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A Combination Chemical and Enzymatic Approach for the Preparation of Azole Carboxamide Nucleoside Triphosphate

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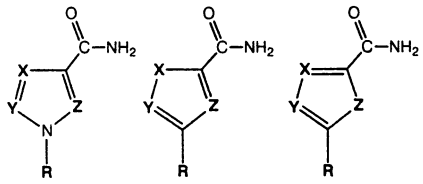
Alternative substrates for DNA and RNA polymerases offer an important set of biochemical tools. Many of the standard methods for nucleoside triphosphate synthesis fail in the cases of nonpurine and nonpyrimidine nucleosides. An efficient preparation of the 5′-*O*-tosylates for both the deoxy- and ribonucleosides enabled preparation of the diphosphate esters by displacement with tris(tetra-*n*-butylammonium) pyrophosphate. Enzymatic synthesis of the azole carboxamide deoxyribonucleoside triphosphate was based on ATP as the phosphate donor, nucleoside diphosphate kinase as the catalyst, coupled with phosphoenol pyruvate (PEP) and pyruvate kinase as an ATP regeneration system. Ribonucleoside triphosphate synthesis required PEP as the phosphate donor and pyruvate kinase as the catalyst. An optimized purification procedure based upon boronate affinity gel was developed to yield highly purified nucleoside triphosphates. The strategy outlined here provides a new and efficient method for preparation of nucleoside 5′-triphosphate and is likely applicable to a broad variety of base and sugar modified nucleoside analogues.

Introduction

The design and synthesis of alternative substrates for DNA and RNA polymerases holds continued promise to create biochemical probes and precursors for the synthesis of nucleic acid mimics. As the number of enzymes in this family and their applications increase, the potential for nonpurine and nonpyrimidine base materials to serve as substrates or inhibitors will be limited by the synthesis and testing of the corresponding nucleoside triphosphates. One class of materials of particular interest is the azole carboxamide nucleotides, which have the interesting property of displaying multiple conformations in the context of DNA replication (Figure 1).[1,2] In addition, the variation in electronic features for these materials offers a range of hydrogen bonding features. The incompatibility of more traditional phosphorylation reagents with the azole carboxamides and the need for additional synthetic approaches for nonstandard nucleoside triphosphates prompted further development.

The general utility of the "one-pot, three-step" procedures for the synthesis of purine or pyrimidine base nucleotides has been established.[3−8] Other multistep methods that rely on activated nucleoside monophosphates have also had long-standing application for these more standard nucleotides.[9−13] These procedures involve the use or variation of the Yoshikawa procedure, which employs conditions for selective 5′-phosphorylation with phosphorus oxychloride as the primary donor.[14] This reactive electrophilic phosphorus reagent presents limi-



X, Y, Z = C, N, O; R = 2'-deoxyribose or ribose

FIGURE 1. General structure of azole carboxamide nucleosides.

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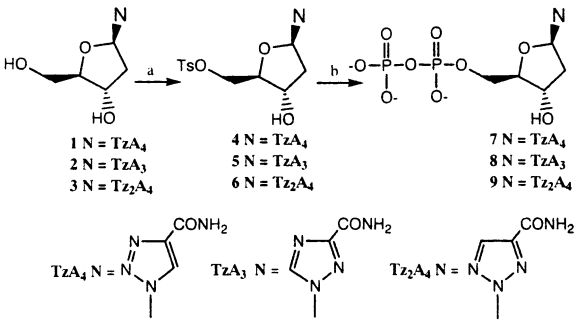
tations with many heterocycles such as the azole carboxamides since attack at the carboxamide led to side reactions with members of this class of nucleosides. Alternative strategies that use other electrophilic P^3 or P^5 reagents are equally limited because many heterocycles are susceptible to addition reactions with these reagents.[15,16]

Enzyme-catalyzed synthesis of nucleoside triphosphates has proven to be a general method for a variety of purine and pyrimidine nucleoside analogues.[17−22] The potential for a lack of base specificity with these enzymes prompted us to explore and optimize this biocatalytical method and its application in the preparation of triphosphates of nucleoside analogues with unnatural bases. In planning a nucleoside triphosphate synthesis strategy, we divided the problem into two stages: first, chemical synthesis of the nucleoside 5′-diphosphate by a nucleophilic displacement reaction,[23] and second, enzymatic conversion of the diphosphate to the triphosphate. Despite the potential of the strategy, its implementation has been limited to date.[22,24] The problems of precursor synthesis, intermediate purification, and substrate specificity are often barriers to success. As reported here, a series of azole carboxamide nucleoside triphosphates that could not be synthesized by the conventional chemical procedures have been prepared with this approach. An optimized purification strategy using boronate affinity gel was also developed to yield highly purified nucleoside triphosphates. It is likely that this strategy will be applicable as a general protocol for preparation of triphosphates of a wide variety of base and sugar modified nucleoside analogues.

