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for

Polymerase Recognition and Stability of Fluoro Substituted Pyridone Nucleobase Analogues

Gil Tae Hwang, Aaron M. Leconte, and Floyd E. Romesberg*

Methods

Chemicals. All commercially available chemicals and solvents were used without further purification. All reactions were carried out with dry glassware under argon atmospheres. Analytical TLC was carried out on Merck 60 F_{254} silica gel plates and column chromatography was performed on silica gel 60 (Geduran, 40-63 µm, Merck). ^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR spectra were taken on a Bruker NMR spectrometer (DRX-500 or DRX-400). The ^{1}H and ^{13}C chemical shifts are referenced relative to TMS, and the ^{31}P and ^{19}F chemical shifts are referenced relative to 85% phosphoric acid in D_2O and neat trichlorofluoromethane, respectively. High-resolution mass spectra were measured on an Agilent ESI-TOF mass spectrometer. Compounds **1a-c** were prepared according to reported procedures.[i] T4 polynucleotide kinase was purchased from New England Biolabs. Klenow fragment exo- and [?-^{33}P]-ATP were purchased from Amersham Biosciences.

General procedure for bistoluoyl nucleoside synthesis: Bis(trimethylsilyl)acetamide (1 equiv) was added to a stirred solution of **1** (1 equiv) in acetonitrile (0.25 M) at room temperature under argon. After stirring at ambient temperature for 40 min, 3,5-bis(toluoyl)-2-deoxyribosyl chloride (1 equiv) was added. The reaction was brought to

0 ºC. To the reaction mixture was added dropwise $SnCl_4$ (20 mmol %). After 1 h, complete dissolution of the 2'-deoxyribofuranoside had occurred. To the reaction was added EtOAc, and the resulting solution was successively extracted with saturated $NaHCO_3$ and brine. The organics were dried over anhydrous Na_2SO_4, and solvents were removed in vacuo. Purification via flash column chromatography on silica gel (5% EtOAc in CH_2Cl_2) afforded the desired product **2**.

1-[2'-Deoxy-3',5'-bis-*O*-(4-methylbenzoyl)-ß-D-erythro-pentofuranosyl]-3-fluoro-2(1*H*)-pyridinone (2a): ^{1}H NMR (400 MHz, $CDCl_3$): *d*?7.96 (d, *J* = 8.4 Hz, 2H; ArH), 7.86 (d, *J* = 8.0 Hz, 2H; ArH), 7.53 (d, *J* = 7.2 Hz, 1H; H-6), 7.28 (d, *J* = 8.0 Hz, 2H; ArH), 7.23 (d, *J* = 8.0 Hz, 2H; ArH), 7.06 (ddd, J_{HH} = 1.7, 7.5 Hz, J_{HF} = 8.0 Hz, 1H; H-4), 6.63 (dd, *J* = 5.8, 7.8 Hz, 1H; H-1'), 6.05–6.01 (m, 1H; H-5), 5.61 (dd, *J* = 2.0, 4.4 Hz, 1H; H-3'), 4.76–4.62 (m, 3H; H-4' and H-5'), 3.05–3.00 (m, 1H; H-2'), 2.44 and 2.41 (2s, 6H; CH_3), 2.31–2.24 (m, 1H; H-2'); ^{13}C NMR (101 MHz, $CDCl_3$): *d* 166.1, 155.6 (d, $^2J_{CF}$ = 26 Hz), 151.8 (d, $^1J_{CF}$ = 248 Hz), 144.5, 144.3, 129.8, 129.5, 129.3 (d, $^3J_{CF}$ = 4 Hz), 126.8, 126.8, 126.5, 126.3, 120.1 (d, $^2J_{CF}$ = 17 Hz), 104.0, 86.5, 83.4, 74.9, 64.1, 39.2, 21.7, 21.7; ^{19}F NMR (376 MHz, $CDCl_3$): *d* -131.4; HRMS (*m/z*): $[M + Na]^+$ calcd for $C_{26}H_{24}F_1N_1O_6Na$, 488.1480; found, 488.1481.

