Synthesis and Separation of Diastereomers of Ribonucleoside 5′-(α-P-Borano)triphosphates

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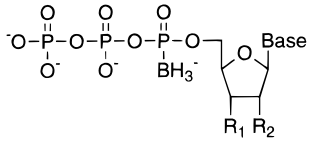
Nucleoside boranophosphates, in which one of the phosphate oxygens is replaced by a borane group, are isoionic and isoelectronic analogues of naturally occurring nucleotides. Boranophosphates also are biochemically important congeners of phosphorothioates and methylphosphonates. We have developed a convenient one-pot method to synthesize the set of ribonucleoside (A, U, G, and C) 5′-(α-P-borano)triphosphates. Phosphitylation of the 2′,3′-protected ribonucleoside with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one gives the 5′-phosphite intermediate **2** which undergoes in situ substitution in the presence of pyrophosphate to give the cyclic intermediate, P^2,P^3-dioxo-P^1-ribonucleosidylcyclotriphosphate **3**. Immediate oxidation of compound **3** with amine•borane complex results in ribonucleoside 5′-(α-P-borano)cyclotriphosphate **4**. Subsequent reaction of compound **4** with water followed by ammonium hydroxide yields the crude product as a diastereomeric mixture of ribonucleoside 5′-(α-P-borano)triphosphate **6**. Pure compound **6** is isolated in 30−45% overall yield using ion-exchange chromatography. The separation of two diastereomers of ribonucleoside 5′-(α-P-borano)triphosphate **6** is achieved by reverse phase HPLC.

Introduction

Deoxyribo- and ribonucleoside triphosphates are the basic building blocks for enzymatic synthesis of DNA and RNA in vitro and in vivo. Modified nucleoside triphosphates have received much attention in searches for potential diagnostic and therapeutic agents, as well as for compounds for studying numerous biochemical and pharmacological processes.[1,2]

The nucleoside 5′-(α-P-borano)triphosphates comprise a new class of modified nucleotides in which one of the α-phosphate oxygens in nucleoside 5′-triphosphate is replaced by a borane group (BH_3) (Scheme 1).[3] The borane moiety in boranophosphate is isoionic and isoelectronic with oxygen in normal phosphate and phosphorothioate[4] and isosteric with the methyl group (CH_3) in methylphosphonate.[5,6] Consequently, boranophosphate analogues have a number of biochemical properties that are similar to those of the phosphorothioates and methylphosphonate analogues. The 2′-deoxy boronated nucleoside triphosphates, like the 2′-deoxyribonucleoside 5′-thiotriphosphates, are substrates for DNA polymerase enzymes and can be successfully incorporated into DNA.[7−9] Once in DNA, the boranophosphate linkage is more resistant to exo- and endo-nucleases than that of phosphate diesters.[9−11] The increased nuclease resistance of boranophosphate may also be useful in stabilizing RNA. In addition, the P−B bond should have a different polarity than a P−O bond, and thereby, the introduction of boranophosphate into RNA could be useful for a variety of studies on RNA structure and function, such as metal−RNA interaction and the mechanism of RNA cleavage.[12]

Scheme 1



dNTPαB: R_1 = OH, R_2 = H
Base = T, A, G, C, U;
NTPαB: R_1 = OH, R_2 = OH
Base = U, A, G, C

One approach for generating boranophosphate oligoribonucleotides is direct chemical synthesis, reported only for synthesis of diuridine 3′,5′-boranophosphate.[13] The other approach is to employ an enzymatic method, but this requires synthesis of a set of 5′-(α-P-borano)triphosphate ribonucleosides (NTPαB) as substrates for polymerases. The availability of NTPαB would also permit investigations where other backbone-modified nucleoside triphosphates have been used,[14,15] since the

