Supporting Information

Protection-free One-pot Synthesis of 2'-Deoxynucleoside 5'-Triphosphate and DNA Polymerization

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1. Materials

Most solvents and reagents were purchased from Sigma, Fluka, Aldrich and ChemGenes without any further purification except in some cases. The nucleoside starting materials (solid reagents) were dried under high vacuum for several hours. Reactions utilizing air or moisture sensitive compounds were performed under an argon atmosphere unless otherwise specified. Analytical thin layer chromatography (TLC, Sorbent Technologies) was run / on Merck 60 F254 plates (0.25 mm thick), and visualized under UV-light. UV spectra were recorded using a Varian Cary 300 Bio (UV/VIS Model 240). ^{1}H-NMR and ^{31}P-NMR spectra were recorded using a Varian EM-400 (400 MHz). High resolution mass spectra (HR-MS) analyses were performed at Georgia State University Mass Spectrometry Facility, Atlanta, Georgia.

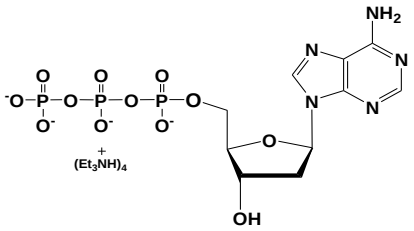
2. Synthesis of deoxynucleoside triphosphates (4a -4e)

General procedure: The unprotected nucleoside: 2'-deoxyadenosine (**1a**), 2'-deoxycytidine (**1b**), 2'-deoxyguanosine (**1c**) or thymidine (**1d**) (0.08 mmol, each), and tributylammonium pyrophosphate (75 mg, 0.16 mmol, 2 eq.) were dried under high vacuum for 1 h at ambient temperature in separate round bottom flasks (15 mL). Throughout the entire experiment, syringes were used to transfer solvent reagents from one flask to another under an argon atmosphere. Dried tributylamine (0.3 mL) was transferred to the septum of the flask containing the tributylammonium pyrophosphate dissolved in 0.25 mL dimethylformamide (DMF). The reaction mixture was injected into the septum of the flask containing the dried 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one **1** (33 mg, 0.16 mmol, 2 eq.) dissolved in DMF (0.25 mL) with vigorous stirring. The progress of the reaction was monitored by phosphorus NMR, where the reference (H_3PO_4) was used as an external standard and thymidine **1d** was chosen as the model compound. Preparation of the reaction for the NMR study was conducted in a glove box using deuterated DMF-d7 solution. After about 30 min into the reaction, a sample (400 µ L) was extracted via syringe and its ^{31}P-NMR (DMF d-7) spectrum showed the appearance of new peaks as a triplet at 98.8 ppm and disappearance of the phosphorous peak of compound **1** (main text) at 124.6 ppm, indicating the formation of a proposed cyclic triphosphite intermediate (**2**).

The reaction mixture was then added to the flask containingan individual nucleoside (dA, dC, dG or T). The reactions continued with stirring for another 1½ h, and TLC analysis (12% methanol in CH_2Cl_2) indicated that approximately 70% of the starting nucleoside was consumed. Extending the stirring to a longer time (16 h) did not result in complete conversion of the

nucleosides. Iodine solution [3% in 1 mL of 9:1 (pyridine:water)] was then injected into each reaction mixture. As the iodine was consumed, dropwise addition of the iodine solution was continued until a permanent brown color of iodine was maintained, affording the nucleoside cyclic 5'-triphosphates. After 15 min, water (2 mL) was added with stirring at room temperature for another 1½ h to afford the desired nucleoside 5'-triphosphates. The progress of the hydrolysis reaction was monitored by TLC (*i*p-OH:NH_3:H_2O = 5:3:2). Each resulting solution was transferred to individual 50 mL centrifuge tube. The reaction volume was measured (approximately 4.5 mL, each reaction) and NaCl (0.5 mL, 3 M) was added to each tube, followed by addition of ethanol (15 mL). After placing at -80 ^{o}C for 1 h, the crude triphosphates were recovered by centrifugation (20 min at 3200 rpm). The supernatants were removed, and the white residues formed were air dried for over 30 min. Preparative reverse-phase HPLC (RP-HPLC) was used to obtain high purity products. Each desired triphosphate was purified with a linear gradient of 0% to 40% ethanol buffer (containing triethylammonium acetate (TEAAc) 20 mM, pH = 6.5) over 27 min on a reverse-phase preparative column (21.2 x 250 mm, XB C18, 10 µm) monitored at 260 nm at a flow rate of 6.0 mL/min. The buffer solutions collected were evaporated by lyophilization, and the nucleoside 5'-triphosphates were re-dissolved in distilled water (600 µL), affording the desired 5'-triphosphates as the triethylammonium salt and were re-precipitated with NaCl/ethanol to afford the 5'-triphosphates as the sodium salt. The integrity of all synthesized nucleoside 5'-triphosphates was confirmed by ^{1}H-NMR, ^{31}P-NMR, and HR-MS analyses and are similar to the literatures.[1] The proton NMR chemical shifts of the ethyl group from the triethylammonium salt present in some of the compounds are not listed in the data section since the salt is present as an impurity. Analytical RP-HPLC analyses of the 5'-triphosphates (Fig. S1& S2) were performed and compared with the commercially available dNTPs. The samples were analyzed on a Welch C18 or XB C18, reverse-phase analytical column (4.6 x 250 mm) as stated in the legends at 260 nm at a flow rate of 1.0 mL/min. Buffer A: 20 mM TEAAc, pH 7.1 and buffer B: 50% acetonitrile in 20 mM TEAAc. The yields were calculated based on the HPLC analysis and the working curve that was established using standard dNTPs, or otherwise stated.

2'-Deoxyadenosine 5′-triphosphate (dATP, 4a)

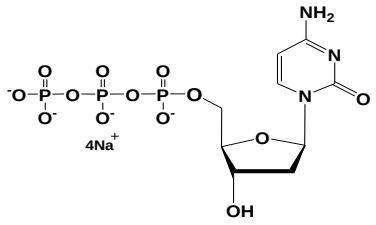


4a

By following the general procedure, **4a** was determined as (19% yield).

^{1}H-NMR (400 MHz, D_2O): δ 8.50 (s, 1H, H-2), 8.27 (s, lH, H-8), 6.54 (t, J = 6.5 Hz, lH, H-1'), 4.41 (m, 1H, H-3'), 4.31 (m, 1H, H-4'), 3.88 (m 1H, H-5'), 3.24 (m 1H, H-5'), 2.84 (m, 1H, H-2′), 2.61 (m, 1H, H-2′); ^{31}P-NMR (162 MHz, D_2O): δ -22.79 (t, J_β = 19.68 Hz, 1P, β-P), -11.21 (d, J_α = 19.60 Hz, 1P, α-P), -9.49 (d, J_γ = 20.57 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 259 nm; HR-MS (ESI-TOF): molecular formula $C_{10}H_{16}N_5O_{12}P_3$; $[M\text{-}H^+]^-$: 489.9938 (calculated: 489.9936).