1-[2'-Deoxy-3',5'-bis-*O*-(4-methylbenzoyl)-ß-D-erythro-pentofuranosyl]-4-fluoro-2(1*H*)-pyridinone (2b): ^{1}H NMR (400 MHz, $CDCl_3$): *d* 7.96 (d, *J* = 7.2 Hz, 2H; ArH), 7.86 (d, *J* = 7.2 Hz, 2H; ArH), 7.76 (t, *J* = 7.6 Hz, 1H; H-6), 7.26 (d, *J* = 8.0 Hz, 2H; ArH), 7.23 (d, *J* = 8.0 Hz, 2H; ArH), 6.53 (t, *J* = 6.6 Hz, 1H; H-1'), 6.16–6.14 (m, 1H; H-3), 6.00–5.97 (m, 1H; H-5), 5.61–5.60 (m, 1H; H-3'), 4.76–4.62 (m, 3H; H-5' and H-4'), 3.04–2.99 (m, 1H; H-2'), 2.42 and 2.41 (2s, 6H; CH_3), 2.27–2.20 (m, 1H; H-2'); ^{13}C NMR (101 MHz, $CDCl_3$): *d* 170.3 (d, $^1J_{CF}$ = 266 Hz), 165.9, 163.2, 163.0, 144.3, 144.2, 133.8 (d, $^3J_{CF}$ = 14 Hz), 129.7, 129.4, 129.2, 129.1, 126.4, 126.2, 102.7 (d, $^2J_{CF}$ = 17 Hz), 98.5 (d, $^2J_{CF}$ = 27 Hz), 86.3, 83.2, 74.8, 64.0, 39.3, 21.6; ^{19}F NMR (376 MHz, $CDCl_3$): *d* -96.7; HRMS (*m/z*): $[M + Na]^+$ calcd for $C_{26}H_{24}F_1N_1O_6Na$, 488.1480; found, 488.1481.

1-[2'-Deoxy-3',5'-bis-*O*-(4-methylbenzoyl)-ß-D-erythro-pentofuranosyl]-5-fluoro-2(1*H*)-pyridinone (2c): ^{1}H NMR (400 MHz, $CDCl_3$): d 7.93 (d, *J* = 8.0 Hz, 2H; ArH), 7.86 (d, *J* = 7.6 Hz, 2H; ArH), 7.66 (t, *J* = 3.6 Hz, 1H; H-6), 7.26–7.18 (m, 5H; ArH and H-4), 6.52–6.49 (m, 2H; H-3 and H-1'), 5.57 (d, *J* = 6.0 Hz, 1H; H-3'), 4.71–4.60

(m, 3H; H-4' and H-5'), 3.00–2.95 (m, 1H; H-2'), 2.38 and 2.36 (2s, 6H; CH_3), 2.24–2.17 (m, 1H; H-2'); ^{13}C NMR (101 MHz, $CDCl_3$): *d* 165.9, 165.8, 160.0, 147.5 (d, $^1J_{CF}$ = 231 Hz), 144.3, 144.1, 131.5 (d, $^2J_{CF}$ = 24 Hz), 129.7, 129.4, 129.1, 129.1, 128.8, 126.3, 126.2, 121.0 (d, $^3J_{CF}$ = 6 Hz), 117.1 (d, $^2J_{CF}$ = 39 Hz), 86.3, 83.3, 74.7, 63.8, 39.0, 21.5; ^{19}F NMR (376 MHz, $CDCl_3$): *d* -146.5; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{26}H_{24}F_1N_1O_6Na$, 488.1480; found, 488.1480.

General procedure for toluoyl deprotection: To a stirred solution of **2** (1 equiv) in methanol (0.2 M) NaOMe (2.4 equiv, 0.5 M in CH_3OH) was added dropwise. After the reaction was complete, it was quenched by addition of saturated aqueous NH_4Cl (excess) and concentrated. Purification via flash column chromatography on silica gel (10% MeOH in CH_2Cl_2) afforded the free nucleoside **3**.

1-(2'-Deoxy-ß-D-erythropentofuranosyl)-3-fluoro-2(1*H*)-pyridinone (3a): ^{1}H NMR (500 MHz, CD_3OD): d 7.88 (d, *J* = 7.0, 1H; H-6), 7.27 (ddd, J_{HH} = 1.9, 7.6 Hz, J_{HF} = 8.6 Hz, 1H; H-4), 6.43 (t, *J* = 6.5 Hz, 1H; H-1'), 6.29 (td, J_{HH} = 7.4 Hz, J_{HF} = 4.7 Hz, 1H; H-5), 4.36–4.33 (m, 1H; H-3'), 3.98–3.96 (m, 1H; H-4'), 3.79–3.69 (m, 2H; H-5'), 2.49–2.44 (m, 1H; H-2'), 2.11–2.06 (m, 1H; H-2'); ^{13}C NMR (126 MHz, CD_3OD): *d* 157.3 (d, $^2J_{CF}$ = 25 Hz), 152.5 (d, $^1J_{CF}$ = 244 Hz), 129.8 (d, $^3J_{CF}$ = 5 Hz), 122.4 (d, $^2J_{CF}$ = 17 Hz), 106.0 (d, $^3J_{CF}$ = 6 Hz), 89.3, 87.6, 71.8, 62.6, 42.5; ^{19}F NMR (376 MHz, CD_3OD): *d* -134.7; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{10}H_{12}F_1N_1O_4Na$: 252.0643; found: 252.0644.