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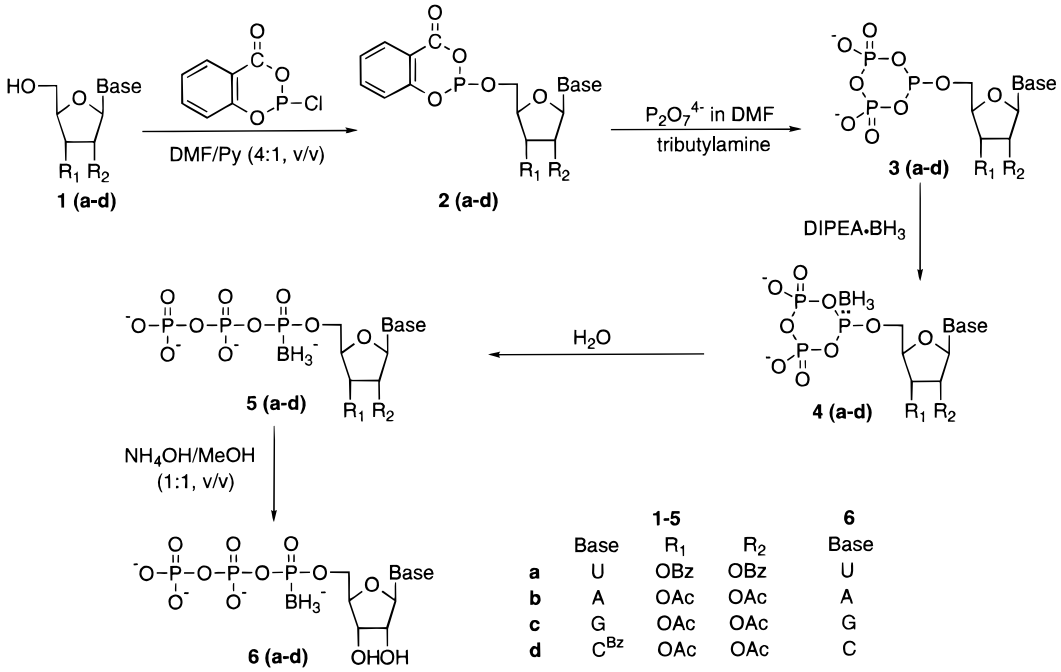
Scheme 2



	1-5 Base	R_1	R_2	6 Base
a	U	OBz	OBz	U
b	A	OAc	OAc	A
c	G	OAc	OAc	G
d	C^{Bz}	OAc	OAc	C

boronated backbone (P−BH_3) resembles several biochemically important modified backbones (P−O, P−S, and P−CH_3). These potential applications of NTPαB led us to develop a chemical synthesis for NTPαB. In this paper, we report for the first time the synthesis and separation of diastereomers of the four ribonucleoside 5′-(α-P-borano)triphosphates (NTPαB) of adenine, uracil, guanine, and cytosine.

Results and Discussion

We initially attempted to synthesize NTPαB by modification of the phosphoramidite approach reported for the synthesis of the deoxy analogues of 5′-(α-P-borano)-triphosphates (dNTPαB).[16] Although one can synthesize NTPαB by using this method, we encountered certain limitations and disadvantages. For example, this method required exocyclic amine protection, isolation of one intermediate compound, and two ion-exchange column chromatography steps, resulting in low overall yield. These limitations led us to explore an alternative synthetic method to increase the yield as well as to decrease the time and cost. Here we have synthesized NTPαB as outlined in Scheme 2 by modification of the method reported by Ludwig and Eckstein for nucleoside 5′-thiotriphosphates.[17]

Phosphitylation of ribonucleoside **1** with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one yielded the two diastereomers of ribonucleoside 5′-(4H-1,3,2-benzodioxaphosphorin-4-one) **2**. They were identified by the appearance of two signals at δ 128.12 and 126.18, observed in the ^{31}P NMR spectra of the reaction mixture. Instead of introduction of the borane group (BH_3) after the phosphitylation stage, as in the phosphoramidite approach,[16] intermediate **2** was treated directly with pyrophosphate to form a cyclic intermediate P^2,P^3-dioxo-P^1-ribonucleosidylcyclotriphosphate **3**. In the ^{31}P NMR spectrum of the reaction mixture containing compound **3**, the upfield shift of peaks from δ 128.12 and 126.18 to a triplet at δ 106.72 (J = 40.48 Hz) along with a doublet at δ −18.55 and −18.81 (J = 42.09 Hz) supported the formation of intermediate **3**.[17]