2'- Deoxycytidine 5′-triphosphate (dCTP, 4b)

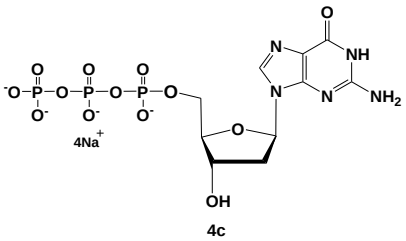


4b

By following the general procedure, 2'- deoxycytidine 5′-triphosphate (dCTP, **4b**), was synthesized from **1b,** which was dissolved in DMF (0.25 mL) and tributylamine (0.1 mL) prior to the addition of the reaction mixture containing compound **2**. **4b** was determined as 46% yield.

^{1}H-NMR (400 MHz, D_2O): δ 7.98 (d, J = 7.6 Hz, 1H, H-6), 6.34 (t, J = 6.0 Hz, lH, H-1'), 6.14 (d, J = 7.6 Hz, 1H, H-5), 4.63 (b, 1H, H-3'), 4.21 (m, 2H, H- 4' & 5'), 3.65 (m, 1H, H-5'), 2.39 (m, 1H, H-2′), 2.33 (m, 1H, H-2′); ^{31}P-NMR (162 MHz, D_2O): δ -22.65 (t, J_β = 19.60 Hz, 1P, β-P), -10.85 (d, J_α = 23.33 Hz, 1P, α-P), -10.16 (d, J_γ = 19.60 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 271 nm; HR-MS (ESI-TOF): molecular formula $C_9H_{16}N_3O_{13}P_3$; $[M\text{-}H^+]^-$: 465.9814 (calculated: 465.9817).

2'-Deoxyguanosine 5′-triphosphate (dGTP, 4c)

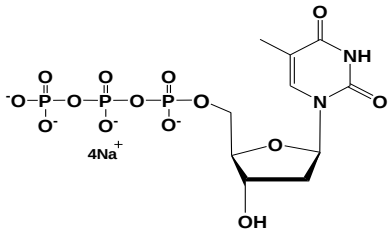


4c

By following the general procedure, **4c** was determined as 30% yield.

^{1}H-NMR (400 MHz, D_2O): δ 8.12 (s, 1H, H-8), 6.33 (t, *J* = 6.8 Hz, lH, H-1'), 4.71 (m, 1H, H-3'), 4.21 (m, 3H, H-4'& 5'), 2.84 (m, 1H, H-2′), 2.52 (m, 1H, H-2′); ^{31}P-NMR (162 MHz, D_2O): δ -22.73 (b, 1P, β-P), -11.10 (d, J_α = 18.31 Hz, 1P, α-P), -9.50 (b, 1P, γ-P); UV (H_2O): λ_{max} = 252 nm; HR-MS (ESI-TOF): molecular formula $C_{10}H_{16}N_5O_{13}P_3$; $[M\text{-}H^+]^-$: 505.9893 and calculated: 505.9885.

Thymidine 5′-triphosphate (TTP, 4d)

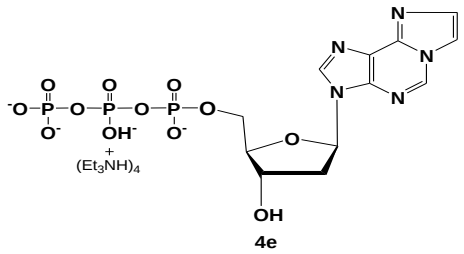


4d

By following the general procedure, **4d** was determined as 39% yield. The reaction was scaled up to 100 mg 70 % converted. The reaction was scaled up to 100 mg and the nucleoside conversion was ca.70 % as determined by analytical TLC after 4 h. Upon further stirring for over 16 h no significant change in nucleoside conversion was observed.

^{1}H-NMR (400 MHz, D_2O): δ 7.75 (s, 1H, H-6), 6.35 (t, *J* = 6.8 Hz, 1H, H-1'), 4.69 (m, 1H, H-3'), 4.22 (m, 3H, H- 4'& -5'), 2.38(m, 2H, H-2'), 1.94 (s, 3H, Me-5); ^{31}P-NMR (162 MHz, D_2O): δ -21.22 (t, J_β = 16.36 Hz, 1P, β-P), -10.50 (d, J_α = 18.47 Hz, 1P, α-P), -6.65 (d, J_γ = 17.17 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 267 nm; HR-MS (ESI-TOF): molecular formula $C_{10}H_{17}N_2O_{14}P_3$; $[M\text{-}H^+]^-$: 480.9831 (calculated: 480.9820).

Modified base: Etheno-deoxyadenosine 5′-triphosphate (EdATP, 4e),



By following the general procedure, etheno-deoxyadenosine 5′-triphosphate (dEATP **4e**), was synthesized from **1e** and determined as 32% yield based on UV-vis analysis.

^{1}H-NMR (400 MHz, D_2O): δ 9.08 (s, 1H, H-2), δ 8.53 (s, 1H, H-8), δ 7.95 (s, 1H, H-11), δ 7.55 (s, 1H, H-10), 6.61 (t, *J* = 6.6 Hz, l H, H-1′), 4.36 (b, 1H, H-3′), 4.26 (m, 3H, H-4'& 5'), 2.94 (m, 1H, H-2′), 2.65 (m, 1H, H-2′); ^{31}P-NMR (161.97 MHz, D_2O): δ -22.89 (b, 1P, β-P,), -11.66 (b, 1P, α-P), - 9.63 (b, 1P, γ-P); UV (H_2O): λ_{max} = 266 and 275 nm; HR-MS (ESI-TOF): molecular formula $C_{12}H_{16}N_5O_{12}P_3$; $[M\text{-}H^+]^-$: 513.9940 (calculated: 513.9936).

3. Analytical RP-HPLC profiles of chemically synthesized dNTPs

a) HPLC profiles of crude dNTPs after NaCl/ethanol precipitation

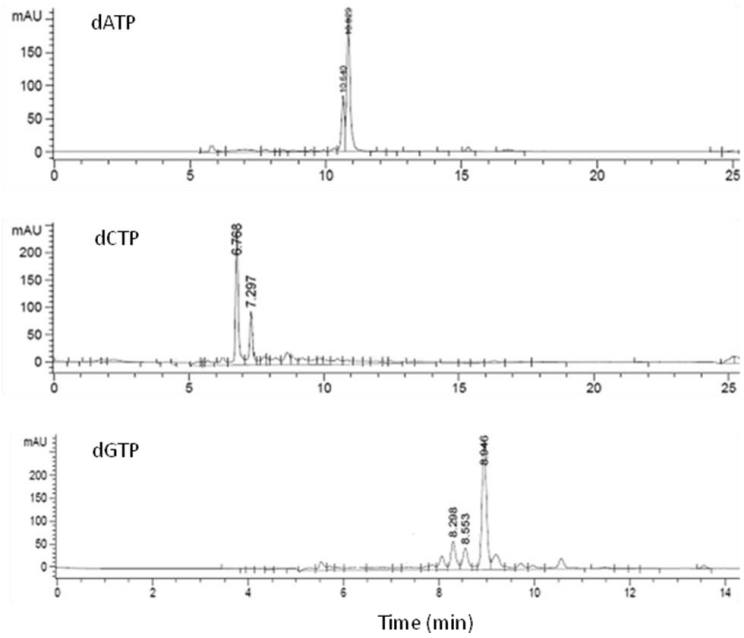


Figure S1: Analytical RP-HPLC profile of crude (dNTPs. a) dATP (RT = 10.8 min. b) dCTP (RT = 6.8 min c) dGTP (RT = 8.9 min). The crudes were eluted at 1 mL/min with a linear gradient from buffer A [10 mM triethylammonium

acetate (TEAAc)] to 25% Buffer B [50% acetonitrile in water, 10 mM TEAAc] in 20 min measured at 260 nm on a Welsch C18 analytical HPLC.

