Supporting Information

Assays to detect the Formation of Triphosphates of Unnatural Nucleotides: Application to *Escherichia coli* nucleoside diphosphate kinase (*Ec*-NDPK)

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Supplementary Figures

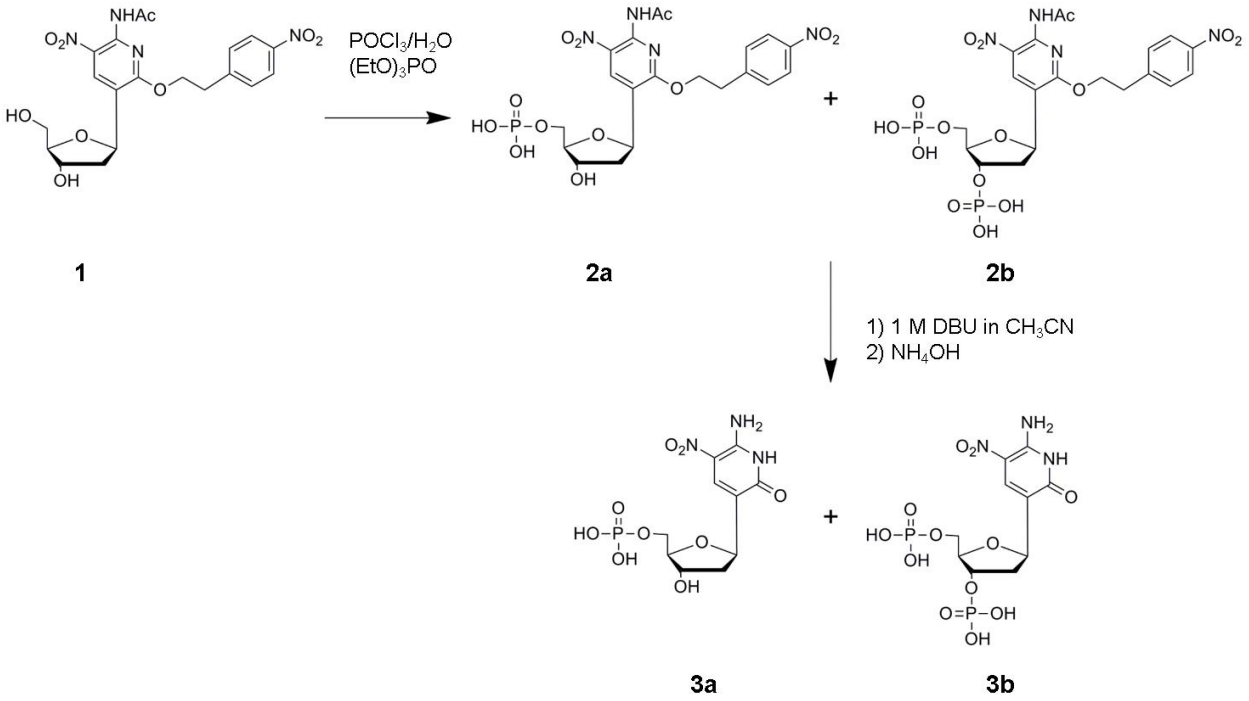

Figure S1: Scheme of **dZ**MP synthesis.

A solution of compound **1** (0.4 mmol) in triethyl phosphate (2 mL) was treated with a mixture of $POCl_3$ (0.12 mL, 3eq) and water (5 µL) in triethyl phosphate (1.6 mL) at 0 °C. This mixture was stirred overnight at 4 °C and poured into 1M TEAB (30 mL). After 1h at rt, this solution was concentrated to give a mixture of **2a** and **2b** in ratio of 3/2. This mixture was treated with 1M DBU in CH_3CN (5 mL). After being stirred overnight at rt, the reaction mixture was concentrated and treated with aq. NH_4OH (30 mL). After 24 h at rt, the mixture was concentrated. The residue was dissolved in water (50 mL) and purified by ion exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 50% B in 20 min, flow rate = 10 mL/min) to give 5'-monophosphate **3a** (Rt=14 min) and 3',5'-diphosphate **3b** (Rt=18 min) as a yellow foam after lyophilization.

3a: ^{1}H-NMR (D_2O, 300 MHz) δ 8.12 (s, 1H), 4.91 (dd, 1H, J = 6.0, 9.9), 4.30 (m, 1H),3.95 (m, 1H), 3.73-3.79 (m, 2H), 2.06 (m, 1H), 1.91 (m, 1H). ^{31}P-NMR (D_2O, 120 MHz) δ -0.06

3b: ^{1}H-NMR (D_2O, 300 MHz) δ 8.14 (s, 1H), 4.93 (dd, 1H, J = 5.7, 10.5), 4.55 (m, 1H), 4.09 (m, 1H), 3.73 (m, 2H), 2.23 (m, 1H), 1.91 (m, 1H). ^{31}P-NMR (D_2O, 121 MHz) δ 1.30 (3'-P), 0.53 (5'-P).

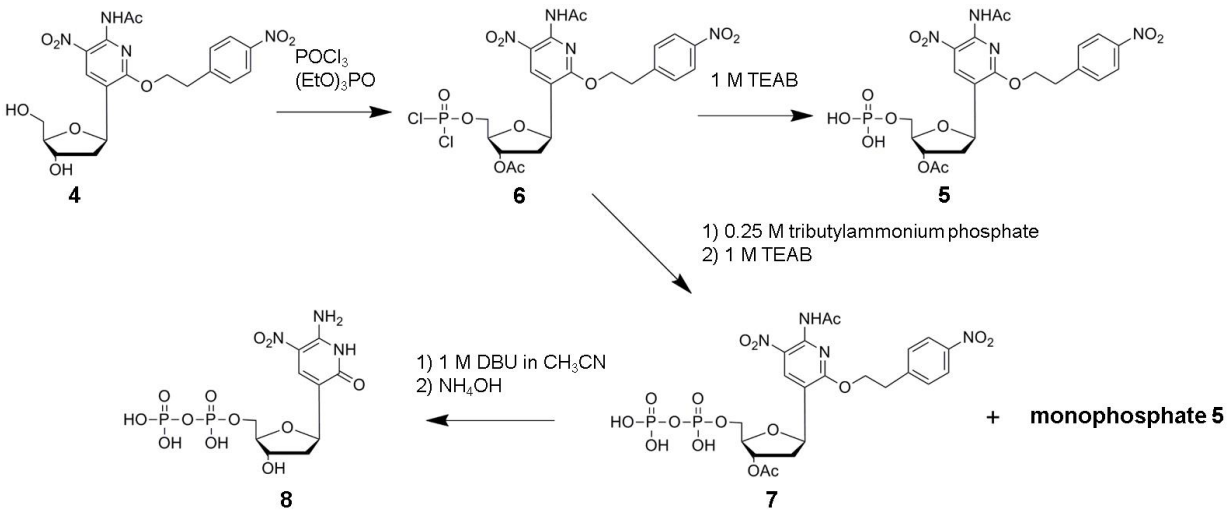

Figure S2: Scheme of **dZ**DP synthesis.