An in situ boronation of intermediate **3** by the exchange reaction with borane−*N*,*N*-diisopropylethylamine complex (DIPEA•BH_3) at room temperature for 6 h resulted in the boranophosphate intermediate, ribonucleoside 5′-(α-P-borano)cyclotriphosphate **4**. According to valence shell electron pair repulsion (VSEPR) theory and molecular orbital (MO) theory, the boron atom in BH_3 carries an empty 2p orbital and, therefore, is extremely susceptible to attack by nucleophiles.[18] Thus, monoborane is willing to accept a pair of electrons. These electrons are provided by a Lewis base, such as an amine or a phosphine. As the Lewis base strength of the tertiary phosphite ($(RO)_3P$) is much greater than that of trialkylamine toward BH_3,[19] the tertiary phosphite intermediate **3** displaced *N*,*N*-diisopropylethylamine in DIPEA•BH_3 to form the boron−phosphorus coordination compound, the phosphite borane **4**. The formation of a P → B bond in intermediate **4** was confirmed on the basis of ^{31}P NMR (Figure 1A), where the triplet at δ 106.72 in compound **3** was completely shifted to a broad peak centered at δ 86.72 in compound **4**. A slight upfield shift of the doublet at δ −21.64 and −21.92 (J = 45.33 Hz) of P^2 and P^3 peaks in compound **4** was also observed. We tried several amine•borane complexes for the boronation step and found that the borane−pyridine complex caused decomposition of intermediate **3** and resulted in nonboronated triphosphate, observed with ^{31}P NMR. In the ^{31}P NMR spectrum, the peak at δ 106.72 in intermediate **3** disappeared, and new peaks at δ −8.40 (pyrophosphate) and at δ −5.90, −12.56, and −20.96 (normal triphosphate) were observed. Furthermore, treatment with the more

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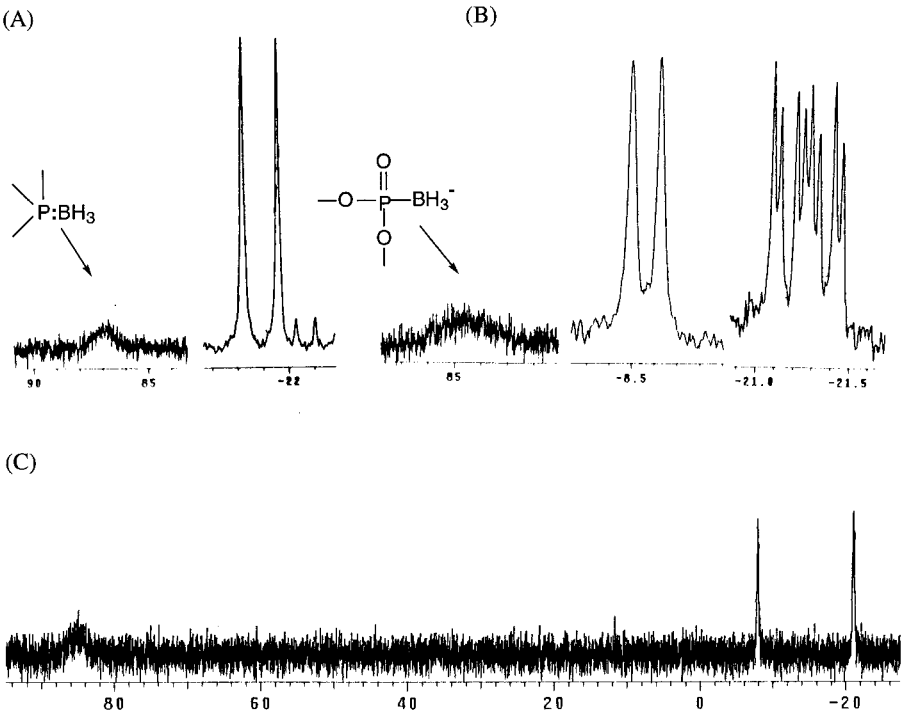
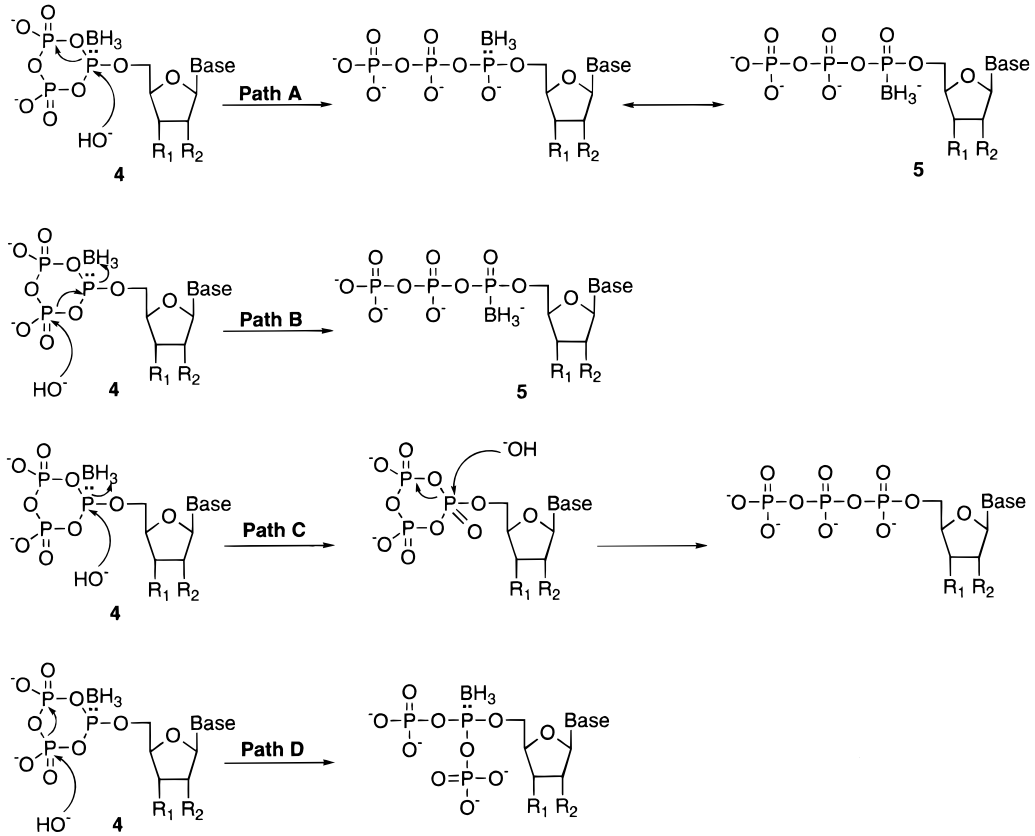


