Supporting Information

Synthesis of β-L-Threofuranosyl Nucleoside Triphosphates (tNTPs)

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General Information

Chemicals and reagents were purchased from Sigma/Aldrich unless otherwise noted. Pyridine and tributylamine were distilled from calcium hydride, DMF from phosphorus pentoxide, and dioxane was dried over sodium. TNA triphosphate syntheses were carried out according to a modified version of the methods developed by Ludwig and Eckstein.[1] Advanced intermediates **1** of thymine, 2,6-diaminopurine, and guanine threofuranosyl nucleosides with 2'-*O*-DMT and heterocyclic protecting groups were a generous gift of the Eschenmoser laboratory. The protecting groups for the heterocyclic 2,6-diaminopurine and guanine bases were removed prior to phosphorylation, but the 2'-*O*-DMT protecting group remained in place. Compound **4** was prepared from L-ascorbic acid and N^4-benzoyl cytosine as reported.[2] Phosphorylation reactions were monitored by TLC on EMD 60 F_{253} silica plates run in 1-propanol/NH_4OH/H_2O (11:7:2) visualized under UV light. Ion exchange chromatography was performed using DEAE Sephadex (Pharmacia) in a Waters 600 HPLC with UV detection eluting with 0-1 M TEAB pH 7.5

followed by reverse phase HPLC purification on a divinyl benzene column eluting with 95% 100 mM TEAA pH 7.0 and a linear gradient from 5% methanol to 100% methanol. The sodium salt was prepared according to the method described by Hoard and Ott.[3]

(β-L-Threofuranosyl)thymine-3'-triphosphate, (β-L-threofuranosyl)-2,6-diaminopurine-3'-triphosphate, and (β-L-threofuranosyl)guanine-3'-triphosphate (tTTP, tDTP and tGTP, 3)

2'-*O*-DMT protected threosyl nucleosides were co-evaporated twice from dry pyridine and stored under high vacuum over P_2O_5. for 1 hr. The nucleosides were dissolved in appropriate solvents and reacted with 2-chlor-4H-1,3,2-benzodiozaphosphorin-4-one in dry dioxane (1 M stock, 1 mol eq.) After 10 minutes tributylammonium pyrophosphate solution in dry DMF (0.5 M stock, 1.5 mol. eq.) and tributylamine (1/3 vol. of pyrophosphate solution) were added and the reaction was allowed to stir for 15 min. I_2 solution in pyridine/Water (98:2) (1% stock, 1 mol. eq.) was added to the reaction mixture with 5% sodium sulfite solution (~ 1/3 volume of I_2 solution) was added after 10 minutes to quench excess iodine. Solvents were removed under high vacuum. The residue was dissolved in 50% acetic acid for 20 min at 0 ^{o}C followed by solvent removal. The residue was then dissolved in water (5 ml) and washed twice with diethyl ether. Ion exchange and reversed-phase HPLC purification followed by conversion to the sodium salts resulted in the threosyl nucleoside 3'-triphosphates in 25-35% yields.

(β-L-Threofuranosyl)thymine-3'-triphosphate

1-{2'-*O*-[(4',4"-dimethoxytriphenyl)methyl]-β-L-threofuranosyl}thymine (10 mg, 19 μmol) was dissolved in 20 μl pyridine and 60 μl dioxane for the phosphorylation reaction performed as described above. Yield 35%. ^{31}P NMR (161.9 MHz, D_2O) δ -8.35 (d, $J_{p,p}$=19 Hz), -10.60 (d, $J_{p,p}$=19 Hz), -21.99 (t, $J_{p,p}$=19 Hz). UV (20 mM Tris-HCl pH 7.5) λ_{max} 267 nm.