Compound **4** was dissolved in triethylphosphate and treated with $POCl_3$ (3 eq) at 0 °C. After 24 h at 4 °C, half of reaction mixture was poured into 1M TEAB and purified by reverse HPLC to give the monophosphate **5**. Another half of mixture was treated with 0.25 M tributylammonium phosphate in DMF (>15 eq) for 30 min, poured into 1M TEAB and purified by reverse HPLC (Sunfire prep C_{18}, OBD 5µm, 30 x 250 mm column, eluent A= 50 mM TEAB pH 8.5, eluent B = CH_3CN, gradient from 40% to 50% B in 10 min, flow rate 15 mL/min) to give the diphosphate **7** (Rt = 7 min) and monophosphate **5** (Rt = 9 min). After deprotection of **7** using 1M DBU and NH_4OH, purification by ion exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0% to 50% B in 15 min, flow rate = 10 mL/min, Rt = 13 min) gave dZDP **8** as a yellow foam after lyophilization.

7: ^{1}H-NMR (D_2O, 300 MHz) δ 8.25 (s, 1H), 7.93 (d, 2H, J = 8.1), 7.30 (d, 2H, J = 8.7), 5.02 (m, 1H), 4.78 (m, 1H), 4.47-4.59 (m, 2H), 4.16 (m, 1H), 3.86-3.91 (m, 2H), 2,90 (m, 2H), 2.17 (s, 3H), 1.98 (s, 3H), 1.72-1.86 (m, 2H). ^{31}P-NMR (D_2O, 120 MHz) δ -5.72 (d, J = 22.32), -10.12 (d, J = 22.32).

8: ^{1}H-NMR (D_2O, 300 MHz) δ 8.10 (s, 1H), 4.92 (m, 1H), 4.38 (m, 1H), 3.87-3.97 (m, 3H), 2.09 (m, 1H), 1.87 (m, 1H). ^{31}P-NMR (D_2O, 121 MHz) δ -6.30 (d, J = 21.72), -9.65 (d, J = 21.84).

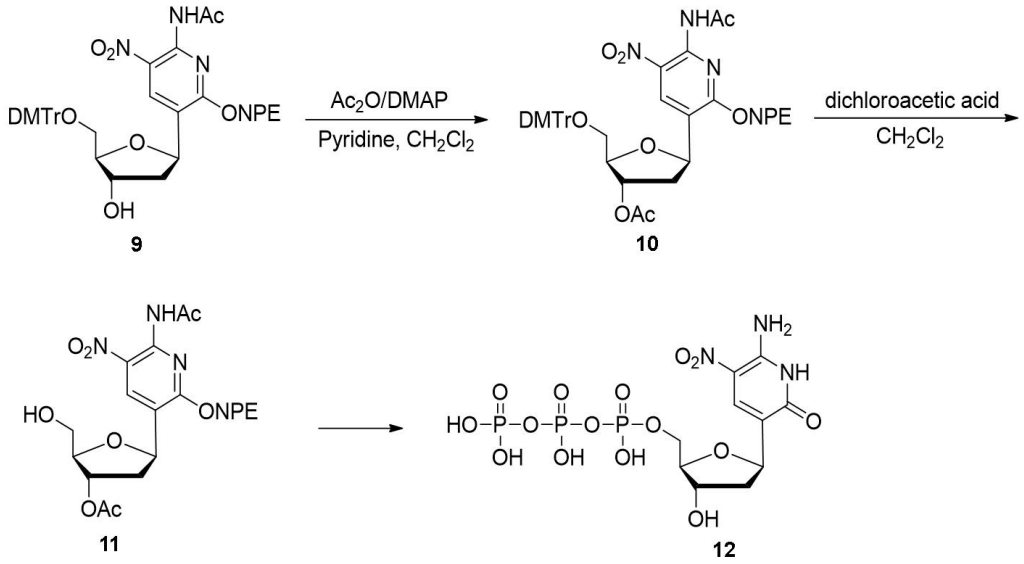

Figure S3: Scheme of **dZ**TP synthesis.

***N*-Acetyl-5-[2'-deoxy-3'-*O*-acetyl-β-D-ribofuranosyl] -3-nitro-6-[2-(4-nitrophenyl)ethoxy]-2-pyridinamine (11)**

To a mixture of solution of compound **9** (3 g, 3.92 mmol), pyridine (0.95 mL, 11.8 mmol) and DMAP (48 mg) in dichloromethane (50 mL) was added Ac_2O (0.601 g, 5.88 mmol) and the mixture was stirred at room temperature for 1 hour. Reaction was quenched with brine and extracted with CH_2Cl_2 (50 mL x 2). The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was resolved by flash chromatography (hexanes : EtOAc = 1: 1) to give a light yellow solid. This was dissolved in dichloromethane (20 mL), dichloroacetic acid as added via syringe, and the mixture was stirred at rt for 10 min. The reaction mixture was neutralized with sat-$NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was resolved by flash chromatography (hexanes: EtOAc = 1 : 2) to give the title compound as a light yellow solid. (1.7 g, 88%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.58 (s, 1H), 8.58 (s, 1H), 8.17 (d, *J* = 8.7 Hz, 2H), 7.29 (d, *J* = 8.4 Hz, 2H), 5.18 (d, *J* = 6.3 Hz, 1H), 5.04 (dd, *J* = 10.4, 5.3 Hz, 1H), 4.75 (t, *J* = 6.6 Hz, 2H), 4.05-4.16 (m, 1H), 3.87 (bs, 2H), 3.28 (t, J = 6.6 Hz, 2H), 2.46 (s, 3H), 2.27 (dd, *J* = 13.7, 5.3 Hz, 2H), 2.16 (s, 3H), 1.79-1.90 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 168.7, 161.5, 146.9, 145.4, 145.3, 134.2, 129.9, 125.6, 123.7, 119.5, 85.4, 76.3, 74.2, 67.8, 62.9, 39.2, 34.9, 26.3, 21.0

6-Amino-3-(2'-deoxy-5'-*O*-triphosphate-β-D-ribofuranosyl)-5-nitro-2(1*H*)-pyridone (12)

To a solution of compound **11** (0.252 g, 0.5 mmol) in pyridine (5 mL) and dioxane (10 mL) was added a solution of 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one (0.152 g, 0.75 mmol) in dioxane (5 mL) at room temperature. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 7.5 mL, 1.5 mmol) and tributylamine (7.5 mL) was added. After 20 min, a solution of iodine (0.190 g, 0.75 mmol) and water (0.315 mL) in pyridine (15.5 mL) was added. After 30 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, until color disappears). The pyridine and dioxane were removed *in vacuo*.

The residue was dissolved in acetonitrile (10 mL) water (10 mL) and kept at room temperature overnight. It was resolved by reversed phase preparative LC (25 mM TEAA to 25 mM TEAA:CH_3CN=45:55 in 25 min, running time 30 min); the fractions containing the desired product were collected and lyophilized. The lyophilized residue was dissolved in CH_3CN (5 mL) and treated with DBU (0.5 mL). The mixture was stirred at room temperature for 24 h. Volatiles were then removed by rotary evaporation. To the residue was added ammonium hydroxide (5 mL) and the mixture was stirred at room temperature for 1 h. Ammonia was removed by rotary evaporation and the residue was diluted with water and lyophilized. The residue was dissolved in water, filtered and resolved by ion exchange HPLC (water to 1.0 M ammonium bicarbonate in 25 min). The collected fraction was lyophilized to give the title compound as a yellow solid. (0.105 mmol, 18%) ^{1}H NMR (300 MHz, D_2O) δ 8.02 (s, 1H), 4.86, (dd, 1H, *J* = 9.8, 5.9 Hz), 4.32 (bs, 1H), 3.87-3.99 (m, 3H), 2.05 (dd, 1H, *J* = 12.9, 5.4 Hz), 1.74-1.87 (m, 1H). ^{31}P NMR (121 MHz, D_2O) δ -9.23 (m, 1P), -10.07 (d, 1P, *J* = 19.0 Hz), -21.8 (m, 1P).