1-(2'-Deoxy-ß-D-erythropentofuranosyl)-4-fluoro-2(1*H*)-pyridinone (3b): ^{1}H NMR (500 MHz, CD_3OD): *d* 8.18 (t, *J* = 7.8 Hz, 1H; H-6), 6.34 (t, *J* = 6.5 Hz, 1H; H-1'), 6.29 (ddd, J_{HH} = 1.9, 5.9 Hz, J_{HF} = 7.3 Hz, 1H; H-3), 6.10 (dd, J_{HH} = 2.8 Hz, J_{HF} = 10.8 Hz, 1H; H-5), 4.33 (td, *J* = 5.0, 3.0 Hz, 1H; H-3'), 3.95 (dd, *J* = 7.0, 3.5 Hz, 1H; H-4'), 3.80–3.68 (m, 2H; H-5'), 2.44 (ddd, *J* = 10.0, 6.3, 4.0 Hz, 1H; H-2'), 2.08–2.03 (m, 1H; H-2'); ^{13}C NMR (126 MHz, CD_3OD): *d* 172.5 (d, $^1J_{CF}$ = 265 Hz), 165.6, 137.6 (d, $^3J_{CF}$ = 14 Hz), 102.8 (d, $^2J_{CF}$ = 18 Hz), 99.9 (d, $^2J_{CF}$ = 27 Hz), 89.3, 87.5, 71.8, 62.5, 42.7; ^{19}F NMR (376 MHz, CD_3OD): *d* -97.3; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{10}H_{12}F_1N_1O_4Na$, 252.0643; found, 252.0645.

1-(2'-Deoxy-ß-D-erythropentofuranosyl)-5-fluoro-2(1*H*)-pyridinone (3c): ^{1}H NMR (500 MHz, CD_3OD): *d* 8.16 (dd, J_{HH} = 3.5 Hz, J_{HF} = 5.0 Hz, 1H; H-6), 7.47 (ddd, J_{HH} = 3.1, 6.9 Hz, J_{HF} = 8.4 Hz, 1H; H-4), 6.44 (dd, J_{HH} = 5.3 Hz, J_{HF} = 9.8 Hz, 1H; H-3),

6.33 (t, *J* = 6.3 Hz, 1H; H-1'), 4.35–4.32 (m, 1H; H-3'), 3.95 (dd, *J* = 7.3, 3.8 Hz, 1H; H-4'), 3.82–3.70 (m, 2H; H-5'), 2.47–2.42 (m, 1H; H-2'), 2.09–2.03 (m, 1H; H-2'); ^{13}C NMR (126 MHz, CD_3OD): *d* 162.1, 149.6 (d, $^1J_{CF}$ = 229 Hz), 133.4 (d, $^2J_{CF}$ = 24 Hz), 121.1 (d, $^3J_{CF}$ = 7 Hz), 120.3 (d, $^2J_{CF}$ = 40 Hz), 89.3, 87.6, 71.6, 62.3, 42.6; ^{19}F NMR (376 MHz, CD_3OD): *d* -147.0; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{10}H_{12}F_1N_1O_4Na$, 252.0643; found, 252.0647.

1-(2'-Deoxy-ß-D-erythro-pentofuranosyl)-3-methoxy-2(1*H*)-pyridinone (3d): ^{1}H NMR (500 MHz, CD_3OD): *d* 7.87 (d, *J* = 8.0 Hz, 1H; H-6), 6.37 (t, *J* = 6.5 Hz, 1H; H-1'), 6.05 (dd, *J* = 2.8, 7.8 Hz, 1H; H-5), 5.81 (d, *J* = 3.0 Hz, 1H; H-3), 4.32 (td, *J* = 4.9, 2.5 Hz, 1H; H-3'), 3.91 (dd, *J* = 7.3, 3.8 Hz, 1H; H-4'), 3.77–3.67 (m, 5H; H-5' and OCH_3), 2.36 (ddd, *J* = 9.8, 6.0, 3.5 Hz, 1H; H-2'), 2.07–2.01 (m, 1H; H-2'); ^{13}C NMR (126 MHz, CD_3OD): *d* 169.7, 165.0, 134.0, 101.7, 96.1, 88.0, 85.7, 71.1, 61.8, 55.4, 41.5; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{11}H_{15}N_1O_5Na$, 264.0842; found, 264.0846.

General procedure for DMT protection: To a solution of free nucleoside **3** in anhydrous pyridine was added 4,4'-dimethoxytrityl chloride (1.5 equiv) and 4-(dimethylamino)pyridine (0.5 equiv) and the mixture was stirred overnight under argon at room temperature. The reaction was quenched by addition of MeOH. The mixture was evaporated, and the crude product was purified by chromatography on a silica gel column (30-40% EtOAc in hexane).

