and storage at −20 °C. Purification of the d_4-ATP (**2**) was accomplished by boronate affinity chromatography. Compound **2** was quantitated by absorption at 259 nm ($\epsilon_{259} = 15\,400$ cm^{-1} mol^{-1}). This reaction yielded 213 μmol of **2** (78% yield). ^{1}H NMR (500 MHz, D_2O) δ 8.55 (s, 1H), 8.26 (s, 1H), 6.14 (d, 1H, $J = 6.0$ Hz), 4.80 (d, 1H, $J = 6.0$ Hz); MS *m*/*z* 510.1 (510.07 calcd for $C_{10}H_{11}{}^2H_4N_5O_{13}P_3$).

Guanosine-*3′,4′,5′,5′-d*$_4$ 5′-Triphosphate (3). Crude D,L-d_4-ribose (±**1**) containing 42.3 mg (275 μmol) of D-d_4-ribose as determined by enzymatic ribose assay, was placed into a three-necked flask containing guanine (77.1 mg, 510 μmol) and sodium 3-phosphoglycerate (2.55 mmol). This was mixed with 50 mL of a solution containing 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium phosphate buffer, pH 7.5. Argon was bubbled through this inhomogeneous solution for 5 min to remove oxygen. To this mixture was added 250 units of phosphoglycerate mutase, 100 units of enolase, 200 units of pyruvate kinase, 38 units of myokinase, 5 units of ribokinase, 2 units of PRPP synthetase, and 0.2 units of xanthine−guanine phosphoribosyltransferase. The reaction was initiated by adding 4.4 μmol of d_4-ATP (**2**). The reaction was stirred at 25 °C and monitored by HPLC. The pH was maintained between 7.0 and 7.9 with 1 M HCl. Additional 3-phosphoglycerate was added to the reaction at a rate of 0.24 mmol every 5 h. After 4 h 2 units of guanylate kinase was added, and after 21 h, an additional 4.4 μmol of **2** was added. The reaction was stopped after 72 h by concentration under vacuum and storage at −20 °C. Purification of the d_4-GTP (**3**) was accomplished by boronate affinity chromatography. Compound **3** was quantitated by the absorbance at 253 nm (GTP $\epsilon_{253} = 13\,700$ cm^{-1} mol^{-1}). This reaction yielded 206 μmol of **3** (75% yield). ^{1}H NMR (500 MHz, D_2O) δ 8.14 (s, 1H), 5.93 (d, 1H, $J = 6.0$ Hz), 4.80 (d, 1H, $J = 6.0$ Hz); MS *m*/*z* 526.2 (526.07 calcd for $C_{10}H_{11}{}^2H_4N_5O_{14}P_3$).

Uridine-*3′,4′,5′,5′-d*$_4$ 5′-Triphosphate (4). Crude D,L-d_4-ribose (±**1**) containing 84.7 mg (550 μmol) of D-d_4-ribose as determined by enzymatic ribose assay, was placed into a three-necked flask containing potassium orotate (198 mg, 1.02 mmol) and sodium 3-phosphoglycerate (10.2 mmol). This was dissolved in 100 mL of a solution that contained 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium phosphate buffer, pH 7.5. Argon was bubbled through this solution for 5 min to remove oxygen. To this solution was added 375 units of phosphoglycerate mutase, 150 units of enolase, 300 units of pyruvate kinase, 38 units of myokinase, 5 units of ribokinase, 2 units of PRPP synthetase, 1.5 units of orotate phosphoribosyltransferase, and 10 units of orotidine-5′-monophosphate decarboxylase. The reaction was initiated by adding 6.5 μmol of d_4-ATP (**2**) and then stirred at 25 °C. The reaction was monitored by HPLC, and the pH was maintained between 7.0 and 7.9 with 1 M HCl. An additional 0.35 mmol of 3-phosphoglycerate was added every 5 h. After 4 h 1 unit of nucleoside monophosphate kinase was added to catalyze the formation of UDP from UMP. After 21 h, 10.9 μmol of **2** was added to stimulate the rate of the reaction. The reaction was stopped after 72 h by concentration under vacuum and storage at −20 °C. Purification of the d_4-UTP (**4**) was accomplished by boronate affinity chromatography. Compound **4** was quantitated by the absorbance at 260 nm ($\epsilon_{260} = 10\,000$ cm^{-1} mol^{-1}). This reaction yielded 495 μmol of **4** (90% yield). ^{1}H NMR (500 MHz, D_2O) δ 7.96 (d, 1H, $J = 8.1$ Hz), 5.99 (d, 1H, $J = 5.4$ Hz), 5.97 (d, 1H, $J = 8.4$ Hz), 4.38 (d, 1H, $J = 5.4$ Hz); MS *m*/*z* 487.1 (487.05 calcd for $C_9H_{10}{}^2H_4N_2O_{15}P_3$).