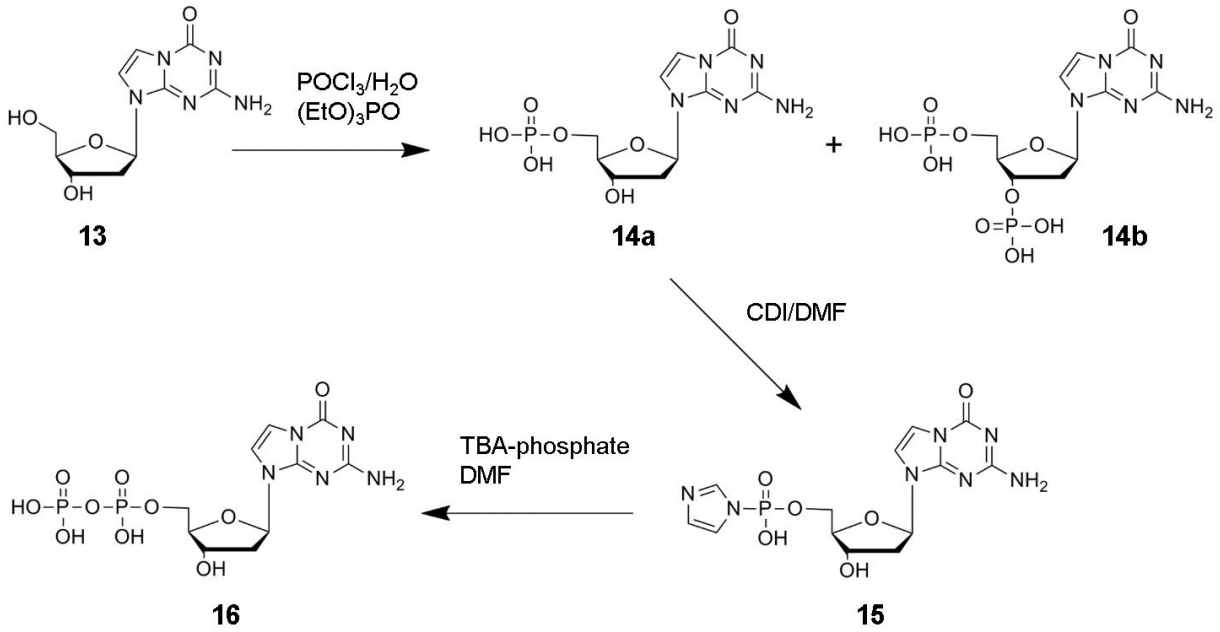

Figure S4: Scheme of **dP**MP and d**P**DP synthesis.

To a cooled mixture of dP nucleoside **13** (0.83 mmol) in triethyl phosphate (4 mL) was added a mixture of $POCl_3$ (0.24 mL) and water (8 μL) in triethyl phosphate (3.2 mL) at 0 °C. The reaction mixture was stirred overnight under 4 °C, poured into 100 mM TEAB (50 mL), and washed with ether. The material in the aqueous extract was resolved by reverse HPLC (Sunfire prep C_{18}, OBD 5 μm, 30 x 250 mm column, eluent A= 50 mM TEAB pH 8.5, eluent B = CH_3CN, gradient from 0% to 20% B in 15 min then 45% B in 5 min, flow rate 15 mL/min) to give monophosphate **14a** (Rt=17 min) and diphosphate **14b** (Rt=14 min). The monophosphate **14a** was further purified by ion exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 25% B in 20 min, flow rate = 10 mL/min, Rt = 13min) to give the monophosphate as a white foam after lyophilization.

14a: ^{1}H-NMR (D_2O, 300 MHz) δ 7.21 (m, 2H), 6.18 (t, 1H, J = 6.9), 4.73 (m, 1H), 4.26 (m, 1H), 3.72 (m, 2H), 2.49-2.62 (m, 2H). ^{31}P-NMR (D_2O, 121 MHz) δ 0.88.
14b: ^{1}H-NMR (D_2O, 300 MHz) δ 7.31 (d, 1H, J = 3.0), 7.12 (d,1H, J =3.0), 6.13 (t, 1H, J = 6.9), 4.68 (m, 1H), 4.15 (m, 1H), 3.76 (m, 2H), 2.40 (m, 2H). ^{31}P-NMR (D_2O, 120 MHz) δ 2.22, 1.85.
A mixture of **14a** (0.2 mmol) and Bu_3N (48 μL) in DMF (6 mL) was evapotared and dried. The residue was resuspended in DMF (8 mL) and CDI (160 mg, 5eq) was added. After stirring for 3h at rt, MeOH (64 μL) was added to the reaction mixture. After 30 min, TBA phosphate (2/1. 0.5 M in DMF, 8 mL) was added at rt. After stirring overnight at rt, the solvent was removed in vacuo. The residue was dissolved in water (40 mL) and purified by ion exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 50% B in 20 min, flow rate = 10 mL/min, Rt=11min) to give dPDP **16** as a white foam after lyophilization.
16: ^{1}H-NMR (D_2O, 300 MHz) δ 7.30 (m, 2H), 6.24 (t, 1H, J = 6.9), 4.90 (m, 1H), 4.35 (m, 1H), 3.76 (m, 2H), 2.60-2.66 (m, 2H). ^{31}P-NMR (D_2O, 121 MHz) δ -9.77 (d), -11.08 (d).

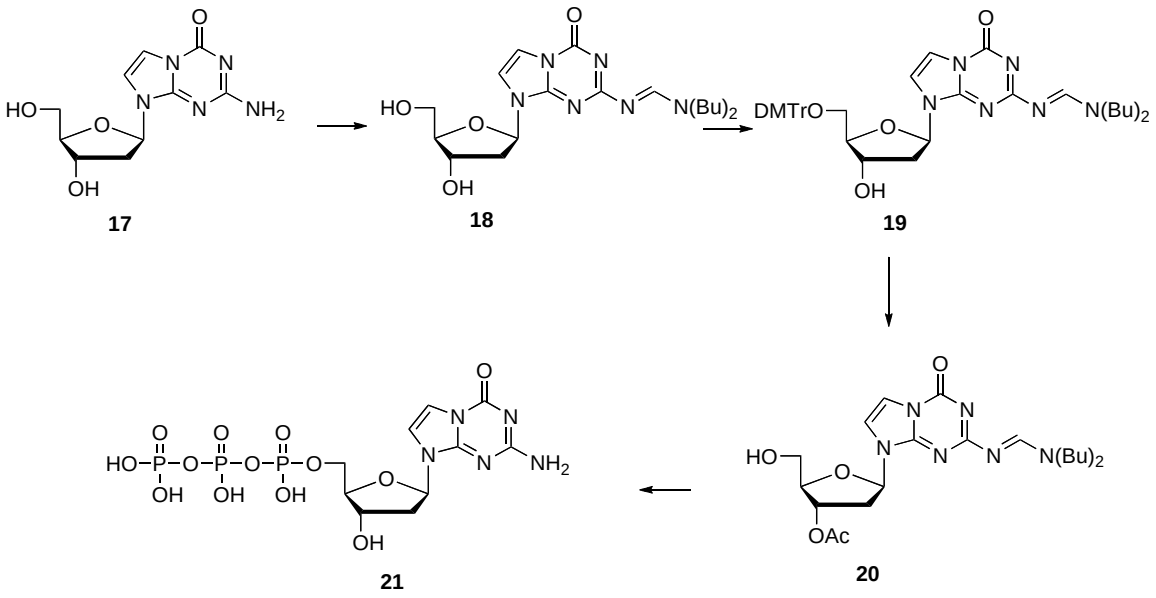

Figure S5: Scheme of **dP**TP synthesis.