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-ß-D-erythropentofuranosyl]-3-fluoro-2(1*H*)-pyridinone (4a): ^{1}H NMR (500 MHz, $CDCl_3$): *d* 7.82(d, *J* = 7.2 Hz, 1H; H-6), 7.42 (d, *J* = 7.2 Hz, 2H; ArH), 7.31 (d, *J* = 8.8 Hz, 4H; ArH), 7.29–7.19 (m, 3H; ArH), 7.06 (ddd, J_{HH} = 1.7, 7.5 Hz, J_{HF} = 8.4 Hz, 1H; H-4), 6.82 (d, *J* = 8.8 Hz, 4H; ArH), 6.59 (t, *J* = 6.2 Hz, 1H; H-1'), 5.91 (td, J_{HH} = 7.2 Hz, J_{HF} = 4.4 Hz, 1H; H-5), 4.57 (dd, *J* = 10.4, 4.4 Hz, 1H; H-3'), 4.19 (dd, *J* = 6.8, 3.2 Hz, 1H; H-4'), 3.77 (s, 6H; OCH_3), 3.51–3.40 (m, 2H; H-5'), 2.79–2.73 (m, 1H; H-2'), 2.25–2.18 (m, 1H; H-2'); ^{13}C NMR (126 MHz, $CDCl_3$): *d* 158.4, 155.6 (d, $^2J_{CF}$ = 26 Hz), 151.4 (d, $^1J_{CF}$ = 247 Hz), 144.4, 135.4, 130.0, 128.0, 127.8, 126.8, 120.3 (d, $^2J_{CF}$ = 16 Hz), 113.0, 103.9 (d, $^3J_{CF}$ = 5 Hz), 89.3, 87.6, 71.8, 62.6, 42.5; ^{19}F NMR (376 MHz, $CDCl_3$): *d* -132.5; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{31}H_{30}F_1N_1O_6Na$, 554.1949; found, 554.1950.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-ß-D-erythropentofuranosyl]-4-fluoro-2(1*H*)-pyridinone (4b): ^{1}H NMR (400 MHz, $CDCl_3$): *d* 8.04 (t, *J* = 7.6

Hz, 1H; H-6), 7.41 (d, *J* = 7.6 Hz, 2H; ArH), 7.32–7.21 (m, 7H; ArH), 6.84 (d, *J* = 8.8 Hz, 4H; ArH), 6.46 (t, *J* = 5.8 Hz, 1H; H-1'), 6.12 (dd, J_{HH} = 2.4 Hz, J_{HF} = 10.8 Hz, 1H; H-3), 5.87–5.83 (m, 1H; H-5), 4.54–4.52 (m, 1H; H-3'), 3.98–3.96 (m, 1H; H-4'), 3.79 (s, 6H; OCH_3), 3.52–3.40 (m, 2H; H-5'), 2.74–2.68 (m, 1H; H-2'), 2.23–2.16 (m, 1H; H-2'); ^{13}C NMR (101 MHz, $CDCl_3$): *d* 170.5 (d, $^1J_{CF}$ = 266 Hz), 163.6, 163.4, 158.5, 144.3, 135.4 (d, $^3J_{CF}$ = 10 Hz), 130.0, 128.0, 127.9, 127.0, 113.2, 102.5 (d, $^2J_{CF}$ = 17 Hz), 98.8 (d, $^2J_{CF}$ = 32 Hz), 86.7, 86.4, 86.1, 71.0, 62.8, 55.2, 42.2; ^{19}F NMR (376 MHz, $CDCl_3$): *d* -96.6; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{31}H_{30}F_1N_1O_6Na$, 554.1949; found, 554.1949.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-ß-D-erythropentofuranosyl]-5-fluoro-2(1*H*)-pyridinone (4c): ^{1}H NMR (500 MHz, $CDCl_3$): *d* 7.85 (t, *J* = 3.8 Hz, 1H; H-6), 7.42 (d, *J* = 7.5 Hz, 2H; ArH), 7.34 (d, *J* = 3.0 Hz, 2H; ArH), 7.32 (d, *J* = 3.0 Hz, 2H; ArH), 7.29–7.23 (m, 3H; ArH and H-4), 7.20 (t, *J* = 7.3 Hz, 1H; ArH), 6.83 (d, *J* = 2.0 Hz, 2H; ArH), 6.82 (d, *J* = 2.5 Hz, 2H; ArH), 6.48–6.45 (m, 2H; H-3 and H-1'), 4.50–4.47 (m, 1H; H-3'), 4.21–4.19 (m, 1H; H-4'), 3.77 (s, 6H; OCH_3), 3.44–3.37 (m, 2H; H-5'), 2.81–2.77 (m, 1H; H-2'), 2.19–2.13 (m, 1H; H-2'); ^{13}C NMR (126 MHz, $CDCl_3$): *d* 160.2, 158.5, 147.7 (d, $^1J_{CF}$ = 230 Hz), 144.4, 135.5, 131.5 (d, $^2J_{CF}$ = 24 Hz), 130.0, 127.9, 127.9, 126.8, 120.5 (d, $^3J_{CF}$ = 7 Hz), 118.4 (d, $^2J_{CF}$ = 38 Hz), 113.2, 86.8, 86.6, 77.2, 71.8, 63.4, 55.1, 42.0; ^{19}F NMR (376 MHz, $CDCl_3$): *d*-146.2; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{31}H_{30}F_1N_1O_6Na$, 554.1949; found, 554.1950.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-ß-D-erythropentofuranosyl]-4-methoxy-2(1*H*)-pyridinone (4d): ^{1}H NMR (400 MHz, $CDCl_3$): *d* 7.78 (d, *J* = 7.6 Hz, 1H; H-6), 7.43 (d, *J* = 7.2 Hz, 2H; ArH), 7.31–7.22 (m, 7H; ArH), 6.82 (d, *J* = 8.8 Hz, 4H; ArH), 6.57 (t, *J* = 6.0 Hz, 1H; H-1'), 5.81 (d, *J* = 2.4 Hz, 1H; H-3), 5.73 (dd, *J* = 7.6, 2.4 Hz, 1H; H-5), 4.54–4.51 (m, 1H; H-3'), 4.18–4.15 (m, 1H; H-4'), 3.76 and 3.71 (2s, 9H; OCH_3), 3.47–3.38 (m, 2H; H-5'), 2.71–2.65 (m, 1H; H-2'), 2.19–2.13 (m, 1H; H-2'); ^{13}C NMR (101 MHz, $CDCl_3$): *d* 168.4, 164.0, 158.5, 144.6, 135.7, 135.6, 130.1, 128.2, 127.9, 126.9, 113.2, 101.0, 96.5, 86.6, 86.3, 85.8, 71.4, 55.5, 55.2, 42.2; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{32}H_{33}N_1O_7Na$, 566.2149; found, 566.2147.