b) HPLC profiles of purified dNTPs

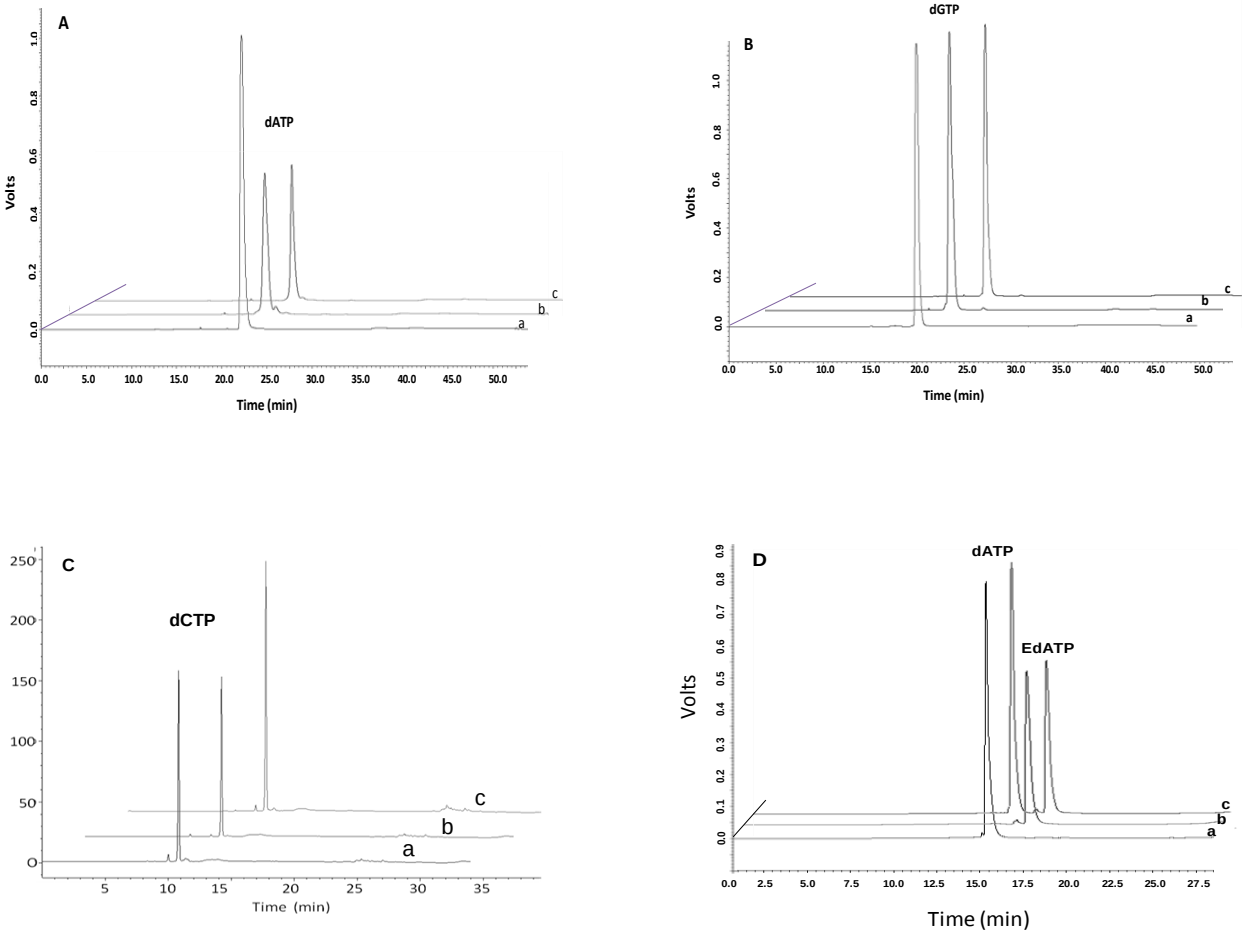


Figure S2: HPLC profiles of chemically synthesized and commercial 2'-deoxynucleoside 5'-triphosphates (dNTPs). **A**: standard dATP (**a**, retention time 22.1 min); synthesized dATP (**b**, retention time 22.0 min); co-injection of synthesized and standard dATP (**c**, retention time 20.0 min). **B**: standard dGTP (**a**, retention time 19.9 min); synthesized dGTP (**b**, retention time 19.9 min); co-injection of synthesized and standard dGTP (**c**, retention time 19.9 min). Profiles A and B were eluted with a linear gradient of 0 to 40% B in 30 min on an XB C18 column. **C**: standard dCTP (**a**, retention time 10.8 min); synthesized dCTP (**b**, retention time 10.8 min); co-injection of synthesized and standard dCTP (**c**, retention time 10.9 min), eluted with a linear gradient of 0 to 25% buffer B in 20 min using a Welsch C18 column. **D**: standard dATP (**a**, retention time 15.2 min); synthesized EdATP (**b**, retention time 17.5 min); co-injection of dATP and EdATP (**c**, retention times 15.2 and 17.2 min), respectively, where samples were eluted with a linear gradient of 0 to 40% B in 30 min using a Welsch C18 column.

4. ^{1}H-, ^{31}P-NMR and HR-MS Spectra of 2'-deoxynucleoside 5'-triphosphates (4a- 4e)

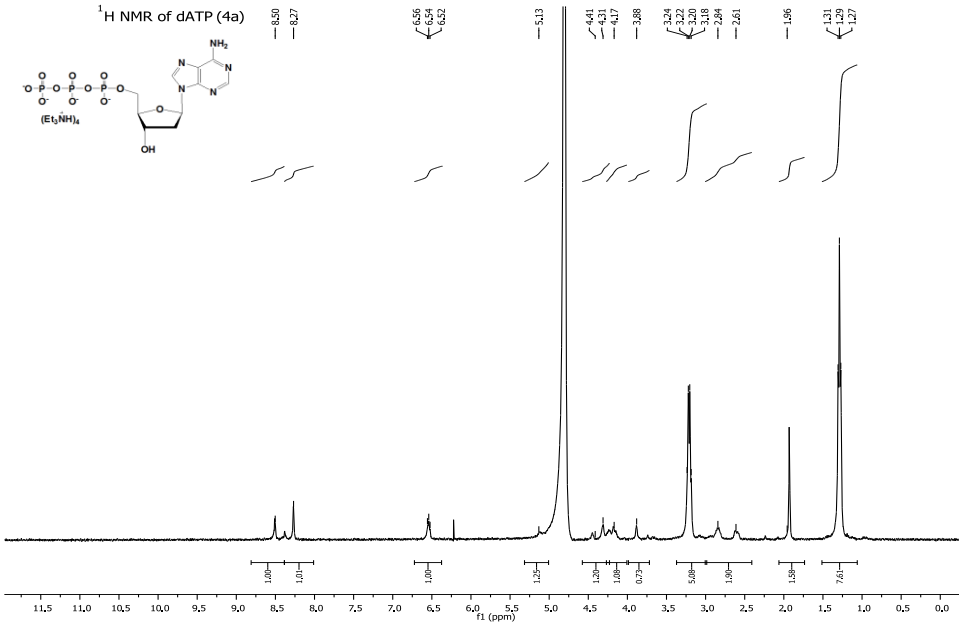


Figure S4. ^{1}H-NMR spectrum (400 MHz, D_2O) of compound (**4a**, the triethylammonium salt).