2-(N,N-dibutylformamidino)-8-(2'-deoxy-5'-O-(4,4-dimethoxytriphenylmethyl)-β-D-ribofuranosyl)-imidazo-[1,21]-1,3,5-triazin-[8H]-4-one (19)

A mixture of compound **17** (7.5 g) and N,N-dibutylformamide dimethylacetal (15 mL) in MeOH (ACS grade, 150 mL) was stirred at rt for 2h. The mixture was evaporated and purified by column chromatography (neutral silica, CH_2Cl_2:MeOH=20:1) to give compound **18** as a white solid (11.0g, 96 %). A mixture of compound **18** (5.7 g) and DMT chloride (5.23 g, 1.1 eq) in anhydrous dichloromethane (150 mL) was treated with triethylamine (3.9 mL, 2.0 eq). The

mixture was stirred at rt for 6h, and washed with water. The water layer was extracted with dichloromethane and the combined organic extracts were dried over Na_2SO_4, filtered, and evaporated to dryness and purified by column chromatography (neutral silica, Ethyl acetate:MeOH=20:1) to give compound **19** as a white solid (80 %).

2-(N,N-dibutylformamidino)-8-(3'-O-acetyl-2'-deoxy-β-D-ribofuranosyl)-imidazo-[1,21]-1,3,5-triazin-[8H]-4-one (20)

To a stirred solution of compound **19** (2 g) in pyridine (40 mL) was added Ac_2O (0.4 mL, 1.5 eq) at room temperature. After being stirred overnight at room temperature, the reaction mixture was evaporated and the residue was purified by column chromatography (neutral silica, Ethyl acetate) to give the acetate (1.9 g, 90 %). A mixture of the 3'-OAc derivative (1.9 g) in 3 % dichloroacetic acid in dichloromethane (50 mL) was stirred for 3h at room temperature and evaporated. The residue was purified by column chromatography (CH_2Cl_2:MeOH=7/1) to give compound **20** (1g, 88 %).

2-(Amino-8-(2'-deoxy-β-D-ribofuranosyl)-imidazo-[1,21]-1,3,5-triazin-[8H]-4-one 5'-triphosphate (21)

To a solution of compound **20** (450 mg, 1.0 mmol) in pyridine (4 mL) and dioxane (3.4 mL) was added a solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (260 mg, 1.4 mmol) in dioxane (2.6 mL) at room temperature. After 10 min, a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 10 mL, 2 mmol) and tributylamine (1.2 mL, 4.8 mmol) were added. After 10 min, a solution of iodine (360 mg, 1.4 mmol) and water (0.56 mL) in pyridine (28 mL) was added. After 20 min, the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 1 mL). The solvents were removed *in vacuo*. The residue was dissolved in 28 % ammonium hydroxide solution (30 mL) and stirred overnight at room temperature. After removing solvent, water (50 mL) was added and the mixture was filtered (0.2 μm). Purification by reverse phase HPLC (Sunfire Prep C_{18} column, 5μm, 30 x 250 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN in A, gradient from 0 to 40% B in 20 min, flow rate = 15 mL/min, R_t = 14 min), followed by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 15 min) gave compound **21** as a colorless foam after lyophilization.

^{1}H-NMR (D_2O, 300 MHz): δ 7.32 (s, 1H), 7.21 (s, 1H), 6.20 (m, 1H), 4.57 (m, 1H), 3.90-4.08 (m, 3H), 2.40 (m, 1H), 2.25 (m, 1H); ^{31}P-NMR (D_2O, 120 MHz): δ -9.93 (d, 1P), -10.50 (d, 1P), -22.38 (t, 1P).

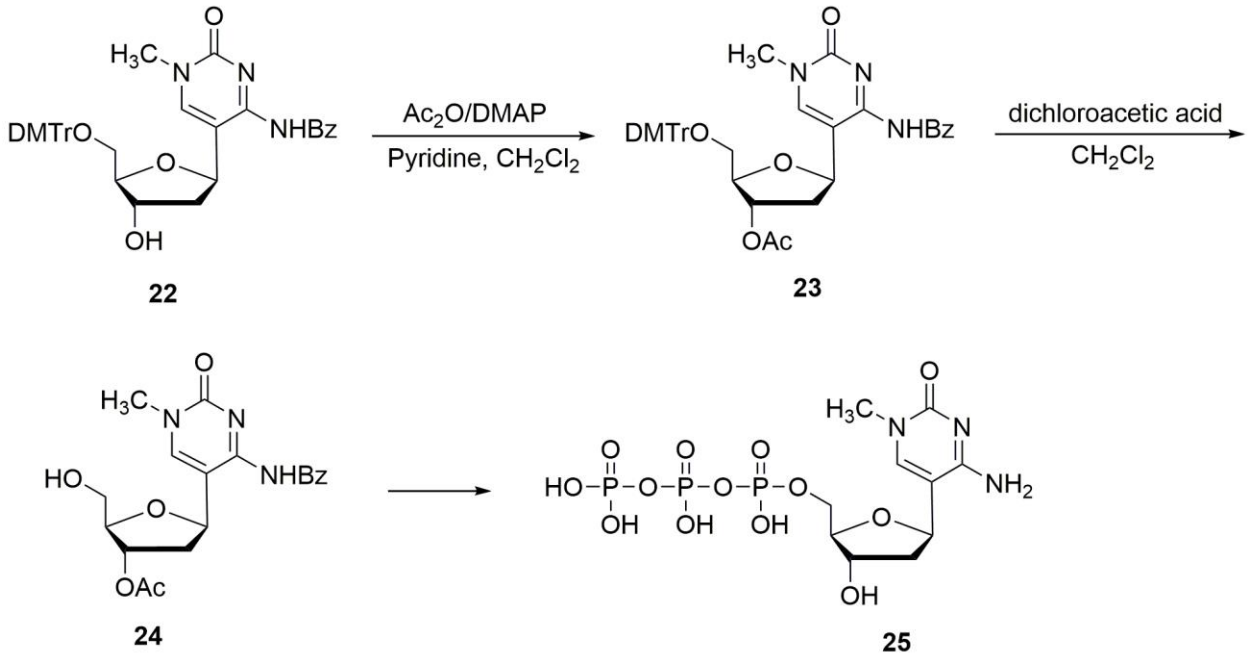

Figure S6: Scheme of **dS**TP synthesis.

