Supplementary Material (ESI) for Chemical Communications

Convenient Synthesis of Nucleoside 5'-Triphosphates for RNA Transcription

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Supplemental Materials

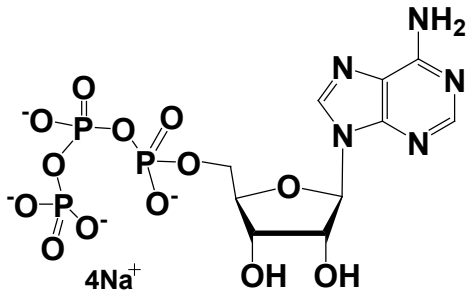
1. Materials: Most solvents and reagents were purchased from Sigma, Fluka, Aldrich (p.a.), Argros and ChemGenes, and no further purification was performed except in some cases. The nucleoside starting materials (solid reagents) were dried under high vacuum for several hours or overnight. Reactions utilizing air- or moisture-sensitive compounds were performed under an argon atmosphere unless otherwise specified. Analytical thin layer chromatography (TLC, Sorbent Technologies) was conducted using Merck Whatman 60 F254 plates (0.25 mm thick; Rf values in the text are for the title products), and visualized under UV-light. UV spectra were recorded using a Varian Cary 300 Bio (UV/VIS Model 240). High-resolution mass spectra (HR-MS) analyses were performed at Georgia State University Mass Spectrometry Facility, Atlanta, Georgia.

2. General procedure: The unprotected nucleoside (adenosine, cytidine, guanosine, or uridine; 10 mg, approximately 0.04 mmol) and tetrabutylammonium pyrophosphate (37 mg, 0.08 mmol, 2 eq.) were dried separately over night under high vacuum at ambient temperature in two round bottom flasks (10 mL each). Under argon, the tetrabutylammonium pyrophosphate was dissolved in dimethylformamide (DMF, 0.15 mL). Tributylamine (0.13 mL) was then added to the pyrophosphate solution, followed by its injection into the flask containing the freshly prepared 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one **1** (9.7 mg, 0.05 mmol, 1.2 eq) dissolved in (DMF, 0.3 mL) under argon. The reaction was stirred for 1 h.

The reaction mixture of **1** and pyrophosphate was added to one of the four nucleosides (A, C, G or U), which was dissolved in DMF (0.2 mL in the case of C and U) or DMF/DMSO (1:1 ratio, 0.2 mL in the case of A and G). The reaction was stirred for another 1 h at room temperature. Alternatively, this reaction was also conducted at 0^{o}C and -10^{o}C for 3 h to maximize the 5'-selectivity. Iodine solution (0.02 M iodine/ pyridine/ water, approximately 0.8 mL) was then injected into the reaction mixture until a permanent brown color was observed, affording the nucleoside cyclic 5'-triphosphate within 15 min. Water (approximately 6.4 mL; 4 fold of the reaction volume) was added, and the hydrolysis was stirred for additional 1.5 h to afford the desired nucleoside 5'-triphosphates. The reaction progress was monitored by TLC (*iso*-propanol:NH_3:H_2O:H_2O = 5:3:2) via comparing with the corresponding known dNTP standard. The resulting solution (approximately 8.0 mL) was transferred to a centrifuge tube (50 mL). NaCl (0.9 mL, 3.0 M) and absolute ethanol (approximately 27 mL; 3 fold of aqueous volume) were added to the tube, followed by vigorous shaking. After being placed at -80^{o}C for 1 h, the tube was centrifuged (40 min at 3200 rpm) to offer the nucleoside 5'-triphosphate as a pellet. The supernatant was poured out, and the tube was up-side down to allow the white precipitate dry naturally.

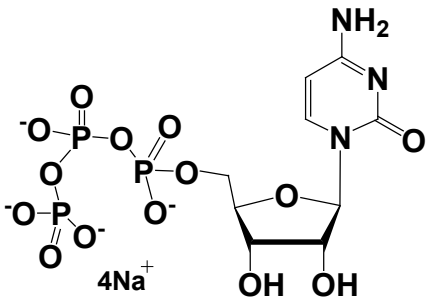
Analytical reverse-phase HPLC was used to analyze the synthesized triphosphates comparing each with the commercially nucleoside 5'-triphosphates as standards. All samples were analyzed on a Welch C18 reversed phase analytical column (4.6 x 250 mm) and measured at 260 nm at a flow rate of 1.0 mL/min and with a linear gradient of 0 to 25% B in 22 min. Buffer A: 20 mM triethylammonium acetate (TEAAc, pH 7.1); Buffer B: 50% acetonitrile in 20 mM TEAAc (pH 7.1). For preparative HPLC purification, the desired peak was collected and the buffers were removed by lyophilization to afford the purified 5'-triphosphates as the triethylammonium salt, a creamy solid, followed by re-precipitation to afford the nucleoside triphosphate sodium salt. The yields were calculated based on UV-vis spectrophotometer. Mass spectrometry data were collected, and HPLC analysis was performed again to confirm the purity of the final dNTPs by co-injection with the corresponding dNTP standards.