Results and Discussion

Synthesis of Nucleoside Diphosphates. Azole carboxamide deoxyribonucleoside diphosphates **7**, **8**, and **9** were synthesized following a previously developed procedure[23] involving displacement of the corresponding nucleoside 5′-*O*-tosylate **4**, **5**, and **6** with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate. The requisite deoxynucleoside 5′-*O*-tosylates **4**−**6** were prepared directly from the nucleoside analogues by selective 5′-*O*-tosylation in pyridine with tosyl chloride at room temperature.[25] Pure 5′-*O*-tosyl-2′-deoxyribonucleosides were obtained after flash chromatography on silica gel in yields of 60−70%. This method was more direct and provided similar yields to those using the 3′-*O*-protected 2′-deoxyribonucleosides.[23] The diphosphate displacement reaction with tosylates **4**, **5**, and **6** was straightforward and the resulting nucleoside diphosphates **7**, **8**, and **9** were purified by flash chromatography on cellulose after the tetra-*n*-butylammonium salts were exchanged to the ammonium salts. The yields of purified diphosphates ranged from 70 to 91% (Scheme 1).

SCHEME 1[a]



[a] Reagents and conditions: (a) TsCl, pyridine, rt; (b) $(NBu_4)_3HP_2O_7$, CH_3CN.

Azole carboxamide ribonucleoside diphosphates **20** and **21** were synthesized by using the same diphosphate displacement procedure used for the deoxyribonucleoside 5′-*O*-tosylates. The protection of compound **13** with the 2′,3′-*O*-methoxymethylidene group[26,27] as previously described[23] proceeded smoothly in tetrahydrofuran (THF) and subsequent treatment of the protected nucleoside with 4-(*N*,*N*-dimethylamino)-pyridine (4-DMAP) and tosyl chloride in dichloromethane provided the protected nucleoside 5′-*O*-tosylate **15** in 70% overall yield. In the case of **14**, both the protection and tosylation reactions did not go to completion because of the low solubility of the nucleoside and its derivative. These problems were overcome by altering the solvent for the protection step to dioxane and subsequent tosylation in pyridine to yield the nucleoside 5′-*O*-tosylate **16** in 52% overall yield. The protecting group in tosylate **15** was removed by hydrolysis in mild acid followed by treatment at pH 8.0. However, the rate for diphosphate displacement reaction on unprotected ribonucleoside 5′-*O*-tosylate **17** was exceptionally slow with completion not achieved even after 4 d. A significant difference in the rates of diphosphate displacement for 5′-*O*-tosyladenosine and its 2′,3′-*O*-isopropylidene derivative (24 h vs 2 h) was observed in the original work.[23] An explanation for this rate difference may be decreased nucleophilicity of the reagent through hydrogen bonding with the exposed hydroxyl groups or the conformational preferences of the ribose ring in the unprotected ribonucleoside. Therefore, the diphosphate displacement reaction was executed prior to removal of the methoxymethylidene protecting group, which proceeded efficiently on **15** and **16** and with completion within 24 h. The deprotection was accomplished after displacement by treating the diphosphate **18** and **19** sequentially with aqueous trifluoroacetic acid (pH 2.0) and ammonium hydroxide (pH 8.5) at room

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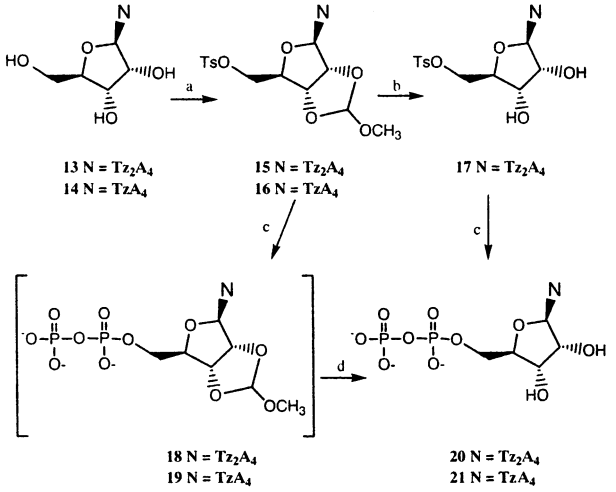
SCHEME 2[a]



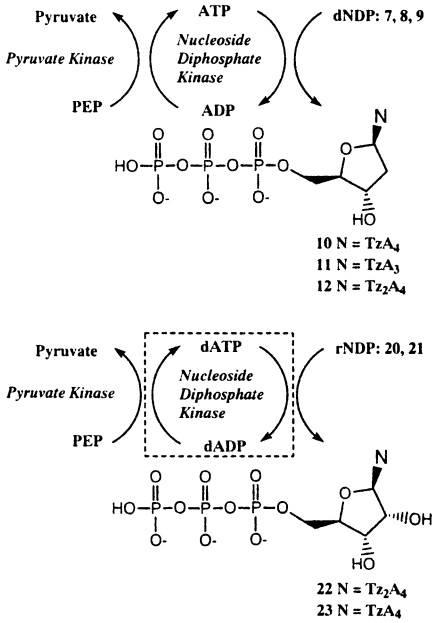
[a] Reagents and conditions: (a) $(CH_3O)_3CH$, *p*-$CH_3C_6H_4SO_3H$, THF, then TsCl, 4-DMAP, CH_2Cl_2, rt; or $(CH_3O)_3CH$, pyridine hydrochloride, dioxane, then TsCl, 4-DMAP, pyridine, rt. (b) HCl/H_2O, pH 1.0, then NH_4OH/H_2O, pH 8.0. (c) $(NBu_4)_3HP_2O_7$, CH_3CN. (d) TFA/H_2O, pH 2.0, then NH_4OH/H_2O, pH 8.5.

temperature. The ribonucleoside diphosphates **20** and **21** were obtained after purification in overall yields of 66−70% for the displacement and deprotection (Scheme 2).