2'-Deoxy-3'-*O*-acetyl-5'-dimethoxytrityl-1-methyl-*N*-benzoylpseudocytidine (23)

To a mixture of solution of compound **22** (1.94 g, 3.92 mmol), pyridoine (0.73 mL, 9 mmol) and DMAP (37 mg) in dichloromethane (50 mL) was added Ac_2O (0.460 g, 4.5 mmol) and the mixture was stirred at room temperature for 1 hour. Reaction was quenched with brine and extracted with CH_2Cl_2 (50 mL x 2). Organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (hexanes: EtOAc = 1: 1) to give a light yellow solid. (2 g, 97%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, *J* = 7.2 Hz, 2H), 7.65 (s, 1H), 7.54 (t, *J* = 7.4 Hz, 1H), 7.44 (t, *J* = 7.4 Hz, 4H), 7.21-7.32 (m, 8H), 6.84 (d, *J* = 8.7 Hz, 4H), 5.34-5.38 (m, 2H), 4.19 (q, *J* = 2.7 Hz, 1H), 3.78 (s, 6H), 3.37 (ddd, *J* = 21.6, 10.2, 3.6 Hz, 2H), 3.18 (s, 3H), 2.73-2.79 (m, 1H), 2.07-2.17 (m, 4H).

2'-Deoxy-3'-*O*-acetyl-1-methyl-*N*-benzoylpseudocytidine (24)

To a solution of compound **23** (2 g, 2.9 mmol) in CH_2Cl_2 (50 mL) was added dichloroacetic acid (2.4 mL, 29 mmol), and the mixture was stirred at rt for 10min. The reaction mixture was quenched with sat-$NaHCO_3$ solution, extracted with CH_2Cl_2. The organic layer was washed with

brine, and dried over Na_2SO_4. Solvent was removed under the reduced pressure, the residue was purified by column chromatography (CH_2Cl_2 : MeOH = 20 : 1) to give a product as a white solid. (0.6 g, 54%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (d, *J* = 7.2 Hz, 2H), 7.60 (s, 1H), 7.54 (t, *J* = 7.4 Hz, 1H), 7.44 (t, *J* = 7.8 Hz, 2H), 5.24 (m, 2H), 4.07 (m, 1H), 3.87 (m, 2H), 3.46 (s, 3H), 2.78(dd, *J* = 15.0, 6.0 Hz, 1H), 2.26 (bs, 1H), 2.16 (s, 3H), 1.98-2.08 (m, 1H).

2'-Deoxy-5'-*O*-triphosphate-1-methylpseudocytidine (25)

To a solution of compound **24** (0.194 g, 0.5 mmol) in pyridine (5 mL) and dioxane (10 mL) was added a solution of 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one (0.152 g, 0.75 mmol) in dioxane (5.0 mL) at room temperature. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 7.5 mL, 1.5 mmol) and tributylamine (0.80 mL) was added. After 20 min a solution of iodine (0.19 g, 0.75 mmol) and water (0.315 mL) in pyridine (15.5 mL) was added. After 30 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, until color disappears). The pyridine and dioxane were removed *in vacuo*. The residue was dissolved in acetonitrile (10 mL) water (10 mL) and kept at room temperature overnight. It was purified by reverse phase prep LC (25 mM TEAA to 25mM TEAA:CH_3CN=30:70 in 20 min, running time 30 min) then the collected fraction was lyophilized. The lyophilized residue was dissolved in NH_4OH (5 mL), and the mixture was stirred at room temperature for 3hr. Ammonia was removed by rotary evaporator and the residue was diluted with water and lyophilized. The residue was dissolved in water, filtered and purified by ion exchange HPLC (water to 1 M ammonium bicarbonate in 25 min). The collected fraction was lyophilized to give a white solid. (0.098 mmol, 20%) ^{1}H NMR (300 MHz, D_2O) δ 7.61 (s, 1H), 4.87 (dd, 1H, *J* = 11.1, 5.1 Hz), 4.45 (d, 1H, *J* = 5.4 Hz), 3.95-4.09 (m, 3H), 3.24 (s, 3H), 2.14-2.24 (m, 1H),1.92 (dd, 1H, *J* = 5.3 Hz). ^{31}P NMR (121 MHz, D_2O) δ -9.60 (d, 1P, *J* = 19.5 Hz), -10.50 (d, 1P, *J* = 19.4 Hz), -22.2 (t, 1P, *J* = 19.1 Hz).

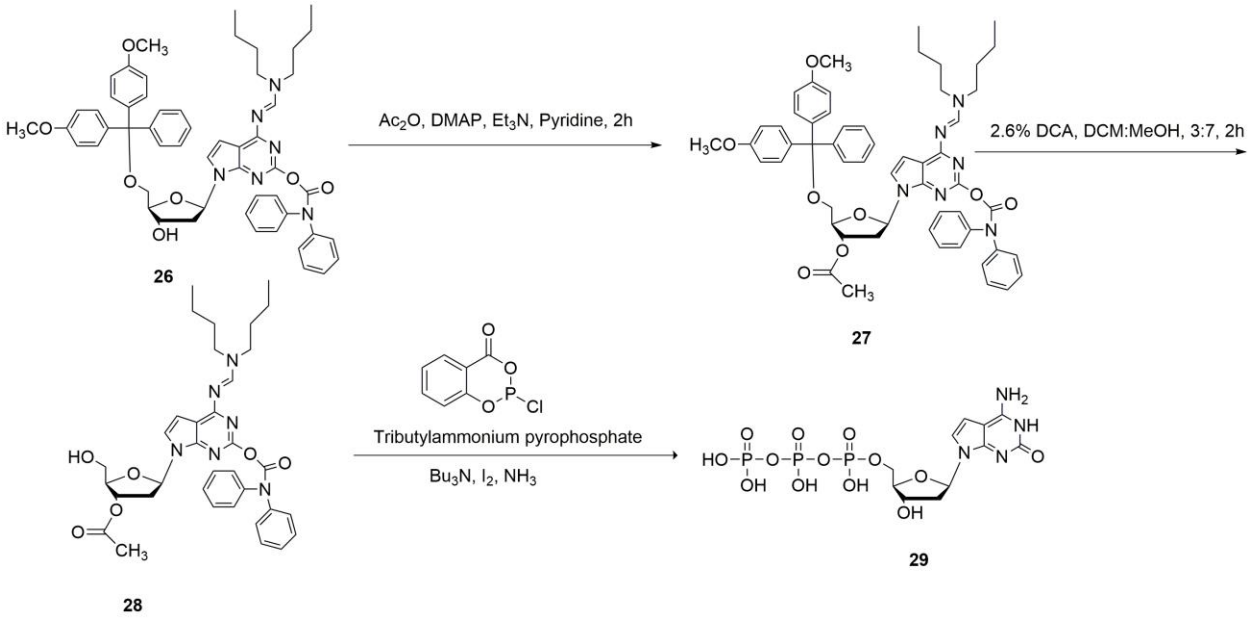

Figure S7: Scheme of **dB**TP synthesis. This scheme was adapted from Karalkar *et al*[20].