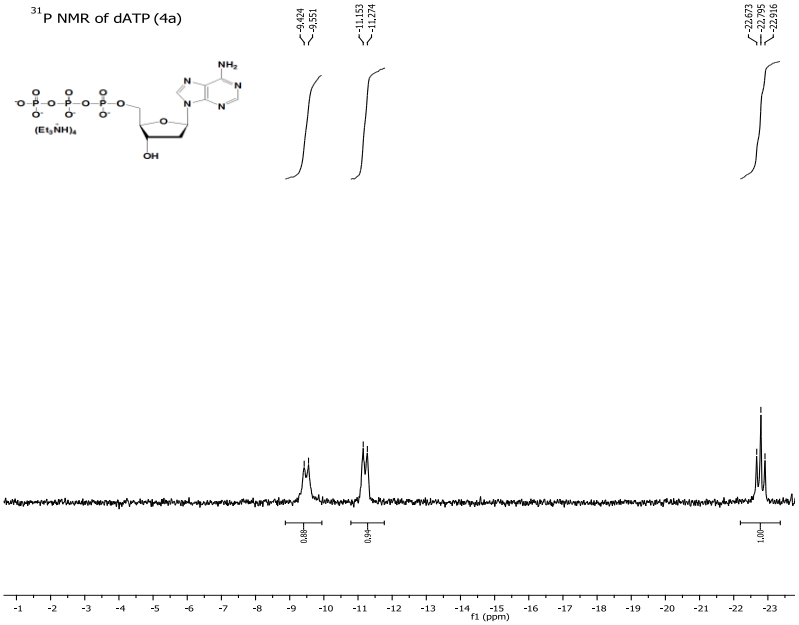


Figure S5. ^{31}P- NMR spectrum (162 MHz, D_2O) of compound (**4a**).

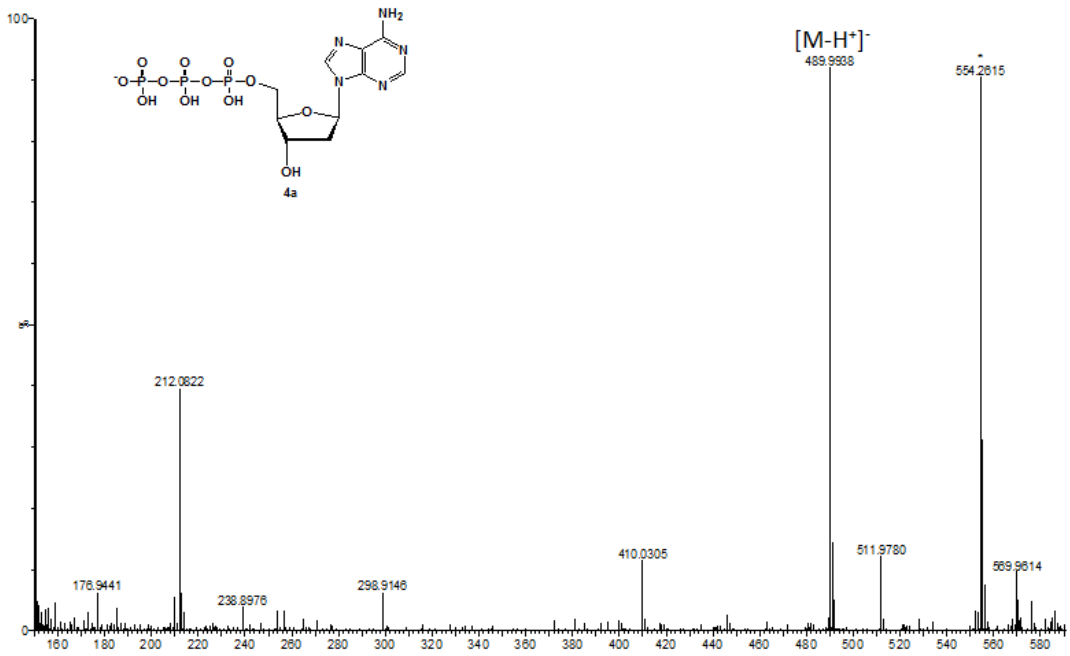


Figure S6. HR-MS (ESI-TOF) of dATP (**4a**): molecular formula $C_{10}H_{16}N_5O_{12}P_3$; $[M\text{-}H^+]^-$: 489.9938 (calculated: 489.9936).

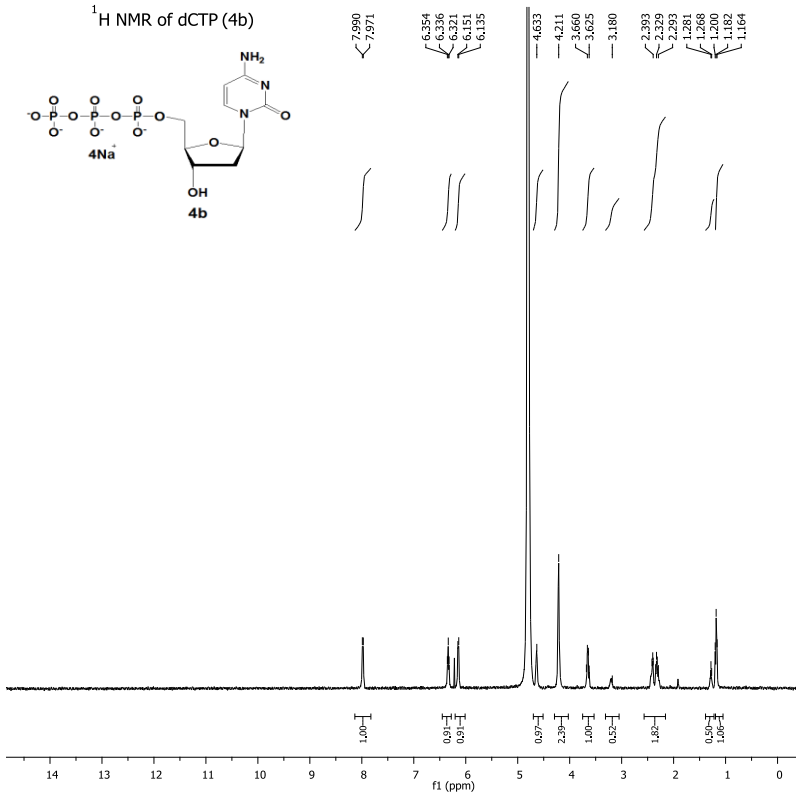


Figure S7. ^{1}H-NMR spectrum (400 MHz, D_2O) of compound (**4b**).