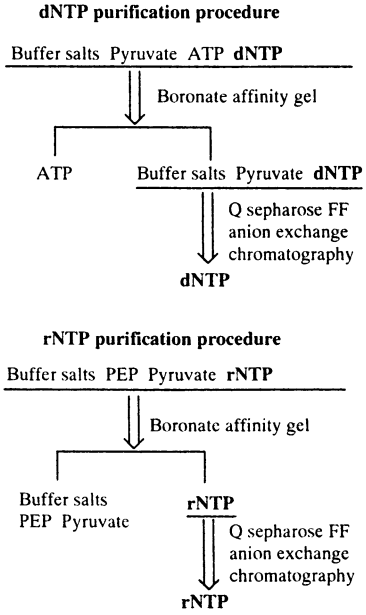
Enzyme Phosphorylation. Nucleoside diphosphate kinase[28] catalyzes the transfer of the γ-phosphate from a nucleoside triphosphate to a nucleoside diphosphate. The enzyme is remarkably nonspecific with regard to the nucleotide substrate; it uses di- and triphosphate nucleotides with either deoxyribose or ribose and any of the natural purine and pyrimidine bases.[29−31] A number of the unnatural purine and pyrimidine analogue nucleotides can also serve as substrates.[20,24] Our initial experiment with NDPK showed that both azole carboxamide 2′-deoxyribo- and ribonucleoside diphosphate esters were substrates for NDPK. The reaction equilibrium offers a general method for preparation of both 2′-deoxyribo- and ribonucleoside triphosphates (Scheme 3). In the case of the azole carboxamide 2′-deoxyribonucleoside diphosphates, incorporation of an ATP regeneration system allowed for efficient conversion to the triphosphates. Therefore pyruvate kinase and PEP were added and ADP was eliminated from the reaction mixture.[32] To eliminate interference during the purification steps and to ensure complete conversion of the diphosphate to triphosphate esters, 2 equiv of PEP were used in the reaction mixtures.

In the case of azole carboxamide ribonucleotide diphosphate esters, preliminary studies showed that NPDK with dATP and a dATP regeneration by pyruvate kinase and PEP could efficiently convert azole carboxamide nucleoside diphosphates to the triphosphates. Later, we found that pyruvate kinase can also directly catalyze the phosphorylation of the azole carboxamide ribonucleoside

SCHEME 3



SCHEME 4. dNTP and rNTP Purification Procedure



diphosphates allowing a simplified system to complete the transformation. Addition of NDPK and dATP is useful when pyruvate kinase is too selective toward the other substrates to effect an efficient transformation.

Purification. Most biochemical applications of nucleoside triphosphates require a high degree of purity especially with respect to other contaminating nucleotides. An efficient purification procedure was optimized for the desired nucleoside triphosphates following enzymatic phosphorylation. In the case of the deoxyribonucleoside triphosphate synthesis, separation from ATP is necessary, which is not readily achieved by conventional ion-exchange chromatography. Since excess PEP interfered with the final purification of the deoxyribonucleoside triphosphates, it was converted to pyruvate by adding 1 equiv of ADP to the reaction mixture after the reaction

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was complete. Boronate affinity gel has been used to separate ribonucleoside 5′-phosphates from deoxyribonucleotides due to the formation of a complex between the borate group and cis-diol of the ribonucleoside.[33,34] The reaction mixtures were passed through the boronate affinity gel at high salt concentration (1 M NH_4HCO_3, pH 8.5). The buffer salts, pyruvate, and deoxyribonucleoside triphosphates were eluted in the void volume, while ATP was bound to the gel. Final purifications of the deoxyribonucleoside triphosphates were achieved by using anion exchange chromatography on Q sepharose FF. The purifications of ribonucleoside triphosphates were more straightforward. The reaction mixtures were passed through boronate affinity gel at high salt concentration (1 M NH_4HCO_3, pH 8.5), and the azole carboxamide ribonucleoside triphosphates were retained on the column. The triphosphates were released from the gel by eluting with deionized water and finally purified by anion exchange chromatography on Q sepharose FF (Scheme 4).

Conclusion

This work has extended the use of the diphosphate displacement procedure to a general route for nucleoside triphosphate synthesis. Purification strategies for both deoxy- and ribonucleotides are demonstrated. This approach has particular utility in cases where the reactivity of the heterocycle precludes the use of electrophilic phosphorylation reagents. The procedure outlined here provides a new and efficient strategy for nucleoside 5′-triphosphate synthesis that is mild, scalable, and very likely applicable to a broad variety of base and sugar modified nucleoside analogues.

Experimental Section

General Methods. Melting points are uncorrected. Routine ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with standard methods. ^{1}H and ^{13}C spectra are referenced to the solvent. ^{31}P spectra are referenced from external phosphoric acid (85%). In the case of ^{31}P NMR spectra, a 10 mM concentration of NaEDTA was used.