Figure 1. (A) Selected regions of the ^{31}P NMR spectrum (ppm) of **4a**. (B) Selected regions of the ^{31}P NMR spectrum (ppm) of **5a**. (C) ^{31}P NMR spectrum (ppm) of **6a**.

Scheme 3



reactive borane−tetrahydrofuran complex under the same conditions resulted in the reduction of nucleoside (unpublished results).

The cyclic intermediate **4** underwent hydrolytic opening by treatment with water to give ribonucleoside 5′-(α-P-borano)triphosphate **5**. Although the nucleophilic attack on intermediate **4** by water might occur at the boranophosphate (paths A and C in Scheme 3) or at the phosphate (paths B and D in Scheme 3), we have not detected any branched triphosphate[20] derived from opening of the pyrophosphate bond (path D). This was to be

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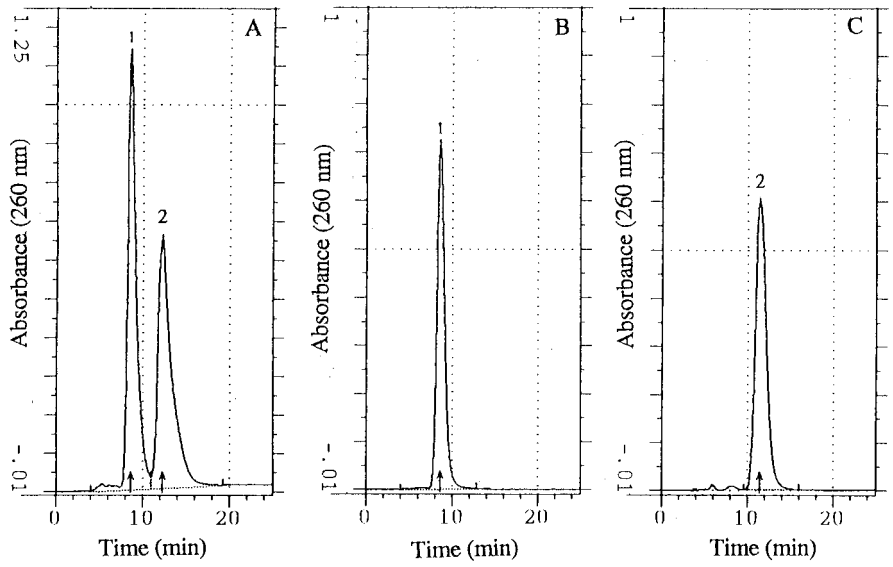