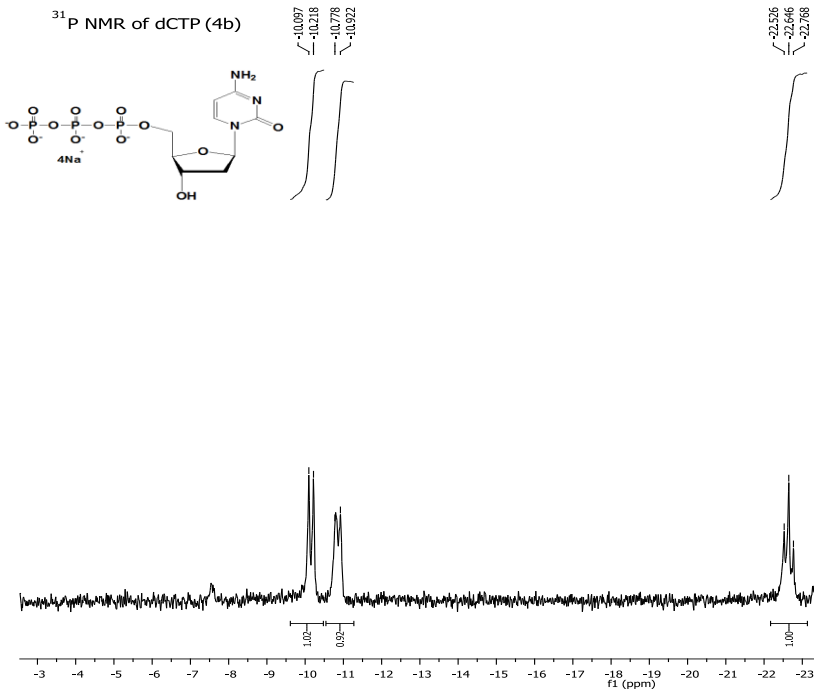


Figure S8. ^{31}P-NMR spectrum (162 MHz, D_2O) of compound (**4b**).

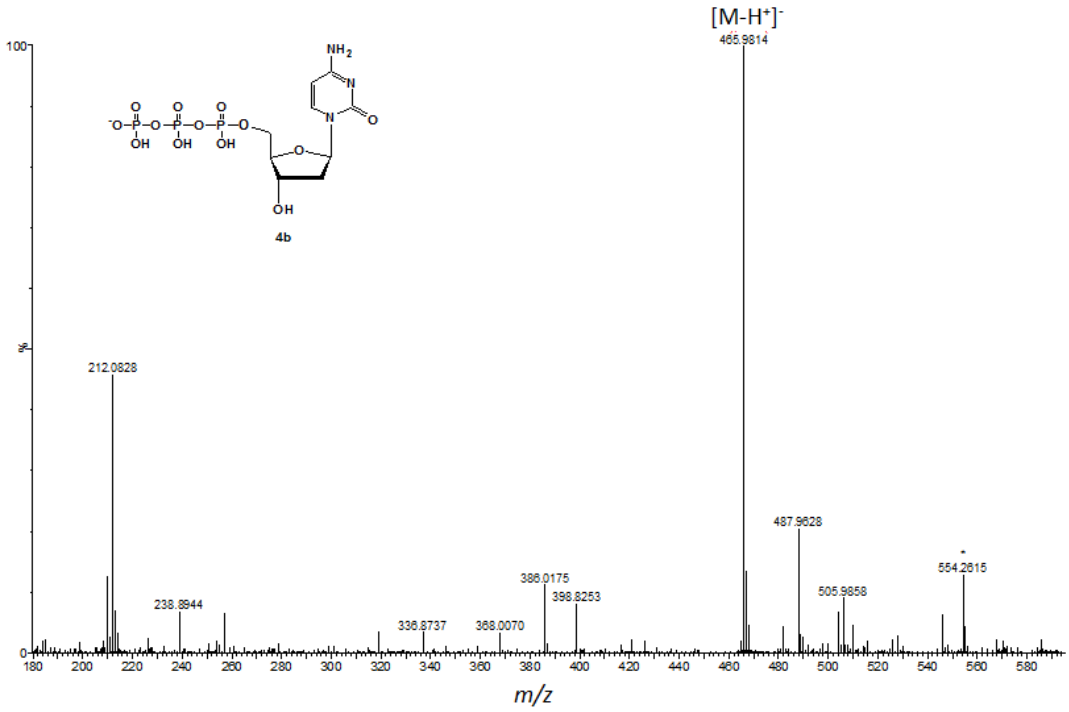


Figure S9. HR-MS (ESI-TOF) of dCTP (**4b**): molecular formula $C_9H_{16}N_3O_{13}P_3$; $[M\text{-}H^+]^-$: 465.9814 (calculated: 465.9817).

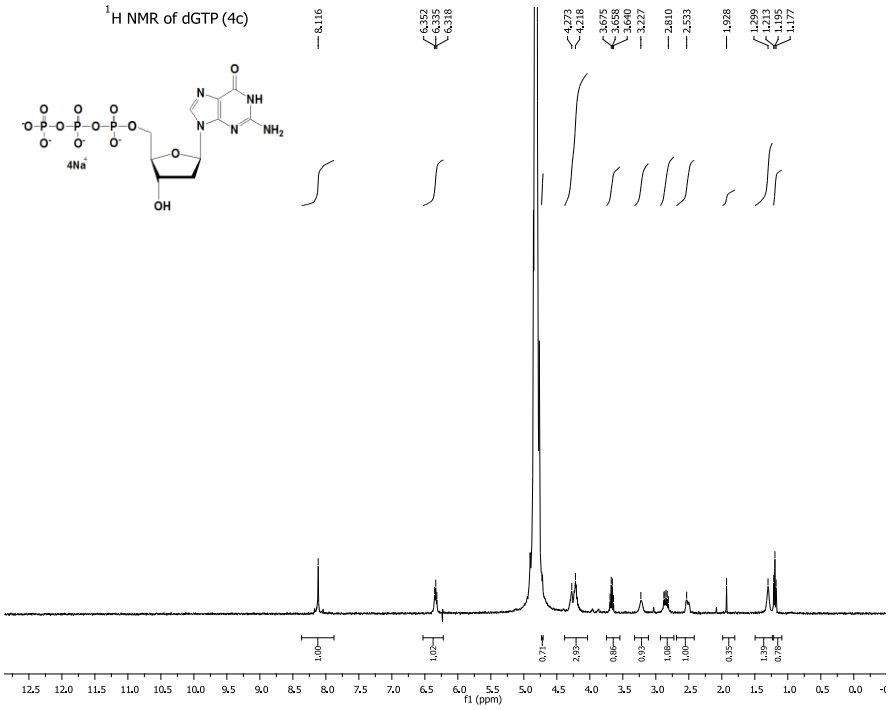


Figure S10. ^{1}H-NMR spectrum (400 MHz, D_2O) of compound (**4c**).