Analytical TLC on silica gel was performed on polyester plates coated with silica gel 60 F_{254}, which were visualized by UV light or 5% phosphomolybdic acid in ethanol. Flash silica gel chromatography was performed on silica gel 60 (230−400 mesh). Analytical TLC on cellulose was performed on EM Science cellulose plates (0.1 mm thickness) which were visualized by sulfosalicylic acid−ferric chloride stain.[35] Flash cellulose chromatography was performed as described[35,36] on Whatman CF-11 fibrous cellulose. Analytical HPLC analyses for monitoring the enzyme phosphorylation process were performed on a SUPELCOSIL LC-18-T column (25 cm × 4 mm, 3 μm) on a Beckman System Gold equipped with 128 Solvent Module and 166 Detector. The elution was carried out at 1.5 mL/min with a gradient from 0.1 M KH_2PO_4/4 mM TBAP, pH 6.0 to 30% methanol/0.1 M KH_2PO_4/4 mM TBAP, pH 7.2, in 10 min with UV detection at 230 nm.

Nucleoside diphosphate kinase (NDPK, EC 2.7.4.6) and pyruvate kinase (EC 2.7.1.40) were purchased from a commercial source.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-tosylate (4). A mixture of compound **1**[37] (100 mg, 0.44 mmol) and tosyl chloride (100 mg, 0.53 mmol) in 2 mL of dry pyridine was stirred for 4 d at room temperature. Silica gel (1.5 g, 60−200 mesh) was added and the mixture was evaporated to dryness. Methanol (5 mL) was added to the residue and evaporated to dryness again, and the procedure was repeated three times to remove pyridine. The residue was applied to a flash silica gel column and eluted with hexane/ethyl acetate (1:1) to yield 105 mg (62%) of **2** as a white solid. TLC R_f 0.5 (ethyl acetate); mp 122−124 °C dec; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.59 (1H, s), 7.88 (1H, s), 7.68 (2H, d), 7.52 (1H, s), 7.39 (2H, d), 6.37 (1H, t, J = 6.0 Hz), 5.58 (1 H, d, D_2O exchangeable), 4.38 (1H, m), 4.20 (1H, m), 4.03 (2H, m), 2.64 (1H, m), 2.40 (4H, m); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 161.3, 145.0, 143.1, 130.1, 127.5, 125.5, 88.0, 84.3, 70.1, 69.9, 21.1; MS (ESI) 383 $[M + H]^+$. Anal. Calcd for $C_{15}H_{18}O_6N_4S$: C, 47.11; H, 4.74; N, 14.65. Found: C, 47.00; H, 4.64; N, 14.52.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,4-triazole-3-carboxamide-5′-tosylate (5). Following the procedure described above for the synthesis of tosylate **4**, compound **2**[38] (200 mg, 0.88 mmol) and tosyl chloride (200 mg, 1.0 mmol) were reacted in dry pyridine (4 mL) for 24 h. Purification on a silica gel column eluting with hexane/acetone (1:1) yielded 198 mg (59%) of **5** as a white solid. TLC R_f 0.38 (acetone); mp 139−140 °C dec; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.68 (1H, s), 7.78 (1H, s), 7.68 (2H, d), 7.62 (1H, s), 7.37 (2H, d), 6.24 (1H, dd, J = 4.2 and 6.9 Hz), 5.50 (1 H, d, D_2O exchangeable), 4.42 (1H, m), 4.21 (1H, m), 4.01 (2H, m), 2.50 (1H, m), 2.38 (3H, s), 2.33 (1H, m); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.7, 157.7, 145.4, 132.3, 130.4, 127.9, 87.5, 84.5, 70.9, 70.3, 21.5; MS (ESI) 383 $[M + H]^+$. Anal. Calcd for $C_{15}H_{18}O_6N_4S$: C, 47.11; H, 4.74; N, 14.65. Found: C, 46.87; H, 4.70; N, 14.43.

2-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-tosylate (6). Following the procedure described above for the synthesis of tosylate **4**, compound **3**[37] (270 mg, 1.2 mmol) and tosyl chloride (267 mg, 1.4 mmol) were reacted in dry pyridine (5 mL) for 24 h. Purification on a silica gel column eluting with ethyl acetate/methanol (20:1) yielded 317 mg (70%) of **6** as a white solid. TLC R_f 0.32 (ethyl acetate); mp 121−122 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.08 (1H, s), 7.86 (1H, s), 7.63 (2H, d), 7.59 (1H, s), 7.36 (2H, d), 6.31 (1H, dd, J = 3.3 and 6.9 Hz), 5.52 (1H, d, D_2O exchangeable), 4.54 (1H, m), 3.96 (3H, m), 2.66 (1H, m), 2.39 (4H, m); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 161.2, 145.3, 143.9, 135.7, 132.2, 130.4, 127.9, 92.1, 84.6, 70.9, 70.4, 21.4; MS (ESI) 383 $[M + H]^+$. Anal. Calcd for $C_{15}H_{18}O_6N_4S$: C, 47.11; H, 4.74; N, 14.65. Found: C, 47.19; H, 4.77; N, 14.45.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-diphosphate (7). Following the published procedure,[23] tosylate **4** (214 mg, 0.56 mmol) and tris-(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.0 g, 1.12 mmol) were reacted in dry acetonitrile (1.5 mL) for 24 h. The reaction mixture was diluted with water (10 mL) and the tetra-*n*-butylammonium cation exchanged for an ammonium cation by passing the solution through a DOWEX AG 50W-X8 column (100−200 mesh, 2.5 cm × 10 cm, 83 equiv, NH_4^+ form) and eluting with two column volumes of deionized water. The eluent was lyophilized and the resultant solid was extracted with CH_3CN/100 mM NH_4HCO_3/NH_4OH (7:3:2). The soluble material was loaded onto a CF-11 cellulose flash column (2.5 cm × 23 cm) and eluted with the same solvent. Cellulose TLC (CH_3CN/100 mM NH_4HCO_3/NH_4OH, 7:3:2) was employed to follow the progress of the chromatography. The fractions containing product were pooled, and acetonitrile was removed by rotary evaporation. The resulting aqueous solution was lyophilized to yield 200 mg (81%) of **7** as a white fluffy solid. TLC R_f 0.24; ^{1}H NMR (D_2O, 300 MHz) δ 8.68 (1H, s), 6.43 (1H,