Adenosine 5′-triphosphate (ATP):



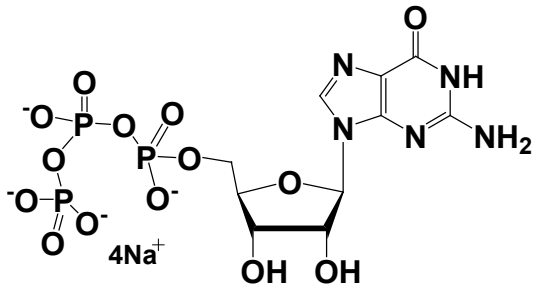
ATP was synthesized (10% crude yield before HPLC purification) in one pot by following the general procedure without any protection. UV (H_2O): λ_{max} = 261 nm; HR-MS (ESI-TOF): molecular formula $C_{10}H_{16}N_5O_{13}P_3$; $[M-H]^-$: 505.9891 (calculated: 505.9885).

Cytidine 5′-triphosphate (CTP):



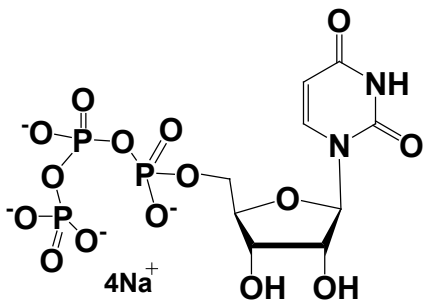
CTP was synthesized (36% crude yield before HPLC purification) in one pot by following the general procedure without any protection. UV (H_2O): λ_{max} = 270 nm; HR-MS (ESI-TOF): molecular formula $C_9H_{16}N_3O_{14}P_3$; [M-H]$^-$: 481.9781 (calculated: 481.9772).

Guanosine 5′-triphosphate (GTP),



GTP was synthesized (50% crude yield before HPLC purification) in one pot by following the general procedure without any protection. UV (H_2O): λ_{max} = 250 nm; HR-MS (ESI-TOF): molecular formula $C_{10}H_{16}N_5O_{14}P_3$; [M-H]$^-$: 521. 9828 (calculated: 521.9834).

Uridine 5′-triphosphate (UTP),



UTP was synthesized (29% crude yield before HPLC purification) in one pot by following the general procedure without any protection. UV (H_2O): λ_{max} = 257 nm; HR-MS (ESI-TOF): molecular formula $C_9H_{15}N_2O_{15}P_3$, $[M-H]^-$: 482.9615 (calculated: 482.9612).

3. Results

HPLC profiles (Figure S1-3): All samples were analyzed on a Welch C18 reversed phase analytical column (4.6 x 250 mm) and measured at 260 nm at a flow rate of 1.0 mL/min and with a linear gradient of 0 to 25% B in 22 min. Buffer A: 20 mM triethylammonium acetate (TEAAc, pH 7.1); Buffer B: 50% acetonitrile in 20 mM TEAAc (pH 7.1).