Figure 2. Isocratic separation of diastereomers of cytidine 5′-(α-P-borano)triphosphate **6d** by HPLC (A) and analysis of purity of HPLC-isolated isomer **I** (B) and isomer **II** (C). The elution was carried out on the Delta Pak C18 column with 100 mM TEAA (pH 6.80) and methanol at a flow rate of 3.0 mL/min. The retention times for two diastereomers were 8.55 and 12.24 min, respectively. Peak 1: isomer **I** of CTPαB. Peak 2: isomer **II** of CTPαB.

expected, since the boranophosphate as a monoanion has a pK_a of 1.5[11] and is thus a better leaving group than the phosphate dianion with a pK_a of 6.8.[17] From the ^{31}P NMR spectrum (Figure 1B) of the reaction mixture, the expected intermediate **5** formed by paths A and B showed a broad peak centered at δ 85.27 (α-P), two quartets (β-P) at δ −21.25 ($J_{\alpha\beta}$ = 32.87 Hz, $J_{\beta\gamma}$ = 20.56 Hz) and δ −21.30 ($J_{\alpha\beta}$ = 33.51 Hz, $J_{\beta\gamma}$ = 19.75 Hz), and a doublet at δ −8.50 and −8.63 (J = 21.05 Hz, γ-P). Another triplet at δ −20.65 and two doublets at δ −8.04 and −3.06 resulted from the normal triphosphate (path C). The singlet at δ −7.02 was due to pyrophosphate. Another singlet at δ 8.12 was attributed to H-phosphonate derived from the hydrolysis of excess phosphitylating agent.[21]

The protecting groups of intermediate **5** were removed by ammonia hydrolysis to afford ribonucleoside 5′-(α-P-borano)triphosphate **6** (Figure 1C) which was isolated in 30−45% yield by ion-exchange column chromatography on QA-52 (HCO_3^-) cellulose. The structure, purity, and homogeneity of compound **6** was confirmed by ^{31}P NMR, ^{1}H NMR, and FAB-MS. In this reaction, in addition to the desired product, three byproducts were also isolated. One was identified as H-phosphonate (about 20% yield) derived from the hydrolysis of excess phosphitylating agent. The other two byproducts were identified as ribonucleoside 5′-monophosphate (about 30% yield) and free pyrophosphate, which likely resulted from decomposition of normal ribonucleoside 5′-triphosphate formed via path C (Scheme 3).

Introduction of a borane group (BH_3) to replace one of the oxygen atoms on the α-phosphate produces a pair of NTPαB diastereomers. These two diastereomers, whose absolute configuration is not yet known, have been separated by preparative reverse phase HPLC and have arbitrarily been named isomer I and isomer II (Table 1). The isomeric purity of each of the individual diastereomers was checked by reverse phase HPLC under the conditions used for separation. The HPLC profiles before and after preparative HPLC separation are presented in Figure 2. The isolated yields of isomers I and II are presented in Table 1. The homogeneity and structure were confirmed by ^{1}H NMR, ^{31}P NMR, and FAB-MS.

Table 1. HPLC Profiles

		retention time (min)	
compound	buffer[a]	isomer I (%)	isomer II (%)
UTPαB	92% TEAA/8% MeOH	8.32 (46.4)	11.80 (53.6)
ATPαB	90% TEAA/10% MeOH	11.04 (42.7)	17.82 (57.3)
GTPαB	92% TEAA/8% MeOH	9.31 (47.5)	14.56 (52.5)
CTPαB	94% TEAA/6% MeOH	8.55 (51.0)	12.24 (49.0)

[a] 100 mM TEAA, pH 6.80, and methanol.