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t, J = 6.0 Hz), 4.14 (1H, m), 3.98 (2H, m), 2.68 (1H, m), 2.52 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.5, 141.9, 125.9, 89.5, 86.7, 70.6, 64.9, 39.9; ^{31}P NMR (D_2O, 121 MHz) δ −9.85 (d, $J_{p,p}$ = 21.8 Hz), −13.38 (d, $J_{p,p}$ = 21.8 Hz). HRMS (ESI): calcd 389.0263 $[M + H]^+$, found 389.0247 $[M + H]^+$.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,4-triazole-3-carboxamide-5′-diphosphate (8). Following the procedure described above for the synthesis of diphosphate **7**, tosylate **5** (300 mg, 0.78 mmol) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.4 g 1.57 mmol) were reacted in dry acetonitrile (1.5 mL) for 24 h to yield 310 mg (91%) of **8** as a white fluffy solid. TLC R_f 0.25; ^{1}H NMR (D_2O, 300 MHz) δ 8.59 (1H, s), 6.23 (1H, t, J = 5.7 Hz), 4.12 (1H, m), 3.94 (2H, m), 2.68 (1H, m), 2.42 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 163.2, 156.4, 145.8, 88.8, 86.5, 71.0, 65.5, 39.1; ^{31}P NMR (D_2O, 121 MHz) δ −9.17 (d, $J_{p,p}$ = 21.3 Hz), −10.90 (d, $J_{p,p}$ = 21.3 Hz). HRMS (ESI): calcd 387.0107 $[M - H]^-$, found 387.0104 $[M - H]^-$.

2-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-diphosphate (9). Following the procedure described above for the synthesis of diphosphate **7,** tosylate **6** (320 mg, 0.84 mmol) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.5 g, 1.7 mmol) were reacted in anhydrous acetonitrile (1.5 mL) for 96 h to yield 260 mg (70%) of **9** as a white fluffy solid. TLC R_f 0.26; ^{1}H NMR (D_2O, 300 MHz) δ 8.01 (1H, s), 6.35 (1H, t, J = 6.0 Hz), 4.13 (1H, m), 3.88 (2H, m), 2.89 (1H, m), 2.44 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.4, 142.9, 136.2, 92.5, 86.4, 71.4, 65.7, 38.4; ^{31}P NMR (D_2O, 121 MHz) δ −9.83 (d, $J_{p,p}$ = 20.6 Hz), −11.00 (d, $J_{p,p}$ = 20.6 Hz). HRMS (ESI): calcd 389.0263 $[M + H]^+$, found 389.0263 $[M + H]^+$.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-triphosphate (10). A 10-mL solution of 83 mM triethanolamine/17 mM $MgCl_2$/67 mM KCl containing compound **7** (180 mg, 0.4 mmol), ATP (220 mg, 0.4 mmol), and PEP (152 mg, 0.8 mmol) was adjusted to pH 7.6 with 6 M NaOH. To this solution were added NDPK (100 unit) and pyruvate kinase (200 unit), and the mixture was incubated at 37 °C for 4 h. The reaction was monitored by analytical HPLC to ensure complete conversion of compound **7** to the corresponding triphosphate. To this mixture ADP (180 mg, 0.4 mmol) was added and incubation was continued for another 1 h to consume the excess PEP before filtration through a 0.2-μm syringe disk and drying by lyophilization.

The residual solid obtained was dissolved in 10 mL of 1 M NH_4HCO_3 (pH 8.5, with concentrated NH_4OH) and applied to a Bio-Rad boronate affinity gel column (2.5 cm × 5.5 cm, in 1 M NH_4HCO_3 , pH 8.5). The column was eluted with the same buffer at 1 mL/min and the fractions containing the first two-column volumes were pooled and dried by lyophilization. Excess NH_4HCO_3 was removed by repeated lyophilization from deionized water after adjustment to pH 7.2 with CO_2.