Cytidine-*3′,4′,5′,5′-d*$_4$ 5′-Triphosphate (5). d_4-UTP (**4**) (247 μmol), glutamine (369 mg, 2.52 mmol), and sodium 3-phosphoglycerate (505 μmol) were placed into a three-necked flask. This was dissolved in 250 mL of a solution containing 10 mM $MgCl_2$ and 50 mM Tris-HCl buffer, pH 7.8. Argon was bubbled through this solution for 5 min to remove oxygen. To this solution was added 250 units of phosphoglycerate mutase, 100 units of enolase, 200 units of pyruvate kinase, and 5.5 units of cytidine-5′-triphosphate synthetase. To initiate the reaction 26.9 μmol of d_4-ATP (**2**) and 2.5 μmol of d_4-GTP (**3**) were added. The reaction was stirred at 25 °C and monitored by HPLC. After 3.5 h, 26.9 μmol of d_4-ATP, 2.5 μmol of d_4-GTP, and 0.86 mmol of 3-phosphoglycerate were added to stimulate the rate of the reaction. The reaction was stopped after 8 h by concentration under vacuum and storage at −20 °C. Purification of the d_4-CTP (**5**) was accomplished by DEAE chromatography. The **5** was eluted from the DEAE resin with a TEABC salt gradient. Boronate affinity chromatography was not possible due to the use of tris buffer, which binds to the boronate affinity resin. Compound **5** was quantitated by absorbance at 259 and 280 nm, using the extinction coefficients ATP $\epsilon_{259} = 15\,400$, ATP $\epsilon_{280} = 1911$, CTP $\epsilon_{259} = 7204$, and CTP $\epsilon_{280} = 6905$. This reaction yielded 215 μmol of **5** (87% yield). ^{1}H NMR (500 MHz, D_2O) δ 8.07 (d, 1H, $J = 7.7$ Hz), 6.21 (d, 1H, $J = 7.7$ Hz), 5.99 (d, 1H, $J = 4.4$ Hz), 4.34 (d, 1H, $J = 4.4$ Hz); MS *m*/*z* 486.1 (486.06 calcd for $C_9H_{11}{}^2H_4N_3O_{14}P_3$).

RNA Synthesis. TAR RNA (5′GGCCAGAUUGAGCCUGGGAGCUCUCUGGCC3′) was synthesized by *in vitro* transcription with T7 RNA polymerase using unlabeled NTPs from Sigma and d_4-NTPs (**2**−**5**).[72,32] The RNA was purified by 20% PAGE, electroeluted, and dialyzed against 50 mM NaCl, 0.1 mM EDTA, and 10 mM sodium phosphate, pH 6.4.

NMR Experiments. NOESY spectra of TAR and d_4-TAR were recorded on a Varian VXR-500 spectrometer with a spectral width of 5500 Hz, acquiring 4096 complex points in t_2 and 644 in t_1, 64 or 32 scans per FID for TAR and d_4-TAR, respectively, a relaxation delay of 1.6 s, and a mixing time of 0.200 s. Sample conditions were 50 mM NaCl, 0.1 mM EDTA, 10 mM sodium phosphate, pH 6.4, 25 °C. RNA concentrations were 0.83 mM for TAR and 1.12 mM for d_4-TAR. Twice the number of scans were acquired per FID for the unlabeled TAR sample to compensate for the difference in concentrations between the two RNA samples.

Nonselective T_1 relaxation rates for individual resonances were measured using inversion recovery, and nonselective T_2 relaxation rates were measured using a CPMG experiment. For both T_1 and T_2 measurements a relaxation delay of 75 s between each of four scans was used.

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(72) Milligan, J. F.; Uhlenbeck, O. C. *Methods Enzymol.* **1989**, *180*, 51−62.