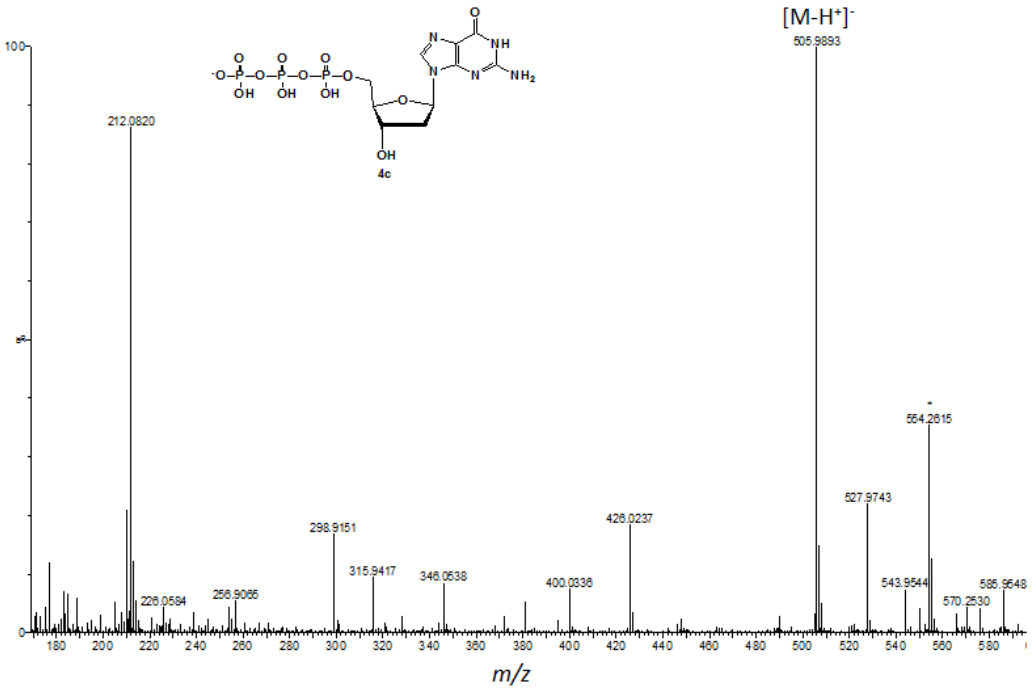


Figure S11. HR-MS (ESI-TOF) of dGTP (**4c**): molecular formula $C_{10}H_{16}N_5O_{13}P_3$; $[M\text{-}H^+]^-$: 505.9893 and calculated: 505.9885.

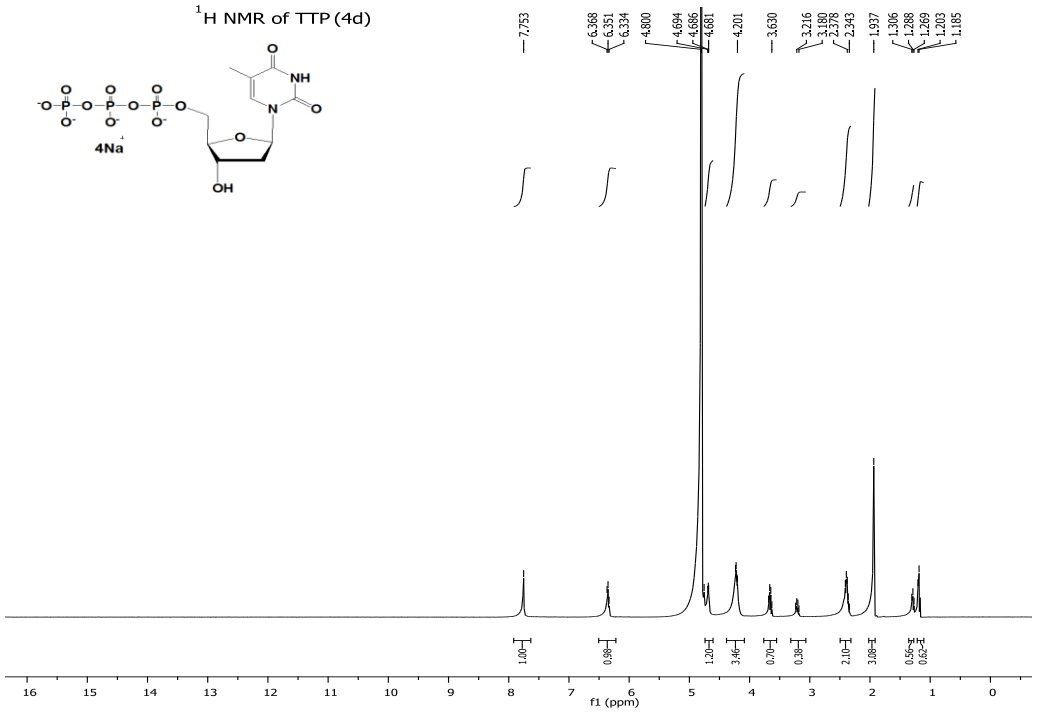


Figure S12. ^{1}H-NMR spectrum (400 MHz, D_2O) of compound (**4d**).

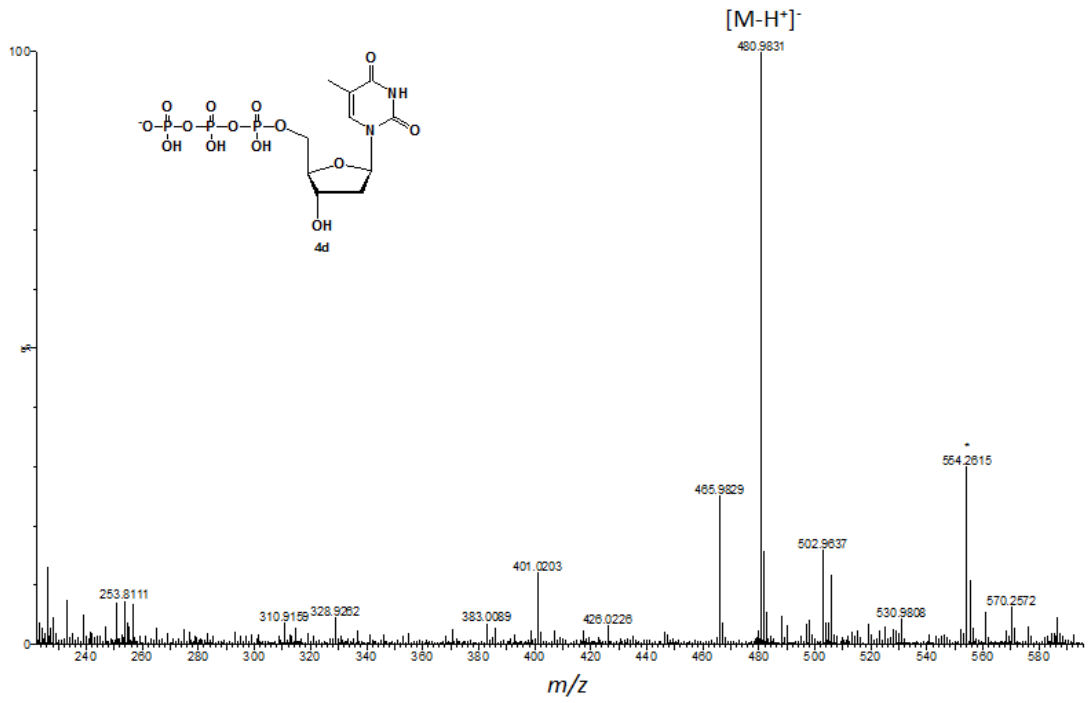


Figure S13. HR-MS (ESI-TOF) of TTP (**4d**): molecular formula $C_{10}H_{17}N_2O_{14}P_3$; $[M\text{-}H^+]^-$: 480.9831 (calculated: 480.9820).