Final purification of the compound was performed on a Q Sepharose FF anion exchange column (2.5 cm × 20 cm, HCO_3^- form) by a linear gradient elution with NH_4HCO_3 (0.05−0.5 M, pH 7.8) at a flow rate of 5 mL/min over 2 h with UV detection at 230 nm. The desired fractions were dried by lyophilization to yield a white fluffy solid (120 mg, NH_4^+ salt, 58%). HPLC 10.74 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.64 (1H, s), 6.42 (1H, t, J = 6.0 Hz), 4.14 (1H, m), 4.01 (2H, m), 2.68 (1H), 2.50 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.5, 141.9, 125.9, 89.5, 86.6, 70.4, 65.2, 39.9; ^{31}P NMR (D_2O, 121 MHz) δ −6.26 (d, $J_{p,p}$ = 19.4 Hz), −10.97 (d, $J_{p,p}$ = 19.4 Hz), −21.88 (t, $J_{p,p}$ = 19.4 Hz). HRMS (ESI): calcd 466.9770 $[M - H]^-$, found 466.9766 $[M - H]^-$.

1-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(1*H*)-1,2,4-triazole-3-carboxamide-5′-triphosphate (11). Following the procedure described above for the synthesis of triphosphate **10**, compound **8** (180 mg, 0.4 mmol) was phosphorylated to yield 120 mg (NH_4^+ salt, 58%) of **11** as a white fluffy solid. HPLC 9.23 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.59 (1H, s), 6.23 (1H, t, J = 5.7 Hz), 4.14 (1H, m), 4.00 (2H, m), 2.68 (1H, m), 2.41 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 163.2,156.4, 145.8, 88.9, 86.5, 70.9, 65.7, 39.1; ^{31}P NMR (D_2O, 121 MHz) δ −8.51 (d, $J_{p,p}$ = 19.8 Hz), −11.07 (d, $J_{p,p}$ = 19.8 Hz), −22.40 (t, $J_{p,p}$ = 19.8 Hz). HRMS (ESI): calcd 466.9770 $[M - H]^-$, found 466.9774 $[M - H]^-$.

2-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-triphosphate (12). Following the procedure described above for the synthesis of triphosphate **10**, compound **9** (180 mg 0.4 mmol) was phosphorylated to yield 190 mg (NH_4 salt, 89%) of **12** as a white fluffy solid. HPLC 11.48 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.04 (1H, s), 6.37 (1H, t, J = 6.0 Hz), 4.16 (1H, m), 3.93 (2H, m), 2.91 (1H, m), 2.46 (1H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.5, 142.9, 136.3, 92.5, 86.4, 71.3, 65.9, 38.5; ^{31}P NMR (D_2O, 121 MHz) δ −8.81 (d, $J_{p,p}$ = 19.4 Hz), −11.09 (d, $J_{p,p}$= 19.4 Hz), −22.53 (t, $J_{p,p}$ = 19.4 Hz). HRMS (ESI): calcd 466.9770 $[M - H]^-$, found 466.9772 $[M - H]^-$.

2-(2′,3′-*O*-Methoxymethylidene-β-D-ribofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-tosylate (15). Compound **13**[39] (250 mg, 1.0 mmol) and *p*-toluenesulfonic acid (210 mg, 1.1 mmol) were stirred in THF (5 mL) at room temperature, trimethyl orthoformate (0.55 mL, 5.0 mmol) was added via syringe, and the solution was stirred for 2 h. Pyridine (0.5 mL) was added and the solvent was removed in vacuo. The residue was then dissolved in dry CH_2Cl_2 (5 mL), and TsCl (230 mg, 1.2 mmol) and 4-DMAP (160 mg, 1.3 mmol) were added. The reaction mixture was stirred for 24 h and purification on silica gel eluting with hexane/ethyl acetate/methanol (10:9:1) yielded 310 mg (70%) of **15** as a white solid. TLC R_f 0.7 (ethyl acetate/methanol, 10:1); mp 122−125 °C; ^{1}H NMR for diastereomer (DMSO-d_6, 300 MHz) δ 8.09, 8.10 (1H, s), 7.89 (1H, s), 7.61 (1H, s), 7.58 (2H, d), 7.35 (2H, d), 6.33 (1H, d, J = 6.0 Hz), 6.03, 6.14 (1H, s), 5.20 (2H, m), 4.49 (1H, m), 4.06 (2H, m), 3.21, 3.29 (3H, s), 2.39 (3H, s); MS (ESI): 463 $[M + Na]^+$. Anal. Calcd for $C_{17}H_{20}O_8N_4S$: C, 46.36; H, 4.58; N, 12.72. Found: C, 46.74; H, 4.55; N, 12.61.