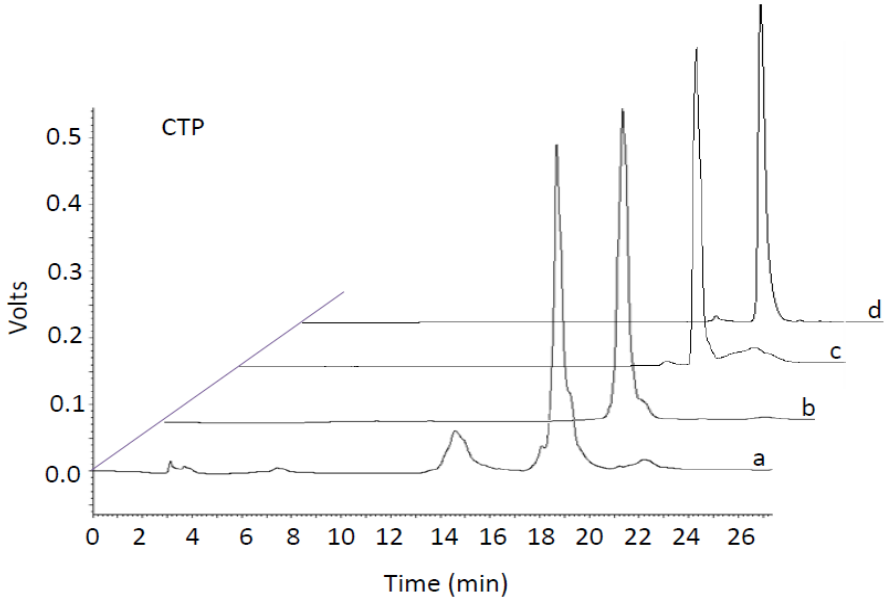


Figure S1. HPLC profiles of chemically synthesized (test) and commercially available cytidine 5'-triphosphate (CTP) as standard. a) HPLC profile of crude CTP at 0°C (injected 40 µL, 1.0 mM, retention time 18.6 min); b) HPLC profile of purified CTP (injected 40 µL, 1.0 mM, retention time 18.8 min); c) co-injection of purified CTP (test) and standard (injected 20 µL each, 1.0 mM, retention time 18.9 min); d) standard CTP (injected 40 µL, 1.0 mM, retention time 18.9 min).

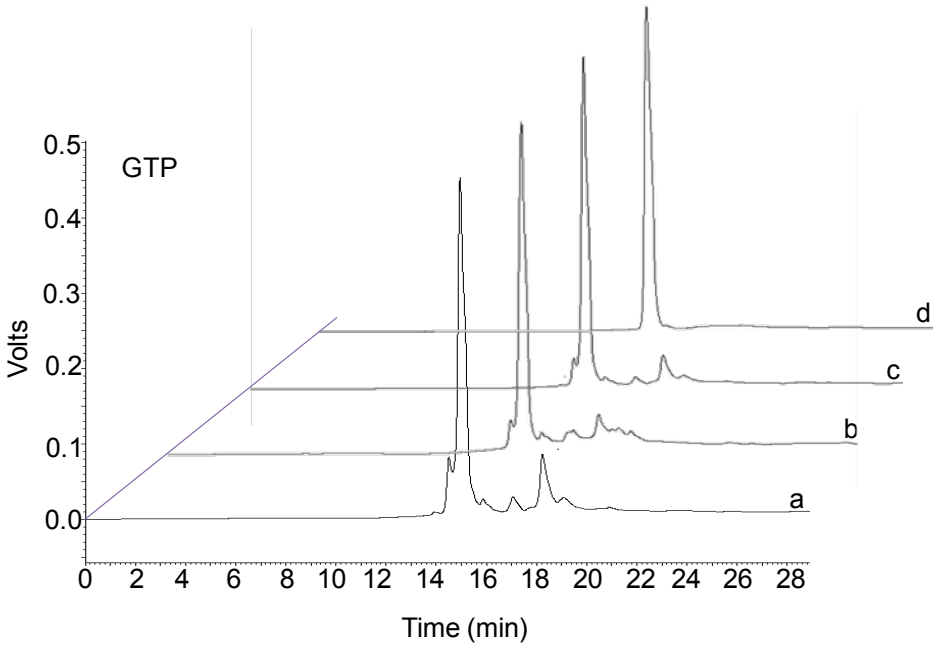


Figure S2. HPLC profiles of chemically synthesized (test) and commercially available guanosine 5'-triphosphate (GTP) at different temperatures (r.t. and 0^{o}C). a) HPLC profile of crude GTP at r.t. (injected 40 µL, 1.0 mM, retention time 14.9 min); b) crude, synthesized (test) GTP at 0^{o}C (injected 40 µL, 1.0 mM, retention time 14.8 min); c) co-injection of GTP (test) at r.t. and standard (injected 20 µL each, 1.0 mM, retention time 14.6 min) and d) standard GTP (injected 40 µL, 1.0 mM, retention time 15.1 min).