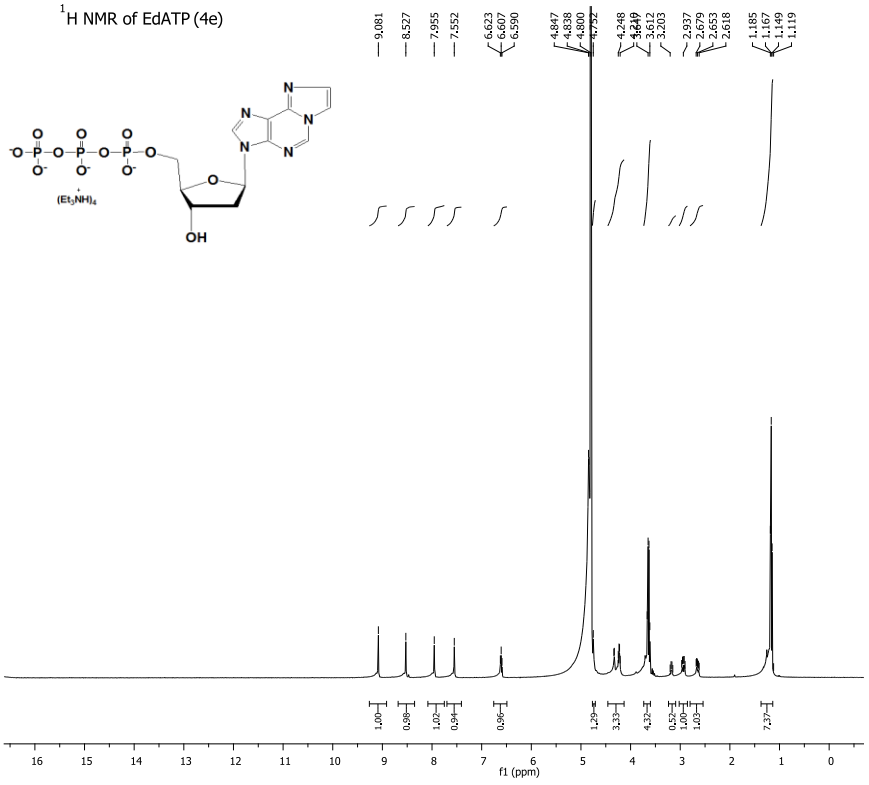


Figure S14. ^{1}H-NMR spectrum (400 MHz, D_2O) of compound (**4e**, triethylammonium salt).

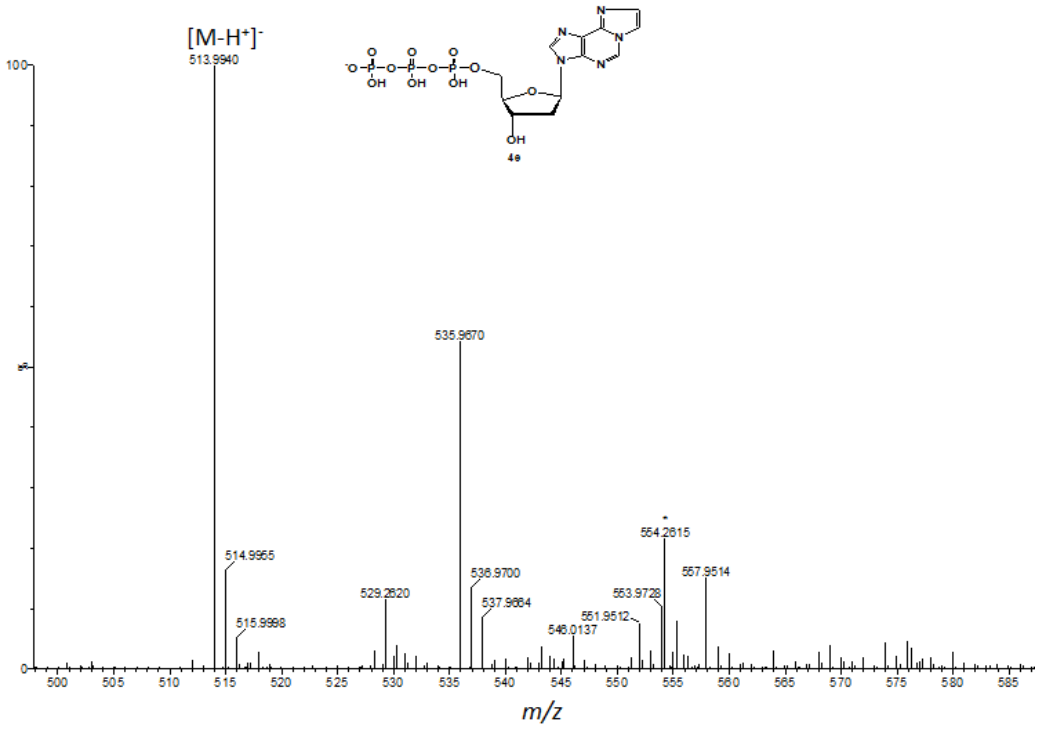


Figure S15. HR-MS (ESI-TOF) of EdATP (**4d**): molecular formula $C_{10}H_{17}N_2O_{14}P_3$; $[M\text{-}H^+]^-$: 480.9831 (calculated: 480.9820).

5. Oligonucleotide synthesis and purification

DNA oligodeoxyribonucleotides were synthesized on an Applied BioSystem 394 Synthesizer employing standard β-cyanoethylphosphoramidite chemistry (1 μmol scale). Oligonucleotides were synthesized with 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 gel was crushed and soaked, follow by NaCl/EtOH precipitation to recover the oligonucleotides (see procedure in Brandt *et al*., 2006)[2]. Oligonucleotides were characterized by MALDI-TOF MS and quantified by UV-vis absorbance at 260 nm. Primer (P) was labeled at the 5'-terminus with [γ-^{32}P] ATP (PerkinElmer Life and Analytical Sciences) by T4 polynucleotide kinase (New England BioLabs).

6. DNA primer extension experiments utilizing the synthesized dNTPs

The primer (P) was 5'-end labeled using T4 polynucleotide kinase and [γ-^{32}-P] ATP. In a buffer of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 0.5 mM dithiothreitol (DTT), primer extension reactions were performed using primer (3.5 μM) and template (5 μM), and equal concentrations (0.1 mM, each) of all standard dNTPs (Epicentre, Inc.) and synthesized dNTPs and 0.05 U/μL of Klenow exo-. A reaction cocktail contained the 5'-^{32}P-labeled primer (3.5 μM, 5'-dGCGTAATACGACTCACTATAG-3') and the template (5 μM, 3'-dCGCATTATGC-TGAGTGATATCCGTTGGACTACTCCGGCTTTCCGGCTTTGCATGT-5'), the polymerase reaction buffer, but without any dNTPs. The cocktail was distributed into eleven tubes to represent the negative controls, the positive controls and test extension reactions. The negative control reactions contained water in lieu of any dNTP (dATP, dCTP, dGTP or TTP) and the polymerase. The positive control reaction contained all standard dNTPs. For the actual test reactions, a standard dNTP in the positive control was substituted for either a corresponding HPLC purified triphosphate, or a crude triphosphate.The polymerase (Kf-) was then added to initiate the reaction. All of the reaction mixtures were incubated at 37 ^{o}C for 1 h. The reactions were quenched by the addition of gel loading dye solution (5 μL each) and the analysis was performed by gel electrophoresis (PAGE, 19% polyacrylamide). The autoradiographic visualization of the reactions utilizing HPLC purified dNTPs and crude dNTPs are presented in Figure 3 in the main text and Figure S16.