1-(2′,3′-*O*-Methoxymethylidene-β-D-ribofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-tosylate (16). Compound **14**[39] (250 mg, 1.0 mmol) and pyridine hydrochloride (140 mg, 1.2 mmol) were stirred in dioxane (10 mL) at room temperature, trimethyl orthoformate (0.55 mL, 5.0 mmol) was added via syringe, and the mixture was stirred at room temperature. After 24 h, the reaction mixture was passed through a silica gel column and eluted with dichloromethane/methanol (20:1). The partially purified compound was then dissolved in anhydrous pyridine (5 mL), and TsCl (210 mg, 1.1 mmol) and 4-DMAP (150 mg, 1.2 mmol) were added. The reaction mixture was stirred at room temperature. After 2 days, silica gel (1.0 g, 60−200 mesh) was added to the reaction mixture and the solvent was evaporated to dryness. Methanol (5 mL) was added to the residue and evaporated to dryness again and the procedure was repeated three times to remove pyridine. Then the residue was applied to a flash silica gel column and eluted with hexane/acetone (1:1) to yield 230 mg (52%) of **16** as a white waxy solid. TLC R_f 0.4 (hexane/acetone 1:1); ^{1}H NMR for diastereomer (DMSO-d_6, 300 MHz) δ 8.63, 8.66 (1H, s), 7.93 (1H, s), 7.62 (2H, d), 7.58 (1H, s), 7.37 (2H, d), 6.42 (1H, t, J = 1.8 Hz), 6.05, 6.16 (1H, s), 5.33 (1H, m), 5.07 (1H, m), 4.47 (1H, m), 4.25 (1H, m), 3.98 (1H, m), 3.21, 3.30 (3, s), 2.38 (3H, s). HRMS (EI): calcd 440.1002 $[M]^+$, found 440.0998 $[M]^+$.

2-(β-D-Ribofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-tosylate (17). Tosylate **15** obtained from compound **13** (400 mg 1.64 mmol) through protection in THF (10 mL) with *p*-toluenesulfonic acid (340 mg, 1.8 mmol) and trimethyl orthoformate (0.9 mL, 8.2 mmol) and subsequent reaction in dichloromethane (10 mL) with TsCl (350 mg, 1.8 mmol) and 4-DMAP (240 mg, 2.0 mmol) was dissolved in a 1:1 (v/v)

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mixture of CH_3CN/0.01 M HCl (10 mL). The solution was stirred for 2 h after adjusting to pH 1.0 with 1 M HCl. Then the pH was adjusted to 9.0 with 1 M NH_4OH, and stirring was continued for an additional 2 h. The solvent was removed in vacuo and the residue was purified by flash chromatography on silica gel, eluting with dichloromethane/methanol (20:1) to yield 470 mg (72%) of **17** as a white solid. TLC R_f 0.42 (ethyl acetate/methanol, 10:1); mp 162−164 °C dec; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.14 (1H, s), 7.88 (1H, s, D_2O exchangeable), 7.67 (2H, d), 7.61 (1H, s, D_2O exchangeable), 7.37 (2H, d), 5.88 (1H, d, J = 3.0 Hz), 5.77 (1H, d, D_2O exchangeable), 5.40 (1H, d, D_2O exchangeable), 4.35 (2H, m), 4.21 (1H, m), 4.09 (2H, m), 2.37 (3H, s); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 161.2, 145.4, 143.9, 135.9, 132.1, 130.4, 127.9, 96.2, 81.8, 74.3, 70.9, 70.4, 21.4; MS (ESI): 399 $[M + H]^+$. Anal. Calcd for $C_{15}H_{18}O_7N_4S$: C, 45.22; H, 4.55; N, 14.06. Found: C, 45.24; H, 4.64; N, 13.99.

2-(β-D-Ribofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-diphosphate (20). A: Following the procedure described above for the synthesis of diphosphate **7**, tosylate **17** (365 mg, 0.92 mmol) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.65 g, 1.8 mmol) were reacted in dry CH_3CN (1.0 mL) for 4 d to yield 250 mg (60%) of **20** as a white fluffy solid. R_f 0.32 (CH_3CN/100 mM NH_4HCO_3/NH_4OH, 7:3:2); ^{1}H NMR (D_2O, 300 MHz) δ 8.01 (1H, s), 5.98 (1H, d, J = 3.6 Hz), 4.49 (1H, m), 4.18 (1H, m), 3.93 (2H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.3, 143.2, 136.5, 95.6, 84.4, 74.6, 70.7, 65.4; ^{31}P NMR (D_2O, 121 MHz) δ −7.78 (d, $J_{p,p}$ = 20.6 Hz), −10.79 (d, $J_{p,p}$= 20.6 Hz). HRMS (ESI): calcd 405.0213 $[M + H]^+$, found 405.0208 $[M + H]^+$.

B: Compound **15** (350 mg, 0.8 mmol) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.4 g, 1.6 mmol) were reacted in anhydrous CH_3CN (0.5 mL) for 2 d. The reaction mixture was diluted with water (5 mL) and was passed through a DOWEX AG 50W-X8 column (100−200 mesh, 2.5 cm × 10 cm, 83 equiv, NH_4^+ form) and eluted with two column volumes of deionized water. The eluent was dried by lyophilization and the resultant solid was dissolved in deionized water (20 mL). The solution was stirred for 3 h after adjusting the pH to 2.0 with trifluoroacetic acid. Then the pH was adjusted to 8.5 with 1.0 M NH_4OH and stirring was continued for another 2 h. The reaction mixture was dried by lyophilization, and the residue was purified by flash cellulose chromatography as described above for the synthesis of diphosphate **7** to yield 254 mg (70%) of **20** as a white fluffy solid.