Conclusions

We have successfully synthesized the set of four 5′-(α-P-borano)triphosphate ribonucleosides (A, U, G, and C) using a one-pot procedure. This method does not require exocyclic amine protection of the nucleobase. Introduction of the borane group (BH_3) was achieved under mild conditions, eliminating the risk of possible reduction of nucleoside. Compared to the previously reported method for synthesis of the deoxy NTPαB analogues, this is a time- and cost-effective approach which resulted in high yields. A two-step purification, ion-exchange chromatography followed by reverse phase HPLC, gave two diastereomers of NTPαB in high purity.

Experimental Section

All solvents, chemicals, and reagents were used without further purification unless otherwise indicated. 2′,3′-Diacetyladenosine, 2′,3′-diacetylguanosine, and 2′,3′-dibenzoyluridine were purchased from Sigma and dried before use in a desiccator over P_2O_5. 2′,3′-Diacetyl-N^4-benzoylcytidine was prepared from acetylation of 5′-dimethoxytrityl-N^4-benzoylcytidine (ChemGenes) followed by detritylation of the 5′-dimethoxytrityl group with 80% acetic acid; the desired compound was isolated in 58% overall yield after chromatography on silica gel. Borane−*N*,*N*-diisopropylethylamine complex, borane−pyridine complex, and borane−tetrahydrofuran complex were purchased from Aldrich and stored over 4 Å molecular sieves.

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QA-52 quaternary ammonium cellulose was purchased from Whatman International. Triethylammonium acetate was prepared from triethylamine and acetic acid. ^{1}H and ^{31}P NMR spectra were acquired at 400.0 and 161.9 MHz, respectively. Ultraviolet (UV) and mass spectroscopy, ion-exchange chromatography, and reverse phase HPLC were performed as described.[22]

Ribonucleoside 5′-(α-P-borano)triphosphates. General Procedure. One of the protected nucleosides [2′,3′-dibenzoyluridine, 2′,3′-diacetyladenosine, 2′,3′-diacetylguanosine, 2′,3′-diacetyl-N^4-benzoylcytidine] (0.50 mmol) was dissolved in anhydrous DMF (1.0 mL) and anhydrous pyridine (0.25 mL) under an argon atmosphere. A freshly prepared solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in anhydrous DMF (0.50 mL, 1.0 M) was added with a syringe. After 10 min, tributylamine (0.30 mL) was added, followed by a solution of tributylammonium pyrophosphate in anhydrous DMF (1.0 mL, 0.5 M). The mixture was stirred for 10 min, borano−*N*,*N*-diisopropylethylamine complex (2.0 mL) was added, and the stirring was continued for 6 h. Deionized water (5.0 mL) was added, and the mixture was stirred for 1 h. The solvent was removed under vacuum, and the resulting residue was treated with a mixture of ammonium hydroxide and methanol (1:1, v/v, 10.0 mL) for 24 h. The solvent was removed, and the residue was taken in water (50 mL) and extracted with diethyl ether (50 mL). The water layer was evaporated, and the residue was applied to a column packed with QA-52 cellulose (HCO_3^-). The column was eluted with a linear gradient of 0.005 and 0.2 M ammonium bicarbonate buffer, pH 9.56 (800 mL each). The desired fraction was dried by lyophilization, and excess salt was removed by repeated lyophilization with deionized water to yield the ammonium salt of ribonucleoside 5′-(α-P-borano)triphosphate.

Uridine 5′-(α-P-borano)triphosphate (UTPαB) (6a). Compound **6a** was prepared in 46% yield (125.4 mg) following the general procedure by using 2′,3′-dibenzoyluridine **1a** (225.5 mg, 0.50 mmol): ^{1}H NMR (D_2O) δ 7.89 (m, 1H), 5.83 (m, 2H), 4.29 (m, 1H), 4.22 (m, 1H), 4.12, 4.08 (2m, 3H), 0.39, 0.13 (2br, 3H); ^{31}P NMR (D_2O) δ 85.05 (br, 1P, α-P), −21.03 (m, 1P, β-P), −7.87 (m, 1P, γ-P); UV (H_2O) λ_{max} 264.0 nm; FAB-MS *m*/*z* 480.97 (M^-, calcd 480.97).