General procedure for phosphoramidation. 2-Cyanoethyldiisopropyl chlorophosphoramidite (1.5 equiv) was added dropwise to a solution of **4** and diisopropyethyla-

mine (4 equiv) in CH_2Cl_2 at room temperature. After the reaction reached completion (30 min), the mixture was concentrated *in vacuo* and purified by chromatography through a short column of SiO_2 (13-25% EtOAc in CH_2Cl_2) to yield **5**.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-3'-*O*-[[bis(1-methylethyl)amino](2-cyanoethoxy)phosphino]-2'-deoxy-ß-D-erythro-pentofuranosyl]-3-fluoro-2(1*H*)-pyridinone (5a): ^{1}H NMR (400 MHz, $CDCl_3$): *d* 7.82 and 7.75 (2d, *J* = 7.2 Hz, 1H; H-6), 7.43–7.40 (m, 2H; ArH), 7.32–7.16 (m, 7H; ArH), 7.07–7.01 (m, 1H; H-4), 6.85–6.81 (m, 4H; ArH), 6.57–6.51 (m, 1H; H-1'), 5.88–5.83 (m, 1H; H-5), 4.67–4.61 (m, 1H; H-3'), 4.20–4.19 (m, 1H; H-4'), 3.80 and 3.79 (2s, 6H; OCH_3), 3.76–3.72 (m, 1H; OCH_2), 3.64–3.35 (m, 5H; NCH, OCH_2, and H-5'), 2.75–2.68 (m, 1H; H-2'), 2.61 and 2.43 (2t, *J* = 6.3 Hz, 2H; CH_2CN), 2.28–2.22 (m, 1H; H-2'), 1.21–1.15 (m, 12H; CH_3); ^{19}F NMR (376 MHz, $CDCl_3$): *d*-132.1, 132.2; ^{31}P NMR (162 MHz, $CDCl_3$): *d* 149.8, 149.1; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{40}H_{47}F_1N_3O_7P_1Na$, 754.3028; found, 754.3025.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-3'-*O*-[[bis(1-methylethyl)amino](2-cyanoethoxy)phosphino]-2'-deoxy-ß-D-erythro-pentofuranosyl]-4-fluoro-2(1*H*)-pyridinone (5b): ^{1}H NMR (400 MHz, $CDCl_3$): *d* 8.06 and 7.98 (2t, *J* = 7.7 Hz, 1H; H-6), 7.42–7.39 (m, 2H; ArH), 7.32–7.24 (m, 7H; ArH), 6.86–6.82 (m, 4H; ArH), 6.46–6.40 (m, 1H; H-1'), 6.13 (dd, J_{HH} = 2.4 Hz, J_{HF} = 10.8 Hz, 1H; H-3), 5.79–5.75 (m, 1H; H-5), 4.67–4.63 (m, 1H; H-3'), 4.19–4.17 (m, 1H; H-4'), 3.79 and 3.77 (2s, 6H; OCH_3), 3.76–3.74 (m, 1H; OCH_2), 3.63–3.37 (m, 5H; NCH, OCH_2, and H-5'), 2.71–2.67 (m, 1H; H-2'), 2.61 and 2.43 (2t, *J* = 6.3 Hz, 2H; CH_2CN), 2.27–2.21 (m, 1H; H-2'), 1.18–1.05 (m, 12H; CH_3); ^{19}F NMR (376 MHz, $CDCl_3$): *d* -97.3; ^{31}P NMR (162 MHz, $CDCl_3$): *d* 149.8, 149.1; HRMS (*m/z*): [*M* + Na]$^+$ calcd for $C_{40}H_{47}F_1N_3O_7P_1Na$, 754.3028; found, 754.3026.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-3'-*O*-[[bis(1-methylethyl)amino](2-cyanoethoxy)phosphino]-2'-deoxy-ß-D-erythro-pentofuranosyl]-5-fluoro-2(1*H*)-pyridinone (5c): ^{1}H NMR (500 MHz, $CDCl_3$): *d* 7.84 and 7.78 (2t, *J* = 4.0 Hz, 1H; H-6), 7.44–7.41 (m, 2H; ArH), 7.34–7.21 (m, 8H; ArH and H-4), 6.84–6.81 (m, 4H; ArH), 6.50–6.38 (m, 2H; H-3 and H-1'), 4.61–4.55 (m, 1H; H-3'), 4.30–4.19 (m, 1H; H-4'), 3.79 and 3.78 (2s, 6H; OCH_3), 3.76–3.72 (m, 1H; OCH_2), 3.68–3.36 (m, 5H; NCH, OCH_2, and H-5'), 2.81–2.56 (m, 2H; H-2' and CH_2CN), 2.44 (t, *J* = 6.4 Hz, 1H;