1-(β-D-Ribofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-diphosphate (21). Compound **16** (220 mg, 0.5 mmol) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (900 mg, 1.0 mmol) were reacted in anhydrous CH_3CN (0.3 mL) for 20 h. The reaction mixture was diluted with water (5 mL) and was passed through a DOWEX AG 50W-X8 column (100−200 mesh, 2.5 cm × 10 cm, 83 equiv, NH_4^+ form) and eluted with two column volumes of deionized water. The eluent was dried by lyophilization and the resultant solid was dissolved in deionized water (10 mL). The solution was stirred for 2 h after adjusting the pH to 2.0 with trifluoroacetic acid. Then the pH was adjusted to 8.5 with 1.0 M NH_4OH and stirring continued for another 2 h. The reaction mixture was dried by lyophilization, and the residue was purified by flash cellulose chromatography as described above for the synthesis of diphosphate **7** to yield 150 mg (66%) of **21** as a white fluffy solid. R_f 0.28 (CH_3CN/100 mM NH_4HCO_3/NH_4OH, 7:3:2); ^{1}H NMR (D_2O, 300 MHz) δ 8.69 (1H, s), 6.07 (1H, d, J = 4.2 Hz), 4.54 (1H, m), 4.39 (1H, m), 4.25 (1H, m), 4.05 (2H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.4, 142.0, 125.9, 92.9, 84.7, 75.7, 70.4, 65.0; ^{31}P NMR (D_2O, 121 MHz) δ −7.98 (d, $J_{p,p}$ = 21.8 Hz), −10.83 (d, $J_{p,p}$ = 21.8 Hz). HRMS (ESI): calcd 403.0056 $[M - H]^-$, found 403.0046 $[M - H]^-$.

2-(β-D-Ribofuranosyl)-(2*H*)-1,2,3-triazole-4-carboxamide-5′-triphosphate (22). A 10-mL solution of 83 mM triethanolamine/17 mM $MgCl_2$/67 mM KCl containing compound **20** (90 mg, 0.2 mmol) and PEP (152 mg, 0.8 mmoL) was adjusted to pH 7.6 with 6 M NaOH. To this solution was added pyruvate kinase (40 units) and the mixture was incubated at 37 °C for 4 h. The reaction was monitored by analytical HPLC to ensure complete conversion of **20** to the corresponding triphosphate. The reaction mixture was adjusted to pH 8.5 by addition of NH_4HCO_3 (790 mg) and finally concentrated NH_4OH. This solution was applied to a Bio-Rad boronate affinity gel column (2.5 cm × 5.5 cm, in 1 M NH_4HCO_3, pH 8.5) and first eluted with 90 mL of 1 M NH_4HCO_3, pH 8.5 at 1 mL/min, then eluted with deionized water until triphosphate **22** was released from the column with UV detection at 230 nm. The desired fractions were dried by lyophilization and excess NH_4HCO_3 was removed by repeated lyophilization from deionized water after adjustment to pH 7.2 with CO_2.

Final purification of the compound was performed on a Q Sepharose FF anion exchange column (2.5 cm × 20 cm, HCO_3^- form) by a linear gradient elution with NH_4HCO_3 (0.05−0.5M, pH 7.8) at a flow rate of 5 mL/min over 2 h with UV detection at 230 nm. The desired fractions were dried by lyophilization to yield a white fluffy solid (78 mg, 72%). HPLC 10.63 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.05 (1H, s), 6.02 (1H, d, J = 3.9 Hz), 4.24 (1H, m), 4.03 (2H, m); ^{13}C NMR (D_2O, 75 MHz) δ 163.9, 142.8, 136.1, 95.1, 84.1, 74.1, 70.4, 65.3; ^{31}P NMR (D_2O, 121 MHz) δ −9.11 (d, $J_{p,p}$ = 19.4 Hz), −11.18 (d, $J_{p,p}$ = 19.4 Hz), −22.56 (t, $J_{p,p}$ = 19.4 Hz). HRMS (ESI): calcd 484.9876 $[M + H]^+$, found 484.9869 $[M + H]^+$.

1-(β-D-Ribofuranosyl)-(1*H*)-1,2,3-triazole-4-carboxamide-5′-triphosphate (23). Following the procedure described above for the synthesis of triphosphate **22**, compound **21** (90 mg 0.2 mmol) was phosphorylated to yield 72 mg (NH_4 salt, 67%) of **23** as a white fluffy solid. HPLC 10.48 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.69 (1H, s), 6.06 (1H, d, J = 4.2 Hz), 4.54 (1H, m), 4.42 (1H, m), 4.25 (1H, m), 4.07 (2H, m); ^{13}C NMR (D_2O, 75 MHz) δ 164.4, 142.0, 125.9, 92.9, 84.7, 75.7, 70.5, 65.3; ^{31}P NMR (D_2O, 121 MHz) δ −9.06 (d, $J_{p,p}$ = 19.4 Hz), −11.18 (d, $J_{p,p}$ = 19..4 Hz), −22.56 (t, $J_{p,p}$ = 19.4 Hz). HRMS (ESI): calcd 482.9719 $[M - H]^-$, found 482.9711 $[M - H]^-$.

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Supporting Information Available: ^{1}H, ^{13}C, and ^{31}P NMR spectra for compounds **7**, **8**, **9**, **10**, **11**, **12**, **20**, **21**, **22**, and **23**, and HPLC trace for compounds **10**, **11**, **12**, **22**, and **23**. This material is available free of charge via the Internet at http://pubs.acs.org.

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