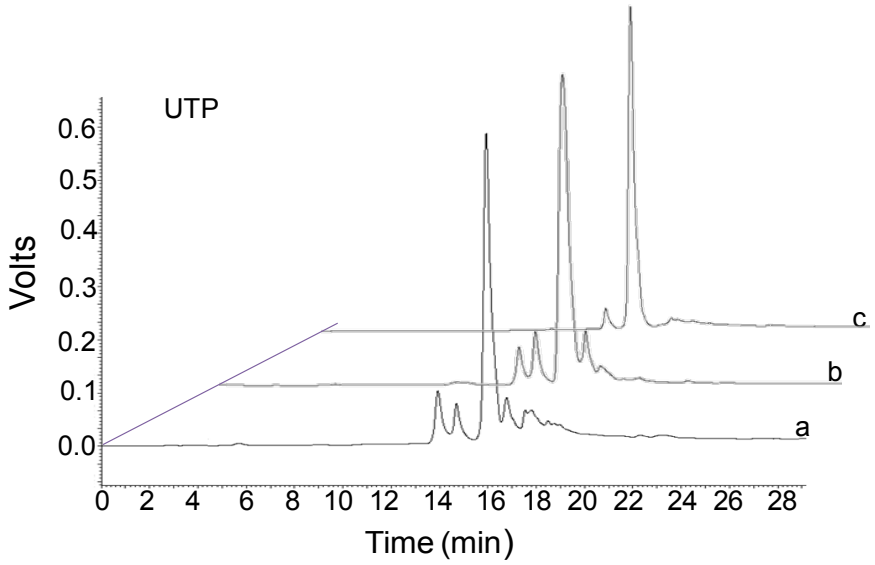


Figure S3. HPLC profiles of chemically synthesized (test) and commercially available uridine 5'-triphosphate (UTP) as standard. a) HPLC profile of crude UTP (at -10^{o}C, injected 40 µL, 1.0 mM, retention time 15.9 min); b) co-injection of crude UTP (test, -10^{o}C) and standard (injected a 20 µL each, 1.0 mM, retention time 15.8 min); c) standard UTP (injected 40 µL, 1.0 mM, retention time 15.8 min).

4. Oligonucleotide synthesis and purification

The DNA promoter and template sequences were synthesized on an Applied BioSystem 394 Synthesizer employing standard β-cyanoethylphosphoramidite chemistry (1 µmol scale). Oligonucleotides were synthesized in DMTr-off mode, and their average coupling efficiencies were greater than 99%. Purification of the oligonucleotides was conducted on 15% polyacrylamide gel electrophoresis. The products were visualized under UV light (254 nm), and the crush-and-soak method was applied, followed by NaCl/EtOH precipitation to isolate the DNA oligonucleotides (see the procedure adapted from Brandt *et al*., 2006). The

oligonucleotides were characterized by MALDI-TOF MS and quantified by UV-vis absorbance at 260 nm.

5. RNA transcription with the synthesized (test) NTPs

AmpliScribe T7 Transcription Kits (Epicentre, Inc.) were used for the *in vitro* transcription. The DNA promoter (final reaction concentration: 1.0 μM; 5'-CGTAATACGACTCACTATAG-3') and the DNA template (final reaction concentration: 1.0 μM; 3'-CGCATTATGCTGAGTGATATCCGTTGGACTACTCCGGCTTTCCGGCTTTGCATGT-5') were added to a cocktail containing the buffer, dithiothreitol (DTT, 10 mM final reaction concentration), pyrophosphatase (0.02 U/μL, final), water and no NTPs. α-[^{32}P]ATP (Perkin-Elmer) was also included in the cocktail to bodily label the RNA transcript. The cocktail was distributed into eleven tubes for preparing the negative and positive controls and the triphosphate tests. The negative control reactions used water to replace the polymerase or one of four NTPs (ATP, CTP, GTP or UTP). The positive control reaction contained all standard NTPs (0.50 mM each; Epicentre, Inc) and the polymerase. For each of the test reactions, one NTP out of the four standard NTPs was replaced each time by the corresponding synthesized (test) NTP out of the four NTPs. T7 RNA polymerase (0.05 U/μL final) was added to initiate the transcription reactions. All of the reaction mixtures were incubated at 37^{o}C for 2 h. The reactions were then quenched by the addition of the gel loading dye solution (5 μL each), and the analysis was performed on 19%, polyacrylamide gel electrophoresis (PAGE). The experimental result from autoradiograph is presented in the main text (Fig. 2).

Reference

G. Brandt, N. Carrasco, Z. Huang, *Biochemistry* **2006**, *45*, 8972-7.