5'-O-(4,4'-Dimethoxytrityl)-N^6[(diisobutylamino)methylidene]-O^2-(diphenylcarbamoyl)-3'-O-acetyl-7-deazaisoguanosine (27)

2'-Deoxy-N^6-[(diisobutylamino)methylidene]-5'-O-(4,4'-dimethoxytrityl)-O^2-(diphenylcarbamoyl)-7-deazaisoguanosine **(26)** (1mmol, 903 mg), DMAP (0.25 mmol, 31 mg), Et_3N (2.52 mmol, 0.351 mL), and Ac_2O (1.21 mmol, 0.114 mL) were added to a solution of dry pyridine (30 mL). The mixture was stirred at room temperature for 2h. MeOH (1 mL) was added, the mixture was diluted with dichloromethane (100 mL) and extracted with 5% $NaHCO_3$ (50 mL). The aqueous layer was back extracted with DCM (100 mL). The combined organic layer was dried over sodium sulfate, filtered and evaporated. The residue was purified by flash chromatography (Hexane: Ethyl acetate, 2:1 to 1:1) (930 mg, 1.02 mmol, 98% yeild).

^{1}H NMR (300 MHz, $CdCl_3$) δ = ppm 8.80 (s, 1 H), 7.14 - 7.50 (m, 21 H), 6.79 - 6.86 (m, 4 H), 6.74 (dd, *J*=9.0, 5.3 Hz, 1 H), 6.58 (d, *J*=3.7 Hz, 1 H), 5.44 (d, *J*=5.9 Hz, 1 H), 4.11 - 4.20 (m, 1 H), 3.77 - 3.82 (m, 6 H), 3.62 (td, *J*=7.4, 3.3 Hz, 2 H), 3.30 - 3.45 (m, 4 H), 2.60 - 2.72 (m, 1 H), 2.41 - 2.51 (m, 1 H), 2.11 (s, 3 H), 1.57 - 1.72 (m, 5 H), 1.23 - 1.46 (m, 5 H), 0.93 - 1.02 (m, 6 H)

^{13}C NMR (300 MHz, $CdCl_3$):
δ=170.76,162.76,158.74,157.28,156.48,153.57,152.90,144.89,142.69,136,130.38,130.35,129.15, 128.48,128.12,127.11,126.48,122.02, 113.40,110.43,102.19,86

3'-O-Acetyl-2'-doexy-N^6[(diisobutylamino)methylidene]-O^2-(diphenylcarbamoyl)-7-deazaisoguanosine (28)

Compound **(27)** (930 mg, 1.02 mmol) was dissolved in a solution of dichloromethane : methanol (3:7) (50 mL) . The solution was cooled to 0^{o}C, and dichloroacetic acid (0.83 mL, 10.2 mmol) was added. Stirring was continued at 0^{o}C for 2 h. The mixture was then neutralized with aqueous sat $NaHCO_3$ (100 mL), and extracted with dichloromethane (200 mL). The resulting organic layer was dried over sodium sulfate, evaporated and the residue was purified by column chromatography (Hexane : Ethyl acetate 1:2) to give a white soild. (460 mg, 0.71 mmol, 70% yield)

^{1}H NMR (300 MHz, $CdCl_3$) δ = ppm 8.79 (s, 1 H), 7.44 - 7.58 (m, 3 H), 7.17 - 7.42 (m, 8 H), 6.97 (d, *J*=3.6 Hz, 1 H), 6.57 (d, *J*=3.6 Hz, 1 H), 6.08 - 6.20 (m, 1 H), 5.75 (dd, *J*=10.6, 3.3 Hz, 1 H), 5.55 (d, *J*=5.4 Hz, 1 H), 4.20 (s, 1 H), 3.88 - 4.04 (m, 2 H), 3.47 - 3.70 (m, 3 H), 3.30 - 3.41 (m, 2 H), 3.13 - 3.30 (m, 1 H), 2.31 (dd, *J*=13.9, 5.3 Hz, 1 H), 2.12 (s, 3 H), 1.54 - 1.70 (m, 9 H), 1.23 - 1.44 (m, 5 H), 0.97 ppm (t, *J*=7.3 Hz, 6 H)

$[M+H]^{+}$= 643.3239

$[M+Na]^{+}$= 665.3058

$[M-H]^{-}$= 504.9932

7-deaza-2'-deoxyisoguanosine 5'-Triphosphate (29)

To a solution of compound **28** (0.160 g, 0.25 mmol) in pyridine (5 mL) and dioxane (10 mL) was added a solution of 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one (0.076 g, 0.375 mmol) in dioxane (5.0 mL) at room temperature. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 3.75 mL, 1.5 mmol) and tributylamine (0.40 mL) was added. After 20 min a solution of iodine (0.095 g, 0.375 mmol) and water (0.315 mL) in pyridine (15.5 mL) was added. After 30 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, until color disappears). The pyridine and dioxane were removed *in vacuo* on a rotavap. The residue was dissolved in acetonitrile (10 mL) / water (10 mL) and kept at room temperature overnight. It was purified by reverse phase prep LC (25 mM TEAA to 25mM TEAA:CH_3CN (1:1) =5:95 in 38 min, running time 46 min) then the collected fraction was lyophilized. The lyophilized residue was dissolved in NH_4OH (5 mL), and the mixture was stirred at room temperature for 3hr. Ammonia was removed by rotary evaporator and the residue was diluted with water and lyophilized. The residue was dissolved in water, filtered and purified by ion exchange HPLC (water to 1 M ammonium bicarbonate in 32 min, running time was 42 min). The collected fraction was lyophilized to give a white solid. (21 mg, 41.73micromole)

^{1}H NMR (300 MHz, D_2O) δ = ppm 7.0 (s,1 H), 6.35 (s,1 H), 6.25 (m, 1 H), 4.0-3.9 (m, 4H), 2.5-2.39 (m,1H), 2.22 -2.18 (m,1H)

^{31}P NMR (121 MHz, D_2O) δ -9.24 (d, *J* = 19.6 Hz,1P), -10.14 (d, *J* = 19.6 Hz, 1P), -21.72 (t, *J* = 19.4 Hz, 1P).