(β-L-Threofuranosyl)-2,6-diaminopurine-3'-triphosphate

N^2,N^6-Dibenzoyl-9-{2'-*O*-[(4', 4"-dimetheoxytriphenyl)methyl]-β-L-threofuranosyl}-2,6-diaminopurine was deprotected in 8 M methylamine in ethanol:12 M methylamine in water (1:1) for 3 hr at 50 °C followed by solvent removal and silica gel flash chromatography (0 to 4% methanol in CH_2Cl_2) yielding 9-{2'-*O*-[(4', 4"-dimethoxytriphenyl)methyl]-β-L-threofuranosyl}-2,6-diaminopurine in near quantitative yield as confirmed by LRMS. This material (10 mg, 18 μmol) was dissolved in 30 μl pyridine and 120 μl DMF for the phosphorylation reaction. Yield 25%. ^{31}P NMR (202.4 MHz, D_2O) δ -6.84 (d, $J_{p,p}$= 18 Hz), -11.73 (d, $J_{p,p}$= 18 Hz), -19.95 (t, $J_{p,p}$= 14 Hz). ESI-MS (neg.) 490.9883 (HRMS calc. 490.9886). UV (20 mM Tris-HCl pH 7.5) λ_{max} 282 nm.

(β-L-Threofuranosyl)guanine-3'-triphosphate

N^2-Acetyl-O^6-diphenylcarbamoyl-9-{2'-*O*-[(4',4"-dimetheoxytriphenyl)methyl]-β-L-threofuranosyl}guanine was deprotected in 8 M methylamine in ethanol:12 M methylamine in water (1:1) for 3 hr at 50 °C followed by solvent removal and silica gel

flash chromatography (0 to 4% methanol in CH_2Cl_2) yielding 9-{2'-*O*-[(4',4''-dimethoxytriphenyl)methyl]-β-L-threofuranosyl}guanine confirmed by LRMS. This material (10 mg, 18 μmol) was dissolved in 30 μl pyridine and 120 μl DMF for the phosphorylation reaction. Yield 25%. ^{31}P NMR (161.9 MHz, D_2O) δ -3.08 (d, $J_{p,p}$= 18 Hz), -10.40 (d, $J_{p,p}$= 18 Hz), -19.07 (t, $J_{p,p}$= 18 Hz). UV (20 mM Tris-HCl pH 7.5) λ_{max} 253 nm.

N^4-Benzoyl-1-{2'-*O*-acetyl-3'-*O*-[(4', 4''-dimetheoxytriphenyl)methyl]-β-L-threofuranosyl}cytosine (5)

N^4-Benzoyl-1-{3'-*O*-[(4',4''-dimetheoxytriphenyl)methyl]-β-L-threofuranosyl}cytosine (**4**, 1 g, 1.62 mmol), prepared starting from L-ascorboc acid and N^4-benzoyl cytosine as described,[2] was co-evaporated twice from dry pyridine (30 ml). Acetic anhydride (275 μl, 2.92 mmol) was added to the solution of **4** in dry pyridine (30 ml). The mixture was stirred under argon at room temperature for 12 hrs, and then evaporated to dryness after MeOH (2 ml) was added to quench the reaction. The residue was dissolved in CH_2Cl_2 and washed with sat. aq. $NaHCO_3$ solution, H_2O and sat. aq. NaCl solution, dried (Na_2SO_4) and filtered. The residue after evaporation was purified by silica gel flash chromatography [hexane/CH_2Cl_2/TEA (1:1:0.0025) to CH_2Cl_2 /TEA(1:0.0025)] to give **5** (974 mg, 91%) as a white foam.