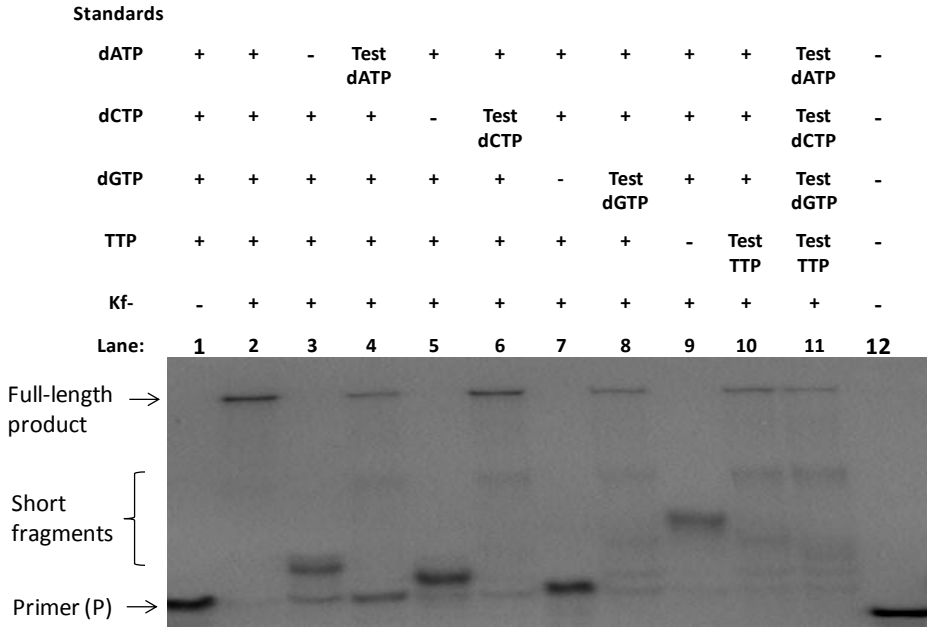


Figure S16. Utilization of crude dNTPs (NaCl-ethanol precipitated) in DNA polymerase extension reaction. Primer extension reaction of chemically synthesized dNTPs and commercially available dNTPs into DNA by Klenow fragment exo(-), Kf-. Primer (P) was 5'-end-labeled using polynucleotide kinase and [γ-^{32}P] ATP. Polymerization reactions were performed with 3.5 µM primer and 5 µM template and equimolar ratios of all dNTPs (0.1 mM) and incubated with 0.05 µL of DNA polymerase per µL at 37 ^{o}C for 1 h. Reaction was analyzed by 19% polyacrylamide gel electrophoresis. Lane 1: the primer and all dNTPs, but no Kf-; Lane 2: P plus template (T) and all commercial dNTPs (Epicentre, Inc) with Kf-; Lane 3: Kf-, P/T and all dNTPs, except dATP; Lane 4: Kf-, P/T, commercial dCTP, dGTP and TTP, and crude dATP*;* Lane 5: Kf-, P/T and all dNTPs, except dCTP; Lane 6: Kf-, P/T and dATP, dGTP, TTP, and crude dCTP; Lane 7: Kf-, P/T and all dNTPs, except dGTP; Lane 8: Kf-, P/T and dATP, dCTP, TTP, and crude dGTP; Lane 9: Kf-, P/T and all dNTPs, except TTP; Lane 10: Kf-, P/T and dATP, dCTP, dGTP, and crude TTP; Lane 11: P/T and all crude dNTPs with Kf-; Lane 12: primer (P).

7. Polymerase Chain Reaction

The PCR reaction was performed according to the procedures in reference 3 but with some modification. Briefly, each reaction was performed with 1.2 µM primers 1 and 2; 0.025 µM template; 1.0 mM of each dNTPs, and 0.5 µL Taq DNA polymerase (QIAGEN) per 20 µL reaction, under conditions of 1 cycle at 94 ^{o}C for 2 min, 30 cycles at 94 ^{o}C for 30 s, 42 ^{o}C for 30 s, 68 ^{o}C for 30 s and 1 cycle at 68 ^{o}C for 7 min. (Primer 1: 5'-GCGTAATACGACTCACTAAG -3',

Primer 2: 5'- TGTACGTTTCGGCC-3'; Template DNA: 3'-dCGCATTATGCTGAGTGATATCCGTTGGACTACTCCGGCTTTCCGGCTTTGCATGT-5). 10 µL of each amplification product was separated by gel electrophoresis on 4% agarose, stained with ethidium bromide and visualized under UV light (Figure S17).

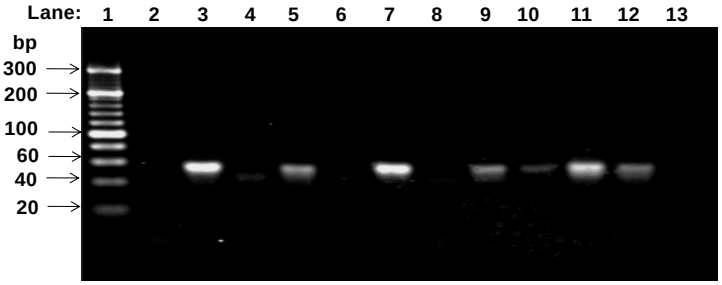


Figure S17. PCR amplification using crude dNTPs (NaCl-ethanol precipitated). They were performed with 1.2 µM primers 1 and 2; 0.025 µM template; 0.2 mM of each dNTPs, and 0.3 µL Taq DNA polymerase per 20 µL reaction. The reaction was performed under conditions of heated at 94 ^{o}C for 2 min, 35 cycles at 94 ^{o}C for 30 s, 42 ^{o}C for 30 s, 68 ^{o}C for 30 s, and the last cycle at 68 ^{o}C for 7 min. Aliquots (10 μL) of each reaction mixture were electrophoresed on a 4.5% agarose gel stained with ethidium bromide to visualize DNA bands: Lane 1: 20 bp DNA ladder; Lane 2: reaction containing primers, template and dNTPs but no enzyme; Lane 3, reaction containing primers, template, all commercial dNTPs and Taq DNA polymerase (as positive control). Lanes 4, 6, 8 and 10 (negative controls): each contains an omitted dATP, dCTP, dGTP and TTP from Lane 3, respectively; Lanes 5, 7, 9 and 11 were compensated with the crude dATP, dCTP, dGTP and TTP (respectively; NaCl-ethanol precipitated) to the corresponding Lane 4, 6, 8 and 10; Lane 12: primers, template, all crude dNTPs, and Taq DNA polymerase; Lane 13: the template 55mer.

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