$[M-H]^-$= 504.9932

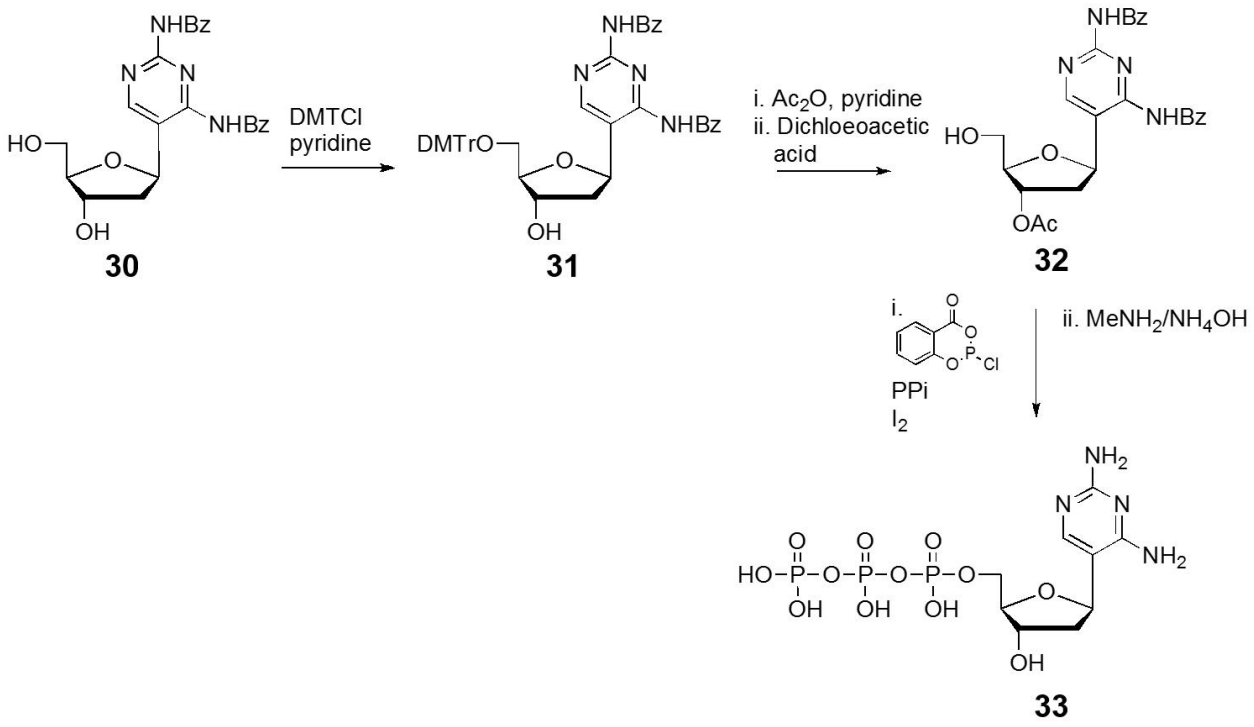

Figure S8: Scheme of **dK**TP synthesis.

2,4-Bisbenzoylamino-5-(1'-β-2'-deoxy-5'-O-(4,4'-dimethoxytripenylmethyl)-D-ribofuranosyl)-pyrimidine (31)

To a stirred solution of compound **30** (1.35 g, 3.1 mmol) in pyridine (40 mL) was added DMTrCl (1.58 g, 4.7 mmol) at room temperature, After being stirred at room temperature overnight, the reaction mixture was queched with MeOH (15 mL) and evaporated. The residue was purified by FLC (neutral silica, Hex/acetone = 2/3) to give compound **31** (1.6 g, 2.17 mmol, 70%).
^{1}H-NMR(300MHz, DMSO-d_6): δ 10.50 (s, 1H), 9.88 (s, 1H), 8.43 (s, 1H), 8.00 (d, 2H, 6.6), 7.81 (d, 2H, J=7.5), 7.15-7.55 (m, 15H), 6.68 (d, 4H, J=8.7). 5.25 (dd, 1H, J =5.1, 10.2), 4.52 (d, J=4.2), 4.29 (m, 1H), 3.70 (s, 6H), 3.16-3.30 (m, 2H), 2.47 (m, 1H), 2.29 (m, 1H),

2,4-Bisbenzoylamino-5-(1'-β-3'-O-acetyl-2'-deoxy-D-ribofuranosyl)-pyrimidine (32)

To a stirred solution of **31** (450 mg, 0.61 mmol) in pyridine (10 mL) was added Ac_2O (86 μL, 0.92 mmol) at room temperature. After being stirred at room temperature overnight, the reaction mixture was and evaporated. The residue was treated with 3% dichloroacetic acid in CH_2Cl_2 (15 mL) for 2 h. Solvents were removed and the residue was purified by by FLC (silica, CH_2Cl_2/MeOH = 7/1) to give compound **32** (230 mg, 0.48 mmole, 79%).
^{1}H-NMR(300MHz, DMSO-d_6): δ11.07 (s, 1H), 10.90 (s, 1H), 8.95 (s, 1H), 7.94 (m, 4H), 7.47-7.66 (m, 6H), 5.15 (d, 1H, J=5.7), 4.97-5.06 (m, 2H), 4.06 (dd, 1H, J=5.1, 10.2), 3.90 (m, 1H), 3.56 (m, 2H), 2.35 (m, 1H), 2.06 (m, 1H), 1.92 (s, 3H).

2,4-Diamino-(1'-β-D-2'-deoxyribofuranosyl)-pyrimidine 5'-triphosphate (33)
To a solution of **32** (210 mg, 0.44 mmol) in pyridine (4 mL) and dioxane (8 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (134 mg, 0.66 mmol) in dioxane (1.4 mL) at RT. After 20 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 6.6 mL, 1.32 mmol) and tributylamine (0.7 mL) was added. After 20 min a solution of iodine (167 mg) and water (0.16 mL) in pyridine (8 mL) was added. After 30 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 1 mL). The solvents were removed *in vacuo*. The residue was treated with $NH_4OH/MeNH_2$ (1/1, 20 mL) overnight at room temperature and the mixture was lyophilized. The residue was dissolved in water (50 mL), and the mixture was filtered (0.2 µm). Purification by reverse phase HPLC (Sunfire Prep C_{18} column, 5µm, 30 x 250 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN in A, gradient from 0 to 40% B in 20 min, flow rate = 15 mL/min, R_t = 14 min), followed by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 15 min) gave compound **33** as a colorless foam after lyophilization.
^{1}H-NMR (D_2O, 300 MHz): δ7.67 (s, 1H), 4.88(dd, 1H, J=6.3, 9.6), 4.71 (m, 1H), 4.42 (m, 1H), 4.00 (m, 2H), 2.02 (m, 2H)); ^{31}P-NMR (D_2O, 120 MHz): δ -5.96 (d, 1P), -10.13 (d, 1P), -21.41 (t, 1P).

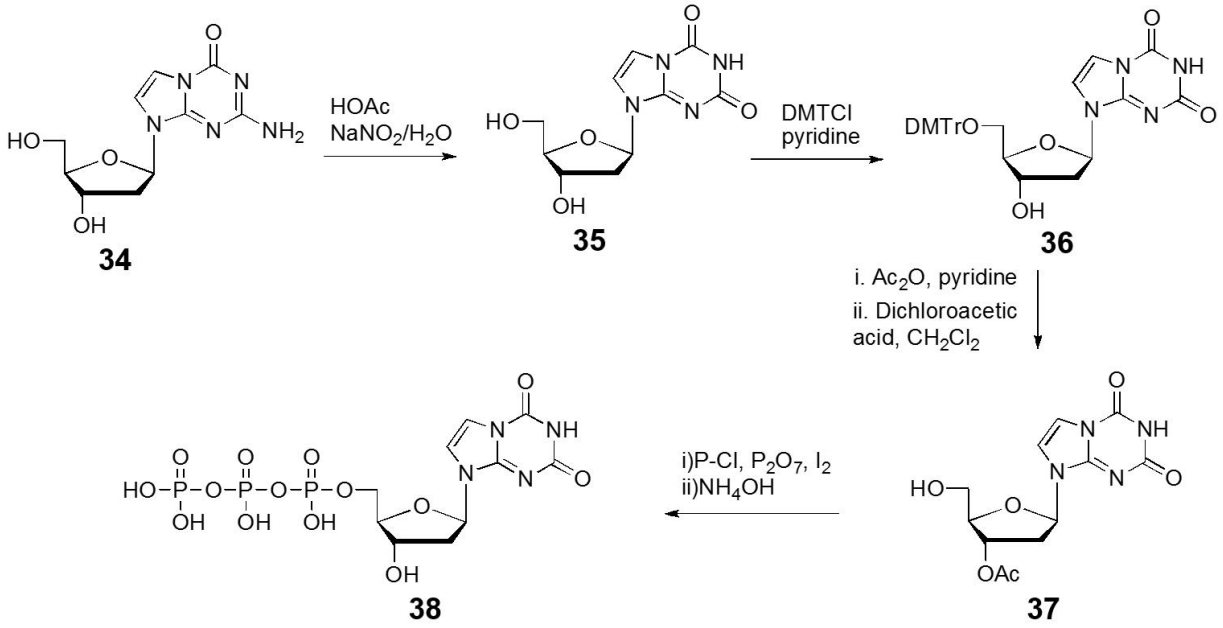

Figure S9: Scheme of **dX**TP synthesis.