CH_2CN), 2.21–2.16 (m, 1H; H-2'), 1.26–1.07 (m, 12H; CH_3); ^{19}F NMR (376 MHz, $CDCl_3$): *d* -147.1; ^{31}P NMR (162 MHz, $CDCl_3$): *d* 149.6, 149.0; HRMS (*m/z*): $[M + Na]^+$ calcd for $C_{40}H_{47}F_1N_3O_7P_1Na$, 754.3028; found, 754.3020.

1-[5'-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-3'-*O*-[[bis(1-methylethyl)amino](2-cyanoethoxy)phosphino]-2'-deoxy-ß-D-erythro-pentofuranosyl]-4-methoxy-2(1*H*)-pyridinone (5d): ^1H NMR (400 MHz, $CDCl_3$): *d* 7.80–7.70 (m, 1H; H-6), 7.43–7.39 (m, 2H; ArH), 7.32–7.21 (m, 7H; ArH), 6.84–6.80 (m, 4H; ArH), 6.53–6.48 (m, 1H; H-1'), 5.82–5.81 (m, 1H; H-3), 5.67 (dd, *J* = 7.8, 2.6 Hz, 1H; H-5), 4.63–4.57 (m, 1H; H-3'), 4.22–4.10 (m, 1H; H-4'), 3.84–3.71 (m, 10H; OCH_2 and OCH_3), 3.61–3.32 (m, 5H; NCH, OCH_2, and H-5'), 2.76–2.58 (m, 2H; H-2' and CH_2CN), 2.41 (t, *J* = 6.4 Hz, 1H; CH_2CN), 2.21–2.16 (m, 1H; H-2'), 1.29–1.03 (m, 12H; CH_3); ^{31}P NMR (162 MHz, $CDCl_3$): *d* 149.6, 149.0; HRMS (*m/z*): $[M + Na]^+$ calcd for $C_{41}H_{50}N_3O_8P_1Na$, 766.3228; found, 766.3231.

General procedure for triphosphate synthesis: Proton sponge (1.5 equiv) and the free nucleoside (1 equiv) were dissolved in trimethyl phosphate (0.3 M) and cooled to -20 °C. $POCl_3$ (1.5 equiv) was added dropwise, and the purple slurry was stirred at -20 °C for 3 h. Tributylamine (6.2 equiv) was added, followed by a solution of tributylammonium pyrophosphate (5.0 equiv) in DMF (0.5 M). After 5 min, the reaction was quenched by addition of 0.5 M aqueous $Et_3NH_2CO_3$ (20 vol-equiv). The resulting solution lyophilized. Purification by reverse-phase (C18) HPLC (4-35% CH_3CN in 0.1 M $Et_3NH_2CO_3$, pH 7.5) followed by lyophilization afforded the triphosphate as a white solid.

1-(2'-Deoxy-ß-D-erythro-pentofuranosyl)-3-fluoro-2(1*H*)-pyridinone-5'-triphosphate (6a): ^1H NMR (400 MHz, D_2O): *d* 7.81 (d, *J* = 6.8 Hz, 1H; H-6), 7.34 (ddd, J_{HH} = 1.6, 7.8 Hz, J_{HF} = 8.7 Hz, 1H; H-4), 6.50–6.41 (m, 2H; H-1' and H-5), 4.58–4.55 (m, 1H; H-3'), 4.19–4.13 (m, 1H; H-4'), 3.13–2.88 (m, 2H; H-5'), 2.47–2.41 (m, 1H; H-2'), 2.27–2.22 (m, 1H; H-2'); ^{19}F NMR (376 MHz, D_2O): *d* -134.6; ^{31}P NMR (162 MHz, D_2O): *d* -5.86 (d, *J* = 21.2 Hz; ?-P), -10.86 (d, *J* = 19.8 Hz; a-P), -22.08 (t, *J* = 20.8 Hz; ß-P).