Adenosine 5′-(α-P-borano)triphosphate (ATPαB) (6b). Compound **6b** was prepared in 31% yield (88.9 mg) following the general procedure by using 2′,3′-diacetyladenosine **1b** (175.8 mg, 0.50 mmol): ^{1}H NMR (D_2O) δ 8.42, 8.40 (2s, 1H), 8.09 (s, 1H), 5.98 (d, J = 5.2 Hz, 1H), 4.57 (s, 1H), 4.45, 4.37 (2m, 1H), 4.23 (m, 1H), 4.12, 4.00 (2m, 2H), 0.37, 0.13 (2br, 3H); ^{31}P NMR (D_2O) δ 85.36 (br, 1P, α-P), −21.15 (dd, J = 21.37 Hz, 30.52 Hz, 1P, β-P), −8.85 (d, J = 18.29 Hz, 1P, γ-P); UV (H_2O) λ_{max} 260.2 nm; FAB-MS *m*/*z* 504.0 (M^-, calcd 504.0).

Guanosine 5′-(α-P-borano)triphosphate (GTPαB) (6c). Compound **6c** was prepared in 29% yield (85.2 mg) following the general procedure by using 2′,3′-diacetylguanosine **1c** (183.3 mg, 0.50 mmol): ^{1}H NMR (D_2O) δ 8.02, 7.99 (2s, 1H), 5.77 (m, 1H), 4.57 (s, 1H), 4.44, 4.36 (2m, 1H), 4.19 (m, 1H), 4.10, 4.00 (2m, 2H), 0.43, 0.19 (2br, 3H); ^{31}P NMR (D_2O) δ 85.72 (br, 1P, α-P), −21.12 (dd, J = 20.56 Hz, 28.33 Hz, 1P, β-P), −8.70 (d, J = 19.91 Hz, 1P, γ-P); UV (H_2O) λ_{max} 255.8 nm; FAB-MS *m*/*z* 520.0 (M^-, calcd 520.0).

Cytidine 5′-(α-P-borano)triphosphate (CTPαB) (6d). Compound **6d** was prepared in 26% yield (71.0 mg) following the general procedure by using 2′,3′-diacetyl-N^4-benzoylcytidine **1d** (216.0 mg, 0.50 mmol): ^{1}H NMR (D_2O) δ 7.95 (d, J = 7.6 Hz, 1H), 6.01 (d, J = 6.0 Hz, 1H), 5.84 (d, J = 4.4 Hz, 1H), 4.57 (m, 1H), 4.25 (m, 1H), 4.16, 4.10 (2m, 3H), −0.20 to +0.80 (2br, 3H); ^{31}P NMR (D_2O) δ 85.20 (br, 1P, α-P), −21.26 (m, 1P, β-P), −8.92 (d, J = 18.29 Hz, 1P, γ-P); UV (H_2O) λ_{max} 272.5 nm; FAB-MS *m*/*z* 480.0 (M^-, calcd 479.96).

Reverse Phase HPLC Separation of Diastereomers of Ribonucleoside 5′-(α-P-borano)triphosphates. The separation of diastereomers of each NTPαB was carried out by ion-pairing chromatography on a reverse phase column (Delta Pak C18, 7.8 × 300 mm, 15 μm, 300 Å) using isocratic elution (100 mM triethylammonium acetate (TEAA), pH 6.80, and methanol as buffer components) with a flow rate 3.0 mL/min. Fractions containing the same isomer (similar retention time) were combined, and the solvent was removed under reduced pressure. The buffer components, triethylammonium acetate (TEAA) and methanol, were removed by repeated lyophilization.

UTPαB, isomer I: ^{1}H NMR (D_2O) δ 7.91 (d, J = 8.4 Hz, 1H), 5.84 (m, 2H), 4.29 (m, 1H), 4.22 (m, 1H), 4.12, 4.05 (2m, 3H), −0.20 to +0.80 (br, 3H); ^{31}P NMR (D_2O) δ 85.40 (br, 1P, α-P), −21.47 (m, 1P, β-P), −9.16 (m, 1P, γ-P); FAB-MS *m*/*z* 480.91 (M^-, calcd 480.95). **Isomer II**: ^{1}H NMR (D_2O) δ 7.89 (d, J = 8.8 Hz, 1H), 5.83 (m, 2H), 4.21 (m, 2H), 4.12 (m, 1H), 4.07 (m, 2H), −0.20 to +0.80 (br, 3H); ^{31}P NMR (D_2O) δ 85.12 (br, 1P, α-P), −21.28 (m, 1P, β-P), −9.14 (m, 1P, γ-P); FAB-MS *m*/*z* 480.91 (M^-, calcd 480.95).