8-(1'-β-D-2'-deoxyribofuranosyl)imidazo[1,2-a]-1,3,5-triazine-2(8H)-4(3H)-dione (35)
Compound **34** (1.34 g, 5 mmol) was dissolved in acetic acid (38 mL) and a solution of $NaNO_2$ (2 g) in H_2O (6 mL) was added at room temperature. The reaction mixture was stirred at room temperature for 2 days and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH=8/1 to 4/1) to give dX nucleoside **35** (900 mg, 3.36 mmol, 67%).

^{1}H NMR(DMSO-d_6, 300MHz) δ7.45 (d, 1H, J=3.0), 7.41 (d, 1H, J=3.0), 6.05 (t, 1H, J=6.6), 4.28 (m, 1H), 3.79 (m, 1H), 3.46-3.58 (m, 2H), 2.28 (m, 1H), 2.13 (m, 1H).

8-(1'-β-2'-deoxy-5'-O-(4,4'-dimethoxytripenylmethyl)-D-ribofuranosyl)imidazo[1,2-a]-1,3,5-triazine-2(8H)-4(3H)-dione (36)

To a stirred solution of 2 (469 mg, 1.75 mmol) in pyridine (25 mL) was added DMTCl (652 mg, 1.92 mmol) at room temperature. The reaction mixture was stirred overnight at room temperzture and evaporated. The residue was purified by silica gel column chromatography (EtOAc to EtOAc/MeOH=9/1) to give compound **36** (580 mg, 1.02 mmol, 58%).
^{1}H NMR(DMSO-d_6, 300MHz) δ 11.26 (s, 1H), 7.42 (d, 1H, J=2.4), 7.18-7.35 (m, 10H), 6.83 (m, 4H), 6.09 (t, 1H, J=6.3), 5.37 (d, 1H, J=4.5), 4.28 (m, 1H), 3.90 (M, 1H), 3.72 (S, 6H), 3.12 (m, 2H), 2.39 (m, 1H), 2.18 (m, 1H).

8-(1'-β-3'-O-acetyl-2'-deoxy-D-ribofuranosyl)imidazo[1,2-a]-1,3,5-triazine-2(8H)-4(3H)-dione (37)

To a stirred solution of **36** (580 mg, 1.02 mmol) in pyridine (15 mL) was added Ac_2O (144 μL, 1.53 mmol) at room temperature. After being stirred at room temperature overnight, the reaction mixture was and evaporated. The residue was treated with 3% dichloroacetic acid in CH_2Cl_2 (25 mL) for 2 h. Solvents were removed and the residue was purified by by FLC (silica, CH_2Cl_2/MeOH = 7/1) to give compound **37** (250 mg, 0.81 mmole, 79%).
^{1}H-NMR(300MHz, DMSO-d_6): δ 11.23 (s, 1H), 7.45 (d, 1H, J=2.7), 7.42 (d, 1H, J=2.4), 6.04 (m, 1H), 5.23 (m, 2H), 4.01 (m, 1H), 3.56 (m, 2H), 2.52 (m, 1H), 2.31 (m, 1H), 2.04 (s, 3H).

8-(1'-β-D-2'-deoxyribofuranosyl)imidazo[1,2-a]-1,3,5-triazine-2(8H)-4(3H)-dione 5'-triphosphate (38)

To a solution of **37** (309 mg, 1 mmol) in pyridine (4 mL) and dioxane (3.4 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (260 mg) in dioxane (2.6 mL) at RT. After 20 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 10 mL, 2 mmol) and tributylamine (1.2 mL, 4.8 mmol) was added. After 20 min a solution of iodine (360 mg) and water (0.56 mL) in pyridine (28 mL) was added. After 30 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 1 mL). The solvents were removed *in vacuo*. The residue was treated with NH_4OH (20 mL) for 3 h at room temperature and the mixture was lyophilized. The residue was dissolved in water (50 mL), and the mixture was filtered (0.2 μm). Purification by reverse phase HPLC (Sunfire Prep C_{18} column, 5μm, 30 x 250 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN in A, gradient from 0 to 40% B in 20 min, flow rate = 15 mL/min), followed by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min) gave compound **38** as a colorless foam after lyophilization.
^{1}H-NMR (D_2O, 300 MHz): δ7.38 (m, 1H), 7.30(m, 1H), 6.15 (t, 1H, J=6.0), 4.54 (m, 1H), 4.01-4.09 (m, 3H), 2.41 (m, 1H), 2.34 (m, 1H); ^{31}P-NMR (D_2O, 120 MHz): δ -10.01 (d, 1P), -10.51 (d, 1P), -22.48 (t, 1P).

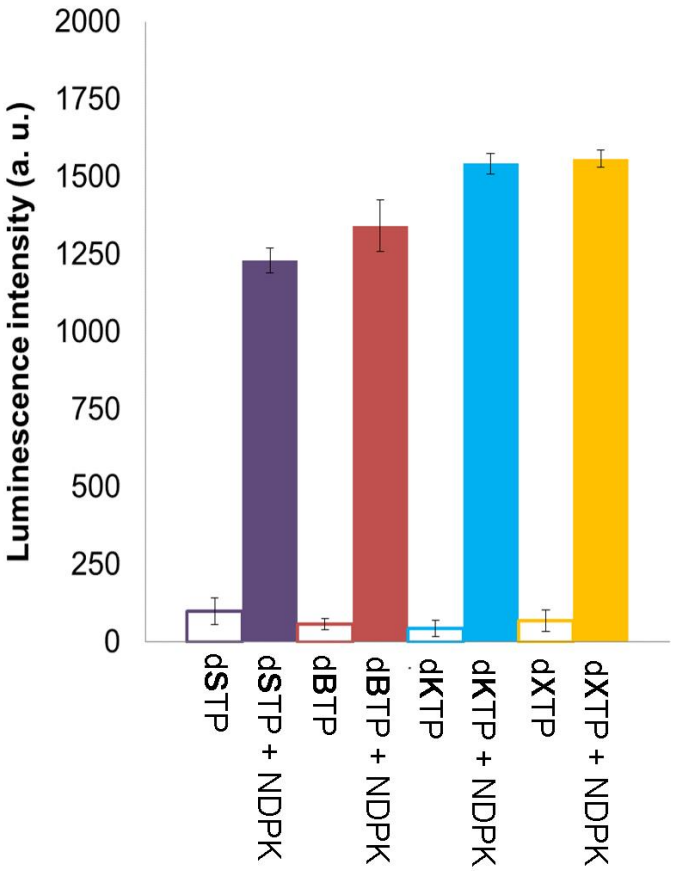

Figure S10: The diagram shows the luminescence intensity (results of ATP production) of each sample. Reaction mixture was prepared by mixing deoxyribonucleoside triphosphate (d**S**TP, d**B**TP, d**K**TP, d**X**TP), ADP, and buffer with or without NDPK. The error bars indicate the standard deviations (n=3).

(20) Karalkar, N. et al., submitted.