TLC (4% MeOH in CH_2Cl_2): R_f 0.32. ^{1}H-NMR (400 MHz; $CDCl_3$) δ 8.82 (bs, 1H), 7.96 (m, 3H), 7.20-7.63 (m, 13H), 6.79-6.84 (m, 4H), 5.93 (bs, 1H), 5.53 (bs, 1H), 4.24 (m, 1H), 3.76-3.78 (2s, 6H), 3.60 (dd, J=14.8, J=7.2, 1H), 3.05 (dd, J=14.8, J=7.6, 1H), 2.08 (s, 3H). ^{13}C NMR (100 MHz; $CDCl_3$) δ 168.7, 162.5, 159.0, 145.4, 144.3, 135.8, 135.3,

133.3, 130.1, 130.0, 129.2, 128.3, 128.1, 127.7, 127.5, 113.8, 113.7, 96.6, 91.4, 88.9, 81.3, 76.4, 75.1, 55.6, 21.2 (hexane residues: 31.6, 22.7, 14.1). ESI-MS (pos.) 684.2324 $[M+Na]^+$ (HSMS calc. 684.2322).

N^4-Benzoyl-1-(2'-O-acetyl-β-L-threofuranosyl)cytosine (6)

N^4-Benzoyl-1-{2'-*O*-acetyl-3'-*O*-[(4',4''-dimetheoxytriphenyl)methyl]-β-L-threofuranosyl}cytosine (**5**, 500 mg, 0.76 mmol) was treated with 3% TCA in CH_2Cl_2 (25 ml) at ambient temperature for 3 min. MeOH was added dropwise to quench released DMT cation until the orange color disappeared. After the acid was neutralized by triethylamine (~10 ml), the mixture was immediately evaporated to avoid deacylation by overexposure to the basic solvent in the presence of MeOH. The residue was purified by silica gel flash chromatography (2% MeOH in CH_2Cl_2). There was still ~ 12% of **5** (tritylated nucleoside) that was subjected to another TCA treatment and column purification. Product fractions were combined to afford **6** (251 mg, 92%) as a white foam. TLC (4% MeOH in CH_2Cl_2): R_f 0.23. ^{1}H NMR (400 MHz; DMSO-d_6) δ 11.26 (bs, 1H), 8.14 (d, J=6.4, 1H), 8.02-7.50 (m, 5H), 7.36 (d, J=6.4, 1H), 5.81 (bs, 1H), 5.72 (bs d,1H), 5.12 (bs, 1H), 4.76 (d, J=9.347, 1H), 4.17 (m, 2H), 2.11 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6) δ 169.7, 168.0, 164.0, 155.0, 146.1, 133.9, 133.3, 129.1, 129.0, 96.4, 91.5, 81.7, 77.2, 73.1, 21.5. ESI-MS (pos.) 382.1015 $[M+Na]^+$ (HSMS calc. 382.1015).

(β-L-Threofuranosyl)cytosine-3'-triphosphate (tCTP, 8)

N^4-Benzoyl-1-(2'-*O*-acetyl-β-L-threofuranosyl)cytosine (**6**, 10 mg, 28 μmol) was dissolved 30 μl dry pyridine and 90 μl dry dioxane for the phosphorylation reaction. The

reaction was carried out as described above except for the treatment with acetic acid. After the excess of iodine was quenched and the solvent was removed under high vacuum, the residue was treated with ammonium hydroxide (28%, 10 ml) at ambient temperature for 3 hours before evaporating to dryness. The residue was dissolved in water (5 ml) and washed twice with CH_2Cl_2. Ion exchange and reverse phase HPLC purification gave **8** in 30% yield.

^{31}P NMR (161.9 MHz, D_2O) δ -3.78 (d, $J_{p,p}$ = 18 Hz), -11.33 (d, $J_{p,p}$ = 18 Hz), -18.87 (t, $J_{p,p}$ = 18 Hz). ESI-MS (neg.) $[M-H]^-$ 452.0. UV (20 mM Tris-HCl pH 7.5) λ_{max} 271 nm.

References for supporting information:

(1) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, 54, 631-635.

(2) Schöning, K.-U., Scholz, P.; Wu X.; Guntha, S.; Delgado, G.; Krishnamurthy, R.; Eschenmoser, A. *Helvetica Chimica Acta* **2002**, *85*, 4111-4153.

(3) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, 87, 1785-1788.