ATPαB, isomer I: ^{1}H NMR (D_2O) δ 8.43 (s, 1H), 8.09 (s, 1H), 5.98 (d, J = 5.6 Hz, 1H), 4.56 (m, 1H), 4.44 (m, 1H), 4.23 (m, 1H), 4.13, 4.00 (2m, 2H), 0.42, 0.18 (2br, 3H); ^{31}P NMR (D_2O) δ 84.79 (br, 1P, α-P), −21.14 (m, 1P, β-P), −8.86 (m, 1P, γ-P); FAB-MS *m*/*z* 503.9 (M^-, calcd 503.99). **Isomer II**: ^{1}H NMR (D_2O) δ 8.40 (s, 1H), 8.06 (s, 1H), 5.96 (d, J = 6.0 Hz, 1H), 4.56 (m, 1H), 4.37 (m, 1H), 4.21 (m, 1H), 4.11, 4.00 (2m, 2H), 0.39, 0.12 (2br, 3H); ^{31}P NMR (D_2O) δ 84.40 (br, 1P, α-P), −20.98 (m, 1P, β-P), −7.71 (m, 1P, γ-P); FAB-MS *m*/*z* 503.95 (M^-, calcd 503.99).

GTPαB, isomer I: ^{1}H NMR (D_2O) δ 8.03 (s, 1H), 5.77 (d, J = 6.0 Hz, 1H), 4.57 (m, 1H), 4.47 (m, 1H), 4.17 (m, 1H), 4.13, 3.98 (2m, 2H), −0.10 to +0.75 (br, 3H); ^{31}P NMR (D_2O) δ 84.38 (br, 1P, α-P), −20.86 (m, 1P, β-P), −7.33 (m, 1P, γ-P); FAB-MS *m*/*z* 520.0 (M^-, calcd 520.0). **Isomer II**: ^{1}H NMR (D_2O) δ 8.00 (s, 1H), 5.76 (d, J = 6.0 Hz, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.18 (m, 1H), 4.10, 4.03 (2m, 2H), −0.10 to +0.75 (br, 3H); ^{31}P NMR (D_2O) δ 85.41 (br, 1P, α-P), −20.99 (m, 1P, β-P), −6.97 (m, 1P, γ-P); FAB-MS *m*/*z* 520.0 (M^-, calcd 520.0).

CTPαB, isomer I: ^{1}H NMR (D_2O) δ 7.93 (d, J = 7.6 Hz, 1H), 5.97 (m, 1H), 5.84 (d, J = 4.0 Hz, 1H), 4.55 (m, 1H), 4.26 (m, 1H), 4.15, 4.09 (2m, 3H), −0.20 to +0.80 (2br, 3H); ^{31}P NMR (D_2O) δ 84.30 (br, 1P, α-P), −21.21 (m, 1P, β-P), −8.68 (m, 1P, γ-P); FAB-MS *m*/*z* 480.0 (M^-, calcd 479.96). **Isomer II**: ^{1}H NMR (D_2O) δ 7.91 (d, J = 6.4 Hz, 1H), 5.98 (d, J = 8.0 Hz, 1H), 5.84 (d, J = 3.6 Hz, 1H), 4.57 (m, 1H), 4.19 (m, 1H), 4.15, 4.10 (2m, 3H), −0.20 to +0.80 (2br, 3H); ^{31}P NMR (D_2O) δ 84.90 (br, 1P, α-P), −21.16 (m, 1P, β-P), −8.63 (m, 1P, γ-P); FAB-MS *m*/*z* 480.0 (M^-, calcd 479.96).

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Supporting Information Available: ^{31}P NMR and ^{1}H NMR spectra for the new compounds **6a**, **6b**, **6c**, and **6d** and for the two diastereomers of **6a**, **6b**, **6c**, and **6d** (24 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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