1-(2'-Deoxy-ß-D-erythro-pentofuranosyl)-4-fluoro-2(1*H*)-pyridinone-5'-triphosphate (6b): ^1H NMR (400 MHz, D_2O): *d* 8.14 (t, *J* = 7.4 Hz, 1H; H-6), 6.59–6.53 (m, 1H; H-3), 6.43 (t, *J* = 6.2 Hz, 1H; H-1'), 6.25 (dd, J_{HH} = 2.0 Hz, J_{HF} = 10.0 Hz, 1H; H-

5), 4.70–4.58 (m, 1H; H-3'), 4.25–4.21 (m, 1H; H-4'), 3.10–2.95 (m, 2H; H-5'), 2.53–2.46 (m, 1H; H-2'), 2.32–2.27 (m, 1H; H-2'); ^{19}F NMR (376 MHz, D_2O): *d* -94.1; ^{31}P NMR (162 MHz, D_2O): *d* -7.23 – -7.25 (m; ?-P), -10.85 (d, *J* = 19.8 Hz; a-P), -22.21 (t, *J* = 20.7 Hz; ß-P).

1-(2'-Deoxy-ß-D-erythro-pentofuranosyl)-5-fluoro-2(1*H*)-pyridinone-5'-triphosphate (6c): ^{1}H NMR (400 MHz, D_2O): *d* 8.07–8.02 (m, 1H; H-6), 7.31–7.28 (m, 1H; H-4), 6.57 (dd, J_{HH} = 5.2 Hz, J_{HF} = 9.6 Hz, 1H; H-3), 6.40 (t, *J* = 6.2 Hz, 1H; H-1'), 4.65–4.54 (m, 1H; H-3'), 4.24–4.20 (m, 1H; H-4'), 3.05–2.97 (m, 2H; H-5'), 2.53–2.50 (m, 1H; H-2'), 2.31–2.26 (m, 1H; H-2'); ^{19}F NMR (376 MHz, D_2O): *d* -142.9; ^{31}P NMR (162 MHz, D_2O): *d* -8.58 – -8.85 (m; ?-P), -11.00 (d, *J* = 20.3 Hz; a-P), -22.52 (t, *J* = 20.3 Hz; ß-P).

1-(2'-Deoxy-ß-D-erythro-pentofuranosyl)-3-methoxy-2(1*H*)-pyridinone-5'-triphosphate (6d): ^{1}H NMR (400 MHz, D_2O): *d* 7.89 (d, *J* = 7.6 Hz, 1H; H-6), 6.49 (t, *J* = 6.8 Hz, 1H; H-1'), 6.37 (dd, *J* = 3.0, 7.8 Hz, 1H; H-5), 5.97 (d, *J* = 2.4 Hz, 1H; H-3), 4.24–4.20 (m, 1H; H-3'), 3.56–3.50 (m, 1H; H-4'), 3.20–3.01 (m, 5H; H-5' and OCH_3), 2.47–2.41 (m, 1H; H-2'), 2.32–2.27 (m, 1H; H-2'); ^{31}P NMR (162 MHz, D_2O): *d* -10.18 to -10.51 (m; ?-P), -10.74 to -10.96 (m; a-P), -22.48 to -23.01 (m; ß-P).

Synthesis of oligonucleotides. Oligonucleotides were prepared by the ß-cyanoethylphosphoramidite method on controlled pore glass supports (1 mmol) using an Applied Biosystems Inc. 392 DNA/RNA synthesizer as standard method. After automated synthesis, the oligonucleotides were cleaved from the support by concentrated aqueous ammonia for 1 h at room temperature, deprotected by heating at 55 °C for 12 h, and purified by denaturing polyacrylamide gel electrophoresis (12-20%, 8 M urea). The primer oligonucleotides containing unnatural bases at the 3'-end were obtained using Universal Support II or 3'-phosphate CPG, which was treated with alkaline phosphatase after deprotection. The oligonucleotides were purified by PAGE, visualized by UV shadowing, and recovered by electroelution. After ethanol precipitation, the concentration of oligonucleotides was determined by UV/Vis absorption.

Thermal stability. The unnatural and natural nucleosides were incorporated into the complementary oligonucleotides 5'-d(GCGTAC**X**CATGCG)-3' and 5'-d(CGCATG**Y**GTACGC)-3' at the positions labeled **X** and **Y**. For UV melting experiments the sample

(3 μM oligonucleotide, 10 mM PIPES, pH 7.0, 100 mM NaCl and 10 mM $MgCl_2$) absorption was monitored at 260 nm from 20 °C to 80 °C at a heating rate of 0.5 °C per min, using a Cary 300 Bio UV/Vis spectrophotometer. Melting temperatures were determined from the derivative method using the Cary Win UV thermal application software.

Steady-state kinetics: The unnatural nucleobases were evaluated as substrates for Kf by measuring initial rates at which a [?-^{33}P]-labeled primer-template, 5'-d(TAATACGACTCACTATAGGGAGA**X**) annealed to the 45-mer template, 5'-d(CGCTAGGACGGCATTGGATCG**Y**TCTCCCTATAGTG AGTCGTATTA), was extended with varying concentrations of natural or unnatural nucleoside triphosphates or a-thiotriphosphate. Each reaction included 40 nM primer template, 0.3-1.2 nM enzyme, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL acetylated BSA. The reactions were initiated by adding a 5 μL 2× dNTP or a-(*S*)dNTP solution to a 5 μL solution containing the polymerase and primer-template and incubating at 25 °C for 3-10 min, and were then quenched with the addition of 20 μL of loading dye (95% formamide, 20 mM EDTA, and sufficient amounts of bromophenol blue and xylene cyanole). The reactions were analyzed by polyacrylamide gel electrophoresis and a Phosphorimager (Molecular Dynamics) was used to quantify gel band intensities corresponding to the extended and unextended primer. The measured velocities were plotted against the concentration of dNTP or a-(*S*)dNTP and fit to the Michaelis-Menten equation to determine V_{max} and K_M. k_{cat} was determined from V_{max} by normalizing by the total enzyme concentration.

Pre-steady-state kinetics: The reactions were carried out by mixing of a solution containing the preincubated complex of 15 nM Kf and 40 nM [?-^{33}P]-labeled primer template with varying concentrations of dGTP in the presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL acetylated BSA at 25 °C. The reactions were quenched at various time points by the addition of 20 μL of loading dye. The reaction mixture was then analyzed by 15% polyacrylamide gel electrophoresis. Radioactivity was quantified using Phosphorimager (Molecular Dynamics). Data were fit to a burst equation: [product] = $A(1 - \exp(-k_{obsd}) + k_{ss}t)$, where A represents the amplitude of the burst, k_{obsd} is the observed first-order rate constant for dGTP incorporation, and k_{ss} is the observed steady-state rate constant.

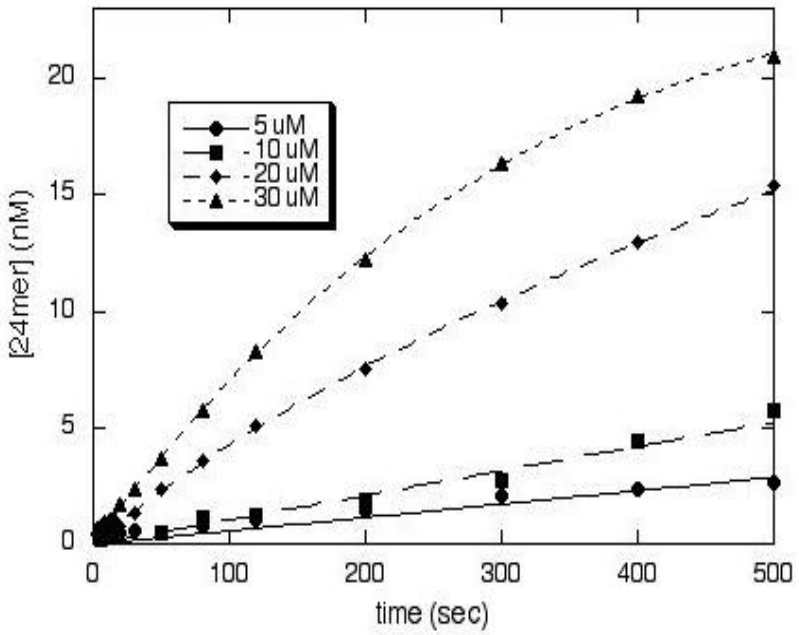

Figure S1. Pre-steady-state kinetics of dGTP incorporation opposite **3FP**. The reaction contained 15 nM Kf and 40 nM DNA (primer-template) and was initiated by the addition of dGTP and incubated at room temperature. After the times indicated, the reaction was quenched with loading buffer (95% formamide, 20 nM EDTA) and analyzed by 15% polyacrylamide gel electrophoresis followed by phosphorimaging.

Table S1. Pre-steady-state rate constant of dGTP incorporation.

5'-d(TAATACGACTCACTATAGGGAGA)

3'-d(ATTATGCTGAGTGATATCCCTCT**X**GCTAGGTTACGGCAGGATCGC)

X	Triphosphate	k_{ss} [s^{-1}]
3FP	G	0.15 ± 0.08

References

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